Supporting Information

Axial hydrogen at C7 position and bumpy tetracyclic core markedly reduce sterol's affinity to amphotericin B in membrane

Yasuo Nakagawa[†], Yuichi Umegawa[†], Kenichi Nonomura[†], Tetsuro Takano[†], Hiroshi Tsuchikawa[†], Shinya Hanashima[†], Nobuaki Matsumori[‡] and Michio Murata[†]**

[†]Department of Chemistry, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, Osaka 560-0043, Japan.

‡Department of Chemistry, Graduate School of Science, Kyushu University, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812-8581, Japan.

Table of contents

MATERIALS

AmB were purchased from Nacalai Tesque (Kyoto, Japan). Ergosterol was from Tokyo Kasei (Tokyo, Japan) and palmitoylleoylphosphatidylcholine (POPC) was from Avanti Polar Lipid Inc. (Alabaster, AL). All other chemicals were obtained from standard venders. Thin-layer chromatography (TLC) was performed on a glass plate precoated with silicagel (Merck Kieselgel 60 F254). Column chromatography was performed with silica gel 60 (Merck, particle size 0.063-0.200 mm, 60-230 mesh). Solution NMR spectra were recorded on ECA-500 and ECS-400 spectrometer (JEOL).

1. Surface plasmon resonance (SPR) analysis

Basically we followed our previous report[1]. For preparing liposome solution, POPC (4 μmol) and sterols, Erg **1**, Bras **2**, **3**, **4**, 7-DHC **5**, Cho **6**, **7**, **8** and **9** (1 μmol) were mixted in $CHCl_3$ (1 mL) in a round-bottom flask. The mixture was evaporated and dried in vacuo overnight, and then hydrated with PBS buffer (1 mL). The mixture was vortexed and sonicated to prepare multilamellar vesicles (MLVs). The resultant suspension was subjected to five cycles of freezing, thawing, and vortexing to form LUVs. The LUVs were passed through 100 nm polycarbonate filters 19 times with LiposoFast-Basic (AVESTIN Inc., Ottawa, Canada) at room temperature and diluted with PBS buffer to furnish a LUV solution with a lipid concentration of 0.25 mM. The experiments were performed at 25 °C using the CM5 sensor chip mounted in a BIAcore T200 analytical system (BIAcore AB, Uppsala, Sweden). Dodecylamine was immobilized to one of the flow cell lanes in the CM5 sensor chip by an amino coupling method, while the other lane was left intact as a control lane. The immobilization reaction was performed at a flow rate of 5 μL/min; briefly, the sensor chip was activated for 7 min by injecting a solution mixture (1:1, v/v, 35 μL) of 390 mM EDC and 100 mM NHS. Dodecylamine (1.0 mg/ mL) in 10 mM acetate

buffer containing 10% DMSO (pH 5.0, 35 μL) was then put on the surface of the sensor chip directly for cross-linking. After 30 minute, dodecylamine on the sensor chip was washed with ethanol and dried under the air. The remaining activated N-hydroxysuccinimide ester groups were converted to amide groups by 1 M ethanolamine hydrochloride (pH 8.5, 35 μL). The sensor chip thus obtained was washed with 10 mM acetate buffer containing 10% DMSO (pH 5.0, 35 μL) to remove nonspecifically bound substances. The running buffer was PBS buffer containing 5% DMSO (pH 7.4). Prior to being used, the buffer was degassed by sonication. The liposome solution (60 μL) was injected to the sensor chip at a flow rate of 2 μL/min, and then 50 mM sodium hydroxide (40 μL) was added at a flow rate of 20 μL/min. The NaOH treatment was repeated three times, resulting in a stable baseline which indicated formation of stable liposome layers on the sensor chip. A solution of AmB (30 μM) in PBS buffer containing 5% DMSO was introduced to the sensor chip at a flow rate of 10 μL/min, and the time course of its association and dissociation was monitored. The Experimental curves were fitted to two state reaction model described by the following equation.[1–3]

$$A+B \underset{k_{d2}}{\overset{k_{a1}}{\leftrightarrows}} AB \underset{k_{a2}}{\overset{k_{a2}}{\leftrightarrows}} AB^*$$

The sensorgrams obtained for each sterol-containing membrane are shown in Figure S1. Previously we reported that SPR sensorgrams for membrane binding of AmB can be analyzed on the basis of two-state reaction model that assumes membrane binding process of AmB and subsequent reorientation process.[1] The sensorgrams were fitted by the two-state reaction model, which well reproduced the experimental sensorgrams as shown in Figure S1 (theoretical in red and experimental in black).

