Supplementary Information

for

Tuning activation and self-immolative properties of the bioorthogonal alkene-azide click-and-release strategy

Jessica M. Fairhall, Madoka Murayasu, Sumit Dadhwal, Sarah Hook, and Allan B. Gamble*

School of Pharmacy, University of Otago, Dunedin, 9054, New Zealand

**Corresponding author: allan.gamble@otago.ac.nz*

Table of Contents:

1 Materials and instrumentation.....S2
1.1 Reagents.....S2
1.2 General Experimental.....S2
2 Synthesis.....S4
3 Rate of [3+2]-Cycloaddition (Click) Reaction (Rate 1).....S16
3.1 *Pseudo*-First Order Rate Plots.....S17
3.2 HPLC Traces.....S20
4 Spectrofluorometry Release Experiments – Rate of Triazoline/Imine Degradation (Rates 2 and 3) and 1,6-/1,8-Self-immolation (Rate 4).....S35
5 $^{1}H/^{19}F$ NMR Mechanistic Investigation.....S37
6 Combined Rates for [3+2]-Cycloaddition Click-and-Release (Rates 1 – 4) under 90% Aqueous Conditions.....S40
6.1 HPLC Traces.....S41
7 Final Compound NMR Spectra.....S47
8 References.....S67

1 Materials and instrumentation

1.1 Reagents

4-Nitrocinnamyl alcohol **14** was purchased from Global Sciences and Technology, NZ. 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, 40-63 μm, 60Å). 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 (TLC) 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.

1.2 General Experimental

^{1}H, ^{13}C and ^{19}F NMR spectra were recorded on a 400 or 500 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 microTOFQ mass spectrometer in the Department of Chemistry, University of Otago, NZ. Attenuated total reflection-fourier transform infrared spectroscopy (ATR-FTIR) was carried out using a Varian 3100 FTIR (Excalibur series) instrument equipped with an attenuated total reflection accessory (GladiATR, Piketech, USA). Samples were clamped directly on to the ATR diamond crystal and the spectra were recorded over a range of 400-4000 cm^{-1} as the mean of 64 scans with a resolution of 4 cm^{-1}. The data were then analysed using Varian Resolution Pro, v4.1.0.101 software.

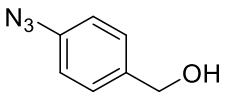
Photochemical reactions were performed in a Southern New England Ultraviolet Company Rayonet® reactor model RPR-100, equipped with eight RPR-2537 Å lamps. Reactions were performed in either the RQV-118 or RQV-218 quartz reaction vessels supplied by Southern New England Ultraviolet Company.

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 an Agilent 1200 system equipped with a Phenomenex Synergi 4 μm Fusion-RP 80A (150 × 4.6 mm) column, and a photodiode array detector. The applied mobile phases used for kinetic studies and purity determinations were: A, H_2O + 0.1% formic acid; and B, MeCN + 0.1% formic acid. Flow speed was 1 mL/min and injection volumes were 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.

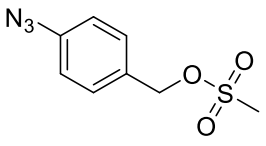
2 Synthesis

2.1 4-Azidobenzyl alcohol (9a):

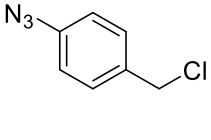


Synthesised as previously described.[1] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.32 (d, *J* = 8.4 Hz, 2H), 7.00 (d, *J* = 8.4 Hz, 2H), 4.63 (s, 2H).

2.2 4-Azidobenzyl mesylate (10a), 4-azidobenzyl chloride (11a):



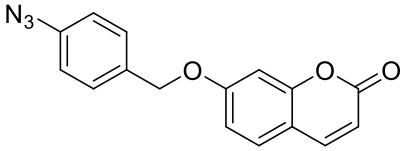
10a



11a

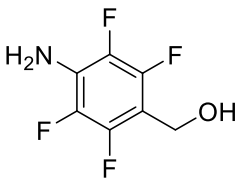
Synthesised as previously described.[2] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.37 (d, *J* = 8.4 Hz, 2H), 7.02 (d, *J* = 8.4 Hz, 2H), 4.57 (s, 2H), 3.67 (s, 1H). Note: As the methyl peak does not integrate to 3 protons, it is suspected that the chloride **11a** has formed alongside the mesylate.

2.3 7-[(4-Azidobenzyl)oxy]-coumarin (1a):



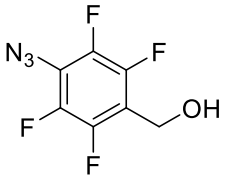
Synthesised as previously described.[2] The spectroscopic data was identical to our previous report.[2] and additional data for the IR of 1a is provided below. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.63 (d, *J* = 9.4 Hz, 1H), 7.42 (d, *J* = 8.5 Hz, 2H), 7.38 (d, *J* = 8.5 Hz, 1H), 7.08 – 7.04 (m, 2H), 6.90 (dd, *J* = 8.5, 2.4 Hz, 1H), 6.87 (d, *J* = 2.4 Hz, 1H), 6.26 (d, *J* = 9.5 Hz, 1H), 5.09 (s, 2H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 161.8, 161.2, 155.9, 143.5, 140.4, 132.5, 129.3, 129.0, 119.5, 113.5, 113.3, 113.0, 102.0, 70.1. HRMS (ESI+) calculated for $C_{16}H_{11}N_3NaO_3$: 316.0693, found: 316.0666. IR: ν_{max}/cm^{-1} 2117, 1720, 1615, 1508, 1352, 1284, 1233, 1128, 834. HPLC-UV (**Figure S9a**): t_R = 8.6 min (λ = 325 nm).

2.4 4-Amino-2,3,5,6-tetrafluorobenzyl alcohol (12b):



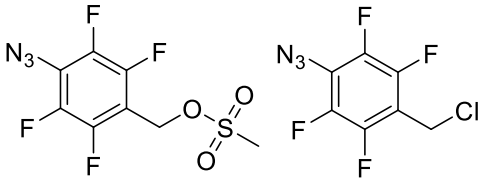
Synthesised as previously described.[2] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.73 – 4.68 (s, 2H).

2.5 4-Azido-2,3,5,6-tetrafluorobenzyl alcohol (9b):



Synthesised as previously described.[2] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.85 – 4.72 (m, 2H).

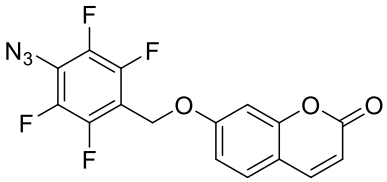
2.6 4-Azido-2,3,5,6-tetrafluorobenzyl mesylate (10b) and chloride (11b):



10b **11b**

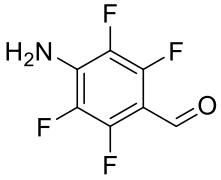
Synthesised as previously described.[2]

2.7 7-[(4-Azido-2,3,5,6-tetrafluorobenzyl)oxy]-coumarin (1b):



Synthesised as previously described.[2] The spectroscopic data was similar to our previous report,[2] and additional data for the ^{19}F NMR and IR spectra are reported here. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.65 (d, *J* = 9.5 Hz, 1H), 7.41 (d, *J* = 8.5 Hz, 1H), 6.92 – 6.86 (m, 2H), 6.29 (d, *J* = 9.5 Hz, 1H), 5.17 (t, *J* = 1.5 Hz, 2H). ^{13}C NMR (125 MHz, Chloroform-*d*) δ 160.90, 160.85, 155.7, 145.6 (dm, $^{1}J_{C\text{-}F}$ = 251.4 Hz, $^{2}J_{C\text{-}F}$ = 11.9 Hz), 143.2, 140.5 (dm, $^{1}J_{C\text{-}F}$ = 250.4 Hz, $^{2}J_{C\text{-}F}$ = 16.4 Hz), 128.9, 121.3 (m), 113.8, 113.3, 112.9, 109.4 (t, *J* = 17.5 Hz), 101.8, 60.6 (t, *J* = 2.1 Hz). ^{19}F NMR (376 MHz, Acetonitrile-d_3) δ -145.1 (m), -153.6 (m). HRMS (ESI+) calculated for $C_{16}H_8F_4N_3O_3$: 366.0496, found: 366.0517. IR: ν_{max}/cm^{-1} 2122, 1722, 1615, 1491, 1277, 1229, 955. HPLC-UV (**Figure S9b**): t_R = 9.1 min (λ = 325 nm).

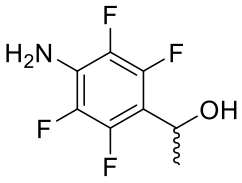
2.8 4-Amino-2,3,5,6-tetrafluorobenzyl aldehyde (13c):



4-Amino-2,3,5,6-tetrafluorobenzyl alcohol (0.797 g, 4.08 mmol) was dissolved in DCM (40 mL). To this was added Celite (4.50 g), followed by PCC (4.38 g, 20.3 mmol). The mixture was allowed to stir at room temperature (25 °C) until the alcohol was consumed (approximately 3 hours; monitored via TLC). The mixture was diluted with DCM (40 mL) then filtered through Celite. The filtered solution was then concentrated *in vacuo* and the resulting crude residue was purified by silica gel flash column chromatography (20% ethyl acetate:hexane) to provide the

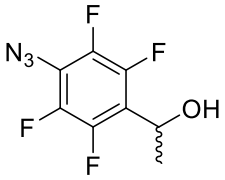
titled compound as a yellow solid (0.575 g, 73%), which was spectroscopically similar to the literature.[3] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 10.12 (s, 1H). HRMS (ESI+) calculated for: $C_7H_3F_4NONa$: 194.0224, found: 194.0221.

2.9 1-(4-Amino-2,3,5,6-tetrafluorophenyl)ethan-1-ol (12c):



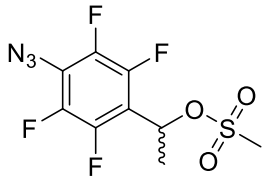
4-Amino-2,3,5,6-tetrafluorobenzyl aldehyde **13c** (0.350 g, 1.81 mmol) was dissolved in dry THF (10 mL) and cooled to 0 °C on ice. Methyl magnesium bromide (3.0 M in diethyl ether, 6 mL, 18.0 mmol) was added dropwise to the solution. The mixture was then allowed to warm to room temperature and allowed to stir under N_2 for 24 hours. The mixture was then quenched with saturated NH_4Cl (2 mL) and diluted with ethyl acetate (50 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to result in a crude residue which upon silica gel flash column chromatography (20% ethyl acetate:hexane) resulted in the titled compound as a brown coloured oil (0.300 g, 79%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.17 (q, *J* = 6.8 Hz, 1H), 1.61 (dt, *J* = 6.8, 0.7 Hz, 3H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 144.6 (dm, $^1J_{C\text{-}F}$ = 242.4 Hz), 136.5 (dm, $^1J_{C\text{-}F}$ = 240.9 Hz), 125.5 (m), 109.9 (t, *J* = 16.2 Hz), 62.2 (p, *J* = 2.2 Hz), 23.4 (t, *J* = 1.8 Hz).

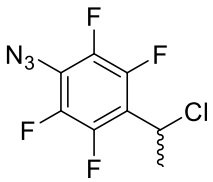
2.10 1-(4-Azido-2,3,5,6-tetrafluorophenyl)ethan-1-ol (9c):



1-(4-Amino-2,3,5,6-tetrafluorophenyl)ethan-1-ol **12c** (0.200 g, 0.956 mmol) and *p*-toluenesulfonic acid (1.48 g, 8.59 mmol) were dissolved in water (4 mL). To this was added sodium nitrite (0.264 g, 3.82 mmol), then slowly sodium azide (0.093 g, 1.43 mmol). The reaction was then left to stir for 1 hour on ice. The reaction mixture was then made basic with solid $NaHCO_3$ and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (3 × 50 mL) and dried over $MgSO_4$ and purified by flash silica gel column chromatography (20% ethyl acetate:hexane) to give the pure title compound as a brown coloured oil (0.042 g, 19%). ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.23 (q, *J* = 6.8 Hz, 1H), 1.63 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 144.6 (dm, $^1J_{C\text{-}F}$ = 247.2 Hz, $^2J_{C\text{-}F}$ = 12.4 Hz, $^3J_{C\text{-}F}$ = 4.5 Hz), 140.5 (dm, $^1J_{C\text{-}F}$ = 250.5 Hz, $^2J_{C\text{-}F}$ = 16.9 Hz, $^3J_{C\text{-}F}$ = 2.9 Hz), 118.7 (m), 107.8 (m), 62.4 (p, *J* = 2.3 Hz), 23.4 (t, *J* = 1.7 Hz).

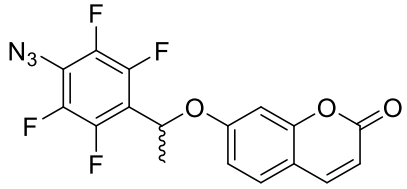
2.11 [(4-Azido-2,3,5,6-tetrafluorobenzyl)ethyl]-mesylate (10c) and chloride (11c):





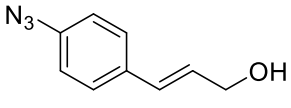
To a mixture of 1-(4-Azido-2,3,5,6-tetrafluorophenyl)ethan-1-ol **4c** (0.042 g, 0.178 mmol) dissolved in DCM (4 mL), was added triethylamine (37 μL, 0.268 mmol) and the mixture was allowed to cool on ice. Mesyl chloride (20 μL, 0.268 mmol) was then added dropwise and the mixture was left to stir for 24 h. After which, the mixture was diluted with DCM (70 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to result in 0.077 g of the title compounds as a brown coloured oil which was used in the next synthetic step without any further purification.

2.12 7-[(4-Azido-2,3,5,6-tetrafluorobenzyl)ethoxy]-coumarin (1c):



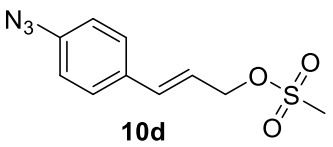
Crude [(4-Azido-2,3,5,6-tetrafluorobenzyl)ethyl]-mesylate/chloride **10c/11c** (0.077 g) dissolved in acetonitrile (2 mL) was added dropwise to a mixture of 7-hydroxycoumarin **1** (0.073 g, 0.456 mmol) and potassium carbonate (0.084 g, 0.608 mmol) in acetonitrile (3 mL). The mixture was allowed to stir in the dark (flask wrapped in foil) at room temperature under nitrogen until mesylate was completely consumed (~5 days; monitored via TLC). Following which, acetonitrile was removed *in vacuo* and the crude residue was dissolved in ethyl acetate (20 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to result in a crude residue which upon silica gel flash column chromatography (20% ethyl acetate:hexane) resulted in the title compound (0.036 g, 53% - yield over 2 steps from **9c**) as a brown coloured crystalline solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.60 (d, *J* = 9.5 Hz, 1H), 7.35 (d, *J* = 8.6 Hz, 1H), 6.85 (dd, *J* = 8.6, 2.4 Hz, 1H), 6.76 (d, *J* = 2.4 Hz, 1H), 6.25 (d, *J* = 9.5 Hz, 1H), 5.74 (q, *J* = 6.6 Hz, 1H), 1.81 (d, *J* = 6.6 Hz, 3H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 160.9, 160.0, 155.7, 144.6 (dm, $^{1}J_{C\text{-}F}$ = 250.2 Hz, $^{2}J_{C\text{-}F}$ = 12.4 Hz, $^{3}J_{C\text{-}F}$ = 3.8 Hz), 143.2, 140.7 (dm, $^{1}J_{C\text{-}F}$ = 251.8 Hz, $^{2}J_{C\text{-}F}$ = 18.4 Hz), 129.0, 120.2 (m), 114.6 (t, *J* = 15.0 Hz), 113.7, 113.4, 113.2, 102.3, 67.6, 20.8 (t, *J* = 1.6 Hz). ^{19}F NMR (376 MHz, Acetonitrile-d_3) δ -145.2 (m), -153.6 (m). HRMS (ESI+) calculated for $C_{17}H_9F_4N_3NaO_3$: 402.0472, found: 402.0459. IR: ν_{max}/cm^{-1} 2131, 1731, 1611, 1489, 1230, 1118, 971, 834, 734. HPLC-UV (**Figure S9c**): t_R = 9.1 min (λ = 325 nm).

2.13 *trans*-4-Azidocinnamyl alcohol (9d):

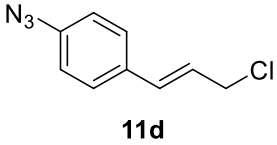


Prepared using a modified literature procedure.[4] *Trans*-4-nitrocinnamyl alcohol **14** (0.300 g, 1.67 mmol) was dissolved in glacial acetic acid (2 mL) and distilled water (3 mL). Zinc powder (0.431 g, 6.59 mmol) was then slowly added to the solution on ice. The mixture was stirred at 25 °C until consumption of 4-nitrocinnamyl alcohol was observed by TLC (approximately 1 hour). The mixture was cooled to 0 °C in an ice bath. Sodium nitrite (0.187 g, 2.71 mmol) in 2 mL of distilled water was then added dropwise to the mixture, followed by sodium azide (0.195 g, 2.99 mmol) in 2 mL of distilled water. The mixture was allowed to stir at room temperature in the dark (flask wrapped in foil) until a significant amount of reduced 4-nitrocinnamyl alcohol had been consumed (approximately 4 hours; monitored via TLC). The reaction mixture was then diluted with water (75 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (3 × 50 mL) and brine (50 mL) and dried over $MgSO_4$. The resulting crude residue was then purified by flash silica gel column chromatography (50% ethyl acetate:hexane) to yield the desired product (0.121 g, 41%) as a dark brown coloured oil. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.33 (m, 2H), 7.00 – 6.95 (m, 2H), 6.58 (dt, *J* = 15.9, 1.6 Hz, 1H), 6.32 (dt, *J* = 15.9, 5.7 Hz, 1H), 4.32 (dd, *J* = 5.7, 1.6 Hz, 2H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 139.3, 133.7, 130.2, 128.5, 127.9, 119.3, 63.8.

2.14 *trans*-4-Azidocinnamyl mesylate (10d) and chloride (11d):



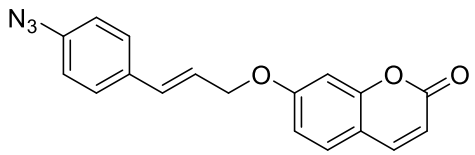
10d



11d

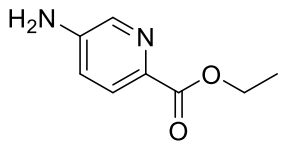
Methanesulfonyl chloride (60 μL, 0.778 mmol) was added dropwise to *trans*-4-azidocinnamyl alcohol **9d** (0.086 g, 0.491 mmol) and triethylamine (100 μL, 0.721 mmol) in DCM (6 mL) on ice and left to stir at 0 °C for 1 hour or until consumption of 4-azidocinnamyl alcohol was observed via TLC. The reaction mixture was then further diluted with DCM (70 mL) and washed with water (3 × 50 mL) and brine (50 mL) and dried over $MgSO_4$. Crude NMR analysis revealed a seemingly clean product although TLC showed the presence of multiple products. With no obvious presence of the methyl group on the mesylate, it was suspected that the alkyl chloride **11d** had formed as the major product, alongside the mesylate **10d**. The crude product (0.071 g), as a brown coloured oil, was then used in the next synthetic step without further purification. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.40 – 7.34 (m, 2H), 7.01 – 6.95 (m, 2H), 6.61 (dt, *J* = 15.7, 1.1 Hz, 1H), 6.27 (dt, *J* = 15.7, 7.2 Hz, 1H), 4.24 (dd, *J* = 7.2, 1.1 Hz, 2H).

2.15 7-[(*trans*-4-Azidocinnamyl)oxy]-coumarin (1d):



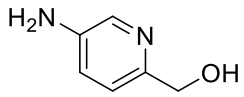
Crude *trans*-4-azidocinnamyl mesylate/chloride **10d/11d** (0.071 g) in 2 mL of acetonitrile was added dropwise to 7-hydroxycoumarin **1** (0.055 g, 0.335 mmol) and potassium carbonate (0.100 g, 0.735 mmol) in 6 mL of acetonitrile. The reaction mixture was allowed to stir in the dark (flask wrapped in foil) at room temperature under nitrogen and monitored by TLC. After 5 days the acetonitrile was removed *in vacuo* and the crude residue was suspended in ethyl acetate (50 mL), washed with water (3 × 50 mL) and brine (50 mL) and dried over $MgSO_4$. The resulting crude residue was then subjected to flash silica gel column chromatography (40% ethyl acetate:hexane) to afford the desired compound (0.081 g, 52% - yield over 2 steps from alcohol **9d**) as a pale-brown crystalline solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 7.64 (d, *J* = 9.5 Hz, 1H), 7.42 – 7.37 (m, 3H), 7.03 – 6.96 (m, 2H), 6.91 – 6.86 (m, 2H), 6.71 (dt, *J* = 16.0, 1.4 Hz, 1H), 6.35 (dt, *J* = 16.0, 5.9 Hz, 1H), 6.26 (d, *J* = 9.5 Hz, 1H), 4.75 (dd, *J* = 5.9, 1.4 Hz, 2H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 161.5, 161.0, 155.6, 143.2, 139.5, 132.8, 132.7, 128.7, 127.9, 122.6, 119.1, 113.1, 113.0, 112.5, 101.5, 68.9. HRMS (ESI+) calculated for $C_{18}H_{13}N_3NaO_3$: 342.0849, found: 342.0837. IR: ν_{max}/cm^{-1} 2112, 2076, 1734, 1609, 1504, 1287, 1229, 1124. HPLC-UV (**Figure S9d**): t_R = 9.1 min (λ = 325 nm).

2.16 Ethyl 5-aminopyridine-2-carboxylate (15e)



Prepared using a modified literature procedure.[5] 5-Aminopyridine-2-carboxylic acid (1.00 g, 7.24 mmol) was dissolved in ethanol (10 mL). H_2SO_4 (0.1 mL) was then added dropwise and the reaction was left to heat at reflux overnight (74 °C). The reaction mixture was then made alkaline (approx. pH 8) with solid $NaHCO_3$ and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (3 × 50 mL) and dried over $MgSO_4$. The crude product (0.377 g, 31%) was then used in the next synthetic step without further purification.

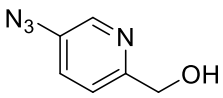
2.17 5-Aminopyridine-2-methanol (12e)



Ethyl 5-aminopyridine-2-carboxylate **15e** (0.377 g, 2.27 mmol) in 5 mL of dry THF was slowly added to lithium aluminium hydride (0.420 g, 11.1 mmol) in dry THF (10 mL) on ice. The reaction was left to stir overnight. The reaction was then quenched with 1 M NaOH and left to stir for 30 minutes then diluted with water (75 mL) and extracted with ethyl acetate (3 × 50 mL). The combined organic layers

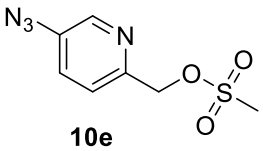
were then washed with water (3 × 50 mL) and brine (50 mL) and dried over $MgSO_4$. The NMR revealed the pure product, as an orange solid, which was spectroscopically similar to that reported in the literature[6]; no further purification was required (0.109 g, 39%). ^{1}H NMR (400 MHz, Methanol-d_4) δ 7.91 (m, 1H), 7.24 (d, J = 8.4 Hz, 1H), 7.10 (dd, J = 8.4, 2.7 Hz, 1H), 4.54 (s, 2H). HRMS (ESI+) calculated for: $C_6H_8N_2ONa$: 125.0710, found: 125.0713.

2.18 5-Azidopyridine-2-methanol (9e)

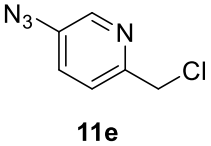


5-Aminopyridine-2-methanol **12e** (0.109 g, 0.878 mmol) and *p*-toluenesulfonic acid (1.36 g, 7.89 mmol) were dissolved in water (3.5 mL). To this was added sodium azide (0.085 g, 1.31 mmol), then slowly sodium nitrite (0.242 g, 3.51 mmol). The reaction was then left to stir for 1 hour on ice. The reaction mixture was then made alkaline with solid $NaHCO_3$ and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were washed with water (3 × 50 mL), dried over $MgSO_4$ and purified by silica gel flash column chromatography (95% ethyl acetate:hexane) to give the title compound as a light brown liquid (0.102 g, 77%). ^{1}H NMR (400 MHz, Acetone-d_6) δ 8.25 (t, J = 1.8 Hz, 1H), 7.55 (m, 1H), 7.54 (m, 1H), 4.68 (s, 2H). ^{13}C NMR (100 MHz, Acetone-d_6): δ 159.3, 140.7, 136.2, 127.5, 121.9, 65.2. HRMS (ESI+) calculated for $C_6H_6N_4NaO$: 173.0434, found: 173.0423.

2.19 [(5-Azidopyridin-2-yl)methyl] mesylate (10e) and chloride (11e)



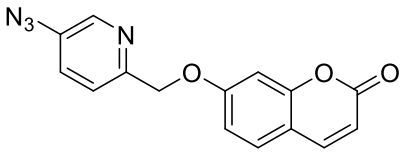
10e



11e

To a mixture of 5-azidopyridine-2-methanol **9e** (0.102 g, 0.679 mmol) dissolved in DCM (6 mL), was added triethylamine (140 μL, 1.01 mmol) and the mixture was allowed to cool on ice. Mesyl chloride (80 μL, 1.04 mmol) was then added dropwise and the mixture was left to stir for 24 h. After which time, the mixture was diluted with DCM (70 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to result in 0.106 g of the title compounds as a brown coloured oil which was used in the next synthetic step without any further purification.

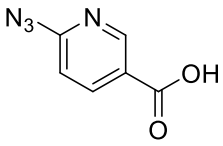
2.20 7-[(5-Azidopyridin-2-yl)methoxy]-coumarin (1e)



Crude [(5-azidopyridin-2-yl)methyl] mesylate/chloride **10e/11e** (0.106 g) was dissolved in acetonitrile (2 mL) and then added dropwise to a mixture of 7-hydroxycoumarin **1** (0.105 g, 0.648 mmol) and potassium carbonate (0.128 g, 0.926 mmol) in acetonitrile (8 mL).

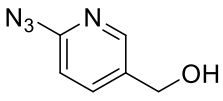
The mixture was then allowed to stir in the dark (flask wrapped in foil) at room temperature and under nitrogen until mesylate/chloride was completely consumed (~5 days; progress monitored via TLC analysis). Next, the acetonitrile was removed *in vacuo* and the crude residue was dissolved in ethyl acetate (20 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The resulting crude residue was purified by silica gel flash column chromatography (30% ethyl acetate:hexane) to afford the title compound (0.038 g, 19% -yield over 2 steps from **9e**) as a pale-brown crystalline solid. ^{1}H NMR (400 MHz, Chloroform-*d*) δ 8.35 – 8.33 (m, 1H), 7.63 (d, *J* = 9.5 Hz, 1H), 7.49 (d, *J* = 8.4 Hz, 1H), 7.41 – 7.36 (m, 2H), 6.96 – 6.90 (m, 1H), 6.89 – 6.86 (m, 1H), 6.25 (d, *J* = 9.5 Hz, 1H), 5.22 (s, 2H). ^{13}C NMR (100 MHz, Chloroform-*d*) δ 161.4, 161.1, 155.8, 152.3, 143.4, 140.8, 136.8, 129.0, 127.0, 122.5, 113.6, 113.1, 113.0, 102.3, 70.7. HRMS (ESI+) calculated for $C_{15}H_{10}N_4NaO_3$: 317.0645, found: 317.0653. IR: ν_{max}/cm^{-1} 2116, 1724, 1612, 1484, 1394, 1300, 1240, 1134, 835. HPLC-UV (**Figure S9e**): t_R = 7.3 min (λ = 325 nm).

2.21 6-Azidopyridine-3-carboxylic acid (16f)



6-azidopyridine-3-carboxylic acid (**16f**) was synthesized via 6-chloropyridine-3-carboxylic acid using a modified literature procedure.[7] 6-Chloropyridine-3-carboxylic acid (0.5 g, 3.17 mmol) was dissolved in 9 mL of 2:1 ethanol:water mixture. To this, was added sodium azide (0.616 g, 6.62 mmol). The reaction mixture was heated to 75 °C and left to heat at reflux overnight. The reaction was allowed to cool to room temperature and then concentrated *in vacuo*. The resulting crude product, as a white solid, which was spectroscopically similar to that reported in the literature[7], was then used in the next synthetic step without further purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.46 (d, *J* = 1.3 Hz 1H), 8.26 (dd, *J* = 9.2, 1.3 Hz, 1H), 8.08 (d, *J* = 9.2 Hz, 1H).

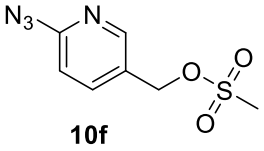
2.22 6-Azidopyridine-3-methanol (9f)



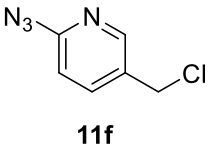
6-Azidopyridine-3-methanol (**9f**) was synthesized via 6-azidopyridine-3-carboxylic acid using a modified literature procedure.[7] Crude 6-azidopyridine-3-carboxylic acid (0.930 g) was dissolved in thionyl chloride (7 mL) and heated to reflux (75 °C) and maintained for 2 hours. The mixture was then allowed to cool to room temperature and the thionyl chloride was removed *in vacuo*. The resulting crude 6-azidopyridine-3-carbonyl chloride was dissolved in acetonitrile (15 mL). The mixture was cooled on ice and sodium borohydride (0.358 g, 9.46 mmol) was slowly added. The reaction

mixture was then allowed to warm to room temperature and left to stir overnight under nitrogen. Next, the acetonitrile was removed *in vacuo* and the crude residue was dissolved in ethyl acetate (75 mL), washed with water (3 × 25 mL), brine (1 × 25 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The resulting crude residue was purified by silica gel flash column chromatography (1-3% methanol:dichloromethane) to afford the title compound (0.125 g, 26% - yield over 3 steps) as a white solid which was spectroscopically similar to that reported.[7] ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (m, 1H), 8.19 (d, J = 9.2 Hz, 1H), 7.83 (dd, J = 9.2, 1.1 Hz, 1H), 5.65 (t, J = 5.7 Hz, 1H), 4.66 (dd, J = 5.7, 1.1 Hz, 2H).

2.23 [(6-Azidopyridin-3-yl)methyl] mesylate (10f) and chloride (11f)



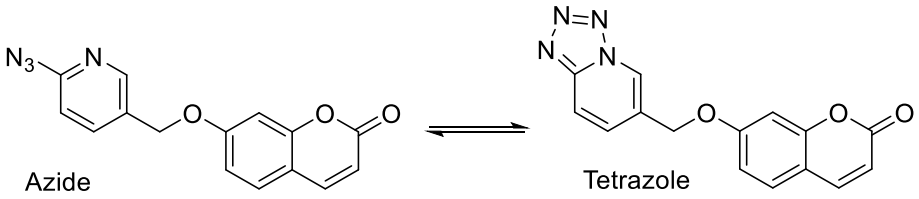
10f



11f

To a mixture of 6-azidopyridine-3-methanol **9f** (0.125 g, 0.833 mmol) dissolved in DCM (8 mL), was added triethylamine (180 μL, 1.29 mmol) and the mixture was allowed to cool on ice. Mesyl chloride (100 μL, 1.29 mmol) was then added dropwise and the mixture was left to stir for 24 h. After which time the mixture was diluted with DCM (70 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo* to result in 0.138 g of the title compounds as a brown coloured oil. The crude oil was used in the next synthetic step without any further purification.

2.24 7-[(6-Azidopyridin-3-yl)methoxy]-coumarin (1f)

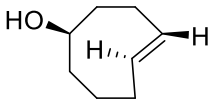


Azide ⇌ Tetrazole

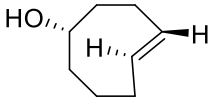
Crude [(6-azidopyridin-3-yl)methyl] mesylate/chloride **10f/11f** (0.138 g) dissolved in acetonitrile (2 mL) was added dropwise to a mixture of 7-hydroxycoumarin **1** (0.078 g, 0.481 mmol) and potassium carbonate (0.167 g, 1.21 mmol) in acetonitrile (8 mL). The mixture was then allowed to stir in the dark (flask wrapped in foil) at room temperature and under nitrogen until mesylate/chloride was completely consumed (~5 days; progress monitored via TLC analysis). Following which, acetonitrile was removed *in vacuo* and the crude residue was dissolved in ethyl acetate (20 mL) and washed with water (3 × 50 mL), brine (1 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The resulting crude residue was purified by silica gel flash column chromatography (40% ethyl acetate:hexane) to afford the title compound (0.093 g, 38% - yield over 2 steps from **9f**) as a pale-brown powder. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.53 – 9.51 (m, 1H), 8.27 (dd, J = 9.2, 1.0 Hz, 1H), 8.01 (d,

J = 9.5 Hz, 1H), 7.97 (dd, *J* = 9.2, 1.5 Hz, 1H), 7.68 (d, *J* = 8.6 Hz, 1H), 7.19 (d, *J* = 2.5 Hz, 1H), 7.10 (dd, *J* = 8.6, 2.5 Hz, 1H), 6.32 (d, *J* = 9.5 Hz, 1H), 5.40 (s, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 161.3, 160.6, 155.7, 148.3, 144.7, 134.0, 130.1, 127.0, 125.5, 115.6, 113.38, 113.37, 113.35, 102.2, 66.8. HRMS (ESI+) calculated for $C_{15}H_{10}N_4NaO_3$: 317.0645, found: 317.0642. IR: ν_{max}/cm^{-1} 1723, 1609, 1229, 1128, 840. HPLC-UV (**Figure S9f**): t_R = 6.1 min (λ = 325 nm).

