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Synthesis of New Glycyrrhetinic Acid (GA) Derivatives and Their Effects on Tyrosinase Activity

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Abstract—To synthesize glycyrrhetinic acid (GA) derivatives (**3**, **4**, **5**, **10**, **13**, **14**, **15**, and **16**), we first removed the ketonic group in the C-11 position, and the carboxylic function at the C-30 position was kept intact, reduced to an alcohol, or transformed to an aldehyde corresponding derivatives **10** and **13**. Glycyrrhetinic acid (GA) derivatives (**3**, **4**, **5**, **15**, and **16**) were coupled with 4-amino piperpyridine derivatives (**12** and **14**) and 4-fluorobenzyl bromide at C-30 carboxylic acid position of glycyrrhetinic acid. In subsequent tyrosinase assays, we found that GA derivatives **4**, **5**, and **16** were not active at early time points, but strongly inhibited tyrosinase activity at late time points. Of the GA derivatives examined, derivative **5** was most active, with an IC_{50} value of 50 μM after 2 h reaction. IC_{50} values of derivatives **4** and **16** were 120 and 170 μM, respectively. Further kinetic data indicated that these derivatives are slow-binding inhibitors of tyrosinase. The time-dependent inhibition was reversed when vitamin C or kojic acid was used, that is, both compounds showed active inhibition at early time points. These results suggest that GA derivatives are much more stable than vitamin C or kojic acid, although their intrinsic inhibitory potentials are relatively low. Higher stability and activity suggest that GA derivative **5** might be a useful candidate for skin whitening.

Introduction

Glycyrrhizin (GR), a triterpenoid saponin found in the roots of licorice (*Glycyrrhiza glabra*), is composed of one molecule of glycyrrhetinic acid (GA) and two molecules of glucuronic acid. The biological activities of GR are associated with 18β-GA, and include anti-inflammatory,[1] anti-viral,[2] anti-allergic,[3] and anti-tumor activities.[4,5] Moreover, GR and GA has been shown to modulate melanogenesis in B16 melanoma cells.[6] Stearyl glycyrrhetinate (SG), a derivative of GA, has also been implicated in skin whitening, which has been used as a sun care and sunscreen agent.[7]

Melanin synthesis, the main process involved in skin pigmentation, is regulated largely by the melanogenic enzyme tyrosinase.[8] This is a bifunctional enzyme that catalyzes the hydroxylation of tyrosine to DOPA and promotes the oxidation of DOPA to DOPA-quinone.[9] As natural compounds, vitamin C and kojic acid are representative tyrosinase-suppressive melanogenic inhibitors.[10,11] Although these substances have long been used as cosmetic skin-whitening agents, their instability limits their broad usage. To increase stability, derivatives of these compounds have recently been developed.[12]

In this study, we synthesized derivatives (**3**, **4**, **5**, **10**, **13**, **14**, **15**, and **16**) starting from GA, another natural compound, and compared their effects on tyrosinase activity with those of vitamin C and kojic acid. In addition, we described the structure and function relationship of the GA derivatives.

Results and Discussion

Chemistry

Although glycyrrhetinic acid (GA, **1**) and stearyl glycyrrhetinate (SG, **2**) have been reported to have inhibitory effects on melanogenesis, our initial assays indicated that these compounds were not or weakly inhibitory on mushroom tyrosinase activity. To increase inhibitory potential of GA, we synthesized its derivatives for which

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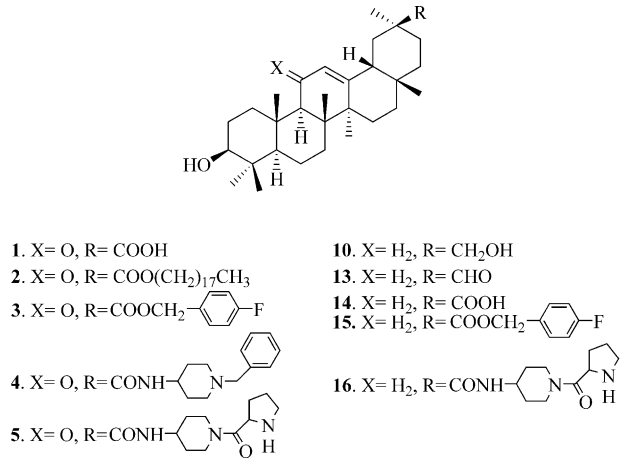


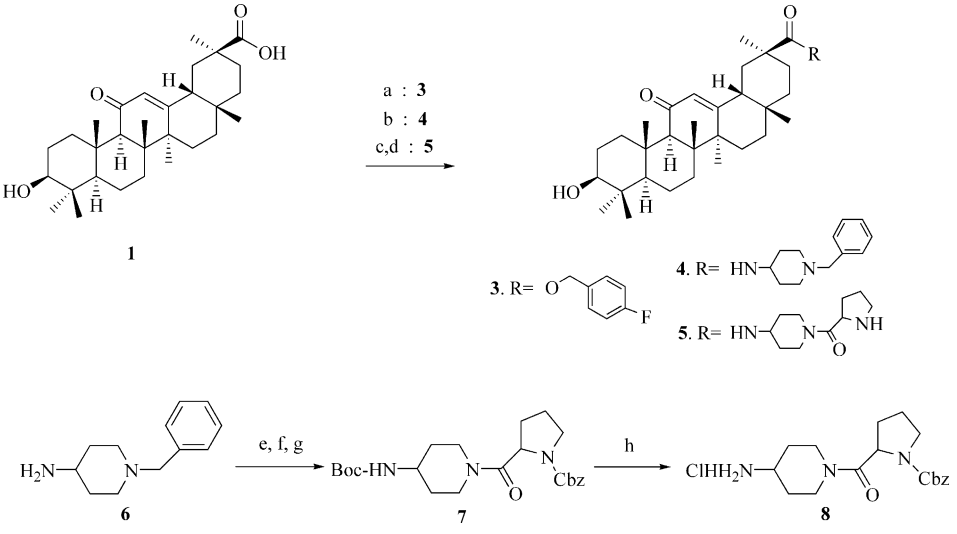
Scheme 1. Structures of glycyrrhetinic acid (GA, **1**) and its derivatives (**2**–**5**, **10**, **13**–**16**).

