Electronic Supplementary Information (ESI)

A Trifunctional Self-Immolative Spacer Enables Drug Release with Two Non-Sequential Enzymatic Reactions

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Synthetic Procedures

Materials and Methods

All manipulations requiring anhydrous conditions were carried out in flame-dried glassware, with magnetic stirring and under a nitrogen atmosphere. All commercially available reagents were used as received. Anhydrous solvents were purchased from commercial sources and withdrawn from the container by syringe, under a slight positive pressure of nitrogen. The reactions were monitored by analytical thin-layer chromatography (TLC) using silica gel 60 F254 pre-coated glass plates (0.25 mm thickness). Visualization was accomplished by irradiation with a UV lamp and/or staining with a ceric ammonium molybdate solution or ninhydrin. Flash column chromatography was performed according to the method of Still and co-workers[1] using Chromagel 60 ACC (40-63 µm) silica gel. Proton chemical shifts are reported in ppm (δ) with the solvent reference relative to tetramethylsilane (TMS) employed as the internal standard ($CDCl_3$ δ = 7.26 ppm; CD_2Cl_2, δ = 5.32 ppm; $[D]_6$-DMSO, δ = 2.50 ppm; CD_3OD, δ = 3.33 ppm, $[D]_8$-THF δ = 3.58 ppm, 1.73 ppm). The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, bs = broad signal, dd = doublet of doublet, ddd = doublet of doublet of doublet, ddt = doublet of doublet of triplet. Carbon NMR spectra were recorded on a spectrometer operating at 100.63 MHz, with complete proton decoupling. Carbon chemical shifts are reported in ppm (δ) relative to TMS with the respective solvent resonance as the internal standard ($CDCl_3$, δ = 77.16 ppm; CD_2Cl_2, δ = 54.00 ppm; d_6-DMSO, δ = 39.51 ppm; CD_3OD, δ = 49.05 ppm; $[D]_8$-THF δ = 67.57 ppm, 25.37 ppm). HPLC purifications were performed on Dionex Ultimate 3000 equipped with Dionex RS Variable Wavelenght Detector (column: Atlantis Prep T3 OBDTM 5 µm 19 x 100 mm; flow 10 ml/min unless stated otherwise). HPLC analysis of carbamate stability was performed on a Waters 515 HPLC pumps equipped with 996 photodiode array detector and Waters Atlantis T3 - 5 µm - 4.6 x 100 mm column. HPLC-MS and high-resolution mass spectrometry analysis (HRMS, 4 decimal places) were performed on a Q-TOF Synapt G2-Si instrument available at the MS facility of the Unitech COSPECT at the University of Milan. Low resolution mass spectra (MS, 1 and 2 decimal places) were recorded on a Thermo Scientific LCQ Fleet Ion Trap Mass Spectrometer (ESI source). Carbonate **Sp1-CPT** was prepared following a published procedure.[2]

General Procedures

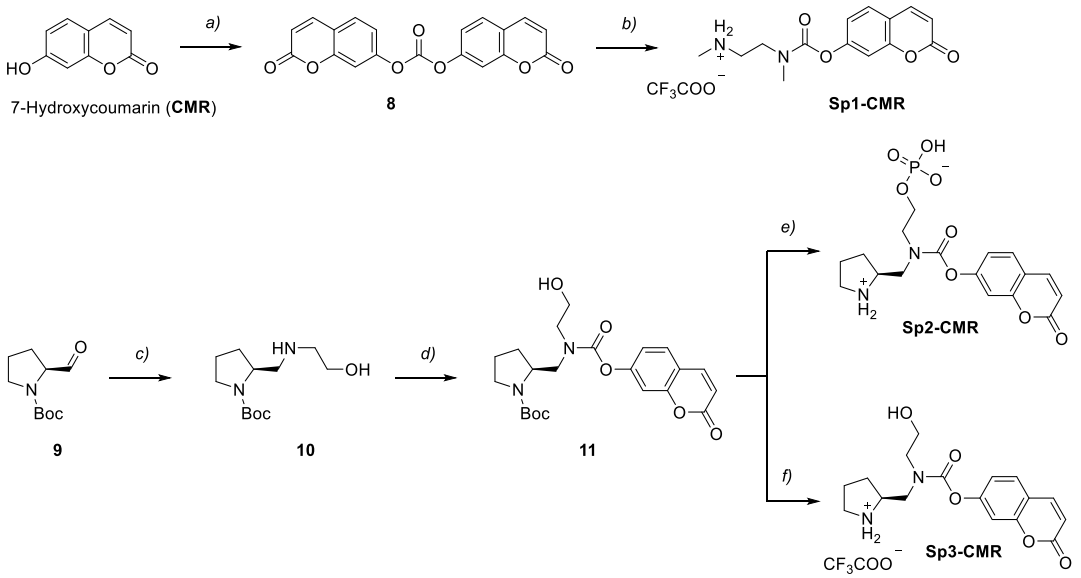
General procedure A for Boc deprotection

To an ice-cold CH_2Cl_2 solution of the *N*-Boc-protected compound, half volume of TFA was added and the mixture was stirred at r.t. for 1 h. The solvent was evaporated and then for two times CH_2Cl_2 was added to the residue followed by evaporation under vacuum, to afford the amine TFA salt.

1 W. C. Still, M. Kahn, A. Mitra, *J. Org. Chem.* 1978, **43**, 2923.

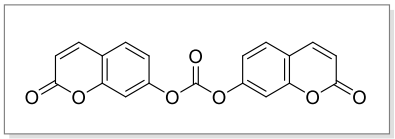
2 E. Riva, D. Comi, S. Borrelli, F. Colombo, B. Danieli, J. Borlak, L. Evensen, J. B. Lorens, G. Fontana, O. M. Gia, L. Dalla Via D. Passarella, *Bioorg. Med. Chem.* 2010, **18**, 8660.

Synthesis of Sp(1-3)-CMR



Scheme S1. REAGENTS AND CONDITIONS: *a)* Triphosgene, *i*Pr$_2$NEt, THF, CH_2Cl_2, 0 °C, 1.5 h; *b)* [1] (*N*-Boc)-*N,N'*-dimethylethylenediamine, DMF, 55 °C, 3h; [2] TFA/ CH_2Cl_2, r.t. 1 h; *c)* Ethanolamine, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t. 16 h; *d)* **8**, *i*Pr$_2$NEt, DMAP, CH_2Cl_2/DMF, 55 °C, 3h; *e)* [1] di-*tert*-butyl *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, r.t. 1 h; [2] *m*CPBA, 0 °C to r.t. 1 h; [3] TFA/ CH_2Cl_2, r.t. 1 h; *f)* TFA/ CH_2Cl_2, r.t. 1 h.

Bis-(7-OH)Coumarin Carbonate (**8**)

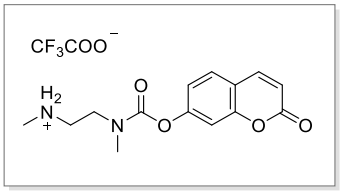


$C_{19}H_{10}O_7$
MW: 350,28 g • mol^{-1}

7-Hydroxy coumarin (500 mg, 3.10 mmol, 1.4 equiv.) was suspended in dry THF (18 ml) and cooled to 0 °C under a nitrogen atmosphere. Dry *i*Pr$_2$NEt (1.9 ml, 11.05 mmol, 5 equiv.) was added. Triphosgene (657 mg, 2.21 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (2 ml) and slowly added to the stirring solution with a syringe, within 5 minutes. The mixture was stirred at 0 °C for 1.5 h, followed by addition of a 0.1 M HCl aq. solution (20 ml). The mixture was stirred for 5 min, the solid was collected through a Buchner filter and washed with a 0.1 M HCl aq. solution. The solid was collected from the filter using MeCN. The resulting suspension was concentrated and volatiles were removed under high vacuum, affording **8** as a pale-brown, poorly soluble solid, which was used in the next synthetic steps without further purification (ca. 500 mg, Y.: quant.).

Mp: 287-292 °C; IR peaks: 1761.65, 1748.16, 1725.98, 1624.73, 1426.10, 1399.10, 1332.57, 1280.50, 1255.43, 1229.40, 1189.86, 1113.69, 1096.33, 985.45, 974.84, 890.95, 876.49. 841.78, 822.49, 756.92, 689.43, 677.86, 613.25 cm^{-1}.

Sp1-CMR

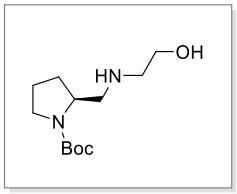


$C_{14}H_{16}N_2O_4$
MW: 276,29 g • mol^{-1} + TFA

Carbonate **8** (25 mg, 71 µmol, 1 equiv.) was suspended in dry DMF (2 ml) and cooled to 0 °C under a nitrogen atmosphere. (*N*-Boc)*N,N'*-dimethylethylenediamine[3] (40 mg, 214 µmol, 3 equiv.) was added. The mixture was warmed to 55 °C and stirred for 3 h. The solvent was removed under high vacuum, then the crude was dissolved in CH_2Cl_2 (1 ml) and treated following General Procedure A. After solvent removal, the crude material was purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 5% B (at min 0.5) to 50% B (at min 8.5), t_R (product): 6.9 min]. The purified product was then lyophilized to give **Sp1-CMR** as a white solid (23 mg, 82% over two steps).

^{1}H NMR (400 MHz, d_6-DMSO + D_2O) δ 8.04 (d, J = 9.6 Hz, 1H), 7.73 (d, J = 8.5 Hz, 1H), 7.30 (bs, 1H), 7.19 (dd, J = 8.5, 2.2 Hz, 1H), 6.44 (d, J = 9.6 Hz, 1H), 3.70 (m, 2H, rotamer A), 3.58 (m, 2H, rotamer B), 3.19 (m, 2H, rotamer A), 3.16 (m, 2H, rotamer B), 3.05 (s, 3H, rotamer A), 2.93 (s, 3H, rotamer B), 2.62 (s, 3H, rotamer A), 2.60 (s, 3H, rotamer B) ppm. HRMS (ESI) *m/z* calcd. for $[C_{14}H_{17}N_2O_4]^+$: 277,1183 $[M+H]^+$, found: 277,1184.

Boc-Pro-OH (**10**)



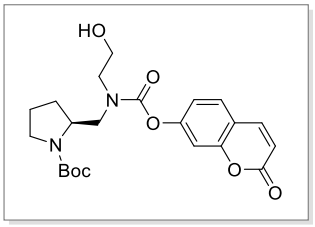
$C_{12}H_{24}N_2O_3$
MW: 244,34 g • mol^{-1}

(L)-Boc-Prolinal (**9**, 190 µl, 1 mmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (20 ml) under a nitrogen atmosphere and acetic acid (2 ml) was added. Ethanolamine (182 µl, 3 mmol, 3 equiv.), followed by solid $NaBH(OAc)_3$ (848 mg, 4 mmol, 4 equiv.) were added and the mixture was stirred overnight at r.t.. The mixture was transferred into a separating funnel and diluted with CH_2Cl_2 (20 ml). A 10% aq. solution of Na_2CO_3 (40 ml) was added. The emulsion was rapidly shaken in the open funnel and flushed with nitrogen. The pH was increased up to 12 with a 2 M NaOH aq. solution (10 ml). The emulsion was shaken, the phases were separated and the aqueous phase was extracted with CH_2Cl_2 (3 × 40 ml). The collected organic phases were washed with brine (1 × 10 ml), dried and concentrated, affording **10** as a pale-yellow oil (240 mg, quant.), which was used in the next steps without further purification.

^{1}H NMR (400 MHz, MeOD) δ 3.89 (m, 1H), 3.65 (t, J = 5.5 Hz, 2H), 3.40 – 3.33 (m, 2H), 2.81 (m, 1H), 2.74 (t, J = 5.5 Hz, 2H), 2.58 (dd, J = 11.8, 7.8 Hz, 1H), 2.03 – 1.79 (m, 4H), 1.47 (s, 9H) ppm; ^{13}C NMR (101 MHz, MeOD) δ 156.9, 81.3 (rotamer A), 80.8 (rotamer B), 61.4, 58.2, 53.5, 52.5, 47.9, 47.4 (rotamer A), 30.5 (rotamer B), 30.1 (rotamer A), 28.8 (rotamer B), 24.6 (rotamer A), 23.8 (rotamer B) ppm; ^{13}C NMR (101 MHz, MeOD) δ 156.9, 81.3, 80.8, 61.4, 58.2, 53.5, 52.5, 47.9, 47.4, 30.5, 30.1, 28.8, 24.6, 23.8 ppm.

3 X. Zhang, K. Tang, H. Wang, Y. Liu, Y. Fang, X. Zhang and W. Lu, *Bioconjugate Chem.* 2016, **27**, 1267.

Boc-Sp3-CMR (**11**)

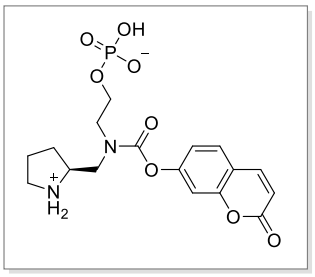


$C_{22}H_{28}N_2O_7$
MW: 432,47 g • mol^{-1}

Amine **10** (81 mg, 0.23 mmol, 1 equiv.) was suspended in dry CH_2Cl_2 (5 ml) and cooled to 0 °C under a nitrogen atmosphere. ADC290 (80 mg, 0.32 mmol, 1.4 equiv.) and *i*Pr_2NEt (200 µl, 1.15 mmol, 5 equiv.) were added. The mixture was warmed to r.t., then DMAP (10 mg, 0.08 mmol, 0.3 equiv.) and dry DMF (2 ml) were added. The mixture was warmed to 55 °C. After ca. 3 h the mixture became limpid. The solvent was removed under high vacuum, then the crude was dissolved in CH_2Cl_2 (100 ml) and washed with $KHSO_4$ aq. 1 M (2 × 20 ml) and brine (1 × 20 ml), dried and concentrated. The crude mixture was purified by flash chromatography (gradient from 1% MeOH in CH_2Cl_2 to 2% MeOH in CH_2Cl_2), obtaining **11** as a pale-yellow solid (40 mg, 40%).

R_f: 0.4 (95:5 CH_2Cl_2/MeOH). ^{1}H NMR (400 MHz, MeOD) δ 7.95 (d, J = 9.6 Hz, 1H), 7.65 (dd, J = 8.4, 2.1 Hz, 1H), 7.27 – 7.11 (m, 2H), 6.40 (d, J = 9.6 Hz, 1H), 4.27 (m, 1H), 3.84 – 3.73 (m, 2H), 3.69 – 3.33 (m, 6H), 2.05 – 1.79 (m, 4H), 1.47 (s, 9H) ppm.

Sp2-CMR

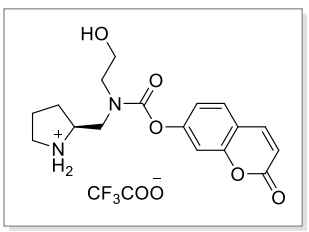


$C_{17}H_{21}N_2O_8P$
MW: 412,33 g • mol^{-1}

Alcohol **11** (13 mg, 30 µmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (3 ml) under a nitrogen atmosphere. A 0.45 M 1*H*-tetrazole solution in MeCN (200 µl, 90 µmol, 3 equiv.) and di-*tert*-butyl *N,N*-diisopropylphosphoramidite (28 µl, 90 µmol, 3 equiv.) were added and the mixture was stirred for 1.5 h at r.t.. The mixture was then cooled to 0 °C and *m*CPBA (18 mg, 105 µmol, 3.5 equiv.) was added. The mixture was warmed to r.t. and stirred for 1 h. Volatiles were removed under vacuum and the solid material was dissolved in CH_2Cl_2 (2 ml) and cooled to 0 °C. Trifluoroacetic acid (1 ml) was added and the mixture was stirred 45 min. at r.t., concentrated and purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 5% B (at min 0.5) to 50% B (at min 8.5), t_R (product): 6.7 min]. The purified product was then lyophilized to give **Sp2-CMR** as a white solid (20 mg, quant. over three steps).

MS (ESI) *m/z* calcd. for $[C_{17}H_{22}N_2O_8P]^+$: 413.11 $[M+H]^+$, found: 412.97. HRMS (ESI) *m/z* calcd. for $[C_{17}H_{22}N_2O_8P]^+$: 413,1108 $[M+H]^+$, found: 413,1113.

Sp3-CMR

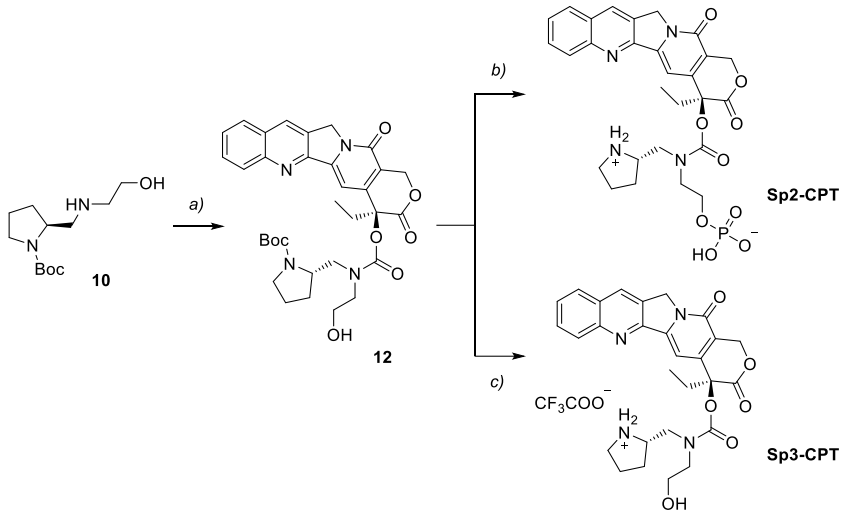


$C_{17}H_{20}N_2O_5$
MW: 332,35 g • mol^{-1} + TFA

Compound **11** (13 mg, 30 µmol, 1 equiv.) was dissolved in CH_2Cl_2 (1 ml) and treated following General Procedure A. After solvent removal, the crude material was purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 5% B (at min 0.5) to 50% B (at min 8.5), t_R (product): 6.7 min]. The purified product was then lyophilized to give **Sp3-CMR** as a white solid (12 mg, quant.).