2.25 *trans*-Cyclooct-4-enol (TCO) 2:



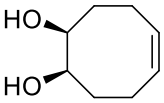
2-equatorial OH



2-axial OH

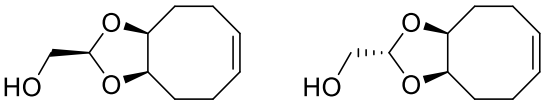
Synthesised as previously described.[1,2] **2-**equatorial ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.56 (m, 1H), 5.37 (m, 1H), 3.47 – 3.41 (m, 1H), 2.37 – 2.21 (m, 3H), 2.00 – 1.86 (m, 4H), 1.72 – 1.49 (m, 3H), 1.36 (s, 1H). **2-**axial ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.63 – 5.50 (m, 2H), 4.03 (m, 1H), 2.37 (m, 1H), 2.28 – 2.18 (m, 2H), 2.17 – 2.04 (m, 2H), 1.92 – 1.74 (m, 3H), 1.65 (m, 1H), 1.30 – 1.21 (m, 2H).

2.26 (1*R*,2*S*,*Z*)-Cyclooct-5-ene-1,2-diol 17.



Prepared as a modified method of general method described in published literature.[8] 1,5-Cyclooctadiene **18** (11.3 mL, 92.4 mmol) was added dropwise to AD-mix-β (33.0 g, 0.001 equivalents with respect to potassium osmate dihydrate) in 150 ml of 1:1 *tert*-butyl alcohol:water cooled on ice. The reaction mixture was allowed to warm to room temperature and stirred for 24 hours. The reaction was then quenched with sodium metabisulfite (1.56 g) and stirred for 30 minutes. Following which, *tert*-butyl alcohol was then removed *in vacuo* and the remaining water partition was extracted with ethyl acetate (3 × 100 mL). The combined organic layers were washed with water (3 × 30 mL), brine (1 × 30 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The resulting crude residue was purified by silica gel flash column chromatography (90% ethyl acetate:hexane) to afford the titled compound (0.987 g, 8%) as a white powder, which was spectroscopically similar to that reported in literature.[9] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.72 – 5.61 (m, 2H), 4.03 – 3.96 (m, 2H), 2.56 – 2.44 (m, 2H), 2.10 – 1.96 (m, 4H), 1.86 – 1.74 (m, 2H). HRMS (ESI+) calculated for: $C_8H_{14}O_2Na$: 165.0886, found: 165.0885.

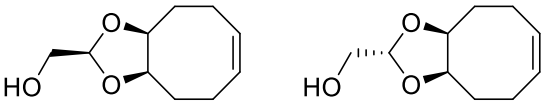
2.27 1:1 dr ((2*s*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*syn*) and ((2*r*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*anti*) (d-CCO) 19:



Syn 1:1 dr ***Anti***

Prepared as described in published literature.[9] (1*R*,2*S*,*Z*)-Cyclooct-5-ene-1,2-diol **18** (0.310 g, 2.18 mmol) and glycolaldehyde dimer (mixture of stereoisomers) (0.125 g, 1.04 mmol) was dissolved in dry toluene (5 mL). To this solution, was added *p*-toluenesulfonic acid (0.079 g, 0.459 mmol) and the mixture was allowed to stir for 90 minutes at 80 °C. The mixture was then diluted with DCM (100 mL) and washed with $NaHCO_3$ (3 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The resulting crude oil was then purified by silica gel column chromatography (30% ethyl acetate:hexane) to afford the title compound, a clear yellow coloured oil, as a mixture of diastereomers 1:1 dr (0.197 g, 49%) which was spectroscopically similar to that reported in the literature.[9] ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.62 – 5.58 (m, 2H) *syn*, 5.58 – 5.54 (m, 2H) *anti*, 5.17 (t, *J* = 3.5 Hz, 1H) *anti*, 4.90 (t, *J* = 3.2 Hz, 1H) *syn*, 4.30 – 4.23 (m, 2H) *anti*, 4.21 – 4.15 (m, 2H) *syn*, 3.65 (dd, *J* = 6.3, 3.2 Hz, 2H) *syn*, 3.55 (dd, *J* = 6.3, 3.5 Hz, 2H) *anti*, 2.57 – 2.43 (m, 4H), 2.18 – 2.01 (m, 8H), 2.00 – 1.88 (m, 6H).

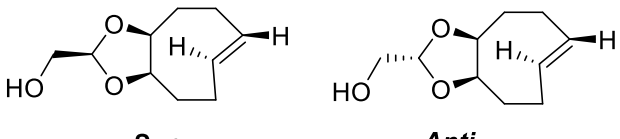
2.28 8:1 dr ((2*s*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*syn*) and ((2*r*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*anti*) (d-CCO) 19-*syn*:



Syn 8:1 dr ***Anti***

Prepared as described in published literature.[9] (1*R*,2*S*,*Z*)-Cyclooct-5-ene-1,2-diol **18** (1.18 g, 8.32 mmol) and glycolaldehyde dimer (mixture of stereoisomers) (0.499 g, 4.15 mmol) was dissolved in THF (80 mL). To this solution, was added *p*-toluenesulfonic acid (0.315 g, 1.66 mmol) and the mixture was allowed to stir at room temperature for 48 hours. The mixture was then diluted with chloroform (150 mL) and washed with $NaHCO_3$ (3 × 50 mL), dried ($MgSO_4$) and concentrated *in vacuo*. The resulting crude oil was then purified by chromatography (30% ethyl acetate:hexane) to afford the title compound, a clear oil, as a mixture of diastereomers d.r. 8:1 *syn*:*anti* (0.720 g, 47%) which was spectroscopically similar to that reported in the literature[9]. Peaks attributable to the *syn* diastereomer: ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.68 – 5.54 (m, 2H), 4.92 (t, *J* = 3.1 Hz, 1H), 4.24 – 4.16 (m, 2H), 3.67 (d, *J* = 3.1 Hz, 2H), 2.58 – 2.45 (m, 2H), 2.17 – 2.02 (m, 4H), 2.02 – 1.92 (m, 2H), 1.89 – 1.82 (m, 1H).

2.29 1.5:1 dr and 14:1 dr ((2*s*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*syn*) and ((2*r*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2- yl)methanol (*anti*) (d-TCO) 3:



14:1 or 1.5:1 dr

d-TCO **3** was synthesized using a modified published procedure.[1,9] ((2r:s,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol 1:1 dr (0.472 g, 2.56 mmol) or 8:1 dr (0.358 g, 1.94 mmol) and methyl benzoate (3 equivalents) was added to diethyl ether (50 mL) into a 250 mL quartz reaction vessel and placed in a Rayonet® photoreactor and irradiated for 20 min at 254 nm. The solution was then passed through $AgNO_3$ impregnated silica and washed with diethyl ether (100 mL). This process was repeated 8 times. Following which, the column was washed with diethyl ether (100 mL), repeatedly until no further *cis*-isomer could be detected via TLC ($KMnO_4$). The dry silica gel was then removed and washed with 10 mL of ammonium hydroxide (25%). The slurry was stirred vigorously for 5 minutes. Diethyl ether (20 mL) was added and stirred vigorously for a further 5 minutes, and then the diethyl ether was decanted from the $AgNO_3$ silica gel. The silica gel was washed with additional diethyl ether (20 mL) until no more *trans*-isomer was detected via TLC ($KMnO_4$). The combined diethyl ether extracts were washed with water (3 × 30 mL), brine (1 × 30 mL), dried ($MgSO_4$) and purified by silica gel flash column chromatography (30% ethyl acetate:hexane) to afford the title compounds, clear oils, as a mixture of diastereomers (determined by integration of dioxolane protons at δ 5.10 (*anti*) and 4.83 (*syn*) ppm dr 1.5:1 (**3**) (0.102 g, 22%) or dr 14:1 (**3**-*syn*) (0.134 g, 37%). The compounds were spectroscopically similar to that reported in literature[9].

d-TCO dr 1.5:1 (*syn*:*anti*) **3** ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.66 – 5.46 (m, 4H), 5.10 (t, *J* = 3.7 Hz, 1H) *anti*, 4.83 (t, *J* = 3.1 Hz, 1H) *syn*, 4.10 – 4.01 (m, 1H) *anti*, 4.01 – 3.89 (m, 3H), 3.66 – 3.59 (m, 2H) *syn*, 3.53 (d, *J* = 3.7 Hz, 2H) *anti*, 2.45 – 2.00 (m, 8H), 1.94 – 1.48 (m, 10H).

d-TCO dr 14:1 (*syn*:*anti*) **3**-*syn* peaks attributable to the *syn* diastereomer ^{1}H NMR (400 MHz, Chloroform-*d*) δ 5.67 – 5.58 (m, 1H), 5.57 – 5.47 (m, 1H), 4.85 (t, *J* = 3.0 Hz, 1H), 4.02 – 3.91 (m, 2H), 3.69 – 3.63 (m, 2H), 2.46 – 2.37 (m, 1H), 2.32 – 2.25 (m, 1H), 2.24 – 2.10 (m, 2H), 1.94 – 1.50 (m, 5H).

3 Rate of [3+2]-Cycloaddition (Click) Reaction (Rate 1)

Table S1. Concentrations of stock A and stock B used for each respective experiment. Final assay concentrations were conducted at 10-fold dilutions of stock concentrations.

Ether Probe	**2**-equatorial	**2**-axial	**3**	**3**-*syn*
1a	A: 100 mM B: 5 mM	A: 100 mM B: 5 mM	A: 50 mM B: 2.5 mM	A: 100 mM B: 5 mM
1b	A: 50 mM B: 2.5 mM	A: 50 mM B: 2.5 mM	A: 10 mM B: 0.5 mM	A: 5 mM B: 0.25 mM
1c	A: 50 mM B: 2.5 mM		A: 10 mM B: 0.5 mM	
1d	A: 100 mM B: 5 mM		A: 50 mM B: 2.5 mM	
1e	A: 100 mM B: 5 mM		A: 50 mM B: 2.5 mM	
1f	A: 100 mM B: 5 mM			

3.1 *Pseudo*-First Order Rate Plots

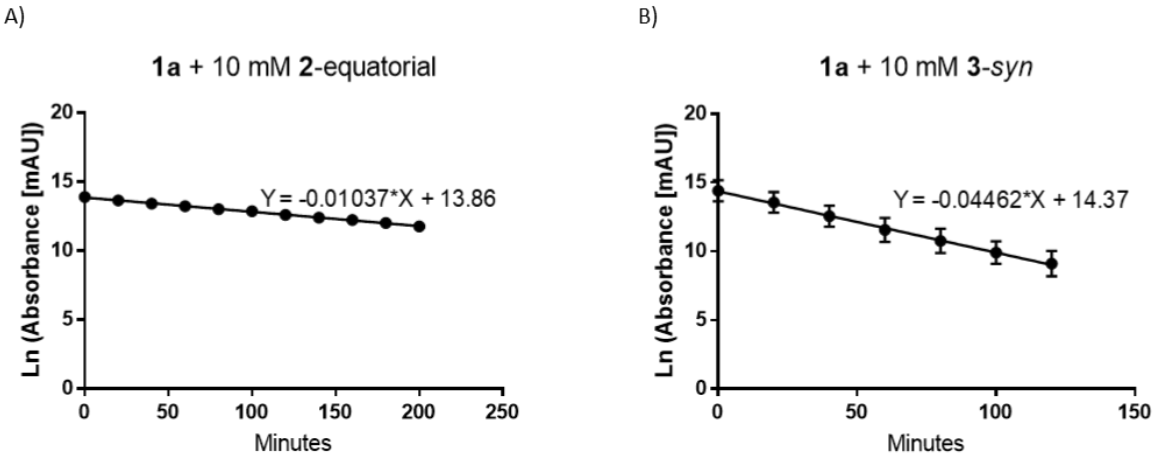


Figure S1.Pseudo-first order rate plots obtained for reactions between **1a** (500 μM) and 10 mM of; A) **2**-equatorial, or B) **3**-*syn* conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

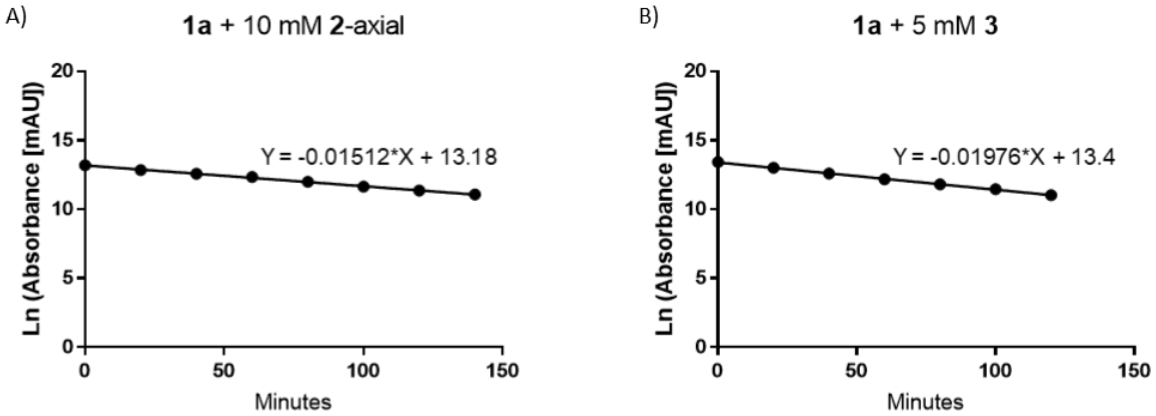


Figure S2. Pseudo-first order rate plots obtained for reactions between **1a** and A) 10 mM **2**-axial, or B) 5 mM **3** conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

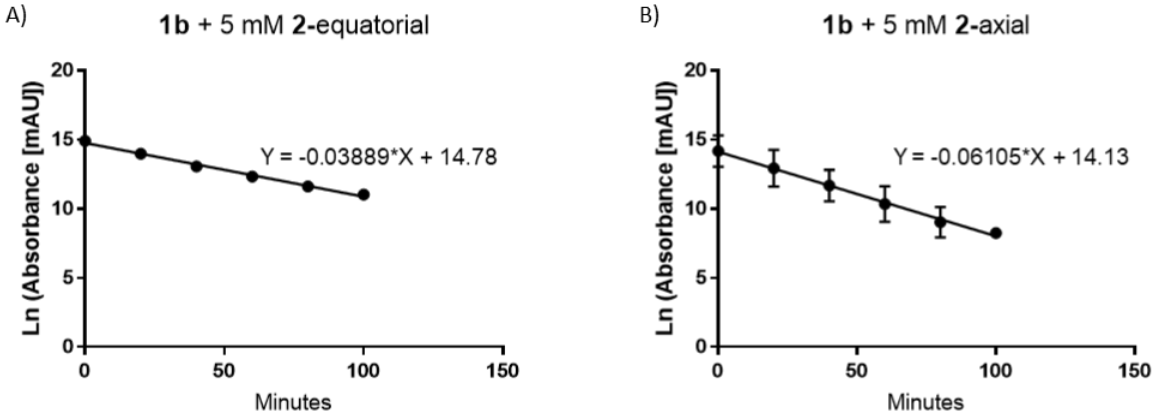


Figure S3. Pseudo-first order rate plots obtained for reactions between **1b** and 5 mM A) **2**-equatorial, or B) **2**-axial conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

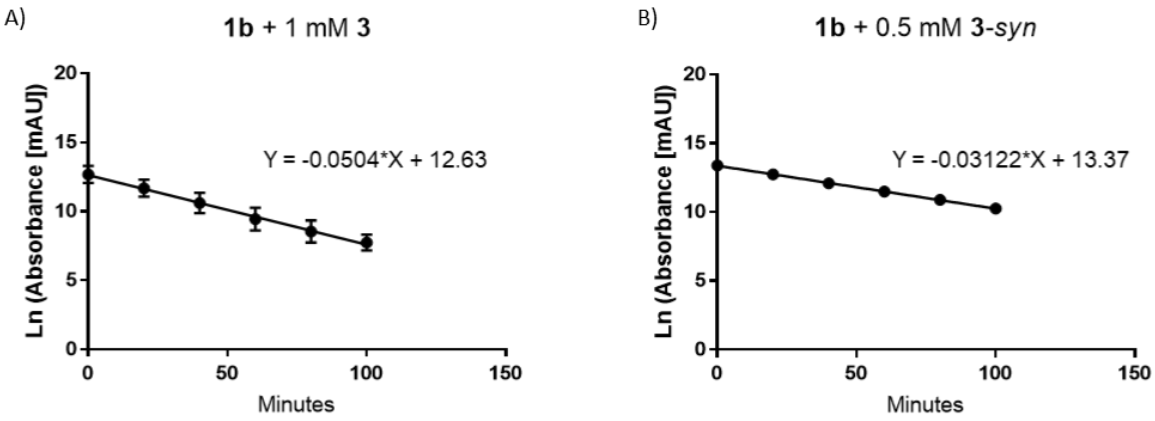


Figure S4. Pseudo-first order rate plots obtained for reactions between **1b** and A) 1 mM **3**, or B) 0.5 mM **3**-*syn* conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

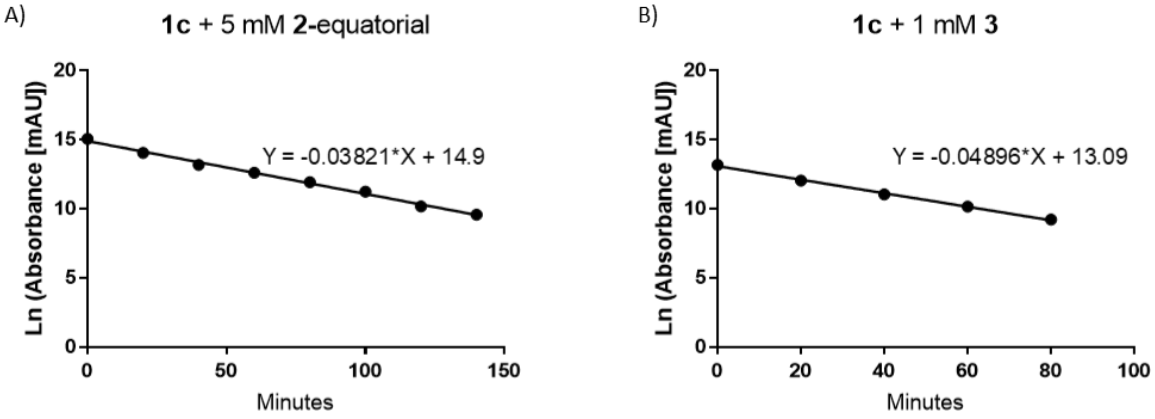


Figure S5. Pseudo-first order rate plots obtained for reactions between **1c** and A) 5 mM **2**-equatorial, or B) 1 mM **3** conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

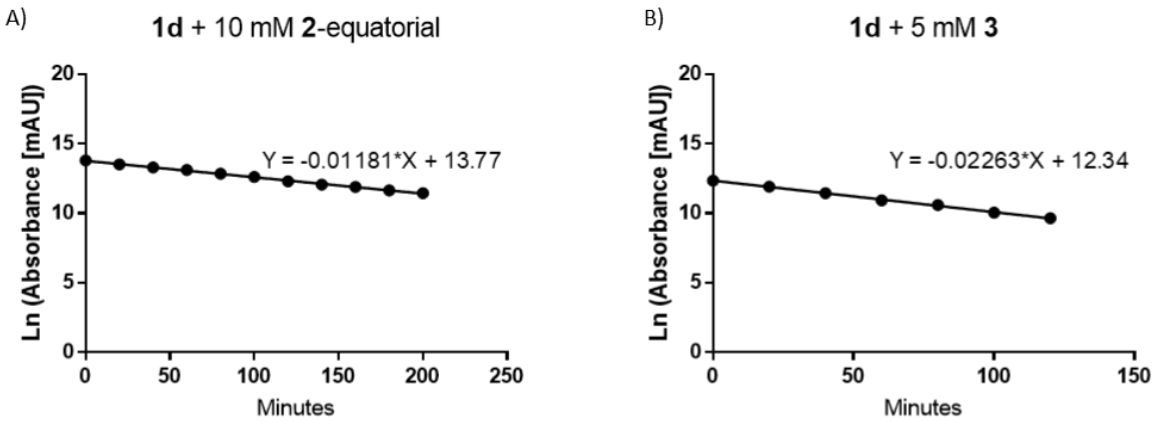


Figure S6. Pseudo-first order rate plots obtained for reactions between **1d** and A) 10 mM **2**-equatorial, or B) 5 mM **3** conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

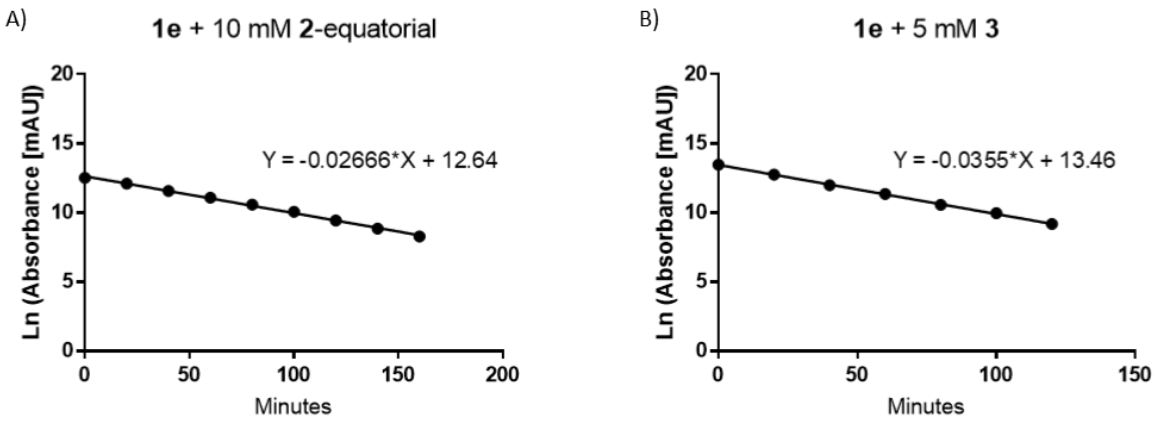


Figure S7. Pseudo-first order rate plots obtained for reactions between **1e** and A) 10mM **2**-equatorial, or B) 5 mM **3** conducted in MeCN:PBS 1:1. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

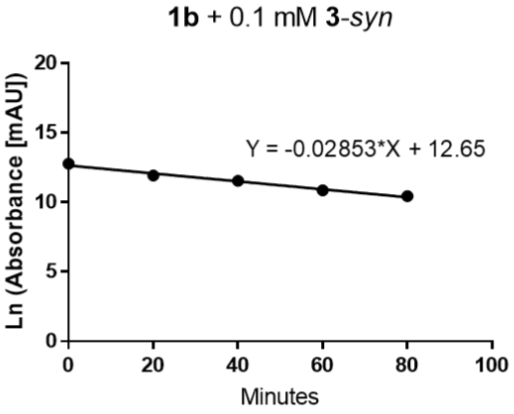
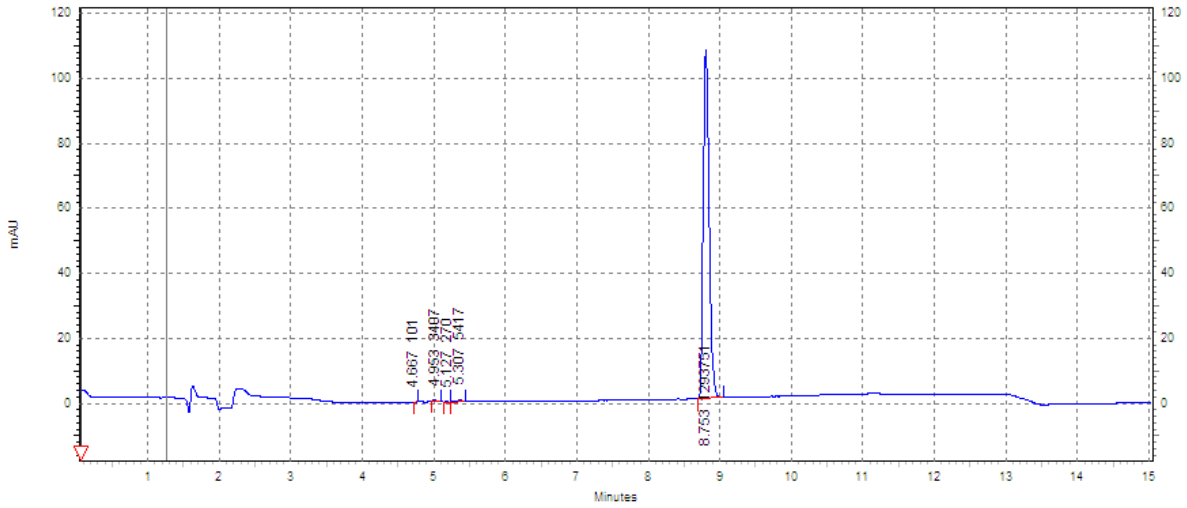


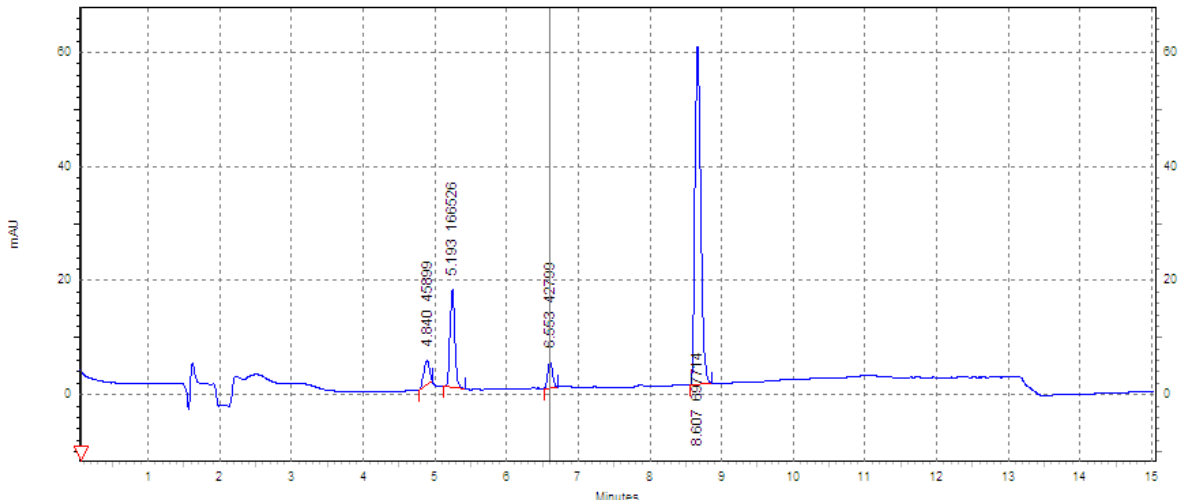
Figure S8. Pseudo-first order rate plots obtained for reactions between **1b** and 0.1 mM **3**-*syn* conducted in 5.25% MeCN in PBS. Represented here as an average and error as ± SD (*n* = 3). For second order rate calculations (Table 1), each experiment was plotted individually and the average of k was calculated.

3.2 HPLC Traces

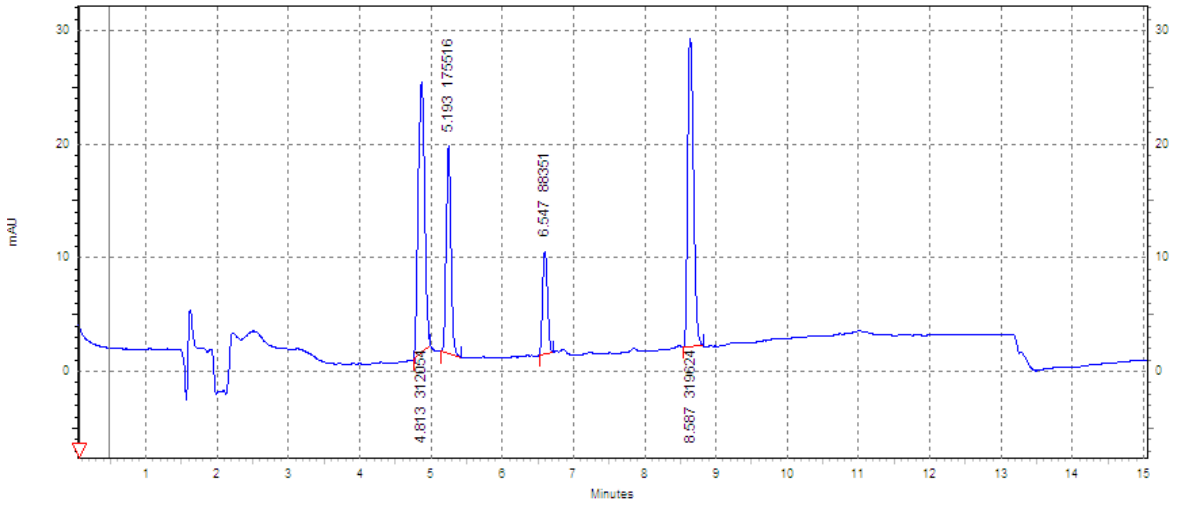
A) **1a** t = 0 minutes post **2**-equatorial addition



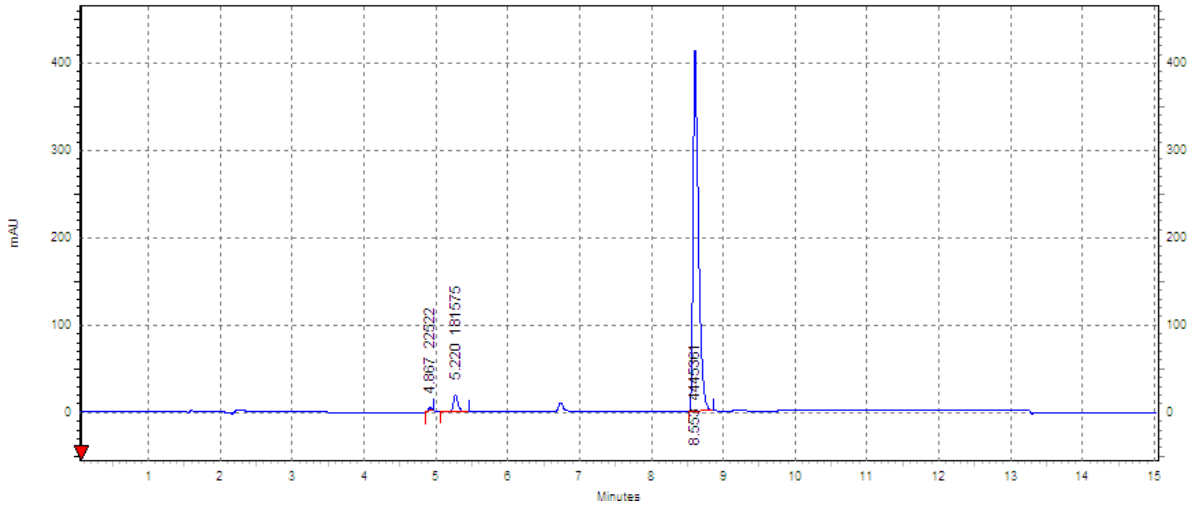
B) **1a** t = 40 minutes post **2**-equatorial addition



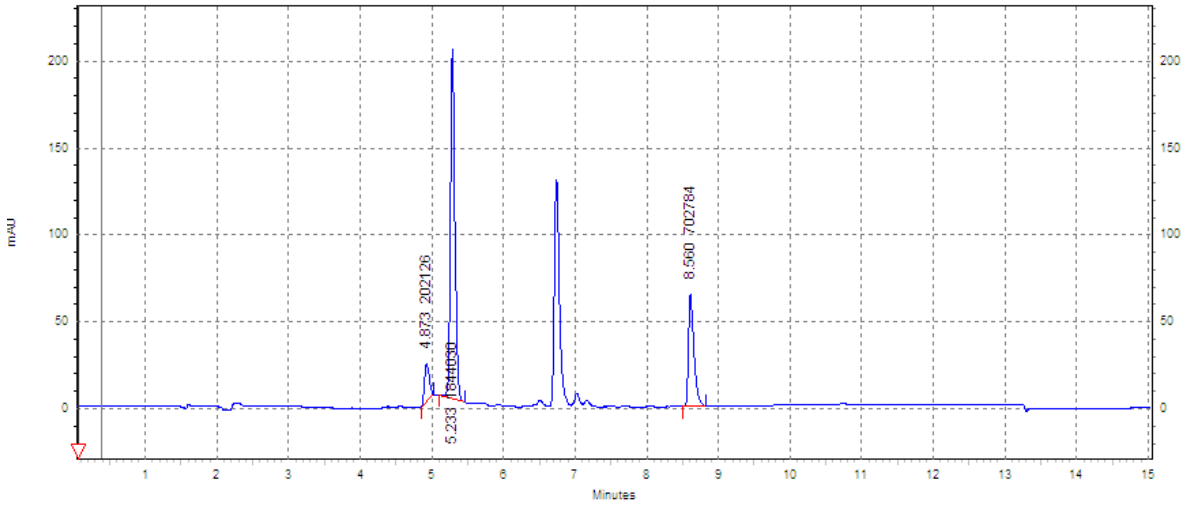
C) **1a** t = 120 minutes post **2**-equatorial addition



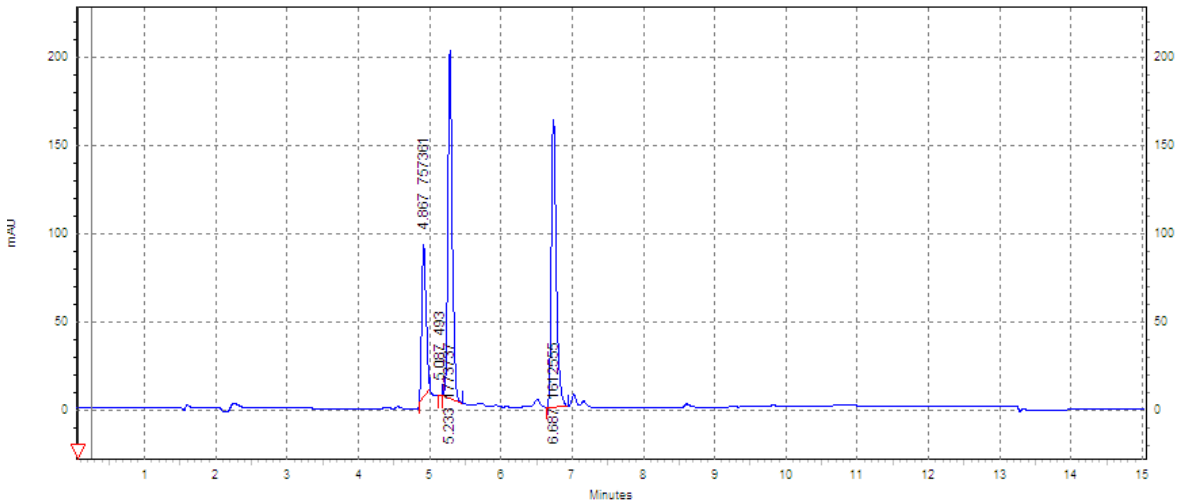
D) **1a** t = 0 minutes post **3**-*syn* addition