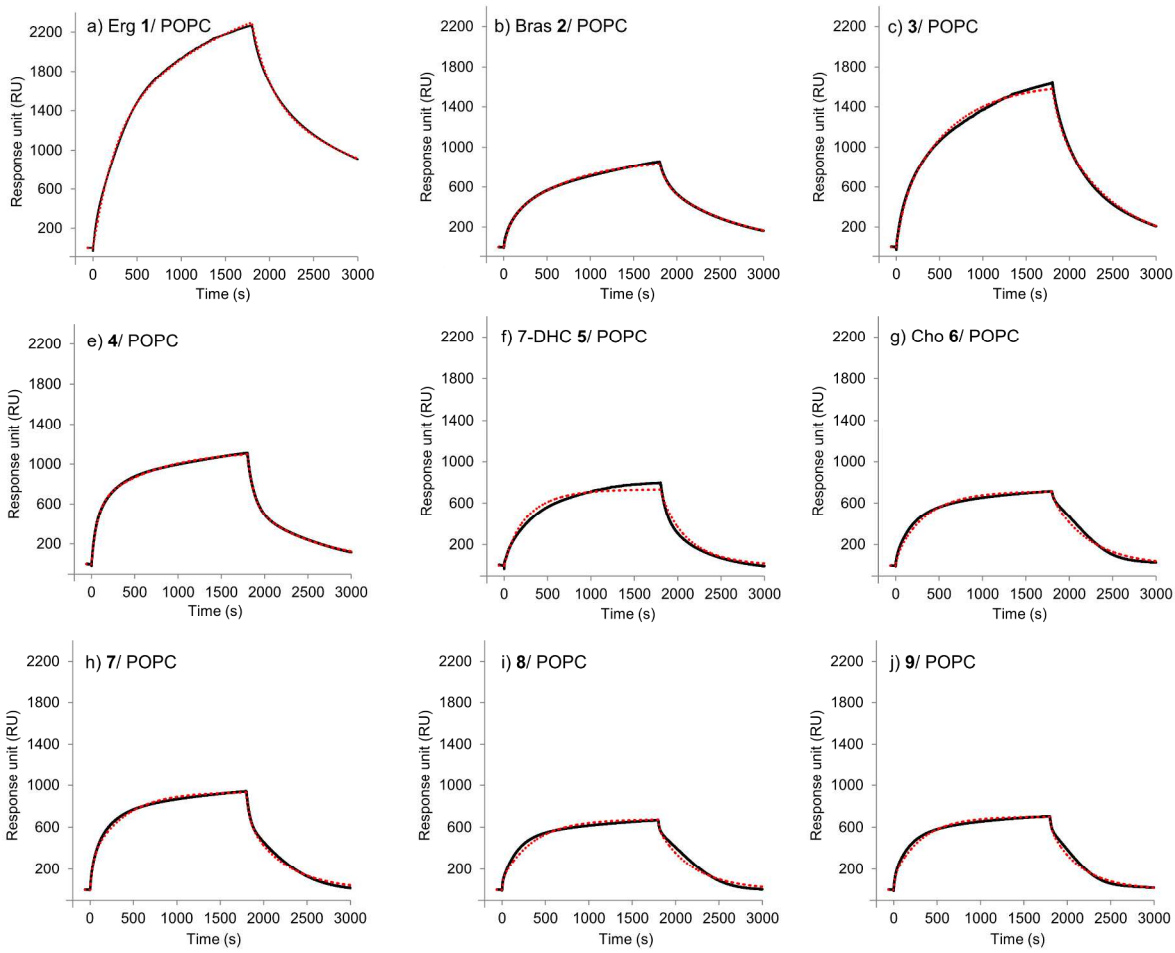


Figure S1. SPR analysis of binding of AmB to immobilized liposome. The liposomes were prepared from POPC and admixed different sterol derivatives (20 mol%). The experimental data were fitted with the two state reaction model, where A+B ⇆ AB ⇆ AB* (see text). Experimental RU values were recorded for 30 μ M AmB. The total fitted curve and experimental sensorgrams were presented as broken red and black solid curve respectively.

