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Synthesis of Ergosterol and 5,6-Dihydroergosterol Glycosides and Their Inhibitory Activities on Lipopolysaccharide-Induced Nitric Oxide Production

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We have synthesized several glycosyl ergosterols and 5,6-dihydroergosterols (DHE) and examined their effects on production of nitric oxide (NO) and iNOS protein expression in LPS-treated RAW264.7 macrophage cells. Our results showed that DHE derivatives inhibited production of NO and iNOS protein expression more strongly than ergosterol derivative. Especially, DHE-Glc exhibited most potent inhibitory activity without cytotoxicity up to the concentration of 100 μM.

Key Words : Glycosyl ergosterol, Glycosyl 5,6-dihydroergosterol, Glycosylation, Inhibitory activity, NO production

Introduction

Nitric oxide (NO), a molecular messenger synthesized by one of three isoforms of nitric oxide synthases from of L-arginine and molecular oxygen, has been implicated in a number of physiological and pathological roles in mammalians.[1] Picomolar concentrations of NO play essential roles in neurotransmission and vascular homeostasis, which were constitutively produced by neuronal NOS (nNOS) and endothelial NOS (eNOS), respectively. Nanomolar concentrations of NO are produced by inducible NOS (iNOS) primarily in macrophages in response to stimuli such as cytokines and bacterial endotoxins, which play an essential role in host defense mechanisms. However, excessive and prolonged production of NO by iNOS can cause inflammatory processes, which leads to generation of various inflammatory diseases such as joint arthritis, atherosclerosis and cancer.[2,3] A number of inhibitors of iNOS have been screened and isolated from a variety of plant extracts to develop *anti*-inflammatory agents to prevent and treat inflammation-related diseases.[5-7]

It has been reported that *Stewartia koreana* leaves have several biological activities, such as inhibitory activities of NO production in LPS-stimulated macrophage cells, and inhibition of HIV protease activity.[4,8,9] Phytosterols such as α-amyrin, isoquercitrin, and 3-*O*-β-D-glucopyranosylspinasterol (spinasterol-Glc, Figure 1) were isolated from methanol extracts of *Stewartia koreana* leaves.[9] Among those compounds, spinasterol-Glc was identified as a compound not only to promote procollagen production but also to exhibit strong *anti*-inflammatory activity.[9,10] However, spinasterol-Glc has not been readily available from both isolation of natural plants and total synthesis. It was found that total synthesis of spinasterol-Glc was very difficult due to the unavailability of steroid backbone, spinasterol. Even though α-spinasterol could be prepared from stigmasterol using double bond migration, its known synthetic methods are inefficient.[11] Therefore, to develop analogues of spinasterol-Glc, we have searched structurally similar sterols to spinasterol, which could be readily obtained. Eventually we found 5,6-dihydroergosterol (DHE), as an alternative of spinasterol, which have a same steroidal backbone, but a different side chain with spinasterol. It could be expected to have equivalent or more potent biological activities and be more readily obtained in large amounts in efficient methods, compared to the synthesis of spinasterol. According to the literature procedures, synthetic methods of DHE have been known, which include the selective reduction of ergosterol.[12] Ergosterol is a major sterol in fungi, which can be commercially available in large quantities. Furthermore, ergosterol itself has been known to exhibit various biological activities, such as degranulation in mBMMCs, inhibitory activities against TPA-induced ear inflammation in mice.[13-15]

In this paper, we will describe the facile synthesis of ergosterol and 5,6-dihydroergosterol (DHE) glycosides (Figure 1) and their biological activities on iNOS inhibition and cell viability in LPS-stimulated macrophage cells.

Results and Discussion

As shown in Scheme 1, in order to investigate biological activities of ergosterol and DHE derivatives, we have synthesized several sugar derivatives of ergosterol and DHE (Compound **2**, **4**, **5** and **6**) (Scheme 1). Up to now, many synthetic methods of glycosyl steroids through the reaction of sterols with glycosyl donors have been reported.[16-18] First, we have tried to prepare a sterol such as DHE from ergosterol. Among known synthetic methods of DHE, the selective reduction of ergosterol by DIBAL (diisobutyl aluminum hydride) has been found most efficient.[12] For the effective

