Isolation and Identification of New Polymethoxyflavonoids from Dancy Tangerine Leaves

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Ten highly oxygenated flavonoids, including four new compounds, were isolated from the leaves of Dancy tangerine. Six were identified as 5,6,7,3′,4′-pentamethoxyflavone (sinensetin) (**1**), 5,6,7,8,4′-pentamethoxyflavone (tangeretin) (**2**), 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin) (**3**), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**4**), 5,7-dihydroxy-6,8,3′,4′-tetramethoxyflavone (**5**), and 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5-*O*-desmethylnobiletin) (**6**) by direct comparison with authentic samples, comparing their spectroscopic data with those reported in the literature or by simply interpreting their spectral data. The new compounds were elucidated as 5,7,8,3′,4′- pentamethoxyflavanone (**7**), 5,6,7,8,3′,4′-hexamethoxyflavanone (**8**), 7-chloro-3,5,6,8,3′,4′-hexamethoxyflavone (**9**), and 7-chloro-3,5,6,8,4′-pentamethoxyflavone (**10**) on the basis of chemical and spectroscopic studies.

Keywords: *Citrus; Dancy tangerine leaves; polymethoxyflavone; chloride polymethoxyflavone; polymethoxyflavanone*

INTRODUCTION

It is generally accepted in many cultures that citrus fruits and juices are beneficial to human health. Citrus fruits and juices are excellent sources of vitamin C, which may be one reason for the health benefits attributed to these fruit. For some time, research on the natural chemical components of citrus has been carried out, and data on the health benefits of citrus phytochemical components continues to accumulate. Polymethoxylated flavonoids (PMFs) are an interesting group of bioactive compounds, present as minor components in crude extracts of citrus bioflavonoids. Robbins (1974) found the PMFs were much more active than the flavanone glycosides in their antiadhesive effects on red blood cells and platelets. These compounds have also been shown by Middleton and co-workers (1982, 1987) to have anti-inflammatory properties and inhibit histamine release to reduce allergic reactions. Bracke and co-workers (1994) found tangeretin to be effective in the inhibition of human breast carcinoma cell invasion. The mode of action was reported to be an inhibition of the breakdown of intercellular cell adhesiveness, thus reducing the motility and spread of cancer cells. Nobiletin and tangeretin were also found by Bracke and co-workers (1991) to have the most potent anti-invasive action of all flavonoids tested on invasion of mouse MO4 tumor cells in normal tissue. Kandaswami and co-workers (1991) also found nobiletin and tangeretin inhibited human squamous cell carcinoma at all concentrations. Lichius and co-workers (1994) found 5,3′-dihydroxy-3,6,7,8,4′-pentamethoxyflavone showed activity against (KB) human nasopharyngeal carcinoma cells ($IC_{50} = 0.04\ \mu g/mL$) and inhibited tubulin assembly into microtubules ($IC_{50} = 12\ \mu M$).

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Citrus fruits and juices are the only commonly consumed foods that contain PMFs. The studies revealed that PMFs have unique biological activities, which have greatly encouraged the discovery of numerous citrus PMFs in recent years (Saxena et al., 1994; Berahia et al., 1994; Kinoshita et al., 1997; Lichius et al., 1994; Machida et al., 1989; Chang et al., 1990; Mizuno et al., 1987; Sugiyama et al., 1993). More recently, this laboratory has isolated and characterized eight known and two new PMFs from cold pressed Dancy tangerine peel oil solids (Chen et al., 1997). In this paper, we report the isolation and structure determination of four novel PMFs from Dancy tangerine leaves using ^1H and ^{13}C NMR and HRMS; included are two chloro-PMFs, which are the first ones discovered in nature.

MATERIALS AND METHODS

General Chromatography. Normal phase TLC was performed on high-performance silica gel plates (Whatman 4870-400), which were visualized by observation under a multiband UV lamp (UV-254/366 nm) and/or by spraying with H_2SO_4/ethanol (1:4) followed by charring with a heat gun. Reversed phase TLC was performed on octadecyl (C_{18}) plates (Whatman 4803-600), which were observed under a multiband UV lamp. Flash chromatography was performed on silica gel (Baker Analyzed 40μ 10t G42353).

