Electronic Supplementary Material (ESI) for Chemical Science.

SUPPORTING MATERIALS

Small Molecules Targeting The NEDD8•NAE Protein-protein Interaction

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A. General Experimental Procedures

All reactions were carried out with dry solvents under anhydrous conditions under an inert atmosphere (argon). Glassware was dried in an oven at 140 ^{o}C for minimum 6 h prior to use for all reactions. Dry solvents were obtained by passing the previously degassed solvents through activated alumina columns. Reagents were purchased at a high commercial quality (typically 97 % or higher) and used without further purification, unless otherwise stated.

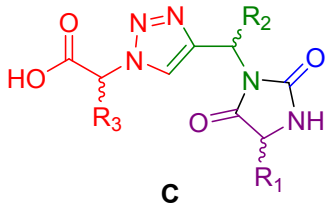
Flash chromatography was performed using silica gel (230 - 400 mesh). If applicable, solution-phase reactions was monitored using analytical thin layer chromatography (TLC) which was carried out on Silicycle silica gel plates and visualized by UV, ninhydrin, and/or potassium permanganate stains. A reverse phase column on preparation high performance liquid chromatography (prepHPLC) is also applied to purify products obtained from solid-phase synthesis in 10-90% MeCN/water gradient with 0.1% trifluoroacetic acid over 20 minutes.

High field NMR spectra were recorded with Bruker Avance III at 400 MHz for ^{1}H, and 100 MHz for ^{13}C for all compounds. All spectra were calibrated using residual non-deuterated solvent as an internal reference ($CDCl_3$: ^{1}H NMR = 7.24, ^{13}C NMR = 77.0, MeOD-d_4: ^{1}H NMR = 3.30, ^{13}C NMR = 49.0, DMSO-d_6: ^{1}H NMR = 2.50, ^{13}C NMR = 39.5). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, quint = quintet, dd = double doublet, dt = double triplet, dq = double quartet, m = multiplet, br = broad. Electrospray ionization mass spectrometry (ESI-MS) data were collected on triple-stage quadrupole instrument in a positive mode. All statistical analyses were carried out by GraphPad Prism version 6.0 (GraphPad Software). Results are represented as means ± SD.

Cell Culture. K562 (ATCC) cells were cultured on 75 cm^2 tissue culture flasks in Roswell Park Memorial Institute (RPMI, Millipore Sigma) 1640 medium with 10% FBS. All cells were cultured in a humidified incubator at 37 °C with 5% CO_2 and 95% air.

B. Computational Studies

QMD & EKO

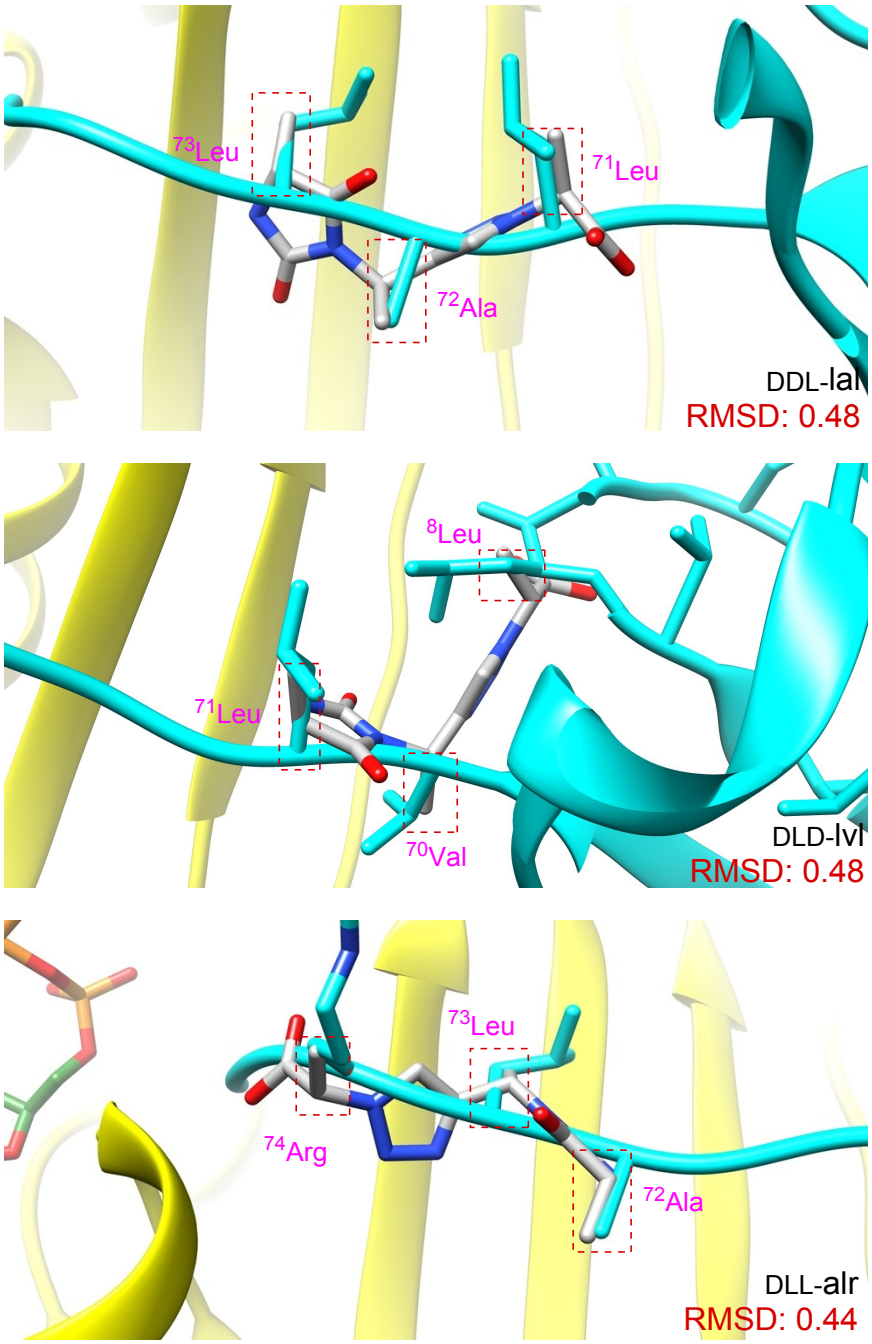


C

QMD was used to generate simulated conformations of all diasteromers of **Caaa**. The procedures had been reported previously.[1] Approximately 1500 conformers of each diastereomer within 3.0 kcal/mol of the global minimum were matched on NAE-NEDD8 protein-protein interaction (PDBID: 1R4N) using EKO analyses.[2] The Cα-Cβ coordinates from the side chains of peptidomimetics were systematically overlaid on Cα-Cβ coordinates of the NEDD8 side chains at protein-protein interface using in-house algorithm. The goodness of fit was reported as root mean square deviation (RMSD). Lower RMSDs mean the Cα-Cβ orientations between chemotypes and protein side chains are similar, thus chemotypes can *mimic* the protein side chains. The conformers within RMSD ≤ 0.50 Å were considered as "potential hits" which were summarized in Table S1. Compounds **C** were further modified to generate compound **1** to **3** series which were synthesized based on the feasibility to obtain the starting materials.

Table S1. Potential NEDD8 mimics **C** from EKO analyses within RMSD ≤ 0.50 Å

Isomer	Residues	RMSD (Å)
DLL	A72-L73-R74	0.44
DLL	L71-V70-L8	0.47
LLL	L71-V70-L8	0.44
DLL	L71-A72-L73	0.46
DLD	L71-V70-L8	0.48
LDL	L73-A72-L71	0.49
DDL	L73-A72-L71	0.48
DLL	V70-L71-A72	0.49


73Leu
71Leu
72Ala
DDL-lal
RMSD: 0.48
8Leu
71Leu
70Val
DLD-lvl
RMSD: 0.48
73Leu
74Arg
72Ala
DLL-alr
RMSD: 0.44

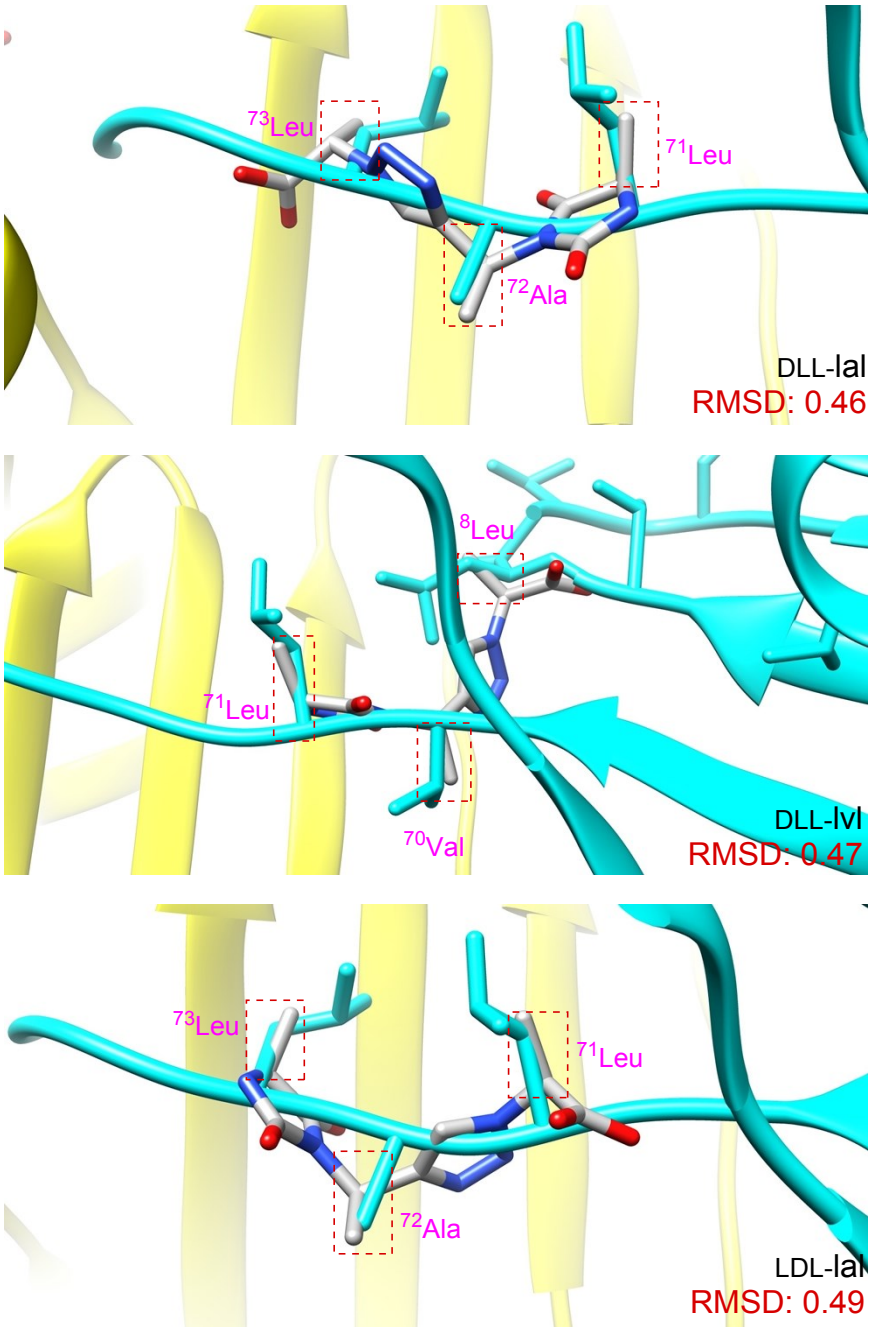


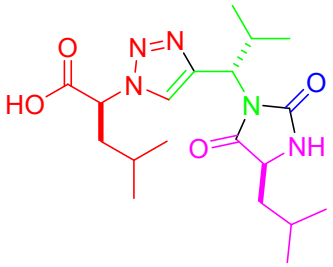
Figure S1. Overlays of featured chemotypes on NEDD8 analyzed by EKO

Glide

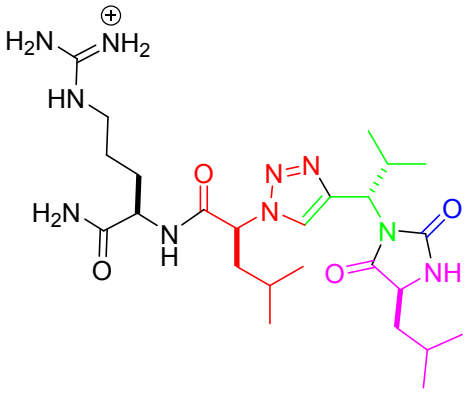
Molecular dockings of the virtual libraries were performed using Glide in Schrödinger package (version 2016-2).[3-6] Selected hits from EKO analyses were used as the templates for optimizations. Side-chain substitutions and C-terminus modifications were enumerated using CombiGlide to obtain virtual combinatorial libraries. NEDD8 protein was obtained by deleting the NAE protein from the same PDB file (1r4n). The docking of virtual compounds was performed using OPLS_2005 force field within 20 Å of the grid box, and the conformations of virtual molecules were restricted within 5 Å from the parental conformers.

R^1-R^3 side chains were replaced with the natural side chains from EKO analysis (Table S1). To enhance the binding ability of the compounds to NAE, incorporation of 4th amino acid was enumerated with other amino-acid side chains to obtain ones that had best docking scores, which were arginine, histidine, and lysine derivatives. After modifying R^1-R^3 side chains, the carboxylic acid was replaced with amino acid moiety, in which R^4 was the library of *D*-, *L*- amino-acid side chains. The top results of compounds **3** , which were having 4th amino acid with positive functional groups, were listed below.

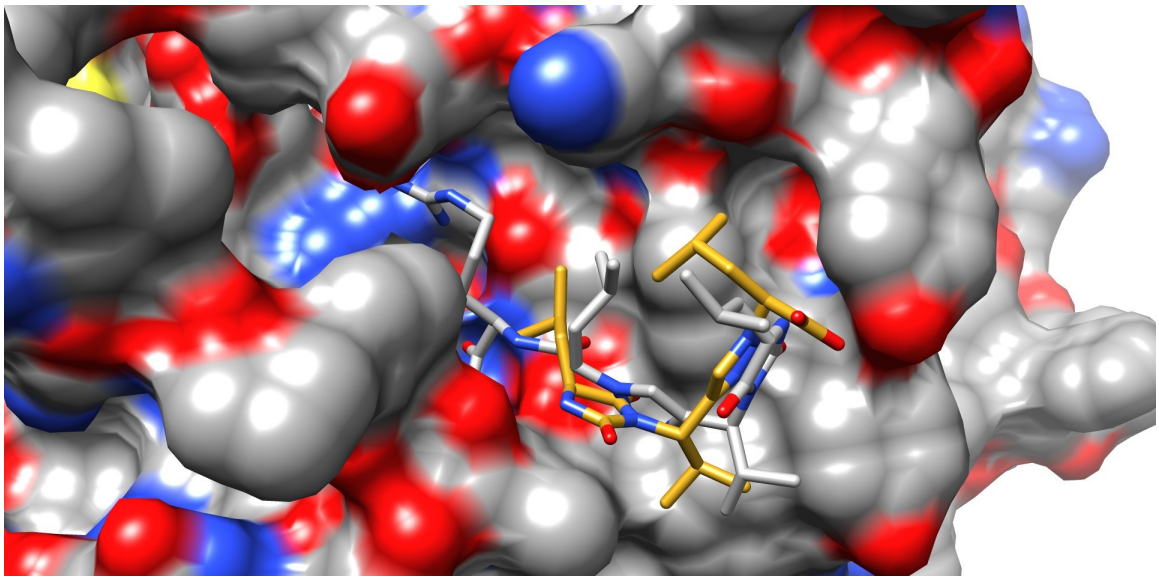
LLLD-**3lvlr**



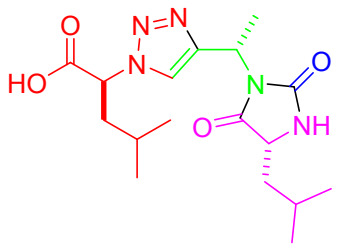
LLL-**Clvl** (gold), ΔG = -1.57 kcal/mol



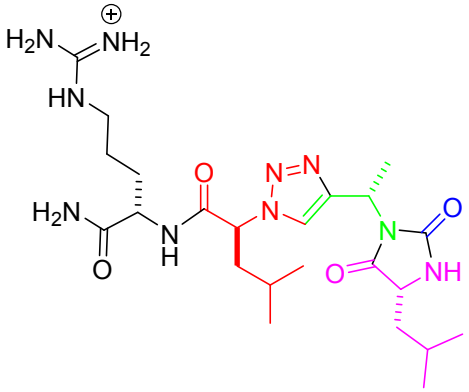
LLLD-**3lvlr** (silver), ΔG = -6.88 kcal/mol



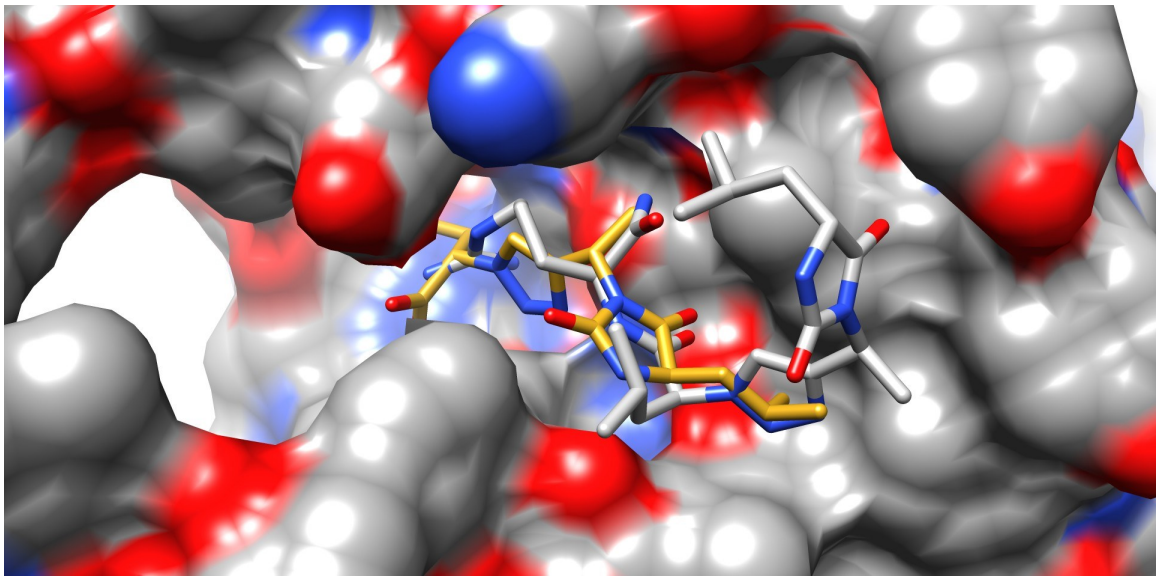
DLLL-**3Ialr**



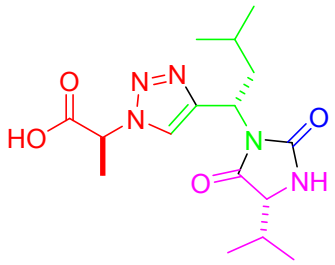
DLL-**CIal** (gold), ΔG = -3.84 kcal/mol



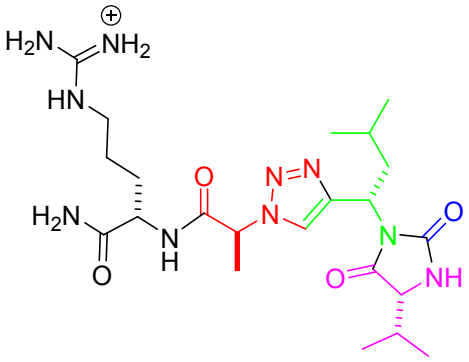
DLLL-**3Ialr** (silver), ΔG = -6.65 kcal/mol



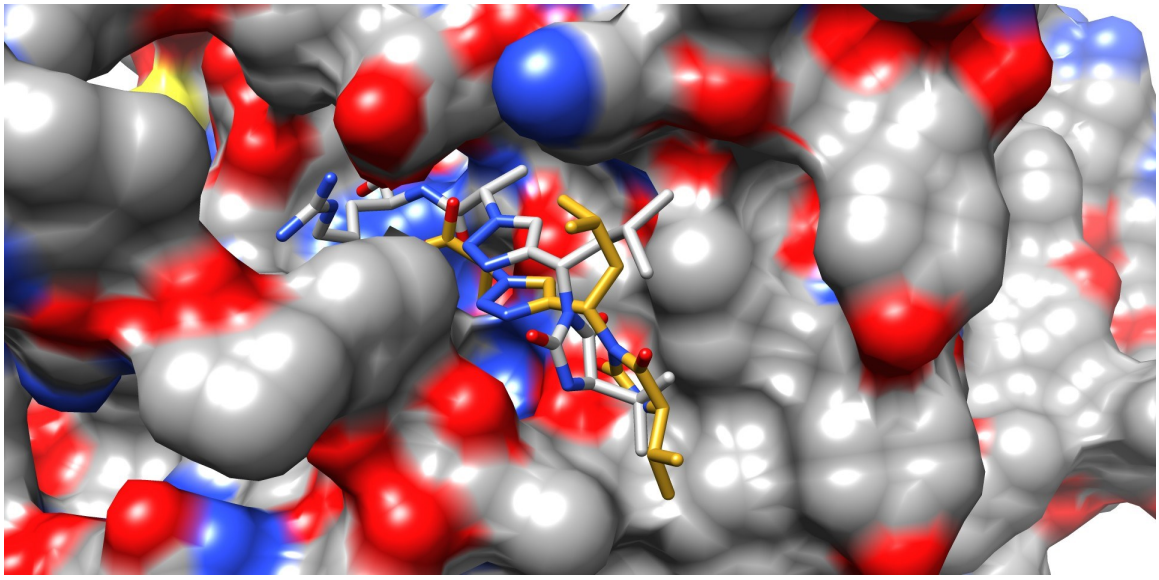
DLLL-**3vlar**



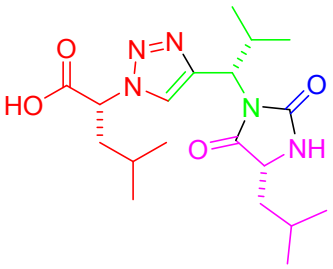
DLL-**Cvla** (gold), ΔG = -3.08 kcal/mol