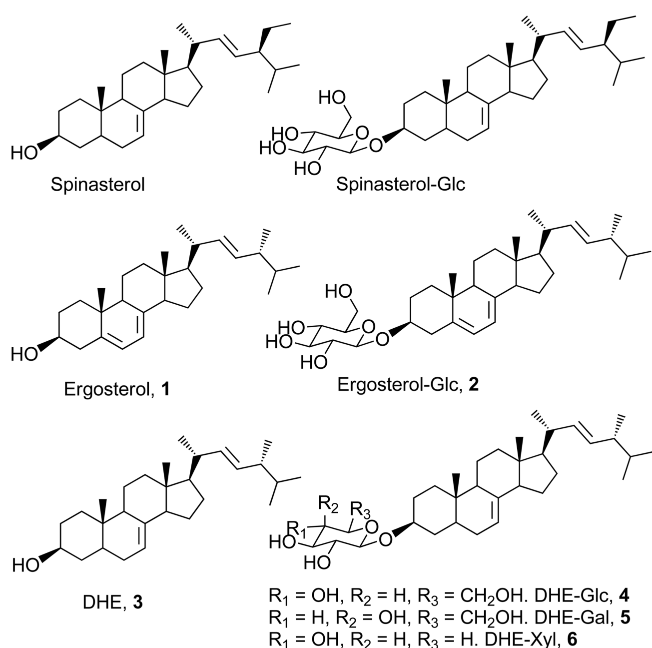


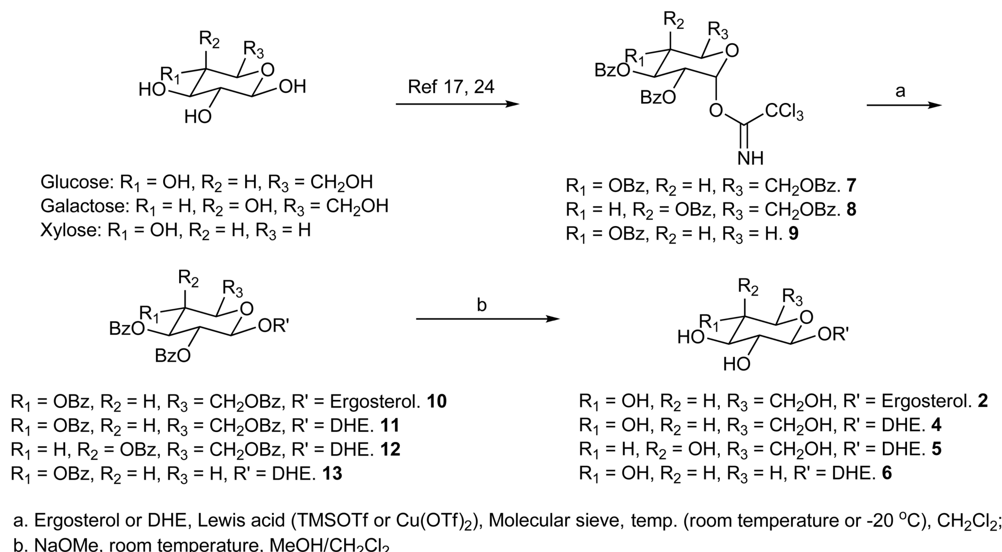
Figure 1. Structures of spinasterol, ergosterol and their derivatives.

glycosylation with a sterol, several glycosyl donors have been developed, which are glycosyl halide,[19] glycosyl sulfide[20] and glycosyl trichloroacetimidate.[18,21,25] In our synthesis, 2-*O*-benzoyl-glycosyl trichloroacetimidates (compound **7**, **8** and **9**) as the glycosyl donor and Lewis acid as the promoter have been chosen for the stereoselective glycosylation of ergosterol and DHE.[18,21,25] It has been expected that the use of 2-*O*-benzoyl-glycosyl donors can generally produce 1,2-*trans*-glycosidic bond due to the participation of C-2 acyl neighboring group.

It was found that the TMSOTf-catalyzed glycosylation of glucosyl trichloroacetimidate **7** with ergosterol gave the β-linked glucosyl ergosterol intermediate **10**, selectively.[22-24] Subsequent debenzoylation of compound **10** was achieved in the presence of NaOMe in methanol and CH_2Cl_2 to afford an Ergosterol-Glc **2**. Synthesis of DHE glycoside derivatives have also been tried by same procedures. However, TMSOTf-catalyzed glycosylation of DHE with 2-*O*-benzoyl-glycosyl trichoroacetimidate did not give a desired product, β-anomer of DHE-Glc, only. From the spectroscopic analysis (^{13}C NMR), we found that α-anomer also was formed in this reaction condition. It could be assumed that the different stereoselectivity between ergosterol and DHE comes from their different reactivity due to the difference of steroidal structure. To overcome this difficulty, a milder glycosylation condition, including $Cu(OTf)_2$ as a catalyst and low temperature (−20 °C), has been adopted. Eventually we could synthesize β-anomers of DHE glycosides (DHE-Glc **4**, DHE-Gal **5** and DHE-Xyl **6**) by a $Cu(OTf)_2$-catalyzed glycosylation of DHE with various perbenzoylated sugars and the subsequent deprotection.

