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Antiplatelet and Anti-Inflammatory Constituents and New Oxygenated Xanthones from *Hypericum geminiflorum*

Abstract

Two new pentaoxygenated xanthones, 2,3-dihydroxy-1,6,7-trimethoxyxanthone (**1**) and 3,6-dihydroxy-1,5,7-trimethoxyxanthone (**2**) were isolated from the leaf of *Hypericum geminiflorum*. The antiplatelet activities of the constituents, 2,6-dimethoxy-*p*-benzoquinone (**3**), gemichalcone A (**4**), gemichalcone B (**5**), and cycloartocarpin (**6**), of this plant, were assessed *in vitro* by determining their inhibitory effects on the aggregation of washed rabbit platelets induced by various inducers. The anti-inflammatory effects of **4** and **5** were assessed *in vitro* by determining their inhibitory effects on the chemical mediators released from mast cells and neutrophils. Of the compounds tested, **4** exhibited the most potent inhibition of platelet aggregation induced by arachidonic acid (AA) and **4** and **5** strongly inhibited the release of β-glucuronidase and lysozyme in formyl-Met-Leu-Phe (fMLP)-stimulated rat neutrophils.

Key words
Hypericum geminiflorum · Clusiaceae · pentaoxygenated xanthone · antiplatelet · anti-inflammatory

Introduction

Hypericum is a large genus of herbs or shrubs, which occurs widely in temperate regions of the world. In China, there are 55 species and eight subspecies and half of them have been used in Chinese herbal medicine, mainly for the treatment of infectious hepatitis. *Hypericum geminiflorum*, an endemic plant in Taiwan, is a Chinese folk medicine used for the treatment of several bacterial diseases, infectious hepatitis, gastrointestinal disorder, and tumor [1].

Recently we have isolated and characterized several constituents of the heartwood, root and leaf of *Hypericum geminiflorum* (Clusiaceae) [2], [3]. Continuing studies on *H. geminiflorum*, two new oxygenated xanthones, 2,3-dihydroxy-1,6,7-trimethoxyxanthone (**1**) and 3,6-dihydroxy-1,5,7-trimethoxyxanthone (**2**) were obtained from the methanol extract of the leaf of this plant. In the present paper we report the isolation and the structure characterization of **1** and **2**. Continual screening for bioactive compounds as inhibitors of platelet aggregation and chemical mediators released from mast cells and neutrophils, the antiplatelet effects of 2,6-dimethoxy-*p*-benzoquinone (**3**), gemichalcones A (**4**) and B (**5**), and cycloartocarpin (**6**), previously isolated from this plant [2], and anti-inflammatory effects of **4** and **5** were also reported.

Materials and Methods

General experimental procedures

Melting points are reported uncorrected. Optical rotation was obtained on JASCO model DIP-370 digital polarimeter; UV spectra were obtained on a JASCO model 7800 UV-vis spectrophotometer; IR spectra were recorded on a Hitachi model 260 – 30

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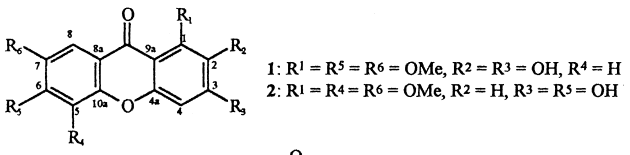

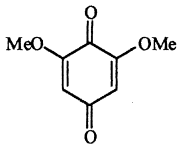

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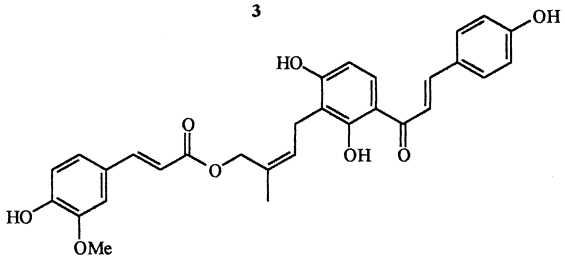

