Production of Triterpene Acids by Cell Suspension Cultures of *Olea europaea*

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Olive (*Olea europaea*) contains large quantity of triterpene acids including oleanolic acid (6) as a major one. Varieties of biological activities exhibited by triterpene acids attracted our attentions, especially from pharmaceutical viewpoints. Cell culture of olive plant was induced and its triterpene constituents were studied. From the cell suspension cultures, six ursane type triterpene acids; ursolic acid (9), pomolic acid (10), rotundic acid (11), tormentic acid (12), 2α-hydroxyursolic acid (13) and 19α-hydroxyasiatic acid (14), and two oleanane type acids; oleanolic acid and maslinic acid (7), have been isolated. Quantity of ursane type triterpene acids produced by cell cultures was larger than that of oleanane type. Further, a multifunctional oxidosqualene cyclase (OSC) named OEA was cloned by homology based PCRs from the same cultured cells. Major product of OEA is α-amyrin (ursane skeleton), showing good accordance to higher content of ursane-type triterpene acids in the cultured cells, and strongly suggesting OEA to be a major contributor OSC for their production.

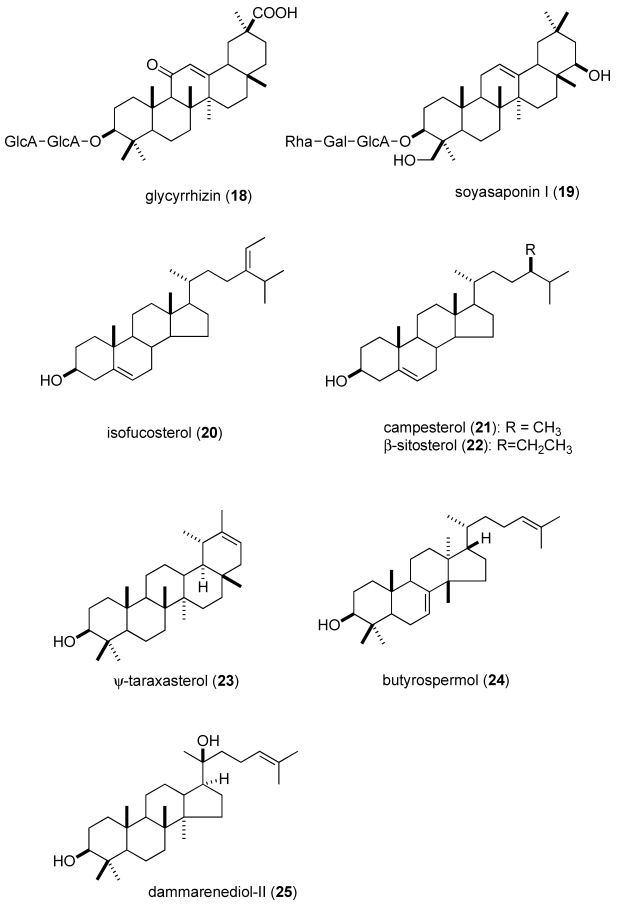
Key words *Olea europaea*; cell suspension culture; triterpene acid; oxidosqualene cyclase; α-amyrin; β-amyrin

Olive (*Olea europaea*) is a well-known evergreen tree native to the Mediterranean coast, whose fruits and oil have been used for foods and cooking. Olive contains large quantity of triterpene acids including oleanolic acid (**6**) as a major one.[1,2] Recently, much attention has been paid for triterpene acids from pharmaceutical viewpoints because of their anti-HIV,[3] anti-inflammatory,[4] anti-tumor-promoting[5] activities and antagonist activity for an endothelin receptor,[6] *etc*. Wax of olive fruits contains predominantly oleanolic acid,[1] whereas that of the leaves contains a mixture of oleanolic acid and betulinic acid (**16**) in a ratio of 7 : 2.[2] In addition to these triterpene acids, triterpene-diols, such as erythrodiol (**8**: oleanane type) and uvaol (**15**: ursane type) are also contained, even though in less quantity than the corresponding acids.[2]

Successful production of triterpene acids by plant cell cultures has been documented. Production of bryonolic acid (**17**), an anti-allergic triterpene acid, by cultured cells of several cucurbitaceous plants[7] is a good example. Cell cultures of plants, however, do not always produce the same secondary metabolites as those in the mother plants. Some callus cultures produce no trace of the metabolites found in the mother plants but produce those metabolites that are not produced by the mother plants. This situation is exemplified by *Glycyrrhiza glabra* cell cultures, which did not produce glycyrrhizin (**18**), a major triterpene saponin of the mother plant, but produced betulinic acid (**16**)[8] and soyasaponin I (**19**).[9] It is quite interesting to know what kinds and how much of triterpenes cultured cells produce. If they produce any particular ones in large quantity, such production system would benefit the supply of triterpene acids for clinical use, since purification and isolation of triterpenes from complex mixtures are generally laborious. Thus we induced cell cultures of *O. europaea* and investigated the production of triterpene metabolites.