HPLC. Preparative HPLC was carried out on an LDC analytical system (Thermo Separation Products Inc., Riviera Beach, FL) consisting of a constaMetric 3200 pump, a model IV refractive index detector, a Rheodyne model 7125 injection valve, and a Gilson FC 203B fraction collector (Gilson Medical Electronics, Middleton, WI). The column used was a Rainin (Woburn, MA) Dynamax 60A column (8 μm C_{18}, 21.4 mm inside diameter × 25 cm) coupled to a Dynamax 60A guard column (8 μm C_{18}, 21.4 mm inside diameter × 5 cm).

Mass and Nuclear Magnetic Resonance Spectroscopy. The high-resolution mass spectral analyses were carried out on a Finnigan MAT 95Q magnetic sector mass spectrometer (Finnigan MAT, San Jose, CA), with electron ionization at 70 eV. ^1H NMR and ^{13}C NMR spectra were recorded on a General

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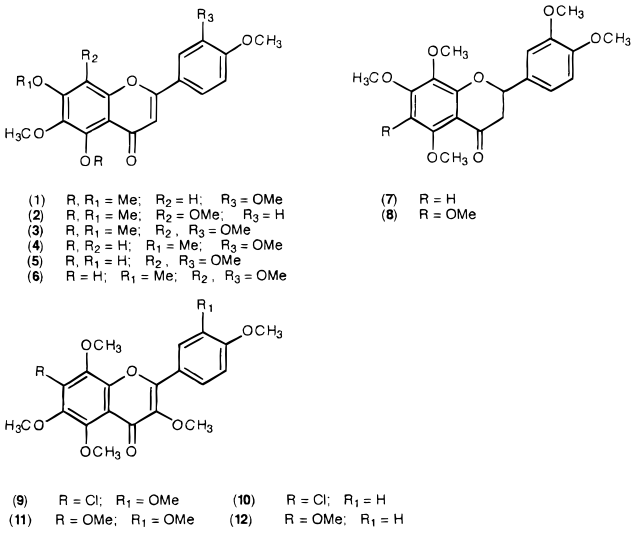

Figure 1. Structures of compounds **1**−**12**.

Electronic QE-300 spectrometer; chemical shifts in $CDCl_3$ are reported relative to internal TMS.

Extraction and Isolation. Six hundred grams of air-dried leaves of Dancy tangerine was extracted with methanol/chloroform (1:1), and the concentrated extract was initially separated using vacuum flash silica gel chromatography, with the following successive solvent: isooctane, isooctane/2-propanol (9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9), 2-propanol, and methanol. Fractions of 300 mL each were collected and combined into 12 pooled fractions on the basis of their TLC composition. Those 12 fractions were each subjected to flash C_{18} column chromatography, with ethanol/water (60% EtOH to 95% EtOH). Compounds **2**, **4**, and **6** were obtained directly from the flash C_{18} column. The remaining fractions were then subjected to C_{18} preparative HPLC (prep-HPLC) purification, with either system A (8:2 methanol/water) or system B (9:1 methanol/water).

RESULTS AND DISCUSSION

The methanol/chloroform (1:1) extract of Dancy tangerine leaves was separated by a combination of silica gel, C_{18} flash column chromatography, and C_{18} preparative HPLC to afford 10 compounds (**1**−**10**, Figure 1) in crystalline form. The spectroscopic data showed that most of these compounds were highly oxygenated flavone derivatives. Compounds **1**−**6**, **9**, and **10** were determined to be polymethoxylated flavones, whereas compounds **7** and **8** were determined to be polymethoxylated flavanones. Compounds **2**, **3**, **5**, **6**, and **8** had fully oxygenated A-rings (5,6,7,8-tetraoxygenated); compounds **1**, **4**, and **7** had trioxygenated A-rings, and compounds **9** and **10** had a chlorine substituent attached to the A-ring. All of the compounds possessed 4′-oxygenated or 3′,4′-dioxygenated B-rings.

