## Supplementary Information

# A Versatile Approach to Site-specifically Install Lysine Acylations in Proteins

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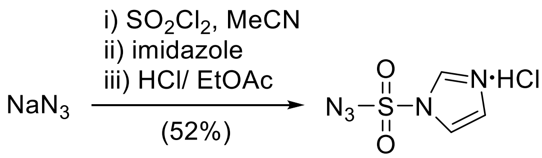
# Contents

# 1. Chemical synthesis of AznL, dPPMT-Ac, dPPMT-Su, and dPPMT-NB-Su

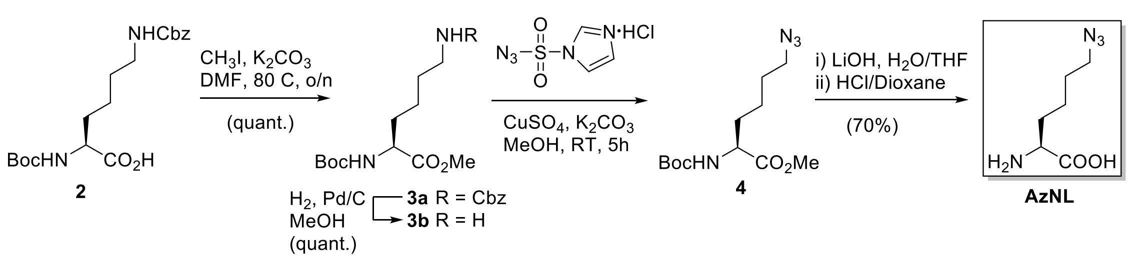
## 1.1 Synthesis of AznL

**SI scheme 1:**




**Synthesis of imidazole-1-sulfonyl azide (1):** To the solution of sodium azide (1.31 g, 20.2 mmol) in anhydrous acetonitrile (30 mL) was added sulfuryl chloride solution (97% solution, 1.7 mL, 20.4 mmol) dropwise in an ice bath. The mixture was then stirred at r.t. for 8 h. Imidazole (2.78 g, 40.9 mmol) was added into the mixture in an ice bath and the afforded solution was stirred at r.t. for another 8 h. The mixture of white slurry was partitioned with EtOAc/$H_2O$ solution and the organic layer was collected and washed with $H_2O$, saturated aqueous $NaHCO_3$, and brine, dried over anhydrous sodium sulfate, and filtered. Hydrogen chloride solution (1 M in EtOAc, 12 mL, 12 mmol) was added into the filtrate in an ice bath. The afforded white suspension was collected by filtration. The filter cake was washed with cold ethanol and EtOAc to afford imidazole-1-fulfonyl azide hydrochloride (**1**) as the desired product (2.2 g, 52%) in white solid. $^1H$ NMR ($D_2O$, 300 MHz) δ 8.88 (br, 1H), 7.87 (br, 1H), 7.44 (br, 1H). MS (Free base + $H^+$): Calc. 174.0, Obs. 174.0362.

**SI scheme 2:**




**Synthesis of compound (3a):** The synthetic procedure of AznL is a modified version from reference[1], which contains four steps. 9.51 g of Boc-Lys(Cbz)-OH (**2**) (25 mmol, Sigma-Aldrich) was dissolved in dry DMF, followed by the addition of $K_2CO_3$ (7.26 g, 52.5 mmol). 3.44 mL of MeI (55 mmol) was gradually added, and was let to stir overnight at 80 $^oC$. Then the reaction

1 Link A., Vink M. K. S., Tirrell D. A., *Nat. Protocols* 2007, 2, 1879-1883

mixture was quenched by the addition of 50 mL $H_2O$, and extracted by EtOAc 30 mL for 3 times. The EtOAc layers were combined, washed with brine, and dried by anhydrous $Na_2SO_4$. The crude product was then purified by the silica gel flash column chromatography (eluted at 10% EtOAc/hexane) to give an oil product (**3a**) (9.6 g, 97%).

**Synthesis of compound (3b):** The solution of Boc-Lys(Cbz)-OMe (**3a**) (3.9 g, 10 mmol) in methanol (100 mL) was added palladium on activated carbon (Pd 10%, 0.6 g, 0.6 mmol). The mixture was stirred at r.t. with hydrogen bubbled through where the reaction progress was monitored by the TLC analysis. The reaction was terminated as the starting protected amino acid on TLC disappeared. Then the reaction mixture was filtered through a celite cake packed on a Buchner funnel and the flowed-through solution was concentrated under reduced pressure to afford colorless oil that was characterized as the desired product (**3b**). The product was directly used in the next step without further purification.

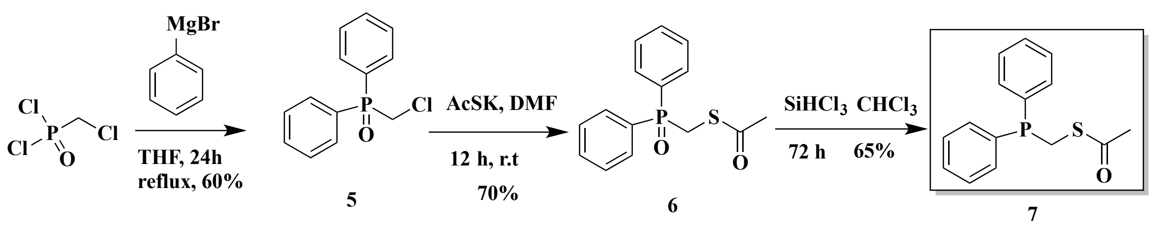
**Synthesis of compound (4):** Boc-Lys-OMe (**3b**) (0.81 g, 3.1 mmol) from the previous step was dissolved in methanol (20 mL). $CuSO_4$ $5H_2O$ (6.8 mg, 0.45 mmol) and $K_2CO_3$ (888 mg, 6.4 mmol) were added into the solution followed by the addition of imidazole-1-sulfonyl azide hydrochloride (**1**) (650 mg, 3.1 mmol) into the mixture in an ice bath. The resulting mixture was stirred at r.t. for 8 h. After addition of EtOAc (10 mL), the diluted mixture was then washed with water, saturated aqueous $NaHCO_3$, brine, dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The crude product was further purified by the silica gel flash column chromatography (eluted at 5% EtOAc/hexane) to give a clear oil as the desired product (**4**) (0.7 g, 79%). $^1$HNMR ($CDCl_3$, 300 MHz). δ 5.09 (d, 1H, J = 1.6 Hz), 4.33 (dd, 1H, J = 1.6, 1.8 Hz), 3.77 (s, 3H), 3.24 (t, 2H, J = 1.2 Hz), 1.99-1.78 (m, 2H), 1.78-1.58 (m, 2H), 1.58-1.32 (m, 12H).

**Synthesis of AznL:** The solution of ester from the previous step (**4**) (0.7 g, 2.4 mmol) in a 1:1 mixture of methanol and THF (24 mL) was added LiOH aqueous solution (0.5 M, 24 mL) and stirred at r.t. The reaction progress was monitored by the TLC analysis. After 5 h, the spot representing the starting ester on TLC plate disappeared. The reaction mixture was then diluted with EtOAc/$H_2O$. After partition, the organic layer was discarded and the aqueous layer was acidified by the addition of 3M HCl until pH was adjusted to 3-4. The mixture was than extracted with EtOAc twice. The organic layers was combined, washed with brine, dried over anhydrous $Na_2SO_4$, and concentrated to afford clear sticky oil as the desired product which was subjected directly to next step without chromatography purification.

The afforded acid from the previous step (0.8 g, 2.9 mmol) in dioxane (5 mL) was added into a HCl solution (4 M in dioxane, 10 mL). The mixture was stirred at r.t. The reaction progress was monitored by TLC. After reaction for 3 h, the HCl solution were removed under reduced pressure. The afforded oil was dissolved in minimal amount of water, neutralized with aqueous sodium hydroxide and subjected to the ion exchange chromatography (Dowex 50WX4) to afford a pale while solid as the desired product (**AznL**) (150 mg, 36% for two steps). $^1$H NMR ($D_2O$, 300 MHz). δ 4.17 (t, 1H, J = 1.4 Hz), 3.35 (t, 2H, J = 1.2 Hz), 2.10-1.79 (m, 2H), 1.78-1.30 (m, 4H). MS (Aznl+$H^+$): Calc. 173.1, Obs. 173.1040.

### 1.2 Synthesis of dPPMT-Ac

**SI scheme 3:**



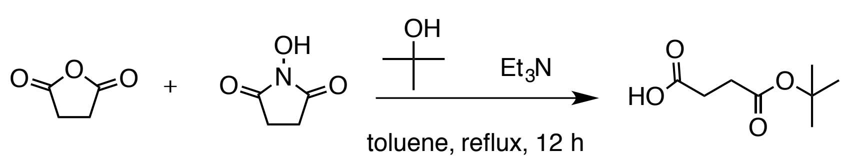

**Synthesis of Compound (5):** Chloromethylphosphonic dichloride (2 g, 12 mmol) was dissolved in freshly distilled THF (30 mL). A solution of phenylmagnesium bromide (1.0 M) in THF (24 mL, 24 mmol) was added dropwise over 30 min. The resulting mixture was stirred at reflux for 24 h. The reaction was then quenched by the addition of water (20 mL), and solvent was removed under reduced pressure. The residue was taken up in $CH_2Cl_2$ and washed once with water (50 mL) and once with brine (50 mL). The organic layer was dried over anhydrous $MgSO_4$ and filtered, and solvent was removed under reduced pressure. The residue was purified by flash chromatography (silica gel, 3% MeOH in $CH_2Cl_2$). Phosphine oxide **5** was isolated as a white solid in 63% yield. $^1$HNMR ($CDCl_3$, 300 MHz) δ 7.84-7.79 (m, 4H), 7.60-7.59 (m, 2H), 7.55-7.49 (m, 4H), 4.05 (d, 2H, J= 4.2 Hz). $^{31}$P NMR ($CDCl_3$, 200 MHz) δ 28.3.

**Synthesis of Compound $AcSCH_2P(O)(C_6H_4)_2$ (6).** Phosphine oxide **5** (1.2 g, 4.8 mmol) was dissolved in DMF (20 mL). Potassium thioacetate (0.829 g, 5.76 mmol) was then added, and the reaction mixture was stirred under Ar(g) for 18 h. The solvent was then removed under reduced pressure. The resulting oil was purified by chromatography (3% v/v MeOH in $CH_2Cl_2$). Phosphine oxide **6** was isolated as a clear, colorless oil in 70 % yield. $^{1}$HNMR ($CDCl_3$, 300 MHz) δ 7.78-7.74 (m, 4H), 7.54-7.53(m, 2H), 7.49-7.45 (m, 4H), 3.77 (d, 2H, J = 4.1 Hz), 2.25 (s, 3H). $^{13}$CNMR ($CDCl_3$, 75MHz) δ 193 (d), 132.3, 132.3, 131.5, 131.1, 131.0, 130.7, 128.7, 128.6, 30.0, 27.6 (d). $^{31}$P NMR ($CDCl_3$, 200 MHz) δ 28.9.

**Synthesis of Compound $AcSCH_2P(C_6H_4)_2$ (7).** Phosphine oxide **6** (1.06 g, 4 mmol) was dissolved in anhydrous chloroform (10 mL). Trichlorosilane (8 mL, 90 mmol) was added, and the resulting solution was stirred under Ar(g) for 72 h. The solvent was then removed under reduced pressure. (CAUTION: Excess trichlorosilane in the removed solvent was quenched by the slow addition of saturated sodium bicarbonate in a well-ventilated hood.) The residue was purified by flash chromatography (silica gel, 3% v/v MeOH in $CH_2Cl_2$). Phosphine **7** was isolated as a white solid in 65%% yield. **Spectral Data.** $^{1}$HNMR ($CDCl_3$, 300 MHz) δ 7.45-7.41 (m, 3H), 7.40-7.33(m, 7H), 3.52 (t, 2H, J = 1.8, 3.3 Hz), 2.29 (s, 3H). $^{13}$CNMR ($CDCl_3$, 75MHz) δ 193, 136.8, 136.6, 132.8, 132.6, 131.0, 129.1, 128.6, 128.5, 30.2, 25.9 (d). $^{31}$P NMR ($CDCl_3$, 200 MHz) δ -15.33.