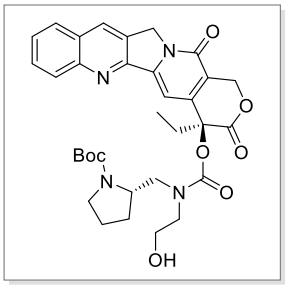
HRMS (ESI) *m/z* calcd. for $[C_{17}H_{21}N_2O_5]^+$: 333,1445 $[M+H]^+$, found: 333,1457.

Synthesis of Sp2-CPT and Sp3-CPT



Scheme S2. REAGENTS AND CONDITIONS: *a)* **CPT-PNP**, *i*Pr_2NEt, DMF, r.t. 16 h; *b)* [1] di-*tert*-butyl *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, r.t. 1 h; [2] *m*CPBA, 0 °C to r.t. 1 h; [3] TFA/CH_2Cl_2, r.t. 1 h; *c)* TFA/ CH_2Cl_2, r.t. 1 h.

Boc-Sp3-CPT (**12**)

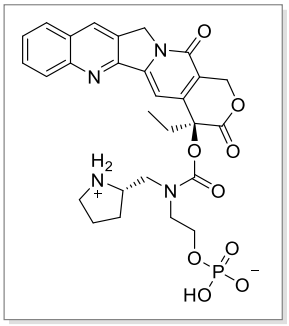


$C_{33}H_{38}N_4O_8$
MW: 618,69 g • mol^{-1}

Camptothecin-*p*-nitrophenyl carbonate (**CPT-PNP**,[2] 50 mg, 93 µmol, 1 equiv.) was dissolved in dry DMF (1 ml) and cooled to 0 °C under a nitrogen atmosphere. A solution of amine **10** (33 mg, 0.14 mmol, 1.5 equiv.) in dry DMF (1 ml) and *i*Pr_2NEt (8 µl, 93 µmol, 1 equiv.) were added to the cooled **CPT-PNP** solution. The mixture was warmed to r.t. and stirred overnight. The solvent was removed under high vacuum, and the crude product was purified by flash chromatography (dry load, gradient from 1% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2), obtaining **SA05** as a pale-yellow solid (56 mg, 97%).

MS (ESI) *m/z* calcd. for $[C_{33}H_{38}N_4NaO_8]^+$: 641.26 $[M+Na]^+$, found: 641.37; *m/z* calcd. for $[C_{33}H_{37}N_4O_8]^-$: 617,26: $[M-H]^-$, found: 617.34.

Sp2-CPT

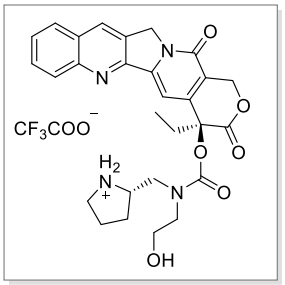


$C_{28}H_{31}N_4O_9P$
MW:598,55 g • mol^{-1}

Alcohol **12** (10 mg, 16 µmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (1 ml) under a nitrogen atmosphere. 1*H*-tetrazole (106 µl of a 0.45 M solution in anhydrous MeCN, 48 µmol, 3 equiv.) and di-*tert*-butyl *N,N*-diisopropylphosphoramidite (15 µl, 48 µmol, 3 equiv.) were added and the mixture was stirred at r.t.. After 1 h, the mixture was cooled to 0 °C and *m*CPBA (14 mg, 80 µmol, 5 equiv.) was added. The reaction mixture was warmed to r.t. and stirred for 1 h. The mixture was concentrated, re-dissolved in CH_2Cl_2 (1 ml) and treated following General Procedure A. After solvent removal, the crude material was purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 10% B (at min 1) to 77% B (at min 14), t_R (product): 8.1 min]. The purified product was then lyophilized to give **Sp2-CPT** as a yellow solid (8 mg, 83%).

^{31}P NMR (162 MHz, d_6-DMSO + D_2O) δ -1.42 ppm (rotamers). MS (ESI) *m/z* calcd. for $[C_{28}H_{31}N_4NaO_9P]^+$: 621.17 $[M+Na]^+$, found: 621.19; MS (ESI) *m/z* calcd. for $[C_{28}H_{30}N_4O_9P]^-$: 597.18 $[M-H]^-$, found: 597.04; HRMS (ESI) *m/z* calcd. for $[C_{28}H_{32}N_4O_9P]^+$: 599,1901 $[M+H]^+$, found: 599,1901.

Sp3-CPT

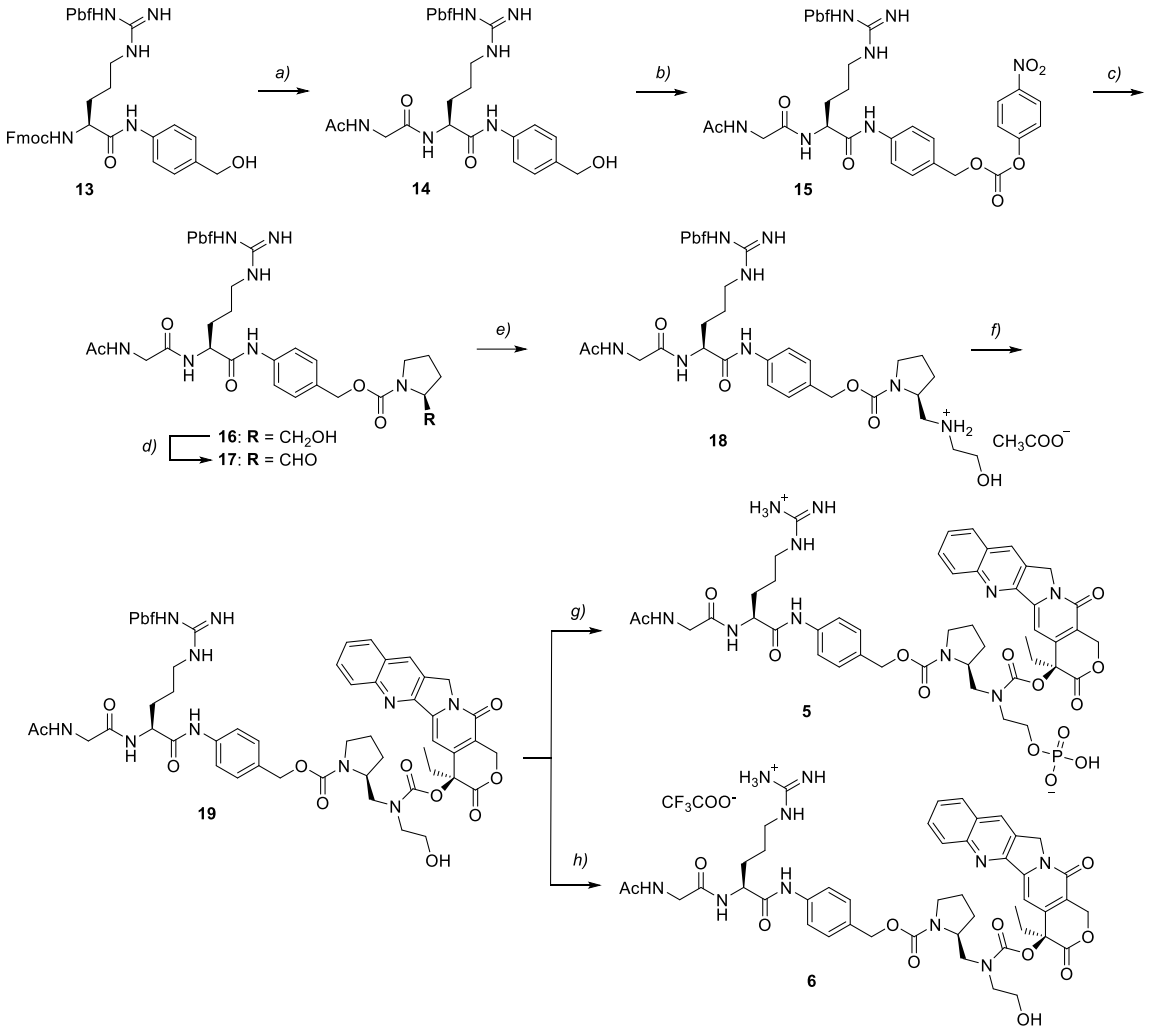


$C_{28}H_{30}N_4O_6$
MW: 518,57g • mol^{-1} + TFA

Compound **12** (56 mg, 90 µmol, 1 equiv.) was dissolved in CH_2Cl_2 (2 ml) and treated following General Procedure A. After solvent removal, the crude material was purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 5% B (at min 1) to 70% B (at min 12), t_R (product): 8.8 min]. The purified product was then lyophilized to give **Sp3-CPT** as a yellow solid (47 mg, 82%).

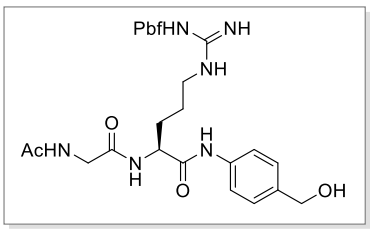
HRMS (ESI) *m/z* calcd. for $[C_{28}H_{31}N_4O_6]^+$: 519,2238 $[M+H]^+$, found: 519,2239.

Synthesis of Camptothecin Prodrugs 5 and 6



Scheme S3. REAGENTS AND CONDITIONS: *a)* [1] Piperidine, DMF, 0 °C to r.t. 1h; [2] Ac-Gly-OH, HATU, *i*Pr$_2$NEt, DMF, 0 °C to r.t. 16h; *b)* bis(4-nitrophenyl) carbonate, *i*Pr$_2$NEt, THF, 50 °C, 6 h; *c)* L-prolinol, THF, 0 °C, 1 h; *d)* Dess-Martin periodinane, CH_2Cl_2, 0 °C to r.t. 1h; *e)* Ethanolamine, $NaBH(OAc)_3$, AcOH, CH_2Cl_2, r.t. 16 h; *f)* **CPT-PNP**, *i*Pr$_2$NEt, CH_2Cl_2, r.t. 48 h; *g)* [1] di-*tert*-butyl *N,N*-diisopropylphosphoramidite, 1*H*-tetrazole, CH_2Cl_2, r.t. 1 h; [2] *m*CPBA, 0 °C to r.t. 1 h; [3] TFA/triisopropylsilane/H_2O 94:3:3 0 °C 2 h; *h)* TFA/triisopropylsilane/H_2O 94:3:3 0 °C 2 h.

Ac-Gly-Arg(Pbf)-PABOH (**14**)



$C_{30}H_{42}N_6O_7S$
MW: 630,76 g • mol^{-1}

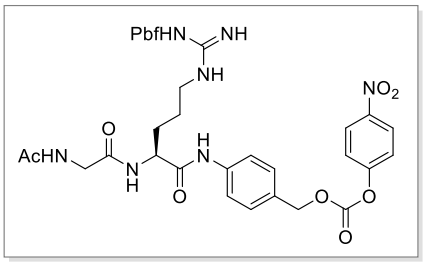
Fmoc-Arg(Pbf)-PABOH[4] (**13**, 550 mg, 0.73 mmol, 1 equiv.) was dissolved in dry DMF (4 ml) and cooled to 0 °C. Piperidine (360 µl, 3.64 mmol, 5 equiv.) was added and the mixture was stirred for 1 h at r.t.. The solvent was removed under high vacuum and the crude was filtered through a plug of silica (dry load, initial elution with 1:1 AcOEt:Hex mixture, followed by 10% MeOH in CH_2Cl_2 + 0.2% Et_3N), obtaining H-Arg(Pbf)-PABOH (360 mg, 93%) as a white foam. Ac-Gly-OH (85 mg, 0.73 mmol, 1 equiv.) was dissolved in dry DMF (3 ml), cooled to 0 °C under a nitrogen atmosphere and HATU (304 mg, 0.80 mmol, 1.1 equiv.) and *i*Pr$_2$NEt (380 µl, 2.19 mmol, 3 equiv.) were added. The mixture was stirred for 15 min. at room temperature, then a solution of H-Arg(Pbf)-PABOH in dry DMF (2 ml) was

4 S. Cazzamalli, A. Dal Corso, D. Neri, *J. Control. Release* 2017, **246**, 39.

added to the reacting solution. The resulting mixture was stirred at r.t. overnight and the solvent was removed under high vacuum. The crude solid was dissolved with CH_2Cl_2 (150 ml) and washed with a 1:1 mixture of brine and a 1 M aq. $KHSO_4$ solution (2 × 40 ml) and with a 1:1 mixture of brine and a sat. aq. $NaHCO_3$ solution (2 × 40 ml). The organic phase was dried and concentrated. The crude was purified by flash chromatography (eluent: 11% MeOH in CH_2Cl_2) to give **14** as a white solid (300 mg, 65%).

^{1}H NMR (400 MHz, MeOD) *δ* 7.56 (d, *J* = 8.6 Hz, 2H), 7.30 (d, *J* = 8.6 Hz, 2H), 4.56 (s, 2H), 4.52 (dd, *J* = 9.0, 5.1 Hz, 1H), 3.88 (Gly AB system, 2H), 3.20 (m, 2H), 2.97 (s, 2H), 2.56 (s, 3H), 2.50 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.90 (m, 1H), 1.73 (m, 1H), 1.59 (m, 2H), 1.44 (s, 6H) ppm; ^{13}C NMR (101 MHz, MeOD) δ 174.1, 172.3, 171.8, 159.9, 158.1, 139.4, 138.9, 138.5, 133.5, 128.5, 126.0, 121.5, 118.4, 87.7, 64.8, 54.9, 43.9, 43.7, 30.5, 28.7, 27.0, 22.5, 19.6, 18.4, 12.5 ppm.

Ac-Gly-Arg(Pbf)-PAB-PNP (**15**)

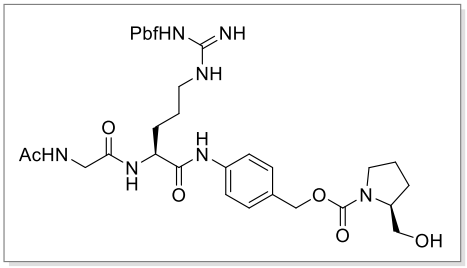


$C_{37}H_{45}N_7O_{11}S$
MW: 795,87 g • mol^{-1}

Alcohol **14** (300 mg, 0.47 mmol, 1 equiv.) was dissolved in dry THF (4 ml) and added, under a nitrogen atmosphere, to a flask containing bis(4-nitrophenyl) carbonate (573 mg, 1.88 mmol, 4 equiv.). *i*Pr_2NEt (329 µl, 1.88 mmol, 4 equiv.) was added and the mixture was stirred at 50 °C for 6 h. The solvent was removed and the crude was dissolved with AcOEt (150 ml) and washed with a 1 M aq. $KHSO_4$ solution (1 × 30 ml). The organic phase was dried and concentrated. The crude solid was mixed with silica and eluted through a pad of silica gel, using this sequence of eluents: 1) 8:2 AcOEt:Hex; 2) AcOEt; 3) 1:1 AcOEt:acetone; 4) acetone. Product eluted between the 3rd and 4th elution step. Product-containing fractions were pooled and concentrated, to give **15** as a white solid (215 mg, 57%).

^{1}H NMR (400 MHz, d_6-DMSO) *δ* 10.06 (s, 1H), 8.31 (d, *J* = 9.2 Hz, 2H), 8.19 (d, *J* = 7.9 Hz, 1H), 8.13 (t, *J* = 5.7 Hz, 1H), 7.68 (d, *J* = 8.5 Hz, 2H), 7.56 (d, *J* = 9.2 Hz, 2H), 7.42 (d, *J* = 8.5 Hz, 2H), 5.25 (s, 2H), 4.41 (dd, *J* = 13.7, 8.0 Hz, 1H), 3.74 (d, *J* = 5.7 Hz, 2H), 3.11 – 3.00 (m, 2H), 2.93 (s, 2H), 2.46 (s, 3H), 2.41 (s, 2H), 1.98 (s, 3H), 1.86 (s, 3H), 1.74 (m, 1H), 1.58 (m, 1H), 1.51 – 1.41 (m, 2H), 1.39 (s, 6H) ppm; ^{13}C NMR (101 MHz, d_6-DMSO) *δ* 170.6, 169.8, 169.1, 157.4, 156.1, 155.3, 152.0, 145.2, 139.2, 137.3, 131.4, 129.4, 125.4, 124.3, 122.6, 119.3, 116.3, 86.3, 70.2, 53.0, 42.4, 42.1, 39.3 (overlapped with solvent signal), 29.4, 28.3, 22.5, 18.9, 17.6, 12.2 ppm.

