Supporting Information

Regulating miRNA-21 Biogenesis by Bi-functional Small Molecules

Hao Yan, Umesh Bhattarai, Zhi-Fo Guo, Fu-Sen Liang*

Department of Chemistry and Chemical Biology, University of New Mexico, 300 Terrace Street NE, Albuquerque, NM 87131, United States

Table of Contents

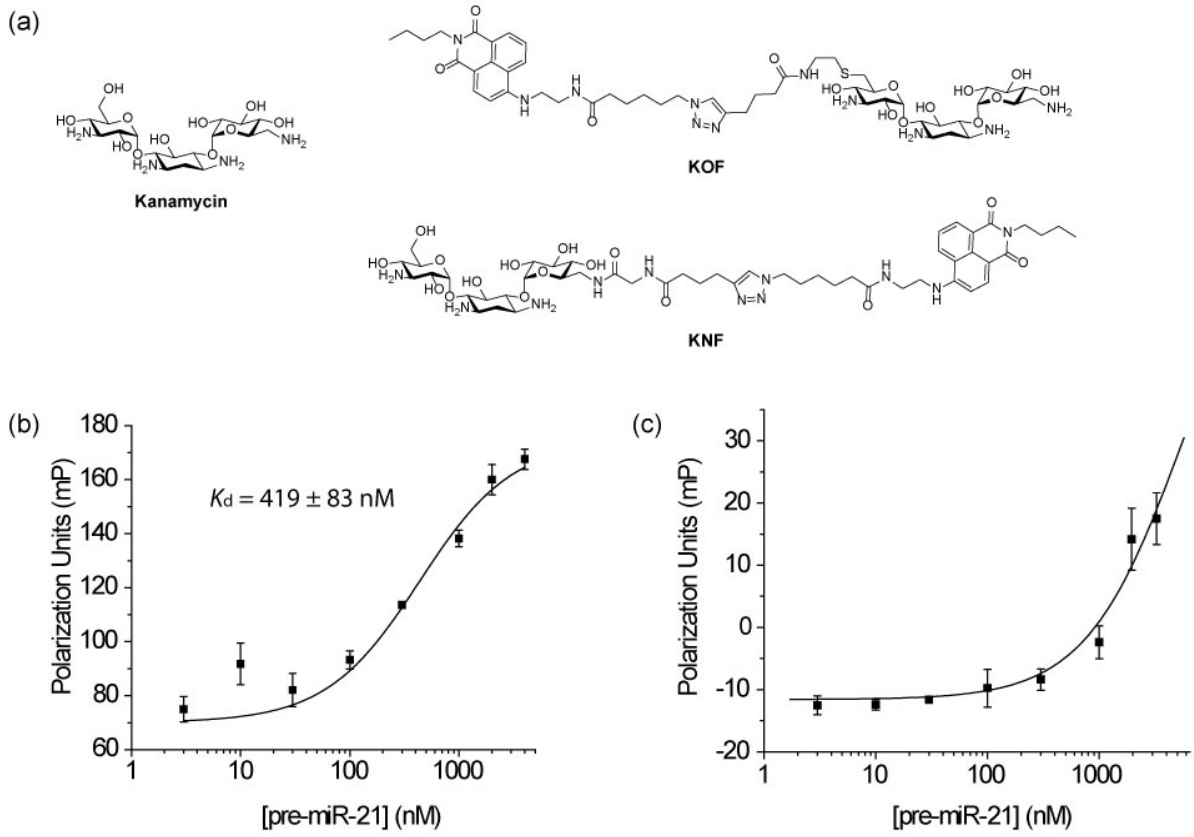


Figure S1. (a) Structures of **Kanamycin**, **KOF** and **KNF**. Fluorescence polarization analysis of **KOF** (b) and **KNF** (c) in the presence of different concentrations of pre-miR-21. The results were the average from 3 independent experiments. The error bars represent the standard error of mean (N = 3).

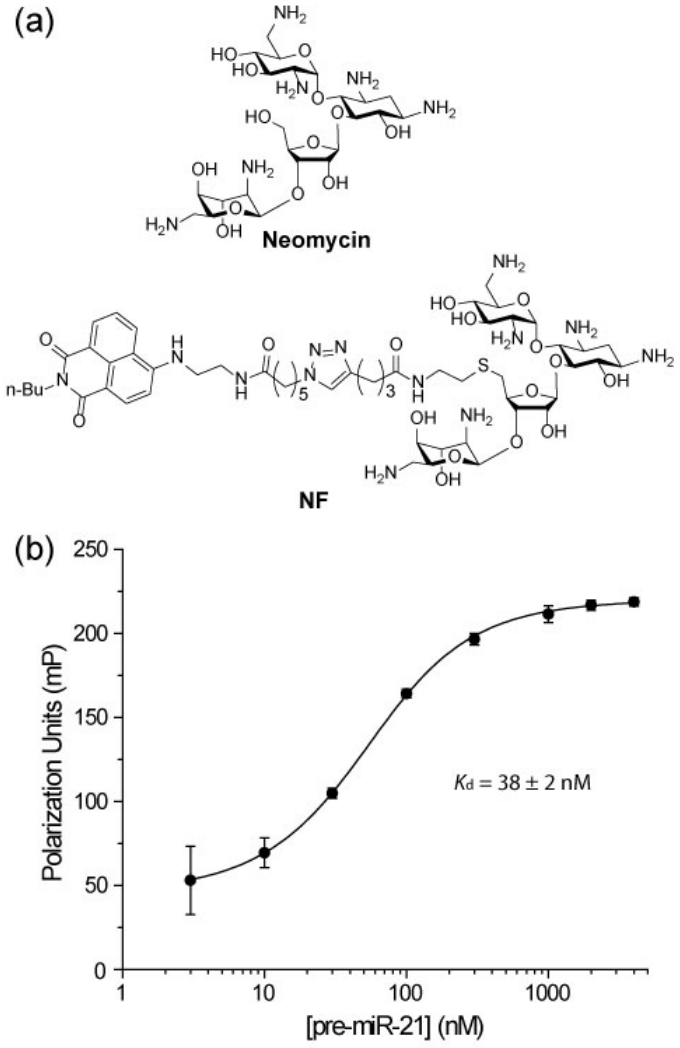


Figure S2. (a) Structures of **Neomycin** and **NF**. (b) Fluorescence polarization analysis of **NF** in the presence of different concentrations of pre-miR-21. The error bars represent the standard error of mean (N = 3).

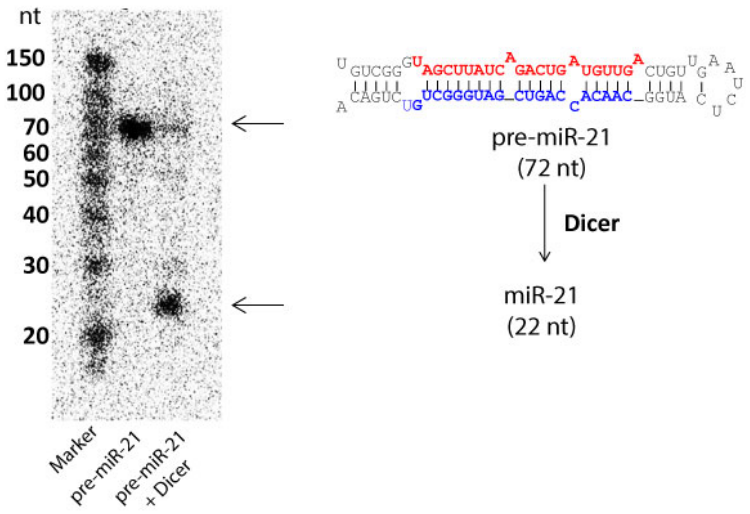


Figure S3. The electrophoresis analysis of Dicer-mediated cleavage of ^{32}P-labeled pre-miR-21.