For evaluating the reducing effect of ergosterol and DHE on NO production, we incubated RAW264.7 macrophage cells with 1 g/mL of LPS, in the presence of various concentrations of ergosterol or DHE. Upon stimulation with LPS, NO production levels increased markedly, by up to 32.6 ± 1.83 μM after 24 h, while the basal level was 2.4 ± 0.2 μM. As shown in Table 1, IC_{50} values of ergosterol and DHE are 16.6 ± 1.3 μM and 13.0 ± 1.6 μM, respectively, indicating that DHE is more potent than ergosterol on inhibition of NO production. Selective reduction of ergosterol to 5,6-DHE is supposed to increase the stability of the chemical structure and then reduce the toxicity.

Ergosterol-Glc (Compound **2** with IC_{50} value of 14.3 ± 1.4 μM) showed slightly higher potency on inhibition of LPS-induced NO production. Sugar derivatives of DHE (compound **4**, **5** and **6**) exhibited strong inhibition of NO production in the LPS-stimulated macrophage RAW264.7 cells with IC_{50} values in the ranges from 8.7 to 23 μM, which were more potent than ergosterol or DHE (Table 1). The data indicate that DHE-Glc exhibited most potent inhibitory activity among



Scheme 1. Synthesis of ergosterol-Glc and DHE-glycoside derivatives.

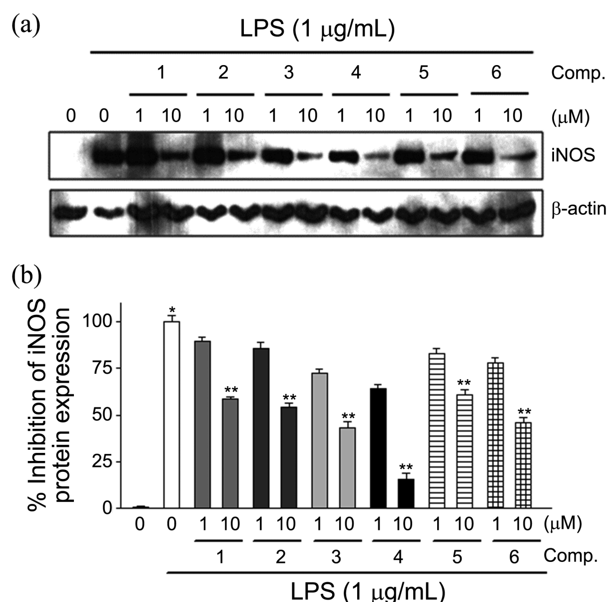


Figure 2. Effects of DHE derivatives on iNOS protein expression levels in LPS-induced RAW264.7 cells. (a) RAW264.7 cells were pretreated with various concentrations of six derivative compounds for 6 h and then stimulated with 1 mg/mL of LPS for 18 h. The Levels of iNOS proteins were measured by Western blot analysis using polyclonal antibodies against murine iNOS. β-actin was used as the internal control. (b) Western blot data were quantified by densitometry. Compounds: Ergosterol, **1**; Ergosterol-Glc, **2**; DHE, **3**; DHE-Glc, **4**; DHE-Gal, **5**; DHE-Xyl, **6**. The Western blot analysis were performed at least by three independent experiments, which were quantified by densitometry. Significance compared with medium alone, *P < 0.01, significance compared with LPS alone, **P < 0.01. The data were expressed as the means ± SD of three individual experiments.

the six compounds. We further investigated the effects of the six compounds on the expression of iNOS protein levels by Western blot analysis (Figure 2(a)), which were quantified by densitometry (Figure 2(b)). Our results showed that the amounts of the iNOS proteins were significantly increased in the RAW264.7 cells upon stimulation of LPS, and these increases were reduced by treatment of cells with ergosterol, DHE and the sugar derivatives of the compounds in a similar way to the inhibition of NO production by the compounds (Table 1).