Compounds **1**−**3** were readily identified as the widely known citrus flavones sinensetin, tangeretin, and nobiletin by comparing their spectral values with those of the corresponding authentic compounds reported in the literature (Chen et al., 1997). For compounds **4**−**6**, the presence of a chelated 5-OH was indicated by a sharp singlet at δ 12.58 in the ^{1}H NMR that disappeared on D_2O exchange. Spectral data of compounds **4** and **6** were in good agreement with those of 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (Sugiyama et al., 1993) and 5-*O*-desmethylnobiletin (Kinoshita et al., 1996), respectively. Compound **5** had a molecular weight $[M]^+$ of 374 for $C_{19}H_{18}O_8$ based on mass spectral data, corresponding to a flavone containing four methoxyl groups and two hydroxy groups. The MS fragments (due to retro-Diels−Alder cleavage) present at 212 (15.14) and 162 (4.30) indicated the two methoxyl groups and two hydroxy groups are attached to the A-ring and the other two methoxyl groups were attached to the B-ring. The ^{1}H NMR spectrum of compound **5** showed that three ABX type aromatic proton signals at δ 7.52 (dd, J = 8.5 and 2.4 Hz), 7.40 (d, J = 2.4 Hz), and 7.10 (d, J = 8.5 Hz) were characteristic of meta and ortho coupling of H-6′, H-2′, and H-5′, respectively, which means the B-ring is 3′,4′-methoxylated. The presence of significant bathochromic shifts by NaOMe shift reagents in the UV spectrum revealed the presence of a free hydroxyl at the 7-position. From the above evidence, the structure of compound **5** was elucidated as 5′,7′-dihydroxy-6,8,3′,4′-tetramethoxyflavone. This compound has been reported as hymenoxin (Thomas et al., 1967); in this paper, we provide complete spectral data and rational assignment.

The ^{1}H NMR spectrum of compound **7** showed three one-proton doublet of doublets at δ 5.35 (J = 3.0 and 13.0 Hz), 3.02 (J = 13.0 and 17.5 Hz), and 2.75 (J = 3.0 and 13.0 Hz), indicating that **7** had a flavanone skeleton; the typical flavanone signals were assigned to H-2, H-3_{ax}, and H-3_{eq}, respectively (Iinuma et al., 1993; Lin et al., 1995). Five methoxyl signals were observed at δ 3.8−4.0; the aromatic protons at δ 6.90 (J = 9.0 Hz), 7.00 (dd, J = 9.0 and 2.5 Hz), and 7.02 (d, J = 2.5 Hz) could be assigned to H-5′, H-6′, and H-2′ of a 3′,4′-dimethoxylated B-ring of a flavonoid. The A-ring aromatic signal at 6.37 was assigned to H-6 of a flavanone. The EI-mass spectrum of **7** showed a $[M]^+$ at *m*/*z* 374 (base peak), and significant peaks at *m*/*z* 344, 211, 210, and 164 were attributed to retro-Diels−Alder fragmentation which supported a structure of a flavanone with a dimethoxylated B-ring and a trimethoxylated A-ring. On the basis of the above evidence, compound **7** was characterized as 5,7,8,3′,4′-pentamethoxyflavanone.

The basic structural similarity of the B- and C-ring for compounds **7** and **8** was suggested by the mutual comparison of ^{1}H NMR spectral data. The flavanone skeleton protons for both compounds were recorded as three one-proton double doublets for H-2, H-3_{ax}, and H-3_{ex} on the C-ring and the ABX type protons for H-2′, H-5′, and H-6′ on the B-ring. Compound **8**, $C_{21}H_{24}O_8$ from EI-MS, differed from **7** in the absence of one A-ring aromatic proton (δ 6.37 with ^{1}H NMR for **7**) and the presence of one more methoxyl group (δ 4.05 with ^{1}H NMR for **8**) which suggested that the A-ring of compound **8** is fully methoxylated. Thus, **8** was determined to be 5,6,7,8,3′,4′-hexamethoxyflavanone.

