Electronic Supplementary Material (ESI) for ChemComm.

Chiral trimethyl lock based on vicinal disubstituent effect: prolonged release of Camptothecin into cancer cells

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Contents

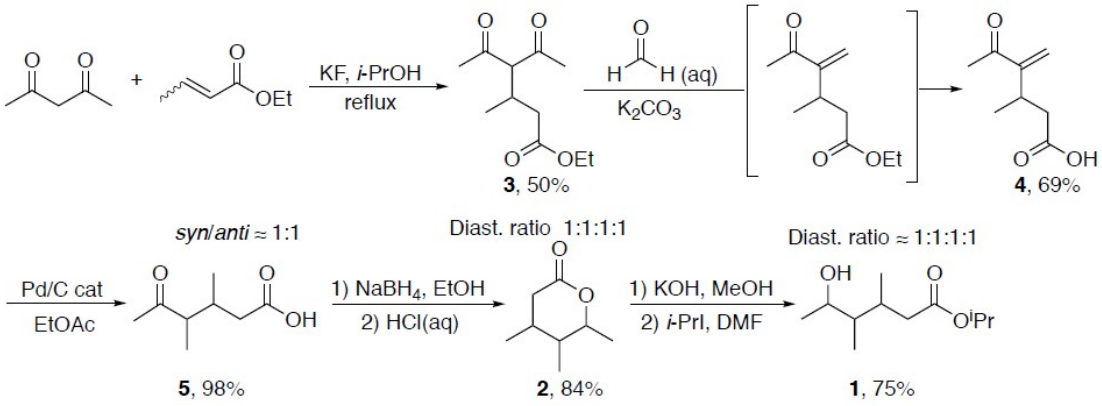
General Information

Chemicals and solvents were purchased from suppliers and used without further purification, while, where required, the solvents were dried over molecular sieves (4 Å). ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz spectrometer at room temperature in deuterated solvents ($CDCl_3$ or C_6D_6). The spectra were calibrated using TMS as an internal standard for ^{1}H and $CDCl_3$ for ^{13}C; chemical shifts δ are expressed in ppm relative to the reference. The coupling constants (*J*) are expressed in Hertz (Hz); all ^{13}C spectra are proton decoupled.
The high-resolution MS spectra of newly synthesized compounds were recorded with a Q-TOF mass spectrometer, equipped with an ESI source.
The ESI-MS experiments of the spots scrapped from the preparative TLC were performed using a: Bruker Esquire 3000 PLUS (ESI Ion Trap LC/MS System), equipped with an ESI source and a quadrupole ion trap detector (QIT). Acquisition parameters: needle: 4.5 kV, N_2 flow rate: 10 L h^{-1}, cone voltage: 40 V, scan resolution and range: 13 000 (m/z) s^{-1} over the mass range m/z 35–600, by direct infusion of methanol solution of compounds at rate: 4 µl min^{-1}. The assignment of some of the species detected was confirmed by collision-activated decomposition (CID) mass spectrometry experiments (MS^2).
The GC-MS analyses of all compounds were performed on a column with a low polarity stationary phase (30 m x 0.25 mm x 0.25 µm). Program temperature: 60 °C (1 min)/6 °C min^{-1}/150 °C (1 min)/12 °C min^{-1}/280 °C (5 min).
HPLC was performed with a Jasco HPLC system PU-2080 *Plus*, equipped UV-2075 *Plus* UV/Vis detector, and using ChromNAV software. Analyses were performed with a column Robusta® C18 5u - 150mm x 4.6mmID.
TLC analyses were performed on precoated silica gel 60 F_{254} plates, and spots were visualized either by UV light (254 nm or 366 nm) or by spraying the phosphomolybdic acid reagent. All chromatographic separations were carried out on silica gel columns (230-400 mesh). MRM analysis was performed on a Xevo G2-XS quadrupole time of flight mass spectrometer coupled to a Waters ACQUITY ultra-performance liquid chromatography (UPLC) H-Class system through an ESI source (Waters Corporation, Milford, MA, USA).

Enzymes and Strains

Lipase from Porcine pancreas (PPL) type II, Sigma-Aldrich, 147 units/mg; *Novozym® 435* (immobilized lipase B from *Candida antarctica*, Cal-B), Novo Nordisk A/S, Denmark, ≥5000 units/g; Lipase from *Pseudomonas Cepacia* (PS), Amano Pharmaceuticals Co., Japan, 30 units/mg; Lipase AK Amano from *Pseudomonas Fluorescens*, Sigma-Aldrich, >700 units/mg; Lipase A *Candida antarctica* immobilized on Immobead 150, recombinant from *Aspergillus oryzae*, Sigma-Aldrich, ≥500 units/g; Lipase from *Aspergillus Niger*, Fluka 200 U/g; Lipase from *Candida cylindracea*, Fluka 37 U/g and Esterase BS3, CLEA Technologies, 170 units/g were employed in this work.

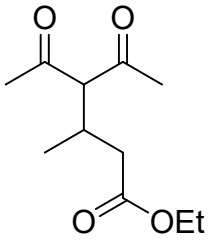
Synthesis of isopropyl 5-hydroxy-3,4-dimethylhexanoate (1)



Scheme S1. Synthesis of substrate **1**.

The synthesis of **1** was accomplished in seven steps with an overall yield of 21% (Scheme 1). The first step consisted of a Micheal addition of the enolate of pentan-2,4-dione on the ethyl crotonate in the presence of KF salt in *i*-PrOH to give **3** in a yield of 50%, after distillation.[1] Then, **3** was treated with an aqueous solution of formaldehyde in the presence of K_2CO_3.[2] During the reaction, the intermediate α,β-unsaturated ketoester was *in situ* hydrolyzed forming the ketoacid **4**. The purification was carried out by column chromatography giving **4** in a yield of 69%. The next step consisted of a hydrogenation of the C=C double bond using a catalytic amount of Pd/C under an H_2 atmosphere, this reaction gave virtually a quantitative yield, achieving **5** (*syn*/*anti* ≈ 1:1, by GC-MS) with a purity sufficiently high to be used in the next step without the need of further purification procedure. Then, the carbonyl group of **5** was reduced with $NaBH_4$ in EtOH to give the hydroxy acid, which underwent to ring-closure reaction by acidification (HCl, 1M), affording the lactone **2**. The crude material was submitted to bulb-to-bulb distillation procedure achieving **2** in a yield of 84%, with an equimolar distribution of the four diastereoisomers (≈ 1:1:1:1, by ^{1}H-NMR). The last stage of synthesis consisted of the alkaline (KOH) ring-opening of lactone **2** to give the intermediate potassium carboxylate salt. Nucleophilic substitution of the latter with *i*-propyl iodine in DMF gave the hydroxy *i*-propyl ester **1** in a yield of 75%, with a purity sufficiently high to be used in the next steps, without the need for further purification.

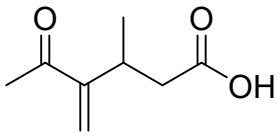
Ethyl 4-acetyl-3-methyl-5-oxohexanoate (3)



3

To a mechanically stirred solution of pentane-2,4-dione (40.0 g, 0.4 mol) and (*E,Z*)-ethyl crotonate (45.6 g, 0.4 mol) in isopropyl alcohol (90 mL) was added KF (11.6 g, 0.2 mol) at 80°C. The slurry reaction mixture was stirred for about 5 days at room temperature. To the ice-cooled reaction mixture was added CH_2Cl_2 (250 mL) and then, it was left at 0° C overnight. The solution was filtered and the solid was washed with CH_2Cl_2 (3x100 mL) then, the solvent was removed under reduced pressure. The crude material was first distilled with no *vacuum* apparatus and then distilled with fractional distillation to give **3**.[1] Yield 50% (42.8 g) as a yellow oil; t_R = 16.51 min; 99% purity by GC-MS; ^{1}H NMR (400 MHz, $CDCl_3$): δ= 4.13 (q, *J* = 7.0 Hz, 2H), 3.73 (d, *J* = 9.8 Hz, 1H), 2.86 - 2.74 (m, 1H), 2.35 - 2.28 (dd, *J* = 15.3, 4.5 Hz, 1H), 2.23 - 2.16 (m, 7H), 1.2 (t, *J* = 7.1 Hz, 3H), 0.97 (d, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 203.90, 203.76, 171.96, 74.06, 60.6238.78, 30.53, 30.12, 29.64, 17.63, 14.31; MS: *m/z* (%) 214 ([M^+], 5), 172 (55), 127 (85), 111 (20), 85 (100), 69 (70); HRMS (ESI) calcd for $C_{11}H_{18}NaO_4^+$ [M + Na]$^+$ 237.1097, found 237.1099.

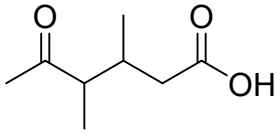
3-Methyl-4-methylene-5-oxohexanoic acid (4)



4

To a mechanically stirred mixture of **3** (14.4 g, 67 mmol) and formaldehyde (37% wt, stabilized with methanol 10-15%, 5.5 mL) was added at room temperature a water solution of K_2CO_3 (18.6 g) in H_2O (13.5 mL).[2] The reaction mixture was stirred for 5 hours at room temperature until the complete formation of **4**; during this time occurs, *in situ*, the hydrolysis of ethyl ester intermediate. Then the slurry reaction mixture was washed with H_2O (3x150 mL). To the ice-cooled aqueous layers was added HCl drop by drop until pH 4. This solution was extracted with EtOAc (10 x 50 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure to give the crude material, which was purified by column chromatography (*n*-hexane/EtOAC 1:1) to give **4**. Yield 69% (7.2 g) as a yellow oil; t_R = 14.13 min; 98% purity by GC-MS; ^{1}H NMR (400 MHz, $CDCl_3$): δ= 6.07 (s, 1H), 5.80 (s, 1H), 3.30 - 3.21 (m, 1H), 2.55 (dd, *J* = 15.6, 6.6 Hz, 1H), 2.41 - 2.34 (m, 4H), 1.13 (dd, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 199.38, 178.49, 152.49, 124.34, 40.35, 30.42, 26.43, 19.90; MS: *m/z* (%)156 ([M^+], 5), 138 (80), 110 (92), 95 (100); HRMS (ESI) calcd for $C_8H_{12}NaO_3^+$ [M + Na]$^+$ 179.0679, found 179.0676.

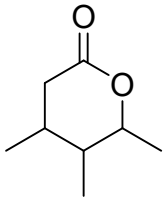
3,4-Dimethyl-5-oxohexanoic acid (5)



5

To a mechanically stirred solution of **4** (3.8 g, 24 mmol) in EtOAc (40 mL) was added Pd/C catalyst powder (10% wt, 0.13 g). The mixture was stirred for about 5 hours under an H_2 atmosphere at room temperature. Then, it was filtered on a celite pad, which was washed with EtOAc (3x50 mL) and concentrated under reduced pressure giving **5**. Yield 98%; (3.7 g) as a yellow oil; t_R = 13.7 min; 99% purity by GC-MS; ^{1}H NMR (400 MHz, $CDCl_3$): δ= 2.64 - 2.22 (m, 4H), 2.20 - 2.09 (m, 3 H), 1.90 - 1.01 (m, 4.5H), 0.93 - 0.91 (d, *J* = 6.8 Hz, 1.5H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 212.03, 211.80, 178.68, 178.43, 51.65, 50.75, 39.48, 37.60, 31.91, 31.27, 29.12, 28.81, 18.57, 15.86, 12.80, 11.53;MS: *m/z* (%)158 ([M^+], 7), 140 (25), 98 (50), 73 (97), 56 (100).

4,5,6-Trimethyltetrahydro-2*H*-pyran-2-one (2)

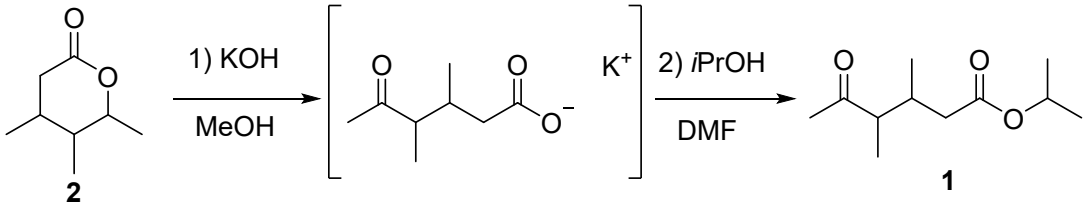


2

To an ice-cooled solution of **5** (7.9 g, 50.0 mmol) in EtOH (75 mL) was portion-added $NaBH_4$ (0.9 g, 25.0 mmol), and the mixture was stirred at room temperature until complete consumption of **5**, checked by TLC (hexane/EtOAc, 7:3). Then, the reaction mixture was acidified at pH=4 with HCl (0.1 M, 20 mL) and left to stir for 4 h. Then, most EtOH was evaporated under reduced pressure. The remaining aqueous phase was neutralized with NaOH (1 M) and extracted with $CHCl_3$ (4x50 mL). The combined organic phase was washed with brine (sat., 50 mL), dried over Na_2SO_4, and the solvent was removed under reduced pressure. The crude material was submitted to bulb-to-bulb distillation purification affording lactone **2**. Yield 84% (5.9 g) as a colorless oil; t_r =12.16 min, t_r =12.44 min, t_r =12.79 min, t_r =13.06 min, 94% purity by GC-MS,

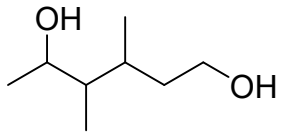
(*cis,cis*)/(*trans,cis*)/(*cis,trans*)/(*trans,trans*) ratio 1:1:1:1 by GC-MS; ^{1}H NMR (C_6D_6, 400 MHz): δ= 3.92 (m, *J*= 6.6, 4.0 Hz, 0.25H), 3.88 (m, *J*= 6.5, 2.5 Hz, 0.25H), 3.71 (dq, *J* = 8.9, 6.3 Hz, 0.25H), 3.42 (dq, *J* = 10.0, 6.2 Hz, 0.25H), 2.33-2.11 (m, 1H), 2.03 (dd, *J*= 17.6, 4.8 Hz, 0.3H), 1.86-1.63 (m, 1H), 1.53-1.30 (m, 0.5H), 1.23-0.86 (m, 3.8H), 0.70-0.60 (m, 0.4H), 0.59-0.31 (m, 6H);^{13}C NMR (100 MHz, C_6D_6): δ= 170.85, 170.09, 169.58, 169.52, 80.50, 79.60, 78.10, 75.81, 42.02, 38.35, 37.97, 37.77, 37.50, 36.70, 35.96, 33.77, 33.00, 31.59, 31.15, 29.75, 20.54, 20.40, 19.97, 19.89, 18.61, 18.30, 16.79, 14.83, 14.47, 13.93, 13.73, 3.85; GC-MS: *m/z* (%): 142 ([M$^+$], 10), 127 (10), 98 (60), 70 (30), 56 (100).

isopropyl 5-hydroxy-3,4-dimethylhexanoate (1)



To an ice-cooled well-stirred solution of **2** (2.8 g, 20 mmol) in MeOH (35 mL) was added a solution of KOH (2.5 g, 45 mmol) in H_2O (3 mL). The heterogeneous mixture was stirred for 16 hours at room temperature and then concentrated under *vacuum* to give a viscous oil. The latter was treated with Et_2O (4x10 mL) and concentrated under reduced pressure. This procedure was repeated at least 4 times in such a way to eliminate all traces of MeOH and H_2O. The crude material was left under a high *vacuum* for 3 hours. Then, to this solution in anhydrous DMF (30 mL) was added isopropyl iodide (50 mmol, 8.5 g). After 16 hours, the reaction mixture was diluted with brine (sat., 30 mL) and then extracted with Et_2O (5x50 mL). The combined organic phases were washed with brine (sat., 50 mL), dried over Na_2SO_4, and concentrated under *vacuum* to give the corresponding hydroxyester **1** which was of sufficient purity for the next step.Yield 75% (3.0 g), as a colorless oil; t_r =15.14 min, t_r =15.28 min, t_r =15.40 min, t_r =15.50 min, 98% purity by GC-MS, (*cis,cis*)/(*trans,cis*)/(*cis,trans*)/(*trans,trans*) ratio 1:1:1:1 by ^{1}H NMR; ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 5.01 (m, *J*= 6.2, 2.6 Hz, 1H), 3.92-3.82 (m, 0.2H), 3.74 (m, 0.2H), 3.66-3.58 (m, 0.2H), 3.58-3.50 (m, 0.2H), 2.49-1.95 (m, 3H), 1.47-1.28 (m, 1.5H), 1.28-1.12 (m, 9H), 0.99 (dt, *J*= 9.4, 7.2 Hz, 2H), 0.94 -0.84 (m, 3H), 0.77 (t, *J*= 7.2 Hz, 1.5); ^{13}C NMR (100 MHz, $CDCl_3$): δ=174.13, 173.38, 173.24, 171.84, 81.74, 80.76, 69.88, 69.51, 67.88, 67.68, 67.55, 64.45, 45.64, 44.54, 44.13, 42.18, 37.78, 37.36, 36.12, 33.61, 33.08, 33.01, 31.85, 31.67, 30.27, 25.44, 22.31, 21.97, 21.94, 21.91, 20.94, 20.12, 20.04, 19.11, 18.79, 18.70, 18.62, 15.04, 11.03, 9.81, 9.64, 4.21; GC-MS: *m/z* (%): 184([M-18]$^+$, 10), 158 (20), 143 (40), 102 (90), 83 (70), 60 (100), 56 (80); HRMS (ESI) calcd for $C_{11}H_{22}NaO_3^+$ [M + Na]$^+$ 225.1461, found 225.1462.

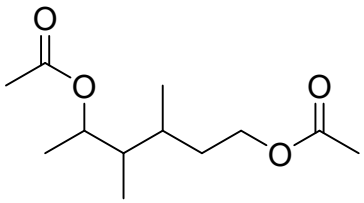
3,4-dimethylhexane-1,5-diol (6)



6

To an iced cooled solution of LAH (760 mg, 20 mmol) in Et_2O (70 mL) was added dropwise **5** (2.8 g, 20 mmol) diluted in Et_2O (60 mL). The reaction mixture was left to stir overnight at room temperature, then, it was quenched with Seignette solution (50 mL), the aqueous phase was extracted with EtOAc (7x70 mL), the organic phase was dried over Na_2SO_4 and concentrated under *vacuum* to give **6**. Yield 98% (2.8 g) as a viscous oil; t_r= 12.56 min, 99% purity by GC-MS; ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 3.92-3.53 (m, 3H, C<u>H</u>-OH + C<u>H</u>$_2$-OH), 1.83-1.19 (m, 7H), 1.01-0.73 (m, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 70.30, 70.03, 69.98, 69.76, 61.54, 61.43, 61.12, 60.87, 46.15, 45.19, 44.05, 44.03, 38.46, 38.35, 35.43, 33.57, 32.03, 30.67, 28.91, 28.34, 22.23, 21.93, 21.73, 21.57, 18.97, 18.65, 15.46, 13.97, 10.86, 10.18, 9.85, 9.70; GC-MS: *m/z* (%): 128 (([M-18]$^+$, 20), 110 (30), 84 (60), 69 (80), 55 (100); HRMS (ESI) calcd for $C_8H_{18}NaO_2^+$ [M + Na]$^+$ 169.1199, found 169.1201.

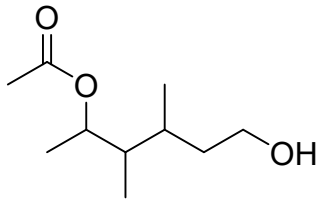
3,4-dimethylhexane-1,5-diyl diacetate (7)



7

To a solution of **6** (2.8 g, 19 mmol) in acetic anhydride (10 mL) were added pyridine (4 mL) and DMAP (50 mg). The reaction mixture was refluxed at 110 °C for three days, then it was washed with H_2O (70 ml), and the aqueous phase was extracted with CH_2Cl_2 (3x60 mL), the organic phase was dried over Na_2SO_4 and concentrated under *vacuum.* The crude material was purified by column chromatography (*n*-hexane/EtOAc 9:1) to give **7**. Yield 97% (4.3 g) as a colorless oil; t_r= 17.75 min, 99% purity by GC-MS; ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 4.95 (m, *J*= 6.2, 0.2 H, C<u>H</u>-OAc), 4.86 (ddq, *J*= 12.8, 8.4, 6.3 Hz, 0.8 H, C<u>H</u>-OAc), 4.16-3.94 (m, 2H, C<u>H</u>$_2$-OAc), 2.02 (dd, *J*= 4.7, 1,7 Hz, 6H, C<u>H</u>$_3$-CO), 1.79-1.22 (m, 4H), 1.18 (ddd, *J* = 10.9, 6.3, 3.4 Hz, 3H), 0.97-0.87 (m, 2H), 0.86-0.75 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 171.17, 170.81, 170.79, 170.74, 170.53, 73.40, 72.56, 72.51, 72.27, 63.29, 63.14, 63.11, 62.94, 43.09, 42.93, 41.68, 41.54, 34.09, 34.05, 31.77, 31.22, 30.91, 30.69, 30.50, 29.60, 21.42, 21.06, 18.68, 18.55, 17.99, 17.88, 17.80, 17.48, 14.57, 14.15, 11.07, 10.72, 10.12, 9.90; GC-MS: *m/z* (%): 171 ([M-59]$^+$, 20), 126 (70), 111 (83), 95 (96), 84 (88), 69 (72), 55 (100); HRMS (ESI) calcd for $C_{12}H_{22}NaO_4^+$ [M + Na]$^+$ 253.1410, found 253.1407.

