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**PAPER**

# Synthesis of 6-F-ergosterol and its influence on membrane-permeabilization of amphotericin B and amphidinol 3

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Two well-known antifungals, amphotericin B (AmB) and amphodinol 3 (AM3), are thought to exert antifungal activity by forming ion-permeable channels or pores together with sterol molecules. However, detailed molecular recognitions for AmB-sterol and AM3-sterol in lipid bilayers have yet to be determined. Toward $^{19}$F NMR-based investigation of the molecular recognition underlying their potent antifungal activity, we synthesized 6-fluoro-ergosterol in five steps *via* ring opening of (5α,6α)-epoxide of ergosterol acetate with using novel combination of $TiF_4$ and *n*-$Bu_4N^+Ph_3SiF_2^-$. Then we evaluated its activity of promoting pore formation of AmB and AM3, and found that pore formation of AmB was barely promoted by 6-F-ergosterol in clear contrast to the dramatic promotion effect of unmodified ergosterol, whereas AM3 activity was markedly enhanced in the presence of 6-F-ergosterol, which was comparable to that of unmodified ergosterol. These results indicate that the introduction of an F atom at C6 position of ergosterol plays an inhibitory role in interacting with AmB, but it is not the case with AM3.

## Introduction

Amphotericin B (AmB, Fig. 1) has been a standard drug for treatment of deep-seated systemic fungal infections for nearly 50 years.[1–3] For lack of better alternatives, as well as the rare occurrence of resistant strains,[4] the clinical importance of AmB has remained unchanged. It is widely accepted that AmB exerts its antifungal activity by forming transmembrane ion-permeable self-assemblies together with ergosterol (Fig. 1), an abundant sterol in fungal membranes. The selective toxicity of AmB is accounted for by its higher affinity to ergosterol than cholesterol (Fig. 1), a component of mammalian membrane. However, detailed molecular recognition between AmB and ergosterol in lipid bilayers has yet to be determined.

Similarly, amphidinol 3 (AM3, Fig. 1) also shows membrane permeabilizing activity.[5–7] AM3 was isolated from marine dinoflagellate *Amphidinium krebsii* as a potent antifungal, hemolytic and cytotoxic substance,[6,8] and then shown to form pores across biological membranes.[5,6] Recently, we have revealed that the pore-formation of AM3 is dramatically promoted in the presence of cholesterol and ergosterol,[7] which is presumably related to the fact revealed by surface plasmon resonance (SPR) experiments that AM3 has much higher affinity to sterol-containing membranes than sterol-free one.[9] As in the case of AmB, however, the mechanism of sterol's promoting AM3 activity is far from understanding.

Meanwhile, $^{19}$F NMR has been of interest for many years in investigating biological systems due in large part to the attractive properties of $^{19}$F, which include 100% natural abundance, spin $I = 1/2$, large magnetogyric ratio, and low background signals in biological samples. In particular, solid-state $^{19}$F NMR has become an important tool for characterizing molecular interactions in lipid bilayers. In the course of our structural studies on the ion-channel formed by AmB and sterol molecules,[10] particularly the molecular recognitions for AmB–AmB[11] and AmB–sterol,[12] we have prepared several fluorinated derivatives of AmB[13,14] and fluorinated cholesterol (Fig. 1),[15] and used them for solid-state NMR measurements.[16] In this study, toward the $^{19}$F NMR investigation of AmB-ergosterol and AM3-ergosterol interactions in membrane, we developed a novel preparation method of a fluorinated derivative of ergosterol, 6-F-ergosterol (Fig. 1). We then assessed its activity of promoting membrane permeabilization by AmB and AM3.

## Results

### Preparation of 6-F-ergosterol

Synthesis of 6-F-ergosterol is shown in Scheme 1. Although synthesis of 6-F-ergosteryl acetate **4** from ergosteryl acetate **1** was reported by Barrett *et al.*[17] by treatment with chromyl fluoride ($CrO_2F_2$)[18] giving fluorohydrin **3** followed by dehydration with

