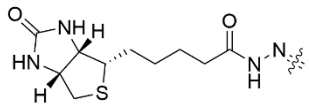
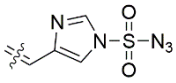
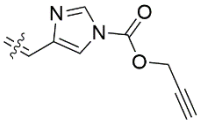
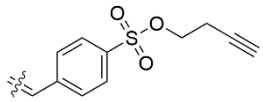
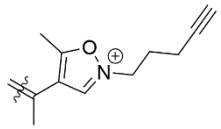
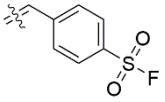
Supporting Information

Table of contents

Table S1. Structures of the *in situ* prepared chemical probes

 **L1**				
				
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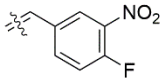	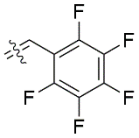	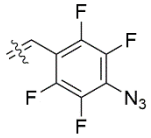	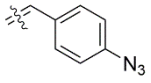	
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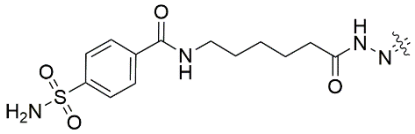
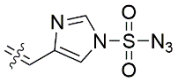
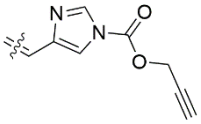
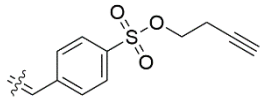
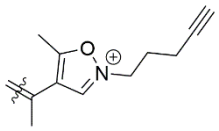
 **L2**				
				
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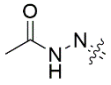
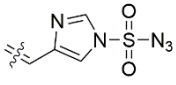
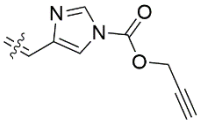
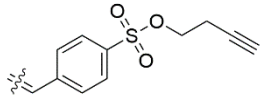
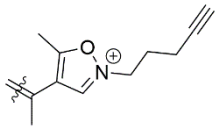
 **L3**				
				
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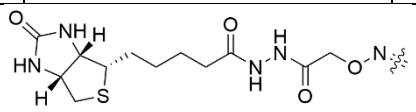
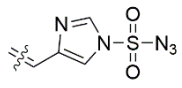
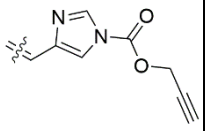
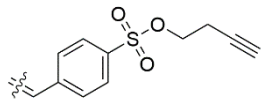
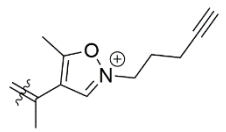
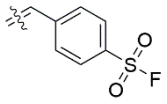
 **L4**				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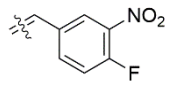	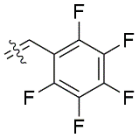	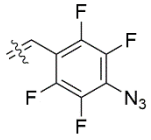	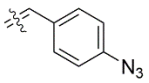	
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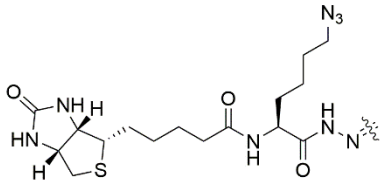				
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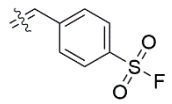	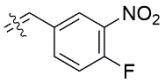	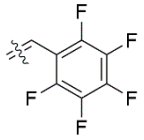	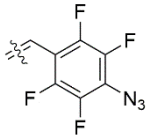	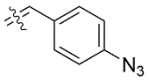
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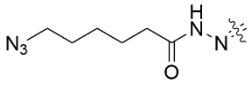				
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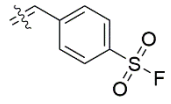	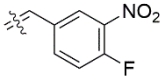	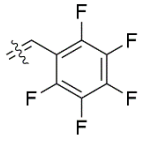	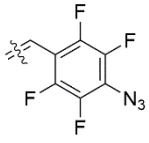	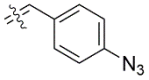
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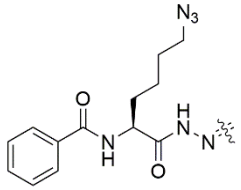				
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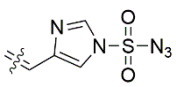	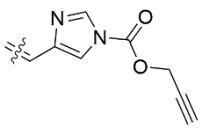	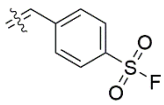	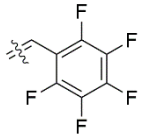	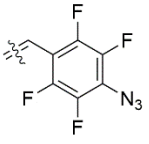	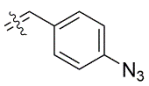
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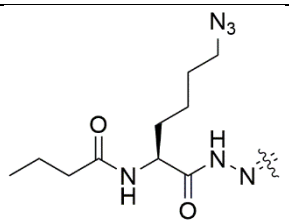
<tr><td colspan="6"> 
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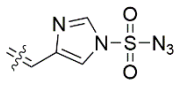
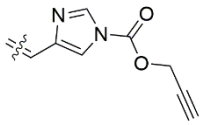
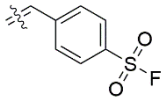
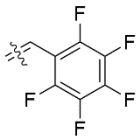
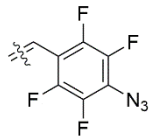
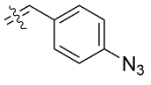
<tr><td>  </td><td>  </td><td>  </td><td>  </td><td>  </td><td>  </td></tr>
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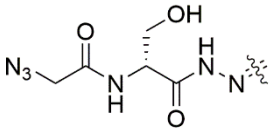
<tr><td colspan="6"> 
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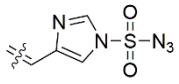
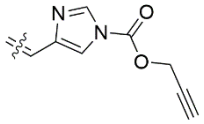
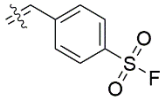
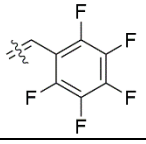
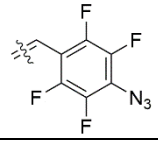
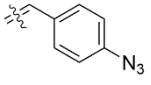
<tr><td>  </td><td>  </td><td>  </td><td>  </td><td>  </td><td>  </td></tr>
<tr><td>L9R1</td><td>L9R2</td><td>L9R5</td><td>L9R7</td><td>L9R8</td><td>L9R9</td></tr>
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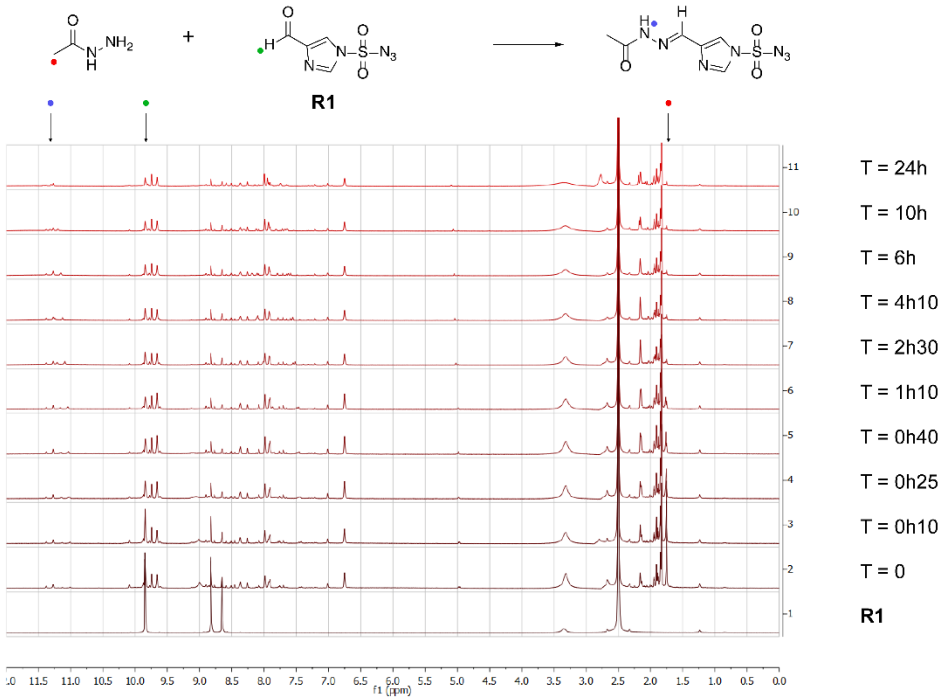
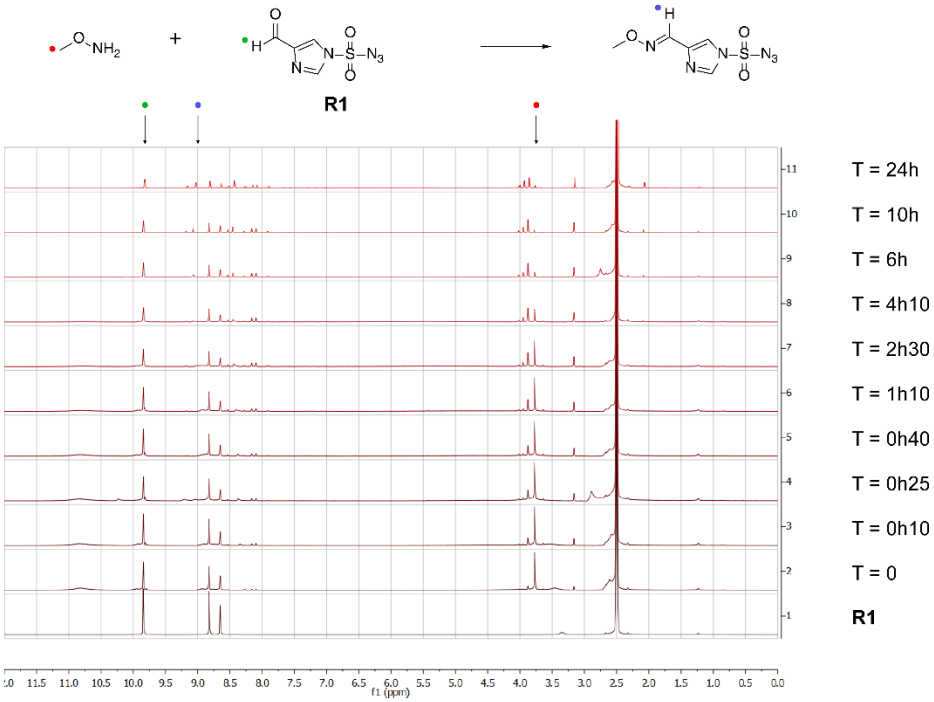
Figure S1. ^{1}H-NMR analysis of probe formation

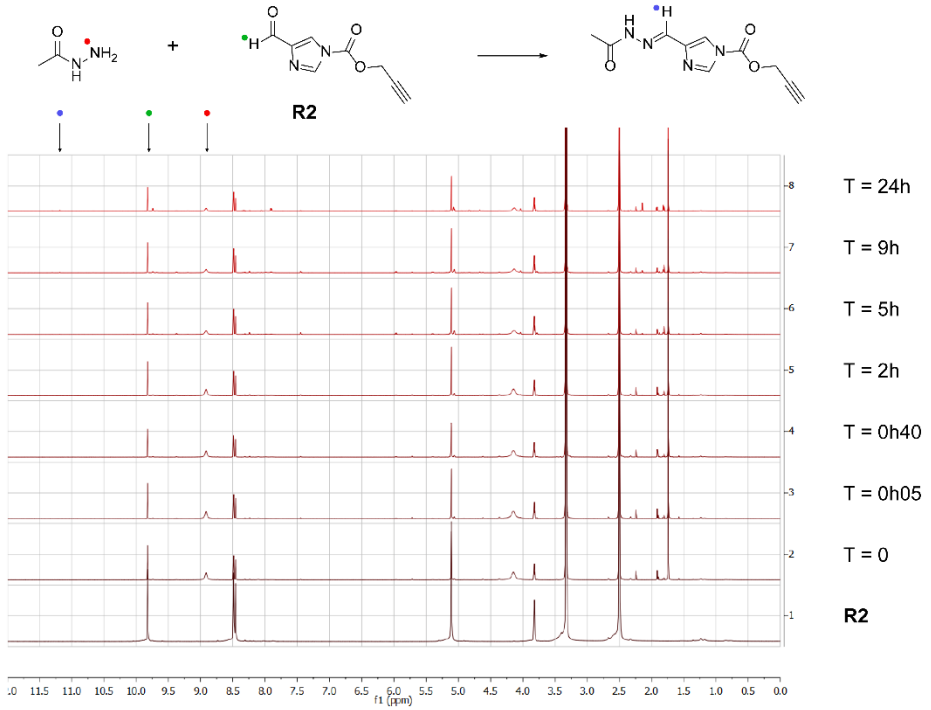
Hydrazone formation of **R1** (400 MHz, DMSO-d_6)



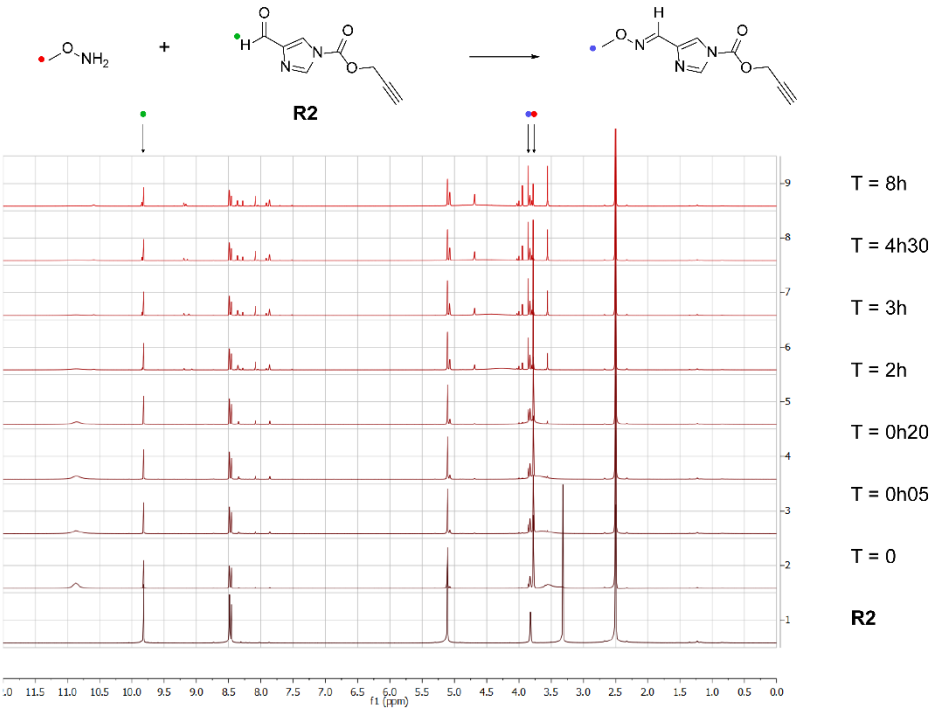
Oxime formation of **R1** (400 MHz, DMSO-d_6)



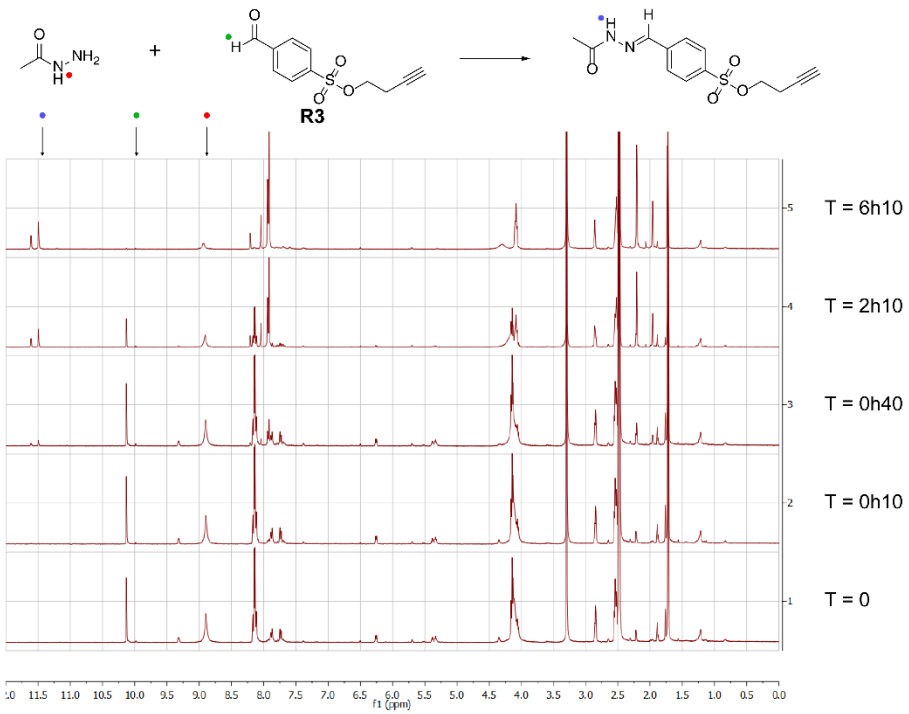
Hydrazone formation of **R2** (400 MHz, DMSO-d_6)



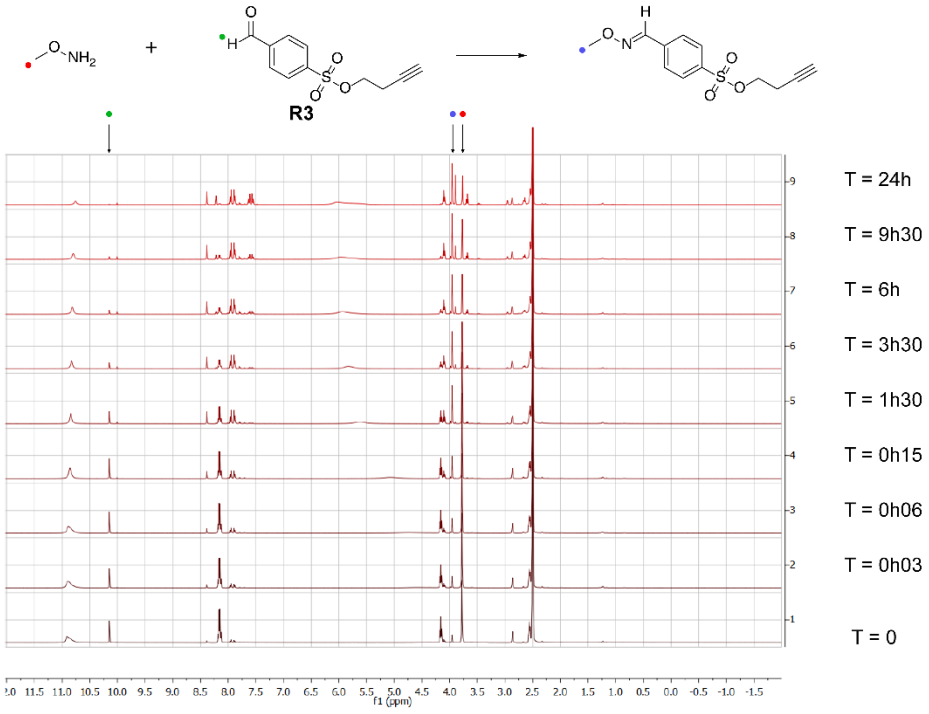
Oxime formation of **R2** (400 MHz, DMSO-d_6)



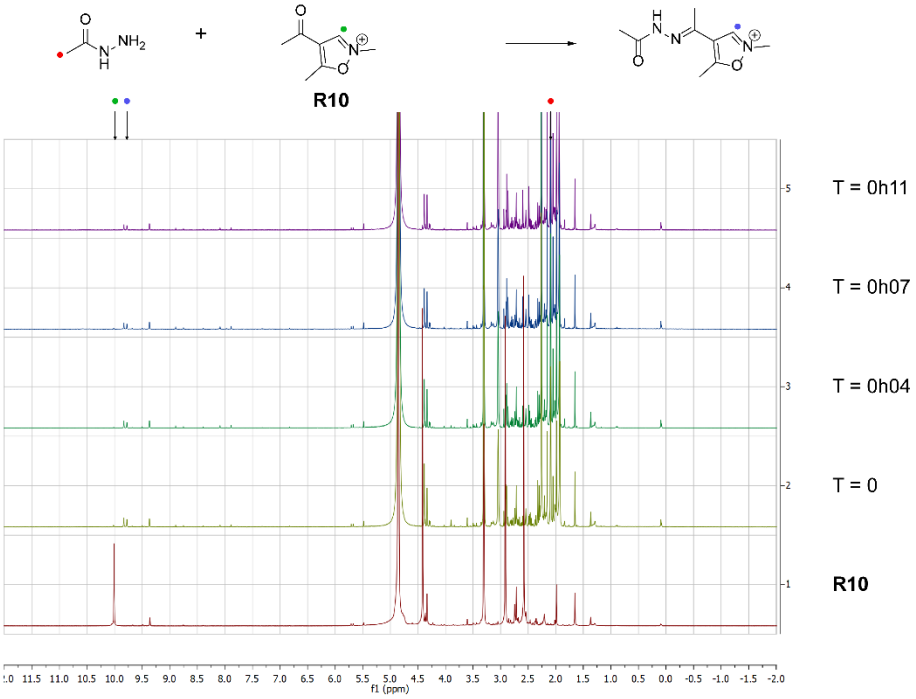
Hydrazone formation of **R3** (400 MHz, DMSO-d_6)



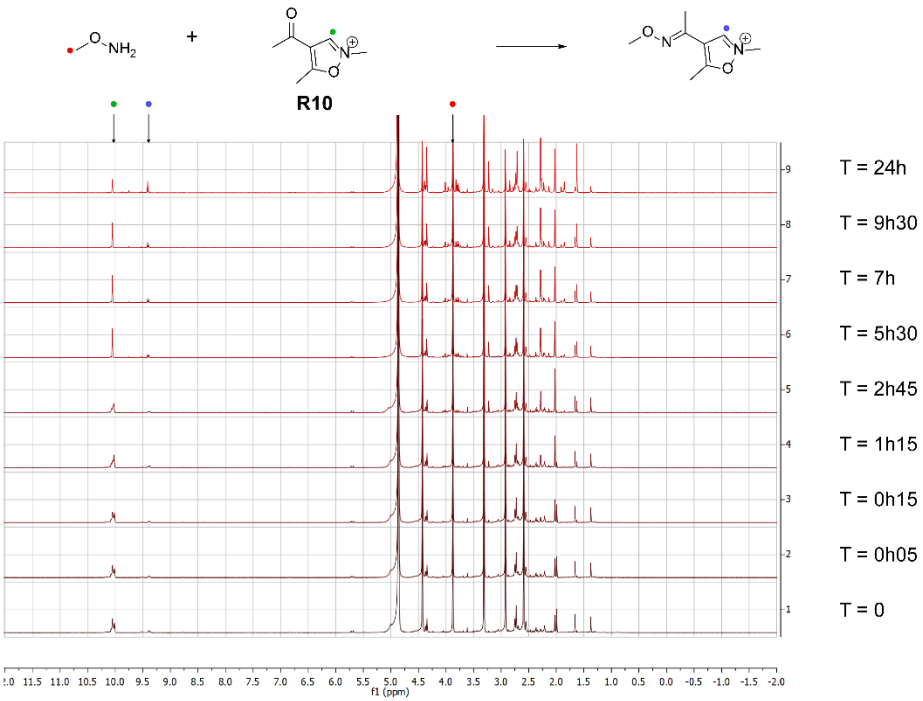
Oxime formation of **R3** (400 MHz, DMSO-d_6)



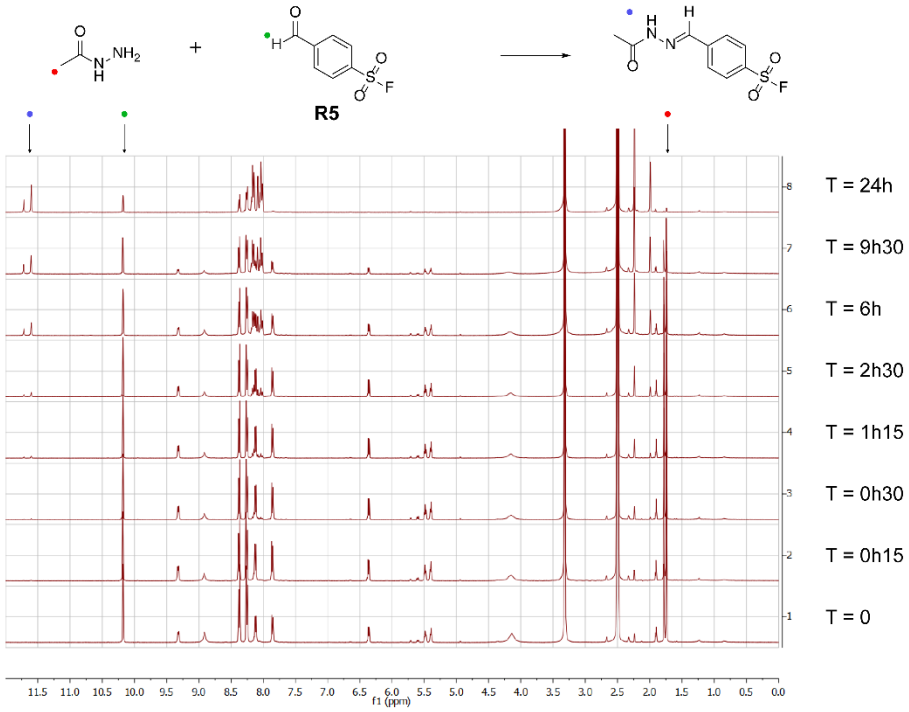
Hydrazone formation of **R10**, a model compound of **R4** (400 MHz, CD_3OD)



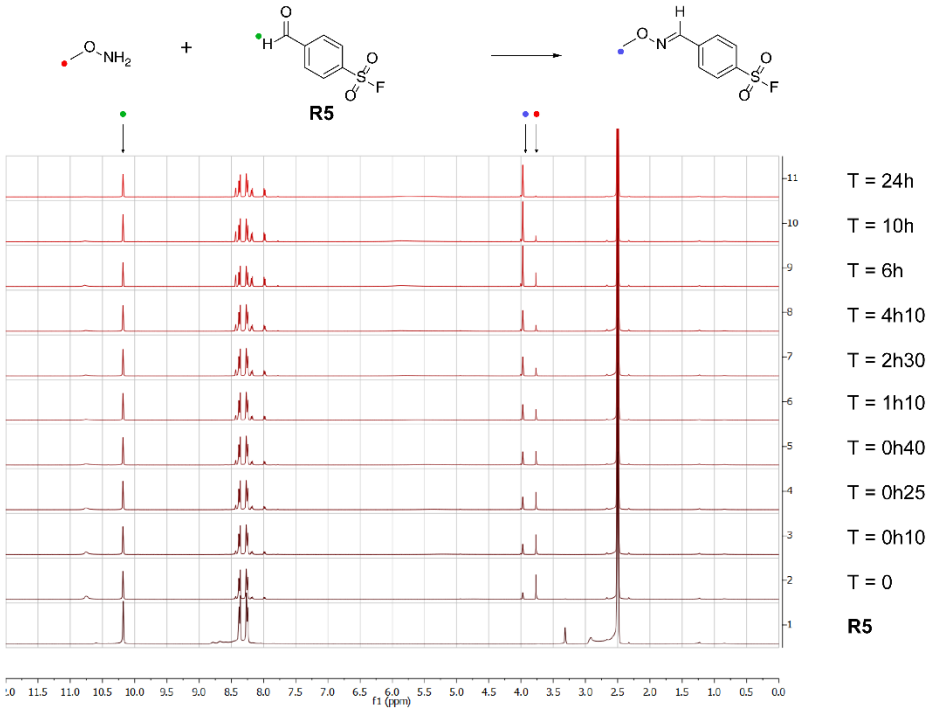
Oxime formation of **R10**, a model compound of **R4** (400 MHz, CD_3OD)



Hydrazone formation of **R5** (400 MHz, DMSO-d_6)



Oxime formation of **R5** (400 MHz, DMSO-d_6)



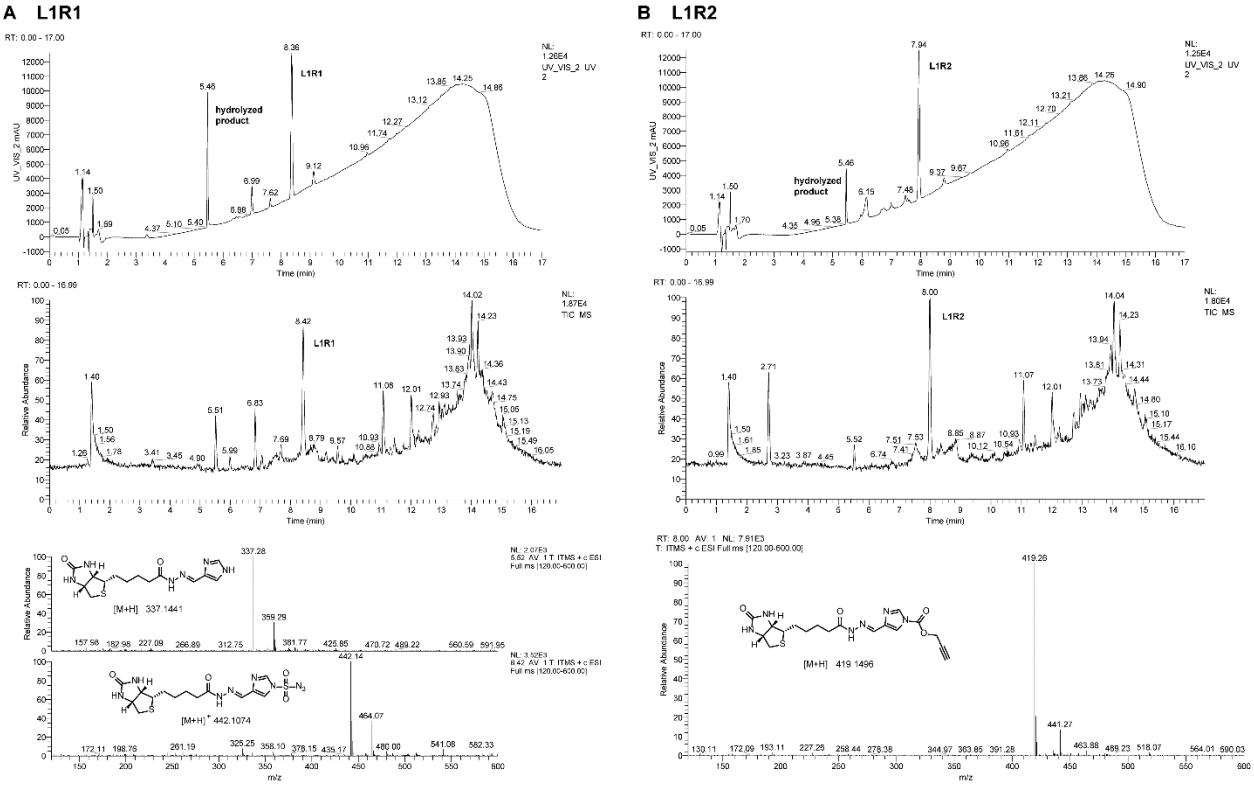


Figure S2. Hydrazone formation of L1 with R1 or R2 analyzed by UPLC-MS. UPLC-MS analysis of the hydrazone probe formation showed the desired products were formed, as well as some hydrolysis of the reactive groups.

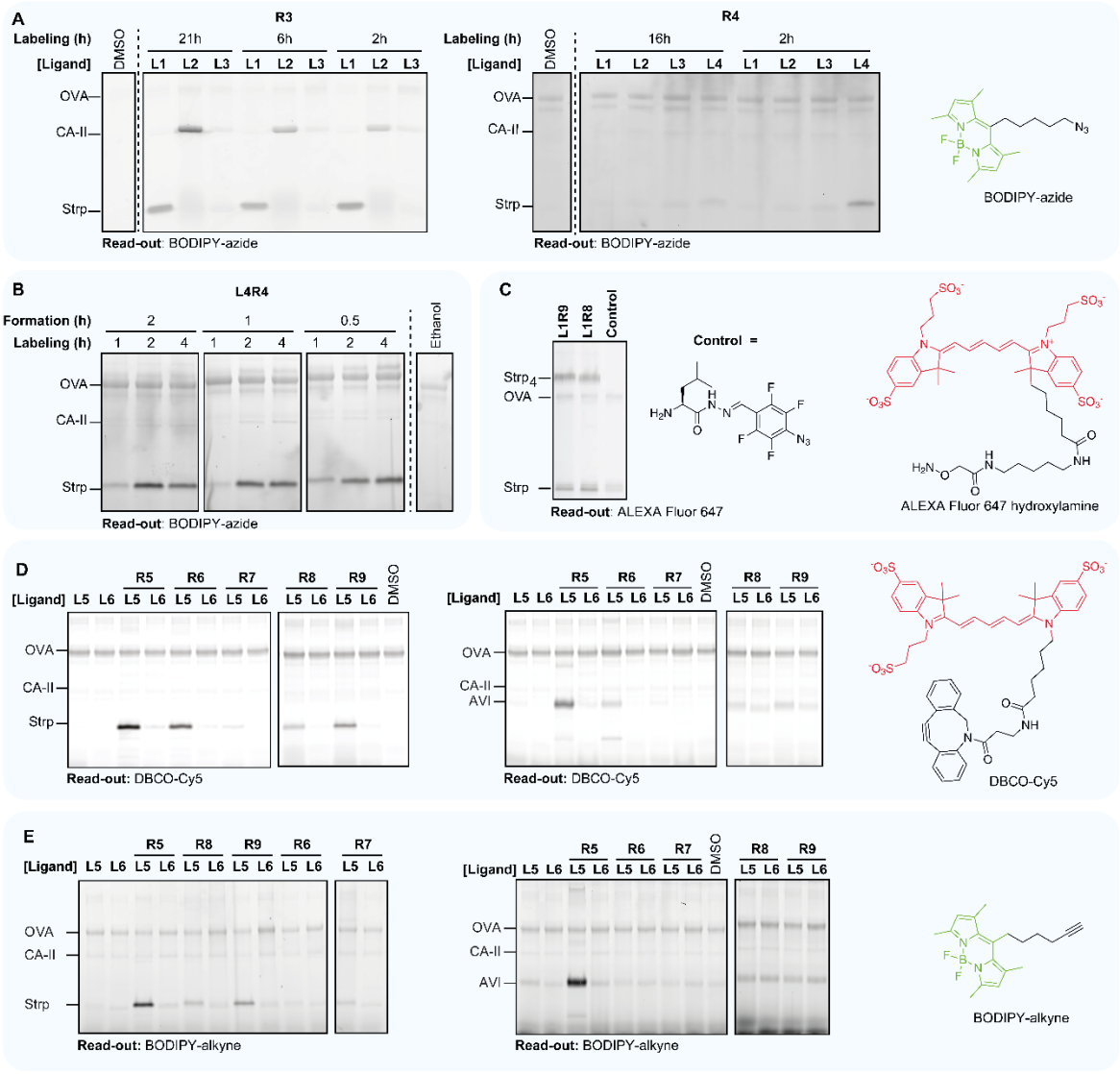


Figure S3. Labeling of the model proteins with *in situ* generated probes. (A) Optimization of the labeling and probe formation time with **L1-L4** with sulfonate **R3** (left panel) and Woodward's Reagent K **R4** (right panel) using BODIPY-azide as read-out. For the sulfonate, labeling seems to saturate after 16 h. For Woodward's Reagent K **R4**, only with the oxime probe **L4R4** some labeling was observed when probe formation was shortened to 2h. Labeling with the other probes was not successful. (B) Further optimization of the probe formation time and labeling time for probes containing Woodward's Reagent K **R4**. (C) Labeling attempt with **R8-R9** using imine exchange with ALEXA fluor 647 hydroxylamine as read-out. (D) Labeling of streptavidin (left panels) and avidin (right panels) with probes prepared from ligands **L5-L6** and reactive groups **R5-R9** using DBCO-Cy5 as fluorescent read-out. (E) Labeling of streptavidin (left

panels) and avidin (right panels) with probes prepared from ligands **L5-L6** and reactive groups **R5-R9** using BODIPY-alkyne as fluorescent read-out.

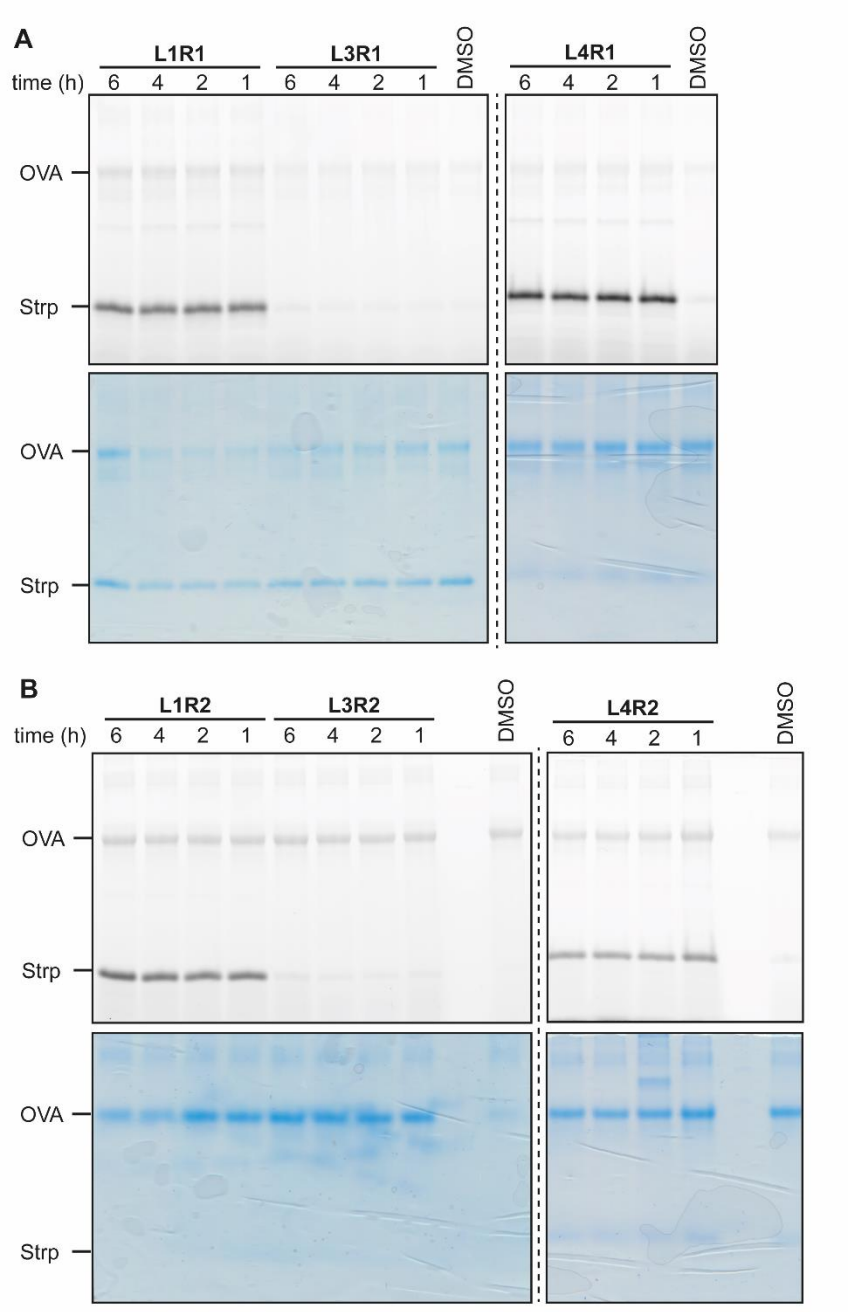


Figure S4. Time-dependent labeling of Streptavidin with *in situ* formed diazotransfer probes (A) and acyl imidazole probes (B). Ligands **L1, L3** and **L4** were preincubated with the reactive groups overnight, diluted with DMSO and added to the protein mixture. The samples were incubated for the indicated time, after which the reaction was quenched by heat-denaturation in the presence of SDS (1%). The proteins were visualized with BODIPY-alkyne (A) and BODIPY-azide (B).