**1.3 Synthesis of dPPMT-Su**

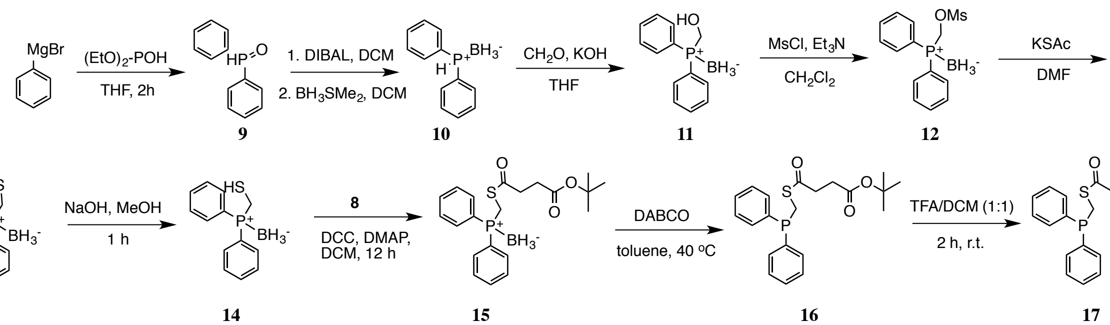
**SI scheme 4:**




**8**

To a mixture of succinic anhydride (3 g, 0.3 mol), *N*-hydroxysuccinimide (1 g, 0.009 mol), and DMAP (0.35 g, 0.003 mol) in toluene (15 mL) were added *tert*-butyl alcohol (3.5 mL, 0.037 mol) and $Et_3N$ (0.009 mol, 1.25 mL). The suspension was refluxed for 24 h. The solution was cooled and diluted with EtOAc (15 mL). The reaction mixture was washed with 10% citric acid and brine, dried over $Na_2SO_4$, and concentrated to give a brown oil. The oil was recrystallized with ether and petroleum ether at -20 °C to give the desired product **8** as a white crystal (4.0 g, 78%).$^1$H NMR ($CDCl_3$, 300 MHz): δ 2.62 (t, 2H, J = 3.3 Hz), 2.54 (t, 2H, J =1.2 Hz), 1.44 (s, 9H), $^{13}$CNMR ($CDCl_3$, 75MHz): 178.5, 171.4, 81.0, 30.0, 29.1, 28.0.

**SI scheme 5:**




**Synthesis of phosphine oxide (9):** A 250 mL round bottom flask equipped with an addition funnel was evacuated and Ar filled for 3 times, charged with 50 mL 1 M PhMgBr/THF (71.7 mL, 71.7 mmol, 3.3 eq.), and the solution cooled to 0 $^o$C under Ar. A solution of 3 g diethylphosphite (21.7 mmol, 1.0 eq) in 10 mL THF was then added dropwise over 15 min. The mixture was let stand for 15 min at 0 $^o$C, then the bath was removed, and the mixture was stirred for two hours at ambient temperature and then cooled again to 0 $^o$C. 75 mL 0.1 N HCl was added dropwise over 20 min, then 75 mL MTBE was added, and the mixture was filtered through a celite pad followed by washing the pad with $CH_2Cl_2$. The filtrate phases were separated, and the organic phase combined with the first organic phase, dried by $MgSO_4$, and the solvents removed *in vacuo*. The residue was purified by flash column chromatography to give **9** (3.0 g, 70%) $^1$H NMR (500 MHz, $CDCl_3$) δ: 8.87 (s. 1H), 7.73-7.50 (m, 4H), 7.49-7.45 (m, 6H). $^{13}$C NMR (75 MHz, $CDCl_3$) δ: 132.5, 132.5, 130.6, 130.5, 128.9, 128.7. $^{31}$PNMR (500MHz, $CDCl_3$): δ: 21.4.

**Phosphine-Borane Complex (10).** A solution of phosphine oxide **9** (2.00 g, 9.90 mmol) in anhydrous $CH_2Cl_2$ (30 mL) was added dropwise slowly to a solution of DIBAL (1 M in $CH_2Cl_2$, 39.6 mL, 39.6 mmol) under Ar(g) in a flame-dried three-neck round-bottom flask. The resulting solution was stirred for 20 min, and then cooled to 0 °C with an ice bath. The solution was then diluted with $CH_2Cl_2$ (30 mL), and a sparge needle of Ar(g) was allowed to blow through the solution for 5 min. A solution of 2 N NaOH (20 mL) was added dropwise slowly to the reaction mixture (*Caution!* Gas evolution!) followed by a saturated solution of Rochelle's salt (20 mL) to dissipate the emulsion that forms. The resulting biphasic solution was transferred to a separatory funnel, and the organic layer was separated, dried over anhydrous $MgSO_4$, filtered, and concentrated under reduced pressure. (1.8 g 100%). Borane-dimethylsulfide (3 M solution in THF, 6.45 mL, 19.35 mmol) was added to a round-bottom flask with freshly distilled THF (20 mL). To this solution, The crude diphenylphosphine (1.8 g, 9.67 mmol) was added in one portion. The solution was stirred under nitrogen for 2 h, and was quenched slowly with ice (~10 g). The resulting solution was diluted with brine (20 mL), and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with brine (2 x 10 mL), dried over sodium sulfate, filtered, and the solvent was removed under reduced pressure and the crude oil was purified by flash chromatography to yield **10** (1.1 g, 60% yield). $^1$H NMR (500 MHz, $CDCl_3$) δ: 7.73-7.66 (m. 4H), 7.54-7.45 (m, 6H). $^{31}$PNMR (500MHz, $CDCl_3$): δ: 22.1.

**Diphenylphosphino(borane)methane alcohol (11): 10** (1.0 g, 5.0 mmol) was dissolved in a mixture of aqueous formaldehyde (37% w/w, 5 mL) and THF (10 mL). To this solution, KOH (700 mg, 10.0 mmol) was added. The solution was stirred for 2 h, and the volatile solvent was removed under reduced pressure. The resulting aqueous solution was extracted with EtOAc (3 x 10 mL), washed with brine (1 x mL), dried over sodium sulfate, filtered, and the solvent was removed under reduced pressure and the crude product was purified by flash chromatography to yield **11** as a colorless liquid (0.861 g, 70%). $^1$H NMR ($CDCl_3$, 300 MHz): δ 7.76-7.72 (m, 4H), 7.55-7.53(m, 2H), 7.50-7.47 (m, 4H), 4.45 (s, 3H), 2.05 (br, 1H), 1.46-0.52 (m, 3H); $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 132.8, 132.7, 131.7, 131.7, 129.0, 128.9, 126.9, 126.4, 60.5, 60.2. $^{31}$P NMR ($CDCl_3$, 300 MHz) δ 25.7.

**Diphenylphosphino(borane)methyl methanesulfonate (12): 11** (1.1 g, 5.0 mmol) was dissolved in freshly distilled methylene chloride (10 mL), followed by addition of triethylamine

(1.4 mL, 10.1 mmol). The solution was cooled to 0 °C with ice-water bath. To this solution, methanesulfonyl chloride (580.0 μL, 7.4 mmol) was added dropwise. The solution was stirred under nitrogen for 15 h. The solvent was removed under reduced pressure. The crude product was purified by flash chromatography (3:1 $C_6H_{14}$:EtOAc) to yield **12** as a white solid (1.0 g, 3.9 mmol, 70%). $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.76-7.72 (m, 4H), 7.59-7.57 (m, 2H), 7.53-7.49 (m, 4H), 4.91 (d, 2H, *J*=1.2 Hz,), 2.89 (s, 3H). $^{31}$P NMR ($CDCl_3$, 300 MHz) δ 18.2.

**Diphenylphosphino(borane)methanethiol acetate (13):** Potassium thioacetate (0.737 g, 5.12 mmol) was added to a solution of **12** (0.8 g, 2.58 mmol) in anhydrous DMF (15 mL) under Ar(g). The resulting solution was stirred overnight at room temperature, after which the solvent was removed under reduced pressure. The residue was dissolved in EtOAc (20 mL), and the resulting solution was washed with water and brine. The combined organic extracts were dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (4:1 hexanes:ethyl acetate) to yield **13** as a white solid (0.487 g, 65%). $^1$H NMR ($CDCl_3$, 300 MHz) δ 7.71-7.67 (m, 4H), 7.51-7.49 (m, 2H), 7.46-7.43 (m, 4H), 3.72 (d, 2H, *J* =4.5 Hz), 2.24 (s, 3H). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 193.2, 132.4, 132.4, 131.8, 131.7, 128.9. 128.8, 127.8, 127.3, 30.0, 23.9 (d).$^{31}$P NMR ($CDCl_3$, 300 MHz) δ 19.0 (d).

**Diphenylphosphino(borane))methanethiol-2-Nitrobenzylsuccinate ester (15)**

Thioacetate **13** (0.4 g, 1.38 mmol) was dissolved in freshly distilled methanol (5 mL), followed by addition of sodium hydroxide (66 mg, 1,66 mmol). After stirring under nitrogen for 10 min, the solution was neutralized by 1 N hydrochloric acid, and was extracted with ethyl acetate (2 x 30 mL). The combined organic layers were washed by brine (1 x 10 mL), dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude thiol (**14**) was dissolved in freshly distilled methylene chloride (10 mL), followed by addition of o-Nitrobenzylsuccinate ester (NBS) (**8**) (0.408 g, 1.58 mmol), 1,3-dicyclohexylcarbodiimide (0.4 g, 0.97 mmol), and cat. *N*,*N*'-dimethyl-4-aminopyridine. The solution was stirred under nitrogen for 4 h, and the solution was filtered through a pad of celite. The solvent was removed under reduced pressure. The crude product was purified by flash chromatography (3:1 hexanes:ethyl acetate) to yield the thioester

**15** as a colorless oil (0.432g, 65%). $^{1}$H NMR ($CDCl_3$, 300MHz) δ 8.10 (t, 1H, J = 1.2 Hz), 7.85-7.83 (m, 1H), 7.82-7.80 (m, 4 H), 7.69-7.43 (m, 8H), 5.61 (s, 2H), 3.73 (d, 2H, J = 1.2 Hz), 2.85 (t, 2H, J = 4.2 Hz), 2.67 (t, 2H, J = 4.2 Hz). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 195 (d), 170, 147.4, 133.9, 132.4, 132.4, 132.0, 131.8, 131.4, 131.3, 129.0, 128.9, 128.9, 128.8, 128.7, 128.6, 127.6, 127.2, 125.0, 63.3, 37.9, 29.0, 23.6 (d). $^{31}$P NMR ($CDCl_3$, 300 MHz) δ 40.4;

**Diphenylphosphino(borane))methanethiol-mono-t-butyl succinate ester (15)**

**13** (0.4 g, 1.38 mmol) was dissolved in freshly distilled methanol (5 mL), followed by addition of NaOH (66 mg, 1,66 mmol). After stirring under nitrogen for 10 min, the solution was neutralized by 1 N HCl, and was extracted with EtOAc (2 x 30 mL). The combined organic layers were washed by brine (1 x 10 mL), dried over sodium sulfate, filtered, and concentrated under reduced pressure to obtain **14**. The crude thiol of **14** was dissolved in freshly distilled $CH_2Cl_2$ (10 mL), followed by the addition of **8** (0.352 g, 2.0 mmol), 1,3-dicyclohexylcarbodiimide (0.5 g, 2,42 mmol), and cat. *N*,*N*'-dimethyl-4-aminopyridine. The solution was stirred under nitrogen for 4 h, and the solution was filtered through a pad of celite. The solvent was removed under reduced pressure. The crude product was purified by flash chromatography (3:1 $C_6H_{14}$:EtOAc) to yield the thioester **15** as a colorless oil (0.33 g, 60%). $^{1}$H NMR ($CDCl_3$, 300 MHz) δ 7.72-7.68 (m, 4H), 7.53-7.45 (m, 6H), 3.75 (d, 2H, J = 3.9 Hz), 2.75 (t, 2H, J = 3.9 Hz), 2.48 (t, 2H, J = 3.3 Hz), 1.43 (s, 9H). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 195 (d), 170.6, 132.5, 132.4, 131.8, 131.7, 128.9, 128.8, 127.8, 127.3, 38.4, 30.3, 28.0, 23.5 (d). $^{31}$PNMR ($CDCl_3$, 75 MHz) δ 41.5.