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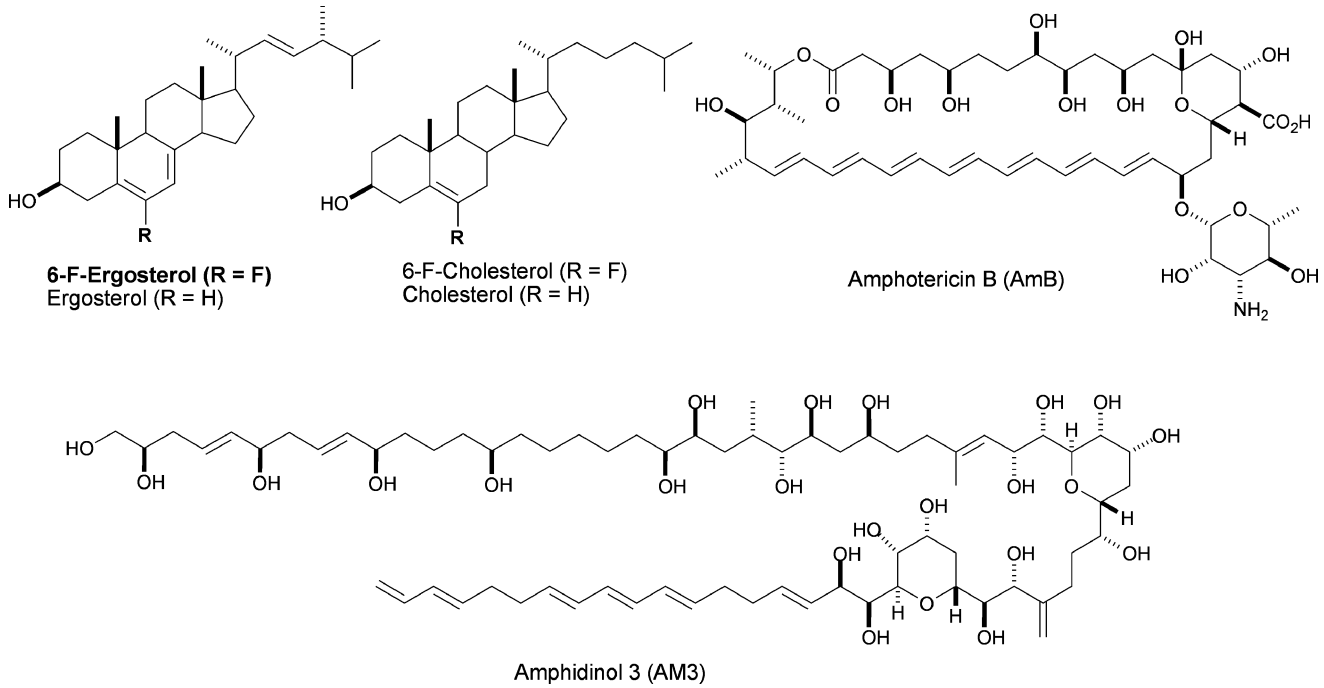
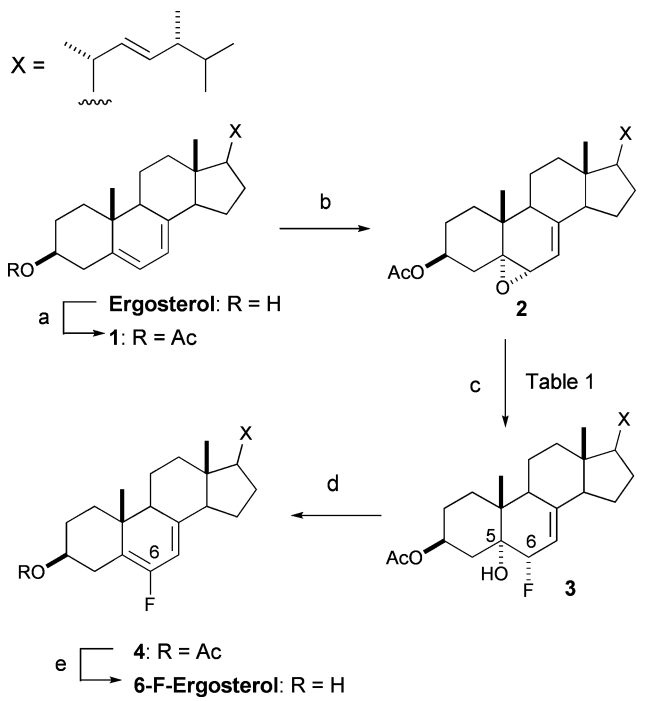



