Electronic Supplementary Material (ESI) for Analyst.

Supplementary Information

Squaraine probes for bimodal staining of lipid droplets and endoplasmic reticulum imaging in live cell

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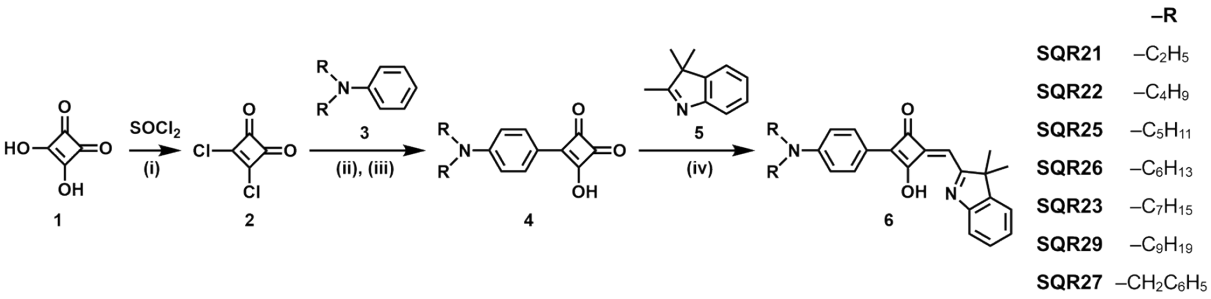
Materials and Methods

Chemicals

All reagents and HPLC grade solvents were purchased from Sigma-Aldrich, Alfa Aesar, and other commercial suppliers and used without further purification. *N*,*N*-dipentylaniline, *N*,*N*-dihexylaniline, *N*,*N*-diheptylaniline, and *N*,*N*-dinonylaniline were prepared based on previously reported procedure.[31] *N*,*N*-dimethylaniline, *N*,*N*-dibutylaniline, and *N*,*N*-dibenzylaniline were obtained from Sigma-Aldrich. All commercial fluorescence organelle trackers were obtained from Thermo Fisher Scientific. Nile Green™, a lipid droplet stain, part of Cell Navigator™ Fluorimetric Lipid Droplet Assay Kit was purchased from AAT Bioquest, Inc.

Synthesis of squaraine probes

In general, the SQR probes were synthesised with a slight modification to the earlier methods as outlined in Scheme S1. [29,30]



Scheme S1. Synthesis of SQR probes. Reagents and conditions: (i) cat. DMF, toluene, reflux, 6 h; (ii) toluene, reflux, 6 h; (iii) 5 N HCl, AcOH : H_2O (1:1), reflux, 2 h; (iv) *n*-BuOH : toluene (4:1), reflux, 3 h.

In brief, squaric acid **1** (2.00 mmol, 1.0 equiv) and thionyl chloride (4.00 mmol, 2.0 equiv) were mixed in toluene (10.0 mL) with a catalytic amount of DMF (10.0 µL) and the mixture was stirred at 70 °C for 12 h. Respective *N*,*N*-dialkylaniline **3** (2.00 mmol, 1.0 equiv) was then added into the reaction mixture and left to stir at 90 °C for 6 h. Excess solvent was removed under reduced pressure, following which the remaining mixture was immediately acidified with an acidic solution of acetic acid (10.0 mL), H_2O (10.0 mL) and 5N HCl (2.0 mL) and left to reflux for another 3 h. The yellow precipitate was filtered off and washed with cold ethyl acetate to obtain intermediate **4** as a crude product which was used without further purification.

4 (1.0 equiv) and 2,3,3-trimethylindolenine **5** (1.0 equiv) were then suspended in a mixture of toluene and *n*-butanol (4:1 v/v) and refluxed for 3 h. The resulting product was then purified through flash chromatography on silica gel (eluted with hexane : ethyl acetate = 2:1). The mixture was then washed with brine, dried over $MgSO_4$, and the solvent was removed under reduce pressure to obtain SQR probes **6**.

Characterization of squaraine probes

Absorbance and fluorescence of the SQR probes were recorded by utilizing Agilent Cary 300 UV-Vis spectrophotometer and Agilent Cary Eclipse fluorescence spectrophotometer respectively. ^{1}H and ^{13}C NMR spectra were recorded on JEOL ECA400 SL (400 MHz) spectrometer at 25 °C (internal standard reference $SiMe_4$ and $CDCl_3$ as solvent). ThermoFinnigan LCQ Fleet Liquid Chromatography

– Mass Spectrometer (LC-MS) was used to analyze the reaction mixture. Calculated log *P* (C log *P*) values were determined using ChemDraw Professional 16.0 software.

SQR21: Dark-blue powder (11% yield) R_f: 0.09 (hexane : ethyl acetate = 1:1). ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 1.21–1.25 (t, J = 7.3 Hz, 6H, -CH_3); 1.49 (s, 6H, -CH_3); 3.43–3.49 (q, J = 7.3 Hz, 4H, -CH_2-); 5.83 (s, 1H, =CH-); 6.70 (d, J = 9.2 Hz, 2H, ArH), 7.18–7.35 (m, 4H, ArH); 8.12 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 12.78, 25.50, 44.82, 50.01, 89.79, 111.64, 113.16, 118.83, 122.61, 125.09, 128.71, 130.91, 139.93, 141.44, 150.63, 169.08, 181.29, 181.62, 185.38, 187.33. MS (ESI): found *m*/*z* $[M+H]^+$ 387.32, calculated for $C_{25}H_{26}N_2O_2$ $[M+H]^+$ 387.50.

SQR22: Dark-blue powder (14% yield) R_f: 0.17 (hexane : ethyl acetate = 1:1). ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.97 (t, J = 7.6 Hz, 6H, -CH_3); 1.33–1.43 (m, 4H, -CH_2-); 1.49 (s, 6H, -CH_3); 1.57–1.65 (m, 4H, -CH_2-); 3.35–3.39 (m, 4H, -CH_2-); 5.83 (s, 1H, =CH-); 6.67 (d, J = 9.2 Hz, 2H, ArH), 7.18–7.35 (m, 4H, ArH); 8.11 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 14.00, 20.34, 25.52, 29.59, 49.98, 50.94, 89.73, 111.79, 113.14, 118.73, 122.61, 125.04, 128.69, 130.81, 139.92, 141.46, 151.04, 169.17, 181.17, 181.62, 185.38, 187.27. MS (ESI): found *m*/*z* $[M+H]^+$ 443.07, calculated for $C_{29}H_{34}N_2O_2$ $[M+H]^+$ 443.61.

SQR25: Dark-blue powder (10% yield) R_f: 0.20 (hexane : ethyl acetate = 1:1). ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.92 (t, J = 6.9 Hz, 6H, -CH_3); 1.34–1.37 (m, 8H, -CH_2-); 1.49 (s, 6H, -CH_3); 1.59–1.66 (m, 4H, -CH_2-); 3.34–3.38 (m, 4H, -CH_2-); 5.83 (s, 1H, =CH-); 6.67 (d, J = 9.2 Hz, 2H, ArH), 7.17–7.35 (m, 4H, ArH); 8.11 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 14.12, 22.59, 25.52, 27.14, 29.27, 49.98, 51.19, 89.73, 111.79, 113.13, 118.72, 122.61, 125.03, 128.69, 130.82, 139.91, 141.46, 151.03, 169.15, 181.16, 181.62, 185.38, 187.22. MS (ESI): found *m*/*z* $[M+H]^+$ 472.54, calculated for $C_{31}H_{38}N_2O_2$ $[M+H]^+$ 471.66.

SQR26: Golden-green powder (26% yield) R_f: 0.23 (hexane : ethyl acetate = 1:1). ^{1}H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.91 (t, J = 6.6 Hz, 6H, -CH_3); 1.25–1.33 (m, 12H, -CH_2-); 1.49 (s, 6H, -CH_3); 1.60–1.63 (m, 4H, -CH_2-); 3.34–3.381 (m, 4H, -CH_2-); 5.83 (s, 1H, =CH-); 6.67 (d, J = 9.2 Hz, 2H, ArH), 7.20–7.33 (m, 4H, ArH); 8.11 (d, J = 8.8 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 14.10, 22.70,

25.52, 26.79, 29.78, 31.68, 49.98, 51.23, 89.72, 111.80, 113.13, 118.71, 122.61, 125.03, 128.69, 130.82, 139.92, 141.46, 151.03, 169.15, 181.16, 181.65, 185.39, 187.17. MS (ESI): found *m*/*z* $[M+H]^+$ 500.83 calculated for $C_{33}H_{42}N_2O_2$ $[M+H]^+$ 499.72.

SQR23: Green-blue powder (21% yield) R_f: 0.26 (hexane : ethyl acetate = 1:1). ^1H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.89 (t, J = 6.9 Hz, 6H, $-CH_3$); 1.24–1.37 (m, 16H, $-CH_2-$); 1.48 (s, 6H, $-CH_3$); 1.62–1.63 (m, 4H, $-CH_2-$); 3.33-3.37 (m, 4H, $-CH_2-$); 5.82 (s, 1H, =CH-), 6.66 (d, J = 9.2 Hz, 2H, ArH), 7.17–7.35 (m, 4H, ArH); 8.11 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 14.15, 22.67, 25.53, 27.08, 27.47, 29.17, 31.87, 49.97, 51.22, 89.71, 111.79, 113.12, 118.73, 122.61, 125.02, 128.69, 130.81, 139.91, 141.47, 151.03, 169.21, 181.14, 181.59, 185.37, 187.27. MS (ESI): found *m*/*z* $[M+H]^+$ 528.81, calculated for $C_{35}H_{46}N_2O_2$ $[M+H]^+$ 527.77.