We next examined the viability of cells treated with either of the compounds *via* MTT assay and found that ergosterol-Glc (LD_{50} = 72.1 ± 2.8 μM) appears to be only slightly cytotoxic, while ergosterol (LD_{50} = 23.8 ± 2.0 μM) exhibited strong cytotoxicity to the cells at the concentrations higher than 20 μM, Our results showed that cytotoxicity was reduced significantly by chemical linkage of a sugar to ergosterol. All sugar derivatives of DHE had no cytotoxic effects at the concentrations up to 100 μM used in the present study. The results indicate that the inhibition of nitrite by the glycosylated DHE was not attributable to cell death. Further studies on the relationship between structure and biological activity will be done.

Table 2. Effects of DHE derivatives on cell viability

Compound	Cell viability (Survival rate%)		$LD_{50}{}^{a}$ (μM)
	1 μM	10 μM	
Ergosterol, **1**	97 ± 1.4	78 ± 1.5	23.8 ± 2.0
Ergosterol-Glc, **2**	98 ± 0.8	84 ± 1.3	72.1 ± 2.8
DHE, **3**	99 ± 1.2	88 ± 0.7	> 100
DHE-Glc, **4**	99 ± 0.6	91 ± 0.9	> 100
DHE-Gal, **5**	99 ± 0.9	90 ± 1.0	> 100
DHE-Xyl, **6**	99 ± 1.0	90 ± 1.0	> 100

$^{a}LD_{50}$ values defined as concentrations that inhibition of 50% cell growth. Data are shown as the mean values ± SD (n = 3) **p* < 0.01 compared to treatment with media alone

Experimental

General Experimental Details. Perbenzoylated glycosyl trichloroacetimidates (Compound **7**, **8** and **9**) have been synthesized by the literature procedure.[18,25] ^{1}H and ^{13}C NMR spectra were recorded on Jeol Lambda-300 instrument. Splitting pattern: s (singlet), d (doublet), t (triplet), dd (doublet of doublet), brs (broad singlet), m (multiplet). Melting point was checked on Barnstead electrothermal 9100 instrument. HR-EI^{+}-MS was recorded on Jeol JMS-700. All chemicals and reagents were purchased from Sigma-Aldrich. All solvents used in reactions were purchased from Honeywell Burdick & Jackson®. The progress of reactions was monitored by TLC (Merck kiesegel $60F_{254}$) and column chromatography was performed on Merck silica gel 60 (230-

Table 1. Effects of DHE derivatives on nitrite production and iNOS protein expression

Compound	NO production (inhibition%)		$IC_{50}{}^{a}$ (μM)	iNOS protein expression (inhibition%)	
	1 μM	10 μM		1 μM	10 μM
Ergosterol	26 ± 0.7	32 ± 0.2	16.6 ± 1.3	21 ± 0.2	42 ± 0.2
Ergosterol-Glc	23 ± 0.2	48 ± 0.4	14.3 ± 1.4	25 ± 0.1	46 ± 0.3
DHE	26 ± 0.3	47 ± 0.5	13.0 ± 1.6	28 ± 0.3	52 ± 0.2
DHE-Glc	23 ± 0.7	77 ± 0.3	8.7 ± 1.2	36 ± 0.3	85 ± 0.2
DHE-Gal	21 ± 0.7	41 ± 0.5	23.0 ± 1.2	17 ± 0.2	40 ± 0.1
DHE-Xyl	23 ± 0.4	45 ± 0.5	16.0 ± 1.4	22 ± 0.3	55 ± 0.3

$^{a}IC_{50}$ values are defined as concentrations that inhibited the activity by 50%. Data are shown as the mean values ± SD (n =3) **p* < 0.05 compared to treatment with LPS alone

400 mesh).

5,6-Dihydroergosterol (3). Ergosterol (0.75 g, 1.90 mmol) and DIBAL (1 M in toluene, 8 mL, 8.00 mmol) in toluene (10 mL) was placed into a sealed tube. The mixture was stirred at 110 °C for 2.5 days. 3 drops of methanol was added to the mixture. The resultant was quickly poured into a 3 N HCl (30 mL) and extracted 3 times with CH_2Cl_2 (50 mL). The combined organic phase was washed with brine and dried over Na_2SO_4. After filtration and evaporation, product was purified by flash column chromatography (ethyl acetate, hexane) to give a white solid. Yield: 96%.

The product was identified by comparing data with literature.[26] ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.54-2.02 (42H), 3.60 (brs, 1H), 5.16-5.20 (m, 3H, olefin protons).

