Synthesis and NO Production Inhibitory Activities of Ursolic Acid and Oleanolic Acid Derivatives

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Structural modifications were performed on the C-3 and C-28 positions of ursolic acid and oleanolic acid and the NO production inhibitory activities of the resulting derivatives were evaluated. The SAR revealed that the ursolic acid and oleanolic acid derivatives **3a** and **4a**, which possessing methoxy group at C-3 position exhibit improved NO production inhibitory activity on LPS-induced RAW247 cells while showing less cytotoxicity than the parent compounds.

Key Words: Ursolic acid, Oleanolic acid, Anti-inflammatory, NO production, Structural modification

Introduction

Ursolic acid and oleanolic acid are pentacyclic triterpenoid compounds with 30 carbon atoms, biosynthetically derived from the cyclization of squalene. Since these triterpenes are widely distributed in food, medicinal herbs and other plants, these compounds can be integral part of the human diet.[1] Consequently, much research interest has been focused on the pharmacological evaluation[2] and efficient isolation of these two compounds from plants.[3]
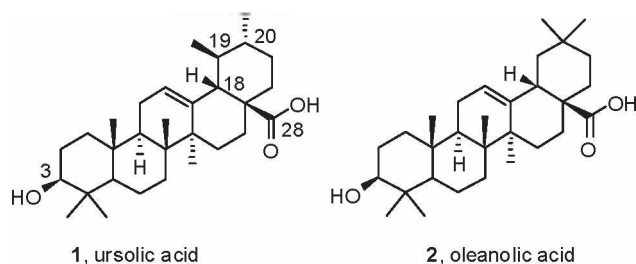
Ursolic acid has been reported to have antioxidant,[4] hepatoprotective,[5] anti-inflammatory, anticancer,[6-8] anti-HIV,[9] and antidiabetic activities.[10] Several mechanisms have been proposed to explain its anti-inflammatory activity, including inhibition of secretory PLA_2 enzymes,[11] IL-1β secretion,[12] and *i*NOS and COX-2.[13] However, in many cases, the potency of ursolic acid and its naturally occurring derivatives is relatively weak. Accordingly, chemical modification of the structure of ursolic acid was required to improve the biological activities while showing less toxicity. To increase its anti-inflammatory activity, considerable structural modifications have been performed mainly on the rings of ursolic acid. Some ursolic acid derivatives with modified rings A and C have been found to have high inhibitory activity against nitric oxide (NO) production in lipopolysaccharide (LPS)-induced macrophages.[14-15] However, the modification of the rings usually require multi-step sequence of reactions. In order to find potent anti-inflammatory derivatives, we synthesized ursolic acid derivatives by the simple synthetic route. Herein, we disclose the synthesis of ursolic acid derivatives, which have

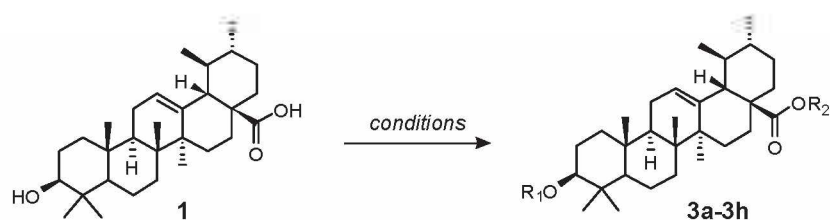1, ursolic acid　　　　　2, oleanolic acid

substituents at C-3 and/or C-28 positions and their evaluation for anti-inflammatory activities. The anti-inflammatory activities of the synthesized compounds were assessed by examining the inhibition of NO production induced by LPS in RAW264.7 cells.[16]

Results and Discussion

The ursolic acid derivatives **3a-3h** were synthesized by alkylation or acylation of hydroxyl group of C-3 position or carboxylic acid at C-28 position as shown in Scheme 1. Treatment of **1** with methyl iodide and NaH in THF provided ursolic acid 3-methyl ether (**3a**) in 59% yield along with methyl ursolate (**3b**) and methyl ursolate 3-methyl ether (**3c**) as minor products. Compound **3b** was also obtained in high yield by using trimethylsilyl diazomethane (TMSCHN₂) in THF/MeOH. The compound **3b** was transformed to **3c** by treatment with methyl iodide and NaH in THF in 68% yield. Acylation at C-3 position of **1** was accomplished by treating the corresponding acyl halides to afford **3d-3f** in 64~92% yields.