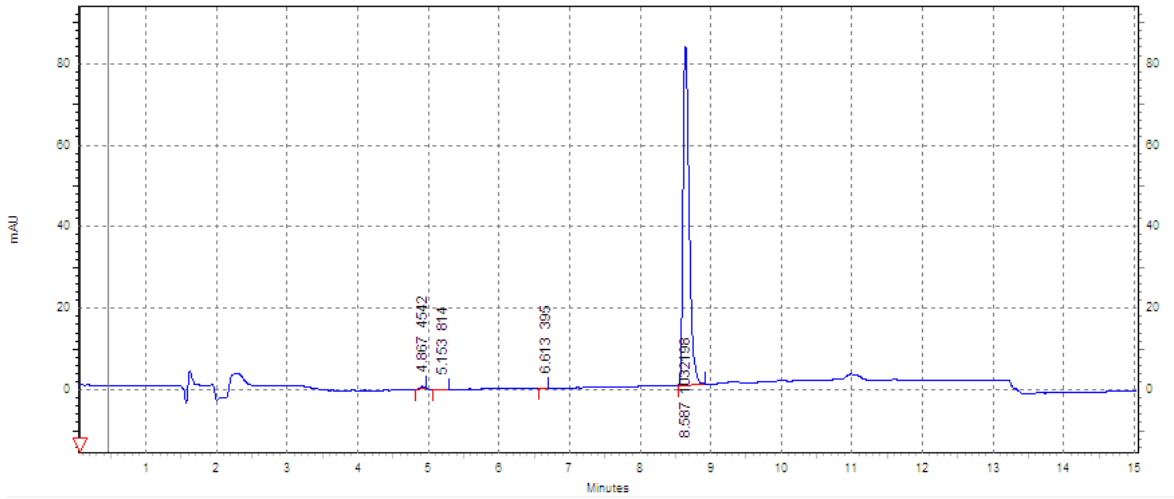
E) **1a** t = 40 minutes post **3**-*syn* addition



F) **1a** t = 120 minutes post **3**-*syn* addition



G) **1a** control t = 240 minutes



H) **1a** control t = 48 hours

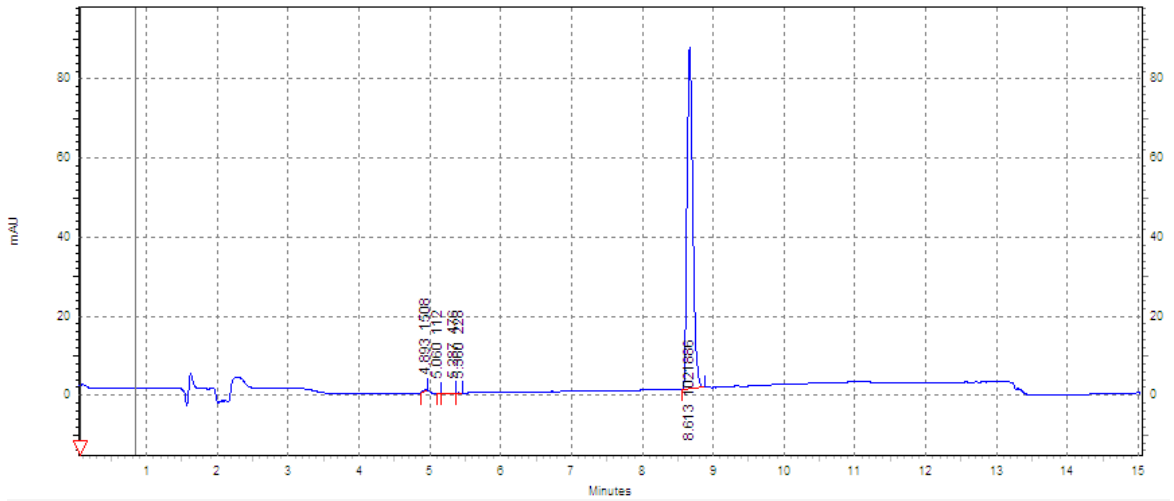
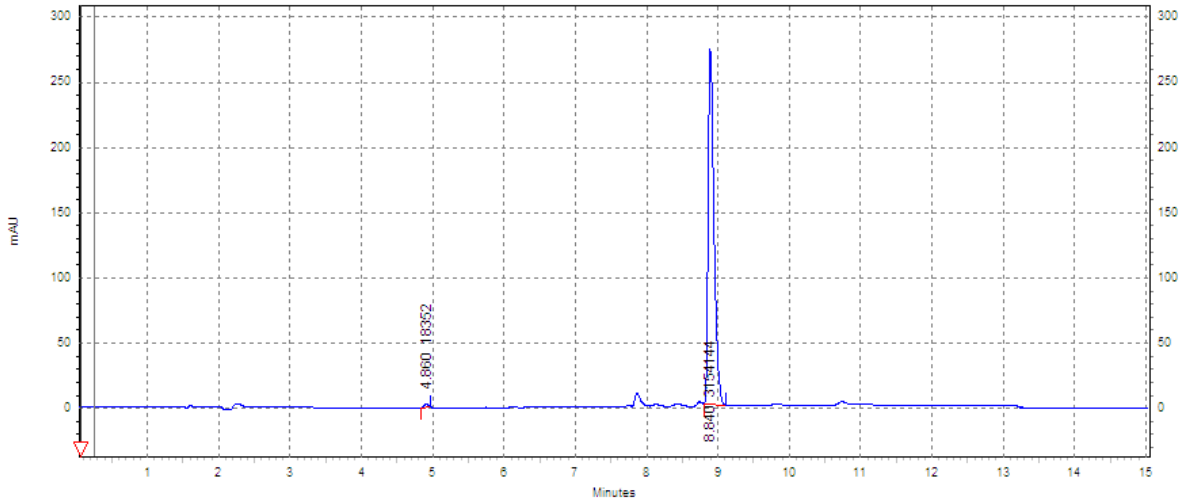
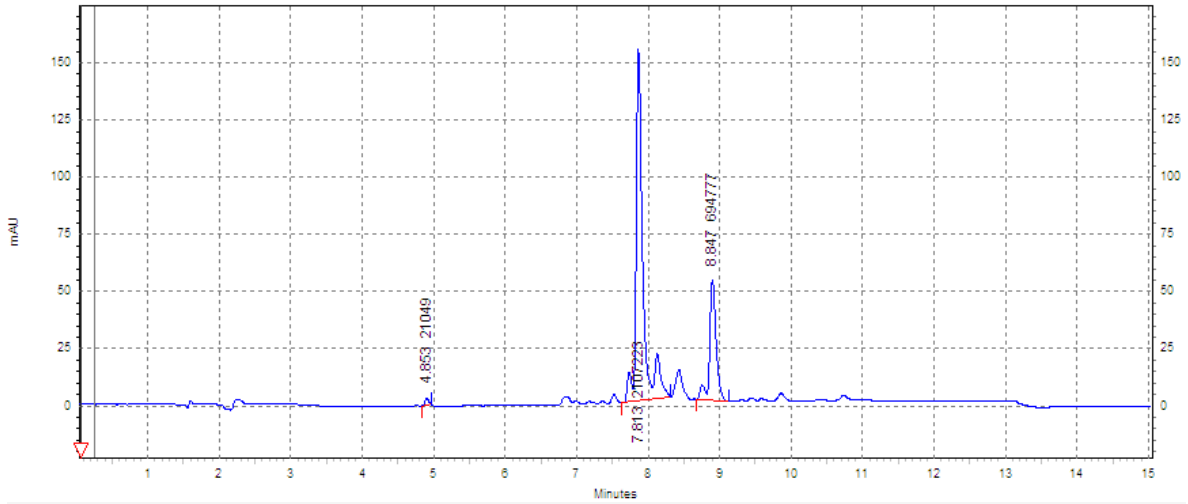


Figure S9a. HPLC-UV traces (λ = 325 nm) of **1a** after reaction with 20-fold excess of **2**-equatorial A) 0 minutes B) 40 minutes C) 120 minutes post TCO addition, or ***3***-syn D) 0 minutes E) 40 minutes F) 120 minutes post TCO addition and concurrent control experiment G) 240 minutes and H) 48 hours. Experiments were conducted in a 1:1 mixture of MeCN:PBS at 37 °C.

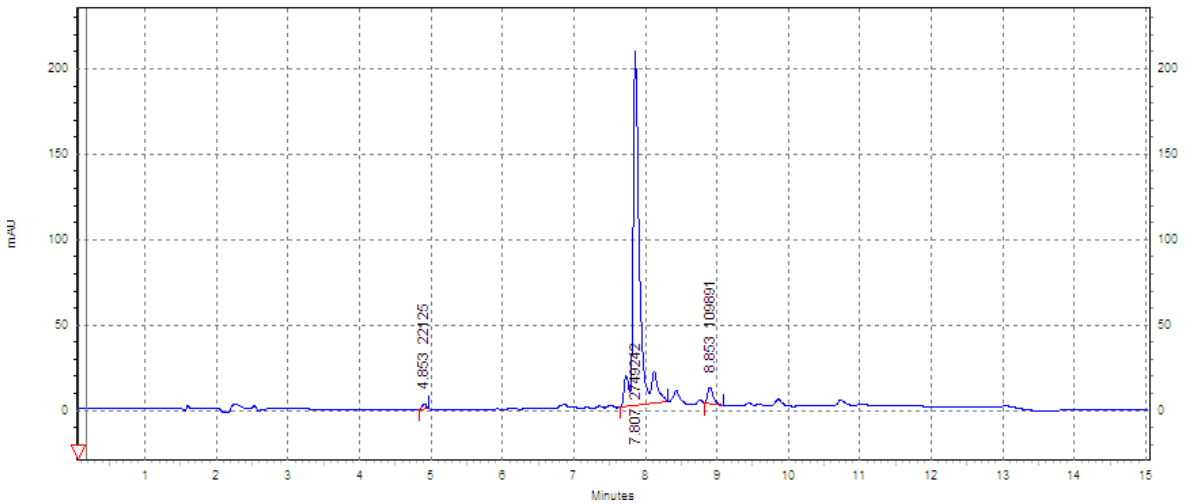
A) **1b** t = 0 minutes post **2**-equatorial addition



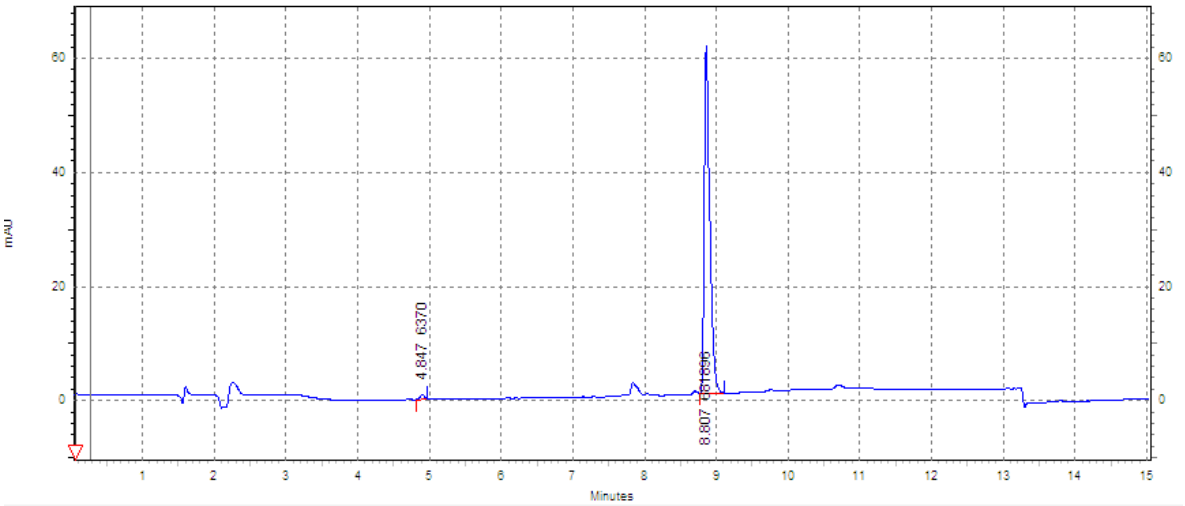
B) **1b** t = 40 minutes post **2**-equatorial addition



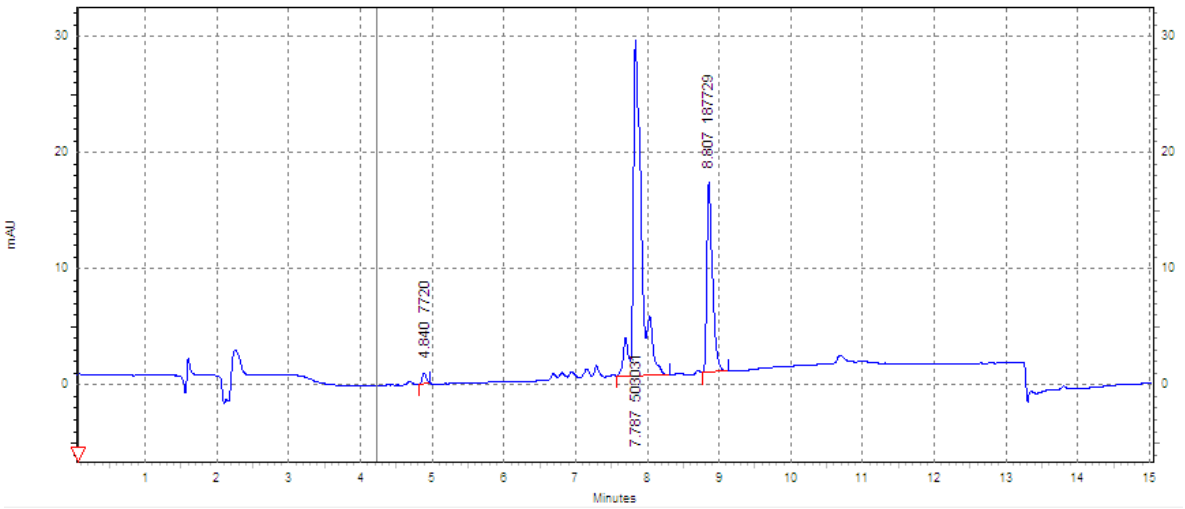
C) **1b** t = 100 minutes post **2**-equatorial addition



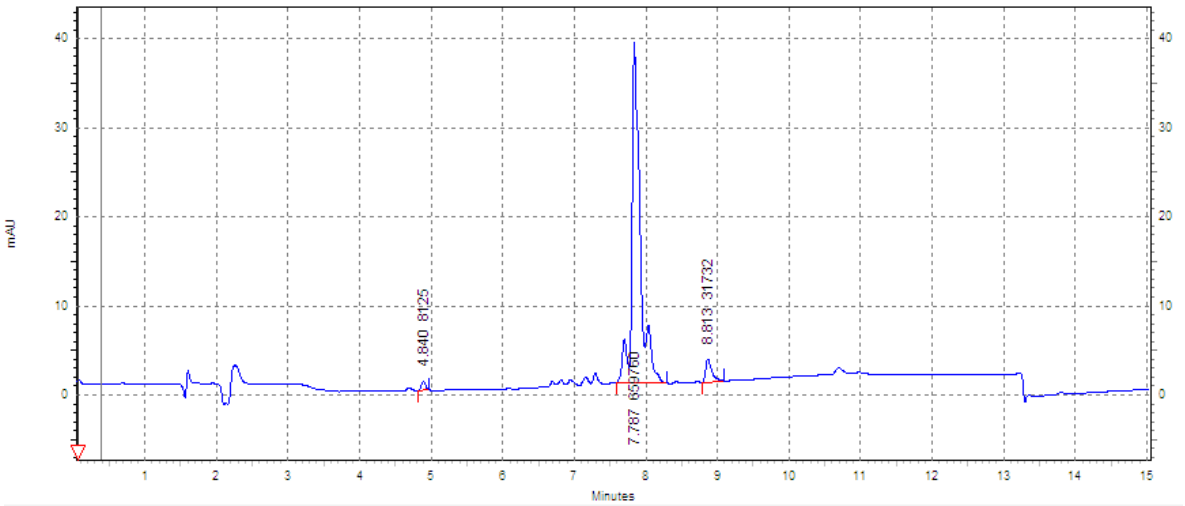
D) **1b** t = 0 minutes post **3**-*syn* addition



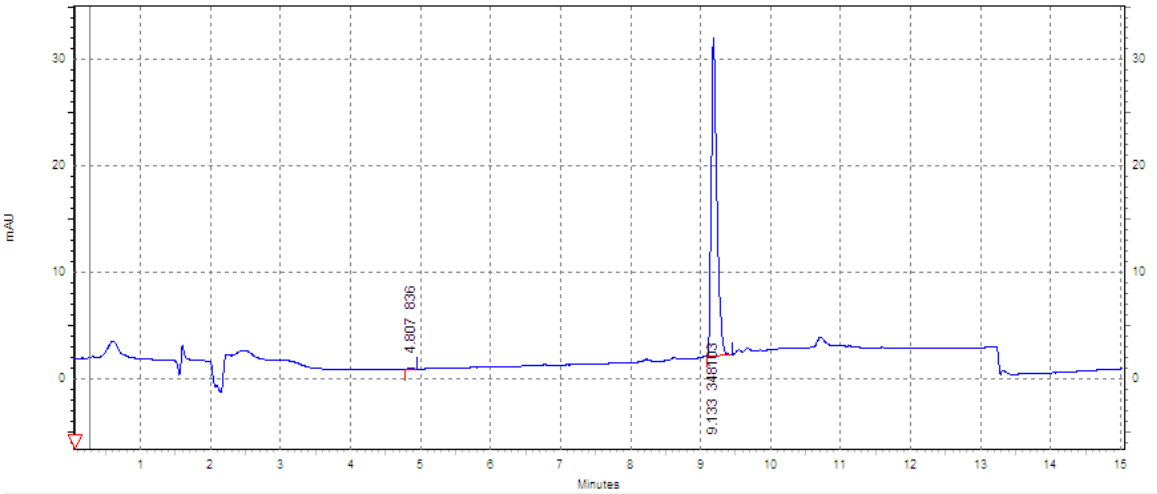
E) **1b** t = 40 minutes post **3**-*syn* addition



F) **1b** t = 100 minutes post **3**-*syn* addition



G) **1b** control t = 240 minutes



H) **1b** control t = 48 hours

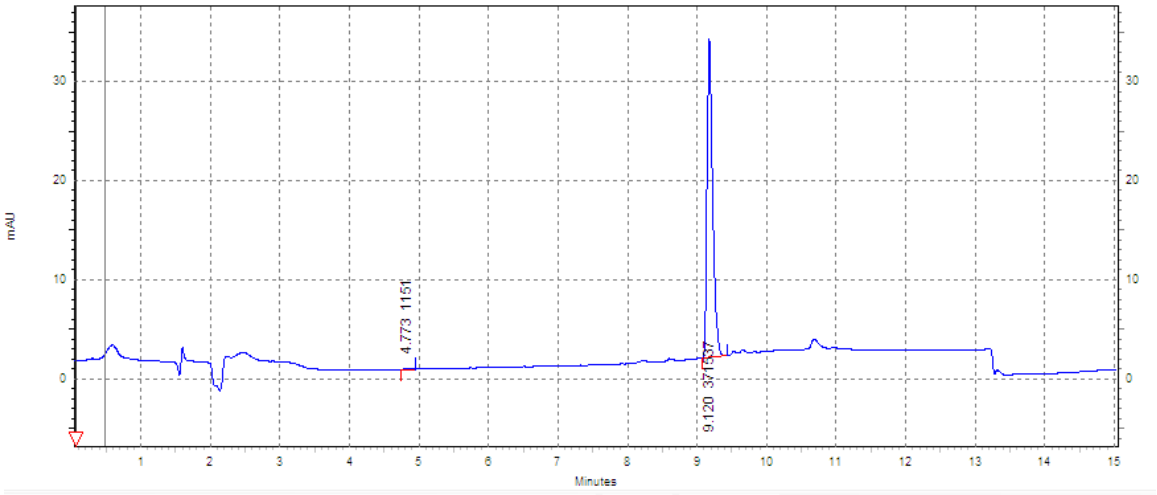
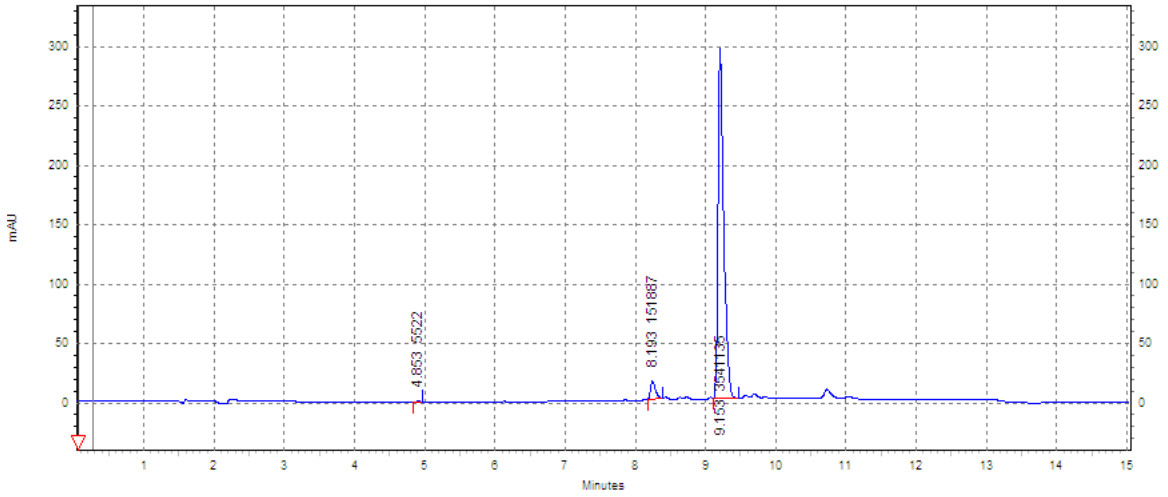
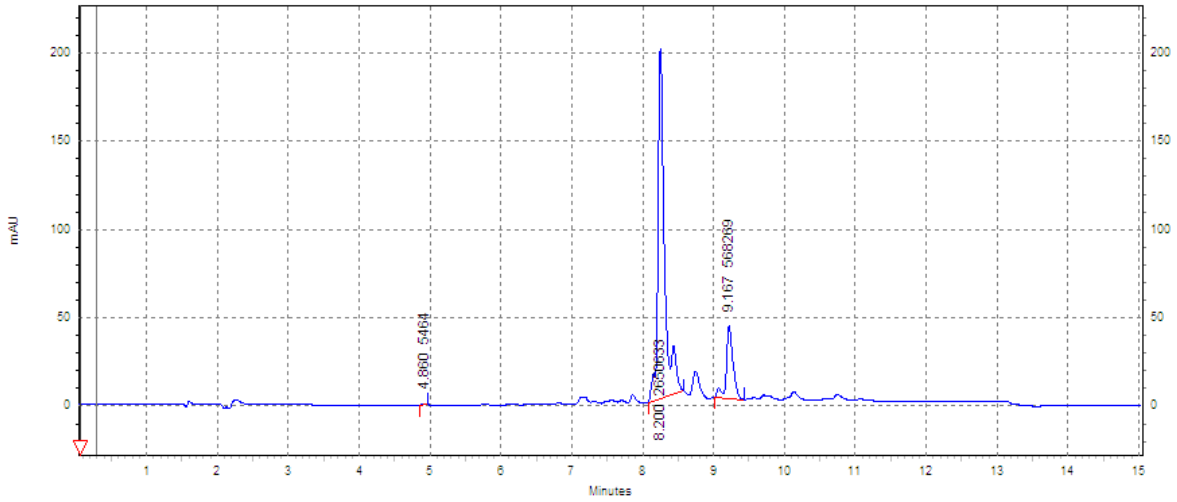


Figure S9b. HPLC-UV traces (λ = 325 nm) of **1b** after reaction with 20-fold excess of **2**-equatorial A) 0 minutes B) 40 minutes C) 100 minutes post TCO addition or **3**-*syn* D) 0 minutes E) 40 minutes F) 100 minutes post TCO addition and concurrent control experiment G) 240 minutes and H) 48 hours. Experiments were conducted in a 1:1 mixture of MeCN:PBS at 37 °C.

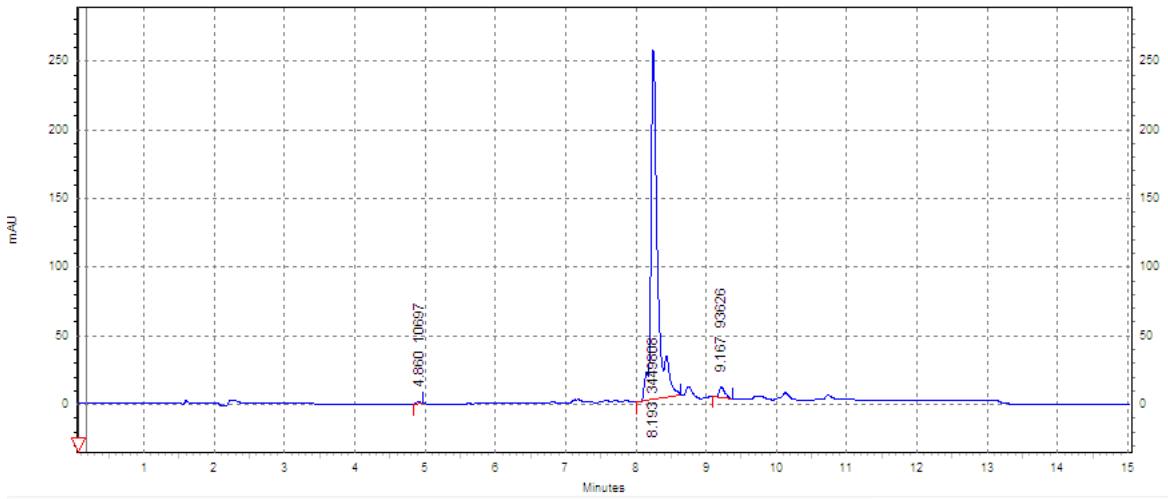
A) **1c** t = 0 minutes post **2**-equatorial addition



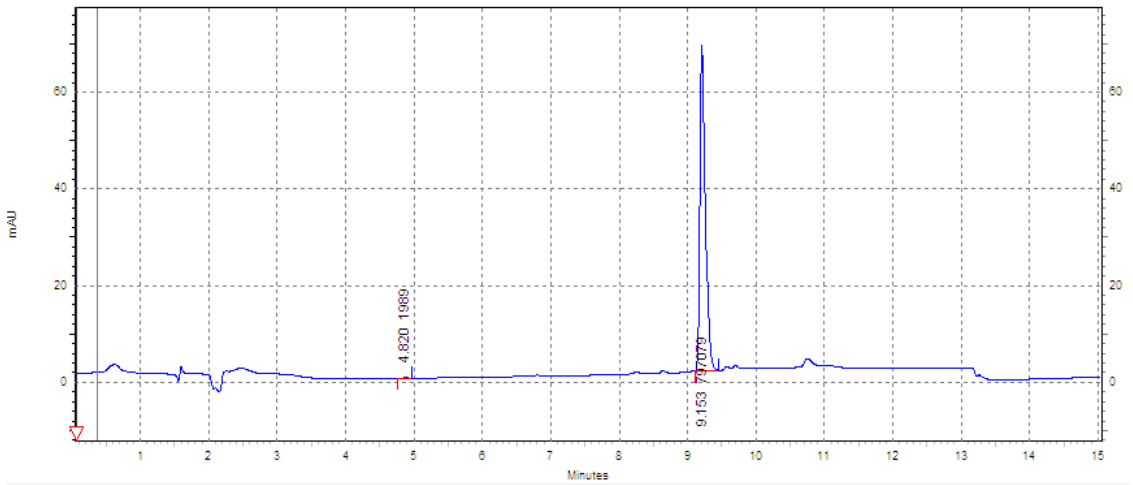
B) **1c** t = 40 minutes post **2**-equatorial addition



C) **1c** t = 100 minutes post **2**-equatorial addition



D) **1c** control t = 240 minutes



E) **1c** control t = 48 hours

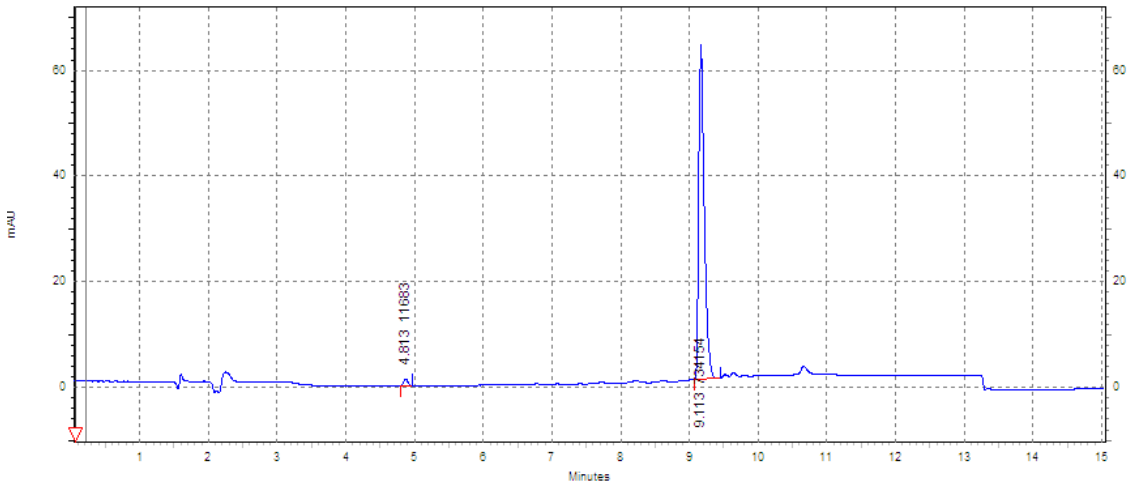
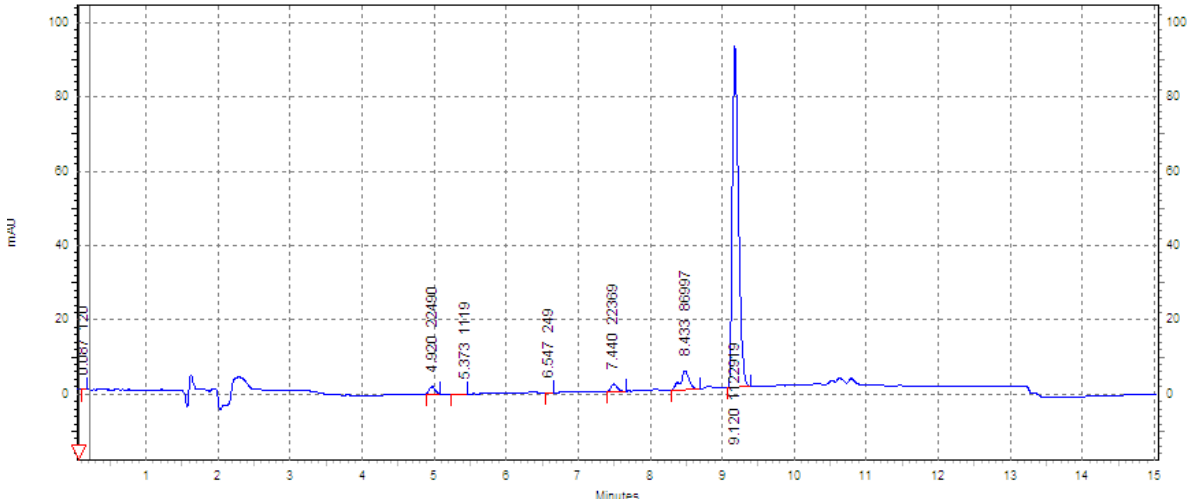
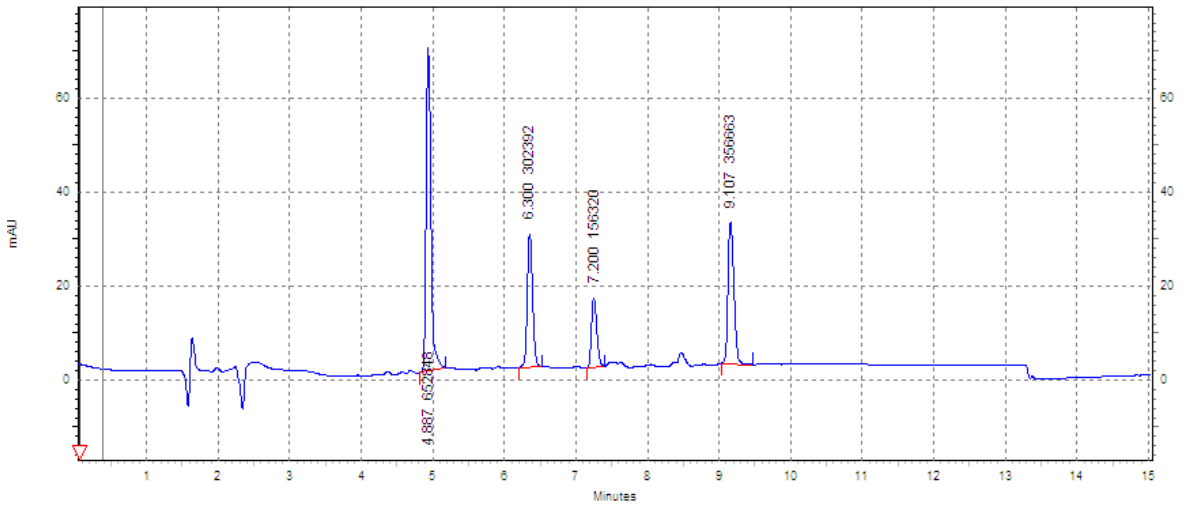


Figure S9c. HPLC-UV traces (λ = 325 nm) of **1c** after reaction with 20-fold excess of **2**-equatorial A) 0 minutes B) 40 minutes C) 100 minutes post TCO addition and concurrent control experiment D) 240 minutes and E) 48 hours. Experiments were conducted in a 1:1 mixture of MeCN:PBS at 37 °C.

