

Electronic Supplementary Information

Identification of new quorum sensing autoinducer binding partners in Pseudomonas aeruginosa using photoaffinity probes

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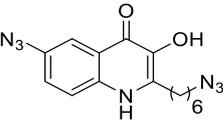
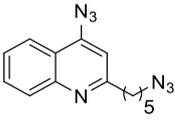
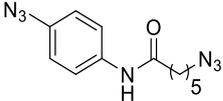
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1 Supplementary discussion

1.1 Evaluation of the probes photoreactivity

Characterisation of probes. The UV absorption spectra for each of the probes were recorded in the range of 200-550 nm under neutral conditions. Whilst all probes had a maximum absorption below 300 nm they all had a second peak in the range of $\lambda = 300-340$ nm except for probe 14 (Supplementary Table 1). The UV traces for the probes are given in Supplementary Appendix 1.

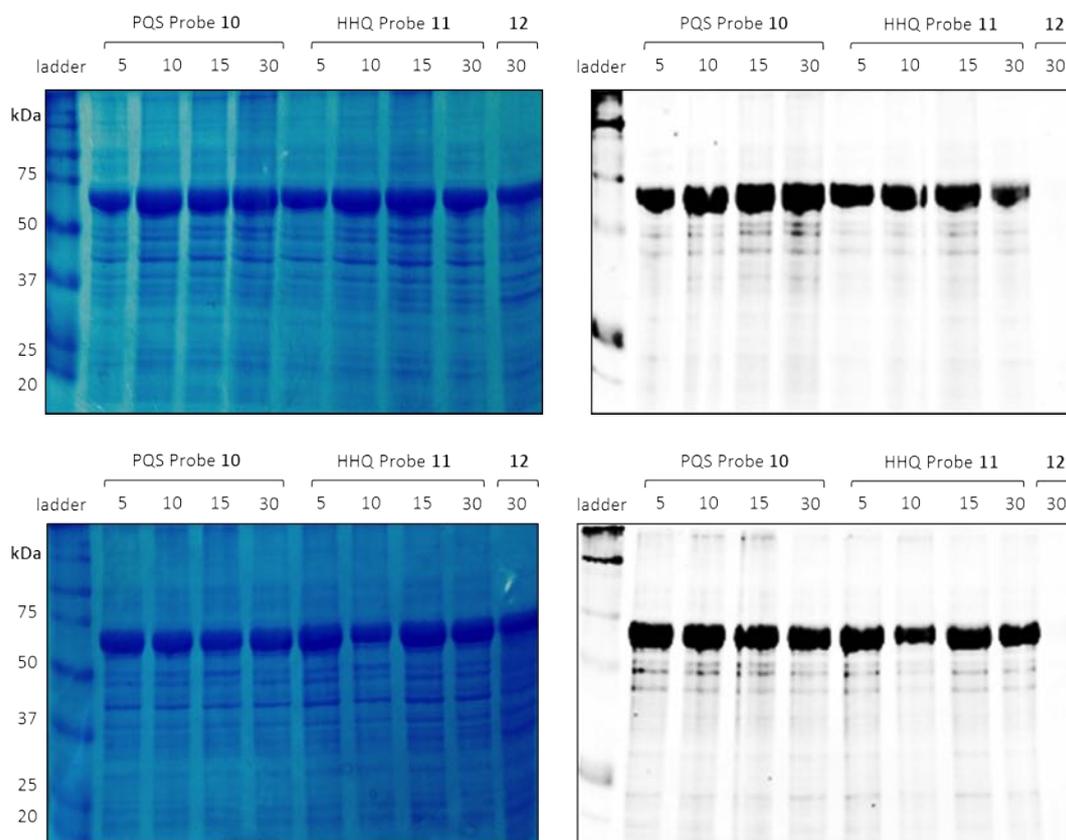
| Probe | λ_{\max} (nm) |
|--|-----------------------|
| 10  | 265 307 336 |
| 11  | 241 271 315 |
| 12  | 267 |

Supplementary Table 1. Quinolone probes and results of photo-reactivity studies using UV light.

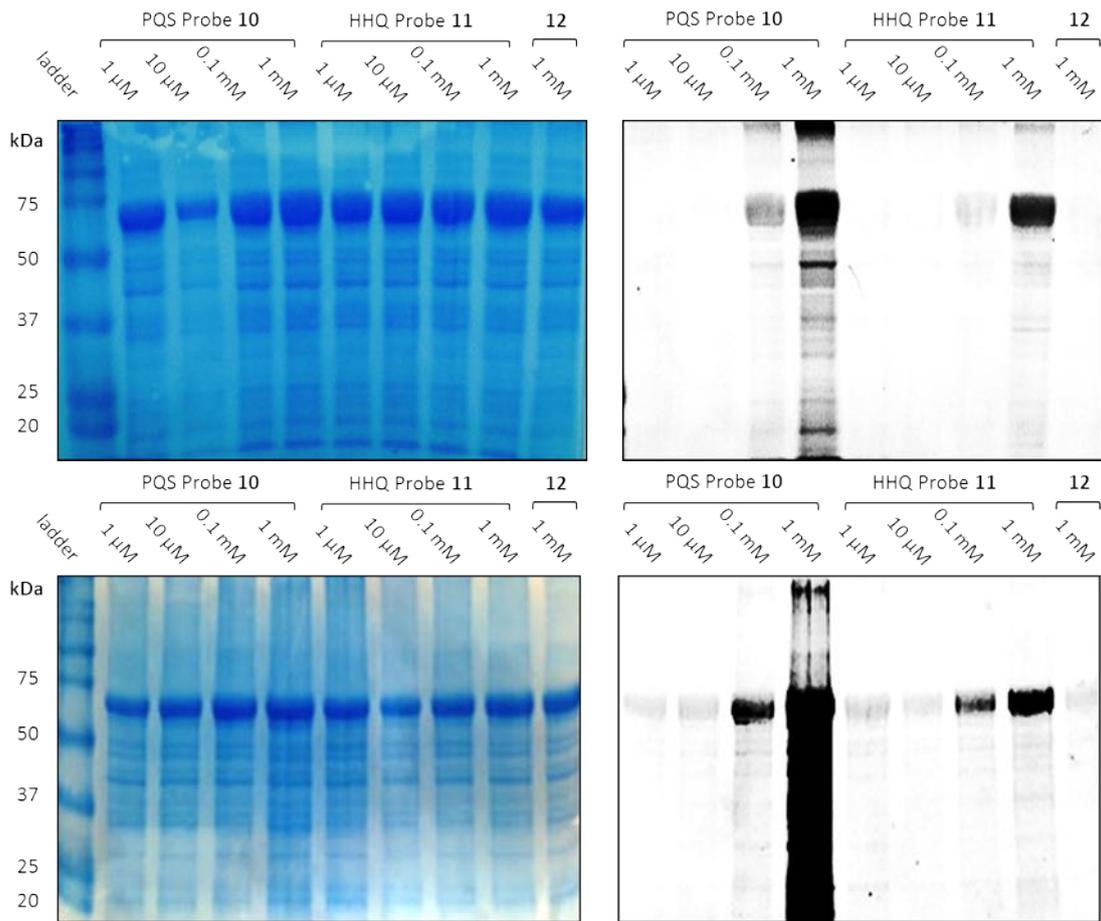
Optimisation of labelling conditions. The labelling protocol was investigated as follows. The ligand binding domain of PqsR was overexpressed as a maltose binding protein fusion (PqsR_{LBD}-MBP) using an *E. coli* strain with the plasmid pMAL-c2X containing a *pqsR* ligand binding domain (LBD) insert.¹ The cells were harvested, lysed in PBS containing a protease inhibitor cocktail tablet (Roche) by sonication and clarified by centrifugation. The cell pellet was discarded and the supernatant used as the cell-lysate samples. The Bradford Assay was then used to determine the protein concentration and the sample was diluted with PBS containing a protease inhibitor cocktail tablet (Roche) to a final concentration of 1 mg/mL.

PQS probe **10** and HHQ probe **11** were added to aliquots of the cell-lysate to a final concentration of 1 μ M in 1% DMSO. The samples were then irradiated using the inverted transilluminator setup keeping the samples at 4 °C. Samples were irradiated with either 302 nm or 365 nm UV light for either 5, 10, 15 or 30 min. Click chemistry was used to add a TAMRA (6-carboxytetramethylrhodamine) dye to the labelled proteins before the proteins were precipitated using TCA. The samples were resolved by SDS PAGE and scanned for

fluorescence prior to Coomassie staining. As a control, Probe **12** was also added to a final concentration of 1 μM in 1% DMSO and irradiated for 30 min using 302 nm. Both the PQS probe **10** and the HHQ probe **11** successfully labelled the overexpressed protein while probe **12** did not, confirming that the probes were capable of labelling the PqsR_{LBD}-MBP in cell-lysate. (Supplementary Fig. 1).

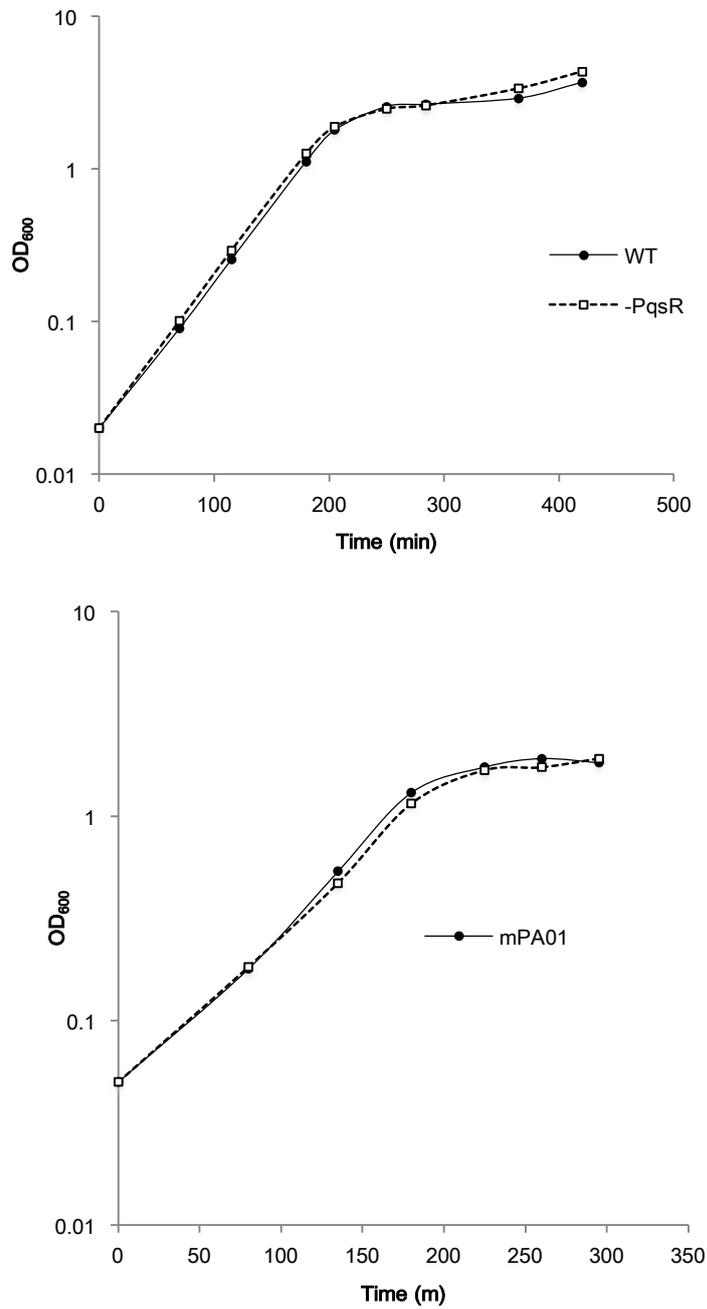


Supplementary Figure 1. Cell-lysate based labelling of PqsR_{LBD}-MBP using $\lambda = 302$ nm (Top) and 365 nm (bottom). Time of irradiation in minutes is given above each lane. 30 min irradiation was used in the case of control probe **12**. Left) gel stained with Coomassie blue; right) visualisation by fluorescent gel imaging. Time of irradiation is given in minutes.



Supplementary Figure 2. Labelling of PqsR_{LBD}-MBP in live *E. coli* using $\lambda = 302$ nm (top) and 365 nm (bottom). Concentration of probe is given above each lane. Left) gel stained with Coomassie blue; right) visualization by fluorescent gel imaging.

1.2 Growth curves and harvesting of the bacterial strains used in this study



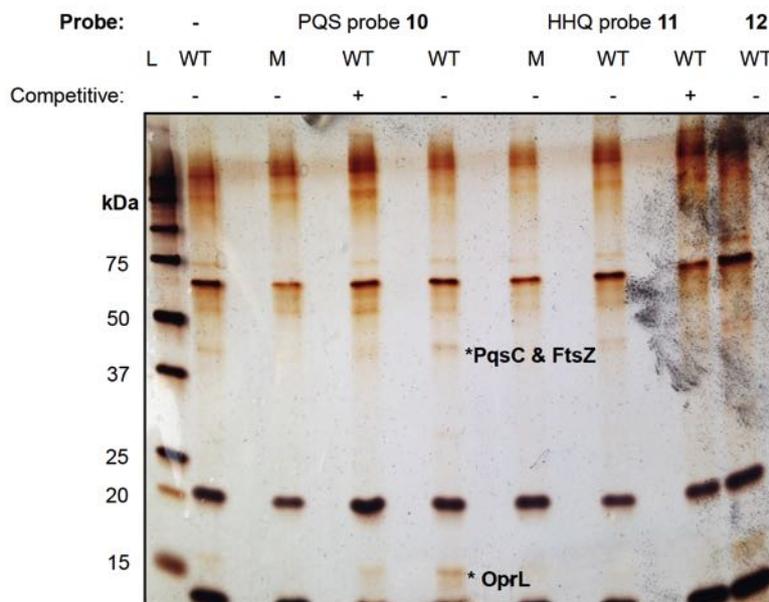
Supplementary Figure 3. Growth curve of cells that were harvested for pull-down experiments. A) Late exponential phase time point; B) Early stationary phase time point.

1.3 SDS PAGE analysis of pull-downs

Late exponential phase pull-downs. Cells were harvested at late exponential phase by centrifugation (Time point A, Supplementary Fig. 3). Late exponential phase was chosen initially as this lies just before the point at which the QS genes are “switched” on. A second reason for this is that Cao et al. reported that PqsR is at maximal levels during late exponential phase growth and is then cleaved and secreted into the supernatant in the stationary phase.² The harvested cells were lysed and centrifuged at low speed, keeping both the pellet and the supernatant.

PQS probe **10**, HHQ probe **11** and control probe **12** were used to prepare samples for MS identification. Cell-lysates were incubated with the probes at 10 μ M for 30 min before irradiation with UV light (302 nm, 15 min). Copper catalysed click chemistry was used to tag a biotin alkyne to the labelled proteins. The protein samples were then incubated with streptavidin-coated magnetic beads. The beads were then washed stringently and the captured proteins eluted by boiling in gel loading buffer containing SDS and free biotin. The proteins were then separated by SDS-PAGE and silver-stained (Supplementary Fig. 4).

In addition competitive binding experiments were also performed. In this case, 100 μ M of PQS was added to a second sample with 10 μ M of PQS probe **10** and 100 μ M of HHQ was added to a second experiments using 10 μ M of HHQ probe **11**. The pull-downs were performed identically to those described above.



Supplementary Figure 4. Silver stain of proteins isolated from late exponential phase labelled using probes **10**, **11** and **12**. Bands marked with an * were analysed by tryptic digest followed by LC-MS. + indicates the competitive control lane. WT = wild type cell-lysate; M = *pqsR* mutant cell-lysate.

For analysis, the proteins identified in the control lane of the gel at the same migration distance and proteins found in on-bead digest negative control experiments (discussed in further detail later) were removed. Ribosomal proteins were also disregarded, as they are known to be highly abundant and stick to the beads used.

The band at around 40 kDa had evidence of FtsZ (Mascot score of 80) and PqsC (Mascot score 39), a protein required for the synthesis of HHQ and PQS. Unfortunately, the LCMS trace for this sample had low levels of material. Despite this, PqsC had the highest scoring peptide (Supplementary Table 9).

The second band analysed at approximately 17 kDa was found to contain 27 potential proteins, many of which are presumably fragments of larger proteins. For this reason only the proteins that were also identified in the later on-bead digest were considered. The only protein from this list with convincing peptide scores was peptidoglycan-associated lipoprotein pal (also referred to as OprL in the literature) with a Mascot score of 85 and the correct mass for the migration by SDS-PAGE.

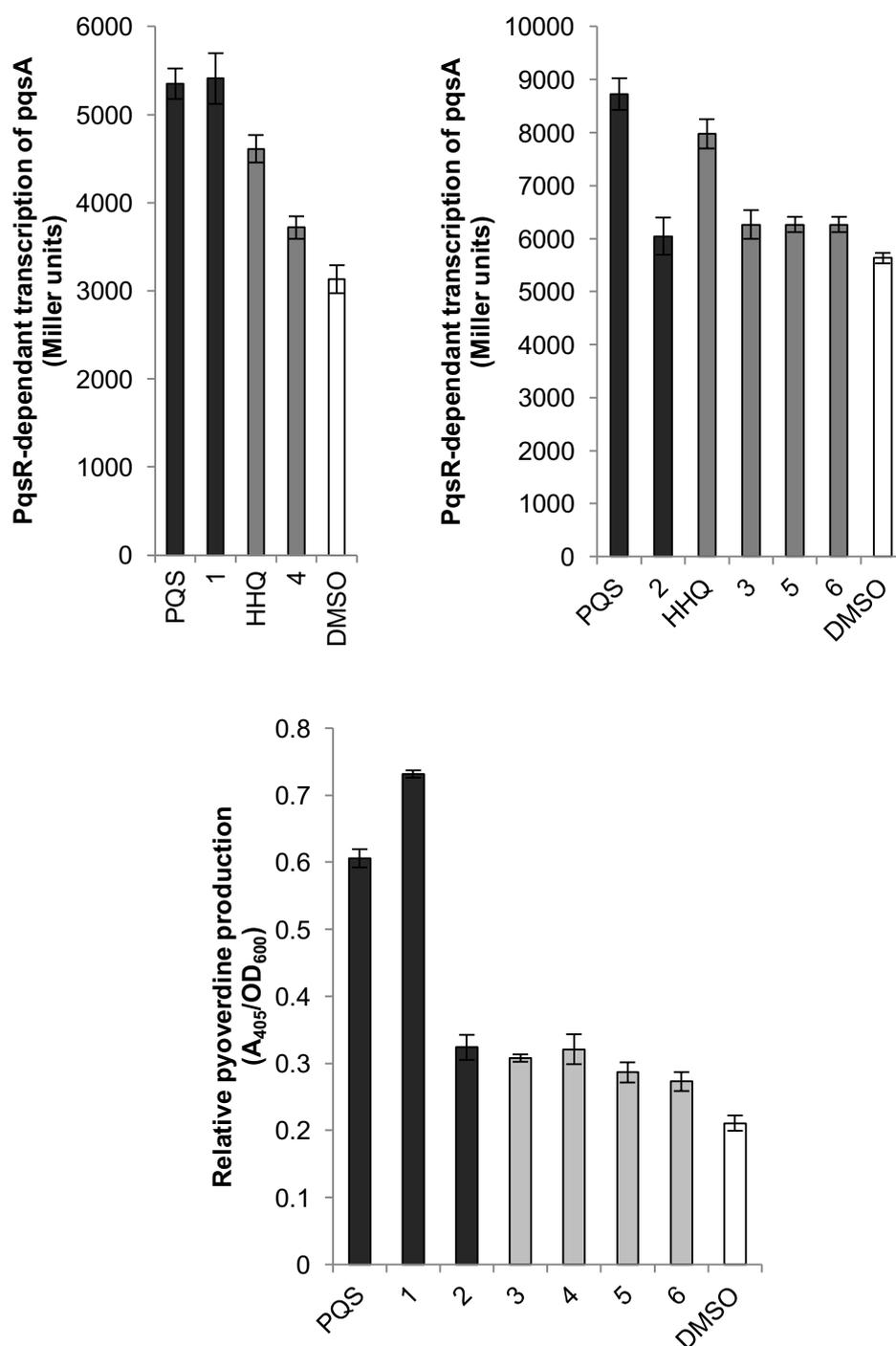
The evidence for the pull-down of OprL using the PQS probe **10** is strong, with three high-scoring peptides and the correct migration distance for the protein's molecular weight. The band is also intense when compared with the corresponding competitive binding assay. The band is not seen for probe **11** suggesting the protein interacts with PQS and not HHQ, again supporting the validity of this result. As the band can be seen with probe **12** it is also possible that it is protein that is under QS control which binds lipophilic compounds. Whilst further biochemical studies are required to confirm that PQS does in fact bind OprL, the pull-down of this protein is promising; one possible hypothesis is that the interaction between PQS and OprL is linked to membrane vesicle (MV) formation.

The role of PQS in membrane vesicle formation has been well documented. It has previously been shown that PQS stimulates membrane vesicle formation and it has been proposed that this is through direct association with the lipopolysaccharide in the outer membrane.³ Multiple studies have identified that MV formation occurs in regions where the membrane is not tethered to the peptidoglycan and it has been suggested that the loss of connections between the peptidoglycan layer and lipoproteins (LP) such as OprL are necessary for MV formation.⁴ In fact, loss of the 18 kDa pal homologue in *E. coli* has been shown to significantly increase MV formation.⁴

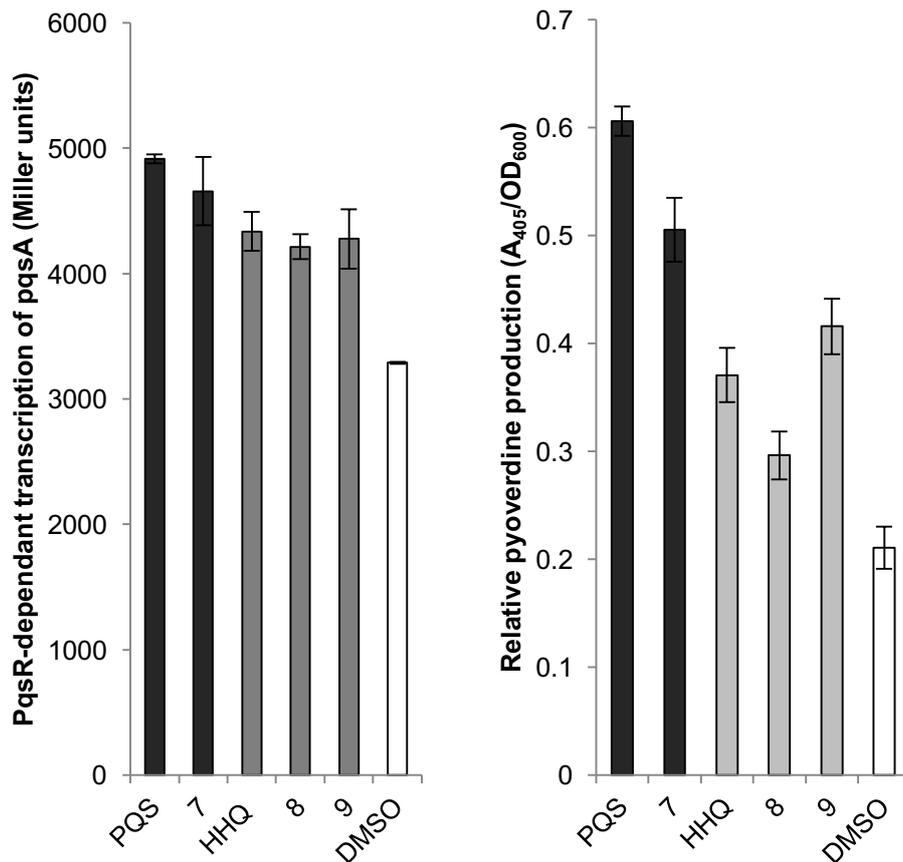
Building on this hypothesis, Wessel et al. demonstrated that deletion of the peptidoglycan-associated outer membrane proteins OprF and OprI, but not OprL, impact production of MVs by the opportunistic pathogen *P. aeruginosa*.⁵ If PQS does bind OprL (as is suggested by this pull-down), the interaction between OprL and PQS might be important in controlling MV formation.

2 Supplementary data

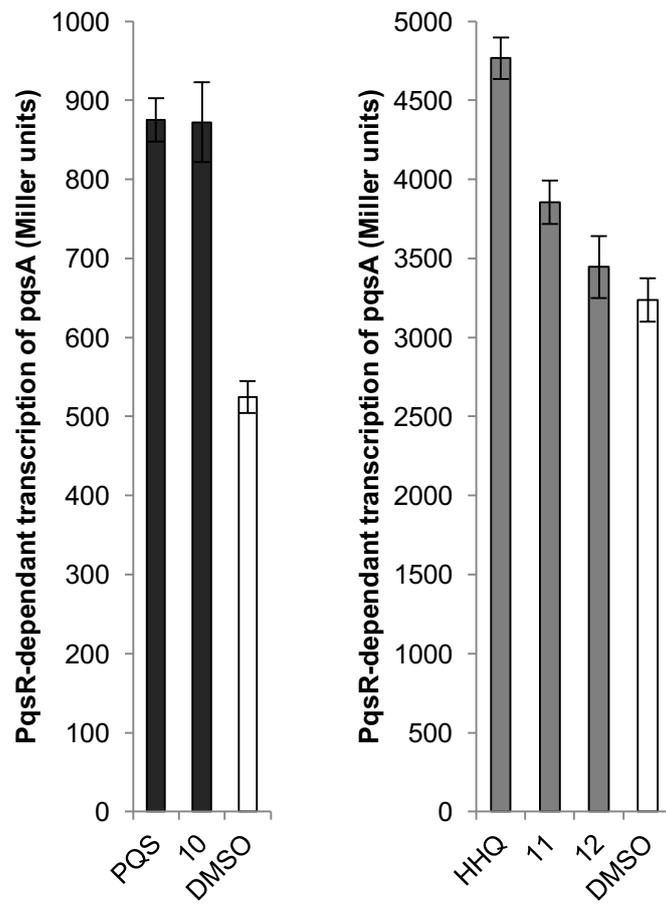
2.1 Biological data



Supplementary Figure 5. Effect of introducing ligation handles groups on quinolone activity. A) PqsR stimulation determined using the method reported by Cugini et al.⁶ These compounds were tested. B) Pyoverdine production determined using the method reported by Welsh et al.⁷ Dark bars show PQS analogues which were tested at 60 nM and light bars show HHQ analogues which were tested at 1 μM. Error bars represent the standard deviation of three independent repeats.



Supplementary Figure 6. Effect of introducing photoreactive groups on quinolone activity. A) PqsR stimulation determined using the method reported by Cugini et al.⁶ B) Pyoverdine production determined using the method reported by Welsh et al.⁷ Dark bars show PQS analogues which were tested at 60 nM and light bars show HHQ analogues which were tested at 1 μ M. Error bars represent the standard deviation of three independent repeats.



Supplementary Figure 7. Biological activity of final probes. PqsR stimulation determined using the method reported by Cugini et al.⁶

3 Supplementary tables

3.1 Peptide tables for proteins in text and supplementary discussion

Supplementary Table 2. Peptide table for proteins identified using PQS probe **10** in the wild type strain.

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|------------------|--|------------|-----------|------------|------------|------------|-----------|-------------|-----------|-----------|------------|---------------------------|------------|
| G3XD23 G3XD23 | WbpB: UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate oxidase | 253 | 35980 | 18 | 635.8074 | 1269.6002 | 2 | 1269.5989 | 0.0013 | 63.04 | 0.00000079 | AAGYLEYEQAR | 0.37 |
| | | | | | 680.3417 | 1358.6689 | 2 | 1358.6718 | -0.0028 | 59.87 | 0.0000033 | YEVDLTYITSR | 0.37 |
| | | | | | 717.3984 | 1432.7823 | 2 | 1432.7827 | -0.0003 | 72.62 | 0.00000018 | NFALIGAAGYIAPR | 0.37 |
| | | | | | 707.7087 | 2120.1042 | 3 | 2120.1014 | 0.0028 | 23.71 | 0.014 | SAVIVPASDNEGHPFV AALAR | 0.37 |
| Q9I246 | PA2069 : Probable carbamoyl transferase | 88 | 64141 | 7.5 | 596.8128 | 1191.6111 | 2 | 1191.6135 | -0.0024 | 66.73 | 0.00000076 | ITGLAAFQDAEK | 0.09 |
| | | | | | 681.6595 | 2041.9567 | 3 | 2041.9606 | -0.0038 | 22.34 | 0.009 | SIVYHPGDASANDWLN QR | 0.09 |
| P47204 | FtsZ: Cell division protein FtsZ | 90 | 41306 | 13.2 | 719.8871 | 1437.7597 | 2 | 1437.7576 | 0.0021 | 59.27 | 0.0000042 | NPLLEDVNLQGAR | 0.07 |
| G3XD12 | HcnC: Hydrogen cyanide synthase subunit HcnC | 85 | 45766 | 5 | 546.7877 | 1091.5609 | 2 | 1091.5611 | -0.0001 | 85.2 | 8.8E-09 | LADAYLEAAR | 0.06 |
| Q7DC80 | PhzD1: Phenazine biosynthesis protein PhzD1 | 83 | 23352 | 12.6 | 506.2501 | 1010.4857 | 2 | 1010.4855 | 0.0002 | 67.97 | 0.00000022 | MALEYAASR | 0.27 |
| | | | | | 955.9904 | 1909.9662 | 2 | 1909.9673 | -0.0011 | 30.52 | 0.0027 | EVVEELAPGPDDWLLT K | 0.27 |
| O69754 | PhzF1 Trans-2,3-dihydro-3-hydroxyanthranilate isomerase | 78 | 30551 | 9.4 | 389.7258 | 777.4371 | 2 | 777.4385 | -0.0014 | 27.93 | 0.0038 | LFLETR | 0.32 |
| | | | | | 770.3634 | 2308.0684 | 3 | 2308.0754 | -0.007 | 39.62 | 0.00011 | EMNLSESTFVLRPQQD GDAR | 0.32 |
| Q9I6D8 | Pfpl : Protease Pfpl OS=Pseudomonas aeruginosa | 69 | 19291 | 11.2 | 713.6606 | 2137.96 | 3 | 2137.9665 | -0.0065 | 45.26 | 0.000031 | DDINNAGGHVVDQEV AVDGK | 0.15 |
| P20582 | PqsD : 2-heptyl-4(1H)-quinolone synthase PqsD | 37 | 36698 | 7.4 | 661.8332 | 1321.6519 | 2 | 1321.6514 | 0.0006 | 37.15 | 0.00052 | INTSDEFIVER | 0.08 |
| | | | | 7.4 | 661.8332 | 1321.6519 | 2 | 1321.6514 | 0.0006 | 37.15 | 0.00052 | INTSDEFIVER | 0.08 |
| O50175 | AstB: N-succinylarginine dihydrolase | 37 | 49300 | 7.1 | 698.3391 | 1394.6637 | 2 | 1394.6678 | -0.004 | 36.69 | 0.00049 | SLGFSGSDEEVIR | 0.06 |

| | | | | | | | | | | | | | |
|--------|--|----|-------|------|--------------|-----------|---|-----------|---------|-------|---------|---------------|------|
| P54292 | RhIR: Regulatory protein RhIR | 26 | 27731 | 27.8 | 774.881 2 | 1547.7479 | 2 | 1547.7521 | -0.0042 | 25.81 | 0.0062 | NDGGFLLWWDGLR | 0.11 |
| Q9I4X1 | PqsC: 2-heptyl-4(1H)-quinolone synthase PqsC | 35 | 38613 | 3.4 | 436.902 | 1307.6843 | 3 | 1307.6833 | 0.0009 | 34.83 | 0.00095 | LPENPTGEAKPR | 0.08 |

Supplementary Table 3. Peptide table for proteins identified using PQS probe **10** in the pqsR mutant strain.

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_p_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|-----------|--|------------|-----------|------------|--------------|------------|-----------|-------------|-----------|-----------|----------------|---------------------------|------------|
| G3XD23 | WbpB: UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate oxidase | 359 | 35980 | 26.9 | 516.812 7 | 1031.6108 | 2 | 1031.6127 | -0.0019 | 49.46 | 0.000016 | LYNILQLR | 0.6 |
| | | | | | 614.807 8 | 1227.601 | 2 | 1227.603 | -0.002 | 34.74 | 0.00077 | HCVETVNTIR | 0.6 |
| | | | | | 635.807 1 | 1269.5997 | 2 | 1269.5989 | 0.0008 | 44.05 | 0.000061 | AAGYLEYEQAR | 0.6 |
| | | | | | 680.341 9 | 1358.6693 | 2 | 1358.6718 | -0.0025 | 65.52 | 0.0000009 | YEVDLTYITSR | 0.6 |
| | | | | | 717.398 8 | 1432.783 | 2 | 1432.7827 | 0.0004 | 52.68 | 0.000017 | NFALIGAAGYIAPR | 0.6 |
| | | | | | 707.708 7 | 2120.1044 | 3 | 2120.1014 | 0.0029 | 43.19 | 0.00015 | SAVIVPASDNEGHPFV AALAR | 0.6 |
| P47204 | FtsZ: Cell division protein FtsZ | 94 | 41306 | 6.3 | 719.885 8 | 1437.7571 | 2 | 1437.7576 | -0.0005 | 63.25 | 0.0000016 | NPLLEDVNLQGAR | 0.07 |
| P54292 | RhIR Regulatory protein RhIR | 100 | 27731 | 32 | 662.321 8 | 1322.629 | 2 | 1322.6295 | -0.0006 | 44.8 | 0.000056 | LGFDYYAYGVR | 0.35 |
| | | | | | 677.396 9 | 1352.7792 | 2 | 1352.7776 | 0.0016 | 64.65 | 0.0000008 6 | APNNLLSVLSVAR | 0.35 |

Supplementary Table 4. Peptide table for proteins identified using PQS probe **10** in the rhIR mutant strain.

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_p_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|-----------|--|------------|-----------|------------|--------------|------------|-----------|-------------|-----------|-----------|------------|---------------------------|------------|
| G3XD23 | WbpB: UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate oxidase | 56 | 35980 | 17.7 | 860.432 8 | 1718.8511 | 2 | 1718.8515 | -0.0004 | 55.8 | 0.0000088 | WFLSVDANDLPESVK | 0.19 |
| | | | | | 707.706 7 | 2120.0984 | 3 | 2120.1014 | -0.003 | 18.12 | 0.054 | SAVIVPASDNEGHPFV AALAR | 0.19 |
| P47204 | FtsZ: Cell division protein FtsZ | 34 | 41306 | 2 | 549.289 2 | 1096.5638 | 2 | 1096.5625 | 0.0013 | 21.31 | 0.017 | GLGAGANPEVGR | 0.08 |
| | | | | | 549.289 4 | 1096.5642 | 2 | 1096.5625 | 0.0017 | 28.3 | 0.0034 | GLGAGANPEVGR | 0.08 |

Supplementary Table 5. Peptide table for proteins identified using HHQ probe 11 in the wild type strain.

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|-----------|--------------------------------------|------------|-----------|------------|------------|------------|-----------|-------------|-----------|-----------|------------|--------------|------------|
| P47204 | FtsZ: Cell division protein FtsZ | 25 | 41306 | 7.4 | 549.2877 | 1096.561 | 2 | 1096.563 | -0.0016 | 24.67 | 0.008 | GLGAGANPEVGR | 0.08 |
| O50175 | AstB: N-succinylarginine dihydrolase | 48 | 49300 | 22.3 | 537.2839 | 1072.553 | 2 | 1072.54 | 0.0131 | 12.34 | 0.25 | DQVAVEDAVK | 0.07 |
| | | | | | 622.8582 | 1243.702 | 2 | 1243.702 | -0.0005 | 34.6 | 0.0012 | TALDELQILK | 0.07 |

Supplementary Table 6. Peptide table for proteins identified using HHQ probe 11 in the pqsR mutant strain.

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|-----------|--|------------|-----------|------------|------------|------------|-----------|-------------|-----------|-----------|------------|--------------|------------|
| P47204 | FtsZ: Cell division protein FtsZ | 25 | 41306 | 7.6 | 549.2889 | 1096.563 | 2 | 1096.563 | 0.0008 | 24.84 | 0.0079 | GLGAGANPEVGR | 0.08 |
| G3XD23 | WbpB: UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate oxidase | 22 | 35980 | 12.7 | 635.8054 | 1269.596 | 2 | 1269.599 | -0.0027 | 18.65 | 0.02 | AAGYLEYEQAR | 0.09 |

Supplementary Table 7. Peptide table for proteins identified using HHQ probe 11 in the rhIR mutant strain.

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|-----------|--|------------|-----------|------------|------------|------------|-----------|-------------|-----------|-----------|------------|-----------------|------------|
| G3XD23 | WbpB: UDP-N-acetyl-2-amino-2-deoxy-D-glucuronate oxidase | 32 | 35980 | 5 | 860.4332 | 1718.852 | 2 | 1718.852 | 0.0003 | 32.42 | 0.0019 | WFLSVDANDLPESVK | 0.09 |
| P47204 | FtsZ: Cell division protein FtsZ | 48 | 41306 | 13.5 | 549.2873 | 1096.56 | 2 | 1096.563 | -0.0024 | 41.79 | 0.00016 | GLGAGANPEVGR | 0.08 |

Supplementary Table 8. Peptide table for proteins identified using PQS probe 10 wild type during late exponential phase as identified from in-gel digestion of band at around 40 kDa (Supplementary Fig. 4).

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | pep_seq | prot_empai |
|-----------|----------------------------------|------------|-----------|------------|------------|------------|-----------|-------------|-----------|-----------|------------|---------------|------------|
| P47204 | FtsZ: Cell division protein FtsZ | 60 | 41306 | 8.2 | 549.2893 | 1096.564 | 2 | 1096.563 | 0.0016 | 46.06 | 0.00057 | GLGAGANPEVGR | 0.07 |
| | | | | | 719.8854 | 1437.756 | 2 | 1437.758 | -0.0014 | 41.86 | 0.00022 | NPLLEDVNLQGAR | 0.07 |

| | | | | | | | | | | | | | |
|--------|---|----|-------|-----|--------------|----------|---|----------|---------|-------|---------|------------|------|
| Q9I4X1 | PqsC 2-heptyl-4(1H)-quinolone synthase PqsC | 39 | 38613 | 3.2 | 632.319 2 | 1262.624 | 2 | 1262.626 | -0.0016 | 38.82 | 0.00044 | NGENEFLVVR | 0.08 |
|--------|---|----|-------|-----|--------------|----------|---|----------|---------|-------|---------|------------|------|

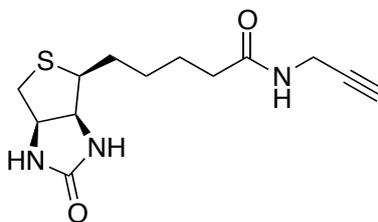
Supplementary Table 9. Peptide table for proteins identified using PQS probe **10** wild type during late exponential phase as identified from in-gel digestion of band at around 17 kDa (Supplementary Fig. 4).

| accession | prot_desc | prot_score | prot_mass | prot_cover | pep_exp_p_mz | pep_exp_mr | pep_exp_z | pep_calc_mr | pep_delta | pep_score | pep_expect | npep_seq | prot_empai |
|-----------|---|------------|-----------|------------|--------------|------------|-----------|-------------|-----------|-----------|------------|--------------------------|------------|
| Q9I4Z4 | Pal: Peptidoglycan-associated lipoprotein | 85 | 17971 | 26.8 | 549.746 5 | 1097.479 | 2 | 1097.481 | -0.0026 | 39.36 | 0.00012 | EYNMALGER | 0.58 |
| | | | | | 385.531 7 | 1153.573 | 3 | 1153.573 | 0.0004 | 19.82 | 0.026 | VVLEHTDER | 0.58 |
| | | | | | 800.707 2 | 2399.1 | 3 | 2399.099 | 0.0007 | 26.56 | 0.0022 | AITTFYFEYDSSDLKPE AMR | 0.58 |

4 Supplementary methods

4.1 Biological experimental section

General. A Jenway 6705 spectrometer and 1 cm path length cuvettes were used for OD₆₀₀, A₄₀₅, A₄₂₀ and A₅₅₀ measurements. cOmplete™, Mini, EDTA-free protease inhibitor cocktail tablets were purchased from Roche and used in accordance with the manufacturers guidelines. TAMRA-alkyne (5/6-Carboxytetramethylrhodamine-PEG₄-Alkyne) was purchased from Jenna Biosciences and was used without further purification. Biotin alkyne without a PEG linker was used (molecular formula C₁₃H₁₉N₃O₂S, Supplementary Figure 8). UV irradiation steps were carried out using an inverted 3UV transilluminator at a distance of 2 cm, keeping the samples cooled on ice. SDS gels were hand cast using a Bio-rad Mini-PROTEAN® Tetra Handcast System with a thickness of 0.75 mm. All fluorescence images of SDS-gels were recorded using a Typhoon 9000 using the 532 nm laser, 800 PMT and 50 μm resolution. Unless otherwise stated all solutions were in water. MilliQ water was used in the preparation of all samples for LC/MS analysis.



