# Novel lobophorin congeners inhibit oral cancer cell growth and induce *Atf4*- and *Chop*- dependent cell death in murine fibroblasts

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**Cell culture and reagents.** The human oral squamous cell carcinoma (OSCC) cell lines UMSCC1 and UMSCC14A were kindly provided by Dr. Thomas Carey at the University of Michigan. Cells were maintained in DMEM containing 10% FBS and 100 g/ml Penicillin and Streptomycin (all from Invitrogen). For gene expression and proliferation analysis 12,500 cells were plated in 96 well plates the day before treatment; cultures were maintained at 37°C, 5% $CO_2$. Data are representative of three independent experiments using triplicate samples; error bars represent standard deviation.

**Quantitative real-time reverse-transcription (qRT-PCR).** Due to the small amount of some of the lobophorin congeners we employed Cells to CT™ (Applied Biosystems) for all gene expression analysis. OSCC cultures were treated with lobophorin congeners in a dose-response fashion (6.25 – 100μM) for six hours. After treatment, the culture medium was removed and the cells were washed with ice-cold PBS. 25μL of Cells to CT™ lysis reagent containing 1% DNase I was added to each well, plates were incubated for 5 minutes at room temperature, and 2.5μL of stop reagent was added. 10μL of the lysis reaction were used as a template with 12.5μL 2X RT Buffer, 1.25μL 20X RT Enzyme Mix and 1.25μL nuclease-free $H_2O$ using random hexamers to generate cDNA in a 25μL reaction volume. For TaqMan gene expression analysis, cDNA was diluted 1:50 in nuclease-free $H_2O$. TaqMan primer probes used for analysis of UPR and cell death genes: 18s, Hs99999901_s1; CHOP/DDIT3, Hs01090850_m1; GADD34, Hs00169585_m1; BIP, Hs99999174_m1; ATF3, HsYYY; ATF4, Hs00909569_g1; ATF5, HsYYY; NOXA, Hs00560402_m1; PUMA, Hs00248075_m1; BIM, Hs00197982_m1; NBK, Hs00154189_m1, BCL2, HsYYY; TRB3, HsYYY; DR5, HsYYY; ERO1α, HsYYY. Data are representative of three independent experiments using triplicate samples; GAPDH was used as an internal housekeeping control and the delta delta CT method was used to calculate fold change; error bars represent standard deviation.

**Semi-quantitative reverse-transcription PCR.** Semi-quantitative reverse transcription PCR (RT-PCR) analysis of spliced and unspliced XBP1 was performed with a single human-specific primer pair ACA CGC TTG GGA ATG GAC AC (forward) and CCA TGG GAA GAT GTT CTG GG (reverse); amplicons were visualized with a Qiaxcel (Qiagen) automated nucleic acid fragment analyzer using a high resolution cartridge on the M500 setting, a 15bp – 1kb alignment marker and a 50bp – 800bp size marker.

**Proliferation.** 12,500 OSCC cells were treated for 24 or 48 hours with 0.8 – 80μM lobophorin and the relative number of proliferating cells was determined using the luminescent Cell Titer-Glo Assay (Promega). All assays were performed at least three times using triplicate samples. Error bars represent standard deviation.

**General Experimental Procedure for Lobophorins.** HPLC separations were performed on a Beckman Coulter system (Fullerton, CA) equipped with a diode-array detector. Optical rotations were recorded on an AUTOPOL III Polarimeter. IR spectra were obtained with a PerkinElmer BX FT-IR infrared spectrometer using NaCl plates. 1H, 13C and 2D NMR spectra were recorded in CD3OD on a Varian INOVA 600 MHz NMR spectrometer at 600 MHz for 1HNMR and 150 MHz for 13C, using TMS as an internal standard.

**Isolation, Characterization and Fermentation of Strain 7790_N4.** Strain 7790_N4 was isolated from a marine sediment collected in Tempisque, Costa Rica, in 2006. After isolation, it was preserved as a spore stock in 20% glycerol at -80 °C. The strain was identified as a *Streptomyces sp.* on basis of its 16s rRNA sequence and phylogenetic analysis. Strain 7790_N4 was cultured in ISP2 medium (10 g of malt extract, 4 g of yeast extract, 4 g of dextrose and 30 g of NaCl) in 1L of medium per 2.8L Fernbach flask.

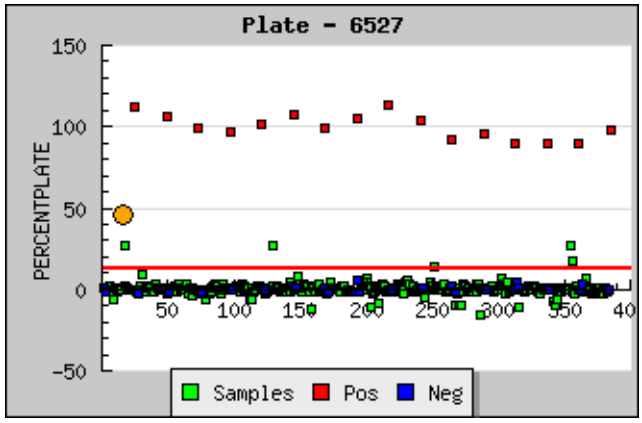
**Extraction and Isolation of Lobophorins.** After three weeks, 7790_N4 cells were harvested by centrifugation at 3000 x g and extracted with MeOH. Broth was shacked overnight with XAD-16 amberlite tea-bags (20 g/L). After that, resin was washed with distill water and later extracted with MeOH. The resulting extracts were filtered and concentrated, obtaining 0.49 g, and 1.23 g from cell and broth extract, respectively. Active extracts were subjected to bioassay guide fractionation by HPLC, using XBridge Prep C18 column (Ø=10 mm x 250 mm) with $CH_3CN/H_2O$ gradient to give: lobophorin A (**1**, 20 mg), lobophorin B (**2**, 15 mg), lobophorin CR1 (**7**, 3 mg), lobophorin E (**4**, 2 mg), lobophorin F (**3**, 1.5 mg), lobophorin CR2 (**5**, 1 mg) and lobophorin CR3 (**6**, 2 mg).

**Lobophorin CR1 (5).** White powder; $[\alpha]^{25}_D$ -83 (c 0.21, MeOH); IR (NaCl) $\nu_{max}$ 3021, 2938, 1725, 1611, 1408, 1235, 1077, 945, 830 $cm^{-1}$; HREIMS $[M+Na]^+$ m/z 1180.5993 (calcd for $C_{61}H_{91}NO_{20}Na$ 1180.6032). $^1H$ NMR (500 MHz, $CD_3OD$) and $^{13}C$ NMR (125 MHz, $CD_3OD$), see Table S1.

**Lobophorin CR2 (6).** White powder. $[\alpha]^{25}_D$ -77 (c 0.06, MeOH); IR (NaCl) $\nu_{max}$ 3452, 2934, 1725, 1599, 1357, 1080, 1022, 945, 838, 719 $cm^{-1}$; HREIMS $[M-H+2Na]^+$ m/z 1247.5697 (calcd for $C_{61}H_{89}N_2O_{22}Na_2$ 1247.5702). $^1H$ NMR (500 MHz, $CD_3OD$) and $^{13}C$ NMR (125 MHz, $CD_3OD$), see Table S2.

**Lobophorin CR3 (7).** White powder. $[\alpha]^{25}_D$ -234 (c 0.1, MeOH); IR (NaCl) $\nu_{max}$ 3475, 2934, 1725, 1641, 1408, 1238, 1074, 945, 838, 703 $cm^{-1}$; HREIMS $[M+Na]^+$ m/z 1241.5809 (calcd $C_{61}H_{90}N_2O_{23}Na$ 1241.5832). $^1H$ NMR (500 MHz, $CD_3OD$) and $^{13}C$ NMR (125 MHz, $CD_3OD$), see Table S3.

**A.** **CHO-Chop-luc**




**B.** **CHO-Xbp1-luc**

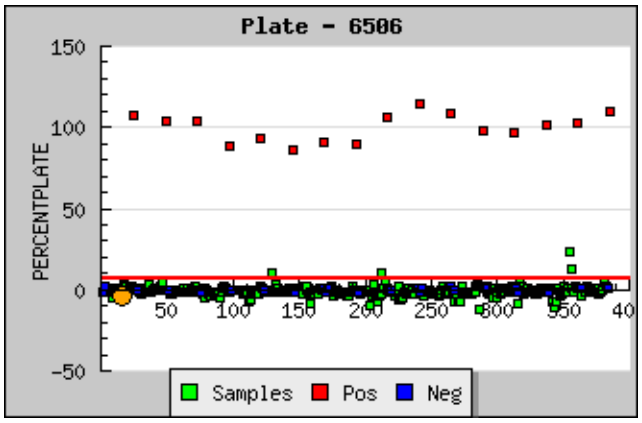



**Figure S1.** High-throughput screen in CHO-K1 cell lines stably transfected with luciferase reporters revealed a natural extract (containing Lobophorin) that robustly activated CHOP (A) and not XBP1 (B).

