SUPPORTING INFORMATION

Click Activated Protodrugs Against Cancer Increase the Therapeutic Potential of Chemotherapy through Local Capture and Activation

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Materials and Methods

All chemicals were received from commercial sources and used without further purification. Chromatographic purifications of synthetic materials were conducted using SiliaSphere™ spherical silica gel with an average particle and pore size of 5 μm and 60 Å, respectively (Silicycle Inc, QC, Canada). Thin layer chromatography (TLC) was performed on SiliaPlate™ silica gel TLC plates with 250 μm thickness (Silicycle Inc, QC, Canada). Preparative TLC was performed using SiliaPlate™ silica gel TLC plates with 1000 μm thickness. Analytical HPLC was performed using Phenomenex Luna 5u C18 (2) analytical column (250 x 5 mm) using a gradient of CH_3CN (0.01% formic acid) in H_2O (0.01% formic acid). HRMS and LC-MS data was acquired using Agilent Technologies 6530 Q-TOF instrument. ^{1}H and ^{13}C NMR spectroscopy was performed on a Bruker NMR at 500 MHz (^{1}H) and 125 MHz (^{13}C). All ^{13}C NMR spectra were proton decoupled.

Cell culture. MC38 cells were purchased from ATCC (American Type Culture Collection, cat.# CRL-2638) and propagated in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum (FBS), supplemented with 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a 5% CO_2 incubator.

Cell proliferation assay. The colorimetric MTT (3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay protocol was used to evaluate proliferation of MC38 cells. On day one, 1×10^3 cells/well in 96-well plates in 100 μL DMEM were plated and incubated for 24 h. On day two, the medium was removed, and the cells were treated with variable concentrations of cytotoxic compounds. On day three, the medium was replaced with 100 μL of fresh medium and cells were incubated for additional 48h. On day five, the medium was removed, and cells were incubated with 100 μL of MTT solution (0.6 mg/mL in DMEM) per well. Cells were incubated in the dark for 4 h at 37 °C. At the end of the incubation, the MTT solution was then replaced with 100 μL of DMSO containing 4% aqueous ammonia per well and agitated for 30 minutes. The absorbance of the purple formazan was recorded using a BioTek Synergy HT multi detection microplate reader at 550 nm. Results were generated from triplicate experiments.

Protodrug activation experiments. Protodrug activation experiments were carried out using Tz-modified alginate gel which has previously been described (*Acta Biomater.* **2014**,*10*, 5099-5105). This material was chosen because of the previously established protodrug activation protocol (*ACS Cent. Sci*. **2018**, *4*, 1624-1632). In particular, the crosslinked hydrogel provides an easy way for separation of small molecules from the biopolymer using centrifugation. The Tz-modified alginate gel (160 μL) was crosslinked using a supersaturated solution of $CaSO_4$ (40 μL) and placed inside of amicon 3K spin columns and treated with 5 nmol of protodrugs of DOX, PTX and ETP in 400 μL of PBS. After 2 h, the supernatant fractions were collected centrifugation at 6,000 RPM and Tz-modified alginate was resuspended in fresh PBS (400 μL). The supernatants were collected after 4, 6, and 24 h to monitor continuous drug release. The supernatant fractions were analyzed by HPLC. All kinetic experiments were carried out in triplicate. Activation of GCB-TCO-acid could not be done using this method because of the sensitivity of GCB to $CaSO_4$. Instead, we performed a ^{1}H NMR experiment where the protodrug was treated with a model Tz compound (3,6-Diisopropyl-1,2,4,5-tetrazine) and the spectra were recorded at different time intervals.

Protodrug plasma stability studies. Mouse blood plasma was purchased from BioIVT (Westbury, NY). Prepared 100 μM protodrug stock solutions in DMSO. Added 2 μL of a protodrug stock solution to 198 μL of mouse plasma and incubated at 37 °C. At different timepoints, 30 μL aliquots were diluted with 60 μL of ice cold Internal Standard Solution (acetonitrile containing 12.5 ng/mL of diclofenac) and mixed. Samples were centrifuged at 6,000g for 30 minutes and subsequently analyzed by LC-MS in the negative mode.

Dox Equivalence Calculations. The molecular weights (MW) of SQP33 (free acid form) and Dox hydrochloride (Dox HCl) are 810.81 g/mol and 579.98 g/mol respectively. SQP33 is composed of TCO-modified Dox. Thus, the molar conversion factor of SQP33 to Dox HCl is 0.7153, based on the MW ratios. All SQP33 protodrug values that were measured in plasma or administered as the dose in the studies reported here have been converted to Dox equivalents (Dox Eq) for a clear comparison of the amount of Dox delivered. The molar conversion factor of SQP33 to Dox HCl for the mouse toxicity study is 0.696 because the sodium salt form of SQP33 was used (MW=833.8 g/mol).

Evaluation of treatment toxicity in mice.

Study Design

6-8-week-old C57BL/6 mice were placed in 9 groups. Groups 1-3 (n = 5 each) received a single IV dose of Dox HCl at 10, 20, or 30 mg/kg, respectively. Groups 4-6 (n = 5 each) evaluated toxicity of SQP33 in the absence of SQL70 biopolymer and received 5 daily IV doses of SQP33 at 55.6 mg/kg/dose Dox Eq (278 mg/kg/cycle Dox Eq), 76.6 mg/kg/dose Dox Eq (383 mg/kg/cycle Dox Eq), or 97.4 mg/kg/dose Dox Eq (487 mg/kg/cycle Dox Eq) without a prior SQL70 biopolymer injection. Groups 7-9 (n = 4 each) evaluated toxicity of SQ3370 treatment and received a single 100 µL SQL70 biopolymer injection on day 1, followed by 5 daily IV doses of SQP33 at 55.6 mg/kg/dose Dox Eq (278 mg/kg/cycle Dox Eq), 76.6 mg/kg/dose Dox Eq (383 mg/kg/cycle Dox Eq), or 97.4 mg/kg/dose Dox Eq (487 mg/kg/cycle Dox Eq). All mice were weighed daily for up to 14 days once treatment started and a mean body weight loss ≥10% per group indicated the maximum tolerated dose (MTD) of the treatment given.

Dosing Procedures

Groups 1-3: Dox HCl was dissolved in saline and administered IV via the tail vein. Groups 4-9: SQP33 was dissolved in sterile water, diluted with sterile saline and administered IV via the tail vein injection. Groups 7-9: 100 µL of SQL70 biopolymer was injected SC 1 hour prior to the first SQP33 protodrug dose.

In Life Assessment

All animals were checked for any effects of treatment on normal behaviors such as mobility, food and water consumption (by visual inspection), body weight gain/loss, eye/hair matting and any other abnormal effect. Mice were weighed daily for up to 14 days once treatment started.

Pharmacokinetics and non-target tissue exposure in rats.

Study Design

Male *Sprague Dawley* (SD) rats were placed in 7 groups. Group 1 (n = 3) served as control and received a single intravenous (IV) dose of Dox HCl at 8.1 mg/kg. Group 2 (n = 3) evaluated SQP33's activity in the absence of SQL70 biopolymer, and received a single IV dose of SQP33, at 43 mg/kg Dox Eq, without a prior SQL70 biopolymer injection. Groups 3-5 (n = 3 per group) assessed the effect of elapsed-time between SQL70 biopolymer injection and SQP33 protodrug IV administration on SQ3370's local activation capacity. These groups received a single IV dose of SQP33 at 1 hour (group 3), 24 hours (group 4), or 96 hours (group 5) after a SC SQL70 biopolymer injection. Group 6 (n = 6) determined the effects of multiple IV SQP33 doses following a single SC SQL70 biopolymer injection, and animals received 5 IV doses of SQP33. The first SQP33 dose was 1 hour after SQL70 biopolymer injection followed by 4 additional daily doses. Lastly, Group 7 (n = 6) evaluated multiple doses of SQP33 in the absence of SQL70 biopolymer. These animals received 5 daily IV doses of SQP33 without a prior SQL70 biopolymer injection.

Dosing Procedures

Group 1: 8.1 mg of Dox per kg of body weight was dissolved in saline. Once a concentration of 1.62 mg/mL was achieved, a total volume of 5 mL per kg of body weight was administered IV via the tail vein. Group 2: 60 mg of SQP33 per kg of body weight was dissolved in saline at pH 7.4, and sodium hydroxide was added dropwise until SQP33 fully dissolved. Once a concentration of

12 mg/mL was achieved, a total volume of 5 mL per kg of body weight was administered IV via the tail vein. Groups 3 to 5: 1 mL of SQL70 was SC injected at the dorsal flank. 60 mg of SQP33 per kg of body weight was dissolved in saline at pH 7.4, and sodium hydroxide was added dropwise until SQP33 fully dissolved. Once a concentration of 12 mg/mL was achieved, a total volume of 5 mL per kg of body weight was administered IV via the tail vein at 1, 24, or 96 hours after SQL70 injection. Group 7: 1 mL of SQL70 was SC injected at the dorsal flank. 30 mg of SQP33 per kg of body weight was dissolved in saline at pH 7.4, and sodium hydroxide was added dropwise until SQP33 fully dissolved. Once a concentration of 6 mg/mL was achieved, a total volume of 5 mL per kg of body weight was administered IV via the tail vein 1 hour after SQL70 injection. Four additional daily doses were given at 24, 48, 72, and 96 hours after the first dose. Group 8: 30 mg of SQP33 per kg of body weight was dissolved in saline at pH 7.4, and sodium hydroxide was added dropwise until SQP33 fully dissolved. Once a concentration of 6 mg/mL was achieved, a total volume of 5 mL per kg of body weight was administered IV via the tail vein 1 hour after SQL70 injection. Four additional daily doses were given at 24, 48, 72, and 96 hours after the first dose.

In Life Assessment

All animals had 200 µL of blood collected via jugular vein catheter at multiple time-points throughout the study. Groups 1-5 received blood-draws pretreatment, and at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, and 120 hours after Dox HCl or SQP33 IV dose. In groups 6 and 7, half of the animals (n = 3 per group) received blood-draws pretreatment, and at 0.5, 1, 2, 3, 4, 6, 8, 12, 24 (before second dose), 48 (before third dose), 72 (before fourth dose), 72.5, 73, 74, 75, 76, 78, 80, 84, 96 (before fifth dose), 120, 144, 168, 192, 216, 240 hours after first SQP33 dose, and the other half (n = 3 per group) received blood-draws pretreatment, and at 24 (before second dose), 48 (before third dose), 48.5, 49, 50, 51, 52, 54, 56, 60, 72 (before fourth dose), 96 (before fifth dose) 96.5, 97, 98, 99, 100, 102, 104, 108, 120, 144, 168, 192, 216, 240 h after first SQP33 dose. The blood-draws for groups 6 and 7 were split in this manner to decrease the amount of blood drawn from each animal to a safe level. All animals were euthanized 120 h after their last IV dose (their only IV dose for groups 1-5). For groups 1, 6, and 7, tissue samples were collected from the liver, heart and kidney, and were homogenized in deionized water and the concentration of active Dox (for tissue and plasma) and SQP33 protodrug (for plasma only) was quantified by LC-MS.

Biopolymer residency in mice.

Study Design

To characterize the biodistribution of the SQL70 biopolymer given as part of SQ3370 treatment, 3 female C57BL/6 mice were treated with a 100 µL SC injection of SQL70 biopolymer. The SQL70 biopolymer used was modified with cyanine-5.5 (Cy5.5) fluorophore to allow for in vivo detection. Biopolymer residency was assessed for 100 days via In Vivo Imaging System (IVIS).

Dosing Procedure

100 µL of Cy5.5-labeled-SQL70 biopolymer was injected SC on Day 1, 0h.

In Life Assessment

Biopolymer residency was assessed for 100 days via IVIS at 1h, 4h, 8h, 24h (Day 2), Day 3, Day 4, Day 7, Day 8, Day 18, and then once weekly until termination and last day. Fluorescence imaging was conducted using 675 nm excitation and 710 nm emission filters to visualize Cy5.5, and the signal was quantified as total radiant efficiency of the biopolymer injection site.

Pharmacokinetics and target tissue exposure in mice.

Study Design

5 female C57BL/6 mice were treated with a 100 µL SC injection of Tz-modified NaHA biopolymer (prototype of SQL70 biopolymer) followed by 15 IV injections of SQP33 protodrug, at 43 mg/kg/dose Dox Eq, into the tail vein over 19 days (one dose per day on weekdays only).

Dosing Procedure

100 µL of Tz-modified NaHA biopolymer was injected SC. 60 mg of SQP33 per kg of body weight was dissolved in saline at pH 7.4, and sodium hydroxide was added dropwise until SQP33 fully dissolved. Once a concentration of 6 mg/mL was achieved, a total volume of 10 mL per kg of body weight was administered IV via the tail vein once a day, on weekdays only, for 15 doses.

In Life Assessment

20 µL of blood was collected at different time points following the first SQP33 protodrug injection. Timepoints were at 1 hour (n = 3), 24 hours (n = 2), and on Days 5, 12, and 19 (n = 5). Biopolymer injection site tissue was collected on Days 19 (n = 1) and 26 (n = 4) after animals were euthanized. Injection site tissue samples were homogenized in deionized water. Tissue and plasma concentration of active Dox and SQP33 protodrug was quantified by LC-MS.

Antitumor activity of SQ3370.

Study Design

Immunocompetent female C57BL/6 mice were inoculated SC in the right flank with $5x10^5$ MC38 tumor cells (Day 0). When the average tumor volume reached approximately 100 mm^3 (Day 7), animals were randomly grouped according to body weight and tumor volume into 2 treatment groups. Treatments started with a 100 µL peritumoral injection of SQL70 biopolymer to both groups, followed by 5 daily IV doses of Saline (n = 5) or SQP33 protodrug at 28.6 mg/kg/dose Dox Eq (n = 10). On Day 38, the remaining animals, which were all from the SQ3370-treatment group, received a second 100 µL peritumoral injection of SQL70 biopolymer followed by another 5 daily doses of SQP33 protodrug at 11.9 mg/kg/dose Dox Eq. Tumor volumes were measured thrice weekly and survival was assessed until all animals died or were sacrificed when tumor volumes reached ≥ 2000 mm^3.

Cell Culture and Inoculation

MC38 cells were cultured with DMEM supplemented with 10% heat inactivated fetal bovine serum (FBS) at 37 ºC in 5% CO_2 incubator. Cells were passaged 2 times a week. Cells were harvested, counted, passaged, and inoculated when around 70% confluent. $5x10^5$ cells suspended in 100 µL PBS mixed with 50% matrix gel were inoculated subcutaneously into the right flank.

Dosing Procedure

SQP33 powder was dissolved in sterile PBS and adjusted to pH 7.2 with NaOH, sterile filtered, and administered IV via the tail vein once daily for 5 consecutive days. An equal volume of Saline was administered IV via the tail vein to the control group once daily for 5 consecutive days. 100 µL of SQL70 biopolymer was injected intra/peritumorally 1 hour before the first IV dose.

In Life Assessment

Tumor volume was measured thrice weekly in two dimensions using a caliper, and the volume was expressed in mm^3 using the formula: $V = 0.5\ a \times b^2$, where *a* and *b* are the long and short diameters of the tumor, respectively. At the time of routine monitoring, the animals were checked for any adverse effects of tumor growth and/or treatment on normal behavior such as effects on mobility, food and water consumption (by observation only), and body weight gain/loss (body weights had measured twice weekly in the pre-dosing phase and daily in the dosing phase), eye/hair matting and any other abnormal effect, including tumor ulceration. Animals were terminated if tumor volume reached ≥ 2000 mm^3.

Table S1. Plasma pharmacokinetic parameters of active Dox and SQP33 protodrug in rats treated with 5 doses of SQP33 without SQL70 biopolymer.

Day	Active Dox		SQP33 Protodrug	
	$AUC_{0.5-24\ h}$# (ng*h/mL)	**C_{max}# (ng/mL)**	**$AUC_{0.5-24\ h}$# (ng*h/mL)**	**C_{max}# (ng/mL)**
1	41.8 ± 6.9	35.2 ± 3.7	1418 ± 176	2095.8 ± 221.8
2	No data recorded	No data recorded	No data recorded	No data recorded
3	41.1 ± 5.4	32.0 ± 5.3	1584 ± 331	1981.4 ± 427
4	46.6 ± .6	41.6 ± 0.5	1297 ± 172.1	1955.2 ± 236
5	54.5 ± 10.6	42.1 ± 6.0	1778 ± 356.3	2517.9 ± 548.2
Avg	36.5	37.7	1519.3	2137.6

AUC = area under the concentration-time curve; Avg = average; C_{max} = maximum observed concentration (time of observing C_{max} = 30 minutes); Dox = doxorubicin; Dox Eq = doxorubicin hydrochloride equivalents; SEM = standard error of mean.
# AUCs were determined using the Riemann Sum method. AUC and C_{max} results show mean ± SEM.

Table S2. Plasma pharmacokinetic parameters of active Dox and SQP33 protodrug in rats treated with 5 doses of SQP33 after a SC SQL70 biopolymer injection.

Day	Active Dox			SQP33 Protodrug		
	C_{max}[#] (ng/mL)	**$AUC_{0.5-24\ h}$[#] (ng*h/mL)**	**AUC $Ratio_1$[§]**	**C_{max}[#] (ng/mL)**	**$AUC_{0.5-24\ h}$[#] (ng*h/mL)**	**AUC $Ratio_2$[†]**
1	1953.3 ± 241.8	1928 ± 104.2	47.0	1.2 ± 0.2	0.6 ± 0.0	2444
2	1206.7 ± 54.4	2015± 25.3	N/A	3.6 ± 0.4	2.7 ± 0.2	N/A
3	644.3 ± 73.4	1221 ± 24.6	29.8	805.4 ± 144.2	413.4 ± 72	3.83
4	190 ± 6.1	655.5 ± 31.2	14.1	1506.9 ± 76.4	1014± 47.5	1.3
5	112.7 ± 3.2	462.9 ± 25.5	8.5	1847.9 ± 263.0	1337 ± 198	1.3

AUC = area under the concentration-time curve; C_{max} = maximum observed concentration (time of observing C_{max} = 30 minutes); Dox = doxorubicin; Dox Eq = doxorubicin hydrochloride equivalents; SEM = standard error of mean.

[#] AUCs were determined using the Riemann Sum method. AUC and C_{max} results are shown as mean ± SEM in each group.

[§] $Ratio_1$ was calculated by: [AUC with SQL70 biopolymer (from this table) ÷ AUC without SQL70 biopolymer (from **Table S1**)].