Ac-Gly-Arg(Pbf)-PABC-Prolinol (**16**)



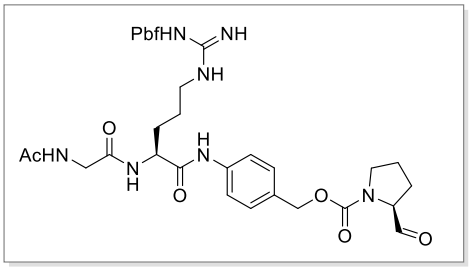
$C_{36}H_{51}N_7O_9S$
MW: 757,90 g • mol^{-1}

Carbonate **15** (200 mg, 0.25 mmol, 1 equiv.) was dissolved in dry THF (4 ml) and cooled to 0 °C. L-Prolinol (65 µl, 0.62 mmol, 2.5 equiv.) was slowly added and the mixture turned yellow immediately. The mixture was stirred at 0 °C for 1 h, when TLC analysis (5% MeOH in CH_2Cl_2) indicated full starting material conversion. The mixture was diluted with CH_2Cl_2 (40 ml) and washed with a 1 M aq. $KHSO_4$ solution (2 × 20 ml). The aqueous phase was extracted with CH_2Cl_2 (2 × 30 ml) and AcOEt (1 × 30 ml). The collected organic phase was dried and concentrated. The crude material was purified by flash chromatography (gradient from 6% MeOH in CH_2Cl_2 to 10% MeOH in CH_2Cl_2) to give **16** as a white solid (160 mg, 85%).

^{1}H NMR (400 MHz, MeOD) *δ* 7.59 (d, *J* = 8.3 Hz, 2H), 7.33 (d, *J* = 8.3 Hz, 2H), 5.17 – 5.01 (m, 2H), 4.52 (dd, *J* = 8.9, 5.1 Hz, 1H), 3.94 – 3.79 (m, 3H), 3.59 (m, 1H), 3.51 – 3.35 (m, 3H), 3.27 – 3.12 (m, 2H), 2.97 (s, 2H), 2.55 (s, 3H), 2.50 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), 1.98 – 1.79 (m, 5H), 1.62 (m, 1H), 1.66 – 1.51 (m, 2H), 1.44 (s, 6H) ppm; ^{13}C NMR (101 MHz, MeOD) *δ* 174.0, 172.3, 171.8, 159.9,

158.1, 157.1, 156.8, 139.4, 134.3, 133.5, 129.8, 129.6, 126.0, 121.5, 118.4, 87.7, 67.7, 67.6, 63.7, 60.7, 60.0, 54.9, 48.1, 43.9, 43.7, 41.3, 30.5, 29.2, 28.7, 28.6, 27.0, 24.7, 23.8, 22.5, 19.6, 18.4, 12.5 ppm.

Ac-Gly-Arg(Pbf)-PABC-Prolinal (**17**)

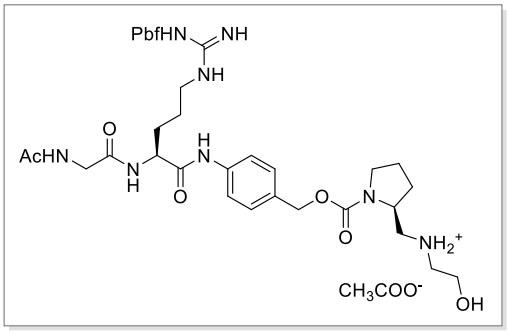


$C_{36}H_{49}N_7O_9S$
MW: 755,89 g • mol^{-1}

Alcohol **16** (135 mg, 0.178 mmol, 1 equiv.) was dissolved in CH_2Cl_2 (9 ml) and cooled to 0 °C. Dess-Martin periodinane (0.3 M solution in CH_2Cl_2, 890 µl, 1.5 equiv.) was added and the mixture was stirred at r.t.. After 1 h, TLC (10% MeOH in CH_2Cl_2) revealed full starting material consumption. The reaction mixture was concentrated and purified by flash chromatography (gradient from 4% to 8% MeOH in CH_2Cl_2), to give aldehyde **17** as a white solid (127 mg, 94%).

MS (ESI) *m/z* calcd. for $[C_{36}H_{50}N_7O_9S]^+$: 756.34 $[M+H]^+$, found: 756.10; *m/z* calcd. for $[C_{36}H_{49}N_7NaO_9S]^+$: 778.32 $[M+Na]^+$, found: 778.28.

Ac-Gly-Arg(Pbf)-PABC-Pro-Ethanolamine Acetate (**18**)

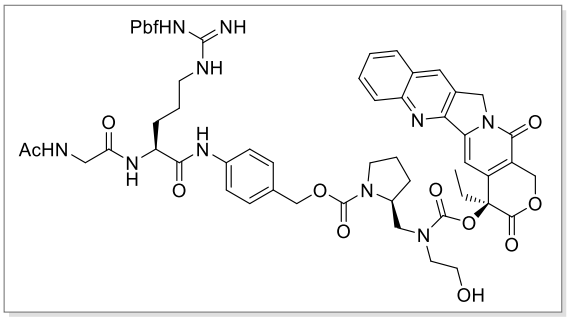


$C_{38}H_{56}N_8O_9S$
MW: 800,97 g • mol^{-1} + AcOH

Aldehyde **17** (65 mg, 86 µmol, 1 equiv.) was dissolved in CH_2Cl_2 (3 ml) under a nitrogen atmosphere. Acetic acid (300 µl) was added, followed by ethanolamine (16 µl, 257 µmol, 3 equiv.) and $NaBH(OAc)_3$ (73 mg, 344 µmol, 4 equiv.). The mixture was stirred at r.t. overnight. The solvent was removed and the mixture was purified by HPLC [eluent A: H_2O + 0.1% AcOH; eluent B: MeCN, ramp from 5% B (at min 1) to 68% B (at min 10), t_R (product): 8.6 min]. The purified product was then freeze dried to give **18** as a white solid (45 mg, 61%).

MS (ESI) *m/z* calcd. for $[C_{38}H_{57}N_8O_9S]^+$: 801.40 $[M+H]^+$, found: 801.27; *m/z* calcd. for $[C_{38}H_{55}N_8O_9S]^-$: 799,38 $[M-H]^-$, found: 799.77. ^{1}H NMR (400 MHz, MeOD) δ 7.61 (d, J = 8.3 Hz, 2H), 7.35 (d, J = 8.3 Hz, 2H), 5.14 (d, J = 12.0 Hz, 1H), 5.10 (d, J = 12.0 Hz, 1H), 4.52 (dd, J = 8.8, 5.1 Hz, 1H), 4.16 (m, 1H), 3.88 (s, 2H), 3.80 (t, J = 5.1 Hz, 2H), 3.55 (m, 1H), 3.43 (m, 1H), 3.27 – 3.02 (m, 6H), 2.98 (s, 2H), 2.55 (s, 3H), 2.50 (s, 3H), 2.17 (m, 1H), 2.05 (s, 3H), 2.01 (s, 3H), 1.99 (s, 3H), 1.96 – 1.84 (m, 3H), 1.79 – 1.67 (m, 2H), 1.66 – 1.53 (m, 2H), 1.44 (s, 6H) ppm; ^{13}C NMR (101 MHz, MeOD) δ 175.2, 174.1, 172.4, 171.9, 158.8, 158.0, 139.5, 133.7, 129.9, 126.1, 121.5, 118.6, 87.8, 68.4, 57.8, 56.4, 54.9, 53.7, 51.0, 48.6 (overlapped with solvent signal), 43.9, 43.8, 41.3, 30.8, 30.4, 28.7, 26.9, 24.8, 22.5, 20.7, 19.6, 18.4, 12.5 ppm.

Ac-Gly-Arg(Pbf)-PABC-Pro(OH)-CPT (**19**)

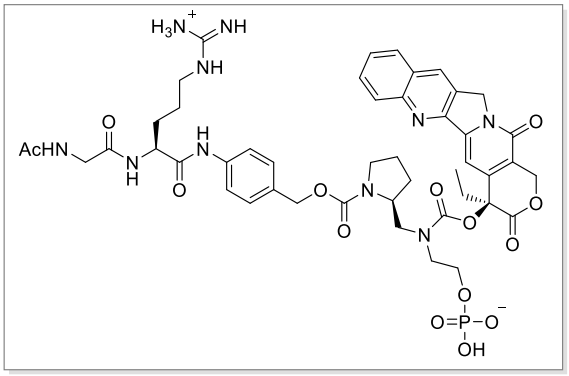


$C_{59}H_{70}N_{10}O_{14}S$
MW: 1175,33 g • mol^{-1}

Dry CH_2Cl_2 (1 ml) was added to a flask containing **18** (10 mg, 11.6 µmol, 1 equiv.) and **CPT-PNP** (8.9 mg, 17.4 µmol, 1.5 equiv.) under a nitrogen atmosphere. *i*Pr_2NEt (5 µl, 29 µmol, 2.5 equiv.) was added and the mixture was stirred at r.t. for 48 h. The solvent was removed and the mixture was purified by flash chromatography (1st eluent: 4% MeOH in CH_2Cl_2; 2nd eluent: 10% MeOH in CH_2Cl_2), to give carbamate **19** as a white solid (10 mg, 72%).

R_f= 0.40 (9:1 CH_2Cl_2/MeOH). MS (ESI) *m/z* calcd. for $[C_{59}H_{70}N_{10}NaO_{14}S]^+$: 1197.47 $[M+Na]^+$, found: 1197.68. ^{1}H NMR (400 MHz, d_6-DMSO) δ 9.97 (bs, 3H), 8.68 (s, 1H), 8.18 – 8.07 (m, 4H), 7.88 – 7.80 (m, 1H), 7.71 (m, 1H), 7.58 (dd, *J* = 13.9, 8.4 Hz, 2H), 7.41 – 7.21 (m, 2H), 7.12 (m, 1H), 6.54 (bs, 3H), 5.50 – 5.41 (m, 2H), 5.29 (s, 2H), 5.11 – 4.96 (m, 2H), 4.91 (t, *J* = 5.1 Hz, 1H), 4.66 (m, 1H), 4.40 (m, 1H), 4.17 (m, 1H), 4.08 (m, 1H), 3.79 – 3.63 (m, 3H), 3.58 – 3.38 (m, 3H), 3.29 – 3.18 (m, 2H), 3.05 (bs, 2H), 2.92 (s, 2H), 2.45 (s, 3H), 2.40 (s, 3H), 2.20 – 2.10 (m, 2H), 1.96 (s, 3H), 1.86 (s, 3H), 1.77 – 1.65 (m, 2H), 1.63 – 1.52 (m, 2H), 1.48 – 1.41 (m, 2H), 1.38 (s, 6H), 0.98 – 0.83 (m, 3H) ppm; ^{13}C NMR (101 MHz, d_6-DMSO) δ 170.4, 169.8, 169.1, 167.9, 167.7, 157.4, 156.6, 156.1, 154.2, 153.8, 152.4, 147.9, 145.8, 145.7, 138.3, 137.3, 132.0, 131.6, 131.4, 130.4, 129.8, 129.0, 128.5, 128.3, 128.0, 127.7, 124.3, 119.3, 116.3, 95.5, 95.2, 86.3, 75.7, 66.4, 66.0, 59.1, 58.1, 55.9, 53.0, 50.2, 49.8, 42.4, 42.1, 39.3 (overlapped with solvent signal), 30.8, 30.2, 29.5, 28.3, 25.6, 25.6, 22.5, 18.9, 17.6, 12.2, 7.8, 7.6 ppm.

Ac-Gly-Arg-PABC-Pro(OPO_3H_2)-CPT (**5**)

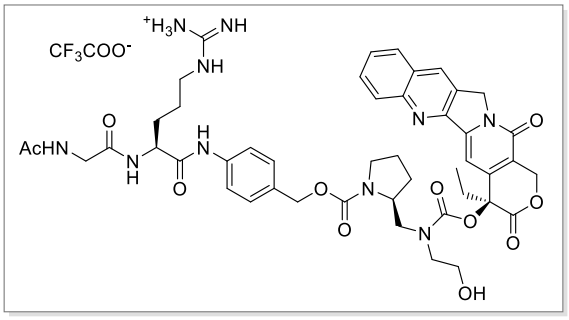


$C_{46}H_{55}N_{10}O_{14}P$
MW: 1002,98 g • mol^{-1}

Alcohol **19** (18 mg, 15.3 µmol, 1 equiv.) was dissolved in dry CH_2Cl_2 (2 ml) under a nitrogen atmosphere. 1*H*-tetrazole (136 µl of a 0.45 M solution in anhydrous MeCN, 61.2 µmol, 4 equiv.) and di-*tert*-butyl *N,N*-diisopropylphosphoramidite (19 µl, 61.2 µmol, 4 equiv.) were added and the mixture was stirred at r.t.. After 1 h, the mixture was cooled to 0 °C and *m*CPBA (16 mg, 76 µmol, 5 equiv.) was added. The reaction mixture was warmed to r.t. and stirred for 1 h. The mixture was concentrated, dissolved in a 94:3:3 mixture of TFA/triisopropylsilane/H_2O (1 ml) and stirred at 0 °C for 2 h. The solution was diluted with ca. 40 ml of cold Et_2O and the resulting yellow precipitate was isolated by centrifugation and purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 10% B (at min 1) to 75% B (at min 12), t_R (product): 7.9 min]. (NOTE: Product **5** was isolated in ca. 90% purity, since its very broad UV peak observed during RP-HPLC purification hindered the complete removal of an impurity, which was identified as the analogue *H*-phosphonate monoester. For more information see "Entry ***0***" in Figure S1/S2). The product was then lyophilized to give **5** as a yellow solid (5 mg, 32%).

HRMS (ESI) *m/z* calcd. for $[C_{46}H_{56}N_{10}O_{14}P]^+$: 1003.3715 $[M+H]^+$, found: 1003.3723.

Ac-Gly-Arg-PABC-Pro(OH)-CPT (**6**)



$C_{46}H_{54}N_{10}O_{11}$
MW: 923,00 g • mol^{-1} + TFA

Compound **19** (9 mg, 7.7 µmol, 1 equiv.) was dissolved in a 94:3:3 mixture of TFA/triisopropylsilane/H_2O (1 ml) and stirred at 0 °C for 2 h. The solution was diluted with ca. 40 ml of cold Et_2O and the resulting yellow precipitate was isolated by centrifugation and purified by HPLC [eluent A: H_2O + 0.1% TFA; eluent B: MeCN, ramp from 5% B (at min 1) to 100% B (at min 20), t_R (product): 10.5 min]. The purified product was then lyophilized to give **6** as a yellow solid (6 mg, 84%).

MS (ESI) *m/z* calcd. for $[C_{46}H_{55}N_{10}O_{11}]^+$: 923.40 $[M+H]^+$, found: 923.59; MS (ESI) *m/z* calcd. for $[C_{46}H_{56}N_{10}O_{11}]^{2+}$: 462.21 $[M+2H]^{2+}$, found: 462.69.

Enzyme-free Carbamate Cleavage Studies

Experimental Procedure

Stock solutions of lyophilized coumarin carbamates (compounds **Sp(1-3)-CMR**; final concentration: 50 mM in DMSO) and Camptothecin prodrugs (compounds **Sp(1-3)-CPT**; final concentration: 25 mM in DMSO) were diluted with further DMSO and 25 mM aqueous buffers according to the following scheme:

	Sp(1-3)-CMR	**Sp(1-3)-CPT**
Sample (in DMSO) (Volume % Total)	5% - [2.5 mM] final	4% - [1 mM] final
Neat DMSO (Volume % Total)	5%	6%
Aq. Buffer (Volume % Total)	90%	90%
Buffer Type	25 mM Acetate Buffer pH 5.0	25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++}

Immediately after preparation, the mixtures were incubated at 37 °C and aliquots were collected at different time points and diluted with a blocking buffer, according to the following scheme:

	Sp(1-3)-CMR	**Sp(1-3)-CPT**
Blocking buffer	H_2O + 0.2% TFA	8:2 H_2O/MeCN + 0.2% TFA
Dilution	1:2.5 - [1 mM] final	1:4 - [0.25 mM] final

The diluted aliquots were injected into an analytical HPLC-PDA system, using the following parameters:

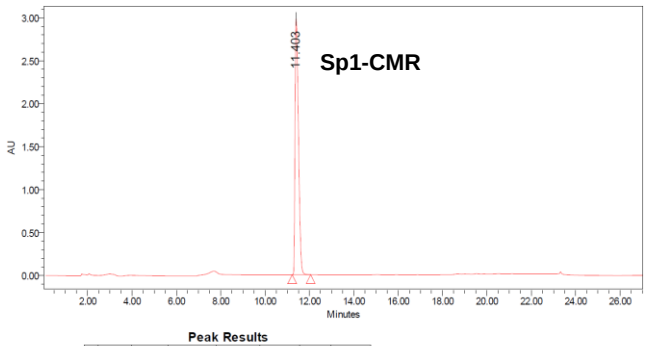
	Sp(1-3)-CMR	**Sp(1-3)-CPT**
Eluent A	H_2O + 0.1% TFA	H_2O + 0.1% TFA
Eluent B	CH_3CN + 0.1% TFA	CH_3CN + 0.1% TFA
Flow Rate	1 ml/min	1 ml/min
Gradient	From 0% B to 50% B in 15 min.	From 10% B to 50% B in 25 min.
UV analysis	254 nm	330 nm

Areas under the curve (AUC) of the detected peaks were measured using Waters Empower software. The rate of free OH release from the starting carbamate were obtained by calculating the relative ratios of AUC values corresponding to the amine-bearing prodrug and the free payload. Data were plotted and half-lives ($t_{1/2}$) were calculated by non-linear fitting.