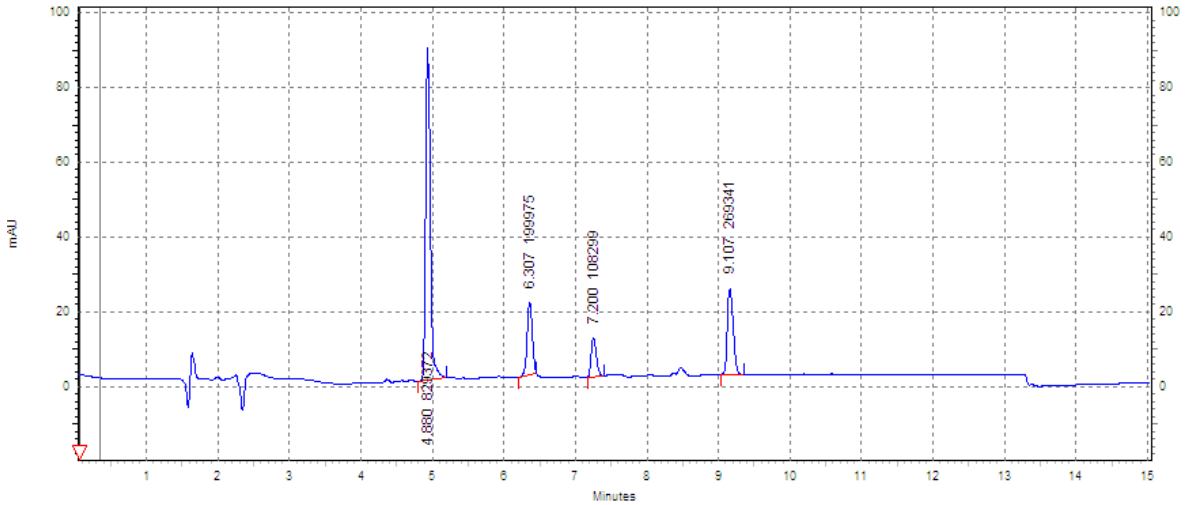
A) **1d** t = 0 minutes post **2**-equatorial addition



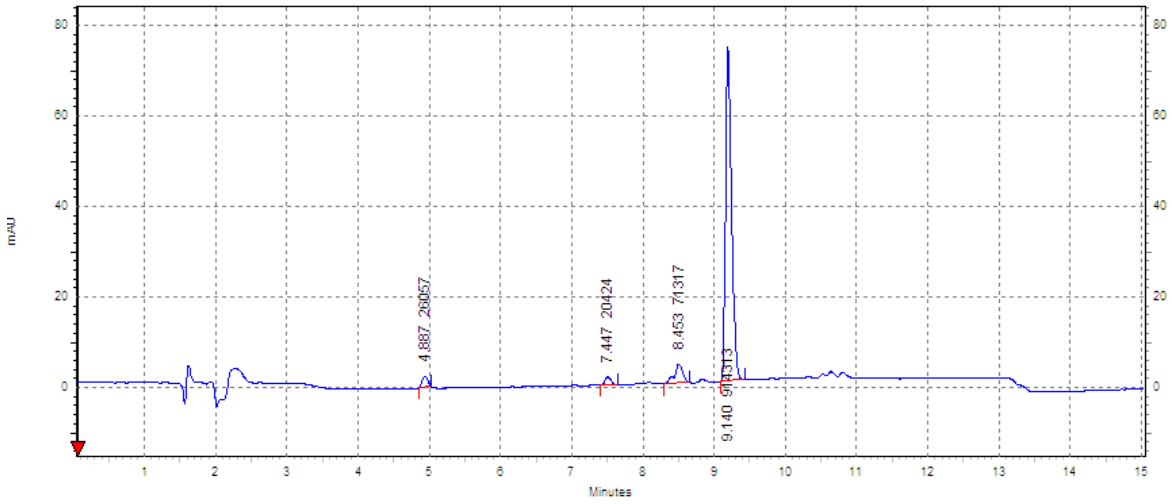
B) **1d** t = 40 minutes post **2**-equatorial addition



C) **1d** t = 120 minutes post **2**-equatorial addition



D) **1d** control t = 240 minutes



E) **1d** control t = 48 hours

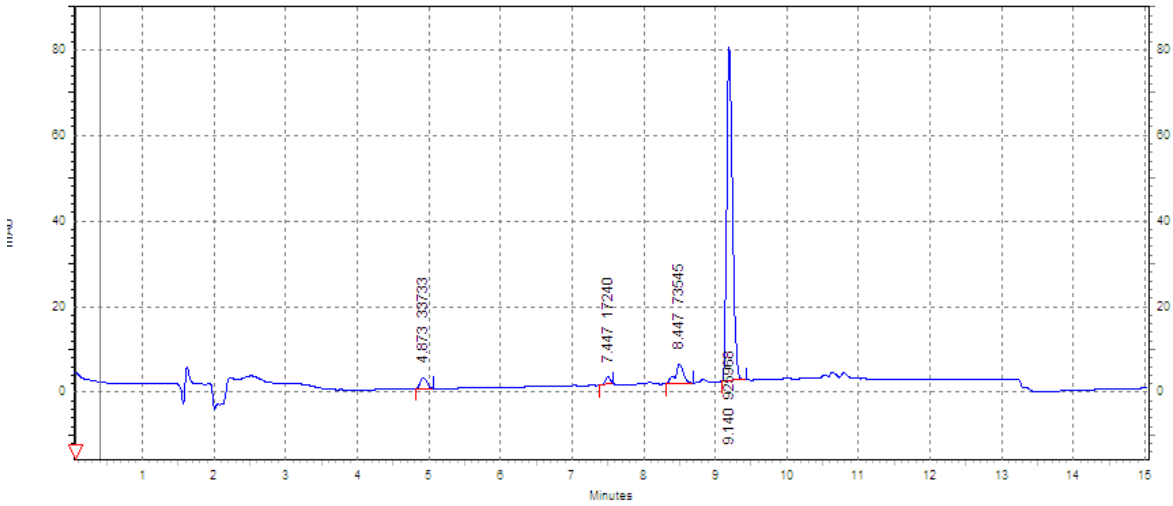
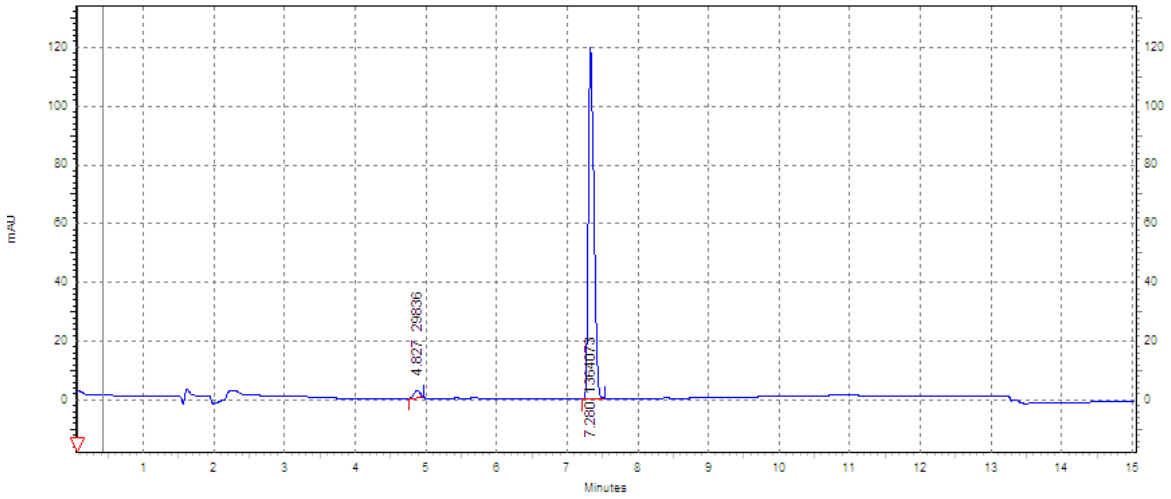
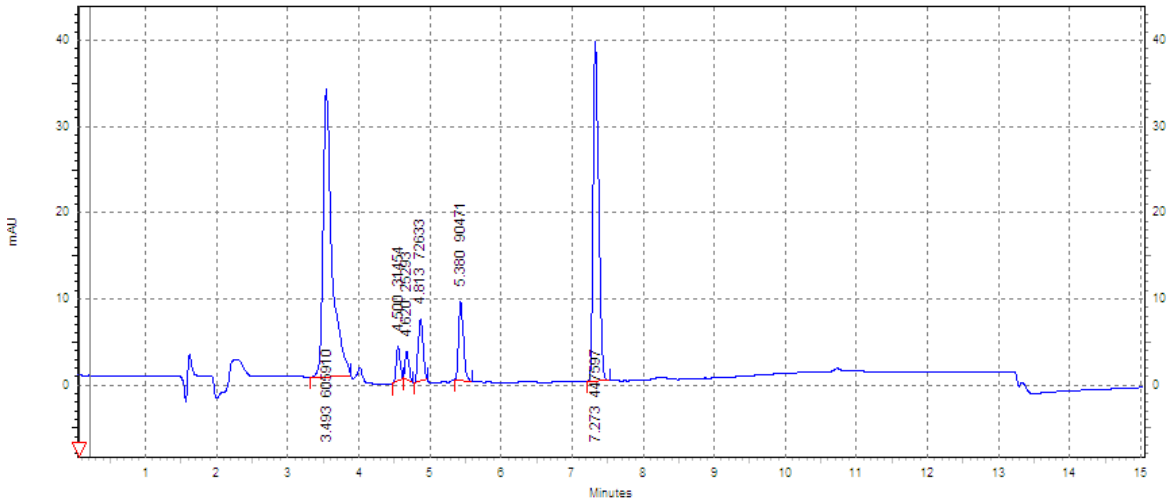


Figure S9d. HPLC-UV traces (λ = 325 nm) of **1d** after reaction with 20-fold excess of **2**-equatorial A) 0 minutes B) 40 minutes C) 120 minutes post TCO addition and concurrent control experiment D) 240 minutes and E) 48 hours. Experiments were conducted in a 1:1 mixture of MeCN:PBS at 37 °C.

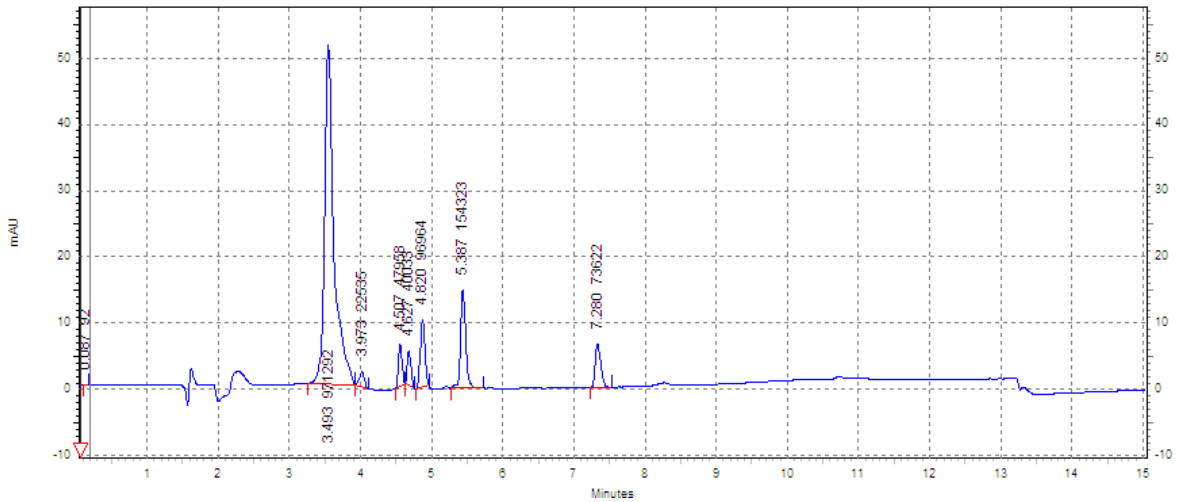
A) **1e** t = 0 minutes post **2**-equatorial addition



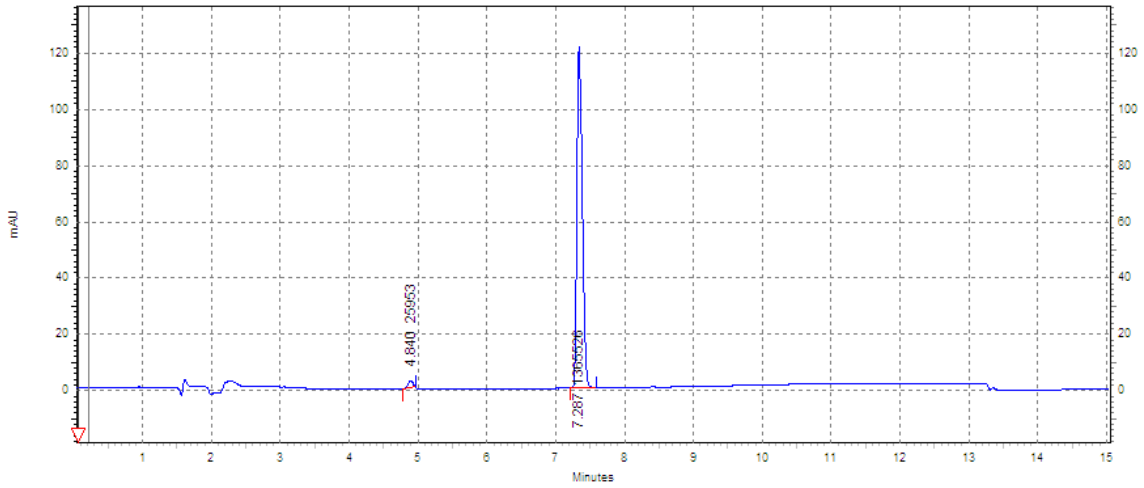
B) **1e** t = 40 minutes post **2**-equatorial addition



C) **1e** t = 120 minutes post **2**-equatorial addition



D) **1e** control t = 240 minutes



E) **1e** control t = 48 hours

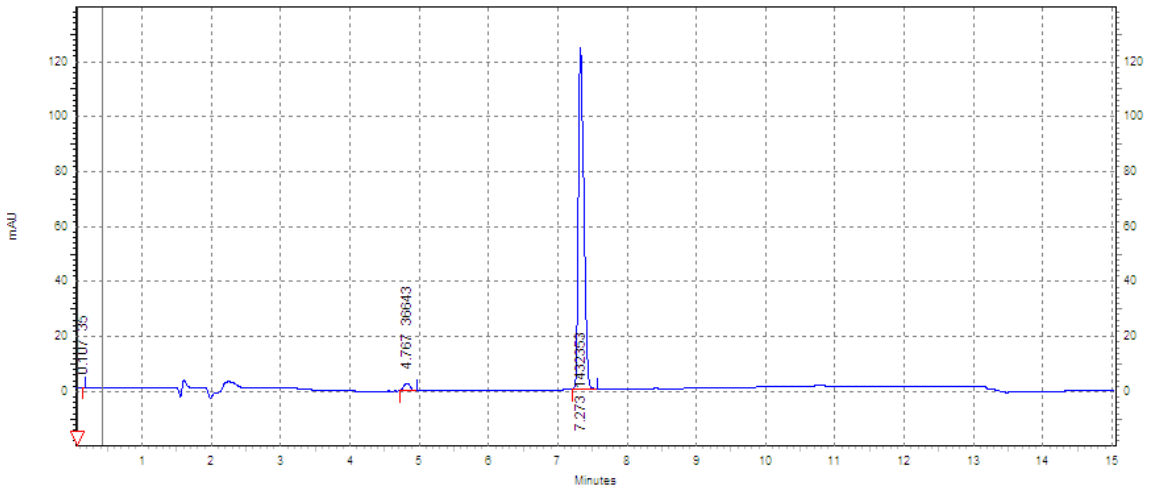
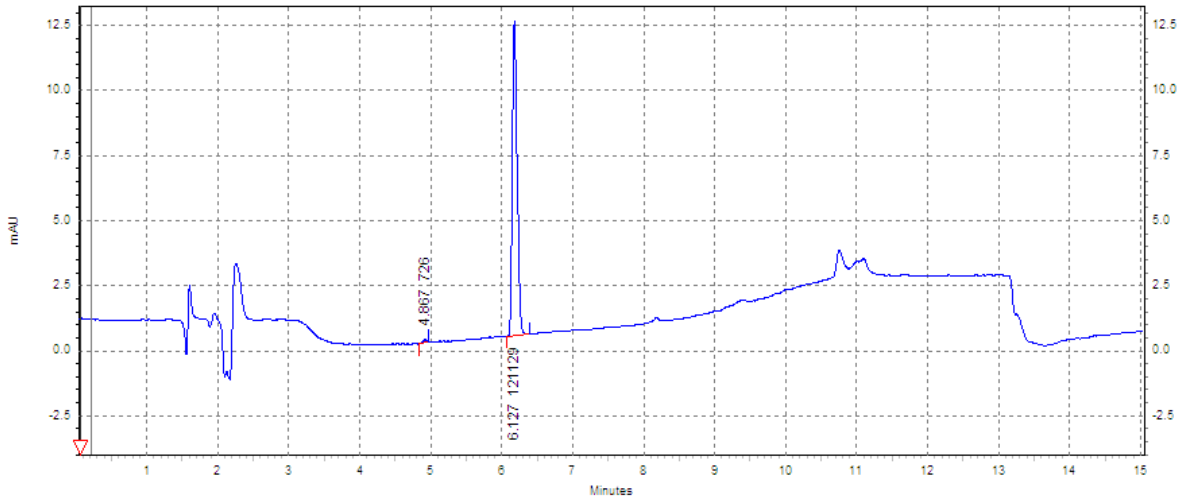
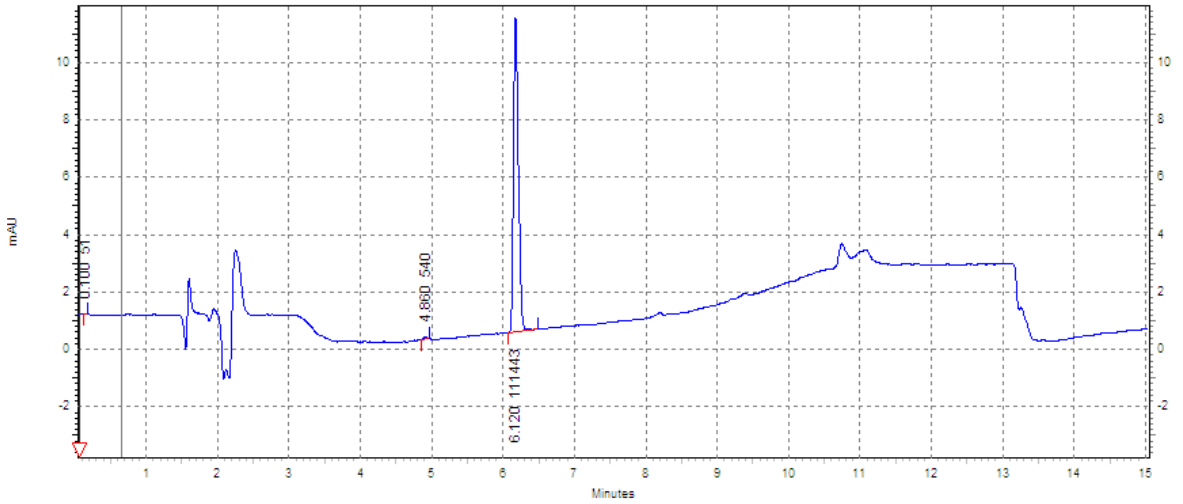


Figure S9e. HPLC-UV traces (λ = 325 nm) of **1e** after reaction with 20-fold excess of **2**-equatorial A) 0 minutes B) 40 minutes C) 120 minutes post TCO addition and concurrent control experiment D) 240 minutes and E) 48 hours. Experiments were conducted in a 1:1 mixture of MeCN:PBS at 37 °C.

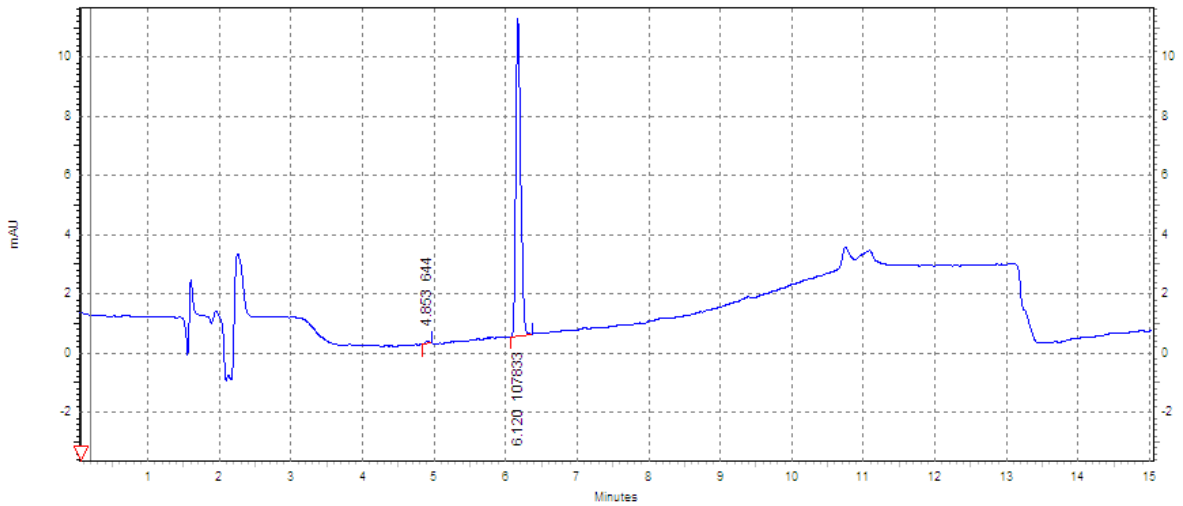
A) **1f** t = 0 minutes post **2**-equatorial addition 1:1 MeCN:PBS



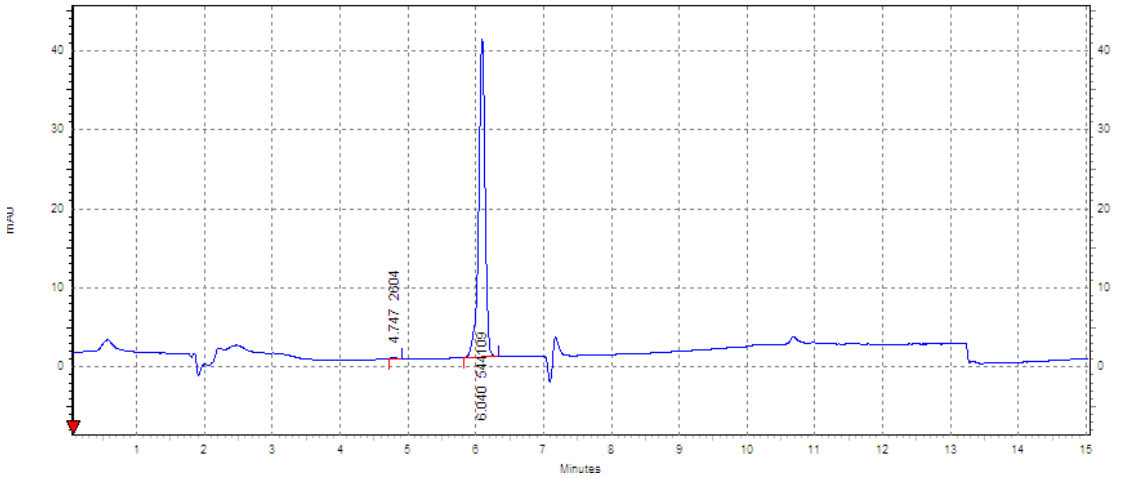
B) **1f** t = 40 minutes post **2**-equatorial addition 1:1 MeCN:PBS



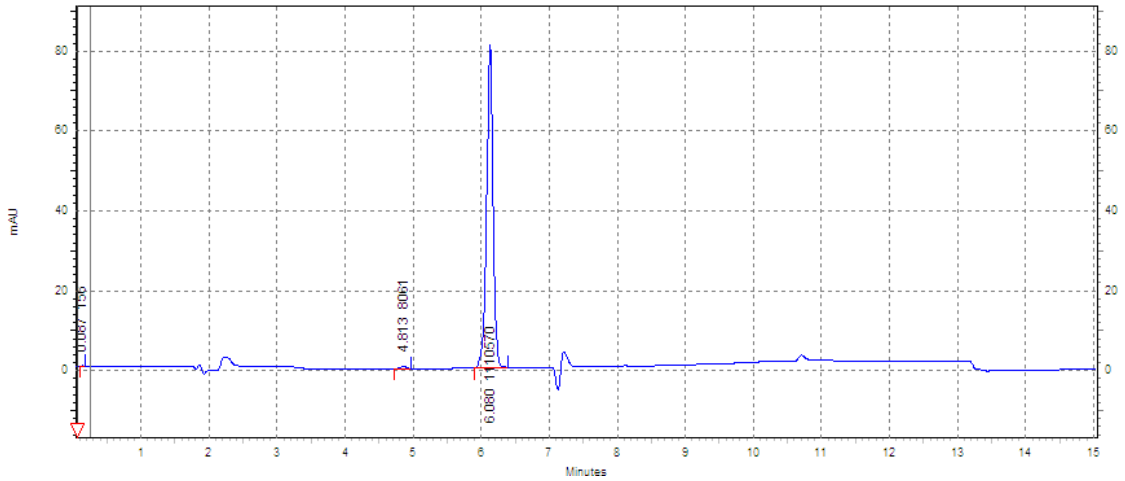
C) **1f** t = 24 hours post **2**-equatorial addition 1:1 MeCN:PBS



D) **1f** t = 0 minutes post **2**-equatorial addition $CHCl_3$



E) **1f** t = 40 minutes post **2**-equatorial addition $CHCl_3$



F) **1f** t = 24 hours post **2**-equatorial addition $CHCl_3$

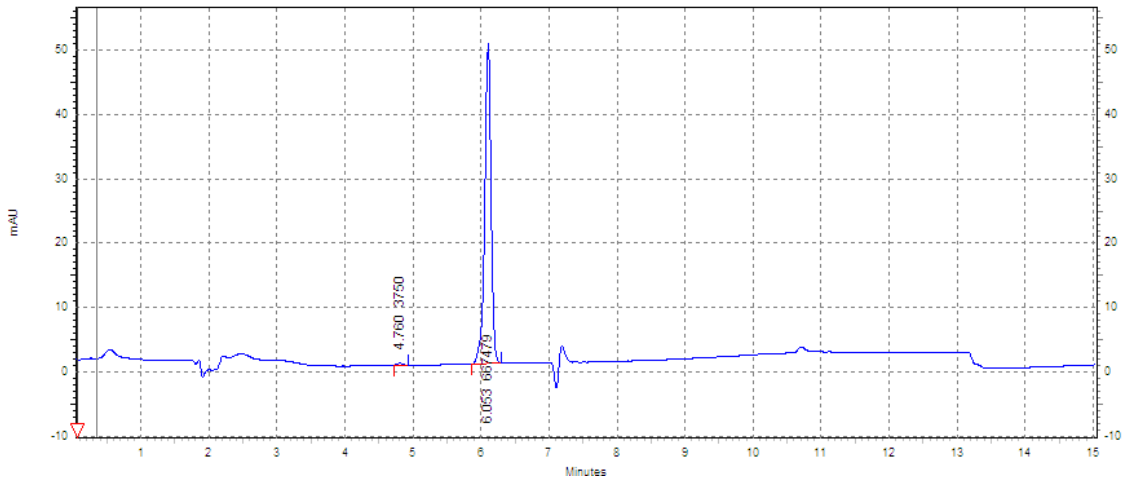


Figure S9f. HPLC-UV traces (λ = 325 nm) of **1f** after reaction with 20-fold excess of **2**-equatorial. Experiments were conducted in a 1:1 mixture of MeCN:PBS A) 0 minutes B) 40 minutes C) 24 hours or 100% $CHCl_3$ D) 0 minutes E) 40 minutes F) 24 hours at 37 °C.

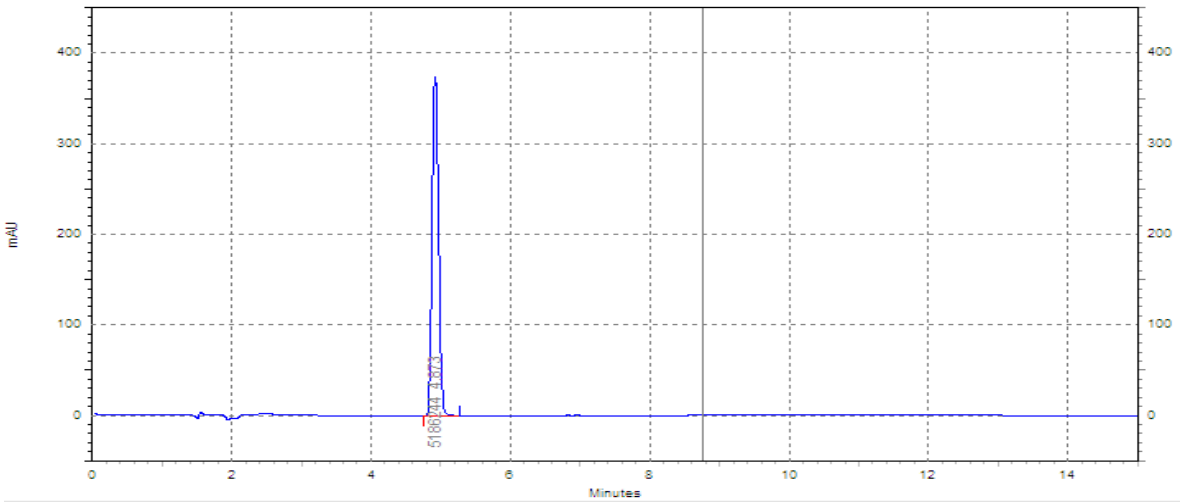


Figure S9g. HPLC-UV trace (λ = 325 nm) of 7-hydroxycoumarin (**1**) showing retention time t_R = 4.873 minutes.

4 Spectrofluorometry Release Experiments – Rate of Triazoline/Imine Degradation (Rates 2 and 3) and 1,6-/1,8-Self-immolation (Rate 4)

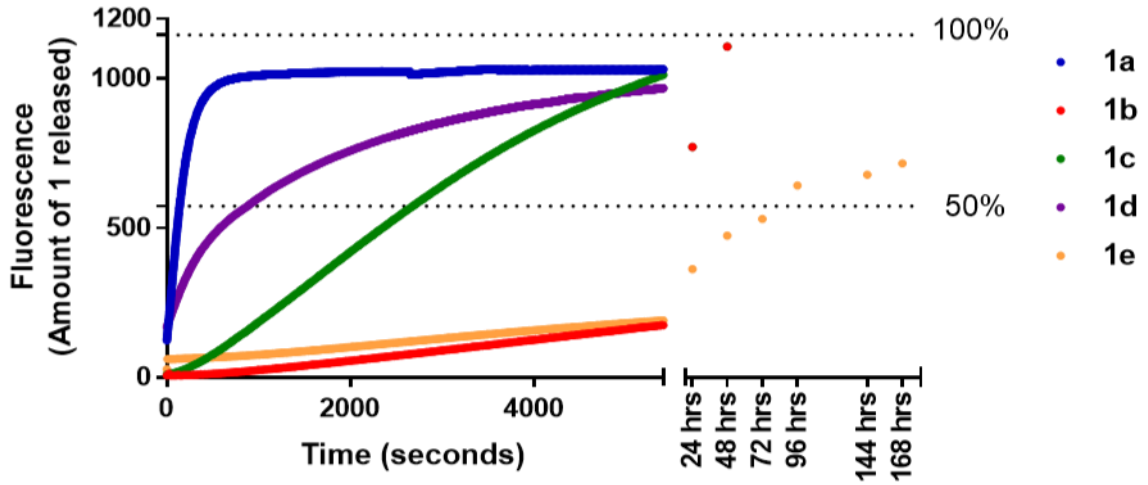


Figure S10. Averaged release of 7-hydroxycoumarin (**1**) from triazoline/imine corresponding to **1a**-**1e** following cycloaddition (rate 1) with **2**-equatorial monitored by spectrofluorometry (ex. 360, em. 455). Experiments run in triplicate (*n* = 3) from one NMR sample. Studies for **1a** and **1b** reported previously.[2]

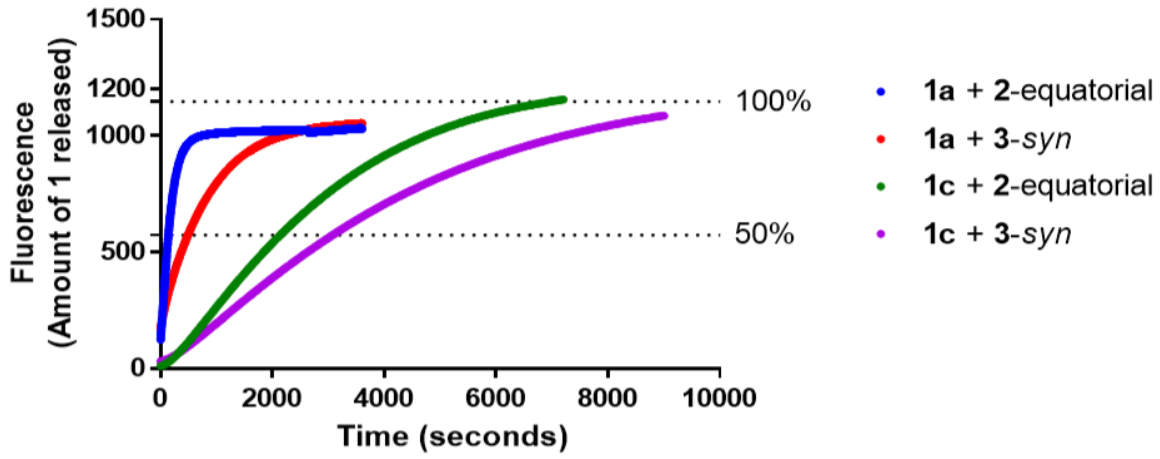


Figure S11. Averaged release of 7-hydroxycoumarin (**1**) from triazoline/imine corresponding to **1a** and **1c** following cycloaddition (rate 1) with **2**-equatorial or **3**-*syn* monitored by spectrofluorometry (ex. 360, em. 455). Experiments run in triplicate (*n* = 3) from one sample. Studies for **1a** + **2**-equatorial reported previously.[2]

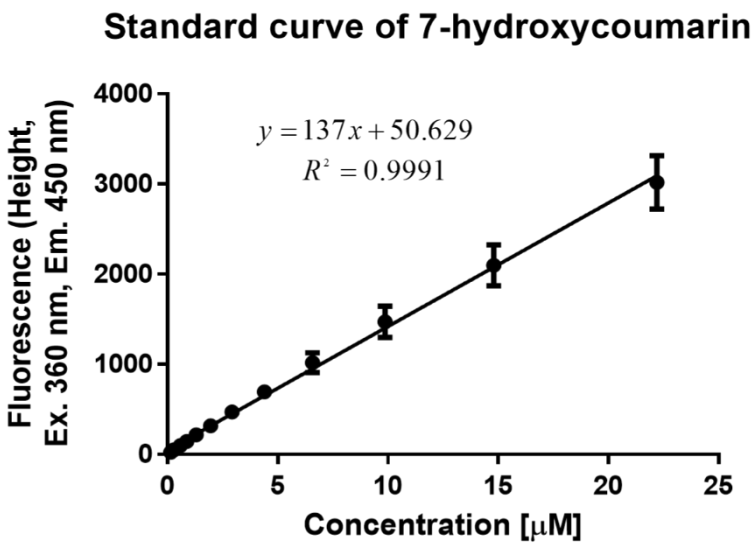


Figure S12 Standard curve of 7-hydroxycoumarin (**1**) measured at an Ex. of 360 nm and an Em. of 455 nm (height). Data shown are the mean ± SD (*n* = 3). Fluorescence expected at 100% release of drug from an 8 μM solution is 1146.63 units.