Triterpenes are biosynthesized from a common precursor, (3*S*)-oxidosqualene (**1**). Over one hundred of diverse triterpene skeletons reported from nature[10] are constructed at the cyclization step of oxidosqualene catalyzed by oxidosqualene cyclase (OSC). Thus, the studies on their product specificity, in particular, would lead to better understanding of the structural diversity of natural triterpenes. As a continuation of our studies on OSCs[11] in this direction, cDNA clonig of OSCs from Olive cell cultures was also conducted.

In this paper, we report induction of cell cultures, isolation



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and identification of triterpene metabolites, and cDNA cloning of OSCs from *O. europaea* cell cultures.

Results and Discussion

Induction of Cell Cultures and Isolation of Triterpene Acids Callus of *O. europaea* was induced from leaf stalk in CM-NH_4 agar media which is a modified Murashige and Skoog[12] media supplemented with K^+ in place of original NH_4^+. Cell suspension culture was established from the callus with CM-NH_4 media at 25 °C. From 4 weeks old liquid culture, the cells (2 kg f.w.) were harvested and extracted with MeOH at room temperature. The MeOH extract was successively partitioned between EtOAc and H_2O, and *n*-BuOH and H_2O. The EtOAc fraction (3.7 g) and *n*-BuOH fraction (6.4 g) were separated by SiO_2 column chromatography and HPLC to yield eleven compounds, which were identified as ursolic acid (**9**, 5 mg), pomolic acid (**10**, 2 mg), rotundic acid (**11**, 17 mg), tormentic acid (**12**, 2 mg), 2α-hydroxyursolic acid (**13**, 3 mg), 19α-hydroxyasiatic acid (**14**, 10 mg), oleanolic acid (**6**, 6 mg), maslinic acid (**7**, 5 mg), isofucosterol (**20**, 7 mg), campesterol (**21**, 5 mg) and β-sitosterol (**22**, 83 mg) by ^{1}H- and ^{13}C-NMR spectra. Compounds **9**—**14** have ursane skeleton, compounds **6** and **7** oleanane skeleton, and compounds **20**—**22** sterol skeleton, respectively. Isolation yields of ursane type triterepene acid were higher than those of oleanane type. HPLC analysis of EtOAc fraction and *n*-BuOH fraction also showed higher content of ursane triterpenes than that of oleanane triterpenes in this cell culture (data not shown). As mentioned above, it is well known that olive contains dominantly oleanane triterpenes and a trace of ursane type. Indeed, our HPLC analysis of the extracts from fresh olive leaves or fruits of the mother plant showed only a trace of ursane type triterpenes (data not shown). That is, the cultured cells of *O. europaea* produce more ursane type triterpenes, which are minor in the mother plant, than oleanane type triterpenes.

cDNA Cloning of Oxidosqualene Cyclase Different spectra of triterpenes produced by cultured cells and the mother plant can be explained by differential expression of oxidosqualene cyclases. β-Amyrin synthase is likely to be expressed highly in the mother plant, whereas α-amyrin synthase in the cultured cells. As olive OSCs, only lupeol synthase OEW and cycloartenol synthase OEX have been reported from its leaves.[13] We carried out cDNA cloning from the cultured cells by homology based PCRs following the reported method.[14] PCR products with degenerate primers were subcloned into plasmid vector and eighteen colonies were picked up and sequenced. They consisted of fourteen cycloartenol synthase (identical to *OEX*[13]), two lupeol synthase (identical to *OEW*[13]) and two new sequences. These new sequences were identical each other and named *OEA*. The full-length sequence of *OEA* was obtained by RACE method.[15] *OEA* is composed of 2289 nucleotide open reading frame that encodes 763 amino acid long protein. *OEA* shows high sequence identities to the recently cloned dammarenediol-II synthase *PNA* (74%) from *Panax ginseng*. ORF of *OEA* was amplified by PCR and ligated to the cloning site of yeast expression vector pYES2. The resulting plasmid was transferred to lanosterol synthase deficient *Saccharomyces cerevisiae* strain GIL77,[14] and products from induction culture were analyzed by LC-APCI-MS. It showed the presence of α-amyrin (**3**), β-amyrin (**2**), ψ-taraxasterol (**23**) and butyrospermol (**24**) (Fig. 2). It is noteworthy that these products are identical to those of PSM, a multi-prod-

