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Article

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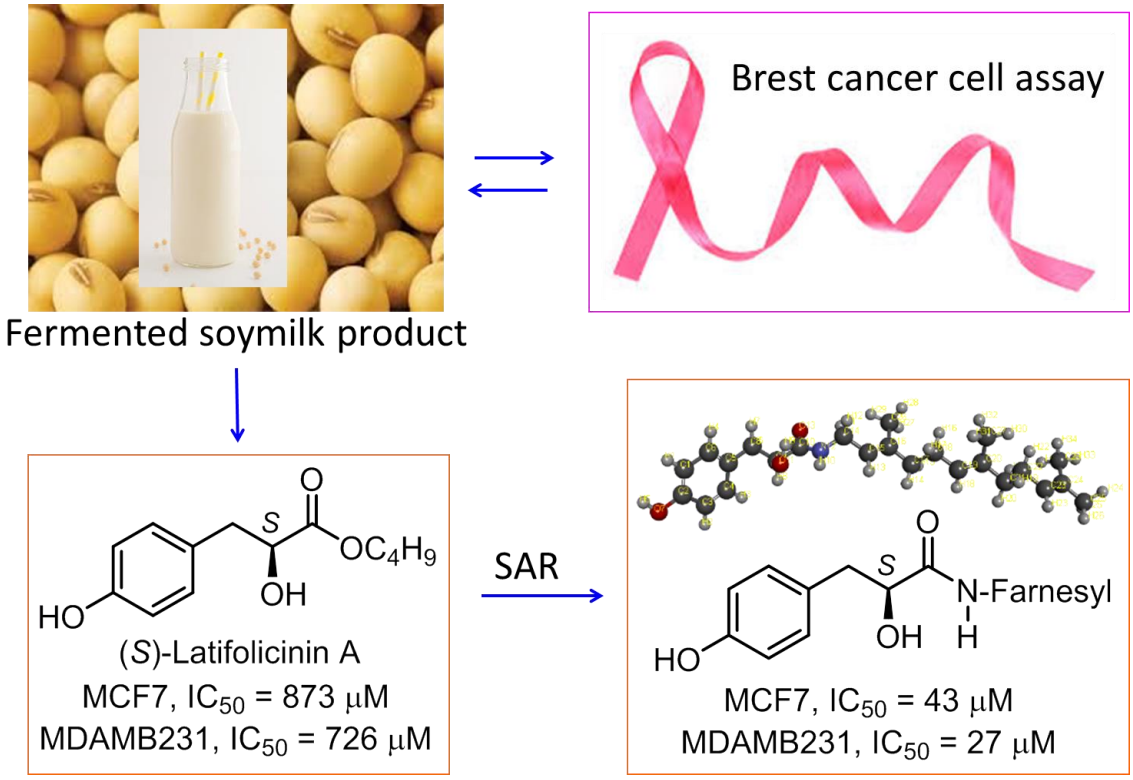

Latifolicinin A from Fermented Soymilk Product and the Structure–Activity Relationship of Synthetic Analogs as Inhibitors of Breast Cancer Cell Growth

Yi-Yu Ke,[†] Chen-Hsuan Tsai,[§] Hui-Ming Yu,[†] Yu-Chen Jao,[†] Jim-Min Fang,*[,†,§] and Chi-Huey Wong*[,†]

[†] The Genomics Research Center, Academia Sinica, 128 Academia Road, Section 2, Nankang, Taipei, 115, Taiwan.

[§] Department of Chemistry, National Taiwan University, 1 Roosevelt Road, Section 4, Taipei, 106, Taiwan.

Table of Contents categories: (1) Bioactive Constituents and Functions; (2) Food and Beverage Chemistry/Biochemistry; (3) Chemical Aspects of Biotechnology/Molecular Biology.

ABSTRACT

The functional components in soymilk may vary depending on the fermentation process. A fermented soymilk product (FSP) obtained by incubation with the microorganisms of intestinal microflora was found to reduce the risk of breast cancer. Guided by the inhibitory activities against breast cancer cells, two cytotoxic compounds, daidzein and (*S*)-latifolicinin A, were isolated from FSP by repetitive extraction and chromatography. Latifolicinin A is the *n*-butyl ester of β-(4-hydroxyphenyl)lactic acid (HPLA). A series of the ester and amide derivatives of (*S*)-HPLA and L-tyrosine were synthesized for evaluation of their cytotoxic activities. In comparison, (*S*)-HPLA derivatives exhibited equal or superior inhibitory activities to their L-tyrosine counterparts, and (*S*)-HPLA amides showed better cytotoxic activities than their corresponding esters. In particular, (*S*)-HPLA farnesyl amide was active to the triple-negative MDA-MB-231 breast cancer cell (IC_{50} = 27 μM) and 10-fold less toxic to Detroit-551 normal cell.

Keywords:

Soymilk; Fermentation; Breast cancer; Latifolicinin; Organic synthesis; Cytotoxicity.

INTRODUCTION

Breast cancer is a heterogeneous disease that often occurs in the pre- and post-menopause period of women. In addition to human epidermal growth factor receptor 2 (HER2), the hormone-sensitive breast cancer cells also contain estrogen receptor (ER) and progesterone receptor (PR) as the biomarkers.[1–3] When ER is stimulated by estrogen, a series of signals are triggered to promote the growth of ER-positive breast tumor. The ER antagonist, e.g. tamoxifen,[4, 5] is used for treatment of the hormone-sensitive breast cancer. MCF-7 is a triple-positive breast cancer cell line because it is ER- and PR-positive with overexpression of HER 2.[2] On the other hand, MDA-MB-231 is coined as a triple-negative breast cancer (TNBC) cell line because it has low expression of HER2 and no expression of ER or PR.[2, 6] Prognosis and treatment of TNBCs are difficult since most drugs only target one of the three receptors.[7–9] The combinatorial therapies are more effective to TNBCs, but may also raise the risk of side effects.

