Natural Product Research: Formerly Natural Product Letters

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gnpl20

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Version of record first published: 27 Jul 2011

To cite this article: Chia-Nan Chen, Che-Jen Hsiao, Shoei-Sheng Lee, Jih-Hwa Guh, Po-Cheng Chiang, Chih-Chiang Huang & Wei-Jan Huang (2012): Chemical modification and anticancer effect of prenylated flavanones from Taiwanese propolis, Natural Product Research: Formerly Natural Product Letters, 26:2, 116-124

To link to this article: http://dx.doi.org/10.1080/14786419.2010.535146

Natural Product Research
Vol. 26, No. 2, January 2012, 116–124

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(*Received 9 November 2009; final version received 17 September 2010*)

Our previous studies demonstrated that eight prenylated flavanones (**1**–**8**), isolated from Taiwanese propolis, were capable of a broad spectrum of biological activities. Among them, nymphaeol A (**3**), nymphaeol B (**4**) and nymphaeol C (**7**), abundant in Taiwanese propolis, exhibited cytotoxicity against cancer cell lines. It therefore seemed interesting to improve their activity *via* a semi-synthetic strategy. In this study, 12 novel prenylated flavanones were synthesised in our laboratory and their activities were assessed for two human prostate cancer cell lines, PC-3 and DU-145, and a human hepatoma cell line, Hep-3B. Of these compounds, **10c**, **11** and **12** showed more potent cytotoxicity against the PC-3 cell line than 5-Fu. Using cytometric analysis followed by double staining with annexin V-FITC and propidium iodide, it was observed that these compounds induced apoptosis as well. This suggests that prenylated flavanones **10c**, **11** and **12** may have anticancer potential for further development.

Keywords: prenylated flavanones; cytotoxicity; PC-3; DU-145; Hep-3B; apoptosis

1. Introduction

Flavanones, widely distributed in the plant kingdom, are found in about 4000 naturally occurring compounds (Rusak, Herwig, & Jutta, 2002), exhibiting a large number of biological effects including anti-inflammatory, antiviral and anticancer properties (Harborne & Williams, 2000). Among various flavanones, prenylated flavanones, characterised by C5 isoprenoid units, attracted interest for their association with several biological targets such as oestrogen receptors (ERs) (Milligan et al., 1999), heat shock proteins (HSP-90) (Hadden, Galam, Matts, & Blagg, 2007), cyclooxygenases (COXs) (Barret, Gordon, & Evans, 1985), P-glycoproteins (PgPs) (Tchamo et al., 2000) and phosphodiesterases (PDEs) (Shin et al., 2002). Prenylated flavanones were reported to inhibit NO production by interfering with inducible nitric oxide synthase (iNOS) expression immediately without the inhibition of iNOS after lipopolysaccharide (LPS)/interferon (IFN)-γ

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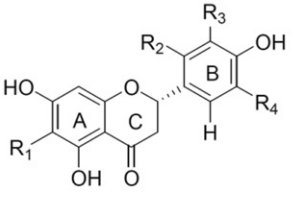
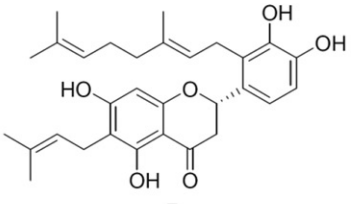
ISSN 1478–6419 print/ISSN 1478–6427 online

http://dx.doi.org/10.1080/14786419.2010.535146
http://www.tandfonline.com

macrophage stimulation (Cerqueira et al., 2008). Pedro et al. reported that a prenylated flavanone, artelastin, demonstrated cytotoxicity against 52 human tumour cell lines ranging from 0.8 to 20.8 μM and showed a dual function as a disrupter of microtubule and inhibitor of DNA replication in cultured human breast cancer cell line MCF-7 (Pedro et al., 2005). In addition, it was reported that 8-prenylated naringenin exhibited strong oestrogenic activity and rapid absorption after oral administration in contrast to the poor bioavailability of general flavanones (Rad et al., 2006). This suggests that the prenyl groups on aromatic rings may increase the lipophilicity and confer a strong affinity to biological membranes (Barron, Balland, Possety, Ravanel, & Desfougeres, 1996).

