Supporting Information

Real-Time Monitoring of Drug Release

Roy Weinstain[a✈], Ehud Segal[b✈], Ronit Satchi-Fainaro[b], and Doron Shabat[a]*

[a]School of Chemistry, Raymond and Beverly Sackler Faculty of Exact Sciences and

[b]Department of Physiology and Pharmacology, Sackler Faculty of Medical Sciences,

Tel Aviv University, Tel Aviv 69978 Israel.

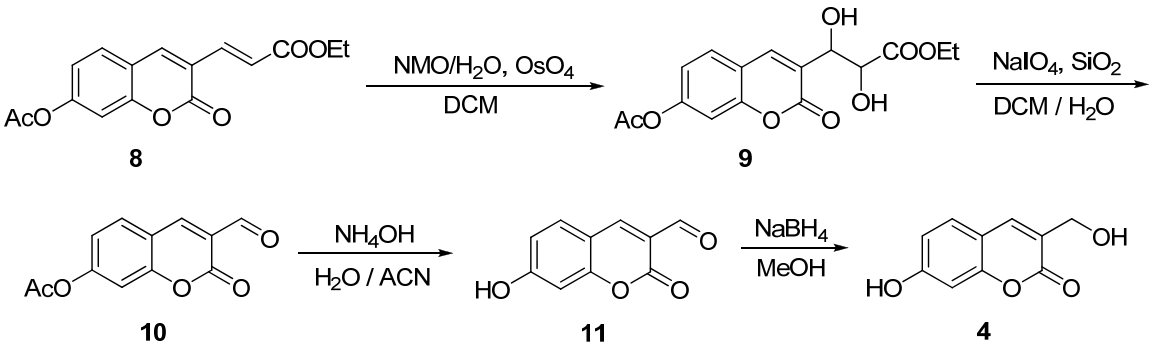
✈These authors contributed equally.

Table of Contents

General methods. All reactions requiring anhydrous conditions were performed under an Ar or N_2 atmosphere. All reactions were carried out at room temperature unless stated otherwise. Chemical and solvents were either A.R. grade or purified by standard techniques. Thin layer chromatography (TLC): silica gel plates Merck 60 F_{254}: compounds were visualized by irradiation with UV light. Flash chromatography (FC): silica gel Merck 60 (particle size 0.040-0.063 mm), eluent given in parentheses. ^{1}H-NMR spectra were measured using Bruker Avance operated at 200 MHz or 400 MHz as mentioned. ^{13}C NMR spectra were measured using Bruker Avance operated at 50 MHz or 100 MHz as mentioned. The chemical shifts are expressed in δ relative to TMS (δ = 0 ppm) and the coupling constants *J* in Hz. The spectra were recorded in $CDCl_3$ as solvent at room temperature unless stated otherwise. All general reagents, including salts and solvents, were purchased from Sigma-Aldrich.

Abbreviations. RP-HPLC – reverse phase high performance liquid chromatography, DCM- Dichloromethane, DMF- Dimethylformamide, EtOAc- Ethyl acetate, Hex- n-Hexanes, PBS- Phosphate buffer saline, THF- Tetrahydrofuran, ACN- Acetonitrile, MeOH- Methanol, NaOH- Sodium hydroxide, AcOH- Acetic acid, DMSO- Dimethylsulfoxide, DBTL- Dibutyltin dilaurate, TFA- Trifluoroacetic acid, PNA- *p*-Nitroaniline, TBS-Cl – *tert*-butyldimethylsilyl chloride, NH_4OH – Ammonium hydroxide, NH_4Cl – Ammonium chloride, DIPEA – N,N-diisopropylethylamine, FITC - Fluorescein isothiocyanate

1. Synthetic schemes and experimental procedures



Compound 8. Compound **8** was synthesized according to a published procedure.[1]

Compound 9. Compound **8** (930 mg, 3.08 mmol, 1 eq) was dissolved in DCM (15 mL). a 1:1 w/w solution of N-oxide morpholine (721 mg, 6.16 mmol, 2 eq) in water (721 µL) was added, followed by 2.5% OsO_4 solution in *tert*-butanol (3.0 mL 0.31 mmol, 0.1 eq). The reaction was stirred at room temperature and followed to completion (10 hours) by TLC (EtOAc:Hex 1:1). The reaction mixture was diluted with EtOAc, washed twice with brine, dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 1:2 to 7:3) to give compound **9** (872 mg, 84%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d^6): δ = 8.03 (1H, s), 7.83 (1H, d, *J* = 8.4 Hz), 7.30 (1H, s), 7.15 (1H, d, *J* = 8.4 Hz), 5.71 (1H, d, *J* = 6.8 Hz), 5.17 (1H, d, *J* = 7.2 Hz), 4.96 (1H, d, *J* = 6.8 Hz), 4.39 (1H, d, *J* = 7.2Hz), 4.16 (2H, q, *J* = 7.2 Hz), 2.49 (3H, s), 1.22 (3H, t, *J* = 7.2 Hz). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 176.3, 173.3, 164.0, 157.5, 156.9, 143.8, 133.6, 132.5, 123.3, 121.4, 114.2, 75.8, 74.1, 64.8, 25.3, 18.8. MS (CI+): calculated for [M+H$^+$] $C_{16}H_{16}O_8$ 337.1; found 337.1.

[1] N. C. Lim, J. V. Schuster, M. C. Porto, M. A. Tanudra, L. Yao, H. C. Freake, C. Bruckner, *Inorg. Chem.* **2005**, 44, 6, 2018.

Compound 10. Silica gel (5 gr) was suspended in DCM (10 mL) and compound **9** (872 mg, 2.59 mmol, 1 eq) was added. A solution of sodium periodate (737 mg, 3.44 mmol, 1.33 eq) in water (10 mL) was slowly added under vigorous stirring (important!). The reaction was followed to completion (10 minutes) by TLC (EtOAc:Hex 1:1). Solids were filtered by Celite layer on sintered glass. The solution was then diluted with EtOAc and washed once with brine. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure to give compound **10** (570 mg, 94%).

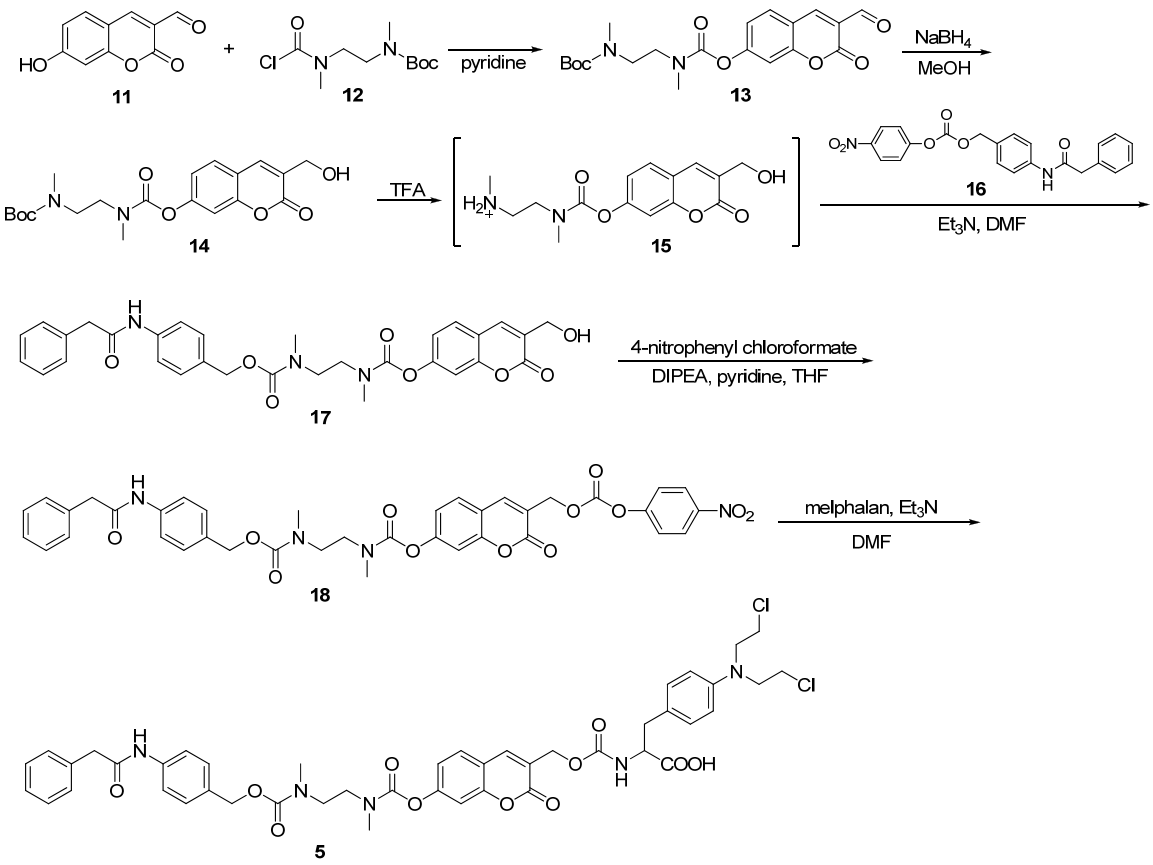
^{1}H NMR (200 MHz, DMSO-d^6): δ = 10.02 (1H, s), 8.67 (1H, s), 8.03 (1H, d, *J* = 8.4 Hz), 7.37 (1H, s), 7.25 (1H, d, *J* = 8.4 Hz), 2.32 (3H, s). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 192.5, 173.1, 163.4, 160.1, 159.8, 150.8, 136.9, 125.5, 124.0, 120.6, 114.8, 25.4. MS (FAB+): calculated for [M+H$^+$] $C_{12}H_8O_5$ 233.0; found 233.0.