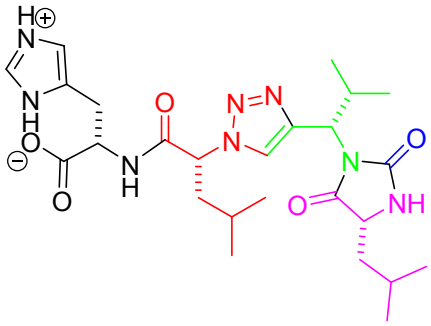
DLLL-**3vlar** (silver), ΔG = -7.60 kcal/mol



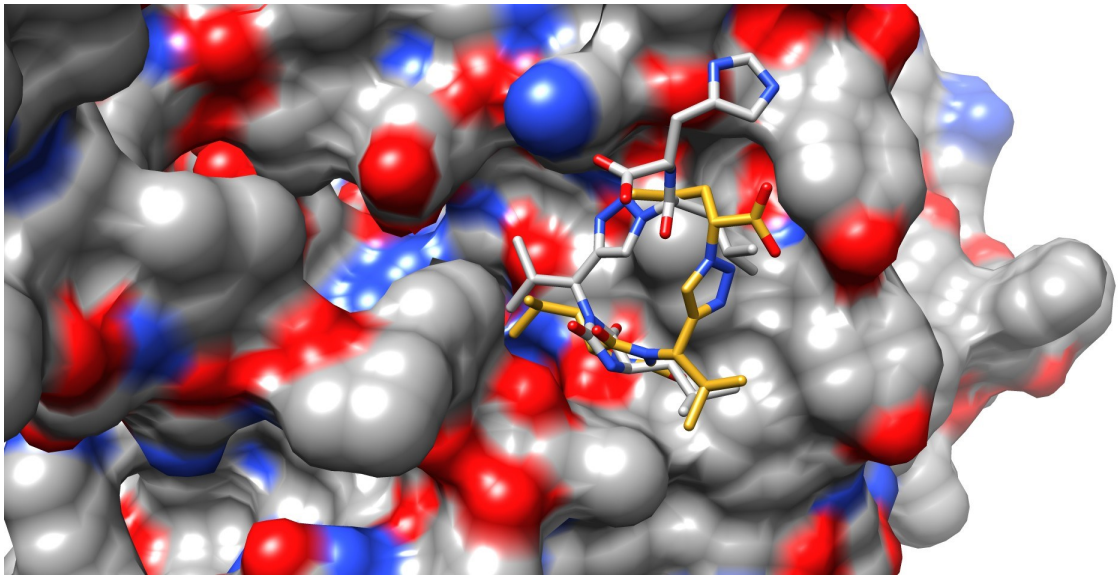
DLDL-**3lvlh**



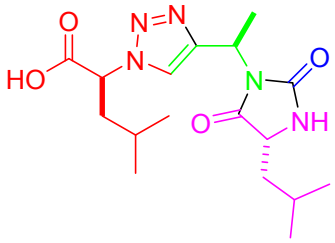
DLD-**Clvl** (gold), ΔG = -2.74 kcal/mol



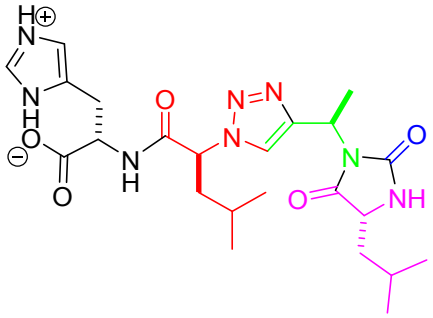
DLDL-**3lvlh** (silver), ΔG = -7.29 kcal/mol



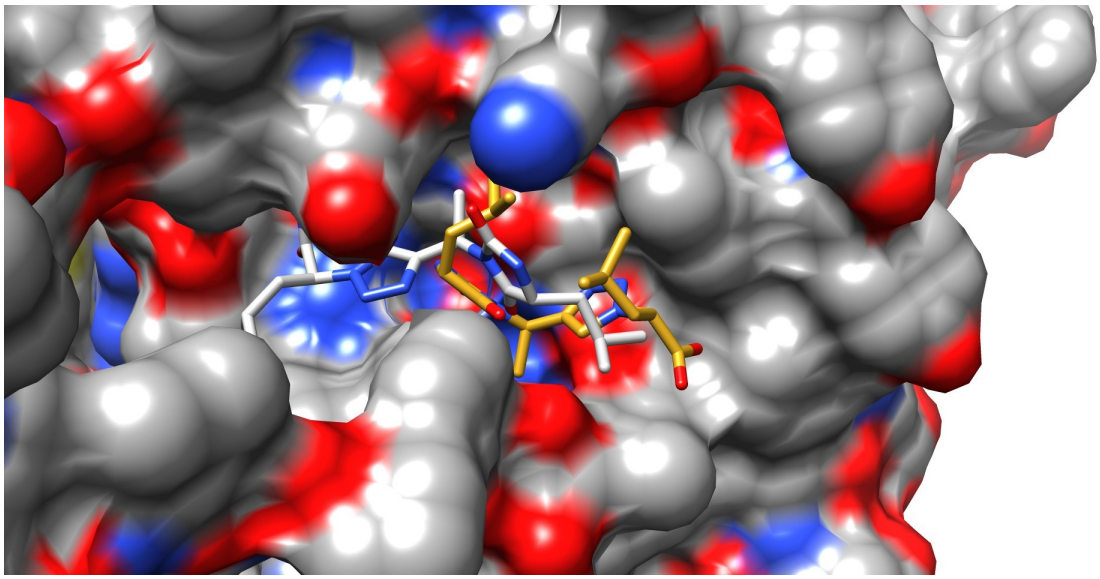
DDLL-**3lalh**



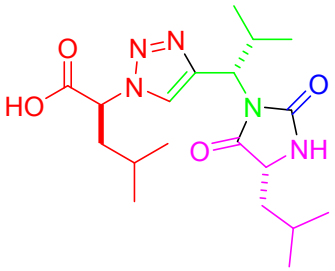
DDL-**Clal** (gold), ΔG = -3.13 kcal/mol



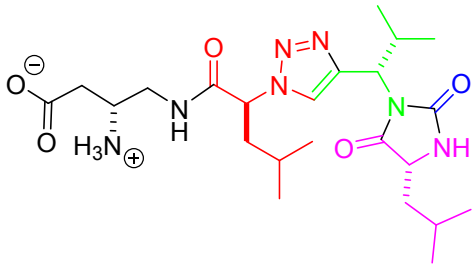
DDLL-**3lalh** (silver), ΔG = -7.36 kcal/mol



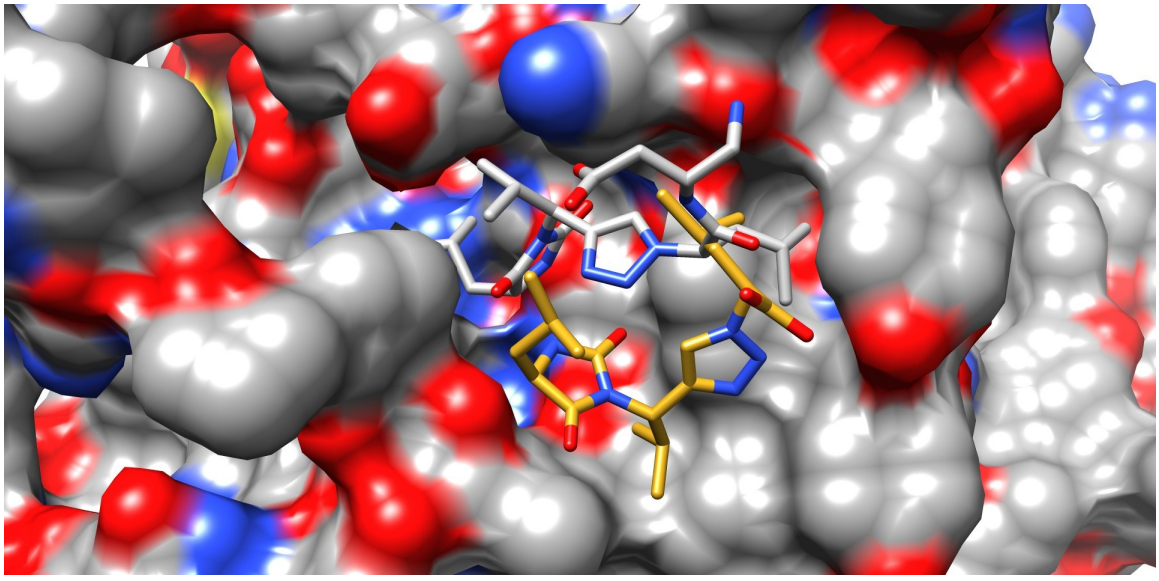
DLLL-**3IvlDbu**



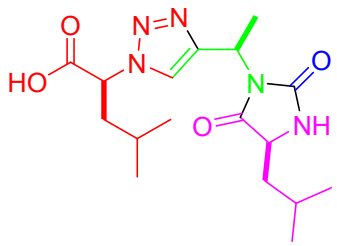
DLL-**CIvl** (gold), ΔG = -2.44 kcal/mol



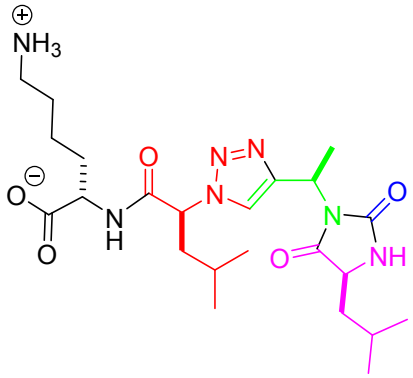
DLLL-**3IvlDbu** (silver), ΔG = -7.95 kcal/mol



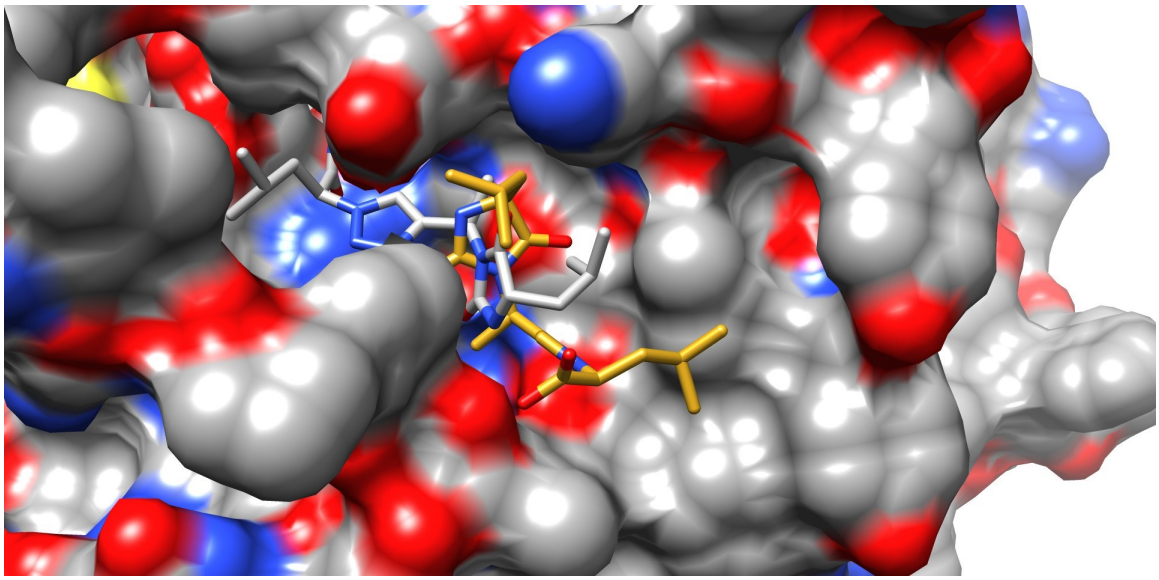
LDLL-**3lalk**



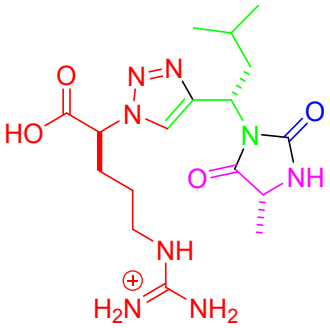
LDL-**ClaI** (gold), ΔG = -3.00 kcal/mol



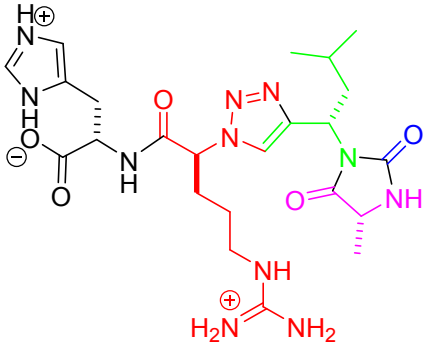
LDLL-**3lalk** (silver), ΔG = -10.3 kcal/mol



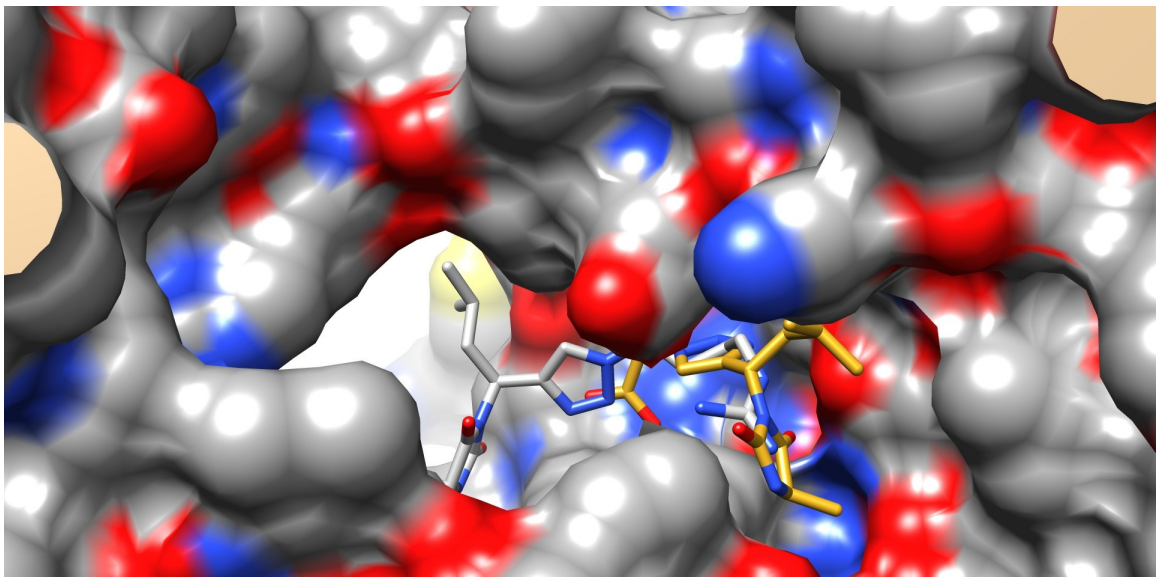
DLLL-**3alrh**



DLL-**Calr** (gold), ΔG = -5.92 kcal/mol

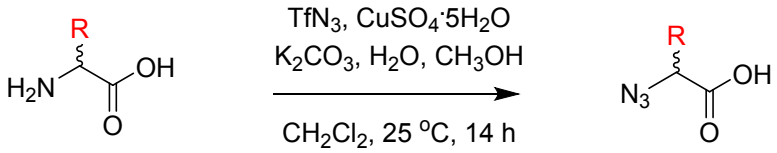


DLLL-**3alrh** (silver), ΔG = -10.2 kcal/mol



C. Preparation of Azido Acid and Fmoc-Amino Alkynes

General Procedure for the Synthesis of Azido Acid



Scheme S1. Synthesis of Azido Acids

Synthesis of TfN_3

Sodium azide (23.7 g, 366 mmol) was dissolved in H_2O/CH_2Cl_2 (1:1; 120 mL) in a 250 mL round-bottom flask, and the solution was cooled to 0 °C. Triflic anhydride (12.4 mL, 74 mmol) was added into the reaction flask slowly over the course of 5 mins, and the reaction was stirred vigorously at 0 °C for 4 h. The organic layer was collected by separation funnel, and the aqueous layer was extracted with CH_2Cl_2 (50 mL x 2). The combined organic layer was washed with sat. Na_2CO_3 aqueous solution (50 mL), and the organic solution was used for the next step without any further purification.

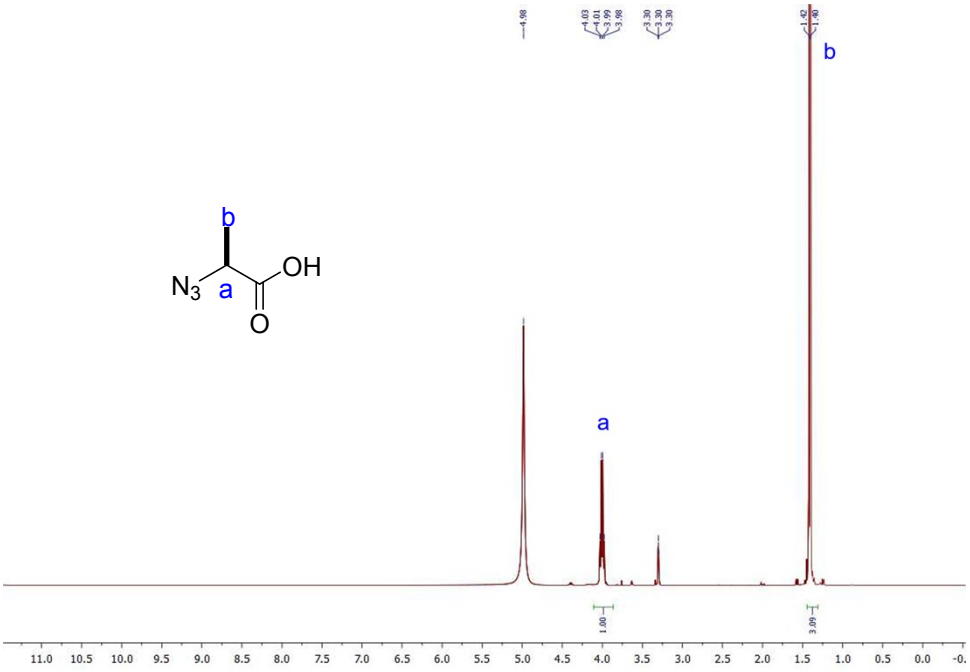
Synthesis of Azido Acids

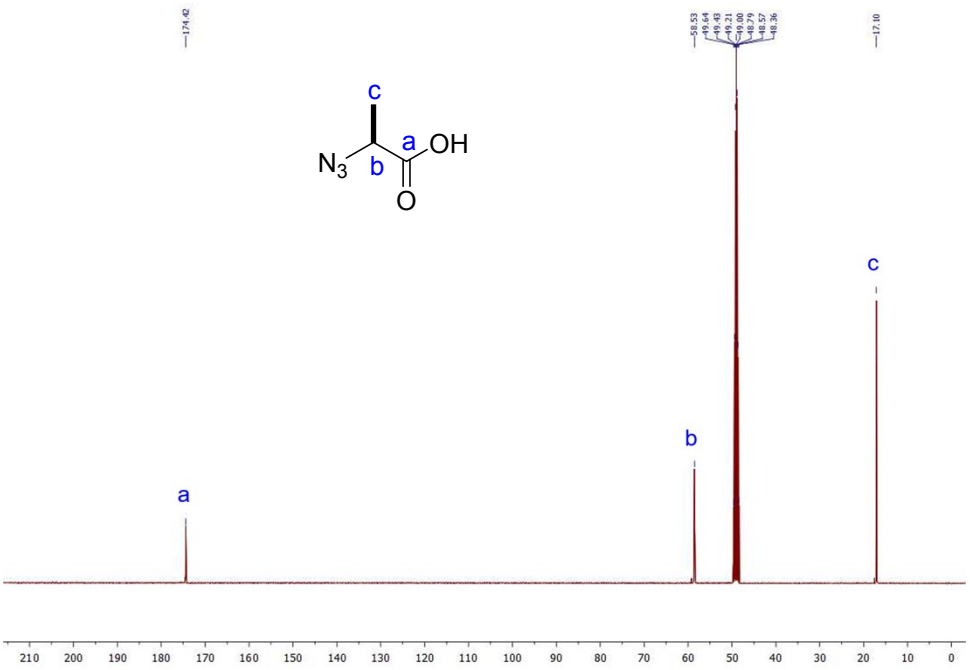
To a 1 L round-bottom flask, Amino acid (37.0 mmol), K_2CO_3 (7.71 g, 55.8 mmol), $CuSO_4 \cdot 5H_2O$ (0.09 g, 0.37 mmol) were dissolved in H_2O/CH_3OH (1:2; 360 mL) solution. The TfN_3 in CH_2Cl_2 solution was added to the reaction flask, and the reaction was stirred at 25 °C for 14 h. Organic solvent was evaporated under reduced pressure at temperature below 35 °C, and the aqueous layer was adjusted to pH = 6 using 2 M HCl aqueous solution. The aqueous solution was then washed with EtOAc (120 mL x 3) to remove any by-product. The aqueous solution was further acidified using 2 M HCl (aq.) solution to pH = 2 to precipitate out the azide product, which was collected by extraction with EtOAc (120 mL x 3). The combined organic layer containing product was washed with brine, dried over anhydrous Mg_2SO_4, filtered, and concentrated to dryness to afford the desired azide as colorless oil.

