Supporting Information

For

Bioorthogonal prodrug activation driven by a strain-promoted 1,3-dipolar cycloaddition

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1. General Experimental

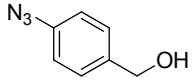
Unless otherwise stated, all chemical reagents and solvents were purchased from commercial sources and used without further purification. Doxorubicin hydrochloride salt was purchased from AK Scientific, California. 5-Hydroxy-1-cyclooctene (*cis*-cyclooct-4-enol) was purchased from Carbosynth Limited, UK. Silver nitrate-impregnated silica gel was purchased from Sigma-Aldrich and Silicycle (SiliaBond® Silver Nitrate, 40-63μm, 60Å). Powdered molecular sieves were activated in an oven at 180 °C overnight before use and then stored at 110 °C. All other reagents were purchased from Sigma-Aldrich or AK Scientific. Reaction solvents were purchased dry from Sigma-Aldrich, Thermo Fischer Scientific or Merck. Thin layer chromatography was performed on 0.2 mm aluminium-backed silica gel plates 60 F_{254}, and visualized with UV light (λ = 254 nm) or basic $KMnO_4$ dip. Flash column chromatography was carried out using 40-63 μm silica gel, with AR or liquid chromatography grade solvents. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz Varian MR spectrometer. Chemical shifts are reported as δ in parts per million (ppm) and coupling constants are reported as J values in Hz. High resolution electrospray ionization mass spectra were recorded on a microTOF$_Q$ mass spectrometer. Photochemical reactions were performed in a Southern New England Ultraviolet Company Rayonet® reactor model RPR-100, equipped with 8 RPR-2537 Å lamps. Reactions were performed in either the RQV-118 or RQV-218 quartz reaction vessels supplied by Southern New England Ultraviolet Company.

Fluorescence data were recorded on a Hitachi F-7000 Fluorescence Spectrofluorometer. Excitation was at 360 nm and emission monitored in the range of 400 – 650 nm (λ_{max} = 455 nm). Default settings were selected for other parameters.

HPLC was performed using either an Agilent 1200 system or Shimadzu 20 series system, equipped with a Phenomenex Synergi 4 μm Fusion-RP 80A (150 x 4.6 mm) column, and a photodiode array detector. The applied mobile phases used for kinetic studies and purity determinations were: A, *aq* H_2O + 0.1% formic acid; and B, *aq* MeCN + 0.1% formic acid. Flow speed was 1 mL/min and injection volumes were 20 μl or 50 μl. Gradient mobile phase, 80% H_2O/20% MeCN with 0.1% formic acid to 100% MeCN with 0.1% formic acid in 10 minutes, 5 minutes at 100% MeCN with 0.1% formic acid, returning to starting conditions by 20 minutes.

2. Synthesis

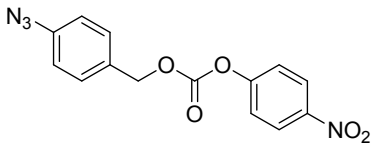
4-azidobenzyl alcohol[1]



The synthesis of 4-azidobenzyl alcohol was performed using a known literature procedure.[1] 4-Aminobenzyl alcohol (0.602 g, 4.89 mmol) was dissolved in 5 mL of 5M HCl. The solution was cooled to 0 °C on ice, followed by dropwise addition of a $NaNO_2$ (0.363 g, 5.26 mmol) solution in 10 mL of water. The reaction was allowed to stir for 30 min at 0 °C, upon which time NaN_3 (1.37 g, 21.1 mmol) was added in portions (solution maintained at 0 °C in an ice bath). The reaction mixture was stirred at 0 °C for 1.5 h, before pouring into an ice-water slurry (~ volume of 50 mL). The solution was adjusted to a pH of ~ 8 with solid $NaHCO_3$, and extracted with ethyl acetate (2 x 50 mL). The combined organic layers were washed with water (3 x 30 mL), dried ($MgSO_4$) and concentrated *in vacuo* (temperature maintained below 30 °C). The oily residue was suspended in petroleum ether (20 mL, b.p. 40-60 °C) and stirred vigorously for 30 min, before being stored in the freezer overnight. Upon removal from the freezer, the precipitate was filtered to provide the azide as a mixture of yellow-brown solid and fine beige colored needles (0.541 g, 73%), which were spectroscopically similar to that previously reported.[1]

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.36 (d, J = 8.4 Hz, 2H); 7.02 (d, J = 8.4 Hz, 2H); 4.67 (s, 2H); 1.65 (br s, 1H).

4-azidobenzyl-4′-nitrophenyl carbonate[1]

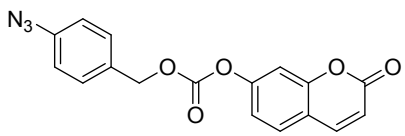


The synthesis of 4-azidobenzyl-4-nitrophenyl carbonate was performed using a modified literature procedure.[1] To a solution of 4-nitrophenyl chloroformate (0.243 g, 1.21 mmol) in anhydrous THF (5 mL) was added pyridine (0.146 mL, 1.81 mmol). The solution was cooled to 0 °C and a solution of 4-azidobenzyl alcohol (0.135 g, 0.905 mmol) in anhydrous THF (8 mL) was added dropwise over 15 min. The solution was allowed to warm to 25 °C and stirred in the dark for 72 h under nitrogen, after which time TLC analysis indicated that the reaction was complete. The THF was removed *in vacuo*, and the crude residue re-dissolved in ethyl acetate (30 mL), which was washed with water (2 x 30 mL), brine (5 x 30 mL), dried ($MgSO_4$) and concentrated *in vacuo* (temperature maintained below 30 °C). The crude residue was subjected to

flash silica gel column chromatography (50% DCM:hexanes) to afford the title compound as a yellow solid (0.161 g, 57%), which was spectroscopically identical to that reported in the literature.[1]

^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.28 (d, *J* = 9.6 Hz, 2H); 7.44 (d, *J* = 8.8 Hz, 2H); 7.38 (d, *J* = 9.2 Hz, 2H); 7.07 (d, *J* = 8.4 Hz, 2H); 5.26 (s, 2H).

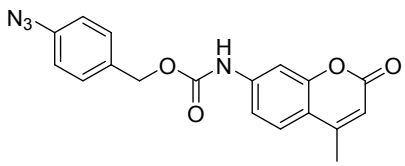
4-azidobenzyl (2-oxo-2H-chromen-7-yl) carbonate (8a)



To a solution of 4-azidobenzyl-4-nitrophenyl carbonate (19.6 mg, 0.0624 mmol) in anhydrous DMF (1 mL) was added 7-hydroxycoumarin (19.4 mg, 0.133 mmol) and triethylamine (35 μL, 0.255 mmol). The reaction mixture was stirred in the dark at 25 °C under an atmosphere of nitrogen for 24 h, after which time the DMF was diluted with water and extracted with ethyl acetate (3 x 20 mL). The combined organic fractions were washed with water (2 x 30 mL), brine (3 x 30 mL), dried ($MgSO_4$), and concentrated *in vacuo* (temperature maintained below 30 °C). The crude residue was subjected to flash silica gel column chromatography (25% ethyl acetate:hexanes) to provide the title compound **8a** as a white solid (10 mg, 48%). Small amounts of 4-nitrophenol co-eluted with the product, but these were removed through rinsing with ice cold methanol (product is partially soluble in methanol) or performing a miniature liquid-liquid extraction (ethyl acetate:water). Following the liquid-liquid extraction, the product was isolated as a white solid (8 mg, 38%), and was used in the spectrofluorometry, HPLC and NMR studies.

^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.69 (d, *J* = 9.6 Hz, 1H); 7.49 (d, *J* = 8.8 Hz, 1H); 7.44 (d, *J* = 8.4 Hz, 2H); 7.21 (d, *J* = 2.4 Hz, 1H); 7.13 (dd, *J* = 8.4 Hz, J = 2.0 Hz, 1H); 7.06 (d, *J* = 8.4 Hz, 2H); 6.40 (d, *J* = 9.6 Hz, 1H); 5.25 (s, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 160.1, 154.6, 153.2, 152.7, 142.7, 140.9, 130.9, 130.4, 128.6, 119.3, 117.6, 116.8, 116.3, 109.8, 70.2. HRMS (ESI+) calculated for $C_{17}H_{11}N_3O_5Na$: 360.0591, found: 360.0588. HPLC-UV: t_r = 9.8 min (λ = 254 nm).

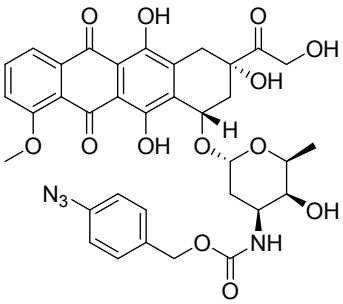
4-azidobenzyl (4-methyl-2-oxo-2H-chromen-7-yl)carbamate (8b)



To a mixture of 7-Amino-4-methylcoumarin (44.6 mg, 0.255 mmol) and DIPEA (123.11 mg, 0.952 mmol) in anhydrous toluene (5 mL) at 0 °C (ice bath) was added dropwise a solution of triphosgene (92.2 mg, 0.310 mmol) in toluene over 1 h. The resultant solution was heated to reflux for 3 h under nitrogen and cooled to room temperature. Further, the reaction mixture was mixed with DCM (6 mL) and stirred for 15 mins to obtain a pale coloured solution. To the resultant solution, 4-azidobenzyl alcohol (50 mg, 0.330 mmol) was added and stirred at room temperature for an additional 3 h. The reaction mixture was then concentrated *in vacuo* (temperature maintained below 30 °C) and subjected to flash silica gel column chromatography (20% DCM:ethyl acetate) to provide the title compound **8b** as a light yellow solid (27 mg, 30%), and was used in the spectrofluorometry, HPLC and NMR studies.

^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.28 (s, 1H); 7.68 (d, J = 8.8 Hz, 1H); 7.54 (d, J = 2.0 Hz, 1H); 7.48 (d, J = 8.4 Hz, 2H); 7.40 (dd, J = 8.4 Hz, 2.0 Hz, 1H); 7.15 (d, J = 8.4 Hz, 2H); 6.23 (s, 1H); 5.17 (s, 2H); 2.38 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 160.0, 153.8, 153.15, 153.10, 142.6, 139.3, 133.1, 130.2, 126.0, 119.2, 114.4, 114.2, 111.9, 104.4, 65.7, 18.0. HRMS (ESI-) calculated for $C_{18}H_{13}N_4O_4$: 349.0942, found: 349.0943. HPLC-UV: t_r = 8.5 min (λ = 254 nm).

4-Azidobenzyl carbamate doxorubicin (9)[1]

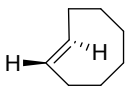


The doxorubicin prodrug **9** was synthesized using a modified procedure.[1] To a solution of 4-azidobenzyl-4-nitrophenyl carbonate (18 mg, 0.057 mmol) in anhydrous DMF (1mL) was added 4 Å molecular sieves (139 mg, crushed) and triethylamine (15 μL, 0.108 mmol). The solution was stirred at 25 °C for 10 min before a solution of doxorubicin hydrochloride salt (40 mg, 0.069 mmol) in anhydrous DMF (1.5 mL) containing triethylamine (16 μL, 0.115 mmol) was added. The solution was stirred under nitrogen at 25 °C for 19 h, at which time TLC analysis (5% methanol:DCM) indicated that all of the 4-azidobenzyl-4-nitrophenyl carbonate had been

consumed. The reaction was diluted with water (70 mL) and extracted with ethyl acetate (4 x 70 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (2 x 50 mL), water (3 x 70 mL), brine (2 x 70 mL), dried ($MgSO_4$), and concentrated *in vacuo* (temperature maintained below 30 °C). The crude red residue was subjected to flash silica gel column chromatography (100% DCM then 2% methanol:DCM), to provide the title compound as a dark red solid (28.6 mg, 69%) (See Figure S30-S32 for NMR and HPLC purity).

^{1}H NMR ($CDCl_3$, 400 MHz): δ 13.90 (s, 1H); 13.13 (s, 1H); 7.97 (d, J = 7.6 Hz, 1H); 7.75 (dd, J = 8.0 Hz, 8.0 Hz, 1H); 7.36 (d, J = 8.4 Hz, 1H); 7.25 (d, J = 9.2 Hz, 2H); 6.92 (d, J = 8.4 Hz, 2H); 5.47 (d, J = 3.6 Hz, 1H); 5.26 (d, J = 8.8 Hz, 1H); 5.22 (br s, 1H); 4.95 (dd, J_{AB} = 15.6 Hz, 2H); 4.74 (d, J = 2.4 Hz, 2H); 4.52 (s, 1H); 4.13 (m, 1H); 4.05 (s, 3H); 3.85 (m, 1H); 3.65 (br s, 1H); 3.15 (d, J_{AB} = 18.8 Hz, 1H); 3.07 (br s, 1H); 2.88 (d, J_{AB} = 18.8 Hz, 1H); 2.31 (d, J = 14.8 Hz, 1H); 2.29 (br s, 1H); 2.14 (dd, J = 14.8 Hz, 4 Hz, 1H); 1.85 (dd, J = 13.2 Hz, 5.2 Hz, 1H); 1.77 (dt, J = 13.2 Hz, 3.6 Hz, 1H); 1.28 (d, J = 6.4 Hz, 3H). ^{13}C NMR: δ 213.8, 186.9, 186.4, 160.9, 156.1, 155.43, 155.41, 139.8, 135.7, 135.3, 133.49, 133.46, 133.0, 129.7, 120.6, 119.8, 119.0, 118.4, 111.4, 111.3, 100.7, 76.5, 69.7, 69.5, 67.3, 66.1, 65.5, 56.6, 47.0, 35.6, 33.8, 30.1, 16.8. HRMS (ESI+) calculated for: $C_{35}H_{34}N_4O_{13}Na$: 741.2015, found: 741.2036. HPLC-UV : t_r = 8.85 min (λ = 254 nm and 480 nm).

trans-Cyclooctene (TCO, 2)[2]

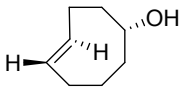


trans-Cyclooctene (TCO) was synthesized using a modified literature procedure.[2] To a 250 mL quartz reaction vessel was added *cis*-cyclooctene (0.602 g, 5.46 mmol), methyl benzoate (2.40 g, 17.6 mmol) and 100 mL of 1:1 diethyl ether:hexanes. The reaction mixture was placed in a Rayonet photoreactor and irradiated for 30 min at 254 nm. The solution was removed from the photoreactor and passed through a silver nitrate ($AgNO_3$) impregnated silica column, washed with one column volume of 1:1 diethyl ether:hexanes and the filtrate re-subjected to the photoreactor for a further 30 min. The process was repeated for a total of seven times. On the final run, the $AgNO_3$-impregnated silica gel was washed with DCM (2 x column volume) and diethyl ether (2 x column volume), and the silica gel allowed to dry under a flow of air pressure. The dry silica gel was then removed from the column and concentrated ammonium

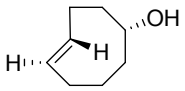
hydroxide (28%) added (10 mL). The slurry was stirred vigorously for 5 min. Pentane (20 mL) was added and stirred vigorously for a further 5 min, and then the pentane was decanted from the $AgNO_3$ silica gel. The silica gel was washed with additional pentane (2 x 20 mL), or until no more *trans*-cyclooctene was observed on the TLC ($KMnO_4$). The combined pentane extracts were washed with water, and dried ($MgSO_4$). The pentane was then removed by distillation at 45 °C on the rotary evaporator (no vacuum) to provide TCO **2** as a clear liquid with some pentane still present (0.178 mg, 29% crude). The product was spectroscopically similar to that reported in the literature.[2]

^{1}H NMR ($CDCl_3$, 400 MHz): 5.55-5.45 (m, 2H); 2.38-2.34 (m, 2H); 2.00-1.91 (m, 4H); 1.86-1.77 (m, 2H); 1.48-1.37 (m, 2H); 0.83-0.75 (m, 2H).

***trans*-Cyclooct-4-enol (major and minor diasteromers)[2-4] (TCO-OH, 10)**



major-**10**
-OH equitorial



minor-**10**
-OH axial

trans-Cyclooct-4-enol (TCO-OH) was synthesized using a modified literature procedure.[2-4] To a 250 mL quartz reaction vessel was added *cis*-cyclooct-4-enol (1.05 g, 8.32 mmol), methyl benzoate (3.40 g, 25.0 mmol) and diethyl ether (50 mL). The reaction mixture was placed in a Rayonet photoreactor and irradiated for 30 min at 254 nm. The solution was removed from the photoreactor and passed through a silver nitrate ($AgNO_3$) impregnated silica column (maintained in dark with aluminium foil), washed with one column volume of diethyl ether and the filtrate re-subjected to the photoreactor for a further 30 min. The process was repeated for a total of six times. On the final run, the $AgNO_3$-impregnated silica gel was washed with DCM (2 x column volume), diethyl ether (2 x column volume), ethyl acetate (1 x column volume), and the silica gel allowed to dry under a flow of air pressure. The dry silica gel was then removed from the column and concentrated ammonium hydroxide (28%) added (20 mL). The slurry was stirred vigorously for 5 min. Diethyl ether (20 mL) was added and stirred vigorously for a further 5 min, and then the diethyl ether was decanted from the $AgNO_3$ silica gel. The silica gel was washed with additional diethyl ether (2 x 20 mL), or until no more *trans*-cyclooct-4-enol was observed on the TLC ($KMnO_4$). The combined diethyl ether extracts were washed with water (3 x 30 mL), brine (3 x 30 mL), dried ($MgSO_4$) and concentrated *in vacuo* to

provide a pure mixture of *trans*-cyclooct-4-enol (1.42:1 mixture of diastereomers) as a clear viscous liquid (0.151 g, 14%). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 135.0, 134.3, 133.0, 132.8, 77.7, 67.4, 44.6, 43.0, 41.1, 34.3, 34.1, 34.0, 32.6, 31.2, 29.3, 27.7. (See figure S33-S36 for ^{1}H and ^{13}C NMR spectra of mixture of diastereomers, and ^{1}H NMR spectra for the major isomer and minor isomer).

The mixture of diastereomers was subjected to flash silica gel column chromatography (10% diethyl ether:pentane) providing the **minor-10** isomer as a clear viscous liquid (0.056 g, 5%).[2] ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.62-5.51 (m, 2H); 4.05-4.02 (m, 1H); 2.40-2.32 (m, 1H); 2.28-2.18 (m, 2H); 2.17-2.06 (m, 2H); 1.92-1.71 (m, 3H); 1.70-1.61 (m, 1H); 1.33 (br s, 1H); 1.30-1.23 (m, 1H).

Continued elution with 20% diethyl ether:pentane provided the **major-10** isomer as a clear viscous liquid (0.087, 8%).[2,4] ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.61-5.53 (m, 1H); 5.42-5.34 (m, 1H); 3.48-3.43 (m, 1H); 2.37-2.23 (m, 3H); 2.00-1.88 (m, 4H); 1.72-1.51 (m, 3H); 1.32 (br s, 1H).