6-hydroxy-3,4-dimethylhexan-2-yl acetate (8)

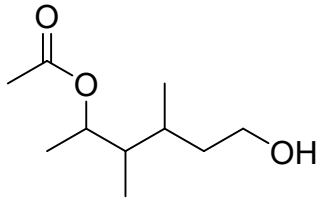


8

A solution of **7** (300 mg, 1.3 mmol) in THF (60 µL) was added to a KP_i buffer solution (3 mL, 50 mM, pH 7.0) containing PPL (150 mg). The reaction mixture was incubated in an orbital shaker (150 rpm, 30 °C) overnight. Then, it was filtered on a celite pad, and the aqueous phase was extracted with EtOAc (3x5 mL).[3] The crude material was purified by column chromatography (*n*-hexane/EtOAc 8:2) to give **8**.

Yield 45% (110 mg) as a colorless oil; t_r= 15.36 min, 99% purity by GC-MS; diastereomeric *ratio* 38:15:22:25 by ^{13}C NMR; ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 5.07-4.76 (m, 1H, C<u>H</u>-OAc), 3.78-3.43 (m, 2H, C<u>H</u>$_2$-OH), 2.02 (t, *J*= 2.5 Hz, 3H, C<u>H</u>$_3$-CO), 1.82-1.41 (m, 4H), 1.22-1.14 (m, 3H), 0.95-0.88 (m, 2H), 0.85-0.78 (m, 4H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 171.15, 171.04, 170.97, 170.86, 73.54, 72.83, 72.60, 72.42, 61.39, 61.31, 61.17, 60.53, 43.35, 43.29. 42.07, 41.69, 38.44, 38.35, 35.53, 34.79, 31.42, 30.44, 29.69, 29.28, 21.55, 21.49, 21.17, 18.82, 18.66, 18.03, 17.95, 17.74, 14.70, 14.33, 14.30, 10.98, 10.93, 10.24, 10.04; GC-MS: *m/z* (%): 129 ([M-59]$^+$, 20), 113 (33), 95 (40), 84 (86), 69 (70), 56 (100); HRMS (ESI) calcd for $C_{10}H_{20}NaO_3^+$ [M + Na]$^+$ 211.1305, found 211.1307.

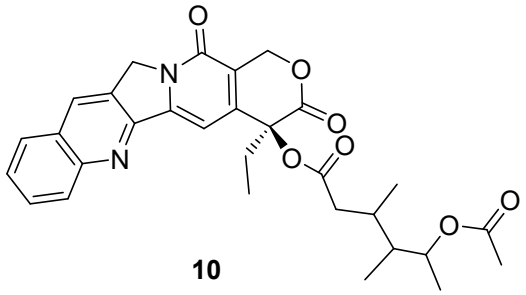
5-acetoxy-3,4-dimethylhexanoic acid (9)



9

A mixture of **8** (110 mg, 0.6 mmol) and TEMPO (5 mg, 0.03 mmol) in toluene (3 mL) and KP_i buffer solution (2 mL, 1 M , pH = 7) was stirred at 25 °C. Then $NaClO_2$ (270 mg, 3 mmol) as aqueous solution (3.5 M) and dilute bleach (2.1 M, 56 µL) were added simultaneously over 1 minute. The mixture was stirred at room temperature overnight. To the reaction mixture was added NaCl and extracted with AcOEt (3x5 mL). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure affording **9**.[4] Yield 85% (103 mg) as a colorless oil; t_r =14.29 min, t_r =14.67 min, t_r =14.75 min, t_r =14.81 min, 99% purity by GC-MS; diastereomeric *ratio* 25:14:34:27 by GC-MS; ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 5.04-4.78 (m, 1H, C<u>H</u>-OAc), 2.52-2.08 (m, 3H), 2.08-2.00 (m, 3H, C<u>H</u>$_3$-CO), 1.75-1.45 (m, 1H), 1.31-1.14 (m, 3H), 1.01 (dd, *J*= 6.7, 1.2 Hz, 1.5H), 0.96-0.78 (m, 4.5H); ^{13}C NMR (100 MHz, $CDCl_3$): δ=179.08, 178.91, 178.61, 178.57, 170.52, 170.49, 170.47, 170.41, 72.68, 72.05, 71.82, 71.75, 42.05, 41.94, 41.01, 40.91, 39.86, 39.66, 37.52, 36.90, 31.77, 30.75, 30.29, 29.42, 20.89, 18.08, 17.94, 17.82, 17.47, 16.88, 14.65, 13.80, 10.87, 10.37, 9.77; GC-MS: *m/z* (%): 153([M-59]$^+$, 15), 113 (33), 95 (40), 84 (86), 69 (70), 56 (100); HRMS (ESI) calcd for $C_{10}H_{18}NaO_4^+$ [M + Na]$^+$ 225.1097, found 225.1295.

(*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl 5-acetoxy-3,4-dimethylhexanoate (10)



10

To an iced cooled solution of Camptothecin (250 mg, 0.72 mmol), **9** (145 mg, 0.72 mmol) in CH_2Cl_2 (30 mL) were added EDCI (145 mg, 1 mmol) and DMAP (231 mg, 0.6 mmol). The reaction mixture was stirred for 16 hours at room temperature, then the solvents were removed under *vacuum* and the crude material was purified by column chromatography (CH_2Cl_2/MeOH 98:2) to give **10**. Yield 72% (275 mg) as a white solid; $[\alpha]_D$=+43.4° (*c* 0.20, CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz): δ= 8.39 (s, 1H), 8.21 (d, *J*= 8.6 Hz, 1H), 7.94 (d, *J*= 8.3 Hz, 1H), 7.83 (ddd, *J*=8.5, 6.8, 1.4 Hz, 1H), 7.67 (ddd, *J*= 8.1, 7.0, 1.1 Hz, 1H), 7.21 (td, *J*= 4.7, 4.2, 2.5 Hz, 1H), 5.67 (dt, *J*= 17.2, 2.4 Hz, 1H), 5.41 (dt, *J*= 17.2, .6 Hz, 1H), 5.29 (d, *J*= 2.5 Hz, 3H), 5.01- 4.80 (m, 1H), 2.69-2.09 (m, 5H), 2.08-1.97 (m, 3H), 1.32-1.08 (m, 5H), 1.05-0.77 (m, 9H); ^{13}C NMR (100 MHz, $CDCl_3$): δ= 172.62, 72,47, 172.29, 172.00, 171.95, 170.80, 170.73, 170.58, 169.92, 167.61, 167.53, 157.45, 152.43, 148.99, 146.36, 146.09, 146.03, 145.92, 145.88, 131.24, 130.72, 129.76, 129.73, 129.71, 128.55, 128.29, 128.10, 120.52, 120.48, 120.37, 120.33, 96.12, 96.02, 95.98, 75.92, 75.87, 73.21, 73.18, 72.49, 72.49, 72.43, 72.27, 72.18, 72.03, 71.80, 67.22, 67.19, 50.02, 42.32, 42.29, 42.23, 41.25, 41.19, 41.19, 41.14, 39.97, 39.85, 39.75, 39.67, 37.92, 37.64, 37.56, 37.23, 32.70, 32.65, 31.96, 31.69, 31.63, 31.58, 21.54, 30.12, 29.87, 29.78, 21.46, 21.41, 21.30, 18.62, 18.43, 18.27, 18.21, 18.15, 18.12, 17.98, 17.91, 17.34, 17.12, 14.91, 14.88, 14,17, 14.13, 11.84, 11.75, 11.16, 11.09, 10.20, 10.10, 10.07, 7.67; HRMS (ESI) calcd for $C_{30}H_{32}N_2NaO_7$ $^+$ $[M + Na]^+$ 555.2102, found 555.2105.

Determination of the relative stereochemical configuration of lactones (2)

The ^{1}H-NMR spectrum of a diastereomeric mixture of **2** in C_6D_6 allowed to assign the relative stereochemical configuration of each isomer by measuring the coupling constants (*J*) of H(6) proton signals. First, in figure S1 we show the conformational analysis of each diastereoisomer. Regardless to the conformation adopted by the *cis*,*trans* and *cis*,*cis* lactones, H(6) is always *gauche* with respect to H(5) (Figure S1a). Therefore, according to the Karplus plot, these two diastereoisomers should have a small $J_{H(6)-H(5)}$ (2-5 Hz). Conversely, the *trans*,*trans* and *trans*,*cis* lactones adopt mainly a conformation in which H(6) is *anti* to H(5) (Figure S1b). In this case, for both diastereoisomers $J_{H(6)-H(5)}$ should be much larger (8-10 Hz).

Hence, the signals H(6) between 3.80 and 3.95 ppm were attributed to the *cis*,*trans* and *cis*,*cis* lactones (Figure S3); more precisely, the doublet of quartet at 3.85 ppm (*J*=6.5 and 2.5 Hz) was assigned to the diastereoisomer with *cis*,*cis* configuration, because its coupling constant values are identical to those reported in the literature.[5]

The *trans*,*trans* diastereoisomer adopts exclusively the conformation in which all the methyl groups are equatorial, and therefore, it should have a higher $J_{H(6)-H(5)}$ than that of the *trans*,*cis* lactone, which, instead is under conformational equilibrium (Figure S1b). Thus, the doublet of quartet at 3.41 ppm with $J_{H(6)-H(5)}$=10.1 Hz was attributed to the *trans*,*trans* diastereoisomer (Figure S2).

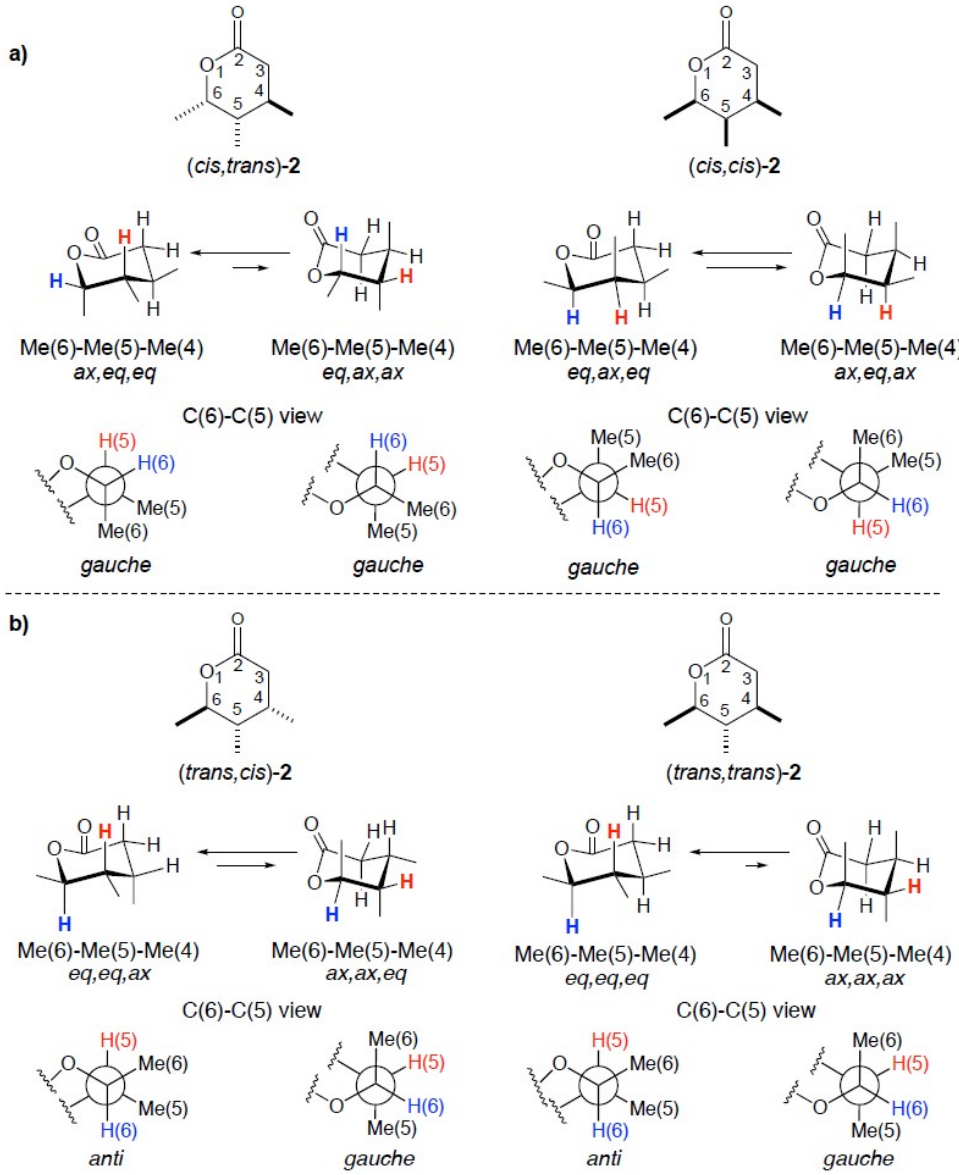


Figure S1. Conformational analysis of lactone **2**: a) *cis*,*cis* and *cis*,*trans* isomers; b) *trans*,*trans* and *trans*,*cis* isomers.

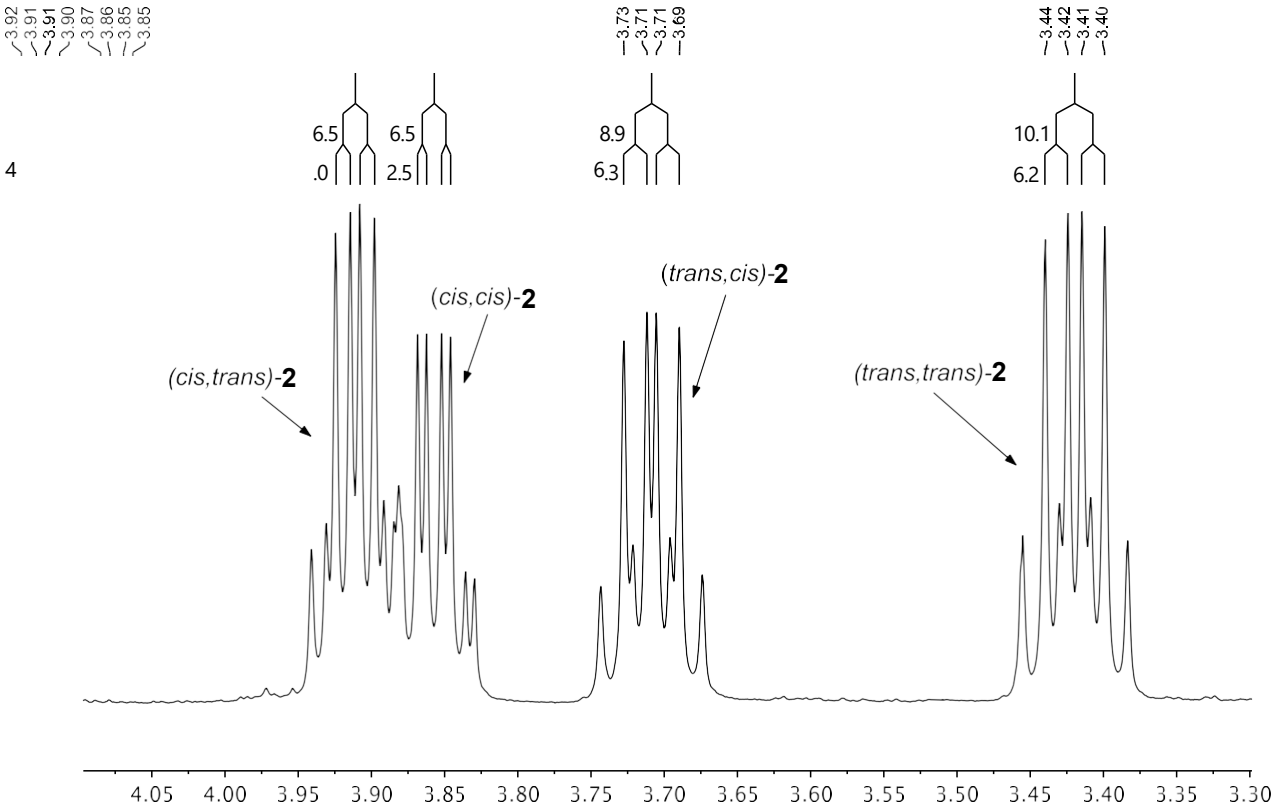


Figure S2. [1]H-NMR spectra expansion relative to the H(6) signals: *cis*,*trans*; *cis,cis; trans*,*cis; trans*,*trans* lactones.

Determination of the relative stereochemistry of hydroxy esters

Following the ring-closure kinetic of hydroxy esters into the corresponding lactones, it was possible to assign to each CHOH signal its diastereoisomer knowing the stereochemistry of products (Figure S3).

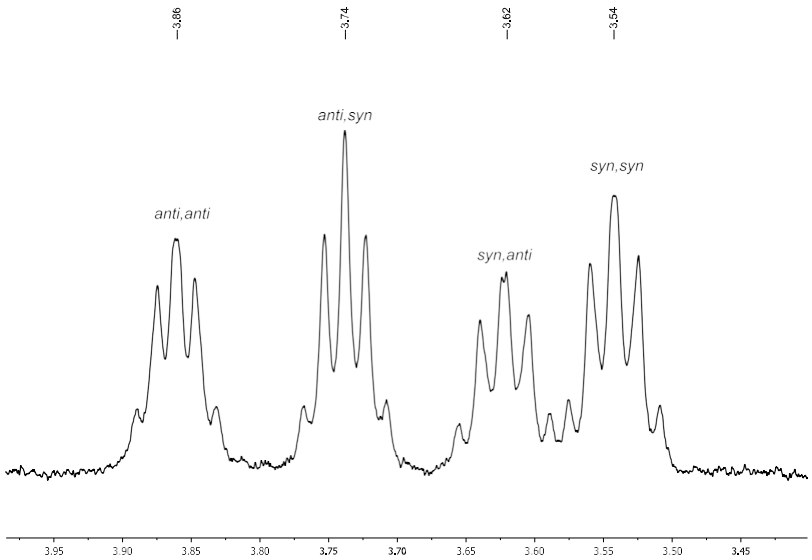


Figure S3. [1]H-NMR spectra expansion relative to the CHOH signals of hydroxy esters: *syn*,*syn*; *syn,anti; anti*,*syn; anti*,*anti.*

Kinetic study of TFA catalyzed ring-closure of *i*-propyl hydroxy ester

The reaction progress was monitored by measuring a series of ^{1}H-NMR (500 MHz) spectra recorded as pseudo 2D of a sample of hydroxy esters in $CDCl_3$ at 303 K allowing a fixed time delay between successive spectra. A pulse width of 30° and a relaxation delay of 2 s were used to avoid errors due to the relaxation. An exponential filter was applied to FID for optimization of the signal-to-noise ratio. Baseline correction was applied to spectra before integration. The bias and slope of integrals were carefully adjusted. The sample was prepared by adding directly, with a gastight Hamilton syringe, a solution of TFA in CH_2Cl_2 (10^{-2} M) to a solution of hydroxy ester (typically ≈8–10 mg each) in $CDCl_3$ (500.0 µL) into an NMR tube. Since the ring closure rates of hydroxy esters were so different, it was necessary to split the kinetic study into different experiments using two different concentrations of TFA. A concentration of TFA allowed us to measure the rate constants of the slowest diastereisomers, *i.e. syn*,*syn* and *syn*,*anti* and *anti*,*anti*, and then a lower concentration of TFA allowed to measure the rate constants of the most rapid diastereoisomers, *i.e. anti*,*anti* and *anti*,*syn*.

The cyclization rate of hydroxyester **E** into lactone **L** (Figure S4) is described by equation 1.

$$-\mathrm{d[E]/dt} = k_{\mathrm{true}}[\mathrm{TFA}][\mathrm{E}] = k_{234}[\mathrm{E}] \quad \text{(eq. 1)}$$

Where $k_{obs} = k_{true}$ [TFA] is the experimental pseudo first-order rate constant and k_{true} is the true second-order catalytic rate constant.

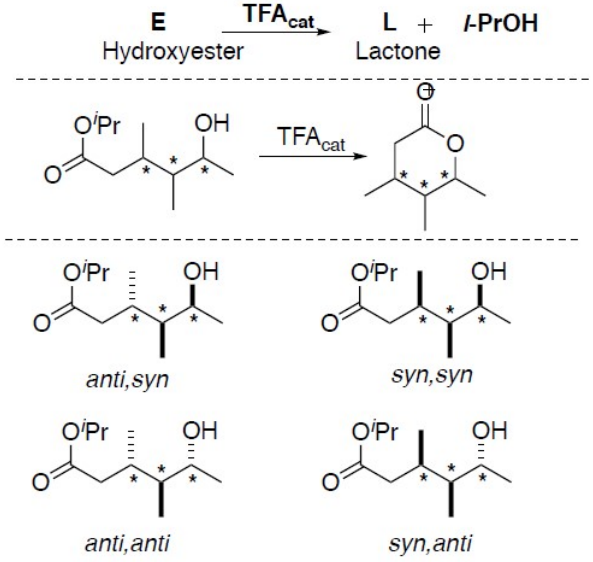


Figure S4. Kinetic scheme and hydroxy esters studied.