**Diphenylphosphinomethanethiol-mono-t-butyl succinate ester (16)**

Phosphine-borane **15** (0.25 g, 0.621 mmol) and 1,4-diazabicyclo[2.2.2]octane (DABCO) (0.139 g mg, 1.24 mmol) was dissolved in freshly distilled toluene (2 mL). The solution was submerged in an oil-bath pre-heated to 60 °C. The solution was stirred under argon for 1 h. After cooling to room temperature, the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (4:1 $C_6H_{14}$:EtOAc) under nitrogen to yield **16** as a colorless oil (0.168 g, 70%). $^{1}$H NMR ($CDCl_3$) δ 7.46-7.43 (m, 4H), 7.37-7.36 (m, 6H), 3.55 (d, 2H, J = 2.1 Hz), 2.80 (t, 2H, J = 4.2 Hz), 2.55 (t, 2H, J = 4.5 Hz), 1.45 (s, 9H). $^{13}$C NMR ($CDCl_3$, 75

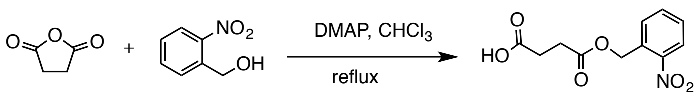
MHz) δ 196 (d), 170.9, 136.8, 136.7, 132.8, 132.6, 129.1, 128.6, 128.5, 80.9, 38.5, 30.5, 28.0, 25.6, 25.4 (d). $^{31}$P NMR ($CDCl_3$, 300 MHz) δ - 15.3.

**Diphenylphosphinomethanethiol-succinic acid (17)**

To a solution of ester **16** (0.15 g, 0.45 mmol) in $CH_2Cl_2$ (3 mL) was added TFA (3 mL). After being stirred at r.t. for 1 h, the reaction mixture was concentrated in vacuum to give a colorless oil **17** (0.098 g, 73%). $^1$H NMR ($CD_3OH$, 300 MHz) δ 7.44-7.42 (m, 4H), 7.41-7.36 (m, 6H), 3.56 (d, 2H, J = 2.4 Hz), 2.82 (t, 2H, J = 4.2 Hz), 2.58 (t, 2H, J = 4.2 Hz). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 196.9 (d), 174.0, 136.8, 132.5, 132.3, 128.8, 128.3, 128.2, 37.8, 28.4, 24.8 (d). $^{31}$P NMR ($CDCl_3$, 300 MHz) δ - 14.9.

**1.4 Synthesis of dPPMT-NB-Su**

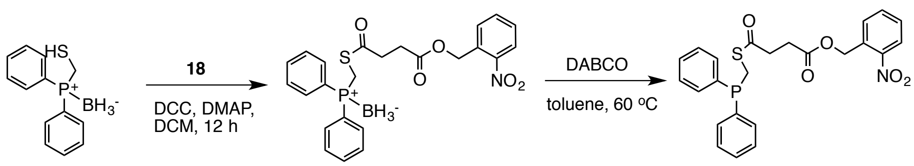
**SI scheme 6:**




**18**

O-nitrobenzyl alcohol (4.0 g, 26.12 mmol), succinic anhydride (5.23 g, 52.24 mmol), and DMAP (1.60 g, 13.06 mmol) were dissolved completely in dried $CHCl_3$ (86 mL) and refluxed under a nitrogen atmosphere for 24 h. After removing partially $CHCl_3$ under reduced pressure, the mixture was washed three times with 10% HCl and then extracted with saturated $NaHCO_3$ solution. The basic aqueous phase was washed with ether and acidified to pH 5.0 with 10% HCl. The white solid precipitate was collected and dried in vacuo at 40 °C overnight to give **18** (6.08 g, 92%). $^1$H NMR ($CDCl_3$): δ 8.09 (dd, 1H, J = 0.4, 1.8 Hz), 7.66−7.59 (m, 2H), 7.50-7.47 (m, 1H), 5.55 (s, 2H), 2.76-2.72 (m, 4H).

**SI scheme 7:**




**19** **20**

**Diphenylphosphino(borane))methanethiol-2-Nitrobenzylsuccinate ester (19)**

**13** (0.4 g, 1.38 mmol) was dissolved in freshly distilled methanol (5 mL), followed by addition of NaOH (66 mg, 1,66 mmol). After stirring under nitrogen for 10 min, the solution was neutralized by 1 N HCl, and was extracted with EtOAc (2 x 30 mL). The combined organic layers were washed by brine (1 x 10 mL), dried over sodium sulfate, filtered, and concentrated under reduced pressure. The crude thiol (**14**) was dissolved in freshly distilled $CH_2Cl_2$ (10 mL), followed by addition of **18** (0.408 g, 1.58 mmol), DCC (0.4 g, 0.97 mmol), and cat. *N,N*'-dimethyl-4-aminopyridine. The solution was stirred under nitrogen for 4 h, and the solution was filtered through a pad of celite. The solvent was removed under reduced pressure. The crude product was purified by flash chromatography (3:1 $C_6H_{14}$:EtOAc) to yield the thioester **19** as a colorless oil (0.432g, 65%). $^1$H NMR ($CDCl_3$, 300MHz) δ 8.10 (t, 1H, J = 1.2 Hz), 7.85-7.83 (m, 1H), 7.82-7.80 (m, 4 H), 7.69-7.43 (m, 8H), 5.61 (s, 2H), 3.73 (d, 2H, J = 1.2 Hz), 2.85 (t, 2H, J = 4.2 Hz), 2.67 (t, 2H, J = 4.2 Hz). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 195 (d), 170, 147.4, 133.9, 132.4, 132.4, 132.0, 131.8, 131.4, 131.3, 129.0, 128.9, 128.9, 128.8, 128.7, 128.6, 127.6, 127.2, 125.0, 63.3, 37.9, 29.0, 23.6 (d). $^{31}$P NMR ($CDCl_3$, 300 MHz) δ 40.4;

**Diphenylphosphinomethanethiol-2-Nitrobenzylsuccinate ester (20)**

**19** (0.2 g, 0.41 mmol) and DABCO (0.139 g mg, 1.24 mmol) was dissolved in freshly distilled toluene (2 mL). The solution was submerged in an oil-bath pre-heated to 60 °C. The solution was stirred under argon for 1 h. After cooling to r.t., the solvent was removed under reduced pressure. The crude product was purified by flash chromatography (3:1 $C_6H_{14}$:EtOAc) under nitrogen to yield **20** as a colorless oil (0.15 g, 80%). $^1$H NMR ($CDCl_3$) δ 8.10 (dd, 1 H, J = 0.6, 4.8 Hz), 7.66-7.63 (m, 2H), 7.46-7.42 (m, 5H), 7.36-7.34 (m, 6H), 5.52 (s, 2H), 3.53 (d, 2H, J= 2.1 Hz), 2.89 (t, 2H, J = 4.2 Hz), 2.73 (t, 2H, J = 4.2 Hz). $^{13}$C NMR ($CDCl_3$, 75 MHz) δ 196 (d), 171. 2, 147.4, 136.7, 136.6, 133.8, 132.8, 132.6, 131.9, 129.2, 128.9, 128.8, 128.7, 128.6, 128.5, 120.0, 63.2, 38.1, 29.1, 25.7 (d). $^{31}$P NMR ($CDCl_3$, 300 MHz) δ - 15.1.

## 2. The identification of AznLRS

### 2.1 Construction of the mmPylRS Library and the selection of pEvol-PylT-AznLRS

AznL was genetically encoded into sfGFP in *E. coli* BL21(DE3) cells using a mutant pyrrolysyl-tRNA synthetase (PylRS)-$tRNA_{CUA}$ pair. An active-site mutant library of the *Methanosarcina mazei* PylRS gene that randomizes at active site residues (Y306NNK, L309NNK, C348NNK, Y/F/W384 and W411NNN). NNK (N=A or C or G or T, K=G or T) was constructed by site-directed mutagenesis PCR. The following pairs of primers were used to generate the mmPylRS gene library, pBK-mmPylRS-348NNK-F: 5'-ACC ATG CTG AAC TTC NNK CAG ATG GGA TCG GGA TGC ACA CGG-3', pBK-mmPylRS-348NNK-R: 5'-AAA CTC TTC GAG GTG TTC TTT GCC GTC GGA CTC-3', pBK-mmPylRS-306-309-NNK-F: 5'-CTT GCT CCA AAC CTT NNK AAC TAC NNK CGC AAG CTT GAC AGG GCC CTG CCT-3', pBK-mmPylRS-306-309-NNK-R: 5'-CAT GGG TCT CAG GCA GAA GTT CTT GTC AAC CCT-3'; pBK-mmPylRS-411NNN-F: 5'-CCG CTT GAC CGG GAA NNN GGT ATT GAT AAA CCC-3', and pBK-mmPylRS-411NNN-R: 5'-TAT GGG TCC GAC TAC TGC AGA GG-3'. The DNA library was cloned into a pBK vector to form a pRSL library.

Positive selection: The pRSL plasmid library was used to transform TOP10 electrocompetent cells containing the positive selection plasmid pY+ to yield a cell library greater than $1\times10^9$ cfu, ensuring complete coverage of the pRSL library. Cells were plated on minimal agar plates containing 12 μg/mL tetracycline (Tet), 25 μg/mL kanamycin (Kan), 102 μg/mL chloramphenicol (Cm) and 1 mM AznL. After incubation at 37 °C for 72 h, colonies on the plates were collected and surviving pRSL plasmids were extracted.

Negative selection: the extracted plasmids from the positive selection were transformed into TOP10 electrocompetent cells containing the negative selection plasmid pY- and plated on LB agar plates containing 50 μg/mL Kan, 200 μg/mL ampicillin (Amp), 0.2% arabinose. After incubation at 37 °C for 16 h. Survived cells from plates were pooled to extract plasmids for further selections.

The alternative positive (3×) and negative (2×) selections were repeated. Final positive selected colonies grew on LB plates with 102 μg/mL Cm, 25 μg/mL Kan, 12 μg/mL Tet, and with or without 1 mM AznL.

### 2.2 The sequencing results of the selected AznLRS

The sequences of the six selected colonies are listed in SI Table 1.

SI Table 1. The mutants for Aznl incorporation and the sequence

| RS mutants | Sequences |
|---|---|
| AznLRS1 | 306L309L348I384F411W |
| AznLRS3 | 306F309M348M384F411W |
| AznLRS4 | 306L309L348V384F411F |
| AznLRS9 | 306L309L348L384F411W |
| AznLRS20 & 45 | 306L309L348I384F411W |

The AznLRS1 was sequenced optimized and cloned into a pEVOL vector that has a gene coding $tRNA^{Pyl}$ to afford the plasmid pEVOL-pylT-AznLRS.