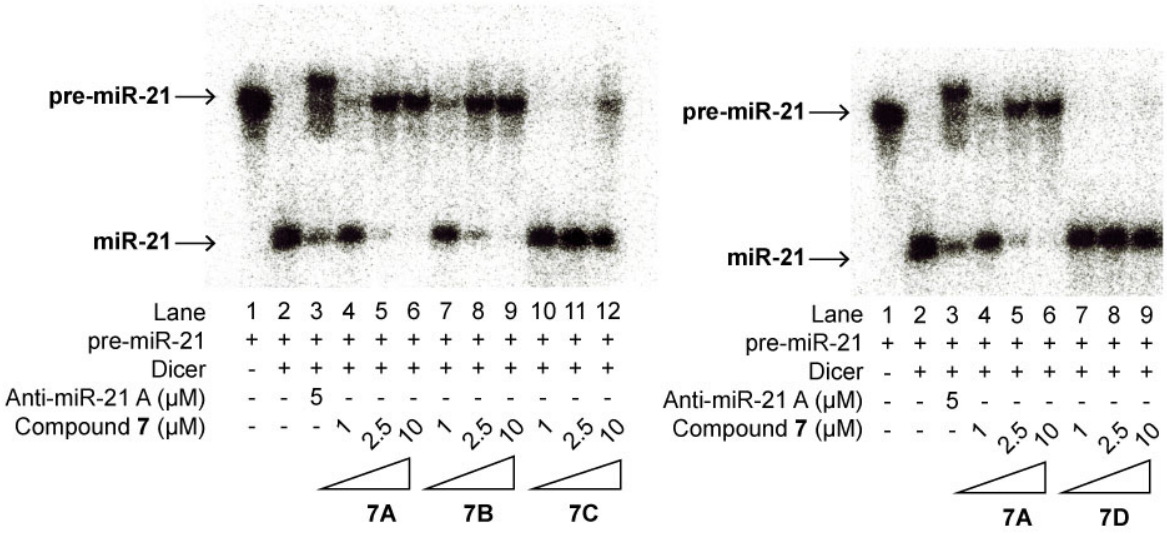


Figure S4. Dicer-mediated pre-miR-21 cleavage in the presence of different concentrations of Compound **7A-D**.

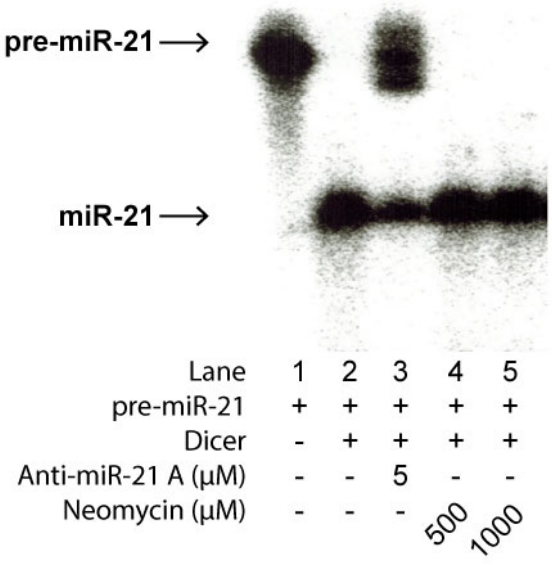


Figure S5. Dicer-mediated pre-miR-21 cleavage in the presence of different concentrations of neomycin.

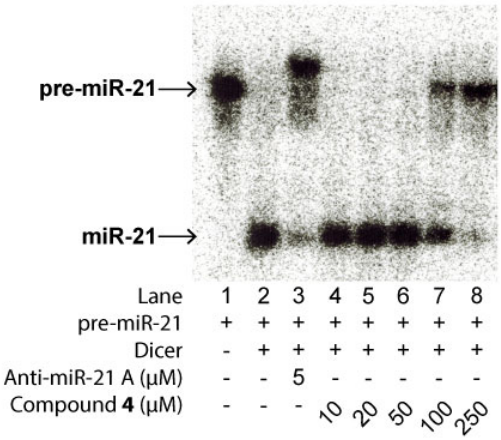


Figure S6. Dicer-mediated pre-miR-21 cleavage in the presence of different concentrations of Compound **4.**

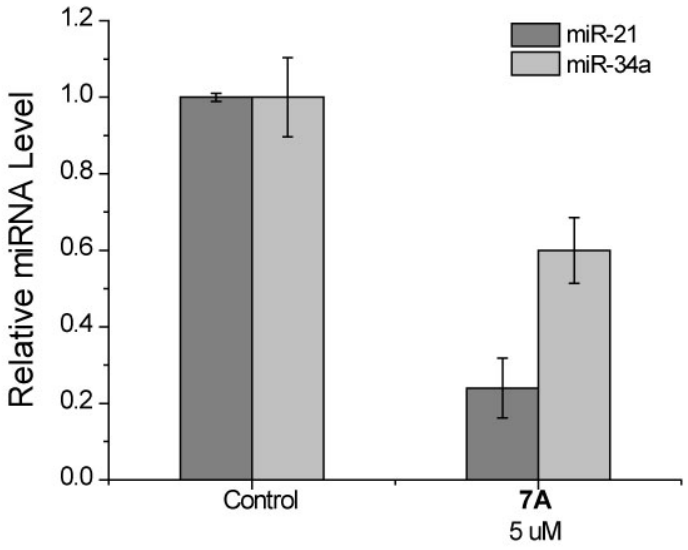


Figure S7. RT-qPCR analysis of mature miR-21 and miR-34a expression level in HEK293T cells expressing corresponding pre-miRNA with or without **7A** treatment. The results were the average from 3 independent experiments. The error bars represent the standard errors of mean (N = 3).

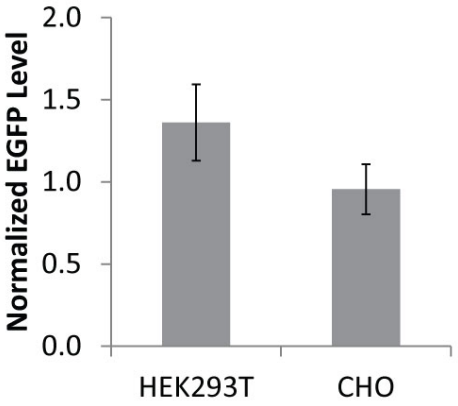


Figure S8. The effects of **7A** on EGFP expression. **7A** does not cause significant change in protein production as measured by the expression of an EGFP reporter plasmid in HEK293T and CHO cells at 5 μM concentration for 24 h that inhibits miR-21 biogenesis. Cells were treated with or without 7A when transfected with the EGFP plasmid. The number of viable cells in each condition (as measured in Figure S9b) was used to normalize for cell number variations. The relative EGFP level was calculated by comparing the condition when cells were treated with **7A** to non-treated cells. The results were the average from 3 independent experiments. The error bars represent the standard deviations (N = 3).

