PERGAMON

Phytochemistry 52 (1999) 623–629

PHYTOCHEMISTRY

Triterpene phytoalexins from nectarine fruits

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Received 28 September 1998; received in revised form 28 April 1999

Abstract

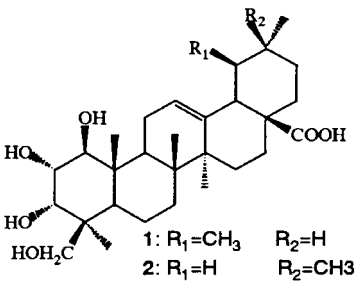
Seven triterpenoid phytoalexins were isolated from peel of unripe fruits of nectarine (*Prunus persica* cv Fantasia) wounded and inoculated with *Colletotrichum musae*. Two were new triterpenoids, identified as 1β,2α,3α,24-tetrahydroxyurs-12-en-28-oic acid and 1β,2α,3α,24-tetrahydroxyolean-12-en-28-oic acid. 2α,3α,24-Trihydroxyolean-12-en-28-oic acid, 2α,3α,24-trihydroxyurs-12-en-28-oic acid, 2α,3β,24-trihydroxyolean-12-en-28-oic acid, 2α,3α-dihydroxyolean-12-en-28-oic acid, and 2α,3α-dihydroxyurs-12-en-28-oic acid were previously reported as constitutive natural products from other plants, but were never described as phytoalexins. All showed antifungal activity against the fungus mentioned.

Keywords: *Prunus persica*; Nectarine; *Colletotrichum musae*; Phytoalexin; Triterpenoid; Antimicrobial activity

1. Introduction

Resistance in many plant-pathogen interactions is accompanied by the rapid deployment of a multicomponent defensive response (Dixon & Harrison, 1994). The individual components of this response include the hypersensitive response, chemical weapons such as antimicrobial phytoalexins and hydrolytic enzymes, and structural defensive barriers such as lignin and hydroxyproline-rich cell proteins (Dixon & Harrison, 1994; Kuc, 1994).

We are evaluating the role of phytoalexins as a part of a research program aimed at understanding the mechanisms of disease resistance of fruits, since some fruits in unripe stage showed resistance to infection by pathogens (Hirai, Ishida & Koshimizu, 1994; Kamo et al., 1998a, b). We have investigated the resistance mechanism in nectarine (*Prunus persica*) fruit, and found that unripe nectarine fruit produced phytoalexins upon wounding and inoculation with *Colletotricum musae*. In this report we describe the induction, isolation and characterisation of seven phytoalexins from unripe fruits of nectarine.



2. Results and discussion

The unripe fruit of nectarine was wounded and inoculated with a conidia suspension of *C. musae* strain No. 1679 since *Colletotrichum* shows a broad host range (Simmonds, 1965). A bioautography with a TLC plate of extracts from peels of the fruit revealed antifungal zones against the fungi mentioned previously, which were not detected in the extracts of

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0031-9422/99/$ - see front matter
PII: S0031-9422(99)00316-7

non-treated fruit. This finding showed that the unripe fruit produced antimicrobial compounds as phytoalexins.

The antifungal compounds **1**–**7** were isolated, of which **3** and **4** were isolated as methyl esters **3a** and **4a** after methylation of a mixture of **3** and **4** since separation of them was difficult. Compounds **1**, **2**, mixtures of **3** and **4**, and **5**–**7** showed antifungal activity against *C. musae* at 30 μg in bioautography, of which **1** and **2** gave large antifungal zones compared to that of **3**–**7**. Compounds **3**–**7** were characterized as 2α,3α,24-trihydroxyolean-12-en-28-oic acid (**3**) (Kojima & Ogura, 1989; Fang & Ying, 1986), 2α,3α,24-trihydroxyurs-12-en-28-oic acid (**4**) (Kojima & Ogura, 1989), 2α,3β,24-trihydroxyolean-12-en-28-oic acid (**5**) (Yamagishi et al., 1988), 2α,3α-dihydroxyolean-12-en-28-oic acid (**6**) (Cheung & Yan, 1970), and 2α,3α-dihydroxyurs-12-en-28-oic acid (**7**) (Kojima & Ogura, 1989) by comparison of their physical constants and spectroscopic data with those reported in the literature. These compounds were previously reported as constitutive natural products from other plants, but were never described as phytoalexins. Compounds **1** and **2** were new triterpenoids.