## 3. The expression of sfGFP-D134AznL and wild type sfGFP

### 3.1 DNA sequences of sfGFP-D134TAG and wild type sfGFP

sfGFP-D134TAG-$His_6$:

atggttagcaaaggtgaagaactgtttaccggcgttgtgccgattctggtggaactggatggtgatgtgaatggccataaatttagcgttcgtggcgaaggcgaaggtgat

gcgaccaacggtaaactgaccctgaaatttatttgcaccaccggtaaactgccggttccgtggccgaccctggtgaccaccctgacctatggcgttcagtgctttagccgc

tatccggatcatatgaaacgccatgatttctttaaaagcgcgatgccggaaggctatgtgcaggaacgtaccattagcttcaaagatgatggcacctataaaacccgtgcg

gaagttaaatttgaaggcgataccctggtgaaccgcattgaactgaaaggtattgattttaaagaataggggcaacattctgggtcataaactggaatataatttcaacagcca

taatgtgtatattaccgccgataaacagaaaaatggcatcaaagcgaactttaaaatccgtcacaacgtggaagatggtagcgtgcagctggcggatcattatcagcagaa

taccccgattggtgatggcccggtgctgctgccggataatcattatctgagcacccagagcgttctgagcaaagatccgaatgaaaaacgtgatcatatggtgctgctgga

atttgttaccgccgcgggcattacccacggtatggatgaactgtataaaggcagccaccatcatcatcaccattga

### 3.2 Protein sequences of sfGFP-D134TAG

sfGFP-D134AznL-$His_6$ (MW: 27866.35):

(M)VSKGEELFTGVVPILVELDGDVNGHKFSVRGEGEGDATNGKLTLKFICTTGKLPVPWPTLVTTLTYGVQ

CFSRYPDHMKRHDFFKSAMPEGYVQERTISFKDDGTYKTRAEVKFEGDTLVNRIELKGIDFKE(Aznl)GNIL

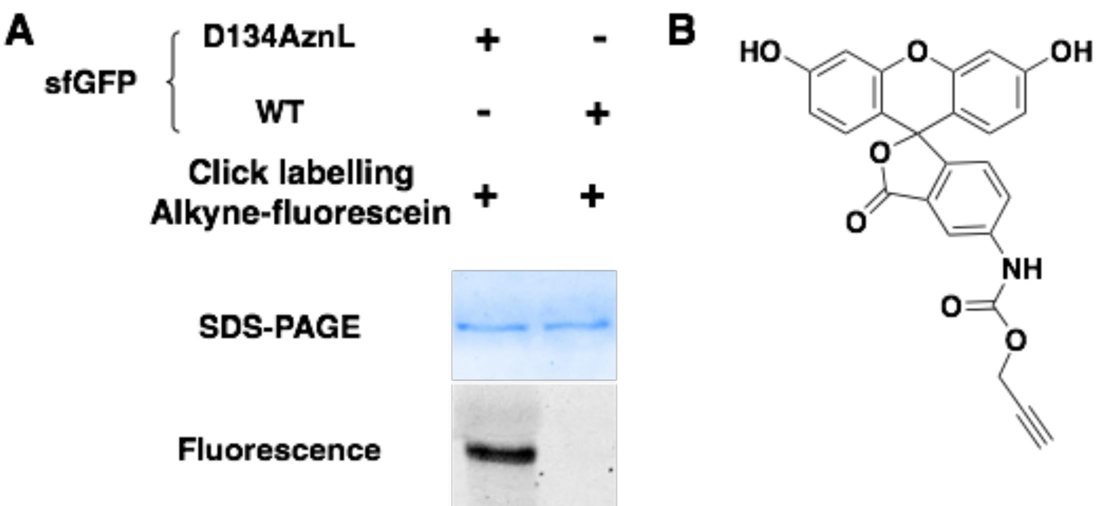
GHKLEYNFNSHNVYITADKQKNGIKANFKIRHNVEDGSVQLADHYQQNTPIGDGPVLLPDNHYLSTQSVLSKDPNEKRDHMVLLEFVTAAGITHGMDELYKGSHHHHHH

**3.3 The expression and purification of sfGFP-D134AznL**

The plasmid of pEvol-PylT-AznLRS mentioned before was used together with pET-sfGFP-D134TAG to co-transform *E.coli* BL21(DE3) cells. The cells were plated on LB agar plate containing 100 μg/mL ampicillin (Amp) and 34 μg/mL chloramphenicol (Cam). A single colony was picked, followed by the growth in a 5 mL LB medium overnight. The overnight culture was further inoculated into a 250 mL 2YT medium with the same concentration of Amp and Cam. Cells were let grow in a 37 $^{o}$C shaker (250 r.p.m.) for 3 h until $OD_{600}$ reached 0.6. The protein expression was induced by the addition of 1 mM IPTG, 0.2% arabinose and 5 mM AznL as the final concentration. After another 8 h incubation, the cells were harvested (4k 20 min, 4 $^{o}$C), washed, and fully resuspended with a lysis buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 10 mM imidazole, pH 7.5). The sonication of cells underwent under ice/water incubation for 3 times (4 min each time with an interval of 4 min). Then the cell lysate was centrifuged (10k 40 min, 4 $^{o}$C), and the precipitate was removed. 1 mL Ni Sepharose$^{TM}$ 6 Fast Flow column (GE Healthcare) was added to the supernatant and incubated for 1 h at 4 $^{o}$C. The mixture was loaded to a column and the flow-through was removed. 15 mL of wash buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 20 mM imidazole, pH 7.5) was used to wash the protein-bound resin in three batches. Around 6 mL of elution buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 250 mM imidazole, pH 7.5) was used to elute the target protein. All the eluted fractions were collected and concentrated by Amicon Ultra-15 Centrifugal Filter Device (10k MWCO, Millipore), and buffer was exchanged to 20 mM phosphate pH 8.5 buffer. FPLC Q-Sepharose anion exchange column was used to further purify the sfGFP. Buffer A is 20 mM phosphate pH 8.5 buffer, while buffer B 20 mM phosphate pH 8.5 buffer containing 1 M NaCl. The wash with 10 column volume (CV) of mobile phase containing 5 % B was followed by the elution of a gradient from 5 % to 50 % B in 10 CV mobile phase. The elution samples were collected and again concentrated by Amicon Ultra-15 Centrifugal Filter Device (10k MWCO, Millipore). The buffer was further exchanged to 200 mM phosphate pH 6.5 buffer for the further Staudinger reaction. The expression of wild type sfGFP followed the similar procedure without the addition of AznL for induction.

### 3.4 Fluorescent labeling of sfGFP-D134AznL

The fluorescent labeling of sfGFP-D134AznL was carried out according to standard CuAAC procedure[2]. 50 μM of $CuSO_4$ was mixed with 300 μM of BTTAA, followed by the addition of 500 μM alkyne-fluorescein, and 15 μM of sfGFP-D134AznL (all the mentioned were final concentrations). The reaction was initialized by the addition of ascorbate stock solution in PBS to a final concentration of 2.5 mM. The same reaction was carried out for wild type sfGFP. The reaction was allowed to be incubated at 25 $^{o}$C for 4 h followed by the SDS-PAGE gel analysis.




**SI Fig. 1**: **A.** fluorescent labeling of sfGFP-D134AznL. **B.** the structure of alkyne-fluorescein.

## 4. The expression of Ub-K48AznL, WT-Ub, and Ub-K48ac

### 4.1 DNA sequences of Ub-K48TAG-$His_6$

Ub-K48TAG-$His_6$

atgcaaatattcgtgaaaaccctaactggtaagaccatcactctcgaagtggagccgagtgacaccattgagaatgtcaaggcaaagatcca agacaaggaaggcatccctcctgaccagcagaggttgatctttgctgggtagcagctggaagatggacgcaccctgtctgactacaacatc cagaaagagtccaccctgcacttggtccttaggctgagaggaggacatcaccatcaccatcactaa

2 C. Besanceney-Webler, H. Jiang, T. Zheng, L. Feng,D. Soriano del Amo, W. Wang, L. M. Klivansky, Prof. Dr. F. L. Marlow, Dr. Y. Liu, Prof. Dr. P. Wu, *Angew. Chem. Int. Ed.* 2011, 50, 8051 –8056

**4.2 Protein sequences of Ub-K48AznL and Ub-K48ac**

Ub-K48AznL-$His_6$

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAG(Aznl)QLEDGRTLSD YNIQKESTLHLVLRLRGGHHHHHH

Ub-K48ac-$His_6$

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAG(AcK)QLEDGRTLSD YNIQKESTLHLVLRLRGGHHHHHH

**4.3 The expression and purification of Ub-K48AznL, Ub-K48ac**

All antibodies were purchased from PTM BioLabs Inc.

The plasmid of pEvol-PylT-AznLRS mentioned before was co-transferred with pET-Ub-K48TAG into *E.coli* BL21(DE3) cells. The cells were plated on LB agar plate containing 100 μg/mL ampicillin (Amp) and 34 μg/mL chloramphenicol (Cam). A single colony was picked, followed by the growth in a 5 mL LB medium overnight culture. The overnight culture was further inoculated 250 mL of 2YT medium with the same concentration of Amp and Cam. Cells grew in a 37 $^{o}$C shaker (250 r.p.m.) for 3 hrs until $OD_{600}$ reached 0.6. The protein expression was induced by the addition of 1 mM IPTG, 0.2% arabinose and 5 mM Aznl as the final concentration. After another 8 h incubation, the cells were harvested (4k 20 min, 4 $^{o}$C), washed, and fully resuspended with PBS. The sonication of cells underwent under ice/water incubation for 3 times (4 min each time with a interval of 4 min). Then the cell lysate was centrifuged (10k 40 min, 4 $^{o}$C), and the precipitate was removed. The supernatant was adjusted pH to 3.5 with acetic acid, and was incubated for 20 min before centrifuging (10k 40 min, 4 $^{o}$C). After the removal of precipitation, 1 mL Ni Sepharose$^{TM}$ 6 Fast Flow column (GE Healthcare) was added to the supernatant and incubated for 1 h at 4 $^{o}$C. The mixture was loaded to a column and the flow-through was removed. 50 mL of wash buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 20 mM imidazole, pH 7.5) was used to wash the protein-bound resin in three batches. Around 6 mL of elution buffer (300 mM NaCl, 50 mM $NaH_2PO_4$, 250 mM imidazole, pH 7.5) was used to elute the target protein. All the eluted fractions were collected and concentrated by Amicon Ultra-15 Centrifugal Filter Device (3.5k MWCO, Millipore), and buffer was exchanged to 200 mM phosphate pH 6.5 buffer for the further traceless Staudinger ligation. For the expression of Ub-K48ac, the pEvol-PylT-AcKRS was used instead, while 5 mM of AcK was provided while induction. All the other purification steps remain the same as Ub-K48AznL.

### 4.4 The expression and purification of wild type Ub

Wild type Ub with a N-terminal 6×his tag was expressed in a 1 L 2YT culture with the addition of 100 μg/mL Amp. Cells were let to grow in a 37 $^{o}$C shaker (250 r.p.m.) for 3 h until $OD_{600}$ reached 0.6. The protein expression was induced by the addition of 0.8 mM IPTG as the final concentration. After another 8 h incubation, cells were harvested. The further purification processes were the same as the purification of Ub-K48AznL.