Compound 11. Compound **10** (620 mg, 2.66 mmol) was dissolved in 32% NH_4OH solution in water and CAN was slowly added until the reaction mixture becomes homogenous. The reaction was followed to completion (20 minutes) by TLC (EtOAc:Hex 1:1). EtOAc was added and the solution was washed twicw with HCl [1M]. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure to give compound **11** (456 mg, 90%).

^{1}H NMR (200 MHz, DMSO-d^6): δ = 9.96 (1H, s), 8.58 (1H, s), 7.81 (1H, d, *J* = 8.4 Hz), 6.87 (1H, d, *J* = 8.4 Hz), 6.77 (1H, s). ^{13}C NMR (50 MHz, DMSO-d^6): δ = 192.3, 169.3, 164.2, 151.8, 137.9, 135.6, 121.6, 119.0, 115.4, 106.7. MS (CI+): calculated for [M+H$^+$] $C_{10}H_6O_4$ 191.3; found 191.3.

Compound 4. Compound **11** (100 mg, 0.53 mmol, 1 eq) was dissolved in MeOH (4 mL) and sodium borohydride (30 mg, 0.79 mmol, 1.5 eq) was added. The reaction was monitored to completion (10 minutes) by TLC (EtOAc:Hex 1:1). The reaction mixture was diluted with EtOAc, washed once with saturated NH_4Cl solution, dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 1:1) to give compound **4** (98 mg, 96%) as a brown solid.

^{1}H NMR (200 MHz, DMSO-d^6): δ = 10.45 (1H, s), 7.85 (1H, s), 7.56 (1H, d, *J* = 8.4 Hz), 6.76 (1H, d, *J* = 8.4 Hz), 6.71 (1H, s), 5.33 (1H, t, *J* = 5.4 Hz), 4.29 (2H, d, *J* = 5.4 Hz). ^{13}C NMR (50 MHz, DMSO-d^6): δ = 164.0, 159.4, 157.8, 141.2, 132.7, 128.3, 123.2, 121.0, 113.8, 62.3. MS (CI+): calculated for [M+H$^+$] $C_{10}H_8O_4$ 193.0; found 193.0.



Compound 12. Compound **12** was prepared according to a published procedure.[2]

Compound 13. Compound **11** (600 mg, 3.15 mmol, 1 eq) was dissolved in pyridine (10 mL) and compound **12** (965 mg, 3.86 mmol, 1.2 eq) was added. The solution was steered at room temperature and the reaction was monitored to completion (12 hours) by TLC chromatography (EtOAc:Hex 1:1). The organic phase was washed twice with HCl [1M] solution, dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 6:4) to give compound **13** (610 mg, 48%) as a yellowish solid.

^{1}H NMR (400 MHz, DMSO-d^6): δ = 10.02 (1H, s), 8.68 (1H, s), 8.01 (1H, d, J = 8.4 Hz), 7.28 (1H, s), 7.20 (1H, d, J = 8.4 Hz), 3.33-3.56 (4H, m), 2.81-3.07 (6H, m),

[2] M. Shamis, D. Shabat, *Chem. Eur. J.*, **2007**, 13, 4253.

1.29 (9H, s). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 192.5, 163.5, 160.1, 157.3, 150.9, 136.7, 125.2, 123.9, 123.5, 119.9, 114.5, 114.0, 83.2, 51.1, 50.0, 39.4, 38.8, 32.5. MS (FAB+): calculated for [M+Na$^+$] $C_{20}H_{24}N_2O_7$ 427.2; found 427.2.

Compound 14. Compound **13** (593 mg, 1.46 mmol, 1 eq) was dissolved in MeOH (7 mL), cooled to 0^{o}C, and sodium borohydride (667 mg, 1.76 mmol, 1.2 eq) was added slowly. The reaction was monitored to completion (20 minutes) by TLC (EtOAc:Hex 3:1). The reaction mixture was diluted with EtOAc, washed once with saturated NH_4Cl solution, dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 3:1) to give compound **14** (582 mg, 97%) as a white solid.
^{1}H NMR (400 MHz, DMSO-d^6): δ = 7.96 (1H, s), 7.79 (1H, d, *J* = 8.4 Hz), 7.18 (1H, s), 7.09 (1H, d, *J* = 8.4 Hz), 5.31 (1H, t, *J* = 8.4 Hz), 4.30 (2H, d, *J* = 8.4 Hz), 3.34-3.58 (4H, m), 2.80-3.05 (6H, m), 1.29 (9H, s). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 164.0, 160.0, 159.4, 157.8, 157.4, 141.2, 132.7, 128.3, 123.2, 121.0, 113.8, 83.1, 62.6, 50.9, 50.1, 39.2, 38.3, 32.3. MS (FAB+): calculated for [M+Na$^+$] $C_{20}H_{26}N_2O_7$ 429.2; found 429.2.

Compound 16. Compound **16** was prepared according to a published procedure.[3]

Compound 17. Compound **14** (160 mg, 0.39 mmol, 1 eq) was dissolved in TFA (0.5 mL) and stirred for one minute. TFA was then immediately removed under reduced pressure to yield ammonium salt **15** as a yellow solid. Compound **15** was immediately used without further purifications. Compound **15** was re-dissolved in DMF (1 mL)

[3] N. Pessah, M. Reznik, M. Shamis, F. Yantiri, H. Xin, K. Bowdish, N. Shomron, G. Ast, D. Shabat, *Bioorg. Med. Chem.*, **2004**, 12, 1859.

and compound **16** (176 mg, 0.43 mmol, 1.1 eq), followed by Et_3N (55 μL, 0.39 mmol, 1 eq) were added. The reaction was monitored to completion (5 minutes) by TLC (EtOAc:Hex 3:1). The reaction mixture was diluted with EtOAc then washed twice with saturated NH_4Cl solution and once with brine. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 4:1) to give compound **17** (125 mg, 55%) as a white solid.