[†] $Ratio_2$ was calculated by: [AUC without SQL70 biopolymer (from **Table S1**) ÷ AUC with SQL70 biopolymer (from this table)].

Table S3. Concentration of active Dox and SQP33 protodrug in plasma or biopolymer injection site in mice treated with 15 SQP33 protodrug doses following a SC biopolymer injection.

Day	Location	Active Dox Conc.	SQP33 Protodrug Conc.	Percent*
19	Plasma	36.2 ng/mL	1325.6 ng/mL	2.7%
19	Injection site	151 ng/g	4.17 ng/g	3621%
26	Injection site	77.5 ng/g	BLOQ	N/A

BLOQ = below limit of quantification; Conc = concentration; Dox = doxorubicin; N/A = not applicable.
* Percent active Dox calculated by [active Dox conc ÷ SQP33 protodrug conc] in the same location and on the same day.

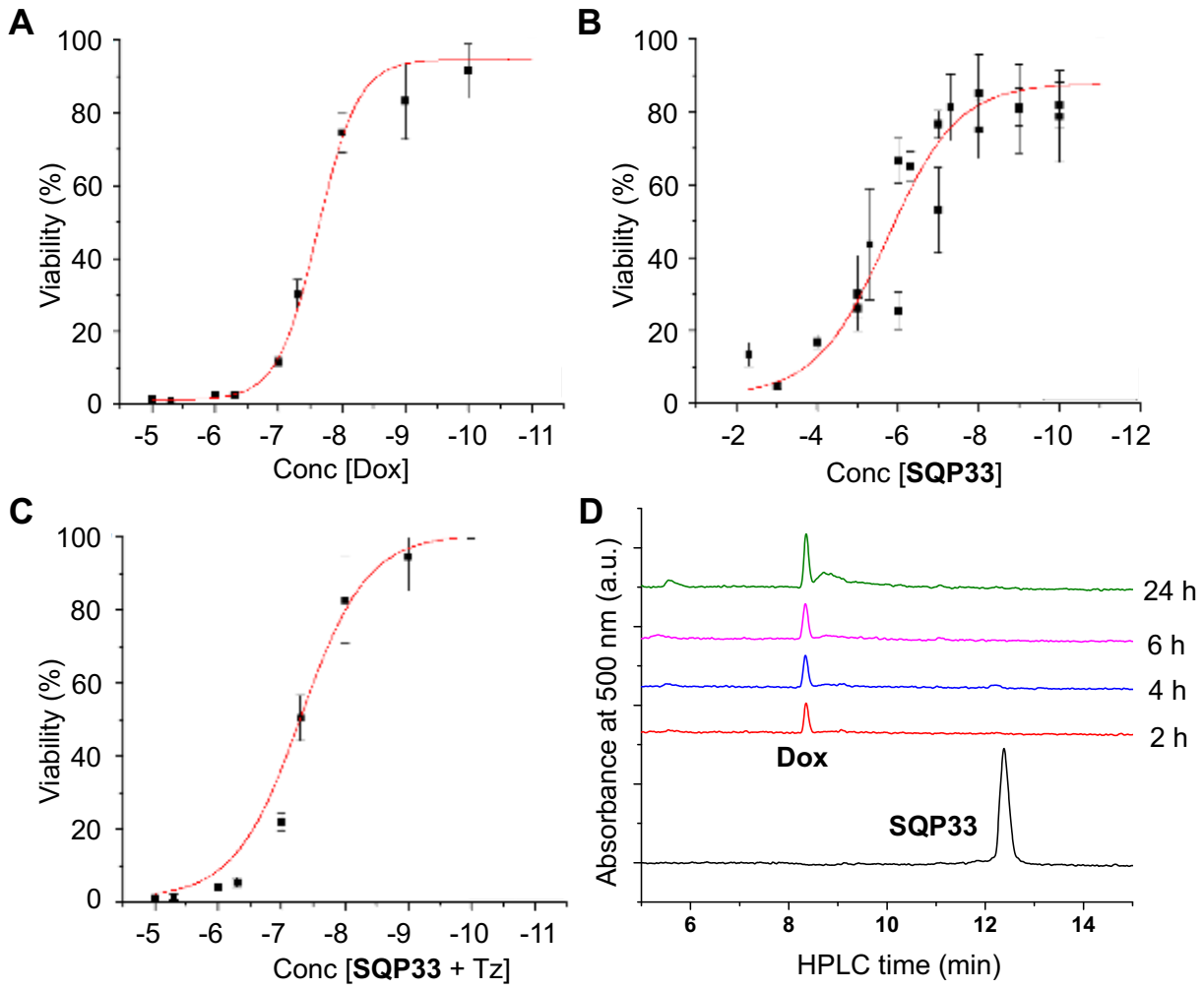


Figure S1. *In vitro* studies: (**A**) Viability of MC38 cells as a function of Dox concentration; (**B**) Viability of MC38 cells as a function of SQP33 concentration; (**C**) Viability of MC38 cells as a function of SQP33 + Tz concentration; (**D**) *in vitro* activation of SQP33 upon addition of the biopolymer.

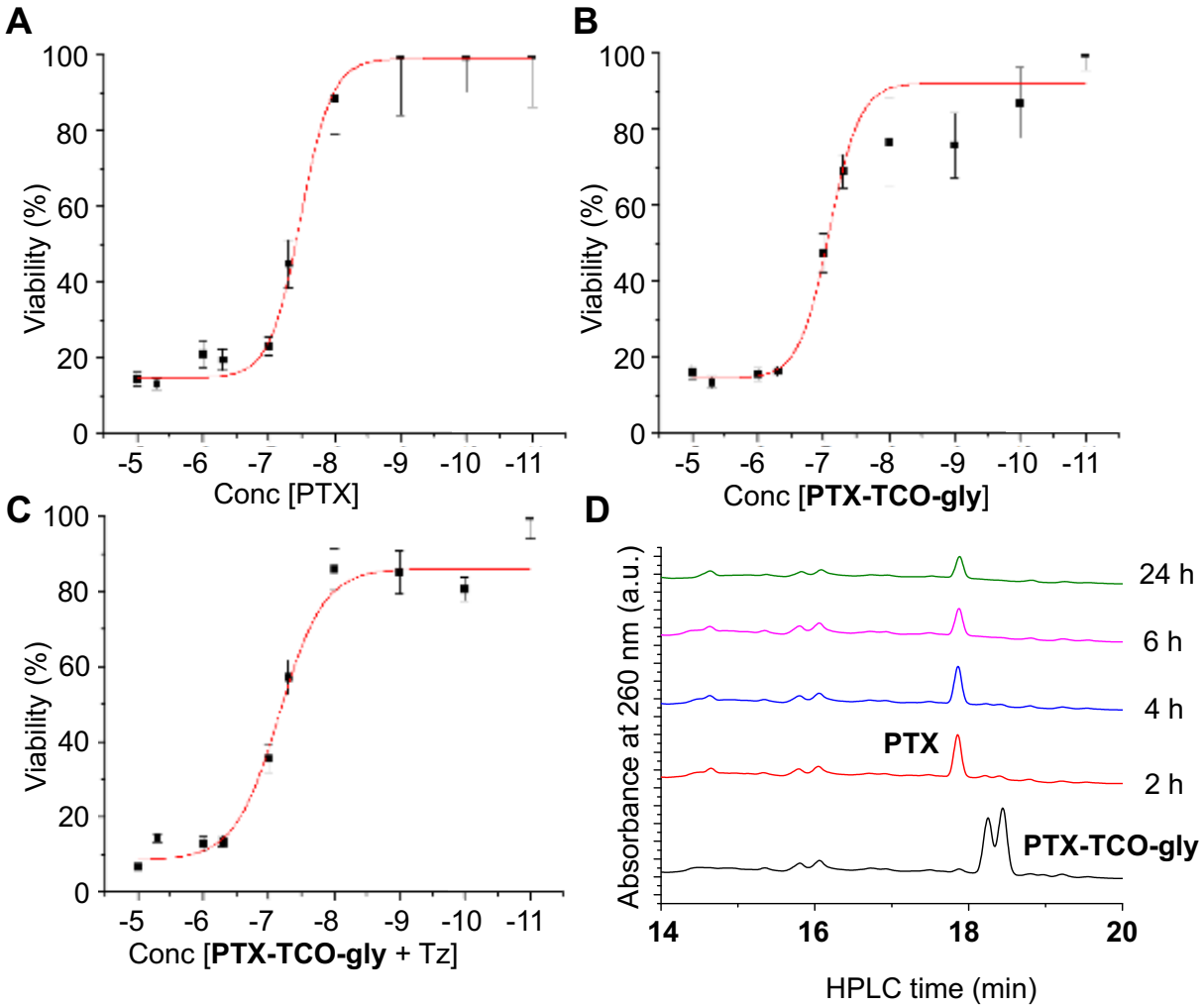


Figure S2. *In vitro* studies: (**A**) Viability of MC38 cells as a function of PTX concentration; (**B**) Viability of MC38 cells as a function of **PTX-TCO-gly** concentration; (**C**) Viability of MC38 cells as a function of **PTX-TCO-gly** + Tz concentration; (**D**) *in vitro* activation of **PTX-TCO-gly** upon addition of the biopolymer.

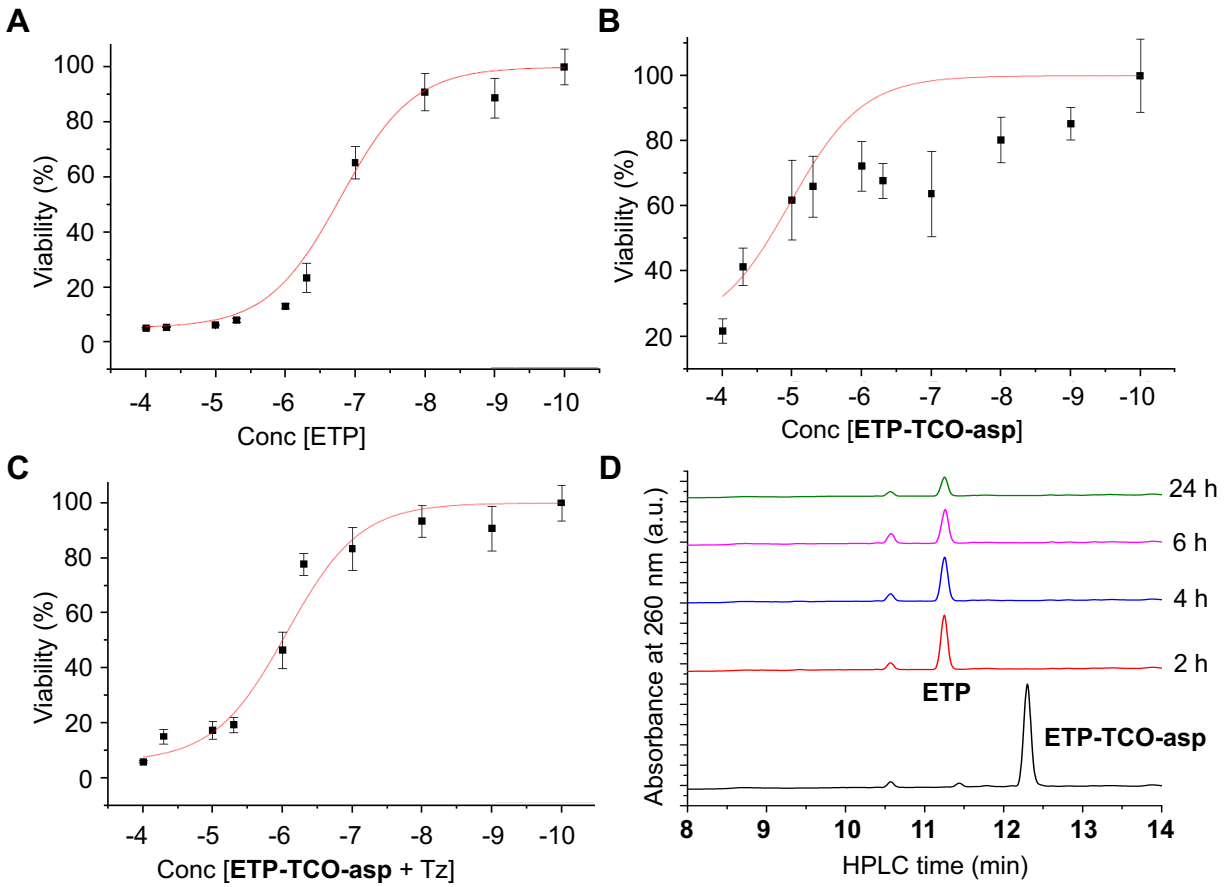


Figure S3. *In vitro* studies: (**A**) Viability of MC38 cells as a function of ETP concentration; (**B**) Viability of MC38 cells as a function of **ETP-TCO-asp** concentration; (**C**) Viability of MC38 cells as a function of **ETP-TCO-asp** + Tz concentration; (**D**) *in vitro* activation of **ETP-TCO-asp** upon addition of the biopolymer.

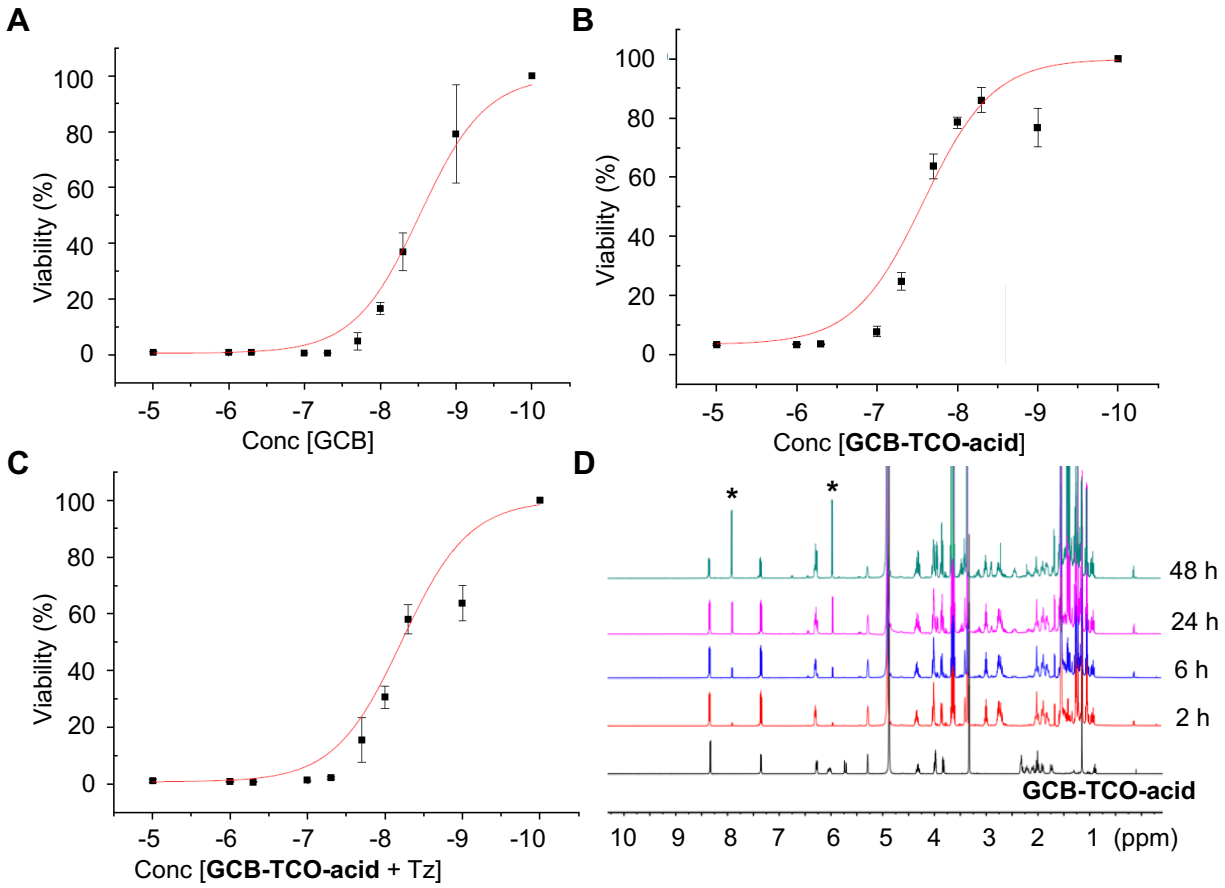


Figure S4. *In vitro* studies: (**A**) Viability of MC38 cells as a function of GCB concentration; (**B**) Viability of MC38 cells as a function of **GCB-TCO-acid** concentration; (**C**) Viability of MC38 cells as a function of **GCB-TCO-acid** + Tz concentration; (**D**) *in vitro* activation of **GCB-TCO-acid** upon addition of the biopolymer. Asterisks indicate the NMR signals corresponding to activated GCB.

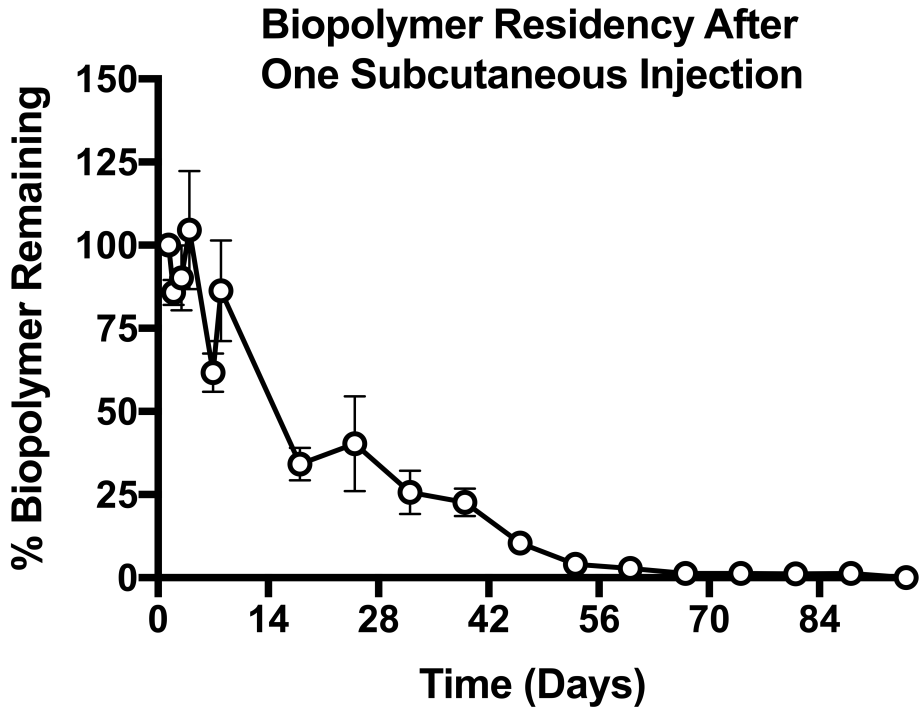


Figure S5. 3 C57BL/6 mice were treated with a 100 µL SC injection of Cy5.5-labeled-SQL70 biopolymer. Biopolymer residency was assessed for 100 days via IVIS at 1h, 4h, 8h, 24h (Day 2), Day 3, Day 4, Day 7, Day 8, Day 18, and then once weekly until termination and last day. Fluorescence imaging was conducted using 675 nm excitation and 710 nm emission filters, and the signal was quantified as total radiant efficiency of the biopolymer injection site. Percent biopolymer remaining was determined by fluorescence radiant efficiency of the injection site relative to the 8-hour post-injection value and is presented as mean ± SEM of n = 3. Fluorescent signal remained evident at the biopolymer injection site for approximately 2 months.