The observed pseudo-first order reaction rate constant k_{obs} was obtained by linear regression of $\ln[E]/[E]_0$ *versus* time. The data were analyzed using Excel software. The relative rate constants k_r were calculated with respect to the *syn*,*syn* diastereoisomer. The relative Gibbs energy barriers ($\delta\Delta G^{\ddagger}$) were calculated with respect to the *syn*,*syn* diastereoisomer (the slowest) following eq. 2, where *R*T at 302 K is 0.602 kcal mol^{-1}. The kinetic parameters are summarized in Table S1.

$$\partial\Delta G^{\ddagger} = -RT\, ln(k_r) \quad \text{(eq. 2)}$$

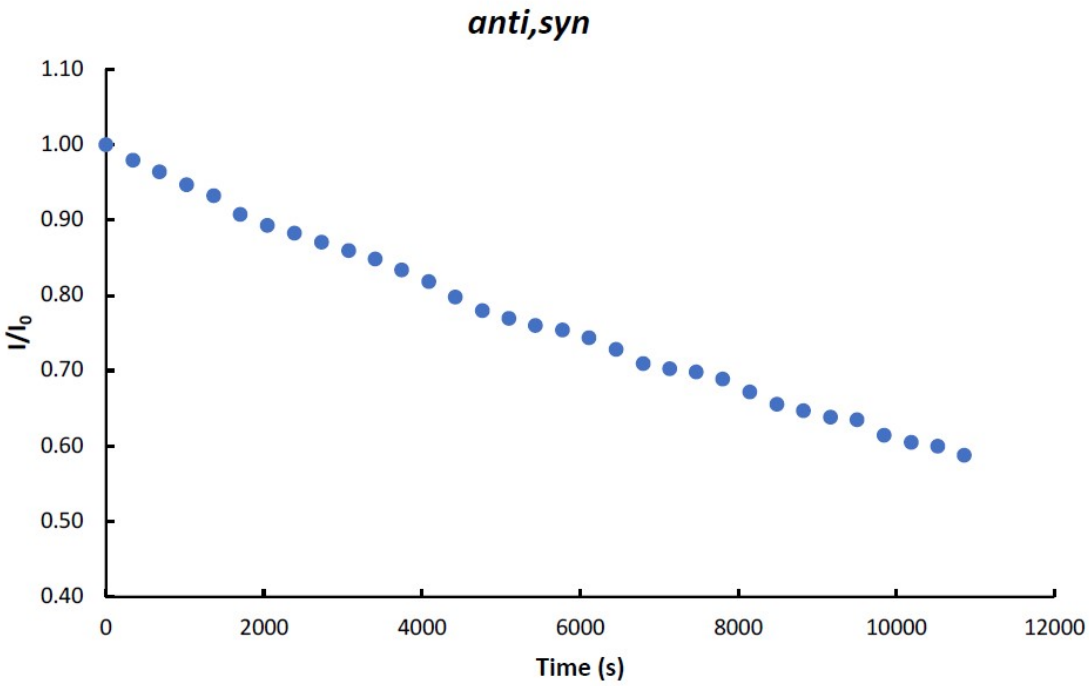
Table S1. Summary of kinetic data of the TFA catalyzed ring closure of hydroxyesters in $CDCl_3$ at 302 K.

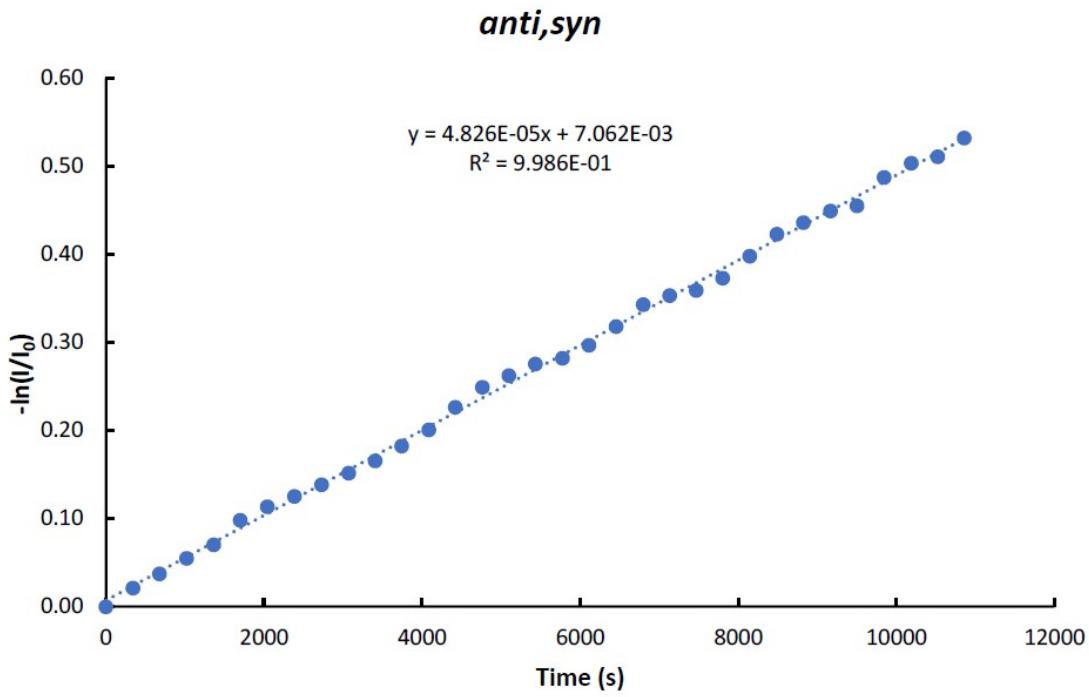
Isomer	$k_{obs(1st)}$ (k_r) [a] [sec^{-1}]	$k_{obs(2nd)}$ (k_r) [b] [sec^{-1}]	k_r	$\delta\Delta G^{\ddagger}$ [kcal mol^{-1}]
anti,syn	$4.83\ 10^{-5}$ (1.0)		1.0	0
syn,syn	$6.77\ 10^{-5}$ (1.5)		1.5	-0.24
anti,anti	$5.89\ 10^{-4}$ (12.2)		12.2	-1.51
anti,anti		$1.54\ 10^{-4}$ (1.0)		
syn,anti		$7.94\ 10^{-4}$ (5.2)	63.4	-2.50

Notes: a) kinetic date with [TFA]= $1.7\ 10^{-4}$; b) a) kinetic date with [TFA]= $4.4\ 10^{-5}$.

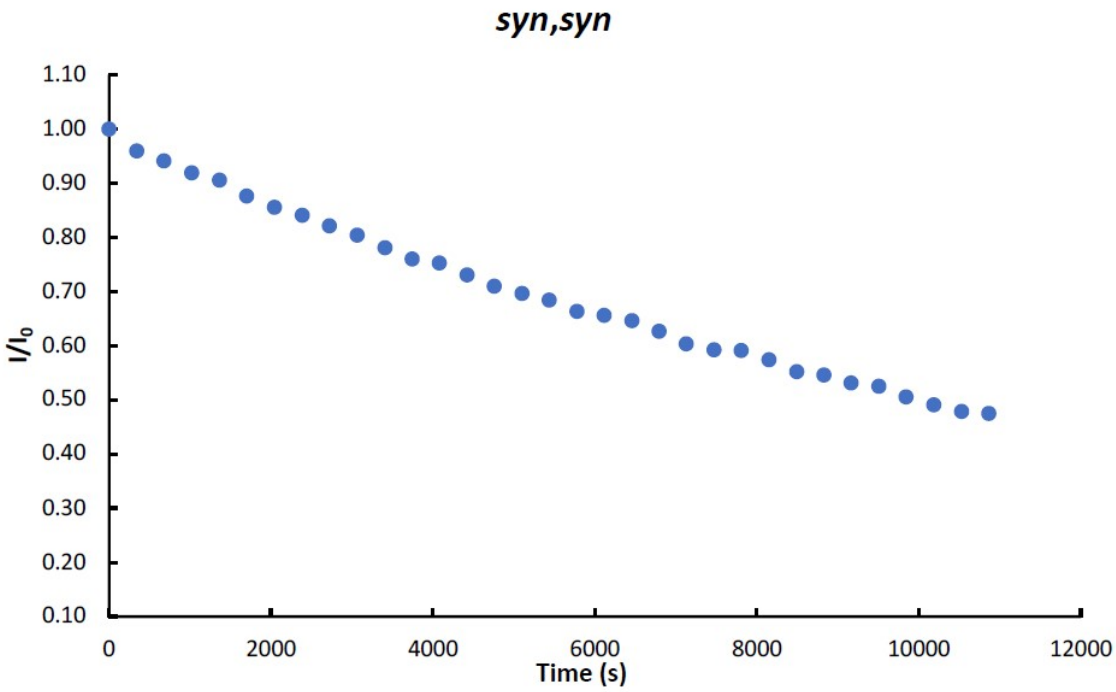
Linear regression plots of TFA catalyzed ring closure in $CDCl_3$ at 303 K first group of kinetics

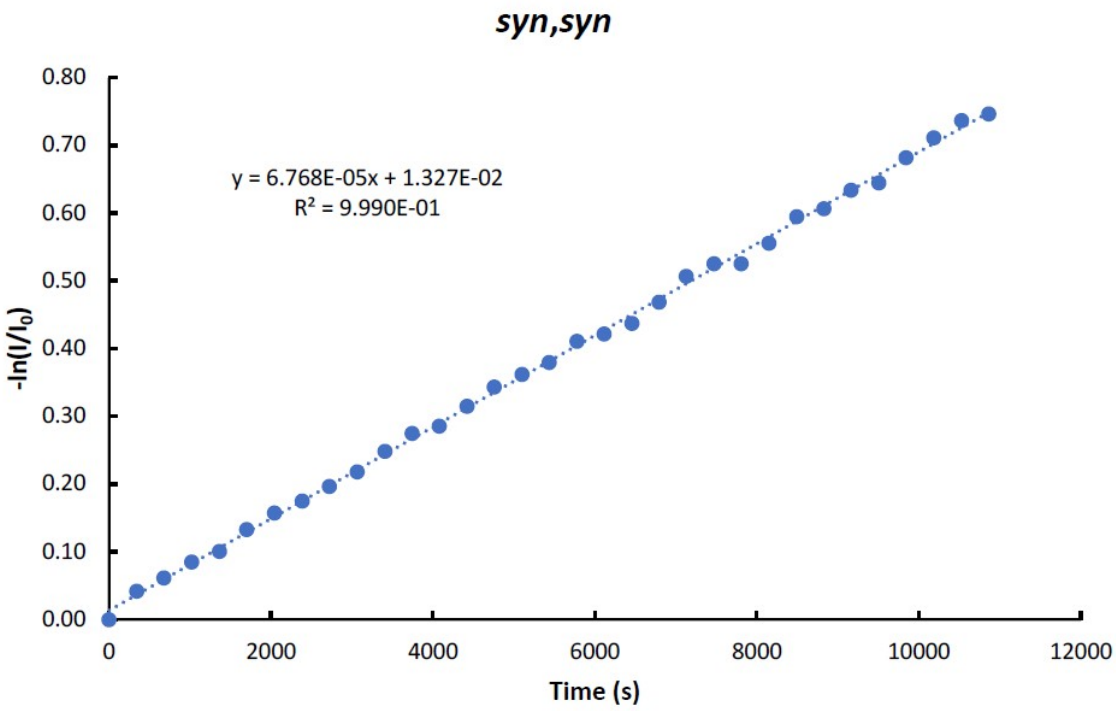
anti,syn $k_{obs}=(4.83)\times10^{-5}\ s^{-1}$, $r^2 = 0.998$, [TFA]= $(1.70\pm0.10)\times10^{-4}$ M
CHOH= 3.74 ppm to *cis*,*cis* H(6) at 4.48 ppm



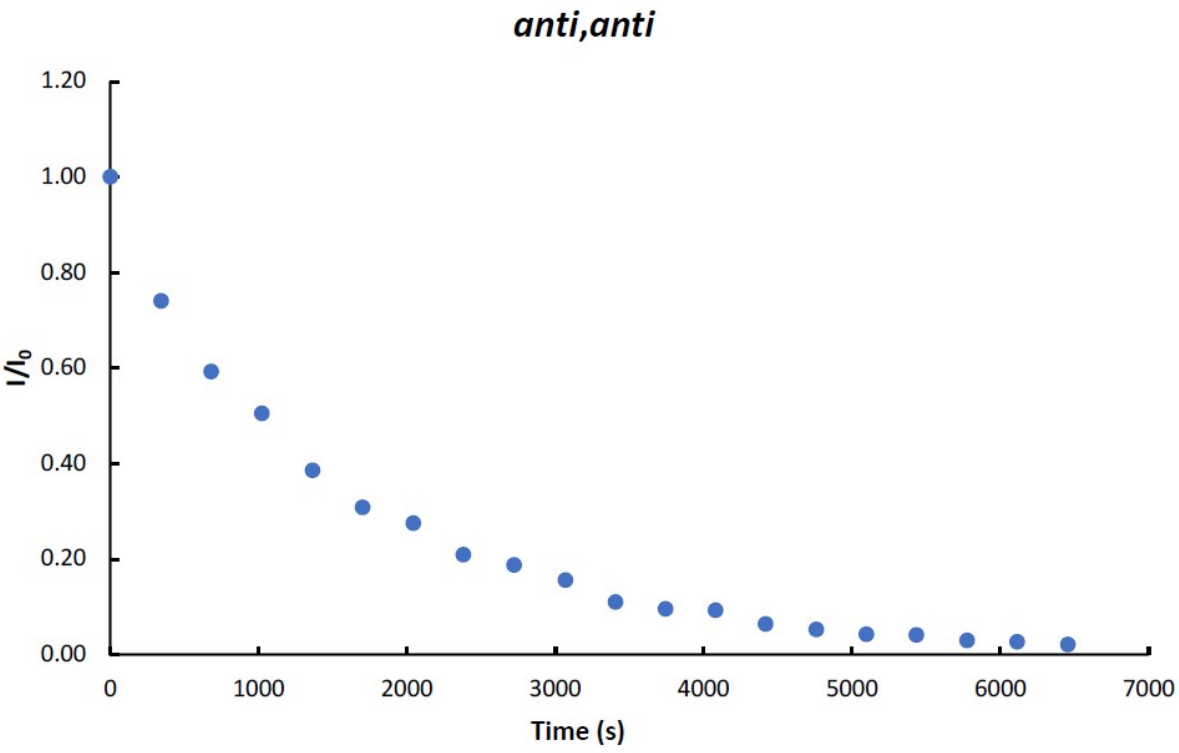


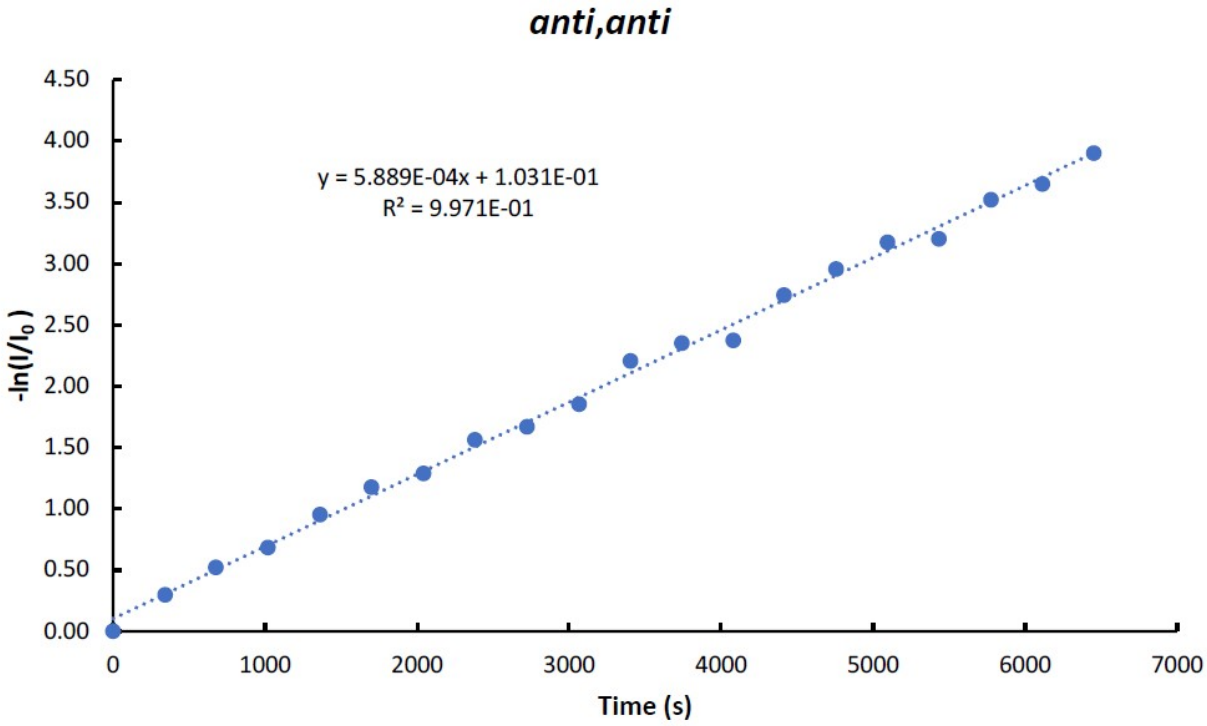
syn*,*syn k_{obs}=(6.77)x$10^{-5}s^{-1}$, r^2 = 0.999, [TFA]= (1.70±0.10)x10^{-4} M
CHOH= 3.54 ppm to *cis*,*trans* H(6) at 4.48 ppm





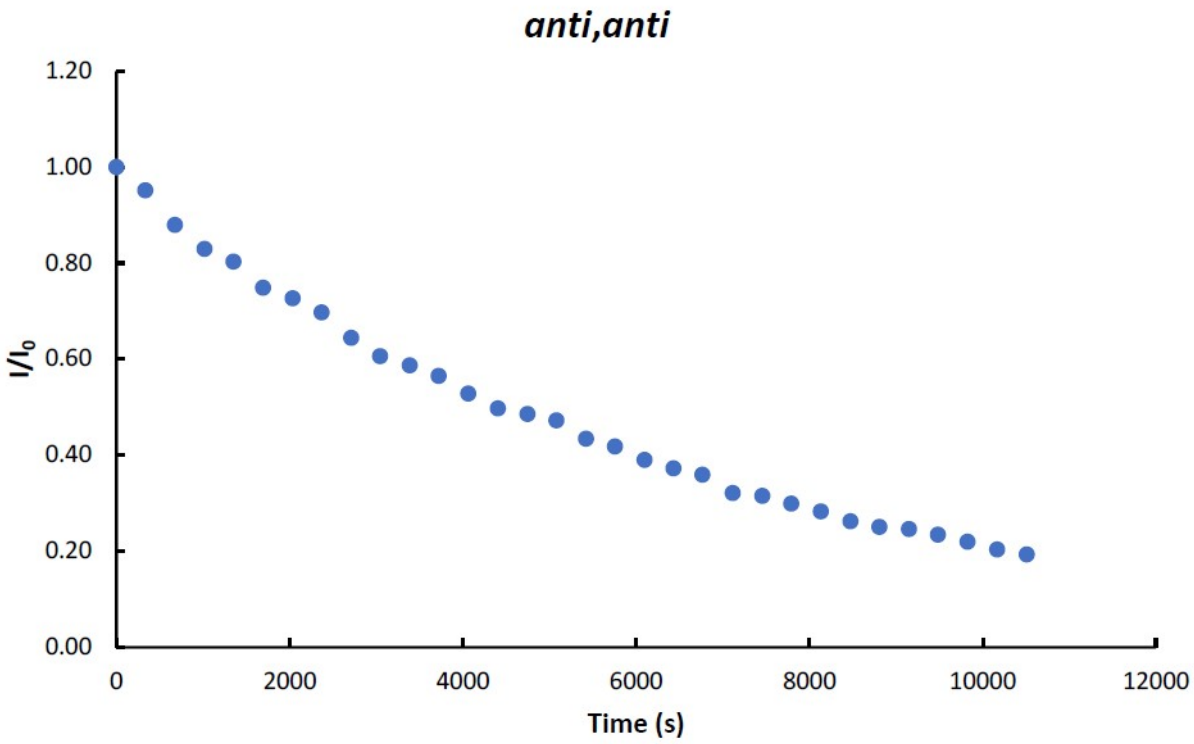
anti,anti $k_{obs}=(5.89)x10^{-4}s^{-1}$, $r^2 = 0.997$, [TFA]= $(1.70\pm0.10)x10^{-4}$ M
CHOH= 3.86 ppm to *trans*,*cis* H(6) at 4.23 ppm