Soy products traditionally taken by Asian people as dietary food and nutrient supplements have also become popular in western world. Moderate consumption of soy foods appears to be safe and may reduce the risk of breast cancer. The isoflavone constituents in soy foods,[10] such as daidzein, **1**,[11] and genistein[10], have chemical structures similar to estrogens. Due to the structural similarity, isoflavones may function as estrogen surrogate or as ER

antagonist.[11–13] It is still disputable whether isoflavones have beneficial or detrimental effects associated with breast cancers.[14]

Soymilk is an aqueous extract of soybean. The functional components in soymilk may vary depending on the fermentation process.[15] Soymilk can be processed with microorganisms such as *Lactobacillus* bacteria to release healthful metabolites.[16] In a previous study,[17] a fermented soymilk product (FSP) obtained by incubation with the microorganisms of intestinal microflora was found to induce apoptosis of MCF-7 breast cancer cells. We report herein the isolation of daidzein, **1**,[12] and latifolicinin A, **4b**,[18, 19] (Figure 1) possessing cytotoxic activity from FSP. A variety of latifolicinin analogs were also synthesized for evaluation of their cytotoxic activities.

Materials and Methods

General. All the reagents were commercially available (Sigma-Aldrich Co., St. Louis, MI) (Acros Organics N.V., Geel, Belgium), and used without further purification unless indicated otherwise. All solvents (Merck Millipore, Darmstadt, Germany) were anhydrous grade unless indicated otherwise. Reactions were magnetically stirred and monitored by thin-layer chromatography on silica gel (Merck Millipore, Darmstadt, Germany). Preparative thin-layer chromatography was performed using 20 cm × 20 cm glass plate of 2 mm SiO_2 thickness (Sigma-Aldrich Co., St. Louis, MI). Flash chromatography was performed on silica

gel (40–63 μm particle size) (Merck Millipore, Darmstadt, Germany) and LiChroprep RP-18 (40–63 μm particle size) (Merck Millipore, Darmstadt, Germany). High-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, CA) was performed using a 4-channel programmable pump and a UV/VIS detector for monitoring at 254 nm wavelength. Melting points were recorded on a melting point apparatus (Yanaco New Science Inc., Kyoto, Japan). ^{1}H and ^{13}C NMR spectra were recorded on 400 or 600 MHz spectrometers (Bruker Corp., Billerica, MA). Chemical shifts are given in δ values relative to tetramethylsilane (TMS); coupling constants *J* are given in Hz. Internal standards were $CHCl_3$ (δ_H = 7.24), $CDCl_3$ (δ_C = 77.0 for the central line of triplet), CH_3OD (δ_H = 3.31), and CD_3OD (δ_C = 49.0). The splitting patterns are reported as s (singlet), d (doublet), q (quartet), m (multiplet), br (broad) and dd (doublet of doublets). Optical rotations were recorded on a digital polarimeter (JASCO International Co. Ltd., Tokyo, Japan). Electrospray ionization high-resolution mass spectra (ESI–HRMS) were recorded on a Daltonics BioTOF III high-resolution mass spectrometer (Bruker Corp., Billerica, MA).

Fermented soymilk product. A concentrated solution of fermented soymilk product (FSP) was prepared and provided by Microbio Co., Ltd., Taipei, Taiwan).[20] In brief, soymilk was fermented by a co-cultural symbiotic system of *Lactobacillus paracasei*, *Lactobacillus bulgaricus* and *Saccharomyces cerevisiae*. The FSP consists of a mixture of soybean extracts

and the secondary metabolites of these microorganisms. The FSP was subjected to a sterilization process to ensure no contamination with any food-borne pathogens.

Extraction and isolation of active compounds. The above-prepared concentrated FSP (0.5 L) was lyophilized to give 47.2 g of powder sample, which was mixed with water (1 L) and extracted with *n*-hexane (1 L) for three times to give the hexane layer (Y-H, 16.3 mg) and water layer (Y-W_1). The Y-W_1 layer was extracted with ethyl acetate (1 L) to give the EtOAc layer (Y-EA, 486.5 mg) and water layer (Y-W_2). The Y-W_2 layer was further extracted with *n*-butanol (1 L) to give the butanol layer (Y-B, 3.6 g) and water layer (Y-W_3, 42.0 g). Finally, the Y-B fraction (3.6 g) was dissolved in $CHCl_3$ and extracted with $CHCl_3$–water (1:1, v/v) for 5 times to give daidzein, **1** (75.5 mg), water layer (Y-BW, 1.2 g), and $CHCl_3$ layer (Y-BC, 1.4 g). The Y-BC fraction was divided into 14 portions and respectively subjected to preparative thin-layer chromatography using *n*-hexane–EtOAc–water (10:5:1) as the mobile phase to give latifolicinin A, **4b** (0.94 mg).

Cell culture. MDA-MB-231, MCF-7 and Detroit-551 cell lines were obtained from the American Type Culture Collection (Rockville, MD). These cells were grown followed by ATCC propagation protocol. In brief, MDA-MB-231 cells were cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum (FBS). MCF-7 cells were cultured in Eagle's minimum essential medium (EMEM) containing 10% FBS and 0.01 mg/mL bovine insulin. Detroit-551 were cultured in EMEM containing 10% FBS. MDA-MB-231 cells were

incubated at 37 °C with 100% air atmosphere without CO_2, whereas MCF-7 and Detroit-551 cells were incubated at 37 °C in 95% air and 5% CO_2 atmosphere. The medium was changed twice a week, and cells were split at about 80% confluence. Second to tenth passage cells were used in experiments.

Determination of cell growth inhibition. Cells were seeded at a density of 3×10^3 (MDA-MB-231), 8×10^3 (MCF-7) or 2×10^3 (Detroit-551) cells per well in 96-well plate for 24 h. Then, the FSP extract or test compound were dosed into the well in triplicate for 72 h incubation. Finally, cell viability was measured using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay reagent (Promega Corporation, Madison, WI) according to the manufacturer's instructions. The absorbance was measured using SpectraMax M5 (Molecular Devices, Sunnyvale, CA) for formazan product at a wavelength of 490 nm with a reference wavelength of 650 nm. The absorbance of each well was corrected with reference to the blank. Percent inhibition of cell survival is expressed as:

[1 – (absorbance of treated cells / absorbance of control cells)] × 100%.

