Supplementary Data

A Conformationally strained *trans*-Cyclooctene with improved stability and excellent reactivity in tetrazine ligation

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Experimental Procedures

General Considerations

Methyl 6-oxohexanoate,[1] *rel*-(1*R*, 4*E*, p*R*)-cyclooct-4-enol[2], *rel*-(1*R*, 4*E*, p*S*)-cyclooct-4-enol[2], and 2,5-dioxopyrrolidin-1-yl 5-oxo-5-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)pentanoate[3] were prepared by known procedures. Anhydrous methylene chloride was dried through a column of alumina using a solvent purification system. Commercial pyridine was redistilled over KOH, and then stored under nitrogen with 4Å molecular sieves. All other reagents were purchased from commercial sources and used without further purification. Chromatography was performed on the normal phase using Silicycle 40-63D, 60Å. An APT pulse sequence was used for ^{13}C NMR, where the secondary and quaternary carbons appear 'up' (u), and tertiary and primary carbons appear 'down' (dn). Kinetic measurements involving 3,6-diphenyl-*s*-tetrazine were measured using a UV-Vis spectrophotometer fitted with a circulating water jacket. Stopped-Flow kinetics were measured using an SX18MV-R stopped-flow spectrophotometer (Applied Photophysics Ltd.) with temperature control. Fluorescence kinetics were measured using a Horiba Yobin Yvon FluoroMax®-4. The phosphate D_2O buffer solution at pD 7.4 was prepared by dissolving sodium dihydrogen phosphate

hydrate ($NaH_2PO_4 \cdot H_2O$, 0.077 g) and disodium hydrogen phosphate (Na_2HPO_4, 0.204 g) in 20 mL D_2O to make a 0.1 M solution, and then adjusting to pD 7.4 by adding DCl. The pD values were measured on an ATI PerpHect LogR pH meter (model 310). pH readings were converted to pD by adding 0.4 units.[4]

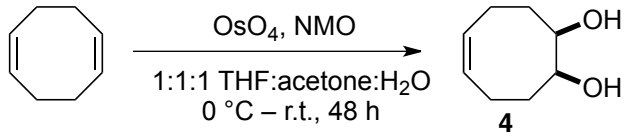
Photoisomerization Apparatus

Photoisomerizations were carried out using a Southern New England Ultraviolet Company Rayonet® reactor model RPR-100 or RPR-200, equipped with 8 low-pressure mercury lamps (2537 Å). Photoisomerizations were carried out in a quartz flask (Southern New England Ultraviolet Company). Biotage® SNAP cartriges (Biotage part No. FSK0-1107) were used to house silica gel and the $AgNO_3$-impregnated silica gel. The bottom of the column was interfaced to PTFE tubing (1/8" OD x 0.063" ID, flanged with a thermoelectric flanging tool), equipped with flangeless nylon fittings (1/4-28 thread, IDEX part no. P-582), using a female luer (1/4-28 thread, IDEX part no. P-628). The top of the column was interfaced using a male luer (1/4-28 thread, IDEX part no. P-675). The pump used for recirculating solvents through the photolysis apparatus was purchased from Fluid Metering, Inc. (FMI pump model RP-D equipped with pumphead FMI R405). Adapters for interfacing the FMI pump to the PTFE tubing were purchased from IDEX (part no. U-510).

Preparation of Silver Nitrate Impregnated Silica Gel

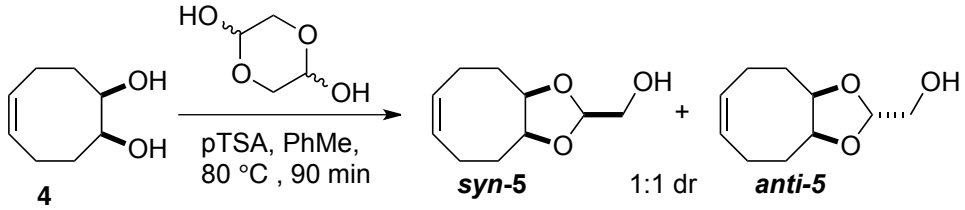
Flash silica gel (90 g, Silicycle cat # R12030B, 60 Å) was suspended in 100 mL of water in a 2 L round bottom flask. The flask was covered with aluminum foil and a silver nitrate (10 g) solution in water (10 mL) was added. The resulting mixture was thoroughly mixed. Water was evaporated under reduced pressure on the rotavap (bath temperature ~ 65 °C) using a bump trap with a coarse fritted disk. To remove the remaining traces of water, toluene (2 x 200 mL) was added and subsequently evaporated by rotary evaporation. The silver nitrate impregnated silica was then dried under vacuum overnight at room temperature.

(1*R*,2*S*,*Z*)-Cyclooct-5-ene-1,2-diol (4)



The following procedure has been modified from Cha et al[5]: Osmium tetroxide (1.85 mL, 4% wt. in water, 0.29 mmol) was added dropwise to a stirring solution of *N*-methylmorpholine-*N*-oxide (10.0 g, 85.5 mmol) and cyclooctadiene (19.5 g, 181 mmol) in THF:acetone:water (1:1:1, 450 mL) at 0 °C. After the addition was complete, the mixture was stirred at room temperature for 48 h. Sodium metabisulfite (0.76 g) and Florisil (9.84 g) slurried in 66 mL water was added and stirred for 30 minutes, and the mixture filtered through a pad of celite. The filtrate was neutralized with 1N H_2SO_4, and concentrated to remove all organics. The resulting aqueous solution was adjusted to pH 2 with 1N H_2SO_4 and extracted with three 400 mL portions of ethyl acetate. The combined ethyl acetate extracts were washed with brine, dried over Na_2SO_4, and concentrated into an off-white solid. Silica gel chromatography (70% ethyl acetate in hexanes) provided diol **4** as a white solid (2.82 g, 19.9 mmol, 23%). ^{1}H NMR spectra agreed with previously reported data.[6] δ_H($CDCl_3$, 400 MHz) 5.76–5.64 (m, 2H), 4.07–3.99 (m, 2H), 2.58–2.45 (m, 2H), 2.15–1.98 (m, 4H), 1.91–1.76 (m, 2H). δ_C($CDCl_3$, 101 MHz) 130.1 (dn), 75.2 (dn), 32.0 (u), 23.0 (u).

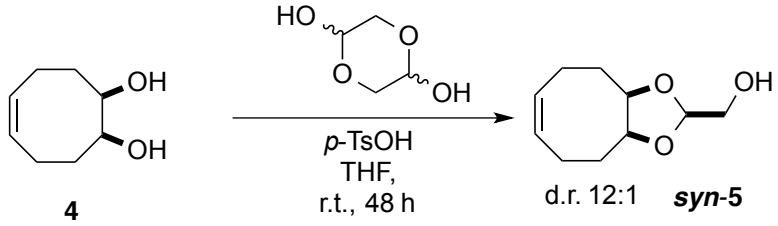
((2*s*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*syn*-5) and ((2*r*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*anti*-5)



p-Toluenesulfonic acid monohydrate (887 mg, 4.66 mmol) was added to a stirring solution of **4** (3.48 g, 24.5 mmol) and glycolaldehyde dimer (1.40 g, 11.7 mmol) in

toluene (40 mL). The mixture was stirred at 80 °C for 90 min and then cooled to room temperature. The mixture was diluted with H_2O (200 mL) and extracted with CH_2Cl_2 (3 x 200 mL). The combined organics were washed with sodium bicarbonate (sat. aq., 300 mL), brine (300 mL), dried over sodium sulfate, filtered and concentrated under reduced pressure. The crude material was purified by silica gel chromatography (10-30% EtOAc in hexane) to afford the dioxolanyl alcohol **5** as a pale yellow oil as a 1:1 mixture of inseparable diastereomers (3.73 g, 83%). NMR peaks attributable to ***syn*-5**: δ_H ($CDCl_3$, 400 MHz) 5.66 – 5.60 (m, 2H), 4.93 (t, 1H, *J* 3.1), 4.24 – 4.17 (m, 2H), 3.68 (dd, 2H, *J* 6.5, 3.0), 2.58 – 2.43 (m, 2H), 2.18 – 2.02 (m, 4H), 2.02 – 1.92 (m, 2H), 1.80 (t, 1H, *J* 6.5). δ_C ($CDCl_3$, 101 MHz) 129.4 (dn), 101.2 (dn), 79.4 (dn), 63.4 (u), 28.6 (u), 23.6 (u). NMR peaks attributable to ***anti*-5:** δ_H ($CDCl_3$, 400 MHz) 5.60 – 5.54 (m, 2H), 5.20 (t, 1H, *J* 3.4), 4.33 – 4.25 (m, 2H), 3.59 (dd, 2H, *J* 6.6, 3.4), 2.66 – 2.58 (m, 2H), 2.24 – 2.18 (m, 4H), 1.92 – 1.88 (m, 2H), 1.75 (t, 1H, *J* 6.6). δ_C($CDCl_3$, 101 MHz) 128.7 (dn), 100.5 (dn), 79.4 (dn), 64.2 (u), 27.1 (u), 23.8 (u). ν_{max}($CDCl_3$)/cm^{-1} 3447, 3013, 2943, 2879, 1136, 1058, 990, 720. HRMS (LIFDI-TOF) *m/z*: [M^+] calcd. for $C_{10}H_{16}O_3^+$ 184.1094; found 184.1104.

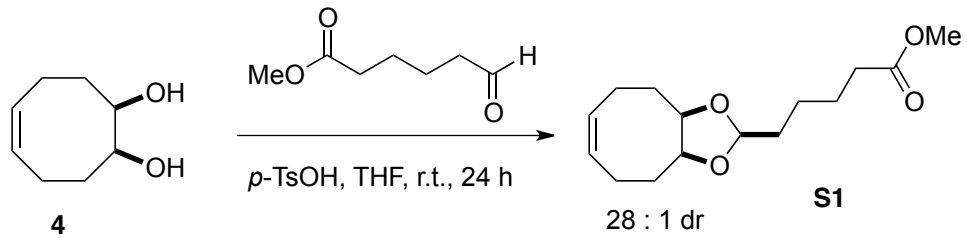
Diastereoselective Synthesis of ((2*s*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*syn*-5)



p-Toluenesulfonic acid monohydrate (0.144 g, 0.759 mmol) was added to a solution of **4** (0.540 g, 3.80 mmol) and glycolaldehyde dimer (0.228 g, 1.90 mmol) in THF (50 mL) and stirred at room temperature for 48 hours. The mixture was diluted with $CHCl_3$ (150 mL) and washed with saturated $NaHCO_3$. The organics were separated and dried over Na_2SO_4, filtered, and concentrated. The crude oil was purified by silica gel chromatography (10-30% EtOAc in hexanes) to afford the title compound as a clear, colorless oil as a 12:1 mixture of diastereomers (0.347 g, 1.88 mmol, 50% yield).

$\nu_{max}(CDCl_3)/cm^{-1}$ 3427, 3013, 2943, 2878, 1653, 1482, 1384, 1229, 1160, 1134, 1058, 991, 882, 721.

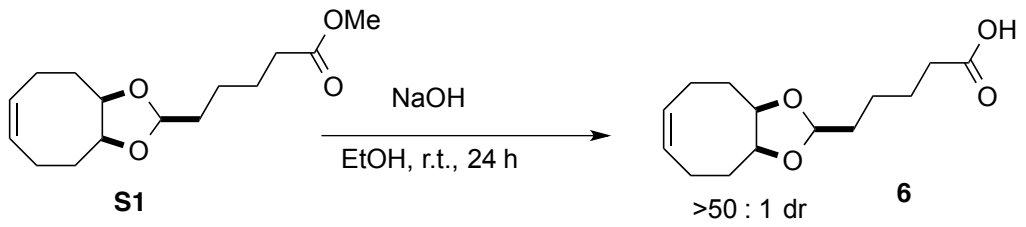
Methyl 5-((2*r*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)pentanoate (S1)



4 28 : 1 dr **S1**

Compound **4** (0.600 g, 4.22 mmol) and methyl 6-oxohexanoate[1] (0.730 g, 5.07 mmol) were dissolved in THF (50 mL). *p*-Toluenesulfonic acid monohydrate (80 mg, 0.42 mmol) was then added and the solution was stirred overnight at room temperature. After 24 hours the starting material was consumed (TLC monitoring). The solution was diluted with $CHCl_3$ (150 mL) and transferred into a separatory funnel and washed with saturated $NaHCO_3$ (15 mL). The organic layer was separated and dried over Na_2SO_4, filtered, and concentrated by rotary evaporation into a pale yellow oil. The oil was purified by flash column chromatography using 15% EtOAc/hexanes to obtain the title compound as a pale yellow oil (0.910 g, 3.40 mmol, 81% yield). $\delta_H(CDCl_3$, 400 MHz) 5.66 – 5.55 (m, 2H), 4.82 (t, 1H, *J* 4.7), 4.16 – 4.07 (m, 2H), 3.66 (s, 3H), 2.55 – 2.40 (m, 2H), 2.31 (t, 2H, *J* 7.5), 2.14 – 2.00 (m, 4H), 1.99 – 1.87 (m, 2H), 1.72 – 1.58 (m, 4H), 1.50 – 1.38 (m, 2H). $\delta_C(CDCl_3$, 101 MHz) 174.2 (u), 129.6 (dn), 102.4 (dn), 79.1 (dn), 51.7 (dn), 34.4 (u), 34.1 (u), 29.0 (u), 25.0 (u), 23.8 (u), 23.6 (u). $\nu_{max}(CDCl_3)/cm^{-1}$ 2947, 2866, 1739, 1435, 1367, 1197, 1163, 1128, 1093, 1041, 722. HRMS (LIFDI-TOF) *m/z*: $[M^+]$ calcd. for $C_{15}H_{24}O_4^+$ 268.1669; found 268.1691.

5-((2*r*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)pentanoic acid (6)



S1 >50 : 1 dr **6**

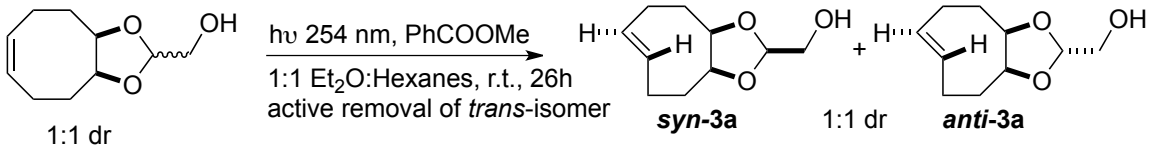
To a solution of **S1** (0.800 g, 2.99 mmol) in ethanol (30 mL) was added 1M NaOH (6 mL). The solution was stirred at room temperature for 24 hours. The solution was concentrated by rotary evaporation and the residue was diluted with $CHCl_3$ (45 mL) and transferred into a separatory funnel. The organics were washed with 1N HCl (45 mL), dried over $MgSO_4$, filtered, and concentrated to give 0.668 g (2.63 mmol, 88% yield) of the title compound as a white solid. The product was used without further purification. Mp 90-93 °C. δ_H($CDCl_3$, 400 MHz) 10.7 – 10.0 (br s, 1H), 5.68 – 5.54 (m, 2H), 4.83 (t, 1H, *J* 4.7), 4.18 – 4.06 (m, 2H), 2.56 – 2.41 (m, 2H), 2.37 (t, 2H, *J* 7.5), 2.15 – 2.00 (m, 4H), 2.00 – 1.87 (m, 2H), 1.74 – 1.62 (m, 4H), 1.53 – 1.42 (m, 2H). δ_C($CDCl_3$, 101 MHz) 178.5 (u), 129.6 (dn), 102.3 (dn), 79.1 (dn), 34.3 (u), 33.8 (u), 29.0 (u), 24.8 (u), 23.7 (u), 23.6 (u). ν_{max}($CDCl_3$)/cm^{-1} 3015, 2946, 2926, 2880, 2860, 1700, 1466, 1314, 1258, 1207, 1128, 1092, 1002, 946. HRMS (LIFDI-TOF) *m/z*: [M+H] calcd. for $C_{14}H_{23}O_4^+$ 255.1591; found 255.1574.

General photoisomerization procedure

The (*Z*)-cyclooctene derivative (0.012-0.024 M in solvent) and methyl benzoate (2.0 equiv.) were dissolved in the chosen solvent in a quartz flask. After sparging the contents with N_2 for 15 minutes, the flask was placed in a Rayonet® reactor and connected via PTFE tubing to a column (Biotage® SNAP) and an FMI pump. The bottom of the column was packed with dry silica gel, and the top of the column was packed with silver nitrate impregnated silica. Any remaining space between the silica and the cartridge cap was filled with cotton. The column was flushed with the reaction solvent, followed by equilibration with the reaction mixture via circulation with the FMI pump (~100 mL per minute). The solution in the quartz flask was then irradiated at 254 nm under continuous flow for the indicated time. After the reaction is complete, the SNAP cartridge was

washed with additional solvent, and then dried by a stream of compressed air. The cartridge contents were emptied into an Erlenmeyer flask and stirred with ammonium hydroxide and methylene chloride for 10 minutes. The silica gel was filtered, and the filtrate transferred to a separatory funnel. The organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2. The organic layers were combined, washed with water, dried ($MgSO_4$), filtered, and concentrated to provide the crude *trans*-cyclooctene products. Flash silica gel chromatography afforded pure *trans*-cyclooctenes.

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



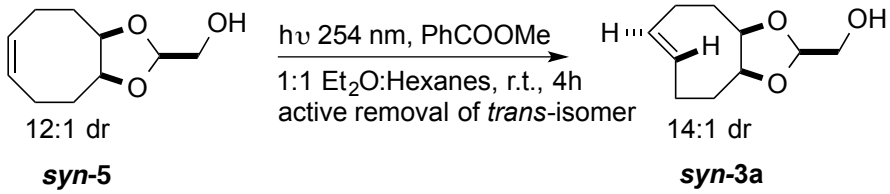
1:1 dr
***syn*/*anti* -5**

***syn*-3a** 1:1 dr ***anti*-3a**

The general photoisomerization procedure was followed using **5** (***syn*/*anti*** = 1:1) (2.02 g, 11.0 mmol) in 1:1 ether/hexanes (500 mL) and methyl benzoate (2.99 g, 22.0 mmol), in a 500 mL quartz tube. A 100 g Biotage® SNAP column was filled with normal silica gel (5 inches) and the remaining space packed with 10% silver impregnated silica (24.2 g). The column was connected to the pump as described in the photoisomerization apparatus and flushed with 1:1 ether:hexanes. The apparatus was then set-up with the quartz tube and equilibrated for 15 min while being degassed. The lamps were turned on and irradiation was carried out for 26 h, at which point only trace starting material remained by TLC. The tubing was disconnected from the quartz flask, and the column was flushed with ether (200 mL) and dried under air flow. After the silica in the column was dry, it was stirred in ammonium hydroxide (200 mL) and methylene chloride (200 mL) for 10 min. The silica was filtered and the layers were separated. The aqueous layer was extracted with additional methylene chloride (200 mL). The combined organic layers were washed with water (100 mL), dried ($MgSO_4$), filtered, and concentrated to give a pale yellow oil. Purification by flash silica gel chromatography (30%-60% Et_2O in hexanes) afforded the title compounds as an oil in a 1:1 mixture of inseparable diastereomers (1.31 g, 7.11 mmol, 65% yield). The oil solidified after overnight storage

at -20 °C. Mp 41-49 °C. NMR peaks attributable to ***syn*-3a**: δ_H($CDCl_3$, 400 MHz) 5.69–5.46 (m, 2H), 4.86 (t, 1H, *J* 3.0), 4.07–3.90 (m, 2H), 3.71–3.61 (m, 2H), 2.48–2.36 (m, 1H), 2.35–2.08 (m, 3H), 1.95–1.80 (m, 2H), 1.77–1.62 (m, 2H), 1.63–1.49 (m, 1H). δ_C($CDCl_3$, 101 MHz) 136.5 (dn), 131.3 (dn), 100.7 (dn), 83.0 (dn), 80.9 (dn), 63.0 (u), 38.9 (u), 33.9 (u), 31.5 (u), 25.7 (u). NMR peaks attributable to ***anti*-3a**: δ_H($CDCl_3$, 400 MHz) 5.69–5.49 (m, 2H), 5.13 (t, 1H, *J* 3.6), 4.13–4.07 (m, 1H), 4.06–3.97 (m, 1H), 3.61–3.53 (m, 2H), 2.47–2.30 (m, 2H), 2.30–2.10 (m, 2H), 1.89–1.73 (m, 2H), 1.73–1.66 (m, 2H), 1.66–1.57 (m, 1H). δ_C($CDCl_3$, 101 MHz,) 136.4 (dn), 131.1 (dn), 100.7 (dn), 84.0 (dn), 79.5 (dn), 63.9 (dn), 39.3 (u), 32.0 (u), 24.9 (u). ν_{max}($CDCl_3$)/cm^{-1} 3323, 2972, 2942, 2922, 2861, 1638, 1542, 1506, 1450, 1364, 1308, 1236, 1179, 1159, 1095, 850, 748, 701, 680. HRMS (LIFDI-TOF) *m/z*: [M^+] calcd. for $C_{10}H_{16}O_3^+$ 184.1094; found 184.1089. The products can be separated by silica gel chromatography after derivatization with *p*-nitrobenzoyl chloride. Subsequent hydrolysis provided the title products in their diastereomerically pure form.

Alternate preparation of ((2*s*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methanol (*syn*-3a)



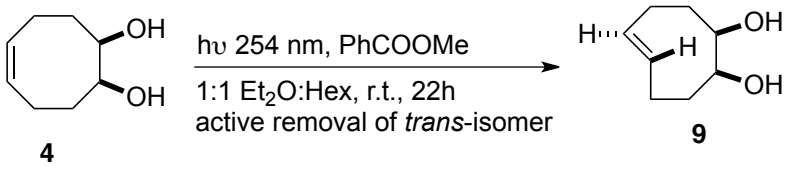
12:1 dr — ***syn*-5**

14:1 dr — ***syn*-3a**

The general photoisomerization procedure was followed using ***syn*-5** (12:1 dr) (0.217 g, 1.18 mmol) in 1:1 ether/hexanes (100 mL) and methyl benzoate (0.321 g, 2.36 mmol), in a 150 mL quartz tube. A 25 g Biotage® SNAP column was filled with normal silica gel (2.5 inches) and the remaining space packed with 10% silver impregnated silica (2.60 g). The column was connected to the pump and flushed with 1:1 ether:hexanes. The apparatus was then set-up and equilibrated. Irradiation was carried out for 4 h, at which point only trace starting material remained by GC monitoring. The tubing was disconnected from the quartz flask, and the column was flushed with 1:1 ether:hexanes (100 mL) and dried under air flow. After the silica in the column was dry, it was stirred in ammonium hydroxide (50 mL) and methylene chloride (50 mL) for 10 min. The silica

was filtered and the layers were separated. The aqueous layer was extracted with additional methylene chloride (20 mL). The combined organic layers were washed with water (50 mL), dried ($MgSO_4$), filtered, and concentrated to give a pale yellow oil. Purification by flash silica gel chromatography (30%-60% Et_2O in hexanes) afforded the title compound as a white solid in a 14:1 mixture of inseparable diastereomers (0.129 g, 0.700 mmol, 59% yield). Mp 62-63 °C. NMR peaks attributable ***syn*-3a**: δ_H($CDCl_3$, 600 MHz) 5.67–5.58 (m, 1H), 5.59–5.48 (m, 1H), 4.86 (t, 1H, *J* 3.0), 4.04–3.92 (m, 2H), 3.71–3.62 (m, 2H), 2.46–2.38 (m, 1H), 2.36–2.24 (m, 1H), 2.25–2.19 (m, 1H), 2.21–2.11 (m, 1H), 1.94–1.77 (m, 2H), 1.75–1.61 (m, 2H), 1.61–1.52 (m, 1H). δ_C($CDCl_3$, 101 MHz) 136.5 (dn), 131.3 (dn), 100.7 (dn), 83.0 (dn), 80.9 (dn), 63.0 (u), 38.9 (u), 33.9 (u), 31.5 (u), 25.7 (u).ν_{max}($CDCl_3$)/cm^{-1} 3462, 2928, 2859, 1637, 1448, 1355, 1206, 1139, 1059, 991, 903, 839.