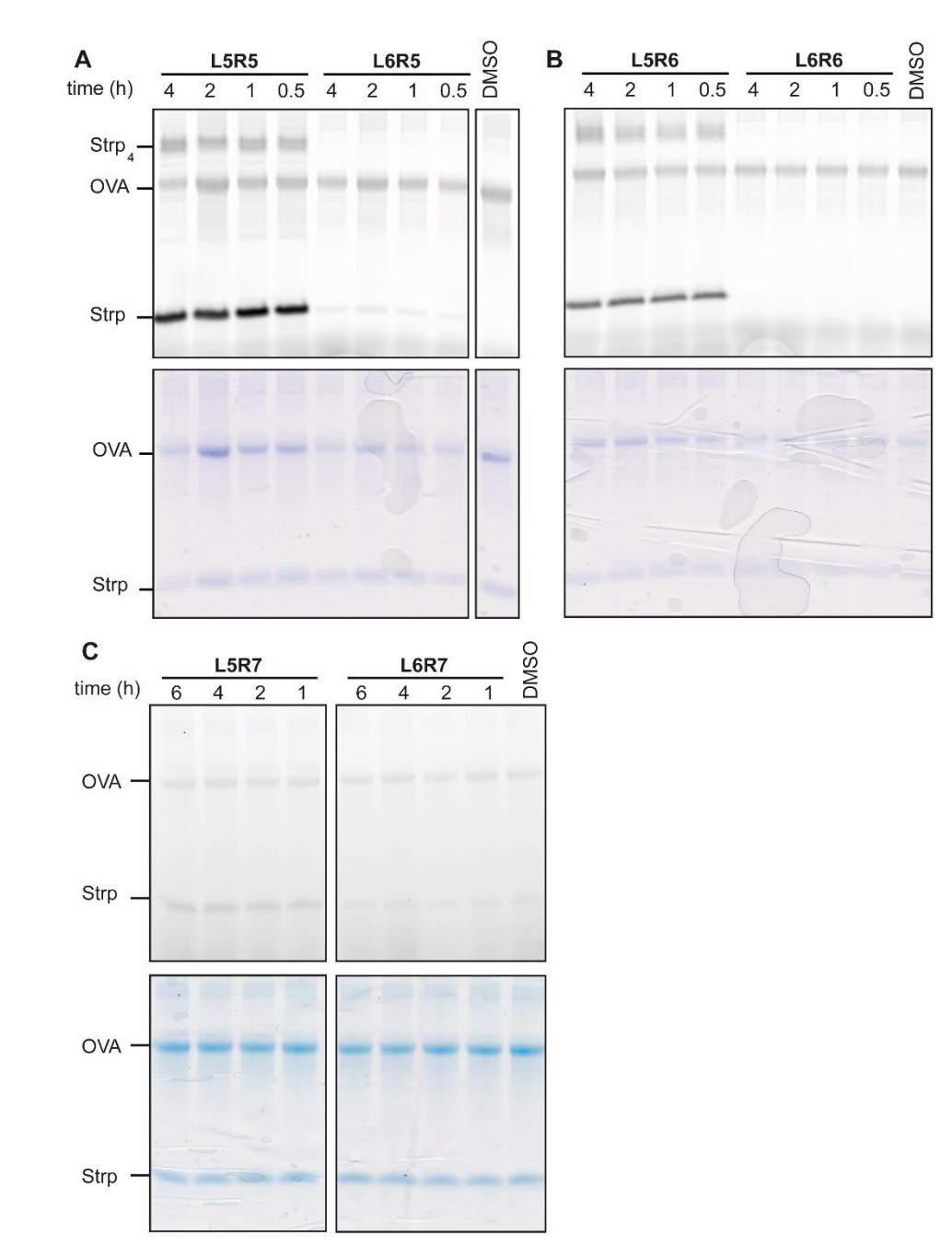


Figure S5. Time-dependent labeling of streptavidin with *in situ* formed sulfonylfluoride R5 (A), nitrobenzylfluoride R6 (B) and pentafluorobenzaldehyde R7 (C). Ligand **L5** and ligand **L6** were preincubated with the reactive groups overnight, diluted with DMSO and added to the protein mixture. The samples were incubated for the indicated time, after which the reaction was quenched by heat-denaturation in the presence of SDS (1%). The proteins were visualized with DBCO-Cy5 (for A and B) and BODIPY-alkyne (for C).

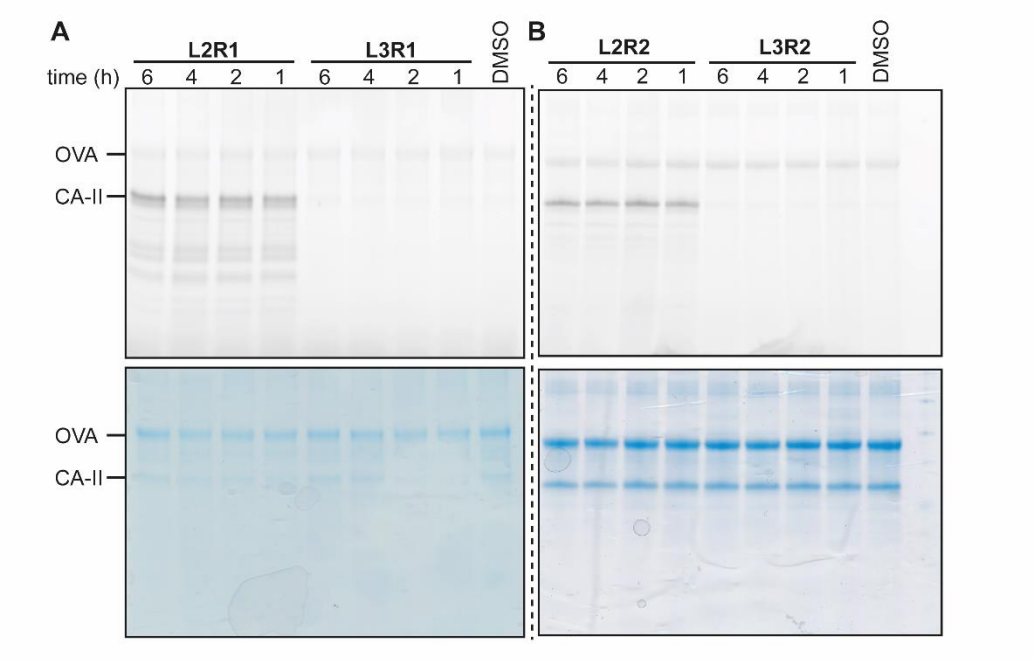


Figure S6. Time-dependent labeling of CA-II with *in situ* formed diazotransfer probes (A) and acyl imidazole probes (B). Ligands **L2** and **L3** were preincubated with the reactive groups overnight, diluted with DMSO and added to the protein mixture. The samples were incubated for the indicated time, after which the reaction was quenched by heat-denaturation in the presence of SDS (1%). The proteins were visualized with BODIPY-alkyne (A) and BODIPY-azide (B).

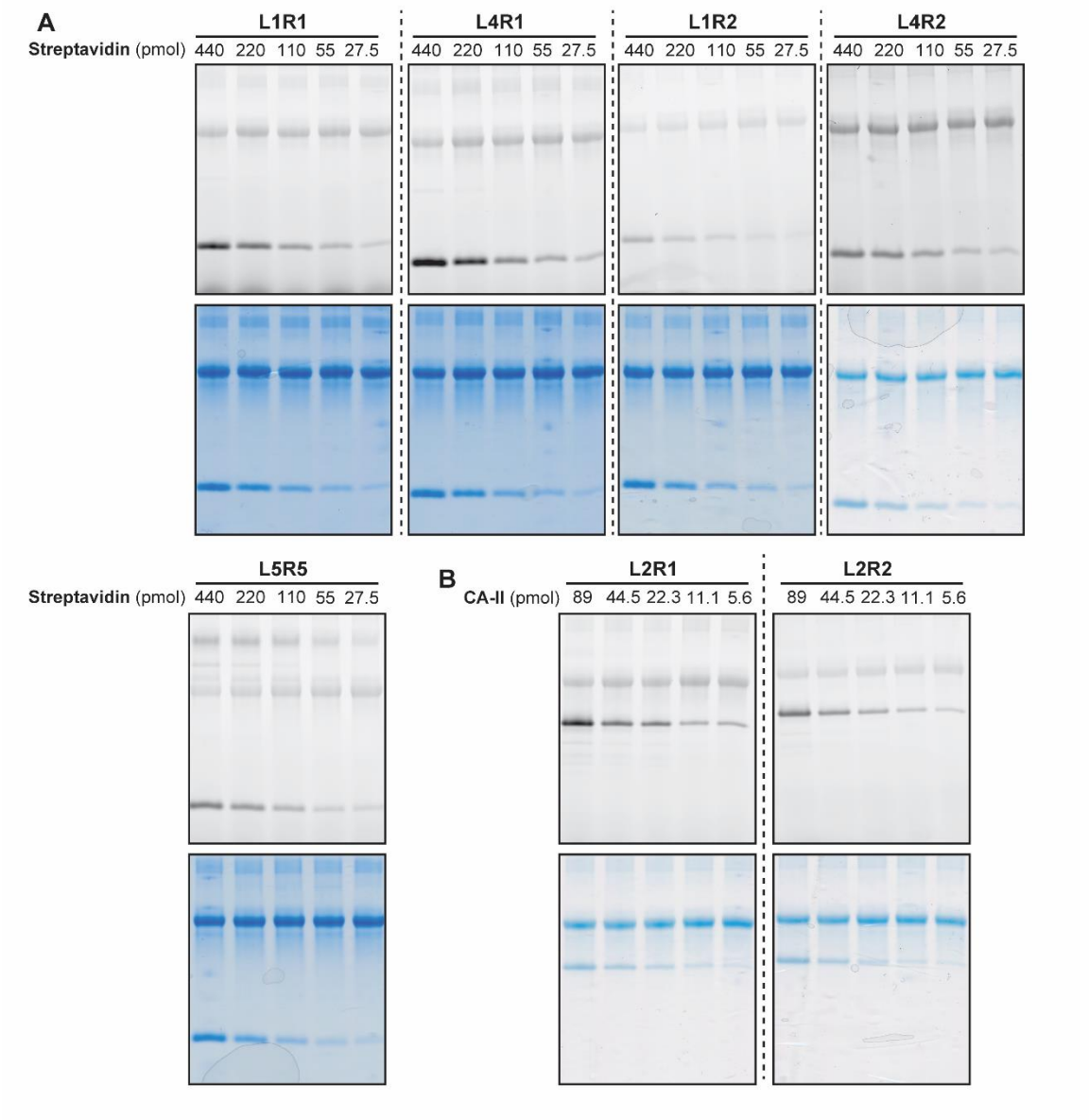


Figure S7. Effect of the target protein concentration on the labeling selectivity of the *in situ* prepared probes. Decreasing amounts of streptavidin (A) or CA-II (B) were incubated with the indicated probe for 2 h, after which the labeled proteins were visualized by BODIPY-alkyne or BODIPY-azide. The labeling experiments in (A) were resolved on different gels, which explains the slight variations in the height of the protein

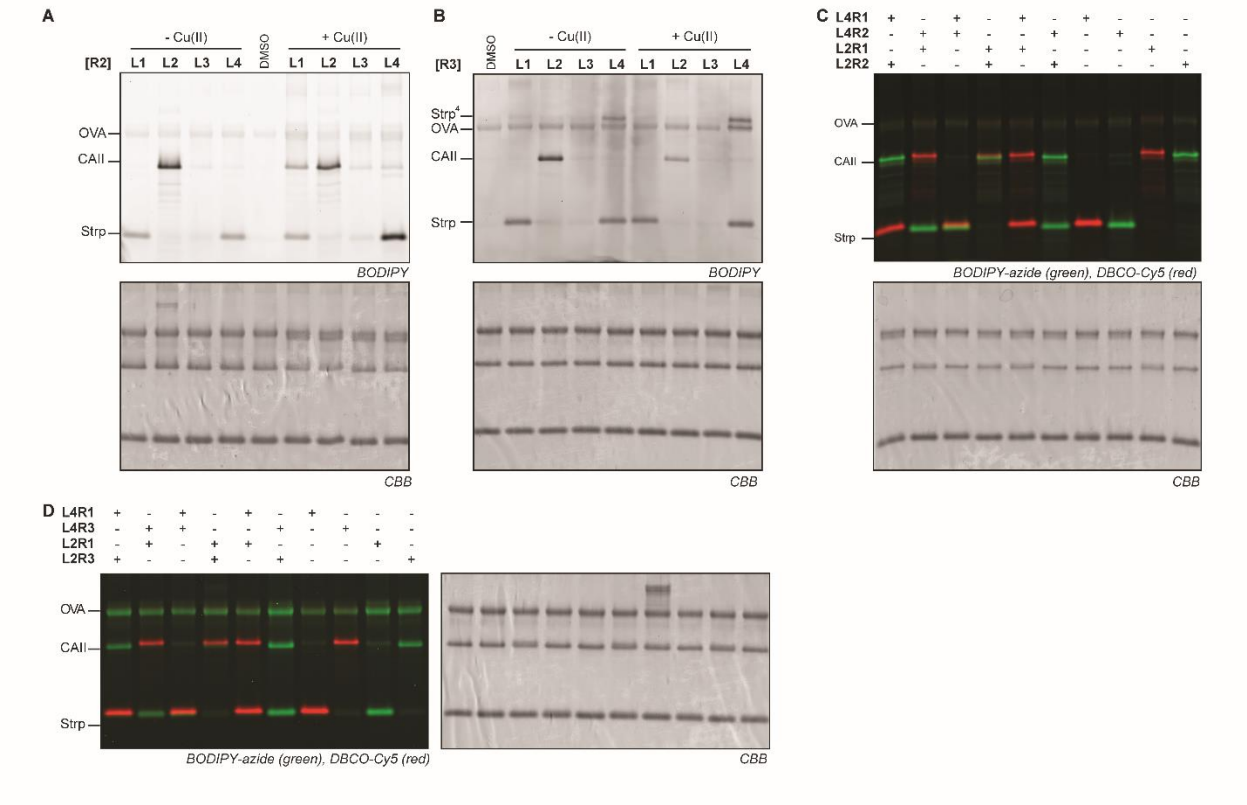


Figure S8. Dual labeling of streptavidin and CA-II. (A) Effect of Cu^{2+} on the labeling efficiency of acyl imidazole **R2** probes. (B) Effect of Cu^{2+} on the labeling efficiency of sulfonate **R3** probes. (C) and (D) Dual labeling of protein mixture with *in situ* prepared diazotransfer probes **L4R1** and **L2R1** and acyl imidazole probes **L4R2** and **L2R2** (C) or sulfonate probes **L4R3** and **L2R3** (D) reveal that exchange of the reactive group during the labeling step is minimal. The protein mixture was simultaneously incubated with the preformed probes. By sequential SPAAC and CuAAC reactions with DBCO-Cy5 and BODIPY-azide, a red and a green fluorophore were appended onto the proteins that reacted with the diazotransfer-based reagents and the acyl imidazole or sulfonate-based reagents respectively. As is clear from the scans, the probes only react with their target proteins. By altering the ligand-group combinations, we could reverse the labeling pattern, perform dual labeling of a single protein or label both proteins with the same bioorthogonal handle.

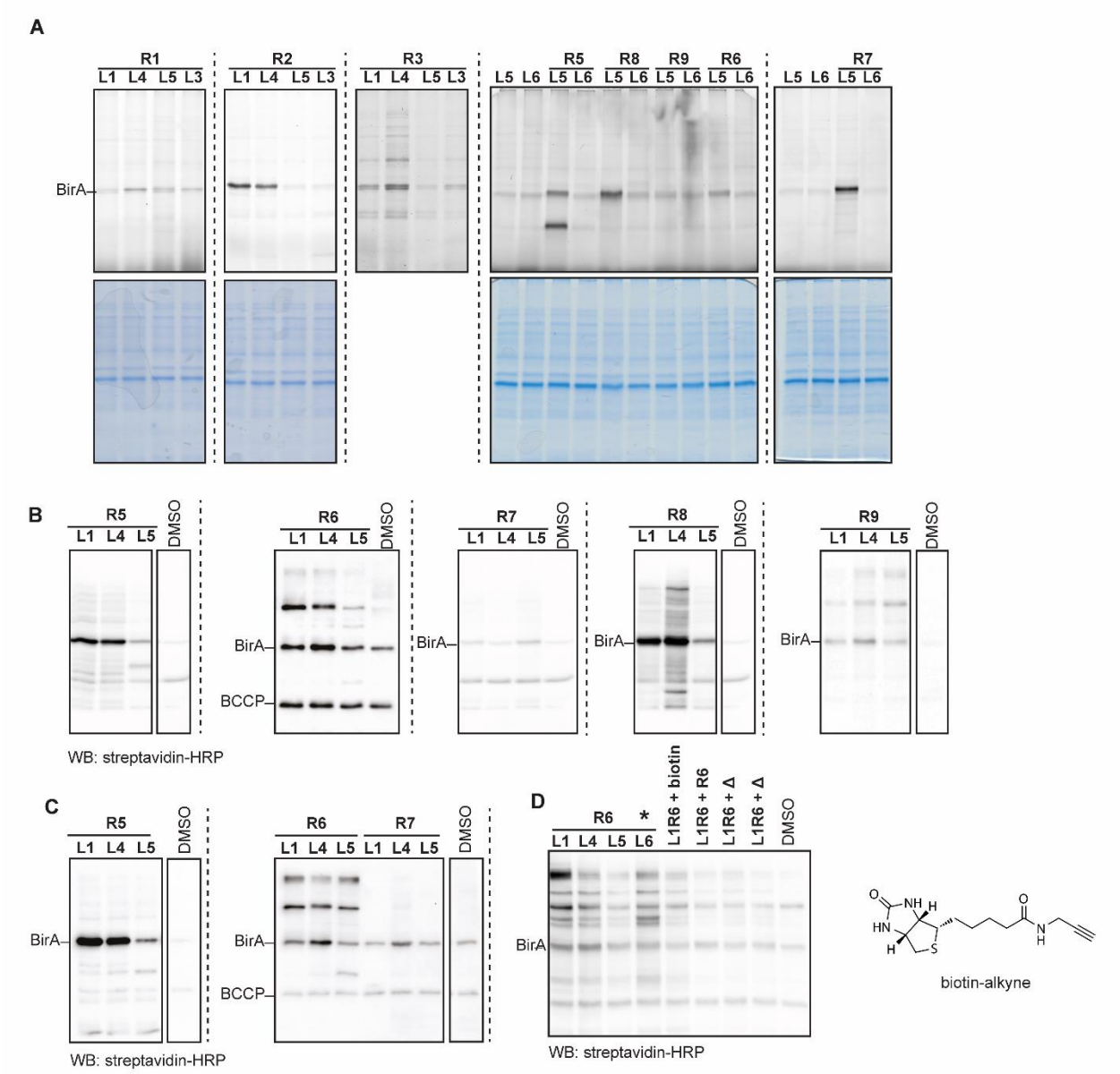
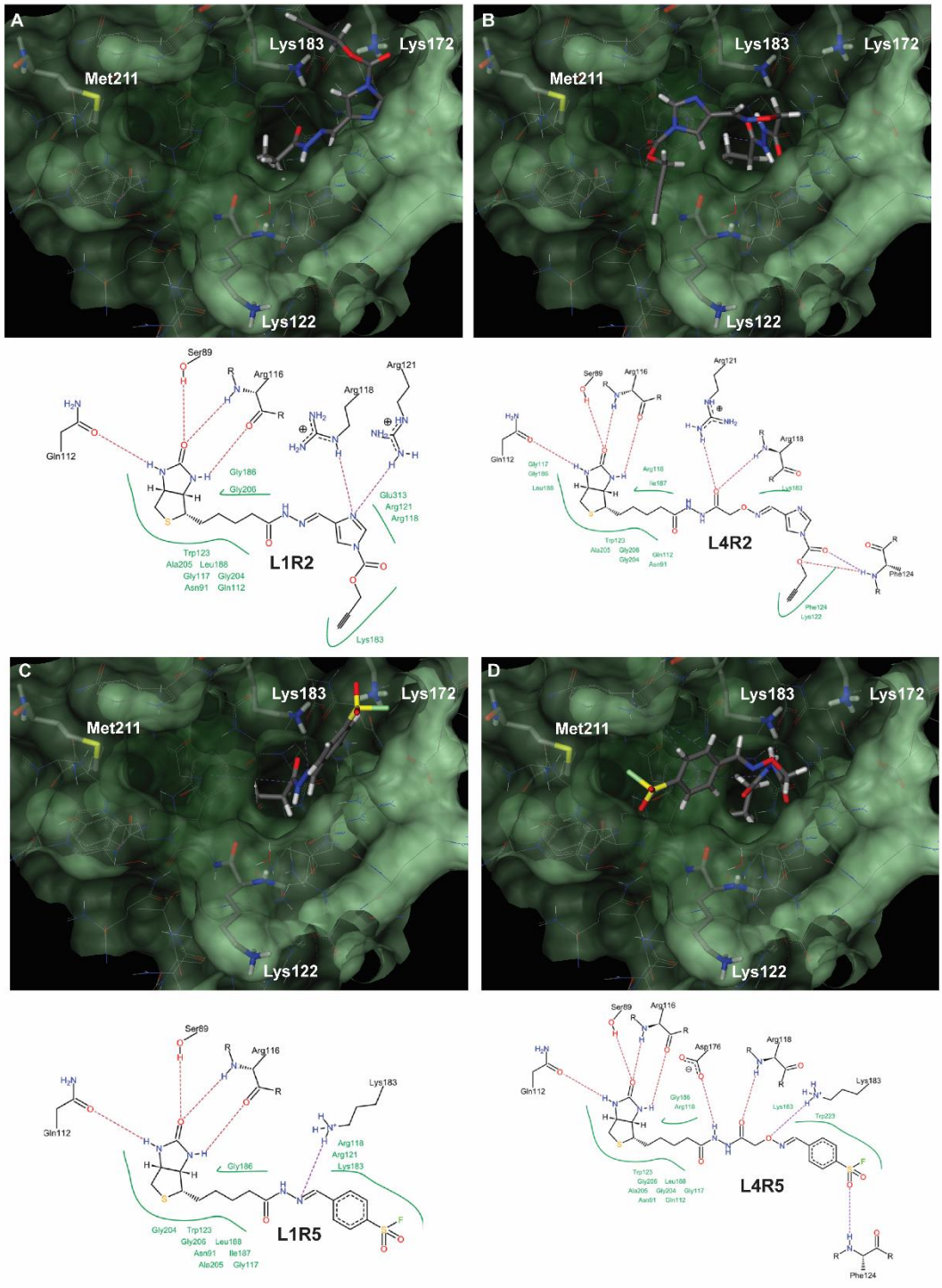


Figure S9. Labeling with the probe library in cell lysate of BirA-overexpressing *E. coli.* (A) In-gel fluorescence analysis of labeling of BirA by probes containing reactive groups **R1**, **R2**, **R3**, **R6**-**R9**. The readout moiety is BODIPY-azide or BODIPY-alkyne. (B,C) Western Blot analysis of lysate incubated with probes based on **R5-R9** for 2 h (B) or 6 h (C). (D) Western Blot analysis of labeling by probes containing **R6** shows that these probes are not selective. Control probe **L6R6** was clicked to biotin-alkyne after the labeling reaction. In this control lane (*), the same labeling profile is observed, indicating that the off-targets are due to the reactivity of the reactive group. Competition experiments with biotin and **R6** further confirm this. Structure of biotin-alkyne.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Gly186
Gly206
Glu313
Arg121
Arg118
Trp123
Ala205
Leu188
Gly117
Gly204
Asn91
Gln112
L1R2
Lys183
L4R2
C
Lys183
Lys172
Met211
Lys122
D
Lys183
Lys172
Met211
Lys122
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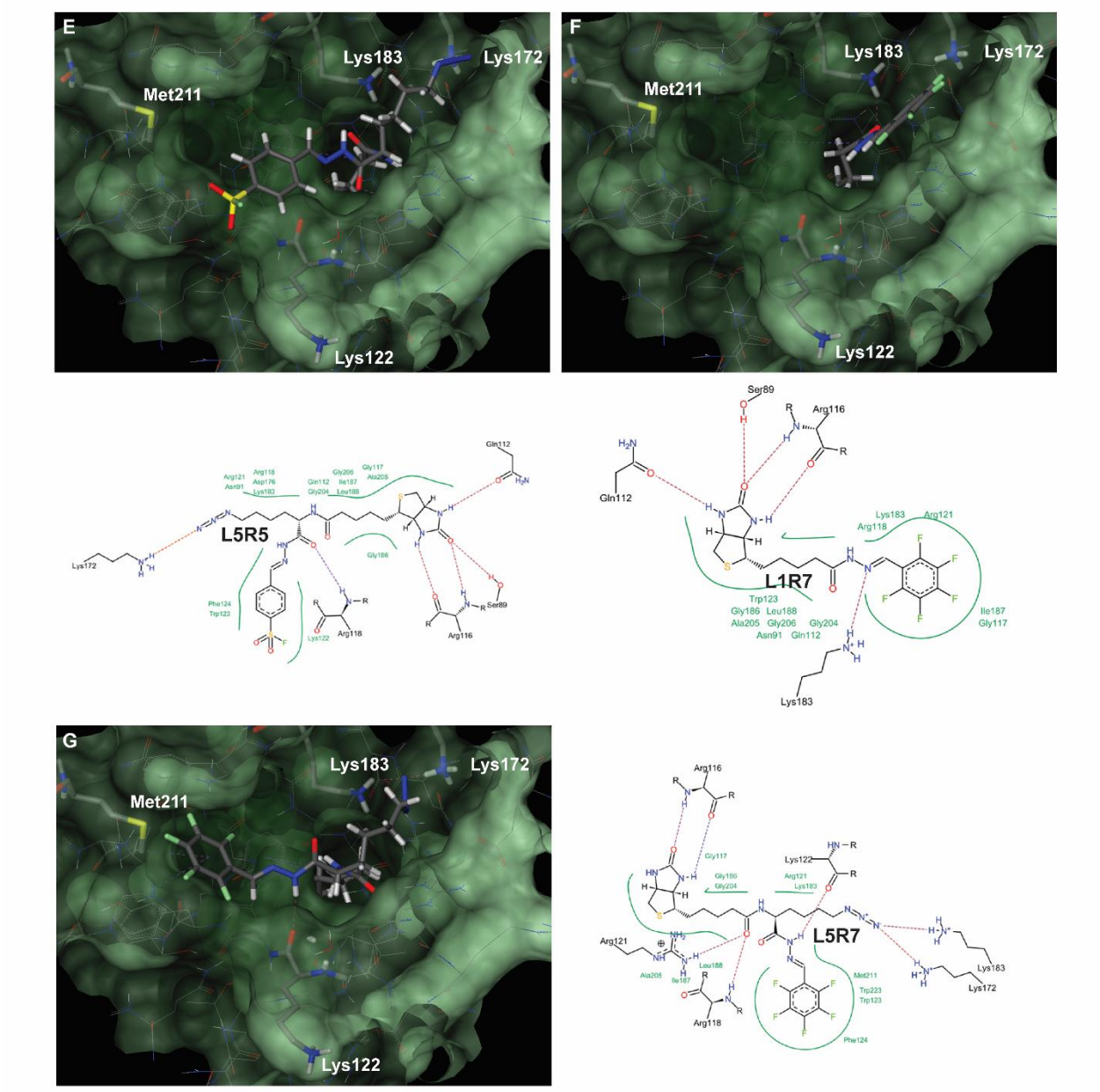


Figure S10. Molecular docking of the probes in BirA. The probes were docked in the crystal structure of BirA (PDB ID: 4WF2) using LeadIT version 2.3.2; BioSolveIT GmbH, Sankt Augustin, Germany, 2017, www.biosolveit.de/LeadIT. In the docking, we used the biotin group as pharmacophore and we restrained the program that at least two out of the four interaction with the pharmacophore should be formed. Representative pose of acyl imidazole **L1R2** (A) and **L4R2** (B), sulfonyl fluoride **L1R5** (C), **L4R5** (D) and **L5R5** (E), and pentafluoroaryl **L1R7** (F) and **L5R7** (G).

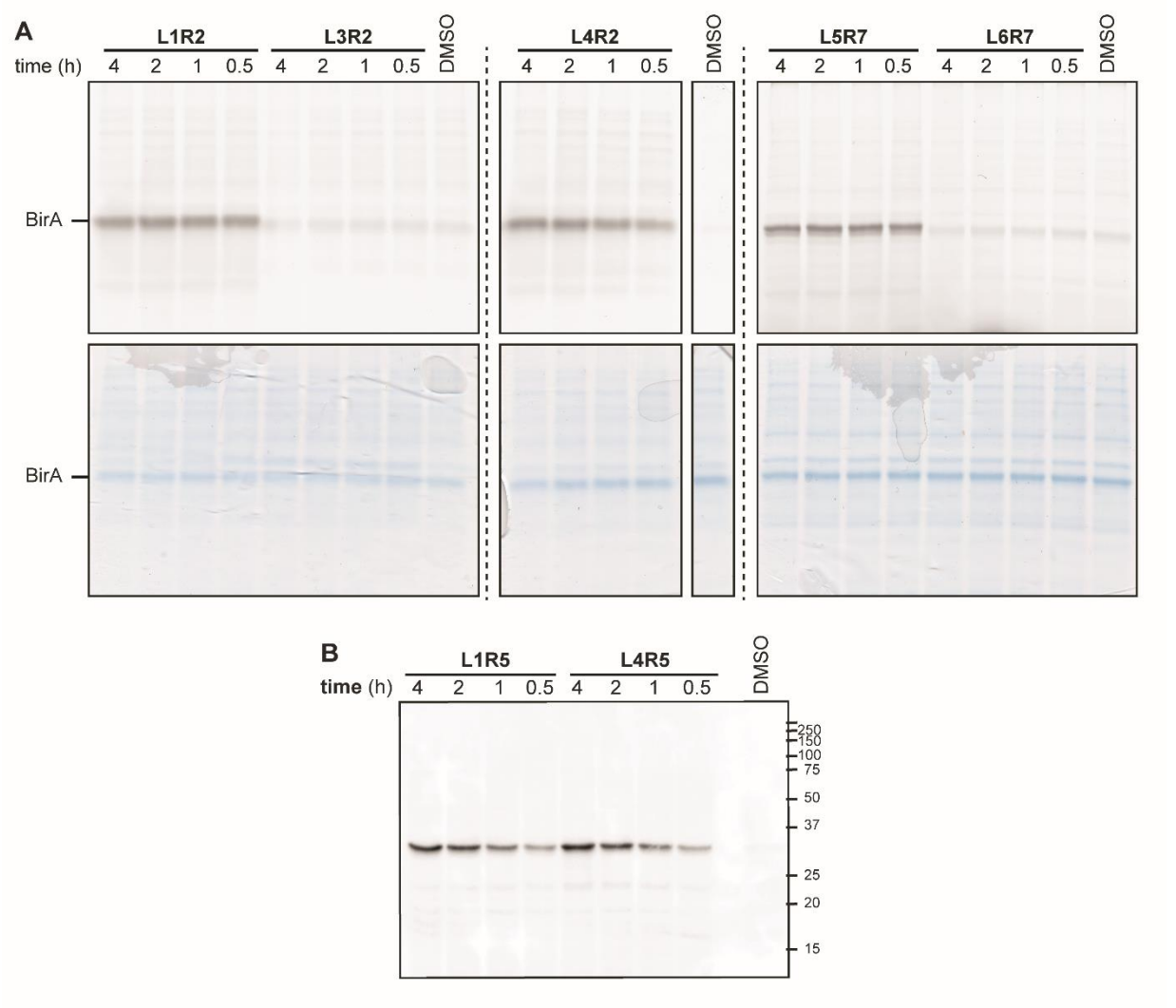


Figure S11. Time-dependent labeling of BirA with probes L1R2, L4R2, L1R5, L4R5 and L5R7, and non-targeted control probes L3R2 and L6R7. Cell lysates of *E. coli* cells overexpressing BirA (8 µL) were incubated with the probes (200 µM, 1 µL) for the indicated time, after which the reaction was quenched by heat-denaturing the protein. Samples that were treated with **L1R2**, **L3R2**, **L4R2**, **L5R7** and **L6R7** were clicked to a BODIPY fluorophore and the labeled proteins were detected by in-gel fluorescence scanning (A). Samples that were incubated with **L1R5** and **L4R5** were directly resolved on an SDS-PAGE and transferred to a PVDF membrane by electroblotting. The labeled proteins were detected using streptavidin-horseradish peroxidase.

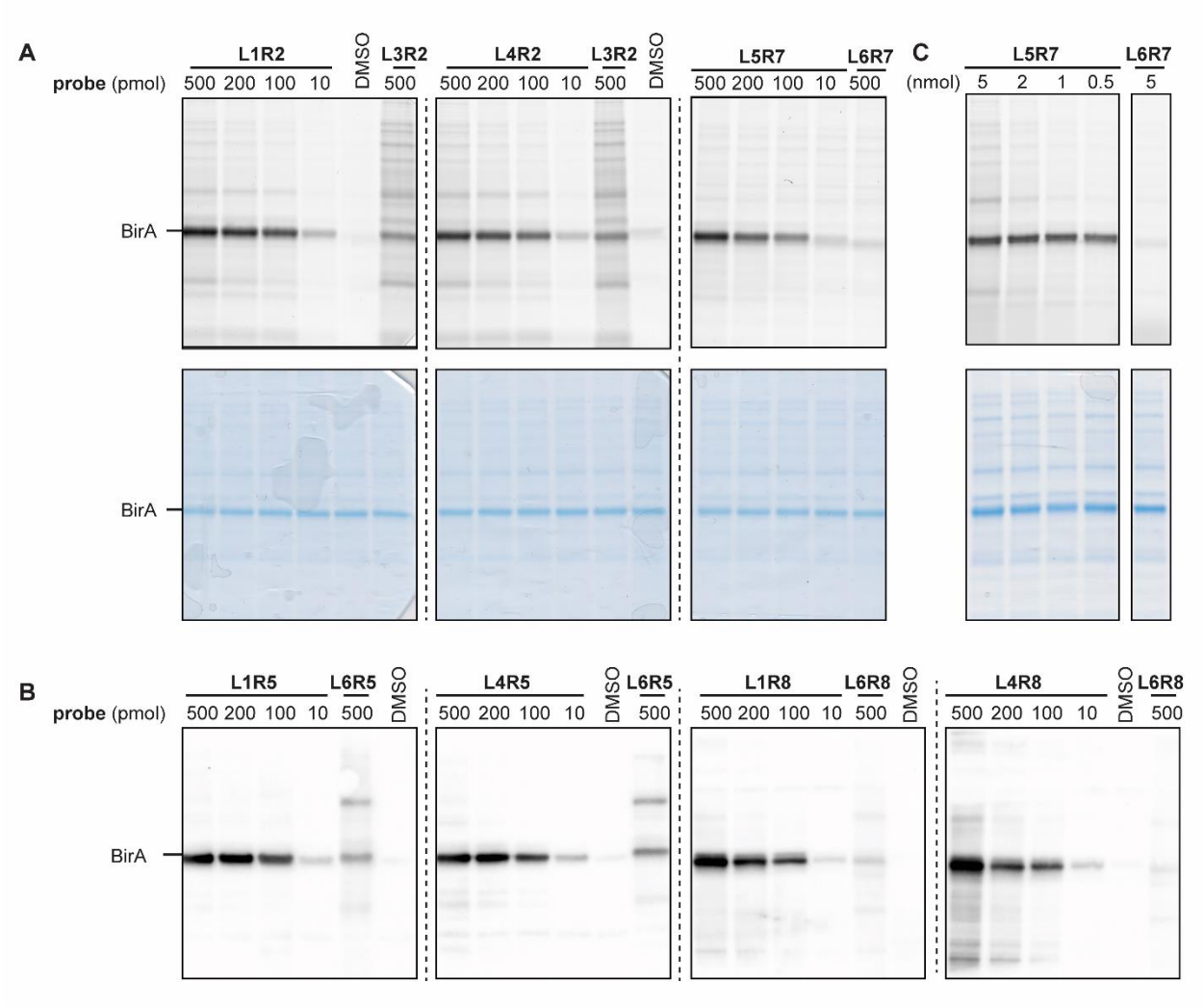


Figure S12. Concentration dependent labeling of BirA. To lysate of *E. coli* overexpressing BirA (8 µL) was added with the indicated amount of probe (1 µL), resulting in a total volume of 9 µL. The mixture was incubated for 2 h, after which the reaction was quenched with SDS. (A) To visualize the labeled proteins, a BODIPY reporter group was clicked to the bioorthogonal handle. The proteins were resolved on a 12% SDS-PAGE and visualized by fluorescence scanning. (B) To visualize the labeling of BirA by sulfonylfluoride probes **L1R5** and **L4R5**, and photocrosslinker probes **L1R8** and **L4R8**, the protein mixture was after the labeling reaction directly subjected to gel electrophoresis on a 12% SDS-PAGE. The proteins were transferred to a PVDF membrane by electroblotting and visualized with Strp-HRP and ECL+. The final concentrations of probe in the reaction mixture ranged from 1.11 µM (lowest concentration) to 55.5 µM (highest concentration). (C) Labeling of BirA with pentafluoroaryl probe **L5R7** at higher concentrations (55.5 µM to 555.5 µM). The labeled proteins were visualized as described for panel (A).

