# Supplementary Information

# Amphotericin forms an extramembranous and fungicidal sterol sponge

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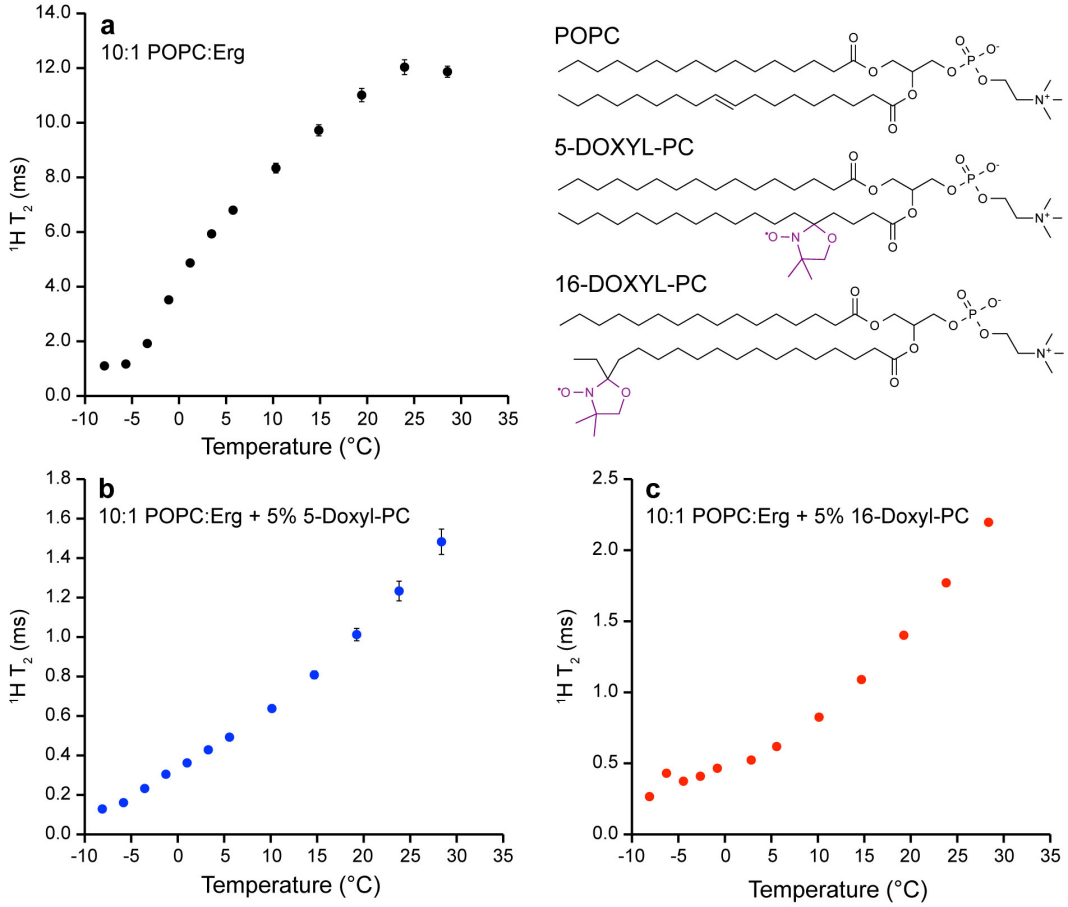
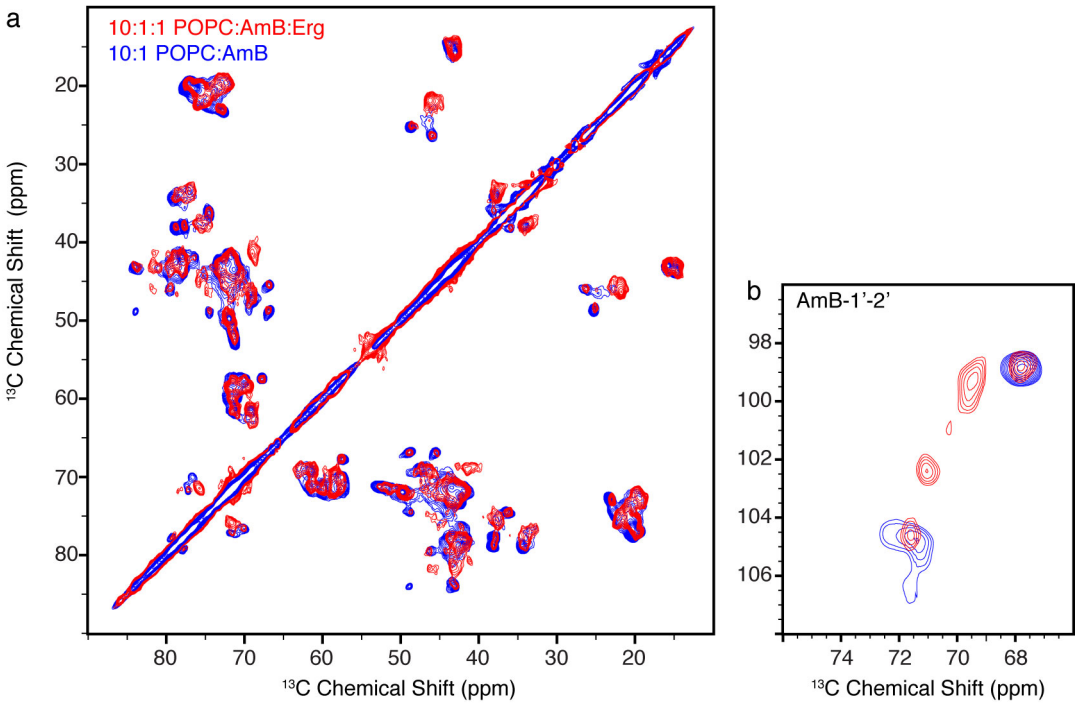
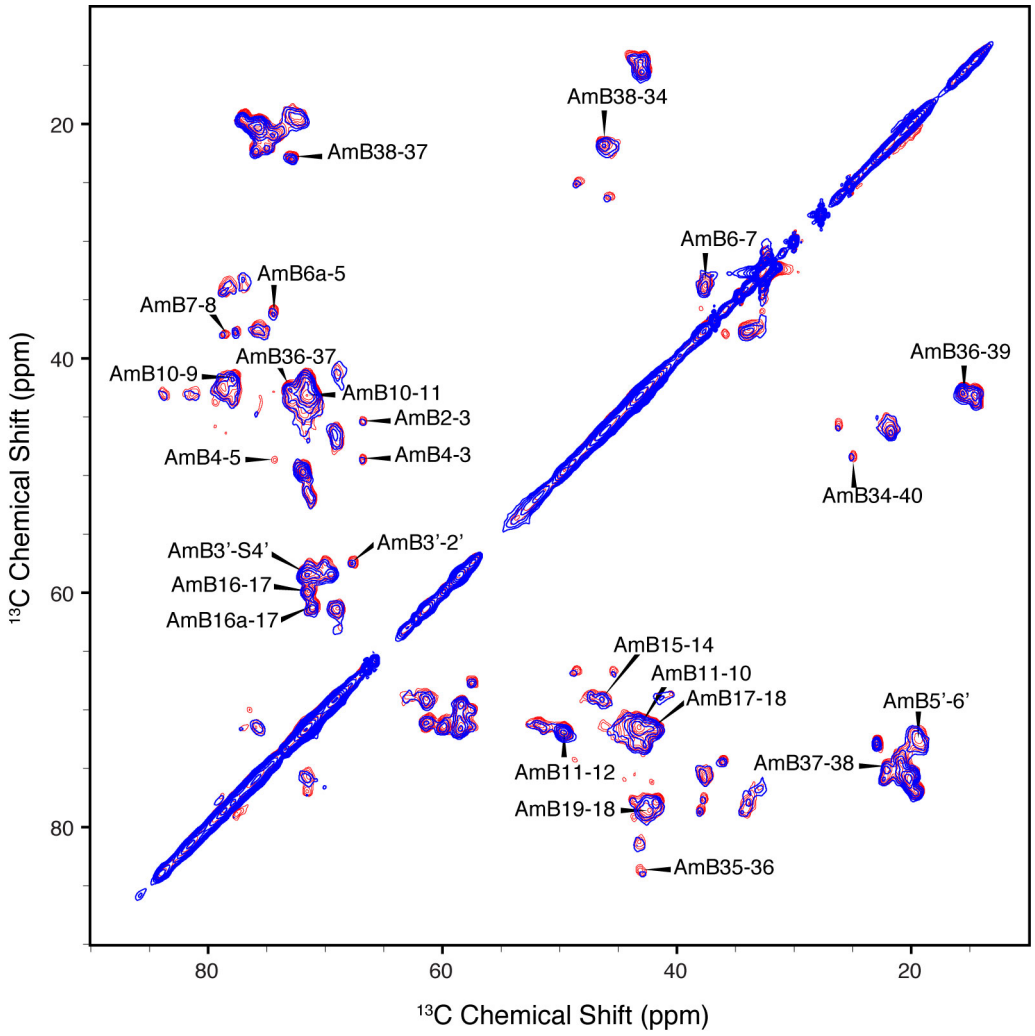
## Supplementary Results




**Supplementary Figure 1:** $^1$H $T_2$ values of the bulk fatty acyl $CH_2$ peaks of molar ratios of (a) 10:1 POPC:Ergosterol (b) 10:1 POPC:Ergosterol + 5% 5-Doxyl-PC and (c) 10:1 POPC:Ergosterol + 5% 16-Doxyl-PC liposomes as a function of temperature. The data were acquired on a 600 MHz spectrometer over a calibrated temperature range of -8 to 28 °C.