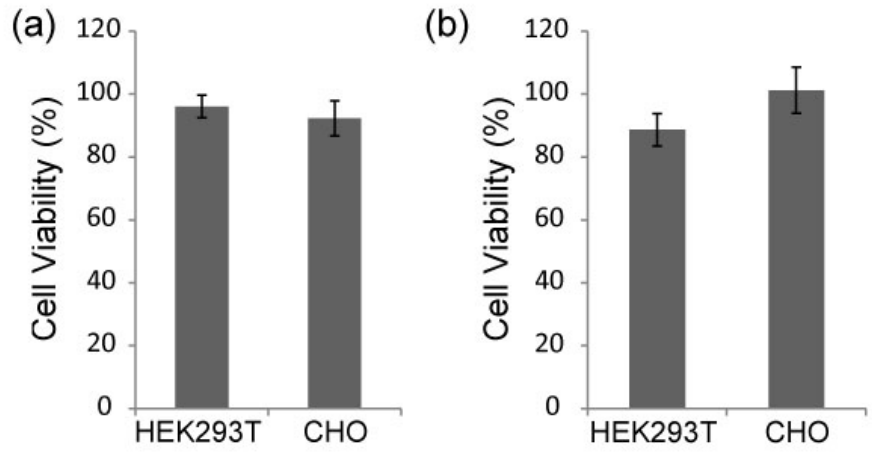
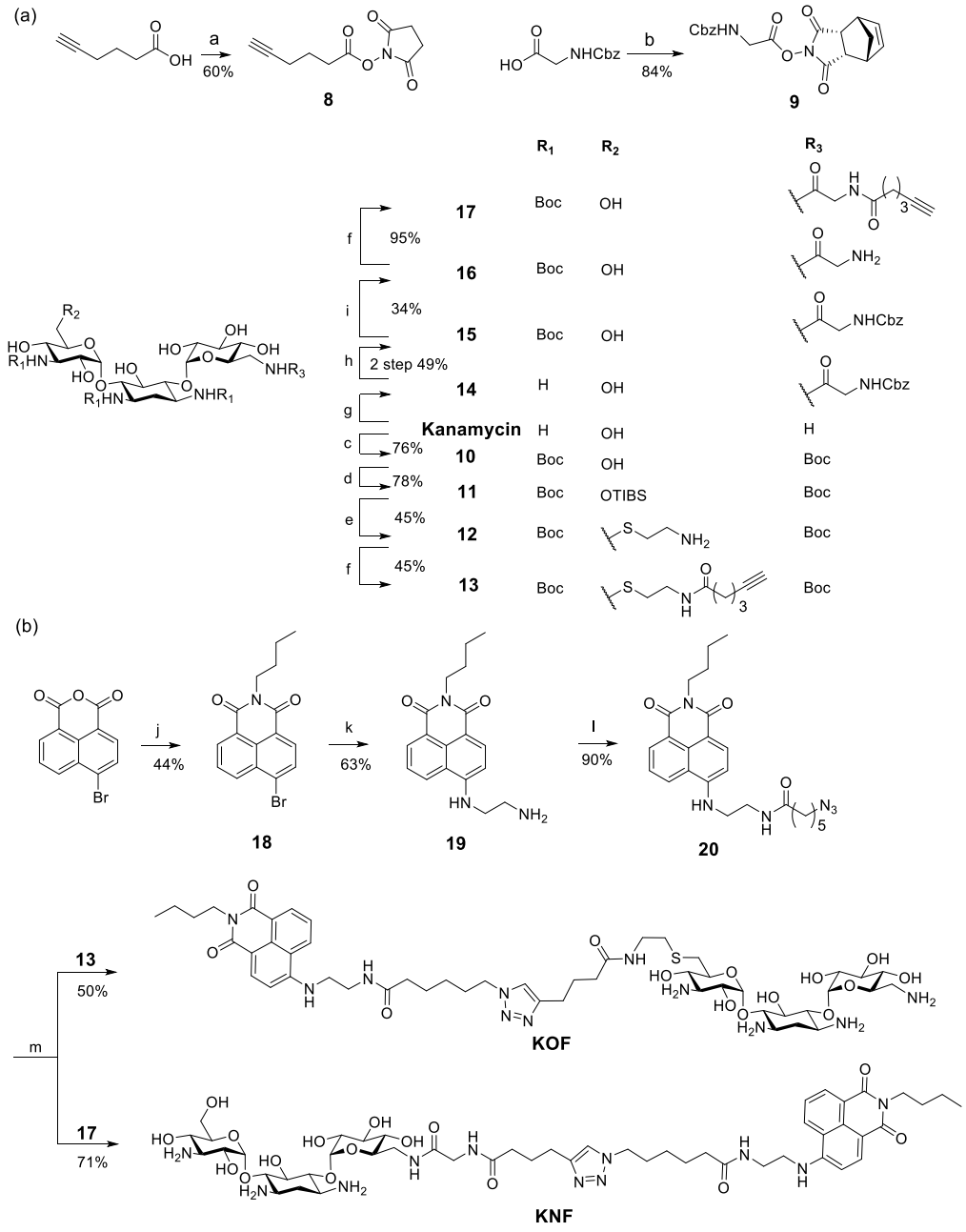
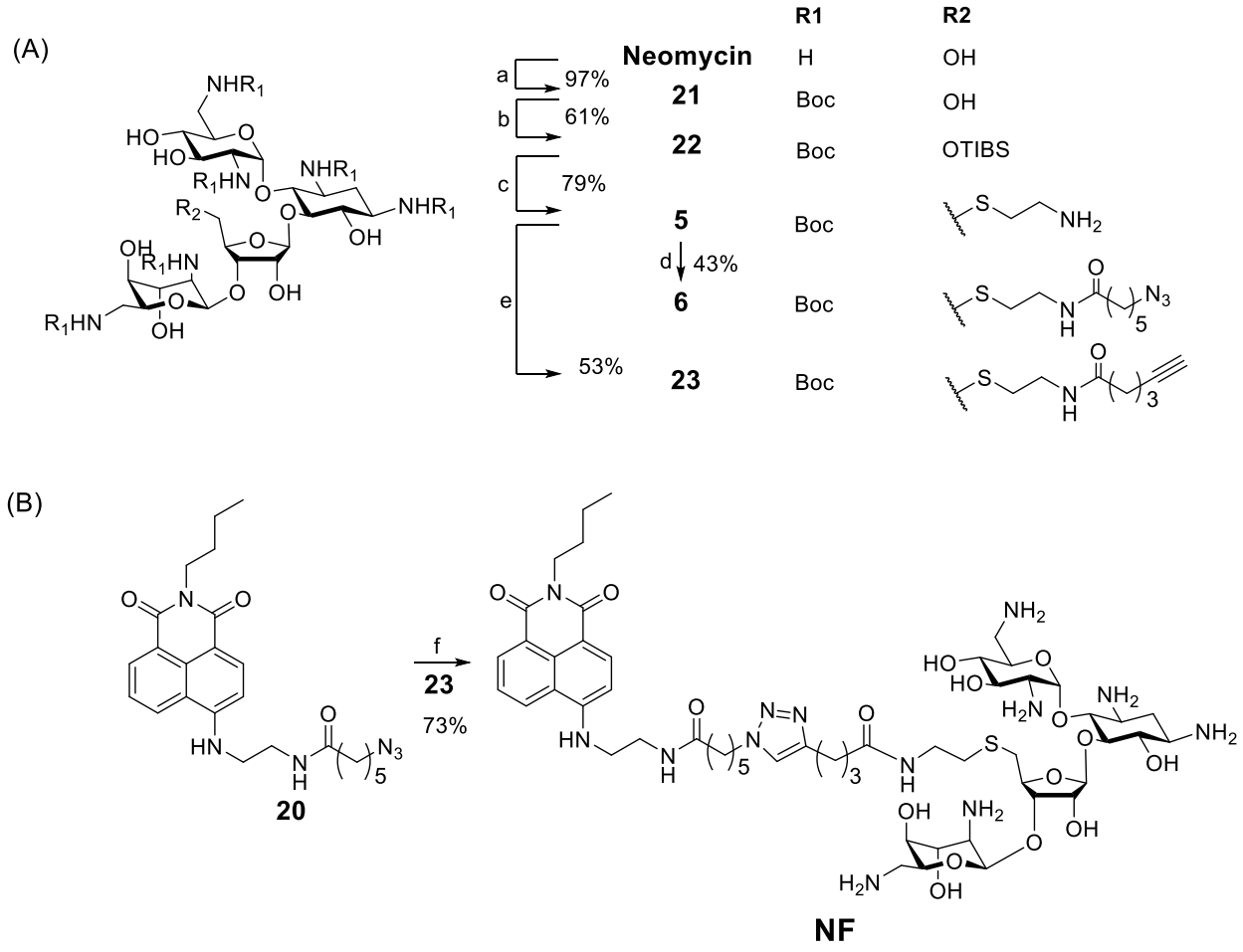