HPLC Data – Coumarin Carbamates

- **Sp1-CMR** 2.5 mM
- 25 mM acetate buffer + 10 % DMSO
- pH 5.0, T: 37 °C

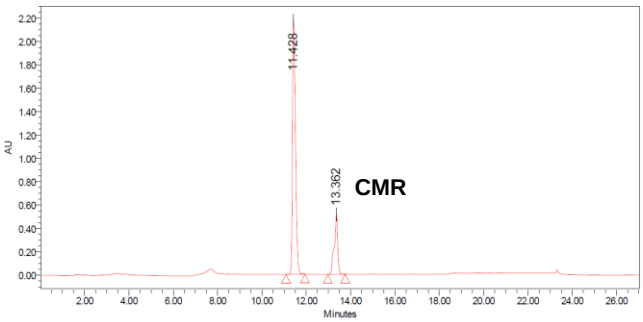
t = 0 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.403	31989963	2984844			100.00

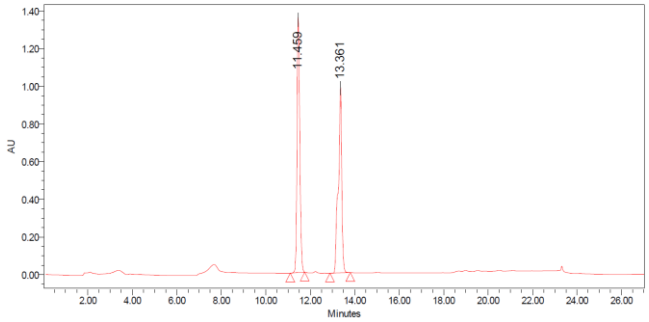
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.428	2060311	2204527			78.84
2		13.362	5531128	516867			21.16

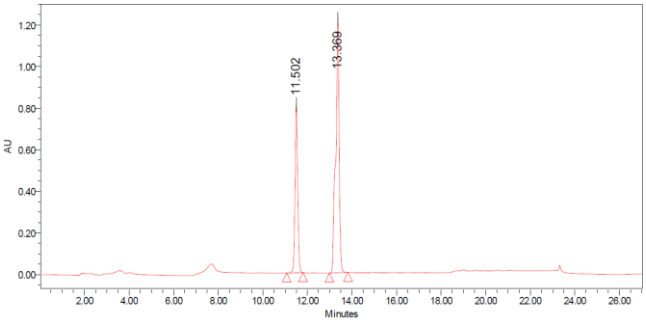
t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.459	11580458	1360714			52.14
2		13.361	10627734	988284			47.86

t = 8 h

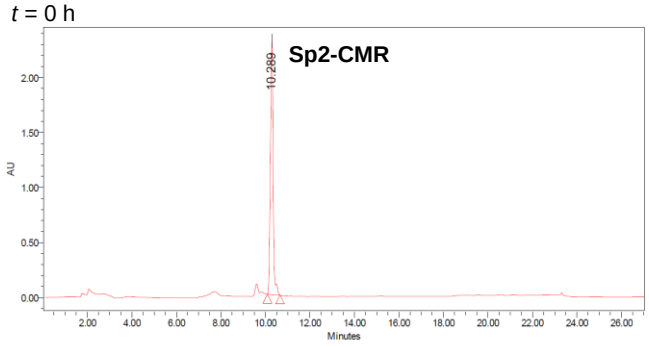


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.502	6678515	812313			33.93
2		13.369	13005695	1225038			66.07

- **Sp2-CMR** 2.5 mM
- 25 mM acetate buffer + 10 % DMSO
- pH 5.0, T: 37 °C

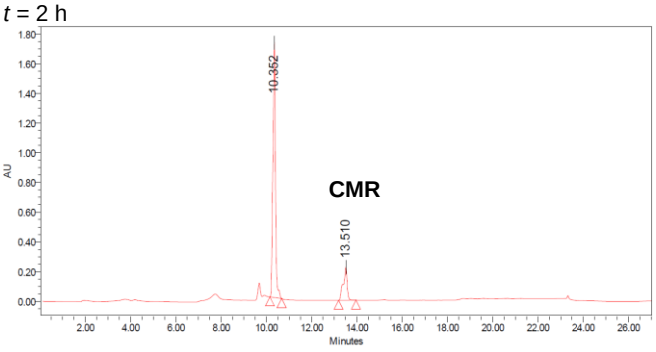
t = 0 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.289	19490659	2307205			100.00

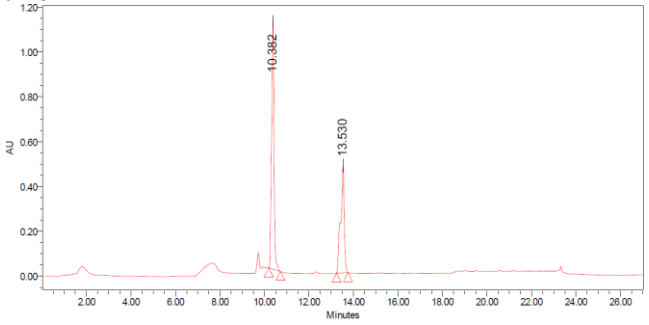
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.352	13304840	1723323			83.60
2		13.510	2609553	223622			16.40

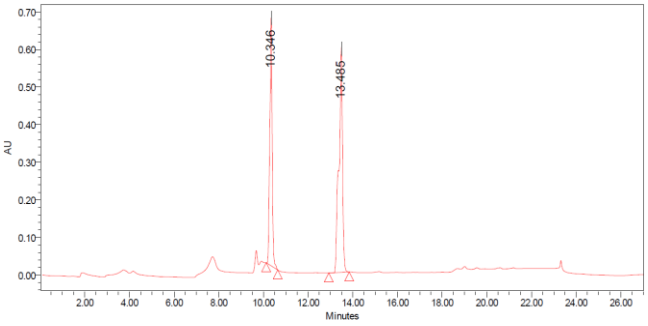
t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.382	8309576	111205			60.79
2		13.530	5358875	482275			39.21

t = 8 h

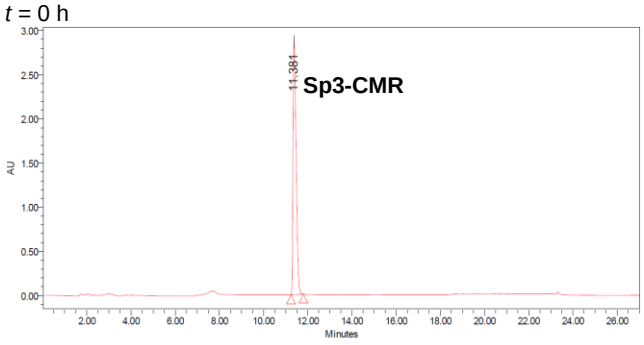


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.346	4925225	666848			42.39
2		13.485	6694804	597949			57.61

- **Sp2-CMR** 2.5 mM
- 25 mM acetate buffer + 10 % DMSO
- pH 5.0, T: 37 °C

t = 0 h



Peak Results

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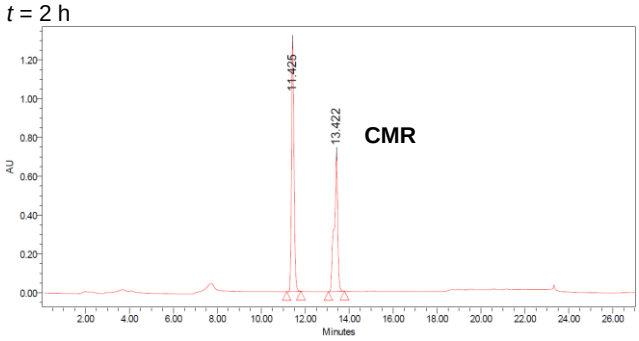
1		11.381	28018998	2919708			100.00

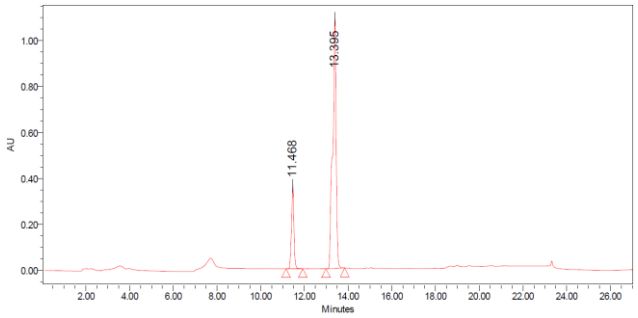
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.425	10112436	1300709			56.38
2		13.422	7823450	708482			43.62

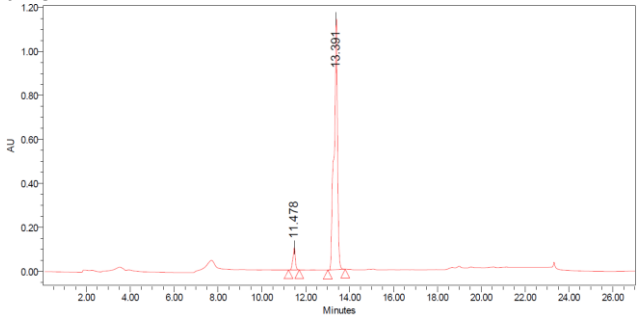
t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.468	2713897	361702			18.55
2		13.395	11912686	1090336			81.45

t = 8 h



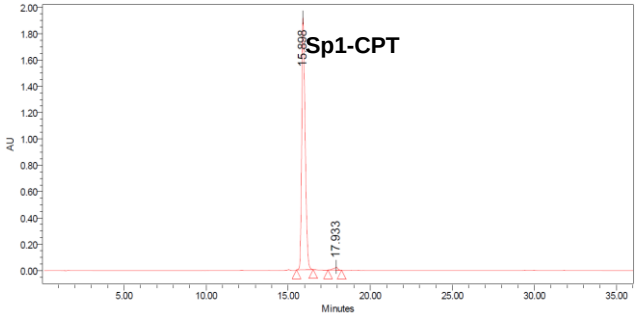
Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.478	780832	103058			5.89
2		13.391	12482665	1144556			94.11

HPLC Data – CPT Prodrugs

- **Sp1-CPT** 1 mM
- 25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++} + 10% DMSO
- pH 7.5, T: 37 °C
-

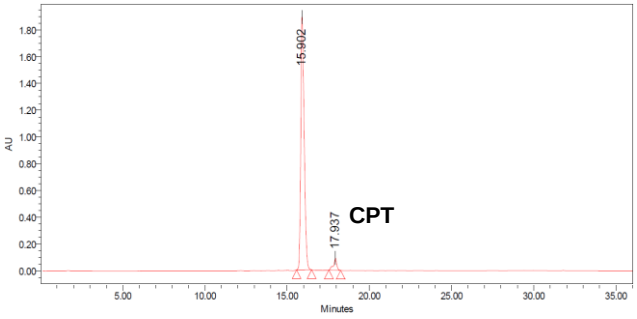
t = 0 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.898	27888006	1933060			98.53
2		17.933	416825	24456			1.47

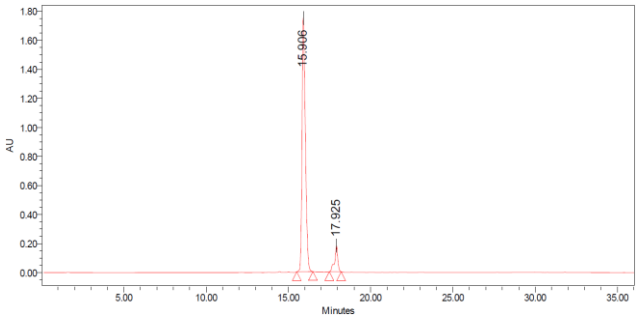
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.902	27057964	1891309			95.61
2		17.937	1242904	90695			4.39

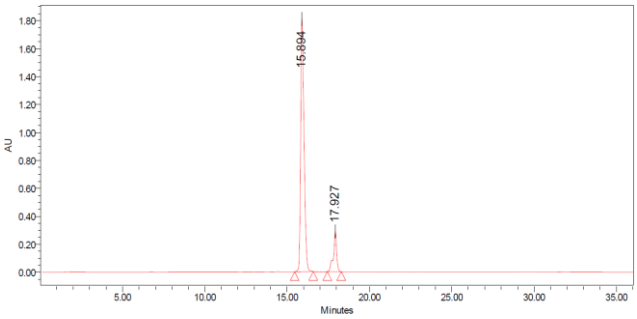
t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.906	24526065	1749015			91.17
2		17.925	2374323	180680			8.83

t = 8 h

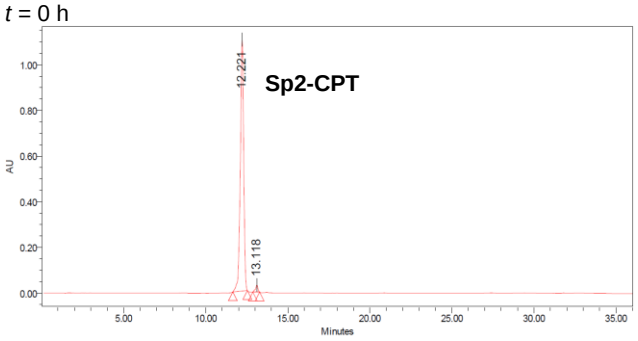


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.894	25739827	1821741			86.95
2		17.927	3862941	288045			13.05

- **Sp2-CPT** 1 mM
- 25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++} + 10% DMSO
- pH 7.5, T: 37 °C

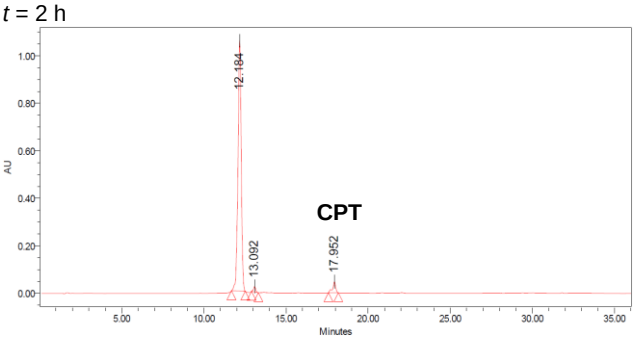
t = 0 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		12.221	15649937	1101763			98.00
2		13.118	319071	30557			2.00

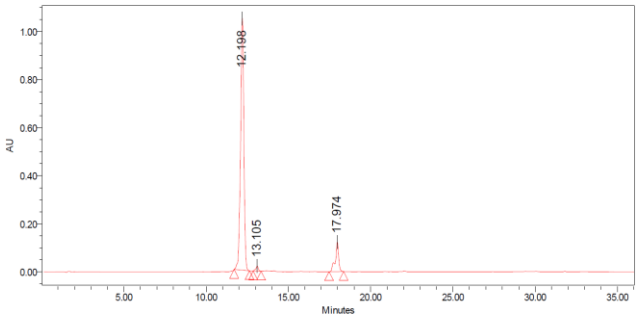
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		12.184	14550772	1055161			94.72
2		13.092	216028	23392			1.41
3		17.952	595465	47170			3.88

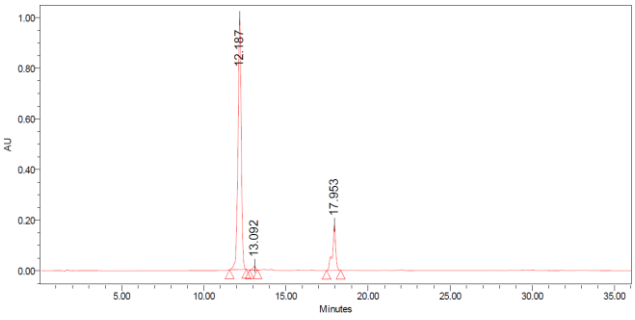
t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		12.198	14566299	1050653			88.37
2		13.105	224852	20862			1.36
3		17.974	1691846	123218			10.26

t = 8 h

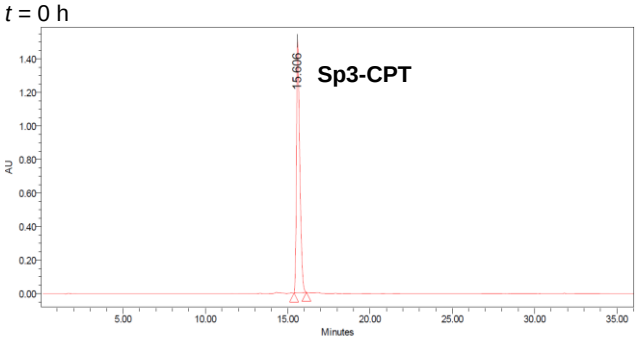


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		12.187	13710183	994137			84.01
2		13.092	167896	15829			1.03
3		17.953	2441616	180927			14.96

- **Sp3-CPT** 1 mM
- 25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++} + 10% DMSO
- pH 7.5, T: 37 °C

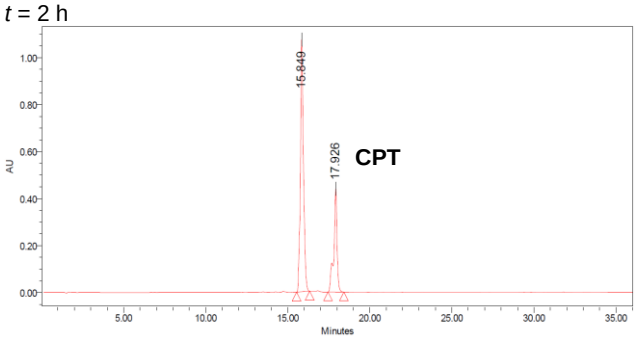
t = 0 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.606	20938804	1510460			100.00

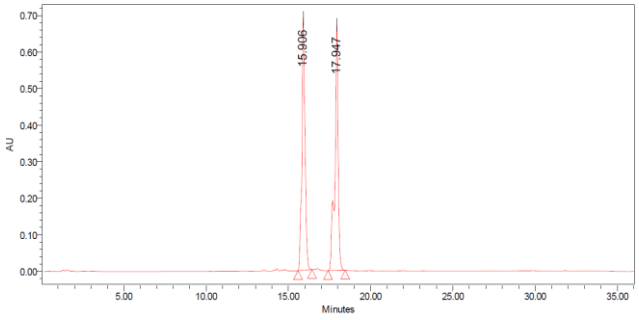
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.849	13930182	1076078			69.89
2		17.926	6000135	442012			30.11

