Identification of Three Triterpenoids in Almond Hulls

Gary Takeoka,* Lan Dao, Roy Teranishi, Rosalind Wong, Stephan Flessa, Leslie Harden, and Richard Edwards

Western Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, 800 Buchanan Street, Albany, California 94710

Three triterpenoids, betulinic acid, oleanolic acid, and ursolic acid, were isolated as their methyl esters (treatment with diazomethane) from diethyl ether extracts of almond hulls (Nonpareil variety) using flash chromatography and preparative high-performance liquid chromatography. The triterpenoids, which comprised ∼1% of the hulls, were characterized using chromatographic and spectroscopic methods. These studies demonstrate that almond hulls are a rich source of these triterpenoids, which have reported anti-inflammatory, anti-HIV, and anti-cancer activities.

Keywords: *Betulinic acid; oleanolic acid; ursolic acid*

INTRODUCTION

Almonds [*Prunus dulcis* (Mill.) D.A. Webb] are a major California commodity, generating $858 million in gross sales in 1995. The annual production of almond hulls is currently in excess of 600000 tons (1.2 billion pounds), with this product mainly used as livestock feed. Previous studies by Buttery et al. (1980) had revealed that direct extraction of almond hulls with diethyl ether gave a white solid (1−1.5% of the hulls) having mass and infrared spectra quite similar to those of oleanolic acid. Triterpenoids such as oleanolic acid and ursolic acid occur especially in the waxy coatings of leaves and on fruits such as apple and pear and may serve as insect antifeedants and antimicrobial agents (Harborne, 1998). Although triterpenoids have had rather limited medicinal use, recent studies indicate their great potential as drugs (Mahato et al., 1992). Betulinic acid has been shown to exhibit significant anti-HIV activity, inhibiting HIV replication in H9 lymphocytes with an EC_{50} value (concentration of the test sample that was able to suppress HIV replication by 50%) of 1.4 mM, whereas its IC_{50} (concentration of test sample that was toxic to 50% of the mock-infected cells) for inhibiting uninfected H9 cell growth was 13 mM [therapeutic index (TI = 9.3); defined as toxicity (IC_{50}) divided by anti-HIV activity (EC_{50})] (Fujioka et al., 1994). Similarly, oleanolic acid was found to inhibit HIV-1 replication in acutely infected H9 cells with an EC_{50} value of 1.7 mg/mL while inhibiting H9 cell growth with an IC_{50} value of 21.8 mg/mL (Kashiwada et al., 1998). These same researchers (Kashiwada et al., 1998) also reported the anti-HIV activity of ursolic acid, which had an EC_{50} value of 2.0 mg/mL while exhibiting slight toxicity (IC_{50} = 6.5 mg/mL, TI = 3.3). Ursolic acid has also shown significant cytotoxicity in the lymphocytic leukemia cells P-388 (ED_{50} = 3.15 mg/mL) and L-1210 (ED_{50} = 4.00 mg/mL) as well as the human lung carcinoma cell A-549 (ED_{50} = 4.00 mg/mL) (Lee et al., 1988). Oleanolic acid has been proposed as an anti-inflammatory and antiarthritic agent (Singh et al., 1994). Betulinic acid has been shown to act as a selective inhibitor of human melanoma in cell culture and animal models that function by induction of apoptosis (Pisha et al., 1995). Preclinical development is currently being conducted to explore the potential use of betulinic acid for the treatment or prevention of human melanoma. In our continuing search for novel phytonutrients in agricultural products, we examined the chemical composition of almond hulls to characterize the unknown triterpenoid(s) and other constituents.

* Author to whom correspondence should be addressed [telephone (510) 559-5668; fax (510) 559-5958; e-mail grt@pw.usda.gov].

EXPERIMENTAL PROCEDURES

Materials. Almond hulls were supplied by the Northern Merced Hulling Association (Ballico, CA). Oleanolic acid was isolated from olives, and ursolic acid was isolated from apple peel. Betulinic acid methyl ester was obtained from Indofine Chemical Co., Inc. (Somerville, NJ). HPLC grade methanol was supplied by Fisher Scientific (Fair Lawn, NJ).

Extraction. Dried almond hulls were ground in a Wiley mill to pass a 6.4 mm screen. The ground hulls were extracted with freshly distilled diethyl ether [containing ∼0.001% Ethyl antioxidant 330 (1,3,5-trimethyl-2,4,6-tris(3,5-di-*tert*-butyl-4-hydroxybenzyl)benzene)] in a Soxhlet extractor.

