Electronic Supplementary Information

Simultaneous determination of deuterium-labelled ergosterol and brassicasterol in stroke-prone spontaneously hypertensive rats by ultra-high performance liquid chromatography electrospray ionization tandem mass spectrometry

Koichi Machida, Yuji Koseki[#], Akira Kotani, Kazuhiro Yamamoto, Tsuyoshi Miura[$], Hideki Hakamata*

Departments of Analytical Chemistry and [$]Pharmaceutical Chemistry, and [#]Center for the Advancement of Pharmaceutical Education, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan

*Corresponding author

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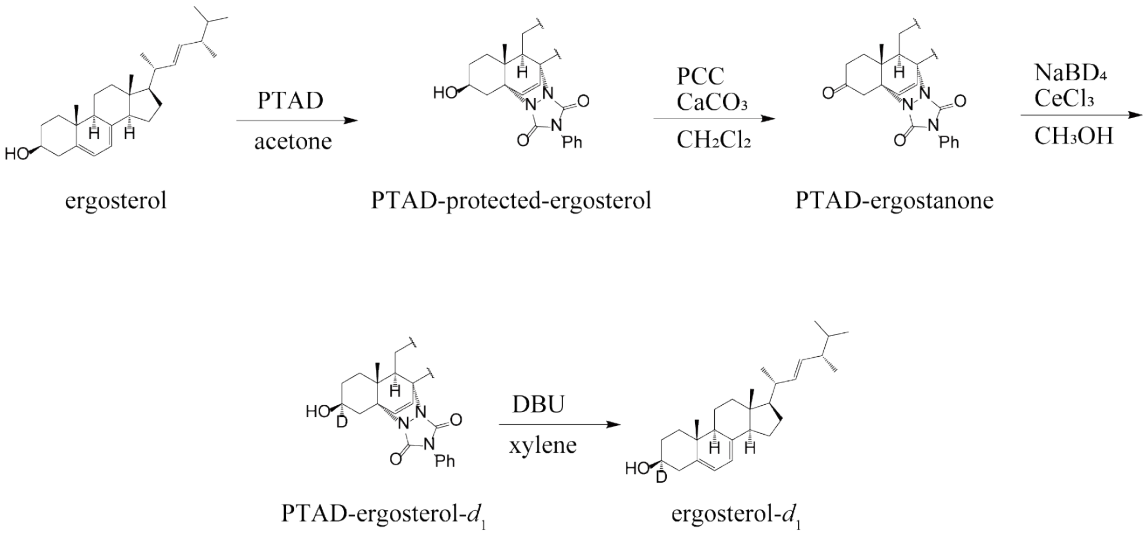
I. Ergosterol-d_1 synthesis

1. Materials and methods

Ergosterol (>95.0%), 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, 97%), and $NaBD_4$ (>90%) were purchased from Sigma-Aldrich Japan. Pyridinium chlorochromate (PCC, >98.0%) was obtained from Tokyo Chemical Industry Co., Ltd. Florisil (60-100 mesh) and molecular sieves 4A were from Nacalai Tesque, Inc, Dichloromethane was from Kanto Chemical Co., Inc. $CaCO_3$ (>99.5%), magnesium sulfate (anhydrous, >98.0%), acetone (>99.5%), hexane (>96.0%), diethyl ether (>99.5%), chloroform (>99.0%), ethyl acetate (>99.5%), methanol (>99.8%), xylene (>80%) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, >98.0%) were from FUJIFILM Wako Pure Chemicals. $CeCl_3 \cdot 7H_2O$ (>99%) was from Kishida Chemical Co., Ltd. Chloroform-*d* (>99.8% D, 0.03 v/v% TMS) was from Acros Organics. All other solvents and reagents were obtained commercially and used without further purification.

TLC was performed on a glass plate precoated with silica gel (Merck 60 F245). Column chromatography was performed with silica gel (Yamazen, particle size 60Å, pore size 40 microns). ^{1}H-NMR spectra were measured in $CDCl_3$ solution using Bruker Avance^{TM}-III HD NanoBay (400 MHz) spectrophotometers and $CHCl_3$ (7.26 ppm for ^{1}H) as a reference. All NMR spectra were recorded in $CDCl_3$ at 25°C. When chemical shifts were multiplicities, the following abbreviations are used: s, singlet; d, doublet; q, quintet; m, multiplet.