3. Spectrofluorometry Release Experiments

*Procedure of a typical experiment for release of 7-hydroxycoumarin **13a** (data in Figure 2):* Stock solutions of coumarin probe **8a** (5 mM, **Stock 1**), TCO-OH mixture-**10** (50 mM, **Stock 2**), and *cis*-cyclooctenol (50 mM, **Stock 3**) were prepared in acetonitrile (HPLC grade). Three solutions of PBS:acetonitrile were prepared to a volume of 800 μL (500 μL PBS and 300 μL acetonitrile). To each of the three solutions was added 100 μL of **stock 1**. To the blank (control) experiments was added an additional 100 μL of acetonitrile, for a total volume of 1 mL (1:1, PBS:acetonitrile) and a 0.5 mM solution of probe **8a** (solution 1). To begin the 1,3-dipolar cycloaddition reaction/release experiments, 100 μL of **stock 2** was added for a final volume of 1 mL (1:1 PBS:acetonitrile), a 0.5 mM solution of probe **8a** and a 5 mM solution of TCO-OH mixture-**10** (solution 2). The final control experiment with *cis*-cyclooctenol was started by addition of 100 μL of **stock 3**, giving a final volume of 1 mL (1:1, PBS:acetonitrile), a 0.5 mM solution of probe **8a** and a 5 mM solution of *cis*-cyclooctenol (solution 3). The solutions were incubated at 37 °C and at the relevant time point, a 50 μl aliquot was taken from each of the three reaction solutions and diluted separately in PBS (2950 μL, 60 x dilution). The PBS solution was vortexed briefly, transferred to a plastic cuvette and the fluorescence measured at 455 nm (Ex 360 nm). The experiment was repeated for a total of three runs (n = 3).

*Procedure of a typical experiment for release of 7-amino-4-methylcoumarin **13b** (data in Figure 2):* Stock solutions of coumarin probe **8b** (1.25 mM, **Stock 4**), and TCO-OH major-**10** (50 mM, **Stock 5**) were prepared in acetonitrile (HPLC grade or CD_3CN). The same experimental procedure as above was carried out with the final concentration of 0.5 mM for **8b** and 5 mM for TCO-OH major-**10**. The solutions were incubated at 37 °C and at the relevant time point, a 50 μl aliquot was taken from each of the three reaction solutions and diluted separately in PBS (2950 μL, 60 x dilution). The PBS solution was vortexed briefly, transferred to a plastic cuvette and the fluorescence measured at 455 nm (Ex 360 nm). The experiment was repeated for a total of three runs (n = 3).

To calculate the amount of fluorescence (relative release from probe **8a/8b**), a standard curve of 7-hydroxycoumarin **13a** and 7-amino-4-methylcoumarin **13b** was measured in 1 x PBS (stocks prepared in acetonitrile:PBS (1:1) and diluted 1000-fold in 1 x PBS). The height at the λ_{max} (455

nm) was used to generate a standard curve for 7-hydroxycoumarin **13a** (Figure S1) and 7-amino-4-methylcoumarin **13b** (Figure S2). From the standard curve, the fluorescence for the maximum amount of coumarin which could be released from probe **8a** was 1191.8 units (8.33 μM in 60 x diluted solution) and for **8b**, 1658.5 units (8.33 μM in 60 x diluted solution).

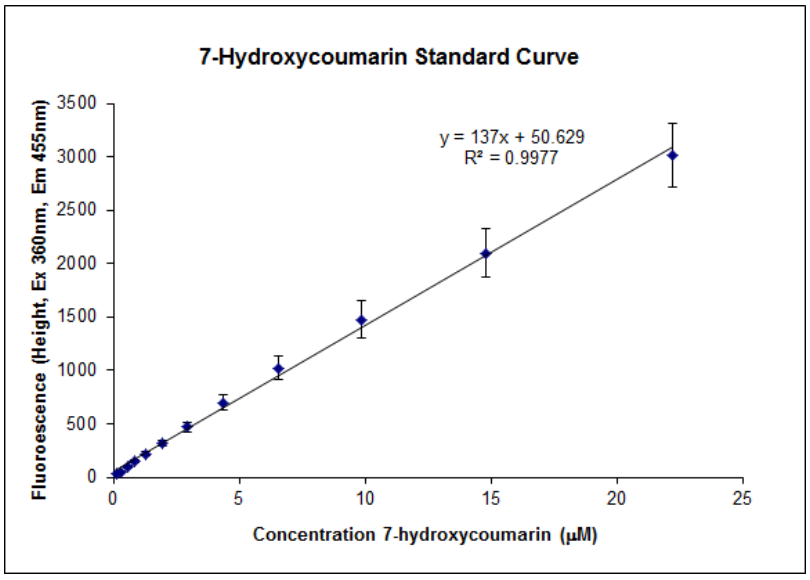


Figure S1. Standard curve of 7-hydroxycoumarin measured at an excitation of 360 nm and an emission of 455 nm (height). Error bars represent ± SD (n = 3).

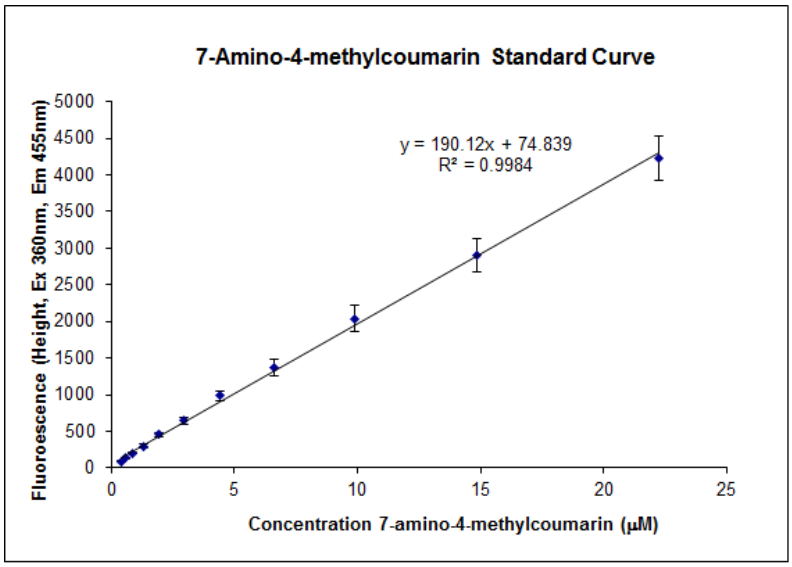


Figure S2. Standard curve of 7-amino-4-methylcoumarin **13b** measured at an excitation of 360 nm and an emission of 455 nm (height). Error bars represent ± SD (n = 3).

4. Spectrofluorometry Kinetic Experiments: Triazoline and Imine Degradation

In a typical experiment: Stock solutions of coumarin probe **8b** (40 mM, **Stock 6**), in DMSO-d_6 and TCO-OH major-**10** (50 mM, **Stock 7**), in CD_3CN were prepared. An aliquot of **Stock 6** (130 µL) was diluted with 195 µL CD_3CN and to this was added 325 µL of **Stock 7,** starting the 1,3-dipolar cycloaddition. The addition of the stocks resulted in a volume of 650 µL and a final concentration of 8 mM for coumarin probe **8b** and 25 mM of TCO-OH major-**10**. The NMR sample was incubated at room temperature and the progress was measured at 0 h, 3 h, 12 h, 19 h (25 °C). After 19 h of incubation, the NMR spectra indicated a complete conversion of the coumarin probe **8b** to the corresponding triazoline **11b** following a 1,3-dipolar cycloaddition reaction. An aliquot of NMR sample containing triazoline **11b** (3 µL) was further diluted into PBS (2997 µL, 1000 x dilution) and was subjected to the triazoline and imine degradation. The PBS solution was vortexed briefly, transferred to a plastic cuvette and the fluorescence was scanned over 3600 seconds at 455 nm (Ex 360 nm). The experiment was repeated for a total of three runs (n = 3).

To calculate the amount of fluorescence (relative release from probe **8b**), a standard curve of coumarin was measured. The height at the λ_{max} (455 nm) was used to generate a standard curve for 7-amino-4-methylcoumarin (Figure S2). From the standard curve (Figure S2), the fluorescence for the maximum amount of coumarin which could be released from probe **8b** was 1595.7 units (8 µM in 1000 x diluted solution). From the *pseudo* first-order plots (Figure S5) of the emitted fluorescence, we could conclude that only one of the two steps is rate determining, with a half-life of 19.25 mins.

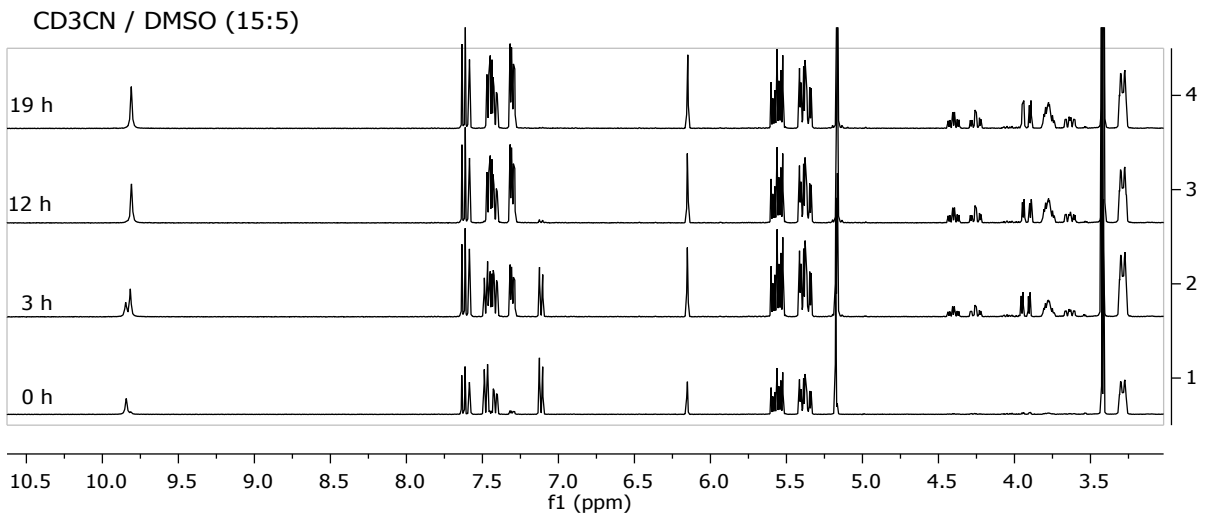


Figure S3. 1,3-Dipolar cycloaddition between probe **8b** and TCO-OH major-**10** monitored by ^{1}H NMR spectroscopy in CD_3CN/DMSO-d_6. At 19 h complete conversion of probe **8b** to the triazoline **11b** diastereomers was observed. Note that the peak at approximately 9.8 ppm is the NH proton of the carbamate linker, not an aldehyde peak.

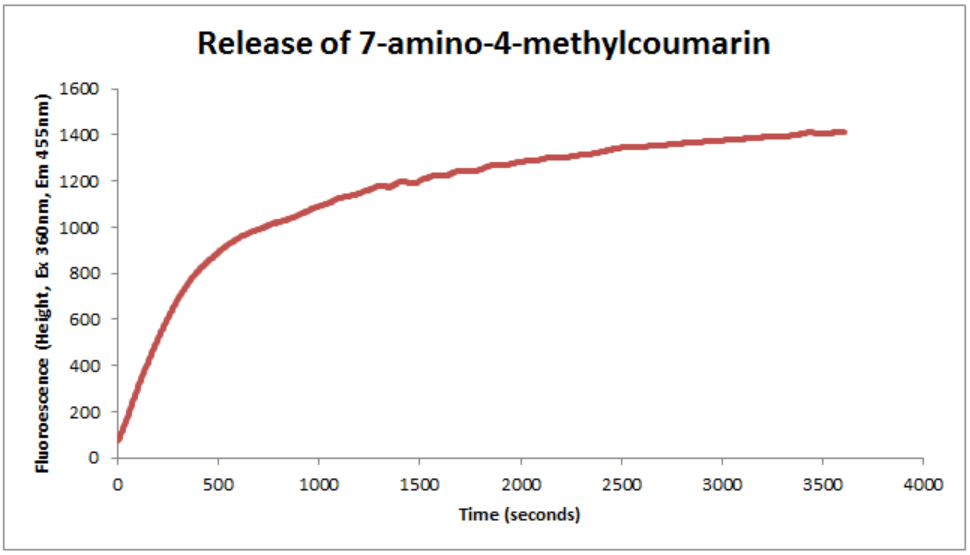


Figure S4. Release of 7-aminocoumarin **13b** from triazoline **11b** monitored by spectrofluorometry (ex. 360, em. 455). Average of triplicate experiments (n = 3).

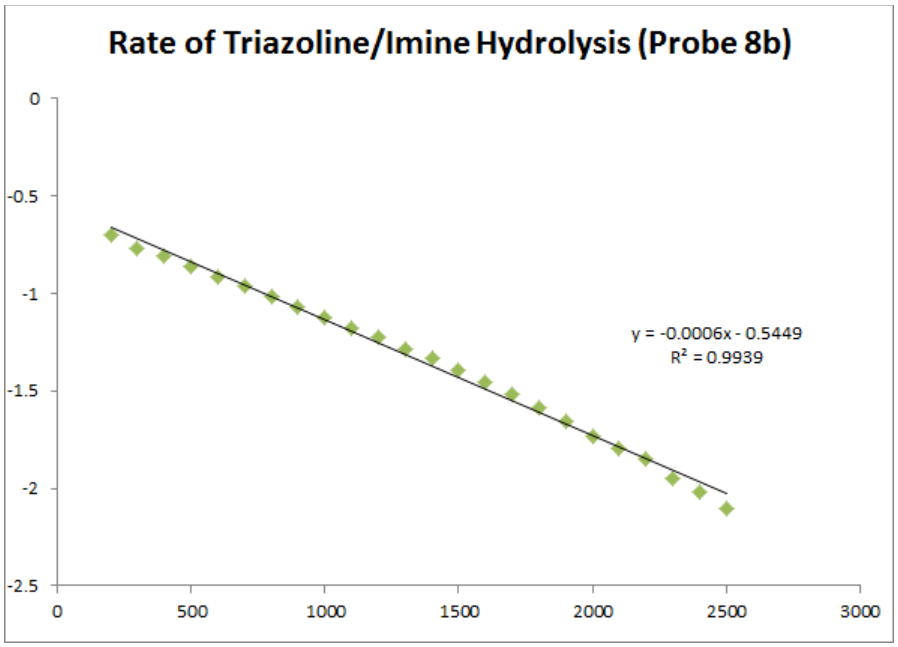


Figure S5. *Pseudo* first-order kinetic data obtained for degradation of azido-coumarin probe **8b** following dilution of 19 h NMR triazoline sample (in CD_3CN) into PBS (1000-fold dilution). Half-life calculated as 19.25 min. Data shown is averaged from triplicate runs (n = 3).

5. HPLC Kinetic Experiments: 1,3-Dipolar Cycloaddition

An acetonitrile stock of *trans*-cyclooctenol **10** was prepared at 100 mM or 200 mM, and a coumarin probe **8a** stock was prepared at 5mM (Note: coumarin probe **8b** experiments were run under similar conditions, a 100 mM stock was prepared with 17% DMSO and was further diluted in acetonitrile 20-fold to 5mM resulting in 0.85% DMSO). For the control experiment (run in triplicate), 100 µl of coumarin probe **8a** stock (in acetonitrile) was added to a solution containing 400 µl acetonitrile and 500 µl PBS (total volume of 1 mL). From this solution was taken a 50 µL aliquot, which was diluted into a 950 µL solution of PBS:acetonitrile (1:1) in an HPLC vial. The solution was injected onto the HPLC (inj. vol. 20 µL or 50 µL) at time = 0 min. The control was then incubated at 37 ^{o}C for 240 min before a further aliquot was taken and measured by HPLC-UV at 254 nm. This was run in at least triplicate and demonstrated that the probe **8a** was stable over the course of the experiment.

To begin the 1,3-dipolar cycloaddition reaction, 100 µl of the *trans*-cyclooctenol **10** stock was added to a vial containing 100 µl of the coumarin probe **8a** stock, 300 µl acetonitrile and 500 µl PBS. The reaction was incubated at 37 °C and at the time points indicated (Figure S6-S8), a 50 µL aliquot was taken and diluted in a 950 µL solution of PBS:acetonitrile (1:1). The solution was injected onto the HPLC (inj. vol. 20 µL or 50 µL), and the disappearance in absorbance for the coumarin probe **8a** was measured at 254 nm (See Figures S6-S10 for examples of *pseudo* first-order rate data and HPLC-UV traces). From the *pseudo* first-order plots, the second-order rates could be calculated using the concentration of TCO-OH **10** (20 or 40-fold excess; i.e. 10 mM or 20 mM).