chemical modifications were mainly directed on ring C, and on the carboxylic function at position 30 (Scheme 1). Our strategy for the synthesis of GA derivatives **3**–**5** is illustrated in Scheme 2. The *O*-alkylation[13] of GA and 4-fluorobenzyl bromide with K_2CO_3 (3 equiv) in DMF at 80 °C for 3 h gave fluorobenzyl glycyrrhetinate **3** in 95% yield. In addition, GA was coupled with 4-amino-1-benzylpiperidine using EDCI/DMAP as a reagent,[14] to prepare benzylpiperidine glycyrrhetin amide **4** in 40% yield. Following our investigation of functionalization at the piperidine ring, we synthesized compound **5** by introducing an L-proline into the *tert*-N of synthetic **4**. Conversion of **4** to its derivative **5** did not proceed by straightforward reduction and subsequent coupling. The preparation of intermediate **8** in four steps started from 4-amino-1-benzylpiperidine **6**. Thus, the protection of **6** with 1 M NaOH and $(Boc)_2O$ afforded *N*-Boc compound in quantitative yield. Subsequent debenzylation[15] of its crude product with 10% Pd/C and H_2 followed by coupling of *N*-Cbz proline with DCC/HOBt/NMM[16] provided the Boc-Cbz amide **7** in 82% overall yield. Further treatment with HCl etherate[17] in anhydrous THF readily afforded the amine **8** in 96% yields. Subsequent coupling of GA and **8** with DCC/HOBt/NMM followed by deprotection of *N*-Cbz compound with 10% Pd/C and H_2 provided glycyrrhetin amide **5** in 76% overall yield.

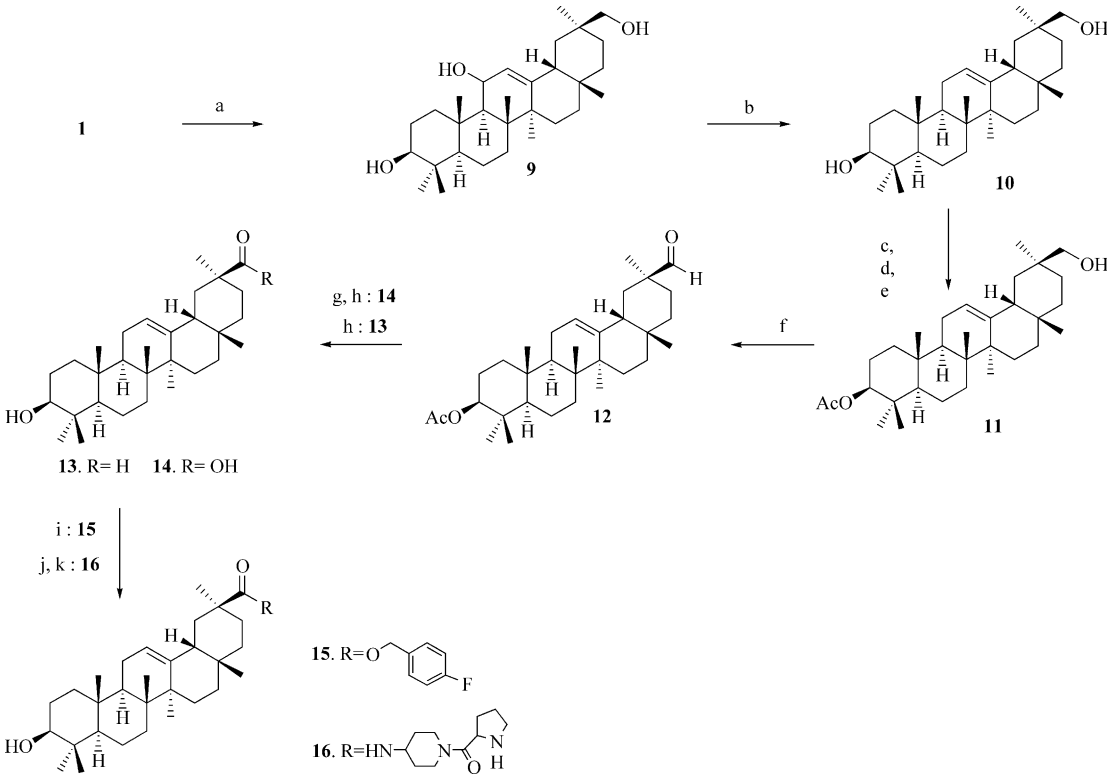
Conversion of GA **1** to the aldehyde **13** was achieved via the diol **10**, as shown in Scheme 3. GA was first reduced the triol **9** with $NaAlH_2(OCH_2CH_2OCH_3)_2$[18] in THF and then catalytically hydrogenated with 10% Pd/C to give 11-deoxo diol **10** in 60% yields. To selectively oxidize the C-30 position, the primary alcohol of **10** was protected with TBDMSCl[19] followed by acetylation of the hydroxyl group on the C-3 position with AcCl/Py/DMAP, and desilylation with TBAF gave 3-acetyl glycyrrhetinol **11** in 62% yields. Subsequent oxidation[20] of **11** was performed with PCC in CH_2Cl_2 to the aldehyde **12** in 85% yields, which was then hydrolyzed in the presence of K_2CO_3/MeOH to the desired derivative **13** in 87% yields. The aldehyde **12** was also oxidized with $KMnO_4$ according to Masamune oxidation[21] and hydrolyzed under basic conditions to give 11-deoxo glycyrrhetinic acid **14** in 87% yields. This compound was then coupled with 4-fluorobenzylbromide using K_2CO_3 as a reagent, to give fluoroglycyrrhetinate **15** in 92% yields. Subsequent coupling of **14** and **8** with DCC/HOBt/NMM followed by deprotection of *N*-Cbz compound with 10% Pd/C and H_2 provided glycyrrhetin amide **16** in 42% overall yield.

Effects on tyrosinase activity

The inhibition of our synthetic GA derivatives on mushroom tyrosinase was initially investigated and compared with those of vitamin C and kojic acid. As shown in Figure 1, the tyrosinase activity, determined using L-DOPA as a substrate, was not much changed by incubating with 100 μM GA or its derivatives for 10 min, whereas was severely inhibited by adding same concentrations of vitamin C or kojic acid. However, reactions for 120 min reversed the inhibition pattern.



Scheme 2. Synthesis of GA derivatives **3**–**5** and intermediates **6**–**8**: (a) 4-Fluorobenzyl bromide, K_2CO_3, DMF, 80 °C, 3 h; (b) 4-Amino benzylpiperidine, EDCI, DMAP, DMF, overnight; (c) **8**, DCC, HOBt, NMM, CH_2Cl_2, rt; 4 h; (d) 10% Pd/C, H_2, MeOH, 1 atm, rt, 3 h; (e) $(Boc)_2O$, 1 M NaOH, *t*-BuOH, rt, overnight; (f) 10% Pd/C, H_2, MeOH, 1 atm, rt, overnight; (g) *N*-Cbz-proline, DCC, HOBt, NMM, CH_2Cl_2, rt, 4 h; (h) HCl etherate, THF, 0 °C, 2 h.