Supplementary Figure 8. Structure of biotin alkyne used throughout this study.

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Supplementary Table 10. Unless otherwise stated the bacteria were grown as follows: Desired strains were struck on a LB agar plate and grown overnight at 37 °C. Single colonies were then used to inoculate 10 mL of LB media and grown overnight at 37 °C. These were used to inoculate cultures to a starting OD₆₀₀ of 0.05 which were then grown in either an incubator or a water bath at 37 °C with good aeration.

| Strain | Description | Source |
|----------------------|---|------------------------------|
| <i>P. aeruginosa</i> | | |
| PAO1 | wild type | Iglewski et al. ⁸ |
| $\Delta pqsR$ | PqsR mutant in a PAO1 background | Pesci et al. ⁹ |
| mPAO1 | wild type | Manoil et al. ¹⁰ |
| $\Delta rhIR/rhII$ | RhIR mutant in a PAO1 background. rhIRI::IRII CNK | Mattick et al. ¹¹ |
| <i>E. coli</i> | | |
| JTH1 | <i>E. coli</i> Contains the plasmid pMAL-c2X with PqsR _{LBD} -MBP insert; ampicillin resistant. | Welch et al. ¹ |
| DH618 | <i>E. coli</i> . Contains the plasmid pEAL08-2 with the PqsR expression vector and <i>pqsA</i> ¹ - <i>lacZ</i> transcriptional fusion; PqsR reporter vector; ampicillin resistant. | Hogan et al. ⁶ |

Supplementary Table 10. Bacterial strains used in this study.

***pqsA*::*lacZ* transcriptional assay.** Quinolone analogues were tested for their PqsR agonist activity by measuring transcription of the *pqsA* promoter region in an *E. coli* DH5 α strain DH618 containing the pEAL08-2 plasmid as previously described.⁶ *E. coli* strain DH618 was always grown in the presence of carbenicillin (50 μ g/mL). Overnight cultures were diluted 1:50 into LB media and grown to an OD₆₀₀ of 0.2 (~2.5 h). Quinolone analogues in DMSO (50 μ L) were added to 5 mL cultures and the cultures grown for a further 2.5 h before measuring the OD₆₀₀ and β galactosidase activity quantified.

Pyoverdine assay. Pyoverdine was quantified as previously described.¹² Overnight cultures of PAO1 were diluted 1:100 into LB medium and quinolone analogues in DMSO (25 μ L) were added to the subcultures (2.5 mL). The cultures were grown with good aeration for 17 h before measuring the OD₆₀₀. The cells were pelleted by centrifugation (13000 xg, 4 °C, 10 min), 200 μ L of the supernatant was transferred to a clear 96 well microtiter plate and the absorbance at 405 nm was measured and the values corrected for the cell density.

PqsRLBD labelling in *E. coli* cell lysate. *E. coli* cells containing a pMAL-c2X plasmid with a PqsRLBD-MBP insert were grown and protein expression induced as previously reported.¹ Cells were harvested by centrifugation (7400 xg, 10 min, 4 °C), washed twice with ice cold

phosphate-buffered saline (PBS) and the pellets stored overnight at -18 °C. Cells were allowed to thaw on ice, re-suspended in ice cold PBS containing a complete, mini, EDTA free protease inhibitor cocktail tablet (Roche) and lysed by ultrasonication (10 x 30 s) taking care to keep the samples below 4 °C. The samples were then clarified using low speed centrifugation (7400 xg, 10 min, 4 °C) and the protein concentration determined using the Bradford protein assay. Protein concentration was adjusted to 5 mg/mL using PBS containing a protease inhibitor cocktail tablet and samples were snap frozen using liquid nitrogen and stored at -80 °C until use. The probes were added as stock solutions in DMSO (2 µL, 0.1 M) to 0.2 mL of cell-lysate and these were incubated at room temperature for 30 min with gentle shaking. The samples were then transferred to a 12 well flat bottomed plate and irradiated with UV light (302 nm) for 15 min at a distance of approximately 0.5 cm using an inverted 3UV benchtop transilluminator whilst being cooled on ice.

PqsRLBD labelling in live *E. coli*. Cultures (10 mL) of *E. coli* containing a pMAL-c2X plasmid with a PqsRLBD-MBP insert were grown and protein expression induced as previously reported.¹ 30 min prior to harvesting the cells the photoreactive probes in DMSO (10 mM, 100 µL) were added to the cultures to a final concentration of 100 µM. Cells were harvested by centrifugation (7400 xg, 20 min, 4 °C) and washed twice with PBS at 4 °C. The cell pellets were re-suspended in PBS (2 mL), transferred to a 6 well plate and irradiated with UV light of 302 nm using a 3UV transilluminator for 15 min at 4 °C. The cells were then collected by centrifugation (7400 xg, 30 min, 4 °C), the supernatant discarded and the cells stored at -80 °C overnight. Cells were thawed on ice, re-suspended PBS (1 mL) containing a protease inhibitor cocktail tablet (Roche). Cells were lysed by ultrasonication (3 x 30 s) while keeping the samples below 4 °C, clarified by centrifugation (15,000 xg, 30 min, 4 °C) and the supernatant containing the PAL proteins was collected and stored at -80 °C until use. These were then labelled with TAMRA or biotin.

Click chemistry tagging of labelled proteins. Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) (5 µL, 1.6 mM solution in DMSO), CuSO₄ (1 µL, 0.1 M in H₂O), tris-(2-carboxyethyl)phosphine (TCEP) (1 µL, 0.1 M in H₂O) and biotin alkyne (1 µL, 2 mM in DMSO) or 5/6-TAMRA-PEG₄-alkyne (Jena Bioscience) (1 µL, 2 mM in DMSO) were added to 100 µL of probe PAL sample. This was incubated with vigorous shaking for 2 h at 25 °C. The reaction was then quenched by the addition of EDTA (10 µL, 0.5 M).

Fluorescence visualisation of TAMRA tagged protein. Proteins were precipitated with trichloroacetic acid (60 µL, 50% (w/v) H₂O) and collected by centrifugation (7400 xg, 20 min, 4 °C). The proteins pellet was washed with acetone/water (1:1, 2 x 200 µL), water (2 x 200 µL) and air dried for 5 min. Proteins were then denatured by heating to 95 °C in sample loading buffer for 10 min and resolved using 12% SDS PAGE. Labelled bands were visualised using gel fluorescence scanning with a Typhoon 9000 using the 532 nm laser, 800 PMT and 50 µm resolution, before staining with Coomassie to visualise total protein content.

Pull-down of biotin tagged PqsR. Biotin tagged proteins were precipitated using the $\text{CHCl}_3/\text{MeOH}$ procedure.¹³ Pull-downs were then performed as outlined previously.¹⁴ Briefly, SDS (10 μL , 10% w/v in H_2O) was added to the biotin labelled protein pellets the sample heated to 100 °C for 5 min. This was diluted with water (4 x 50 μL) with vortexing after each addition. The proteins were precipitated using the $\text{CHCl}_3/\text{MeOH}$ procedure¹³ and the resulting protein pellet was solubilised in 1% SDS in 100 mM NH_4HCO_3 (200 μL) with vigorous shaking for 30 min. Any remaining insoluble protein was removed by centrifugation (7400 xg, 10 min, 4 °C) and the samples were then separated by SDS-PAGE using a 12% gel and stained with Coomassie to visualise the pull-down.

Preparation of *P. aeruginosa* cell lysates. *P. aeruginosa* strains were struck on a LB agar plate and grown overnight at 37 °C. Single colonies were then used to inoculate 10 mL of LB media and grown overnight at 37 °C. These were used to inoculate cultures to a starting OD_{600} of 0.05 which were then grown to early stationary phase in a water bath at 37 °C with good aeration. Cells were harvested by centrifugation (7400 xg, 10 min, 4 °C), washed twice with ice cold phosphate-buffered saline (PBS) and the cell pellets stored over night at -18 °C. Cells were allowed to thaw on ice before being resuspended in ice cold PBS containing a cComplete, mini, EDTA free protease inhibitor cocktail tablet and lysed by ultrasonication (10 x 30 s) taking care to keep the samples below 4 °C. The protein samples were then clarified using low speed centrifugation (7400 xg, 10 min, 4 °C) and the protein concentration determined using the Bradford protein assay before being snap frozen using liquid nitrogen and stored at -80 °C until use.

Cell-lysate photocrosslinking and labelling with biotin. Cell-lysates were adjusted to a protein concentration of 5 mg/mL using PBS containing a protease inhibitor cocktail tablet (Roche). The probes were then added (10 μM final concentration from 1 mM stock solution in DMSO) to 0.2 mL of protein sample, which were then incubated at room temperature for 30 min with gentle shaking. The samples were then irradiated with UV light ($\lambda = 302 \text{ nm}$) for 15 min. Click chemistry with biotin alkyne was used to install biotin (as described earlier) and the proteins were precipitated using a Chloroform/Methanol precipitation.¹³

4.2 Pull-down experiments for protein identification

Pull-down of biotin tagged proteins. Biotin tagged proteins were precipitated using the $\text{CHCl}_3/\text{MeOH}$ procedure.¹³ Pull-downs were then performed as outlined previously.¹⁴ Briefly, SDS (10 μL , 10% w/v in H_2O) was added to the biotin labelled protein pellets the sample heated to 100 °C for 5 min. This was diluted with water (4 x 50 μL) with vortexing after each addition. The proteins were precipitated using the $\text{CHCl}_3/\text{MeOH}$ procedure¹³ and the resulting protein pellet was solubilised in 1% SDS in 100 mM NH_4HCO_3 (200 μL) with vigorous shaking for 30 min. Any remaining insoluble protein was removed by centrifugation (7400 xg, 10 min, 4 °C) and the samples were reduced by the addition of DTT (1 μL , 1 M in H_2O) and incubating

the samples at 37°C for 30 min. Iodoacetamide (15 µL, 200 mM in 1% (w/v) SDS in 100 mM NH₄HCO₃) was added and the samples incubated at 37 °C for 30 min in the dark. Any insoluble protein was removed by centrifugation (7400 xg, 10 min, 4 °C) and a second CHCl₃/MeOH precipitation was performed.¹³ The protein pellet was solubilised in 2% (w/v) SDS in 100 mM NH₄HCO₃ (50 µL) using a sonic water bath and heating to 70 °C. This was diluted with 50 mM NH₄HCO₃ (50 µL) and vortexed for 2 min. The dilution was repeated twice more before the addition of 50 mM NH₄HCO₃ (800 µL). Any insoluble protein was removed by centrifugation (7400 xg, 10 min, 4 °C) and the protein sample incubated for 2 h with inversion at room temperature with pre-equilibrated MyOne T1 Streptavidin grafted magnetic beads (Invitrogen) (50 µL) pre-equilibrated according to manufacturer's instructions. The beads were stringently washed with H₂O (2 x 200 µL), PD buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) with 0.1% SDS (2 x 100 µL), PD buffer (2 x 200 µL), wash buffer I (4 M urea, 50 mM NH₄HCO₃) (1 x 200 µL), wash buffer II (50 mM Tris-HCl, 10 mM NaCl, pH 7.5) (1 x 200 µL) and water (1 x 200 µL). In the case of the PqsRLBD live cell labelling experiment, the bound proteins were eluted from the beads by boiling in loading buffer containing 10 µM biotin (50 µL) for 10 min. The sample was then separated by SDS-PAGE using a 12% gel and stained with Coomassie to visualise pull-down.

LC/MS protein identification and analysis. The beads from the pull-down experiment were washed twice with PBS, twice with digestion buffer (100 mM NH₄HCO₃ / 50 mM NaCl), taken up in 100 µL digestion buffer with 200 ng MS-grade trypsin (Promega) and digested overnight at 37°C. Formic acid (2 µL) was added to achieve a pH of 2-3 for inhibiting the trypsin activity and the peptides were desalted with Stagetips prior to LC-MS analysis. Tryptic peptides were analysed on a Surveyor nanoLC system (Thermo) hyphenated to an LTQ-Orbitrap mass spectrometer (Thermo). Gold and carbon coated emitters (OD/ID = 360/25 µm tip ID = 5 µm), trap column (OD/ID = 360/100 µm packed with 25 mm robust Poros10R2/ 15 mm BioSphere C18, 5 µm, 120 Å beads) and analytical columns (OD/ID = 360/75 µm packed with 20 cm BioSphere C18, 5 µm, 120 Å beads) were from Nanoseparations (Nieuwkoop, The Netherlands). The mobile phases (A: 0.1% formic acid/H₂O, B: 0.1% formic acid/MeCN) were made with ULC/MS grade solvents (Biosolve). The emitter tip was coupled end-to-end with the analytical column via a 15 mm long TFE Teflon tubing sleeve (OD/ID 0.3 x 1.58 mm, Supelco, USA) and installed in a stainless steel holder mounted in a nano-source base (Upchurch scientific, Idex, USA). General mass spectrometric conditions were as follows: an electrospray voltage of 1.8 kV was applied to the emitter, no sheath and auxiliary gas flow, ion transfer tube temperature 150 °C, capillary voltage 41 V, tube lens voltage 150 V. Internal mass calibration was performed with air-borne protonated polydimethylcyclsiloxane (m/z = 445.12002) and the plasticiser protonated dioctyl phthalate ions (m/z = 391.28429) as lock mass. For shotgun proteomics analysis, 10 µL of the samples was pressure loaded on the trap column with a 10 µL/min flow for 5 min followed by peptide separation with a gradient of 35 min 5%–30% B, 15 min 30%–60% B, 5 min A at a flow of 300 µL/min split to 250 nL/min

by the LTQ divert valve. For each data-dependent cycle, one full MS scan (300–2000 m/z) acquired at high mass resolution (60,000 at 400 m/z, AGC target 1x10⁶, maximum injection time 1000 ms) in the Orbitrap was followed by three MS/MS fragmentations in the LTQ linear ion trap (AGC target 5x10⁴, maximum injection time 120 ms) from the three most abundant ions. MS2 settings were as follows: collision gas pressure 1.3 mT, normalised collision energy 35%, ion selection threshold of 750 counts, activation $q = 0.25$ and activation time of 30 ms. Fragmented precursor ions that were measured twice within 10 s were dynamically excluded for 120 s and ions with $z < 2$ or unassigned were not analysed. For peptide identification, mascot generic files (mgf) were generated from the .raw data files and matched with the Mascot (Matrix science) search engine to the *Pseudomonas aeruginosa* database extracted from Uniprot, using a reversed decoy database to estimate the false discovery rate, 20 ppm peptide tolerance for the selection of the parent ion, 0.8 Da MS/MS tolerance, trypsin cleavage allowing for two miss-cleavages, mono-isotopic peptide selection, carbamidomethyl addition to cysteines (57.0513 Da) as fixed and oxidation of methionine (15.9994 Da) as variable modifications. The protein lists were then compared using Microsoft Excel and proteins present in the control samples were identified and removed.

4.3 Synthetic experimental

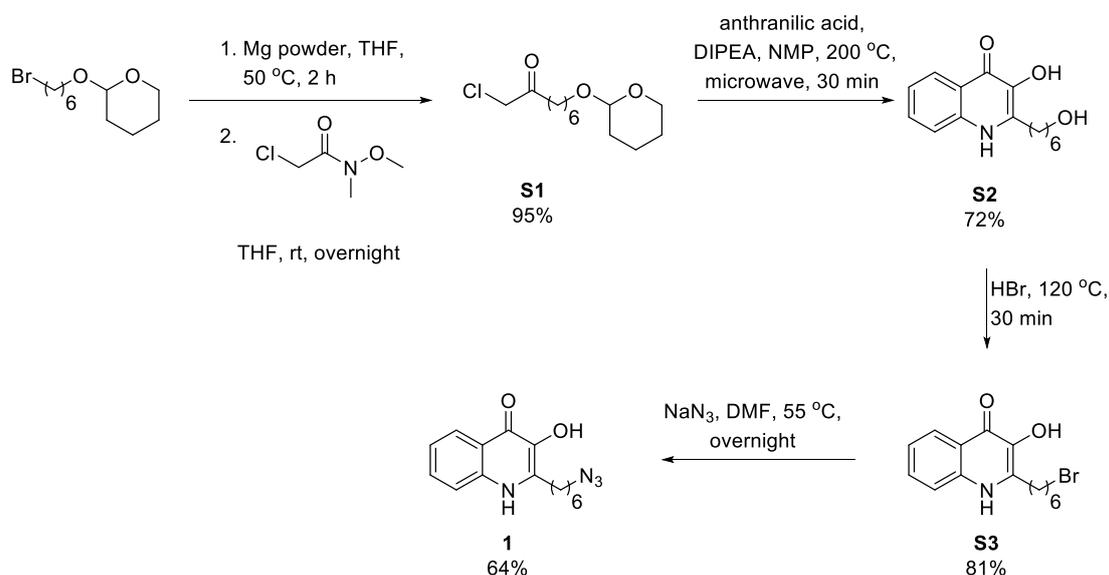
General. Unless otherwise stated, reactions were performed in oven-dried glassware under nitrogen with dry, freshly distilled solvents. THF was distilled from LiAlH₄ in the presence of triphenyl methane indicator. CH₂Cl₂, hexane, MeOH, pyridine and MeCN were distilled from calcium hydride. Anhydrous DMF (Acros) and DCE (Aldrich) were used without further purification. All other chemicals were used as obtained from commercial sources. Biotin alkyne without a PEG linker was used (molecular formula C₁₃H₁₉N₃O₂S, see Supplementary Figure 8). Yields refer to analytically pure compounds. TLC was performed using Merck pre-coated 0.23 mm thick plates of Keisegel 60 F₂₅₄ and visualised using UV ($\lambda = 254$ nm) or by staining with KMnO₄. All retention factors (R_f) are given to 0.01. All column chromatography was carried out using Merck 9385 Keisegel 60 silica gel (230-400 mesh). ¹H NMR spectra were recorded on Bruker DPX 400 or 500 spectrometers operating at 400 or 500 MHz respectively using an internal deuterium lock at ambient probe temperatures. Chemical shifts (δ) are quoted to the nearest 0.01 ppm and are reference relative to residual solvent peak.¹⁵ Coupling constants (J) are given to the nearest 0.1 Hz. The following abbreviations are used to indicate the multiplicity of signals: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and br = broad. Data is reported as follows: chemical shift (multiplicity, coupling constant(s), integration). ¹³C NMR spectra were recorded on Bruker 400 or 500 spectrometers operating at 100 and 125 MHz respectively using an internal deuterium lock at ambient probe temperatures. Chemical shifts (δ) are quoted to the nearest 0.1 ppm and are reference relative to the deuterated solvent peak.¹⁵ High resolution mass spectra (HRMS) were recorded using either a Micromass Q-TOF or a Micromass LCT Premier spectrometer and reported mass values are within ± 5 ppm mass units unless otherwise stated. Low

resolution mass spectra were recorded on an HP/Agilent LCMS APCI 120-1000 full gradient machine. IR spectra were recorded using neat sample on a Perkin-Elmer 1600 FT IR spectrometer. Selected absorption maxima are reported in wavenumbers (cm^{-1}). Melting points (mp) were measured using a Buchi B-545 melting point apparatus and are uncorrected.

Synthetic procedures

PQS and HHQ were synthesised according to literature procedures.^{16,17}

4.3.1 Synthesis of PQS analogue 1



1-Chloro-8-((tetrahydro-2H-pyran-2-yl)oxy)octan-2-one **S1**

The title compound was prepared following the procedure outlined by Hodgkinson et al.¹⁶ 2-((6-Bromohexyl)oxy)tetrahydro-2H-pyran (1.6 mL, 7.3 mmol, 1.0 eq) was added to a suspension of magnesium turnings (532 mg, 21.9 mmol, 3.0 eq) in THF (15 mL) under argon. The reaction was stirred at room temperature for 1 h, then heated to 50 °C for a further 2 h and cooled to room temperature to give a ~0.5 M solution of the Grignard reagent in THF. The Grignard solution was then added to a solution of 2-chloro-*N*-methoxy-*N*-methylacetamide (500 mg, 3.6 mmol, 0.5 eq) in THF (25 mL) and the mixture was stirred overnight at room temperature. The reaction was diluted two-fold with toluene, cooled to 0 °C and quenched with an equal volume of 0.1 M HCl. The organic phase was then washed with brine, dried over MgSO₄, filtered and the solvent removed under reduced pressure to give the resulting crude α -chloroketone **S1** as a pale yellow oil in 95% yield which was then used without further purification.

TLC (EtOAc:hexane, 4:6 v/v): $R_f = 0.27$; ^1H NMR (400 MHz, CDCl_3): δ 4.55 (dd, $J = 4.6, 2.7$ Hz, 1H), 4.06 (s, 2H), 3.85 (ddd, $J = 11.1, 7.4, 3.2$ Hz, 1H), 3.72 (dt, $J = 9.6, 6.8$ Hz, 1H), 3.54 – 3.42 (m, 1H), 3.37 (dt, $J = 9.6, 6.5$ Hz, 1H), 2.58 (t, $J = 7.4$ Hz, 2H), 1.82 – 1.44 (m, 10H), 1.44 – 1.28 (m, 4H); ^{13}C NMR (101 MHz, CDCl_3): δ 202.9, 99.0, 67.6, 62.5, 48.3, 39.8, 30.9, 29.7, 29.0, 26.1, 25.6, 23.7, 19.9; IR (neat)/ cm^{-1} : 1727; HRMS (ESI-TOF) m/z : $[\text{M} + \text{Na}]^+$ calcd. for $\text{C}_{13}\text{H}_{23}\text{O}_3\text{ClNa}$, 285.1228; found, 285.1217.

3-Hydroxy-2-(6-hydroxyhexyl)quinolin-4(1H)-one **S2**

The title compound was prepared following the procedure outlined by Hodgkinson et al.¹⁶ DIPEA (0.46 mL) and α -chloroketone **S1** (578 mg, 2.2 mmol, 1.0 eq), were added to a solution of anthranilic acid (300 mg, 2.2 mmol, 1.0 eq), in anhydrous NMP (4.5 mL). The mixture was then heated under microwave irradiation at 200 °C for 30 min. After cooling to room temperature the reaction was quenched by pouring into ice/water. The resulting mixture was stirred for 5 min before being allowed to settle for 30 min and the precipitate was collected and dried under vacuum to give the title compound **S2** (421 mg, 1.58 mmol) as a white precipitate in 72% yield.

Mp: 179.6-180.8 °C (EtOAc); TLC (MeOH: CH_2Cl_2 , 1:9 v/v, KH_2PO_4 treated silica): $R_f = 0.5$; ^1H NMR (400 MHz, $\text{DMSO}-d_6$): δ 11.42 (s, 1H), 8.09 (d, $J = 8.1$ Hz, 1H), 7.53 (d, $J = 3.9$ Hz, 2H), 7.21 (dt, $J = 8.1, 4.0$ Hz, 1H), 4.33 (s, 1H), 3.37 (t, $J = 6.4$ Hz, 2H), 2.73 (t, $J = 7.7$ Hz, 2H), 1.66 (p, $J = 7.3$ Hz, 2H), 1.54 – 1.23 (m, 6H); ^{13}C NMR (101 MHz, $\text{DMSO}-d_6$): δ 168.9, 137.8, 137.4, 135.5, 124.5, 122.2, 121.5, 117.8, 60.7, 32.4, 28.8, 28.1, 27.9, 25.3; IR (neat)/ cm^{-1} : 3227, 1628, 1588, 1557; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{15}\text{H}_{19}\text{NO}_3$: 262.1438; found 262.1429;

2-(6-Bromohexyl)-3-hydroxyquinolin-4(1H)-one **S3**

Quinolone **S2** (15 mg, 0.057 mmol, 1.0 eq) was dissolved in 48% aqueous HBr (0.3 mL) and heated to 120 °C for 30 min with stirring. The reaction was cooled to room temperature and added slowly to a stirring saturated aqueous solution of NaHCO_3 . The aqueous phase was extracted with EtOAc (3 x 10 mL), dried over MgSO_4 , filtered and evaporated to dryness to give the title compound **S3** (15 mg, 0.046 mmol) as dark brown needles in 81% yield.

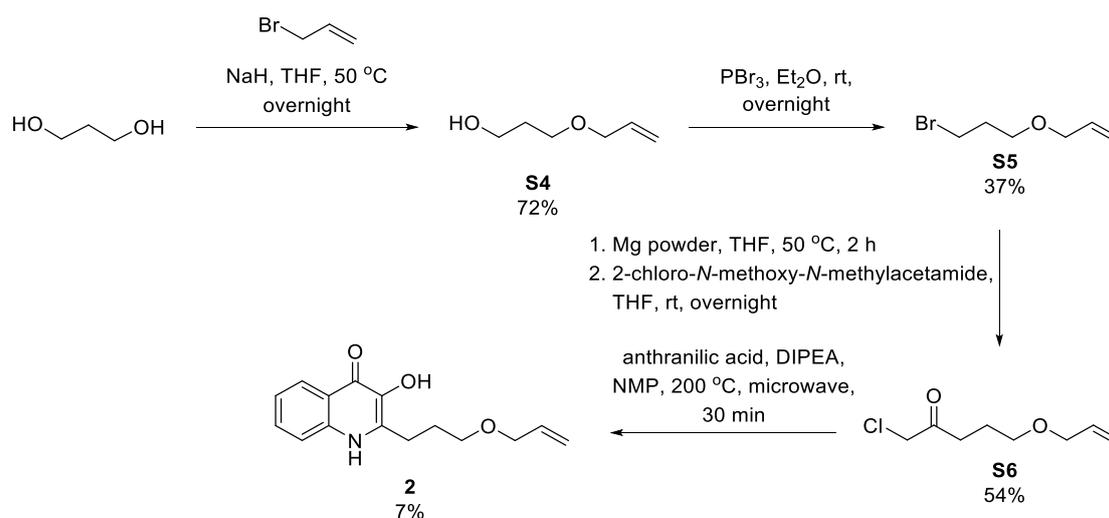
Mp: 157.3-158.5 °C (EtOAc); TLC (MeOH: CH_2Cl_2 , 1:9 v/v, KH_2PO_4 treated silica): R_f : 0.59; ^1H NMR (500 MHz, $\text{DMSO}-d_6$): δ 11.44 (s, 1H), 8.09 (d, $J = 8.1$ Hz, 1H), 7.53 (d, $J = 3.2$ Hz, 2H), 7.21 (app dt, $J = 8.1, 4.0$ Hz, 1H), 3.52 (t, $J = 6.7$ Hz, 2H), 2.74 (t, $J = 7.7$ Hz, 2H), 1.80 (app p, $J = 6.8$ Hz, 2H), 1.72 – 1.63 (m, 2H), 1.48 – 1.31 (m, 4H); ^{13}C NMR (126 MHz, $\text{DMSO}-d_6$): δ 168.8, 137.8, 137.4, 135.4, 130.0, 124.5, 122.2, 121.5, 117.8, 35.1, 32.1, 28.0, 27.9, 27.7, 27.3; IR (neat)/ cm^{-1} : 1638, 1599, 1557, 757; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{15}\text{H}_{19}\text{NO}_2\text{Br}$: 324.0599; found 324.0600;

2-(6-Azidohexyl)-3-hydroxyquinolin-4(1H)-one **1**

A solution of **S3** (10 mg, 0.03 mmol, 1.0 eq) and sodium azide (4 mg, 0.06 mmol, 2.0 eq) in DMF (1 mL) was heated to 55 °C overnight with stirring. The reaction was quenched by pouring into ice/water (20 mL) and the mixture was stirred for 5 min. The resulting precipitate was collected by filtration, washed with water and dried under vacuum. The crude produce was further purified by crystallisation from EtOAc to give the title compound **1** (5.7 mg, 0.02 mmol) as dark brown needles in 64% yield.

Mp: 167.2-168.1 °C (EtOAc); TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.41; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.43 (s, 1H), 8.09 (d, *J* = 8.1 Hz, 1H), 7.53 (app d, *J* = 4.2 Hz, 2H), 7.21 (app dt, *J* = 7.9, 3.8 Hz, 1H), 3.30 (t, *J* = 6.8 Hz, 2H), 2.73 (t, *J* = 7.6 Hz, 2H), 1.67 (t, *J* = 7.2 Hz, 2H), 1.53 (t, *J* = 6.8 Hz, 2H), 1.46 – 1.26 (m, 4H); ¹³C NMR (101 MHz, DMSO-*d*₆) δ 168.9, 137.8, 137.4, 135.4, 130.0, 124.5, 122.2, 121.5, 117.8, 50.6, 28.3, 28.2, 28.0, 27.7, 25.9; IR (neat)/cm⁻¹: 3260, 2800 (br), 2093, 1640, 1597; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₅H₁₉N₄O₂: 287.1494; found 287.1503.

4.3.2 Synthesis of PQS analogue **2**



3-(Allyloxy)propan-1-ol **S4**

NaH (60% dispersion in mineral oil, 1.05 g, 26.3 mmol, 2.1 eq) was added piecewise to a solution of allyl bromide (2.17 mL, 12.8 mmol, 1.0 eq) and 1,3-propanediol (1.9 mL, 24 mmol, 1.9 eq) in anhydrous THF at 0 °C. The reaction was then heated at 50 °C overnight. The solvent was removed under vacuum and the residue was partitioned between EtOAc (50 mL) and H₂O (50 mL). The organic layer was dried over MgSO₄, filtered and evaporated to dryness under reduced pressure. The crude product was purified by column chromatography (stepwise gradient of 20–40% Et₂O in 30-40 PE) to give the title compound **S4** (2.08g, 18 mmol) as a colourless oil in 72% yield.

TLC (Et₂O:40-60 PE, 4:6 v/v): R_f = 0.10; ¹H NMR (400 MHz, CDCl₃): δ 5.89 (ddt, *J* = 17.3, 10.4, 5.6 Hz, 1H), 5.26 (ddd, *J* = 17.2, 3.3, 1.4 Hz, 1H), 5.17 (ddd, *J* = 10.4, 2.9, 1.3 Hz, 1H), 3.98 (dt, *J* = 5.6, 1.4 Hz, 2H), 3.77 (t, *J* = 5.7 Hz, 2H), 3.62 (t, *J* = 5.8 Hz, 2H), 1.84 (tt, *J* = 5.8, 5.8 Hz, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 134.7, 117.2, 72.2, 69.5, 62.0, 32.2; IR (neat)/cm⁻¹: 3372 (br), 1647. Spectroscopic data consistent with literature values.¹⁸

3-(3-Bromopropoxy)prop-1-ene S5

Phosphorus tribromide (426 μL, 4.6 mmol, 0.5 eq) was added dropwise to a solution of compound **S4** (1.05 g, 9 mmol, 1.0 eq) in Et₂O (10 mL) at -5 °C. The reaction was allowed to warm to room temperature and left stirring overnight where it turned a yellow colour. The reaction was cooled to 0 °C, quenched by the addition of H₂O (15 mL) and diluted with hexane (30 mL). The organic phase was washed with water (2 x 30 mL), dried over Na₂SO₄, filtered and the solvent was removed under reduced pressure. The resulting oil was purified by column chromatography (0-5% Et₂O in hexane) to give the title compound **S5** (0.587 g, 3.3 mmol) as a colourless solution in ~37% yield. (Not all hexane was removed as product is volatile and the yield was calculated from ¹H NMR ratio of product:hexane).

TLC (Et₂O:hexane, 5:95 v/v): R_f = 0.30; ¹H NMR (400 MHz, CDCl₃): δ 5.91 (ddt, *J* = 17.3, 10.4, 5.6 Hz, 1H), 5.28 (ddd, *J* = 17.2, 3.3, 1.7 Hz, 1H), 5.19 (ddd, *J* = 10.3, 2.9, 1.4 Hz, 1H), 3.98 (dt, *J* = 5.6, 1.4 Hz, 2H), 3.56 (t, *J* = 5.9 Hz, 2H), 3.52 (t, *J* = 6.5 Hz, 2H), 2.12 (tt, *J* = 6.3, 6.2 Hz, 2H); ¹³C NMR (101 MHz, CDCl₃): δ 134.8, 117.1, 72.1, 67.8, 33.1, 31.7, 30.8. No spectroscopic data reported.¹⁹

5-(Allyloxy)-1-chloropentan-2-one S6

The title compound was prepared following the procedure outlined by Hodgkinson et al.¹⁶ Alkyl bromide **S5** (250 mg, 1.4 mmol, 1.0 eq) was added to a suspension of magnesium turnings (50 mg, 4.2 mmol, 3.0 eq) in THF (3 mL) under argon. The reaction was stirred at room temperature for 1 h, then heated to 50 °C for a further 2 h and cooled to room temperature to give a ~0.5 M solution of the Grignard reagent in THF. The Grignard solution was then added to a solution of 2-chloro-*N*-methoxy-*N*-methylacetamide (96 mg, 0.7 mmol, 0.5 eq) in THF (5 mL) and the mixture was stirred overnight at room temperature. The reaction was diluted two-fold with toluene, cooled to 0 °C and quenched with an equal volume of 0.1 M HCl. The organic phase was then washed with brine, dried over MgSO₄, filtered and the solvent removed under reduced pressure to give a pale yellow oil. This was purified by column chromatography (stepwise gradient of 0-40% EtOAc in hexane) to afford **S6** (66 mg, 0.375 mmol) as a colourless oil in 54% yield. Contains the bromoketone as an impurity in the NMR ~25% Br ketone, 75% Cl ketone

TLC (EtOAc:hexane, 30:70 v/v): R_f = 0.30; ¹H NMR (400 MHz, CDCl₃): δ 5.88 (ddt, *J* = 17.3, 10.4, 5.6 Hz, 1H), 5.25 (dq, *J* = 17.2, 1.7 Hz, 1H), 5.17 (dq, *J* = 10.4, 1.4 Hz, 1H), 4.10 (s,

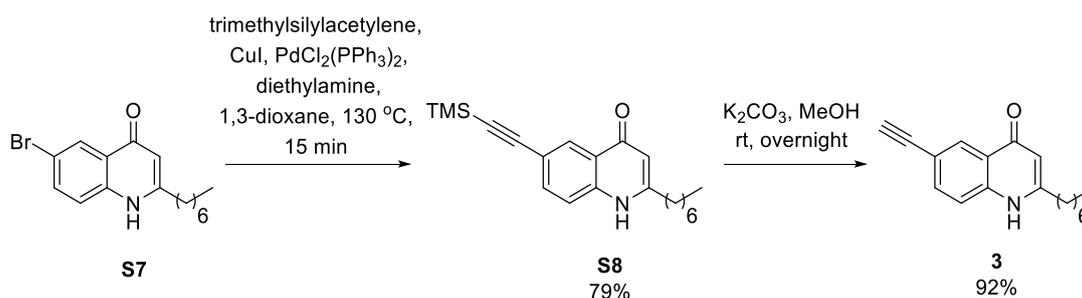
1.5H), 3.93 (dt, $J = 5.6, 1.5$ Hz, 2H), 3.91 (s, 0.5H (Br ketone)), 3.45 (td, $J = 6.0, 1.0$ Hz, 1.5H), 2.76 (t, $J = 7.0$ Hz, 0.5H (Br ketone)), 2.69 (t, $J = 7.1$ Hz, 2H), 1.96-1.89 (m, 2H); ^{13}C NMR (101 MHz, CDCl_3): δ 202.6, 134.8, 117.1, 71.9, 69.0, 48.4, 36.9 (Br ketone), 36.7, 34.5, (Br ketone) 24.4 (Br ketone), 24.1; CDCl_3 IR (neat)/ cm^{-1} : 2934, 2858, 1716, 1646.

2-(3-(Allyloxy)propyl)-3-hydroxyquinolin-4(1H)-one **2**

The title compound was prepared following the procedure outlined by Hodgkinson et al.¹⁶ DIPEA (194 μL , 1.1 mmol, 1.2 eq) and the required α -chloroketone **S6** (100 mg, 0.93 mmol, 1.0 eq) were added to a 0 solution of anthranilic acid (128 mg, 0.93 mmol, 1.0 eq) in anhydrous NMP (2 mL). The mixture was then heated under microwave irradiation at 200 $^\circ\text{C}$ for 30 min. After cooling to room temperature the reaction was quenched by pouring into ice/water. The resulting mixture was stirred for 5 min before being allowed to settle for 30 min and the precipitate was collected and dried under vacuum. The crude product was purified by column chromatography (stepwise gradient of 0-20% MeOH in CH_2Cl_2) to give **2** as an amorphous brown residue (17 mg, 0.066 mmol) in 7% yield.

TLC (MeOH: CH_2Cl_2 , 7:93 v/v): $R_f = 0.30$ streaks; ^1H NMR (500 MHz, MeOD): δ 8.22 (dd, $J = 8.2, 0.9$ Hz, 1H), 7.63 – 7.53 (m, 2H), 7.32 (ddd, $J = 8.1, 6.4, 1.5$ Hz, 1H), 5.89 (ddt, $J = 17.3, 10.4, 5.6$ Hz, 1H), 5.24 (ddd, $J = 17.2, 3.4, 1.7$ Hz, 1H), 5.12 (ddd, $J = 10.4, 1.8, 1.3$ Hz, 1H), 3.98 (dt, $J = 5.6, 1.5$ Hz, 2H), 3.55 (t, $J = 6.2$ Hz, 2H), 2.97 (t, $J = 7.7$ Hz, 2H), 2.08 – 2.02 (m, 2H); ^{13}C NMR (126 MHz, MeOD): δ 170.6, 139.0, 138.8, 136.1, 131.7, 130.8, 125.5, 123.9, 123.7, 118.9, 117.2, 72.8, 70.5, 29.2, 27.0; IR (neat)/ cm^{-1} : 3067, 1672, 1640, 1592, 1555; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{15}\text{H}_{18}\text{NO}_3$: 260.1281; found 260.1274.