2. Synthesis of ergosterol-d_1



Scheme S1 Synthesis of ergosterol-d_1

Conversion of ergosterol to PTAD-protected ergosterol

Ergosterol (10.8 g, 27.2 mmol) was dissolved in acetone (456 mL). To a solution of ergosterol was added a solution of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD, 5.1 g) in acetone (273 mL), the mixture stirred for 40 h at room temperature. The reaction mixture was quenched with water and extracted three times with ethyl acetate. The ethyl acetate layers were combined,

washed with brine, dried over anhydrous $MgSO_4$, and then filtered. Removal of solvent from the extract under reduced pressure gave a product, which was PTAD-protected ergosterol (16.15 g, 27.5 mmol) as a yellow solid in 103.7% yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.40 (m, 4H, Ph), 7.29 (m, 1H, Ph), 6.38 (d, 1H, *J* =8.4 Hz, H6), 6.21 (d, 1H, *J* =8.3 Hz, H7), 5.19 (m, 2H, H22 and 23), 4.43 (m, 1H, H3), 3.15 (dd, 1H, *J* =13.8, 13.9 Hz, H4e), 1.02 (d, 3H, *J* =6.6 Hz, H21), 0.95 (s, 3H, H19), 0.89 (d, 3H, *J* =6.8 Hz, H28), 0.81 (m, 6H, H26 and 27), 0.79 (s, 3H, H18).

Conversion of PTAD-protected ergosterol to PTAD-ergostanone

PTAD-protected ergosterol (16.15 g, 28.34 mmol) was dissolved in dichloromethane (800 mL). To this solution was added $CaCO_3$ (9.09 g), molecular sieve 4A (11.7 g) and pyridinium chlorochromate (PCC, 18.3 g), and stirred at room temperature. After stirring for 2 h, the mixture was purified with florisil and silica gel layered dry column vacuum chromatography passed through diethyl ether 1500 mL and chloroform 2500 mL and collect each solution. These solutions were concentrated under reduced pressure to give a product, which was PTAD-ergostanone (14.7 g, 25.1 mmol) in 91.3% yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.39 (m, 4H, Ph), 7.28 (m, 1H, Ph), 6.57 (d, 1H, *J* =8.2 Hz, H6), 6.23 (d, 1H, *J* =8.3 Hz, H7), 5.20 (m, 2H, 22 and 23), 3.58 (d, 1H, *J* =18.5 Hz, H4e), 2.78 (d, 1H, *J* =18.5 Hz, H4a), 1.08 (s, 3H, H19), 1.01 (d, 3H, *J* =6.6 Hz, H21), 0.90 (d, 3H, *J* =6.8 Hz, H28), 0.88 (s, 3H, H18), 0.83 (m, 6H, H26 and 27)

Conversion of PTAD-ergostanone to PTAD-ergosterol-d_1

To a stirred solution of PTAD-ergostanone (14.7 g, 25.7 mmol) in methanol (900 mL) was added $NaBD_4$ (2.15 g) and $CeCl_3 \cdot 7H_2O$ (19.1 g), and the mixture was stirred at 0°C for 30 min. The reaction mixture was quenched with water and extracted three times with $CHCl_3$. Removal of the solvent from the $CHCl_3$ layer under reduced pressure gave a mixture containing PTAD-ergosterol-d_1. The residue was purified by SiO_2 column chromatography to give PTAD-ergosterol-d_1 (3.36 g, 5.71 mmol) in 22.7% yield. SiO_2 column chromatography conditions: mobile phase, ethyl acetate(A)-hexane(B) mixture, 0-103 min 60% B; detection wave length, 220 nm. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.38 (m, 4H, Ph), 7.27 (m, 1H, Ph), 6.38 (d, 1H, *J* =8.3 Hz, H6), 6.22 (d, 1H, *J* =8.3 Hz, H7), 5.19 (m, 2H, H22 and 23), 3.15 (d, 1H, *J* =14.0 Hz, H4e), 1.02 (d, 3H, *J* =6.6 Hz, H21), 0.96 (s, 3H, H19), 0.88 (d, 3H, *J* =6.8 Hz, H28), 0.80 (m, 6H, H26 and 27), 0.79 (s, 3H, H18)

Conversion of PTAD-ergosterol-d_1 to ergosterol-d_1

To a solution of PTAD-ergosterol-d_1 (3.36 g, 4.16 mmol) in xylene (100 mL) was added 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 3.10 g), and the mixture was stirred at 150°C for 2 h. The reaction mixture was evaporated under reduced pressure to give a mixture containing ergosterol-d_1. The residue was purified by SiO_2 column chromatography and recrystallized from aqueous hexane to give ergosterol-d_1 (1.74 g, 4.38 mmol) in 74.6% yield. Eventually, ergosterol-d_1 was obtained by 4 steps (16% overall yield) from ergosterol. SiO_2 column chromatography conditions: mobile phase, ethyl acetate(A)-hexane(B) mixture, 0-90 min 50% B; flow rate, 20 mL/min;

detection wave length, 254 nm. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.53 (dd, 1H, *J* =5.6, 5.6 Hz, H6), 5.34 (m, 1H, H7), 5.16 (m, 2H, H22 and 23), 2.44 (dd, 1H, *J* =2.2, 2.2 Hz, H4e), 2.29 (d, 1H, *J* =0.48 Hz, H4a), 0.99 (s, 3H, H19), 0.89 (d, 3H, *J* =6.8 Hz, H28), 0.80 (m, 6H, H26 and 27), 0.59 (s, 3H, H18).