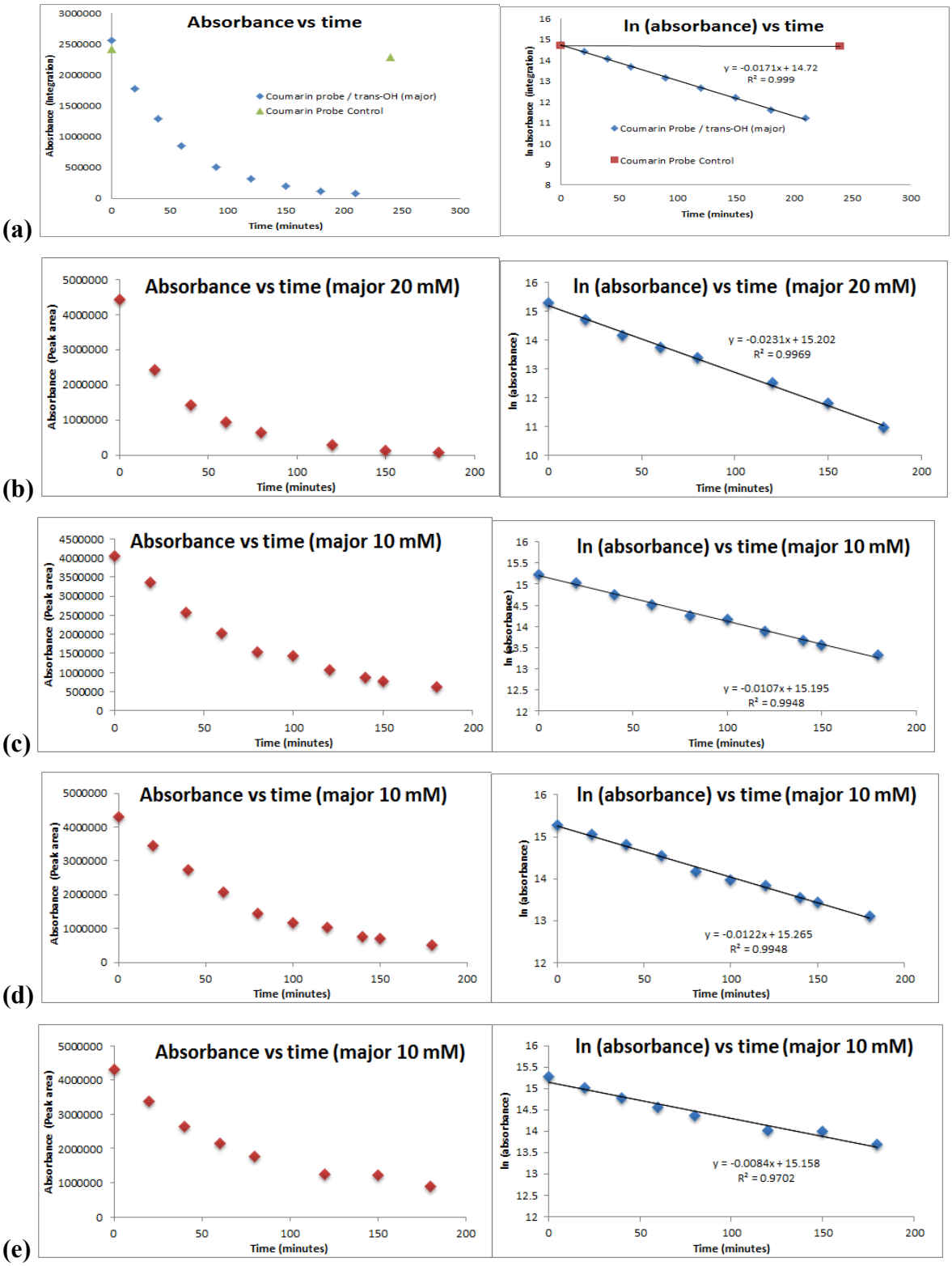


Figure S6. *Pseudo* first-order kinetic data obtained for reaction of azido-coumarin probe **8a** (0.5 mM) and TCO-OH **major-10**. **(a)** 20 mM; **(b)** 20 mM; **(c)** 10 mM; (d) 10 mM; and **(e)** 10 mM. Second-order rate constant calculated as: 0.017 ± 0.003 $M^{-1}s^{-1}$ (n = 5).

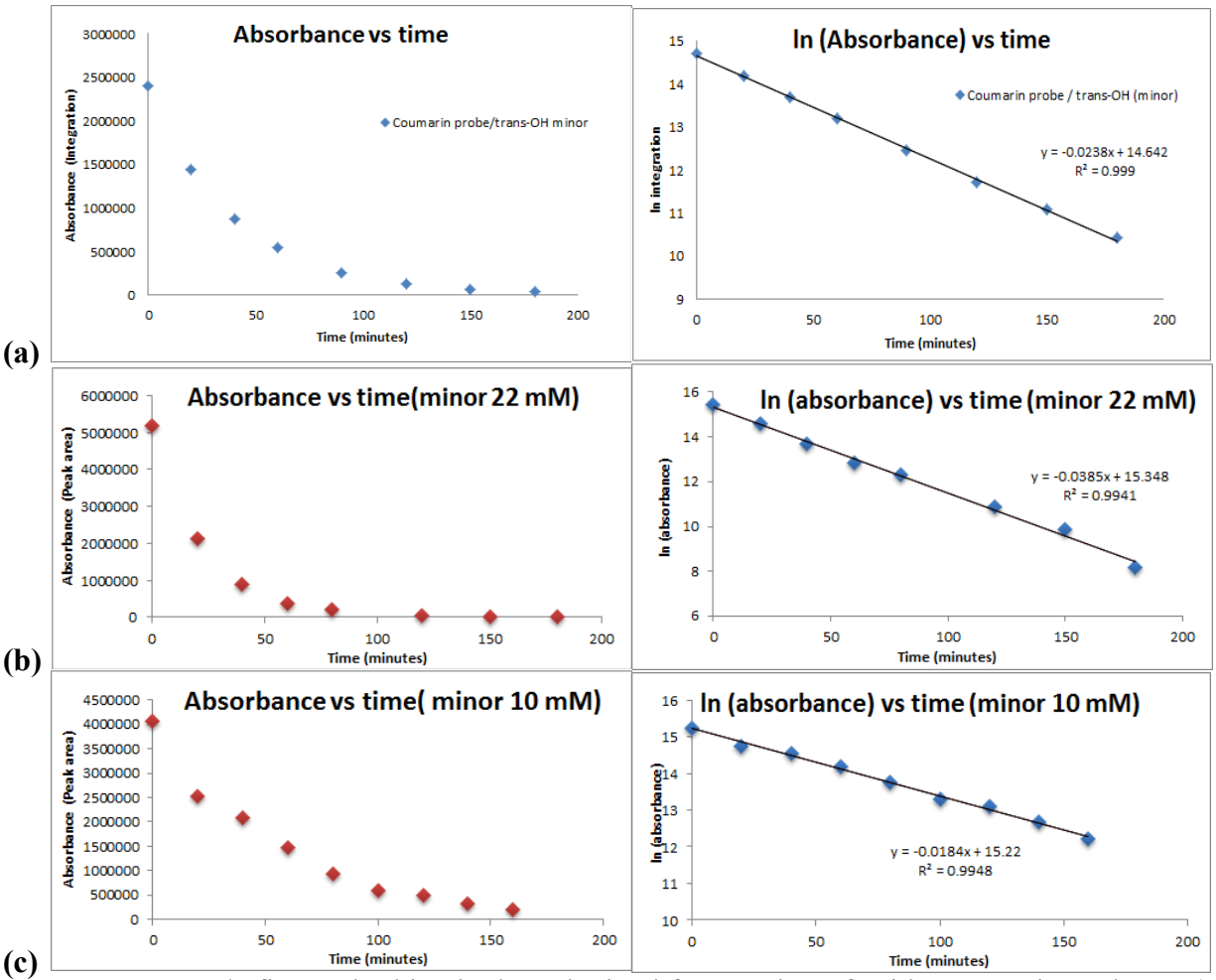


Figure S7. *Pseudo* first-order kinetic data obtained for reaction of azido-coumarin probe **8a** (0.5 mM) and TCO-OH **minor-10**. **(a)** 20 mM; **(b)** 22 mM; and **(c)** 10 mM. Second-order rate constant calculated as: 0.027 ± 0.006 $M^{-1}s^{-1}$ (n = 3).

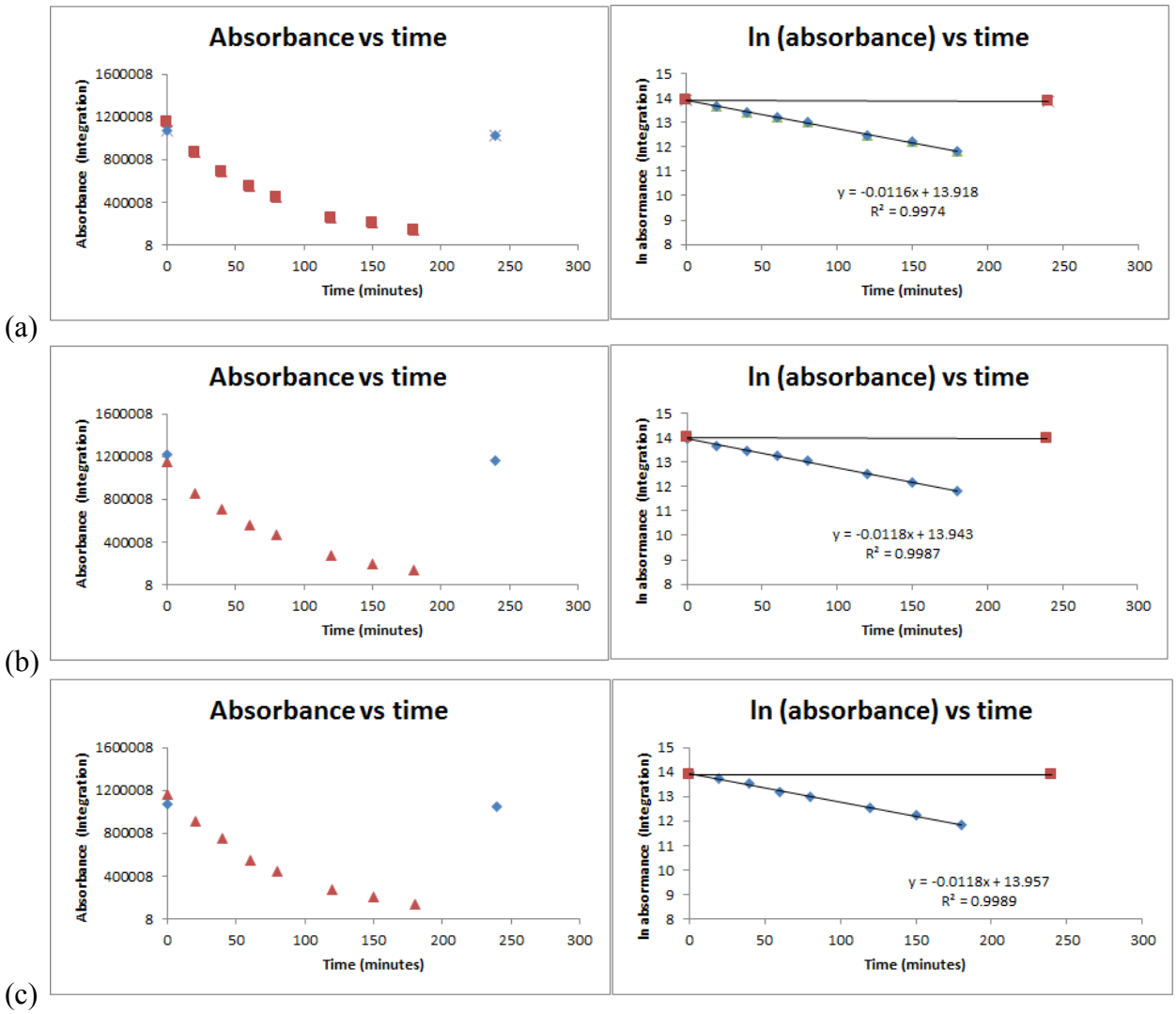


Figure S8. *Pseudo* first-order kinetic data obtained for reaction of azido-(7-amino-4-methyl) coumarin probe **8b** (0.5 mM) and TCO-OH **major-10. (a)** 10 mM; **(b)** 10 mM; **(c)** 10 mM; Second-order rate constant calculated as: 0.020 ± 0.0002 $M^{-1}s^{-1}$ (n = 3).

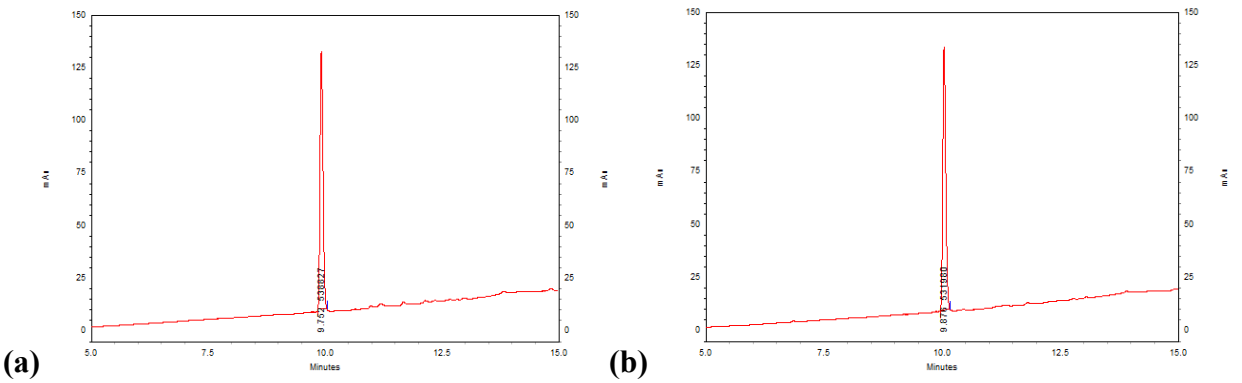


Figure S9. Example of probe **8a** control experiment (t_R = 9.8 min) measured at **(a)** 0 min and (b) 240 min. Absorbance is measured at 254 nm, and the area under curve was used for *pseudo* first-order calculations.

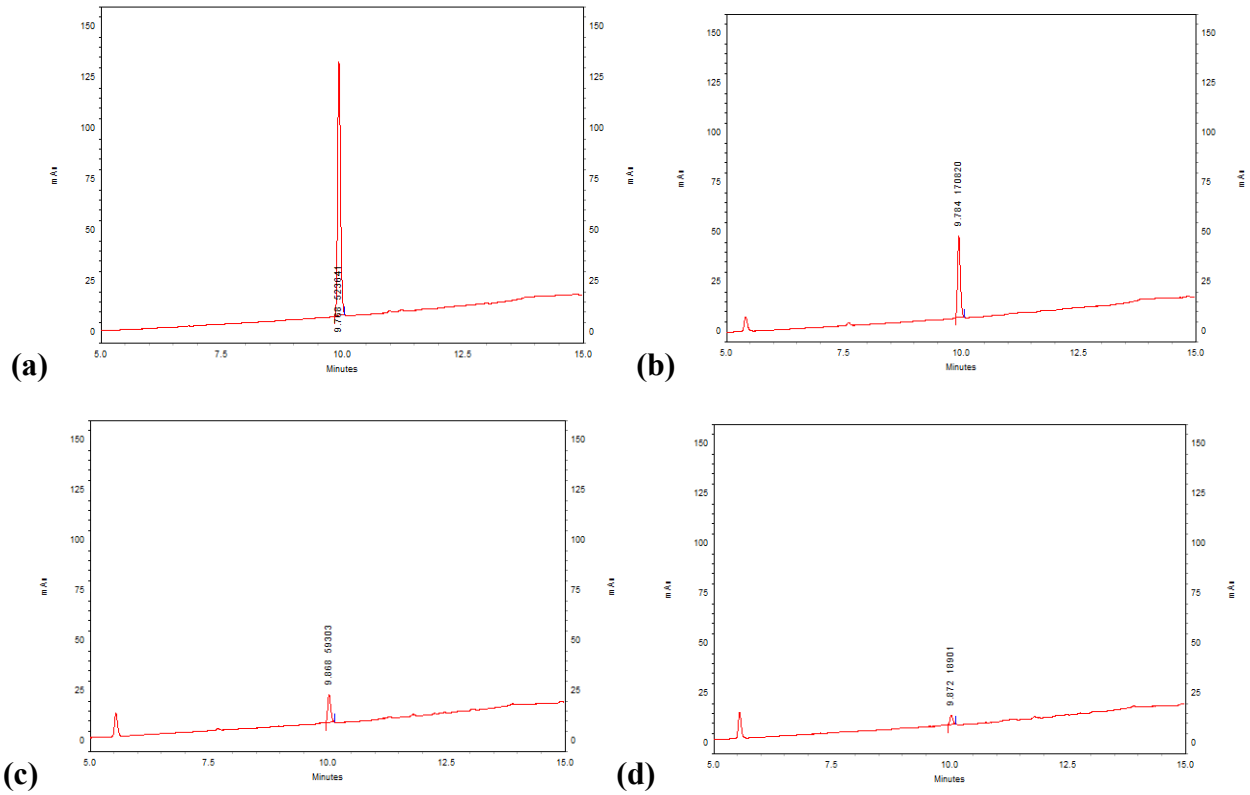


Figure S10. Examples of 1,3-dipolar cycloaddtion kinetic experiment between probe **8a** (t_R = 9.8 min) and *trans*-cyclooctenol **10** measured at **(a)** 0 min, **(b)** 60 min, **(c)** 120 min, and **(d)** 180 min. Absorbance is measured at 254 nm, and the area under curve was used for *pseudo* first-order calculations.

6. ^{1}H NMR Product Distribution Experiments

In a typical ^{1}H NMR experiment: A stock solution of coumarin probe **8a** (10 mM), 4-iodonitrobenzene (~175 mM, internal standard) in either $CDCl_3$ (filtered through basic alumina) or CD_3CN were prepared (Note: the concentration of the 4-iodobenzene could not be accurately determined due to lower solubility in CD_3CN, hence the internal standard was used solely as a visual guide to determine changes in the size of other peaks). An aliquot was taken from the coumarin probe **8a** stock solution and added to an NMR tube so that the final concentration when diluted to 750 μL would be 6.67 mM. To this solution was added the internal standard (20 μL) and $CDCl_3$ (or CD_3CN and D_2O) to give a volume of 735 μL. The reaction was started (time = 0 h) upon addition of a 15 μL solution of *trans*-cyclooctene **2**, which gave a final volume of 750 μL and a *trans*-cyclooctene **2** concentration of 18.7 mM in the $CDCl_3$ experiment and 10.6 mM in the CD_3CN experiment (Note: traces of pentane from work-up were present in the *trans*-cyclooctenol **2**, therefore, the final concentration of **2** was determined by integration against the known concentration of coumarin probe **8a**). The NMR sample was incubated at 37 °C and measured at the indicated time points (25 °C).

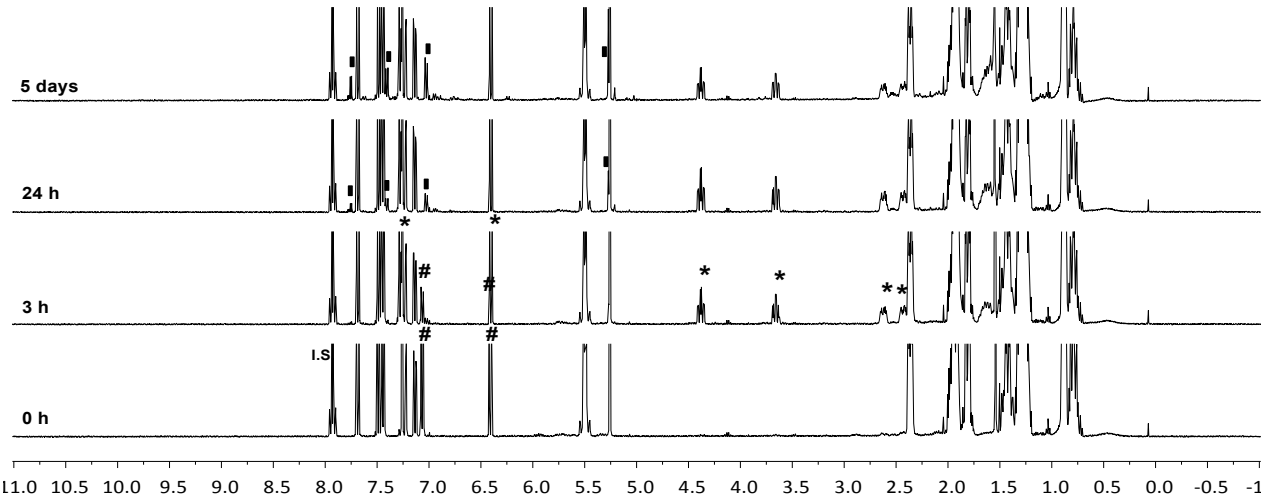


Figure S11. Full ^{1}H NMR spectrum (in $CDCl_3$) monitoring the 1,3-dipolar cycloaddition between TCO **2** and coumarin probe **8a**. Legend: Key structural proton shifts illustrated; #: coumarin probe **8a**; *: triazoline **11a**; ■: aldimine **12a**; !: TCO; I.S.: Internal Standard, 4-iodonitrobenzene.