L-Ala Azido Acid ($C_3H_5N_3O_2$, 98% yield)

^{1}H NMR (400 MHz, MeOD) δ 4.00 (q, *J* = 7.1 Hz, 1H), 1.41 (d, *J* = 7.1 Hz, 3H).

^{13}C NMR (100 MHz, MeOD) δ 174.42, 58.53, 17.10.

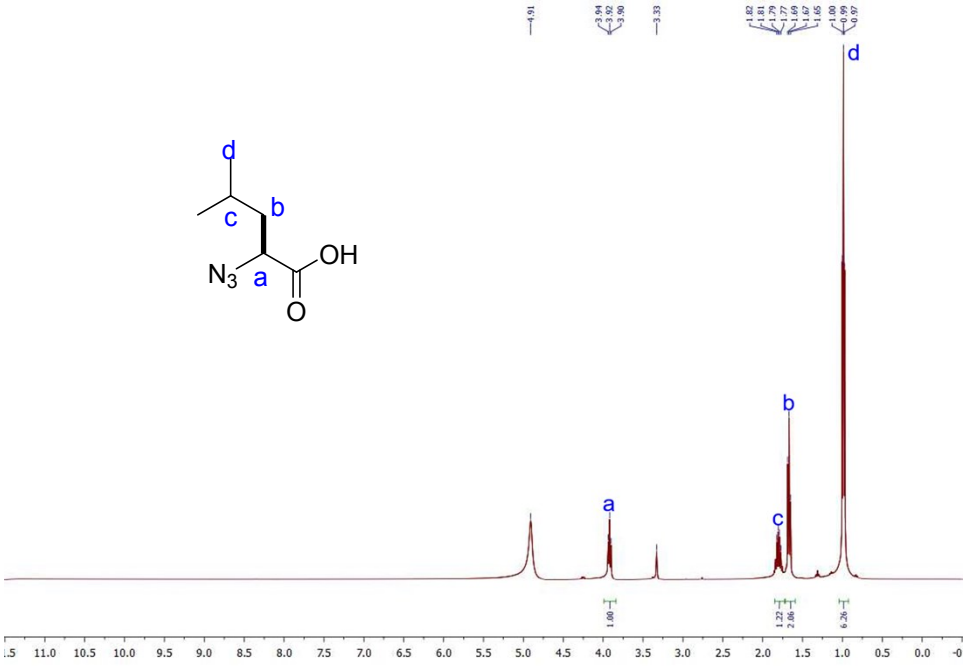


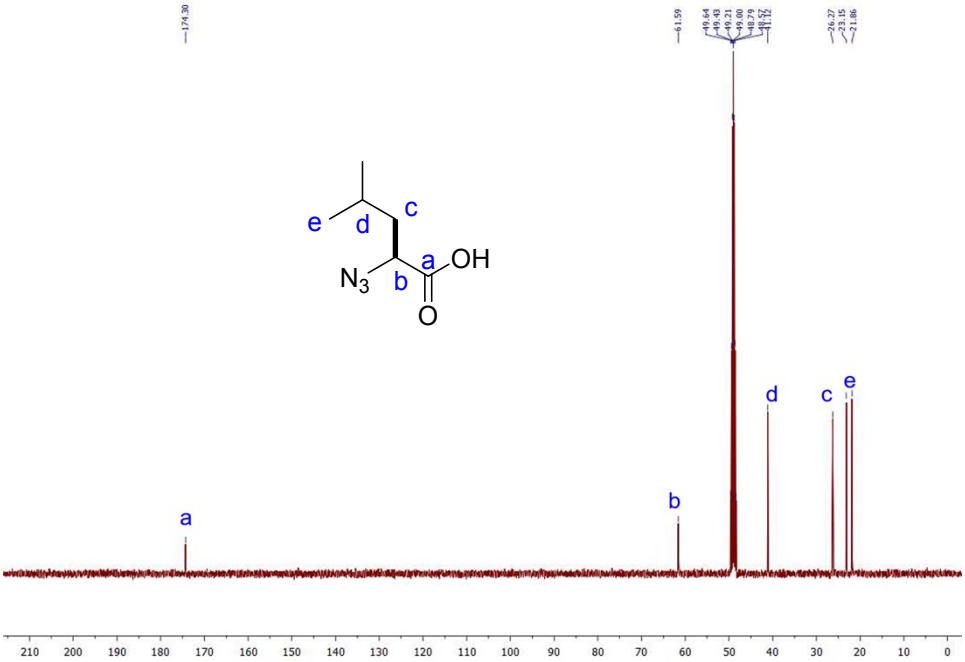


L-Leu Azido Acid ($C_5H_{11}N_3O_2$, quantitative)

^{1}H NMR (400 MHz, MeOD) δ 3.92 (t, *J* = 7.2 Hz, 1H), 1.80 (td, *J* = 13.4, 6.7 Hz, 1H), 1.67 (t, *J* = 7.1 Hz, 2H), 0.99 (t, *J* = 6.2 Hz, 6H).

^{13}C NMR (100 MHz, MeOD) δ 174.30, 61.59, 41.12, 26.27, 23.15, 21.86.

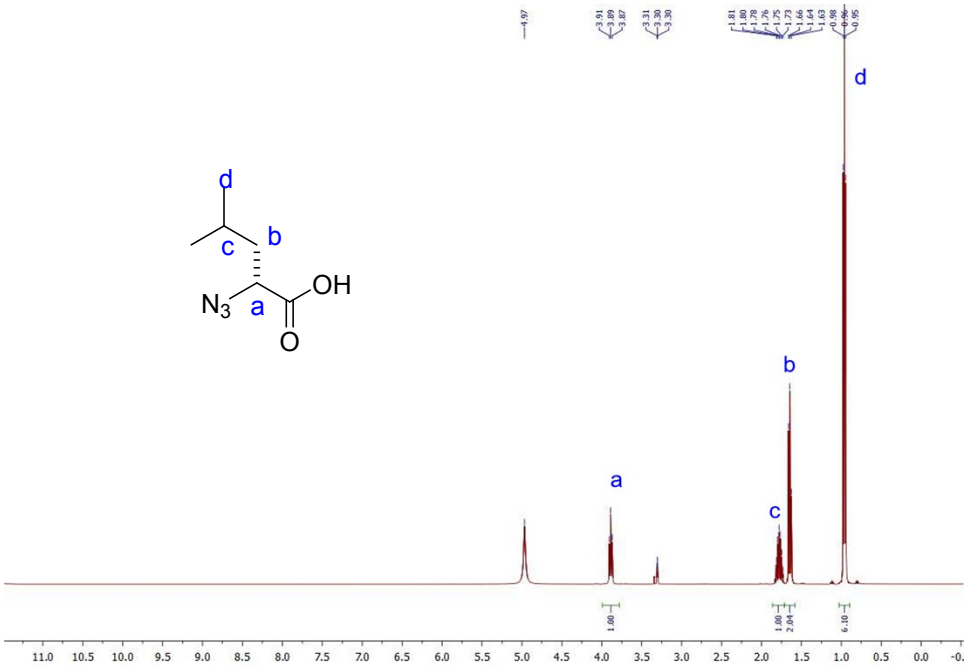


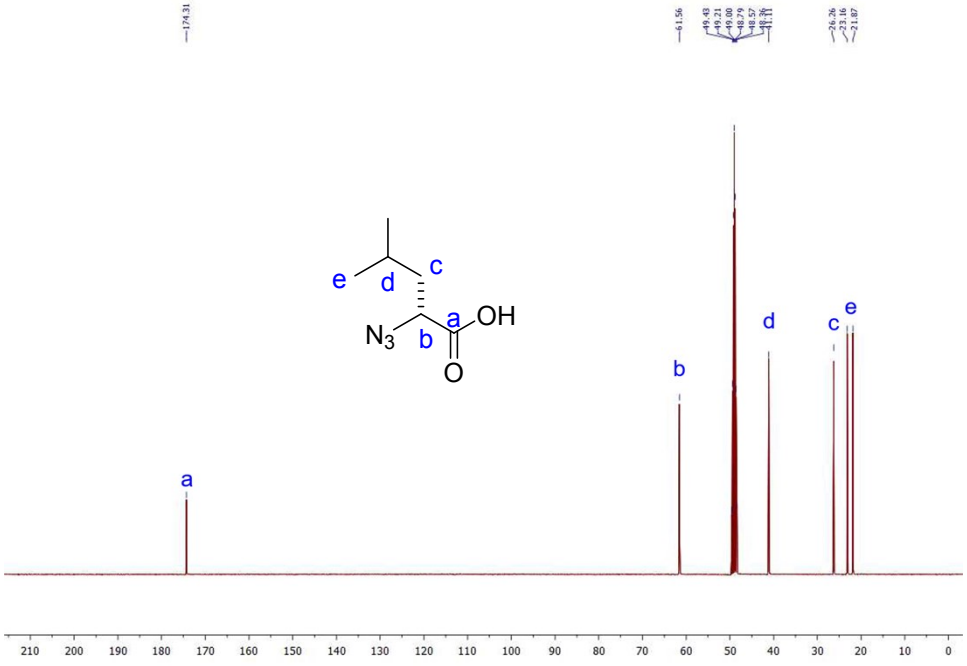


D-Leu Azido Acid ($C_5H_{11}N_3O_2$, quantitative)

^{1}H NMR (400 MHz, MeOD) δ 3.89 (t, *J* = 7.3 Hz, 1H), 1.77 (td, *J* = 13.4, 6.7 Hz, 1H), 1.64 (t, *J* = 7.0 Hz, 2H), 0.96 (t, *J* = 6.3 Hz, 6H).

^{13}C NMR (100 MHz, MeOD) δ 174.31, 61.56, 41.11, 26.26, 23.16, 21.87.

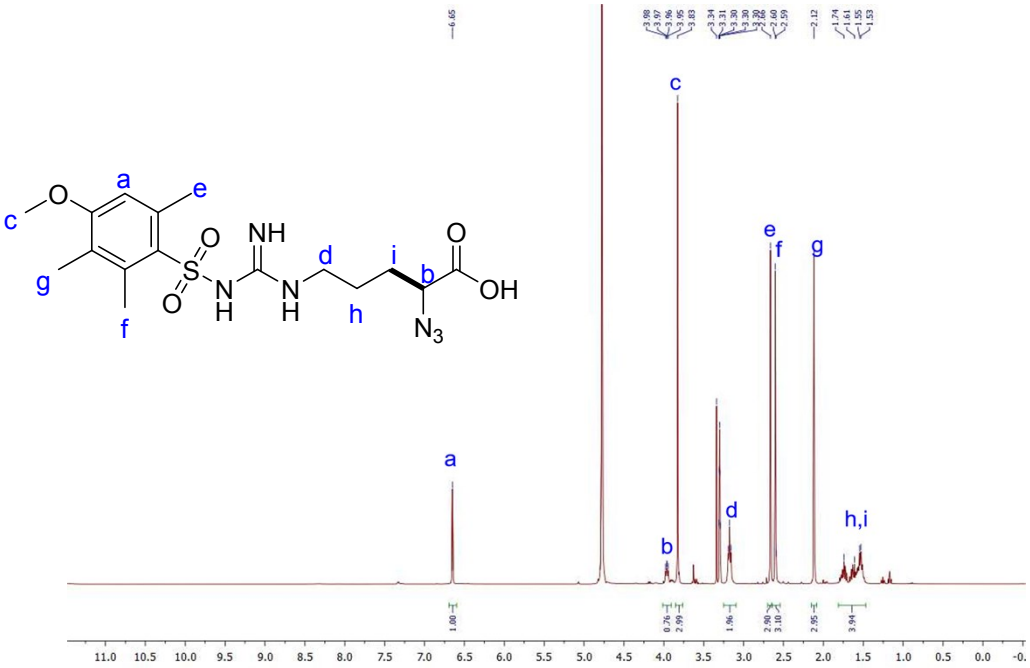


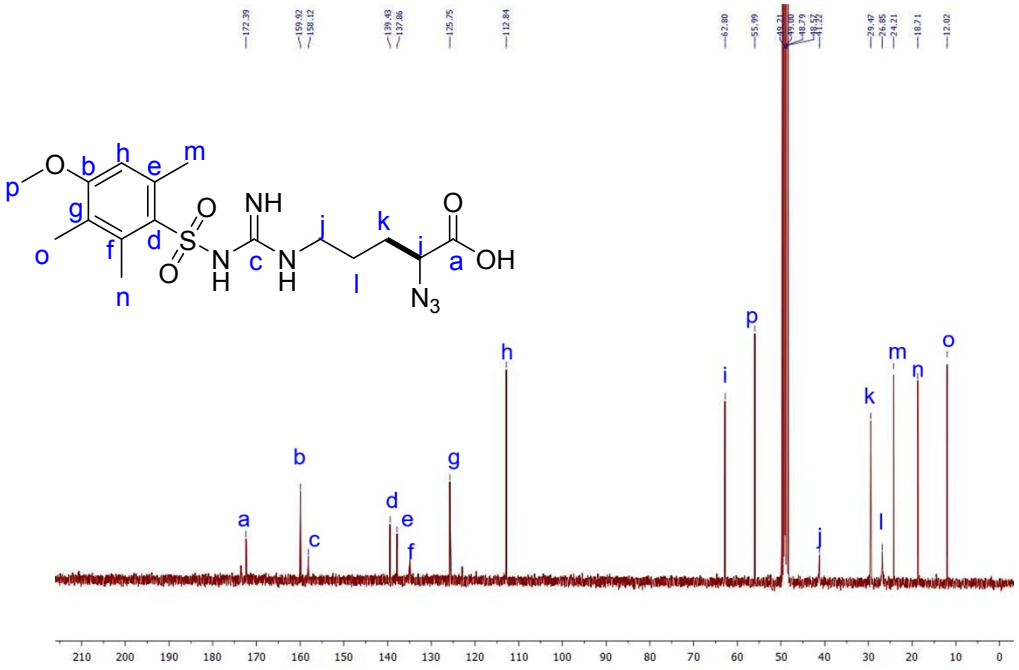


L-Arg(mtr) Azido Acid ($C_{16}H_{24}N_6O_5S$, 99% yield)

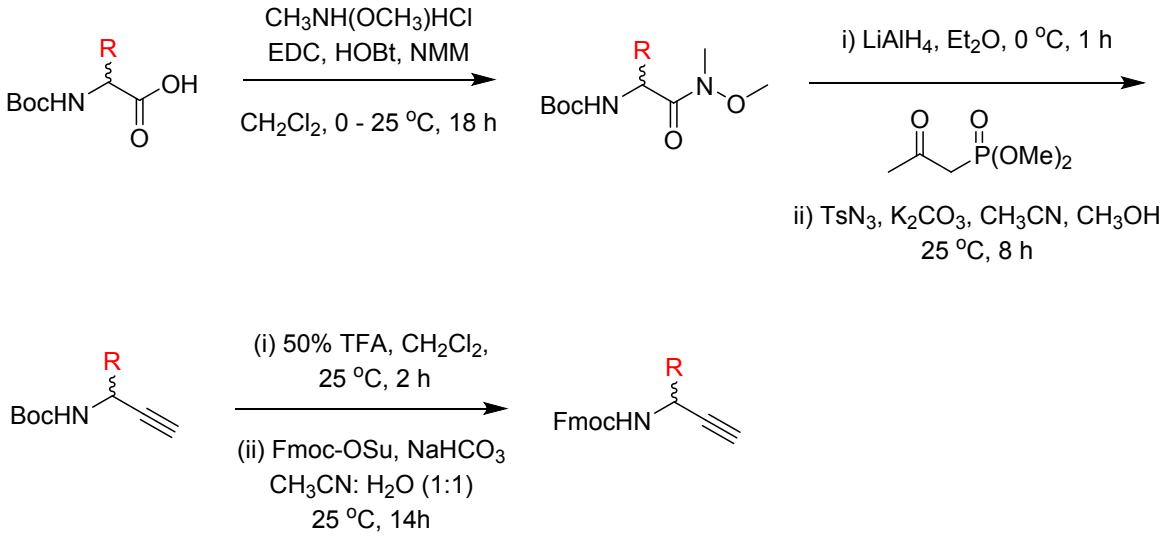
^{1}H NMR (400 MHz, MeOD) δ 6.68 (s, 1H), 3.98 – 3.88 (m, 1H), 3.86 (s, 3H), 3.22 (t, *J* = 6.6 Hz, 2H), 2.69 (s, 3H), 2.63 (s, 3H), 2.15 (s, 3H), 1.92 – 1.45 (m, 4H).

^{13}C NMR (100 MHz, MeOD) δ 173.51, 160.01, 157.99, 139.47, 138.01, 134.92, 125.81, 112.88, 62.87, 56.00, 41.22, 29.57, 26.85, 24.23, 18.69, 12.02.





General Procedure for the Synthesis of Fmoc-amino Alkynes



Scheme S2. Synthesis of Fmoc-amino Aklynes

Synthesis of Boc-Weinreb Amide

To a 250 mL round-bottom flask, Boc-amino acid (10.0 mmol) was dissolved in CH_2Cl_2 solution, and the solution was cooled to 0 °C. HOBt (11.0 mmol), EDC (12.0 mmol) were added into reaction solution, which was stirred at 0 °C for 15 mins. Weinreb amine (11.5 mmol) and *N*-methyl-morphine (12.0 mmol) were added into reaction flask which was stirred 18 h warming to room temperature. Reaction solvent was removed under reduced pressure, and the resultant mixture was participated in EtOAc (75 mL) and 1 M HCl aqueous solution (75 mL). The organic layer was further washed with 1 M HCl aqueous solution (75 mL), sat. aqueous $NaHCO_3$ (75 mL), and brine. The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated to dryness under reduce pressure to perform the desired Weinreb amide without any further purification.

Synthesis of Boc-Amino Alkynes

TsN_3 was synthesized following the protocol previously described.[ref] To a 250 mL round-bottom flask, TsN_3 (12.5 mmol) was dissolved in CH_3CN (120 mL). K_2CO_3 (30 mmol) and 2-oxopropyl phosphonate (12.5 mmol) were added into reaction flask, which was stirred

for 4 h at 25 °C prior to use. To another 250 mL round-bottom flask, Weinreb amide (10 mmol) was dissolved in anhydrous Et_2O (70 mL) at 0 °C. $LiAlH_4$ (20.0 mmol; 1.0 M in THF) was slowly added into reaction flask over the course of 15 mins. The reaction was stirred at 0 °C for 1 h (monitored by $KMnO_4$ TLC staining) and the reaction was quenched by adding 5 % aqueous $KHSO_4$ solution dropwisely till the generation of bubbles stopped. The organic layer was collected, and the aqueous layer was extracted with Et_2O (30 mL x 3). The combined organic layer was dried with anhydrous $MgSO_4$, filtered, and concentrated to dryness to afford Boc-amino aldehyde. Boc-amino aldehyde was dissolved in dry CH_3OH (25 mL) solution, which was added into the reaction flask containing Bestmann reagent, and the reaction was stirred for another 8 h at 25 °C. The solvent was removed under reduced pressure, and the resultant mixture was participated in water and Et_2O (60 mL/60 mL). The organic layer was collected, and the aqueous layer was extracted with Et_2O (30 mL x 3). The combined organic layer was dried with anhydrous $MgSO_4$, filtered, concentrated to dryness, and purified using flash column chromatography (Hex:EtOAc; 6:1) to afford desired Boc-amino alkynes as colorless oil or white solid.