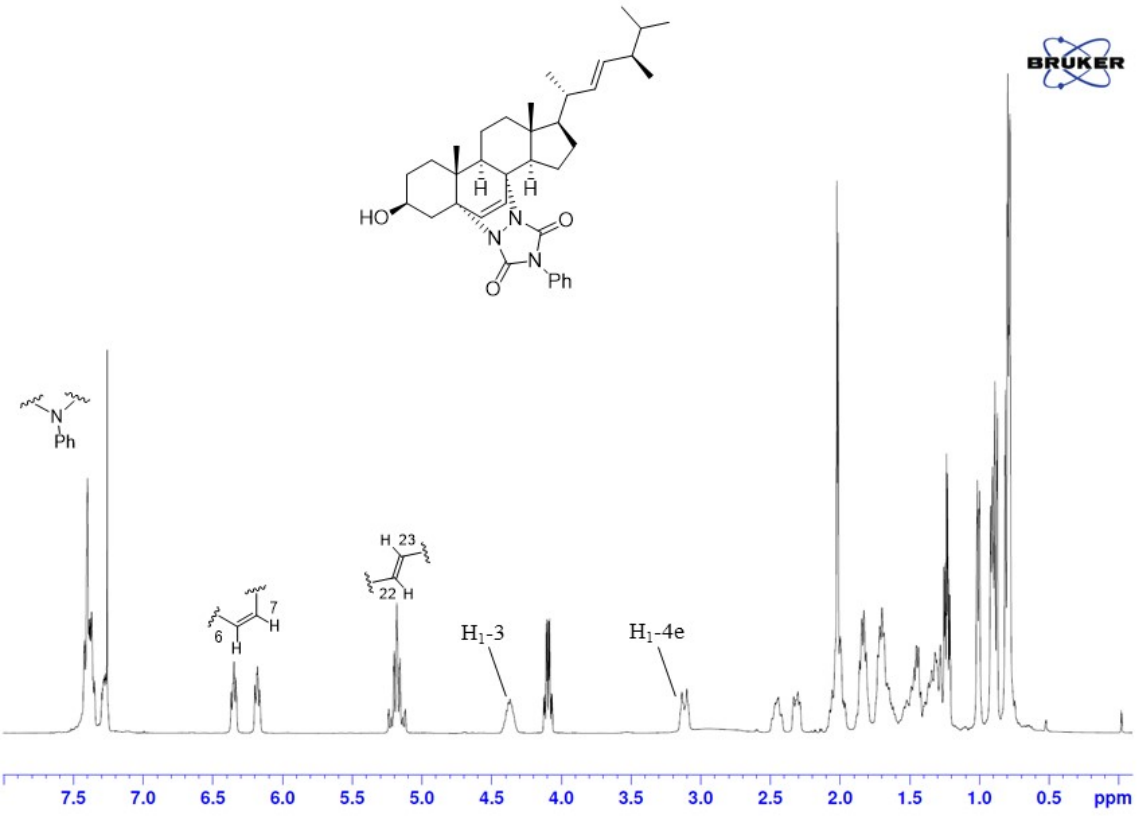


Figure S1 ^{1}H-NMR spectrum of PTAD-protected-ergosterol

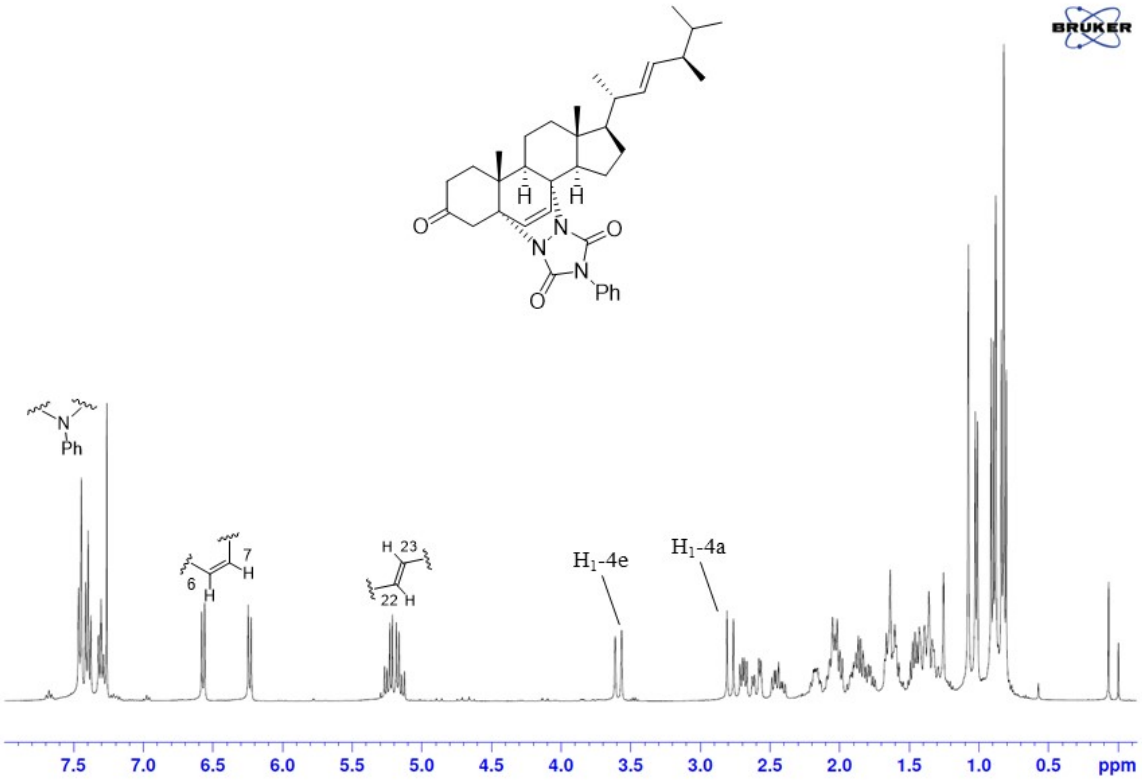


Figure S2 ^{1}H-NMR spectrum of PTAD-protected-ergostanone

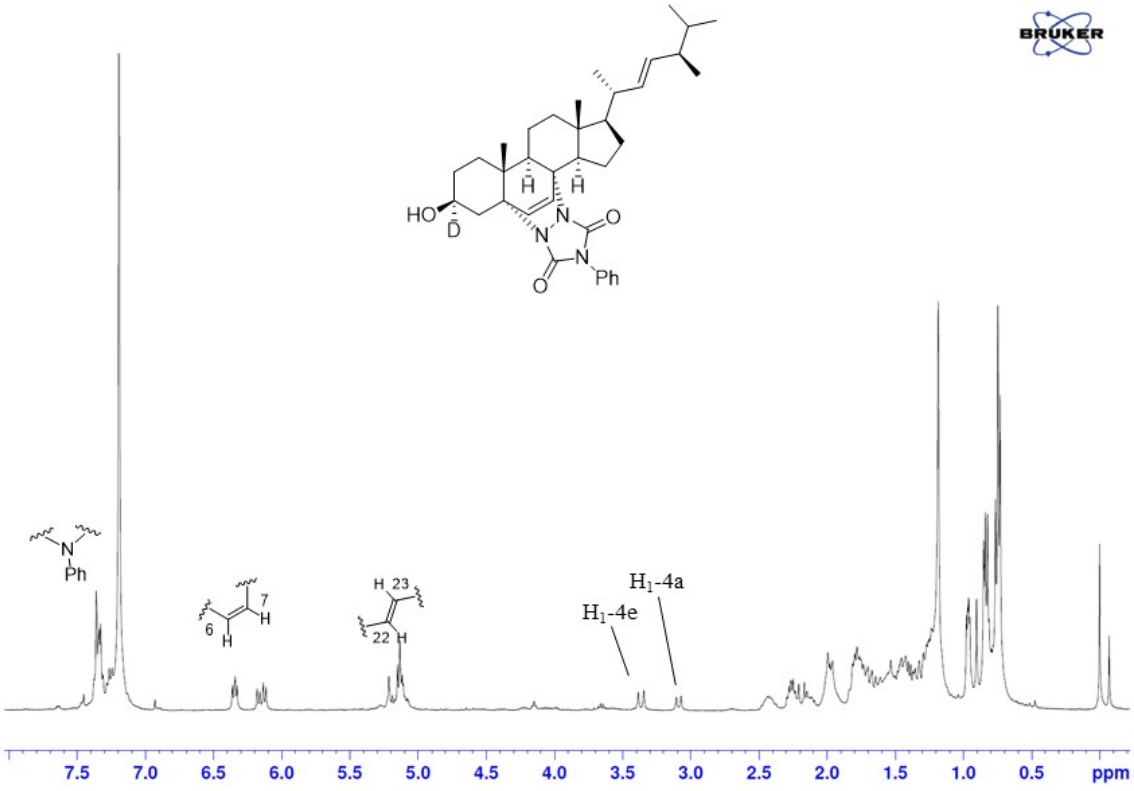