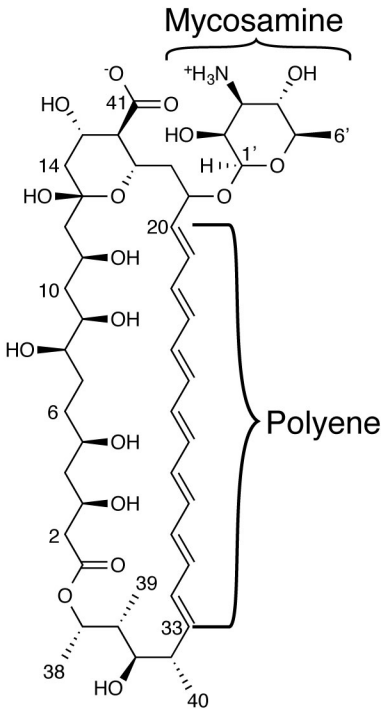

**Supplementary Figure 2:** $^{13}$C-$^{13}$C 2D spectra of (1.44 ms $SPC5_3$ mixing) of 10:1:1 POPC:U-$^{13}$C-AmB:Erg (red) and 10:1:0 POPC:U-$^{13}$C-AmB:Erg (blue) at 600 MHz with SPINAL $^{1}$H decoupling.[53] Irrespective of the presence of Erg, the peak positions do not change substantially. However, as shown in panel b, some interesting changes in the mycosamine resonances upon the binding of Erg are noted.

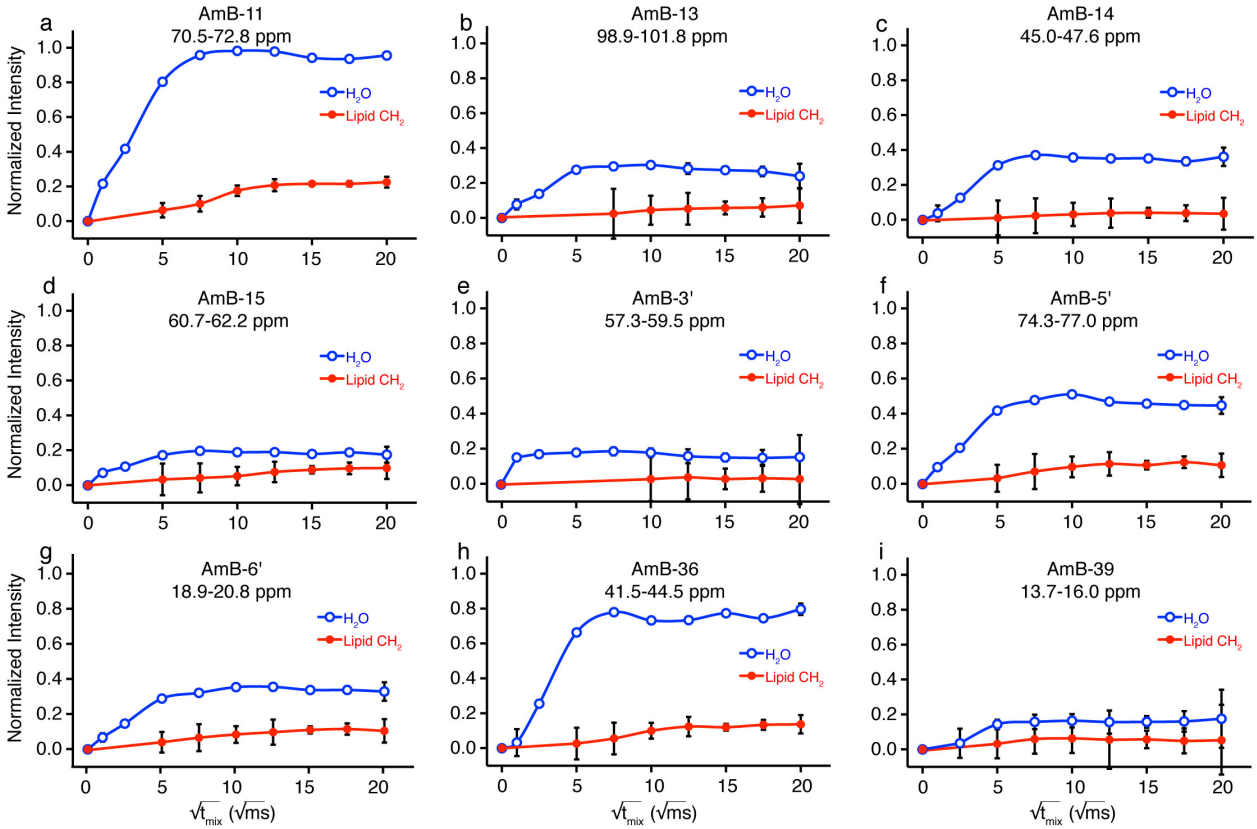



**Supplementary Figure 3:** $^{13}$C-$^{13}$C 2D spectra of (1.44 ms SPC5 mixing) of 10:1:1 POPC:U-$^{13}$C-AmB:Erg (red) and 10:1:1 POPC:U-$^{13}$C-AmB:Erg with 5 mol% 5-DOXYL-PC used to make site-specific assignments of the AmB and confirm that the addition of the DOXYL spin labels has no significant effect on AmB. Spectrum was acquired on a 600 MHz spectrometer, at 20 °C with and MAS rate of 10 kHz, and 75 kHz SPINAL $^{1}$H decoupling.[53]