(1*R*,2*S*,*E*)-Cyclooct-5-ene-1,2-diol (9)

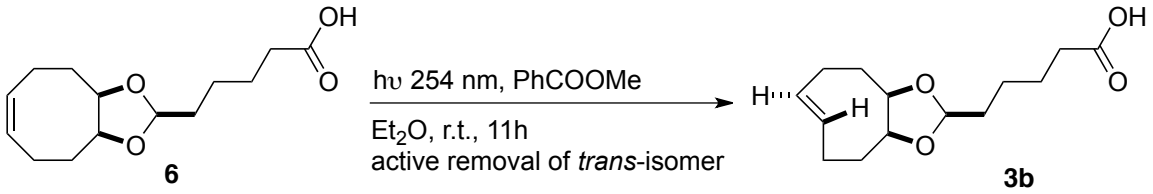


4 **9**

The general photoisomerization procedure was followed using compound **4** (0.502 g, 3.53 mmol) in 1:1 hexanes:Et_2O (300 mL), and methyl benzoate (0.961 g, 7.06 mmol) in a 500 mL quartz tube. A 25 g Biotage® SNAP column was filled with normal silica gel (2.5 inches), then topped with 10% silver impregnated silica (7.80 g). Irradiation was carried out for 22 h. The tubing was disconnected from the quartz flask, and the column was flushed with ether (100 mL) and dried under air flow. After the silica in the column was dry, it was stirred in ammonium hydroxide (100 mL) and methylene chloride (100 mL) for 10 min. The silica was filtered and the layers were separated. The aqueous layer was extracted with additional methylene chloride (100 mL). The combined organic layers were washed with water (50 mL), dried ($MgSO_4$), filtered, and concentrated. After isolation, the residue was dissolved in ether and passed through a short plug of silica to provide the title compound as a white solid (0.196 g, 1.39 mmol, 39% yield). Mp 130-131°C. δ_H($CDCl_3$, 400 MHz) 5.72 (ddd, 1H, *J* 14.7, 9.4, 5.0), 5.43 (ddd, 1H, *J* 15.5, 11.3,

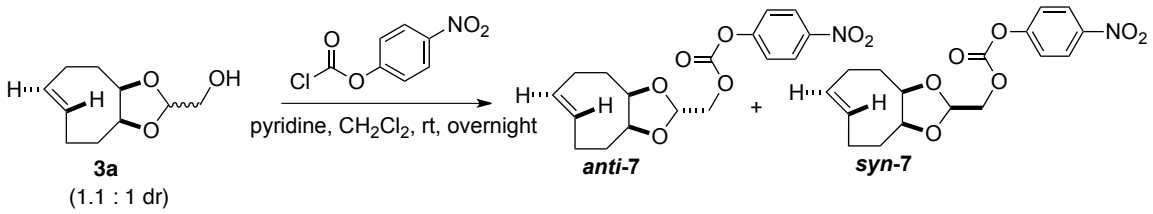
3.3), 4.01–3.92 (m, 1H), 3.69 (d, 1H, *J* 10.1), 2.42–2.09 (m, 5H), 2.07–1.97 (m, 1H), 1.83–1.66 (m, 3H), 1.56 (s, 1H). δ_C($CDCl_3$, 101 MHz) 135.2 (dn), 130.5 (dn), 79.2 (dn), 77.6 (dn), 38.0 (u), 37.9 (u), 32.2 (u), 27.9 (u). ν_{max}($CDCl_3$)/cm^{-1} 3583, 3287, 2921, 1441, 1262, 1043, 992, 979, 971, 883, 794. HRMS (LIFDI-TOF) *m/z*: [M^+] calcd. For $C_8H_{14}O_2^+$ 142.0988; found 142.0986.

5-((2*r*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)pentanoic acid (3b)



The general photoisomerization procedure was followed using 5-((2*r*,3a*R*,9a*S*,*Z*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)pentanoic acid (0.500 g, 1.97 mmol) and methyl benzoate (0.535 g, 3.93 mmol) in ether (150 mL) in a 250 mL quartz tube. A 25 g Biotage® SNAP column was filled with normal silica gel (2.5 inches), then topped with 10% silver impregnated silica (4.34 g). Irradiation was carried out for 11 h. The tubing was disconnected from the quartz flask, and the column was flushed with ether (100 mL), then ethanol (300 mL), followed by saturated methanolic ammomia (100 mL, produced by bubbling ammonia in methanol for 30 min). The methanolic ammonia solution was then concentrated by rotavap to obtain the title compound as an oily residue. The crude residue was purified by flash silica gel chromatography (50% Et_2O in hexanes) to provide the title compound as a white solid (0.180 g, 0.708 mmol, 36% yield). Mp 78-80 °C. δ_H($CDCl_3$, 400 MHz) 10.58 (s, 1H), 5.68–5.56 (m, 1H), 5.56–5.44 (m, 1H), 4.74 (t, 1H, *J* 4.6), 3.96–3.78 (m, 2H), 2.44–2.32 (m, 3H), 2.30–2.22 (m, 1H), 2.20–2.07 (m, 2H), 1.94–1.78 (m, 2H), 1.74–1.60 (m, 5H), 1.59–1.41 (m, 3H). δ_C($CDCl_3$, 101 MHz) 178.7 (u), 136.5 (dn), 131.4 (dn), 101.7 (dn), 82.7 (dn), 80.6 (dn), 38.9 (u), 34.0 (u), 33.8 (u), 33.5 (u), 31.5 (u), 26.0 (u), 24.7 (u), 23.5 (u). ν_{max}($CDCl_3$)/cm^{-1} 2928, 2860, 1734, 1707, 1419, 1134, 990. HRMS (LIFDI-TOF) *m/z*: [M^+] calcd. For $C_{14}H_{22}O_4^+$ 254.1513; found 254.1506.

((2*r*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methyl (4-nitrophenyl) carbonate (*syn*-7) and ((2*s*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methyl (4-nitrophenyl) carbonate (*anti*-7)



3a
(1.1 : 1 dr)

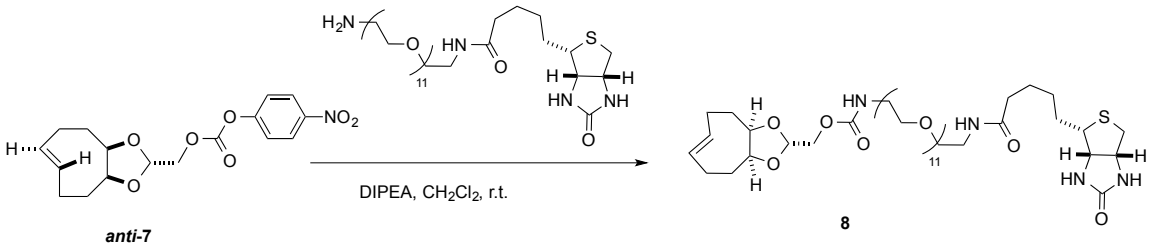
A solution of 4-nitrophenyl chloroformate (60 mg, 0.299 mmol) in dry CH_2Cl_2 (1 mL) was added dropwise by syringe to a 1.1 : 1 mixture of isomers of **3a** (50 mg, 0.271 mmol) and dry pyridine (54 mg, 0.678 mmol) in 5 mL CH_2Cl_2. The mixture was stirred for 20 h at room temperature and then quenched by addition of saturated NH_4Cl. The resultant layers were separated and the aqueous layer was extracted 2 times with CH_2Cl_2. The combined organic layers were dried over $MgSO_4$, filtered, and concentrated into a residue. The crude residue was purified by flash silica gel chromatography (10% Et_2O in hexanes) to afford ***syn*-7** (25 mg, 0.072 mmol) and ***anti*-7** (28 mg, 0.080 mmol) as white solids (53 mg total, 0.152 mmol, 56% yield).

Anti diastereomer: mp 79-82 °C. $\delta_H(CDCl_3$, 400 MHz) 8.28 (d, 2H, *J* 9.2), 7.39 (d, 2H, *J* 9.2), 5.71–5.50 (m, 2H), 5.38–5.30 (m, 1H), 4.22 and 4.21 (*ABX* multiplet, 2H, J_{AB} = 11.4, J_{AX} = 4.7, J_{BX} = 3.7), 4.16–4.10 (m, 1H), 4.09–4.02 (m, 1H), 2.49–2.30 (m, 2H), 2.31–2.12 (m, 2H), 1.93–1.75 (m, 2H), 1.74–1.58 (m, 2H). $\delta_C(CDCl_3$, 101 MHz) 155.6 (u), 152.5 (u), 145.6 (u), 136.5 (dn), 131.0 (dn), 125.5 (dn), 121.9 (dn), 98.2 (dn), 84.0 (dn), 79.2 (dn), 68.5 (u), 39.1 (u), 32.1 (u), 31.8 (u), 24.9 (u). $\nu_{max}(CDCl_3)/cm^{-1}$ 2930, 1770, 1722, 1617, 1594, 1525, 1492, 1349, 1266, 1217, 1142, 1063, 980, 860.

Syn diastereomer: mp 93-96 °C. $\delta_H(CDCl_3$, 400 MHz) 8.28 (d, 2H, *J* 9.2), 7.38 (d, 2H, *J* 9.2), 5.71–5.59 (m, 1H), 5.59–5.48 (m, 1H), 5.06 (t, 1H, *J* 3.6), 4.31 (d, 2H, *J* 3.6), 4.07–3.92 (m, 2H), 2.50–2.36 (m, 1H), 2.36–2.22 (m, 2H), 2.22–2.09 (m, 1H), 1.97–1.79 (m, 2H), 1.79–1.56 (m, 1H). $\delta_C(CDCl_3$, 101 MHz) 155.6 (u), 152.5 (u), 145.5 (u), 136.6 (dn), 131.2 (dn), 125.5 (dn), 122.0 (dn), 98.3 (dn), 83.2 (dn), 81.1 (dn), 68.6 (u), 38.8 (u), 33.7 (u), 31.5 (u), 25.5 (u). $\nu_{max}(CDCl_3)/cm^{-1}$ 2931, 2861, 1771, 1723, 1617, 1594, 1525,

1493, 1443, 1349, 1218, 1156, 1067, 990, 860, 774. HRMS (LIFDI-TOF) *m/z*: [M^+] calcd. for $C_{17}H_{19}NO_7^+$ 349.1156; found 349.1161.

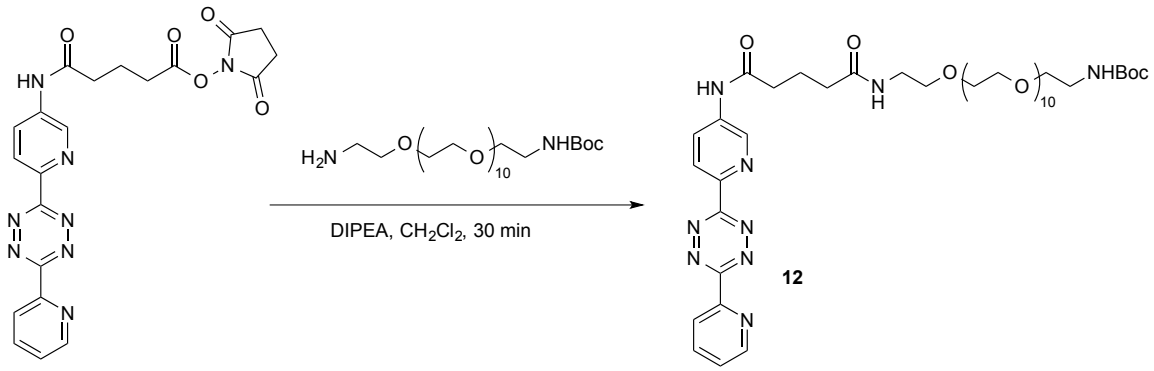
((2*r*,3a*R*,9a*S*,*E*)-3a,4,5,8,9,9a-Hexahydrocycloocta[*d*][1,3]dioxol-2-yl)methyl (37-oxo-41-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)-3,6,9,12,15,18,21,24,27,30,33-undecaoxa-36-azahentetracontyl)carbamate (8)



anti-7 **8**

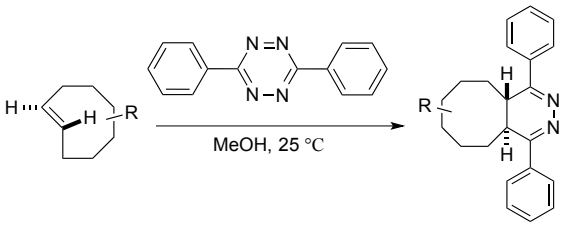
To a 5 mL round bottomed flask equipped with a stir bar was added ***anti*-7** (5.2 mg, 0.015 mmol) and CH_2Cl_2 (2 mL) . Amine-PEG11-Biotin (14.7 mg, 0.018 mmol, purchased from Thermo Scientific) and diisopropylethylamine (3.8 mg, 0.030 mmol) were added sequentially and the mixture was stirred at room temperature for 11 h. The solution was concentrated by rotary evaporation into a yellow residue. The residue was purified by flash silica gel chromatography (10% MeOH in CH_2Cl_2) to provide the title compound as a colorless residue (10.8 mg, 0.011 mmol, 73% yield). δ_H(MeOD, 600 MHz) 5.68–5.56 (m, 2H), 5.16 (t, 1H, *J* 4.2), 4.49 (dd, 1H, *J* 7.8, 4.7), 4.31 (dd, 1H, *J* 7.9, 4.5), 4.14–4.08 (m, 1H), 4.08–4.02 (m, 1H), 3.96 and 3.95 (*ABX* multiplet, 2H, J_{AB} = 11.5, J_{AX} = 4.9, J_{BX} = 3.6), 3.67–3.62 (m, 39H), 3.65–3.59 (m, 5H), 3.54 (dt, 5H, *J* 7.9, 5.5), 3.36 (t, 2H, *J* 5.5), 3.28 (t, 2H, *J* 5.5), 3.24–3.17 (m, 1H), 2.93 (dd, 1H, *J* 12.7, 5.0), 2.71 (d, 1H, *J* 12.6), 2.37 (dt, 1H, *J* 13.3, 6.8), 2.33–2.26 (m, 1H), 2.25–2.19 (m, 2H), 2.22–2.13 (m, 2H), 1.91–1.81 (m, 1H), 1.79–1.70 (m, 2H), 1.72–1.56 (m, 4H), 1.45 (p, 2H, *J* 7.8). ν_{max}(MeOD)/cm^{-1} 3738, 3584, 3342, 2918, 2860, 1702, 1543, 1452, 1350, 1259, 1102. HRMS (LIFDI-TOF) *m/z*: $[M+Na]^+$ calcd. For $C_{45}H_{80}N_4O_{17}SNa^+$ 1003.5131; found 1003.5143.

***tert*-Butyl (37,41-dioxo-41-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)-3,6,9,12,15,18,21,24,27,30,33-undecaoxa-36-azahentetracontyl)carbamate (12)**



Diisopropylethylamine (11 mg, 0.087 mmol) was added to *O*-(2-aminoethyl)-*O*'-[2(Boc-amino)ethyl]decaethylene glycol (33 mg, 0.052 mmol) in 4 mL dry CH_2Cl_2. 2,5-Dioxopyrrolidin-1-yl 5-oxo-5-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)amino)pentanoate (20 mg, 0.043 mmol) was added in one portion and the mixture stirred for 30 min, after which TLC indicated the reaction was complete. The solution was concentrated by rotary evaporation and the residue purified by silica gel chromatography (3%-5% MeOH in CH_2Cl_2) to obtain compound **12** as a purple solid (31 mg, 0.031 mmol, 72% yield). ^{1}H and ^{13}C NMR spectra agreed with previously reported data.[3] δ_H($CDCl_3$, 400 MHz) 9.68 (s, 1H), 8.98 (m, 2H), 8.73 (dd, 2H), 8.64 (dd, 1H), 8.01 (td, 1H), 7.58 (ddd, 1H), 6.70 (s, 1H), 5.07 (s, 1H), 3.62 (m, 42H), 3.52 (t, 2H), 3.47 (q, 2H), 3.30 (q, 2H), 2.58 (t, 2H), 2.37 (t, 2H), 2.10 (m, 2H), 1.43 (s, 9H). δ_C($CDCl_3$, 151 MHz) 173.1 (u), 172.7 (u), 163.5 (u), 163.4 (u), 150.9 (dn), 150.2 (u), 143.8 (u), 142.1 (dn), 138.7 (u), 137.5 (dn), 126.5 (dn), 126.4 (dn), 125.1 (dn), 124.3 (dn), 70.5 (u), 70.5 (u), 70.5 (u), 70.5 (u), 70.5 (u), 70.4 (u), 70.4 (u), 70.2 (u), 70.1 (u), 69.7 (u), 53.7 (dn), 42.0 (u), 40.4 (u), 39.3 (u), 36.1 (u), 35.0 (u), 28.4 (dn), 25.6 (u), 21.5 (u), 12.0 (dn). ν_{max}($CDCl_3$)/cm^{-1} 3324, 2916, 1701, 1662, 1582, 1538, 1436, 1393, 1385, 1250, 1105, 954, 593. HRMS (LIFDI-TOF) *m/z*: $[M+Na]^+$ calcd. for $C_{46}H_{73}N_9O_{15}Na^+$ 1014.5118; found 1014.5119.

General procedure for UV-Vis kinetic analysis between *trans*-cyclooctenes and 3,6-diphenyl-*s*-tetrazine



The reaction between *trans*-cyclooctenes and 3,6-diphenyl-*s*-tetrazine was monitored by UV-Vis spectroscopy at 282 nm under pseudo-first order conditions. The *trans*-cyclooctene (0.7 mL, 200 μM in methanol) was added to the tetrazine (0.7 mL, 20 μM in methanol) and quickly mixed in a 1 mL cuvette. The final concentrations were 100 μM for the *trans*-cyclooctene and 10 μM for the tetrazine. Analysis was carried out in triplicate at 298 K. For each run, UV-Vis spectra were acquired every 5 seconds for 600-1200 seconds. The k_{obs} was determined by nonlinear regression analysis of the data points using Prism software (v. 6.00, GraphPad Software Inc.). Results are displayed in table 1.

A) **B)**

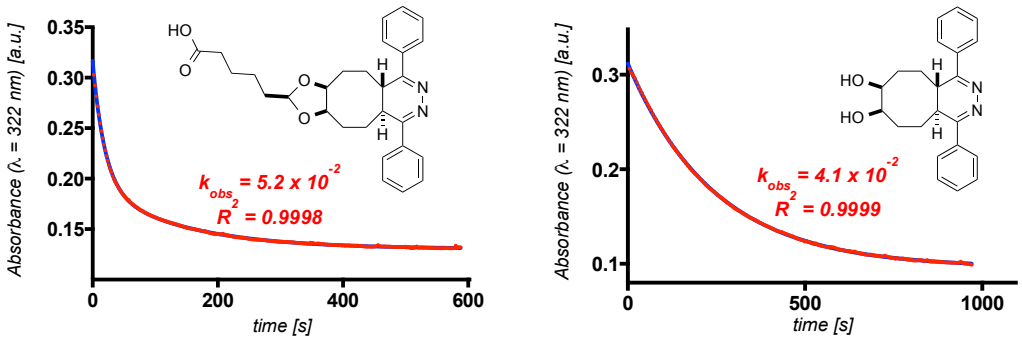
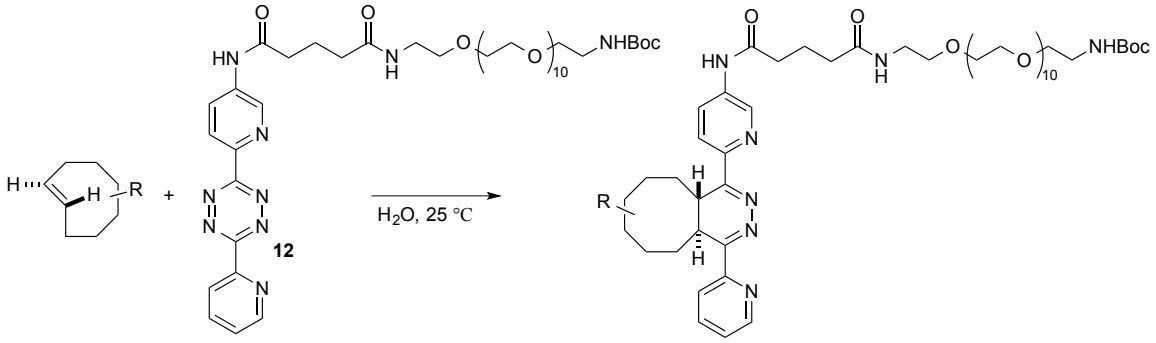


Fig. 1 (A) Reaction of **3b** with 3,6-diphenyl-*s*-tetrazine at 298 K monitored at 282 nm. **(B)** Reaction of **9** with 3,6-diphenyl-*s*-tetrazine at 298 K monitored at 282 nm.

TCO	k_{obs} (s^{-1})	k_2 ($M^{-1}s^{-1}$)	$t_{1/2}$
9	4.1×10^{-3} +/- 8.1×10^{-6}	41 +/- 8.1×10^{-2}	170.3 +/- 3.6
3b	5.2×10^{-2} +/- 3.0×10^{-4}	520 +/- 3.0	13.3 +/- 1.4

Table 1. Rate constants for the reaction of *trans*-cyclooctenes with 3,6-diphenyl-*s*-tetrazine at 25°C in methanol measured under pseudo first order conditions using a UV-Vis spectrophotometer. Values were determined from the average of three runs.

General Procedure for the stopped-flow kinetic analysis of *trans*-cyclooctenes and 12



The reaction between *trans*-cyclooctenes and the PEGylated tetrazine **12** was measured under pseudo-first order conditions with 10 equivalents of TCO in water by following the exponential decay of the tetrazine at 322 nm over time using an SX 18MV-R stopped-flow spectrophotometer (Applied Photophysics Ltd.). Solutions were prepared for the TCO (1 mM in water) and the tetrazine (0.1 mM in water) and thermostatted in the syringes of the spectrophotometer before measuring. An equal volume of each was mixed by the stopped flow device resulting in a final concentration of 0.05 mM tetrazine and 0.5 mM TCO. The only exception was PEGylated s-TCO **14** (0.32 mM in water), which was measured with tetrazine (0.062 mM) at a final concentration of 0.160 mM and 0.031 mM respectively. Data was recorded for 0.1-5 seconds, depending on the *trans*-cyclooctene used, and performed in triplicate at 298 K. The k_{obs} was determined by nonlinear regression analysis of the data points using Prism software (v. 6.00, GraphPad Software Inc.). Results are displayed in table 2 and figure 2.