The low water solubility of ursolic acid derivatives can limit their further development as anti-inflammatory agents. In this regard, the attachment of amine moiety at C-3 of **1** followed by acidification with HCl was carried out to increase water solubility of the ursolic acid derivatives by salt formation.[1] When ursolic acid was treated with 1-(2-chloroethyl) pyrrolidine, catalytic amount of NaI and K_2CO_3 in DMF, the C-28 alkylated product **3g** rather than expected C-3 alkylated product was obtained. In the ¹H NMR of ursolic acid 3-methyl ether (**3a**), the signal of H-3 (δ 2.65) was shifted downfield significantly by 0.80 ppm when compared to that of **1** upon alkylation of C-3 hydroxyl group. On the other hand, ¹H NMR of **3g** shows only a slight change in chemical shift of H-3 at δ 3.17 indicating no alkylation occurred at C-3 position. Furthermore, in the HMBC spectrum of **3g**, the signal at δ 4.17 (H-1') was correlated with the signal at δ 176.2 (C-28) (Figure 1). On the basis obtained, the alkylation of **1** with 1-(2-chloroethyl)pyrrolidine occurred at the carboxylic acid site to afford compound **3g**. Compound **3g** was converted to

Scheme 1

Compounds	R₁, R₂	Conditions	Yields (%)
3a	R₁=CH₃, R₂=H	CH₃I, NaH, THF	59
3b	R₁=H, R₂=CH₃	TMSCHN₂, THF/MeOH	77
3c[a]	R₁=R₂=CH₃	CH₃I, NaH, THF	68
3d	R₁=Ac, R₂=H	AcCl, pyridine, THF	78
3e	R₁=CH₃OCH₂CO, R₂=H	CH₃OCH₂COCl, pyridine	64
3f	R₁=(CH₃)₂NCO, R₂=H	(CH₃)₂NCOCl, pyridine	92
3g	R₁=H, R₂=2-(pyrrolidin-1-yl)ethyl	i) 1-(2-chloroethyl)pyrrolidine, NaI, K₂CO₃, DMF, ii) 3N HCl, MeOH/CH₂Cl₂	15[b]
3h	R₁=2-(pyrrolidin-1-yl)acetyl, R₂=H	i) ClCH₂COCl, pyridine, CH₂Cl₂, ii) pyrrolidine, NEt₃, CH₂Cl₂, iii) AcCl, EtOH/THE	30[b]

[a]Prepared from **3b**. [b]Overall yield to obtain as a HCl salt.
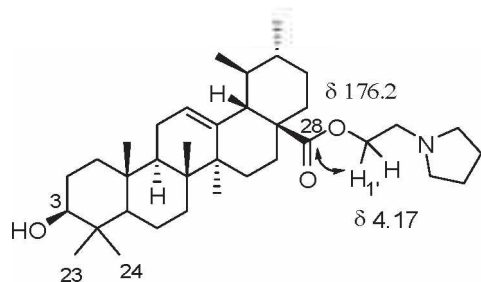
Figure 1. HMBC correlation of **3g**

Table 1. NO production inhibition and cell viability of ursolic acid derivatives **3a-3h**.

Compds	NO production inhiition (%)			Cell viability (%)		
	2 μM	10 μM	20 μM	2 μM	10 μM	20 μM
3a	25.3	26.8	39.7	79.3	69.9	55.7
3b	27.8	27.8	36.6	61.5	56.9	54.8
3c	41.8	43.5	50.2	61.7	34.9	30.6
3d	29.6	33.1	36.2	60.6	54.6	45.5
3e	32.1	34.4	51.6	61.7	33.2	28.5
3f	13.1	17.7	25.2	86.0	67.1	49.4
3g	28.9	45.0	93.7	82.5	54.8	19.9
3h	4.5	36.2	42.8	63.2	35.6	28.1
1	0.3	9.3	16.0	48.7	43.8	12.7

HCl salt in overall 15% yield by treating the free base with 3N HCl in MeOH/ CH₂Cl₂. It was thought that the difficulty of alkylation at C-3 might be caused by the steric hinderance of neighboring dimethyl groups at C-23 and 24 positions. To overcome this we decided to use a linker to introduce the amino group. Compound **1** was first acylated with chloroacetyl chloride and then alkylated with pyrrolidine followed by acidification with excess acetyl chloride in THF/EtOH to provide **3h**, which has an ammonium moiety at C-3 position.