SQR29: Golden-green powder (23% yield) R_f: 0.32 (hexane : ethyl acetate = 1:1). ^1H NMR ($CDCl_3$, 400 MHz, δ ppm): 0.89 (t, J = 6.9 Hz, 6H, $-CH_3$); 1.25–1.33 (m, 24H, $-CH_2-$); 1.49 (s, 6H, $-CH_3$); 1.59–1.63 (m, 4H, $-CH_2-$); 3.34–3.38 (m, 4H, $-CH_2-$); 5.83 (s, 1H, =CH-); 6.67 (d, J = 9.2 Hz, 2H, ArH), 7.18–7.33 (m, 4H, ArH); 8.11 (d, J = 8.7 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 13.95, 22.49, 25.21, 26.86, 27.22, 29.08, 29.27, 29.38, 31.67, 49.72, 50.97, 89.52, 111.55, 112.86, 118.38, 122.36, 124.84, 128.41, 130.60, 139.62, 141.11, 150.78, 168.54, 180.88, 181.55, 185.09, 186.42. MS (ESI): found *m*/*z* $[M+H]^+$ 584.12, calculated for $C_{39}H_{54}N_2O_2$ $[M+H]^+$ 583.88.

SQR27: Dark blue powder (11% yield) R_f: 0.13 (hexane : ethyl acetate = 1:1). ^1H NMR ($CDCl_3$, 400 MHz, δ ppm): 1.48 (s, 6H, $-CH_3$); 4.73 (s, 4H, $-CH_2-$); 5.87 (s, 1H, =CH-); 6.82 (d, J = 9.2 Hz, 2H, ArH), 7.20–7.34 (m, 14H, ArH); 8.08 (d, J = 9.2 Hz, 2H, ArH). ^{13}C NMR ($CDCl_3$, 400 MHz, δ ppm): 25.07, 50.13, 53.86, 90.39, 112.45, 113.40, 120.15, 122.52, 125.39, 126.48, 127.31, 128.64, 128.81, 130.31, 137.01, 139.93, 141.12, 151.73, 167.49, 181.27, 182.12, 185.13, 188.10. MS (ESI): found *m*/*z* $[M+H]^+$ 512.35, calculated for $C_{35}H_{30}N_2O_2$ $[M+H]^+$ 511.64.

Confocal-microscope setup and data analysis

Confocal fluorescence imaging was performed with a Carl Zeiss LSM 800 microscope (Germany) equipped with oil immersion objective lenses (Plan-APOCHROMAT 60×, NA = 1.4 or Plan-APOCHROMAT 100×, NA = 1.4). For a multi-colour imaging, the following filter sets were used: ER Tracker Blue-White and Hoechst (Ex = 405 nm; Em = 410-700 nm), Nile Green™ and other organelle trackers (Ex = 488 nm; Em = 500-550 nm), SQR probes (Ex = 640 nm; Em = 656-700 nm). Two-dimensional images were acquired with an exposure time of 30 msec and time-series images were obtained with a rate of 1 frame per minute. ImageJ software (National Institutes of Health) was used to analyze the captured confocal images. Colocalization analysis was performed using JACoP plugin on ImageJ by calculating the Pearson correlation coefficient (*r*).

Cell culture

HeLa (ATCC® CCL-2™) and MCF-7 (ATCC® HTB-22™) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS, 10%) and penicillin-streptomycin (1%). 3T3-L1 (ATCC® CL-173™) cell was cultured in DMEM supplemented with newborn calf serum (NBCS, 10%) and penicillin-streptomycin (1%). SKOV-3 (ATCC® HTB-77™) and U-2 OS (ATCC® HTB-96™) cells were cultured in McCoy's 5A medium supplemented with FBS (10%) and penicillin-streptomycin (1%). The cells were grown and kept at 37 °C under 5% CO_2 environment. Cells were grown on glass-based dishes for confocal observation.

Colocalization experiments

For colocalization experiments, HeLa cells (80% confluent) on a 3.5 cm glass-based dish were first washed and replaced with 1 mL unsupplemented DMEM. To examine the colocalization with the ER and mitochondria, cells were co-incubated with 1 μM SQR22, 1 μM of ER-Tracker™ Blue-White DPX, and 200 nM of MitoTracker™ Green for 30 minutes. Colocalization with the lipid droplets (LDs) were performed by first incubating the cells with 1 μM of SQR22 probe for 30 minutes followed by washing and subsequent incubation with Nile Green™. For colocalization with Endosomes, the cells were first

incubated overnight with 10 µL of either CellLight™ Early Endosomes-GFP, BacMam 2.0 or CellLight™ Late Endosomes-GFP, BacMam 2.0. The cells were then washed with DMEM and followed by incubation with 1 µM of SQR22 for 30 minutes. To observe the colocalization with the lysosomes, the cells were co-incubated with 1 µM SQR22 and 50 nM of LysoTracker™ Green DND-26 for 30 minutes.

Cell cytotoxicity assay

SQR22 cell cytotoxicity was evaluated using MTT assay. The mitochondrial dehydrogenase enzyme in living cell reduces MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to form formazan crystals. HeLa cells were seeded in a 96-well plate at a density of 2.5×10^4 cells/mL in DMEM supplemented with FBS (10%) and penicillin-streptomycin (1%) and grown at 37 °C under 5% CO_2 environment. After 48 hours, the cells were washed with 100 µL phosphate buffered saline (PBS) in each well. The cells were then incubated with 100 µL unsupplemented DMEM mixed with SQR22 at different concentrations (0.1, 0.5, 1, 5, 10 µM) and varying incubation duration (4, 24, 48 hours). Upon completion of incubation with SQR22, 10 µL MTT solution was added into each well and incubated at 37 °C under 5% CO_2 condition. After 2 hours incubation, 200 µL DMSO was added into each well to dissolve the formed purple formazan crystals. The absorbance of the formazan crystal was then measured at 570 nm while background absorbance was measured at 630 nm using Tecan 200 microplate reader. The corrected signal from each SQR-treated well was then normalized to that from the control well to obtain relative percentage cell viability.

Cell fixation

HeLa cells (80% confluent) on a 3.5 cm glass-based dish were first washed with 1 mL cold PBS (4 °C) thrice. The cells were then incubated with 4% paraformaldehyde (PFA) in PBS for 20 minutes or ice-cold MeOH for 10 minutes at 4 °C. The cells were then washed thrice with 1 mL cold PBS (4 °C) before further incubation with SQR probes and further observations.

Supplementary Tables

SQR	21	22	25	26	23	29	27
60 minutes	0.865	0.695	0.736	0.572	0.393	0.602	0.900
30 minutes	0.798	0.689	0.516	0.428	0.535	0.670	0.620
15 minutes	0.879	0.652	0.511	0.275	0.745	0.587	0.518
5 minutes	0.729	0.464	0.454	0.199	0.593	0.432	0.450
1 minute	0.685	0.467	0.250	0.246	0.364	0.563	0.229

Table S1. Pearson correlation coefficient (*r*) of SQR probes and ER-Tracker™ Blue-White DPX in HeLa cells as depicted in Figure 2. *r* values are calculated using JACoP plugin on ImageJ software.

SQR	3 hours	6 hours	9 hours	12 hours	15 hours	18 hours	21 hours	24 hours
26	0.595	0.715	0.752	0.812	0.806	0.785	0.729	0.697
23	0.450	0.561	0.378	0.474	0.571	0.594	0.735	0.660
29	0.565	0.687	0.572	0.515	0.691	0.596	0.450	0.452

Table S2. Pearson correlation coefficient (*r*) of SQR probes and ER-Tracker™ Blue-White DPX in HeLa cells as depicted in Figure S2. *r* values are calculated using JACoP plugin on ImageJ software.

Supplementary Figures

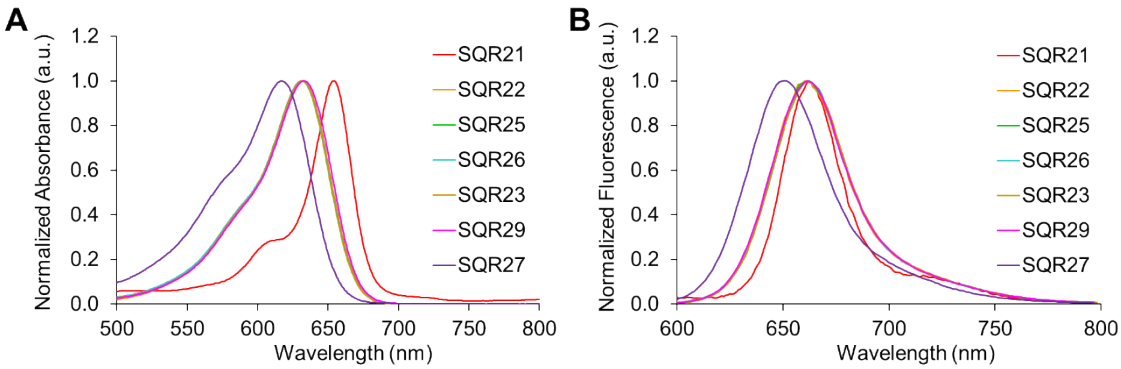


Figure S1. (A) Absorption and (B) emission spectra of the synthesized SQR probes in ethanol.