4.3.3 Synthesis of HHQ analogue **3**



2-Heptyl-6-((trimethylsilyl)ethynyl)quinolin-4(1H)-one **S8**

Based on a procedure by Mugnaini et al.²⁰ solution of **S7** prepared as previously reported²¹ (128 mg, 0.4 mmol, 1.0 eq) in diisopropylamine (14 mL) and 1,3-dioxane (10 mL) was degassed by bubbling argon through the solution for 10 min before the addition of CuI (16 mg, 20 mol%), PdCl₂(PPh₃)₂ (28 mg, 10 mol%) and trimethylsilylacetylene (80 μL , 0.56 mmol, 1.8 eq). The mixture was heated in a sealed tube at 130 $^\circ\text{C}$ for 15 min after which the reaction

was diluted with CH₂Cl₂ (10 mL), filtered through celite and washed with 1 M HCl (10 mL) and brine (10 mL). The organic phase was dried over MgSO₄, filtered and evaporated to dryness under reduced pressure and the resulting residue purified by column chromatography (stepwise gradient of 0-4% MeOH in CH₂Cl₂) to give the title compound **S8** (108 mg, 0.32 mmol) as an off white amorphous powder in 79% yield (~95% pure by ¹H NMR).

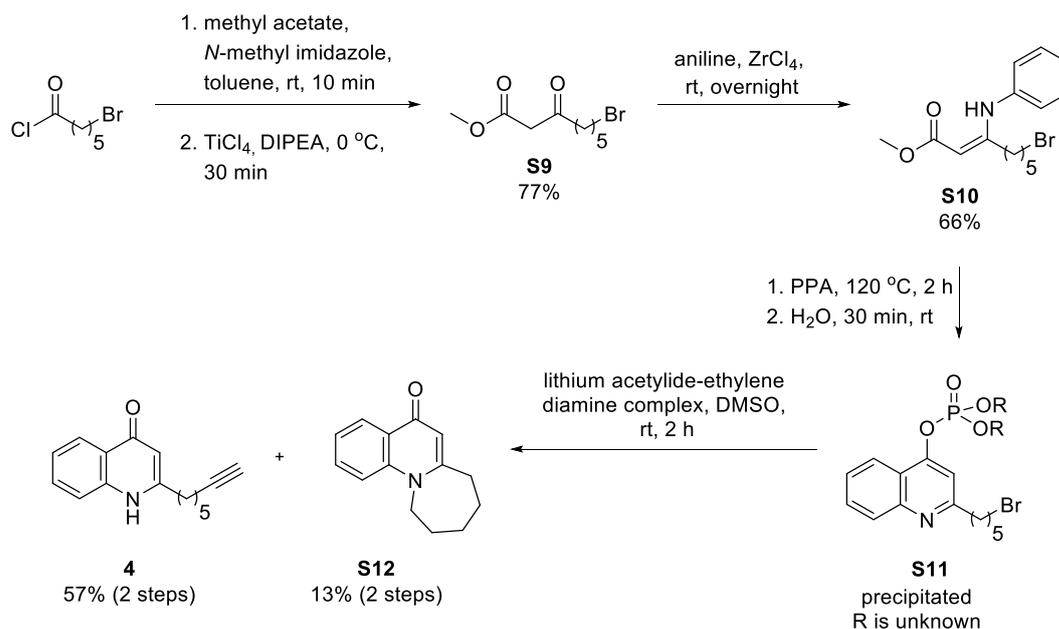
TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f = 0.37; ¹H NMR (400 MHz, MeOD): δ 8.29 (d, *J* = 1.9 Hz, 1H), 7.72 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 6.25 (s, 1H), 2.74 (t, *J* = 7.7 Hz, 2H), 1.80 (app p, *J* = 7.6 Hz, 2H), 1.45 – 1.30 (m, 8H), 0.94 (t, *J* = 6.9 Hz, 3H), 0.29 (s, 9H); ¹³C NMR (126 MHz, CDCl₃): δ 178.4, 154.8, 140.1, 134.8, 129.9, 124.77, 118.6, 118.3, 108.9, 94.6, 51.02, 34.5, 31.8, 29.3, 29.1, 29.0, 22.7, 14.2, 0.1; IR (neat)/cm⁻¹: 3060, 2159, 1636, 1586, 1548, 836; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₂₁H₃₀NOSi, 340.2091; found, 340.2087.

6-Ethynyl-2-heptylquinolin-4(1H)-one **3**

A solution of compound **S8** (40 mg, 0.12 mmol, 1.0 eq) and K₂CO₃ (120 mg, 0.89 mmol, 7.2 eq) in MeOH (4 mL) was stirred at room temperature overnight. The solvent was then removed under reduced pressure and the resulting mixture was partitioned between CH₂Cl₂ (10 mL) and H₂O (10 mL). The aqueous phase was extracted twice more with CH₂Cl₂ (10 mL) and the combined organic phases were dried over MgSO₄, filtered and the solvent removed under reduced pressure. The resulting residue was purified by column chromatography (stepwise gradient of 0-5% MeOH in CH₂Cl₂) to give the title compound **3** (29.6 mg, 0.1 mmol) in 92% yield. This was further purified by recrystallisation from CH₂Cl₂.

mp: 142.1-143.9 °C (CH₂Cl₂); TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f: 0.37; ¹H NMR (400 MHz, MeOD): δ 8.34 (d, *J* = 1.9 Hz, 1H), 7.76 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.58 (dd, *J* = 8.6, 0.6 Hz, 1H), 6.26 (s, 1H), 3.60 (s, 1H), 2.75 (t, *J* = 7.7 Hz, 2H), 1.80 (app p, *J* = 7.6 Hz, 2H), 1.46 – 1.32 (m, 8H), 0.96 – 0.90 (m, 3H); ¹³C NMR (126 MHz, CDCl₃): δ 178.3, 153.7, 139.8, 135.1, 130.4, 124.9, 117.8, 117.6, 109.5, 82.6, 77.2, 34.6, 31.8, 29.2, 29.1, 28.7, 22.7, 14.2; IR (neat)/cm⁻¹: 3056, 2924, 2576, 1636, 1586, 1548; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₈H₂₂NO, 268.1696; found, 268.1962.

4.3.4 Synthesis of HHQ analogue 4



Methyl 8-bromo-3-oxooctanoate S9

Based on the reported procedure by Hashimoto et al.²² 6-Bromohexanoyl chloride (15 g, 70 mmol, 1.0 eq) was added dropwise to a stirring solution of methyl acetate (7.62 mL, 96 mmol, 1.4 eq) and 1-methyl imidazole (5.74 mg, 72 mmol, 1.0 eq) in toluene (180 mL) forming a white precipitate. After 10 min the suspension was cooled to 0 °C before TiCl₄ (21.72 mL, 198 mmol, 2.8 eq) was added dropwise over 20 min with vigorous stirring and the mixture turned bright orange. DIPEA (37.6 mL, 216 mmol, 3.0 eq) was added dropwise over 20 min resulting in a dark red solution and the reaction was allowed to warm to room temperature over 30 min. The reaction was quenched by the addition of water (250 mL) and extracted with Et₂O (2 x 250 mL). The combined organic phases were dried over Na₂SO₄, filtered and evaporated to dryness yielding **S9** as a pale yellow oil (13.3 g, 53.6 mmol) in 77% yield without the need for purification.

TLC (Et₂O:40-60 PE, 3:7 v/v): R_f = 0.35; ¹H NMR (400 MHz, CDCl₃): δ 3.74 (s, 3H), 3.45 (s, 2H), 3.40 (t, *J* = 6.7 Hz, 3H), 2.57 (t, *J* = 7.2 Hz, 2H), 1.91 – 1.83 (m, 2H), 1.65-1.57 (m, 2H), 1.50 – 1.38 (m, 2H); ¹³C NMR (101 MHz, CDCl₃): δ 202.7, 167.5, 124.8, 52.1, 48.8, 42.9, 28.8, 28.8, 23.3, 22.4; IR (neat)/cm⁻¹: 1735, 1623; HRMS (ESI-TOF) *m/z*: [M + Na]⁺ calcd. for C₉H₁₅OBrNa, 273.0094; found, 273.0097.

Methyl (Z)-8-bromo-3-(phenylamino)oct-2-enoate S10

A mixture of β-keto ester **S9** (1.7 g, 6.9 mmol, 1.0 eq), aniline (632 μL, 6.9 mol, 1.0 eq) and ZrCl₄ (50 mg, 0.21 mmol, 3 mol%) was stirred at room temperature overnight. The reaction was diluted with Et₂O (20 mL), filtered and the organic phase washed with 0.1 M HCl (2 x 20

mL). The solvent was removed under reduced pressure and the resulting mixture was purified by column chromatography (stepwise gradient of 0-30% Et₂O in 40-60 PE) to give the title compound **S10** (1.13 g, 3.48 mmol) as an amorphous solid in 66% yield (96% based on recovered starting material).

TLC (Et₂O:40-60 PE, 3:7 v/v): R_f = 0.24; ¹H NMR (400 MHz, CDCl₃): δ 10.26 (s, 1H), 7.38 – 7.30 (m, 2H), 7.10 (t, *J* = 7.4 Hz, 1H), 7.09 (d, *J* = 7.5 Hz, 2H), 4.72 (s, 1H), 3.69 (s, 3H), 3.30 (t, *J* = 6.7 Hz, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 1.74 (app p, *J* = 6.9 Hz, 2H), 1.48 – 1.32 (m, 4H); ¹³C NMR (101 MHz, CDCl₃): δ 171.1, 163.2, 139.3, 129.3, 125.6, 125.3, 84.9, 50.5, 33.5, 32.3, 32.2, 27.7, 27.2; IR (neat)/cm⁻¹: 1588, 1552, 760.

2-(Hept-6-yn-1-yl)quinolin-4(1H)-one **4 and 8,9,10,11-Tetrahydroazepino[1,2-a]quinolin-5(7H)-one **S12****

Imine **S11** (325 mg, 1.0 mmol, 1.0 eq) was added to a stirring solution of polyphosphoric acid (115% H₃PO₄ basis, 1.0 g) and heated to 90 °C with for 1 h. The reaction was cooled to room temperature and H₂O (20 mL) was then slowly added to the stirring mixture which was stirred for a further 30 min, and formed a white precipitate. The supernatant was then decanted and the precipitate was washed twice with H₂O and dried under vacuum to give the crude protected product as a white sticky solid. The precipitate was washed twice with water and dried under reduced pressure to give a gummy white solid which was then dissolved in DMSO (10 mL) and lithium acetylide-ethylenediamine complex (643 mg, 7.0 mmol, 7.0 eq) was added piecewise and stirred at room temperature overnight. The reaction was poured into an ice/brine mixture (1:1, 100 mL), acidified with 1 M HCl, extracted with CH₂Cl₂ (3 x 50 mL), dried over Na₂SO₄, filtered and evaporated to dryness. The resulting residue was purified by column chromatography (stepwise gradient of 0-5% MeOH in CH₂Cl₂) to give the title compound **4** (166 mg 0.57 mmol) as colourless needles in 57% yield over two steps. Side product **S12** (27.6 mg, 0.13 mmol) was isolated in 13% yield.

Data for 2-(Hept-6-yn-1-yl)quinolin-4(1H)-one **4**

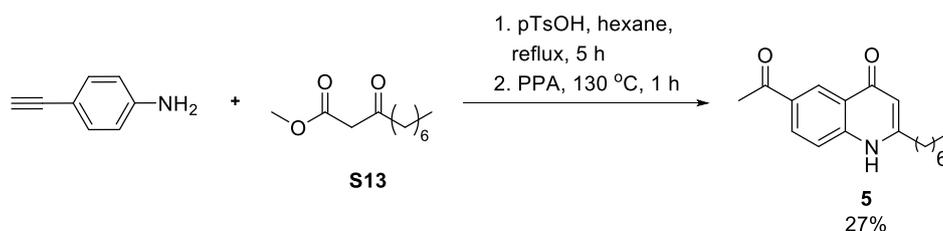
TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f = 0.43; ¹H NMR (400 MHz, CDCl₃): δ 11.23 (s, 1H), 8.35 (dd, *J* = 8.1, 1.4 Hz, 1H), 7.64 (dd, *J* = 8.4, 1.3 Hz, 1H), 7.59 (ddd, *J* = 8.4, 6.8, 1. Hz, 1H), 7.33 (ddd, *J* = 8.1, 6.8, 1.3 Hz, 1H), 6.22 (d, *J* = 1.5 Hz, 1H), 2.69 (t, *J* = 7.8 Hz, 2H), 2.12 (td, *J* = 6.7, 2.6 Hz, 2H), 1.90 (t, *J* = 2.6 Hz, 1H), 1.76 (app q, *J* = 7.7 Hz, 2H), 1.54 – 1.36 (m, 4H); ¹³C NMR (101 MHz, CDCl₃): δ 179.1, 154.3, 140.5, 132.0, 125.6, 125.2, 123.8, 118.2, 108.6, 84.3, 68.7, 34.4, 28.5, 28.3, 28.1, 18.4; IR (neat)/cm⁻¹: 3261, 1666, 1629, 1595, 1519; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₆H₁₇NO, 240.1388; found, 240.1383.

Data for 8,9,10,11-Tetrahydroazepino[1,2-a]quinolin-5(7H)-one **S12**

mp: 99.9-101.3 °C (CDCl₃); TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f = 0.40; ¹H NMR (500 MHz,

CDCl₃): δ 8.46 (dd, *J* = 8.0, 1.7 Hz, 1H), 7.64 (ddd, *J* = 8.7, 7.0, 1.7 Hz, 1H), 7.52 (d, *J* = 8.6 Hz, 1H), 7.35 (ddd, *J* = 8.0, 6.9, 0.9 Hz, 1H), 6.24 (s, 1H), 4.35 – 4.23 (m, 2H), 2.91 (app dd, *J* = 7.3, 3.2 Hz, 2H), 1.91 – 1.81 (m, 6H); ¹³C NMR (126 MHz, CDCl₃): δ 178.3, 157.0, 141.5, 132.2, 127.1, 126.8, 123.2, 115.0, 110.6, 47.3, 35.7, 28.5, 27.4, 26.7; IR (neat)/cm⁻¹: 1616, 2594, 1553; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₄H₁₆NO, 214.1226; found, 214.1223.

4.3.5 Synthesis of HHQ analogue 5

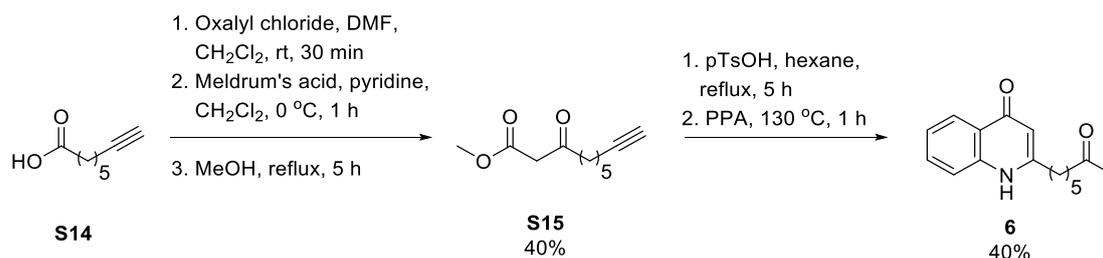


6-Acetyl-2-heptylquinolin-4(1H)-one 5

4-Ethynylaniline (146 mg, 1.24 mmol, 1.0 eq), methyl-3-oxydecanoate **S13** prepared following previously reported procedure²³ (250 mg, 1.25 mmol, 1.0 eq) and *p*-TsOH (5.5 mg, 2.5 mol%) were dissolved in hexane (2.5 mL) and heated to reflux with stirring using Dean-Stark apparatus for 5 h. The solvent was then removed under reduced pressure and the resulting imine was added to polyphosphoric acid (115% H₃PO₄ basis, 1.8 g) at 130 °C and stirred vigorously for 1 h. The resulting mixture was added slowly to a saturated solution of NaHCO₃ and stirred at 0 °C until a precipitate formed. The precipitate was collected by filtration and purified by column chromatography (stepwise gradient of 0-5% MeOH in CH₂Cl₂) to yield **5** (96 mg, 0.34 mmol) as a white amorphous solid in 27% yield.

mp: 206.5-208.5 °C (MeOH); TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f = 0.36; ¹H NMR (500 MHz, MeOD): δ 8.88 (d, *J* = 2.8 Hz, 1H), 8.33 (d, *J* = 8.7 Hz, 1H), 7.63 (dd, *J* = 9.2, 2.8 Hz, 1H), 6.27 (s, 1H), 2.73 (t, *J* = 7.9 Hz, 2H), 2.70 (s, 3H), 1.78 (app p, *J* = 7.5 Hz, 2H), 1.45 – 1.30 (m, 8H), 0.91 (t, *J* = 7.0 Hz, 3H); ¹³C NMR (126 MHz, MeOD): δ 199.0, 180.7, 157.9, 144.4, 133.7, 131.8, 128.4, 124.7, 119.6, 110.0, 35.0, 32.9, 30.2, 30.1, 30.0, 26.5, 23.7, 14.4. IR (neat)/cm⁻¹: 3148, 3080, 1682, 1634, 1552; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₈H₂₄NO₂, 286.1802; found, 286.1793.

4.3.6 Synthesis of HHQ analogue 6



Methyl 3-oxodec-9-ynoate S15

The title compound was prepared based on a published procedure by Oikawa et al.²⁴ Oct-7-ynoic acid **S14** has been prepared as reported by Garner and Janda.²⁵ Oxalyl chloride (1 mL, 2 M in CH₂Cl₂, 2 mmol, 2.0 eq) was added dropwise to a solution of acid **S14** (140 mg, 1.0 mmol, 1.0 eq) and DMF (1 drop) in CH₂Cl₂ at 0 °C and the reaction was stirred for 1 h. Then the reaction was allowed to warm to room temperature and stirred for a further 2 h. After this the solvent was removed under reduced pressure and the acid chloride was dissolved in CH₂Cl₂ (100 μL). A solution of Meldrum's acid (131 mg, 0.91 mmol, 1.0 eq) in dry CH₂Cl₂ (2 mL) was cooled to 0 °C and pyridine (150 μL) was added drop-wise over 20 min. The acid chloride in CH₂Cl₂ was then added and the mixture was stirred at 0 °C for a further 2 h. The reaction mixture was allowed to warm to room temperature, diluted with CH₂Cl₂ (x 2) and poured into an equal volume of cold HCl (2 N). The organic phase was washed with saturated NaCl, dried over MgSO₄, filtered and evaporated to dryness. The resulting orange/brown oil was heated to reflux in anhydrous MeOH (5 mL) for 5 h, evaporated to dryness and purified by (stepwise gradient of 5%-10% Et₂O in 40/60 PE) to give **S15** (78 mg, 0.4 mmol) as a colourless oil in 40% yield.

TLC (Et₂O:hexane, 2:8 v/v): R_f: 0.31; ¹H NMR (400 MHz, CDCl₃): δ 3.74 (s, 3H), 3.45 (s, 2H), 2.55 (t, *J* = 7.3 Hz, 2H), 2.19 (dt, *J* = 7.3, 2.7 Hz, 2H), 1.94 (t, *J* = 2.7 Hz, 1H), 1.65 – 1.58 (m, 2H), 1.56 – 1.49 (m, 2H), 1.47 – 1.36 (m, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 202.6, 167.8, 84.4, 68.5, 52.5, 49.2, 43.0, 28.3, 28.2, 23.0, 18.4; IR (neat)/cm⁻¹: 3283, 1743, 1714; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₁H₁₇O₃, 197.1178, found: 197.1178.

Spectroscopic data consistent with literature values.²⁵

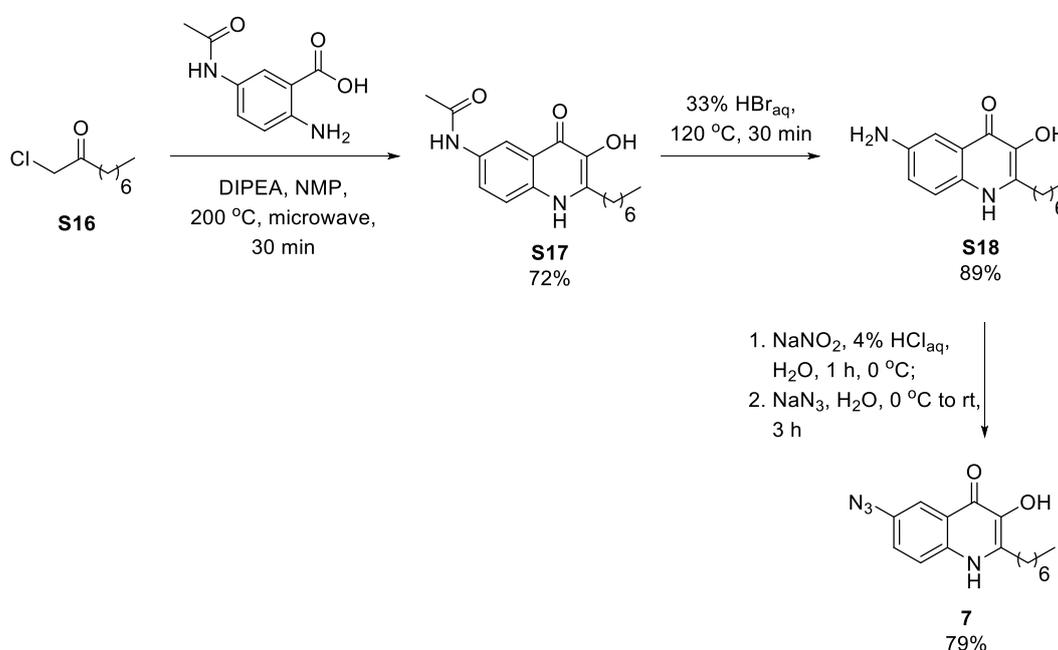
2-(6-Oxoheptyl)quinolin-4(1H)-one 6

A solution of aniline (47.2 μL, 0.5 mmol, 1.0 eq), *p*-TsOH (2.42 mg, 2.5 mol%) and β-keto ester **S15** (100 mg, 0.5 mmol, 1.0 eq) in hexane (0.5 mL) was stirred at room temperature overnight. The solvent was removed under reduced pressure and the residue was added to polyphosphoric acid (115% H₃PO₄ basis, 1 g) heated to 120 °C with stirring for 1 h. The reaction was then allowed to cool to room temperature and added slowly to a saturated

solution of NaHCO₃ (15 mL) at 0 °C. The resulting precipitate was collected by filtration and purified by column chromatography (stepwise gradient of 0-5% MeOH in CH₂Cl₂) to yield **6** (90 mg, 0.23 mmol) as a white amorphous solid in 70% yield.

Mp: 123.4-124.5 °C (CDCl₃); TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f = 0.56; ¹H NMR (500 MHz, CDCl₃): δ 8.34 (d, *J* = 7.8 Hz, 1H), 7.60 – 7.51 (m, 2H), 7.32 (ddd, *J* = 8.1, 4.7, 3.4 Hz, 1H), 6.18 (d, *J* = 1.6 Hz, 1H), 2.67 (t, *J* = 7.4 Hz, 2H), 2.44 (t, *J* = 6.8 Hz, 2H), 2.14 (s, 3H), 1.72 (app p, *J* = 7.3 Hz, 2H), 1.65 – 1.52 (m, 2H), 1.33 (tt, *J* = 10.5, 6.4 Hz, 2H); ¹³C NMR (126 MHz, CDCl₃): δ 209.8, 179.1, 153.6, 140.2, 131.9, 125.9, 125.2, 123.7, 117.9, 109.0, 43.3, 33.7, 30.3, 28.7, 28.1, 22.5; IR (neat)/cm⁻¹: 1705, 1640, 1594; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. C₁₆H₂₀NO₂: 258.1489; found: 258.1483.

4.3.7 Synthesis of PQS analogue 7



N-(2-heptyl-3-hydroxy-4-oxo-1,4-dihydroquinolin-6-yl)acetamide **S17**

The title compound was prepared following the procedure outlined by Hodgkinson et al¹⁶ DIPEA (0.46 mL, 2.6 mmol, 1.2 eq) and the required α-chloro ketone **S16** prepared according as previously reported¹⁶ (578 mg, 2.2 mmol, 1.0 eq), were added to a solution of 5-(acetamino)-2-aminobenzoic acid (427 mg, 2.2 mmol, 1.0 eq), in anhydrous NMP (4.5 mL). The mixture was then heated under microwave irradiation at 200 °C for 30 min. After cooling to room temperature the reaction was quenched by pouring into ice/water. The resulting mixture was stirred for 5 min before being allowed to settle for 30 min and the precipitate was collected and dried under vacuum to give **S17** (421 mg, 1.58 mmol) as a beige amorphous solid in 72% yield.

mp: degrades ~300 °C (EtOAc); TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.5; ¹H NMR (500 MHz, DMSO-*d*₆): δ 11.39 (s, 1H), 10.04 (s, 1H), 8.32 (d, *J* = 2.4 Hz, 1H), 7.74 (dd, *J* = 9.0, 2.5 Hz, 1H), 7.48 (d, *J* = 9.0 Hz, 1H), 2.71 (t, *J* = 7.7 Hz, 1H), 2.06 (s, 3H), 1.75 – 1.56 (m, 2H), 1.37 – 1.16 (m, 8H), 0.84 (t, *J* = 6.8 Hz, 3H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ 168.4, 168.1, 137.4, 135.2, 133.7, 133.6, 123.1, 122.4, 118.2, 112.7, 31.2, 28.8, 28.5, 28.1, 27.8, 24.0, 22.1, 13.9; IR (neat)/cm⁻¹: 3397, 3284, 3144, 1658, 1645, 1536; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₈H₂₅N₂O: 317.1860; found 317.1856.

6-Amino-2-heptyl-3-hydroxyquinolin-4(1H)-one **S18**

Intermediate **S16** (400 mg, 1.3 mmol, 1.0 eq) was dissolved in HBr (33% aqueous solution, 4 mL) and heated to 120 °C for 30 min with stirring. The reaction was cooled to room temperature and a crystalline solid formed. The solid was collected by filtration and partitioned between EtOAc/NaHCO₃. The organic layer was dried over Na₂SO₄, filtered and the solvent removed under reduced pressure to give **S18** (308 mg, 1.12 mmol) as an amorphous solid in 89% yield.

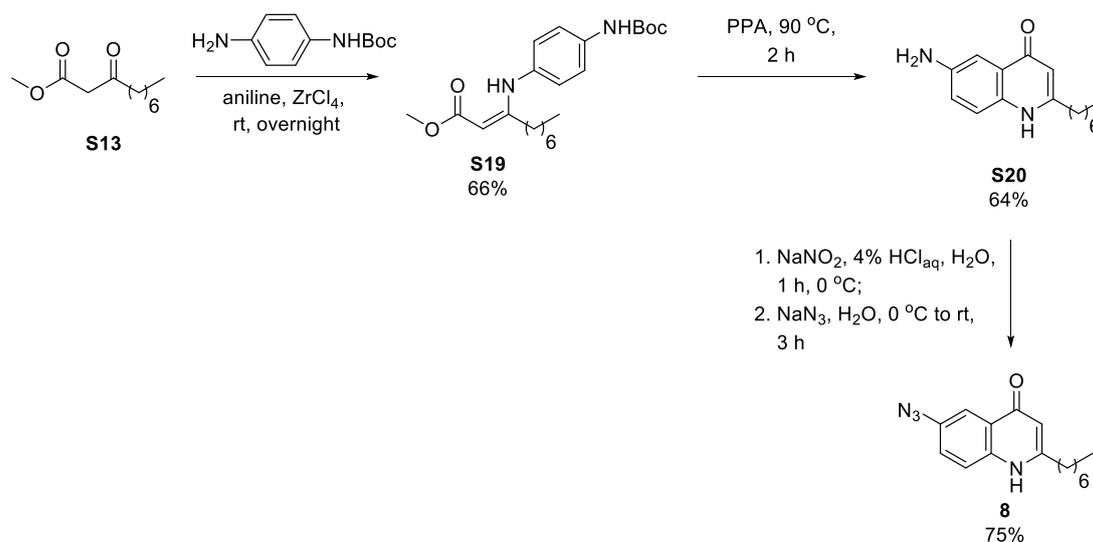
mp: decomposes ~360 °C (EtOAc); TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.45; ¹H NMR (400 MHz, MeOD): δ 8.15 (d, *J* = 2.3 Hz, 1H), 8.04 (d, *J* = 9.1 Hz, 1H), 7.76 (dd, *J* = 9.1, 2.4 Hz, 1H), 3.14 (t, *J* = 8.0 Hz, 2H), 1.83 (app p, *J* = 7.6 Hz, 1H), 1.53 – 1.26 (m, 9H), 0.95 – 0.86 (t, *J* = 6.9 Hz, 3H); ¹³C NMR (126 MHz, MeOD) δ 159.9, 152.6, 137.6, 135.4, 134.4, 127.5, 122.4, 121.9, 115.1, 32.8, 31.0, 30.5, 30.0, 29.4, 23.7, 14.4; IR (neat)/cm⁻¹: 3000, 1607, 1556; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₆H₂₃N₂O₂: 275.1767; found 275.1760.

6-Azido-2-heptyl-3-hydroxyquinolin-4(1H)-one **7**

Compound **S18** (274 mg, 1 mmol, 1.0 eq) was dissolved in 4% HCl (5 mL) and the solution was cooled to 0 °C. A solution of NaNO₂ (140 mg, 3 mmol, 2.0 eq) in H₂O (1 mL) was added dropwise and the reaction stirred at 0 °C for 1 h. A solution of NaN₃ (163 mg, 2.5 mmol, 2.5 eq) in H₂O, (1 mL) was then added dropwise and the reaction was allowed to warm to room temperature and stirred for a further 3 h. The reaction was diluted with H₂O (10 mL) and extracted with EtOAc (3 x 20 mL). The combined organic extracts dried over MgSO₄, filtered and evaporated to dryness to give **7** (211 mg, 0.70 mmol) as brown crystals in 70% yield.

mp: decomposes ~136.7 °C (CH₂Cl₂); TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.67; ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.06 (s, 1H), 7.74 (d, *J* = 2.6 Hz, 1H), 7.67 (d, *J* = 8.9 Hz, 1H), 7.33 (dd, *J* = 8.9, 2.7 Hz, 1H), 2.77 (t, *J* = 7.7 Hz, 2H), 1.67 (app p, *J* = 7.3 Hz, 2H), 1.35 – 1.21 (m, 8H), 0.85 (t, *J* = 6.7 Hz, 3H); ¹³C NMR (101 MHz, DMSO-*d*₆): δ 166.2, 137.9, 137.8, 134.5, 133.9, 122.7, 122.6, 120.4, 112.4, 31.2, 28.8, 28.4, 28.3, 27.8, 22.1, 14.0; IR (neat)/cm⁻¹: 3089, 2108, 1632, 1591, 1551; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₆H₂₁N₄O₂: 301.1659; found: 301.1649.

4.3.8 Synthesis of HHQ analogue 8



Methyl (Z)-8-bromo-3-((4-((tert-butoxycarbonyl)amino)phenyl)amino)oct-2-enoate **S19**

A mixture of β -keto ester **S13**²³ (2.0 g, 8 mmol, 1.0 eq), *N*-Boc-*p*-phenylenediamine (1.6 g, 8 mmol, 1.0 eq) and $ZrCl_4$ (60 mg, 0.26 mmol, 3 mol%) was stirred at room temperature overnight. The reaction was diluted with Et_2O (20 mL), filtered and the solvent was removed under reduced pressure. The crude product was purified by column chromatography (stepwise gradient of 0-40% Et_2O in 40-60 PE) to give the title compound **S19** (1.7 g, 4.4 mmol) as a pale yellow-white amorphous solid in 66% yield (98% based on recovered starting material).

TLC (Et_2O :hexane, 3:7 v/v): R_f = 0.45; 1H NMR (500 MHz, $CDCl_3$): δ 10.15 (s, 1H), 7.33 (d, J = 8.5 Hz, 2H), 7.02 (d, J = 8.7 Hz, 2H), 6.48 (s, 1H), 4.69 (s, 1H), 3.68 (s, 3H), 2.21 (t, J = 7.8 Hz, 2H), 1.52 (s, 9H), 1.41 – 1.14 (m, 10H), 0.84 (t, J = 7.1 Hz, 3H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 171.2, 164.4, 152.8, 136.1, 134.3, 126.4, 119.2, 84.0, 80.9, 50.4, 32.3, 31.7, 29.2, 29.0, 28.5, 28.1, 22.7, 14.2; IR (neat)/ cm^{-1} : 3340, 3259, 1724, 1634, 1610, 1583, 1519; HRMS (ESI-TOF) m/z : $[M + H]^+$ calcd. for $C_{22}H_{35}N_2O_4$: 391.2587; found: 391.2587.

6-Amino-2-heptylquinolin-4(1H)-one **S20**

Enamine **S19** (195 mg, 0.50 mmol, 1.0 eq) was added slowly to polyphosphoric acid (115% H_3PO_4 basis, 2.0 g) at 90 °C and stirred for 2 h. The mixture was then added slowly to a stirring solution of saturated $NaHCO_3$ and, once the bubbling had stopped, extracted with CH_2Cl_2 to yield the target compound **S20** (81.5 mg, 0.32 mmol) in 64% yield as an amorphous brown solid without need for further purification.

TLC ($MeOH:CH_2Cl_2$, 5:95 v/v): R_f = 0.39; 1H NMR (400 MHz, $DMSO-d_6$): δ 7.26 (d, J = 8.7 Hz, 1H), 7.14 (d, J = 2.5 Hz, 1H), 6.94 (dd, J = 8.7, 2.5 Hz, 1H), 5.73 (s, 1H), 5.14 (s, 2H),

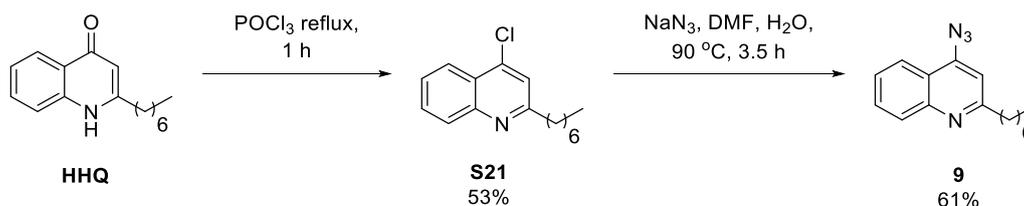
1.63 (app p, $J = 7.4$ Hz, 2H), 1.31-1.23 (m, 8H), 0.85(t, $J = 6.9$ Hz, 3H); ^{13}C NMR (101 MHz, DMSO- d_6): δ 176.3, 151.3, 144.7, 132.0, 126.2, 120.7, 118.6, 105.7, 105.4, 33.2, 31.2, 28.6, 28.5, 28.4, 22.1, 14.0; IR (neat)/ cm^{-1} : 3340, 3224, 1659, 1623, 1506; LRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{16}\text{H}_{23}\text{N}_2\text{O}_2$: 258.2; found: 258.4.

6-Azido-2-heptylquinolin-4(1H)-one **8**

Compound **S20** (258 mg, 1 mmol, 1.0 eq) was dissolved in 4% HCl (5 mL) and the solution was cooled to 0 °C. A solution of NaNO_2 (140 mg, 3 mmol, 2.0 eq) in H_2O (1 mL) was added dropwise and the reaction stirred at 0 °C for 1 h. A solution of NaN_3 (163 mg, 2.5 mmol, 2.5 eq) in H_2O , (1 mL) was then added dropwise and the reaction was allowed to warm to room temperature and stirred for a further 3 h. The reaction was diluted with H_2O (10 mL) and extracted with EtOAc (3 x 20 mL) The combined organic extracts dried over MgSO_4 , filtered and evaporated to dryness to give **8** (213 mg, 0.75 mmol) as orange brown crystals in 75% yield.

Mp: decomposes 172.6-177.4 °C (EtOAc); TLC (MeOH: CH_2Cl_2 , 5:95 v/v): $R_f = 0.42$; ^1H NMR (500 MHz, MeOD): δ 7.89 (d, $J = 2.6$ Hz, 1H), 7.62 (d, $J = 8.9$ Hz, 1H), 7.38 (dd, $J = 8.9, 2.6$ Hz, 1H), 6.23 (s, 1H), 2.72 (t, $J = 7.7$ Hz, 2H), 1.76 (app p, $J = 7.7$ Hz, 2H), 1.46 – 1.26 (m, 8H), 0.90 (t, $J = 7.7$ Hz, 3H); ^{13}C NMR (126 MHz, MeOD): δ 179.5, 157.1, 138.9, 138.0, 126.5, 125.6, 121.3, 114.4, 108.8, 35.0, 32.9, 30.2, 30.2, 30.1, 23.7, 14.4; IR (neat)/ cm^{-1} : 2108, 1592, 1551, 1505; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{16}\text{H}_{20}\text{N}_3\text{O}$: 285.1715; found: 285.1721.

4.3.9 Synthesis of HHQ analogue **9**



4-Chloro-2-heptylquinoline **S21**

HHQ prepared following a literature procedure¹⁷ (14 mg, 0.058 mmol, 1.0 eq) was dissolved in POCl_3 (1 mL) and the mixture was heated to reflux for 1 h. The excess POCl_3 was removed under reduced pressure and the resulting residue was dissolved in CH_2Cl_2 (5 mL) and washed with saturated NaHCO_3 (3 x 5 mL). The organic layer was then dried over MgSO_4 , filtered and evaporated to dryness to give **S21** (10 mg, 0.3 mmol) as a colourless oil in 53% yield without further purification.

TLC (Et_2O :hexane, 2:8 v/v): $R_f = 0.58$; ^1H NMR (500 MHz, CDCl_3): δ 8.18 (ddd, $J = 8.4, 1.6, 0.6$ Hz, 1H), 8.05 (ddd, $J = 8.5, 1.6, 0.9$ Hz, 1H), 7.73 (ddd, $J = 8.4, 6.9, 1.4$ Hz, 1H), 7.58

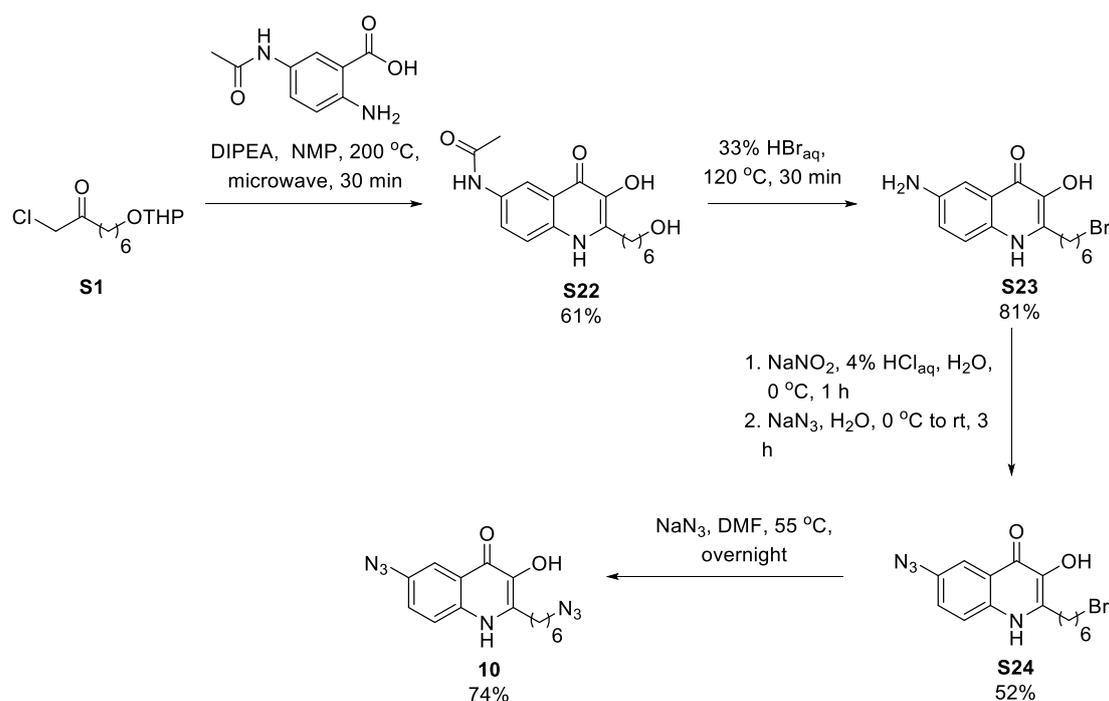
(ddd, $J = 8.2, 6.9, 1.2$ Hz, 1H), 7.40 (s, 1H), 2.94 (t, $J = 7.9$ Hz, 2H), 1.84 – 1.77 (m, 2H), 1.44 – 1.25 (m, 8H), 0.88 (t, $J = 7.0$ Hz, 3H); ^{13}C NMR (126 MHz, CDCl_3): δ 163.2, 148.9, 142.6, 130.4, 129.3, 126.8, 125.1, 124.1, 121.5, 39.3, 31.9, 30.0, 29.6, 29.3, 22.8, 14.2; IR (neat)/ cm^{-1} : 1616, 1589, 1552, 757; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{16}\text{H}_{21}\text{NCl}$: 262.1363; found: 262.1367.