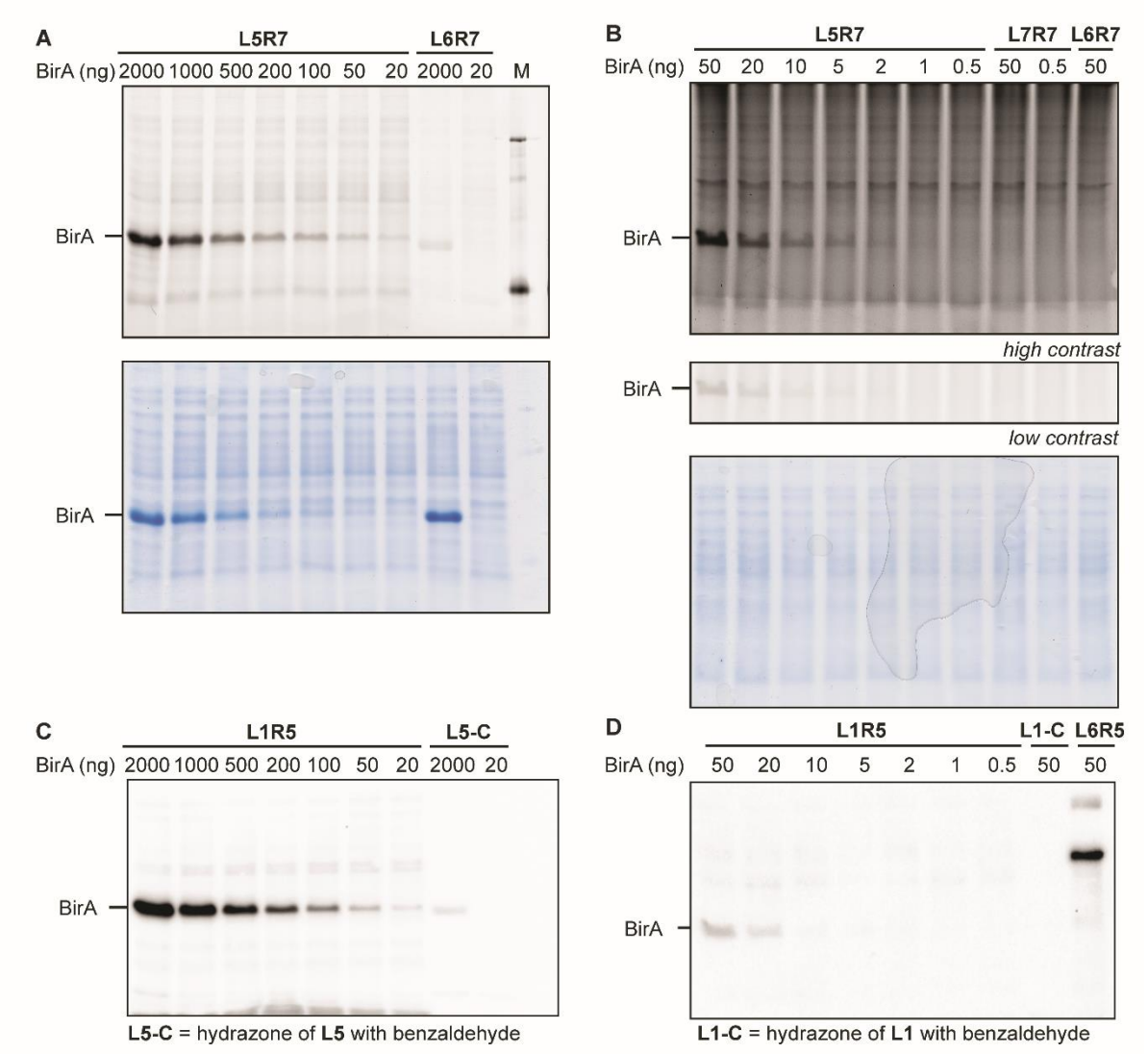


Figure S13. Detection limit for BirA of **L5R7** (A,B) and **L1R5** (C,D). (A,B) The indicated amount of BirA was added to *E. coli* lysate of BL21-CodonPlus (DE3)-RIL (8 µL) followed by the addition of **L5R7** (2 mM, 1 µL (A) or 200 µM, 1 µL (B)). After 2 hours, the proteins were denatured and clicked to BODIPY alkyne. The proteins were resolved on a 12% SDS-PAGE and visualized by fluorescence scanning. (C,D) The indicated amount of BirA was added to *E. coli* lysate of BL21-CodonPlus (DE3)-RIL (8 µL) followed by the addition of **L1R5** (1 mM, 1 µL (C) or 200 µM, 1 µL (D)). After 2 hours, the proteins were denatured, resolved on a 12% SDS-PAGE and visualized by western blotting.

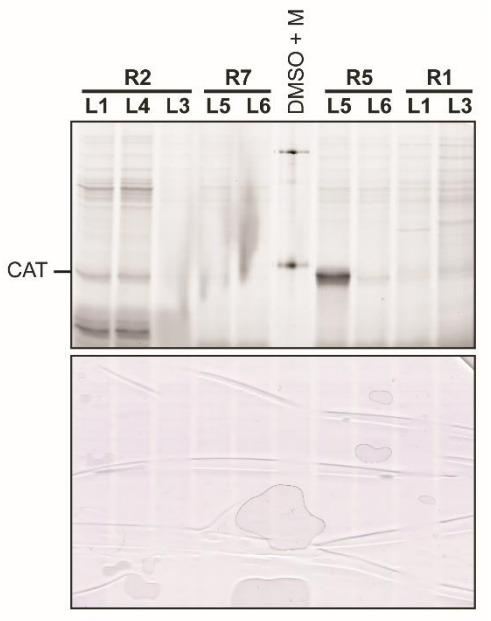


Figure S14. Labeling in lysate *E. coli* of BL21-CodonPlus (DE3)-RIL. Lysate (8 µL) was incubated with the indicated probe (200 µM, 1 µL). After 2 hours, the proteins were denatured and clicked to BODIPY alkyne. The proteins were resolved on a 12% SDS-PAGE and visualized by fluorescence scanning.

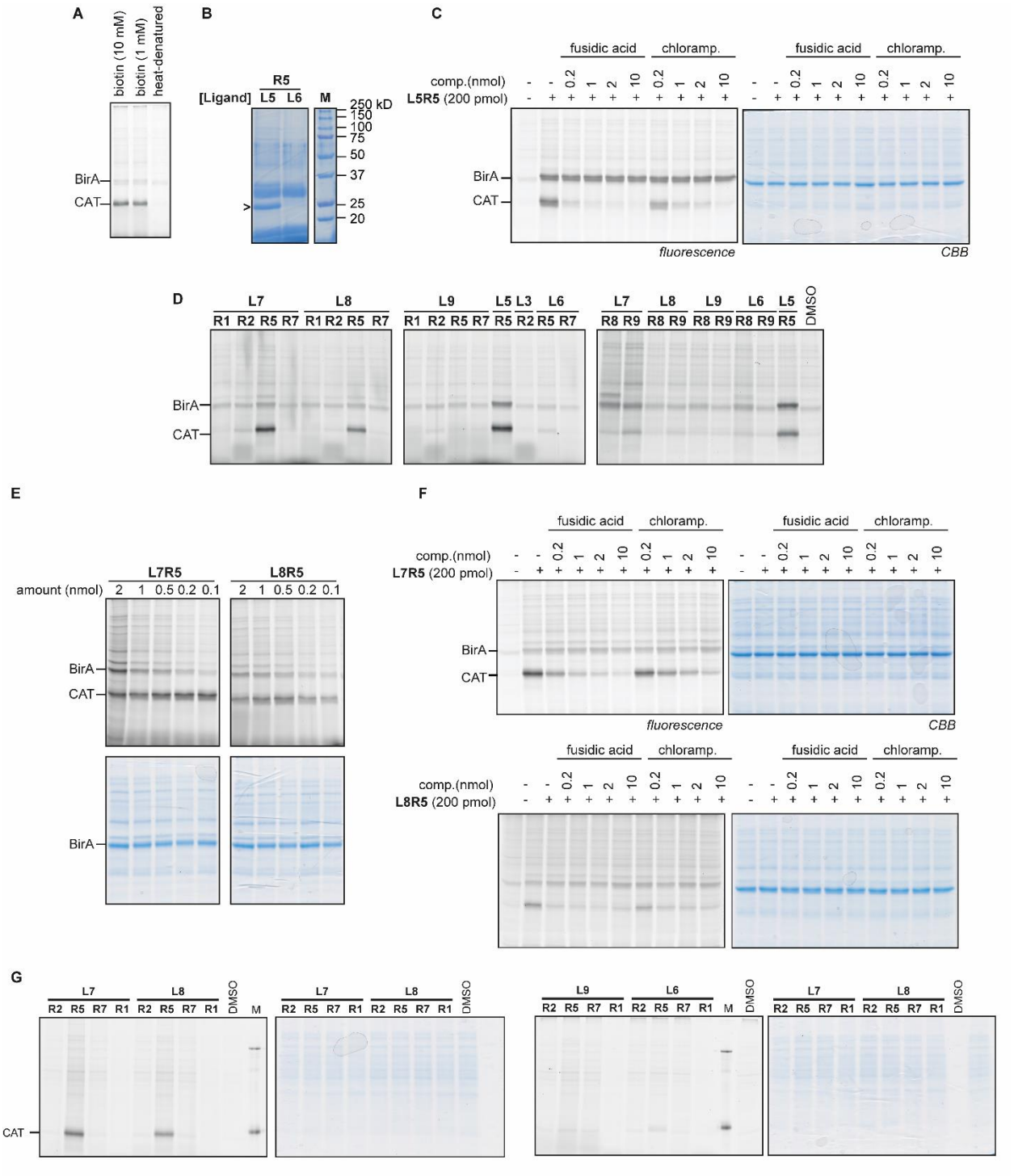
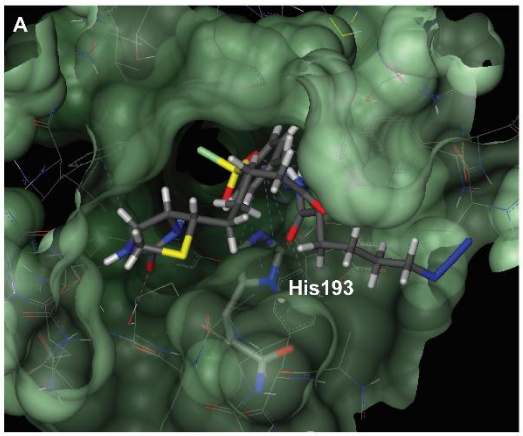


Figure S15. (A) Effect of biotin and heat-denaturing on the labeling CAT by **L5R5**. Upon treating heat denatured lysate with **L5R5**, no labeling of CAT is observed. Thus, the structural integrity of CAT is important for binding of **L5R5**. (B) Coomassie brilliant blue stain of the retrieved proteins in the presence of **L5R5** and **L6R6**. The band indicated with arrow was excised and subjected to MS analysis. (C)

Competition between fusidic acid or chloramphenicol and **L5R5**. (D) Profiling experiment with azidolysine and azidoserine derived hydrazone probes in *E. coli* lysate overexpressing BirA. (E) Concentration dependent labeling of CAT by **L7R5** and **L8R5**. (F) Competition between fusidic acid or chloramphenicol and **L7R5** or **L8R5**. (G) Profiling in native lysate *E. coli* of BL21-CodonPlus (DE3)-RIL. Lysate (8 µL) was incubated with the indicated probe (200 µM, 1 µL). After 2 hours, the proteins were denatured and clicked to BODIPY alkyne. The proteins were resolved on a 12% SDS-PAGE and visualized by fluorescence scanning.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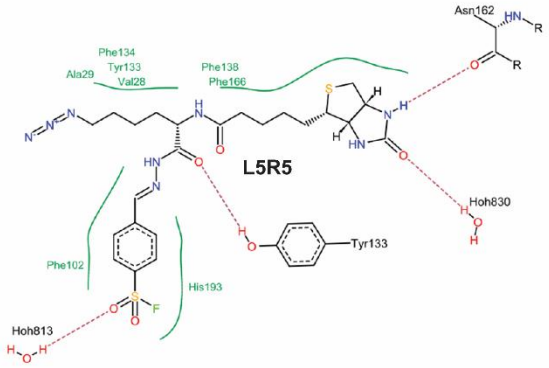
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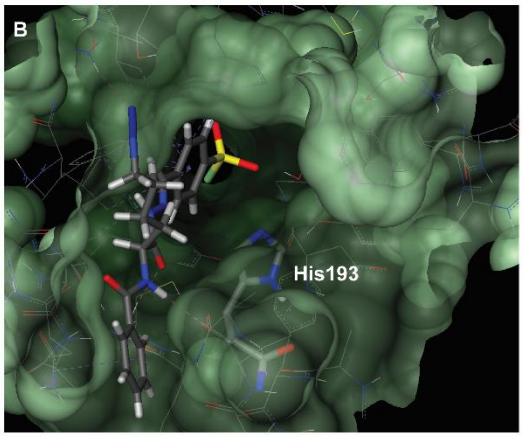
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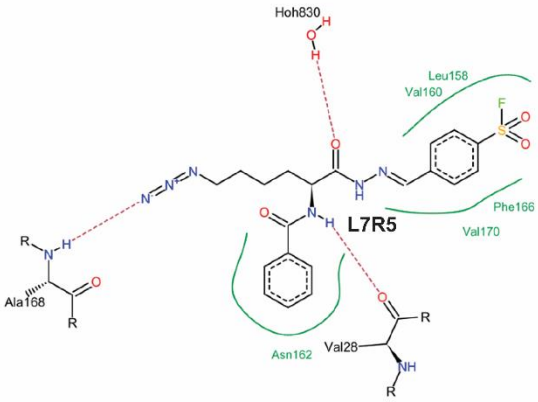
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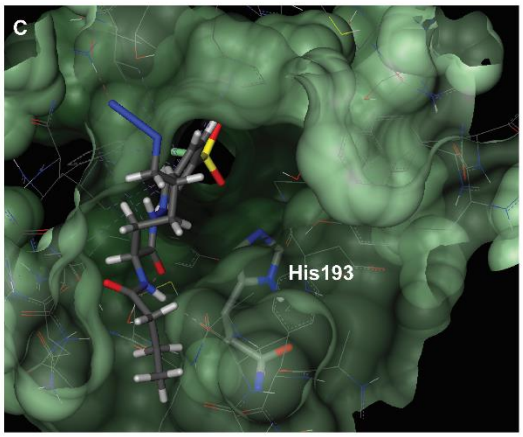
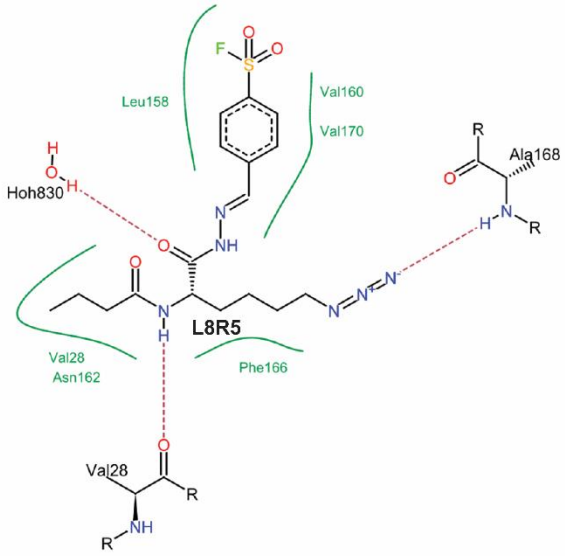
Val170
L7R5
Ala168
Asn162
Val28


C
His193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Val160
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Hoh830
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Val28

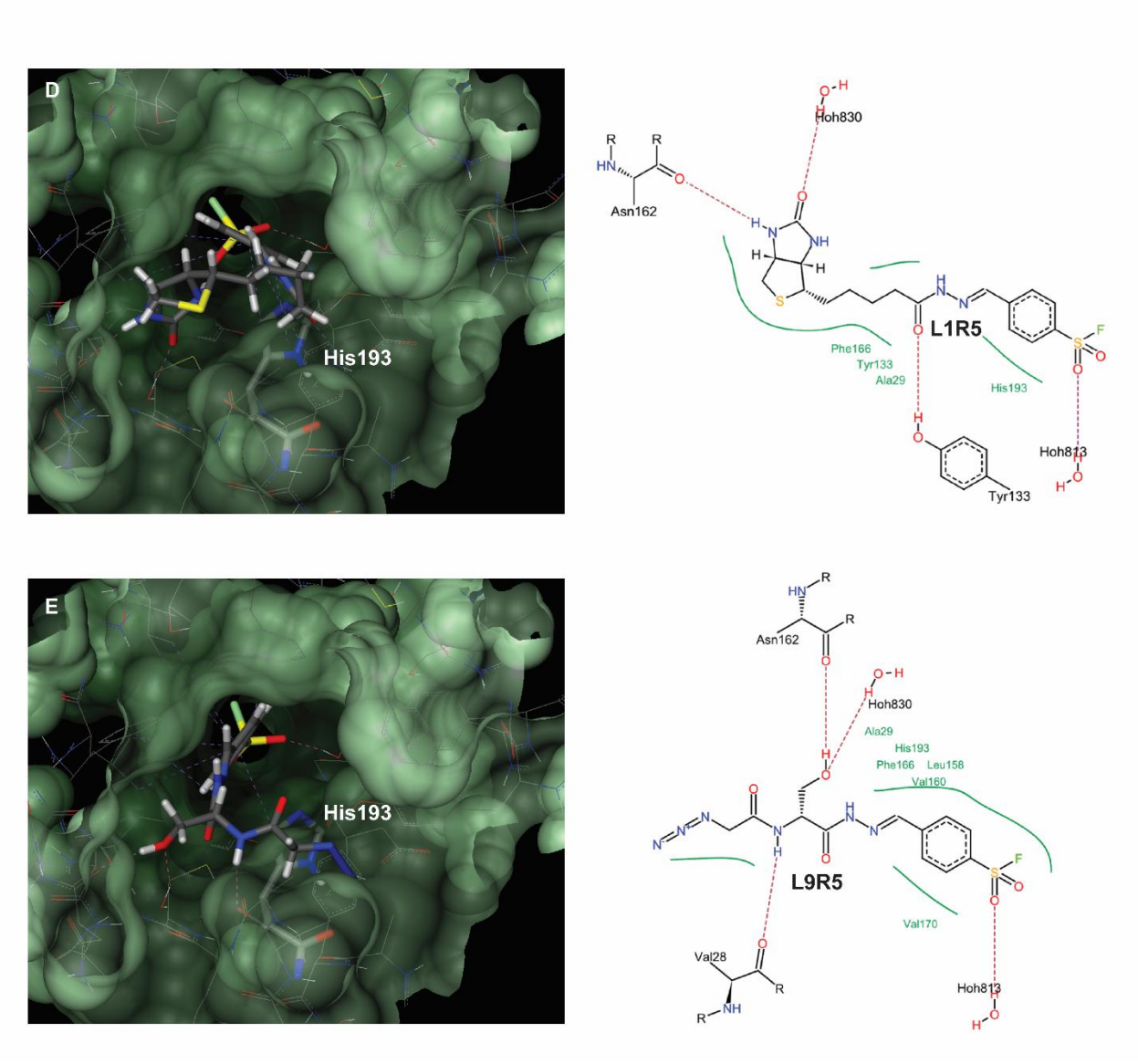


Figure S16. Molecular docking of the probes in the crystal structure of CAT complexed with fusidic acid (PDB ID: 1Q23). Representative pose of sulfonyl fluoride **L5R5** (A), **L7R5** (B), **L8R5** (C), **L1R5** and **L9R5** (G).

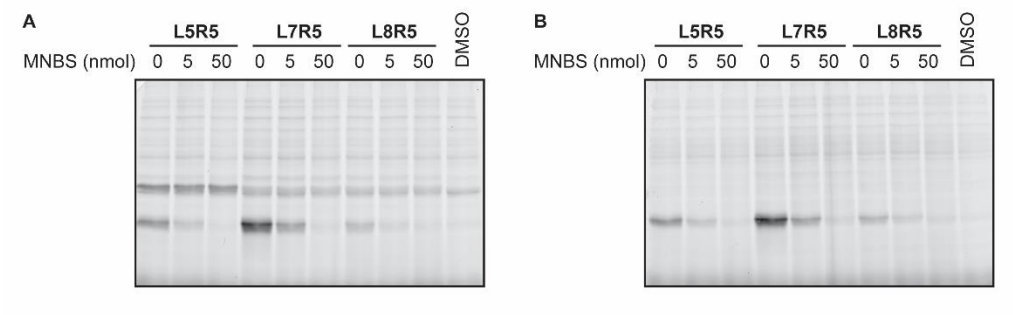


Figure S17. Competition experiment between methyl 4-nitrobenzenesulfonate and probes **L5R5**, **L7R5** and **L8R5** in lysate of BirA overexpressing *E. coli* (A) and lysate *E. coli* of BL21-CodonPlus (DE3)-RIL (B). To the lysate (8 µL) was added the indicated amount of methyl 4-nitrobenzenesulfonate (MNBS) in DMSO (0.5 µL) and incubated for 30 minutes. Then, the indicated probes (200 µM, 1 µL) were added. After 30 minutes, the proteins were denatured and clicked to BODIPY alkyne. The proteins were resolved on a 12% SDS-PAGE and visualized by fluorescence scanning. Labeling of CAT is inhibited, while labeling of BirA was unaffected.

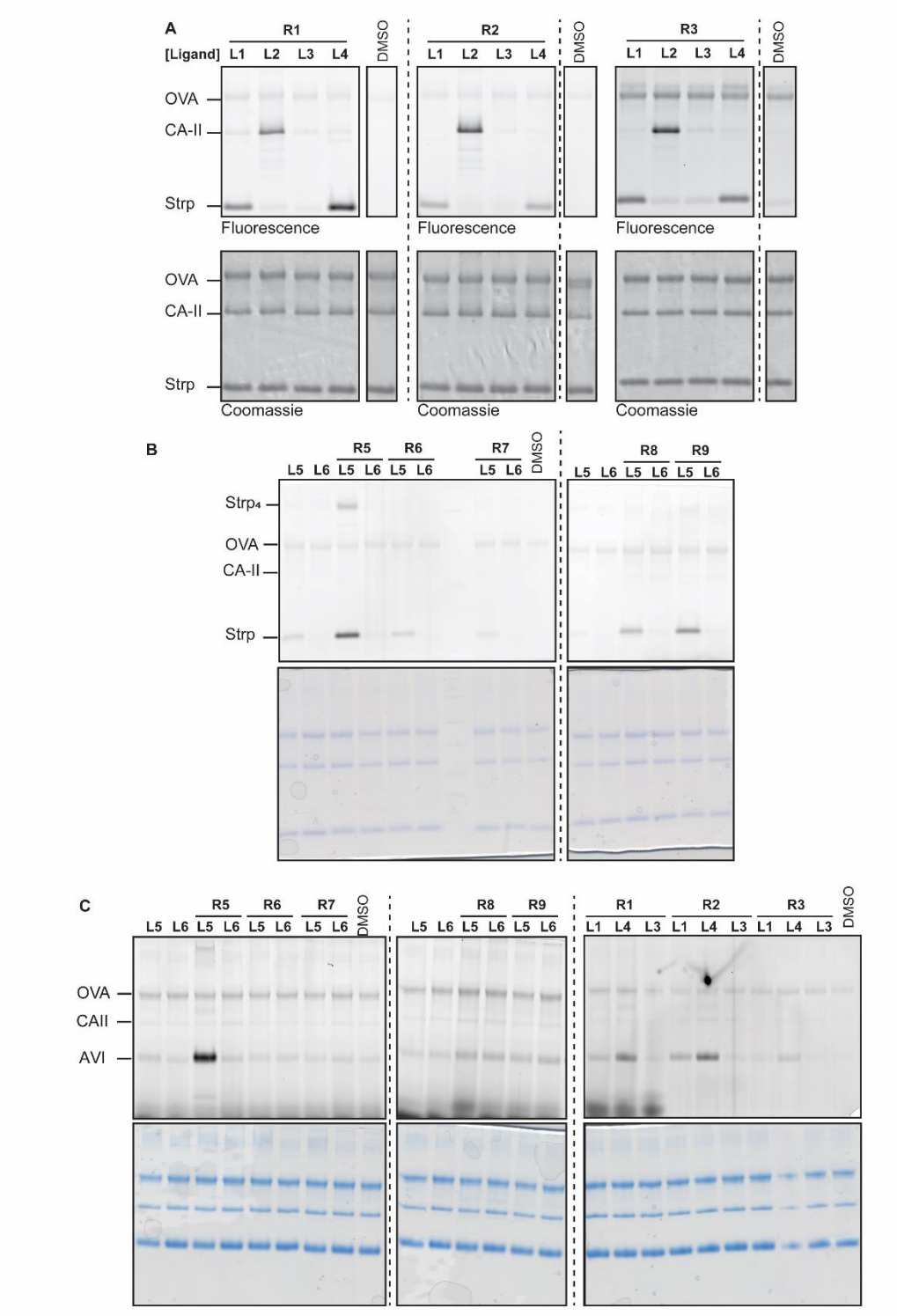


Figure S18. Representative screening of **R1-R3** on streptavidin and carbonic anhydrase (A). Screening of **R5-R9** on streptavidin (B) and avidin (C).

^{1}H NMR measurements of hydrazone and oxime formation

The carbonyl-containing reactive groups **R1**, **R2**, **R3**, **R5** and **R10** (50 mM in DMSO-d_6) were mixed with equal amounts of acethydrazide or methoxyamine hydrochloride (50 mM in DMSO-d_6) within an NMR tube. Starting immediately after addition of the reagents, ^{1}H NMR measurements were performed to follow hydrazone and oxime formation over time.

Biochemical procedures

General biochemical procedures

Proteins

Streptavidin (Strp) and avidin (Avi) were purchased from Thermo Fisher (catalog numbers 434302 and 21121, respectively). Bovine carbonic anhydrase II (CA-II) was purchased from Serva and chicken egg ovalbumin (OVA) was purchased from Sigma-Aldrich. Lysate of BirA-overexpressing *E. coli* BL21-CodonPlus(DE3)-RIL (16 mg/mL) was produced as described previously.[1] PBS (10×) was purchased from Thermo Fisher. Stock solutions of streptavidin (100 μM monomer), avidin (100 μM monomer), CA-II (100 μM) and OVA (400 μM) were prepared in HEPES (100 mM, pH 7.4, 150 mM NaCl) and stored at -20 °C. For the labeling experiments, protein solutions were prepared as follows. For the Strp/CA-II protein mixture, streptavidin (210 μL, 100 μM), CA-II (42.5 μL, 100 μM) and OVA (26.5 μL, 400 μM) were mixed with HEPES (151 μL, 100 mM, pH 7.4, 150 mM NaCl). For the Avi/CA-II protein mixture, avidin (210 μL, 100 μM), CA-II (42.5 μL, 100 μM) and OVA (26.5 μL, 400 μM) were mixed with HEPES (151 μL, 100 mM, pH 7.4, 150 mM NaCl). For BirA lysate, the lysate of BirA-overexpressing *E. coli* BL21-CodonPlus(DE3)-RIL (30 μL, 16 mg/mL) was diluted with HEPES (141 μL, 100 mM, pH 7.4, 150 mM NaCl). For native *E. coli* lysate, the lysate of *E. coli* BL21-CodonPlus(DE3)-RIL (60 μL, 18 mg/mL) was diluted with HEPES (480 μL, 100 mM, pH 7.4, 150 mM NaCl).

SDS-PAGE, fluorescence scanning, Western Blotting

Labeling experiments on streptavidin, avidin and CA-II were resolved on 12% TRIS-tricine type SDS-PAGE gel according to standard literature procedures. Labeling experiments on BirA were resolved on 12% Laemmli-type SDS-PAGE gel according to standard procedures.[2] Gels were prepared using acrylamide-bis ready-to-use solution 40% (37.5:1) (Merck Millipore) and separated on a Mini-PROTEAN Tetra cell (Bio-Rad). In-gel fluorescence scanning of the SDS-PAGE gels was performed on a Typhoon FLA 9500 (GE Healthcare) using the Cy2-settings for BODIPY (laser excitation at 473 nm and emission filter 515-545 nm) and the Cy5-settings for Cy5 and Alexa Fluor 647 (laser excitation at 635 nm and emission filter ≥665). Western Blot was performed using a Mini-PROTEAN Tetra cell (Bio-rad), using standard procedures. Visualization after electroblotting was performed using Pierce High Sensitivity streptavidin-HRP (Thermo Fisher). After fluorescent scanning of the gel, the proteins were stained with a Coomassie brilliant blue R250 solution or Roti®-Blue Colloidal Coomassie staining solution (Carl Roth) according to standard

protocols. Centrifugation of samples during pull-down experiments was performed using a Dragon Lab 24R centrifuge.

Probes and bio-reagents

Biotin hydrazide **L1** and 4-fluoro-3-nitrobenzaldehyde **R6** were purchased from Combi-Blocks (catalog numbers QB-7914 and OS-7911, respectively). 2,3,4,5,6-pentafluorobenzaldehyde **R7** and DBCO-Cy5 were purchased from Sigma-Aldrich. Biotin alkoxyamine **L2** and Alexa Fluor 647 hydroxylamine were purchased from Thermo Fisher. Streptavidin-agarose beads were purchased from Sigma-Aldrich. NeutrAvidin-agarose beads were purchased from Thermo Fisher. All ligands and reactive groups were prepared as stock solutions (50 mM) in DMSO and stored at -20 °C, except for biotin alkoxyamine **L4** (40 mM). Woodward's reagent K **R4** (50 mM) is unstable in DMSO and was therefore freshly dissolved in ethanol at the start of each experiment. In order to generate the probes, the ligand stocks (1 μL) were mixed with the reactive group stocks (1 μL) and incubated at room temperature overnight, except for **R4**, which was only incubated for 2 h. Subsequently, the probes were diluted in DMSO until the desired concentration was obtained for biochemical testing. The probe solutions were stored at -20 °C and could be used over the course of two months, until fresh probe solutions had to be prepared again. A stock solution of SDS (20% w/v) was prepared in water and stored at room temperature. Stock solutions of iodoacetamide (IAA; 100 mM), dithiothreitol (DTT; 90 mM), THPTA (100 mM) and $CuSO_4$ (100 mM) were prepared in water and stored at -20 °C. Solutions of sodium ascorbate (20 mM) in water were always prepared fresh from the salt. Stock solutions of BODIPY-azide (5 mM), BODIPY-alkyne (5 mM), DBCO-Cy5 (5 mM), biotin-alkyne and Alexa Fluor 647 hydroxylamine (10 mM) were prepared in DMSO and stored at -20 °C.

Click mixtures for CuAAC reactions were prepared as follows: BODIPY-azide, BODIPY-alkyne or biotin-alkyne (16 μL, 5 mM in DMSO), DMSO (48 μL), THPTA/$CuSO_4$ (20 μL, 20 mM in water) and water (20 μL) were added together in this exact order, followed by addition of sodium ascorbate (20 μL, 20 mM in water; freshly prepared). Stock solutions of chloramphenicol (100 mM) and fusidic acid (100 mM) were prepared in DMSO and stored at -20 °C. UV irradiation was performed with a Spectroline ENB-280C/FE UV lamp (312 nm).

Optimization of labeling experiments

Optimization of labeling with **R3** and **R4**: The effect of the labeling time on the labeling of **R3** was investigated by incubating the strp/CA-II protein mixture (8 μL) with probe (1 μL, 200 μM) for 2, 6 or 21 h followed by addition of BODIPY-azide click mixture (5 μL) and incubation for another 1 h (Figure S3A).

After addition of reducing sample buffer (17 μL, 2×) and boiling of the samples at 100 °C for 15 minutes, the samples (17 μL) were loaded onto a 12% TRIS-TRICINE PAGE gel, resolved and the in-gel fluorescence was analyzed. The results show that the labeling intensity of CA-II increases over time. However, the intensity of streptavidin labeling did not seem to increase and it was decided to use 6 h labeling time for further studies.

Optimization of the probe formation time with **R4**: Due to the observed instability of Woodward's Reagent K **R4**, we first assessed the influence of incubation time for probe formation (Figure S3A). The probes were formed as described above, with incubation times of 16 h (overnight) or 2 h. Then, probe (1 μL, 200 μM) was incubated with Strp/CA-II mixture (8 μL) for 1 h and BODIPY-azide click mixture (5 μL) was added. After 1 h, reducing sample buffer (17 μL, 2×) was added, the samples were boiled at 100 °C for 15 minutes, half of the sample (17 μL) was loaded onto a 12% TRIS-TRICINE PAGE and resolved. In-gel fluorescence scanning showed that maximal labeling with **R4** was achieved with a probe formation time of 2 hours prior to the labeling reaction. Presumably, degradation of the reactive group causes a decrease in labeling for longer incubation times, which corroborates the results of the NMR experiments. The experiment was then repeated, but now the probe formation times were ½ h, 1 h or 2 h (Figure S3B). Also, longer labeling times of 2 h and 4 h were tested. The results show that after 1 h of labeling, almost no fluorescent signal is observed. Increase of labeling time leads to higher labeling intensity. With shorter probe formation times, labeling was still observed. A probe formation time of 2 h with a labeling time of 2 h seemed to give the most intense signal and therefore these conditions were used to test the probes with **R4**.

Hydrazone/oxime exchange with Alexa Fluor 647 hydroxylamine:

Strp and OVA were diluted in PBS (1×) and to this was added the photocrosslinker probes **L1R9** in such a way that the total concentrations were 8.9 μM Strp, 10 μM OVA and 10 μM probe in a total volume of 20 μL. As a control leucine-hydrazide tethered to **R9** was used. After incubation for 30 minutes, the samples were irradiated at 312 nm for 10 minutes, followed by addition of Alexa Fluor 647 hydroxylamine (2 μL, 1.1 mM) and incubation overnight. Reducing sample buffer was added, the samples were boiled at 96 °C for 10 minutes, loaded on 15% SDS-PAGE, resolved and analyzed by in-gel fluorescence (Figure S3C).

Labeling of CA-II, streptavidin and avidin

Labeling with **R1-R4**: Strp/CA-II or Avi/CA-II protein mixture (8 μL) was incubated with the probe (1 μL, 200 μM; in case of **R1** also $CuSO_4$ (1 μL, 1 mM) was added) according to the optimized labeling times (1 h for **R1** and **R2**; 6 h for **R3**; 2 h for **R4**). Subsequently, BODIPY-azide or BODIPY-alkyne click mixture (5 μL)

was added and the samples were left to react for 2 h, followed by addition of reducing sample buffer (17 μL, 2×) and boiling at 100 °C for 15 minutes. Half of the sample (14 μL) was loaded on 12% TRIS-TRICINE PAGE, resolved and analyzed by in-gel fluorescence.

Labeling with **R5-R9**: Strp/CA-II or Avi/CA-II protein mixture (8 μL) was incubated with probe (1 μL, 200 μM) for 2 h. After 90 minutes of incubation time, the samples containing photocrosslinkers **R8** and **R9** were irradiated at 312 nm for 15 minutes. After the 2 h incubation, IAA (1 μL, 100 mM) was added and the samples were incubated for 15 minutes. Subsequently, BODIPY-alkyne click mixture (5 μL) was added, the samples were left to react for 2 h. Then, reducing sample buffer (17 μL, 2×) was added, the samples were boiled at 100 °C for 15 minutes and half of the sample (14 μL) was loaded on 12% Tris-Tricine PAGE, resolved and analyzed by in-gel fluorescence scanning (Figure S3E).

Alternatively, instead of the BODIPY-alkyne click mixture (5 μL), DBCO-Cy5 (1 μL, 200 μM) was used. In this case, the reaction time was increased from 2 h to overnight (Figure S3D).

Time-dependent labeling: A mixture of Strp and OVA or CA-II and OVA in HEPES (8 μL) was incubated with probe (1 μL, 200 μM) for 1 h, 2 h, 4 h, 6 h. Then, SDS (0.5 μL, 20% w/v in H_2O) was added and the samples were heated to 100 °C for 15 minutes. The samples were cooled to room temperature and IAA (1 μL, 100 mM) was added and the samples were incubated for 15 minutes. Subsequently, either BODIPY click mixture (5 μL) or DBCO-Cy5 (1 μL, 200 μM) was added, the samples were left to react for 2 h. Then, reducing sample buffer (17 μL, 2×) was added, the samples were boiled at 100 °C for 15 minutes and half of the sample (14 μL) was loaded on 12% Tris-Tricine PAGE, resolved and analyzed by in-gel fluorescence scanning (Figure S4-6).

Effect of the protein concentration: A mixture of Strp (105 μL, 100 μM) and OVA (13.25 μL, 400 μM) and HEPES (96.75 μL) or CA-II (42.5 μL, 100 μM) and OVA (26.5 μL, 400 μM) and HEPES (361 μL) was diluted with HEPES containing the same concentration OVA as the parent stocks. Of the serial dilution, 9 μL was added to Eppendorf cups. Then probe (1 μL, 200 μM) was added and the mixture was incubated for 2 h. To the samples that contained diazotransfer probes, $CuSO_4$ (1 μL, 200 μM) was added prior to the incubation step. After 2 hours, BODIPY click mixture (5 μL) was added and the reactions were incubated for 2 hours. Then, reducing sample buffer (17 μL, 2×) was added, the samples were boiled at 100 °C for 15 minutes and half of the sample (14 μL) was loaded on 12% Tris-Tricine PAGE, resolved and analyzed by in-gel fluorescence scanning (Figure S7).