Scheme 3. Synthesis of GA derivatives **10** and **13**–**16**: (a) $NaAlH_2(OCH_2CH_2OCH_3)_2$, THF, rt, 4 h; (b) 10% Pd/C, H_2, EtOH, 1 atm, rt, overnight; (c) TBDMCl, imidazole, DMF, rt, 2 h; (d) AcOCl, Py., DMAP, CH_2Cl_2, 0 °C, rt, 4 h; (e) TBAF, THF, 0 °C, rt, overnight; (f) PCC, CH_2Cl_2, 0 °C, rt, 2 h; (g) $KMnO_4$, 1.25 M NaH_2PO_4, 0 °C, rt, 2 h; (h) K_2CO_3, MeOH, 24 h; (i) 4-Fluorobenzylbromide, K_2CO_3, DMF, 80 °C, 3 h; (j) **14**, DCC, HOBt, NMM, CH_2Cl_2, rt; 4 h; (k) 10% Pd/C, H_2, MeOH, 1 atm, rt, 3 h.

Although GA and some derivatives (**2**, **3**, **10**, **13**, **14**, and **15**) were still inactive, other GA derivatives (**4**, **5**, and **16**) strongly inhibited tyrosinase activity, whereas vitamin C and kojic acid were no longer inhibitory. Interestingly, kojic acid increased tyrosinase activity, which will be discussed late.

The inhibitory potentials of GA derivatives **4**, **5**, **16** were further determined by measuring their IC_{50} values, the concentrations showing 50% inhibition. The IC_{50} values of GA derivatives, vitamin C, and kojic acid at different reaction times are summarized in Table 1. At 10 min, only vitamin C and kojic acid showed strong, dose-dependent suppressive effects on tyrosinase activity. At 60 min, the inhibitory potentials of vitamin C and kojic acid were markedly reduced, whereas those of GA derivatives began to become evident. At 120 min, the pattern of inhibition was completely reversed. Moreover, at reaction times longer than 2 h, only GA derivatives showed inhibitory effects (data not shown). Why are only derivatives **4**, **5** and **16** active and derivative **5** the most active? Structurally, derivative **4** contains benzylpiperidine linked to GA via amide bond. Other two compounds **5** and **16** also contain piperidines that coupled to L-proline instead of benzyl group. Therefore, piperidine appears to be important for the inhibition activity. The difference between **5** and **16** is present in the ring C, where compound **5** contains ketone. Since compound **5** is more active than **16**, a ketone in the ring C may also contribute on the increased activity. Taken together, both piperidine and ketone groups are related to inhibitory function against tyrosinase.

In subsequent experiments, we investigated the effects of reaction time on the tyrosinase activity. For this purpose, we incubated mushroom tyrosinase with 100 μM of GA derivatives **4**, **5**, **16**, vitamin C, and kojic acid for different time points. As shown in Figure 2, the inhibitory potentials of GA derivatives increased gradually, whereas those of vitamin C and kojic acid were highest at early time points (20 and 10 min, respectively), but afterwards became sharply decreased with time. To rule out any possibility that the inhibition may result from quenching the absorbance generated by a reaction between tyrosinase and L-DOPA, similar reactions were performed in the absence of tyrosinase. Although data are not shown, no changes in absorbance were detected at 475 nm, implying that the observed inhibition is specific for tyrosinase. Therefore, these results suggest that our GA derivatives could be nonclassical and slow-binding inhibitors, which are different from a classical and fast-binding inhibitor like vitamin C and kojic acid. After the kinetics on the slow-binding inhibition of enzyme reaction were well-defined,[22] a similar mode of inhibition was also found by other groups using tyrosinase and its inhibitors such as *m*-coumaric acid and L-mimosine.[23,24] However, our experimental conditions cannot exclude the possibility that such inhibition may result from the secondary reaction of the product by our GA derivatives. Of interest, kojic acid appeared to

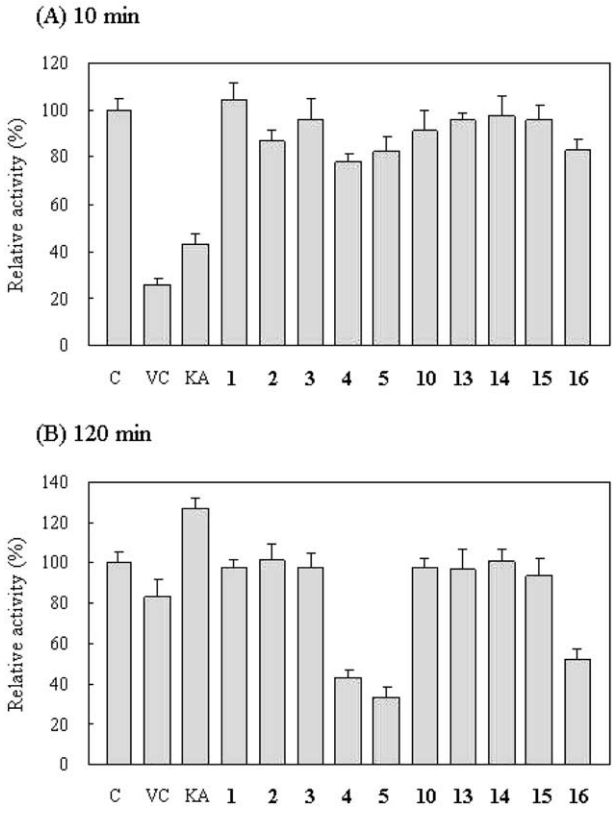


Figure 1. Effect of GA derivatives on tyrosinase activity. To measure tyrosinase activity, the DOPA-oxidase assay was employed in the presence of 1 mM of L-DOPA as described in the Experimental. Assays were performed with 100 μM of inhibitors and GA derivatives at two different time points: (A) 10 min; (B) 120 min. Relative activity (%) means percentage of relative absorbance at 475 nm in the presence versus in the absence of inhibitor. Symbols indicated as follows: C, control; VC, vitamin C; KA, kojic acid; **1**, Stearyl glycyrrhetinate (SG); **2**, glycyrrhetinic acid (GA); **3**–**16**; GA derivatives.

increase tyrosinase activity after 30 min reaction. Since this effect is specific for tyrosinase as described above, we assume that DOPA-quinone and/or DOPA-chrome, reaction products of tyrosinase may cooperate with kojic acid, which leads to shift equilibrium to the activation of tyrosinase under an excess condition of substrate, L-DOPA.