5 $^{1}H/^{19}F$ NMR Mechanistic Investigation

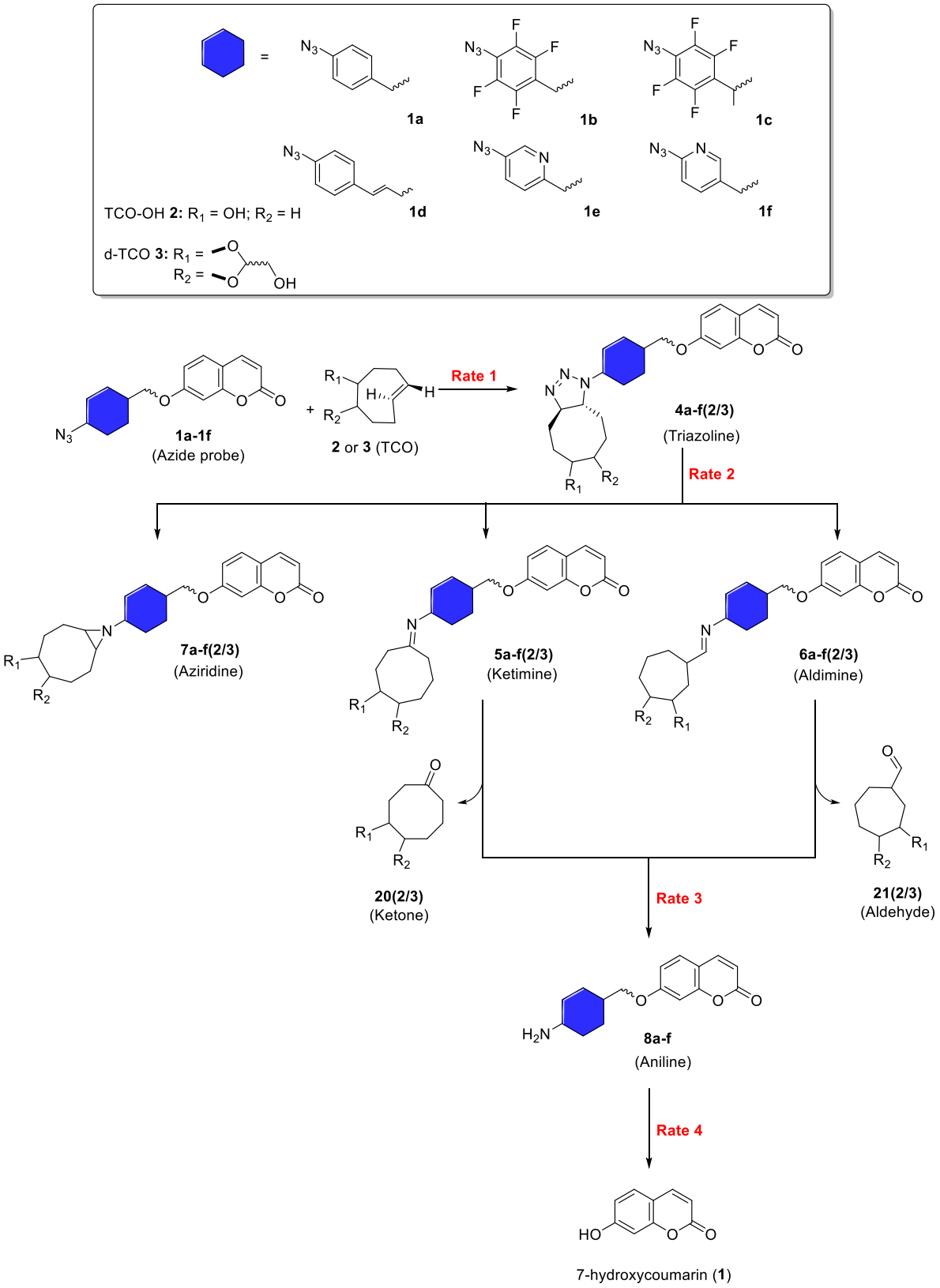


Figure S13 Possible product distribution for ether probes **1a-1f** after reaction with TCO (**2**-equatorial or **3**-*syn*). Products of the reaction were monitored by ^{1}H NMR and ^{19}F NMR (for **1b** and **1c** only).

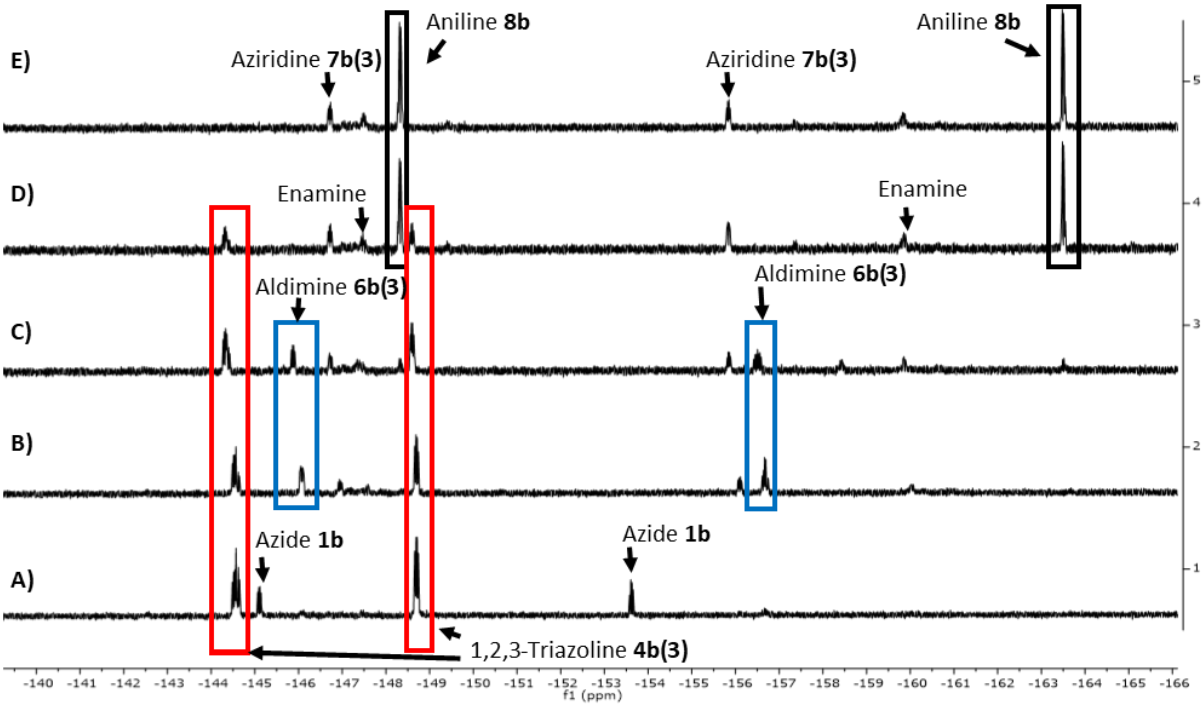


Figure S14 ^{19}F NMR product distribution experiments (containing internal standard: 1-fluoro-2,4-dinitrobenzene δ -109.41 ppm – not shown) for reaction of **3**-*syn* with **1b**: A) t = 5 min (CD_3CN); B) t = 72 h (CD_3CN); C) t = 5 min after adding 10% D_2O; D) t = 24 h post D_2O addition; E) t = 96 h post D_2O addition. (Note the slight change in chemical shift following D_2O addition observed due to change in solvent).

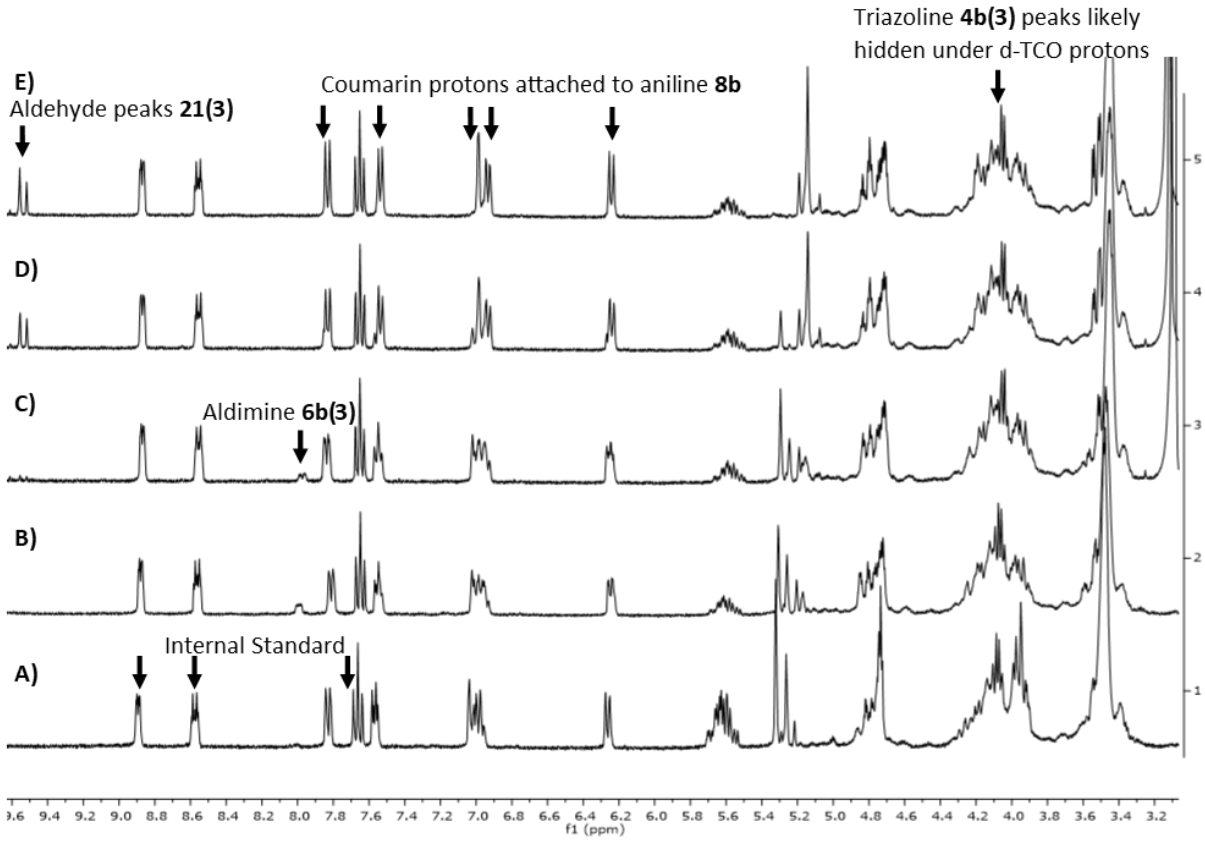


Figure S15 ^{1}H NMR product distribution experiments (containing internal standard: 1-fluoro-2,4-dinitrobenzene) for reaction of d-TCO **3**-*syn* with **1b**: A) t = 5 min (CD_3CN); B) t = 72 h (CD_3CN); C) t = 5 min after adding 10% D_2O; D) t = 24 h after adding 10% D_2O; E) t = 96 h after adding 10% D_2O.

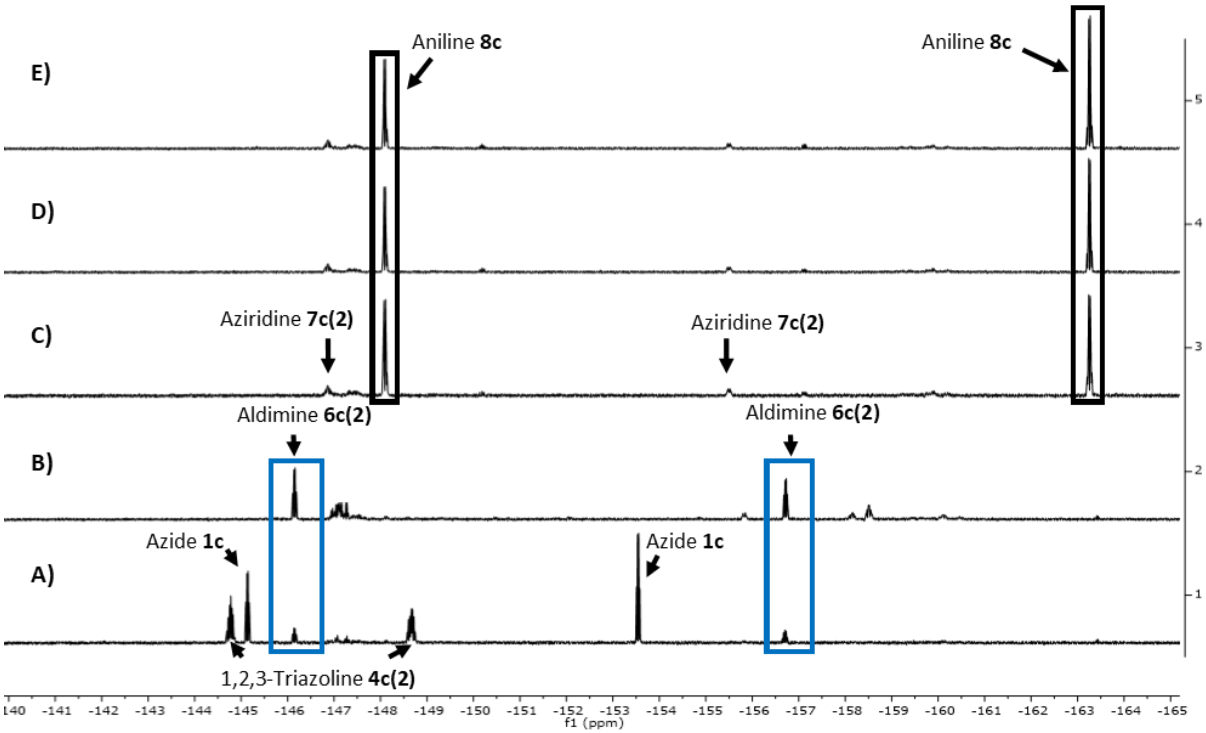


Figure S16 ^{19}F NMR product distribution experiments (containing internal standard: 1-fluoro-2,4-dinitrobenzene δ -109.41 ppm – not shown) for reaction of **2**-equatorial with **1c**: A) t = 5 min (CD_3CN); B) t = 72 h (CD_3CN); C) t = 5 min after adding 10% D_2O; D) t = 24 h post D_2O addition; E) t = 96 h post D_2O addition. (Note the slight change in chemical shift following D_2O addition observed due to change in solvent).

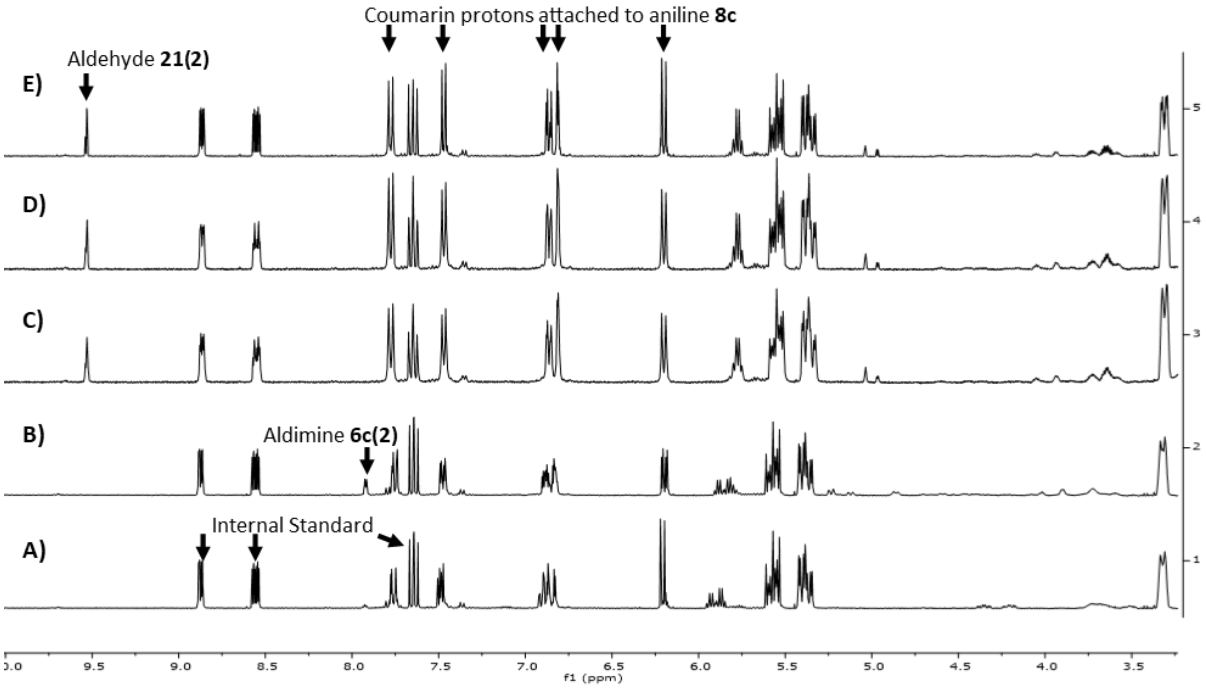


Figure S17 ^{1}H NMR product distribution experiments (containing internal standard: 1-fluoro-2,4-dinitrobenzene) for reaction of **2**-equatorial with **1c**: A) t = 5 min (CD_3CN); B) t = 72 h (CD_3CN); C) t = 5 min after adding 10% D_2O; D) t = 24 h after adding 10% D_2O; E) t = 96 h after adding 10% D_2O.

6 Combined Rates for [3+2]-Cycloaddition Click-and-Release (Rates 1 – 4) under 90% Aqueous Conditions

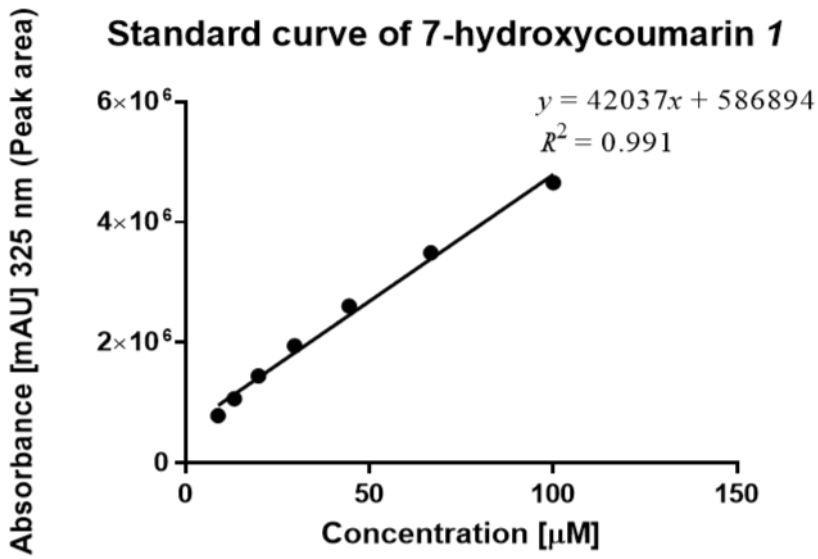
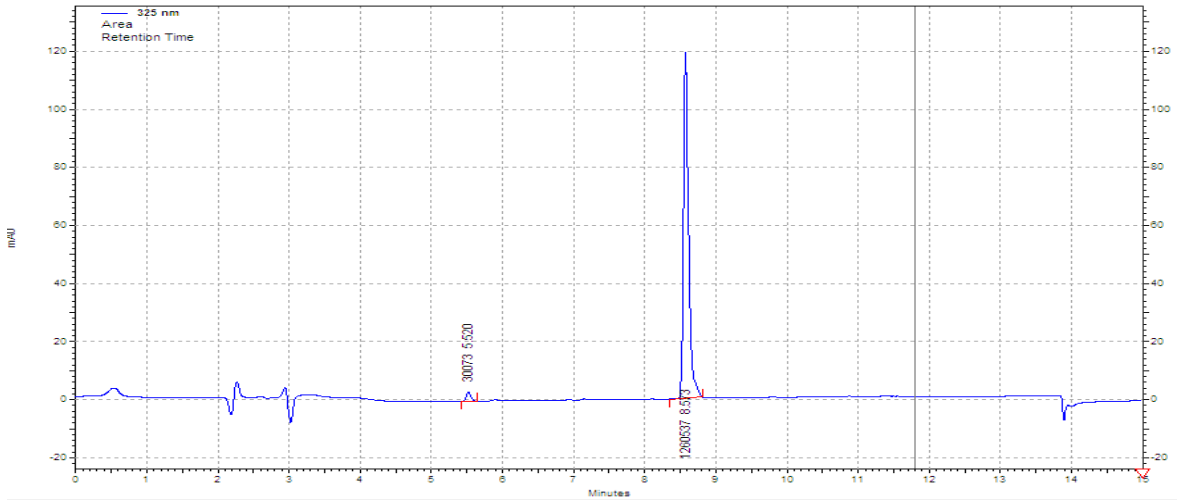


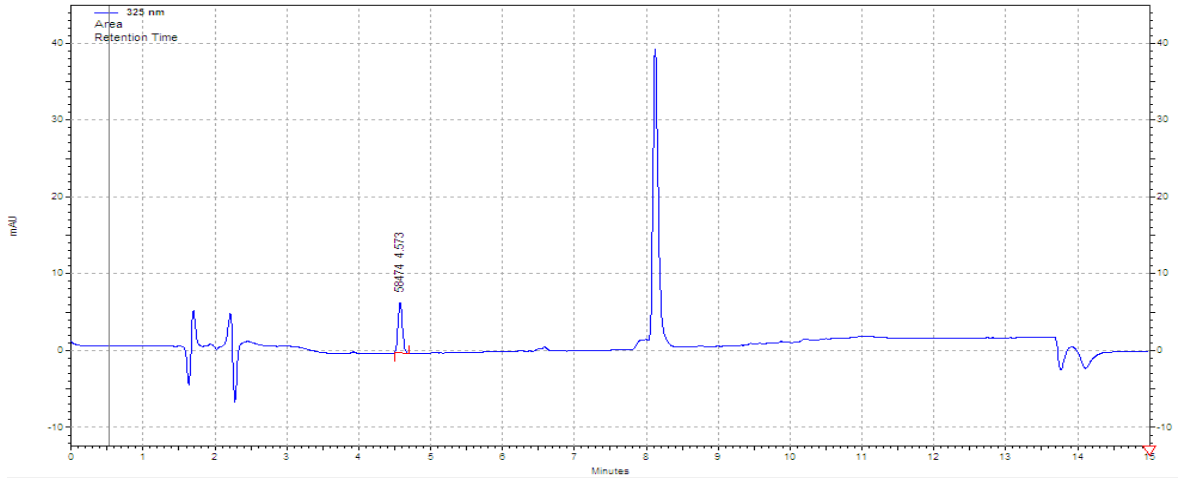
Figure S18 Standard curve of 7-hydroxycoumarin (**1**) measured (t_R = 4.87 minutes) in 1:9 MeCN:PBS, peak area measured at 325 nm. Data shown are the mean ± SD (*n* = 3).

6.1 HPLC Traces

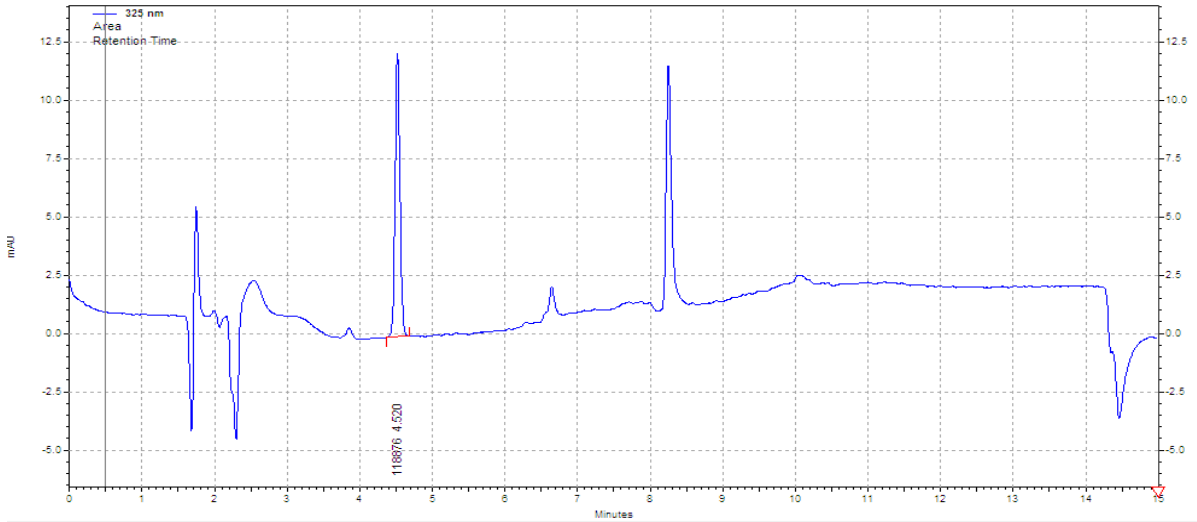
A) **1a** t = 0 hours



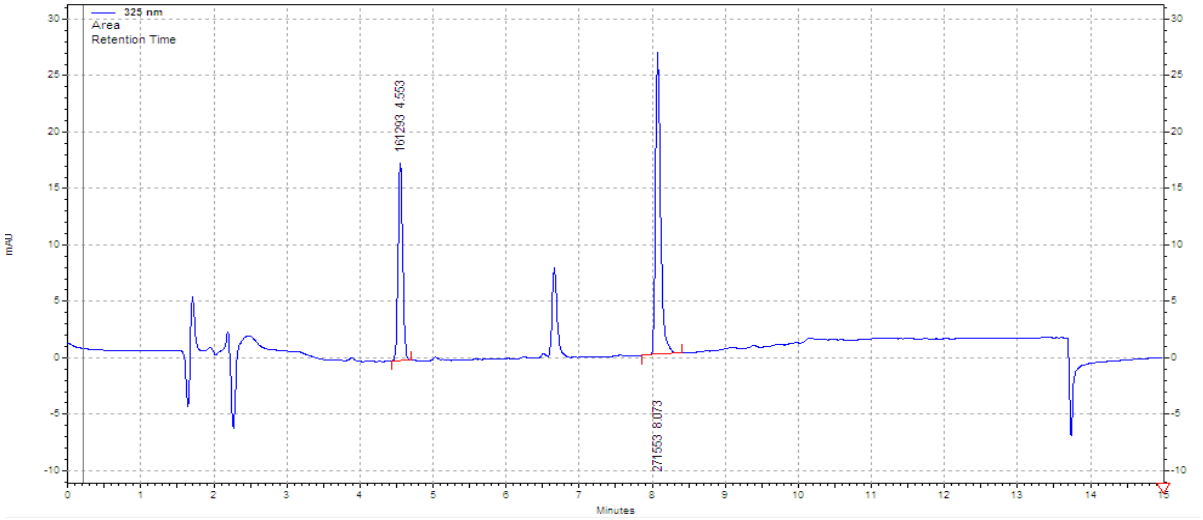
B) **1a** t = 24 hours post **2**-equatorial addition



C) **1a** t = 96 hours post **2**-equatorial addition



D) **1a** t = 24 hours post **3**-*syn* addition



E) **1a** t = 96 hours post **3**-*syn* addition

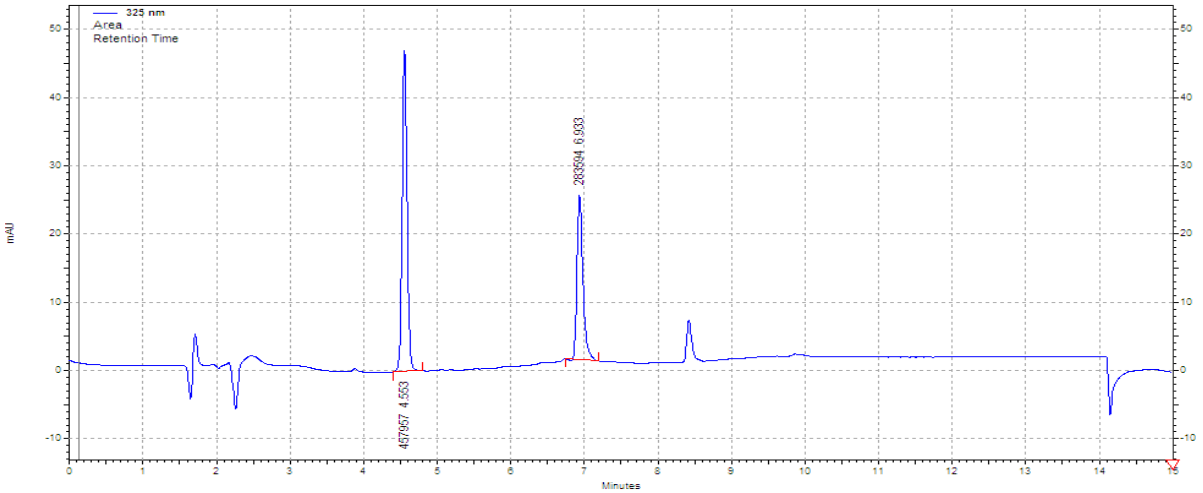
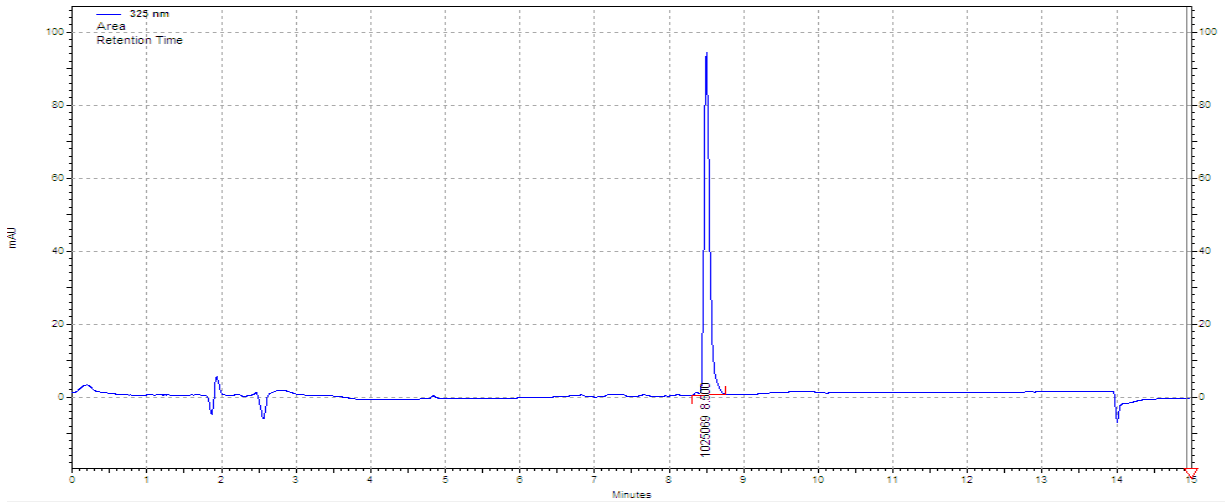
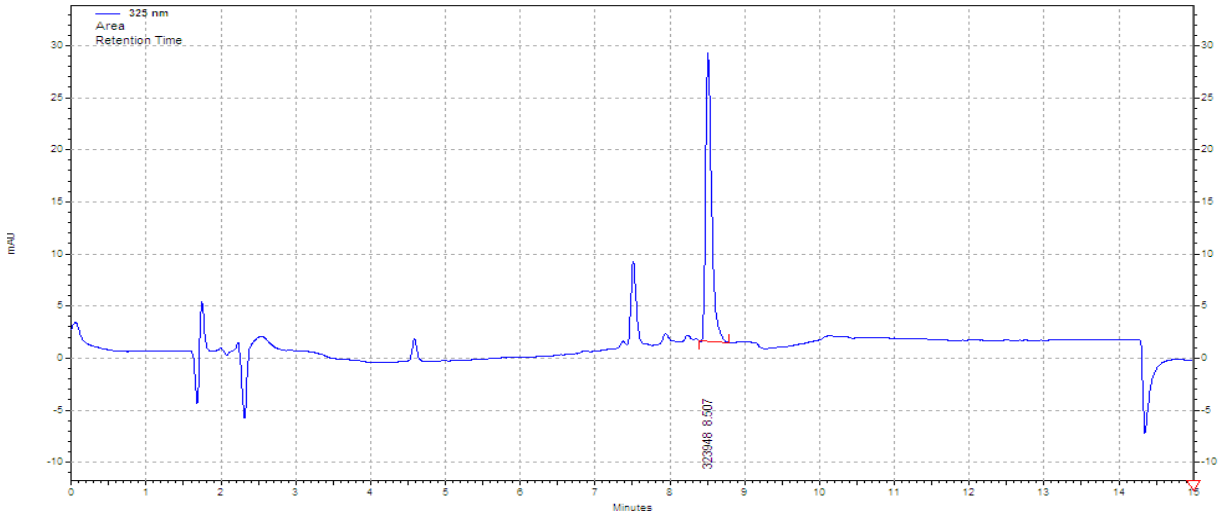


Figure S19a HPLC-UV traces (λ = 325 nm) of **1a** before reaction A) 0 hours; then after reaction with 10-fold excess of **2**-equatorial B) 24 hours C) 96 hours post TCO addition; or **3**-*syn* D) 24 hours E) 96 hours post TCO addition. Experiments were conducted in a 1:9 mixture of MeCN:PBS at 37 °C.