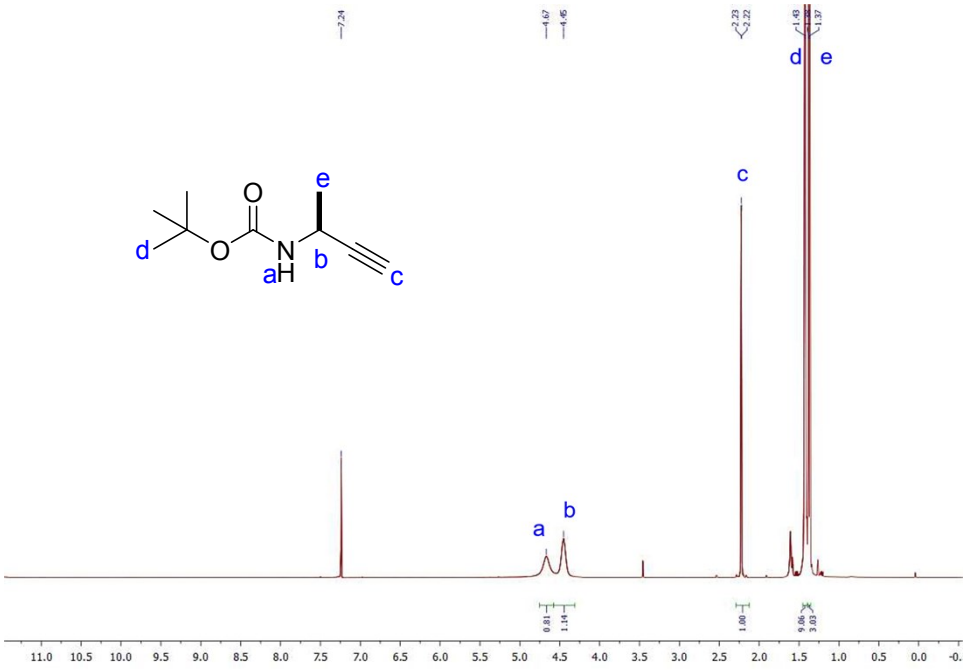
Synthesis of Fmoc-Amino Alkynes

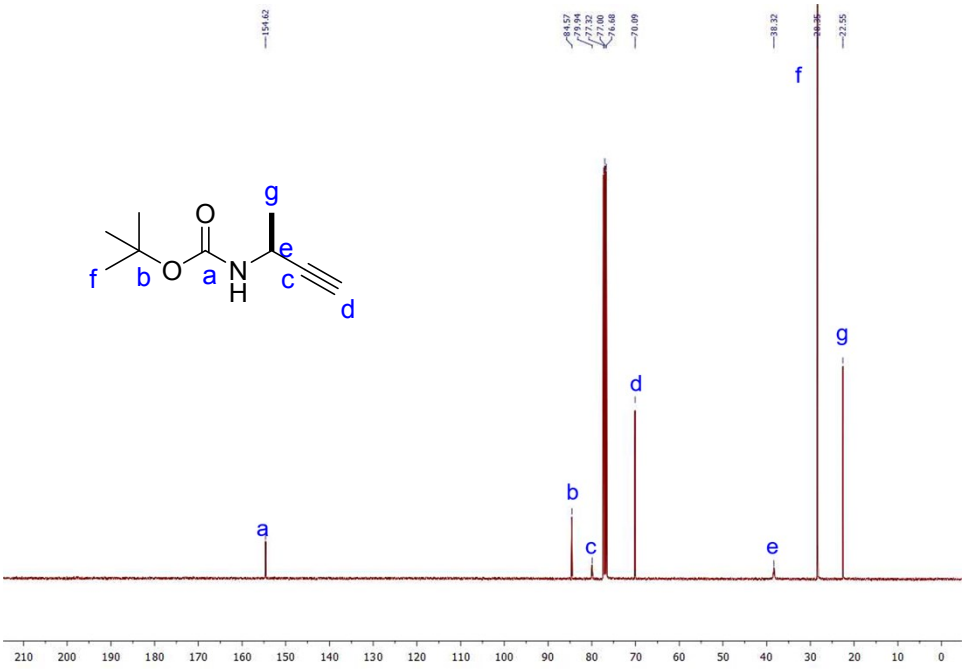
Boc-deprotection reaction was conducted by dissolving Boc-amino alkynes (6.00 mmol) in CH_2Cl_2/TFA (1:1; 50 mL) solution, and the reaction was stirred at 25 °C for 2 h. Solvent was removed, and the residue was re-dissolved in CH_3CN/water (1:1; 60 mL). $NaHCO_3$ (15.0 mmol) and Fmoc-OSu (6.60 mmol) were added into reaction flask, and the reaction was stirred at 25 °C for 14 h. EtOAc (100 mL) was added, and the organic layer was washed with 1 M HCl aq. solution (20 mL x 3), sat. aq. $NaHCO_3$ (30 mL), and brine (10 mL). The organic layer was dried with anhydrous $MgSO_4$, filtered, concentrated to dryness, and purified using flash column chromatography (Hex:EtOAc; 6:1) to afford desired Fmoc-amino alkynes as white solid.

Boc-L-Ala-Alkyne volatile ($C_9H_{15}NO_2$, white solid, 63% yield over three steps)

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.67 (s, 1H), 4.45 (m, 1H), 2.22 (d, *J* = 2.3 Hz, 1H), 1.43 (s, 9H), 1.37 (d, *J* = 6.9 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 154.62, 84.57, 79.94, 70.09, 38.32, 28.35, 22.55.

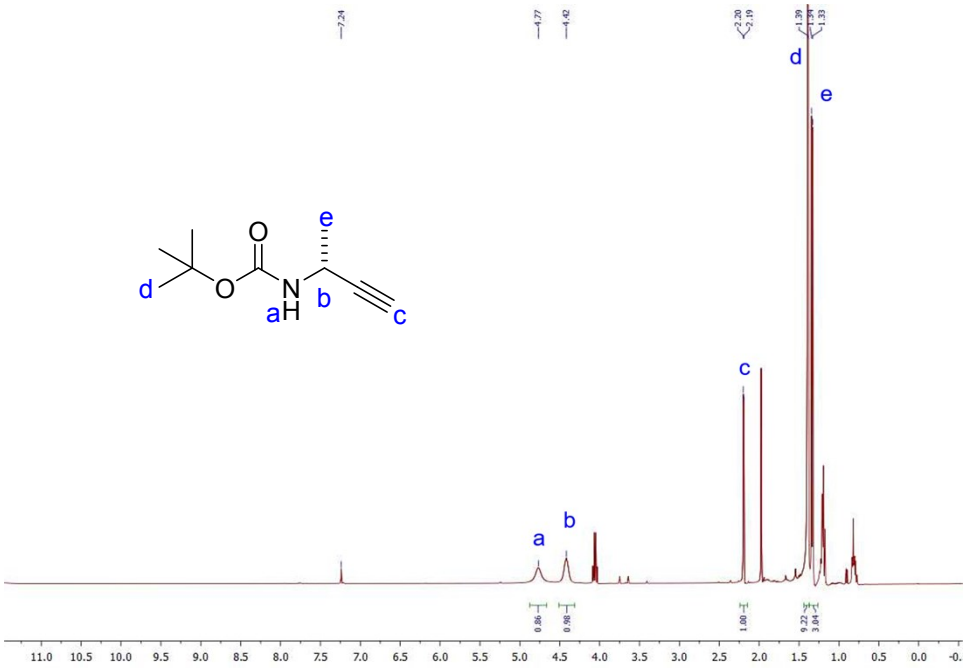


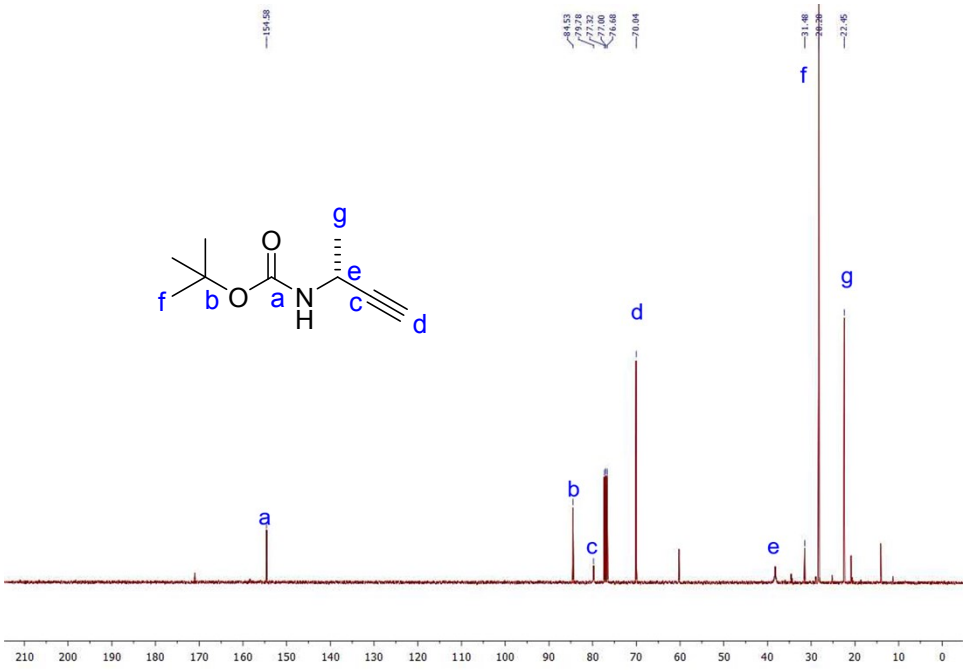


Boc-D-Ala-Alkyne volatile ($C_9H_{15}NO_2$, white solid, 34% yield over three steps)

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.77 (s, 1H), 4.42 (m, 1H), 2.20 (d, *J* = 2.3 Hz, 1H), 1.39 (s, 9H), 1.34 (d, *J* = 6.9 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 154.58, 84.53, 79.78, 70.04, 38.35, 28.28, 22.45.

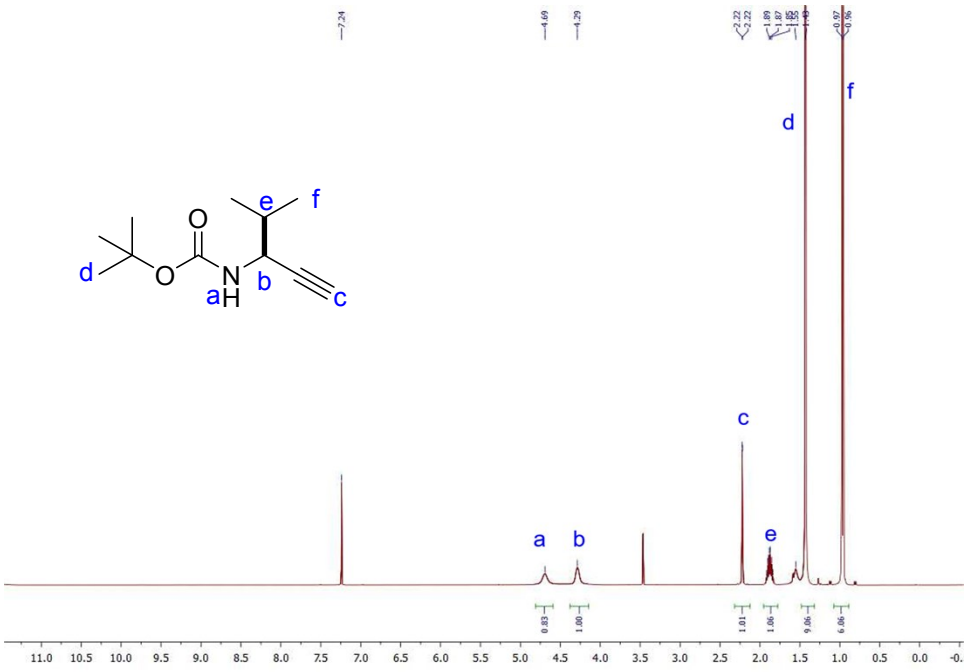


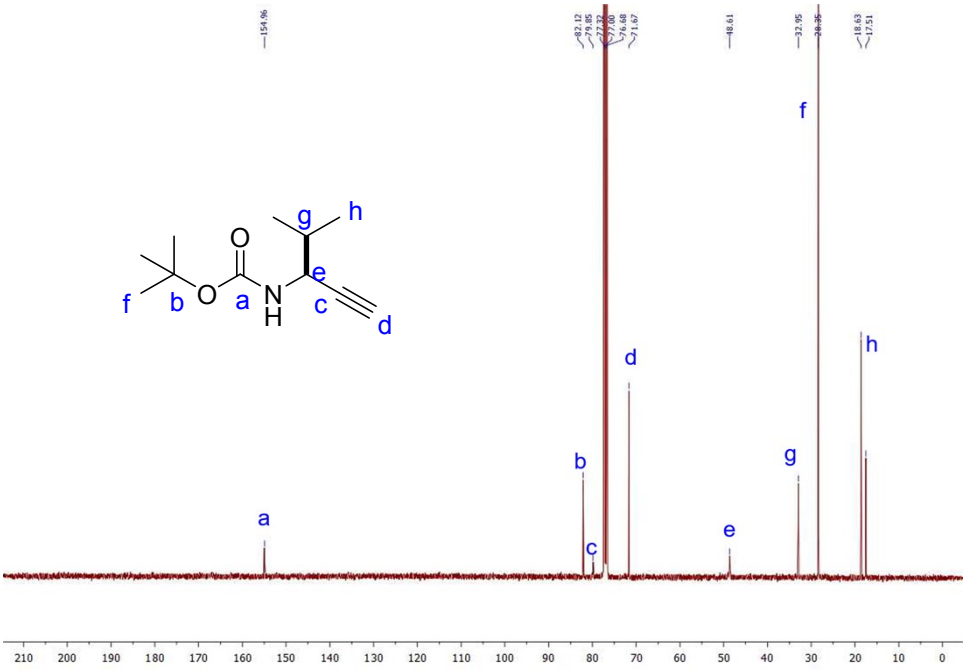


Boc-L-Val-Alkyne ($C_{11}H_{19}NO_2$, white solid, 42% yield over three steps)

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.69 (s, 1H), 4.39 – 4.09 (m, 1H), 2.22 (d, *J* = 2.4 Hz, 1H), 1.88 (dq, *J* = 13.2, 6.6 Hz, 1H), 1.43 (s, 9H), 0.96 (d, *J* = 6.8 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 154.96, 82.12, 79.85, 71.67, 48.61, 32.95, 28.35, 18.63, 17.51.

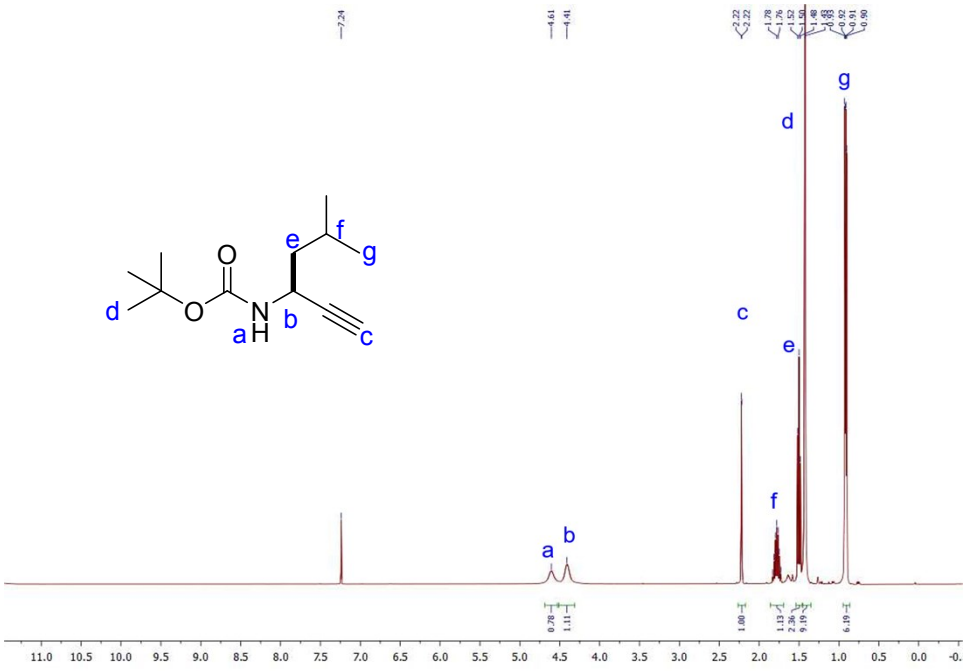


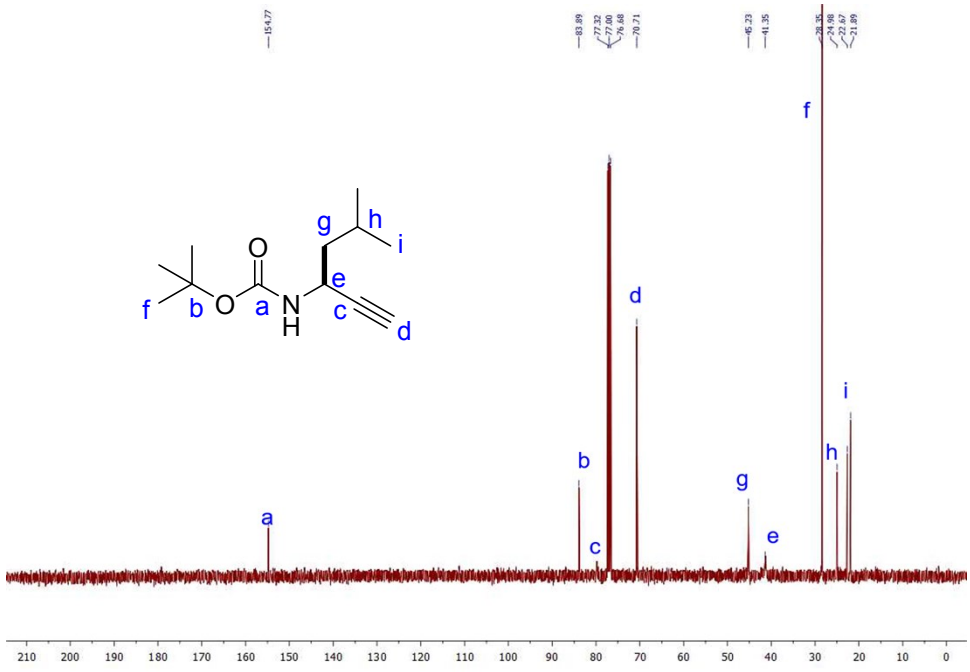


Boc-L-Leu-Alkyne ($C_{12}H_{21}NO_2$, colorless oil, 38% yield over three steps)

^{1}H NMR (400 MHz, $CDCl_3$) δ 4.61 (s, 1H), 4.41 (s, 1H), 2.22 (d, *J* = 2.3 Hz, 1H), 1.85 – 1.71 (m, 1H), 1.50 (t, *J* = 7.4 Hz, 2H), 1.43 (s, 9H), 0.92 (dd, *J* = 6.6, 3.6 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 154.77, 83.89, 79.83, 70.71, 45.23, 41.35, 28.35, 24.98, 22.67, 21.89.

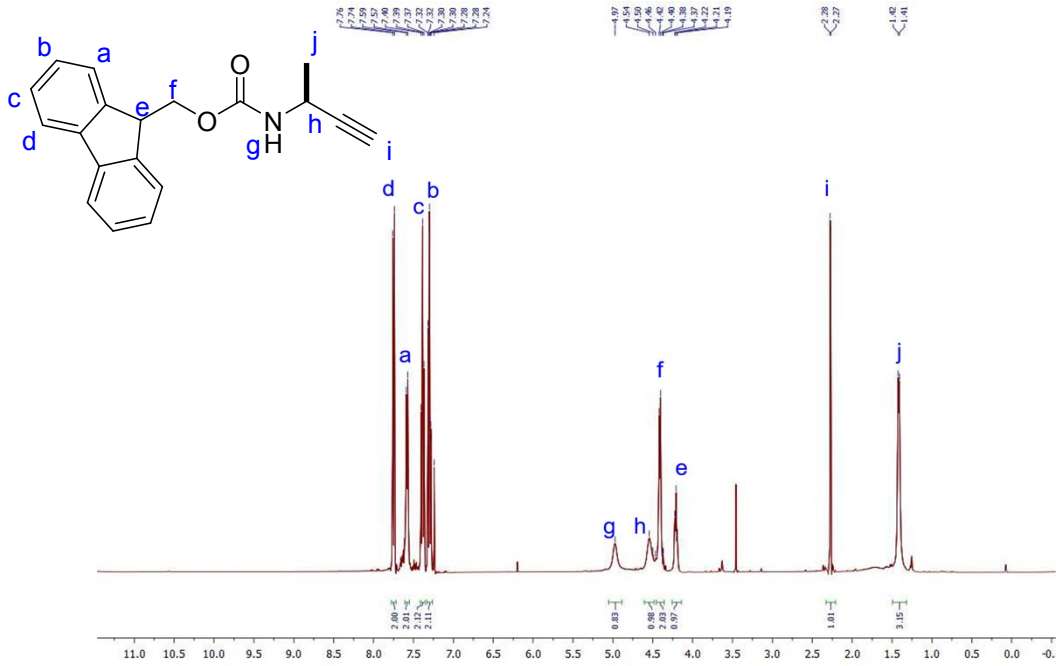


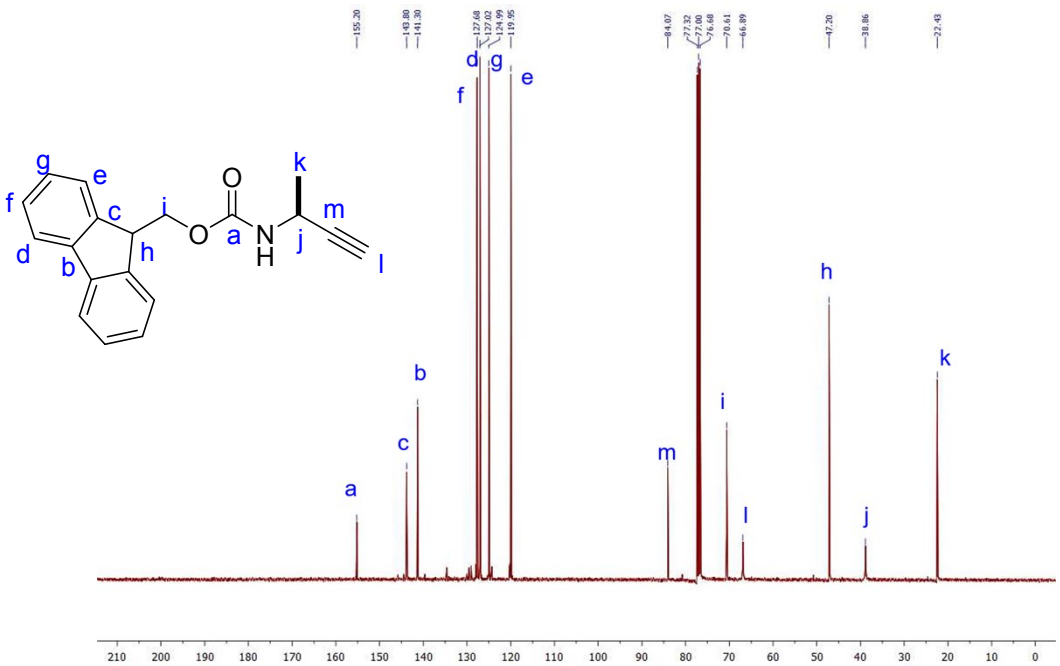


Fmoc-L-Ala-Alkyne ($C_{19}H_{17}NO_2$, 70% yield)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (td, *J* = 7.5, 1.0 Hz, 2H), 4.97 (s, 1H), 4.52 (m, 1H), 4.41 (d, *J* = 6.6 Hz, 2H), 4.21 (t, *J* = 6.6 Hz, 1H), 2.27 (d, *J* = 2.3 Hz, 1H), 1.42 (d, *J* = 6.3 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.20, 143.80, 141.30, 127.68, 127.02, 124.99, 119.95, 84.07, 70.61, 66.89, 47.20, 38.86, 22.43.