Interestingly, our data appear to be opposite to what was reported by other group.[25] In this report, they suggested that glycyrrhizin (GR), composed of one molecule of GA and two molecules of glucuronic acid, increases the cellular melanin content and tyrosinase activity. However, they found that GA had no or weakly inhibited melanogenesis, suggesting that glucuronic acid may have a certain role in stimulating melanogenesis. In addition, another group has reported that glabridin, a main constituent of the hydrophobic fraction of licorice extracts, shows inhibitory effects on melanogenesis.[26] In our study, GR was not analyzed and GA had no clear effect on tyrosinase activity as reported. Therefore, it is still possible that some GA derivatives may inhibit tyrosinase activity. As indicated in our study, GA derivatives with piperidine and ketone groups showed the inhibitory activity against tyrosinase.

Table 1. IC_{50} values of GA derivatives on tyrosinase activity

Derivatives	IC_{50} (μM)[a]		
	10 min	60 min	120 min
Glycyrrhetinic acid (GA)	> 500[b]	> 500	> 500
GA derivative **4**	> 500	100	70
GA derivative **5**	> 500	80	35
GA derivative **16**	> 500	150	100
Vitamin C	30	300	> 500
Kojic acid	60	> 500	> 500

[a]IC_{50} values were determined by interpolation of the dose–response curves obtained by DOPA-oxidase assays using 5 mM of L-DOPA at three different time points as described in the Experimental.
[b]> 500 means undetectable in our concentration range tested.

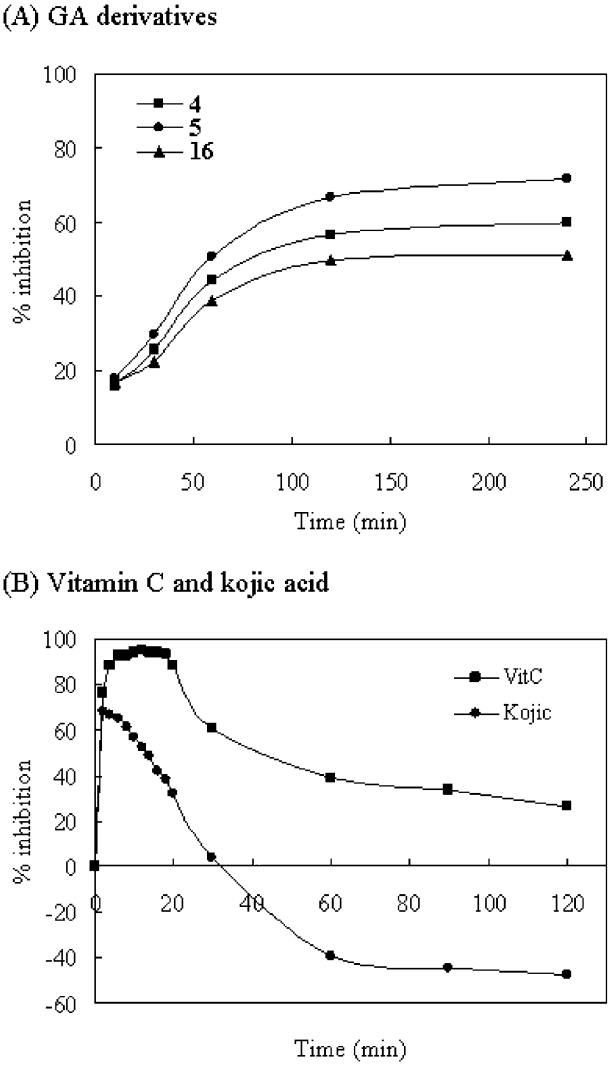


Figure 2. Comparison of the time-dependent responses of GA derivatives (**4**, **5**, and **16**), vitamin C, and kojic acid. The DOPA-oxidase assay was employed in the presence of 5 mM of L-DOPA as described in the Experimental. Assays were performed with 100 μM of GA derivatives (A), and vitamin C and kojic acid (B) by increasing reaction time. Data are expressed as a percentage (%) inhibition that means level of inhibition in the presence of inhibitor.

Conclusions

In this study, we synthesized some derivatives glycyrrhetinic acid (GA) and determined their anti-tyrosinase activities. Of the GA derivatives synthesized, compounds **4**, **5**, and **16** inhibited L-DOPA xidation of mushroom tyrosinase, whereas its parental GA and other derivatives including commercially available SG were not active in inhibition. Our analysis on structure and function relationship suggests that both piperidine and ketone groups are critical for the inhibitory activity of GA derivatives against tyrosinase. Of note, the inhibition was time-dependent, suggesting that these compounds could be slow-binding inhibitors as reported for other compounds like *m*-coumaric acid and L-mimosine. In our study, vitamin C and kojic acid, broadly used whitening agents in cosmetic formulation, strongly inhibited the tyrosinase activity for 20 and 10 min, respectively. Afterwards, their activities sharply decreased with time, indicating that these compounds are very unstable during prolonged reaction. Among three GA derivatives, compound **5** was most active with IC_{50} of 35 μM at 120-min reaction. In addition, time-course experiments indicated that compound **5** could be much more stable than vitamin C and kojic acid. Taken together, GA derivative **5** might be a useful candidate as a skin-whitening agent for cosmetic use, although its cytotoxicity remains to be determined in normal skin cells and in animals.

Experimental

The 18β-glycyrrhetinic acid was purchased from Sigma Chemical Co. (Sigma-Aldrich Korea, Seoul, Korea). Dry CH_2Cl_2, DMF were obtained by distillation from CaH_2. THF was distilled from sodium, benzophenone. The other commercially available reagents and solvents were used without further purification. All reactions were conducted under an Ar atmosphere, except for those reactions utilizing water as a solvent. They were monitored by TLC (Merck Kieselgel 60, F254). All the products prepared were purified by flash column chromatography on silica gel 60 (Merck, 230–400 mesh). The ^1H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM EX-400 using $CDCl_3$ or CD_3OD as a solvent. All chemical shifts (δ) are quoted in ppm downfield from TMS and coupling constants (*J*) are given in Hz. Low-resolution mass spectra were measured on a Agilent 1100 LC/MSD (API-ES) mass spectrometer and High-resolution electron impact mass spectra were obtained on VG Auto-spec Ultma mass spectrometer (EI 70 eV.)