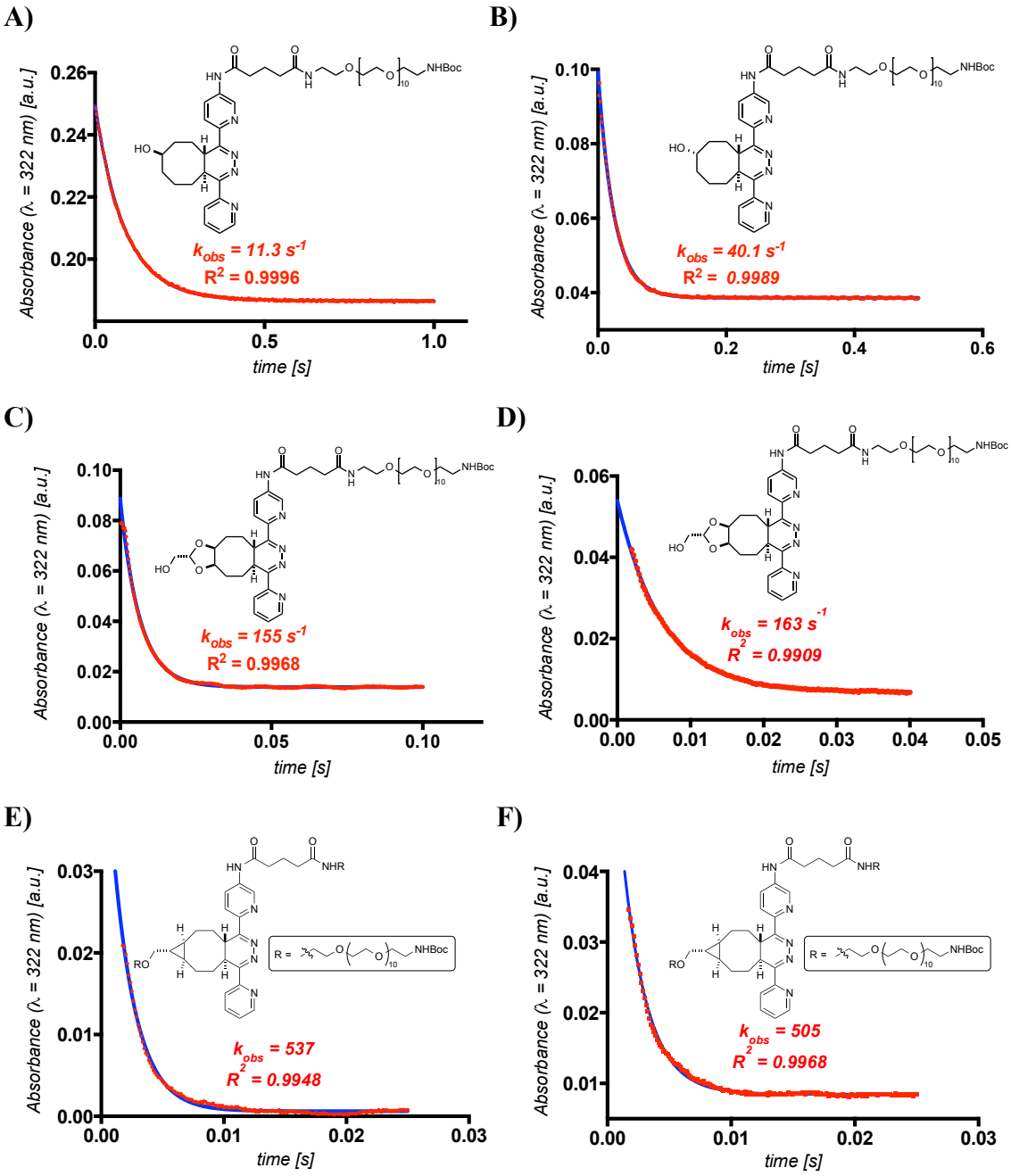
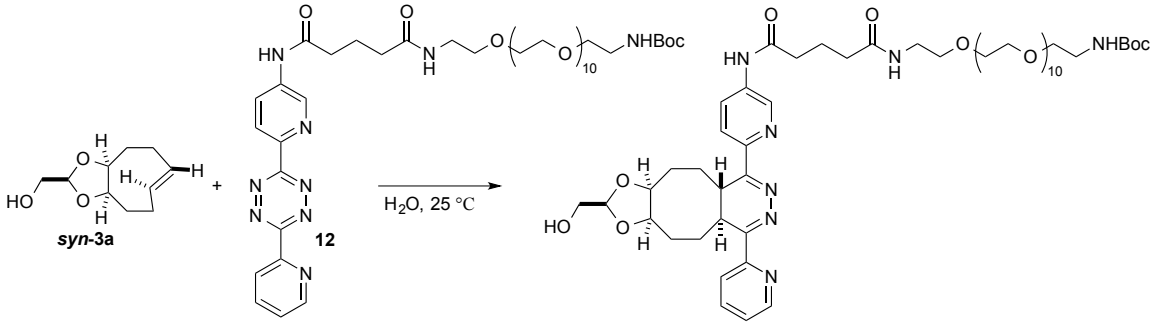


Fig. 2 Stopped-flow monitored reaction of **12** with **(A) 11**, **(B) 13**, **(C)** ***anti*-3a**, and **(E) 14** at 298K monitored at 322 nm. **(D)** Duplicate experiment of ***anti*-3a** with **12**. **(F)** Duplicate experiment of **14** with **12**.

Stopped-flow kinetic analysis of *syn*-3a and 12



The general procedure for the stopped-flow analysis of *trans*-cyclooctenes was followed using ***syn*-3a** (1 mM to 6 mM in water) and **12** (0.1 mM in water). Final concentrations were 0.05 mM for the tetrazine and 0.5 mM to 3.0 mM TCO. Each measurement was carried out in triplicate and the average of the observed rates k' was plotted against the concentration of ***syn*-3a** to obtain the bimolecular rate constant k_2 from the slope of the plot. This set was then duplicated and the mean k_2 reported as 366,000 (+/- 15,000) $M^{-1}s^{-1}$ [1] (Figure 3, Table 2).

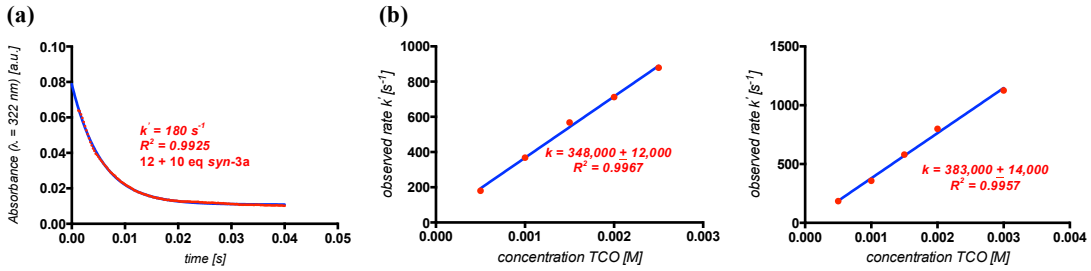
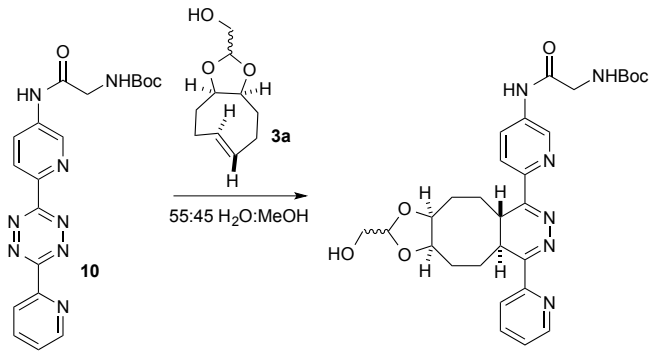


Fig 3. Determination of the bimolecular rate constant k from the stopped-flow monitored reaction of ***syn*-3a** and **12**. **(a)** The exponential plot of the reaction of **12** with 10 equivalents of ***syn*-3a** at 298 K monitored at 322 nm. **(b)** The observed rates k' of duplicate sets of measurements were plotted against the concentration of ***syn*-3a** to obtain the bimolecular rate constant k_2 from the slope of the plot. The mean of the two sets of measurements is reported.

TCO	k_2 ($M^{-1}s^{-1}$)
11 (equatorial)	22,600 +/- 40
13 (axial)	80,200 +/- 200
***syn*-3a**[a,b]	366,000 +/- 15,000
***anti*-3a**[b]	318,000 +/- 2900
14[b]	3,300,000 +/- 40,000

Table 2. Rate constants k_2 for the reaction of various *trans*-cyclooctenes with PEGylated tetrazine **12** at 25°C in water measured in triplicate under pseudo first order conditions (10 equivalents of TCO) using a stopped-flow spectrophotometer. [a]Value determined from plot of observed rate constant, k', and concentration of TCO using 10-60 equivalents of TCO. [b]Values were determined from the average of two runs.

Stopped flow kinetic analysis of 3a and 10



The general procedure for the stopped flow analysis of *trans*-cyclooctenes was followed using **3a** (1 mM to 5 mM) and **10** (0.1 mM) in 55:45 H_2O:MeOH. Final concentrations were 0.5 mM to 2.5 mM for the TCO and 0.05 mM for the tetrazine. Each measurement was carried out in triplicate and the average of the observed rates k' was plotted against the concentration of **3a** to obtain the bimolecular rate constant k_2 from the slope of the plot. This set was then duplicated and the average k_2 reported as 167,000 (+/- 7,000) $M^{-1}s^{-1}$ (Figure 4).

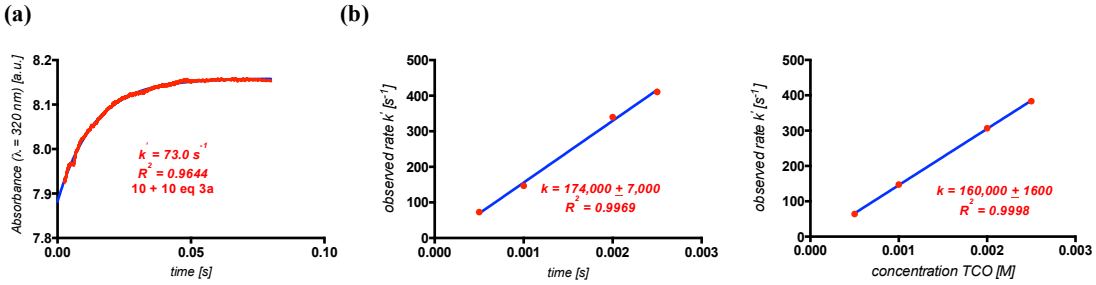


Fig 4. Determination of the bimolecular rate constant k from the stopped-flow monitored reaction of **3a** and **10**. **(a)** The exponential plot of the reaction of **10** with 10 equivalents of **3a** at 298 K monitored at 320 nm. **(b)** The observed rates k' of duplicate sets of measurements were plotted against the concentration of **3a** to obtain the bimolecular rate constant k from the slope of the plot. The mean of the two sets of measurements is reported.

***In vitro* kinetic analysis of tetrazine containing GFP (2) with 3b**

Compound **3b** (4.52 mg, 0.178 μmol) was dissolved in 10 mL 1:4 EtOH:1x PBS to make a 1.78 mM solution. A solution of GFP-tetrazine **2** (1 mL, 3.32 mg/mL in 1x PBS) was diluted with 10 mL 1x PBS to make a 0.012 mM solution. The TCO and GFP solutions were further diluted 10x with 1x PBS to obtain 0.178 mM and 0.0012 mM solutions respectively. Kinetic trials were initiated by adding 30 μL TCO solution to 20 μL of GFP-tetrazine solution in a 50 μL fluorescence cuvette and monitored by observing the fluorescence increase of the cuvette using a Horiba Jobin Yvon FluoroMax®-4 fluorometer. Fluorescence was measured at 22°C in 2 second increments using the following instrument parameters: excitation 488 nm with 2 nm slit, emission 506 nm with 5 nm slit, 0.1 second integration time. Measurements were carried out in triplicate and rate constants were determined using Prism software (v. 6.00, Graphpad Software Inc). The k_{obs} was 1.05 x 10^{-2} (+/- 6.74 x 10^{-5}) s^{-1} and the second order rate constant was calculated to be 99 (+/- 0.6) $M^{-1}s^{-1}$. An identical procedure was performed with ***syn*-3a** in which the k_{obs} was 1.01 x 10^{-2} (+/- 3.32 x 10^{-5}) with a second order rate constant k_2 = 95 (+/- 0.3) $M^{-1}s^{-1}$.

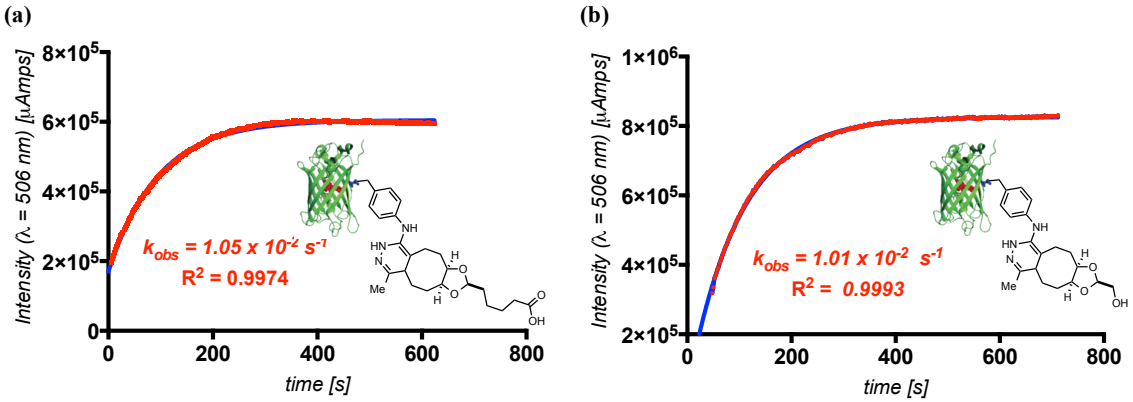
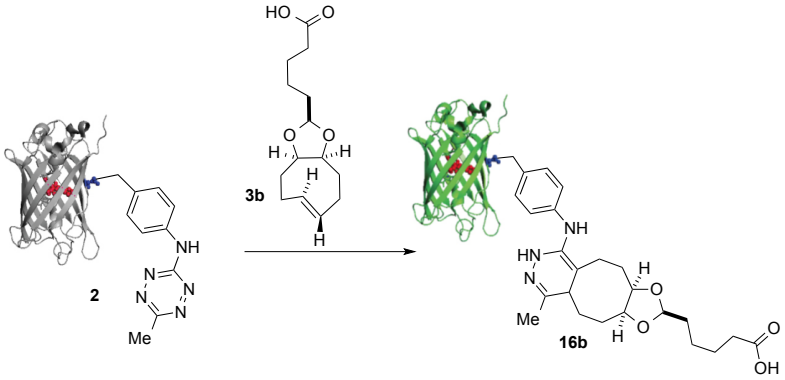


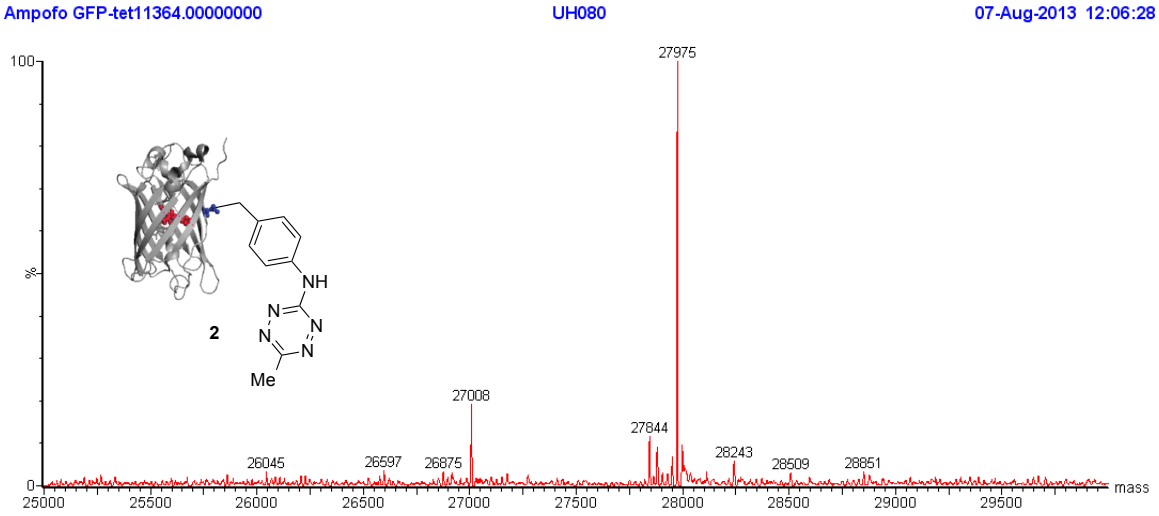
Fig. 4 *In vitro* reaction of GFP-tetrazine **2** with **(a) 3b** and **(b) *syn*-3a** monitored by fluorescence increase of product

***In vitro* labeling of GFP-tetrazine 2 with 3b**



GFP-tetrazine **2** (20 μL, 0.2 mg/mL in water), was incubated with **3b** (30 μL, 0.3 mg/mL in 1:1 water:1x PBS) for 5 minutes at room temperature. The sample was desalted using a protein spin desalting column and analyzed by mass spectrometry (Q-TOF MS). A sample with GFP-tetrazine without *trans*-cyclooctene was run in parallel (Figure 5).

A)



B)

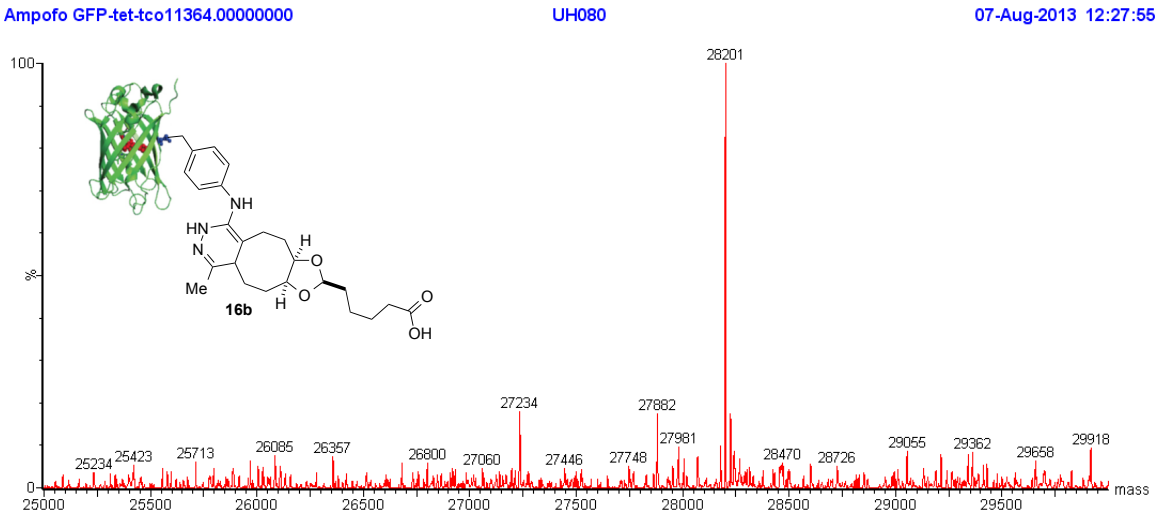
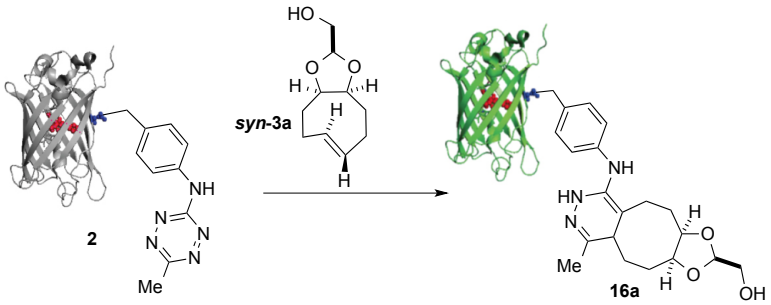


Fig. 5 *In vitro* labeling of GFP-1 with **3b**. **(A)** Q-TOF MS analysis shows a single major peak at 27975 Da which signifies GFP-1 without the incubation of TCO while **(B)** shows a single major peak at 28201 Da which shows the expected molecular weight difference of 226 Da from GFP-1 demonstrating specific conversion to GFP-1-TCO.

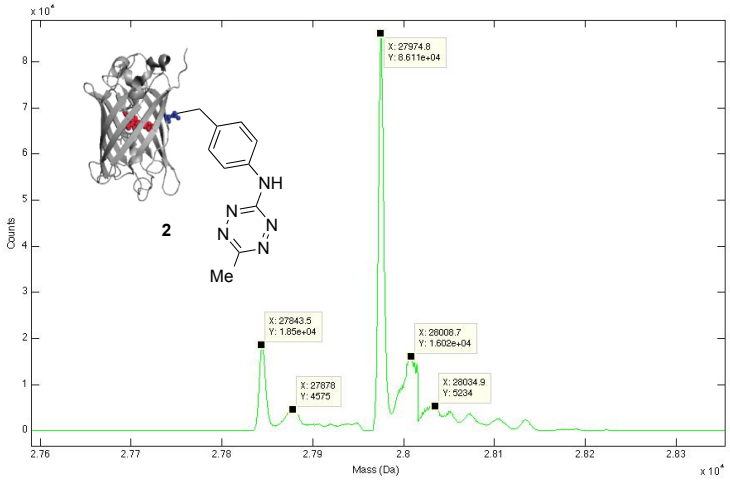
***In vivo* labeling of GFP-tetrazine 2 with *syn*-3a**



GFP-tetrazine **2** was expressed in DH10b *E. coli* as previously described.[7] A cell pellet from 100 mL of expression was thawed on ice and resuspended in 50 mL of PBS. The cells were washed by centrifugation at 5000 rcf and suspended in 5 mL PBS buffer pH 7.5. Washed cells (2.5 mL) containing GFP-tetrazine **2** in PBS buffer was incubated with ***syn*-3a** at a final concentration of 900 μM (final total reaction volume was 2.6 mL). The reaction was run for 2 hrs at room temperature. The unreacted ***syn*-3a** was removed by pelleting the cells before the GFP-tetrazine **2** was purified with BD-TALON cobalt ion-exchange chromatography as described below. Control reactions with GFP-tetrazine **2** (no ***syn*-3a** added**)** were run in parallel and purified under identical conditions.

For BD-TALON cobalt ion-exchange chromatography, the cell pellet was resuspended in wash buffer (50 mM sodium phosphate, 300 mM sodium chloride, pH 7). Cells were lysed using a microfluidizer while cooled on ice. The lysate was clarified by centrifugation, applied to 0.3 mL bed-volume resin, and bound for 30 min. Bound resin was washed with >50 volumes wash buffer. Protein was eluted from the bound resin with 0.5 mL aliquots of elution buffer (50 mM sodium phosphate, 300 mM sodium chloride, 150 mM imidazole pH 7) until the resin turned pink and the color of the eluent the column was no longer green. The elution concentrations were checked with a Bradford protein assay. The protein was desalted into 20 mM ammonium acetate using PD10 columns and analyzed by mass spectrometry (ESI-MS-Tof, Figure 6).

A)



B)

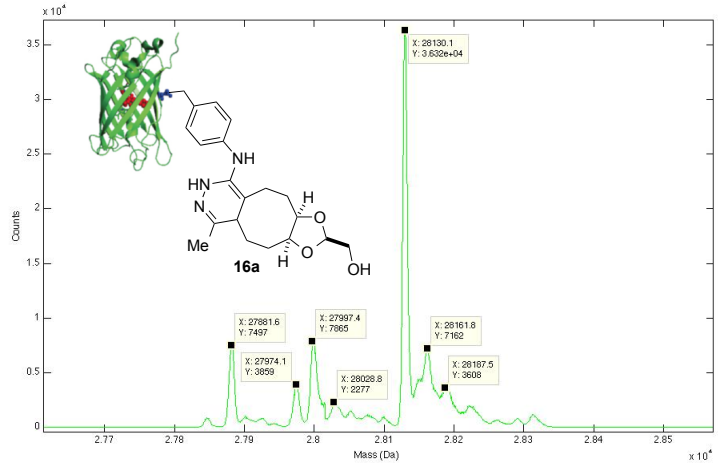


Fig. 6 MS characterization of GFP-tetrazine labeled with ***syn*-3a** *in vivo*. ESI-MS of proteins; GFP-tetrazine **2**, and GFP-tetrazine-***syn*-3a**, **16a**, demonstrates specific and near quantitative labeling of GFP-tetrazine. (A) ESI-MS-Tof analysis of GFP-tetrazine shows a single major peak at 27975.0 Da ±1 Da. This shows the expected molecular weigh difference of 144 Da from native indicating a single efficient incorporation of **15** at the expected site. (B) ESI-MS-Tof analysis of GFP-tetrazine incubated with *syn*-**3a** shows a single major peak at 28130.0 Da ±1 Da. This shows the expected molecular weight difference of 155 Da from GFP-tetrazine demonstrating specific and near quantitative conversion to **16a**. Each sample did show a small peak at -131 ±1 Da indicating minor amounts of peptidase-based removal of N-terminal methionines and +22 sodium adducts.