## 5. The expression of histone H3-K4Aznl, WT-H3, and H4-$His_6$-SUMO

### 5.1 DNA sequences of WT-H3

WT-H3(C110A):

gctcgcaccaaacagactgctcgtaagtccactggcggtaaagcgccgcgtaaacagctggcaaccaagatggcgcgtaaaagcgctccagctactggcggcgtgaa
gaagccgcaccgttatcgcccgggtactgtggctctgcgtgaaatccgccgctaccagaaaagcaccgaactgctgattcgcaaactgccatttcaacgtctggttcgcg
aaattgctcaggatttcaaaaccgacctgcgcttccagtctagcgctgtgatggcactgcaagaggcgtctgaggcatatctggttggcctgttcgaagataccaacctgtg
cgcaatccatgcaaagcgtgtaaccattatgccgaaagacatccaactggctcgtcgtatccgtggtgagcgtgcgtga

### 5.2 Protein sequences of $His_6$-TEV-H3-K4AznL(C110A)

$His_6$-TEV-H3-K4AznL(C110A):

MGSSHHHHHHSQDPENLYFQART(AznL)QTARKSTGGKAPRKQLATKAARKSAPATGGVKKPHRYRPGT
VALREIRRYQKSTELLIRKLPFQRLVREIAQDFKTDLRFQSSAVMALQEASEAYLVGLFEDTNLAAIHAKRV
TIMPKDIQLARRIRGERA

### 5.3 The expression and purification of H3-K4AznL

The plasmid of pEvol-PylT-AznLRS mentioned before was used together with pETduet-H3K4TAG to co-transform *E.coli* BL21(DE3) cells. The cells were plated on LB agar plate containing 100 μg/mL ampicillin (Amp) and 34 μg/mL chloramphenicol (Cam). A single colony was picked, followed by the growth in a 5 mL LB medium with same Amp Cam concentrations overnight culture. The overnight culture was further inoculated into a 250 mL 2YT medium with the same concentration of Amp and Cam. Cells were grown in a 37 $^{o}$C shaker (250 r.p.m.) for 3 hrs until $OD_{600}$ reached 0.6. The protein expression was induced by the addition of 1 mM IPTG, 0.2 % arabinose and 5 mM AznL. After another 8 h incubation, the cells were harvested by centrifugation (4k 20 min, 4 $^{o}$C), washed, and fully resuspended in a 40 mL lysis buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% Triton X-100, 0.1% $NaN_3$, pH 7.5). The sonication of cells underwent under ice/water incubation for 3 times (4 min each time with an interval of 4 min). Then the cell lysate was centrifuged (6k 20 min, 4 $^{o}$C), and the supernatant was removed. The precipitate was washed with 30 mL lysis wash buffer (20 mM Tris-HCl, 500 mM NaCl, 0.1% $NaN_3$, pH 7.5) twice. The supernatant was removed again, and the inclusion body was dissolved in 6 M urea histone solublization buffer (6 M urea, 20 mM Tris, 500 mM NaCl, pH 7.5). The solution was centrifuged at 10 k for 40 min to remove all the precipitate. The supernatant was loaded to 1 mL Ni Sepharose$^{TM}$ 6 Fast Flow column (GE Healthcare) and the flow-through was removed. 15 mL of wash buffer (6 M urea, 20 mM Tris, 500 mM NaCl, 20 mM imidazole, pH 7.5) was used to wash the protein-bound resin in three batches. Around 6 mL elution buffer (6 M urea, 20 mM Tris, 500 mM NaCl, pH 7.5) was used to elute the target protein.

All the eluted fractions were then collected and concentrated by Amicon Ultra-15 Centrifugal Filter Device (3 k MWCO, Millipore), and buffer was exchanged to histone solublization buffer for the further traceless Staudinger ligation reaction.

### 5.4 The expression and purification of wild type H3 and H4

Both histone proteins with a N-terminal 6×his tag were expressed in a 1 L 2YT culture with the addition of 100 μg/mL Amp. Cells were let to grow in a 37 $^{o}$C shaker (250 r.p.m.) for 3 h until $OD_{600}$ reached 0.6. The protein expression was induced by the addition of 0.8 mM IPTG. After another 8 h incubation, cells were harvested. The further purification processes were the same as the purification of H3-K4AznL.

## 6. General process of time-dependent traceless-Staudinger ligation of Ub-K48AznL and H3K4AznL

~5 mg of dPPMT-Ac, dPPMT-Su, and dPPMT-NB-Su were dissolved in DMSO solution to reach 100 mM. The Ub-K48AznL, wild type Ub, and Ub-K48ac were all prepared in 200 mM phosphate buffer, pH 6.0 with 10-20 µM concentrations. The phosphine reagents in DMSO stock solution were slowly added into the Ub-K48AznL (~20 µM) sample to reach to a final concentration of 5 mM. Reactions were gently pipetted to make solutions well mixed and then let stand at 37 $^{o}$C. A same amount of wild type Ub with the addition of same amount of phosphine reagent was used as the negative control. For the acetylation modification, Ub-K48ac without phosphine was used as the positive control. Samples of reaction mixtures were taken 12 h intervals. 5 µL of azidoethanol was added to each sample as the quencher, followed by the addition of 10× PBS buffer. The quenched samples were centrifuged at 14K for 5 min to remove any precipitations, dialyzed over 10× PBS buffer for 3 times, and then concentrated to small volumes. For dPPMT-NB-Su, the quenched samples were treated with UV light at 365 nm for 30 min. With the addition of a SDS loading buffer, final samples were applied to two 15 % SDS-PAGE gel separately. The first gel was stained with Coomassie blue and applied for imaging. The second gel was further transferred to nitrocellulose membrane for Western blot. The pan anti-Kac or pan anti-Ksu antibody from PTM BioLabs Inc. was first applied and then followed by the blotting with secondary antibody and visualization. For H3K4su, the degassed histone solublization buffer at pH 6.0 instead of a PBS buffer was used when it was necessary.

**7. MS analysis of sfGFP-D134AznL, Ub-K48ac, and Ub-K48su**

The purified sfGFP-D134AznL was dialyzed against 20 mM ammonium bicarbonate (ABC) buffer, followed by the complete removal of solvent with lyophilization. The solid protein powder was then dissolved in 40 % ACN/water with 0.1 % formic acid, and injected to ESI-MS (Applied Biosystems QSTAR Pulsar, Concord, ON, Canada, equipped with a nanoelectrospray ion source). The deconvoluted data was analyzed by Protein Deconvolution Software from the same company. The products Ub-K48ac and Ub-K48su from traceless-Staudinger ligation were directly applied to Ziptip (Reversed-phase, pipette tips for sample preparation, Millipore Corporation) sample preparation, followed by the analysis of MALDI-TOF-MS (Applied Biosystems Voyager-DE STR) with data explorer software from the same company.

## 8. Tandem MS/MS analysis of Ub-K48ac, Ub-K48su, and H3-K4su

Liquid Chromatography—tandem Mass Spectrometry (LC-MS/MS) was applied to identify the specific modifications to Ub-K48 and histone H3-K4 proteins. The protein digestion, LC-MS/MS analysis, and data analysis were conducted following the method described previously[3]. 10 µg of each protein was used for the analysis. Briefly, protein sample was denatured by 8 M urea dissolved in Tris buffer (50 mM Tris-HCl, 10mM $CaCl_2$, pH 7.6), supplemented with 5 mM dithiothreitol (DTT) for reduction of potential disulphide bonds. The denatured protein was diluted into a final concentration of 1 M urea with the same Tris buffer, followed by peptide digestion. Ubiquitin was digested by Mass Spectrometry Grade Trypsin Gold (Promega, Madison, WI) with 1:50 w/w at 37 °C overnight, while histone H3 was digested by chymotrypsin (Promega, Madison, WI) at 25 °C. The digested peptides were then cleaned up using a Sep-Pak Plus C18 column (Waters Corporation, Milford, MA), followed by loading onto a biphasic 2D reversed phase (RP)/strong cation exchange (SCX) capillary column with a pressure cell. The column was then washed with 100% aqueous solvent (99.9 % $H_2O$, 0.1 % formic acid) for 5 min, followed by a 1-hour wash by ramping up the solvent to 100% organic solvent (0.1 % formic acid, 80% acetonitrile). This washing step migrates all the peptides into the SCX phase, where peptides were eluted and separated in a 15-cm-long 100 µm-ID C18 capillary column connected to the 2D column. LTQ ion trap mass spectrometer (Thermo Finnegan, San Jose, CA) was used to analyze the peptides. The full scan was set to the range of 300-1700 m/z, in which the top 5 abundant peptides in each scan were subjected to collision induced dissociation (CID) fragmentation for tandem mass spectrometry (MS/MS) analysis. Peptide identity was processed by the in-house pipeline as described previously. Peptide searching was based on ProLuCID (version 1.0) search algorism. For the peptide modification search, mass increase was added in order to identify the modified peptides. More specifically, 42.0367 was added for lysine acetylation modification, and 100.0648 was added for lysine succinylation modification.

## 9. The preparation of H3-K4su

3 Z. Wang, Y. Zeng, Y. Kurra, X. Wang, J. M. Tharp, E. C. Vatansever, W. W. Hsu, S. Y. Dai, X. Fang, W. R. Liu, *Angew. Chem. Int. Ed.* **2016**, accepted

The preparation of H3-K4su was carried out in large batch with the procedure similar to part 6. In each reaction, about 1.5 mg H3-K4AznL was applied to the traceless Staudinger reaction with dPPMT-NB-Su, followed by the same reaction work-up process. After concentrating to lower volume, the sample was then treated with UV photolysis for 30 min, followed by another dialysis against histone solubilization buffer, pH 7.5 for 3 times. The solution was concentrate again down to 100 μl and stored for the next step.

**10. Refolding of H3K4su-H4 tetramer**

The H3-K4su was assembled into tetramer with wild type H4. Both histone proteins with a N-terminal 6×His tag (H4 with SUMO) were dissolved with equimolar ratios in the histone solubilization buffer (6 M urea, 20 mM Tris, 500 mM NaCl, pH 7.5). The protein mixture was dialyzed against 500 mL refolding buffer 1 (20 mM Tris, 2 M NaCl, pH 7.5) for 30 min once, 500 mL refolding buffer 2 (20 mM Tris, 1 M NaCl, pH 7.5) for 30 min once, and 500 mL refolding buffer 3 (20 mM Tris, 500 mM NaCl, pH 7.5) for 30 min twice by using Slide-A-Lyzer MINI Dialysis devices (3,500-Da cutoff). After the refolding process, tetramers were put into a microcentrifuge tube and spun down by 14000 rpm for 1 min to remove all the precipitation during the refolding process, and the tetramer concentration was about 16 mg/mL. SDS-PAGE gel was used to confirm the tetramer folding, and the tetramer was stored on ice for further usage.

**11. Sirtuin enzyme expression and deacylation assay**

**11.1 Sirtuin 1 and Sirtuin 5 expression**

The Sirt1 and Sirt5 were expressed according to the procedure reported[4]. Both enzymes were expressed in *E.coli* BL21(DE3) cells with 1 L LB media at 30 $^{o}$C overnight, after the addition of IPTG 0.1 mM as an inducer. After collecting cells and their sonication, the precipitate was removed by centrifugation. The supernatant was subjected to purification with the GST resin. The mixture was directly loaded to a column and the flow-through was removed. 50 mL PBS washing buffer (100 mM NaCl, 200 mM $NaH_2PO_4$, pH 7.4) was used to wash the protein-bound resin in three batches. 10 mL of elution buffer (15 mM Glutathione in reduced form, 100 mM

4 Du J., Jiang H., Lin H., *Biochemistry* 2009, 48, 2878-2890

NaCl, 200 mM $NaH_2PO_4$, pH 7.4) was used to elute the target protein. After concentration measurement, the enzymes were split into several portions with glycerol added. The final solution was frozen by ethanol-dry ice mixture and stored into -80 $^{o}$C freezer for future usage.