**Fig. 1** Structures of sterols including 6-F-ergosterol, AmB, and AM3.



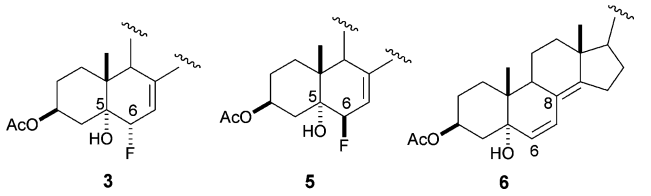

**Scheme 1** Synthesis of 6-F-ergosterol. Reagents and conditions: a) $Ac_2O$, DMAP, pyridine, $CH_2Cl_2$, rt, 1 h; b) MCPBA, $Na_2CO_3$, $CH_2Cl_2$, rt, 1 h, 88% (2 steps); c) $TiF_4$, $n$Bu$_4$NPh$_3$SiF$_2$, $CH_2Cl_2$, 0 °C, 40 min, 38% (**6**: 45%); d) $SOCl_2$, pyridine, $CH_2Cl_2$, −30 °C to 0 °C, 1 h; e) $K_2CO_3$, MeOH, THF, rt, 1 h, 42% (2 steps).

$SOCl_2$, careful handling is required for hazardous chromyl fluoride generated from $CrO_3$ and $CoF_3$ by heating at 450 °C under the stream of nitrogen gas. Therefore, we developed an alternative method using conventional synthetic operations with tractable reagents.

Epoxidation of ergosteryl acetate **1** with MCPBA in the presence of $Na_2CO_3$ gave (5α,6α)-epoxide **2** as a single diastereomer in 88% for two steps from ergosterol.[19] Treatment of **2** with triethylamine trihydrofluoride ($Et_3N{\cdot}3HF$)[20] resulted in the formation of desired 6α,5α-fluorohydrin **3** and 6β-epimer **5** with concomitant formation of conjugated diene **6** in a 15 : 10 : 75 ratio (Table 1, entry 1). The structures of these compounds were determined by NMR experiments.[17,19,21] Presumably, the fluorohydrins **3** and **5** were formed *via* allylic cation generated from acid catalyzed ring opening of the vinyl epoxide **2**, followed by nucleophilic attack of fluoride anion at the less hindered C6 position (Fig. 2A, path a), whereas deprotonation at C14 resulted in the formation of diene **6** (Fig. 2A, path b). We next carried out the reaction using $TiF_4$ and fluorinating reagents: tetra-n-butylammonium fluoride $n$-Bu$_4$N$^+$F$^-$ (TBAF, entry 2 in Table 1),[22] tris(dimethylamino)sulfonium difluorotrimethylsilicate $(Me_2N)_3S^+Me_3SiF_2^-$ (TASF, entry 3 in Table 1),[23] and tetra-n-butylammonium difluorotriphenylsilicate $n$-Bu$_4$N$^+$Ph$_3$SiF$_2^-$ (TBAT, entry 4 in Table 1),[24] to give comparable results as entry 1. After considerable experimentation, we found that the reaction using novel combination of reagents, $TiF_4$/TBAT in dichloromethane, gave the best result (**3** : **5** : **6** = 43 : 0 : 57, entry 5 in Table 1) to afford fluorohydrin **3** as a single diastereomer, presumably *via* activation of the epoxide with titanium species[25] and subsequent internal fluoride ion delivery (Fig. 2B). Purification by flash column chromatography afforded **3** in 38% yield which was isolated from diene **6** (45%). Finally, dehydration of *cis*-fluorohydrin **4** with $SOCl_2$/pyridine[17] and methanolysis of the acetate furnished 6-F-ergosterol in 42% for two steps. In contrast to **3**, dehydration of *trans*-fluorohydrin **5** using $SOCl_2$/pyridine or Burgess reagent[26] was unsuccessful to give a mixture of byproducts containing C4-C5 unsaturated counterpart.