We previously isolated and structurally characterised three novel prenylated flavanones: propolins A (**1**), B (**2**) and E (**5**) (Chen, Wu, Shy, & Lin, 2003; Chen, Weng, Wu, & Lin, 2004; Chen, Wu, & Lin, 2004) together with five known ones: nymphaeol A (**3**), nymphaeol B (**4**), nymphaeol C (**7**) (Yakushijin, Shibayama, Murata, & Furukawa, 1980), isonymphaeol B (**6**) (Kumazawa et al., 2004) and 3′-geranyl-naringenin (**8**) (Jayasinghe, Rupasinghe, Hara, & Fujimoto, 2006; Kumazawa et al., 2007) from Taiwanese propolis (Figure 1). Most of them exhibited a broad spectrum of biological activities such as anticancer, antioxidant and antimicrobial functions (Chen et al., 2003, Chen, Weng, Wu & Lin, 2004, 2008; Chen, Wu, & Lin, 2007; Huang et al., 2007; Weng et al., 2007). Of these compounds,





7

	R_1	R_2	R_3	R_4
1	H	R	OH	H
2	H	H	OH	R
3	R′	H	OH	H
4	H	R′	OH	H
5	H	H	R	H
6	H	H	OH	R′
8	H	H	R′	H

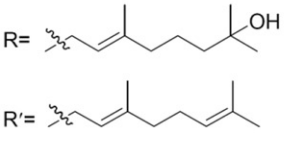
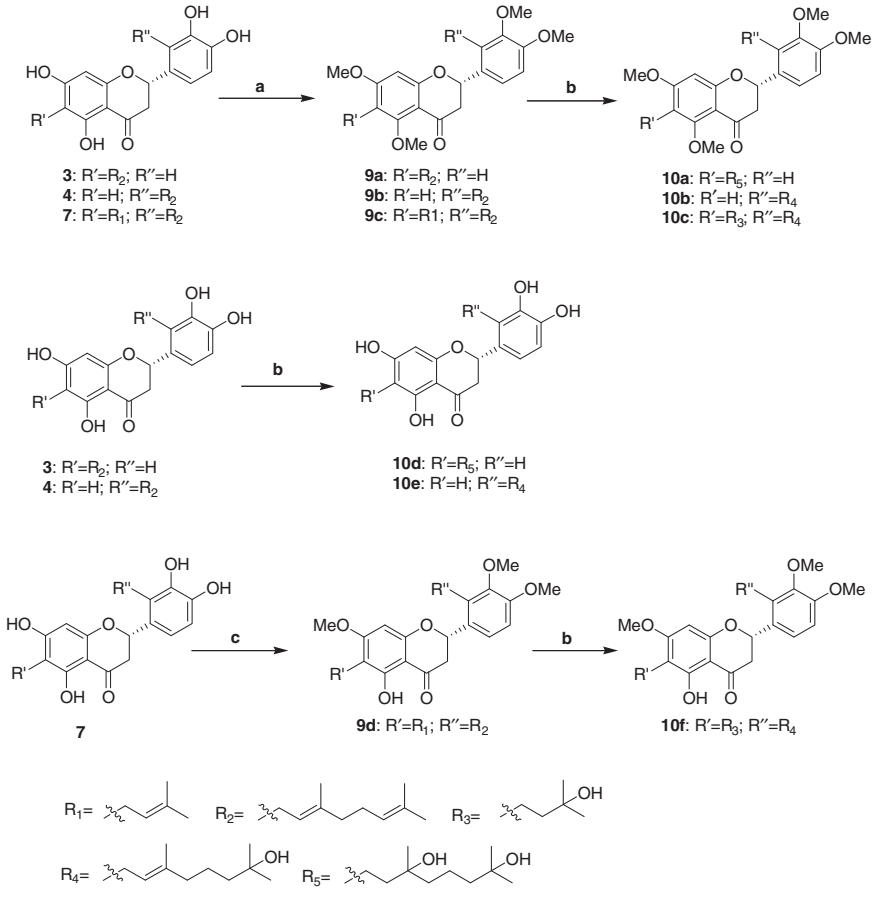


Figure 1. Structures of the prenylated flavanones (**1–8**) isolated from Taiwanese propolis.