Biological Evaluation. The inhibitory activities of synthetic triterpenoids **3a-3h** and **4a-4c** on NO production induced by LPS in RAW264.7 cells are shown in Table 1 and 2. For comparisons, the activities of ursolic acid (**1**) and oleanolic acid (**2**) were included as positive controls. The cytotoxicities of compounds in RAW264.7 cells were also assessed using MTT assay.[17] In most cases, ursolic acid derivatives exhibited more potent NO production inhibitory activities and less cytotoxicity on RAW264.7 cells than the parent compound, ursolic acid **1**. The 3-methyl ether of ursolic acid (**3a**) and methyl ursolate (**3b**) exhibited about 3 times more potent NO production inhibitory activity than **1** at 10 μM concentration. Methylation on both C-3 hydroxyl and C-28 carboxylic acid groups (**3c**) also increased the NO production inhibitory activity. However, inhibitions by this compound appeared to

be due in part to its cytotoxic effects because it shows only 34.9% cell viability at 10 μM concentration. The introduction of acetyl or methoxyacetyl group at C-3 (**3d-3f**) increased NO production inhibitory activity in similar levels to **3a** and **3b**. Compounds **3g**, which contains an ammonium moiety at C-3 position, exhibited the most potent NO production inhibition by 93.7% at 20 μM concentration. However, compound **3g** also showed strong cytotoxicity at this concentration (19.9% cell viability) and therefore, the observed NO production inhibition seems to be due to its cytotoxicity. In terms of cytotoxicity and NO production inhibition, compounds **3a**, which possesses methyl group at C-3 position, was best. i.e., it inhibited NO production by ~27% with RAW264.7 cells survival of ~70% at 10 μM while ursolic acid showed 9.3% NO production inhibition with 43.8% cell viability at the same concentration.

Since the structure of oleanolic acid (**2**) is closely related to that of ursolic acid (**1**) and differs only in the position of methyl groups at C-19 and C-20, the structures-activity relationship of oleanolic acid derivatives was expected to be similar to

Table 2. NO production inhibition and cell viability of oleanolic acid derivatives 4a-4c.

4a-4c
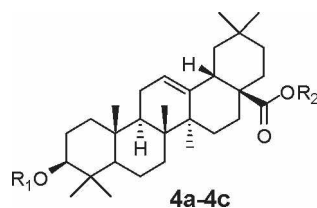
Compds	R_1, R_2	NO production inhibition (%)			Cell viability (%)		
		2 μM	10 μM	20 μM	2 μM	10 μM	20 μM
4a	R_1=CH₃, R_2=H	18.4	24.0	29.5	67.9	60.5	44.9
4b	R_1=H, R_2=CH₃	13.2	19.5	23.1	67.6	55.4	45.1
4c	R_1=R_2=CH₃	19.5	23.1	25.0	49.2	40.6	38.2
1, ursolic acid		0.3	9.3	16.0	48.7	43.8	12.7
2, oleanolic acid		4.3	17.9	20.4	70.5	51.7	42.8

that of ursolic acid. Therefore, we synthesized oleanolic acid derivatives 4a-4c using the similar procedures used for ursolic acid derivatives 3a-3c as shown in Scheme 1 and evaluated their NO production inhibition activities (Table 2). As was found in ursolic acid derivatives, the 3-methyl ether derivative 4a exhibited the most potent NO production inhibitory activity (24.0% inhibition at 10 μM) and weaker cytotoxicity than the parent compound, oleanolic acid (2, 17.9% inhibition at 10 μM) although the improvement was not as much as in ursolic acid derivatives.