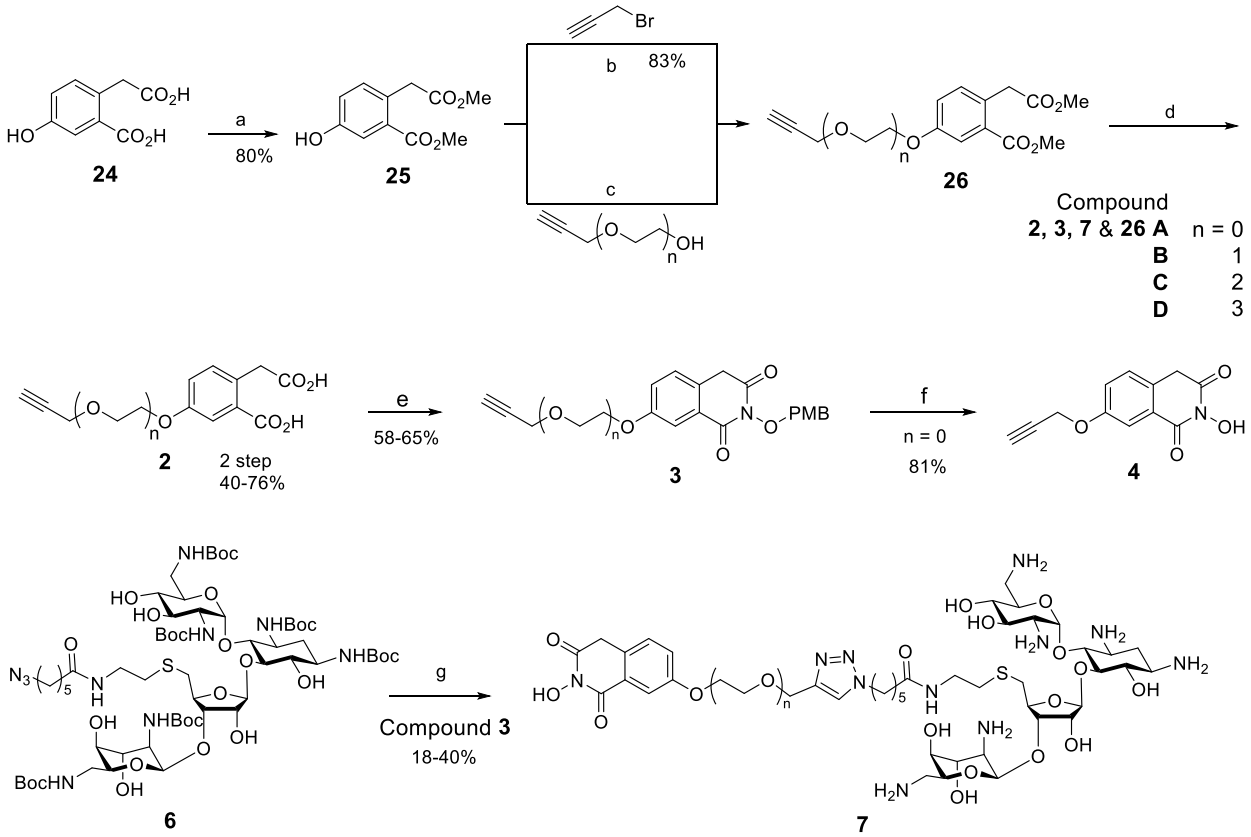
Figure S9. Cytotoxicity of 7A. The cytotoxicity of **7A** (5 μM) toward HEK293T and CHO cells was evaluated by the MTT assay. (a) Cells were directly treated with **7A**. (b) Cells were transfected with an EGFP reporter plasmid and treated with **7A** at the same time. The cell viability percentages were calculated by comparing treated cells to non-treated ones. The results were the average from 3 independent experiments. The error bars represent the standard deviations (N = 3).



Scheme S1. (a) Synthesis of Kanamycin building blocks. (b) Synthesis of **KOF** and **KNF**. *Reagents and conditions*: (a) DCC, HOSu, DCM; (b) DCC, endo-N-hydroxy-5-norbornene-2,3-dicarboximide, DCM; (c) Boc_2O, TEA, DMF/H_2O, 60 ℃; (d) TPS-Cl, pyridine, R.T., 16 h; (e) Cysteamine, Cs_2CO_3, DMF, 16 h; (f) Compound **8**, TEA, DCM; (g) Compound **9**, H_2O/acetone; (h) Boc_2O, H_2O/acetone; (i) H_2, Pd/C, MeOH; (j) n-Butylamine, EtOH, reflux; (k) Ethylenediamine, dioxane, reflux; (l) 6-Azidohexanoic, EDCI, HOBt, DMF; (m) i) $CuSO_4$, Sodium ascorbate, DMSO/H_2O; ii) TFA, DCM.



Scheme S2. Synthesis of neomycin building blocks (A) and **NF** (B). *Reagents and conditions*: (a) Boc_2O, TEA, DMF/H_2O, 60 ℃; (b) TPS-Cl, pyridine, R.T., 16 h; (c) Cysteamine, Cs_2CO_3, DMF, 16 h; (d) 6-Azidohexanoic, EDCI, DIPEA, DCM; (e) 6-hexynoic, EDCI, DIPEA, DCM; (f) i) $CuSO_4$, Sodium ascorbate, DMSO/H_2O; ii) TFA, DCM.



Scheme S3. Synthesis of bi-functional inhibitors. *Reagents and conditions*: (a) H_2SO_4, MeOH, reflux; (b) Propargyl bromide, K_2CO_3, DMF; (c) DIAD, Ph_3P, THF; (d) i) LiOH, MeOH/H_2O; ii) HCl; (e) *O*-(4-Methoxybenzyl)-hydroxylamine, toluene, reflux; (f) TFA, DCM; (g) i) $CuSO_4$, Sodium ascorbate, DMSO/H_2O; ii) TFA, DCM.

Experimental Details

In vitro Transcription of pre-miR-21.[1]

The pre-miR-21 RNA was made from template oligonucleotides using transcription kit (Ambion). Briefly, forward primer:

5'GAAATTAATACGACTCACTATAGGTGTCGGGTAGCTTATCAGACTGATGTTGACTGTTGAATCTCA**T**GGC

and reverse primer:

5' TGTCAGACAGCCCATCGACTGGTGTTGCC**A**TGAGATTCAACAGTCAAC

2 μM of each were subjected to primer extension using Taq polymerase per the manufacture's protocol. The hybrid template with T7 promoter was used for in vitro transcription following manufacturer's instructions (Ambion). Right before use, the RNA prepared was refolded as follows: RNA was heated to 94 ^{o}C for 2 min and then cooled to 4 ^{o}C at a rate of 1 ^{o}C/s.