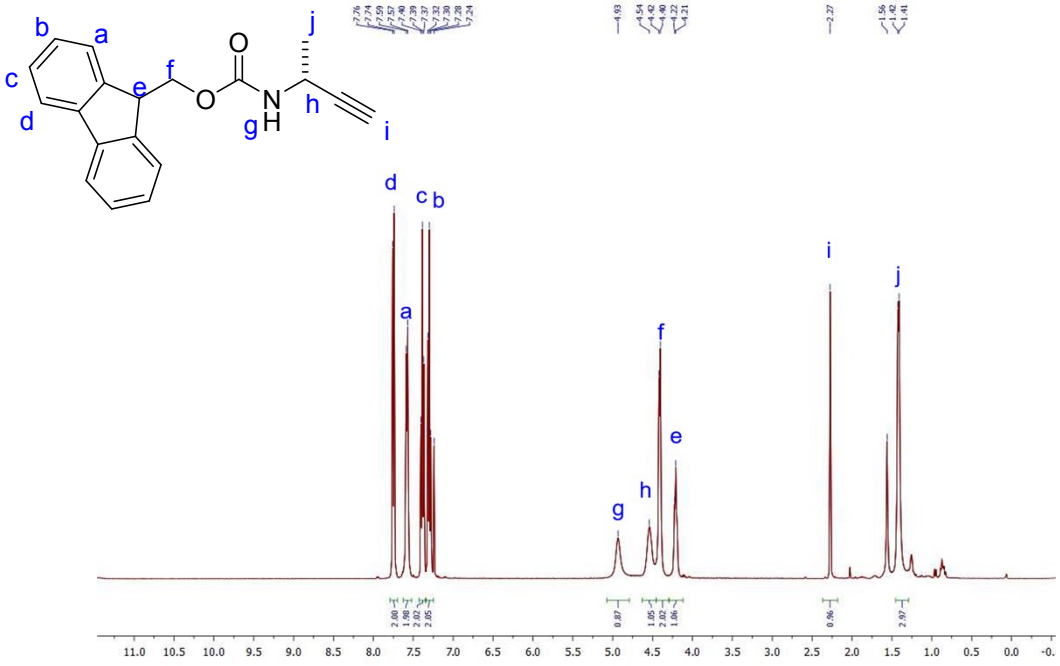


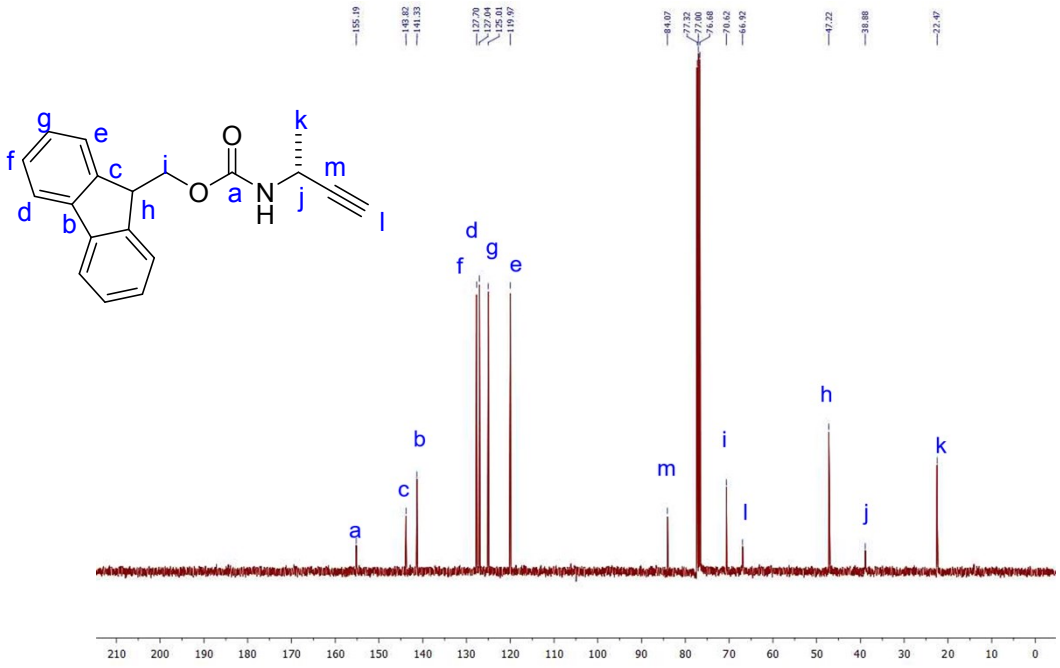


Fmoc-D-Ala-Alkyne ($C_{19}H_{17}NO_2$, 69% yield)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.2 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 4.93 (s, 1H), 4.64 – 4.47 (m, 1H), 4.41 (d, *J* = 6.3 Hz, 2H), 4.29 – 4.14 (m, 1H), 2.27 (s, 1H), 1.42 (d, *J* = 5.7 Hz, 3H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 155.19, 143.82, 141.33, 127.70, 127.04, 125.01, 119.97, 84.07, 70.62, 66.92, 47.22, 38.88, 22.47.

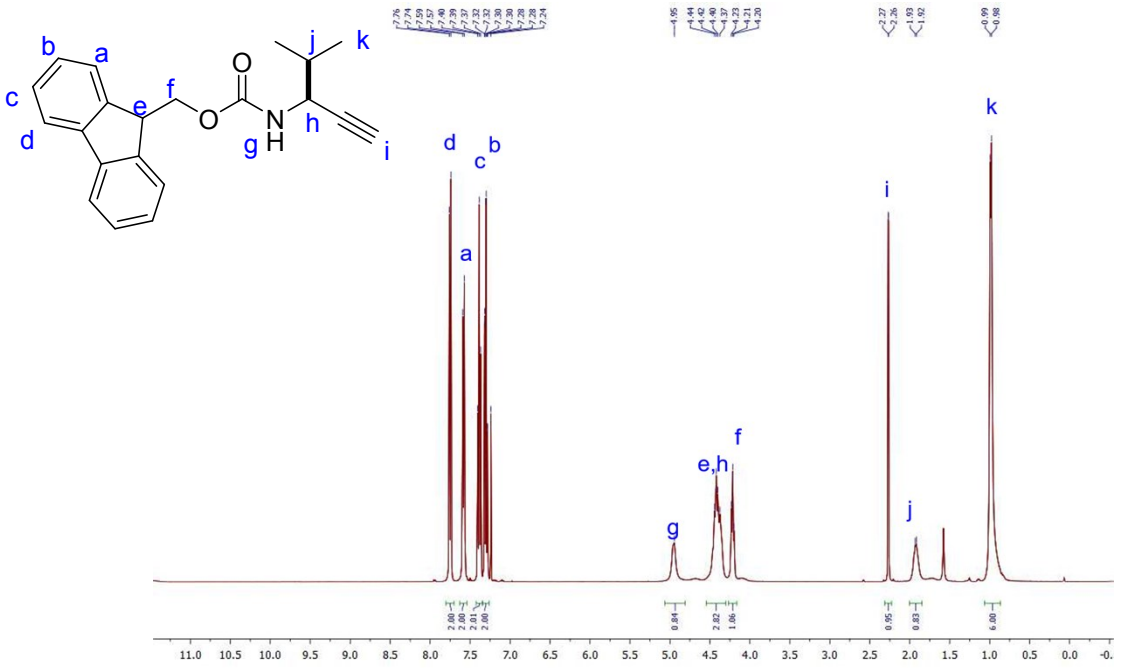


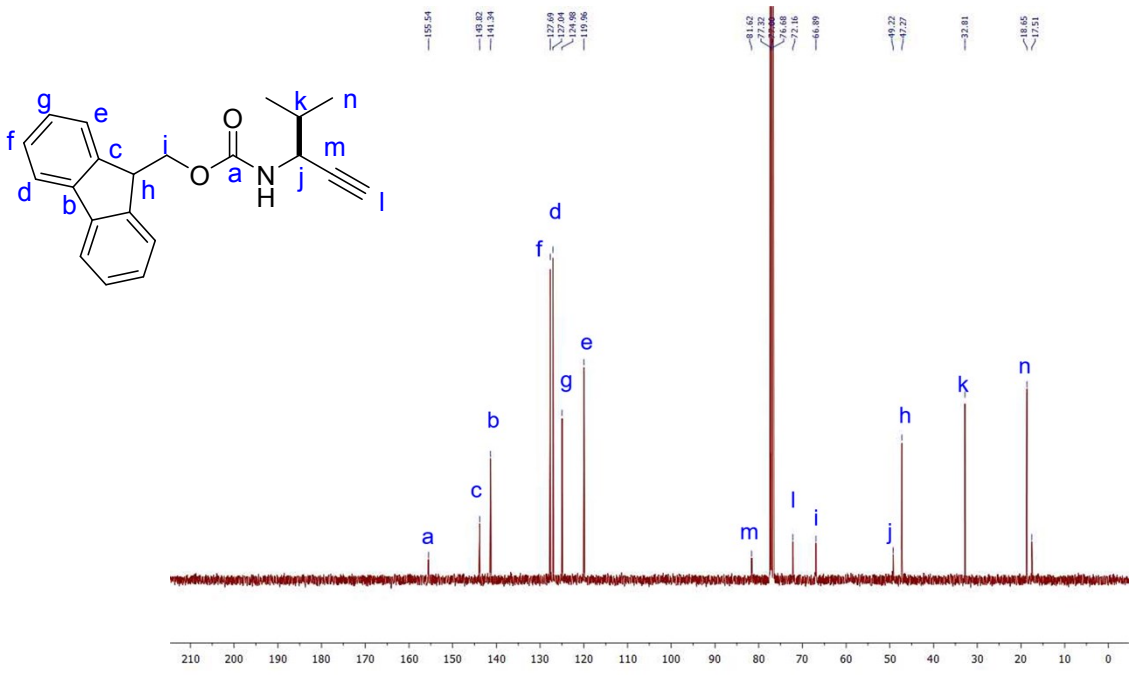


Fmoc-L-Val-Alkyne ($C_{21}H_{21}NO_2$, 76% yield)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.58 (d, *J* = 7.4 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.30 (t, *J* = 7.4 Hz, 2H), 4.95 (s, 1H), 4.41 (m, 3H), 4.21 (t, *J* = 6.7 Hz, 1H), 2.27 (d, *J* = 2.4 Hz, 1H), 1.92 (d, *J* = 5.6 Hz, 1H), 0.99 (d, *J* = 6.1 Hz, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.54, 143.82, 141.34, 127.69, 127.04, 124.98, 119.96, 81.62, 72.16, 66.89, 49.22, 47.27, 32.81, 18.65, 17.51.

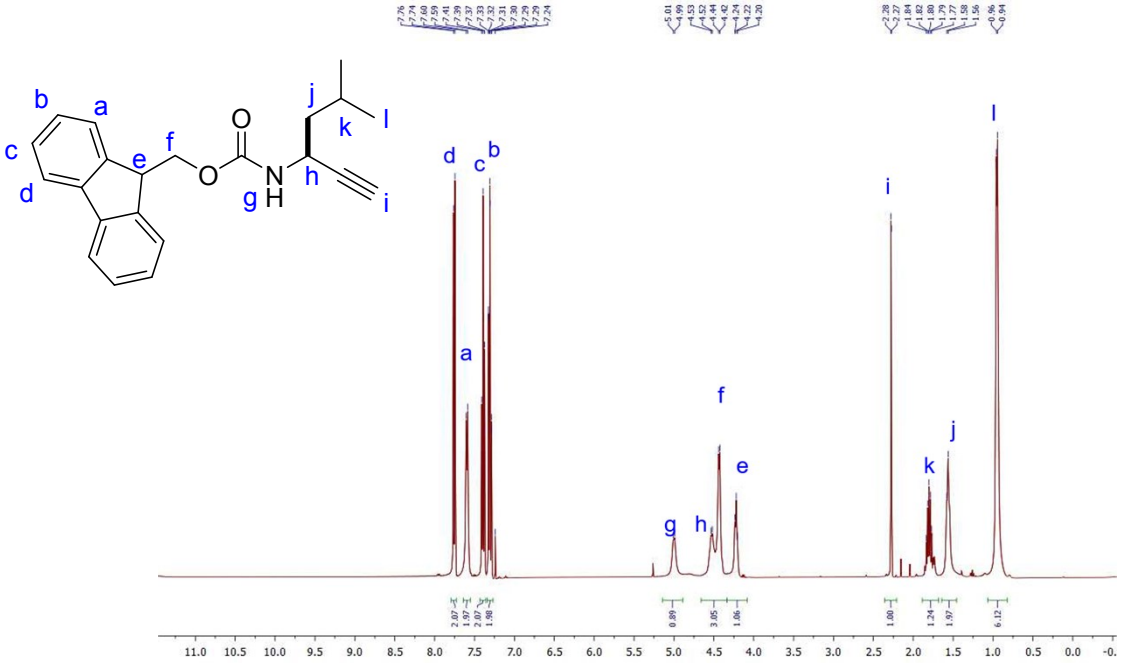


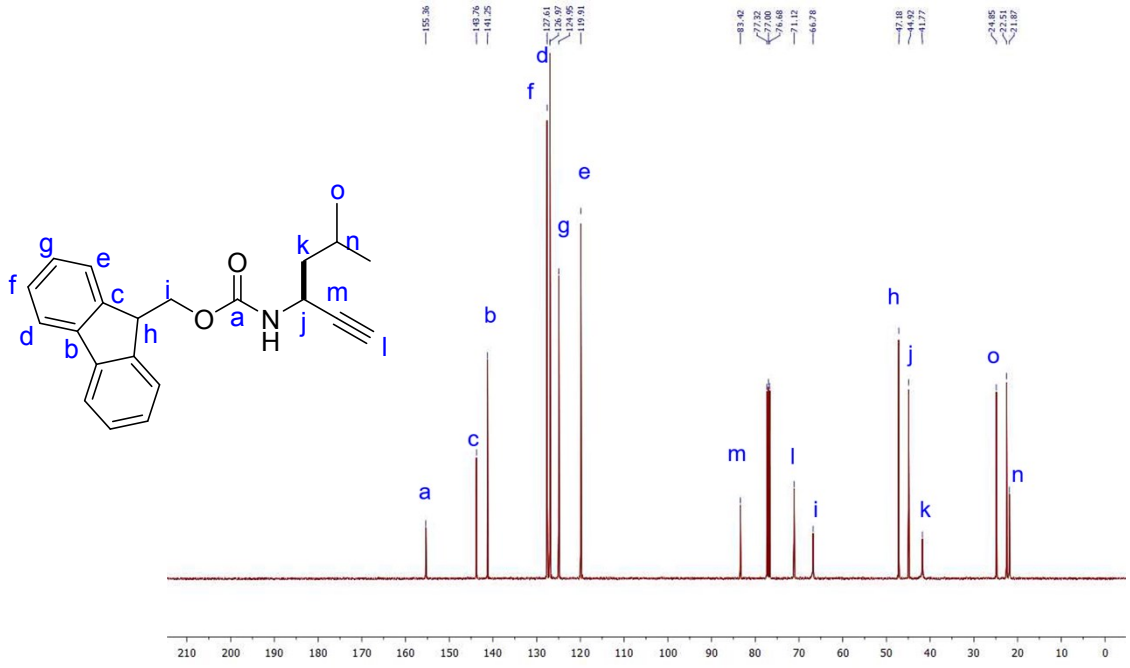


Fmoc-L-Leu-Alkyne ($C_{22}H_{23}NO_2$, 80% yield)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.59 (d, *J* = 7.1 Hz, 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.31 (td, *J* = 7.4, 0.9 Hz, 2H), 5.00 (d, *J* = 6.1 Hz, 1H), 4.48 (m, 3H), 4.22 (t, *J* = 6.3 Hz, 1H), 2.28 (d, *J* = 2.3 Hz, 1H), 1.80 (dt, *J* = 13.5, 6.7 Hz, 1H), 1.57 (m, 2H), 0.95 (d, *J* = 5.8 Hz, 3H).

^{13}C NMR (100 MHz, $CDCl_3$) δ 155.36, 143.76, 141.25, 127.61, 126.97, 124.95, 119.91, 83.42, 71.12, 66.78, 47.18, 44.92, 41.77, 24.85, 22.51, 21.87.





D. Solid-Phase Syntheses and Characterization of Compound LLL-1lvl

Solid-Phase Syntheses

Loading methionine linker on resin: TentaGel-amine resin (600 mg, 0.26 mequiv/g) was swollen with CH_3OH (2 mL) for 5 min, CH_2Cl_2 (5 mL) for 15 min, and then in DMF for at least 1 h in a fritted syringe. After removing DMF, Fmoc-Met-OH (232 mg, 0.62 mmol), $^{i}Pr_2NEt$ (0.22 mL, 1.25 mmol) dissolved 2.5 mL of 0.25 M HATU in DMF solution were added into the fritted syringe. The reaction was heated while stirring under microwave irradiation (100 W, 75 ºC, 10 min). Solution was drained, and washed with DMF (4 mL x 5). The completeness of loading was determined by negative Kaiser test.

Fmoc deprotection: The Fmoc deprotection was conducted by shaking beads in 2 mL of 20% piperidine in DMF for 1 min, and the solvent was drained. Another fresh 4 mL of 20% piperidine solution was added and heated under microwave irradiation (160 W, 75 ºC, 3 min). The solution was drained, and beads were washed with DMF (4 mL x 5), CH_2Cl_2 (4 mL x 3), CH_3OH (4 mL x 3) and DMF (4 mL x 3).

Amide coupling: Fmoc-dansyl-lysine, Fmoc-ethylene glycol linker were prepared following the protocol previously described.[7-9] Fmoc-ethylene glycol linker, Azido acid or Fmoc-amino acid (4 equiv.) and $^{i}Pr_2NEt$ (8 equiv.) were dissolved in 0.25 M HATU in DMF solution (2.5 mL) which were added into the resin containing primary amine. Beads with azide solution were shaken at 25 ^{o}C for 2 h whether other beads solutions were heated under microwave irradiation (100 W, 75 ºC, 10 min), then washed with DMF (4 mL x 5). For arginine azido/amino acid, beads were shaken in the coupling solution for 20 min at room temperature, and then irradiated with microwave (100 W, 75 ºC, 10 min) or shaken for another 2 h at 25 ^{o}C (for azide solution). The solution was replaced with another fresh arginine coupling solution and irradiated again with the same condition. For histidine amino acid, the coupling reaction was performed under microwave at lower temperature to avoid epimerization (100 W, 50 ºC, 15 min).

Click Reaction: Fmoc-amino alkyne (4 equiv.), $^{i}Pr_2NEt$ (8 equiv.), 2,6-lutidine (8 equiv.), and CuI (4 eq.) were dissolved in THF (2.5 mL) which were added into the resin containing azide. The beads were shaken in the dark at 25 ^{o}C for 16 h, and the beads

were washed with 3 mL of 0.05 M EDTA (aq.)/THF (1:1) solution till all CuI was removed (disappearance of green color of the bead). The beads were washed with DMF (4 mL x 5) to remove water.

Hydantoin cyclization: For TentaGel resin, after the Fmoc deprotection at the R^1 position, beads were washed with CH_3CN (2 mL x 3), drained, and treated twice with the solution of *p*-nitrophenyl chloroformate (3 equiv.) and of $^iPr_2NEt$ (6 equiv.) in CH_3CN at ~ 0.05 M concentration. Beads were shaken at room temperature for 15 min each. After coupling step, beads were washed with CH_3CN (4 mL x 3), CH_2Cl_2 (4 mL x 3) and NMP (4 mL x 3). Beads were shaken 5 times with 5 mL of 5 % $^iPr_2NEt$ /NMP (v/v) solution for 1 h each to remove any yellowish byproducts. After the reaction, beads were washed with NMP (4 mL x 5), CH_2Cl_2 (4 mL x 5) and CH_3OH (4 mL x 5) and dried. For 2-chlorotrityl chloride resin, CH_3CN was replaced by CH_2Cl_2 for the reaction.