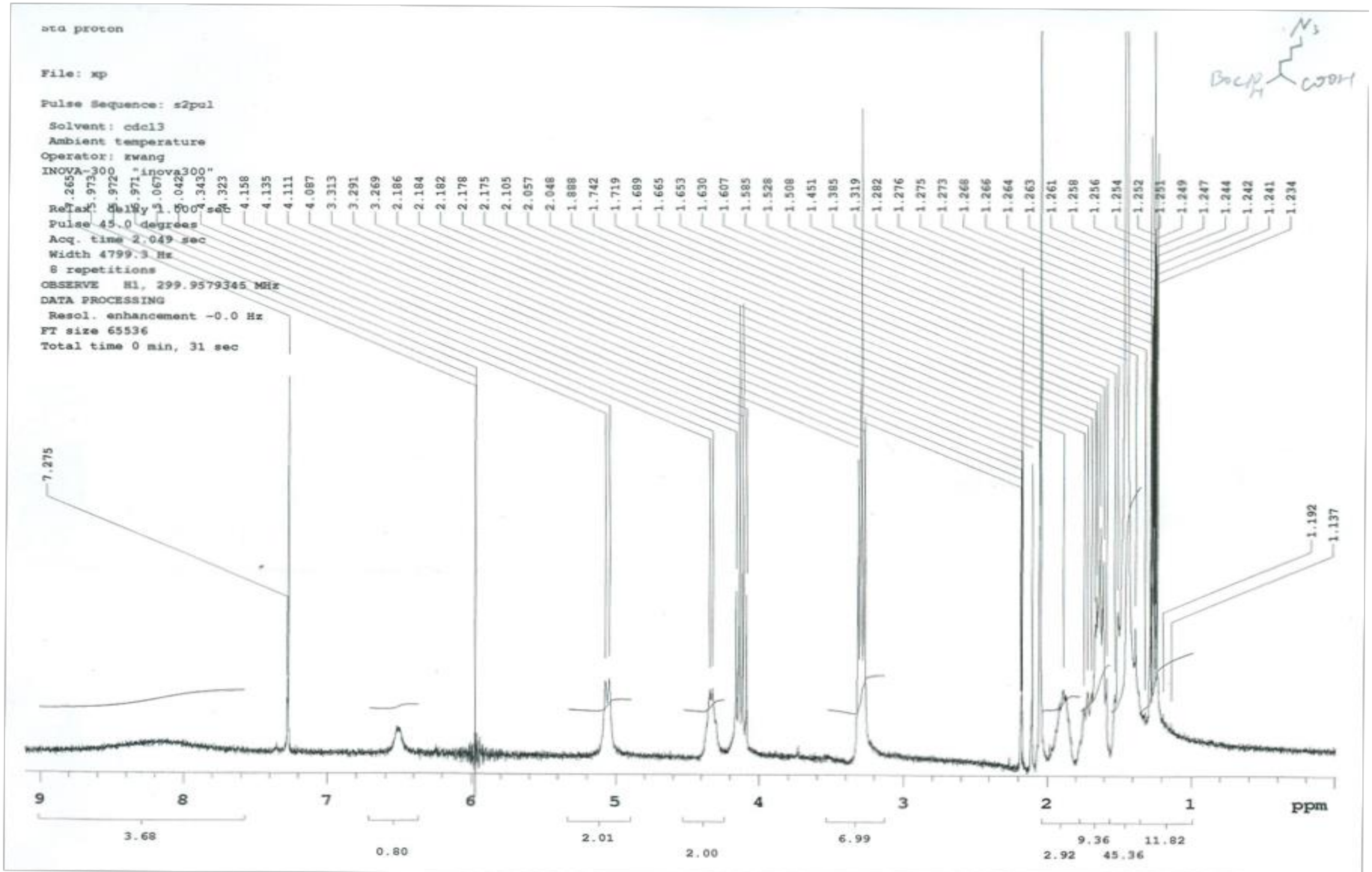
### 11.2 Sirtuin enzyme deacylation assay on H3K4su-H4 tetramer

The H3K4su-H4 tetramer was freshly assembled according to Part 10 at concentration of about 16 mg/mL or 180 μM. Sirtuin enzyme stock solution was added to each sample as the final concentration of 500 nM for Sirt1 or Sirt5. After 4 h incubation at 37 $^{o}$C, the samples were subjected to the SDS-PAGE analysis. SDS-PAGE separated proteins were transferred to nitrocellulose membranes for Western blot. The pan anti-Ksu antibody was used for the membrane blotting, followed by the Stripping-off[5] of the antibody for probing with a generally used anti-H3 antibody (Abcam).