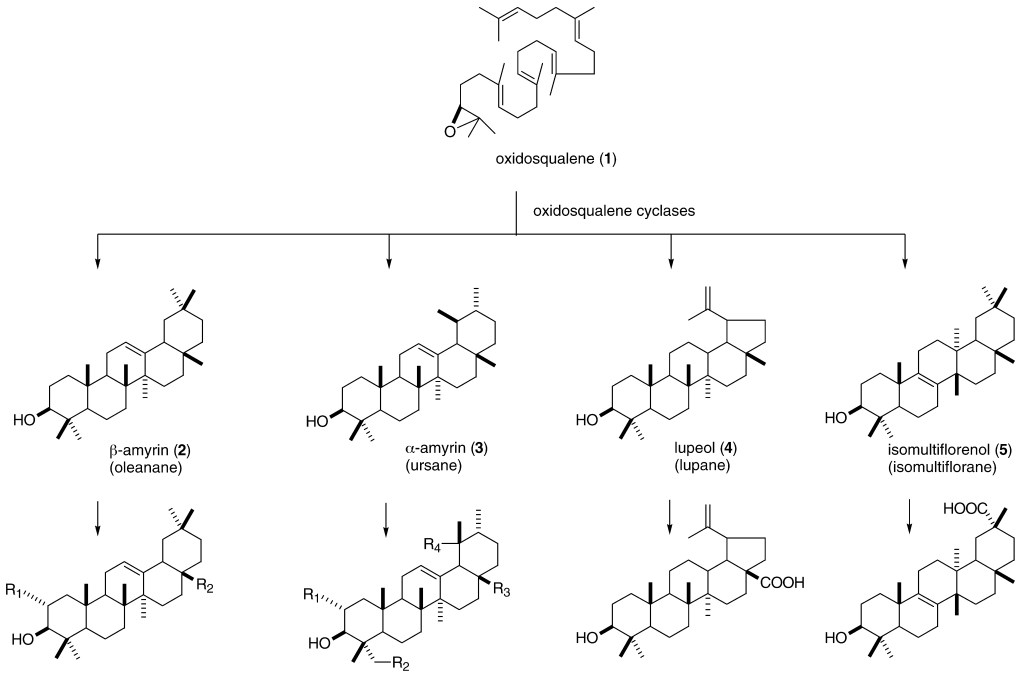

Fig. 1. Biosynthesis of Triterpenes

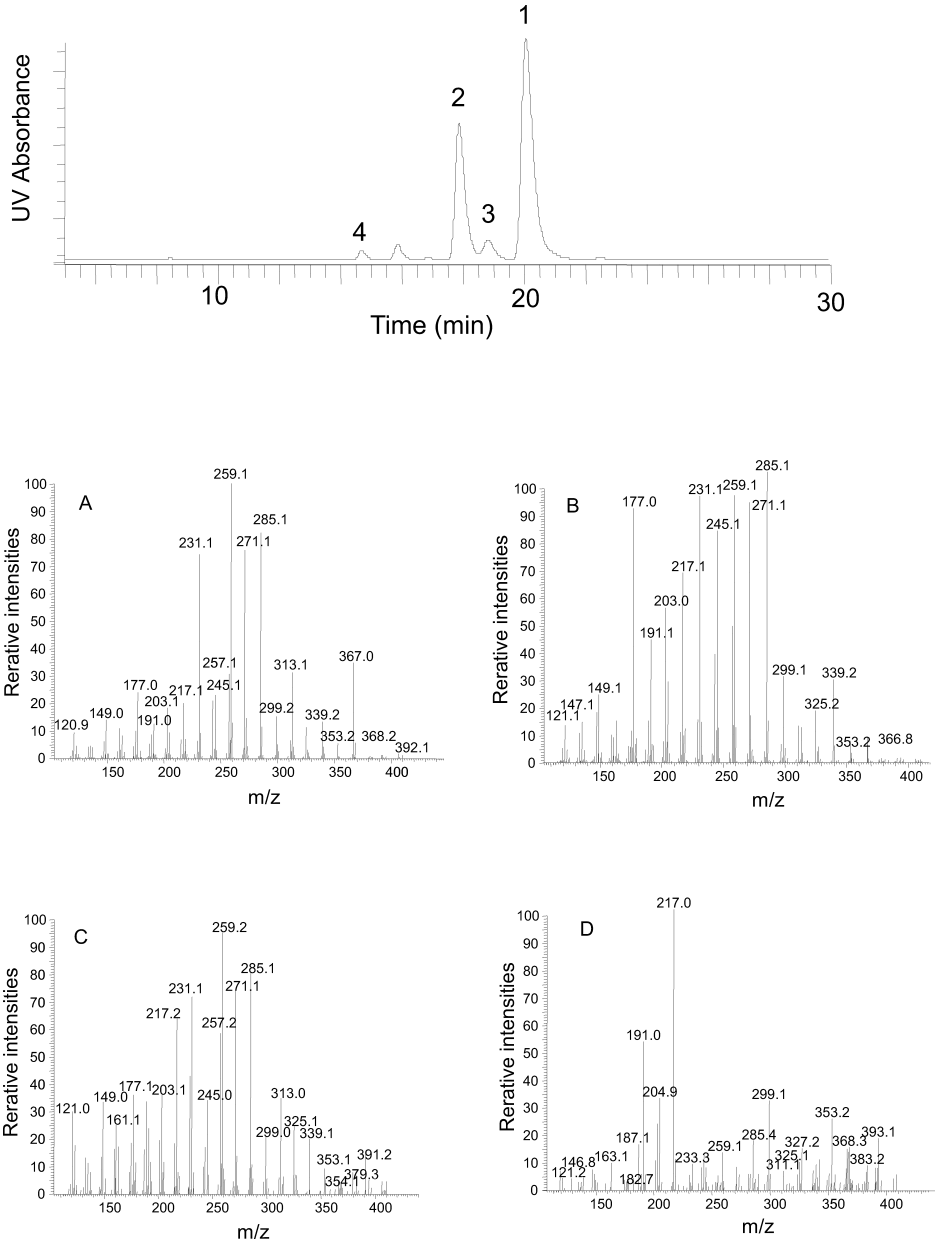

Fig. 2. Product Analysis of OEA by LC-APCI-MS

HPLC profile monitored by UV absorbance at 202 nm was shown in a top. MS/MS at m/z=409 ($M+H^+-H_2O$) for peaks 1—4 were shown in A—D, respectively. Peak 1—4 was identical to the authentic α-amyrin, β-amyrin, ψ-taraxasterol, and butyrospermol, in retention times and MS fragmentations, respectively.

uct-OSC from *Pisum sativum*. Major product of OEA is α-amyrin, showing good accordance to high production of ursane-type triterpene acids in the cultured cells. These facts strongly suggest that OEA is highly expressed in the cultured cells and a major contributor OSC for production of ursane-type triterpenes.

Origin of Ursane-Type Triterpenes in Higher Plants More than one hundred triterpene skeletons are constructed at the cyclization step of oxidosqualene catalysed by OSCs.[10] So far, over thirty OSCs of different product specificity have been cloned from the plant kingdom.[16] Some OSCs show high catalytic fidelity to produce single cyclization product, suggesting the existence of more than 100 different OSCs. However, this estimate should be greatly reduced by rather common occurrence of multiproduct synthases with leaky product specificity.[16] No OSC yielding α-amyrin as a sole product has been reported and all OSCs whose products include α-amyrin are multifunctional. They are OEA from *O. europaea* (this study), PSM from *Pisum sativum*,[17] At1g78960[18] and At1g78500[19,20] from *Arabidopsis thaliana*, *KcMS* from *Kandelia candel*,[21] and two OSCs from *Taraxacum officinale* (our unpublished results). Furthermore, most of the phytochemical studies reported the presence of ursane type triterpenes together with oleanane type triterpenes. These facts strongly suggest that α-amyrin is produced only by multifunctional enzymes like OEA and there may not be product specific α-amyrin synthase in nature. This is a sharp contrast to oleanane and lupane triterpenes, as β-amyrin and lupeol are produced not only by monofunctional enzymes but also by multifunctional en-

zymes, although ursane type triterpenes widely distribute in higher plants as well as oleanane and lupane type triterpenes.