Side-chain deprotection: TentaGel-amine resin containing side-chain protecting groups were stirred in TFA/Et_3SiH/water (95:2.5:2.5 v/v) cocktail for 4 h at room temperature. Beads were washed with DMF (4 mL x 5), CH_2Cl_2 (4 mL x 3), CH_3OH (4 mL x 3) and DMF (4 mL x 3) before cleavage.

Cleavage from the resin: For TentaGel-amine resin, compounds were cleaved off from the beads by treating with cyanogen bromide (30 mg/mL) in CH_3CN/acetic acid/water (5:4:1 v/v) solution. Beads were shaken at room temperature for at least 24 h. For TentaGel Sram or 2-chlorotrityl chloride resin, compounds were cleaved off from the beads by treating with 4 mL of TFA/Et_3SiH/water (95:2.5:2.5 v/v) cocktail for 4 h at room temperature. After filtration, the crude material in the filtrate was dried under a stream of nitrogen gas and purified by preparative reversed-phase HPLC (10% - 95% CH_3CN/water containing 0.05% TFA). Compounds were lyophilized to obtain white to yellowish powders.

Solid-Phase Syntheses Efficiency Analysis: The solid-phase syntheses of the chemotype is very clean and efficient by using HPLC (monitored at 340 nm) to analyze the crude material (before prep. reversed-phase HPLC purification) with addition of dansyl-derivative as internal standard (Figure S2.)

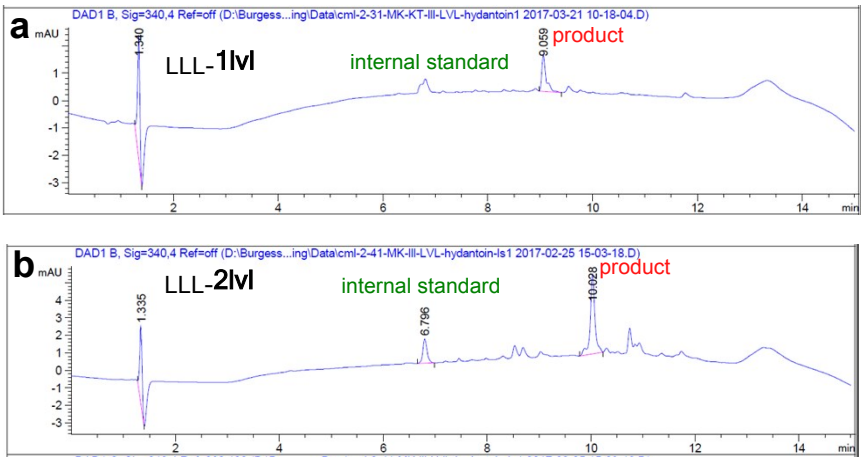
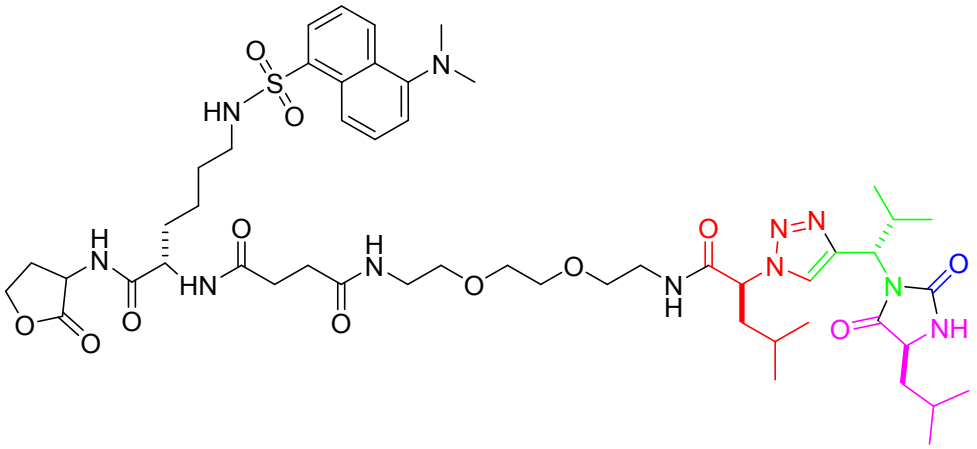


Figure S2. HPLC spectra of crude material from solid-phase syntheses **a** LLL-**1lvl** **b** LLL-**2lvl**

*LLL-**1lvl*** ($C_{51}H_{77}N_{11}O_{12}S$, khaki solid, 49 %)

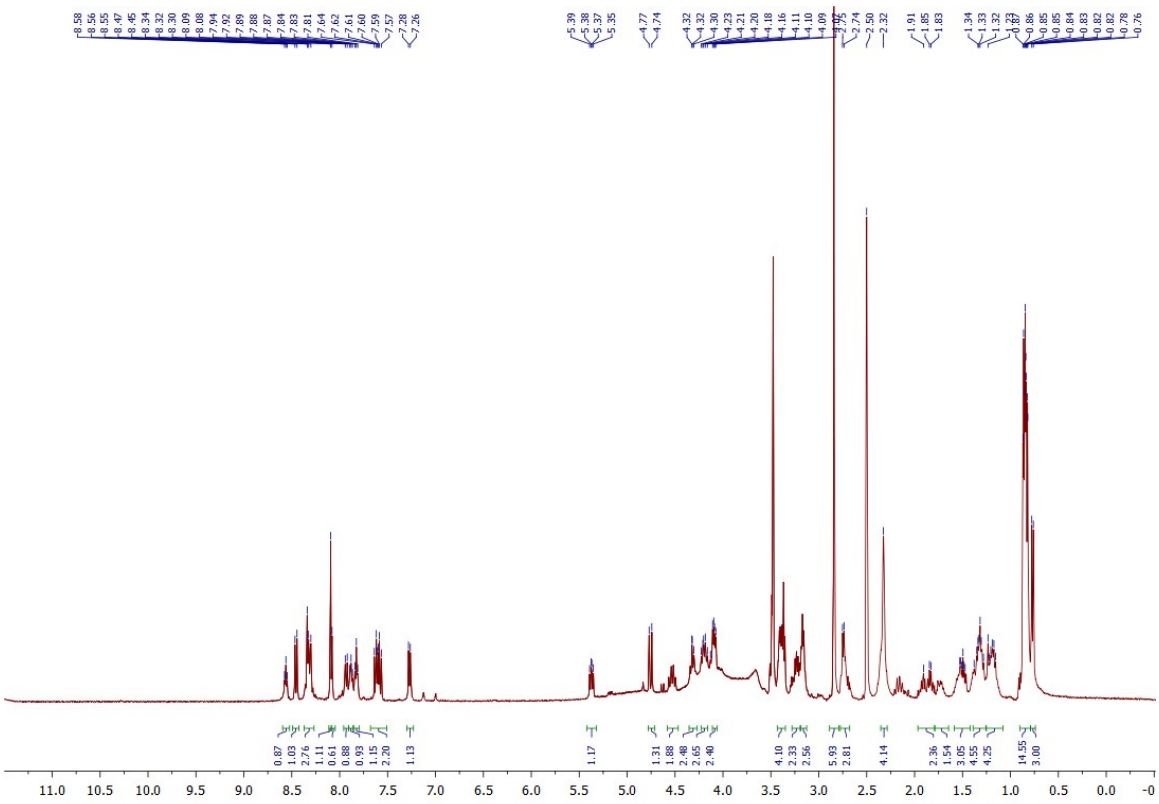


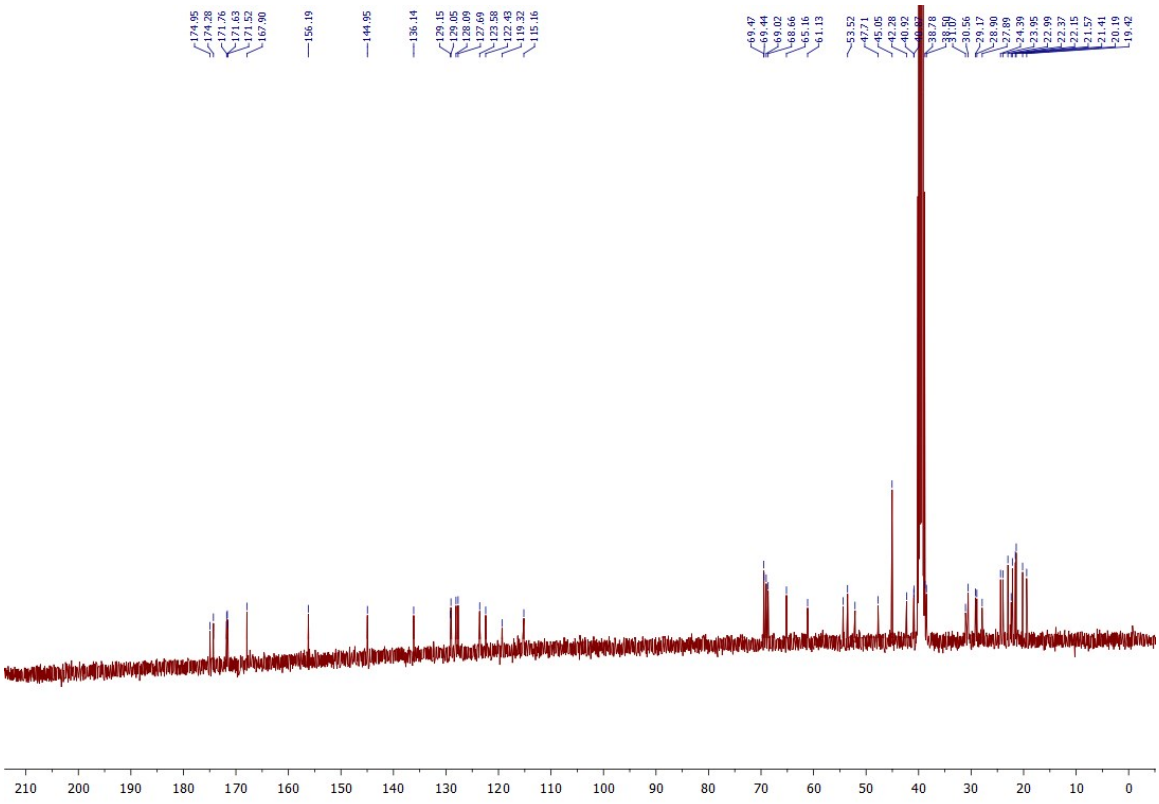
^{1}H NMR (400 MHz, DMSO) δ 8.56 (t, *J* = 5.5 Hz, 1H), 8.46 (d, *J* = 8.4 Hz, 1H), 8.37 – 8.26 (m, 3H), 8.09 (d, *J* = 5.8 Hz, 2H), 7.93 (d, *J* = 8.2 Hz, 1H), 7.91 – 7.85 (m, 1H), 7.83 (t, *J* = 5.8 Hz, 1H), 7.68 – 7.53 (m, 2H), 7.27 (d, *J* = 7.5 Hz, 1H), 5.37 (dd, *J* = 9.3, 6.5 Hz, 1H), 4.76 (d, *J* = 11.1 Hz, 1H), 4.53 (dd, *J* = 18.3, 9.7 Hz, 2H), 4.36 – 4.26 (m, 2H), 4.27 – 4.15 (m, 2H), 4.15 – 4.05 (m, 2H), 3.43 – 3.34 (m, 4H), 3.30 – 3.12 (m, 4H), 2.84 (s, 6H), 2.78 – 2.68 (m, 3H), 2.40 – 2.25 (m, 4H), 1.98 – 1.78 (m, 2H), 1.73 (dd, *J* = 15.3, 8.6 Hz, 2H), 1.57 – 1.43 (m, 3H), 1.43 – 1.27 (m, 4H), 1.27 – 1.07 (m, 4H), 0.84 (ddd, *J* = 8.0, 6.5, 2.1 Hz, 15H), 0.77 (d, *J* = 6.6 Hz, 3H).

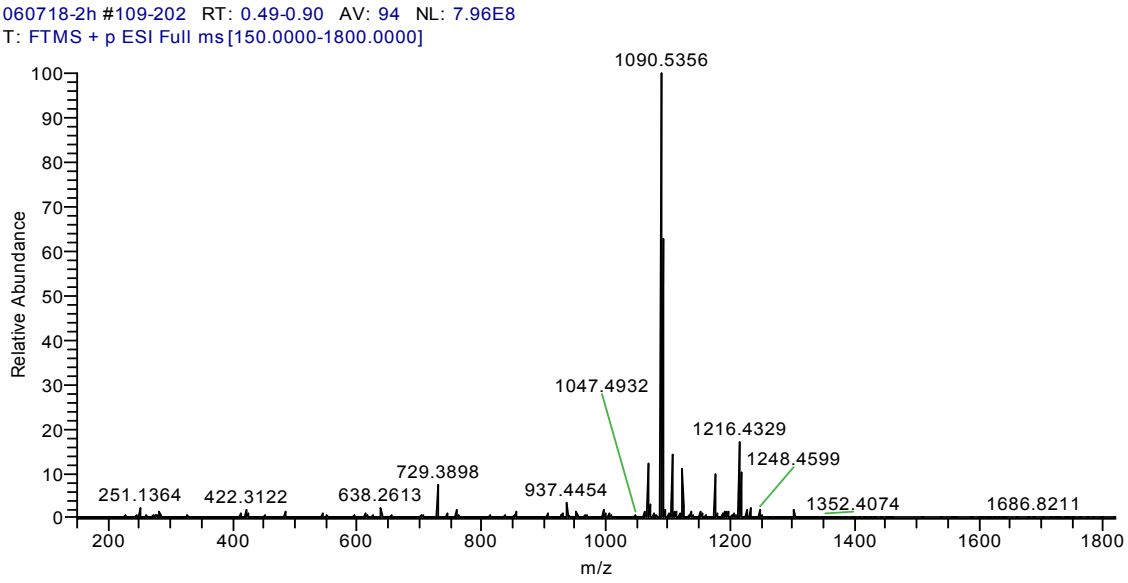
^{13}C NMR (100 MHz, DMSO) δ 174.95, 174.28, 171.76, 171.63, 171.52, 167.90, 156.19, 144.95, 136.14, 129.15, 129.05, 128.09, 127.69, 123.58, 122.43, 119.32, 115.16, 69.47,

69.44, 69.02, 68.66, 65.16, 61.13, 54.38, 53.52, 52.11, 47.71, 45.05, 42.28, 40.92, 40.87, 38.78, 38.50, 31.07, 30.56, 29.17, 28.90, 27.89, 24.39, 23.95, 22.99, 22.37, 22.15, 21.57, 21.41, 20.19, 19.42.

HRMS (ESI) m/z calcd for $C_{51}H_{77}N_{11}NaO_{12}S^+$ 1090.5366; found 1090.5356



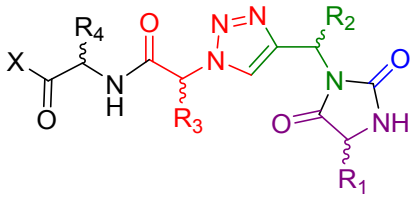




E. QikProp Calculation

QikProp[10, 11] from Schrödinger package (2016) was used to evaluate pharmaceutically properties of compounds **3**

Table S2. QikProp calculations of compounds **3**



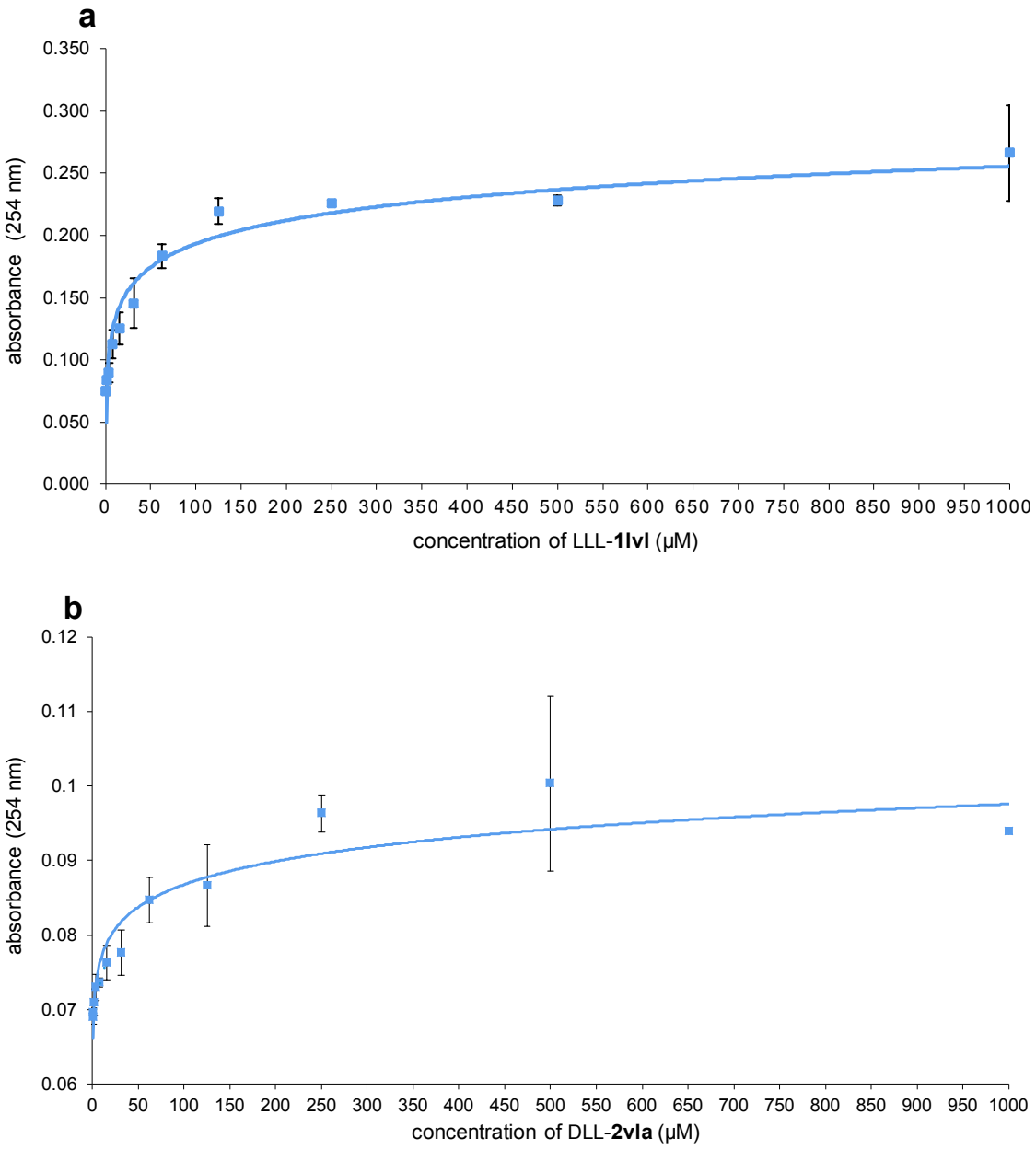
3

mimic Configuration	LLLD-**3lvlr**	DLLL-**3lalr**	DLLL-**3vlar**	DLDL-**3lvlh**	DDLL-**3lalh**	DLLL-**3lvlDbu**	LDLL-**3lalk**
X =	NH_2			OH			
MW	549.36	521.33	507.32	530.30	502.27	493.30	493.30
log $P_{o/w}$	0.126	-0.422	-0.486	-2.551	2.145	0.161	-0.179
rule-of-five violations	3	3	3	2	2	1	1
PSA (Å^2)	240.109	241.779	247.512	193.747	195.188	196.356	178.046
PCaco-2 (nm/s)	1.458	4.177	0.834	5.474	5.429	2.630	1.543

log$P_{o/w}$ – predicted octanol/water partition coefficient; Rule of Five – number of violations of Lipinski's rule of five; PSA – Van der Waals surface area of polar nitrogen and oxygen atoms; PCaco-2 – predicted Caco-2 cell permeability (ideally 25 to 500 nm/s)

F. Determination of Water Solubility

Compound solubility was measured by UV absorbance following the literature.[12, 13] Briefly, stock solution (10 mM) of LLL-**1lvl**, DLD-**2lvl**, and DLL-**2vla** was prepared in methanol, To a 96-well plate was added 0 to 20 µL of stock solution (triplicate of each concentration) which was further dried with nitrogen stream to remove methanol. 200 µL of PBS buffer (pH 7.4) containing 0.5% DMSO was added to each well to make final concentration of each sample from 0 to 1000 µM. The plate was shaken on a horizontal orbital shaker in dark for 6 h at room temperature, and kept steady overnight for equilibrium. Then the plate was centrifuged at 600x g for 10 min. 100 µL supernatant was transferred into 96-well UV transparent plate (Corning® 96 Well Clear Flat Bottom UV-Transparent Microplate) and read compound absorbance at 254 nm against blank using microplate reader (BioTek Synergy H4).


a
absorbance (254 nm)
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
0 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
concentration of LLL-1lvl (μM)
b
absorbance (254 nm)
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
concentration of DLL-2vla (μM)

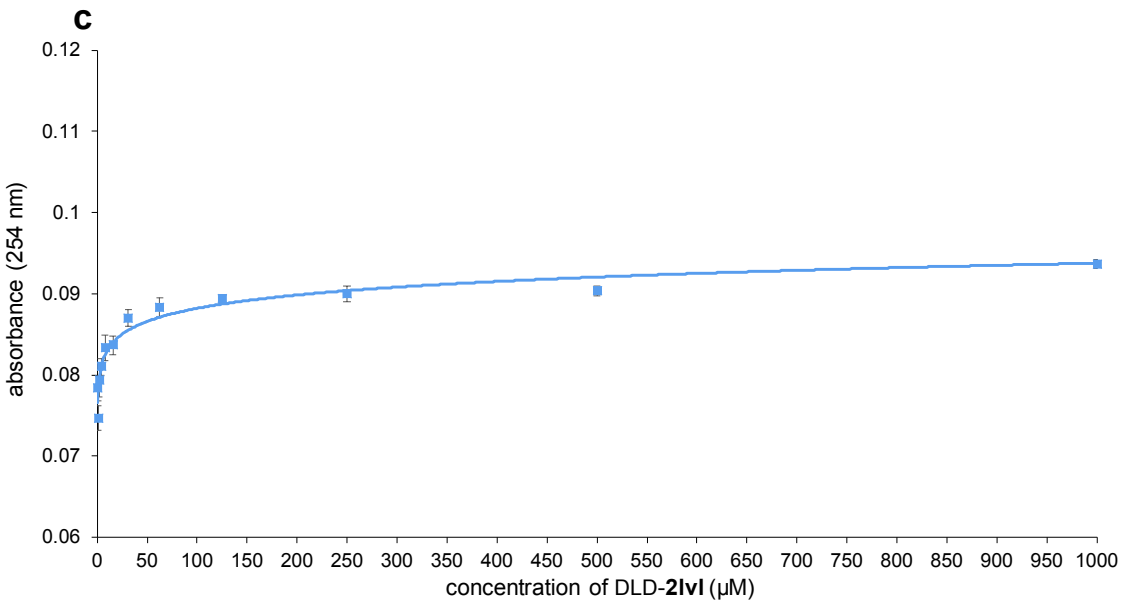


Figure S3. Solubility of **a** LLL-**1lvl b** DLL-**2vla c** DLD-**2lvl** in pH 7.40 PBS 0.5% DMSO. The solubility in PBS buffer were 77±2.5; 56±15; 35±3 μM respectively.