Since the binding process as shown in the rate constant k_{a1} is known to heavily depend on the experimental conditions such as the concentrations of AmB[1,4] and the amount of immobilized lipids on the sensor chip, we compared the SPR results of the membranes containing each sterol by dissociation constant k_{d2} obtained in the two state reaction model, which is known to reproduce the SPR data of AmB very well.[1] Previously we demonstrated that lifetime of the AmB-sterol complex could be assumed by using the disassociation time constant T_{d2} ($1/k_{d2}$). Hence we examined how alicyclic structure of the sterols influence on the stabilization of the complex by its lifetime (Figure 2S). The results showed similar tendency between T_{d2} and eflux activity r (Figure 3), which indicate the alicyclic structure affected the channel stability as well as sidechain.

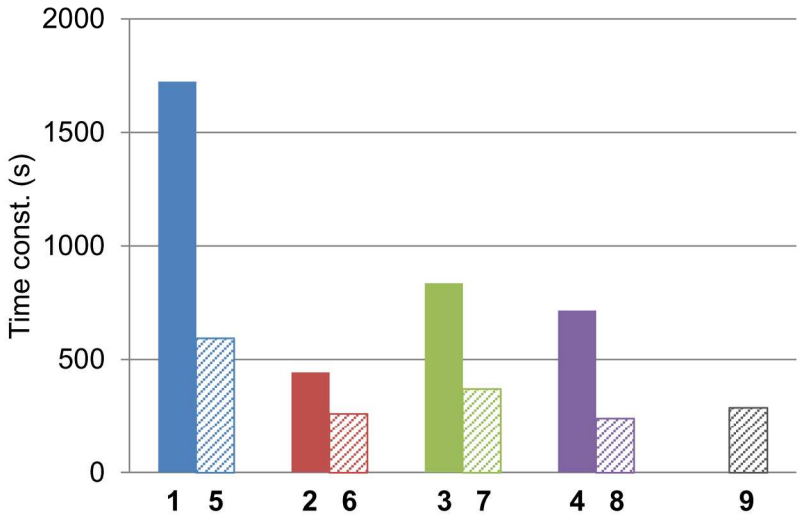


Figure S2. Life time of AmB-sterol complex as evaluated by the time constant of dissociation T_{d2}