4-Azido-2-heptylquinoline 9

Compound **S21** (40 mg, 0.15 mmol, 1.0 eq) and NaN_3 (52.5 mg, 0.8 mmol, 5.3 eq) were dissolved in a mixture of DMF and H_2O (8:3, 1 mL) and heated to 90°C for 4 h. The reaction was cooled to rt, diluted with saturated NH_4Cl (30 mL) and extracted with CH_2Cl_2 (3 x 30 mL). The combined organic phases were dried over MgSO_4 , filtered and the solvent was removed under reduced pressure. The crude product was triturated with Et_2O , filtered and the solvent removed under vacuum to give **9** (24.5 mg, 0.09 mmol) as a waxy amorphous solid in 61% yield.

TLC (Et_2O :hexane, 2:8 v/v): $R_f = 0.51$; ^1H NMR (500 MHz, MeOD): δ 8.07 (dd, $J = 8.4, 1.4$ Hz, 1H), 7.96 (dt, $J = 8.6, 0.8$ Hz, 1H), 7.97 – 7.95 (m, 1H), 7.54 (ddd, $J = 8.2, 6.8, 1.2$ Hz, 1H), 7.29 (s, 1H), 2.97 (t, $J = 7.9$ Hz, 2H), 1.85 – 1.80 (m, 2H), 1.47 – 1.28 (m, 8H), 0.91 (t, $J = 6.3$ Hz, 3H); ^{13}C NMR (126 MHz, MeOD) δ 165.1, 149.4, 148.6, 131.9, 128.3, 127.1, 123.3, 121.5, 110.2, 39.7, 32.9, 31.3, 30.3, 23.7, 14.4. IR (neat)/ cm^{-1} : 2109, 1619, 1590, 1559, 1502; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{16}\text{H}_{21}\text{N}_4$: 269.1766; found: 269.1763.

4.3.10 Synthesis of PQS probe 10



N-(3-hydroxy-2-(6-hydroxyhexyl)-4-oxo-1,4-dihydroquinolin-6-yl)acetamide S22

The title compound was prepared following the procedure outlined by Hodgkinson et al.¹⁶ DIPEA (0.23 mL, 1.3 mmol, 1.2 eq) and α -chloroketone **S1** (282 mg, 1.1 mmol, 1.0 eq) were added to a solution of acetamidoanthranilic acid (212 mg, 1.1 mmol, 1.0 eq) in anhydrous NMP (2.25 mL). The mixture was then heated under microwave irradiation at 200 °C for 30 min. After cooling to room temperature the reaction was quenched by pouring into acetonitrile (20 mL) to give **S22** (214 mg, 0.67 mmol) in 61% yield as a pale yellow powder which was used without further purification.

mp: 276.3-277.8 °C (EtOAc); TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.27; ¹H NMR (500 MHz, DMSO-*d*₆): δ 11.40 (s, 1H), 10.04 (s, 1H), 8.32 (d, *J* = 2.4 Hz, 1H), 7.74 (dd, *J* = 9.0, 2.4 Hz, 1H), 7.48 (d, *J* = 9.0 Hz, 1H), 4.33 (s, 1H), 3.36 (t, *J* = 6.5 Hz, 2H), 2.71 (t, *J* = 7.7 Hz, 2H), 2.06 (s, 3H), 1.65 (app q, *J* = 7.7 Hz, 2H), 1.44 – 1.38 (m, 2H), 1.37 – 1.30 (m, 4H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ 168.4, 168.1, 137.4, 135.2, 133.7, 133.6, 123.1, 122.4, 118.2, 112.7, 60.6, 32.4, 28.8, 28.1, 27.9, 27.8, 25.3, 24.0; IR (neat)/cm⁻¹: 3269, 3127, 3100, 1661, 1643, 1613, 1539; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₇H₂₃N₂O₄: 319.1658; found 319.1661.

6-Amino-2-(6-bromohexyl)-3-hydroxyquinolin-4(1H)-one S23

Compound **S22** (738 mg, 2.46 mmol, 1.0 eq) was dissolved in HBr (33% aqueous solution, 2.4 mL) and heated to 120 °C for 30 min with stirring. The reaction was cooled to room temperature and a crystalline solid was formed. The solid was collected by filtration and partitioned between EtOAc/NaHCO₃. The organic layer was dried over Na₂SO₄, filtered and the solvent removed under reduced pressure to give **S23** (840 mg, 2.0 mmol) as an amorphous solid in 81% yield.

TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.43; ¹H NMR (400 MHz, DMSO-*d*₆): δ 7.38 (d, *J* = 8.8 Hz, 1H), 7.24 (d, *J* = 2.5 Hz, 1H), 7.02 (dd, *J* = 8.8, 2.5 Hz, 1H), 3.51 (t, *J* = 6.7 Hz, 2H), 2.72 (t, *J* = 7.8 Hz, 2H), 1.79 (app p, *J* = 6.8 Hz, 2H), 1.69 – 1.61 (m, 2H), 1.46-1.30 (m, 4H); ¹³C NMR (101 MHz, DMSO-*d*₆): δ 165.7, 142.8, 136.5, 135.7, 130.4, 123.3, 121.4, 118.9, 104.5, 35.2, 32.1, 28.1, 27.9, 27.8, 27.3; IR (neat)/cm⁻¹: 2800 (Br), 1605, 1557; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₅H₂₀N₂O₂Br: 339.0708; found: 339.0708.

6-Azido-2-(5-bromopentyl)-3-hydroxyquinolin-4(1H)-one S24

A solution of NaNO₂ (31 mg, 0.45 mmol, 1.3 eq) in water (1 mL) was added dropwise to a stirring solution of compound **S23** (100 mg, 0.31 mmol, 1.0 eq) in a 4% aqueous solution of HCl (3 mL) at 0 °C. A solution of NaN₃ (42 mg, 0.649 mmol, 2.1 eq) in water (1 mL) was then added dropwise, the mixture allowed to warm to room temperature and left to stir for 3 h. The

pH of the reaction was adjusted to pH 10-12 with 10% NaOH and the aqueous layer was extracted with EtOAc (3 x 10 mL). The combined organic phases washed with brine (20 mL), dried over Na₂SO₄, filtered and evaporated to dryness to give the title compound S24 (84 mg, 0.24 mmol) as pale brown crystals in 52% yield.

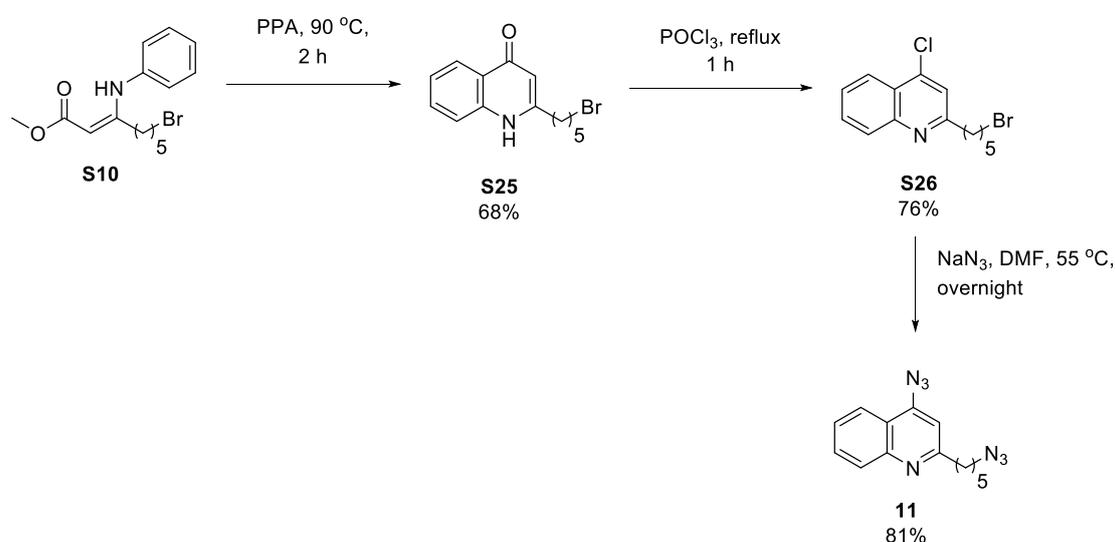
mp: decomposes ~72.5 °C (EtOAc); TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.47; ¹H NMR (500 MHz, DMSO-*d*₆): δ 11.59 (s, 1H), 7.72 (d, *J* = 2.7 Hz, 1H), 7.60 (d, *J* = 9.0 Hz, 1H), 7.29 (dd, *J* = 8.9, 2.7 Hz, 1H), 3.52 (t, *J* = 6.7 Hz, 2H), 2.73 (t, *J* = 7.7 Hz, 2H), 1.80 (app p, *J* = 6.8 Hz, 2H), 1.70-1.64 (m, 2H), 1.47 – 1.34 (m, 4H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ 167.8, 138.0, 135.9, 134.8, 133.2, 122.9, 122.5, 120.2, 112.6, 35.1, 32.1, 28.0, 27.9, 27.6, 27.3; IR (neat)/cm⁻¹: 2116, 1630, 1590, 1552, 815; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₅H₁₈BrN₄O₂: 365.0608; found: 365.0594.

6-Azido-2-(6-azidohexyl)-3-hydroxyquinolin-4(1H)-one 10

A solution of compound **S24** (50 mg, 0.14 mmol, 1.0 eq) and sodium azide (16.8 mg, 0.28 mmol, 2.0 eq) in DMF (1 mL) was stirred overnight at room temperature. The reaction was quenched by pouring into ice/water (20 mL) and the mixture was stirred for 5 min. The resulting precipitate was collected by filtration, washed with water and dried under vacuum. The crude product was further purified by crystallisation from EtOAc to give the title compound **10** (34.5 mg, 0.11 mmol) as an amorphous dark brown solid in 74% yield.

TLC (MeOH:CH₂Cl₂, 1:9 v/v, KH₂PO₄ treated silica): R_f = 0.67; ¹H NMR (400 MHz, DMSO-*d*₆): δ 11.58 (s, 1H), 8.20 (s, 1H), 7.72 (d, *J* = 2.6 Hz, 1H), 7.60 (d, *J* = 8.9 Hz, 1H), 7.29 (dd, *J* = 8.9, 2.6 Hz, 1H), 3.31 (t, *J* = 6.5 Hz, 3H, partially obscured by H₂O peak), 2.73 (t, *J* = 7.7 Hz, 2H), 1.79 – 1.63 (m, 2H), 1.60 – 1.49 (m, 4H), 1.37 (t, *J* = 3.8 Hz, 2H); ¹³C NMR (126 MHz, DMSO-*d*₆): δ 167.8, 138.0, 135.9, 134.8, 133.2, 129.6, 122.9, 122.4, 120.2, 112.6, 50.6, 28.3, 28.1, 28.0, 25.9; IR (neat)/cm⁻¹: 2119, 2097, 1717, 1519; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₅H₁₈N₇O₂: 328.1516; found: 328.1503.

4.3.11 Synthesis of HHQ probe 11



2-(5-Bromopentyl)quinolin-4(1H)-one **S25**

Enamine **S10** (325 mg, 1.0 mmol, 1.0 eq) was added to a stirring solution of polyphosphoric acid (115% H₃PO₄ basis, 1.0 g) and heated to 90 °C for 1 h. The reaction was then added slowly to a saturated solution of NaHCO₃ (15 mL) at 0 °C. The resulting precipitate was collected by filtration and purified by column chromatography (stepwise gradient of 0-5% MeOH in CH₂Cl₂) to yield **S11** (199 mg, 0.68 mmol) as a white amorphous solid in 68% yield.

mp: 128.4-129.9 °C (CH₂Cl₂/MeOH); TLC (MeOH:CH₂Cl₂, 5:95 v/v): R_f = 0.46; ¹H NMR (400 MHz, CDCl₃): δ 12.71 (s, 1H), 8.35 (d, *J* = 8.1 Hz, 1H), 7.81 (d, *J* = 8.4 Hz, 1H), 7.61 (t, *J* = 7.3, 1H), 7.34 (t, *J* = 7.6 Hz, 1H), 6.25 (s, 1H), 3.26 (t, *J* = 6.7 Hz, 2H), 2.71 (t, *J* = 7.6 Hz, 2H), 1.76 – 1.71 (m, 4H), 1.44 – 1.37 (m, 2H); ¹³C NMR (101 MHz, CDCl₃): δ 179.0, 155.2, 140.9, 132.0, 125.2, 125.0, 123.8, 118.8, 108.2, 34.2, 33.5, 32.4, 28.4, 27.8; IR (neat)/cm⁻¹: 1628, 1591, 1547, 760; HRMS (ESI-TOF) *m/z*: [M + H]⁺ calcd. for C₁₄H₁₇NOBr, 294.0488; found, 294.0483.

2-(5-Bromopentyl)-4-chloroquinoline **S26**

A solution of compound **S25** (50 mg, 0.17 mmol, 1.0 eq) in phosphorus oxychloride (1 mL) was heated to reflux for 1 h. The excess phosphorus oxychloride was removed under reduced pressure and the resulting residue was dissolved in CH₂Cl₂ (5 mL) and washed with saturated NaHCO₃ (3 x 5 mL). The organic layer was dried over MgSO₄, filtered and evaporated to dryness to give the title compound **S26** (37 mg, 0.13 mmol) as a colourless oil. The mixture was used in the next step without further purification.

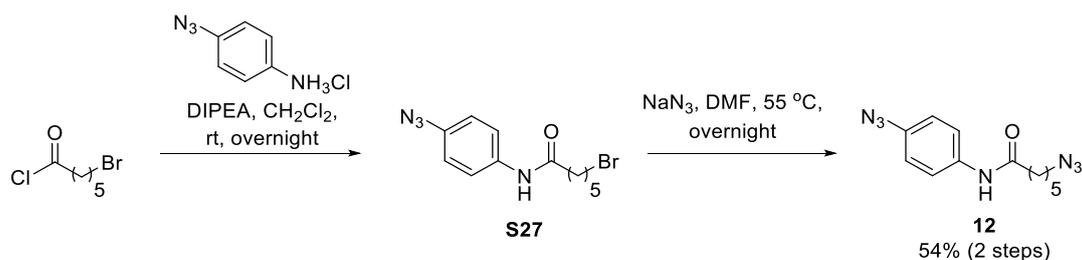
TLC (EtOAc:hexane, 4:6 v/v): $R_f = 0.19$; $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 8.19 (d, $J = 8.5$ Hz, 1H), 8.05 (d, $J = 8.4$ Hz, 1H), 7.74 (dd, $J = 8.4, 6.9$ Hz, 1H), 7.59 (dd, $J = 8.3, 7.0$ Hz, 1H), 7.40 (s, 1H), 3.42 (t, $J = 6.8$ Hz, 2H), 2.96 (t, $J = 7.8$ Hz, 2H), 1.97 – 1.82 (m, 4H), 1.61-1.55 (m, 2H); $^{13}\text{C NMR}$ (126 MHz, CDCl_3) δ 162.5, 148.9, 142.8, 130.5, 129.3, 126.9, 125.1, 124.1, 121.5, 77.2, 38.9, 33.8, 32.7, 29.9, 28.9, 28.1; IR (neat)/ cm^{-1} : 1589, 1553, 839, 760; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{14}\text{H}_{16}\text{BrClN}$: 312.0155; found: 312.0416.

4-Azido-2-(5-azidopentyl)quinolone **11**

A solution of compound **S26** (31 mg, 0.1 mmol, 1.0 eq) and sodium azide (30 mg, 0.5 mmol, 2.5 eq) in DMF (0.5 mL) was heated to 55 °C overnight with stirring. The reaction was quenched by the addition of water and the mixture was stirred for 5 min. The resulting mixture was extracted with EtOAc (3 x 20 mL), the organic phase was washed with water (2 x 20 mL), dried over MgSO_4 , filtered and evaporated to dryness. The crude product was purified by column chromatography (stepwise gradient of 0-20% EtOAc in hexane) to give **11** (21 mg, 0.074 mmol) a colourless oil in 74% yield.

TLC (EtOAc:hexane, 2:8 v/v): $R_f = 0.17$; $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 8.02 – 7.99 (m, 2H), 7.71 (ddd, $J = 8.4, 6.9, 1.5$ Hz, 1H), 7.48 (ddd, $J = 8.3, 6.9, 1.2$ Hz, 1H), 7.02 (s, 1H), 3.29 (t, $J = 6.9$ Hz, 2H), 2.97 (t, $J = 7.9$ Hz, 2H), 1.93 – 1.82 (m, 2H), 1.73 – 1.64 (m, 2H), 1.55 – 1.47 (m, 2H); $^{13}\text{C NMR}$ (126 MHz, CDCl_3): δ 162.8, 149.1, 146.4, 130.6, 128.8, 125.9, 122.2, 120.4, 108.7, 51.5, 39.3, 29.5, 28.9, 26.8; IR (neat)/ cm^{-1} : 2107, 2091, 1681, 1591, 1560, 1502.

4.3.12 Synthesis of negative control probe **12**



N-(4-Azidophenyl)-6-bromohexanamide **S27**

A solution of 4-azidoaniline hydrochloride (200 mg, 1.17 mmol, 1.0 eq), 6-bromohexanoyl chloride (180 μL , 1.16 mmol, 1.0 eq) and DIPEA (205 μL , 2.33 mmol, 2.0 eq) in CH_2Cl_2 (5 mL) was stirred overnight at room temperature. The reaction was quenched by pouring into saturated aqueous solution of NaHCO_3 and the mixture was stirred for 5 min. The resulting mixture was extracted with CH_2Cl_2 (3 x 20 mL) and the organic phase was washed with 0.1 M HCl (1 x 20 mL), saturated NaHCO_3 (1 x 20 mL), water (1 x 20 mL) and then dried over MgSO_4 , filtered and evaporated to dryness to give **S27** (288 mg) as an impure brown

amorphous solid (100% yield assumed, contaminated with free acid chlorogide) which was used without further purification.

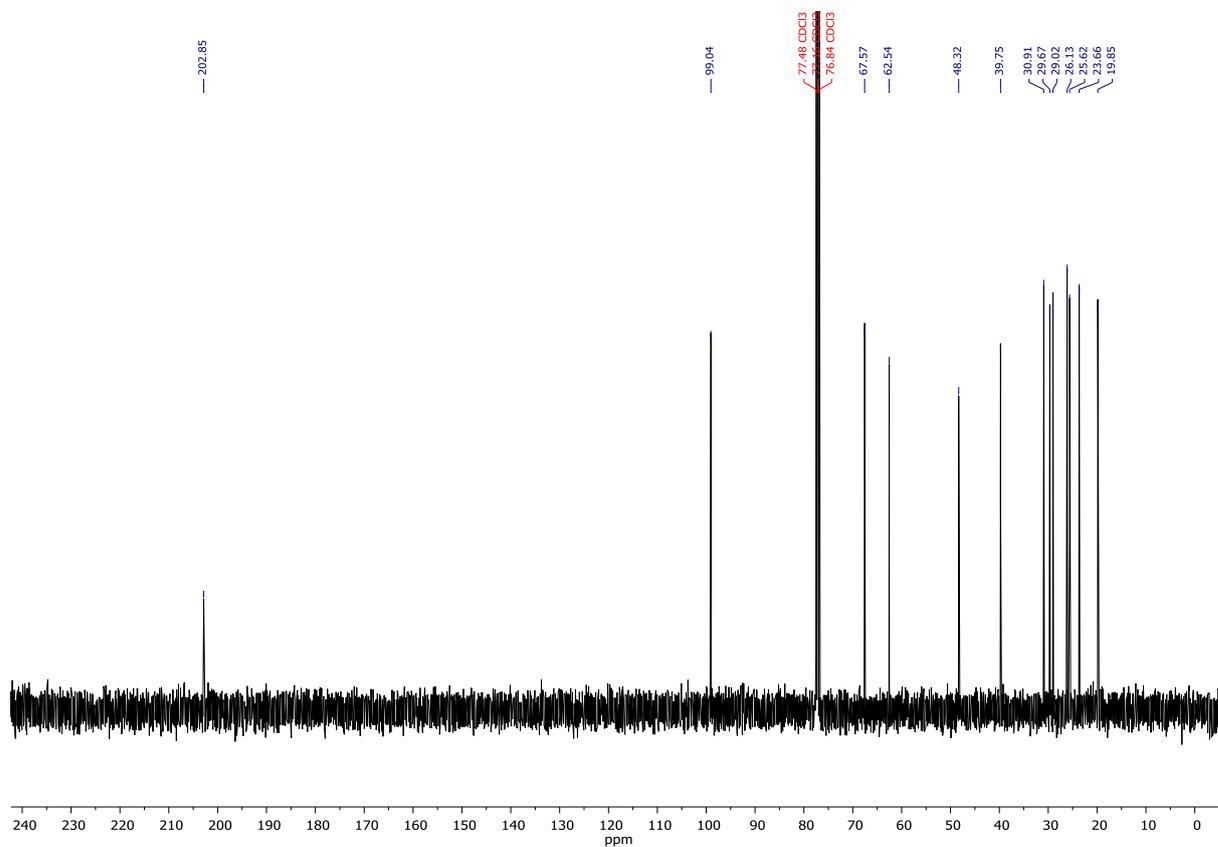
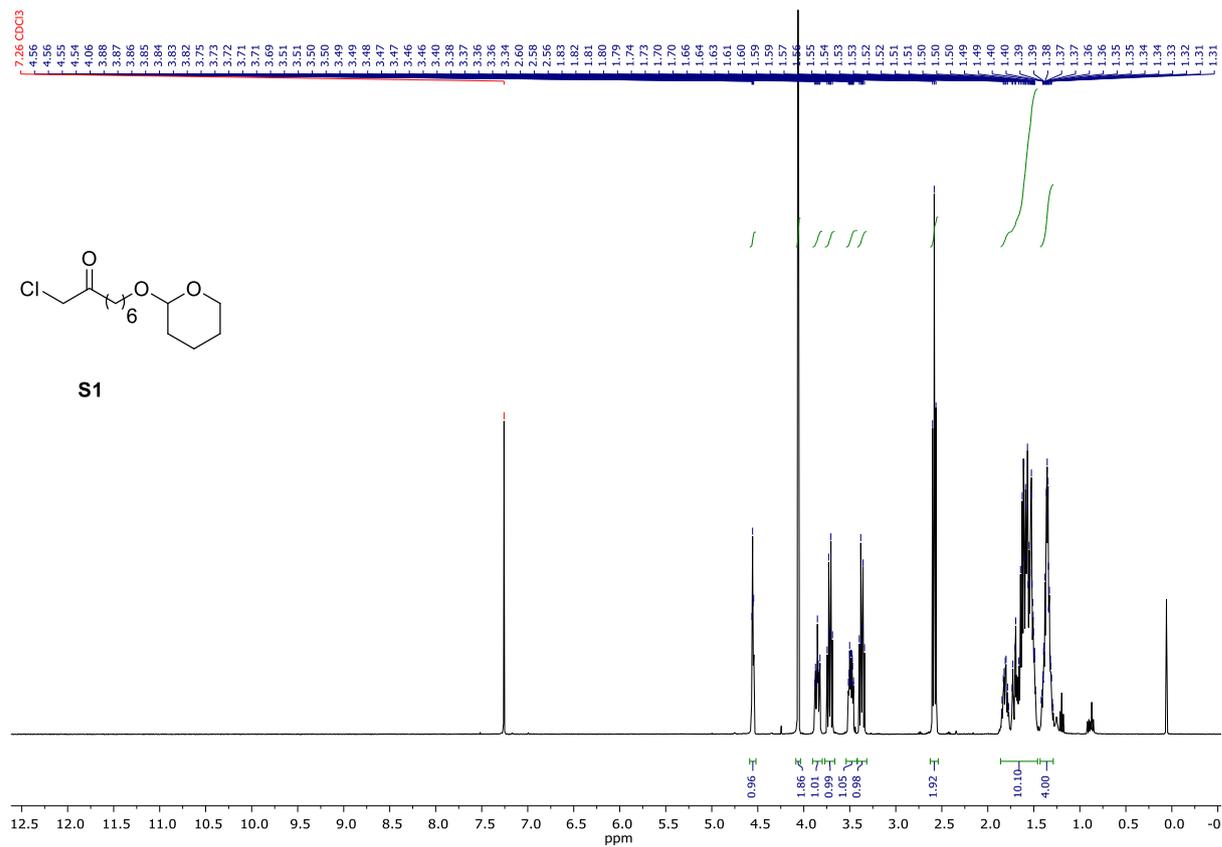
TLC (EtOAc:hexane, 2:8 v/v): $R_f = 0.37$; $^1\text{H NMR}$ (500 MHz, $\text{DMSO-}d_6$): δ 9.95 (s, 1H), 7.66 – 7.60 (m, 2H), 7.08 – 7.02 (m, 2H), 3.54 (t, $J = 6.7$ Hz, 2H), 2.30 (t, $J = 7.4$ Hz, 2H), 1.84 – 1.80 (m, 2H), 1.61 (app p, $J = 7.6$ Hz, 2H), 1.44 - 1.40 (m, 2H); $^{13}\text{C NMR}$ (126 MHz, $\text{DMSO-}d_6$): δ 174.3 (acid chloride), 171.0, 136.6, 133.5 (acid chloride), 120.5, 119.4, 39.5, 35.1, 32.0, 27.2, 24.2; IR (neat)/ cm^{-1} : 3323, 2108, 1655, 1598, 1507; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{12}\text{H}_{16}\text{N}_4\text{OBr}$: 311.0502; found: 311.0492.

6-Azido-N-(4-azidophenyl)hexanamide **12**

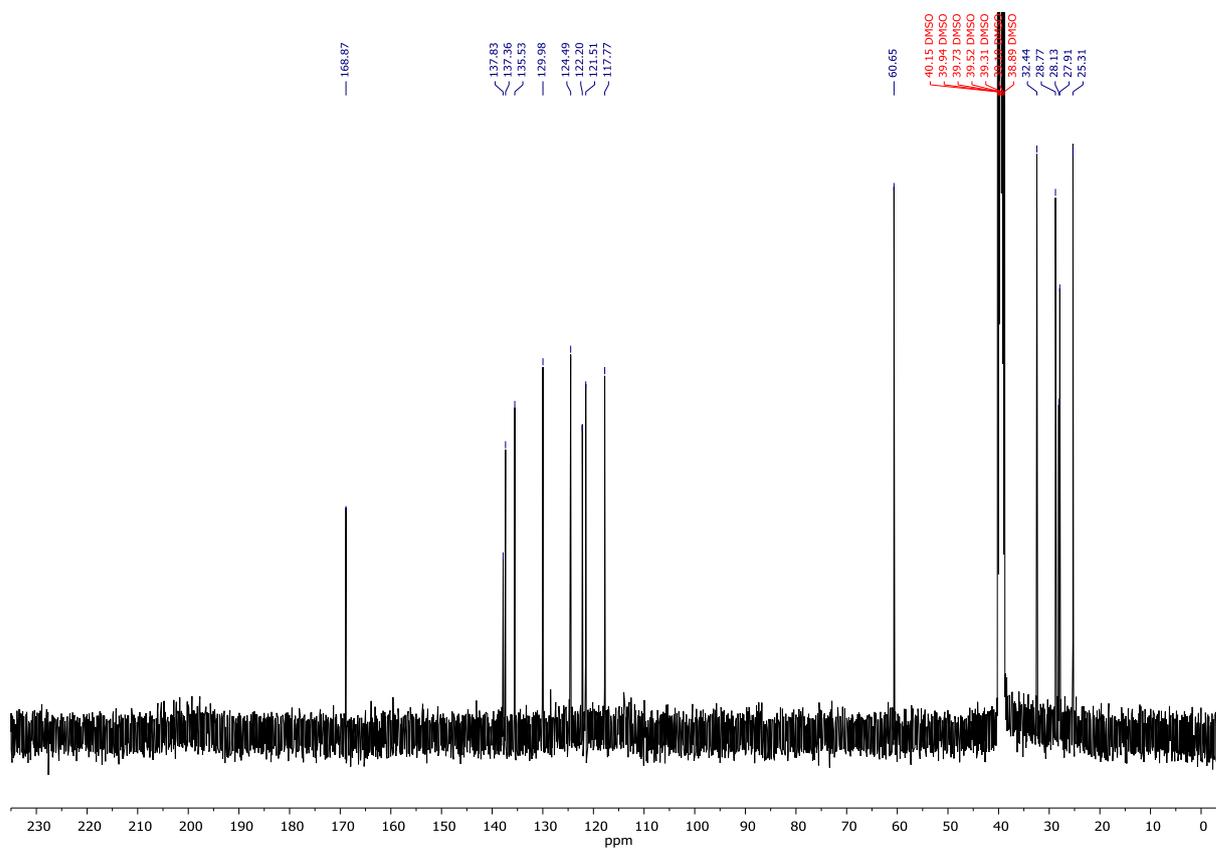
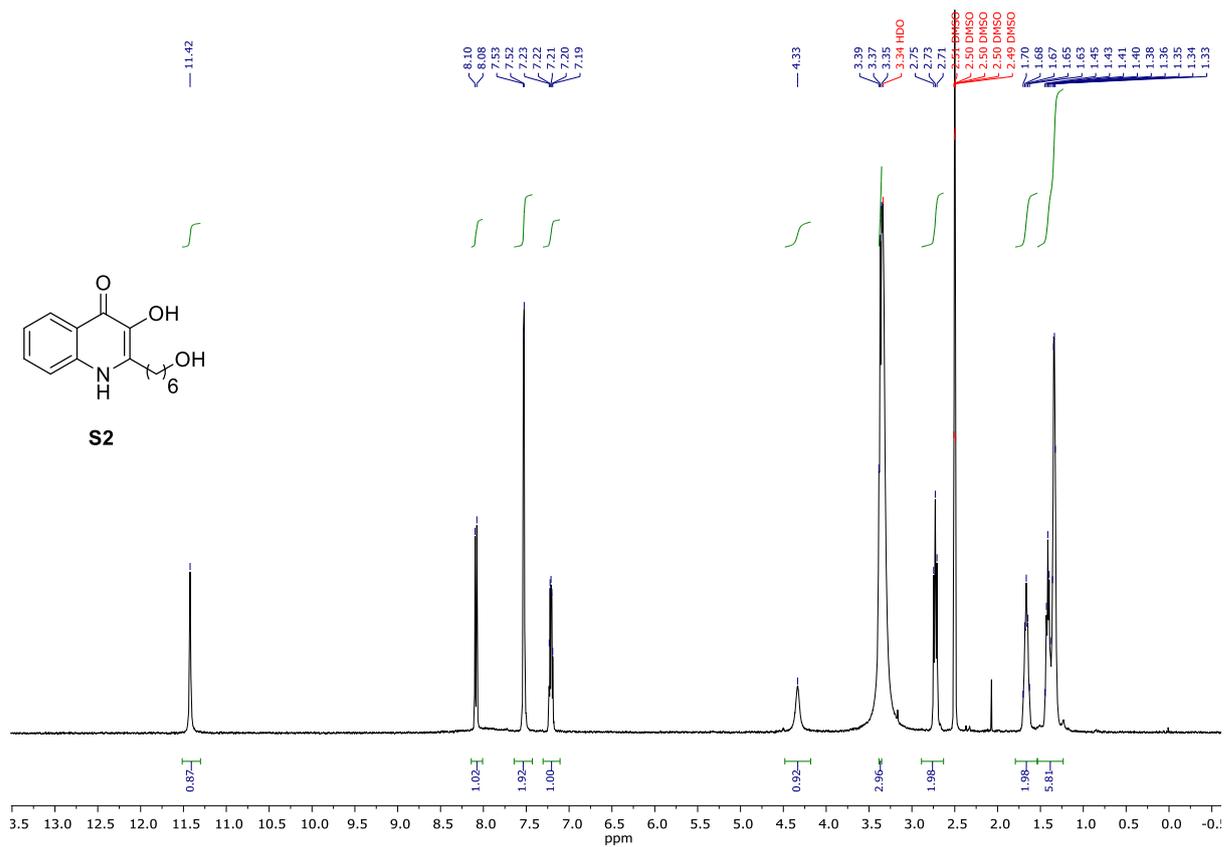
A solution of crude **S27** (200 mg, ~ 0.4 mmol, 1.0 eq) and NaN_3 (72 mg, 1.2 mmol, 3.0 eq) in DMF (1 mL) was heated to 55 °C overnight with stirring. The reaction was quenched by pouring into water and the resulting mixture was extracted with EtOAc (3 x 20 mL). The combined organic phases were washed with water (2 x 20 mL), dried over MgSO_4 , filtered and evaporated to dryness to give a yellow oil. The crude product was purified by column chromatography (stepwise gradient of 0-20% EtOAc in hexane) to give a **12** (64 mg, 0.22 mmol) as a colourless oil which crystallised on standing in 54% yield.

mp: 51.5-52.3 °C (EtOAc); TLC (EtOAc:hexane, 2:8 v/v): $R_f = 0.37$; $^1\text{H NMR}$ (500 MHz, CDCl_3): δ 7.51 (d, $J = 8.9$ Hz, 2H), 7.11 (s, 1H), 6.98 (d, $J = 8.8$ Hz, 2H), 3.29 (t, $J = 6.8$ Hz, 2H), 2.37 (t, $J = 7.4$ Hz, 2H), 1.77 (app p, $J = 7.5$ Hz, 2H), 1.65 (dt, $J = 14.6, 6.9$ Hz, 2H), 1.51 – 1.43 (m, 2H); $^{13}\text{C NMR}$ (126 MHz, CDCl_3): δ 170.9, 135.9, 135.0, 121.4, 119.7, 51.4, 37.5, 28.8, 26.5, 25.1; IR(neat)/ cm^{-1} : 3286, 2083, 1654, 1596, 1533, 1505; HRMS (ESI-TOF) m/z : $[\text{M} + \text{H}]^+$ calcd. for $\text{C}_{12}\text{H}_{15}\text{N}_7\text{ONa}$: 296.1230; found: 296.1219.