The ^{1}H NMR spectrum of compound **1** was similar to that of **4**, and displayed four tertiary methyl groups at δ 0.87 (H-26), 1.02 (H-25), 1.10 (H-23 or H-24) and 1.17 (H-27), and two secondary methyl groups at δ 0.92 (H-29) and 1.01 (H-30) on an ursane skeleton. A doublet of one proton at δ 2.23 and a triplet of one proton at δ 5.12 were assigned to H-18 and H-12, respectively, suggesting an urs-12-ene skeleton. A methylene proton at δ 2.50 was assigned to one of H-11, the low field chemical shift will be explained later. Three methine protons at δ 3.50 (*d*, J = 9.6 Hz), δ 3.65 (*dd*, J = 9.6 and J = 3.1 Hz) and δ 3.85 (*d*, J = 3.1 Hz), and methylene protons at δ 3.44 and 3.70 (1H each, *d*, J = 11.4 Hz) suggested that **1** has at least four hydroxyl groups. Compound **1** gave a monomethyl ester (**1a**) on treatment with diazomethane, and acetylation of **1a** afforded a tetra-acetate (**1b**), confirming that **1** has one carboxyl and four hydroxyl groups. A mass spectrum of **1a** showed a molecular ion at *m*/*z* 518, and its high resolution mass spectrum gave a molecular formula $C_{31}H_{50}O_6$ for **1a**. Fragment ions at *m*/*z* 262, 203, 189, and 133 were also observed in the mass spectrum. The fragment ion at *m*/*z* 262 which had a formula $C_{17}H_{26}O_2$ would be formed by a retro-Diels-Alder fragmentation between C-9 and C-11, and between C-8 and C-14, as shown in Fig. 1, since such cleavage is characteristic of pentacyclic triterpenoids having a double bond at C-12 like a urs-12-ene skeleton (Budzikiewicz, Wilson & Djerassi, 1963; Ngninzeko, David & Lucas, 1987). Formation of the other fragment ions was explained by fragmentation of this ion. Further, the fragment ion at *m*/*z* 262 revealed that neither of the four hydroxyl groups were present in the rings C, D and E, consequently these hydroxyl groups must be attached on the rings A and/or B. The position of the hydroxyl groups was established on the basis of HMBC and HMQC spectra of **1**.

In the HMBC spectrum (Fig. 2), the hydroxymethylene protons H-24 or H-23 showed a correlation with

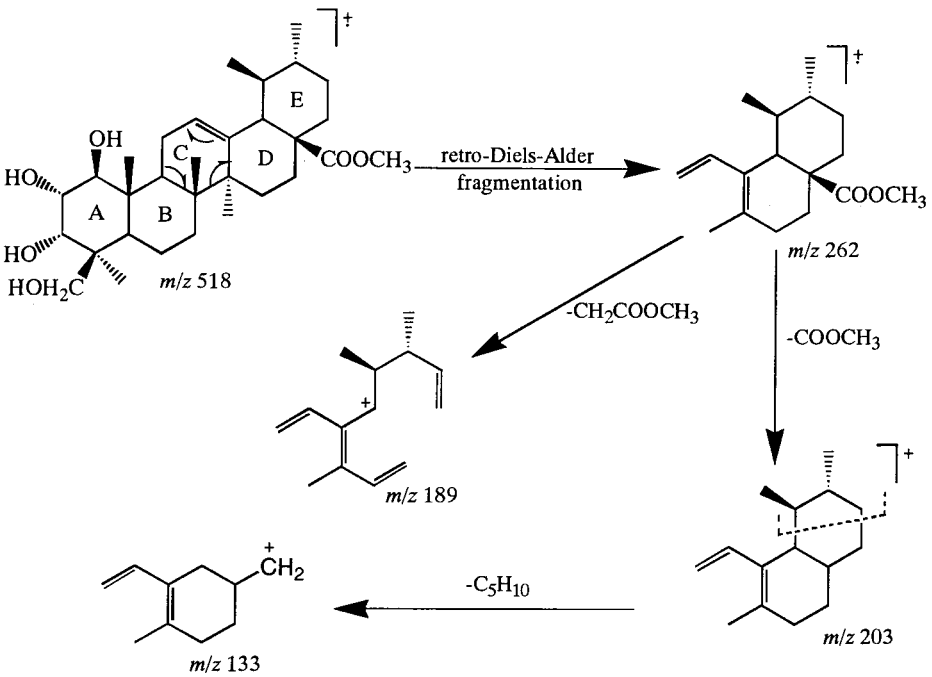

Fig. 1. Proposed EI mass spectral fragmentation of compound **1a**.

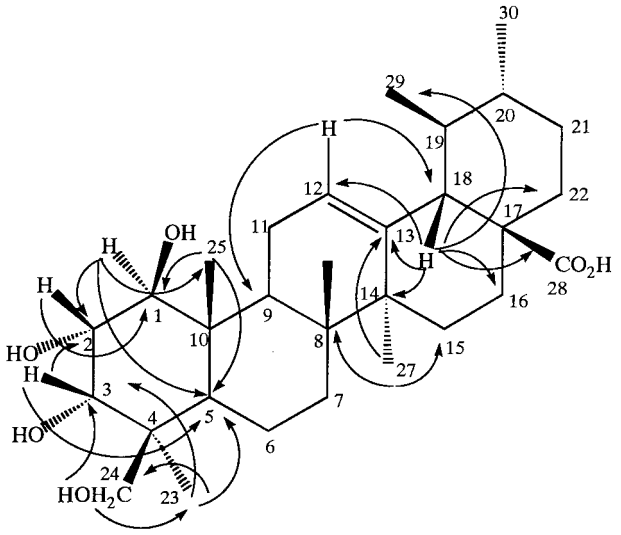

Fig. 2. Three and two-bond correlations for compound **1** in the HBMC spectrum.