G. Biological Studies

Cell Viability

AlarmarBlue was used to evaluate the cell viability of K562 cells. RPMI-1640 with 10 % FBS medium was used to make K562 cell suspensions which were seeded to 96-well plates as 6000 cells/well (50 μL). Various concentrations of **MLN4924**, **1** – **3**, **N8-peptide** were prepared in the same medium and added to 96-well plates containing cells (50 μL/well) to make final concentrations from 0 to 100 μM. After 72 h incubation at 37 ˚C in 5% CO_2, all plates were added with AlarmarBlue working solution (10 μL/well), and incubated for another 2 h. The . fluorescence intensity (Ex/Em 560/590 nm) was measured using microplate reader (BioTek Synergy H4). Results were analyzed and the survival curves were processed via GraphPad Prism 6.0 software.

Flow Cytometry for Apoptosis/Necrosis Assay

Apoptosis/necrosis of K562 cells was also evaluated using FITC annexin V/PI apoptosis detection kit (Invitrogen V13242). K562 cells were seeded at 3 x 10^5 cells/well in 6-well plates, and incubated with different concentrations of LLL-**1lvl** (0 μM, 12.5 μM, 25 μM, 50 μM and 75 μM) and **MLN4924** (5 μM) for 36 h in RPMI-1640 medium containing 10% FBS (1.5 mL/well). Cells were washed with ice-cold PBS buffer twice, and then

approximative 10^5 cells per sample were resuspended in 100 μL 1X binding buffer, followed by the addition of 5 μL FITC annexin V staining solution and 1 μL PI 1X staining solution. The cell samples were incubated for 15 min at room temperature in the dark, and followed by addition of another 400 μL 1×binding buffer. The cell samples were analyzed immediately by flow cytometry.

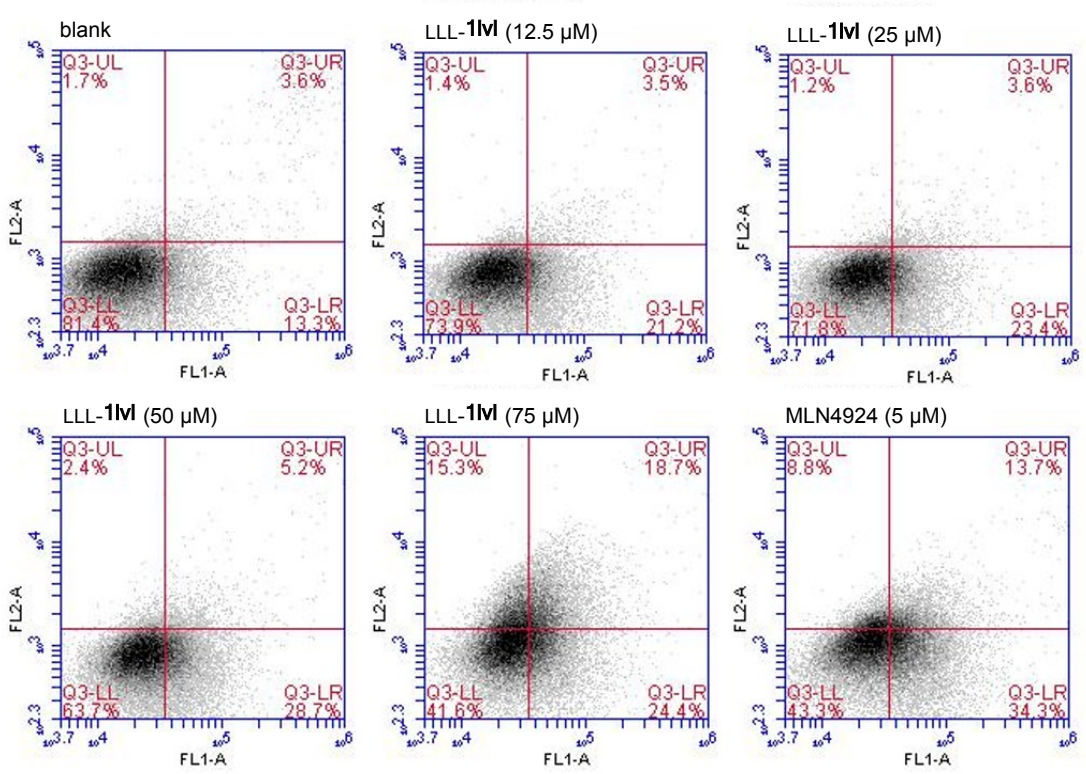


Figure S4. Apoptosis/necrosis analysis of K562 cells treated with LLL-**1lvl** or MLN4924 by flow cytometry

In Vitro NEDDylation Protein Assay

NAE (cat no.E312), Ubc12 (E2-656), fluorescein-NEDD8 (UL-830) proteins, and conjugation conjugation reaction buffer kit (SK-20) were purchased from BostonBiochem® and the protocol followed the instructions of the manufacturer. 1 μL NAE (2.5 μM), 1 μL Ubc12 (25 μM), 1 μL Mg2+/ATP solution (1 mM), 1 μL 10X reaction buffer and 5 μL water solution of compounds (0 μM, 10 μM, 50 μM, 100 μM, and 200 μM) were added into reaction eppendorf tube in order, and the reaction solution was incubated at 37 ˚C for 1 h. The reaction was initiated by addition of 1 μL fluorescein-

NEDD8 (100 μM), and the mixture was incubated at 37 ˚C for another 60 min. 10 μL of EDTA stop buffer (2X) was added to quench NEDDylation in each reaction tube which was further subjected to a non-reducing gel electrophoresis. To a non-reducing conditions on a 15% SDS-PAGE gel, protein samples were made by diluting 2 μL of each reaction samples with 8 μL of Milli-Q H_2O and 2 μL of non-reducing gel loading buffer (6X). Ubc12-NEDD8 levels were determined by fluorescent intensity using Typhoon FLA 9500 fluorescent gel scanner (Alexa-488).

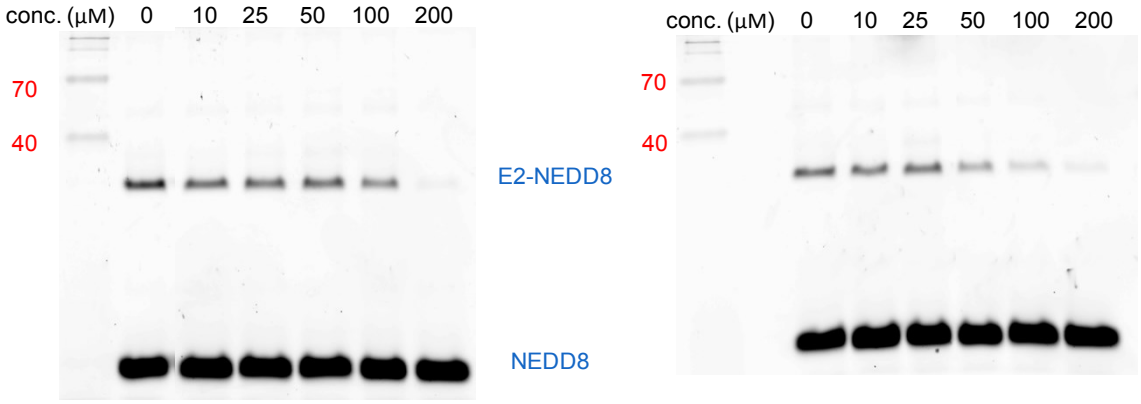


Figure S5. a Representative fluorescence gel imaging of NEDDylation protein assay. DLD-**2lvl** (left); DLL-**2vla** (right)

In Vitro ATP Competitive Assay

The protocol was similar to *In Vitro* NEDDylation Protein Assay, each sample reaction contained NAE (0.25 μM), Ubc12 (2.5 μM), 1X reaction buffer, **LLL-1lvl** (0 μM, 100 μM, and 200 μM) or **MLN4924** (50 μM), fluorescein-NEDD8 (10 μM) and different concentration of Mg2+/ATP solution (10 to 500 μM), The mixture was incubated at 37 ˚C for 60 min, and EDTA stop buffer was added to quench NEDDylation. The NEDD8-Ubc12 complex formation indicated the lead compound **LLL-1lvl** was not ATP competitive inhibitor compared to the result of **MLN4924**.

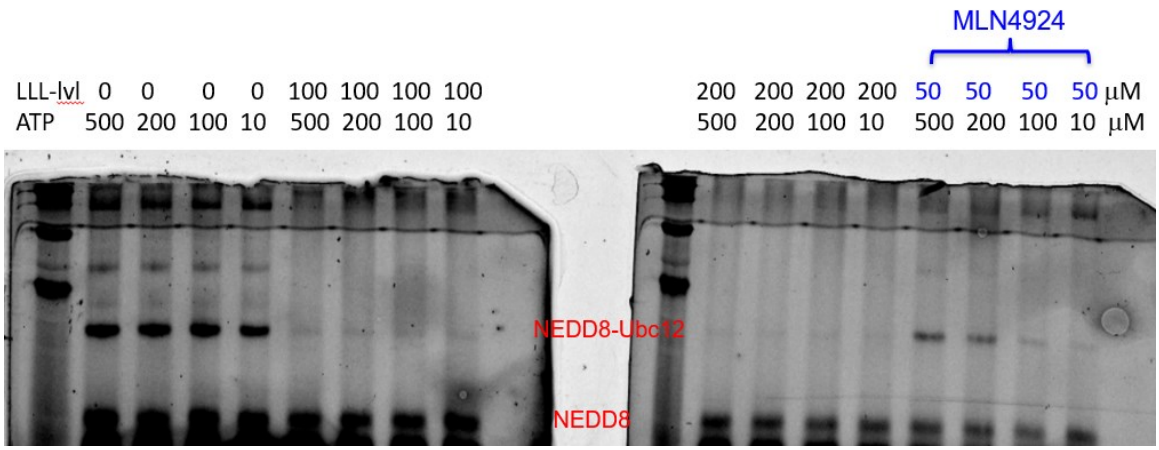
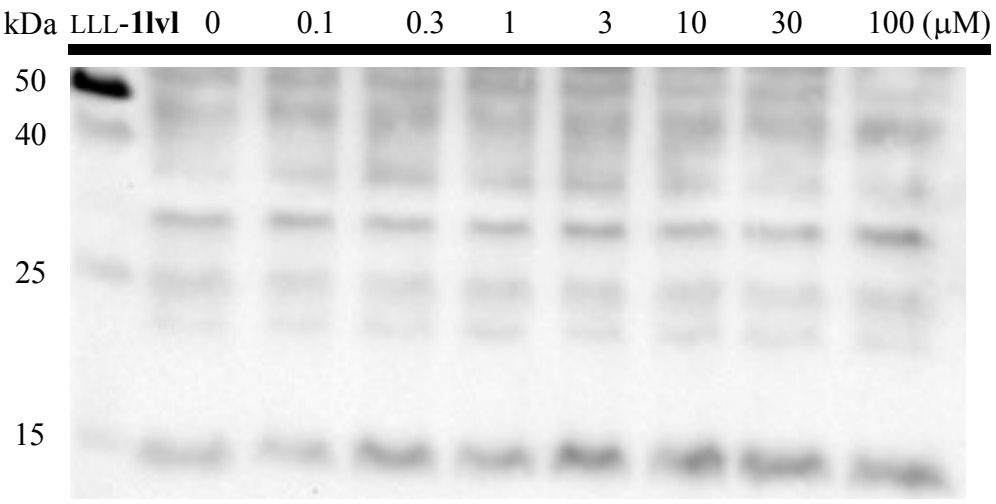


Figure S5. b Representative fluorescence gel imaging of NEDDylation protein ATP-competitive assay.

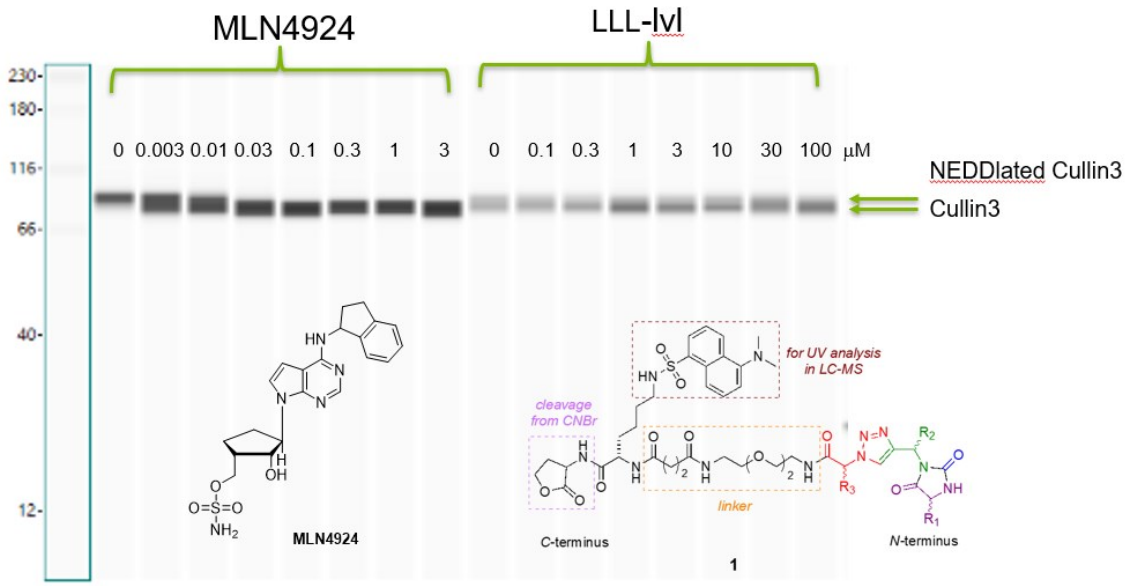
Cell-Based ImmunoBlot

To a 24-well plate, K562 cells (2 x 10^5 cells/well) were incubated to the indicated concentrations of LLL-**1lvl** (0.1 – 100 μM) or 0.1% (v/v) DMSO in RPMI-1640 medium containing 10%FBS for 18 h. Cells were washed twice with ice-cold PBS, resuspended in RIPA lysis buffer with 1% protease inhibitor (~30 μL per sample), and gently shaken on ice for 30 min. Lysate samples were centrifuged at 19000 rcf for 25 min at 4 ˚C to remove cell debris. The supernatant was collected and the protein concentration was determined using Pierce BSA protein assay kit (cat. #23225). Equal total protein amounts (30 μg) were electrophoresed under non-reducing conditions for NEDDylation and Ubiquitination analysis and reducing condition for substrate proteins (Nrf2 and P27) detection on a 15% SDS-PAGE. Protein samples were transferred to a PVDF membrane which was blocked with blocking buffer (ThermoScientific #37536) for 1 h at room temperature, and probed with either NEDD8 antibody (Abcam #ab81264), Ubc9 antibody (CST #4786S), Ubc10 antibody (Abcam #ab187181), Nrf2 antibody (Abcam #ab62352), P27 antibody (CST #3686S), Culin3 antibody (CST #2759S) or Ub antibody (CST #43124S) in blocking buffer (1:1000) overnight at 4 ˚C. The membrane was washed 5 times with TBS/0.1% (v/v) Tween 20 (TBST) and incubated with horseradish peroxide-conjugated secondary antibody in blocking buffer (1:5000) for 1.5 h at room temperature. After washing 5 times with TBST buffer, NEDD8-conjugated proteins were detected using Western blotting substrate (Pierce #32106).

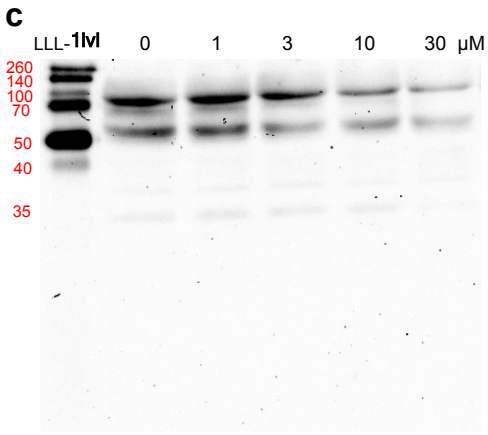
a


kDa
LLL-1lvl
0
0.1
0.3
1
3
10
30
100 (μM)
50
40
25
15

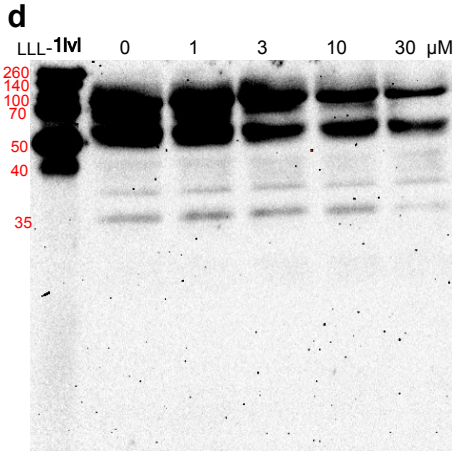
b


MLN4924
LLL-lvl
230-
180-
116-
66-
40-
12-
0 0.003 0.01 0.03 0.1 0.3 1 3
0 0.1 0.3 1 3 10 30 100 μM
NEDDlated Cullin3
Cullin3
MLN4924
for UV analysis in LC-MS
cleavage from CNBr
linker
C-terminus
N-terminus
1

c


LLL-1lvl
0
1
3
10
30 μM
260
140
100
70
50
40
35

d


LLL-1lvl
0
1
3
10
30 μM
260
140
100
70
50
40
35

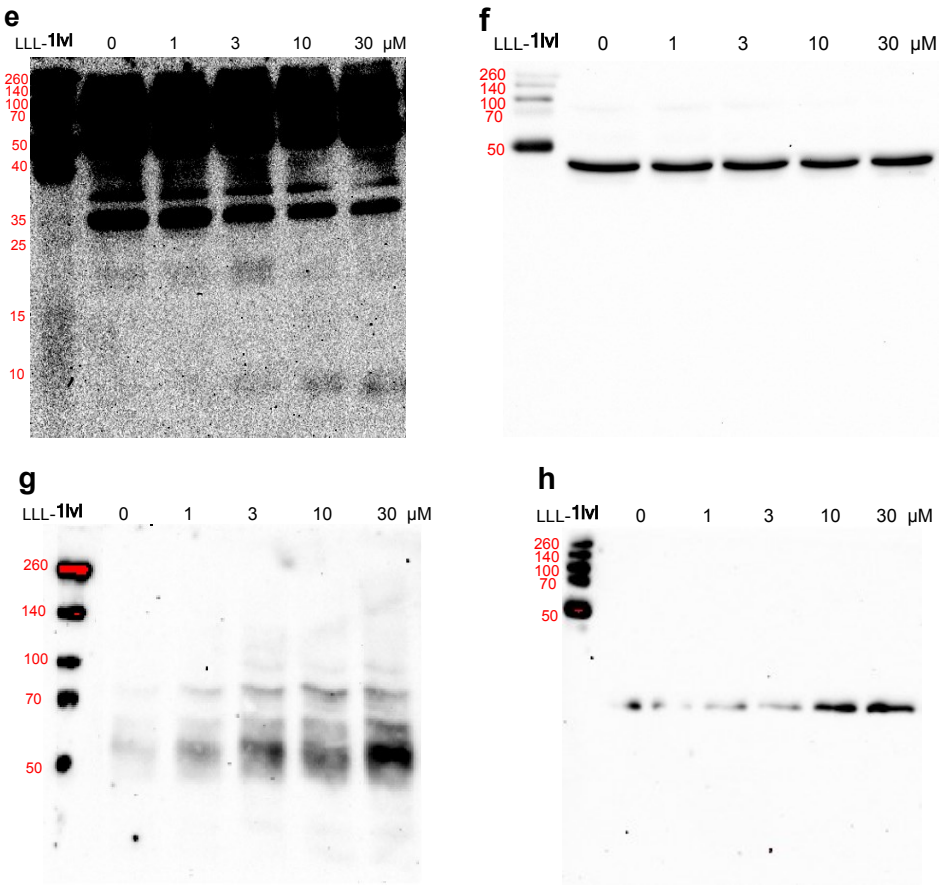


Figure S6. a Immunoblot imaging with Ub antibody. No reduction of Ub conjugate signal was observed in the mass region of most of the E2s, indicating LLL-**1lvl** did not interfere E2-Ub formation. **b** using Cullin3 antibody, lead compound LLL-**1lvl** inhibited NEDDylation of Cullin3 in a dose response manner. **c-e** whole blot imaging using NEDD8 antibody with increased contrast. **f** whole blot imaging using β-actin antibody. **g** whole blot imaging using P27 antibody. **h** whole blot imaging using NRF2 antibody.