**Table 1** Epoxide opening reaction




**3** **5** **6**

| Entry | Conditions | Ratio (**3** : **5** : **6**)[a] |
|---|---|---|
| 1 | $Et_3N{\cdot}3HF$, $CH_2Cl_2$, 0 °C, 1 h | 15 : 10 : 75 |
| 2 | $TiF_4$, TBAF, THF, 0 °C to rt, 9 h | 12 : 22 : 66 |
| 3 | $TiF_4$, TASF, $CH_2Cl_2$, −50 °C to −30 °C, 2 h | 19 : 15 : 66 |
| 4 | $TiF_4$, TBAT, THF, 0 °C to rt, 2 d | 17 : 14 : 69 |
| 5 | $TiF_4$, TBAT, $CH_2Cl_2$, 0 °C, 40 min | 43 : 0 : 57 |

[a] Ratio was determined by $^1H$ NMR of the crude product.

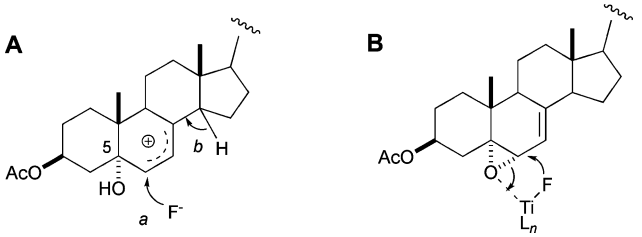



**Fig. 2** Plausible reaction pathways of the epoxide opening.

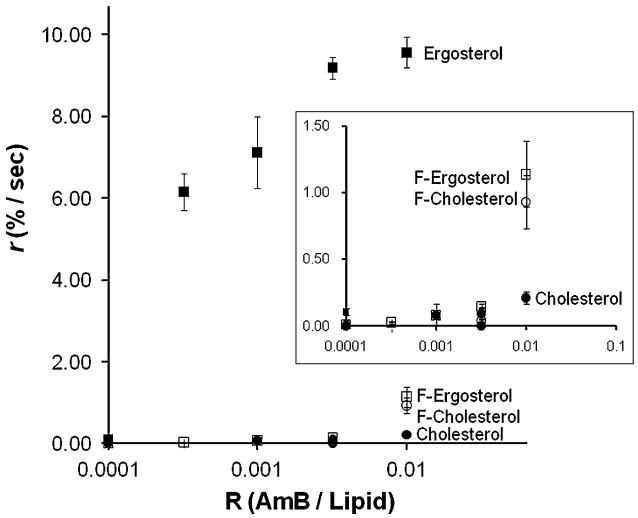



**Fig. 3** AmB-induced $K^+/H^+$ exchange monitored by liposome-encapsulated pH dependent fluorescent dye. Liposomes were prepared from POPC containing 10 mol% ergosterol (filled square), 10 mol% 6-F-ergosterol (open square), 10 mol% cholesterol (filled circle), and 10 mol% 6-F-cholesterol (open circle). The *r* values of sterol-free POPC membrane stayed near 0 over the R values tested. Three experiments were done for each point, and average values are shown in the figure with error bars. *Inlet*: the enlarged graph for 6-F-erogosterol, 6-F-cholesterol, and cholesterol.

### $K^+$ Flux assay of AmB in the presence of 6-F-ergosterol

To examine the effect of 6-F-ergosterol on the ion channel formation of AmB molecules, we then assessed the membrane-permeabilizing activity of AmB using artificial liposomes composed of 1-palmitoyl-2-oleoyl-*sn-glycero*-3-phosphocholine (POPC).[27] The liposomes possessed a higher external $K^+$ concentration and *trans*-membrane pH gradient with pH 5.8 inside and pH 7.8 outside. $K^+$ influx into the liposomes through AmB ion channels elicited $H^+$ leakages by a proton-transporter carbonyl cyanid-*p*-trifluoro-methoxyphenyl hydrazone (FCCP), consequently raising the inner pH. The pH change in the liposomes was monitored by the increase in the fluorescent intensity of liposome-entrapped BCECF, a pH dependent fluorescent dye. In this method, $K^+/H^+$ exchange rate *r*, which grossly corresponds to the conductance of AmB-induced ion channels, was calculated from the initial rate of fluorescence intensity change. The *r* value was expressed as percentage of $[K^+]$ entering into liposomes per second with respect to the initial $[K^+]$ difference between outside and inside of the liposomes.