The ^{1}H NMR spectrum of **9** had six methoxyl signals observed at δ 4.12−3.97 and ABX type coupled aromatic protons at δ 7.68 (dd, J = 9.0 and 2.2 Hz), 7.58 (d, J = 2.2 Hz), and 7.02 (d, J = 9.0 Hz) which could be assigned to H-6′, H-2′, and H-5′, respectively. Signals in the ^{13}C NMR spectrum (δ 61.7−62.3) were characteristic for four aromatic methoxyl groups having substituents in both ortho positions (Panichpol et al., 1978; Roitman et al., 1985) and suggested that those methoxyl groups are located on A-ring and C-3. EI-mass spectrum of **9** gave $[M]^+$ at *m*/*z* 436. An isotope M + 2 peak was observed; the ratio of the M + 2 peak to the molecular ion peak was 1:3 which suggested a chlorine atom in compound **9**. HRMS established the molecular formula of **9** as $C_{21}H_{21}ClO_8$ from the experimentally measured masses

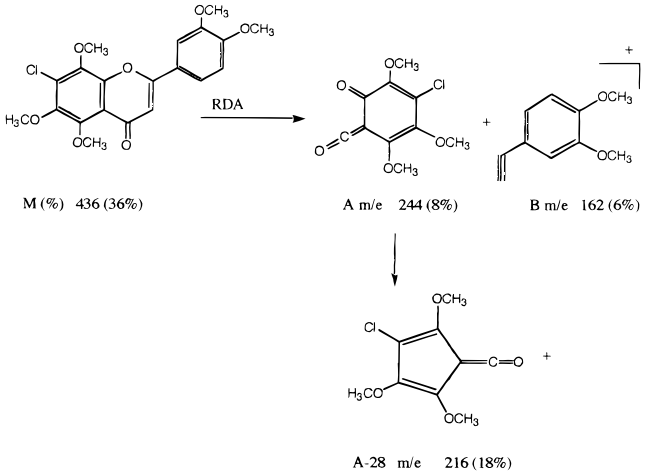

Figure 2. Retro-Diels–Alder fragmentation of compound **9**.

Table 1. ^{13}C NMR Data of Compounds 9–12 (δ)[a]

C	compound **11**[b]	compound **9**	compound **12**[c]	compound **10**
C-2	151.1	151.7	151.3	151.7
C-3	140.8	141.0	140.7	143.8
C-4	173.9	172.3	174.0	173.5
C-5	143.9	144.7	143.9	144.6
C-6	**137.8**	**158.1**	**138.0**	**156.8**
C-7	**151.3**	**117.3**	**153.5**	**117.3**
C-8	**137.8**	**157.2**	**138.0**	**157.7**
C-9	148.2	149.2	148.2	148.5
C-10	115.1	113.4	115.2	114.0
C-1′	123.5	123.9	123.4	123.8
C-2′	110.9	110.9	130.0	130.9
C-3′	148.8	147.0	114.2	114.7
C-4′	153.0	151.8	161.5	161.9
C-5′	111.0	111.1	114.2	114.6
C-6′	121.9	123.1	130.0	130.8
OCH_3	62.3	62.3	62.4	62.3
	61.9	62.2	62.1	61.9
	61.8	61.9	61.9	61.8
	61.7	61.7	61.7	61.6
	59.9	(3,5,6,8)	58.0	(3,5,6,8)
	(3,5,6,7,8)	56.1	(3,5,6,7,8)	55.4
	56.0	56.0	55.0	(4′)
	55.9	(3′,4′)	(4′)	
	(3′,4′)			

[a] Solvent was $CDCl_3$. [b] Data for ^{13}C NMR from Chen et al. (1997). [c] Data for ^{13}C NMR from Sugiyama et al. (1993).

of ^{35}- and ^{37}Cl isotopes; they are 436.091 (calcd 436.092) and 438.089 (calcd 438.090), respectively. The fragments of the retro-Diels–Alder pathway indicated that the chlorine is attached to the A-ring (Figure 2). Comparing the ^{13}C NMR spectrum of known compound 3,5,6,7,8,3′,4′-heptamethoxyflavone (**11**) with that of **9** shows only three major changes of chemical shifts were found in C-6, C-7, and C-8 (Table 1); the symmetrical shifts of C-6 and C-8 indicated that the chlorine is attached to C-7. Thus, compound **9** was identified as 7-chloro-3,5,6,8,3′,4′-hexamethoxyflavone.