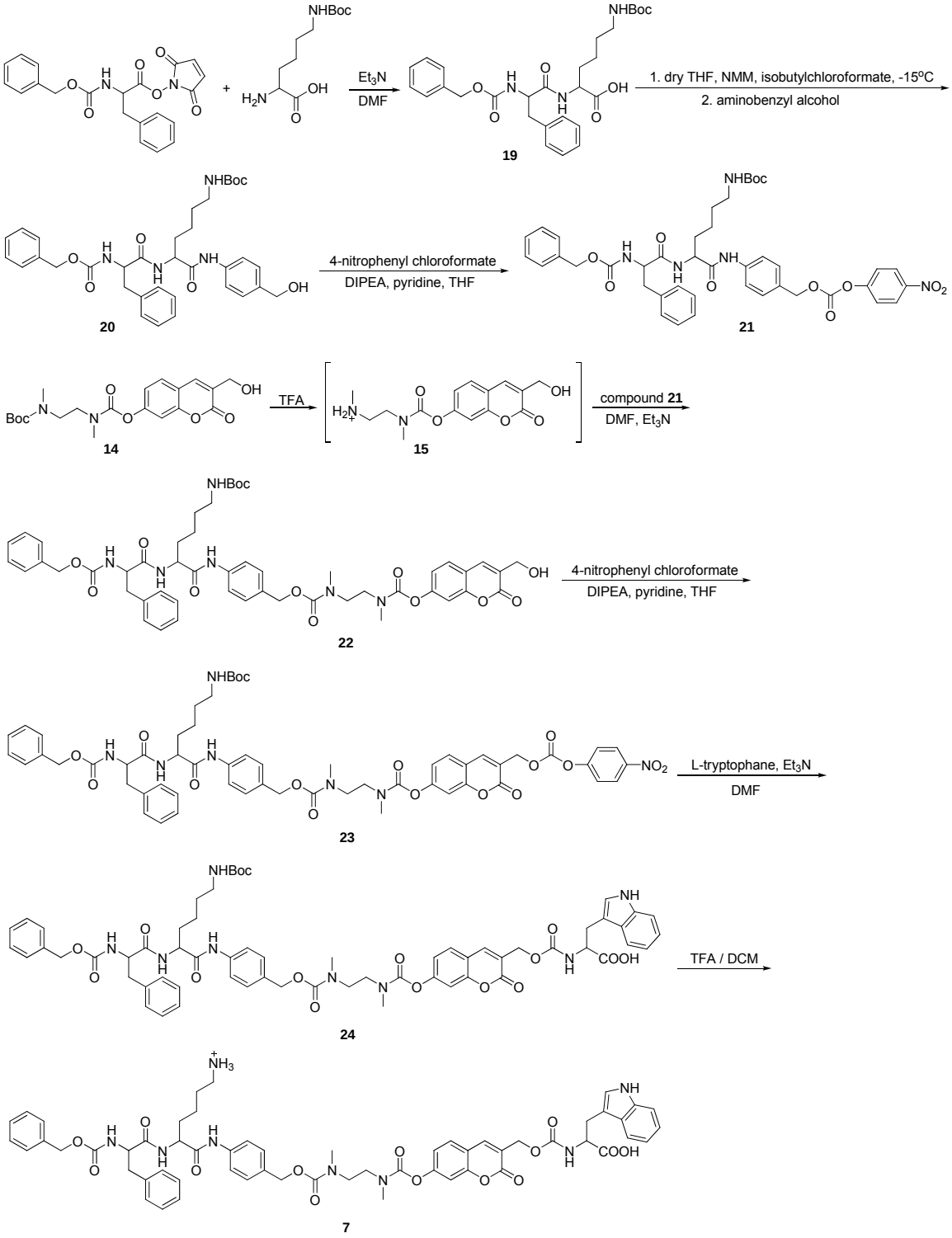
^{1}H NMR (200 MHz, DMSO-d^6): δ = 10.15 (1H, s), 7.94 (1H, s), 7.54 (2H, d, *J* = 8.4 Hz), 7.32 (1H, d, *J* = 8.4 Hz), 7.23-7.26 (7H, m), 7.15 (1H, s), 7.00 (1H, d, *J* = 8.4 Hz), 5.75 (1H, t, *J* = 4.8 Hz), 5.00 (2H, s), 4.36 (2H, d, *J* = 4.8 Hz), 3.61 (2H, s), 3.45-3.56 (4H, m), 2.84-3.03 (6H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 173.5, 164.0, 157.3, 143.4, 141.2, 140.4, 135.9, 135.7, 133.6, 133.1, 132.9, 132.8, 132.6, 131.0, 123.3, 122.9, 120.9, 114.0, 113.8, 70.5, 62.3, 51.2, 50.9, 47.7, 39.2, 35.8. MS (FAB+): calculated for [M+Na$^+$] $C_{31}H_{31}N_3O_8$ 596.2; found 596.2.

Compound 18. Compound **17** (118 mg, 0.2 mmol, 1 eq) was dissolved in dry THF (3 mL) under argon atmosphere and cooled to 0^{o}C. DIPEA (143 μL, 0.82 mmol, 4 eq) and 4-nitrophenyl chloroformate (124 mg, 0.62 mmol, 3 eq) were added, followed by pyridine (5 μL, 0.06 mmol, 0.3 eq). The reaction was monitored to completion (1 hour) by TLC (EtOAc:Hex 1:1). The reaction mixture was diluted with EtOAc then washed twice with saturated NH_4Cl solution and once with brine. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 1:1) to give compound **18** (110 mg, 75%) as a white solid.

^{1}H NMR (200 MHz, DMSO-d^{6}): δ = 10.15 (1H, s), 8.31 (2H, d, *J* = 8.8 Hz), 7.98 (1H, s), 7.64 (2H, d, *J* = 8.8 Hz), 7.53 (2H, d, *J* = 8.4 Hz), 7.32 (1H, d, *J* = 8.4 Hz), 7.21-7.26 (7H, m), 7.15 (1H, s), 7.00 (1H, d, *J* = 8.4 Hz), 5.75 (1H, t, *J* = 4.8 Hz), 5.00 (2H, s), 4.83 (2H, s), 3.61 (2H, s), 3.45-3.56 (4H, m), 2.84-3.03 (6H, m). ^{13}C NMR (100 MHz, DMSO-d^{6}): δ = 173.5, 164.0, 157.3, 143.4, 141.2, 140.4, 135.9, 135.7, 133.6, 133.1, 132.9, 132.8, 132.6, 131.0, 123.3, 122.9, 120.9, 114.0, 113.8, 70.5, 62.3, 51.2, 50.9, 47.7, 39.2, 35.8. MS (FAB+): calculated for [M+Na^{+}] $C_{38}H_{34}N_4O_{12}$ 761.2; found 761.2.

Compound 5. Compound **18** (20 mg, 0.027 mmol, 1 eq) was dissolved in dry DMF (1 mL) under argon atmosphere. Melphalane (8.5 mg, 0.027 mmol, 1 eq) and Et_3N (9.5 μL, 0.067 mmol, 2.5 eq) were added and the reaction was stirred for 12 hours. Solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc:Hex 9:1, 1% AcOH) to give compound **5** (21 mg, 83%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d^{6}): δ = 10.15 (1H, s), 7.94 (1H, s), 7.54 (2H, d, *J* = 8.4 Hz), 7.32 (1H, d, *J* = 8.4 Hz), 7.23-7.26 (7H, m), 7.15 (1H, s), 7.09 (2H, d, *J* = 8.4 Hz), 7.00 (1H, d, *J* = 8.4 Hz), 6.63 (2H, d, *J* = 8.4 Hz), 5.01 (2H, s), 4.87 (2H, s), 4.01-4.09 (1H, m), 3.62 (2H, s), 3.43-3.60 (12H, m), 2.84-3.03 (8H, m). ^{13}C NMR (100 MHz, DMSO-d^{6}): δ = 175.3, 173.5, 164.0, 157.3, 145.9, 143.4, 141.2, 140.4, 135.9, 135.7, 133.6, 133.1, 132.9, 132.8, 132.6, 131.0, 129.5, 124.4, 123.3, 122.9, 120.9, 117.3, 114.0, 113.8, 70.5, 62.3, 57.3, 53.5, 51.2, 50.9, 47.7, 40.8, 39.2, 37.5, 35.8. MS (FAB+): calculated for [M+Na^{+}] $C_{45}H_{47}Cl_2N_5O_{11}$ 926.2; found 926.2.



Compound 19. Commercially available Z-Phe-NHS ester (2.03 gr, 5.01 mmol, 1 eq) was dissolved in DMF (7 mL). Commercially available H_2N-lys(Boc)-OH hydrochloride (1.26 gr, 5.13 mmol, 1.025 eq) and Et_3N (2.77 mL, 20.04 mmol, 4 eq) were added and the reaction was monitored to completion (12 hours) by TLC

(EtOAc:Hex 1:1, 1% AcOH). Solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc:Hex 1:1, 1% AcOH) to give compound **19** (3.64 gr, 73%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d^{6}): δ = 8.21 (1H, d, *J* = 7.6 Hz), 7.45 (1H, d, *J* = 8.8 Hz), 7.18-7.34 (10H, m), 6.76 (1H, t, *J* = 7.2 Hz), 4.93 (2H, s), 4.28-4.33 (1H, m), 4.15-4.18 (1H, m), 2.69-3.03 (4H, m), 1.58-1.72 (2H, m), 1.25-1.41 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^{6}): δ = 171.8, 164.6, 156.2, 156.0, 137.8, 137.4, 128.6, 128.0, 127.8, 127.3, 126.6, 119.4, 77.7, 62.6, 56.4, 53.8, 32.4, 29.7, 28.6, 23.1. MS (FAB+): calculated for [M+H^{+}] $C_{28}H_{37}N_3O_7$ 528.3; found 528.3.