Fig. 3 shows the relation between AmB concentration and *r* value. We used POPC liposomes containing 10 mol% of 6-F- and unmodified ergosterols, as well as 6-F- and unmodified cholesterols for comparison. As shown in Fig. 3, while ergosterol naturally promoted membrane-permeabilization of AmB, the activity of 6-F-ergosterol was unexpectedly diminished as compared with unmodified ergosterol. These results indicate that the introduction of an F atom at C6 position of ergosterol plays an inhibitory role in interacting with AmB. Intriguingly, the promoting activity of 6-F-cholesterol is comparable to that of 6-F-ergosterol, which implies that introduction of a fluorine atom at C6 of the sterol skeleton largely eliminates the ergosterol selectivity of channel formation by AmB.

### Calcein leakage assay of AM3 in the presence of 6-F-ergosterol

Then we examined the membrane-permeabilizing activity of AM3 in the presence of fluorinated and unmodified sterols. Although AM3 also forms pores in membranes as AmB does, its pore size was reported to be around 2 nm in diameter,[28] which is about three times larger that of AmB (0.7 nm in diameter).[29] Due to its larger pore size, the $K^+$-influx assay used for AmB was not applicable to the evaluation of AM3 activity, because pH-dependent dye BCECF, which should be entrapped into liposomes even after pore formation, leaks from the AM3 pore. Therefore, the membrane-permeabilizing activity of AM3 was evaluated by the leakage of a fluorescent dye, calcein, entrapped in POPC liposomes.[7] Since the fluorescence of liposome-encapsulated calcein is very weak due to self-quenching while that of calcein outside liposomes is strong, the AM3 pore formation can be evaluated by the increase in fluorescent intensity of calcein. On the contrary, the calcein leakage assay is not suitable to the evaluation of AmB activity because AmB pores are not large enough to pass through calcein molecules.

Table 2 lists $t_{90}$ values that are elapsed times until 90% of liposome-entrapped calcein was leaked at 2.5 μM of AM3. As reported previously,[7] AM3 showed no activity to sterol-free liposomes, whereas addition of 5% cholesterol or ergosterol to liposomes dramatically elevated the activity of AM3 (Table 2). Interestingly, calcein was leaked more rapidly from ergosterol-containing liposomes than from cholesterol-liposomes. This is consistent to our recent finding in SPR experiments that AM3 has higher affinity to ergosterol-membrane than cholesterol-membrane.[9] Noteworthy is that fluorinated sterols show comparable or even higher promoting effects than unmodified ones; the

**Table 2** Times for 90% calcein leakage from liposomes by 2.5 μM of AM3

| | POPC liposomes[a] | | | | |
|---|---|---|---|---|---|
| | Ergosterol[b] | 6-F-Ergosterol[b] | Cholesterol[b] | 6-F-Cholesterol[b] | Sterol-free |
| $t_{90}$ (*sec*)[c] | 56 | 64 | 203 | 22 | n.d.[d] |

[a] POPC concentration of liposome suspensions was set to 27 μM. [b] Sterols were contained at 5 mol% of POPC. [c] Times for 90% calcein leakage from liposomes at 2.5 μM of AM3. [d] Leakage was not observed at all for 15 min.

effect of 6-F-ergosterol was almost identical to that of unmodified ergosterol, and 6-F-cholesterol showed even higher promoting activity than unmodified cholesterol. These data suggest that, in the case of AM3, the introduction of a fluorine atom at C6 of sterol skeleton does not hinder, or in some cases rather enhances, the AM3-sterol interactions.

## Discussion

In this study, we first developed a practical route to synthesize 6-F-erogosterol, and examined its activity of promoting pore formations of AmB and AM3. The results unexpectedly showed that the activity of AmB was barely promoted by 6-F-ergosterol, while it promoted AM3 activity as efficacious as unmodified ergosterol. Here, we try to explain this different response of AmB and AM3 to 6-F-ergosterol from the viewpoint of pore formation mechanisms of AmB and AM3.

AmB is thought to form barrel-stave type pores,[30] in which AmB molecules penetrate cell membranes while interacting with ergosterol in a parallel manner.[12b] Accordingly, in the pore complex, AmB recognizes multiple points of ergosterol molecule,[31] such as 3-OH group, steroid skeleton including conjugated double bonds and the terminal side chain. In particular, the conjugated double bonds of ergosterol are thought to be of critical importance for interacting with the heptaene portion of AmB.[31,32] In this context, the introduction of electronegative fluorine atom on C6 of ergosterol should considerably affect the electronic state of the conjugated diene in ergosterol, which probably weakens the interaction with the polyene portion of AmB and consequently reduces the AmB activity.