the C-23 or C-24 and with a methine carbon at δ 73.2. The methine carbon was linked to a methine proton at δ 3.85 in the HMQC spectrum, which was assigned to the H-3. The H-3 was correlated with the methine carbon signals at δ 47.6 (C-5) and δ 69.7 (C-2) through three bonds and two bonds, respectively, in the HMBC spectrum, so the methine carbon was assigned to the C-2. The double doublets at δ 3.65, assigned to H-2, were two bonds away from a methine carbon at δ 79.4 (C-1). The methine proton at δ 3.50 which was assigned to H-1 was confirmed to be attached to the methine carbon C-1 in the HMQC spectrum. Both the C-5 and the C-25 showed a correlation with the H-1 through three bonds in the HMBC spectrum, and this proton had also a correlation with the C-2. All these correlations indicated clearly that the four hydroxyl groups were attached to C-1, C-2, C-3, and C-24 or C-23 of the ring A. In the ^{13}C NMR spectrum of **1**, the signal corresponding to the hydroxymethylene carbon C-24 or C-23 appeared at δ 63.7. It is known that in the ^{13}C NMR spectra of cyclic triterpenoids the signal due to an axial hydroxymethylene carbon at C-4 appears upfield shifted at δ 63–66 than an equatorial hydroxymethylene carbon at C-4, 68–71 (Kojima & Ogura, 1989; Zhang & Yang, 1994; Ahmed, Bano & Bano, 1986). Therefore, the hydroxymethylene carbon was assigned to the axial C-24, meaning that the tertiary methyl group at δ 1.10 in the ^{1}H NMR is the H-23. The chemical shifts for the protons H-2 and H-3 were within the range for a β axial-β equatorial position (Kojima & Ogura, 1989), and the values of the coupling constant for the H-1 and H-2, and H-2 and H-3 showed α axial-β axial and β axial-β equatorial couplings, respectively. A calculation using the MNDO program showed that the 1β-hydroxyl group is close to the H-11β, the distance is about 2.13 Å, which explains the low chemical shift δ 2.50 observed for the methylene proton in the ^{1}H NMR spectrum. Thus, the structure of a new phytoalexin **1** was identified as 1β,2α,3α,24-tetrahydroxyurs-12-en-28-oic acid.

Another triterpene **2** gave a monomethyl ester **2a** on treatment with diazomethane. Compound **2a** had the same molecular formula $C_{31}H_{50}O_6$ as compound **1a**, and its mass spectrum was identical to that of **1a**. A ^{1}H NMR spectrum of **2** was similar to that of **1**, but six methyl singlets were observed instead of the four methyl singlets and two secondary methyl groups as in **1**, and H-18 appeared as double doublets instead of a doublet in **1**. These showed that **2** has an olean-12-ene skeleton. Signals corresponding to H-1, H-2, H-3 and H-24 showed the similar chemical shifts and the same multiplicities as **1** in the ^{1}H NMR spectrum, indicating that **2** has the same substitutions at the same position on the ring A as those of **1**. From these data, the structure of **2** was elucidated as 1β,2α,3α,24-tetrahydroxyolean-12-en-28-oic acid.

Triterpenoids with four hydroxyl groups in the ring A have been already reported in the literature, but those compounds were accompanied with a 19α-hydroxyl group in the ring E in the case of compounds with an ursane skeleton (Zhang & Yang, 1994), and with a 3β-hydroxyl group in the case of compounds with oleanane and ursane skeletons (Zhang & Yang, 1994; Gupta & Singh, 1989). The antimicrobial activity reported here for tetrahydroxy-triterpenoids is the first example as far as we now.

Compound **3** is known to occur as a constitutive glucosyl ester in *Polygala japonica Houtt* (Fang & Ying, 1986), so compounds **1**–**7** might be present as glucosyl esters or glucosides in the fruits before treatment, and released by action of β-glucosidase after infection by fungi (Schonbeck & Schlosser, 1976). Aqueous material from non-treated fruits was hydrolysed with β-glucosidase to examine the presence of the conjugates. However, compounds **1**–**7** were not detected, indicating that the conjugates do not occur in the non-treated fruits. The activities of **1**–**7** meant that 1,2,3,24-tetrahydroxytriterpenoids have higher activities than 2,3,24-trihydroxytriterpenoids and 2,3-dihydroxytriterpenoids. This suggested another possibility that the triterpenoids with low degree of oxidation may occur as precursors in the fruits before treatment. In order to examine this possibility, material that was soluble in an EtOAc fraction from non-treated fruits was analysed, and ursolic and oleanolic acids were found (Kojima & Ogura, 1986). This finding suggested that the fruit might induce enzymes hydroxylating C-1, C-2 and C-24 when the fruit is exposed to micro-organism, in order to produce more active triterpenoids than triterpenoids with low degree of oxidation.

A pentacyclic triterpenoid has been reported as a phytoalexin from the sapwood of apple infected with *Chondrostereum purpureum Pouzar* (Kemp, Holloway & Burden, 1985). However, triterpenoids are rarely recognised as phytoalexins. The phytoalexins from nectarine fruit are the second example as far as we know.

3. Experimental

3.1. Instruments

^{1}H and ^{13}C NMR, and HMQC and HMBC: TMS as international standard using Bruker ARX 500 (500 MHz) and AC 300 (300 MHz); MS: JEOL JMS-600H; IR: Shimadzu FTIR-8100 AI spectrometer; Optical Rotation: JASCO DIP-1000 polarimeter.

3.2. Materials

Unripe fruits of *P. persica* cv Fantasia were collected in Okayama prefecture, Japan on 16 July 1997. *C. musae* Berk and Curt Arx. Strain No. 1679 was obtained from Department of Scientific and Research, Mount Albert Research Centre, Auckland, New Zealand, and cultured on potato–sucrose–agar medium at 23°C in darkness.