In conclusion, the ursolic acid derivatives 3a-3h and oleanolic acid derivatives 4a-4c were synthesized and examined in terms of their abilities to inhibit NO production in LPS-induced RAW264.7 cells. Of the various derivatives synthesized, compounds 3a and 4a possessing methyl groups at C-3 position, were found to inhibit NO production most potently with less cytotoxicities. These results suggest that the simple modification at C-3 position of ursolic acid and oleanolic acid can improve the NO production inhibition activities on LPS-induced RAW264.7 cells while decreasing the toxicity.

Experimental Section

¹H NMR spectra were recorded on a Gemini Varian-400 (400 and 100 MHz, respectively). Analytical thin layer chromatographies (TLC) were carried out by pre-coated silica gel (E. Merck, Kiesegel 60F₂₅₄ layer thickness 0.25 mm). All solvents used were purified according to standard procedures.

Ursolic acid 3-methyl ether (3a): To a suspension of NaH (526 mg, 13.10 mmol) in THF (5 mL) was added a solution of ursolic acid (500 mg, 1.09 mmol) in THF (20 mL) at 0 °C, and the mixture was stirred at the same temperature for 1 h. MeI (1.5 mL, 24.0 mmol) was added dropwise, and the solution was stirred in ice-bath for 16 h. The solution was diluted with EtOAc and washed with water and saturated NH₄Cl. The water phases were washed with EtOAc and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated. The residue was purified by column chromatography (EtOAc/n-hexane = 1:5) to provide 3a as a white solid (314 mg, 59%). ¹H NMR (400 MHz, pyridine-d₅) δ 5.50 (1H, br s, olefinic-H), 3.31 (3H, s, -OCH₃), 2.64 (1H, m, H₃); ¹³C NMR (100 MHz,

pyridine-d₅) δ 179.6, 139.1, 125.4, 88.0, 56.8, 55.7, 53.3, 47.8, 47.7, 42.3, 39.7, 39.3, 39.2, 38.7, 38.3, 37.2, 36.9, 33.3, 30.8, 28.4, 28.1, 24.7, 23.7, 23.4, 21.9, 21.2, 18.3, 17.3, 17.2, 16.6, 15.3.

Methyl ursolate (3b): To a solution of the ursolic acid (50 mg, 0.11 mmol) in THF/MeOH (2 mL, 3:2) was added dropwise an etherial solution of TMSCHN₂ until yellow color persisted (0.024 mL, 0.16 mmol). The mixture was stirred at room temperature for 1 h and concentrated. The residue was purified by column chromatography (EtOAc/n-hexane = 1:3) to provide 3b as a white solid (40 mg, 77%). ¹H NMR (400 MHz, pyridine-d₅) δ 5.39 (1H, br s, olefinic-H), 3.69 (3H, s, -COOCH₃), 3.46 (1H, m, H₃), 2.43 (1H, d, J = 11.1 Hz, H₁₈); ¹³C NMR (100 MHz, pyridine-d₅) δ 178.6, 139.6, 126.9 79.0, 56.7, 54.3, 52.4, 49.2, 48.9, 43.2, 40.8, 40.3, 40.2, 40.1, 38.2, 37.9, 34.3, 31.7, 31.4, 29.7, 29.3, 29.0, 25.5, 24.8, 24.5, 22.2, 19.7, 18.3, 18.2, 17.5, 16.6.