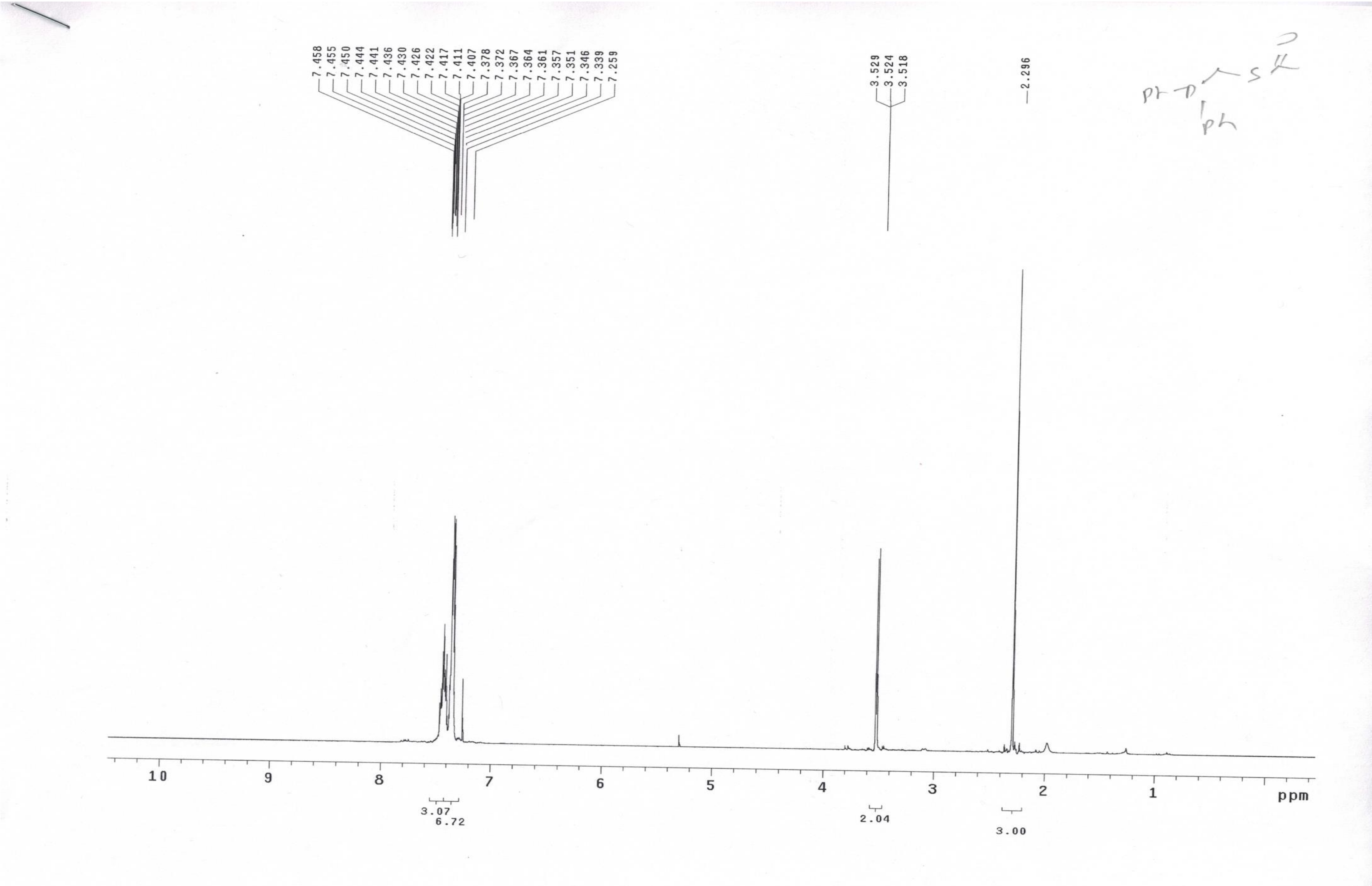
**Appendix:**

[5] http://www.abcam.com/ps/pdf/protocols/stripping%20for%20reprobing.pdf

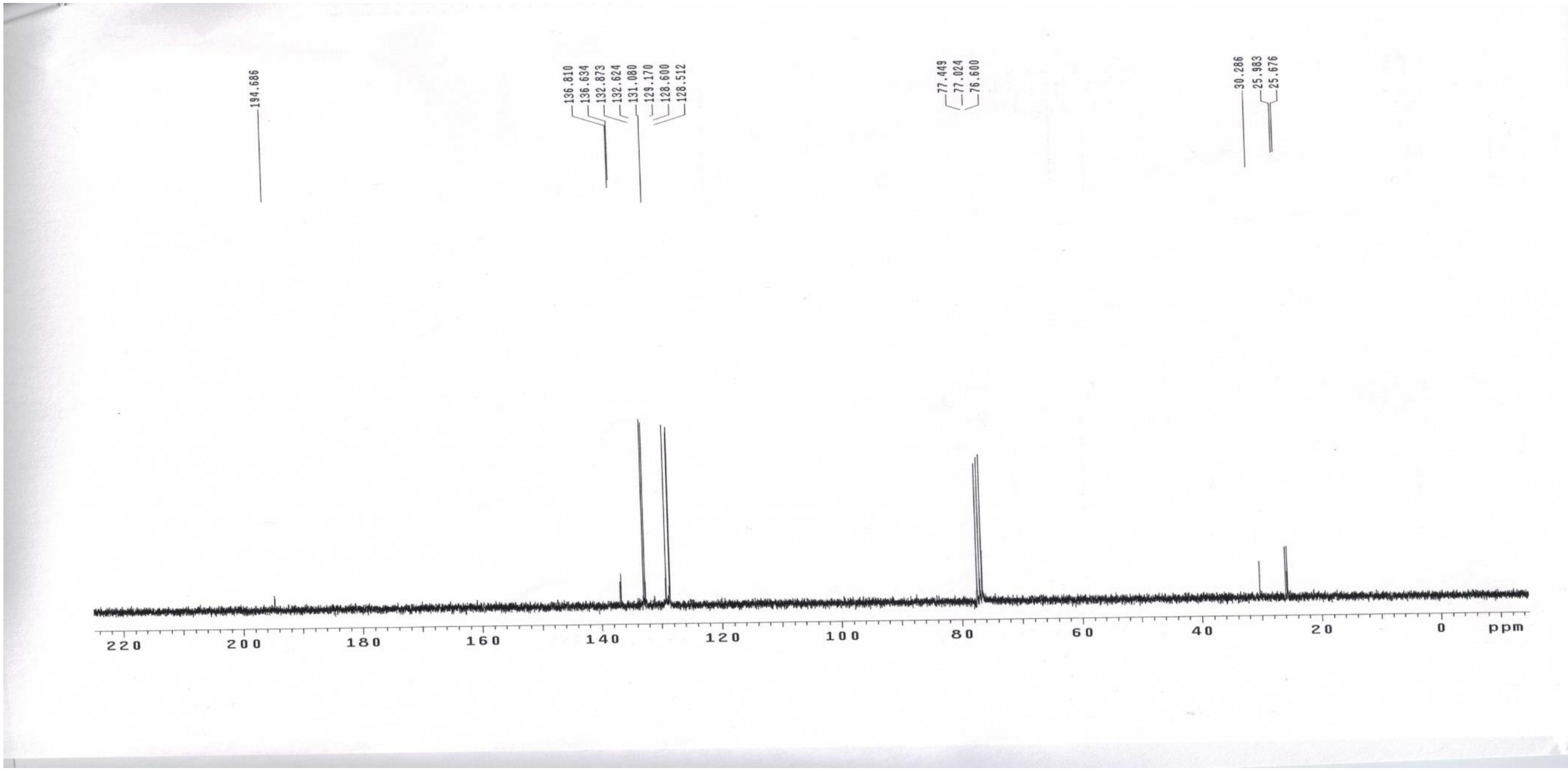
# [1]HNMR Spectrum of **AznL**



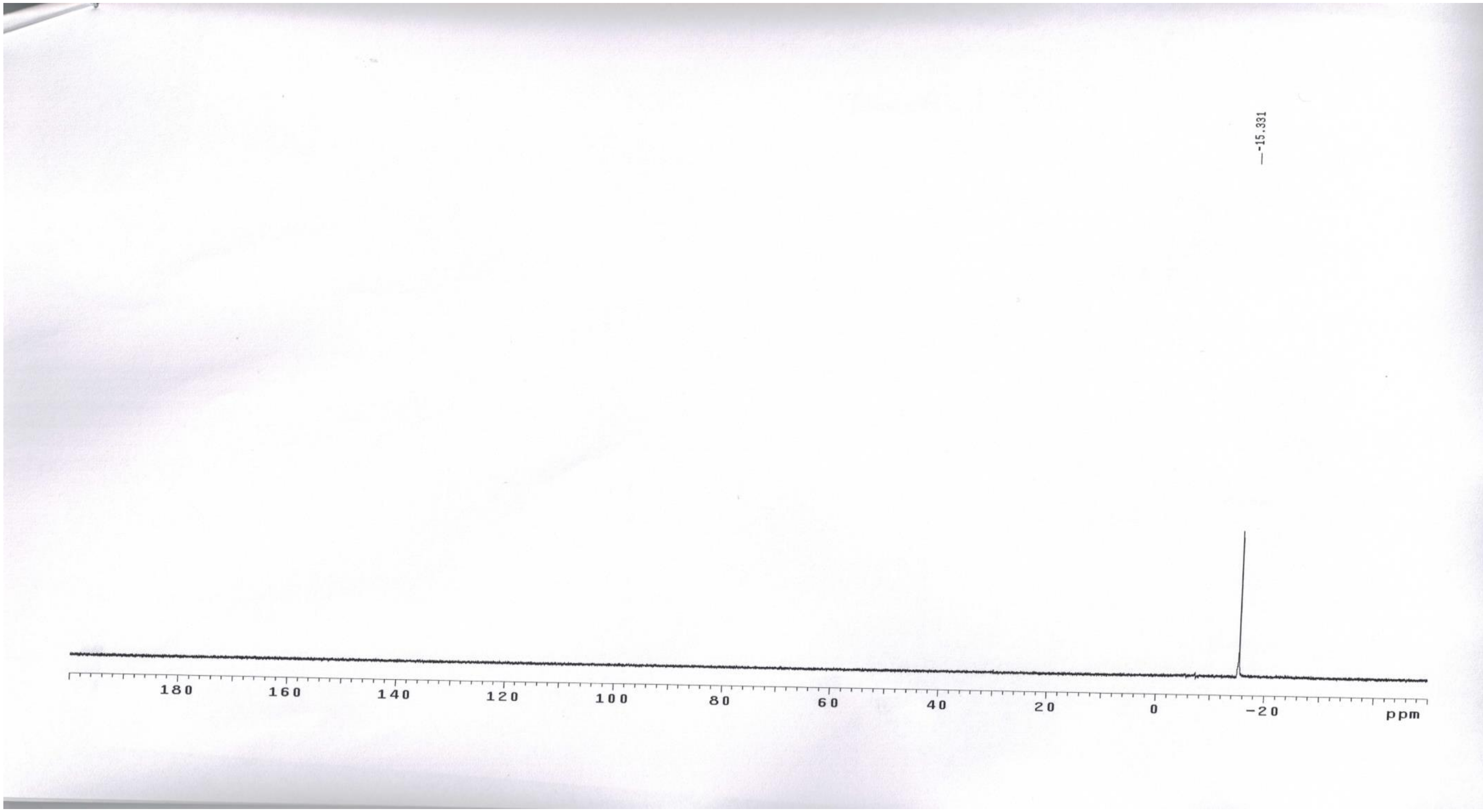
[1]HNMR Spectrum of **dPPMT-Ac**



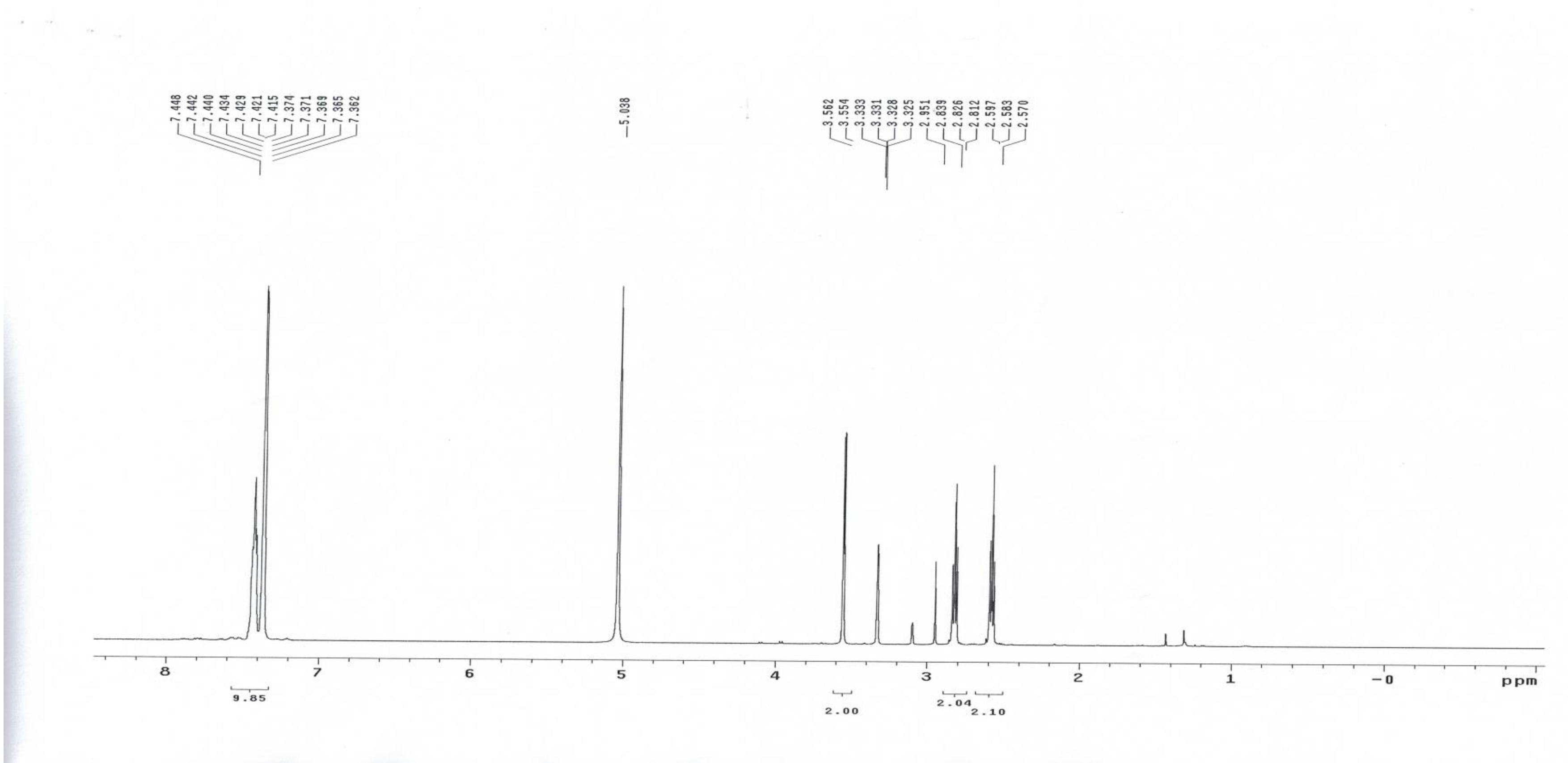
$^{13}$CNMR Spectrum of **dPPMT-Ac**



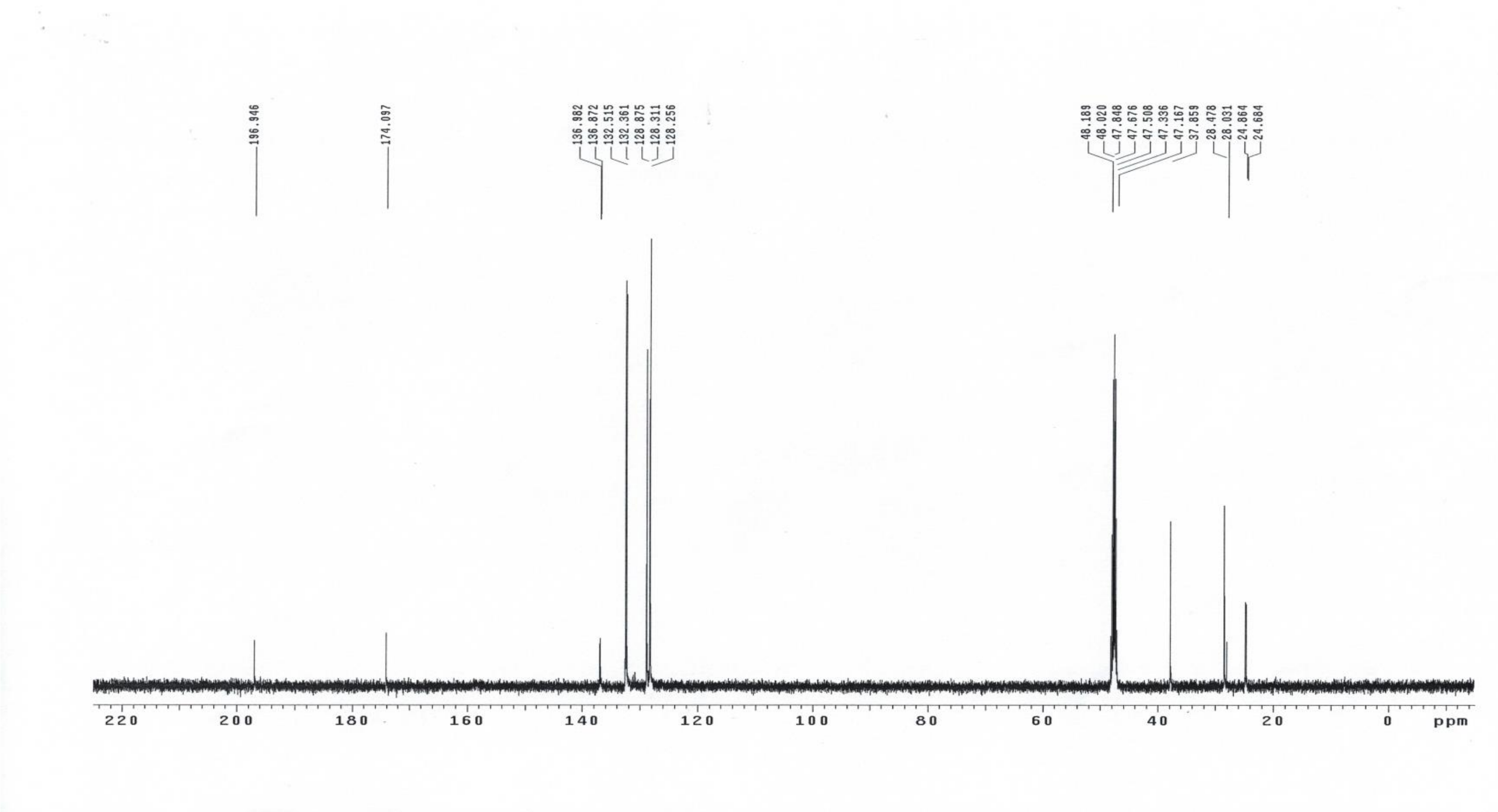