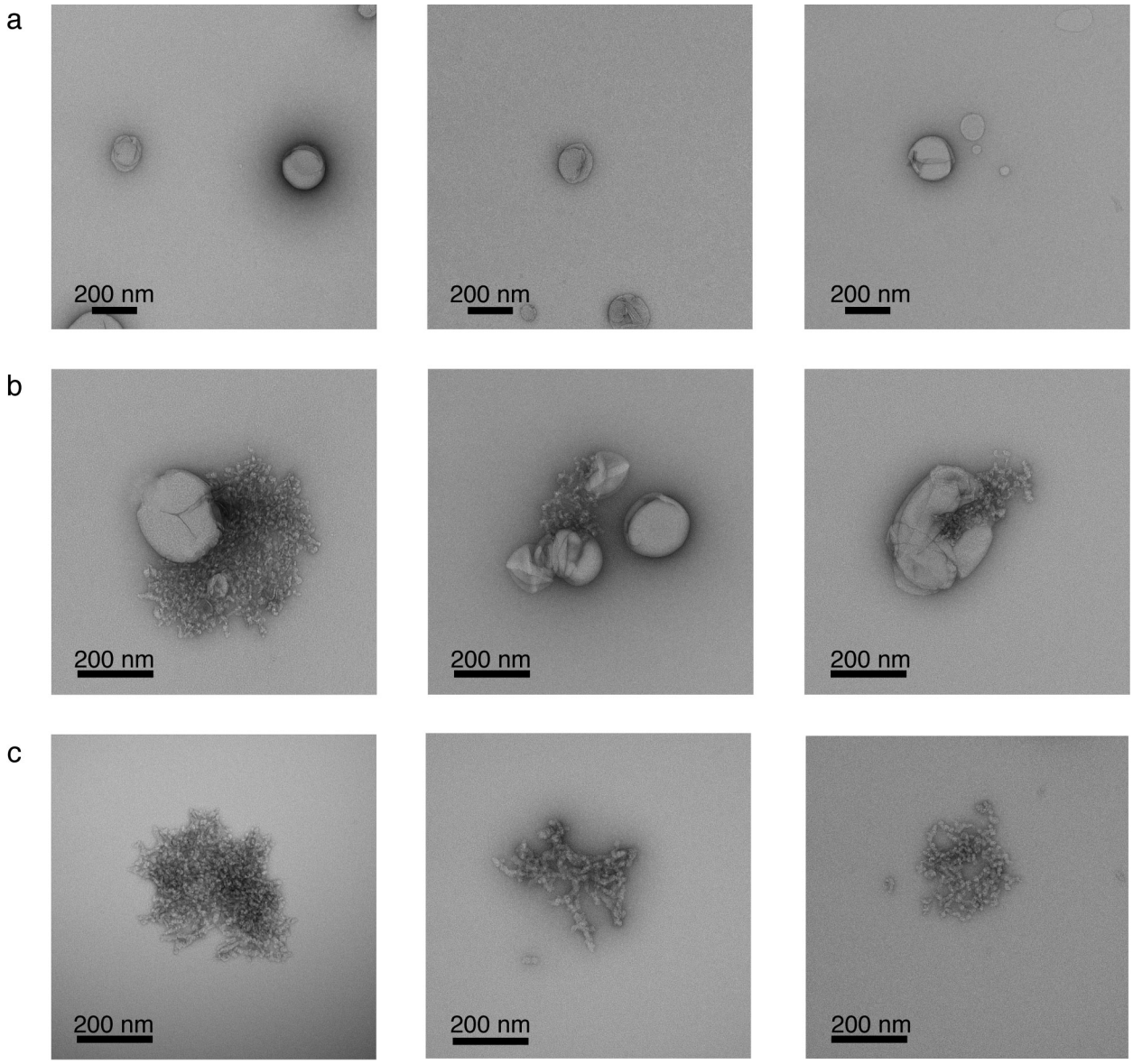
**Supplementary Table 1**: $^{13}C$ resonance assignments for AmB. Carbon atoms are numbered sequentially starting with the macrolactone carbonyl, denoted "AmB-1." The mycosamine appendage is numbered starting with "AmB-1' " carbon atoms 20 to 33 are identified as "AmB-Polyene". Asterisk (*) indicates resonances that overlap with lipid resonances and therefore were excluded from PRE and line shape analysis.

| Assignment | $^{13}C$ Chemical Shift (ppm) | | | | | |
|---|---|---|---|---|---|---|
| AmB-1 | 174.6 | 175.4 | | | | |
| AmB- | 45.5 | 40.6 | 44.2 | | | |
| AmB-3 * | 66.8 | 68.6 | 73.2 | | | |
| AmB-4 | 48.8 | 42.6 | | | | |
| AmB-5 | 74.4 | 78.9 | 74.6 | 77.9 | 75.7 | |
| AmB-6 * | 37.8 | 36.1 | 34.4 | 33.0 | 33.9 | |
| AmB-7 * | 33.9 | 34.1 | 38.1 | 37.9 | 37.7 | |
| AmB-8 | 78.0 | 77.8 | 78.8 | 78.4 | | |
| AmB-9 | 77.8 | 77.9 | 79.4 | 78.9 | | |
| AmB-10 | 41.8 | 44.2 | 43.7 | 42.5 | | |
| AmB-11 | 71.8 | 71.6 | 71.3 | 71.7 | | |
| AmB-12 | 49.7 | 51.8 | 51.3 | 52.7 | | |
| AmB-13 | 99.9 | 100.4 | 100.7 | 100.2 | 101.3 | 99.6 |
| AmB-14 | 46.4 | 46.9 | 47.4 | 47.9 | | |
| AmB-15 * | 69.0 | 69.1 | 69.2 | | | |
| AmB-16 * | 61.2 | 59.9 | 61.5 | 63.0 | | |
| AmB-17 | 71.5 | 71.0 | 71.3 | 71.6 | | |
| AmB-18 | 43.2 | 42.2 | 43.3 | | | |
| AmB-19 | 78.5 | 79.3 | | | | |
| AmB-Polyene | 134.5-141.1 | | | | | |
| AmB-34 | 46.2 | 48.7 | 46.0 | 45.2 | | |
| AmB-35 | 81.5 | 83.9 | 84.0 | 79.5 | 83.5 | |
| AmB-36 | 43.1 | 42.9 | 43.8 | 43.4 | 42.1 | |
| AmB-37 * | 72.7 | 73.0 | 76.5 | 74.2 | 74.9 | 75.9 |
| AmB-38 | 22.9 | 22.3 | 21.8 | 23.2 | 21.2 | 19.5 |
| AmB-39 | 15.5 * | 14.6 | 14.6 | | | |
| AmB-40 * | 25.1 | 26.3 | 21.8 | 22.2 | | |
| AmB-41 | 182.1 | 183.9 | 182.0 | 184.8 | | |
| AmB-1' | 102.3 | 104.6 | 98.7 | 106.4 | 99.2 | 99.9 |
| AmB-2' * | 71.5 | 67.7 | 70.9 | 69.6 | 69.4 | |
| AmB-3' | 58.4 | 57.5 | 61.2 | 59.8 | | |
| AmB-4' | 71.5 | 69.2 | 70.1 | 70.8 | 69.6 | |
| AmB-5' | 75.6 | 75.8 | 76.1 | 76.8 | 77.1 | |
| AmB-6' | 20.3 | 19.6 | 22.4 | 20.0 | 20.1 | |





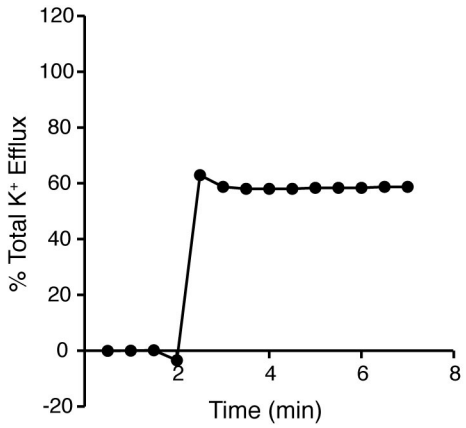

**Supplementary Figure 4:** $T_2$-filtered (1 ms) spin diffusion studies of 10:1:1 POPC:U-$^{13}$C-AmB:Erg MLVs show rapid transfer from lipid $^{1}$H to lipid $^{13}$C signals and from water to U-$^{13}$C-AmB, but much slower transfer of lipid polarization to AmB. The selected $^{1}$H-$^{13}$C correlations were collected with 1 ms $T_2$ filter and $^{1}$H-$^{1}$H spin diffusion times of 1 ms to 400 ms. Cross peaks from the lipid acyl chain $CH_2$ protons (~1.35 ppm) and water (~4.7 ppm) to the various regions on U-$^{13}$C-AmB are shown in red and blue, respectively. The rates show that the majority of the AmB is closely associated with water and >15-20 Å from the lipid acyl chains.[28] The polarization transfer is normalized based on the maximum observed intensity after correction of the $^{1}$H $T_1$ relaxation. Spectra were acquired on a 600 MHz spectrometer, at 20 °C with and MAS rate of 10 kHz, and 75 kHz SPINAL $^{1}$H decoupling.[53]




**Supplementary Figure 5:** Supplemental transmission electron microscopy images. a) 10:1 POPC:Erg liposomes. Average LUV size was ≈ 200 nm b) POPC:Erg 10:1 liposomes with 1 equivalent of added AmB. The average LUV size was ≈ 200 nm. c) 1 equivalent AmB added in HEPES Buffer.

