Electronic Supplementary Material (ESI) for Chemical Science.

Supplementary information

Short-Chain Reactive Probes as Tools to Unravel the *Pseudomonas aeruginosa* Quorum Sensing Regulon

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Abbreviations

3-oxo-C_{12}-HSL- *N*-3-oxododecanoyl homoserine lactone
ABPP - activity-based protein profiling
AHL- *N*-acylhomoserine lactone
AOCHC- 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid
BSA- bovine serum albumin
BODIPY- boron-dipyrromethene
C_4-HSL- *N*-butyryl-L-homoserine lactone
CI- Confidence intervals
CuAAC- copper(I)-catalyzed azide-alkyne cycloaddition
DCM- dichloromethane
DHPCA- 5,10-dihydro-PCA
DHPDC- 5,10-dihydro- PDC
DMSO- Dimethyl Sulfoxide
DMF- dimethylformamide
EC_{50}- effective concentration that gives a half-maximal response
EDC- 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA- ethylenediaminetetraacetic acid
ESI- electrospray ionization
EtOAc- ethyl acetate
FA- formic acid
HHPDC- hexahydrophenazine-1,6-dicarboxylic acid
HPLC- high-performance liquid chromatography
HSL- homoserine lactone
IAA- Iodoacetamide
IMAC- Immobilized Metal Affinity Chromatography
IPTG- Isopropyl β-D-1-thiogalactopyranoside
kDa- kilodaltons (molecular mass)
LB- Luria-Bertani broth
LC-MS- liquid chromatography-mass spectrometry
MS- mass spectrometry
NHS- *N*-hydroxysuccinimide
Ni-NTA- nickel-nitrilotriacetic acid charged affinity resin
OD_{600}- optical density of a sample measured at a wavelength of 600 nm
OD_{520}- optical density of a sample measured at a wavelength of 520 nm
PBS- phosphate buffered solutions
PCA- phenazine-1-carboxylic acid
PDC- phenazine-1,6-dicarboxylic acid
PP- polypropylene
PQS- *Pseudomonas* quinolone signal (2-heptyl-3-hydroxy-4-quinolone)
QS- quorum Sensing
RPM- revolutions per minute
SDS-PAGE- sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SDS- sodium dodecyl sulfate
StageTips- Stop and Go Extraction Tips
TBTA- tris [(1-benzyl-1*H*-1,2,3-triazol-4-yl) methyl] amine
TCEP- tris (2-carboxyethyl) phosphine
TEA- trimethylamine
TEV- tobacco etch virus
THF- tetrahydrofuran
TLC- thin-layer chromatography
UV- ultraviolet
WT- wild type

Supplemental Experimental section

Synthetic procedures

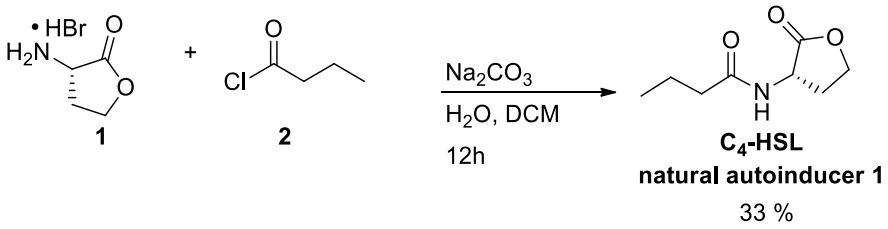
Synthesis of C_4-HSL - natural autoinducer 1

(*S*)-(−)-α-amino-γ-butyrolactone hydrobromide (**1**), (176 mg, 9.6 mmol), and Na_2CO_3, (254 mg, 2.4 mmol) were partitioned between DCM (2.2 mL) and H_2O (2.2 mL). Butyryl chloride (**2**), (0.17 mL, 1.7 mmol) was added and the mixture was stirred vigorously at room temperature overnight. The two phase mixture was separated and the aqueous layer extracted with DCM until there was no spot on the TLC plate (EtOAc:DCM, 50/50 v/v). The organic layers were combined, washed with brine, dried over $MgSO_4$, and filtered. The solvent was removed by rotary evaporation and separated by column chromatography (EtOAc:DCM, 50/50 v/v) to give *N*-butyryl-*L*-homoserine lactone (**C_4-HSL**) as a white solid (55 mg, 33%), (**Scheme S1**).

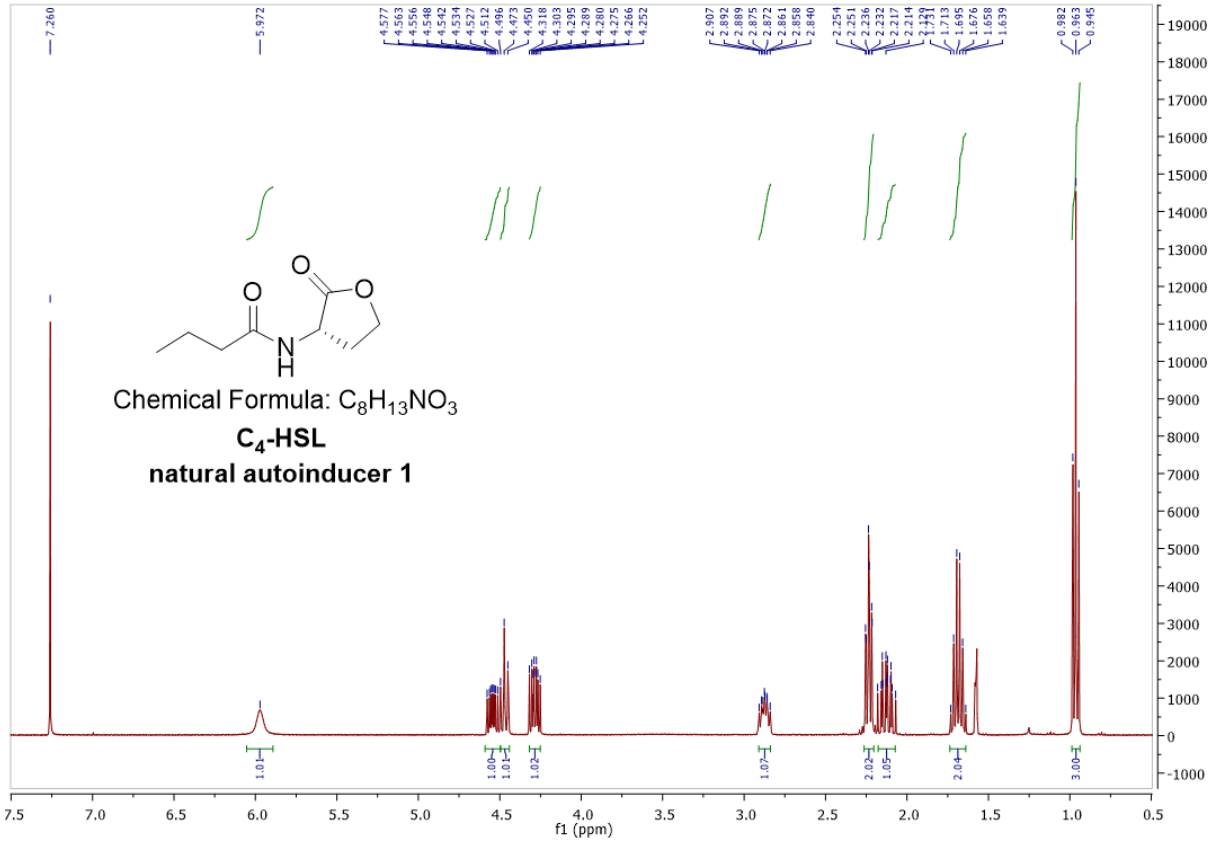
^{1}H NMR (400 MHz, $CDCl_3$): δ 5.97 (br s, 1H), 4.51-4.57 (ddd, *J* = 11.6, 8.4, 5.6 Hz, 1H), 4.45-4.49 (t, *J* = 9.2 Hz, 1H), 4.25-4.31 (ddd, *J* = 11.6, 9.2, 6 Hz, 1H), 2.84-2.90 (m, 1H), 2.21-2.25 (td, *J* = 7.2, 1.2 Hz, 2H), 2.06-2.18 (dtd, *J* = 12.4, 11.6, 8.8 Hz, 1H), 1.63-1.73 (m, 2H), 0.96 (t, *J* = 7.6 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 175.65, 173.70, 66.26, 49.43, 38.20, 30.88, 19.02, 13.83.

LC-MS *m/z* calculated for $C_8H_{13}NO_3$ (M+H): 172.09. Found: 172.02.



Scheme S1. Synthesis of *N*-butyryl-*L*-homoserine lactone (**C_4-HSL**), the natural autoinducer of the Rhl system.



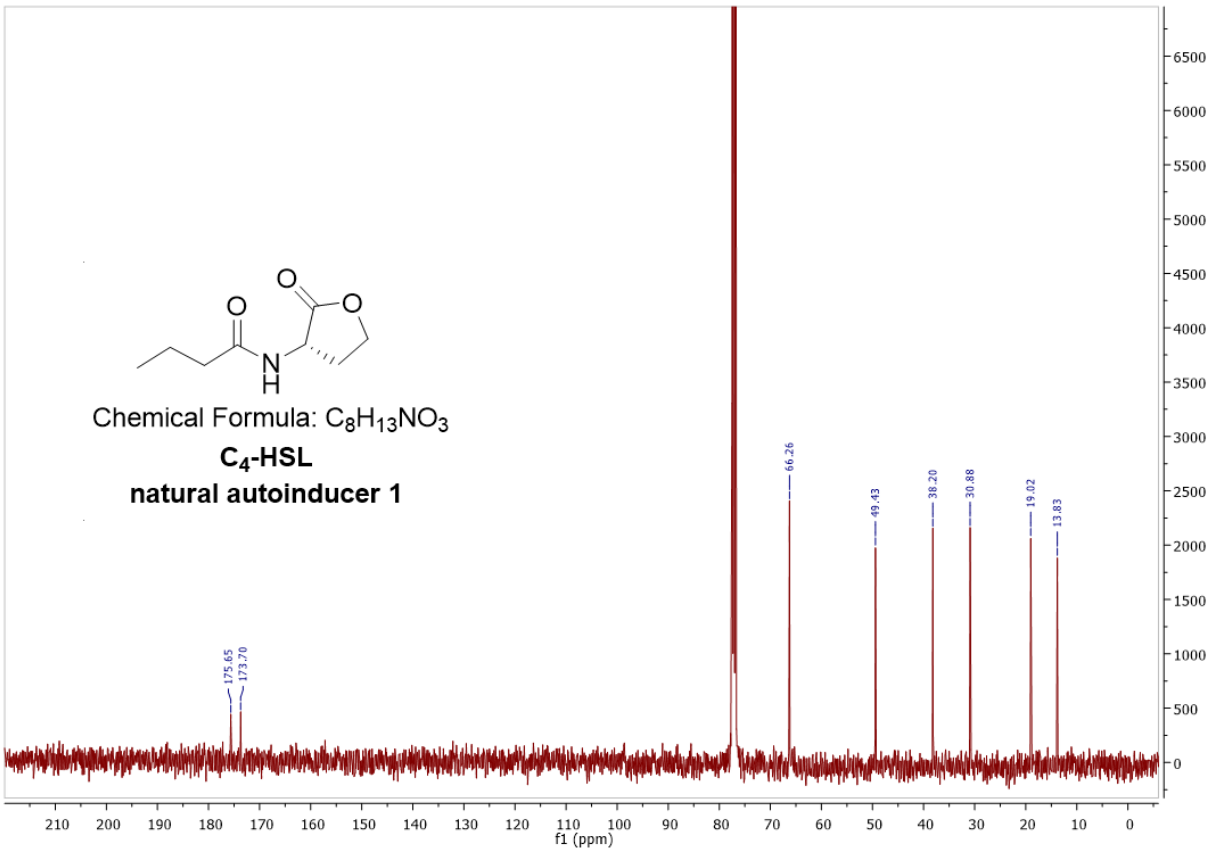


Figure S1. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of C_4-HSL - natural autoinducer **1**.

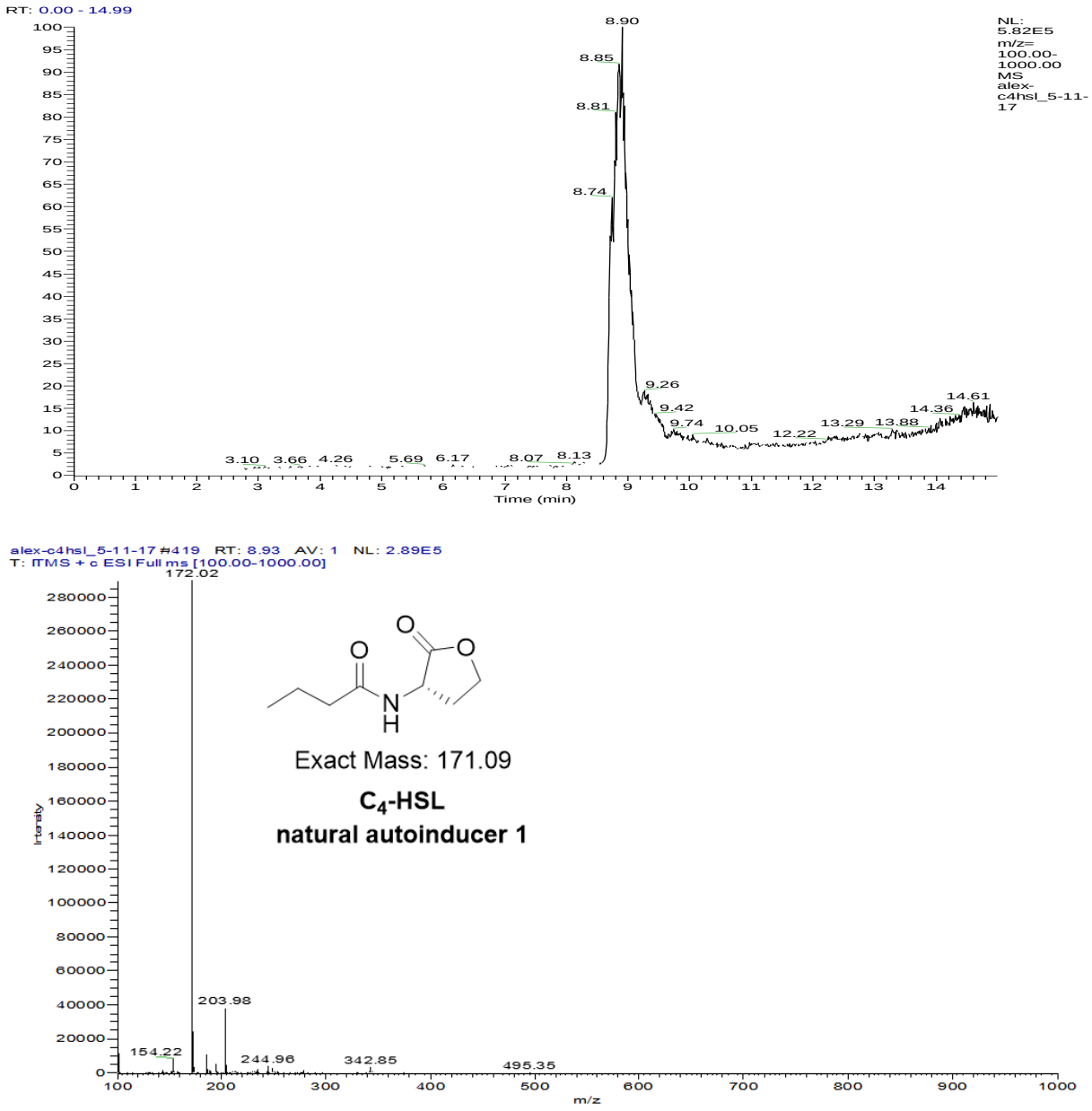


Figure S2. LC-MS of C_4-HSL - natural autoinducer **1**.

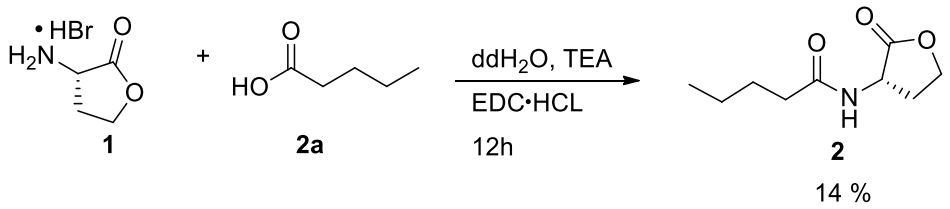
Synthesis of 2

To a 50 mL round-bottom flask with a magnetic stirrer were added ultrapure Milli-Q water (34.3 mL), TEA, (0.13 mL, 0.96 mmol), (*S*)-(−)-α-amino-γ-butyrolactone hydrobromide (**1**), (176 mg, 0.96 mmol) and pentanoic acid (**2a**), (0.156 mL, 1.44 mmol). EDC•HCl, (276 mg, 1.44 mmol) was added. The reaction mixture was stirred at room temperature for 24 h and extracted with EtOAc. The combined organic layers were extracted with 5% aqueous $NaHCO_3$, 1 M $KHSO_4$ and brine, dried over $MgSO_4$ and filtered. The solvent was removed by rotary evaporation and separated by column chromatography (EtOAc:DCM, 50/50 v/v) to give *N*-pentanoyl-*L*-homoserine lactone (**2**) as a white solid (24 mg, 14%), (**Scheme S2**).

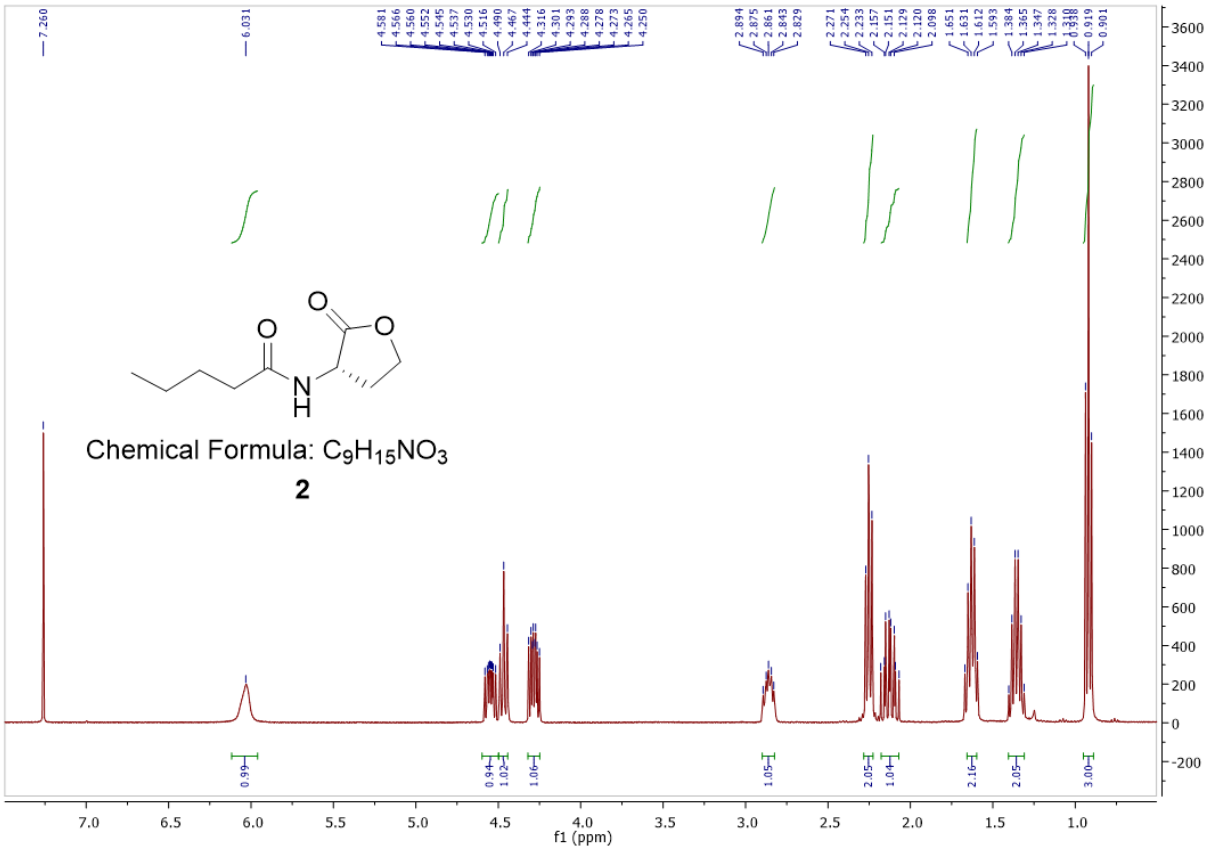
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.03 (br s, 1H), 4.51-4.58 (ddd, *J* = 11.6, 8.4, 6 Hz, 1H), 4.44-4.49 (t, *J* = 9.2 Hz, 1H), 4.25-4.31 (ddd, *J* = 11.2, 9.2, 6 Hz, 1H), 2.82-2.89 (m, 1H), 2.23-2.27 (t, *J* = 6.8 Hz, 1H), 2.06-2.18 (ddd, *J* = 12.8, 11.6, 9.2 Hz, 1H), 1.59-1.66 (quint, *J* = 7.2 Hz, 2H), 1.31-1.40 (sext, *J* = 7.6 Hz, 2H), 0.90-0.93 (t, *J* = 7.6 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 175.70, 173.88, 66.26, 49.41, 36.05, 30.83, 27.63, 22.46, 13.91.

LC-MS *m/z* calculated for $C_9H_{15}NO_3$ (M+H): 186.11. Found: 186.02.



Scheme S2. Synthesis of **2**.



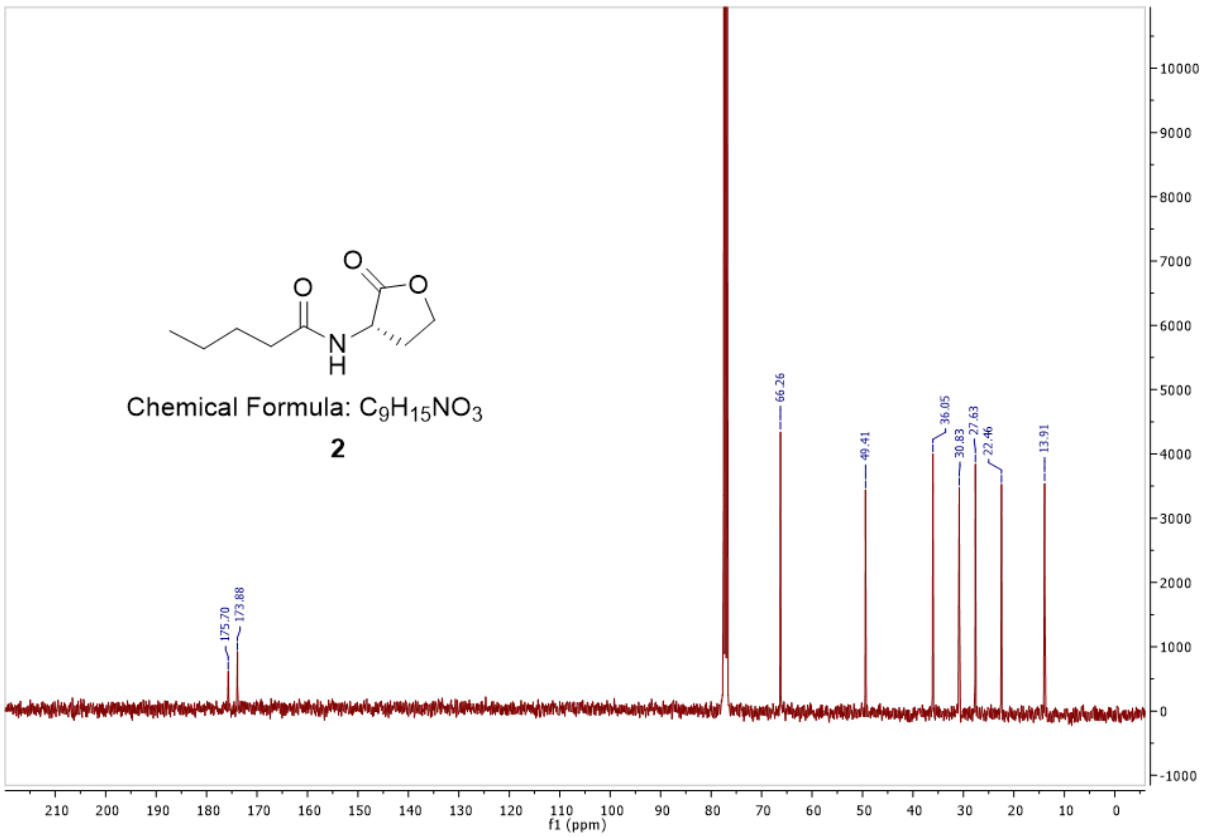


Figure S3. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **2**.

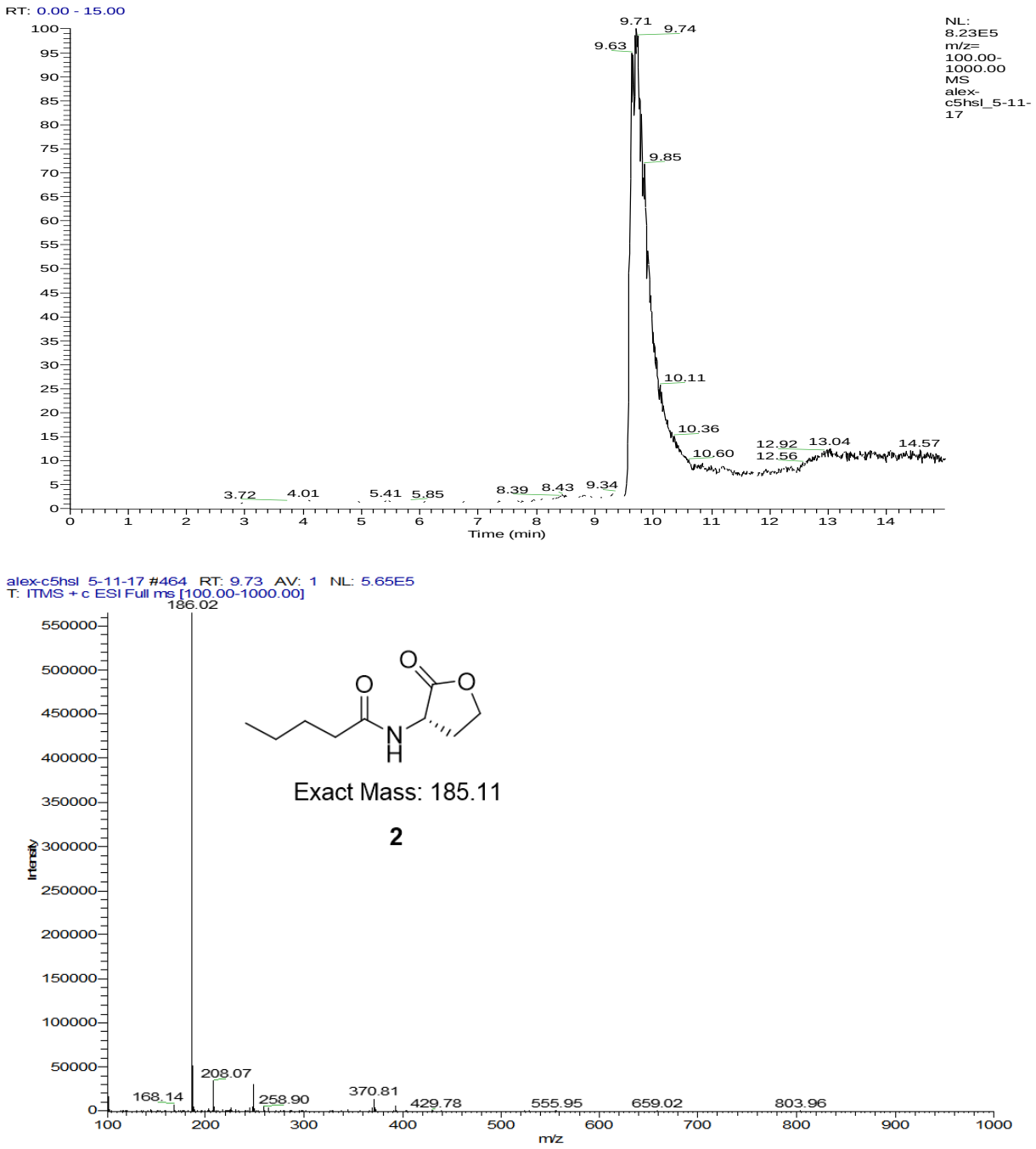


Figure S4. LC-MS of **2**.

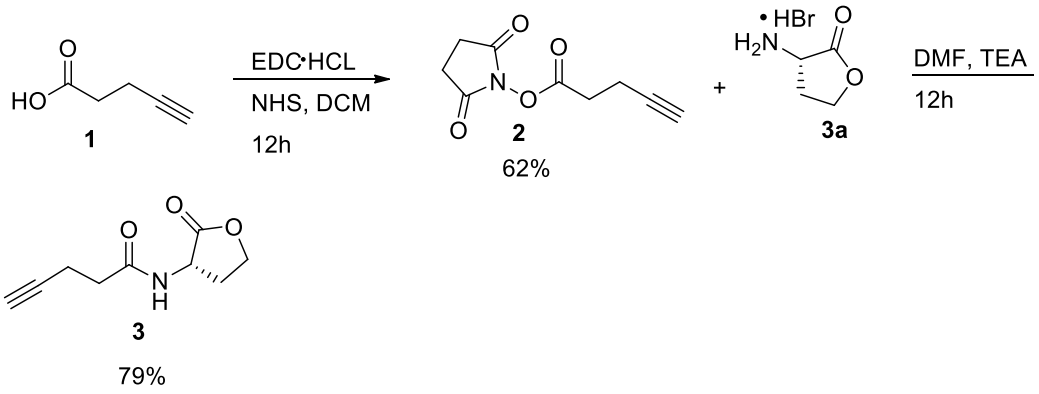
Synthesis of 3

To a 20 mL vial with a stirring magnet was added 4-pentynoic acid (**1**), (100 mg, 1.02 mmol) and dissolved in 0.6 mL of DCM. To another 20 mL vial were added EDC•HCl, (215 mg, 1.12 mmol), NHS, (130 mg, 1.12 mmol) and dissolved in 0.78 mL of DCM. The two solutions were combined and left stirred overnight. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate (**2**), (115.4 mg, 62%) as white solid. To a 20 mL vial with a stirring magnet was added (*S*)-(−)-α-amino-γ-butyrolactone hydrobromide (**3a**), (78.8 mg, 0.42 mmol) and dissolved in 1.5 mL of DMF. To another 20 mL vial were added 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate (**2**), (55 mg, 0.28 mmol) and dissolved in 1.5 mL of DMF. The two solutions were combined and TEA, (0.08 mL, 0.56 mmol) was added and left stirred overnight. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:DCM, 25/75 v/v) to yield (*S*)-*N*-(2-oxotetrahydrofuran-3-yl) pent-4-ynamide (**3**) as a white solid (40.4 mg, 79%), (**Scheme S3**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.27 (br s, 1H), 4.53-4.59 (ddd, *J* = 11.6, 8.8, 6 Hz, 1H), 4.45-4.49 (td, *J* = 8.8, 0.8 Hz, 1H), 4.25-4.32 (ddd, *J* = 11.6, 9.6, 6 Hz, 1H), 2.83-2.90 (m, 1H), 2.46-2.57 (m, 4H), 2.09-2.20 (dtd, *J* = 12.8, 11.6, 9.2 Hz, 1H), 2.01-2.02 (t, *J* = 2.8 Hz, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 175.43, 171.70, 82.63, 69.80, 66.28, 49.54, 34.95, 30.73, 14.78.