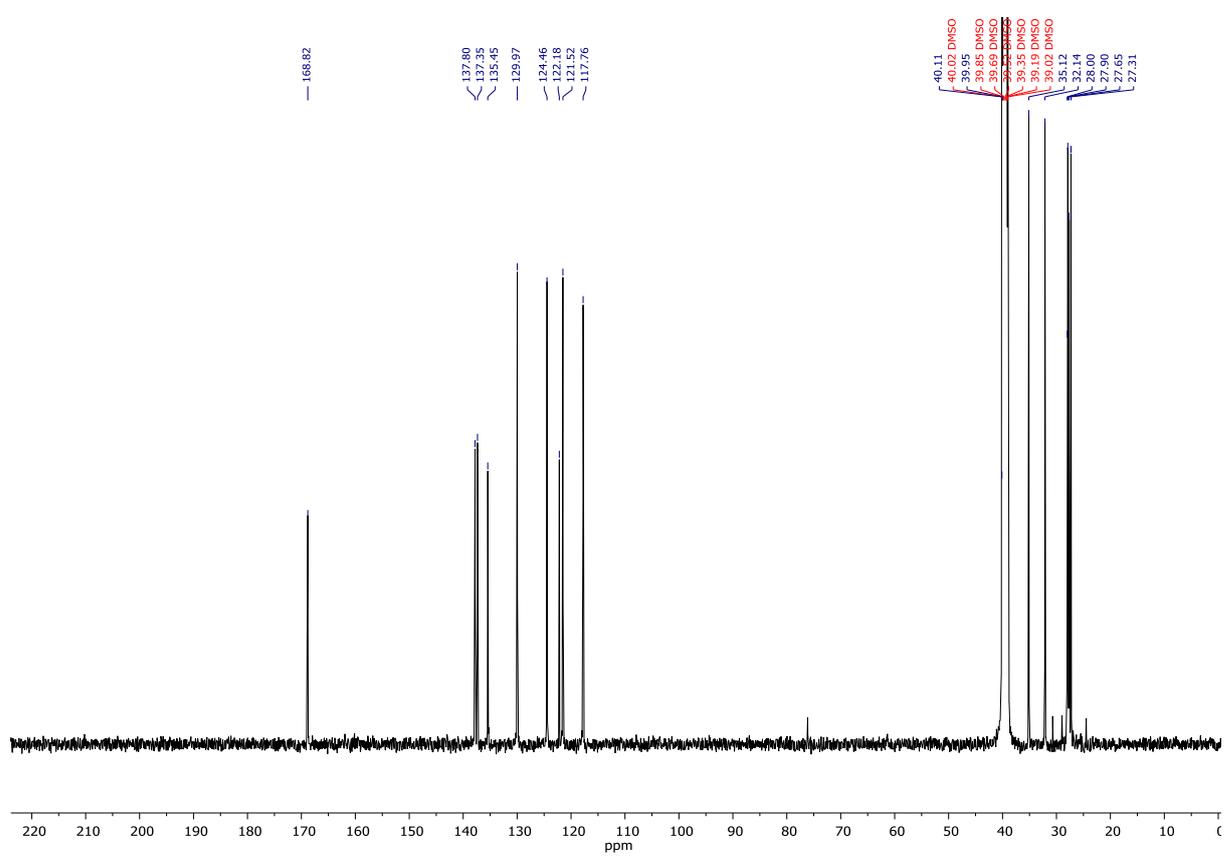
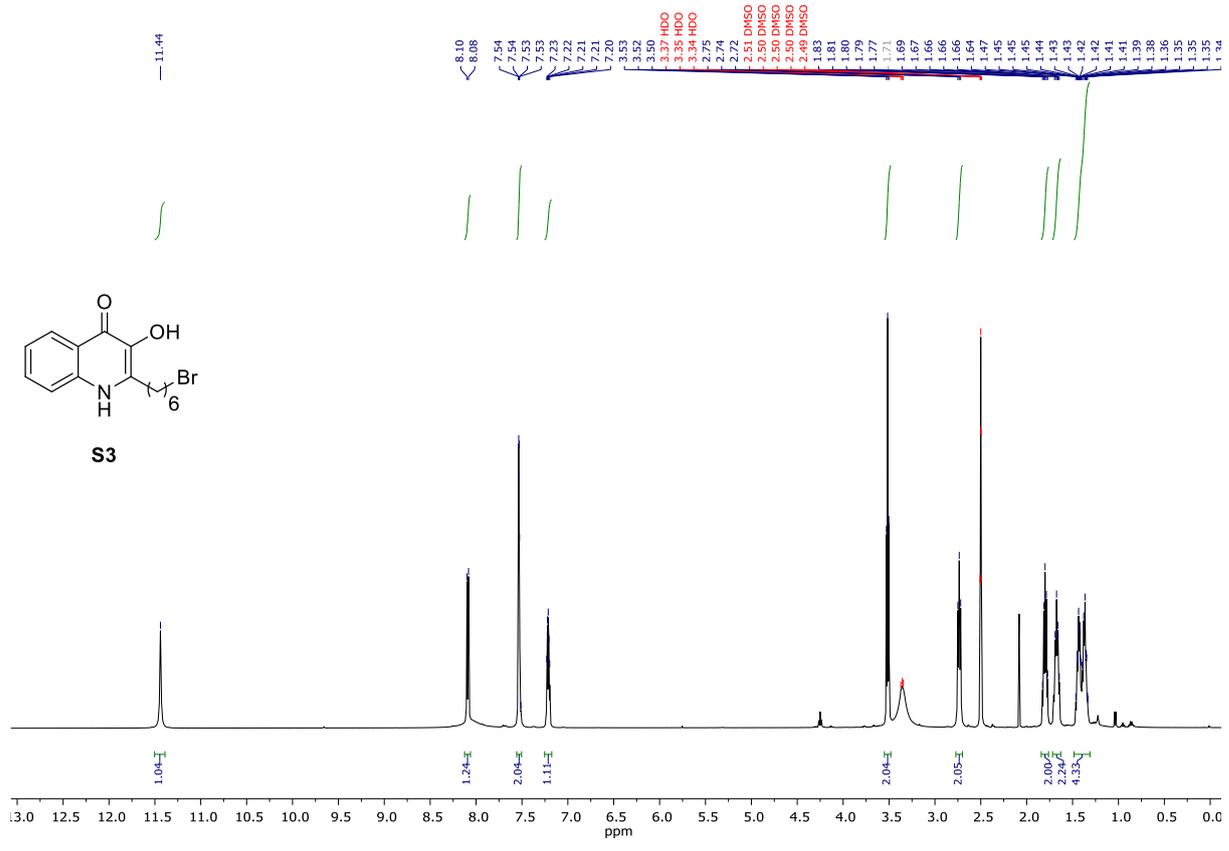
4.4 NMR spectra



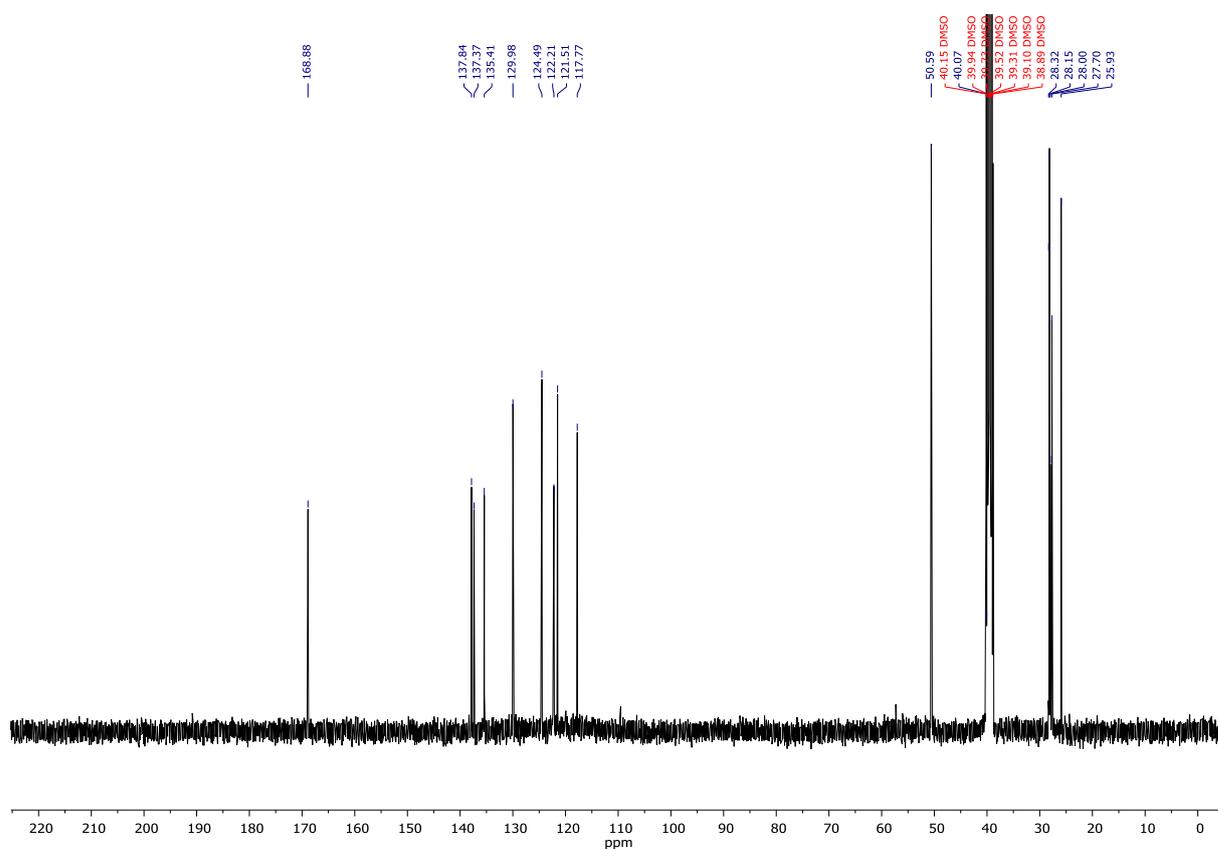
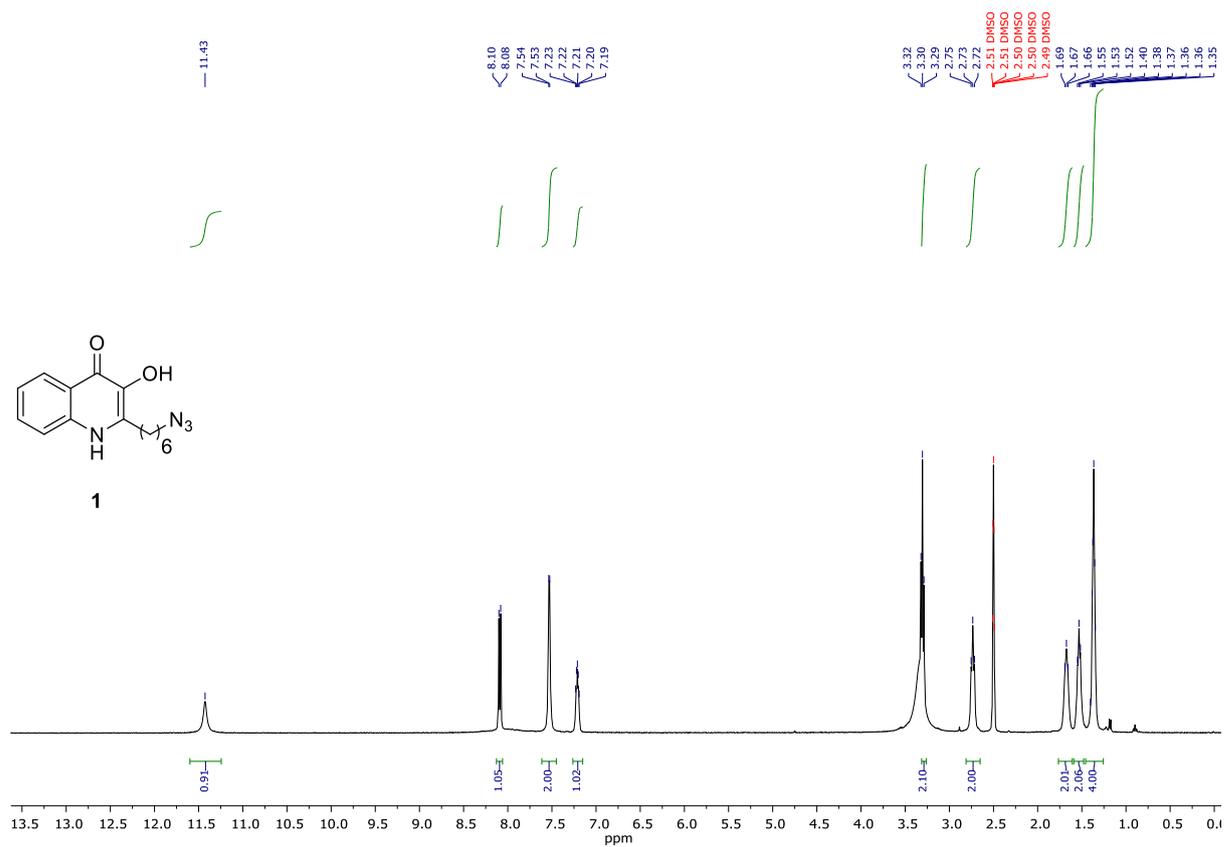
Supplementary Figure 9. ¹H and ¹³C NMR of S1.



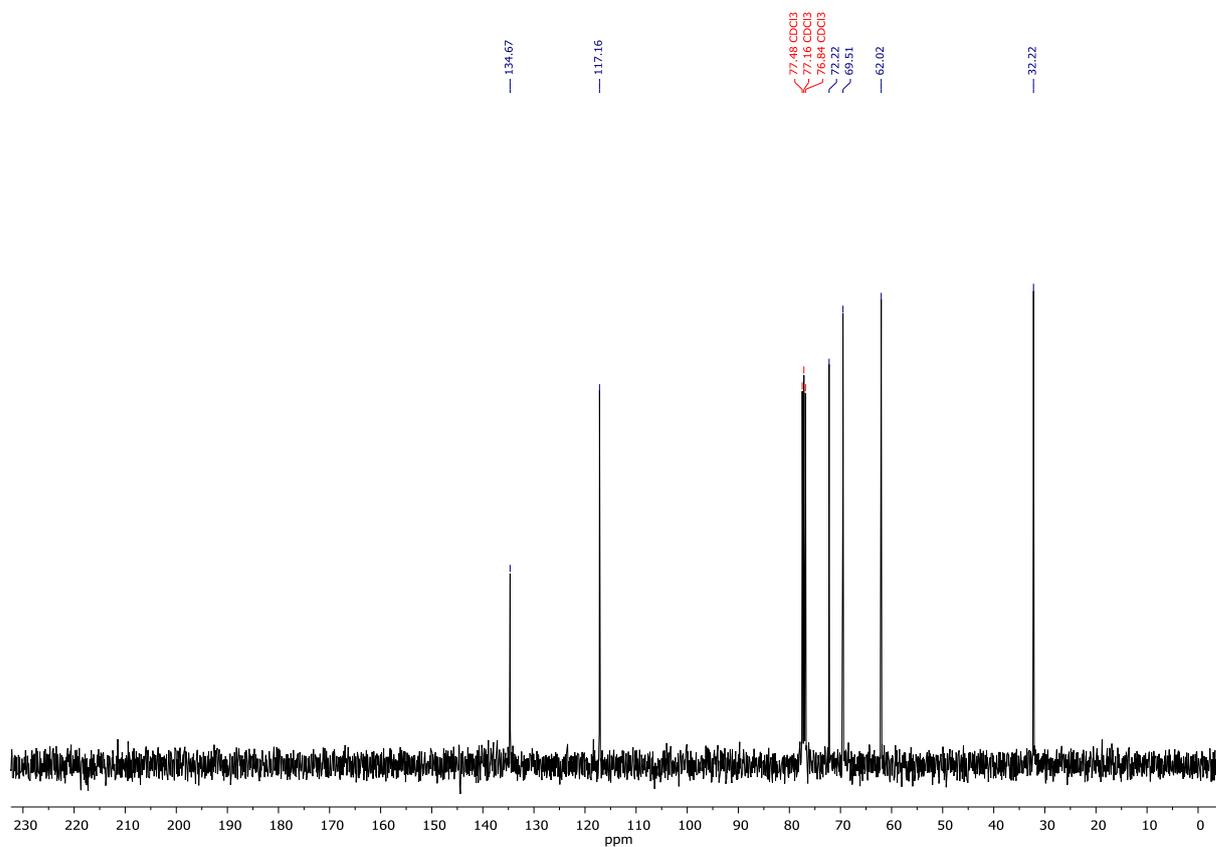
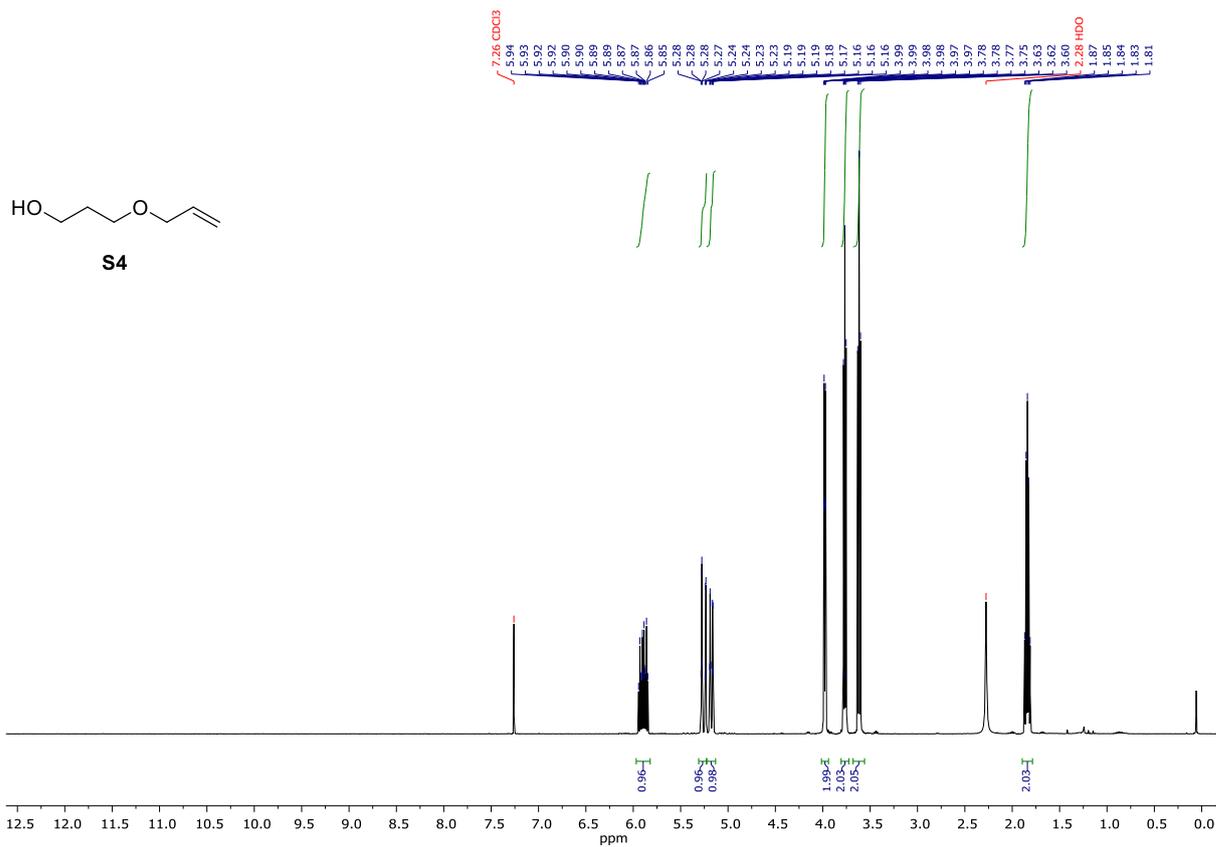
Supplementary Figure 10. ¹H and ¹³C NMR of S2.



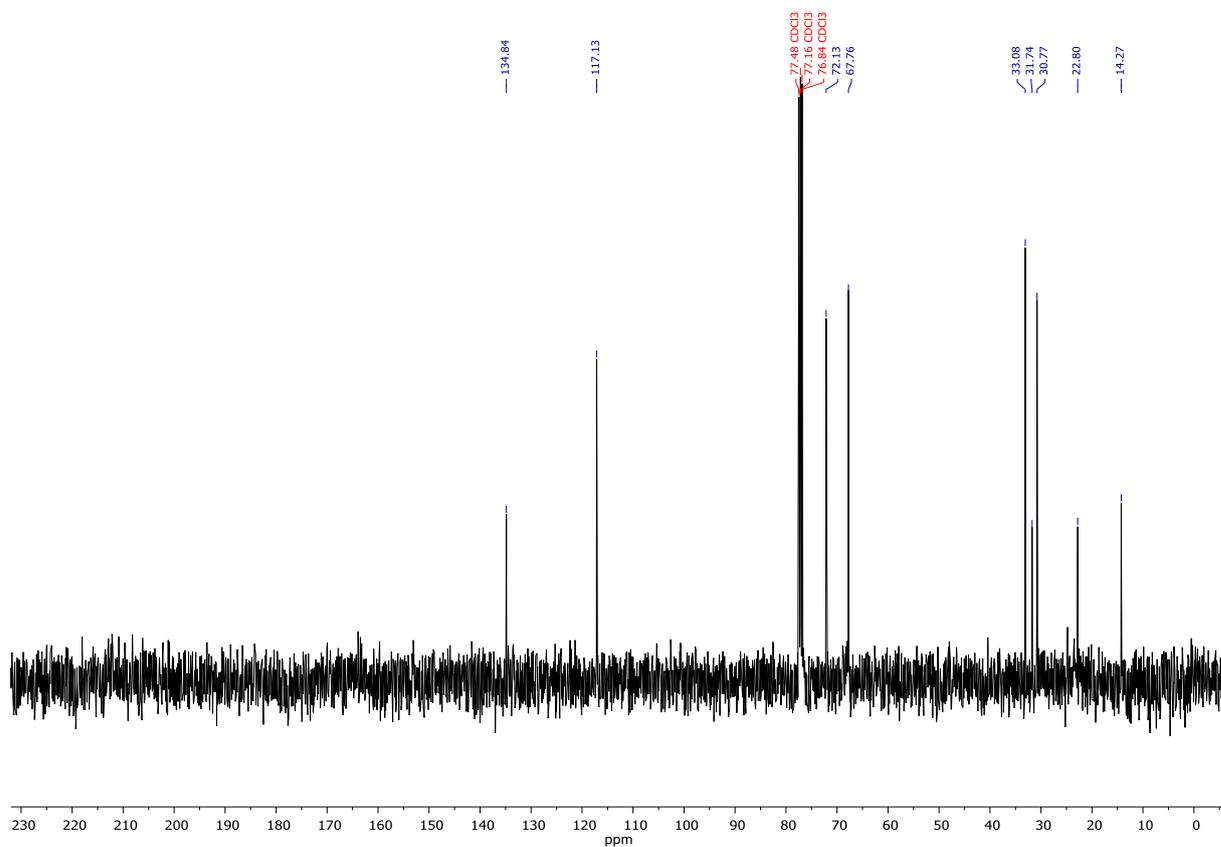
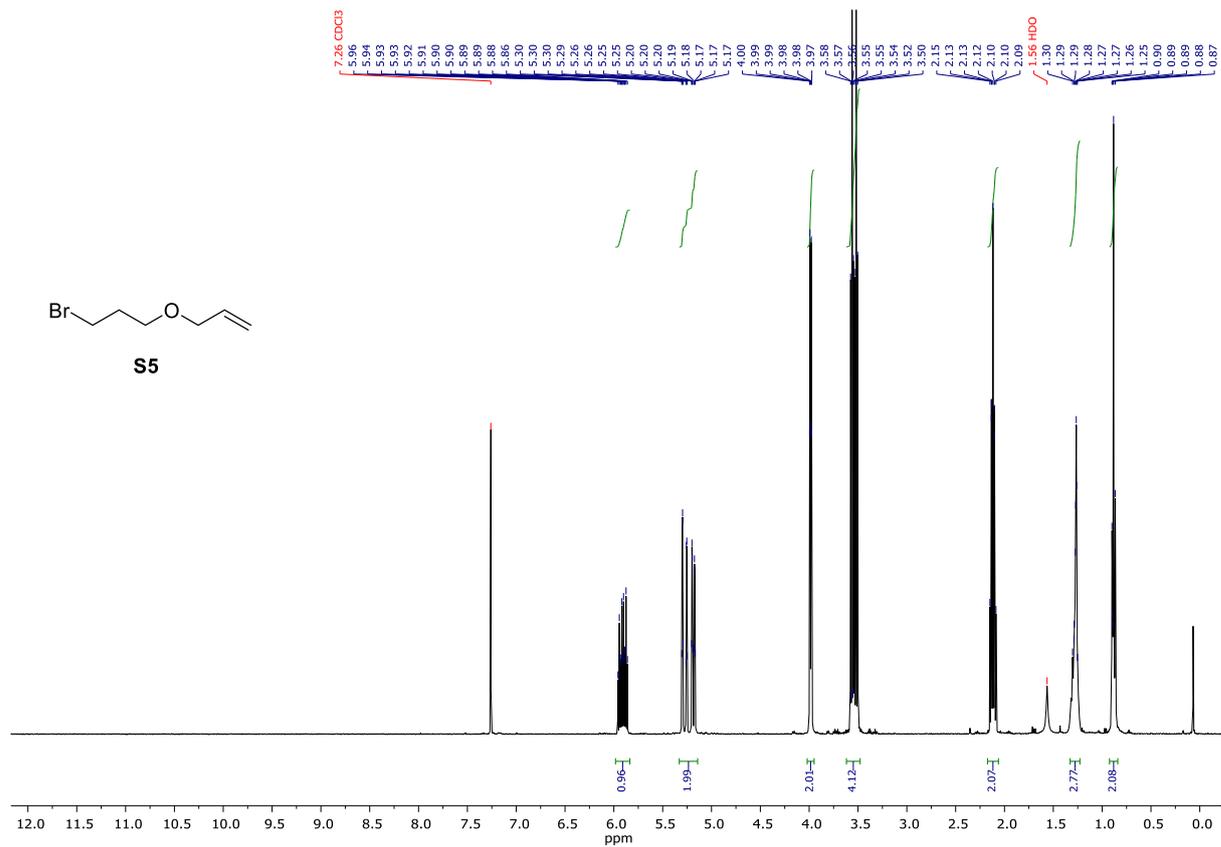
Supplementary Figure 11. ¹H and ¹³C NMR of S3.



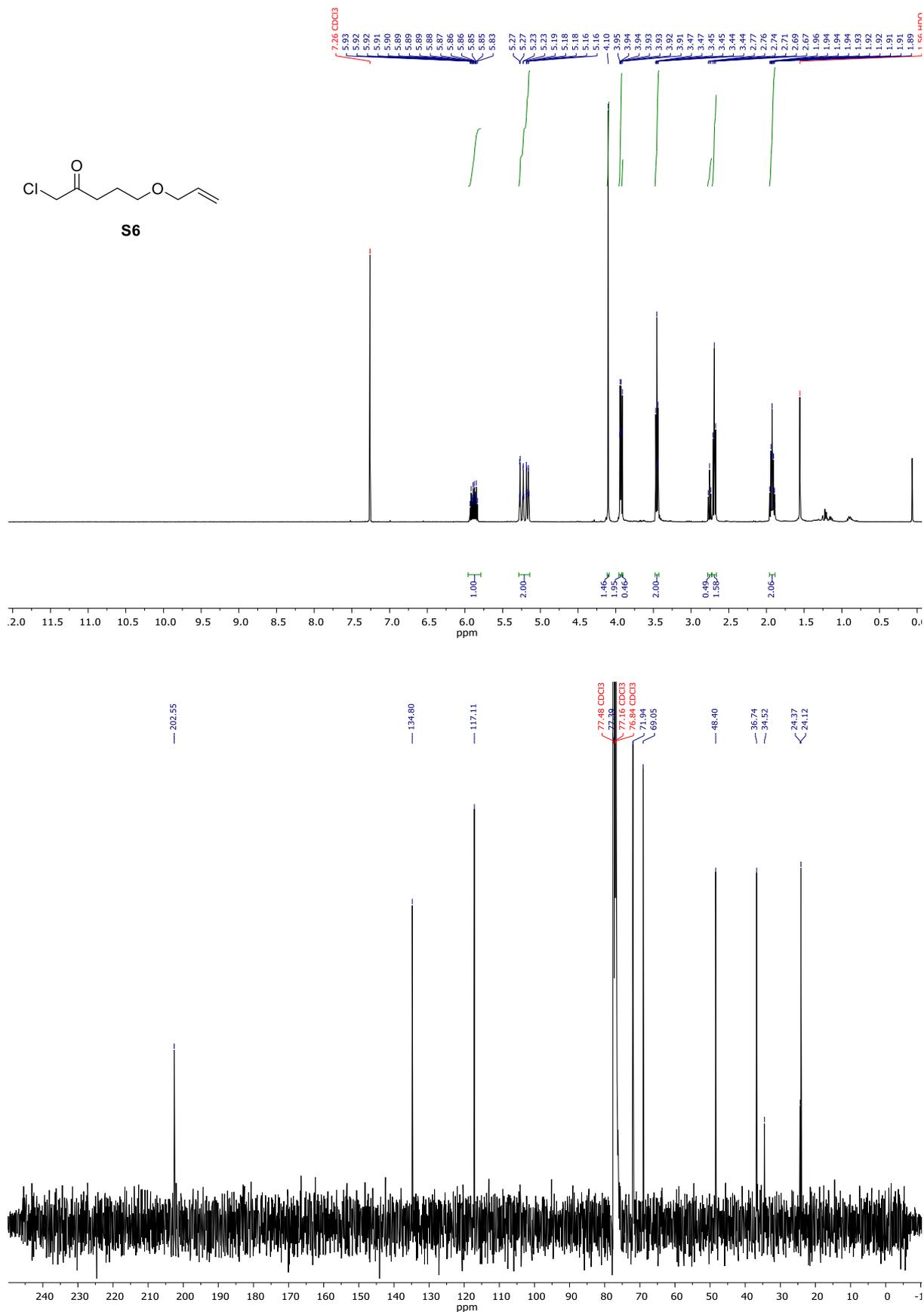
Supplementary Figure 12. ¹H and ¹³C NMR of 1.



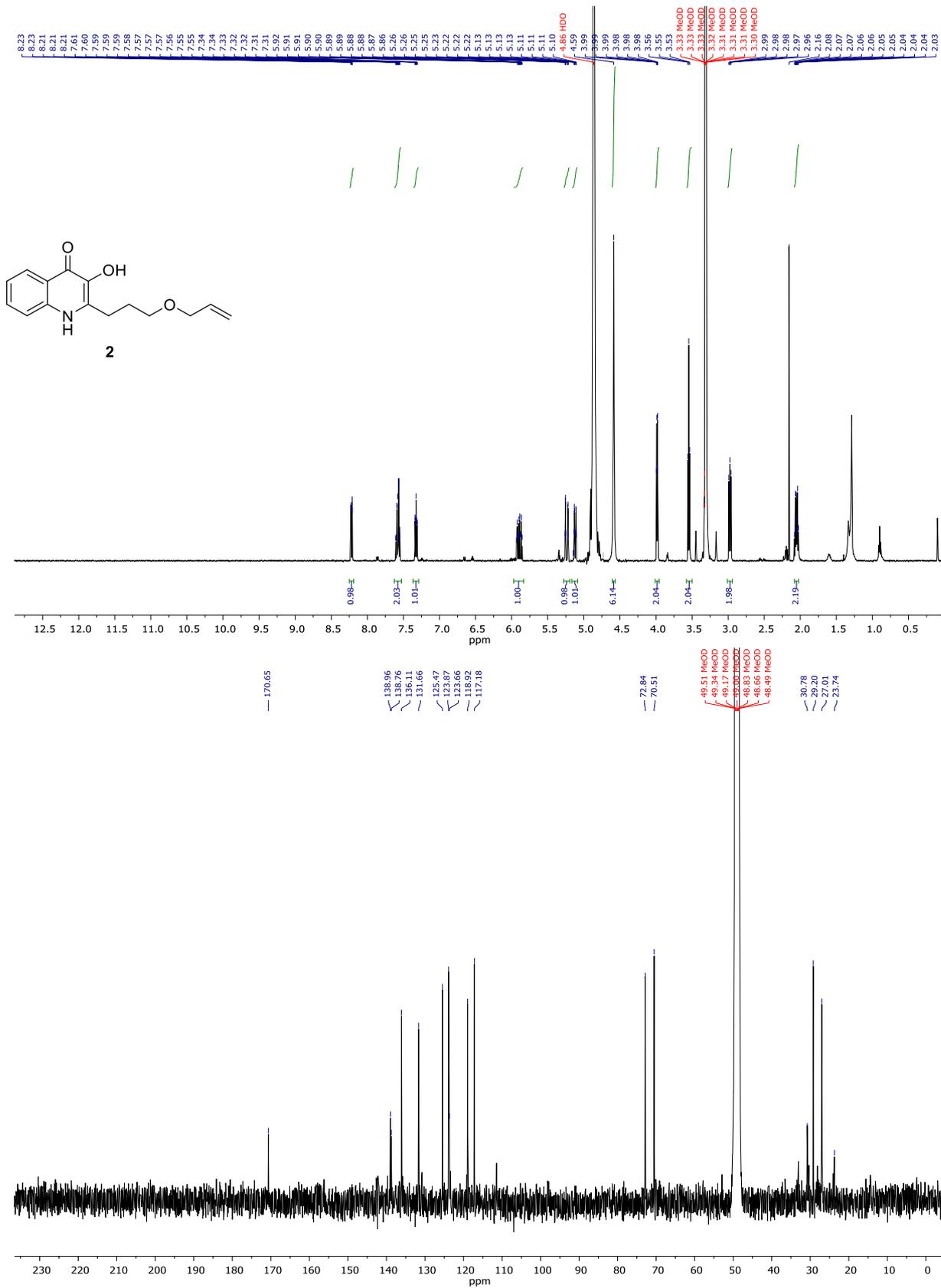
Supplementary Figure 13. ¹H and ¹³C NMR of S4.



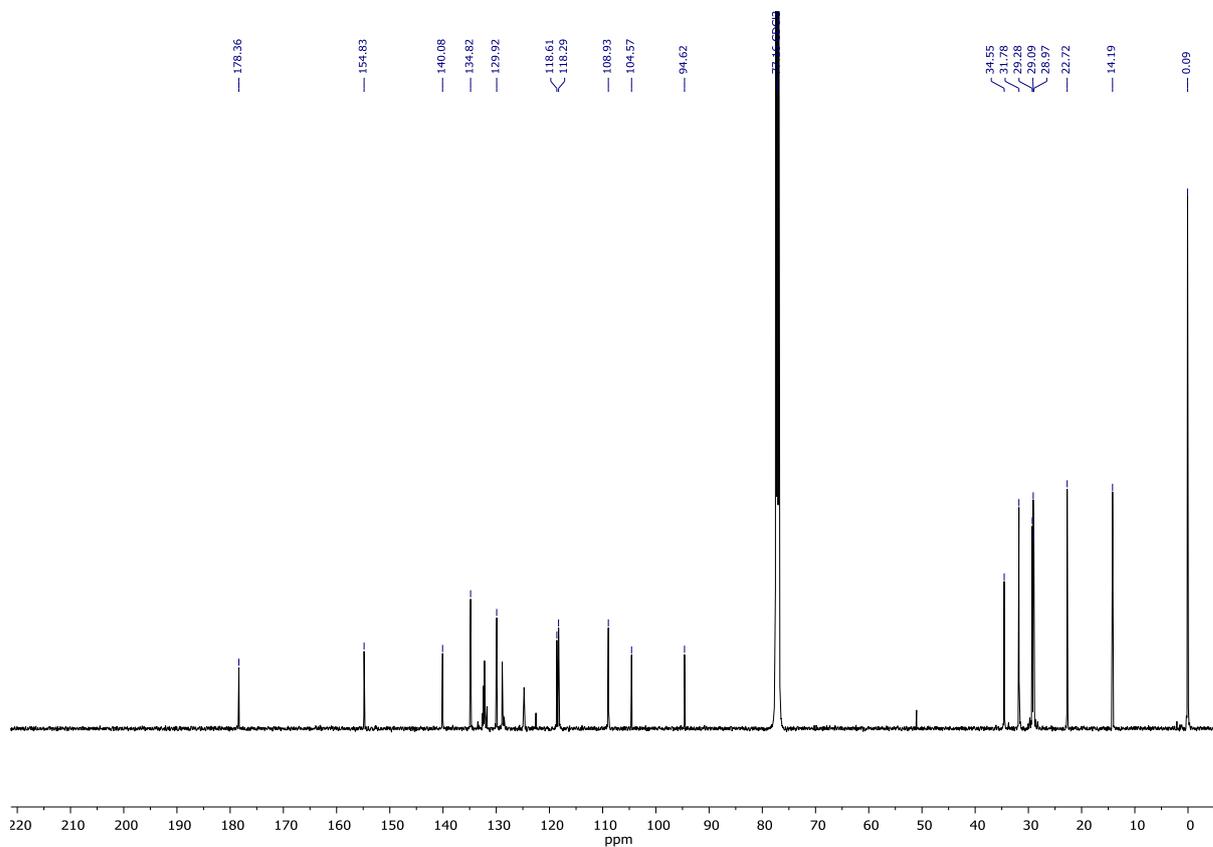
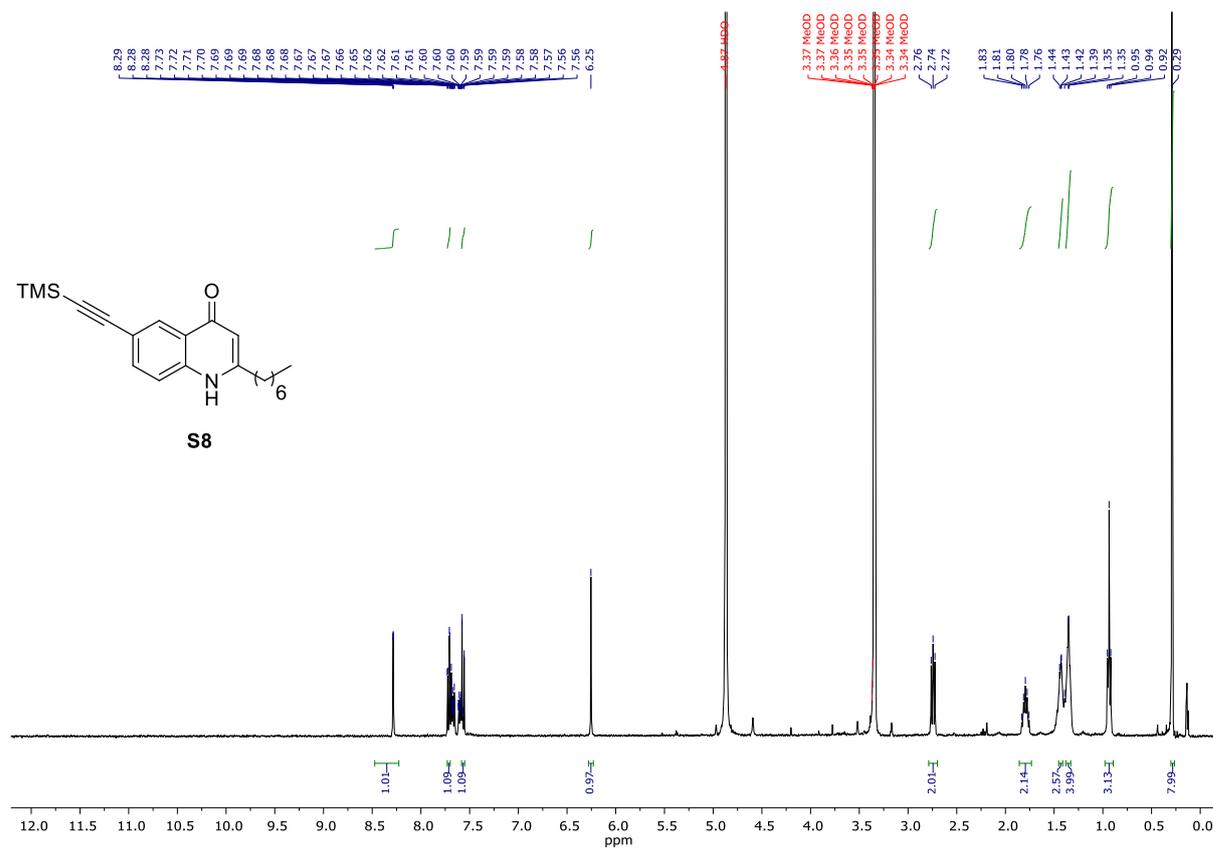
Supplementary Figure 14. ¹H and ¹³C NMR of S5.



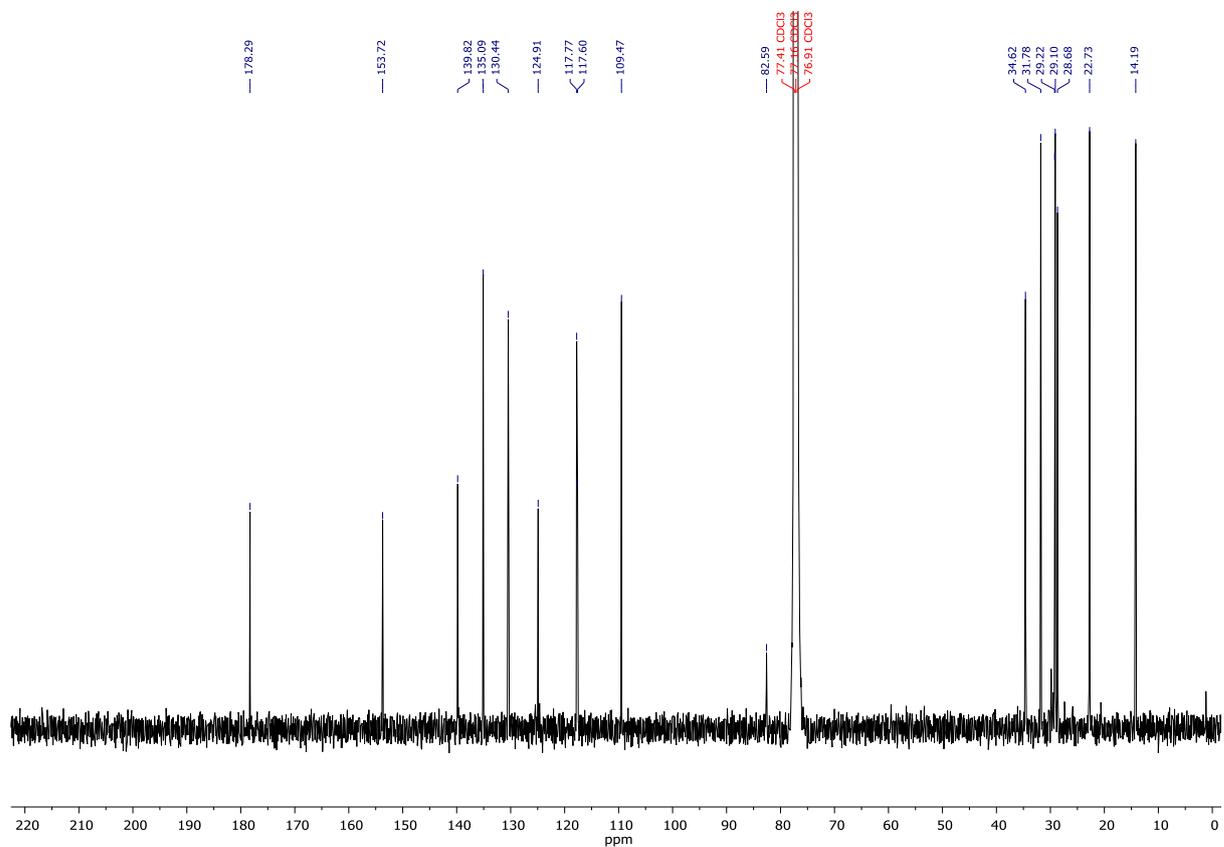
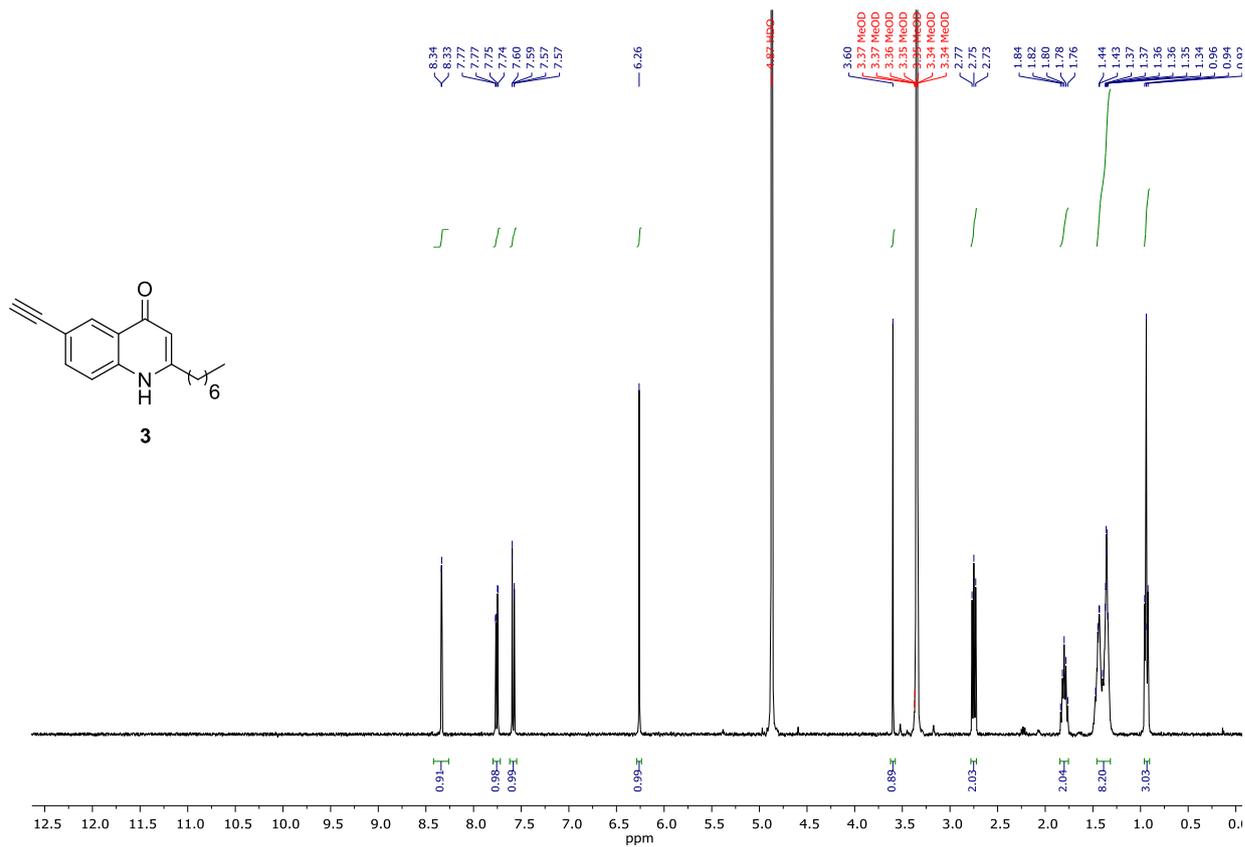
Supplementary Figure 15. ¹H and ¹³C NMR of S6.