10-Hydroxy - 2,4a,6a,6b,9,9,12a - heptamethyl - 13 - oxo - 1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10, 11,12,12a,12b,13,14b-eicosahydro-picene-2-carboxylic acid 4-fluoro-benzyl ester (3). A mixture of 18β-glycyrrhetinic acid (0.30 g, 0.64 mmol), K_2CO_3 (0.26 g, 1.91 mmol) and 4-fluorobenzylbromide (0.13 g, 0.70 mmol) was added DMF (15 mL). The reaction mixture was heated 3 h at 80 °C and cooled to room temperature. The crude was treated with H_2O (200 mL), the aqueous layer was extracted with EtOAc (2×20 mL). The joined organic extracts were washed with H_2O (100 mL), brine (50 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 1:3) afforded **3** as a white powder (0.35 g, 95%). Mp 142–144 °C; ^1H NMR (400 MHz, $CDCl_3$) : δ 7.36 (m, 2H), 7.07 (m, 2H), 5.57 (s, 1H), 5.11 (dd, *J* = 12.30, 3.87 Hz, 2H), 3.21 (m, 1H), 2.80 (m, 1H), 2.33 (s, 1H), 2.18 (m, 4H), 1.65 (m, 6H), 1.60 (s, 3H), 1.36 (s, 3H), 1.34 (m, 8H), 1.15 (s, 3H), 1.12 (s, 3H), 1.10 (s, 3H), 1.01 (m, 2H), 0.81 (s, 3H), 0.74 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.12, 176.15, 169.00, 132.00, 131.96, 130.29, 130.21, 128.50, 115.61, 115.40, 78.69, 65.45, 61.77, 60.37, 54.91, 48.19, 45.32, 43.93, 43.14, 41.01, 39.11, 37.60, 37.04, 32.72, 31.73, 31.12, 28.41, 28.21, 28.07, 27.27, 26.41, 23.33, 18.63, 17.45, 16.35, 15.59,14.18; MS: *m*/*z* (100%) = 601 ($[M+Na]^+$, 100), 579 (M^+, 13), 269 (13), 105 (8) ; HREIMS *m*/*z* 578.3773 (calcd for $C_{37}H_{51}FO_4$ 578.3771).

10-Hydroxy - 2,4a,6a,6b,9,9,12a - heptamethyl - 13 - oxo - 1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10, 11,12,12a,12b,13,14b-eicosahydro-picene-2-carboxylic acid (1-benzyl-piperidin-4-yl)-amide (4). A mixture of 18β-glycyrrhetinic acid (0.30 g, 0.64 mmol), EDCI (0.37 g, 1.91 mmol) and DMAP (0.08 g, 0.64 mmol) was added DMF (15 mL). The reaction mixture was added 4-aminobenzylpiperidine (0.14 g, 0.70 mmol) and stirred for overnight at room temperature. The crude was treated with H_2O (200 mL), the aqueous layer was extracted with EtOAc (2×50 mL). The joined organic extracts were washed with H_2O (100 mL), brine (50 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (10% MeOH–EtOAc) afforded **4** as a white powder (0.16 g, 40%); mp 122–125 °C; ^1H NMR (400 MHz, $CDCl_3$): δ 7.26 (m, 5H), 5.62 (s, 1H), 5.57 (s, 1H), 5.11 (m, 1H), 4.10 (m, 1H), 3.49 (s, 2H), 3.22 (m, 1H), 2.80 (m, 4H), 2.32 (s, 1H), 2.12 (m, 4H), 1.91 (m, 4H), 1.79 (m, 7H), 1.44 (m, 6H), 1.13 (s, 3H), 1.12 (s, 3H), 1.10 (s, 3H), 1.04 (m, 2H), 1.00 (s, 3H), 0.93 (s, 3H), 0.80 (s, 3H), 0.79 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 199.98, 174.93, 169.13, 138.19, 129.06, 128.43, 128.20, 127.04, 78.72, 62.92, 61.81, 60.37, 54.95, 52.27, 48.17, 46.27, 48.27, 45.33, 43.36, 43.19, 41.83, 39.19, 39.13, 37.43, 37.06, 32.75, 32.30, 32.11, 31.86, 31.48, 29.50, 28.51, 28.11, 27.26, 26.45, 26.37, 23.32, 21.04, 18.63, 17.46, 16.32, 15.58; MS: *m*/*z* (100%) = 643 (M^+, 100), 425 (12), 174 (63), 91 (74); HREIMS *m*/*z* 642.4765 (calcd for $C_{42}H_{62}N_2O_3$ 642.4760).

10-Hydroxy - 2,4a,6a,6b,9,9,12a - heptamethyl - 13 - oxo - 1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10, 11,12,12a,12b,13,14b-eicosahydro - picene - 2 - carboxylic acid [1 - (pyrrolidine - 2 - carbo nyl)-piperidin-4-yl]-amide (5). To a solution of **8** (1.25 g, 3.77 mmol) in CH_2Cl_2 (60 mL) was added drop by drop NMM (0.87 mL, 7.92 mmol) at 0 °C. After stirring for 10 min at 0 °C, a reaction mixture was added 18β-glycyrrhetinic acid (2.66 g, 5.65 mmol) and DCC (1.16 g, 5.65 mmol) at 0 °C. After stirring for 10 min at 0 °C, the reaction mixture warmed up room temperature and stirred for 4 h. The appearing precipitated was filtered off and filtrate was evaporated in vacuo. Purification by flash

chromatography (CH_2Cl_2–MeOH = 45:1→40:1) afforded coupling compound as a white powder (2.36 g, 80%). Continuously, to a solution of coupling compound (2.36 g, 3.01 mmol) in MeOH (30 mL) was added Pd/C (10%, 0.23 g) at room temperature. After stirring for 3 h under H_2, a reaction mixture was filtered with Celite pad and evaporated. Purification by recrystallization ($MeOH/CH_2Cl_2$) afforded **5** as a white powder (1.86 g, 95%, overall yield: 76%); mp 182–185 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.61 (d, J = 3.7 Hz,1H), 5.48 (m, 1H), 4.58 (d, J = 3.7 Hz, 1H), 4.04 (m, 1H), 3.88 (m, 2H), 3.16 (m, 3H), 2.71 (m, 3H), 2.32 (s, 1H), 2.20 (m, 6H), 1.56 (m, 12H), 1.36 (s, 6H), 1.43 (m, 5H), 1.12 (s, 6H), 1.10 (m, 4H), 0.99 (s, 3H), 0.85 (m, 2H), 0.80 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 200.00, 175.13, 172.37, 162.6, 128.47, 78.63, 61.78, 57.98, 52.91, 48.06, 46.56, 45.34, 43.93, 43.73, 43.38, 43.20, 41.77, 41.58, 39.13, 37.41, 36.02, 34.63, 32.60, 31.84, 31.55, 31.42, 31.24, 30.88, 29.43, 29.01, 28.56, 28.09, 26.87, 26.42, 25.24, 22.62, 17.44, 15.09, 14.09, 11.41; MS: m/z (100%) = 650 (M^+, 100), 306 (15); HREIMS m/z 649.4820 (calcd for $C_{40}H_{63}N_3O_4$ 649.4819).