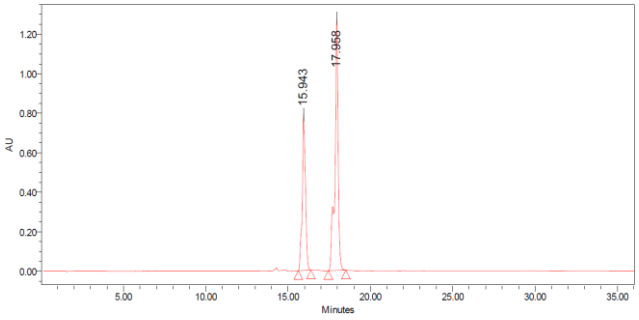
t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.906	9083274	691012			49.30
2		17.947	9340551	675096			50.70

t = 8 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.943	10757968	794070			38.07
2		17.958	17498640	1280589			61.93

Carbamate Cleavage Studies in the presence/absence of Phosphatase

Experimental Procedure

Stock solutions of lyophilized coumarin carbamates (compounds **Sp(1/2)-CMR**; final concentration: 50 mM in DMSO) and Camptothecin prodrugs (compounds **Sp(1/2)-CPT**; final concentration: 25 mM in DMSO) were added to 25 mM aqueous buffer (devoid or containing phosphatase) according to the following scheme:

	Sp(1/2)-CMR	**Sp(1/2)-CPT**
Sample (in DMSO) (Volume % Total)	5% - [2.5 mM] final	10% - [2.5 mM] final
Aq. Buffer (Volume % Total)	95%	90%
Buffer Type	25 mM Acetate Buffer pH 5.0	25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++}
Phosphatase Type	lyophilized Phosphatase, Acid, from potato - [50 µM] final	lyophilized Phosphatase, Alkaline from bovine intestinal mucosa - [50 µM] final

Immediately after preparation, the mixtures were incubated at 37 °C and aliquots were collected at different time points and diluted 1:10 with a 0.2% TFA solution in MeCN. Diluted aliquots were cooled at -20 °C for at least 0.5 h and then centrifuged at 13000 rpm at 4 °C for 15 min. The supernatant was transferred in glass vials, volatiles were removed under vacuum, and the concentrated DMSO solution was diluted with aqueous buffers for HPLC analysis, according to the following scheme:

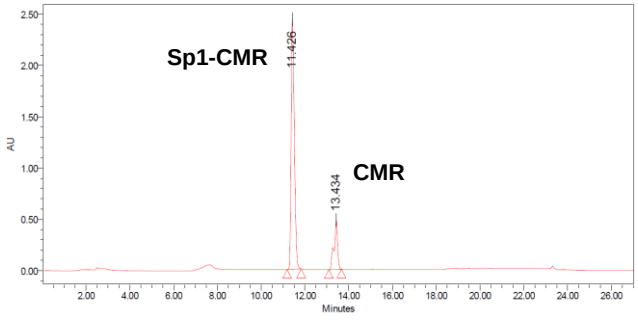
	Sp(1/2)-CMR	**Sp(1/2)-CPT**
Buffer for HPLC analysis	H_2O + 0.2% TFA	8:2 H_2O/MeCN + 0.2% TFA
Final sample concentration	1 mM	0.25 mM

The diluted aliquots were subjected to HPLC analysis as described before.

HPLC Data – Coumarin Carbamates

- **Sp1-CMR** 2.5 mM
- 25 mM acetate buffer + 5% DMSO
- pH 5.0, T: 37 °C

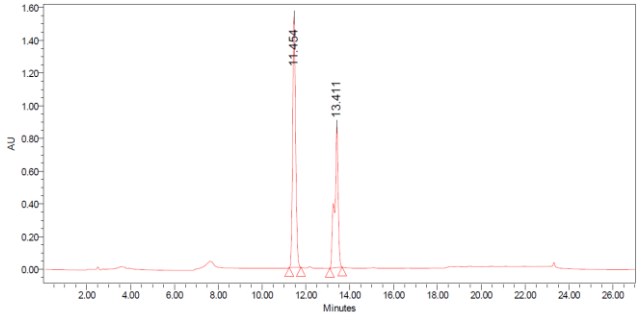
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.426	24643644	2462619			82.11
2		13.434	5367917	484521			17.89

t = 5 h

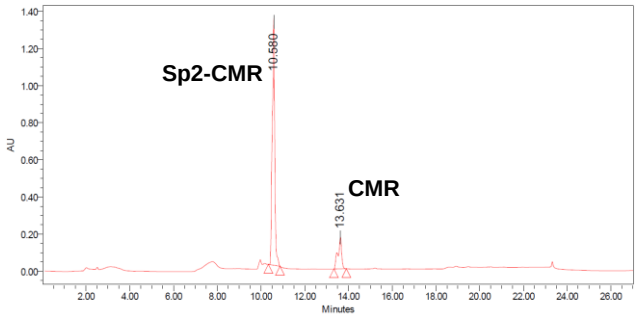


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.454	14325320	1529002			59.40
2		13.411	9791854	867113			40.60

- **Sp2-CMR** 2.5 mM
- 25 mM acetate buffer + 5% DMSO
- pH 5.0, T: 37 °C

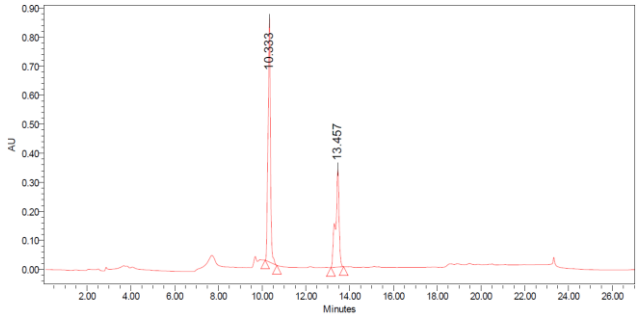
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.580	10512885	1323449			84.30
2		13.631	1957177	170920			15.70

t = 5 h

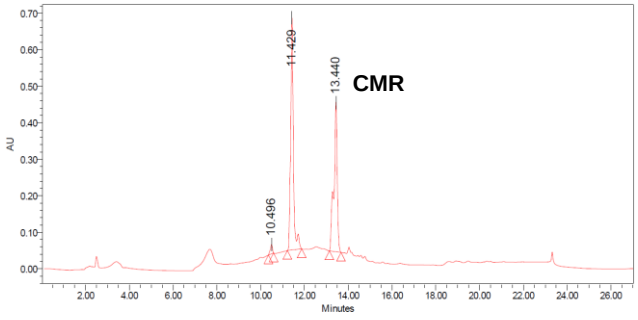


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.333	6383451	838639			62.86
2		13.457	3772248	336931			37.14

- **Sp2-CMR** 2.5 mM
- 50 μM phosphatase in 25 mM acetate buffer + 5% DMSO
- pH 5.0, T: 37 °C

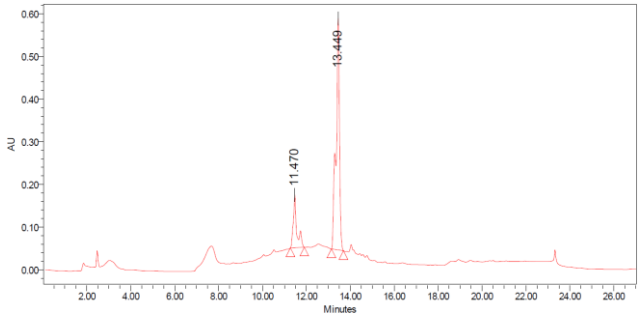
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		10.496	166193	25246			1.66
2		11.429	5479413	639770			54.84
3		13.440	4346049	407634			43.50

t = 5 h



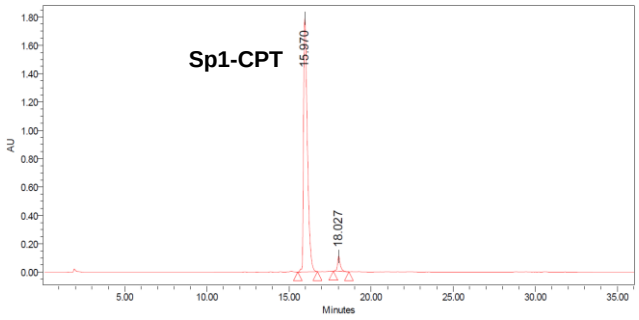
Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		11.470	1350579	122401			18.66
2		13.449	5885384	545847			81.34

HPLC Data – CPT Prodrugs

- **Sp1-CPT** 2.5 mM
- 25 mM HEPES Buffer + 0.5 mM Mg^{++} + 10% DMSO
- pH 7.5, T: 37 °C

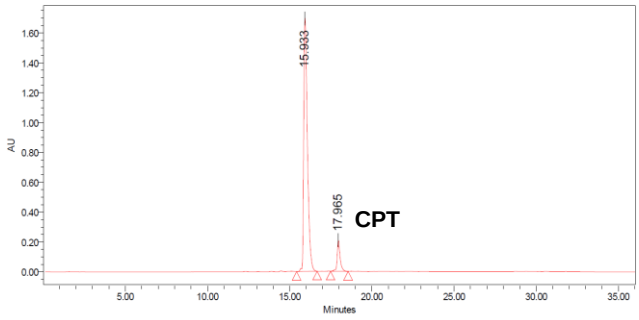
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.970	28924617	1788419			95.67
2		18.027	1307994	107295			4.33

t = 5 h

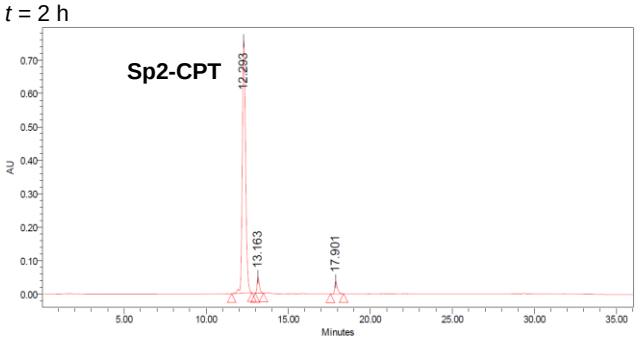


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		15.933	26418342	1699555			91.20
2		17.965	2548907	208847			8.80

- **Sp2-CPT** 2.5 mM
- 25 mM HEPES Buffer + 0.5 mM Mg^{++} + 10% DMSO
- pH 7.5, T: 37 °C

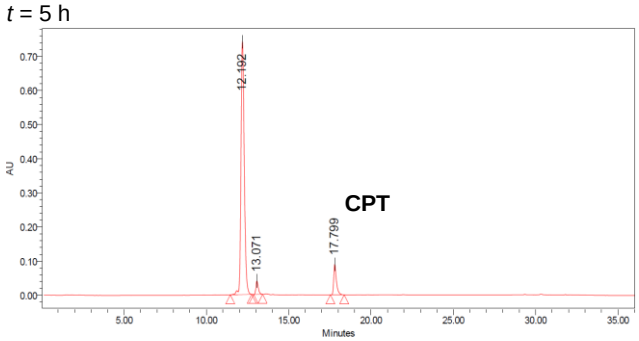
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		12.293	10610300	757540			92.17
2		13.163	483895	48374			4.20
3		17.901	417749	37391			3.63

t = 5 h

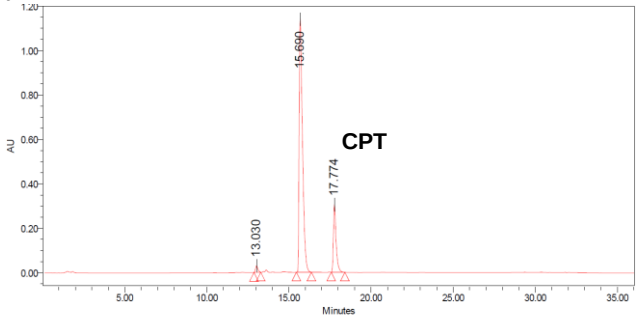


Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		12.192	10267479	744284			87.87
2		13.071	403525	39968			3.45
3		17.799	1013704	90015			8.68

- **Sp2-CPT** 2.5 mM
- 50 µM phosphatase in 25 mM HEPES Buffer + 0.5 mM Mg^{++} + 10% DMSO
- pH 7.5, T: 37 °C

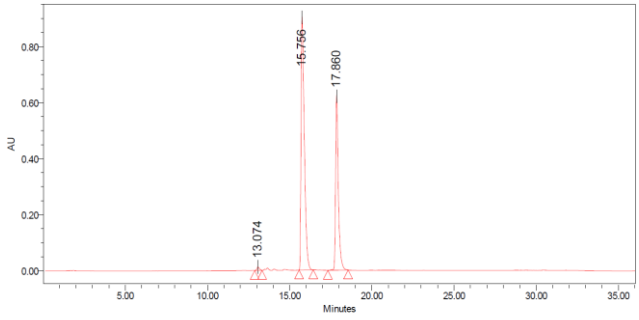
t = 2 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		13.030	301452	30248			1.52
2		15.690	16059187	1139917			81.16
3		17.774	3427576	305330			17.32

t = 5 h



Peak Results

	Name	RT	Area	Height	Amount	Units	% Area
1		13.074	127537	12677			0.64
2		15.756	12446516	903295			62.92
3		17.860	7206230	624911			36.43

Camptothecin Release from Prodrug 5 in the presence/absence of Phosphatase and Trypsin

Experimental Procedure

The following stock solutions were prepared:

5	Lyophilized **5** dissolved in DMSO at 20 mM concentration
HEPES Buffer	25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++}
Phosphatase	1 mg of lyophilized Phosphatase, Alkaline from bovine intestinal mucosa (Sigma-Aldrich) dissolved in 78 µl of HEPES Buffer. [80 µM] final
Trypsin	1 mg of lyophilized Trypsin from porcine pancreas (Sigma-Aldrich) dissolved in 525 µl of HEPES Buffer. [80 µM] final

The solutions were mixed according to the following scheme:

Entry *0*) No Enzyme	• **5** (10% volume, [2 mM] final) • HEPES Buffer (90% volume)
Entry *I*) Phosphatase only	• **5** (10% volume, [2 mM] final) • HEPES Buffer (33% volume) • Phosphatase (45% volume, [0.04 mM] final) • MeCN (12% volume, added to prevent precipitation of dephosphorylated prodrug **6**)
Entry *II*) Trypsin only	• **5** (10% volume, [2 mM] final) • HEPES Buffer (45% volume) • Trypsin (45% volume, [0.04 mM] final)
Entry *III*) Phosphatase + Trypsin	• **5** (10% volume, [2 mM] final) • Trypsin (45% volume, [0.04 mM] final) • Phosphatase (45% volume, [0.04 mM] final)

Immediately after preparation, entry ***0*** was diluted with 8:2 H_2O/MeCN + 0.2% TFA mixture up to 0.25 mM concentration of **5** (sample used as reference for HPLC analysis). Samples of entries ***I***, ***II*** and ***III*** were incubated at 37 °C. After 5 h, the samples were diluted 1:10 with a 0.2% TFA solution in MeCN. Diluted aliquots were cooled to -20 °C for at least 0.5 h and then centrifuged at 13000 rpm at 4 °C for 15 min. The supernatant was transferred in glass vials, volatiles were removed under vacuum, and the concentrated DMSO solution was diluted with 8:2 H_2O/MeCN + 0.2% TFA mixture up to 0.25 mM concentration of initial substrate **5**. The diluted aliquots were subjected to HPLC-MS analysis (Data shown in Figure 3B and Figure S1-S2). The stability of **5** in the absence of enzymes was confirmed in an independent experiment, where a 2 mM solution of **5** in 25 mM HEPES Buffer (pH 7.5 + 0.5 mM Mg^{++}) was incubated at 37 °C and then analyzed by HPLC.

Cell Growth Inhibition Assays

Experimental Procedure

The human ovarian carcinoma cell lines IGROV-1 was cultured in RPMI-1640 medium (Lonza, Basel, Switzerland), supplemented with 10% Fetal Bovine Serum (FBS, Gibco Life Technologies, Carlsbad, California). Cells were thawed from frozen stocks and cultured for no more than 20 passages. CPT and all compounds were diluted in water. Cell sensitivity to compounds was measured by growth inhibition assays, evaluated in keeping with cell pharmacology guidelines.[5] Twenty-four hours after seeding, cells were exposed to serial dilutions of the tested compounds according to different schedules of treatments (72 h *in continuo* or 24 h following cell washout and incubation with fresh RPMI-1640 medium for 48 h). For cell growth inhibition assays in the presence of phosphatase, cells were in medium added with 100 nM concentration of alkaline phosphatase (Sigma-Aldrich, dissolved in 25 mM HEPES Buffer pH 7.5 + 0.5 mM Mg^{++}) while exposed to serial dilutions of prodrug **5**. Cells were counted with a cell counter 72 h after treatment start. IC_{50} is defined as the drug concentration producing 50% decrease of cell growth.

5 P. Perego, G. Hempel, S. Linder, T. D. Bradshaw, A. K. Larsen, G. J. Peters, R. M. Phillips, *Cancer Chemother. Pharmacol*. 2018, **81**, 427.