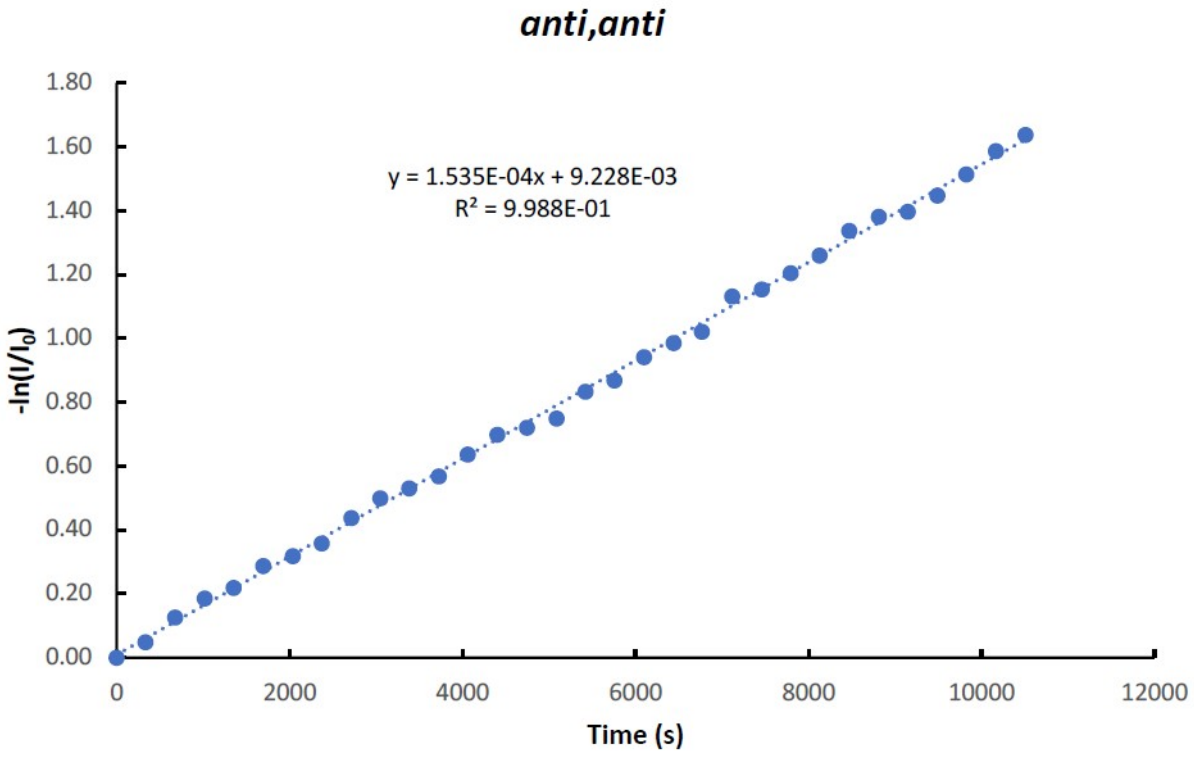




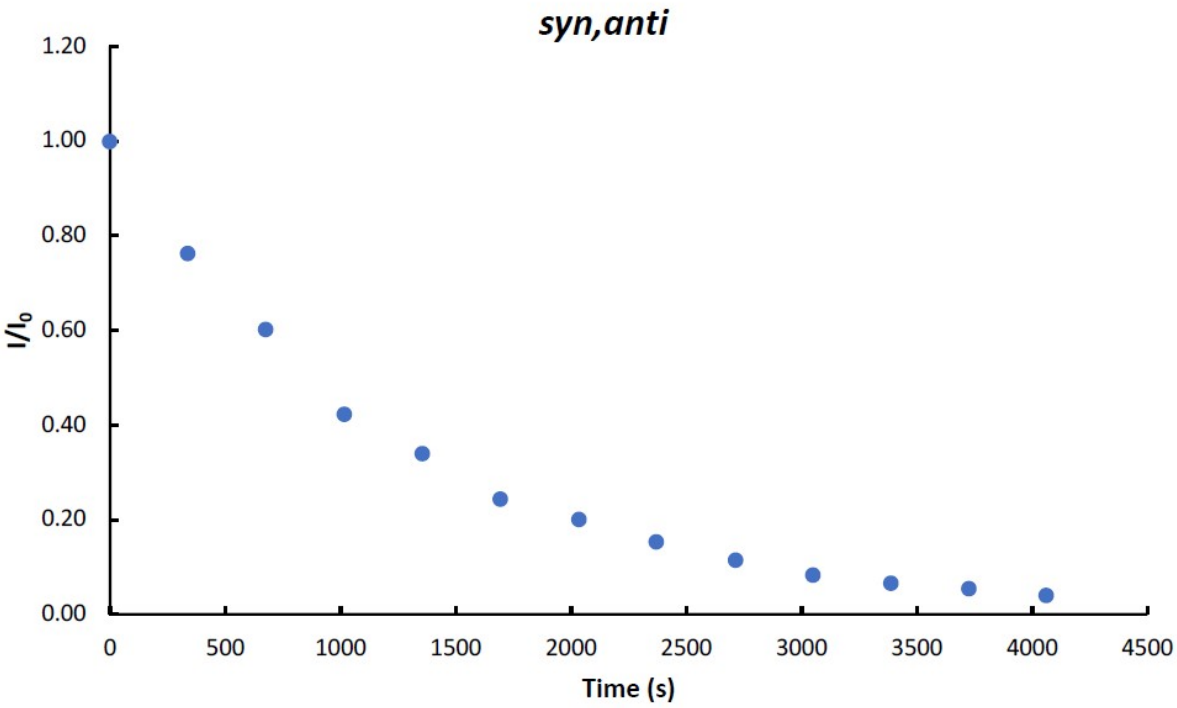
Linear regression plots of TFA catalyzed ring closure in $CDCl_3$ at 303 K second group of kinetics

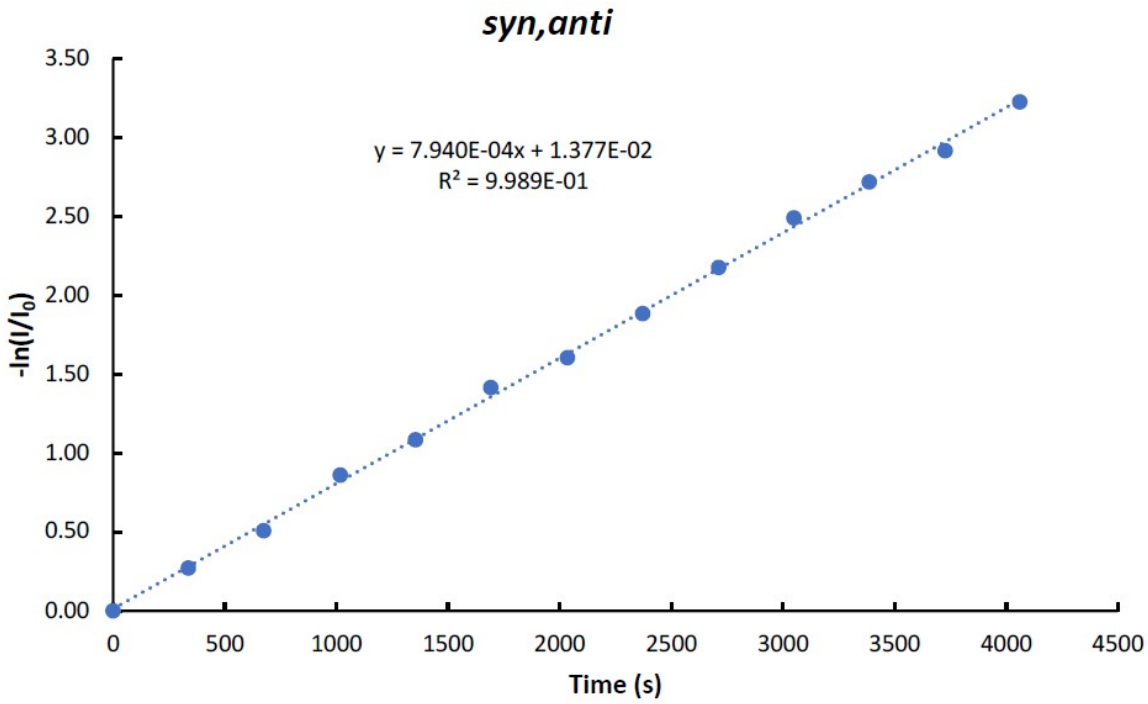
anti,anti k_{obs}=(1.54)x10^{-4} s^{-1}, r^2 = 0.999, [TFA]= (4.44±0.10)x10^{-5} M
CHOH= 3.86 ppm to *trans*,*cis* H(6) at 4.23 ppm





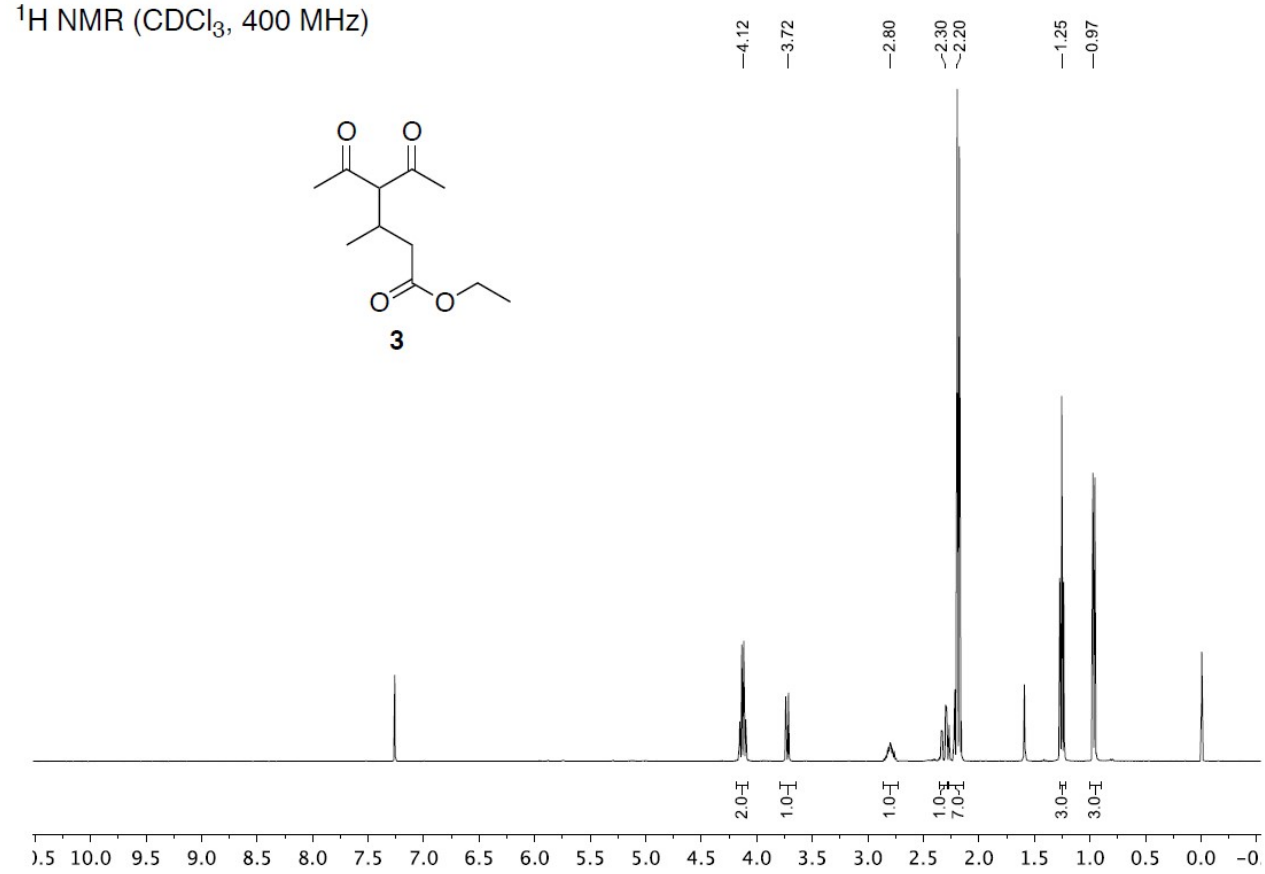
syn,anti $k_{obs}=(7.94)\times10^{-4}\ s^{-1}$, $r^2 = 0.999$, [TFA]= $(4.44\pm0.10)\times10^{-5}$ M
CHOH= 3.62 ppm to *trans*,*trans* H(6) at 4.00 ppm



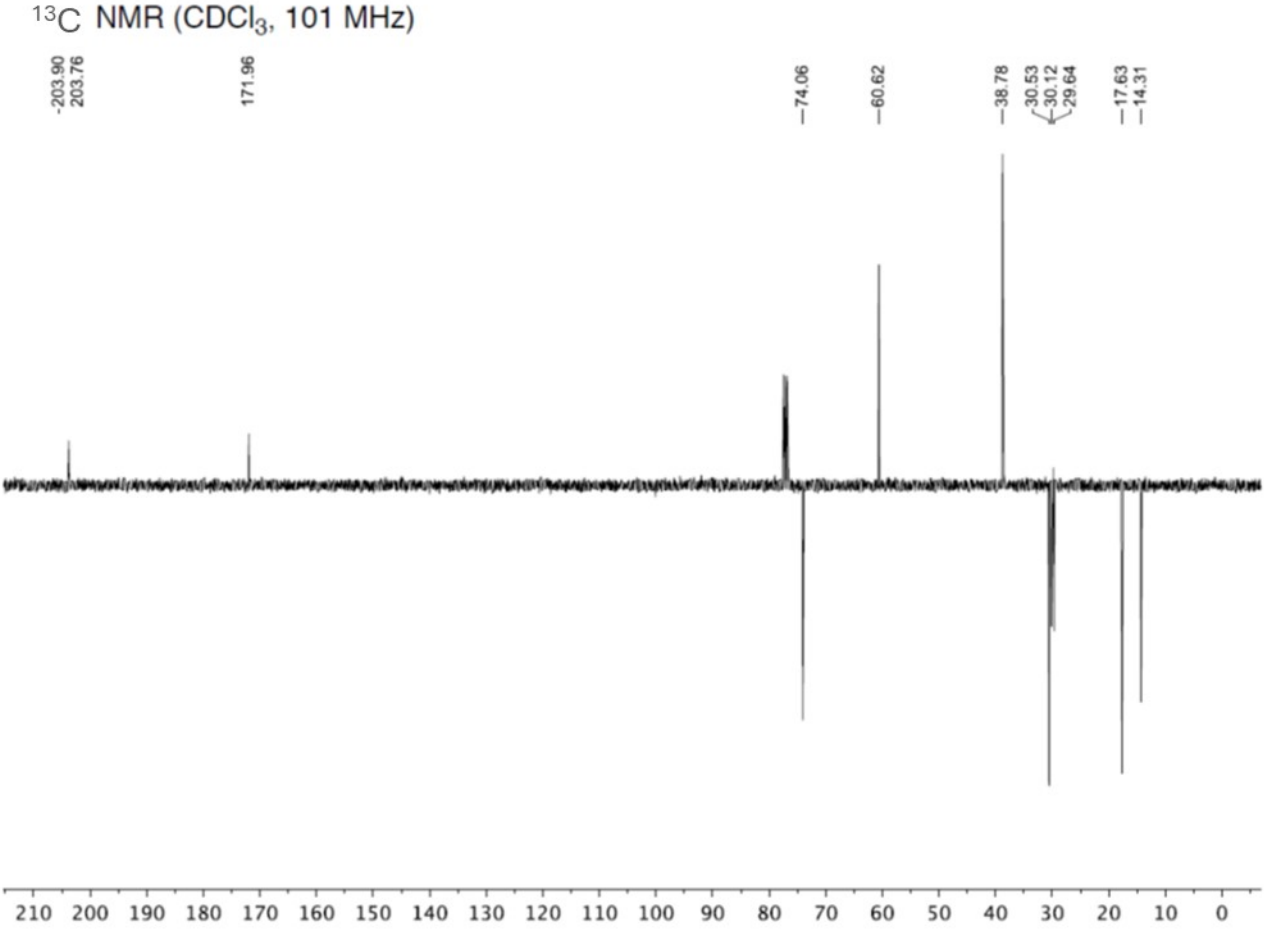


Copies of ^{1}H and ^{13}C spectra

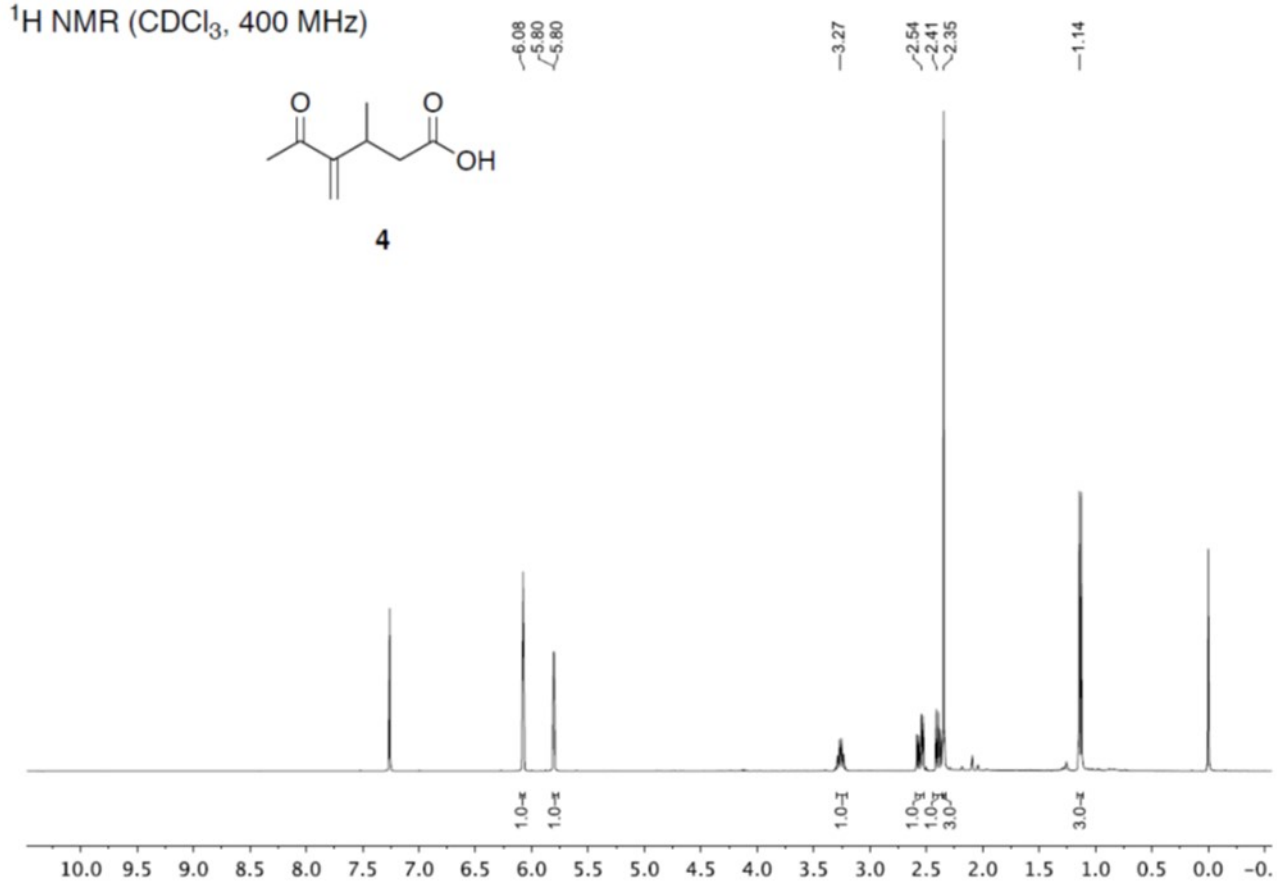
Ethyl 4-acetyl-3-methyl-5-oxohexanoate (3)

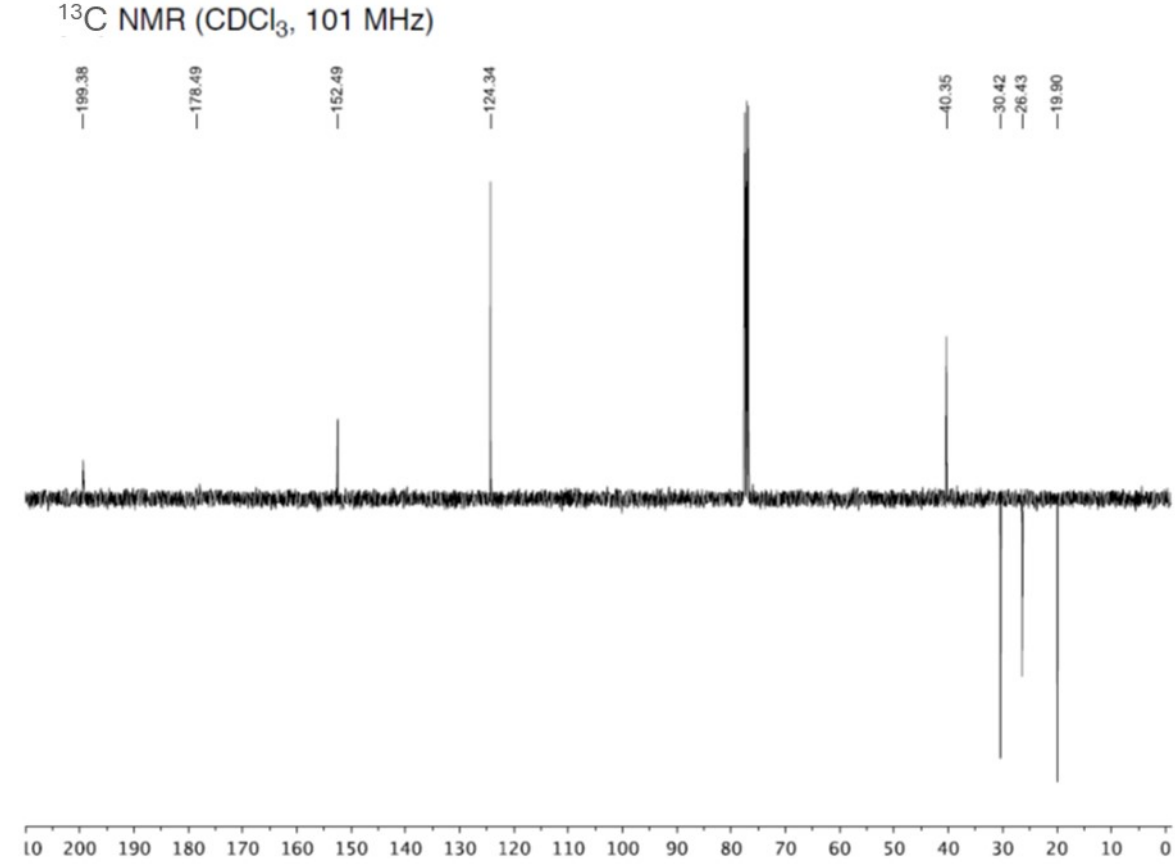


3



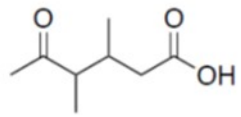
3-methyl-4-methylene-5-oxohexanoic acid (4)





