Supplementary Information

Optimizing Aromatic Oligoamide Foldamer Side-Chains for Ribosomal Translation Initiation

Christos Tsiamantas,[†a] Sunbum Kwon,[†b,c] Céline Douat,[b,d] Hiroaki Suga*[a] and Ivan Huc*[b]

[a] Department of Chemistry, Graduate School of Science, The University of Tokyo, Tokyo, Japan

[b] Department of Pharmacy and Center for Integrated Protein Science, Ludwig-Maximilians-Universität, Butenandtstraße 5-13, D-81377 München, Germany

[c] Department of Chemistry, Chung-Ang University, 84 Heukseok-ro, Dongjak-gu, Seoul 06974, Republic of Korea

[d] CBMN (UMR5248), Université de Bordeaux - CNRS - IPB, Institut Européen de Chimie et Biologie, 2 rue Escarpit, 33600 Pessac, France

Table of Contents

1. Supplementary Figures

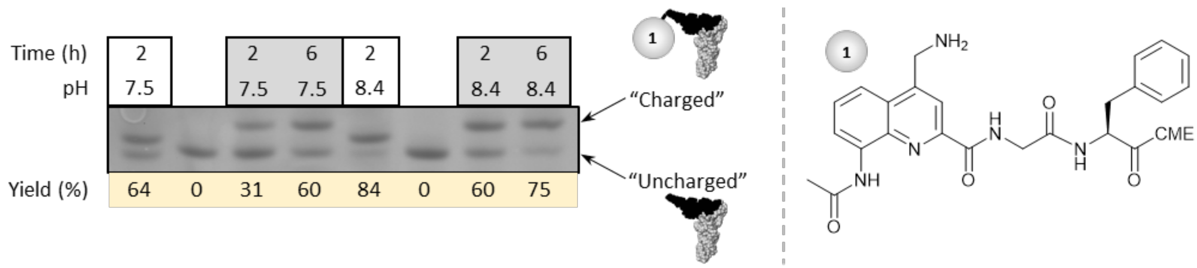


Figure S1. Charging of foldamer **1** on µ-helix RNA. Two different pH conditions were tested for the aminoacylation reaction (pH = 7.5 and 8.4). Data suggest that maximum aminoacylation efficiency for compound **1** is obtained using 50 mM HEPES-KOH, pH 8.4 for 6 hours. The fidelity of the setup was confirmed by aminoacylating a known substrate, ClAc-L-Trp-CME (white frames). The non-framed lane corresponds to an aminoacylation reaction in which no activated amino acid was added, thus the band corresponds to "uncharged" RNA.

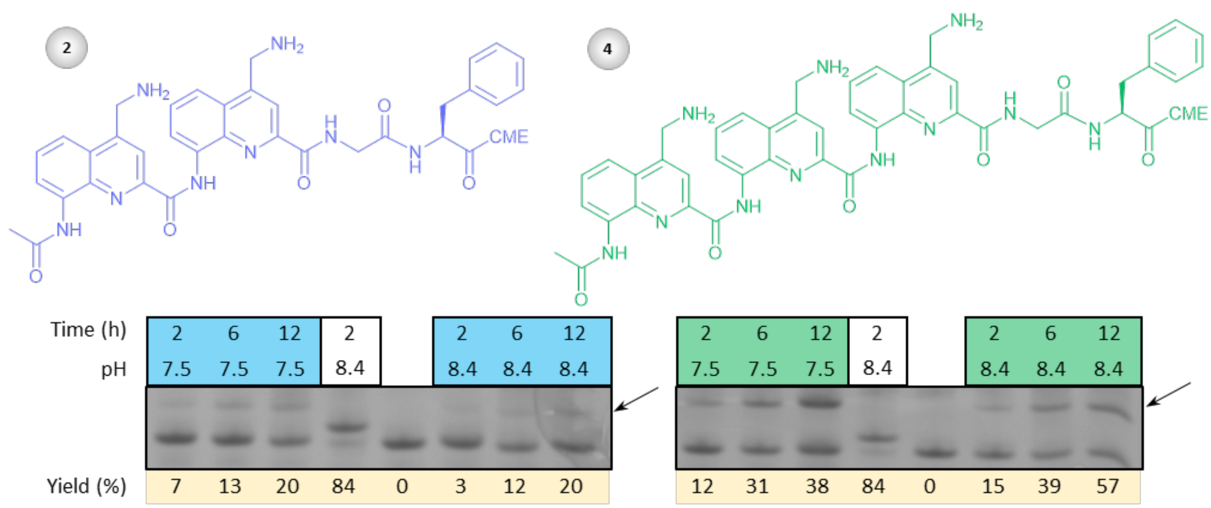


Figure S2. Charging of foldamer **2** and **4** on µ-helix RNA. Two different pH conditions were tested for the aminoacylation reaction (pH = 7.5 and 8.4). Data suggest that maximum aminoacylation efficiency for both compounds **2** and **4** is obtained using 50 mM HEPES-KOH, pH 8.4 for 12 hours. The fidelity of the setup was confirmed as mentioned in Figure S1.

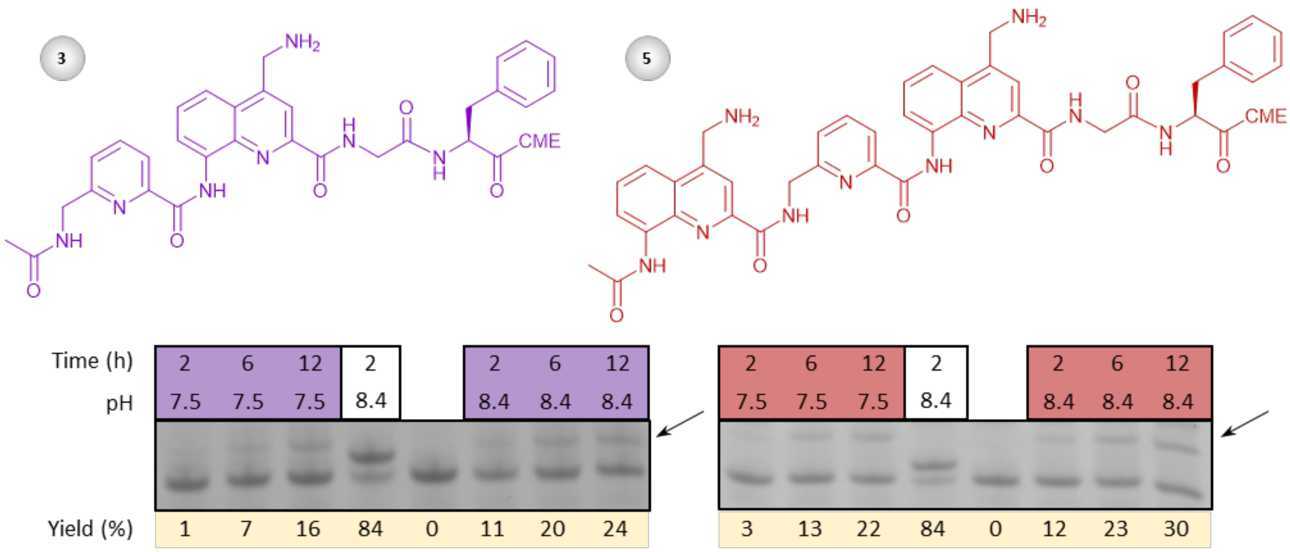


Figure S3. Charging of foldamer **3** and **5** on µ-helix RNA. Two different pH conditions were tested for the aminoacylation reaction (pH = 7.5 and 8.4). Data suggest that maximum aminoacylation efficiency for both compounds **3** and **5** is obtained using 50 mM HEPES-KOH, pH 8.4 for 12 hours. The fidelity of the setup was confirmed as mentioned in Figure S1.

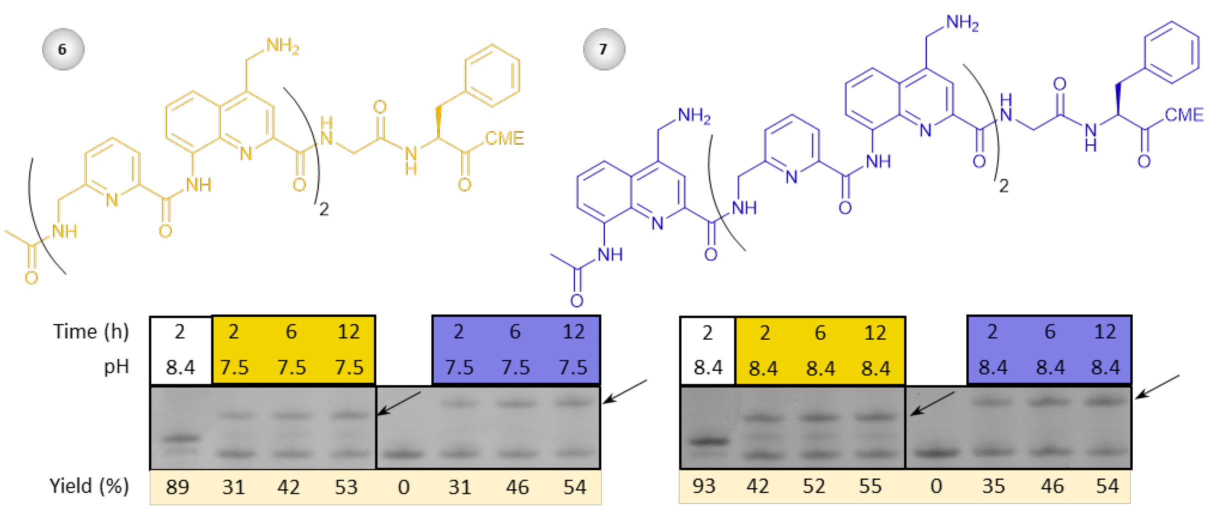


Figure S4. Charging of foldamer **6** and **7** on µ-helix RNA. Two different pH conditions were tested for the aminoacylation reaction (pH = 7.5 and 8.4). Data suggest that maximum aminoacylation efficiency for both compounds **6** and **7** is obtained using 50 mM HEPES-KOH, pH 8.4 for 12 hours. The fidelity of the setup was confirmed as mentioned in Figure S1.

2. Experimental section

2.1. Methods for *in vitro* translation

Preparation of eFx and $tRNA^{fMet}_{CAU}$: All oligonucleotides were purchased from Operon (Japan). DNA templates were assembled using reported protocols and after transcription, using T7 RNA polymerase, they resulted in the desired sequences.[1, 2]

Microhelix: Microhelix RNA was purchased from GeneDesign (Japan), being a mimic of the acceptor stem of tRNA (the site of aminoacylation), originally based on the acceptor stem of *E.coli* Asn tRNA.[1]

Aminoacylation of μ-helix: 3 μL H_2O, 1 μL microhelix (250 μM), 1 μL eFx (250 μM) and1 μL HEPES-KOH pH 8.4 (500 mM) were mixed, heated to 95 °C for 2 min and cooled down to room temperature for 5 min. 2 μL $MgCl_2$ (3 M) added and left for 5 min at room temperature. Solution was placed on ice until cold and the aminoacylation was initiated by adding 2 μL cyanomethyl substrate (25 μM) in DMSO. Reaction was incubated in ice for 2-12 hours, depending on the substrate. (Final concentrations: 25 μM microhelix, 25 μM eFx and 5 mM cyanomethyl ester in 50 mM HEPES-KOH pH 8.4, 600 mM $MgCl_2$, 20% DMSO). The reaction was stopped by pelleting any insoluble substrate, collecting the substrate, adding 4 reaction volumes (40 μL) of 0.3 M NaOAc pH 5.2, and the product precipitated using 10 reaction volumes of EtOH (100 μL). The pellet was washed with 0.1 M NaOAc pH 5.2, 70% EtOH and analyzed by 20% denaturing acid PAGE (50 mM sodium acetate, 6 M urea). The RNA was stained with ethidium bromide and analyzed by FLA-5100 (Fuji, Japan).

Aminoacylation of $tRNA^{fMet}_{CAU}$ with foldamer substrates: 25 μM $tRNA^{fMet}_{CAU}$, 25 μM eFx and 5 mM cyanomethyl ester substrate were incubated in 50 mM HEPES-KOH pH 8.4, 600 mM $MgCl_2$ in 20% DMSO using the time originating from the "*Aminoacylation of μ-helix*" for each substrate (see Supporting Figures, above).

Model mRNA template, (MGGGTYYDYKDDDDK): The following primers were purchased by Eurofins genomics (Japan).

P1: GGCGTAATACGACTCACTATAG
P2: TAATACGACTCACTATAGGGTTAACTTTAACAAGGAGAAAAACATGGGC
P3: CGTCGTCGTCCTTGTAGTCGTAGTAGGTGCCGCCGCCCATGTTTTTCTCCTTGTTAAAG
P4: CGAAGCTTACTTGTCGTCGTCGTCCTTGTAGTC

P2 was annealed with P3 and extended using Taq DNA polymerase. The resulting product was diluted 200 times with PCR reaction buffer and amplified by using P1 and P4 as the 5'- and 3'-primers, respectively. The DNA product was transcribed by T7 RNA polymerase and purified by 10% denaturing PAGE. The mRNA template was dissolved in water and its concentration was adjusted to 10 μM.

mRNA template:	AUG	GGC	GGC	GGC	ACC	UAC	UAC	GAC	UAC	AAG	GAC	GAC	GAC	GAC	AAG	UAA
Wild type peptide:	fM	G	G	G	T	Y	Y	D	Y	K	D	D	D	D	K	(stop)

Preparation of EF-P: EF-P was expressed, modified and purified according to previously published protocol.[3]

In vitro translation and MALDI-TOF-MS: A custom-made *in vitro* translation mixture was used, with the final concentrations of individual components: 1.2 μM ribosome, 0.1 μM T7 RNA polymerase, 4 μg/mL creatine kinase, 3 μg/mL myokinase, 0.1 μM pyrophosphatase, 0.1 μM nucleotidediphosphatase kinase, 2.7 μM IF1, 0.4 μM IF2, 1.5 μM IF3, 30 μM EF-Tu, 30 μM EFTs, 0.26 μM EF-G, 0.25 μM RF2, 0.17 μM RF3, 0.5 μM RRF, 0.6 μM MTF, 0.73 μM AlaRS, 0.03 μM ArgRS, 0.38 μM AsnRS, 0.02 μM CysRS, 0.06 μM GlnRS, 0.23 μM GluRS, 0.09 μM GlyRS, 0.02 µM HisRS, 0.4 µM IleRS, 0.04 µM LeuRS, 0.03 µM MetRS, 0.68 µM PheRS, 0.16 µM ProRS, 0.04 µM SerRS, 0.09 µM ThrRS, 0.03 µM TrpRS, 0.02 µM ValRS, 0.13 µM AspRS, 0.11 µM LysRS, 0.02 µM TyrRS. Additionally, 50 mM HEPES-KOH (pH 7.6), 100 mM potassium acetate, 2 mM GTP, 2 mM ATP, 1 mM CTP, 1 mM UTP, 20 mM creatine phosphate, 12 mM $Mg(OAc)_2$, 2 mM spermidine, 2 mM DTT, and 1.5 mg/mL *E. coli* total tRNA (Roche).