**Table S1.** $^{13}C$ and $^{1}H$ NMR Data for lobophorin **CR1** ($CD_3OD$, TMS, δ in ppm).

| Lobophorin CR1 | | | | | |
|---|---|---|---|---|---|
| **Carbon** | **δ $^{13}C$** | **δ $^{1}H$** | **Carbon** | **δ $^{13}C$** | **δ $^{1}H$** |
| **1** | | - | **D1** | 98.4 | 4.67 |
| **2** | 100.7 | - | **D2** | 39.3 | 1.50; 1.68 |
| **3** | 201.1 | - | **D3** | 71.5 | - |
| **4** | 52.3 | - | **D4** | 57.4 | 3.25 |
| **5** | 43.8 | 2.06 | **D5** | 67.9 | 4.08 |
| **6** | 31.2 | 1.54 | **D6** | 16.1 | 1.07 |
| **7** | 41.5 | 1.48; 1.56 | **D3-$CH_3$** | 26.9 | 1.16 |
| **8** | 34.5 | 2.19 | **D4-NH** | | - |
| **9** | 84.8 | 3.39 | **D4-C0** | 158.4 | - |
| **10** | 38.6 | 2.05 | **D4-$OCH_3$** | 51.4 | 3.63 |
| **11** | 125.5 | 5.75 | **A1** | 98.2 | 4.75 |
| **12** | 127.1 | 5.34 | **A2** | 29.5 | 1.71; 2.36 |
| **13** | 51.6 | 3.71 | **A3** | 67.9 | 3.97 |
| **14** | 136.4 | - | **A4** | 72.0 | 3.23 |
| **15** | 122.6 | 5.20 | **A5** | 64.7 | 4.08 |
| **16** | 31.0 | 2.21; 2.40 | **A6** | 16.9 | 1.19 |
| **17** | 79.1 | 4.17 | **B1** | 91.8 | 5.15 |
| **18** | 137.1 | - | **B2** | 34.5 | 1.97; 2.06 |
| **19** | 119.3 | 5.13 | **B3** | 66.5 | 4.15 |
| **20** | 40.1 | 3.48 | **B4** | 81.8 | 3.26 |
| **21** | 122.3 | 5.38 | **B5** | 62.2 | 4.04 |
| **22** | 140.4 | - | **B6** | 16.7 | 1.17 |
| **23** | 27.7 | 2.56 | **C1** | 99.2 | 4.93 |
| **24** | 34.8 | 1.69; 2.31 | **C2** | 37.3 | 1.70; 2.03 |
| **25** | 83.7 | - | **C3** | 62.9 | 4.26 |
| **26** | | - | **C4** | 82.1 | 2.83 |
| **27** | 14.1 | 1.50 | **C5** | 68.0 | 3.79 |
| **28** | 21.7 | 0.63 | **C6** | 16.9 | 1.24 |
| **29** | 13.2 | 1.11 | **C4-$OCH_3$** | 55.6 | 3.36 |
| **30** | 13.2 | 1.35 | | | |
| **31** | 13.9 | 1.39 | | | |
| **32** | 63.7 | 4.05; 4.13 | | | |
| **33** | 18.9 | 1.26 | | | |