Compound 20. Compound **19** (500 mg, 0.95 mmol, 1 eq) was dissolved in dry THF (5 mL) under argon atmosphere. N-methyl morpholine (130 μL, 1.18 mmol, 1.25 eq) was added and the solution was cooled to -15^{o}C. Isobutylchloroformate (135 μL, 1.04 mmol, 1.1 eq), dissolved in dry THF (2 mL), was added drop-wise to the cooled solution. The reaction was stirred at -15^{o}C and monitored to completion (30 min) by TLC (EtOAc:Hex 1:1, 1% AcOH). Then, 4-aminobenzyl alcohol (175 mg, 1.8 mmol, 1.5 eq) was added and the reaction was slowly warmed to room temperature. Upon disappearance of starting material (30 minutes), as determined by TLC, the reaction mixture was diluted with EtOAc then washed twice with saturated NH_4Cl solution and once with brine. The organic phase was dried over $MgSO_4$, filtered and the solvent was removed under reduced pressure. The crude product was loaded on silica gel and purified by column chromatography on silica gel (EtOAc:Hex 6:4 to 9:1) to give compound **20** (509 mg, 80%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d^{6}): δ = 10.02 (1H, s), 8.23 (1H, d, *J* = 7.6 Hz), 7.45-7.60 (3H, m), 7.18-7.32 (12H, m), 6.75 (1H, t, *J* = 7.2 Hz), 5.09 (1H, t, *J* = 5.6 Hz),

4.95 (2H, s), 4.43 (2H, d, *J* = 5.6 Hz), 4.20-4.38 (2H, m), 2.49-2.90 (4H, m), 1.55-1.78 (2H, m), 1.23-1.41 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 171.9, 170.7, 156.2, 156.0, 138.4, 137.8, 137.4, 129.6, 128.6, 128.4, 128.0, 127.8, 127.3, 126.6, 119.4, 77.7, 65.6, 62.6, 56.4, 53.8, 32.4, 29.7, 28.6, 23.1. MS (FAB+): calculated for [M+H$^+$] $C_{35}H_{44}N_4O_7$ 633.3; found 633.3.

Compound 21. Compound **20** (353 mg, 0.55 mmol, 1 eq) was dissolved in dry THF (3 mL) under argon atmosphere and cooled to 0^{o}C. DIPEA (385 μL, 2.21 mmol, 4 eq) and 4-nitrophenyl chloroformate (334 mg, 1.66 mmol, 3 eq) were added, followed by pyridine (9 μL, 0.11 mmol, 0.2 eq). The reaction was monitored to completion (1 hour) by TLC (EtOAc:Hex 1:1). The reaction mixture was diluted with EtOAc then washed twice with saturated NH_4Cl solution and once with brine. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 1:1 to 2:1) to give compound **21** (347 mg, 78%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d^6): δ = 10.14 (1H, s), 8.31 (2H, d, *J* = 8.8 Hz), 8.23 (1H, d, *J* = 7.6 Hz), 7.65 (2H, d, *J* = 8.8 Hz), 7.45-7.60 (3H, m), 7.18-7.32 (12H, m), 6.75 (1H, t, *J* = 7.2 Hz), 5.25 (2H, s), 4.95 (2H, s), 4.33-4.41 (2H, m), 2.71-3.05 (4H, m), 1.59-1.73 (2H, m), 1.19-1.37 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 176.1, 175.2, 160.3, 160.0, 159.8, 156.4, 149.6, 143.8, 142.5, 141.5, 133.9, 133.7, 132.8, 132.5, 132.1, 131.9, 130.7, 129.8, 127.1, 123.7, 120.3, 81.8, 74.7, 69.7, 60.5, 58.0, 43.4, 41.9, 36.4, 33.8, 33.5, 32.7, 27.2. MS (FAB+): calculated for [M+H$^+$] $C_{42}H_{47}N_5O_{11}$ 798.3; found 798.3.

Compound 22. Compound **14** (104 mg, 0.25 mmol, 1 eq) was dissolved in TFA (0.5 mL) and stirred for one minute. TFA was then immediately removed under reduced pressure to yield ammonium salt **15** as a yellow solid. Compound **15** was immediately used without further purifications. Compound **15** was re-dissolved in DMF (800 µL) and Compound **21** (246 mg, 0.30 mmol, 1.2 eq), followed by Et_3N (69 µL, 0.50 mmol, 2 eq) were added. The reaction was monitored to completion (5 minutes) by TLC (EtOAc:Hex 9:1). The reaction mixture was diluted with EtOAc then washed twice with saturated NH_4Cl solution and once with brine. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 9:1) to give compound **22** (88 mg, 36%) as a yellowish solid.

^{1}H NMR (400 MHz, DMSO-d^6): δ = 10.05 (1H, s), 8.18 (1H, d, *J* = 7.6 Hz), 7.94 (1H, s), 7.73 (1H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 8.8 Hz), 7.45 (1H, d, *J* = 8.8 Hz), 7.22-7.33 (12H, m), 7.17 (1H, s), 7.03 (1H, d, *J* = 8.4 Hz), 6.75 (1H, t, *J* = 7.6 Hz), 5.43 (1H, t, *J* = 4.4 Hz), 5.02 (2H, s), 4.95 (2H, s), 4.25-4.40 (4H, m), 3.35-3.61 (4H, m), 2.67-3.07 (10H, m), 1.51-1.77 (2H, m), 1.20-1.41 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 171.8, 170.6, 164.0, 160.0, 159.4, 157.8, 157.4, 156.2, 156.0, 141.2, 138.4, 137.8, 137.4, 132.7, 129.6, 128.8, 128.4, 128.3, 128.0, 127.6, 127.3, 126.6, 123.2, 121.0, 119.4, 113.8, 77.7, 65.6, 62.3, 56.4, 53.8, 50.9, 50.1, 39.0, 38.3, 32.4, 29.7, 28.6, 23.1. MS (FAB+): calculated for [M+Na$^+$] $C_{51}H_{60}N_6O_{13}$ 987.4; found 987.4.

Compound 23. Compound **22** (60 mg, 0.0.06 mmol, 1 eq) was dissolved in dry THF (3 mL) under argon atmosphere and cooled to 0^{o}C. DIPEA (43 µL, 0.25 mmol, 4 eq) and 4-nitrophenyl chloroformate (38 mg, 0.19 mmol, 3 eq) were added, followed by

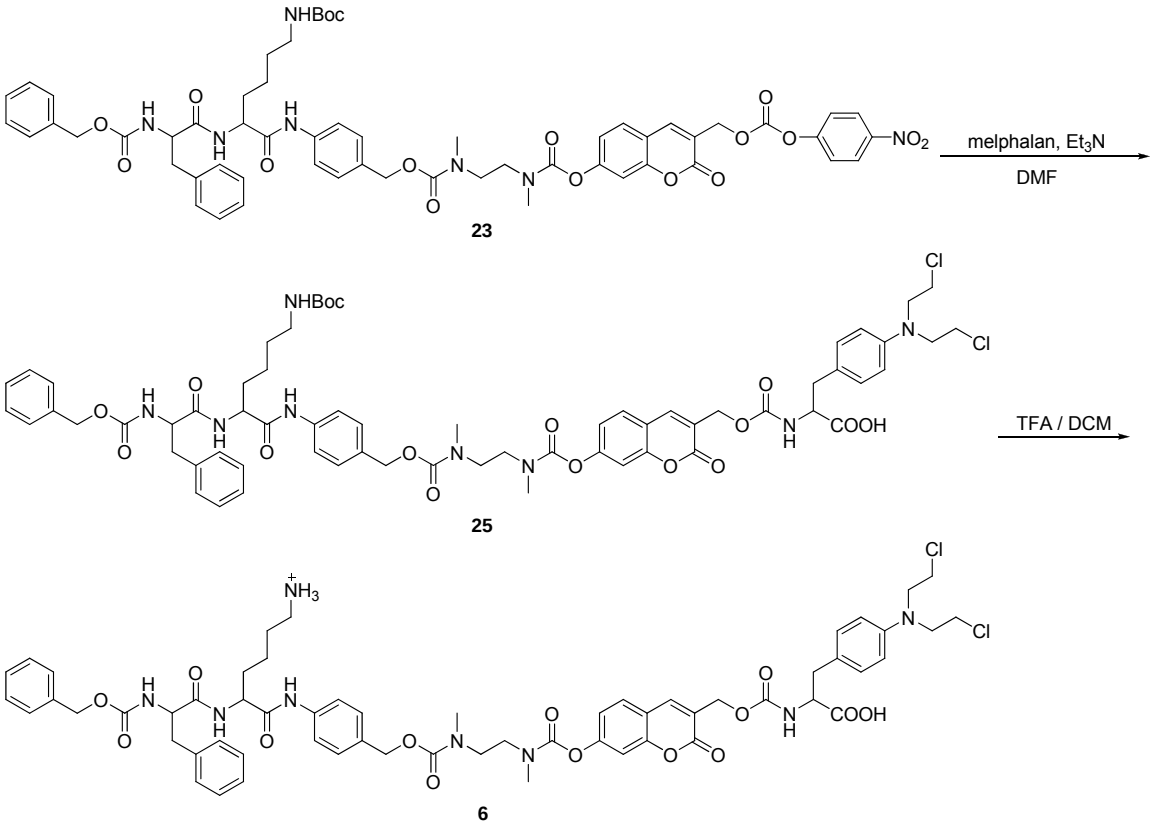
pyridine (5 μL, 0.05 mmol, 0.8 eq). The reaction was monitored to completion (3 hour) by TLC (EtOAc:Hex 9:1). The reaction mixture was diluted with EtOAc then washed twice with saturated NH_4Cl solution and once with brine. The organic phase was dried over $MgSO_4$, then filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography on silica gel (EtOAc:Hex 3:1 to 9:1) to give compound **23** (36 mg, 50%) as a white solid. ^{1}H NMR (400 MHz, DMSO-d^6): δ = 10.05 (1H, s), 8.33 (2H, d, *J* = 8.8 Hz), 8.18 (1H, d, *J* = 7.6 Hz), 7.94 (1H, s), 7.73 (1H, d, *J* = 8.4 Hz), 7.67 (2H, d, *J* = 8.8 Hz), 7.57 (2H, d, *J* = 8.8 Hz), 7.45 (1H, d, *J* = 8.8 Hz), 7.22-7.33 (12H, m), 7.17 (1H, s), 7.03 (1H, d, *J* = 8.4 Hz), 6.75 (1H, t, *J* = 7.6 Hz), 5.43 (1H, t, *J* = 4.4 Hz), 5.22 (2H, s), 5.02 (2H, s), 4.95 (2H, s), 4.32-4.40 (2H, m), 3.35-3.61 (4H, m), 2.67-3.07 (10H, m), 1.51-1.77 (2H, m), 1.20-1.41 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 176.0, 172.1, 170.9, 164.0, 160.1, 159.8, 159.4, 157.8, 157.4, 156.2, 156.0, 141.2, 138.4, 137.8, 137.4, 132.7, 132.5, 129.6, 128.9, 128.4, 128.2, 128.0, 127.6, 127.3, 126.6, 123.2, 121.0, 119.4, 113.8, 82.0, 77.7, 62.3, 56.4, 53.8, 50.9, 50.1, 39.0, 38.3, 32.4, 29.7, 28.6, 23.1. MS (FAB+): calculated for [M+H$^+$] $C_{58}H_{63}N_7O_{17}$ 1130.4; found 1130.4.

