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Monoacylglycerol from *Punica granatum* Seed Oil

MAJEKODUNMI O. FATOPE,*,† SUAD KHAMIS S. AL BURTOMANI,† AND YOSHIO TAKEDA‡

Chemistry Department, College of Science, Sultan Qaboos University, P.O. Box 36, Postal Code 123, Al-Khod, Sultanate of Oman, and Faculty of Integrated Arts and Sciences, The University of Tokushima, Minamijosanjimacho 1-Chome Tokushima 770-8502, Japan

The seeds of *Punica granatum*, known as *hap roman* in the Arabian Peninsula, are commonly eaten as a dessert. As part of an ongoing project to find nonnutritional natural products which have health benefits, or that can be exploited to protect crops, the chloroform-soluble extract of the fermented seeds of *P. granatum* was found to be rich in 1-*O*-*trans,cis,trans*-9,11,13-octadecatrienoyl glycerol (**1**). The seed oil is not lethal to brine shrimp larvae. 1-*O*-isopentyl-3-*O*-octadec-2-enoyl glycerol (**2**) and the known *cis*-9-octadecenoic, octadecanoic, and eicosanoic acids were also detected in small amounts in the seed oil by LC and MS. The structure of **1** was determined from NMR and MS spectral data.

KEYWORDS: ***Punica*; Punicaceae; pomegranate; polyunsaturated oil; brine shrimp; monoacylglycerol; C_{18} − C_{20} fatty acids**

INTRODUCTION

Punica granatum L. (Punicaceae), or pomegranate, is well-known in the Arabian Peninsula as a fruit-bearing shrub. In the Sultanate of Oman, *Punica* trees are cultivated and the fruits are sold in the markets. The fleshy parts of the fruits are not edible. However, the seeds, which have a sweet taste and pleasant odor, are consumed as a dessert. The pericarp of ripe fruits is dried and used to treat stomach ache in traditional medicine. In previous work, the seed oil of *P. granatum* afforded polyunsaturated triacylglycerols (*1*). Yellow-colored hydrolyzable tannins were reported (*2*−*4*) as constituents of the pericarp, leaves, and bark of *P. granatum*. *N*- (2′,5′- dihydroxyphenyl) pyridinium chloride, brevifolin, polyphenols, apigenin, and luteolin glycosides were isolated from the leaves by Nawwar and co-workers (*5*, *6*). The alkaloid pelletierine was also isolated from this plant (*7*). Pelletierine is toxic to tapeworm and is listed in the Merck Index as an anthelmintic (*8*). The dark staining of skin and clothes following contact with the juice from *Punica* fruit attests to the high level of tannins in the fleshy part of the fruit.

The composition of the seed oil of pomegranate is of nutritional interest. It was found that 1-*O*-*trans,cis,trans*-9,11,13-octadecatrienoyl glycerol (**1**), 1-*O*-isopentyl-3-*O*-octadec-2-enoyl glycerol (**2**), and *cis*-9-octadecenoic, octadecanoic, and eicosanoic acids were present in the seed oil. Compound **2**, and octadecanoic and eicosanoic acids were detected in trace amounts. Compound **2** is a rare glyceride and its structural assignment is tentatively based on mass spectral data. The structure of **1** was determined from NMR and MS data. In this paper, the isolation of **1**, and the spectral evidence for the presence of **1**, **2**, and some fatty acids in pomegranate seed oil are described.

MATERIALS AND METHODS

Apparatus and Reagents. IR was measured on a Nicolet FT-IR spectrometer. UV was obtained with a UV−visible HP-8453 spectrophotometer. ^{1}H and ^{13}C NMR (DEPT, homo- and hetero-nuclear COSY) were run on a JEOL JNM-EX400 or a Bruker Avance 400 spectrometer. Compounds were analyzed in $CDCl_3$ with tetramethylsilane (TMS) as internal standard. Mass spectra were obtained with a JEOL JMS-SX102A (EIMS, 70 eV; HREIMS, 3.0 KV; FABMS, *m*-nitrobenzoic acid matrix, at 85.5 °C). Open-column chromatography was performed on Kieselgel S (Riedel-deHaen) 70−230 mesh, and TLC was performed on Whatman precoated silica gel (60A K6F) plates. TLC bands were visualized under UV lamp or by exposure to iodine vapor.