Methylation. A portion of the white solid obtained from the diethyl ether extraction (1.97 g) was treated with CH_2N_2 in diethyl ether; the methylation products were further purified by flash chromatography and by HPLC. Oleanolic acid (0.31 g) and ursolic acid (0.10 g) were treated with CH_2N_2 in diethyl ether and purified by HPLC. CH_2N_2 was generated according to the procedure described by Black (1983).

Flash Chromatography. A portion of the methylated extract (1.21 g) was separated by flash chromatography on a glass column (33 cm × 2.3 cm i.d.; bed height = 23 cm) packed with silica gel (Merck grade 9385, 230−400 mesh). Using pentane/diethyl ether mixtures, the following fractions were obtained: fraction 1 (pentane; 150 mL), fraction 2 (95:5 v/v; 150 mL), fraction 3 (70:30 v/v; 150 mL), fraction 4 (50:50 v/v; 150 mL), fraction 5 (30:70 v/v; 150 mL), and fraction 6 (diethyl ether; 150 mL).

Analytical HPLC-MS. An HP 1100 liquid chromatograph equipped with a manual injector (Rheodyne model 1725) fitted with a 20 mL sample loop and an HP 1100 diode array detector (DAD) was coupled with an HP 1100 mass selective detector (MSD). Atmospheric pressure chemical ionization (APCI) was utilized with the following mass spectrometer operating conditions: gas temperature, 350 °C, with a nitrogen flow rate of 4.2 L/min; nebulizer pressure, 60 psi; vaporizer temperature,

10.1021/jf9908289
Published on Web 07/08/2000

315 °C; capillary voltage, 2500 V; and fragmentor, 60 V. The column was a 250 mm × 4.6 mm i.d. Luna C18(2) (particle size = 5 mm; Phenomenex, Torrance, CA) equipped with a Supelguard LC-18 guard column (2 cm × 2.1 mm i.d.; Supelco, Bellefonte, PA). Methanol was used as the mobile phase at a flow rate of 0.7 mL/min. The instrument was controlled and the data were processed by an HP ChemStation (rev. A.06.01 [403]).

Preparative HPLC. The preparative HPLC system consisted of Gilson model 305 and 306 pumps, a Gilson 806 manometric module, a Gilson 811C dynamic mixer (Gilson Medical Electronics, Inc., Middleton, WI), a manual injector (Rheodyne model 1725) fitted with a 900 μL sample loop (PEEK tubing), and an HP 1047A refractive index detector. A C18 reversed phase Rainin Dynamax preparative column (250 mm × 21.4 mm i.d., 8 mm, 100 Å; Varian Associates, Walnut Creek, CA) was directly coupled to a guard column (50 mm × 21.4 mm i.d.) containing the same packing material. The mobile phase was 100% methanol. The flow rate was 4.4 mL/min. The three triterpenoids contained in fraction 4 were isolated, and their purity was checked by analytical LC-MS. When necessary, the compounds were rechromatographed using a mobile phase of 98:2 v/v methanol/water. Oleanolic acid (obtained from olives) and ursolic acid (obtained from apple peel) were methylated (diazomethane) and subsequently purified using the above HPLC conditions.

Capillary GC. An HP 5890 series II gas chromatograph equipped with a flame ionization detector (FID) was used. A DB-1HT fused silica capillary column (30 m × 0.25 mm i.d.; d_f = 0.1 mm) was also used. The linear velocity of helium carrier gas was 34.4 cm/s (150 °C). The oven temperature was programmed from 150 °C (4 min isothermal) to 290 °C at 2 °C/min. The injector and detector temperatures were 250 and 310 °C, respectively.

Mass Spectrometry. The spectra were obtained via DCI using a VG70/70 mass spectrometer. The ionization energy was 70 eV, and the EI source temperature was 205 °C. Methyl betulinate had the following mass spectrum: 470 (M^+, 19), 452 (32), 411(15), 410 (19), 262 (38), 220 (20), 207 (36), 189 (100). Methyl oleanolate had the following mass spectrum: 470 (M^+, 5), 452 (4), 411 (3), 410 (3), 262 (70), 203 (100), 189 (28). Methyl ursolate had the following mass spectrum: 470 (M^+, 6), 452 (8), 411 (5), 410 (5), 262 (69), 203 (100), 189 (34).