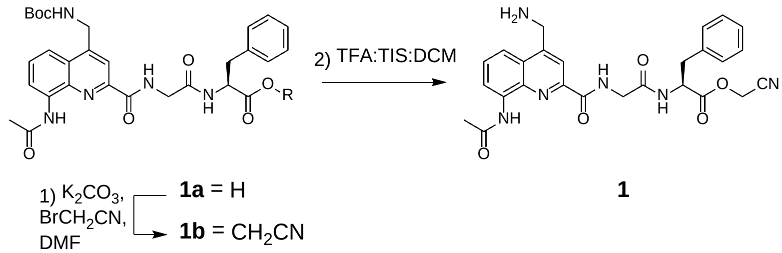
- For MALDI-TOF analysis: 19 of the 20 canonical amino acids were included at 500 µM: Met was not added, neither the formyl donor (10-formyl-5,6,7,8-tetrahydrofolic acid) required for initiation using formyl methionine (fMet). Solutions containing the above plus 1.5 µM mRNA template and 50 µM foldamer-$tRNA^{fMet}_{CAU}$ (prepared using eFx, above) were incubated for 30 min at 37 °C. The foldamer-peptide hybrid was isolated using anti-FLAG M2 affinity agarose gel (Sigma) and eluted using 0.2% TFA. Solution was mixed, 1:1, with a half-saturated solution (80% MeCN, 19.5% H_2O, 0.5% AcOH) of α-cyano-4-hydrocinnamic acid prior to spotting on a MALDI plate. Foldamer-peptide MALDI-TOF-MS analysis was performed by an UltrafleXtreme (Bruker Daltonics) in reflector/positive mode.
- For radioisotope quantification: 18 of the 20 canonical amino acids were included at 500 µM: Met aspartic acid (Asp) were excluded from the mixture. Instead 0.05 mM [^{14}C]Asp was added. *In vitro* translation was carried out as above. Translation reactions were stopped by adding an equal volume of stop solution [0.9 M Tris-HCl (pH 8.45), 8% SDS, 30% glycerol and 0.001% xylene cyanol] and incubating at 95 °C for 2 min. Then, the samples were analyzed by 15% tricine SDS-PAGE and autoradiography using Typhoon FLA 7000 (GE Healthcare). Peptide yield was normalized by intensity of [^{14}C]-Asp band. Note that FLAG-tag purification is not carried out during this experiment.

2.2. Materials and methods for chemical synthesis and characterizations

General: Chemical reagents were purchased from commercial suppliers (Sigma-Aldrich, Alfa-aesar or TCI) and used without further purification. H-(*L*)-Phe-2CT resin (manufacturer's loading: 0.54 mmol g^{-1}) was purchased from Iris biotech. Tetrahydrofuran (THF) and dichloromethane (CH_2Cl_2) were dried over alumina columns. *N*,*N*-diisopropylethylamine (DIPEA) was distilled over CaH_2 prior to use. Column chromatography purifications were performed on silica gel (230-400 mesh, 40-63 µm, Merck). Reactions were monitored by thin layer chromatography on silica gel 60-F254 plates (Merck). ^{1}H NMR spectra were recorded on Avance III HD 400 MHz Bruker BioSpin and Avance III HD 500 MHz Bruker BioSpin spectrometers. Chemical shifts are reported in ppm relative to residual solvent signals of $CDCl_3$ (δ 7.26) and DMSO-d_6 (δ 2.50). High-resolution electrospray mass spectra were recorded on a Thermo Exactive orbitrap instrument. Molecular modelling was carried out using MacroModel (Schrödinger Release 2019-1). Energy minimization was conducted with manually pre-organized structures using the following parameters: force field, MMFFs; solvent, water; charge from, force field; cutoff, extended; method, PRCG; max. iterations, 2500; converge on, gradient; convergence threshold, 0.05.

Preparation of monomers: 8-Amino-2-quinolinecarboxylic acid monomer (Q^{Dap}) with an aminomethyl group in position 4 and 6-aminomethyl-2-pyridinecarboxylic acid monomer (P) were prepared by following the reported synthetic procedures.[4,5]

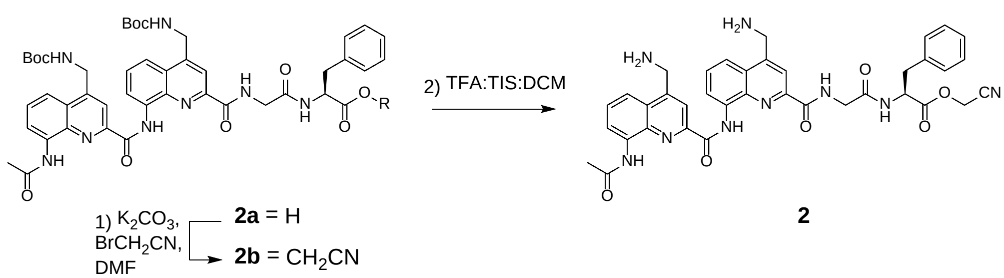
Preparation of cyanomethyl ester-activated foldamer substrates: Solid phase aromatic foldamer synthesis (including Fmoc deprotection, acid chloride activation and HBTU coupling, acetylation and resin cleavage), cyanomethyl ester installation and final TFA labile side chain-deprotection were carried out by following the reported procedures.[6]



Compound 1a: Compound **1a** was synthesized on a H-(*L*)-Phe-2CT resin (12.1 µmol).[6] Cleavage of the resin gave compound **1a**, which was used without further purification (4.0 mg, 59%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.36 (s, 1H), 9.81 (t, *J* = 6.3 Hz, 1H), 8.76 (d, *J* = 7.7 Hz, 1H), 8.25 (d, *J* = 7.8 Hz, 1H), 8.11 (s, 1H), 7.86 (d, *J* = 8.4 Hz, 1H), 7.66-7.73 (m, 2H), 7.20-7.26 (m, 5H), 4.66-4.73 (m, 2H), 4.40-4.49 (m, 1H), 3.93-4.10 (m, 2H), 3.04-3.10 (m, 1H), 2.88-2.95 (m, 1H), 2.33 (s, 3H), 1.43 (s, 9H). HRMS (ESI$^-$): *m/z* calcd for $C_{29}H_{32}N_5O_7$ $[M-H]^-$ 562.2307 found 562.2314.

Compound 1b. Compound **1b** was synthesized from compound **1a** (4.0 mg, 7.1 µmol).[6] The crude residue was purified by silica gel column chromatography eluting with EtOAc/cyclohexane (9:1, v/v) to give **1b** as a white solid (2.1 mg, 49%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.35 (s, 1H), 8.65-8.78 (m, 2H), 8.13 (s, 1H), 7.50-7.59 (m, 2H), 7.20-7.30 (m, 3H), 7.13-7.17 (m, 2H), 6.61 (d, *J* = 7.8 Hz, 1H), 5.21 (t, *J* = 5.3 Hz, 1H), 4.95-5.01 (m, 1H), 4.63-4.83 (m, 4H), 4.05-4.29 (m, 2H), 3.11-3.22 (m, 2H), 2.31 (s, 3H), 1.51 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{31}H_{35}N_6O_7$ $[M+H]^+$ 603.2562 found 603.2561.

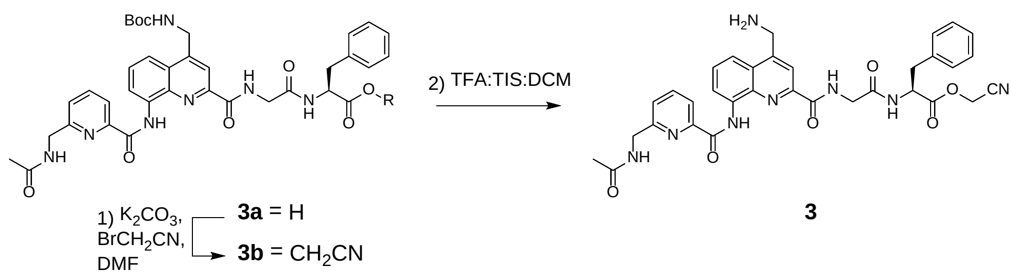
Compound 1. Compound **1** was synthesized from compound **1b** (2.0 mg, 3.32 µmol) as a white powder (1.45 mg, 86.8%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.39 (s, 1H), 9.88 (t, *J* = 7.9 Hz, 1H), 8.81 (d, *J* = 9.7 Hz, 1H), 8.72 (d, *J* = 9.3 Hz, 1H), 8.45-8.62 (br, 3H), 8.34 (s, 1H), 7.87 (dd, *J* = 10.6, 1.2 Hz, 1H), 7.77 (t, *J* = 10.2 Hz, 1H), 7.21-7.33 (m, 5H), 4.93-5.03 (m, 2H), 4.64-4.76 (m, 2H), 4.54-4.62 (m, 1H), 3.98-4.13 (m, 2H), 2.94-3.13 (m, 2H), 2.34 (s, 3H). HRMS (ESI$^+$): *m/z* calcd for $C_{26}H_{27}N_6O_5$ $[M+H]^+$ 503.2037 found 503.2032.



Compound 2a: Compound **2a** was synthesized on a H-(*L*)-Phe-2CT resin (9.83 µmol).[6] Cleavage of the resin gave compound **2a**, which was used without further purification (7.9 mg, 93%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.99 (s, 1H), 10.10 (s, 1H), 9.33 (t, *J* = 5.7 Hz, 1H), 8.88 (d, *J* = 7.6 Hz, 1H), 8.69 (d, *J* = 7.6 Hz, 1H), 8.23 (s, 1H), 8.15-8.21 (m, 1H), 8.11 (s, 1H), 8.02 (d, *J* = 8.4 Hz, 1H), 7.94 (d, *J* = 8.5 Hz, 1H), 7.78-7.87 (m, 2H), 7.71-7.77 (m, 2H), 7.09-7.18 (m, 5H), 4.71-4.81 (m, 4H), 4.19-4.27 (m, 1H), 3.77-3.94 (m, 2H), 2.75-2.95 (m, 2H), 2.08 (s, 3H), 1.45 (s, 9H), 1.44 (s, 9H). HRMS (ESI$^-$): *m/z* calcd for $C_{45}H_{49}N_8O_{10}$ $[M-H]^-$ 861.3577 found 861.3575.

Compound 2b. Compound **2b** was synthesized from compound **2a** (7.9 mg, 9.15 µmol).[6] The crude residue was purified by silica gel column chromatography eluting with EtOAc/cyclohexane (7:3, v/v) to give **2b** (2.2 mg, 27%). ^{1}H NMR (400 MHz, $CDCl_3$, 318 K): δ 11.73 (s, 1H), 9.62 (s, 1H), 8.87 (d, *J* = 7.5 Hz, 1H), 8.76 (d, *J* = 7.5 Hz, 1H), 8.49-8.54 (m, 1H), 8.29-8.34 (m, 2H), 7.72-7.82 (m, 2H), 7.62-7.70 (m, 2H), 7.18-7.27 (m, 3H), 7.01-7.06 (m, 2H), 6.17-6.25 (m, 1H), 5.07-5.25 (m, 2H), 4.83-4.94 (m, 4H), 4.53-4.70 (m, 3H), 4.02-4.16 (m, 2H), 2.92-3.05 (m, 2H), 2.16 (s, 3H), 1.50-1.53 (m, 18H). HRMS (ESI$^+$): *m/z* calcd for $C_{47}H_{52}N_9O_{10}$ $[M+H]^+$ 902.3832 found 902.3830.

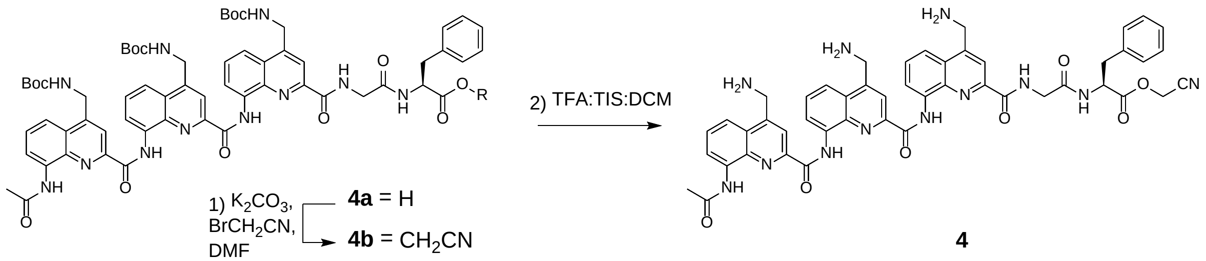
Compound 2. Compound **2** was synthesized from compound **2b** (2.2 mg, 2.44 μmol) as a white powder (1.43 mg, 84%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.02 (s, 1H), 10.17 (s, 1H), 9.38 (t, *J* = 6.0 Hz, 1H), 8.93 (d, *J* = 7.6 Hz, 1H), 8.75 (d, *J* = 7.7 Hz, 1H), 8.65-8.71 (m, 4H), 8.58-8.65 (br, 3H), 8.47 (s, 1H), 8.36 (s, 1H), 8.04 (d, *J* = 8.6 Hz, 1H), 7.91-7.98 (m, 2H), 7.83 (t, *J* = 8.2 Hz, 1H), 7.15-7.27 (m, 5H), 4.84-4.92 (m, 2H), 4.74-4.83 (m, 4H), 4.34-4.42 (m, 1H), 3.86-3.96 (m, 2H), 2.86-3.00 (m, 2H), 2.10 (s, 3H). HRMS (ESI$^+$): *m/z* calcd for $C_{37}H_{36}N_9O_6$ $[M+H]^+$ 702.2783 found 702.2781.



