

Synthesis of D-desthiobiotin-AI-2 as a novel chemical probe for autoinducer-2 quorum sensing receptors

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ABSTRACT

In processes regulated by quorum sensing (QS) bacteria respond to the concentration of autoinducers in the environment to engage in group behaviours. Autoinducer-2 (AI-2) is unique as it can foster interspecies communication. Currently, two AI-2 receptors are known, LuxP and LsrB, but bacteria lacking these receptors can also respond to AI-2. In this work we present an efficient and reproducible synthesis of a novel chemical probe, D-desthiobiotin-AI-2. This probe binds both LuxP- and LsrB-class receptors from different species of bacteria. Thus, this probe is able to bind receptors that recognise the two known biologically active forms of AI-2. A protocol to pull down receptors bound to D-desthiobiotin-AI-2 with anti-biotin antibodies has also been established. This work highlights the potential of conjugating chemical signals to biotinylated derivatives to identify and tag signal receptors involved in quorum sensing or other chemical signalling processes.

Keywords: AI-2, AI-2 receptors, Chemical Probe, Quorum sensing

1. INTRODUCTION

Quorum sensing (QS) is a process mediated by the production and detection of small extracellular signalling molecules, named autoinducers, which accumulate in the environment in proportion to cell density. This process allows bacteria to monitor population density and regulate gene expression accordingly.¹⁻³ Antibiotic production, biofilm formation and bioluminescence are examples of group behaviours that become effective when cells are working in unison and thus are regulated by QS.¹⁻³ In Gram-negative bacteria, intra-species communication is achieved mainly by production and detection of acyl-homoserine lactones (AHL) whereas in Gram-positive bacteria, oligopeptides are the most common autoinducers. However, a universal QS signalling molecule, autoinducer-2 (AI-2), has been identified both in Gram-negative and -positive bacteria and is involved in inter-species communication.^{2,4}

AI-2 is comprised of a family of molecules that exist in chemical equilibrium (Fig. 1). (S)-4,5-Dihydroxypentane-2,3-dione (DPD) is the linear precursor that is the product of the reaction catalysed by the LuxS enzyme. This linear molecule can then acquire different

cyclic forms when in solution.⁵ To date two DPD derivate molecules have been identified as signalling molecules: S-THMF-borate ((2S,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran-borate) and R-THMF ((2R,4S)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran).^{4,5}

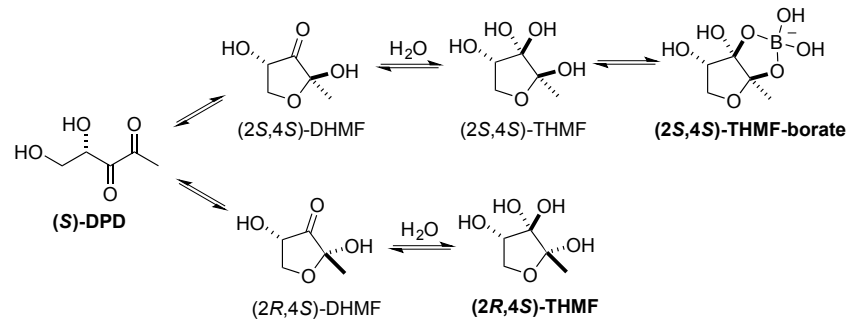


Figure 1. Linear and cyclic forms of Al-2.

Although the Al-2 synthase, LuxS, has been identified in more than 500 bacterial species⁶ only two receptors have been identified, LuxP and LsrB.² LuxP binds the S-THMF-borate form of Al-2 and, upon binding, interacts with a membrane-spanning sensor histidine kinase regulating a phosphorylation signal transduction cascade and the production of bioluminescence in *Vibrio harveyi*. LsrB, however, binds R-THMF and, upon binding, interacts with the membrane components of an adenosine triphosphate binding cassette (ABC) transporter to facilitate Al-2 internalisation and processing.^{4,7-11} The distinct amino acid composition of the binding site of these receptors is reported to explain the recognition of the different Al-2 adducts.^{7,12}

Additional classes of Al-2 receptors are hypothesised to exist as phenotypes regulated by Al-2 have been identified in species where LuxP and LsrB are absent². For example, in *Enterococcus faecalis*, an opportunistic pathogen that is among the leading causes of nosocomial infections due to its strong ability to form biofilms, high concentrations of Al-2 induce phage release and, consequently, virulence.¹³ Another example was identified in *Staphylococcus aureus*, a human pathogen able to cause an assortment of diseases ranging from minor skin infections to life-threatening syndromes, in which biofilm formation is regulated by Al-2.¹⁴ Nevertheless, no known receptor has been identified in these bacterial species and identification of new classes of Al-2 receptors has proven a challenge. The low sequence homology between LsrB and LuxP (11%)^{2,5,7,15} raises the possibility that other types of receptors, which also share low homology with LuxP and LsrB, might exist but cannot be identified through classical bioinformatic approaches using sequence comparisons. Moreover, although these receptors share the same structural fold, the class I fold of periplasmic binding proteins, so do many sugar binding proteins. Therefore, simple identification of fold is thus not sufficient to differentiate between potential Al-2 receptors and other ligand binding proteins. This makes the

development of new strategies to identify novel AI-2 receptors crucial for the study of quorum sensing processes in bacteria lacking LuxP or LsrB homologues.

A chemical probe to identify AI-2 receptors, based on the structure of rhodamine, was previously developed. This probe was able to bind LsrB receptors which recognise *R*-THMF, but not the LuxP receptor which binds the *S*-THMF-borate form of AI-2.¹⁰ To the best of our knowledge, there are no chemical probes able to recognise both AI-2 receptors. Other classes of QS signal analogues, for AHLs, were explored to target LuxR-type receptors;^{15,16} however, these molecules are only inhibitors and/or activators of known QS receptors. As a biological tool, the system biotin/desthiobiotin with streptavidin or avidin is widely used for different applications, taking advantage of the ability of creating non-covalent bonds such as ionic, hydrogen-bonding and hydrophobic interactions.¹⁷ These molecules are used for example to: study protein-protein or protein-ligand interactions,¹⁷ immobilise DNA on the surface of microarrays,¹⁸ study of new RNA-protein interactions¹⁹ and to purify and/or immobilise proteins.²⁰ Therefore, many commercially available tools exist to study compounds covalently linked to these molecules. D-desthiobiotin is a derivative of D-biotin and is also used as a biological tool for similar applications.^{21,22}

Here, we present the synthesis of a new chemical probe, based on the structures of DPD and D-desthiobiotin (DB-AI-2, Fig. 2). D-desthiobiotin was used instead D-biotin because the sulfur atom was not compatible with the oxidation step of the synthesis. When D-biotin was used, the corresponding sulfone was obtained instead of the desired di-ketone.

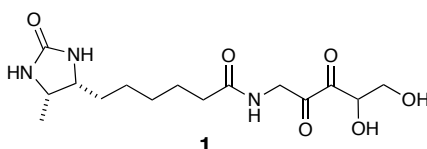
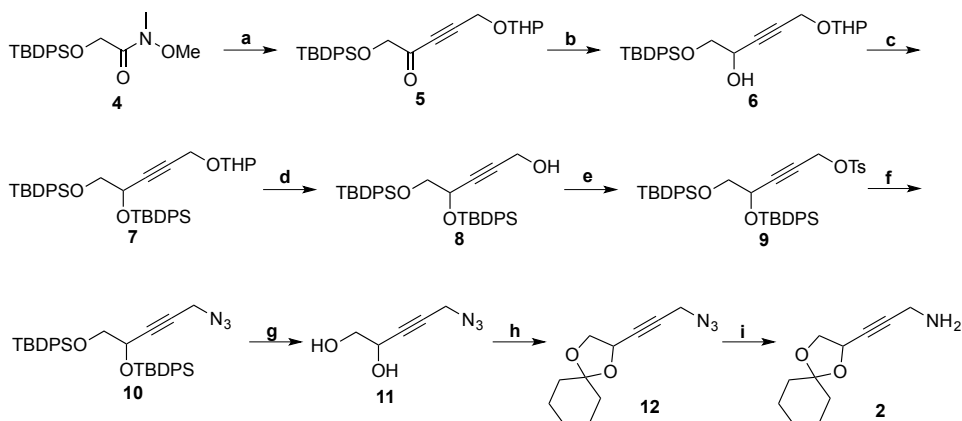


Figure 2. Structure of the synthesised chemical probe D-desthiobiotin-AI-2 **1**.

2. RESULTS AND DISCUSSION

2.1 Chemical Synthesis

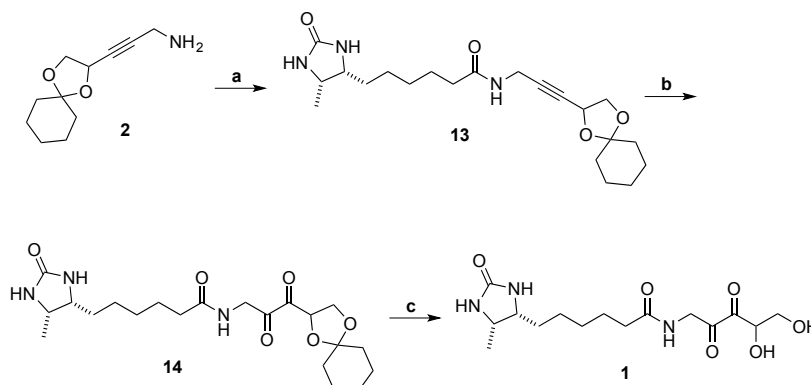
Due to the biological relevance of the different DPD isomers it is important to develop probes that have the flexibility needed for DPD to acquire different conformations as the conformation that will be recognised by new receptors is not known.



a) 2-(prop-2-yn-1-yloxy)oxane (**3**), LDA, THF, -78 to 0 °C, 90%. b) NaBH₄, MeOH, 0 °C to r.t., quantitative yield. c) TBDPSCI, DIPEA, DMAP, CH₂Cl₂, r.t., 95%. d) *p*-TsOH.H₂O, MeOH, r.t., 95%. e) *p*-TsCl, KOH, Et₂O, 0 °C to r.t., 95%. f) NaN₃, DMF, r.t., 95%. g) TBAF, THF, r.t., 76%. h) 1,1-dimethoxycyclohexanone, H₂SO₄, DMF, r.t., 84%. i) PPh₃, H₂O, THF, r.t., 94%.

Scheme 1. Synthesis of linker **2** to coupling with D-desthiobiotin.

To prepare D-Desthiobiotin-Al-2 (**1**) we first synthesised linker (**2**, Scheme 1). Linker **2** was specifically designed to bond to D-desthiobiotin at the C-1 position of the DPD derivative. In this way, no interference with the cyclisation would occur, this is crucial for the interaction of the DPD with the biological receptors. Although we are able to efficiently synthesise the enantiomeric pure form of DPD,²³ in this work we synthesised the racemic form to avoid the exclusion of any new receptor which can recognise different DPD adducts. The alkylation of the Weinreb amide **4**²³ with propyne **3** afforded the ketone **5** in 90% yield (Scheme 1). The non-stereoselective reduction of ketone **5** with NaBH₄ gave alcohol **6** that after protection with the silyl group afforded the alkyne **7** in excellent yield. The C-1 position had to be converted into an amine for the coupling with the carboxylic acid of the D-desthiobiotin. Alkyne **7** was converted to alcohol **8** in 95% yield and protection with tosyl chloride afforded compound **9**. After azide **10** formation and silyl deprotection, diol **11** was achieved. Finally, linker **2** was obtained after protection with 1,1-dimethoxycyclohexanone and conversion of the azide via Staudinger reaction to the corresponding amine (Scheme 1). The cyclohexylidene group is easily hydrolysed to obtain the free form of the DPD.²³ The linker **2** could be prepared in large scale, maintaining the efficiency of the synthesis. The synthesis of this compound is a good example of the use of the strategy previously developed²³ as an efficient method to prepare DPD analogues for different applications. Also, different linkers based on the DPD structure could be easily prepared to be coupled with D-desthiobiotin.



a) D-desthiobiotin, EDC, HOBT, MeCN, r.t., 50%. b) OsO₄ (2% aq. sol.), KClO₃, H₂O:Et₂O, r.t., quantitative yield. c) D₂SO₄, D₂O:DMSO-*d*₆, r.t.

Scheme 2. Synthesis of the chemical probe D-desthiobiotin-Al-2 **1**.

The coupling reaction between linker **2** and D-desthiobiotin was achieved in 50% yield (Scheme 2). Di-ketone **14** was obtained successfully in quantitative yield using KClO_3 and OsO_4 .²⁴ The oxidation of compound **13** was attempted using $\text{RuO}_2 \cdot \text{H}_2\text{O}$ and NaIO_4 described in the DPD synthesis,²³ however compound **14** was not isolated. The final chemical probe **1** was obtained after an hydrolysis protocol which consisted of preparing a solution of the compound **14** in $\text{DMSO-}d_6$: D_2O (1:4) and adding 10% D_2SO_4 .^{25,26} After 16 hours of stirring, the mixture was washed with deuterated chloroform to remove the cyclohexanone in solution and no further purification was need.

2.2 D-desthiobiotin-AI-2 binds both LuxP and LsrB receptors

To determine the functionality of this chemical probe we tested binding of D-desthiobiotin-AI-2 (DB-AI-2 **1**) to known AI-2 receptors, including the well-characterised LuxP from *V. harveyi* and the canonical LsrB receptor from *Escherichia coli* (EcLsrB). For LuxP we used LuxP bound to two fluorescent proteins, CLPY, which is routinely used for AI-2 detection assays.²⁷ Recently, an LsrB receptor with a different binding site was identified in *Clostridium saccharobutylicum*,²⁸ and we also tested this non-canonical LsrB receptor (CsLsrB).

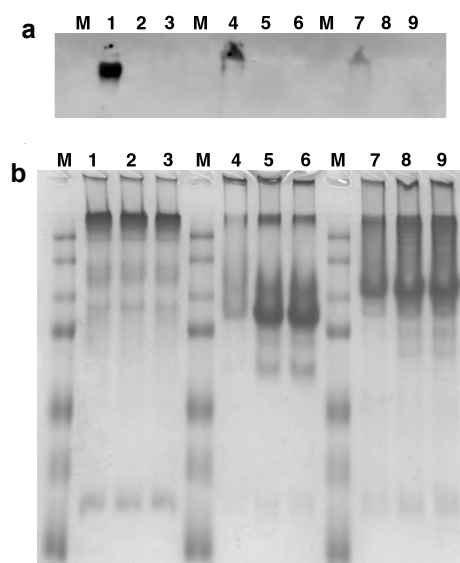


Figure 3. D-desthiobiotin-AI-2 **1** is able to bind both LuxP and LsrB receptors. **(a)** Western-blot membrane incubated with an anti-biotin antibody able to recognise D-desthiobiotin. Protein standards (M), CLPY-LuxP incubated with DB-AI-2 (1), D-desthiobiotin (2) and buffer (3); CsLsrB incubated with DB-AI-2 (4), with D-desthiobiotin (5) and buffer (6); EcLsrB incubated with DB-AI-2 (7), D-desthiobiotin (8) and buffer (9). **(b)** Native PAGE gel of the same samples.

After an affinity purification, these receptors were incubated with DB-AI-2 **1**. To test for specificity, as controls, receptor proteins were also incubated with non-conjugated D-desthiobiotin and with buffer alone. Next, these samples were run in a native gel to prevent denaturation of the receptors. The native gel was used for immunoblotting using an anti-biotin antibody able to recognize D-desthiobiotin²⁹ (Fig. 3a). A native gel run in parallel with the same samples was stained with Coomassie to visualize the bands (Fig. 3b). In the wells of LsrB proteins incubated with DB-AI-2 (Fig. 3b, wells 4 and 7) some bands are fainter, indicating that

standards (M), unbound antibody (1), protein sample that is not retained by the complex beads/antibody (2), washes to eliminate unbound sample (3-5) and elution of the receptor protein (6). Since denaturing conditions were used the anti-biotin antibody was also eluted.

Using this approach, we were able to pull-down LuxP through one-step purification. Moreover, magnetic separation improved the process as washes and elutions are faster and consume fewer reagents. The denaturing conditions used caused the antibody to elute with LuxP (Fig. 4b). However, all the known AI-2 receptors have around 30-40 kDa and thus, other receptors are expected to have a similar molecular weight. This means that bands of interest should appear in between the heavy and light chains of the antibody. Nevertheless, if this presents a problem going forward, covalent linking of the antibody to the beads can readily be accomplished. Additionally, specific elution using synthetic AI-2,²³ which should have higher affinity to AI-2 receptors, can also be optimised.

3. CONCLUSION

In summary, a new chemical probe able to bind both LuxP and LsrB receptors was successfully designed and synthesised. In this work, we show that DB-AI-2 is the first chemical probe able to bind the two different types of known receptors, demonstrating that the molecule of AI-2 in this probe has the plasticity necessary to acquire the different conformations needed to bind different types of receptors. It has, therefore, great potential as a tool for the discovery of new receptors, which might bind different forms of AI-2. New classes of AI-2 receptors are hypothesised to exist as bacteria such as *E. faecalis* and *S. aureus*, where no proteins with significant similarity to the known receptors were identified, possess phenotypes regulated by AI-2. Thus, DB-AI-2 will be an important tool for the identification of new AI-2 receptors in relevant commensals and pathogenic bacteria where no AI-2 receptors have been identified. The acquired knowledge will be extremely important for the development of strategies to modulate AI-2 mediated quorum sensing and the collective behaviours that contribute for the establishment of bacterial infections.

Moreover, the synthetic process developed for DB-AI-2 is flexible and efficient in terms of yields and atom economy. Thus, it can be further applied to other compounds like AI-2 analogues or other quorum sensing molecules originating new chemical probes. Broadly, the use of the probe developed here exemplifies the advantage of using biotinylated derivatives as conjugates to study chemical binding proteins involved in signalling transduction pathways or other biological processes.

4. MATERIALS AND METHODS

4.1 Chemical Synthesis

4.1.1 Materials

¹H-NMR spectra were obtained with a Bruker Avance II+ (400 MHz) spectrometer, at 400 MHz in CDCl₃ or D₂O with chemical shift values (δ) in ppm downfield from

tetramethylsilane in the case of CDCl_3 and ^{13}C -NMR spectra were obtained at 100 MHz in CDCl_3 or D_2O . Assignments are supported by 2D correlation NMR spectroscopy studies. Preparative chromatographic separations were carried out by flash chromatography using Kieselgel 60, (0.032–0.063mm). Analytical TLC was performed on aluminum-backed Merck 60 F₂₅₄ silica gel plates. Reagents and solvents were purified and dried according to ref.³⁰ All the reactions were carried out under an inert atmosphere (argon), except when the solvents were not dried.