It is interesting to note here that *OEA* shares significant amino acid sequence homology (74%) with *PNA*, a product specific dammarenediol-II synthase from *P. ginseng*. In contrast, it shows less homology (55%) to *PSM*, a multifunctional OSC from *P. sativum*, which yields the same products as PNA. Considering a trace occurrence of dammarenediol-II (**25**) in the fruits of *O. europaea*[22] and also in a closely related species *O. madagascariensis*,[23] *OEA* might be an intermediate OSC in the evolutional process from β-amyrin synthase to dammarenediol-II synthase.

Experimental

General Procedure HPLC was performed on a LC-10 system equipped with a SPD-10Avp photodiode array detector and an RID-10A refractive index detector (Shimadzu, Kyoto, Japan). ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra were measured by a JNM-LA500 spectrometer (JEOL, Tokyo, Japan). Electrospray ionization (ESI) MS were obtained by an Esquire 3000 plus (Bruker Daltonics, MA, U.S.A.).

Plant Material Leaf stalks of *O. europaea* were obtained from the olive tree which is cultivated in Experimental Station for Medicinal Plant Studies, the Univ. of Tokyo (Chiba, Japan).

Callus Induction of *O. europaea* and Subculture Sterilized fresh leaf stalks were placed on CM-NH_4 agar medium, which was a modified Murashige and Skoog (MS) agar medium with 30 g/l sucrose, 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.1 mg/l kinetin, 7% coconut milk, 9 g/l agar and supplemented with K^+ instead of NH_4^+ from MS agar medium, and kept at 25 °C in the dark for 4 weeks, to induce callus. Induced calli were subcultured every 4 weeks on the same medium at 25 °C in the dark.

Extraction and Isolation of Triterpenes from the Cultured Cells of *O. europaea* The olive cells (about 10 g) cultured for 4 weeks on CM-NH_4 agar medium were inoculated into 500 ml flasks containing 250 ml CM-NH_4 liquid medium and cultured on a rotary shaker (120 rpm) at 25 °C in the dark. After 4 weeks culture, the cells were harvested and fresh cells from twenty flasks (2 kg f.w.) were homogenized and extracted with MeOH twice at room temperature. The MeOH extract was concentrated and partitioned between EtOAc and H_2O. The EtOAc layer was evaporated to give EtOAc fraction (3.7 g). The H_2O layer was then partitioned between *n*-BuOH to give *n*-BuOH fraction (6.4 g). Both fractions were separated by SiO_2 column chromatography (MeOH/$CHCl_3$ gradient) and HPLC using MILLIPORE μ BONDSPHERE (5 μm, C_{18}, 100 Å, 19×150 mm) and MILLIPORE Prep Nova-Pak HR (6 μm, C_{18}, 60 Å, 7.8×300 mm), 75—100% MeOH (v/v), 1.6—5.0 ml/min to yield compounds **6**, **7**, **9**—**14** and **20**—**22**. ^{1}H- and ^{13}C-NMR spectra showed the identical data as reported.[24—31]