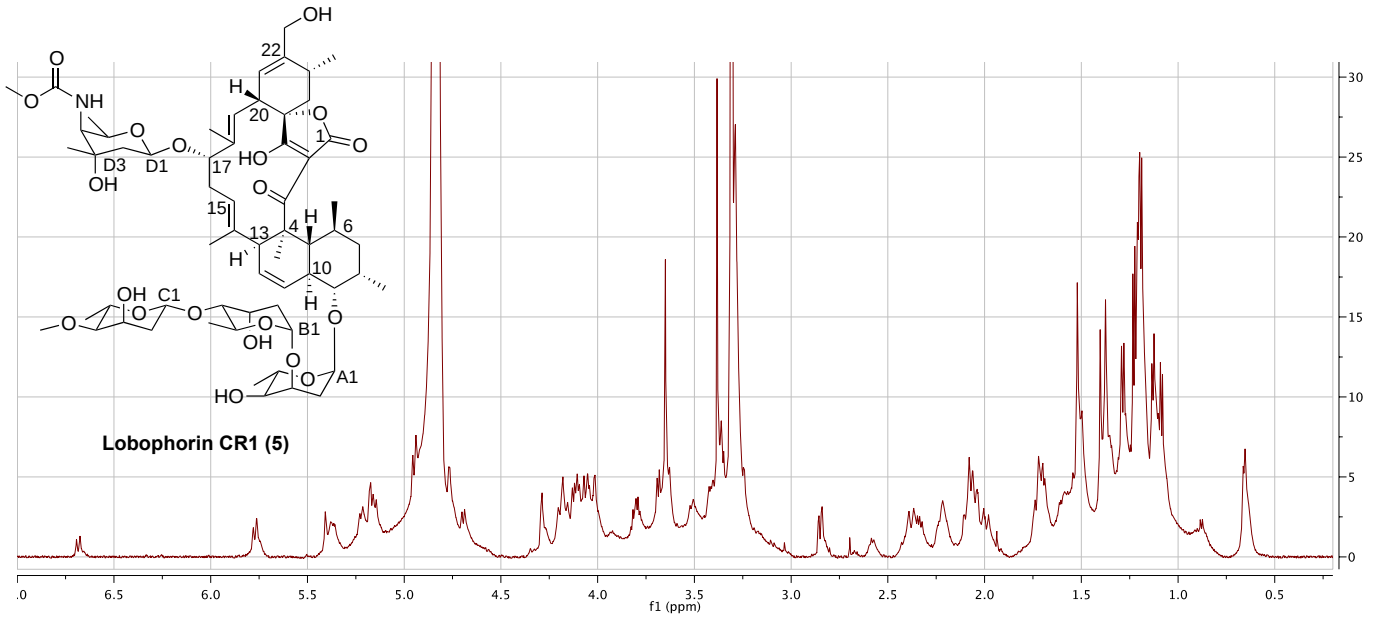



**Figure S2.** $^{1}$HNMR spectrum of lobophorin CR1 (**6**; 600 MHz, $CD_3OD$)

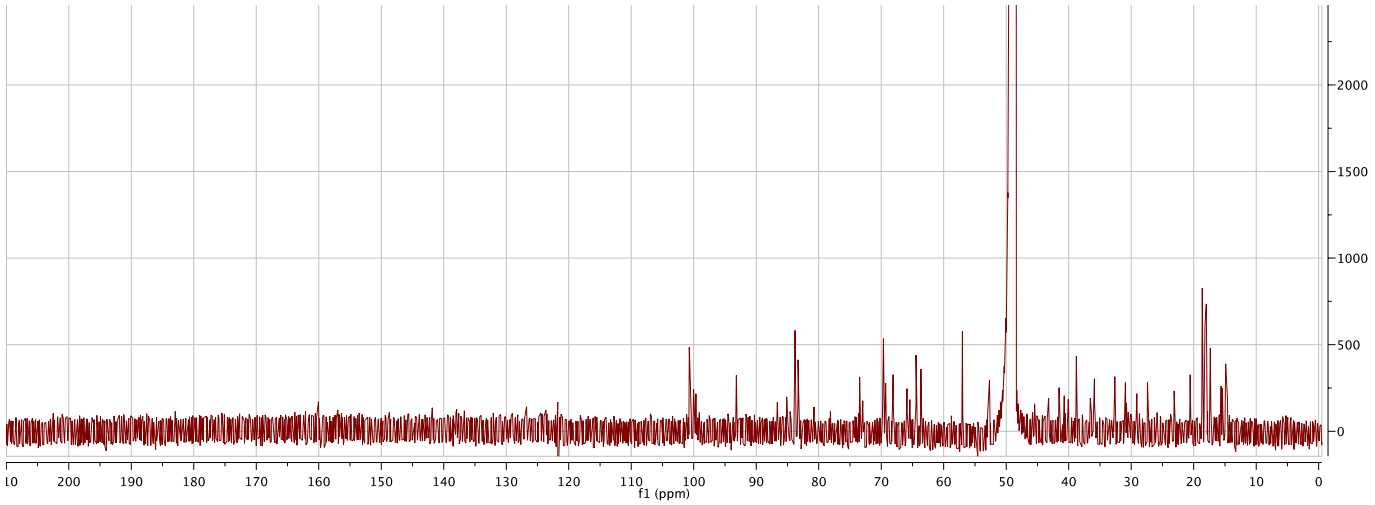



**Figure S3.** $^{13}$CNMR spectrum of lobophorin CR1 (**6**; 150 MHz, $CD_3OD$)

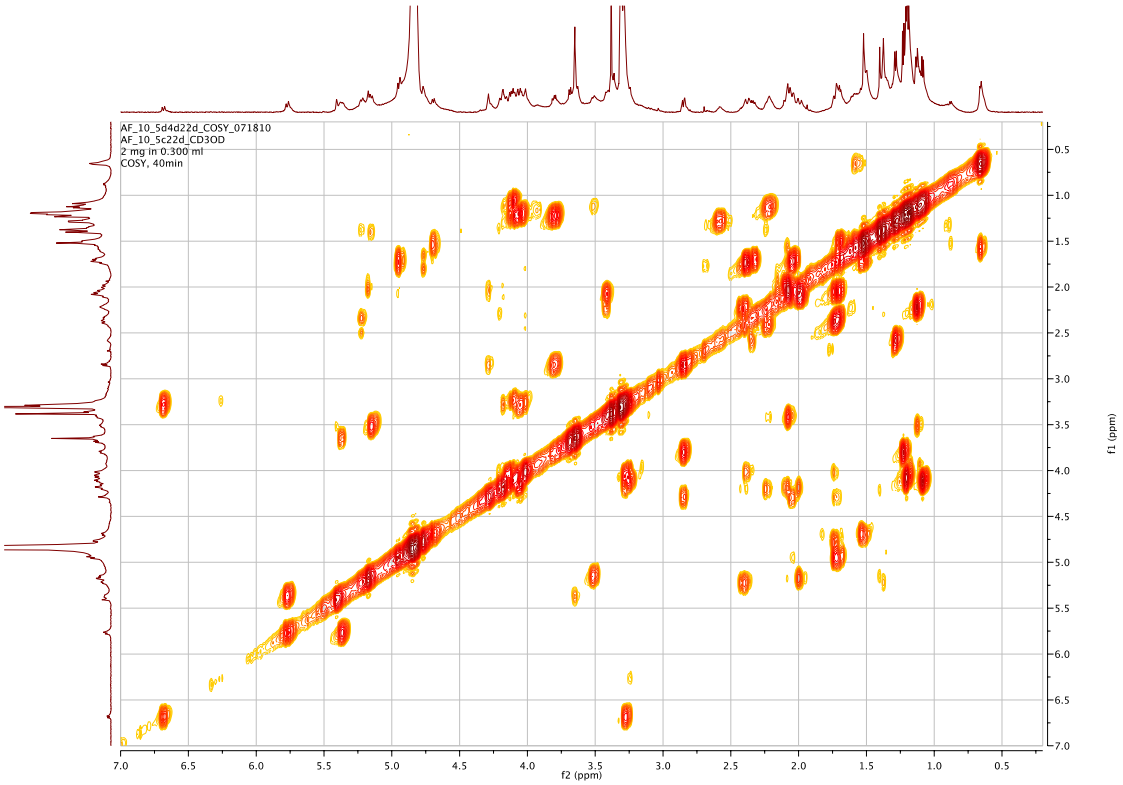



**Figure S4.** COSY spectrum of lobophorin CR1 (**6**; 600 MHz, $CD_3OD$)

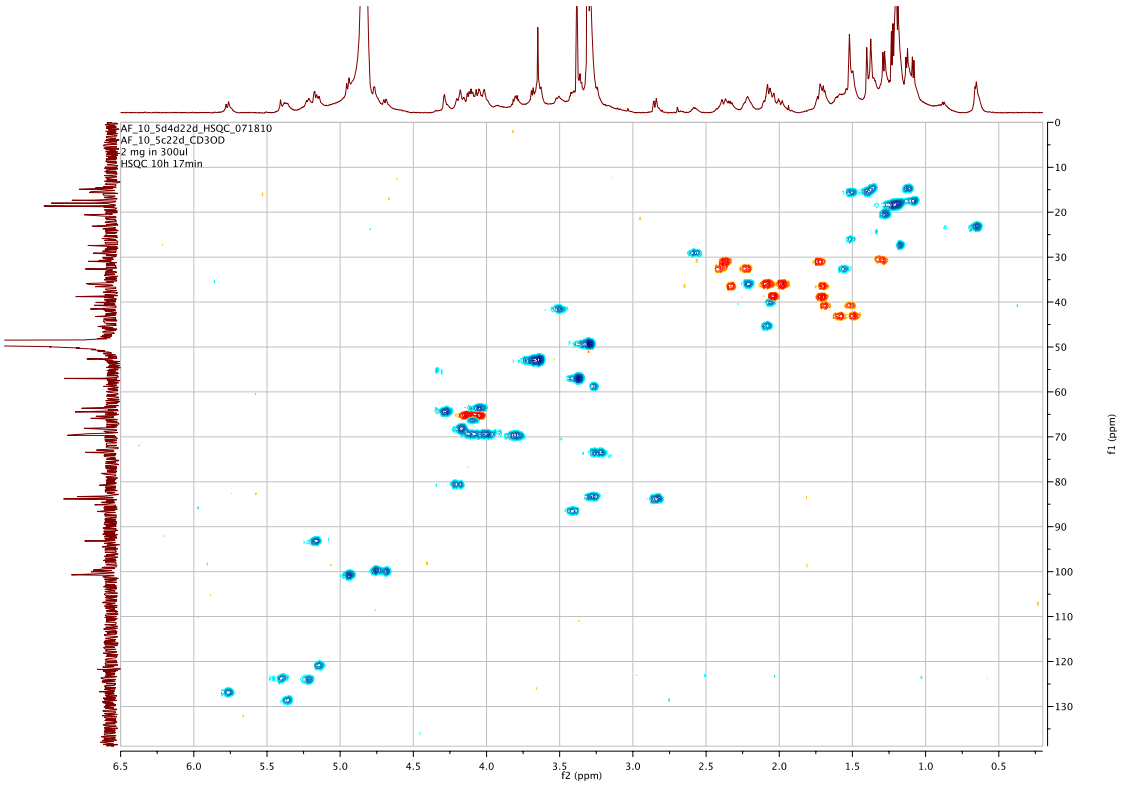



**Figure S5.** HSQC spectrum of lobophorin CR1 (**6**; 600 MHz, $CD_3OD$)

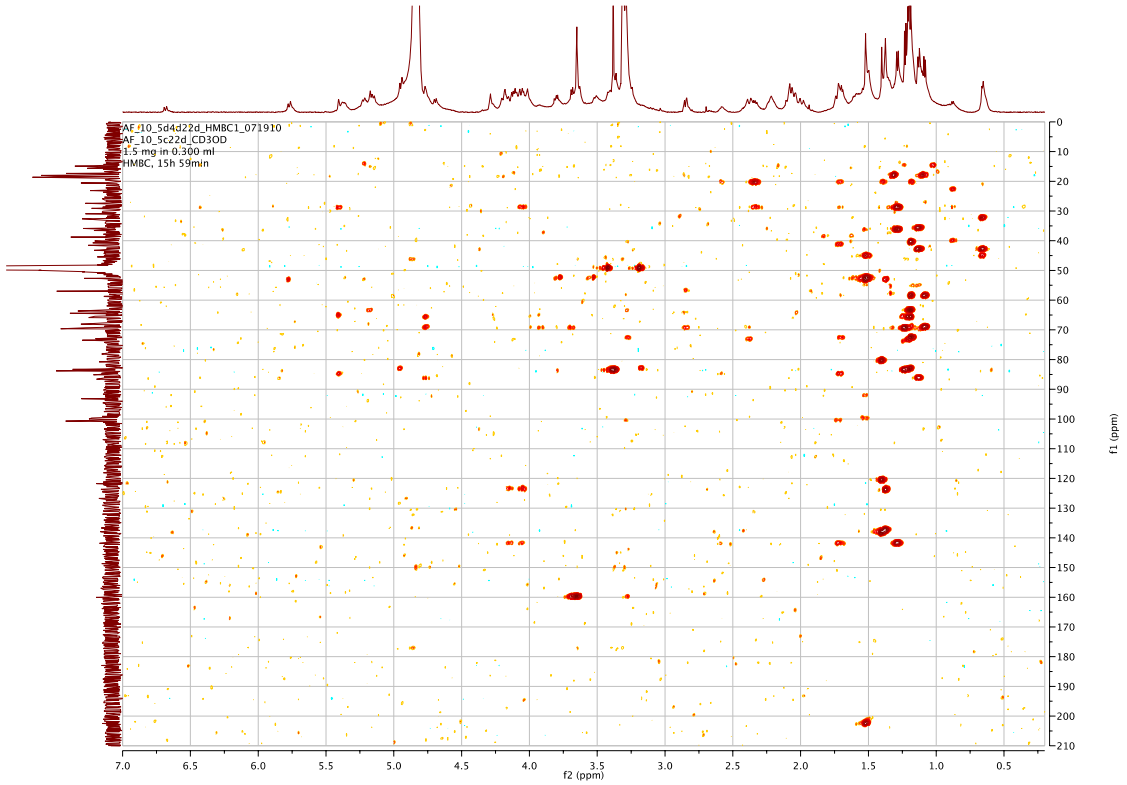



**Figure S6.** HMBC spectrum of lobophorin CR1 (**6**; 600 MHz, $CD_3OD$)

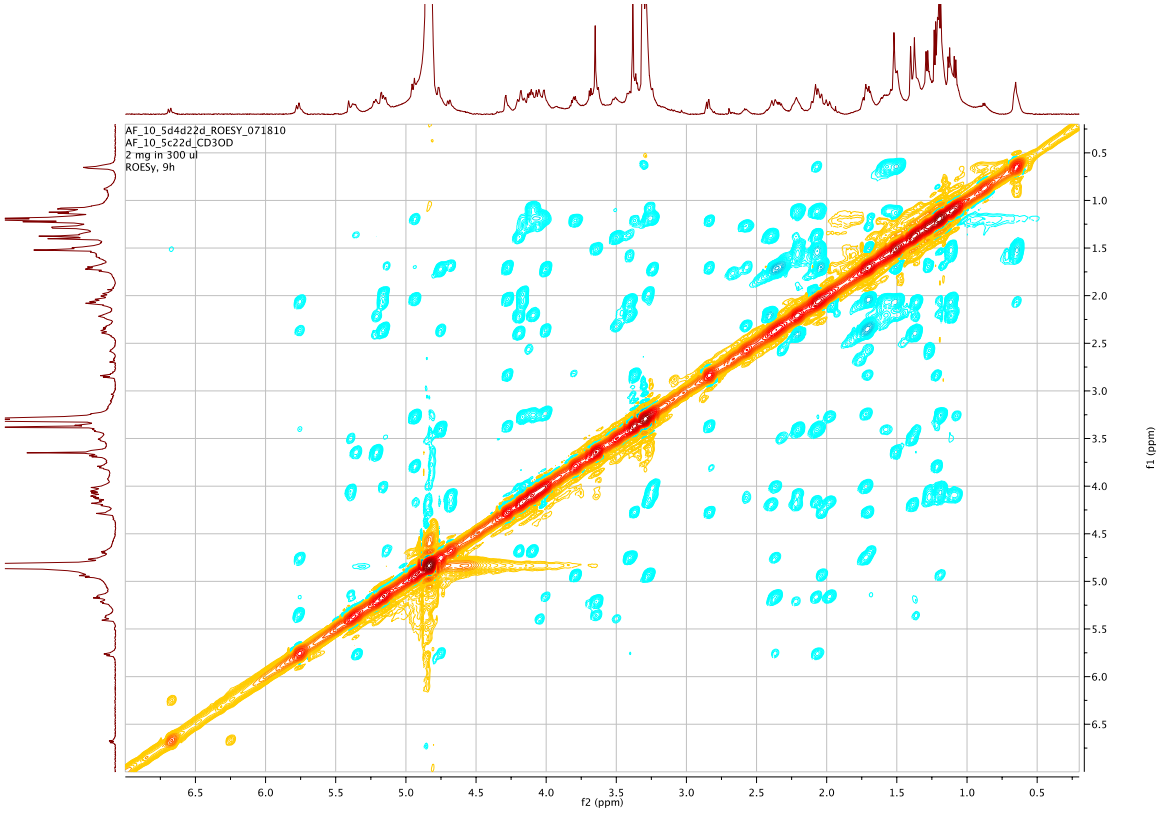



**Figure S7.** ROESY spectrum of lobophorin CR1 (**6**; 600 MHz, $CD_3OD$)

**Table S2.** $^{13}C$ and $^{1}H$ NMR Data for lobophorin **CR2** ($CD_3OD$, TMS, δ in ppm).

| Lobophorin CR2 | | | | | |
|---|---|---|---|---|---|
| **Carbon** | **δ $^{13}C$** | **δ $^{1}H$** | **Carbon** | **δ $^{13}C$** | **δ $^{1}H$** |
| **1** | | - | **D1** | 96.9 | 4.47 |
| **2** | | - | **D2** | 35.0 | 1.74; 2.67 |
| **3** | 199.2 | - | **D3** | 90.8 | - |
| **4** | 50.5 | - | **D4** | 53.6 | 4.33 |
| **5** | 42.8 | 1.86 | **D5** | 68.7 | 3.50 |
| **6** | 31.7 | 1.51 | **D6** | 16.1 | 1.13 |