Compound 24. Compound **23** (31 mg, 0.027 mmol, 1 eq) was dissolved in dry DMF (1 mL) under argon atmosphere. L-Tryptophane (11 mg, 0.054 mmol, 2 eq) and Et_3N (10 μL, 0.071 mmol, 2.5 eq) were added and the reaction was stirred for 12 hours. Solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc:MeOH 95:5, 1% AcOH) to give compound **24** (27 mg, 83%) as a white solid.

^{1}H NMR (400 MHz, DMSO-d^6): δ = 10.80 (1H, s), 10.05 (1H, s), 8.18 (1H, d, *J* = 7.6 Hz), 7.94 (1H, s), 7.73 (1H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 8.8 Hz), 7.45 (1H, d, *J* =

8.8 Hz), 7.22-7.33 (13H, m), 7.17 (1H, s), 7.05-7.11 (4H, m), 7.03 (1H, d, *J* = 8.4 Hz), 6.75 (1H, t, *J* = 7.6 Hz), 5.43 (1H, t, *J* = 4.4 Hz), 5.02 (2H, s), 4.95 (2H, s), 4.25-4.40 (4H, m), 4.01-4.11 (1H, m), 3.35-3.61 (6H, m), 2.67-3.07 (10H, m), 1.51-1.77 (2H, m), 1.20-1.41 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 175.1, 171.8, 166.2, 164.0, 160.0, 159.4, 157.8, 157.4, 156.2, 156.0, 141.2, 138.4, 137.8, 137.4, 136.6, 132.7, 129.6, 128.8, 128.4, 128.3, 128.0, 127.6, 127.3, 126.9, 126.6, 123.9, 123.2, 121.7, 121.0, 120.0, 119.4, 118.7, 116.6, 113.8, 111.0, 77.7, 65.6, 62.3, 60.2, 56.4, 53.8, 50.9, 50.1, 39.0, 38.3, 32.4, 29.7, 28.6, 26.0, 23.1. MS (FAB+): calculated for [M+H$^+$] $C_{63}H_{70}N_8O_{16}$ 1195.5; found 1195.5.

Compound 7. Compound **24** (3 mg) was dissolved in a mixture of TFA and DCM 1:1 (0.5 mL) and stirred for one and a half minutes. TFA and DCM were then immediately removed under reduced pressure to give compound **7** as a yellow solid. Complete conversion of compound **24** into compound **7** was monitored by RP-HPLC. Compound **7** was used without further purification.



Compound 25. Compound **23** (39 mg, 0.034 mmol, 1 eq) was dissolved in dry DMF (800 12 µL) under argon atmosphere. Melphalane (21 mg, 0.069 mmol, 2 eq) and Et_3N (12 µL, 0.086 mmol, 2.5 eq) were added and the reaction was stirred for 12 hours. Solvent was removed under reduced pressure and the crude product was purified by column chromatography on silica gel (EtOAc:MeOH 95:5, 1% AcOH) to give compound **25** (32 mg, 72%) as a white solid.

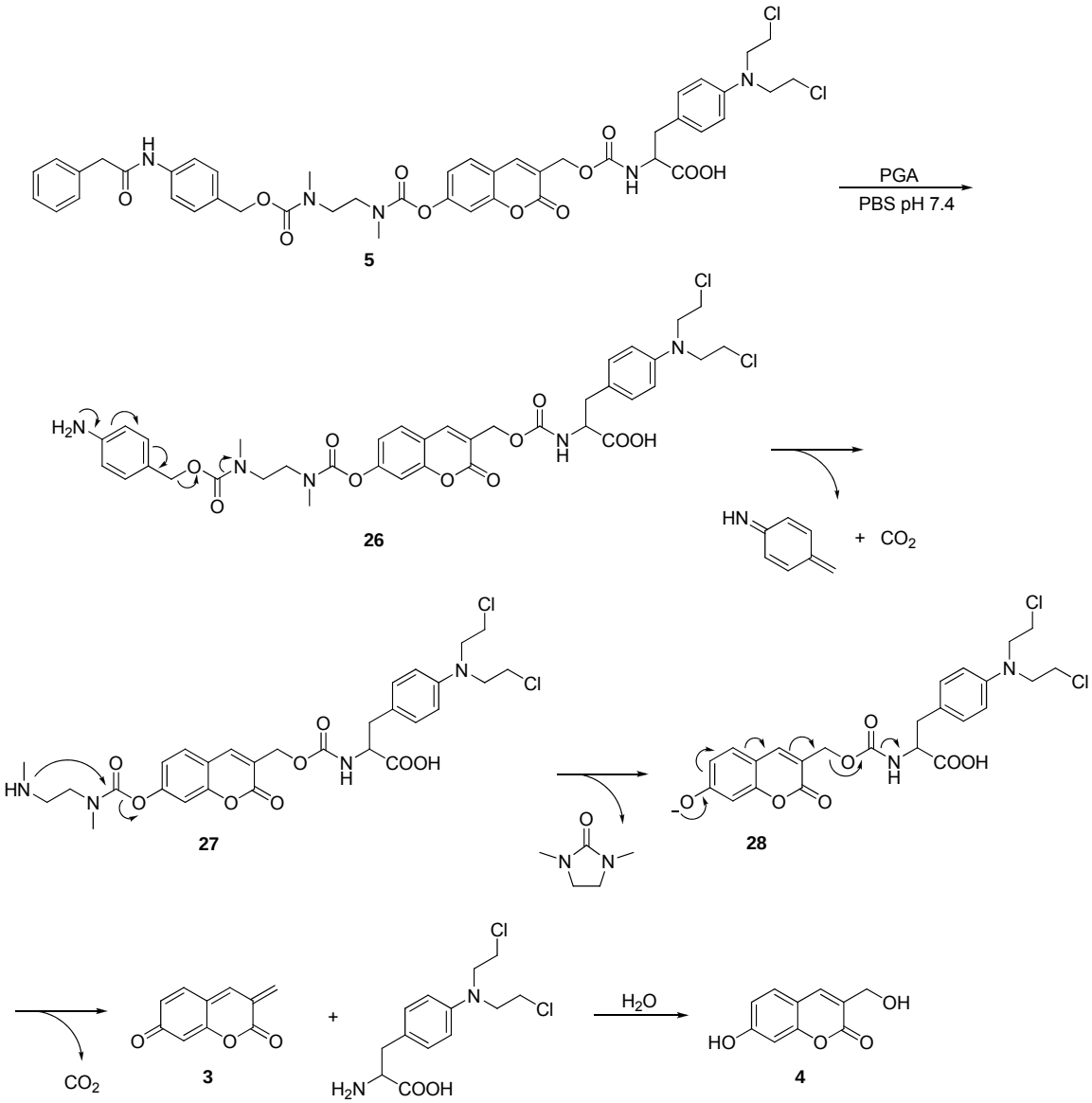
^{1}H NMR (400 MHz, DMSO-d^6): δ = 10.12 (1H, s), 10.05 (1H, s), 8.18 (1H, d, *J* = 7.6 Hz), 7.94 (1H, s), 7.73 (1H, d, *J* = 8.4 Hz), 7.57 (2H, d, *J* = 8.8 Hz), 7.45 (1H, d, *J* = 8.8 Hz), 7.38 (2H, d, *J* = 8.4Hz), 7.22-7.33 (12H, m), 7.17 (1H, s), 7.03 (1H, d, *J* = 8.4 Hz), 6.75 (1H, t, *J* = 7.6 Hz), 6.63 (2H, d, *J* = 8.4 Hz), 5.43 (1H, t, *J* = 4.4 Hz), 5.02 (2H, s), 4.95 (2H, s), 4.25-4.40 (4H, m), 3.35-3.61 (4H, m), 2.58-3.21 (20H, m), 1.51-1.77 (2H, m), 1.20-1.41 (13H, m). ^{13}C NMR (100 MHz, DMSO-d^6): δ = 174.7,