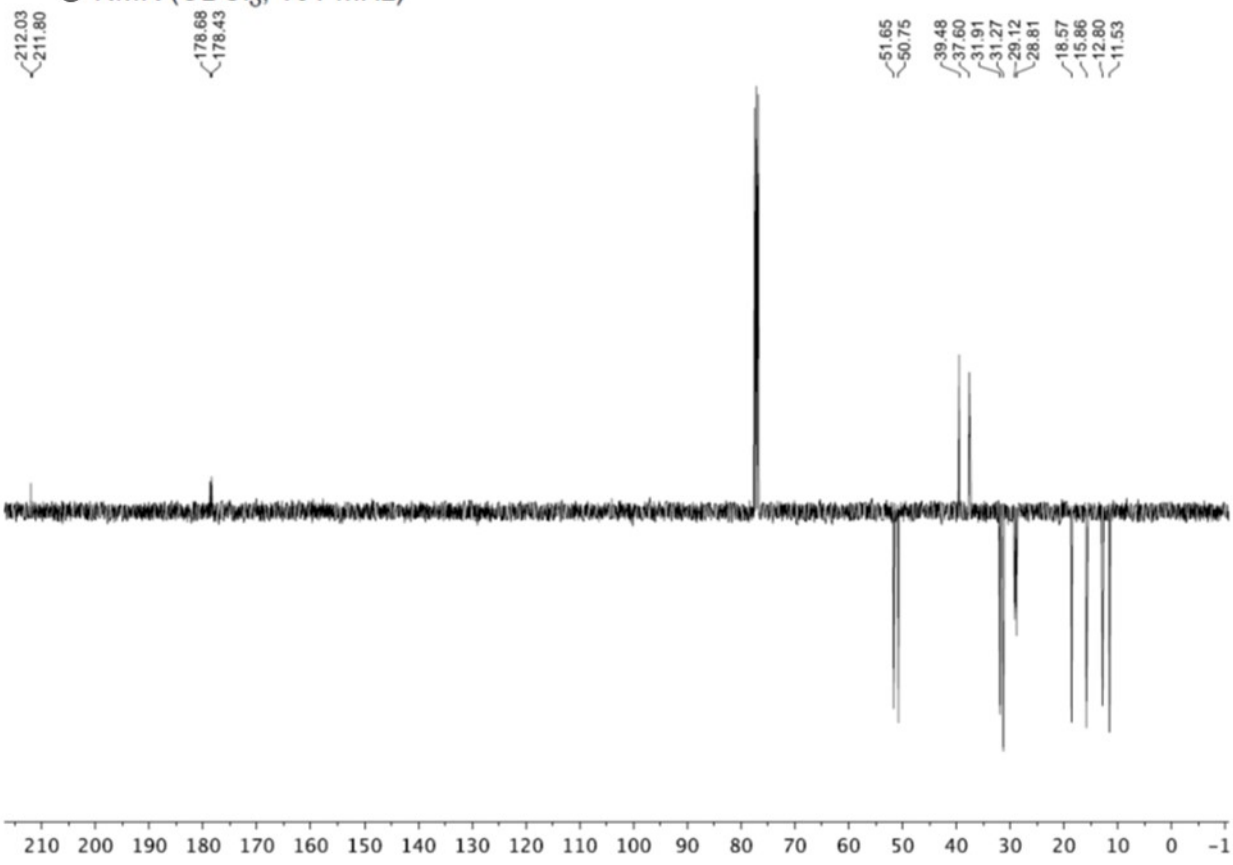
3,4-dimethyl-5-oxohexanoic acid (5)

^{1}H NMR ($CDCl_3$, 400 MHz)

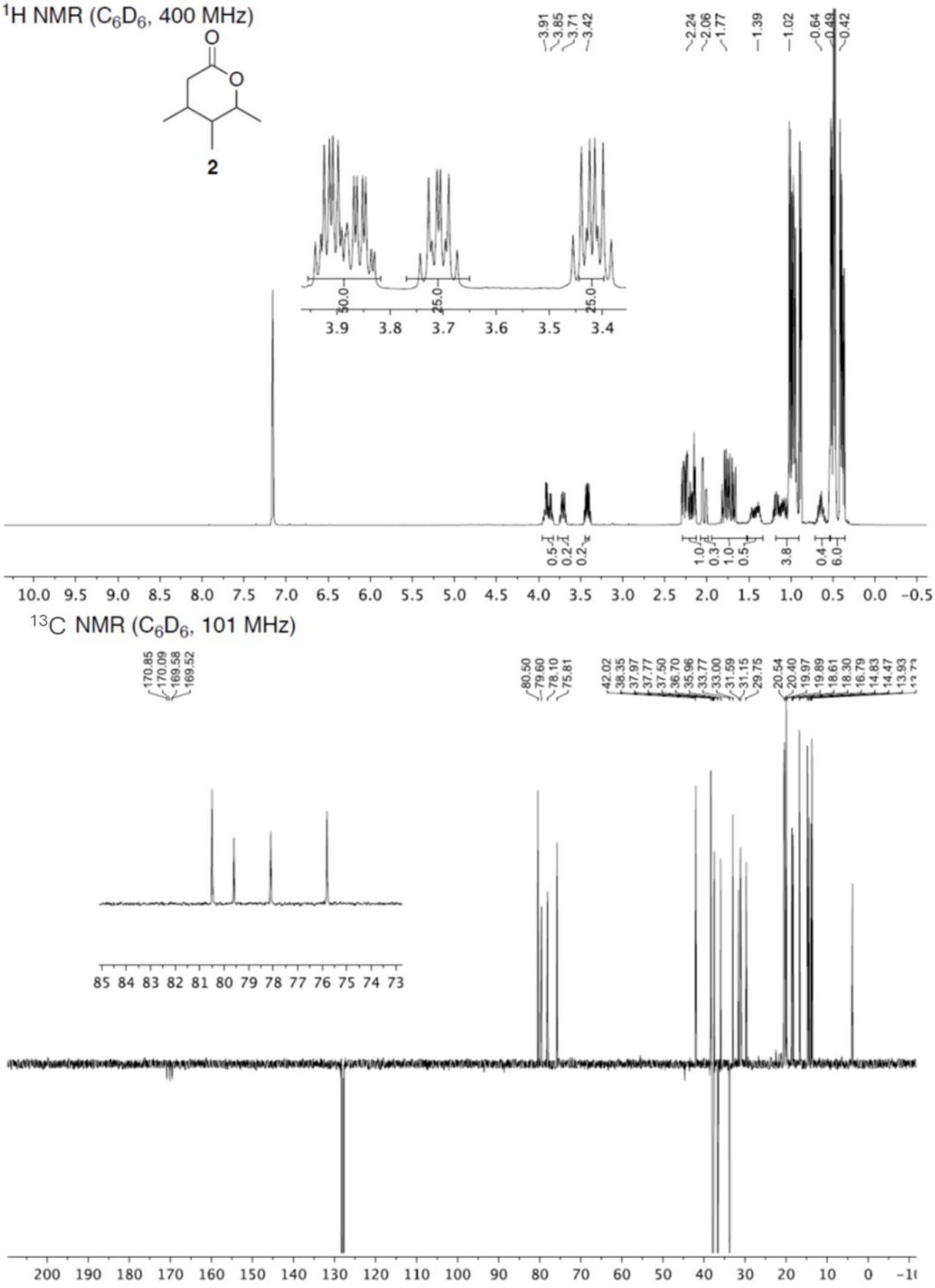


5

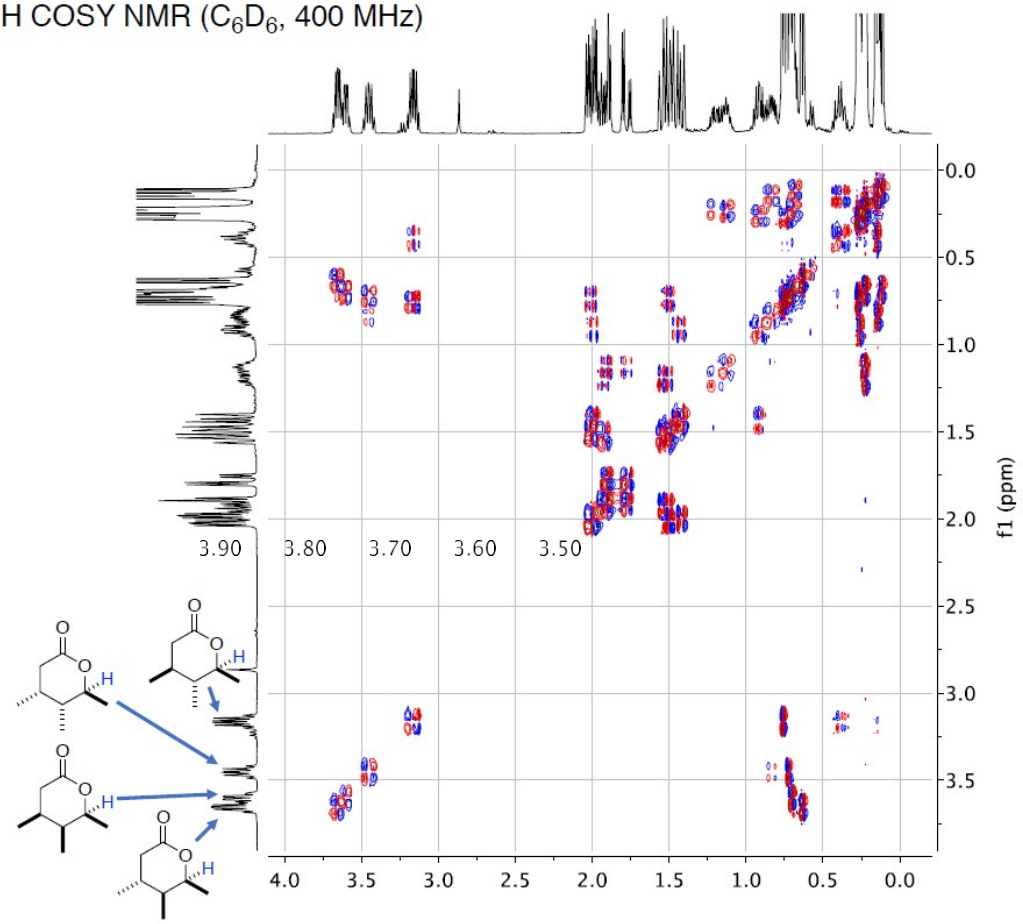
^{13}C NMR ($CDCl_3$, 101 MHz)



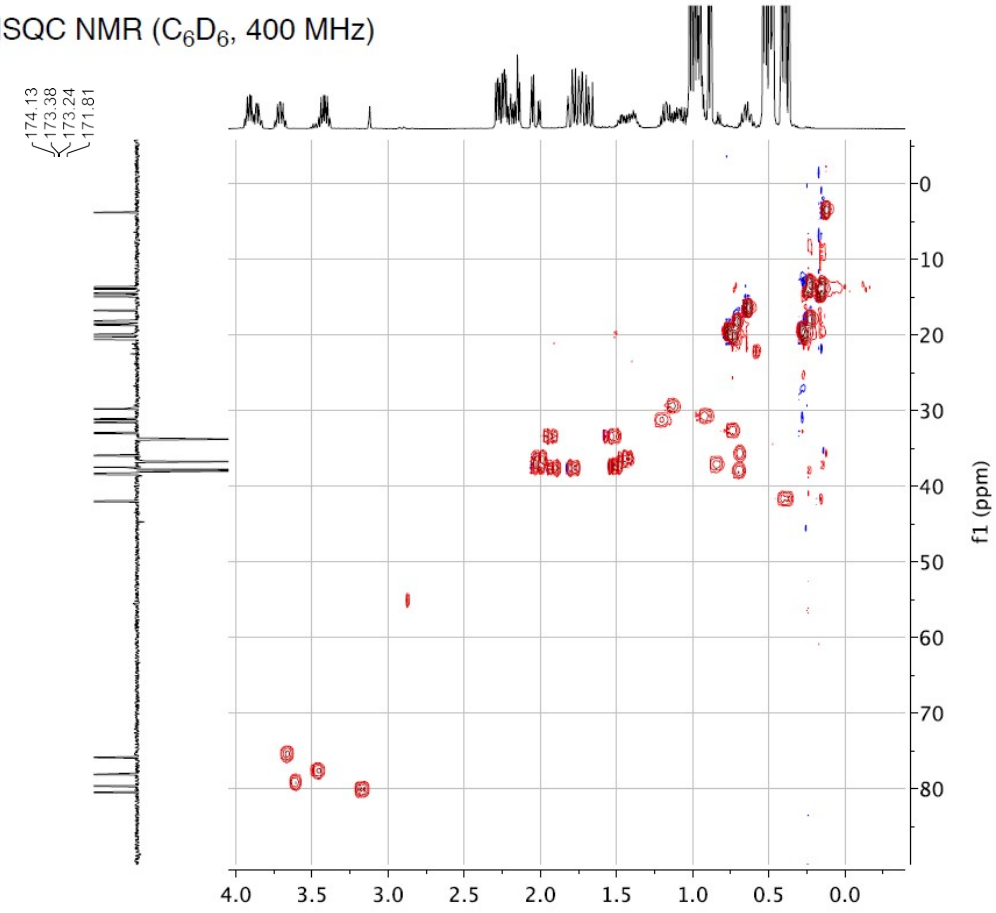
4,5,6-trimethyltetrahydro-2*H*-pyran-2-one (2)



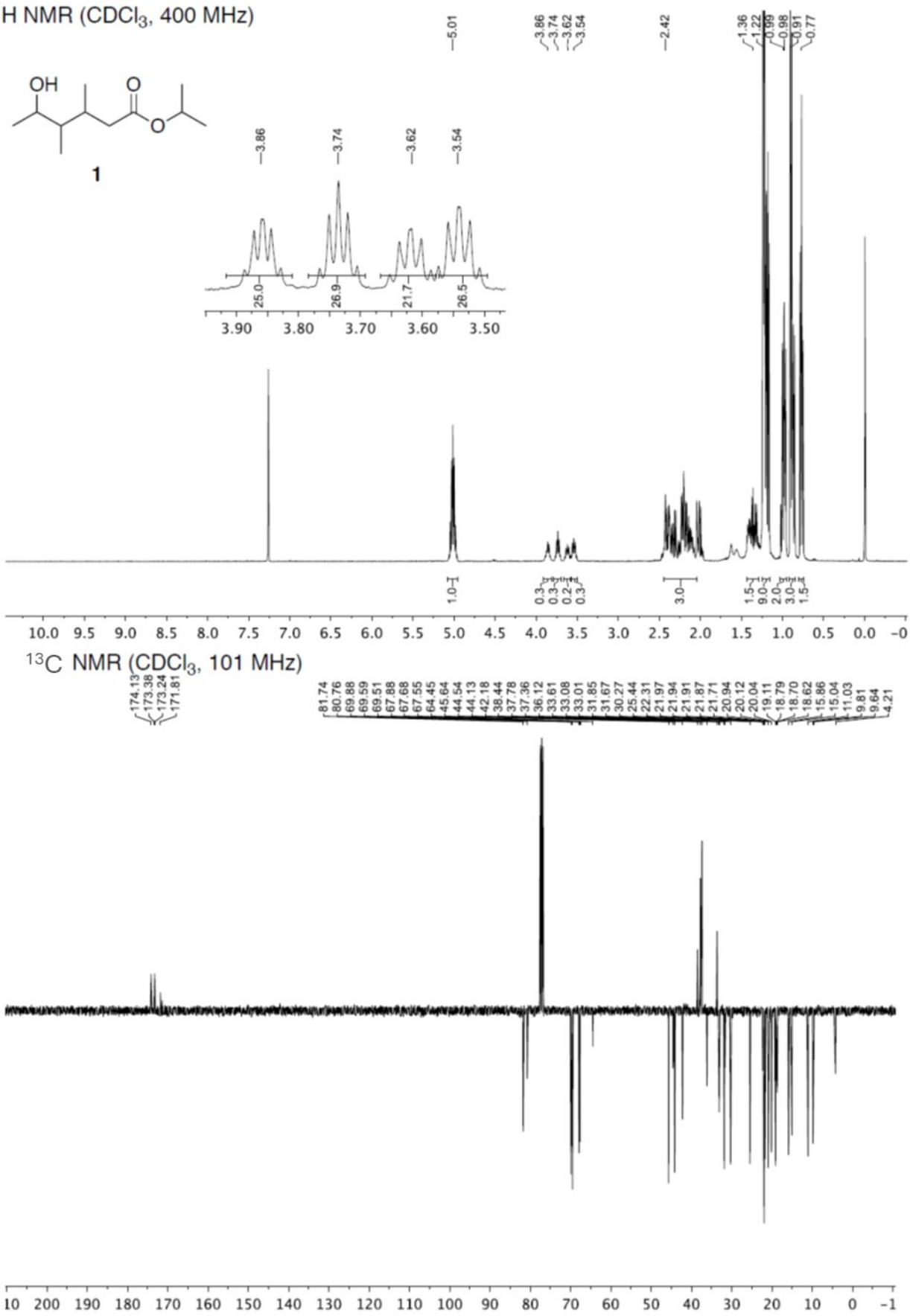
^{1}H-^{1}H COSY NMR (C_6D_6, 400 MHz)



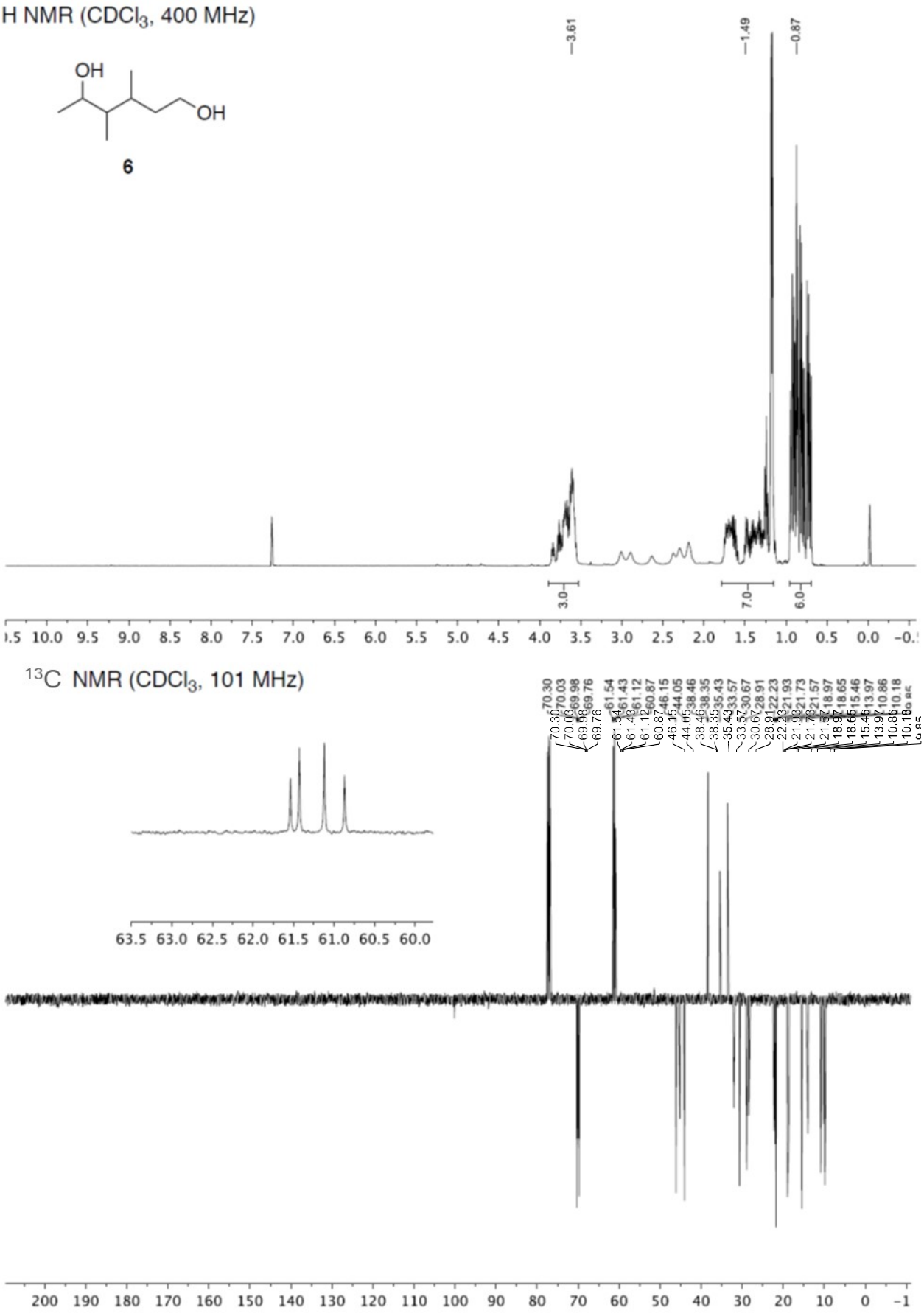
^{1}H-^{13}C HSQC NMR (C_6D_6, 400 MHz)