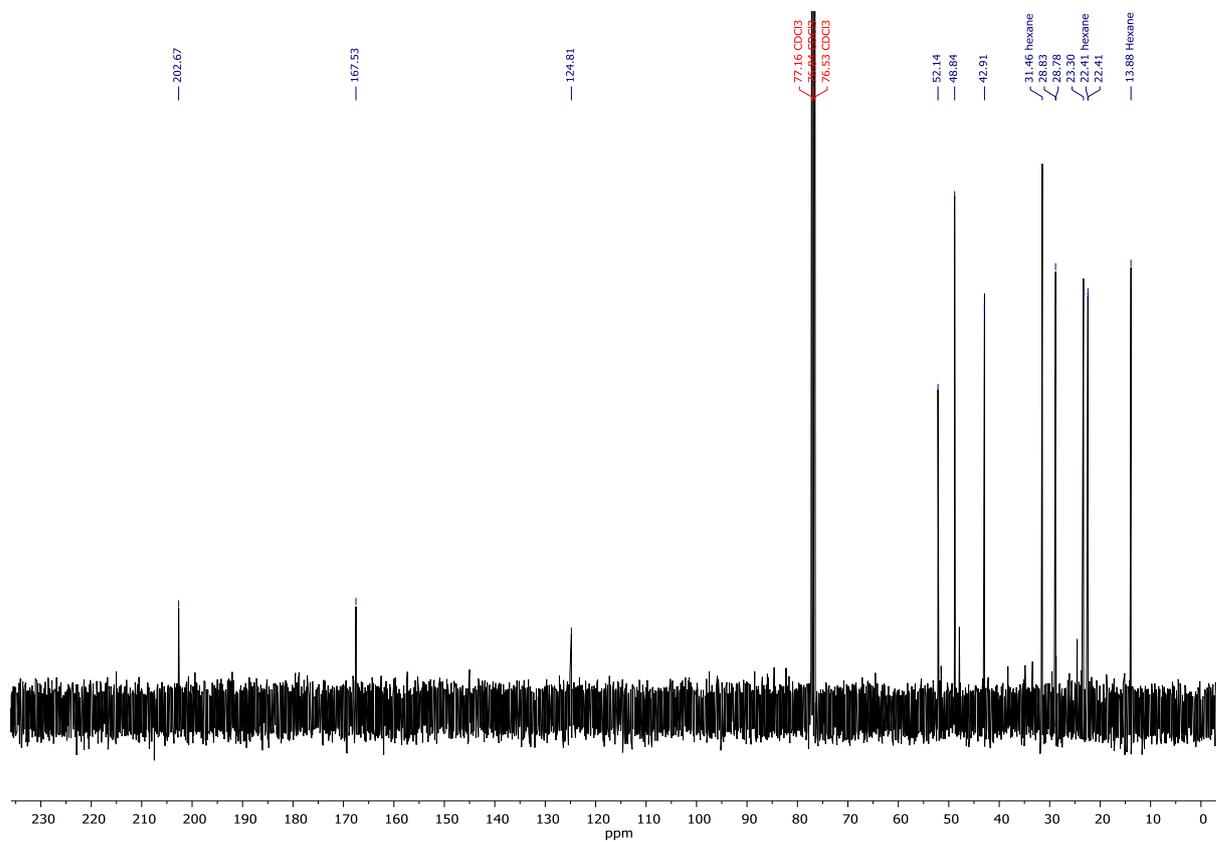
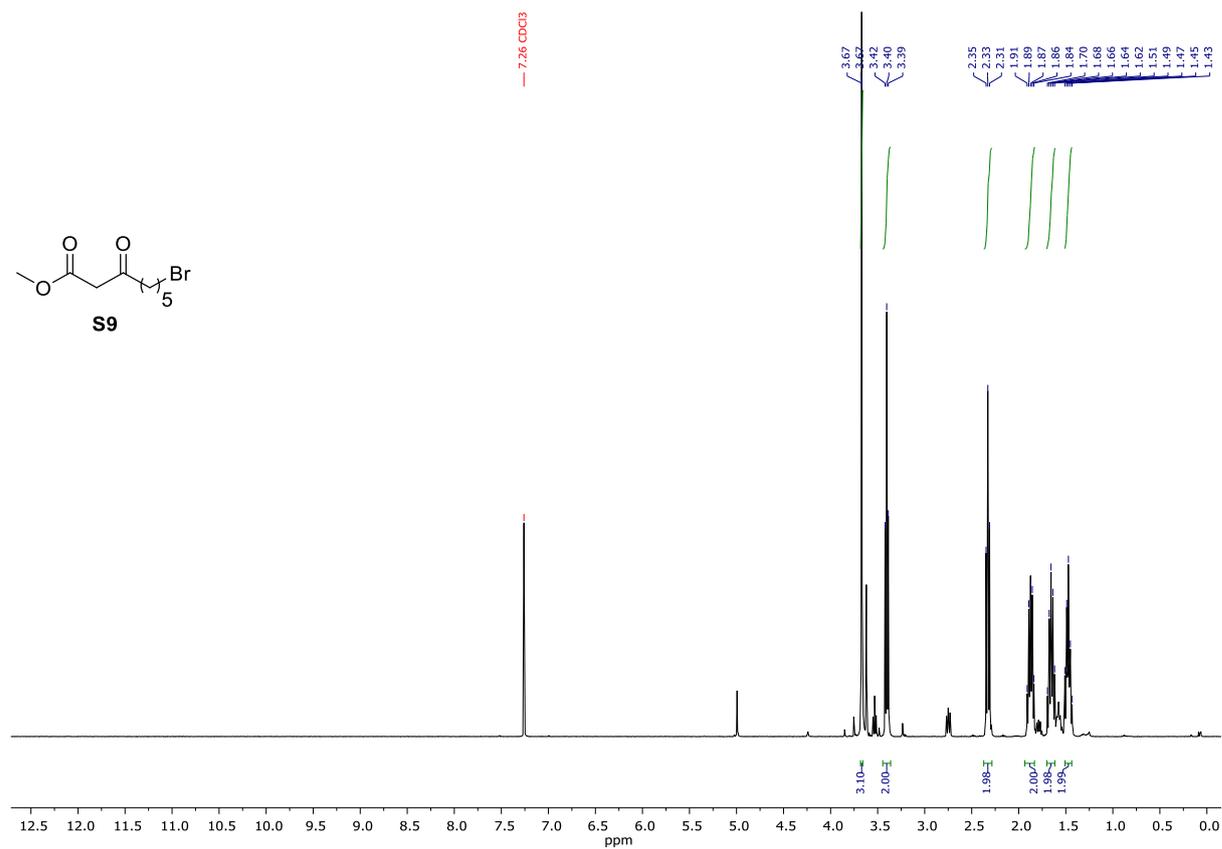
Supplementary Figure 16. ¹H and ¹³C NMR of 2.



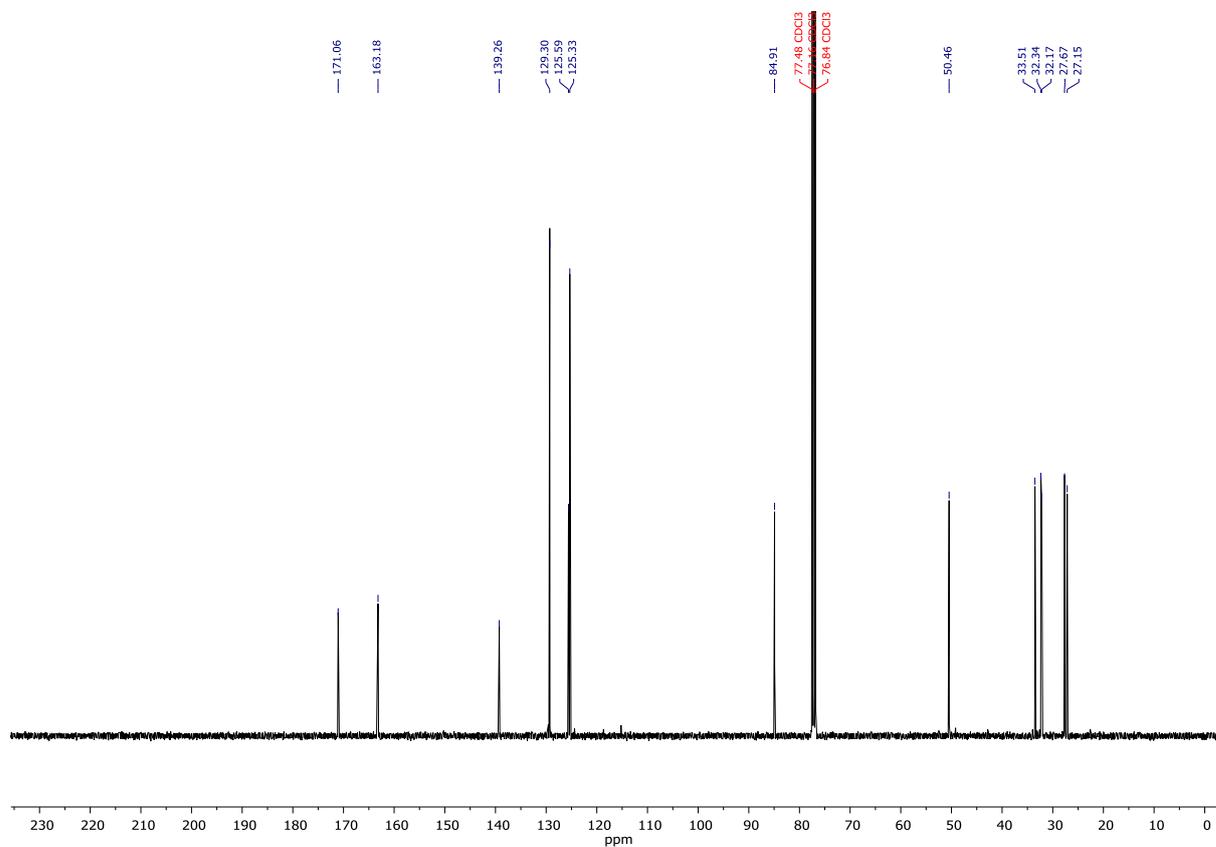
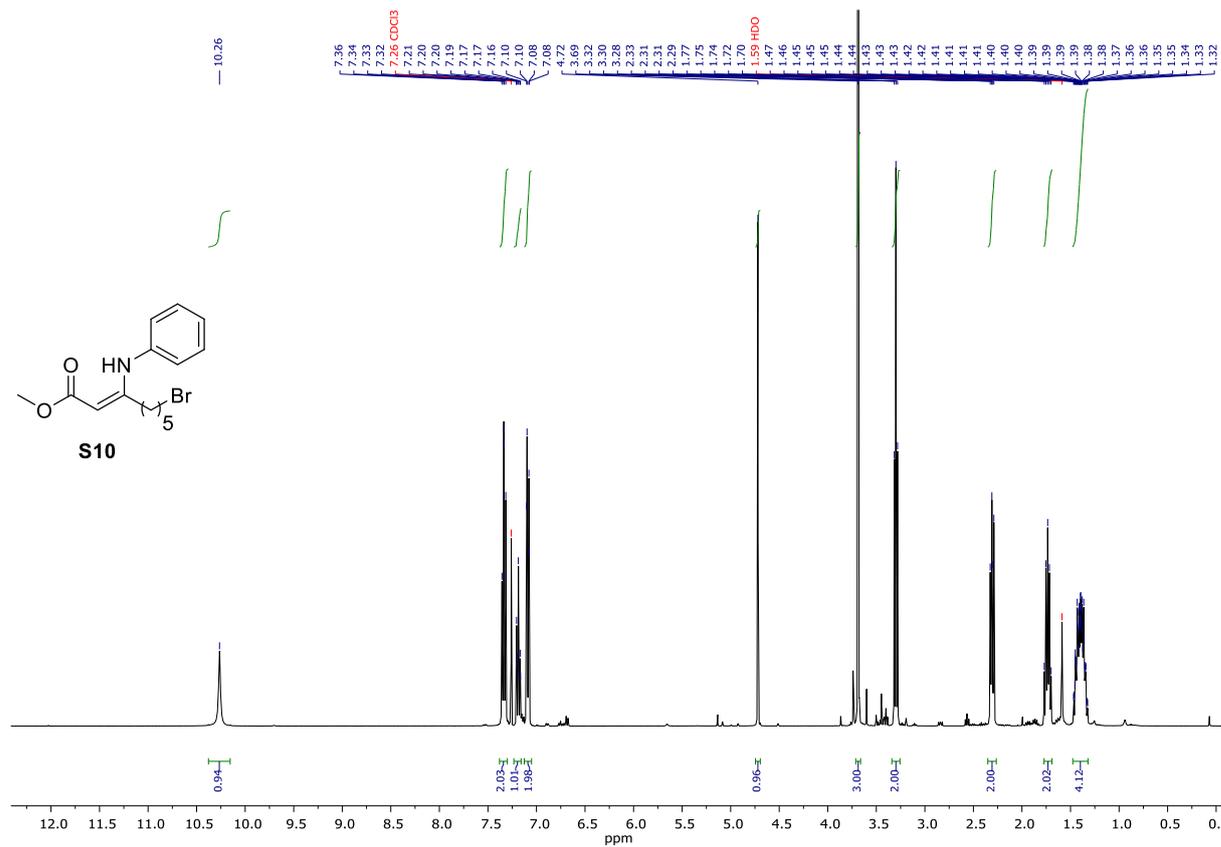
Supplementary Figure 17. ¹H and ¹³C NMR of S8.



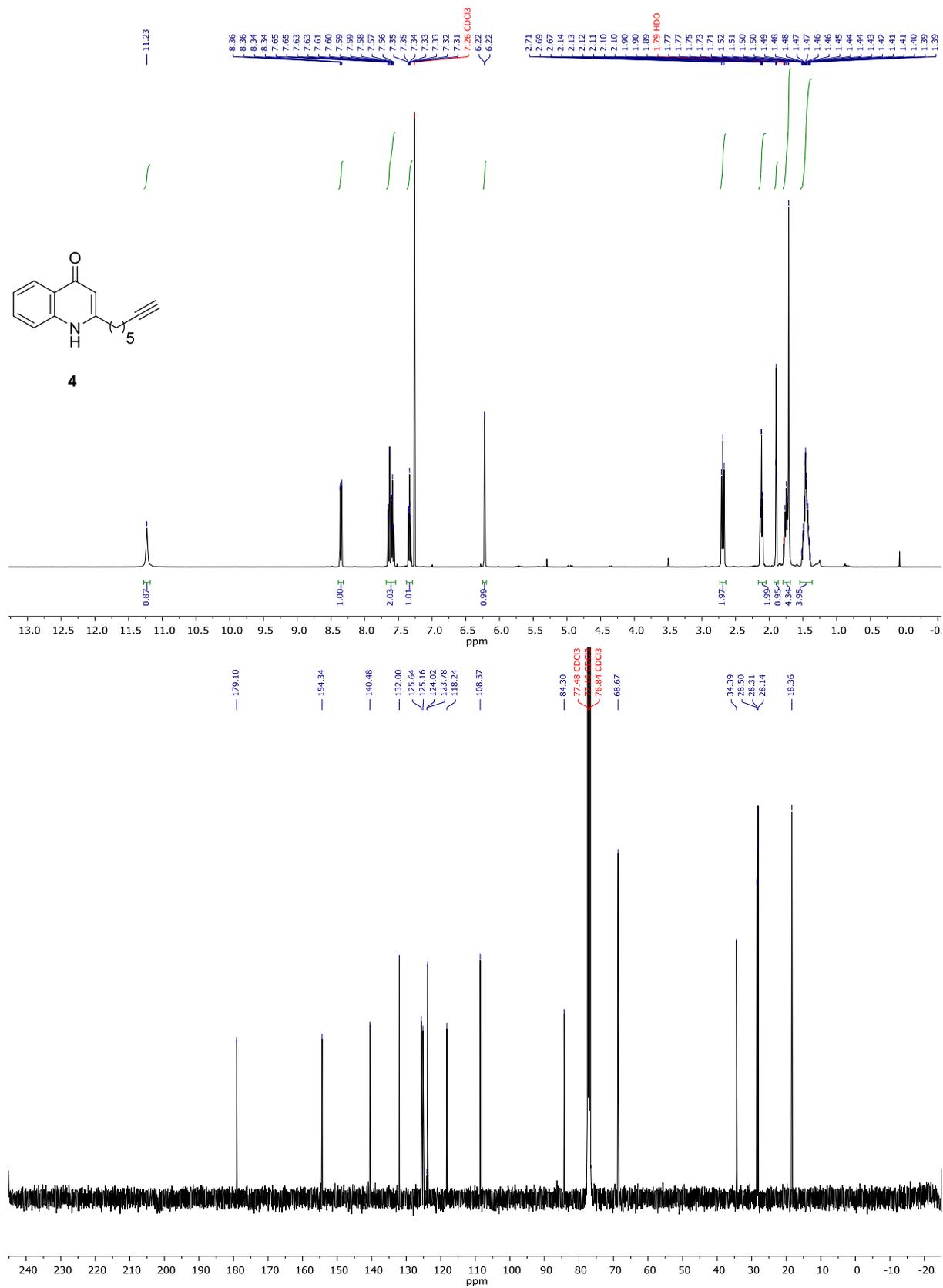
Supplementary Figure 18. ^1H and ^{13}C NMR of **3**.



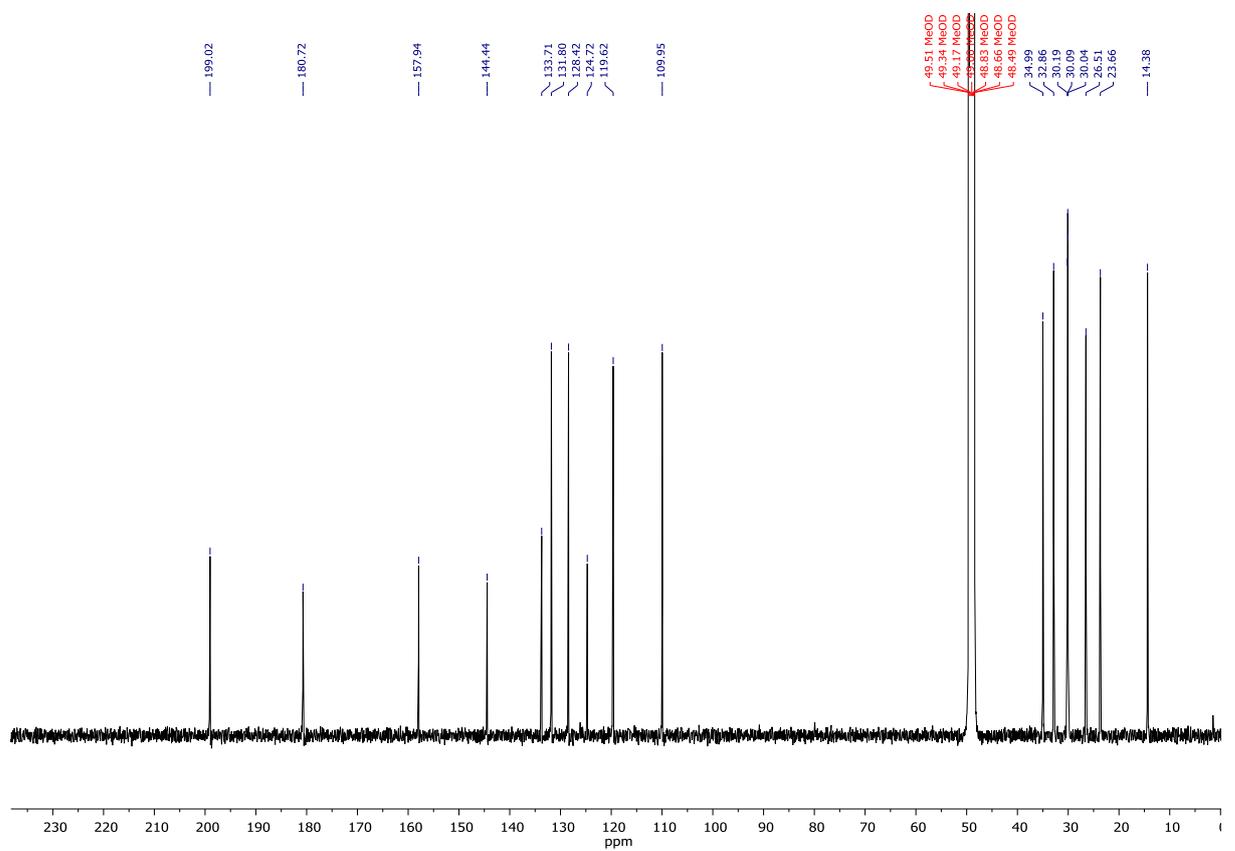
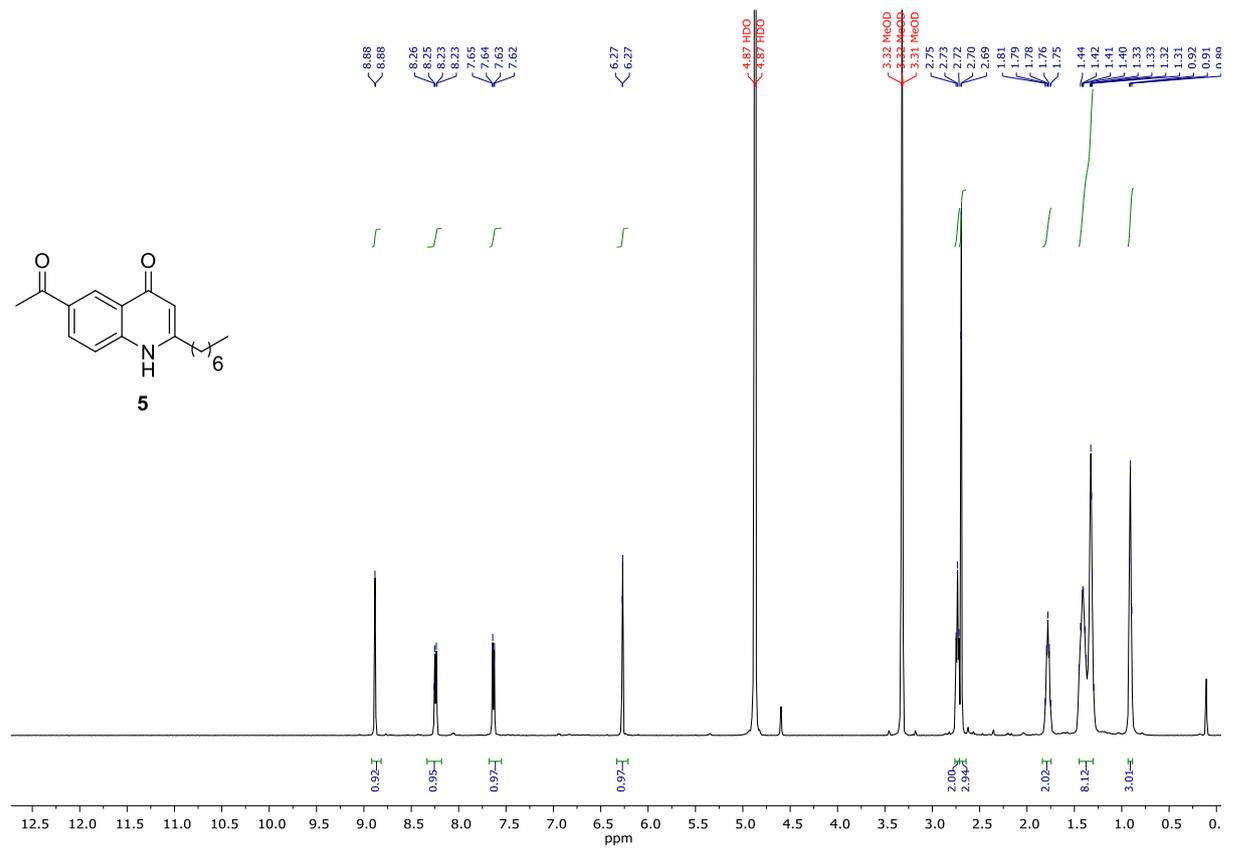
Supplementary Figure 19. ¹H and ¹³C NMR of S9.



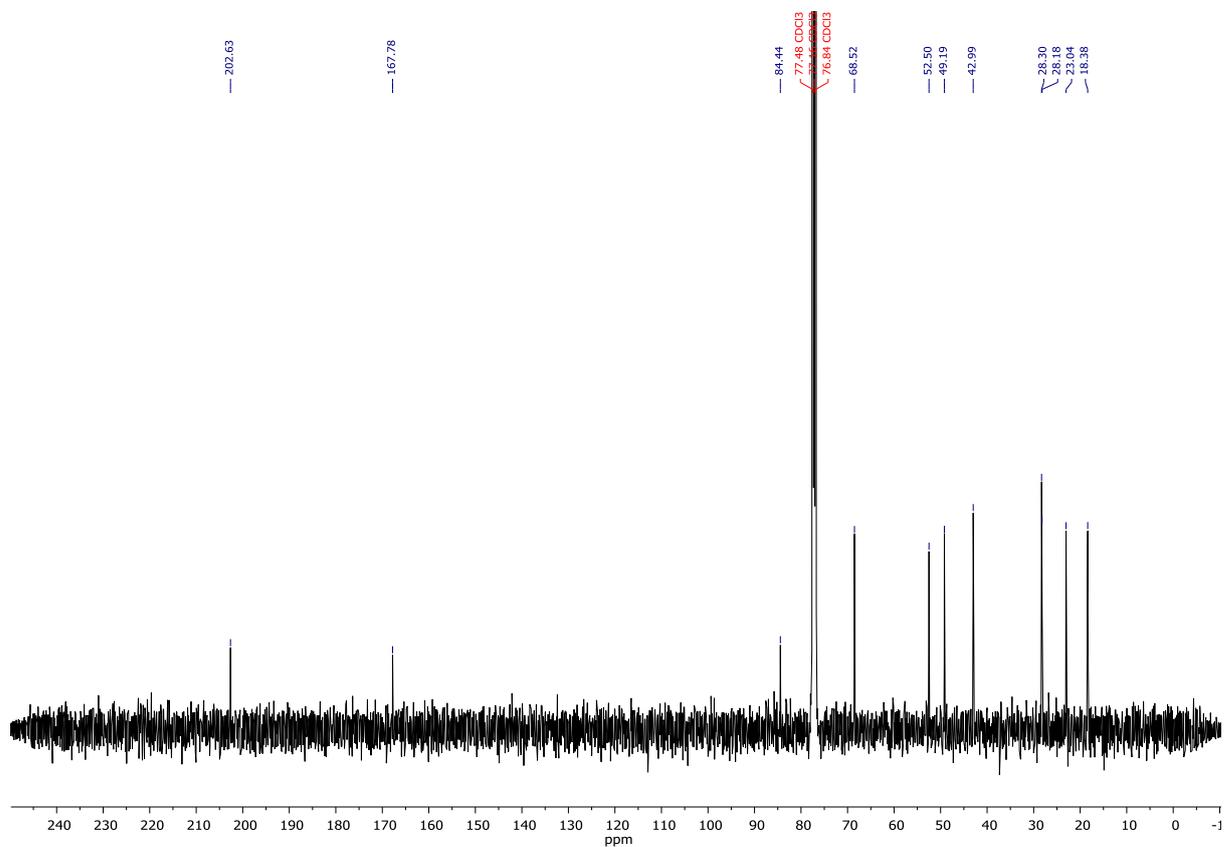
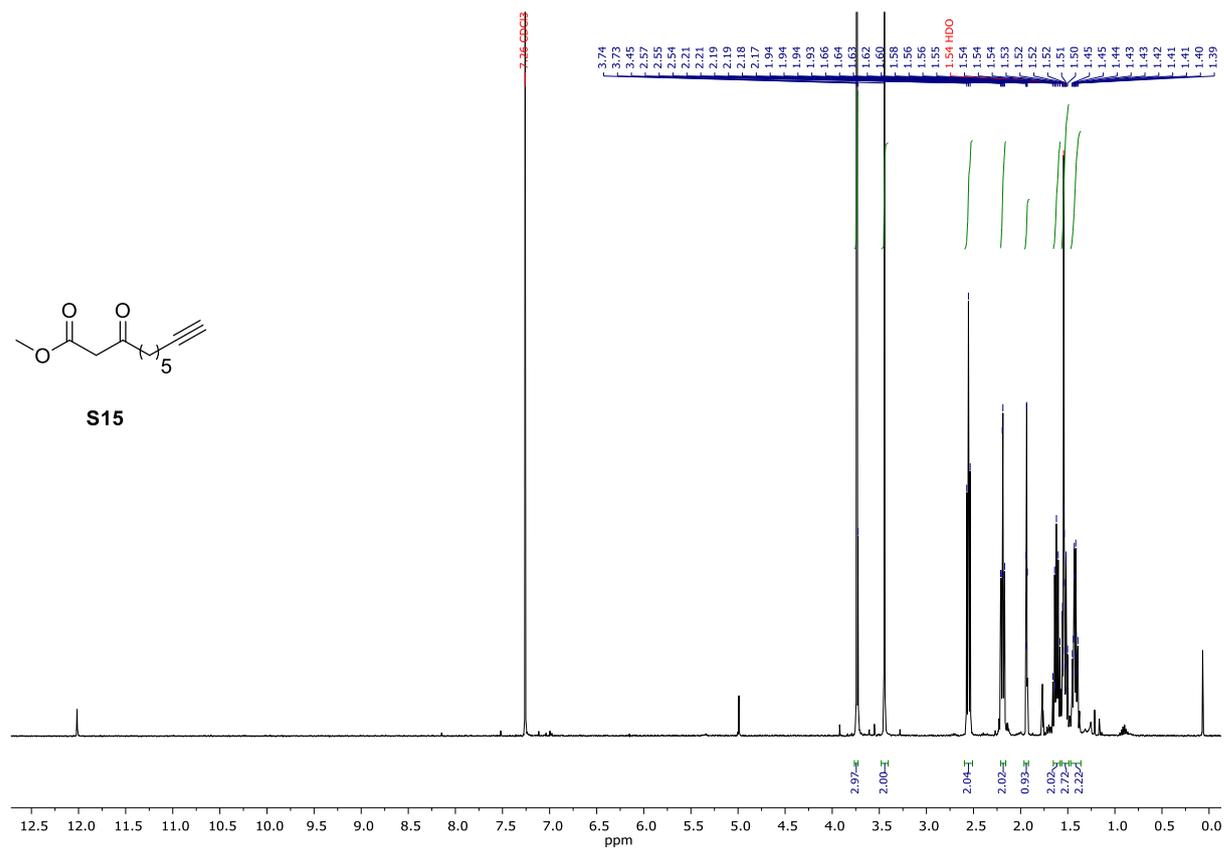
Supplementary Figure 20. ¹H and ¹³C NMR of S10.



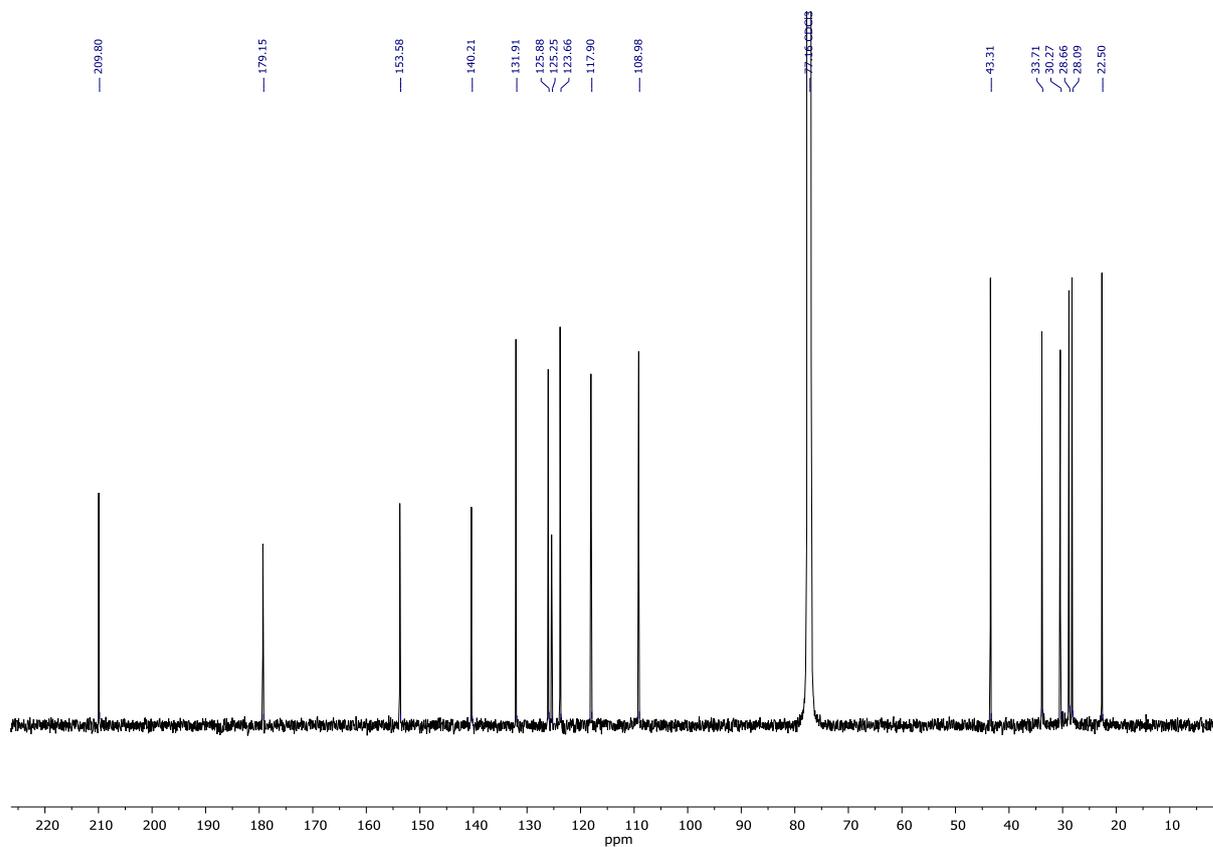
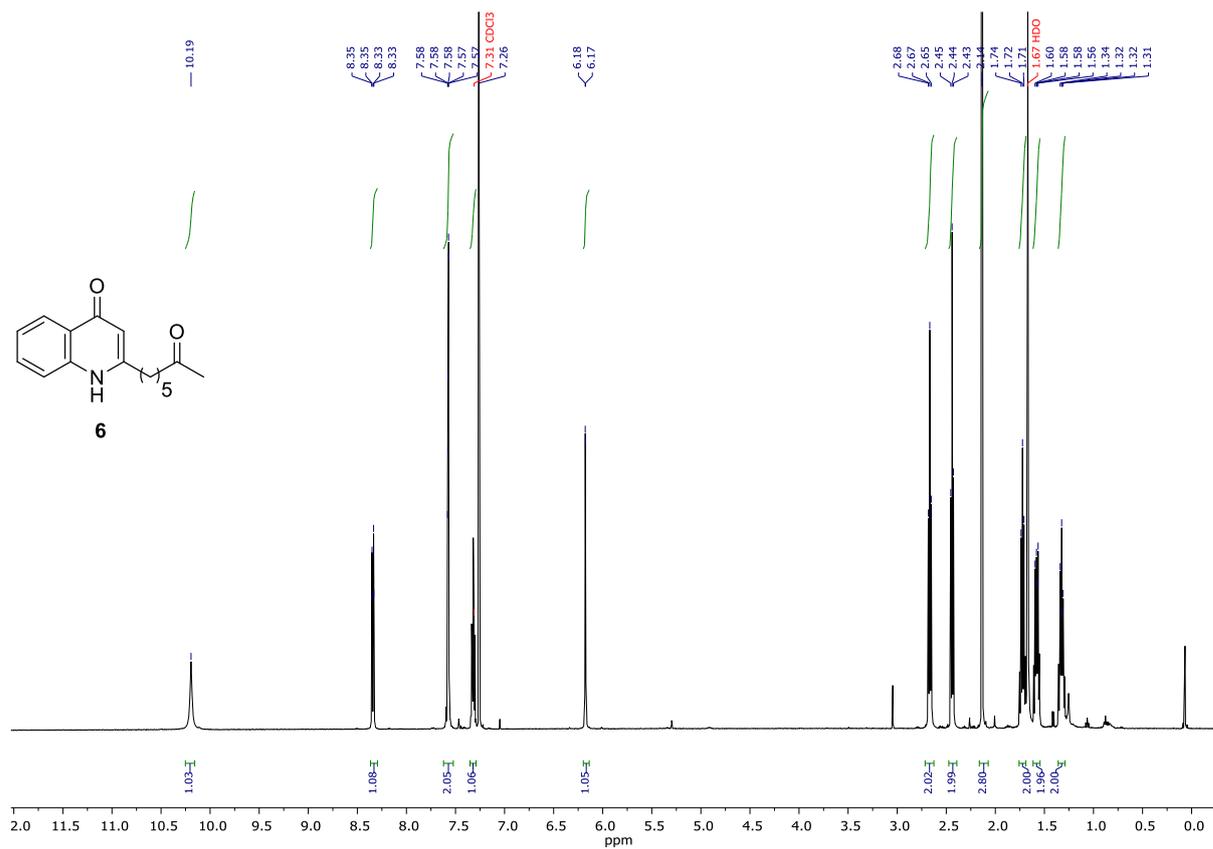
Supplementary Figure 21. ¹H and ¹³C NMR of 4.