171.7, 170.4, 164.5, 164.0, 160.0, 159.4, 157.8, 157.4, 156.2, 156.0, 144.1, 141.2, 138.4, 137.8, 137.4, 132.7, 129.7, 128.8, 128.6, 128.3, 128.0, 127.8, 127.6, 127.1, 126.6, 123.2, 121.0, 119.4, 113.8, 112.2, 77.7, 65.9, 62.3, 59.2, 56.3, 53.8, 53.3, 50.9, 50.1, 43.0, 39.0, 38.3, 36.4, 32.4, 29.7, 28.6, 23.1. MS (FAB+): calculated for $[M+Na^+]$ $C_{65}H_{76}Cl_2N_8O_{16}$ 1317.5; found 1317.5.

Compound 6. Compound **25** (3 mg) was dissolved in a mixture of TFA and DCM 1:1 (0.5 mL) and stirred for one and a half minutes. TFA and DCM were then immediately removed under reduced pressure to give compound **6** as a yellow solid. Complete conversion of compound **25** into compound **6** was monitored by RP-HPLC. Compound **6** was used without further purification.

2. Disassembly mechanisms of molecules 5, 6 and 7

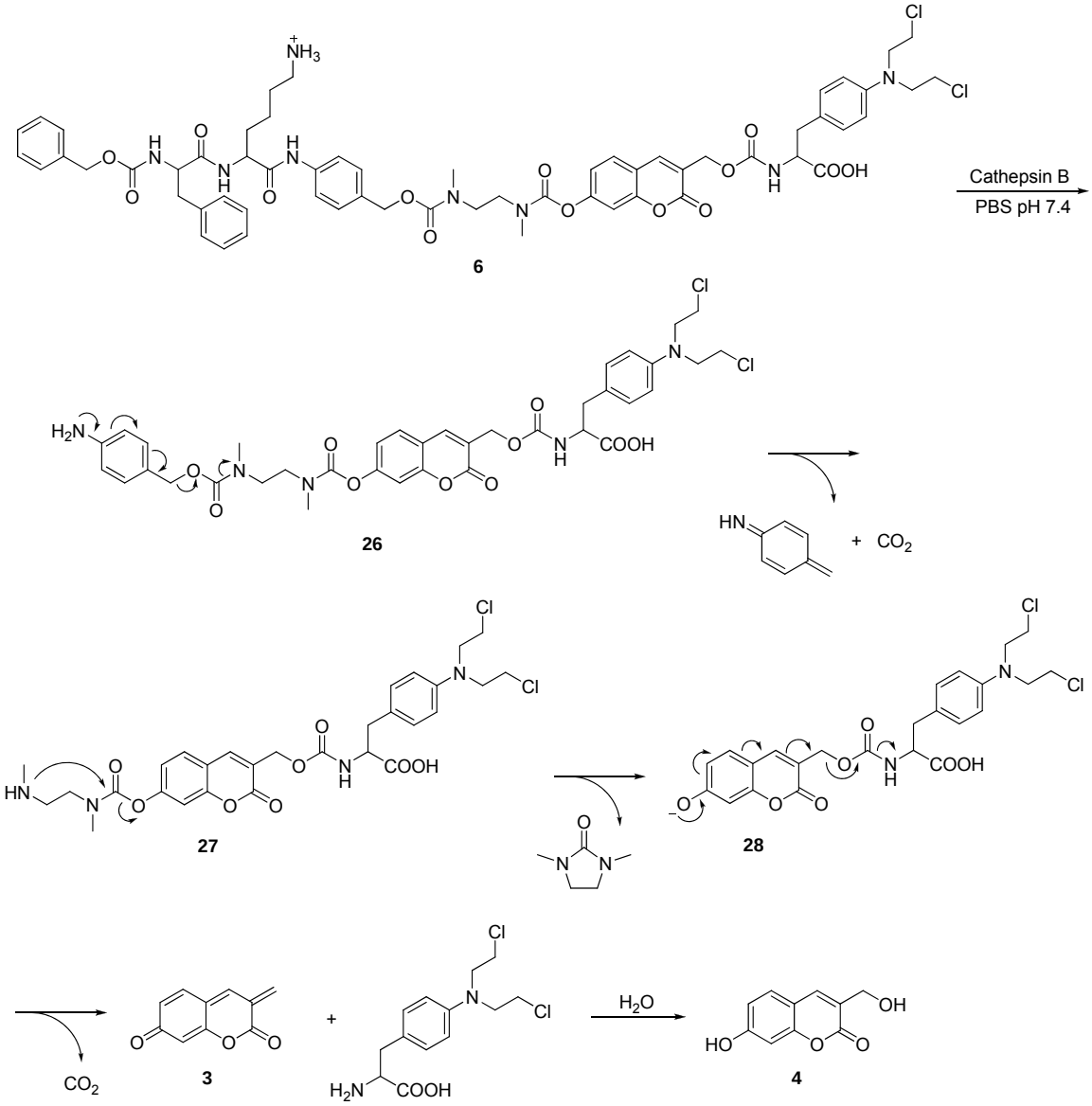
Disassembly mechanisms of molecule 5:



Scheme 1S: Cleavage of the phenylacetamide moiety by PGA generates an aniline derivative (**26)**, which undergoes spontaneous 1,6-elimination followed by decarboxylation. The exposed secondary amine (**27**) then undergoes an intramolecular cyclization to release the phenolate of coumarin (**28**). A spontaneous 1,8-elimination reaction then takes place, leading to the release of melphalane and generation of coumarin quinone-methide derivative **3**. Addition of a water molecule to the reactive

quinone-methide **3** leads to formation of the highly fluorescence coumarin derivative **4**.