NAE protein SiRNA Knockdown

SF cell line 4D-Nucleofector X kit was applied to deliver SiRNA to K562 cells to knock down NAE. Briefly, 1×10^6 cells were suspended in 100 μL transfection solution, prepared by mixing 82 μL Nucleofector solution with 18 μL supplement. The suspended solution was then added with SiRNA (ID# SASI_Hs01_00071904 from sigma) prior to transfer to a single Nucleocuvette. Mission SiRNA universal negative control was served as a scrambled negative control whereas pMAX-GFP was used as a positive control. After the cells were electroporated via Nucleofector core unit, they were incubated for 10 min at room temperature. Cell suspension was then transferred into 6-well plate with 2 mL

pre-warmed media in each well, and was allowed to proliferate for 48 h before usage. To validate the success of knockdown of NAE, 2×10^5 cells were collected and lysed by 30 μL RIPA buffer with 1% v/v Halt protease and phosphatase inhibitor. 0.2 mg of each cell lysate, of which concentration is determined by BCA assay, was loaded on Jess and blotted by NAE antibody (1 to 250 dilution; Abcam ab81264, lot# GR292610-14). To test the effect of LLL-1lvl, wild-type and NAE(-) K562 cells were seeded to a 24-well plate, respectively and dosed with increasing concentrations of LLL-1lvl (1 μM to 30 μM) or DMSO vehicle. The cells were incubated for 18 h before being lysed. 0.2 mg of lysates were loaded on Jess and blotted by NEDD8 antibody (1/250).

Intracellular Imaging

Intracellular localization of the K562 cells was measured using Olympus Fluoview FV1000. The images were taken at 60x/1.20 water immersed objective. Lysosome, Mitochondria and Nucleus were stained using Lysotracker Green DND 26, Mitotracker Green FM and, NucRed respectively were bought from Life Technologies. 405 nm laser was used for organelle staining, 633 nm laser was used for nucleus and 543 nm laser was used for compound LLL-**1lvl**.

Briefly, 10^5 cells were seeded on 4 well chambers (Nunc Lab-Tek) and incubated with 10 μM of compound LLL-**1lvl** for 60 mins. Cells were washed twice with ice-cold PBS, incubated with organelle stains according to manufacturer's instructions. Cells were washed twice again using ice-cold PBS buffer and stained with NucRed for 10 mins.

H. Fluorescence Polarization

Syntheses of FITC-N8-Peptide

N8-peptide was synthesized by solid phase synthesis using TentaGel sram resin via Liberty Blue Automated Microwave Peptide synthesizer (CEM cooperation) with the sequence of (*C*-terminus) GGRLALVLHLV-β-Ala (*N*-terminus). The extra β-Ala spacer avoided the auto-degradation of the peptide. To a fritted syringe, DMF-swollen TentaGel sram resin (200 mg) containing N8-peptide-NH_2 was treated with a DMF solution (2 mL) containing fluorescein isothiocyanate isomer (FITC) (2 eq.) and $^{i}Pr_2NEt$ (4 eq.). The reaction was stirred at room temperature for 8 h, and the beads were washed with 2 mL of DMF (5 times) and were stirred in TFA/Et_3SiH/water (95:2.5:2.5 v/v) cocktail for 4 h at room temperature. After filtration, FITC-N8-peptide crude solution was dried under

nitrogen stream and purified by preparative reversed-phase HPLC (10% - 95% MeCN/water containing 0.05% TFA).

Determination of Optimal Concentration of Fluorescent Probe for FP binding assay[14]

DMSO stock solution of FITC-N8-peptide (20 mM) was diluted from 5 nM to 100 nM in 50 mM HEPES, 50 mM NaCl pH 8.0 buffer. 20 μL of each concentration sample was added in corning 384-well black plate. The polarized fluorescence (EX485/EM528 filter) was measured using microplate reader (BioTek Synergy H4). Figure SXX indicated the stable anisotropy of FITC-N8-peptide was observed after 40 nM which was chosen as the ligand concentration. The experiment was performed in triplicate.

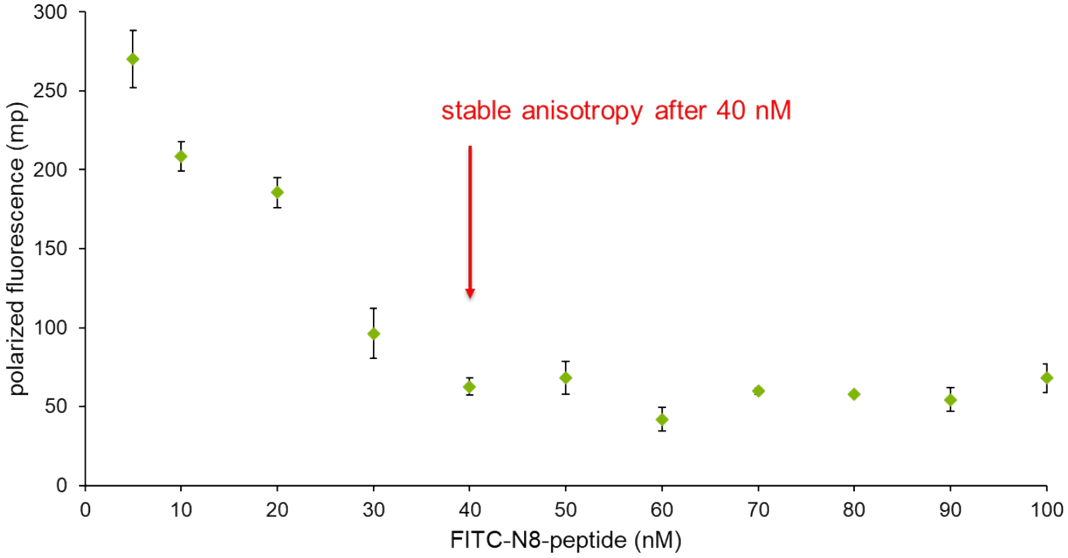
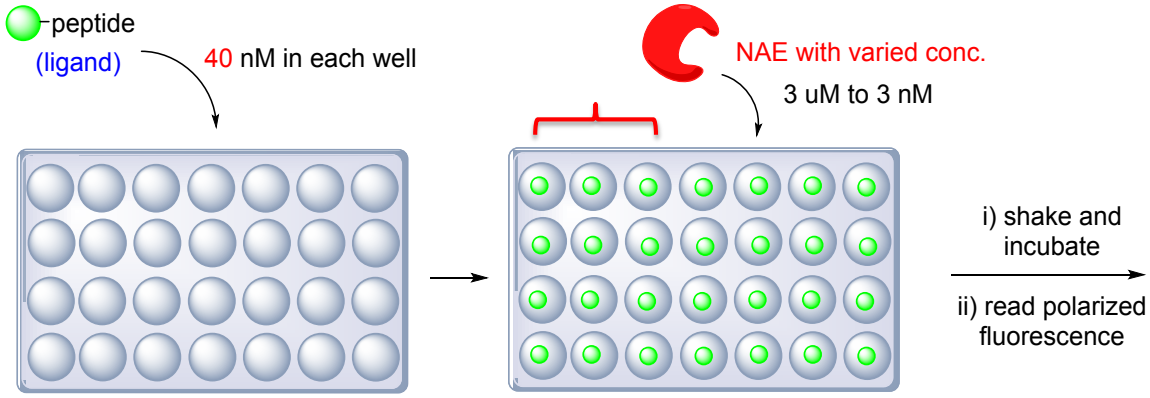


Figure S7. Anisotropy of FITC-N8-peptide in different concentrations

Determination of the FITC-N8-Peptide Direct Binding Affinity to NAE



Briefly, to a corning 384-well black plate, 10 μL of FITC-N8-peptide (80 nM) in 50 mM HEPES, 50 mM NaCl pH 8.0 buffer was added in each well, which was then added with 10 μL of various concentrations of NAE protein in the same buffer to make a final concentration from 0 to 3 μM. The plate was shaken in the dark for 3 h (300 rpm), and the polarized fluorescence was measured using microplate reader (BioTek Synergy H4). The K_d value of FITC-N8-peptide to NAE was calculated using the equation below, where L_T is the total added concentration of ligand; A is the experimental anisotropy; A_f and A_b are the anisotropy of the free ligand and fully bound ligand; R_T is the total NAE concentration.

$$L_T \times \frac{A - A_f}{A_b - A_f} = \frac{L_T + K_d + R_T - \sqrt{[(L_T + K_d + R_T)^2 - 4L_T R_T]}}{2}$$

Determination of the Assay Z-factor

50 mM HEPES, 50 mM NaCl pH 8.0 buffer containing 40 nM of FITC-N8-peptide and 500 nM of NAE protein in the absence (negative control) and presence (positive control) of 10 μM of N8-peptide were incubated at room temperature for 3 h in the dark. Each solution was added to octuplicate wells in a 384-well black plate with final volumes of 20 μL. The Z- factors were calculated using the following equation where σ_- and σ_+ represent the standard deviation of negative and positive control; μ_- and μ_+ represent the mean anisotropy value of the negative and positive control.

$$Z' = 1 - \frac{3(\sigma_- + \sigma_+)}{\mu_- - \mu_+}$$

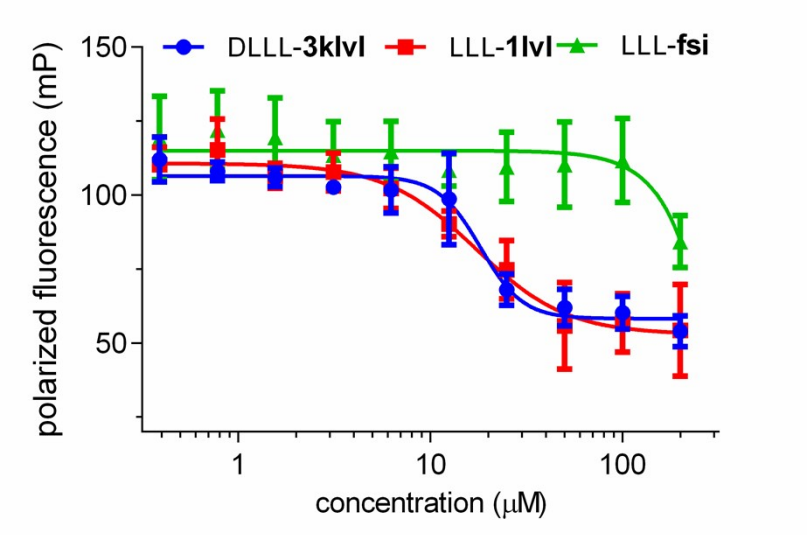
Competitive Binding Assay Using LLL-1lvl

To a corning 384-well plate, each well was added a solution (10 μL) containing 80 nM of FITC-N8-peptide, 1 μM of NAE protein in 50 mM HEPES, 50 mM NaCl pH 8.0 buffer. Each well was then added the same buffer solution (10 μL) containing various concentrations of LLL-**1lvl** (370 nM to 200 μM), and the plate was shaken in the dark for 3 h. The anisotropy value was measured using microplate reader (BioTek Synergy H4), and the competitive binding IC_{50} value can be calculated using the equation below, where A represents the experimental anisotropy; A_{min} and A_{max} represent minimum and maximum anisotropy; C is the concentration of LLL-**1lvl**.

$$A = A_{min} + \frac{(A_{max} - A_{min})C^{slope}}{IC_{50}^{Slope} + C^{slope}}$$

K_i value of LLL-**1lvl** to NAE was calculated by fitting the IC_{50} value to the following equation, where I indicated the concentration of FITC-N8-peptide; K_d indicated the binding affinity of FITC-N8-peptide to NAE.

$$K_i = \frac{IC_{50}}{1 + (I/K_d)}$$



K_i (μM)	no detergent	w/ 0.01 % Tween-20
LLL-**3klvl**	3.8 ± 0.2	9.5 ± 0.4
LLL-**1lvl**	6.4 ± 0.3	9.0 ± 0.4

Figure S8. Fluorescence polarization competitive binding assays with addition of 0.01 % Tween-20. Error Bars show s.d.; n = 6.

I. Morphology Imaging

The cell morphology imaging was taken using Evos FL Imaging System (Thermofisher).

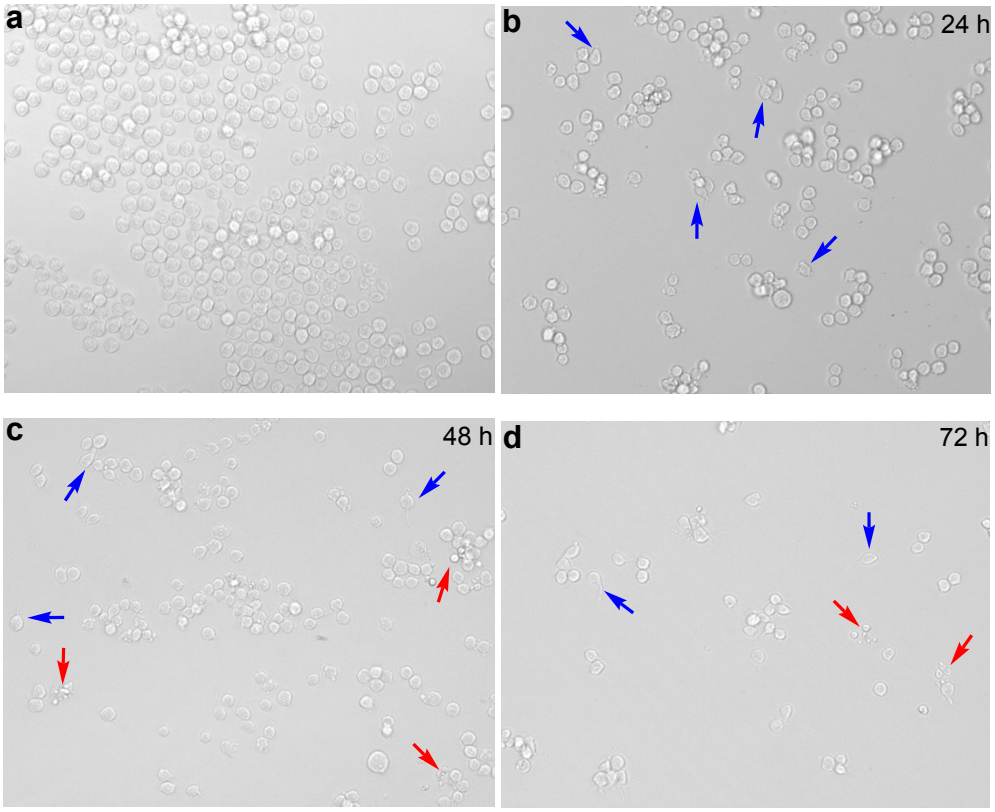


Figure S9. 20x Magnification cell morphology imaging of K562 cells **a** blank **b-d** incubated with 25 μM of LLL-**1lvl** from 24 to 72 h. blue arrow indicated K562 cells with significantly changed morphology. red arrow indicated the dead cells

J. Mass Spec Binding Analysis

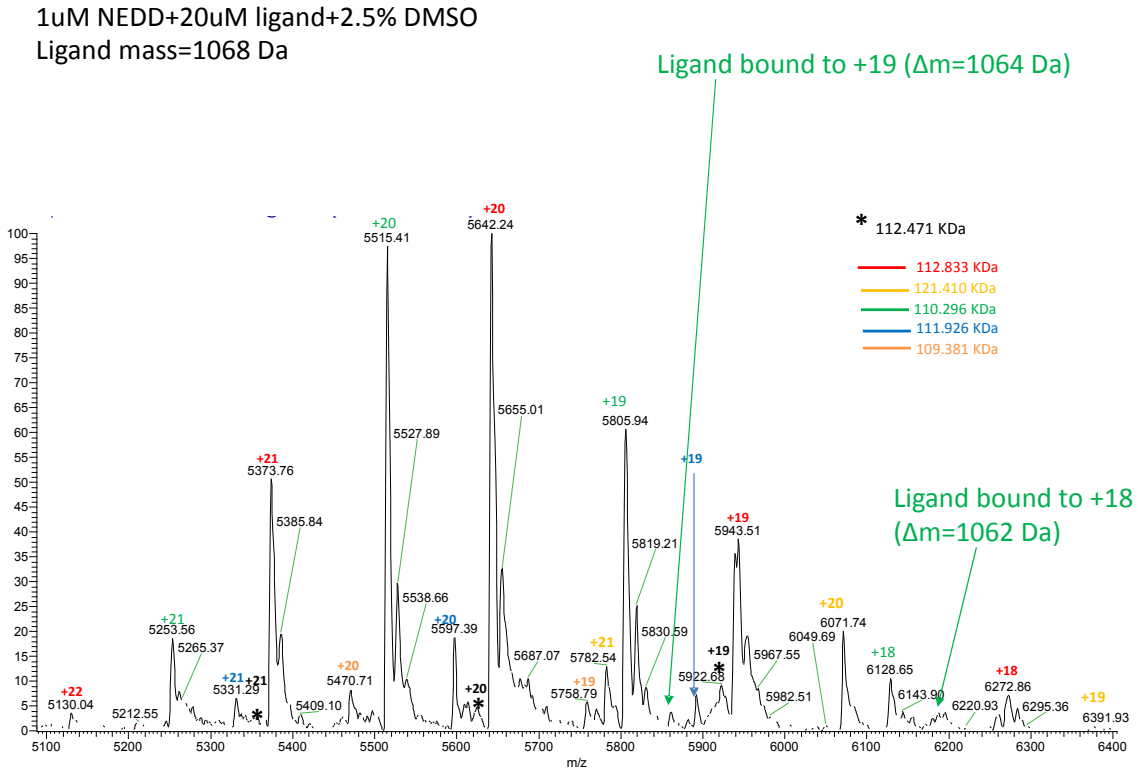


Figure S10. Mass Spec analysis indicated the ligand LLL-**1lvl** bound to NAE heterodimer

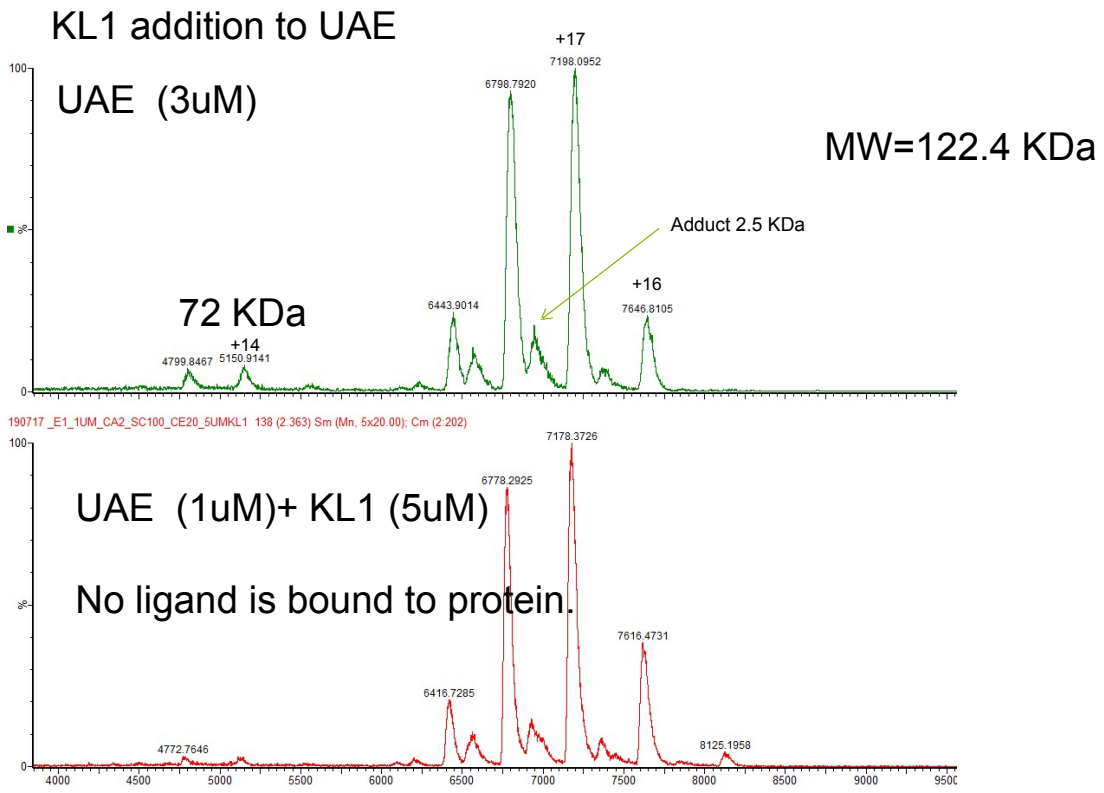


Figure S11. Mass Spec analysis indicated the ligand LLL-**1lvl** didn't bind to UAE

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