Compound 3a: Compound **3a** was synthesized on a H-(*L*)-Phe-2CT resin (11.44 μmol).[6] Cleavage of the resin gave compound **3a**, which was used without further purification (6.8 mg, 85%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.16 (s, 1H), 8.98 (d, *J* = 7.7 Hz, 1H), 8.89 (t, *J* = 6.1 Hz, 1H), 8.60-8.66 (br, 1H), 8.23 (d, *J* = 6.6 Hz, 1H), 8.16 (s, 1H), 8.13 (d, *J* = 7.4 Hz, 1H), 8.09 (t, *J* = 7.7 Hz, 1H), 7.98 (d, *J* = 8.4 Hz, 1H), 7.81 (t, *J* = 8.2 Hz, 1H), 7.76 (t, *J* = 6.1 Hz, 1H), 7.58 (d, *J* = 7.6 Hz, 1H), 7.17-7.22 (m, 5H), 4.72-4.79 (m, 2H), 4.44-4.55 (m, 2H), 4.34-4.44 (m, 1H), 4.04-4.17 (m, 2H), 2.87-3.06 (m, 2H), 1.89 (s, 3H), 1.44 (s, 9H). HRMS (ESI$^-$): *m/z* calcd for $C_{36}H_{38}N_7O_8$ $[M-H]^-$ 696.2787 found 696.2803.

Compound 3b. Compound **3b** was synthesized from compound **3a** (6.8 mg, 9.75 μmol).[6] The crude residue was purified by silica gel column chromatography eluting with EtOAc/cyclohexane (95:5, v/v) to give **3b** as a white solid (1.4 mg, 20%). ^{1}H NMR (500 MHz, 2% DMSO-d_6/$CDCl_3$ (v/v), 313 K): δ 11.99 (s, 1H), 9.01 (d, *J* = 9.4 Hz, 1H), 8.65 (t, *J* = 7.4 Hz, 1H), 8.30 (s, 1H), 8.21 (d, *J* = 9.7 Hz, 1H), 7.89 (t, *J* = 9.7 Hz, 1H), 7.66-7.79 (m, 2H), 7.52-7.62 (m, 1H), 7.36-7.43 (m, 1H), 6.99-7.17 (m, 6H), 5.29-5.41 (br, 1H), 4.75-4.90 (m, 3H), 4.60-4.75 (m, 2H), 4.50-4.57 (m, 2H), 4.20-4.33 (m, 2H), 3.00-3.16 (m, 2H), 1.98 (s, 3H), 1.47 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{38}H_{41}N_8O_8$ $[M+H]^+$ 737.3042 found 737.3041.

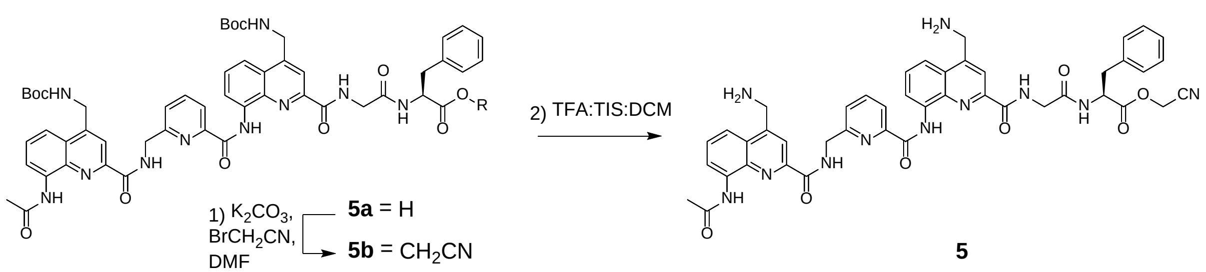
Compound 3. Compound **3** was synthesized from compound **3b** (1.4 mg, 1.90 μmol) as a white powder (1.02 mg, 84%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.17 (s, 1H), 9.04 (d, *J* = 9.5 Hz, 1H), 8.98 (t, *J* = 7.9 Hz, 1H), 8.74 (d, *J* = 9.1 Hz, 1H), 8.54-8.62 (br, 3H), 8.50 (t, *J* = 7.5 Hz, 1H), 8.39 (s, 1H), 8.08-8.17 (m, 2H), 7.99 (d, *J* = 10.5 Hz, 1H), 7.90 (t, *J* = 10.2 Hz, 1H), 7.62 (d, *J* = 9.1 Hz, 1H), 7.19-7.31 (m, 5H), 4.90-5.00 (m, 2H), 4.72-4.81 (m, 2H), 4.56-4.63 (m, 1H), 4.47-4.55 (m, 2H), 4.08-4.23 (m, 2H), 2.95-3.10 (m, 2H), 1.91 (s, 3H). HRMS (ESI$^+$): *m/z* calcd for $C_{33}H_{33}N_8O_6$ $[M+H]^+$ 637.2518 found 637.2511.



Compound 4a: Compound **4a** was synthesized on a H-(*L*)-Phe-2CT resin (14.06 µmol).[6] Cleavage of the resin gave compound **4a**, which was used without further purification (6.5 mg, 40%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.33 (s, 1H), 11.88 (s, 1H), 9.44 (s, 1H), 8.98 (d, *J* = 7.6 Hz, 1H), 8.86 (d, *J* = 7.6 Hz, 1H), 8.34-8.40 (m, 1H), 8.25 (s, 1H), 8.21 (s, 1H), 8.09-8.17 (m, 1H), 8.06 (d, *J* = 8.5 Hz, 1H), 7.83-7.92 (m, 3H), 7.75 (t, *J* = 8.1 Hz, 1H), 7.70 (t, *J* = 5.6 Hz, 1H), 7.56-7.63 (m, 2H), 7.34 (t, *J* = 8.1 Hz, 1H), 7.16-7.31 (m, 2H), 6.98-7.12 (m, 5H), 5.10-5.22 (m, 2H), 4.79-4.85 (m, 2H), 4.67-4.79 (m, 2H), 4.56-4.65 (m, 2H), 4.03-4.15 (m, 1H), 2.73-2.86 (m, 2H), 1.75 (s, 3H), 1.50 (s, 9H), 1.47 (s, 9H), 1.46 (s, 9H). HRMS (ESI$^-$): *m/z* calcd for $C_{61}H_{66}N_{11}O_{13}$ [M-H]$^-$ 1160.4847 found 1160.4849.

Compound 4b. Compound **4b** was synthesized from compound **4a** (6.8 mg, 5.59 µmol).[6] The crude residue was purified by silica gel column chromatography eluting with EtOAc/cyclohexane (7:3, v/v) to give **4b** (3.2 mg, 48%). ^{1}H NMR (500 MHz, 2% DMSO-d_6/$CDCl_3$ (v/v), 313 K): δ 12.11 (s, 1H), 11.79 (s, 1H), 9.03 (s, 1H), 9.00 (d, *J* = 8.7 Hz, 1H), 8.83 (d, *J* = 9.5 Hz, 1H), 8.41 (s, 1H), 8.20 (s, 1H), 7.85 (d, *J* = 10.6 Hz, 1H), 7.67-7.81 (m, 5H), 7.53 (d, *J* = 10.6 Hz, 1H), 7.35 (s, 1H), 7.31 (t, *J* = 10.2 Hz, 1H), 7.13-7.24 (m, 3H), 7.01-7.08 (m, 2H), 6.90-6.99 (m, 1H), 6.24-6.30 (m, 1H), 5.46-5.55 (m, 1H), 5.00-5.13 (m, 1H), 4.91-4.98 (m, 2H), 4.77-4.87 (m, 2H), 4.55-4.73 (m, 4H), 4.45-4.53 (m, 1H), 3.47-3.60 (m, 2H), 2.84-3.04 (m, 2H), 1.83 (s, 3H), 1.55 (s, 9H), 1.52 (s, 9H), 1.50 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{63}H_{69}N_{12}O_{13}$ [M+H]$^+$ 1201.5102 found 1201.5099.

Compound 4. Compound **4** was synthesized from compound **4b** (3.2 mg, 2.66 µmol) as a yellow powder (2.0 mg, 83%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.38 (s, 1H), 11.98 (s, 1H), 9.58 (s, 1H), 9.03 (d, *J* = 7.6 Hz, 1H), 8.87 (d, *J* = 7.6 Hz, 1H), 8.68-8.82 (m, 6H), 8.64 (t, *J* = 6.1 Hz, 1H), 8.44-8.51 (m, 4H), 8.38-8.43 (m, 2H), 8.08 (d, *J* = 8.6 Hz, 1H), 7.92-8.01 (m, 2H), 7.85 (t, *J* = 8.1 Hz, 1H), 7.75 (d, *J* = 7.8 Hz, 1H), 7.70 (d, *J* = 8.4 Hz, 1H), 7.43 (t, *J* = 8.1 Hz, 1H), 7.39 (s, 1H), 7.07-7.25 (m, 5H), 4.83-4.91 (m, 2H), 4.77-4.85 (m, 2H), 4.67-4.75 (m, 2H), 4.45-4.58 (br, 2H), 4.24-4.32 (m, 1H), 3.37-3.58 (br, 2H), 2.82-2.93 (m, 2H), 1.79 (s, 3H). HRMS (ESI$^+$): *m/z* calcd for $C_{48}H_{45}N_{12}O_7$ [M+H]$^+$ 901.3529 found 901.3524.

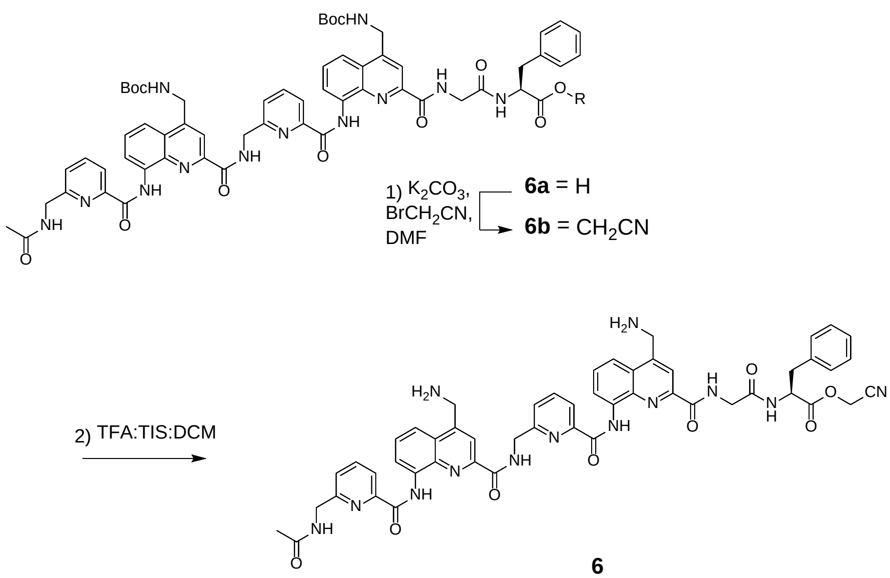


Compound 5a: Compound **5a** was synthesized on a H-(*L*)-Phe-2CT resin (7.40 µmol scale).[6] Cleavage of the resin gave compound **5a**, which was used without further purification (3.5 mg, 47%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.19 (s, 1H), 8.99 (d, *J* = 7.7 Hz, 1H), 8.91 (t, *J* = 5.6 Hz, 1H), 8.73 (d, *J* = 7.7 Hz, 1H), 8.43-8.51 (m, 1H), 8.07-8.19 (m, 4H), 7.96 (t, *J* = 7.6 Hz, 1H), 7.79-7.85 (m, 2H), 7.76 (t, *J* = 6.1 Hz, 1H), 7.72 (d, *J* = 7.5 Hz, 1H), 7.63-7.69 (m, 2H), 7.18-7.35 (m, 2H), 7.00-7.09 (m, 5H), 4.72-4.82 (m, 4H), 4.65-4.71 (m, 2H), 4.42-4.50 (m, 1H), 4.10-4.27 (m,

2H), 2.75-2.98 (m, 2H), 2.14 (s, 3H), 1.45 (s, 9H), 1.40 (s, 9H). HRMS (ESI$^-$): *m/z* calcd for $C_{52}H_{55}N_{10}O_{11}$ [M-H]$^-$ 995.4057 found 995.4061.

Compound 5b. Compound **5b** was synthesized from compound **5a** (3.5 mg, 3.51 µmol).[6] The crude residue was purified by silica gel column chromatography eluting with EtOAc/cyclohexane (7:3, v/v) to give **5b** (1.1 mg, 30%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.21 (s, 1H), 10.25 (s, 1H), 10.14 (t, *J* = 6.2 Hz, 1H), 8.99 (d, *J* = 7.6 Hz, 1H), 8.94 (t, *J* = 6.2 Hz, 1H), 8.79 (d, *J* = 7.5 Hz, 1H), 8.71 (d, *J* = 7.9 Hz, 1H), 8.15-8.20 (m, 2H), 8.09-8.14 (m, 2H), 7.97 (d, *J* = 8.3 Hz, 1H), 7.79-7.86 (m, 2H), 7.73-7.78 (m, 2H), 7.64-7.70 (m, 2H), 7.02-7.11 (m, 5H), 4.80-4.89 (m, 4H), 4.72-4.78 (m, 2H), 4.66-4.71 (m, 2H), 4.55-4.61 (m, 1H), 4.12-4.29 (m, 2H), 2.84-2.98 (m, 2H), 2.14 (s, 3H), 1.45 (s, 9H), 1.41 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{54}H_{58}N_{11}O_{11}$ [M+H]$^+$ 1036.4312 found 1036.4309.

Compound 5. Compound **5** was synthesized from compound **5b** (1.1 mg, 1.06 µmol) as a white powder (0.7 mg, 79%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.22 (s, 1H), 10.29 (s, 1H), 10.21 (t, *J* = 6.3 Hz, 1H), 9.04-9.09 (m, 2H), 8.86 (d, *J* = 7.5 Hz, 1H), 8.78 (d, *J* = 7.8 Hz, 1H), 8.52-8.67 (m, 6H), 8.38 (s, 1H), 8.36 (s, 1H), 8.19 (d, *J* = 7.6 Hz, 1H), 8.13 (t, *J* = 7.7 Hz, 1H), 8.00 (d, *J* = 8.6 Hz, 1H), 7.85-7.93 (m, 2H), 7.73-7.79 (m, 2H), 7.04-7.15 (m, 5H), 4.81-4.90 (m, 4H), 4.74-4.79 (m, 2H), 4.67-4.73 (m, 2H), 4.57-4.63 (m, 1H), 4.16-4.32 (m, 2H), 2.86-3.00 (m, 2H), 2.14 (s, 3H). HRMS (ESI$^+$): *m/z* calcd for $C_{44}H_{42}N_{11}O_7$ [M+H]$^+$ 836.3263 found 836.3258.