| **7** | 41.8 | 1.46; 1.60 | **D3-$CH_3$** | 24.4 | 1.51 |
| **8** | 34.5 | 2.18 | **D4-NH** | | - |
| **9** | 85.3 | 3.39 | **D4-C0** | 158.5 | - |
| **10** | 39.3 | 2.02 | **D4-$OCH_3$** | 51.4 | 3.68 |
| **11** | 123.5 | 5.65 | **A1** | 98.0 | 4.76 |
| **12** | 128.8 | 5.50 | **A2** | 29.5 | 1.71; 2.37 |
| **13** | 45.4 | 3.94 | **A3** | 67.9 | 4.00 |
| **14** | | - | **A4** | 71.8 | 3.23 |
| **15** | 67.9 | 4.06 | **A5** | 64.5 | 4.10 |
| **16** | 34.0 | 1.80; 1.91 | **A6** | 16.6 | 1.19 |
| **17** | 80.1 | 4.41 | **B1** | 91.8 | 5.17 |
| **18** | 135.9 | - | **B2** | 34.4 | 1.99; 2.09 |
| **19** | 121.5 | 5.22 | **B3** | 66.7 | 4.16 |
| **20** | 41.0 | 3.50 | **B4** | 81.8 | 3.26 |
| **21** | 124.0 | 5.51 | **B5** | 62.0 | 4.03 |
| **22** | 140.5 | - | **B6** | 16.6 | 1.18 |
| **23** | 27.4 | 2.65 | **C1** | 99.0 | 4.94 |
| **24** | 35.0 | 1.67; 2.35 | **C2** | 37.3 | 1.70; 2.03 |
| **25** | | | **C3** | 62.9 | 4.28 |
| **26** | | | **C4** | 82.1 | 2.83 |
| **27** | 14.7 | 1.53 | **C5** | 68.2 | 3.79 |
| **28** | 22.2 | 0.63 | **C6** | 16.9 | 1.23 |
| **29** | 13.2 | 1.11 | **C4-$OCH_3$** | 55.6 | 3.38 |
| **30** | 113.8 | 4.78; 5.10 | | | |
| **31** | 14.7 | 1.54 | | | |
| **32** | 64.2 | 4.02; 4.18 | | | |
| **33** | 18.9 | 1.30 | | | |