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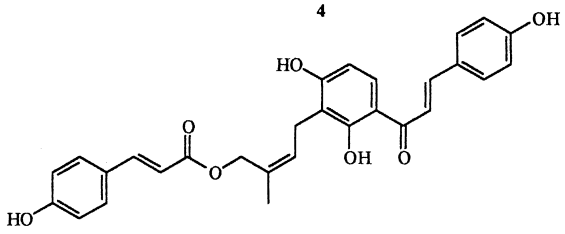

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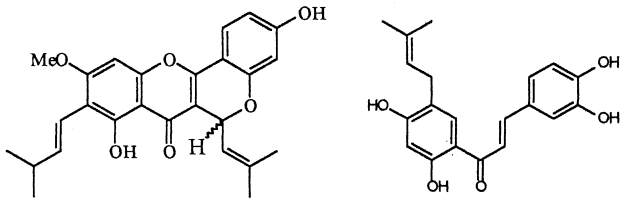

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spectrophotometer; ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer; mass spectra were run on a Jeol JMS-SX 102 mass spectrometer. Plants of *H. geminiflorum* Hemsl. were collected at Ping Tung Hsieng, Taiwan, during November 1993 and a voucher specimen (9302) is deposited at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical University.

Extraction and isolation

The fresh leaves (3 kg) were chopped and extracted with MeOH. The MeOH extract (50 g) was separated by column chromatography (CC) on silica gel (100 g, 2 × 50 cm, CH_2Cl_2/MeOH, 6 : 1, 1 ml/min; t_R of **1**: between 2 and 6 ml, **2**: 7 – 10 ml) yielded **1** (2 mg) (R_f 0.6 – 0.8, UV detection) and **2** (2.5 mg) (R_f 0.3 – 0.5, UV detection).

Isolates

2,3-Dihydroxy-1,6,7-trimethoxyxanthone (**1**): Yellow needles (CH_2Cl_2-MeOH), m.p. 210 – 213 °C. UV (MeOH): λ_{max} (log ε) = 256 (4.11), 306 (3.84), 342 (sh, 3.78), 405 (2.85); ($AlCl_3$): unchanged; (NaOAc): 256, 291, 338; (NaOAc-H_3BO_3): 256, 280 (sh), 310 (sh), 350 (sh), 400 nm. IR: ν_{max} = 3150 (OH), 1625 (-CO), 1600 cm^{-1}(aromatic ring C = C stretch). EI-MS: *m/z* (rel. int.) = 318 (M^+, 100), 303 ([M-15]$^+$, 43), 289 ([M-CHO]$^+$, 35), 272 ([289-OH]$^+$, 19); HR/EIMS: $C_{16}H_{14}O_7$, found: 318.0735 [M]$^+$, calcd: 318.0738.). ^{1}H-NMR (pyridine-d_5, 400 MHz): **see text**. ^{13}C-NMR (CD_3OD, 100 MHz): δ = 56.4 (OMe), 56.5 (OMe), 61.4 (OMe), 96.4 (C-4), 96.5 (C-5), 97.6 (C-8), 106.4 (C-9a), 119.3 (C-8a), 140.2 (C-2), 142.1 (C-10a), 143.0 (C-4a), 151.4 (C-7), 161.2 (C-3), 163.6 (C-6), 165.7 (C-1), 176.9 (CO).

3,6-Dihydroxy-1,5,7-trimethoxyxanthone (**2**): Yellow powder (CH_2Cl_2-MeOH). UV (MeOH): λ_{max} (log ε) = 254 (4.46), 308 (4.10), 340 (sh, 3.75), 401 (sh, 3.02); ($AlCl_3$): unchanged; (NaOAc-H_3BO_3): unchanged; (NaOAc) : 256 (4.47), 291 (4.23), 340.0 nm (4.27); IR: ν_{max} = 3300 (OH), 1630 (-CO), 1600 cm^{-1} (aromatic ring C = C stretch). EI-MS : *m/z* (rel. int.) = 318 (M^+, 100), 289 ([M-CHO]$^+$, 31), 272 ([289-OH]$^+$, 20), 245 (14). HR/EI-MS : $C_{16}H_{14}O_7$, found: 318.0740 [M]$^+$, calcd: 318.0738.); ^{1}H-NMR (pyridine-d_5, 400 MHz), **see text**. ^{13}C-NMR (pyridine-d_5, 100 MHz): δ = 55.8 (OMe), 56.1 (OMe), 60.6 (OMe), 96.0 (C-2), 96.6 (C-4), 97.1 (C-8), 106.4 (C-9a), 119.6 (C-8a), 140.8 (C-6), 141.6 (C-10a), 142.2 (C-5), 150.6 (C-7), 160.5 (C-4a), 163.0 (C-3), 164.8 (C-1), 174.5 (CO) .