3.3. Treatments and extraction

Unripe fruits, 120, were washed with water, wiped with 70% ethanol, and rubbed with sand-paper (G 60 or 80). Conidia of the fungus were suspended in sterilised water at density 6×10^6 ml^{-1}. The injured fruits were soaked in the suspension of conodia in a plastic bag for 10 s, and incubated in the plastic box at 25°C in darkness for 4 days. Ten unripe fruits were incubated under the same conditions without wounding and inoculation. After incubation, the peels of non-treated and treated fruits, 80 and 768 g, respectively, were soaked in MeOH for 3 days at room temp. The MeOH extracts were filtered, and used for the bioautography and isolation of compounds.

3.4. Bioautography

The filtered MeOH extracts corresponding to 0.2 g fresh weight of the peels, 0.4 mg for the non-treated fruits, 2.2 mg for the wounded fruits and 2.1 mg for the wounded and inoculated fruits, were applied to thin layer silica gel plate, and developed with $CHCl_3$–acetone (4:6) until 11 cm. After drying the solvent, conidia of *C. musae* strain No. 1679 suspended in a Czapek-Dox medium at a density of 10^6 ml^{-1} were sprayed on the thin layer plate, which was then incubated in a moist chamber at 23°C for 2 days in darkness. Antifungal zones lacking aerial mycelia were detected by the absence of hyphae turning brown on exposure of the plate to iodine vapour.

3.5. Isolation

The MeOH extract from the treated fruits was concentrated to give 100 ml of an aqueous solution, and the solution was extracted with EtOAc three times. The EtOAc extract was concentrated to dryness to give 1.9 g of crude material which was subjected to silica gel (100 g) column. Elution was carried out using gradients of *n*-hexane–EtOAc, EtOAc, and MeOH.

The material eluted with *n*-hexane–EtOAc (4:6) was chromatographed on silica gel (8 g) column using gradient of *n*-hexane–EtOAc (6:4), and the fraction containing compounds **6** and **7** was applied to prep. TLC developed with *n*-hexane–EtOAc (1:1). The material at an R_f 0.7 was recovered and purified by HPLC in an A-311 column (ODS, 6 × 100 mm; YMC, Kyoto, Japan), eluting with CH_3CN–H_2O (6:4) at a flow rate of 1.0 ml min^{-1} with detection at 205 nm. The materials eluted at R_t 14.6 min and at R_t 16.0 min were collected, and concentrated to afford **6** (4.8 mg) and **7** (2.8 mg), respectively, as white powder.

The material eluted with 100% EtOAc was subjected to a silica gel (50 g) column eluted with gradients of $CHCl_3$–acetone to give three fractions. The first one which was eluted with 40% acetone was chromatographed on silica gel (5 g) column using gradients of $CHCl_3$–MeOH. The material eluted with 5% MeOH was applied to a Sephadex LH-20 (8 g) column, previously equilibrated with *n*-hexane–$CHCl_3$–MeOH (2:1:1), and was eluted with the same mixture. Fractions containing **5** were concentrated, and purified by prep. TLC developed with a mixture of $CHCl_3$–MeOH (9:1) to give **5** (7 mg). The second fraction which was eluted with 100 ml of 60% acetone was submitted to a chromatographic separation on a silica gel (8 g) column eluted with gradients of $CHCl_3$–MeOH. The material eluted with 5% MeOH was subjected to a Sephadex LH-20 (8 g) column eluted with *n*-hexane–$CHCl_3$–MeOH (2:1:1). Fractions containing **1** and **2** were combined, and injected to an A-311 column (ODS, 6 × 100 mm; YMC) eluted with CH_3CN–H_2O (35:65) at a flow rate of 1.0 ml min^{-1} with detection at 205 nm. The materials eluted at R_t 20.8 min and at R_t 23.3 min were collected, and concentrated to give **1** (1.2 mg) and **2** (1.0 mg), respectively, as white powder. The third fraction which was eluted with further 50 ml of 60% acetone was concentrated, methylated with CH_2N_2, and chromatographed in an A-311 column (ODS, 6 × 100 mm; YMC) eluted with CH_3CN–H_2O (55:45) at a flow rate of 1.0 ml min^{-1} with detection at 205 nm. The materials eluted at R_t 25.8 min and at R_t

26.8 min were collected to give **3a** (2.8 mg) and **4a** (0.7 mg), respectively.

3.6. 1β,2α,3α,24-Tetrahydroxyurs-12-en-28-oic acid (*1*)