4.1.2 Preparation of 2-(ethynyloxy)oxane 3

Propyne 3 was prepared and characterised according to the literature.³¹

4.1.3 Preparation of *N*-Methoxy-*N*-methyl-2-(*tert*-butyldiphenylsilyloxy)acetamide 4

Weinreb amide 4 was prepared and characterised according to the literature.²³

4.1.4 Preparation of 1-[(*tert*-butyldiphenylsilyloxy)-5-(oxan-2-yloxy)pent-3-yn-2-one 5

To a solution of diisopropylamine (1.6 mL, 11.2 mmol) in THF (8 mL), 1.6M solution of *n*-BuLi in hexane (6.3 mL, 10.7 mmol) was added dropwise at -78°C and the mixture stirred at 0°C for 15 minutes. At -78°C , a solution of propyne 3 (1.57 g, 11.2 mmol) in THF (8 mL) was added and the mixture stirred for 30 minutes. Still at -78°C , a solution of Weinreb amide 4 (2.00 g, 5.6 mmol) in THF (10 mL) was added. The mixture was stirred at -78°C for 20 minutes and then at 0°C until all starting material had been consumed (TLC), approximately 2 hours. The mixture was quenched with a saturated aqueous NH_4Cl solution (20mL) and extracted with CH_2Cl_2 (3x20mL). The combined organic layers were dried with anhydrous Na_2SO_4 , filtered and concentrated under vacuum. The residue was purified by flash column chromatography (90:10 Hexane/EtOAc) to afford the ketone 5 (2.20 g, 90%) as a colourless viscous oil.

^1H -NMR (CDCl_3), 400 MHz: δ 7.68-7.66 (m, 4H, Ar), 7.44-7.37 (m, 6H, Ar), 4.77 (t, 1H, $J=3.2$ Hz, CH from THP), 4.39 (d, 2H, $J=1.7$ Hz, $\text{C}\equiv\text{CCH}_2$), 4.32 (s, 2H, $\text{TBDPSOCH}_2\text{C}=\text{O}$), 3.81-3.76 (m, 1H, $1\times\text{CH}_2$ from THP), 3.54-3.49 (m, 1H, $1\times\text{CH}_2$ from THP), 1.80-1.50 (6H, m, $3\times\text{CH}_2$ from THP), 1.10 (s, 9H, $\text{C}(\text{CH}_3)_3$) ppm. **^{13}C -NMR** (CDCl_3), 100 MHz: δ 185.4 ($\text{C}=\text{O}$), 135.6 (Ar), 132.6 (C_q Ar), 130.0 (Ar), 127.9 (Ar), 97.2 (CH from THP), 91.0 ($\text{C}\equiv\text{C}$), 83.0 ($\text{C}\equiv\text{C}$), 70.5 ($\text{TBDPSOCH}_2\text{C}=\text{O}$), 62.0 (CH_2 from THP), 53.8 ($\text{C}\equiv\text{CCH}_2$), 30.0 (CH_2 from THP), 26.7 ($\text{C}(\text{CH}_3)_3$), 25.2 (CH_2 from THP), 19.3 ($\text{C}(\text{CH}_3)_3$), 18.8 (CH_2 from THP). **FTIR** (Neat): 1720 ($\text{C}=\text{O}$), 2155 ($\text{C}\equiv\text{C}$) cm^{-1} . **HRMS**: calcd. for $\text{C}_{26}\text{H}_{32}\text{O}_4\text{SiNa}^+$ $[\text{M}+\text{Na}]^+$ 459.2070; found 459.1962.

4.1.5 Preparation of 1-[(*tert*-butyldiphenylsilyloxy)-5-(oxan-2-yloxy)pent-3-yn-2-ol 6

To a solution of ketone 5 (0.790 g, 1.8 mmol) in dry CH_2Cl_2 (5 mL), at 0°C , *tert*-butyldimethylsilylchloride (0.60 mL, 2.7 mmol), diisopropylethylamine (0.63 mL, 3.6 mmol) and a catalytic amount of DMAP were added. The reaction mixture was stirred

allowing the temperature to rise until r.t., after 16 hours all the starting material was consumed and water was added. The mixture was extracted with CH₂Cl₂, dried with Na₂SO₄, filtered and concentrated under vacuum. Without further purification, alcohol **6** was obtained (0.819 g, quantitative yield) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.69-7.65 (m, 4H, Ar), 7.44-7.38 (m, 6H, Ar), 4.77 (t, 1H, J=3.37 Hz, CH from THP), 4.52-4.50 (m, 1H, CHC≡C), 4.31 (ddd, 1H, J=15.7 Hz, J=3.5 Hz, J=1.8 Hz, C≡CCH₂), 4.23 (ddd, 1H, J=15.7 Hz, J=3.5 Hz, J=1.8 Hz, C≡CCH₂), 3.82-3.70 (m, 3H, TBDPSOCH₂CH and 1xCH₂ from THP), 3.51-3.47 (m, 1H, 1xCH₂ from THP), 1.63-1.49 (m, 6H, 3xCH₂ from THP), 1.07 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 135.6 (Ar), 135.5 (Ar), 132.9 (C_q Ar), 132.8 (C_q Ar), 129.92 (Ar), 129.91 (Ar), 127.84 (Ar), 127.82 (Ar), 96.8 (CH from THP), 67.4 (TBDPSOCH₂CH), 63.2 (CHC≡C), 61.9 (CH₂ from THP), 54.2 (C≡CCH₂), 30.2 (CH₂ from THP), 26.8 (C(CH₃)₃), 25.3 (C(CH₃)₃), 19.3 (C(CH₃)₃) ppm. **FTIR** (Neat): 2158 (C≡C), 1022 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₂₆H₃₄O₄SiNa⁺ [M+Na]⁺ 461.2226; found 461.2119.

4.1.6 Preparation of 2,2,9,9-tetramethyl-5-[3-(oxan-2-yloxy)prop-1-yn-1-yl]-3,3,8,8-tetraphenyl-4,7-dioxa-3,8-disiladecane **7**

To a solution of alcohol **6** (0.815 g, 1.9 mmol) in dry MeOH (5 mL), at 0°C, NaBH₄ (0.141 g, 3.7 mmol) was added. The reaction mixture was stirred allowing the temperature to rise to r.t., after 3 hours all the starting material was consumed and water was added. The mixture was extracted with EtOAc, dried with Na₂SO₄, filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (90:10 Hexane/EtOAc) to afford the alkyne **7** (1.160 g, 95%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.75 (d, 2H, J=6.90 Hz, Ar), 7.71 (d, 2H, J=7.3 Hz, Ar), 7.65 (t, 4H, J=6.2 Hz, Ar), 7.40-7.32 (m, 12H, Ar), 4.64-4.56 (m, 2H, CH from THP and CHC≡C), 4.11-4.02 (m, 2H, C≡CCH₂), 3.81-3.68 (m, 3H, TBDPSOCH₂CH and 1xCH₂ from THP), 3.44-3.40 (m, 1H, 1xCH₂ from THP), 1.78-1.45 (m, 6H, 3xCH₂ from THP), 1.08 (s, 9H, C(CH₃)₃), 1.02 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 136.1 (Ar), 135.9 (Ar), 135.7 (Ar), 135.6 (Ar), 133.6 (C_q Ar), 133.5 (C_q Ar), 133.4 (C_q Ar), 133.3 (C_q Ar), 129.62 (Ar), 129.58 (Ar), 127.6 (Ar), 127.5 (Ar), 127.4 (Ar), 96.5 (CHC≡C), 85.1 (C≡C), 81.6 (C≡C), 68.2 (TBDPSOCH₂CH), 65.3 (CHC≡C), 61.9 (CH₂ from THP), 54.0 (C≡CCH₂), 30.2 (CH₂ from THP), 26.9 (C(CH₃)₃), 26.7 (C(CH₃)₃), 25.4 (CH₂ from THP), 19.3 (C(CH₃)₃), 19.2 (C(CH₃)₃), 19.1 (CH₂ from THP) ppm. **FTIR** (Neat): 2170 (C≡C), 1111 and 1079 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₄₂H₅₂O₄Si₂Na⁺ [M+Na]⁺ 699.3404; found 699.3296.

4.1.7 Preparation of 4,5-bis[(tert-butyl)diphenylsilyloxy]pent-2-yn-1-ol **8**

To a stirred solution of alkyne **7** (0.57 g, 0.84 mmol) in dry MeOH (5 mL) at r.t., *p*-toluenesulfonic acid monohydrate (11 mg, 0.06 mmol) was added. After 2 hours all the starting material had been consumed and the reaction mixture was quenched with a

saturated aqueous NaHCO₃ solution (10mL) and extracted with EtOAc (3x10mL). The combined organic layers were dried with anhydrous Na₂SO₄, filtered and concentrated under vacuum. The residue was purified by flash column chromatography (95:05 Hexane/EtOAc) to afford the alcohol **8** (473 mg, 95%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.78-7.65 (m, 8H, Ar), 7.44-7.33 (m, 12H, Ar), 4.62-4.59 (m, 1H, CHC≡C), 3.93 (d, 2H, J=1.4 Hz, C≡CCH₂OH), 3.81 (dd, 1H, J=6.9 Hz, J=10.2 Hz, 1xTBDPSOCH₂CH), 3.71 (dd, 1H, J=4.7 Hz, J=10.1 Hz, 1xTBDPSOCH₂CH), 1.08 (s, 9H, C(CH₃)₃), 1.05 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 136.3 (Ar), 136.0 (Ar), 135.7 (Ar), 134.1 (C_q Ar), 133.3 (C_q Ar), 129.71 (Ar), 129.66 (Ar), 129.64 (Ar), 129.61 (Ar), 127.7 (Ar), 127.6 (Ar), 127.3 (Ar), 85.1 (C≡C), 84.1 (C≡C), 68.2 (TBDPSOCH₂CH), 65.2 (CHC≡C), 50.9 (C≡CCH₂OH), 26.9 (C(CH₃)₃), 26.7 (C(CH₃)₃), 19.2 (C(CH₃)₃) ppm. **FTIR** (Neat): 2156 (C≡C), 3673 (OH) cm⁻¹. **HRMS**: calcd. for C₃₇H₄₄O₃Si₂Na⁺ [M+Na]⁺ 615.2829; found 615.2721.

4.1.8 Preparation of 4,5-bis[(tert-butylidiphenylsilyl)oxy]pentyl 4-methylbenzene-1-sulfonate **9**

To a stirred solution of alcohol **8** (870 mg, 1.47 mmol) in dry ether (10 mL) at 0°C, *p*-toluenesulfonyl chloride (336 mg, 1.76 mmol) and potassium hydroxide (412 mg, 7.34 mmol) were added. The reaction mixture was stirred and the temperature allowed to rise to r.t., after 16 hours all the starting material was consumed and water was added. The mixture was extracted with ether, dried with Na₂SO₄, filtered and concentrated under vacuum. Without further purification alkyne **9** was obtained (1.04 g, 95%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.69-7.61 (m, 10H, Ar), 7.42-7.33 (m, 12H, Ar), 7.17 (d, 2H, J=8.1 Hz, Ar), 4.44 (s, 2H, C≡CCH₂OTs), 4.43-4.40 (m, 1H, CHC≡C), 3.65 (dd, 1H, J=6.6 Hz, J=10.1 Hz, 1xTBDPSOCH₂CH), 3.55 (dd, 1H, J=5.1 Hz, J=10.0 Hz, 1xTBDPSOCH₂CH), 2.32 (s, 3H, PhCH₃), 1.05 (s, 9H, C(CH₃)₃), 1.01 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 144.9 (C_q Ar), 136.1 (Ar), 135.9 (Ar), 135.61 (Ar), 135.59 (Ar), 133.24 (C_q Ar), 133.2 (C_q Ar), 133.18 (C_q Ar), 133.0 (C_q Ar), 129.8 (Ar), 129.7 (Ar), 128.1 (Ar), 127.7 (Ar), 127.6 (Ar), 127.4 (Ar), 88.6 (C≡C), 77.2 (C≡C), 67.7 (TBDPSOCH₂CH), 64.9 (CHC≡C), 57.8 (C≡CCH₂OTs), 26.8 (C(CH₃)₃), 26.7 (C(CH₃)₃), 21.6 (PhCH₃), 19.22 (C(CH₃)₃), 19.19 (C(CH₃)₃) ppm. **FTIR** (Neat): 2158 (C≡C), 1111 and 1079 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₄₄H₅₀O₅SSi₂Na⁺ [M+Na]⁺ 769.2917; found 769.2810.

4.1.9 Preparation of 5-(3-azidopropyl)-2,2,9,9-tetramethyl-3,3,8,8-tetraphenyl-4,7-dioxa-3,8-disiladecane **10**

To a stirred solution of alkyne **9** (1.04 g, 1.39 mmol) in dry DMF (10 mL) at r.t., sodium azide (0.45 g, 6.96 mmol) was added. After 2 hours all the starting material was consumed. The reaction mixture was diluted with CH₂Cl₂ and the obtained solid was

filtered, the solvent was concentrated under vacuum. The residue was purified by flash column chromatography (90:10 Hexane/EtOAc) to afford the azide **10** (817 mg, 95%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.75-7.64 (m, 8H, Ar), 7.42-7.36 (m, 12H, Ar), 4.59-4.56 (m, 1H, CHC≡C), 3.80 (dd, 1H, J=6.5 Hz, J=10.1 Hz, 1xTBDPSOCH₂CH), 3.71 (dd, 1H, J=5.3 Hz, J=10.0 Hz, 1xTBDPSOCH₂CH), 3.66 (d, 2H, J=1.2 Hz, C≡CCH₂N₃), 1.08 (s, 9H, C(CH₃)₃), 1.03 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 136.1 (Ar), 135.9 (Ar), 135.64 (Ar), 135.60 (Ar), 133.5 (C_q Ar), 133.33 (C_q Ar), 133.29 (C_q Ar), 133.26 (C_q Ar), 129.73 (Ar), 129.68 (Ar), 129.6 (Ar), 127.7 (Ar), 127.6 (Ar), 127.4 (Ar), 86.7 (C≡C), 77.8 (C≡C), 68.1 (TBDPSOCH₂CH), 65.1 (CHC≡C), 39.9 (C≡CCH₂N₃), 26.9 (C(CH₃)₃), 26.7 (C(CH₃)₃), 19.3 (C(CH₃)₃), 19.2 (C(CH₃)₃) ppm. **FTIR** (Neat): 2121 (N=N=N) cm⁻¹. **HRMS**: calcd. for C₃₇H₄₃N₃O₂Si₂Na⁺ [M + Na]⁺ 640.2787; found 640,2786.

4.1.10 Preparation of 5-azidopent-3-yne-1,2-diol **11**

To a stirred solution of azide **10** (815 mg, 1.32 mmol) in dry THF (8 mL) at room temperature, a 1M solution of TBAF in THF (2.6 mL, 2.64 mmol) was added. After 3 hours all the starting material was consumed and the mixture was quenched with water (saturated with NaCl, 10 mL). The mixture was extracted with EtOAc (3x10 mL), dried with Na₂SO₄, filtered and concentrated under vacuum. The residue was purified by flash column chromatography (20:80 Hexane/EtOAc) to afford the diol **11** (142 mg, 76%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.57-4.53 (m, 1H, CHC≡C), 3.98 (d, 2H, J=1.4 Hz, C≡CCH₂N₃), 3.79 (dd, 1H, J=3.7 Hz, J=11.5 Hz, 1xHOCH₂CH), 3.71 (dd, 1H, J=6.6 Hz, J=11.5 Hz, 1xHOCH₂CH) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 85.2 (C≡C), 78.3 (C≡C), 66.3 (HOCH₂CH), 63.1 (CHC≡C), 39.9 (C≡CCH₂N₃) ppm. **FTIR** (Neat): 2112 (N=N=N), 3283 (OH) cm⁻¹.

4.1.11 Preparation of 2-(3-azidoprop-1-yn-1-yl)-1,4-dioxaspiro[4.5]decane **12**

To a stirred solution of diol **11** (94 mg, 0.67 mmol) in dry DMF (3 mL) at r.t., cyclohexanone dimethyl ketal (0.20 mL, 1.33 mmol) and two drops of sulfuric acid were added. After 16 hours all the starting material was consumed and the mixture was quenched with a saturated aqueous NaHCO₃ solution (5 mL) and extracted with EtOAc (3x5mL). The combined organic layers were dried with anhydrous Na₂SO₄, the solvent was filtered and concentrated under vacuum. The residue was purified by flash column chromatography (95:05 Hexane/EtOAc) to afford the acetal **12** (123 mg, 84%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.80-4.76 (m, 1H, CHC≡C), 4.19 (dd, 1H, J=6.4 Hz, J=8.1 Hz, 1xHOCH₂CH), 3.96 (s, 2H, C≡CCH₂N₃), 3.94 (dd, 1H, J=6.3 Hz, J=8.0 Hz, 1xHOCH₂CH), 1.76-1.39 (m, 10H, CH₂ from ketal) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 111.3 (C_q), 85.3 (C≡C), 77.8 (C≡C), 69.6 (HOCH₂CH), 65.0 (CHC≡C), 39.9

(C≡CCH₂N₃), 35.7 (CH₂ from ketal), 35.4 (CH₂ from ketal), 25.0 (CH₂ from acetal), 23.9 (2xCH₂ from acetal) ppm. **FTIR** (Neat): 2120 (N=N=N), 1040 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₁₁H₁₅N₃O₂⁺ [M]⁺ 222.1236; found 222.1237.