Stability of 3a in methanol-d_4

A solution of **3a** (1:1 syn:anti, 0.092 g, 0.500 mmol) in methanol-d_4 (1 mL) was monitored by ^{1}H NMR to observe the stability of **3a**. After 3 days, 5% of the *cis*-isomers were observed. After 7 days, 85% of ***syn*-3a** remain and 83% of ***anti*-3a** remain. A waterfall plot of the NMR spectra is displayed.

Stability of *syn*-3a in phosphate buffered D_2O (pD = 7.4)

A solution of ***syn*-3a** (3.2 mg, 0.017 mmol) in phosphate buffered D_2O (1 mL, pD = 7.4) was added 4-methoxybenzoic acid (2.8 mg, 0.018 mmol) as an internal standard and monitored by ^{1}HNMR to observe the stability of ***syn*-3a**. No isomerization was observed after 14 days. A waterfall plot of the NMR spectra is displayed.

Stability of *syn*-3a in mercaptoethanol in D_2O (pD = 7.4)

To a solution of ***syn*-3a** (5.5 mg, 0.030 mmol) in phosphate buffered D_2O (pD = 7.4, 1 mL) was added 2-mercaptoethanol (2.0 μL, 0.030 mmol) and transferred to an NMR tube. 4-Methoxybenzoic acid (4.5 mg, 0.030 mg) was added as an internal standard. The solution was monitored by ^{1}HNMR to observe the isomerization of ***syn*-3a** to ***syn*-5**. After 24 hours, 45 % of ***syn*-3a** remained. Results were plotted using Prism software (v. 6.00, Graphpad Software Inc, Fig 7). A waterfall plot of the NMR spectra is displayed.

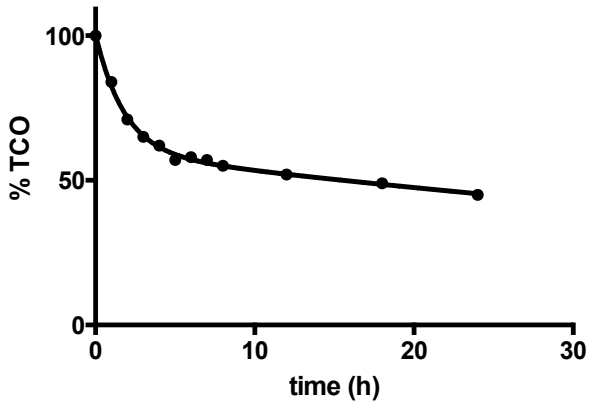


Fig. 7 Stability profile of ***syn*-3a** (30 mM) in the presence of mercaptoethanol (30 mM) in phosphate buffered D_2O (pD = 7.4).

Stability of 3a in mercaptoethanol in phosphate buffer (pH = 6.8)

To a solution of **3a** (1:1 syn:anti, 11.8 mg, 0.064 mmol) in 2.1 mL phosphate buffer (pH 6.82) was added an equimolar amount of mercaptoethanol (5.0 mg, 4.4 µL, 0.064 mmol) at room temperature. Aliquots of the solution were taken and titrated with 3,6-di-(2-pyridyl)tetrazine (5.0 mM, 0.2 mL) to determine the concentration of *trans*-cyclooctene in the mixture. The *trans*-cyclooctene **3a** was stable for 6 hours and gradually isomerized thereafter (Table 3).

Time (h)	Cis:trans
1	0:100
3	0: 100
6	0:100
8	16:84
24	16:84
28	20:80
31	32:68
48	44:56
6 days	80:20

Table 3. Stability of **3a** (30 mM) in the presence of mercaptoethanol (30 mM) in phosphate buffer (pH = 6.82). Cis:trans ratios were measured by titration of the reaction mixture with 3,6-di-(2-pyridyl)-*s*-tetrazine.

Stability of *syn*-3a and s-TCO with mercaptoethanol in methanol-d_4

To a solution of ***syn*-3a** (5.5 mg, 0.030 mmol) in methanol-d_4 (1 mL) was added 2-mercaptoethanol (2.1 µL, 0.30 mmol) with mesitylene (4.14 µL, 0.030 mmol) as an internal standard. Isomerization of ***syn*-3a** to ***syn*-5** was monitored by ^{1}HNMR. After an induction period of 10 hours, 92% of ***syn*-3a** isomerized to ***syn*-5** over a 14 hour period, with a half-life of 5.3 hours. An identical experiment was performed with s-TCO (4.6 mg, 0.030 mmol) in parallel. After an 8 hour induction period, s-TCO completely isomerized in 4 hours with a half-life of 1.4 hours. Results were plotted using Prism software (v. 6.00, Graphpad Software Inc, Fig 8). Waterfall plots of the NMR spectra is displayed.

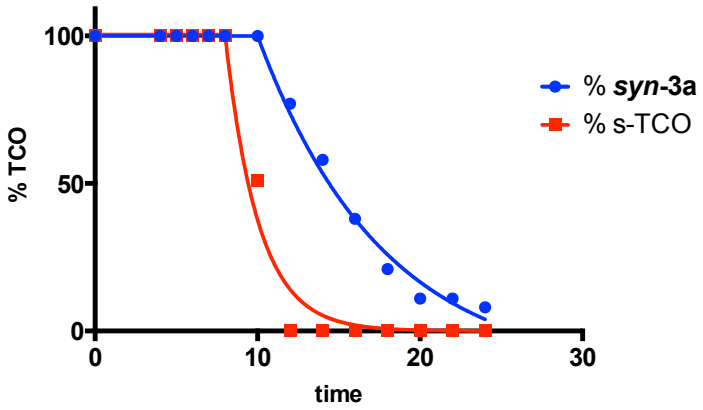


Fig. 8 Stability profiles of ***syn*-3a**, and s-TCO with mercaptoethanol (30 mM) in methanol-d_4

Stability of TCOs with glutathione in D_2O (pD 7.4)

To a solution of ***syn*-3a** (1.86 mg, 0.010 mmol) in phosphate buffered D_2O (pD 7.4, 1 mL) was added glutathione (3.08 mg, 0.010 mmol) and transferred to an NMR tube. 4-Methoxybenzoic acid (1.53 mg, 0.010 mmol) was used as an internal standard. The solution was monitored by ^{1}HNMR to observe the isomerization of ***syn*-3a** to ***syn*-5**. An identical experiment was performed with **9** (1.41 mg, 0.010 mmol) and **3b** (2.60 mg, 0.010 mmol), monitoring for isomerization to **4** and **6**, respectively. Results were plotted using Prism software (v. 6.00, Graphpad Software Inc, Fig 9). Waterfall plots of the NMR spectra are displayed.

(a) **(b)**

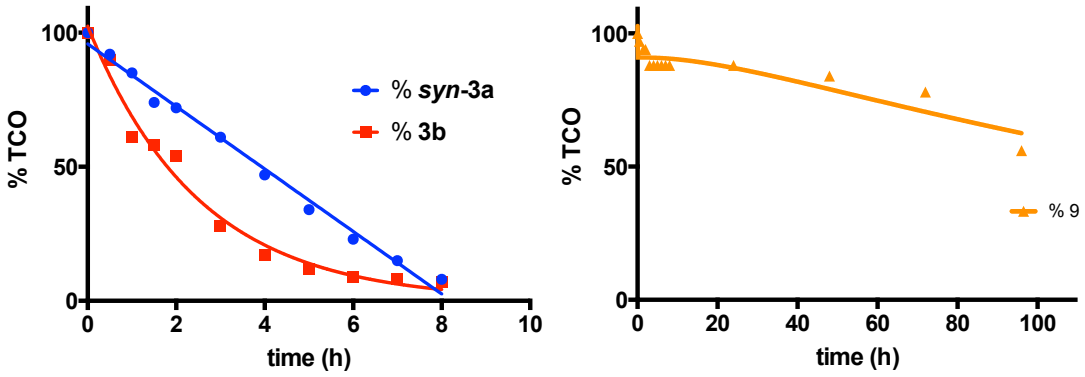


Fig. 9 Stability profiles of (a) ***syn*-3a**, **3b**, and (b) **9** with glutathione (10 mM) in phosphate buffered D_2O (pD = 7.4)

Stability of *syn*-3a in rabbit reticulocyte lysate

***Syn*-3a** (1.9 mg, 0.010 mmol) was dissolved in a solution of rabbit reticulocyte lysate (1 mL, 10% solution in phosphate buffered D_2O, pD = 7.4) and transferred to an NMR tube. 4-Methoxybenzoic acid (1.6 mg, 0.011 mmol) was added as an internal standard. Isomerization of ***syn*-3a** to ***syn*-5** was monitored by ^{1}HNMR and results plotted using Prism software (v. 6.00, Graphpad Software, Fig 10). A waterfall plot of the NMR spectra is displayed.

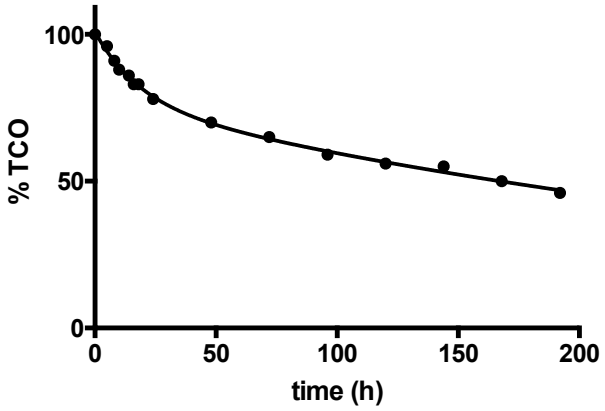


Fig. 10 Stability profiles of ***syn*-3a** (10 mM) with rabbit reticulocyte lysate (10% in phosphate buffered D_2O, pD = 7.4)

Stability of *syn*-3a in Human Blood Serum

Compound ***syn*-3a** (10.0 mg, 0.054 mmol) was added to human blood serum (10 mL, from human male AB plasma) and stirred for 24 hours. Stirring was stopped and the solution extracted with CH_2Cl_2 (4 x 5 mL), dried with $MgSO_4$, filtered, and concentrated. The residue was purified by silica gel chromatography using a pipet column, recovering 8 mg of ***syn*-3a.** An ^{1}H NMR spectrum showed ***syn*-3a** without any sign of isomerization. Another experiment was performed where ***syn*-3a** (10.0 mg, 0.054 mmol) was allowed to stir in human blood serum (10 mL) for 4 days. An ^{1}H NMR analysis of the crude residue after extraction showed 3% isomerization of ***syn*-3a**. The ^{1}H NMR spectra are displayed.

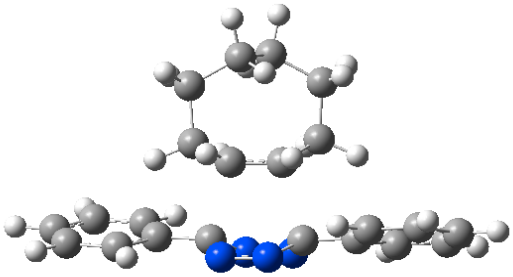
Partition Coefficient (log*P*) of *syn*-3a

***Syn*-3a** (5.30 mg, 0.029 mmol) was added to a 1:1 solution of octanol:water (2 mL) and stirred vigorously for 12 hours. The phases were separated and the organic phase was dried with Na_2SO_4. Both layers were then titrated with a 5 mM solution of 3,6-di-(2-pyridyl)-*s*-tetrazine. The organic phase was found to contain 27 mM of ***syn*-3a**, while the aqueous phase contained 3 mM. Log*P* was calculated to be 0.95.

Computational Studies

1a

M06L/6-311+G(d,p) RESULTS



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Thermal correction to Gibbs Free Energy= 0.364152
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Sum of electronic and thermal Enthalpies= -1071.393514
Sum of electronic and thermal Free Energies= -1071.470561
Charge = 0 Multiplicity = 1

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TS

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Thermal correction to Enthalpy= 0.440588

Thermal correction to Gibbs Free Energy= 0.368585

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Charge = 0 Multiplicity = 1

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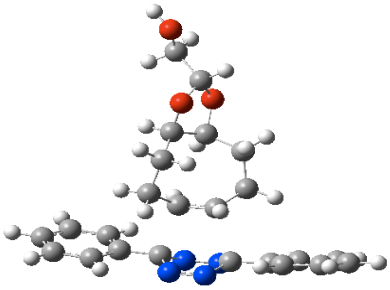

3a

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TS
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H 5.0356364167,-2.4046531263,2.261110654
H 5.193199615,-2.5861717446,-2.023216436
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H 1.5808258652,5.4322033563,0.215732125

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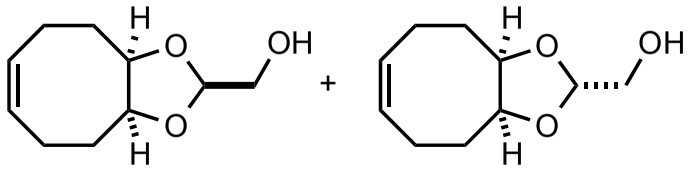
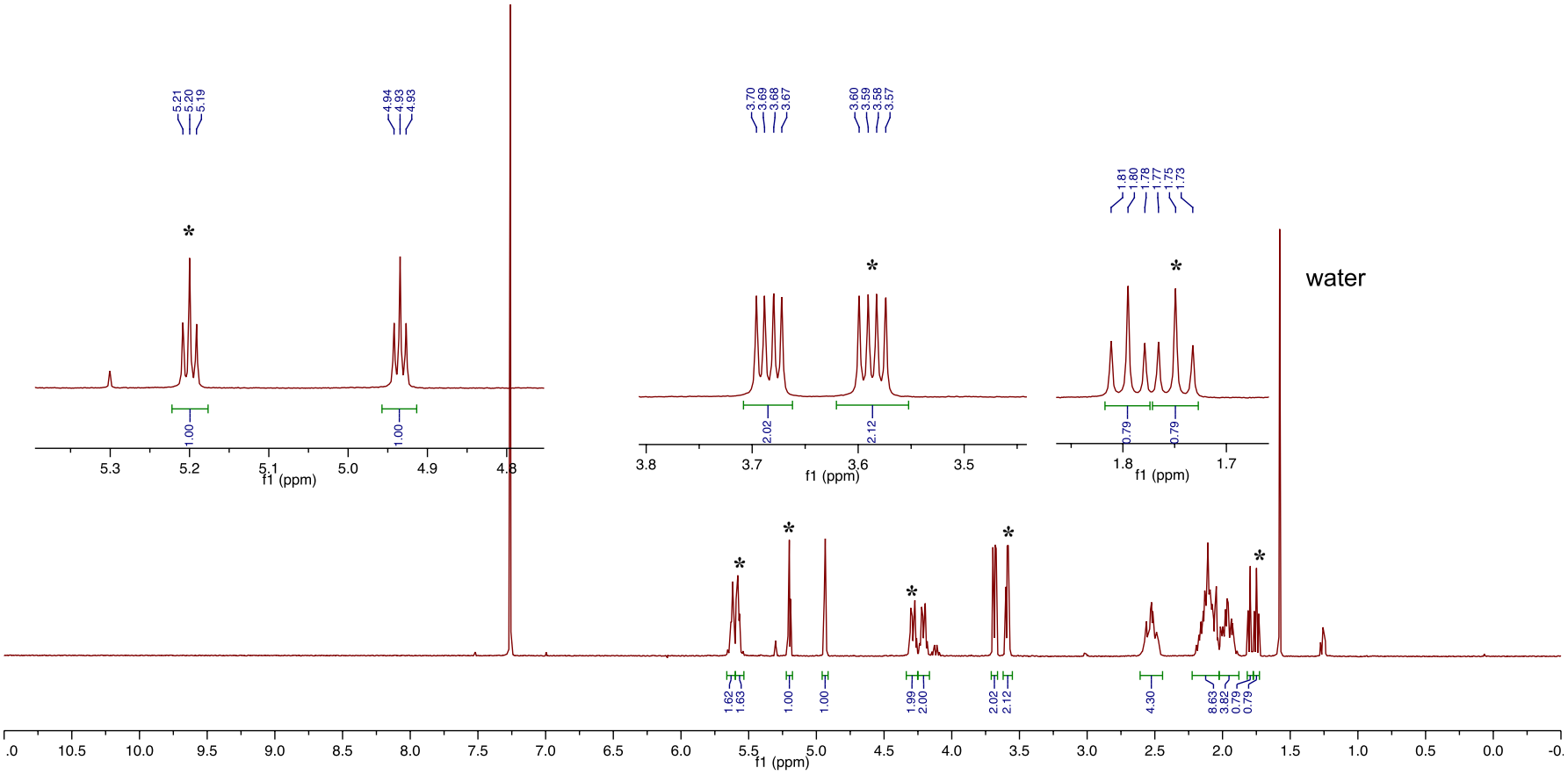
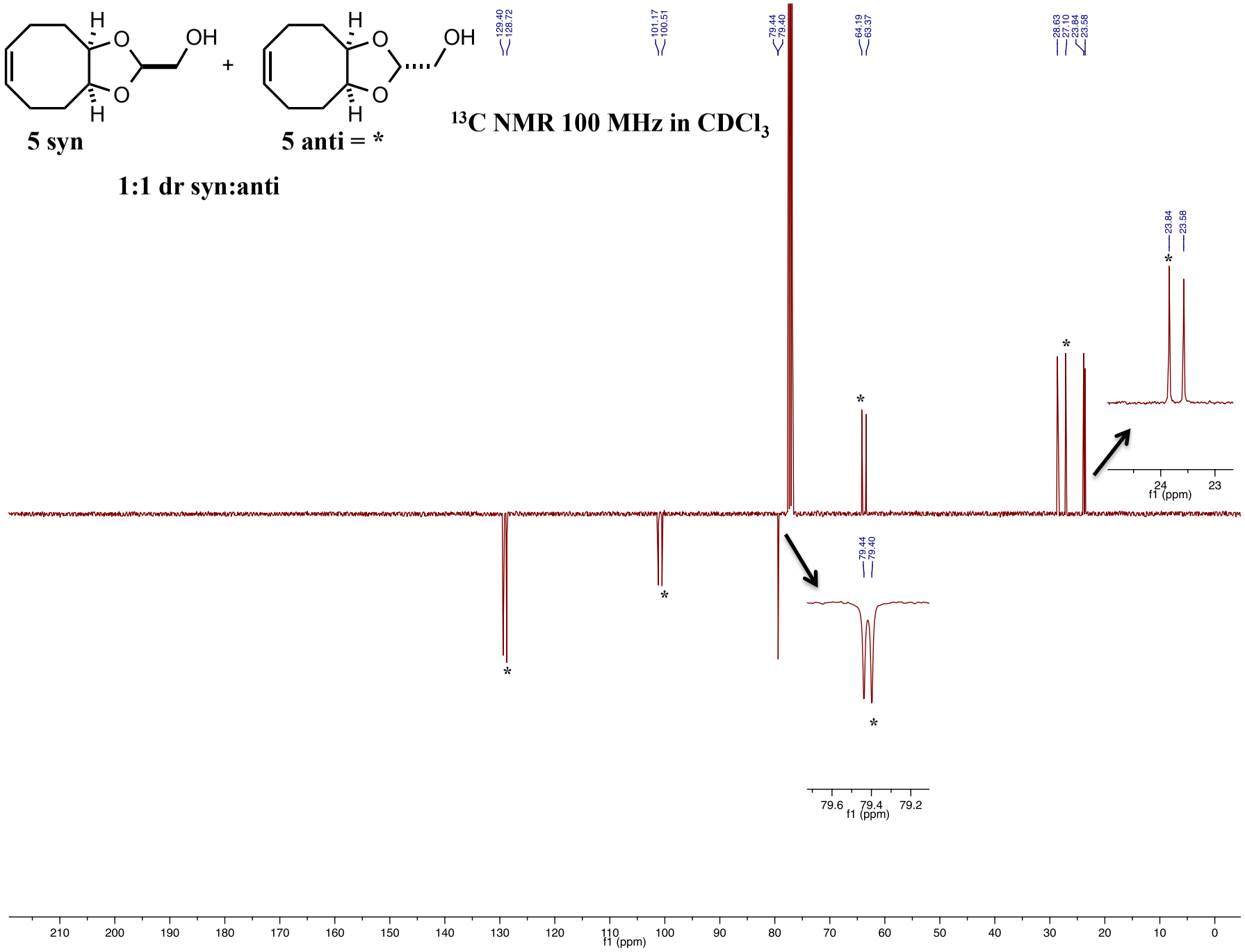


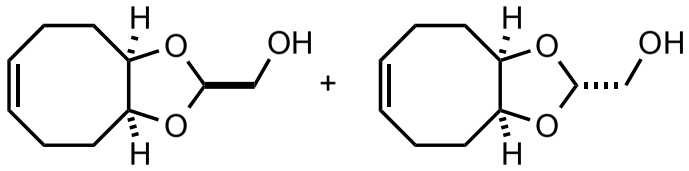
5 syn + **5 anti = ***

1:1 dr syn:anti

^{1}H NMR 400 MHz in $CDCl_3$



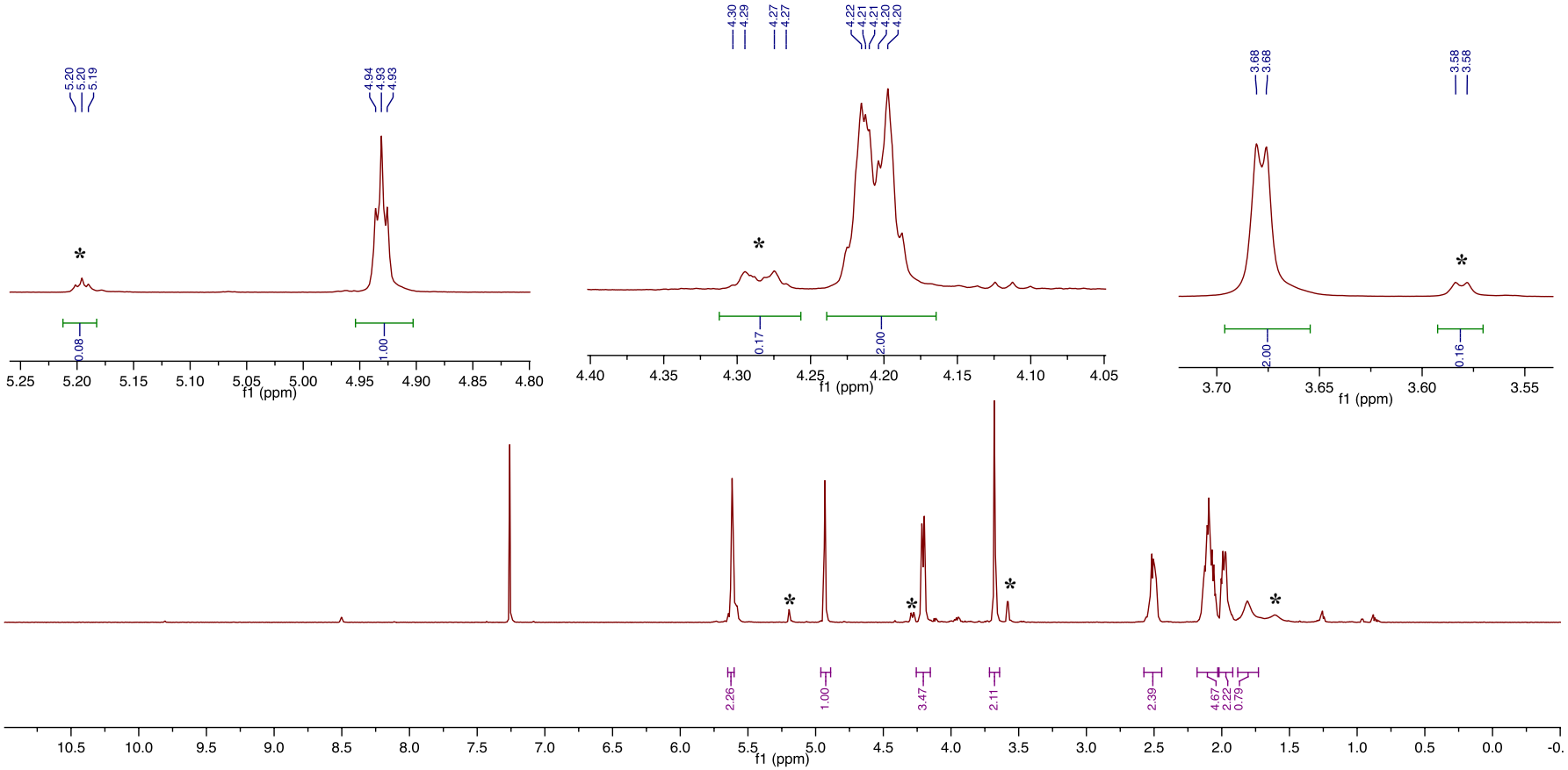

5 syn
5 anti = *
1:1 dr syn:anti
^{13}C NMR 100 MHz in CDCl$_3$
129.40
128.72
101.17
100.51
79.44
79.40
64.19
63.37
28.63
27.10
23.84
23.58
f1 (ppm)