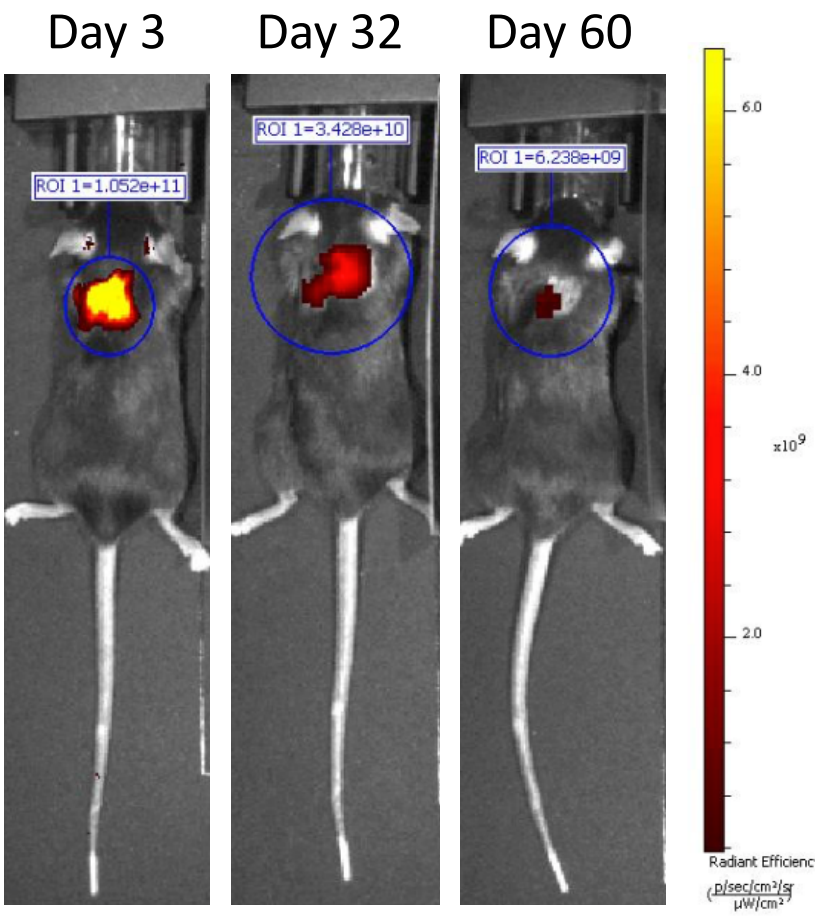


Figure S6. Representative in vivo images of SQL70 biopolymer residency in mice. 3 C57BL/6 mice were treated with a 100 µL SC injection of Cy5.5-labeled-SQL70 biopolymer. Biopolymer residency was assessed for 100 days via IVIS at 1h, 4h, 8h, 24h (Day 2), Day 3, Day 4, Day 7, Day 8, Day 18, and then once weekly until termination and last day. Fluorescence imaging was conducted using 675 nm excitation and 710 nm emission filters, and the signal was quantified as total radiant efficiency of the biopolymer injection site. Biopolymer remained evident at the injection site for approximately 2 months.

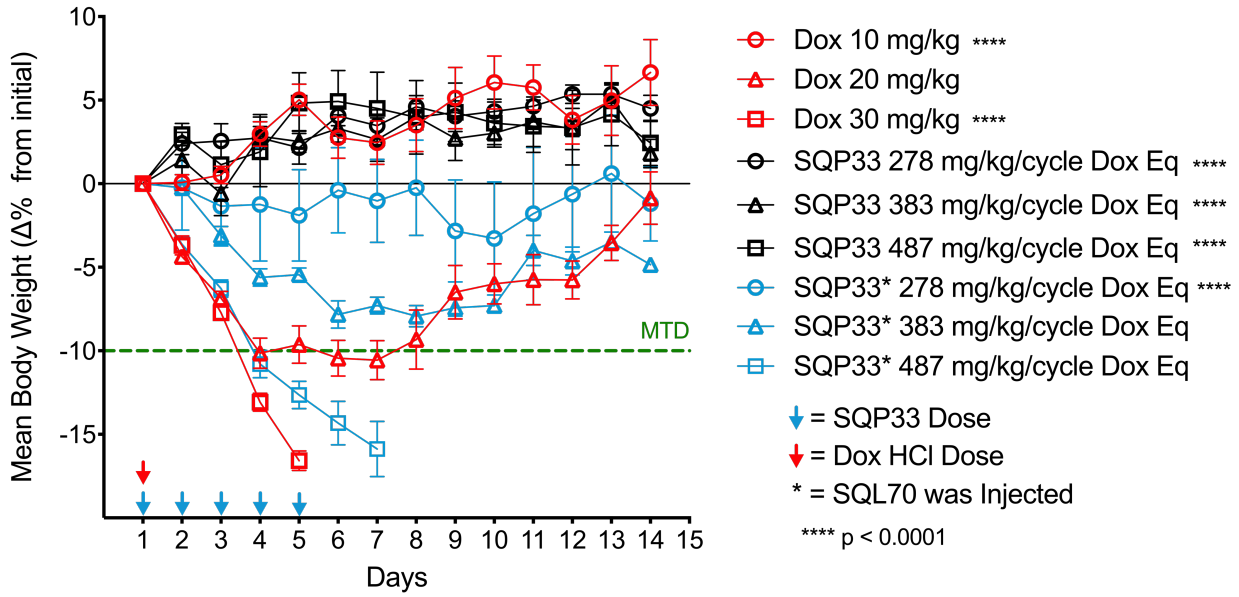


Figure S7. Systemic toxicity of SQ3370 vs. conventional Dox HCl in mice. C57BL/6 mice (n = 4-5 per group) were treated with one dose of Dox HCl, at 10, 20, or 30 mg/kg, and compared to mice treated with 5 daily doses of SQP33, at 278, 383, or 487 mg/kg/cycle Dox Eq, in the presence or absence of SQL70 biopolymer. Mice were weighed daily for up to 14 days once treatment started. Mean body weight loss ≥10% per group indicated the maximum tolerated dose (MTD) of the treatment given. Data shows group mean ± SEM of percent change in body weights and significance was established by unpaired t-test with Welch's correction as compared to the 20 mg/kg Dox HCl group (the MTD of Dox HCl).

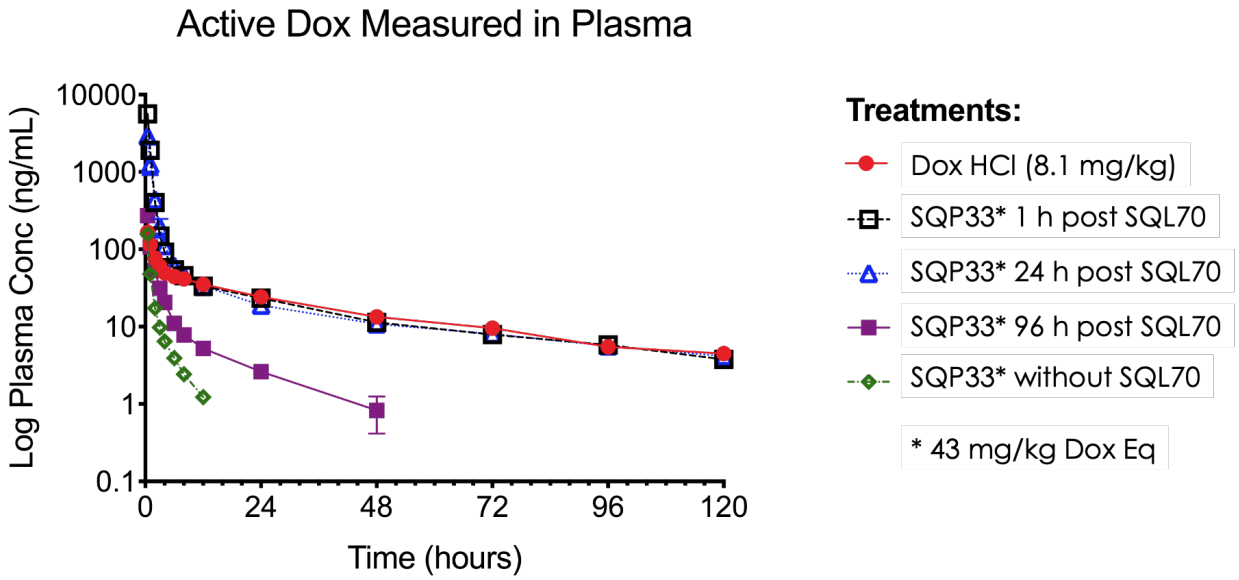


Figure S8. Sprague Dawley rats were treated with 1 IV dose of SQP33 protodrug, at 43 mg/kg Dox Eq, in the presence or absence of SC SQL70 biopolymer injection. SQP33 protodrug was administered at different timepoints post SQL70 injection for each group. Control animals were given 1 IV dose of Dox HCl at 8.1 mg/kg. Plasma samples were assessed for active Dox concentrations using LC-MS. Blood was drawn for analysis at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 48, 72, 96, and 120 hours following IV dose. Data is plotted as mean ± SEM for each group (n = 3). Plots end when the measurements following that time are BLOQ (1 ng/mL).

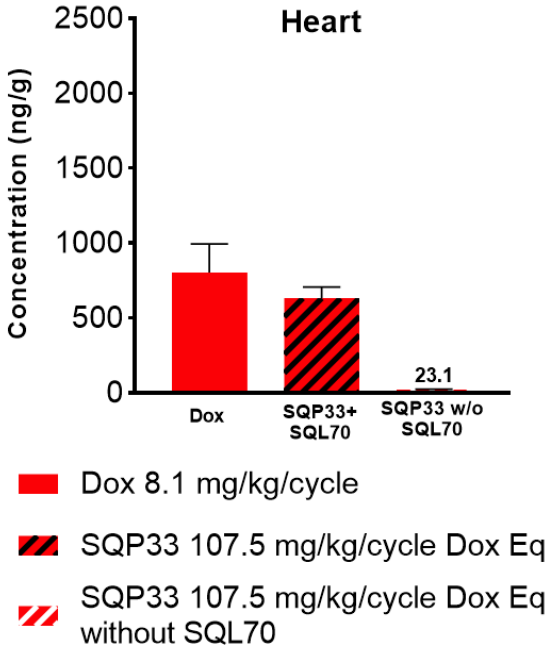


Figure S9. Sprague Dawley rats were treated with 5 doses of SQP33 protodrug in the presence or absence of SQL70 biopolymer. Animals given a single dose of Dox HCl were used as control. Heart tissue samples were collected 120 hours after the final SQP33 protodrug or Dox HCl dose was administered. Tissue samples were assessed for active Dox concentration using LC-MS. Data is plotted as mean ± SEM (n = 3-6 per group).

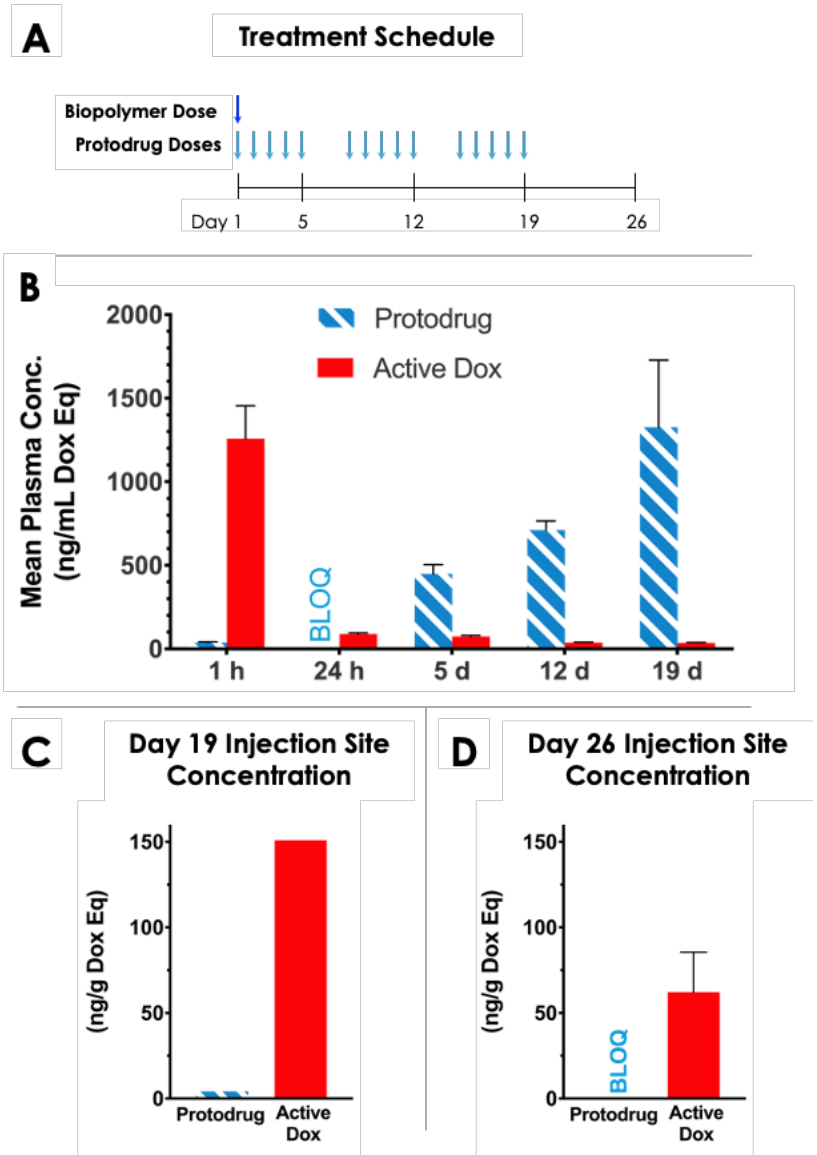
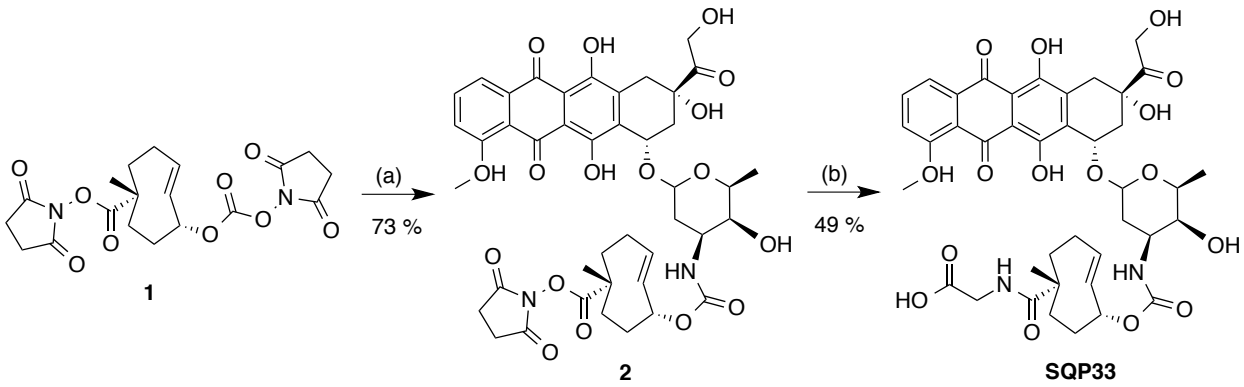


Figure S10. Plasma and biopolymer injection site concentrations of SQP33 protodrug and active Dox in mice. C57BL/6 mice (n = 5) received (A) one biopolymer injection followed by 15 SQP33 protodrug doses given over 19 days (5 daily doses per week, on weekdays only). Plasma was collected at 1 h, 24 h, Day 5, 12, and 19. Plasma concentrations of SQP33 protodrug and active Dox are shown in (B). At 1 h (on Day 19; n = 1) and 7 days (on Day 26; n = 4) after the last IV dose, tissue from the biopolymer injection site was collected, with local distribution of SQP33 protodrug and active Dox shown in (C) and (D). All samples were analyzed by LC-MS for both analytes and are displayed as mean + SEM.

Synthetic Procedures:



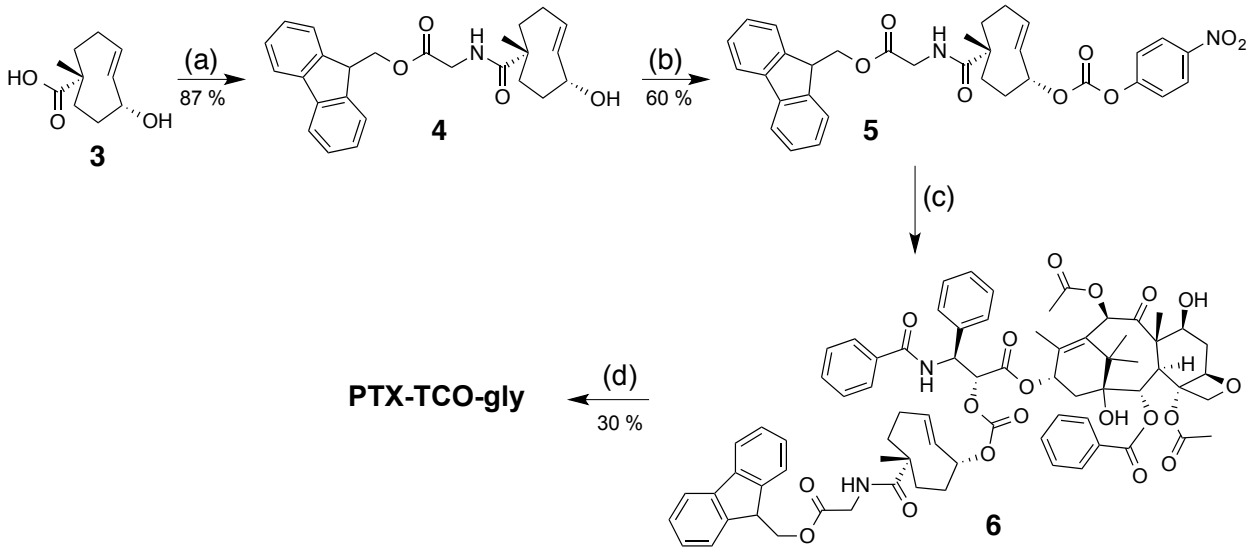
Synthesis of the compound 2

Compound **1** was synthesized using previously reported method [*Bioconj. Chem.* **2016**, *27*, 1697-1706]. Compound **1** (1.75 g, 4.14, 1.2 equiv) was added in one portion to DMF (75 mL) solution of Dox·HCl (2 g, 3.45 mmol) and DIPEA (1.34 g, 10.35 mmol, 3 equiv). The resulting solution was stirred for 12 h and quenched with water (150 mL). The product was extracted with EtOAc (3x200 mL) and the combined organic phases were washed with brine and dried over Na_2SO_4. After removal of the organic solvents, the residue was purified by flash chromatography using a gradient of MeOH in CH_2Cl_2 (0-5%) to afford compound **2**. Yield = 2.16 g, 73%. The NMR spectrum is identical to the previously published spectra [*Bioconj. Chem.* **2016**, *27*, 1697-1706].