Methyl ursolate 3-methyl ether (3c): To a suspension of NaH (107 mg, 2.68 mmol) in THF (5 mL) was added a solution of methyl ursolate (3b, 105 mg, 0.22 mmol) in THF (5 mL) at 0 °C, and the mixture was stirred at the same temperature for 1 h. MeI (305 μL, 4.91 mmol) was added dropwise, and the solution was stirred in ice-bath for 16 h. The solution was diluted with EtOAc and washed with water and aqueous saturated NH₄Cl. The aqueous layer was washed with EtOAc and the combined organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by silica gel column chromatography (EtOAc/n-hexane = 1:5) to give 3c as a white solid (72 mg, 68%). ¹H NMR (400 MHz, CDCl₃) δ 5.24 (1H, t, J = 3.5 Hz, olefinic-H), 3.60 (3H, s, -COOCH₃), 3.35 (3H, s, -OCH₃), 2.65 (1H, dd, J = 11.7, 4.3 Hz, H₃), 2.23 (1H, d, J = 11.3 Hz, H₁₈); ¹³C NMR (100 MHz, CDCl₃) δ 178.1, 138.1, 125.6 88.7, 57.6, 55.8, 52.9, 51.5, 48.1, 47.7, 47.6, 41.9, 39.5, 39.1, 38.9, 38.7, 38.5, 37.0, 36.6, 33.0, 30.6, 28.2, 28.0, 24.2, 23.6, 23.3, 22.0, 21.2, 18.2, 17.1, 16.9, 16.4.

3-O-Acetylursolic acid (3d): To a solution of ursolic acid (100 mg, 0.22 mmol) in THF (5 mL) was added acetyl chloride (18.7 μL, 0.26 mmol), pyridine (38.9 μL, 0.48 mmol) and 4-dimethylaminopyridine (5.3 mg, 0.04 mmol) at 0 °C. After stirring for 10 min at the same temperature, the reaction mix-

ture was warmed up to rt and further stirred for 2 days. The crude mixture was treated with 1N KHSO₄. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with water, brine, dried over anhydrous Na₂SO₄ and evaporated. The residue was purified by column chromatography (EtOAc/n-hexane = 1:5) to provide **3d** (85 mg, 78%). ¹H NMR (400 MHz, CDCl₃) δ 5.22 (1H, br s, olefinic-H), 4.49 (1H, m, H₃), 2.17 (1H, d, *J* = 11.2 Hz, H₁₈), 2.04 (3H, s, -COO*CH₃*); ¹³C NMR (100 MHz, CDCl₃) δ 184.1, 171.0, 137.9, 125.7, 80.9, 55.3, 52.5, 48.0, 47.5, 41.9, 39.5, 39.0, 38.8, 38.2, 37.7, 36.9, 36.7, 32.8, 30.6, 29.7, 28.1, 28.0, 24.0, 23.6, 23.3, 21.3, 21.2, 18.1, 17.1, 17.0, 16.7, 15.5.

3-*O*-Methoxyacetylursolic acid (3e): To a solution of ursolic acid (100 mg, 0.22 mmol) in THF (4 mL) was added methoxyacetyl chloride (143 μL, 1.58 mmol), pyridine (141 μL, 1.75 mmol) and 4-dimethylaminopyridine (8 mg, 0.07 mmol) at 0 °C. After stirring for 10 min at the same temperature, the reaction mixture was warmed up to rt and further stirred for 30 min. The reaction mixture was treated with 1N KHSO₄. The aqueous layer was extracted with EtOAc. The combined organic layer was washed with water, brine, dried over anhydrous Na₂SO₄ and evaporated. The residue was purified by column chromatography (EtOAc/n-hexane = 1:3) to provide **3e** (75 mg, 64%). ¹H NMR (400 MHz, CDCl₃) δ 5.23 (1H, br s, olefinic-H), 4.62 (1H, m, H₃), 4.04 & 4.15 (2H, two d, *J* = 14.6 Hz, -COO*CH₂*OCH₃), 3.45 (3H, s, -CH₂O*CH₃*), 2.18 (1H, d, *J* = 11.2 Hz, H₁₈); ¹³C NMR (100 MHz, CDCl₃) δ 183.8, 169.17, 138.0, 125.7, 81.6, 70.0, 59.4, 55.3, 52.5, 48.0, 47.4, 41.9, 39.5, 39.0, 38.8, 38.2, 37.8, 36.9, 36.7, 32.8, 30.6, 29.7, 28.1, 28.0, 24.0, 23.6, 23.2, 21.2, 18.1, 17.1, 17.0, 16.7, 15.5.