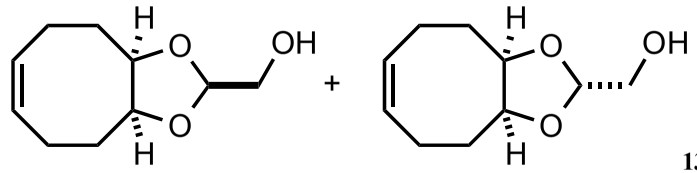


5 syn **5 anti = ***

12:1 dr syn:anti

^{1}H NMR 600 MHz in $CDCl_3$

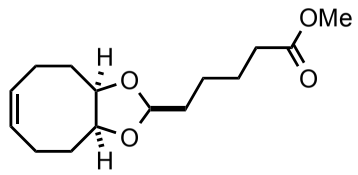




5 syn + **5 anti = ***

12:1 dr syn:anti

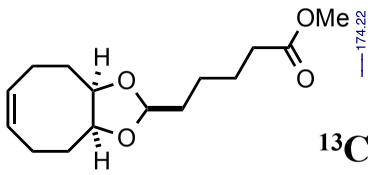
^{13}C NMR 100 MHz in $CDCl_3$



^{1}H NMR 400 MHz in $CDCl_3$

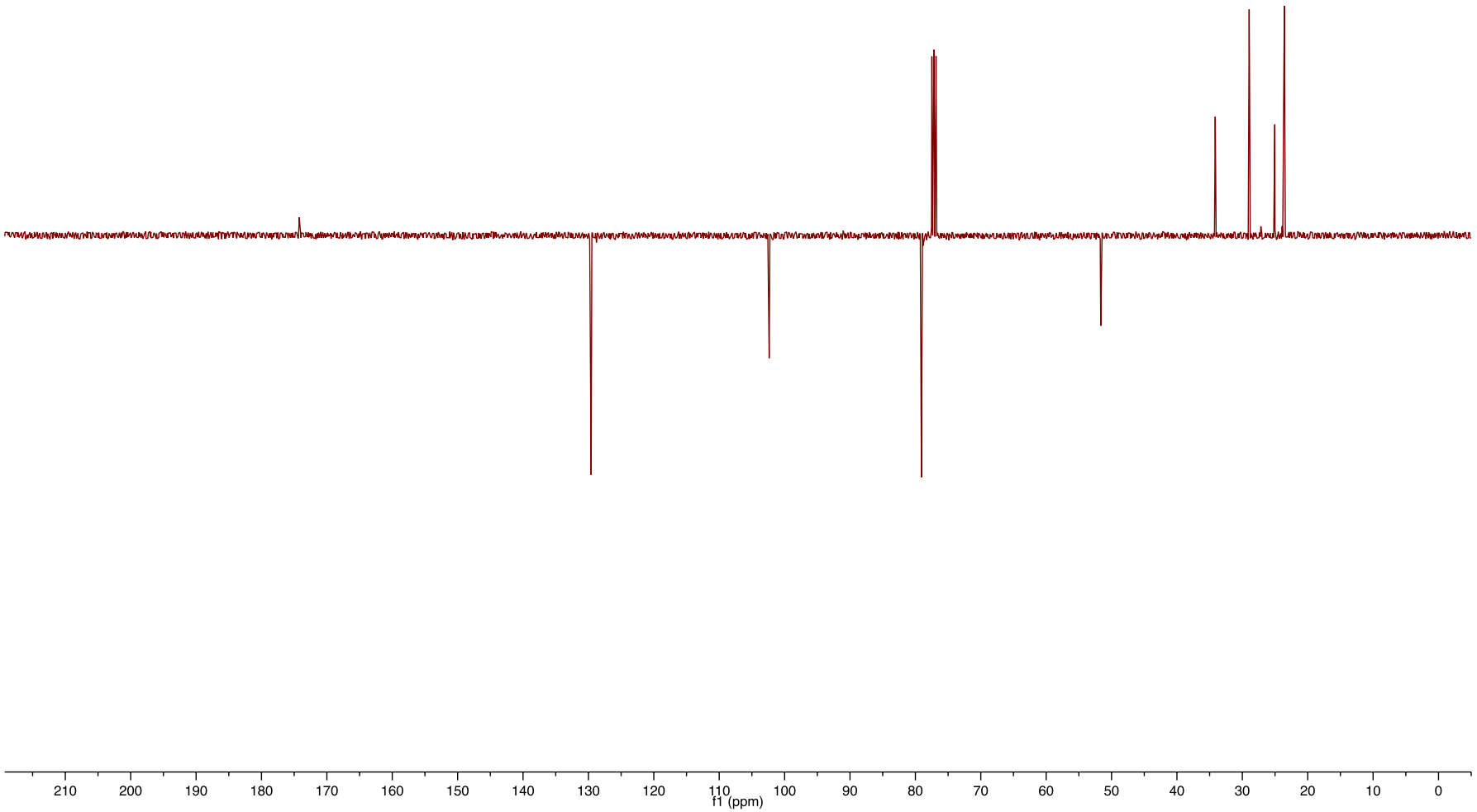
*** = anti**

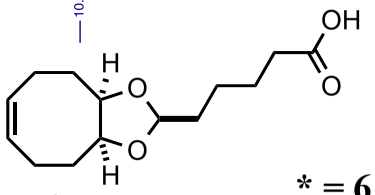
>25:1 dr syn : anti



^{13}C NMR 100 MHz in $CDCl_3$

>25:1 dr



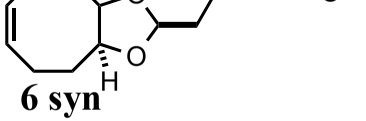


6 syn

^{1}H NMR400 MHz in $CDCl_3$

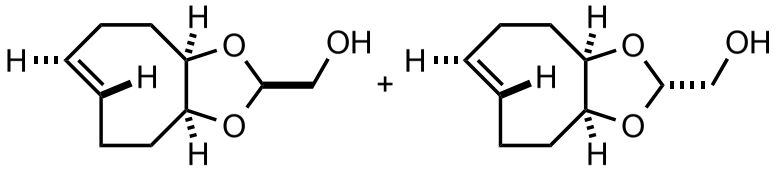
*** = 6 anti**

>50:1 dr



6 syn

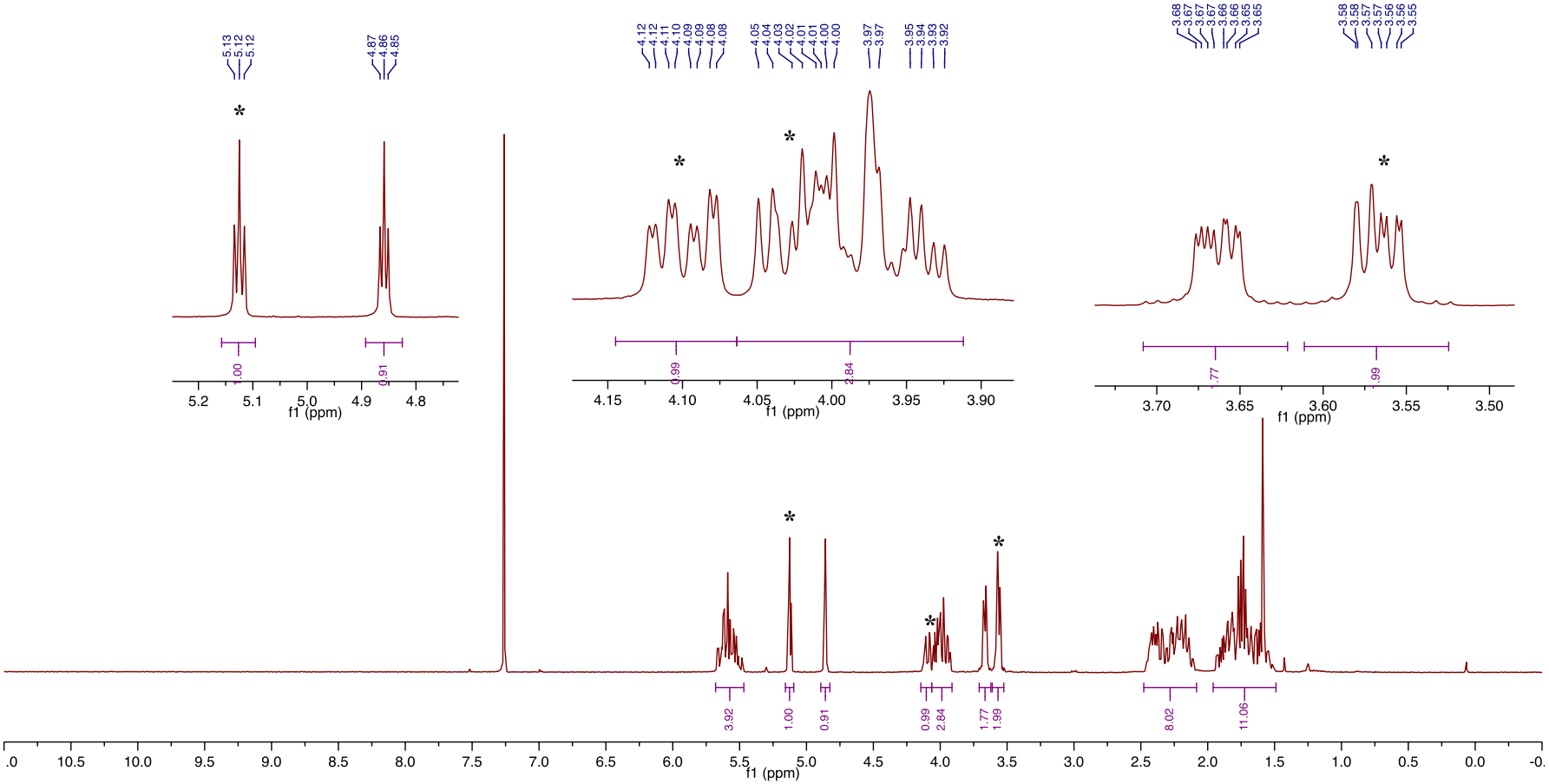
>50:1 dr **100 MHz in $CDCl_3$**

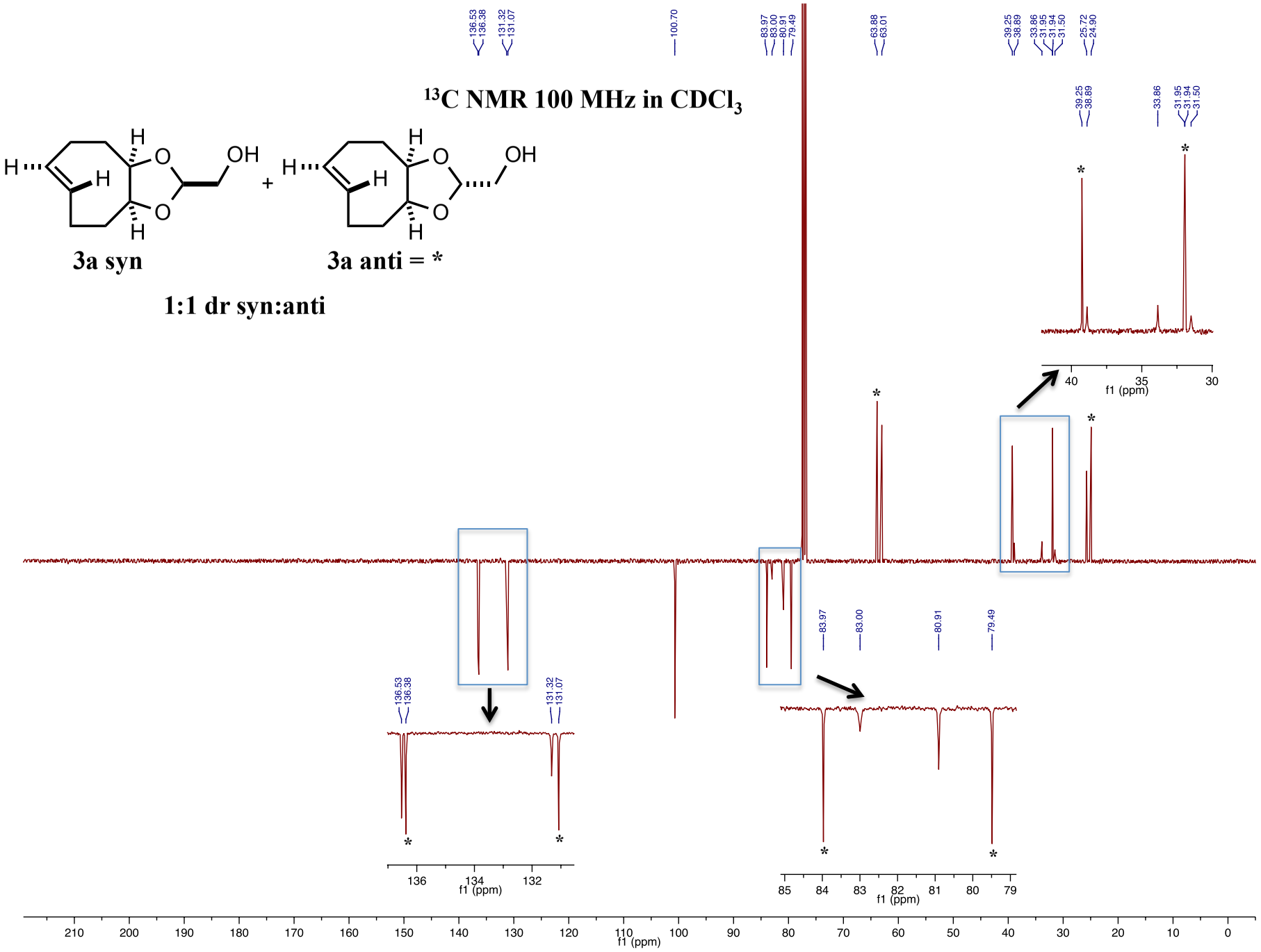


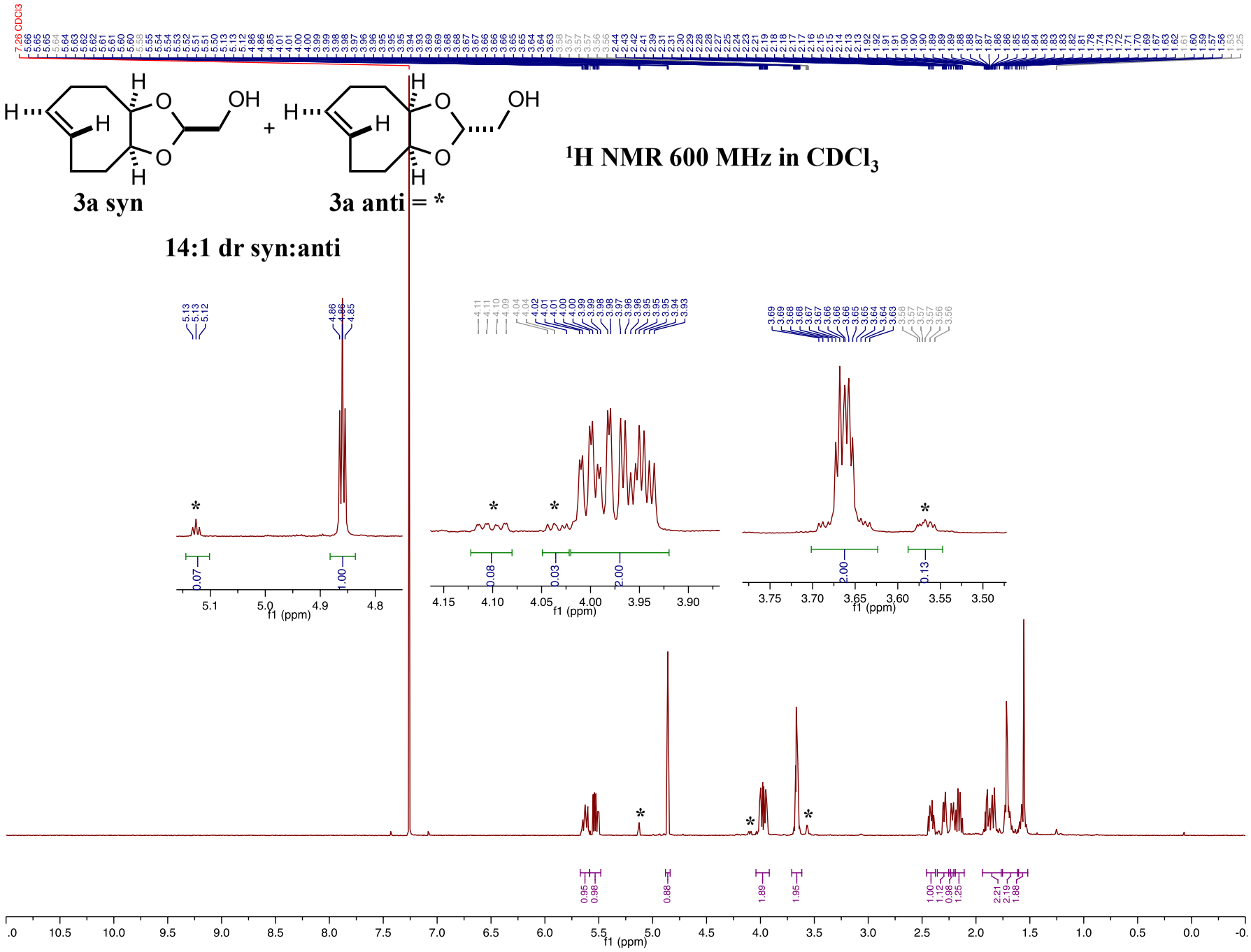
3a syn + 3a anti = *

^{1}H NMR 400 MHz in $CDCl_3$

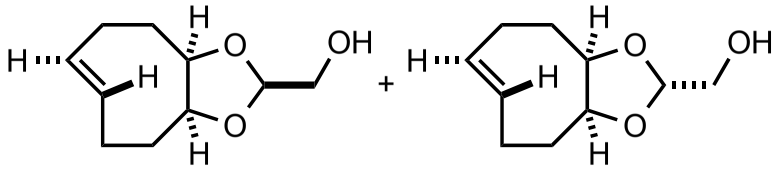
1:1 dr syn:anti




136.53
136.38
131.32
131.07
100.70
83.97
83.00
80.91
79.49
63.88
63.01
39.25
38.89
33.86
31.95
31.94
31.50
25.72
24.90
^{13}C NMR 100 MHz in CDCl$_3$
3a syn
3a anti = *
1:1 dr syn:anti
f1 (ppm)

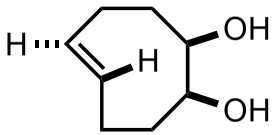

3a syn
3a anti = *
14:1 dr syn:anti
^{1}H NMR 600 MHz in CDCl$_3$
f1 (ppm)