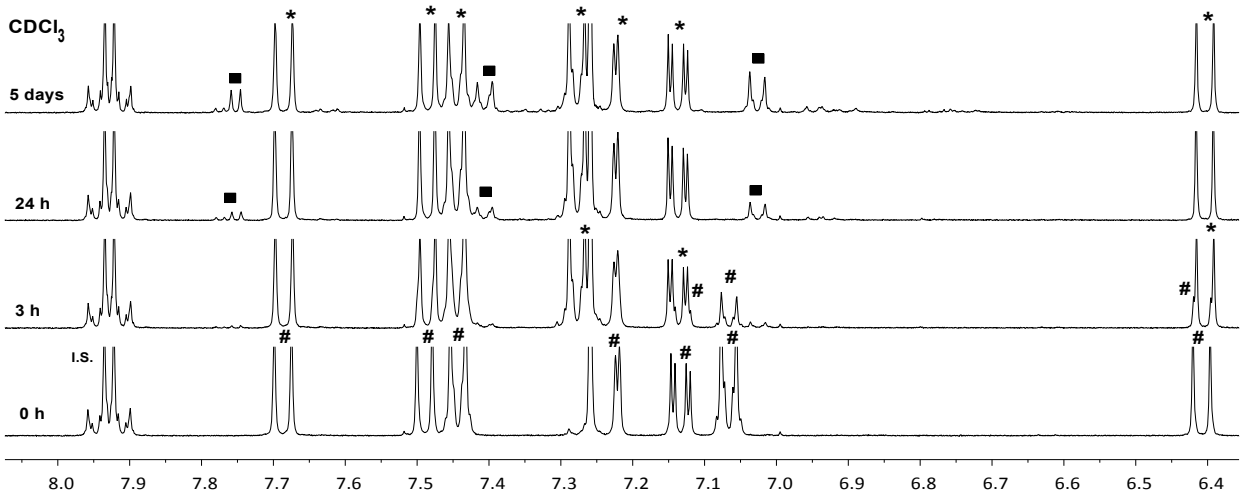


Figure S12. Aromatic region of $CDCl_3$ ^1H NMR spectrum monitoring the 1,3-dipolar cycloaddition between TCO **2** and coumarin probe **8a**. Legend: Key structural proton shifts illustrated; #: coumarin probe **8a**; *: triazoline **11a**; ■: aldimine **12a**; !: TCO; I.S.: Internal Standard, 4-iodonitrobenzene.

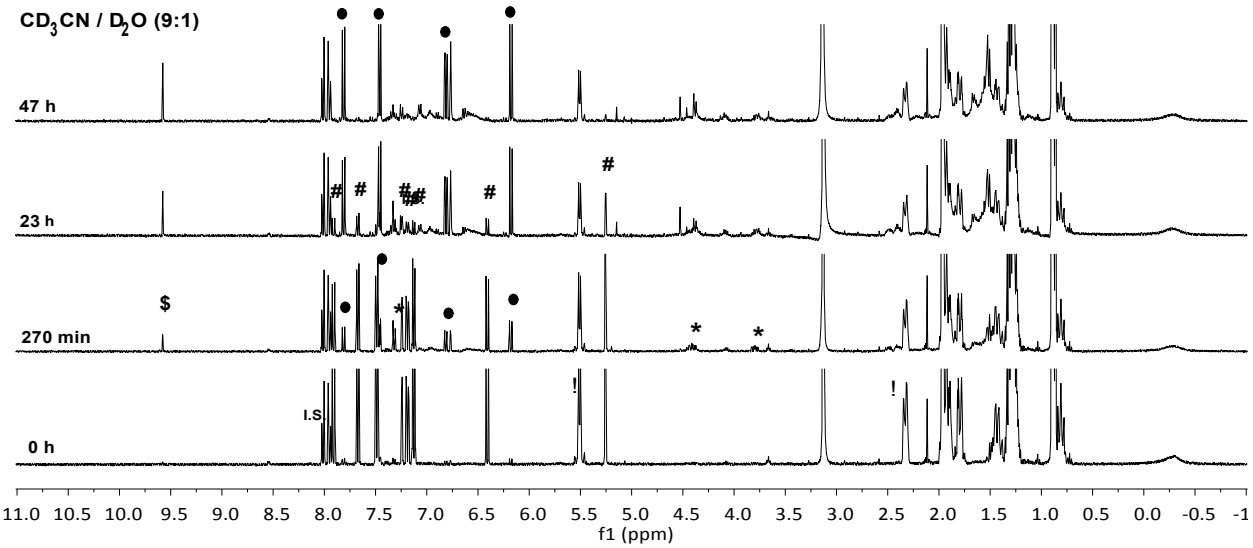


Figure S13. Full ^1H NMR spectrum (in CD_3CN:D_2O) monitoring the 1,3-dipolar cycloaddition between TCO **2** and coumarin probe **8a**. Legend: Key structural proton shifts illustrated; #: coumarin probe **8a**; *: triazoline **11a**; ●: 7-hydroxycoumarin **13**; $: exocyclic aldehyde **14a**; !: TCO; I.S.: Internal Standard, 4-iodonitrobenzene.

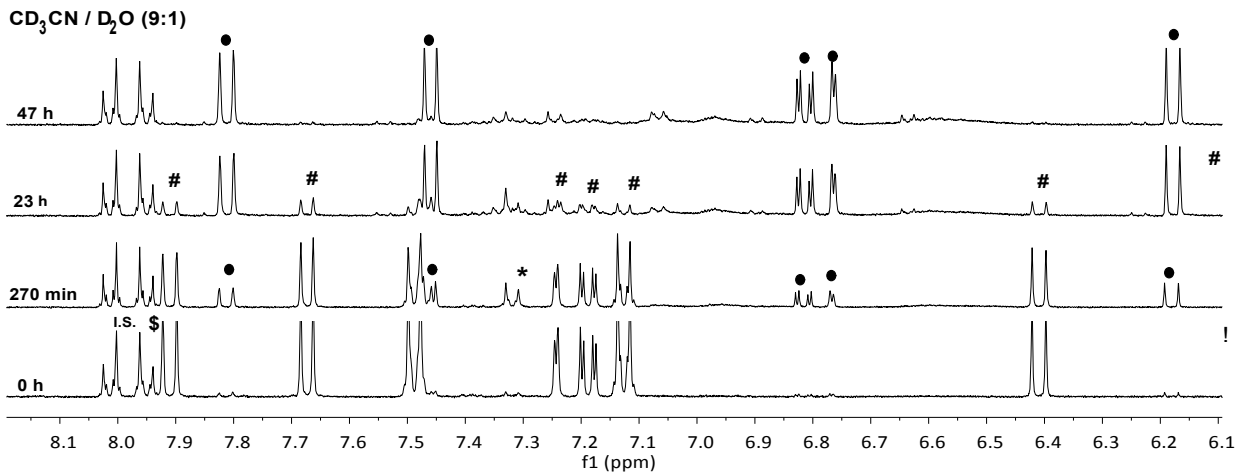


Figure S14. Aromatic region of $CD_3CN:D_2O$ (9:1) spectrum monitoring the 1,3-dipolar cycloaddition between TCO **2** and coumarin probe **8a**. Legend: Key structural proton shifts illustrated; #: coumarin probe **8a**; *: triazoline **11a**; ●: 7-hydroxycoumarin **13**; $: exocyclic aldehyde 14a; !: TCO; I.S.: Internal Standard, 4-iodonitrobenzene.

7. Cell Culture and Proliferation Assay

Murine B16-OVA melanoma cell line was kindly donated from Prof Sarah Hook (School of Pharmacy, University of Otago). The murine B16-OVA melanoma cells were maintained in a humidified CO_2 (5%) incubator (Heraeus) at 37° C in RPMI-1640 media (Gibco), supplemented with 10% fetal bovine serum (FBS) (Gibco), 1% GlutaMAX (Gibco), 1% Pen Strep (Gibco) and 0.1% 2-Mercaptoethanol (55mM) (Gibco). Cells were plated out in flat bottom 96-well plates (BD Falcon) at a density of 7500 cells/well and allowed to attach for 24 h. The doxorubicin prodrug **9** was dissolved in DMSO at 10 mM and subsequently serial-diluted in pre-warmed culture media. *trans*-Cyclooctenol **10** was dissolved in DMSO at 100 mM and diluted to 200 μM in pre-warmed culture media. After 24 h cell attachment, cell culture medium was replaced by 50 μl pre-warmed media containing the compounds in different concentrations and 50 μl prewarmed media containing TCO-OH **10** at 200 μM (final concentration in each well was 100 μM). After 72 h incubation, cell proliferation was assessed by means of a MTT assay. Thiazolyl Blue Tetrazolium Bromide (MTT) (Sigma Aldrich) was freshly dissolved in PBS at 5 mg/mL, passed through a 0.22 μm filter, and 10 μL were added to each well. After an incubation period of 4 h, the medium was gently removed. The formed formazan crystals were dissolved in 100 μL DMSO (Sigma Aldrich), subsequently the absorbance was measured with a plate reader (PolarStar Omega, BMG Labtech)) at 570 nm. As a reference wavelength (background), 690 nm was chosen and subtracted from the absorbance at 570 nm. The cytotoxicity assay was performed in at least triplicate independent experiments, with duplicate experiments on each plate (i.e. 2 x 3 wells per drug/prodrug concentration in each of the independent experiments for a total of $n \geq 6$). IC_{50} values were calculated using the normalized (variable) dose-response parameters in GraphPad Prism 5 software (Figure S16). The cytotoxicity of the TCO-OH **10** (mixture, major, and minor) and CCO-OH against the B16-OVA cell line at 100 μM were determined from at least three independent experiments (Figure S15).

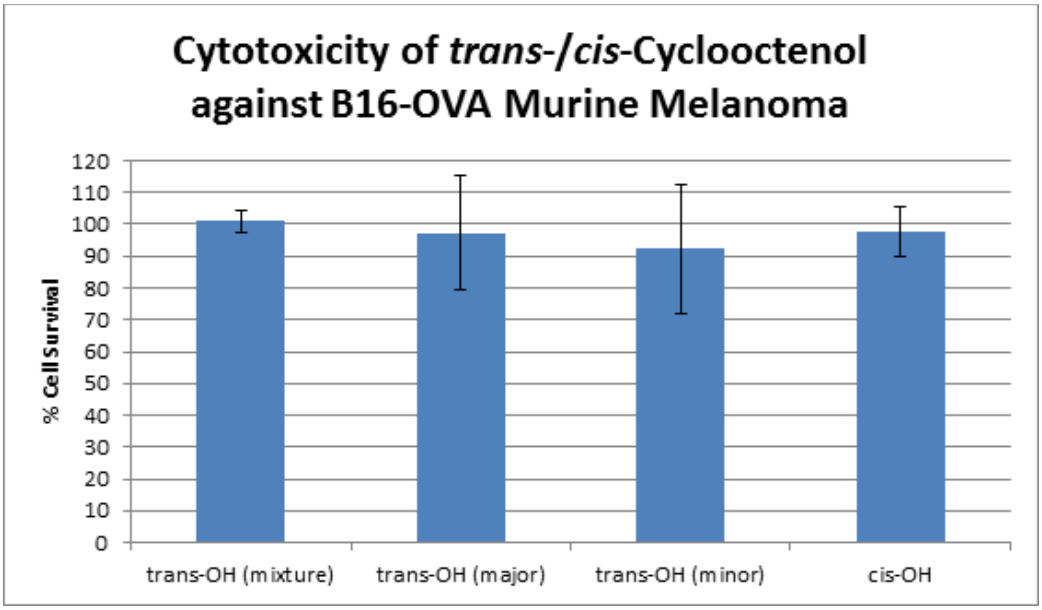


Figure S15. Cytotoxicity of *trans*-cyclooctenol **10** and *cis*-cyclooctenol against B16-OVA murine melanoma cells. Error bars represent ±SD ($n \geq 3$).

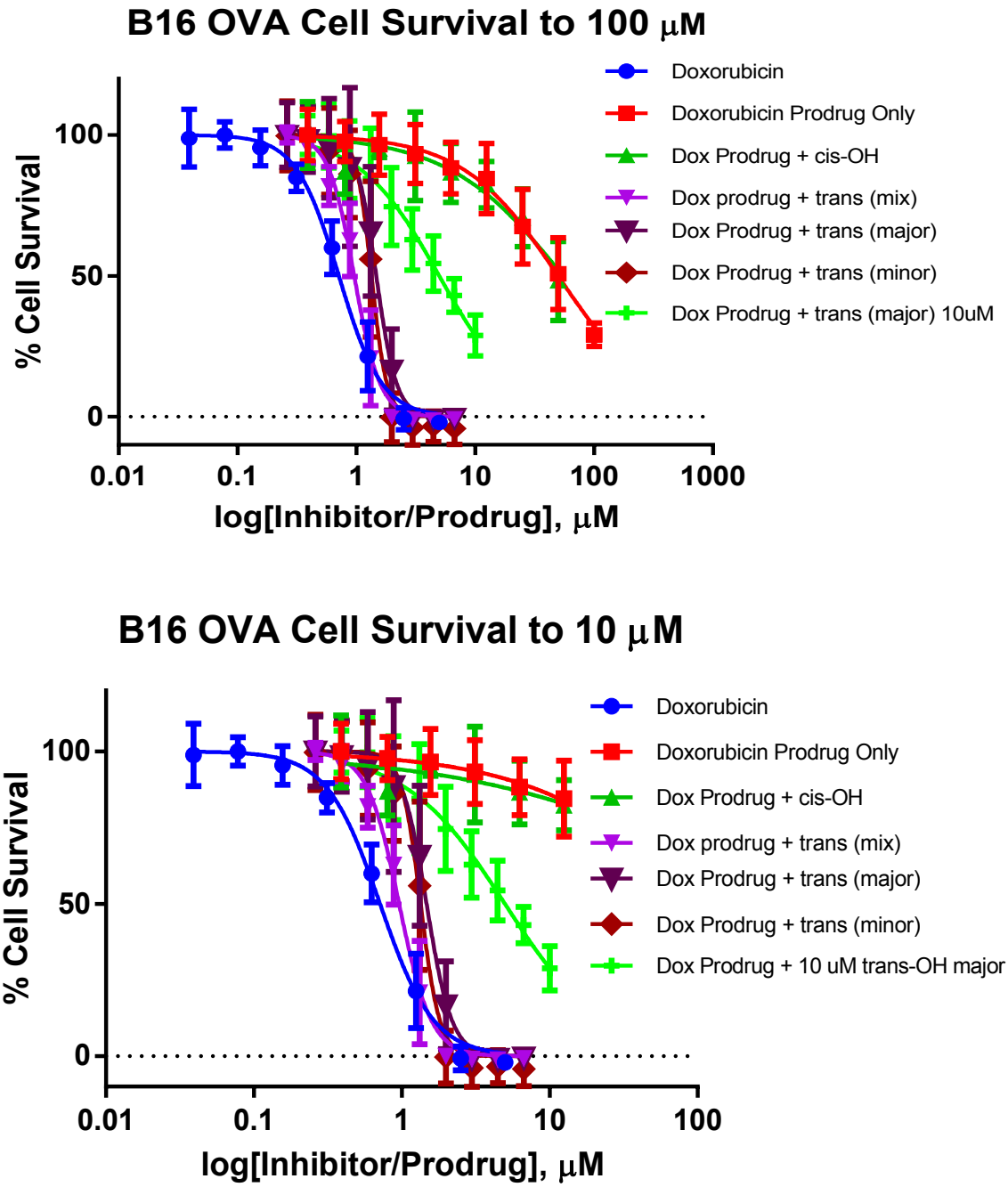


Figure S16. Cytotoxicity assay against the B16-OVA murine melanoma cell line. Error bars represent ±SD ($n \geq 6$).

8. Stability, Release and Rate Studies on Doxorubicin Prodrug 9

Fresh mouse serum (from C57B1/6 mice) was collected and provided by Prof Sarah Hook in the School of Pharmacy, University of Otago.

***A typical stability experiment*:** A stock solution of doxorubicin prodrug **9** (10 mM) in DMSO was prepared. This was diluted in PBS to a concentration of 200 μM. 100 μL of the PBS solution was added to 100 μL of mouse serum (or PBS for control experiments), vortexed and incubated at 37 °C. A 20 μL aliquot was taken at the indicated time points (0 h, 4 h, 24 h, 48 h) and diluted with 80 μL of cold acetonitrile (5-fold dilution). The diluted sample was centrifuged (13,400 rpm) for 5 min, and the supernatant added to an HPLC vial (150 μL insert). 50 μL of the supernatant was injected onto the HPLC and analysed at 254 nm and 480 nm using the general HPLC method (see section 1 of S.I.). The stability in 50% serum:PBS and PBS was then calculated by the decrease in peak area of the prodrug **9** (t_R = 8.7 min), relative to the area at 0 h (100%). The percentage of doxorubicin released at each time point was calculated from a standard curve of doxorubicin (Figure S17). The results for the stability studies are shown in Table S1, Table S2, Figure S18-S20. A representative example of the raw HPLC data is illustrated in Figure S21 and Figure S22.

A typical release experiment: Stock solutions of doxorubicin prodrug **9** (10 mM in DMSO) and TCO-OH major-**10** (50 mM in DMSO) was prepared. 2 μL of the prodrug **9** stock was added to 196 μL of serum:PBS (1:1) or PBS only (control). To start the reaction 2 μL of the TCO-OH stock was added (final volume 200 μL), and the reaction incubated at 37 °C. A 20 μL aliquot was taken at the indicated time points (0 h, 4 h, 24 h, 48 h) and diluted with 80 μL of cold acetonitrile (5-fold dilution). The diluted sample was centrifuged (13,400 rpm) for 5 min, and the supernatant added to an HPLC vial (150 μL insert). 50 μL of the supernatant was injected onto the HPLC and analysed at 254 nm and 480 nm using the general HPLC method (see section 1 of S.I.). The amount of reacted prodrug **9** was determined by the decrease in peak area (t_R = 8.7 min), relative to the area at 0 h (100%). The percentage of doxorubicin **15** released at each time point was calculated from a standard curve of doxorubicin (Figure S17). The results for the activation/release studies are shown in Table S1, Table S2, Figure S19, and Figure S20. A representative example of the raw HPLC data is illustrated in Figure S23 and Figure S24.

Table S1. Stability and release studies of doxorubicin relative to peak area at time = 0 h.