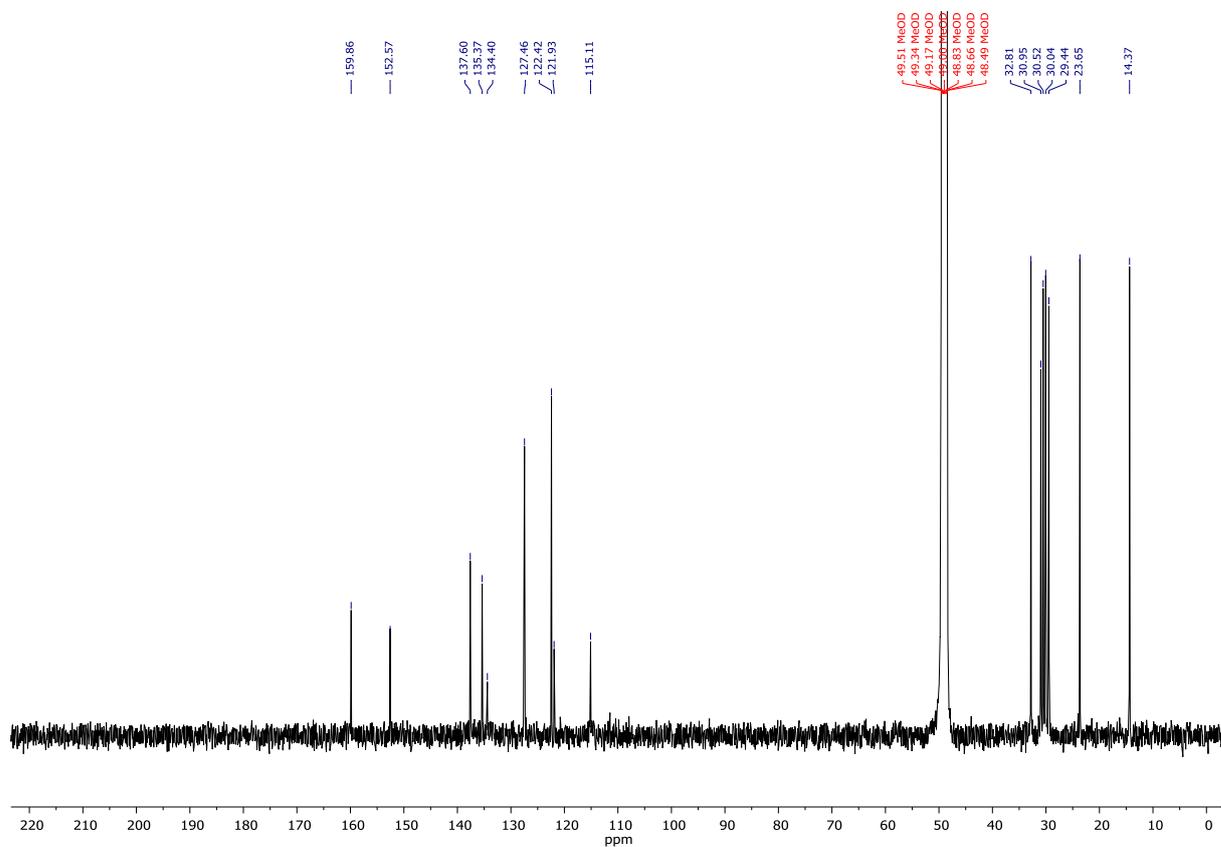
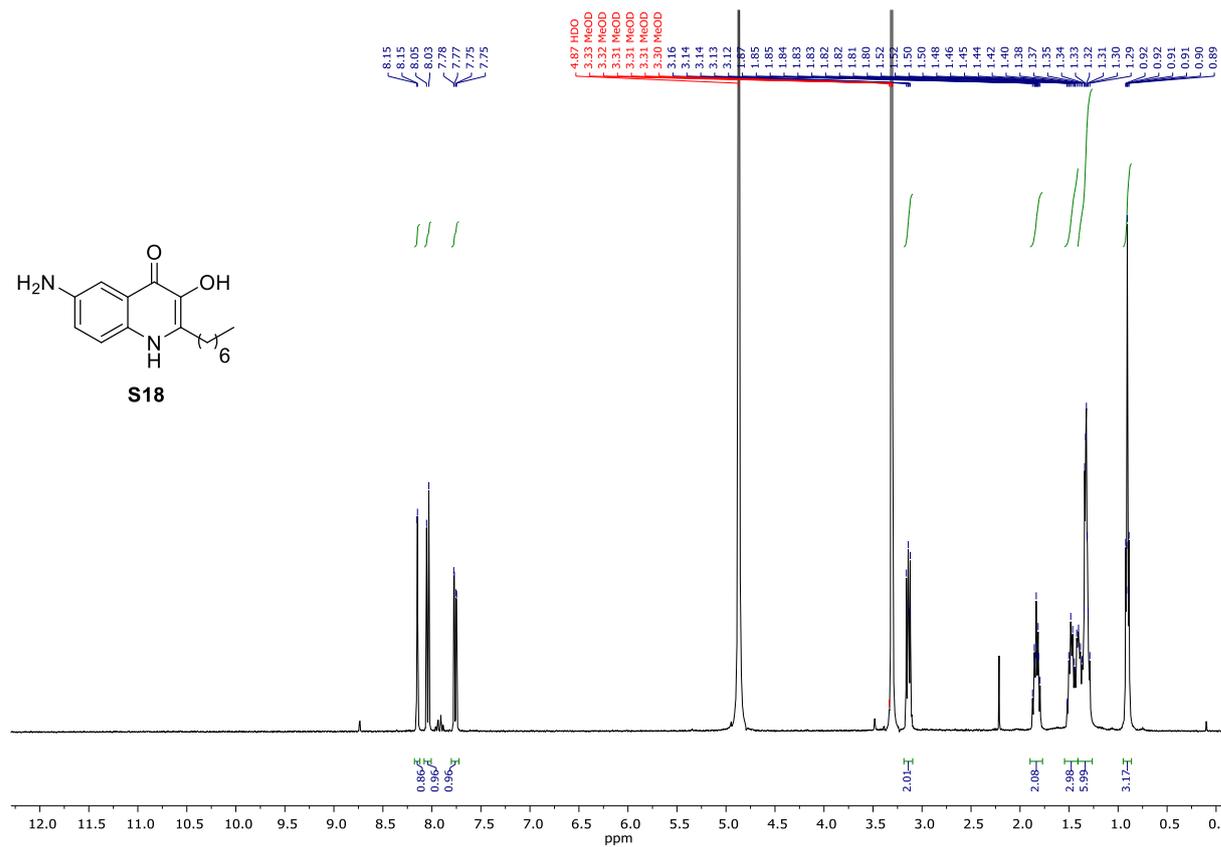
Supplementary Figure 23. ^1H and ^{13}C NMR of 5.



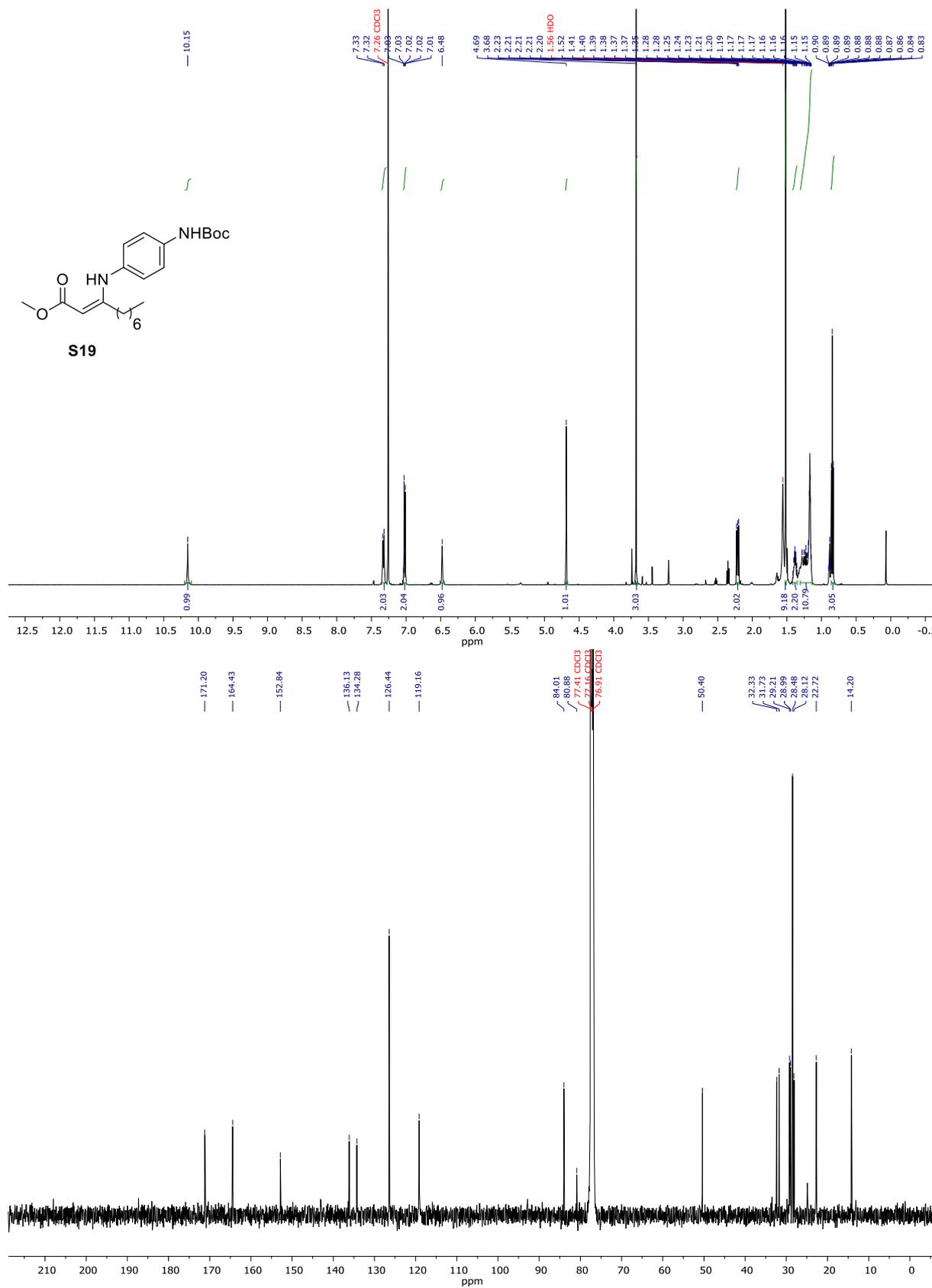
Supplementary Figure 24. ^1H and ^{13}C NMR of S15.



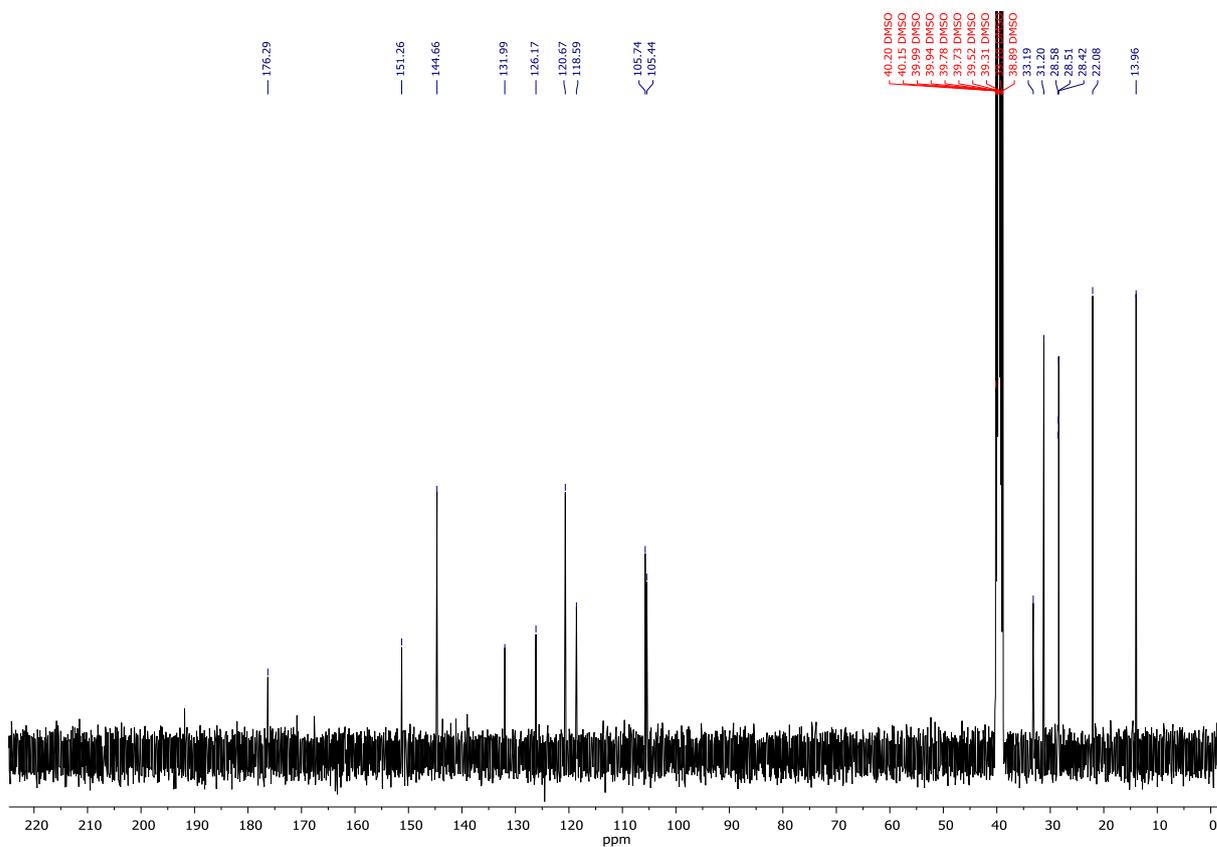
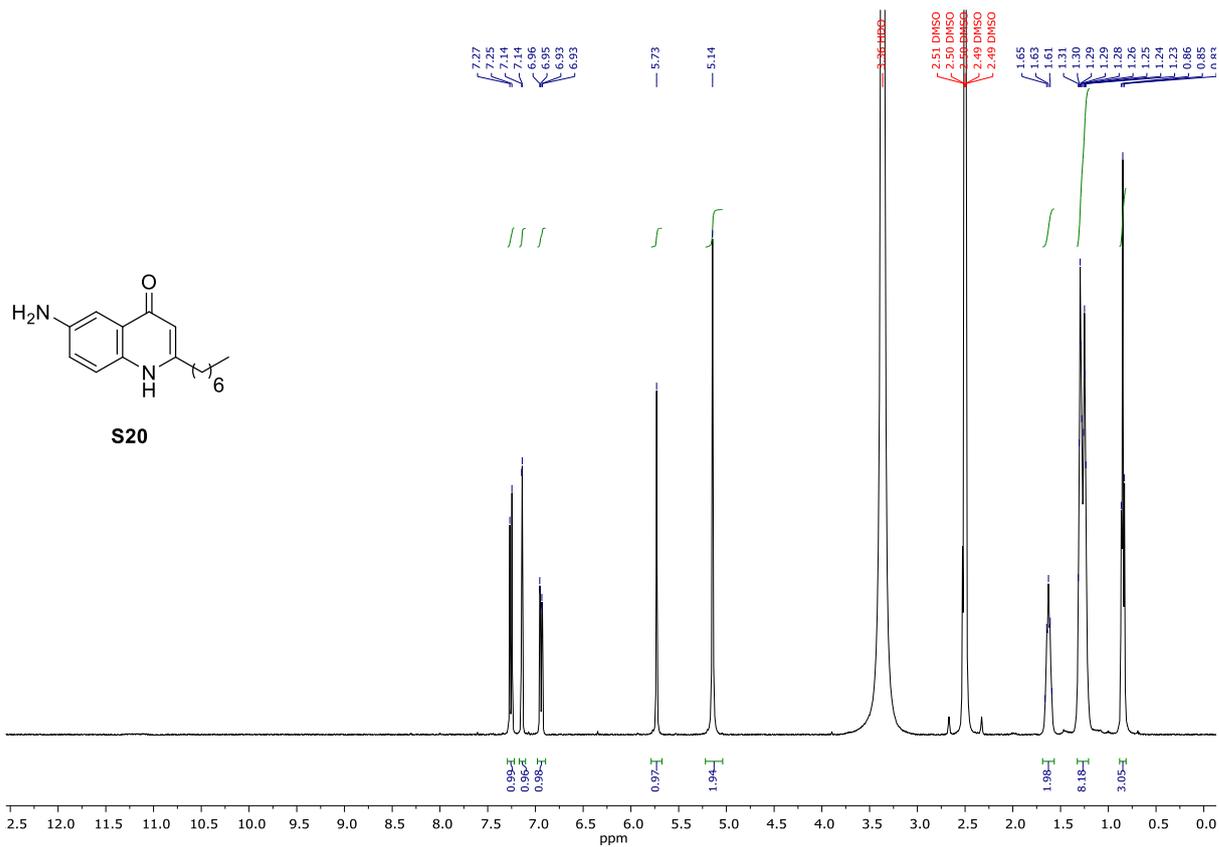
Supplementary Figure 25. ¹H and ¹³C NMR of 6.



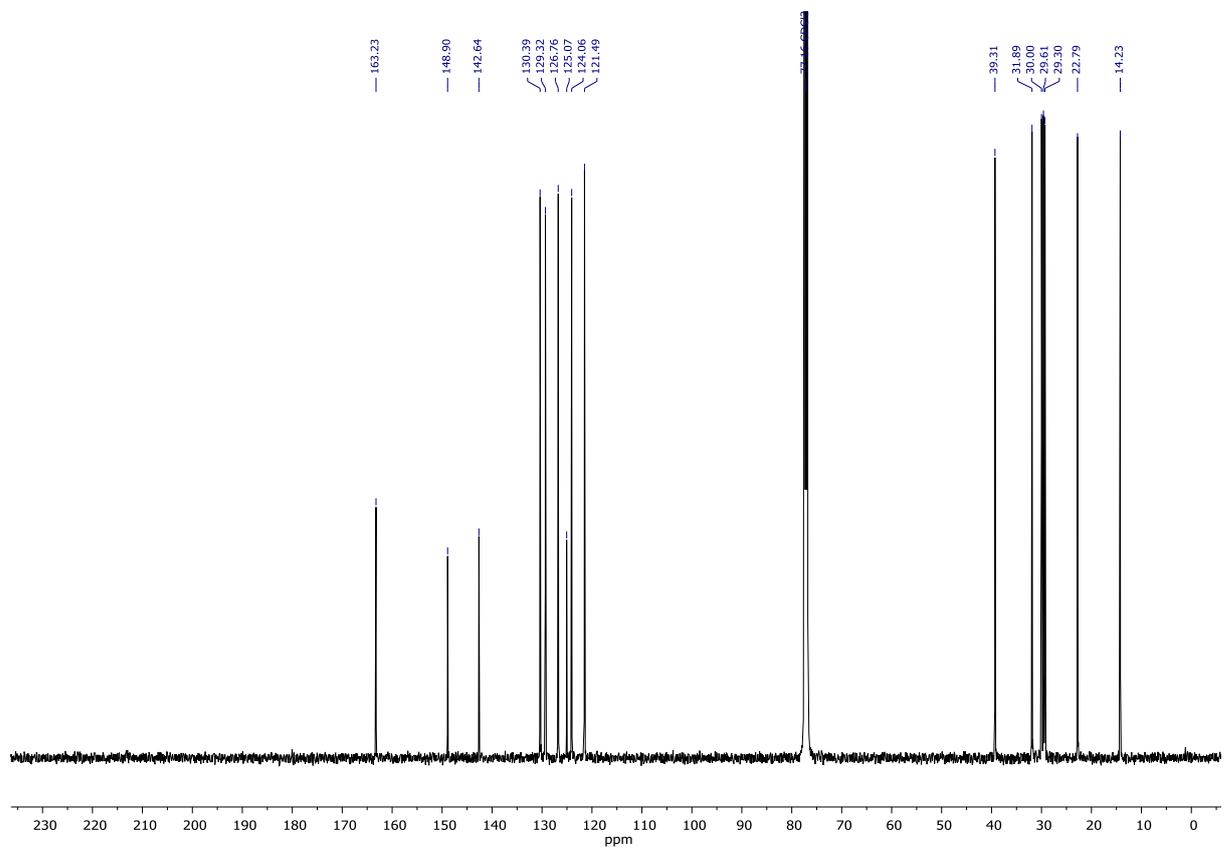
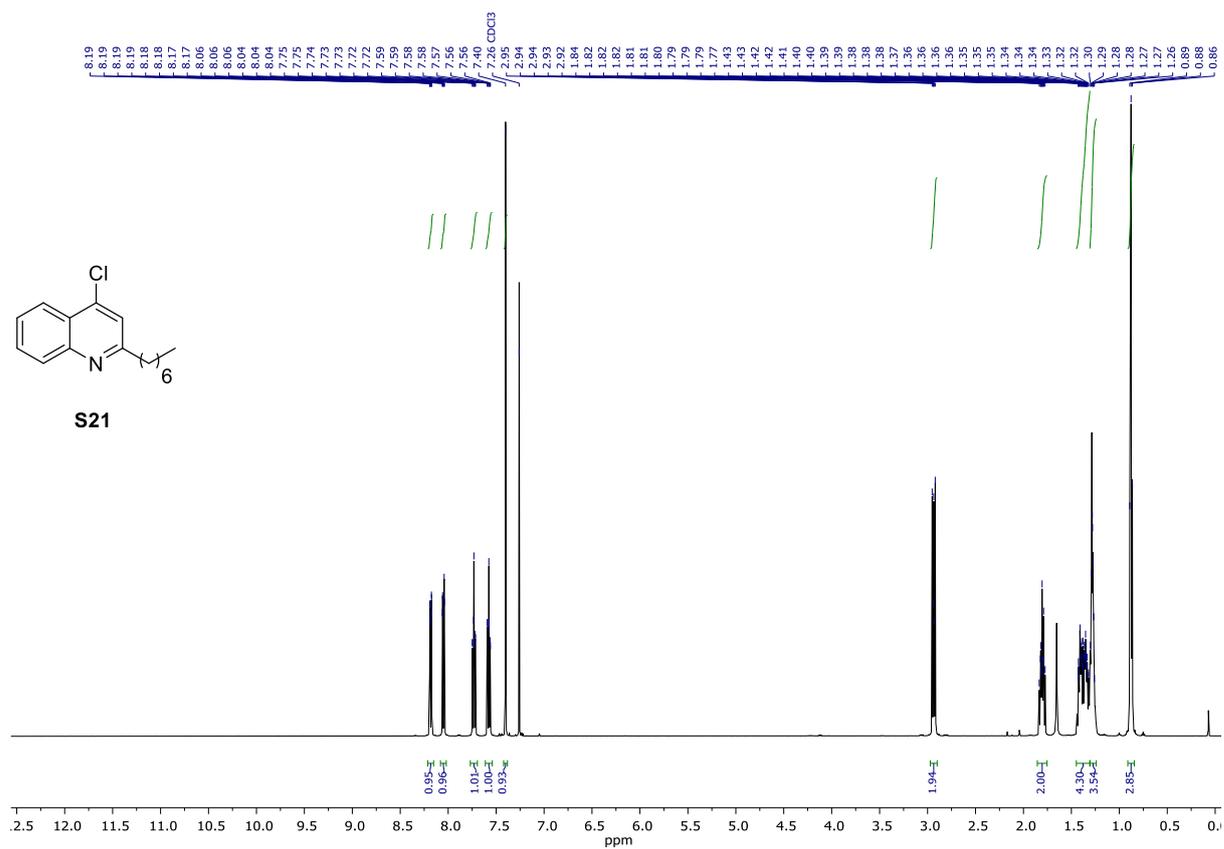
Supplementary Figure 27. ^1H and ^{13}C NMR of S18



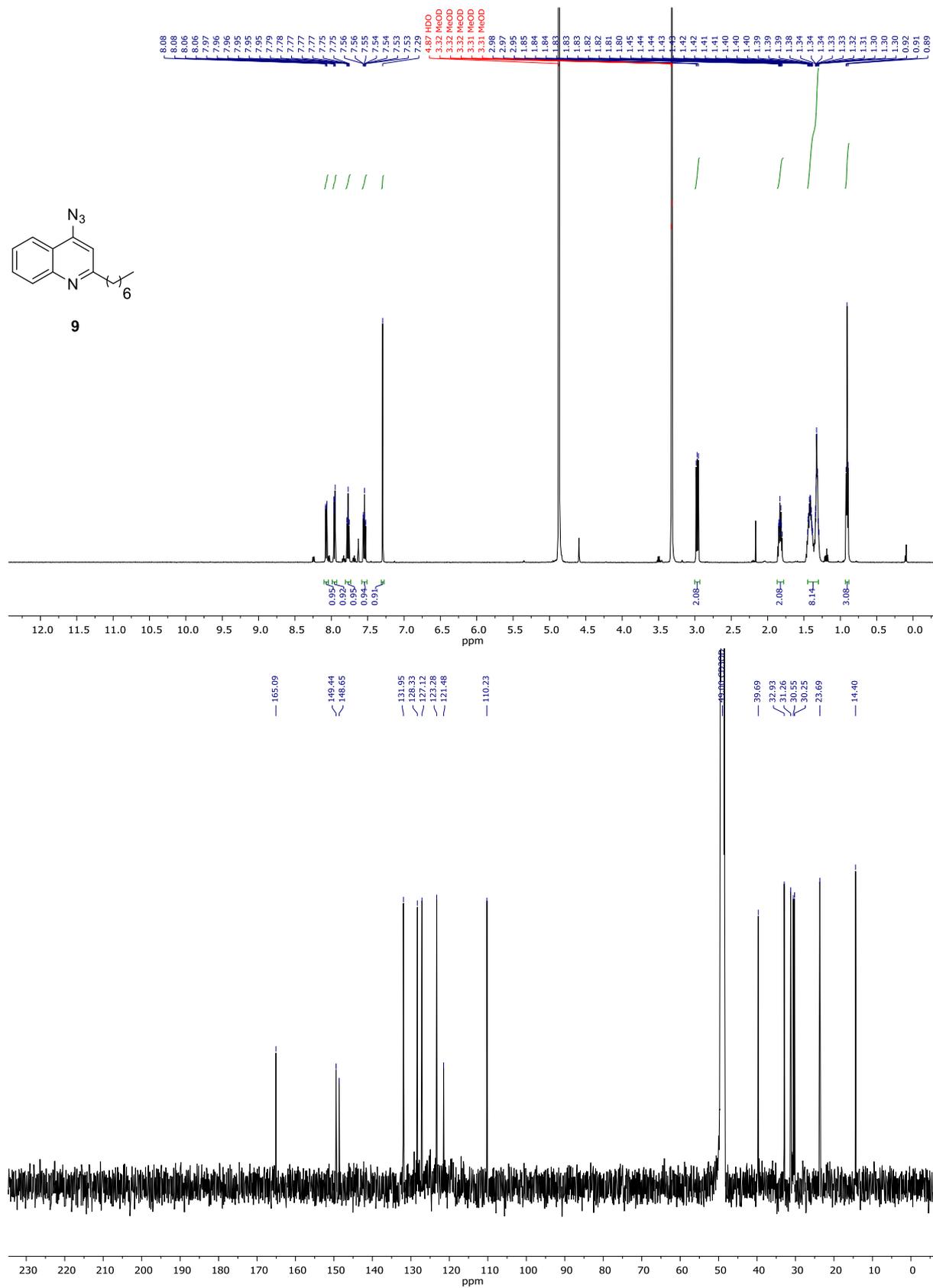
Supplementary Figure 29. ^1H and ^{13}C NMR of S19.



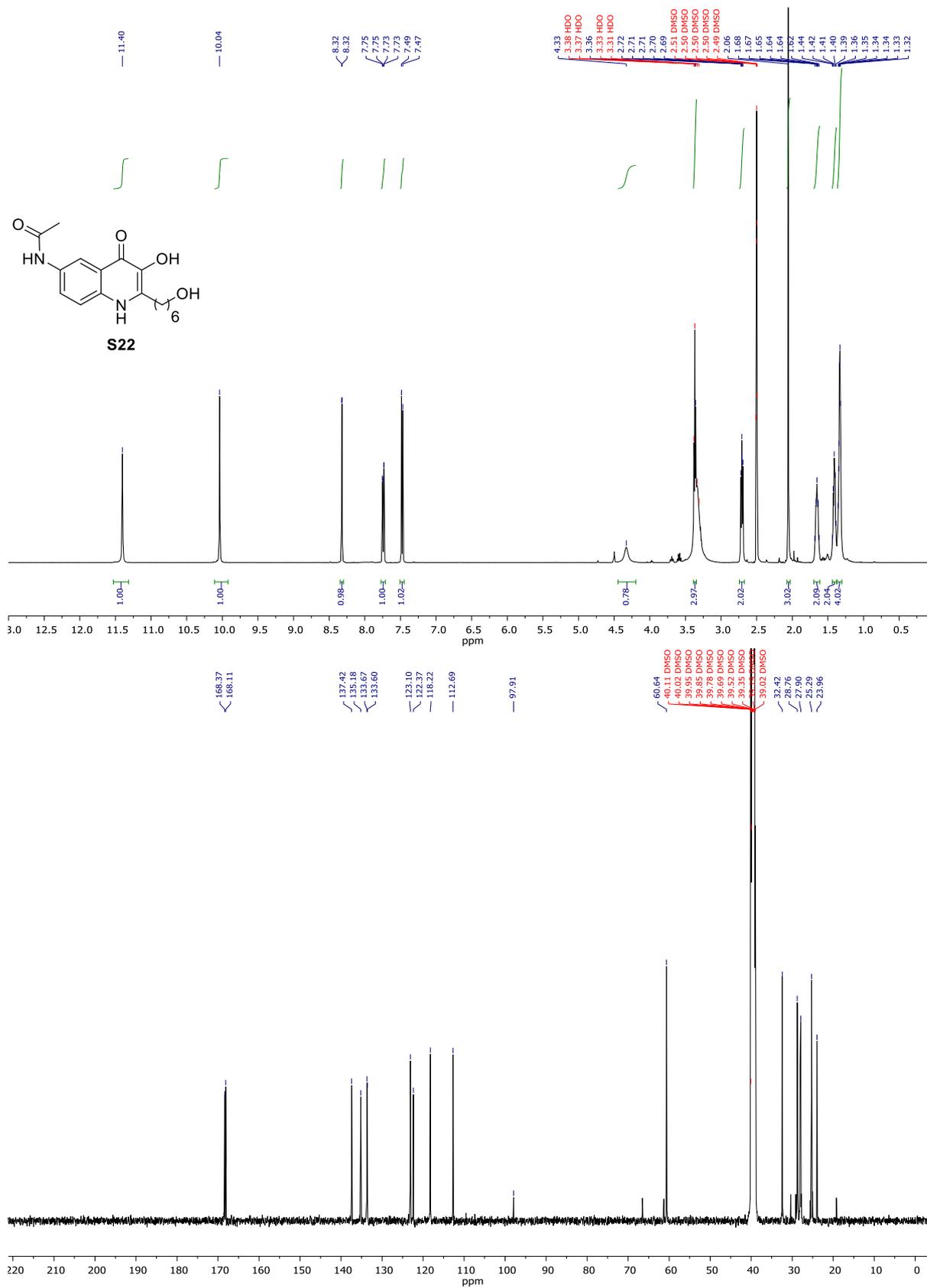
Supplementary Figure 30. ¹H and ¹³C NMR of S20.



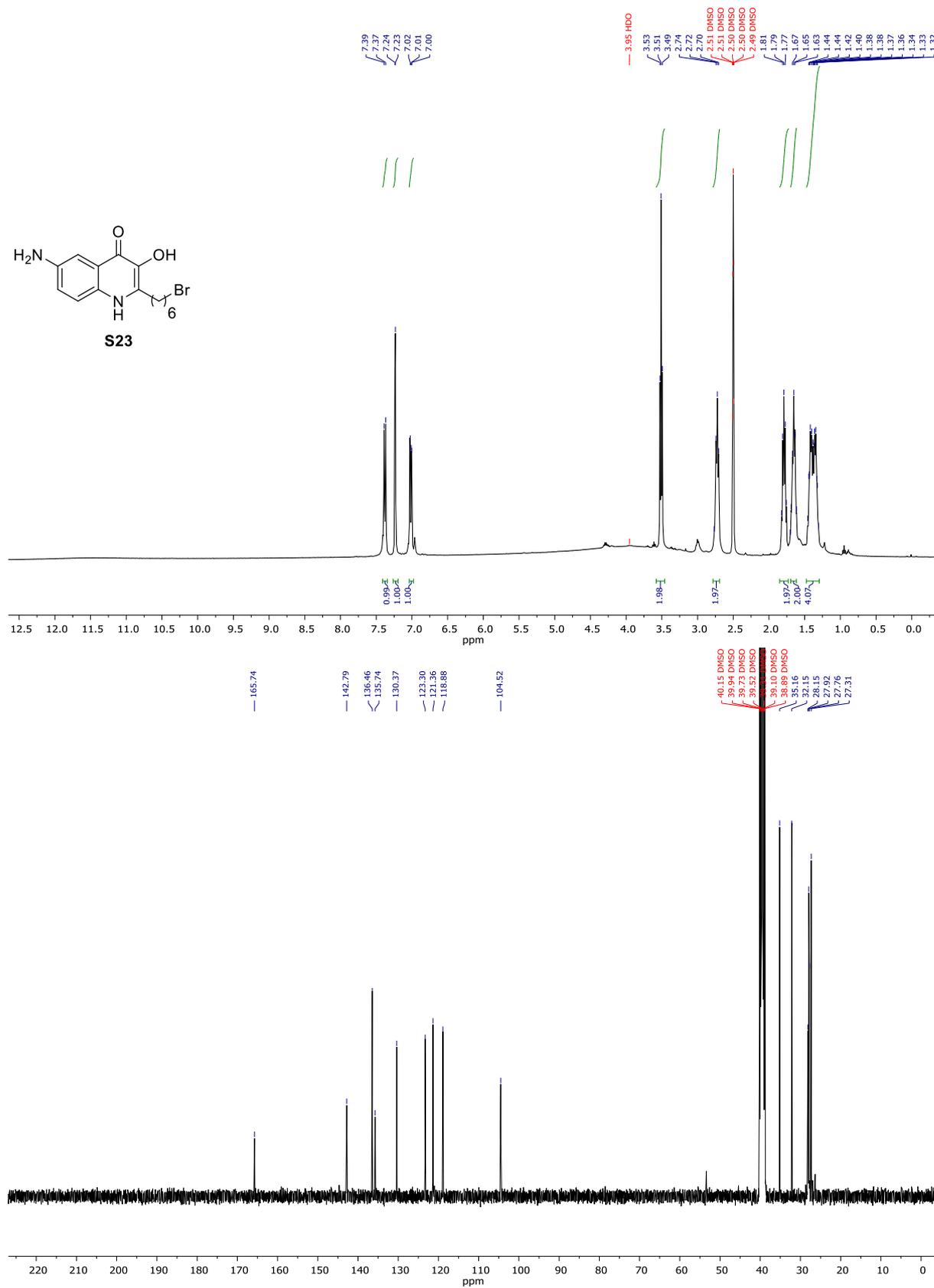
Supplementary Figure 32. ¹H and ¹³C NMR of S21.



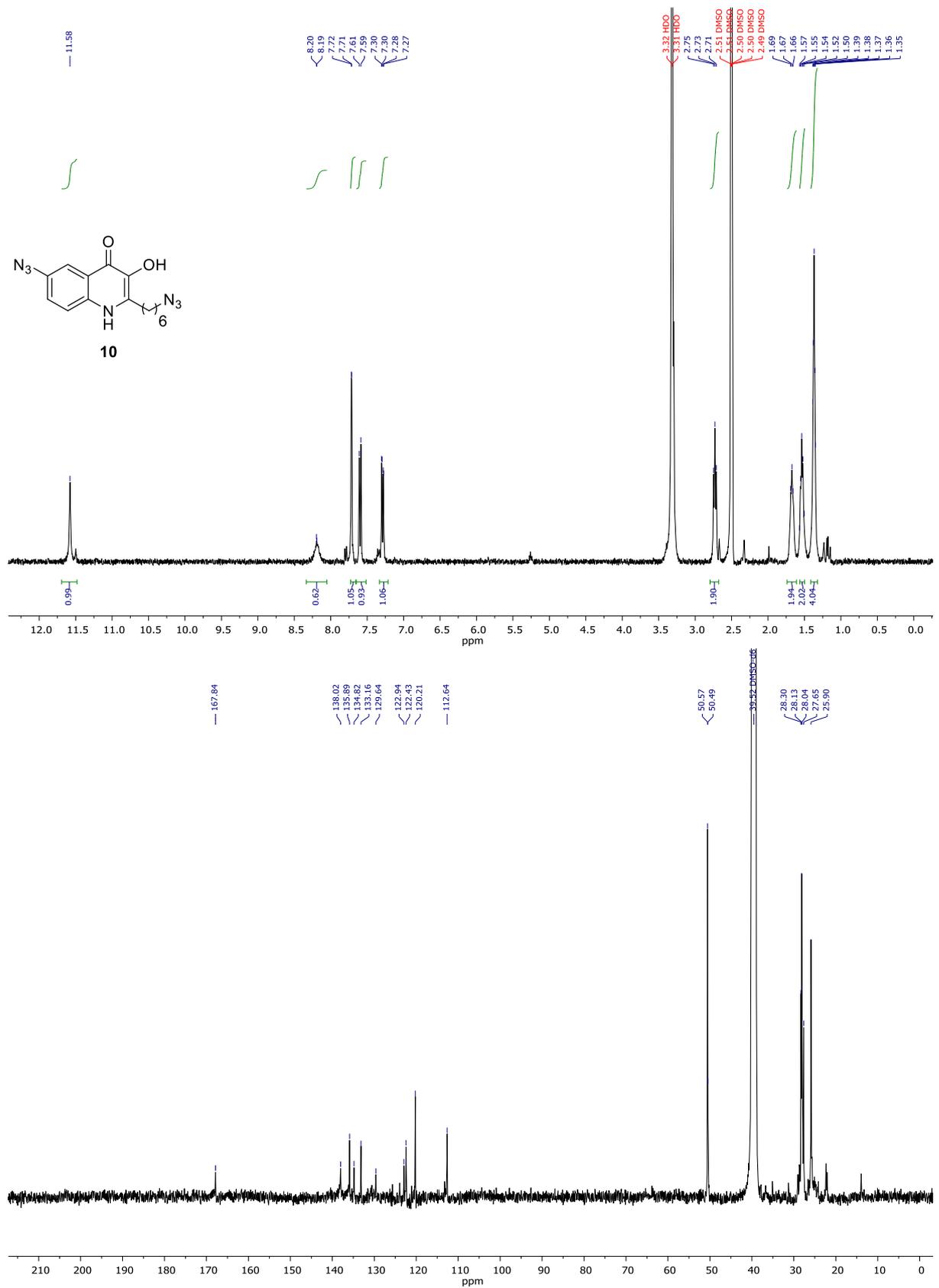
Supplementary Figure 33. ^1H and ^{13}C NMR of **9**.