The isolation, purification, and identification of **3 – 6** were reported previously [2], [16]. (Copies of the original spectra are obtainable from the author of correspondence).

Platelet aggregation

Washed rabbit platelets were obtained from ethylenediaminetetraacetic acid (EDTA)-anticoagulated platelet-rich plasma (PRP) according to procedures described previously [5]. Platelet numbers were counted by use of a Coulter Counter (Model ZM) and adjusted to 4.5 × 10^8 platelet/ml. The platelet pellets were suspended in Tyrode's solution containing (mM): NaCl 136.8, KCl 2.8, $NaHCO_3$ 11.9, $MgCl_2$ 2.1, NaH_2PO_4 0.33, $CaCl_2$ 1.0, and glucose 11.2 with 0.35% bovine serum albumin. All glassware was siliconized. PRP or the platelet suspension was stirred at 1200 rev/min one min before addition of the aggregation inducer. Aggregation was measured by a turbidimetric method [6]. The absorbance of PRP or platelet-poor plasma or platelet-free Tyrode solution was taken as 100% aggregation. The aggregation was measured by means of Lumi-aggregometer (Chrono-Log Co., USA) connected to dual channel recorders.

Mast cell degranulation

Heparinized Tyrode solution was injected into the peritoneal activity of exsanguinated rats (Sprague-Dawley, 250 – 300 g). After abdominal massage, the cells in the peritoneal fluid were harvested and then separated through 38% bovine serum albumin. The cells were washed and suspended in Tyrode solution. The cell suspension was pre-incubated with DMSO or drugs at 37 °C for 3 min.. Fifteen minutes after addition of compound 48/80 (10 mg/ml), β-glucuronidase (phenolphthalein-β-D-glucuronide as substrate, 550 nm) and histamine (*o*-phthaldialdehyde condensation, 350 and 450 nm) in the supernatant were determined [7].

Neutrophil degranulation

Blood was withdrawn from the rat and mixed with EDTA. After dextran sedimentation, Ficoll-hypaque separation, and hypotonic lysis of the residual erythrocytes, neutrophils were washed and suspended in Hank's balanced salt solution [8]. The cell suspension was pre-incubated with dimethyl sulphoxide (DMSO) or drugs at 37 °C for 3 min, then challenged with fMLP (1 μM). Lyso-

zyme (*Micrococcus lysodeikticus* as substrate, 450 nm) and β-glucuronidase in the supernatant were determined 45 min later [9]. The total content was measured after treatment of the cell suspension with Triton X-100. The percent released was determined [7].

Superoxide anion formation

Superoxide anion formation was measured in terms of superoxide dismutase- inhibitable cytochrome *c* reduction [10]. Neutrophil suspension was pre-incubated with 0.5% DMSO or drugs for 3 min, and then superoxide dismutase or HBSS was added into the test and blank wells, respectively. After addition of cytochrome *c*, the reaction was initiated by challenge with fMLP/CB (0.3 μM/5 μg/ml) or phorbol 12-myristate B acetate (PMA) (3 nM). The reaction was terminated after 30 min by centrifugation and the absorbance changes of supernatant were monitored at 550 nm in a microplate reader. The final concentration of drugs in DMSO was fixed at 0.5%.

Statistical analysis

Data are presented as the mean ± s. e. m. Statistical analyses were performed using the Least Significant Difference Test method after analysis of variance. $P < 0.05$ was considered to be significant. Analysis of the regression line was used to calculate IC_{50} values.