Figure S3 ^{1}H-NMR spectrum of PTAD-protected-ergosterol-d_1

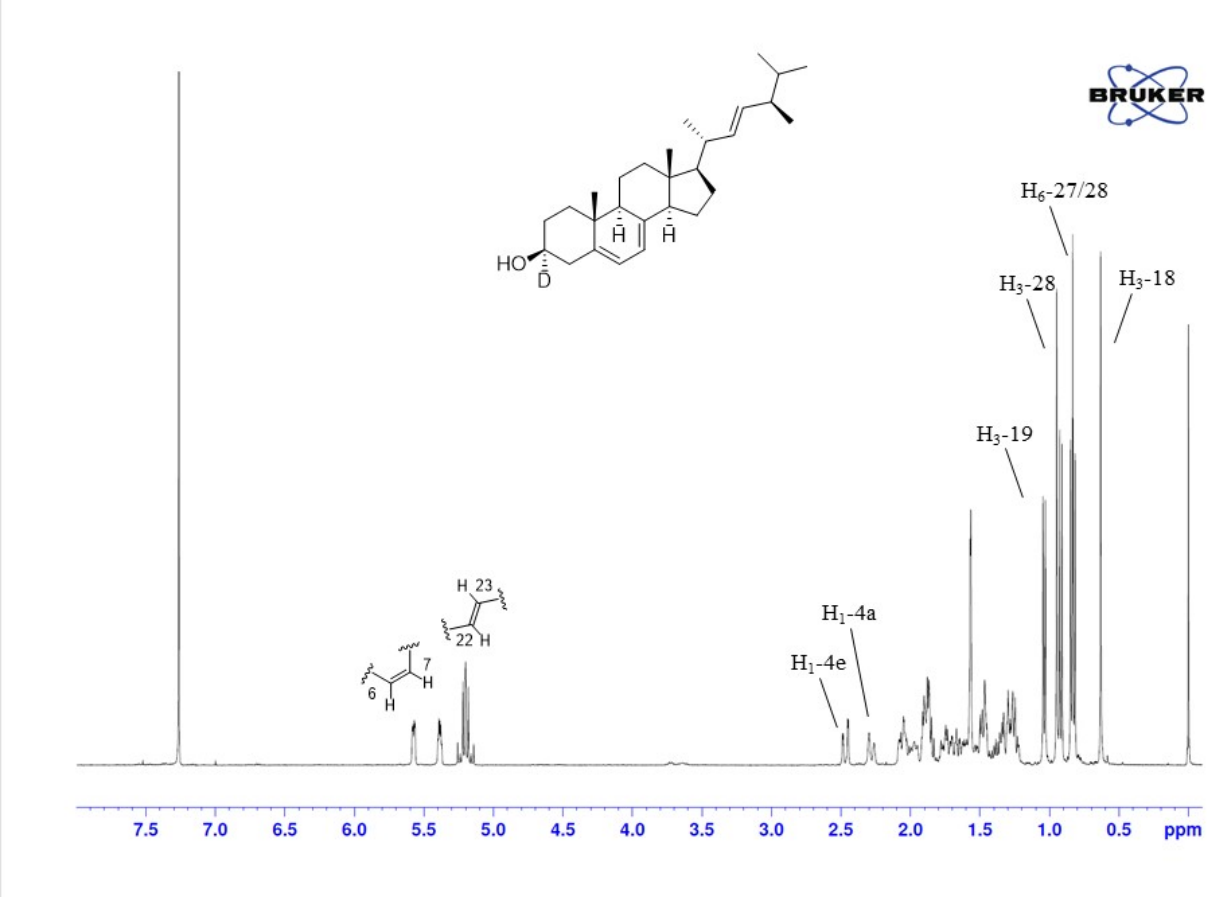


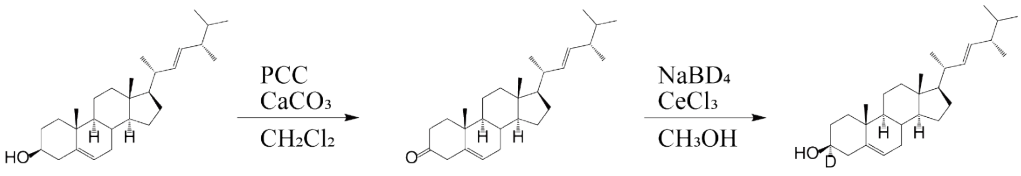
Figure S4 ^{1}H-NMR spectrum of ergosterol-d_1

II. Brassicasterol-d_1 synthesis

1. Materials and methods

Brassicasterol (>98.3%) was purchased from Tama Biochemical Co., Ltd (Tokyo, Japan). Other reagents used were obtained from the same manufactures as described in materials and methods of Ergosterol-d_1 synthesis. TLC and ^{1}H-NMR were also performed as described in materials and methods of Ergosterol-d_1 synthesis.