3-*O*-Dimethylcarbamoylursolic acid (3f): Ursolic acid (100 mg, 0.22 mmol) was dissolved in pyridine (3.8 mL) and dimethylcarbamyl chloride (200 μL, 2.19 mmol) was added. After heating at reflux for 15 h, the reaction mixture was cooled and treated with aqueous CuSO₄. The mixture was extracted with EtOAc and the organic layer was dried over anhydrous Na₂SO₄ and concentrated *in vacuo*. The residue was purified by column chromatography (EtOAc/ n-hexane = 1:3) to provide **3f** (106 mg, 93%). ¹H NMR (400 MHz, CDCl₃) δ 5.28 (1H, t, *J* = 3.4 Hz, olefinic-H), 3.20 (1H, dd, *J* = 10.7, 5.0 Hz, H₃), 2.98 (3H, s, -COON*CH₃*), 2.91 (3H, s, -COON*CH₃*), 2.21 (1H, d, *J* = 11.2 Hz, H₁₈); ¹³C NMR (100 MHz, CDCl₃) δ 173.2, 150.9, 137.5, 126.3, 79.0, 55.3, 52.7, 49.0, 47.6, 42.2, 39.7, 39.1, 38.8, 37.0, 36.9, 36.8, 36.0, 33.2, 31.6, 30.6, 29.7, 29.0, 28.2, 27.9, 27.2, 24.4, 23.4, 21.1, 18.3, 17.3, 16.9, 15.6, 15.5.

2-(Pyrrolidin-1-yl)ethyl ursolate hydrochloride (3g): A mixture of ursolic acid (200 mg, 0.44 mmol), 1-(2-chloroethyl)pyrrolidine (149 mg, 0.88 mmol), NaI (131 mg, 0.88 mmol), K₂CO₃ (242 mg, 1.75 mmol), and DMF (5 mL) was stirred at 75 °C for 3 h. After stirring at rt for 12 h, EtOAc was added to the mixture. The EtOAc layer was washed with water and brine, dried over anhydrous Na₂SO₄, and evaporated. The residue was purified by column chromatography (CH₂Cl₂/MeOH = 20:1) to provide **3g** as a free base (107 mg, 43%).

The above free base (107 mg, 0.19 mmol) was dissolved in MeOH/CH₂Cl₂ (1:1, 5 mL) and treated with 3N HCl (2 mL)

solution at 0 °C. The reaction was stirred at rt for 2 h. After addition of anhydrous Et₂O, the resulting precipitate was filtered, washed with ether and dried *in vacuo* to provide **3g** as a HCl salt (40 mg, 35%). ¹H NMR (400 MHz, methanol-*d₄*) δ 5.17 (1H, br s, olefinic-H), 4.23 (2H, t, *J* = 5.2 Hz, -COO*CH₂*CH₂-), 3.36~3.36 (6H, m, -*CH₂*N(*CH₂*)₂-), 3.05 (1H, m, H₃), 2.17 (d, 1H, *J* = 11.2 Hz, H₁₈), 1.99~2.03 (4H, m, -*CH₂CH₂*-); ¹³C NMR (100 MHz, methanol-*d₄*) δ 176.8, 138.2, 125.9, 78.2, 59.5 (2C), 55.3, 54.6, 53.1, 52.8, 47.4, 41.8, 39.5, 38.9, 38.8, 38.5, 38.4, 36.7, 36.3, 32.8, 30.2, 27.7, 27.4, 26.5, 25.5, 23.9, 22.9, 22.7, 22.5 (2C), 20.1, 18.0, 16.6, 16.2, 15.0, 14.6.