nymphaeol A (**3**), nymphaeol B (**4**) and nymphaeol C (**7**), abundant in Taiwanese propolis, showed activity against human melanoma cells. So, it seemed interesting to improve their cytotoxicity *via* a semi-synthetic strategy. The study of the cytotoxic effect of these flavanone derivatives was carried out using three human cancer cell lines of PC-3, DU-145 and Hep-3B. Here we show that compounds **10c**, **11** and **12** exhibited higher cytotoxicity against PC-3 cancer cells than 5-Fu and induced apoptosis, observed through cell cycle progression, followed by double staining with annexin V-FITC and propidium iodide (PI). The following section describes the outcome of these efforts.

2. Results and discussion

Our previous result indicated that the hydration of the terminal olefin on a prenyl side chain of prenylated flavanones can increase their cytotoxicity against melanocyte (Chen, Weng, Wu & Lin, 2004; Chen, Wu & Lin, 2004). Hydration of the terminal olefin either on a prenyl or geranyl group was achieved as described in Scheme 1. Tetramethylation of nymphaeol A (**3**), nymphaeol B (**4**) and nymphaeol C (**7**) in the presence of dimethyl sulphate and potassium carbonate under a general condition afforded **9a–c**, respectively. Treatment of **9b–c** with 49% $H_2SO_{4(aq)}$ using THF as a co-solvent gave the corresponding monohydrated geranyl products **10b–c**. However, under the similar condition, **9a** afforded the dihydrated geranyl product **10a**. The different reactivity of the geranyl group at C-6 of **9a** might be attributed to its steric hindrance, which is less than that of **10b–c**. Direct hydration of nymphaeol A (**3**) and B (**4**) also yielded **10d–e**, respectively. However, hydration of nymphaeol C (**7**) under conditions similar to those described above had failed. In addition, a selective trimethylation of nymphaeol C (**7**) at C-7, C-3′ and C-4′ according to the reported method (Chu, Wu, & Lee, 2004) gave the expected product **9d** (Phommart, P. Sutthivaiyakit, Chimnoi, Ruchirawat, & S. Sutthivaiyakit, 2005) and when followed by acidic hydration afforded **10f**. Increased nymphaeol C (**7**) lipophilicity was achieved as described in Scheme 2. The conversion of two tertiary alcohols of **10c** into carbonate in the treatment of ethyl chloroformate and triethylamine had failed, while leading to the C-ring opened product chalcone **11**. The ^{1}H-NMR of **11** showed the characteristic chemical shift at δ 8.11 (1H, d, $J = 15.4$ Hz) and 7.76 (1H, d, $J = 15.4$ Hz), which supports this structure. The HRESIMS spectrum of **11** showed a quasi-molecular ion $(M+H)^+$ at m/z 585.3412, consistent with that calculated for $C_{34}H_{49}O_8$. Atmospheric hydrogenation of **10c** using palladium on carbon as a catalyst produced saturated product **12**. It is noted that **12** has a R_f similar to that of **10c**. The HREIMS spectrum of **12** showed a molecular ion $(M)^+$ at m/z 586.3506, consistent with the expected formula $C_{34}H_{50}O_8$. Tetra-acetylation of nymphaeol B (**4**) and **C** (**7**) with acetic anhydride and pyridine provided **13a** (Yakushijin et al., 1980) and **13b**, respectively. Investigation of the effect of the prenyl side chain on flavanone skeletons was conducted as described in Scheme 2. Ozonolysis of **9c** and subsequent treatment of thiourea split the olefins into the dialdehyde **14** (Huang, Chen, & Lee, 2003). The ^{1}H-NMR of **14** showed two additional signals at δ 9.73 (1H, s) and 9.63 (1H, s) compared to **9c**, indicating the presence of aldehyde. The HREIMS spectrum of **14** showed a molecular ion $(M)^+$ at m/z 428.1469, consistent with the expected formula $C_{23}H_{24}O_8$. Sodium chlorite