	Stability (% Intact)[a,b] of Prodrug 9 (100 μM)		Reaction (% Intact)[a,b] of Prodrug 9 (100 μm) with TCO-OH major-10 (500 μM)	
Time (h)	**50% Serum:PBS**	**PBS only**	**50% Serum:PBS TCO-OH (500 μM)**	**PBS only TCO-OH (500 μM)**
0	100	100	100	100
4	95.3 ± 8.4	105.7 ± 20.6	46.7 ± 2.5	84.4 ± 14.2
24	68.0 ± 14.7	121.1 ± 36.0	11.5 ± 4.2	38.8 ± 16.3
48	55.6 ± 13.2[c]	111.9 ± 56.2	1.8 ± 0.4	11.7 ± 5.6

a) Values represent the percentage of intact prodrug **9** based on peak area (t_R = 8.7 min) at 254 nm (triplicate runs). b) Error shown as ± standard deviation (n = 3). c) Serum measurement taken at 53 h.

Table S2. Doxorubicin 15 release from prodrug 9 (100 μM) in the presence and absence of TCO-OH major-10 (500 μM). Concentration of doxorubicin release was determined using the standard curve (Figure S17) and used to calculate the percentage release (from the expected maximum concentration of doxorubicin).

	Release (%)[a,b] of Doxorubicin 15 (Stability)		Release (%)[a,b] of Doxorubicin 15 (Activation)	
Time (h)	**50% Serum:PBS**	**PBS only**	**50% Serum:PBS TCO-OH (500 μM)**	**PBS only TCO-OH (500 μM)**
0	0	0	0	0
4	0	0	51.4 ± 5.5	33.9 ± 11.3
24	0	0	48.5 ± 14.4	77.4 ± 15.0
48	5.7 ± 5.6[c]	0	4.7 ± 4.6[d]	78.5 ± 12.0

a) Values represent the percentage of doxorubicin **15** detected at 254 nm (t_R = 4.2 min) relative to the maximum amount of doxorubicin release possible (triplicate runs). b) Error shown as ± standard deviation (n = 3). c) Serum stability measurement taken at 53 h. d) Unidentified doxorubicin-related metabolite was observed at t_R = 7.6 min.

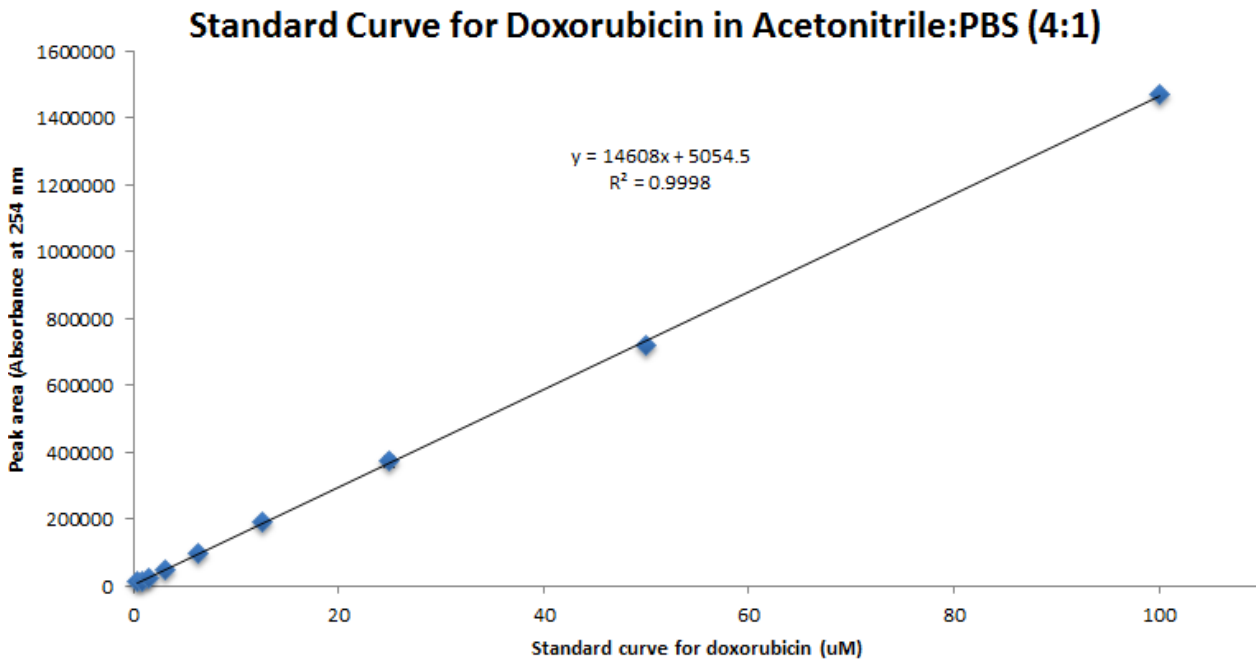


Figure S17. Doxorubicin (t_R = 4.2 min) standard curve in acetonitrile:PBS (4:1) at 254 nm. Error bars represent ± SD (n = 2). Note: Error bars too small to observe.

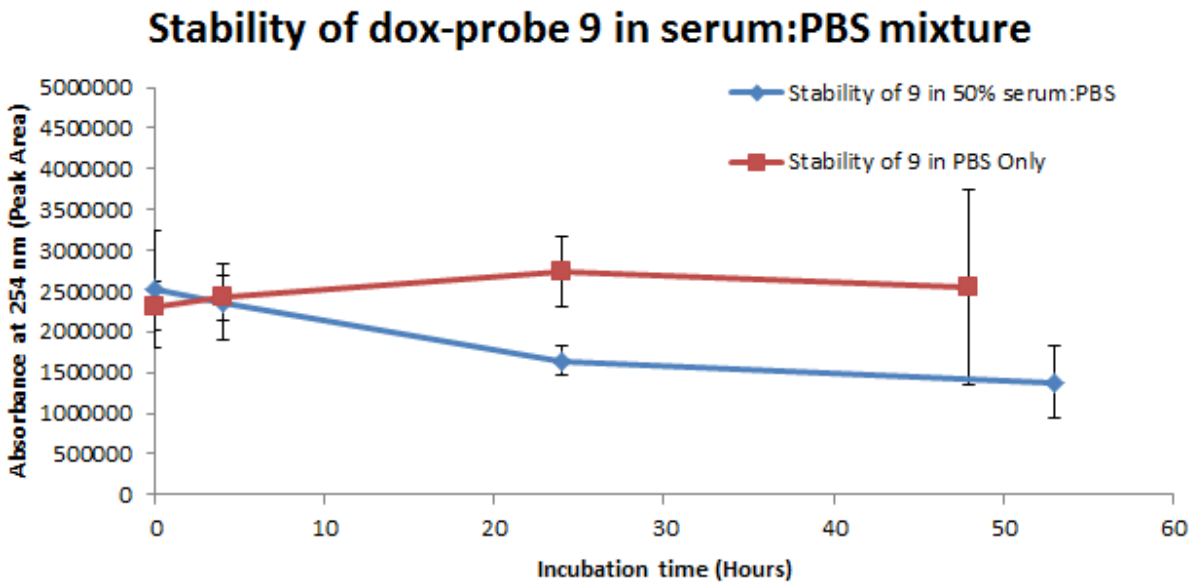


Figure S18. Stability of doxorubicin prodrug **9** in 50% serum:PBS and PBS only (control). Error bars represent ± SD (n = 3).

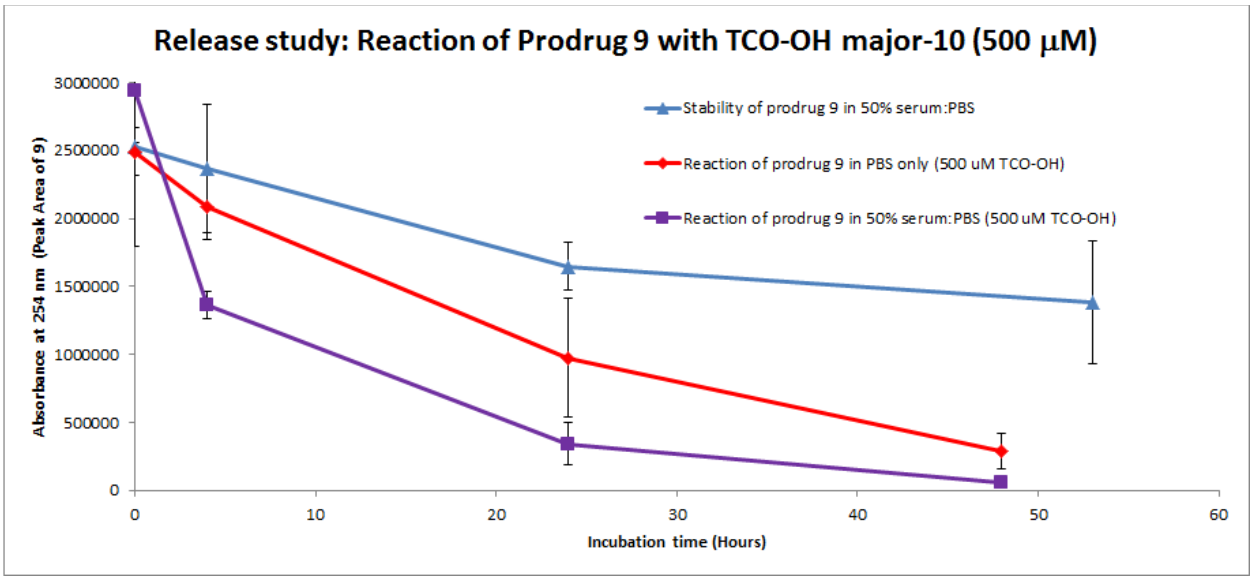


Figure S19. Reaction of doxorubicin prodrug **9** (100 µM) with TCO-OH major-**10** (500 µM) in 50% serum:PBS and PBS only. Shown for comparison is stability of **9** in 50% serum:PBS (also see Figure S18). Error bars represent ± SD (n = 3).

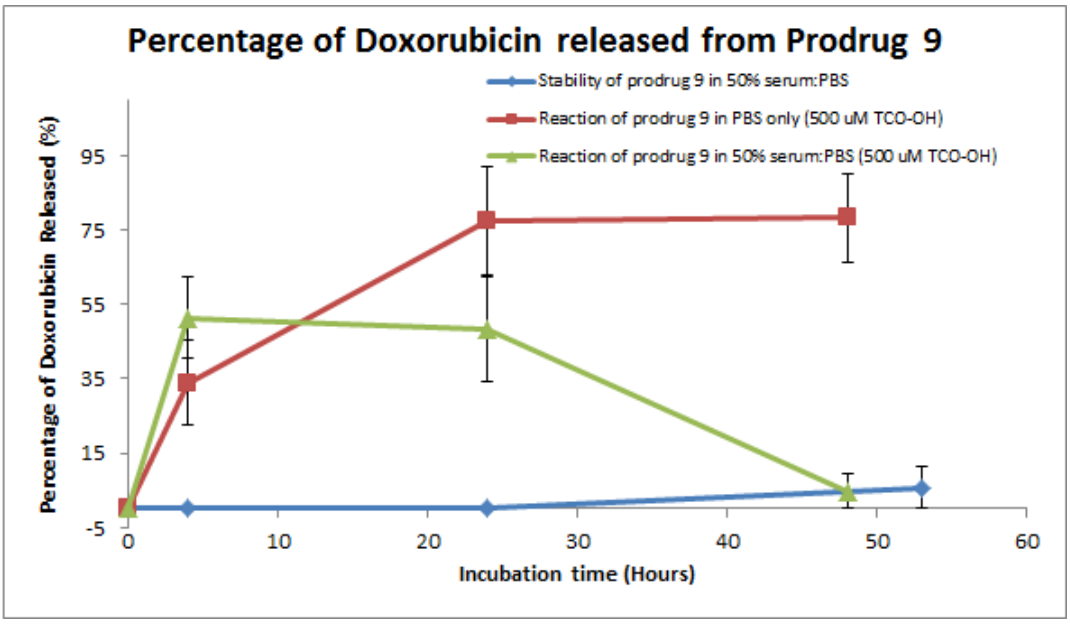


Figure S20. Percentage of doxorubicin **15** released from prodrug **9** in the stability and release (reaction with TCO-OH) experiments. The concentration of doxorubicin **15** released was determined from the doxorubicin standard curve (Figure S17 – measurements at 254 nm), and the percentage release calculated from maximum expected concentration (20 µM). Error bars represent ± SD (n = 3).

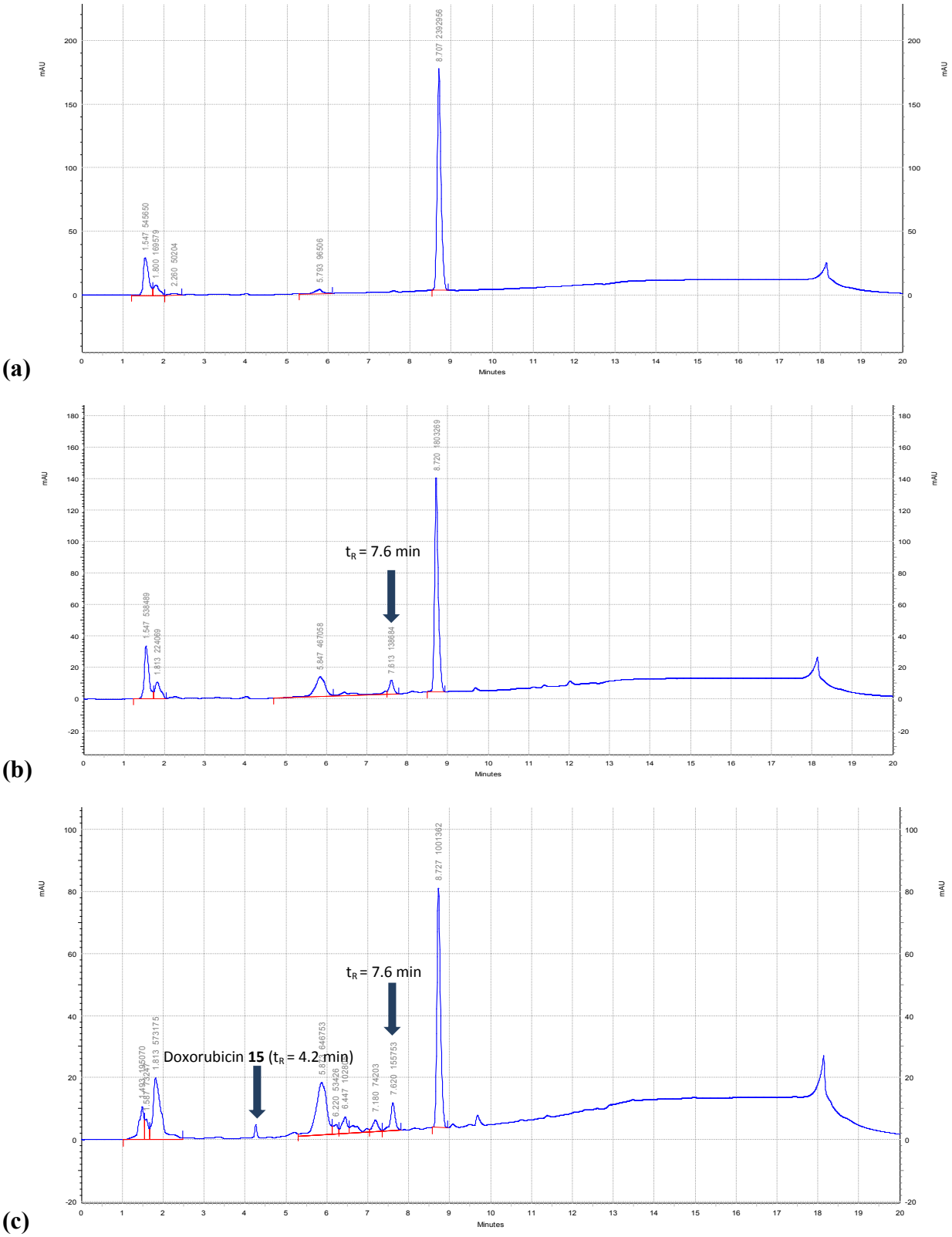
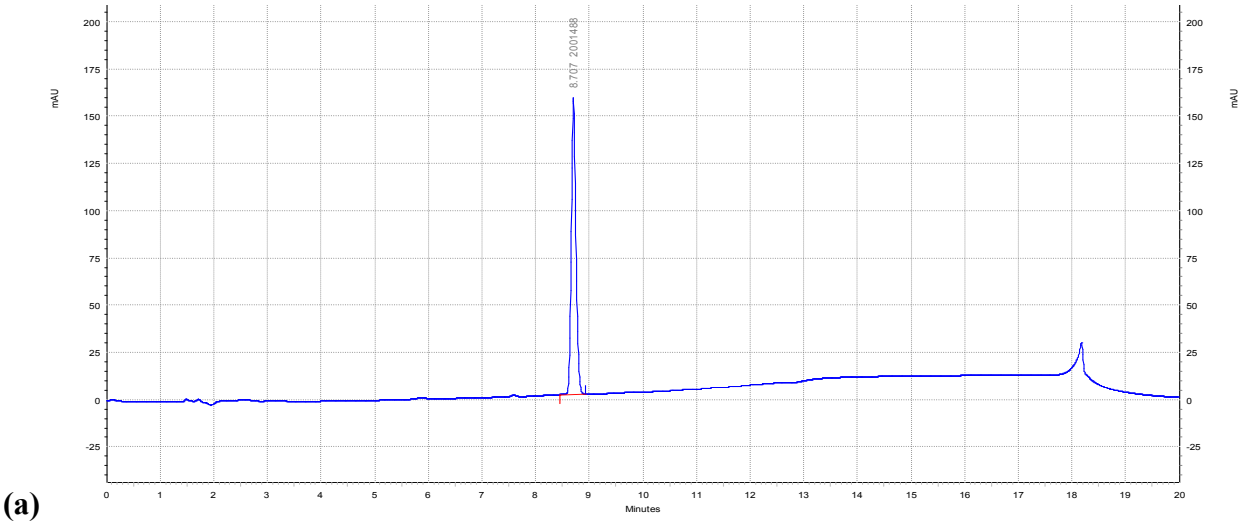
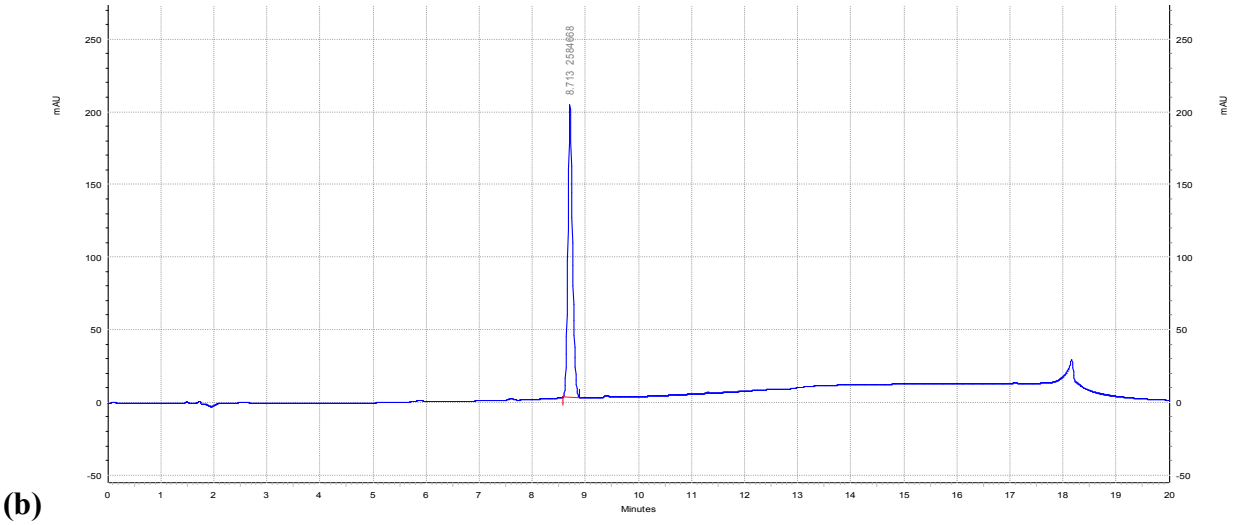