2. Synthesis of brassicasterol-d_1



Scheme S2 Synthesis of brassicasterol-d_1

Conversion of brassicasterol to brassicasterone (compound 1)

Brassicasterol (100 mg, 0.251 mmol) was dissolved in dichloromethane (8 mL). $CaCO_3$ (20 mg) and molecular sieve 4A (96 mg) was added to the substance solution, and stirred at room

temperature. Then, pyridinium chlorochromate (PCC, 156 mg) was added to the solution and stirred at the same temperature. After 60 min, the mixture was purified with florisil and silica gel layered dry column vacuum chromatography passed through CH_2Cl_2 20 mL and diethyl ether 30 mL and collect each solution. These solutions were concentrated under reduced pressure to give a crude product, which was compound **1** (18.1 mg, 0.0456 mmol) in 18.2% yield. The amount of compound **1** was not enough, so the above steps were repeated to give a compound **1** (87.5 mg, 0.221 mmol) in 87.9% yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.26 (m, 1H, H6), 5.12 (m, 2H, H22 and 23), 3.22 (d, 1H, *J* =16.4 Hz, H4e), 2.45 (d, 1H, *J* =16.4 Hz, H4a), 0.94 (s, 3H, H19), 0.85 (d, 3H, *J* =6.8 Hz), 0.77 (m, 6H, H26 and 27), 0.65 (s, 3H, H18).

Conversion of 1 to brassicasterol-d_1

To a stirred solution of **1** (105.6 mg, 0.266 mmol) in methanol (10 mL) was added $NaBD_4$ (26 mg) and $CeCl_3 \cdot 7H_2O$ (235 mg), and the mixture was stirred at 0°C for 45 min. The reaction mixture was quenched with water, and extracted with $CHCl_3$. Removal of the solvent from the $CHCl_3$ layer under reduced pressure gave a mixture containing brassicasterol-d_1. The residue was purified by SiO_2 column chromatography to give brassicasterol-d_1 (62.8 mg, 0.157 mmol) as clear crystal in 59.0% yield. Eventually, brassicasterol-d_1 was obtained by 2 steps (31% overall yield) from brassicasterol. SiO_2 column chromatography conditions: mobile phase, acetone(A)-hexane(B) mixture, 0-100 min 95% B, 100-110 min 0% B; detection wave length, 220 nm. ^{1}H-NMR (400 MHz, $CDCl_3$) δ 5.35 (m, 1H, H6), 5.20 (m, 2H, H22 and 23), 2.24 (m, 2H, H4a and 4e), 0.92 (m, 3H, H19), 0.82 (m, 6H, H26 and 27), 0.69 (s, 3H, H18).