2,3,4,6-Tetrabenzoyl Glucopyranosyl Ergosterol (10). 2,3,4,6-Tetra-*O*-benzoyl-β-D-glucopyranosyl trichloroacetimidate (**7**) (0.60 g, 0.81 mmol), ergosterol (0.27 g, 0.68 mmol) and 4 Å molecular sieve in methylene chloride (6.0 mL) were stirred at 0 °C for 0.5 h. After addition of TMSOTf (12.3 μL, 0.08 mmol), the mixture was stirred at 0 °C for 0.5 h and at room temperature for another 0.5 h. After neutralization by Et_3N, the mixture was filtered through celite. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (ethyl acetate, hexane) to give a light yellow solid. Yield: 79%. mp 157-160 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.56 (s, 1H), 0.69 (s, 1H), 0.77-0.85 (m, 9H), 0.89-0.93 (m, 4H), 1.00-1.06 (m, 3H), 1.10-1.17 (m, 3H), 1.21-1.29 (m, 2H), 1.33-1.35 (m, 1H), 1.41-1.71 (m, 9H), 1.83-2.00 (m, 4H), 2.10-2.17 (m, 3H), 3.57-3.62 (m, 1H), 4.13-4.18 (m, 1H), 4.52 (dd, 1H, *J* = 6.1 Hz, 11.9 Hz), 4.61 (d, 1H, *J* = 11.7 Hz), 4.92-4.96 (m, 1H), 5.10-5.32 (m, 2H), 5.49 (t, 1H, *J* = 9.7 Hz), 5.62 (t, 1H, *J* = 9.7 Hz), 5.90 (t, 1H, *J* = 9.7 Hz), 7.26-7.43 (m, 9H), 7.46-7.57 (m, 3H), 7.83 (d, 2H, *J* = 7.3 Hz), 7.91 (d, 2H, *J* = 7.3 Hz), 7.96 (d, 2H, *J* = 7.5 Hz), 8.01 (d, 2H, *J* = 7.9 Hz).[27]

(3β, 22*E*)-Ergosta-5,7,22-trien-3-yl D-glucopyranoside (ergosterol-glucose, 2). To a solution of 2,3,4,6-tetrabenzoyl glucopyranosyl ergosterol (**10**) (0.30 g, 0.31 mmol) in a mixture solution (6.0 mL, CH_2Cl_2:MeOH = 5.0 mL:1.0 mL) was added NaOMe (66 mg, 1.20 mmol). The mixture was stirred at room temperature for 4 h. After addition of methanolic HCl, the mixture was filtered through celite. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (CH_2Cl_2, MeOH) to give a white solid. Yield: quantitative. mp 260-262 °C (literature: 259-261 °C). HR-EI$^+$-MS *m/z*: 558.3923 (Calcd for $C_{34}H_{54}O_6$: 558.3920). ^{1}H NMR (300 MHz, Pyridine-d^5) δ 0.59-2.19 (41H, ergosterol peaks), 4.04 (m, 2H), 4.33-4.36 (m, 2H), 4.47 (dd, 1H, *J* = 5.0, 11.4 Hz), 4.62 (d, 1H, *J* = 10.6 Hz), 5.08-5.11 (m, 3H), 5.27 (t, 2H, *J* = 6.0 Hz). ^{13}C NMR (75 MHz, Pyridine-d^5) δ 16.12, 17.84, 18.32, 19.80, 20.13, 21.20, 21.39, 22.05, 25.61, 26.92, 30.21, 33.29, 34.97, 35.50, 36.90, 37.12(2C), 39.26, 40.87, 43.03, 45.05, 57.35, 62.90, 71.73, 75.36, 76.93, 78.55, 78.65, 102.24, 117.81, 128.74, 132.23, 141.07, 151.22.[27]

2,3,4,6-Tetrabenzoyl Glucopyranosyl Dihydroergosterol (11). 2,3,4,6-Tetra-*O*-benzoyl-β-D-glucopyranosyl trichloroacetimidate (**7**) (1.21 g, 1.63 mmol), dihydroergosterol (0.50 g, 1.36 mmol) and 4 molecular sieve in methylene chloride (27.0 mL) were stirred at −20 °C for 0.5 h. After addition of $Cu(OTf)_2$ (49 mg, 0.14 mmol), the mixture was stirred at −20 °C for overnight. After neutralization by Et_3N, the mixture was filtered through celite. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (ethyl acetate, hexane) to give a white solid. Yield: 68%. mp 160-163 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.51-1.86 (42H, dihydroergosterol peaks), 3.59 (brs, 1H), 4.14-4.20 (m, 1H), 4.52 (dd, 1H, *J* = 5.9, 12.1 Hz), 4.61 (dd, 1H, *J* = 3.3, 11.9 Hz), 4.95 (d, 1H, *J* = 7.9 Hz), 5.09 (brs, 1H), 5.18 (t, 1H, *J* = 6.3 Hz), 5.47-5.53 (m, 1H), 5.63 (t, 1H, *J* = 9.7 Hz), 5.90 (t, 1H, *J* = 9.7 Hz), 7.29-7.57 (m, 12H), 7.82-8.03 (m, 8H).