4.1.12 Preparation of 3-{1,4-dioxaspiro[4.5]decan-2-yl}prop-2-yn-1-amine, **2**

To a stirred solution of acetal **12** (120 mg, 0.54 mmol) in dry THF (5 mL) at r.t., triphenylphosphine (313 mg, 1.19 mmol) was added. After 3 hours 45 μL of water were added and the mixture was stirred overnight. The reaction mixture was concentrated under vacuum and the residue was purified by flash column chromatography (100% EtOAc followed by 90:10 CH₂Cl₂/MeOH) to afford the amine **2** (100 mg, 94%) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.74 (t, 1H, J=6.2 Hz, CHC≡C), 4.14 (dd, 1H, J=6.2 Hz, J=8.0 Hz, 1xHOCH₂CH), 3.88 (dd, 1H, J=6.6 Hz, J=8.0 Hz, 1xHOCH₂CH), 3.56 (d, 2H, C≡CCH₂NH₂), 1.74-1.40 (m, 10H, CH₂ from ketal) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 111.0 (C_q), 84.6 (C≡C), 80.9 (C≡C), 69.5 (HOCH₂CH), 65.2 (CHC≡C), 35.8 (CH₂ from ketal), 35.4 (CH₂ from ketal), 31.2 (C≡CCH₂NH₂), 25.0 (CH₂ from ketal), 23.9 (CH₂ from ketal), 23.8 (CH₂ from ketal) ppm. **FTIR** (Neat): 2106 (C≡C), 1099 (C-N), 1590 (N-H) cm⁻¹. **HRMS**: calcd. for C₁₁H₁₆NO₂⁺ [M]⁺ 195.1259; found 196.1332.

4.1.13 Preparation of N-(3-{1,4-dioxaspiro[4.5]decan-2-yl}prop-2-yn-1-yl)-6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamide **13**

To a solution of D-desthiobiotin (110 mg, 0.51 mmol) in dry MeCN were added EDC (147 mg, 0.77 mmol) and HOBt (69 mg, 0.51 mmol). After stirred for 2h30min the amine **2** (100 mg, 0.51 mmol) was added in dry MeCN and the reaction mixture was stirred overnight, at room temperature. The mixture was concentrated under vacuum and the crude residue was purified by flash column chromatography (95:05 CH₂Cl₂/MeOH) to afford the amide **13** (91 mg, 46%).

¹H-NMR (CDCl₃), 400 MHz: δ 6.40 (s, 1H, NH), 4.71 (t, 1H, J=6.4 Hz, C≡CCHCH₂), 4.13 (dd, 1H, J=4.3 Hz, J=8.0 Hz, 1xC≡CCHCH₂), 4.08 (s, 2H, HNCH₂C≡C), 3.88-3.84 (m, 2H, 1xC≡CCHCH₂ and CH₃CHNH D-desthiobiotin), 3.74-3.67 (m, 1H, HNCHCH₂ D-desthiobiotin), 2.20 (t, 2H, J=7.4 Hz, CH₂(C=O)NH), 1.73-1.24 (m, CH₂ from D-desthiobiotin and ketal), 1.14 (d, 3H, J=6.5 Hz, CH₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 172.7 (C=O), 163.9 (C=O), 111.0 (C_q from ketal), 82.00 (C≡C), 81.96 (C≡C), 80.52 (C≡C), 80.49 (C≡C), 69.4 (C≡CCHCH₂), 65.1 (C≡CCHCH₂), 56.1 (CH₃CHNH), 51.5 (HNCHCH₂), 35.8 (CH₂), 35.62 (CH₂), 35.59 (CH₂), 35.3 (CH₂(C=O)NH), 29.4 (CH₂), 29.3 (CH₂), 29.2 (HNCH₂C≡C), 28.5 (CH₂), 28.4 (CH₂), 25.73 (CH₂), 25.71 (CH₂), 25.03 (CH₂), 24.99 (CH₂), 23.9 (CH₂), 23.8 (CH₂), 15.7 (CH₃) ppm. **HRMS**: calcd. for C₂₀H₃₄N₃O₄⁺ [M]⁺ 392.2543; found 392.2544.

4.1.14 Preparation of *N*-(3-{1,4-dioxaspiro[4.5]decan-2-yl}-2,3-dioxopropyl)-6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamide **14**

To a solution of amide **13** (15 mg, 0.38 mmol) in 2 mL of H₂O:Et₂O (1:1.5) was added KClO₃ (12 mg, 0.96 mmol), OsO₄ (195 μL, 0.15 mmol, 2% aqueous solution) and *t*-BuOH (195 μL). After 24h, at room temperature, the reaction mixture was quenched with a saturated aqueous NH₄Cl solution (2 mL) and extracted with CH₂Cl₂ (3x2mL). The combined organic layers were dried with anhydrous Na₂SO₄, filtered and concentrated under vacuum. The amide **14** (16 mg, quant. yield) was afforded without further purification as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.92 (t, 1H, J=6.5 Hz, (C=O)CHCH₂), 4.26 (t, 1H, J=8.0 Hz, 1xHNCH₂(C=O)), 4.15-4.08 (m, 2H, 1xHNCH₂(C=O) and 1x(C=O)CHCH₂), 3.90-3.85 (1x(C=O)CHCH₂ and CH₃CHNH D-desthiobiotin), 3.76-3.65 (m, 1H, HNCHCH₂ D-desthiobiotin), 2.45-2.18 (m, CH₂(C=O)NH), 1.74-1.25 (m, CH₂ from D-desthiobiotin and ketal), 1.13 (d, 3H, J=6.3 Hz, CH₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 206.7 (C=O), 192.4 (C=O), 174.7 (C=O), 164.8 (C=O), 134.1 (C_q from ketal), 76.7 ((C=O)CHCH₂), 69.4 ((C=O)CHCH₂), 66.9 (HNCH₂(C=O)), 56.2 (HNCHCH₂ D-desthiobiotin), 56.1 (CH₃CHNH D-desthiobiotin), 42.0 (CH₂(C=O)NH), 35.8 (CH₂), 35.4 (CH₂), 35.3 (CH₂), 34.8 (CH₂), 29.7 (CH₂), 29.3 (CH₂), 28.6 (CH₂), 27.0 (CH₂), 26.6 (CH₂), 25.7 (CH₂), 25.0 (CH₂), 24.6 (CH₂), 23.9 (CH₂), 23.8 (CH₂), 23.0 (CH₂), 15.7 (CH₃) ppm. **HRMS**: calcd. for C₂₁H₃₃N₃O₆⁺ [M]⁺ 424.2440; found 424.2442.

4.1.15 Preparation of *N*-(4,5-dihydroxy-2,3-dioxopentyl)-6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamide **1**

To a solution of amide **14** (9.6 mg, 0.023 mmol) in 750 μL of DMSO-*d*₆:D₂O (1:4) was added D₂SO₄ (10 μL of a 10% solution in D₂O). The solution was sonicated during 15 minutes, in a cooled ultrasounds bath, and stirring for 24h at 800 rpm. The mixture was quenched with CDCl₃ (3x500 μL) and the aqueous phase was recovered. The complex D-desthiobiotin-AI-2 **1** (7.8 mg, quant. yield) was obtained in solution without further purification.

¹H-NMR (D₂O), 400 MHz: δ 8.15 (s, NH), 4.27 (t, J=4.1 Hz, CH from AI-2), 3.86-3.76 (m, 1H, CH₃CHNH D-desthiobiotin), 3.76 (d, J=4.2 Hz, CH₂ from AI-2), 3.72-3.65 (m, 1H, HNCHCH₂ D-desthiobiotin), 3.58-3.49 (m, (C=O)HNCH₂(C=O)), 2.21-2.16 (m, 2H, CH₂(C=O)NH), 1.62-1.50 (m, 2H, CH₂ from D-desthiobiotin), 1.47-1.39 (m, 2H, CH₂ from D-desthiobiotin), 1.35-1.18 (m, 4H, CH₂ from D-desthiobiotin), 1.03 (d, 3H, J=6.5 Hz, CH₃) ppm. Other peaks are present, come from the cyclic forms of AI-2. **¹³C-NMR** (D₂O), 100 MHz: δ 71.4 (CH from AI-2), 65.1 ((C=O)HNCH₂(C=O)), 63.3 (CH₂ from AI-2), 55.9 (HNCHCH₂ D-desthiobiotin), 51.4 (CH₃CHNH D-desthiobiotin), 35.4 (CH₂), 35.0 (CH₂), 28.8 (CH₂), 28.7 (CH₂), 28.2 (CH₂), 28.1 (CH₂), 25.3 (CH₂), 25.1 (CH₂), 25.0 (CH₂), 14.6 (CH₃) ppm.

4.1.16 Protein expression and purification

Escherichia coli BL21 ($\Delta luxS$) transformed with the plasmid pQE30 (carrying LuxP protein from *Vibrio harveyi* fused to cyan (CFP) and yellow (YFP) fluorescent proteins) – pQE30-CLPY- was kindly provided by Richard Sayre (The Ohio State University, Ohio, USA). Expression and purification of this protein was carried out as previously described.²⁷ Briefly, protein expression was induced at an optical density (OD_{600}) of 0.6 with 0.3 mM of isopropyl β -D-thiogalactopyranoside (IPTG) and grown for 6h, at 28 °C before cell harvesting by centrifugation. The resulting pellet was resuspended in 25 mM sodium phosphate buffer pH 8.0, 35 mM NaCl, 1:100 protease inhibitor cocktail (Roche) and cells were lysed by passing them three times through a french press. Cell debris were removed by centrifugation and LuxP was purified from clarified supernatants through Nickel-nitrilotriacetic (Ni^{+} -NTA) acid affinity chromatography (Qiagen). The protein was eluted in 25 mM sodium phosphate buffer pH 8.0, 35 mM NaCl, 40% glycerol, 250 mM imidazole, 1:100 protease inhibitor cocktail. The expression and purification of *Clostridium saccharobutylicum* DSM13864 and *E. coli* K-12 MG1655 LsrB proteins (CsLsrB and EcLsr, respectively) were carried out as previously described.^{12,28} Concisely, *E. coli* BL21 ($\Delta luxS$) cells transformed with the constructs pPro-EcLsrB and pDEST-CsLsrB were grown in LB with 1 μ g/mL ampicillin at 37°C until the OD_{600} was 0.3. At this point, temperature was decreased to 22°C. At $OD_{600} = 0.9$, protein expression was induced with 0.1 mM or 0.3 mM of IPTG for the expression of EcLsr and CsLsrB, respectively. Cells grew for 6h after induction and were harvested by centrifugation. The resulting pellets were resuspended in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, 2.5 μ g/mL DNase and 2.5 μ g/mL leupeptin and cells were lysed by passing thrice through a french press. Centrifugation was employed to remove cell debris and the tagged-proteins were purified by Ni^{+} -NTA affinity chromatography. EcLsrB and CsLsrB were eluted in 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 250 mM imidazole and subsequently dialyzed (Slide-A-Lyzer, ThermoFisher Scientific) into 25 mM Tris buffer pH 8.0, 150 mM NaCl, 1 mM DTT. CLPY-LuxP, EcLsrB and CsLsrB have a molecular weight of approximately 98, 40 and 37.5 kDa, respectively. The higher molecular weight of CPLY-LuxP when compared with the other receptors is due to its fusion to CFP and YFP.

4.1.17 Native PAGE and western-blot

Approximately 125 μ g of partially purified CPLY (CFP-LuxP-YFP; \sim 1 nmol), CsLsrB (\sim 3 nmol), and EcLsrB (\sim 3 nmol) were incubated with approximately 59 μ g of desthiobiotin-AI-2 (\sim 172 nmol) overnight under gentle agitation at 4°C. These quantities ensured the presence of at least 50 times more probe than receptors. As a control 125 μ g of the protein samples were incubated with desthiobiotin alone (59 μ g) or with buffer in the same conditions (25 mM sodium phosphate buffer pH 8.0, 35 mM NaCl, 1 mM boric acid for CPLY and 25 mM Tris buffer pH 8.0, 150 mM NaCl, 1 mM DTT for CsLsrB and EcLsrB). 20 μ L of all the samples were mixed with 5 μ L of 5X sample buffer (0.31 M Tris-HCl buffer pH 6.8, 0.05% bromophenol blue and 50% glycerol) and 11 μ L of these mixes were loaded into each of two 10% polyacrylamide non-denaturing gels. 5 μ L of protein standards (All Blue Standards, Bio-Rad) was also added. The gel was run in Tris-

glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3), at 160 V, 4°C for approximately 1h30. One of the gels was stained with Comassie blue and the presence of the bands assessed. The second gel was used for a western-blot. Transfer was performed at 90 mA, 4°C, overnight in 25 mM Tris, 192 mM glycine and 0.05% SDS to a PVDF membrane. The membrane was incubated in a 1:5000 dilution of an anti-biotin, HRP conjugated antibody (PA1-30595, Invitrogen) for 1h at room temperature. An ECL chemiluminescent substrate was added (SuperSignal West Pico, Pierce) and signal detection was performed in a blot imager (Amersham Imager 680).

4.1.18 Pull-down of LuxP receptor

Magnetic Dynabeads with protein G (Invitrogen, 10003D) were conjugated with the anti-biotin antibody (#31852, Pierce) as per the manufacturers instructions. Briefly, 50 µL of Dynabeads (1.5 mg) were added to a 1.5 mL eppendorf and the storage solution was removed through magnetic separation. 10 µg of antibody diluted in 200 µL of wash buffer (PBS buffer pH 7.4, 0.02% Tween-20) was added to the beads and the mixture was incubated for 10 min, at room temperature with agitation. The unbound antibody was then removed by magnetic separation and the beads were washed with 200 µL of wash buffer. Next, the sample was added to the anti-biotin antibody conjugated magnetic beads. The sample used for the pull down of CLPY was incubated following the conditions mentioned above and included approximately 76,5 µg of DB-AI-2 and 265 µg of partially purified CLPY. The mixture sample/antibody-conjugated beads was incubated for 1h at room temperature with agitation followed by three washes with 200 µL of wash buffer to remove unbound protein. CLPY, complexed with the AI-2 probe and retained by the antibody conjugated magnetic beads, was eluted by denaturation. The final volume was heated for 10 minutes at 70°C in 20 µL of 50 mM glycine pH 2.8 and 10 µL of 240 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 0.04% bromophenol blue and 5% β-mercaptoethanol. 10 µL of the wash and elution steps were mixed with 5 µL of 200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue and 40% glycerol and added to a denaturing gel composed of 15% acrylamide in the separating gel and 5% in the stacking gel. As protein standards, 5 µL of All Blue Standards (Bio-Rad) were loaded. The gel was run in SDS running buffer at room temperature, 180 V for approximately 70 minutes and stained with Comassie blue.

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Synthesis of D-desthiobiotin-AI-2 as a novel chemical probe for autoinducer-2 quorum sensing receptors

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Electronic Supporting Information

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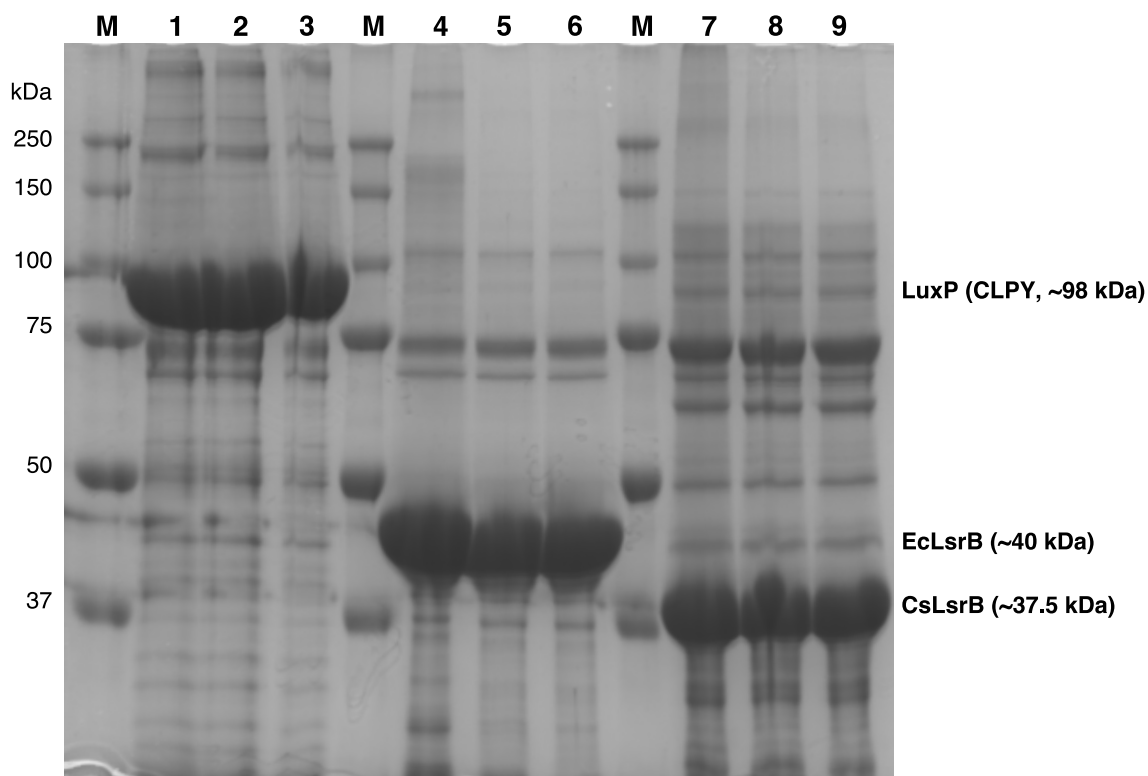


Figure S1 Loading control of the samples ran in the native gel. The samples were heated at 95°C for 5 min and loaded into a denaturing gel composed of 15% acrylamide in the separating gel and 5% in the stacking gel. 5 μ L of All Blue Standards (Bio-Rad) were loaded as protein standards. The gel was run in SDS running buffer at room temperature, 180 V for approximately 70 minutes and stained with Coomassie blue. Protein standards (M), CLPY-LuxP incubated with DB-AI-2 (1), desthiobiotin (2) and buffer (3); EcLsrB incubated with DB-AI-2 (4), with desthiobiotin (5) and buffer (6); CsLsrB incubated with DB-AI-2 (7), desthiobiotin (8) and buffer (9).

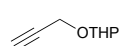
1. Material and Methods

1.1 Synthesis

^1H NMR spectra were obtained with a Bruker Avance II+ (400 MHz) spectrometer, at 400 MHz in CDCl_3 or D_2O with chemical shift values (δ) in ppm downfield from tetramethylsilane in the case of CDCl_3 , and ^{13}C NMR spectra were obtained at 100 MHz in CDCl_3 or D_2O . Assignments are supported by 2D correlation NMR spectroscopy studies. Preparative chromatographic separations were carried out by flash chromatography using Kieselgel 60, (0.032–0.063mm). Analytical TLC was performed on aluminum-backed Merck 60 F_{254} silica gel plates. Reagents and solvents were purified and dried according to ref.¹ All the reactions were carried out under an inert atmosphere (argon), except when the solvents were not dried.