A) **1b** t = 0 hours



B) **1b** t = 24 hours post **2**-equatorial addition



C) **1b** t = 96 hours post **2**-equatorial addition

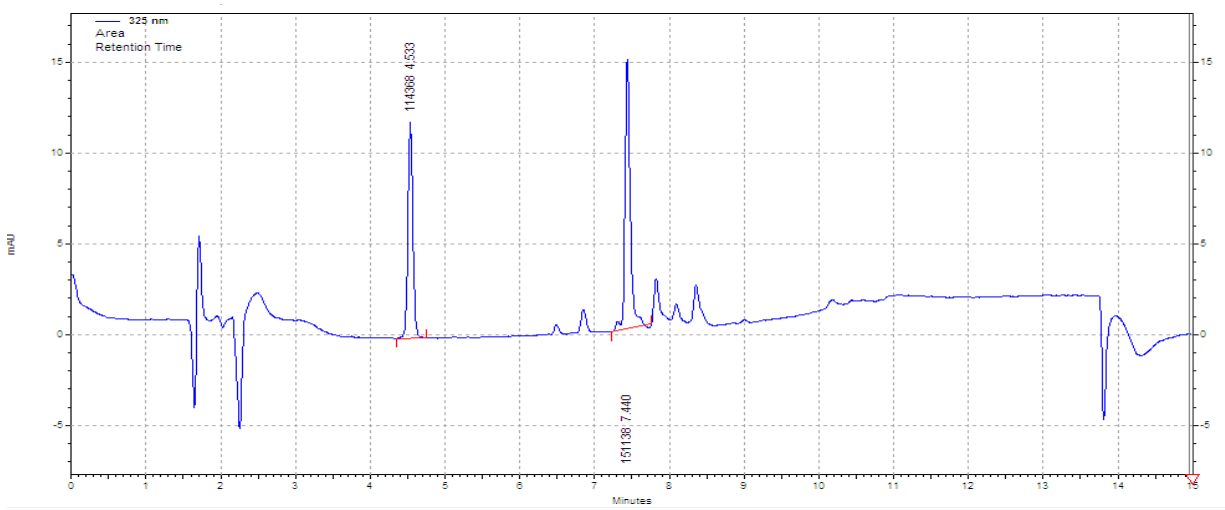
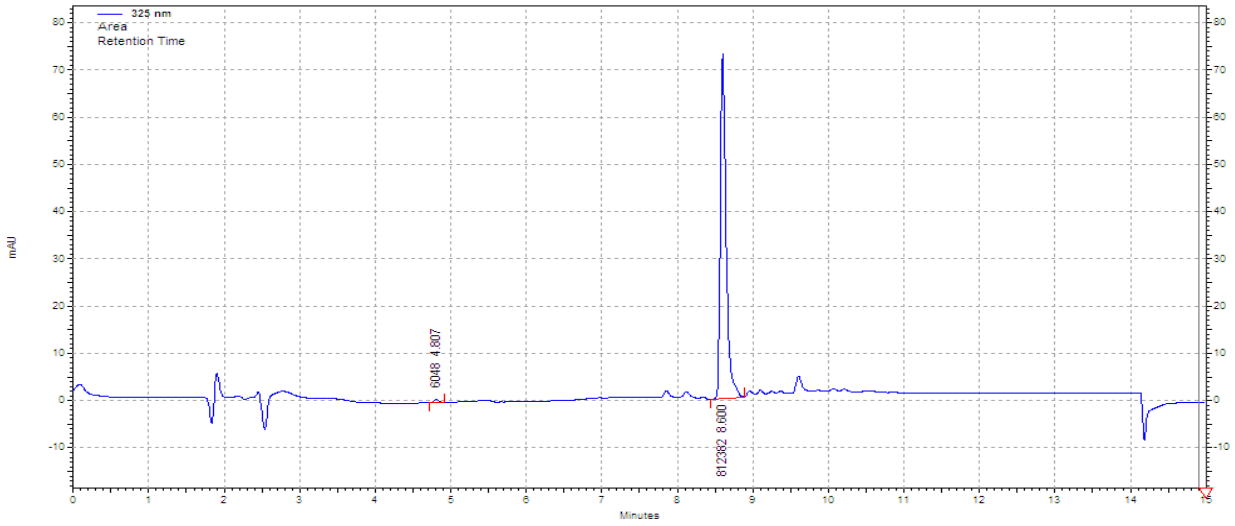
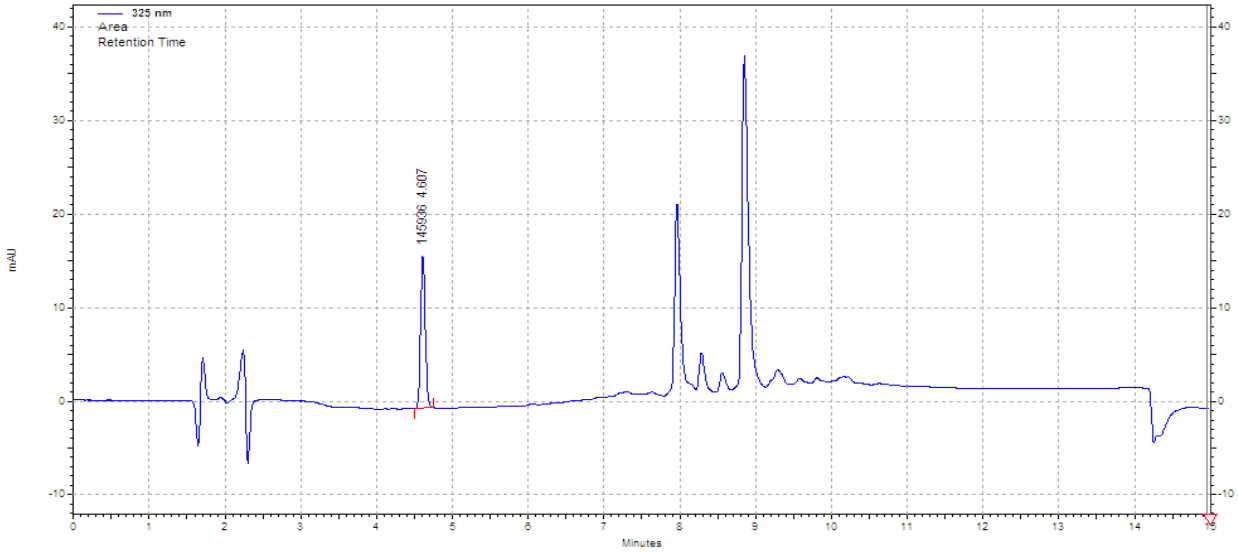


Figure S19b HPLC-UV traces (λ = 325 nm) of **1b** before reaction A) 0 hours; then after reaction with 10-fold excess of **2**-equatorial B) 24 hours C) 96 hours post TCO addition. Experiments were conducted in a 1:9 mixture of MeCN:PBS at 37 °C.

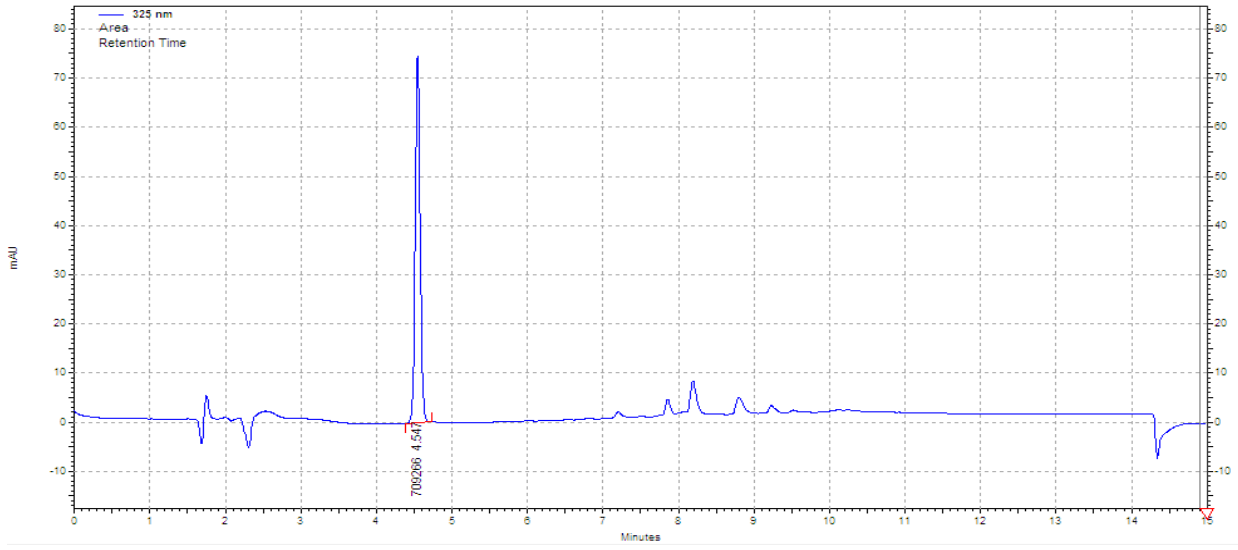
A) **1c** t = 0 hours



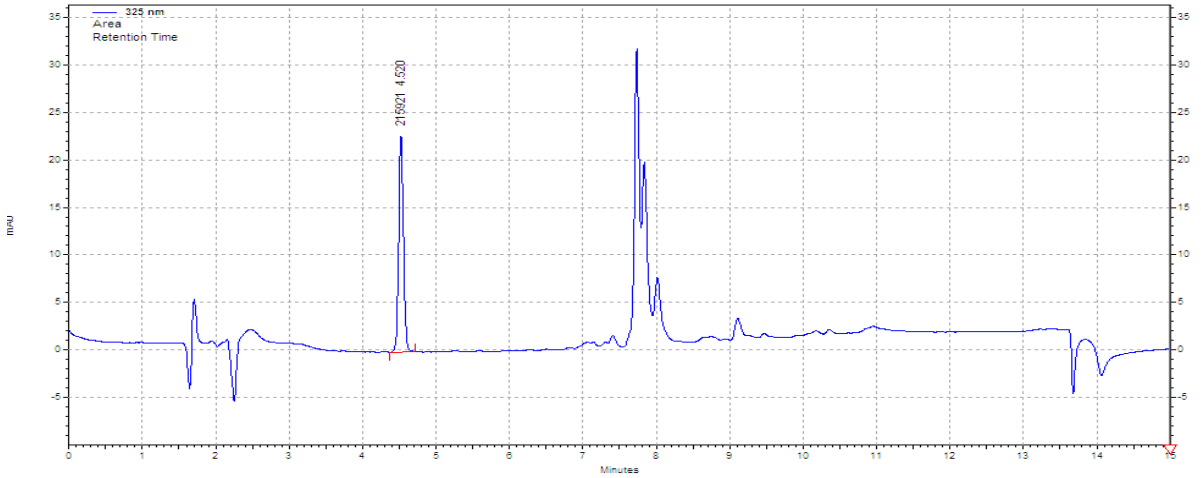
B) **1c** t = 4 hours post **2**-equatorial addition



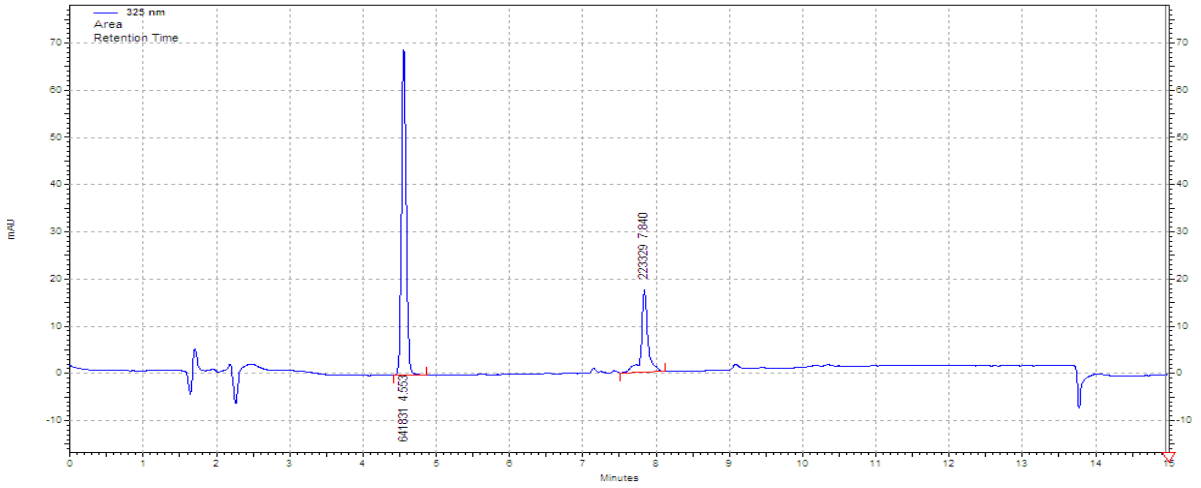
C) **1c** t = 24 hours post **2**-equatorial addition



D) **1c** t = 4 hours post **3**-*syn* addition



E) **1c** t = 24 hours post **3**-*syn* addition



F) **1c** t = 96 hours post **3**-*syn* addition

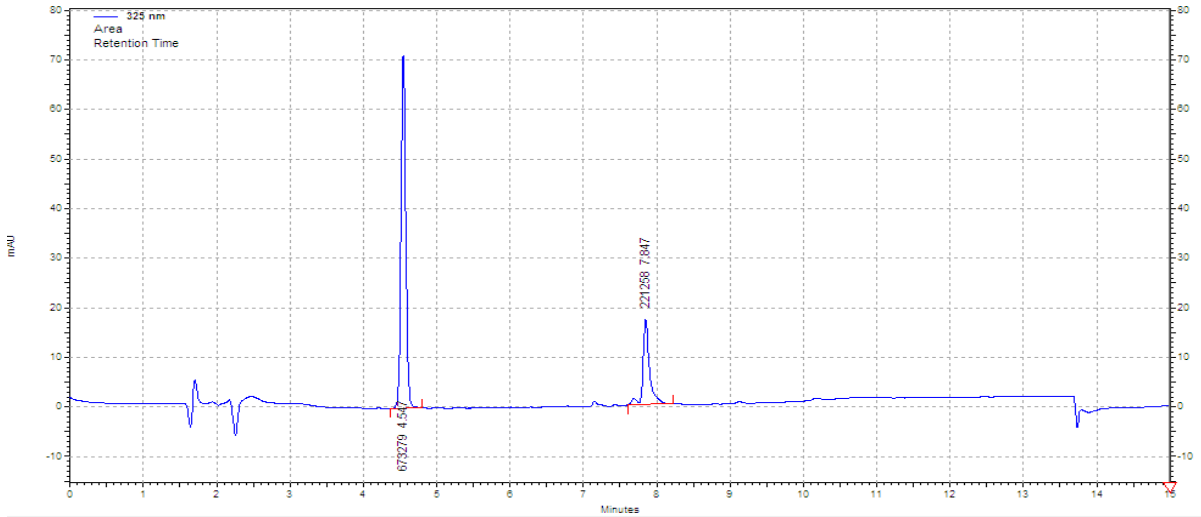
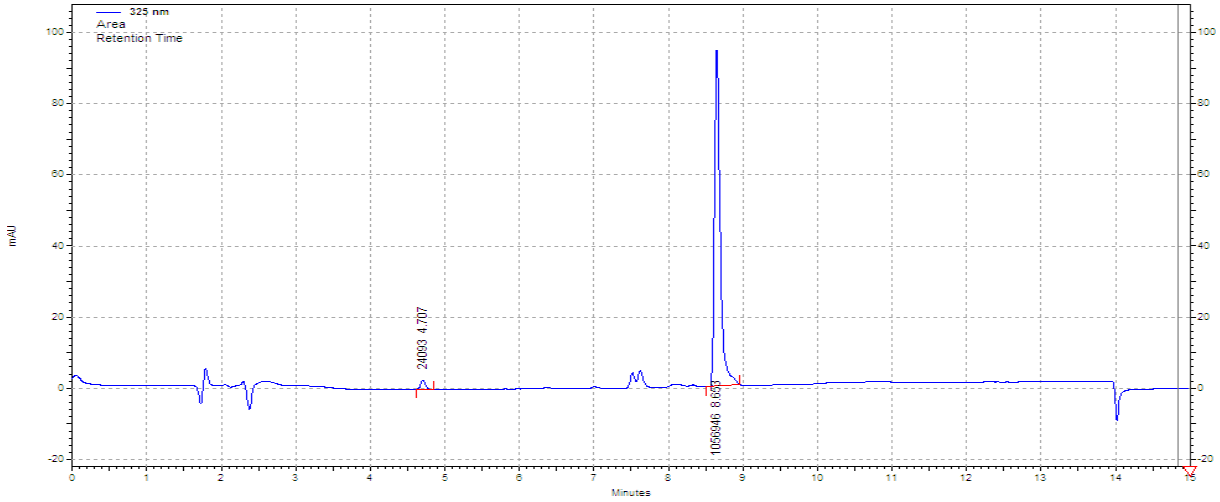
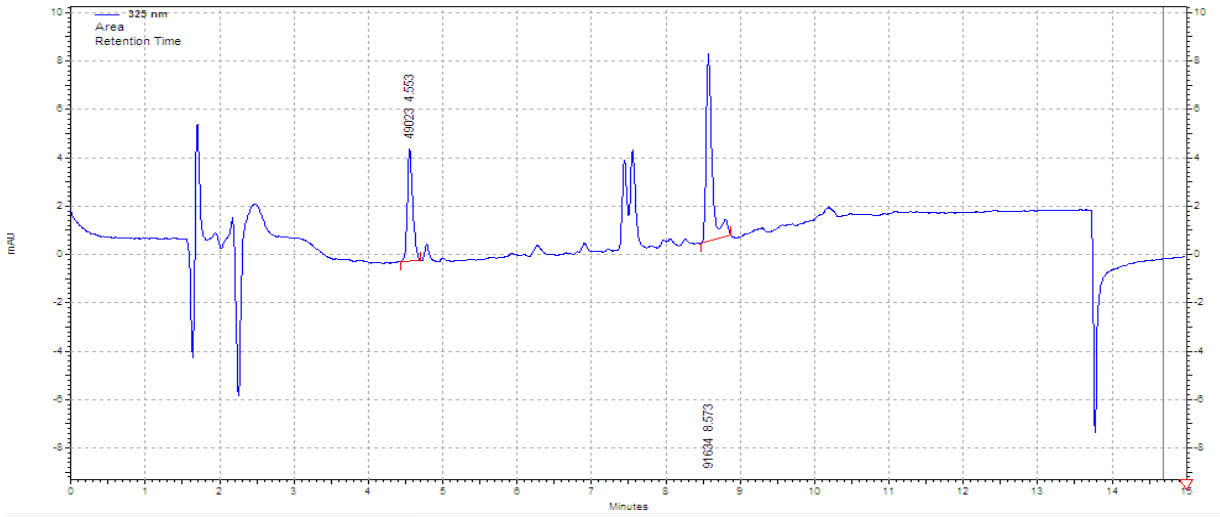


Figure S19c HPLC-UV traces (λ = 325 nm) of **1c** before reaction A) 0 hours; then after reaction with 10-fold excess of **2**-equatorial B) 24 hours C) 96 hours post TCO addition; or **3**-*syn* D) 24 hours E) 96 hours post TCO addition. Experiments were conducted in a 1:9 mixture of MeCN:PBS at 37 °C.

A) **1d** t = 0 hours



B) **1d** t = 24 hours post **2**-equatorial addition



C) **1d** t = 24 hours post **2**-equatorial addition

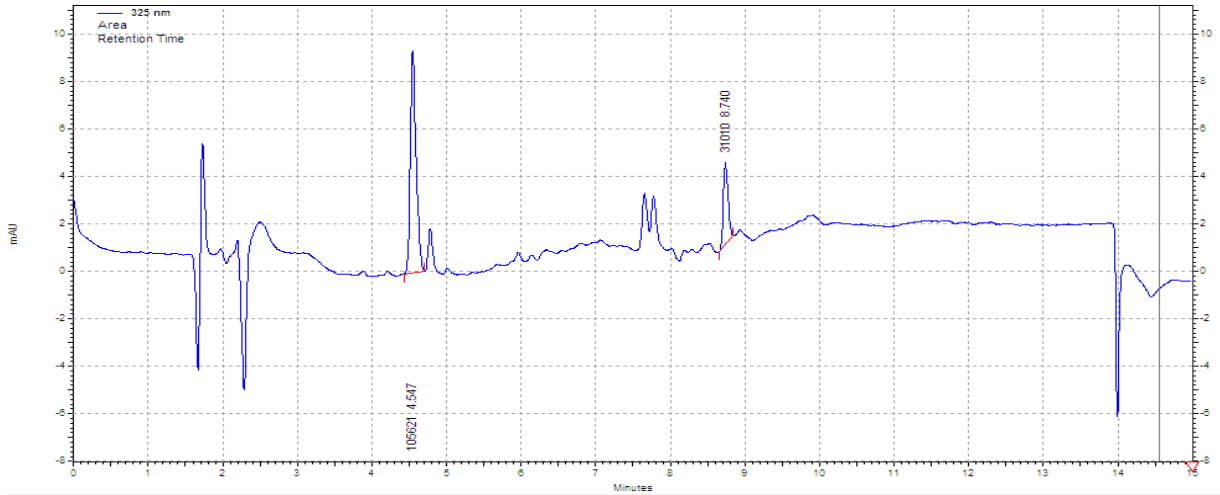


Figure S19d HPLC-UV traces (λ = 325 nm) of **1d** before reaction A) 0 hours; then after reaction with 10-fold excess of **2**-equatorial B) 24 hours C) 96 hours post TCO addition. Experiments were conducted in a 1:9 mixture of MeCN:PBS at 37 °C.

7 Final Compound NMR Spectra

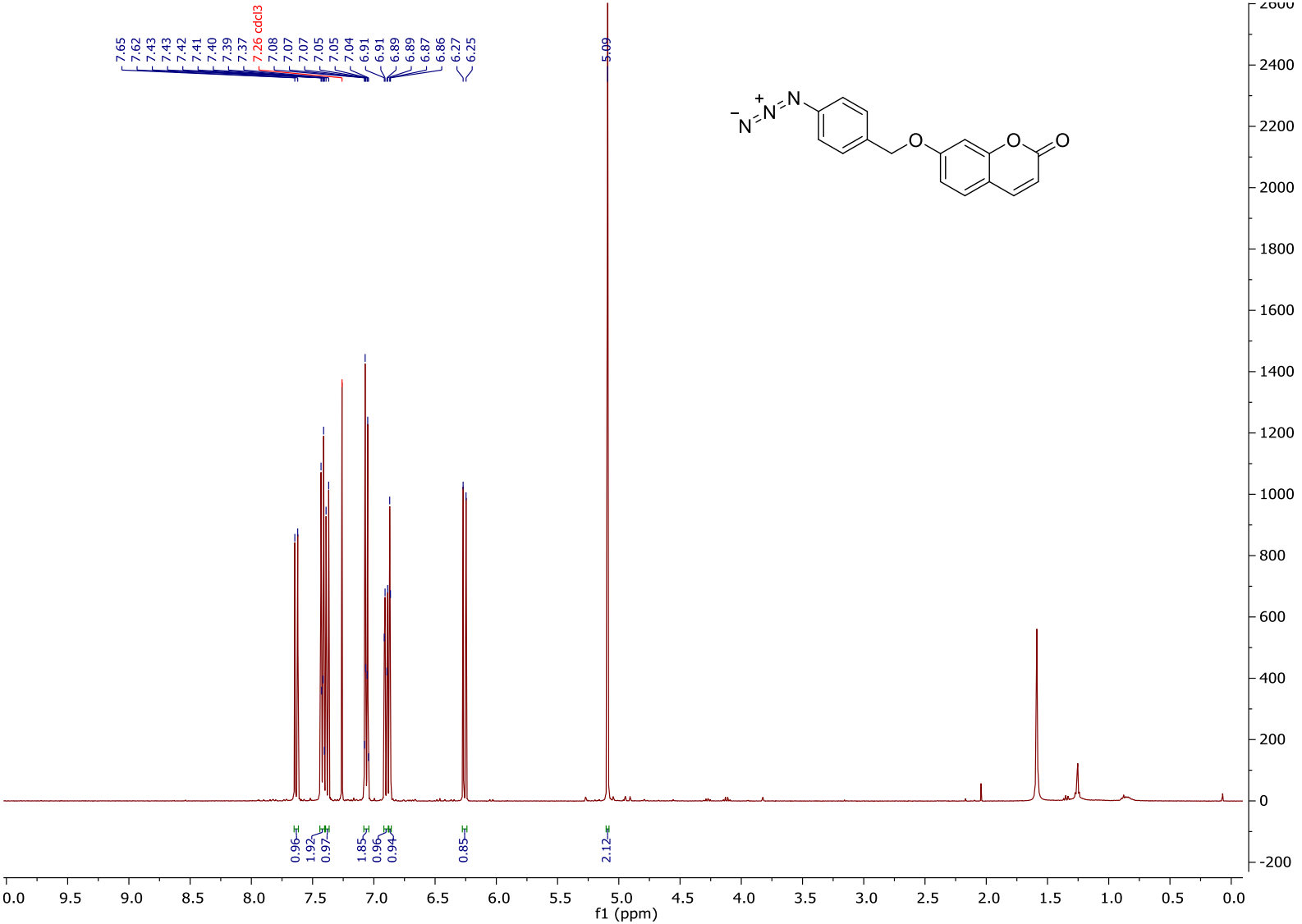


Figure S20 [1]H NMR Spectra of 7-[(4-azidobenzyl)oxy]-coumarin **1a**.[2]

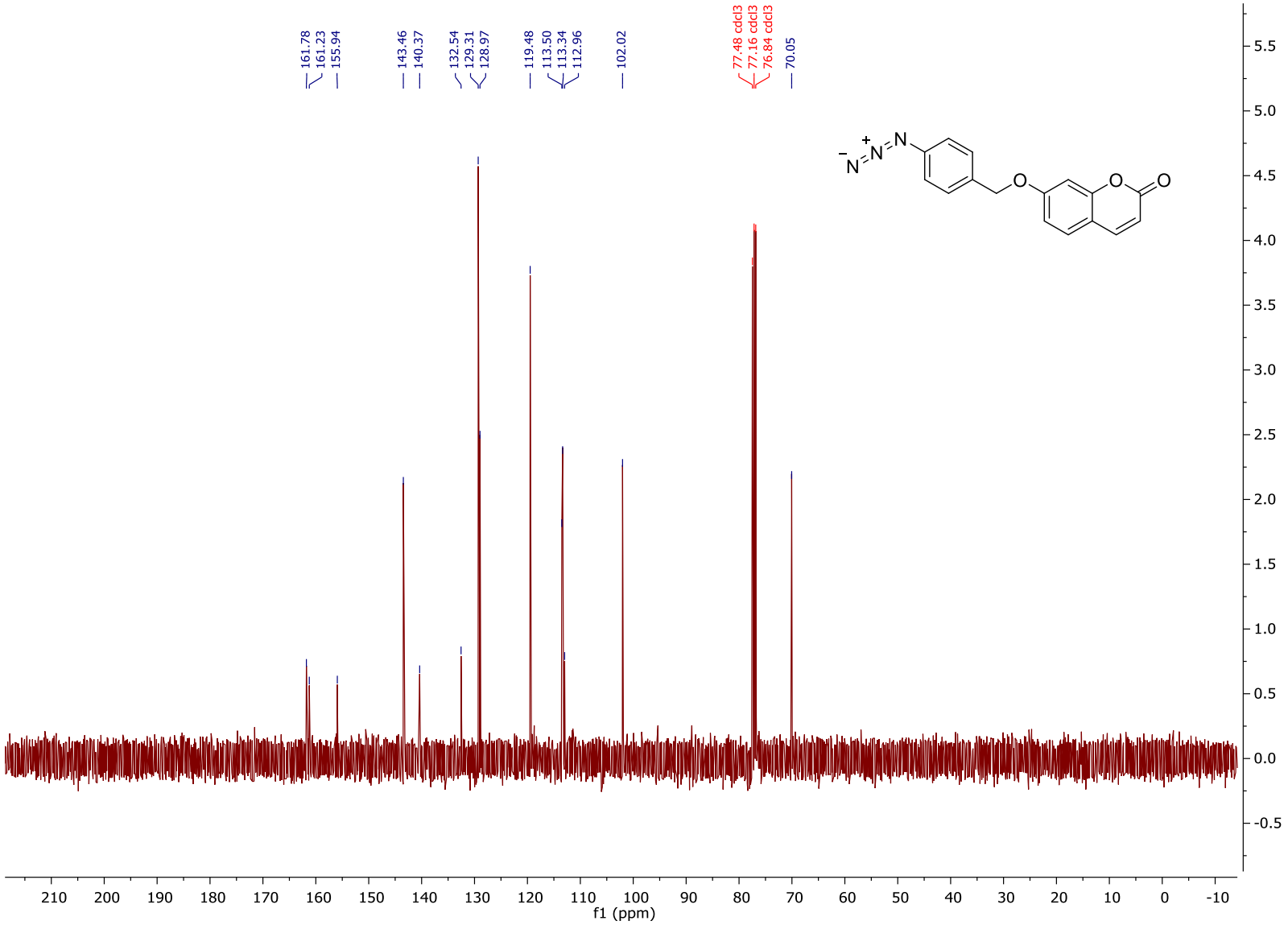


Figure S21 ^{13}C NMR Spectra of 7-[(4-azidobenzyl)oxy]-coumarin **1a**.[2]

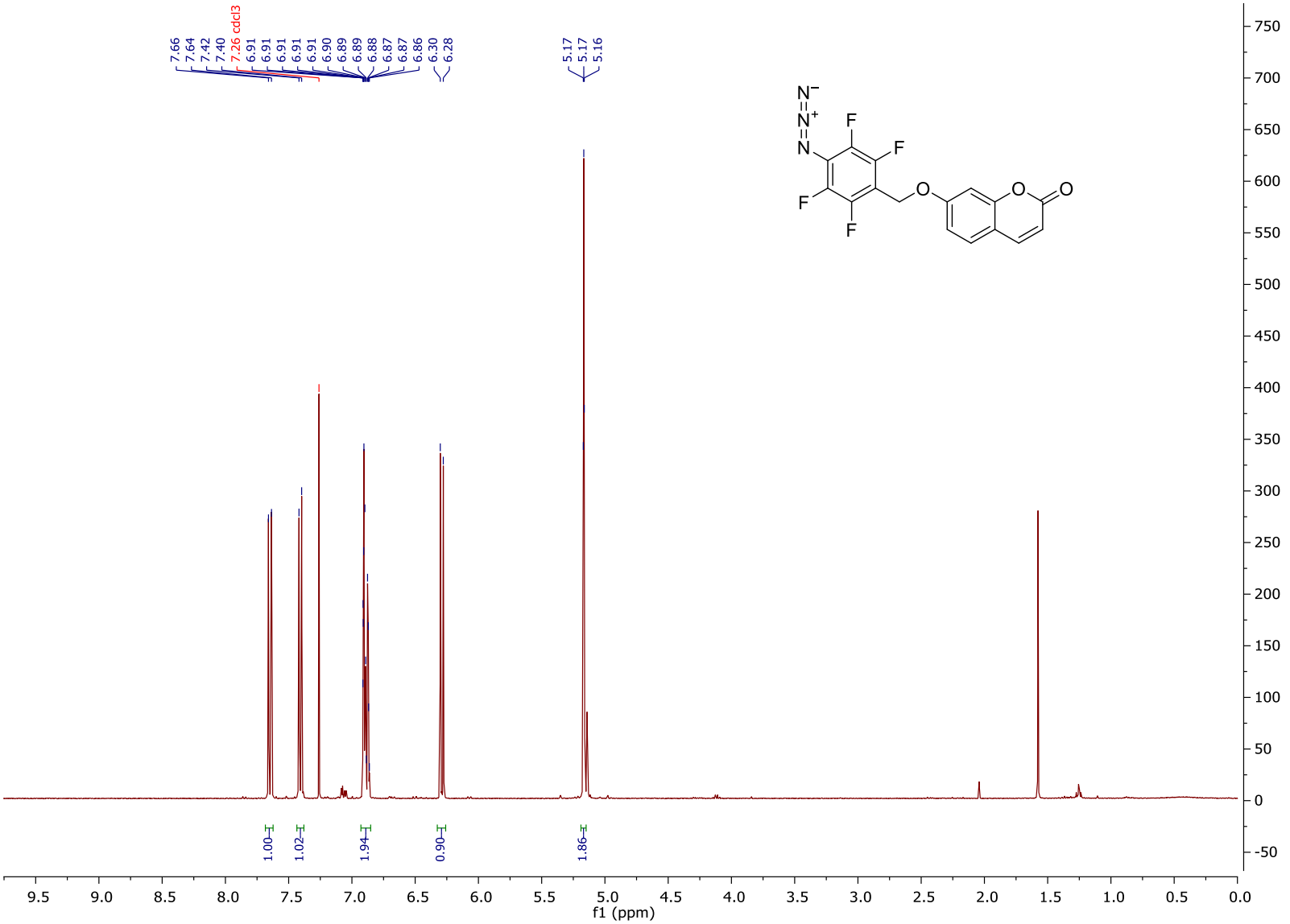


Figure S22 [1]H NMR Spectra of 7-[(4-azido-2,3,5,6-tetrafluorobenzyl)oxy]-coumarin **1b**.[2]