NMR and HRMS data of compound **10** suggested that it is also a 7-chloropolymethoxylated flavone. Compound **10** has only one methoxyl group attached to the B-ring at C-4′, indicated in the ^{1}H NMR spectrum by a pair of two-proton ortho coupled doublets, typical of a para-substituted benzene ring. The A-ring chlorine attachment was determined by comparing the ^{13}C NMR spectrum of 3,5,6,7,8,4′-hexamethoxyflavone **12** (Sugiyama et al., 1993) with that of compound **10** (Table 1). The chemical structure of compound **10** was thus established as 7-chloro-3,5,6,8,3′-pentamethoxyflavone.

Four new natural products were discovered in Dancy tangerine leaf extracts. The chlorinated PMFs (**9** and **10**) are a very interesting discovery, because chlorinated flavonoid are very rare; these are the first chlorinated flavonoids found in citrus. To prove these chlorinated PMFs are real natural products, a variety of single and mixed forms of PMFs were treated with methanol/chloroform (1:1), to imitate the chloroform extraction conditions (varied concentrations, different temperatures, and various lengths of time). After those compounds were evaluated by HPLC and mass spectra, no artificial chlorinated PMFs were found.

CHEMICAL COMPOUND LISTING

5,7-Dihydroxy-6,8,3′,4′-tetramethoxyflavone (5): yellow needles (MeOH); mp 210–211 °C; t_R 21.5 min (prep-HPLC with system A); R_f 0.45 (TLC with system A); UV (MeOH) λ_{max} 364, 274 (sh), 206 nm; UV (MeOH and NaOMe) λ_{max} 397, 330 (sh), 208 nm; ^{1}H NMR δ 1.5 (1H, s, OH-5, D_2O exchangeable), 7.52 (1H, dd, J = 8.5 and 2.4 Hz, H-6′), 7.40 (1H, d, J = 2.4 Hz, H-2′), 7.10 (1H, d, J = 8.5 Hz, H-5′), 6.60 (1H, s, H-3), 6.05 (1H, br, OH-7, D_2O exchangeable), 4.10 (3H, s, OMe), 3.99 (3H, s, OMe), 3.97 (3H, s, OMe), 3.94 (3H, s, OMe); ^{13}C NMR δ 160.2 (C-2), 106.8 (C-3), 177.4 (C-4), 150.0 (C-5), 134.0 (C-6), 148.9 (C-7), 137.5 (C-8), 144.5 (C-9), 110.4 (C-10), 123.7 (C-1′), 114.6 (C-2′), 145.5 (C-3′), 149.5 (C-4′), 110.5 (C-5′), 121.5 (C-6′), 62.3 (OMe), 61.7 (OMe), 56.1 (OMe), 56.0 (OMe); EIMS *m/z* (relative intensity) 374 (M^+, 70.66), 359 (M − CH_3, 84.97), 343 (M − OCH_3, 4.00), 212 (15.14), 183 (21.75), 162 (4.30).

5,7,8,3′,4′-Pentamethoxyflavanone (7): pale yellow needles (MeOH); mp 177–178 °C; t_R 14.5 min (prep-HPLC with system B); R_f 0.23 (TLC with system B); ^{1}H NMR δ 7.02 (1H, d, J = 2.5 Hz, H-2′), 7.00 (1H, dd, J = 9.0 and 2.5 Hz, H-6′), 6.90 (1H, d, J = 9.0 Hz, H-5′), 6.37 (1H, s, H-6), 5.35 (1H, dd, J = 13.0 and 3.0 Hz, H-2), 3.02 (1H, dd, J = 17.5 and 13.0 Hz, H-3_{ax}), 2.75 (1H, dd, J = 13.0 and 3.0 Hz, H-3_{eq}), 3.95 (3H, s, OMe), 3.92 (3H, s, OMe), 3.90 (3H, s, OMe), 3.87 (3H, s, OMe), 3.82 (3H, s, OMe); ^{13}C NMR δ 79.0 (C-2), 45.6 (C-3), 189.2 (C-4), 156.2 (C-5), 89.5 (C-6), 157.7 (C-7), 132.0 (C-8), 156.8 (C-9), 107.5 (C-10), 131.6 (C-1′), 114.7 (C-2′), 147.0 (C-3′), 148.2 (C-4′), 112.1 (C-5′), 118.0 (C-6′), 61.4 (OMe), 56.5 (OMe), 56.2 (OMe), 56.1 (OMe), 56.0 (OMe); EIMS *m/z* (relative intensity) 374 (M^+, 100), 343 (M − OCH_3, 2.51), 237 (4.50), 211 (10.5), 210 (63.76), 195 (62.17), 167 (53.63), 164 (50.44).