(3β, 22*E*)-Ergosta-7,22-dien-3-yl D-glucopyranoside (DHE-Glc, 4). To a solution of 2,3,4,6-tetrabenzoyl glucopyranosyl dihydroergosterol (**11**) (0.70 g, 0.72 mmol) in a mixed solvent (CH_2Cl_2:MeOH = 7.0 mL:7.0 mL) was added NaOMe (0.5 M in MeOH, 8.6 mL, 4.30 mmol). The mixture was stirred at room temperature for overnight. After neutralization by Dowex Mac-3, the mixture was filtered. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (CH_2Cl_2, MeOH) to give a white solid. Yield: 89%. mp 267-270 °C. HR-EI$^+$-MS *m/z*: 560.4075 (Calcd for $C_{34}H_{56}O_6$: 560.4077). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.59 (s, 3H), 0.74 (s, 3H), 0.89 (d, 6H, *J* = 6.8 Hz), 0.99 (d, 4H, *J* = 6.8 Hz), 1.08 (d, 3H, *J* = 6.6 Hz), 1.15-1.28 (m, 4H), 1.35-1.62 (m, 8H), 1.66-1.73 (m, 4H), 1.73-1.83 (m, 2H), 1.86-1.95 (m, 2H), 1.99-2.06 (m, 3H), 3.95-4.08 (m, 3H), 4.24-4.34 (m, 2H), 4.41-4.44 (m, 1H), 4.60 (d, 1H, *J* = 11.5 Hz), 4.86 (brs, -OH peak), 5.04 (d, 1H, *J* = 7.7 Hz), 5.19 (brs, 1H), 5.26-5.29 (m, 1H). ^{13}C NMR (75 MHz, Pyridine-d^5) δ 12.27, 13.06, 17.84, 19.83, 20.14, 21.37, 21.73, 23.27, 28.53, 30.02, 33.33, 34.53, 34.72, 37.31, 39.60, 40.15, 40.85, 43.06, 43.46, 49.56, 55.28, 56.05, 62.90, 71.75, 75.38, 77.04, 78.54, 78.67, 102.24, 117.86, 132.06, 136.18, 139.54.

2,3,4,6-Tetrabenzoyl galactopyranosyl dihydroergosterol (12). 2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl trichloroacetimidate (**8**) (2.23 g, 3.01 mmol), dihydroergosterol (1.0 g, 2.51 mmol) and 4 molecular sieve in methylne chloride (36.0 mL) were stirred at −20 °C for 0.5 h. After addition of $Cu(OTf)_2$ (90 mg, 0.25 mmol), the mixture was stirred at −20 °C for 4 h. After neutralization by Et_3N, the mixture was filtered through celite. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (ethyl acetate, hexane) to give a white solid. Yield: 74%. mp 158-161 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.52-2.33 (42H, dihydroergosterol peaks), 3.67 (brs, 1H), 4.31-4.36 (m, 1H), 4.41-4.47 (m, 1H), 4.66-4.72 (m, 1H), 4.93 (d, 1H, *J* = 7.7 Hz), 5.10-5.21 (m, 2H), 5.60 (dd, 1H, *J* = 3.2, 10.4 Hz), 5.75-5.81 (m, 1H), 5.99 (d, 1H, *J* = 2.8 Hz), 7.21-7.26 (m, 2H), 7.35-7.63 (m, 10H), 7.79 (d, 2H, *J* = 7.5 Hz), 7.96 (d, 2H, *J* = 7.5 Hz), 8.03 (d, 2H, *J* = 8.1 Hz), 8.11 (d, 2H, *J* = 7.3 Hz).