2-(4-*tert*-Butoxycarbonylamino-piperidine-1-carbonyl)-pyrrolidine-1-carboxylic acid benzyl ester (7). A mixture of 1 M NaOH (11.56 mL, 11.56 mmol) and *t*-BuOH (5 mL) was stirred at room temperature for 30 min. 4-Amino benzylpiperidine (1.07 mL, 5.26 mmol) was added dropwise to a reaction mixture. Di-*tert*-dibutylcarbonate (1.26 g, 5.78 mmol) was added to a reaction mixture. The reaction was stirred at room temperature for overnight. The reaction mixture was acidified with 1 N HCl (< pH1), extracted EtOAc (2×30 mL) and washed with H_2O (500 mL), Brine, dried with Na_2SO_4, filtered, concentrated to give *N*-BOC compound as white powder solid (1.50 g, 98%). To a solution of *N*-BOC compound (1.46 g, 11.10 mmol) in MeOH (110 mL) was added Pd/C (10%, 0.15 g) at room temperature. After stirring for overnight under H_2, the reaction mixture was filtered with Celite pad and evaporated. Purification by recrystallization ($MeOH/CH_2Cl_2$) afforded debenzylation compound as a white powder (0.96 g, 95%). Continuously, to a solution of *N*-CBZ-Proline (1.19 mmol, 4.79 mmol) in CH_2Cl_2 (50 mL) were added HOBt (0.78 g, 5.75 mmol) and DCC (1.19 g, 5.75 mmol) at 0 °C. The solution was stirred for 30 min at 0 °C. To this mixture was added debenzylation compound (0.96 g, 4.79 mmol) in dry DMF (5 mL) and NMM (1.10 mL, 10.06 mmol), stirred 4 h at room temperature. The crude was treated with H_2O (300 mL), the aqueous layer was extracted with EtOAc (2×50 mL). The joined organic extracts were washed with H_2O (150 mL), brine (80 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography ($MeOH–CH_2Cl_2$ = 30:1→25:1) afforded **7** as a white powder (1.82 g, 88%, overall yield: 82%); mp 142–142 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.33 (m, 5H), 5.08 (dd, J = 12.16, 11.9 Hz, 2H), 4.60 (m, 2H), 3.90 (m, 1H), 3.61 (m, 4H), 2.03 (m, 8H), 1.44 (s, 9H), 1.31 (m, 2H); MS: m/z (100%) = 454 ($[M+Na]^+$, 100), 332 (81), 288 (10); HREIMS m/z 431.2421 (calcd for $C_{23}H_{33}N_3O_5$ 431.2420).

2-(4-Amino-piperidine-1-carbonyl)-pyrrolidine-1-carboxylic acid benzyl ester (8). To a solution of 13 (1.32 g 3.06 mmol) in CH_2Cl_2 (13 mL) was added 1.0 M solution HCl in diethylether (200 mL) at 0 °C. After stirring for 10 min at 0 °C, a reaction mixture was warmed up room temperature and stirred for 2 h. The reaction mixture was concentrated to give **8** as white powder solid (0.97 g, 96%); mp 79–82 °C; ^{1}H NMR (400 MHz, DMSO-d_6): δ 7.33 (m, 5H), 5.97 (m, 2H), 4.72 (m, 1H), 4.18 (m, 1H), 3.82 (m, 1H), 3.42 (m, 4H), 3.07 (m, 2H), 2.89 (m, 2H), 2.20 (m, 1H), 1.82 (m, 4H), 0.86 (m, 2H); MS: m/z (100%) = 332 (M^+, 100), 288 (18); HREIMS m/z 331.1896 (calcd for $C_{18}H_{25}N_3O_3$ 331.1895).

11-Hydroxymethyl-4,4,6a,6b,8a,11,14b-heptamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10, 11,12,12a,14,14a,14b-eicosahydro-picene-3,14-diol (9). To a solution of 18β-glycyrrhetinic acid (3.00 g, 6.37 mmol) in THF (63 mL) was added drop by drop $NaAl(OCH_2CH_2OCH_3)_2H_2$ (8.90 mL, 31.87 mmol) at 0 °C. After stirring for 10 min at 0 °C, a reaction mixture was warmed up room temperature and stirred for 4 h. The reaction quenched with 1 N HCl (aq) and extracted with EtOAc. The extracts were washed with brine, dried (Na_2SO_4) and evaporated in vacuo. Purification by recrystallization (EtOAc–*n*-hexane) afforded **9** as a white powder (1.84 g, 63%); mp 232–235 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.30 (d, J = 3.80 Hz, 1H), 4.30 (t, J = 4.20 Hz, 1H), 3.45 (m, 3H), 3.21 (m, 1H), 1.95 (m, 4H), 1.54 (m, 9H), 1.40 (s, 3H), 1.34 (m, 8H), 0.99 (m, 2H), 1.17 (s, 3H), 1.14 (s, 3H), 0.96 (s, 3H), 0.82 (s, 3H), 0.78 (s, 3H),0.69 (s, 3H); MS: m/z (100%) = 481 ($[M+Na]^+$, 100), 268 (17), 186 (8), 105 (37); HREIMS m/z 458.3760 (calcd for $C_{30}H_{50}O_3$ 458.3759).

11-Hydroxymethyl-4,4,6a,6b,8a,11,14b-heptamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10, 11,12,12a,14,14a,14b-eicosahydro-picen-3-ol (10). To a solution of **9** (1.84 g, 4.01 mmol) in EtOH (20 mL) was added Pd/C (10%, 0.27 g) at room temperature. After stirring for overnight under H_2, a reaction mixture was filtered with Celite pad and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 4:1→2:1) and recrystallization (EtOAc–*n*-hexane) afforded **10** as a white powder (1.70 g, 95%); mp 241–244 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.18 (t, J = 3.46 Hz, 1H), 3.50 (dd, J = 10.63, 8.66 Hz, 2H), 3.22 (m, 1H), 2.07 (m, 4H), 1.69 (m, 7H), 1.41 (m, 12H), 1.14 (s, 3H), 0.99 (s, 3H), 0.96 (s, 3H), 0.93 (s, 3H), 0.89 (s, 3H), 0.83 (s, 3H),0.79 (s, 3H);); ^{13}C NMR (100 MHz, $CDCl_3$): δ 146.83, 125.88, 77.89, 68.25, 62.57, 56.51, 47.59, 45.72, 44.72, 43.99, 43.78, 42.21, 39.12, 38.48, 36.56, 33.72, 32.63, 32.46, 29.81, 27.88, 27.47, 27.56, 27.52, 25.20, 24.45, 19.63, 18.57, 16.65, 15.98, 15.51; MS: m/z (100%) = 465 ($[M+Na]^+$, 100), 252 (20), 170 (12), 89 (45); HREIMS m/z 442.3815 (calcd for $C_{30}H_{50}O_2$ 442.3811).