2. The UV spectrum of the POPC

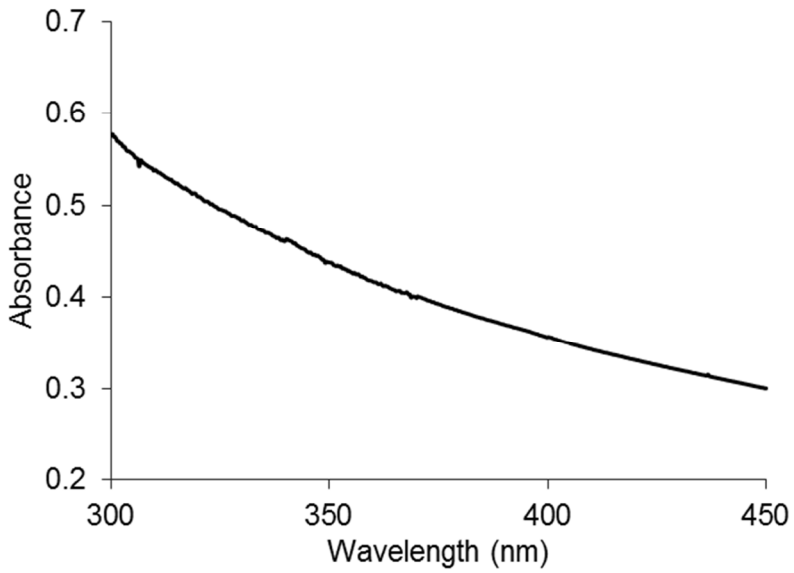
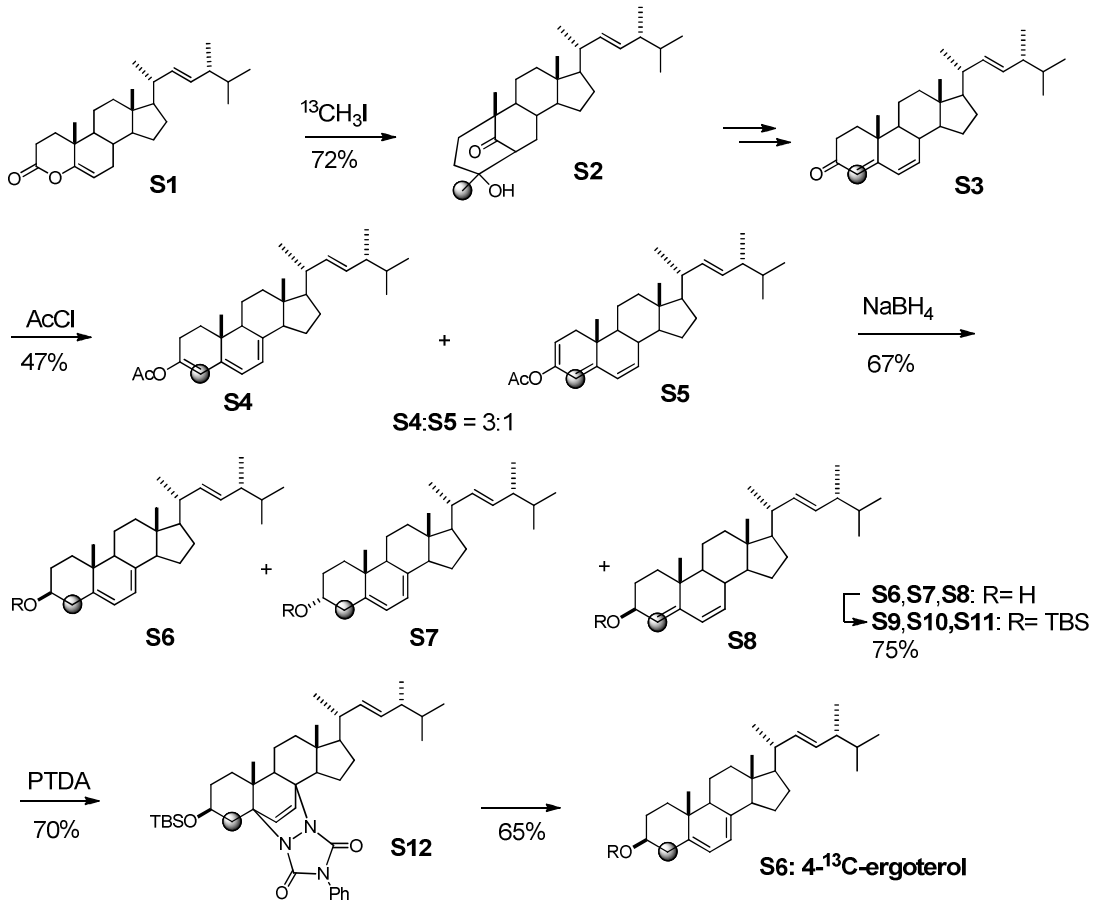


Figure S3. UV spectra of POPC liposome in sucrose buffer at 125 μM.

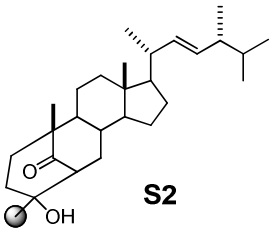
3. Synthesis of 4-^{13}C-ergosterol



Scheme 1. Synthesis of 4-^{13}C-ergosterol

The 4-^{13}C-ergosterol was synthesized by basically following Pelc's group report which synthesized 4-^{14}C-ergosterol[5], that is, the ^{13}C atom was introduced into latone **S1** by Grignard reaction using $^{13}CH_3I$ instead of $^{14}CH_3I$. The acetylation of the dienone **S3**[5,6], however provided the inseparable mixture of olefin isomers **S4** and **S5**, and following reduction by sodium brohydride produced inseparable mixture of 4-^{13}C-ergosterol **S6**, its diastereomer **S7** and olefin isomer derived from **S5**. These byproduct weren't mentioned in previous paper[5]. Since these compounds couldn't be separated by silicagel column chromatography, it was purified after