LC-MS *m/z* calculated for $C_9H_{11}NO_3$ (M+H): 182.07. Found: 182.04.



Scheme S3. Synthesis of **3**.

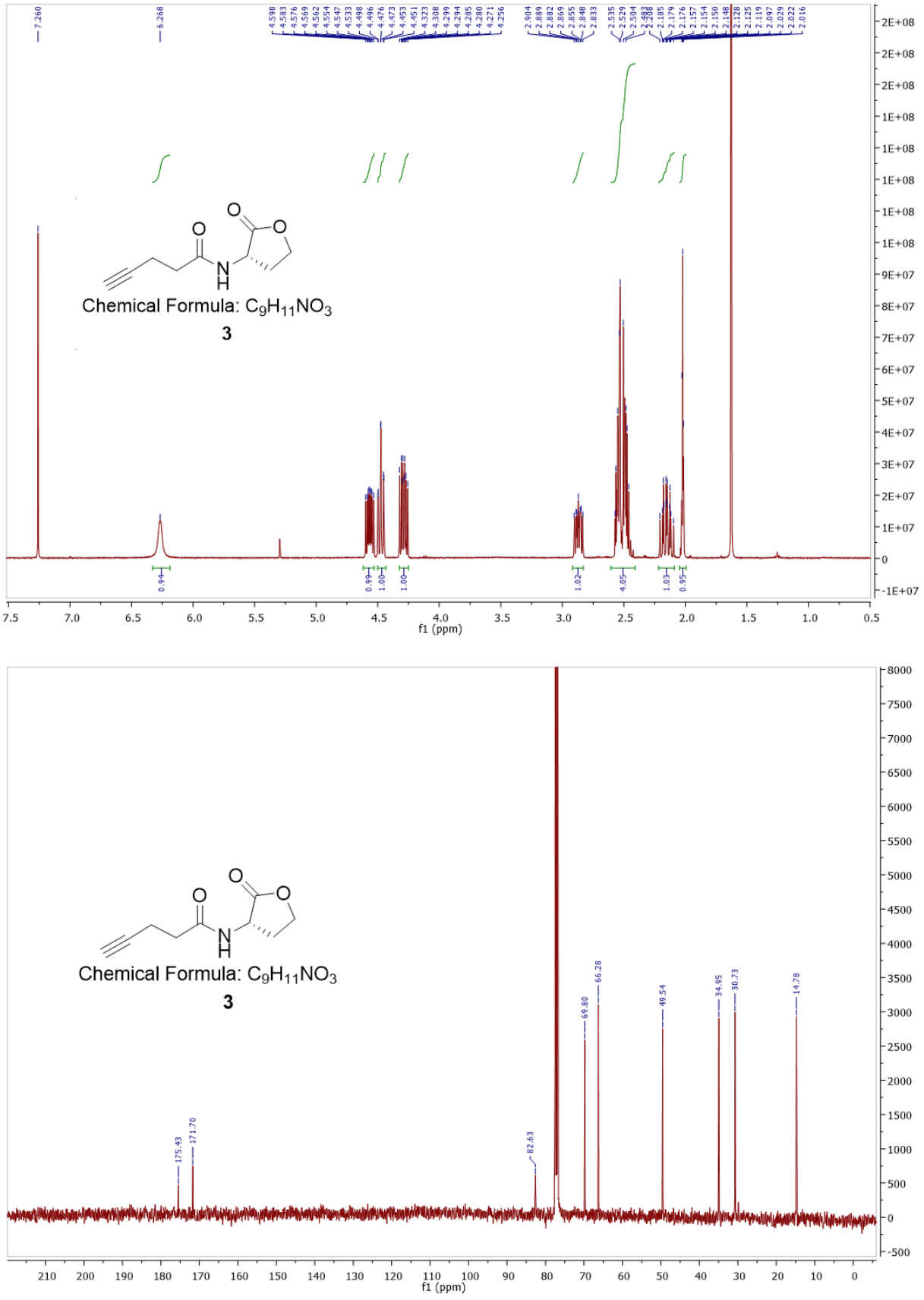


Figure S5. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **3**.

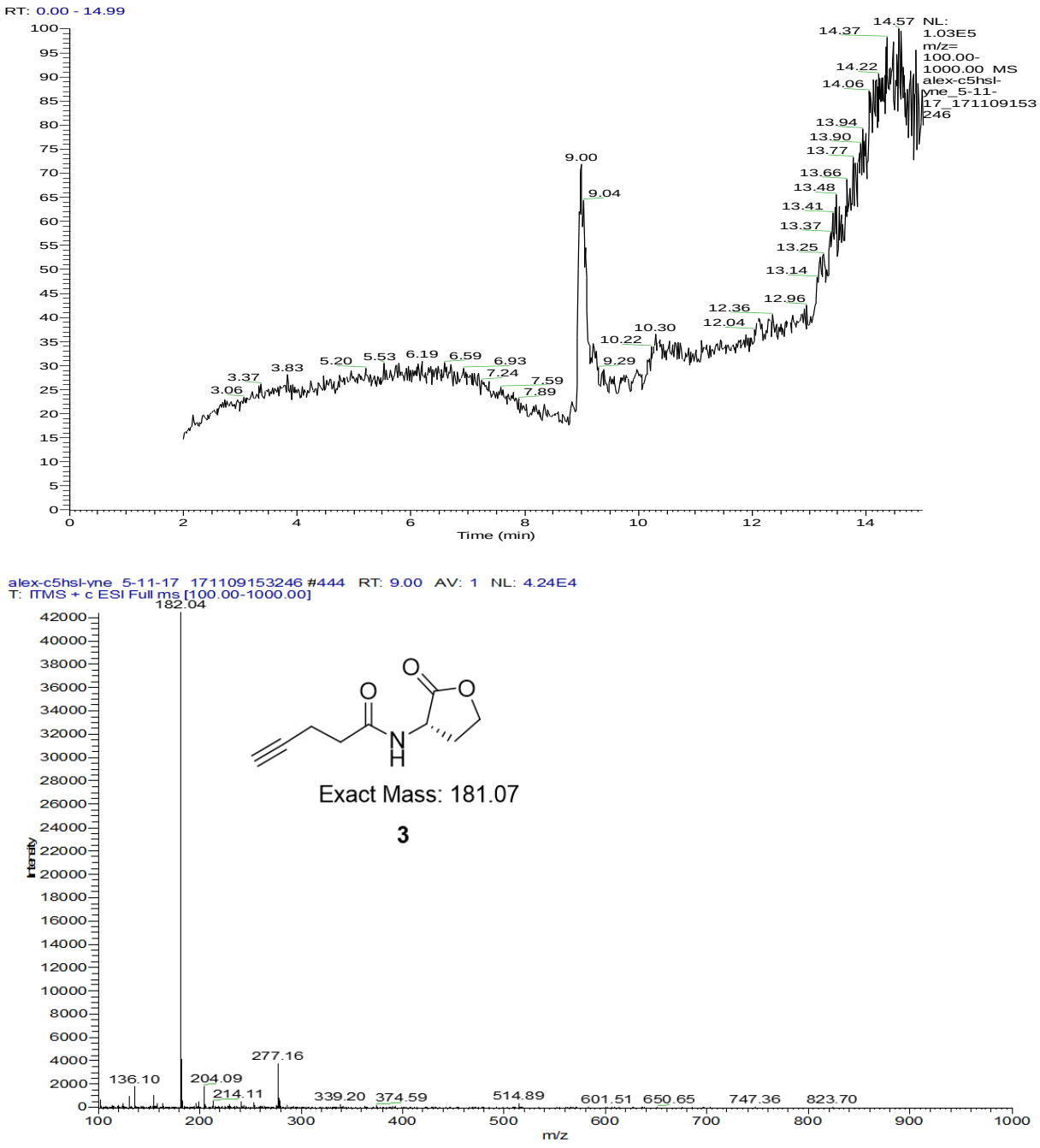


Figure S6. LC-MS of **3**.

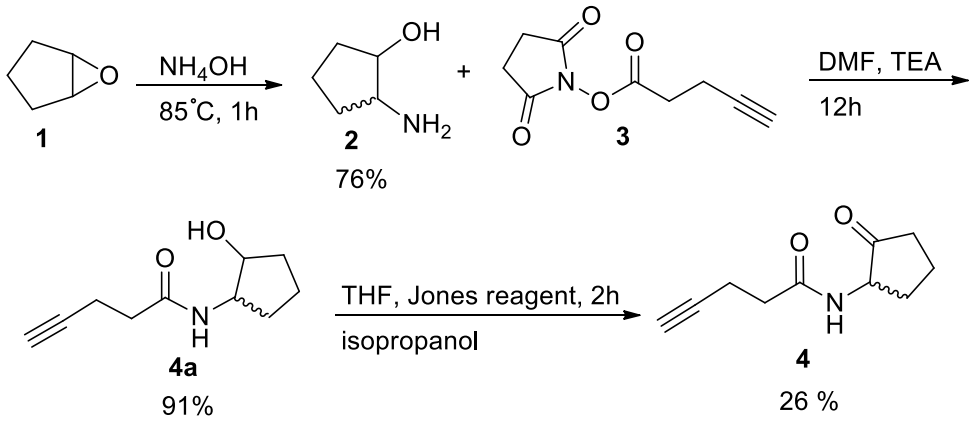
Synthesis of 4

To a microwave reaction tube were added cyclopentene oxide (**1**), (1 gr, 11.8 mmol) and aqueous NH_4OH, (25%, 15.74 mL). The tube was sealed and irradiated under microwave at 85°C for 1 h. The reaction was cooled to room temperature. The solvent was removed by rotary evaporation to yield 2-amino-cyclopentanol (**2**), (902.65 mg, 76%) as a white liquid. To a 20 mL vial with a stirring magnet was added 2-amino-cyclopentanol (**2**), (225 mg, 2.23 mmol) and dissolved in 1.25 mL of DMF. To another 20 mL vial was added 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate (**3**), (289.9 mg, 1.48 mmol), and dissolved in 1.25 mL of DMF. The two solutions were combined, stirred for 10 min, and TEA, (0.42 mL, 2.96 mmol), was added and left stirred overnight. The solvent was removed by rotary evaporation to yield brown liquid which was purified by column chromatography (EtOAc:Hexane, 75/25 v/v) to yield *N*-(2-hydroxycyclopentyl) pent-4-ynamide (**4a**), (244.86 mg, 91%) as a white solid. To a 20 mL vial with a stirring magnet was added *N*-(2-hydroxycyclopentyl) pent-4-ynamide (**4a**), (244.86 mg, 1.35 mmol) and dissolved in 1 mL of THF. 1.5 mL of Jones reagent (CrO_3/H_2SO_4) was added drop-wise and the mixture was stirred for 2 h. The reaction was quenched with 1 mL of isopropanol. Water and EtOAc were added to extract the product. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:DCM, 50/50 v/v) to yield *N*-(2-oxocyclopentyl) pent-4-ynamide (**4**) as a white solid (61.7 mg, 26%), (**Scheme S4**).

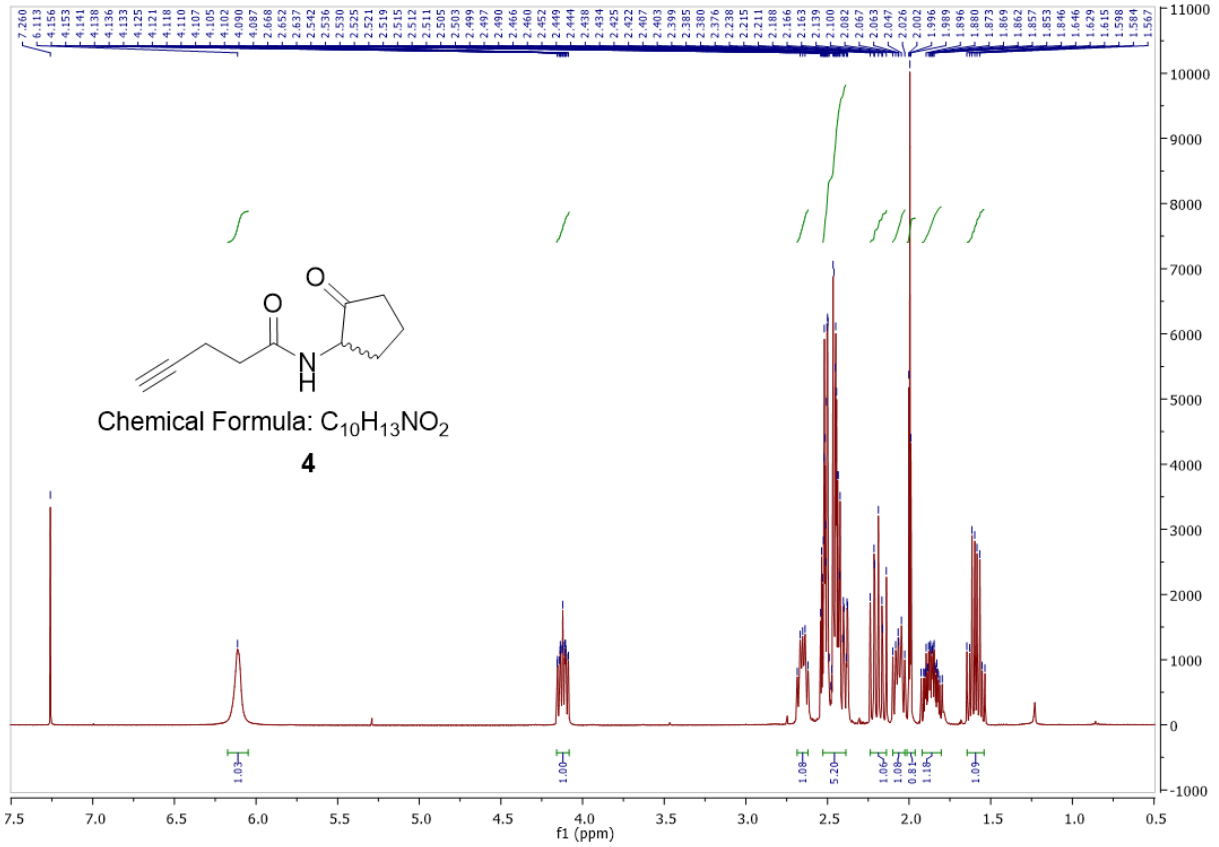
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.11 (br s, 1H), 4.09-4.16 (m, 1H), 2.62-2.68 (br m, 1H), 2.37-2.54 (m, 5H), 2.13-2.23 (m, 1H), 2.02-2.10 (br m, 1H), 2.00 (t, *J* = 2.4 Hz, 1H), 1.79-1.92 (m, 1H), 1.53-1.64 (qd, *J* = 12.4, 6.8 Hz, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 215.24, 171.40, 82.85, 69.51, 58.33, 35.03, 34.99, 30.19.

LC-MS *m/z* calculated for $C_{10}H_{13}NO_2$ (M+H): 180.09. Found: 180.06.



Scheme S4. Synthesis of **4**.



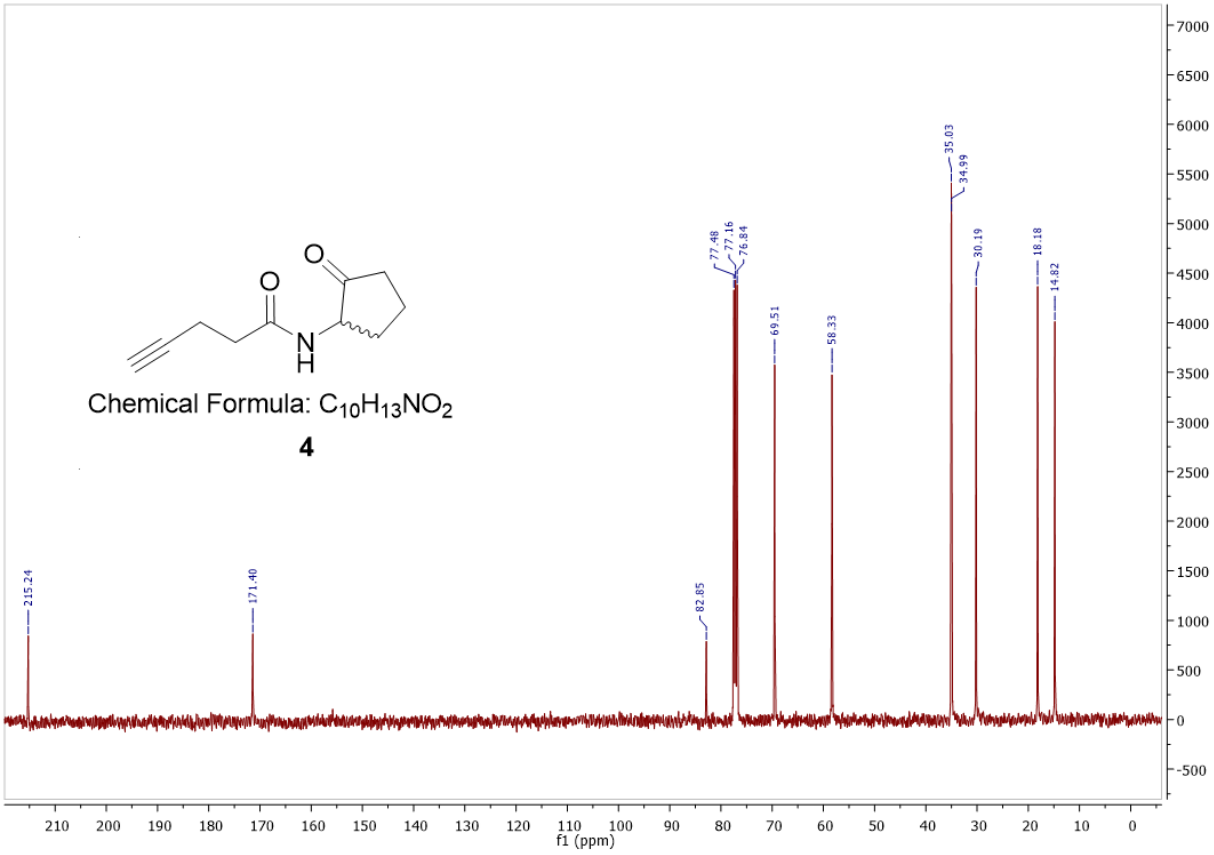
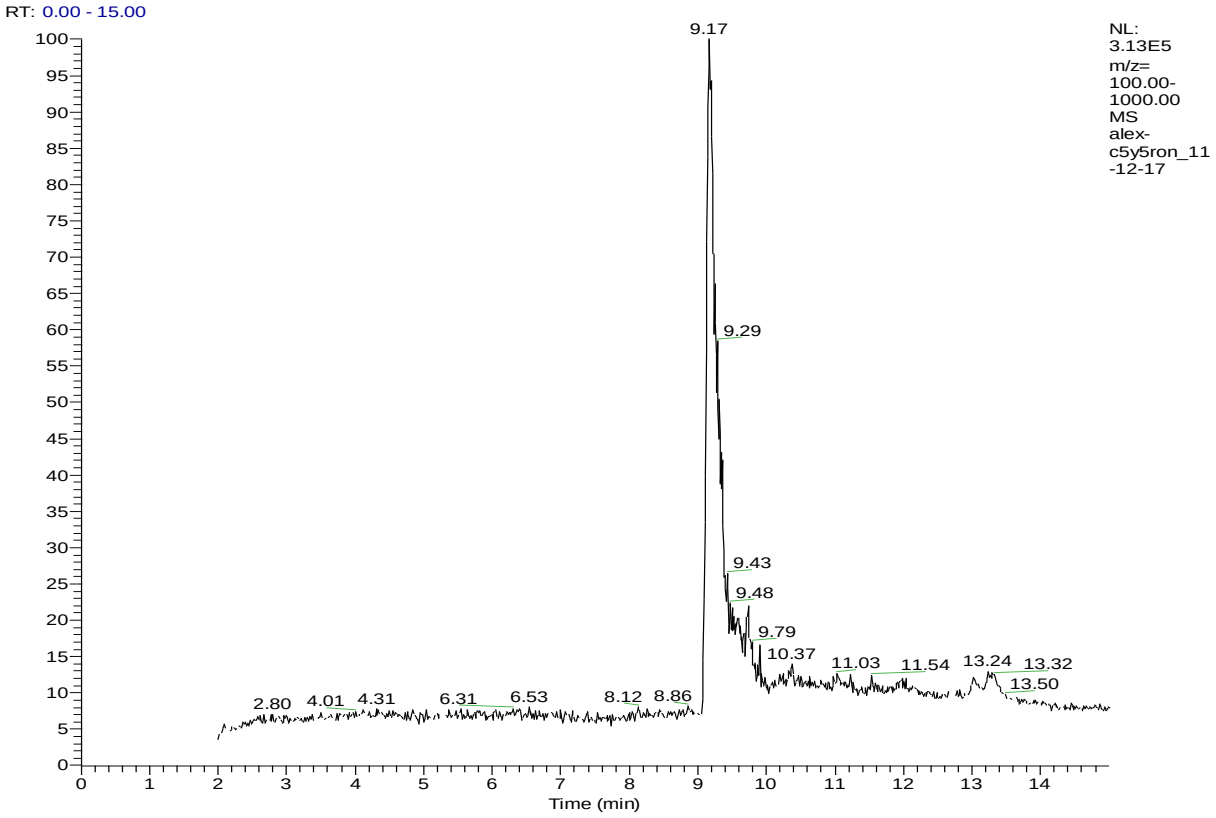
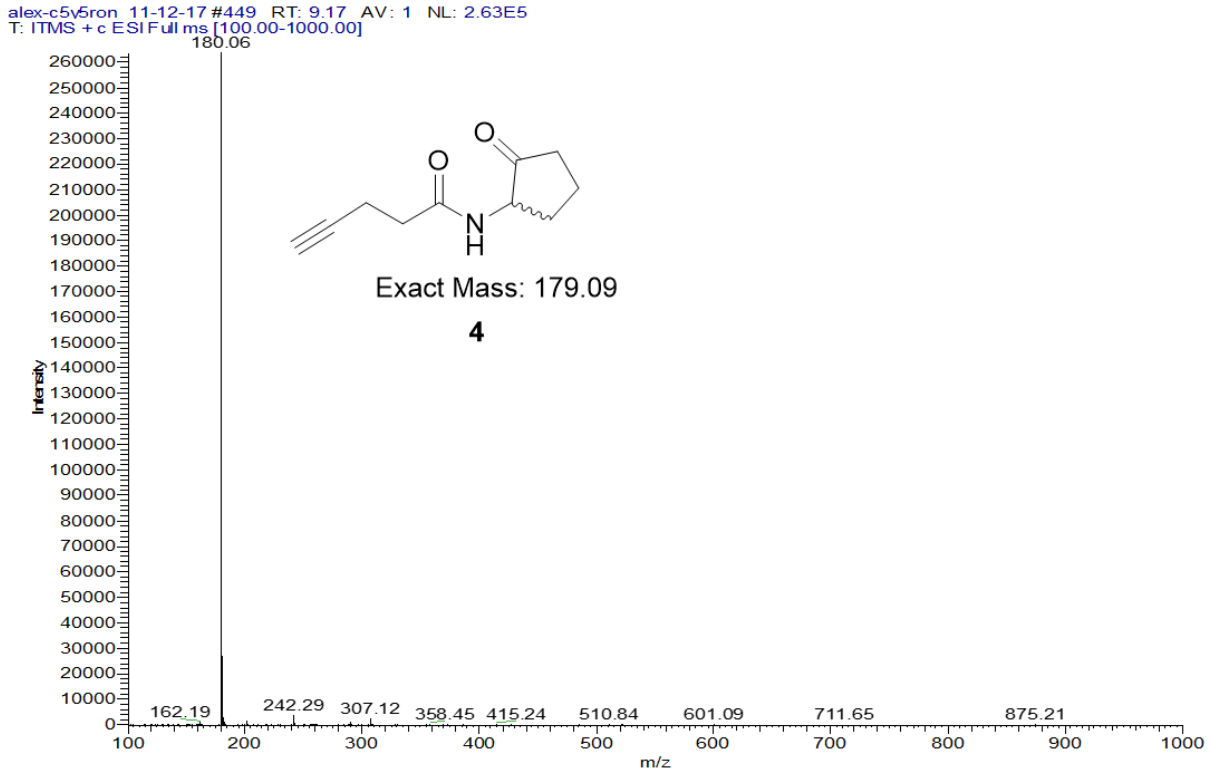


Figure S7. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **4**.





Exact Mass: 179.09

4

Figure S8. LC-MS of **4**.

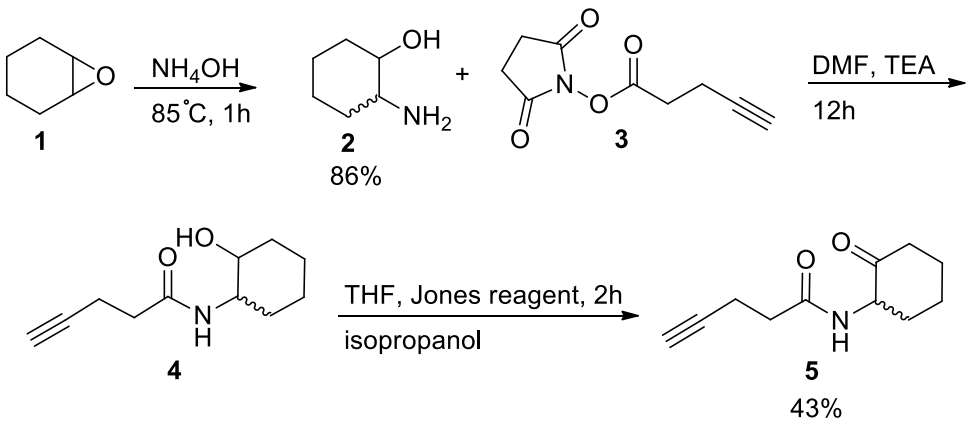
Synthesis of 5

To a microwave reaction tube were added cyclohexene oxide (**1**), (0.5 gr, 5 mmol) and aqueous NH_4OH, (25%, 7 mL). The tube was sealed and irradiated under microwave at 85°C for 1 h. The reaction was cooled to room temperature. The solvent was removed by rotary evaporation to yield 2-amino-cyclohexanol (**2**), (0.49 gr, 86%) as white solid. To a 20 mL vial with a stirring magnet was added 2-amino-cyclohexanol (**2**), (180 mg, 1.56 mmol) and dissolved in 1.1 mL of DMF. To another 20 mL vial was added 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate (**3**), (254.48 mg, 1.3 mmol), and dissolved in 1.1 mL of DMF. The two solutions were combined, stirred for 10 min and TEA, (0.36 mL, 2.6 mmol), was added and left stirred overnight. The solvent was removed by rotary evaporation and the crude was purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield *N*-(2-hydroxycyclohexyl) pent-4-ynamide (**4**), as a white solid. To a 20 mL vial with a stirring magnet was added *N*-(2-hydroxycyclohexyl) pent-4-ynamide (**4**), (320.54 mg, 1.64 mmol) and dissolved in 1 mL of THF. 1.5 mL of Jones reagent (CrO_3/H_2SO_4) was added drop-wise and the mixture was stirred for 2 h. The reaction was quenched with 1 mL of isopropanol. Water and EtOAc were added to extract the product. The solvent was removed by rotary evaporation and the crude was purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield *N*-(2-oxocyclohexyl) pent-4-ynamide (**5**), as a white solid (136.9 mg, 43%), (**Scheme S5**).