$^{31}$PNMR Spectrum of **dPPMT-Ac**



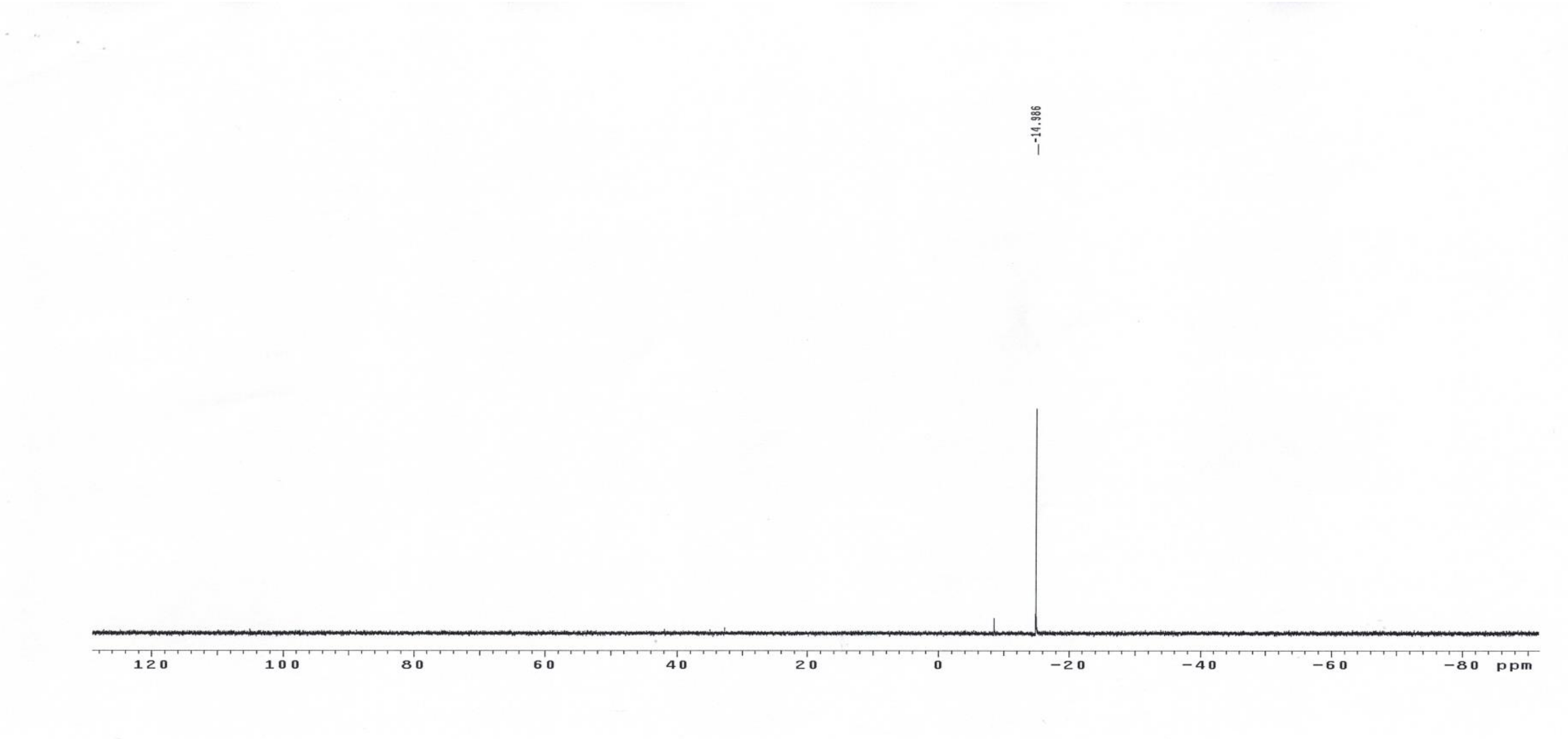
[1]HNMR Spectrum of **dPPMT-Su**



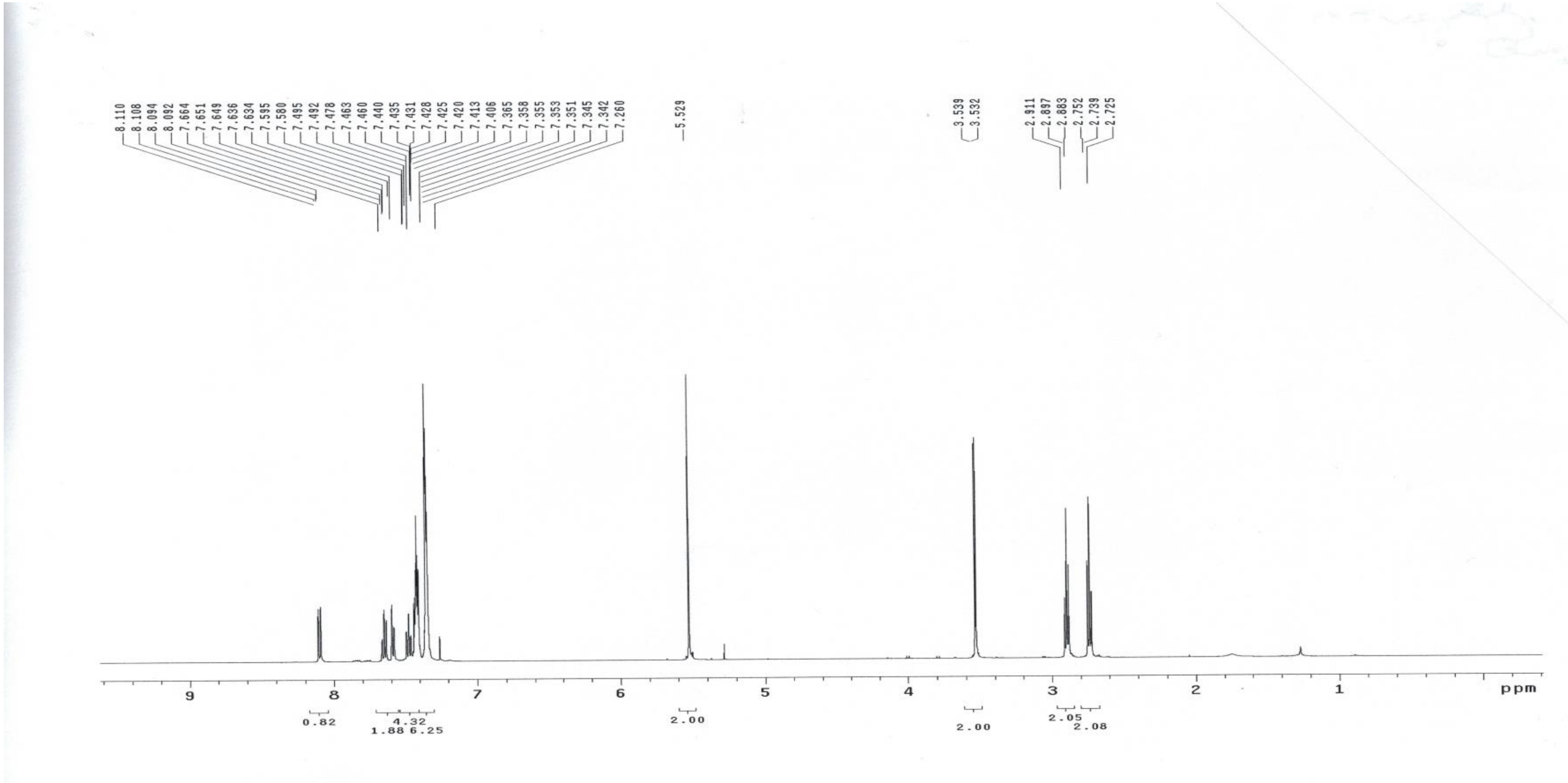
[13]CNMR Spectrum of **dPPMT-Su**



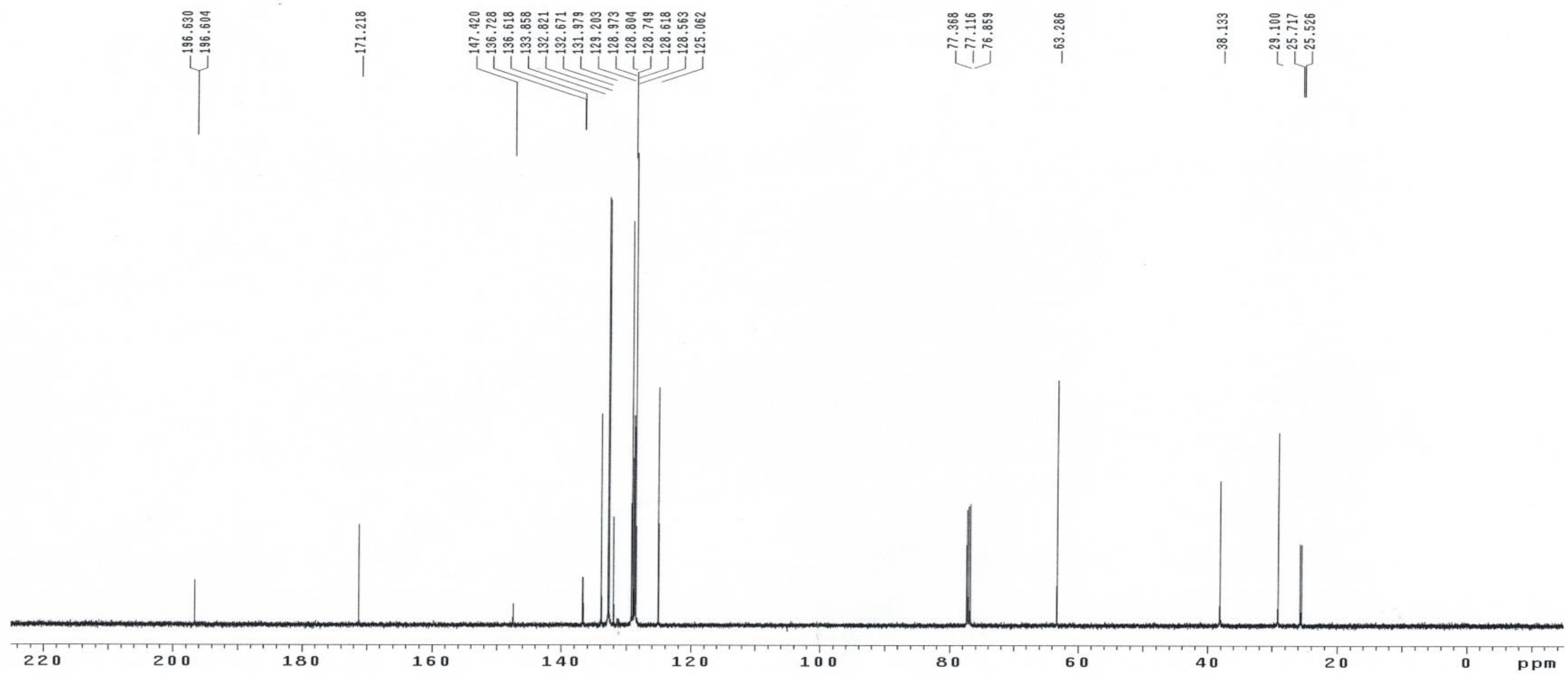
$^{31}$PNMR Spectrum of **dPPMT-Su**



[1]HNMR Spectrum of **dPPMT-NB-Su**



[13]CNMR Spectrum of **dPPMT-NB-Su**



[31]PNMR Spectrum of **dPPMT-NB-Su**