^{13}C NMR 100 MHz in $CDCl_3$



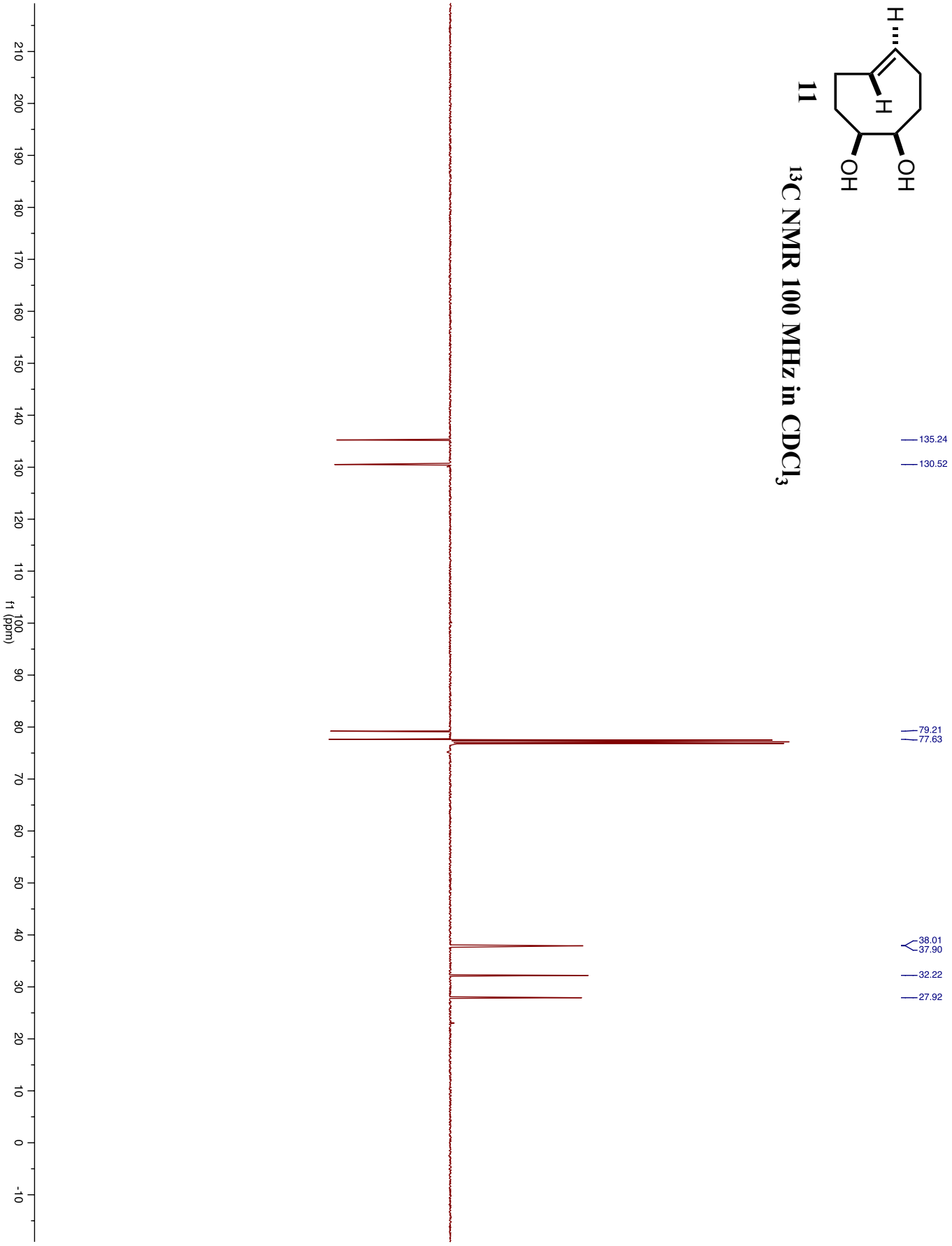
3a syn + **3a anti = ***

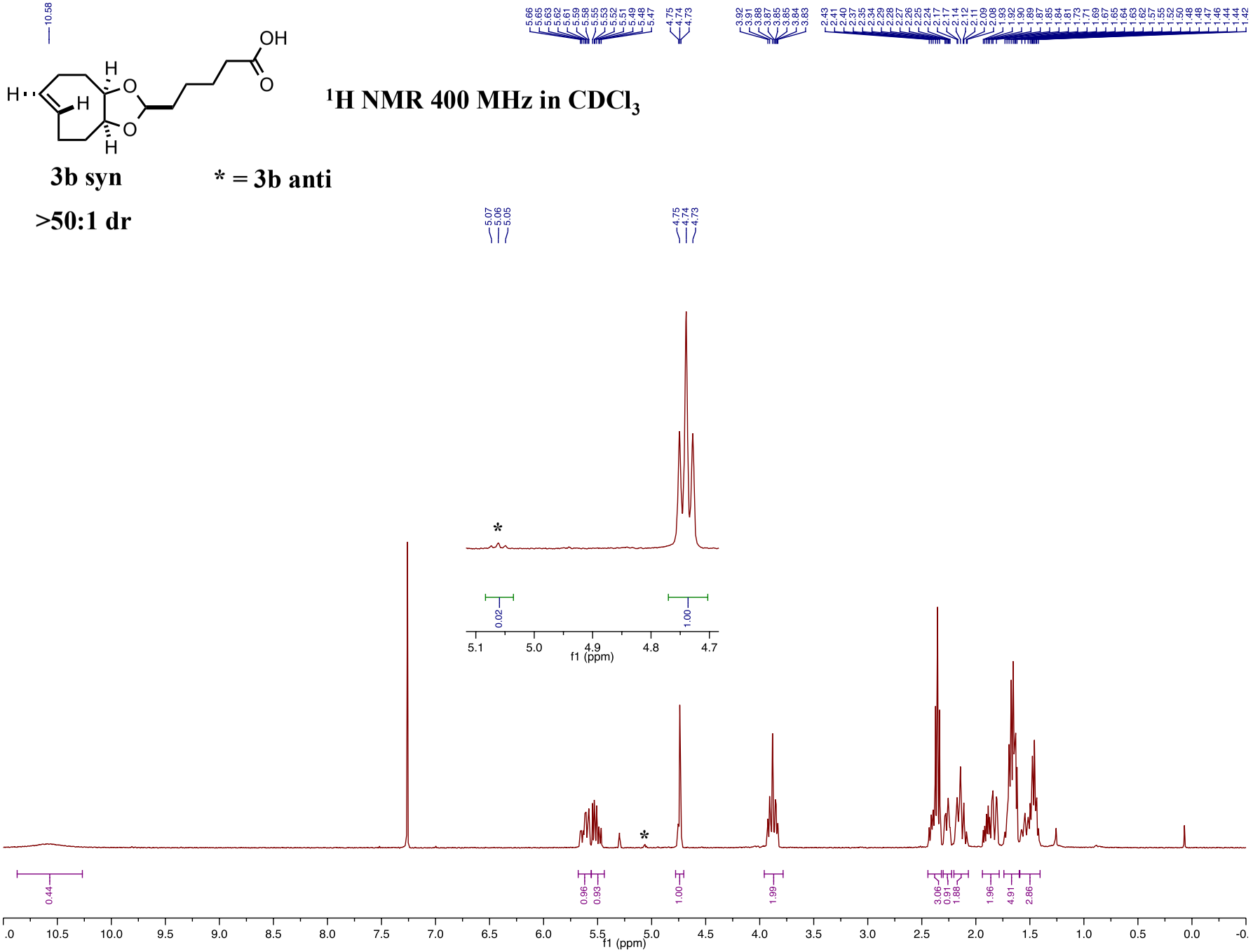
14:1 dr syn:anti



11

^{1}H NMR 400 MHz in $CDCl_3$


11
^{13}C NMR 100 MHz in CDCl$_3$
135.24
130.52
79.21
77.63
38.01
37.90
32.22
27.92
f1 (ppm)

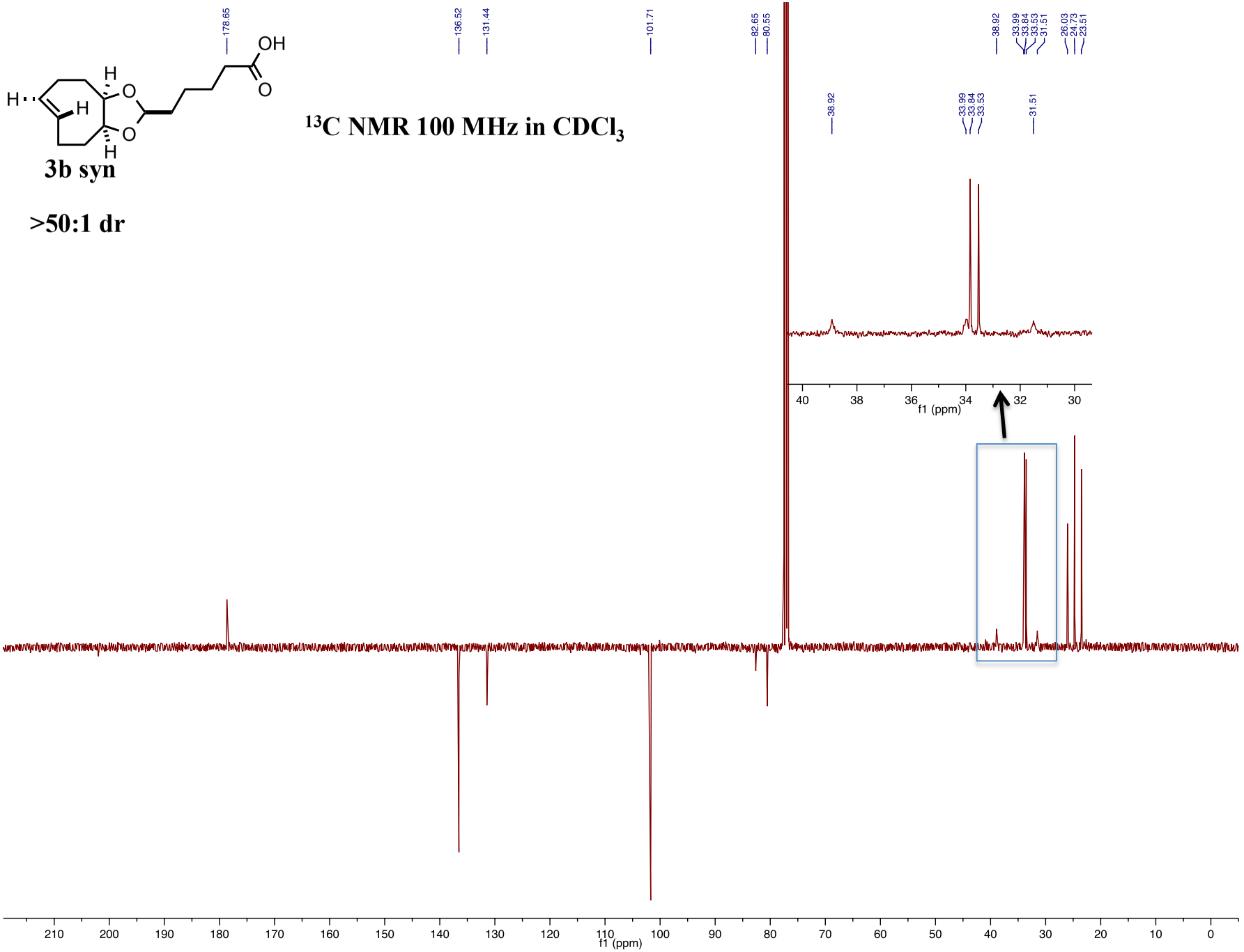


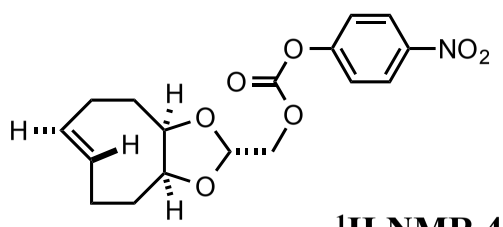
^{1}H NMR 400 MHz in $CDCl_3$

3b syn

>50:1 dr

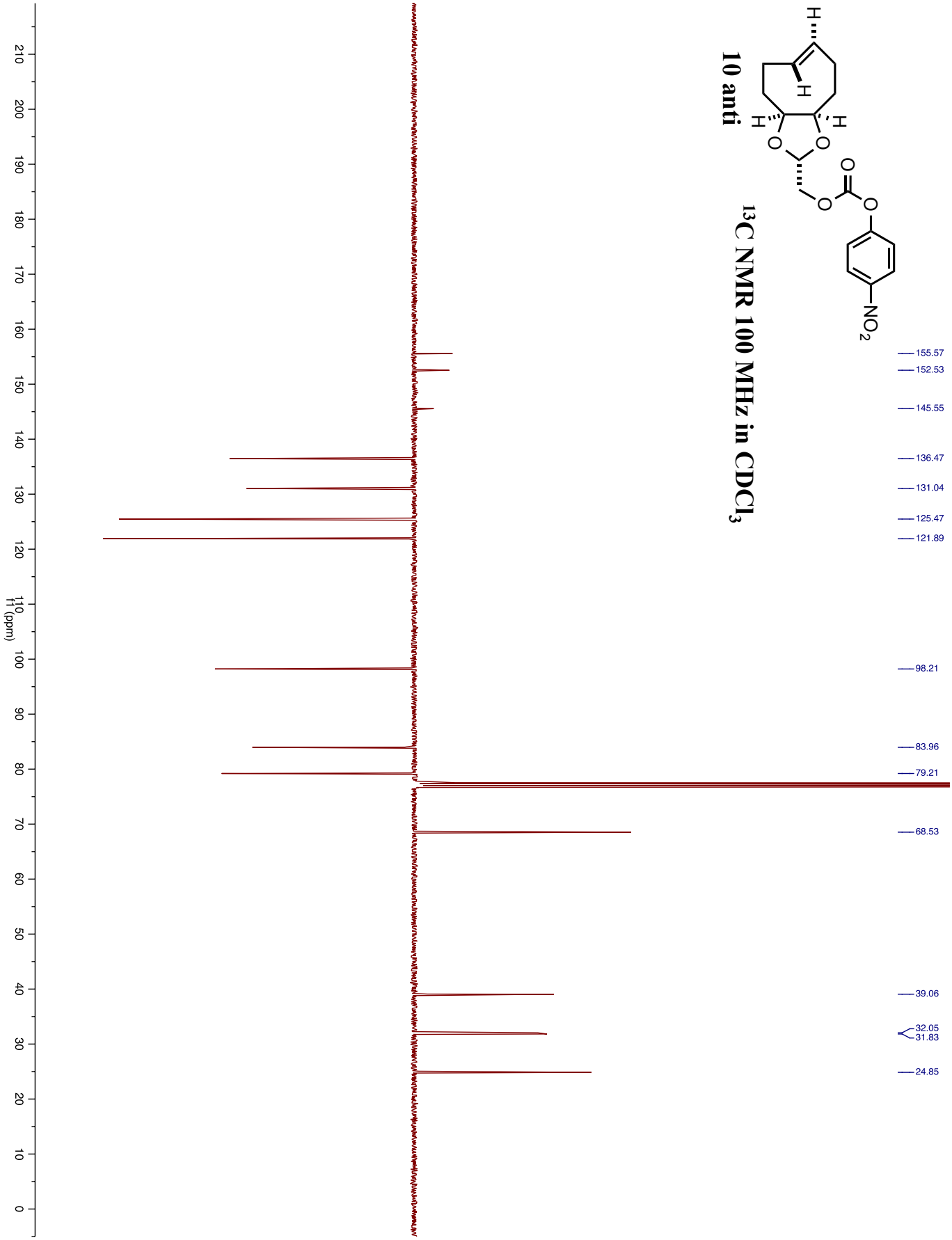
*** = 3b anti**

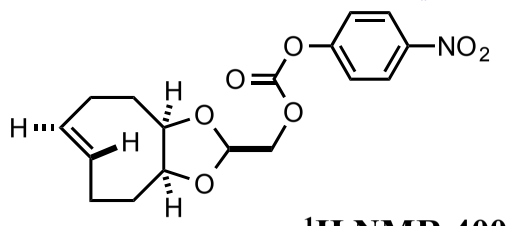

178.65
136.52
131.44
101.71
82.65
80.55
38.92
33.99
33.84
33.53
31.51
26.03
24.73
23.51
OH
O
H
O
O
H
H
H
3b syn
>50:1 dr
^{13}C NMR 100 MHz in CDCl$_3$
f1 (ppm)



10 anti

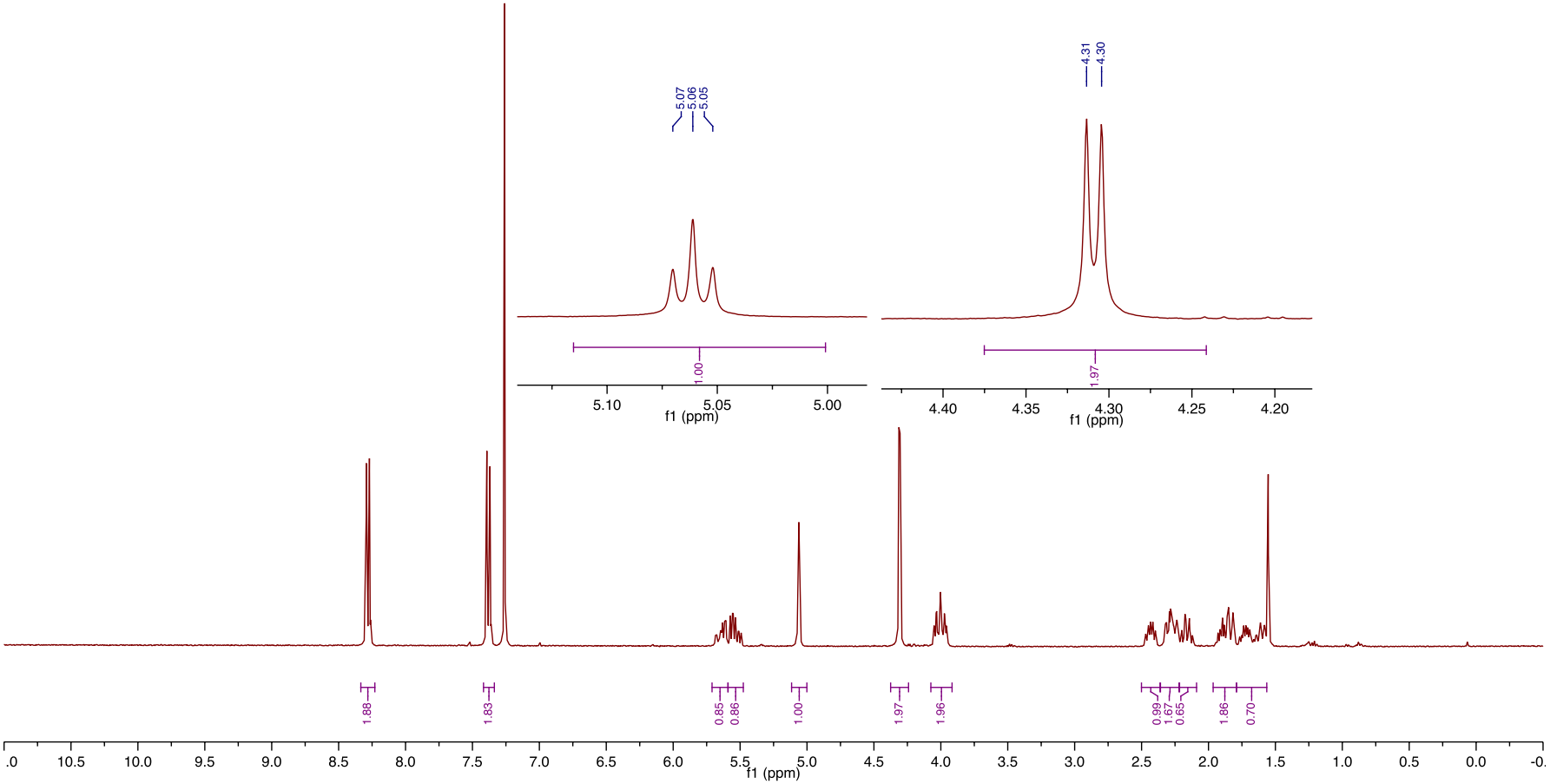
^{1}H NMR 400 MHz in $CDCl_3$

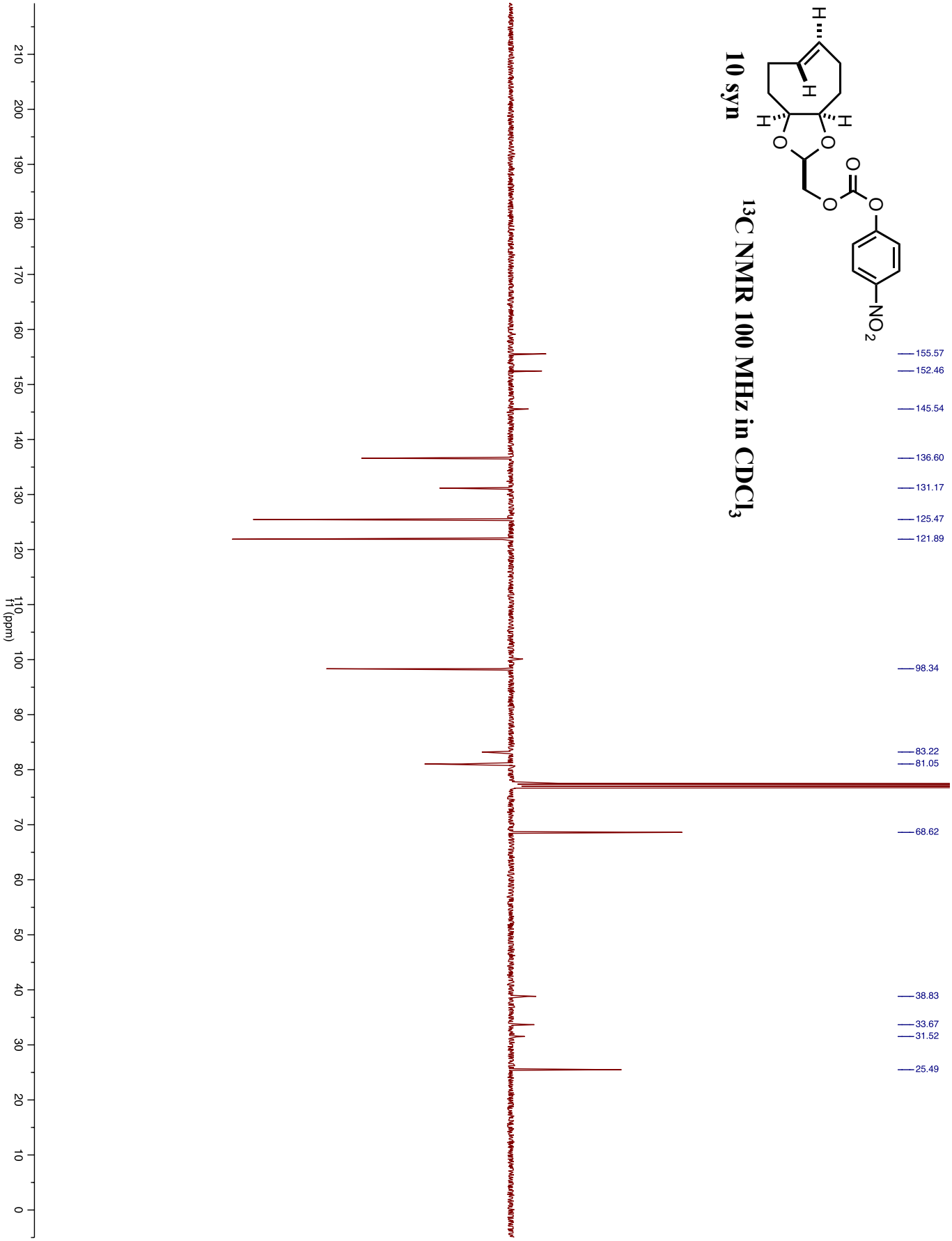

10 anti
^{13}C NMR 100 MHz in $CDCl_3$
155.57
152.53
145.55
136.47
131.04
125.47
121.89
98.21
83.96
79.21
68.53
39.06
32.05
31.83
24.85
f1 (ppm)