Inhibitor IC_{50} values, i.e. the concentrations of the compound required for 50% cell viability were determined from dose–response curves by plotting the percent inhibition of cell viability versus inhibitor concentrations using Prism 5 (GraphPad Software, Inc., San Diego, CA).

Synthesis of (*S*)-3-(4-hydroxyphenyl)lactic acid (HPLA), (*S*)-3, (*R*)-enantiomer and racemic mixture. A solution of (4-hydroxyphenyl)pyruvic acid, **2**, (3.0 g, 16.7 mmol) and Et_3N (2.3 mL, 16.7 mmol) in *N*,*N*-dimethylformamide (DMF, 100 mL) was stirred at –40 ^oC for 10 min. A solution of (+)-diisopinocampheylchloroborane[21, 22] ((+)-Ipc_2BCl) (16.0 g, 50.0 mmol) in THF (50 mL) was injected over a period of 30 min. The mixture was stirred and warmed from –40 ^oC to room temperature over a period of 12 h. The mixture was quenched with saturated $NaHCO_{3(aq)}$, and concentrated under reduced pressure. The residue was acidified with 1 M $HCl_{(aq)}$ to pH 3, and extracted with EtOAc (50 mL × 3). The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column (5 cm inner diameter (i.d.) × 30 cm length) with elution of MeOH/CH_2Cl_2 [1:9 v/v (300 mL) to 1:4 (500 mL)] to afford the reduction product HPLA, **3**,[21, 23] (2.16 g, 71%) predominating in the (*S*)-enantiomer (>91% ee as inferred from the HPLC analysis of the related (*S*)-Mosher ester[24] of (*S*)-**5b**). $C_9H_{10}O_4$; $[\alpha]^{20}_D$ = –10.6 (*c* 1.2, MeOH); TLC (MeOH/CH_2Cl_2, 1:4) R_f = 0.4; ^1H NMR (CD_3OD, 400 MHz) δ 7.07 (2 H, d, *J* = 8.5 Hz), 6.69 (2 H, d, *J* = 8.5 Hz), 4.26 (1 H, dd, *J* = 7.8, 4.5 Hz), 2.99 (1 H, dd, *J* = 13.9, 4.4 Hz), 2.81 (1 H, dd, *J* = 14.0, 7.9 Hz); ^{13}C NMR (CD_3OD, 150 MHz) δ 177.6, 157.1, 131.7 (2 ×), 129.7, 116.1 (2 ×), 73.2, 40.9; ESI–HRMS calcd. for $C_9H_{11}O_4$: 183.0652, found: *m/z* 182.0648 $[M + H]^+$.

By a similar procedure, reduction of (4-hydroxyphenyl)pyruvic acid, **2**, with (–)-Ipc_2BCl gave (*R*)-**3** (>90% ee as inferred from the HPLC analysis of the (*S*)-Mosher ester of (*R*)-**5b**). The physical and spectral properties of (*R*)-**3** were the same as that described for (*S*)-**3** except for the optical rotation $[\alpha]^{20}_{D}$ = +10.6 (*c* 1.2, MeOH). The racemic sample (±)-**3** was prepared by catalytic hydrogenation of a solution of oxoacid **2** in EtOAc (4 atm of H_2, Pd/C, room temperature, 24 h).

General procedure for the synthesis of (*S*)-HPLA esters 4a–4i. *Method A*. A mixture of HPLA, (*S*)-**3**, (26 mg, 0.14 mmol)) and a drop of concentrated H_2SO_4 in *n*-butanol (4 mL) was heated at 80 ^{o}C for 2 h. The mixture was extracted with $NaHCO_{3\,(aq)}$ and EtOAc. The organic layer was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column (1 cm i.d. × 8 cm) with elution of EtOAc/*n*-hexane (3:7 v/v, 50 mL) to afford the *n*-butyl ester, latifolicinin A,[18] (*S*)-**4b**, (28 mg, 82%).

Method B. A mixture of (*S*)-**3** (50 mg, 0.27 mmol), K_2CO_3 (38 mg, 0.27 mmol) and farnesyl bromide (81 μL, 0.30 mmol) in DMF (5 mL) was stirred at room temperature for 8 h. The mixture was concentrated under reduced pressure. The residue was added to H_2O (2 mL), and extracted with EtOAc (5 mL × 3). The organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduce pressure. The residue was purified by

chromatography on a silica gel column (1 cm i.d. × 8 cm) with elution of EtOAc/*n*-hexane (v/v 3:7, 50 mL) to afford the farnesyl ester (*S*)-**4h** (44 mg, 42%).

(*S*)-3-(4-Hydroxyphenyl)lactic acid farnesyl ester (4h): $C_{24}H_{34}O_4$; syrup; $[\alpha]^{28}_D$ = –34.8 (*c* 2.2, CH_2Cl_2); IR ν_{max} (neat) 3403, 2965, 2924, 2855, 1732, 1615, 1516, 1446, 1377, 1217, 1107, 1085, 931, 829 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 7.01 (2 H, d, *J* = 8.4 Hz), 6.67 (2 H, dd, *J* = 5.6, 2.8 Hz), 5.35–5.31 (1 H, m), 5.09–5.05 (2 H, m), 4.67 (3 H, d, *J* = 7.2 Hz), 4.39 (1 H, s), 3.03 (1 H, dd, *J* = 14.2, 4.4 Hz), 2.87 (2 H, dd, *J* = 13.2, 6.6 Hz), 2.20–2.00 (6 H, m), 1.98–1.94 (2 H, t, *J* = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 174.3, 154.7, 143.5, 135.6, 131.4, 130.6 (2 ×), 127.8, 124.2, 123.4, 117.4, 115.3 (2 ×), 71.4, 62.5, 39.6, 39.5, 39.4, 26.7, 26.1, 25.7, 17.7, 16.5, 16.0; ESI–HRMS calcd for $C_{24}H_{35}O_4$: 385.2379, found: *m*/*z* 385.2391 $[M + H]^+$.