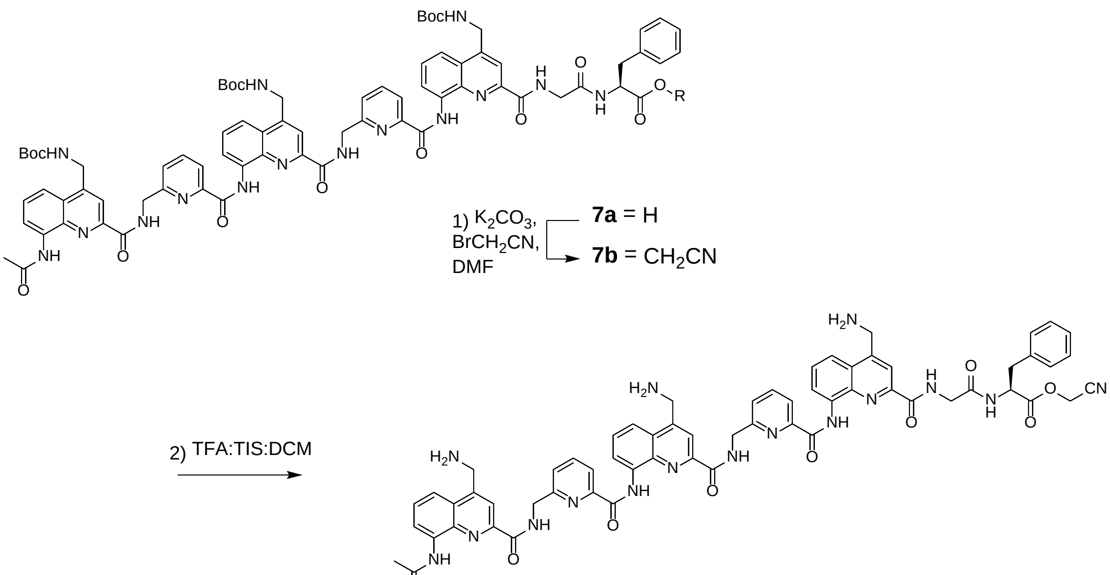
6

Compound 6a: Compound **6a** was synthesized on a H-(*L*)-Phe-2CT resin (7.67 µmol).[6] Cleavage of the resin gave compound **6a**, which was used without further purification (6.4 mg, 74%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 11.96 (s, 1H), 11.90 (s, 1H), 9.51-9.58 (m, 1H), 8.65-8.71 (m, 1H), 8.57-8.63 (m, 1H), 8.25-8.33 (m, 1H), 8.05-8.22 (m, 3H), 7.91-8.03 (m, 4H), 7.63-7.87 (m, 5H), 7.37 (d, *J* = 7.6 Hz, 1H), 7.17-7.32 (m, 2H), 6.96-7.07 (m, 6H), 4.95-5.04 (m, 2H), 4.63-4.79 (m, 6H), 4.26-4.36 (m, 1H), 3.78-3.98 (m, 2H), 2.74-2.91 (m, 2H), 1.64 (s, 3H), 1.47 (s, 9H), 1.43 (s, 9H). HRMS (ESI$^-$): *m/z* calcd for $C_{59}H_{61}N_{12}O_{12}$ [M-H]$^-$ 1129.4537 found 1129.4535.

Compound 6b. Compound **6b** was synthesized from compound **6a** (6.4 mg, 5.66 µmol). The crude residue was purified by silica gel column chromatography eluting with MeOH/DCM (5:95, v/v) to give **6b** (3.2 mg, 48%).[6] ^{1}H NMR (500 MHz, 2% DMSO-d_6/$CDCl_3$ (v/v), 313 K): δ 11.52 (s, 1H), 11.46 (s, 1H), 9.30-9.38 (m, 1H), 8.38-8.46 (m, 2H), 8.27 (s, 1H), 8.12 (d, *J* = 9.4 Hz, 1H), 8.01 (t, *J* = 9.7 Hz, 1H), 7.85-7.91 (m, 1H), 7.63-7.82 (m, 4H), 7.48-7.62 (m, 3H), 7.06-7.25 (m, 7H), 6.31-6.38 (m, 1H), 6.00-6.07 (m, 1H), 5.30-5.44 (m, 1H), 4.93-5.06 (m, 2H), 4.59-4.88

(m, 7H), 3.58-3.77 (m, 4H), 2.99-3.11 (m, 2H), 1.82 (s, 3H), 1.55 (s, 9H), 1.54 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{61}H_{64}N_{13}O_{12}$ $[M+H]^+$ 1170.4792 found 1170.4788.

Compound 6. Compound **6** was synthesized from compound **6b** (3.2 mg, 2.73 µmol) as a white powder (2.1 mg, 79%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.01 (s, 1H), 11.98 (s, 1H), 9.61 (t, *J* = 5.8 Hz, 1H), 8.80-8.85 (m, 2H), 8.74 (d, *J* = 7.7 Hz, 1H), 8.54-8.71 (m, 7H), 8.41 (s, 1H), 8.23 (t, *J* = 6.1 Hz, 1H), 8.08-8.15 (m, 3H), 7.99-8.05 (m, 2H), 7.97 (d, *J* = 8.6 Hz, 1H), 7.92 (d, *J* = 8.5 Hz, 1H), 7.86 (t, *J* = 8.0 Hz, 1H), 7.79 (t, *J* = 8.1 Hz, 1H), 7.75 (dd, *J* = 7.0, 1.6 Hz, 1H), 7.40 (dd, *J* = 6.9, 1.9 Hz, 1H), 7.01-7.14 (m, 5H), 4.96-5.09 (m, 2H), 4.79-4.87 (m, 2H), 4.64-4.78 (m, 4H), 4.47-4.53 (m, 1H), 3.93-4.09 (m, 2H), 3.80-3.90 (m, 2H), 2.84-2.96 (m, 2H), 1.64 (s, 3H). HRMS (ESI$^+$): *m/z* calcd for $C_{51}H_{48}N_{13}O_8$ $[M+H]^+$ 970.3743 found 970.3738.



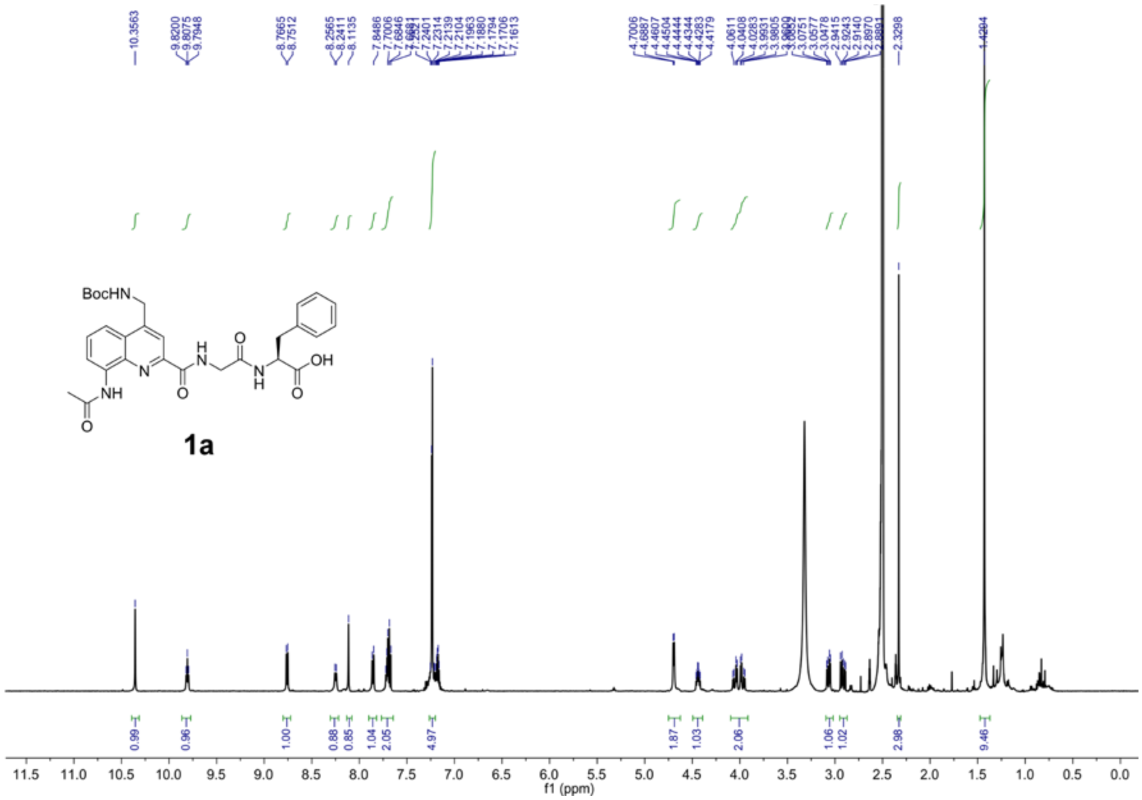
7

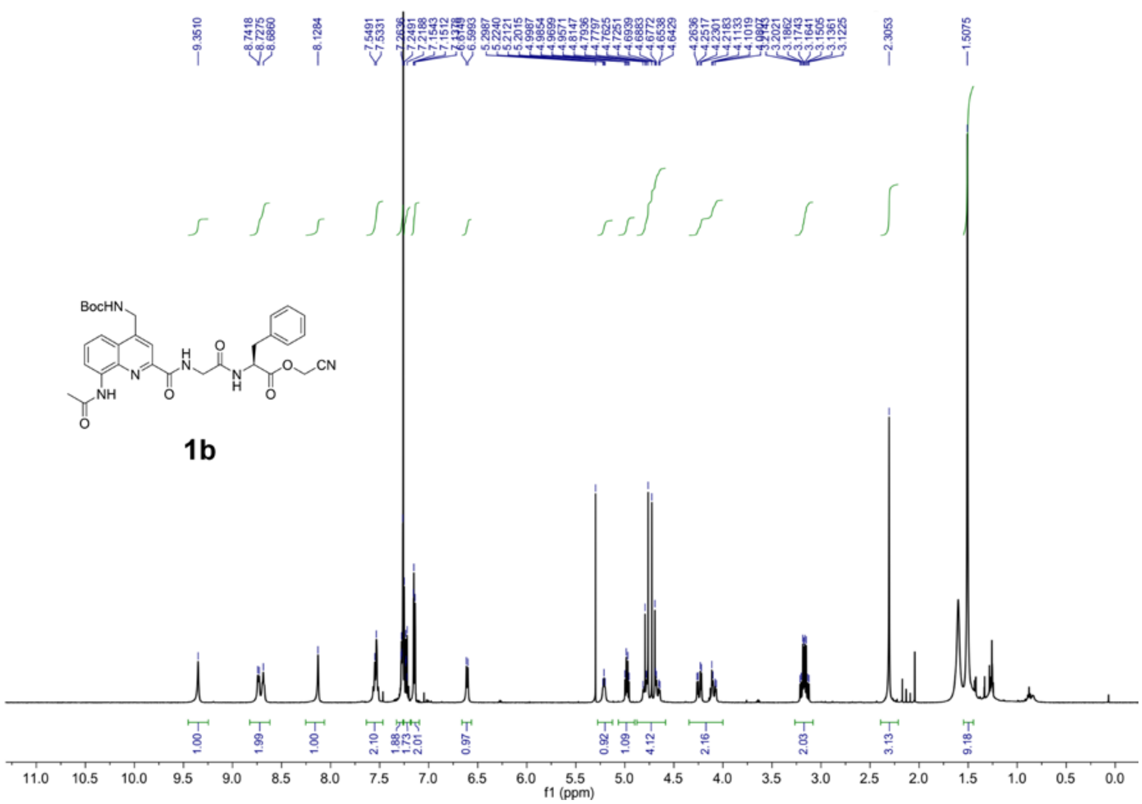
Compound 7a: Compound **7a** was synthesized on a H-(*L*)-Phe-2CT resin (7.67 µmol).[6] Cleavage of the resin gave compound **7a**, which was used without further purification (7.0 mg, 64%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.31 (s, 1H), 11.61 (s, 1H), 9.38 (t, *J* = 5.6 Hz, 1H), 8.82 (d, *J* = 7.4 Hz, 1H), 8.44-8.54 (m, 3H), 8.23 (s, 1H), 8.01-8.20 (m, 6H), 7.91-8.01 (m, 3H), 7.65-7.80 (m, 4H), 7.53-7.59 (m, 1H), 7.42-7.52 (m, 2H), 7.33-7.41 (m, 2H), 7.18-7.23 (m, 1H), 7.10 (d, *J* = 4.3 Hz, 1H), 6.87-6.97 (m, 5H), 5.05-5.21 (m, 4H), 4.72-4.79 (m, 2H), 4.46-4.56 (m, 2H), 4.35-4.43 (m, 2H), 4.14-4.26 (m, 2H), 4.01-4.11 (m, 1H), 2.70-2.90 (m, 2H), 2.11 (s, 3H), 1.47 (s, 9H), 1.46 (s, 9H), 1.40 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{75}H_{78}N_{15}O_{15}$ $[M-H]^-$ 1428.5807 found 1428.5814.