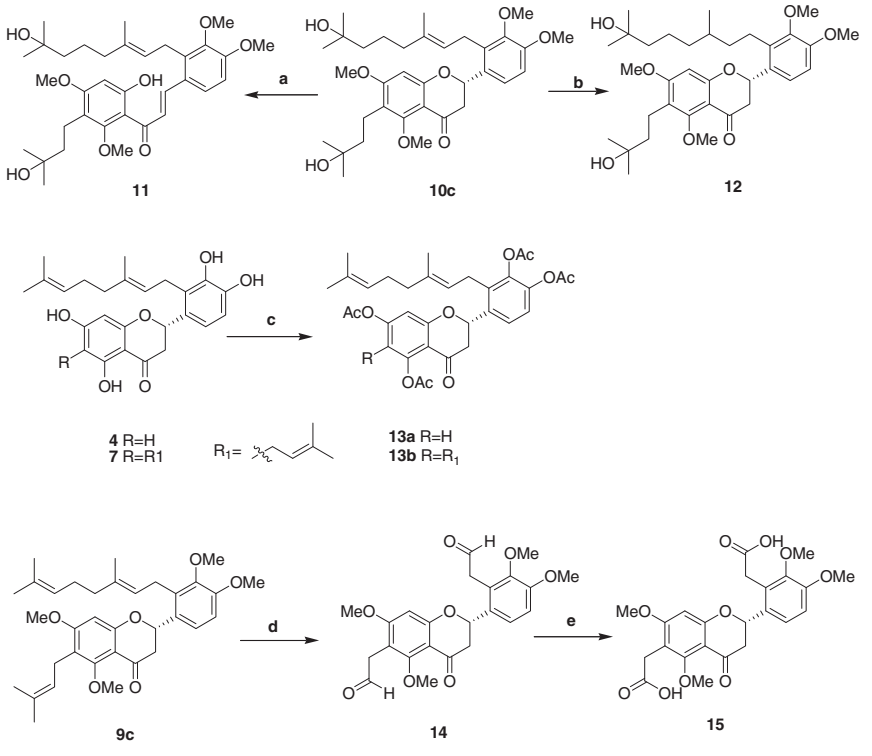


Reagents and conditions: (**a**) K_2CO_3, DMS, acetone, Δ, 24 h (**b**) H_2SO_4-THF, rt; (**c**) K_2CO_3, DMS, acetone, Δ, 6h;

Scheme 1. Syntheses of prenylated flavanones **10a-f**.

oxidation (Andrus, Asgari, & Sclafani, 1997) of **14** gave the diacid **15**. The HREIMS spectrum of **15** showed a molecular ion $(M)^+$ at *m/z* 460.1382, consistent with the expected formula $C_{23}H_{24}O_{10}$. The physical data for all compounds are provided as supplementary material (available online only).

The cytotoxic effect of flavanones was assessed against two human prostate cancer cell lines, PC-3 and DU-145, and a human hepatoma cell line, Hep-3B, using 5-Fu as a control compound (Supplementary Table S1 – online only). It indicated that most compounds were not capable of selective cytotoxicity against these three cancer cells. Of these compounds, compounds **10c**, **11** and **12** showed higher cytotoxic effect on PC-3 than 5-Fu. Compared to **7**, compound **10c** displayed stronger potency, suggesting that this two-step chemical modification made a good contribution to activity, whereas it showed a marginal effect on compounds **10a–b**. Compared to **12**, compound **10c** showed selective cytotoxicity towards PC-3, which might be attributable to the importance of C2′–C3′ double bond on the geranyl side chain. The stronger cytotoxicity of compound **10c** compared to **10b** might be



Reagents and conditions: (**a**) $ClCO_2Et$, Et_3N, toluene, Δ; (**b**) H_2, Pd-C, rt; (**c**) $(Ac)_2O$, pyr, rt; (**d**)**1**)O_3, CH_2Cl_2-MeOH, -40°C, **2**)$(NH_2)_2CS$, 0°C~rt; (**e**) $NaClO_2$, NaH_2PO_4, rt;

Scheme 2. Syntheses of prenylated flavanones **11–12, 13a–b, 15**.

attributed to the presence of a prenyl group at C6 position. The decrease in activity of compound **10e** compared to **10b** indicated the importance of methoxy groups in the hydrated nymphaeol B (**4**). The weaker activity of compounds **13a–b** suggested that acetyl groups showed a marginal effect on nymphaeol B (**4**) and C (7).