Dual labeling of CA-II and streptavidin

*Effect of Cu^{2+} on labeling with **R2** and **R3** based probes*: it has been shown that addition of a catalytic amount of copper(II) is required to efficiently label proteins with diazotransfer reagents like **R1**.[3] Likewise, catalytic copper(II) might also affect labeling by **R2** and **R3** and we therefore first determined its effect on the labeling efficiency and selectivity (Figure S4).[4] To Strp/CA-II protein mixture (8 μL) was added the respective probe (1 μL, 200 μM) and $CuSO_4$ (1 μL, 1 mM) where indicated. The reaction was incubated for 1 h, followed by addition of BODIPY-azide click mixture (5 μL) and additional incubation for 1 h. Subsequently, reducing sample buffer (17 μL, 2×) was added and the samples were boiled at 100 °C for 15 minutes. Half of the sample (17 μL) was loaded and resolved on 12% TRIS-tricine PAGE. In-gel fluorescent scanning showed that copper did not seem to enhance labeling of the target by the **R2**-based probes but it did result in significant amounts of off-target labeling. The oxime-based probe **L4R2** was more selective in the presence of copper. It was therefore decided to perform the labeling reactions with **R2** in absence of copper(II) (Figure S4A). The copper catalyst also affected labeling by **R3** based reagents (Figure S4B). Addition of Cu^{2+} led to reduced labeling of CA-II.

Dual labeling: **L1R1** or **L2R1** (1 μL, 200 μM) and **L2R2** or **L4R2** (1 μL, 200 μM) followed by $CuSO_4$ (1 μL, 1 mM) were added to Strp/CA-II protein mixture (8 μL) to determine if remaining aldehyde affected the selectivity when using multiple probes. After 1 h, DBCO-Cy5 (1 μL, 200 μM) was added and the mixture was incubated for an additional 1 h. Finally, BODIPY-azide click mixture (5 μL) was added and the mixture was incubated for another hour. Subsequently, reducing sample buffer (17 μL, 2×) was added and the samples were boiled at 100 °C for 15 minutes. Half of the sample (14 μL) was loaded on 12% TRIS-TRICINE PAGE, resolved and analyzed by in-gel fluorescence scanning.

Alternatively, the experiment could also be performed using the same conditions for dual labeling where **L2R2** and **L4R2** were replaced with **L4R3** and **L2R3** (Figure S4C).

Labeling of BirA-overexpressing *E. coli* lysate

BirA-lysate protein mixture (8 μL) was incubated with probe (1 μL, 200 μM) for 2 h (in case of **R1** also 1 μL of 1 mM $CuSO_4$ was added). At minute 90 of incubation time, the samples containing photocrosslinkers **R8** and **R9** were irradiated at 312 nm for 15 minutes. After the 2 h of incubation, SDS (0.5 μL, 20%) was added and the samples were boiled at 100 °C for 15 minutes, followed by addition of IAA (1 μL 100 mM) and incubation for 15 minutes. Subsequently, BODIPY-azide or BODIPY-alkyne click mixture (5 μL) was added and the samples were left to react for 2 h. Then, reducing sample buffer (5 μL, 4×) was added, the

samples were boiled at 100 °C for 15 minutes and the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and analyzed by in-gel fluorescence.

Alternatively, the samples could be loaded directly on 12% Laemmli-type SDS-PAGE without addition of BODIPY-azide or BODIPY-alkyne click mixture but, resolved, transferred to a PVDF membrane and visualized with streptavidin-HRP using the biotin ligand as readout moiety. In case of the non-targeted ligand **L6** that lacks a biotin moiety for streptavidin-HRP readout, the samples were incubated with biotin-alkyne click mixture (5 μL) for 2 h before they were loaded on gel.

Time-dependent labeling: To determine the effect of the labeling time, the reactions were carried out as described for the initial screen, but the incubation with the probe was varied between 30 minutes to 4 hours. After incubating with the probes, the samples were quenched, clicked to reporter groups and visualized as described above (Figure S11).

Concentration-dependent labeling: To determine the effect of the concentration, the reactions were carried out as described for the initial screen, but the amount of probe was varied between 10 pmol and 5 nmol (1.11 μM and 555 μM). After incubating with the probes, the samples were quenched, clicked to reporter groups and visualized as described above (Figure S12).

Pull-down experiment

Optimization of off-target labeling with **L5R5**: Before the pull-down experiment, first the optimal concentration of probe for labeling was determined. BirA lysate mixture (8 μL) was incubated with different concentrations of **L5R5** (1 μL, concentration range 5 mM, 1 mM, 500 μM, 200 μM, 100 μM, 10 μM, 1 μM, 0.1 μM) for 30 minutes. Subsequently, BODIPY-alkyne click mixture (5 μL) was added and the samples were left to react for 2 h. Then, reducing sample buffer (5 μL, 4×) was added, the samples were boiled at 100 °C for 15 minutes and the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and analyzed by in-gel fluorescence. Based on these results, it was decided to use 200 μM probe (20 μM final concentration) for the pull-down experiment.

Similarly, the optimal labeling time was determined. BirA lysate mixture (8 μL) was incubated with **L5R5** (1 μL, 100 μM) and biotin (100 μM) for 5, 10, 20, 30 or 60 minutes. Subsequently, 1% SDS (1 μL) was added and the samples were boiled to prevent labeling during the click step. BODIPY-alkyne click mixture (5 μL) was added and the samples were left to react for 2 h. Then, reducing sample buffer (5 μL, 4×) was added,

the samples were boiled at 100 °C for 15 minutes and the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and analyzed by in-gel fluorescence.

Pull-down: BirA lysate (3 mL, 16 mg/mL) was incubated with prewashed streptavidin-agarose beads (100 μL) for 45 minutes to remove endogenous biotinylated proteins. After centrifugation, the pre-cleared solution was transferred to another vial. 100 μL of the supernatant was incubated with **L5R5** or **L6R5** (10 μL, 200 μM) for 90 minutes, followed by addition of SDS (5 μL, 20%), boiling at 100 °C for 15 minutes and incubation with biotin-alkyne click mixture (50 μL) for 2 h. The proteins were precipitated with chloroform/methanol[5], redissolved in SDS (100 μL, 2% in 1× PBS) and incubated with DTT (10 μL, 90 mM) for 30 minutes. Subsequently, the samples were incubated with IAA (30 μL, 100 mM) for 30 minutes and diluted with PBS (1.86 mL, 1×). NeutrAvidin-agarose beads (100 μL of a slurry) were added and after 90 minutes the samples were centrifuged, the supernatant was removed, the beads were washed three times with SDS (2 mL, 0.2% in 1× PBS) and two times with PBS (2 mL, 1×). Reducing sample buffer containing biotin (55 μL, 2× sample buffer, 20 μM biotin) was added and the beads were boiled at 100 °C for 15 minutes. The samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and stained with Roti®-Blue Colloidal Coomassie staining solution. The bands around 25 kDa were excised and cut in small pieces. The gel pieces were completely de-stained with ammonium carbonate in acetonitrile (50 mM, 1:1 v/v water/acetonitrile), dehydrated with acetonitrile (150 μL), reduced with DTT (10 mM, 30 min at 55 °C) and alkylated with IAA (40 mM, 45 min at RT, in the dark) and overnight digested with trypsin (10 μL of a 10 ng/μL solution, V5111; Promega) at 37 °C. Peptides were extracted from the gel pieces by adding, sonicating and collecting sequentially in the same tube TFA (40 μL, 2%), ACN (40 μL, 33%); TFA (1.7%) and ACN (40 μL, 67%), TFA (0.7%). The samples were dried under vacuum. The peptide samples were reconstituted with TFA (1%) and cleaned with Pierce® C18 tips (87784; Thermo) according to the instruction manual. The eluted fractions were dried under vacuum and reconstituted with ACN (20 μL, 2%), formic acid (0.1%). Peptide separation was performed with peptide sample (2 μL) using a nano-flow chromatography system (EASY nLC II; Thermo) equipped with a reversed phase HPLC column (75 μm, 15 cm) packed in-house with C18 resin (ReproSil-Pur C18–AQ, 3 μm resin; Dr. Maisch) using a linear gradient from 95% solvent A (0.1% FA, 2% acetonitrile) and 5% solvent B (99.9% acetonitrile, 0.1% FA) to 28% solvent B over 45 min at a flow rate of 200 nL/min. The peptide and peptide fragment masses were determined by an electrospray ionization mass spectrometer (LTQ-Orbi-trap XL; Thermo). Raw files were imported into the Peaks Studio software (Bioinformatics Solutions) or the Scaffold software (Proteome Software Inc., version 4.8.4) analyzed against forward and reverse peptide sequences of *E. coli* and the

over-expressed constructs. The search criteria were set as follows: one end tryptic specificity was required (cleavage after lysine or arginine residues but not when followed by a proline); three missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) and deamination (NQ) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.5 Da for fragment ions.

Competition experiments of **L5R5, L7R5, L8R5, L9R5** on BirA lysate

8 μL BirA-lysate protein mixture was incubated with probe (1 μL, 200 μM) or with probe (0.5 μL, 400 μM) and competitor (0.5 μL, different amounts of chloramphenicol, fusidic acid or biotin) for 30 minutes. Then, 20% SDS (0.5 μL) was added and the samples were boiled at 100 °C for 15 minutes, followed by addition of IAA (1 μL 100 mM) and incubation for 15 minutes. Subsequently, BODIPY-alkyne click mixture (5 μL) was added and the samples were left to react for 2 h. Then, reducing sample buffer (5 μL, 4×) was added, the samples were boiled at 100 °C for 15 minutes and the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and analyzed by in-gel fluorescence.

Labeling experiments on native *E. coli* lysate

Native *E. coli* lysate protein mixture (8 μL) was incubated with probe (1 μL, 200 μM or 2 mM) for 2 h. After incubation, SDS (0.5 μL, 20%) was added and the samples were boiled at 100 °C for 15 minutes, followed by addition of IAA (1 μL 100 mM) and incubation for 15 minutes. Subsequently, BODIPY-azide or BODIPY-alkyne click mixture (5 μL) was added and the samples were left to react for 2 h. Then, reducing sample buffer (5 μL, 4×) was added, the samples were boiled at 100 °C for 15 minutes and the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and analyzed by in-gel fluorescence.

Alternatively, the samples could be loaded directly on 12% Laemmli-type SDS-PAGE without addition of BODIPY-azide or BODIPY-alkyne click mixture but, resolved, transferred to a PVDF membrane and visualized with streptavidin-HRP using the biotin ligand as readout moiety. In case of the non-targeted ligand **L6** that lacks a biotin moiety for streptavidin-HRP readout, the samples were incubated with biotin-alkyne click mixture (5 μL) for 2 h before they were loaded on gel.

Determination of the detection limit of BirA

Purified BirA was diluted in native *E. coli* lysate such that the total lysate concentration was constant at 2 mg/mL while the amount of BirA would be as indicated in Figure S13 at 8 μL of mix. Then, probe (1 μL, 2 mM or 200 μM for **L5R7** and 1 mM or 200 μM for **L1R5**) was added to the mix and the samples were

incubated for 2 h. After incubation, SDS (0.5 μL, 20%) was added and the samples were boiled at 100 °C for 15 minutes, followed by addition of IAA (1 μL, 100 mM) and incubation for 15 minutes.

For **L5R7**: BODIPY-alkyne click mixture (5 μL) was added and the samples were left to react for 2 h. Then, reducing sample buffer (5 μL, 4×) was added, the samples were boiled at 100 °C for 15 minutes and the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved and analyzed by in-gel fluorescence.

For **L1R5**: Either reducing sample buffer (9.5 μL, 2×) was added, or the samples were incubated with biotin-alkyne click mix (5 μL) for 2 h and then reducing sample buffer (5 μL, 4×) was added. After addition of the reducing sample buffer, all samples were boiled at 100 °C for 15 minutes. Subsequently, the samples were loaded on 12% Laemmli-type SDS-PAGE, resolved, transferred to a PVDF membrane and visualized with streptavidin-HRP using biotin as readout moiety.

Molecular Docking of the probes in BirA and CAT

For BirA: To determine the binding mode of the probes, we downloaded the PDB file of BirA bound to biotin-AMP from the RCSB PDB data bank (PDB ID 4WF2) and docked eight probes using LeadIT (version 2.3.2; BioSolveIT GmbH, Sankt Augustin, Germany, 2017, www.biosolveit.de/LeadIT). We selected the crystal structure 4WF2, because of its resolution (2.3 Å) and because the probes should mimic the bound ligand. In the docking, water molecule 652A is automatically included. The biotin in the crystal structure makes hydrogen bonds with Gln112, 2× Arg116 and Ser89. Since we considered that the binding of the biotin moiety is essential, we set the docking parameters such that the docked molecules make at least 2 out of these 4 interactions. Thirty poses were generated per molecule, if feasible. The docking of the eight probes depicted in Figure S10 resulted in a total of 218 binding poses (out of 240). Of the docked molecules, **L1R2, L2R2, L1R5, L4R5, L5R5**, and **L1R7** adopt binding poses in which the biotin group is fixed in the appropriate position. **L6R5** lacks a biotin and adopts several different binding poses including many poses in which the sulfonylfluoride is bound in the biotin pocket. In the binding poses for **L5R7**, the biotin pharmacophore has slightly moved, but the different binding modes are very similar in all cases. The distance between para CF and Met211 is around 3.0 – 3.8 Å. Docking of the probes without constrains gave similar binding poses for all the probes (240/240), with more flexibility in the binding of the biotin moiety.

For CAT: To determine the binding mode in CAT3, we downloaded the crystal structure of CAT3 bound to CLM-221-A (fusidic acid) from the RCSB PDB data bank (PDB ID: 1Q23). This crystal structure was selected since binding of fusidic acid results in opening of the binding pocket and therefore results in a more spacious active site. CAT3 forms a homotrimeric structure. The active site is located at the interface of two monomers and the peptide chains of both contribute to the active site. Therefore, the receptor was prepared by selecting Chains A and B in LeadIT (version 2.3.2; BioSolveIT GmbH, Sankt Augustin, Germany, 2017, www.biosolveit.de/LeadIT). The binding site was defined around reference ligand CLM-221-A (radius 6.5Å). Water molecules with more than two interactions were included (813-A, 814-A and 830-A). In the docking, we fixed histidine 193 of the B chain in the $N^{\delta 1}$ tautomer, since this residue forms a catalytic dyad with a neighboring aspartic acid residue. Thirty poses were generated per molecule, if feasible. Docking of the six ligands depicted in Figure S16 resulted in 180/180 poses. According to the docking, **L7R5** and **L9R5** adopt three different binding modes. **L6R5** adopts many poses in which the reactive group is not in proximity of the catalytic histidine. Both **L5R5** and **L8R5** have one main binding mode. In this binding mode, the sulfonylfluoride is in close proximity of the catalytic histidine.

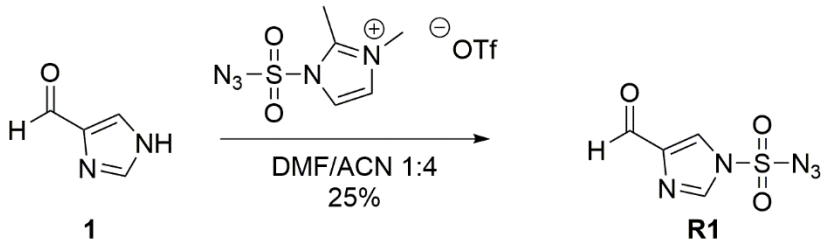
Synthetic procedures

General synthetic procedures

All solvents used were of commercial grade and used without further purification. Dry DCM was taken from a MBraun SPS 800 solvent purification system. Reagents were purchased from Sigma-Aldrich, TCI, or Fluorochem, unless otherwise noted, and were used without further purification. BODIPY-azide[1], BODIPY-alkyne[6], 1-(azidosulfonyl)-2,3-dimethyl-1*H*-imidazol-3-ium trifluoromethylsulfonate[7], 2-methyl-1*H*-imidazole-1-sulfonyl azide hydrochloride[7], azidohexanehydrazide **L6**[8], 4-azido-2,3,5,6-fluorobenzaldehyde **R8**[9], 4-azidobenzaldehyde **R9**[10] and biotin-alkyne[11] were synthesized according to literature procedures. TLC was performed on Merck TLC Silica gel 60/Kieselguhr F_{254} plates and visualization was done by UV light, iodine (I_2 crystals in silica), ninhydrin, potassium permanganate or dinitrophenylhydrazine staining solutions. Manual flash column chromatography was performed using silica (SilicaFlash P60, 230-400 mesh, Silicycle, Canada) as the stationary phase. ^{1}H-, ^{13}C-, ^{19}F-, APT and HSQC NMR were recorded on a Varian AMX400 spectrometer (400, 101 and 376 MHz, respectively) or on a Bruker Avance NEO 600 (600

and 150 MHz, respectively) using, $CDCl_3$, CD_3OD, DMSO-d_6 or D_2O as solvent. Chemical shift values are reported in ppm with the solvent resonance as the internal standard ($CDCl_3$: δ7.26 for ^1H, δ 77 for ^{13}C; CD_3OD: δ3.31 for ^1H, δ 49.15 for ^{13}C; DMSO-d_6: δ2.50 for ^1H δ 39.52 for ^{13}C; D_2O: δ 4.79 for ^1H). Data are reported as follows: chemical shifts (δ), multiplicity (s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, p = quintet, m = multiplet, apparent quartet = app q), coupling constants *J* (Hz), and integration. UPLC-MS was performed on an LCQ Fleet mass spectrometer coupled to a Vanquish UHPLC system. High resolution mass measurements were performed using a ThermoScientific LTQ OrbitrapXL spectrometer. *WARNING: Diazotransfer reagents may be shock sensitive and should be handled using appropriate precautions.*

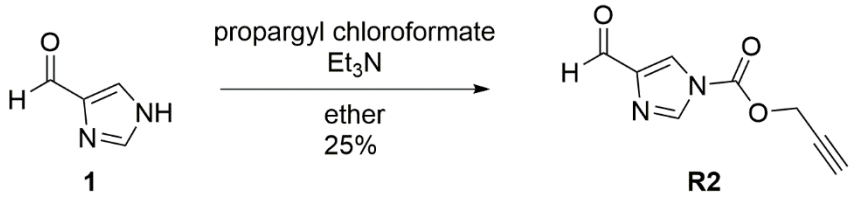
Synthesis reactive group **R1**



4-formyl-*1H*-imidazole-1-sulfonyl azide (R1)

A solution of 1-(azidosulfonyl)-2,3-dimethyl-1*H*-imidazol-3-ium trifluoromethylsulfonate (386 mg, 1.1 mmol) in dry DMF (2 mL) was added dropwise to an ice water-bath-cooled solution of 4-imidazolecarboxaldehyde **1** (96 mg, 1.0 mmol) in dry acetonitrile (8 mL) over 1 h under nitrogen atmosphere. After stirring at room temperature for 4 h, the solution was concentrated and dry-loaded onto celite. Subsequently, the crude was purified by automated column chromatography (1% MeOH/DCM to 10% MeOH/DCM) to yield **R1** (51 mg, 0.24 mmol, 25%) as a white solid. ^1H NMR (400 MHz, CD_3OD) δ 9.87 (s, 1H), 8.52 (s, 1H), 8.46 (s, 1H). ^{13}C NMR (101 MHz, CD_3OD) δ 185.7, 146.5, 138.6, 126.5. HRMS *m/z* calculated for $[M+H]^+$ 202.0029, found 202.0032. In the H-NMR, the hydrate of R1 is also observed.

Synthesis reactive group **R2**

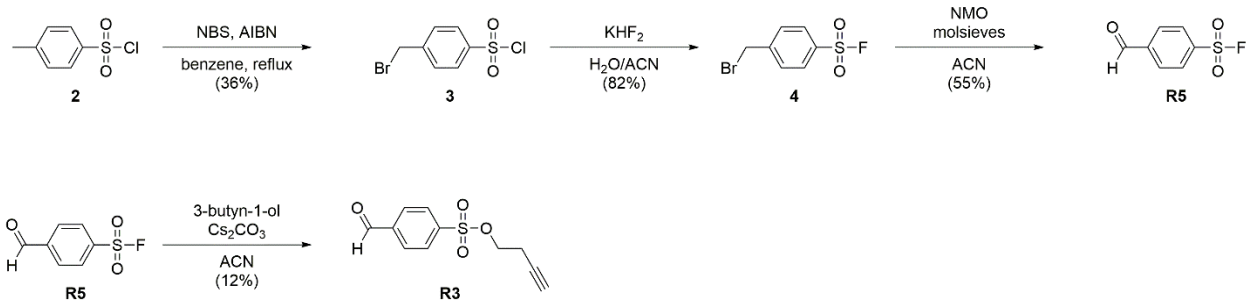


prop-2-yn-1-yl 4-formyl-1*H*-imidazole-1-carboxylate (R2)

Propargyl chloroformate (0.11 mL, 1.1 mmol) was slowly, dropwise, added to an ice-water bath cooled suspension of 4-imidazolecarboxaldehyde **1** (221 mg, 2.3 mmol) in dry ether (5 mL) under nitrogen atmosphere. After stirring at room temperature for 4 h, the suspension was purified by column chromatography (100% pentane -> 60% ether/pentane, R_f 0.39 in 60% ether/pentane) the crude was loaded directly from the flask onto the column) to yield **R2** (57 mg, 0.32 mmol, 28%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.95 (s, 1H), 8.23 (s, 1H), 8.10 (s, 1H), 5.05 (m, 2H), 2.68 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 185.6, 147.4 142.8, 138.0, 121.9, 77.7, 75.1, 56.3. HRMS *m/z* calculated for $[M+H]^+$ 179.0451, found 179.0454.

Note of caution: this compound is sensitive to hydrolysis and protocols that included an aqueous workup resulted in lower yields. The product was stored under nitrogen atmosphere at -20 ℃.

Synthesis reactive group **R3** and **R5**



α-bromo-*p*-toluenesulfonyl choride (3)[12]

Tosyl chloride (1.91 g, 10 mmol), NBS (1.81 g, 10 mmol) and AIBN (7 mg, 0.03 mmol) were dissolved in benzene (12 mL) and refluxed for 23 h. After cooling to room temperature, the red suspension was filtered and the residue rinsed with toluene. Subsequently the filtrate was washed with aqueous $NaHCO_3$ (sat., 30 mL, 2×) and brine (50 mL, 2×), dried over Na_2SO_4 and concentrated under reduced pressure. Heptane (2 mL) was added to the crude and the resultant precipitate was filtered over a glass filter and rinsed with heptane to yield *α*-bromo-*p*-toluenesulfonyl choride **3** (965 mg, 3.6 mmol, 36%) as a slightly pink solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.02 (d, *J* = 8.1 Hz, 2H), 7.64 (d, *J* = 8.2 Hz, 2H), 4.52 (s, 2H).

α-bromo-*p*-toluenesulfonyl fluoride (4) *procedure based on*[13], *for NMR see*[14]

A solution of **3** (965 mg, 3.6 mmol) in acetonitrile (4 mL) was added to a Falcon 50 mL Conical Centrifuge Tube containing a saturated solution of KHF_2 (0.65 g, 8.3 mmol) in H_2O (1.8 mL). The biphasic reaction mixture was vigorously stirred at room temperature overnight, followed by dilution with EtOAc (4 mL) and separation of the layers. The water layer was extracted with EtOAc (2 mL, 3×) and the combined organic layers were washed with brine (10 mL, 2×), dried over Na_2SO_4 and concentrated under reduced pressure to yield *α*-bromo-*p*-toluenesulfonyl fluoride **4** (747 mg, 2.95 mmol, 82%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.6 Hz, 2H), 7.65 (d, *J* = 8.1 Hz, 2H), 4.52 (s, 2H). ^{19}F NMR (376 MHz, $CDCl_3$) δ 66.05 (s).

4-formylbenzenesulfonyl fluoride (R5)

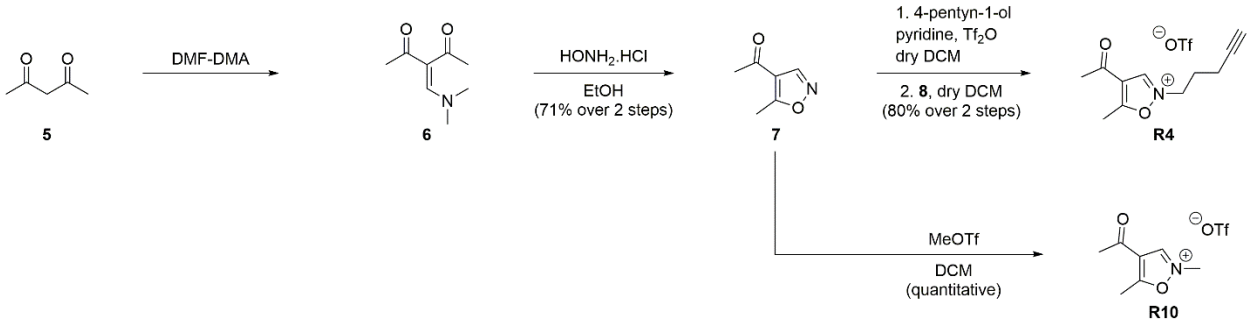
NMO (428 mg, 3.7 mmol) was added to a solution of **4** (453 mg, 1.8 mmol) and molecular sieves (4 Å) in acetonitrile (11 mL) under nitrogen atmosphere and stirred at room temperature for 2 h. Subsequently, EtOAc was added (50 mL) and the resultant precipitate was filtered over a glass filter. The filtrate was

washed with H_2O (30 mL, 2×), HCl (1 M, 30 mL, 2×) and brine (30 mL, 1×), dried over Na_2SO_4 and concentrated under reduced pressure. Purification by column chromatography (8% EtOAc/pentane, R_f 0.26) yielded **R5** (184 mg, 0.98 mmol, 55%) as an off-white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1H), 8.20 (d, *J* = 8.4 Hz, 2H), 8.14 (d, *J* = 8.6 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.1, 140.8, 137.7 (d, *J* = 26 Hz), 130.4, 129.2. ^{19}F NMR (376 MHz, $CDCl_3$) δ 66.82 (s). HRMS *m/z* calculated for $[M\text{-}SO_2F+H]^+$ 107.0491, found 107.0405.

but-3-yn-1-yl 4-formylbenzenesulfonate (R3)

R5 (55 mg, 0.29 mmol) and 3-butyn-1-ol (24.5 μL, 0.32 mmol) were dissolved in acetonitrile (1.5 mL), followed by addition of Cs_2CO_3 (188 mg, 0.58 mmol) and the reaction mixture was stirred at room temperature for 4 h. After concentration under reduced pressure, the crude was purified by column chromatography (15% EtOAc/pentane, R_f 0.14) to yield **R3** (8 mg, 0.03 mmol, 12%) as a white solid. The final product contains 10% but-3-ynol based on NMR. ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.14 (s, 1H), 8.11 (d, *J* = 8.3 Hz, 2H), 8.07 (d, *J* = 8.4 Hz, 2H), 4.20 (t, *J* = 6.9 Hz, 2H), 2.60 (td, *J* = 6.8, 2.6 Hz, 2H), 1.97 (t, *J* = 2.6 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 190.5, 140.9, 139.7, 130.2, 128.7, 78.1, 71.0, 68.1, 19.5. HRMS *m/z* calculated for $[M+H]^+$ 239.0373, found 239.0372.

Synthesis reactive group **R4** and **R10**



1-(5-methylisoxazol-4-yl)ethan-1-one (7)[15]

A mixture of acetylacetone **5** (1.6 mL, 15.6 mmol) and DMF-DMA (4.0 mL, 30.1 mmol) was heated to reflux for 2 h until completion and then concentrated under reduced pressure to yield 3-((dimethylamino)methylene)pentane-2,4-dione **6** (2.46 g, 15.6 mmol, quantitative) as a dark yellow oil.

6 (2.46 g, 15.6 mmol) was dissolved in EtOH (30 mL), followed by addition of hydroxylamine hydrochloride (1.09 g, 15.7 mmol) and the red solution was refluxed for 3 h until completion. The now orange mixture was concentrated under reduced pressure, H_2O (70 mL) was added and the mixture was extracted with DCM (80 mL, 2×). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. Purification by column chromatography (10% -> 30% ether/pentane, R_f 0.38 (10% ether/pentane)) yielded 1-(5-methylisoxazol-4-yl)ethan-1-one **7** (1.40 g, 11.2 mmol, 72%) as a thin yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.47 (s, 1H), 2.71 (s, 3H), 2.45 (s, 3H).

4-acetyl-5-methyl-2-(pent-4-yn-1-yl)isoxazol-2-ium triflate (R4)

4-Pentyn-1-ol (0.28 mL, 3.0 mmol) and pyridine (0.24 mL, 3.0 mmol) were dissolved in dry DCM (3 mL) under nitrogen atmosphere and cooled with an ice-water bath, followed by slow, dropwise addition of a Tf_2O (0.58 mL, 3.4 mmol) solution in dry DCM (4 mL). After 30 minutes, the mixture was diluted with H_2O (15 mL), the layers were separated and the organic layer was washed with H_2O (10 mL), dried over Na_2SO_4 and concentrated under reduced pressure (≥ 700 mbar). The crude pent-4-yn-1-yl triflate was redissolved in dry DCM (5 mL) under nitrogen atmosphere. Subsequently, a solution of 1-(5-methylisoxazol-4-yl)ethan-1-one (376 mg, 3.0 mmol) dissolved in dry DCM (4 mL) was added dropwise and the reaction mixture was stirred at room temperature overnight, followed by concentration under reduced pressure and trituration with ether (10 mL, 5×) to yield **R4** (817 mg, 2.4 mmol, 80%) as a very dark oil. ^{1}H NMR (400 MHz, D_2O) δ 10.01 (s, 1H), 4.89 (t, *J* = 6.7 Hz, 2H), 2.95 (s, 3H), 2.64 (s, 3H), 2.42-2.43 (m, 3H), 2.34 – 2.27 (m, 2H). ^{13}C NMR (101 MHz, D_2O) δ 191.8, 178.6, 148.8, 112.0, 119.7 (q, *J* = 318 Hz, CF_3 of triflate), 82.4, 70.8, 54.5, 28.8, 25.7, 14.7, 13.0. HRMS *m/z* calculated for $[M+H]^+$ 192.1019, found 192.1022. *Major peak found in HRMS belonged to the hydrolysis product 3-oxo-N-(pent-4-yn-1-yl)butanamide, HRMS m/z calculated for $[M+H]^+$ 168.1019, found 168.1021.*

Note of caution: this compound is sensitive to hydrolysis and was stored under nitrogen atmosphere at -20 °C.

4-acetyl-2,5-dimethylisoxazol-2-ium triflate (R10)

1-(5-methylisoxazol-4-yl)ethan-1-one (263 mg, 2.1 mmol) was dissolved in dry DCM (10 mL) and cooled with an ice-water bath for 30 minutes, followed by slow dropwise addition of MeOTf (0.36 mL, 3.2 mmol). The reaction mixture was stirred at room temperature for 3 h until completion, at which point it was concentrated under reduced pressure to yield 4-acetyl-2,5-dimethylisoxazol-2-ium triflate (617 mg, 2.1 mmol, quantitative) as a yellow oil. ^{1}H NMR (400 MHz, CD_3OD) δ 10.02 (s, 1H), 4.42 (s, 3H), 2.92 (s, 3H),

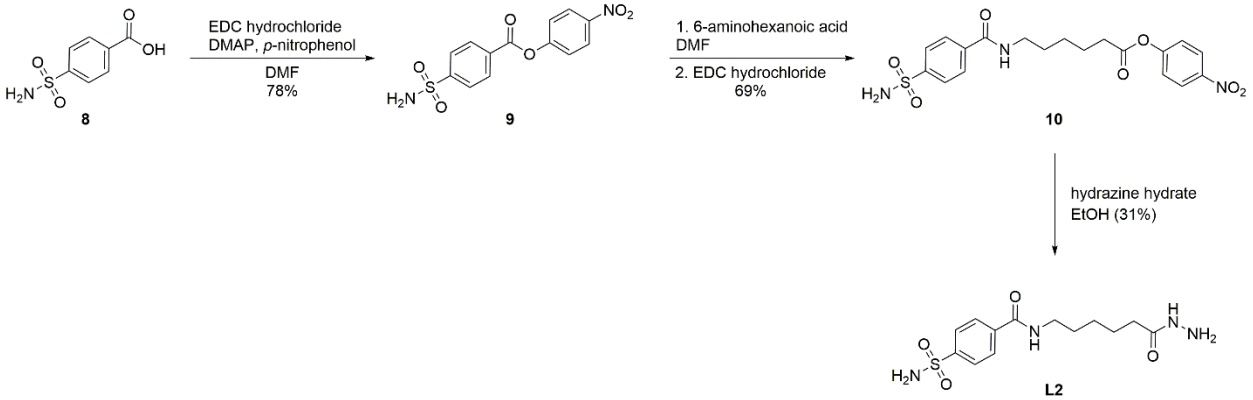
2.58 (s, 3H). ^{13}C NMR (101 MHz, CD_3OD) δ 190.3, 178.9, 150.5, 121.7 (q, *J* = 318.6 Hz, CF_3 of triflate), 121.3, 41.8, 29.4, 13.4. HRMS *m/z* calculated for $[M+H]^+$ 140.0706, found 140.0707. *Major peak found in HRMS belonged to the hydrolysis product N-methyl-3-oxobutanamide, HRMS m/z calculated for* $[M+H]^+$ *116.0706, found 116.0706.*

Note of caution: this compound is sensitive to hydrolysis and was stored under nitrogen atmosphere at -20 °C.

Synthesis ligand **L2**

4-nitrophenyl 4-sulfamoylbenzoate (9)

4-Sulfamoylbenzoic acid **8** (2.01 g, 10.0 mmol) and EDC hydrochloride (2.30 g, 12 mmol) were suspended



in anhydrous DMF (50 mL). After one hour at room temperature under constant stirring, *p*-nitrophenol (1.53 g, 11.0 mmol) was added. The reaction mixture was stirred at room temperature until 4-sulfamoylbenzoic acid was consumed according to TLC (24 h). The reaction volume was concentrated under reduced pressure. Then 1 M HCl (100 mL) was stirred into the concentrate. The resulting pale precipitate was filtered off and successively washed with 1 M HCl (50 mL), H_2O (50 mL) and ether (50 mL), dried under high vacuum and subsequently recrystallized from EtOH to yield **9** (2.51 g, 7.8 mmol, 78%) as white crystals. ^{1}H NMR (400 MHz, CD_3OD) δ = 8.37 (d, *J* = 9.1 Hz, 2H), 8.36 (d, *J* = 8.4 Hz, 2H), 8.10 (d, *J* = 8.5, 2H), 7.57 (d, *J* = 9.1, 2H). ^{13}C NMR (101 MHz, CD_3OD) δ = 164.5, 156.9, 150.1, 147.1, 133.2, 131.9, 127.6, 126.2, 124.0. Elemental analysis [Found: C, 48.1; H, 3.2; N, 8.9%. $C_{13}H_{10}N_2O_6S$ calculated: C, 48.4; H, 3.1; N, 8.7%].

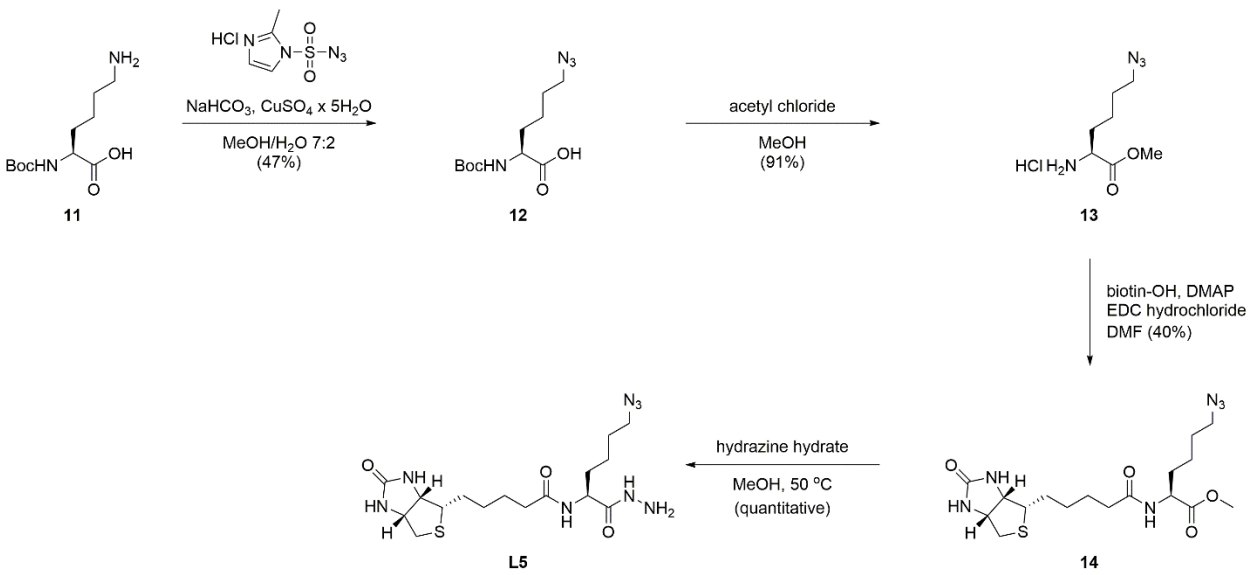
4-nitrophenyl 6-(4-sulfamoylbenzamido)hexanoate (10)

To a solution of *p*-nitrophenyl ester **9** (1.61 g, 5.0 mmol) in anhydrous DMF (50 mL), 6-aminohexanoic acid (0.59 g, 4.5 mmol) dissolved in H_2O (2 mL) was added dropwise over 6 h and the mixture was stirred at room temperature for 24 h, followed by addition of EDC hydrochloride (1.44 g, 7.5 mmol). After stirring for 24 h, the reaction mixture was concentrated under reduced pressure and resuspended in 1 M HCl (100 mL). The resulting white precipitate was filtered off and successively washed with 1 M HCl (50 mL), H_2O (50 mL) and ether (50 mL). The resulting solid was dissolved in acetone, dry-loaded onto celite and purified with automated flash column chromatography (2% MeOH/DCM to 10% MeOH/DCM) to yield **10** (1.35 g, 3.5 mmol, 69%) as an off-white powder. ^{1}H NMR (400 MHz, CD_3OD) δ = 8.29 (d, *J* = 9.2 Hz, 2H), 7.97 (d, *J* = 8.7 Hz, 2H), 7.97 (d, *J* = 8.9 Hz, 2H), 7.36 (d, *J* = 9.2 Hz, 2H), 3.44 (t, *J* = 7.1, 7.1, 2H), 2.68 (t, *J* = 7.4, 7.4, 2H), 1.82 (app p, *J* = 7.4 2H), 1.72 (app p, *J* = 7.2 Hz, 2H), 1.54 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ = 171.2, 165.1, 155.4, 146.1, 145.0, 137.5, 127.8, 125.6, 125.2, 123.2, 33.4, 28.6, 25.8, 23.9. HRMS *m/z* calculated for $[M+H]^+$ 463.117, found 463.117.