Plant Material. Ripe and fresh fruits were harvested from *P. granatum* trees at Jaber Al Akhader-Said, Sultanate of Oman, in September 1999. Twigs of the plant were also collected. The plant material was authenticated by comparison with a voucher specimen at Sultan Qaboos University Herbarium, Oman (Voucher Reference: Collection number 006, Ahmed Al Sabahi and Shahina Ghazanfar). The seeds were removed from 20 pieces of fruits, fermented for two weeks in an open tray, drained, washed with water, dried, and milled to give 250 g of powdered seed.

Brine Shrimp Lethality Test (BST). Fractions from the chloroform extracts of the twigs and seeds were evaluated for lethality to brine shrimp larvae (*9*, *10*). In this test, a drop of DMSO was added to vials of the test and control substances to enhance the solubility of test materials.

Extraction and Isolation. Dried twigs of *P. granatum* were extracted and fractionated following a previously reported procedure (*11*). Dried powdered seeds (250 g) were extracted by maceration with chloroform

* Corresponding author. Tel: 968 515491. Fax: 968 513415. E-mail: majek@squ.edu.om.
† Sultan Qaboos University.
‡ University of Tokushima.

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Table 1. NMR Data for Compound **1**

position	^{13}C[a]		^{1}H (J in Hz)[b]	
1	173.24	C		
2	34.16	CH_2	2.31	(2H, t, 7.2)
3	24.85	CH_2	1.61	(2H, m)
4	29.13	CH_2	1.38–1.22	m
5	29.13	CH_2	1.38–1.22	m
6	29.08	CH_2	1.38–1.22	m
7	29.70	CH_2	1.38–1.22	m
8	27.83	CH_2	2.29	(2H, m)
9	132.48	CH	5.44–5.41	(1H, ddd, 18.4, 10.0, 7.6)
10	128.84	CH	6.02–6.08	(1H, dd, 18.4, 5.6)
11	127.80	CH	6.40–6.47	(1H, dd, 9.6, 5.6)
12	127.94	CH	6.44–6.48	(1H, dd, 9.6, 5.2)
13	128.73	CH	6.02–6.08	(1H, dd, 18.4, 5.6)
14	132.72	CH	5.44–5.41	(1H, ddd, 18.4, 10.0, 7.6)
15	27.58	CH_2	2.21	(2H, m)
16	31.84	CH_2	1.35	(2H, m)
17	22.33	CH_2	1.38–1.32	(2H, m)
18	13.97	CH_3	0.92	(3H, t, 7.3)
1′	62.09	CH_2	4.29	(2H, dd, 11.9, 4.4)
2′	68.85	CH	5.26	(1H, m)
3′	62.09	CH_2	4.14	(2H, dd, 11.6, 6.0)

[a] DEPT ^{13}C NMR,100 MHz, $CDCl_3$. [b] 400 MHz, $CDCl_3$.

(2 × 2.5 L) for two weeks. The chloroform extract was evaporated under vacuum at 25−30 °C to give an oil (58 g). A sample (40 g) of the residue was chromatographed on silica gel (120 g) and eluted successively with hexane (3.2 L), chloroform−hexane (1:1, 4.4 L), chloroform (2.4 L), chloroform−acetone (2:1, 1.0 L), and chloroform−acetone (1:2, 1.0 L) with collection of 400-mL fractions. Fractions 9−13, eluted with chloroform−hexane (1:1), gave a chromatographically identical oil (35 g) with R_f value of 0.8 (eluent $CHCl_3$−hexane, 3:2). Fractions 22−25, eluted with $CHCl_3$, gave an oil (800 mg) with R_f value of 0.2 (eluent $CHCl_3$−hexane, 3:2).