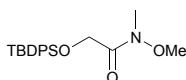
1.2 Characterisation of compounds

Synthesis of 2-(ethynyloxy)oxane 3



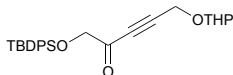
Propyne **3** was prepared and characterised according to the literature.²

Synthesis of *N*-Methoxy-*N*-methyl-2-(*tert*-butyldiphenylsilyloxy)acetamide 4



Weinreb amide **4** was prepared and characterised according to the literature.³

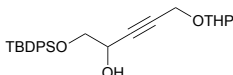
Synthesis of 1-[(*tert*-butyldiphenylsilyl)oxy]-5-(oxan-2-yloxy)pent-3-yn-2-one **5**



To a solution of DIPA (1.6 mL, 11.2 mmol) in THF (8 mL), 1.6M solution of *n*-BuLi in hexane (6.3 mL, 10.7 mmol) was added dropwise at -78 °C and the mixture stirred at 0 °C for 15 minutes. At -78 °C, a solution of propyne **3** (1.57 g, 11.2 mmol) in THF (8 mL) was added and the mixture stirred for 30 minutes. Still at -78 °C, a solution of Weinreb amide **4** (2.00 g, 5.6 mmol) in THF (10 mL) was added. The mixture was stirred at -78 °C for 20 minutes and then at 0 °C until all starting material had been consumed (TLC), approximately 2 hours. The mixture was quenched with a saturated aqueous NH₄Cl solution (20mL) and extracted with CH₂Cl₂ (3x20mL). The combined organic layers were dried with anhydrous Na₂SO₄, the solvent was filtered and concentrated under vacuum. The residue was purified by flash column chromatography (90:10 Hexane/EtOAc) to afford the ketone **5** (2.20 g, 90 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.68-7.66 (m, 4H, Ar), 7.44-7.37 (m, 6H, Ar), 4.77 (t, 1H, J=3.2 Hz, CH from THP), 4.39 (d, 2H, J=1.7 Hz, C≡CCH₂), 4.32 (s, 2H, TBDPSOCH₂C=O), 3.81-3.76 (m, 1H, 1xCH₂ from THP), 3.54-3.49 (m, 1H, 1xCH₂ from THP), 1.80-1.50 (6H, m, 3xCH₂ from THP), 1.10 (s, 9H, C(CH₃)₃) ppm. ¹³C-NMR (CDCl₃), 100 MHz: δ 185.4 (C=O), 135.6 (Ar), 132.6 (C_q Ar), 130.0 (Ar), 127.9 (Ar), 97.2 (CH from THP), 91.0 (C≡C), 83.0 (C≡C), 70.5 (TBDPSOCH₂C=O), 62.0 (CH₂ from THP), 53.8 (C≡CCH₂), 30.0 (CH₂ from THP), 26.7 (C(CH₃)₃), 25.2 (CH₂ from THP), 19.3 (C(CH₃)₃), 18.8 (CH₂ from THP). FTIR (Neat): 1720 (C=O), 2155 (C≡C) cm⁻¹. HRMS: calcd. for C₂₆H₃₂O₄SiNa⁺ [M + Na]⁺ 459.2070; found 459.1962.

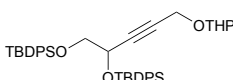
Synthesis of 1-[(*tert*-butyldiphenylsilyl)oxy]-5-(oxan-2-yloxy)pent-3-yn-2-ol **6**



To a solution of ketone **5** (0.790 g, 1.8 mmol) in dry CH₂Cl₂ (5 mL), at 0 °C, was added *tert*-butyldimethylsilylchloride (0.60 mL, 2.7 mmol), diisopropylethylamine (0.63 mL, 3.6 mmol) and a catalytic amount of DMAP. The reaction mixture was stirred allowing the temperature to rise until r.t., after 16 hours all the starting material was consumed and water was added. The mixture was extracted with CH₂Cl₂, dried with Na₂SO₄, the solvent was filtered and concentrated under vacuum. Without further purification, alcohol **6** was obtained (0.819 g, quantitative yield) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.69-7.65 (m, 4H, Ar), 7.44-7.38 (m, 6H, Ar), 4.77 (t, 1H, J=3.37 Hz, CH from THP), 4.52-4.50 (m, 1H, CHC≡C), 4.27 (qdd, 2H, J=16.0 Hz, J=1.7 Hz, J=3.5 Hz, C≡CCH₂), 3.82-3.70 (m, 3H, TBDPSOCH₂CH and 1xCH₂ from THP), 3.51-3.47 (m, 1H, 1xCH₂ from THP), 1.63-1.49 (m, 6H, 3xCH₂ from THP), 1.07 (s, 9H, C(CH₃)₃) ppm. ¹³C-NMR (CDCl₃), 100 MHz: δ 135.6 (Ar), 135.5 (Ar), 132.9 (C_q Ar), 132.8 (C_q Ar), 129.92 (Ar), 129.91 (Ar), 127.84 (Ar), 127.82 (Ar), 96.8 (CH from THP), 67.4 (TBDPSOCH₂CH), 63.2 (CHC≡C), 61.9 (CH₂ from THP), 54.2 (C≡CCH₂), 30.2 (CH₂ from THP), 26.8 (C(CH₃)₃), 25.3 (C(CH₃)₃), 19.3 (C(CH₃)₃) ppm. FTIR (Neat): 2158 (C≡C), 1022 (C-O-C) cm⁻¹. HRMS: calcd. for C₂₆H₃₄O₄SiNa⁺ [M + Na]⁺ 461.2226; found 461.2119.

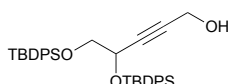
Synthesis of 2,2,9,9-tetramethyl-5-[3-(oxan-2-yloxy)prop-1-yn-1-yl]-3,3,8,8-tetraphenyl-4,7-dioxa-3,8-disiladecane **7**



To a solution of alcohol **6** (0.815 g, 1.9 mmol) in dry MeOH (5 mL), at 0 °C, was added NaBH₄ (0.141 g, 3.7 mmol). The reaction mixture was stirred allowing the temperature to rise to r.t., after 3 hours all the starting material was consumed and water was added. The mixture was extracted with EtOAc, dried with Na₂SO₄, the solvent was filtered and concentrated under vacuum. The crude residue was purified by flash column chromatography (90:10 Hexane/EtOAc) to afford the alkyne **7** (1.160 g, 95 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.75 (d, 2H, J=6.90 Hz, Ar), 7.71 (d, 2H, J=7.3 Hz, Ar), 7.65 (t, 4H, J=6.2 Hz, Ar), 7.40-7.32 (m, 12H, Ar), 4.64-4.56 (m, 2H, CH from THP and CHC≡C), 4.11-4.02 (m, 2H, C≡CCH₂), 3.81-3.68 (m, 3H, TBDPSOCH₂CH and 1xCH₂ from THP), 3.44-3.40 (m, 1H, 1xCH₂ from THP), 1.78-1.45 (m, 6H, 3xCH₂ from THP), 1.08 (s, 9H, C(CH₃)₃), 1.02 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 136.1 (Ar), 135.9 (Ar), 135.7 (Ar), 135.6 (Ar), 133.6 (C_q Ar), 133.5 (C_q Ar), 133.4 (C_q Ar), 133.3 (C_q Ar), 129.62 (Ar), 129.58 (Ar), 127.6 (Ar), 127.5 (Ar), 127.4 (Ar), 96.5 (CHC≡C), 85.1 (C≡C), 81.6 (C≡C), 68.2 (TBDPSOCH₂CH), 65.3 (CHC≡C), 61.9 (CH₂ from THP), 54.0 (C≡CCH₂), 30.2 (CH₂ from THP), 26.9 (C(CH₃)₃), 26.7 (C(CH₃)₃), 25.4 (CH₂ from THP), 19.3 (C(CH₃)₃), 19.2 (C(CH₃)₃), 19.1 (CH₂ from THP) ppm. **FTIR** (Neat): 2170 (C≡C), 1111 and 1079 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₄₂H₅₂O₄Si₂Na⁺ [M + Na]⁺ 699.3404; found 699.3296.

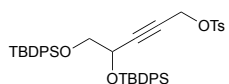
Synthesis of 4,5-bis[(tert-butyl-diphenylsilyl)oxy]pent-2-yn-1-ol **8**



To a stirred solution of alkyne **7** (0.57 g, 0.84 mmol) in dry MeOH (5 mL) at r.t., *p*-toluenesulfonic acid monohydrate (11 mg, 0.06 mmol) was added. After 2 hours all the starting material had been consumed and the reaction mixture was quenched with a saturated aqueous NaHCO₃ solution (10 mL) and extracted with EtOAc (3x10 mL). The combined organic layers were dried with anhydrous Na₂SO₄, the solvent was filtered and concentrated under vacuum. The residue was purified by flash column chromatography (95:05 Hexane/EtOAc) to afford the alcohol **8** (473 mg, 95 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.78-7.65 (m, 8H, Ar), 7.44-7.33 (m, 12H, Ar), 4.62-4.59 (m, 1H, CHC≡C), 3.93 (d, 2H, J=1.4 Hz, C≡CCH₂OH), 3.81 (dd, 1H, J=6.9 Hz, J=10.2 Hz, 1xTBDPSOCH₂CH), 3.71 (dd, 1H, J=4.7 Hz, J=10.1 Hz, 1xTBDPSOCH₂CH), 1.08 (s, 9H, C(CH₃)₃), 1.05 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 136.3 (Ar), 136.0 (Ar), 135.7 (Ar), 134.1 (C_q Ar), 133.3 (C_q Ar), 129.71 (Ar), 129.66 (Ar), 129.64 (Ar), 129.61 (Ar), 127.7 (Ar), 127.6 (Ar), 127.3 (Ar), 85.1 (C≡C), 84.1 (C≡C), 68.2 (TBDPSOCH₂CH), 65.2 (CHC≡C), 50.9 (C≡CCH₂OH), 26.9 (C(CH₃)₃), 26.7 (C(CH₃)₃), 19.2 (C(CH₃)₃) ppm. **FTIR** (Neat): 2156 (C≡C), 3673 (OH) cm⁻¹. **HRMS**: calcd. for C₃₇H₄₄O₃Si₂Na⁺ [M + Na]⁺ 615.2829; found 615.2721.

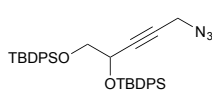
Synthesis of 4,5-bis[(tert-butyl-diphenylsilyl)oxy]pentyl 4-methylbenzene-1-sulfonate **9**



To a stirred solution of alcohol **8** (870 mg, 1.47 mmol) in dry ether (10 mL) at 0 °C, *p*-toluenesulfonyl chloride (336 mg, 1.76 mmol) and potassium hydroxide (412 mg, 7.34 mmol) were added. The reaction mixture was stirred and the temperature allowed to rise to r.t., after 16 hours all the starting material was consumed and water was added. The mixture was extracted with ether, dried with Na₂SO₄, the solvent was filtered and concentrated under vacuum. Without further purification alkyne **9** was obtained (1.04 g, 95 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.69-7.61 (m, 10H, Ar), 7.42-7.33 (m, 12H, Ar), 7.17 (d, 2H, J=8.1 Hz, Ar), 4.44 (s, 2H, C≡CCH₂OTs), 4.43-4.40 (m, 1H, CHC≡C), 3.65 (dd, 1H, J=6.6 Hz, J=10.1 Hz, 1xTBDPSOCH₂CH), 3.55 (dd, 1H, J=5.1 Hz, J=10.0 Hz, 1xTBDPSOCH₂CH), 2.32 (s, 3H, PhCH₃), 1.05 (s, 9H, C(CH₃)₃), 1.01 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 144.9 (C_q Ar), 136.1 (Ar), 135.9 (Ar), 135.61 (Ar), 135.59 (Ar), 133.24 (C_q Ar), 133.2 (C_q Ar), 133.18 (C_q Ar), 133.0 (C_q Ar), 129.8 (Ar), 129.7 (Ar), 128.1 (Ar), 127.7 (Ar), 127.6 (Ar), 127.4 (Ar), 88.6 (C≡C), 77.2 (C≡C), 67.7 (TBDPSOCH₂CH), 64.9 (CHC≡C), 57.8 (C≡CCH₂OTs), 26.8 (C(CH₃)₃), 26.7 (C(CH₃)₃), 21.6 (PhCH₃), 19.22 (C(CH₃)₃), 19.19 (C(CH₃)₃) ppm. **FTIR** (Neat): 2158 (C≡C), 1111 and 1079 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₄₄H₅₀O₅Si₂Na⁺ [M + Na]⁺ 769.2917; found 769.2810.

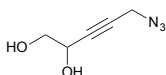
Synthesis of 5-(3-azidopropyl)-2,2,9,9-tetramethyl-3,3,8,8-tetraphenyl-4,7-dioxaspiro[3,8]undecane **10**



To a stirred solution of alkyne **9** (1.04 g, 1.39 mmol) in dry DMF (10 mL) at r.t., sodium azide (0.45 g, 6.96 mmol) was added. After 2 hours all the starting material was consumed. The reaction mixture was diluted with CH₂Cl₂ and the obtained solid was filtered, the solvent was concentrated under vacuum. The residue was purified by flash column chromatography (90:10 Hexane/EtOAc) to afford the azide **10** (817 mg, 95 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 7.75-7.64 (m, 8H, Ar), 7.42-7.36 (m, 12H, Ar), 4.59-4.56 (m, 1H, CHC≡C), 3.80 (dd, 1H, J=6.5 Hz, J=10.1 Hz, 1xTBDPSOCH₂CH), 3.71 (dd, 1H, J=5.3 Hz, J=10.0 Hz, 1xTBDPSOCH₂CH), 3.66 (d, 2H, J=1.2 Hz, C≡CCH₂N₃), 1.08 (s, 9H, C(CH₃)₃), 1.03 (s, 9H, C(CH₃)₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 136.1 (Ar), 135.9 (Ar), 135.64 (Ar), 135.60 (Ar), 133.5 (C_q Ar), 133.33 (C_q Ar), 133.29 (C_q Ar), 133.26 (C_q Ar), 129.73 (Ar), 129.68 (Ar), 129.6 (Ar), 127.7 (Ar), 127.6 (Ar), 127.4 (Ar), 86.7 (C≡C), 77.8 (C≡C), 68.1 (TBDPSOCH₂CH), 65.1 (CHC≡C), 39.9 (C≡CCH₂N₃), 26.9 (C(CH₃)₃), 26.7 (C(CH₃)₃), 19.3 (C(CH₃)₃), 19.2 (C(CH₃)₃) ppm. **FTIR** (Neat): 2121 (N=N=N) cm⁻¹. **HRMS**: calcd. for C₃₇H₄₃N₃O₂Si₂Na⁺ [M + Na]⁺ 640.2787; found 640,2786.

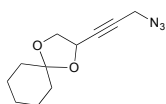
Synthesis of 5-azidopent-3-yne-1,2-diol **11**



To a stirred solution of azide **10** (815 mg, 1.32 mmol) in dry THF (8 mL) at r.t., sodium azide (0.45 g, 6.96 mmol) was added. After 2 hours all the starting material was consumed and the mixture was quenched with water. The mixture was extracted with EtOAc, dried with Na₂SO₄, the solvent was filtered and concentrated under vacuum. Without further purification diol **11** (142 mg, 76 %) was obtained as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.57-4.53 (m, 1H, CHC≡C), 3.98 (d, 2H, J=1.4 Hz, C≡CCH₂N₃), 3.79 (dd, 1H, J=3.7 Hz, J=11.5 Hz, 1xHOCH₂CH), 3.71 (dd, 1H, J=6.6 Hz, J=11.5 Hz, 1xHOCH₂CH) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 85.2 (C≡C), 78.3 (C≡C), 66.3 (HOCH₂CH), 63.1 (CHC≡C), 39.9 (C≡CCH₂N₃) ppm. **FTIR** (Neat): 2112 (N=N=N), 3283 (OH) cm⁻¹. **HRMS**: calcd. for C₄₄H₅₀O₅Si₂Na⁺ [M + Na]⁺.

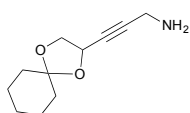
Synthesis of 2-(3-azidoprop-1-yn-1-yl)-1,4-dioxaspiro[4.5]undecane **12**



To a stirred solution of diol **11** (94 mg, 0.67 mmol) in dry DMF (3 mL) at r.t., cyclohexanone dimethyl ketal (0.20 mL, 1.33 mmol) and two drops of sulfuric acid were added. After 16 hours all the starting material was consumed and the mixture was quenched with a saturated aqueous NaHCO₃ solution (5 mL) and extracted with EtOAc (3x5mL). The combined organic layers were dried with anhydrous Na₂SO₄, the solvent was filtered and concentrated under vacuum. The residue was purified by flash column chromatography (95:05 Hexane/EtOAc) to afford the acetal **12** (123 mg, 84 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.80-4.76 (m, 1H, CHC≡C), 4.19 (dd, 1H, J=6.4 Hz, J=8.1 Hz, 1xHOCH₂CH), 3.96 (s, 2H, C≡CCH₂N₃), 3.94 (dd, 1H, J=6.3 Hz, J=8.0 Hz, 1xHOCH₂CH), 1.76-1.39 (m, 10H, CH₂ from ketal) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 111.3 (C_q), 85.3 (C≡C), 77.8 (C≡C), 69.6 (HOCH₂CH), 65.0 (CHC≡C), 39.9 (C≡CCH₂N₃), 35.7 (CH₂ from ketal), 35.4 (CH₂ from ketal), 25.0 (CH₂ from acetal), 23.9 (2xCH₂ from acetal) ppm. **FTIR** (Neat): 2120 (N=N=N), 1040 (C-O-C) cm⁻¹. **HRMS**: calcd. for C₁₁H₁₅N₃O₂⁺ [M]⁺ 222.1236; found 222.1237.