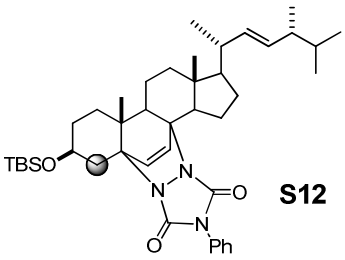
protection of the hydroxyl groups and conjugated double bond. The desired protected compound **S12** was finally converted to the 4-^{13}C-ergosterol **S6** as a single isomer by removal of TBS and 4-phenyl-1,2,4- triazoline-3,5-dione (PTDA) groups.



S2

Conversion of S1 to S2. A solution of $^{13}CH_3I$ in Et_2O was slowly added dropwise to the stirred solution of lactone **S1** [5–7](5.2 g, 13.05 mmol) in CH_2Cl_2 (90 ml) at 0 °C, and it was stirred for 4 h at room temperature. The reaction was quenched with 1 M H_2SO_4 aq. and extracted by Et_2O. The organic phase was washed by H_2O and brine, and it was dried over Na_2SO_4. After evaporation of the filtrate, the resultant residue was purified by silicagel column chromatography, eluting with (1:9 MeOH/$CHCl_3$) to provide alcohol **S2** (3.9 g, 72%) as a white solid.

White solid ; R_f = 0.05 (silica, hexane/AcOEt=6/1); ^{1}H NMR (500MHz, $CDCl_3$) δ 5.15 (2H, ddd, *J* = 15.5, 8.0, 7.5 Hz, H22, H23), 2.31 (1H, d, *J* = 11.0 Hz), 2.14 (1H, td, *J* = 17.0, 5.5 Hz), 1.92-2.04 (m), 1.82 (1H, q, *J* = 7.0 Hz), 1.61-1.76 (m), 1.41-1.52 (m), 1.24 (3H, d, *J* = 125.5 Hz, ^{13}Cme), 0.97 (3H, s, Me19), 0.97 (3H, d, *J* = 6.0 Hz, Me25), 0.89 (3H, d, *J* = 7.0 Hz, Me21), 0.80 (6H, dd, *J* = 8.0 Hz, Me27, Me28), 0.60 (3H, s, Me18).



S12

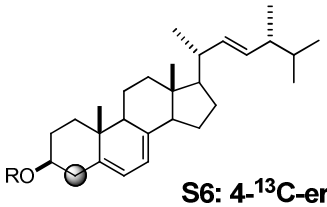
Conversion of S3 to S12. Dienone **S3** (1.2 g, 3.03 mmol), which was converted from **S1** as described in previous report[5–7], and AcCl (8.6 ml, 121 ml) was added to Ac_2O (20 ml) at room temperature. The mixture was warmed up to 75 °C and stirred for 7 h. After cooling to room temperature, the reaction was quenched with MeOH and extracted with Et_2O. The organic phase was washed with H_2O and brin and then dried over Na_2SO_4. After concentration, the crude was purified by Florisil® column chromatography (1:9 MeOH/$CHCl_3$) to provide inseparable mixture acetylated alcohol **S4** and **S5**.

$NaBH_4$ (425 mg, 0.56 mmol) was added to a stirred solution of a mixture of **S4** and **S5** (246 mg, 0.56 mmol) in MeOH (24 ml) and THF (12 ml) at room temperature. After stirring for 2 h at same temperature, the reaction was quenched with AcOH and H_2O and extracted with Et_2O. The organic phase was washed with H_2O and brine and then dried over Na_2SO_4. After evaporation, the crude was purified by silicagel column chromatography (1:9 EtOAc/ hexane) to provide a mixture of 4-^{13}C-ergosterol **S6** and byproducts **S7** and **S8** (149 mg, 67%).

2,6-lutidine (0.41 ml, 3.52 mmol) and TBSOTf (0.49 ml, 2.11 mmol) was added to a solution of **S6**, **S7** and **S8** (280 mg, 0.70 mmol) in CH_2Cl_2(28 ml) at 0 °C. The reaction was stirred for 30 min at same temperature and then quenched with sat. aq. $NaHCO_3$. The resultant mixture was extracted with Et_2O and washed with H_2O and brine. the organic phase was dried over $MgSO_4$ and concentrated *in vacuo*. The resultant residue was purified by silicagel column (1:9 EtOAc/ hexane) to give a TBS ether **S9**, **S10** and **S11.** chromatography (1:9 EtOAc/ hexane).