***Cis*-9-Octadecenoic acid.** Oil, 800 mg, obtained from fractions 22−25, eluted with chloroform. ^1H NMR ($CDCl_3$, 400 MHz) δ 5.37 (2H, dt, J = 11.0 and 6.9 Hz, H-9 and H-10), 2.36 (2H, t, J = 7.5 Hz, H-2), 2.02 (4H, m, H-8 and H-11), 1.20−1.80 (22H, m), 0.88 (3H, t, J = 6.9 Hz, H-18); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 179.7 (C-1), 34.0 (C-2), 24.7 (C-3), 29.2 (C-4), 29.2 (C-5), 29.2 (C-6), 29.7 (C-7), 27.2 (C-8), 129.7 (C-9), 130.0 (C-10), 27.2 (C-11), 29.6 (C-12), 29.5 (C-13), 27.2 (C-14), 29.5 (C-15), 31.9 (C-16), 22.7 (C-17), 14.13 (C-18); FABMS (positive mode) m/z (rel int.) 283 (100) $[M + H]^+$.

1-*O*-*Trans,cis,trans*-9,11,13-octadecatrienoyl glycerol (1). Yellow oil, 35 g. IR ν_{max} (film) 3007, 2932, 2857, 1745, 1461, 1219, 1152, 1001, 759 cm^{-1}; UV λ_{max} (Log ϵ) ($CHCl_3$) 268 (4.45), 278 (4.56), 289 (4.45) nm; FABMS m/z (rel int.) $[M + Na]^+$ 391 (3), 305 (25), 277 (25), 278 (20), 279 (23), 255 (10), 199 (10), 152 (70), 153 (100), 154 (20), 122 (30), 87 (25), 46 (40); ^1H NMR ($CDCl_3$, 400 MHz); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1.

1-*O*-Isopentyl-3-*O*-octadec-2-enoyl glycerol (2). Detected from MS analysis of a portion of fraction 13. EIMS m/z (rel int.) $[M^+]$ 426 (30), 408 (18) $[M^+ - H_2O]$, 396 (15) $[M^+ - 2 \times CH_3]$, 365 (10) $[M^+ - H_2O - CH(CH_3)_2]$, 313 (5) $[M^+ - (CH_2)_7CH_3]$, 273 (5), 234 (22), 214 (80), 189 (30), 161 (10), 162 (10), 102 (100), 91 (55), 61 (13). HREIMS m/z 426.3826 $[M^+]$ (calcd 426.3709 for $C_{26}H_{50}O_4$).

Hydrolysis of Oil. A sample (2.01 g) of oil, from which **1** was isolated as a major product, was refluxed with KOH (5 g) in 20% aqueous MeOH (100 mL) for 8 h, and kept overnight. The entire reaction mixture formed a soft solid, to which water, chloroform, and ice chips were added, and was acidified with cold HCl−H_2O (1:1). The organic layer was separated, washed with H_2O, dried (Na_2SO_4), and evaporated to give a residue (1.96 g). A portion of the residue (1.8 g) was chromatographed on silica gel (60 g) and eluted successively with hexane (2.4 L), hexane−chloroform (9:1, 1.0 L), hexane−chloroform (1:1, 2.6 L), chloroform (1.2 L), chloroform−acetone (1:1, 1.2 L), and chloroform−ethylacetate (1:1, 2.2 L). The chloroform−acetone fraction was collected as four fractions, each with R_f value of 0.6 (eluent chloroform−hexane 3:2). The four fractions gave a residue (0.74 g). EIMS analysis of the residue showed the presence of octadecanoic and eicosanoic acids.

Octadecanoic Acid. EIMS m/z (rel int.) $[M^+]$ 284 (40), 256 (100), 213 (25), 185 (24), 129 (55), 111 (40), 97 (50), 69 (90), 57 (88), 43 (75). HREIMS m/z 284.2747 $\{M^+\}$ (calcd 284.2715 for $C_{18}H_{36}O_2$).

Eicosanoic Acid. EIMS m/z (rel int.) $[M^+]$ 312 (8), 284 (100), 264 (48), 256 (96), 185 (34), 171 (28), 157 (25), 152 (23), 143 (20), 195 (95), 124, (22), 115 (38), 111 (77), 98 (77), 97 (93), 96 (46), 95 (47), 87 (46), 84 (67), 82 (54), 69 (64), 57 (62), 43 (52). HREIMS m/z 312.3039 $[M^+]$ (calcd 312.3028 for $C_{20}H_{40}O_2$).