Synthesis of SQP33

TMS-Cl (638 mg, 5.9 mmol, 10 equiv) was added in one portion to a suspension of glycine (440 mg, 5.9 mmol, 10 equiv) in 50 mL of $CHCl_3$-MeCN (60%-40%). The resulting mixture was stirred at reflux (80 ^oC) for 2 h and then cooled to rt. DIPEA (2 mL, 12 mmol, 20 equiv) and Dox-TCO-NHS **2** (500 mg, 0.58 mmol) were added at room temperature and the mixture was then stirred at 65 ^oC for 6 h. After removal of the solvents, the residue was dissolved in water (5 mL) and purified by HPLC using a 0-50% gradient of MeCN (0.1% formic acid) in water (0.1% formic acid) to afford SQP33 (235 mg, 49%) as orange solid.

^1H NMR (500 MHz, DMSO) δ 13.93 (s, 1H), 13.17 (s, 1H), 7.77 (dd, *J* = 42.5, 36.0 Hz, 2H), 7.56 (s, 2H), 6.72 – 6.56 (m, 1H), 5.94 – 5.72 (m, 1H), 5.65 – 5.51 (m, 1H), 5.40 (s, 1H), 5.21 (s, 1H), 4.96 – 4.82 (m, 2H), 4.59 (s, 2H), 4.17 (d, *J* = 6.5 Hz, 1H), 3.94 (s, 3H), 3.79 – 3.52 (m, 4H), 3.52 – 3.36 (m, 2H), 3.06 – 2.88 (m, 1H), 2.88 – 2.73 (m, 1H), 2.28 – 1.94 (m, 5H), 1.91 – 1.77 (m, 3H), 1.77 – 1.56 (m, 3H), 1.48 (t, *J* = 11.5 Hz, 2H), 1.12 (t, *J* = 21.0 Hz, 3H), 0.97 (s, 3H).
^{13}C NMR (125 MHz, DMSO) δ 213.32, 186.11, 185.81, 182.18, 172.19, 160.73, 155.92, 155.75, 154.52, 135.64, 134.52, 134.06, 133.56, 131.26, 131.10, 119.66, 118.97, 118.64, 110.74, 110.51, 100.87, 75.93, 72.20, 69.73, 68.84, 68.68, 67.25, 64.36, 55.62, 45.01, 43.96, 40.93, 38.97, 35.72, 35.39, 32.55, 30.66, 30.46, 29.46, 16.80, 15.93, 15.78.
HRMS (*ESI*) *m/z*: calcd. for $C_{40}H_{46}N_2O_{16}$ $[M+Na]^+$ 833.2740, found 833.2743.



Synthesis of the compound 4

Compound **3** (1.0 g, 5.4 mmol) and Fmoc-protected glycine·HCl (3.15 g, 10.8 mmol) were dissolved in anhydrous DMF (25 mL). HATU (4.13 g, 10.8 mmol mmol) and DIPEA (3.5 g, 27.1 mmol) were added and the resulting mixture was stirred overnight. The reaction mixture was poured onto water (50 mL). The product was extracted with EtOAc (3 x 30 mL). The combined organic phase was dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography using a gradient of EtOAc in hexanes (20-40%) to give compound **4** (2.0 g, 87%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 7.6 Hz, 2H), 7.57 (d, *J* = 7.5 Hz, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.36 – 7.24 (m, 2H), 6.26 (t, *J* = 4.8 Hz, 1H), 6.14 – 6.00 (m, 1H), 5.62 (d, *J* = 16.6 Hz, 1H), 4.44 (d, *J* = 7.1 Hz, 3H), 4.21 (t, *J* = 7.0 Hz, 1H), 4.06 (t, *J* = 12.1 Hz, 2H), 2.79 (t, *J* = 5.2 Hz, 3H), 2.64 (d, *J* = 14.7 Hz, 1H), 2.36 – 2.16 (m, 2H), 2.15 – 1.96 (m, 2H), 1.85 (ddd, *J* = 32.9, 21.9, 10.1 Hz, 3H), 1.58 (dd, *J* = 15.6, 6.2 Hz, 1H), 1.13 (d, *J* = 21.4 Hz, 3H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 181.01, 170.31, 143.44, 143.42, 141.27, 135.17, 130.11, 127.92, 127.22, 125.02, 120.10, 69.51, 67.14, 46.62, 45.76, 44.38, 41.63, 38.63, 38.40, 31.03, 30.10, 17.69.
HRMS (*ESI*) *m/z*: calcd. for $C_{26}H_{29}NO$ $[M+H]^+$ 420.2169, found 420.2166.

Synthesis of the compound 5

Pyridine (1.13 mg, 14.3 mmol) was added to a solution of **8** (2.0 g, 4.77 mmol) in freshly distilled CH_2Cl_2 (20 mL). A solution of p-nitrophenyl chloroformate (1.24 g, 6.2 mmol) in CH_2Cl_2 (5 mL) was slowly added via syringe at 0 °C. The mixture was stirred at rt for 12 h. The solvent was removed under reduced pressure. The product was purified by flash chromatography using a gradient of EtOAc in hexanes (20-40%) to give compound **5** (1.65 g, 60%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.32 – 8.26 (m, 2H), 7.79 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.5 Hz, 2H), 7.47 – 7.38 (m, 4H), 7.34 (td, *J* = 7.5, 0.9 Hz, 2H), 6.13 – 5.98 (m, 2H), 5.67 (dd, *J* = 16.7, 2.3 Hz, 1H), 5.31 (s, 1H), 4.50 (d, *J* = 7.1 Hz, 2H), 4.25 (t, *J* = 7.0 Hz, 1H), 4.20 – 4.03 (m, 2H), 2.46 – 2.38 (m, 1H), 2.38 – 2.26 (m, 1H), 2.18 (tdd, *J* = 27.2, 17.0, 10.1 Hz, 2H), 2.07 – 1.85 (m, 3H), 1.77 (dd, *J* = 15.3, 6.3 Hz, 2H), 1.22 (d, *J* = 28.2 Hz, 3H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 180.09, 170.17, 155.55, 151.58, 145.37, 143.35, 141.31, 132.87, 129.43, 127.98, 127.24, 125.33, 124.97, 121.79, 120.15, 67.24, 46.63, 45.50, 44.33, 41.63, 35.75, 31.16, 31.04, 17.86.
HRMS (*ESI*) *m/z*: calcd. for $C_{33}H_{32}N_2O_8$ $[M+H]^+$ 585.2231, found 585.2237.

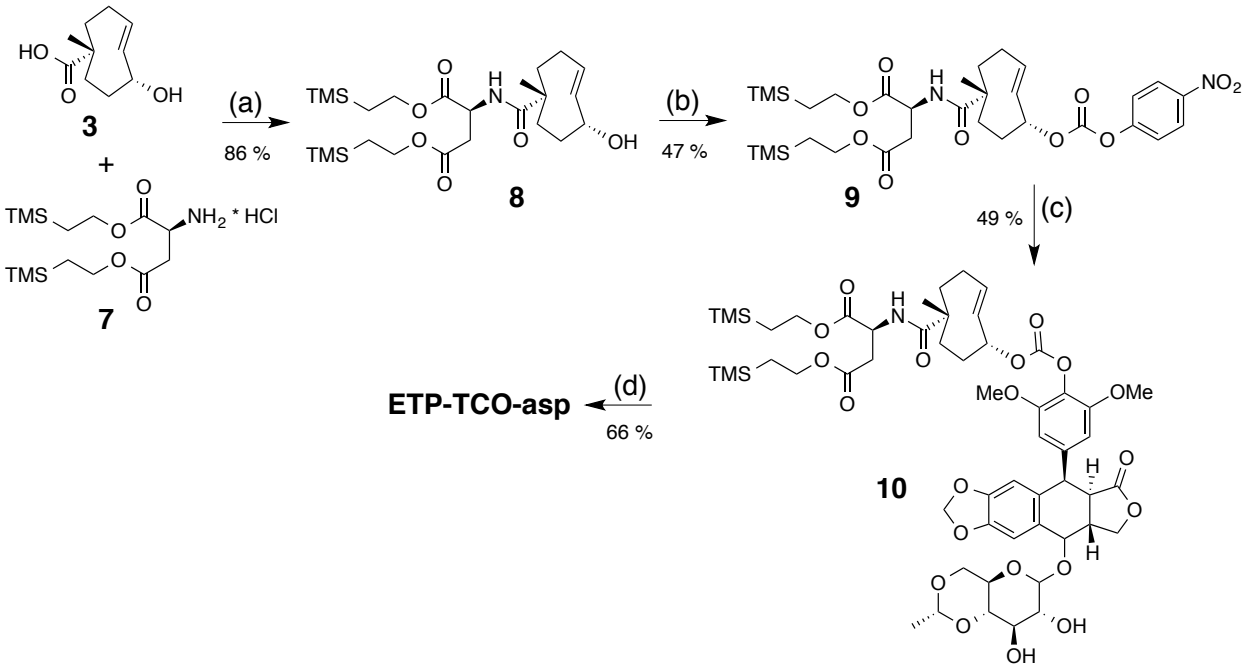
Synthesis of PTX-TCO-gly

Compound **9** (47.2 mg, 0.082 mmol) and DMAP (14 mg, 0.117 mmol) were added to a solution of PTX (50.0 mg, 0.058 mmol) in CH_2Cl_2 (10 mL). The resulting solution was stirred for 3 d. The reaction was quenched with water (30 mL). The product was extracted with CH_2Cl_2 (3 x 50 mL) and the combined organic phase was dried with Na_2SO_4 and concentrated to give crude compound **6** which was used without further purification. Compound **6** was added to a solution of 10% piperidine in DMF (8 mL). The resulting mixture was stirred overnight at rt. The reaction was quenched with water (20 mL) and the product was extracted with EtOAc (3 x 30 mL). The combined organic phase was washed with water (3 x 50 mL) and brine, and concentrated under reduced pressure. The residue was purified by flash chromatography using a gradient of MeOH in CH_2Cl_2 (5-10%) to give **PTX-TCO-gly** (20 mg, 30% over 2 steps).

^{1}H NMR (500 MHz, MeOD) δ 8.13 (d, *J* = 8.3 Hz, 2H), 7.85 (d, *J* = 8.3 Hz, 2H), 7.69 (t, *J* = 7.0 Hz, 1H), 7.60 (td, *J* = 7.7, 3.0 Hz, 2H), 7.58 – 7.51 (m, 3H), 7.47 (t, *J* = 7.6 Hz, 4H), 7.29 (dd, *J* = 16.0, 7.5 Hz, 1H), 6.46 (d, *J* = 3.6 Hz, 1H), 6.09 (dd, *J* = 16.2, 8.0 Hz, 1H), 6.04 – 5.79 (m, 2H), 5.73 – 5.61 (m, 2H), 5.52 (dd, *J* = 16.8, 6.8 Hz, 1H), 5.17 (d, *J* = 15.5 Hz, 1H), 5.01 (d, *J* = 9.6 Hz, 1H), 4.39 – 4.31 (m, 1H), 4.20 (s, 2H), 3.82 (dd, *J* = 7.0, 4.3 Hz, 2H), 2.56 – 2.45 (m, 1H), 2.43 (d, *J* = 1.7 Hz, 3H), 2.33 – 2.11 (m, 7H), 2.11 – 1.88 (m, 7H), 1.88 – 1.73 (m, 3H), 1.67 (d, *J* = 2.3 Hz, 4H), 1.15 (s, 8H).

^{13}C NMR (126 MHz, MeOD) δ 203.77, 170.29, 169.96, 168.99, 168.83, 166.27, 153.62, 153.55, 140.94, 140.79, 136.74, 134.02, 133.58, 133.48, 133.25, 131.99, 131.73, 131.58, 129.99, 129.81, 128.73, 128.35, 128.20, 127.28, 84.51, 80.84, 77.62, 77.04, 76.96, 76.77, 76.62, 76.07, 75.40, 74.85, 71.76, 70.91, 57.82, 54.14, 54.05, 46.47, 45.04, 44.00, 43.19, 36.11, 35.26, 35.11, 34.99, 30.59, 30.51, 29.35, 25.57, 21.89, 21.01, 19.43, 16.83, 13.57, 9.08.

HRMS (*ESI*) *m/z*: calcd for $C_{60}H_{68}N_2O_{19}$ $[M+H]^+$ 1121.4489, found 1121.4495.



Synthesis of the compound 8
Compound **3** (300 mg, 1.63 mmol) and TMS ethyl aspartate **4** (1.21 g, 3.26 mmol) were dissolved in anhydrous DMF (15 mL). HATU (1.24 g, 3.26 mmol) and DIPEA (1.05 g, 8.56 mmol) were added and the resulting mixture was stirred overnight. The reaction mixture was poured onto water (50 mL). The product was extracted with EtOAc (3 x 30 mL). The combined organic phase was dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography using a gradient of EtOAc in hexanes (20-40%) to give compound **8** (700 mg, 86%).

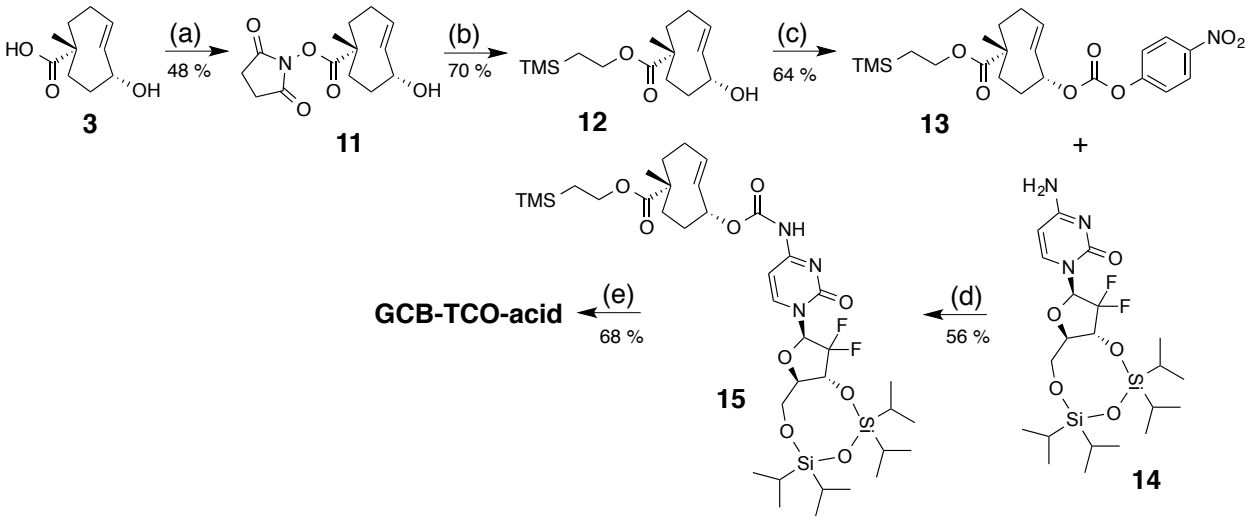
^{1}H NMR (500 MHz, $CDCl_3$) δ 6.57 (dd, *J* = 7.9, 2.9 Hz, 1H), 6.10 – 6.00 (m, 1H), 5.63 (dd, *J* = 16.6, 2.4 Hz, 1H), 4.73 (qd, *J* = 8.4, 4.3 Hz, 1H), 4.45 (d, *J* = 4.8 Hz, 1H), 4.26 – 4.18 (m, 2H), 4.18 – 4.13 (m, 2H), 2.94 (dq, *J* = 16.2, 4.1 Hz, 1H), 2.75 (dt, *J* = 16.9, 4.8 Hz, 1H), 2.26 (dqd, *J* = 35.4, 11.9, 3.7 Hz, 2H), 2.14 – 2.04 (m, 2H), 1.98 (ddd, *J* = 19.3, 9.7, 5.9 Hz, 1H), 1.93 – 1.85 (m, 1H), 1.85 – 1.76 (m, 2H), 1.56 (ddd, *J* = 28.0, 15.5, 6.2 Hz, 1H), 1.10 (s, 3H), 1.00 – 0.95 (m, 4H), 0.04 – 0.01 (m, 18H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 180.20, 171.34, 171.12, 135.12, 130.12, 69.47, 64.17, 63.28, 48.65, 45.82, 44.32, 38.38, 36.15, 30.96, 29.88, 17.60, 17.33, 17.29, -1.55, -1.56.
HRMS (*ESI*) *m/z*: calcd for $C_{24}H_{45}NO_6Si_2$ $[M+H]^+$ 500.2858, found 500.2856.