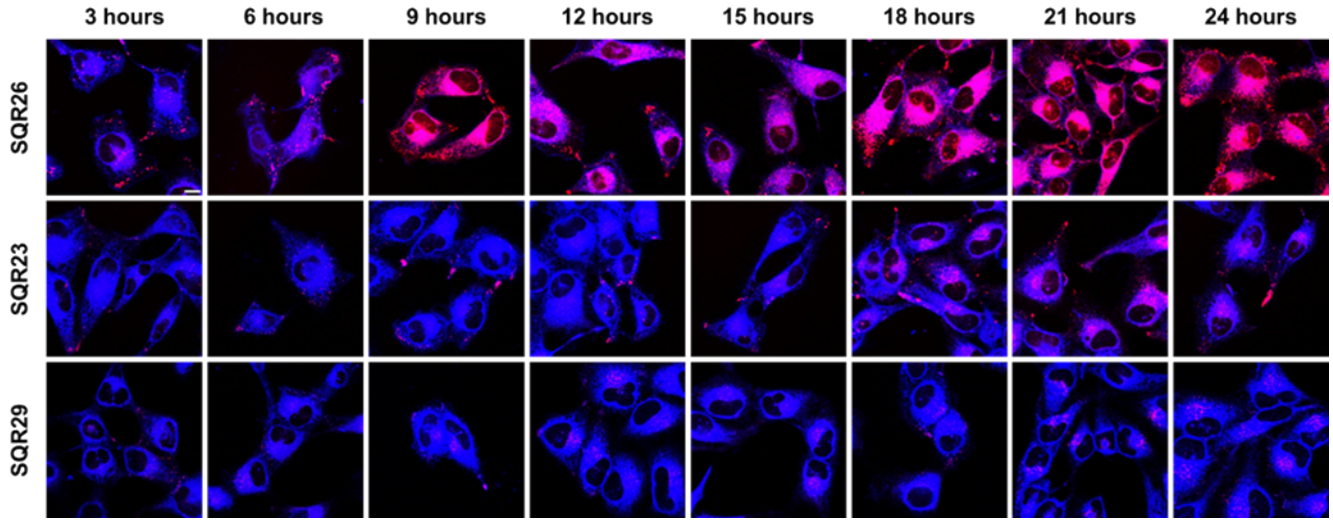


Figure S2. Time dependent staining of endoplasmic reticulum (ER) with SQR probes in HeLa cell. The cells were co-stained with SQR probes (1 μM, red) and ER-Tracker™ Blue-White DPX (1 μM, blue). Scale bar = 10 μm.

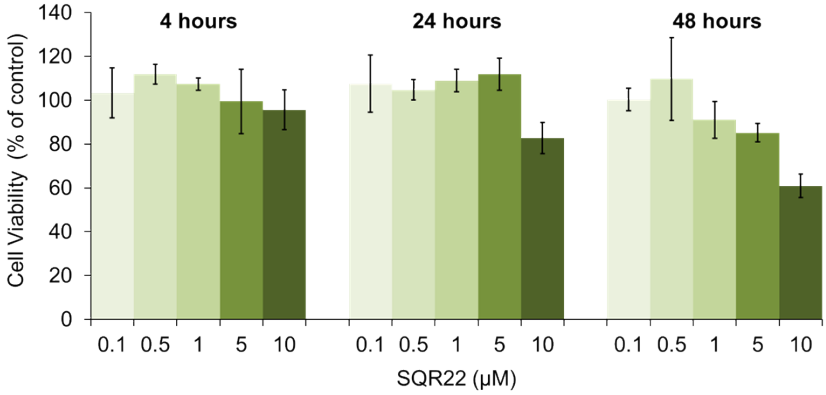


Figure S3. MTT Cytotoxicity assay of SQR22.

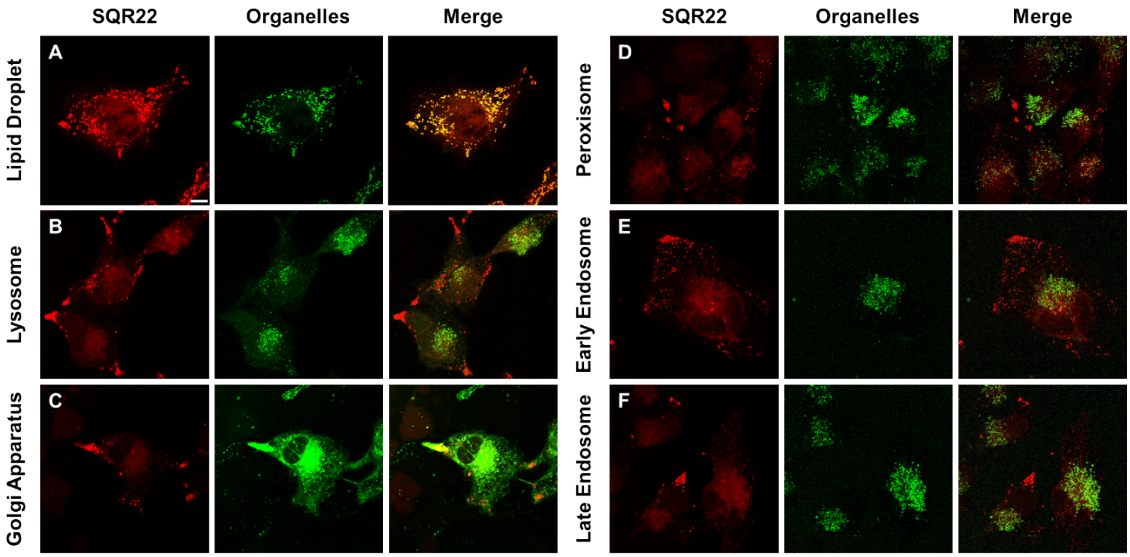


Figure S4. Colocalization of SQR22 (1 μM, red) with other targeted organelles (green) in HeLa cells. (A) Lipid Droplet, Nile Green™ and Nucleus (blue), Hoechst (B) Lysosome, LysoTracker™ Green DND-26 (C) Golgi Apparatus, BODIPY™ FL C5-Ceramide (D) Peroxisome, CellLight™ Peroxisome-GFP (to update) (E) Early Endosome, CellLight™ Early Endosomes-GFP (F) Late Endosome, CellLight™ Late Endosomes-GFP. Scale bar = 10 μm.

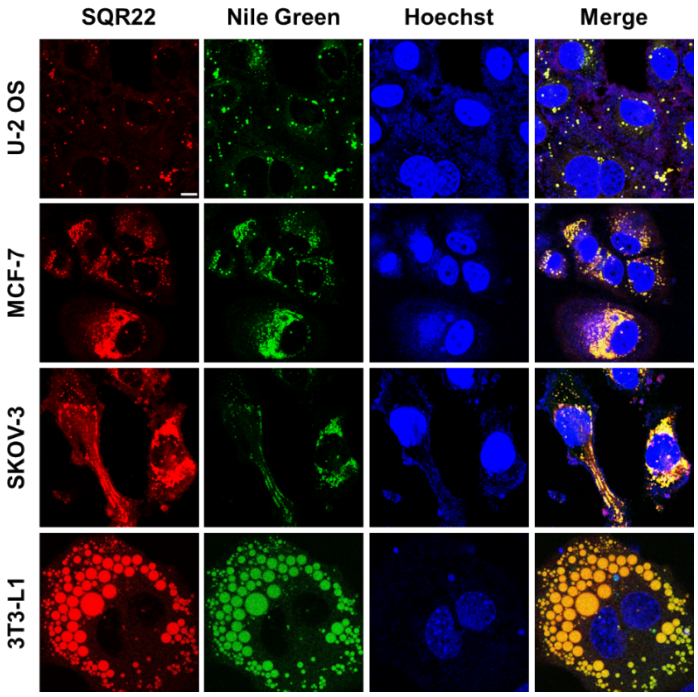


Figure S5. Colocalization of SQR22 (1 μM, red) with Lipid Droplet (Nile Green™) in different cancer cells and non-malignant cell 3T3-L1. Scale bar = 10 μm.

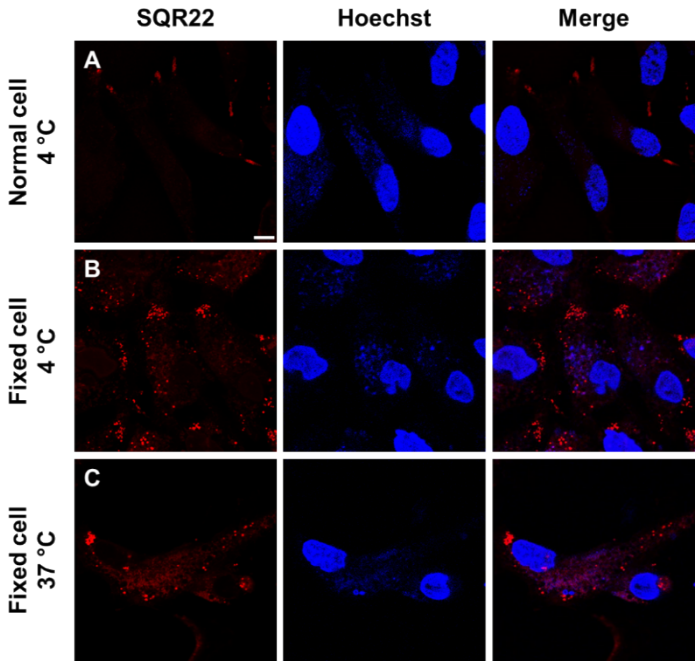


Figure S6. Simple diffusion of SQR22 into lipid droplet in HeLa cell fixed with Paraformaldehyde. (A) Normal HeLa cells or (B, C) fixed HeLa cells were co-stained with SQR probes (1 μM, red) and Hoechst (0.5 μg mL^{-1}, blue) at different incubation temperatures: (A, B) 4 °C or (C) 37 °C. Scale bar = 10 μm.