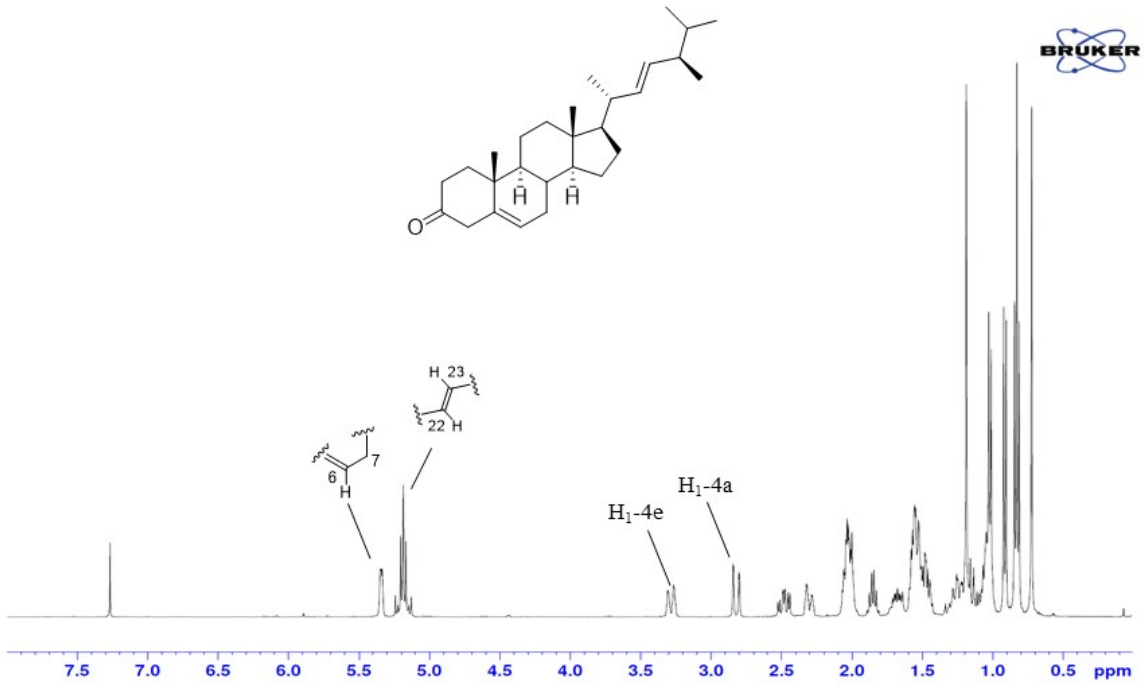


Figure S5 ^{1}H-NMR spectrum of brassicasterone (compound 1).