***N*-(6-hydrazineyl-6-oxopentyl)-4-sulfamoylbenzamide (L2)**

4-nitrophenyl 6-(4-sulfamoylbenzamido)hexanoate **10** (100 mg, 0.23 mmol) was dissolved in DMF (1 mL) and slowly, dropwise, added to a solution of hydrazine hydrate (50-60%; 0.13 mL, 2.4 mmol) in EtOH (3.1 mL) under nitrogen atmosphere. The mixture was stirred at room temperature overnight, followed by addition of ether (25 mL). The resultant suspension was stirred for 2 h, filtered over a glass filter, rinsed with ether (5 mL, 2×) and dried under vacuum for 3 h. A second crop of product could be obtained by concentrating the filtrate under reduced pressure, resuspension with ether (10 mL), filtration over a glass filter, rinsing with ether (5 mL, 2×) and drying under vacuum for 3 h. The two crops were combined to yield **L2** (23 mg, 0.07 mmol, 31%) as an off-white solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.90 (s, 1H), 8.61 (t, *J* = 6.0 Hz, 1H), 7.97 (d, *J* = 8.1 Hz, 2H), 7.88 (d, *J* = 8.1 Hz, 2H), 7.46 (s, 2H), 4.13 (s, 2H), 3.25 (q, *J* = 6.4 Hz, 2H), 2.01 (t, *J* = 7.5 Hz, 2H), 1.52 (p, *J* = 7.5 Hz, 4H), 1.23-1.31 (m, 2H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.5, 165.0, 146.1, 137.6, 127.8, 125.6, 33.4, 28.9, 28.8, 26.2, 25.0. HRMS *m/z* calculated for $[M+H]^+$ 329.128, found 329.129.

Synthesis ligand **L5**



Boc-Lys(N_3)-OH (12) *adapted from*[16]

To a suspension of Boc-Lys-OH **11** (357 mg, 1.45 mmol), 2-methyl-1*H*-imidazole-1-sulfonyl azide hydrochloride (453 mg, 2.0 mmol) and $NaHCO_3$ (499 mg, 5.9 mmol) in MeOH (9 mL) was added $CuSO_4 \times 5H_2O$ (14 mg, 0.09 mmol) dissolved in H_2O (2.6 mL). The resultant blue suspension was stirred at room temperature for 5 h, then extra 2-methyl-1*H*-imidazole-1-sulfonyl azide hydrochloride (64 mg, 0.29 mmol) was added and the reaction mixture was stirred for another 1 h. The mixture was acidified to pH 3 with $KHSO_4$ (1 M), diluted with H_2O (40 mL) and extracted with EtOAc (25 mL, 3×). The combined organic layers were washed with brine (30 mL, 1×), dried over Na_2SO_4, concentrated under reduced pressure and purified by column chromatography (1% AcOH/DCM -> 0.5% MeOH/1% AcOH/DCM, R_f 0.16 in 1% AcOH/DCM) to yield **12** (187 mg, 0.69 mmol, 47%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.03 – 4.94 (m, 1H), 4.32 (m, 1H), 3.30 (t, *J* = 6.6 Hz, 2H), 1.98 – 1.83 (m, 1H), 1.74-1.60 (m, 3H), 1.50 (m, 2H), 1.46 (s, 9H).

HCl.H_2N-Lys(N_3)-OMe (13) *adapted from*[17]

MeOH (10 mL) was cooled with an ice-water bath under nitrogen atmosphere, followed by addition of acetyl chloride (0.15 mL, 2.1 mmol). The mixture was warmed to room temperature, a suspension of Boc-Lys(N_3)-OH **12** (187 mg, 0.69 mmol) in MeOH (2 mL) was added, the mixture was refluxed for 8 h and subsequently concentrated under reduced pressure to yield HCl.H_2N-Lys(N_3)-OMe **13** (139 mg, 0.62 mmol,

91%) as a white solid. ^{1}H NMR (400 MHz, D_2O) δ 4.19 (t, *J* = 6.5 Hz, 1H), 3.87 (s, 3H), 3.38 (t, *J* = 6.6 Hz, 2H), 2.10 – 1.90 (m, 2H), 1.66 (m, 2H), 1.60 – 1.42 (m, 2H).

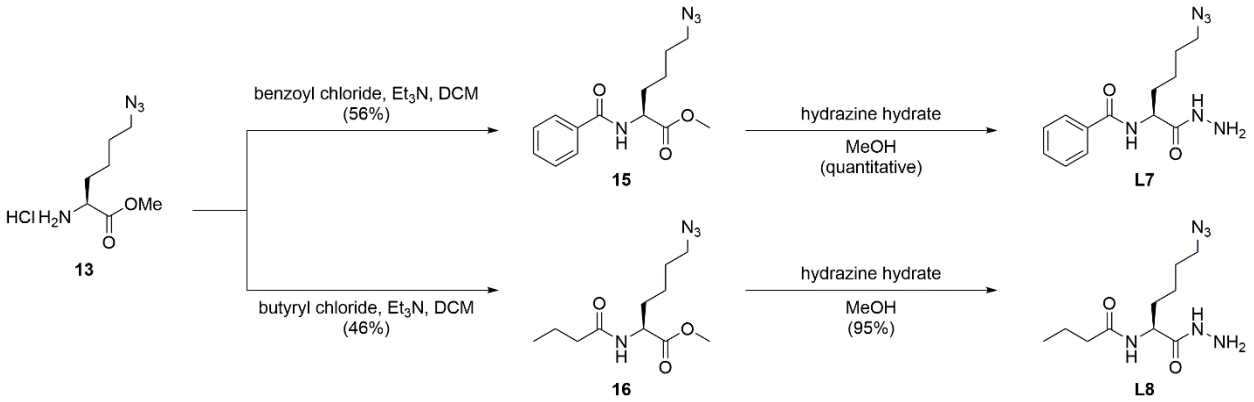
Biotin-Lys(N_3)-OMe (14)

To a solution of biotin-OH (155 mg, 0.63 mmol) and DMAP (7 mg, 0.06 mmol) in DMF (10 mL) was added EDC hydrochloride (124 mg, 0.65 mmol). After 5 minutes, a solution of HCl.H_2N-Lys(N_3)-OMe **13** (139 mg, 0.62 mmol) in DMF (2 mL) was added, the mixture was stirred at room temperature overnight and then concentrated under reduced pressure. After dilution with DCM (50 mL), the resultant suspension was washed with 1 M $KHSO_4$ (30 mL, 2×), aqueous $NaHCO_3$ (sat., 40 mL, 2×) and water (30 mL, 1×). The separatory funnel was rinsed with MeOH to remove any remaining solids, this rinsing solution was combined with the other organic layer and concentrated under reduced pressure to yield biotin-Lys(N_3)-OMe **14** (104 mg, 0.25 mmol, 40%) as an off-white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 4.51 (m, 1H), 4.39 (dd, *J* = 9.2, 5.0 Hz, 1H), 4.32 (m, 1H), 3.72 (s, 3H), 3.22 (m, 1H), 3.30 (m, 2H), 2.94 (dd, *J* = 12.7, 5.0 Hz, 1H), 2.73 (d, *J* = 12.6 Hz, 1H), 2.28 (t, *J* = 7.3 Hz, 2H), 1.90 – 1.55 (m, 8H), 1.46 (m, 4H). ^{13}C NMR (101 MHz, CD_3OD) δ 176.3, 174.3, 166.0, 63.3, 61.7, 57.0, 53.5, 52.7, 52.2, 41.0, 36.2, 31.9, 29.6, 29.4, 29.4, 26.7, 24.2. HRMS *m/z* calculated for $[M+H]^+$ 413.197, found 413.196.

Biotin-Lys(N_3)-$NHNH_2$ (L5)

Biotin-Lys(N_3)-OMe **14** (82 mg, 0.20 mmol) was suspended in MeOH (5 mL), followed by addition of hydrazine hydrate (50-60%, 0.3 mL, 5 mmol) and heating at 50 °C overnight. Subsequently, the mixture was concentrated under reduced pressure and co-evaporated with MeOH (2×) to yield biotin-Lys(N_3)-$NHNH_2$ **L1** (82 mg, 0.20 mmol, quantitative) as a white solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 9.12 (s, 1H), 7.91 (m, 1H), 6.41 (s, 1H), 6.35 (s, 1H), 4.31 (m, 1H), 4.21 (m, 3H), 4.13 (m, 1H), 3.31 (m, 2H), 3.10 (m, 1H), 2.83 (dd, *J* = 12.4, 5.1 Hz, 1H), 2.58 (d, *J* = 12.5 Hz, 1H), 2.12 (m, 2H), 1.67 – 1.57 (m, 2H), 1.56 (m, 6H), 1.39 – 1.20 (m, 4H). ^{13}C NMR (150 MHz, DMSO-d_6) δ 171.9, 171.0, 162.7, 61.0, 59.2, 55.4, 50.7, 50.5, 40.1, 34.8, 31.7, 28.1, 28.0, 27.8, 25.2, 22.6 (note: the ppm value of the peak at 40.1 could not exactly be determined due to overlapping DMSO-d_6 signals but was confirmed by HSQC to be present). HRMS *m/z* calculated for $[M+H]^+$ 413.208, found 413.207.

Synthesis ligand **L7** and **L8**



Bz-Lys(N_3)-OMe (15)

A mixture of HCl.H_2N-Lys(N_3)-OMe **13** (45 mg, 0.20 mmol) and Et_3N (0.7 mL, 5 mmol) in dry DCM (2 mL) was cooled with an ice-water bath while under nitrogen atmosphere. Subsequently, benzoyl chloride (0.03 mL, 0.26 mmol) was added and the reaction was left stirring at room temperature overnight. The crude reaction mixture was directly purified by column chromatography (0 -> 1% MeOH/DCM) to yield Bz-Lys(N_3)-OMe **15** (33 mg, 0.11 mmol, 56%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.79 (dd, *J* = 8.1, 1.6 Hz, 2H), 7.52 – 7.46 (m, 1H), 7.42 (m, 2H), 6.82 (d, *J* = 7.8 Hz, 1H), 4.82 (dd, *J* = 12.9, 7.4 Hz, 1H), 3.77 (s, 2H), 3.25 (m, 2H), 1.98 (m, 1H), 1.80 (m, 1H), 1.68 – 1.56 (m, 2H), 1.46 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.0, 167.2, 133.9, 131.9, 128.7, 127.2, 52.6, 52.4, 51.1, 32.2, 28.5, 22.6. HRMS *m/z* calculated for $[M+H]^+$ 313.127, found 313.127.

Bz-Lys(N_3)-$NHNH_2$ (L7)

Hydrazine hydrate (50-60%; 0.17 mL, 2.7 mmol) was added to a solution of Bz-Lys(N_3)-OMe **15** (33 mg, 0.11 mmol) in MeOH (3 mL) and the reaction mixture was stirred at room temperature overnight. Concentration under reduced pressure, followed by co-evaporation with MeOH (1×), yielded Bz-Lys(N_3)-$NHNH_2$ **L7** (35 mg, 0.11 mmol, quantitative) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 7.87 (m, 2H), 7.56 (m, 1H), 7.48 (m, 2H), 4.55 (dd, *J* = 8.6, 6.1 Hz, 1H), 3.32 (m, 2H), 1.88 (m, 2H), 1.65 (m, 2H), 1.60 – 1.36 (m, 2H). ^{13}C NMR (100 MHz, CD_3OD) δ 173.6, 170.2, 135.2, 132.9, 129.5, 128.5, 53.9, 52.2, 32.6, 29.5, 24.3. HRMS *m/z* calculated for $[M+H]^+$ 313.138, found 313.138.

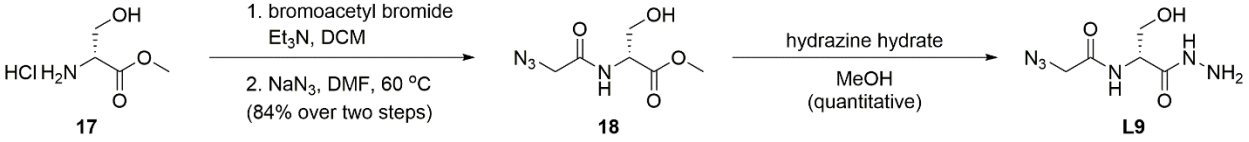
butyryl-Lys(N_3)-OMe (16)

A mixture of HCl.H_2N-Lys(N_3)-OMe **13** (79 mg, 0.36 mmol) and Et_3N (0.1 mL, 0.75 mmol) in dry DCM (4 mL) was cooled with an ice-water bath while under nitrogen atmosphere. Subsequently, butyryl chloride (0.04 mL, 0.36 mmol) was added and the reaction was left stirring at room temperature overnight. The crude

reaction mixture was directly purified by column chromatography (0 -> 1% MeOH/DCM) to yield butyryl-Lys(N_3)-OMe **16** (42 mg, 0.16 mmol, 46%) as a colorless oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.12 (d, *J* = 8.0 Hz, 1H), 4.60 (td, *J* = 7.8, 5.3 Hz, 1H), 3.71 (s, 3H), 3.23 (td, *J* = 6.8, 1.5 Hz, 2H), 2.17 (m, 2H), 1.90 – 1.78 (m, 2H), 1.71 – 1.51 (m, 4H), 1.45 – 1.29 (m, 2H), 0.92 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 173.1, 172.9, 52.5, 51.7, 51.1, 38.5, 32.1, 28.4, 22.5, 19.1, 13.7. HRMS *m/z* calculated for $[M+H]^+$ 279.143, found 279.143.

butyryl-Lys(N_3)-$NHNH_2$ (L8)

Hydrazine hydrate (50-60%; 0.25 mL, 3.9 mmol) was added to a solution of butyryl-Lys(N_3)-OMe **16** (42 mg, 0.16 mmol) in MeOH (4 mL) and the reaction mixture was stirred at room temperature overnight. Concentration under reduced pressure, followed by co-evaporation with MeOH (1×), yielded butyryl-Lys(N_3)-$NHNH_2$ **L8** (40 mg, 0.16 mmol, quantitative) as a white solid. ^{1}H NMR (400 MHz, CD_3OD) δ 4.31 (dd, *J* = 8.6, 5.9 Hz, 1H), 3.32 (m, 2H), 2.24 (m, 2H), 1.87 – 1.57 (m, 6H), 1.56 – 1.33 (m, 2H), 0.97 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 176.1, 173.5, 53.1, 52.2, 38.7, 32.7, 29.5, 24.1, 20.3, 14.0. HRMS *m/z* calculated for $[M+H]^+$ 279.154, found 279.153.



N_3Ac-D-Ser-OMe (18)

Et_3N (1.5 mL, 10.5 mmol) was added to D-Serine methyl ester hydrochloride (772 mg, 5.0 mmol) in dry DCM (17 mmol) and the mixture was cooled with an ice-salt bath to -15 °C. Then, a solution of bromoacetyl bromide (0.43 mL, 5.0 mmol) in DCM (11 mL) was added dropwise over the course of 30 minutes. The cooling bath was removed and after 3 h of stirring at room temperature the reaction mixture was concentrated under reduced pressure. The resultant brown solid was redissolved in DMF (15 mL), followed by addition of sodium azide (1.34 g, 20 mmol) and heated at 60 °C overnight. The DMF was removed by concentrating under reduced pressure and subsequent purification by column chromatography (0 -> 3% MeOH/DCM) yielded N_3Ac-D-Ser-OMe **18** (846 mg, 4.2 mmol, 84%) as a yellow oil. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.19 (d, *J* = 7.6 Hz, 1H), 4.68 (dt, *J* = 7.5, 3.6 Hz, 1H), 4.04 (s, 2H), 4.01 (m,

1H), 3.92 (m, 1H), 3.80 (s, 3H), 2.64 (t, *J* = 5.7 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.5, 167.4, 63.1, 54.6, 53.1, 52.6. HRMS *m/z* calculated for $[M+H]^+$ 225.0594, found 225.059.

N_3Ac-D-Ser-$NHNH_2$ (L9)

Hydrazine hydrate (50-60%; 1.7 mL, 327 mmol) was added to a solution of N_3Ac-D-Ser-OMe **18** (225 mg, 1.1 mmol) in MeOH (11 mL) and the reaction mixture was stirred at room temperature for 2 h. Concentration under reduced pressure, followed by co-evaporation with MeOH (2×), yielded N_3Ac-D-Ser-$NHNH_2$ **L9** (230 mg, 1.1 mmol, quantitative) as a yellow solid. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.18 (s, 1H), 8.13 (d, *J* = 8.1 Hz, 1H), 4.91 (t, *J* = 5.6 Hz, 1H), 4.30 – 4.17 (m, 3H), 3.94 – 3.81 (m, 2H), 3.54 (t, *J* = 5.5 Hz, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 168.8, 167.4, 61.6, 54.0, 50.6. HRMS *m/z* calculated for $[M+H]^+$ 225.071, found 225.070.

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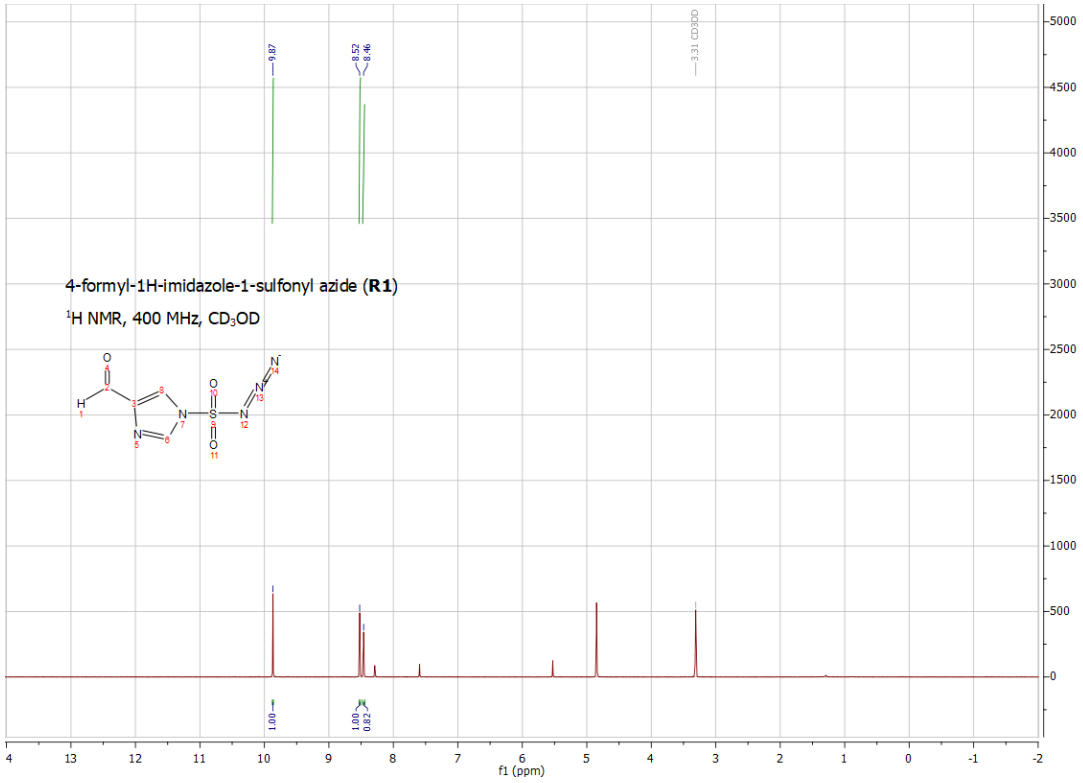
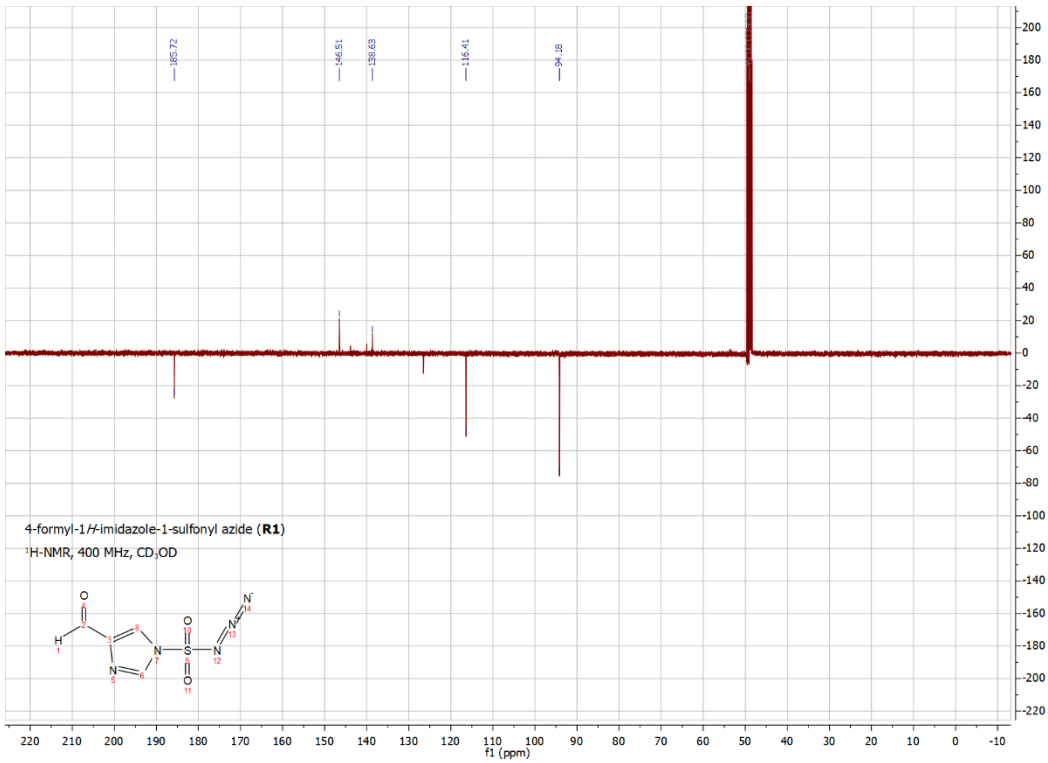
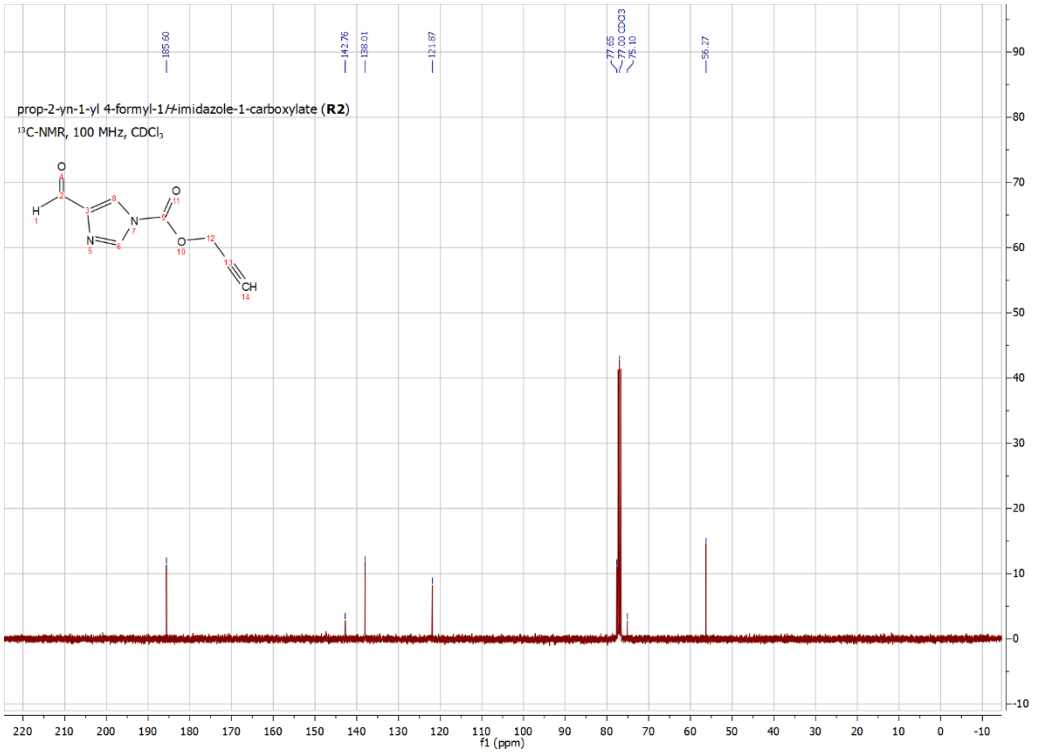
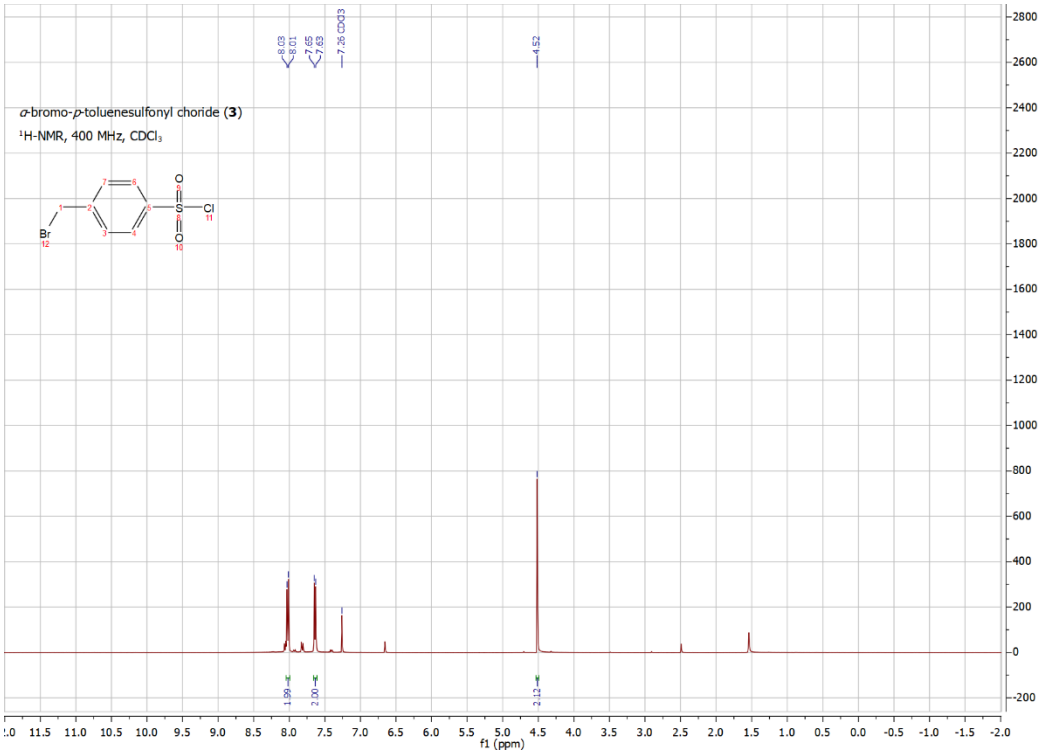
NMR spectra

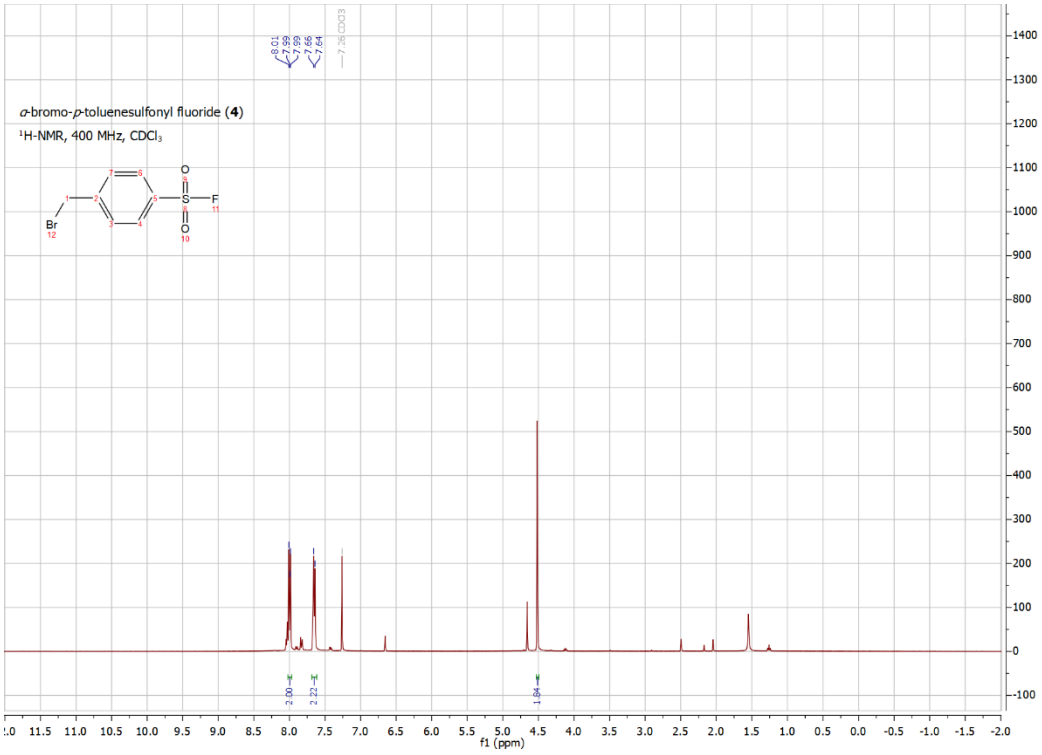

9.87
8.52
8.46
3.31 CD3OD
4-formyl-1H-imidazole-1-sulfonyl azide (**R1**)
^{1}H NMR, 400 MHz, CD_3OD
1.00
1.00
0.82
f1 (ppm)

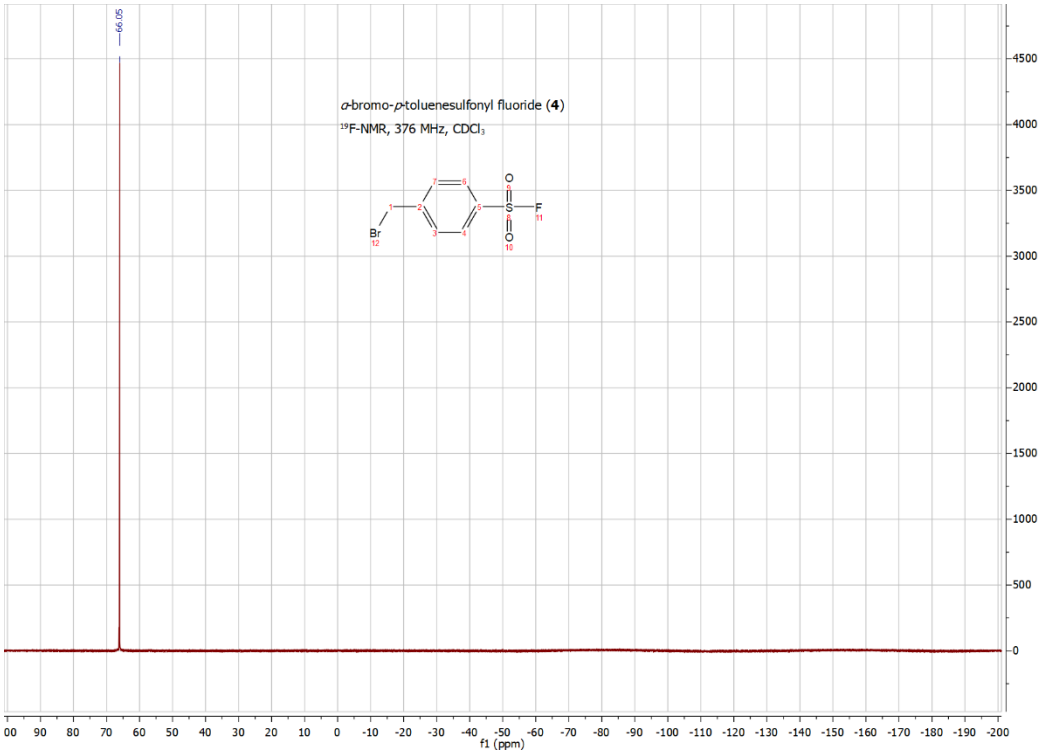

185.72
146.51
136.63
116.41
94.18
4-formyl-1*H*-imidazole-1-sulfonyl azide (**R1**)
^{1}H-NMR, 400 MHz, CD_3OD
f1 (ppm)

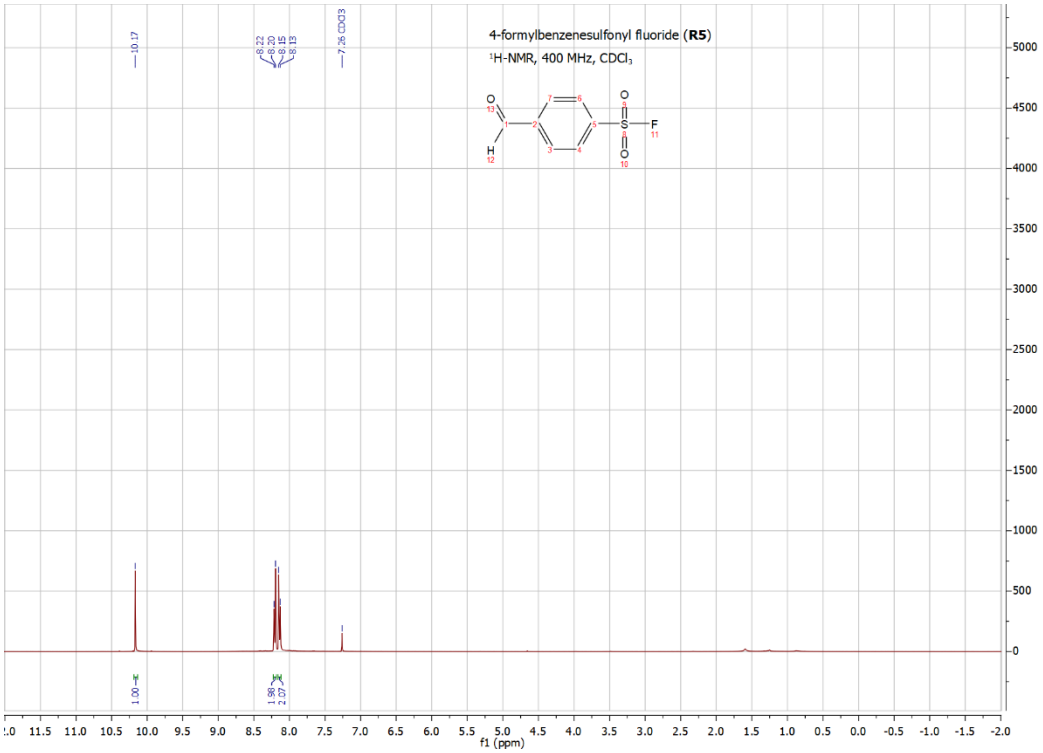

prop-2-yn-1-yl 4-formyl-1H-imidazole-1-carboxylate (R2)
1H-NMR, 400 MHz, CDCl3
9.95
8.23
8.10
7.26 CDCl3
5.06
5.05
5.05
2.68
2.68
2.67
1.00
0.96
0.94
2.11
0.95
f1 (ppm)

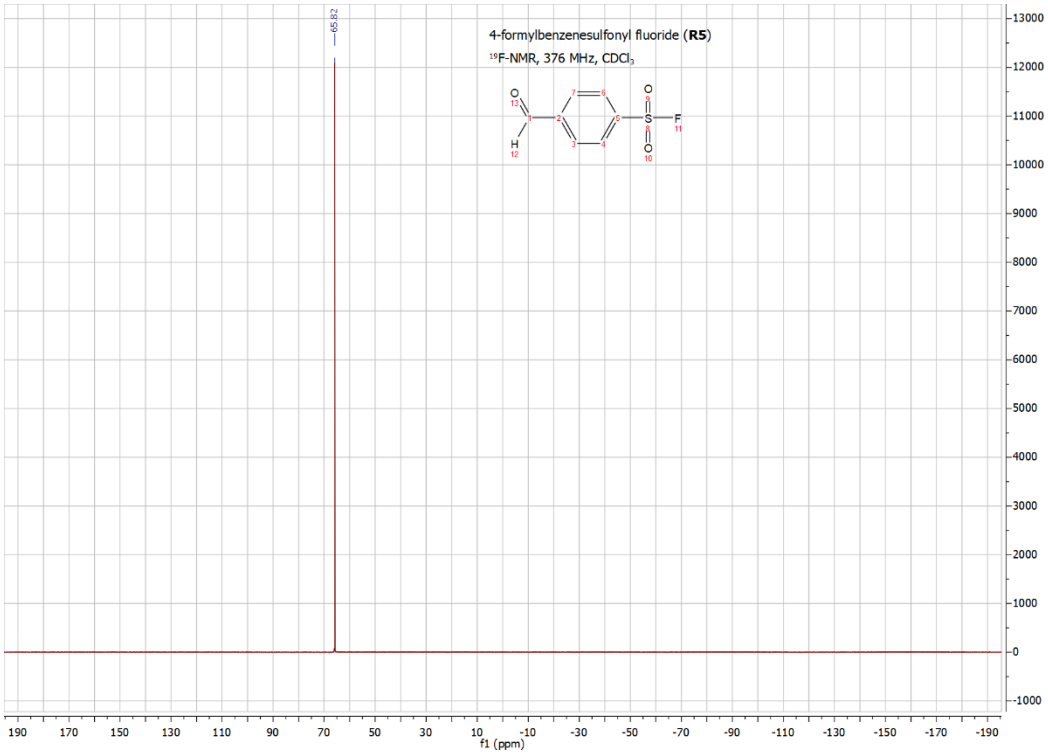

prop-2-yn-1-yl 4-formyl-1H-imidazole-1-carboxylate (R2)
13C-NMR, 100 MHz, CDCl3
185.60
142.76
138.01
121.87
77.65
77.00 CDCl3
75.10
56.27
f1 (ppm)