NMR Spectroscopy. NMR spectra were obtained at 298 K from samples in $CDCl_3$ with TMS as an internal standard on a Bruker model ARX400 spectrometer at a frequency of 100.62 MHz for carbon and 400.13 MHz for proton. One- and two-dimensional experiments were run for both nuclei. The number of attached protons for ^{13}C signals was determined from DEPT90 and DEPT135 assays.

RESULTS AND DISCUSSION

Soxhlet extraction of almond hulls with diethyl ether gave a white solid (yield = 3.04 ± 0.14%). A portion of the solid (1020 mg) was methylated with diazomethane prior to fractionation by flash chromatography. Fraction 4 (29.4%, 300 mg) displayed three peaks by capillary gas chromatography that were subsequently identified as methyl oleanolate (15.6%, $I^{DB\text{-}1}$ = 3419), methyl betulinate (40.9%, $I^{DB\text{-}1}$ = 3433), and methyl ursolate (43.5%, $I^{DB\text{-}1}$ = 3473) (Figure 1). This mixture was separated by preparative HPLC to yield three purified triterpenoids, which all had apparent molecular weights of 470 as evidenced by APCI mass spectrometry. In contrast to the retention order observed by capillary GC, the compounds had the following retention order (in order of increasing retention time) by reversed-phase (C18) HPLC: methyl betulinate, methyl oleanolate, and methyl ursolate. The three triterpenoids were identified by comparison of the Kovats indices, EI mass spectra, and ^1H and ^{13}C NMR spectra (Table 1) with those of authentic reference standards. The mass spectrum of methyl betulinate was similar to that reported by Budzikiewicz et al. (1963), whereas the mass spectra of methyl oleanolate and methyl ursolate closely matched

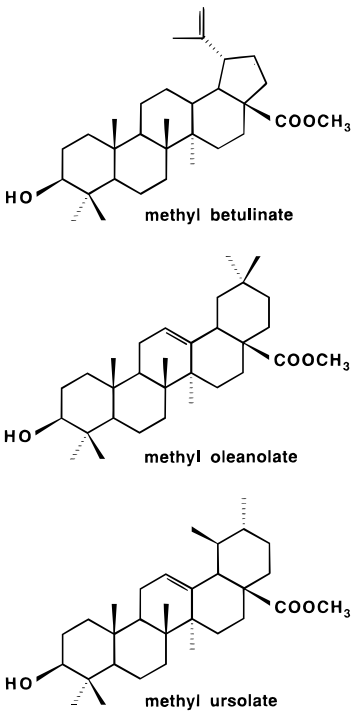

Figure 1. Chemical structures of methylated almond hull triterpenoids.

Table 1. ^{13}C NMR Data of Almond Hull Triterpenoids (All Measured in $CDCl_3$)

C	methyl betulinate		methyl oleanolate[a]		methyl ursolate[b]	
1	CH_2	38.77	CH_2	38.48	CH_2	38.67
2	CH_2	27.45	CH_2	27.24	CH_2	27.26
3	CH	79.00	CH	79.05	CH	79.03
4	C	38.89	C	38.78	C	38.77
5	CH	55.41	CH	55.28	CH	55.27
6	CH_2	18.33	CH_2	18.37	CH_2	18.34
7	CH_2	34.38	CH_2	32.72	CH_2	33.01
8	C	40.72	C	39.32	C	39.53
9	CH	50.60	CH	47.68	CH	47.60
10	C	37.24	C	37.08	C	37.00
11	CH_2	20.92	CH_2	23.44	CH_2	23.32
12	CH_2	25.57	CH	122.40	CH	125.60
13	CH	38.31	C	143.82	C	138.17
14	C	42.43	C	41.69	C	42.02
15	CH_2	30.66	CH_2	27.74	CH_2	28.06
16	CH_2	32.21	CH_2	23.12	CH_2	24.26
17	C	56.60	C	46.76	C	48.12
18	CH	47.00	CH	41.35	CH	52.92
19	CH	49.53	CH_2	45.93	CH	39.08
20	CH	150.58	C	30.71	CH	38.90
21	CH_2	29.71	CH_2	33.90	CH_2	30.68
22	CH_2	36.99	CH_2	32.42	CH_2	36.66
23	CH_3	28.01	CH_3	28.13	CH_3	28.16
24	CH_3	15.37	CH_3	15.58	CH_3	15.45
25	CH_3	15.98	CH_3	15.32	CH_3	15.63
26	CH_3	16.13	CH_3	16.87	CH_3	16.93
27	CH_3	14.74	CH_3	25.96	CH_3	23.63
28	C=O	176.67	C=O	178.29	C=O	178.05
29	CH_2	109.58	CH_3	33.12	CH_3	16.93
30	CH_3	19.40	CH_3	23.66	CH_3	21.18
CO_2Me		51.25		51.52		51.43