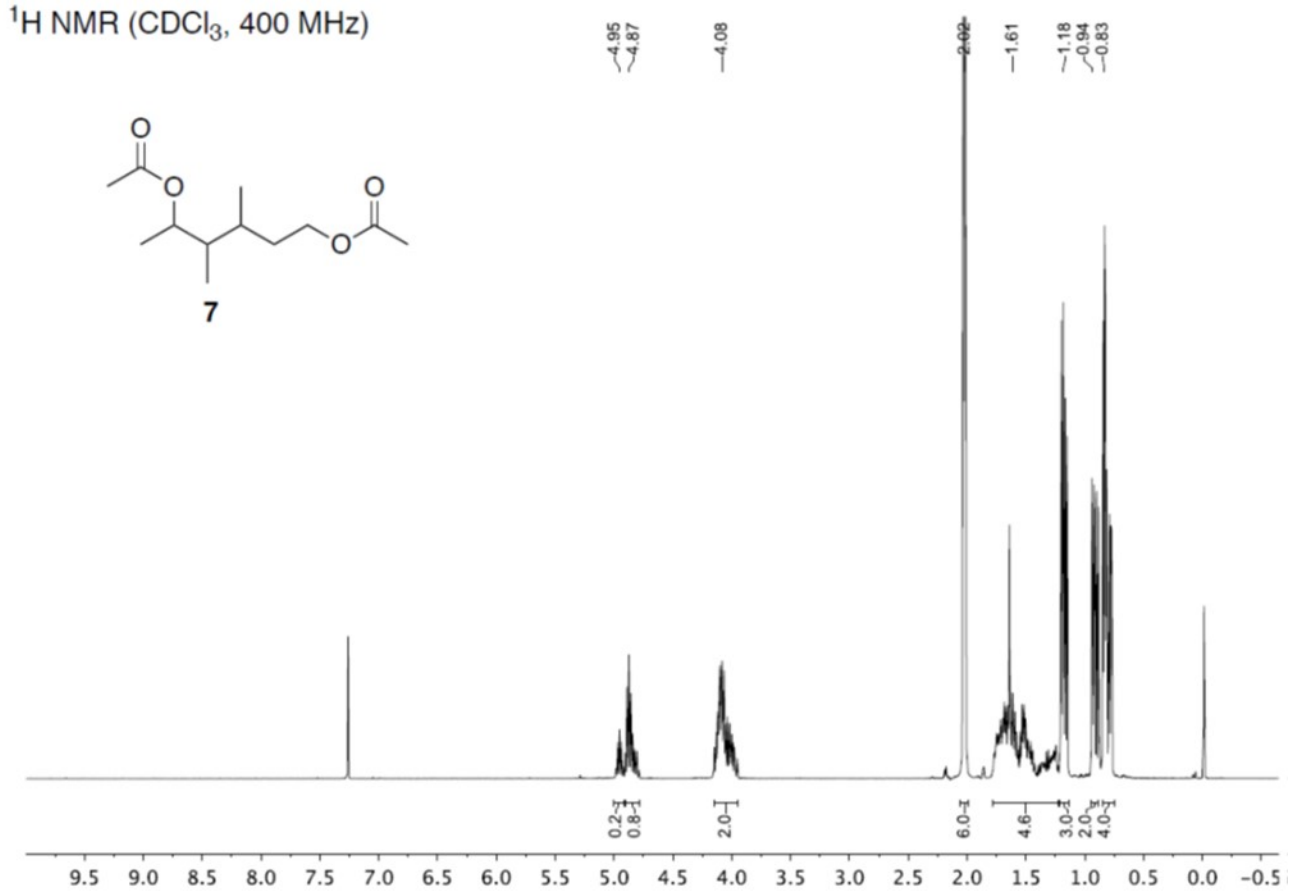
isopropyl 5-hydroxy-3,4-dimethylhexanoate (1)

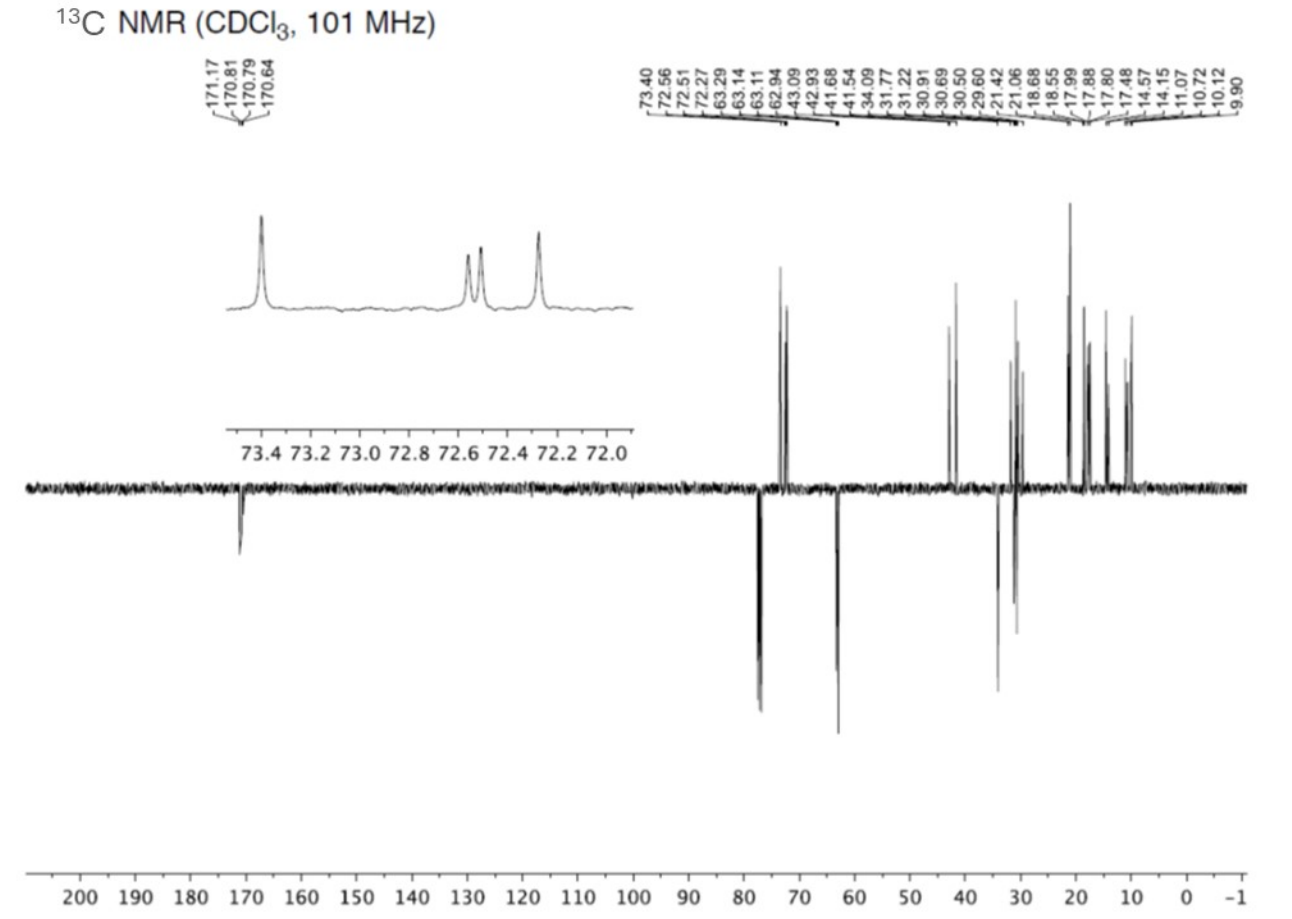


3,4-dimethylhexane-1,5-diol (6)

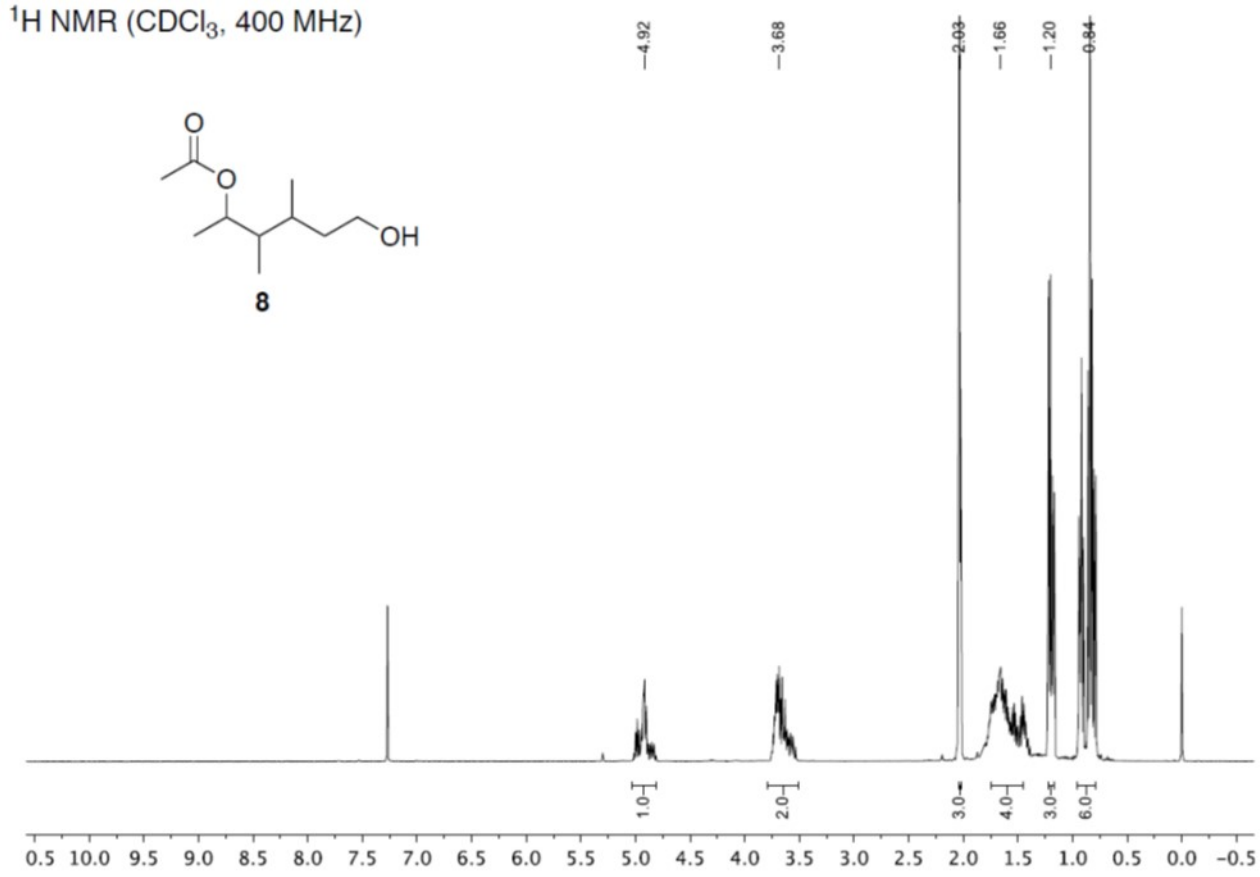


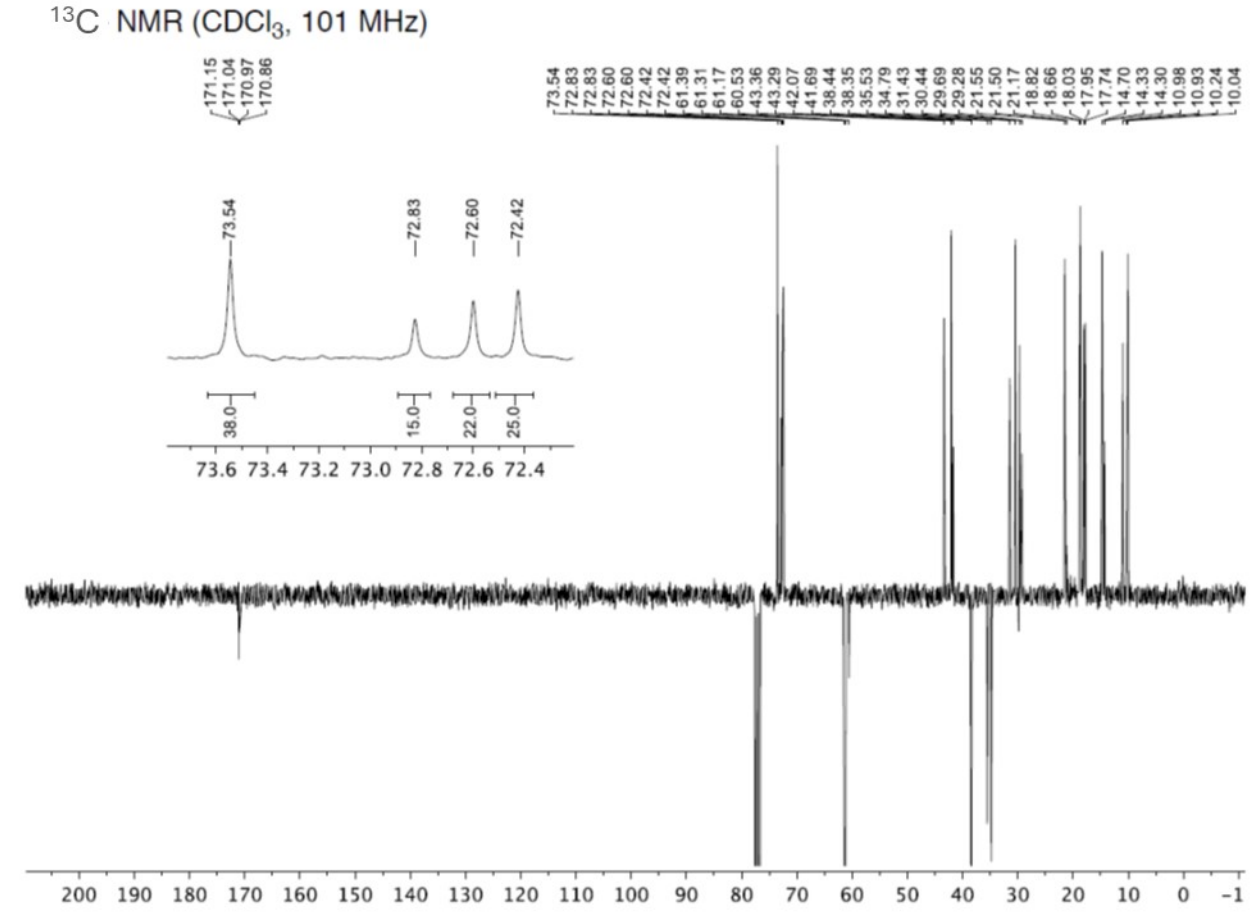
3,4-dimethylhexane-1,5-diyl diacetate (7)



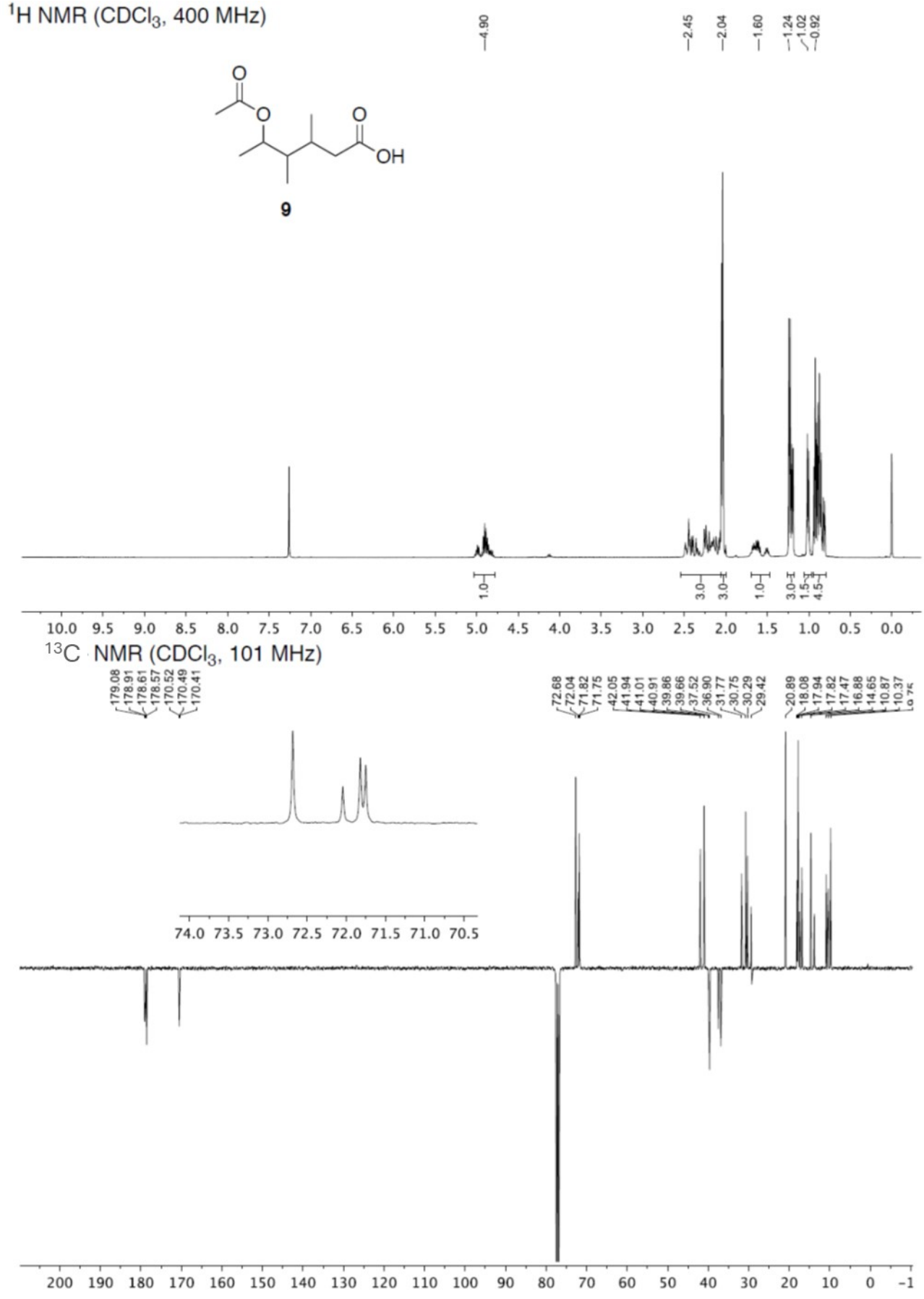


6-hydroxy-3,4-dimethylhexan-2-yl acetate (8)