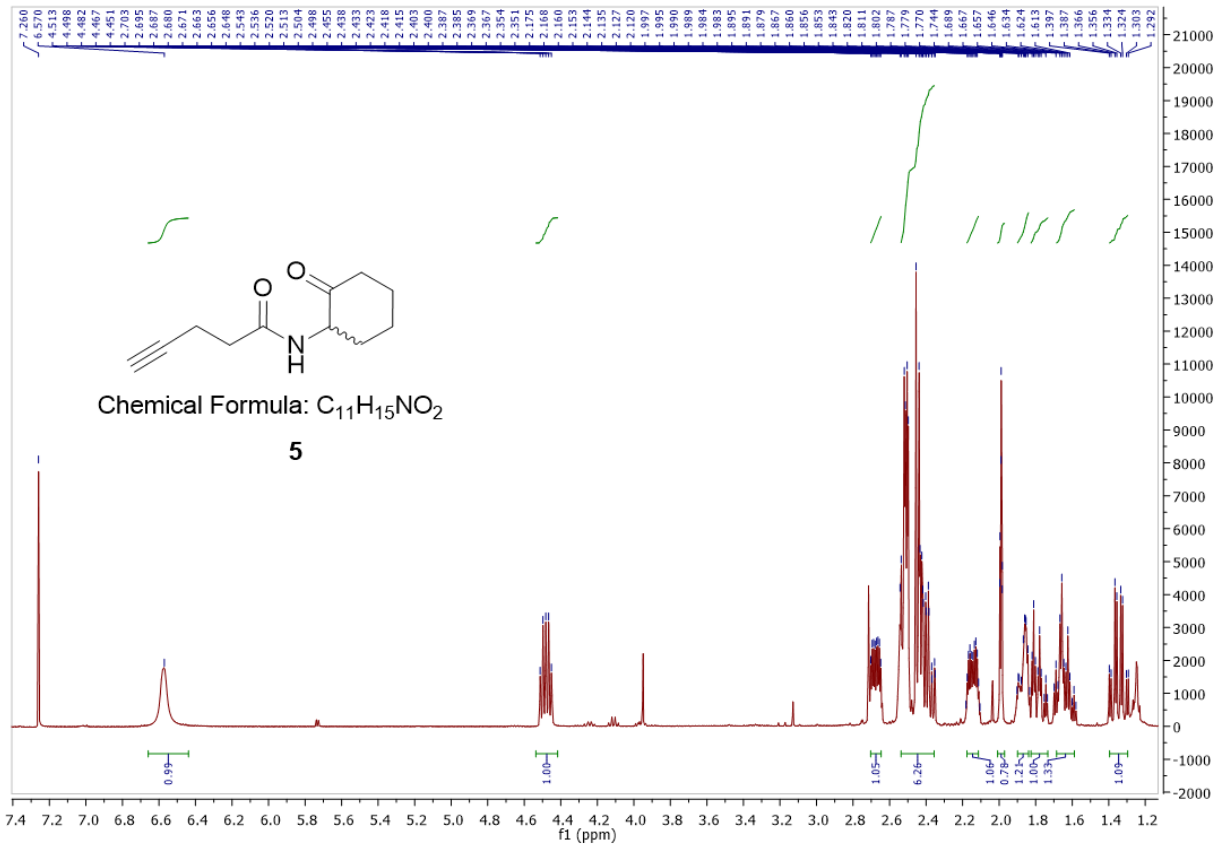
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.57 (br s, 1H), 4.45-4.51 (m, 1H), 2.64-2.70 (m, 1H), 2.35-2.54 (m, 6H), 2.10-2.18 (m, 1H), 1.98-1.99 (td, *J* = 2.8, 0.8 Hz, 1H), 1.83-1.90 (br m 1H), 1.73-1.82 (br m, 1H), 1.57-1.69 (br m, 1H), 1.29-1.39 (qd, *J* = 12.4, 4.0 Hz, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 207.85, 170.61, 82.89, 69.44, 58.34, 41.26, 35.64, 35.37, 28.20, 24.13, 14.94.

LC-MS *m/z* calculated for $C_{11}H_{15}NO_2$ (M+H): 194.11. Found: 194.02.



Scheme S5. Synthesis of **5**.



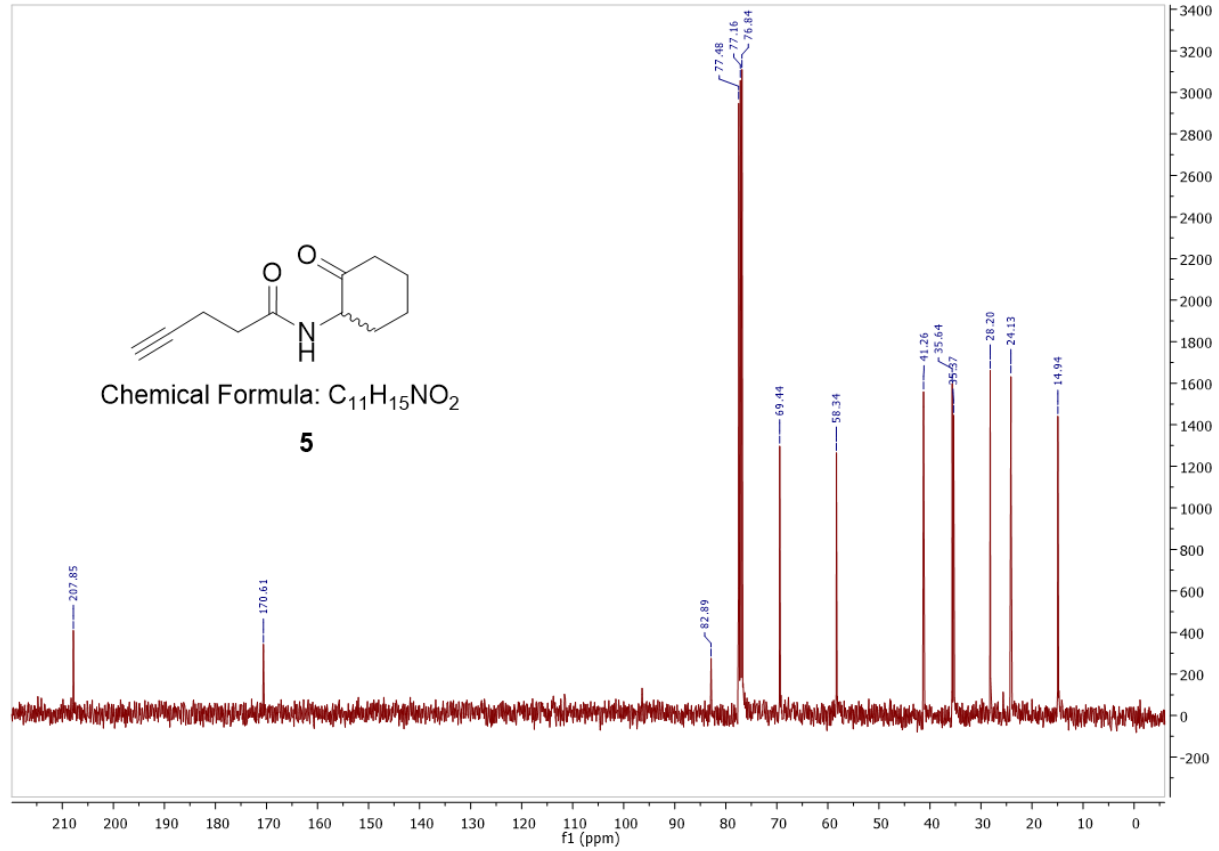


Figure S9. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **5**.

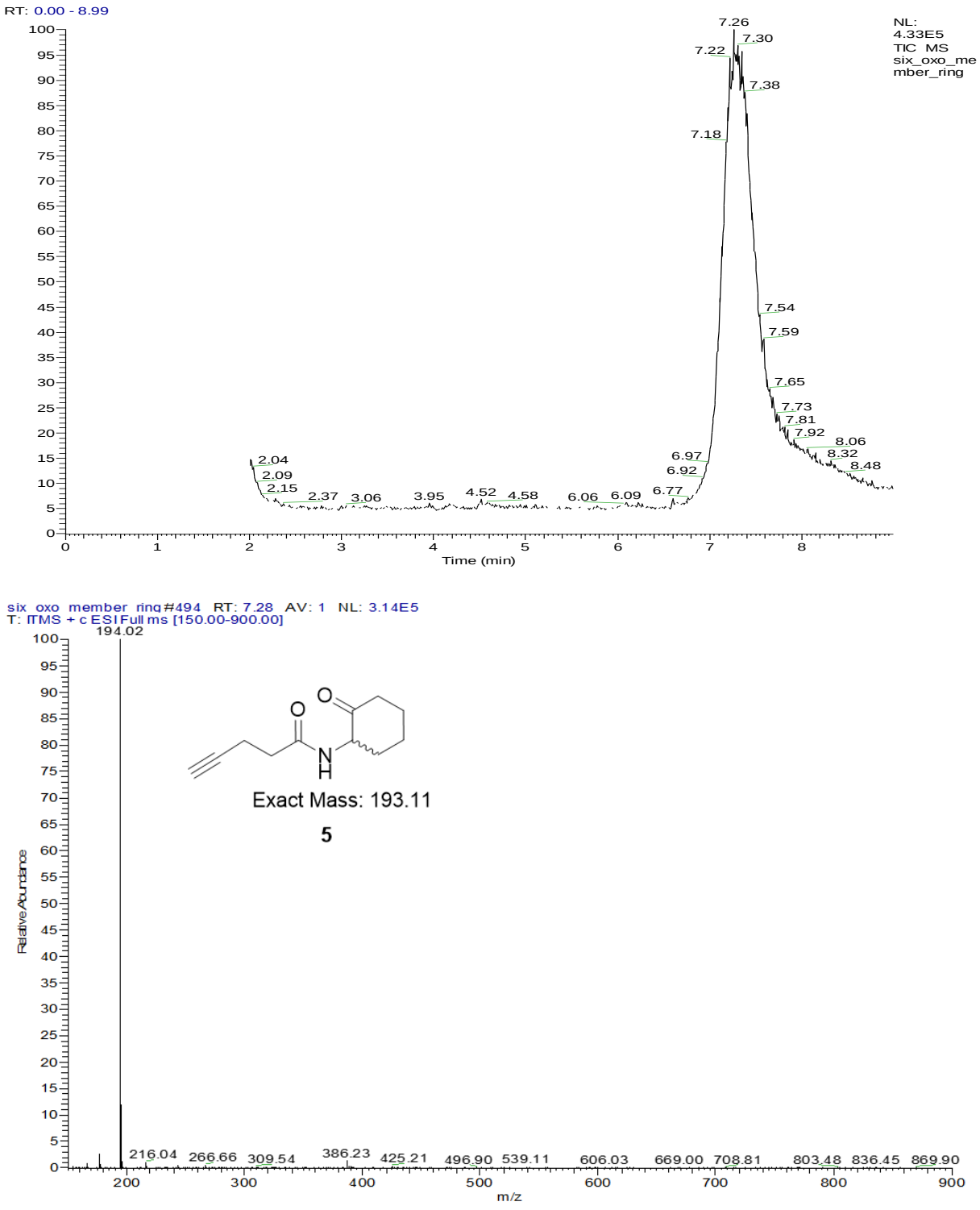


Figure S10. LC-MS of **5**.

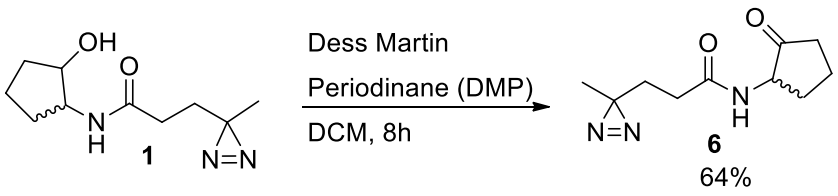
Synthesis of 6

To a 4 mL vial was added *N*-(2-hydroxycyclopentyl)-3-(3-methyl-3*H*-diazirin-3-yl) propanamide (**1**), (35 mg, 0.166 mmol) and dissolved in 0.4 mL of DCM. Dess-Martin periodinane, (84.3 mg, 0.198 mmol) was added to the vial and the mixture was stirred with a magnet and left overnight. To a 100 mL flask was added 25 mL water and the reaction mixture, 50 mL of 10% $NaHCO_3$ and equal amount of DCM. The two phases were separated and the organic phase was dried over $MgSO_4$. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 60/40 v/v) to yield 3-(3-methyl-3*H*-diazirin-3-yl)-*N*-(2-oxocyclopentyl) propanamide (**6**), as a white solid (22.5 mg, 64%), (**Scheme S6**).

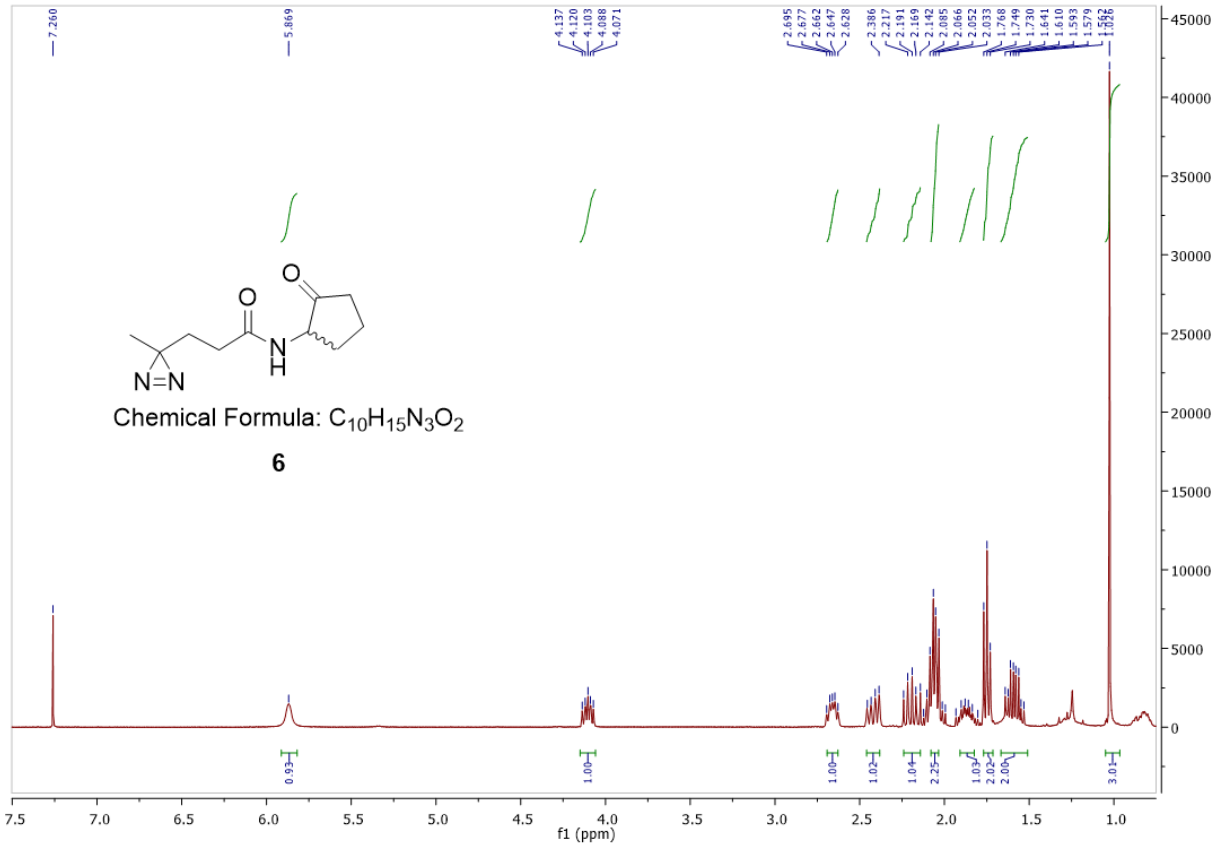
^{1}H NMR (400 MHz, $CDCl_3$): δ 5.86 (br s, 1H), 4.07-4.13 (m, 1H), 2.62-2.69 (br m, 1H), 2.38-2.45 (dd, *J* = 18.5, 9.1 Hz, 1H), 2.14-2.24 (m, 1H), 1.99-2.24 (m, 2H), 1.83-1.93 (m, 1H), 1.73-1.76 (t, *J* = 7.6 Hz, 2H), 1.53-1.64 (qd, *J* = 12.4, 6.8, Hz, 2H), 1.03 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 215.20, 171.79, 58.36, 34.98, 30.43, 30.18, 30.06, 25.50, 20.00, 18.18.

LC-MS *m/z* calculated for $C_{10}H_{15}N_3O_2$ (M+H): 210.12. Found: 209.98.



Scheme S6. Synthesis of **6**.



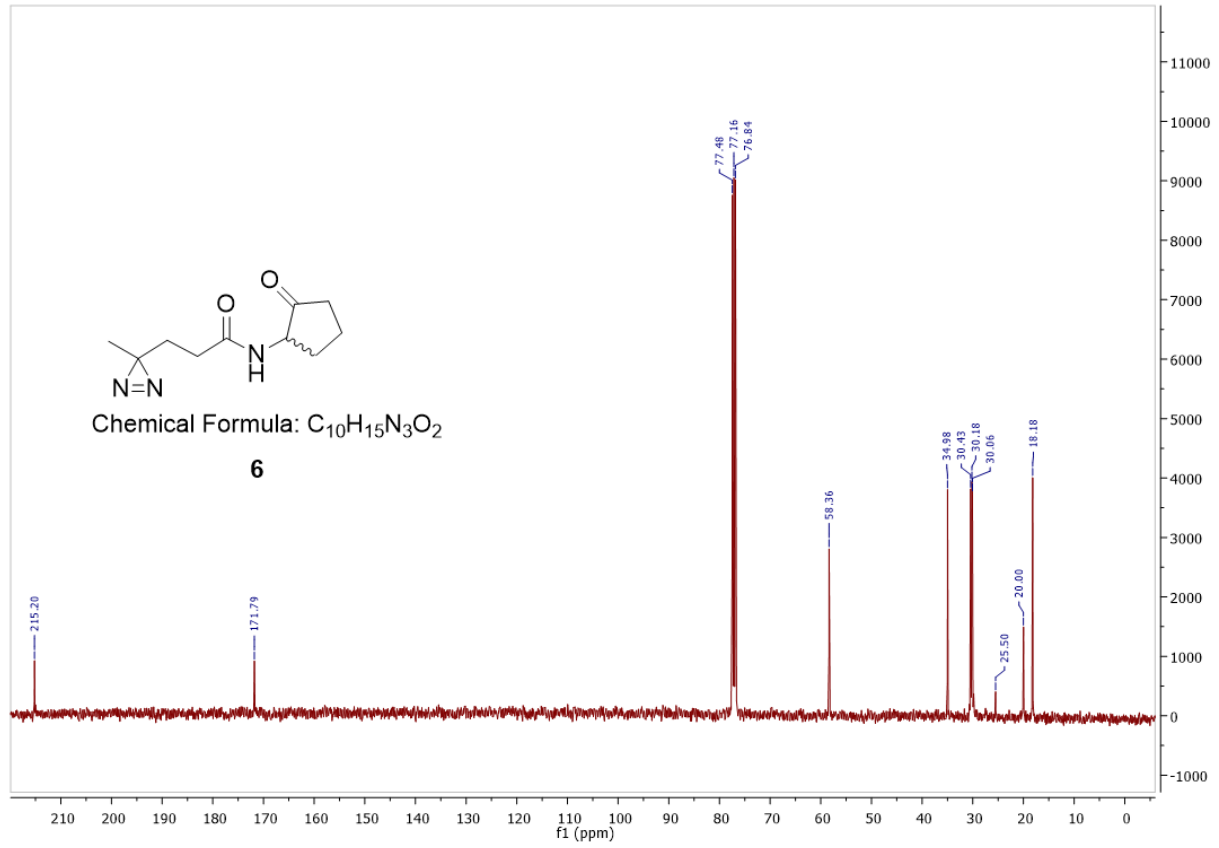


Figure S11. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **6**.

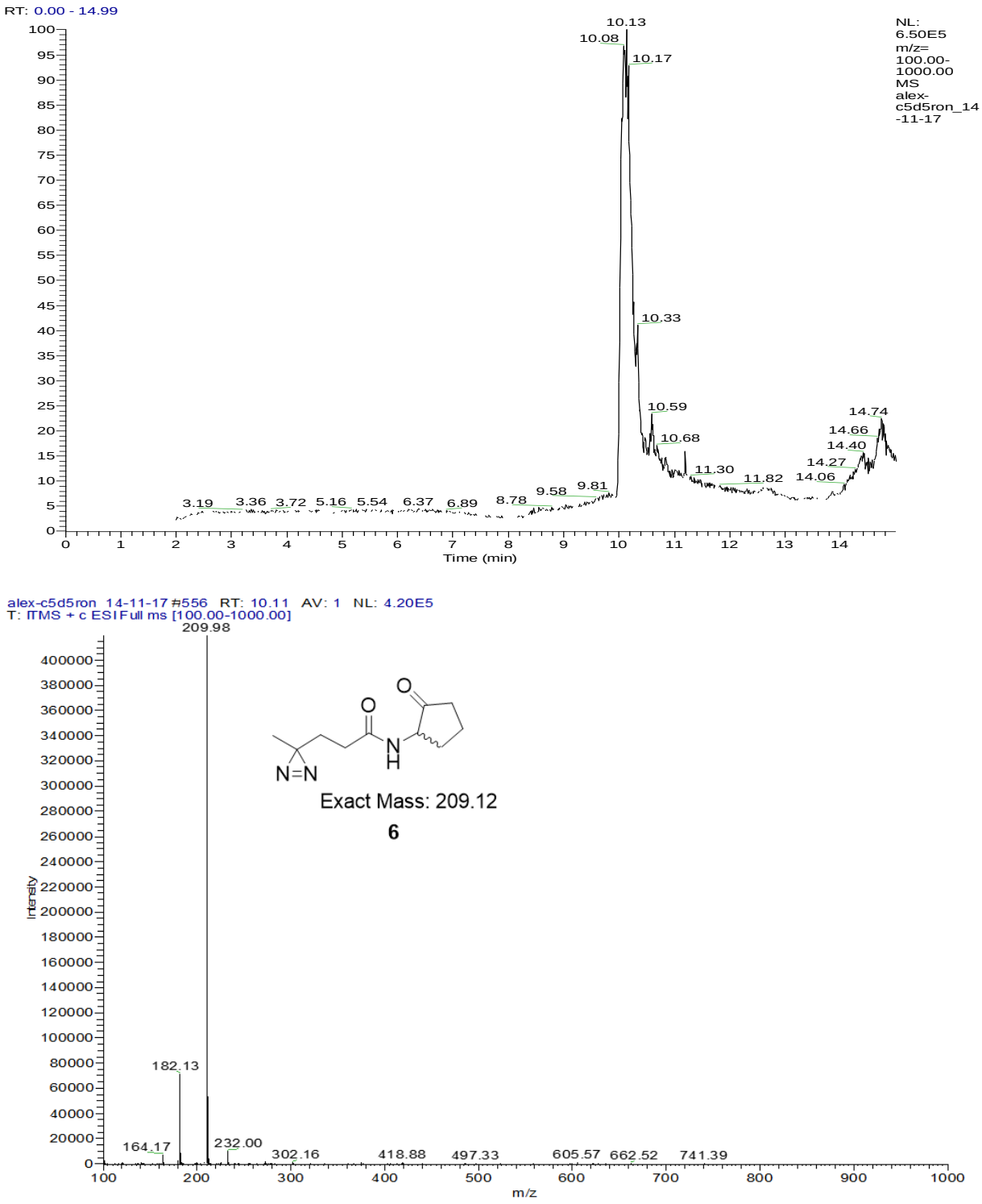


Figure S12. LC-MS of **6**.

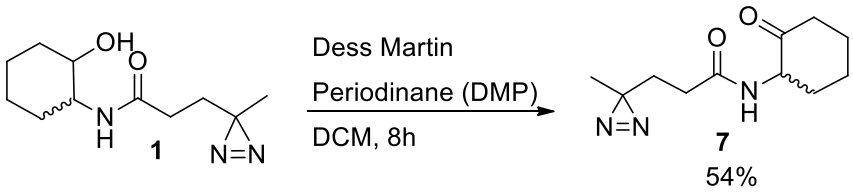
Synthesis of 7

To a 4 mL vial was added *N*-(2-hydroxycyclohexyl)-3-(3-methyl-3*H*-diazirin-3-yl) propanamide (**1**), (59.6 mg, 0.265 mmol) and dissolved in 0.53 mL of DCM. Dess-martin periodinane, (135 mg, 0.318 mmol) was added to the vial and the mixture was stirred with a magnet and left overnight. To a 100 mL flask was added 25 mL water and the reaction mixture, 50 mL of 10% $NaHCO_3$ and equal amount of DCM. The two phases were separated and the organic phase was dried over $MgSO_4$. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 60/40 v/v) to yield 3-(3-methyl-3*H*-diazirin-3-yl)-*N*-(2-oxocyclohexyl) propanamide (**7**) as a white solid (31.8 mg, 54%), (**Scheme S7**).

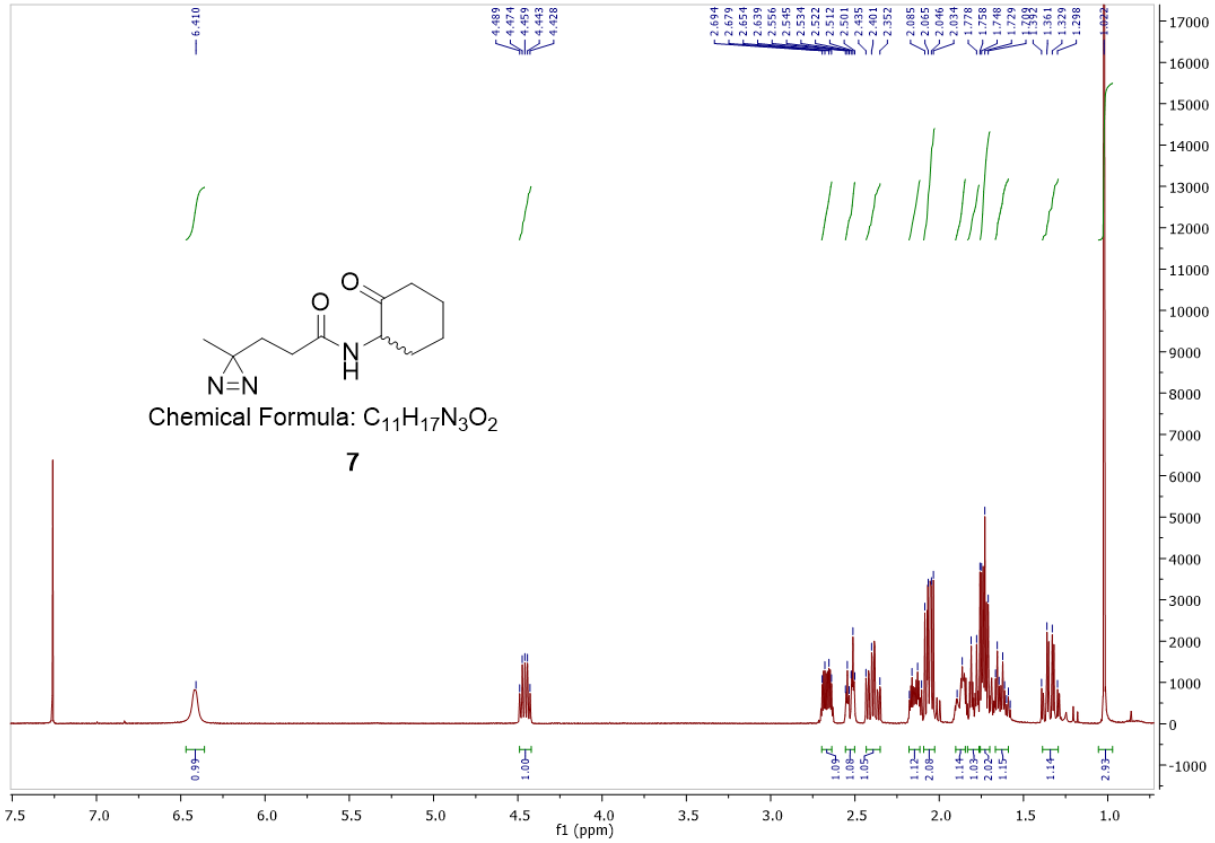
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.42 (br s, 1H), 4.42-4.48 (m, 1H), 2.63-2.70 (m, 1H), 2.50-2.55 (m, 1H), 2.34-2.43 (m, 1H), 2.10-2.18 (m, 1H), 1.99-2.08 (m, 2H), 1.84-1.90 (m, 1H), 1.76-1.81 (m, 1H), 1.68-1.75 (m, 2H), 1.57-1.67 (m, 1H), 1.28-1.39 (qd, *J* = 12.4, 4.0 Hz, 1H), 1.02 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 207.88, 171.00, 58.27, 41.25, 35.59, 30.72, 30.14, 28.19, 25.50, 24.13, 19.98.

LC-MS *m/z* calculated for $C_{11}H_{17}N_3O_2$ (M+H): 224.13. Found: 223.95.



Scheme S7. Synthesis of **7**.