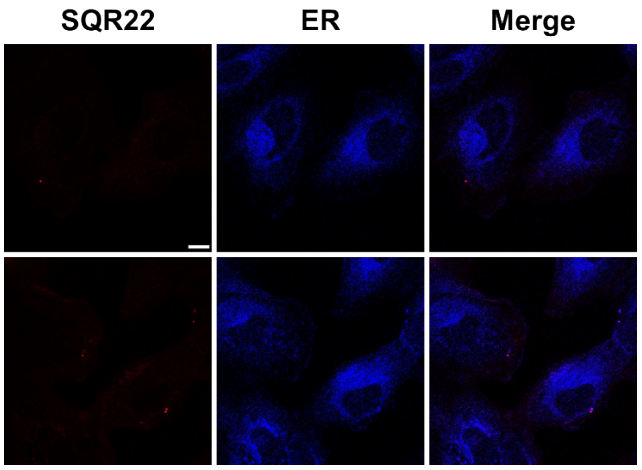


Figure S7. Simple diffusion of SQR22 into lipid droplet in HeLa cell fixed with methanol. Cells were co-stained with SQR probes (1 μM, red) and ER-Tracker™ Blue-White DPX (1μM, blue). Scale bar = 10 μm.

^{1}H and ^{13}C NMR Spectra

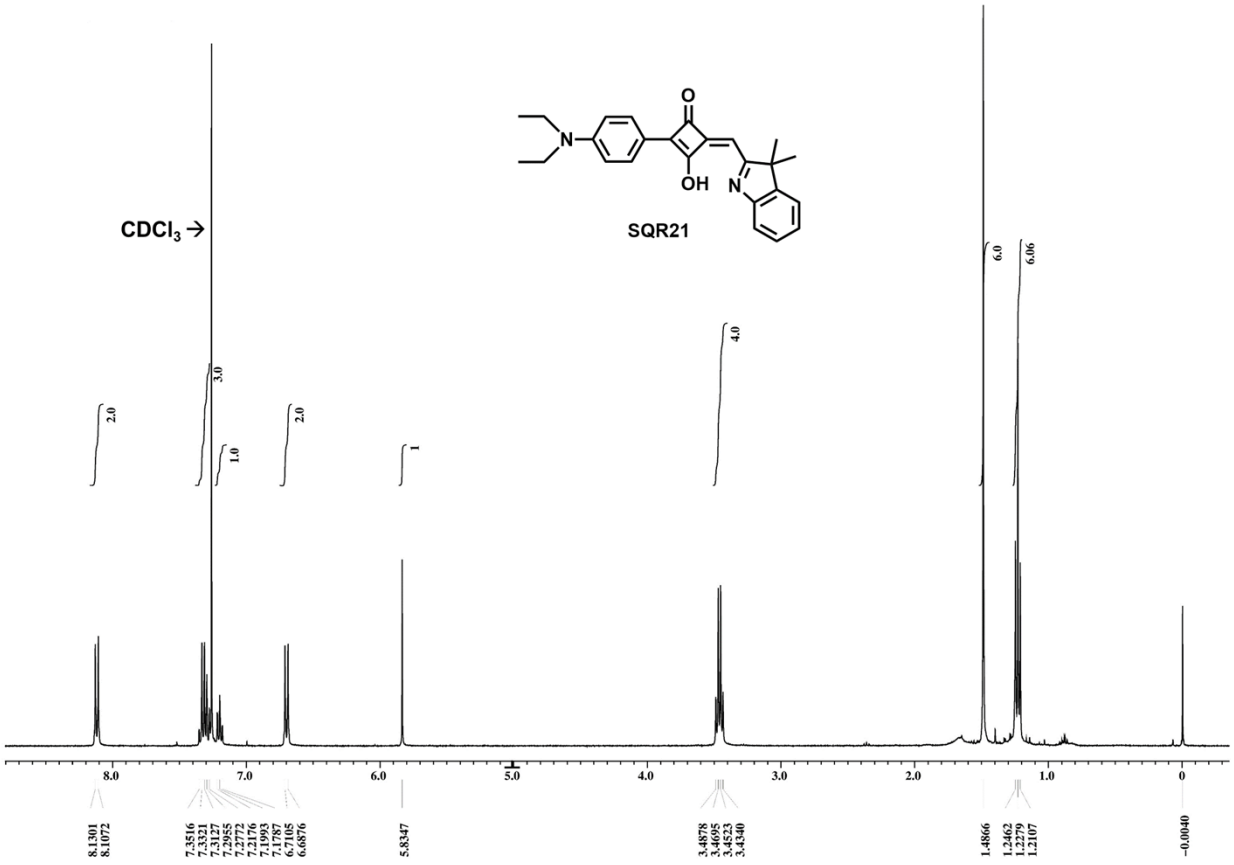


Figure S8. ^{1}H NMR spectra of SQR21 ($CDCl_3$, 400 MHz).

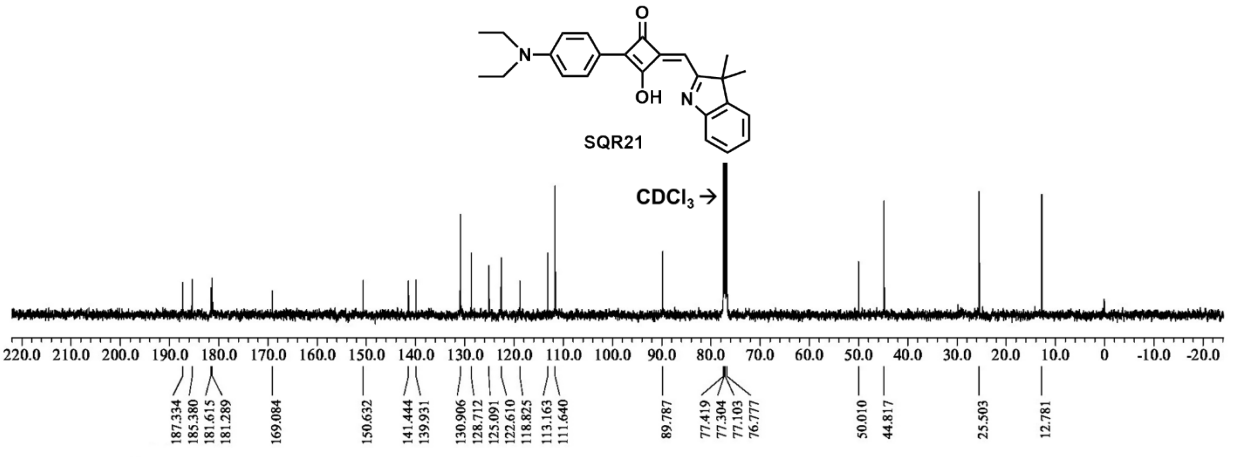


Figure S9. ^{13}C NMR spectra of SQR21 ($CDCl_3$, 400 MHz).

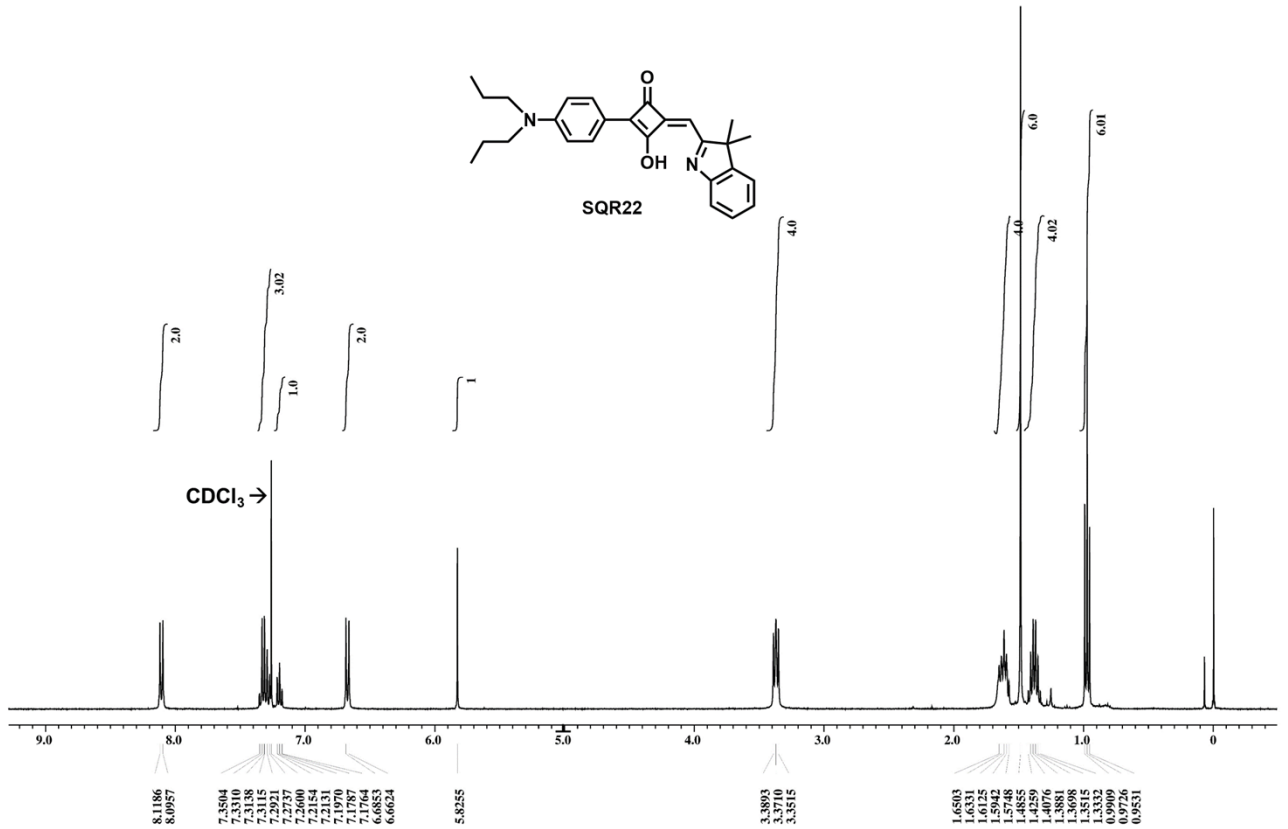


Figure S10. ^{1}H NMR spectra of SQR22 ($CDCl_3$, 400 MHz).