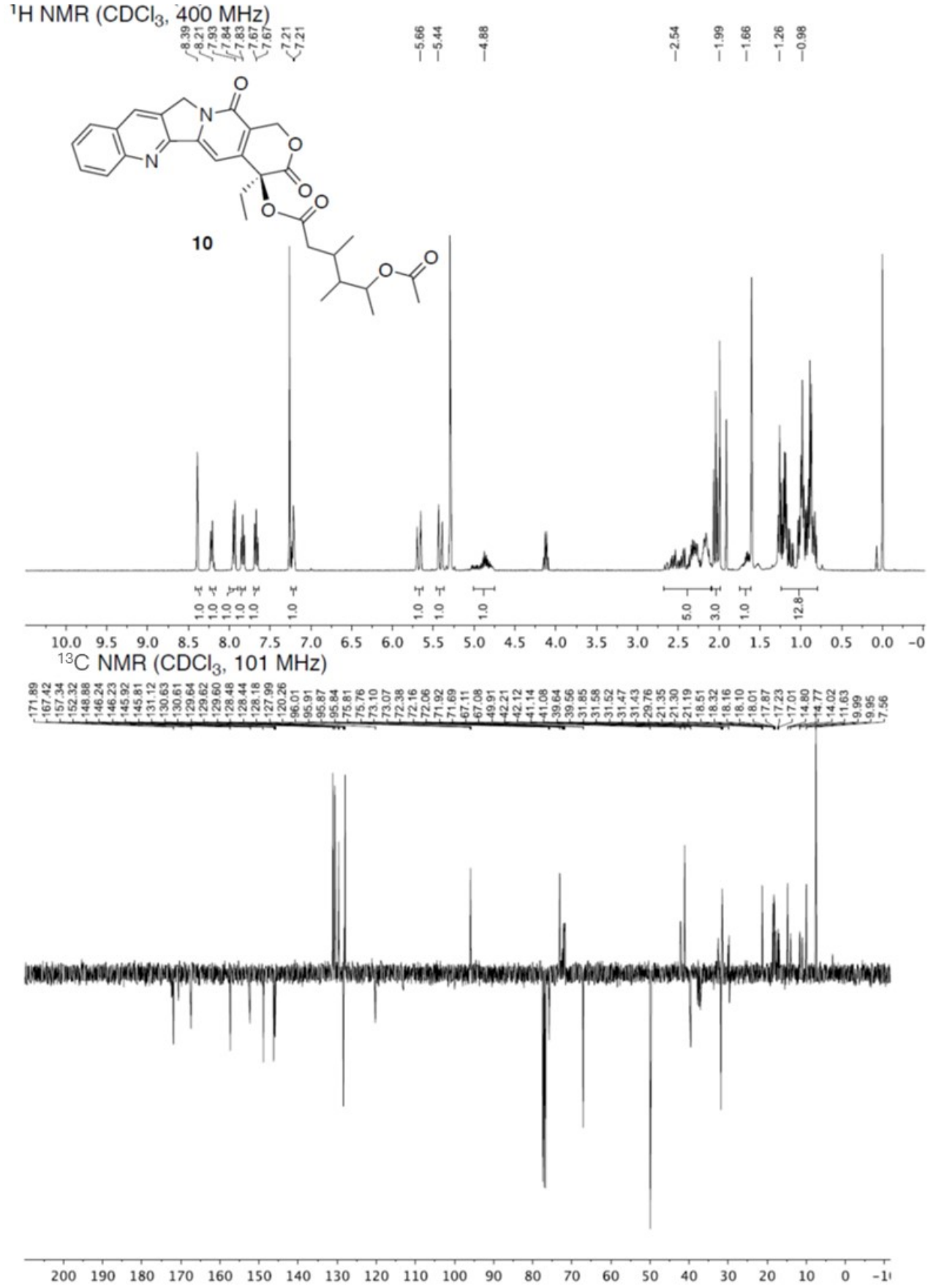




5-acetoxy-3,4-dimethylhexanoic acid (9)



(*S*)-4-ethyl-3,14-dioxo-3,4,12,14-tetrahydro-1*H*-pyrano[3',4':6,7]indolizino[1,2-*b*]quinolin-4-yl 5-acetoxy-3,4-dimethylhexanoate (10)



Screening of enzymatic hydrolysis of 10 with a panel of commercially available lipases and esterases

A solution of **1** in THF (200 µL, 3.2 mM) was added to KP_i buffer solution (800 µL, 50 mM, pH 7.0) containing lipase according to Table S2 (20 mg). The mixture was incubated in an orbital shaker (150 rpm, 35 °C) and it was monitored every day by TLC (CH_2Cl_2/MeOH 5:0.2), and in all cases, no conversion was observed.

Table S2. Lipase catalyzed release of Camptothecin.

Enzyme	Conversion[a)]
Lipase PPL	-
Novozym® 435	-
Lipase PS	-
Lipase AK	-
Lipase A	-
Lipase from *A. Niger*	-
Lipase from *C. Cylindracea*	-
Esterase BS3	-

[a)] After seven days

Acidic trigger of CPTOH release, qualitative TLC analysis

A solution of **1** in THF (200 µL, 3.2 mM) was added to NaOAc buffer solution (800 µL, 0.1 M, pH 5.0), and the mixture was incubated in an orbital shaker (150 rpm, 35 °C). The reaction was monitored by TLC (eluent: CH_2Cl_2/MeOH 96:4, Uv lamp ℵ= 366 nm), Figure S5 shows the time-course release of Camptothecin with the formation of the hydroxy ester intermediate **Int**.

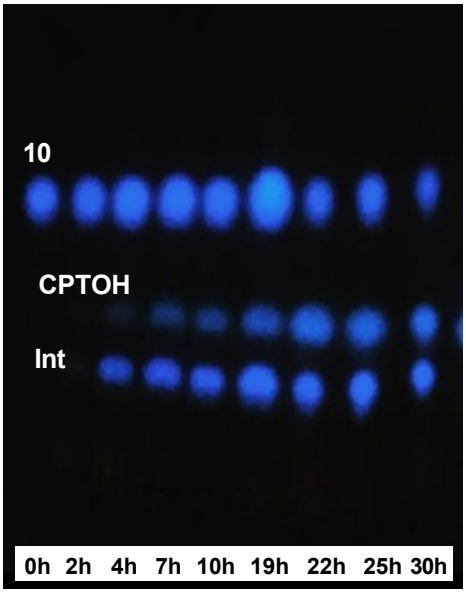
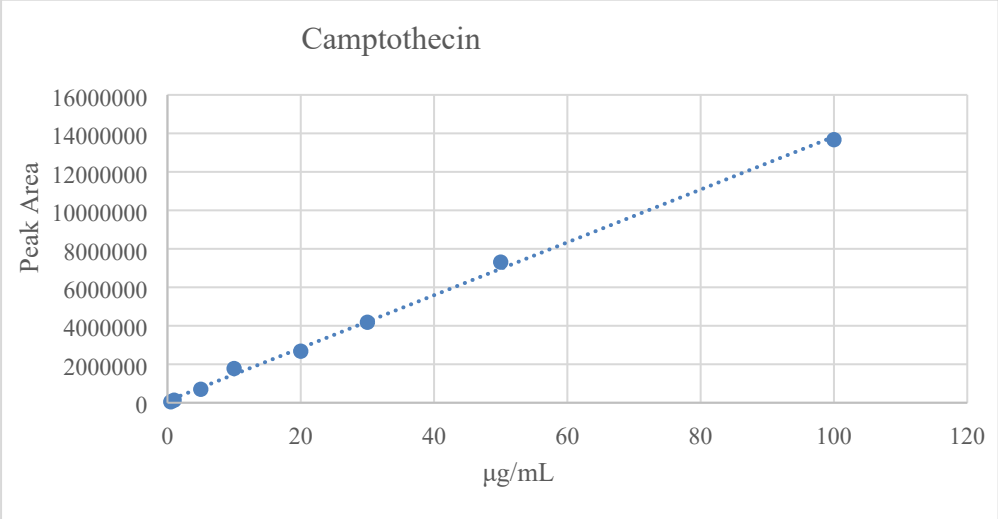


Figure S5. Time-course CPTOH release triggered by acid hydrolysis of acetyl.

Acidic trigger of CPTOH release: HPLC analysis

HPLC conditions were as follows: column Robusta® C18 5u - 150mm x 4.6mmID; solvents: water (A) and acetonitrile (B). Gradient elution A/B 60/40 to 0/100 in 10 min, isocratic 0/100 for 10 min then 0/100 to 60/40 in 5 min; flow rate: 1.0 mL/min, detection: 360 nm.

HPLC calibration curves:
A stock solution of Camptothecin 1mg/mL ($CHCl_3$/MeOH 1/1) and standard calibration solutions were prepared performing cascade dilutions using MeOH, to the following final concentrations 100, 50, 30, 20, 10, 5 µg/mL.
For the analysis 20µl of each standard solution was injected. The retention time (tR) of standard Camptothecin was 7,5 min. A calibration curve using Camptothecin 100-5 µg/mL was set up for the quantification of the produced Camptothecin based on the peak areas.



Camptothecin Calibration curve

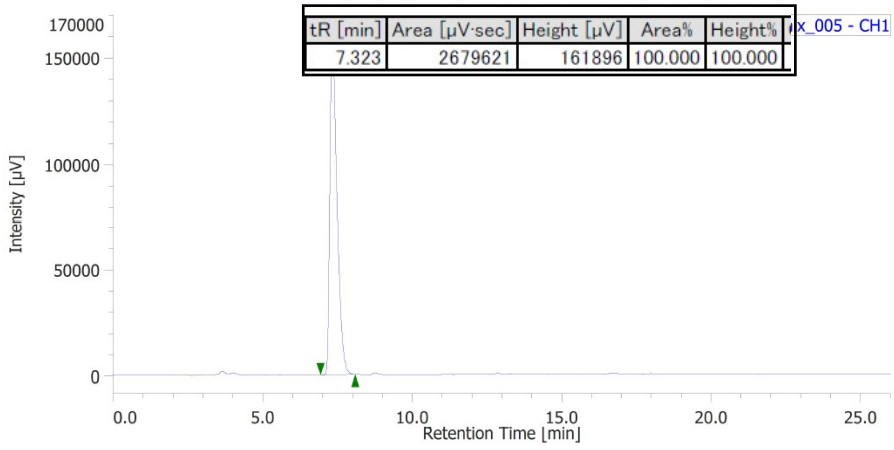
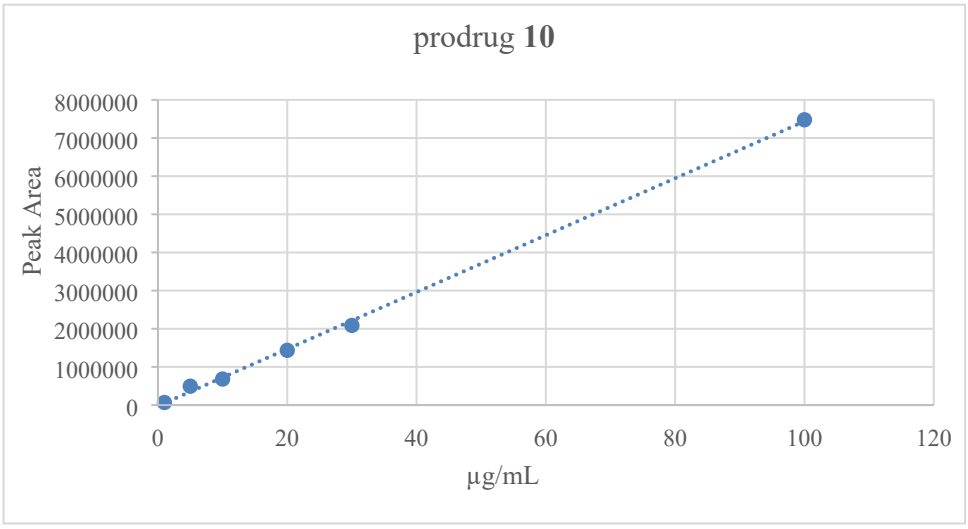


Figure S6. HPLC sample chromatogram of Camptothecin.

A stock solution of **10** was prepared in $CHCl_3$/MeOH 2/8 (0,94 mg/mL) and standard calibration solutions were prepared performing cascade dilutions using MeOH, to the following standard solutions 100, 30, 20, 10 µg/mL.
For the analysis 20µl of each standard solution was injected. The diastereoisomeric mixture **10** presented two partially overlaying peaks with retention time (tR) of 15.0 and 15.3 min. A calibration curve using 10 100-10 µg/mL was set up for the quantification of **10** based on the peak areas.



Prodrug **10** calibration curve

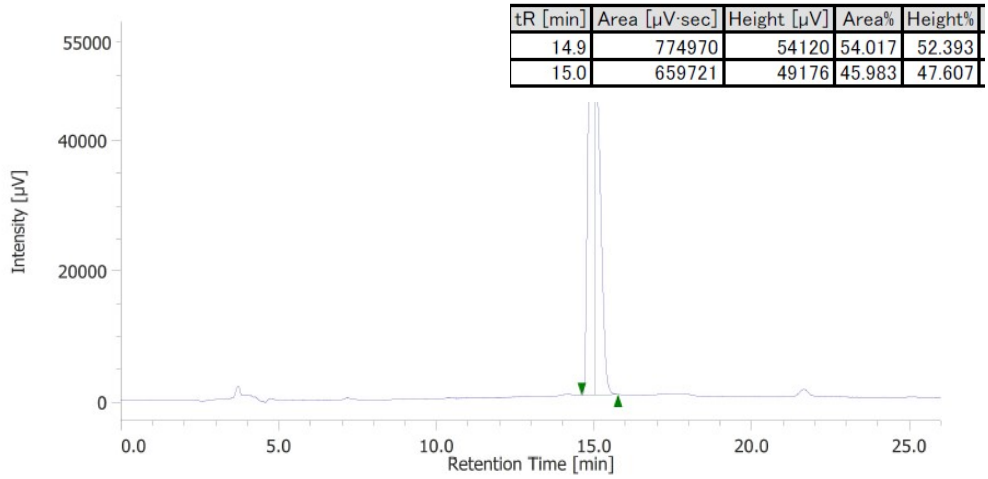


Figure S7. HPLC sample chromatogram of prodrug **10**.

Acidic trigger of CPTOH release

A solution of **10** in $CHCl_3$/MeOH 2/8 (850 µL, 0,94 mg/mL) was added to NaOAc buffer solution (1150 µL, 0.1 M, pH 5.0), the mixture was kept under constant stirring in the dark (150 rpm, 35 °C). The reaction was monitored by HPLC as follows: 10µL were withdrawn from the reaction vessel and diluted with 90µL of MeOH, then for the analysis 20µl of this solution was injected.
Acid-catalyzed release of Camptothecin and the prodrug **10** consumption were monitored and quantified for 280 h, using the calibration curves previously prepared, the intermediate peaks (**Int**) have not been quantified.

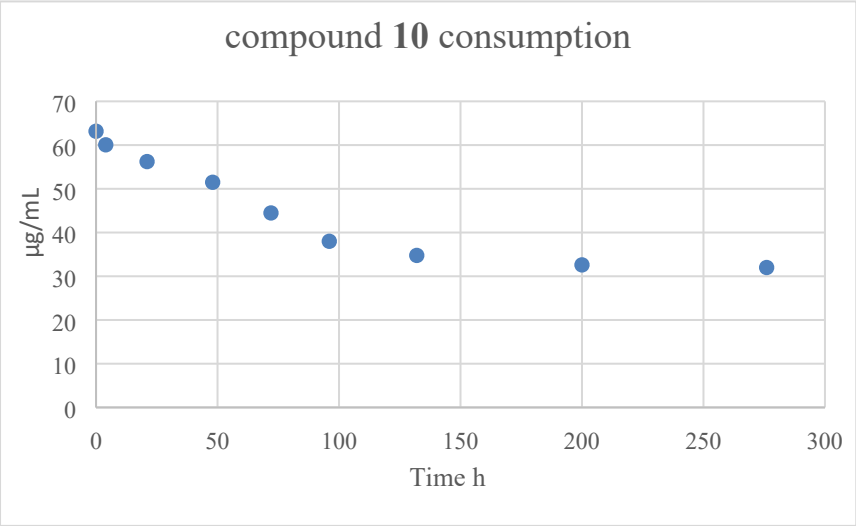


Figure S8. Time course quantification of prodrug **10** consumption.

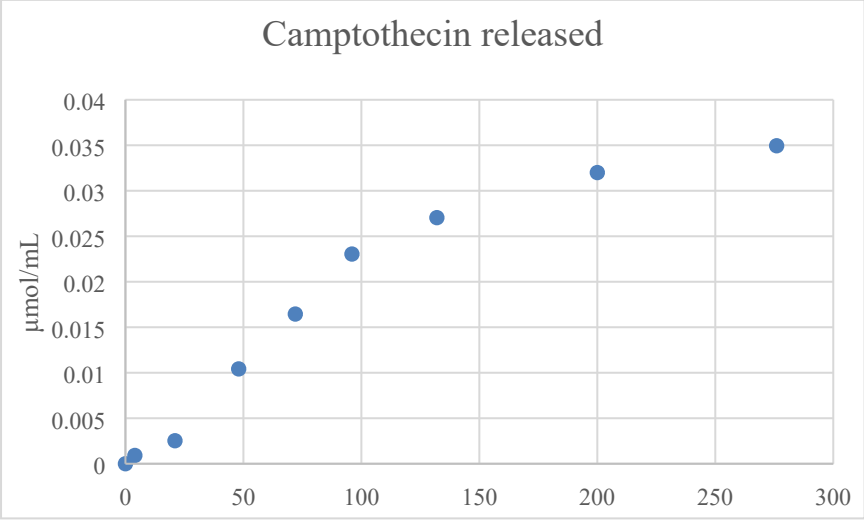


Figure S9. Time course quantification of Camptothecin release.

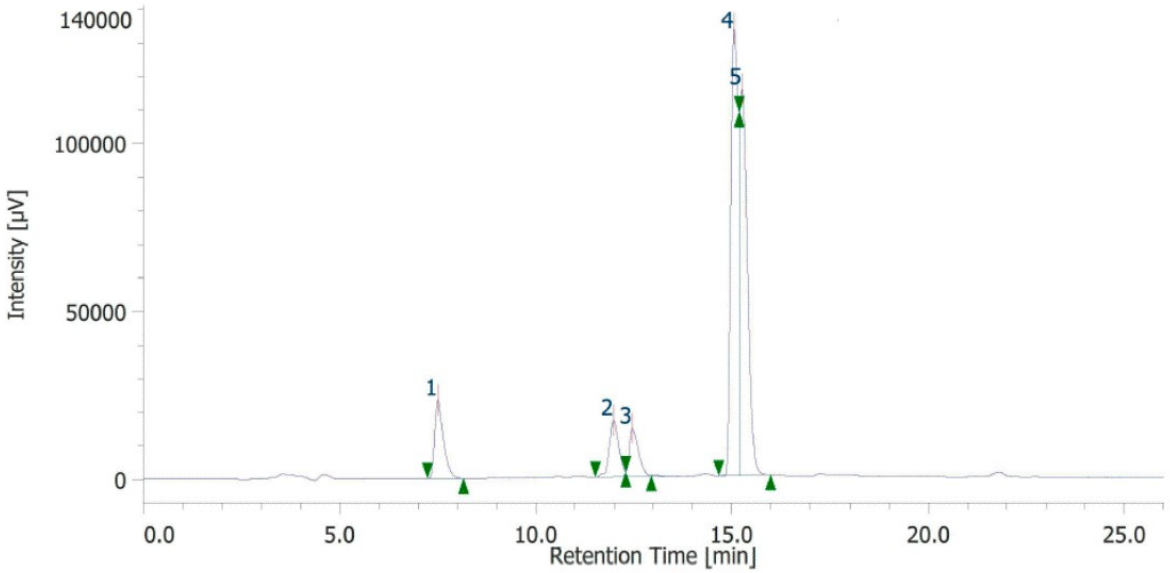
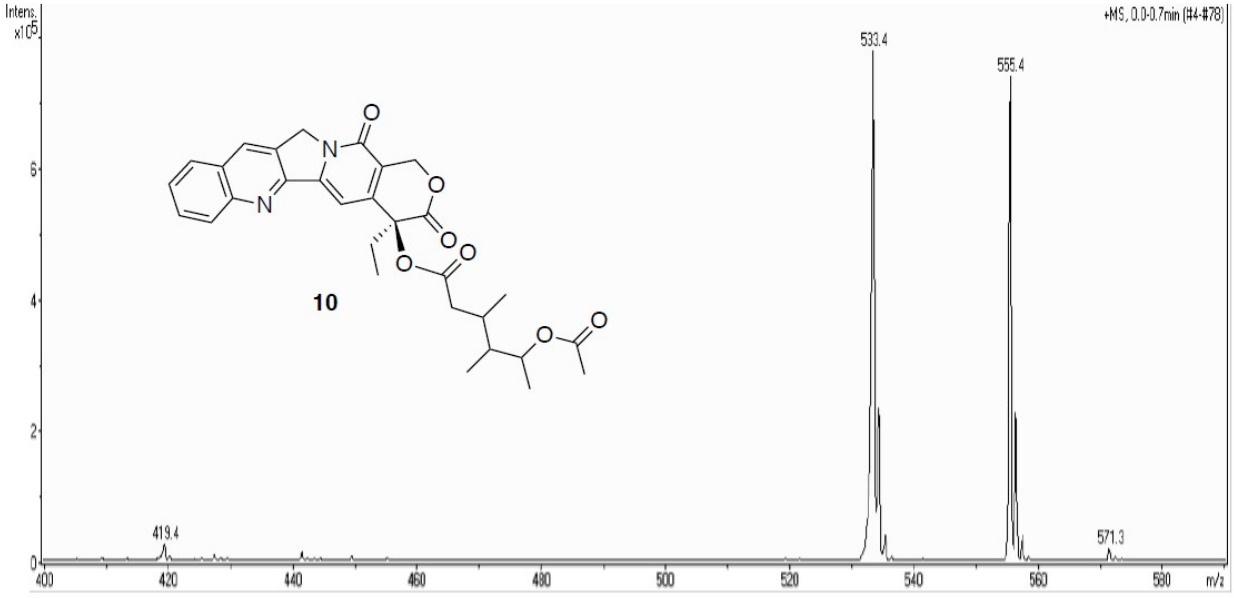
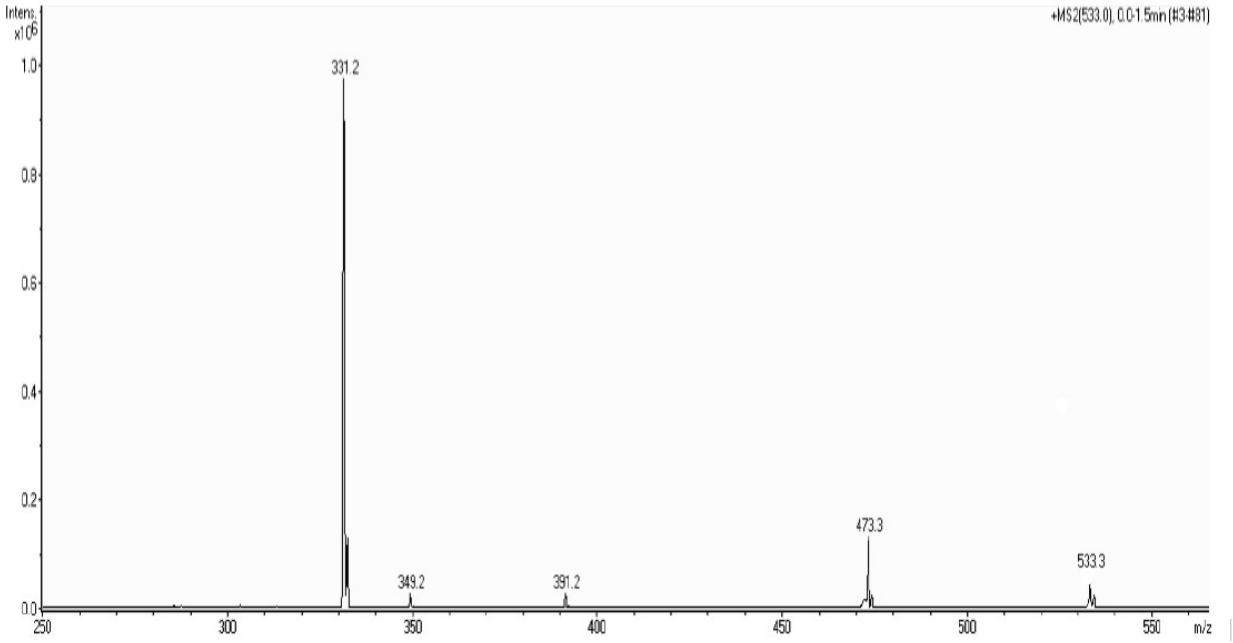