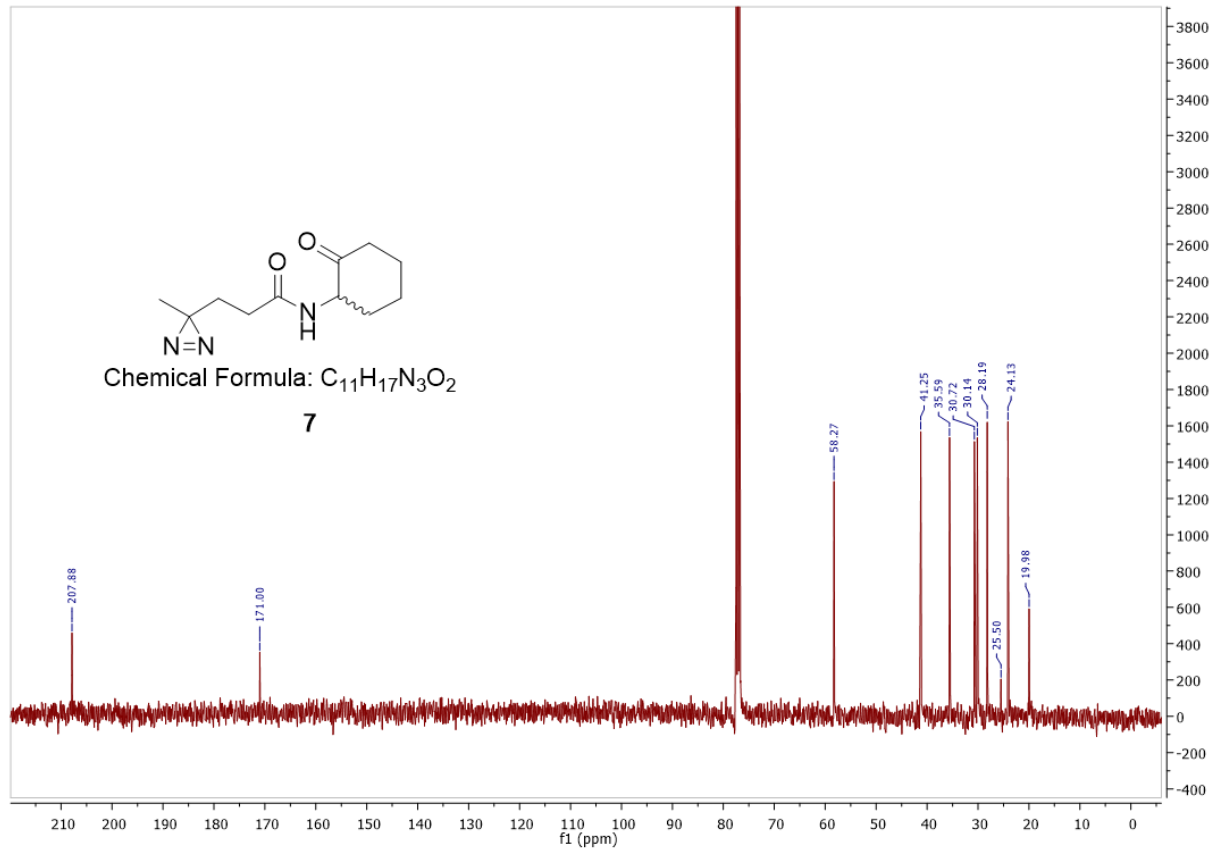
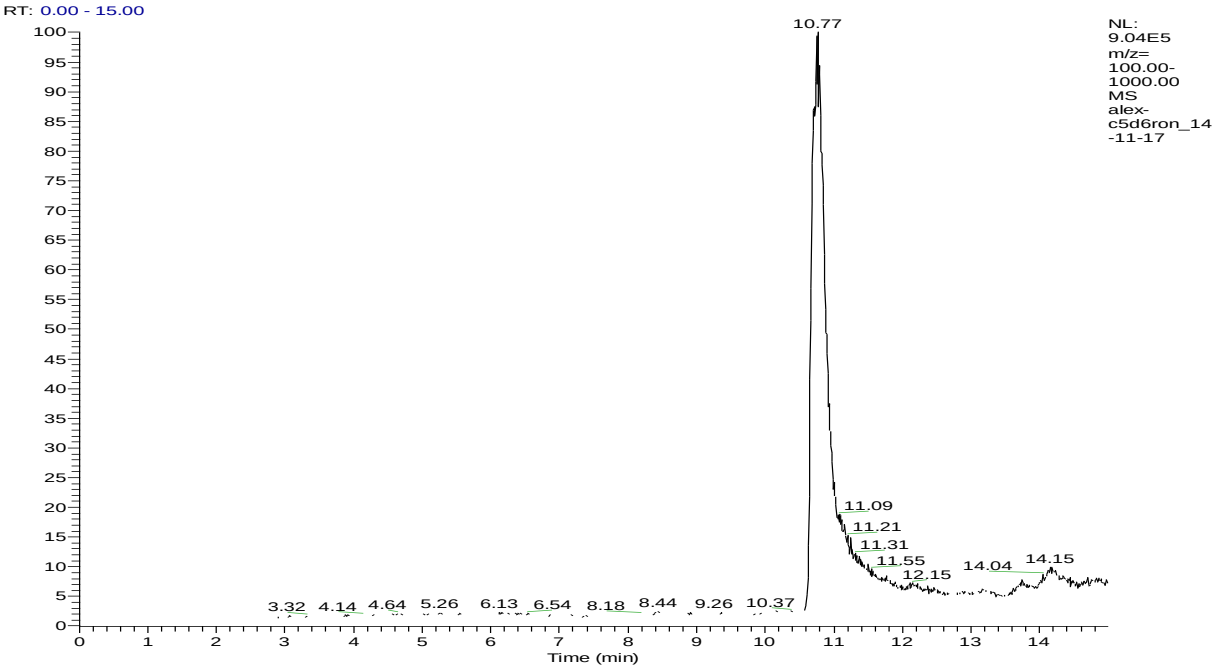


Figure S13. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **7**.



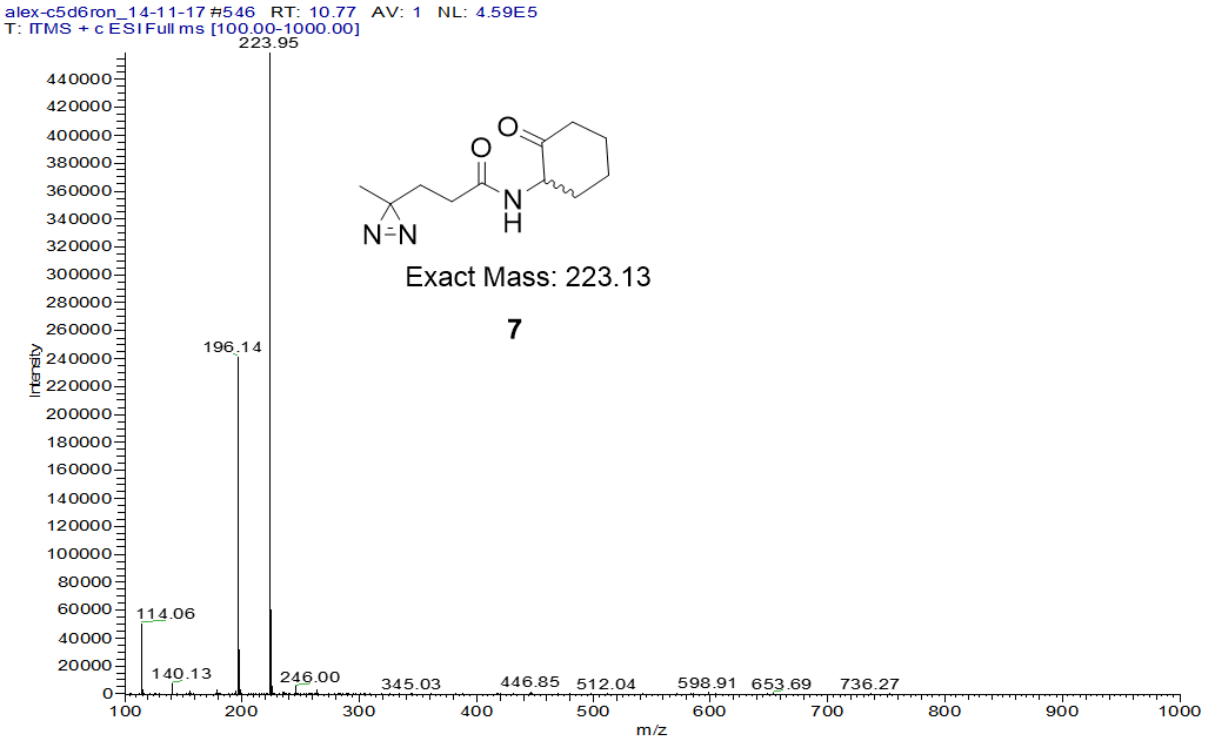


Figure S14. LC-MS of **7**.

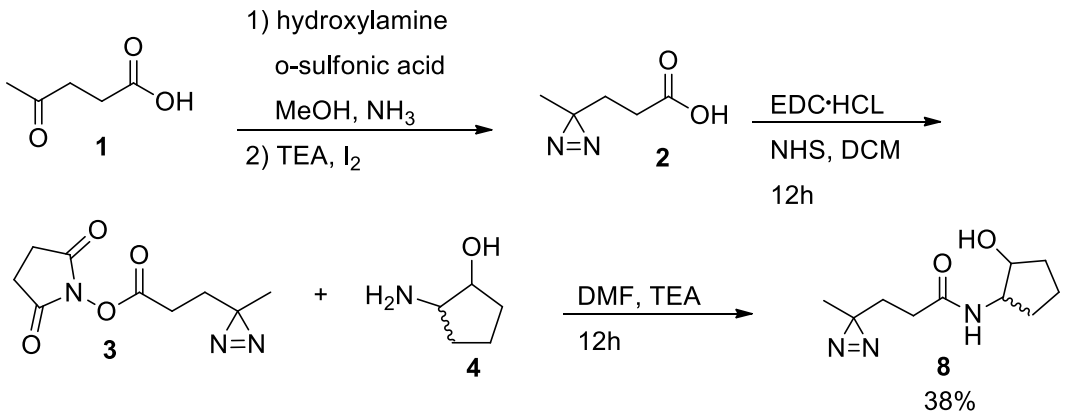
Synthesis of 8

To a 50 mL round bottom flask with a stirring magnet, septum and N_2 atmosphere was added levulinic acid (**1**), (476 mg, 4.1 mmol). To the flask was added 8 N methanolic ammonia (4.6 mL) and the mixture was allowed to be stirred for 3 h at 0°C. A balloon was used to maintain the ammonia environment and allowing the release of pressure. Hydroxylamine-O-sulfonic acid 97%, (625 mg, 5.52 mmol) was dissolved in 4 mL of MeOH and added to the reaction mixture dropwise. The mixture was allowed to be stirred overnight. The ammonia was removed by blowing air through the flask. The solvent was concentrated by rotary evaporation to give a yellowish liquid. The reaction flask was covered with aluminum foil to minimize exposure to light and 25 mL of MeOH was added to re-dissolve the diaziridine acid. The reaction flask was placed in an ice-water bath and the mixture was allowed to stir for 5 min with a rubber septum cap. TEA, (0.9 mL, 4.1 mmol) was added drop-wise and the mixture was allowed to stir for 5 min. Using a spatula, 642 mg of iodine chips were added slowly to the stirring reaction mixture until the solution stayed persistently red-brown for more than 5 min. The reaction mixture was mixed with 25 mL of EtOAc and transferred to a separatory funnel, washed with 100 mL of 1 M aqueous HCl, 100 mL of 10% $Na_2S_2O_3$, 50 mL of saturated aqueous NaCl and dried with anhydrous $MgSO_4$. The reaction was filtered and evaporated by rotary evaporation to yield 3-(3-methyl-3*H*-diazirin-3-yl) propanoic acid (**2**) as a yellowish-white liquid. To the reaction mixture of 3-(3-methyl-3*H*-diazirin-3-yl) propanoic acid (**2**) was added 0.86 mL of DCM, to another 20 mL vial were added EDC·HCl, (345 mg, 1.8 nmol), NHS, (207 mg, 1.8 mmol) and dissolved in 1.2 mL of DCM. The two solutions were combined and left stirred overnight. The solvent was removed by rotary evaporation and the product was purified by column chromatography (Hexane:EtOAc, 60/40 v/v) to yield 2, 5-dioxopyrrolidin-1-yl 3-(3-methyl-3*H*-diazirin-3-yl) propanoate (**3**), (265 mg) as a white-yellowish solid. To a 20 mL vial was added 2-aminocyclopentanol (**4**), (64.6 mg, 0.64 mmol) and dissolved in 0.88 mL of DMF. To another 20 mL vial flask with a stirring magnet was added 2, 5-dioxopyrrolidin-1-yl 3-(3-methyl-3*H*-diazirin-3-yl) propanoate (**3**), (120 mg, 0.53 mmol). The vials were combined and TEA, (0.07 mL, 0.53 mmol) was added and left stirred overnight. The solvent was removed by rotary evaporation purified by column chromatography (EtOAc:Hexane, 90/10 v/v) to yield *N*-(2-hydroxycyclopentyl)-3-(3-methyl-3*H*-diazirin-3-yl) propanamide (**8**), as a white solid (43.3 mg, 38%), (**Scheme S8**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.62 (br s, 1H), 4.34 (br s, 1H), 3.94-3.99 (dd, *J* = 13.6, 6.8 Hz, 1H), 3.78-85 (m, 1H), 2.08-2.17 (m, 1H), 1.98-2.05 (m, 3H), 1.63-1.7483 (m, 5H), 1.36-1.46 (ddd, *J* = 16.4, 12.8, 8.0 Hz, 1H), 1.04 (s, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 173.55, 79.80, 61.15, 32.75, 30.63, 30.47, 30.02, 25.55, 21.47, 20.13.

LC-MS *m/z* calculated for $C_{10}H_{17}N_3O_2$ (M+H): 212.13. Found: 212.01.



Scheme S8. Synthesis of **8**.

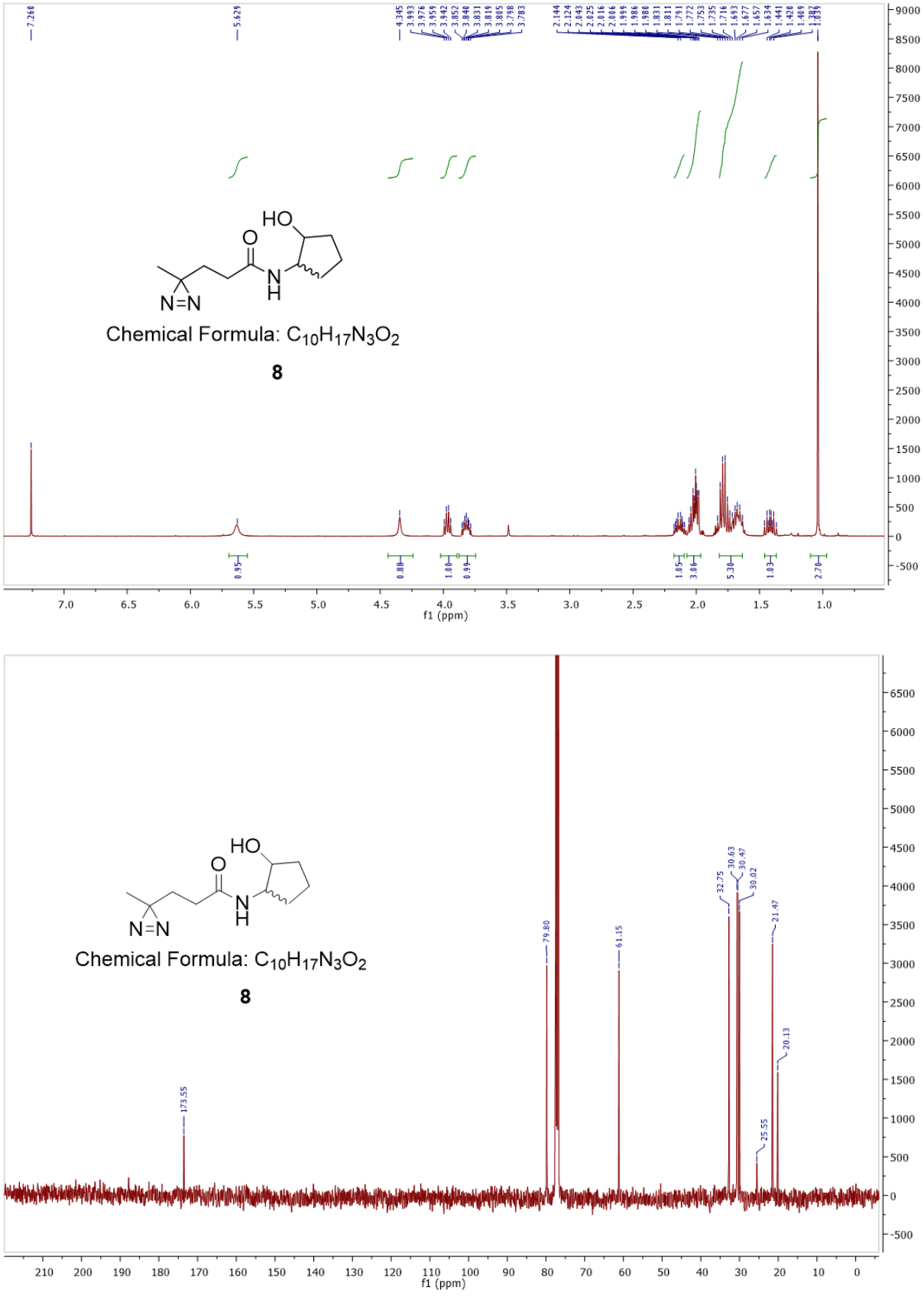


Figure S15. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **8**.

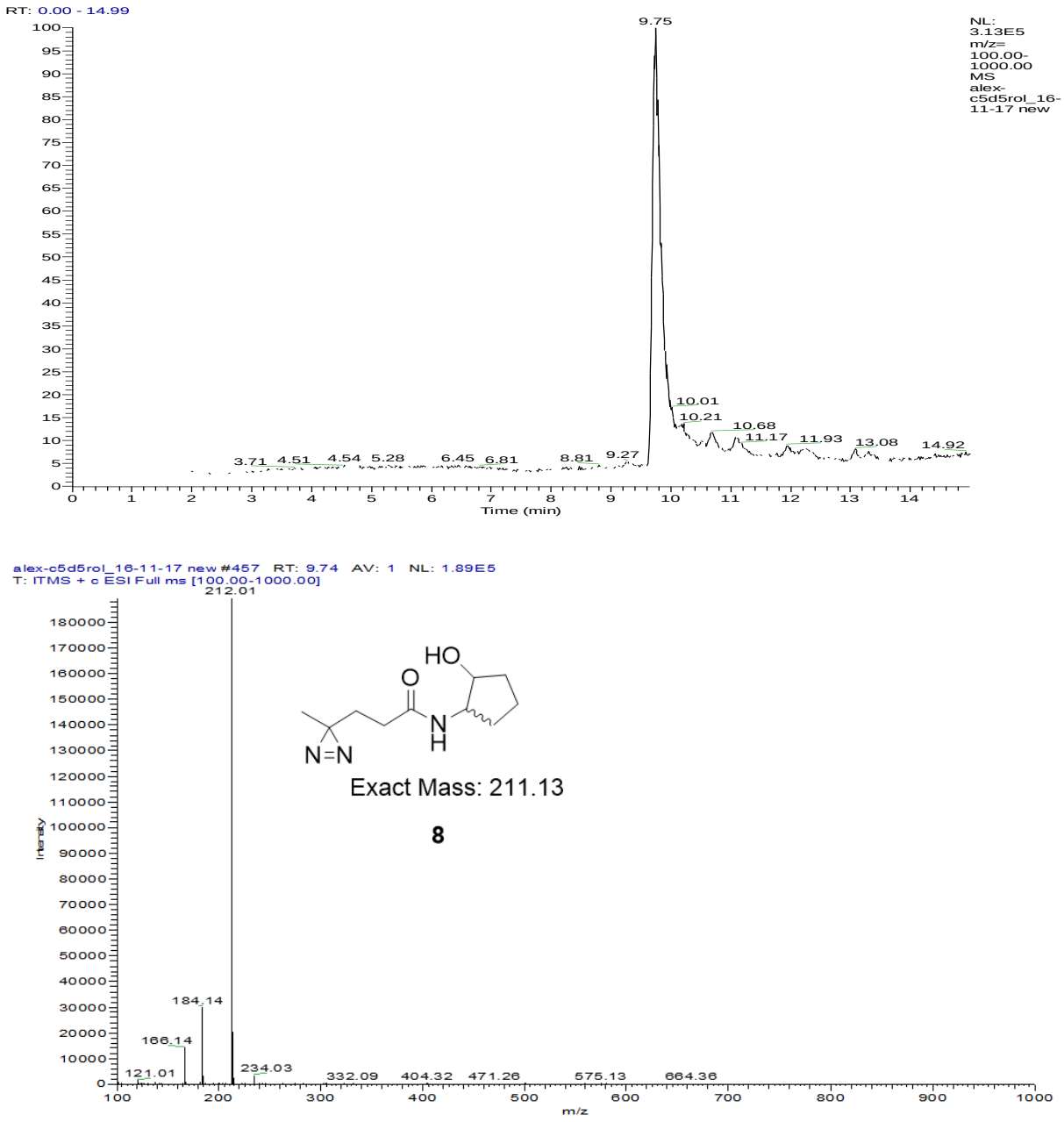


Figure S16. LC-MS of **8**.

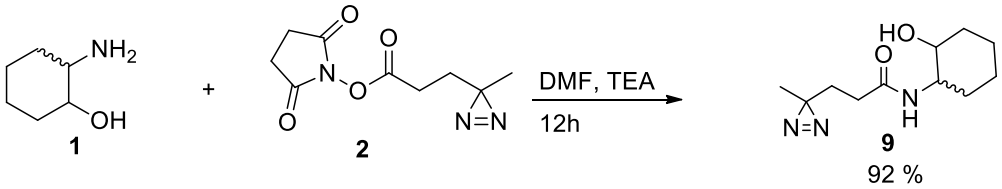
Synthesis of 9

To a 20 mL vial was added 2-aminocyclohexanol (**1**), (73.6 mg, 0.64 mmol) and dissolved in 0.88 mL of DMF. To another 20 mL vial flask with a stirring magnet was added 2, 5-dioxopyrrolidin-1-yl 3-(3-methyl-3*H*-diazirin-3-yl) propanoate (**2**), (120 mg, 0.53 mmol). The vials were combined and TEA, (0.07 mL, 0.53 mmol) was added and left stirred overnight. The solvent was removed by rotary evaporation and the crude was purified by column chromatography (EtOAc:Hexane, 60/40 v/v) to yield *N*-(2-hydroxycyclohexyl)-3-(3-methyl-3*H*-diazirin-3-yl) propanamide (**9**) as a white solid (111 mg, 92%), (**Scheme S9**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.45 (br s, 1H), 3.59-3.67 (m, 1H), 3.28-3.34 (m, 2H), 2.04-2.07 (m, 1H), 2.02 (t, *J* = 7.6 Hz, 2H), 1.93-1.97 (m, 1H), 1.76-1.86 (m, 2H), 1.68-1.75 (m, 2H), 1.13-1.38 (m, 4H), 1.04 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 173.22, 75.56, 55.99, 34.55, 31.62, 30.91, 30.06, 25.67, 24.70, 24.12, 20.11.

LC-MS *m/z* calculated for $C_{11}H_{19}N_3O_2$ (M+H): 226.15. Found: 226.03.



Scheme S9. Synthesis of **9**.

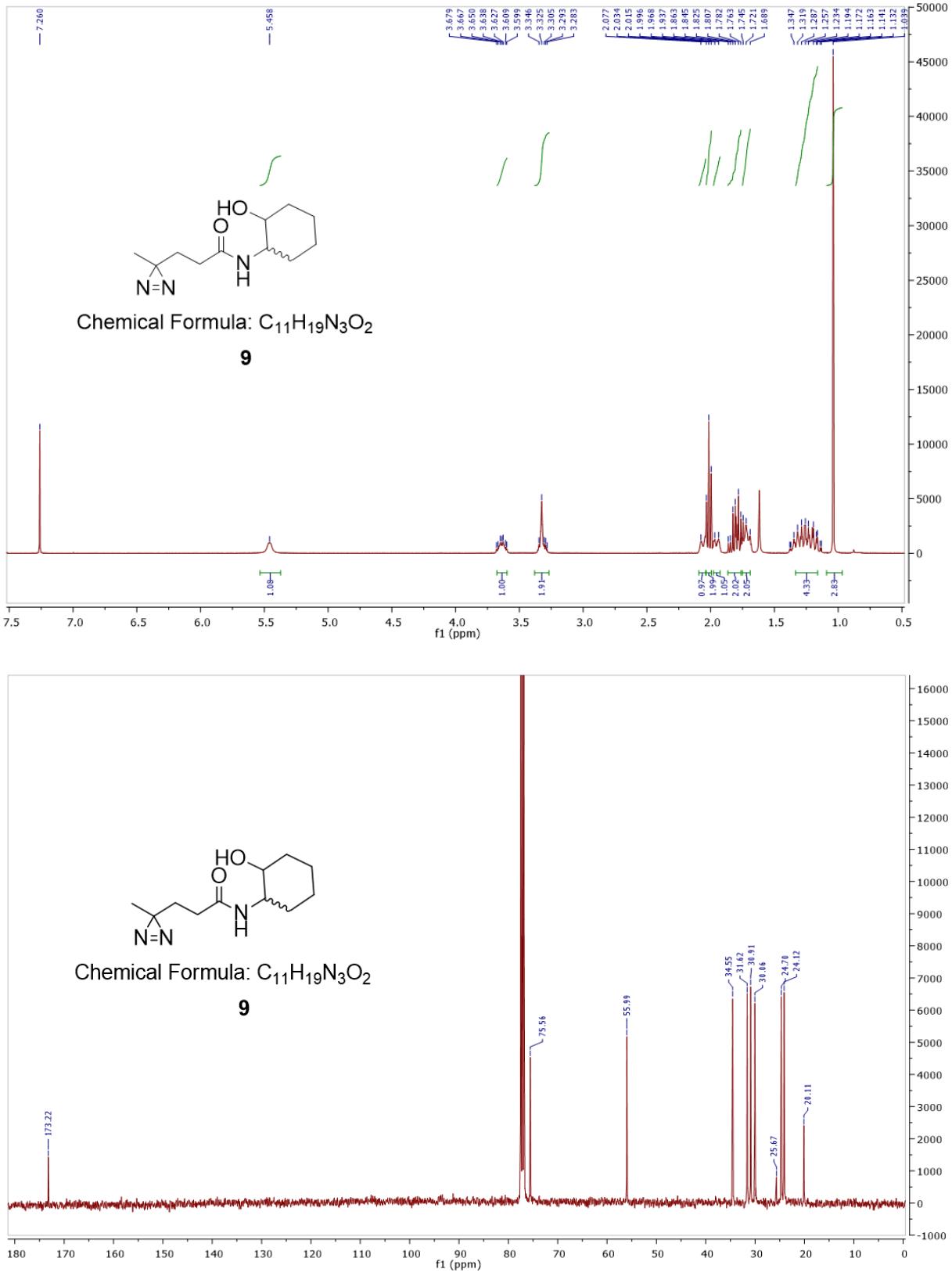


Figure S17. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **9**.

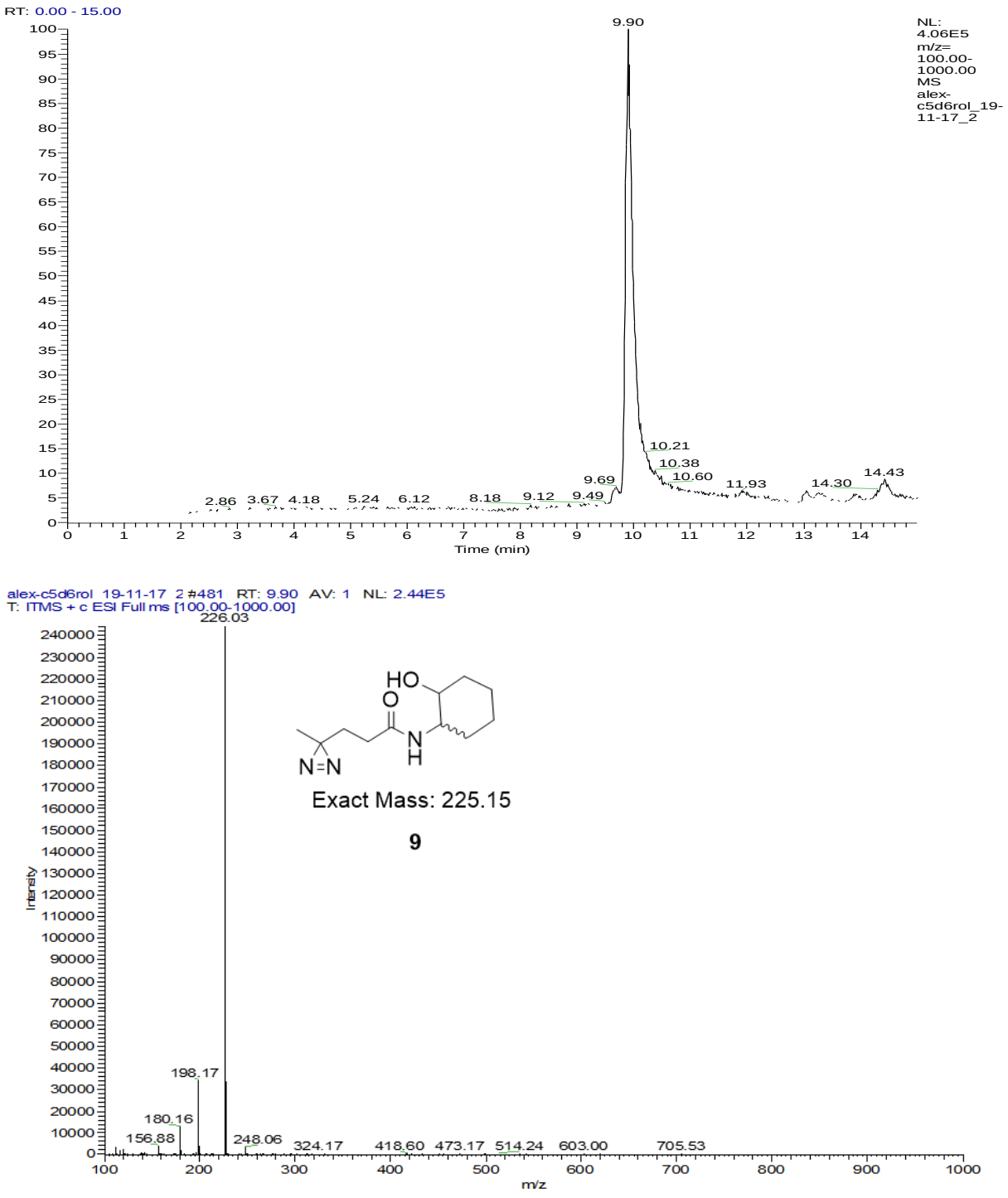


Figure S18. LC-MS of **9**.