**Supplementary Table 2:** $^{13}C$ Line widths and order parameters

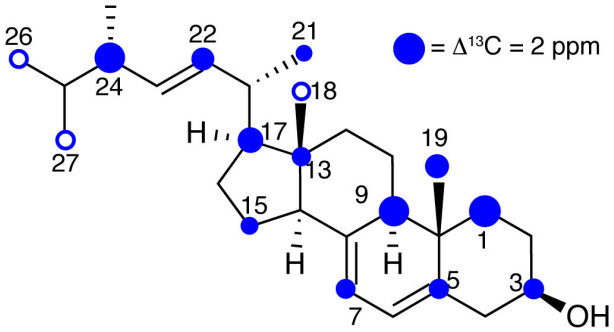
| | - AmB | |
|---|---|---|
| Peak Assignment | Line Width (Hz) | S |
| Erg-1 | 41 | 0.443 |
| Erg-3 | 42 | 0.412 |
| Erg-7 | 45 | 0.219 |
| Erg-9 | 45 | 0.456 |
| Erg-17 | 60 | 0.414 |
| Erg-19 | 43 | 0.393 |
| Erg-22 | 45 | 0.377 |
| Erg-24 | 57 | 0.287 |
| | + AmB | |
| Erg-1 | 153 | 0.943 |
| Erg-3 | 149 | 0.938 |
| Erg-7 | 167 | 0.976 |
| Erg-9 | 179 | 0.948 |
| Erg-17 | 176 | 0.989 |
| Erg-19 | 184 | 0.803 |
| Erg-22 | 207 | 0.912 |
| Erg-24 | 228 | 0.921 |
| A1' | 110 | 0.981 |
| A1' | 138 | 0.988 |
| AmB-Polyene | | 0.981 |
| A8 | 111 | 0.988 |
| A9 | 158 | 0.994 |
| A11, A17 | 140 | 0.999 |
| A35 | 161 | 0.994 |

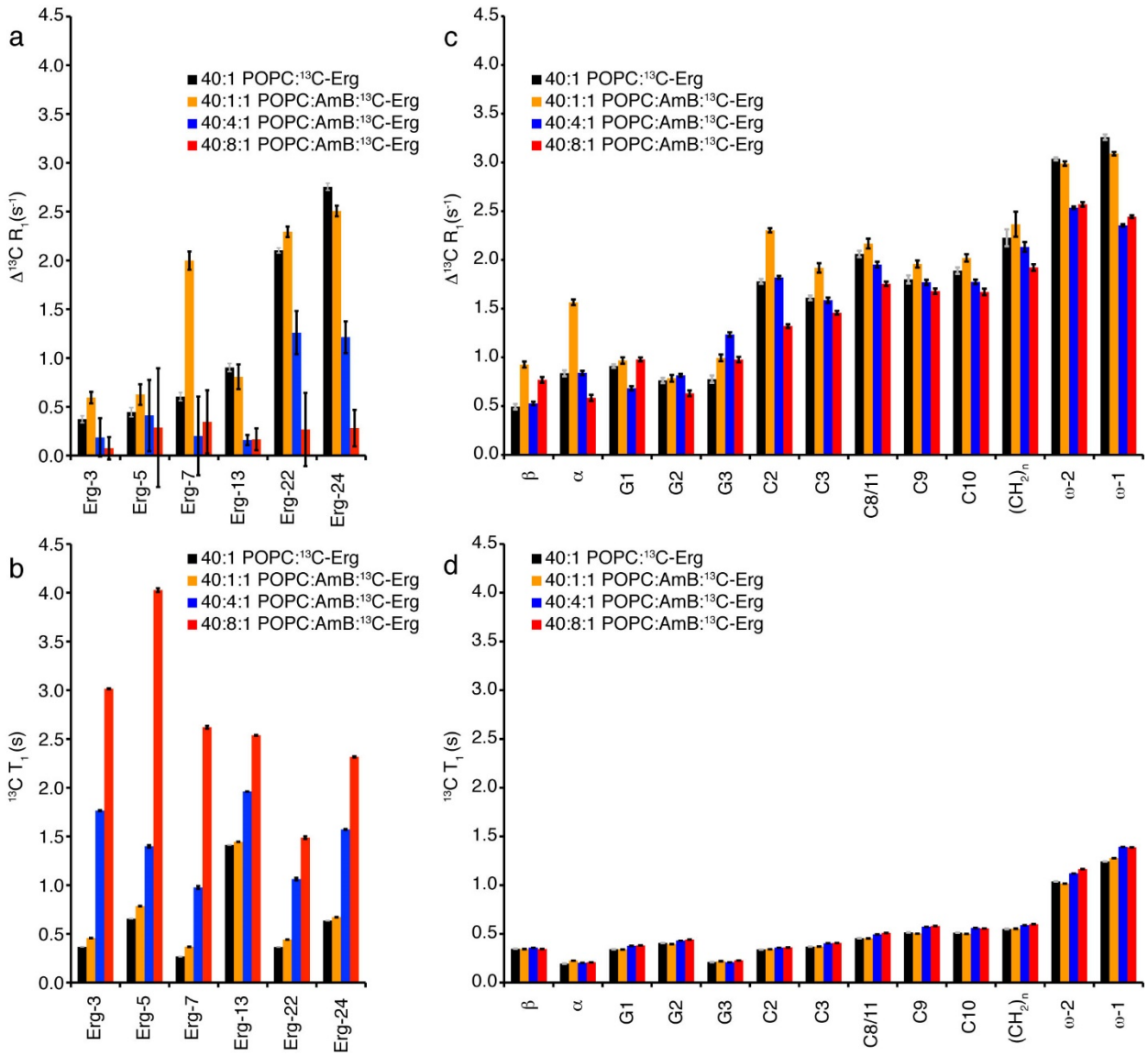



**Supplementary Figure 6:** AmB promotes robust $K^+$ efflux from 200 mm LUV's composed of 10:1 POPC:Erg. This activity mirrors both the *in vitro* and *in vivo* efflux activity of AmB previously reported by our laboratories.[25,27] 10 mol% AmB was added as a DMSO solution (final AmB concentration of 1 μM) at t = 1.5 min, and efflux is reported as a percentage of total efflux observed upon addition of Triton X-100. The observed plateau at 60% efflux is likely reflective of equilibrium between the bulk solution and the vesicle interior.

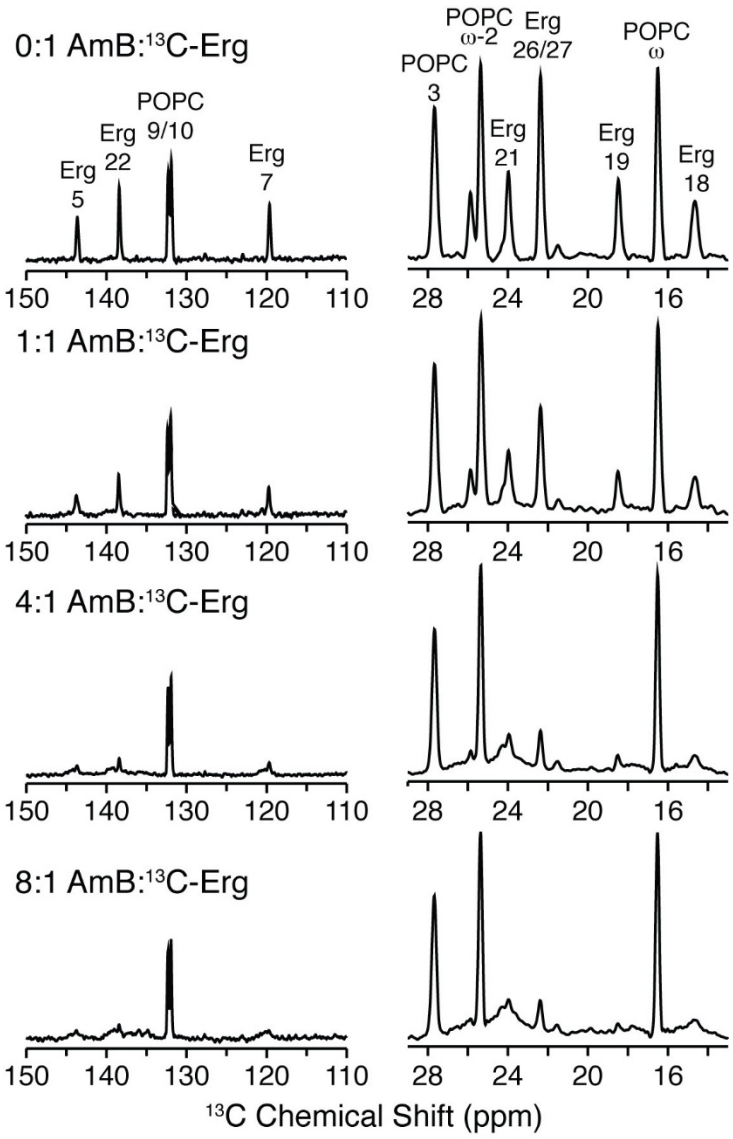
**Supplementary Table 3:** $^{13}C$ Chemical Shifts of $^{13}C$ Skip Labeled Ergosterol ($^{13}C$-Erg)

| | - AmB | + AmB | |
|---|---|---|---|
| Assignments | $^{13}C$ (ppm) | $^{13}C$ (ppm) | $\Delta^{13}C$ (ppm) |
| Erg-1 | 41.2 | 39.3 | 1.9 |
| Erg-3 | 72.0 | 71.5 | 0.6 |
| Erg-5 | 143.7 | 144.3 | -0.6 |
| Erg-7 | 119.7 | 120.1 | -0.4 |
| Erg-9 | 48.9 | 47.2 | 1.7 |
| Erg-13 | 45.3 | 45.0 | 0.3 |
| Erg-15 | 26.0 | 26.2 | -0.2 |
| Erg-17 | 58.5 | 57.4 | 1.1 |
| Erg-18 | 14.7 | 14.7 | 0.0 |
| Erg-19 | 18.6 | 17.8 | 0.8 |
| Erg-21 | 24.0 | 24.2 | -0.2 |
| Erg-22 | 138.4 | 139.4 | -0.9 |
| Erg-24 | 45.9 | 47.5 | -1.6 |
| Erg-26 | 20.3 | 20.3 | 0.0 |
| Erg-27 | 22.4 | 22.4 | 0.0 |