RESULTS AND DISCUSSION

Chloroform extracts of the twigs of *P. granatum* were partitioned between 10% aqueous methanol and hexane. Preliminary screening of the polar fraction showed a high lethality to brine shrimp larvae (BST LC_{50} = 48 μg/mL). This result could be correlated with the presence of tannins in the plant. The high tannin content makes *P. granatum* extracts unattractive for investigation as a source of pesticide. The seed oil, unlike the twigs, is not toxic to brine shrimp larvae (BST LC_{50} > 1000 μg/mL), and thus may have no effect on solid tumor cells (*12*). MS and NMR analysis of the oil revealed the presence of octadecanoic acid, *cis*-9-octadecenoic acid, 1-*O*-*trans,cis,trans*-9,11,13-octadecatrienoyl glycerol (**1**), and 1-*O*-isopentyl-3-*O*-octadec-2-enoyl glycerol (**2**). The EIMS of unhydrolyzed fraction 13 from the column showed two compounds with m/z 279 (retention time of 0.39 min) and m/z 426 (retention time of 3.09 min) as dominant peaks in the highest mass region of their respective spectra. The m/z 279 is not a molecular ion. It was attributed to *trans,cis,trans*-9,11,13-octadecatrienoic acid residue from a fragmented glyceride. In the FABMS, the same fraction gave a molecular ion peak at m/z 391 $[M + Na]^+$ (retention time of 1.34 min), suggesting a compound with a nominal molecular mass of 368. This signal established the presence of **1**, the most abundant component of the seed oil. The loss of the $NaOCH_2CH(OH)CH_2OH$ fragment from m/z 391 gave an ion at m/z 277. Peaks at m/z 278 and 279, arising from sequential donations of hydrogen atoms to m/z 277, were also observed in the FABMS. The loss of 126 amu, corresponding to fragment $-CO(CH_2)_7-$, from this cluster of peaks gave ions at m/z 151, 152, and 153, respectively. The ^1H NMR spectra of fraction 13 (Table 1) revealed the presence of 1-monoacylglyceryl and conjugated triene substructures in **1**. Three well-resolved signals observed at δ 5.26, 4.14, and 4.29 were attributed to H-2′, H-1′, and H-3′ of the glyceryl substructure, respectively. These signals and a molecular mass of 368 excluded the possibility that **1** could be a 2-mono-, 1,2-di-, 1,3-di-, or 1,2,3-triacyl-glycerol (*13*). The cross-peaks in the HETCOR NMR spectra and the connectivity of signals in the $^1H/^1H$ COSY allowed the structure **1** to be assigned. In the $^1H/^1H$ COSY, signals at δ 4.14 (H-1′) and δ 4.29 (H-3′) were correlated with each other and with the signal at δ 5.26 (H-2′). Signals at δ 2.21−2.29 (H-8 and H-15) showed cross-peaks to signals at δ 1.3 (H-7 and H-16) and to signals at δ 5.41−5.44 (H-9 and H-14). Additional connectivity of signals at δ 6.02−6.08 (H-10 and H-13) with those at δ 5.41−5.44 (H-9 and H-14) and δ 6.44−6.48 (H-11 and H-12) were readily observed in the $^1H/^1H$ COSY NMR. The stereochemistry of the conjugated triene was inferred from the coupling constant values ($J_{9,10}$ and $J_{13,14}$ = 18.4 Hz, and $J_{11,12}$ = 9.6 Hz) as *trans,-cis,trans*. The ^{13}C NMR signals of the fatty acid moiety of **1** are similar to those reported (*14*) for the methyl ester of *cis,-trans,cis*-9,11,13-octadecatrienoic acid, except for signals assigned to C-9, C-10, C-13, and C-14. Direct correlations from the HETCOR NMR allowed the carbon atoms connected by one bond to H-9, H-10, H-13, and H-14 to be unambiguously assigned.