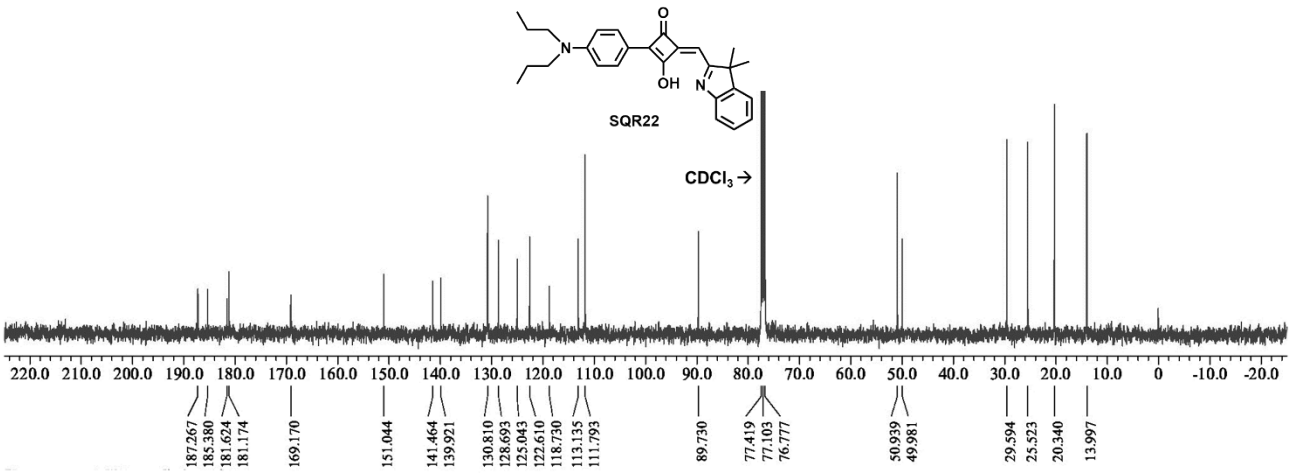


Figure S11. ^{13}C NMR spectra of SQR22 ($CDCl_3$, 400 MHz).

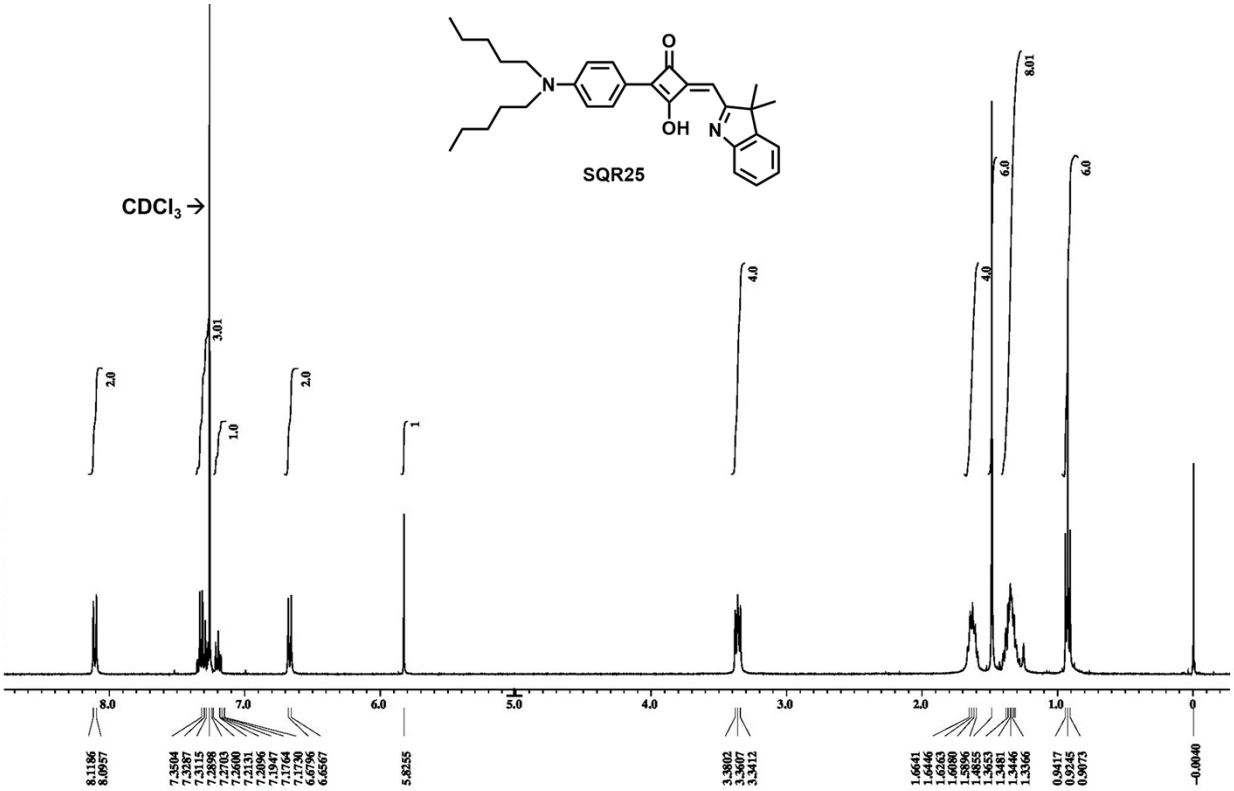


Figure S12. ^{1}H NMR spectra of SQR25 ($CDCl_3$, 400 MHz).

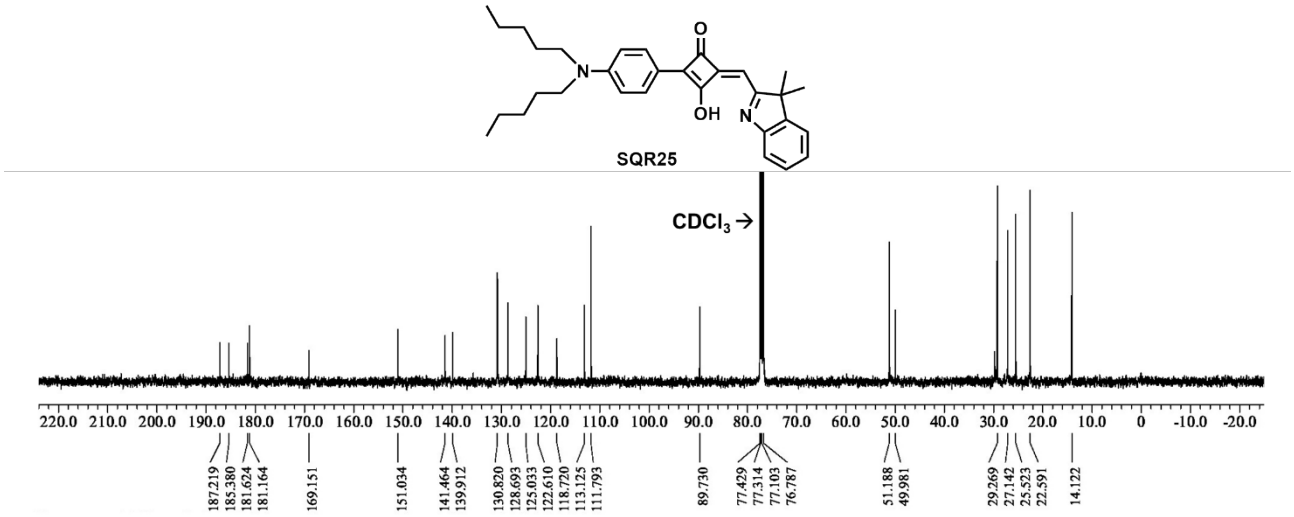


Figure S13. ^{13}C NMR spectra of SQR25 ($CDCl_3$, 400 MHz).