Acetic acid 11-hydroxymethyl-4,4,6a,6b,8a,11,14b-heptamethyl-1,2,3,4,4a,5,6,6a,6b, 7,8,8a,9,10,11,12,12a,14,14a, 14b-eicosahydro-picen-3-yl ester (11). To a solution of **10** (0.97 g, 2.09 mmol) in DMF (20 mL) was added TBDMSCl (0.63 g, 4.18 mmol) and imidazole (0.57 g, 8.37 mmol) at 0 °C. After stirring for 10 min at 0 °C, a

reaction mixture was warmed up room temperature and stirred for 2 h. The crude was treated with 1 N HCl (200 mL), the aqueous layer was extracted with diethylether (2×40 mL). The joined organic extracts were washed with H_2O (100 mL), brine (50 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 12:1) afforded TBDMS protection compound as a colorless oil (1.05 g, 90%). To a solution of TBDMS protection compound (1.05 g, 1.88 mmol) in CH_2Cl_2 (20 mL) was added acetylchloride (0.16 mL, 2.25 mmol), pyridine (0.30 mL, 4.13 mmol) and DMAP (7 mg, 0.37 mmol) at 0 °C. After stirring for 10 min at 0 °C, a reaction mixture was warmed up room temperature and stirred for 4 h. The crude was treated with 1 N $KHSO_4$ (200 mL), the aqueous layer was extracted with EtOAc (2×40 mL). The joined organic extracts were washed with H_2O (100 mL), brine (50 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 30:1) afforded acetyl compound as a white powder (0.84 g, 75%). Continuously, to a solution of acetyl compound (0.84 g, 1.41 mmol) in THF (14 mL) was added 1.0 M TBAF (2.5 mL, 2.52 mmol) at 0 °C. After stirring for 10 min at 0 °C, a reaction mixture was warmed up room temperature and stirred for overnight. The reaction mixture was concentrated and the residue was purified by flash chromatography (*n*-hexane–EtOAc = 4:1) afforded compound **11** as a white powder (0.63 g, 92%, overall yield: 62%); mp 210–215 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.18 (t, J = 3.40 Hz, 1H), 4.50 (m, 1H), 3.49 (dd, J = 10.60, 8.70 Hz, 2H), 2.05 (s, 3H), 1.94 (m, 4H), 1.66 (s, 3H), 1.62 (m, 5H), 1.57(s, 3H), 1.32 (m, 9H), 1.21 (s, 3H), 1.06 (m, 2H), 1.01 (s, 3H), 0.90 (s, 3H), 0.87 (s, 3H), 0.86 (s, 3H), 0.83 (s, 3H); MS: m/z (100%) = 507 ($[M+Na]^+$, 100), 268 (18), 105 (12); HREIMS m/z 484.3913 (calcd for $C_{32}H_{52}O_3$ 484.3916).

Acetic acid 11-formyl-4,4,6a,6b,8a,11,14b-heptamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a, 9,10,11,12,12a,14,14a,14b-eicosahydro-picen-3-yl ester (12). To a solution of **11** (0.13 g, 0.27 mmol) in CH_2Cl_2 (5 mL) was added PCC (0.09 g, 0.40 mmol) at 0 °C. After stirring for 1 h at 0 °C, a reaction mixture was warmed up room temperature and stirred for 1 h. The reaction mixture was filtered with Celite pad and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 14:1) afforded **12** as a white powder (0.10 g, 85%); mp 212–215 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 9.45 (s, 1H), 5.20 (t, J = 3.37 Hz, 1H), 4.50 (m, 1H), 2.07 (s, 3H), 1.92 (m, 4H), 1.67 (s, 3H), 1.63 (m, 5H), 1.53(s, 3H), 1.35 (m, 9H), 1.23 (s, 3H), 1.08 (m, 2H), 1.02 (s, 3H), 0.90 (s, 3H), 0.84 (s, 3H), 0.86 (s, 3H), 0.83 (s, 3H); MS: m/z (100%) = 505 ($[M+Na]^+$, 100), 268 (15), 191 (8), 105 (12); HREIMS m/z 482.374 (calcd for $C_{32}H_{50}O_3$ 482.3759).

Acetic acid 11-formyl-4,4,6a,6b,8a,11,14b-heptamethyl-1,2,3,4,4a,5,6,6a,6b, 7,8,8a,9, 10,11,12,12a,14,14a,14b-eicosahydro-picen-3-yl ester (13). To a solution of **12** (0.10 g, 0.26 mmol) in MeOH (5 mL) was added K_2CO_3 (0.30 g, 2.20 mmol) at room temperature. A reaction mixture was stirred for 24 h. The crude was treated with H_2O (200 mL), the aqueous layer was extracted with EtOAc (2×20 mL). The joined organic extracts were washed with H_2O (100 mL), brine (50 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 5:1) afforded **13** as a white powder (0.08 g, 87%); mp 235–237 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.18 (t, J = 3.57 Hz, 1H), 3.23 (m, 1H), 3.22 (m, 1H), 1.75 (m, 6H), 1.60 (m, 5H), 1.37 (m, 5H), 1.25 (m, 5H), 1.16 (s, 3H), 0.99 (s, 3H), 0.90 (s, 3H), 0.85 (m, 2H), 0.83 (s, 6H), 0.79 (s, 3H), 0.77 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 206.30, 148.23, 127.08, 69.25, 65.59, 57.57, 47.49, 45.52, 44.22, 43.75, 43.45, 42.51, 39.42, 38.40, 36.45, 33.82, 32.53, 32.42, 28.88, 28.27, 27.87, 27.84, 27.52, 25.20, 24.85, 19.93, 18.77, 16.75, 15.68, 15.41; MS: m/z (100%) = 463 ($[M+Na]^+$, 100), 236 (21), 159 (15), 73 (19); HREIMS m/z 440.3651 (calcd for $C_{30}H_{48}O_2$ 440.3654).