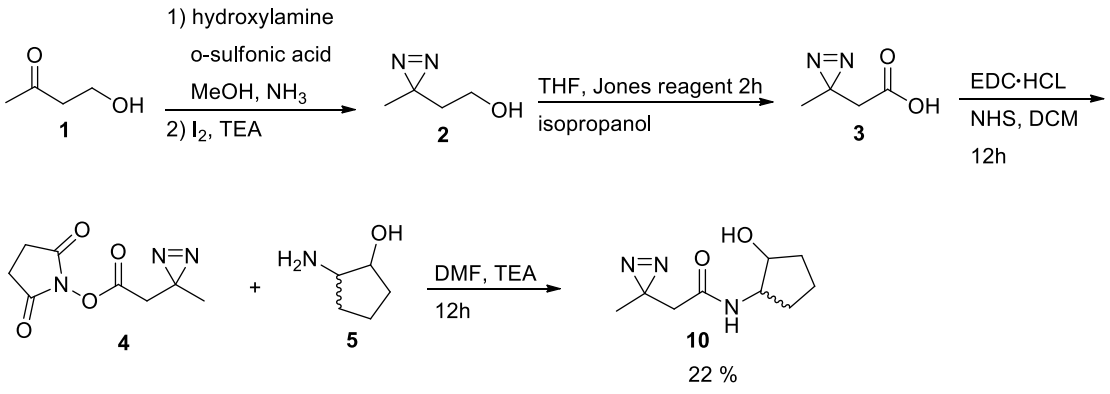
Synthesis of 10

To a 500 mL round bottom flask with a stirring magnet, septum and N_2 atmosphere was added 4-hydroxy-2-butanone (**1**), (3 gr, 34 mmol). A balloon with a syringe was added through a rubber septum to maintain the ammonia environment and allowing the release of pressure. To the flask was added 8 N methanolic ammonia (33.8 mL) and the mixture was allowed to be stirred for 3 h at 0°C. Hydroxylamine-O-sulfonic acid 97%, (4.52 gr, 40 mmol) was dissolved in 28.9 mL of MeOH and added to the reaction mixture dropwise. The mixture was allowed to be stirred overnight. The ammonia was removed by blowing air into the flask. The solvents were concentrated by rotary evaporation to give yellowish liquid. The reaction flask was covered with aluminum foil to minimize exposure to light and 50 mL of MeOH was added to re-dissolve the diaziridine acid. The reaction flask was placed in an ice-water bath and the mixture was allowed to be stirred for 5 min with a rubber septum cap. TEA, (7.2 mL, 34 mmol) was added drop-wise and the mixture was allowed to be stirred for 5 min. Using a spatula, 6.4 gr' of iodine chips were added slowly to the stirring reaction mixture until the solution stayed persistently red-brown more than 5 min. The reaction mixture was mixed with 100 mL of EtOAc and transferred to a separatory funnel, washed with 100 mL of 1 M aqueous HCl, 100 mL of 10% $Na_2S_2O_3$, 50 mL of saturated aqueous NaCl and dried with anhydrous $MgSO_4$. The reaction was filtered and evaporated by rotary evaporation to yield 2-(3-methyl-3*H*-diazirin-3-yl) ethanol (**2**), as a yellowish-white liquid. To the reaction mixture of 2-(3-methyl-3*H*-diazirin-3-yl) ethanol (**2**) was added 6.2 mL of THF and drop-wise 22.6 mL of Jones reagent (CrO_3/H_2SO_4) and the mixture was allowed to be stirred for 2 h. The reaction was quenched with 100 mL of isopropanol. Water and EtOAc 1:1 were added to extract the product. The aqueous layer was extracted with EtOAc several times until the acid spot vanishes from TLC. The layers were separated and the organic layer was dried over $MgSO_4$. The solvent was removed under reduced pressure, yielding 2-(3-methyl-3*H*-diazirin-3-yl) acetic acid (**3**), as a yellowish oil. To the reaction mixture of 2-(3-methyl-3*H*-diazirin-3-yl) acetic acid (**3**) was added 19.1 mL of DCM. To another 50 mL flask were added EDC·HCL, (6.51 gr', 34 mmol), NHS, (3.91 gr', 34 mmol) and dissolved in 23.8 mL of DCM. The two solutions were combined and left stirred overnight. The solvent was removed by rotary evaporation and the product was purified by column chromatography (EtOAc:Hexane, 75/25 v/v) to yield 2, 5-dioxopyrrolidin-1-yl 2-(3-methyl-3*H*-diazirin-3-yl) acetate (**4**), (2.033 gr') as a white-yellowish solid. To a 20 mL vial was added 2-aminocyclopentanol (**5**), (344 mg, 3.4 mmol) and dissolved in 5 mL of DMF. To another 20 mL flask with a stirring magnet was added 2, 5-dioxopyrrolidin-1-yl 2-(3-methyl-3*H*-diazirin-3-yl) acetate (**4**), (600 mg, 2.84 mmol). The vial was added to the 50 mL flask and TEA (0.07 mL, 0.53 mmol) was added and left stirred overnight. The solvent was removed by rotary evaporation and diluted with EtOAc. DMF was removed by aqueous $CuSO_4$ and the product was extracted with EtOAc. The solvent was removed by rotary evaporation and the crude was purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield *N*-(2-hydroxycyclopentyl)-2-(3-methyl-3*H*-diazirin-3-yl) acetamide (**10**) as a white solid. (122 mg, 22%), (**Scheme S10**).

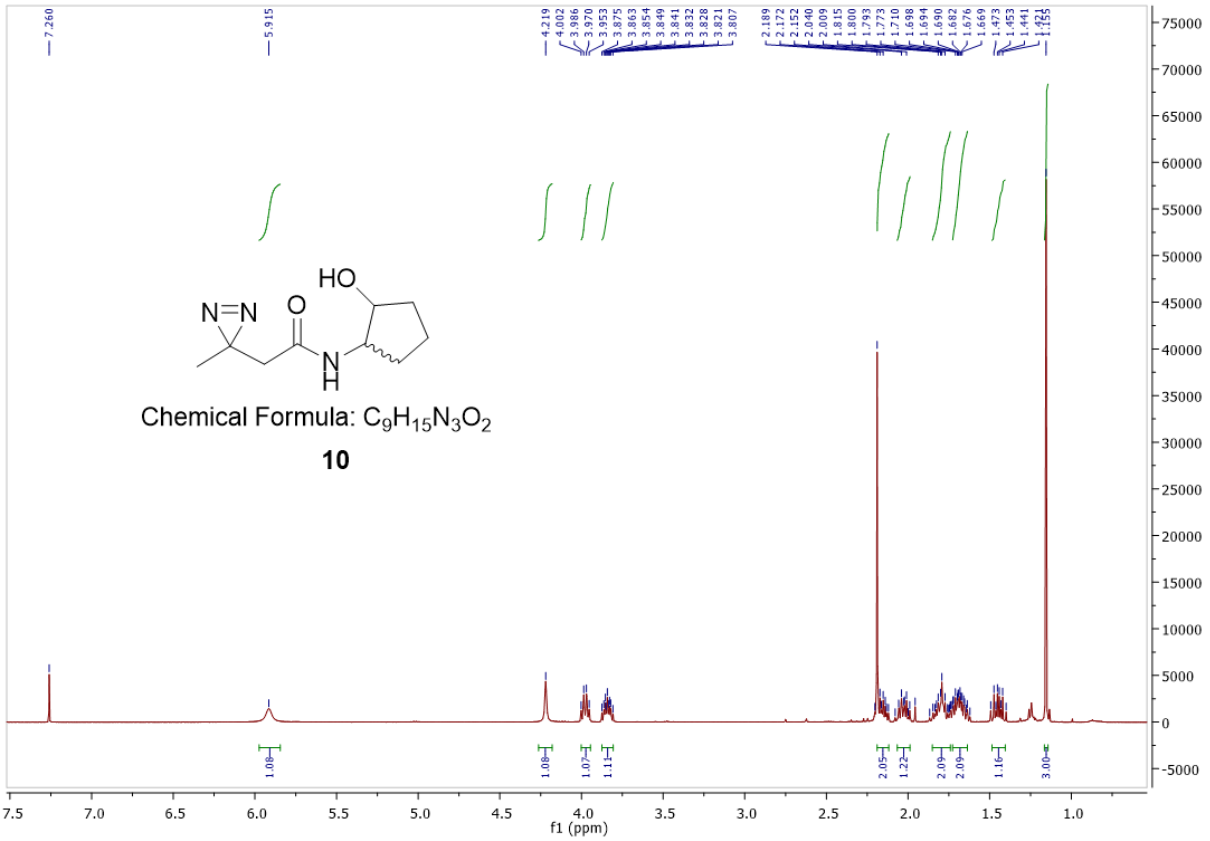
^{1}H NMR (400 MHz, $CDCl_3$): δ 5.91 (br s, 1H), 4.22 (br s, 1H), 3.95-4.00 (dd, *J* = 12.8, 6.4 Hz, 1H), 3.80-3.87 (m, 1H), 2.12 – 2.20 (m, 2H), 1.99 – 2.07 (m, 1H), 1.62 – 1.86 (m, 4H), 1.40-1.49 (ddd, *J* = 16.4, 12.8, 8.4 Hz, 1H), 1.15 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.11, 79.71, 61.18, 42.70, 32.76, 30.65, 23.85, 21.51, 19.95.

LC-MS *m/z* calculated for $C_9H_{15}N_3O_2$ (M+H): 198.12. Found: 198.00.



Scheme S10. Synthesis of **10**.



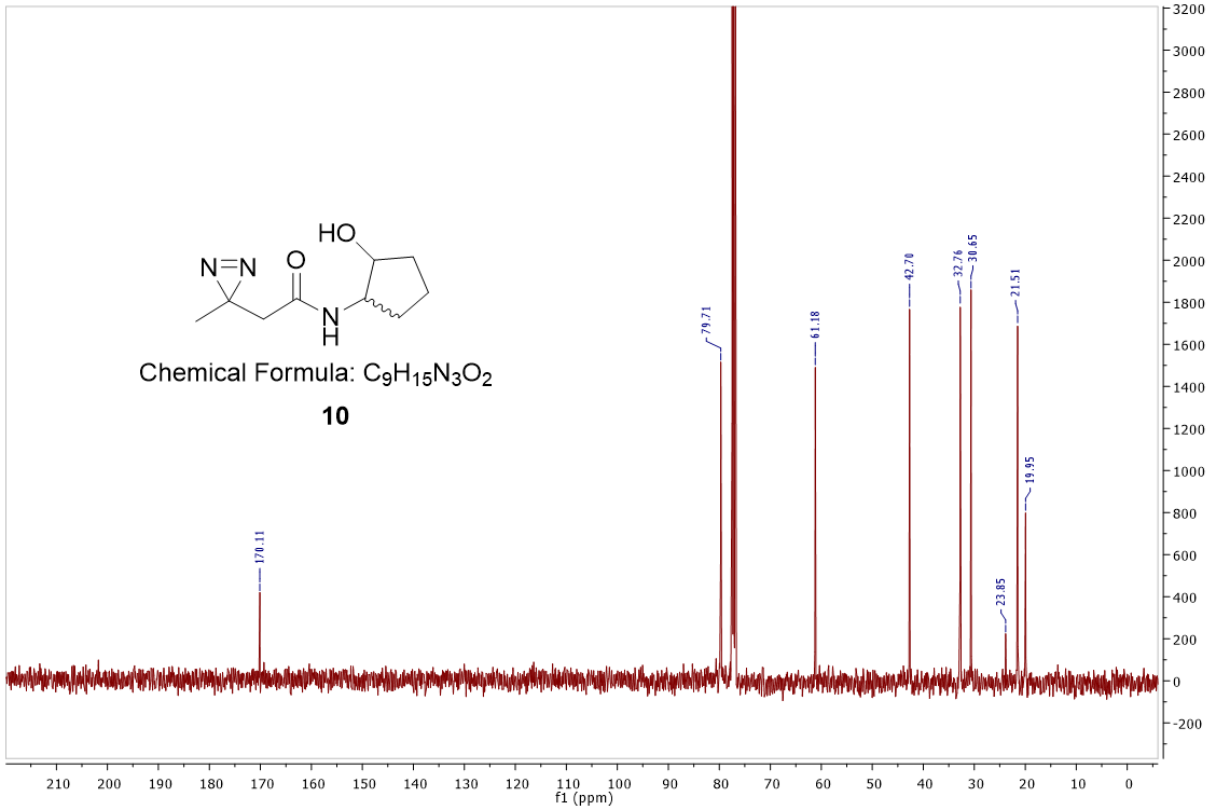


Figure S19. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **10**.

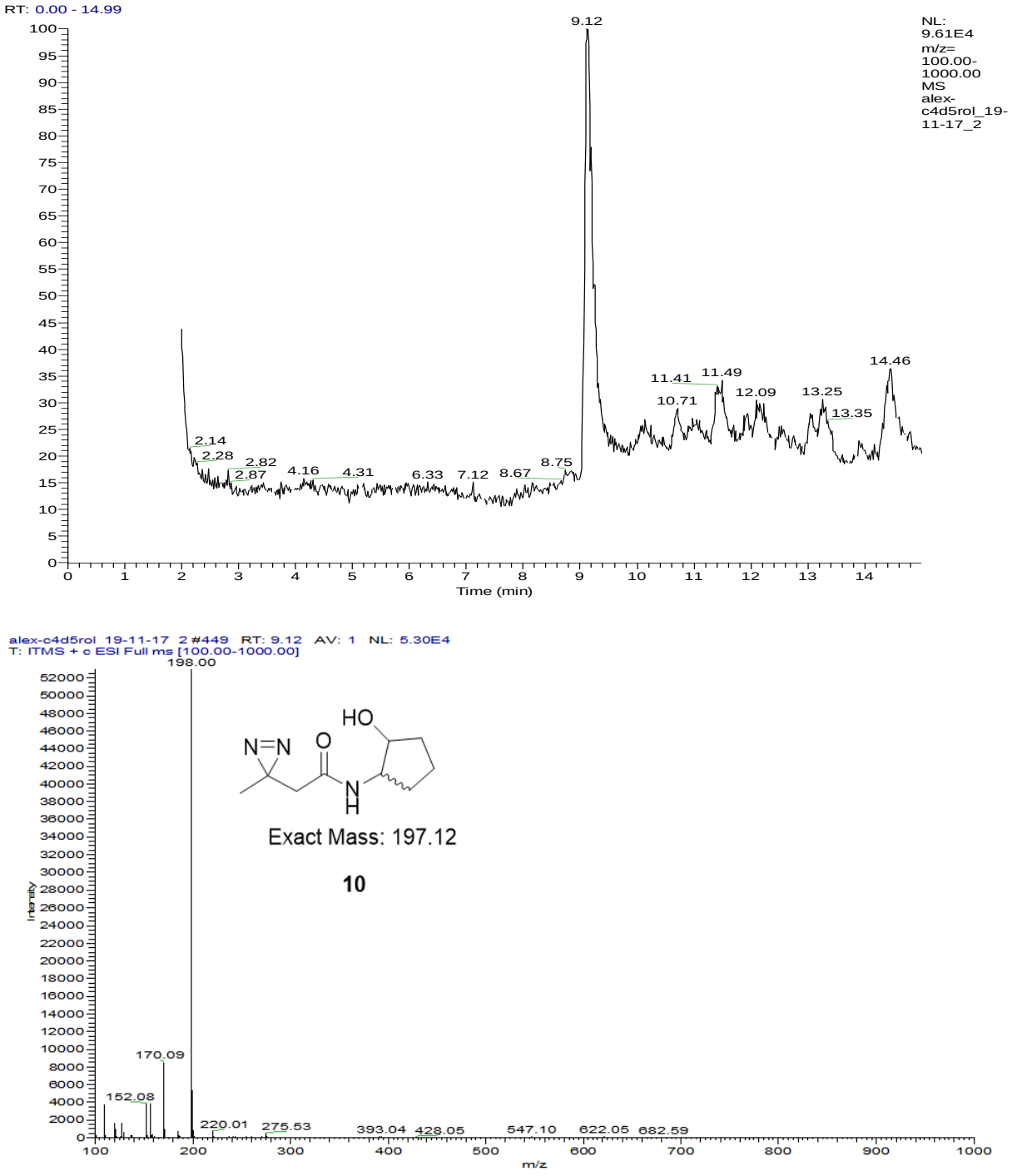


Figure S20. LC-MS of **10**.

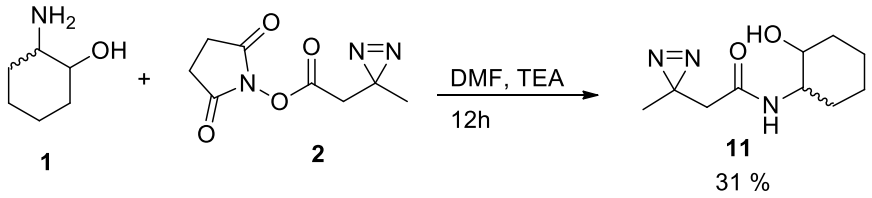
Synthesis of 11

To a 20 mL vial was added 2-aminocyclohexanol (**1**), (393 mg, 3.4 mmol) and dissolved in 5 mL of DMF. To a 50 mL flask with a stirring magnet was added 2, 5-dioxopyrrolidin-1-yl 2-(3-methyl-3*H*-diazirin-3-yl) acetate (**2**), (600 mg, 2.84 mmol). The vial was added to the 50 mL flask, and TEA, (0.47 mL, 3.4 mmol) was added and left stirred overnight. The solvent was removed by rotary evaporation and the crude was purified by column chromatography (EtOAc:DCM, 90/10 v/v) to yield *N*-(2-hydroxycyclohexyl)-2-(3-methyl-3*H*-diazirin-3-yl) acetamide (**11**) as a white solid (188.6 mg, 31%), (**Scheme S11**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 5.71 (br s, 1H), 3.62-3.71 (m, 1H), 3.31-3.37 (td, *J* = 10.0, 4.4 Hz, 1H), 2.15-2.25 (AB q, *J* = 23.6, 15.2 Hz, 2H), 1.97-2.08 (m, 2H), 1.70-1.76 (m, 2H), 1.20-1.38 (m, 5H), 1.16 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 169.67, 75.35, 56.09, 43.15, 34.70, 31.68, 24.67, 24.15, 23.95, 19.88.

LC-MS *m/z* calculated for $C_{10}H_{17}N_3O_2$ (M+H): 212.13. Found: 212.01.



1 **2** **11**

31 %

Scheme S11. Synthesis of **11**.

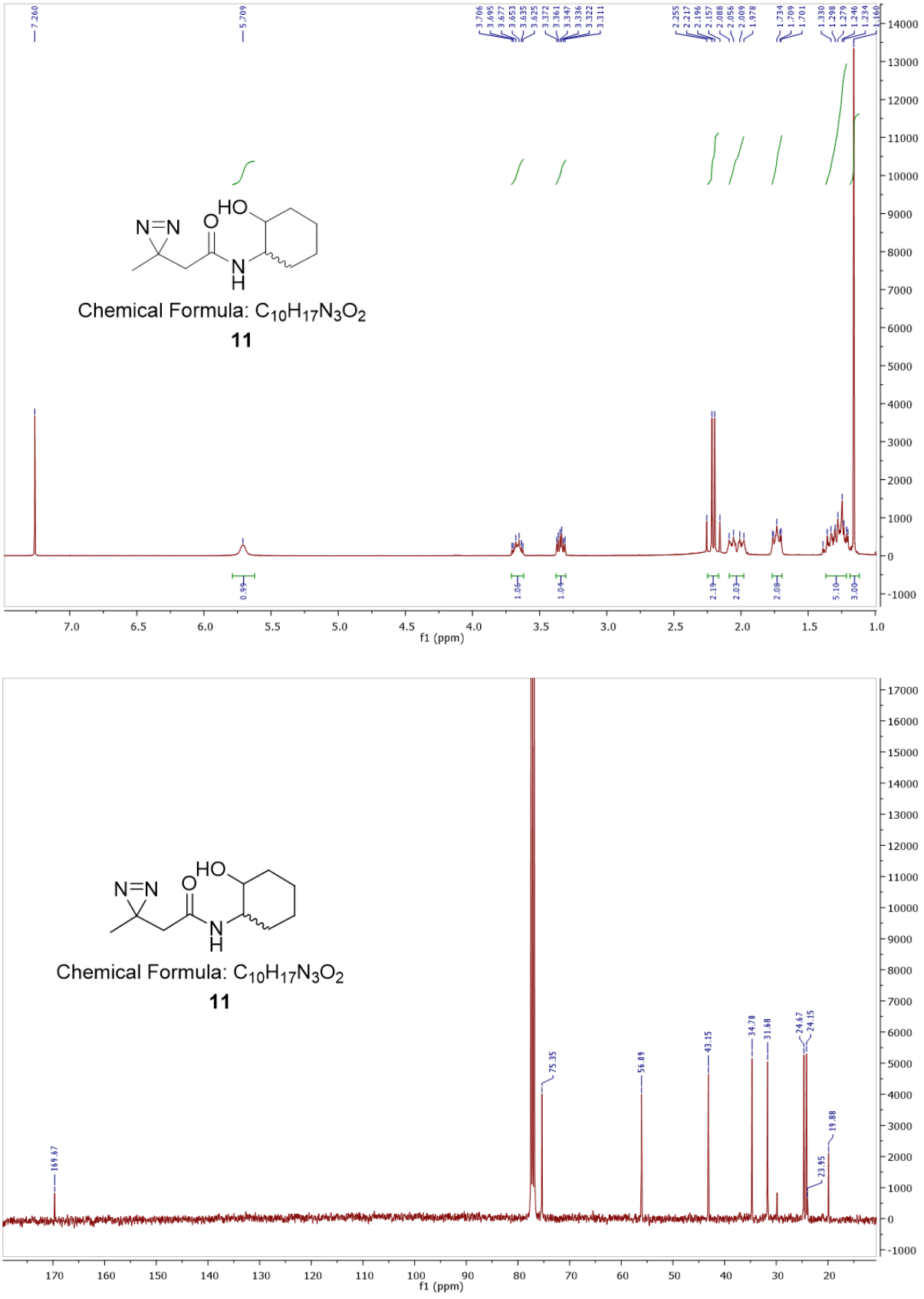


Figure S21. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **11**.

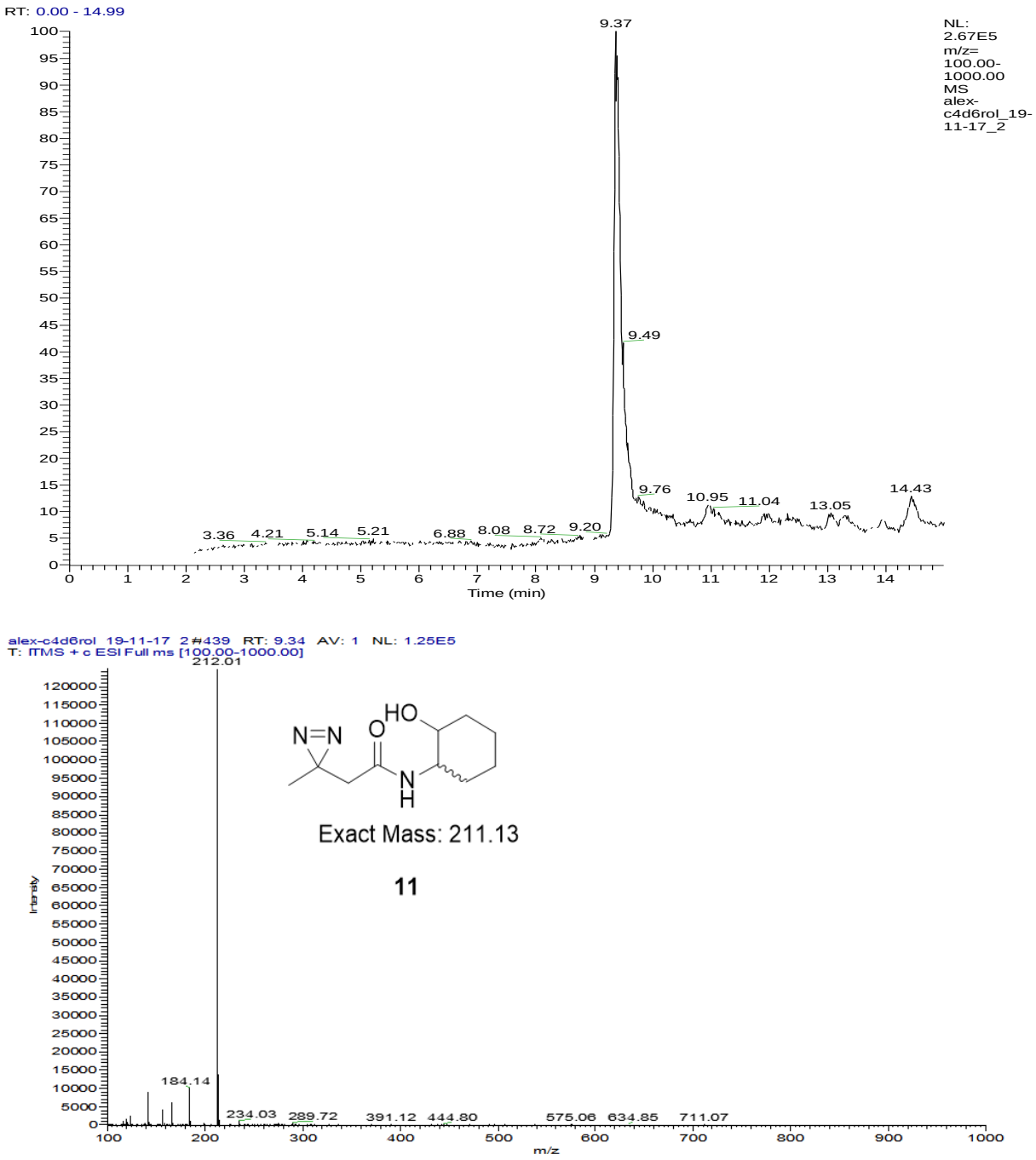


Figure S22. LC-MS of **11**.

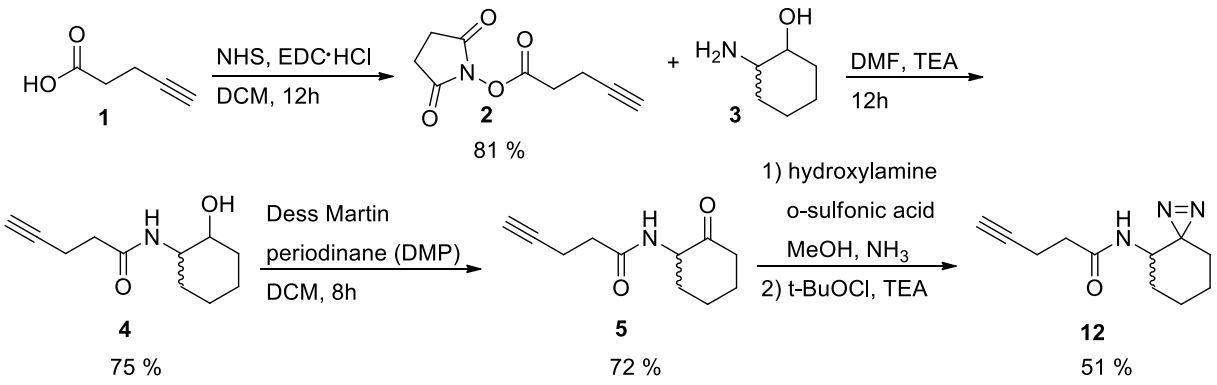
Synthesis of 12

To a vial, 20 mL with a stirring magnet was added 4-pentynoic acid (**1**), (153.55 mg, 1.56 mmol) and dissolved in 1 mL of DCM. To another 20 mL vial were added EDC·HCl, (198 mg, 1.72 mmol), NHS, (330 mg, 1.72 mmol) and dissolved in 1.5 mL of DCM. The two solutions were combined and left stirred overnight. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate (**2**), (248.2 mg, 81%) as white solid. To a 20 mL vial was added 2-aminocyclohexanol (**3**), (175 mg, 1.52 mmol) and dissolved in 1.1 mL of DMF. To another 20 mL vial flask with a stirring magnet was added 2, 5-dioxopyrrolidin-1-yl pent-4-ynoate (**2**), (248.8 mg, 1.27 mmol) and dissolved in 1.1 mL of DMF. The vials were combined and TEA, (0.35 mL, 2.54 mmol) was added and left stirred overnight. The solvent was removed by rotary evaporation and diluted with EtOAc. DMF was removed by aqueous $CuSO_4$ and the product was extracted with EtOAc. The solvent was removed by rotary evaporation and the crude was purified by column chromatography with EtOAc to yield *N*-(2-hydroxycyclohexyl) pent-4-ynamide (**4**), (224 mg, 75%) as a white solid. To a 20 mL vial was added *N*-(2-hydroxycyclohexyl) pent-4-ynamide (**4**), (224 mg, 1.14 mmol) and dissolved in 1.63 mL of DCM. Dess-martin periodinane, (725 mg, 1.71 mmol) was added to the vial and the mixture was stirred with a magnet and left overnight. To a 100 mL flask was added 25 mL water and the reaction mixture, 50 mL of 10% $NaHCO_3$, and an equal amount of DCM. The two phases were separated and the organic phase was dried over $MgSO_4$. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield *N*-(2-oxocyclohexyl) pent-4-ynamide (**5**) as a white solid (158.7 mg, 72%). To a 4 mL vial with a stirring magnet, septum and N_2 atmosphere was added *N*-(2-oxocyclohexyl) pent-4-ynamide (**5**), (49.8 mg, 0.26 mmol). A balloon with a syringe was added through a rubber septum to maintain the ammonia environment and allowing the release of pressure. To the flask was added 0.7 mL of 8 N methanolic ammonia and the mixture was allowed to be stirred for 3 h at 0°C. Hydroxylamine-O-sulfonic acid 97%, (44.2 mg, 0.39 mmol) was dissolved in 0.22 mL of MeOH and added to the reaction mixture dropwise. The mixture was allowed to be stirred overnight. The ammonia was removed by blowing air into the flask. The solvents were concentrated by rotary evaporation to give yellowish liquid. The reaction flask was covered with aluminum foil to minimize exposure to light and 0.5 mL of MeOH was added to re-dissolve the diaziridine acid. The reaction flask was placed in an ice-water bath and the mixture was allowed to be stirred for 5 min with a rubber septum cap. TEA, (0.055 mL, 0.26 mmol) was added drop-wise and the mixture was allowed to be stirred for 5 min. Tert-butyl hypochlorite, (32 μL, 0.28 mmol) was added dropwise and the mixture was allowed to be stirred for 24 h. The reaction was evaporated by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 25/75 v/v) to yield *N*-(1,2-diazaspiro [2.5] oct-1-en-4-yl) pent-4-ynamide (**12**) as a yellowish-white solid (26.9 mg, 51%), (**Scheme S12**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 4.91 (br s, 1H), 4.02-4.08 (m, 1H), 2.42-2.46 (m, 2H), 2.23-2.26 (t, *J* = 7.2 Hz, 2H), 2.00-2.02 (br t, *J* = 2.4 Hz, 1H), 1.83-1.87 (m, 1H), 1.69-1.76 (m, 2H), 1.52-1.60 (m, 4H), 0.74-0.81 (m, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 170.20, 82.89, 69.70, 48.05, 35.25, 32.07, 30.74, 30.12, 24.21, 24.06, 14.92.

LC-MS *m/z* calculated for $C_{11}H_{15}N_3O$ (M+H): 206.12. Found: 205.93.



Scheme S12. Synthesis of **12**.