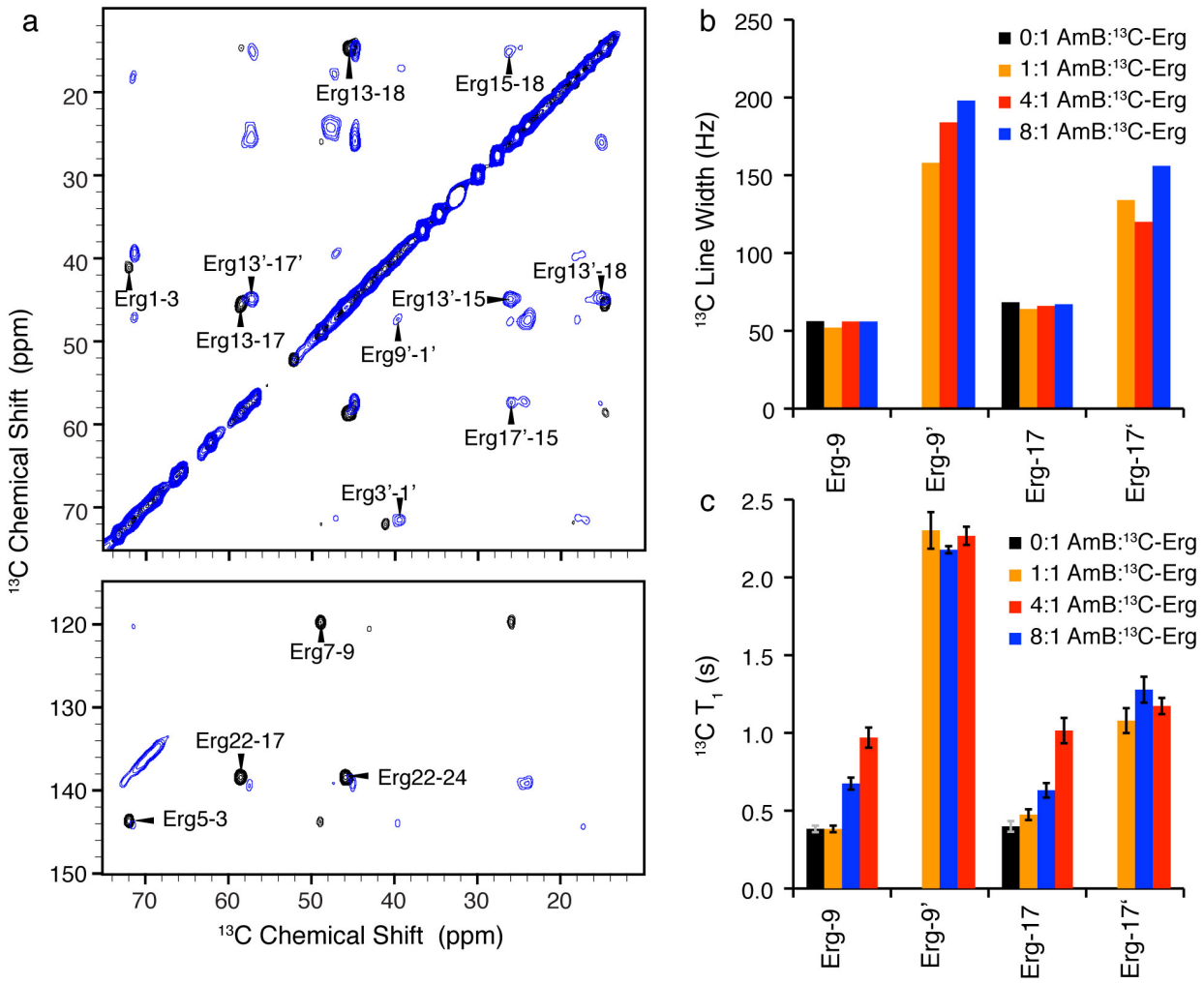




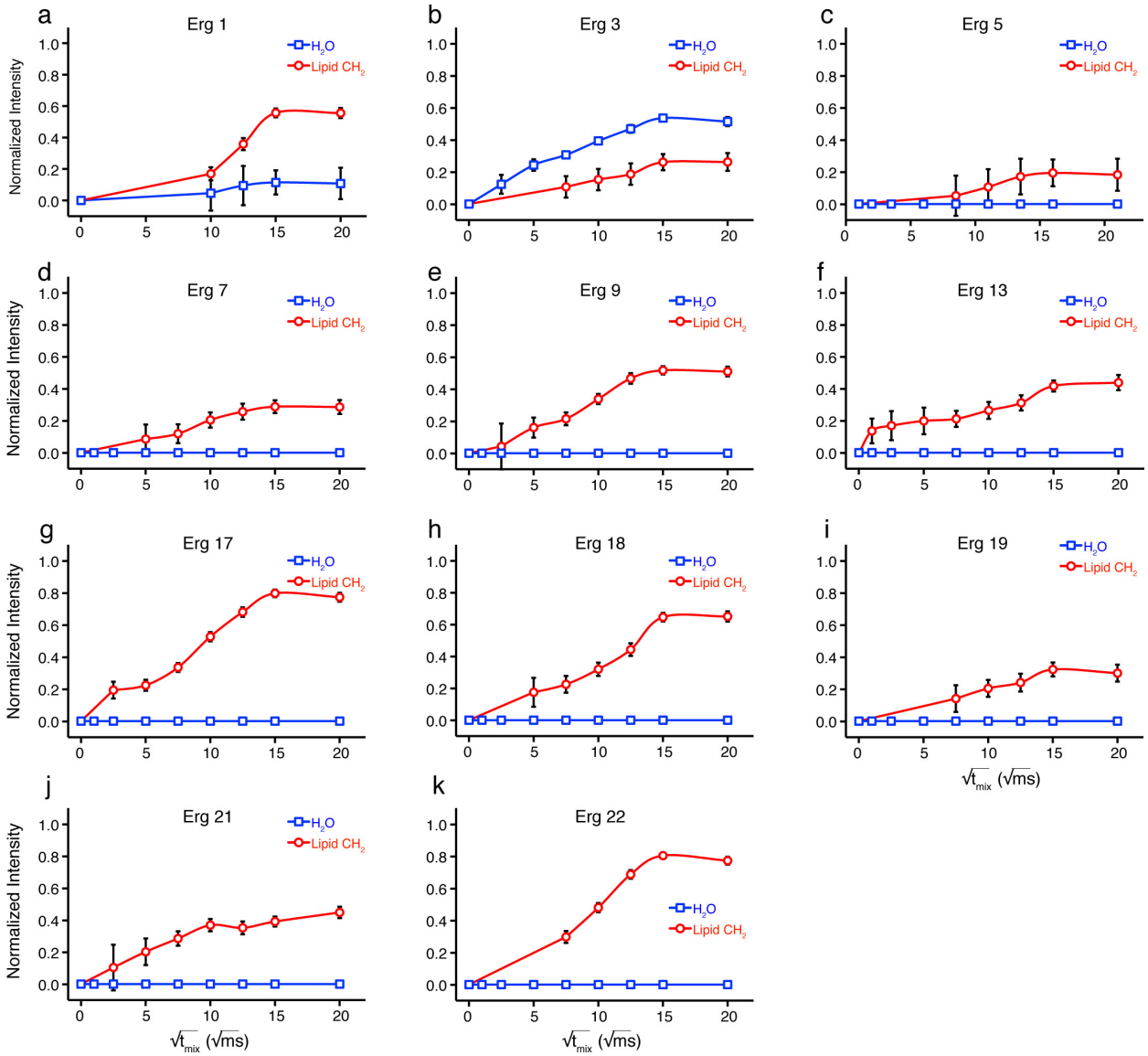

**Supplementary Figure 7:** The addition of AmB to POPC:Erg liposomes has a large effect on Erg, but a relatively small effect on POPC. The combination of the decrease in (a) PRE and substantial increase in the (b) longitudinal relaxation times ($T_1$) of Erg are consistent with the formation of an AmB:Erg complex that is separated from the lipid bilayer. Note the small relative change in the POPC (c) PRE (~20% on average) and (d) $^{13}C$ $T_1$ values (~ 10% on average). (40:1 POPC:$^{13}C$-Erg (black), 40:1:1 POPC:AmB:$^{13}C$-Erg (orange), 40:4:1 POPC:AmB:$^{13}C$-Erg (blue), and 40:8:1 POPC:AmB:$^{13}C$-Erg (red) liposomes ± 5 mol% 16-DOXYL-PC.) Only methine (CH) and methylene ($CH_2$) sites that do not shift significantly (< 0.5 ppm) upon addition of AmB are shown. SSNMR experiments were performed on a 600 MHz spectrometer, at 20 °C with an MAS rate of 10 kHz and 75 kHz $^{1}H$ decoupling.[53]



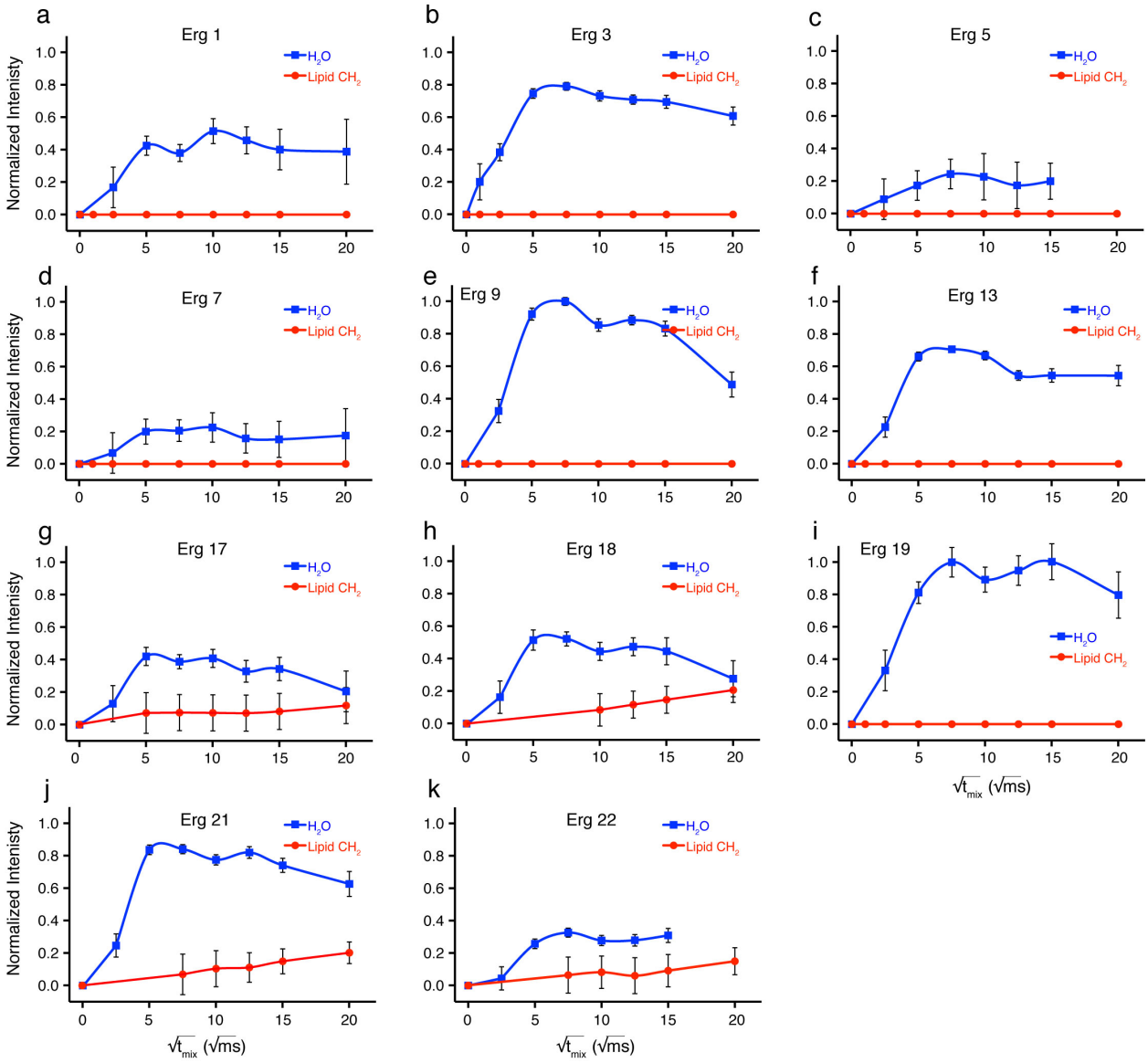

**Supplementary Figure 8: AmB extracts Erg from lipid bilayers**. 1D $^{13}C$ spectra of $^{13}C$-Erg titrated with natural abundance AmB. As a function of AmB:Erg ratio, the $^{13}C$-Erg resonances broaden from an average linewidth of 0.3 ppm to >1.0 ppm, consistent with formation of an AmB-Erg complex Spectra were acquired at 14.1 T (600 MHz $^{1}H$ frequency), at 20 °C and 10 kHz MAS rate.