Ursolic Acid[24] (**9**, 5 mg): ^{1}H-NMR δ ($CDCl_3$): 0.71 (3H, s), 0.74 (3H, s), 0.79 (3H, d, J=6.0 Hz), 0.79 (3H, s), 0.84 (3H, d, J=6.0 Hz), 0.93 (3H, s), 1.01 (3H, s), 2.22 (1H, d, J=11.0 Hz, H-18), 3.14 (1H, dd, J=7.0, 6.5 Hz, H-3), 5.20 (1H, dd, J=3.5, 3.5 Hz, H-12). ^{13}C-NMR δ ($CDCl_3$): 15.0 (q), 15.4 (q), 16.5 (q), 16.7 (q), 18.0 (t), 20.8 (q), 22.9 (t), 23.2 (q), 24.0 (t), 26.9 (t), 27.8 (t), 27.9 (q), 30.4 (t), 32.7 (t), 36.5 (s), 36.5 (t), 38.3 (s), 38.4 (t), 38.5 (d), 38.8 (d), 39.1 (s), 41.7 (s), 47.2 (d), 47.3 (s), 52.6 (d), 54.9 (d), 78.0 (d), 124.8 (d), 138.2 (s), 179.9 (s). ESI-MS *m/z*: 457 $[M+H]^+$.

Pomolic Acid[25] (**10**, 2 mg): ^{1}H-NMR δ (C_5D_5N): 0.92 (3H, s, H-25), 1.02 (3H, s, H-24), 1.11 (3H, s, H-26), 1.11 (3H, d, J=6.5 Hz, H-30), 1.22 (3H, s, H-23), 1.44 (3H, s, H-29), 1.72 (3H, s, H-27), 3.06 (1H, s, H-18), 3.11 (1H, ddd, J=13.0, 12.5, 4.5 Hz, H-16), 3.42 (1H, dd, J=10.5, 4.5 Hz, H-3), 5.60 (1H, br dd, H-12). ^{13}C-NMR δ (C_5D_5N): 15.5 (q), 16.4 (q), 16.7 (q), 17.1 (q), 18.9 (t), 24.0 (t), 24.6 (q), 26.4 (t), 26.9 (t), 27.1 (q), 28.1 (t), 27.7 (q), 29.3 (t), 33.6 (t), 37.3 (s), 38.5 (t), 39.0 (t), 39.3 (s), 40.3 (s), 42.1 (s), 42.3 (d), 47.7 (d), 48.3 (s), 54.6 (d), 55.8 (d), 72.7 (s), 78.1 (d), 128.0 (d), 140.0 (s), 180.7 (s). ESI-MS *m/z*: 473 $[M+H]^+$.

Rotundic Acid[26] (**11**, 17 mg): ^{1}H-NMR δ (C_5D_5N): 1.00 (3H, s, H-26), 1.06 (3H, s, H-23), 1.10 (3H, s, H-25), 1.10 (3H, d, J=7.0 Hz, H-30), 1.42 (3H, s, H-29), 1.64 (3H, s, H-27), 2.32 (1H, ddd, J=13.5, 13.5, 5.0 Hz, H-15), 3.06 (1H, s, H-18), 3.71 (1H, d, J=10.0 Hz, H-24a), 4.16 (1H, d, J=10.0 Hz, H-24b), 4.17 (1H, dd, J=9.5, 5.0 Hz, H-3), 5.58 (1H, dd, J=3.5, 3.5 Hz, H-12). ^{13}C-NMR δ (C_5D_5N): 13.0 (q), 16.0 (q), 16.7 (q), 17.3 (q), 18.7 (t), 24.0 (t), 24.6 (q), 26.4 (t), 27.0 (t), 27.1 (q), 27.6 (t), 29.3 (t), 33.3 (t), 37.2 (s), 38.5 (t), 38.8 (t), 40.3 (s), 42.1 (s), 42.3 (d), 42.8 (s), 47.8 (d), 48.3 (s), 48.7 (d), 54.6 (d), 68.1 (t), 72.7 (s), 73.5 (d), 127.8 (d), 140.1 (s), 181.0 (s). ESI-MS *m/z*: 489 $[M+H]^+$.