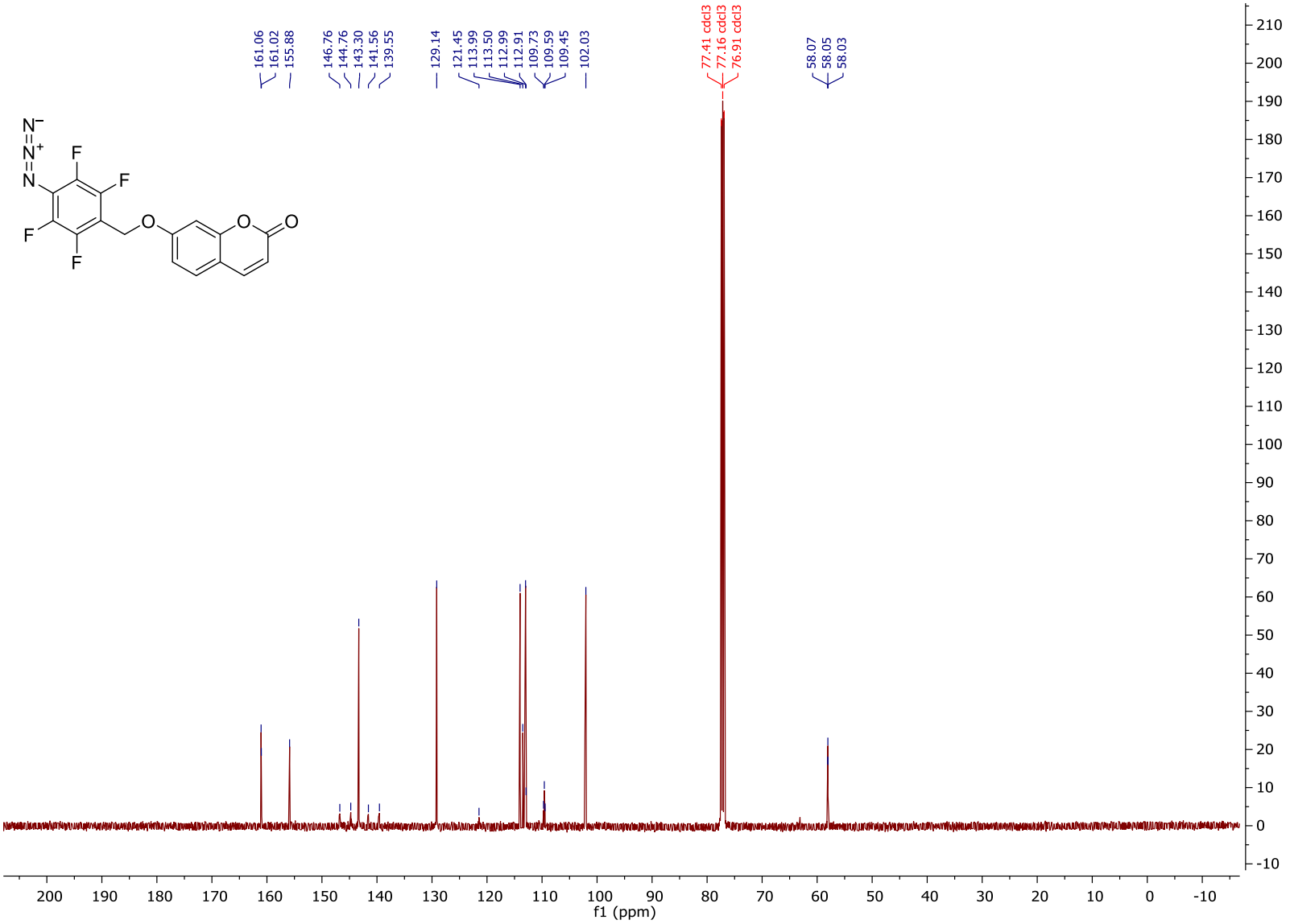


Figure S23 ^{13}C NMR Spectra of 7-[(4-azido-2,3,5,6-tetrafluorobenzyl)oxy]-coumarin **1b**.[2]

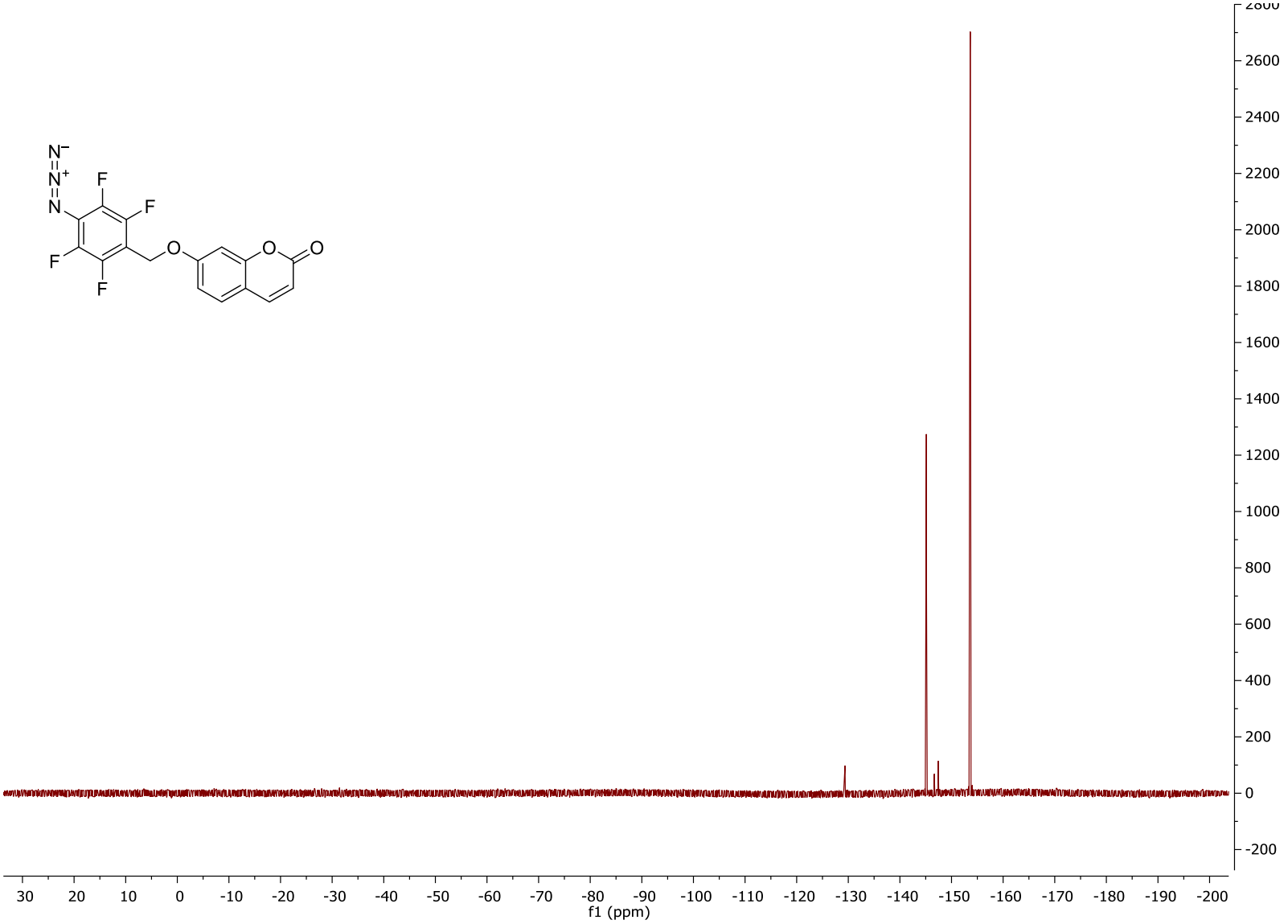


Figure S24 ^{19}F NMR Spectra of 7-[(4-azido-2,3,5,6-tetrafluorobenzyl)oxy]-coumarin **1b**.[2]

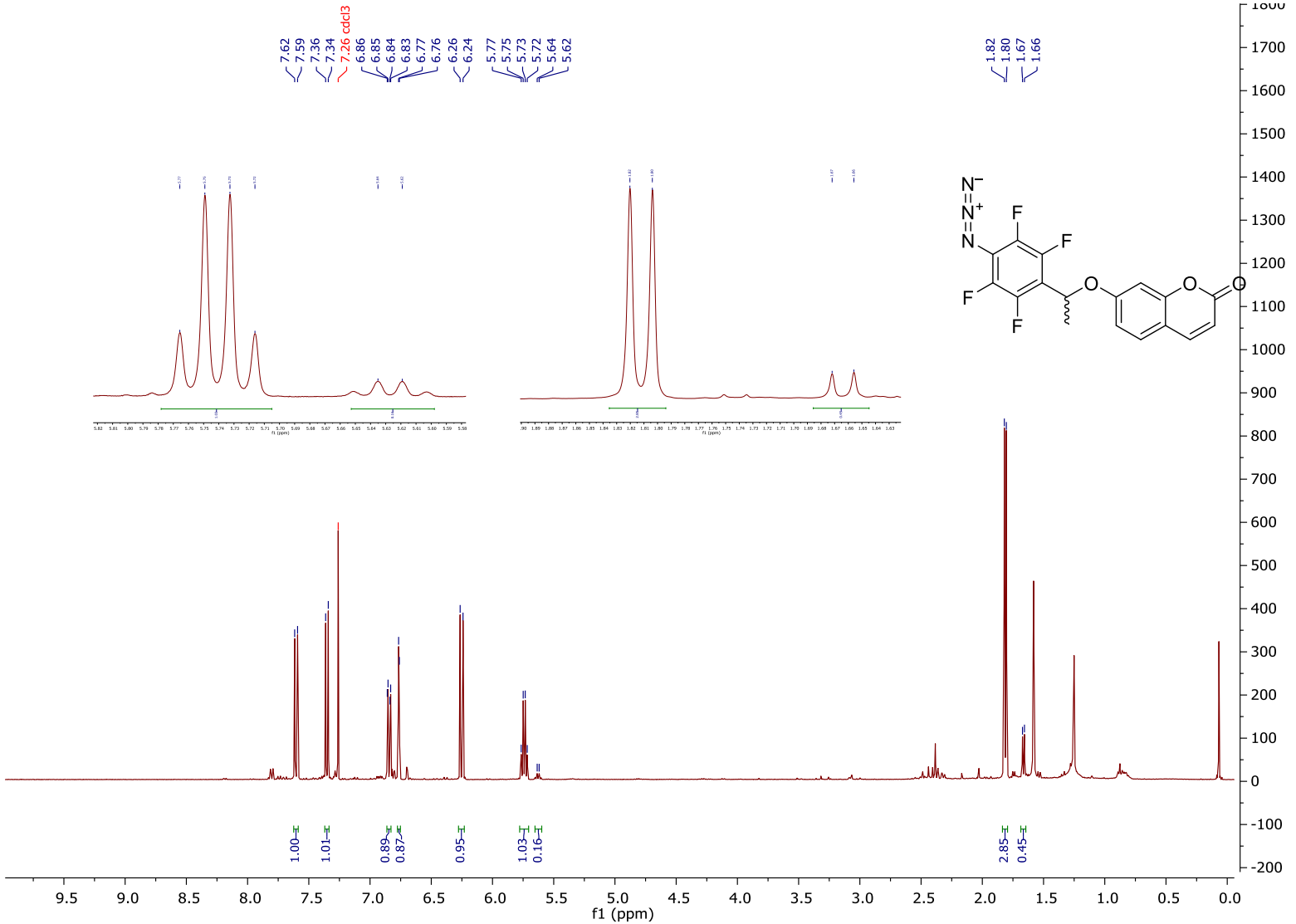


Figure S25 [1]H NMR Spectra of 7-[(4-azido-2,3,5,6-tetrafluorobenzyl)ethoxy]-coumarin **1c**.

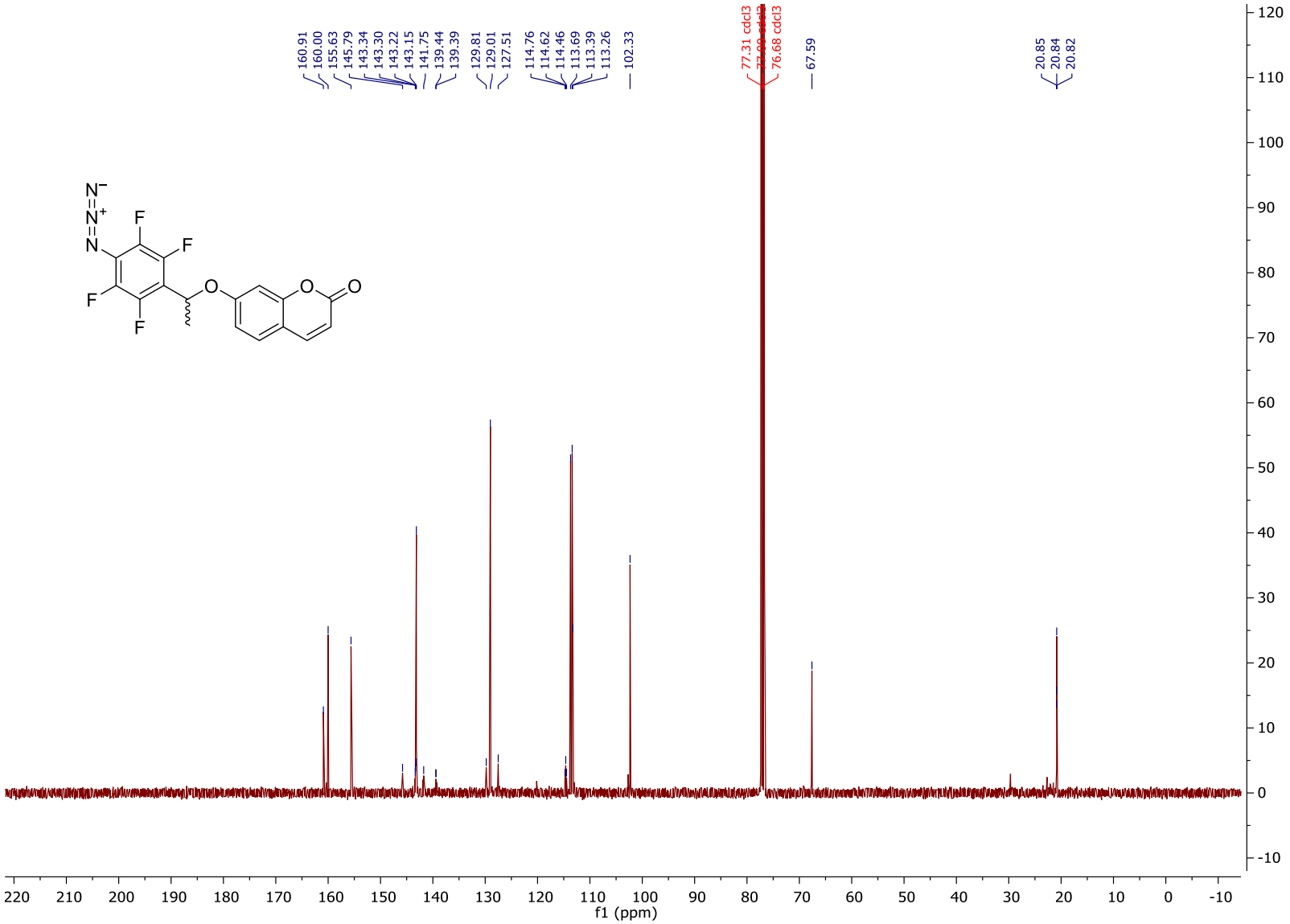


Figure S26 ^{13}C NMR Spectra of 7-[(4-azido-2,3,5,6-tetrafluorobenzyl)ethoxy]-coumarin **1c**.

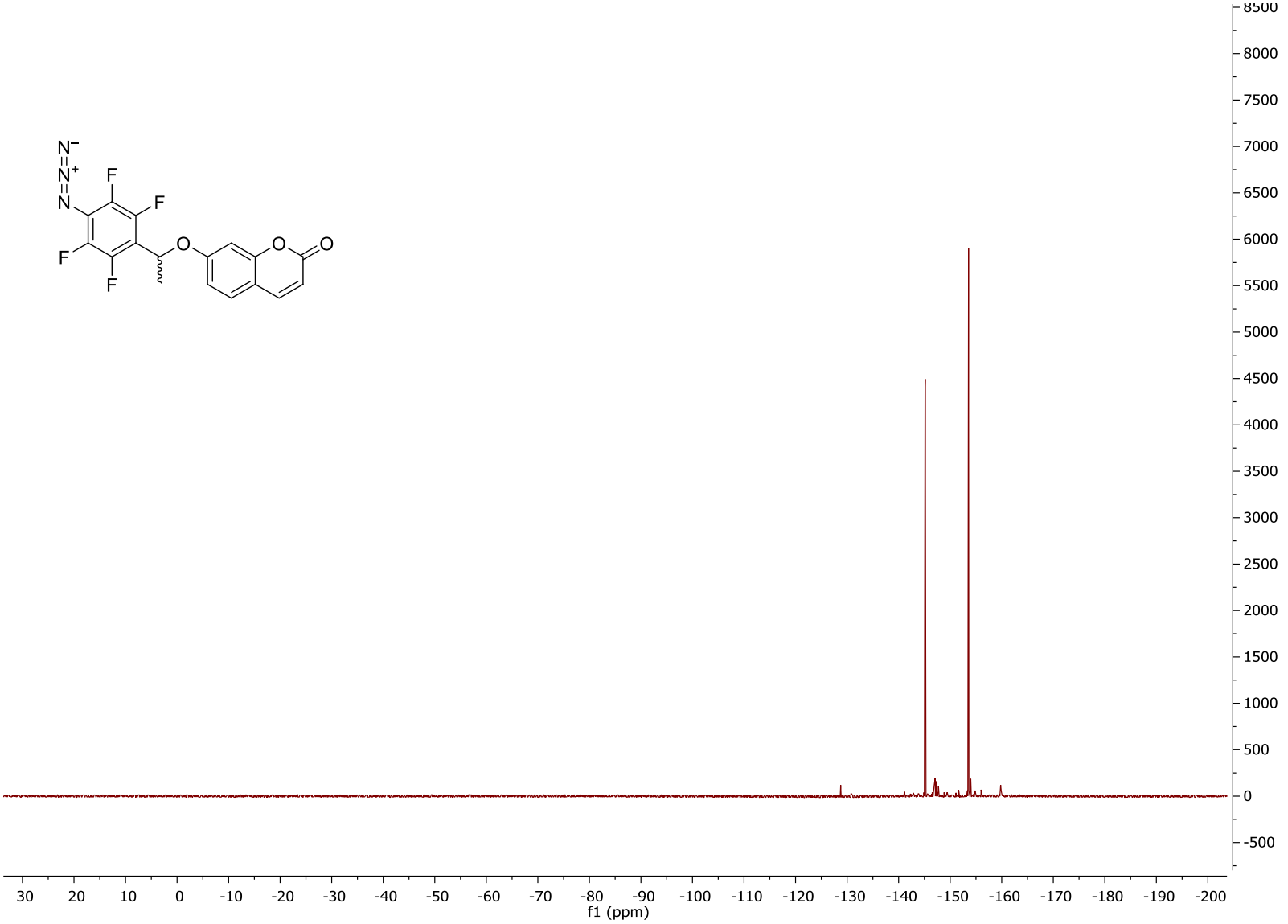


Figure S27 ^{19}F NMR Spectra of 7-[(4-azido-2,3,5,6-tetrafluorobenzyl)ethoxy]-coumarin **1c**.

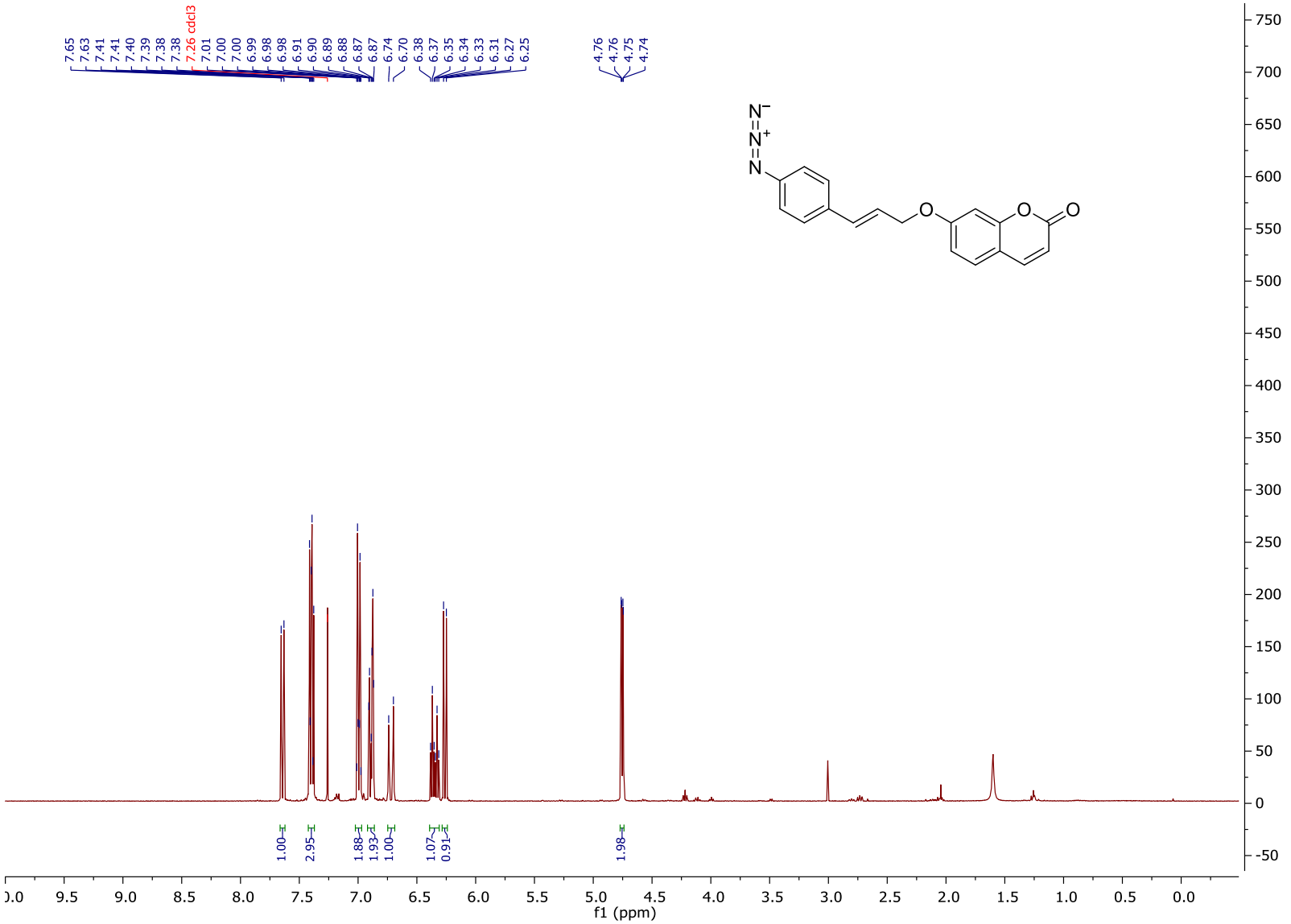


Figure S28 ^{1}H NMR Spectra of 7-[(*trans*-4-azidocinnamyl)oxy]-coumarin **1d**.

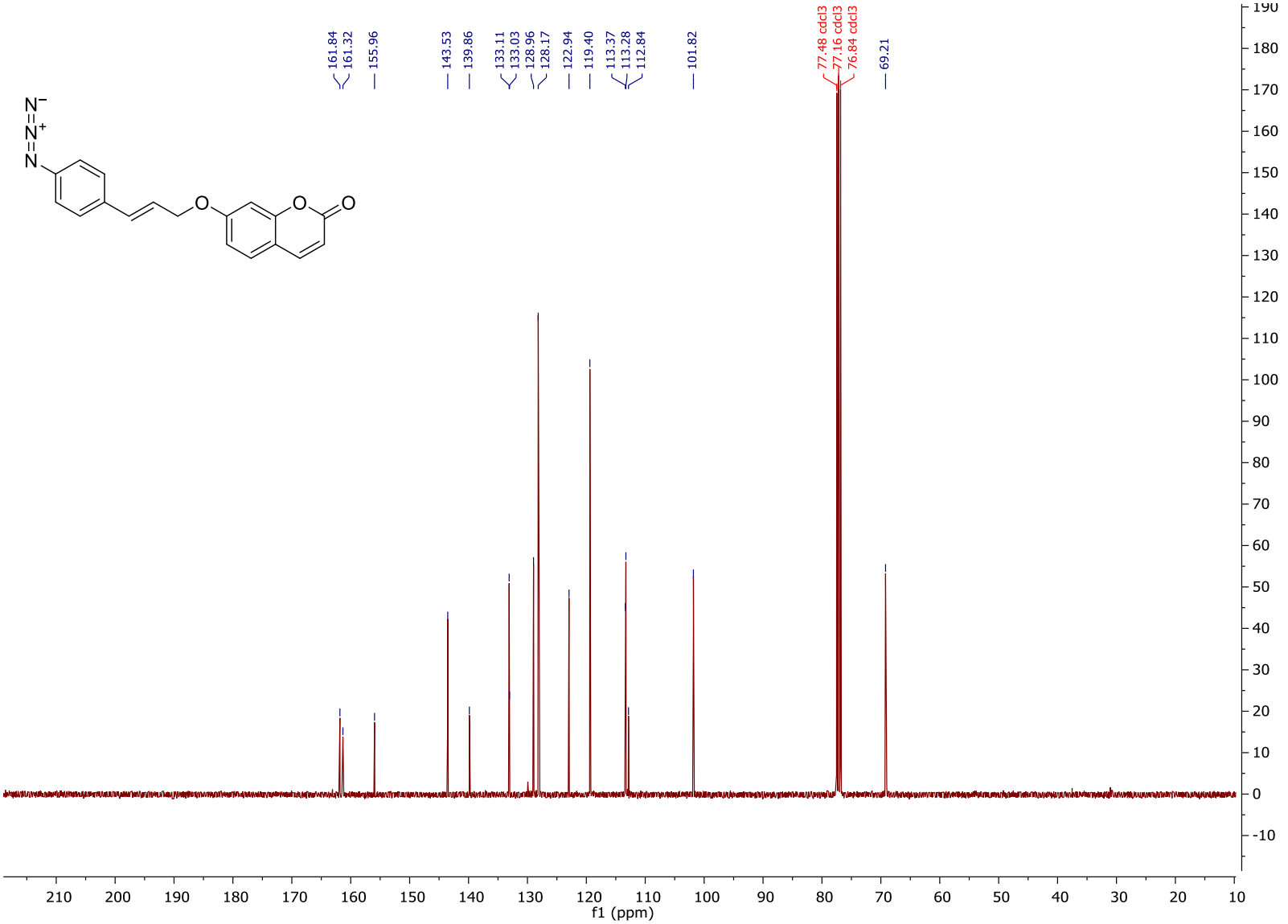


Figure S29 ^{13}C NMR Spectra of 7-[(*trans*-4-azidocinnamyl)oxy]-coumarin **1d**.

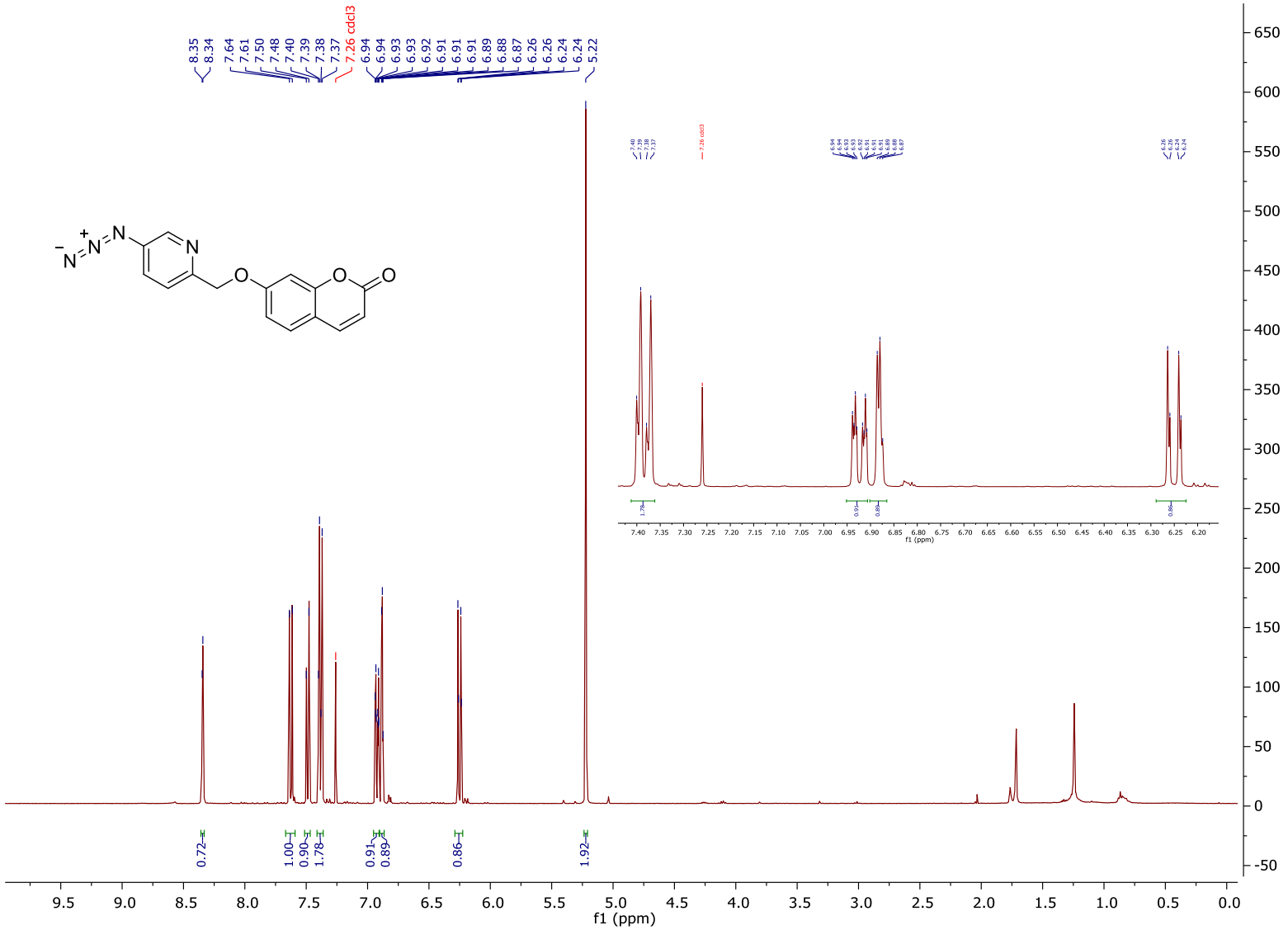


Figure S30 ^{1}H NMR Spectra of 7-[(5-Azidopyridin-2-yl)methoxy]-coumarin **1e**.

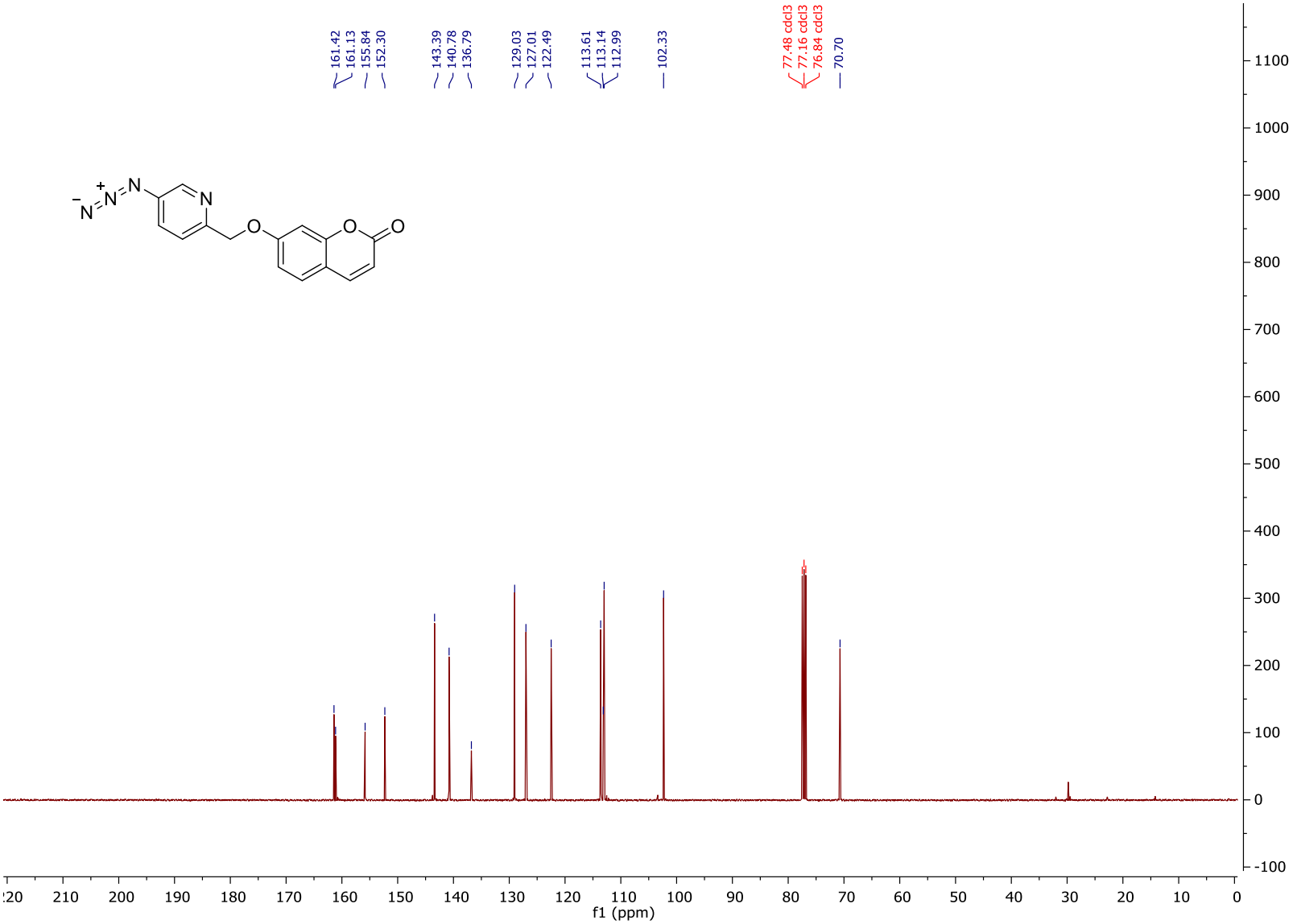


Figure S31 ^{13}C NMR Spectra of 7-[(5-Azidopyridin-2-yl)methoxy]-coumarin **1e**.