Fluorescence polarization binding assay.[2]

The affinity of fluorophore tagged aminoglycosides to pre-miR-21 was determined as follows. 30 nM of fluorophore tagged aminoglycoside was incubated with various concentrations of pre-miR-21 (3 nM to 4 μM) in cacodylate buffer (10 mM, pH 7.4, 0.01% Triton X-100) at room temperature for 1 hour. The polarization values were obtained with a microplate reader (SpectraMax i3X, Molecular Devices) equipped with a fluorescence polarization detection cartridge. Polarization units (mP) were plotted against pre-miR-21 concentration and fit in the following equation to determine K_d:

$$\mathrm{P} = \mathrm{P}_0 + \Delta\mathrm{P}\,\frac{[\mathrm{RNA}]_{\mathrm{total}} + [\mathrm{F}]_{\mathrm{total}} + K_{\mathrm{d}} - \sqrt{([\mathrm{RNA}]_{\mathrm{total}} + [\mathrm{F}]_{\mathrm{total}} + K_{\mathrm{d}})^2 - 4[\mathrm{RNA}]_{\mathrm{total}}[\mathrm{F}]_{\mathrm{total}}}}{2[\mathrm{F}]_{\mathrm{total}}}$$

Where P_0 is the polarization of free fluorescent small molecule, P is the measured polarization at each pre-miR-21 concentration $[RNA]_{total}$, ΔP is the total change of polarization upon saturation, $[F]_{total}$ is the

total concentration of fluorescent compound. For the competition binding experiments, various competitors (3 μM) were incubated with fluorophore tagged aminoglycosides (30 nM) and pre-miR-21 (1 μM) for 1 h before reading. The experiment was performed in triplicate.

Dicer-mediated pre-miRNA cleavage assay

The Dicer-mediated pre-miR-21 cleavage assay was performed at 37 °C for 2.5 h using Dicer buffer (Genlantis). Each reaction mixture contained 1 μL of ^{32}P labeled pre-miR-21 (~20 ng, prepared by *in vitro* transcription), 0.5 unit of Dicer (1 μL, Genlantis) and indicated compounds with a final volume of 10 μL. A morpholino anti-miR-21 nucleotide (Anti-miR-21 A, Sequence: 5'-AGTCAACATCAGTCTGATAAGCTAC-3') was used as a positive control. The reaction was stopped by boiling with equal volume of 95% formamide with dyes (Thermo Fisher Scientific) and then separated in 15% denaturing polyacrylamide gel. The gel was imaged with phosphorimager and analyzed by Quantity one software (Bio-rad).

Cell culture and Transfection

pCMV-miR21 (Addgene plasmid # 20381) and MSCV-miR-34a (Addgene plasmid # 63932) were obtained from Addgene. HEK293T and Chinese hamster ovary (CHO) cells were cultured in DMEM medium (Gibco) without antibiotics, supplemented with 10% FBS and 2 mM GlutaMAX (Life Technologies) at 37 ℃ in a humidified atmosphere containing 5% CO_2. HEK293T cells were plated 1 d before transfection in 24-well plates at 2.0 × 10^5 cells/well. Transfections with DNA plasmid mixtures were carried out at ~70% cell confluency using Lipo2000 transfection reagent (Invitrogen). Briefly, DNA 200 ng, Lipofectamine 2000 (Thermo Fisher Scientific) 1 μL and indicated amount of tested compounds were incubated in Opti-MEM reduced serum medium (50 μL) at room temperature for 5 min before transfection. A phosphorothioate-based anti-miR-21 ASO (Anti-miR-21 B, Integrated DNA Technologies, Sequence: 5'-

CAACATCAGTCTGATAAGCTAC-3') was used as a positive control. Cells were harvested for RNA extraction after 22 h or for western blot analysis after 84 h.

RNA extraction and RT-qPCR

Total RNA was extracted using miRNeasy Mini Kit (Qiagen) per the manufacturer's protocol. Approximately 10 ng of total RNA was used in reverse transcription reactions, which were completed using a Taqman MicroRNA RT Kit (Applied Biosystems) per the manufacturer's protocol. RT-qPCR was performed on CFX96 Real Time PCR System (Bio-Rad) using Taqman Universal PCR Master Mix (Applied Biosystems) with 1 µL of the RT product. All primer sets for mature miRNAs were purchased from Applied Biosystems. The triplicate threshold cycles (*Ct*) obtained for each treatment were used to determine the relative levels of miRNA normalized to U6 small nuclear RNA using the $2^{-\Delta\Delta Ct}$ method.[3] The result presented was based on three independent assays.

Western Blotting

Total protein was extracted using RIPA buffer and quantified using a Bio-Rad Protein Assay. Approximately 20 µg of total protein was resolved on a 4-15% SDS-polyacrylamide gel, and then transferred to a PVDF membrane. The membrane was briefly washed with 1× Tris-buffered saline (TBS), and then blocked in 5% milk dissolved in 1× TBST (1× TBS containing 0.1% Tween-20) for 1 h at room temperature. The membrane was then incubated in 1:1000 PDCD4 (Cell Signaling Technology, PDCD4 (D29C6) XP® Rabbit mAb) or GAPDH primary antibody (Cell Signaling Technology, GAPDH (D16H11) XP® Rabbit mAb) in 1 × TBST containing 3% (w/v) BSA overnight at 4 °C. The membrane was washed with 1 × TBST and incubated with 1:10000 anti-rabbit IgG horseradish-peroxidase conjugate in 1 × TBS for 1 h at room temperature. After washing with 1× TBST, protein expression was quantified using Clarity Western

ECL Substrate (Bio-Rad) per the manufacturer's protocol. The image was quantified with Image lab software. The quantification data provided were based on three independent assays.

Cytotoxicity Assay

The cytotoxicity of **7A** toward HEK293T and CHO cells were tested by MTT assays (MTT = 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). HEK293T (50,000 cells/well) and CHO (25,000 cells/well) cells were seeded in 96-well plates and grown overnight to around 70% confluency. For the cell viability under non-transfected condition, the cells were treated with or without **7A** (5 μM) for 24 h. For the cell viability under transfection condition, the cells were transfected with 100 ng of EGFP reporter plasmid (Actin-IRES-eGFP)[4] following the method mentioned above and treated with or without **7A** (5 μM) for 24 h. Then the medium was removed and 10 μL of MTT stock solution (12 mM) was added to wells along with 100 μL of phenol free DMEM. The cells were incubated at 37 ^{o}C for 4 h. The MTT containing medium was replaced with 50 μL of DMSO. The solution was mixed thoroughly with the pipette and incubated 37 ^{o}C for 10 min. The absorbance at 540 nm was recorded with a plate reader (SpectraMax i3X, Molecular Devices). The relative viability of the cells was calculated based on 6 parallel tests by comparing to the controls. The result presented was based on three independent assays.

Translational Inhibition Assay[5]

HEK293T (2×10^5 cells/well) and CHO cells (1×10^5 cells/well) were plated into 24-well plates and grown overnight to around 70% confluency. The cells were then transfected with 400 ng of eGFP reporter plasmid (Actin-IRES-eGFP)[4] following the method mentioned above and treated with or without **7A** (5 μM). After 24 h, the cells were collected by trypsinizing and centrifugation. The GFP expression was quantified with a microplate reader (SpectraMax i3X, Molecular Devices). The relative cell viability

obtained from the cytotoxicity assay was used for normalizing the GFP level. The assay was performed in triplicate. The result presented was based on three independent assays.