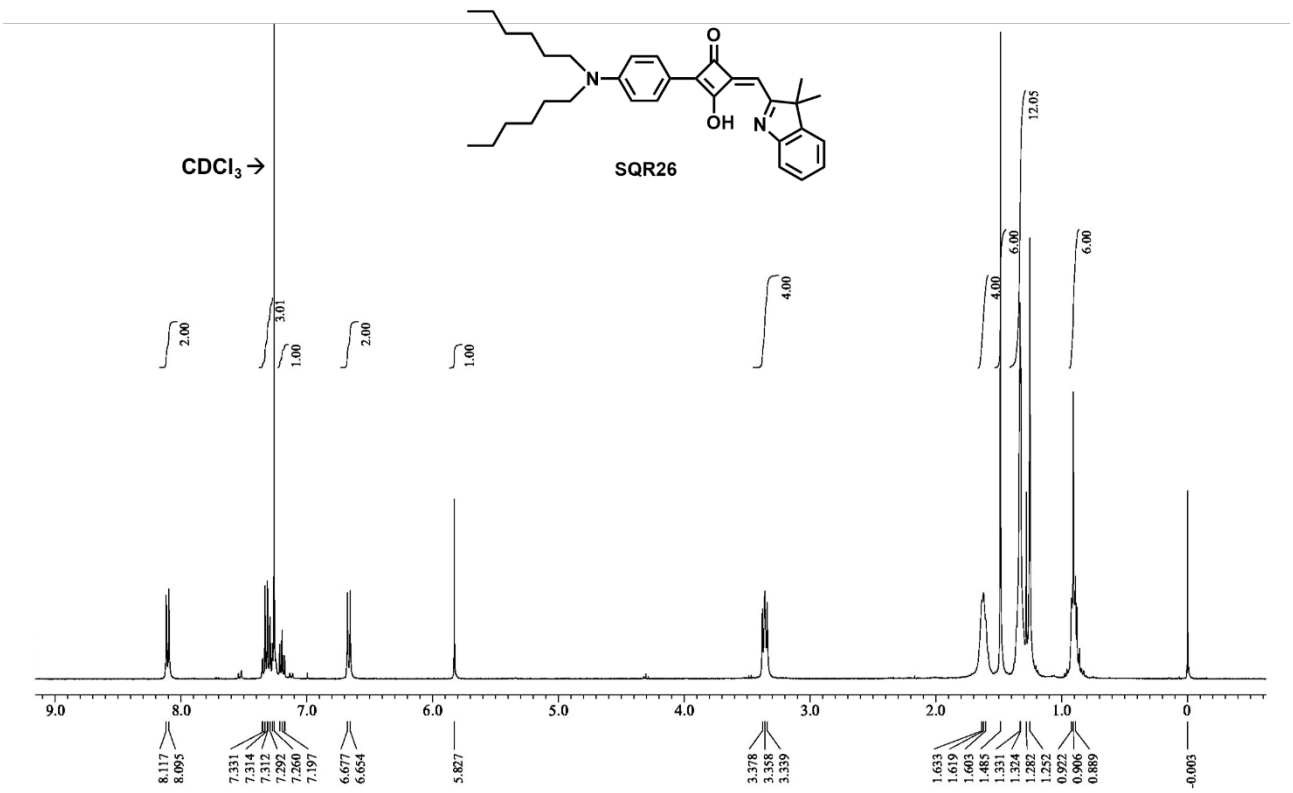


Figure S14. ^{1}H NMR spectra of SQR26 ($CDCl_3$, 400 MHz).

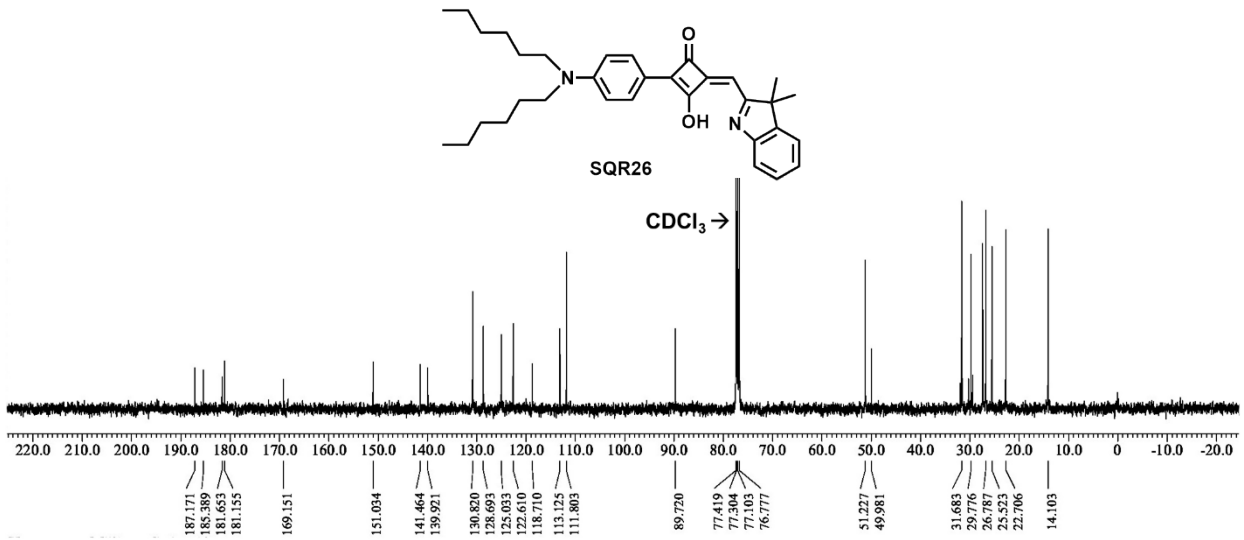


Figure S15. ^{13}C NMR spectra of SQR26 ($CDCl_3$, 400 MHz).

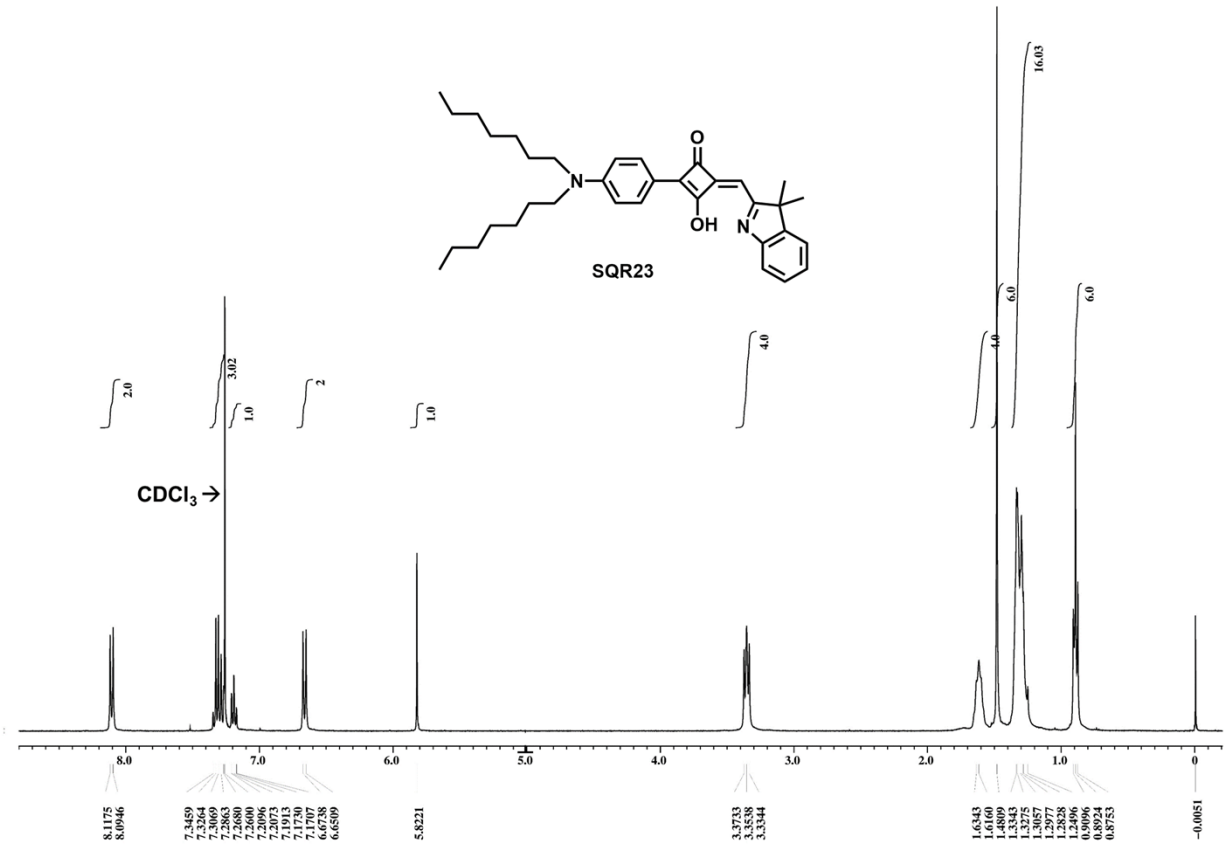


Figure S16. ^{1}H NMR spectra of SQR23 ($CDCl_3$, 400 MHz).