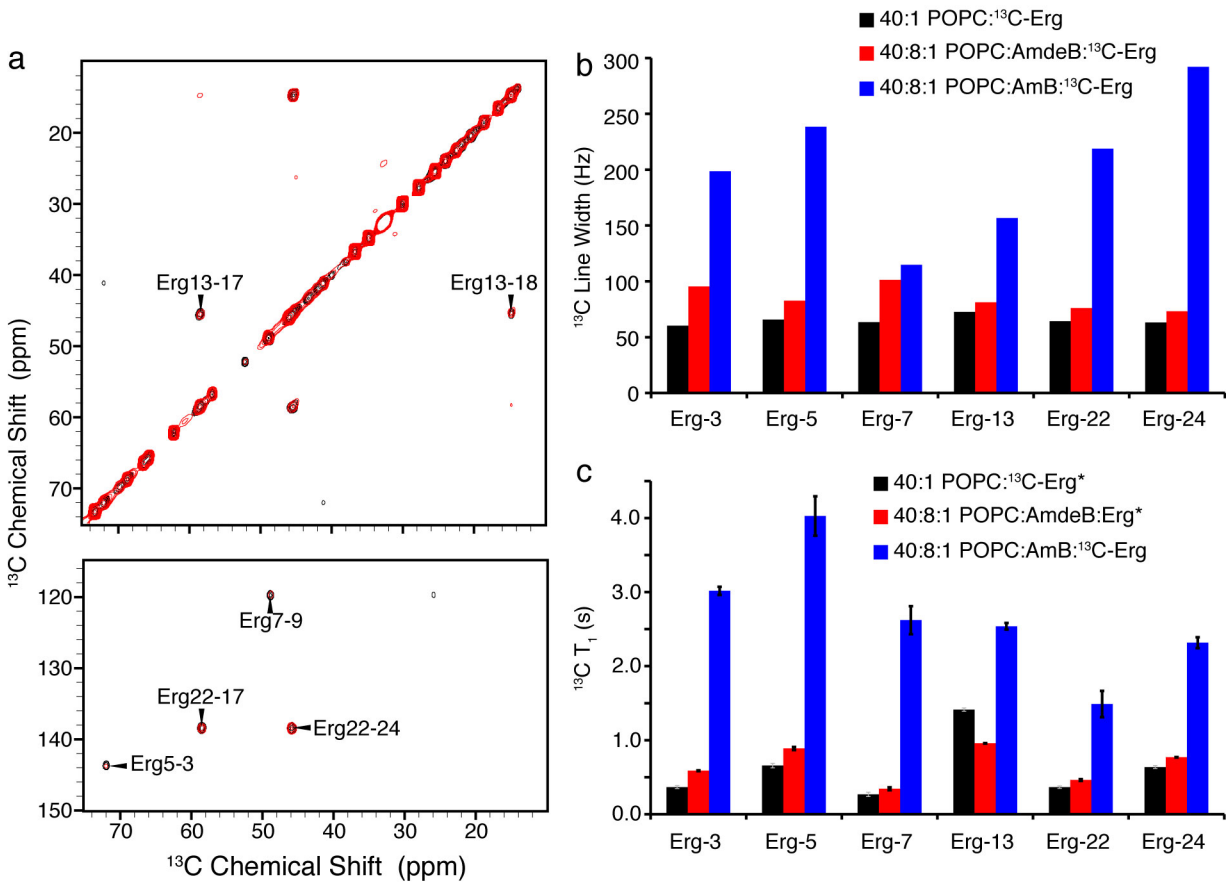

**Supplementary Figure 9:** The addition of increasing amounts of AmB to 40:1 POPC:$^{13}$C-Erg membranes induces substantial changes in the spectroscopic properties of Erg. (a) The $^{13}$C-$^{13}$C 2D DARR spectra (500 ms mixing) of POPC:$^{13}$C-Erg (black) and 40:8:1 POPC:AmB:$^{13}$C-Erg (blue) show dramatic changes in not only the postion but number of cross peaks. This coupled with the increase in (b) line width (c) $^{13}$C $T_1$ values for the bound state (denoted with a ') versus the free state indicates a large reduction in the mobility of Erg in the presence of AmB, consistent with the formation of an AmB:Erg complex. (40:1 POPC:$^{13}$C-Erg (black), 40:1:1 POPC:AmB:$^{13}$C-Erg (orange), 40:4:1 POPC:AmB:$^{13}$C-Erg (blue), and 40:8:1 POPC:AmB:$^{13}$C-Erg (red) liposomes.) Spectra were acquired on a 600 MHz frequency spectrometer, at 20 °C with an MAS rate of 10 kHz and 75 kHz $^{1}$H decoupling.[53]



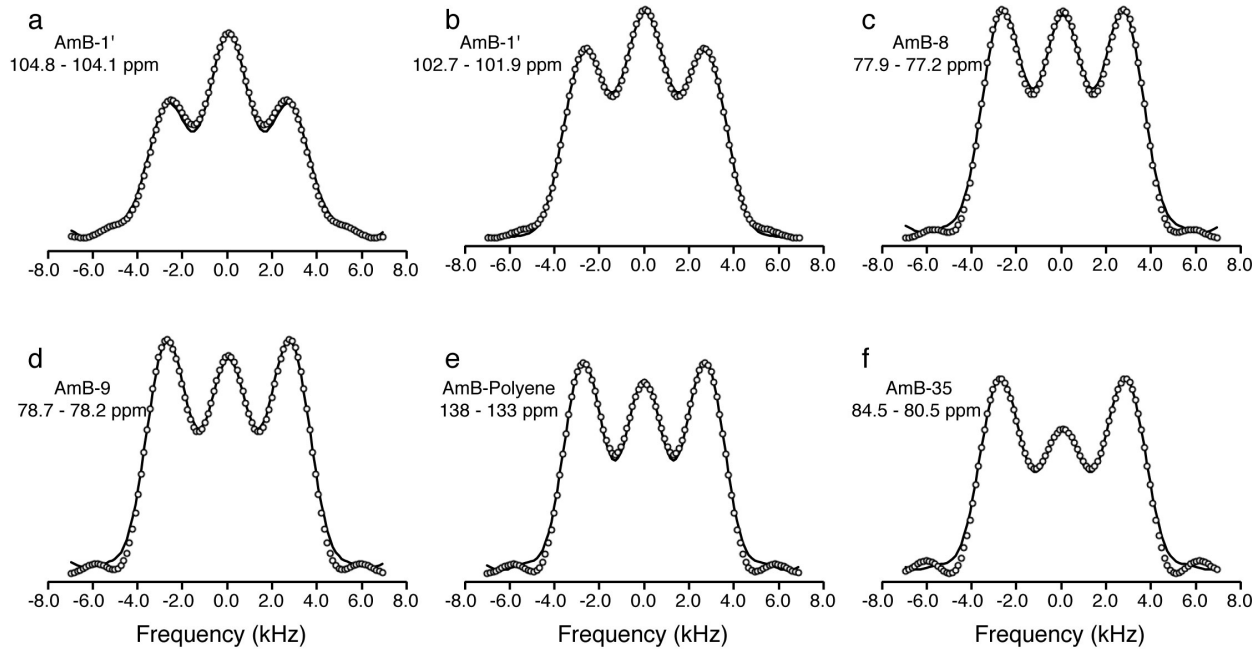

**Supplementary Figure 10:** $T_2$-filtered (1 ms) spin diffusion studies of 40:0:1 POPC:AmB:$^{13}$C-Erg MLVs show that in the absence of AmB Erg is embedded in the lipid membrane, with the C3 site on the A ring located close to the membrane water interface. The selected $^{1}$H-$^{13}$C correlations were collected with 1 ms $T_2$ filter and $^{1}$H-$^{1}$H spin diffusion times of 1 ms to 400 ms. Cross peaks from the lipid acyl chain $CH_2$ protons (~1.35 ppm) and water (~4.7 ppm) to all resolved sites of $^{13}$C-Erg are shown in red and blue, respectively. The polarization transfer is normalized based on the maximum observed intensity after correction of the $^{1}$H $T_1$ relaxation. Spectra was acquired on a 600 MHz spectrometer, at 20 °C with and MAS rate of 10 kHz, and 75 kHz SPINAL $^{1}$H decoupling.[53]



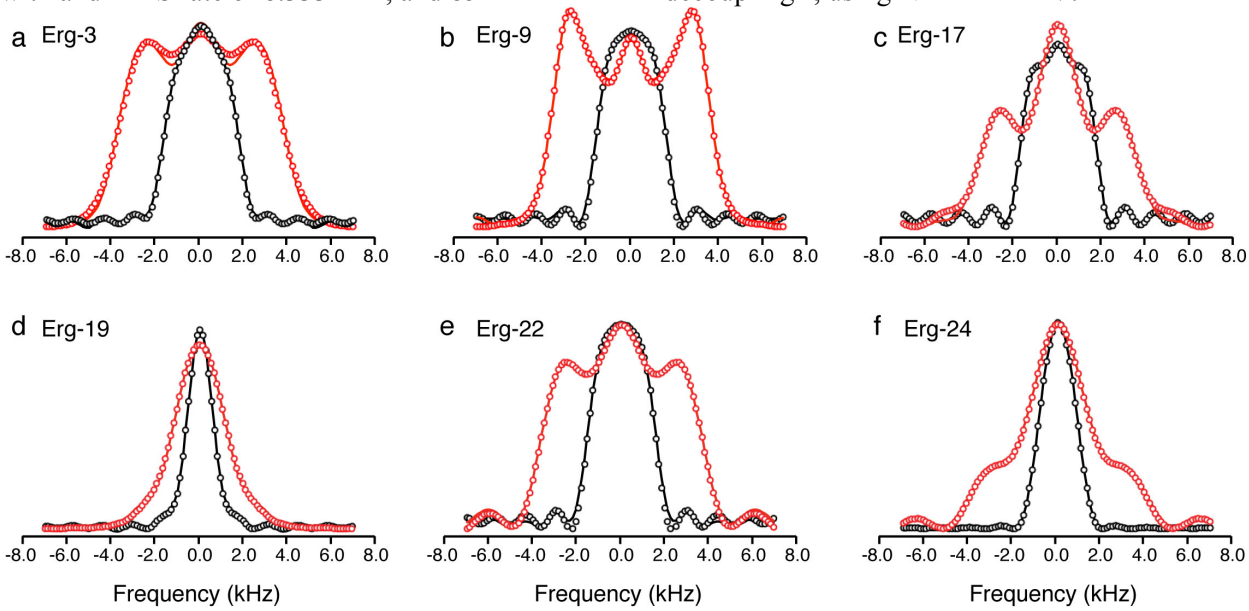

**Supplementary Figure 11:** $T_2$-filtered (1 ms) spin diffusion studies of 40:4:1 POPC:AmB:$^{13}$C-Erg MLVs show that in the presence of AmB Erg has been removed from the lipid bilayer, with the majority of Erg located more than 15-20 Å away from the lipid acyl chains. The polarization transfer from water is very similar to that observed for AmB (Supplementary Information Fig. 4, Fig. 2f), supporting the fact that the Erg has been absorbed by the AmB sterol sponge. The selected $^{1}$H-$^{13}$C correlations were collected with 1 ms $T_2$ filter and $^{1}$H-$^{1}$H spin diffusion times of 1 ms to 625 ms. Cross peaks from the lipid acyl chain $CH_2$ protons (~1.35 ppm) and water (~4.7 ppm) to all resolved sites of $^{13}$C-Erg are shown in red and blue, respectively. The polarization transfer is normalized based on the maximum observed intensity after correction of the $^{1}$H $T_1$ relaxation. Spectra was acquired on a 600 MHz spectrometer, at 20 °C with and MAS rate of 10 kHz, and 75 kHz SPINAL $^{1}$H decoupling.[53]