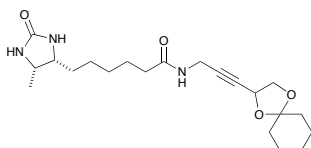
Synthesis of 3-{1,4-dioxaspiro[4.5]decan-2-yl}prop-2-yn-1-amine, **2**



To a stirred solution of acetal **12** (120 mg, 0.54 mmol) in dry THF (5 mL) at r.t., triphenylphosphine (313 mg, 1.19 mmol) was added. After 3 hours 45 μ L of water were added and the mixture was stirred overnight. The reaction mixture was concentrated under vacuum and the residue was purified by flash column chromatography (100% EtOAc followed by 90:10 CH₂Cl₂/MeOH) to afford the amine **2** (100 mg, 94 %) as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.74 (t, 1H, J=6.2 Hz, CHC \equiv C), 4.14 (dd, 1H, J=6.2 Hz, J=8.0 Hz, 1xHOCH₂CH), 3.88 (dd, 1H, J=6.6 Hz, J=8.0 Hz, 1xHOCH₂CH), 3.56 (d, 2H, C \equiv CCH₂NH₂), 1.74-1.40 (m, 10H, CH₂ from ketal) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 111.0 (C_q), 84.6 (C \equiv C), 80.9 (C \equiv C), 69.5 (HOCH₂CH), 65.2 (CHC \equiv C), 35.8 (CH₂ from ketal), 35.4 (CH₂ from ketal), 31.2 (C \equiv CCH₂NH₂), 25.0 (CH₂ from ketal), 23.9 (CH₂ from ketal), 23.8 (CH₂ from ketal) ppm. **FTIR** (Neat): 2106 (C \equiv C), 1099 (C-N), 1590 (N-H) cm⁻¹. **HRMS**: calcd. for C₁₁H₁₆NO₂⁺ [M]⁺ 195.1259; found 196.1332.

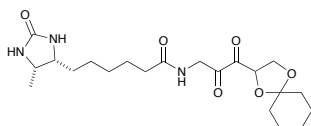
Synthesis of N-(3-{1,4-dioxaspiro[4.5]decan-2-yl}prop-2-yn-1-yl)-6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamide **13**



To a solution of D-desthiobiotin (110 mg, 0.51 mmol) in dry MeCN were added EDC (147 mg, 0.77 mmol) and HOBt (69 mg, 0.51 mmol). After stirred for 2h30min the amine **2** (100 mg, 0.51 mmol) was added in dry MeCN and the reaction mixture was stirred overnight, at room temperature. The mixture was concentrated under vacuum and the crude residue was purified by flash column chromatography (95:05 CH₂Cl₂/MeOH) to afford the amide **13** (91 mg, 46%).

¹H-NMR (CDCl₃), 400 MHz: δ 6.40 (s, 1H, NH), 4.71 (t, 1H, J=6.4 Hz, C \equiv CCHCH₂), 4.13 (dd, 1H, J=4.3 Hz, J=8.0 Hz, 1xC \equiv CCHCH₂), 4.08 (s, 2H, HNCH₂C \equiv C), 3.88-3.84 (m, 2H, 1xC \equiv CCHCH₂ and CH₃CHNH D-desthiobiotin), 3.74-3.67 (m, 1H, HNCHCH₂ D-desthiobiotin), 2.20 (t, 2H, J=7.4 Hz, CH₂(C=O)NH), 1.73-1.24 (m, CH₂ from D-desthiobiotin and ketal), 1.14 (d, 3H, J=6.5 Hz, CH₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 172.7 (C=O), 163.9 (C=O), 111.0 (C_q from ketal), 82.00 (C \equiv C), 81.96 (C \equiv C), 80.52 (C \equiv C), 80.49 (C \equiv C), 69.4 (C \equiv CCHCH₂), 65.1 (C \equiv CCHCH₂), 56.1 (CH₃CHNH), 51.5 (HNCHCH₂), 35.8 (CH₂), 35.62 (CH₂), 35.59 (CH₂), 35.3 (CH₂(C=O)NH), 29.4 (CH₂), 29.3 (CH₂), 29.2 (HNCH₂C \equiv C), 28.5 (CH₂), 28.4 (CH₂), 25.73 (CH₂), 25.71 (CH₂), 25.03 (CH₂), 24.99 (CH₂), 23.9 (CH₂), 23.8 (CH₂), 15.7 (CH₃) ppm. **HRMS**: calcd. for C₂₀H₃₄N₃O₄⁺ [M]⁺ 392.2543; found 392.2544.

Synthesis of N-(3-{1,4-dioxaspiro[4.5]decan-2-yl}-2,3-dioxopropyl)-6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamide **14**

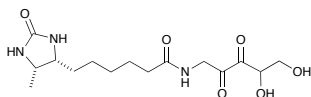


To a solution of amide **13** (15 mg, 0.38 mmol) in 2mL of H₂O:Et₂O (1:1.5) was added KClO₃ (12 mg, 0.96 mmol), OsO₄ (195 μ L, 0.15 mmol, 2% aqueous solution) and *t*-BuOH (195 μ L). After 24h, at room temperature, the reaction mixture was quenched with a saturated aqueous NH₄Cl solution (2 mL) and extracted with CH₂Cl₂ (3x2mL). The combined organic layers were dried with anhydrous Na₂SO₄, the solvent was filtered and concentrated under vacuum. The amide **14** (16 mg, quant. yield) was afforded without further purification as a colourless viscous oil.

¹H-NMR (CDCl₃), 400 MHz: δ 4.92 (t, 1H, J=6.5 Hz, (C=O)CHCH₂), 4.26 (t, 1H, J=8.0 Hz, 1xHNCH₂(C=O)), 4.15-4.08 (m, 2H, 1xHNCH₂(C=O) and 1x(C=O)CHCH₂), 3.90-3.85 (1x(C=O)CHCH₂ and CH₃CHNH D-desthiobiotin), 3.76-3.65 (m, 1H, HNCHCH₂ D-desthiobiotin), 2.45-2.18 (m, CH₂(C=O)NH), 1.74-1.25 (m, CH₂ from D-desthiobiotin and ketal), 1.13 (d, 3H, J=6.3 Hz, CH₃) ppm. **¹³C-NMR** (CDCl₃), 100 MHz: δ 206.7 (C=O), 192.4 (C=O), 174.7 (C=O), 164.8 (C=O), 134.1 (C_q from ketal), 76.7 ((C=O)CHCH₂), 69.4 ((C=O)CHCH₂), 66.9

(HNCH₂(C=O)), 56.2 (HNCH₂ D-desthiobiotin), 56.1 (CH₃CHNH D-desthiobiotin), 42.0 (CH₂(C=O)NH), 35.8 (CH₂), 35.4 (CH₂), 35.3 (CH₂), 34.8 (CH₂), 29.7 (CH₂), 29.3 (CH₂), 28.6 (CH₂), 27.0 (CH₂), 26.6 (CH₂), 25.7 (CH₂), 25.0 (CH₂), 24.6 (CH₂), 23.9 (CH₂), 23.8 (CH₂), 23.0 (CH₂), 15.7 (CH₃) ppm. **HRMS**: calcd. for C₂₁H₃₃N₃O₆⁺ [M]⁺ 424.2440; found 424.2442.

Synthesis of *N*-(4,5-dihydroxy-2,3-dioxopentyl)-6-(5-methyl-2-oxoimidazolidin-4-yl)hexanamide **1**



To a solution of amide **14** (9.6 mg, 0.023 mmol) in 750 μ L of DMSO-*d*₆:D₂O (1:4) was added D₂SO₄ (10 μ L of a 10% solution in D₂O). The solution was sonicated during 15 minutes, in a cooled ultrasounds bath, and stirring for 24h at 800 rpm. The mixture was quenched with CDCl₃ (3x500 μ L) and the aqueous phase was recovered. The complex D-desthiobiotin-Al-2 **1** (7.8 mg, quant. yield) was obtained in solution without further purification.

¹H-NMR (D₂O), 400 MHz: δ 8.15 (s, NH), 4.27 (t, J=4.1 Hz, CH from Al-2), 3.86-3.76 (m, 1H, CH₃CHNH D-desthiobiotin), 3.76 (d, J=4.2 Hz, CH₂ from Al-2), 3.72-3.65 (m, 1H, HNCH₂ D-desthiobiotin), 3.58-3.49 (m, (C=O)HNCH₂(C=O)), 2.21-2.16 (m, 2H, CH₂(C=O)NH), 1.62-1.50 (m, 2H, CH₂ from D-desthiobiotin), 1.47-1.39 (m, 2H, CH₂ from D-desthiobiotin), 1.35-1.18 (m, 4H, CH₂ from D-desthiobiotin), 1.03 (d, 3H, J=6.5 Hz, CH₃) ppm. Other peaks are present, come from the cyclic forms of Al-2. **¹³C-NMR** (D₂O), 100 MHz: δ 71.4 (CH from Al-2), 65.1 ((C=O)HNCH₂(C=O)), 63.3 (CH₂ from Al-2), 55.9 (HNCH₂ D-desthiobiotin), 51.4 (CH₃CHNH D-desthiobiotin), 35.4 (CH₂), 35.0 (CH₂), 28.8 (CH₂), 28.7 (CH₂), 28.2 (CH₂), 28.1 (CH₂), 25.3 (CH₂), 25.1 (CH₂), 25.0 (CH₂), 14.6 (CH₃) ppm.

1.3 Protein expression and purification

Escherichia coli BL21 (Δ luxS) transformed with the plasmid pQE30 (carrying LuxP protein from *Vibrio harveyi* fused to cyan (CFP) and yellow (YFP) fluorescent proteins) – pQE30-CLPY- was kindly provided by Richard Sayre (The Ohio State University, Ohio, USA). Expression and purification of this protein was carried out as previously described.⁴ Briefly, protein expression was induced at an optical density (OD₆₀₀) of 0.6 with 0.3 mM of isopropyl β -D-thiogalactopyranoside (IPTG) and grown for 6h, at 28 °C before cell harvesting by centrifugation. The resulting pellet was resuspended in 25 mM sodium phosphate buffer pH 8.0, 35 mM NaCl, 1:100 protease inhibitor cocktail (Roche) and cells were lysed by passing them three times through a french press. Cell debris were removed by centrifugation and LuxP was purified from clarified supernatants through Nickel-nitrilotriacetic (Ni⁺-NTA) acid affinity chromatography (Qiagen). The protein was eluted in 25 mM sodium phosphate buffer pH 8.0, 35 mM NaCl, 40% glycerol, 250 mM imidazole, 1:100 protease inhibitor cocktail. The expression and purification of *Clostridium saccharobutylicum* DSM13864 and *E. coli* K-12 MG1655 LsrB proteins (CsLsrB and EcLsr, respectively) were carried out as previously described.^{5,6} Concisely, *E. coli* BL21 (Δ luxS) cells transformed with the constructs pPro-EcLsrB and pDEST-CsLsrB were grown in LB with 1 μ g/mL ampicillin at 37°C until the OD₆₀₀ was 0.3. At this point, temperature was decreased to 22°C. At OD₆₀₀ = 0.9, protein expression was induced with 0.1 mM or 0.3 mM of IPTG for the expression of EcLsr and CsLsrB, respectively. Cells grew for 6h after induction and were harvested by centrifugation. The resulting pellets were resuspended in 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 10 mM imidazole, 2.5 μ g/mL DNase and 2.5 μ g/mL leupeptin and cells were lysed by passing thrice through a french press. Centrifugation was employed to remove cell debris and the tagged-proteins were purified by Ni⁺-NTA affinity chromatography. EcLsrB and CsLsrB were eluted in 50 mM NaH₂PO₄ (pH 8.0), 300 mM NaCl, 250 mM imidazole and subsequently dialyzed (Slide-A-Lyzer, ThermoFisher Scientific) into 25 mM Tris buffer pH 8.0, 150 mM NaCl, 1 mM DTT. CLPY-LuxP, EcLsrB and CsLsrB have a molecular weight of approximately 98, 40 and 37.5 kDa, respectively. The higher molecular weight of CPLY-LuxP when compared with the other receptors is due to its fusion to CFP and YFP.

1.4 Native PAGE and western-blot

Approximately 125 µg of partially purified CPLY (CFP-LuxP-YFP; ~1 nmol), CsLsrB (~3 nmol), and EcLsrB (~3 nmol) were incubated with approximately 59 µg of desthiobiotin-AI-2 (~172 nmol) overnight under gentle agitation at 4°C. These quantities ensured the presence of at least 50 times more probe than receptors. As a control 125 µg of the protein samples were incubated with desthiobiotin alone (59 µg) or with buffer in the same conditions (25 mM sodium phosphate buffer pH 8.0, 35 mM NaCl, 1 mM boric acid for CPLY and 25 mM Tris buffer pH 8.0, 150 mM NaCl, 1 mM DTT for CsLsrB and EcLsrB). 20 µL of all the samples were mixed with 5 µL of 5X sample buffer (0.31 M Tris-HCl buffer pH 6.8, 0.05% bromophenol blue and 50% glycerol) and 11 µL of these mixes were loaded into each of two 10% polyacrylamide non-denaturing gels. 5 µL of protein standards (All Blue Standards, Bio-Rad) was also added. The gel was run in Tris-glycine buffer (25 mM Tris, 192 mM glycine, pH 8.3), at 160 V, 4°C for approximately 1h30. One of the gels was stained with Coomassie blue and the presence of the bands assessed. The second gel was used for a western-blot. Transfer was performed at 90 mA, 4°C, overnight in 25 mM Tris, 192 mM glycine and 0.05% SDS to a PVDF membrane. The membrane was incubated in a 1:5000 dilution of an anti-biotin, HRP conjugated antibody (PA1-30595, Invitrogen) for 1h at room temperature. An ECL chemiluminescent substrate was added (SuperSignal West Pico, Pierce) and signal detection was performed in a blot imager (Amersham Imager 680).

1.5 Pull-down of LuxP receptor

Magnetic Dynabeads with protein G (Invitrogen, 10003D) were conjugated with the anti-biotin antibody (#31852, Pierce) as per the manufacturers instructions. Briefly, 50 µL of Dynabeads (1.5 mg) were added to a 1.5 mL eppendorf and the storage solution was removed through magnetic separation. 10 µg of antibody diluted in 200 µL of wash buffer (PBS buffer pH 7.4, 0.02% Tween-20) was added to the beads and the mixture was incubated for 10 min, at room temperature with agitation. The unbound antibody was then removed by magnetic separation and the beads were washed with 200 µL of wash buffer. Next, the sample was added to the anti-biotin antibody conjugated magnetic beads. The sample used for the pull down of CLPY was incubated following the conditions mentioned above and included approximately 76,5 µg of DB-AI-2 and 265 µg of partially purified CLPY. The mixture sample/antibody-conjugated beads was incubated for 1h at room temperature with agitation followed by three washes with 200 µL of wash buffer to remove unbound protein. CPLY, complexed with the AI-2 probe and retained by the antibody conjugated magnetic beads, was eluted by denaturation. The final volume was heated for 10 minutes at 70°C in 20 µL of 50 mM glycine pH 2.8 and 10 µL of 240 mM Tris-HCl pH 6.8, 40% glycerol, 8% SDS, 0.04% bromophenol blue and 5% β-mercaptoethanol. 10 µL of the wash and elution steps were mixed with 5 µL of 200 mM Tris-HCl pH 6.8, 8% SDS, 0.4% bromophenol blue and 40% glycerol and added to a denaturing gel composed of 15% acrylamide in the separating gel and 5% in the stacking gel. As protein standards, 5 µL of All Blue Standards (Bio-Rad) were loaded. The gel was run in SDS running buffer at room temperature, 180 V for approximately 70 minutes and stained with Coomassie blue.

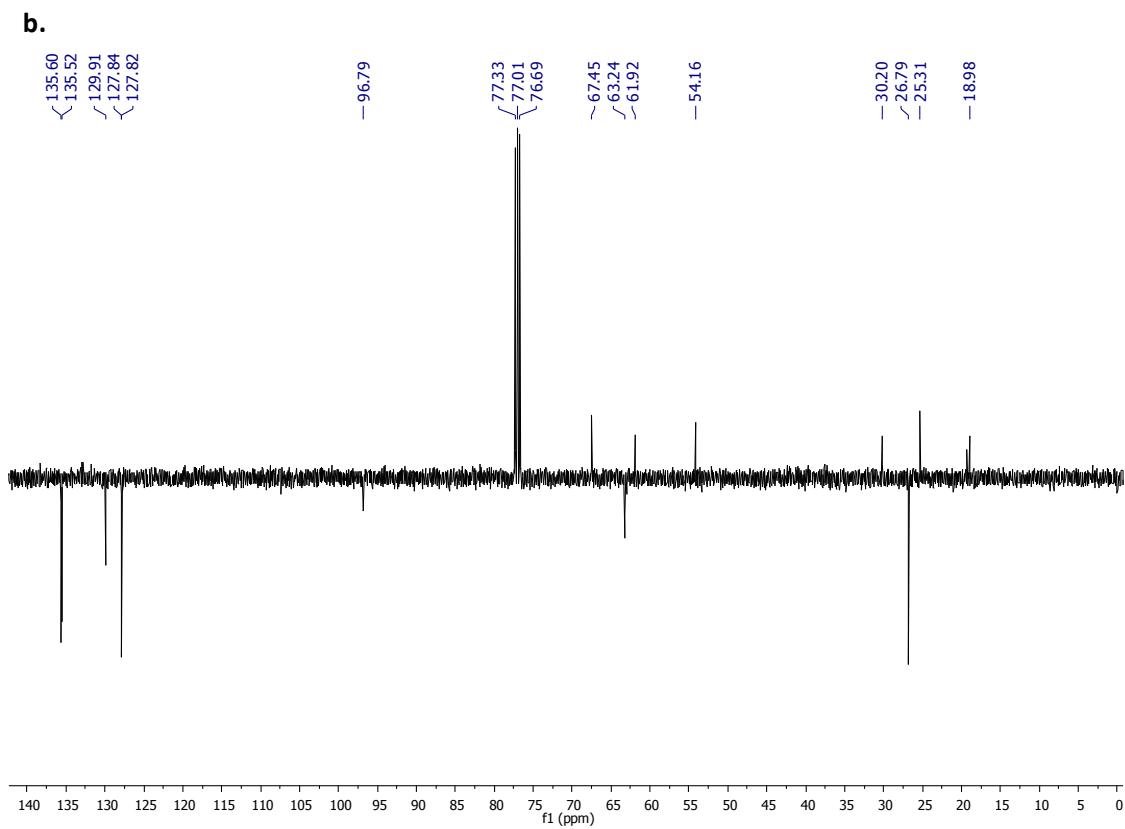
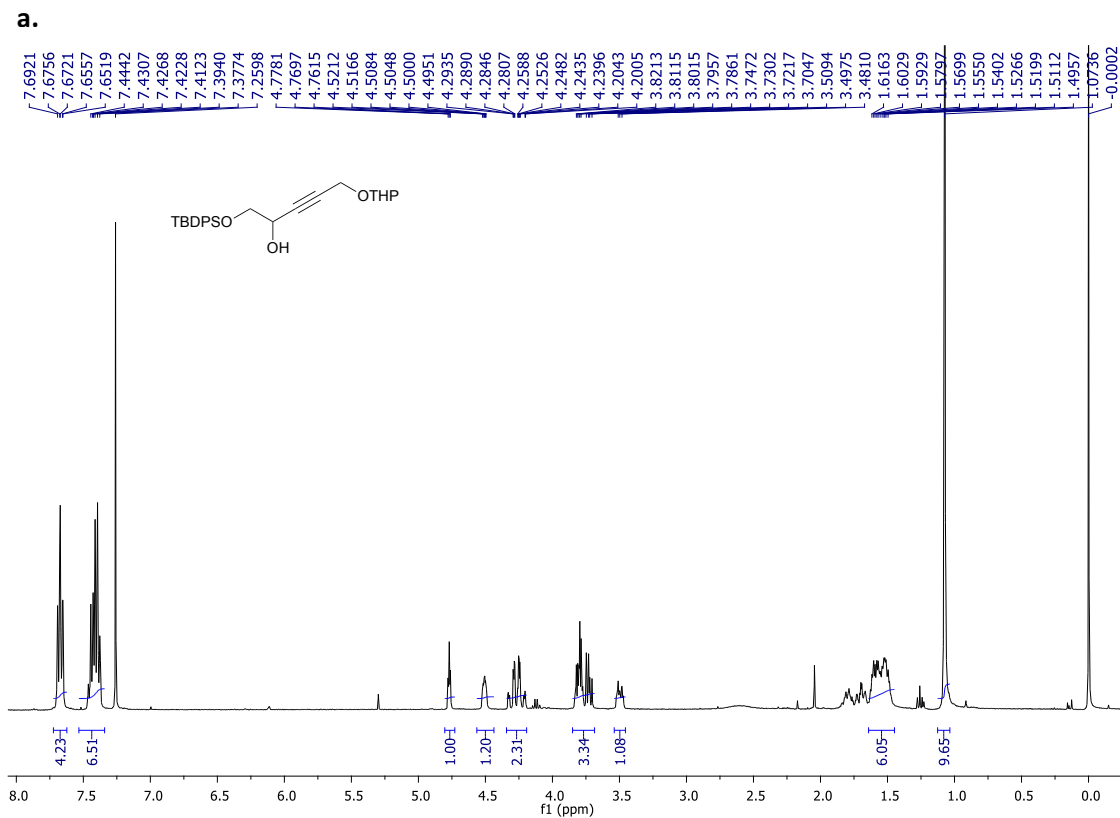