Tormentic Acid[27] (**12**, 2 mg): ^{1}H-NMR δ (C_5D_5N): 1.00 (3H, s, H-25), 1.08 (3H, s, H-24), 1.09 (3H, s, H-26), 1.10 (3H, d, J=6.5 Hz, H-30), 1.25 (3H, s, H-23), 1.42 (3H, s, H-29), 1.70 (3H, s, H-27), 3.08 (1H, s, H-18), 3.37 (1H, d, J=10.0 Hz, H-3), 4.09 (1H, ddd, J=10.0, 10.0, 4.5 Hz, H-2), 5.56 (1H, br dd, H-12),. ^{13}C-NMR δ (C_5D_5N): 16.7 (q), 16.8 (q), 17.3 (q), 17.6 (q), 18.9 (t), 24.1 (t), 24.7 (q), 26.4 (t), 27.0 (t), 27.1 (q), 29.3 (q), 29.3 (t), 33.5 (t), 38.4 (s), 38.5 (t), 39.8 (s), 40.3 (s), 42.1 (s), 42.4 (d), 47.8 (d), 47.9 (t), 48.3 (s), 54.6 (d), 55.9 (d), 68.5 (d), 72.7 (s) , 83.8 (d), 127.7 (d), 140.1 (s), 181.2 (s). ESI-MS *m/z*: 489 $[M+H]^+$.

2α-Hydroxyursolic Acid[27] (**13**, 3 mg): ^{1}H-NMR δ (C_5D_5N): 0.94 (3H, d, J=7.0 Hz, H-30), 0.96 (3H, s, H-25), 0.97 (3H, d, J=6.5 Hz, H-29), 1.03 (3H, s, H-26), 1.06 (3H, s, H-24), 1.20 (3H, s, H-27), 1.26 (3H, s, H-23), 1.73 (1H, dd, J=10.5, 6.5 Hz, H-9), 2.10 (1H, ddd, J=12.5, 12.5, 4.0 Hz, H-6), 2.22 (1H, dd, J=12.5, 4.0 Hz, H-1), 2.32 (1H, ddd, J=14.0, 14.0, 5.0 Hz, H-15), 2.62 (1H, d, J=12.0 Hz, H-18), 3.38 (1H, d, J=10.0 Hz, H-3), 4.08 (1H, ddd, J=10.5, 10.0, 4.0 Hz, H-2), 5.45 (1H, dd, J=4.0, 3.0 Hz, H-12). ^{13}C-NMR δ (C_5D_5N): 16.9 (q), 17.4 (q), 17.4 (q), 17.6 (q), 18.8 (t), 21.3 (q), 23.7 (t), 23.9 (q), 24.9 (t), 28.6 (t), 29.3 (q), 31.0 (t), 33.4 (t), 37.4 (t), 38.4 (s), 39.3 (d), 39.4 (d), 39.8 (s), 40.0 (s), 42.5 (s), 47.9 (d), 48.0 (s), 48.0 (d), 53.5 (d), 55.8 (d), 68.5 (d), 83.9 (d), 125.5 (d), 139.2 (s), 179.9 (s). ESI-MS *m/z*: 473 $[M+H]^+$.

19α-Hydroxyasiatic Acid[28] (**14**, 10 mg): ^{1}H-NMR δ (C_5D_5N): 1.05 (3H, s, H-24), 1.08 (3H, s, H-25), 1.09 (3H, d, J=6.5 Hz, H-30), 1.11 (3H, s, H-26), 1.06 (3H, s, H-25), 1.40 (3H, s, H-29), 1.63 (3H, s, H-27), 3.02 (1H, s, H-18), 3.06 (1H, ddd, J=13.0, 13.0, 5.0 Hz, H-16), 3.70 (1H, d, J=10.0 Hz, H-23a), 4.17 (1H, d, J=10.0 Hz, H-23b), 4.18 (1H, d, J=11.0 Hz, H-3), 4.22 (1H, ddd, J=11.0, 10.0, 4.0 Hz, H-2), 5.56 (1H, br dd, H-12). ^{13}C-NMR δ (C_5D_5N): 14.3 (q), 16.7 (q), 17.3 (q), 17.3 (q), 18.6 (t), 24.1 (t), 24.6 (q), 26.3 (t), 26.9 (t), 27.0 (q), 29.2 (t), 33.1 (t), 38.3 (s), 38.4 (t), 40.4 (s), 42.1 (s), 42.3 (d), 43.6 (s), 47.8 (t), 47.8 (d), 48.0 (d), 48.2 (s), 54.5 (d), 66.6 (t), 68.8 (d), 72.6 (s), 78.3 (d), 127.9 (d), 139.9 (s), 180.7 (s). ESI-MS *m/z*: 505 $[M+H]^+$.

Oleanolic Acid[29] (**6**, 6 mg): ^{1}H-NMR δ ($CDCl_3$): 0.70 (3H, s), 0.72 (3H, s), 0.80 (3H, s), 0.82 (3H, s), 0.92 (3H, s), 1.07 (3H, s), 2.85 (1H, dd, J=14.0, 4.0 Hz, H-18), 3.14 (1H, dd, J=8.0, 8.0 Hz, H-3), 5.24 (1H, dd, J=3.5, 3.5 Hz, H-12). ^{13}C-NMR δ ($CDCl_3$): 15.1 (q), 15.5 (q), 16.7 (q), 18.2 (t), 23.1 (t), 23.2 (t), 23.5 (q), 25.7 (q), 27.0 (t), 27.7 (t), 28.0 (q), 30.6 (s), 32.5 (t), 32.5 (t), 33.0 (q), 33.9 (t), 36.9 (q), 38.3 (t), 38.6 (s), 39.1 (s), 41.1 (d), 41.6 (s), 46.0 (t), 46.2 (s), 47.5 (d), 55.1 (d), 78.4 (d), 121.8 (d), 144.2 (s), 180.6 (s). ESI-MS *m/z*: 457 $[M+H]^+$.