On the other hand, AM3 is assumed to form toroidal-type pores,[7,33] in which AM3 polyol chains always interact with lipid polar headgroups, and consequently the lipid monolayer continuously curves from the outer leaflet to the inner in the fashion of a toroidal hole.[33] The aforementioned larger pore size of AM3 as compared with AmB is consistent to the toroidal pore formation, because toroidal pores are generally said to be larger than barrel-stave pores.[34] If the toroidal pore formation is true for AM3, it is likely that the interaction of AM3 and sterol predominantly occurs between the polar regions. In fact, in the previous paper[7] we have revealed that esterification of 3-OH of cholesterol almost eliminate the AM3 activity. The paper also reported that the AM3 activity was efficaciously enhanced not only by cholesterol and ergosterol but also by 25-hydroxycholesterol,[7] which commonly share the A-ring structure in steroid skeleton. Taken together, it can be reasonably assumed that the molecular recognition between AM3 and sterol predominantly takes place around the A-ring of sterol skeleton including 3-OH group, and therefore the introduction of F atom on the B-ring has small influence on the AM3 activity.

In summary, the different response of AmB and AM3 to 6-F-ergosterol probably reflects the difference in pore formation mechanism of AmB and AM3, and appears to be consistent with the proposed pore models, barrel-stave pore for AmB and toroidal one for AM3.

## Conclusion

In conclusion, 6-F-ergosterol was synthesized from ergosterol in 14% overall yield for five steps through epoxide opening reaction using a novel combination of $TiF_4$ and *n*-$Bu_4N^+Ph_3SiF_2^-$ in dichloromethane. We then assessed its activity of promoting membrane-permeabilization of AmB and AM3. The assays showed that the activity of AmB was barely promoted by 6-F-ergosterol, while it promoted AM3 activity as efficacious as unmodified ergosterol. This suggests that the introduction of a F atom at C6 position of ergosterol plays an inhibitory role in interacting with AmB, but it is not the case with AM3. The different response of AmB and AM3 to 6-F-ergosterol is likely attributed to the difference in the pore formation mechanism of AmB and AM3. For comparison, we also assayed 6-F- and unmodified cholesterols, and found that 6-F-cholesterol is more effective than cholesterol in promoting AM3 activity. The current study demonstrates that the fluorinated derivative of ergosterol and cholesterol should serve as powerful molecular probes for $^{19}F$ NMR studies on the detailed mechanism of AM3 pore formation.

## Experimental

### Preparation of 6-F-ergosterol

**Chemistry.** Anhydrous methylene chloride ($CH_2Cl_2$) and tetrahydrofuran (THF) were purchased from Kanto Chemical Co. Inc., and used without further drying. $TiF_4$ was purchased from Sigma-Aldrich, and *n*-$Bu_4N^+Ph_3SiF_2^-$ was from TCI. All other chemicals were obtained from local venders, and used as supplied unless otherwise stated. Analytical thin-layer chromatography (TLC) was performed using E. Merck silica gel 60 $F_{254}$ pre-coated plates (0.25-mm thickness). For column chromatography, Kanto silica gel 60 N (spherical, neutral, 100–210 μm) was used. Optical rotations were recorded on a JASCO P-1010 polarimeter. IR spectra were recorded on a JASCO FT-IR-300E Fourier transform infrared spectrometer. $^1H$ and $^{19}F$ NMR spectra were recorded on a JEOL GSX500 or ECA500 spectrometer. ESI-MS spectra were measured on an LCQ-deca (Thermo Finnigan).

**6-F-ergosterol.** To a solution of *n*-$Bu_4N^+Ph_3SiF_2^-$ (642 mg, 1.19 mmol, 2.0 eq) and $TiF_4$ (147 mg, 1.19 mmol, 2.0 eq) in $CH_2Cl_2$ (1.0 mL) was added a solution of epoxide **2** (271 mg, 0.60 mmol) in $CH_2Cl_2$ (2.0 mL) at 0 °C. After 40 min of stirring, the reaction mixture was quenched with saturated aqueous $NaHCO_3$, and extracted with AcOEt. The organic layer was washed with saturated aqueous NaCl, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue was purified by flash column chromatography (hexane–AcOEt = 15/1) to afford fluorohydrin **3** (108 mg, 0.228 mmol, 38%) and conjugated diene **6** (121 mg,