Supplementary Figures

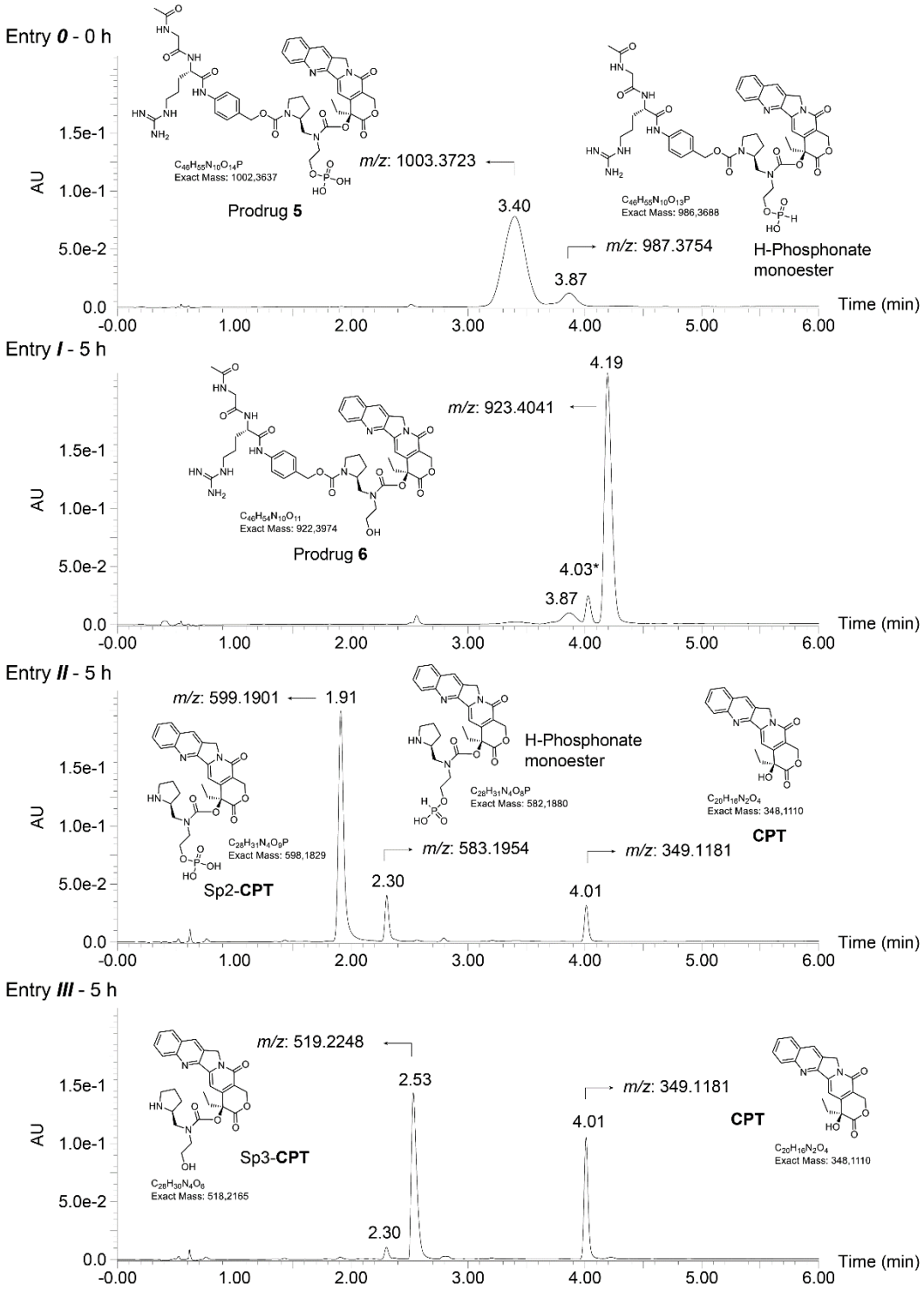
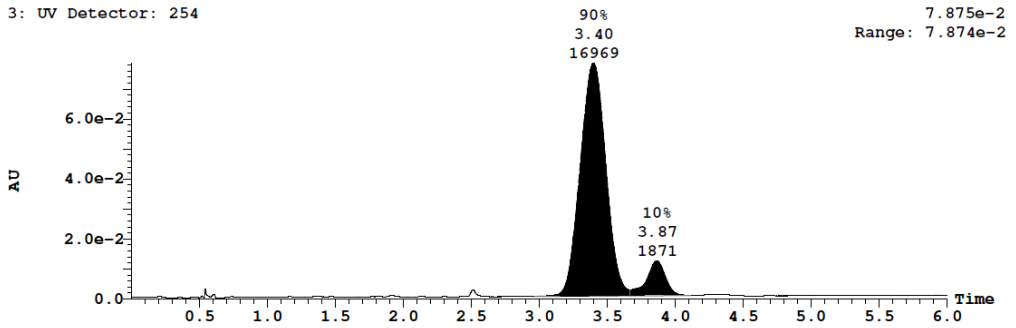


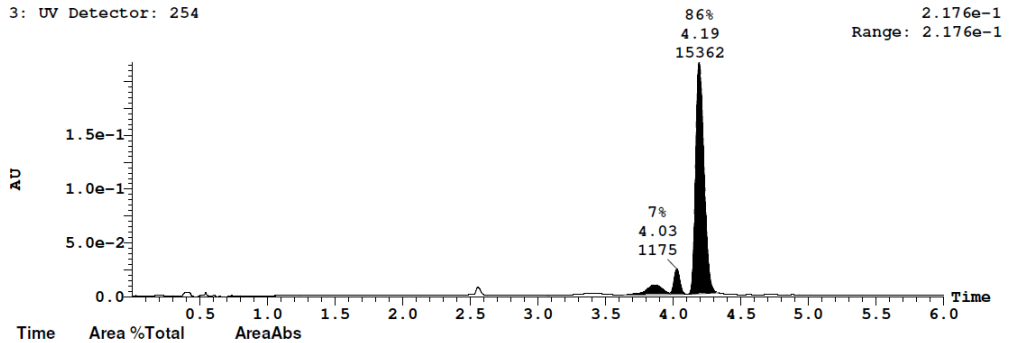
Figure S1. Assigned HPLC peaks relative to the analysis of prodrug **5** (2.5 mM in HEPES buffer at pH 7.5) at *t* = 0 (entry ***0***) and upon 5 h incubation at 37 °C with 50 mM alkaline phosphatase (entry ***I***), 50 µM trypsin (entry ***II***) or 50 µM alkaline phosphatase + 50 µM trypsin (entry ***III***). This analysis highlighted the presence of ca. 10% impurity in the batch of **5**, characterized by a molecular ion M(**5**)-16 Da (see UV peak at r.t. = 3.87 in Figure S1, entry ***0***). This impurity was interpreted as the H-phosphonate analogue of **5**, accounting for an incomplete oxidation of the *tert*-butyl phosphite intermediate during the synthesis. * Not assigned.

Entry ***0***



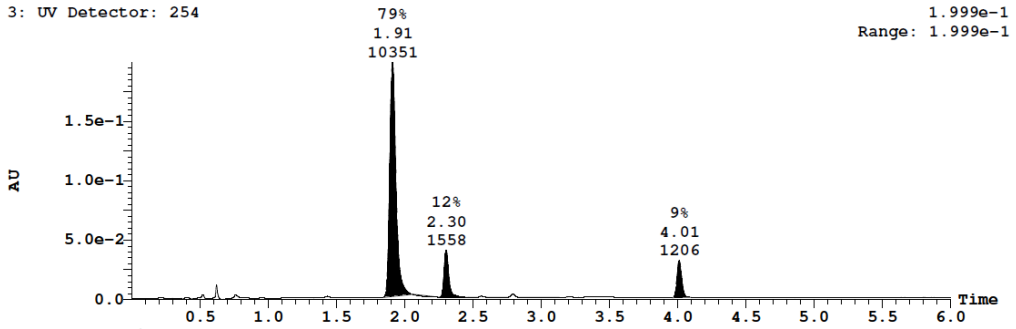
Time	Area %Total	AreaAbs
3.40	90.07	1.6969e+004
3.87	9.93	1.8712e+003

Entry ***I***



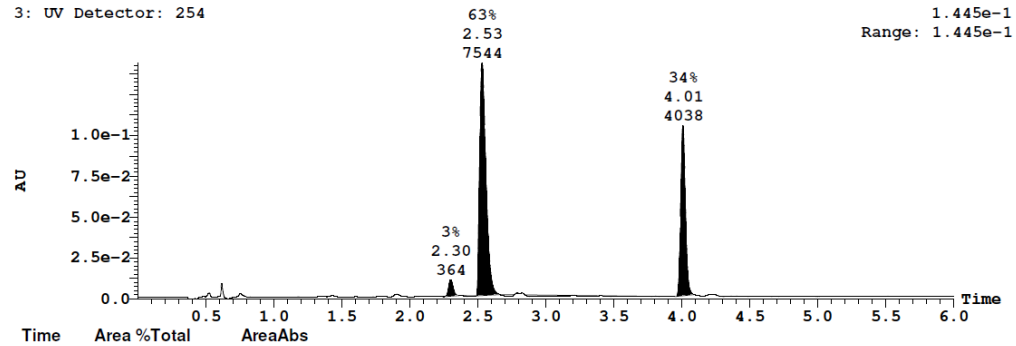
Time	Area %Total	AreaAbs
3.87	7.29	1.2997e+003
4.03	6.59	1.1748e+003
4.19	86.13	1.5362e+004

Entry ***II***



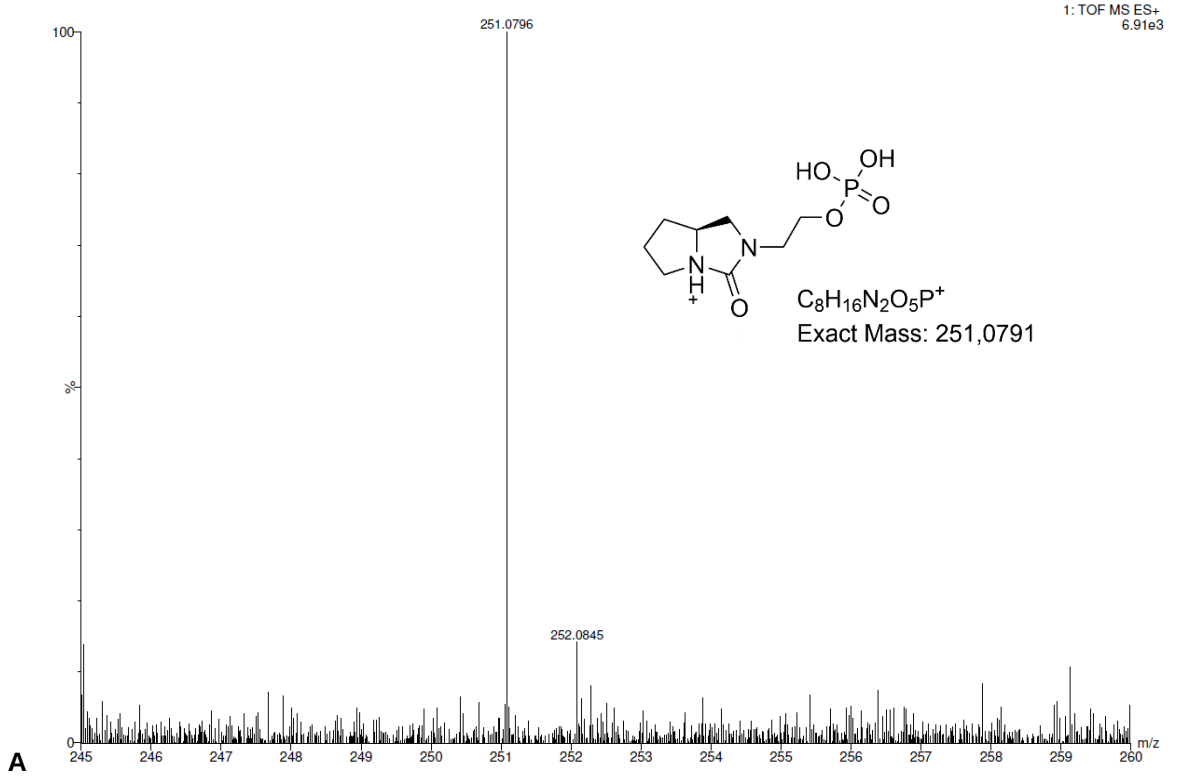
Time	Area %Total	AreaAbs
1.91	78.92	1.0351e+004
2.30	11.88	1.5578e+003
4.01	9.20	1.2062e+003

Entry ***III***



Time	Area %Total	AreaAbs
2.30	3.05	3.6376e+002
2.53	63.15	7.5436e+003
4.01	33.80	4.0379e+003

Figure S2. HPLC peaks integrals relative to the stability analysis of prodrug **5** (see Figure S1).



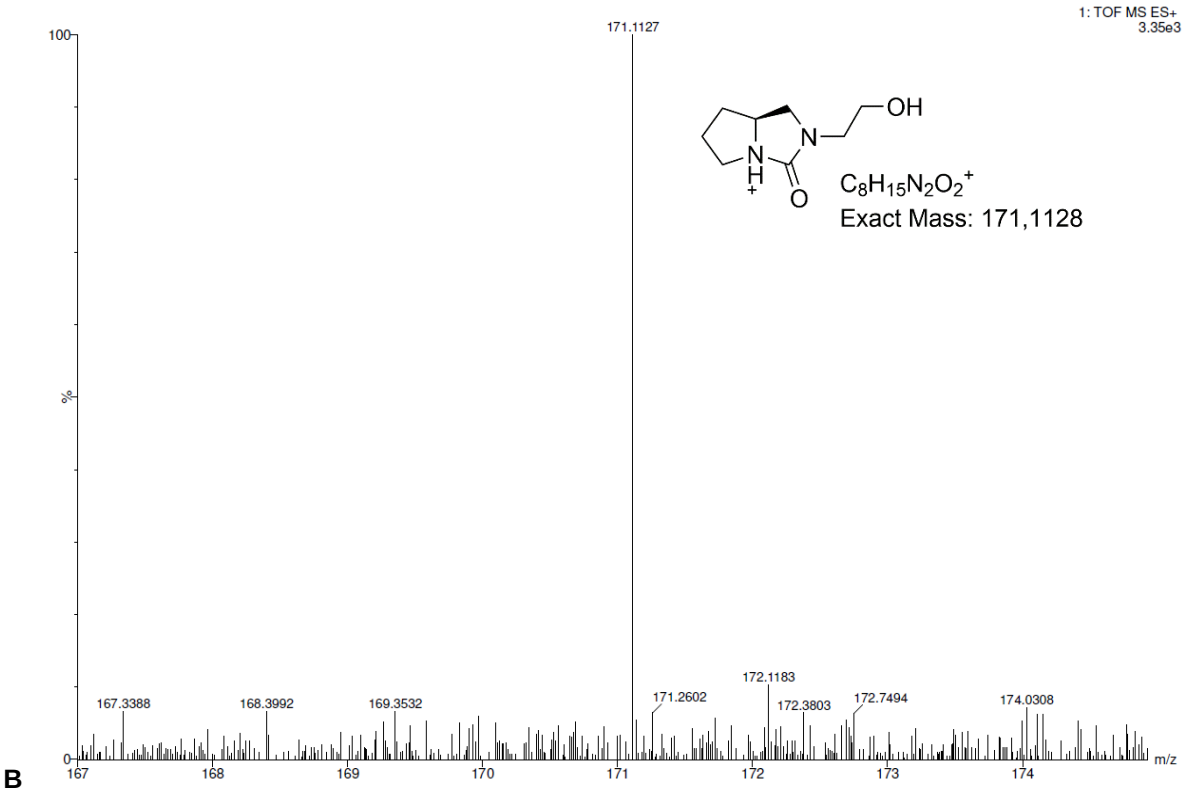
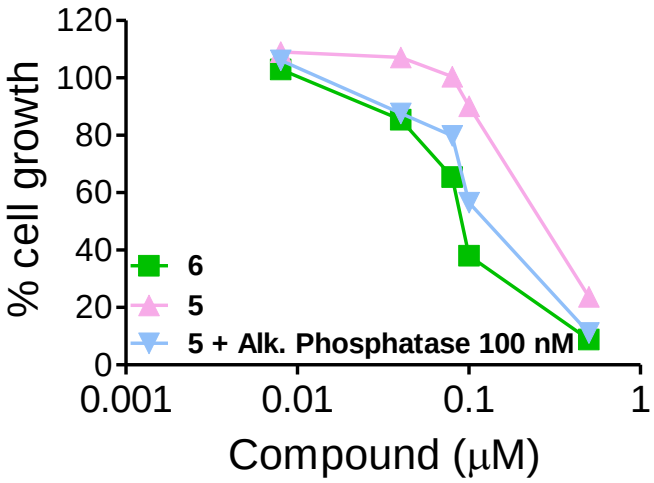


Figure S3. MS spectra of cyclic urea products derived from the cyclization of the **Sp2** (**A**) and **Sp3** (**B**) SI spacers, as detected from the HPLC-MS stability analysis of prodrug **5**. A and B spectra were detected during the analysis of entry ***II*** and ***III***, respectively (see Figure S1).