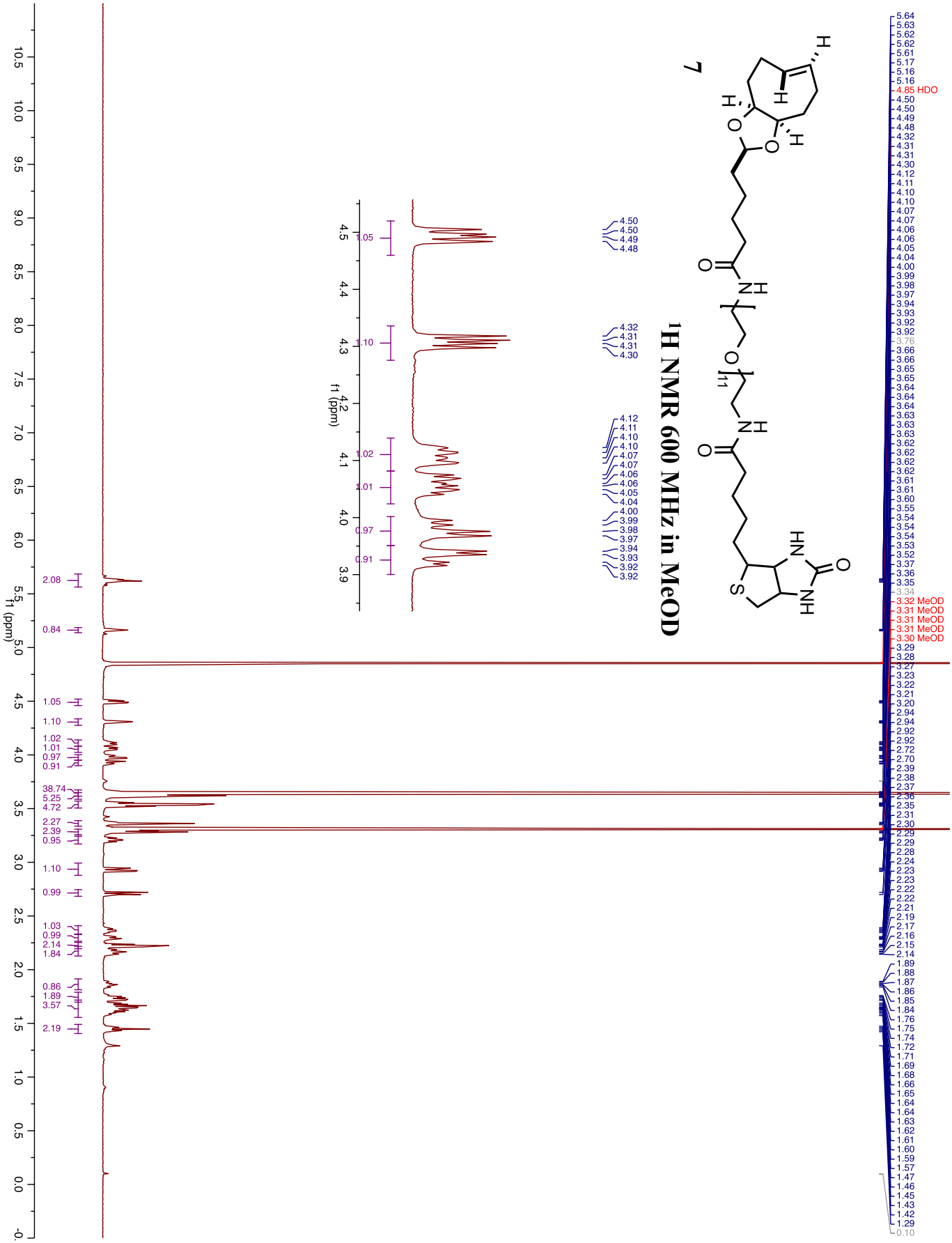


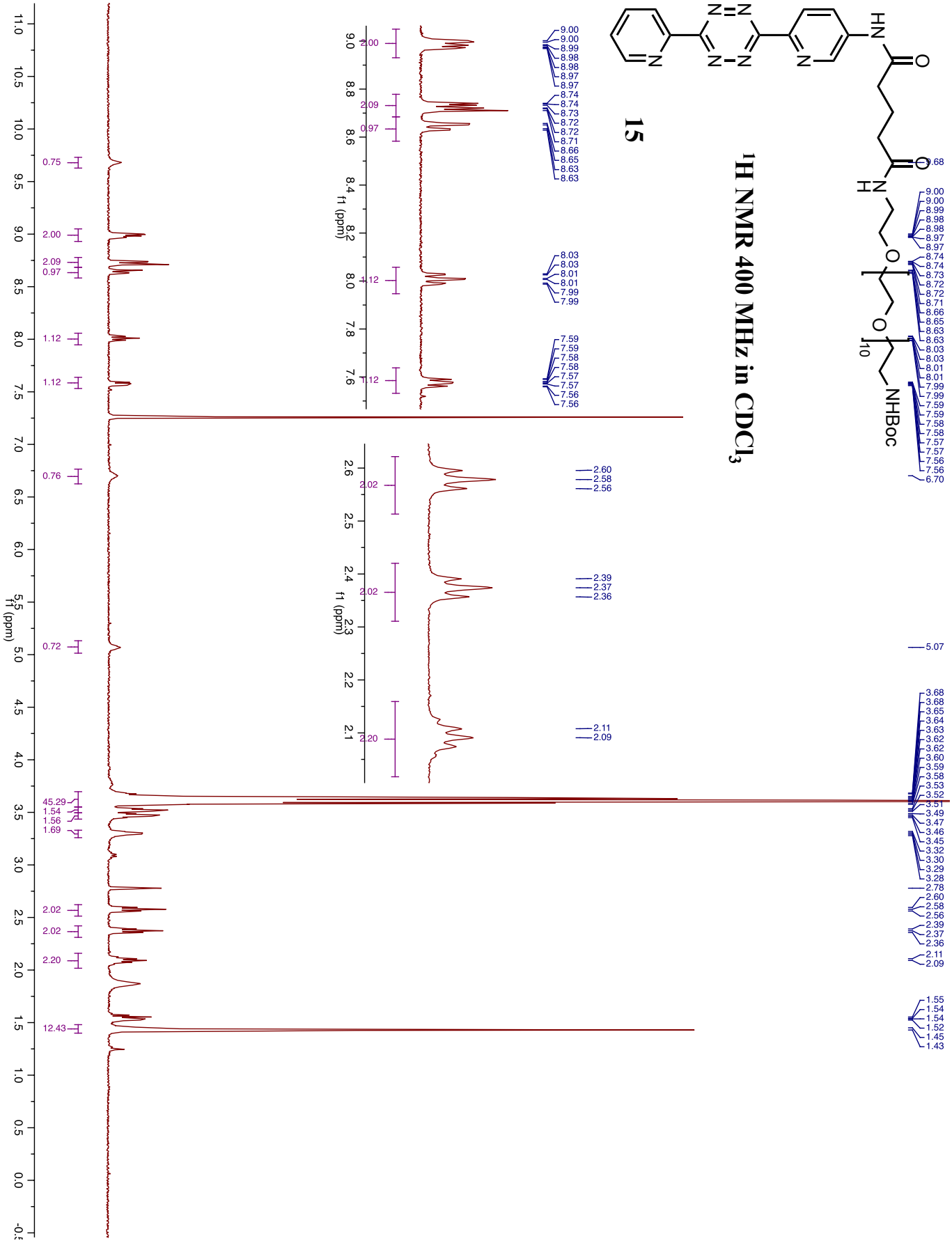
10 syn

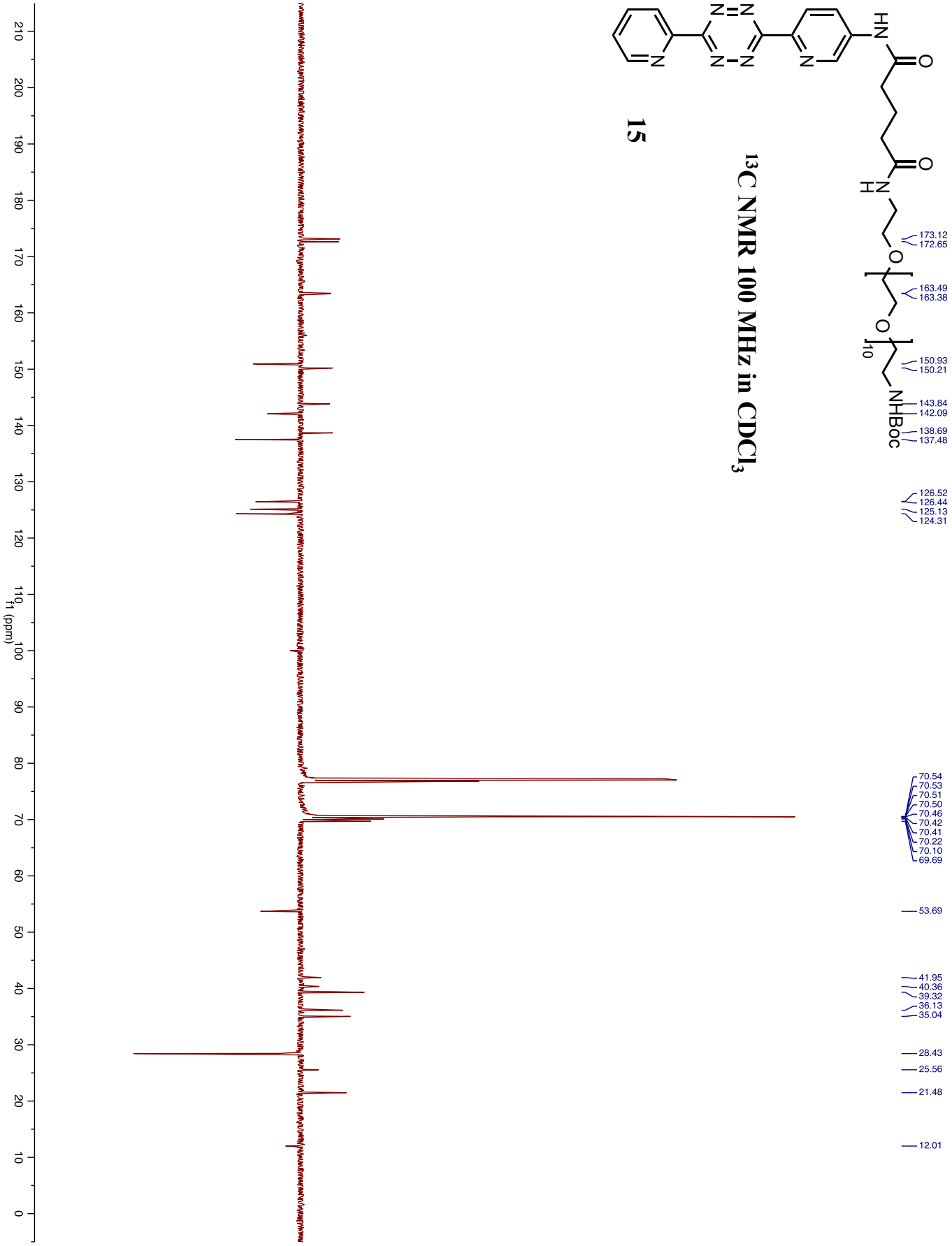
^{1}H NMR 400 MHz in $CDCl_3$



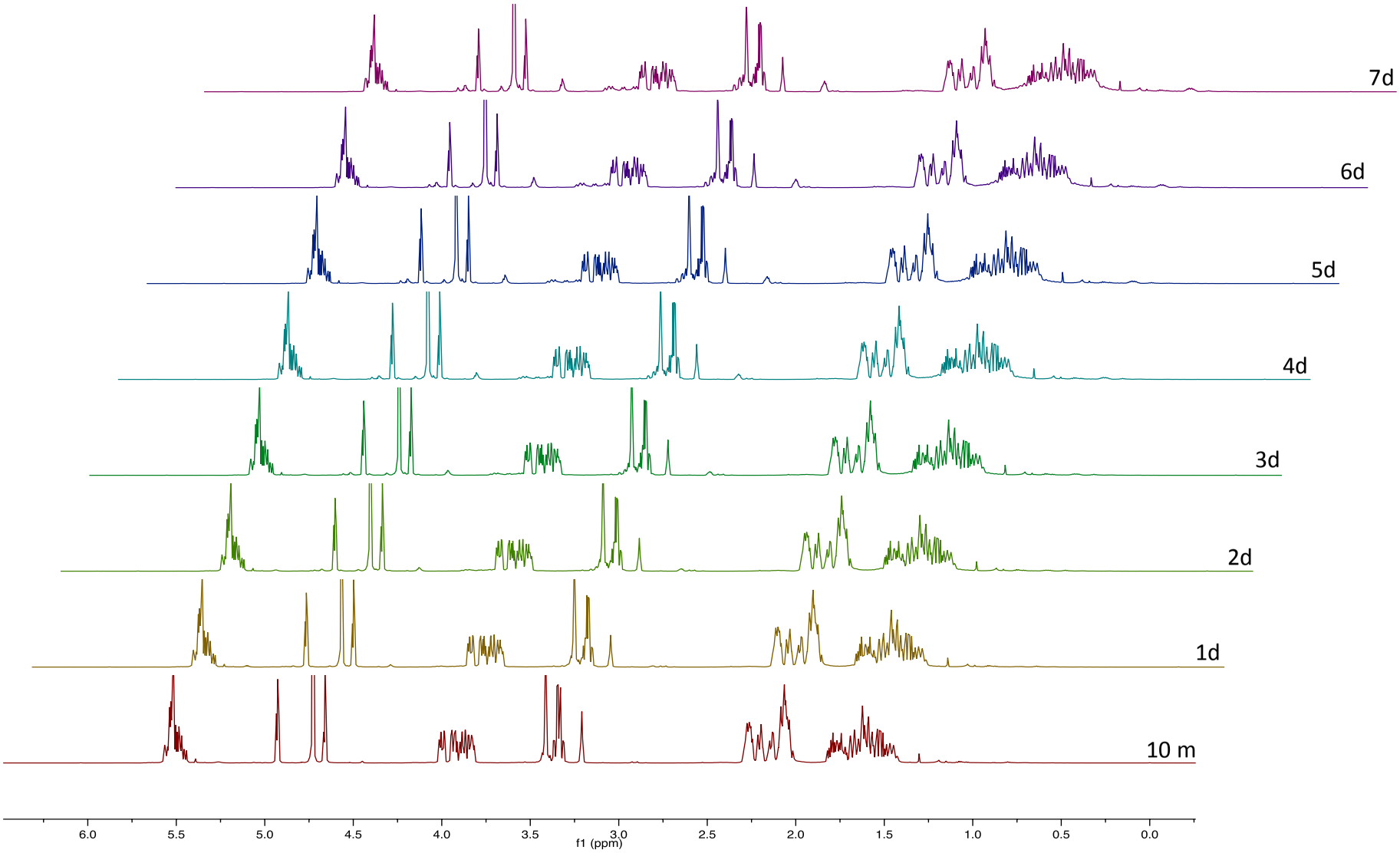

10 syn
^{13}C NMR 100 MHz in $CDCl_3$
155.57
152.46
145.54
136.60
131.17
125.47
121.89
98.34
83.22
81.05
68.62
38.83
33.67
31.52
25.49
f1 (ppm)


7
1H NMR 600 MHz in MeOD
f1 (ppm)
4.85 HDO
3.32 MeOD
3.31 MeOD
3.31 MeOD
3.31 MeOD
3.30 MeOD


15
^{1}H NMR 400 MHz in $CDCl_3$
NHBoc
f1 (ppm)

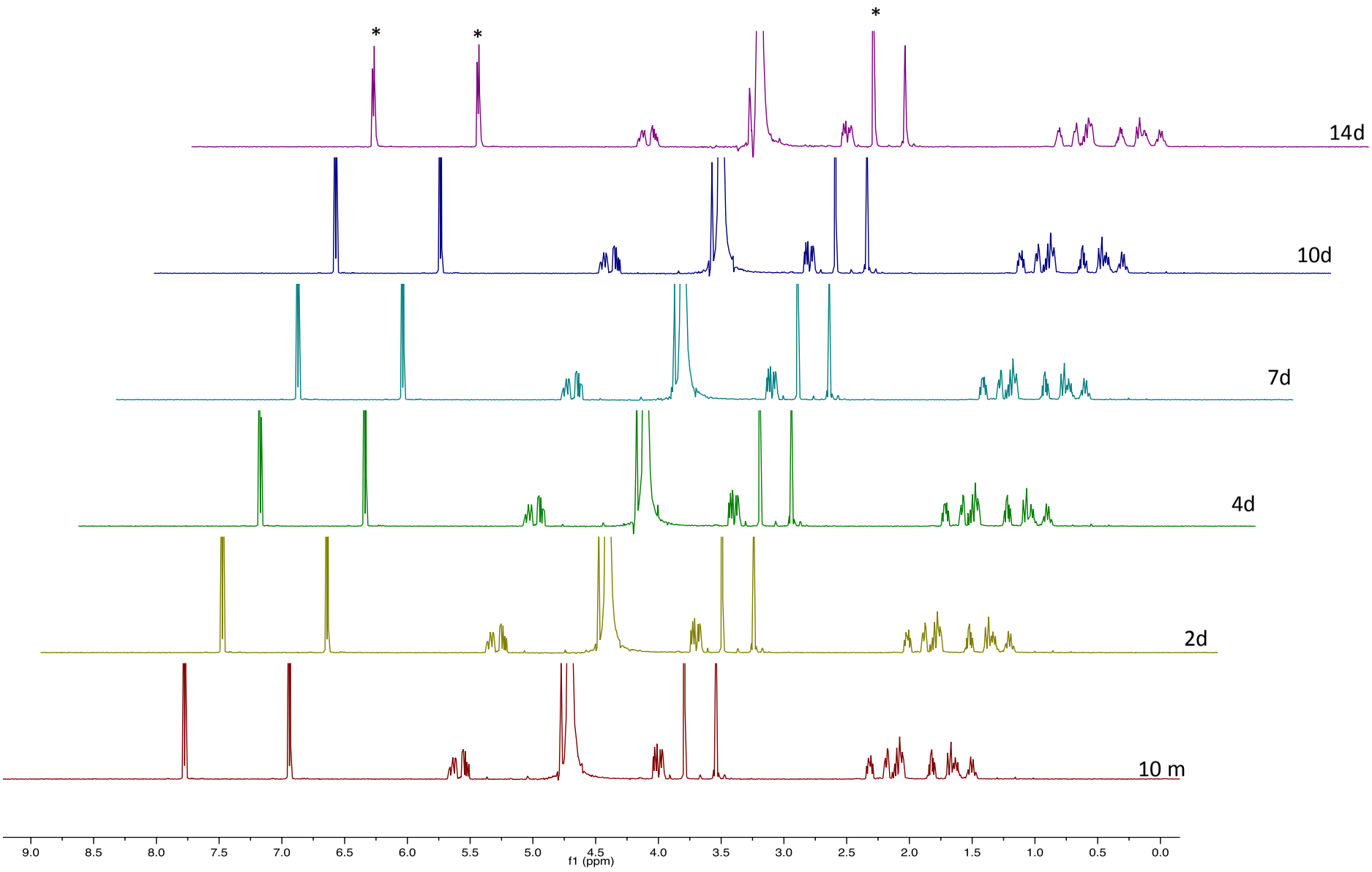

15
^{13}C NMR 100 MHz in $CDCl_3$
173.12
172.65
163.49
163.38
150.93
150.21
143.84
142.09
138.69
137.48
126.52
126.44
125.13
124.31
70.54
70.53
70.51
70.50
70.46
70.42
70.41
70.22
70.10
69.69
53.69
41.95
40.36
39.32
36.13
35.04
28.43
25.56
21.48
12.01
f1 (ppm)

^{1}HNMR analysis of the stability of 3a (1:1 syn:anti)
600 mHz in MeOH-d_4



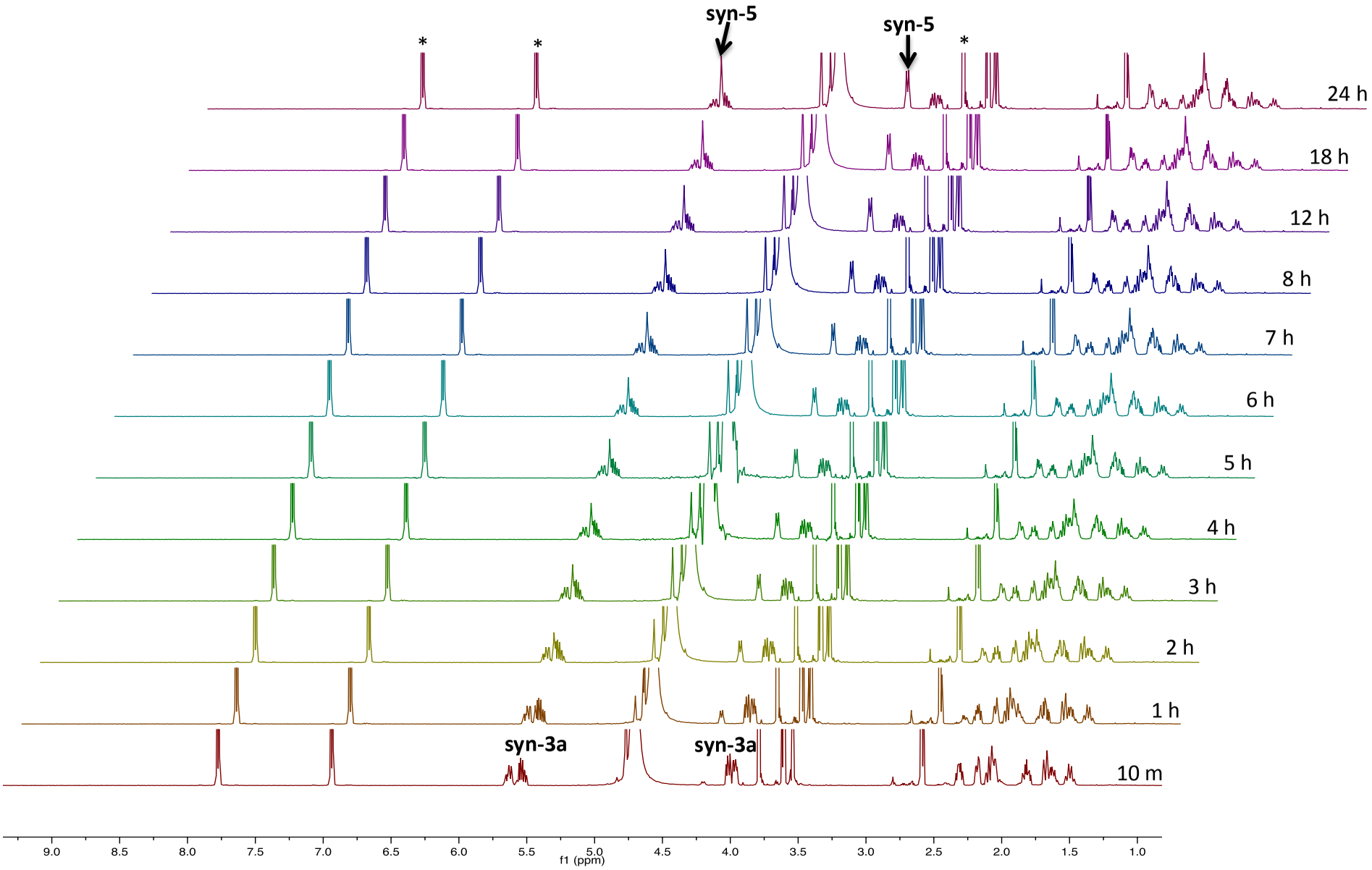
^{1}HNMR analysis of the stability of syn-3a in D_2O (pD = 7.4) 600 mHz

*** = 4-methoxybenzoic acid**



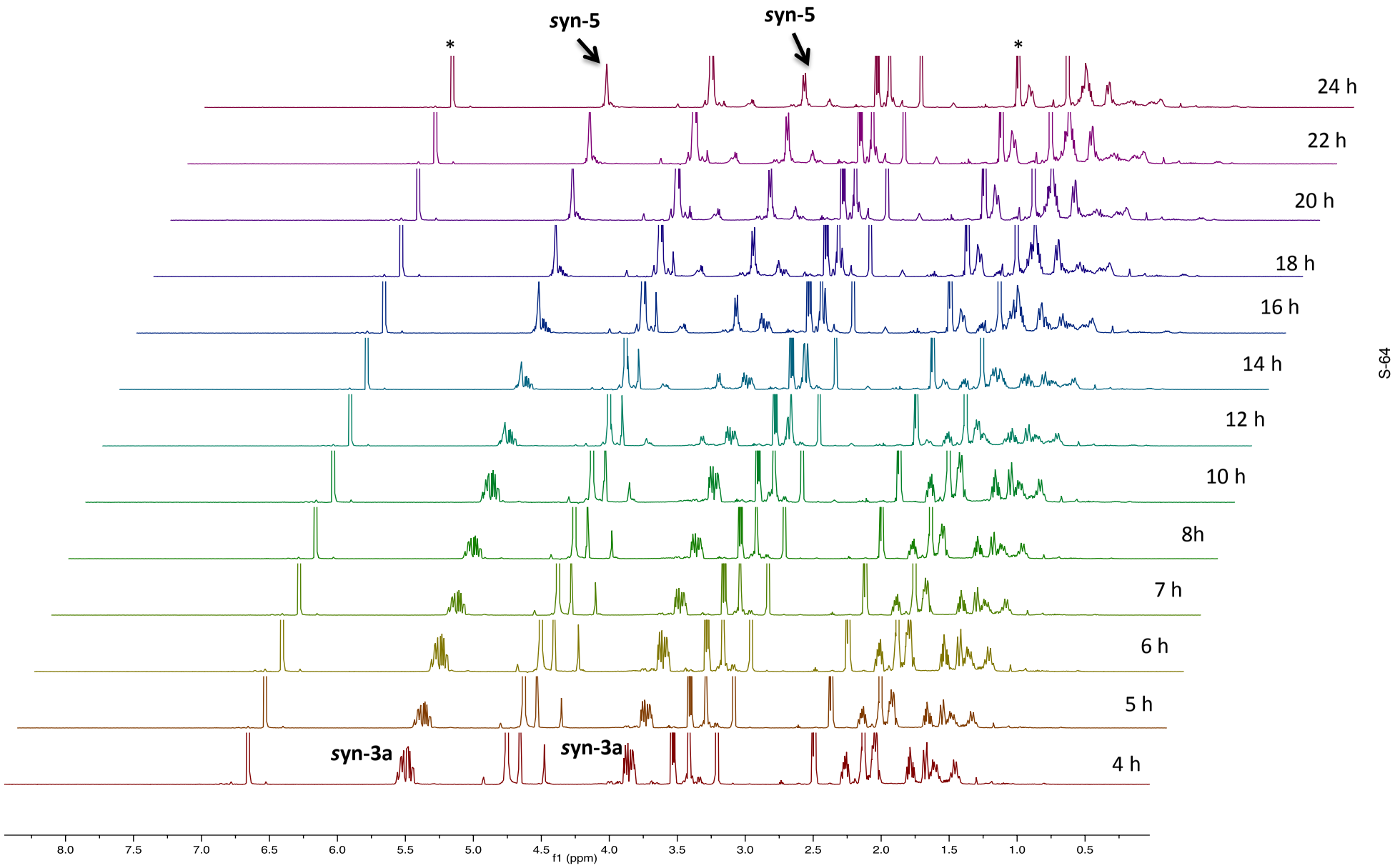
^{1}HNMR analysis of the stability of syn-3a in mercaptoethanol
600 mHz in D_2O (pD = 7.4)