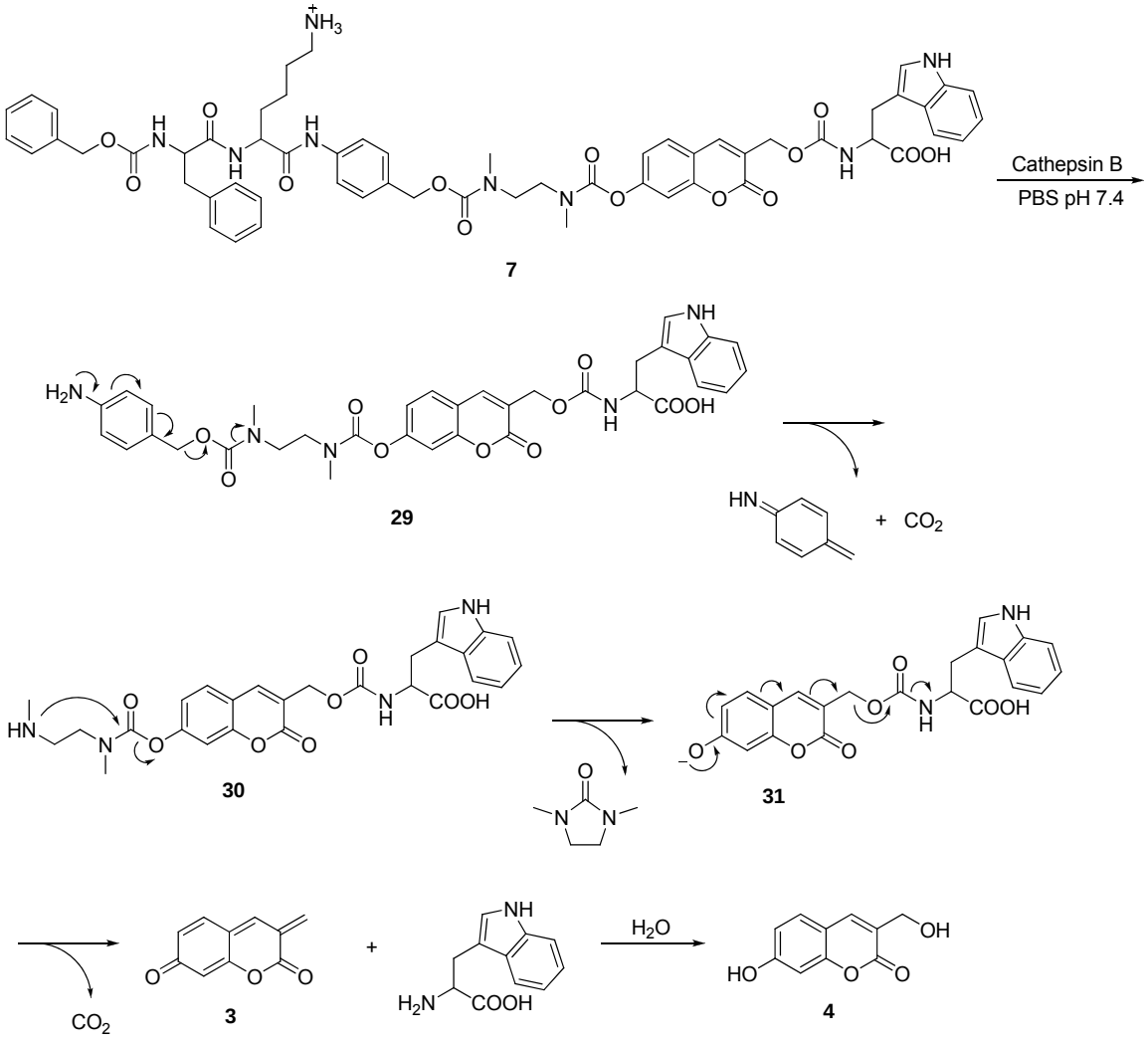
Disassembly mechanisms of molecule 6:



Scheme 2S: Cleavage of the amide bond at the C-terminus of the lysine by cathepsin-B generates an aniline derivative (**26)**, which undergoes spontaneous 1,6-elimination followed by decarboxylation. The exposed secondary amine (**27**) then undergoes an intramolecular cyclization to release the phenolate of coumarin (**28**). A spontaneous

1,8-elimination reaction then takes place, leading to the release of melphalane and generation of coumarin quinone-methide derivative **3**. Addition of a water molecule to the reactive quinone-methide **3** leads to formation of the highly fluorescence coumarin derivative **4**.

Disassembly mechanisms of molecule 7:



Scheme 3S: Cleavage of the amide bond at the C-terminus of the lysine by cathepsin-B generates an aniline derivative (**29)**, which undergoes spontaneous 1,6-elimination followed by decarboxylation. The exposed secondary amine (**30**) then undergoes an intramolecular cyclization to release the phenolate of coumarin (**31**). A spontaneous

1,8-elimination reaction then takes place, leading to the release of melphalane and generation of coumarin quinone-methide derivative **3**. Addition of a water molecule to the reactive quinone-methide **3** leads to formation of the highly fluorescence coumarin derivative **4**.

3. Additional spectroscopic data and comments

Fluorescent emission spectra of coumarin derivatives

Sample solutions of compound **4** or **14** [500 µM] were prepared by adding 5 µL from a corresponding compound **4** or **14** stock solution (10 mM in DMSO) into 95 µL PBS pH 7.4 in a 96 wells plate. The resulting solutions were excited at λ = 315 nm and emission spectra were recorded.

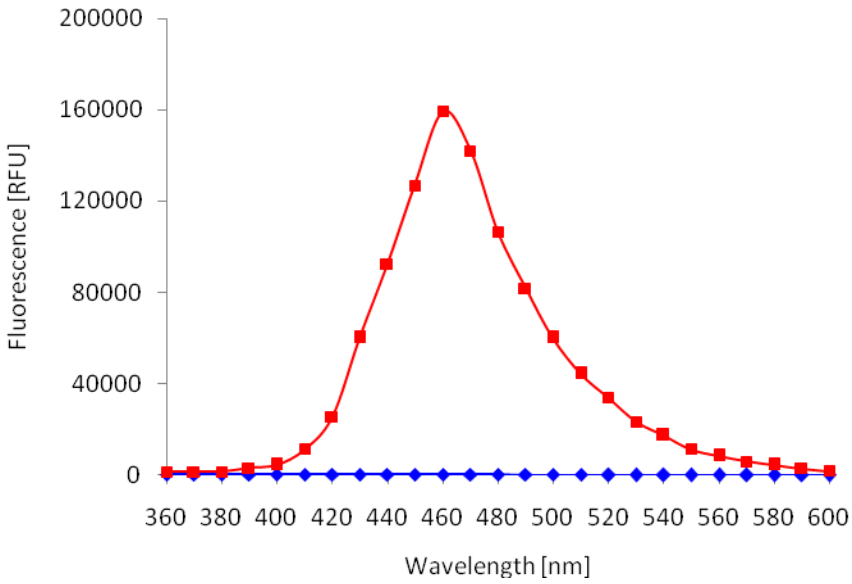


Figure 1S: Fluorescence emission spectra of (■) compound **14** [500 µM] and (♦) compound **14** [500 µM] in 95 µL PBS pH 7.4 upon excitation at 315 nm.

Fluorescence emitted from leukemia MOLT-3 cell line treated with RDDS 6

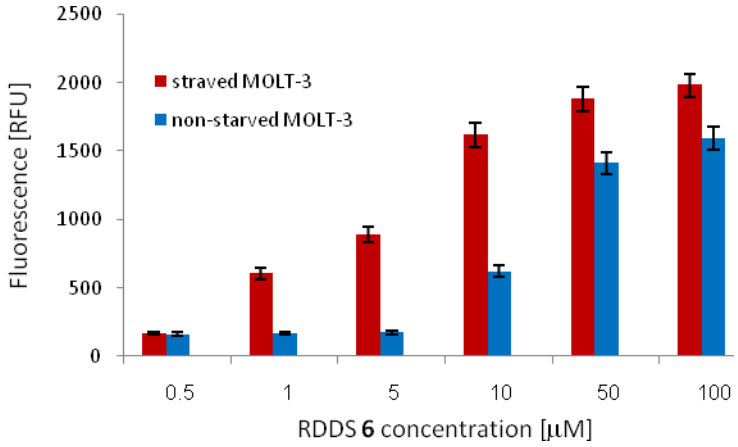


Figure 2S: Fluorescence emitted from starved and non-starved leukemia MOLT-3 cell line solutions in 100 µL RPMI treated with RDDS **6** at the indicated concentrations. Fluorescence was measured 12 hours after treatment at λ_{ex} = 315 nm, λ_{em} = 460 nm. Error bars represent mean ± SD.

Cell viability and fluorescence emission of HUVEC treated with RDDS 7

Cell culture

Human acute lymphoblastic leukemia MOLT-3 cells were a kind gift from Holger Lode (Charlte´ Children's Hospital, Berlin). Human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (ATCC). MOLT-3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 µg/ml Penicillin, 100 U/ml Streptomycin, 12.5 U/ml Nystatin and 2 mM L-glutamin (Biological Industries, Israel). HUVEC were cultured in EGM-2 medium (Lonza Switzerland). All cells were grown at 37°C in 5% CO_2.

Cell proliferation assay

MOLT-3 cells were harvested from culture flasks, resuspended in cell culture medium, and plated at a density of $5x10^3$ cells/well in 100 µL onto 96-well culture plate in RPMI 1640 medium supplemented with 10% FBS. Cells were treated with melphalan, coumarin **4** and RDDS **5** at the indicated concentrations in RPMI medium (100–100,000 nM) in the presence or absence of PGA enzyme (1 µM). For cell growth inhibition experiments by RDDS **6** and RDDS **7**, MOLT-3 and HUVEC cells were grown in complete medium (RPMI supplemented with 10% fetal bovine serum or EGM-2 respectively) or in starvation medium (RPMI supplemented with 2% fetal bovine serum or EBM-2 supplemented with 2.5% FBS respectively) 24 h prior to treatment with RDDS **6** and RDDS **7** at serial concentrations (100-100,000nM). For cell growth inhibition experiments with HUVEC, cells were plated at 15,000 cells/well onto 24-well culture. Following treatments with RDDS **6** and RDDS **7** cells were incubated for 72 h (5% CO_2). MOLT-3 and HUVEC viable cells were counted by XTT reagent or by Z1 Coulter® Particle Counter (Beckman Coulter™) respectively.