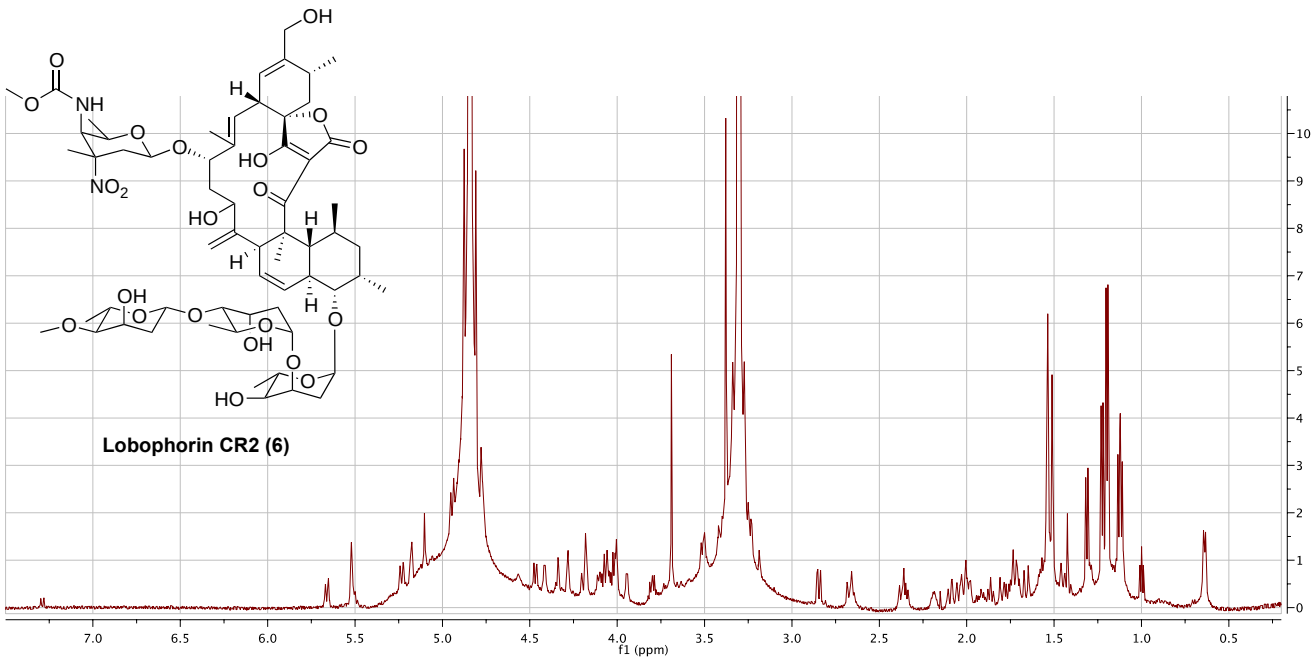



**Figure S8.** $^{1}$HNMR spectrum of lobophorin CR2 (600 MHz, $CD_3OD$)

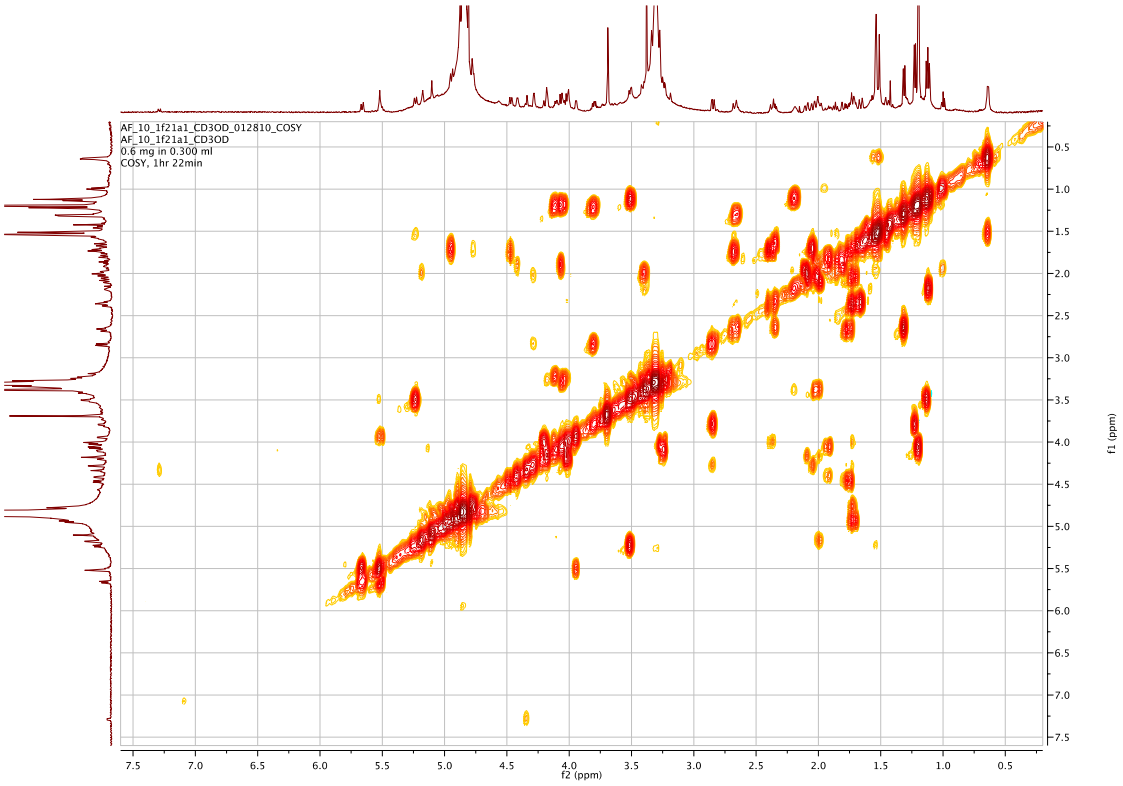



**Figure S9.** COSY spectrum of lobophorin CR2 (600 MHz, $CD_3OD$)

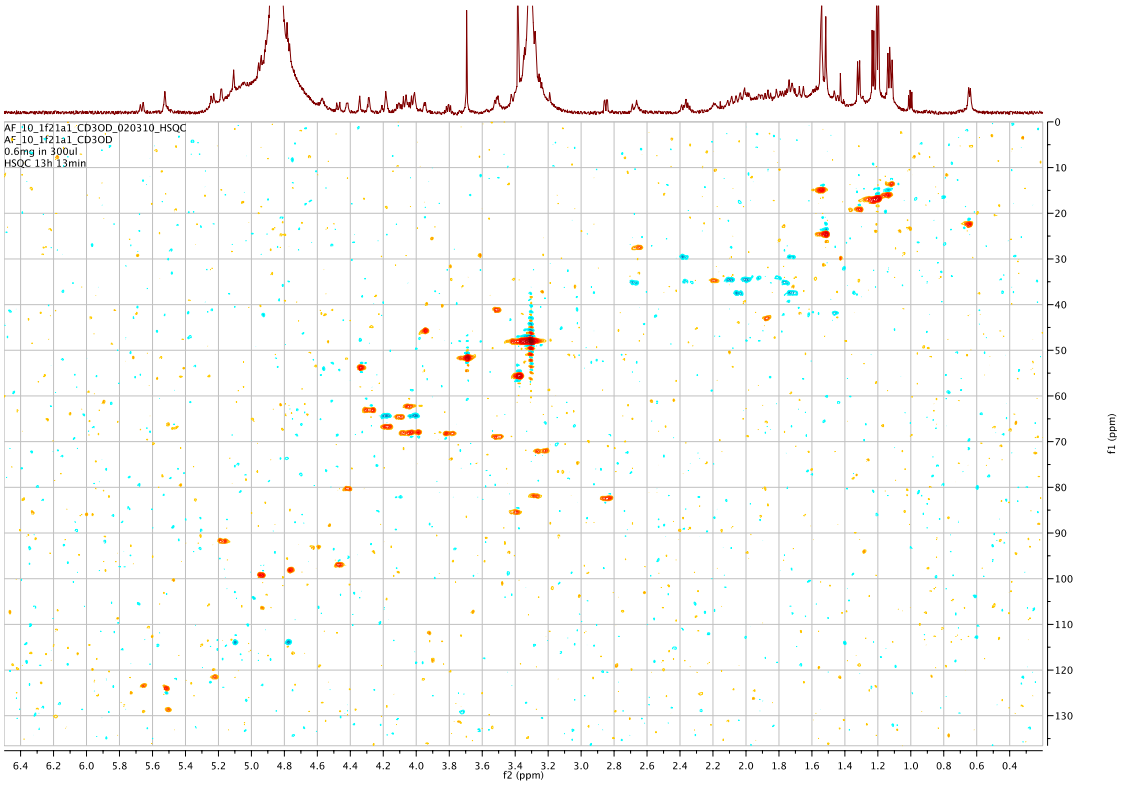



**Figure S10.** HSQC spectrum of lobophorin CR2 (600 MHz, $CD_3OD$)

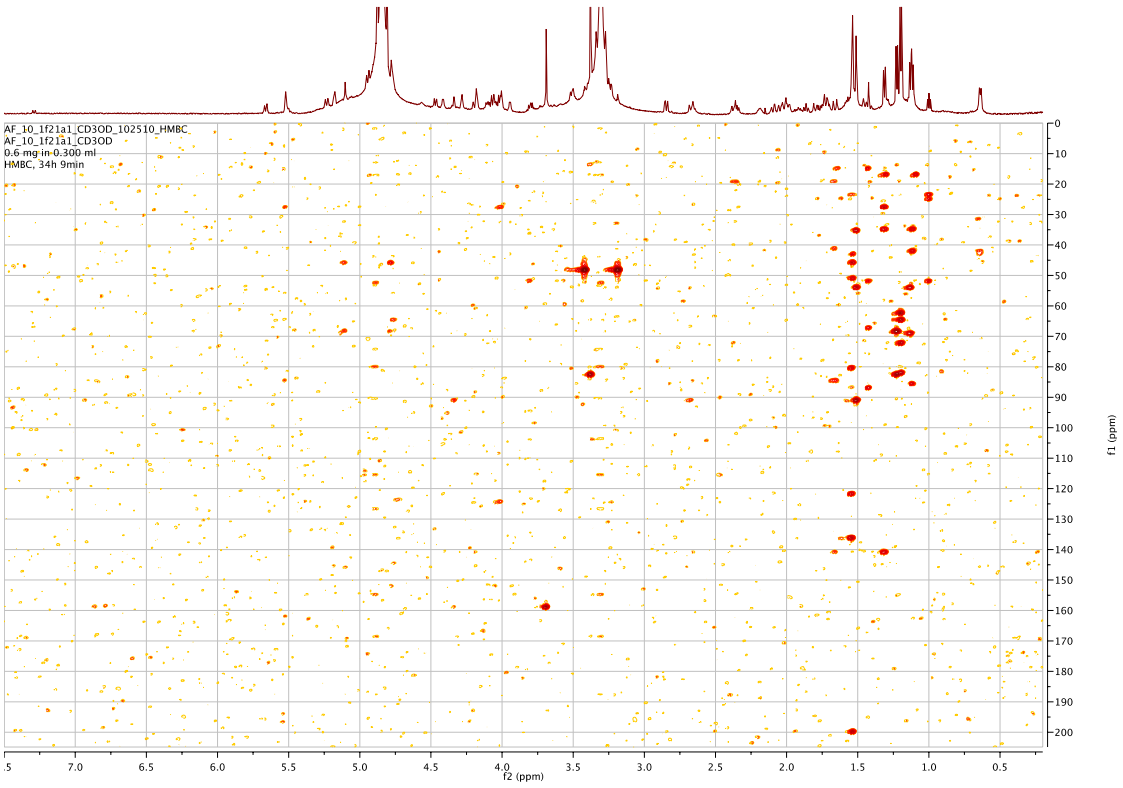



**Figure S11.** HMBC spectrum of lobophorin CR2 (600 MHz, $CD_3OD$)

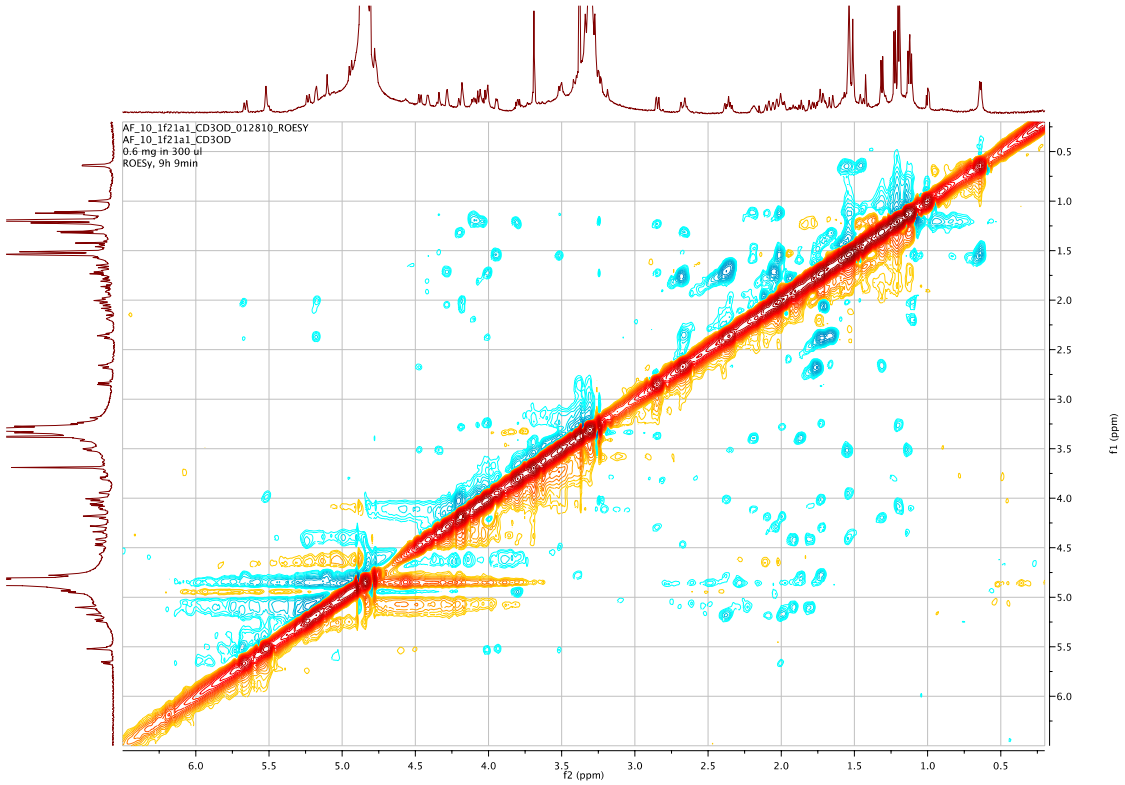



**Figure S12.** ROESY spectrum of lobophorin CR2 (600 MHz, $CD_3OD$)

**Table S3.** $^{13}C$ and $^{1}H$ NMR Data for lobophorin **CR3** ($CD_3OD$, TMS, δ in ppm).

| Lobophorin CR3 | | | | | |
|---|---|---|---|---|---|
| **Carbon** | **δ $^{13}C$** | **δ $^{1}H$** | **Carbon** | **δ $^{13}C$** | **δ $^{1}H$** |
| **1** | 165.6 | - | **D1** | 97.1 | 4.51 |
| **2** | 100.7 | - | **D2** | 34.8 | 1.82; 2.58 |
| **3** | 199.9 | - | **D3** | 90.7 | - |
| **4** | 50.6 | - | **D4** | 53.6 | 4.31 |
| **5** | 42.8 | 1.86 | **D5** | 68.5 | 3.49 |
| **6** | 31.3 | 1.51 | **D6** | 15.9 | 1.12 |
| **7** | 41.8 | 1.45; 1.59 | **D3-$CH_3$** | 24.4 | 1.50 |
| **8** | 34.5 | 2.18 | **D4-NH** | | - |