Figure S3 – ¹H NMR spectra of compound 6 (a); ¹³C NMR spectra of compound 6 (b).

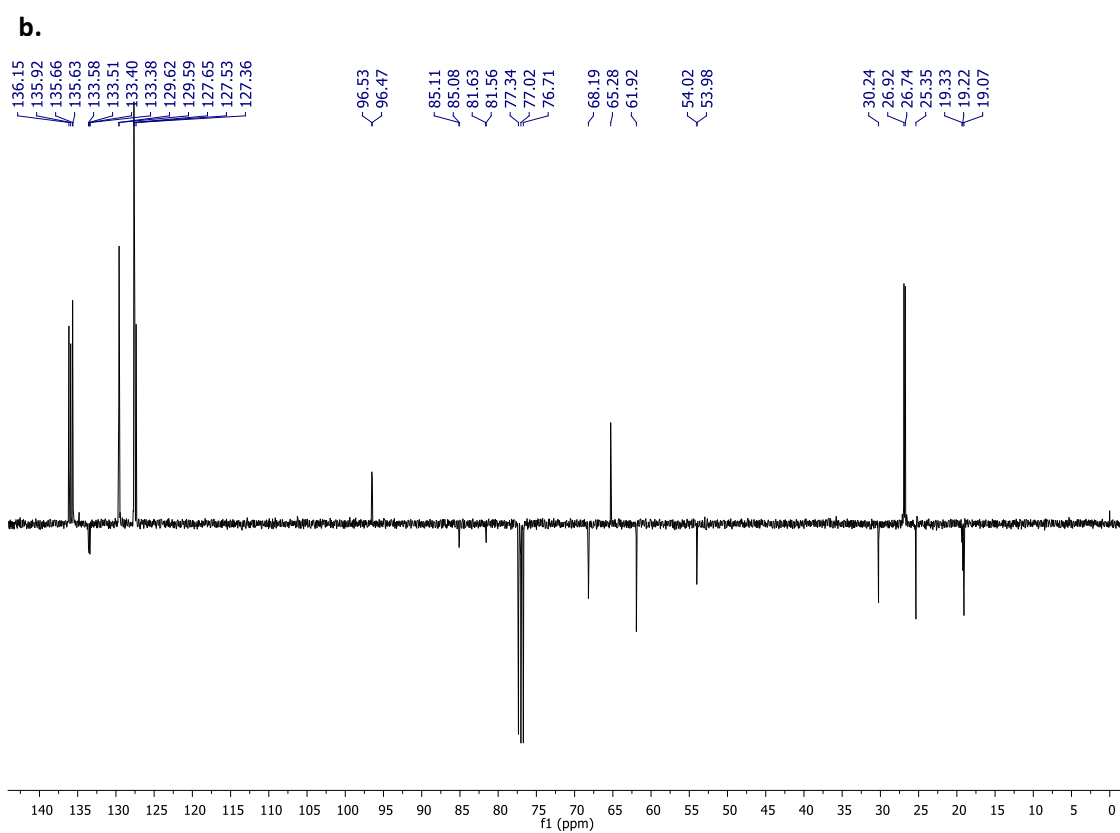
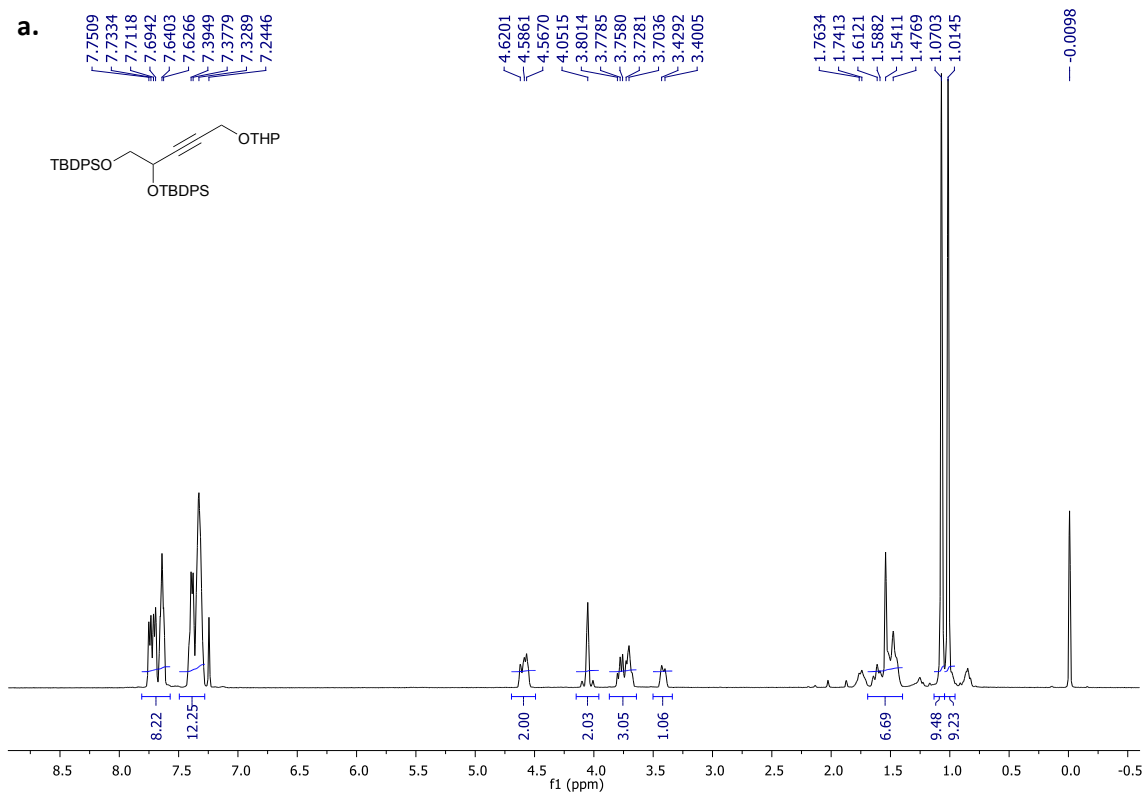


Figure S4 – ^1H NMR spectra of compound 7 (a); ^{13}C NMR spectra of compound 7 (b).

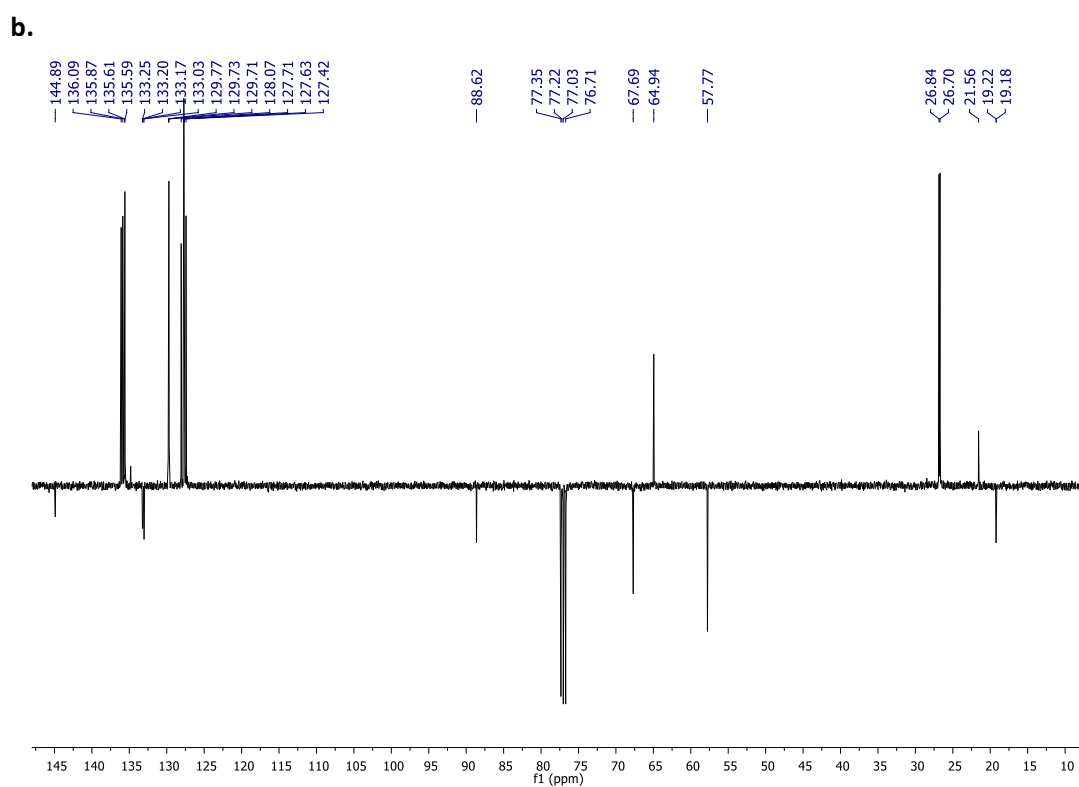
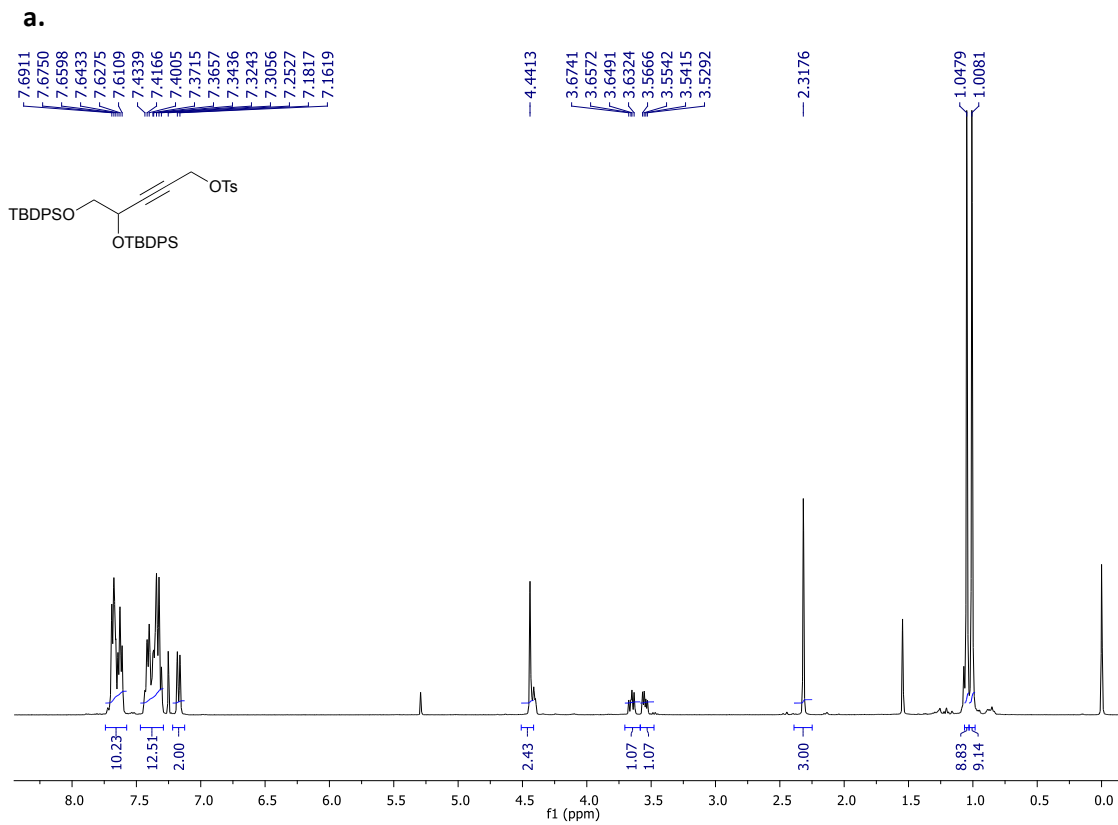


Figure S6 – ¹H NMR spectra of compound 9 (a); ¹³C NMR spectra of compound 9 (b).

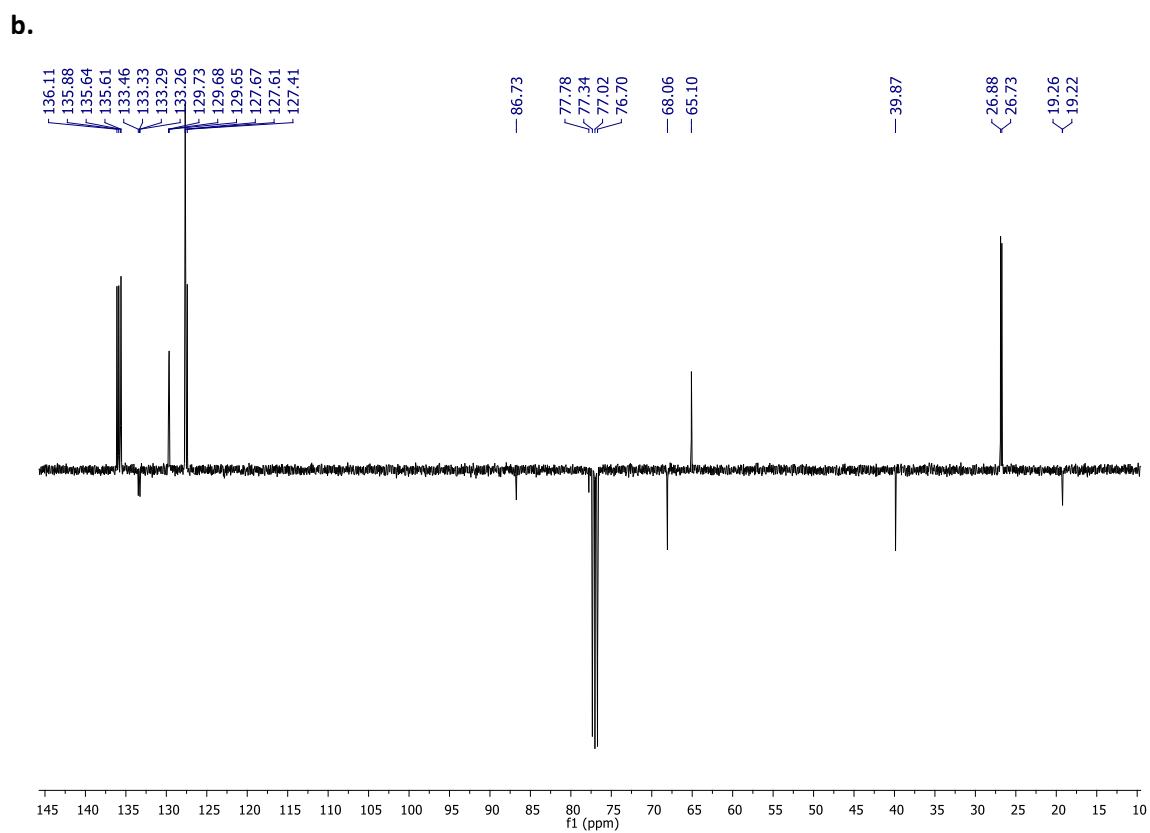
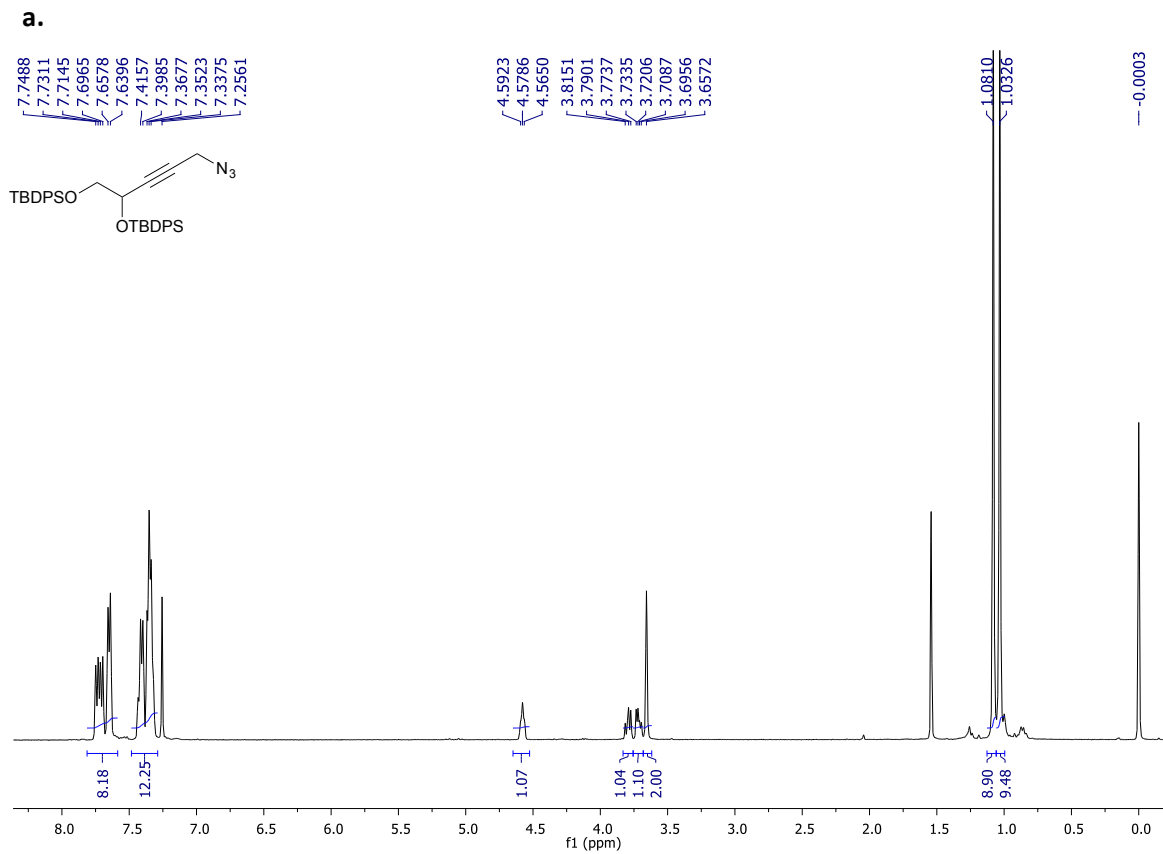


Figure S7 – 1H NMR spectra of compound **10 (a)**; ^{13}C NMR spectra of compound **10 (b)**.