Maslinic Acid[30] (**7**, 5 mg): ^{1}H-NMR δ (C_5D_5N): 0.93 (3H, s, H-29), 0.97 (3H, s, H-26), 0.98 (3H, s, H-30), 1.00 (3H, s, H-24), 1.06 (3H, s, H-25), 1.25 (3H, s, H-23), 1.26 (3H, s, H-27), 1.80 (1H, m, H-6), 2.24 (1H, dd, J=12.0, 4.5 Hz, H-1), 2.32 (1H, ddd, J=14.0, 14.0, 5.0 Hz, H-15), 3.28 (1H, dd, J=8.5, 5.0 Hz, H-18), 3.38 (1H, d, J=9.5 Hz, H-3), 4.10 (1H, ddd, J=10.5, 9.5, 4.5 Hz, H-2), 5.42 (1H, dd, J=4.5, 3.5 Hz, H-12). ^{13}C-NMR δ (C_5D_5N): 16.7 (q), 17.4 (q), 17.6 (q), 18.8 (t), 23.6 (t), 23.7 (q), 23.9 (t), 26.1 (q), 28.2 (t), 29.3 (q), 30.9 (s), 33.1 (t), 33.1 (t), 33.2 (q), 34.1 (t), 38.5 (s), 39.8 (s), 39.8 (s), 41.9 (d), 42.1 (s), 46.4 (t), 46.6 (s), 47.7 (t), 48.1 (d), 55.8 (d), 68.5 (d), 83.8 (d), 122.4 (d), 144.8 (s), 180.1 (s). ESI-MS *m/z*: 473 $[M+H]^+$.

Isofucosterol[31] (**20**, 7 mg): ^{13}C-NMR δ (C_5D_5N): 11.8 (q), 12.8 (q), 18.8 (q), 19.4 (q), 21.0 (q), 21.1 (t), 21.1 (t), 21.1 (t), 24.3 (t), 27.9 (t), 28.2 (t), 28.6 (d), 31.7 (t), 31.9 (t), 31.9 (t), 35.9 (t), 36.1 (d), 36.5 (s), 37.2 (t), 39.8 (t), 42.3 (t), 42.3 (t), 50.1 (d), 56.0 (d), 71.8 (d), 116.4 (d), 121.7 (d), 140.7 (s), 145.9 (d). ESI-MS *m/z*: 413 $[M+H]^+$.

Campesterol[31] (**21**, 5 mg): ^{13}C-NMR δ (C_5D_5N): 11.9 (q), 15.4 (q), 18.2 (q), 18.7 (q), 19.4 (q), 20.2 (q), 21.1 (t), 24.3 (t), 28.2 (t), 30.3 (t), 31.7 (t), 31.9 (d), 31.9 (t), 32.4 (t), 33.7 (t), 35.9 (d), 36.5 (s), 37.2 (t), 38.8 (d), 39.8 (t), 42.3 (t), 42.4 (d), 50.1 (d), 56.1 (d), 56.8 (d), 71.8 (d), 121.7 (d), 140.8 (s). ESI-MS *m/z*: 401 $[M+H]^+$.

β-Sitosterol[31] (**27**, 83 mg): ^{13}C-NMR δ (C_5D_5N): 11.8 (q), 12.0 (q), 18.8 (q), 19.0 (q), 19.4 (q), 19.8 (q), 21.1 (t), 23.1 (t), 24.3 (t), 26.1 (t), 28.2 (t), 29.1 (t), 31.7 (t), 31.9 (t), 31.9 (d), 33.9 (t), 36.1 (d), 36.5 (s), 37.2 (t), 39.8 (t), 42.3 (t), 42.3 (s), 45.8 (d), 50.1 (d), 56.0 (d), 56.8 (d), 71.8 (d), 121.7 (d), 140.8 (s). ESI-MS *m/z*: 415 $[M+H]^+$.