| **9** | 85.2 | 3.39 | **D4-C0** | 158.7 | - |
| **10** | 39.1 | 2.00 | **D4-$OCH_3$** | 51.6 | 3.68 |
| **11** | 123.1 | 5.66 | **A1** | 98.1 | 4.77 |
| **12** | 128.6 | 5.57 | **A2** | 29.5 | 1.72; 2.37 |
| **13** | 45.4 | 3.98 | **A3** | 67.7 | 3.99 |
| **14** | 148.7 | - | **A4** | 71.8 | 3.23 |
| **15** | 81.0 | 4.22 | **A5** | 64.4 | 4.10 |
| **16** | 29.8 | 1.71; 2.32 | **A6** | 16.7 | 1.19 |
| **17** | 80.0 | 4.30 | **B1** | 91.6 | 5.17 |
| **18** | 136.4 | - | **B2** | 34.5 | 1.99; 2.08 |
| **19** | 121.8 | 5.21 | **B3** | 66.5 | 4.16 |
| **20** | 40.6 | 3.49 | **B4** | 81.8 | 3.27 |
| **21** | 123.8 | 5.51 | **B5** | 62.2 | 4.05 |
| **22** | 140.7 | - | **B6** | 16.6 | 1.18 |
| **23** | 27.5 | 2.62 | **C1** | 99.2 | 4.94 |
| **24** | 34.7 | 1.63; 2.34 | **C2** | 37.3 | 1.70; 2.03 |
| **25** | 84.1 | - | **C3** | 62.9 | 4.27 |
| **26** | 201.1 | - | **C4** | 82.3 | 2.84 |
| **27** | 14.6 | 1.52 | **C5** | 67.9 | 3.79 |
| **28** | 21.9 | 0.64 | **C6** | 16.9 | 1.23 |
| **29** | 13.4 | 1.10 | **C4-$OCH_3$** | 55.6 | 3.37 |
| **30** | 116.0 | 4.86; 5.10 | | | |
| **31** | 15.1 | 1.56 | | | |
| **32** | 64.0 | 4.00; 4.18 | | | |
| **33** | 18.9 | 1.29 | | | |