3-*O*-(Pyrrolidin-1-yl)acetylursolic acid hydrochloride (3h): To a solution of ursolic acid (100 mg, 0.22 mmol) in THF (4 mL) was added 2-chloroacetyl chloride (172 μL, 1.75 mmol), pyridine (160 μL, 1.97 mmol) and DMAP (8 mg, 0.07 mmol) at 0 °C. After stirring at the same temperature for 10 min, the reaction mixture was warmed up to rt and further stirred for 40 min. The crude mixture was treated with 1N KHSO₄ and the aqueous layer was extracted with EtOAc. The combined organic layer was washed with water, brine, dried over anhydrous Na₂SO₄ and evaporated. The residue was purified by column chromatography (EtOAc/n-hexane = 1:5) to provide 3-*O*-chloroacetylursolic acid (109 mg, 93%). ¹H NMR (400 MHz, CDCl₃) δ 5.23 (1H, br s, olefinic-H), 4.58 (1H, m, H₃), 4.03 & 4.07 (2H, two d, *J* = 14.6 Hz, -COO*CH₂*Cl), 2.17 (1H, d, *J* = 11.2 Hz, H₁₈); ¹³C NMR (100 MHz, CDCl₃) δ 184.2, 167.2, 138.0, 125.6, 83.3, 55.2, 52.5, 48.0, 47.4, 41.9, 41.3, 39.5, 39.0, 38.8, 38.2, 37.9, 36.9, 36.7, 32.8, 30.6, 29.1, 28.1, 28.0, 24.0, 23.6, 23.4, 23.3, 21.2, 18.1, 17.1, 17.0, 16.6.

To the compound above obtained (109 mg, 0.20 mmol) in DMF (3 mL) was added the pyrrolidine (51.4 μL, 0.61 mmol) and triethylamine (114.4 μL, 0.82 mmol) at rt and the mixture was stirred for 4 h. The reaction mixture was diluted with EtOAc and water. The EtOAc layer was separated and washed with H₂O and brine, dried over anhydrous Na₂SO₄, and evaporated. The residue was purified by column chromatography (CH₂Cl₂/MeOH = 20:1) to provide **3h** (60 mg, 52%). ¹H NMR (400 MHz, methanol-*d₄*) δ 5.23 (1H, br s, olefinic-H), 4.59 (1H, m, H₃), 3.59 (2H, s, -COO*CH₂*N-), 2.87 (4H, br s, -CH₂N(*CH₂*)₂-), 2.20 (1H, d, *J* = 11.2 Hz, H₁₈).

The above free base (67 mg, 0.12 mmol) was dissolved in EtOH/THF (1:1, 4 mL) and treated with acetyl chloride (84 μL, 1.18 mmol) at 0 °C. After stirring at rt for 2 h, the reaction mixture was poured into diethyl ether (4 mL). The resulting precipitate was filtered, washed with ether and dried *in vacuo* to provide **3h** as a HCl salt (45 mg, 63%). ¹H NMR (400 MHz, methanol-*d₄*) δ 5.23 (1H, br s, olefinic H), 4.68 (1H, m, H₃), 4.25 & 4.32 (2H, two d, *J* = 16.9 Hz, -COO*CH₂*N-), 3.31 (4H, br s, -CH₂N(*CH₂*)₂-), 2.20 (1H, d, *J* = 11.2 Hz, H₁₈)); ¹³C NMR (100 MHz, methanol-*d₄*) δ 180.2, 166.1, 138.3, 125.3, 83.8, 55.2, 54.7 (2C), 54.6, 52.9, 41.8, 39.4, 39.0 (2C), 38.0, 37.5, 36.7, 36.6, 32.7, 30.3, 27.8, 27.2, 23.9, 23.1, 22.9, 22.8 (2C), 22.7, 20.2, 17.8, 16.3, 16.2, 15.7, 14.6.

Oleanolic acid 3-methyl ether (4a): To a suspension of NaH (105 mg, 2.63 mmol) in THF (5 mL) was added a solution of oleanolic acid (100 mg, 0.22 mmol) in THF (5 mL) at 0 °C, and the mixture was stirred at the same temperature for 1 h. MeI (300 μL, 4.82 mmol) was added dropwise, and the solu-

tion was stirred in ice-bath for 16 h. The solution was diluted with EtOAc and washed with water and saturated NH_4Cl. The water phases were washed with EtOAc and the combined organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by column chromatography (EtOAc/*n*-hexane = 1:6) to provide **4a** as a white solid (35 mg, 33%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.21 (1H, br s, olefinic-H), 3.29 (3H, s, -OCH_3), 2.76 (1H, m, H_3), 2.59 (1H, m, H_{18}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 183.2, 142.6, 121.6, 87.7, 56.5, 54.7, 46.6, 45.5, 44.8, 40.5, 39.8, 38.2, 37.7, 37.3, 36.1, 32.7, 32.1, 31.5, 31.4, 29.6, 27.1, 26.6, 24.9, 22.6, 22.4, 21.8, 20.9, 17.1, 16.1, 15.2, 14.3.