(3β, 22*E*)-Ergosta-7,22-dien-3-yl D-galactopyranoside (DHE-Gal, 5). To a solution of 2,3,4,6-Tetrabenzoyl galac-

topyranosyl dihydroergosterol (**12**) (0.20 g, 0.20 mmol) in a mixed solvent (CH_2Cl_2:MeOH = 2.0 mL:2.0 mL) was added NaOMe (0.5 M in MeOH, 2.5 mL, 1.23 mmol). The mixture was stirred at room temperature for 5 h. After neutralization by Dowex Mac-3, the mixture was filtered. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (CH_2Cl_2, MeOH) to give a white solid. Yield: 87%. mp 267-269 °C. HR-EI$^+$-MS *m/z*: 560.4075 (Calcd for $C_{34}H_{56}O_6$: 560.4077). ^{1}H NMR (300 MHz, Pyridine-d^5) δ 0.59 (s, 3H), 0.73 (s, 3H), 0.83 (dd, 1H, J = 2.9, 7.0 Hz), 0.89 (dd, 6H, J = 1.9, 6.7 Hz), 0.99 (d, 4H, J = 6.8 Hz), 1.08 (d, 3H, J = 6.4 Hz), 1.18-1.30 (m, 4H), 1.34-1.62 (m, 9H), 1.68-1.72 (m, 4H), 1.77-1.86 (m, 1H), 1.88-1.94 (m, 1H), 1.98-2.12 (m, 3H), 4.01 (br s, 1H), 4.14 (t, 1H, J = 5.9 Hz), 4.24 (dd, 1H, J = 3.0, 9.4 Hz), 4.47-4.52 (m, 3H), 4.62 (d, 1H, J = 2.4 Hz), 4.81 (d, 1H, J = 7.5 Hz), 5.18 (brs, 1H), 5.27 (t, 1H, J = 5.8 Hz). ^{13}C NMR (75MHz, Pyridine-d^5) δ 12.27, 13.05, 17.83, 19.82, 20.14, 21.36, 21.73, 23.27, 28.52, 30.02, 33.33, 34.52, 34.75, 37.33, 39.61, 40.17, 40.84, 43.06, 43.47, 49.59, 55.28, 56.07, 62.62, 70.39, 72.73, 75.46, 76.93, 76.99, 102.86, 117.86, 132.06, 136.19, 139.55.

2,3,4-Tribenzoyl xylopyranosyl dihydroergosterol (13). 2,3,4-Tri-*O*-benzoyl-β-D-xylopyranosyl trichloroacetimidate (**9**) (0.50 g, 0.82 mmol), dihydroergosterol (0.30 g, 0.75 mmol) and 4 molecular sieve in methylene chloride (15.0 mL) were stirred at −20 °C for 0.5 h. After addition of $Cu(OTf)_2$ (27 mg, 0.08 mmol), the mixture was stirred at −20 °C for 10.5 h. After neutralization by Et_3N, the mixture was filtered through celite. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (ethyl acetate, hexane) to give a white solid. Yield: 65%. mp 190-192 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.62-2.15 (42H, dihydroergosterol peaks), 3.65-3.71 (m, 2H), 4.44 (d, 1H, J = 11.7 Hz), 4.96 (brs, 1H), 5.26-5.35 (m, 2H), 5.75 (t, 1H, J = 7.1 Hz), 7.33-7.40 (m, 6H), 7.49-7.53 (m, 3H), 7.98-8.05 (m, 6H).

(3β, 22*E*)-Ergosta-7,22-dien-3-yl D-xylopyranoside (DHE-Xyl, 6). To a solution of 2,3,4-Tribenzoyl xylopyranosyl dihydroergosterol (**13**) (0.70 g, 0.83 mmol) in a mixed solvent (CH_2Cl_2:MeOH = 12.0 mL:6.0 mL) was added NaOMe (0.5 M in MeOH, 6.6 mL, 3.32 mmol). The mixture was stirred at room temperature for 3 h. After neutralization by Dowex Mac-3, the mixture was filtered. The filtrate was concentrated *in vacuo* and purified by flash column chromatography (CH_2Cl_2, MeOH) to give a white solid. Yield: 86%. mp 220-224 °C. HR-EI$^+$-MS *m/z*: 530.3975 (Calcd for $C_{33}H_{54}O_5$: 530.3971). ^{1}H NMR (300 MHz, Pyridine-d^5) δ 0.59 (s, 3H), 0.75 (s, 3H), 0.89 (dd, 6H, J = 2.2, 6.8 Hz), 0.99 (d, 3H, J = 6.8 Hz), 1.08 (d, 3H, J = 6.4 Hz), 1.19-1.53 (m, 11H), 1.62-1.79 (m, 8H), 1.88-1.94 (m, 2H), 1.99-2.02 (m, 2H), 2.12-2.16 (m, 1H), 3.80 (t, 1H, J = 10.2 Hz), 3.97 (brs, 1H), 4.09 (t, 1H, J = 8.1 Hz), 4.24-4.37 (m, 2H), 4.42-4.47 (m, 1H), 4.95 (d, 1H, J = 7.5 Hz), 5.19 (brs, 1H), 5.26 (t, 1H, J = 7.5 Hz). ^{13}C NMR (75 MHz, Pyridine-d^5) δ 12.28, 13.06, 17.83, 19.83, 20.14, 21.37, 21.75, 23.27, 28.54, 30.03, 30.17, 33.33, 34.56, 34.81, 37.41, 39.59, 40.31, 40.86, 43.05, 43.47, 49.56, 55.28, 56.06, 67.22, 71.26, 75.16, 77.36, 78.61, 103.20, 117.85, 132.06, 136.19, 139.59.