Subcellular confocal imaging of activated coumarin 4 in HUVEC cells treated with RDDS 5 and RDDS 6

HUVEC were seeded on sterile 13 mm cover glasses in 35 mm culture dishes 24h prior to incubation with RDDS **5** or RDDS **6.** Cells were incubated with RDDS **5** or RDDS **6** for 4 h, then washed several times with cold PBS, fixed with 3.5% paraformaldehyde for 15 min at room temperature (RT) and washed with PBS again.

For counter staining, cells were permeabilized with 0.1% Triton-X100 for 3 min and rinsed with PBS again. Actin filaments were labeled using phalloidin-FITC conjugate (50 mg/ml, 40 min at RT), Nuclei were labeled using propidium iodide (10 mg/ml) and cover glasses were mounted by AntifadeH mounting media. All slides were kept at 4^0C in dark until confocal microscopy analysis was preformed.

Confocal microscopy

Cellular uptake and internalization of activated coumarin **4** monitored utilizing a Leica TCS SP5 confocal imaging systems with 20X objectives. All images were taken using a multi-track channel acquisition to prevent emission cross-talk between fluorescent dyes. Single XY plane-images were acquired at minimum of 1024x1024 resolution. Images from Z stack acquisition were processed as separate channels using Huygens deconvolution software and merged as a single image.

A B

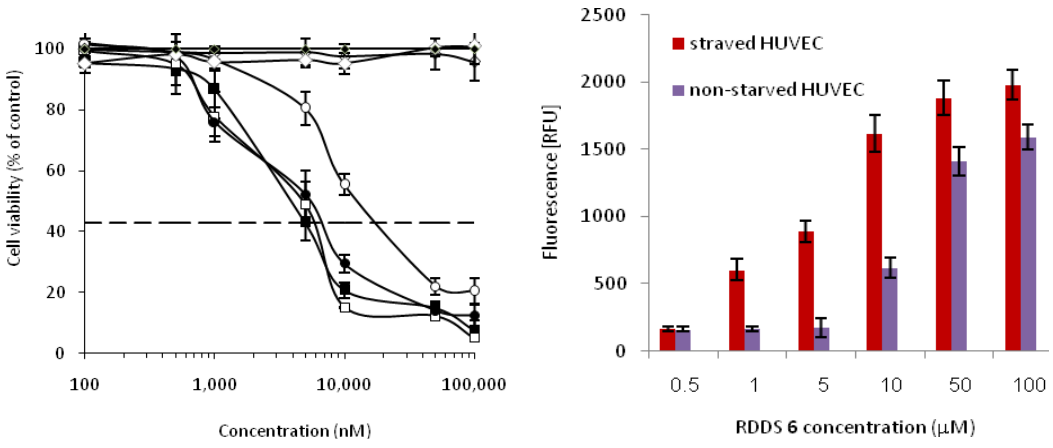


Figure 3S: A. Cell viability of non-starved HUVEC treated with (□) melphalan, (○) RDDS **6** or (◊) coumarin **4**, and starved HUVEC treated with (■) melphalan, (●) RDDS **6** or (♦) coumarin **4**. Cells were incubated for 72 hours. Symbols represent mean ± SD. B. Fluorescence emitted from starved and non-starved HUVEC immersed in 500 µL RPMI treated with RDDS **7** at the indicated concentrations.

Fluorescence was measured 12 hours after treatment at λ_{ex} = 315 nm, λ_{em} = 460 nm. Bars represent mean ± SD.

Cellular localization of activated coumarin in HUVEC using confocal microscopy:

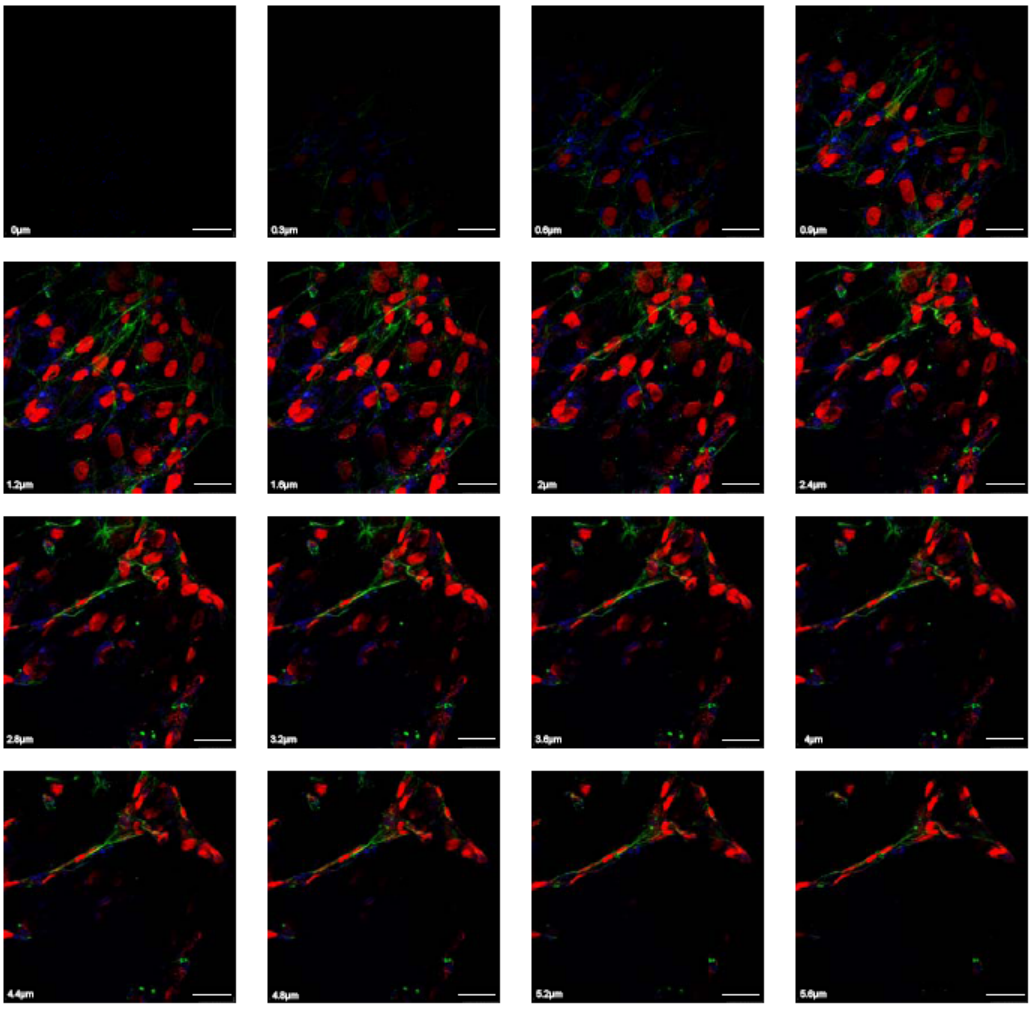


Figure 4S: Cellular localization of activated coumarin in HUVEC using confocal microscopy. 5.6 µm Z-stack of 16 slices of HUVC incubated with RDDS **6** revealed activated coumarin **4** at similar focal plane localization with actin fibers (green) and nuclei (red). Scale bars represent 50 µm.

Additional Comments:

We observed that cell viability was decreased in the non-starved cells at high concentration of RDDS **6**. This could be explained by the fact that similar to other tumor cells, MOLT-3 cells overexpress basal levels of cathepin B at normal non-

starved conditions. Since drug release is proportional to the amount of cathepsin B in the cells, there is a sufficient cathepsin B in non-starved cells to trigger the drug release at high concentrations.

Cathepsin B has been associated with angiogenesis processes and is observed at high levels in lysosomes of tumor endothelial cells. HUVEC are grown *in vitro* under stimulating conditions and as such imitate the clinical setting where tumor endothelial cells, unlike normal healthy ones, proliferate in the adult. Therefore, HUVEC are used as a standard *in vitro* model for studying angiogenesis. Similar to tumor endothelial cells, serum-starved HUVEC express high levels of cathepsin B. Consequently, HUVEC were chosen as an appropriate model for further examination of our ability to monitor cathepsin B-released coumarin in real-time using molecular imaging techniques.