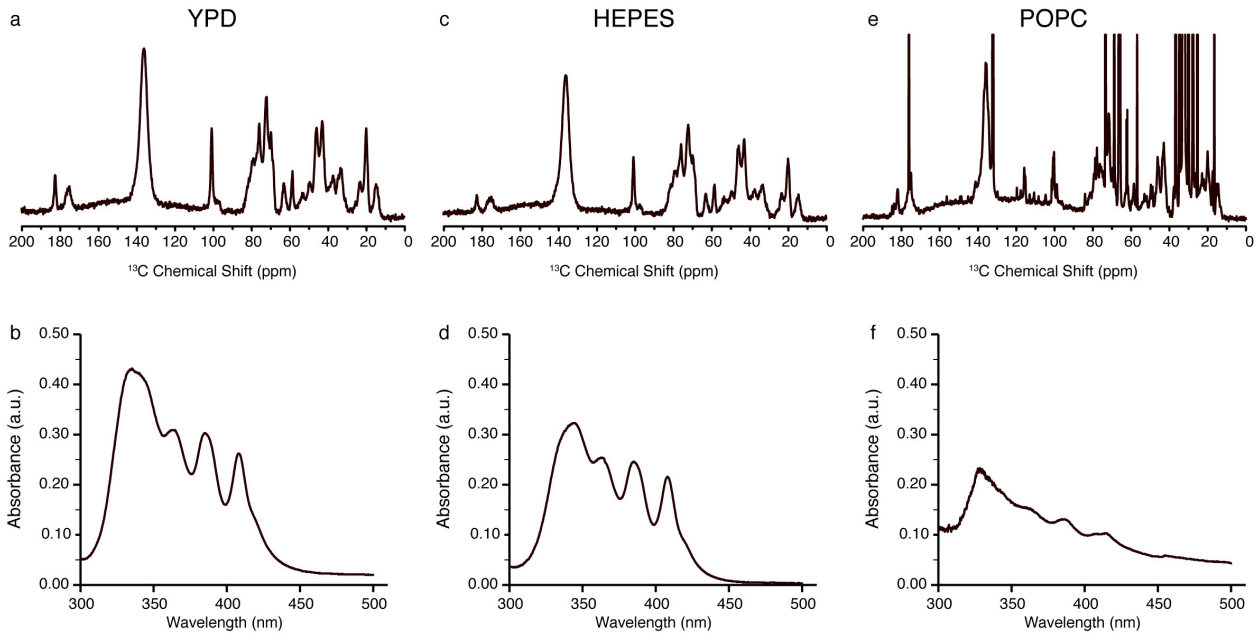

**Supplementary Figure 12:** As a control experiment we also examined the effect of AmdeB, a derivative of AmB lacking the mycosamine appendage that does not bind Erg.[25,27] There were no chemical shift perturbations observed upon the addition of AmdeB, as shown in (a) the over lay of $^{13}$C-$^{13}$C 2D DARR (500ms mixing) spectra of 40:1 POPC:$^{13}$C-Erg (black), 40:8:1 POPC:AmdeB:$^{13}$C-Erg (red). This is in stark contrast to the dramatic changes observed with the addition of AmB to POPC:$^{13}$C-Erg membranes, as shown in Supplementary Fig. 4a. Furthermore there were only slight perturbations of the (b) $^{13}$C linewidth, and (c) $^{13}$C longitudinal ($T_1$) relaxation times in comparison to those changes induced by the addition of an equivalent amount of AmB (40:8:1 POPC:AmB:$^{13}$C-Erg (blue)). Spectra were acquired on a 14.1 T (600 MHz $^{1}$H frequency) spectrometer, at 20 °C with an MAS rate of 10 kHz and 75 kHz $^{1}$H decoupling.[53]



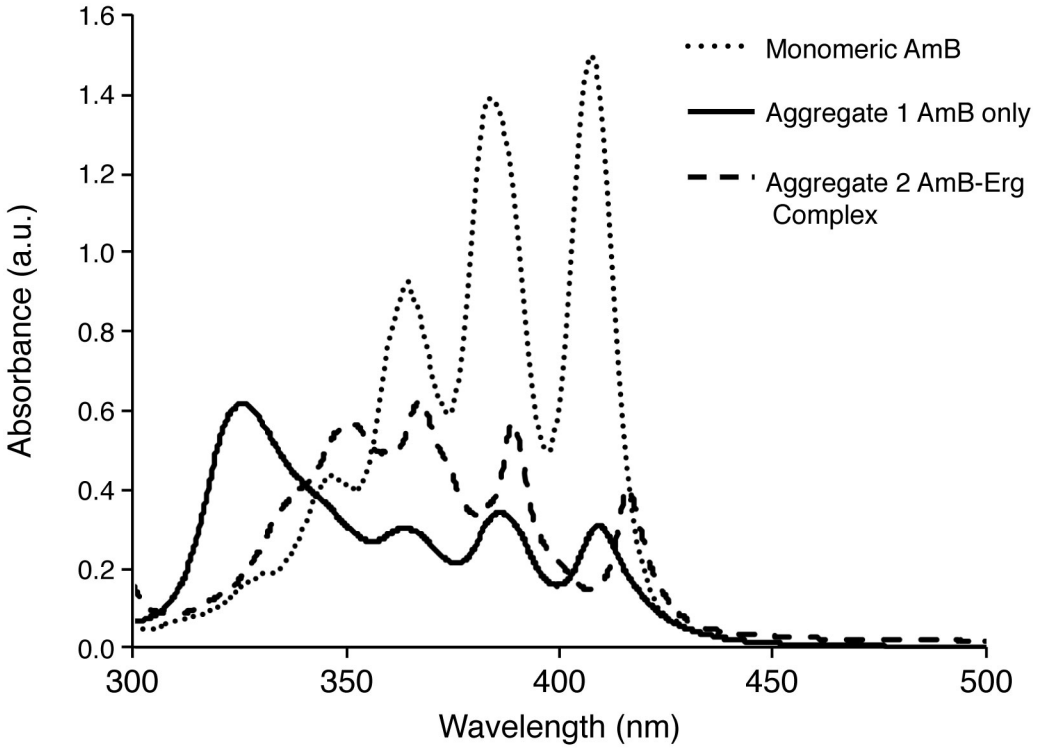

**Supplementary Figure 13:** The T-MREV dipolar lines shapes and order parameter measured select AmB resonances in 10:1:1 POPC:U-$^{13}$C-AmB:Erg MLVs (black, experimental solid line, simulation open circle) confirm that sterol sponge is rigid. Spectra was acquired on a 600 MHz spectrometer, at 20 °C with and MAS rate of 8.333 kHz, and 85 kHz TPPM $^{1}$H decoupling[59], using N=4 T-MREV.[44]




**Supplementary Figure 14:** The substantial broadening of the T-MREV dipolar lines shapes and order parameter measured for select Erg resonances in 40:0:1 (black, experimental solid line, simulation open circle) and 40:4:1 POPC:AmB:$^{13}$C-Erg (red, experimental solid line, simulation open circle) further confirms the absorption of Erg by the AmB Sterol sponge. Spectra was acquired on a 600 MHz spectrometer, at 20 °C with and MAS rate of 8.333 kHz, and 85 kHz TPPM $^{1}$H decoupling,[59] N=4 T-MREV.[44]




**Supplementary Figure 15:** $^{13}$C NMR and UV-Vis spectra of (a, b) AmB precipitated in YPD buffer used for *in vivo* studies, (c,d) AmB precipitated in HEPES buffer used for TEM studies, and (e, f) 10:1 mole ratio of POPC:AmB MLVs lyophilized and rehydrated for SSNMR studies.




**Supplementary Figure 16:** Monomeric AmB in PBS:methanol and AmB aggregate in PBS buffer demonstrate spectral signatures by UV spectroscopy. [60]

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