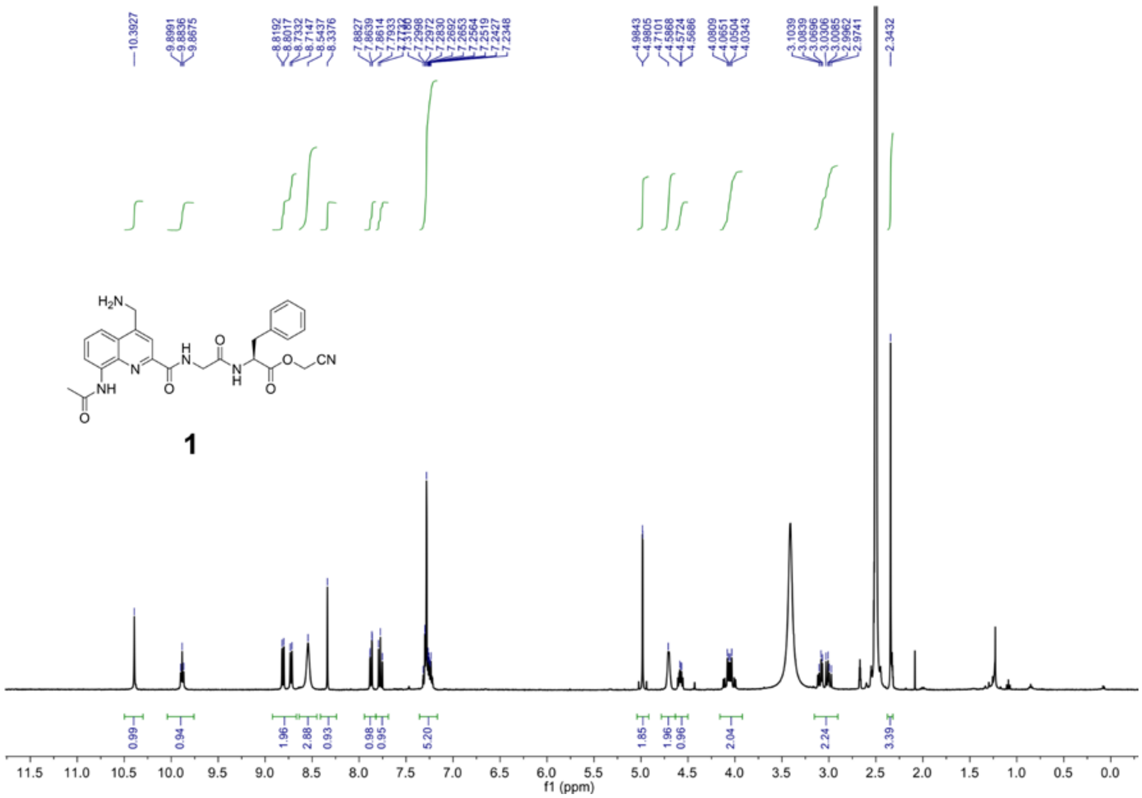
Compound 7b. Compound **7b** was synthesized from compound **7a** (7.0 mg, 4.89 µmol).[6] The crude residue was purified by silica gel column chromatography eluting with EtOAc/cyclohexane (95:5, v/v) to give **7b** (2.1 mg, 29%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.26 (s, 1H), 11.60 (s, 1H), 9.74 (t, *J* = 5.9 Hz, 1H), 9.69 (s, 1H), 9.36 (t, *J* = 6.0 Hz, 1H), 8.81 (d, *J* = 7.6 Hz, 1H), 8.74 (d, *J* = 7.2 Hz, 1H), 8.49 (d, *J* = 6.8 Hz, 1H), 8.38-8.44 (m, 2H), 8.26 (s, 1H), 8.11-8.19 (m, 2H), 8.04 (d, *J* = 7.5 Hz, 1H), 7.93-8.00 (m, 3H), 7.70-7.82 (m, 4H), 7.58-7.67 (m, 2H), 7.42-7.54 (m, 3H), 7.29-7.41 (m, 2H), 6.96-7.06 (m, 5H), 5.00-5.11 (m, 2H), 4.73-4.83 (m, 4H), 4.50-4.60 (m, 3H), 4.32-4.39 (m, 2H), 4.04-4.24 (m, 4H), 2.81-2.90 (m, 2H), 2.21 (s, 3H), 1.48 (s, 9H), 1.46 (s, 9H), 1.38 (s, 9H). HRMS (ESI$^+$): *m/z* calcd for $C_{77}H_{84}N_{17}O_{15}$ $[M+NH_4]^+$ 1486.6327 found 1486.6327.

Compound 7. Compound **7** was synthesized from compound **7b** (2.1 mg, 1.41 µmol) as a white powder (1.2 mg, 73%).[6] ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.25 (s, 1H), 11.66 (s, 1H), 9.90 (t, *J* = 6.2 Hz, 1H), 9.79 (s, 1H), 9.51 (t, *J* = 6.3 Hz, 1H), 8.90 (d, *J* = 7.8 Hz, 1H), 8.80 (d, *J* = 7.3 Hz, 1H), 8.65-8.74 (br, 3H), 8.52-8.60 (m, 4H), 8.47-8.51 (m, 2H), 8.36-8.45 (m, 3H), 8.15-8.21 (m, 2H), 8.08 (d, *J* = 7.2 Hz, 1H), 7.99-8.05 (m, 3H), 7.88 (t, *J* = 8.2 Hz, 1H), 7.77 (dd, *J* = 6.7, 2.1 Hz, 1H), 7.63 (d, *J* = 8.8 Hz, 1H), 7.53-7.59 (m, 2H), 7.50 (t, *J* = 8.1 Hz, 1H), 7.40 (d, *J* = 8.1 Hz, 1H), 7.01-7.10 (m, 5H), 5.04-5.17 (m, 2H), 4.75-4.88 (m, 4H), 4.49-4.63 (m, 3H), 4.35-4.44 (m, 2H), 4.09-4.26 (m, 4H), 2.86-2.97 (m, 2H), 2.21 (s, 3H). HRMS (ESI^+): *m/z* calcd for $C_{62}H_{57}N_{16}O_9$ $[M+H]^+$ 1169.4489 found 1169.4482.