0.265 mmol, 45%). To a solution of **3** (17.8 mg, 37.5 μmol) and pyridine (0.3 mL, 3.75 mmol, 100 eq) in $CH_2Cl_2$ (1.0 mL) was added a solution of $SOCl_2$ (13.7 μL, 18.7 μmol, 5.0 eq) in $CH_2Cl_2$ (0.5 mL) at −30 °C and allowed to warm to 0 °C. After 1 h of stirring at 0 °C, the reaction mixture was diluted with $Et_2O$ and quenched with $H_2O$. The organic layer was separated and washed with 0.2 M HCl, saturated aqueous $NaHCO_3$, and saturated aqueous NaCl, dried over anhydrous $MgSO_4$, filtered and concentrated. The residue containing **4** was used for next step without further purification. A solution of the crude **4** in THF (0.5 mL) and MeOH (0.5 mL) was added solid $K_2CO_3$ (10.4 mg, 75 μmol, 2.0 eq), and stirred for 1 h at room temperature. The solvent was removed under reduced pressure and the residue was purified by silica gel column chromatography to afford 6-F-ergosterol (6.5 mg, 15.7 mmol, 42% for two steps): pale yellow amorphous solid; m.p. 158.0–159.0 °C; $R_f$ 0.18 (hexane–AcOEt = 5/1); $[\alpha]_D^{21}$ −119.1 (*c* 0.42, $C_6H_6$); IR (KBr) ν 3434, 2956, 2871, 1677, 1459, 1369, 1255, 1128, 1058, 1008, 995, 970, 844, 806 $cm^{-1}$; $^1H$ NMR (500 MHz, $CDCl_3$) $\delta$ 5.38 (1H, ddd, $J$ = 8.6, 2.6, 2.6 Hz), 5.20 (1H, dd, $J$ = 15.2, 7.5 Hz), 5.15 (1H, dd, $J$ = 15.2, 7.5 Hz), 3.59 (1H, dddd, $J$ = 11.2, 11.2, 4.9, 4.9 Hz), 2.96 (1H, ddd, $J$ = 14.6, 4.8, 1.7 Hz), 2.07–1.95 (3H, m), 1.90–1.80 (5H, m), 1.78–1.56 (6H, m), 1.50–1.42 (2H, m), 1.40–1.21 (5H, m), 1.01 (3H, d, $J$ = 6.9 Hz), 0.95 (3H, s), 0.90 (3H, d, $J$ = 6.9 Hz), 0.81 (3H, d, $J$ = 7.1 Hz), 0.80 (3H, d, $J$ = 7.1 Hz), 0.60 (3H, s); $^{13}C$ NMR (125 Hz, $CDCl_3$) $\delta$ 151.9 (d, $^1J_{CF}$ = 241.5 Hz), 145.1 (d, $^3J_{CF}$ = 10.4 Hz), 135.2, 132.0, 113.1 (d, $^2J_{CF}$ = 9.3 Hz), 112.6 (d, $^2J_{CF}$ = 36.4 Hz), 69.6, 55.6, 54.5, 46.2, 42.8, 40.4, 38.8, 38.5, 36.9, 33.1, 31.4, 30.4 (d, $^3J_{CF}$ = 4.7 Hz), 28.2, 22.8, 21.1, 20.9, 20.0, 19.6, 17.6, 16.7, 12.1; $^{19}F$ NMR (470.4 MHz, DMSO-$d_6$) $\delta$ −126.2 (br d, $J$ = 8.0 Hz); MS (ESI) $m/z$ 415 $[M+H]^+$.

### Assays

**Chemistry.** AmB, carbonyl cyanid-p-trifluoro-methoxyphenyl hydrazone (FCCP), 20,70-bis(carboxyethyl)-4 or 5-carboxyfluorescein (BCECF), valinomycin, and calcein were purchased from Nacalai Tesque (Kyoto, Japan). 1-Palmitoyl-2-oleoyl-sn-*glycero*-3-phosphocholine (POPC) was obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). Cholesterol and ergosterol were purchased from Sigma–Aldrich (St. Louis, MO). AM3 was isolated as previously reported.[6] All the other chemicals were standard and analytical quality reagents. Polycarbonate filters were from Nuclepore (Pleasanton, CA). Fluorescence spectra were recorded on a JASCO FP-6600 fluorophotometer.