Chemical Synthesis

Chemicals and instrumentation. Neomycin tri-sulfate was purchased from Sigma Aldrich (Neomycin B > 85%). Kanamycin A was purchased from Amresco. All other reagents and solvents were purchased from BroadPharm, Aldrich or Alfa Aesar and used without further purification. Reactions involving air- or moisture-sensitive reagents were performed under an argon atmosphere. Analytical thin-layer chromatography (TLC) was conducted on silica gel plates with fluorescent indicator 254 nm, and compounds were visualized by irradiation (254 nm) or by staining with ninhydrin stain or cerium ammonium molybdate stain. Column chromatography was carried out on silica gel (pore size 60 Å, 200-425 mesh particle size, Sigma Aldrich). HPLC was performed using a Thermo Scientific UltiMate 3000 semi-preparative system coupled with a Thermo Scientific Acclaim 120 C18 column (100 mm × 4.6 mm, 3 μm). All HPLC analyses were run at RT and monitored at 260 nm. A gradient of CH_3CN containing 0.1% TFA in water containing 0.1% TFA was used at a flow rate of 1 mL/min. For analytical HPLC, acetonitrile was increased from 5% to 90% in 25 min. For Prep-HPLC, acetonitrile was increased from 5% to 19% in 10 min, 19% to 90% in 1 min and then kept constant for 3 min. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance III 300 spectrometer. Chemical shifts are reported in parts per million (ppm, δ) referenced to the residual ^{1}H resonance of the solvent.[6] Splitting patterns are designated as follows: s (singlet), d (doublet), t (triplet), m (multiplet), br (broad). Coupling constants (J values) are listed in hertz (Hz). High resolution mass spectra (HRMS) were obtained with a Waters Micromass mass spectrometer with electrospray ionization probe.

*Synthesis of **KOF** and **KNF**.*

Compound **8**[7] To a solution of 5-hexynoic acid (2.0 g, 17.8 mmol) in anhydrous THF (50 mL) was added N-hydoxysuccinimide (2.05 g, 17.8 mmol) and DCC (3.68 g, 17.8 mmol). The reaction was stirred at room temperature for overnight. The DCU solid was filtered out and the product was purified by column chromatography (EA: Hexane = 1:2) to give **8** as a colorless oil (2.2 g, 60%). ^{1}H NMR (300MHz, $CDCl_3$, δ) 2.84 (br, 4H), 2.78 (t, *J* = 7.5 Hz, 2H), 2.46 (m, 2H), 2.02 (t, *J* = 2.7 Hz, 1H), 1.97 (t, *J* = 7.2 Hz, 2H).

Compound **10**[8]

A solution of kanamycin (2.0 g, 3.43 mmol) in a mixture of DMF (40 mL), water (8 mL) and triethylamine (1 mL) was treated with di-*tert*-butyldicarbonate (3.1 g, 14.1 mmol). The reaction solution was heated to 60 ºC for 6 h, then cooled to room temperature. The volatiles were removed in vacuo. The residue was partitioned between water (300mL) and ethyl acetate (600 mL). The aqueous layer was separated and extracted with ethyl acetate (2 × 150 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (MeOH: DCM = 1:10) to give **10** as a white solid (2.3 g, 76%). Rf = 0.2 (10% methanol in dichloromethane). ^{1}H NMR(300 MHz, MeOD, δ): 5.10 (br, 1H), 5.07 (d, *J* = 3.3 Hz, 1H), 4.07 (d, *J* = 8.7 Hz, 1H), 3.80-3.52 (m, 10H), 3.47-3.35 (m, 5H), 3.19(t, *J* = 9.3 Hz, 1H), 2.09 (d, *J* = 12.6 Hz, 1H), 1.45 (m, 37H).

Compound **11**[8]

A solution of **10** (0.45 g, 0.51 mmol) in pyridine (10 mL) was treated with 2,4,6-triisopropylbenzenesulfonyl chloride (2.5 g, 8.3 mmol). The reaction mixture was stirred at room temperature for overnight and then neutralized by adding hydrochloric acid (1.0 N). The mixture was partitioned between water (100 mL) and ethyl acetate (200 mL). The aqueous layer was separated and extracted with ethyl acetate (2 × 100 mL). The combined organic layer was washed with brine, dried

over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (50% to 100% EA in hexane) to give **11** a white solid (0.46 g, 78%). Rf = 0.35 (Methanol: DCM = 1:10). ^{1}H NMR (300 MHz, MeOD, δ) 7.28 (s, 2H), 5.04 (br, 2H), 4.38 (t, *J* = 10.2 Hz, 2H), 4.15 (m, 3H), 3.69 (br, 2H), 3.61-3.35 (m, 11H), 3.16 (m, 1H), 2.95 (m, 1H), 2.01 (m, 1H), 1.45 (m, 36H), 1.25 (m, 19H).

Compound **12**[8]

A solution of **11** (328 mg, 0.285 mmol) and cesium carbonate (1.10 g, 3.42 mmol) in DMF (5 mL) was treated with cysteamine hydrochloride (0.65 g, 5.7 mmol). The reaction mixture was stirred at 80 ºC for 4 h and then partitioned between water (100 mL) and ethyl acetate (200 mL). The aqueous layer was separated and extracted with ethyl acetate (2 × 150 mL). The combined organic layer was washed with brine, dried over Na_2SO_4, and concentrated in vacuo. The residue was purified by column chromatography (MeOH: DCM = 1:1) to give **12** as a white solid (120 mg, 45%). R_f = 0.15 (15% methanol in dichloromethane). ^{1}H NMR (300 MHz, MeOD, δ) 5.09 (br, 1H), 5.03 (br, 1H), 4.15 (br, 1H), 3.67 (m, 2H), 3.59 (m, 3H), 3.53 (br, 2H), 3.52-3.32 (m, 5H), 3.17 (m, 1H), 3.02 (m, 2H), 2.97 (d, *J* = 7.8 Hz, 1H), 2.80 (m, 3H), 2.67 (m, 1H), 2.05 (m, 1H), 1.42 (m, 36H), 1.25 (m, 1H).

Compound **13**

To a solution of **12** (100 mg, 0.106 mmol) in DCM (2 mL) was added TEA (50 µL) and **8** (22.2 mg, 0.106 mmol). The reaction was stirred at 40 ^{o}C for 10 h. After removal of the solvents, the residue was purified by column chromatography (MeOH: DCM = 1:20) to give **13** as a white solid (50 mg, 45%). R_f = 0.55 (15% MeOH in DCM). ^{1}H NMR (300 MHz, MeOD, δ) 5.11 (br, 1H), 5.05 (br, 1H), 4.20 (br, 1H), 3.65 (m, 3H), 3.59 (m, 2H), 3.47 (m, 4H), 3.38 (m, 4H), 3.29 (m, 2H), 2.95 (m, 1H), 2.61 (m, 4H), 2.30 (m, 5H), 2.08 (br, 1H), 1.79 (m, 2H), 1.42 (m, 36H), 1.26 (m, 1H). ^{13}C NMR (75 MHz, MeOD, δ): 175.44, 174.90, 159.41, 157.94, 157.72, 102.69, 99.91, 85.15, 84.25, 80.57, 80.38, 80.12, 77.02, 74.60, 74.05, 73.48, 72.33, 72.18,

71.76, 70.31, 57.13, 52.21, 50.89, 41.87, 40.21, 35.88, 34.57, 33.24, 29.95, 28.81, 28.40, 26.27, 25.92, 18.68. HRMS (ESI): calculated for $C_{46}H_{79}N_5O_{19}S$ $[M + H]^+$ 1038.5168, found 1038.5146.