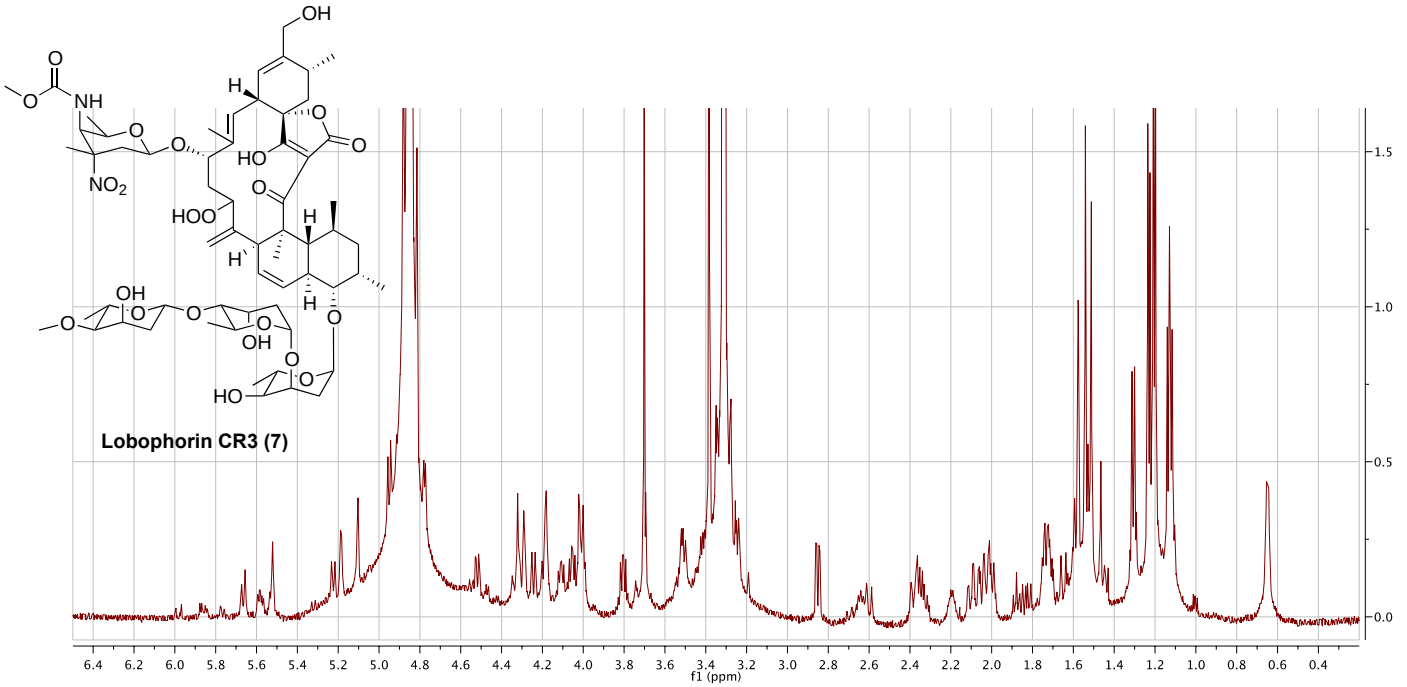



**Figure S13.** $^{1}$HNMR spectrum of lobophorin CR3 (600 MHz, $CD_3OD$)

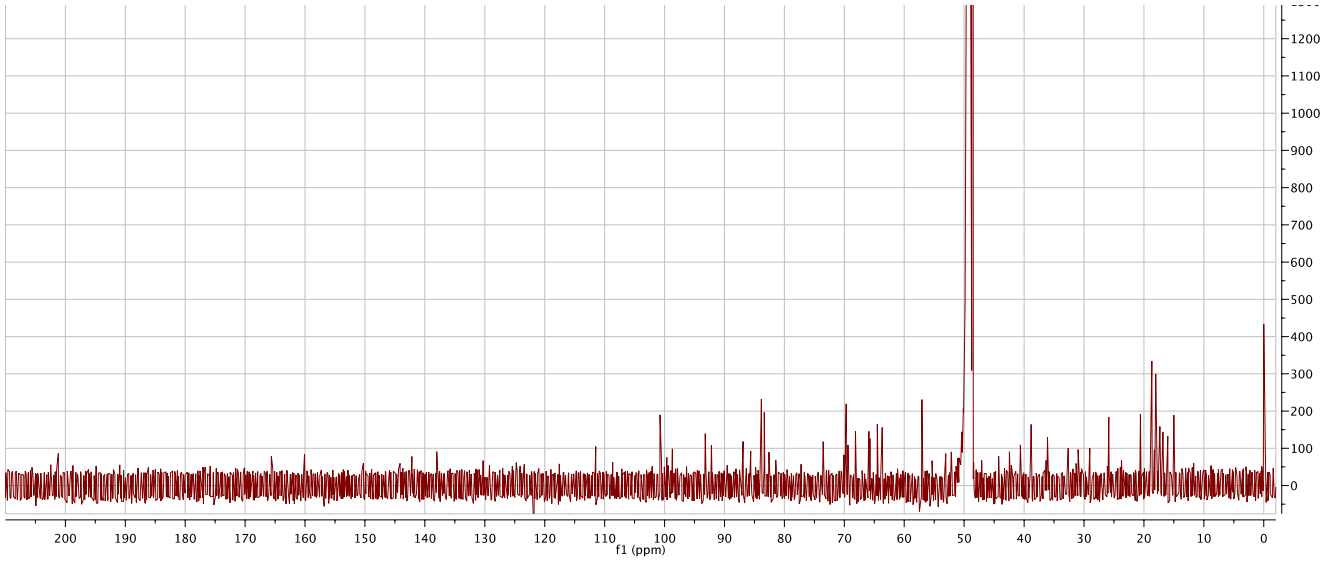



**Figure S14.** $^{13}$CNMR spectrum of lobophorin C3 (150 MHz, $CD_3OD$)

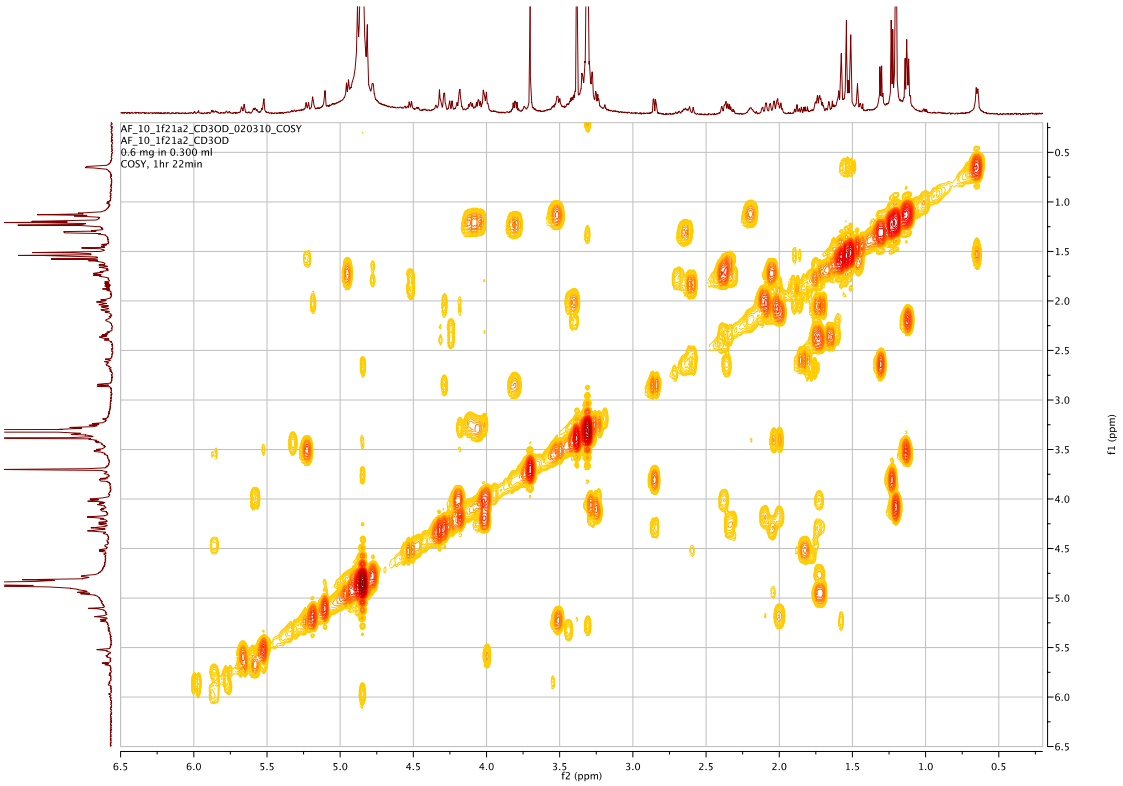



**Figure S15.** COSY spectrum of lobophorin CR3 (600 MHz, $CD_3OD$)

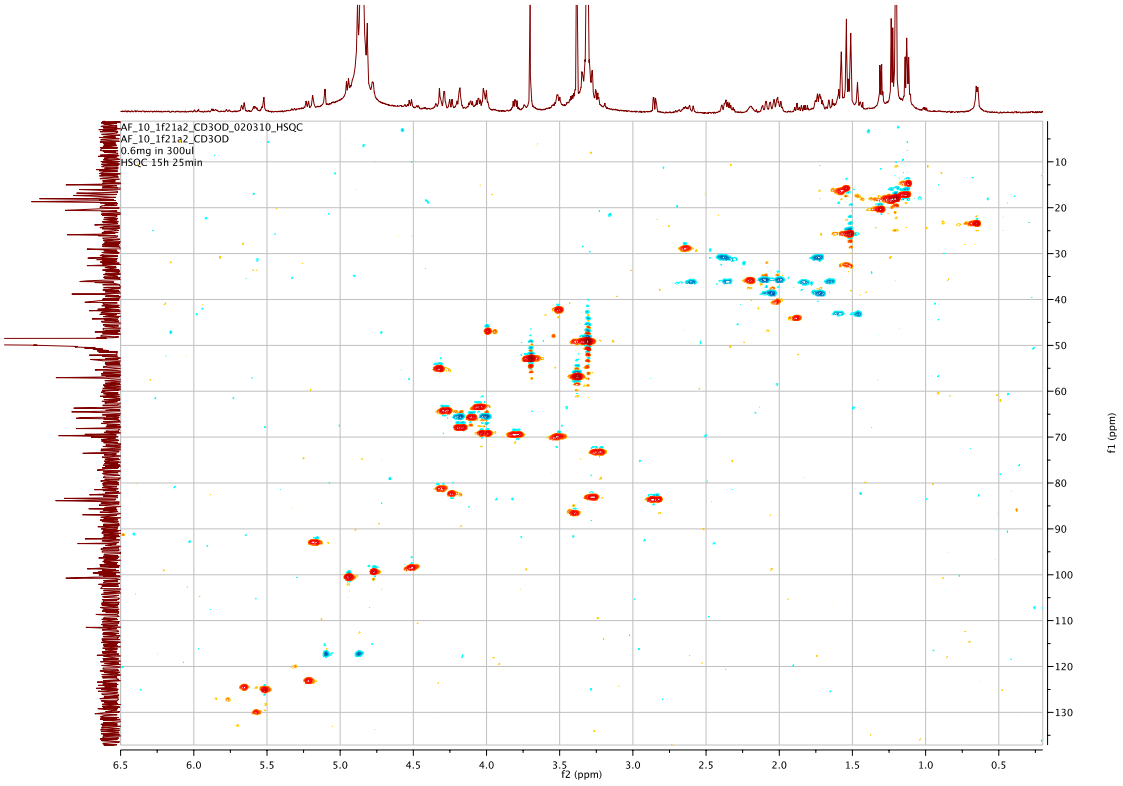



**Figure S16.** HSQC spectrum of lobophorin CR3 (600 MHz, $CD_3OD$)

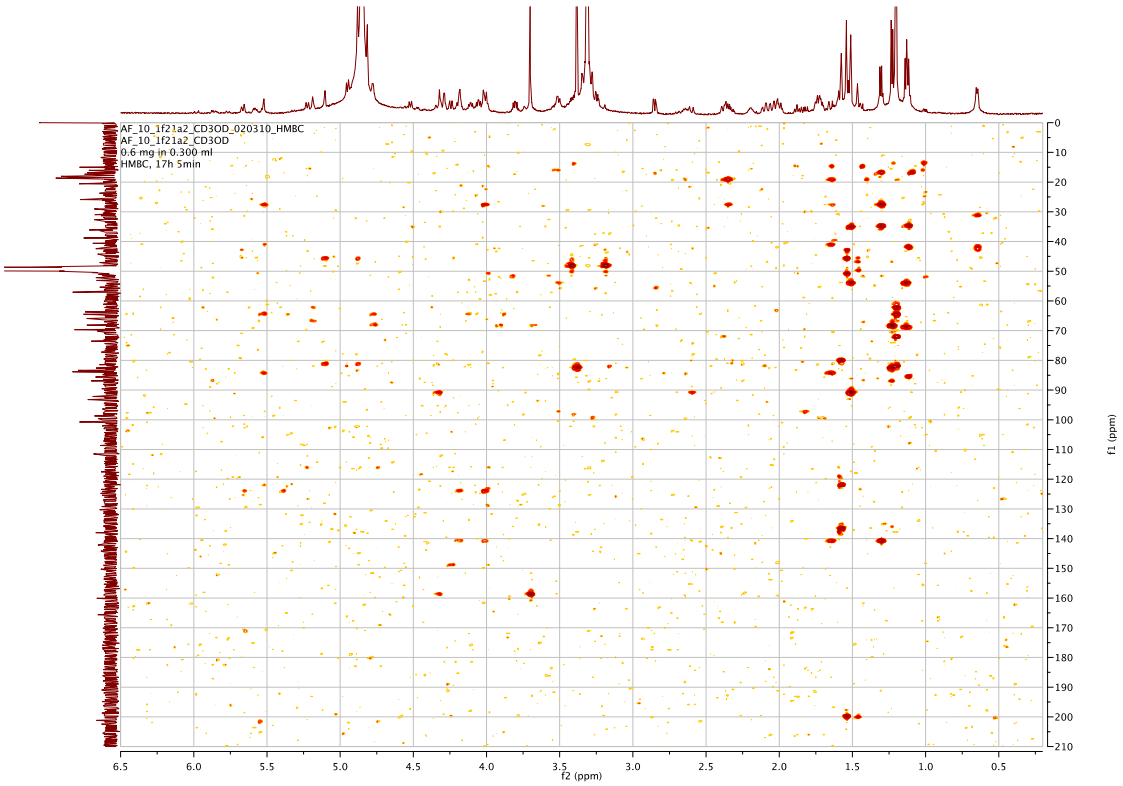



**Figure S17.** HMBC spectrum of lobophorin CR3 (600 MHz, $CD_3OD$)

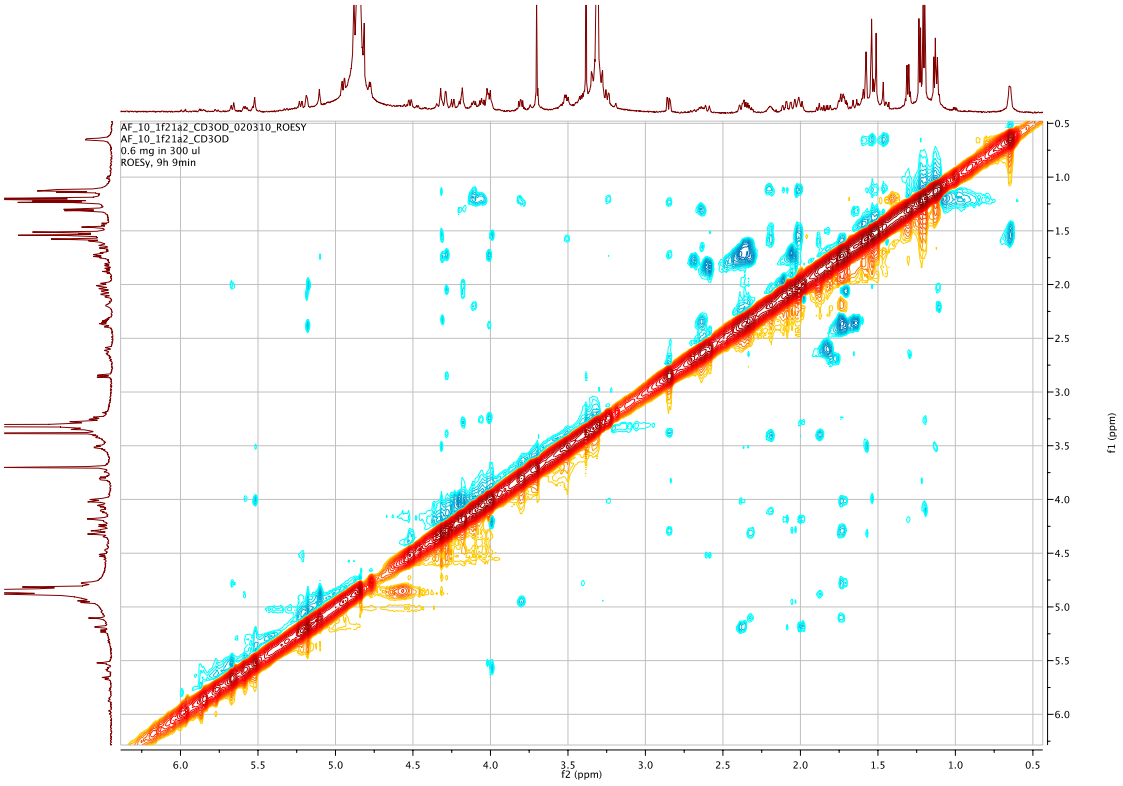



**Figure S18.** ROESY spectrum of lobophorin CR3 (600 MHz, $CD_3OD$)

**Table S4a.** Calculated ic50 values for OSCC and normal cells.

| | | ic50 (µM) | |
|---|---|---|---|
| | | **24h** | **48h** |
| **Lob. A** | **nHEK** | > 80.0 | > 80.0 |
| | **SCC-1** | > 80.0 | > 80.0 |
| | **SCC-14A** | > 80.0 | > 80.0 |
| **Lob. B** | **nHEK** | > 80.0 | 63.3 |
| | **SCC-1** | 79.5 | 44.9 |
| | **SCC-14A** | 68.6 | 44.8 |
| **Lob. CR-1** | **nHEK** | > 80.0 | > 80.0 |
| | **SCC-1** | > 80.0 | > 80.0 |
| | **SCC-14A** | > 80.0 | 66.1 |
| **Lob. E** | **nHEK** | > 80.0 | > 80.0 |
| | **SCC-1** | > 80.0 | 78.8 |
| | **SCC-14A** | > 80.0 | 74.3 |
| **Lob. F** | **nHEK** | > 80.0 | 41.0 |
| | **SCC-1** | 56.6 | 35.7 |
| | **SCC-14A** | 59.1 | 32.9 |

**Table S4b.** Two-way ANOVA for OSCC vs. nHEK.

| | | | **24h** | **48h** |
|---|---|---|---|---|