General procedure for the synthesis of (*S*)-HPLA amides 10b, **10d**, **10f**, **10h and 10i.** A mixture of (*S*)-**3** (317 mg, 1.74 mmol) and acetic anhydride (0.95 mL, 10 mmol) in pyridine (5 mL) was stirred at room temperature for 3 h. The mixture was concentrated under reduced pressure. The residue was added to 1 M $HCl_{(aq)}$ (5 mL), and extracted with EtOAc (15 mL × 3). The organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford a diacetylation product (*S*)-**7a** (413 mg, 90%).

The above-prepared diacetate (*S*)-**7a** (413 mg, 1.57 mmol) was treated with $NaHCO_3$ (252 mg, 3 mmol) in MeOH–H_2O solution (5 mL, 1:1 v/v) at room temperature for 8 h. The

residue was added to 1 M $HCl_{(aq)}$ to pH 3, and then extracted with EtOAc (15 mL × 4). The organic phase was washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford monoacetate (*S*)-**7** (309 mg, 88%).

A mixture of (*S*)-**7** (32 mg, 0.14 mmol), diisopropylethylamine (DIPEA) (0.05 mL, 0.15 mmol) and isopropyl chloroformate (*i*-PrOCOCl) (0.15 mL, 0.15 mmol) in DMF (2 mL) was stirred for 0.5 h at 0 ^{o}C. Dodecylamine (0.03 mL, 0.14 mmol) was added, and the mixture was stirred for 1 h. The mixture was concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column (1 cm i.d. × 8 cm length) with elution of EtOAc/*n*-hexane [1:9 v/v (30 mL) to 2:1 (50 mL)] to afford compound (*S*)-**9f** (32 mg), which was treated with 1 M NaOH (0.5 mL) in MeOH (2 mL) for 20 min at room temperature. The mixture was concentrated under reduced pressure. The residue was neutralized with 1 M $HCl_{(aq)}$, and extracted with EtOAc (5 mL × 3). The combined organic layers were washed with brine, dried over $MgSO_4$, filtered and concentrated under reduced pressure to afford amide (*S*)-**10f** (25 mg, 50% overall yield from (*S*)-**7**).

***N*-Dodecyl (*S*)-3-(4-hydroxyphenyl)lactamide, 10f:** $C_{21}H_{35}NO_3$; white solid; mp 93.4–94.6 ^{o}C; $[\alpha]^{23}_{D}$ = –4.6 (*c* 1.6, MeOH); IR ν_{max} (neat) 3301, 2922, 2851, 1744, 1658, 1369, 1221, 1194, 911, 760 cm^{-1}; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.66 (1 H, t, *J* = 5.5 Hz), 7.05 (2 H, d, *J* = 8.5 Hz), 6.68 (2 H, d, *J* = 8.5 Hz), 4.16 (1 H, dd, *J* = 7.2, 4.1 Hz), 3.23–3.05 (2 H, m), 2.96 (1 H, dd, *J* = 14.0, 4.1 Hz), 2.75 (1 H, dd, *J* = 14.0, 7.3 Hz), 1.41 (2 H, quint), 1.36–1.16

(18 H, m), 0.90 (3 H, t, J = 6.7 Hz); ^{13}C NMR (CD_3OD, 100 MHz) δ 176.4, 157.0, 131.7 (2 ×), 129.6, 115.9 (2 ×), 74.1, 41.0, 40.1, 33.1, 30.8, 30.7, 30.6, 30.5, 30.4, 27.9, 23.7, 14.4; ESI–HRMS calcd for $C_{21}H_{36}NO_3$: 350.2690, found: m/z 350.2687 $[M + H]^+$.

Synthesis of L-tyrosine ester: *N*-dodecyl (*S*)-2-amino-3-(4-hydroxyphenyl)propanoate, 12f. A mixture of compound (*S*)-**11** (100 mg, 0.35 mmol), Cs_2CO_3 (60 mg, 0.19 mmol) and 1-iodododecane (0.01 mL, 0.39 mmol) in DMF (5 mL) was stirred for 8 h at room temperature. The mixture was concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column (1 cm i.d. × 10 cm length) with elution of EtOAc/*n*-hexane [1:9 v/v (40 mL) to 1:1 (60 mL)] to afford the ester product (130 mg). The ester product was treated with 2 M HCl in EtOAc for 1 h at room temperature to remove the Boc protecting group, and then concentrated under reduced pressure to afford (*S*)-**12f** as the hydrochloride salt (107 mg, 83% overall yield). $C_{21}H_{36}NO_3Cl$; white solid; mp 129.2–131.8 °C; $[\alpha]^{23}_D$ = +20.7 (c = 1.0, MeOH); IR ν_{max} (neat) 3297, 2958, 2921, 2856, 2623, 1740, 1618, 1517, 1356, 1234, 849, 735; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.07 (2 H, d, J = 8.4 Hz), 6.78 (2 H, d, J = 8.4 Hz), 4.22–4.15 (3 H, m), 3.12 (1 H, d, J = 6.8 Hz), 1.63–1.58 (2 H, m), 1.63–1.58 (2 H, m), 1.34–1.25 (18 H, m), 0.90 (3 H, t, J = 6.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 170.8, 158.9, 132.0 (2 ×), 126.2, 117.4 (2 ×), 68.1, 55.9, 37.3, 33.6, 31.3, 31.2, 31.1, 31.0, 30.8, 27.4, 24.2, 14.9; ESI–HRMS of (*S*)-**12f** (as the HCl salt) calcd for $C_{21}H_{36}NO_3$: 350.2695 $[M - Cl]^+$, found: m/z 350.2691.