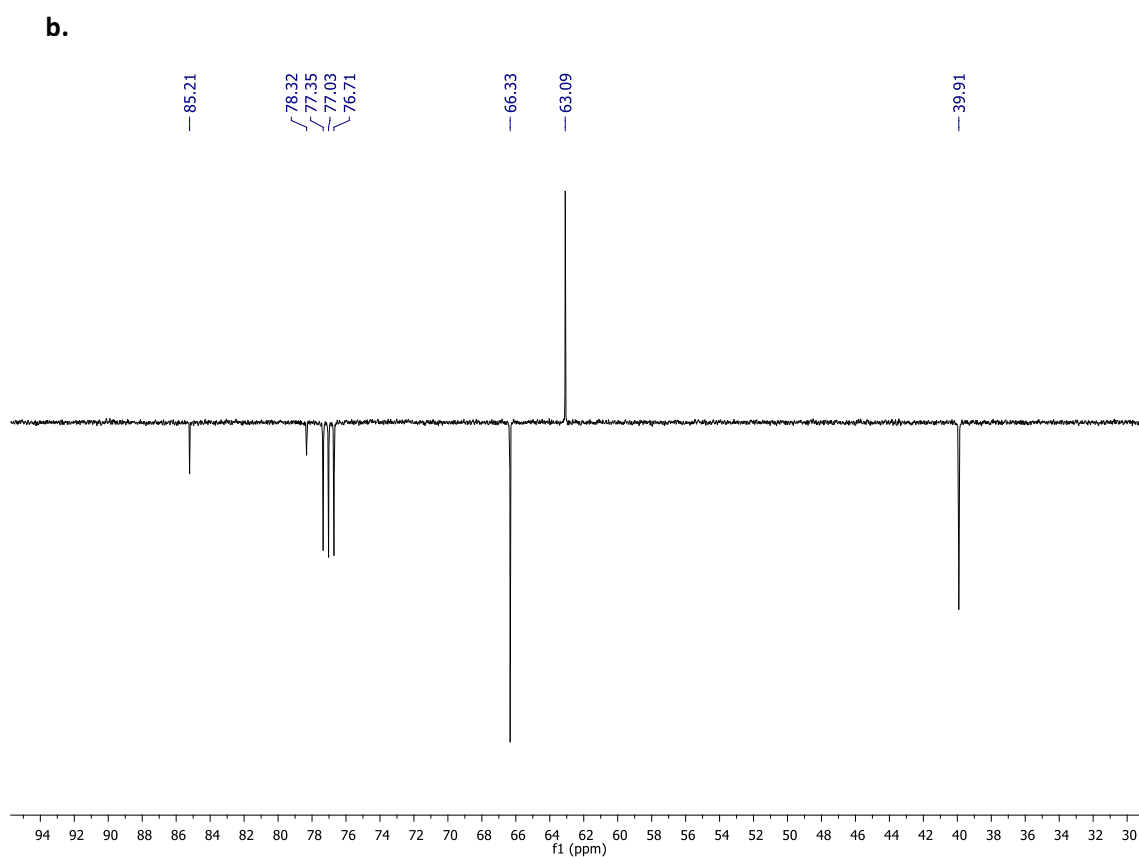
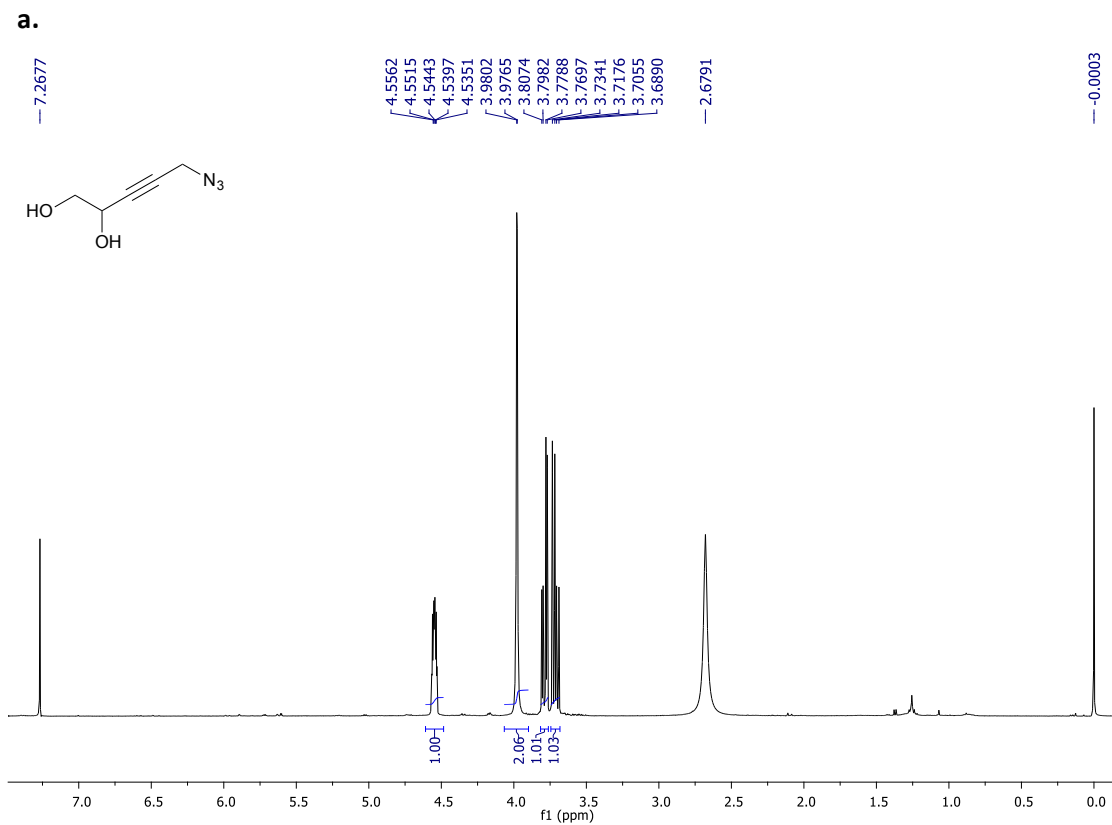


Figure S8 – ^1H NMR spectra of compound **11** (a); ^{13}C NMR spectra of compound **11** (b).

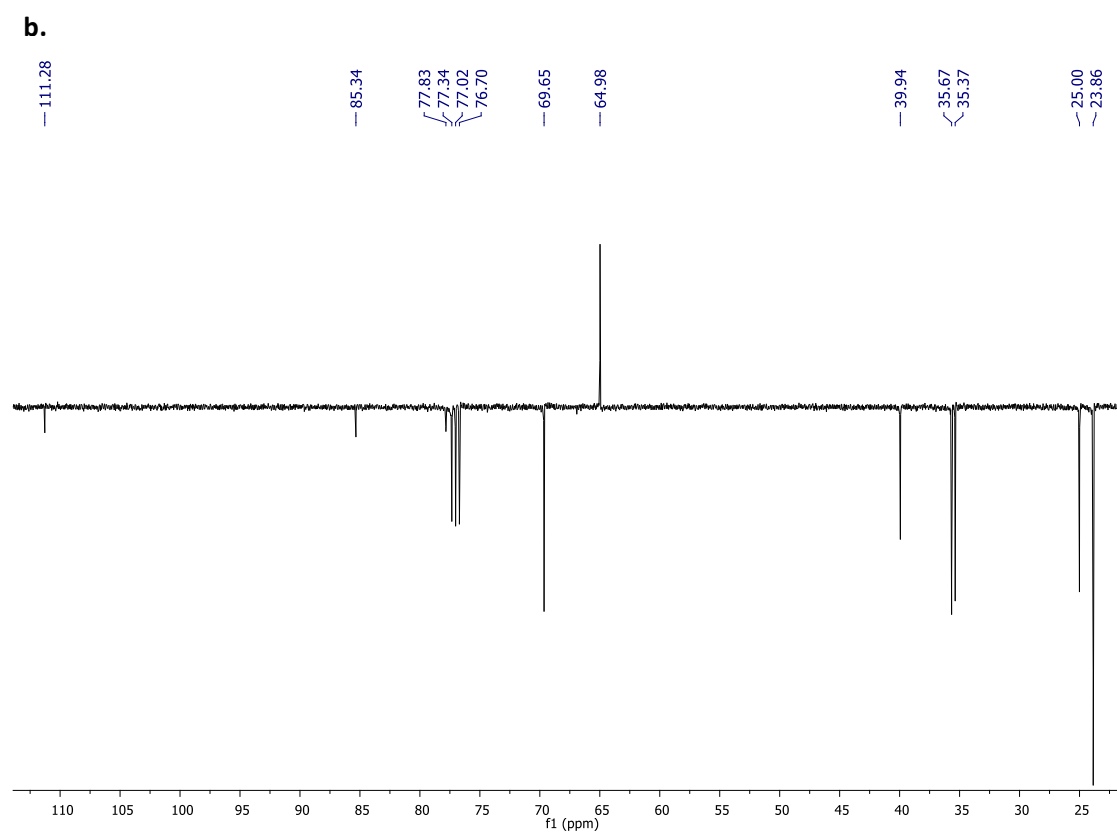
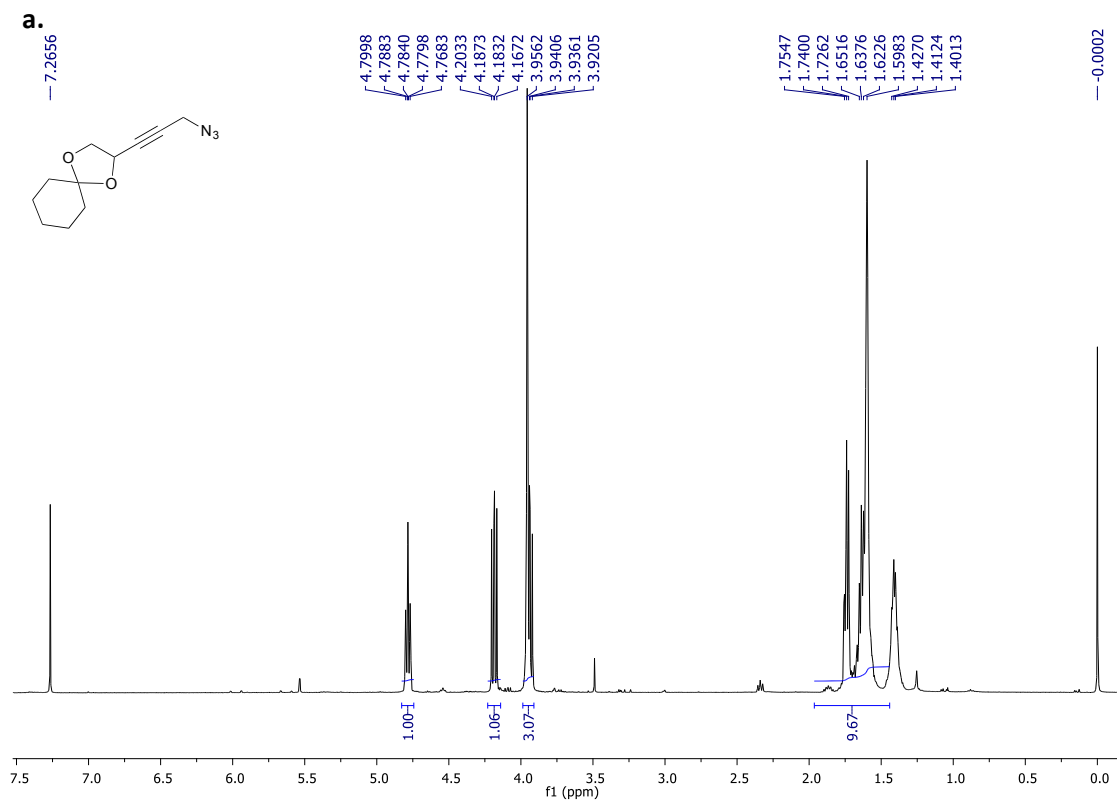


Figure S9 – ^1H NMR spectra of compound **12** (a); ^{13}C NMR spectra of compound **12** (b).

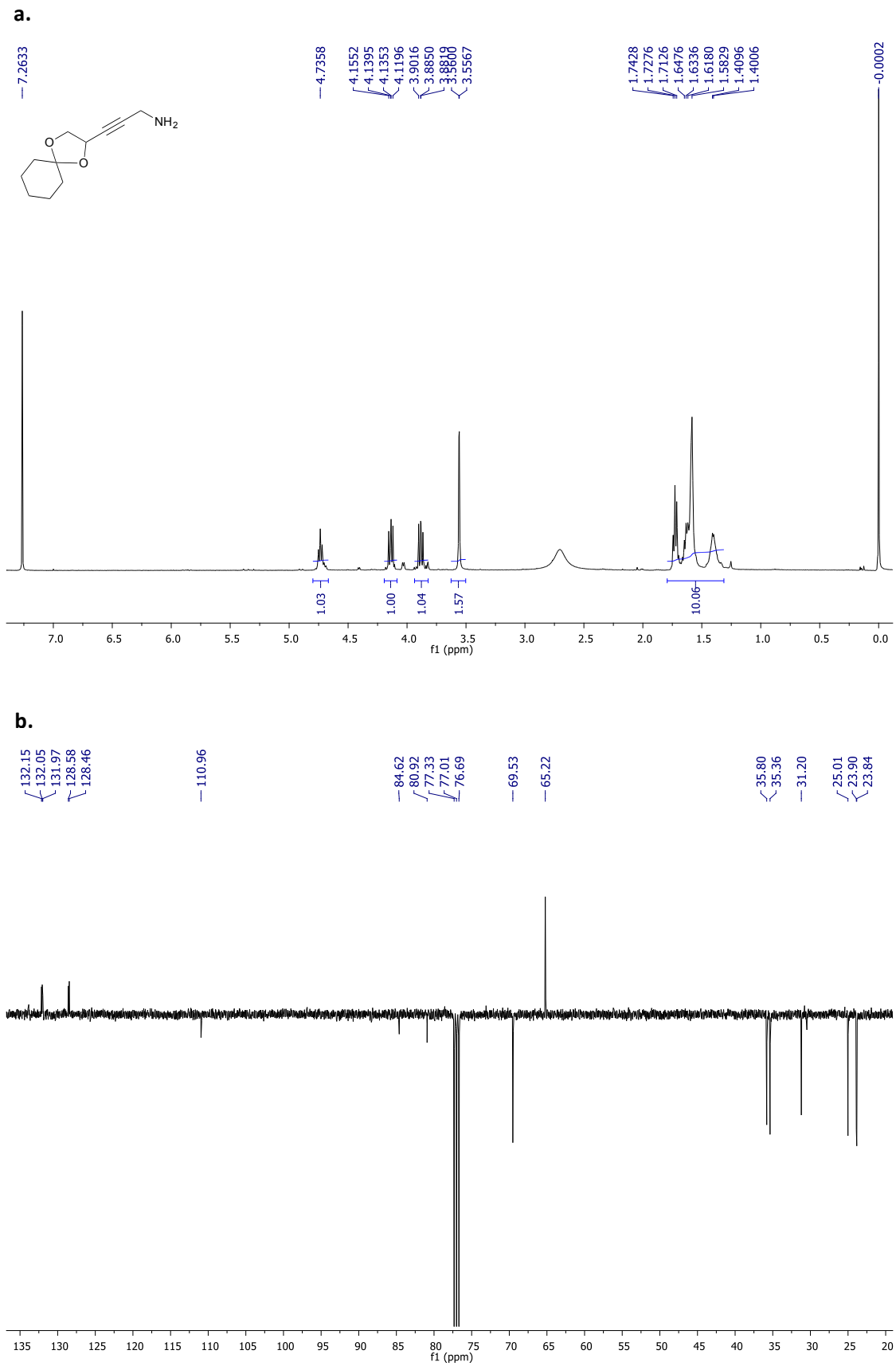


Figure S10 – ^1H NMR spectra of compound 2 (a); ^{13}C NMR spectra of compound 2 (b).