Synthesis of compound 9
Pyridine (332 mg, 4.2 mmol) was added to a solution of compound **8** (700 mg, 1.4 mmol) in freshly distilled CH_2Cl_2 (20 mL). A solution of p-nitrophenyl chloroformate (366 mg, 1.8 mmol) in DCM (5 mL) was slowly added via syringe at 0 °C. The mixture was stirred at rt for 12 h. The solvent was removed under reduced pressure. The product was purified by flash chromatography using a gradient of EtOAc in hexanes (0-20%) to give compound **9** (440 mg, 47%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 8.33 – 8.22 (m, 2H), 7.45 – 7.37 (m, 2H), 6.63 (d, *J* = 7.8 Hz, 1H), 6.14 – 6.03 (m, 1H), 5.67 (dd, *J* = 16.7, 2.5 Hz, 1H), 5.30 (s, 1H), 4.79 – 4.69 (m, 1H), 4.32 – 4.14 (m, 4H), 2.99 (dd, *J* = 17.0, 4.4 Hz, 1H), 2.79 (dd, *J* = 17.0, 4.5 Hz, 1H), 2.43 – 2.15 (m, 4H), 2.06 – 1.86 (m, 3H), 1.24 – 1.10 (m, 4H), 1.05 – 0.94 (m, 4H), 0.09 – 0.01 (m, 18H).
^{13}C NMR (125 MHz, $CDCl_3$) δ 179.54, 171.42, 171.09, 155.56, 151.56, 145.38, 132.91, 129.38, 125.30, 121.75, 64.32, 63.40, 48.70, 45.48, 44.33, 36.13, 35.75, 31.11, 30.94, 17.83, 17.37, -1.54.
HRMS (*ESI*) *m/z*: calcd for $C_{31}H_{48}N_2O_{10}Si_2$ $[M+H]^+$ 665.2920, found 665.2919.

Synthesis of ETP-TCO-asp
Compound **9** (1.17 g, 1.77 mmol) and Cs_2CO_3 (1.3 g, 4.1 mmol) were added to a solution of ETP (800 mg, 1.36 mmol) in DMF (10 mL). The resulting solution was stirred for 3 d. The reaction was quenched with water (30 mL). The product was extracted with EtOAc (3 x 50 mL) and the combined organic phase was dried with Na_2SO_4 and concentrated. The residue was purified by flash chromatography using a gradient of EtOAc in hexanes (20-50%) to give compound **10** (210 mg, 49%).
1M TBAF solution in THF (2 mL) was added to a solution of compound **10** (400 mg, 0.36 mmol) in anhydrous THF (8 mL). The resulting mixture was stirred overnight at rt. The reaction was quenched with water (20 mL) and the product was extracted with EtOAc (3 x 30 mL). The combined organic phase was washed with water (6 x 50 mL, to get rid of excess TBAF) and brine, and concentrated under reduced pressure. The residue was recrystallized from EtOAc/Hexane to give **ETP-TCO-asp** (212 mg, 66%).

^{1}H NMR (500 MHz, MeOD) δ 7.15 (d, *J* = 3.9 Hz, 1H), 6.66 – 6.60 (m, 2H), 6.52 (d, *J* = 6.0 Hz, 1H), 6.03 – 5.92 (m, 3H), 5.71 (dd, *J* = 16.7, 2.4 Hz, 1H), 5.19 (s, 1H), 4.76 (q, *J* = 5.0 Hz, 1H), 4.67 (dd, *J* = 13.5, 6.7 Hz, 1H), 4.52 – 4.38 (m, 3H), 4.30 (d, *J* = 7.8 Hz, 1H), 4.12 (dd, *J* = 10.3, 4.3 Hz, 1H), 3.67 (dd, *J* = 10.6, 5.1 Hz, 1H), 3.60 – 3.41 (m, 3H), 3.31 – 3.17 (m, 5H), 2.93 – 2.76 (m, 2H), 2.41 – 2.26 (m, 2H), 2.18 (ddd, *J* = 25.2, 16.8, 6.0 Hz, 2H), 1.97 (dd, *J* = 17.5, 6.5 Hz, 2H), 1.84 (d, *J* = 13.3 Hz, 1H), 1.69 (dt, *J* = 20.7, 10.9 Hz, 2H), 1.51 – 1.30 (m, 4H), 1.37 – 1.24 (m, 4H), 1.17 (d, *J* = 13.1 Hz, 3H). ^{13}C NMR (125 MHz, MeOD) δ 182.62, 181.74, 174.57, 153.76, 153.66, 149.30, 148.16, 142.30, 133.37, 133.22, 131.23, 130.06, 129.38, 110.21, 108.88, 106.40, 102.98, 102.61, 100.72, 89.04, 81.63, 77.92, 76.45, 75.92, 74.80, 70.14, 69.21, 67.52, 64.92, 56.81, 55.43, 54.89, 50.58, 46.52, 46.43, 45.49, 45.02, 40.70, 36.78, 36.60, 32.01, 31.91, 20.63, 18.19.
HRMS (*ESI*) *m/z*: calcd for $C_{44}H_{52}NO_{20}$ $[M + H]^+$ 914.3077, found 914.3078.



Synthesis of compound 11
DSC (1.6 g, 6.5 mmol) was added in one portion to a stirring solution of compound **3** (1.2 g, 6.5 mmol) and DIPEA (2.5 g, 19.5 mmol) in CH_3CN (50 mL). The resulting solution was allowed to stir for 12 h at rt. After evaporation of solvents under reduced pressure, the residue was purified by flash chromatography using a gradient of EtOAc in hexanes (30-60%) to give compound **11** (800 mg, 48%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 6.10 – 5.92 (m, 1H), 5.60 (dd, *J* = 16.6, 2.0 Hz, 1H), 4.43 (s, 1H), 2.77 (d, *J* = 3.3 Hz, 5H), 2.46 (s, 1H), 2.41 – 2.30 (m, 1H), 2.30 – 2.17 (m, 2H), 2.15 – 1.99 (m, 2H), 1.99 – 1.91 (m, 1H), 1.88 – 1.77 (m, 2H), 1.25 (s, 3H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 174.67, 169.65, 135.33, 129.88, 69.30, 44.45, 44.36, 38.30, 30.49, 29.29, 25.63, 17.76.
HRMS (*ESI*) *m/z*: calcd for $C_{14}H_{19}NO_5$ $[M+H]^+$ 282.1136, found 282.1135.

Synthesis of compound 12
NaH (994 mg, 24.9 mmol) was added in three portions to a solution of TMS ethanol (1.47 g, 12.4 mmol) in anhydrous THF (50 mL) at 0 °C. The mixture was stirred at 0 °C for 30 min and a solution of **11** (700 mg, 2.49 mmol) in anhydrous THF (15 mL) was added slowly via syringe. The reaction was warmed to rt and the stirring was continued for 5 h. The reaction mixture was poured into ice water and extracted with EtOAc (3×50 mL). The combined extracts were washed with sat. NH_4Cl_{aq} and dried over Na_2SO_4. After concentration, the residue was purified by flash chromatography using a gradient of EtOAc in hexanes (0-25%) to give compound **12** (570 mg, 70%).

^{1}H NMR (400 MHz, CDCl3) δ 6.15 – 5.94 (m, 1H), 5.64 (dd, J = 16.6, 2.0 Hz, 1H), 4.48 (s, 1H), 4.19 – 4.01 (m, 2H), 2.36 – 2.10 (m, 3H), 1.87 (dd, J = 24.9, 11.3 Hz, 3H), 1.61 – 1.48 (m, 3H), 1.10 (s, 3H), 0.98 (d, J = 8.4 Hz, 2H), 0.04 (s, 9H).
^{13}C NMR (100 MHz, $CDCl_3$) δ 179.99, 134.93, 130.42, 69.66, 62.73, 44.87, 44.35, 38.19, 30.84, 29.63, 18.12, 17.26, -1.49.
HRMS (*ESI*) *m/z*: calcd for $C_{15}H_{28}O_3Si$ $[M+H]^+$ 285.1880, found 285.1885.

Synthesis of compound 13
Pyridine (750 mg, 9.5 mmol) was added to a solution of compound **12** (900 mg, 3.16 mmol) in freshly distilled CH_2Cl_2 (20 mL). The resulting mixture was cooled to 0 °C and a solution of p-nitrophenyl chloroformate (956 mg, 4.7 mmol) in CH_2Cl_2 (5 mL) was slowly added via syringe. The mixture was stirred at rt for 12 h. The solvents were removed under reduced pressure and the resulting residue was purified by flash chromatography using a gradient of EtOAc in hexanes (0-10%) to give compound **13** (900 mg, 64%).
^{1}H NMR (400 MHz, CDCl3) δ 8.28 (d, J = 9.1 Hz, 2H), 7.40 (d, J = 9.1 Hz, 2H), 6.13 – 5.96 (m, 1H), 5.64 (d, J = 16.7 Hz, 1H), 5.29 (s, 1H), 4.12 (dd, J = 11.2, 6.0 Hz, 2H), 2.42 – 2.12 (m, 4H), 2.04 – 1.83 (m, 3H), 1.79 – 1.68 (m, 1H), 1.14 (s, 3H), 1.04 – 0.93 (m, 2H), 0.05 (s, 9H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 179.37, 155.57, 151.56, 145.36, 133.10, 129.34, 125.27, 121.76, 62.92, 44.78, 44.38, 35.63, 30.95, 30.57, 18.33, 17.34, -1.51.
HRMS (*ESI*) *m/z*: calcd for $C_{22}H_{31}NO_7Si$ $[M+H]^+$ 450.1943, found 450.1949.

Synthesis of compound 14
1,3-Dichloro-1,1,3,3-tetraisopropyldisiloxane (3.47 g, 11.0 mmol) was added to a solution of GCB (3 g, 10.0 mmol) in pyridine (25 mL) and the mixture was stirred at rt overnight. After removal of the solvent, the residue was purified by flash chromatography using a gradient of MeOH in CH_2Cl_2 (0-10%) to give compound **14** (3.9 g, 77%).

^{1}H NMR (400 MHz, CDCl3) δ 8.75 – 8.26 (m, 1H), 7.58 (t, J = 8.3 Hz, 1H), 6.16 (t, J = 9.5 Hz, 2H), 4.37 – 4.11 (m, 2H), 4.02 (d, J = 13.4 Hz, 1H), 3.92 (d, J = 9.2 Hz, 1H), 1.04 (dd, J = 22.1, 17.2 Hz, 28H).
^{13}C NMR (100 MHz, $CDCl_3$) δ 165.70, 155.37, 139.67, 123.52, 121.46, 119.40, 95.52, 84.71, 84.48, 84.20, 79.14, 69.34, 69.16, 68.99, 59.65, 17.37, 17.27, 17.23, 17.20, 16.91, 16.79, 16.64, 13.39, 12.89, 12.73, 12.38.
HRMS (*ESI*) *m/z*: calcd for $C_{21}H_{37}F_2N_3O_5Si_2$ $[M+H]^+$ 506.2313, found 506.2315.

Synthesis of the compound 15
NaH (65 mg, 1.6 mmol) was added in three portions to a solution of compound **14** (330 mg, 0.65 mmol) in DMF (15 mL) at 0 °C. The mixture was allowed to stir for 30 min at 0 °C. A solution of **13** (350 mg, 0.78 mmol) in DMF (3 mL) was added via syringe under at 0 °C. The reaction was slowly warmed up to rt and stirred for 12 h. The reaction was quenched with water (20 mL). The product was extracted with EtOAc (3x30 mL) and the combined organic phase was dried Na_2SO_4 and concentrated. The residue was purified by flash chromatography using a gradient of EtOAc in hexanes (10-50%) to give compound **15** (300 mg, 56%).

^{1}H NMR (400 MHz, CDCl3) δ 8.15 (d, J = 5.0 Hz, 1H), 7.34 – 7.21 (m, 1H), 6.34 (s, 1H), 6.06 – 5.88 (m, 1H), 5.61 (d, J = 16.8 Hz, 1H), 5.25 (s, 1H), 4.44 (dd, J = 21.6, 12.4 Hz, 1H), 4.16 – 4.05 (m, 4H), 2.19 (ddd, J = 45.1, 35.6, 16.7 Hz, 4H), 1.97 – 1.79 (m, 3H), 1.71 – 1.56 (m, 1H), 1.08 (dd, J = 16.8, 9.7 Hz, 30H), 0.98 – 0.91 (m, 3H), 0.04 (d, J = 11.2 Hz, 9H).
^{13}C NMR (126 MHz, $CDCl_3$) δ 179.79, 163.26, 155.27, 151.78, 143.96, 132.67, 129.84, 124.35, 122.27, 120.21, 95.78, 85.06, 84.84, 84.55, 81.10, 74.02, 68.31, 68.12, 67.95, 62.98, 59.44, 44.63, 44.35, 35.60, 31.88, 30.93, 30.58, 22.69, 18.19, 17.39, 17.29, 17.25, 17.19, 14.11, 13.47, 13.38, 13.10, 12.87, -1.52.
HRMS (*ESI*) *m/z*: calcd for $C_{37}H_{63}F_2N_3O_9Si_3$ $[M+H]^+$ 816.3913, found 816.3916.

Synthesis of GCB-TCO-acid
Stirred compound **15** (300 mg, 0.56 mmol) in 1M THF solution of TBAF (3 mL) for 18 h. The reaction was quenched with water (20 mL) and extracted with EtOAc (3 x 30 mL). The combined organic phase was washed with water and brine, and concentrated. The residue was recrystallized in EtOAc/Hexane to give **GCB-TCO-acid** (120 mg, 68%).
^{1}H NMR (400 MHz, MeOD) δ 8.31 (d, J = 7.7 Hz, 1H), 7.32 (d, J = 7.6 Hz, 1H), 6.31 – 6.21 (m, 1H), 6.08 – 5.92 (m, 1H), 5.69 (d, J = 16.7 Hz, 1H), 5.27 (s, 1H), 4.31 (dd, J = 20.7, 12.1 Hz, 1H), 4.04 – 3.90 (m, 2H), 3.81 (dd, J = 12.3, 2.5 Hz, 1H), 2.31 – 2.15 (m, 3H), 2.15 – 1.95 (m, 3H), 1.89 (d, J = 13.4 Hz, 1H), 1.13 (s, 3H);
^{13}C NMR (125 MHz, MeOD) δ 182.56, 164.08, 156.06, 152.33, 144.25, 132.00, 130.26, 130.21, 124.58, 123.09, 122.52, 121.28, 120.46, 117.59, 95.67, 84.77, 81.43, 73.81, 68.88, 68.69, 58.94, 44.80, 44.28, 35.16, 30.57, 30.38, 17.56
ESI HRMS calcd for $C_{20}H_{25}F_2N_3O_8$ $[M + H]^+$ 474.1682, found 474.1681.

Synthesis of SQL70
Synthesis of SQL70 follows an optimized 3-step process that includes: (i) condensing the sodium salt of HA with a tetrazine derivative ((4-(6-methyl-1,2,4,5-tetrazin-3-yl)phenyl)methanamine HCl) in the presence of excess carbodiimide activating agent, (ii) purification using tangential flow filtration (TFF) to remove low molecular weight reagents and by-products, and (iii) sterilization by ultrafiltration. The derivatization reaction occurs in N-morpholinoethanesulfonic acid (MES) buffered aqueous solvent with activation of the HA carboxylic acid group by N-hydroxysulfosuccinimide and N,N-(dimethylaminopropyl)-N-ethyl carbodiimide HCl. The resulting product is conjugated with tetrazine at ~19 weight%. Specific analytical methods were developed to characterize SQL70. Size exclusion chromatography was used to determine molecular weight. Purity and percent tetrazine modification were determined by HPLC-UV and UV-Vis Spectroscopy at ~520 nm (peak absorbance of the tetrazine moiety). Further, pH, osmolality, endotoxin and bioburden tests were performed to ensure that the biopolymer was safe for animal use.

General procedure to determine solubility

A standard solution was prepared by dissolving 10 mg of **SQP33** in 1 mL of DMSO. After 100-fold dilution in PBS, the standard solution was analyzed by HPLC (*spectrum 1*). To determine the solubility in PBS, **SQP33** was continuously added to 1 mL PBS until significant precipitate was observed. The resulting solution was agitated for 5 min. The solids were removed by centrifugation and the supernatant was diluted 100-fold in PBS. The diluted solution was analyzed by HPLC (*spectrum 2*), while injecting the same volume as the one used for the standard solution. The solubility of **SQP33** was determined to be 28 mg/mL by comparison of the main peak's integration values.