| **Lob. A** | **SCC-1** | *Interaction* | < 0.0001 | 0.0011 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| | **SCC-14A** | *Interaction* | 0.0014 | 0.0513 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| **Lob. B** | **SCC-1** | *Interaction* | < 0.0001 | 0.001 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| | **SCC-14A** | *Interaction* | < 0.0001 | < 0.0001 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| **Lob. CR-1** | **SCC-1** | *Interaction* | < 0.0001 | 0.0008 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| | **SCC-14A** | *Interaction* | < 0.0001 | < 0.0001 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| **Lob. E** | **SCC-1** | *Interaction* | < 0.0001 | < 0.0001 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| | **SCC-14A** | *Interaction* | < 0.0001 | 0.0004 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| **Lob. F** | **SCC-1** | *Interaction* | 0.0001 | 0.0045 |
| | | *Dose* | < 0.0001 | < 0.0001 |
| | **SCC-14A** | *Interaction* | 0.0037 | < 0.0001 |
| | | *Dose* | < 0.0001 | < 0.0001 |

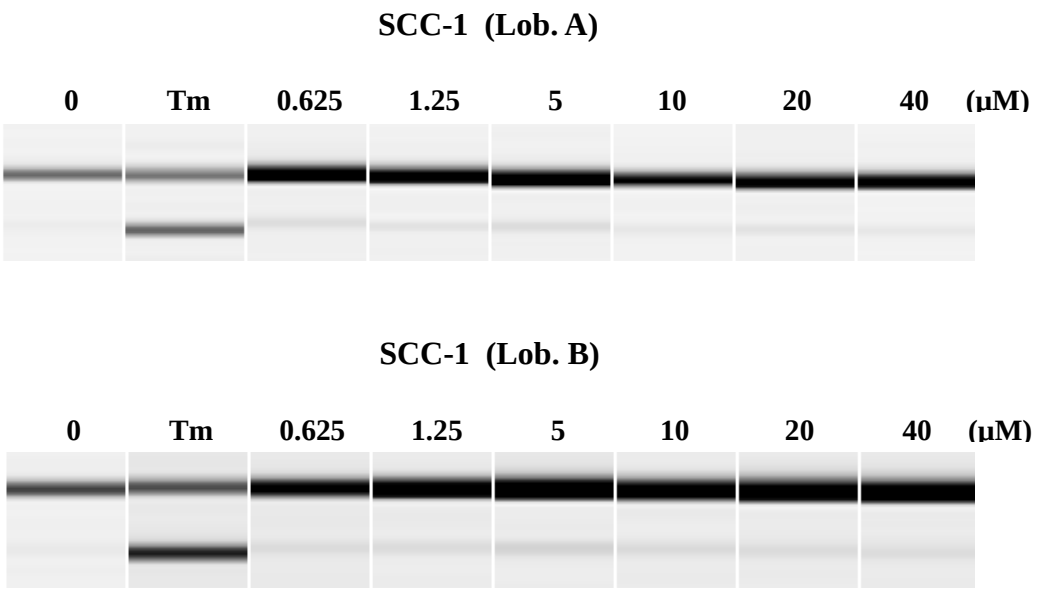



**Figure S19.** RT-PCR analysis of XBP1 slicing in UMSCC-1 cells treated in dose response with Lobophorin A and B.

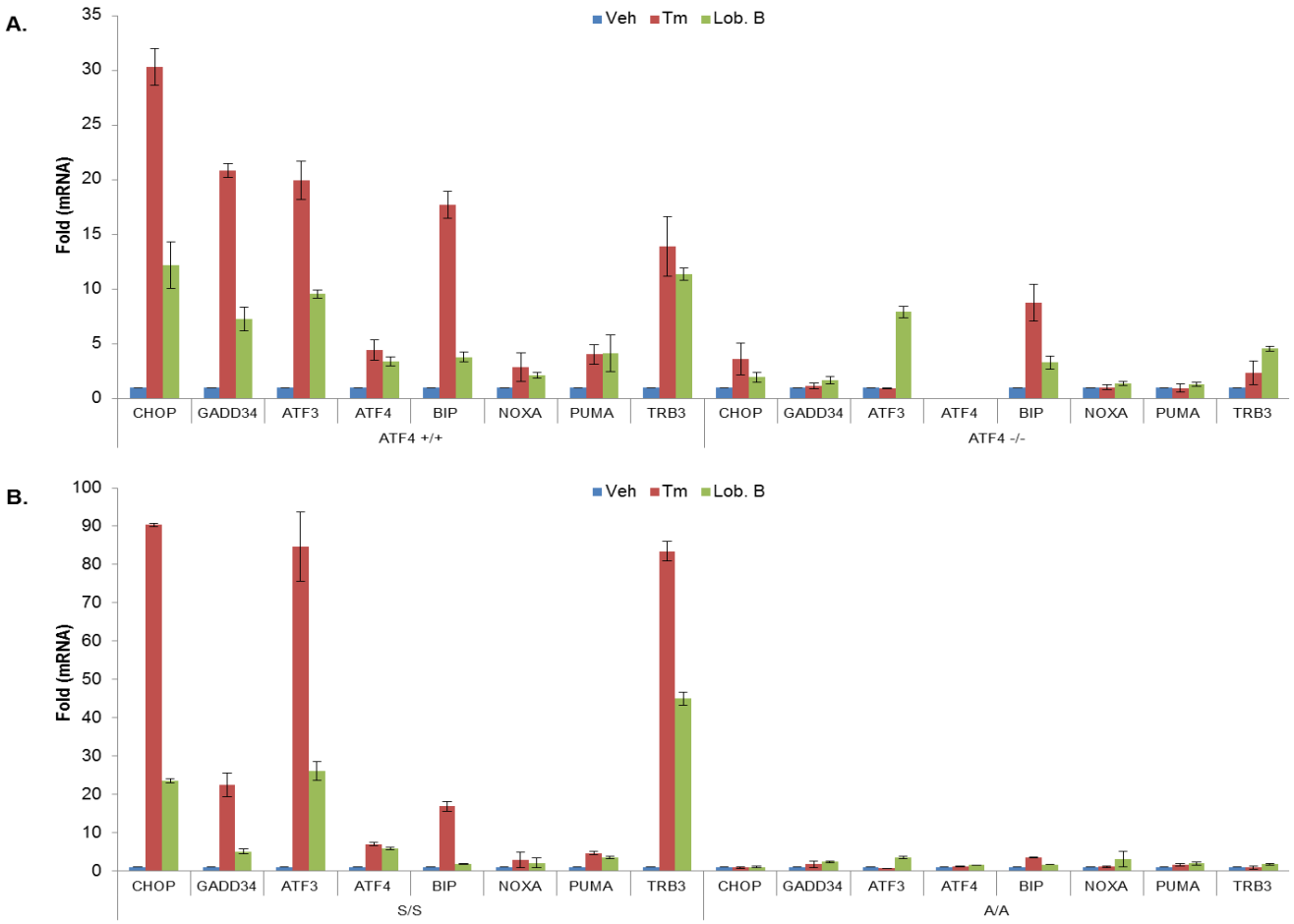



**Figure S20.** qRT-PCR analysis of Atf4 wt and Atf4 -/- murine fibroblasts (A) and s/s and a/a murine fibroblasts (B) following 6 hours treatment with 80µM lobophorin B. Error bars represent standard deviations for technical replicates.