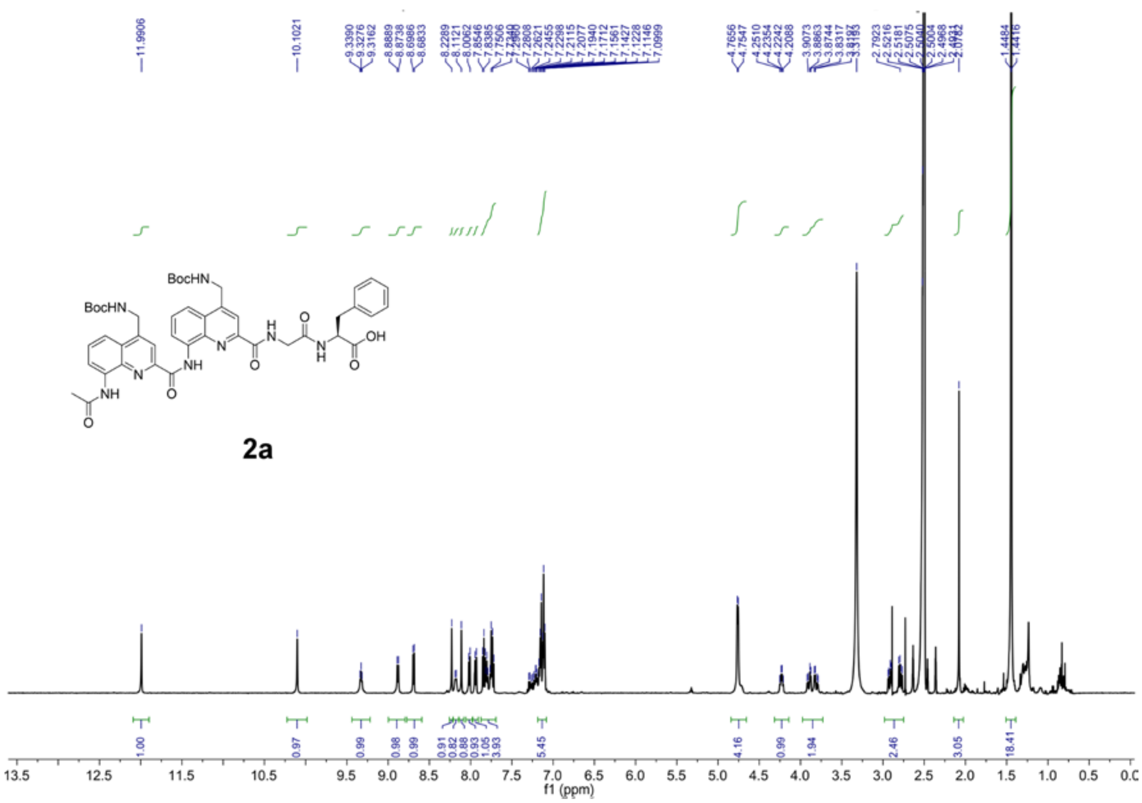
2.3. ^{1}H NMR spectra



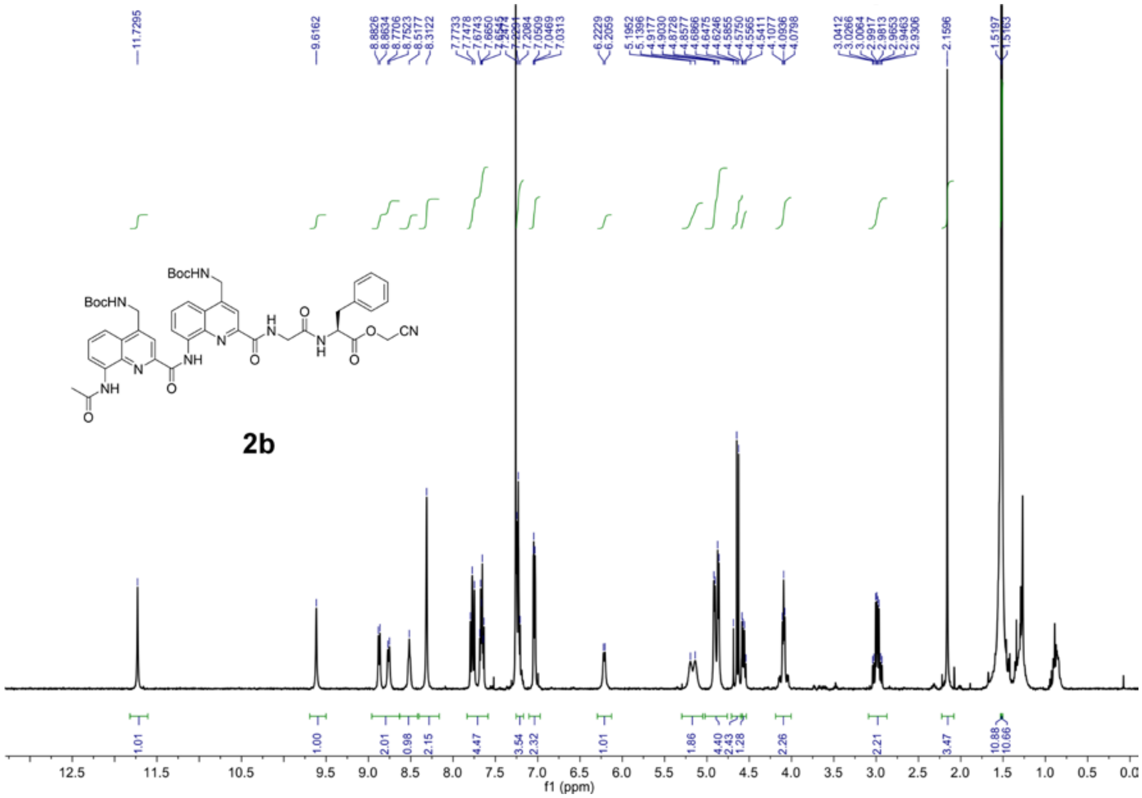




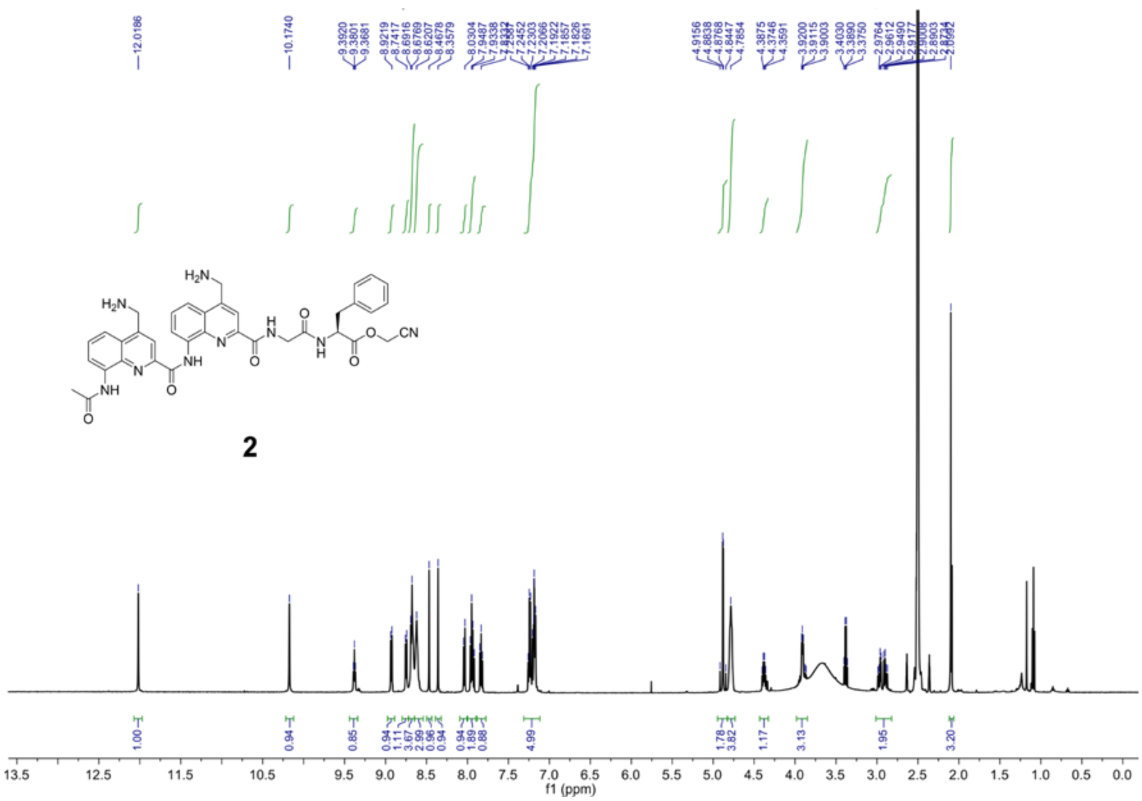
1



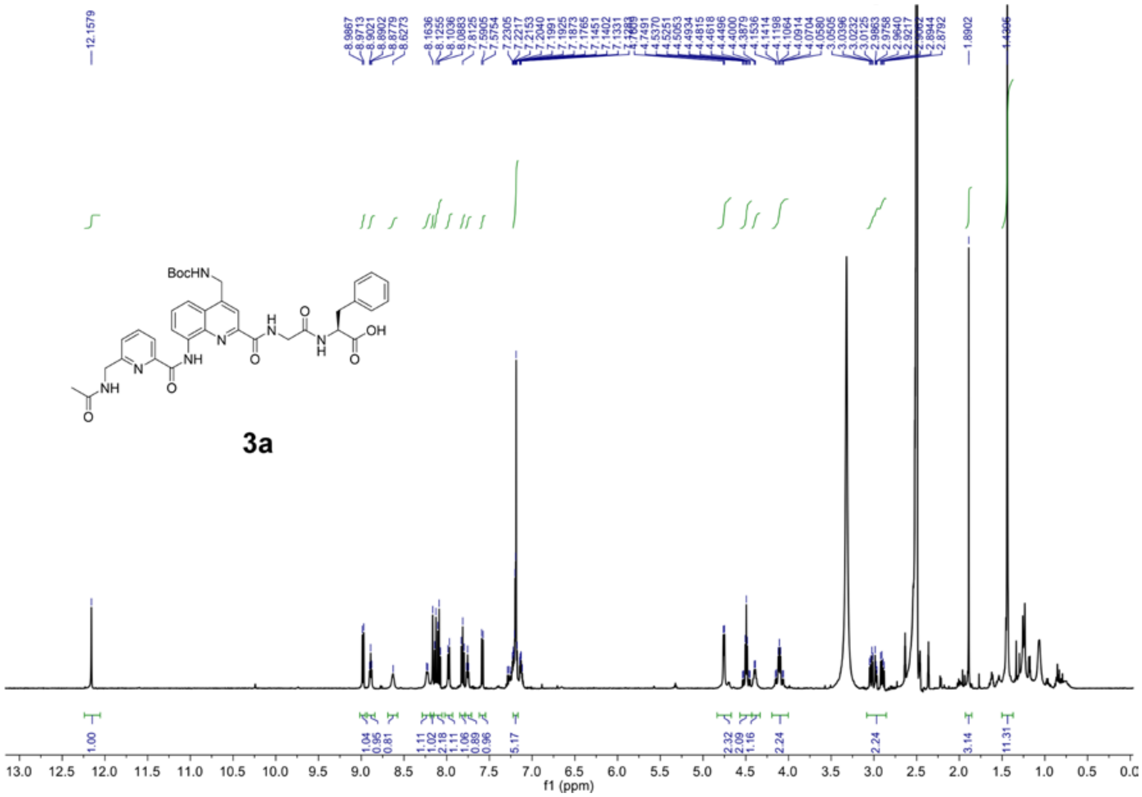
2a

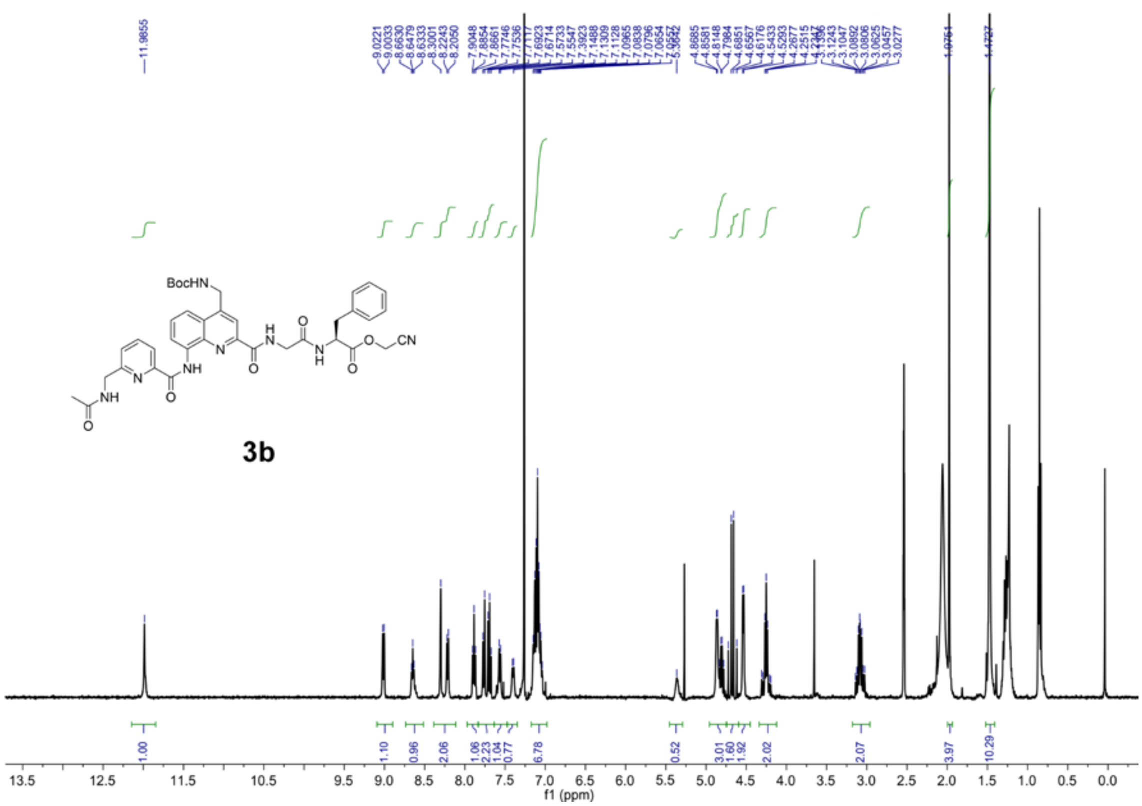


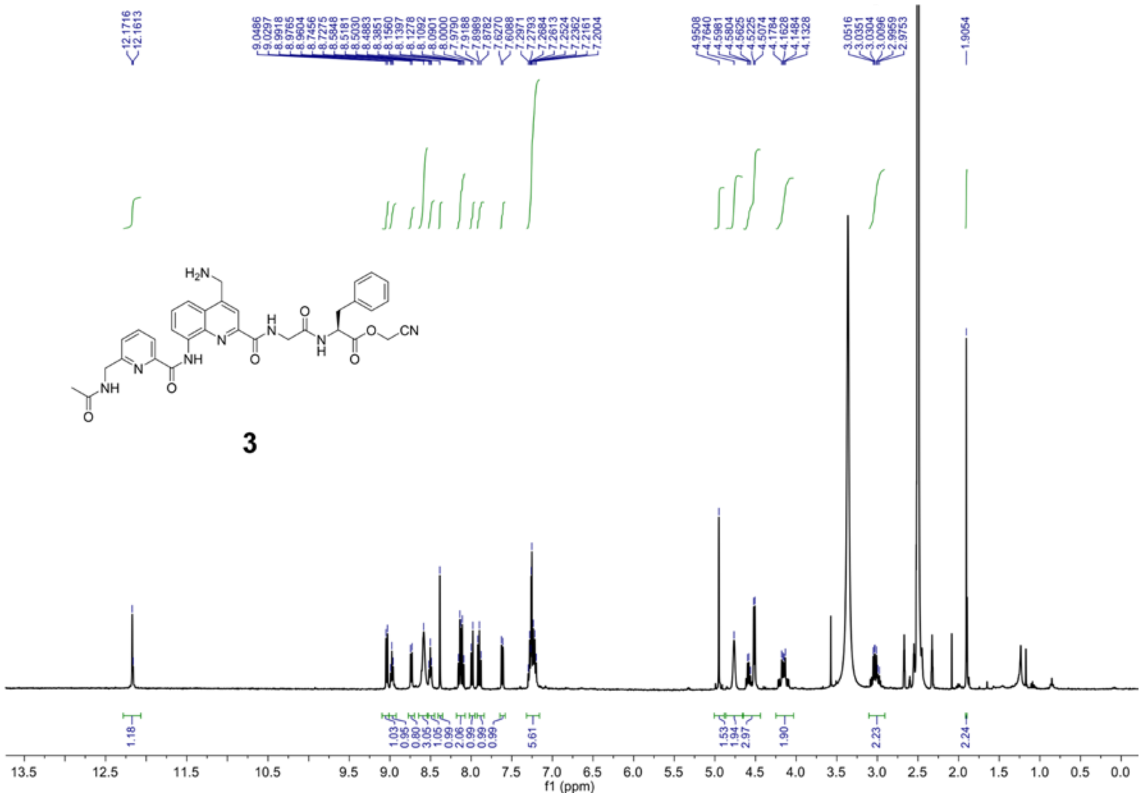
2b

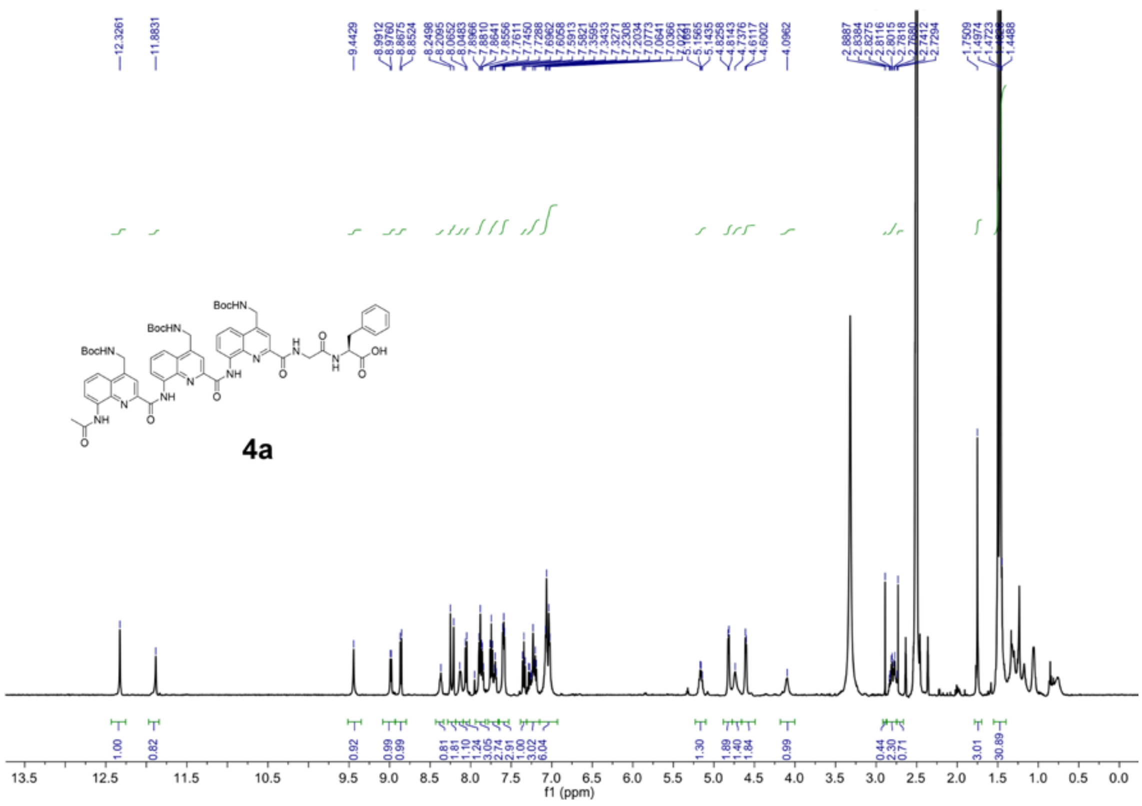


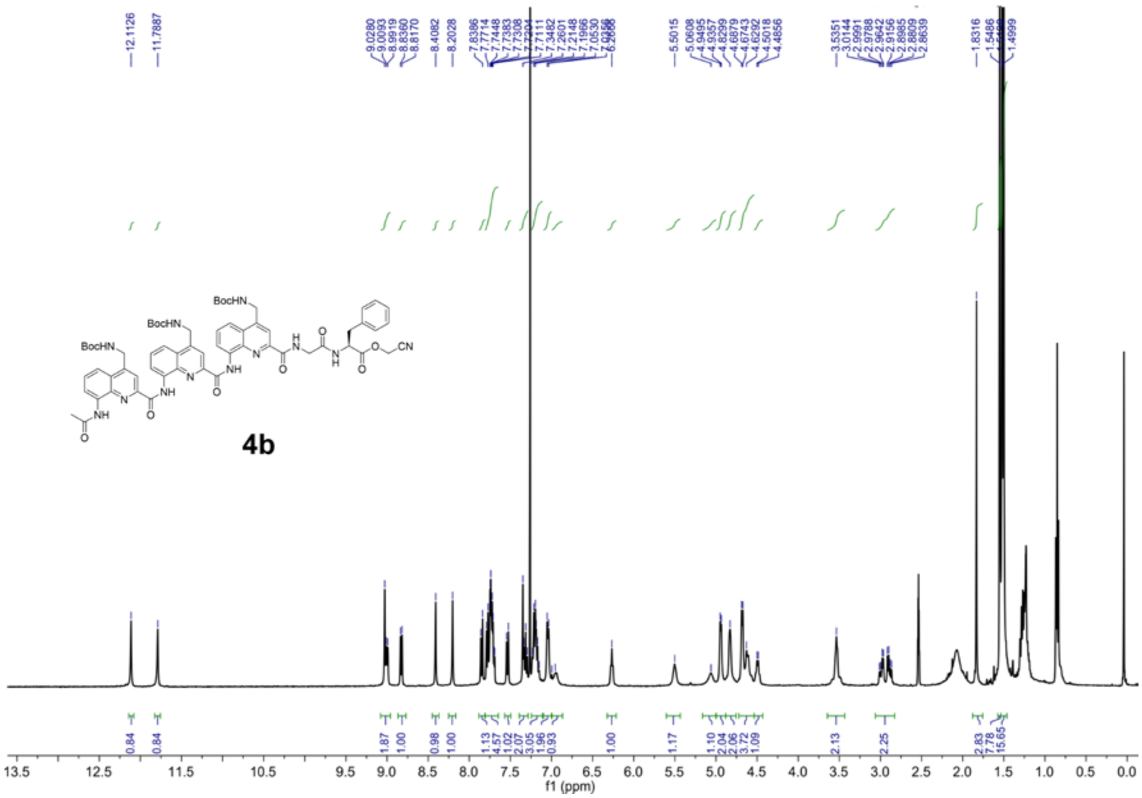
2

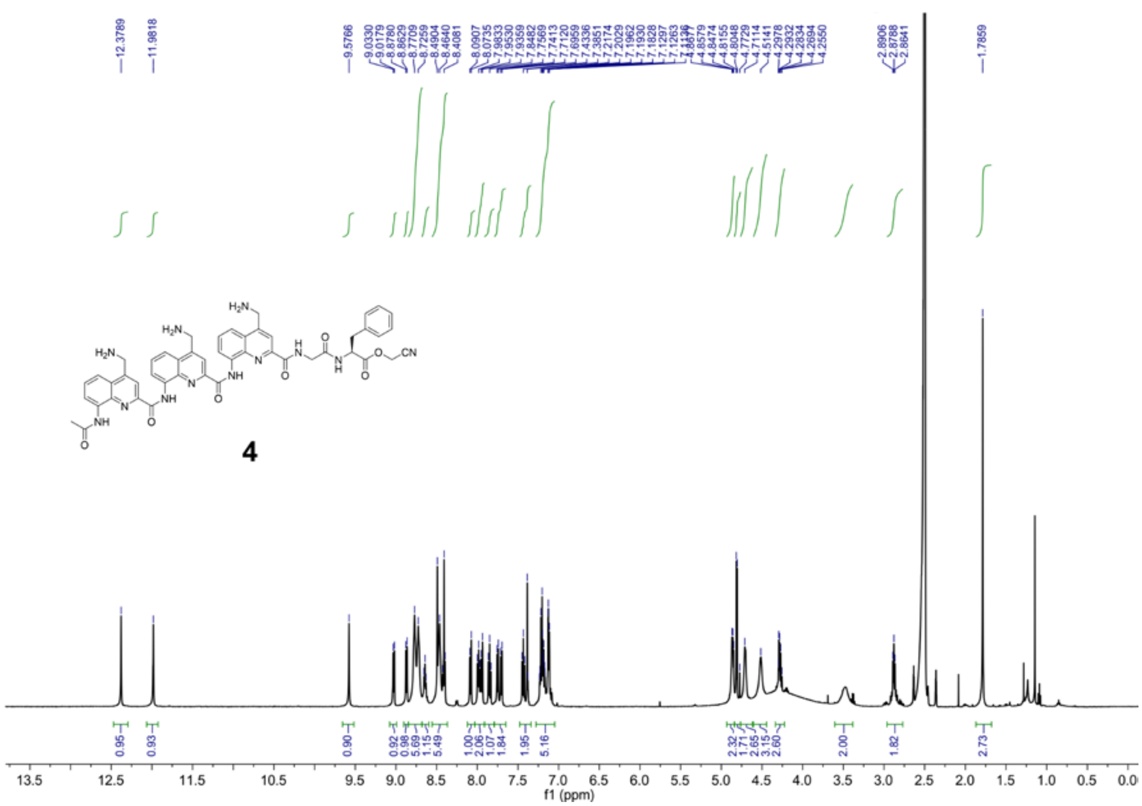

3a
f1 (ppm)