Methyl oleanolate (4b): To a stirred solution of the oleanolic acid (200 mg, 0.44 mmol) in THF and MeOH (3:2, 10 mL) was added dropwise an etherial solution of $TMSCHN_2$ (97 μL, 0.66 mmol) until yellow color persisted. The mixture was stirred at rt for 1 h and then concentrated. The residue was purified by column chromatography (EtOAc/*n*-hexane = 1:10) to provide **4b** as a white solid (180 mg, 87%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.21 (1H, br s, olefinic-H), 3.55 (3H, s, -$COOCH_3$), 3.14 (1H, m, H_3), 2.79 (1H, m, H_{18}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 178.3, 143.8, 122.3, 79.0, 55.2, 51.6, 47.6, 46.7, 45.9, 41.6, 41.3, 39.2, 38.7, 38.4, 37.0, 33.8, 33.1, 32.6, 32.4, 30.7, 28.1, 27.7, 27.2, 25.9, 23.7, 23.4, 23.1, 18.3, 16.8, 15.6, 15.3.

Methyl oleanolate 3-methyl ether (4c): THF (5 mL) was suspended in NaH (81 mg, 2.04 mmol) at 0 °C. Methyl oleanolate (**4b**, 80 mg, 0.17 mmol) dissolved in THF (5 mL) was added, and the mixture was stirred at 0 °C for 1 h. MeI (232 μL, 3.74 mmol) was added dropwise, and the solution was stirred in ice-bath for 16 h. The solution was diluted with EtOAc and washed with water and saturated NH_4Cl. The water phases were washed with EtOAc and the combine organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue mixture was purified by column chromatography (EtOAc/*n*-hexane = 1:20) to provide **4c** as a white solid (62 mg, 75%). ^1H NMR (400 MHz, $CDCl_3$) δ 5.22 (1H, br s, olefinic-H), 3.55 (3H, s, -$COOCH_3$), 3.29 (3H, s, -OCH_3), 2.79 (1H, m, H_3), 2.59 (1H, m, H_{18}); ^{13}C NMR (100 MHz, $CDCl_3$) δ 177.2, 142.7, 121.3, 87.6, 56.5, 54.7, 50.5, 46.6, 45.7, 44.8, 40.6, 40.2, 38.2, 37.6, 37.3, 35.9, 32.8, 32.1, 31.6, 31.3, 29.7, 27.1, 26.6, 24.9, 22.6, 22.4, 22.0, 20.9, 17.2, 15.8, 15.3, 14.2.

Assay of NO production in LPS-induced RAW264.7 cells[16]: RAW 264.7 cells were seeded at 5×10^4 cells per well in flat-bottomed 96-well plates. LPS (1 μg/mL) and test agents were added to the culture medium, and incubated at 37 °C for 16 h, briefly centrifuged, and then 150 μl of cell culture supernatant was mixed with 150 μl of Griess reagent and incubated for 10 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm and compared to a standard calibration curve prepared from sodium nitrite.

Cytotoxicity assay of test agents against RAW264.7 cells: RAW264.7 cells donated from the Korean Cell Bank (Seoul, Korea) were cultured in DMEM containing 10% FBS, 1% antibiotic-antimycotic solution, 1mM sodium pyruvate and 1.5 g/L sodium bicarbonate under 5% CO_2 at 37 °C. The cytotoxic effect of test agents on RAW264.7 cells was measured using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay.[17] Briefly, RAW 264.7 cells were dispensed into 96 well plates at the concentration of 1×10^4 cells per well. The test compounds were added into RAW264.7 cells, and preincubated for 16 h and then rinsed with phosphate-buffered saline. MTT reagent (0.25 mg/mL) was added into the cells, incubated for 1 h, and then added 100 μL of dimethylsulfoxide. Absorbance at 540 nm was measured to estimate survived cells.

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