In order to investigate the effect of compounds **10c**, **11** and **12** and the reference compound 5-Fu on cell cycle progression, a flow cytometric analysis was performed against PC-3 cells at time periods 12 and 24 h, respectively (Supplementary Figure S1 – online only). With a treatment at 30 μM, compounds **10c**, **11** and **12** increased the sub-G1 population by 10-, 3- and 7-fold, respectively, at 12 h. Furthermore, after incubation for 24 h, compounds **10c**, **11** and **12** significantly increased the sub-G1 population by 17-, 4-, and 8-fold, respectively. However, 5-Fu had only a slight effect on cell cycle progression at each time period. In order to investigate whether compounds **10c**, **11** and **12** could induce cell death related to apoptosis, a cytofluorometric analysis using annexin V-FITC and PI double staining was carried out at time periods of 12 and 24 h. Annexin V (+)/PI (−) and annexin V (+)/PI (+) showed that cells were at the early and the late stages of apoptosis, respectively (Supplementary Figure S2 – online only). At a concentration of 30 μM, compounds **10c**, **11** and **12** increased the population of annexin V (+)/PI (−) compared to

annexin V (+)/PI (+) by 27.48%, 40.32% and 16.32% compared to 6.84%, 4.56% and 9.18%, respectively, at 12 h. In addition, treatment with compounds **10c**, **11** and **12** for 24 h, resulted in an increased population of annexin V (+)/PI (−) compared to annexin V (+)/PI (+) by 50.73%, 57.72% and 50.49% compared to 30.26%, 19.96% and 20.14%, respectively. This suggested that compounds **10c**, **11** and **12** may induce apoptosis in a time-dependent manner. In conclusion, this study reveals the cytotoxicity of prenylated flavanones derived from three isolated Taiwanese propolis towards several cancer cell lines and further investigates their effect on cancer cell deaths, which may be useful for therapeutic applications. Further experiments to study the mechanisms are being undertaken in this laboratory.

3. Experimental

3.1. *General*

The physical and spectral data of prepared compounds were obtained from the following instruments: Fisher–Johns melting point apparatus (uncorrected); JEOL JMX-HX110 (HREIMS and HRFABMS), JMS-SX102A (EIMS and FABMS) and Finnigan MAT 95S (HRESIMS and ESIMS) mass spectrometers; and Bruker AV-400 and AV-500 spectrometers using solvent peak as reference standard.

3.2. *Nymphaeol A (3), B (4) and C (7)*

Further information about nymphaeol A (**3**), B (**4**) and C (**7**), such as propolis origin, extraction and isolation is supplied as supplementary material (available online only).

3.3. *General procedure for preparation of 9a–c*

To the mixtures of prenylated flavanones (**3**, **4** or **7**, 0.31 mmol), K_2CO_3 (431 mg, 3.1 mmol) and acetone (15 mL), Me_2SO_4 (0.25 mL, 2.48 mmol) was added and the resulting final solution was heated and refluxed under N_2 for 24 h. After removal of the organic solvent, the residue was dissolved in CH_2Cl_2 (50 mL) and washed with distilled H_2O (50 mL × 3). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue obtained was purified by a silica gel column (EtOAc: *n*-hexane = 1:6) to give **9a–c**.

3.4. *General procedure for preparation of (10a–f)*

To a solution of (**9a–d** or **3** or **4**, 0.17 mmol) in THF (6 mL), 49% H_2SO_4 (4 mL) was added drop-wise in an ice bath. After complete addition, the reaction mixture was stirred at room temperature (RT) for 8 h and then diluted with H_2O. The aqueous layer was extracted with CH_2Cl_2 (50 mL × 3). The combined organic layers were dried over Na_2SO_4 and evaporated under reduced pressure to give a residue, which was purified by a silica gel column (EtOAc: *n*-hexane = 1:6∼1:1) to give the pure oil **10a–f**.