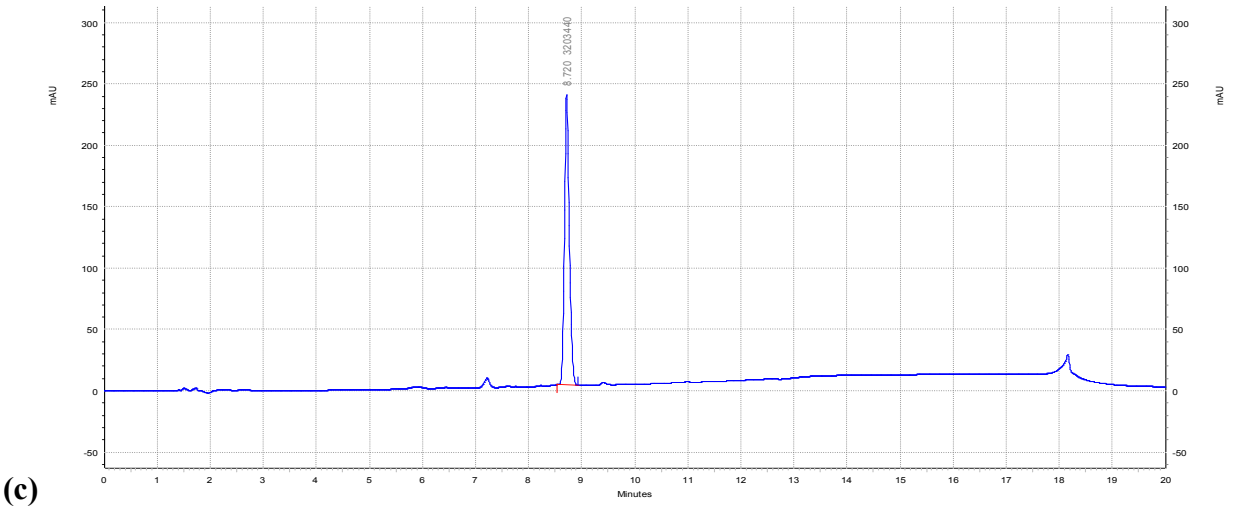
Figure S21. Stability of doxorubicin prodrug **9** in 50% mouse serum:PBS. Representative time points: (a) 0 h, (b) 24 h, (c) 53 h. Prodrug **9** t_R = 8.7 min, doxorubicin **15** t_R = 4.2 min.



(a)



(b)



(c)

Figure S22. Stability of doxorubicin prodrug **9** in PBS Only (Control). Representative time points: (a) 0 h, (b) 24 h, (c) 48 h. Prodrug **9** t_R = 8.7 min, doxorubicin **15** t_R = 4.2 min.

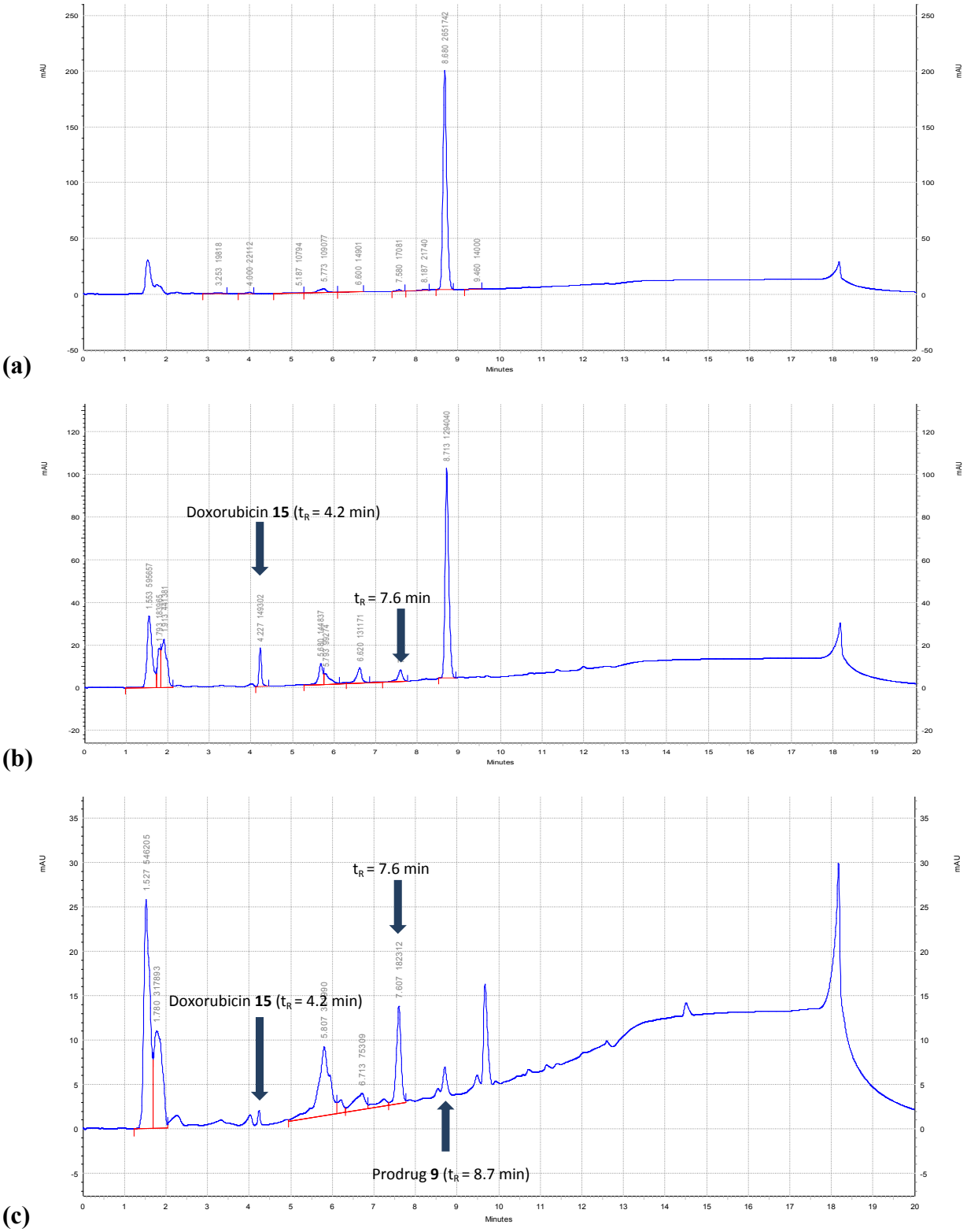


Figure S23. Release studies of doxorubicin **15** from doxorubicin prodrug **9** in 50% Serum:PBS with 500 µM of TCO-OH major-**10**. Representative time points: (a) 0 h, (b) 4 h, (c) 48 h. Prodrug **9** t_R = 8.7 min, doxorubicin **15** t_R = 4.2 min.

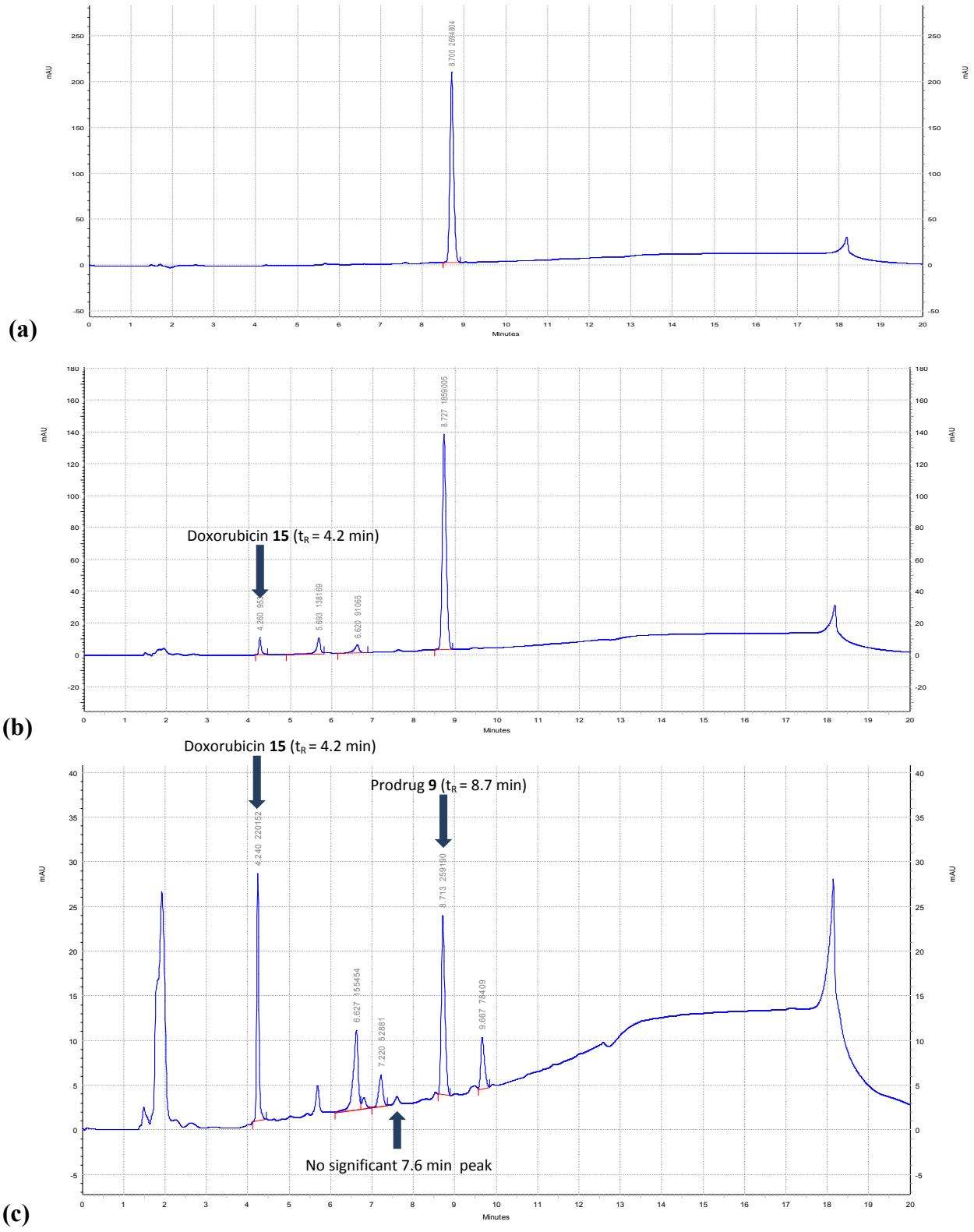


Figure S24. Release studies of doxorubicin **15** from doxorubicin prodrug **9** in PBS only (control) with 500 µM of TCO-OH major-**10**. Representative time points: (a) 0 h, (b) 4 h, (c) 48 h. Prodrug **9** t_R = 8.7 min, doxorubicin **15** t_R = 4.2 min.

*Typical rate determination assay for doxorubicin prodrug **9** in 50% mouse serum:PBS.*

The second-order rate constant for doxorubicin prodrug **9** was measured using *pseudo* first-order kinetics (similar to section 5 rate calculations with coumarin probes **8a** and **8b**). In a typical experiment, the reaction of prodrug **9** (0.1 mM) with TCO-OH major-**10** (10 mM) was incubated at 37 °C. A 15 μL aliquot was taken at the indicated time points (0 h, 4 h, 24 h, 48 h) and diluted with 60 μL of cold acetonitrile (5-fold dilution). The diluted sample was centrifuged (13,400 rpm) for 5 min, and the supernatant added to an HPLC vial (100 μL insert). 50 μL of the supernatant was injected onto the HPLC and analysed at 254 nm and 480 nm using the general HPLC method (see section 1 of S.I.). The experiment was repeated in triplicate (n = 3) to provide a second-order rate constant of 0.137 ± 0.012 $M^{-1}s^{-1}$ (Figure S25).

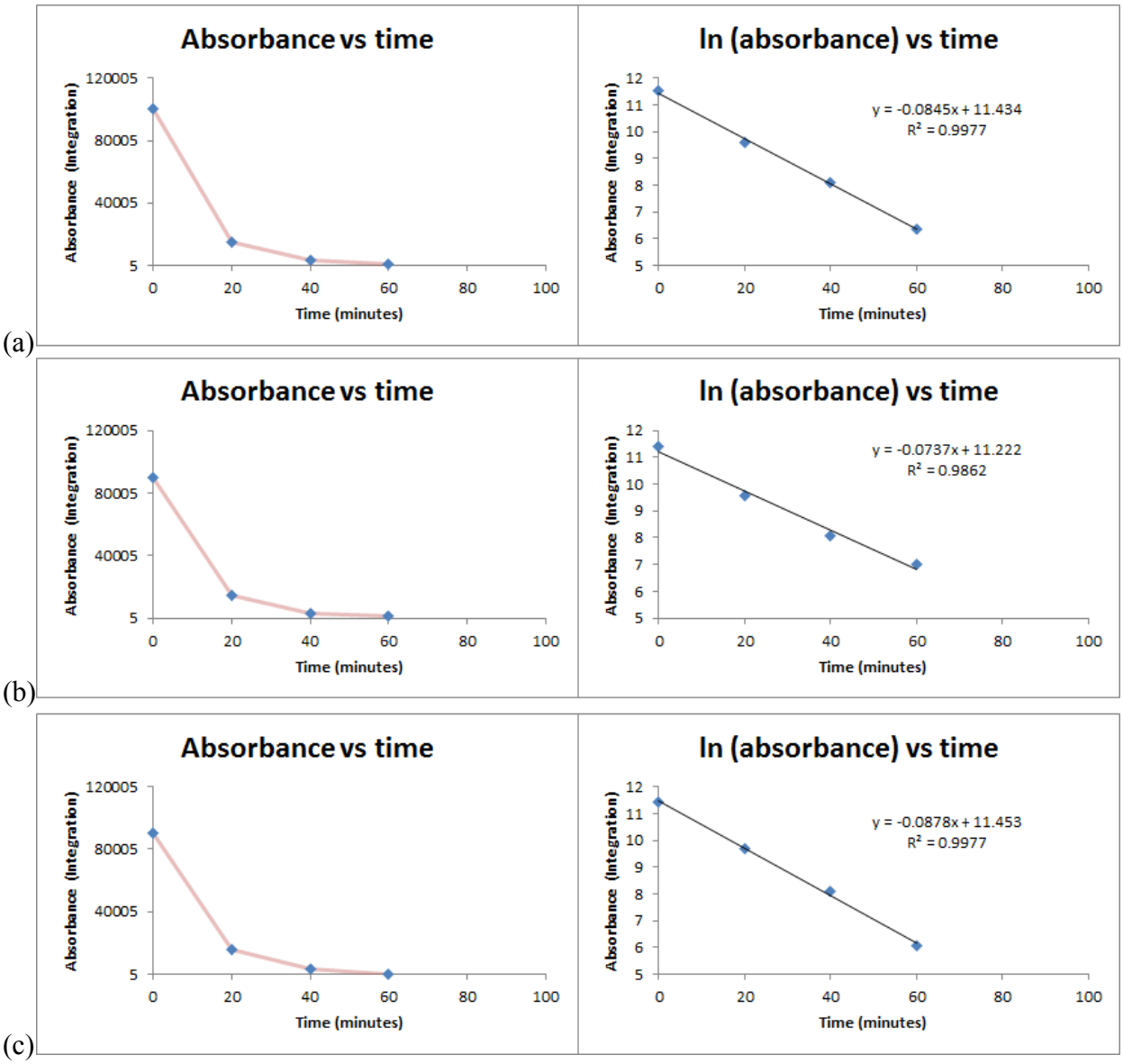


Figure S25. *Pseudo* first-order kinetic data obtained for reaction of azido-doxorubicin probe **9** (0.1 mM) and TCO-OH major-**10** in serum:PBS (1:1)**. (a)** 10 mM; **(b)** 10 mM; **(c)** 10 mM; Second-order rate constant calculated as: 0.137 ± 0.012 $M^{-1}s^{-1}$ (n = 3).