RNA Preparation and Amplification of the Fragments of OSCs Total RNA was prepared from cultured cells of *O. europaea* (5 g) by phenol-SDS extraction and lithium chloride precipitation following the reported method.[14] Total RNA solution (20 ml) and RACE32 primer (5′-GACTC-GAGTCGACATCGATTTTTTTTTTTTTT-3′, 1 μg) were mixed and heated at 70 °C for 3 min, then quickly chilled on ice. The RNA mixture was reverse transcribed by a reverse transcriptase (SuperScript II 100 unit, Gibco-BRL) following the manufacturer's protocol. Reaction volume was adjusted

by TE buffer to 50 μl after incubation. The resulting *O. europaea* cDNA pool was used as a template for PCR. The first PCR was performed in a final volume of 100 μl, with 10x Ex-Taq buffer (10 μl), 2.5 mM dNTP mix (8 μl), 162S (5′-GAYGGIGGITGGGGIYTICA-3′), and 711A (5′-CKRTAYTCIC-CIARIGCCCADATIGGRAA-3′) primers (1 μg each) and the cDNA pool (3 μl) as a template with the program, 30 cycles of 94 °C 1 min, 42 °C 2 min, 72 °C 3 min, and final extension at 72 °C 10 min, using Robocycler Gradient 40 (Stratagene). Ex-Taq DNA polymerase (Takara Biochemicals) (0.5 μl) was added to PCR mixture after pre-incubation at 94 °C for 5 min. After the first PCR, the reaction mixture was filtered with Suprec02™ to remove the primers, and adjusted to a final volume of 100 μl. The second PCR was carried out using 463S and 623A primers (1 μg each) and the first PCR product (3 μl) as a template. The second PCR was performed in the same manner as described in the first PCR. The resulting 480-bp fragment was ligated to a plasmid vector pT7Blue (Novagen). Nucleotide sequences were determined by infrared dye chain termination method using Thermo Sequenase™ Cycle Sequencing Kit (USB78500) (Amersham) with DNA Sequencer Long Read IR 4200 (LI-COR). Among 18 clones picked up, fourteen clones were identical to OEX, a cycloartenol synthase.[13] Two clones were identical to OEW, a lupeol synthase.[13] Remaining two clones showed identical new sequence and were named OEA.

3′- and 5′-Amplification of cDNA Ends 3′- and 5′ ends of cDNA were amplified following the reported method. For 5′ amplification, OEA534S (5′-ATTTGGGAGCCCCCAGTTCCACAG-3′) primer was used. For 3′, OEA544A (5′-CAAATATGGCTGTGGAACTGGGGG-3′), OEA360A (5′-TGCTCCGTAGCGCATATATTTGAT-3′), OEA252A (5′-ATAAGGTAA-AAATGAAGGGAA-3′), OEA204A (5′-GGCTCTAGCAATCGAACCATT-3′) primers. The sequence was deposited to DDBJ (accesion nunber AB291240).

PCR Amplification for Open Reading Frame The full-length cDNA for OEA was obtained by Nested PCR using N-terminal and C-terminal primers. The first PCR was carried out with OEA-5′N-32 (5′-CAGAGAT-CACTAAAGAACAGT-3′) and OEA-3′C+30 (5′-CACTCAAAAGGAATA-CATCCA-3′) primers, and the second PCR with Kpn-OEA-N (5′-AATG-GTACCATGTGGAAGCTTAAGATTGCTGAA-3′) and Xho-OEA-C (5′-ATATCTCGAGTTAAAGATTAATGTGATGATT-3′) primers with the same condition as described above except that the annealing temperature was 58 °C.

Expression of PNA in *ERG7* Deficient Yeast Mutant GIL77 The 2.3-kb PCR product of full-length OEA was digested with *Kpn* I and *Xho* I and ligated into the corresponding sites of pYES2 (Invitrogen) to construct the plasmid pYES2-OEA. The plasmid pYES2-OEA was transferred to mutant yeast strain GIL77 by Frozen-EZ Yeast Transformation II Kit (Zymo Reseach). The transformants were inoculated in 20 ml synthetic complete medium without uracil (SC-U), containing ergosterol (20 μg/ml), hemin chloride (13 μg/ml) and Tween 80 (5 mg/ml), and incubated at 37 °C for 2 d. Then, media were changed to SC-U with the same supplements and 2% galactose in place of glucose. Cells were incubated at the same condition for one day, harvested by centrifugation at 500×***g*** for 5 min. Collected cells were refluxed with 2 ml of 20% KOH/50% EtOH aq. for 5 min. After extraction with the same volume of hexane, the extract was concentrated and applied onto TLC plate (Merck #11798) which was developed with benzene/acetone=19/1. The band corresponding to triterpene monoalcohol was scraped off and extracted with acetone. The extract was concentrated and applied to LC-APCIMS (LCQ, Thermo Quest). HPLC was carried out with Tosoh Super ODS (2 μm, C_{18}, 4.6×200 mm), 95% acetonitrile (v/v), 1.0 ml/min, at 40 °C, monitoring UV absorbance at 202 nm. All triterpene monoalcohols gave the base peak ion at *m/z* 409 $[M+H-H_2O]^+$ in APCIMS analysis (data not shown). For rigorous identification of triterpene products, MS/MS spectrum was measured (*m/z* 409 as the parent ion). Retention time and mass fragmentation pattern of OEA products were compared with those of authentic triterpene alcohols (the stock of our laboratory), butyrospermol (14.7 min), β-amyrin (17.9 min), ψ-taraxasterol (18.9 min), and α-amyrin (20.3 min).

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