Results and Discussion

The HREI-MS of **1** indicated $[M]^+$ at *m/z* 318.0735, which corresponded to a molecular formula of $C_{16}H_{14}O_7$. Its IR spectrum showed absorption bands for hydroxy (3150 cm^{-1}), conjugated carbonyl (1625 cm^{-1}), and aromatic rings (1600 cm^{-1}), and the UV spectrum exhibited absorption maxima characteristic of xanthones [4]. The ^{1}H-NMR spectrum showed three methoxy signals at δ = 3.92 (9H, s) and three aromatic proton signals at δ = 6.37 (1H, s, H-4), 6.53 (1H, s, H-5), and 7.20 (1H, s, H-8). In addition to the above evidence, the UV spectrum showing a bathochromic shift with NaOAc and NaOAc-H_3BO_3, but not on addition of $AlCl_3$, the presence of an *ortho*-dimethoxy group (δ = 56.4, 56.5, and 61.4) in the ^{13}C-NMR spectrum [11], and a correlation between H-8 (δ = 7.20) and the methoxy signal at δ = 3.92 in the NOESY experiment indicated that the compound is 2,3-dihydroxy-1,6,7-trimethoxyxanthone (**1**). The ^{13}C-NMR spectrum was assigned by DEPT and comparison with corresponding data in the literature [11], [12]. The ^{13}C-NMR and MS data also supported structure **1**.

The HREI-MS of **2** indicated $[M]^+$ at *m/z* 318.0740, which corresponded to the molecular formula $C_{16}H_{14}O_7$. Its IR spectrum showed absorption bands for hydroxy (3300 cm^{-1}), conjugated carbonyl (1630 cm^{-1}), and aromatic rings (1600 cm^{-1}), and the UV spectrum exhibited absorption maxima characteristic of xanthones [4]. The ^{1}H-NMR spectrum showed three methoxy signals at δ = 3.74 (3H, s), 3.86 (3H, s), and 3.93 (3H, s) and three aromatic proton signals at δ = 6.69 (1H, d, J = 2.4 Hz, H-2), 6.79 (1H, d, J = 2.4 Hz, H-4), and 7.66 (1H, s, H-8). In addition to the above evidence, the UV spectrum showing a bathochromic shift with NaOAc, but not on addition of $AlCl_3$ and NaOAc-H_3BO_3, the presence of methoxy carbonyl signal at δ = 60.6 in the ^{13}C-NMR spectrum, and correlations between H-8 (δ 7.66) and the methoxy signal at δ = 3.74, and H-2 (δ = 6.69) and the methoxy signal at δ = 3.86 in the NOESY experiment, indicated that this compound is 3,6-dihydroxy-1,5,7-trimethoxyxanthone (**2**). The ^{13}C-NMR spectrum was assigned by DEPT and comparison with corresponding data in the literature [11], [12]. The ^{13}C-NMR and MS data also supported structure **2**. Because platelet aggregation is an important pathogenic factor in the development of atherosclerosis and associated thrombosis [13], one rational approach in the research for antithrombotic drugs is to search for inhibitors of platelet aggregation. Compounds **3 – 6** inhibited the aggregation of washed rabbit platelets (Table **1**). The degree of this inhibitory effect varied depending upon the types of aggregation inducers. Aggregation induced by AA was most easily inhibited by **4**, that by collagen was inhibited by **3** and **6**, and that by PAF and thrombin was inhibited by **3**.

More experiments were performed to study the antiplatelet effects of **3 – 6** on AA-, collagen-, PAF-, and thrombin-induced platelet aggregation at various concentrations (Fig. **1** and **2**). These inhibitions appeared to be concentration dependent. Aspirin was used in this study as a positive control. It was found (Table **1**) that aspirin (50 μM) inhibited completely the platelet aggregation induced by AA but not that induced by collagen, PAF, or thrombin.