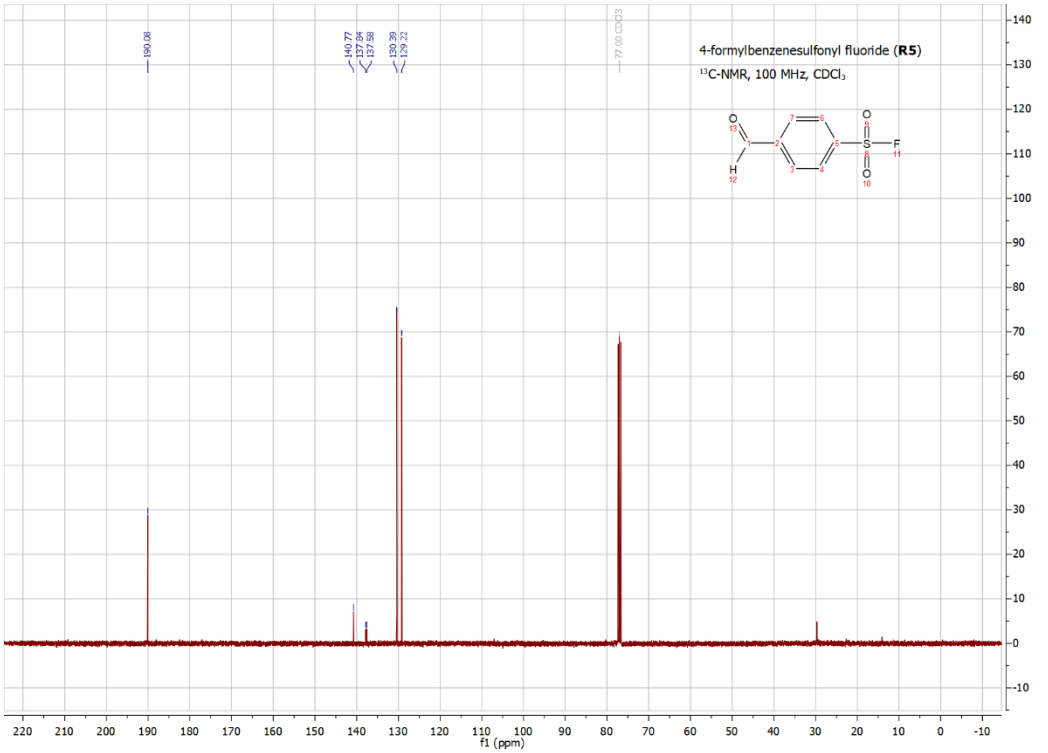

α-bromo-p-toluenesulfonyl choride (3)
¹H-NMR, 400 MHz, CDCl₃
8.03
8.01
7.65
7.63
7.26 CDCl3
4.52
1.99
2.00
2.12
f1 (ppm)

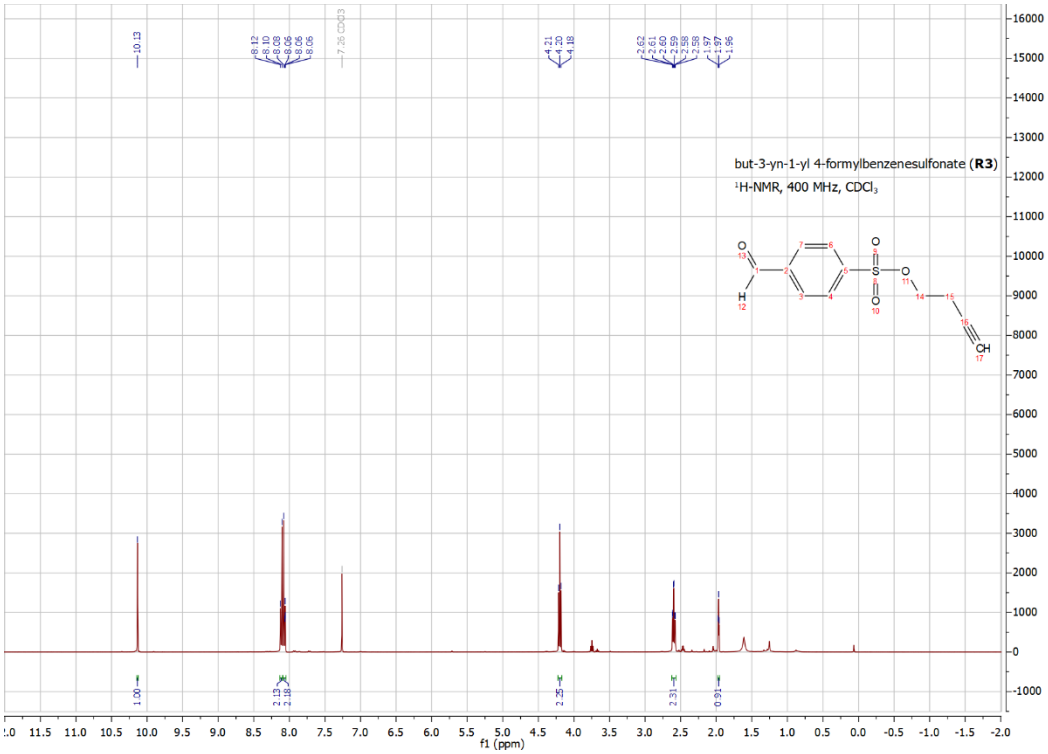

α-bromo-p-toluenesulfonyl fluoride (4)
¹H-NMR, 400 MHz, CDCl₃
8.01
7.99
7.66
7.64
7.26 CDCl3
2.00
2.22
1.94
f1 (ppm)

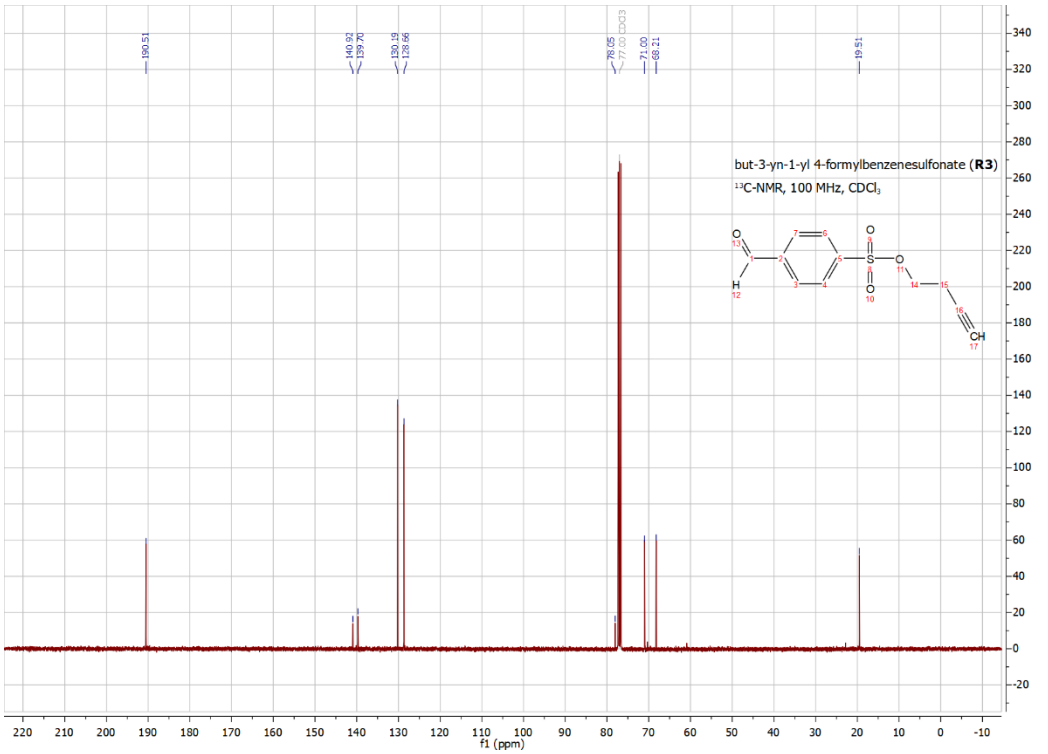

-66.05
o-bromo-p-toluenesulfonyl fluoride (4)
19F-NMR, 376 MHz, CDCl3
f1 (ppm)

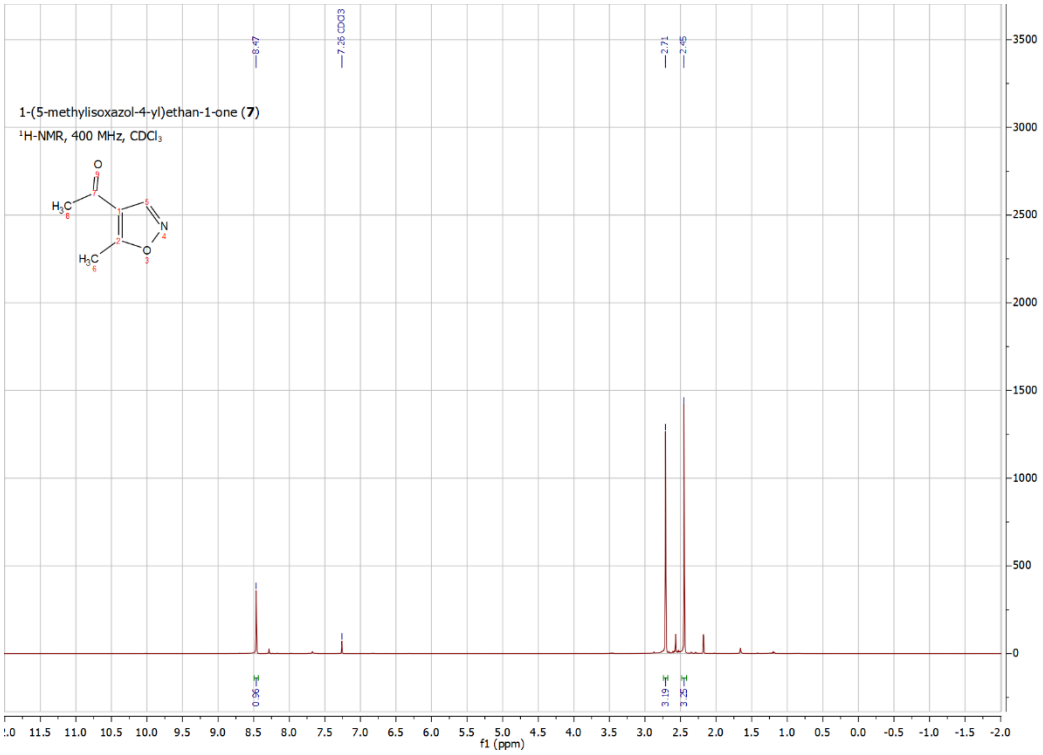

10.17
8.22
8.20
8.15
8.13
7.26 CDCl3
4-formylbenzenesulfonyl fluoride (R5)
1H-NMR, 400 MHz, CDCl3
1.00
1.98
2.07
f1 (ppm)

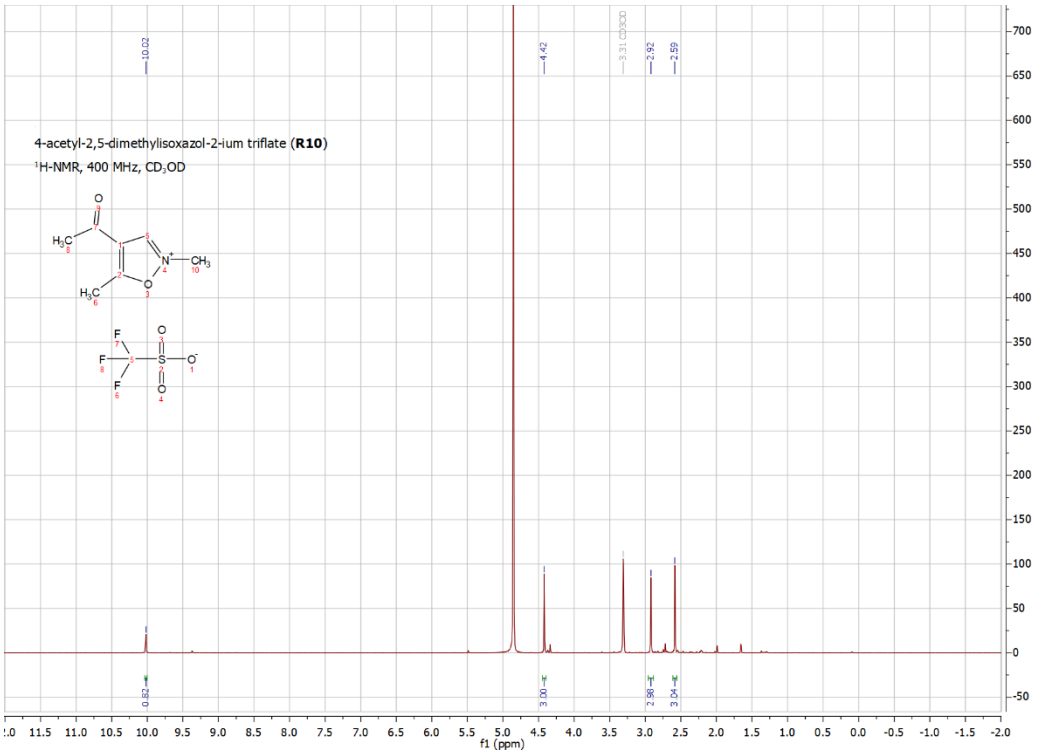

4-formylbenzenesulfonyl fluoride (**R5**)
^{19}F-NMR, 376 MHz, $CDCl_3$
-65.82
f1 (ppm)


4-formylbenzenesulfonyl fluoride (**R5**)
^{13}C-NMR, 100 MHz, $CDCl_3$
190.08
140.77
137.94
137.58
130.39
129.22
77.00 CDCl3
f1 (ppm)


but-3-yn-1-yl 4-formylbenzenesulfonate (**R3**)
^{1}H-NMR, 400 MHz, $CDCl_3$
f1 (ppm)


but-3-yn-1-yl 4-formylbenzenesulfonate (**R3**)
^{13}C-NMR, 100 MHz, $CDCl_3$
f1 (ppm)


1-(5-methylisoxazol-4-yl)ethan-1-one (7)
¹H-NMR, 400 MHz, CDCl₃
8.47
7.26 CDCl3
2.71
2.45
0.96
3.19
3.25
f1 (ppm)


4-acetyl-2,5-dimethylisoxazol-2-ium triflate (R10)
¹H-NMR, 400 MHz, CD₃OD
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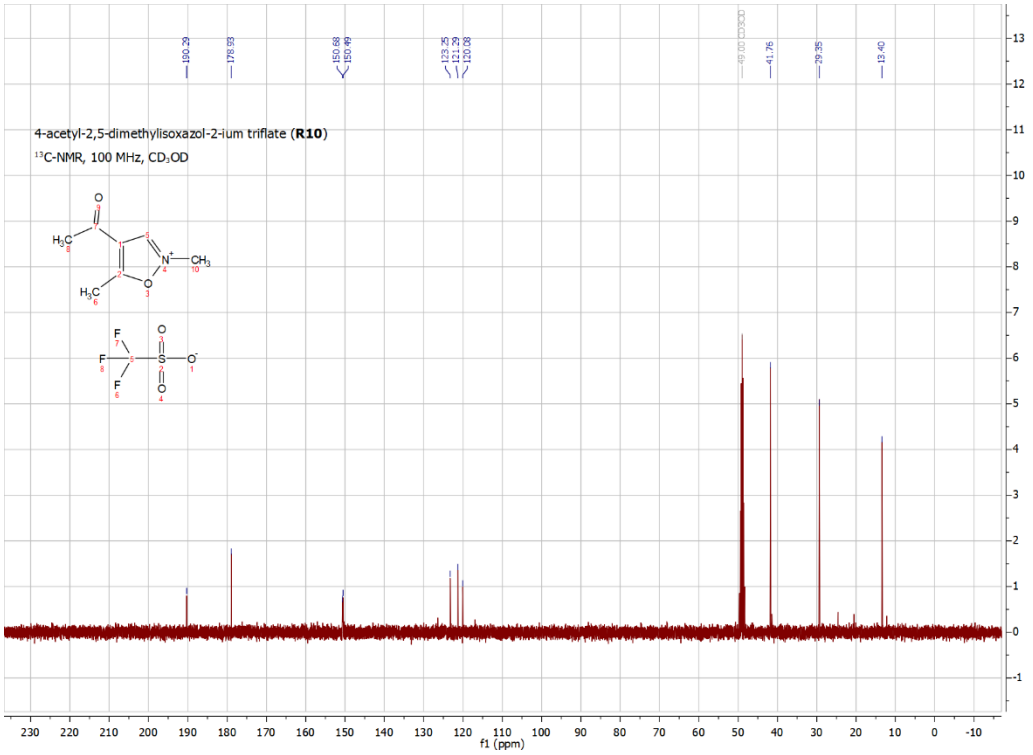
4.42
3.31 CD3OD
2.92
2.59
0.82
3.00
2.98
3.04
f1 (ppm)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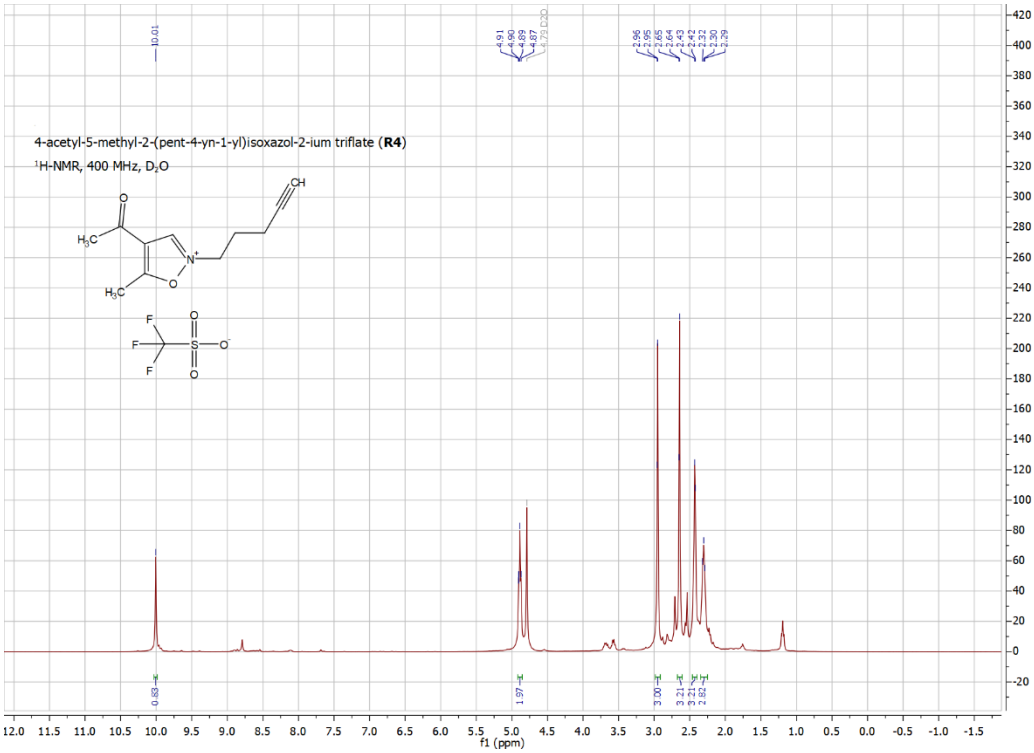
f1 (ppm)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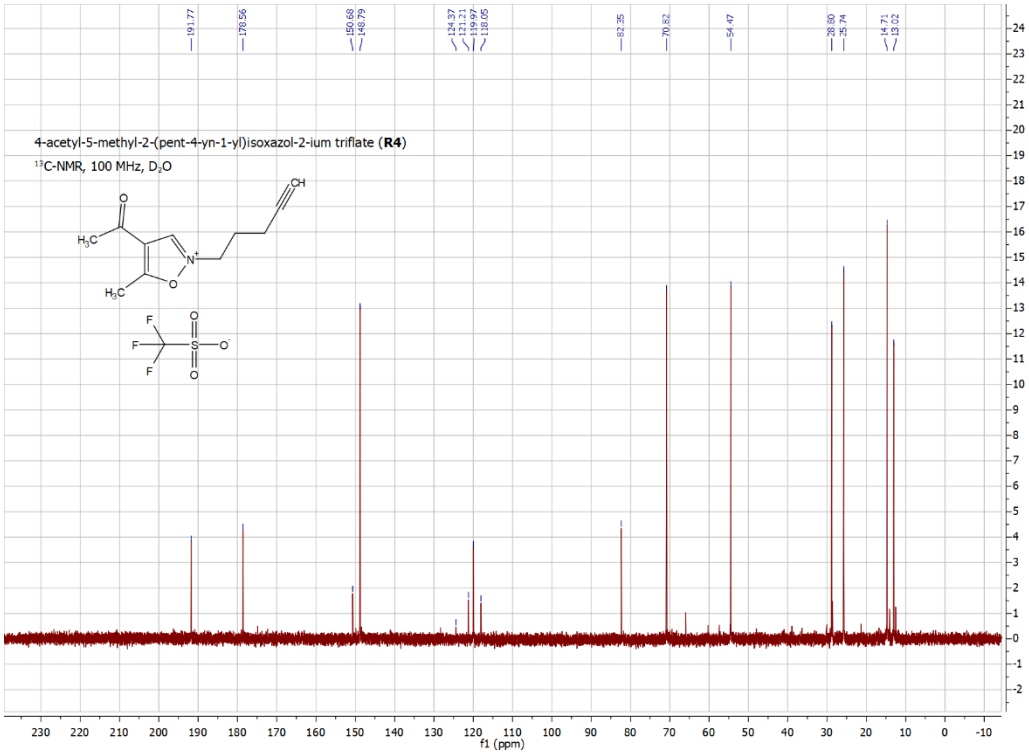
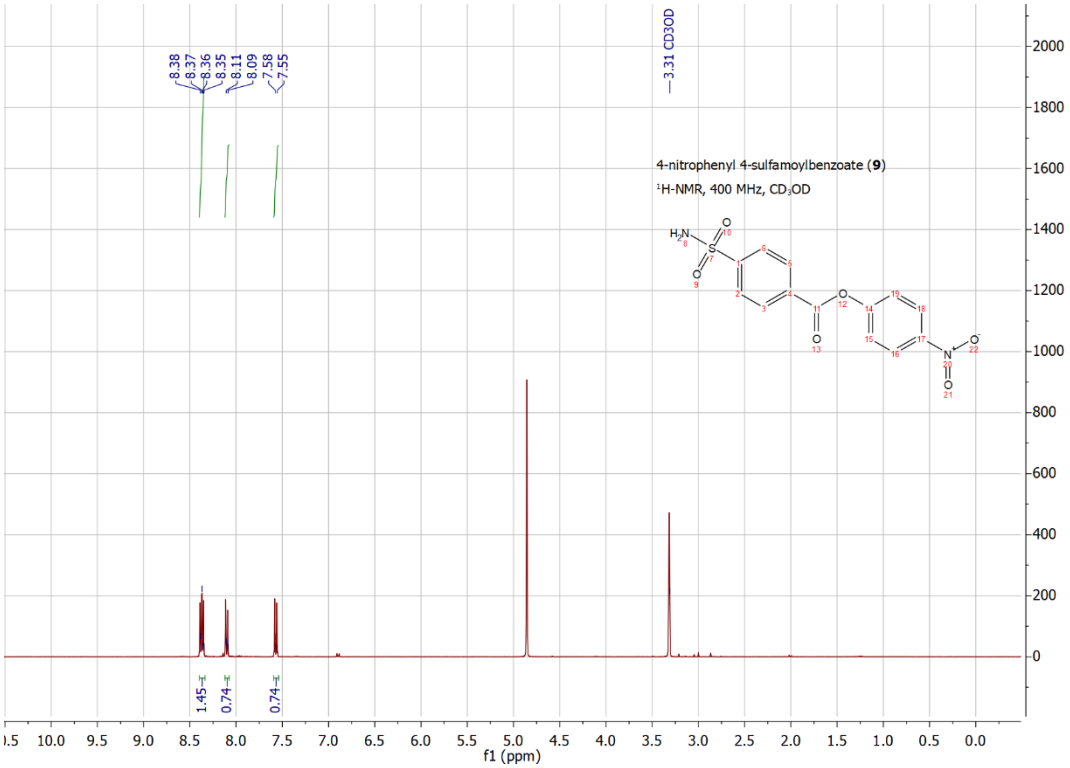
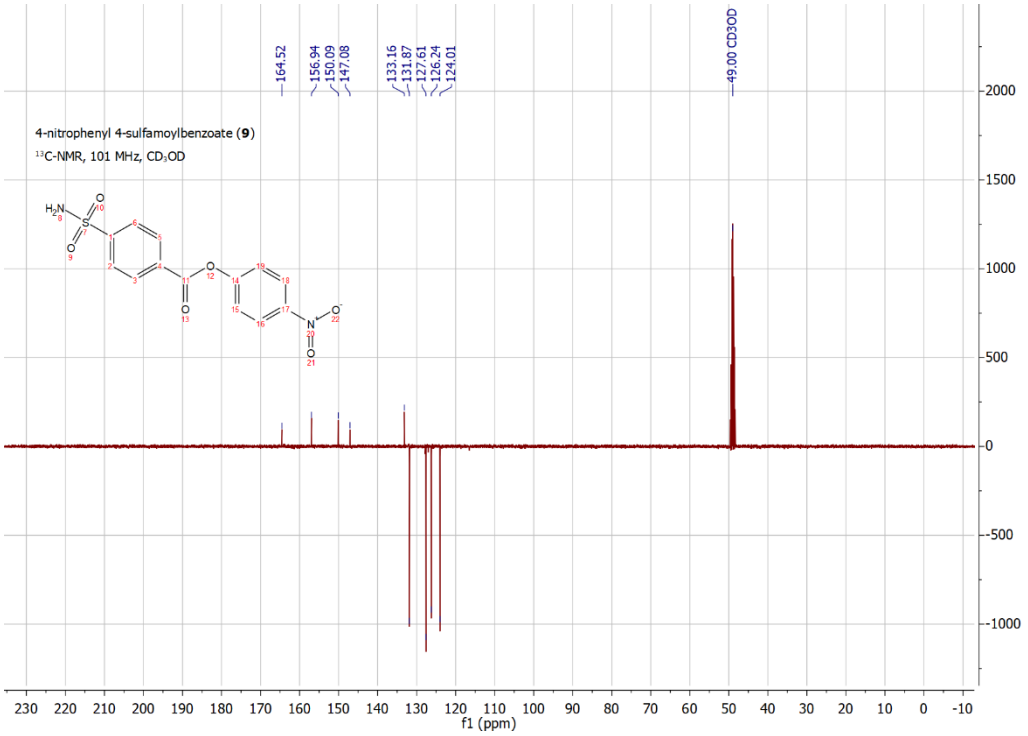
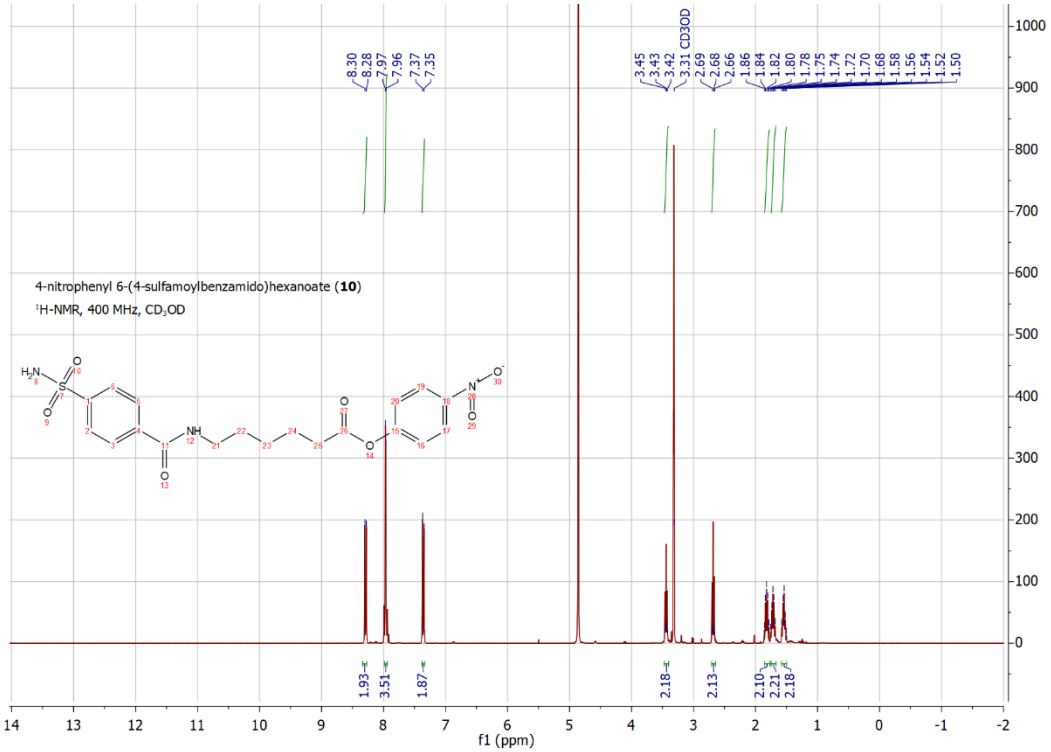
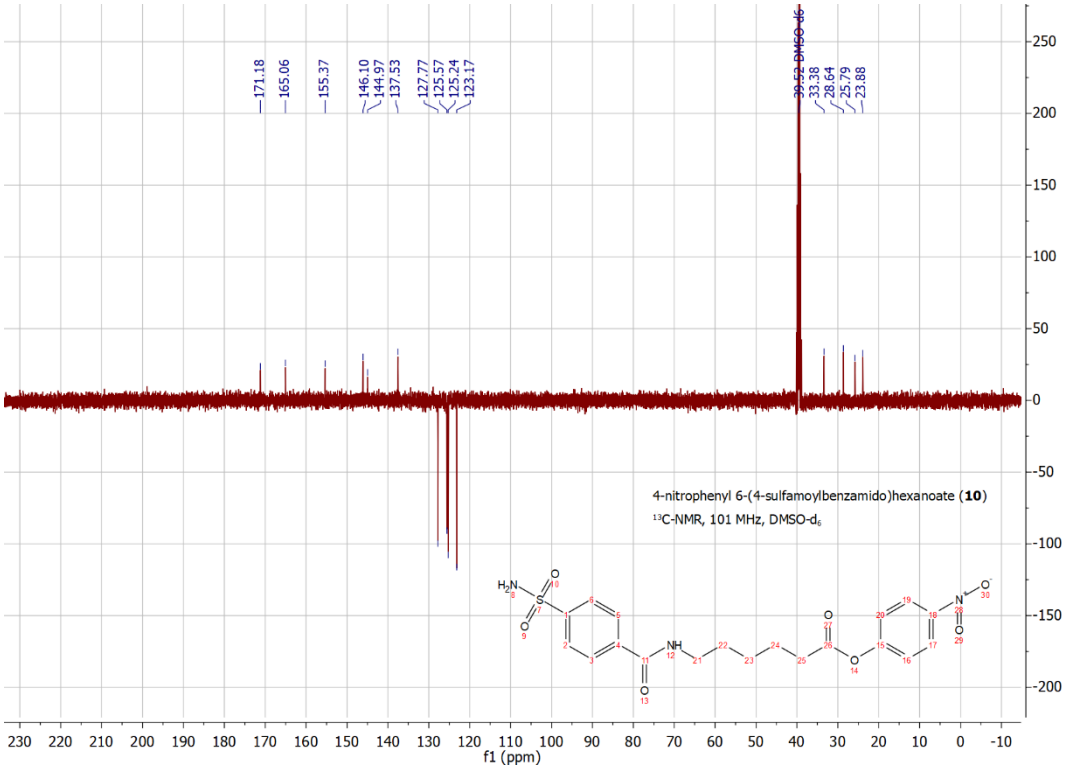
f1 (ppm)

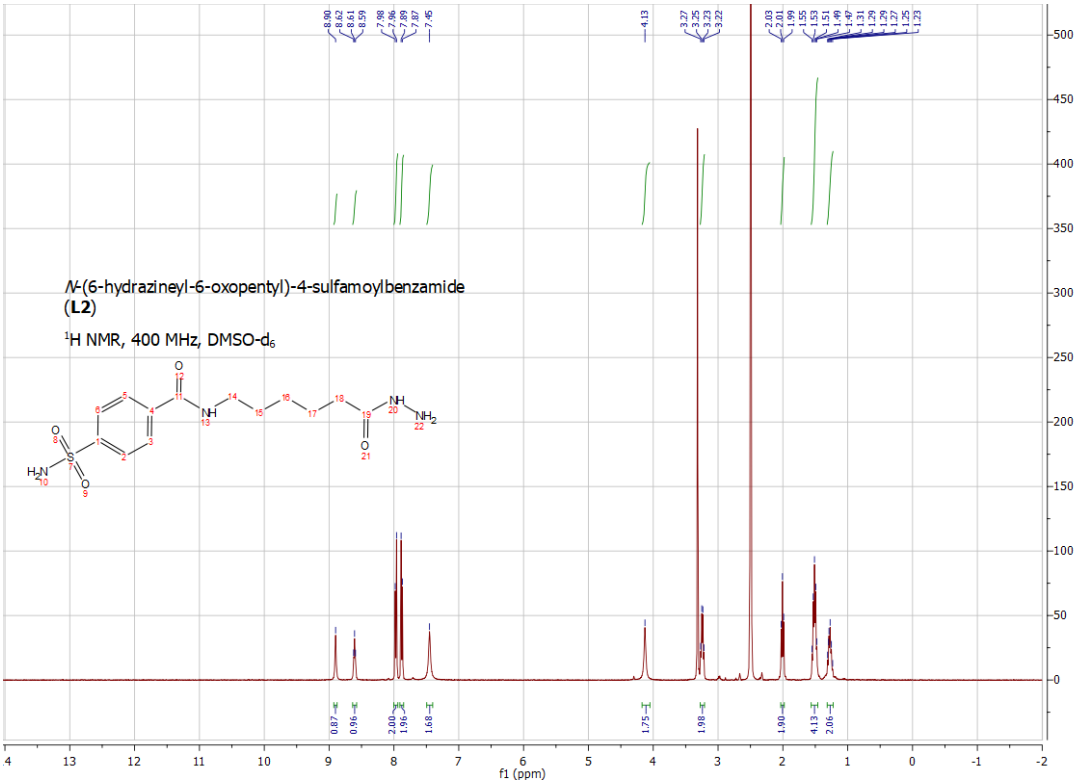

191.77
176.56
150.68
146.79
134.97
121.21
119.97
118.05
82.35
70.82
54.47
28.80
25.74
14.71
13.02
4-acetyl-5-methyl-2-(pent-4-yn-1-yl)isoxazol-2-ium triflate (R4)
13C-NMR, 100 MHz, D2O
f1 (ppm)

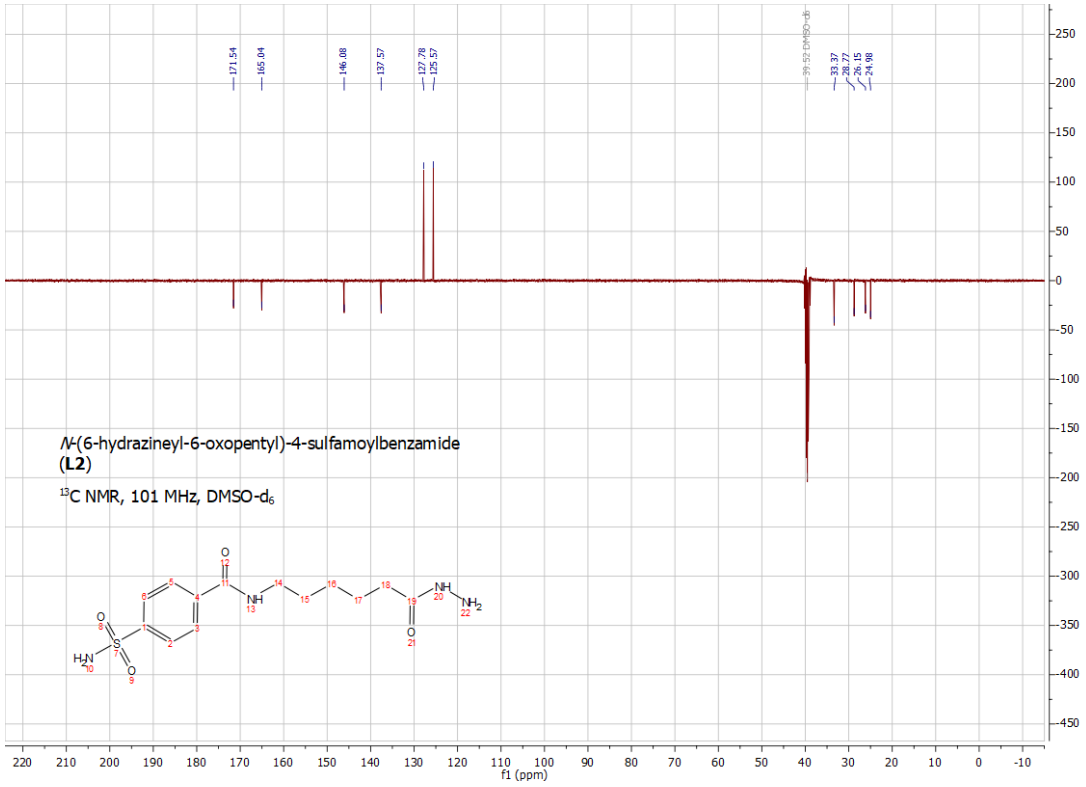

8.38
8.37
8.36
8.35
8.11
8.09
7.58
7.55
3.31 CD3OD
4-nitrophenyl 4-sulfamoylbenzoate (9)
1H-NMR, 400 MHz, CD3OD
1.45
0.74
0.74
f1 (ppm)