Compound **15**[9]

Compound **9** was first synthesized with a similar procedure as compound **8**. To a solution of kanamycin (free base, 0.243g, 0.5 mmol) in H_2O (25 mL) was added a solution of **9** (0.185 g, 0.5 mmol) in acetone (25 mL) dropwise at 15 ºC. The reaction was stirred at room temperature for 1 h to produce compound **14** (not isolated). Boc_2O (0.437 g, 2 mmol) was then added and the reaction was stirred for 48 h. The precipitate was collected by filtration and purified by column chromatography (MeOH: $CHCl_3$: $NH_3{\cdot}H_2O$ = 1:4:0.1) to give **15** as a white solid (0.240 g, 49%). Rf = 0.4 (MeOH: DCM: $NH_3{\cdot}H_2O$ = 1:5:0.1). ^{1}H NMR (300 MHz, DMSO and D_2O, δ) 7.54 (br, 1H), 7.48 (br, 1H), 7.36 (m, 5H), 6.90 (br, 1), 6.54 (d, *J* = 4.8 Hz, 1H), 6.50 (d, *J* = 5.4 Hz, 1H), 5.03 (s, 2H), 4.94 (s, 1H), 4.91 (s, 1H), 3.80 (br, 1H), 3.67 (m, 2H), 3.60 (m, 1H), 3.50 (m, 2H), 3.47-3.21 (m, 12H), 3.04 (m, 1H), 1.79 (br, 1H), 1.35 (m, 27H), 1.23 (m, 1H). ^{13}C NMR (75 MHz, DMSO and D_2O, δ): 170.11, 156.85, 156.75, 155.71, 155.29, 137.19, 128.68, 128.15, 127.99, 101.19, 98.07,84.04, 80.48, 78.35, 78.31, 77.71, 75.25, 73.15, 72.77, 72.27, 70.44, 70.34, 70.19, 67.57, 65.86, 60.49, 56.03, 50.31, 49.26, 43.59, 40.94, 29.29, 28.59, 28.48, 28.42. HRMS (ESI): calculated for $C_{43}H_{69}N_5O_{20}$ $[M + H]^+$ 976.4614, found 976.4660.

Compound **16**

To a suspension of **15** (0.2g, 0.2 mmol) in MeOH (20 mL) was added Pd/C (10%, 0.05g). The mixture was stirred under H_2 atmosphere (30 psi) for 4 h. The Pd/C was filtered away and the solvents were evaporated in vacuum. The residue was purified by column chromatography (MeOH: $CHCl_3$: $NH_3{\cdot}H_2O$ = 1:1:0.1) to give the product as a white solid (56 mg, 34%). R_f = 0.2 (MeOH: DCM: $NH_3{\cdot}H_2O$ = 1:1:0.1). ^{1}H NMR (300 MHz, DMSO, δ) 7.77 (br, 1H), 6.92 (br, 1H), 6.61 (br, 1H), 6.51 (br, 1H), 4.91 (br, 2H), 4.23 (br,

1H), 3.80 (br, 1H), 3.63 (br, 1H), 3.50 (m, 6H), 3.35 (m, 5H), 3.24 (m, 3H), 3.03 (m, 2H), 1.79 (br, 1H), 1.38 (m, 27H), 1.23 (br, 1H). ^{13}C NMR (75 MHz, DMSO, δ): 172.45, 156.38, 155.37, 154.94, 101.16, 97.75, 84.16, 80.22, 77.87, 77.24, 74.99, 72.95, 72.78, 72.29, 70.34, 70.24, 70.10, 70.00, 67.35, 60.25, 55.90, 50.04, 49.02, 43.83, 34.79, 28.36, 28.25, 28.19. HRMS (ESI): calculated for $C_{35}H_{63}N_5O_{18}$ $[M + H]^+$ 842.4246, found 842.4245.

Compound **17**

Following the procedure for the synthesis of **13**, Compound **16** (56 mg, 0.067 mmol), TEA (60 μL) and compound **8** (50 mg, 0.24 mmol), were used to give **17** (61 mg, 95%) as a white solid. R_f = 0.2 (DCM: MeOH: $NH_3 \cdot H_2O$ = 5:1:0.1). ^{1}H NMR (300 MHz, DMSO, δ) 8.14 (br, 1H), 7.53 (br, 1H), 6.92 (br, 1H), 6.58 (br, 1H), 6.52 (br, 1H), 4.97 (br, 1H), 4.92 (br, 1H), 4.23 (br, 1H), 3.80 (m, 2H), 3.62 (m, 1H), 3.50 (m, 1H), 3.35-3.22 (m, 13H), 3.05 (br, 1H), 2.78 (s, 1H), 2.23 (br, 2H), 2.16 (br, 2H), 1.78 (br, 1H), 1.67 (br, 2H), 1.38 (m, 27H), 1.09 (br, 1H). ^{13}C NMR (75 MHz, DMSO, δ): 172.09, 169.67, 156.39, 155.407, 154.95, 101.11, 97.79, 84.12, 80.26, 77.92, 77.26, 75.07, 72.96, 72.69, 72.19, 71.50, 70.36, 70.12, 67.37, 64.96, 60.29, 55.93, 50.09, 48.98, 45.67, 42.04, 34.82, 33.97, 28.37, 28.25, 28.20, 24.28, 17.39, 15.21. HRMS (ESI): calculated for $C_{41}H_{69}N_5O_{19}$ $[M + Na]^+$ 958.4484, found 958.4487.

Compound **20**

To a solution of **19** (0.15 g, 0.48 mmol) (synthesized following a reported method[10] and characterized by ^{1}H NMR) in DMF (5 mL) was added 6-azidohexanoic (0.076g, 0.48 mmol), EDCI·HCl (0.10g, 0.528 mmol), HOBt (0.072 g, 0.528 mmol) and TEA (0.097 mg, 0.96 mmol). The reaction was stirred at room temperature for overnight. The mixture was partitioned between water (20 mL) and EA (20 mL). The aqueous phase was separated and extracted with EA (2 × 20 mL). The combined organic phase was dried with Na_2SO_4 and filtered. After removing the solvents, the residue was purified by column

chromatography (MeOH: DCM = 1:25) to give **20** as a yellow solid (0.20 g, 90%). R_f = 0.4 (5% MeOH in DCM). ^{1}H NMR (300 MHz, $CDCl_3$, δ) 8.57 (d, *J* = 7.5 Hz, 1H), 8.42 (d, *J* = 8.4 Hz, 1H), 8.22 (d, *J* = 8.4 Hz, 1H), 7.65 (t, *J* = 7.5 Hz, 1H), 7.15 (s, 1H), 6.54 (d, *J* = 8.4 Hz, 1H), 6.17 (t, *J* = 6.0 Hz, 1H), 4.16 (t, *J* = 7.5 Hz, 2H), 3.77 (m, 2H), 3.48 (m, 2H), 3.13 (t, *J* = 6.6 Hz, 2H), 2.30 (t, *J* = 7.5 Hz, 2H), 1.73 (m, 4H), 1.55 (m, 2H), 1.48 (m, 2H), 1.42 (m, 2H), 0.95 (t, *J* = 7.5 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$, δ): 176.15, 164.55, 164.12, 150.12, 134.21, 130.87, 129.45, 127.20, 124.55, 122.28, 120.04, 109.05, 103.06, 50.98, 46.21, 39.83, 38.60, 36.08, 30.23, 28.43, 26.18, 25.17, 20.34, 13.81. HRMS (ESI): calculated for $C_{24}H_{30}N_6O_3$ $[M + H]^+$ 451.2458, found 451.2464.