General procedure for the synthesis of L-tyrosine esters: *N*-dodecyl (*S*)-2-amino-3-(4-hydroxyphenyl)propylamide, 13f. A mixture of compound (*S*)-**11** (281 mg, 1.00 mmol), DIPEA (0.19 mL, 1.10 mmol) and *i*-PrOCOCl (1.10 mL, 0.56 mmol) in DMF (10 mL) was stirred for 0.5 h at 0 ^{o}C. After addition of *n*-dodecylamine (0.25 mL, 1.10 mmol), the mixture was stirred for 1 h, and concentrated under reduced pressure. The residue was purified by chromatography on a silica gel column (1.5 cm i.d. × 15 cm length) by elution with EtOAc/*n*-hexane [1:9 v/v (50 mL) to 1:1 (100 mL)] to afford an amide product (332 mg). The amide product was treated with 2 M HCl in EtOAc for 1 h at room temperature to remove the Boc protecting group, and then concentrated under reduced pressure to afford (*S*)-**13f** as the hydrochloride salt (300 mg, 74% overall yield). $C_{21}H_{37}N_2O_2Cl$; white solid; mp 129.2–131.8 ^{o}C; $[\alpha]^{23}_{D}$ = +34.1 (*c* 1.0, MeOH); IR ν_{max} (neat) 3343, 2920, 2851, 1661 ,1613, 1551, 1517 ,1467, 1452, 1273, 1205, 837; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.01 (2 H, d, *J* = 8.8 Hz), 6.70 (2 H, d, *J* = 8.4 Hz), 3.46 (1 H, t, *J* = 7.0 Hz), 3.19–3.12 (1 H, m), 3.09–3.02 (1 H, m), 2.86 (1 H, dd, *J* = 13.4, 7.0 Hz), 2.74 (1 H, dd, *J* = 13.6, 6.8 Hz), 1.43–1.35 (2 H, m), 1.35–1.15 (18 H, m), 0.90 (3 H, t, *J* = 6.8 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 170.0, 158.7, 132.1 (2 ×), 126.6, 117.3 (2 ×), 56.6, 41.1, 38.5, 33.6, 31.2 (2 ×), 31.1 (2 ×), 31.0, 30.9, 30.6, 28.4, 24.2, 14.9; ESI–HRMS of (*S*)-**13f** (as the HCl salt) calcd for $C_{21}H_{37}N_2O_2$: 349.2855 $[M - Cl]^+$, found: *m*/*z* 349.2847.

***N*-Dodecyl (*S*)-2-dodecylamino-3-(4-hydroxyphenyl)propylamide, 14f.** To a mixture of compound (*S*)-**13f** (63 mg, 0.16 mmol) and 1-dodecanal (0.04 mL, 0.18 mmol) in EtOH (3 mL) a solution of $NaBH_3CN$ (12 mg, 0.18 mmol) in EtOH (0.6 mL) was added in three portions at room temperature. The solution was adjusted to pH 4–6 by addition of HCl solution (2 M in EtOH, 0.1 mL). The mixture was stirred for 20 min, neutralized with saturated $NaHCO_{3(aq)}$ (1 mL), and extracted with Et_2O (15 mL × 3). The combined organic phase was dried over $MgSO_4$, concentrated under reduced pressure, and purified by chromatography on a silica gel column (1 cm i.d. × 10 cm length) with elution of EtOAc/*n*-hexane [1:9 v/v (30 mL), 2:3 (30 mL), to 4:1 (50 mL)] to afford compound (*S*)-**14f** (43 mg, 52%). $C_{33}H_{60}N_2O_2$; yellow oil; $[\alpha]^{25}_D$ = +11.7 (*c* 2.0, MeOH); IR ν_{max} (neat) 3305, 2958, 2925, 2856, 1650 , 1569, 1516 ,1467, 1254, 825; ^{1}H NMR (CD_3OD, 400 MHz) δ 7.37 (NH, t, *J* = 5.6 Hz), 7.00 (2 H, d, *J* = 8.4 Hz), 6.80 (2 H, d, *J* = 8.4 Hz), 3.30–3.16 (3 H, m), 3.08 (1 H, dd, *J* = 14.0, 4.0 Hz), 2.54 (1 H, dd, *J* = 14.0, 9.6 Hz), 2.38 (2 H, t, *J* = 7.0 Hz), 1.46 (2 H, *J* = 6.4 Hz), 1.26–1.15 (40 H, m), 0.85 (6 H, t, *J* = 7.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 174.5, 155.6, 130.0, 129.8 (2 ×), 116.7 (2 ×), 64.3, 48.9, 39.1, 38.6, 33.6, 31.9 (2 ×), 29.9 (2 ×), 29.63 (2 ×), 29.60 (2 ×), 29.5 (2 ×), 29.4 (2 ×), 29.3 (2 ×), 29.2 (2 ×), 27.1, 27.0, 22.7 (2 ×), 14.1 (2 ×); ESI–HRMS calcd for $C_{33}H_{61}N_2O_2$: 517.4733, found: *m*/*z* 517.4741 [M + H]$^+$.

RESULTS AND DISCUSSION

Isolation of cytotoxic compounds from fermented soymilk product. The powder sample of FPS was partitioned between *n*-hexane and water. The aqueous layer (Y-W_1) was extracted successively with ethyl acetate and *n*-butanol. The hexane-, EtOAc- and butanol layers were found to possess modest inhibitory activity against the growth of MDA-MB-231 cancer cells with the IC_{50} values of 0.49, 0.48 and 0.43 mg/mL, respectively. The butanol layer (Y-B) was further extracted with $CHCl_3$–water, and the active compounds daidzein, **1**,[12] and (*S*)-latifolicinin A, **4b**,[18, 19] were isolated from the chloroform layer (Y-BC).

Identification of (*S*)-latifolicinin A as the bioactive enantiomer. Latifolicinin A is the *n*-butyl ester of 2-hydroxy-3-(4-hydroxyphenyl)propanoic acid, **3**, which is also known as β-(4-hydroxyphenyl)lactic acid (HPLA).[23, 25] Latifolicinin A was firstly isolated from the fruit of *Cordia latifolia* (Boraginaceae, forget-me-not family) along with its analogs, including the ethyl ester **4a** (latifolicinin B), the methyl ester (latifolicinin C) and latifolicinin D that is a derivative of latifolicinin acid having an anisole moiety instead of the phenol group.[18, 19] Latifolicinins A–D were found to have modest larvicidal activities against the yellow-fever-transmitting mosquito, but no significant antibacterial activity.[18, 19] HPLA and latifolicinin C have been reported to possess antioxidant activity.[26, 27] The HPLA fragment has also been found as a moiety in the structures of aquatic peptides isolated from the

cyanobacterium *Microcystis aeruginosa*.[23] However, the absolute configuration of active HPLA derivatives was not clearly defined in these studies.