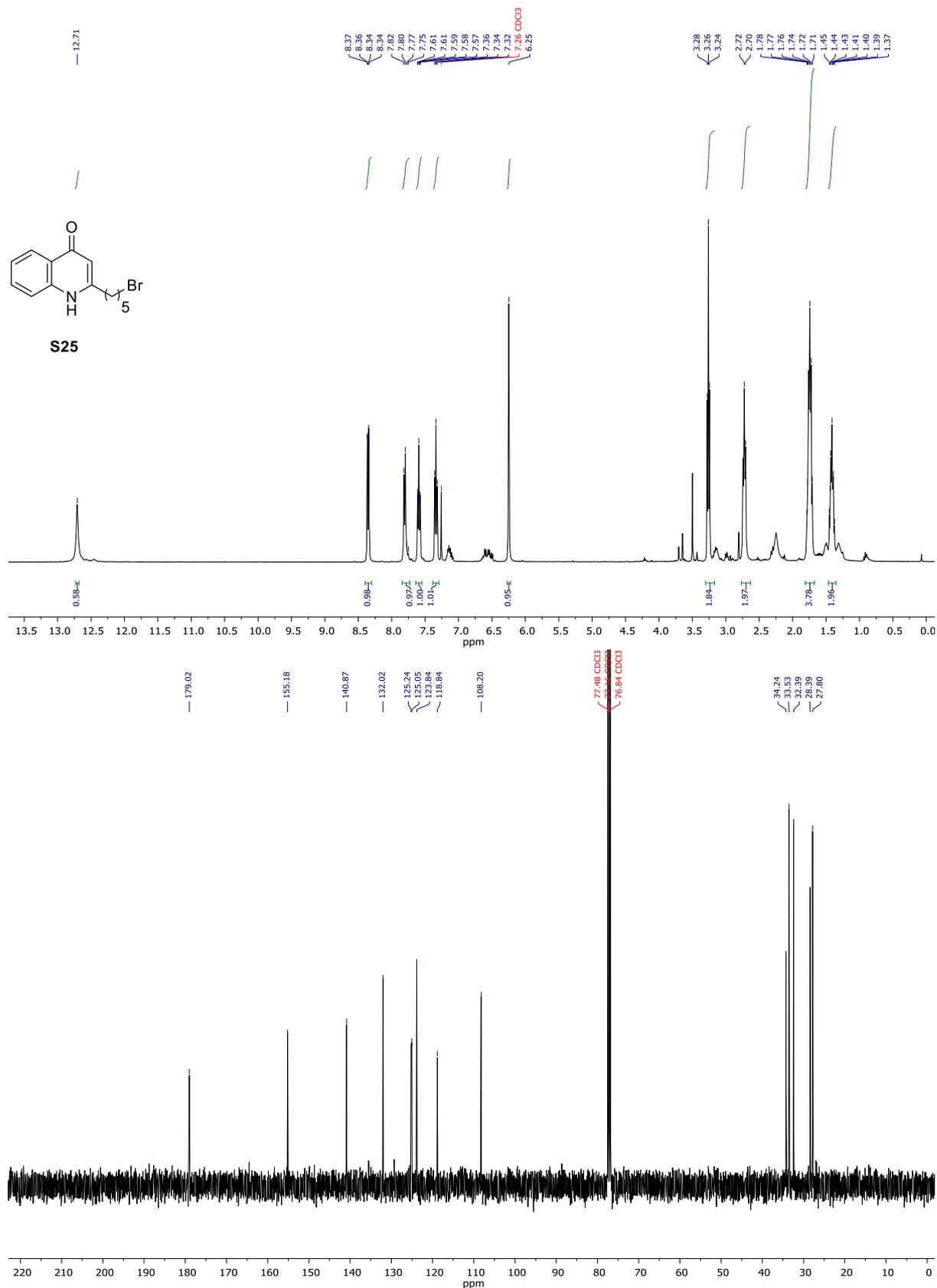
Supplementary Figure 34. ¹H and ¹³C NMR of S22.



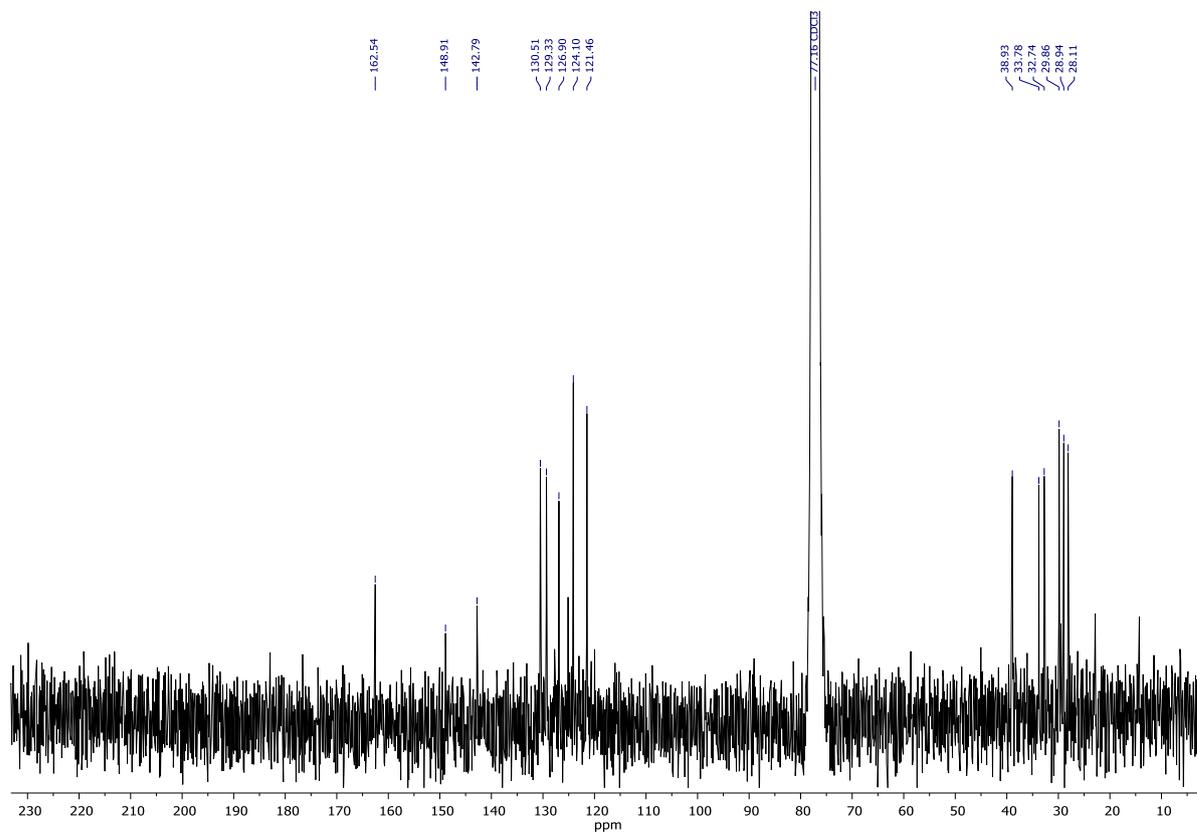
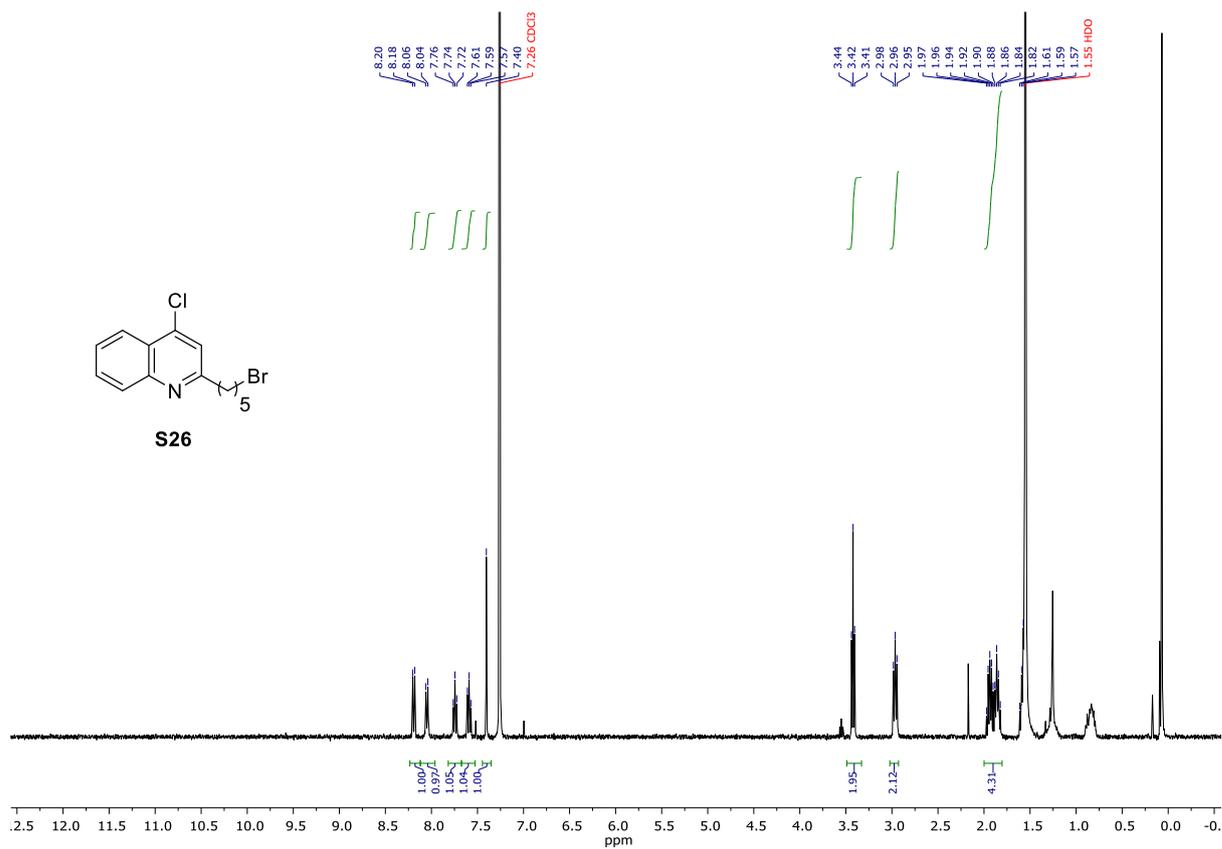
Supplementary Figure 35. ^1H and ^{13}C NMR of S23.



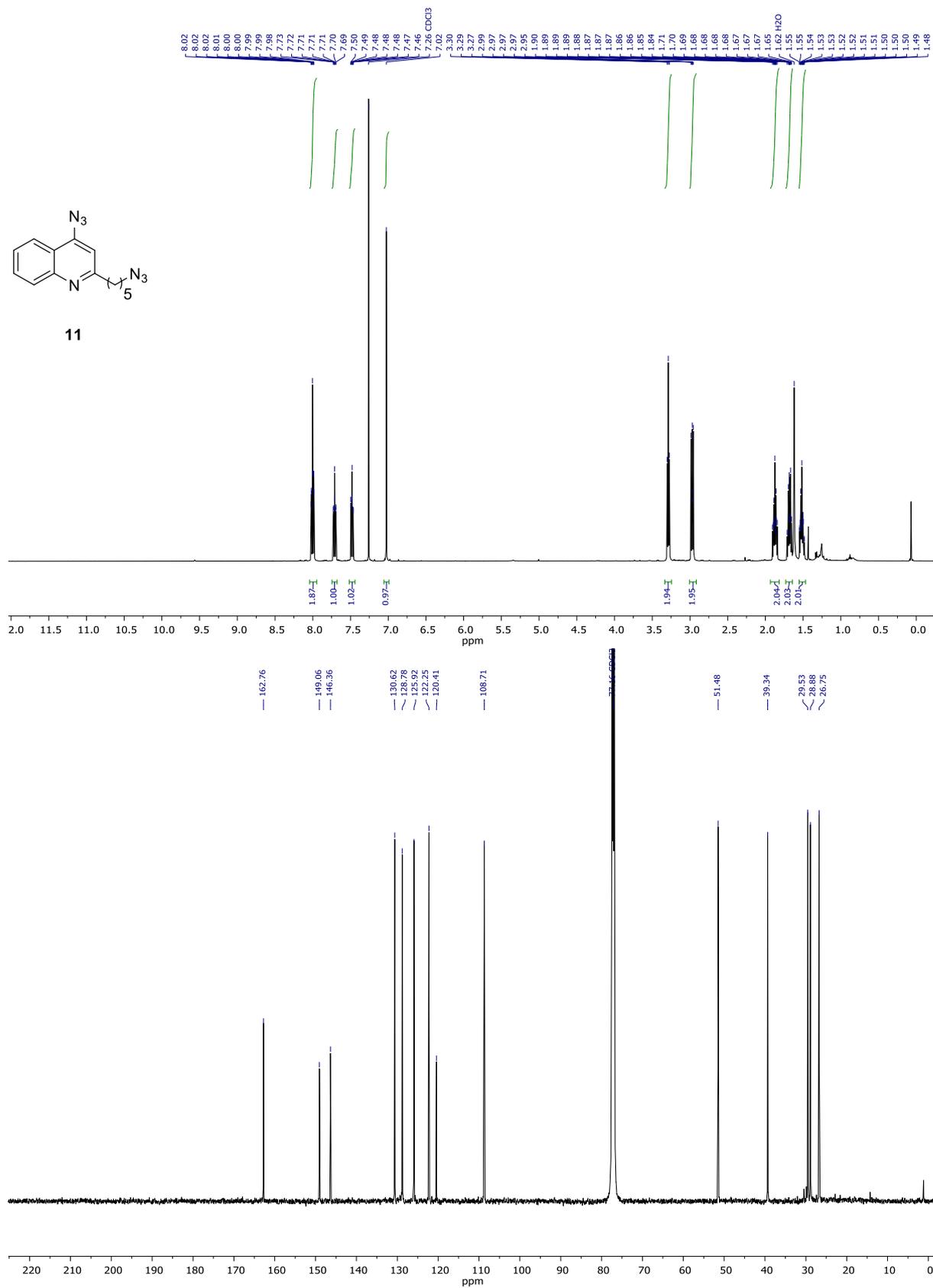
Supplementary Figure 37. ¹H and ¹³C NMR of 10.



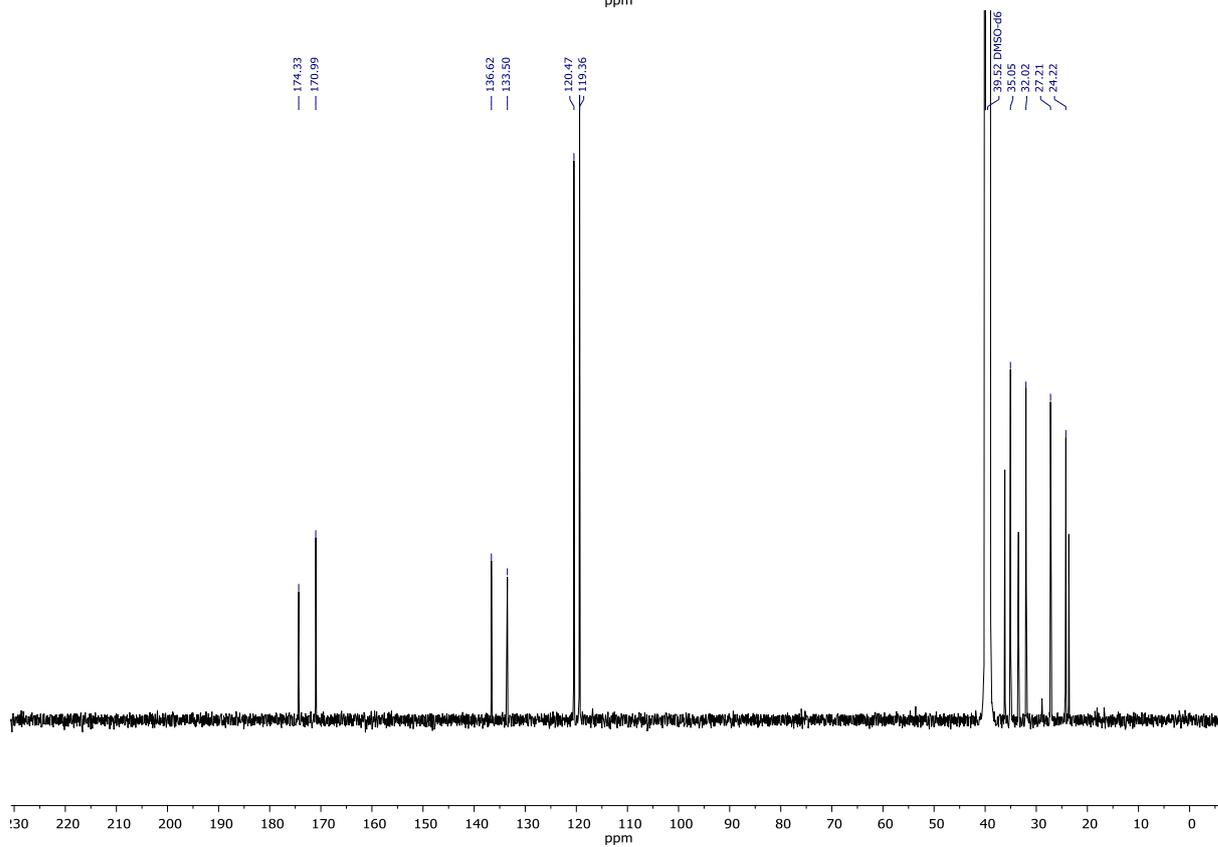
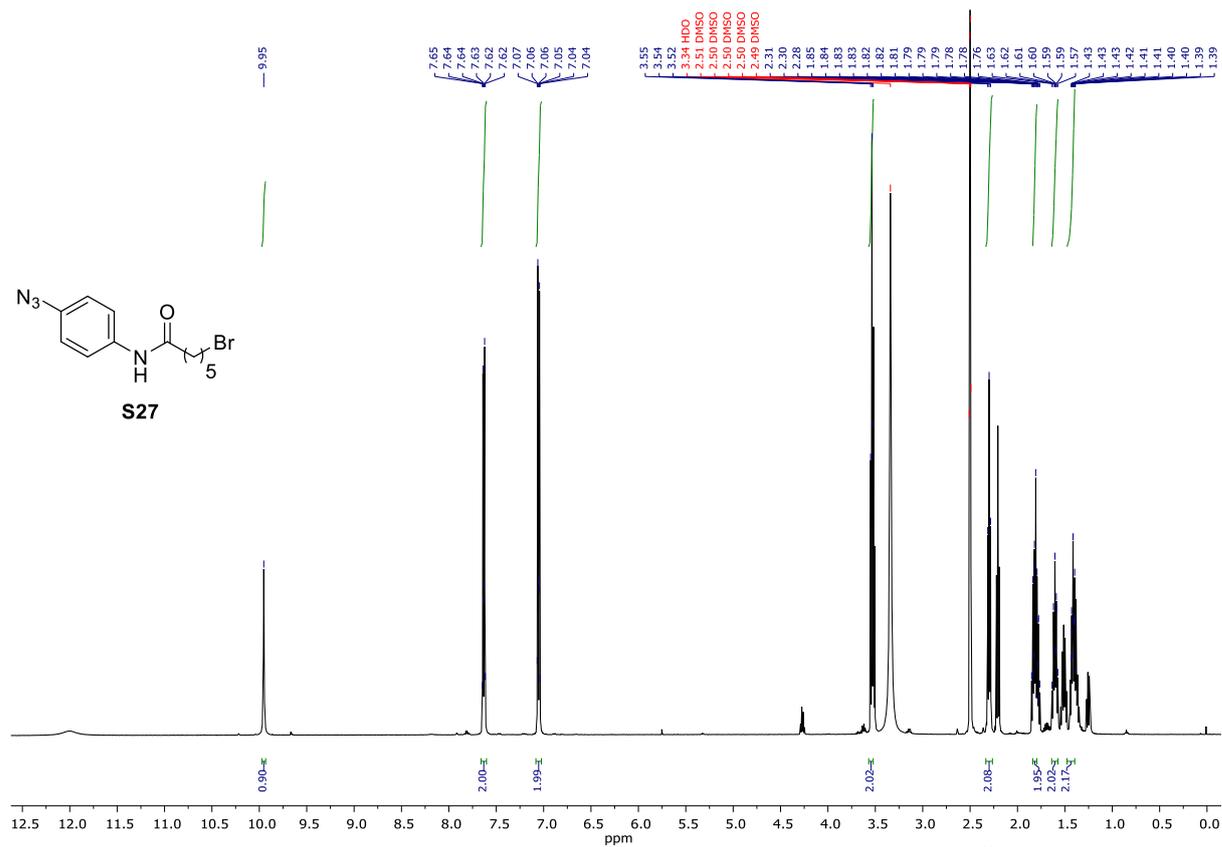
Supplementary Figure 38. ¹H and ¹³C NMR of S25.



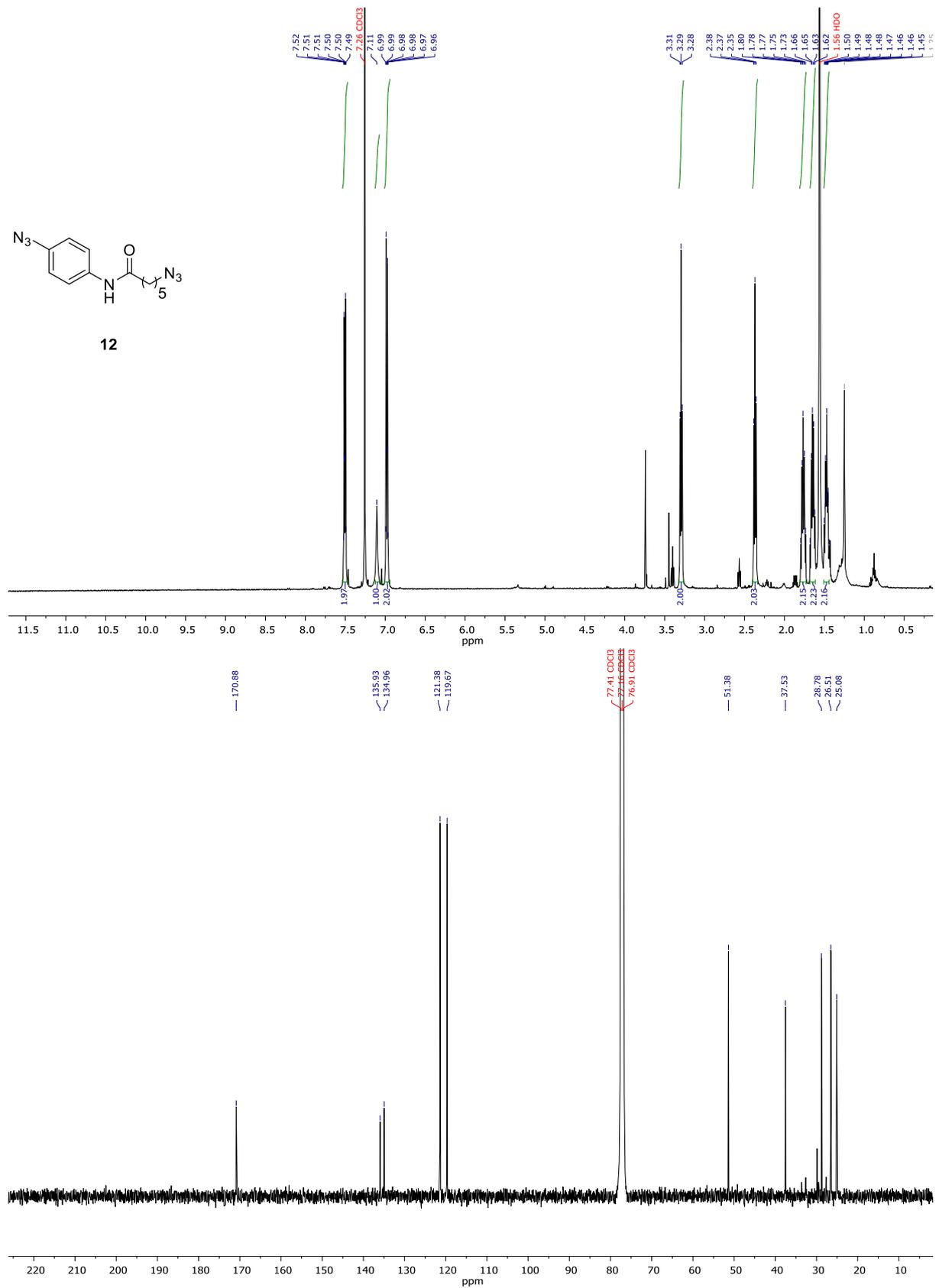
Supplementary Figure 39. ¹H and ¹³C NMR of S25.



Supplementary Figure 40. 1H and ^{13}C NMR of **11**.



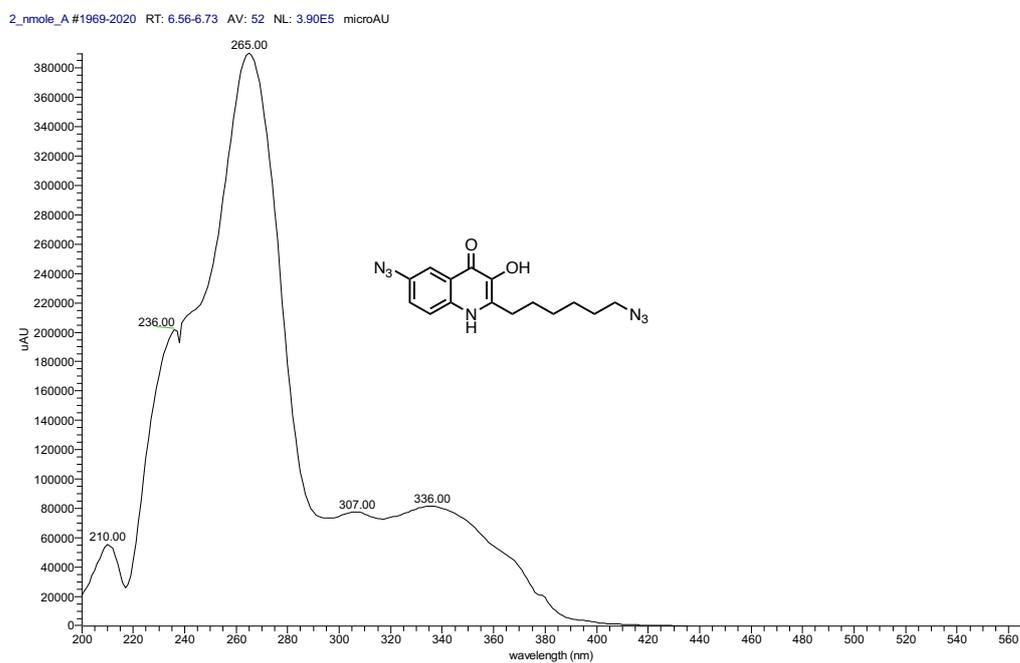
Supplementary Figure 41. ¹H and ¹³C NMR of S27.



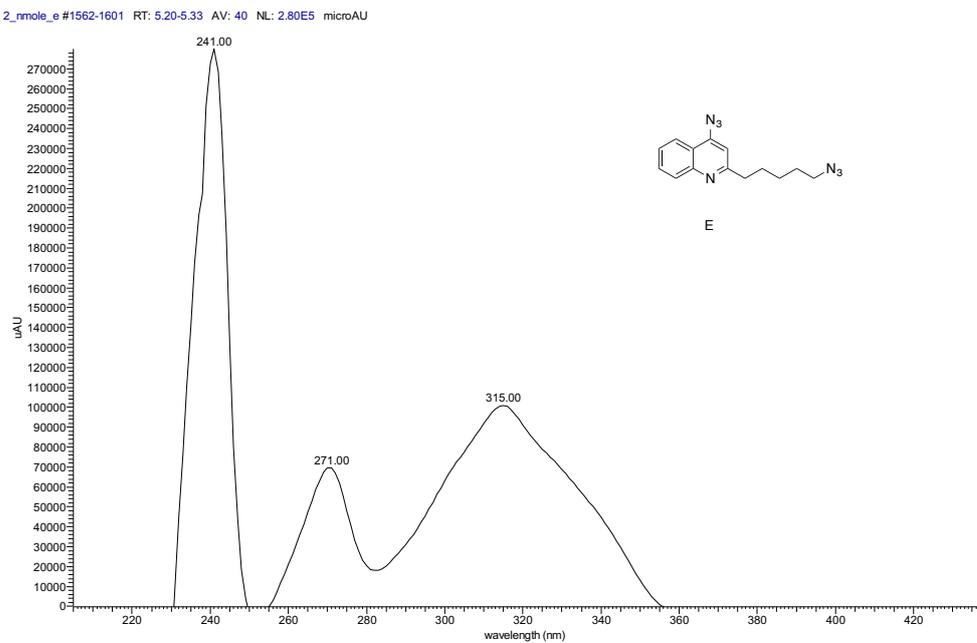
Supplementary Figure 42. ¹H and ¹³C NMR of **12**.

5 Supplementary Appendix 1- UV traces for probes 10-12.

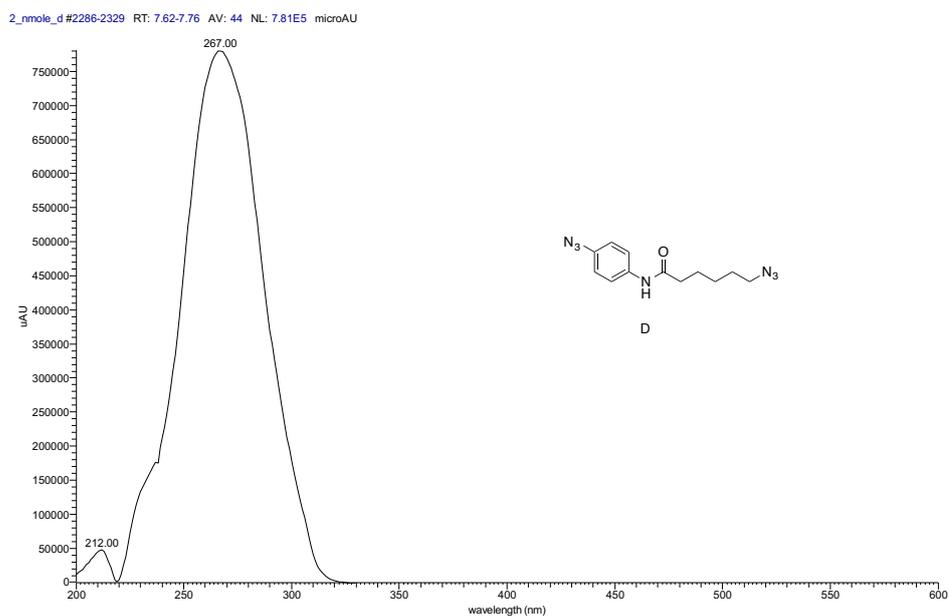
UV trace for probe 10:



UV trace for probe 11:



UV trace for probe 12:



6 Supplementary Appendix 2 - PqsD cloning and purification and fluorimetry protocol

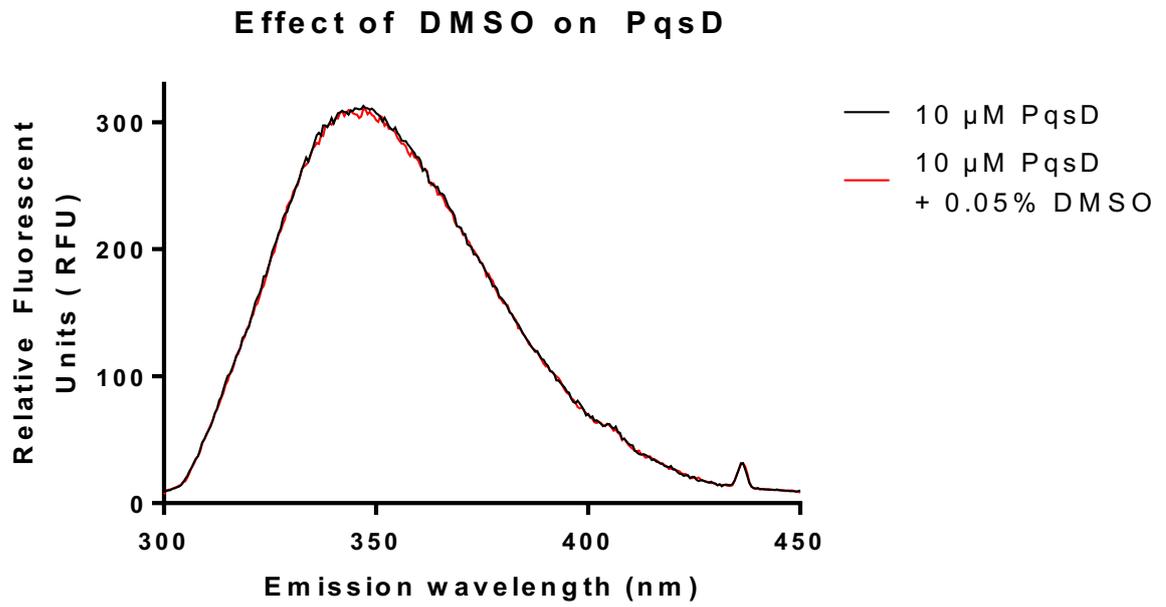
Cloning. The coding region of *pqsD* was amplified by PCR from *P. aeruginosa* genomic DNA using synthetic primers (5'-ATATACTACATATGATGGGTAATCCGATCCTGGC-3' and 5'-ATATGGATCCTCAACATGGCCGGTTCACCT-3') designed according to the published nucleotide sequence of *pqsD*. The PCR product was digested with NdeI and BamHI. The digested PCR product was subsequently cloned into the expression vector, pET-19m.²⁶ *E. coli* BL21 (DE3) cells containing the plasmid pRARE2 were then transformed with the resulting pET-19m-*pqsD* plasmid.

Protein expression. To overexpress PqsD, overnight cultures of *E. coli* strain BL21 (DE3) carrying pRARE 2 and pET-19m-*pqsD* were inoculated into 2 x 1L of LB containing 50 µg/ml carbenicillin and 25 µg/ml chloramphenicol. Cell cultures were grown at 37°C with agitation until they reached an absorbance of 0.6 at 600 nm. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the cultures at a final concentration of 0.3 mM. The cultures were incubated overnight with agitation at 20°C. Cells were harvested by centrifugation (11,899 x g, 20 min, 4°C), and stored at -20°C for future use. Cell pellets were thawed, and suspended in 10 ml of ice-cold lysis buffer (NaCl 300 mM, 50 mM Tris-HCl pH 8.0, 5% glycerol (v/v), 10 mM imidazole, 5 mM β-mercaptoethanol) containing an EDTA-free protease inhibitor cocktail™ tablet (Roche). Samples were lysed by sonication (5 rounds of 30 seconds at 13 ampere with 1 minute rest on ice between pulses) and clarified by centrifugation (20,000 x g, 30 min, 4°C). The supernatant was collected and filtered through 0.45 µm membrane filters (Sartorius).

Purification of PqsD. Unless otherwise stated, all purification steps were carried out at 4°C. The PqsD supernatant was loaded onto a Ni-NTA agarose column (Qiagen) pre-equilibrated with lysis buffer. The column was subsequently washed O/N with approximately 200 ml of lysis buffer, after which PqsD was eluted with elution buffer (NaCl 300 mM, 50 mM Tris-HCl pH 8.0, 5% glycerol (v/v), imidazole 250 mM, 5 mM β-mercaptoethanol). PqsD was concentrated using Sartorius' Vivaspinn 2 ultrafiltration column (MWCO: 10,000) and washed several times in dialysis buffer (NaCl 100 mM, 50 mM Tris-HCl pH 8.0, glycerol 5% (v/v)). PqsD was judged pure by SDS-PAGE analysis. The protein was separated into aliquots, flash-frozen in liquid nitrogen, and stored at -80°C.

Fluorimetry analysis. All fluorescence spectra were generated on a Perkin Elmer LS 55 luminescence spectrometer. PqsD protein (10 µM final concentration) was added to 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10% (v/v) glycerol to a final volume of 1 ml in a quartz, optical cuvette with a 1 cm path length. Spectra illustrated (Fig. 1 below) represent an average of three traces, with no significant variation between each of the three replicates. The excitation wavelength used was 292 nm. At a rate of 10 nm/min, emission spectra were recorded between 300 and 450 nm with the excitation and emission slit widths set at 2.5 nm.

A



B

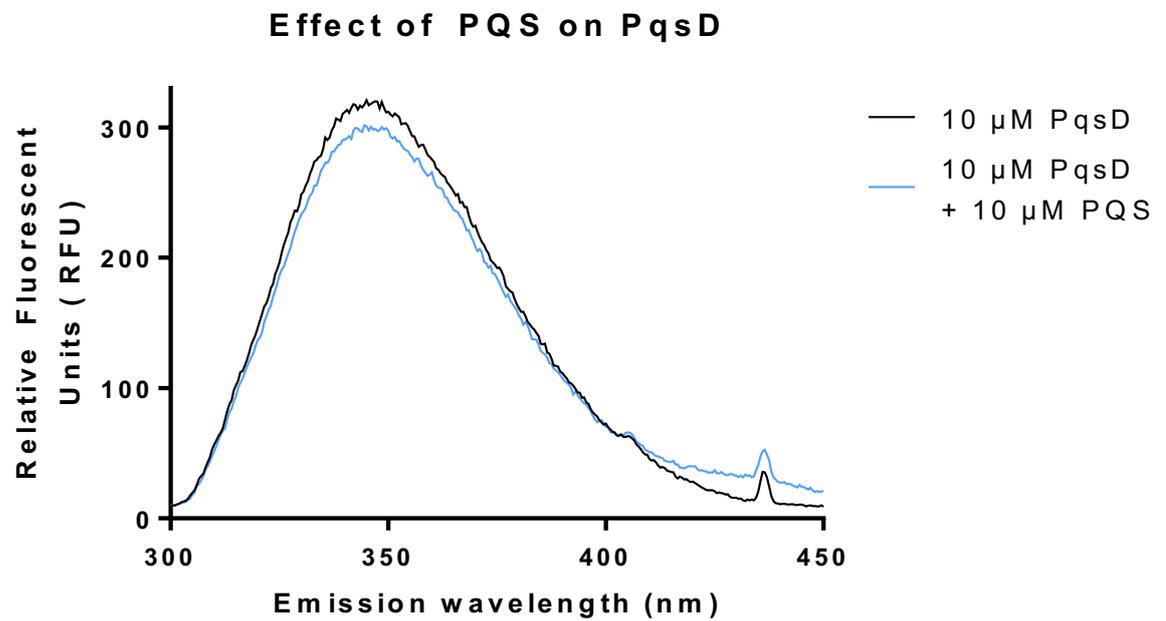


Figure 1. **A)** 10 μ M PqsD with 0.05 % DMSO (vehicle control) and **B)** 10 μ M PqsD with 10 μ M PQS

7 References

- 1 M. Welch, J. T. Hodgkinson, J. Gross, D. R. Spring and T. Sams, *Biochemistry*, 2013, **52**, 4433–4438.
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