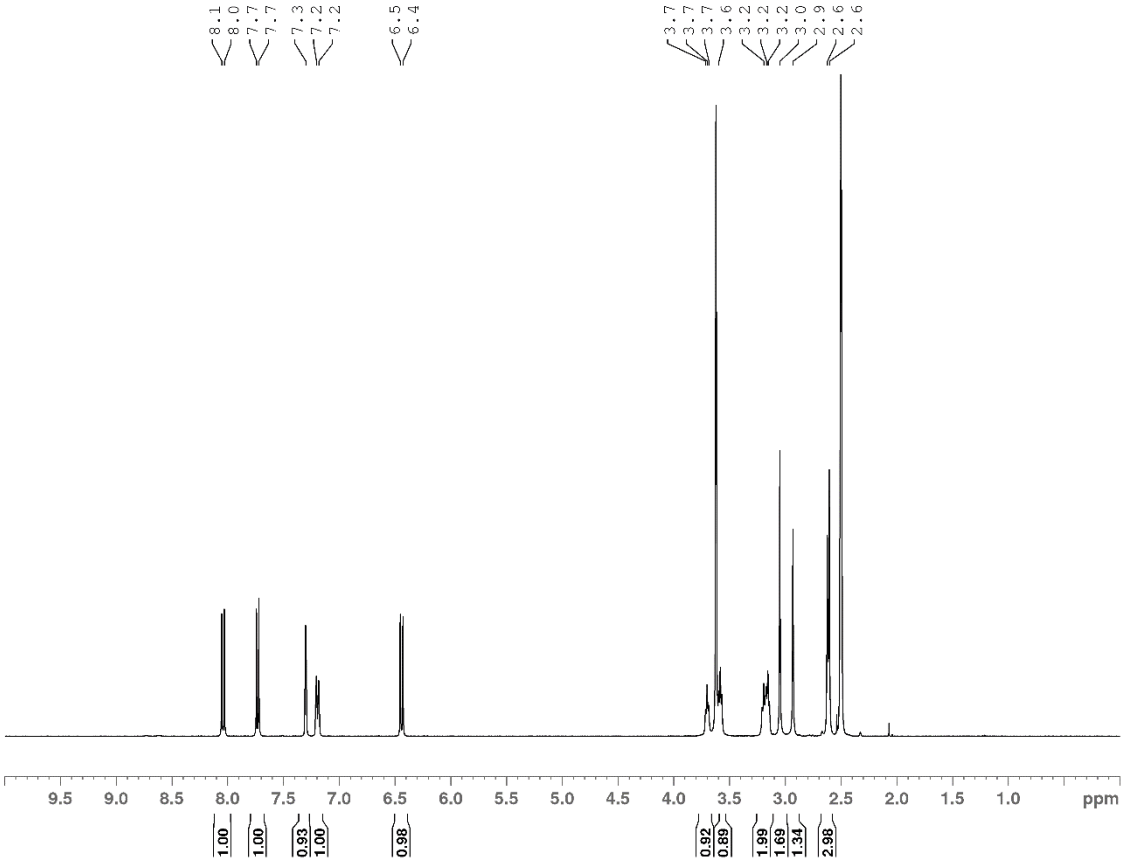
Compound	IC_{50} (nM)[a]
Prodrug **5**	264
Prodrug **6**	90
Prodrug **5** + 100 nM Akaline phosphatase	125

Figure S4. Cell growth-inhibition assays of IGROV-1 cells upon incubation with prodrug **5** (with or w/o phosphatase) and **6**. IC_{50} values are reported in the Table. Graph and Table report the mean of duplicate data from a single representative experiment. [a] Cells were incubated for 24 h with serial dilutions of prodrugs **5**-**6**, followed by cell washout and incubation for additional 48 h in fresh medium.

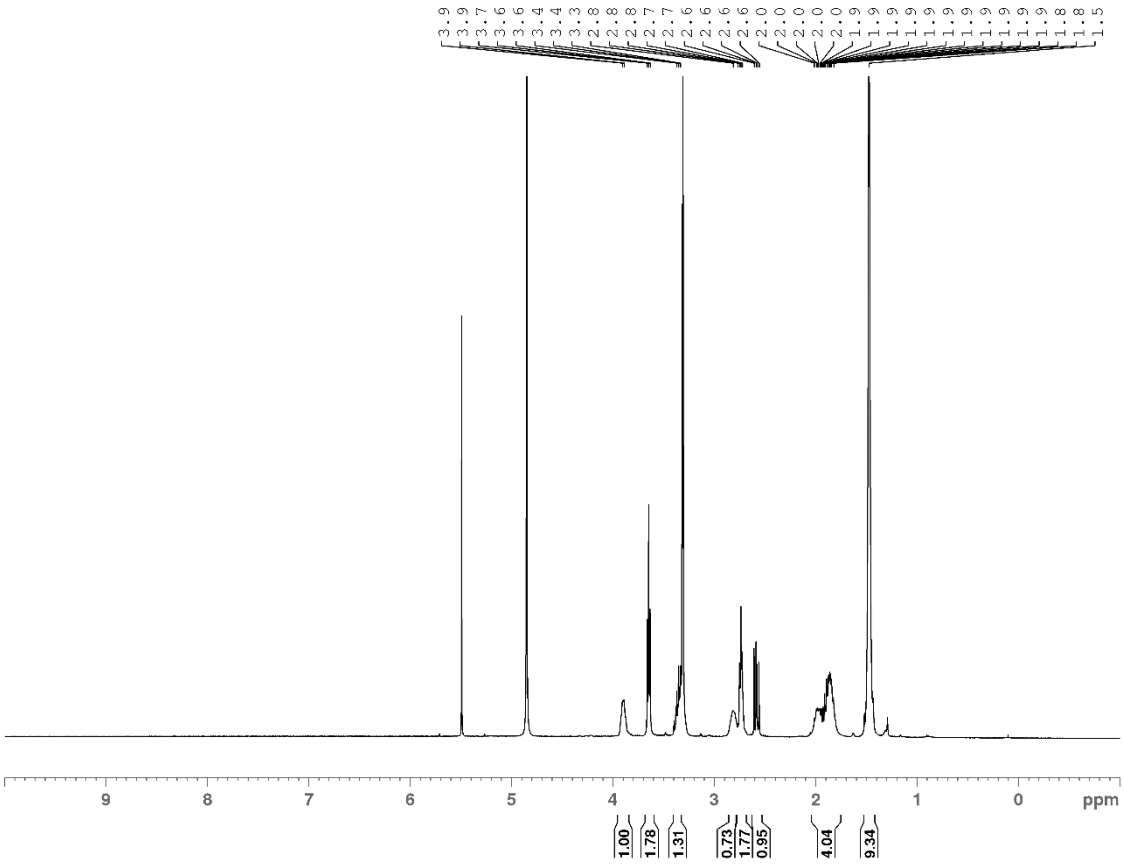
Appendix

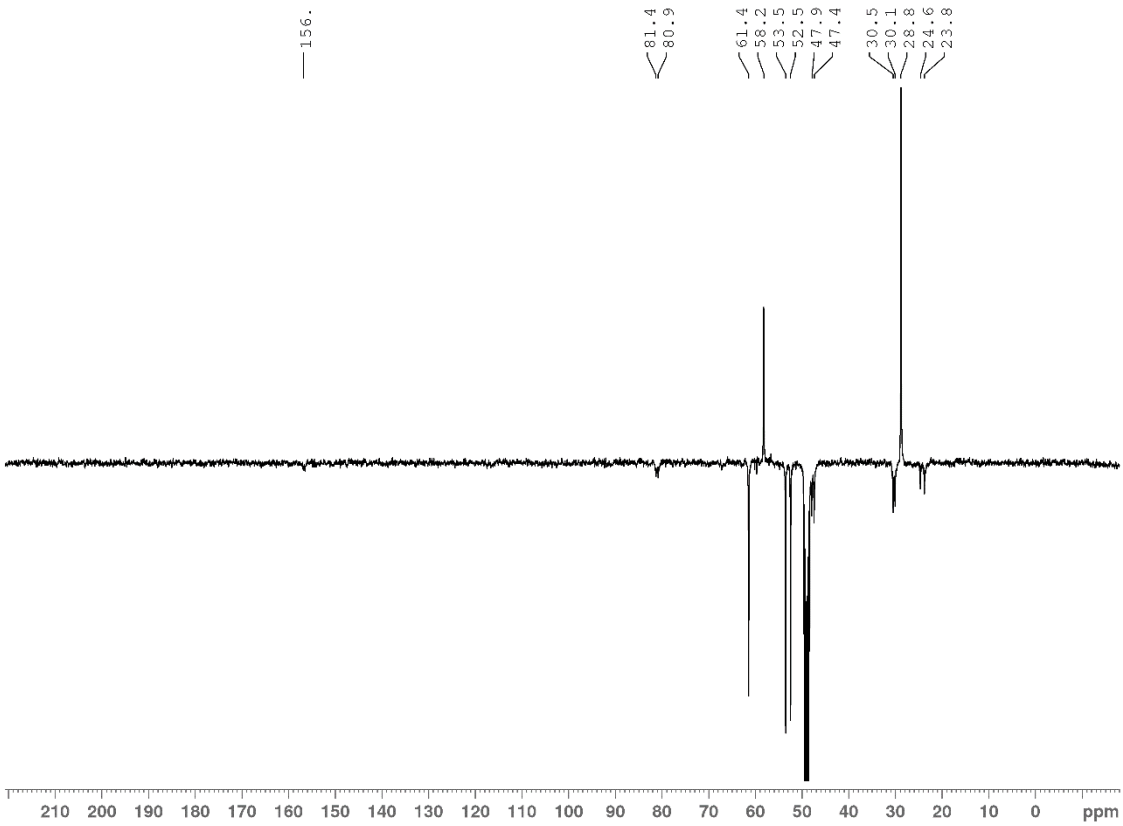
NMR Spectra

Sp1-CMR

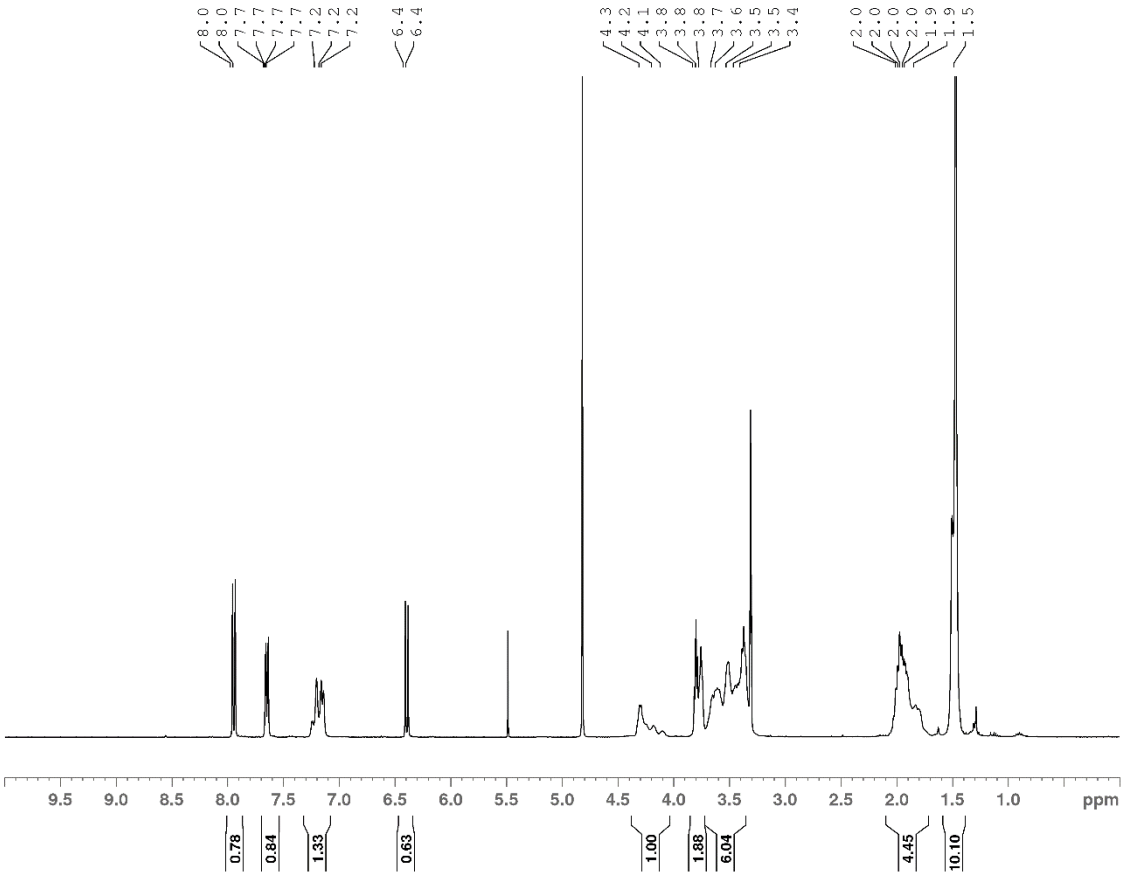


Boc-Pro-OH (**10**)

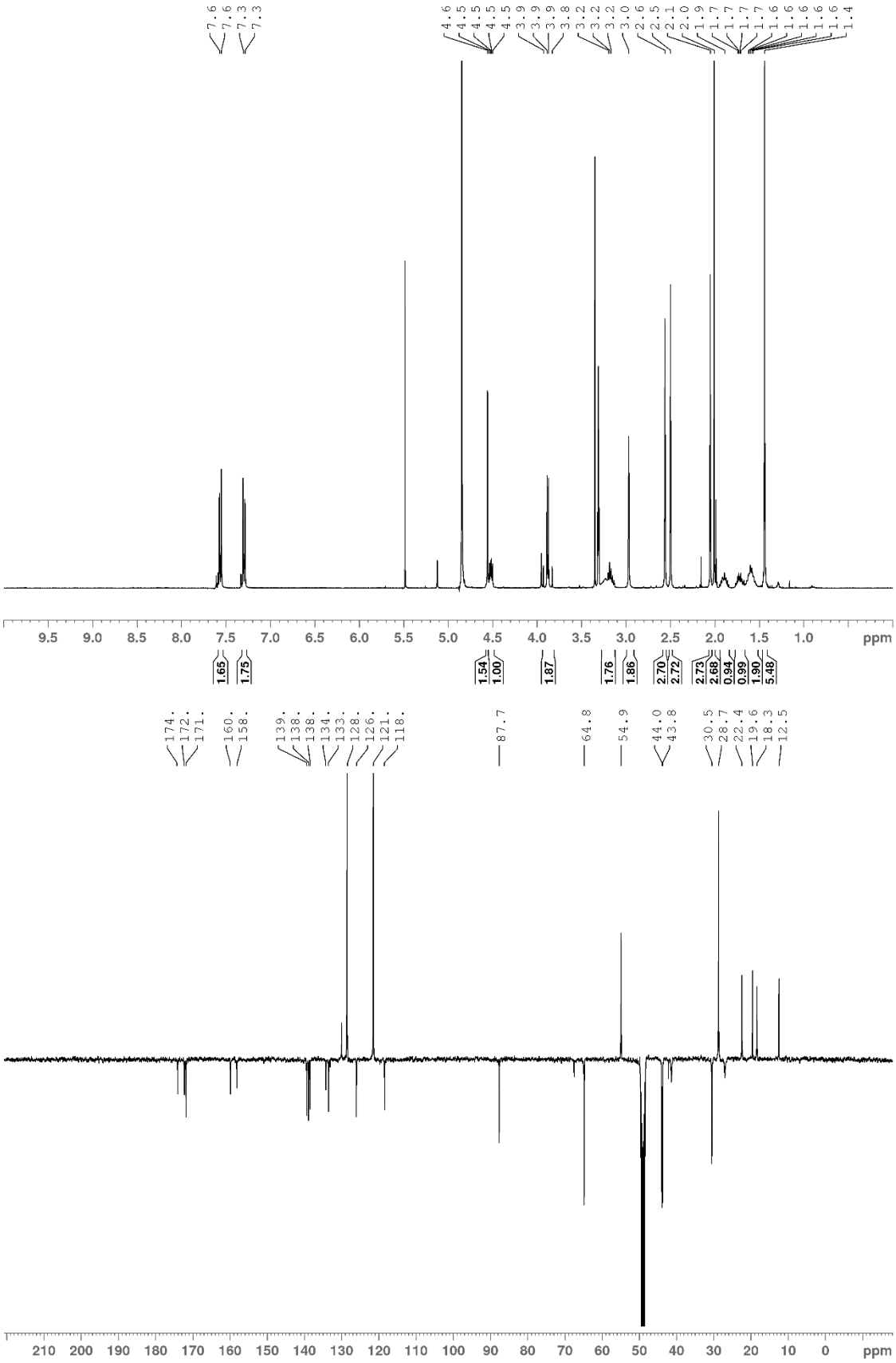




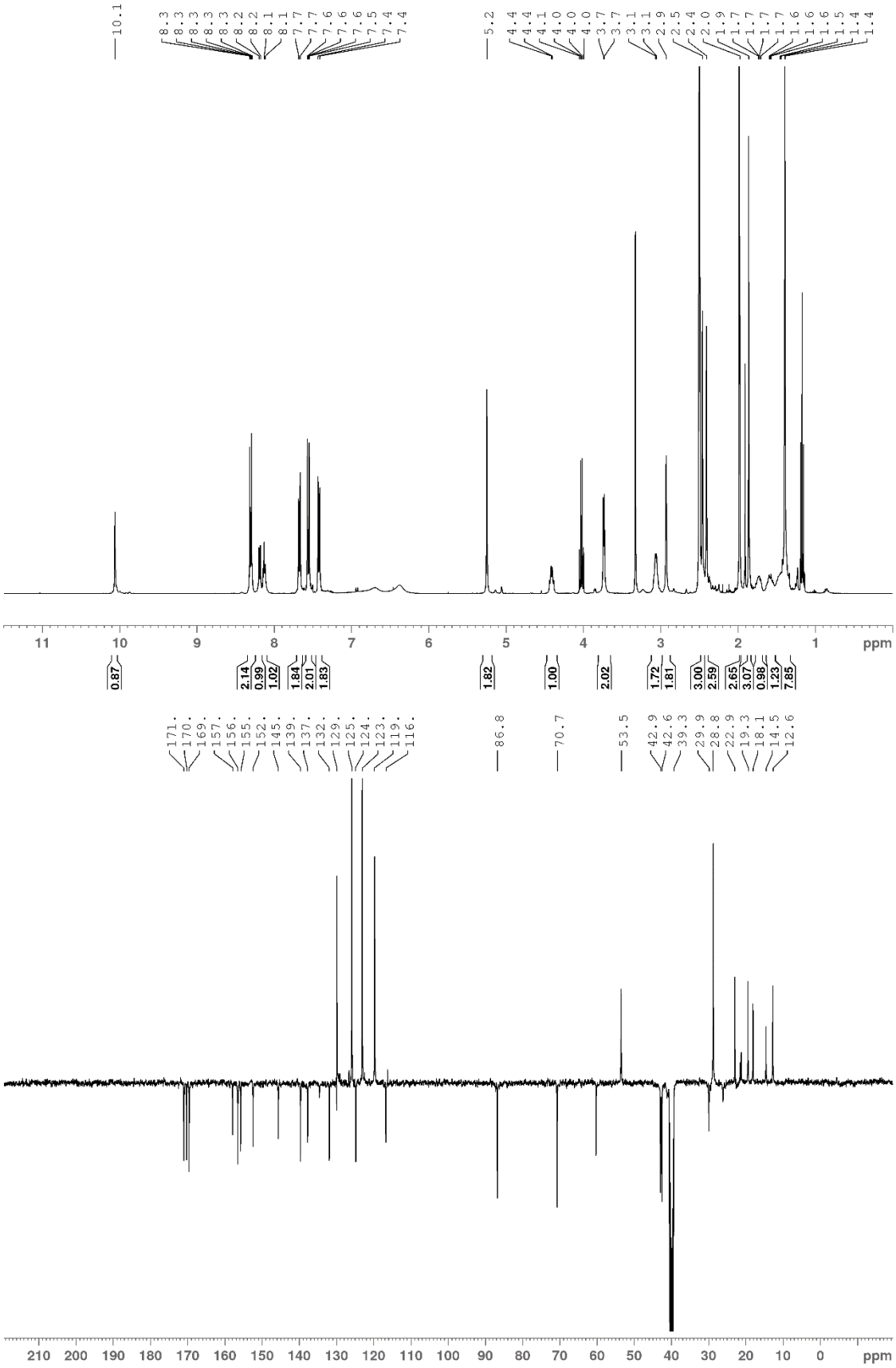
Boc-Sp3-CMR (**11**)