^{1}H NMR (500 MHz, CD_3OD): δ 0.87 (3H, *s*, H-26), 0.92 (3H, *d*, *J* = 6.4 Hz, H-29), 1.01 (3H, *d*, *J* = 7.5 Hz, H-30), 1.02 (3H, *s*, H-25), 1.10 (3H, *s*, H-23), 1.17 (3H, *s*, H-27), 1.19–2.00 (17H, *m*, H-5, 6, 7, 9, 11α, 15, 16, 19, 20, 21, 22), 2.23 (1H, *d*, *J* = 9.6 Hz, H-18), 2.50 (1H, *m*, H-11β), 3.44 (1H, *d*, *J* = 11.4 Hz, H-24a), 3.50 (1H, *d*, *J* = 9.6 Hz, H-1), 3.65 (1H, *dd*, *J* = 9.6 and 3.1 Hz, H-2), 3.70 (1H, *d*, *J* = 11.4 Hz, H24b), 3.85 (1H, *d*, *J* = 3.1 Hz, H-3), 5.12 (1H, *t*, *J* = 3.3 Hz, H-12); ^{13}C NMR (125 MHz, CD_3OD): δ 79.4 (C-1), 69.7 (C-2) 73.2 (C-3), 42.9 (C-4), 47.6 (C-5), 16.2 (C-6), 32.8 (C-7), 39.5 (C-8), 42.3 (C-9), 38.6 (C-10), 26.1 (C-11), 125.6 (C-12), 137.0 (C-13), 47.9 (C-14), 27.4 (C-15), 36.3 (C-16), 41.2 (C-17), 52.5 (C-18), 30.0 (C-19), 23.5 (C-20), 27.4 (C-21), 38.5 (C-22), 21.1 (C-23), 63.7 (C-24), 11.6 (C-25), 15.7 (C-26), 22.1 (C-27), 178.0 (C-28), 19.8 (C-29), 17.4 (C-30). The distance between 1β-OH and 11β-H of **1** was measured after minimisation of its energy by an MNDO method of CS Chem3D Pro version 3.5.1 (Cambridge Soft Corporation, Massachusetts, USA).

3.7. Methylation of compound *1*

Compound **1** (1.2 mg) was dissolved in 0.8 ml of MeOH, and treated with an ether soln of CH_2N_2 at room temp. for 2 h. After evaporation of the solvent, 1.2 mg of methyl ester **1a** was obtained. IR ν_{max} cm^{-1}: 3400, 2700, 1750, 1600, 1450; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 518 $[M]^+$ (7), 500 (50), 262 (92), 203 (100), 189 (35), 133 (47); HR–EIMS (probe) 70 eV: $[M]^+$ at *m*/*z* 518.3605 ($C_{31}H_{50}O_6$ requires 518.3607); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.73 (3H, *s*, H-25), 0.87 (3H, *d*, *J* = 7.2 Hz, H-29), 0.88 (3H, *s*, H-23 and H-26), 0.97 (3H, *d*, *J* = 11.6 Hz, H-30), 1.09 (3H, *s*, H-23), 1.13 (3H, *s*, H-27), 1.20–2.02 (17H, *m*, H-5, 6, 7, 9, 11α, 15, 16, 19, 20, 21, 22), 3.47 (1H, *d*, *J* = 9.5 Hz, H-1), 3.51 (1H, *d*, *J* = 12.0 Hz, H-24a), 3.66 (3H, *s*, 17-COOMe), 3.70 (2H, *m*, H-24b and H-2), 3.97 (1H, *br s*, H-3), 5.25 (1H, *br s*, H-12).

3.8. Acetylation of compound *1a*

Compound **1a** (1.0 mg) was treated with 1 ml of Ac_2O–pyridine (1:1). The reaction mixture was kept for 36 h at room temp. The solution was treated as usually and the organic fraction was concentrated to give a tetra-acetate (**1b**). $[\alpha]_D^{31} + 27.0^\circ$ (MeOH; *c* 0.06); EIMS (probe) 70 eV, *m*/*z* (rel. int.): 686 $[M]^+$ (3), 627 (60), 626 (100), 566 (58), 506 (62), 262 (39), 203 (51); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, *s*, H-25), 0.26 (3H, *s*,H-26), 0.85 (3H, *d*, *J* = 6.4 Hz, H-29), 0.96 (3H, *d*, *J* = 6.7 Hz, H-30), 1.11 (3H, *s*, H-23), 1.18 (3H, *s*, H-27), 1.25–1.78 (18H, *m*, H-5, 6, 7, 9, 11, 15, 16, 19, 20, 21, 22), 1.93, 1.98, 2.09 and 2.16 (3H each, *s*, 4 × OAc), 3.60 (3H, *s*, 17-COOMe), 4.07 (1H, *d*, *J* = 11.6 Hz, H-24a), 4.25 (1H, *d*, *J* = 11.6 Hz, H-24b), 5.15 (2H, *bs*, H-2 and H-1), 5.36 (2H, *br s*, H-12 and H-3).

3.9. 1β,2α,3α,24-Tetrahydroxyolean-12-en-28-oic acid (*2*)