Cell Culture. Murine macrophage RAW264.7 cells were cultured at 37 °C in Dulbecco's modified Eagles's medium containing 10% fetal bovine serum, 2 mM glutamate, 100 unit/mL of penicillin, and 100 μg/mL of streptomycin in a humidified incubator with 5% CO_2. Cells were incubated with 1 g/mL LPS along with various concentrations of ergosterol or its derivatives for 24 h as indicated.

MTT Assay. Cell viability was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-thetazolium bromide (MTT) assay. RAW264.7 cells were maintained in culture media, washed three times with PBS and then seeded into 96-well plates (5 × 10^3 cells/well). Cells were then treated with various concentrations of ergosterol or its derivatives for 48 h. Cell viability was evaluated by the addition of 50 μL of MTT. After 1 h of incubation, the cell-free supernatants were removed completely from each well, and 100 mL of dimethyl sulfoxide was added. Absorbance was measured at 540 nm using a spectrophotometric multiwell microplate reader (Multiskan MS; Thermo Electron Corporation, Waltham, MA, USA).

Measurements of Nitric Oxide. Nitric oxide was detected by measuring the amount of nitrite, a stable oxidized product, in cell culture supernatants, as previously described (Lee *et al*., 2007). To test the effect of ergosterol or its derivative on iNOS activity, RAW264.7 cells (1 × 10^4 cells/well) were grown in culture media in 96-culture well plate for 24 h. Cells were washed with PBS twice and incubated in serum-free medium for 6 h. Cells were then stimulated with 1 μg/mL of LPS (Sigma, St. Louis, MO, USA) in the presence of various concentrations of ergosterol or its derivatives for 18 h. 100 mL of cell culture supernatant was mixed with 100 μL of Griess reagent (Sigma, USA) in a 96-well plate, and absorption was read at 550 nm with a spectrophotometer. Nitrite concentrations were determined by comparison with a sodium nitrite standard curve.

Western Blot Analysis. RAW264.7 cells were lysed in RIPA buffer (150 mM sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 7.5, 2 mM EDTA) containing proteinase inhibitor cocktails (Roche, Indianapolis, IN, USA). Protein concentration was quantified with a protein assay kit (Bio-Rad Laboratories, Philadelphia, PA, USA). Proteins (20 μg/lane) were resolved with SDS-polyacrylamide gel electrophoresis, and Western blot analysis was performed as described previously (Lee *et al*., 2007). Mouse *anti*-iNOS (R&D Systems, Minneapolis, MN, USA) were utilized as primary antibodies, and peroxidase-conjugated antibody was used as a secondary antibody. The membranes were developed with an enhanced chemiluminescence system from GE healthcare (Buckinghamshire, UK) exposed to X-ray film (Fuji photo Film Co., Ltd) for 30 s.

Statistical Analysis. Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. The data are presented as means ± SD and statistical comparisons between groups were performed using 1-way ANOVA followed by Student's *t*-test.

Conclusion

In conclusion, we have found 5,6-dihydroergosterol (DHE) as an highly efficient alternative of spinasterol. We have studied the synthesis of several Ergosterol and 5,6-dihydroergosterol glycosides and their inhibitory activities of NO production. 5,6-Dihydroergosterol was made from commercially available steroid, ergosterol, by the selective DIBAL reduction of double bond. Ergosterol and 5,6-Dihydroergosterol was linked with various sugar moieties by the trichloroacetimidate method. We found that 5,6-dihydroergosterol-Glucose showed strong inhibitory activity of NO production and low toxicity.

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