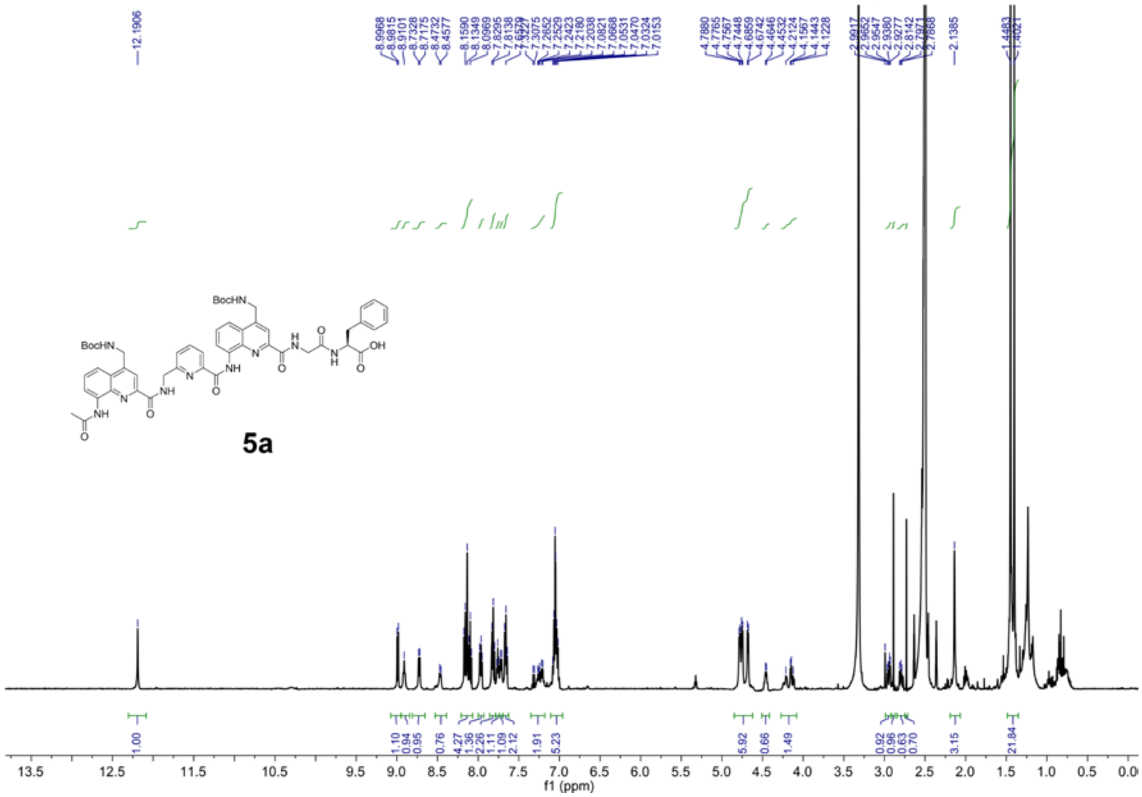

3b
f1 (ppm)

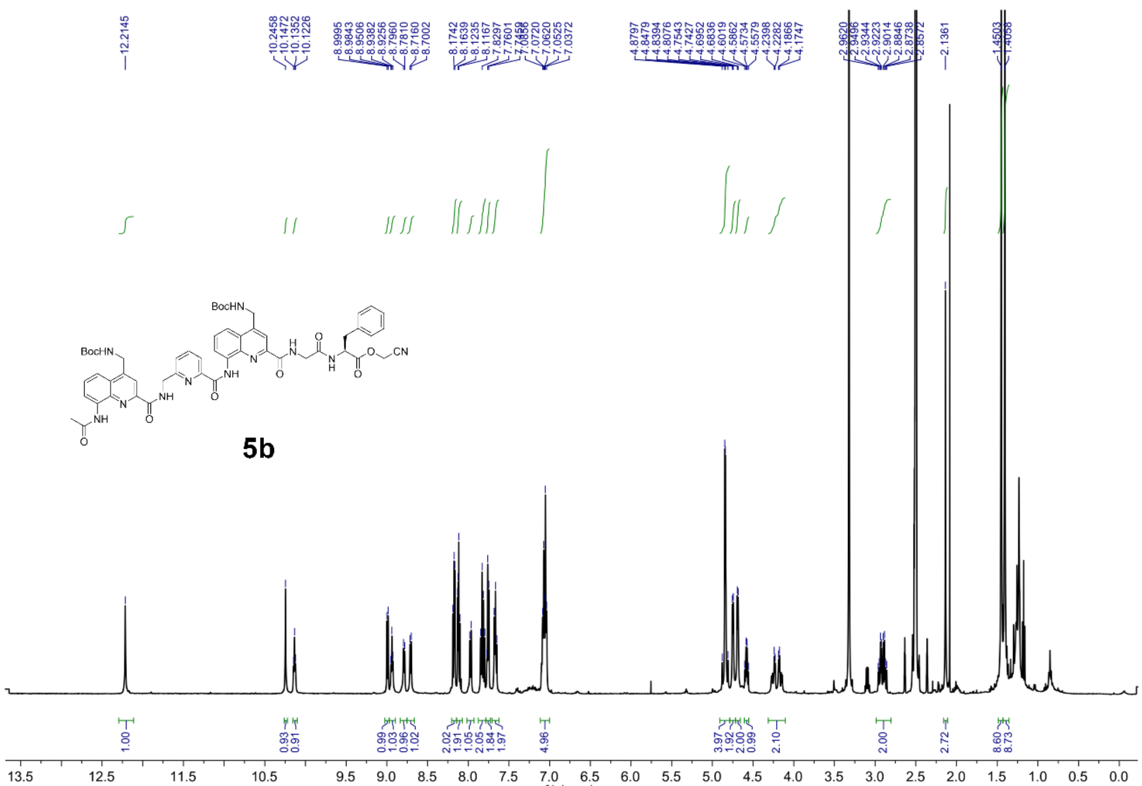

3
f1 (ppm)

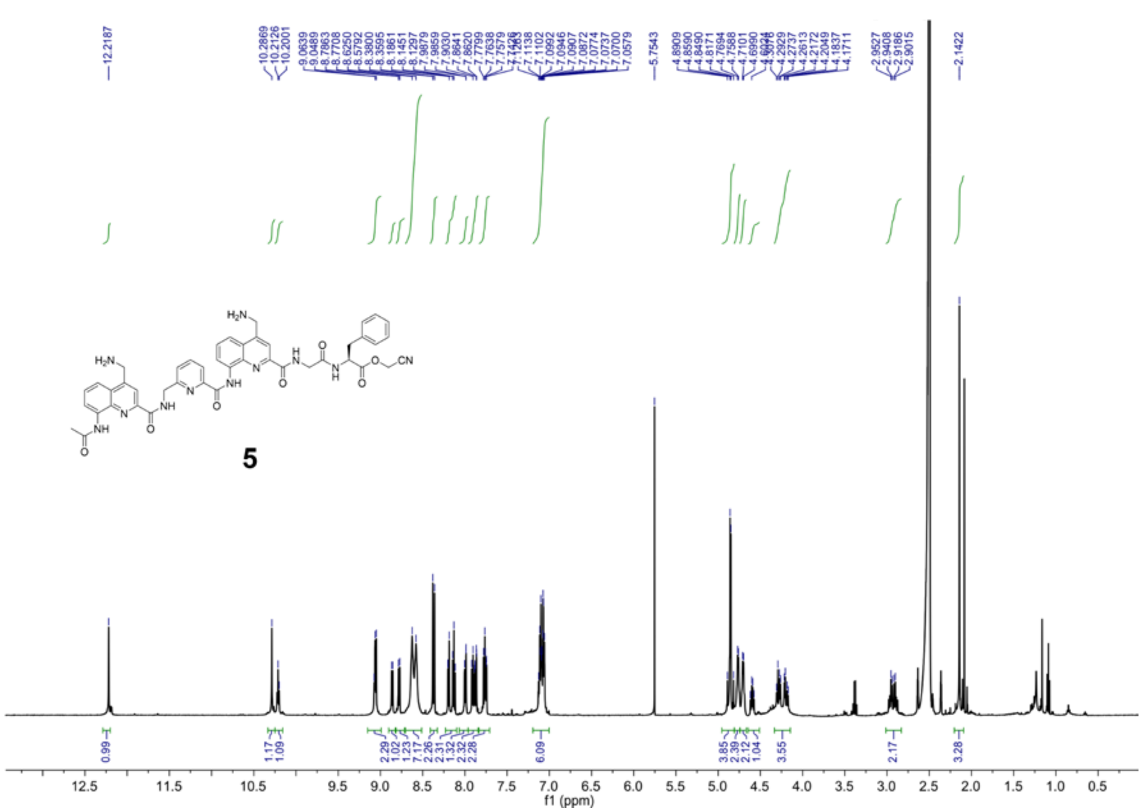

4a
f1 (ppm)


4b
f1 (ppm)

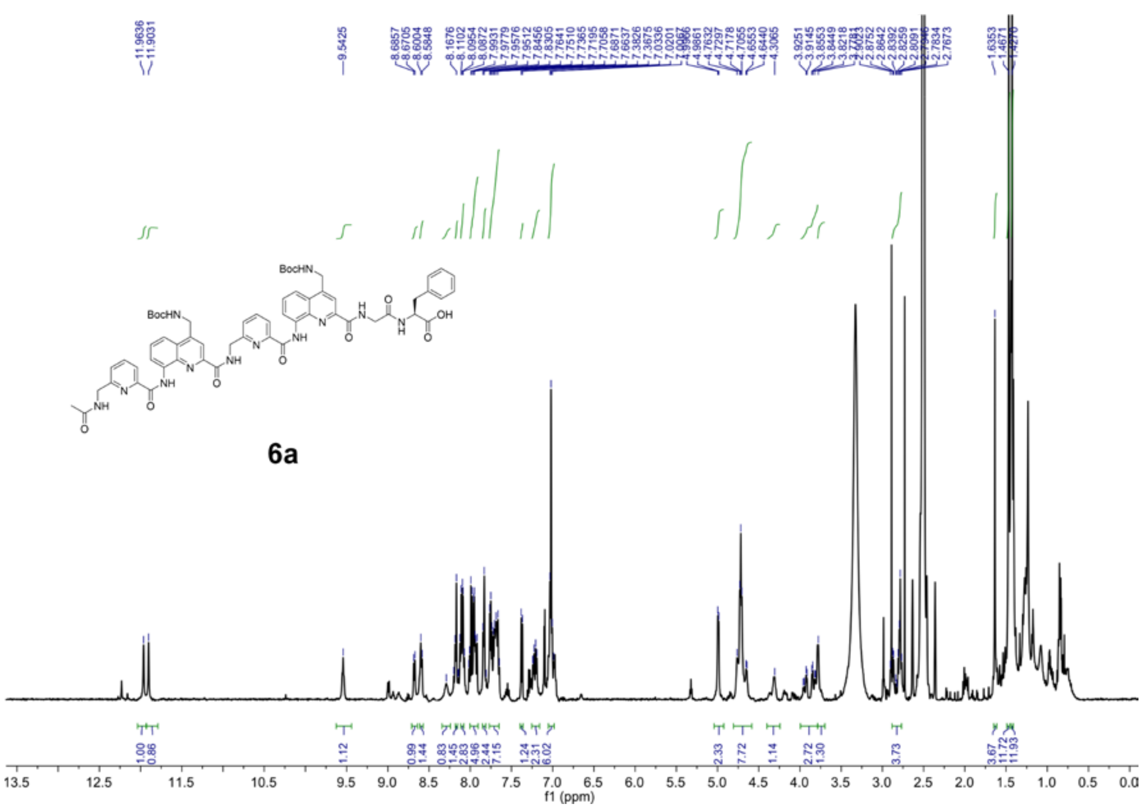

4
f1 (ppm)


5a
f1 (ppm)