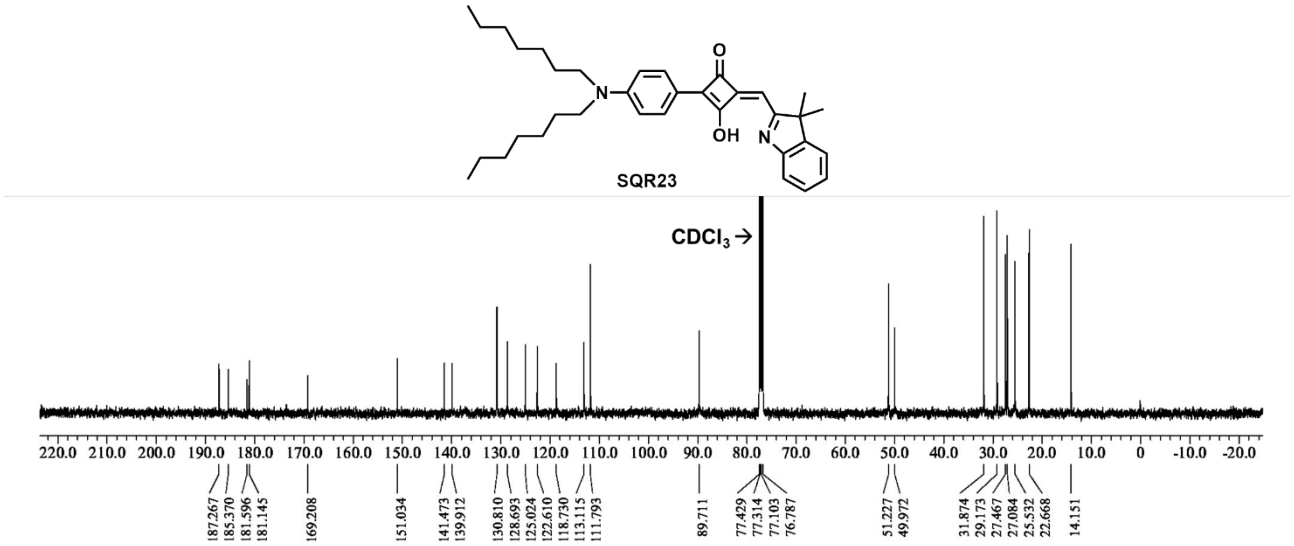


Figure S17. ^{13}C NMR spectra of SQR23 ($CDCl_3$, 400 MHz).

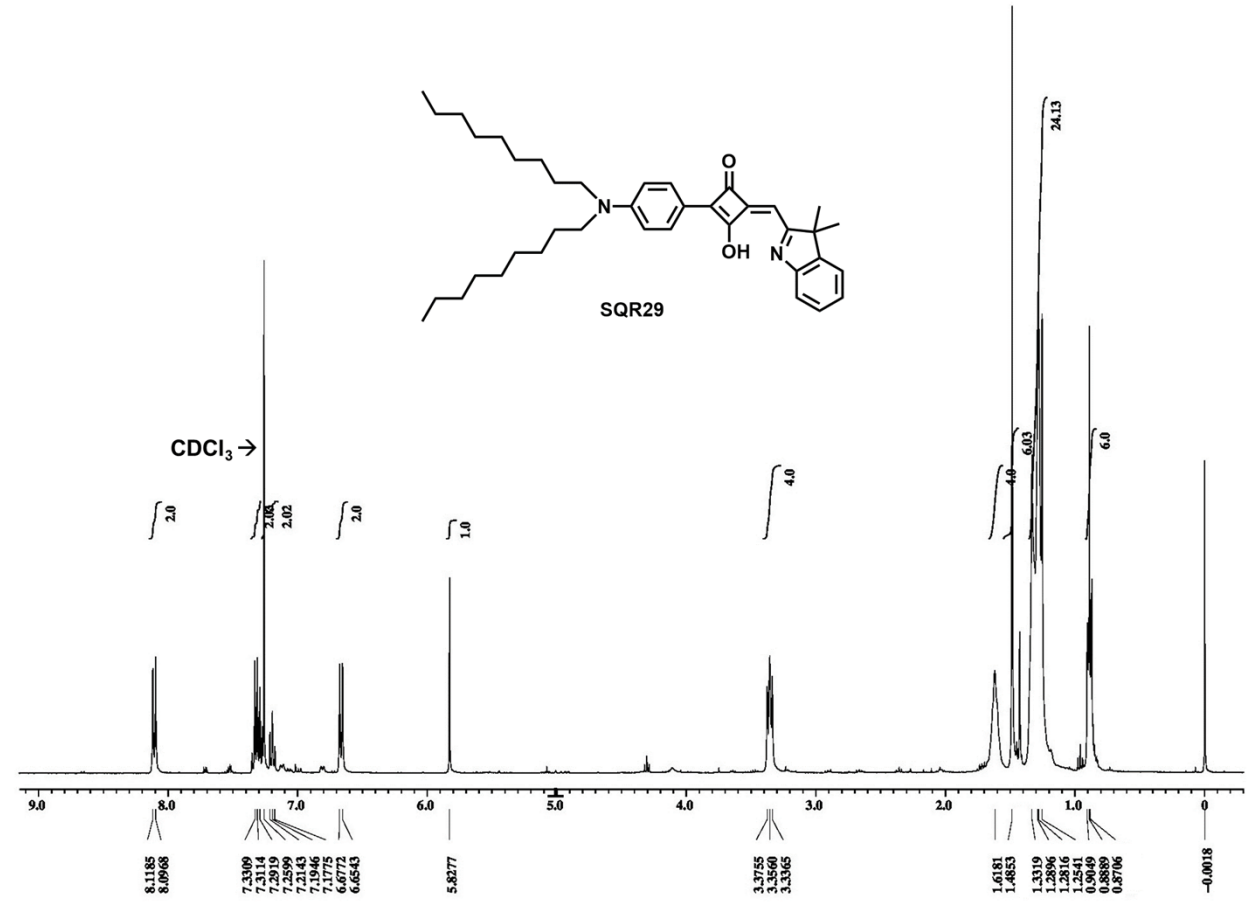


Figure S18. ^{1}H NMR spectra of SQR29 ($CDCl_3$, 400 MHz).

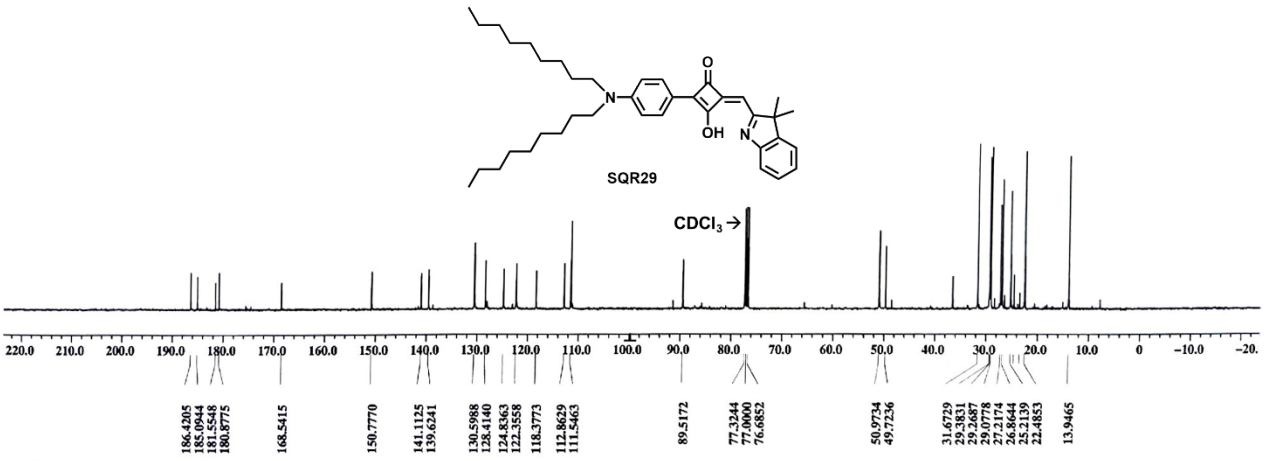


Figure S19. ^{13}C NMR spectra of SQR29 ($CDCl_3$, 400 MHz).

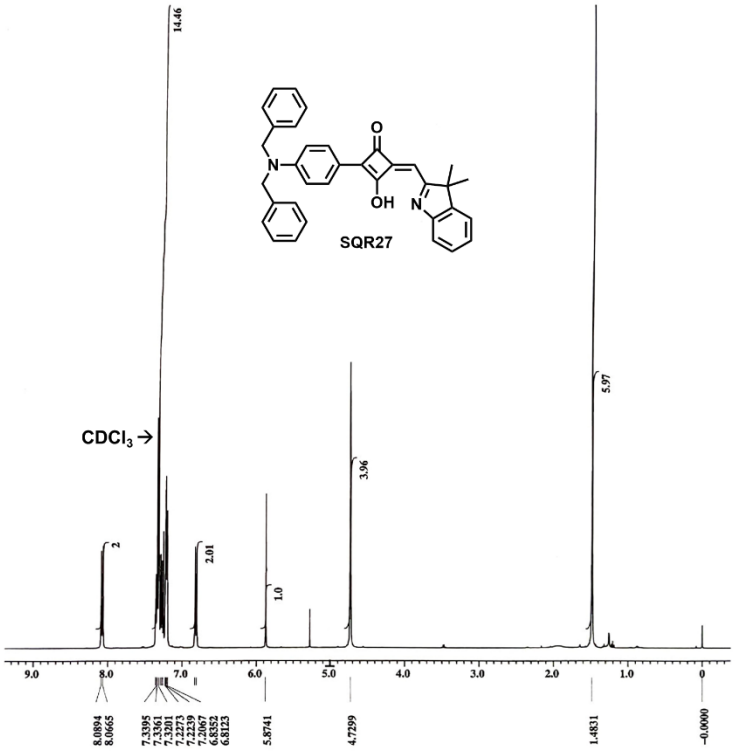


Figure S20. ^{1}H NMR spectra of SQR27 ($CDCl_3$, 400 MHz).