10-Hydroxy-2,4a,6a,6b,9,9,12a-heptamethyl-1,2,3,4,4a,5, 6,6a,6b,7,8,8a,9,10,11,12, 12a,12b,13,14b-eicosahydro-picene-2-carboxylic acid (14). To a solution of **12** (0.23 g, 0.49 mmol) in mixture solvent (*t*-BuOH/CH_2Cl_2/1.25 M NaH_2PO_4 = 7.5 mL/1 mL/6 mL) was added 1.0 M $KMnO_4$ (2.06 mL, 1.96 mmol) at 0 °C. After stirring for 1 h at 0 °C, a reaction mixture was warmed up room temperature and stirred for 30 min. The reaction quenched with satd Na_2SO_3 (aq) and extracted with diethylether. The extracts were washed with brine, dried (Na_2SO_4) and evaporated in vacuo. The residue was purified by flash chromatography (*n*-hexane–EtOAc = 5:1) afforded acid compound as a white powder (0.16 g, 70%). Continuously, to a solution of acid compound (0.16 g, 0.33 mmol) in MeOH (3 mL) was added K_2CO_3 (0.46 g, 3.30 mmol) at room temperature. The reaction was stirred for 24 h. The crude was treated with H_2O (200 mL), the aqueous layer was extracted with EtOAc (2×50 mL). The joined organic extracts were washed with H_2O (150 mL), brine (50 mL), dried (Na_2SO_4) and evaporated. Purification by flash chromatography (*n*-hexane–EtOAc = 5:1) afforded **14** as a white powder (0.13 g, 87%, overall yield: 61%); mp 289–231 °C; ^{1}H NMR (400 MHz, CD_3OD): δ 5.15 (t, J = 3.44 Hz, 1H), 3.01 (m, 1H), 1.85 (m, 8H), 1.55 (m, 8H), 1.35 (m, 5H), 0.93 (m, 2H), 1.05 (s, 3H), 1.01 (m, 3H), 0.88 (s, 3H), 0.85 (s, 6H), 0.67 (s, 3H), 0.66 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 181.08, 145.23, 125.08, 68.25, 64.59, 56.87, 47.38, 45.32, 45.22, 43.85, 43.52, 42.51, 39.82, 38.25, 36.25, 33.72, 32.83, 32.22, 28.79, 28.37, 27.97, 27.79, 27.72, 25.30, 24.75, 19.89, 18.87, 16.87, 15.78, 15.41; MS: m/z (100%) = 479 ($[M+Na]^+$, 100), 413 (53), 381 (31), 148 (22); HREIMS m/z 456.3605 (calcd for $C_{30}H_{48}O_3$ 456.3603).

10-Hydroxy-2,4a,6a,6b,9,9,12a-heptamethyl-1,2,3,4,4a, 5,6,6a,6b,7,8,8a,9,10,11,12, 12a,12b, 13,14b-eicosahydro-picene-2-carboxylic acid 4-fluoro-benzyl ester (15) followed by the method of compound 3. Yield; 96% as a white powder; mp 243–245 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.34 (m, 2H), 7.04 (m, 2H), 5.18 (t, J = 3.43 Hz, 1H), 5.13 (dd, J = 12.30, 9.96 Hz, 2H), 3.22 (m, 1H), 1.92 (m, 6H), 1.63 (m, 9H), 1.28 (m, 8H), 1.12 (s, 6H), 0.99 (s, 3H), 0.94 (m, 3H), 0.93 (s, 3H), 0.76 (s, 3H), 0.72 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 176.23, 149.00, 132.02, 131.86, 130.49, 130.45, 128.48, 115.63, 115.38,

78.89, 65.25, 60.37, 54.71, 48.39, 45.82, 44.52, 43.93, 43.14, 41.21, 39.32, 38.60, 36.54, 33.82, 32.83, 32.52, 29.81, 28.88, 28.37, 27.56, 26.59, 25.20, 23.45, 19.63, 18.35, 16.85, 15.58, 15.18; MS: m/z (100%) = 587 ($[M+Na]^+$, 100), 565 (M^+, 13), 255 (13), 91 (8); HREIMS m/z 564.3975 (calcd for $C_{37}H_{53}FO_3$ 564.3979).

10-Hydroxy-2,4a,6a,6b,9,9,12a-heptamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12, 12a, 12b,13,14b-eicosahydropicene-2-carboxylic acid [1-(pyrrolidine-2-carbonyl)-piperidin-4-yl]-amide (16) followed by the method of compound 5. Yield; 42% as a white powder; mp 188–191 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.41 (m, 1H), 5.38 (m, 1H), 4.45 (m, 1H), 5.13 (m, 2H), 4.04 (m, 1H), 3.88 (m, 2H), 3.16 (m, 3H), 2.71 (m, 3H), 2.32 (s, 1H), 2.20 (m, 6H), 1.56 (m, 12H), 1.36 (s, 6H), 1.33 (m, 5H), 1.11 (s, 6H), 1.09 (m, 4H), 0.89 (s, 3H), 0.75 (m, 2H), 0.63 (s, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ 175.13, 172.37, 128.47, 78.63, 61.78, 57.98, 52.91, 48.06, 46.56, 45.34, 43.93, 43.73, 43.38, 43.20, 41.77, 41.58, 39.13, 37.41, 36.02, 34.63, 33.50, 32.60, 31.84, 31.55, 31.42, 31.24, 30.88, 29.43, 29.01, 28.56, 28.09, 26.87, 26.42, 25.24, 22.62, 17.44, 15.09, 14.09, 11.41; MS: m/z(100%) = 636 (M^+, 100), 291 (17); HREIMS m/z 635.5028 (calcd for $C_{40}H_{65}N_3O_3$ 635.5026).

Tyrosinase assay

The DOPA-oxidase assay measured the second major catalytic reaction of tyrosinase, the conversion of DOPA to DOPA-chrome via DOPA-quinone. This reaction was performed in the chromogenic appearance of DOPA-chrome from 1 or 5 mM DOPA at 475 nm. Forty microliters of 5 mM DOPA, 100 μL of 100 mM phosphate buffer (pH 6.8), and 10 μL of the same buffer with GA derivatives, vitamin C, or kojic acid were added to a 96-well plate, and then 40 μL of mushroom tyrosinase (0.01 mg/mL) (Sigma Chemical Co., St. Louis, MO, USA) were mixed. Following incubation at 37 °C for the indicated time, the absorbance of reaction mixture was determined at wavelength 475 nm. Dose-response curves were obtained by performing assays in the presence of increasing concentrations of inhibitors (0, 1.6, 3.2, 6.3, 12.5, 25, 50, 100, 200 μM). IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves.

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