[a] Data are in agreement with that of Seo et al. (1981) with the exception of the value for C-17, where we suspect there was a typographical error in the paper. [b] Data are in accordance with that of Seo et al. (1981).

those reported by Furuya et al. (1987). The three triterpenoids together comprised ~1% of the hulls, thus revealing almond hulls as a rich source of these promising anti-inflammatory, anti-HIV, and anticancer agents.

LITERATURE CITED

Black, T. H. The preparation and reactions of diazomethane. *Aldrichim. Acta* **1983**, *16*, 3−10.

Budzikiewicz, H.; Wilson, J. M.; Djerassi, C. Mass spectrometry in structural and stereochemical problems. XXXII. Pentacyclic triterpenes. *J. Am. Chem. Soc.* **1963**, *85*, 3688−3699.

Buttery, R. G.; Soderstrom, E. L.; Seifert, R. M.; Ling, L. C.; Haddon, W. F. Components of almond hulls: possible navel orangeworm attractants and growth inhibitors. *J. Agric. Food Chem.* **1980**, *28*, 353−356.

Fujioka, T.; Kashiwada, Y.; Kilkuskie, R. E.; Cosentino, L. M.; Ballas, L. M.; Jiang, J. B.; Janzen, W. P.; Chen, I.-S.; Lee, K.-H. Anti-AIDS agents. 11. Betulinic acid and platanic acid as anti-HIV principles from *Syzigium clavisflorum*, and the anti-HIV activity of structurally related triterpenoids. *J. Nat. Prod.* **1994**, *57*, 243−247.

Furuya, T.; Orihara, Y.; Hayashi, C. Triterpenoids from *Eucalyptus perriniana* cultured cells. *Phytochemistry* **1987**, *26*, 715−719.

Harborne, J. B. In *Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis*, 3rd ed.; Chapman & Hall: London, U.K., 1998; p 129.

Kashiwada, Y.; Wang, H.-K.; Nagao, T.; Kitanaka, S.; Yasuda, I.; Fujioka, T.; Yamagishi, T.; Cosentino, L. M.; Kozuka, M.; Okabe, H.; Ikeshiro, Y.; Hu, C.-Q.; Yeh, E.; Lee, K.-H. Anti-AIDS agents. 30. Anti-HIV activity of oleanolic acid, pomolic acid, and structurally related triterpenoids. *J. Nat. Prod.* **1998**, *61*, 1090−1095.

Lee, K. H.; Lin, Y. M.; Wu, T. S.; Zhang, D. C.; Yamaguchi, T.; Hayashi, T.; Hall, I. H.; Chang, J. J.; Wu, R. Y. Yang, T. H. The cytotoxic principles of *Prunella vulgaris*, *Psychotria serpens*, and *Hyptis capitata*: ursolic acid and related derivatives. *Planta Med.* **1988**, *54*, 308.

Mahato, S. B.; Nandy, A. K.; Roy, G. Triterpenoids. *Phytochemistry* **1992**, *31*, 2199−2249.

Pisha, E.; Chai, H.; Lee, I.-K.; Chagwedera, T. E.; Farnsworth, N. R.; Cordell, G. A.; Beecher, C. W. W.; Fong, H. H. S.; Kinghorn, A. D.; Brown, D. M.; Wani, M. C.; Wall, M. E.; Hieken, T. J.; Gupta, T. K. D.; Pezzuto, J. M. Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nat. Med.* **1995**, *1*, 1046−1051.

Seo, S.; Tomita, Y.; Tori, K. Biosynthesis of oleanene- and ursene-type triterpenes from [4-^{13}C]mevalonolactone and [1,2-$^{13}C_2$]acetate in tissue cultures of *Isodon japonicus* Hara. *J. Am. Chem. Soc.* **1981**, *103*, 2075−2080.

Singh, G. B.; Singh, S.; Bani, S. Oleanolic acid. *Drugs Future* **1994**, *19*, 450−451.

Received for review July 26, 1999. Accepted May 21, 2000.

JF9908289