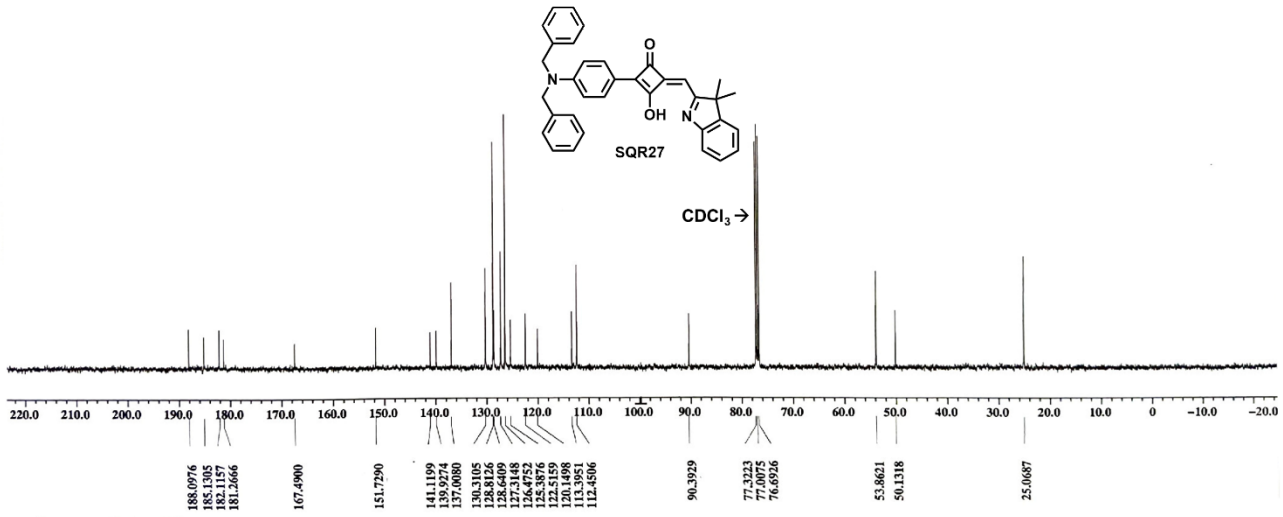


Figure S21. ^{13}C NMR spectra of SQR27 ($CDCl_3$, 400 MHz).

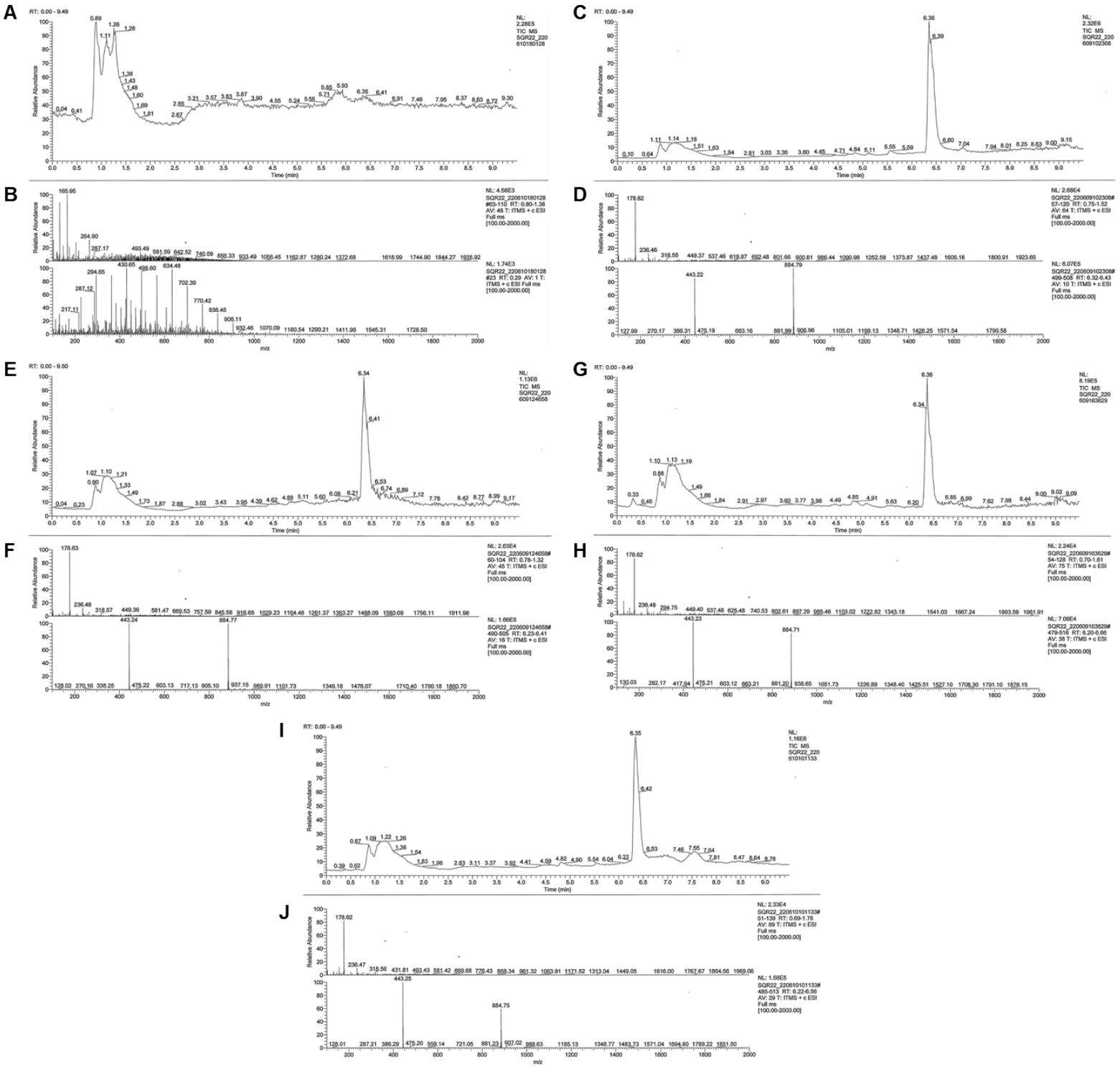

A
RT: 0.00 - 9.49
Relative Abundance
Time (min)
NL: 2.28E5 TIC MS SQR22_220610180128
B
NL: 4.58E3 SQR22_220610180128 #63-110 RT: 0.80-1.38 AV: 48 T: ITMS + c ESI Full ms [100.00-2000.00]
NL: 1.74E3 SQR22_220610180128 #23 RT: 0.29 AV: 1 T: ITMS + c ESI Full ms [100.00-2000.00]
m/z
C
RT: 0.00 - 9.49
Relative Abundance
Time (min)
NL: 2.32E6 TIC MS SQR22_220609102308
D
NL: 2.68E4 SQR22_220609102308# 57-120 RT: 0.75-1.52 AV: 64 T: ITMS + c ESI Full ms [100.00-2000.00]
NL: 6.07E5 SQR22_220609102308# 499-508 RT: 6.32-6.43 AV: 10 T: ITMS + c ESI Full ms [100.00-2000.00]
m/z
E
RT: 0.00 - 9.50
Relative Abundance
Time (min)
NL: 1.13E6 TIC MS SQR22_220609124658
F
NL: 2.63E4 SQR22_220609124658# 60-104 RT: 0.78-1.32 AV: 45 T: ITMS + c ESI Full ms [100.00-2000.00]
NL: 1.66E5 SQR22_220609124658# 490-505 RT: 6.23-6.41 AV: 16 T: ITMS + c ESI Full ms [100.00-2000.00]
m/z
G
RT: 0.00 - 9.49
Relative Abundance
Time (min)
NL: 8.19E5 TIC MS SQR22_220609163629
H
NL: 2.24E4 SQR22_220609163629# 54-128 RT: 0.70-1.61 AV: 75 T: ITMS + c ESI Full ms [100.00-2000.00]
NL: 7.06E4 SQR22_220609163629# 479-516 RT: 6.20-6.66 AV: 38 T: ITMS + c ESI Full ms [100.00-2000.00]
m/z
I
RT: 0.00 - 9.49
Relative Abundance
Time (min)
NL: 1.15E8 TIC MS SQR22_220610101133
J
NL: 2.33E4 SQR22_220610101133# 51-139 RT: 0.69-1.78 AV: 89 T: ITMS + c ESI Full ms [100.00-2000.00]
NL: 1.58E5 SQR22_220610101133# 485-513 RT: 6.22-6.56 AV: 29 T: ITMS + c ESI Full ms [100.00-2000.00]
m/z

Figure S22. Chromatogram and mass spectra of major peaks of SQR22 (0.1 mg mL^{-1}) incubated in DMEM medium. (A, B) DMEM medium only (C, D) SQR22 in DMEM at t = 0 (E, F) SQR22 in DMEM at t = 2 hours (G, H) SQR22 in DMEM at t = 6 hours (I, J) SQR22 in DMEM at t = 24 hours.

Movie S1. Timelapse image of ER staining with SQR22 in HeLa cell. The cells were co-stained with SQR probes (1 μM, red) and ER-Tracker™ Blue-White DPX (1 μM, blue).