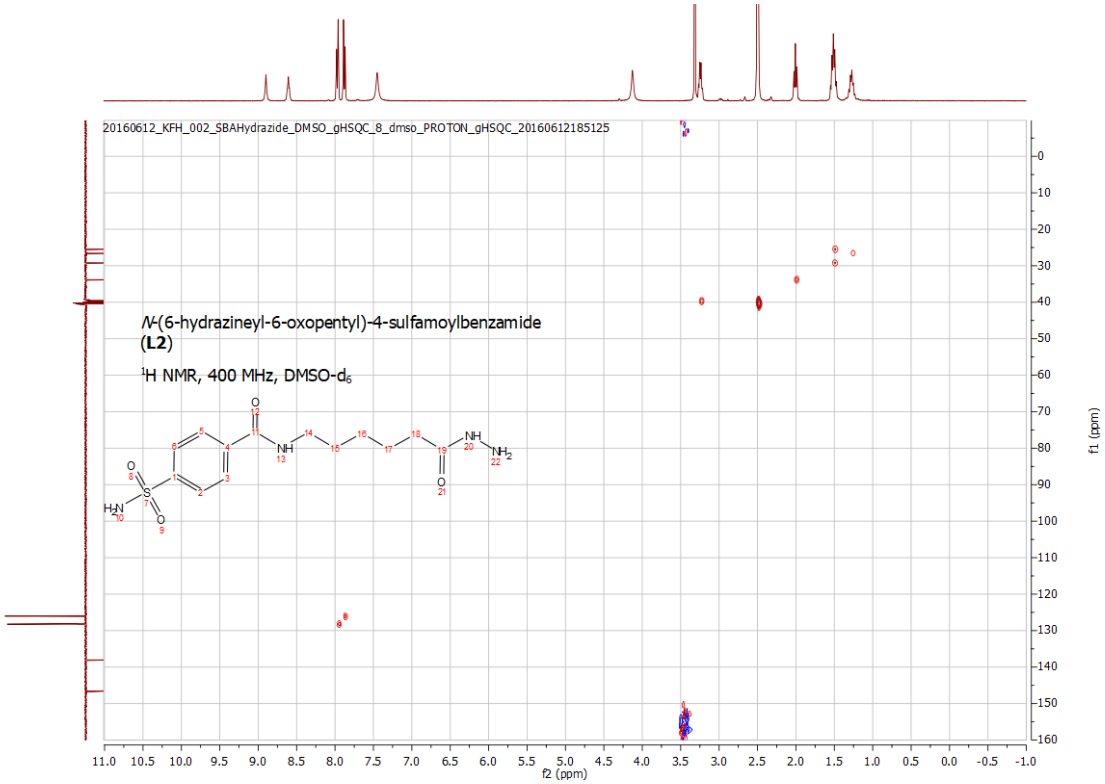

164.52
156.94
150.09
147.08
133.16
131.87
127.61
126.24
124.01
49.00 CD3OD
4-nitrophenyl 4-sulfamoylbenzoate (9)
13C-NMR, 101 MHz, CD3OD
f1 (ppm)

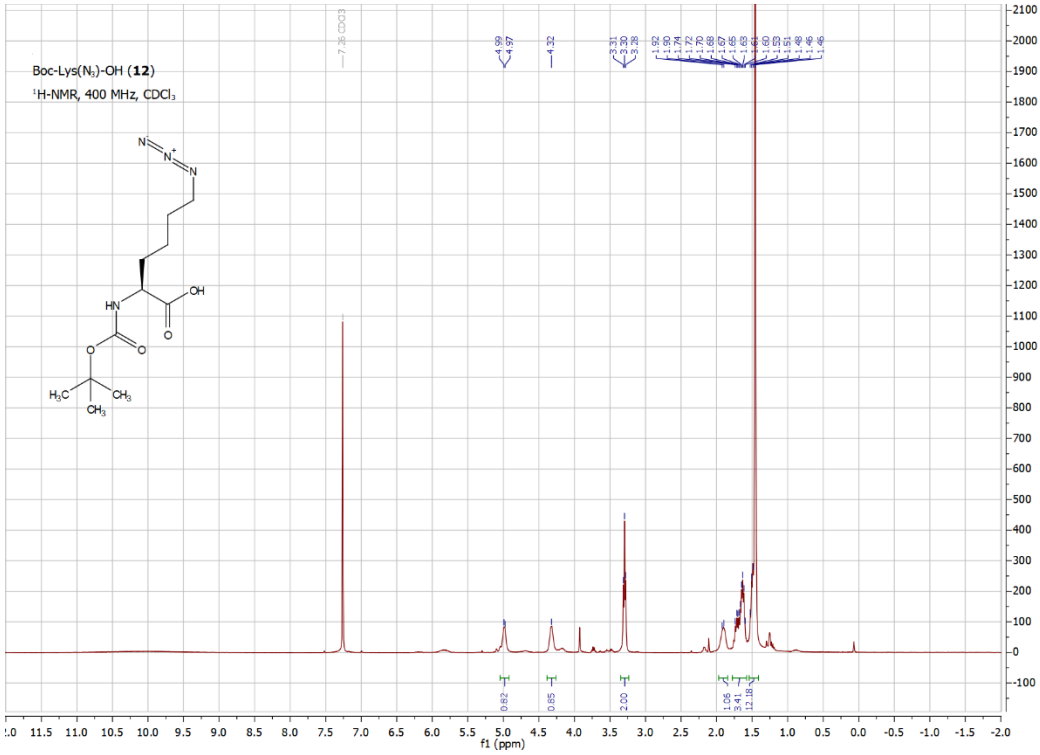

8.30
8.28
7.97
7.96
7.37
7.35
3.45
3.43
3.42
3.31 CD3OD
2.69
2.68
2.66
1.86
1.84
1.82
1.80
1.78
1.75
1.74
1.72
1.70
1.68
1.58
1.56
1.54
1.52
1.50
4-nitrophenyl 6-(4-sulfamoylbenzamido)hexanoate (10)
1H-NMR, 400 MHz, CD3OD
1.93
3.51
1.87
2.18
2.13
2.10
2.21
2.18
f1 (ppm)

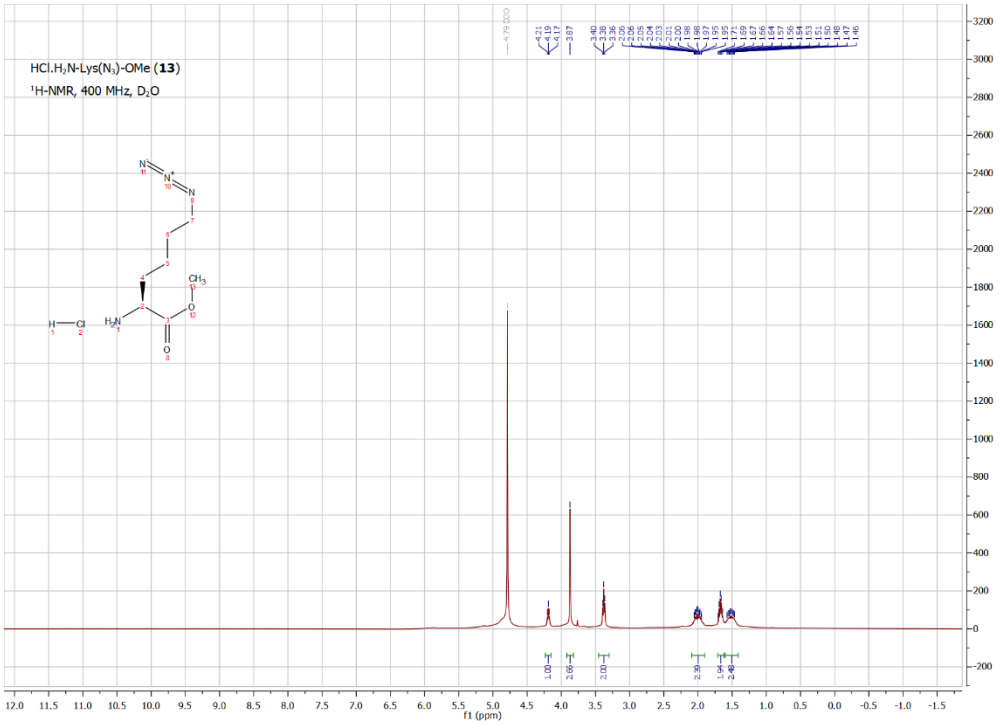

171.18
165.06
155.37
146.10
144.97
137.53
127.77
125.57
125.24
123.17
39.52 DMSO-d6
33.38
28.64
25.79
23.88
4-nitrophenyl 6-(4-sulfamoylbenzamido)hexanoate (10)
13C-NMR, 101 MHz, DMSO-d6
f1 (ppm)

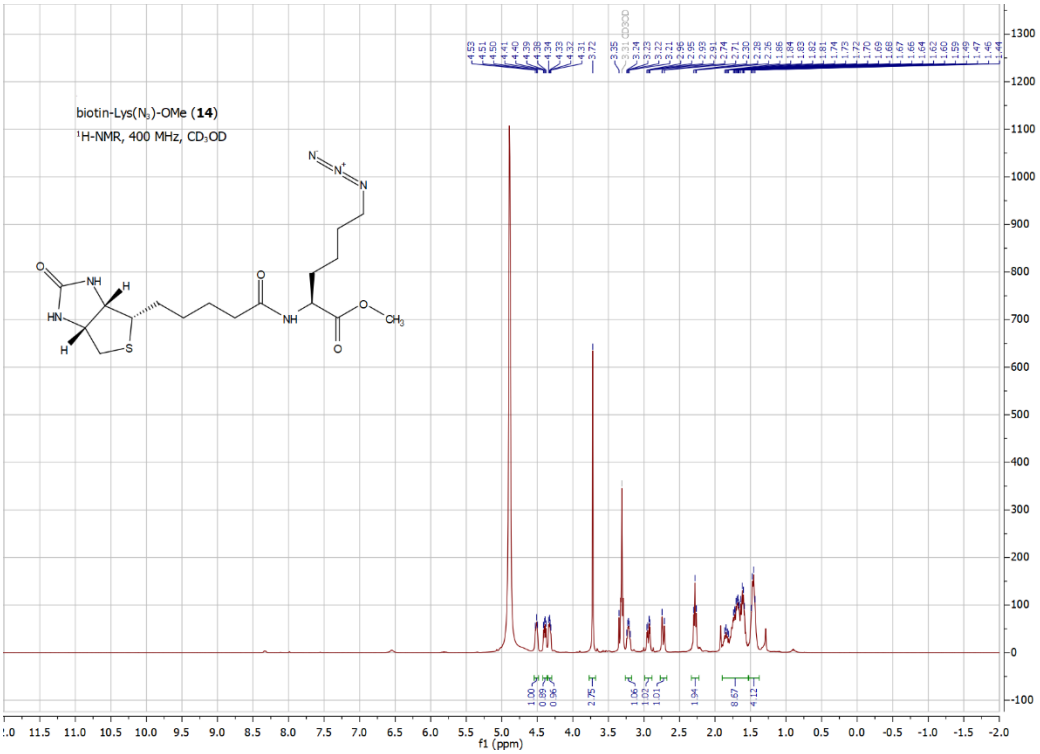

N-(6-hydrazineyl-6-oxopentyl)-4-sulfamoylbenzamide
(L2)
1H NMR, 400 MHz, DMSO-d6
f1 (ppm)

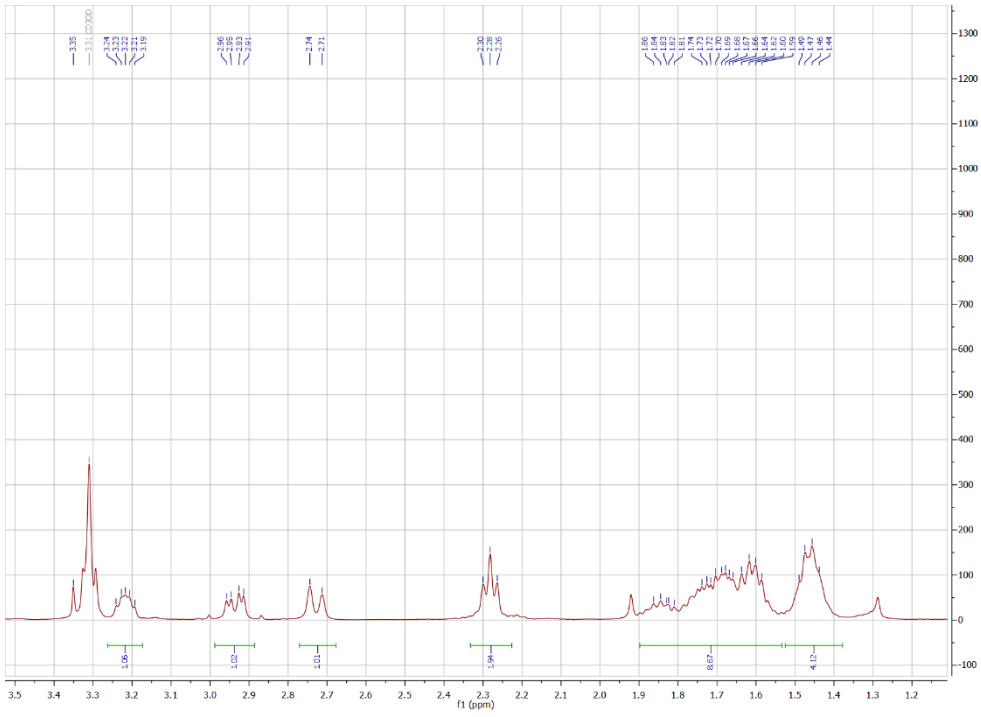

171.54
165.04
146.08
137.57
127.78
125.57
39.52 DMSO-d6
33.37
28.72
26.15
24.98
N-(6-hydrazineyl-6-oxopentyl)-4-sulfamoylbenzamide
(L2)
13C NMR, 101 MHz, DMSO-d6
f1 (ppm)

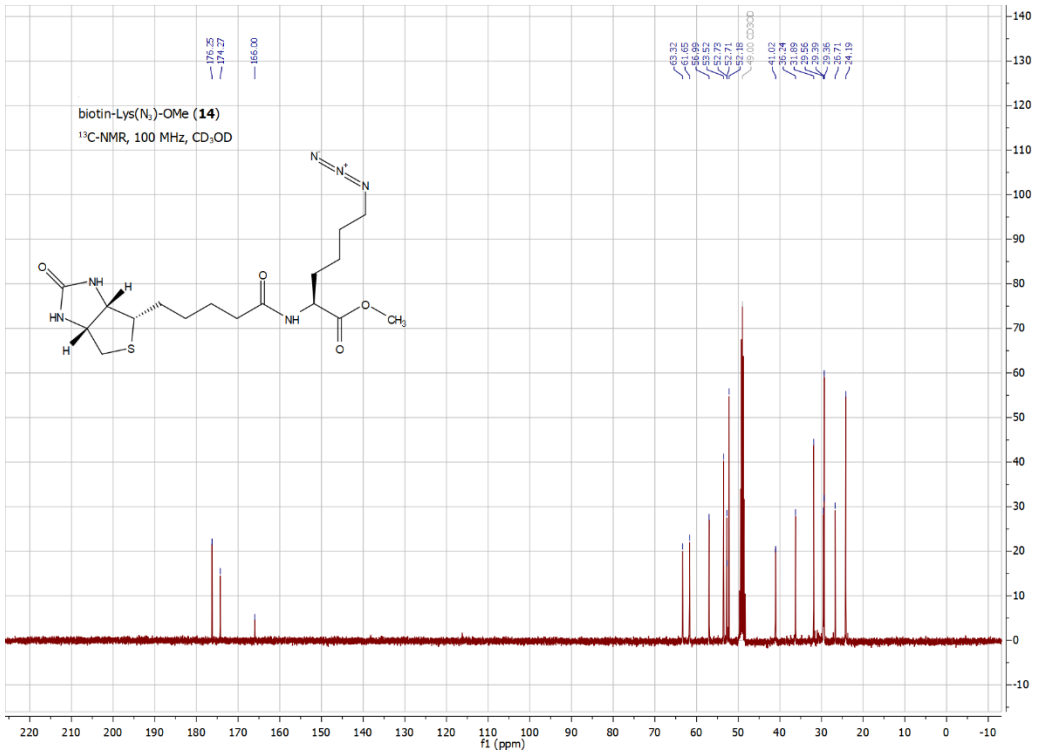

20160612_KFH_002_SBAHydrazide_DMSO_gHSQC_8_dmso_PROTON_gHSQC_20160612185125
N-(6-hydrazineyl-6-oxopentyl)-4-sulfamoylbenzamide
(L2)
1H NMR, 400 MHz, DMSO-d6
f2 (ppm)
f1 (ppm)

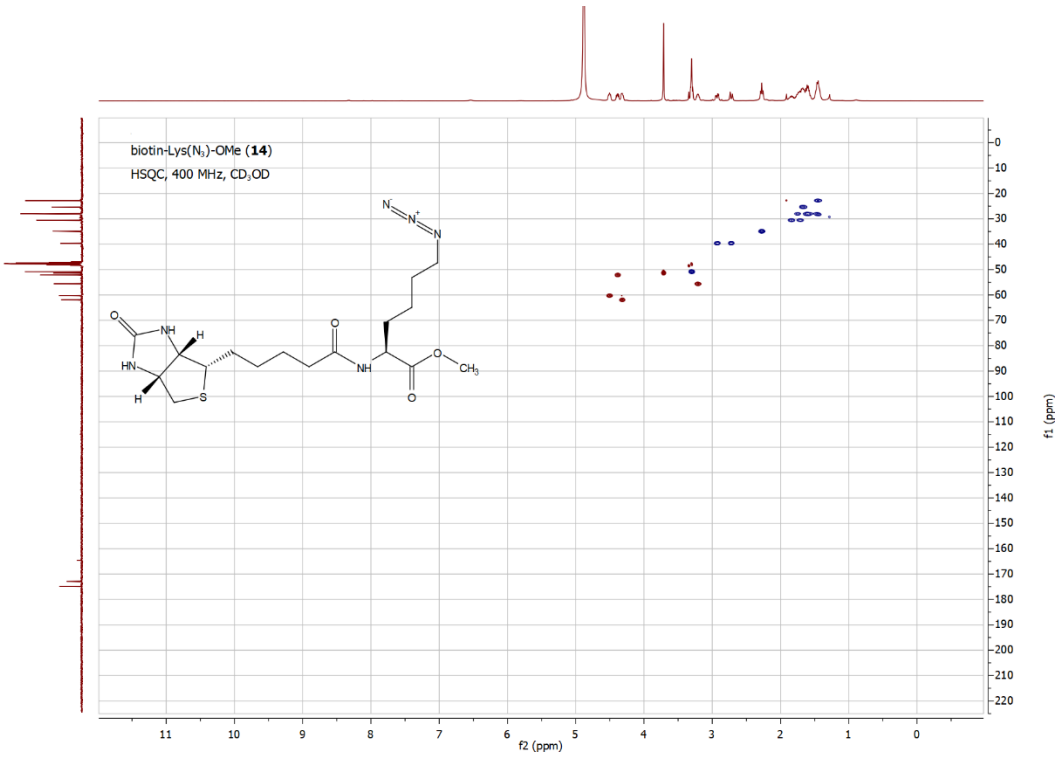

Boc-Lys(N3)-OH (12)
1H-NMR, 400 MHz, CDCl3
f1 (ppm)

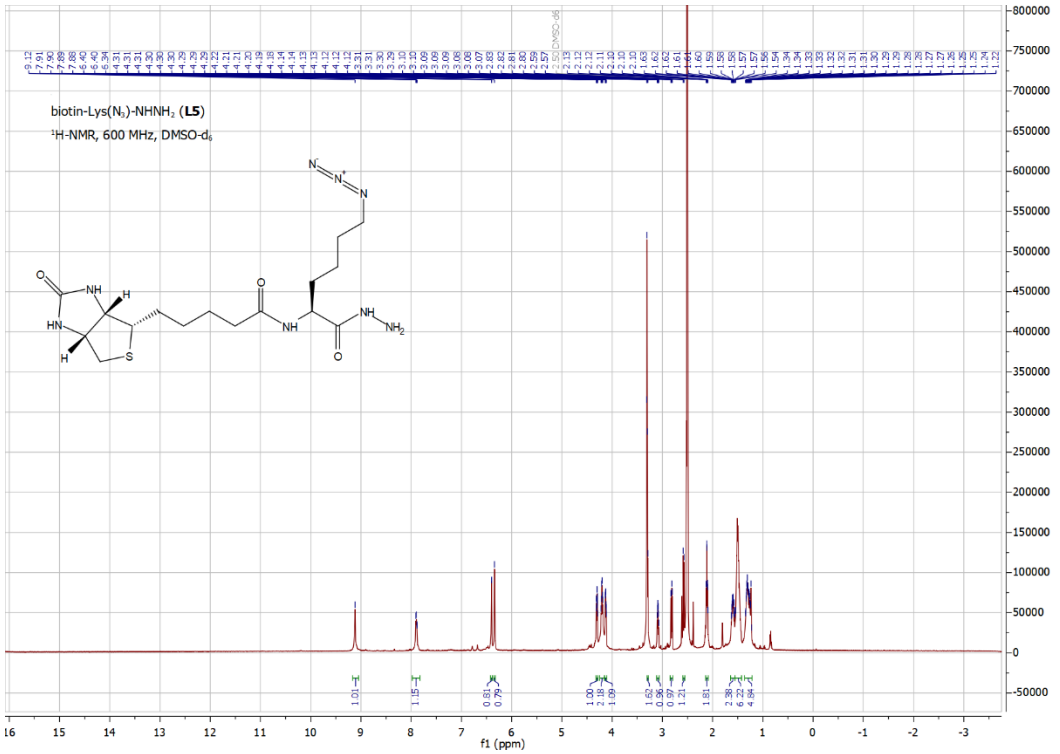

HCl.H2N-Lys(N3)-OMe (13)
1H-NMR, 400 MHz, D2O
f1 (ppm)

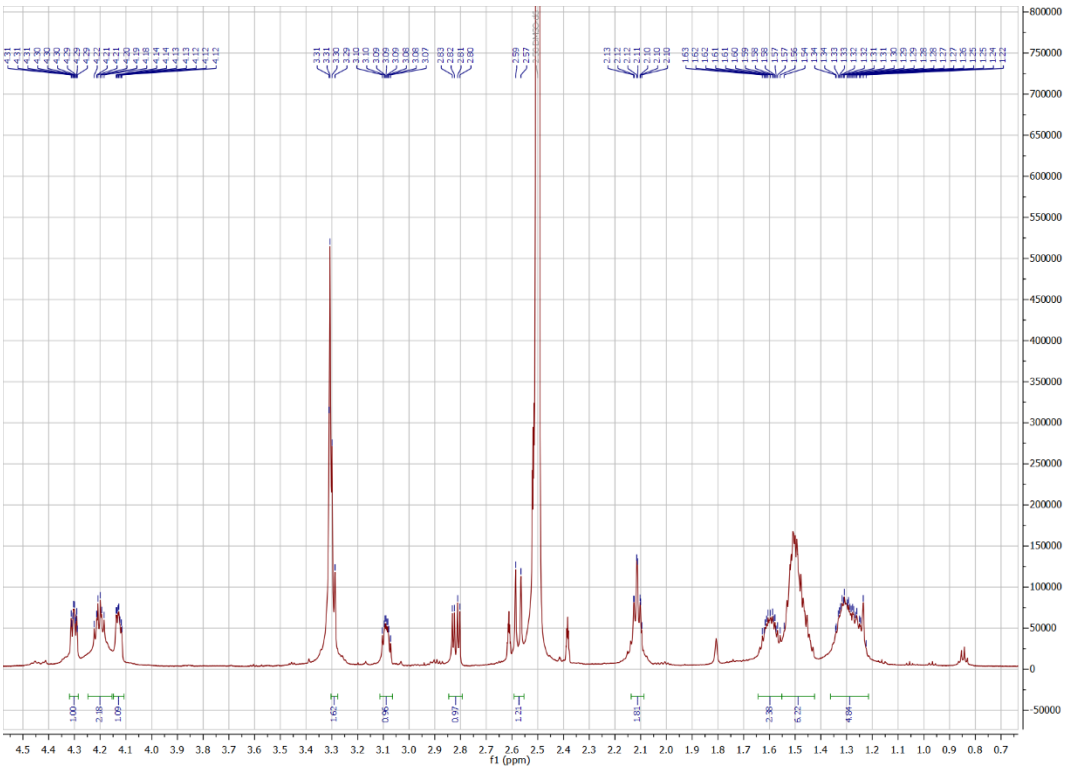

biotin-Lys(N3)-OMe (14)
1H-NMR, 400 MHz, CD3OD
f1 (ppm)

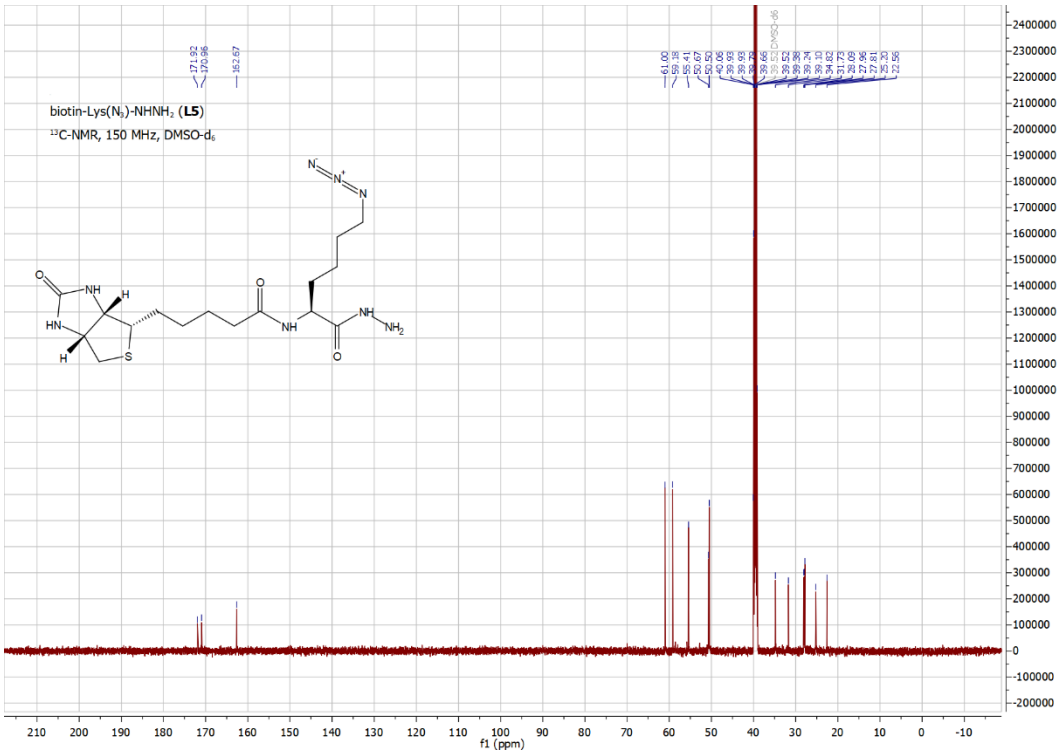

f1 (ppm)

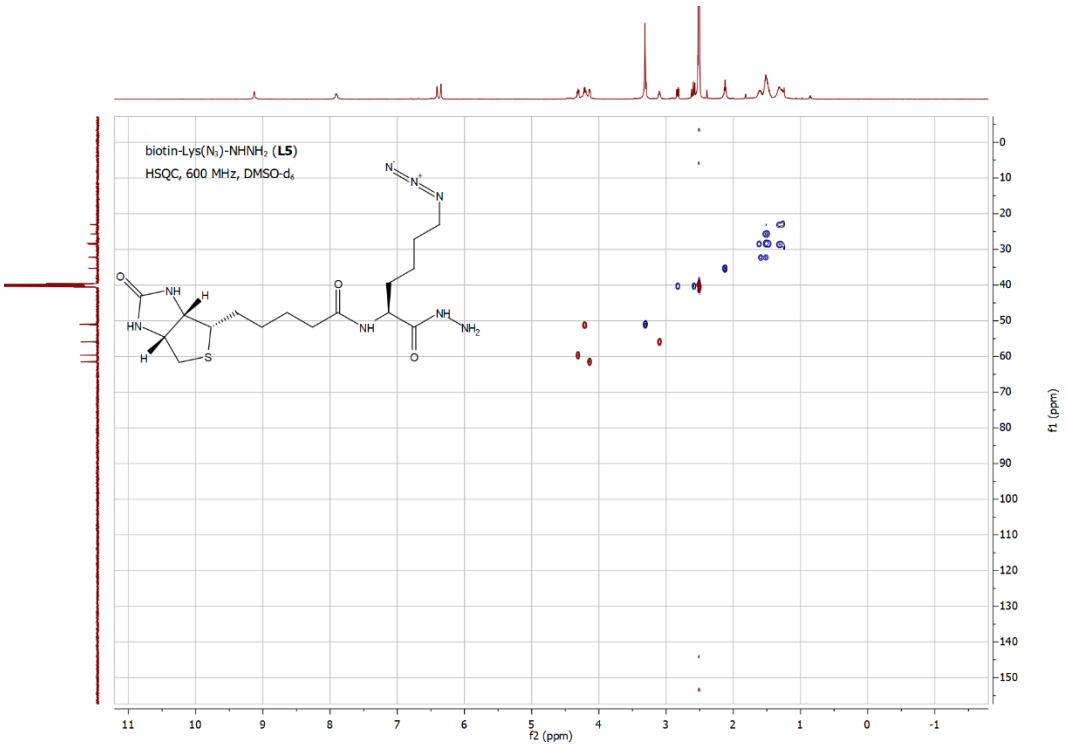

biotin-Lys(N3)-OMe (14)
13C-NMR, 100 MHz, CD3OD
176.25
174.27
166.00
63.32
61.65
56.99
55.52
53.73
52.71
52.18
49.00 CD3OD
41.02
36.24
31.89
29.56
29.39
29.36
26.71
24.19
f1 (ppm)

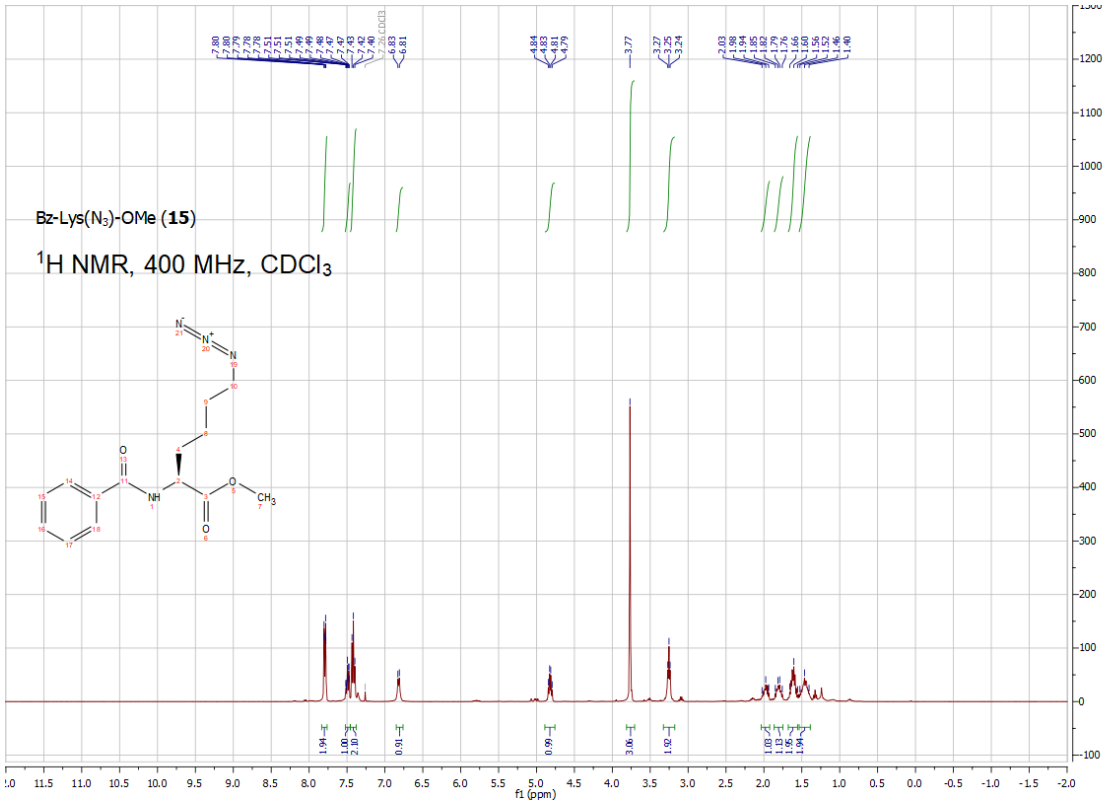

biotin-Lys(N3)-OMe (14)
HSQC, 400 MHz, CD3OD
f2 (ppm)
f1 (ppm)

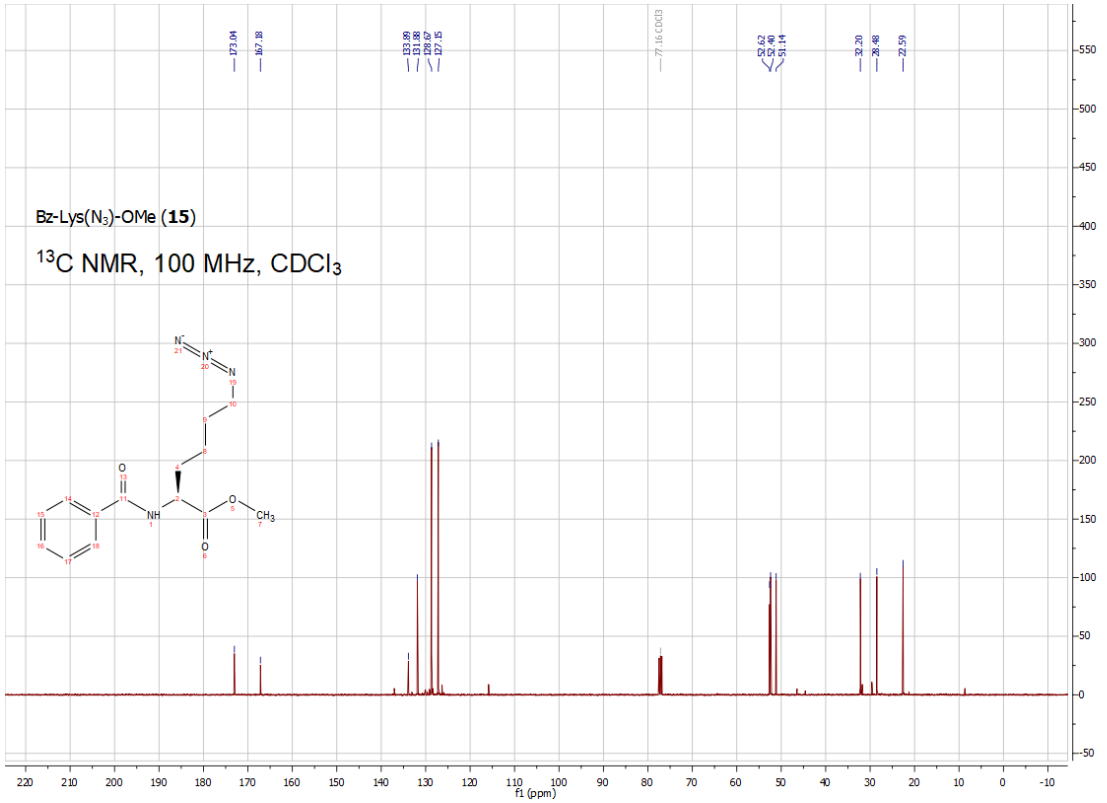

biotin-Lys(N3)-NHNH2 (L5)
1H-NMR, 600 MHz, DMSO-d6
f1 (ppm)


f1 (ppm)

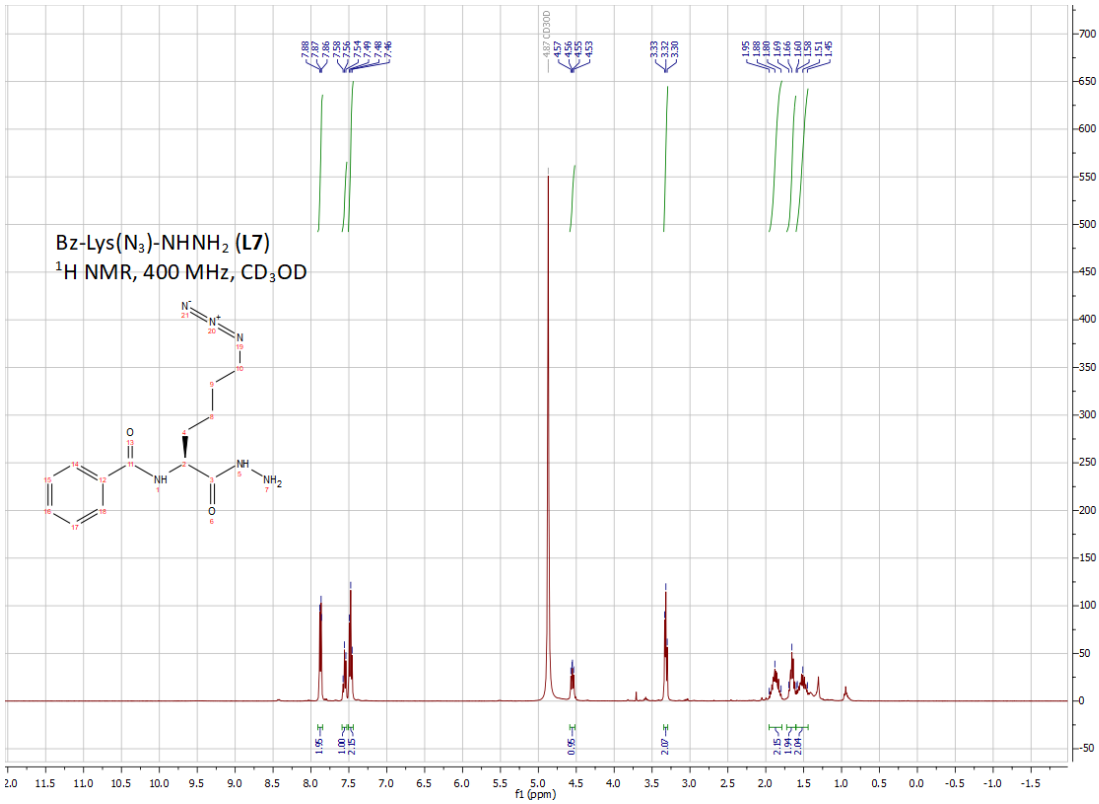

biotin-Lys(N3)-NHNH2 (L5)
13C-NMR, 150 MHz, DMSO-d6
f1 (ppm)