3.5. *3′,4′,7-O-Trimethylnymphaeol C (9d)*

To the mixture of **7** (300 mg, 0.61 mmol), K_2CO_3 (252 mg, 1.83 mmol) and acetone (20 mL), Me_2SO_4 (0.20 mL, 2.1 mmol) was added and the resulting solution was heated to reflux under N_2 for 6 h. After removal of acetone, the residue was dissolved in CH_2Cl_2 (50 mL) and washed with distilled H_2O (50 mL × 3). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by a silica gel column (EtOAc: *n*-hexane = 1:6) to give **9d** (120 mg, yield: 37%).

3.6. *3-(3-Hydroxy-3-methylbutyl)-2′-(7-hydroxy-3,7-dimethyloct-3-enyl)-6-hydroxy- 3′,4′,2,4-tetramethoxychalcone (11)*

To a solution of **9c** (100 mg, 0.17 mmol) in toluene (10 mL), ethyl chloroformate (0.4 mL) and pyridine (0.1 mL) was added and the resulting solution was heated to 120°C under N_2 for 48 h. After removal of the solvent, the residue was purified by a silica gel column (EtOAc: *n*-hexane = 1:1) to afford **11** (75 mg, yield: 75%).

3.7. *6-(3-Hydroxy-3-methylbutyl)-2′-(7-hydroxy-3,7-dimethyloctyl)-3′,4′,5,7-tetra methoxyflavanone (12)*

To the mixture of **9c** (100 mg, 0.17 mmol) and EtOH (10 mL), Pd-C (30 mg) was added, and the resulting solution was stirred at RT under atmospheric H_2 overnight. The reaction mixture was filtered through celite and concentrated under reduced pressure. The residue was purified by a silica gel column (EtOAc: *n*-hexane = 1:6) to give pure oil **12** (80 mg, yield: 79%).

3.8. *General procedure for the preparation of 13a–b*

To a solution of **7** or **4** (0.61 mmol) in pyridine (4 mL), acetic anhydride (2 mL) was added and the reaction mixture was stirred at RT for 6 h. The reaction mixture was diluted with EtOAc (25 mL) and washed with 0.1 N HCl (10 mL × 3). The organic layer was dried over Na_2SO_4 and evaporated under reduced pressure. The residue was purified by a silica gel column (EtOAc: *n*-hexane = 1:5) to yield pure **13a–b**.

3.9. *2′,6-Di-(2-oxo-ethyl)-3′,4′,5,7-tetramethoxyflavanone (14)*

The mixture of **9c** (5.00 g, 9.12 mmol), CH_2Cl_2 (100 mL) and MeOH (100 mL) was cooled to −20°C and then treated with ozone until all of the starting material was exhausted. After addition of $(NH_2)_2CS$ (1.04 g, 13.68 mmol) to the above solution, the reaction mixture was warmed to RT for 30 min, washed with 1% $NaHCO_3$ (50 mL × 3) and dried over Na_2SO_4. The organic solvents were removed under reduced pressure to give the residue, which was subjected to a silica gel column (EtOAc: *n*-hexane = 1:1) to yield pure **14** (2.93 g, yield: 74%).

3.10. *2′,6-Di-(2-carboxylethyl)-3′,4′,5,7-tetramethoxyflavanone (15)*

Compound **14** (2.31 g, 4.40 mmol) was dissolved in *t*-BuOH (15 mL), and then 2-methyl-2-butene (3 mL) and 11 mL of an aqueous solution of $NaClO_2$ (3.78 g, 44 mmol) and NaH_2PO_4-H_2O (1.52 g, 10.93 mmol) were added drop-wise over a period of 20 min. The reaction mixture was stirred at RT overnight. The *t*-BuOH was removed under reduced pressure and then the residue was partitioned between CH_2Cl_2 (50 mL) and water (30 mL × 3). The CH_2Cl_2 layer was dried over Na_2SO_4 and evaporated under reduced pressure to give a residue, which was purified by a silica gel column (EtOAc: *n*-hexane = 1:1) to give **15** (1.64 g, yield: 81%).

Supplementary material

Physical data for all compounds and further experimental details regarding nymphaeol A (**3**), B (**4**) and C (**7**) are available online, alongside Supplementary Table S1 and Supplementary Figures S1–S2.

Acknowledgements

We gratefully acknowledge the financial support from the Ministry of Economics and the National Science Council, Taiwan, Republic of China under grants IZ-950295 and NSC97-2320-B-038-010-MY3, respectively.

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