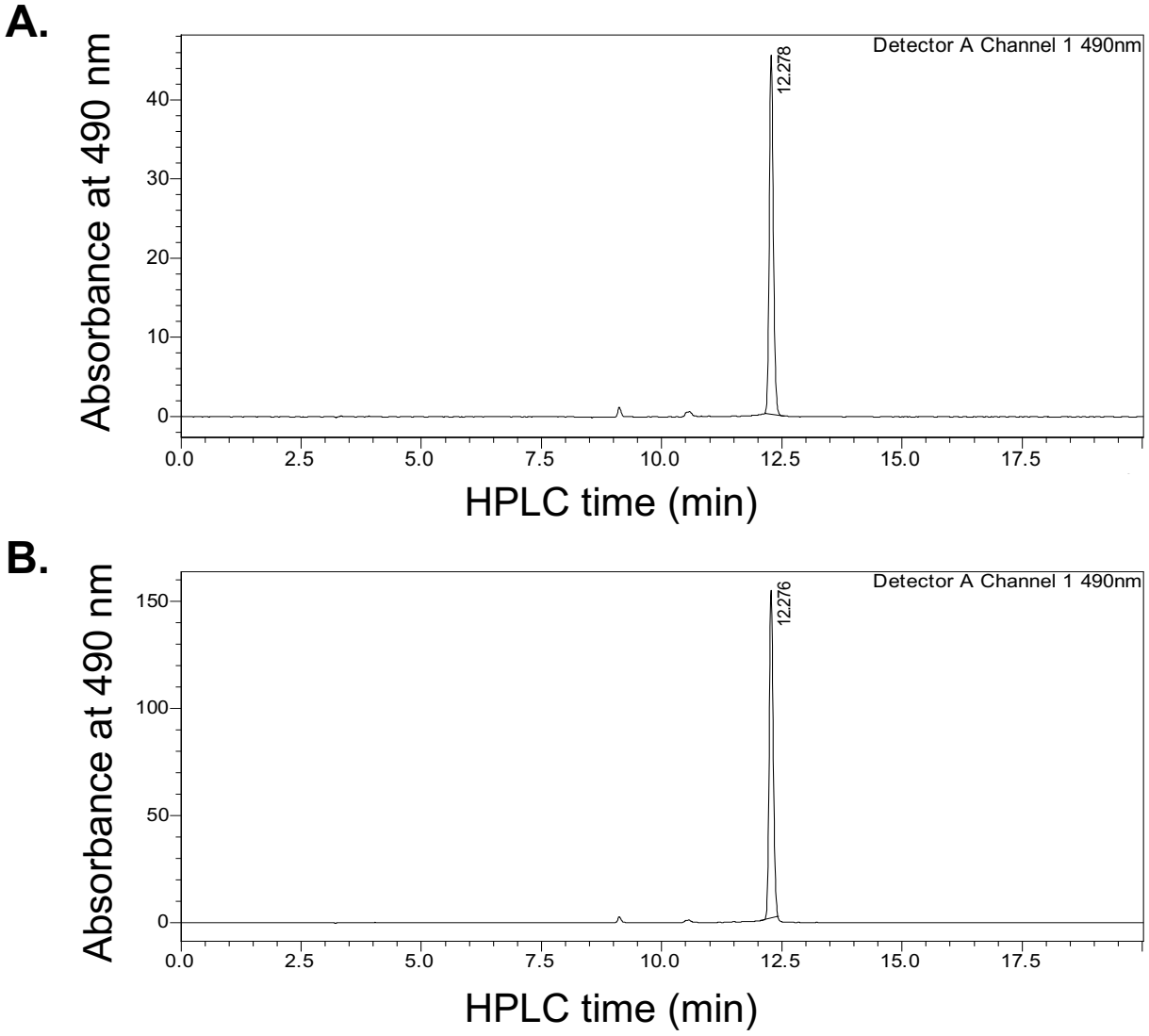
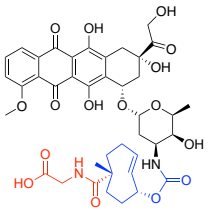
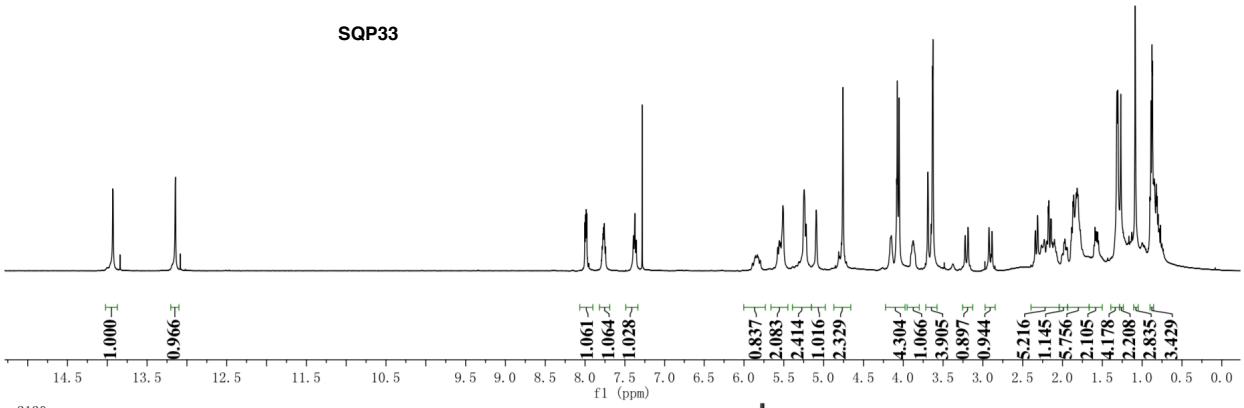
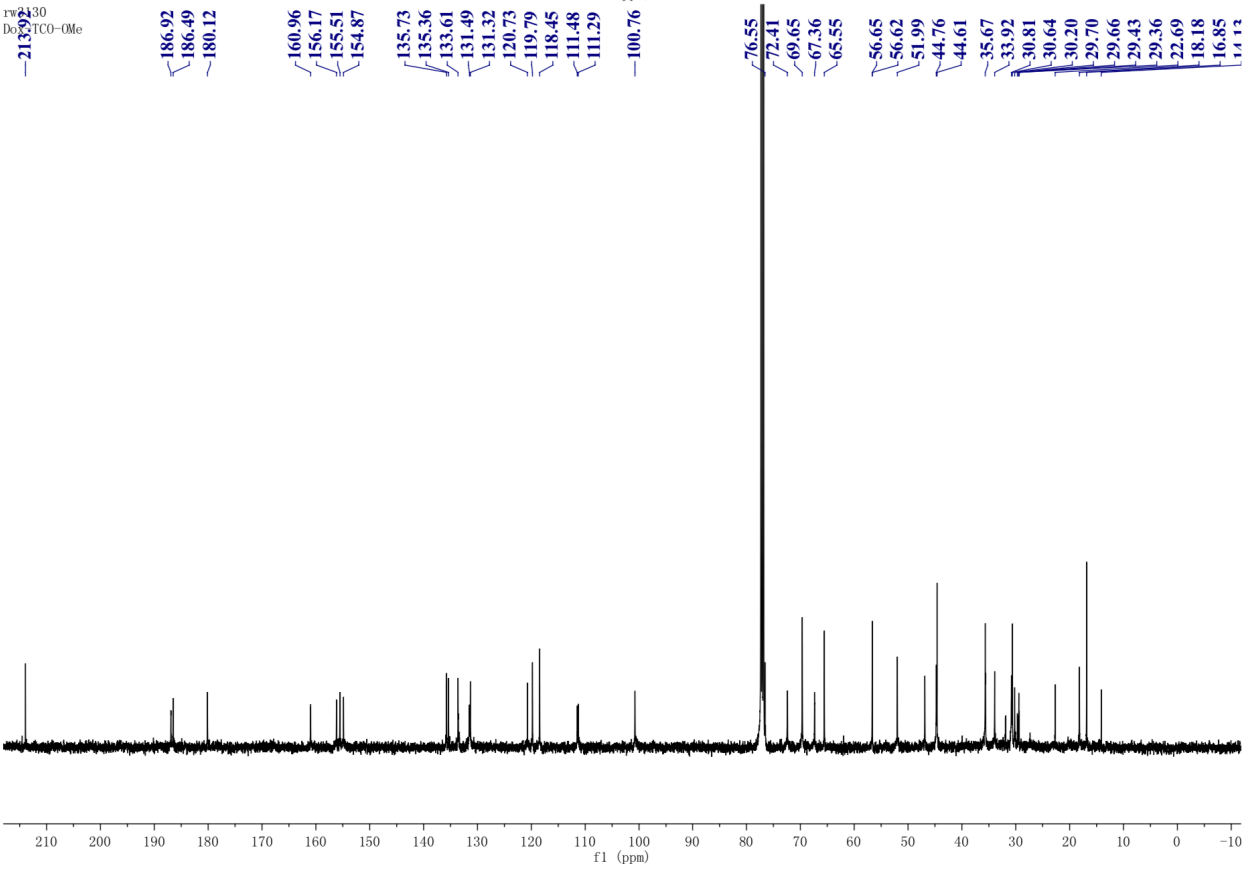


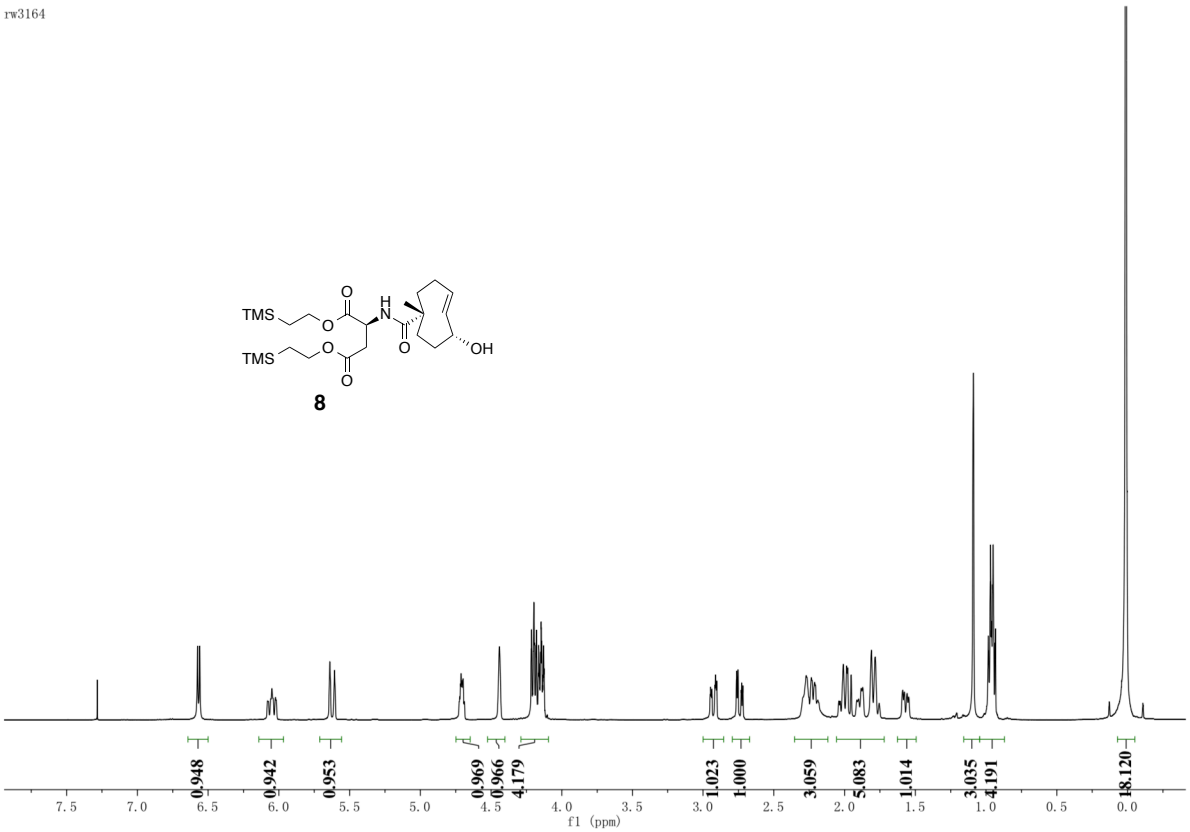
Figure S11. Determination of **SQP33** solubility in PBS: (**A**) HPLC spectrum of a standard **SQP33** solution in PBS; (**B**) HPLC spectrum of PBS saturated with **SQP33**.



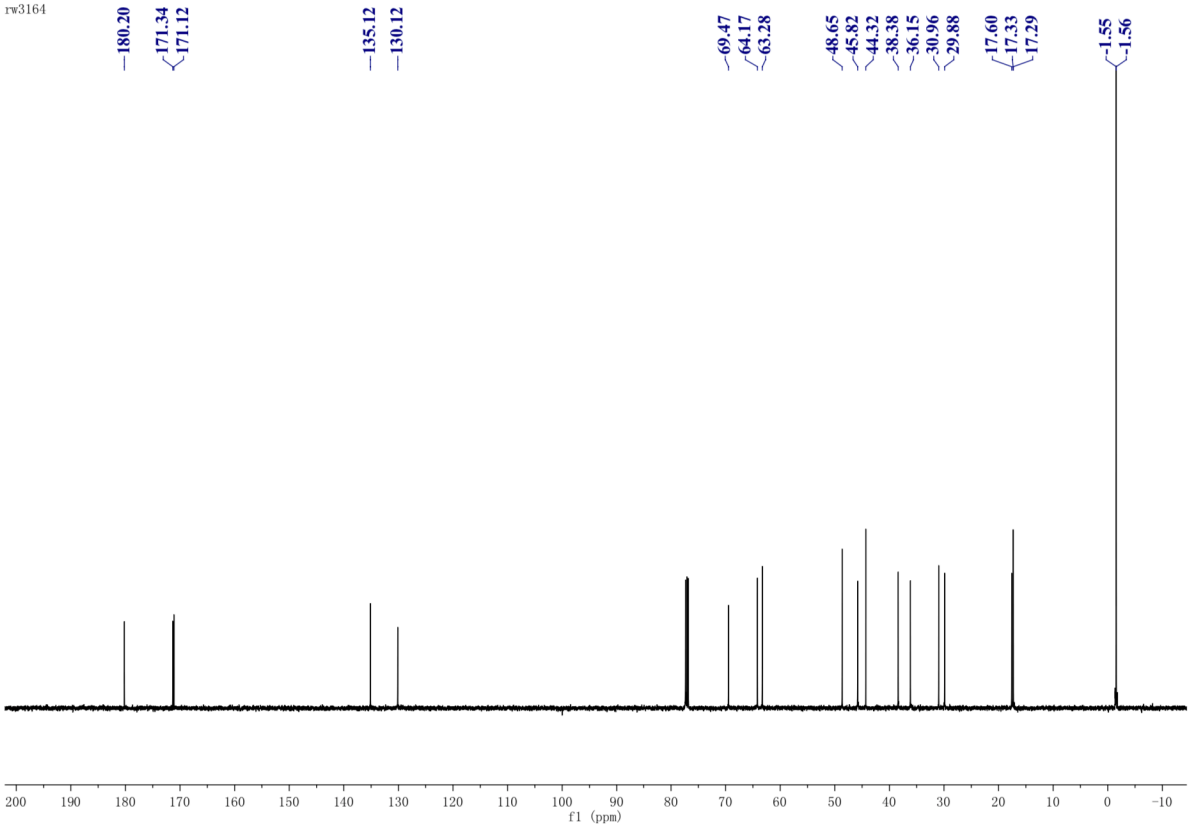
SQP33


1.000
0.966
1.061
1.064
1.028
0.837
2.083
2.414
1.016
2.329
4.304
1.066
3.905
0.897
0.944
5.216
1.145
5.756
2.105
4.178
2.208
2.835
3.429
f1 (ppm)

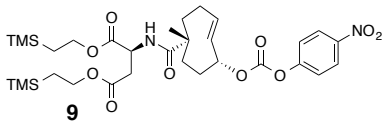

213.02
186.92
186.49
180.12
160.96
156.17
155.51
154.87
135.73
135.36
133.61
131.49
131.32
120.73
119.79
118.45
111.48
111.29
100.76
76.55
72.41
69.65
67.36
65.55
56.65
56.62
51.99
44.76
44.61
35.67
33.92
30.81
30.64
30.20
29.70
29.66
29.43
29.36
22.69
18.18
16.85
f1 (ppm)


rw3164
TMS
TMS
O
O
O
O
O
H
N
O
OH
0.948
0.942
0.953
0.969
0.966
4.179
1.023
1.000
3.059
5.083
1.014
3.035
4.191
18.120
f1 (ppm)

8


rw3164
180.20
171.34
171.12
135.12
130.12
69.47
64.17
63.28
48.65
45.82
44.32
38.38
36.15
30.96
29.88
17.60
17.33
17.29
1.55
1.56
f1 (ppm)

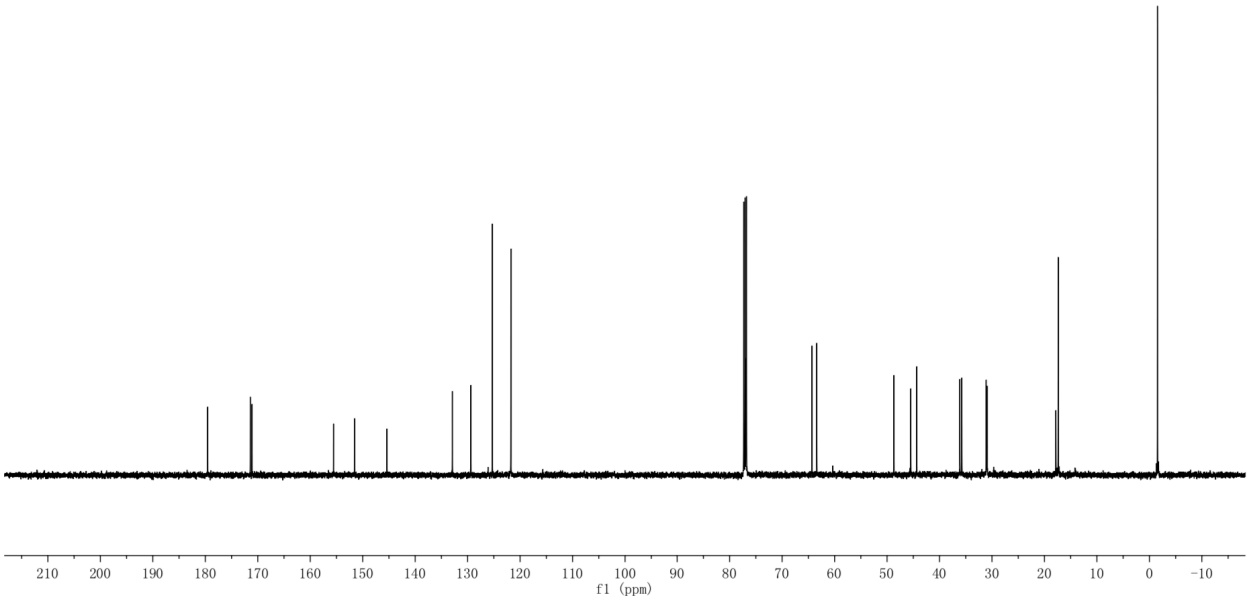
rw3011
1H rw3011

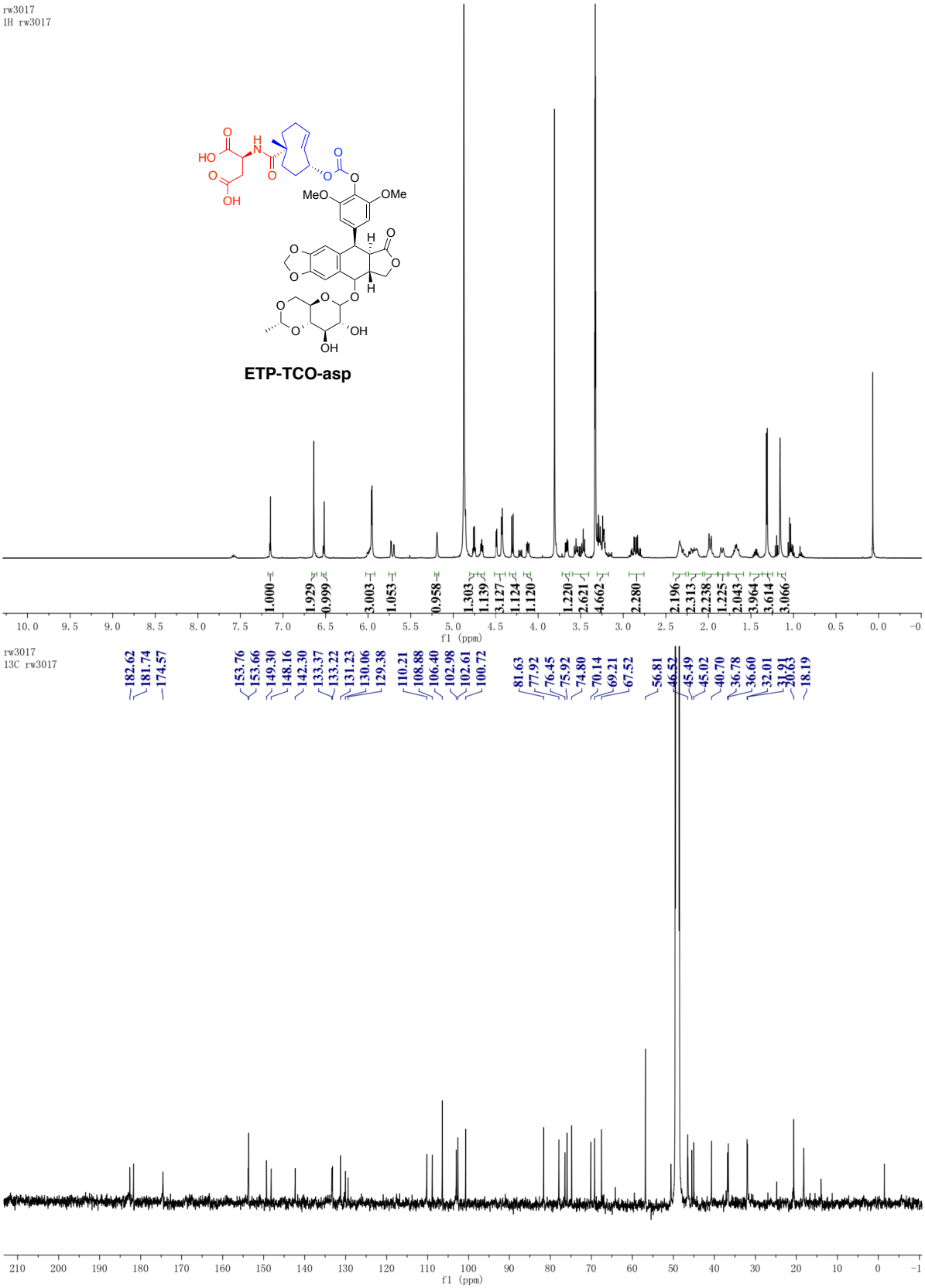


9

rw3011
13C rw3011

179.54
171.42
171.09
155.56
151.56
145.38
132.91
129.38
125.30
121.75
64.32
63.40
48.70
45.48
44.33
36.13
35.75
31.11
30.94
17.83
17.37
−1.54