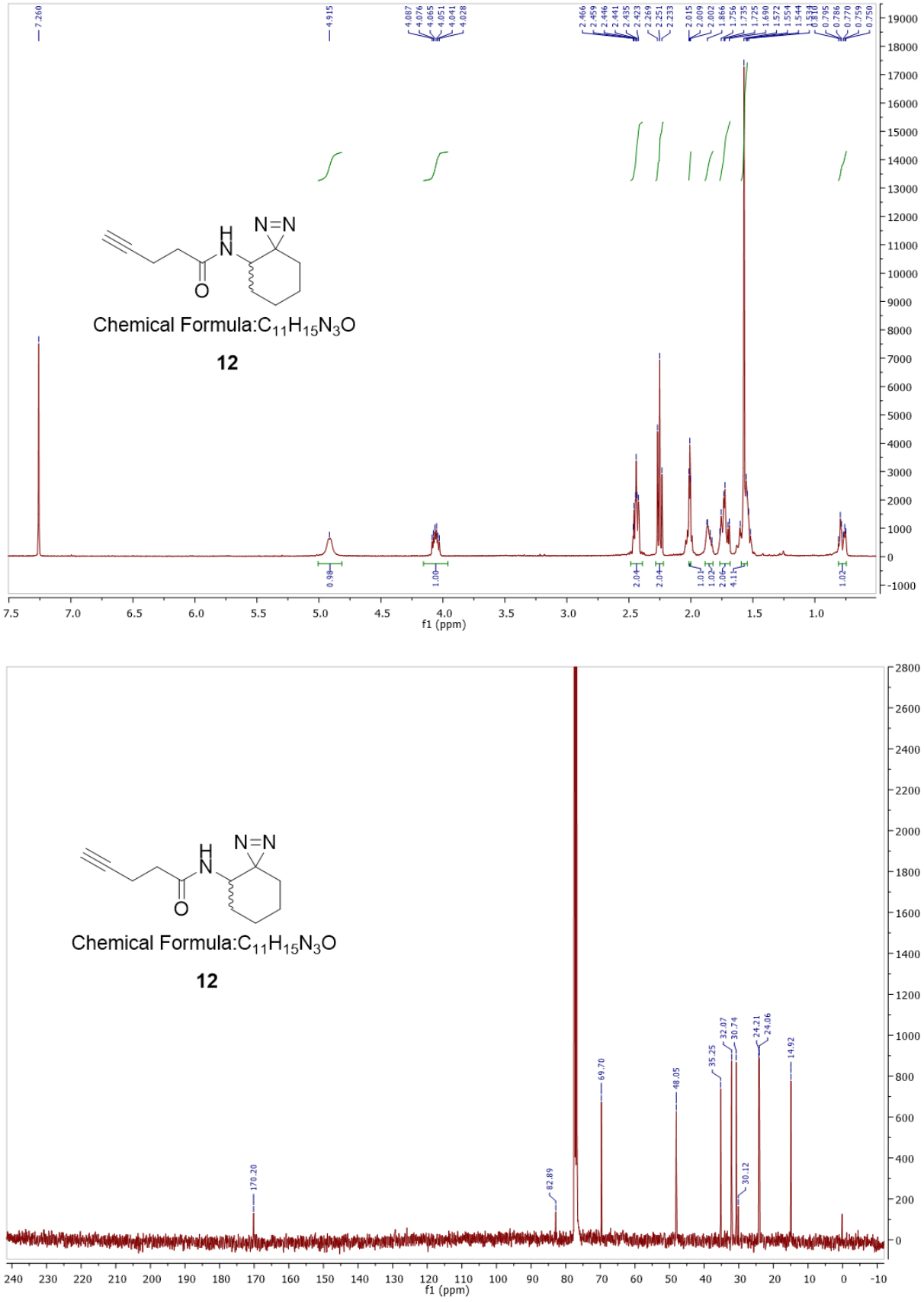
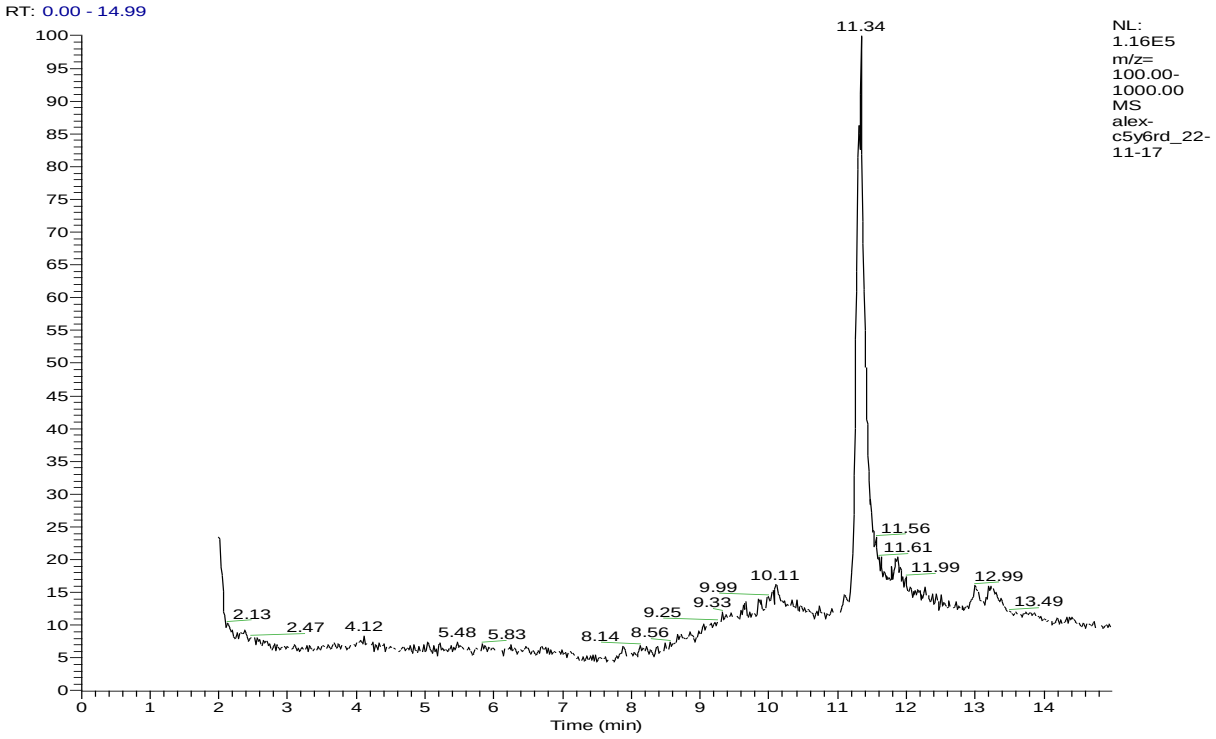


Figure S23. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **12**.



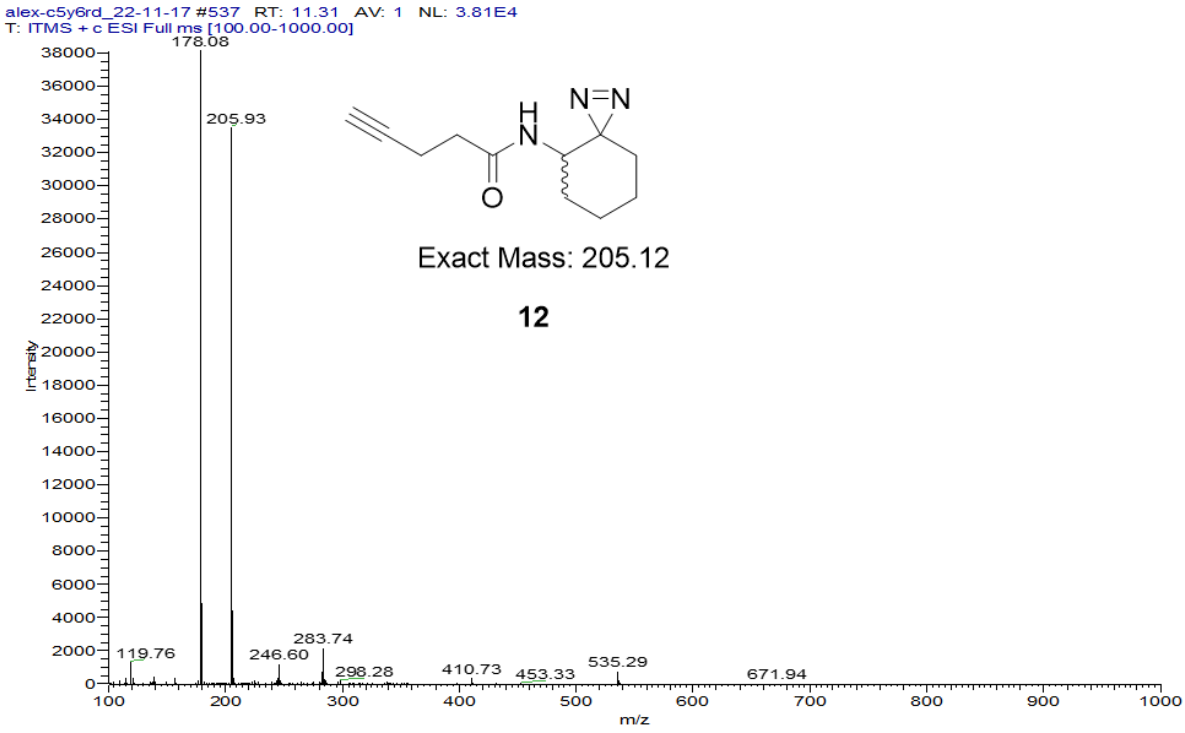


Figure S24. LC-MS of **12**.

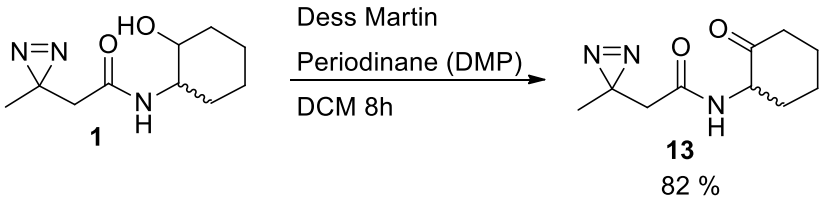
Synthesis of 13

To a 4 mL vial was added *N*-(2-hydroxycyclohexyl)-2-(3-methyl-3*H*-diazirin-3-yl) acetamide (**1**), (150 mg, 0.71 mmol) and dissolved in 2 mL of DCM. Dess-martin periodinane, (451 mg, 1.065 mmol) was added to the vial and the mixture was stirred with a magnet and left overnight. To a 100 mL flask was added 25 mL water and the reaction mixture, 50 mL of 10% $NaHCO_3$ and equal amount of DCM. The two phases were separated and the organic phase was dried over $MgSO_4$. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield 2-(3-methyl-3*H*-diazirin-3-yl)-*N*-(2-oxocyclohexyl) acetamide **(13)** as a white solid (122.4 mg, 82%), (**Scheme S13**).

^{1}H NMR (400 MHz, $CDCl_3$): δ 6.61 (br s, 1H), 4.45-4.51 (m, 1H), 2.65-2.71 (m, 1H), 2.52-2.57 (m, 1H), 2.40 (td, *J* = 13.6, 6.4 Hz, 1H), 2.16-2.24 (app AB q, *J* = 21.2, 15.6 Hz, 2H), 2.13-2.15 (m, 1H), 1.85-1.91 (m, 1H), 1.74-1.83 (m, 1H), 1.59-1.71 (m, 1H), 1.37 (qd, *J* = 12.4, 4.0 Hz, 1H), 1.15 (s, 1H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 207.40, 167.49, 58.22, 42.83, 41.07, 35.37, 28.00, 23.99, 23.60, 19.82.

LC-MS *m/z* calculated for $C_{10}H_{15}N_3O_2$ (M+H): 210.12. Found: 210.01.



Scheme S13. Synthesis of **13**.

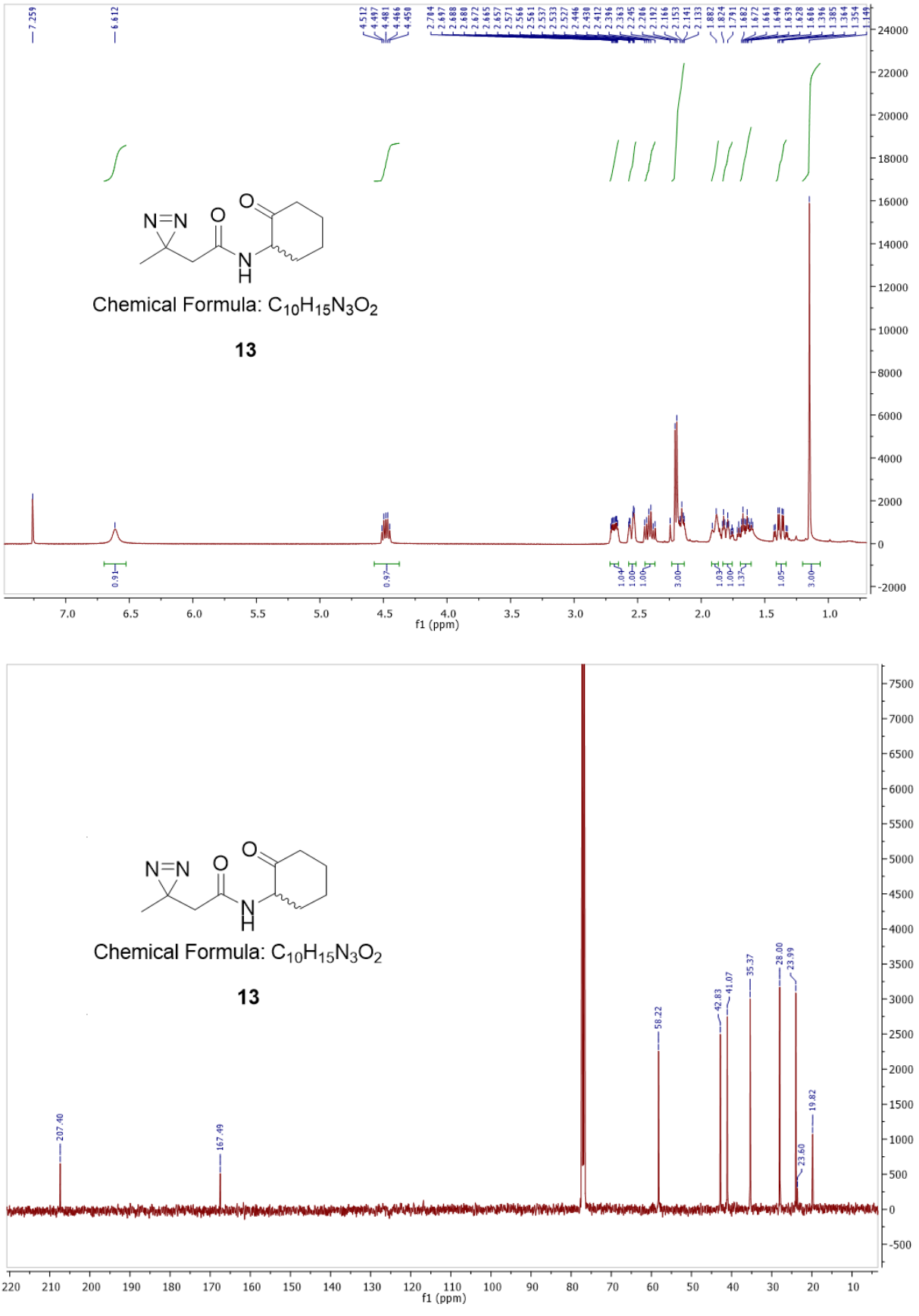
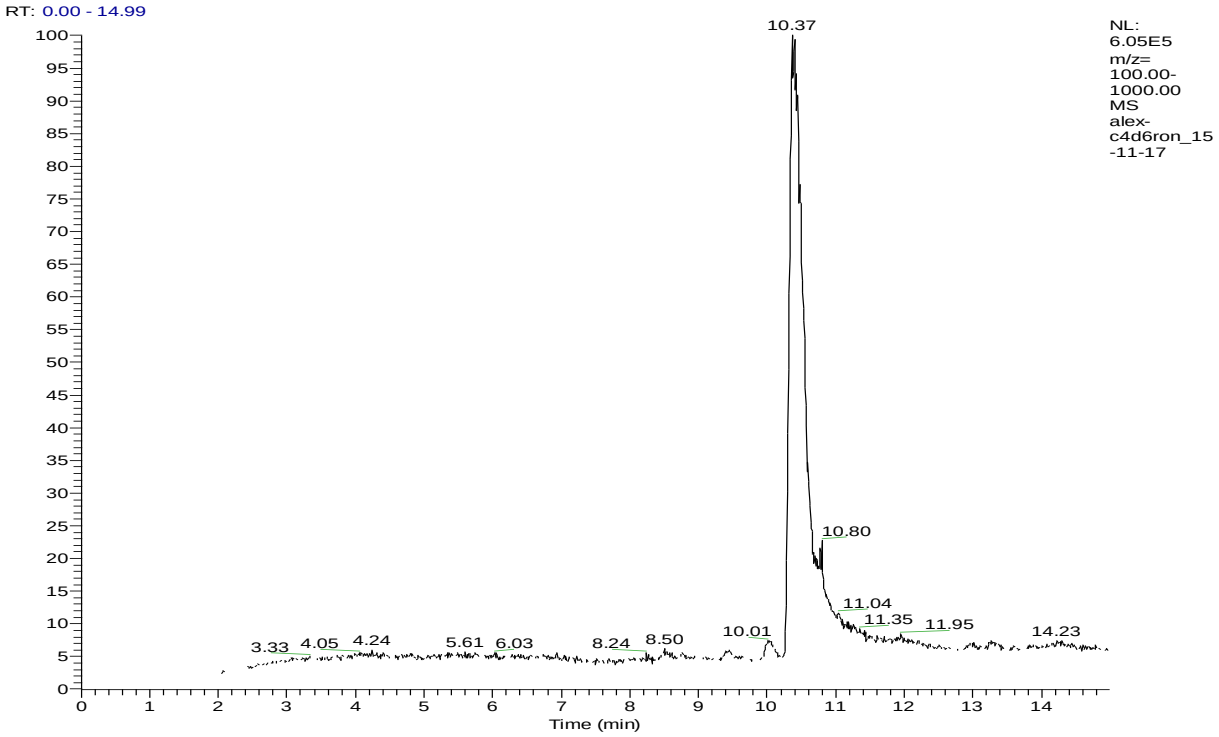
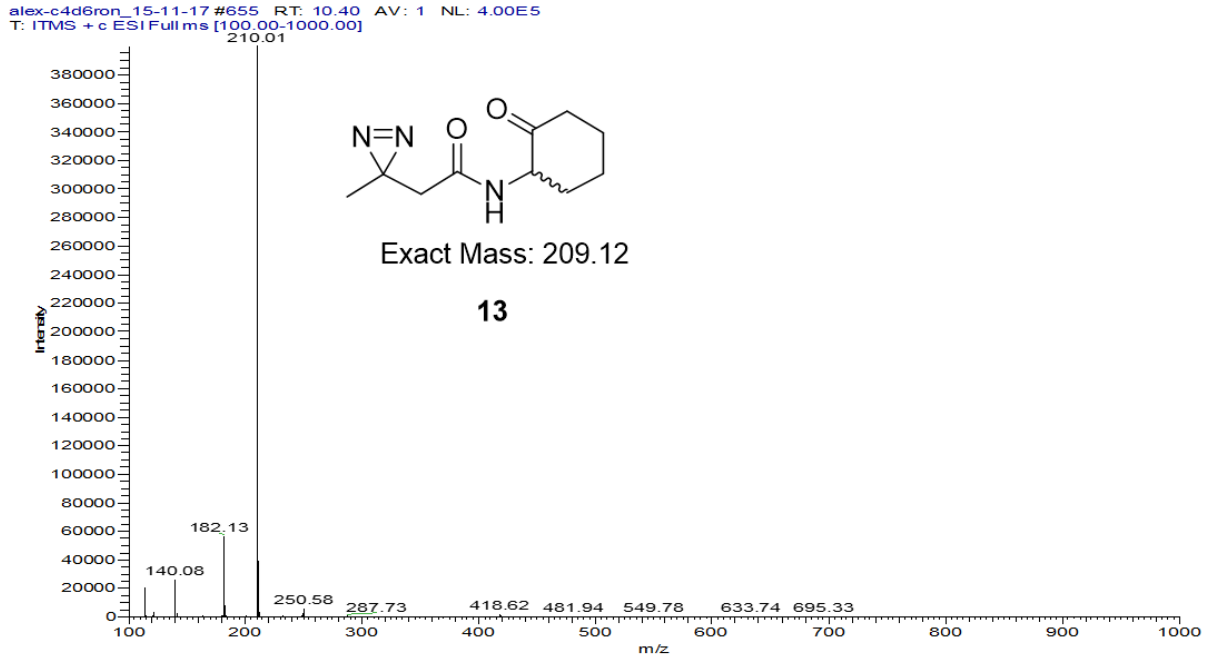


Figure S25. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **13**.





Exact Mass: 209.12

13

Figure S26. LC-MS of **13**.

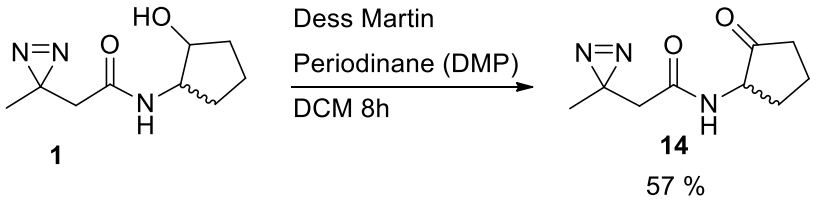
Synthesis of 14

To a 4 mL vial was added *N*-(2-hydroxycyclopentyl)-2-(3-methyl-3*H*-diazirin-3-yl) acetamide (**1**), (100 mg, 0.507 mmol) and dissolved in 1.6 mL of DCM. Dess-martin periodinane, (354 mg, 0.835 mmol) was added to the vial and the mixture was stirred with a magnet and left overnight. To a 100 mL flask was added 25 mL water and the reaction mixture, 50 mL of 10% $NaHCO_3$ and equal amount of DCM. The two phases were separated and the organic phase was dried over $MgSO_4$. The solvent was removed by rotary evaporation and purified by column chromatography (EtOAc:Hexane, 50/50 v/v) to yield 2-(3-methyl-3*H*-diazirin-3-yl)-*N*-(2-oxocyclopentyl) acetamide (**14**) as a white solid (56.5 mg, 57%), (**Scheme S14**).

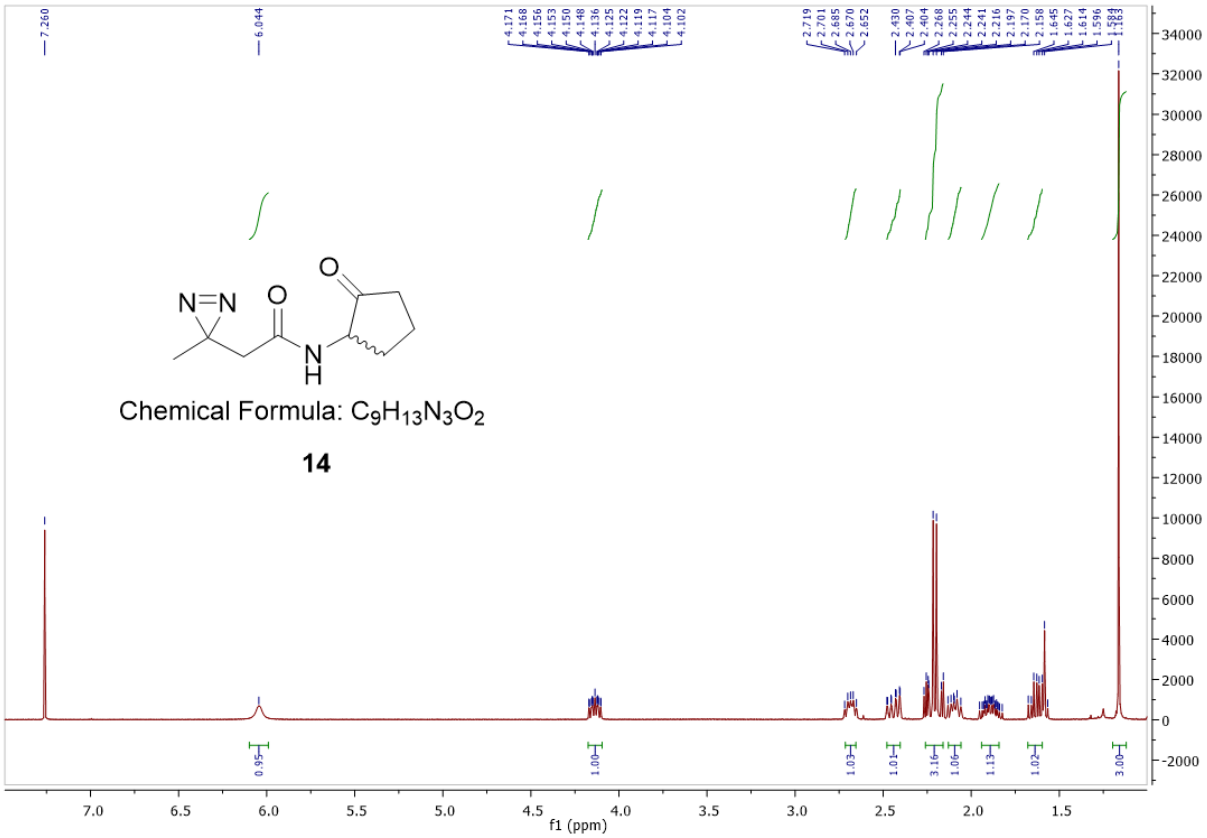
^{1}H NMR (400 MHz, $CDCl_3$): δ 6.04 (br s, 1H), 4.10-4.17 (m, 1H), 2.65-2.71 (m, 1H), 2.40-2.47 (m, 1H), 2.15-2.26 (app AB q, *J* = 17.6, 10 Hz, 3H), 2.05-2.13 (m, 1H), 1.82-1.95 (m, 1H), 1.56-1.67 (dq, *J* = 12.4, 6.8, Hz, 1H), 1.16 (s, 3H).

^{13}C NMR (101 MHz, $CDCl_3$): δ 214.96, 168.45, 58.29, 42.62, 35.00, 29.99, 23.81, 19.89, 18.20.

LC-MS *m/z* calculated for $C_9H_{13}N_3O_2$ (M+H): 196.10. Found: 196.01.



Scheme S14. Synthesis of **14**.



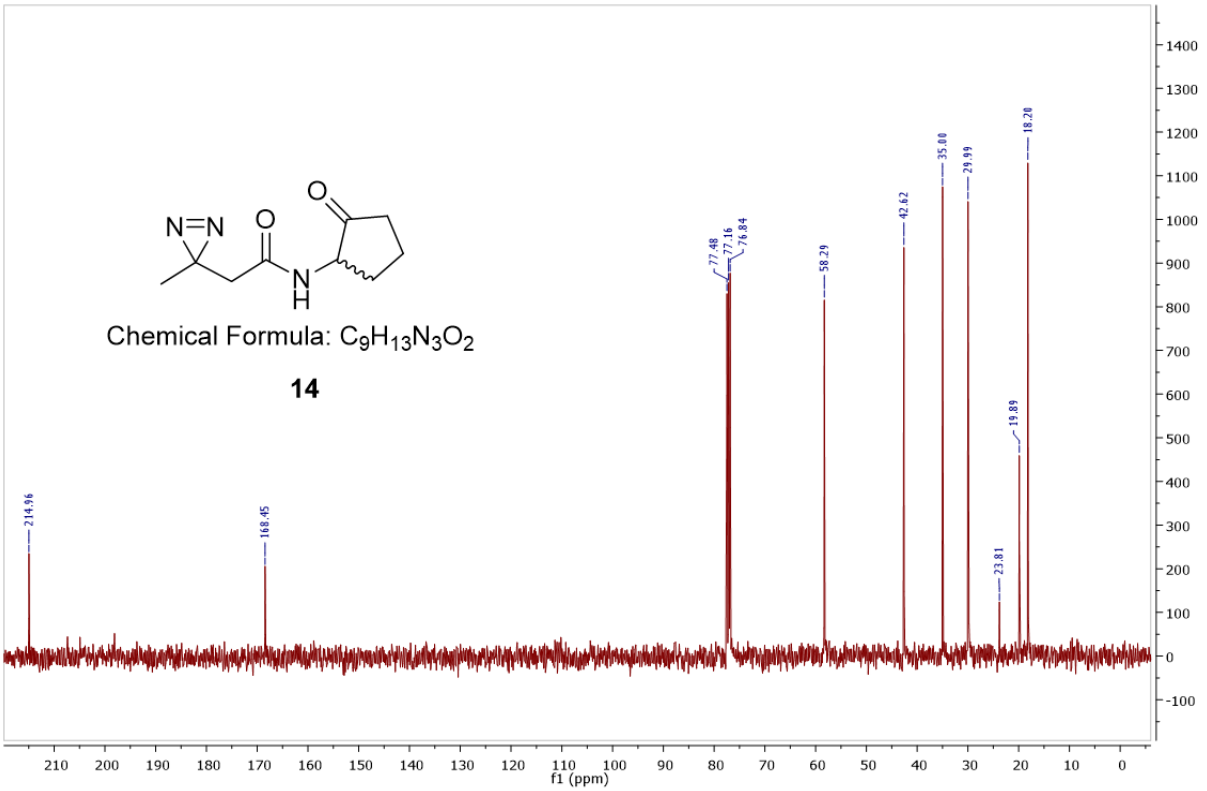


Figure S27. ^{1}H NMR (upper spectrum) and ^{13}C NMR (lower spectrum) of **14**.