9. Final Compound NMR Spectra and Purity of Doxorubicin Prodrug 9.

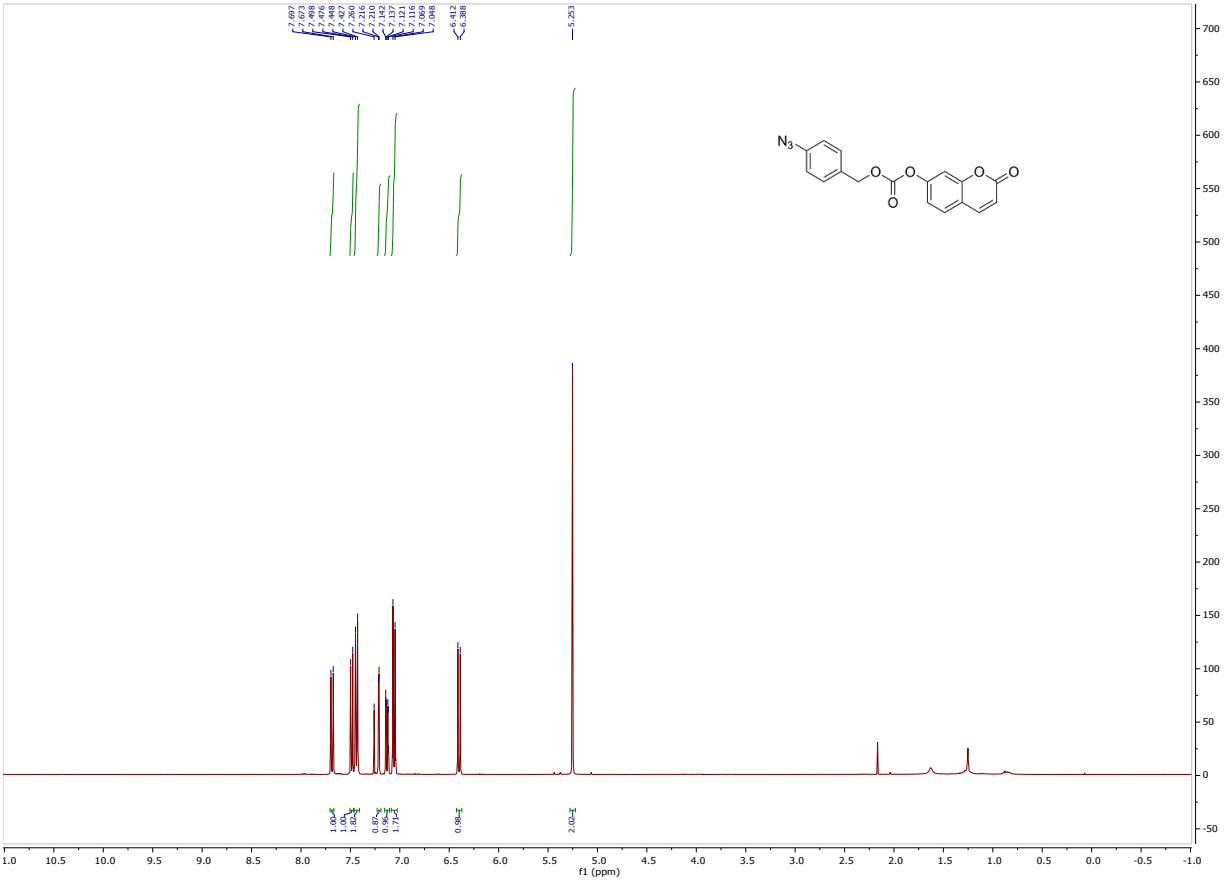


Figure S26. ^{1}H NMR Spectrum of coumarin carbonate probe **8a**.

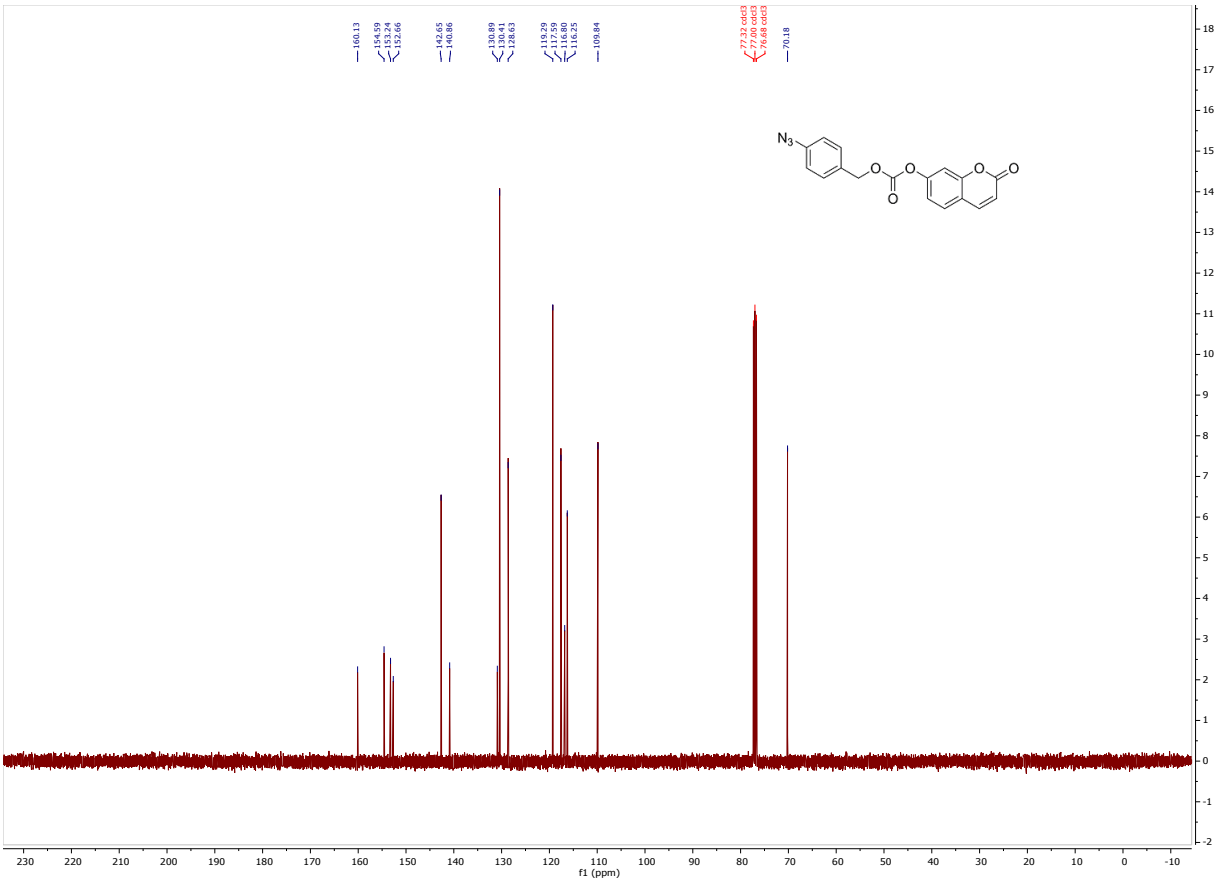


Figure S27. ^{13}C NMR Spectrum for coumarin carbonate probe **8a**.

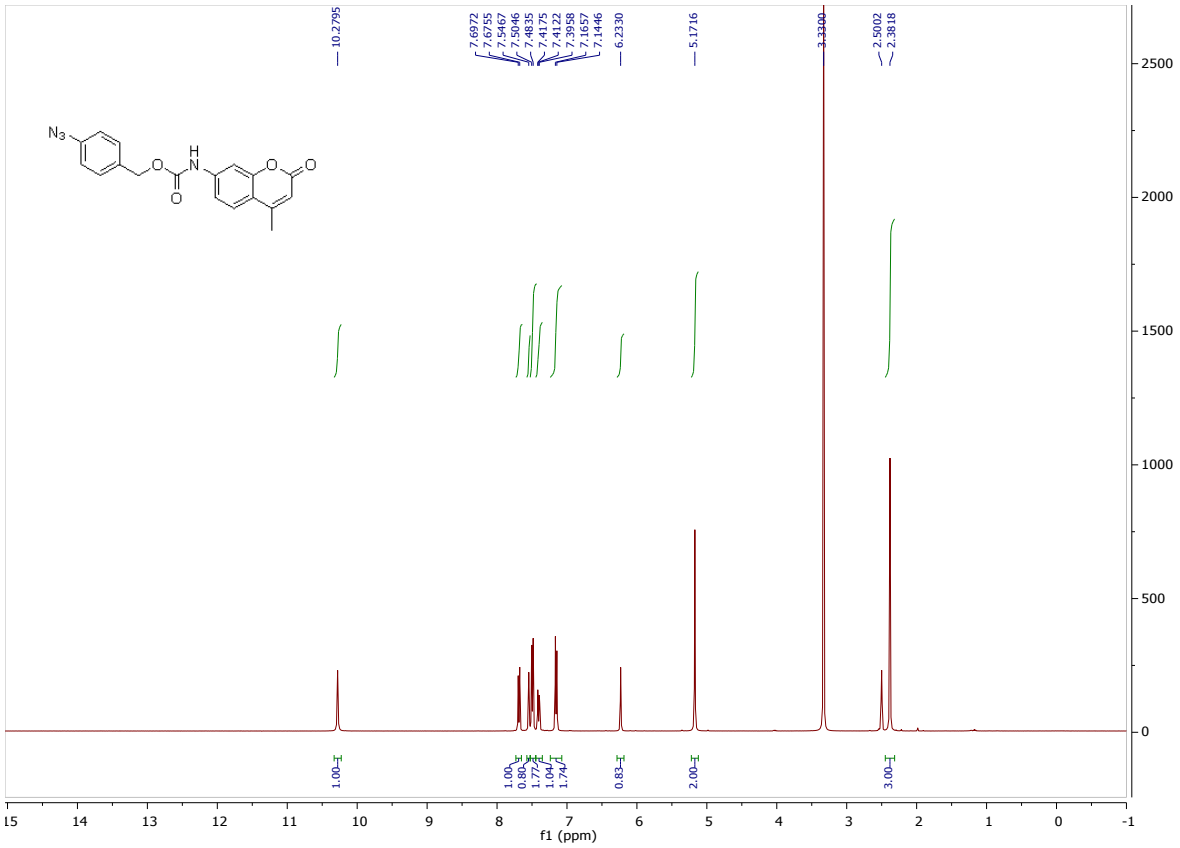


Figure S28. ^{1}H NMR Spectrum of coumarin carbamate probe **8b**.

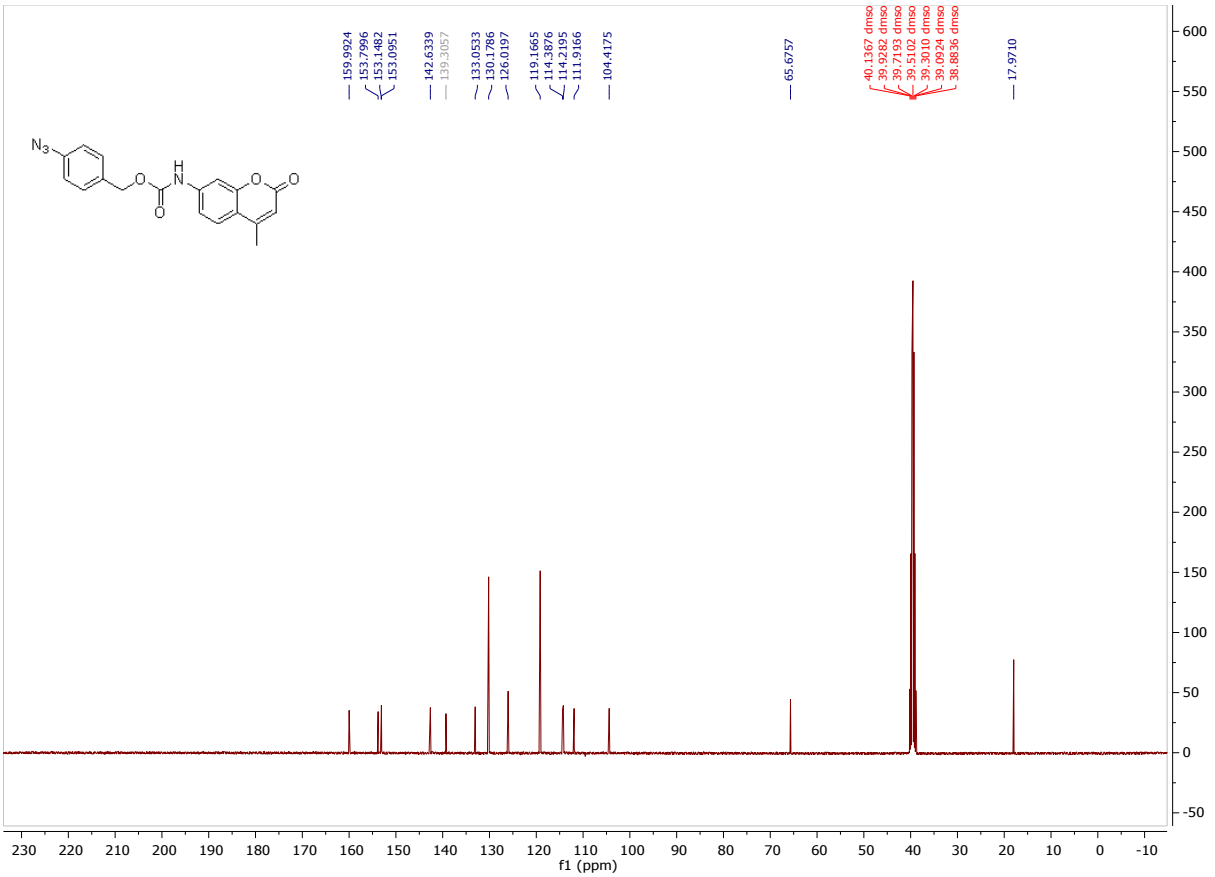


Figure S29. ^{13}C NMR Spectrum for coumarin carbamate probe **8b**.

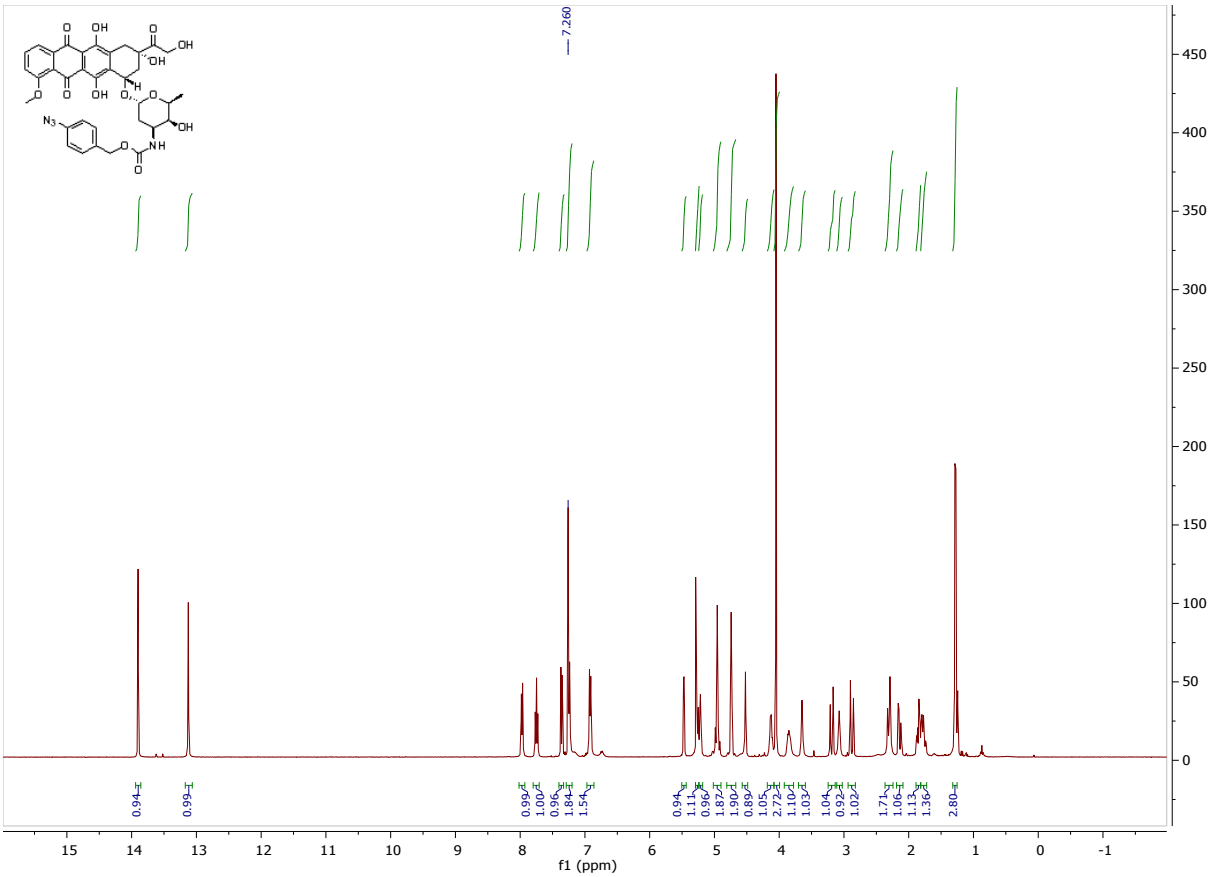


Figure S30. ^{1}H NMR Spectrum for Doxorubicin Prodrug **9**.

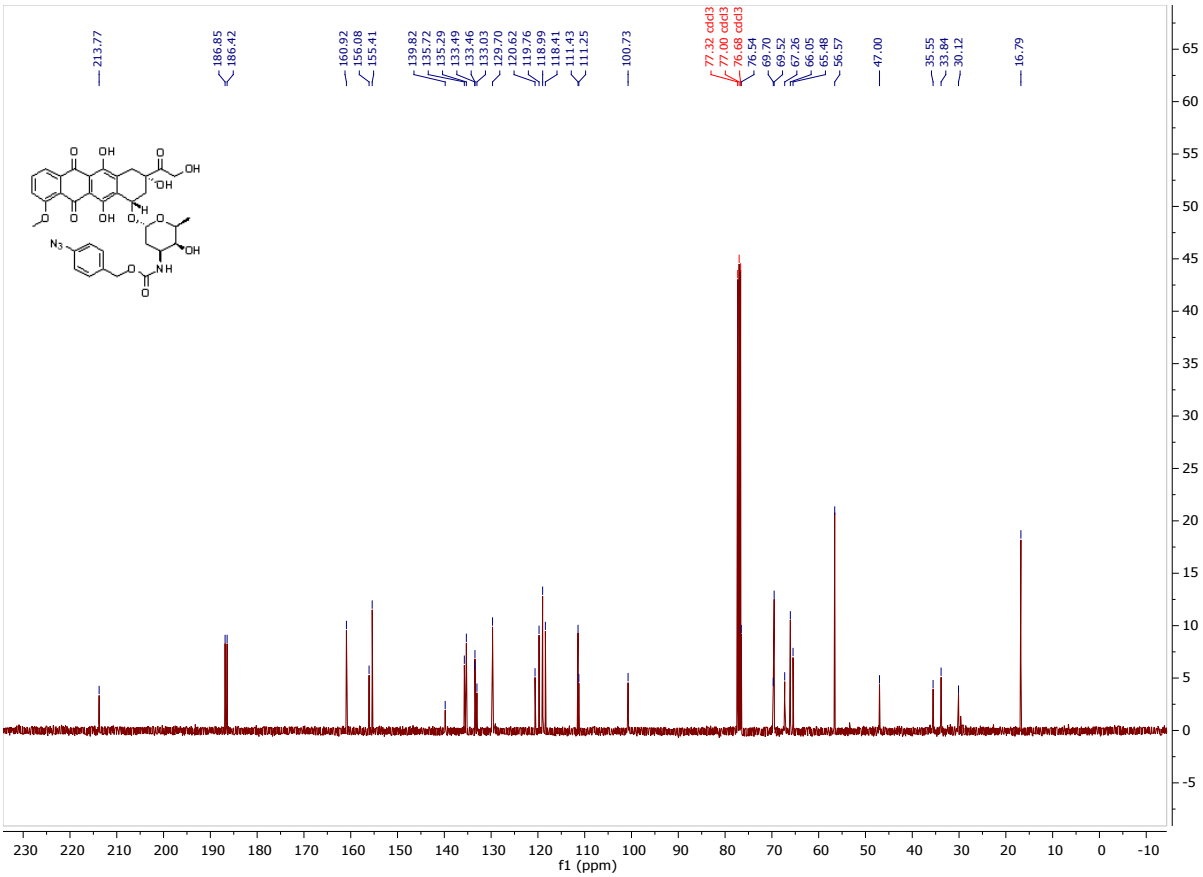
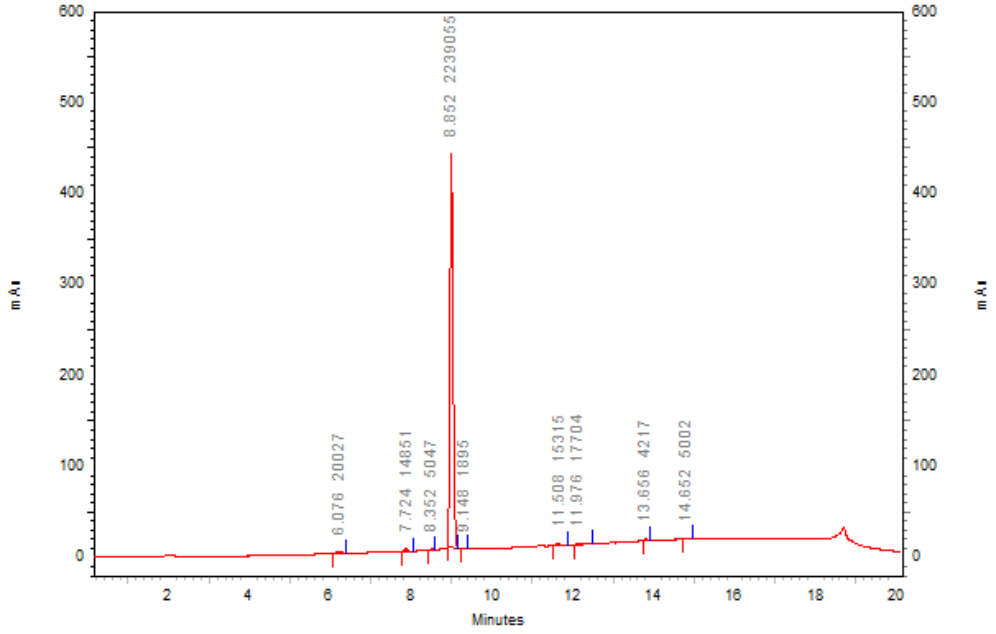


Figure S31. ^{13}C NMR for Doxorubicin Prodrug **9**.