We thus prepared (*S*)-HPLA (Figure 1B) by an enantioselective reduction of 3-(4-hydroxyphenyl)-2-oxopropanoic acid, **2**, with (+)-diisopinocampheylchloroborane ((+)-Ipc_2BCl) according to the previously reported method.[21, 22, 27, 28] The product (*S*)-**3b** was subjected to an acid-catalyzed esterification in *n*-butanol to give (*S*)-latifolicinin A, (*S*)-**4b**, which was treated with iodomethane in the presence of K_2CO_3 for selective alkylation of the phenol group to yield (*S*)-**5b**. The Mosher ester[24] (*S*,*S*)-**6b** derived from (*S*)-**5b** and (*S*)-MTPA-Cl was determined to have 91.2% diastereomeric excess (de) by the NMR and HPLC analyses (Figure 2).[29] Accordingly, the above-prepared HPLA, **3**, and latifolicinin A, **4b**, should have at least 91.2% enantiomeric excess (ee) predominating in the (*S*)-enantiomers because the alkylation and ester formation under both basic and neutral conditions would hardly change the structural configuration.[28]

By a similar procedure, reduction of α-oxoacid **2** with (–)-Ipc_2BCl afforded (*R*)-HPLA,[23, 30] which underwent esterification in *n*-butanol to give (*R*)-latifolicinin A in >90% ee. Our cell viability assays indicated that (*S*)-latifolicinin A possessed inhibitory activities against MDA-MB-231 and MCF-7 cancer cells with IC_{50} in sub-millimolar range (Table 1), whereas the enantiomer (*R*)-**4b** was inactive (IC_{50} > 2 mM). We also found that (*S*)-**5b** having an anisole moiety instead of the phenol group in (*S*)-**4b** did not show any significant inhibition

294 against MDA-MB-231 or MCF-7 cells, indicating the phenol group was essential for the
295 cytotoxic activity.

296 **Synthesis of (*S*)-HPLA esters and amides.** In order to explore more potent cytotoxic
297 agents, we synthesized a series of (*S*)-latifolicinin analogs **4a** and **4c**–**4i** (Figure 1B). Thus, the
298 above-prepared (*S*)-**3b** was treated with appropriate *n*-alkyl alcohols using concentrated
299 H_2SO_4 as the catalyst to give the desired alkyl esters **4a** and **4c**–**4f** (method A). In another
300 approach (method B), alkylation reactions of (*S*)-**3b** with geranyl chloride, farnesyl bromide
301 and 3-(5-bromopentoxy)-estra-1,3,5(10)-triene-17β-ol in the presence of a base (K_2CO_3 or
302 Cs_2CO_3) occurred selectively at the secondary hydroxyl group to afford esters **4g**, **4h** and **4i**.

303 Furthermore, (*S*)-HPLA amides **10b**, **10d**, **10f**, **10h** and **10i** bearing butyl, octyl, dodecyl,
304 farnesyl and (estradiol-3-yl)pentyl substituents, respectively, were prepared as shown in
305 Figure 3. The above-prepared (*S*)-**3b** was first reacted with acetic anhydride in pyridine to
306 give the diacetylation product (*S*)-**7a**, which was treated with $NaHCO_3$, a weak base, to give
307 monoacetate (*S*)-**7** by selective removal of the acetyl group on the phenyl moiety. Compound
308 (*S*)-**7** was activated with isopropyl chloroformate to a mixed anhydride, and then treated with
309 appropriate amines (**8b**, **8d**, **8f**, **8h** and **8i**) to produce the corresponding amide derivatives.
310 Compounds **10b**, **10d**, **10f**, **10h** and **10i** predominating in the (2*S*)-configuration were
311 obtained in 50–72% overall yields after saponification of the acetyl group.

Synthesis of L-tyrosine esters and amides. As L-tyrosine bearing α-amino group is structurally similar to (*S*)-HPLA that has α-hydroxyl group, the ester and amide derivatives of L-tyrosine were also prepared (Figure 4) for comparison of their cytotoxic activities. The alkylation reaction of *N*-Boc-tyrosine (*S*)-**11** with dodecyl iodide was carried out, followed by removal of the Boc protecting group in acidic conditions, to provide the tyrosine ester (*S*)-**12f** in 83% yield. Alternatively, *N*-Boc-tyrosine was subjected to amidation reactions with dodecylamine and phytylamine[31], respectively, to give the amide derivatives (*S*)-**13f** and (*S*)-**13j** after removal of the Boc group. Compound (*S*)-**13f** was further treated with dodecanal in the presence of $NaBH_3CN$ to give the reductive amination product (*S*)-**14f**.

Measurement of cell viability and determination of IC_{50} values. Table 1 shows the cytotoxic activities of the above-prepared ester and amide derivatives of (*S*)-HPLA and L-tyrosine. As the alkyl chains elongated from 2 to 12 carbons, the (*S*)-HPLA esters **4a**–**4f** appeared to possess increasing cytotoxicity against MDA-MB-231 and MCF-7 breast cancer cells (entries 1–6). The cytotoxic activities of (*S*)-HPLA amides **10b**, **10d** and **10f** also increased as the lengths of their alkyl substituents increased (entries 10–12). In a similar trend, the farnesyl ester (*S*)-**4h** was about 5-fold more potent than the geranyl ester (*S*)-**4g** against both MDA-MB-231 and MCF-7 cancer cells (entries 7 and 8). In comparison, the (*S*)-amide derivatives **10d**, **10f** and **10h** showed somewhat better cytotoxic activities than their corresponding (*S*)-esters **4d**, **4f** and **4h**. The L-tyrosine dodecyl amide (**13f**) was about 11-fold

more potent than the L-tyrosine dodecyl ester (**12f**) against both breast cancer cells (entries 15 and 16). However, the cytotoxic activities of the double-alkylated compound (*S*)-**14f** greatly decreased by >30 fold when another dodecyl substituent was introduced to the N^2-position of L-tyrosine (cf. entries 16 and 18). The (*S*)-HPLA derivatives **4f** and **10f** appeared to have the cytotoxic activities superior or equal to their L-tyrosine counterparts **12f** and **13f** (cf. entries 6 vs. 15, and 12 vs. 16, respectively).