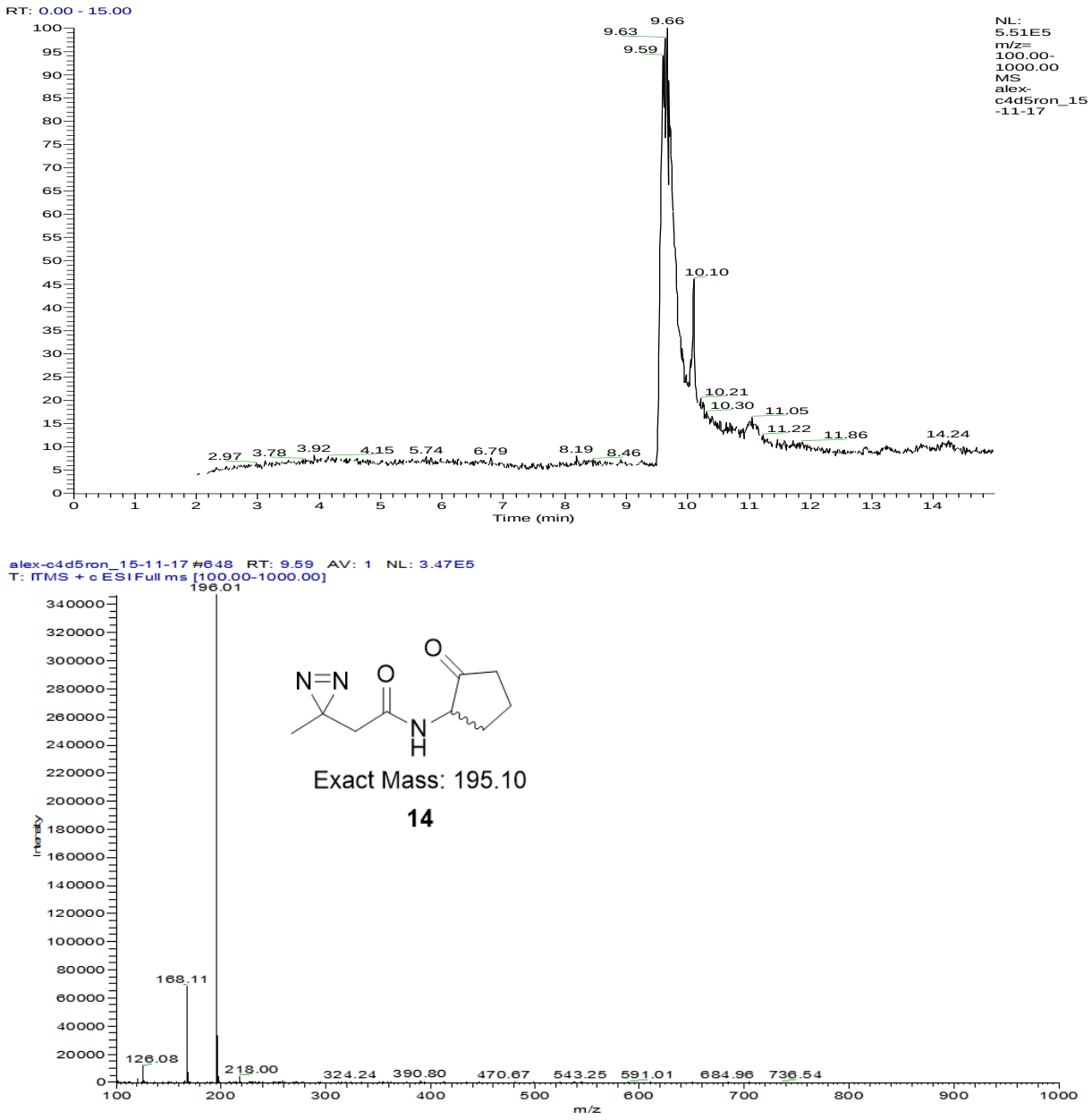
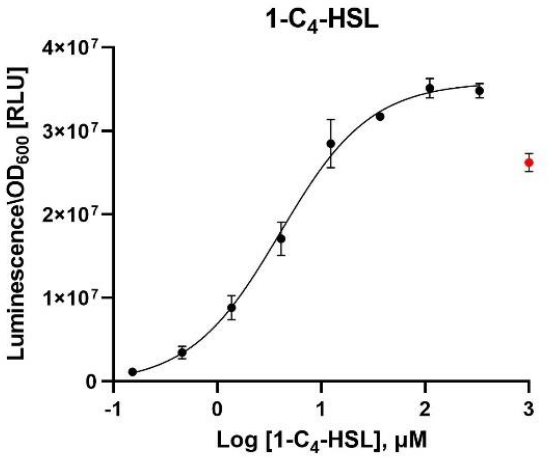
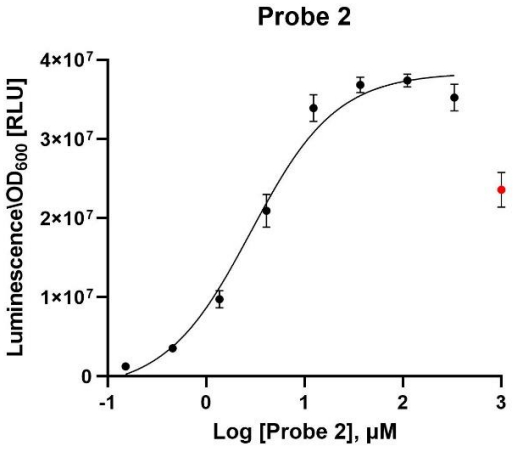


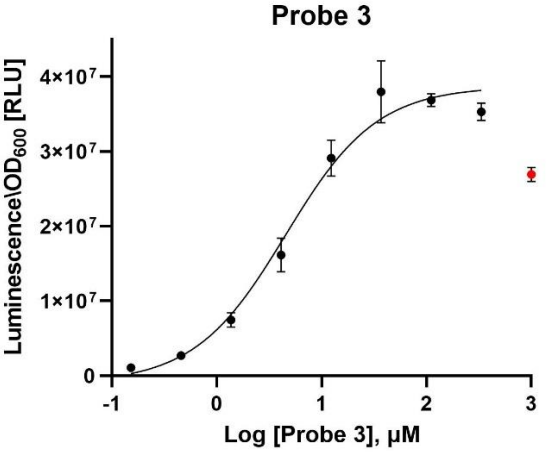
Figure S28. LC-MS of **14**.

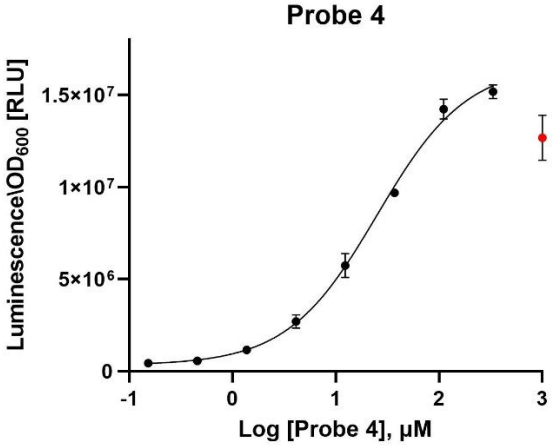
Biological evaluation

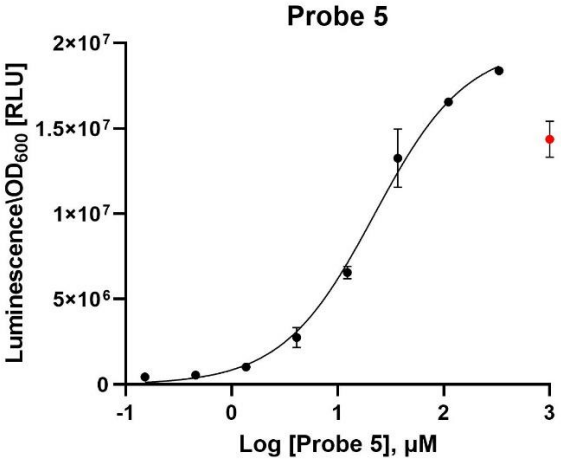
Activation assays – Luminescent Reporter Strain PAO-JP2 (pKD-*rhlA*)

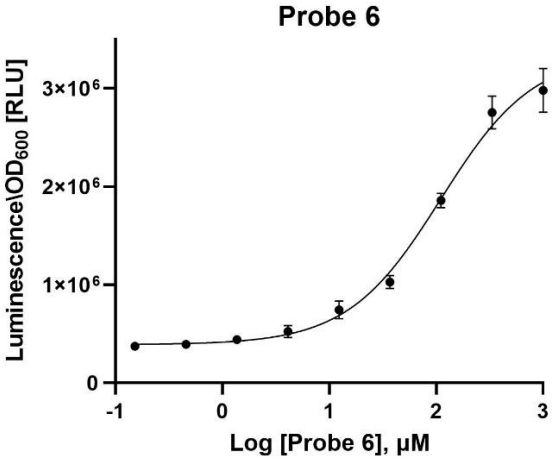


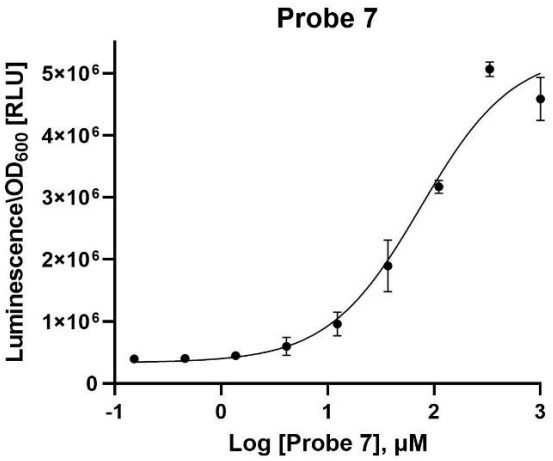


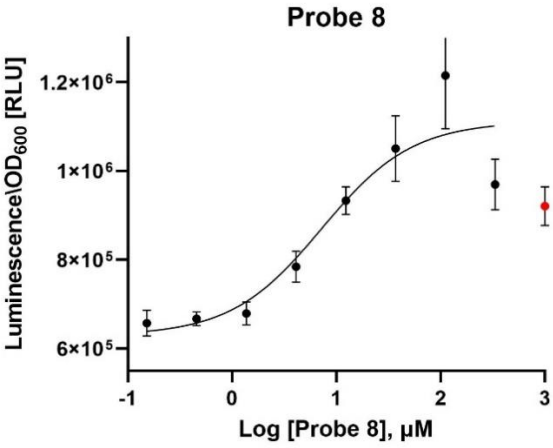


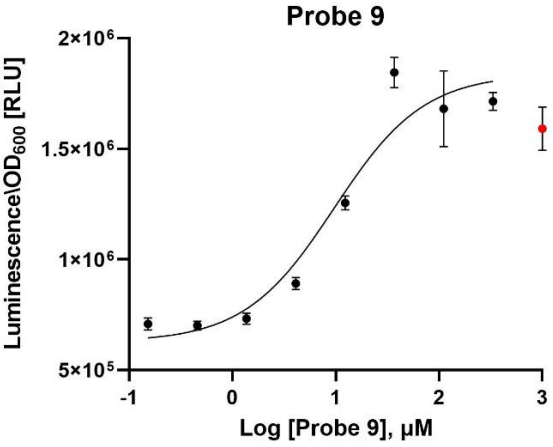


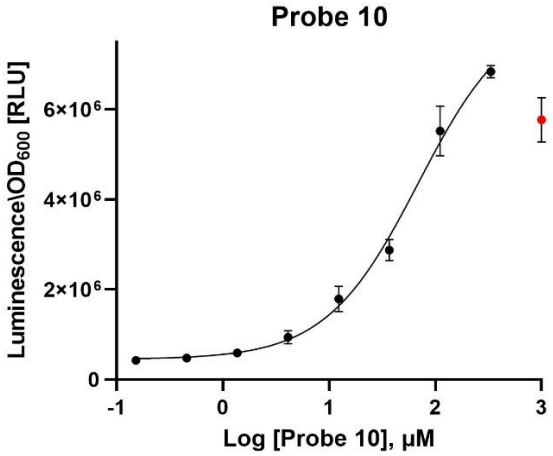


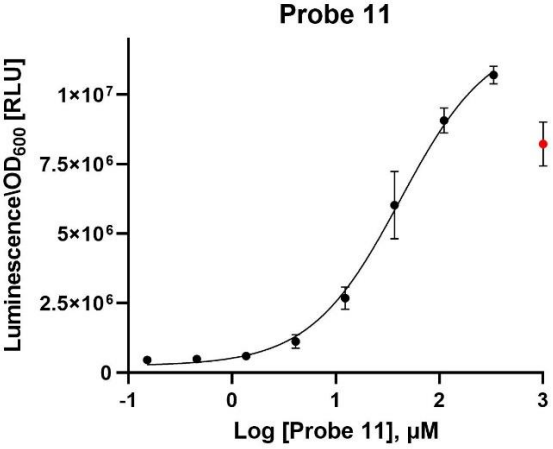


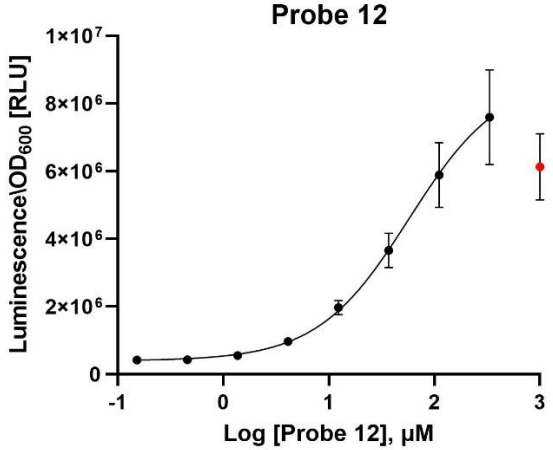

Probe 7
Luminescence\OD600 [RLU]
5×10^6
4×10^6
3×10^6
2×10^6
1×10^6
0
-1
0
1
2
3
Log [Probe 7], µM


Probe 8
Luminescence\OD600 [RLU]
1.2×10^6
1×10^6
8×10^5
6×10^5
-1
0
1
2
3
Log [Probe 8], µM


Probe 9
Luminescence\OD600 [RLU]
2×10^6
1.5×10^6
1×10^6
5×10^5
-1
0
1
2
3
Log [Probe 9], µM


Probe 10
Luminescence\OD600 [RLU]
6×10^6
4×10^6
2×10^6
0
-1
0
1
2
3
Log [Probe 10], µM


Probe 11
Luminescence\OD600 [RLU]
1×10^7
7.5×10^6
5×10^6
2.5×10^6
0
-1
0
1
2
3
Log [Probe 11], µM


Probe 12
Luminescence\OD600 [RLU]
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
-1
0
1
2
3
Log [Probe 12], µM

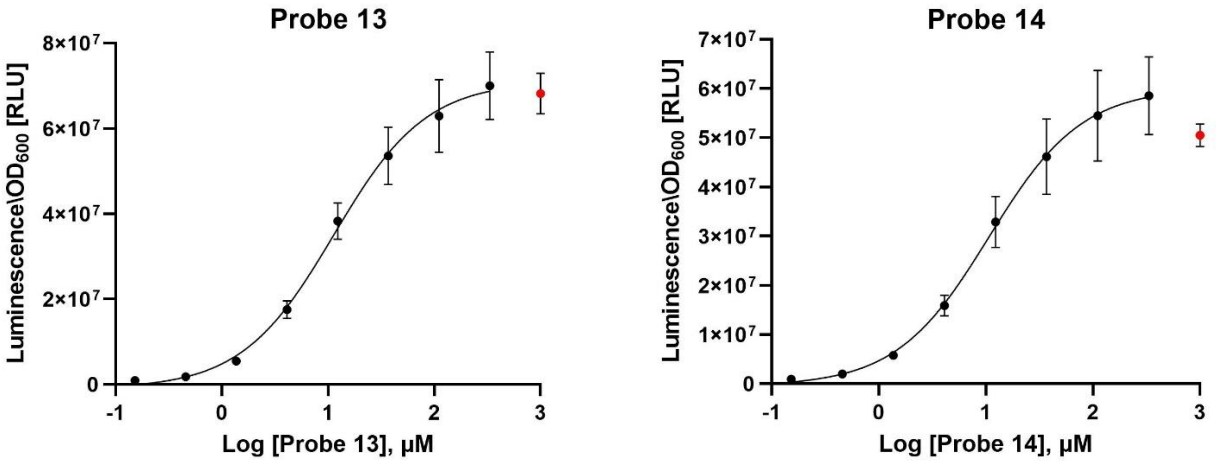
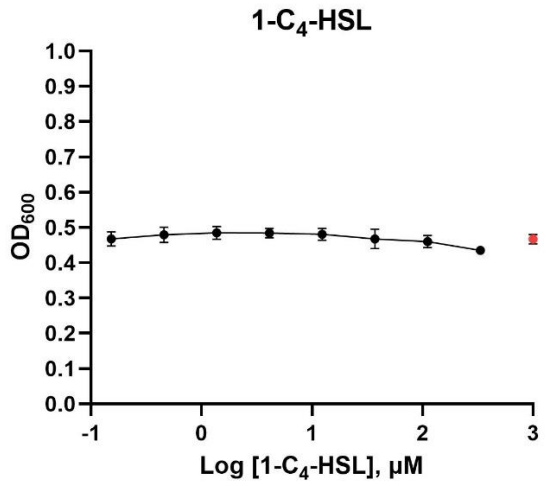
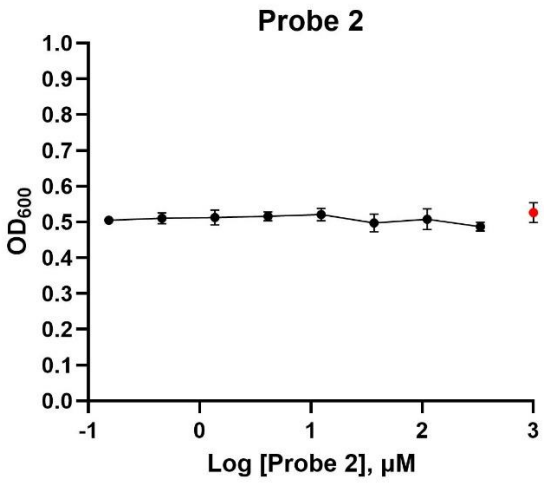
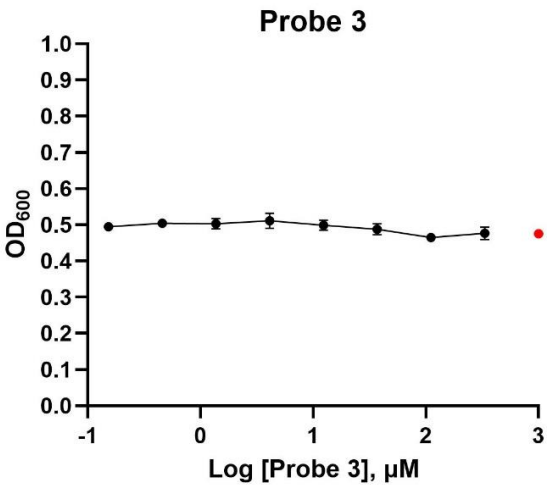


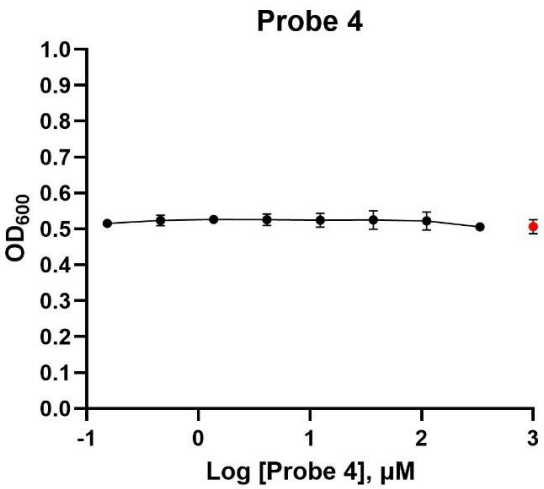
Figure S29. Activation assays showing the concentration-dependent, luminescence/OD_{600} Rhl QS system activation in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*) reporter strain by the different synthesized probes. All compounds were tested in triplicates and data represent the average ± SD of three independent experiments per probe. Values for the highest concentrations were excluded from the curve fitting process for all compounds, since for most compounds 1 mM of probe resulted in lower activation of the Rhl system, most likely caused by membrane perturbation. Three parameter fits were used to calculate EC_{50} values.

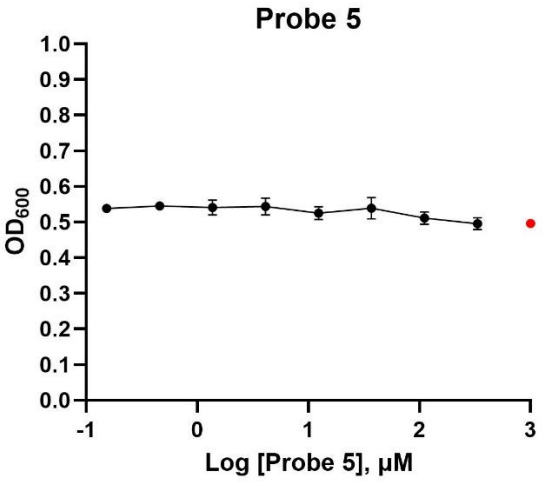
Growth assays (OD_{600}) - *P. aeruginosa* PAO-JP2 (pKD-*rhlA*)

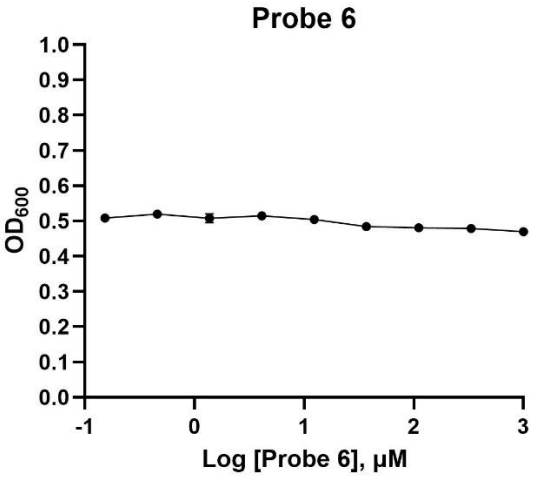


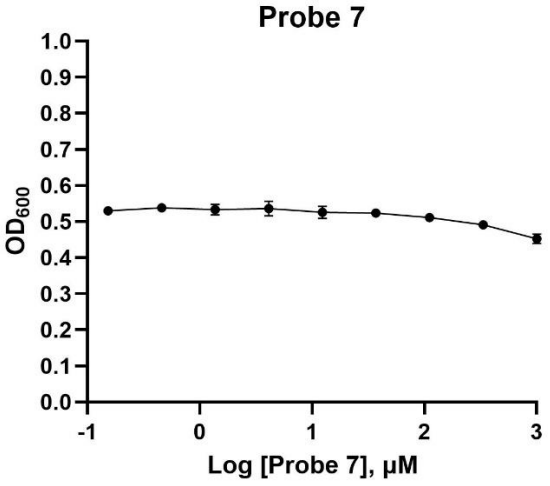


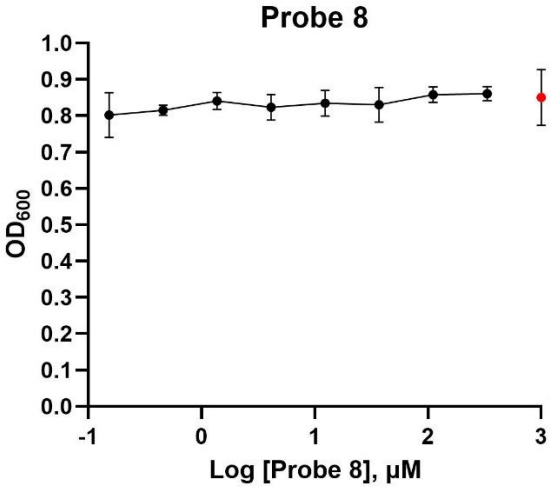


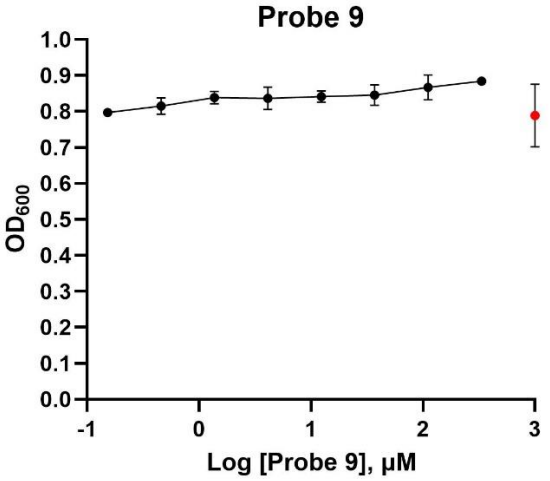


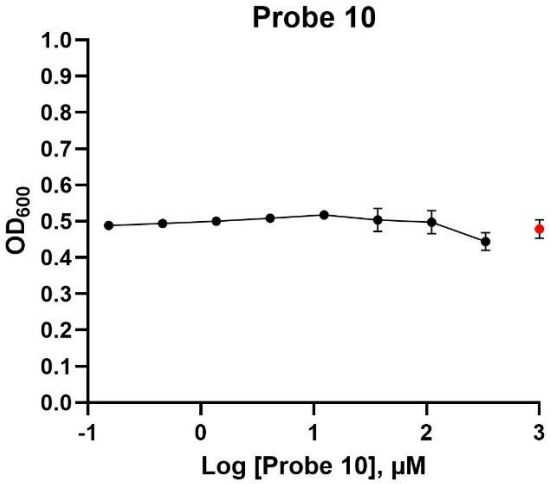


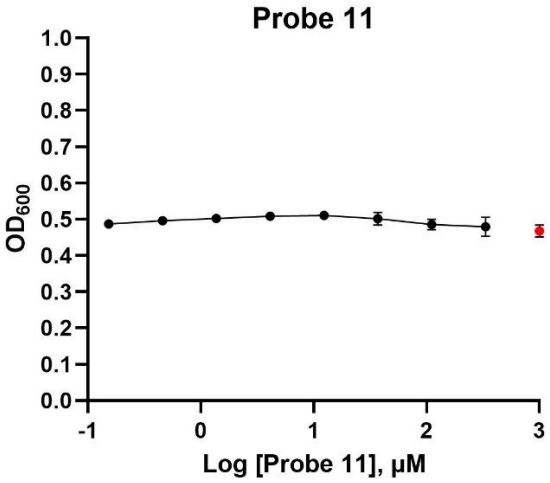


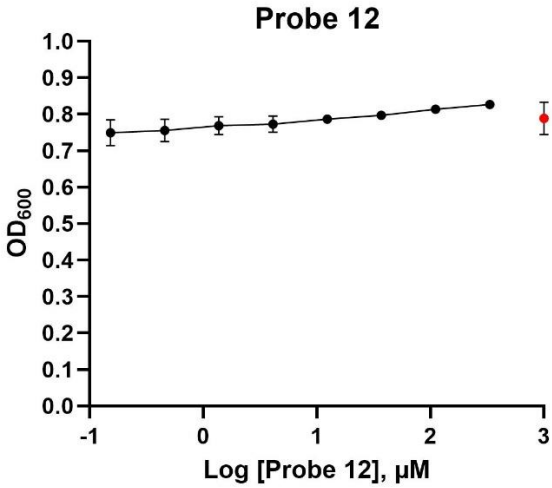

Probe 7
OD600
Log [Probe 7], μM


Probe 8
OD600
Log [Probe 8], μM


Probe 9
OD600
Log [Probe 9], μM


Probe 10
OD600
Log [Probe 10], μM


Probe 11
OD600
Log [Probe 11], μM


Probe 12
OD600
Log [Probe 12], μM

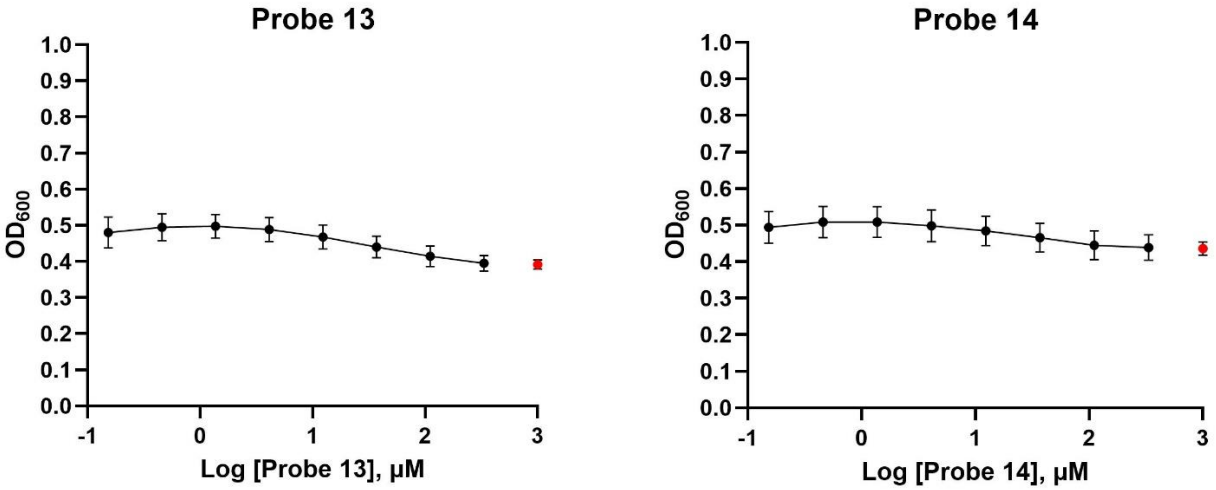


Figure S30. Concentration-dependent growth (OD_{600}) data for the different probes in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*) reporter strain. All compounds were tested in triplicates and data represent the average ± SD of three independent experiments per probe. Values for the highest concentrations were excluded from the curve fitting process for all compounds, since for most compounds 1 mM of probe resulted in lower activation of the Rhl system, most likely caused by membrane perturbation.