Table **1** Effects and IC_{50} values of **3 – 6** on the platelet aggregation induced by arachidonic acid, collagen, PAF, and thrombin in washed rabbit platelets[a]

Compound	*Aggregation (%)/IC_{50} (μM)*[e]			
	AA (100 μM)	*Collagen (10 μg/ml)*	*PAF (2 ng/ml)*	*Thrombin (0.1 U/ml)*
Control	87.2 ± 2.3	92.6 ± 1.4	90.8 ± 1.4	91.5 ± 0.7
3	2.2 ± 1.0[d]/42.2	0.0 ± 0.0[d]/9.0	0.0 ± 0.0[d]/42.5	30.4 ± 3.2[b]/< 300 (6.63)
4	0.0 ± 0.0[d]/8.4	24.8 ± 2.7[d]/< 300 (73.2)	19.2 ± 6.2[d]/< 300 (78.9)	84.9 ± 1.1[d]/> 300 (7.2)
5	4.2 ± 2.1[d]/34.8	28.9 ± 3.1[d]/< 300 (68.8)	11.8 ± 1.7[d]/> 300 (70.0)	86.2 ± 1.9[b]/> 300 (5.8)
6	0.0 ± 0.0[d]/38.5	0.0 ± 0.0[c]/36.3	59.3 ± 5.8[c]/> 300 (30.3)	76.0 ± 4.7[b]/> 300 (13.3)
Aspirin	0.0 ± 0.0/19.7	85.4 ± 3.9	90.5 ± 1.2	91.9 ± 2.5

[a] Plateltes were preincubated with DMSO (0.5%, control), compounds (each at 300 μM) or aspirin (50 μM) at 37 °C for 3 min, and the inducer was then added. Values are presented as means ± s. e. m. (n = 3 – 4).
[b] $P < 0.05$.
[c] $P < 0.01$.
[d] $P < 0.001$ as compared with the respective control.
[e] When 50% inhibition could not reached at the highest concentration, the % of inhibition is given in parentheses.

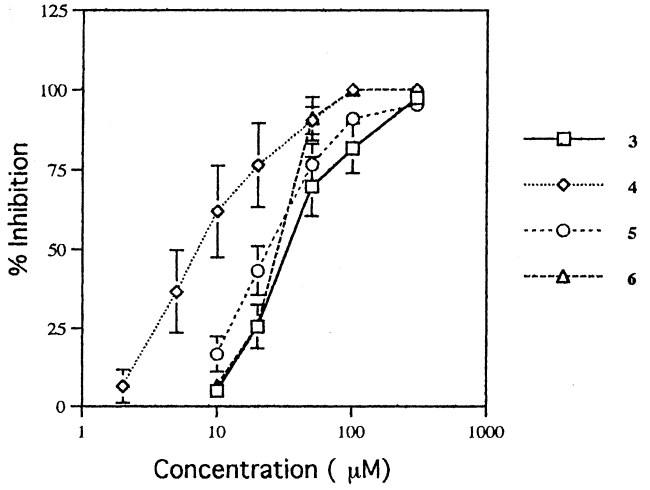

Fig. **1** The effects of **3 – 6** on AA-induced platelet aggregation. Washed rabbit platelets were incubated with various concentrations of **3 – 6** and then AA (100 μM) was added to stimulate platelet aggregation.

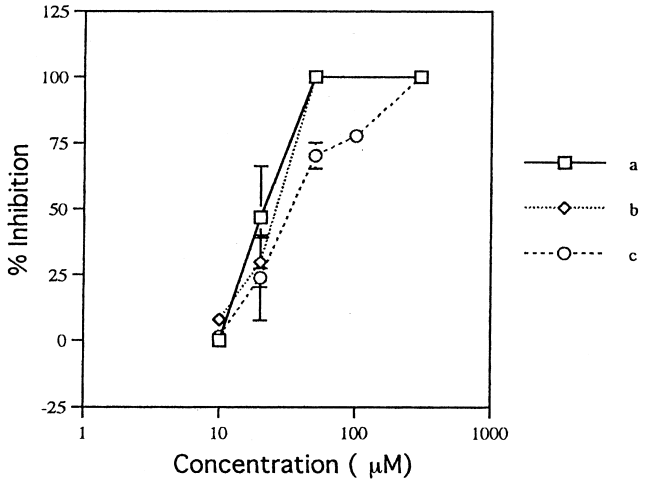

Fig. **2** The effects of **3** (a) and **6** (b) on collagen induced platelet aggregation and **3** (c) on PAF induced platelet aggregation. Washed rabbit platelets were incubated with various concentrations of **3** or **6** and then collagen (10 μg/ml) or PAF (2 ng/ml) was added to stimulate platelet aggregation.