The (*S*)-HPLA derivatives **4i**, **10f**, **10h** and **10i** bearing geranyl, farnesyl and estradiol moieties were found to be less toxic to Detroit-551 fibroblast cells derived from a skin biopsy of a normal embryo (Table 1). Among them, (*S*)-HPLA farnesyl amide, **10h**, showed 6–10 fold less toxic to Detroit-551 normal cell (IC_{50} = 261 μM) than MDA-MB-231 (IC_{50} = 27 μM) and MCF-7 cells (IC_{50} = 43 μM) cancer cells.

It was interesting to note that the (*S*)-HPLA ester **4i** and amide **10i**, prepared by incorporation of an estradiol moiety, also exhibited good inhibitory activities ($IC_{50} \approx$ 10–15 μM) against both ER-negative MDA-MB-231 and ER-positive MCF-7 breast cancer cells (entries 9 and 14). L-Tyrosine phytyl amide (**13j**) also exhibited high activities ($IC_{50} \approx$ 5–9 μM, entry 17) against the two breast cancer cells, but it was equally toxic to Detroit-551 cell (IC_{50} = 4.7 μM). However, one may not overlook the possible surfactant effect of the aliphatic phytyl substituent on cell membranes, even though the mechanism of function is unclear. Though (*S*)-HPLA esters (e.g. **4b**, **4h** and **4**i) and (*S*)-HPLA amides (e.g. **10f**, **10h** and **10i**)

contain a structural scaffold similar to that of L-tyrosine, none of them at 400 μM showed appreciable inhibition against tyrosine kinase in our preliminary test. Further studies are needed to elucidate the real target protein(s) of (*S*)-HPLA esters and amides.

In summary, we have identified (*S*)-latifolicinin A, **4b**, as a cytotoxic constituent in the fermented soymilk product prepared by incubation with microorganisms of intestinal microflora. (*S*)-Latifolicinin A possessed moderate inhibitory activities ($IC_{50} \approx 0.8$ mM) against triple-negative MDA-MB-231 and triple-positive MCF-7 breast cancer cells, whereas its (*R*)-enantiomer and the anisole surrogate (*S*)-**5b** were inactive to the cancer cells. To understand the structure–activity relationship, we have synthesized a series of (*S*)-HPLA esters and amides. The cytotoxic activities of the (*S*)-HPLA derivatives enhanced as the lengths of alkyl substituents increased. Among the examined compounds, (*S*)-HPLA farnesyl amide, **10h**, was active to the triple-negative MDA-MB-231 breast cancer cell ($IC_{50} = 27$ μM) and 10-fold less toxic to Detroit-551 normal cell. The mechanism for the cytotoxic activity of (*S*)-HPLA derivatives awaits further studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at http://pubs.acs.org. DOI:

Flow chart for isolation of daidzein and (*S*)-latifolicinin A, additional synthetic procedure and characterization of compounds, ^{1}H and ^{13}C NMR spectra, and HPLC diagram.

AUTHOR INFORMATION

Corresponding Author

*(J.-M.F) Phone: 8862-33661663. Fax: 8862-23637812. E-mail: jmfang@ntu.edu.tw.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Boc, *tert*-butoxycarbonyl; de, diastereomeric excess; ee, enantiomeric excess; EMEM, Eagle's minimum essential medium; ER, estrogen receptor; FBS, fetal bovine serum; FSP, fermented soymilk product; HER2, human epidermal growth factor receptor 2; HPLA,

388 β-(4-hydroxyphenyl)lactic acid; Ipc_2BCl, diisopinocampheylchloroborane; PR, progesterone

389 receptor; TNBC, triple-negative breast cancer.

390

391

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472 reductive aminations. *Tetrahedron* **2001**, *57*, 4147–4160.
473
474

475 **Figure Captions:**

476 **Figure 1.** (A) Chemical structure of daidzein, **1**. (B) Synthesis of (*S*)-HPLA esters **4a**–**4i**.

477 **Figure 2.** Conversion of the synthesized sample of (*S*)-latifolicinin A, (*S*)-**4b**, to Mosher ester

478 for optical purity determination.

479 **Figure 3.** Synthesis of (*S*)-HPLA amides.

480 **Figure 4.** Synthesis of L-tyrosine ester and amides.

481

Table 1. Growth inhibition of cells after treatment with the ester and amide derivatives of (*S*)-HPLA and L-tyrosine.

entry	compound (R =)	IC_{50} (μM)[a]		
		MDA-MB-231	MCF-7	Detroit-551
1	**4a** (C_2H_5)	>1000	>1000	ND[b]
2	**4b** (C_4H_9)	726 ± 113	873 ± 26	ND[b]
3	**4c** (C_6H_{13})	633 ± 129	534 ± 22	ND[b]
4	**4d** (C_8H_{17})	179 ± 42	299 ± 31	ND[b]
5	**4e** ($C_{10}H_{21}$)	131 ± 39	175 ± 38	ND[b]
6	**4f** ($C_{12}H_{25}$)	53.9 ± 2.6	92.8 ± 11.7	ND[b]
7	**4g** (geranyl)	150 ± 21	265 ± 8	ND[b]
8	**4h** (farnesyl)	35.4 ± 3.9	51.9 ± 12.0	ND[b]
9[c]	**4i** (estradiol-pentyl)	10.1 ± 0.2	12.8 ± 0.8	53.9 ± 0.9
10	**10b** (C_4H_9)	>500	ND[b]	ND[b]
11	**10d** (C_8H_{17})	130 ± 10	269 ± 32	ND[b]
12[c]	**10f** ($C_{12}H_{25}$)	8.3 ± 0.4	17.2 ± 0.4	45.2 ± 10.8
13[c]	**10h** (farnesyl)	26.9 ± 2.2	43.3 ± 1.2	261 ± 37
14[c]	**10i** (estradiol-pentyl)	11.0 ± 0.8	14.5 ± 2.5	30.1 ± 5.2
15	**12f** ($C_{12}H_{25}$)	76.1 ± 28.7	183 ± 4	150 ± 5
16	**13f** ($C_{12}H_{25}$)	8.7 ± 0.1	16.1 ± 0.9	26.9 ± 0.4
17	**13j** (phytyl)	5.4 ± 1.3	8.8 ± 0.6	4.7 ± 0.7
18	**14f** ($C_{12}H_{25}$)	277 ± 19	936 ± 47	516 ± 10
19	Daidzein	>1000	>1000	>1000
20	Benzethonium chloride	19 ± 1.2	380 ± 42	>1000

[a] Data are shown as mean ± SD of three experiments.