**$K^+$ Flux assays of AmB.** POPC and sterol were dissolved in chloroform or in methanol to prepare stock solutions. A series of liposomes was prepared by adding aliquots of the stock solutions into round-bottom flasks. The solvent was evaporated to form lipid films at the bottom of flask, which were then left under vacuum for 6 h to completely remove the solvent. Lipid films thus obtained were hydrated with phosphate buffer (0.15 M potassium phosphate and 40 μg $ml^{-1}$ BCECF, pH 5.8) by sonication, vortex mixing, and subsequently five cycles of frozen/thawed to furnish large vesicles. After the sizing of the liposomes using Liposofast extruder (Avestin Inc., Ottawa, Canada) by filtering 31 times through a polycarbonate filter of 200 nm pore size, the resultant BCECF-entrapping LUV liposomes were separated from the excess amount of BCECF by gel filtration using Sepharose 4B (10 mm × 150 mm, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 0.15 M potassium phosphate buffer (pH 5.8). The lipid concentration in liposome suspension was determined by HPLC (SCL-10AVP, Shimadzu) with an evaporative light scattering detector (ELSD-LT, Shimadzu). Liposome suspension was diluted with the same buffer to lipid concentration of 0.5 mM. To monitor the potassium–proton exchange, AmB in DMSO–MeOH and a 0.5 μl aliquot of 1 mM FCCP was added to 200 μl of liposome suspension, which was then incubated for 3 h at 6 °C. The suspension (200 μl) was added to a cuvette containing 1.8 ml of $K_2HPO_4$ (0.15 M) that was set to a fluorescence spectrometer in advance, and the time course of pH change in the liposomes was monitored at 6 °C by the increase in the fluorescent intensity (excitation 500 nm and emission 535 nm). The initial pH of the liposome suspension was pH 7.8 while that of liposome lumen was pH 5.8. The background fluorescence change ($I_0$) was obtained by the extrapolation of the tangent of the chart. The exchange rate $r$ (%/s) was calculated from the tangent as $r = (I_t - I_0) \times 100/(I_{100} - I_0)\ t$ where $t$ is the time after the addition of the liposomes (*sec*) and the $I_t$ is the fluorescent intensity at $t$. After the time-course measurements, a valinomycin aqueous solution (10 μl, 5 mM) was added to the suspension to obtain the fluorescent intensity corresponding to the 100% exchange ($I_{100}$).

**Calcein leakage experiments of AM3.** Large unilamellar vesicles (LUVs) were prepared as follows. POPC (20 mg) with or without 5 mol% sterol was dissolved in 3 mL of $CHCl_3$. After evaporation of $CHCl_3$ at 30 °C under vacuum for 2 h, it was dried *in vacuo* overnight. The lipid film was suspended in 3 mL of 1 mM Tris–HCl (pH 7.5) containing 60 mM calcein and agitated for 30 min. A freeze-thaw cycle was repeated three times to obtain multilamellar vesicles (MLV). Subsequently, the suspension was passed through a polycarbonate membrane filter (pore size, 200 nm) 19 times using a Liposofast extruder (Avestin Inc., Ottawa, Canada) above 5 degree of $T_m$. The resultant calcein-entrapping LUVs were separated from the excess amount of calcein by gel filtration using Sepharose 4B (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with 10 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 150 mM NaCl. The lipid and cholesterol concentration in the LUV fraction were measured using a phospholipid C-Test (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and cholesterol *E*-Test Wako (Wako Pure Chemical Industries, Ltd, Osaka, Japan), respectively. The resulting stock solution was stored at 4 °C under nitrogen gas. To monitor calcein leakage from LUV, 20 μl of the LUV suspension in a cuvette was diluted with 980 μl of the same buffer to give a lipid concentration of 27 μM. A 20-μl aliquot of AM3 in MeOH was added to the LUV suspension. The time courses of liposome leakage were measured on a spectrofluorometer with excitation at 490 nm (slit 1.5 nm), emission at 517 nm (slit 5 nm) at 25 °C in a final volume of 1 ml buffer with vesicles. Triton X-100 (10%, 20 μl) was added to obtain the condition corresponding to the 100% leakage.

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