^{1}H NMR (300 MHz, CD_3OD): δ 0.84 (3H, *s*, H-26), 0.93 (3H, *s*, H-29), 0.98 (3H, *s*, H-30), 1.01 (3H, *s*, H-23), 1.21 (3H, *s*, H-27), 1.32-2.13 (17H, *m*, H-5, 6, 7, 9, 11α, 15, 16, 19, 21, 22), 2.88 (1H, *dd*, *J* = 9.0 and 3.5 Hz, H-18), 2.46 (1H, *m*, H-11β), 3.44 (1H, *d*, *J* = 11.4 Hz, H-24a), 3.48 (1H, *d*, *J* = 9.7 Hz, H-1), 3.64 (1H, *dd*, *J* = 9.7 and 3.1 Hz, H-2), 3.70 (1H, *d*, *J* = 11.4 Hz, H-24b), 3.85 (1H, *d*, *J* = 3.1 Hz, H-3), 5.26 (1H, *t*, *J* = 3.2 Hz, H-12); ^{13}C NMR (75 MHz, CD_3OD): δ 79.2 (C-1), 69.7 (C-2), 73.2 (C-3), 42.9 (C-4), 47.8 (C-5), 22.3 (C-6), 33.2 (C-7), 39.2 (C-8), 42.3 (C-9), 31.7 (C-10), 26.2 (C-11), 122.2 (C-12), 142.7 (C-13), 47.6 (C-14), 27.0 (C-15), 32.0 (C-16), 40.8 (C-17), 52.5 (C-18), 24.5 (C-19), 32.4 (C-20), 28.8 (C-21), 29.7 (C-22), 21.1 (C-23), 63.7 (C-24), 11.4 (C-25), 16.1 (C-26), 12.1 (C-27), 178.0 (C-28), 17.5 (C-29), 16.1 (C-30).

3.10. Methylation of compound *2*

Compound **2** (1.0 mg) was methylated by the same method as that for **1** to give a methyl ester **2a**. IR ν_{max} cm^{-1}: 3300, 1730, 1600, 1100; EIMS (probe) 70 eV, *m*/*z* (rel. int.): 518 $[M]^+$ (10), 500 (20), 262 (62), 203 (100), 189 (36), 133 (21); HR-EIMS (probe) 70 eV: $[M]^+$ at *m*/*z* 518.3548 ($C_{31}H_{50}O_6$ requires 518.3607); ^{1}H NMR (300 MHz, $CDCl_3$); δ 0.70 (3H, *s*, H-26), 0.85 (3H, *s*, H-29), 0.89 (3H, *s*, H-30), 0.93 (3H, *s*, H-25), 0.97 (3H, *s*, H-23), 1.14 (3H, *s*, H-27), 1.32–2.13 (17H, *m*, H-5, 6, 7, 9, 11α, 15, 16, 19, 21, 22), 2.85 (1H, *br d*, *J* = 11.0 Hz, H-18), 3.47 (1H, *d*, *J* = 9.5 Hz, H-1), 3.51 (1H, *d*, *J* = 12.0 Hz, H-24a), 3.66 (3H, *s*, 17-COOMe), 3.70 (2H, *m*, H-2 and H-24b), 3.97 (1H, *br s*, H-3), 5.28 (1H, *br s*, H-12).

3.11. Acetylation of compound *2a*

Compound **2a** (0.8 mg) was acetylated by the same method as that for **1a** to give a tetra-acetate **2b**. $[\alpha]_D^{31} + 25.0^\circ$ (MeOH; *c* 0.07); EIMS (probe) 70 eV, *m*/*z* (rel. int.): 686 $[M]^+$ (7), 627 (77), 626 (100), 566 (71), 506 (68), 262 (45), 203 (53); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.72 (3H, *s*, H-26), 0.88 (3H, *s*, H-29), 0.90 (6H, *s*, H-30 and H-25), 0.93 (3H, *s*, H-23), 0.95 (3H, *s*, H-27), 1.25–1.78 (18H, *m*, H-5, 6, 7, 9, 11, 15, 16,

19, 20, 21, 22), 1.93, 1.97, 2.01, 2.08 (3H each, *s*, 4 × OAc), 2.85 (1H, *br d, J* = 8.4 Hz, H-18), 3.63 (3H, *s*, 17-COOMe), 4.07 (1H, *d, J* = 11.6 Hz, H-24a), 4.21 (1H, *d, J* = 11.6 Hz, H-24b), 5.14 (2H, *bs*, H-2 and H-1), 5.36 (2H, *br s*, H-12 and H-3).

3.12. 2α,3α,24-Trihydroxyolean-12-en-28-acid methyl ester (**3a**)

$[\alpha]_D^{23} + 48.1^\circ$ (MeOH; *c* 0.28); EIMS (probe) 70 eV, *m/z* (rel. int.): 502 $[M]^+$ (2), 425 (5), 368 (5), 262 (93), 203 (100), 189 (32), 133 (26); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.69 (3H, *s*, H-26), 0.92 (3H, *s*, H-29), 0.92 (6H, *s*, H-30 and H-25), 1.13 (3H, *s*, H-23), 1.15 (3H, *s*, H-27), 2.86 (1H, *dd, J* = 13.8 and 4.2 Hz, H-18), 3.51 (1H, *d, J* = 11.2 Hz, H-24a), 3.62 (3H, *s*, 17-COOMe), 3.69 (1H, *d, J* = 11.2 Hz, H-24b), 3.85 (1H, *d, J* = 2.4 Hz, H-3), 3.97 (1H, *m*, H-2), 5.28 (1H, *t, J* = 3.5 Hz, H-12).

3.13. 2α,3α,24-Trihydroxyurs-12-en-28-acid methyl ester (**4a**)

$[\alpha]_D^{23} + 56.5^\circ$ (MeOH; *c* 0.04); EIMS (probe) 70 eV, *m/z* (rel. int.): 502 $[M]^+$ (14), 262 (100), 203 (90), 189 (19), 133 (45); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, *s*, H-26), 0.85 (3H, *d, J* = 6.4 Hz, H-29), 0.94 (3H, *s*, H-25), 0.95 (3H, *d, J* = 2.7 Hz, H-30), 1.09 (3H, *s*, H-23), 1.15 (3H, *s*, H-27), 2.23 (1H, *d, J* = 11.7 Hz, H-18), 3.51 (1H, *d, J* = 11.0 Hz, H-24a), 3.62 (3H, *s*, 17-COOMe), 3.69 (1H, *d, J* = 11.0 Hz, H-24b), 3.86 (1H, *s*, H-3), 3.97 (1H, *m*, H-2), 5.26 (1H, *t, J* = 3.4 Hz, H-12).