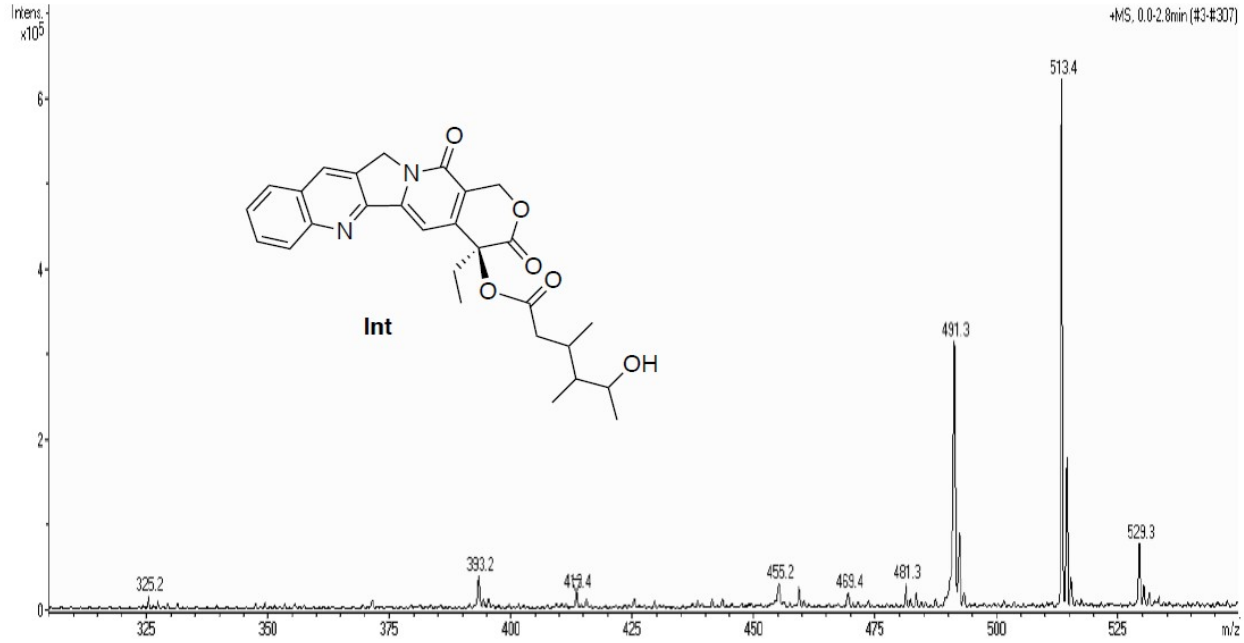
Figure S10. HPLC sample chromatogram after 96h of the acidic trigger of Camptothecin (CPTOH) release.

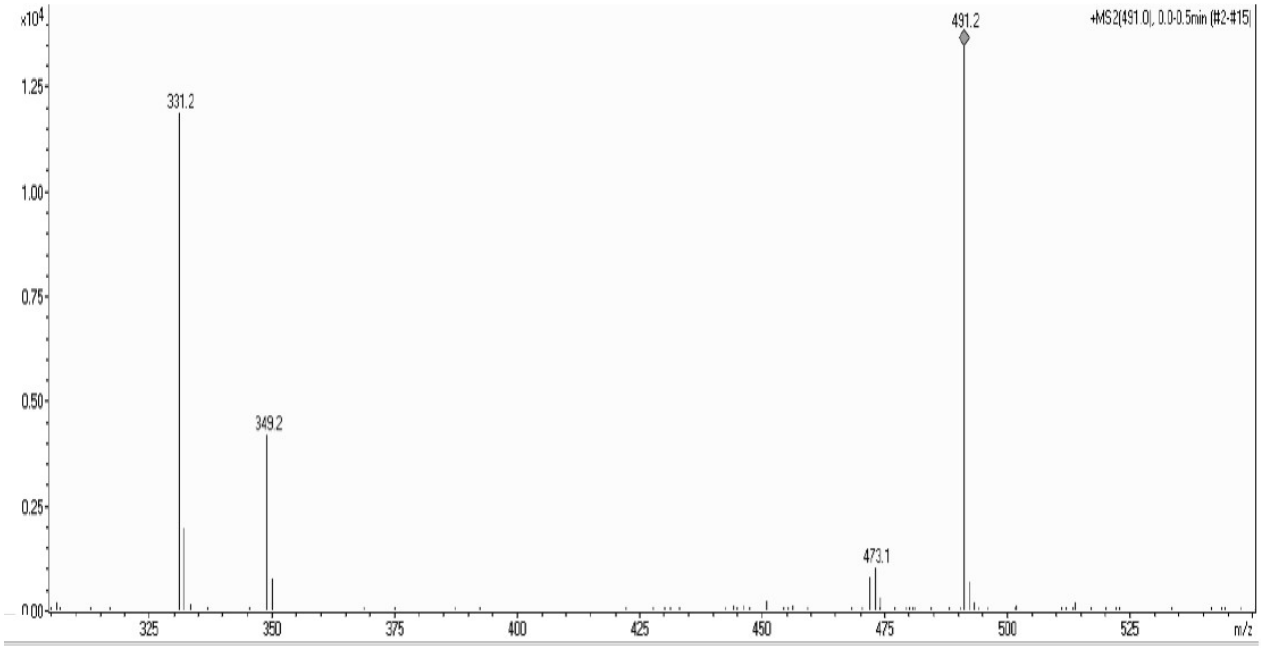
ESI-MS experiments



10




Intens.
x10^5
+MS, 0.0-2.8min (#3-#307)
513.4
491.3
529.3
325.2
393.2
415.4
455.2
469.4
481.3
m/z
Int
OH


x10^4
+MS2(491.0), 0.0-0.5min (#2-#15)
491.2
331.2
349.2
473.1
m/z

Materials and Methods for *in vitro* biological assays

The human pancreatic ductal adenocarcinoma cell lines MIAPaCa-2 were originally obtained from ATCC. MIAPaCa-2 cells were maintained in DMEM medium supplied with 10% fetal bovine serum (Euroclone), L-glutamine (2mM), penicillin (100 units/mL), and streptomycin (0.1 µg/mL) (Sigma-Aldrich). Cells were routinely subcultured twice a week and maintained in a humidified incubator with 5% CO_2 at 37 °C. Camptothecin was purchased from Sigma-Aldrich and it was dissolved in DMSO. Prodrug **10** and lactone linker **2** were also dissolved in DMSO. Phospho-Histone H2A.X (Ser139) (γ-H2AX) (20E3) Rabbit was purchased from Cell Signaling. DAPI was purchased from Sigma-Aldrich. Trypan blue vital assay was performed by plating 30.000 viable cells in 12-well dishes. For the immunofluorescence experiment, the cells were subcultured onto coverslips in 12-well dishes. After overnight incubation, cells were treated with DMSO for the control sample (-) and with Camptothecin (CPTOH 200nM), prodrug **10** (200nM), and lactone **2** (200nM). To measure cell proliferation and death, after 24, 48, 72 and 96 hours, harvested cells were counted using the Burker chamber. Where indicated, cell viability and cell death count were performed using Trypan Blue Stain 0.4% (Thermo Fisher Scientific). To measure γ-H2AX staining, the coverslips were fixed with 4% paraformaldehyde for 10 minutes at room temperature. After that, the coverslips were blocked in PBS/3% BSA, incubated with primary antibody (1:600), with secondary antibody (1:400), and then with DAPI 2µg/mL. Coverslips were mounted with Fluorimount-G (Invitrogen) and then examined under a Nikon90i fluorescence microscope at a magnification of 40X.

Statistics

For cell experiments, statistical analysis was performed using GraphPad Prism v 8.0.2 (GraphPad Software Inc., La Jolla, CA). Data are presented as mean ± SD from six independent experiments (N=6), and statistical significance (**p < 0.01, ***p < 0.001 ****p < 0.0001) is determined using two-way repeated measures ANOVA with Tukey's multiple comparisons test.

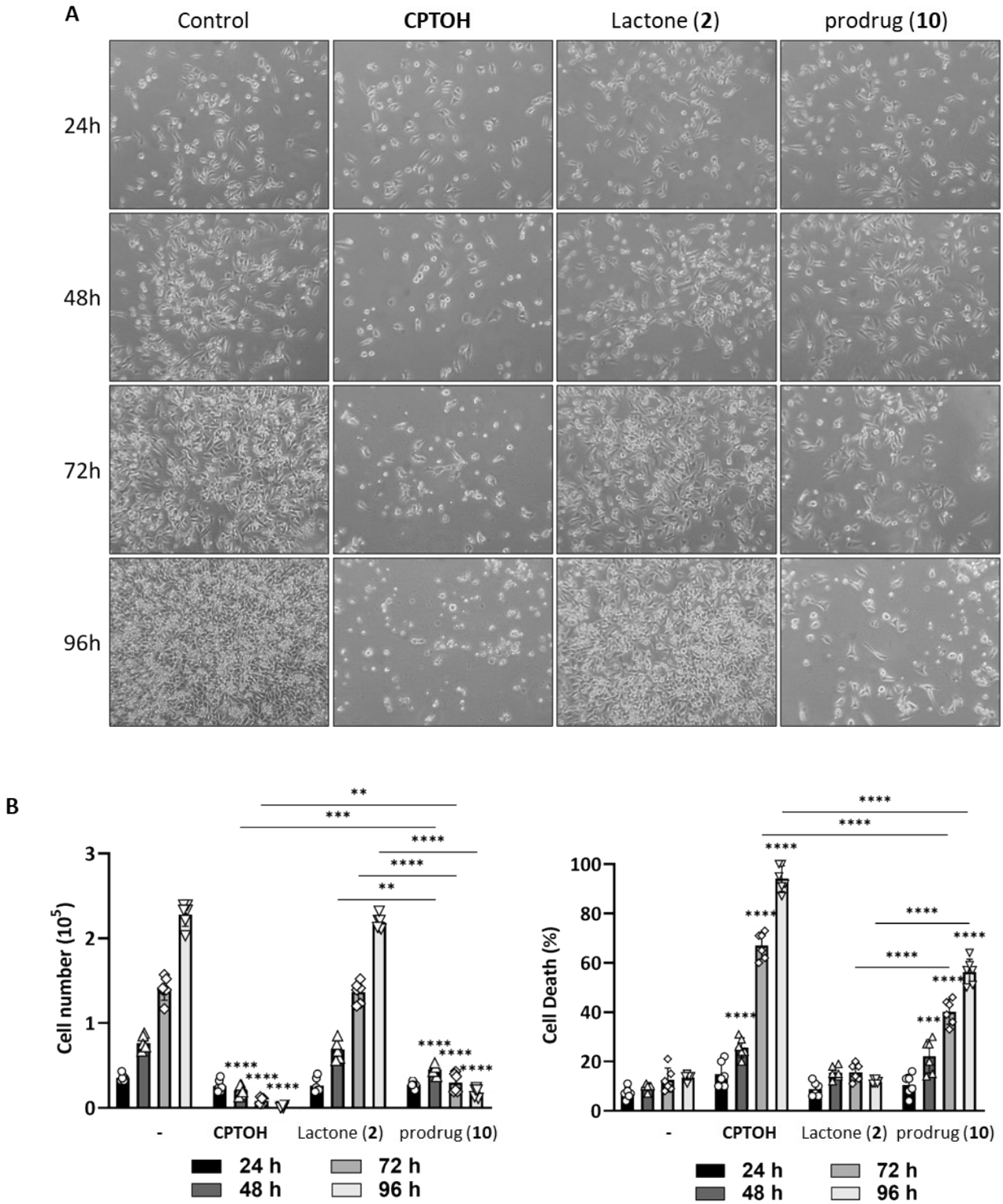


Figure S11. MIAPaCa-2 cells treated with prodrug 10 show a phenotype similar to MIAPaCa-2 CPT-treated cells. (**A**) Phase contrast microscopy and (**B**) analysis of cell number and cell death (% of the total population) of MIAPaCa-2 cells upon control treatment with DMSO (Control) or treatment with the linker (Lactone (2)), Camptothecin (CPTOH), and Prodrug (10) at 24, 48, 72 and 96 hours.

Metabolite Extraction

MIAPaCa-2 cells (7,5 x 10 5) were seeded onto 100-mm dishes in the complete growth medium, and after 24h, cells were washed twice with phosphate buffer saline 1X (PBS 1X, Euroclone, Milan, Italy) and incubated in a complete medium without or with prodrug **10** (200nM).

At 48h post-treatment, the cells were washed twice with cold PBS 1X and disrupted in a cold solution of 80% methanol for 5 minutes in dry ice. Once collected, cells were sonicated on ice and centrifuged at 14,000g for 30 minutes at 4°C. Finally, supernatants were collected and centrifugated in Amicon® ultra 0.5 centrifugal filter devices (MerckMillipore, Milan, Italy) at 14000g for 2h at 4°C to remove soluble protein and other contaminants.

Multiple Reaction Monitoring (MRM) Analysis

MRM is a highly specific and sensitive label-free acquisition mode for detecting/quantifying targeted analytes. It refers to a tandem MS (MS/MS) scan mode based on the selection of precursor ions in the first quadrupole and the analysis of specific fragmentations in the high-resolution time of flight analyzer (TOF). Based on the predesigned transition lists, the first quadrupole (Q1) of the MS will be able to select and transmit the precursor ions to the collision cell for further fragmentation. The resultant product ions will be transmitted to the TOF analyzer, which detects product ions with enhanced transmission of selected predefined *m*/*z*. For the identification of CPTOH transitions 349.1>249.1 and 349.1>305.1 (precursor ion - product ion) were selected: for prodrug **10** transitions 533.2>331.1, 533.2>303.1, 533.2>287.1 were selected.

Two microliters of each sample were injected into a Xevo G2-XS quadrupole time of flight mass spectrometer coupled to a Waters ACQUITY ultra-performance liquid chromatography (UPLC) H-Class system through an ESI source (Waters Corporation, Milford, MA, USA). The instrument was operated in positive ion current in sensitivity mode. The capillary voltage was maintained at 1 kV with the source temperature held constant at 140 °C.

An ACQUITY UPLC® BEH C18 1.7µm, 2.1mm x 50mm, (Waters Corporation, Milford, MA, USA) was used for analyte separation. The flow rate was set to 400 µL/min with a column temperature of 40 °C. A gradient of solvent A (99.9% LC-MS-grade water with 0.1% formic acid) and solvent B (LC-MS-grade 99.9% acetonitrile with 0.1% formic acid) was applied with a total run time of 12 min as follows: 0–0.3 min at 300% B; 0.3-6.3 min linear increase from 30% to 80% B; 6.3-8.3 80% B; 9–12 min 30% B.

Transition selection and optimization of the collision energy were performed manually with pure compounds, and data acquisition in MRM mode was performed with Masslynx (v4.2). The transitions monitored were m/z 349.1 (CE 20V) → m/z 305.1(quantifier) and 249.1 at CE 20V for Camptothecin, and m/z 533.2 → 331.1 (quantifier, CE 15V), 303.1, and 287.1 at CE of 30V for the prodrug **10**.

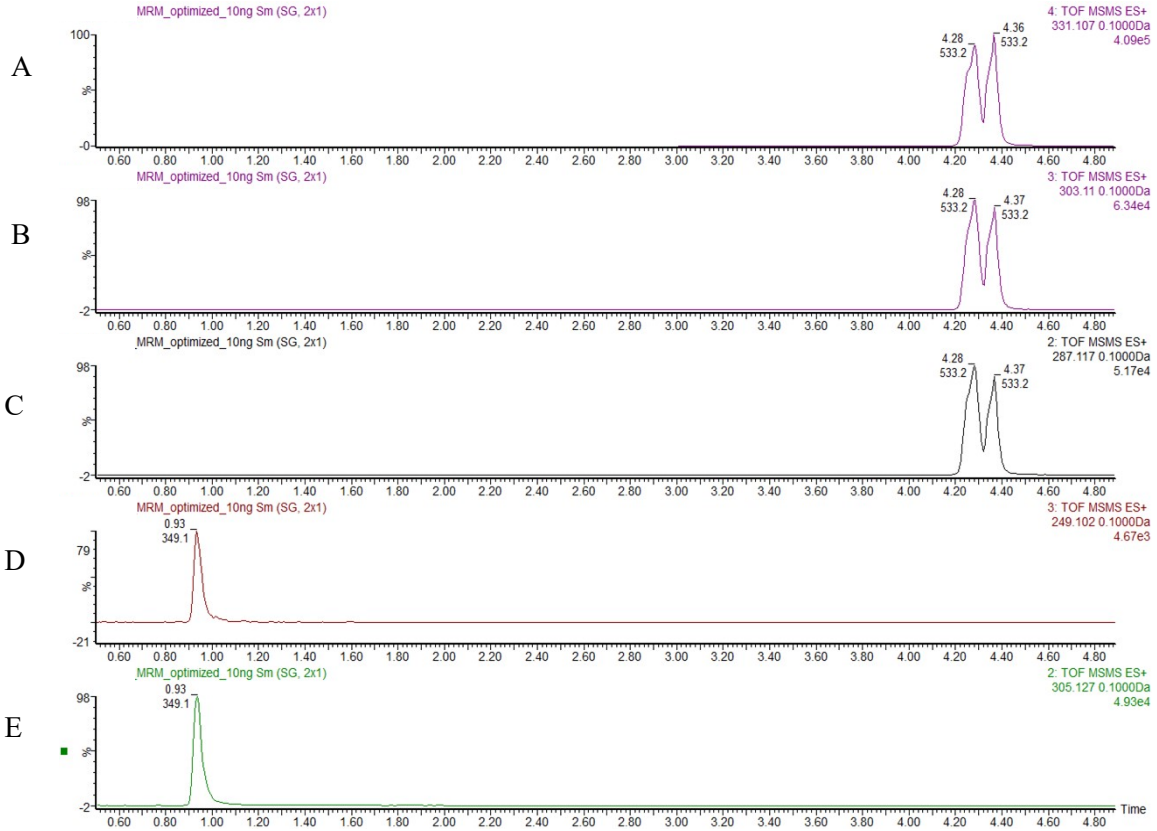


Figure S12. Representative MRM chromatograms were obtained from the analysis of pure standard prodrug **10** and Camptothecin (CPTOH) in solution at 10ng/ml. CPTOH transitions 349.1>249.1 and 349.1>305.1, in panels D and E, respectively. Prodrug 10 transitions 533.2>331.1, 533.2>303.1, 533.2>287.1, in panels A, B, and C, respectively.

References

[1] S. E. Boiadjiev, R. V. Person, G. Puzicha, C. Knobler, E. Maverick, K. N. Trueblood and D. A Lightner, *J. Am. Chem. Soc.*, 1992, **114**, 10123.
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[4] K.Furokawa, M. Shibuya and Y. Yamamoto, *Org. Lett*., 2015, **17**, 2282.
[5] M. J. Fehr, G. Consiglio, M. Scalone and R. Schmid, *J. Org. Chem.*, 1999, **64**, 5768.