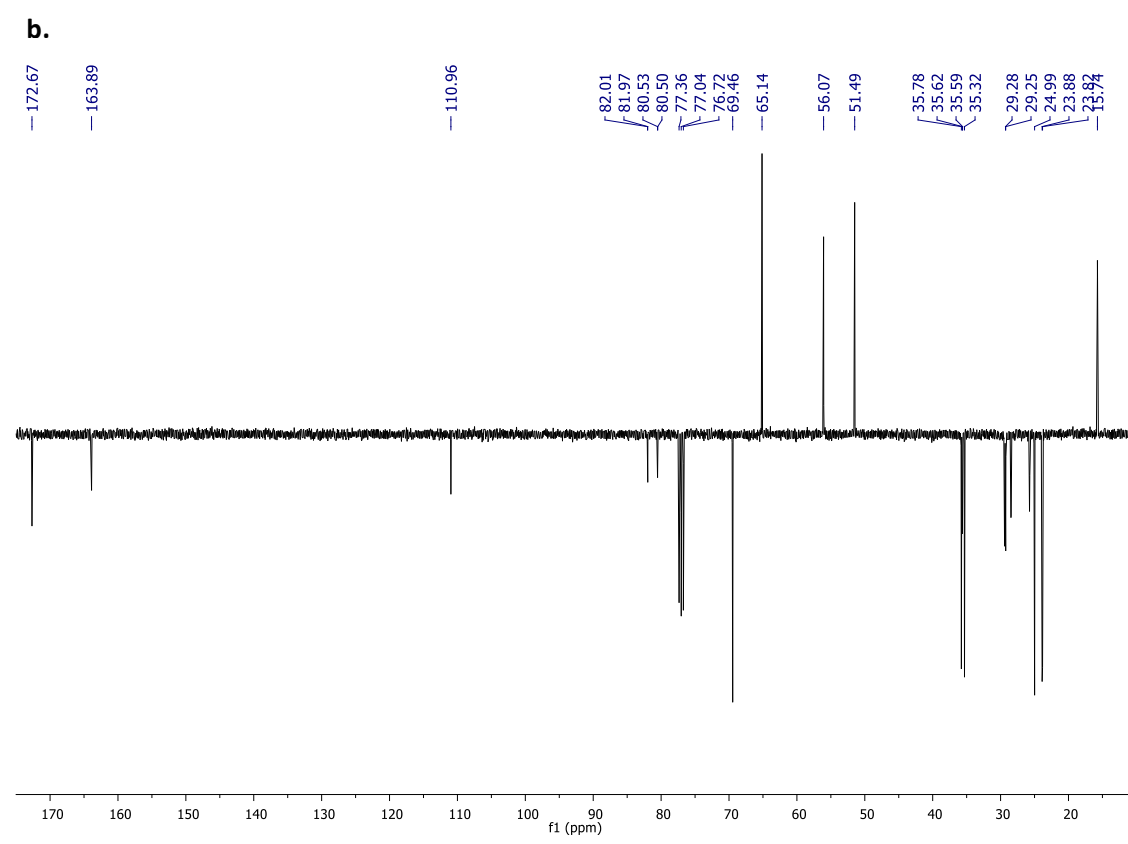
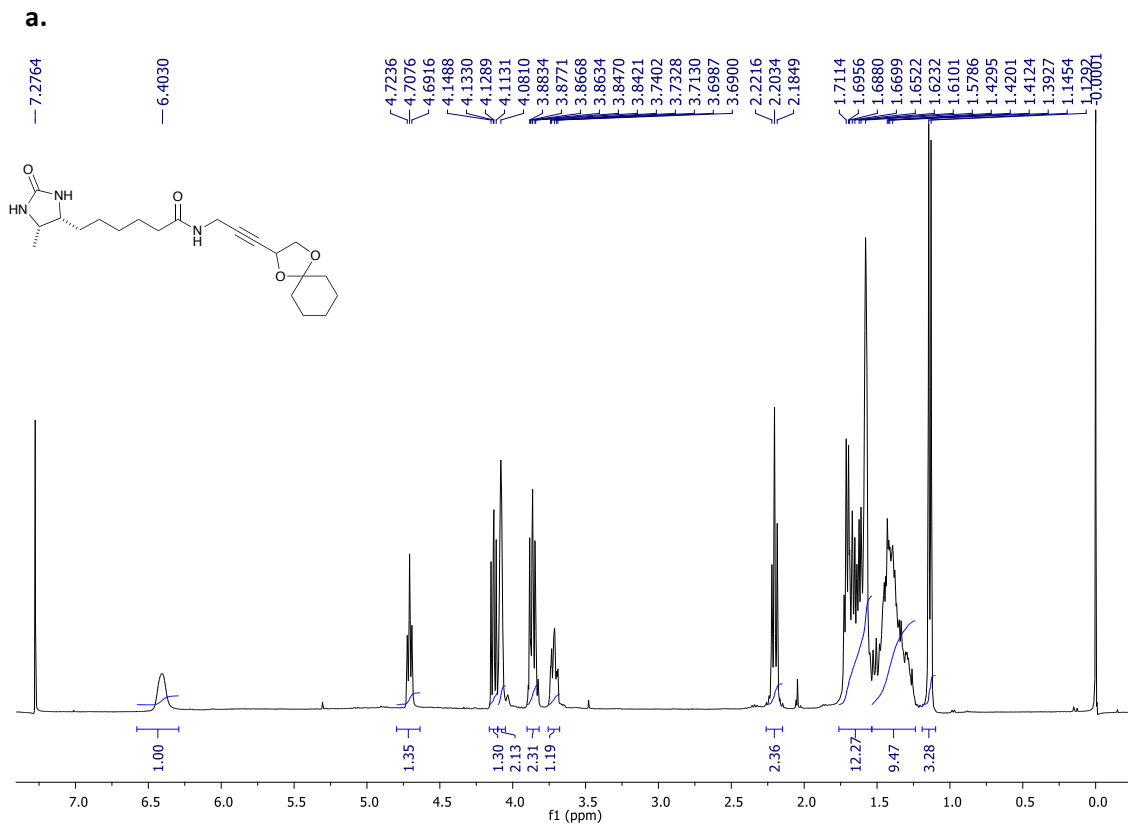


Figure S11 – ¹H NMR spectra of compound 13 (a); ¹³C NMR spectra of compound 13 (b).

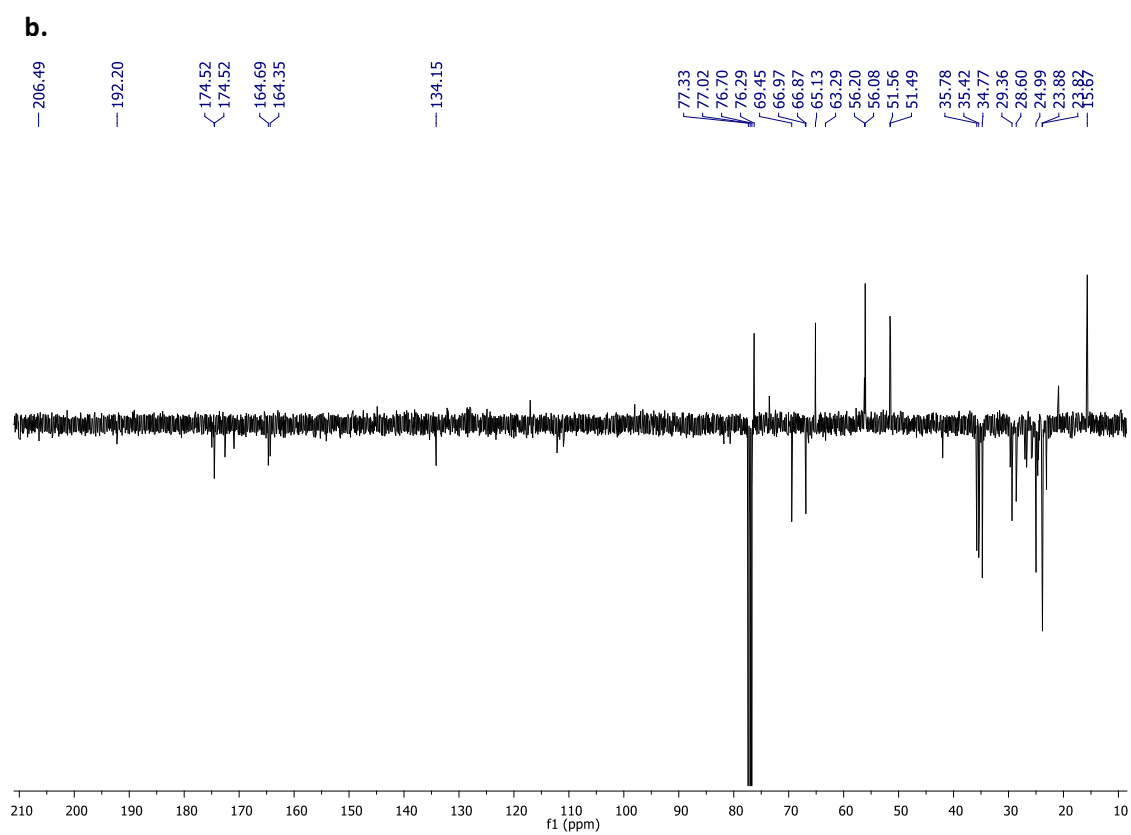
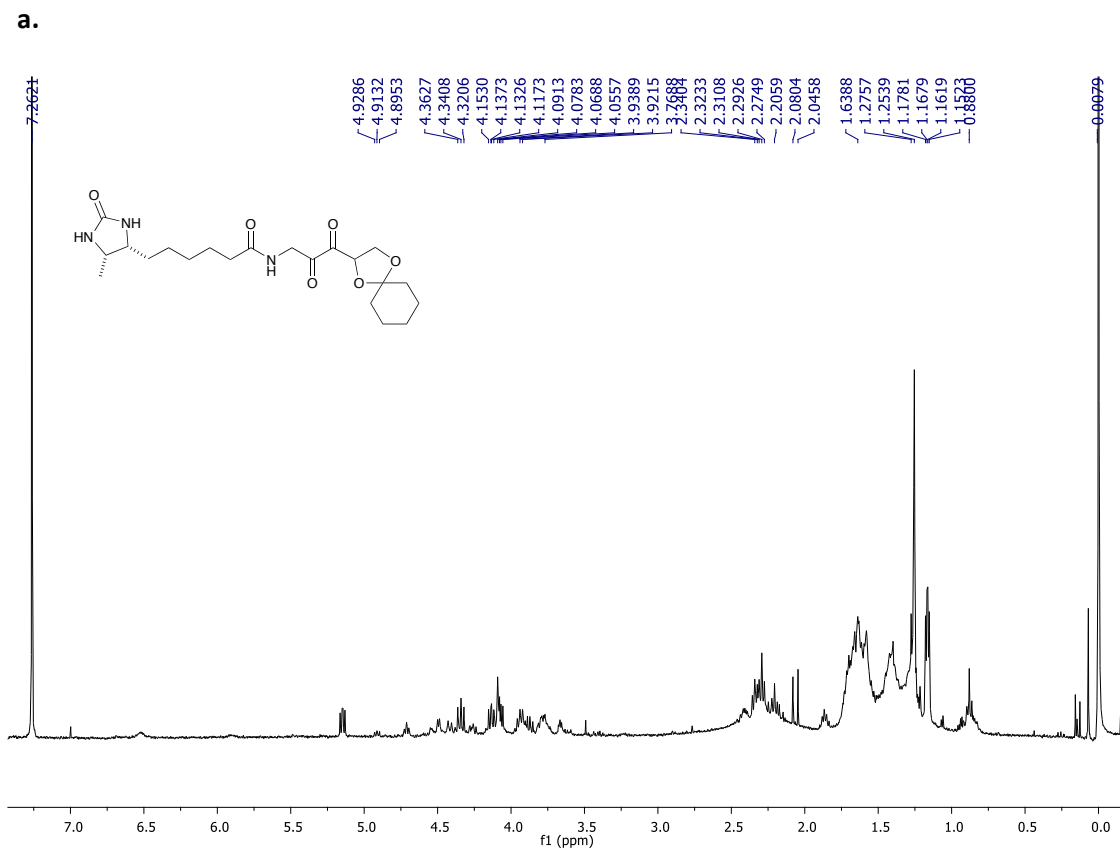


Figure S12 – ¹H NMR spectra of compound 14 (a); ¹³C NMR spectra of compound 14 (b).

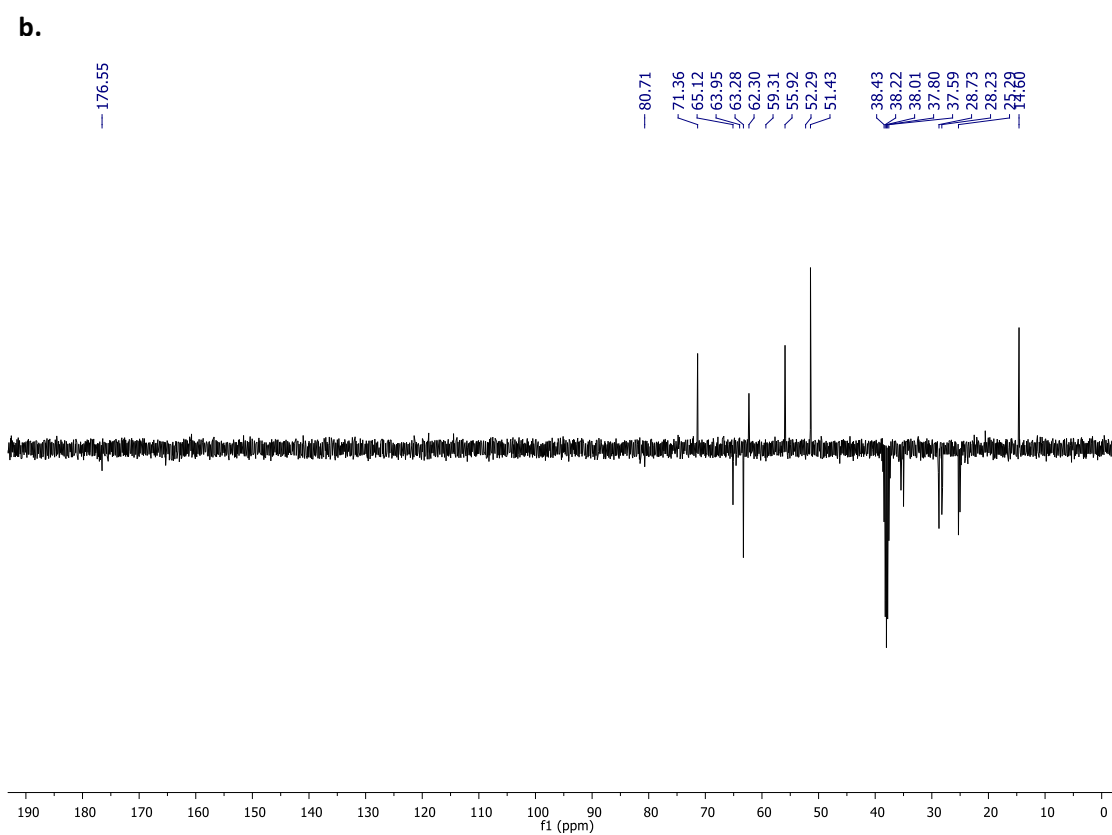
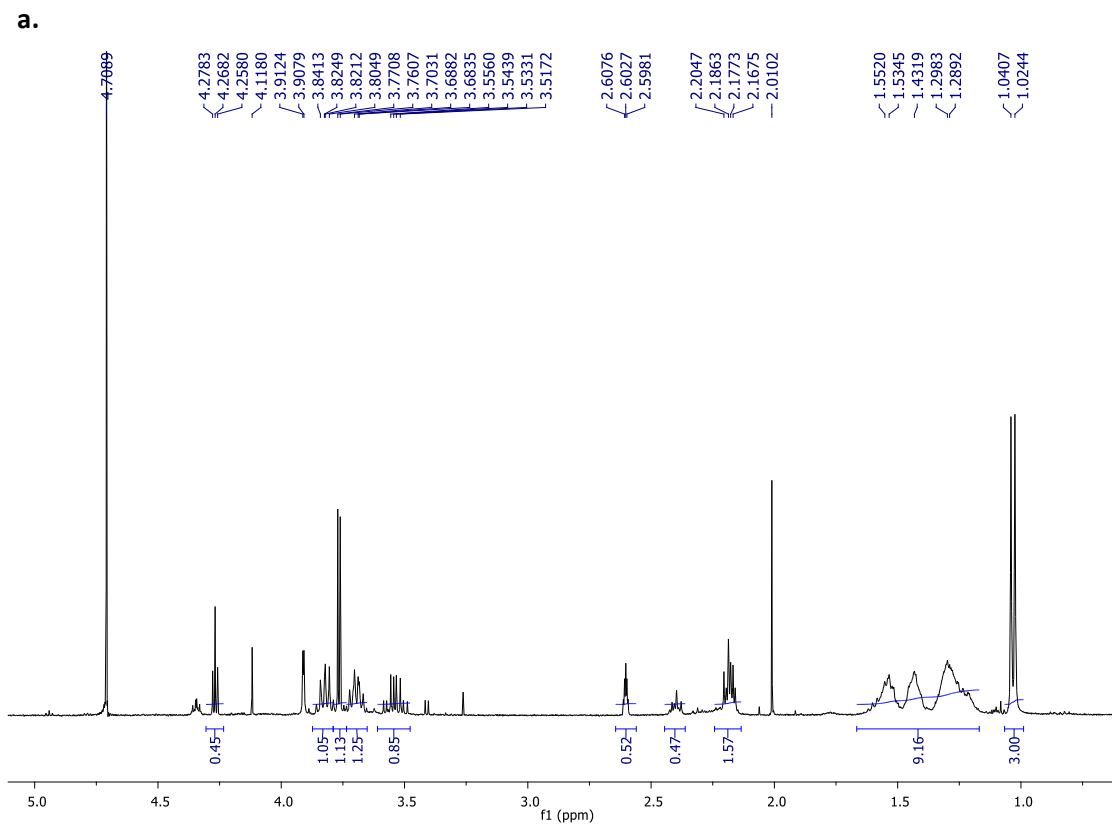


Figure S13 – ^1H NMR spectra of compound **1** (a); ^{13}C NMR spectra of compound **1** (b).

3. References

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