5,6,7,8,3′,4′-Hexamethoxyflavanone (8): pale yellow needles (MeOH); mp 164–165 °C; t_R 15.2 min (prep-HPLC with system B); R_f 0.26 (TLC with system B); ^{1}H NMR δ 7.02 (1H, d, J = 2.5 Hz, H-2′), 7.00 (1H, dd, J = 9.0 and 2.5 Hz, H-6′), 6.90 (1H, d, J = 9.0 Hz, H-5′), 5.40 (1H, dd, J = 13.0 and 3.0 Hz, H-2), 3.05 (1H, dd, J = 17.5 and 13.0 Hz, H-3_{ax}), 2.84 (1H, dd, J = 13.0 and 3.0 Hz, H-3_{eq}), 4.05 (3H, s, OMe), 3.90–3.88 (9H, 3 x OMe), 3.85–3.82 (6H, 2 x OMe); ^{13}C NMR δ 78.0 (C-2), 45.6 (C-3), 190.2 (C-4), 151.2 (C-5), 139.5 (C-6), 154.7 (C-7), 141.6 (C-8), 150.5 (C-9), 112.0 (C-10), 131.4 (C-1′), 114.3 (C-2′), 146.7 (C-3′), 148.1 (C-4′), 112.1 (C-5′), 118.0 (C-6′), 62.0 (OMe), 61.8 (OMe), 61.7 (OMe), 60.8 (OMe), 56.1 (OMe), 56.0 (OMe); EIMS *m/z* (relative intensity) 404 (M^+, 38.50), 374 (M − OCH_3, 4.50), 241 (9.62), 240 (100), 225 (61.75), 210 (10.85), 197 (43.23), 164 (36.01), 149 (17.41).

7-Chloro-3,5,6,8,3′,4′-hexamethoxyflavone (9): yellow needles (MeOH); mp 145–146 °C; t_R 15.8 min (prep-HPLC with system B); R_f 0.35 (TLC with system B); ^{1}H NMR δ 7.68 (1H, dd, J = 9.0 and 2.2 Hz, H-6′), 7.58 (1H, d, J = 2.2 Hz, H-2′), 7.02 (1H, d, J = 9.0 Hz, H-5′), 4.12 (3H, s, OMe), 4.0–3.97 (15H, 5 x OMe); EIMS *m/z* (relative intensity) 436 (M^+, 35.68), 438 (M + 2, 12.01), 421 (M − 15, 100), 244 (8.50), 216 (18.23), 165 (14.02); HRMS *m/z* (relative intensity) 436.091 (M^+, 25.06), calcd for $C_{21}H_{21}ClO_8$ 436.092, 438.089 (M + 2, 8.24), calcd for ^{37}Cl 438.090.

7-Chloro-3,5,6,8,4′-pentamethoxyflavone (10): yellow needles (MeOH); mp 161–162 °C; t_R 14.7 min (prep-HPLC with system B); R_f 0.28 (TLC with system B); ^{1}H NMR δ 7.94 (2H,

d, J = 9.0 Hz, H-2′ and H-6′), 7.02 (2H, d, J = 9.0 Hz, H-3′ and H-5′), 4.10 (3H, s, OMe), 3.95 (3H, s, OMe), 3.94 (6H, 2 x OMe), 3.89 (3H, s, OMe); EIMS *m/z* (relative intensity) 406 (M^+, 36.42), 408 (M + 2, 12.40), 391 (M − 15, 100), 348 (9.01), 197 (29.1), 135 (26.25); HRMS *m/z* (relative intensity) 406.082 (M^+, 28.04), calcd for $C_{20}H_{19}ClO_7$ 406.082, 408.077 (M + 2, 9.21), calcd for ^{37}Cl 408.079.

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