rw3017
1H rw3017
ETP-TCO-asp
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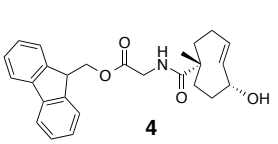
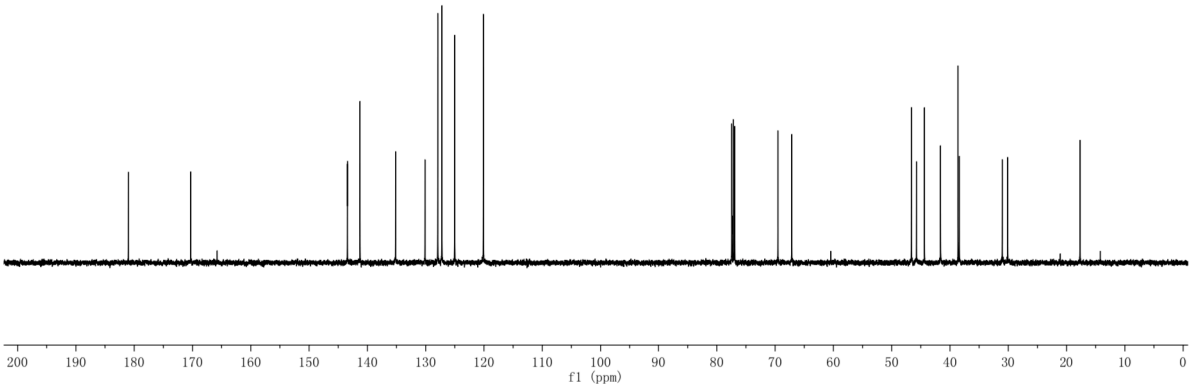
1.929
0.999
3.003
1.053
0.958
1.303
1.139
3.127
1.124
1.120
1.220
2.621
4.662
2.280
2.196
2.313
2.238
1.225
2.043
3.964
3.614
3.066
f1 (ppm)
rw3017
13C rw3017
182.62
181.74
174.57
153.76
153.66
149.30
148.16
142.30
133.37
133.22
131.23
130.06
129.38
110.21
108.88
106.40
102.98
102.61
100.72
81.63
77.92
76.45
75.92
74.80
70.14
69.21
67.52
56.81
46.52
45.49
45.02
40.70
36.78
36.60
32.01
31.91
30.23
18.19
f1 (ppm)

rw3055
1H rw3055

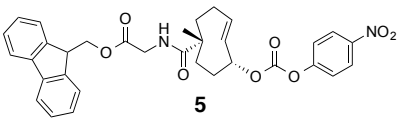


4

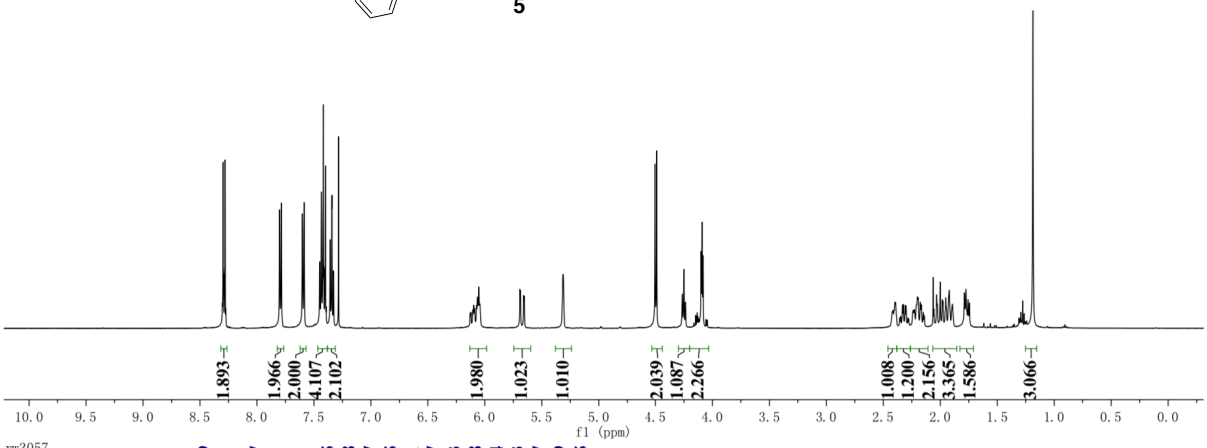
rw3055
1H rw3055



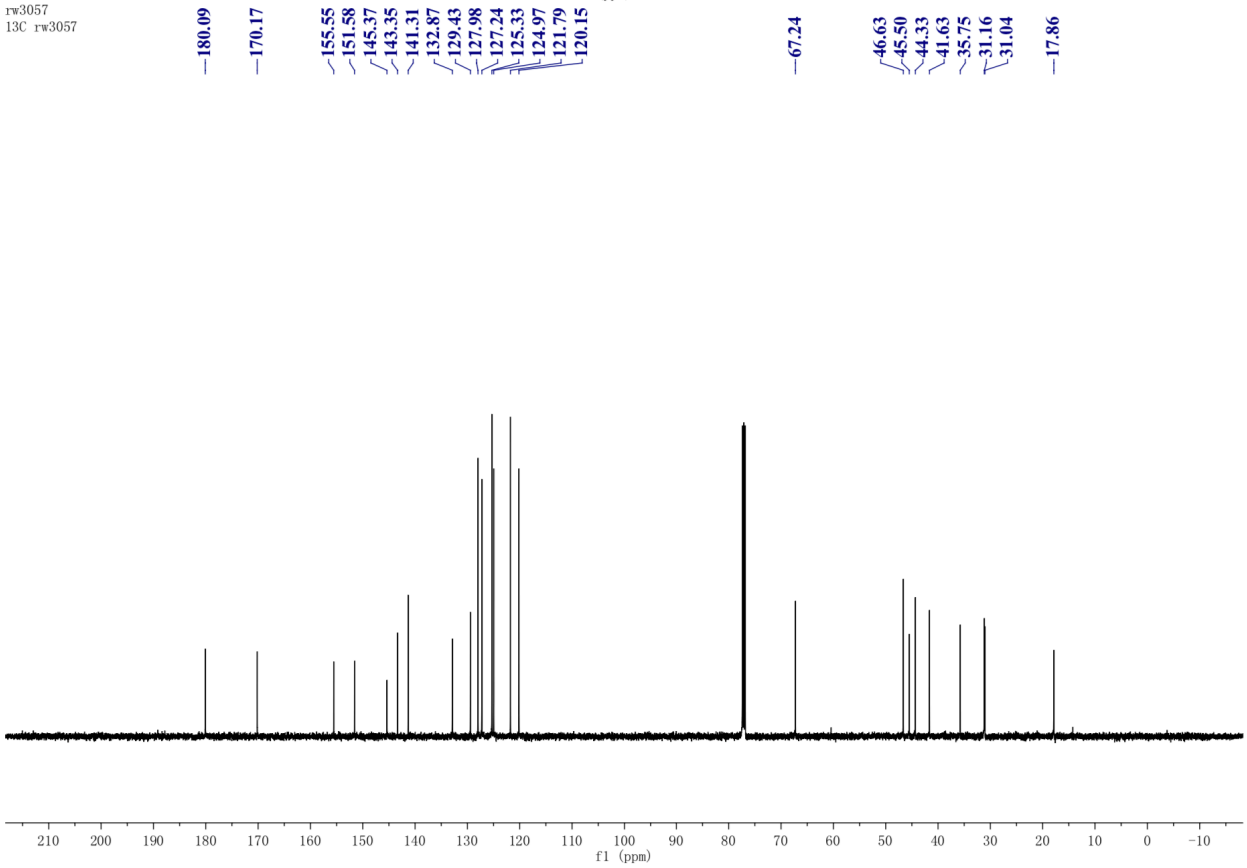
rw3057
1H rw3057



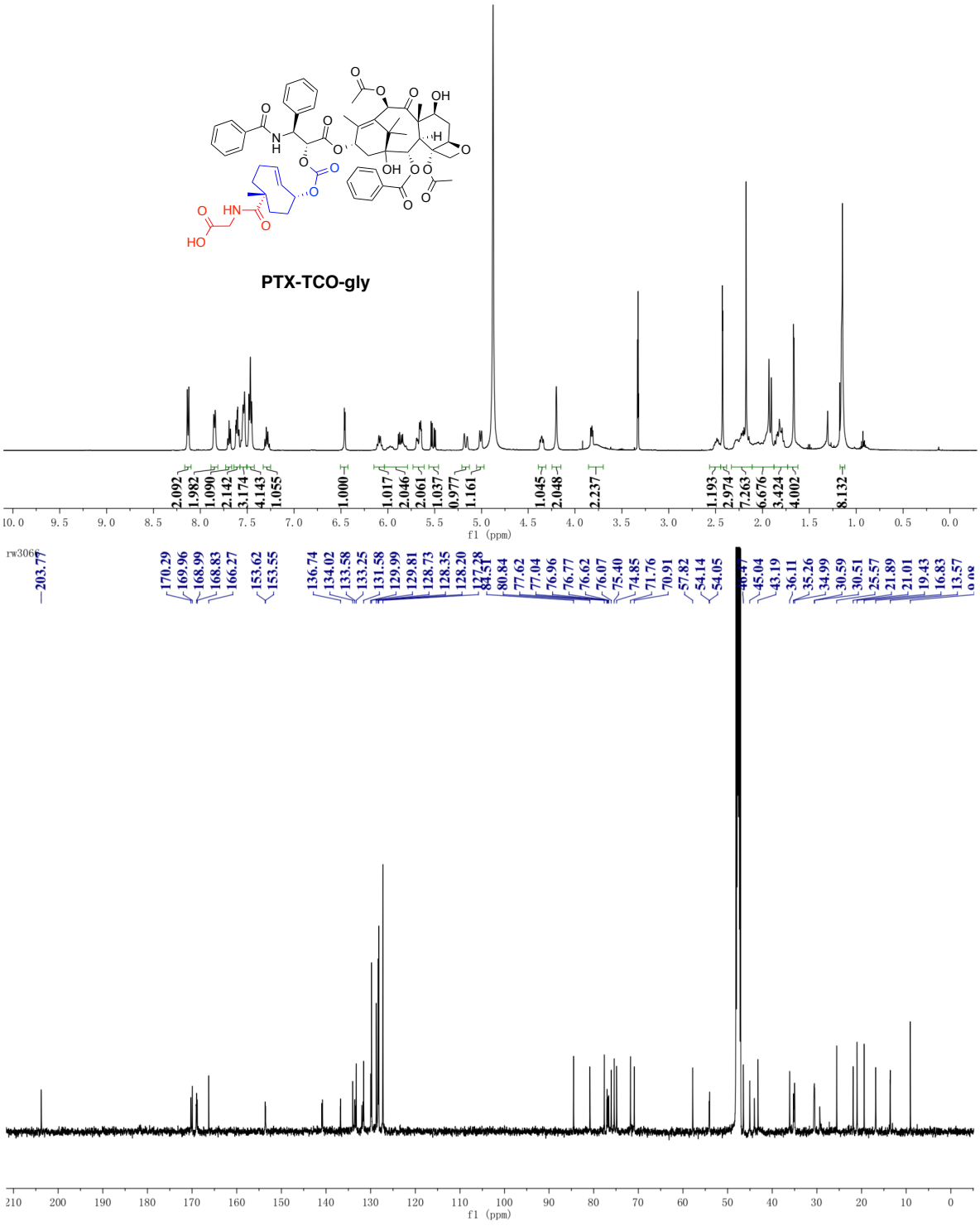
5



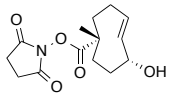
rw3057
13C rw3057



rw3066

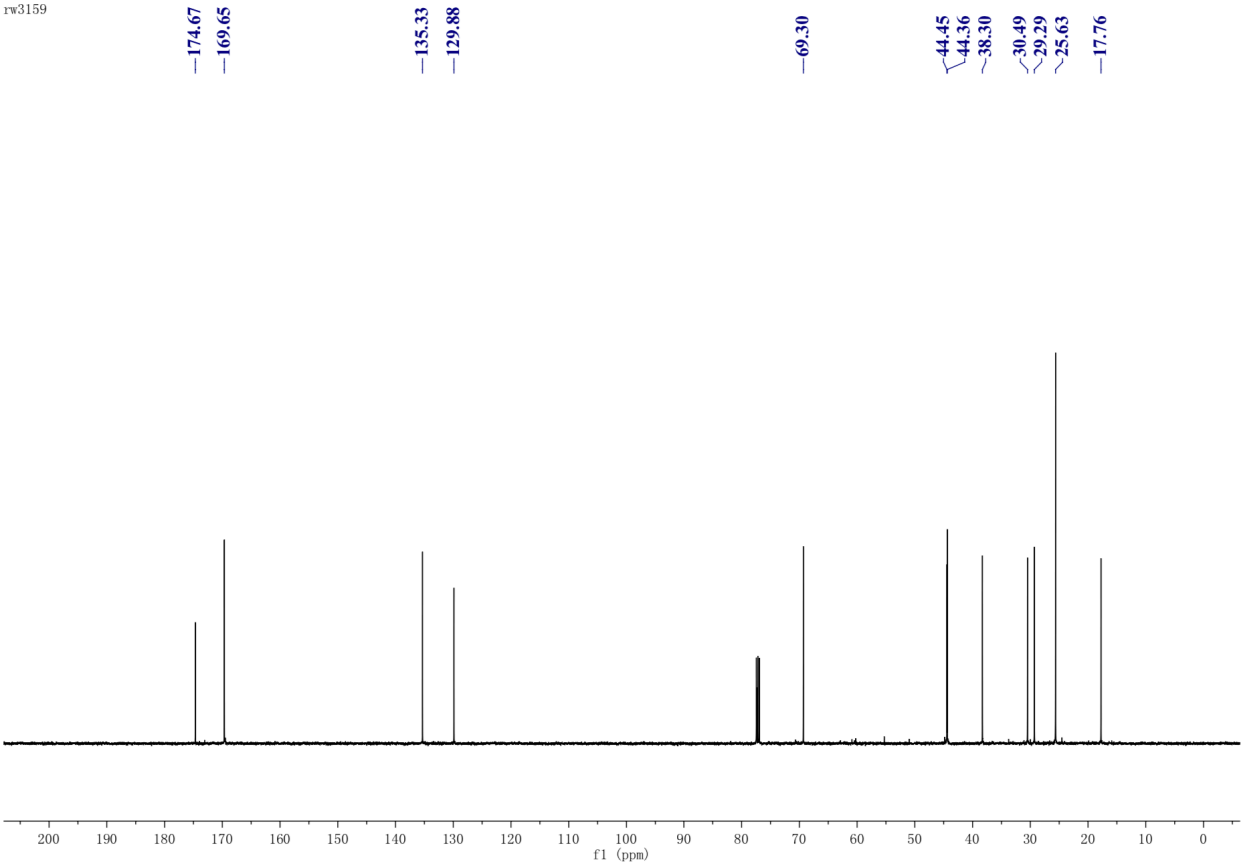

PTX-TCO-gly
2.092
1.982
1.090
2.142
3.174
4.143
1.055
1.000
1.017
2.046
2.061
1.037
0.977
1.161
1.045
2.048
2.237
1.193
2.974
7.263
6.676
3.424
4.002
8.132
f1 (ppm)
rw3066
203.77
170.29
169.96
168.99
168.83
166.27
153.62
153.55
136.74
134.02
133.58
133.25
131.58
129.99
129.81
128.73
128.35
128.20
127.28
84.51
80.84
77.62
77.04
76.96
76.77
76.62
76.07
75.40
74.85
71.76
70.91
57.82
54.14
54.05
45.04
43.19
36.11
35.26
34.99
30.59
30.51
25.57
21.89
21.01
19.43
16.83
13.57
f1 (ppm)