(a)



(b)

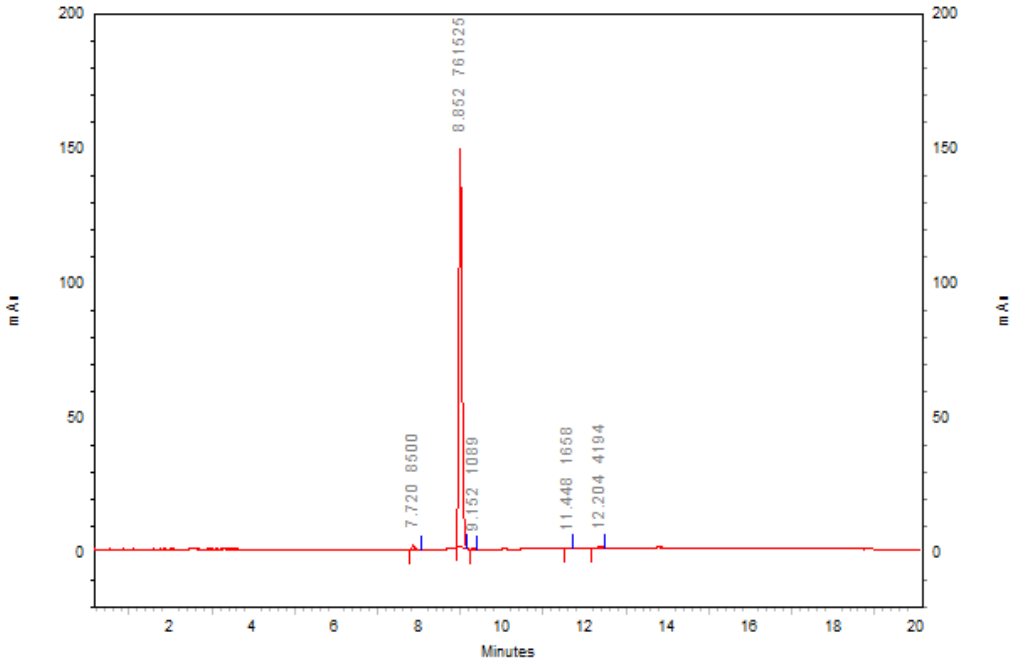


Figure S32. HPLC Purity of doxorubicin prodrug **9** used in the cytotoxicity assays (t_R = 8.85 min). **(a)** 254 nm (purity ≥ 96%) and **(b)** 480 nm (purity ≥ 98%). Note: Doxorubicin.HCl, t_R = 4.3 min.

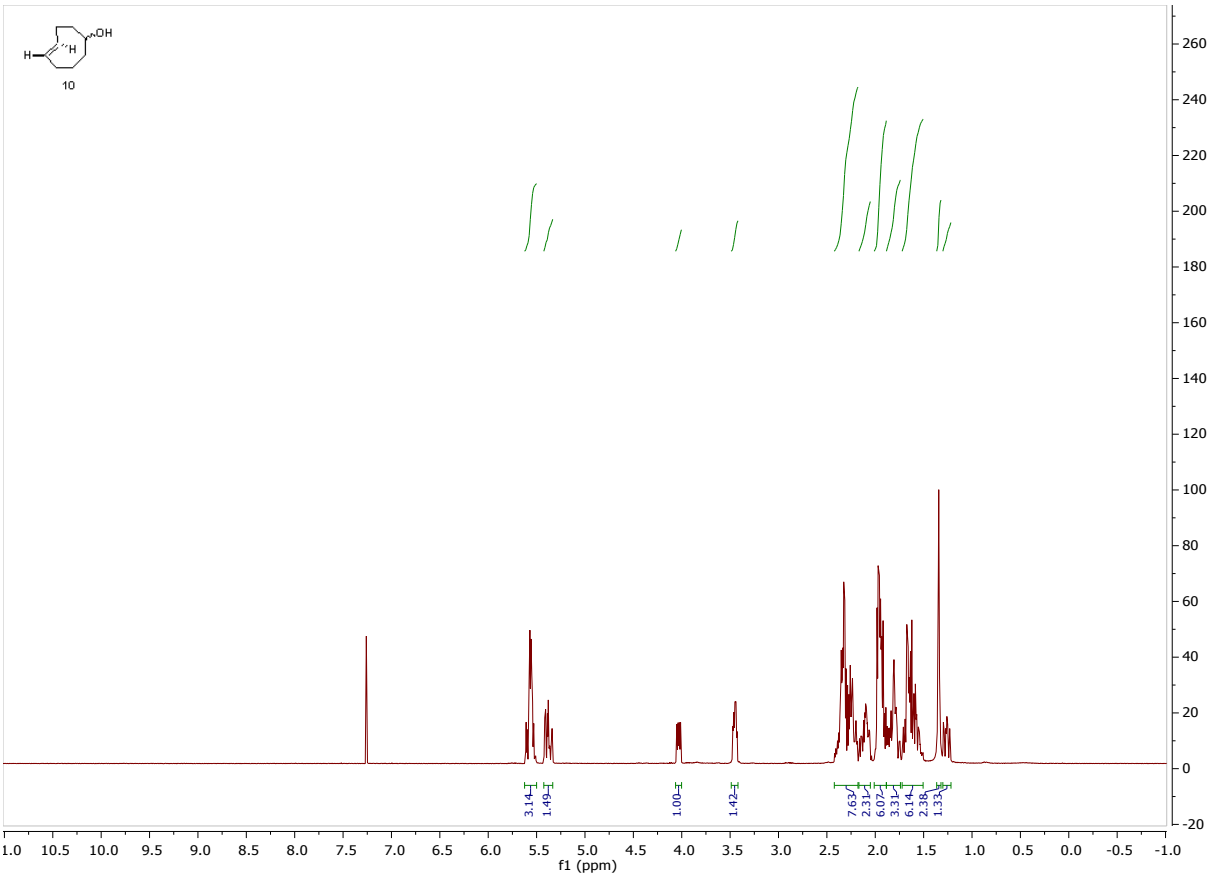


Figure S33. ^{1}H NMR Spectrum for *trans*-cyclooctenol **10** (mixture of diastereomers).

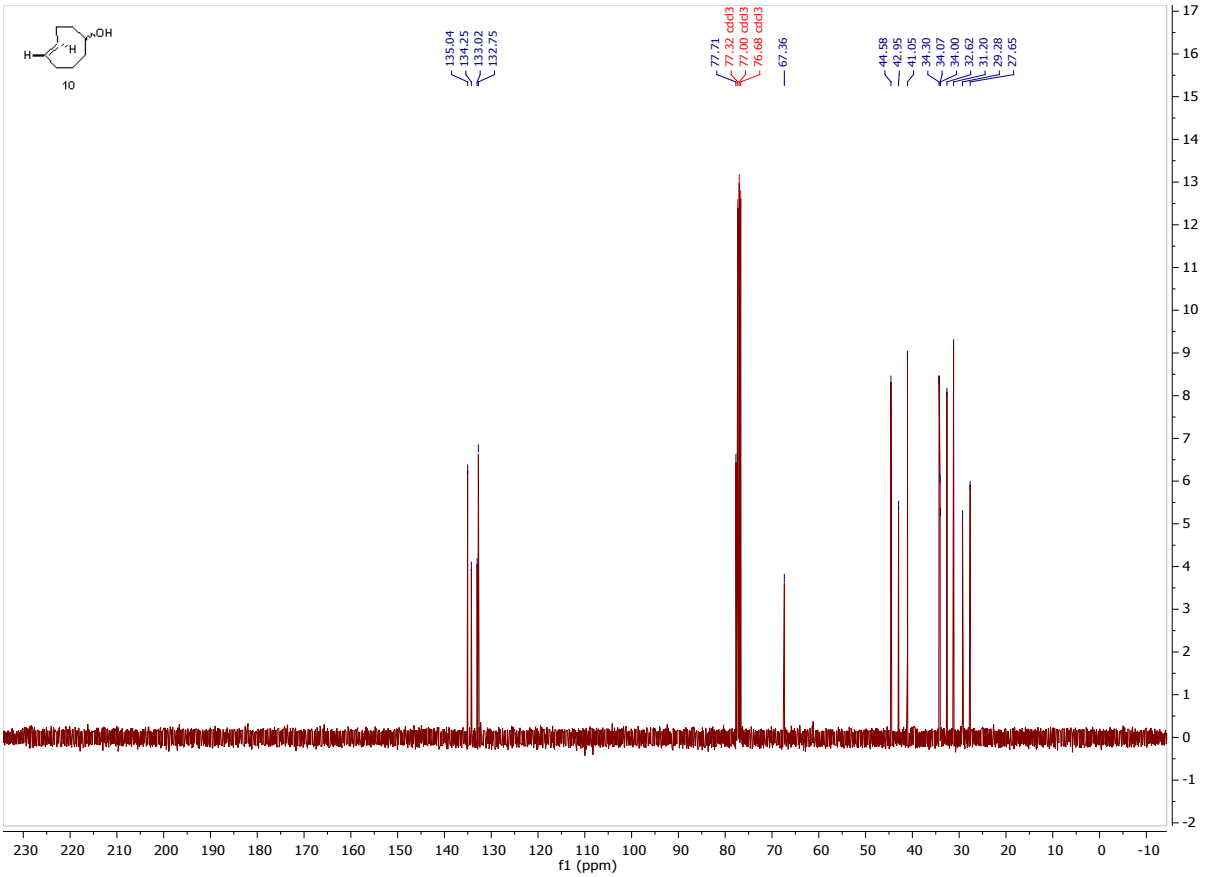


Figure S34. ^{13}C NMR Spectrum for *trans*-cyclooctenol **10** (mixture of diastereomers).

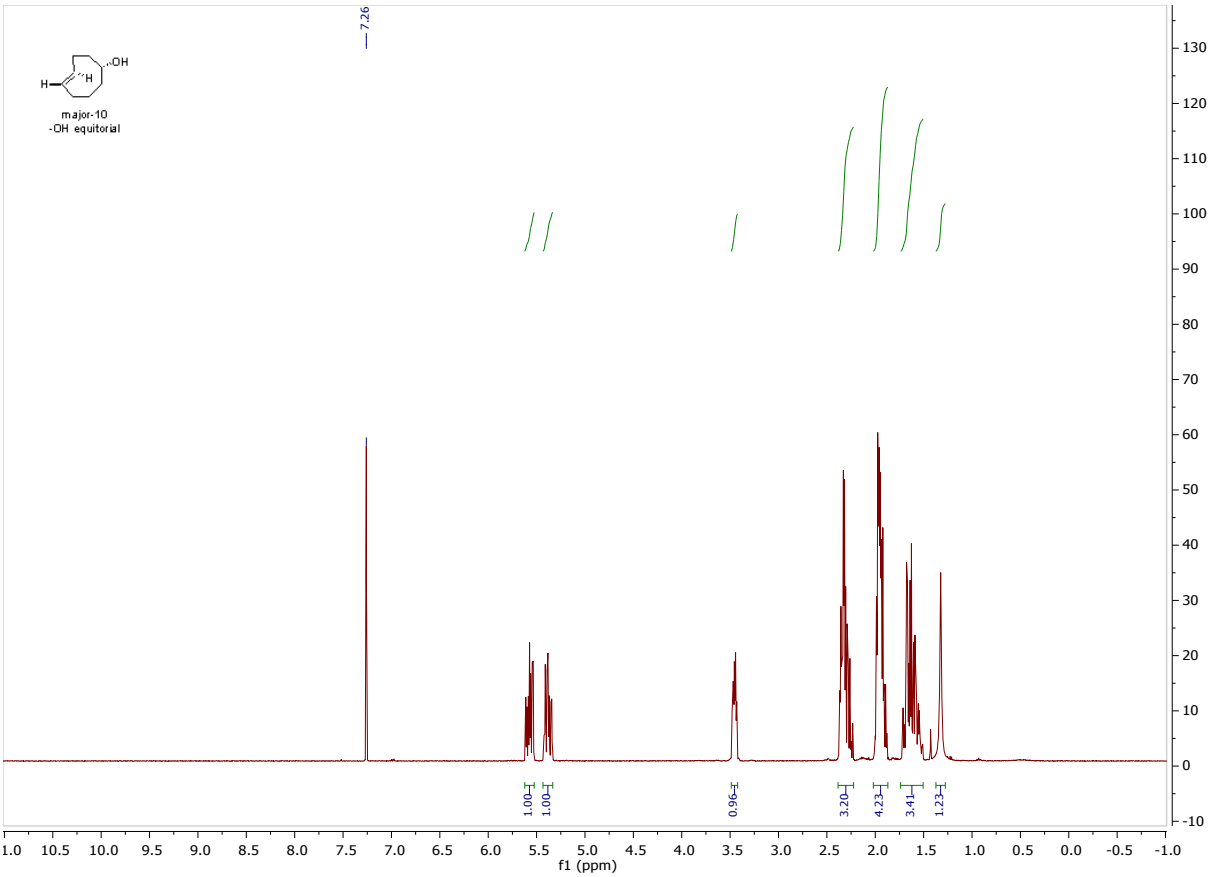


Figure S35. ^{1}H NMR Spectrum for *trans*-cyclooctenol **10** (major).

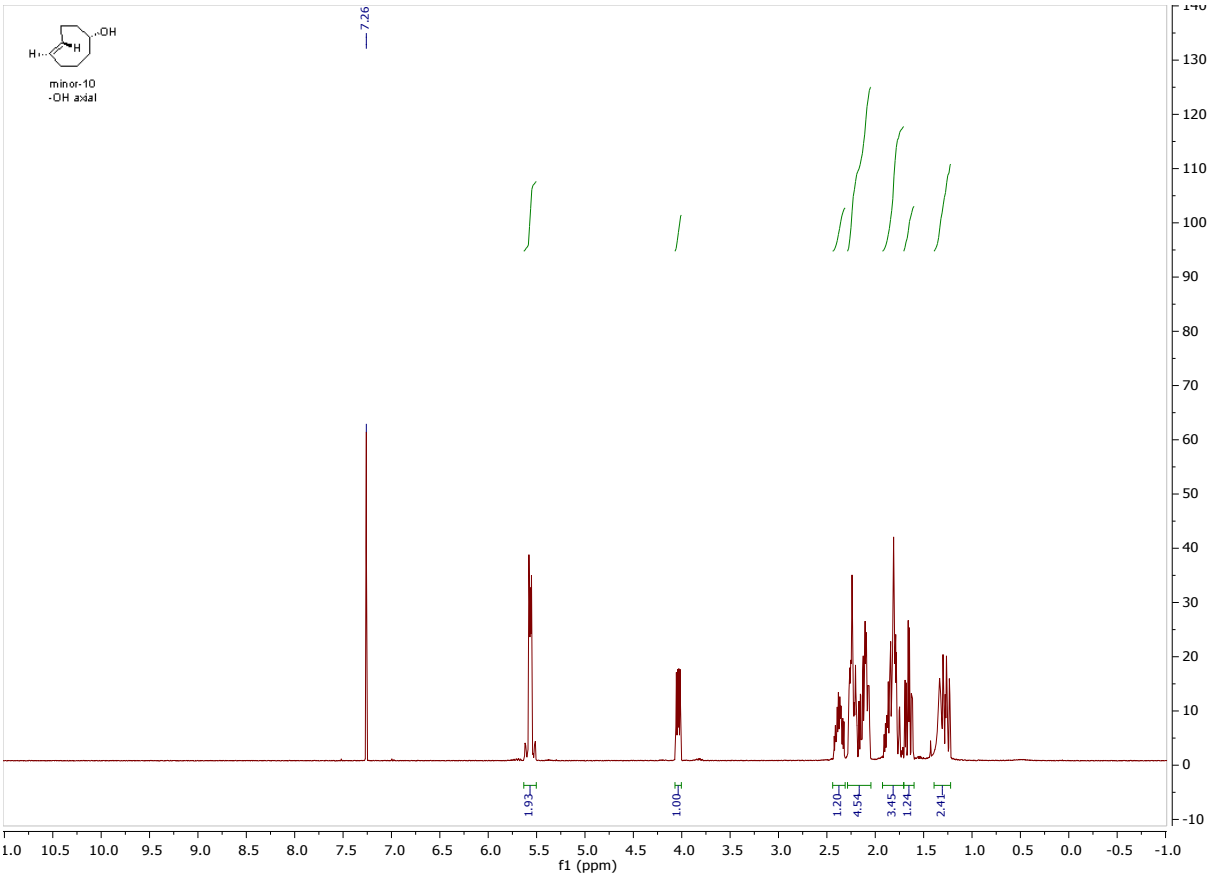


Figure S36. ^{1}H NMR Spectrum for *trans*-cyclooctenol **10** (minor).

10. References.

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