A solution of PTAD (278 mg, 1.59 mmol) in acetone (13 ml) was added stirred solution of TBS ether **S9**, **S10** and **S11** (271 mg, 0.53 mg) in CH_2Cl_2 (8 ml) at room temperature. It was stirred for 30 min and then solvent was removed by ecvaporation. The resultant crude was purified by silicagel column (1:9 EtOAc/ hexane) to provide desired compound **S12** (253 mg, 70%) as a single isomer.

White solid ; R_f = 0.4 (silica, hexane/AcOEt=6/1); ^{1}H NMR (500MHz, $CDCl_3$) δ 7.37-7.44 (4H, m, Ph), 7.28 (1H, t, *J* = 6.5 Hz, Ph), 6.35 (1H, d, *J* = 8.5 Hz, H7), 6.18 (1H, dd, *J* = 8.0, 3.0 Hz, H6), 5.18 (2H, ddd, *J* = 15.5, 7.5 Hz, H22, H23), 4.38 (1H, m, 3H), 3.09 (1H, ddd, *J* = 135.0, 14.0, 5.0 Hz, H4), 2.47 (1H, m), 2.32(1H, dd, *J* = 14.0, 4.0 Hz), 1.99-2.12 (m), 1.75-1.87 (m), 1.64-1.71 (m), 1.23-1.53 (m), 1.96-2.03 (3H, m), 1.01 (3H, d, *J* = 7.0 Hz, Me25), 0.94 (3H, s, Me19), 0.88 (3H, d, *J* = 7.0 Hz, Me21), 0.86 (9H, m, TBS), 0.81 (3H, s, Me18), 0.79 (6H, dd, *J* = 6.5 Hz, Me27, Me28).



S6: 4-^{13}C-ergoterol

Conversion of S12 to 4-^{13}C-ergosterol S6. DIBAL-H (1.0 M, 5.5 ml) was added to a stirred solution of **S12** in toluene (12 ml) at 0 °C, and it was stirred for 90 min. The reaction was quenched with sat. aq. NH_4Cl and sat. aq. tart-K^+Na^+ and extracted with Et_2O. The organic phase was washed with H2O and brine and dried over MgSO4. After evaporation, the residue was purified by by silicagel column (1:5 EtOAc/ hexane) to provide 4-^{13}C-ergosterol **S6** (95 mg, 65%) as a white solid.

White solid ; R_f = 0.3 (silica, hexane/AcOEt=6/1); ^{1}H NMR (400MHz, $CDCl_3$) δ 5.57 (1H, td, *J* = 5.8, 2.5 Hz, H6), 5.38 (1H, m, H7), 5.18 (2H, ddd, *J* = 15.5, 7.5 Hz, H22, H23), 3.62 (1H, m, H3), 2.47 (1H, dddd, *J* = 129.6, 14.5, 4.7, 2.3 Hz, $H4_{eq}$), 2.28 (1H, brtd, *J* = 124.7, 12.6 Hz, $H4_{ax}$), 2.02-2.08 (1H, m, H20), 1.97 (1H, m, H24), 1.83-1.91 (m), 1.63-1.77 (m), 1.45-1.51 (m), 1.23-1.37 (m), 1.03 (3H, d, *J* = 7.0 Hz, Me25), 0.94 (3H, s, Me19), 0.92 (3H, d, *J* = 6.5 Hz, Me21), 0.81 (6H, dd, *J* = 6.0 Hz, Me27, Me28), 0.61 (3H, s, Me18).