rw3159

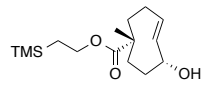


11

rw3159

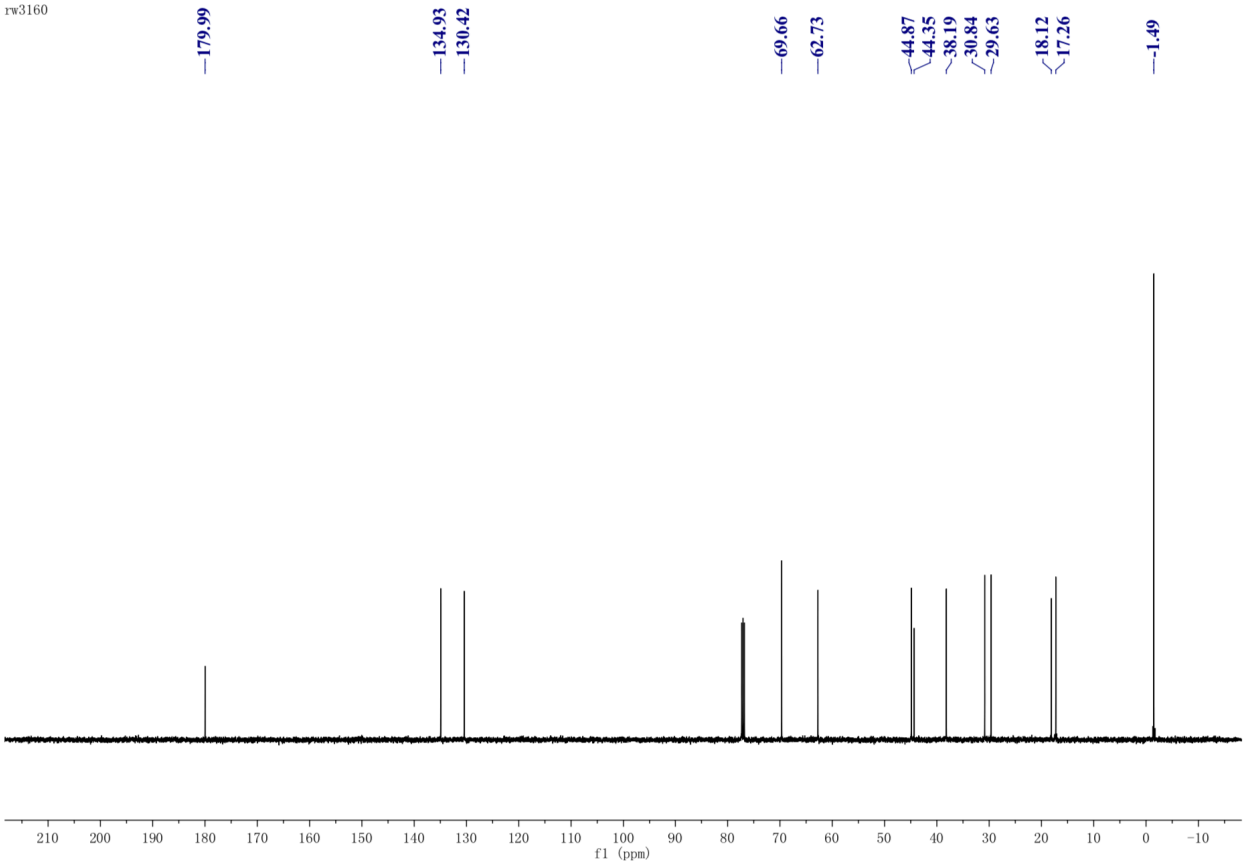


rw3160

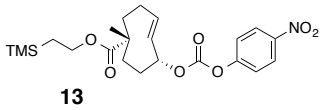


12

rw3160

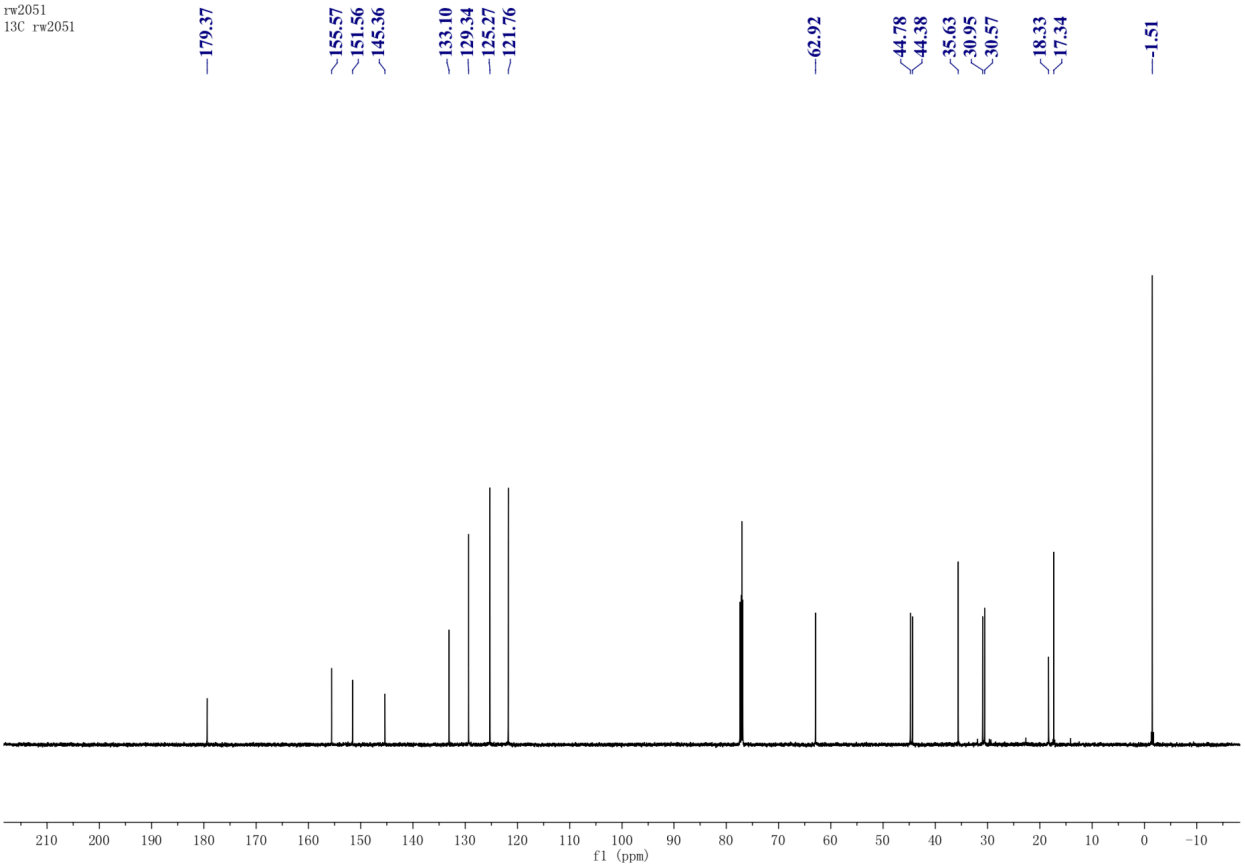


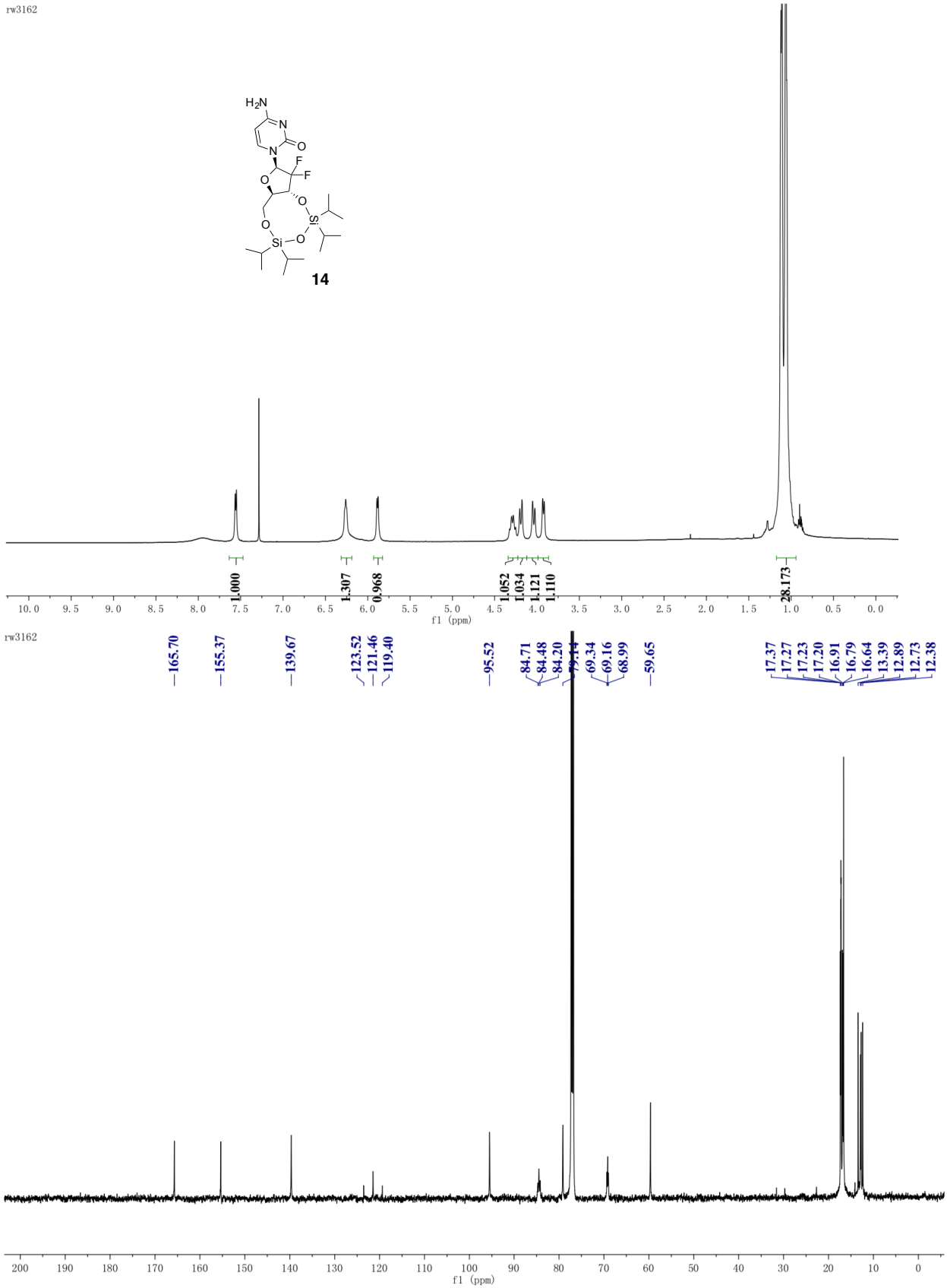
rw2051
Proton rw2051



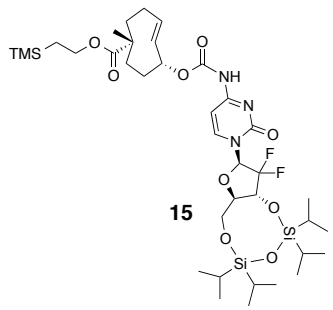
13

rw2051
13C rw2051



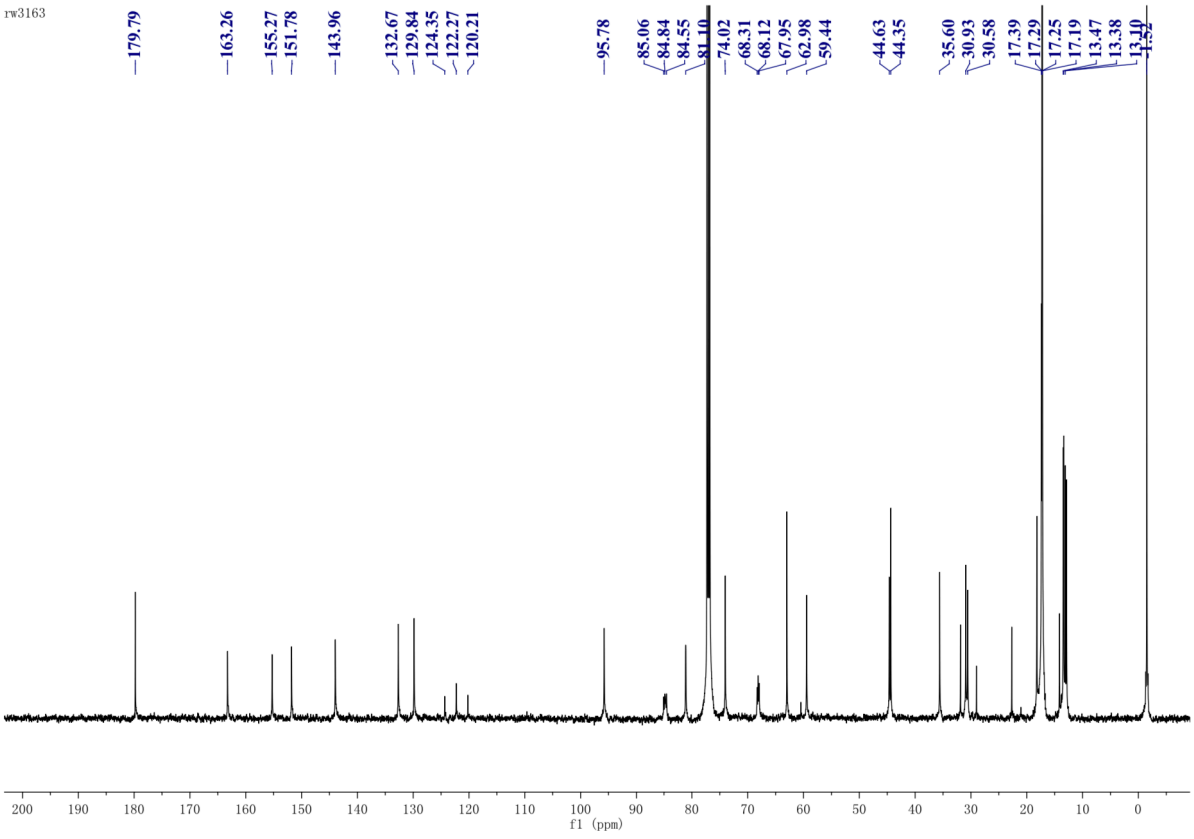

rw3162
14
1.000
1.307
0.968
1.052
1.034
1.121
1.110
28.173
f1 (ppm)
rw3162
165.70
155.37
139.67
123.52
121.46
119.40
95.52
84.71
84.48
84.20
69.34
69.16
68.99
59.65
17.37
17.27
17.23
17.20
16.91
16.79
16.64
13.39
12.89
12.73
12.38
f1 (ppm)

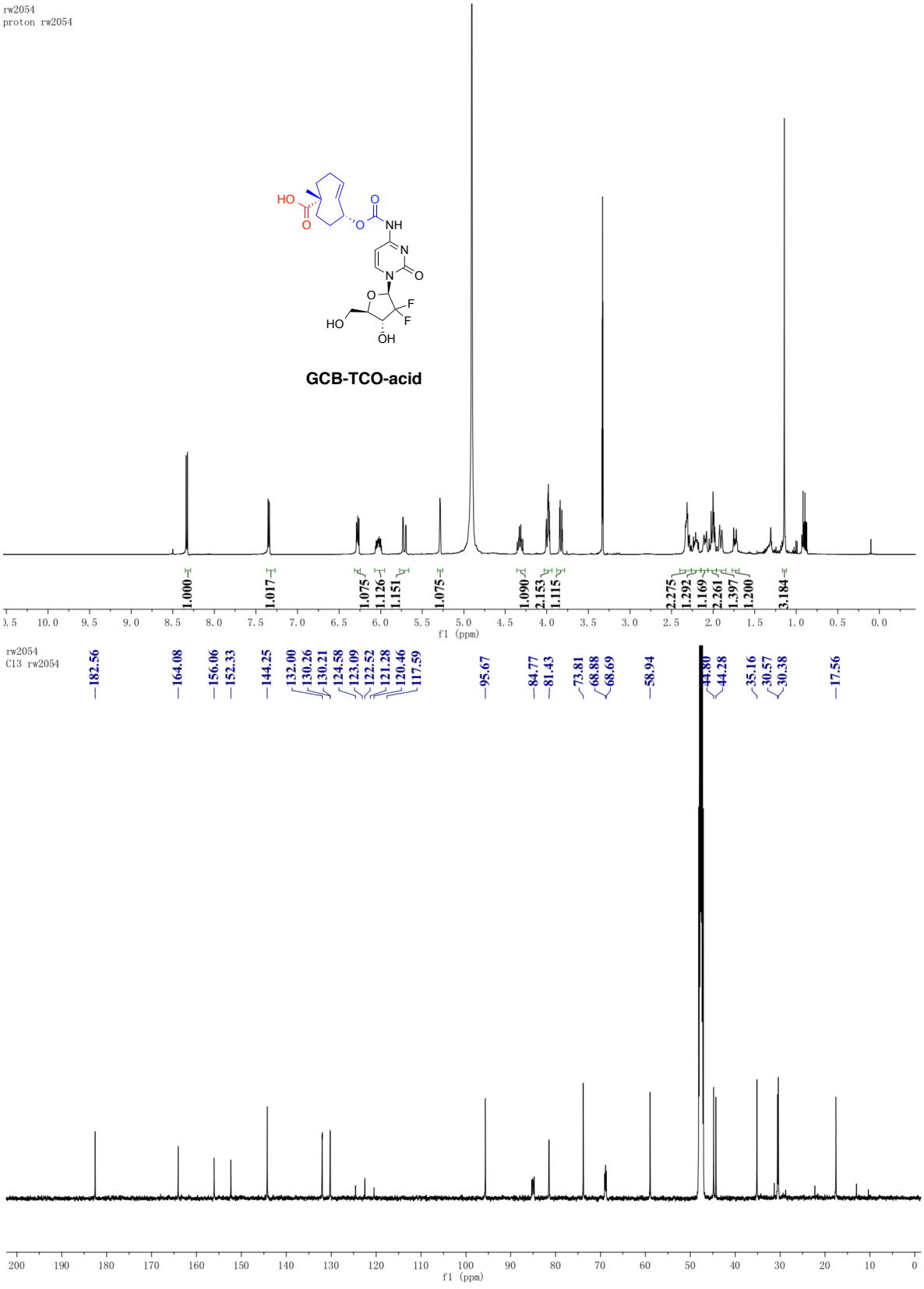
rw2038
Proton
rw2038



15

rw3163




rw2054
proton rw2054
GCB-TCO-acid
1.000
1.017
1.075
1.126
1.151
1.075
1.090
2.153
1.115
2.275
1.292
1.169
2.261
1.397
1.200
3.184
f1 (ppm)
rw2054
C13 rw2054
182.56
164.08
156.06
152.33
144.25
132.00
130.26
130.21
124.58
123.09
122.52
121.28
120.46
117.59
95.67
84.77
81.43
73.81
68.88
68.69
58.94
44.80
44.28
35.16
30.57
30.38
17.56
f1 (ppm)