*** = 4-methoxybenzoic acid**



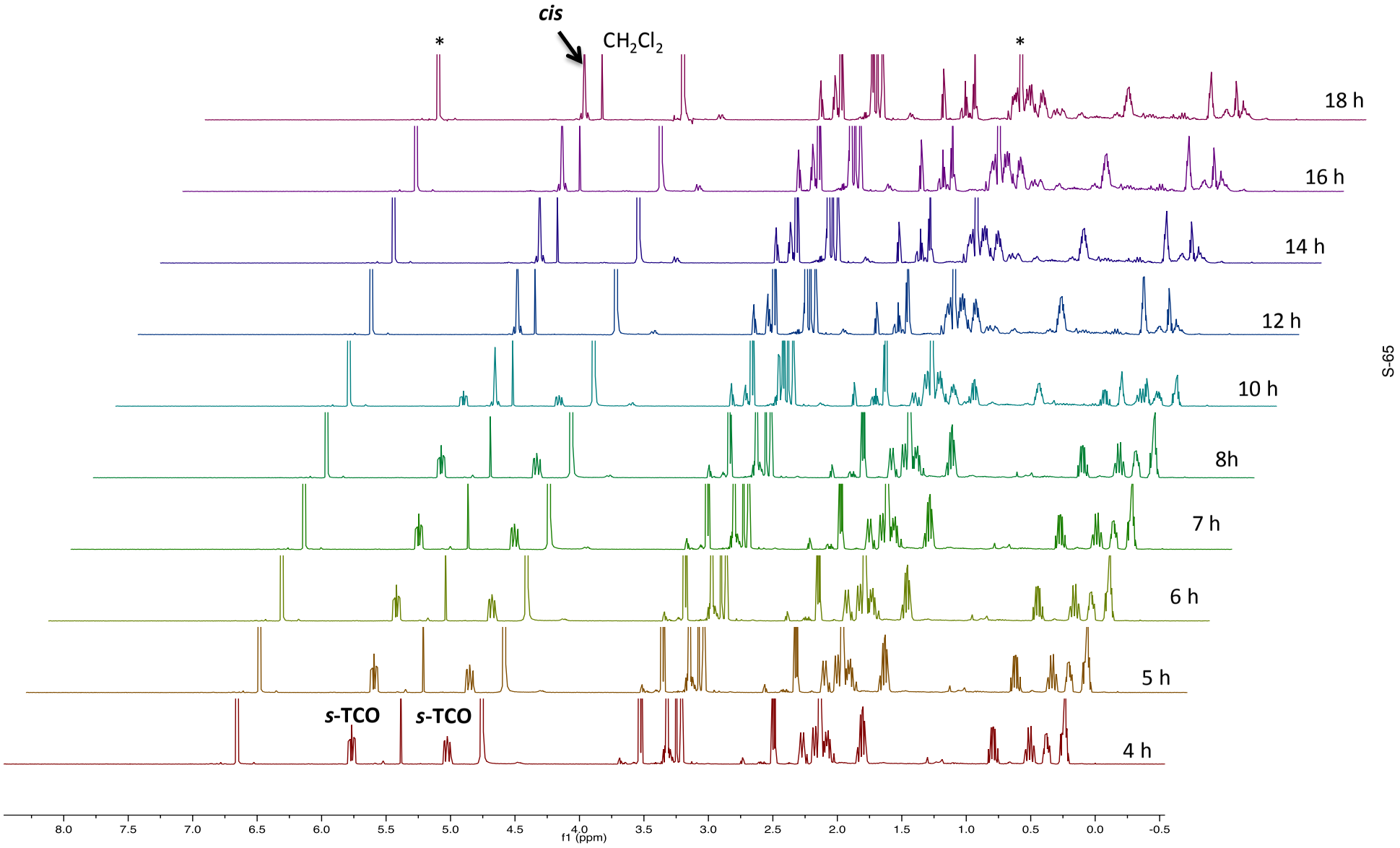
^{1}HNMR analysis of the stability of **syn-3a** in mercaptoethanol
600 mHz in MeOH-d_4

* = mesitylene



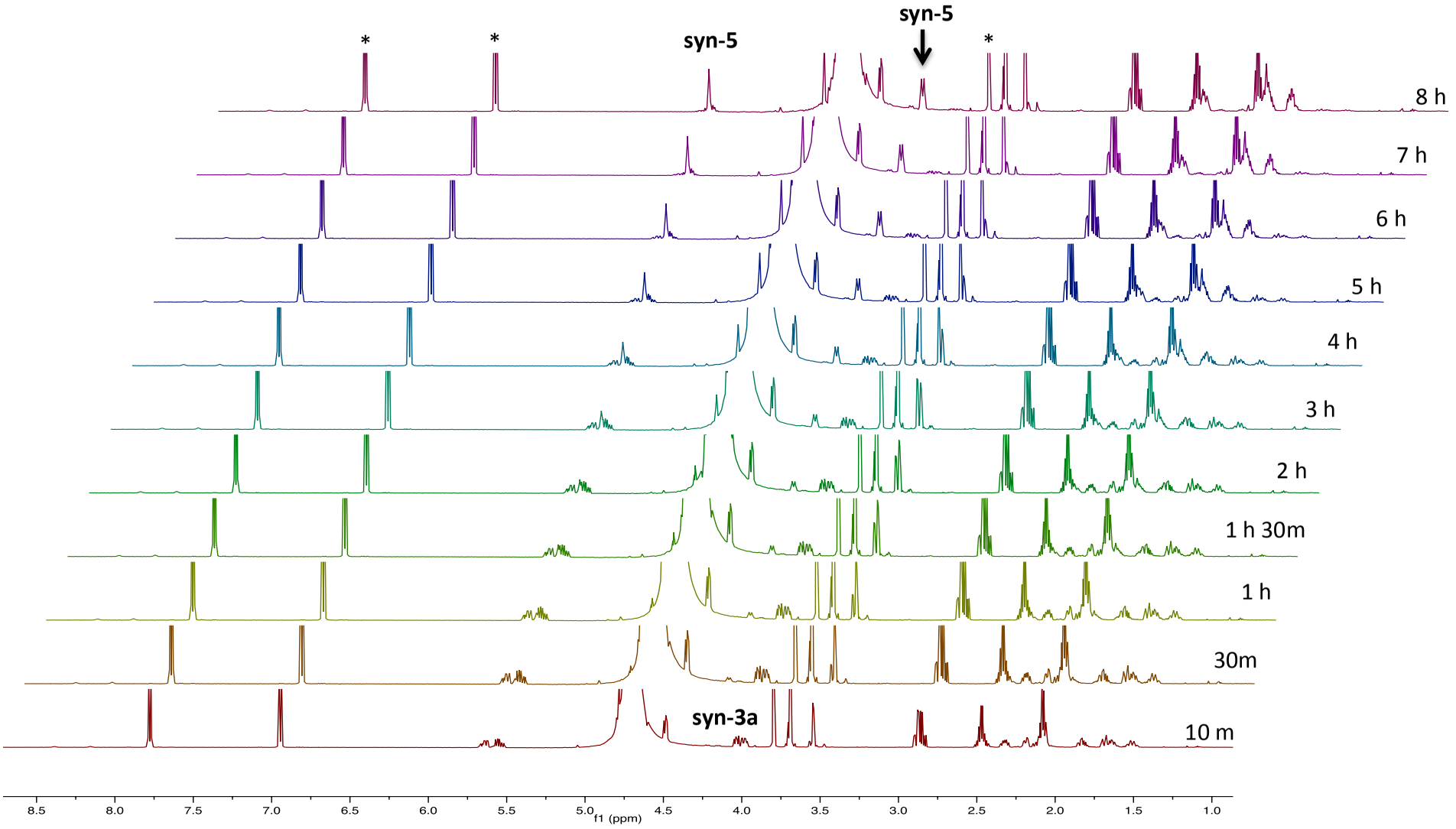
^{1}HNMR analysis of the stability of s-TCO in mercaptoethanol
600 mHz in MeOH-d_4

*** = mesitylene**



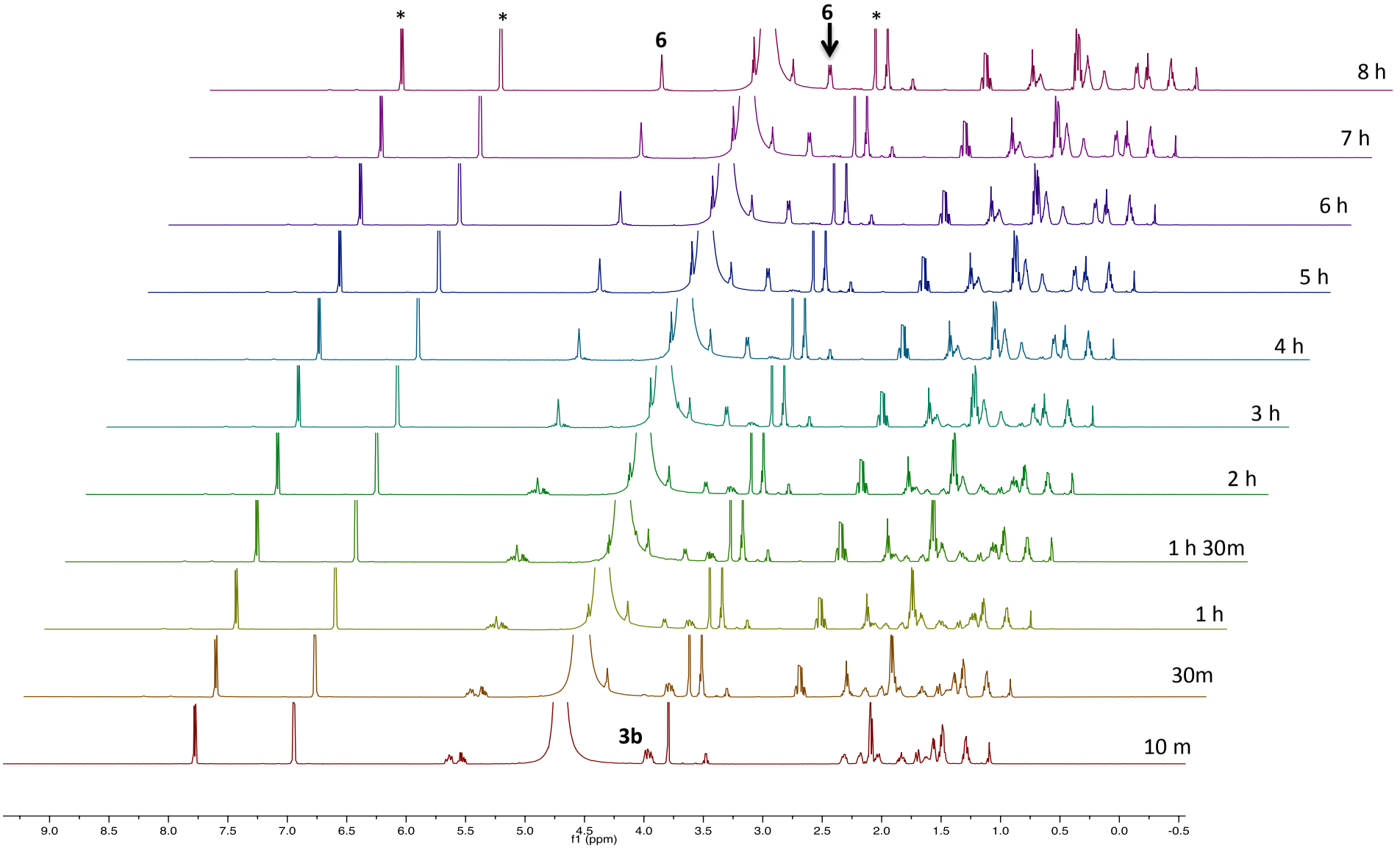
^{1}HNMR analysis of the stability of syn-3a with glutathione in D_2O (pD = 7.4) 600 mHz

*** = 4-methoxybenzoic acid**



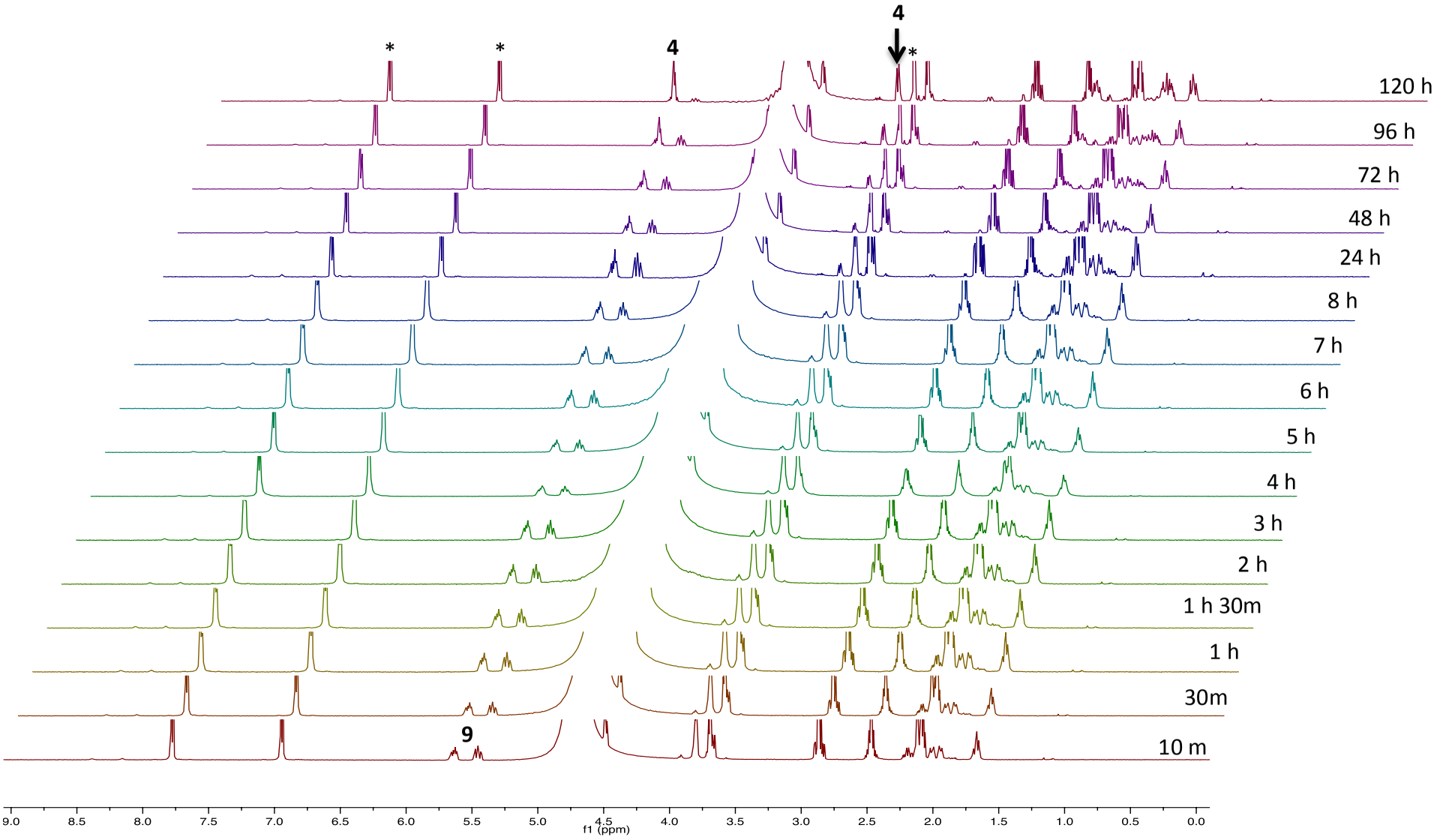
^{1}HNMR analysis of the stability of **3b** with glutathione in D_2O (pD = 7.4) 600 mHz

* = 4-methoxybenzoic acid



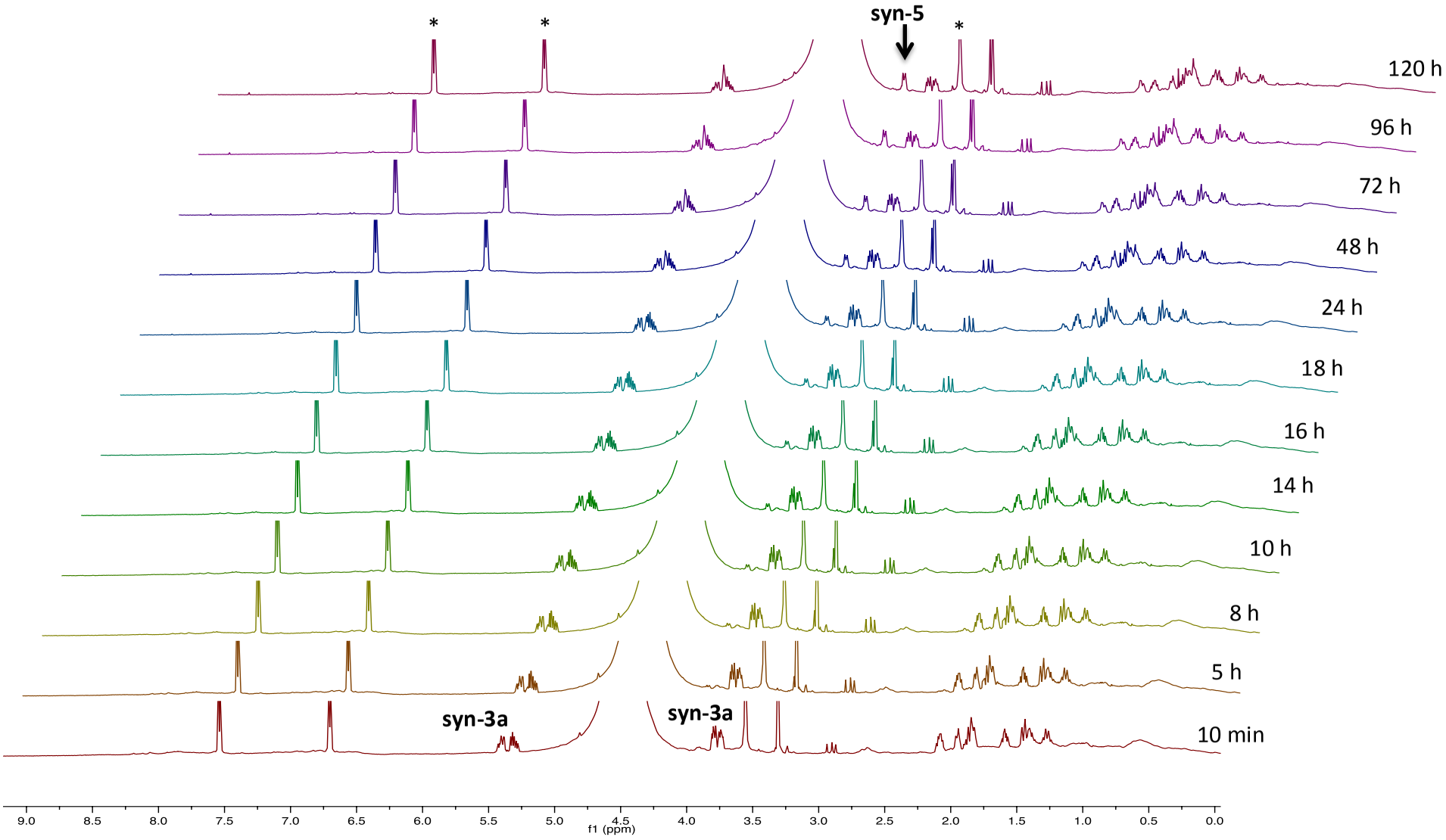
^{1}HNMR analysis of the stability of 9 with glutathione in D_2O (pD = 7.4) 600 mHz

*** = 4-methoxybenzoic acid**



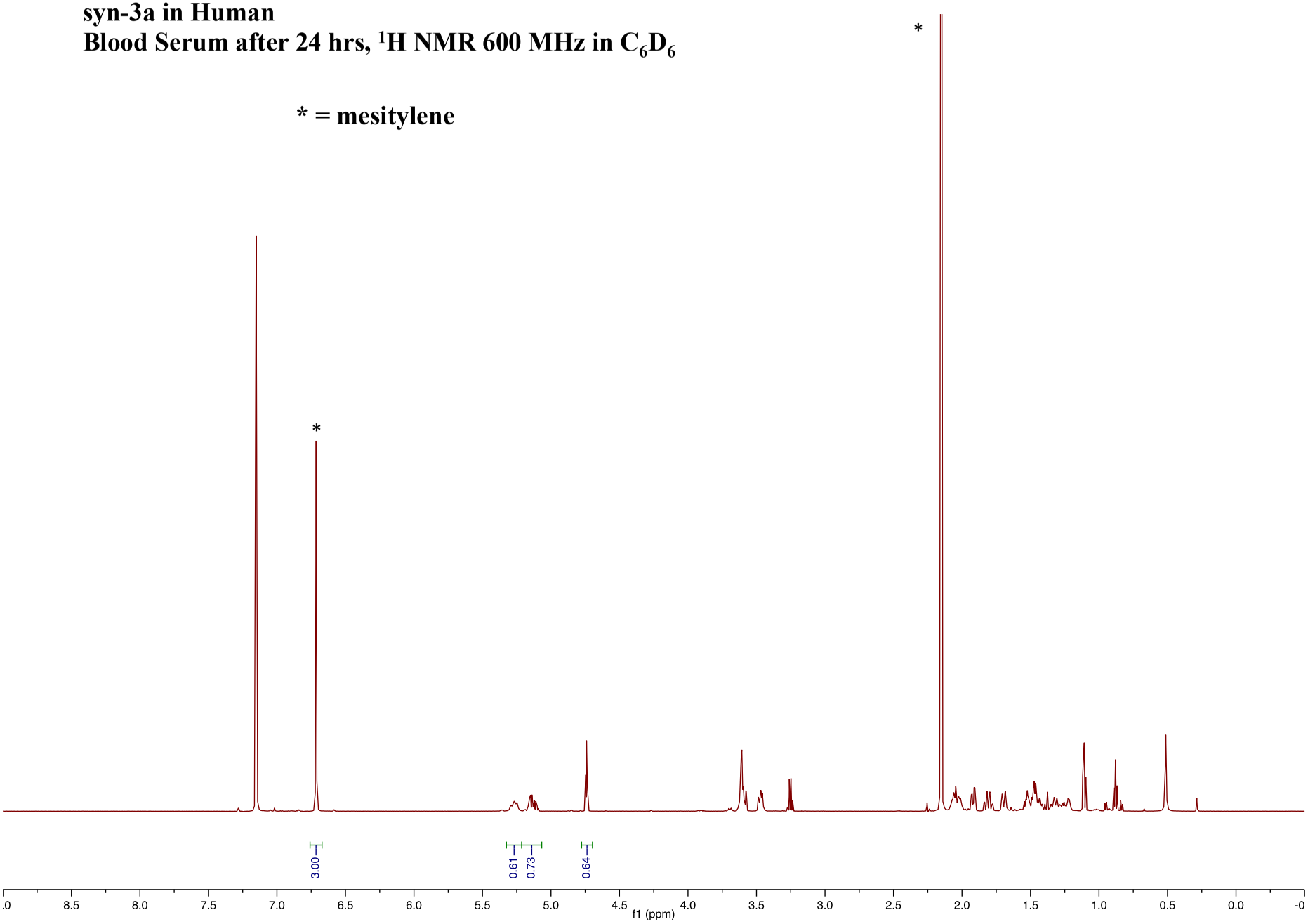
^{1}HNMR analysis of the stability of syn-3a with rabbit reticulocyte lysate (10% in D_2O , pD = 7.4) 600 mHz

*** = 4-methoxybenzoic acid**



syn-3a in Human
Blood Serum after 24 hrs, ^{1}H NMR 600 MHz in C_6D_6

*** = mesitylene**



syn-3a in Human
Blood Serum after 4 days, ^{1}H NMR 600 MHz in C_6D_6