3.14. 2α,3β,24-Trihydroxyolean-12-en-28-oic acid (**5**) and its methyl ester (**5a**)

Compound **5**: $[\alpha]_D^{24} + 38.3^\circ$ (MeOH; *c* 0.30); ^{1}H NMR (300 MHz, CD_3OD): δ 0.87 (3H, *s*,H-26), 0.91 (3H, *s*, H-29), 0.96 (3H, *s*, H-30), 0.98 (3H, *s*, H-25), 1.17 (3H, *s*, H-23), 1.26 (3H, *s*, H-27), 2.94 (1H, *dd, J* = 9.3 and 3.8 Hz, H-18), 3.07 (1H, *d, J* = 9.3 Hz, H-3), 3.41 (1H, *d, J* = 11.2 Hz, H-24a), 3.67 (1H, *m*, H-2), 4.06 (1H, *d, J* = 11.2 Hz, H-24b), 5.25 (1H, *br s*, H-12). Compound **5** (0.5 mg) was methylated with CH_2N_2 to give a methyl ester **5a**. EIMS (probe) 70 eV, *m/z* (rel. int.): 502 $[M]^+$ (12), 484 (6), 466 (5), 262 (100), 203 (100), 189 (80), 133 (75), 119 (43); HR-EIMS (probe) 70 eV: $[M]^+$ at *m/z* 502.3669 ($C_{31}H_{50}O_5$ requires 502.3658); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H, *s*, H-25), 0.90 (3H, *s*,H-26), 0.91(3H, *s*, H-29), 0.93 (3H, *s*,H-30), 1.07 (3H, *s*, H-23), 1.21 (3H, *s*, H-27), 2.87 (1H, *br d, J* = 13.9 Hz, H-18), 3.16 (1H, *d, J* = 8.6 Hz, H-3), 3.38 (1H, *d, J* = 10.5 Hz, H-24a), 3.60 (3H, *s*, 17-COOMe), 3.88 (1H, *m*, H-2), 4.12 (1H, *d, J* = 10.5 Hz, H-24b), 5.28 (1H, *br s*, H-12).

3.15. 2α,3α-Dihydroxyolean-12-en-28-oic acid (**6**) and its methyl ester (**6a**)

Compound **6**: $[\alpha]_D^{23} + 146^\circ$ (MeOH; *c* 0.070); ^{1}H NMR (300 MHz, CD_3OD): δ 0.86 (3H, *s*, H-26), 0.89 (3H, *s*, H-29), 0.93 (3H, *s*, H-23), 0.98 (3H, *s*, H-30), 1.02 (6H, *s*, H-24 and H-25), 1.20 (3H, *s*, H-27), 2.91 (1H, *br d, J* = 10.4 Hz, H-18), 3.34 (1H, overlapping with methanol), 3.95 (1H, *m*, H-2), 5.27 (1H, *br s*, H-12). Compound **6** (0.7 mg) was treated with CH_2N_2 to give a monomethyl ester **6a**. EIMS (probe) 70 eV, *m/z* (rel. int.): 486 $[M]^+$ (6), 262 (90), 203 (100), 189 (25), 129 (25); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.71 (3H,*s*, H-26), 0.88 (3H, *s*, H-29), 0.92 (3H, *s*, H-23), 0.95 (3H, *s*, H-30), 1.01 (3H, *s*, H-24), 1.13 (3H, *s*, H-25), 1.17 (3H, *s*, H-27), 2.86 (1H, *dd, J* = 13.6 and 4.1 Hz, H-18), 3.43 (1H, *br s*, H-3), 3.62 (3H, *s*, 17-COOMe), 4.00 (1H, *m*, H-2), 5.28 (1H, *t, J* = 3.6 Hz, H-12).

3.16. 1α, 2α-Dihydroxyurs-12-en-28-oic acid (7) and its methyl ester (**7a**)

Compound **7**: $[\alpha]_D^{21} + 25.6^\circ$ (MeOH; *c* 0.050); ^{1}H NMR (300 MHz, CD_3OD): δ 0.89 (3H, *s*, H-26), 0.90 (3H, *s*, H-23), 0.92 (3H, *d, J* = 6.6 Hz, H-29), 1.01 (3H, *d, J* = 7.2 Hz, H-30), 1.02 (3H, *s*, H-24), 1.04 (3H, *s*, H-25), 1.16 (3H, *s*, H-27), 2.26 (1H, *d, J* = 11.1 Hz, H-18), 3.34 (1H, overlapping with CD_3OD), 3.96 (1H, *m*, H-2), 5.26 (1H, *t, J* = 3.4 Hz, H-12). Compound **7** (1.0 mg) was methylated with CH_2N_2 to give a monomethyl ester **7a**. EIMS (probe) 70 eV, *m/z* (rel. int.): 486 $[M]^+$ (21), 262 (100), 263 (100), 203 (100), 189 (95), 133 (100), 119 (65); ^{1}H NMR (300 MHz, $CDCl_3$): δ 0.73 (3H, *s*, H-26), 0.85 (6H, *d, J* = 5.6 Hz, H-29 and H-23), 0.96 (3H, *d, J* = 5.4 Hz, H-30), 1.02 (3H, *s*, H-24), 1.09 (3H, *s*, H-25); 1.23 (3H, *s*, H-27), 2.33 (1H, *d, J* = 9.6 Hz, H-18), 3.43 (1H, *br s*, H-3), 3.60 (3H, *s*, 17-COOMe), 4.00 (1H, *m*, H-2), 5.25 (1H, *t, J* = 3.5 Hz, H-12).