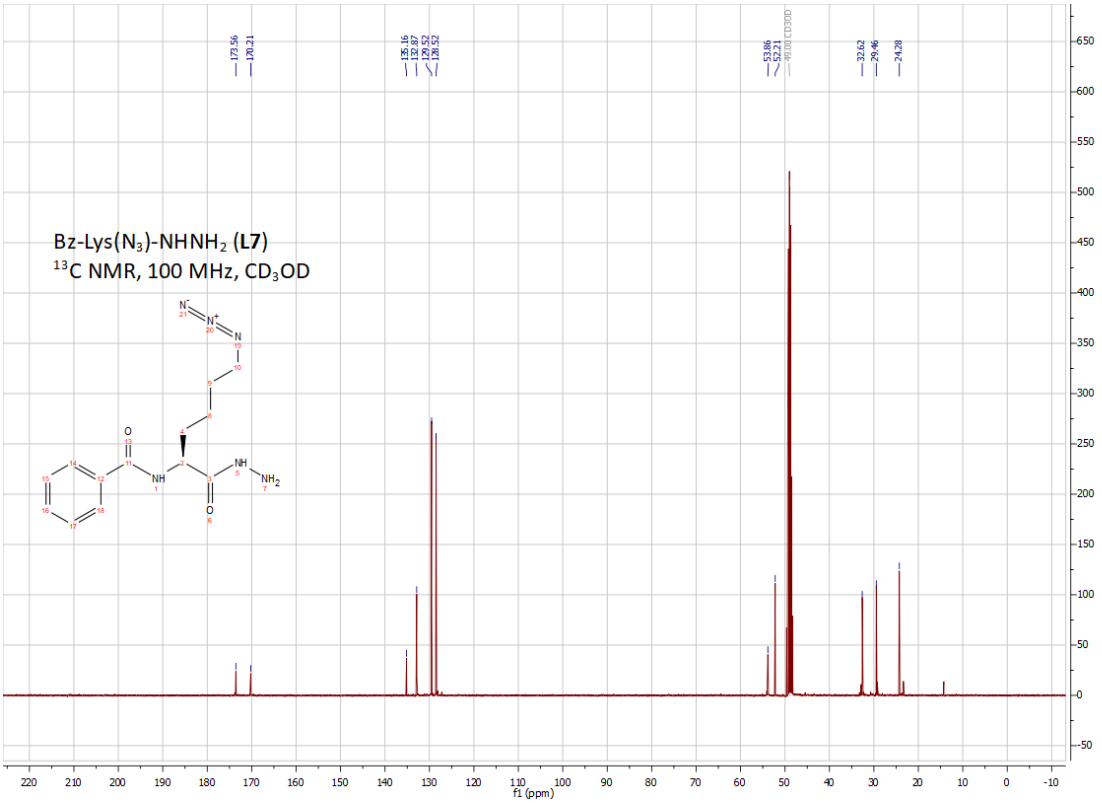

biotin-Lys(N3)-NHNH2 (L5)
HSQC, 600 MHz, DMSO-d6
f2 (ppm)
f1 (ppm)

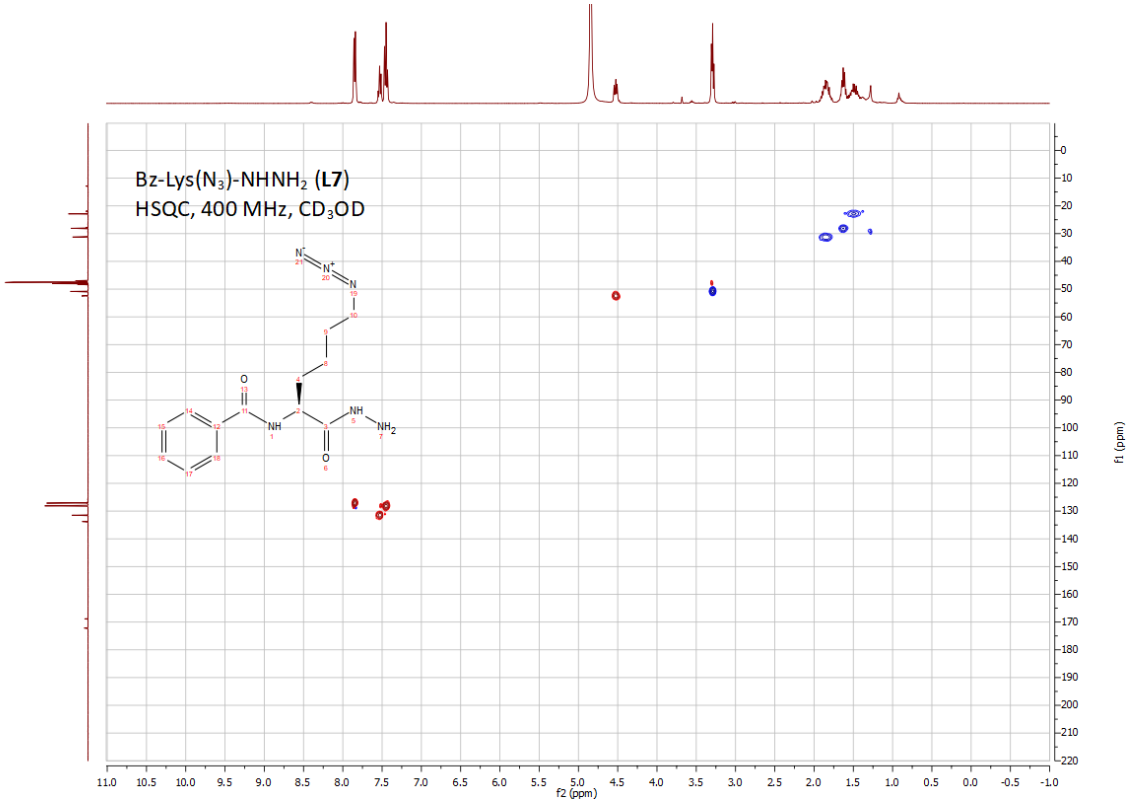

Bz-Lys(N3)-OMe (15)
1H NMR, 400 MHz, CDCl3
f1 (ppm)

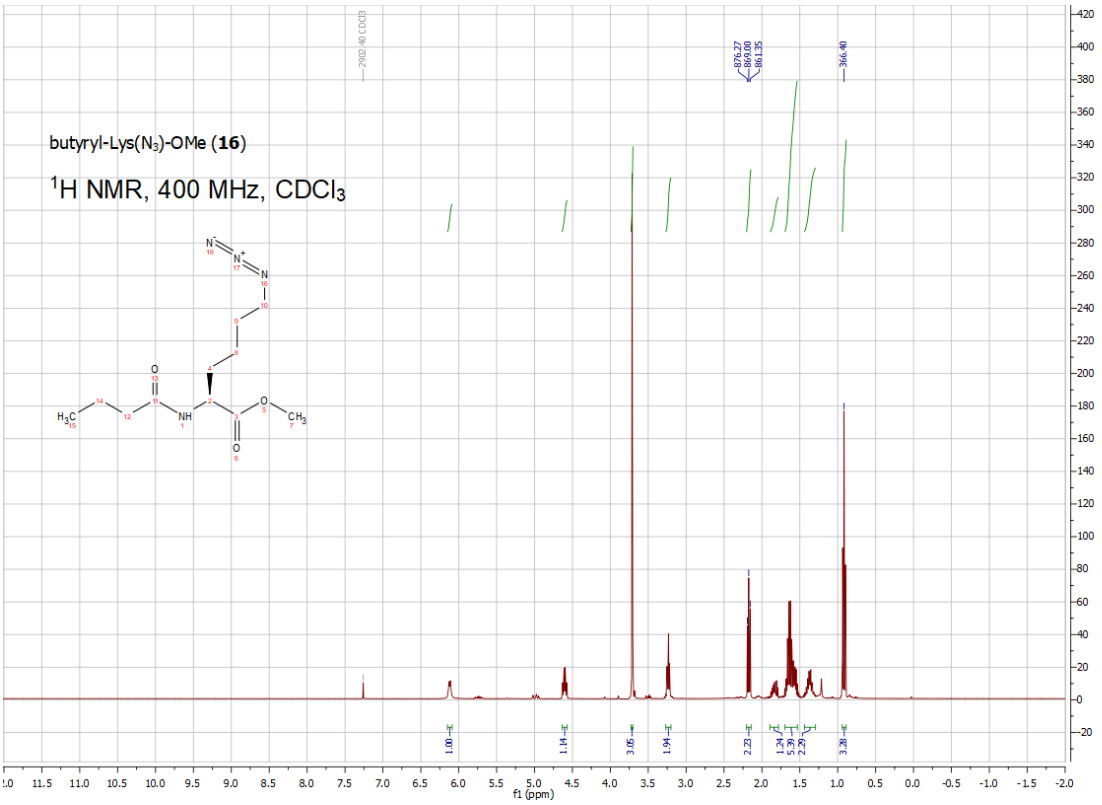

Bz-Lys(N3)-OMe (15)
13C NMR, 100 MHz, CDCl3
f1 (ppm)

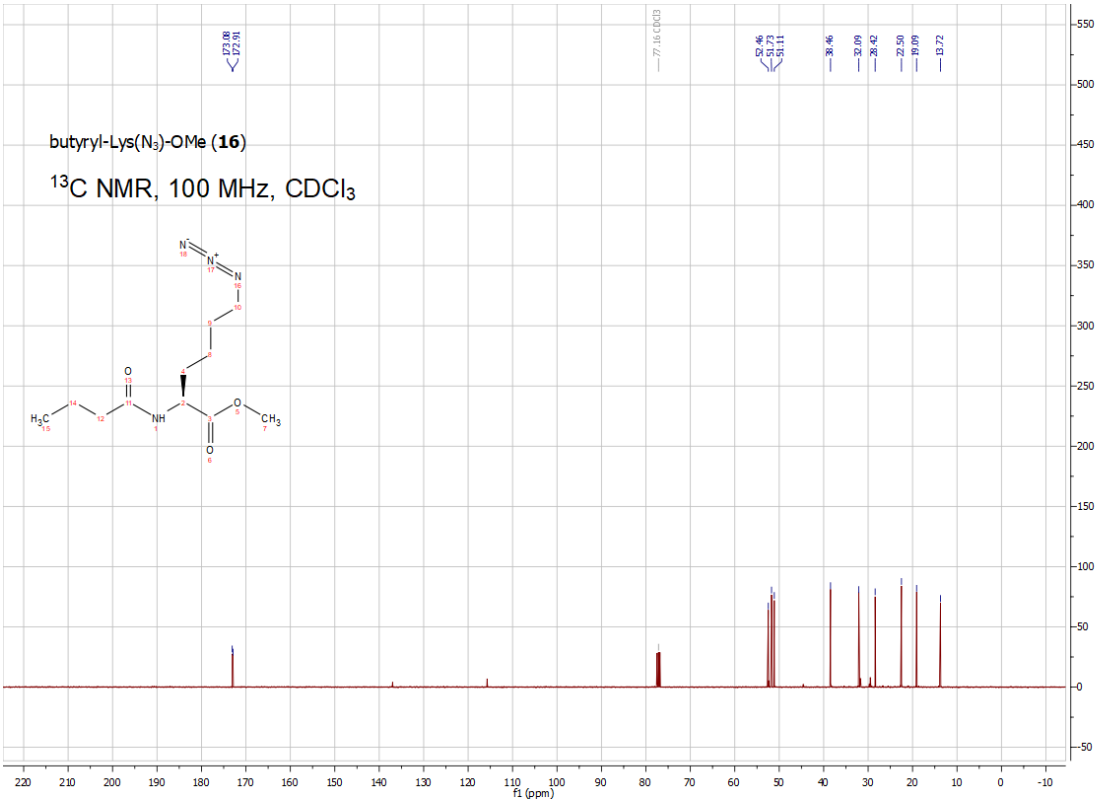

Bz-Lys(N3)-OMe (15)
HSQC, 400 MHz, CDCl3
f2 (ppm)
f1 (ppm)

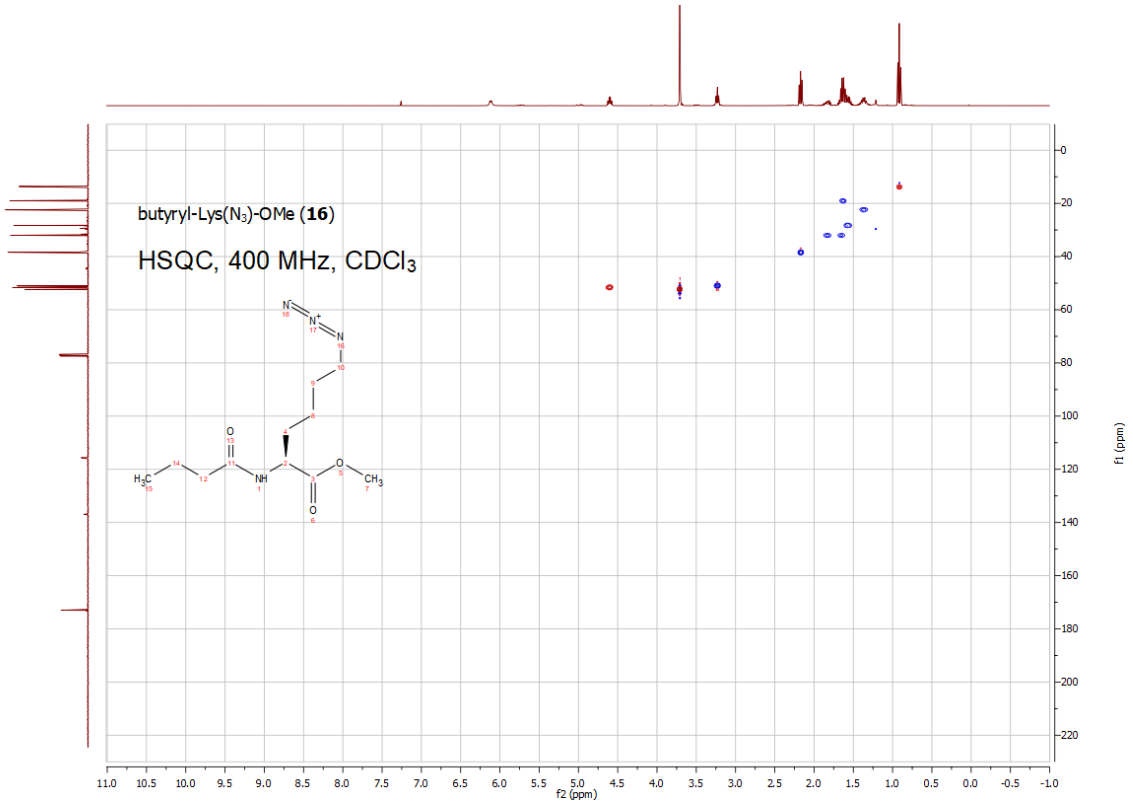

Bz-Lys(N3)-NHNH2 (L7)
1H NMR, 400 MHz, CD3OD
f1 (ppm)

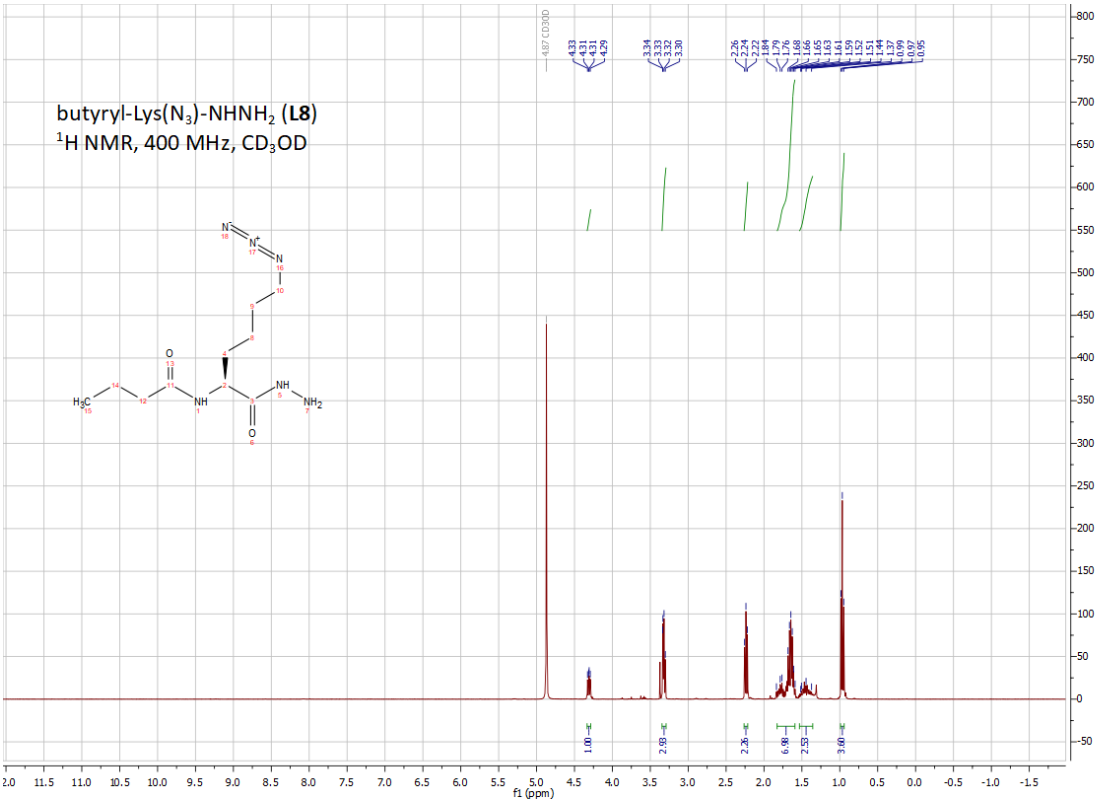

Bz-Lys(N3)-NHNH2 (L7)
13C NMR, 100 MHz, CD3OD
173.56
170.21
135.16
129.27
128.52
126.52
53.86
53.71
49.00 CD3OD
32.62
29.46
24.28
f1 (ppm)

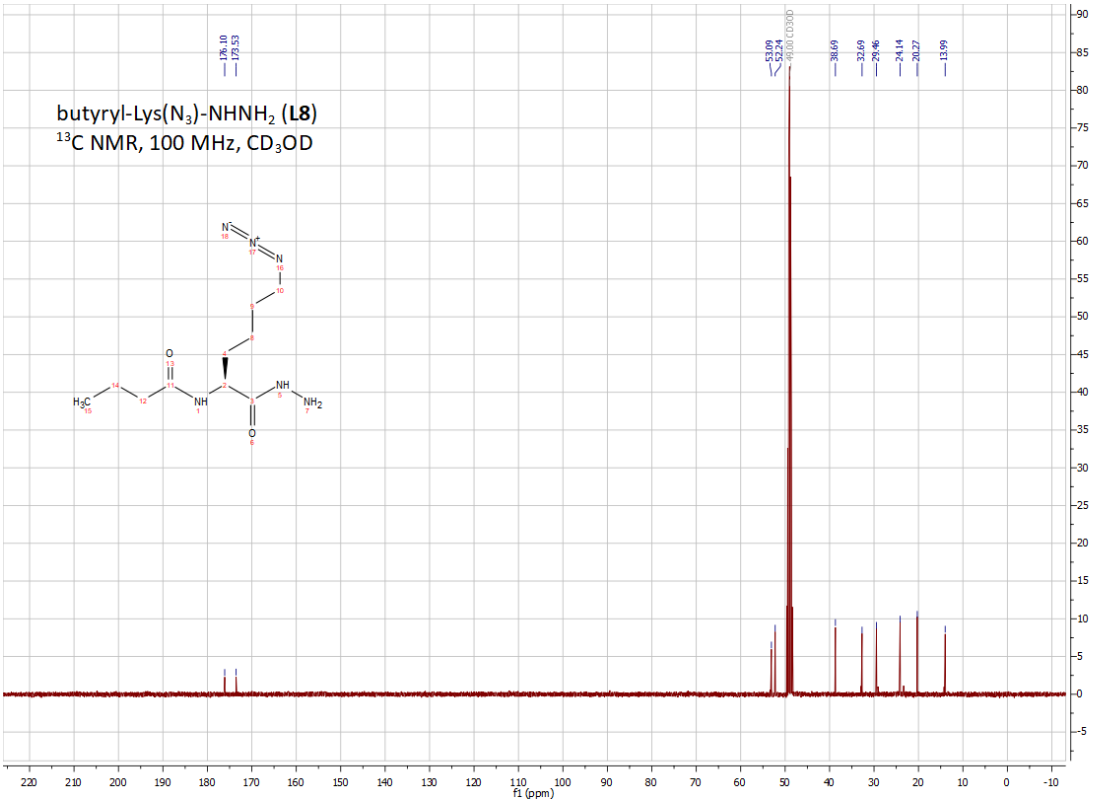

Bz-Lys(N3)-NHNH2 (L7)
HSQC, 400 MHz, CD3OD
f2 (ppm)
f1 (ppm)

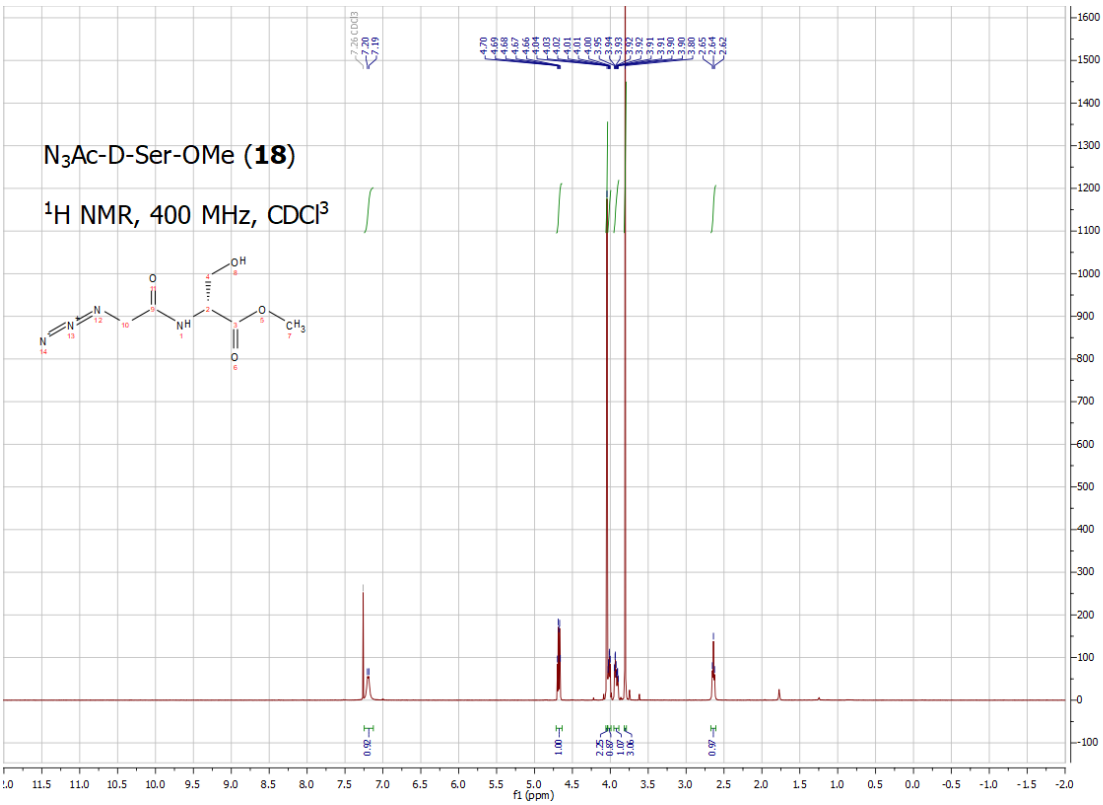

butyryl-Lys(N3)-OMe (16)
1H NMR, 400 MHz, CDCl3
f1 (ppm)

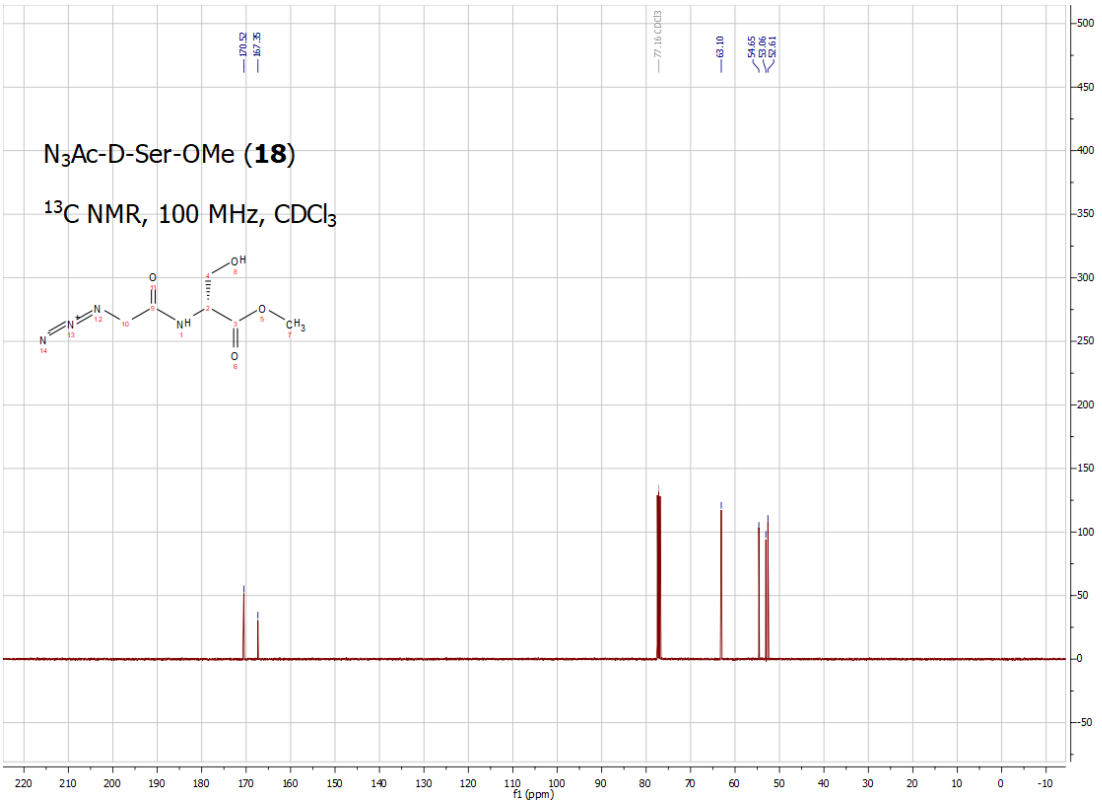

butyryl-Lys(N3)-OMe (16)
13C NMR, 100 MHz, CDCl3
f1 (ppm)

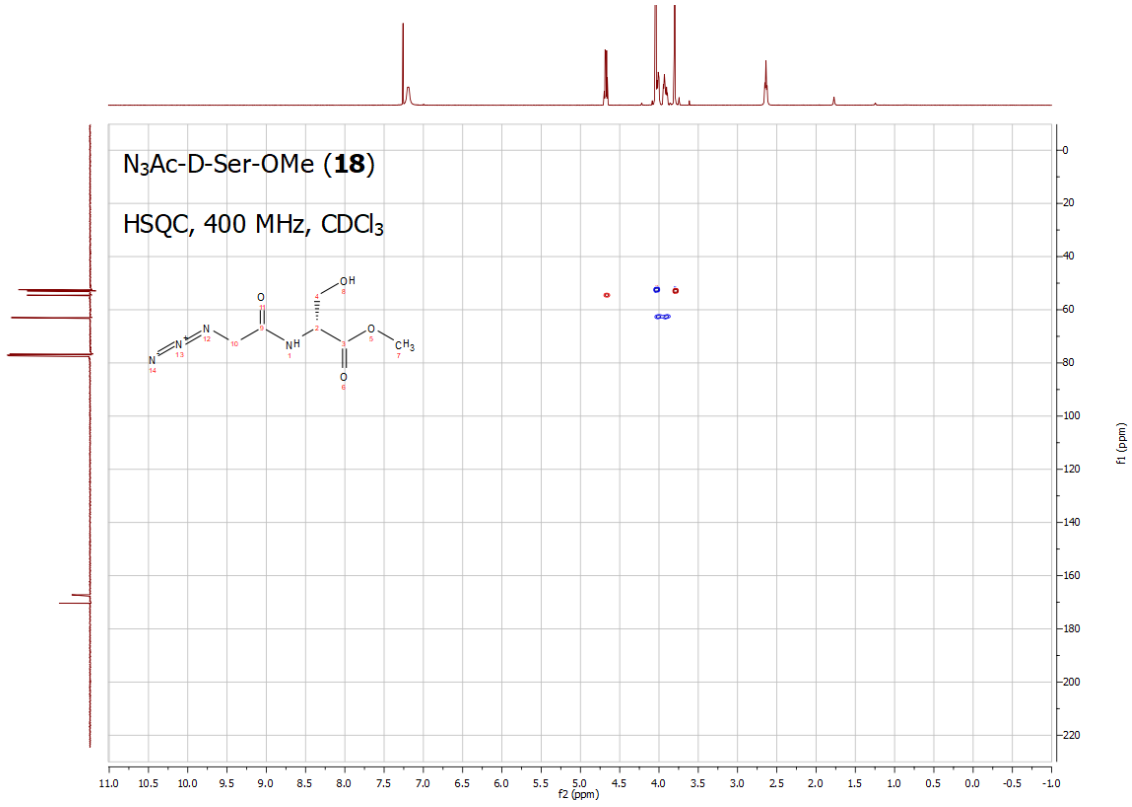

butyryl-Lys(N3)-OMe (16)
HSQC, 400 MHz, CDCl3
f2 (ppm)
f1 (ppm)

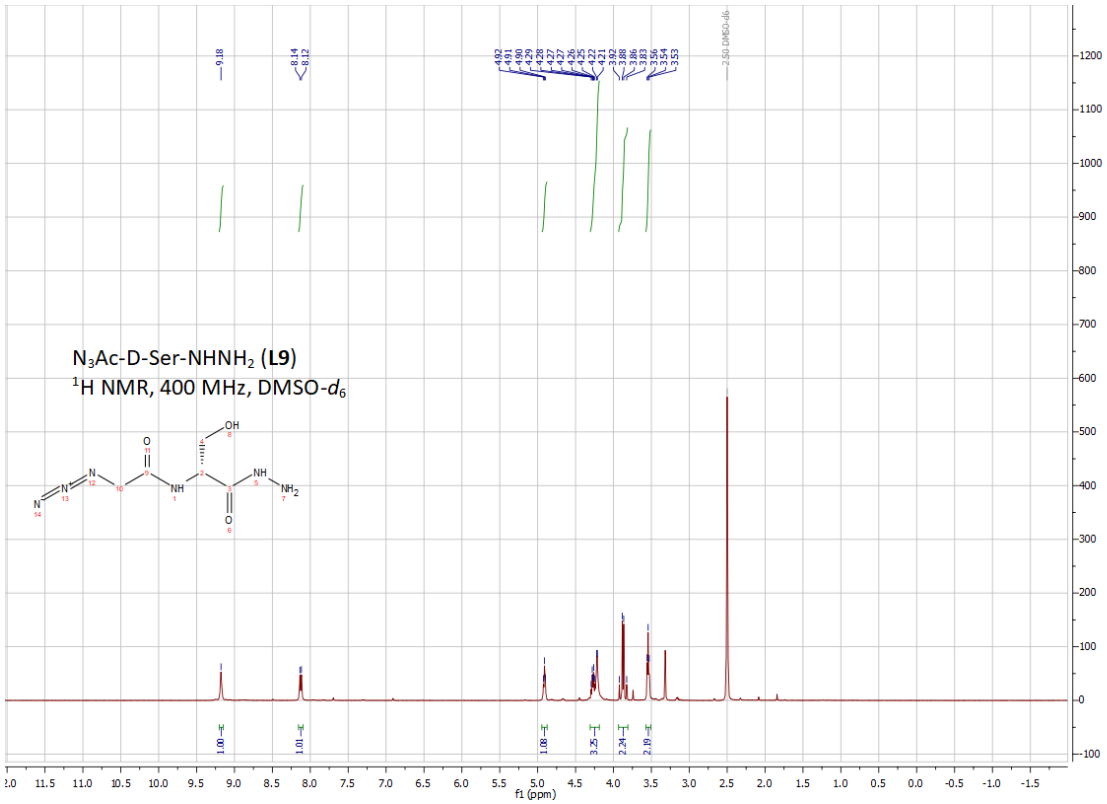

butyryl-Lys(N3)-NHNH2 (L8)
1H NMR, 400 MHz, CD3OD
f1 (ppm)

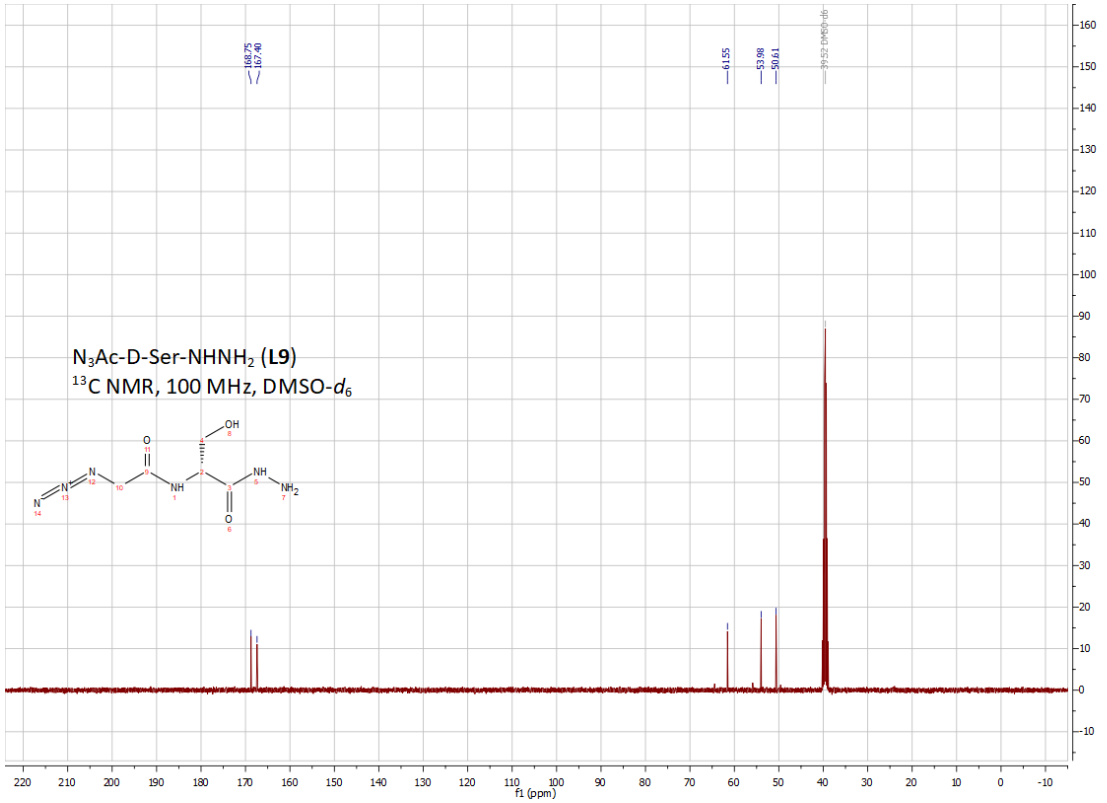

butyryl-Lys(N_3)-$NHNH_2$ (**L8**)
^{13}C NMR, 100 MHz, CD_3OD
f1 (ppm)

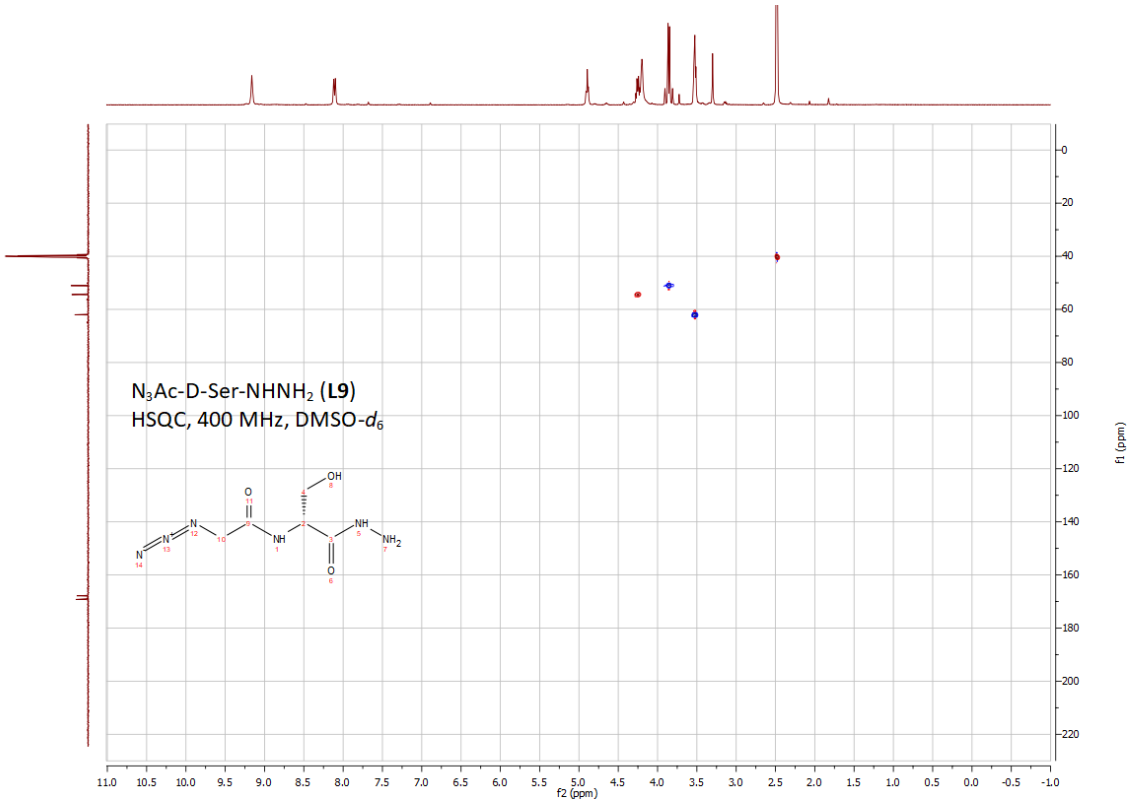

N_3Ac-D-Ser-OMe (**18**)
^{1}H NMR, 400 MHz, $CDCl^3$
f1 (ppm)


N3Ac-D-Ser-OMe (18)
13C NMR, 100 MHz, CDCl3
170.52
167.35
77.16 CDCl3
63.10
54.65
53.06
52.61
f1 (ppm)


N3Ac-D-Ser-OMe (18)
HSQC, 400 MHz, CDCl3
f2 (ppm)
f1 (ppm)


N3Ac-D-Ser-NHNH2 (L9)
1H NMR, 400 MHz, DMSO-d6
f1 (ppm)


N3Ac-D-Ser-NHNH2 (L9)
13C NMR, 100 MHz, DMSO-d6
f1 (ppm)


N3Ac-D-Ser-NHNH2 (L9)
HSQC, 400 MHz, DMSO-d6
f2 (ppm)
f1 (ppm)