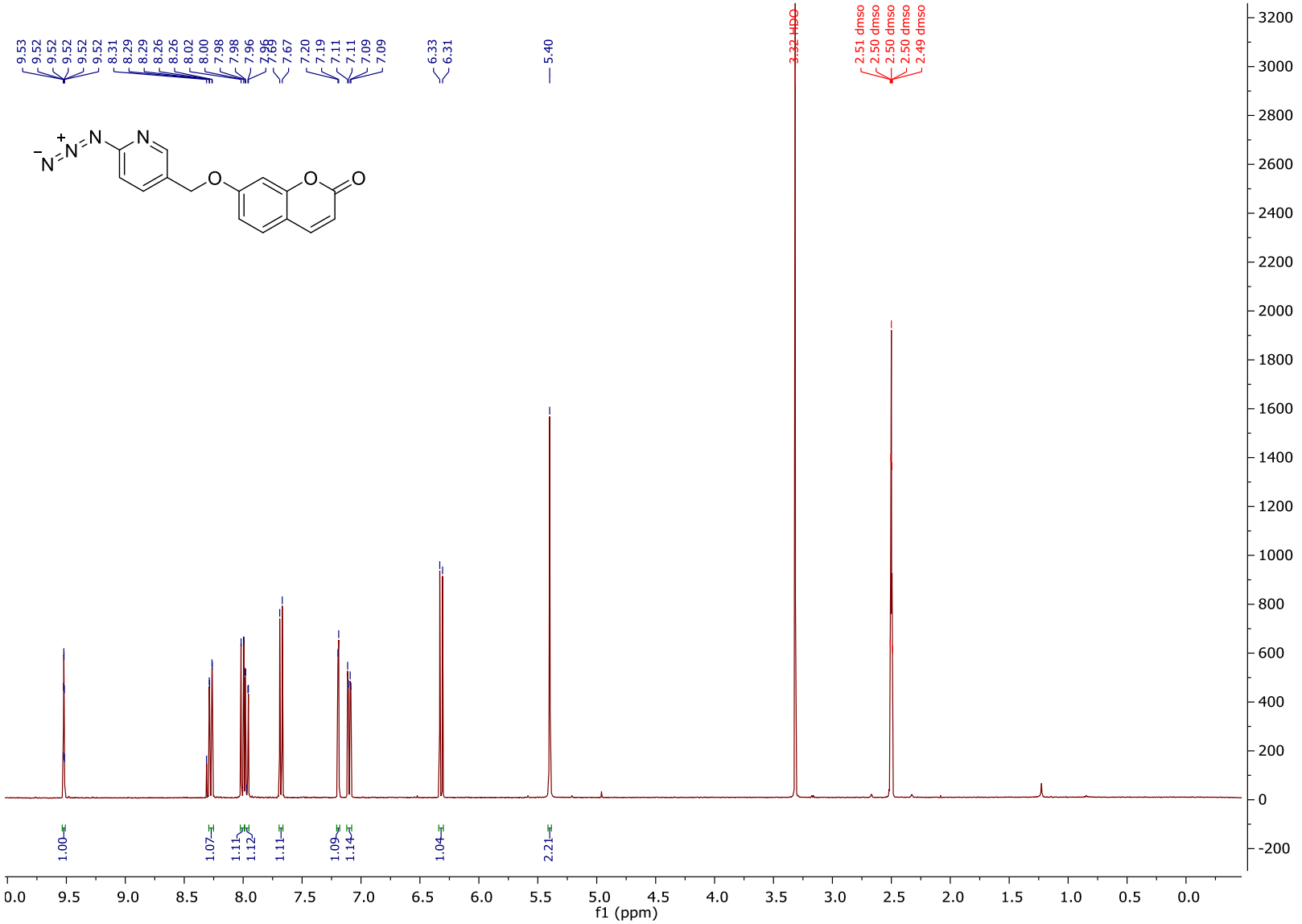


Figure S32 ^{1}H NMR Spectra of 7-[(6-Azidopyridin-3-yl)methoxy]-coumarin **1f**.

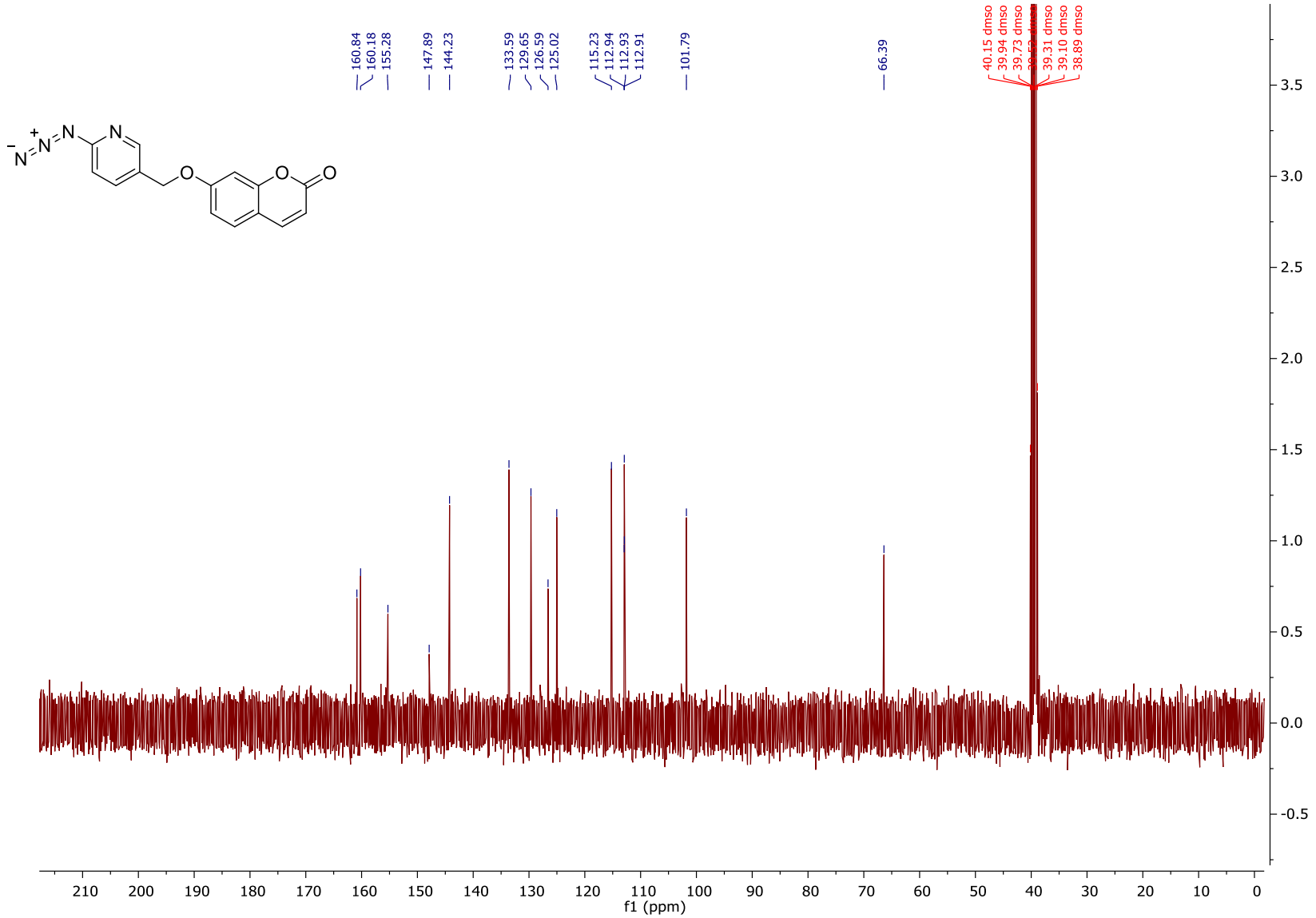


Figure S33 ^{13}C NMR Spectra of 7-[(6-Azidopyridin-3-yl)methoxy]-coumarin **1f**.

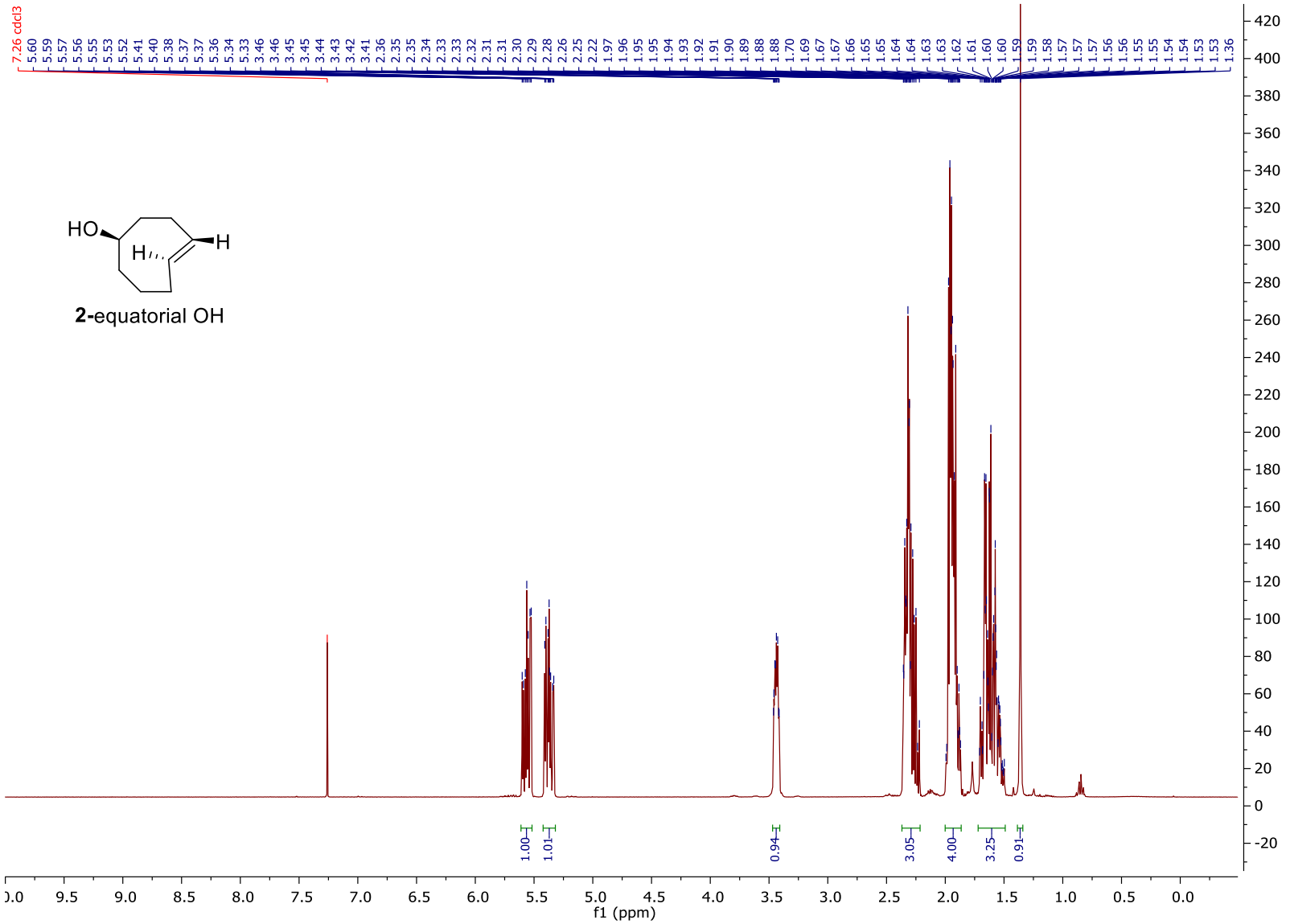


Figure S34 ^{1}H NMR Spectra of the equatorial-OH isomer of *trans*-cyclooct-4-enol (TCO) **2**.

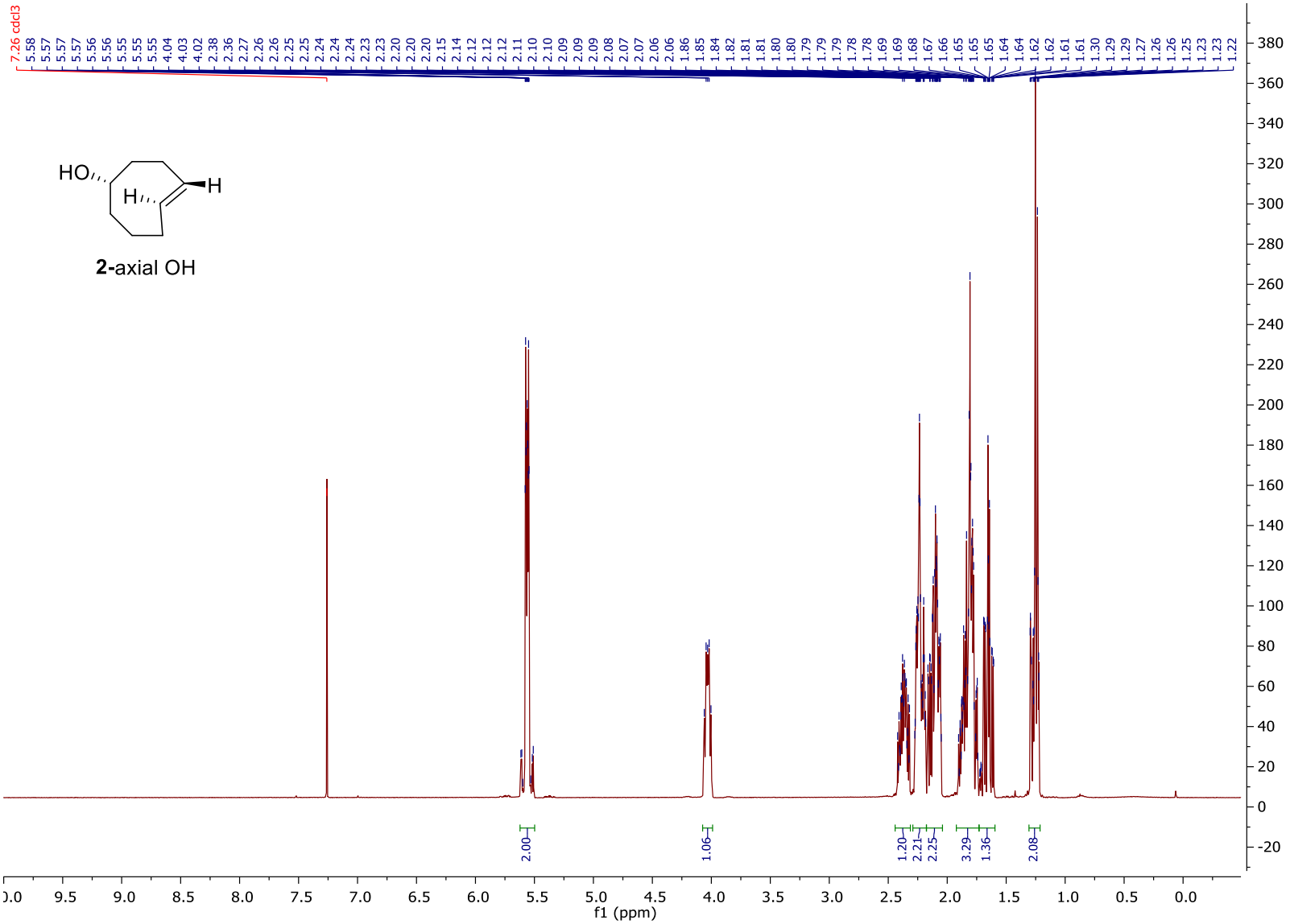


Figure S35 ^{1}H NMR Spectra of the axial-OH isomer of *trans*-cyclooct-4-enol (TCO) **2**.

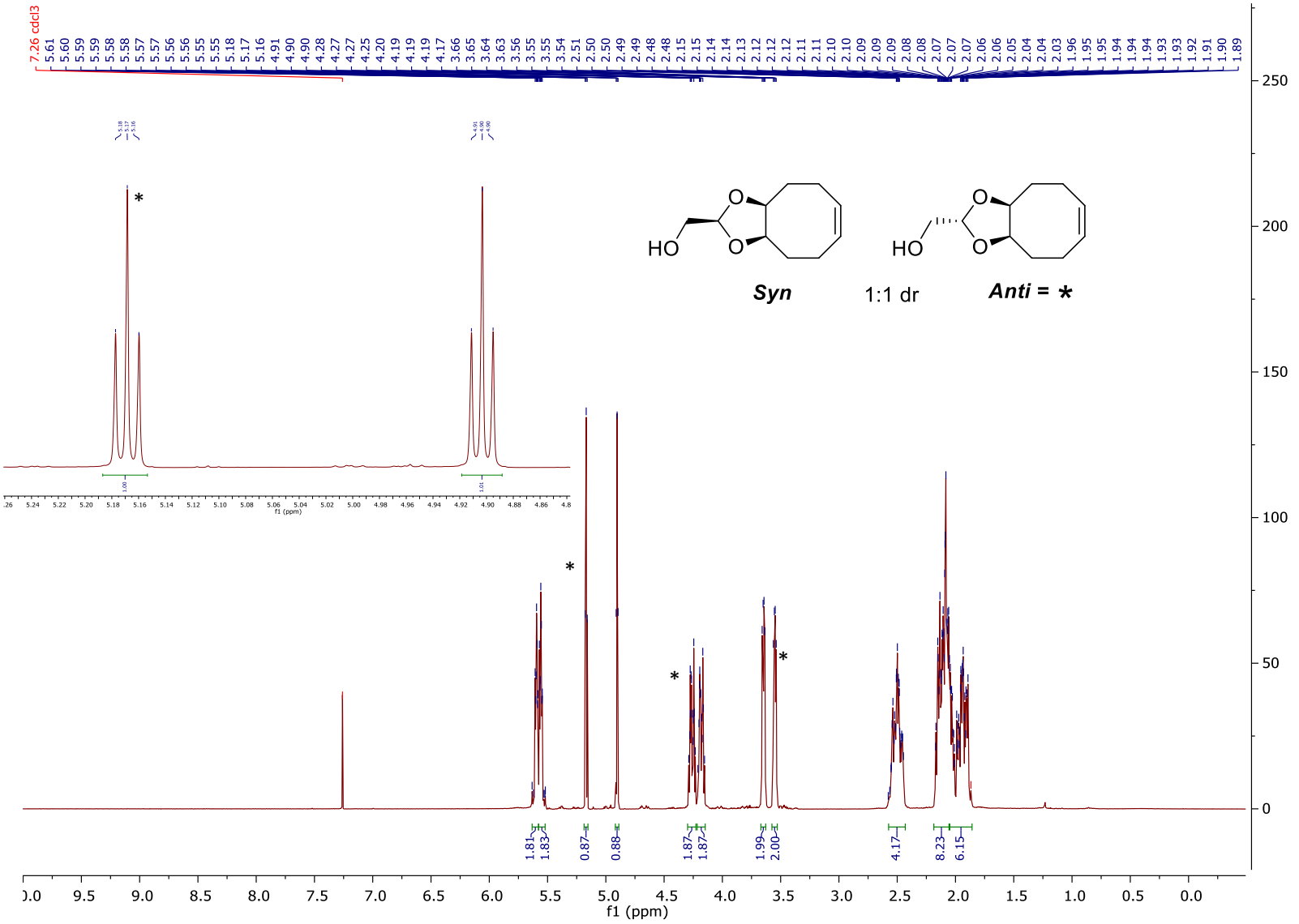


Figure S36 ^{1}H NMR Spectra of 1:1 dr d-CCO **19**.

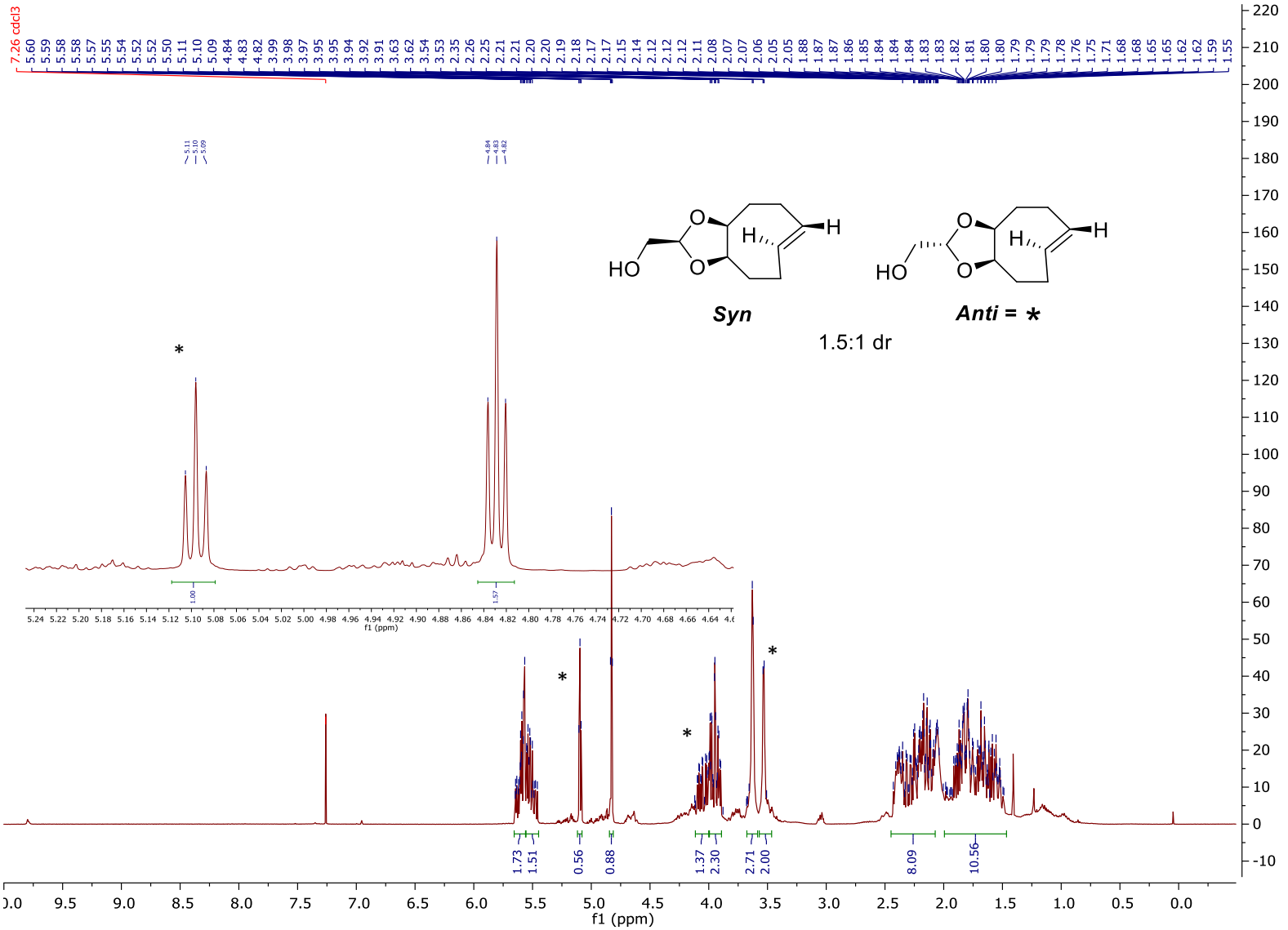


Figure S37 ^{1}H NMR Spectra of 1.5:1 dr d-TCO **3**.

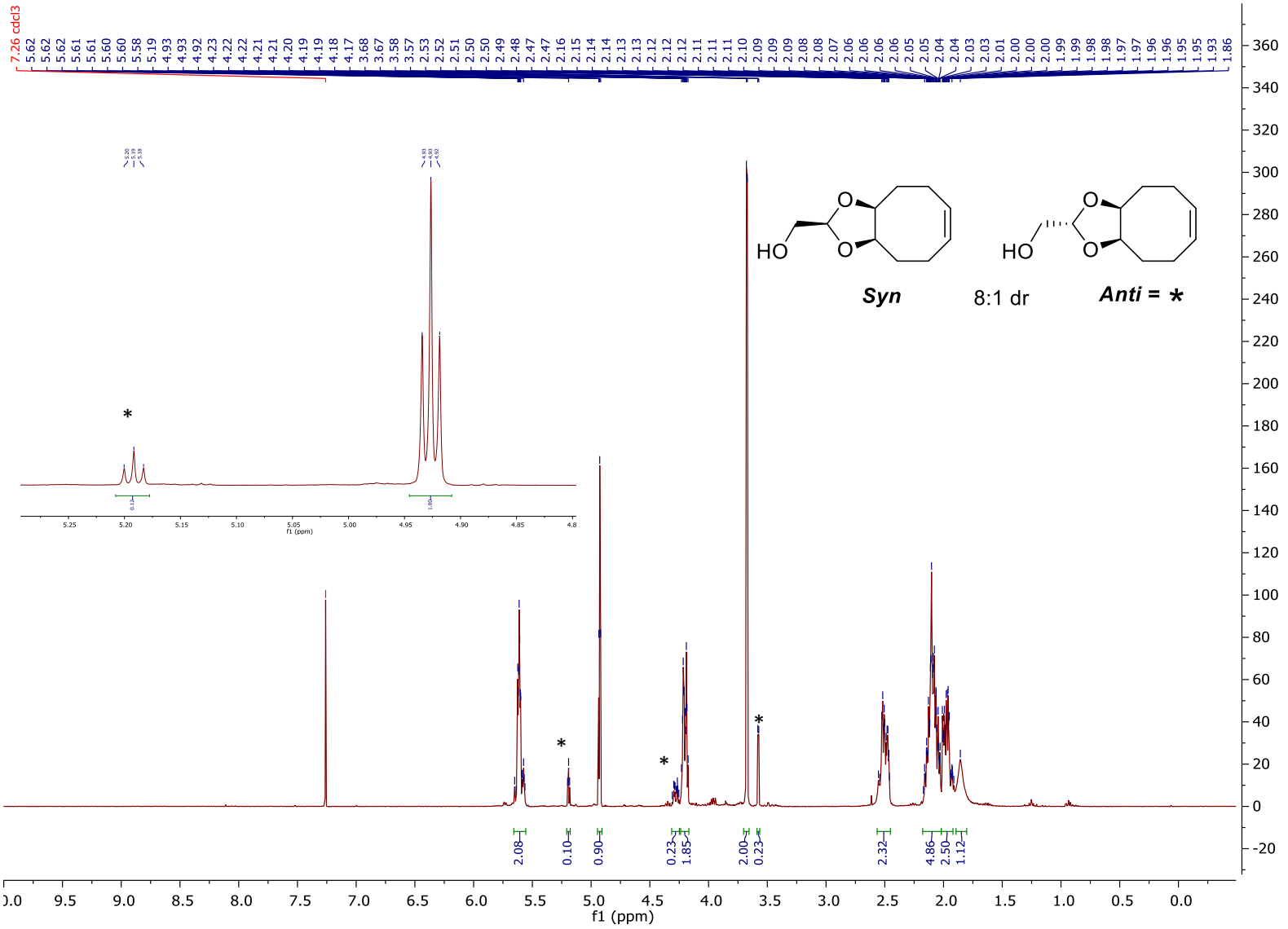


Figure S38 ^{1}H NMR Spectra of 8:1 dr d-CCO **19**-*syn*.

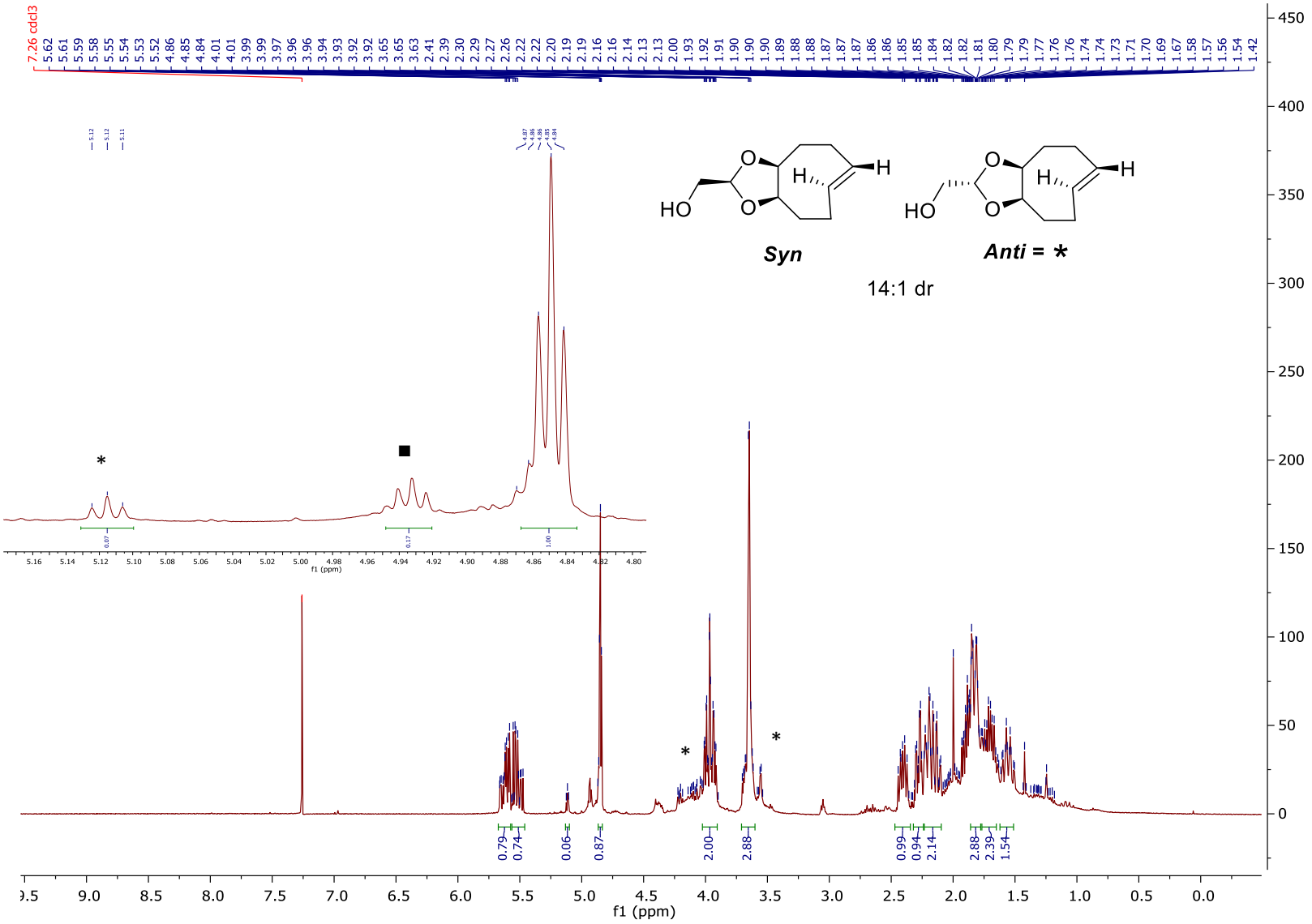


Figure S39 ^{1}H NMR Spectra of 14:1 dr d-TCO (**3**-*syn*). ■ = *syn* **19** impurity, possible isomerization back to *cis.*

8 References

1 S. S. Matikonda, D. L. Orsi, V. Staudacher, I. A. Jenkins, F. Fiedler, J. Chen and A. B. Gamble, *Chem. Sci*., 2015, **6**, 1212–1218.

2 S. S. Matikonda, J. M. Fairhall, F. Fiedler, S. Sanhajariya, R. A. J. Tucker, S. Hook, A. L. Garden and A. B. Gamble, *Bioconjugate Chem.*, 2018, **29**, 324–334.

3 B. R. Branchini, E. W. Adams, L. A. Egan and M. H. Murtiashaw, J. Label. *Compd. Radiopharm.*, 1992, **31**, 387–395.

4 F. Zhao, Z. Chen, P. Lei, L. Kong and Y. Jiang, *Tetrahedron Lett.*, 2015, **56**, 2197–2199.

5 K. Bleicher, S. M. Ceccarelli, O. Chomienne, P. Mattei, T. Schulz-Gasch, C. M. Stahl, US Pat., 20060276494, 2006, 1–26.

6 S. Ulrich, A. Petitjean and J. M. Lehn, Eur. J. *Inorg. Chem.*, 2010, 1913–1928.

7 S. L. Bender, D. Bhumralkar, M. R. Collins, S. J. Cripps, J. G. Deal, M. D. Nambu, C. L. Palmer, Z. Peng, M. D. Varney, L. Jia, US Pat., WO2001053274A1, 2001, 1–237.

8 E. J. Corey, A. Guzman-Perez and M. C. Noe, *JACS*, 1995, **117**, 10805–10816.

9 A. Darko, S. Wallace, O. Dmitrenko, M. M. Machovina, R. A. Mehl, J. W. Chin and J. M. Fox, *Chem. Sci*., 2014, **5**, 3770–3776.