3.17. Enzymatic hydrolysis

The MeOH extract from non-treated fruits was concentrated, and partitioned between H_2O and EtOAc by the same method as for the treated fruits. The aqueous layer was concentrated to give a gummy syrup (1.4 g), and this was divided into two equal parts. One part was dissolved in 40 ml of 100 mM AcOH–AcONa buffer soln (pH 5.0), and 10 ml of the buffer soln containing 1000 units of β-glucosidase (Sigma, from almonds) was added into the soln. This soln was incubated for 3 h at 37°C, and then its pH was adjusted to 3 with 2 N HCl. The solution was saturated with NaCl, and partitioned with 70 ml of EtOAc three times. The EtOAc layer was washed with a small amount of H_2O, and concentrated to give an oil

(2.3 mg). Another part was dissolved in 50 ml of the same buffer, and then partitioned with EtOAc after acidification by the same method as for the first part to give an oil (2.1 mg). These materials from the EtOAc layers were analysed with a silica gel TLC developed with *n*-hexane–EtOAc (3:2).

3.18. Isolation of oleanolic and ursolic acids from non-treated fruits

The organic layer (160 mg) from the MeOH extract of non-treated fruits was subjected to a silica gel (10 g) column eluted with gradients of *n*-hexane–EtOAc, and MeOH. The material eluted with *n*-hexane–EtOAc (9:1) was applied to a Sephadex LH-20 (4 g) column, previously equilibrated with *n*-hexane–$CHCl_3$–MeOH (2:1:1), using as eluent the same mixture of solvent, and the mixture of ursolic and oleanolic acids was purified by HPLC in an A-311 column (ODS, 6 × 100 mm; YMC), eluting with CH_3CN–H_2O (85:15) at a flow rate of 1.0 ml min^{-1} with detection at 205 nm. The materials eluted at R_t 7.8 min and at R_t 8.4 min were collected to give oleanolic acid (3.8 mg) and ursolic acid (4.8 mg), respectively. Ursolic and oleanolic acids were identified on the basis of ^1H NMR data and by EI mass spectra of their monomethyl esters (Kojima & Ogura, 1986).

Acknowledgements

We thank Dr Hideo Nasu (the Agricultural Experimental Station of Okayama Prefecture, Okayama, Japan) for supplying nectarine fruits.

References

Ahmad, V. U., Bano, S., & Bano, N. (1986). *Phytochemistry*, *25*, 1487.
Budzikiewicz, H., Wilson, J. M., & Djerassi, C. (1963). *Journal of American Chemical Society*, *85*, 3688.
Cheung, H. T., & Yan, T. C. (1970). *Journal of Chemical Society Chemical Communications*, 369.
Dixon, R. A., & Harrison, M. J. (1994). *Annual Review of Phytopathology*, *32*, 479.
Fang, Z., & Ying, G. (1986). *Zhiwu Xuebao*, *28*, 196.
Gupta, D., & Singh, J. (1989). *Phytochemistry*, *28*, 1197.
Hirai, N., Ishida, H., & Koshimizu, K. (1994). *Phytochemistry*, *37*, 383.
Kamo, T., Kato, N., Hirai, N., Tsuda, M., Fujioka, D., & Ohigashi, H. (1998a). *Bioscience, Biotechnology and Biochemistry*, *62*, 95.
Kamo, T., Kato, N., Hirai, N., Tsuda, M., Fujioka, D., & Ohigashi, H. (1998b). *Phytochemistry*, *49*, 1617.
Kemp, M. S., Holloway, P. J., & Burden, R. S. (1985). *Journal of Chemical Research (S)*, 154.
Kojima, H., & Ogura, H. (1986). *Phytochemistry*, *25*, 729.
Kojima, H., & Ogura, H. (1989). *Phytochemistry*, *28*, 1703.
Kuc, J. (1994). *Acta Horticulturae*, *381*, 526.
Ngninzeko, F. N., David, L., & Lucas, S. (1987). *Phytochemistry*, *27*, 301.
Schonbeck, F., & Schlosser, E. (1976). Heitefuss, R., & Williams, P. H. (Ed.), *Encyclopedia of plant physiology* (4, p. 662). Springer Berlin.
Simmonds, J. H. (1965). *Queensland Journal of Agriculture and Animal Sciences*, *22*, 437.
Yamagishi, T., Zhang, D., Chang, J., Mcphail, D. R., Mcphail, A. T., & Lee, K. (1988). *Phytochemistry*, *27*, 3213.
Zhang, Y., & Yang, C. (1994). *Phytochemistry*, *36*, 997.