Final activation assays for compounds 12-14 (Luminescence/OD_{600}) - *P. aeruginosa* PAO-JP2 (pKD-*rhlA*)

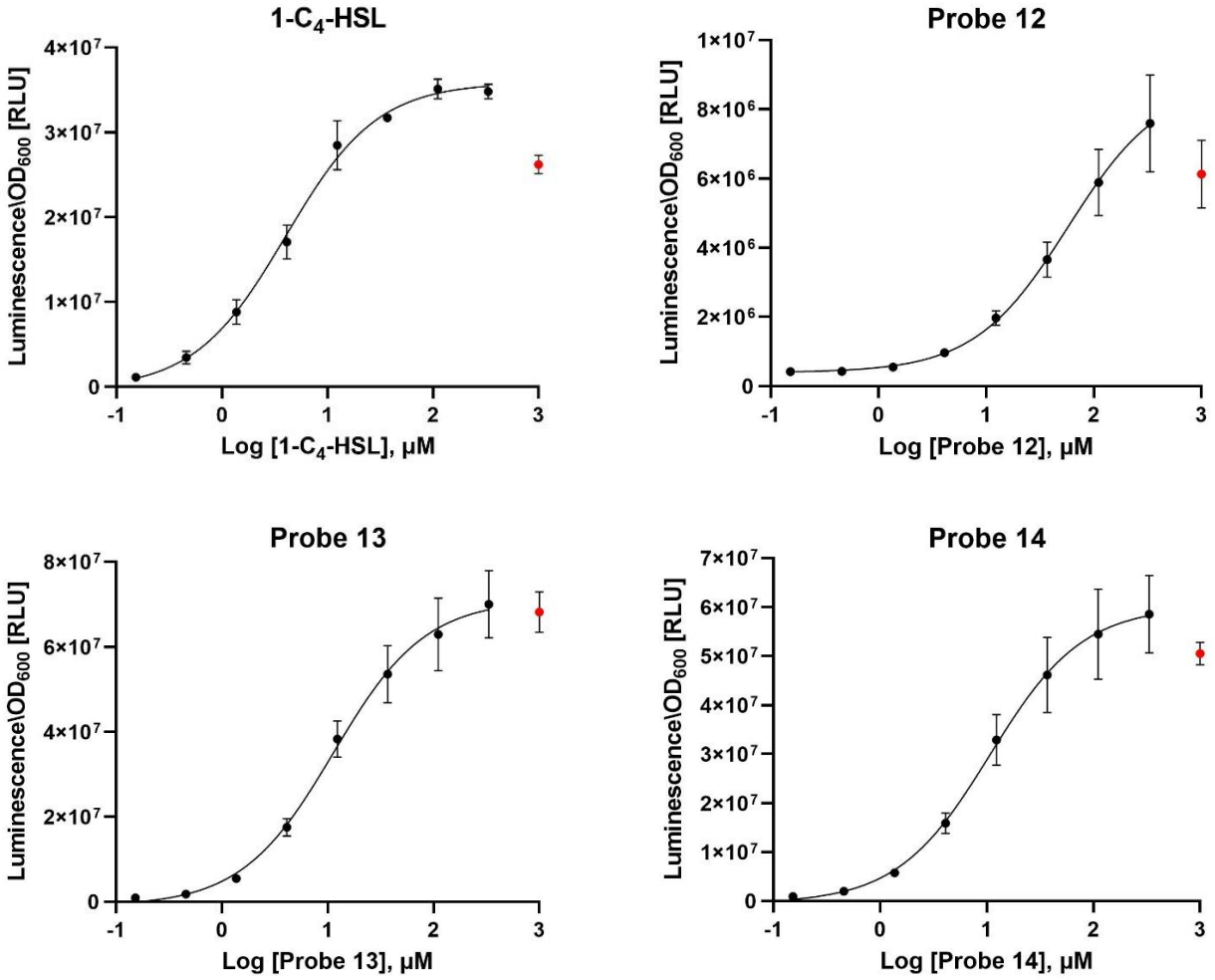


Figure S31. Final concentration-dependent luminescence/OD_{600} results for the synthetically optimized probes as shown in fig. 3 in the article manuscript. All compounds were tested in triplicates and data represent the average ± SD of three independent experiments per probe. Values for the highest concentrations were excluded from the curve fitting process for all compounds, since for most compounds 1 mM of probe resulted in lower activation of the Rhl system, most likely caused by membrane perturbation. Three parameter fits were used to calculate EC_{50} values (Table 1 in the article manuscript).

Luminescence/OD_{600} values at 75% activation for each probe compared to C_4-HSL

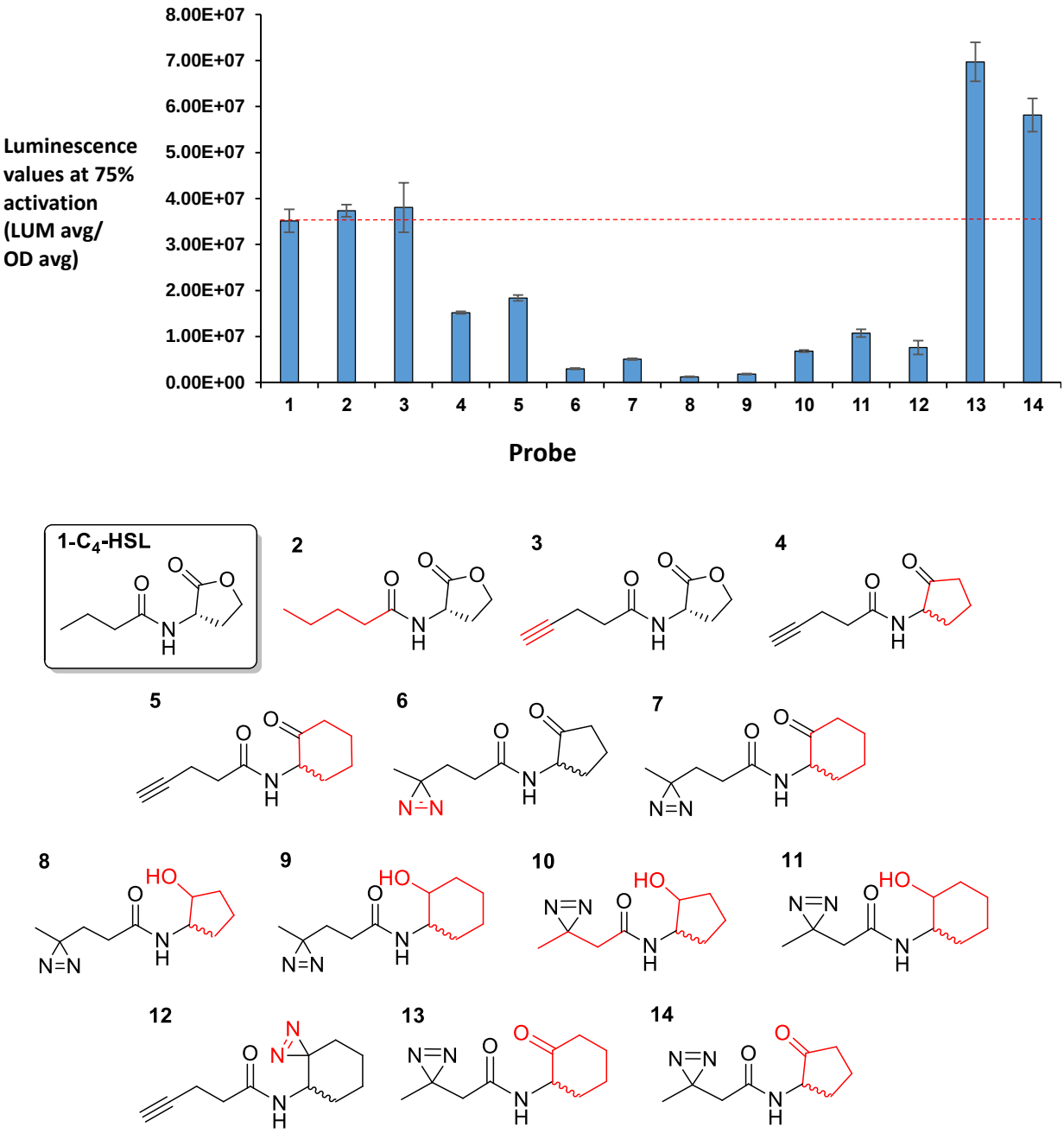


Figure S32. Structure-activity relationship results for the activation of the Rhl QS system in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*) by the different probes compared to the activation of the natural autoinducer C_4-HSL. All compounds were tested in triplicates and data represent the average ± SD of three independent experiments per probe.

Coomassie images of probes 12 and 13 fluorophore labeling

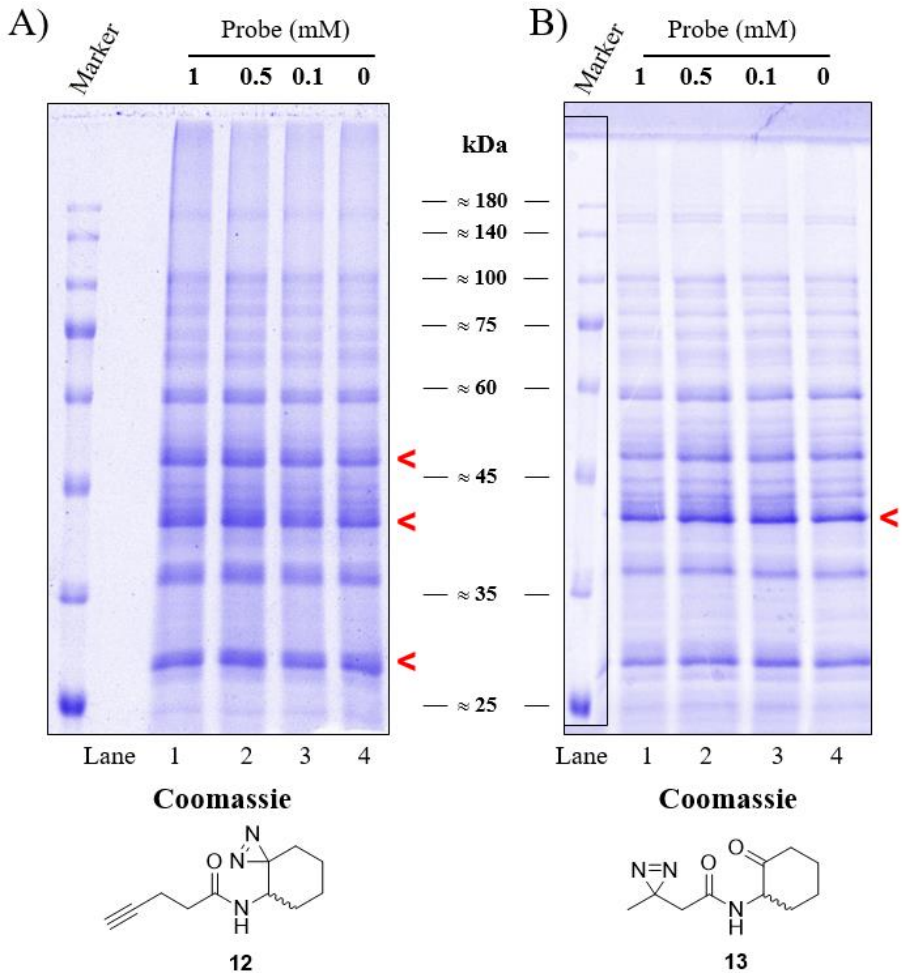


Figure S33. **(A)** In situ Coomassie gel images in the presence (lane 1-3) or absence (lane 4) of probe **12** in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*). A full SDS-PAGE separation is shown, ≈ 29, 42, 50 kDa regions are marked in arrows indicating probe labeling. **(B)** In situ Coomassie gel images in the presence (lane 1-3) or absence (lane 4) of probe **13** in *P. aeruginosa* PAO-JP2 (pKD-*rhlA*). Marker bands in image B are copied from the same gel.

Strain activity comparison

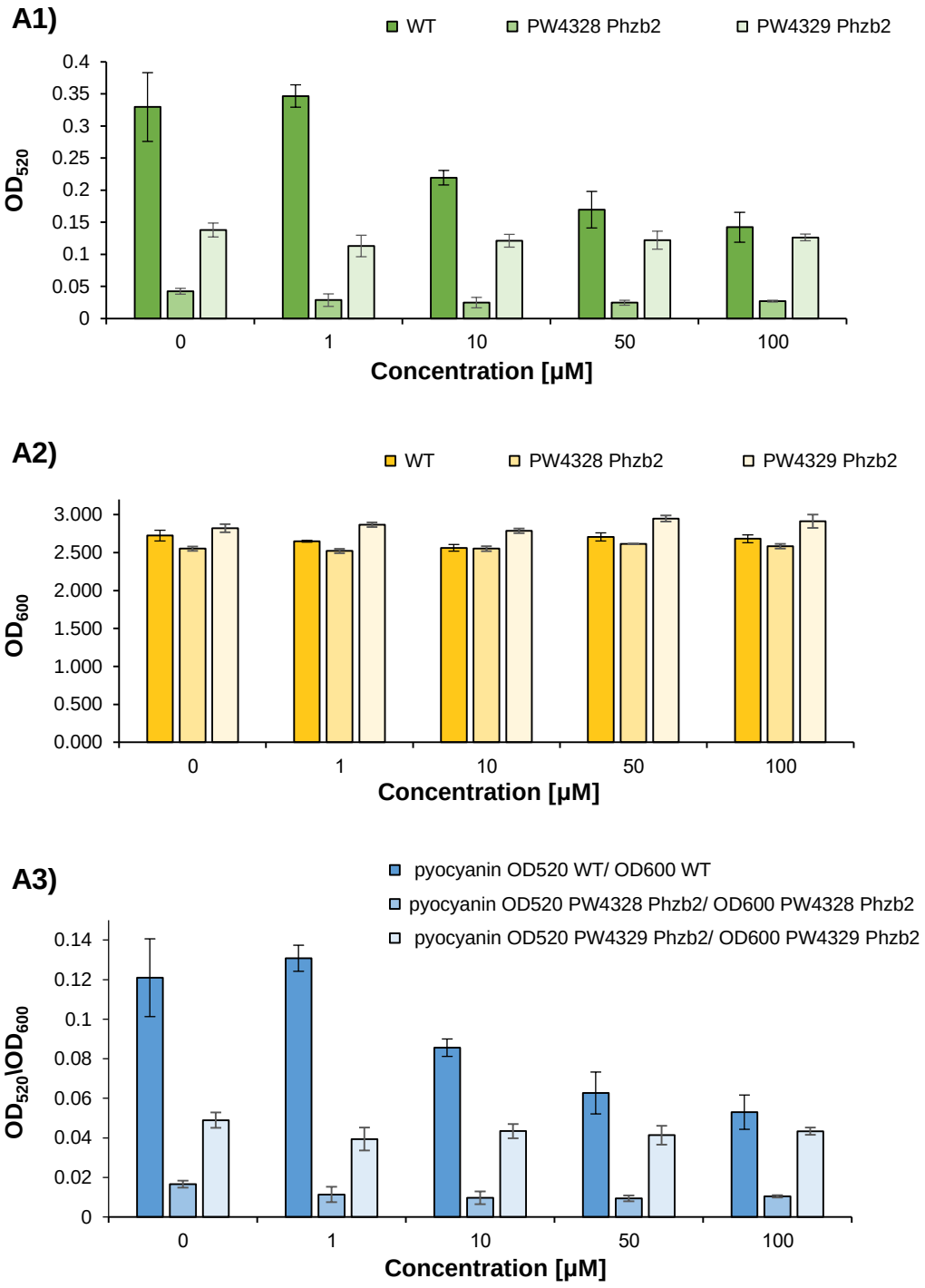


Figure S34. Activity comparison towards C_4-HSL increasing concentration. **(A1)** Pyocyanin production by wild-type *P. aeruginosa* strain PAO1, transposon mutants PW4328 (PhzB2), and PW4329 (PhzB2). **(A2)** Growth response for wild-type *P. aeruginosa* strain PAO1, PW4328 (PhzB2), and PW4329 (PhzB2). **(A3)** $OD_{520}\backslash OD_{600}$ for wild-type *P. aeruginosa* strain PAO1, PW4328 (PhzB2), and PW4329 (PhzB2). All bacteria were tested in triplicates and data represent the average ± SD of three independent experiments.

Purification of His_6-TEV-PhzB1

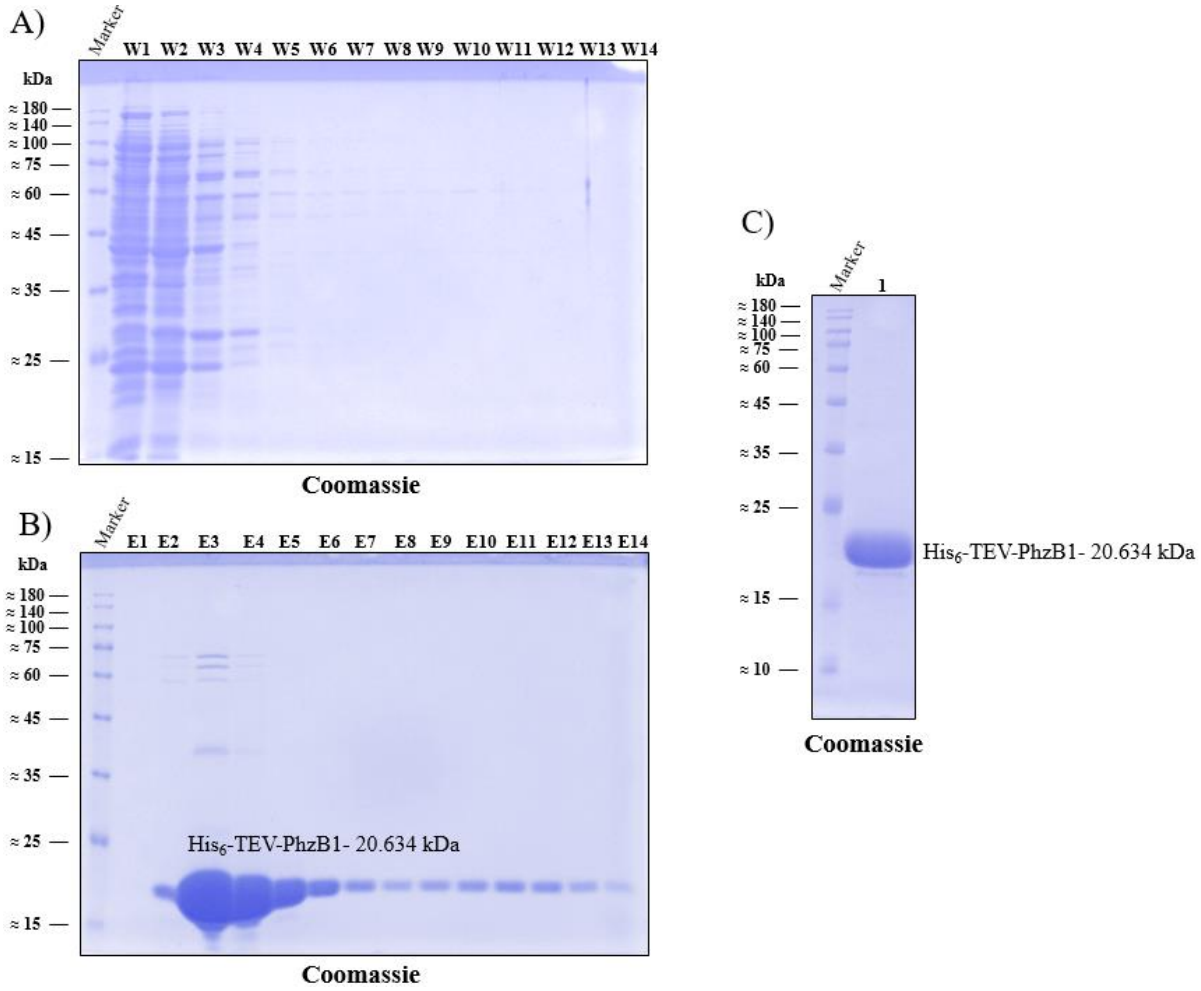


Figure S35. **(A)** Coomassie image of the washed (W1-W14) proteins. **(B)** The His_6-TEV tagged protein that was retained on the Ni-NTA beads and eluted with elution buffer (E1-E14). The molecular mass of His_6-TEV-PhzB1 predicted based on the DNA sequence and corresponds to 20,634 kDa. **(C)** His_6-TEV-PhzB1 after overnight dialysis at 4°C.

Concentration-dependent His_6-TEV-PhzB1 labeling

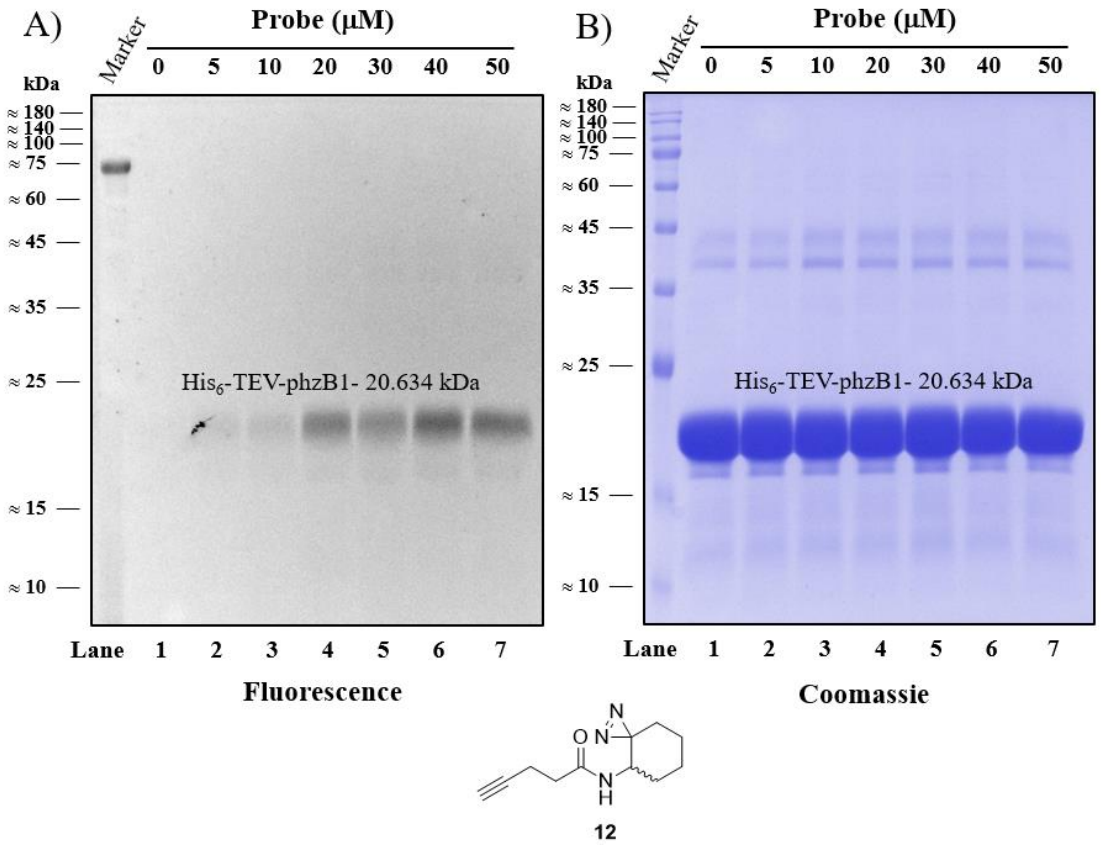


Figure S36. **(A)** The fluorescent image of His_6-TEV-PhzB1 concentration-dependent labeling (≈20 kDa). The figure shows the increase in labeling as the concentration of probe **12** increases from 0-50 μM. **(B)** The Coomassie image of the labeled protein.

The specificity of labeling toward His_6-TEV-PhzB1

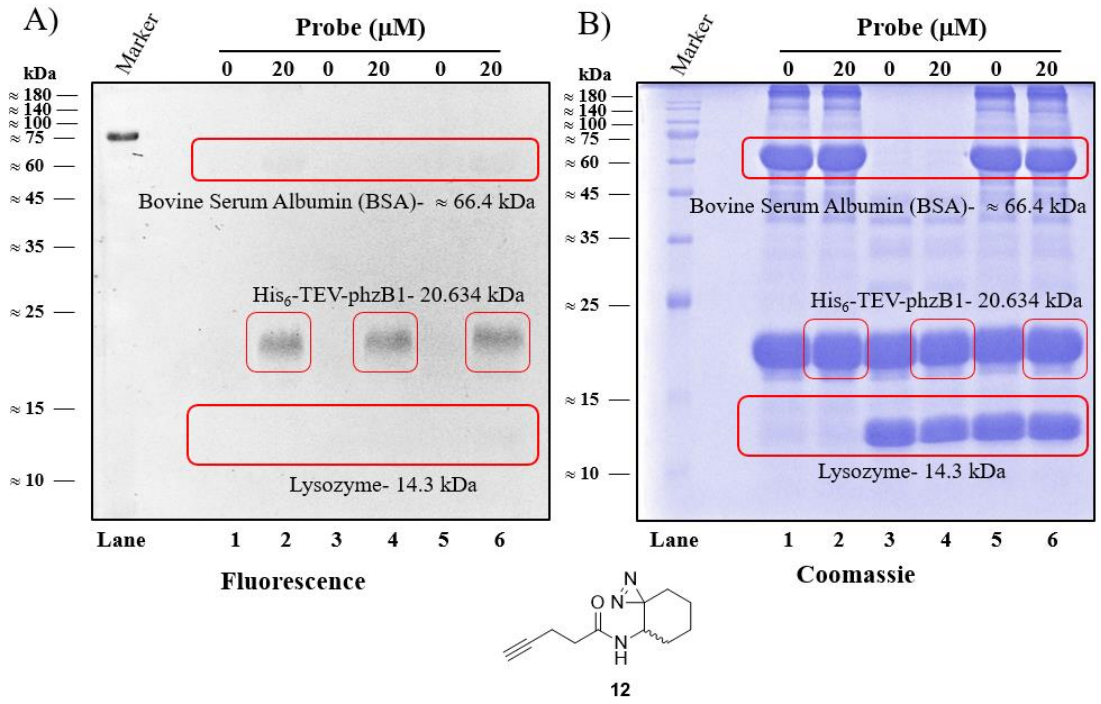


Figure S37. **(A)** Fluorescent image of the specific labeling of His_6-TEV-PhzB1 protein (≈20 kDa) by probe **12** compared to the control proteins, BSA (≈60-62 kDa), and Lysozyme (≈14 kDa). The specificity towards the His_6-TEV-PhzB1 protein can be seen in lanes 2, 4 and 6. Lanes 1, 3, and 5 served as a negative control. **(B)** The Coomassie image of the proteins.

C_4-HSL concentration-dependent competitive labeling of His_6-TEV-PhzB1

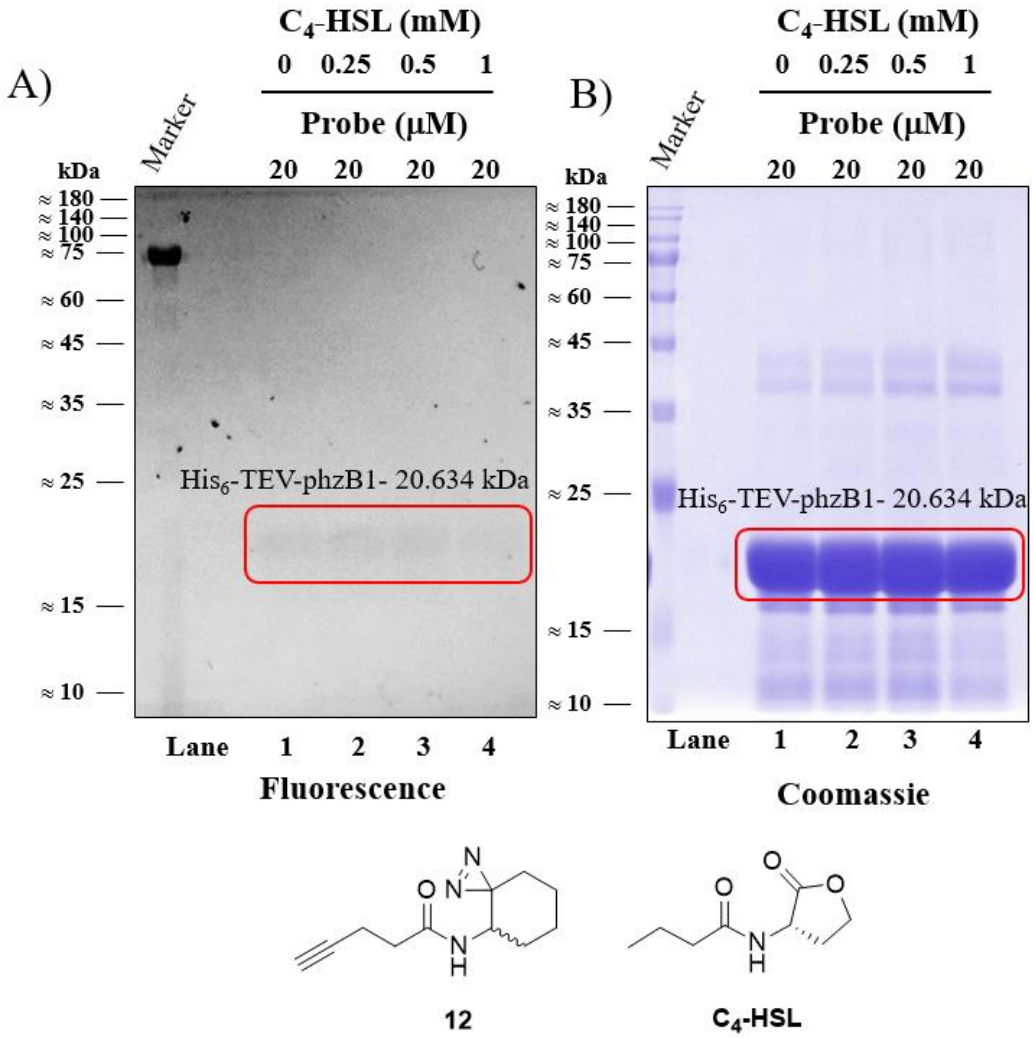


Figure S38. Repeat experiment of competitive labeling of the purified His_6-TEV-PhzB1 **(A)** Competitive labeling of His_6-TEV-PhzB1 with constant probe **12** concentration of 20 µM and increasing concentrations of C_4-HSL of 0-1000 µM. **(B)** The corresponding Coomassie gel image.

Phenazine biosynthetic pathway

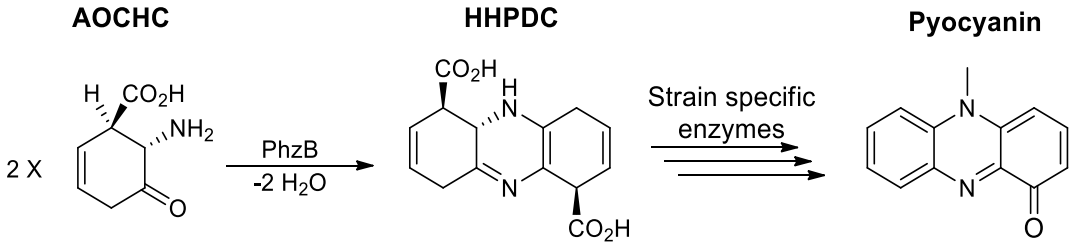


Figure S39. The role of PhzB in the biosynthetic pathway of strain-specific phenazines. Modified from N. Guttenberger et al.[1] and Blankenfeldt and Parsons.[2] The effect of C_4-HSL as a direct inhibitor of PhzB in the synthesis of hexahydrophenazine-1,6-dicarboxylic acid (HHPDC) from 6-amino-5-oxocyclohex-2-ene-1-carboxylic acid (AOCHC) is currently examined in more details.

Supplemental References

1. N. Guttenberger, W. Blankenfeldt and R. Breinbauer, *Bioorg. Med. Chem.,* 2017, **25**, 6149-6166.
2. W. Blankenfeldt, J.F. Parsons, *Curr. Opin. Struct. Biol.*, 2014, **29**, 26-33.