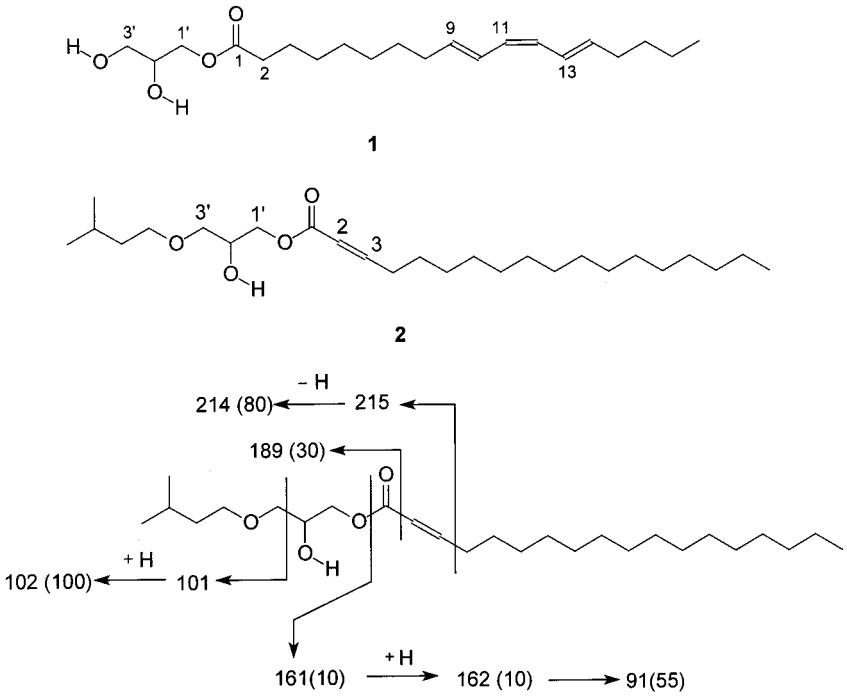

Figure 1. Structures of glycerol esters and mass spectrometric fragmentation pattern of compound **1**.

The second compound was assigned structure **2** on the basis of MS spectral data (Figure 1). The molecular ion at *m*/*z* 426 lost H_2O to give *m*/*z* 408. The peaks at *m*/*z* 313, 214, and 189 arose from the sequential truncation of the unsaturated fatty acid moiety of **2** (Figure 1). The cleavage of oxygen-acyl bond of the ester gave *m*/*z* 161. This ion could lose $-OCH_2CHO$ to give *m*/*z* 102, or abstract a proton to give *m*/*z* 162. The latter could lose 71 amu, corresponding to $(CH_3)_2CHCH_2CH_2-$ to give *m*/*z* 91. HREIMS indicated a molecular formula of $C_{26}H_{50}O_4$ for **2**.

The yellowish substance (800 mg) eluted by chloroform from the silica gel column was identified as *cis*-9-octadecenoic acid. The stereochemistry of the double bond was determined cis on the basis of the coupling constant of the signal at δ 5.37 ($J_{9,10}$ = 11.0 Hz), and the resonances of H-8 and H-11 at δ 2.02. The ^{13}C NMR data of this compound agreed with literature values (*15*).

The EIMS of the hydrolyzed oil gave two compounds with molecular ion at *m*/*z* 284 (retention time of 0.30 min) and *m*/*z* 312 (retention time of 0.45 min), respectively. HREIMS suggested molecular formulas of $C_{18}H_{36}O_2$ and $C_{20}H_{40}O_2$. The two compounds were identified as octadecanoic and eicosanoic acids. In addition, the FABMS detected a compound (retention time of 3.67 min) that had *m*/*z* 543 (100) $[M + Na]^+$ and daughter ions at *m*/*z* 513 (20), 410 (50), 281 (25), 152 (60), 153 (82), and 46 (25). A molecular mass this high was not observed in the unhydrolyzed fraction 13. We did not detect *trans*,*cis*,*trans*-9,11,13-octadecatrienoic acid in the hydrolysis product. Hydrolysis of **1** with lipase (*16*) may well be a better method for recovering *trans*,*cis*,*trans*-9,11,13-octadecatrienoic acid from **1**.

The seed oil of *P. granatum* could be a rich source of *trans*,-*cis*,*trans*-9,11,13-octadecatrienoic acid. Pomegranate yielded about 20% oil. Compound **1** constitutes about 88% of the oil. Polyunsaturated acylglycerols are readily oxidized through a radical chain reaction leading to an unpleasant taste or smell in food products prepared with pomegranate oil. Polyunsaturated fatty acids are precursors to several metabolites and hydroxy fatty acids that regulate critical biological functions (*17*, *18*).

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