We previously reported that prenylflavonoids and prenylchalcones had potent antiplatelet actions on AA- and collagen-induced platelet aggregation with little or no effect on PAF-induced platelet aggregation and that their antiplatelet actions are partially due to an inhibitory effect on cyclooxygenase activity and diminishing thromboxane formation [14]. In the present study, we found that the prenylflavonoids and prenylchalcones, listed in Table **1**, had the same antiplatelet action as those of prenylflavonoids and prenylchalcones reported previously [14]. Cycloartocarpin A indicated potent antiplatelet effects on AA- and collagen-induced platelet aggregation while O-methylation at 7-OH of cycloartocarpin A (i.e., **6**) did not enhance the antiplatelet effects. From the results in Tables **1** and our previous report [14], it is further supported that prenylation at C-6 or C-8 of flavonoids may modulate the antiplatelet effects [14].

The anti-inflammatory activities of compounds **4** and **5** (Tables **2 – 4**) were studied *in vitro* for their inhibitory effects on the activation of mast cells and neutrophils. Compound 48/80 (10 μg/ml) induced the release of histamine and β-glucuronidase from rat peritoneal mast cells. Compound **4** produced a significant and dose-dependent inhibition of mast cell degranulation caused by compound 48/80 (10 μg/ml) (Table **2**). Mepacrine was used in this study as a positive control. FMLP induced the release of β-glucuronidase and lysozyme from rat neutrophils. Compounds **4 – 5** all indicated potent and concentration dependent inhibition of the neutrophil degranulation stimulated with fMLP (Table **3**) and all of them showed stronger inhibitory effects than trifluoperazine.

FMLP/CB and PMA stimulated superoxide anion formation from rat neutrophils. Compound **4** significantly inhibited the superoxide anion formation from rat neutrophils stimulated with PMA in a concentration-dependent manner with an IC_{50} values of 66.3 ± 8.1 μM (Table **4**). Previously we reported that broussochalcone A (**7**), a prenylated chalcone isolated from *Broussonetia papyrifera* (Moraceae), inhibited O_2 consumption in fMLP- and PMA-stimulated rat neutrophils in a concentration-dependent

Table **2** The inhibitory effects of **4** and **5** on the release of β-glucuronidase and histamine from rat peritoneal mast cells stimulated with compund 48/80

Compound	*IC_{50} (μM)[a]*	
	β-glucuronidase	*Histamine*
4	70.0 ± 9.9	70.5 ± 8.9
5	> 30 (38.7 ± 5.6)	> 30 (22.3 ± 1.6)
Mepacrine	22.3 ± 6.2	15.1 ± 3.0

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Average ± s.e.m. (n = 3 – 5) of at least three separate determinations.

Table **3** The inhibitory effects of **4** and **5** on the release of β-glucuronidase and lysozyme from rat neutrophils stimulated with fMLP.

Compound	*IC_{50} (μM)[a]*	
	β-glucuronidase	*lysozyme*
4	6.6 ± 0.5	5.2 ± 0.4
5	5.8 ± 0.5	5.4 ± 0.5
Trifluoperazine	15.2 ± 2.6	13.8 ± 2.2

Table **4** The inhibitory effects of **4** and **5** on superoxide anion formation from rat neutrophils stimulated with fMLP/CB or PMA.

Compound	*IC_{50} (μM)[a]*	
	FMLP/CB	*PMA*
4	> 100 (43.3 ± 7.7)	66.3 ± 8.1
5	> 100 (36.1 ± 1.8)	> 100 (46.6 ± 9.3)
Trifluoperazine	6.3 ± 0.7	5.3 ± 0.5

[a] When 50% inhibition could not be reached at the highest concentration, the % of inhibition is given in parentheses. Average ± s.e.m. (n = 3 – 5) of at least three separate determinations.

manner with IC_{50} values of 70.3 ± 4.9 and 63.9 ± 7.1 μM, respectively [15]. This indicated that **4**, an ester of γ-hydroxymethylallylchalcone, may inhibit O_2 consumption in PMA-stimulated rat neutrophils.

Acknowledgements

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