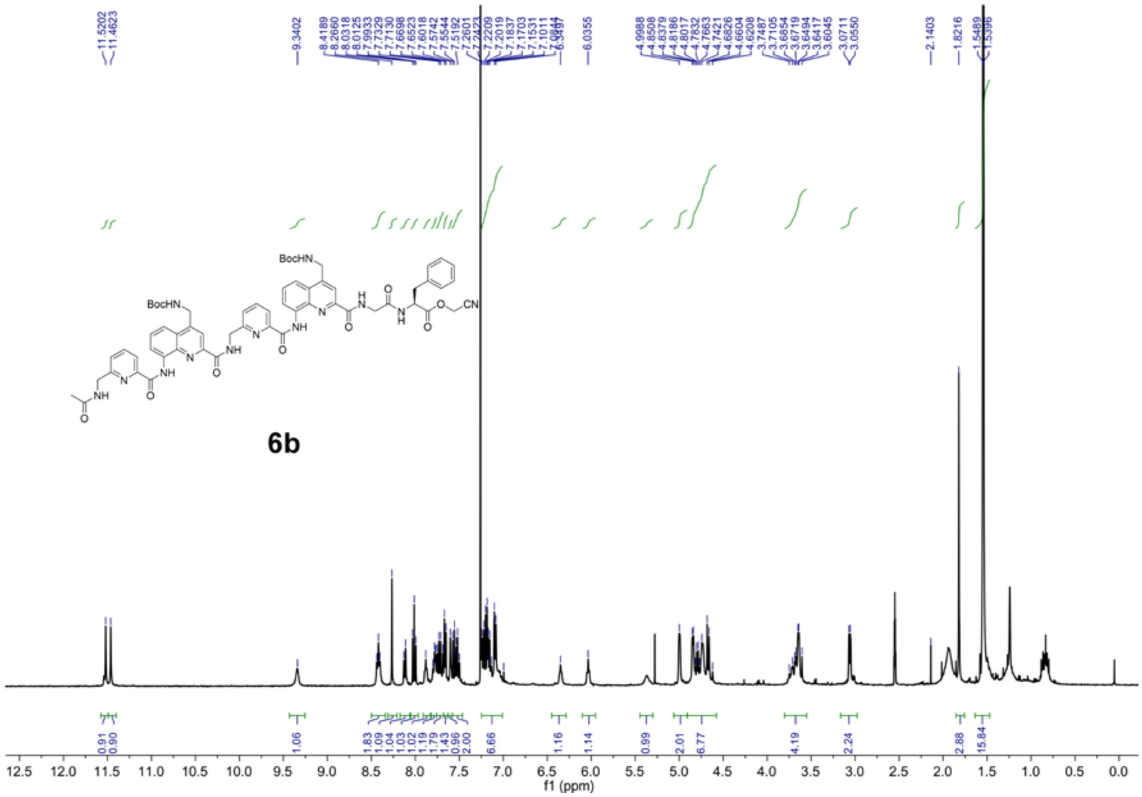

5b
f1 (ppm)

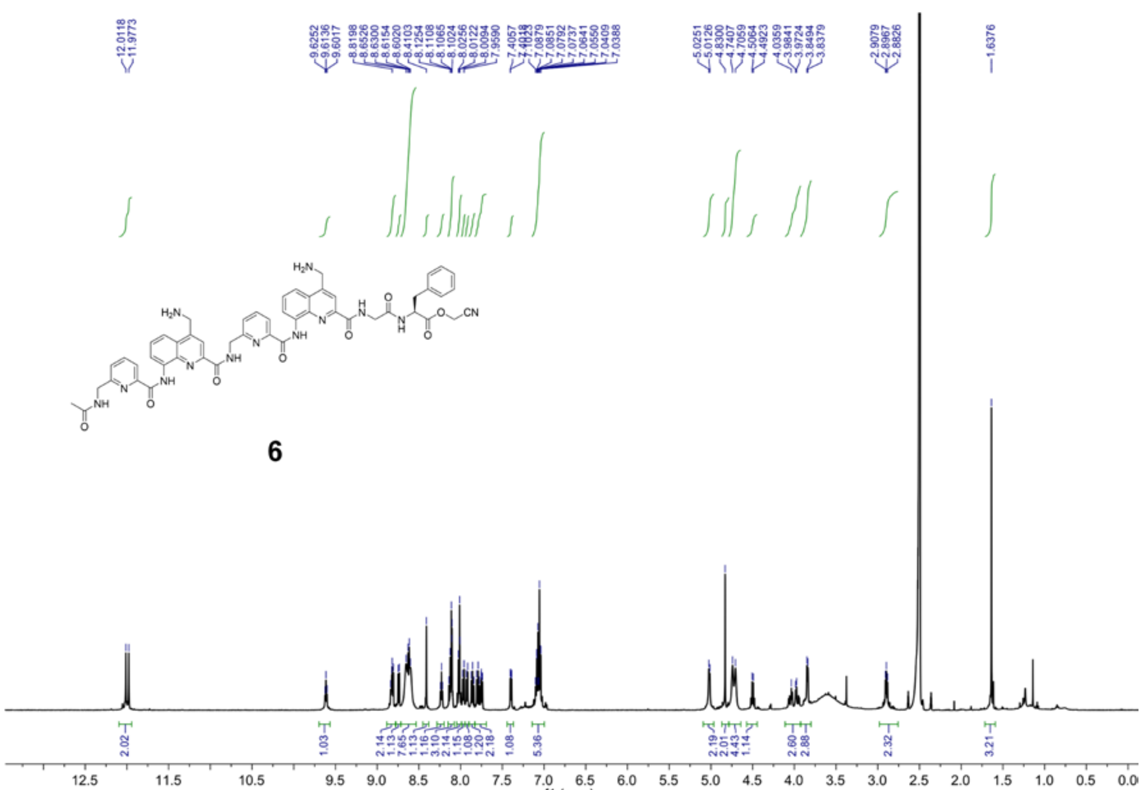


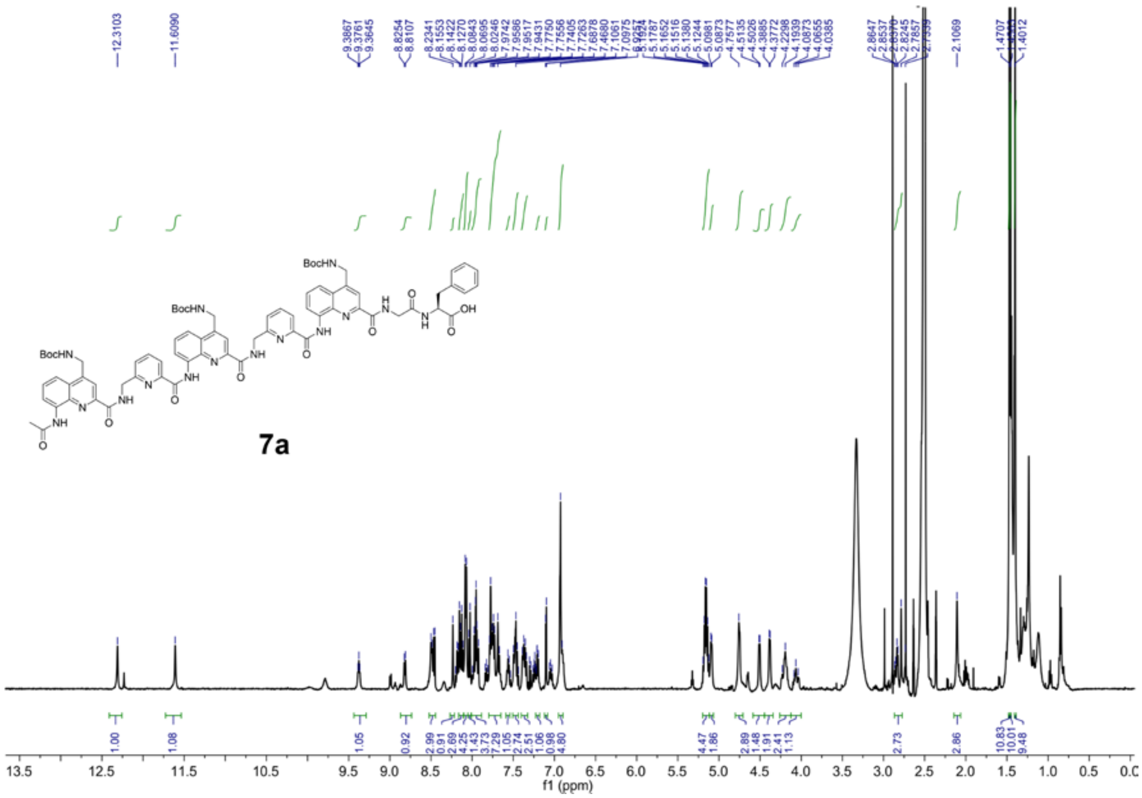
5



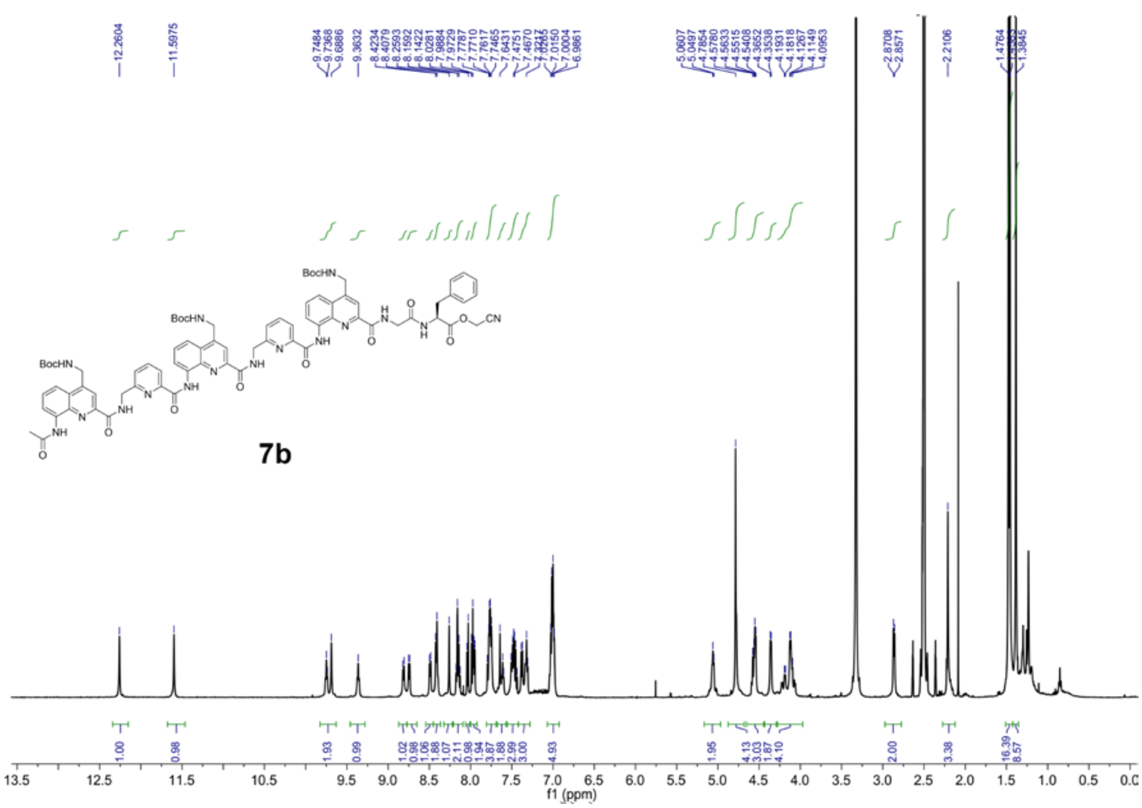
6a


BocHN
6b
f1 (ppm)

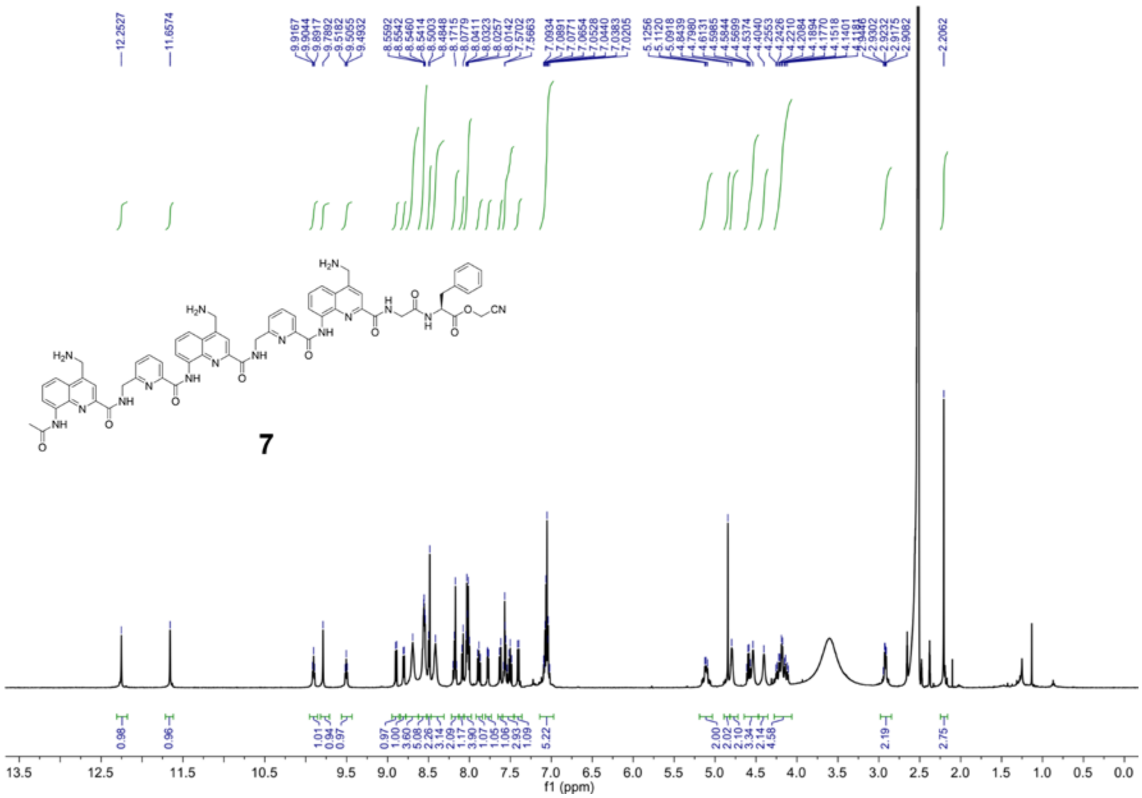

H_2N
6
f1 (ppm)



7a



7b



3. References

1. H. Murakami, A. Ohta, H. Ashigai and H. Suga, *Nat. Methods* **2006**, *3*, 357.
2. Y. Goto, A. Ohta, Y. Sako, Y. Yamagishi, H. Murakami and H. Suga, *ACS Chem. Biol.* **2008**, *3*, 120.
3. T. Katoh, Y. Iwane and H. Suga, *Nucleic Acids Res.* **2017**, *45*, 12601.
4. X. Hu, S. J. Dawson, P. K. Mandal, X. de Hatten, B. Baptiste and I. Huc, *Chem. Sci.* **2017**, *8*, 3741.
5. M. Vallade, P. S. Reddy, L. Fischer and I. Huc, *Eur. J. Org. Chem.* **2018**, 5489.
6. J. M. Rogers, S. Kwon, S. J. Dawson, P. K. Mandal, H. Suga and I. Huc, *Nat. Chem.* **2018**, *10*, 405.