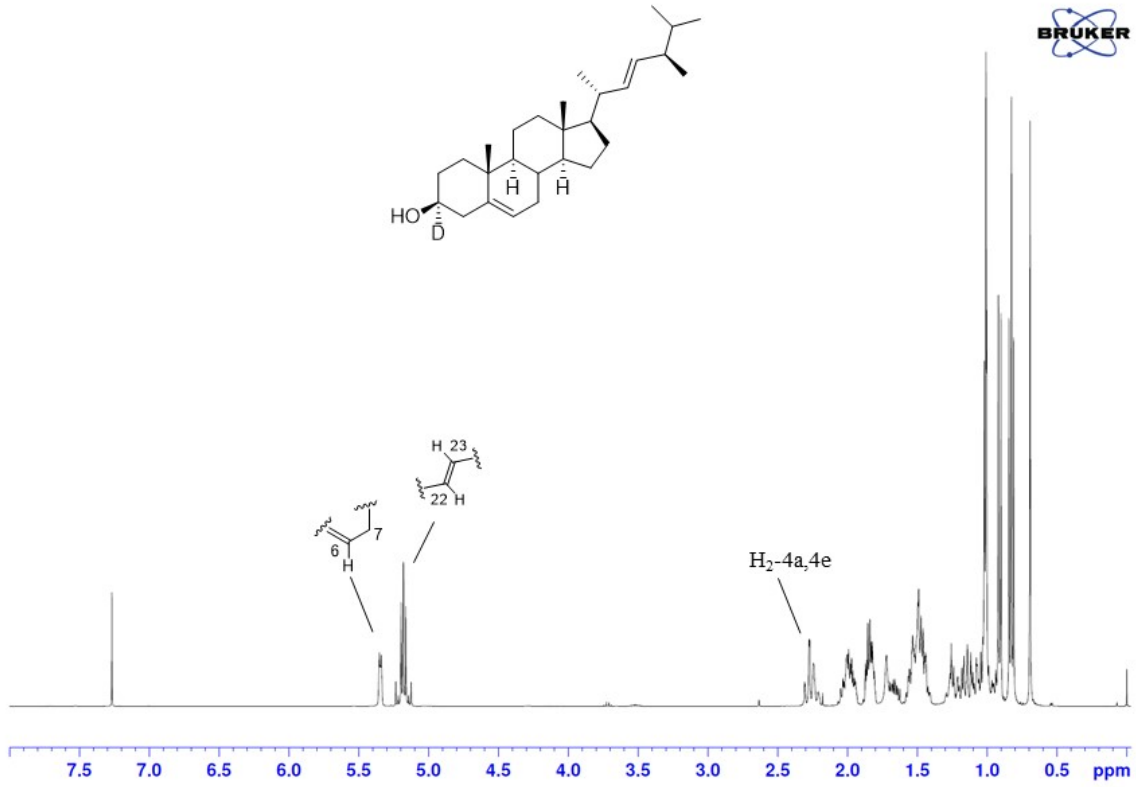


Figure S6 ^{1}H-NMR spectrum of brassicasterol-d_1

III. Calibration curves of ergosterol-d_1 and brassicasterol-d_1

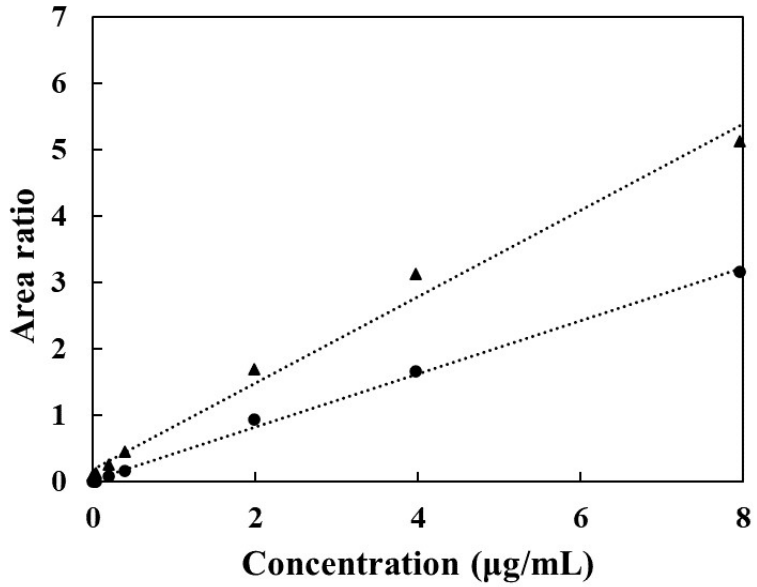


Figure S7 Calibration curves of ergosterol-d_1 (●) and brassicasterol-d_1 (▲) based on the peak area ratio to IS (6-ketocholestanol). The linearity and range of each curve were shown in Table 2. The corresponding chromatograms for the concentration point of 4 μg/mL ergosterol-d_1 and brassicasterol-d_1 were shown in Figures 2D and 2E, respectively. Figures S8A and S8B shows the chromatograms of 0.04 μg/mL ergosterol-d_1 and 0.02 μg/mL brassicasterol-d_1, respectively.

IV. MRM Chromatograms of the lower limit of quantification

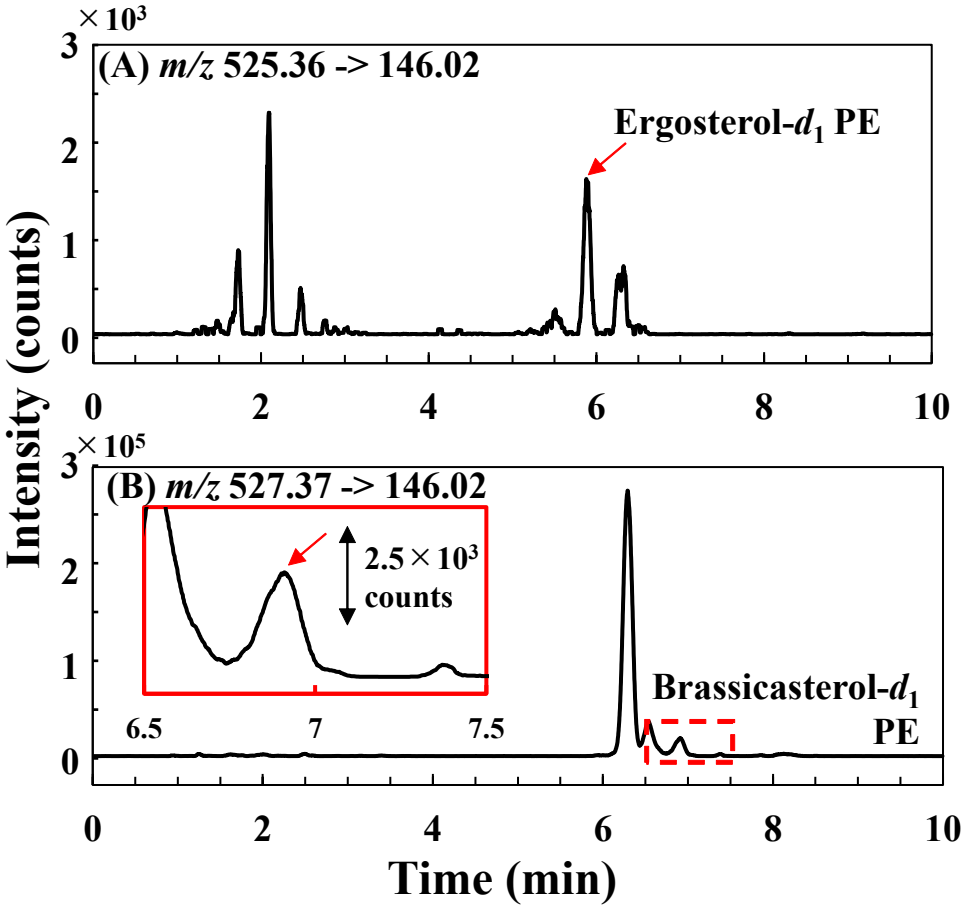


Figure S8 Multiple reaction monitoring chromatograms of the lower limit of quantification (LLOQ). (A) 0.04 μg/mL ergosterol-d_1 in the presence of the pooled control SHRSP serum. (B) 0.02 μg/mL brrasicasterol-d_1 in the presence of the pooled control SHRSP serum. The inset indicates an expanded chromatogram of a boxed area. Sample preparation and LC-MS conditions were same as Figure 2.