8.0
8.0
7.7
7.7
7.7
7.7
7.2
7.2
7.2
6.4
6.4
4.3
4.2
4.1
3.8
3.8
3.7
3.6
3.5
3.5
3.4
2.0
2.0
2.0
2.0
1.9
1.9
1.5
9.5
9.0
8.5
8.0
7.5
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
ppm
0.78
0.84
1.33
0.63
1.00
1.88
6.04
4.45
10.10

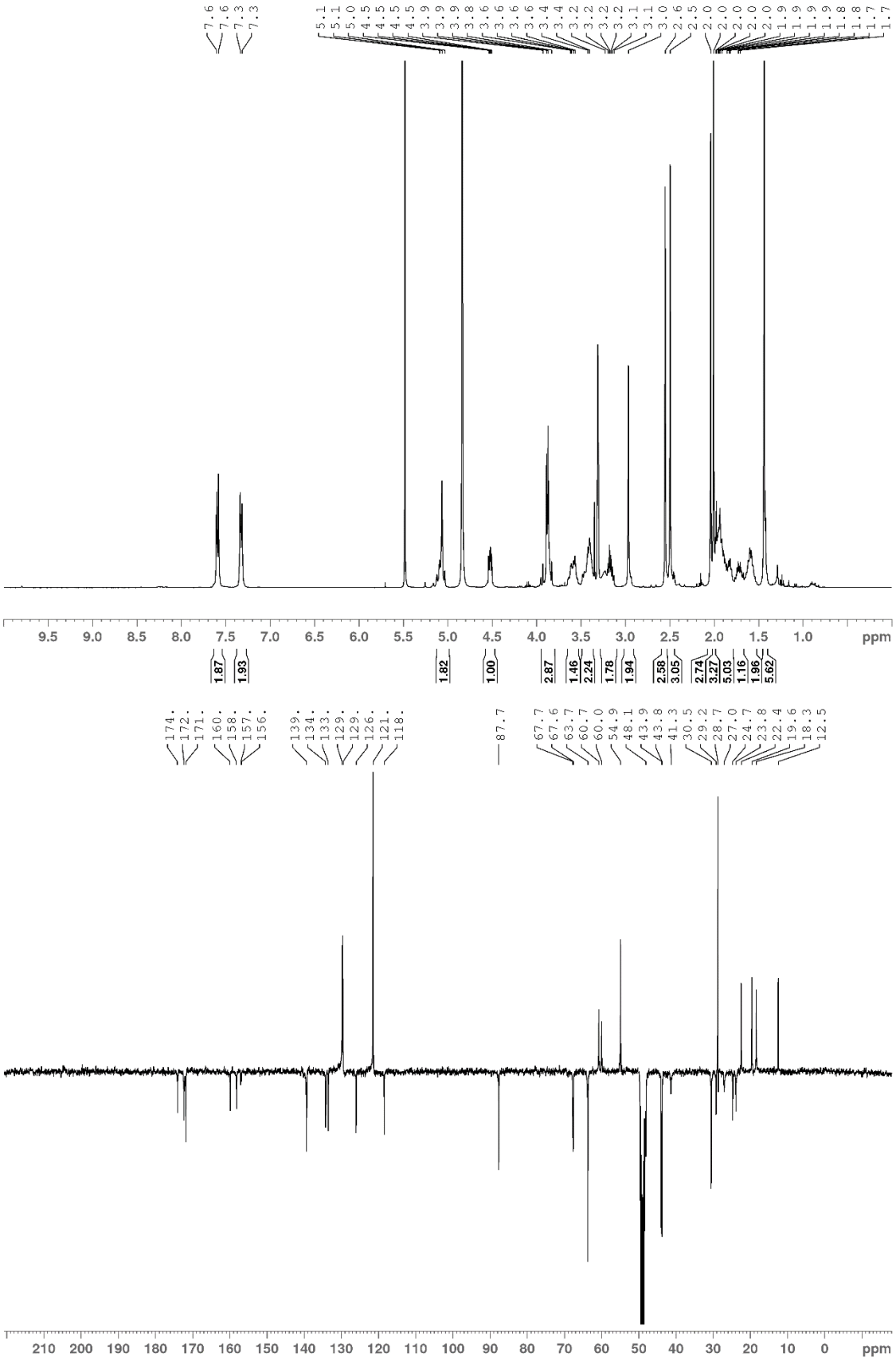
Ac-Gly-Arg(Pbf)-PABOH (**14**)



Ac-Gly-Arg(Pbf)-PAB-PNP (**15**)

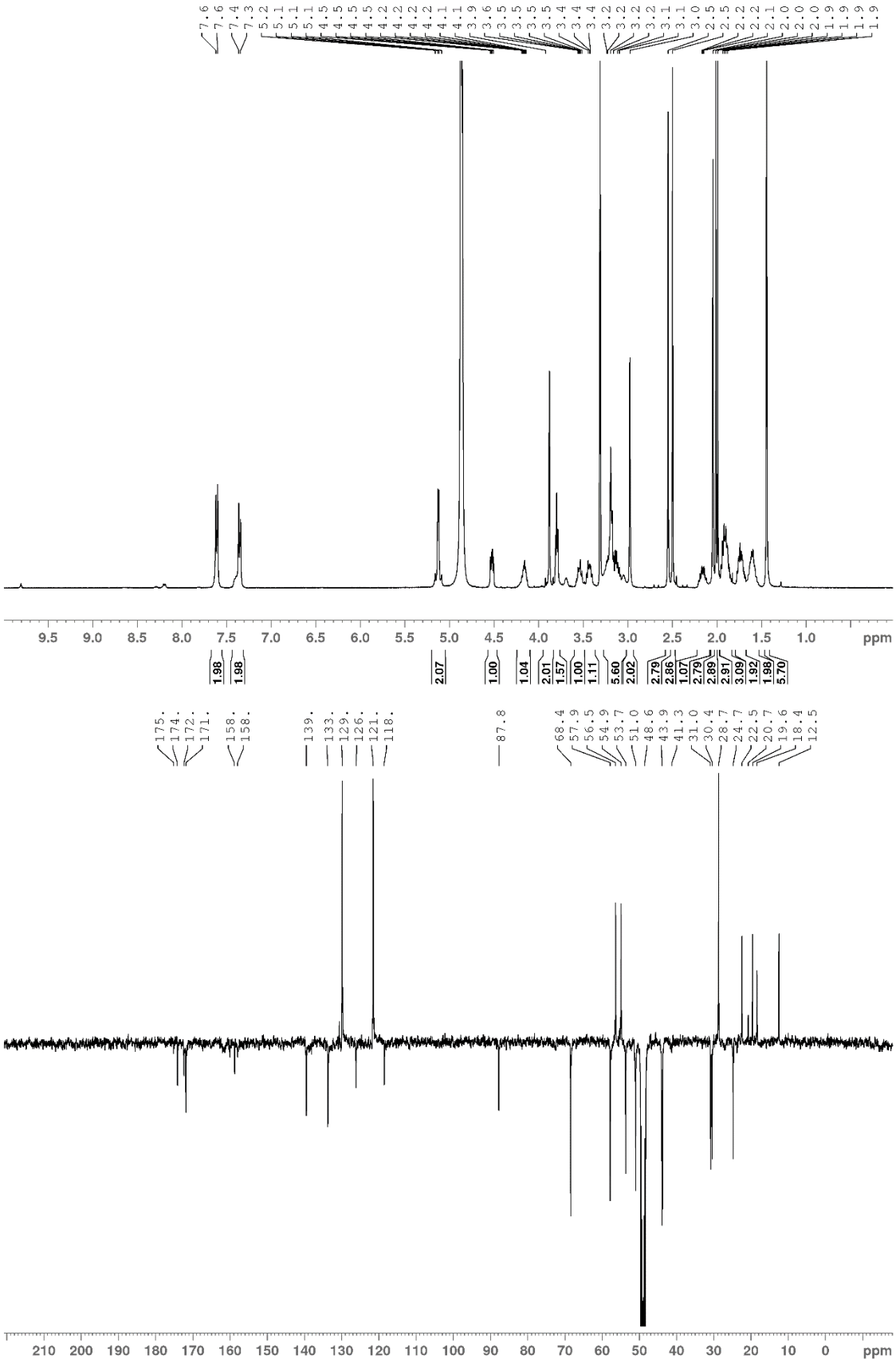


Arg(Pbf)-PABC-Prolinol (**16**)

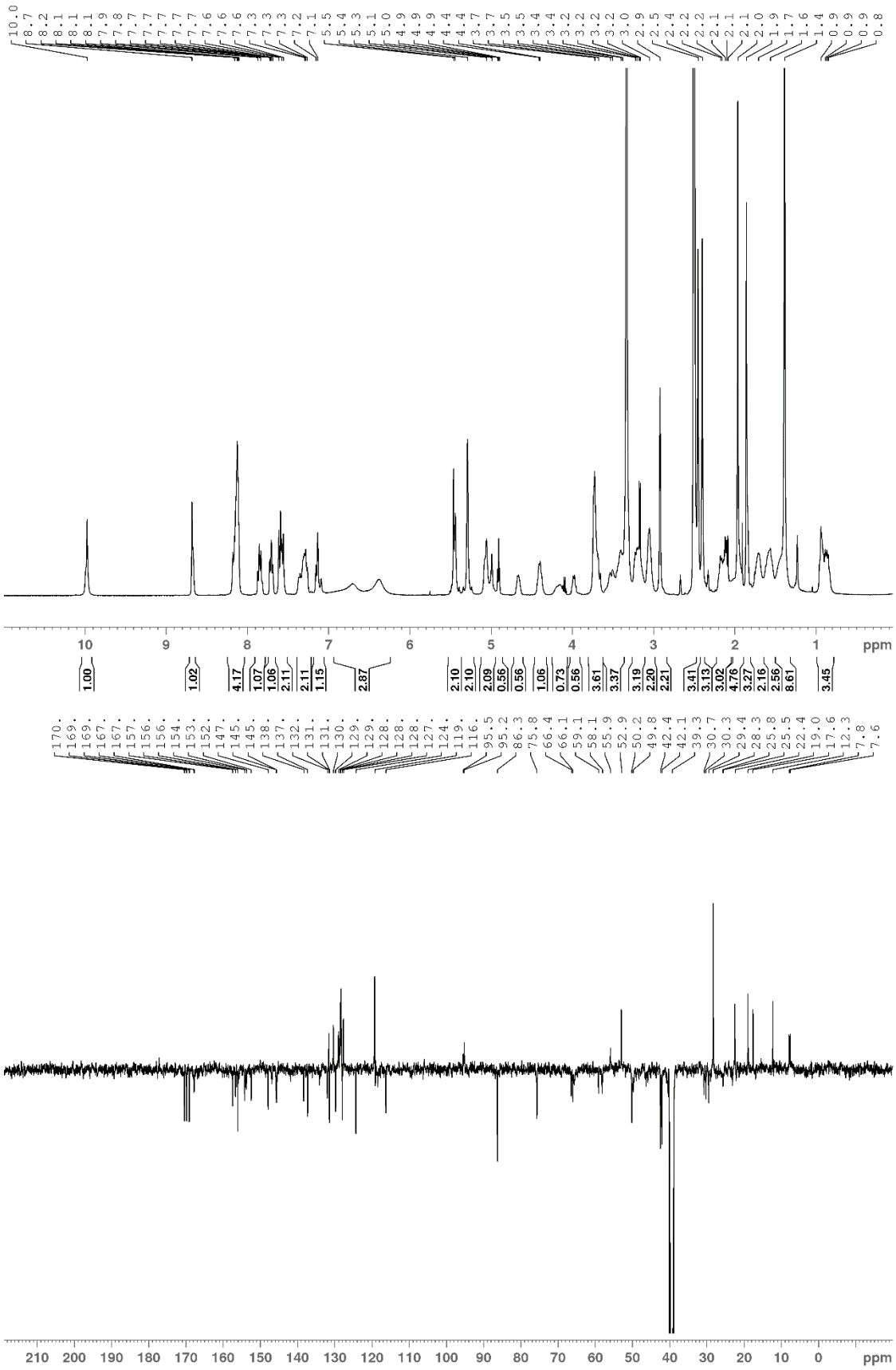


Ac-Gly-

Ac-Gly-Arg(Pbf)-PABC-Pro-Ethanolamine Acetate (**18**)

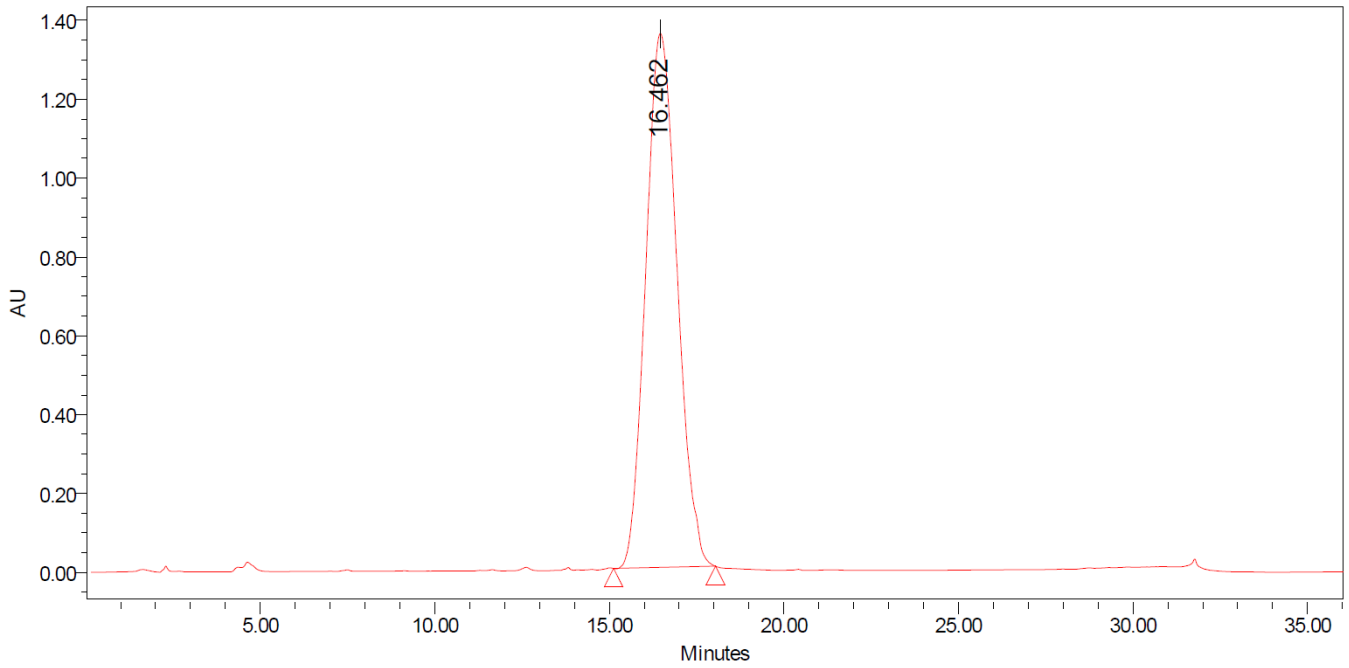


Ac-Gly-Arg(Pbf)-PABC-Pro(OH)-CPT (**19**)



HPLC traces of purified prodrugs 5 and 6

Ac-Gly-Arg-PABC-Pro(OPO$_3$H$_2$)-CPT (**5**, 254 nm)



Ac-Gly-Arg-PABC-Pro(OH)-CPT (**6**, 254 nm)