[b] Not determined.

Figure 1.

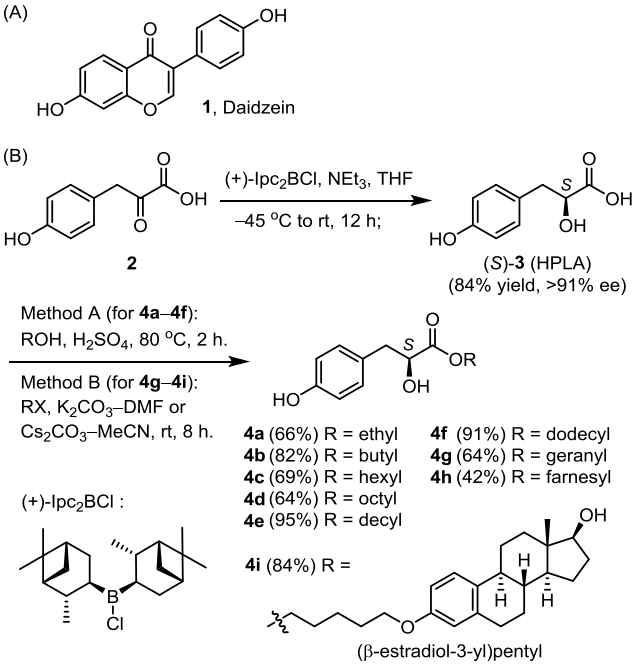

(A)
1, Daidzein
(B)
(+)-Ipc2BCl, NEt3, THF
–45 °C to rt, 12 h;
2
(S)-3 (HPLA)
(84% yield, >91% ee)
Method A (for 4a–4f):
ROH, H2SO4, 80 °C, 2 h.
Method B (for 4g–4i):
RX, K2CO3–DMF or
Cs2CO3–MeCN, rt, 8 h.
4a (66%) R = ethyl
4b (82%) R = butyl
4c (69%) R = hexyl
4d (64%) R = octyl
4e (95%) R = decyl
4f (91%) R = dodecyl
4g (64%) R = geranyl
4h (42%) R = farnesyl
(+)-Ipc2BCl :
4i (84%) R =
(β-estradiol-3-yl)pentyl

Figure 2.

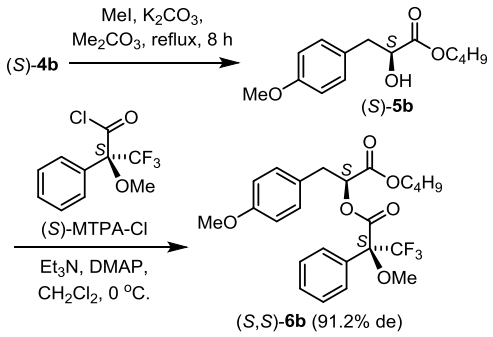
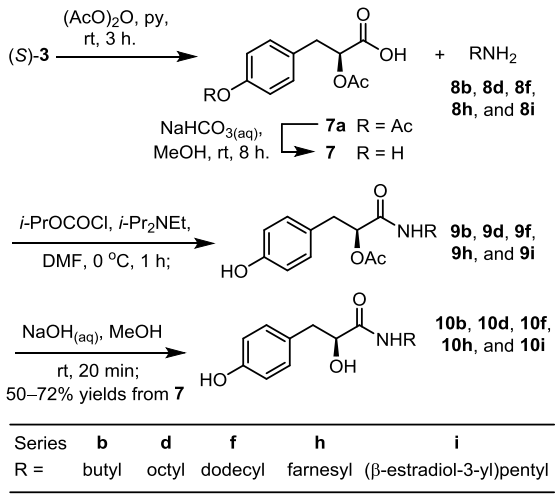

MeI, K_2CO_3,
Me_2CO_3, reflux, 8 h
(S)-4b
(S)-5b
(S)-MTPA-Cl
Et_3N, DMAP,
CH_2Cl_2, 0 °C.
(S,S)-6b (91.2% de)

Figure 3.



Series	b	d	f	h	i
R =	butyl	octyl	dodecyl	farnesyl	(β-estradiol-3-yl)pentyl

Figure 4.

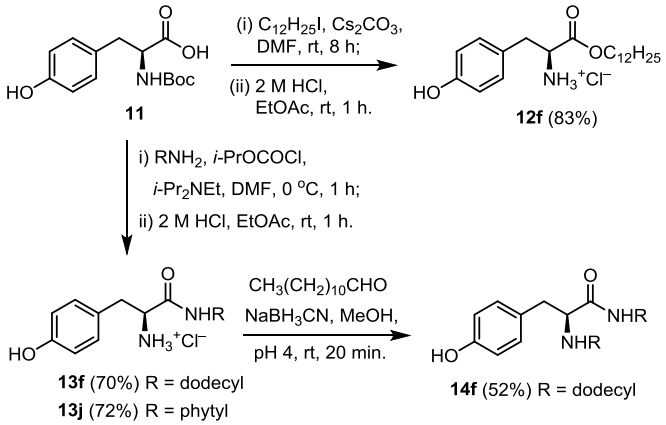

(i) $C_{12}H_{25}I$, Cs_2CO_3, DMF, rt, 8 h;
(ii) 2 M HCl, EtOAc, rt, 1 h.
11
12f (83%)
i) RNH_2, *i*-PrOCOCl,
i-Pr_2NEt, DMF, 0 °C, 1 h;
ii) 2 M HCl, EtOAc, rt, 1 h.
$CH_3(CH_2)_{10}CHO$
$NaBH_3CN$, MeOH,
pH 4, rt, 20 min.
13f (70%) R = dodecyl
13j (72%) R = phytyl
14f (52%) R = dodecyl

Table of Contents Graphic