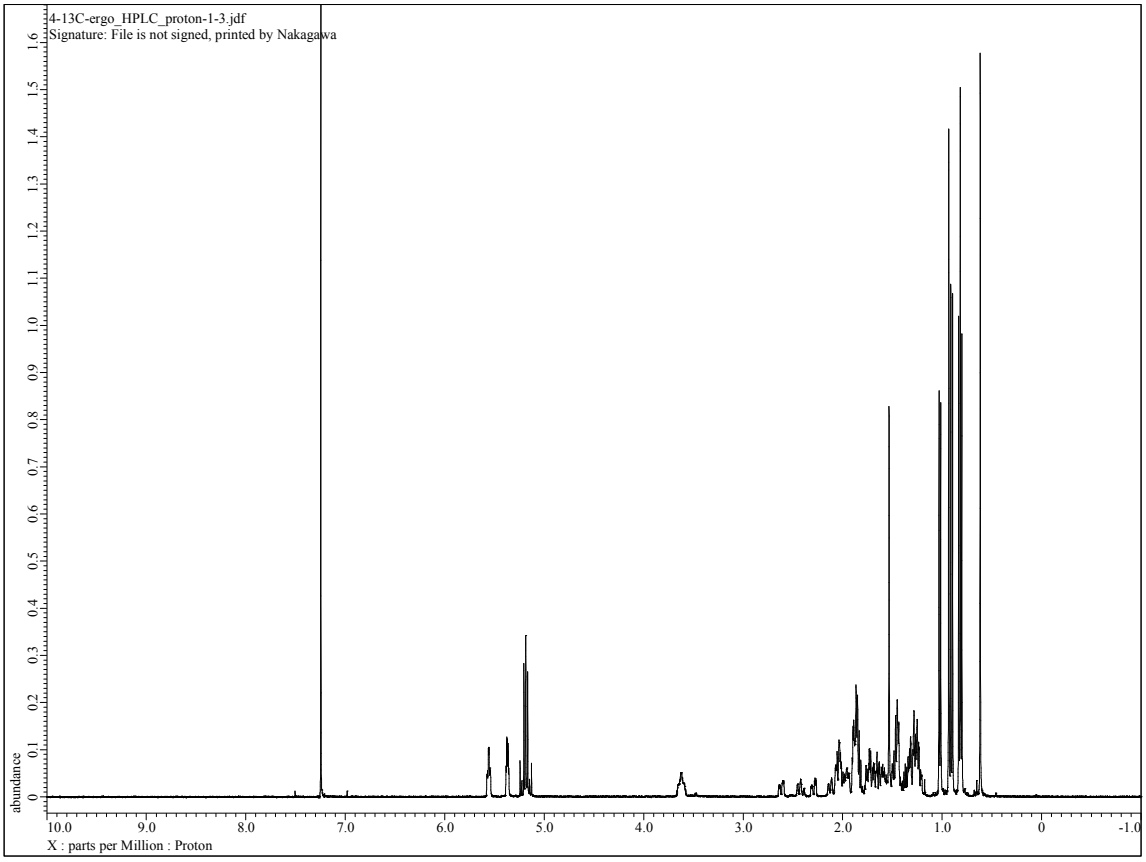


Figure S4. ^{1}H spectrum of 4-^{13}C-ergosterol **S6**

4. The preferable conformation of sterol analogues

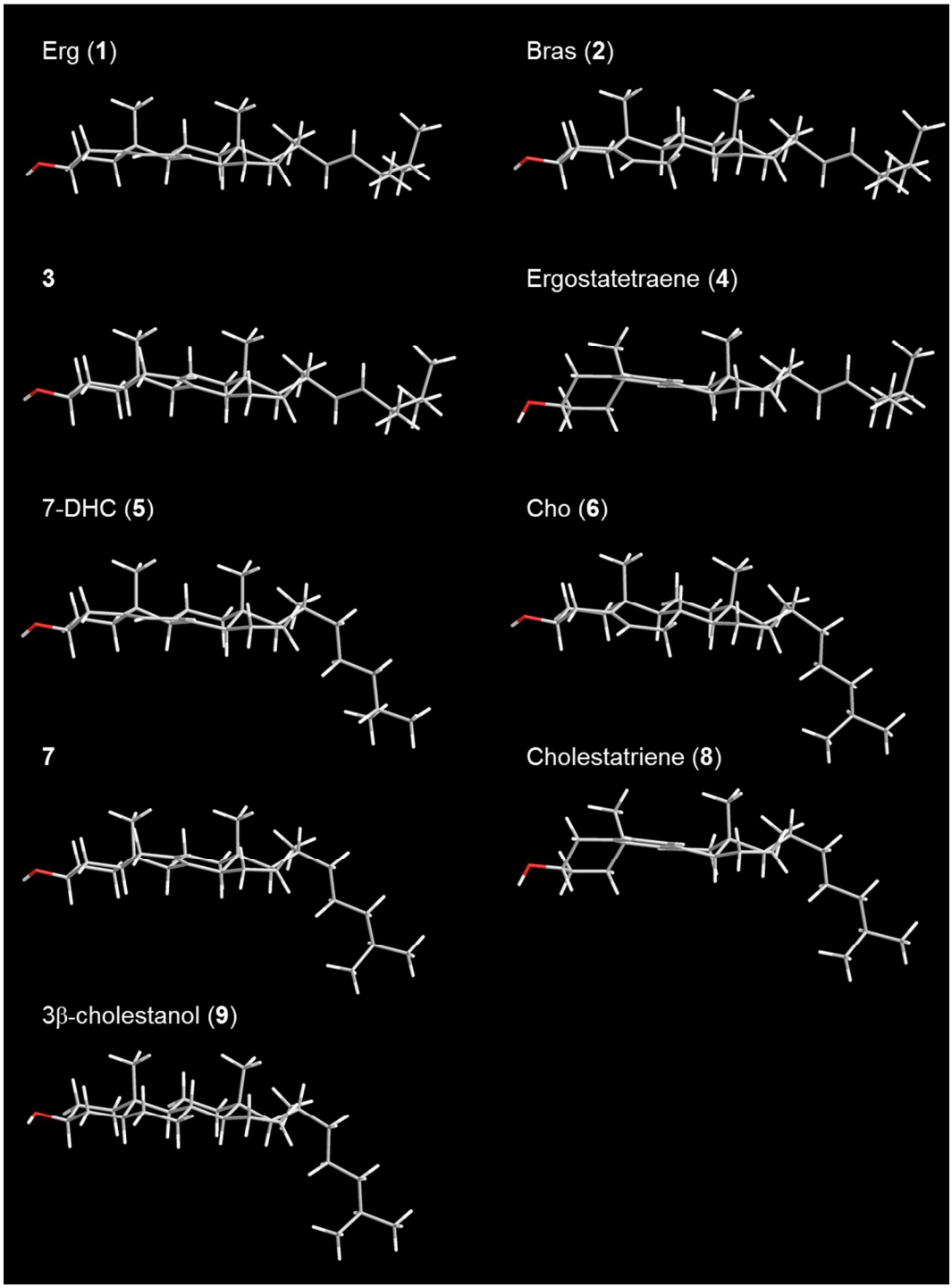


Figure S5. The most stable conformations of the sterol analogues generated by DFT optimization.

REFERENCES

(1) Mouri, R., Konoki, K., Matsumori, N., Oishi, T., and Murata, M. (2008) Complex formation of amphotericin B in sterol-containing membranes as evidenced by surface plasmon resonance. *Biochemistry 47*, 7807–7815.

(2) Papo, N., and Shai, Y. (2003) Exploring peptide membrane interaction using surface plasmon resonance: differentiation between pore formation versus membrane disruption by lytic peptides. *Biochemistry 42*, 458–66.

(3) Morton, T., Myszka, D., and Chaiken, I. (1995) Interpreting complex binding kinetics from optical biosensors: a comparison of analysis by linearization, the integrated rate equation, and numerical integration. *Anal. Biochem. 227*, 176–185.

(4) Onishi, M., and Kamimori, H. (2013) High-throughput and sensitive assay for amphotericin B interaction with lipid membrane on the model membrane systems by surface plasmon resonance. *Biol. Pharm. Bull. 36*, 658–63.

(5) Pelc, B., and Kodicek, E. (1972) Synthesis of [4-^{14}C] Ergosterol and [4-^{14}C] Ergocalcifero. *J. Chem. Soc. Perkin Trans. 1* 2980–2981.

(6) Pelc, B., and Kodicek, E. (1972) The Location of Tritium in [1-^{3}H]Ergostero. *J. Chem. Soc. Perkin Trans. 1* 244–246.

(7) Pelc, B., and Kodicek, E. (1971) Synthesis of ergosterol and ergocalciferol. *J. Chem. Soc. C Org.* 3415–3418.