*General procedure for the synthesis of **KOF** and **KNF***

Procedure A. To a solution of kanamycin alkyne (1 equiv) in DMSO (280 μL) and H_2O (40 μL) was added **20** (1 equiv), $CuSO_4$ (0.1 equiv) and sodium ascorbate (0.2 equiv). The reaction was stirred at room temperature for overnight. The crude product was extracted with ethyl acetate and purified by chromatography on a silica gel column using a DCM/MeOH mixture to give the Boc protected conjugates.

Procedure B. The Boc protected conjugates was treated with TFA (1 mL) and DCM (1 mL) for 24 h. The solvent and TFA were removed under reduced pressure. The residue was purified by precipitation from a solution in MeOH by Et_2O (repeat 5 times), leading to the final product as solids (TFA salts).

KOF. General procedure A was employed for the reaction between **13** (10 mg, 0.01 mmol) and compound **20** (4.3 mg, 0.01 mmol), leading to the Boc protected **KOF** as a yellow solid (8 mg, 52%). ^{1}H NMR (300 MHz, DMSO, δ) 8.61 (d, *J* = 8.7 Hz, 1H), 8.44 (d, *J* = 7.2 Hz, 1H), 8.26 (d, *J* = 8.7 Hz, 1H), 8.10 (br, 1H), 7.90 (br, 1H), 7.85 (br, 1H), 7.81 (s, 1H), 7.70 (t, *J* = 7.5 Hz, 1H), 6.93 (br, 1H), 6.83 (d, *J* = 8.7 Hz, 1H), 6.62 (br, 1H), 6.53 (d, *J* = 8.7 Hz, 1H), 6.37 (br, 1H), 5.48 (s, 1H), 5.26 (s, 1H), 4.22 (t, *J* = 7.5 Hz, 2H), 4.14 (m, 1H), 4.01 (t, *J* = 7.5 Hz, 2H), 3.60-3.10 (m, 13H), 2.57 (m, 4H), 2.08 (m, 4H), 1.77 (m, 5H), 1.55 (m, 4H),

1.37 (m, 36H), 1.25 (m, 5H), 0.93 (t, *J* = 7.5 Hz, 3H). HRMS (ESI): calculated for $C_{70}H_{109}N_{11}O_{22}S$ $[M + Na]^+$ 1510.7367, found 1510.7410. General procedure B was further applied to remove Boc and give **KOF** as a yellow solid (8.2 mg, 97%). ^{1}H NMR (300 MHz, D_2O, δ) 7.98 (d, *J* = 7.5 Hz, 1H), 7.82 (d, *J* = 7.8 Hz, 1H), 7.77 (d, *J* = 8.7 Hz, 1H), 7.31 (t, *J* = 7.8 Hz, 1H), 7.21 (s, 1H), 6.41 (d, *J* = 8.7 Hz, 1H), 5.51 (d, *J* = 3.6 Hz, 1H), 5.01 (d, *J* = 3.3 Hz, 1H), 3.92 (m, 2H), 3.85 (m, 2H), 3.69 (m, 4H), 3.61-3.32 (m, 9H), 3.27 (m, 2H), 3.17 (m, 2H), 2.95 (m, 1H), 2.63 (m, 2H), 2.47 (m, 2H), 2.11 (m, 4H), 1.84 (m, 1H), 1.71 (br, 2H), 1.26 (br, 7H), 0.85 (t, *J* = 7.5 Hz, 3H). HRMS (ESI): calculated for $C_{50}H_{77}N_{11}O_{14}S$ $[M + H]^+$ 1088.5450, found 1088.5485.

KNF General procedure A was employed for the reaction between **17** (10 mg, 0.01 mmol) and compound **20** (4.3 mg, 0.01 mmol), leading to the Boc protected **KNF** as a yellow solid (10 mg, 72%). ^{1}H NMR (300 MHz, DMSO, δ) 8.58 (d, *J* = 5.4 Hz, 1H), 8.44 (d, *J* = 5.1 Hz, 1H), 8.26 (d, *J* = 8.4 Hz, 1H), 8.10 (br, 2H), 7.85 (s, 1H), 7.81 (s, 1H), 7.70 (m, 1H), 7.58 (s, 1H), 6.92 (m, 1H), 6.84 (d, *J* = 8.1 Hz, 1H), 6.59 (m, 2H), 5.39 (s, 1H), 5.22 (s, 1H), 5.0-2.3 (very poor resolution), 2.17 (m, 2H), 2.08 (m, 2H), 1.77 (m, 5H), 1.55 (m, 4H), 1.35 (m, 28H), 0.91 (t, *J* = 7.5 Hz, 3H). HRMS (ESI): calculated for $C_{65}H_{99}N_{11}O_{22}$ $[M + Na]^+$ 1408.6864, found 1408.6891. General procedure B was applied to remove Boc and give **KNF** as a yellow solid (9.9 mg, 98%). ^{1}H NMR (300 MHz, D_2O, δ) 7.88 (d, *J* = 6.0 Hz, 1H), 7.72 (d, *J* = 6.9 Hz, 1H), 7.68 (d, *J* = 7.5 Hz, 1H), 7.27 (s, 1H), 7.24 (d, *J* = 8.7 Hz, 1H), 6.33 (d, *J* = 6.0 Hz, 1H), 5.40 (d, *J* = 3.6 Hz, 1H), 5.05 (d, *J* = 3.6 Hz, 1H), 3.93 (m, 3H), 3.75 (m, 7H), 3.68 (m, 7H), 3.55 (m, 7H), 3.46 (m, 3H), 3.25 (t, *J* = 9.3 Hz, 1H), 2.48 (br, 3H), 2.20 (m, 2H), 2.18 (m, 2H), 1.92 (m, 1H), 1.76 (br, 2H), 1.41 (br, 5H), 1.32 (m, 4H), 0.91 (t, *J* = 7.2 Hz, 3H). HRMS (ESI): calculated for $C_{50}H_{75}N_{11}O_{16}$ $[M + H]^+$ 1086.5472, found 1086.5488.

*Synthesis of **NF***

Compound **5** was prepared according to a reference method.[8]

Compound **6**.

To a solution of **5** (200 mg, 0.157 mmol) in DCM (2 mL) was added 6-azidohexanoic (30 mg, 0.188 mmol), *N*-(3-Dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (36 mg, 0.188 mmol) and *N,N*-diisopropylethylamine (49 mg, 0.377 mmol). The reaction was stirred at room temperature for overnight. The mixture was partitioned between water (50 mL) and ethyl acetate (50 mL). The aqueous phase was separated and extracted with ethyl acetate (2 × 50 mL). The combined organic phase was washed with brine (3 X 50 mL) and dried over Na_2SO_4. After removing the solvents, the residue was purified by column chromatography (4.5% MeOH in DCM) to give **6** as a white solid (96 mg, 43%). R_f = 0.5 (10% MeOH in DCM). ^{1}H NMR (300 MHz, CD_3OD, δ) 5.38 (s, 1H), 5.15 (s, 1H), 4.94 (s, 1H), 4.61 (s, 1H), 4.25 (br, 2H), 4.09 (m, 1H), 3.90 (m, 2H), 3.77 (s, 1H), 3.71 (m, 1H), 3.59 (m, 3H), 3.48 (m, 4H), 3.43 (m, 3H), 3.21 (m, 2H), 2.89 (d, *J* = 5.4 Hz, 2H), 2.76 (t, *J* = 6.6 Hz, 2H), 2.24 (t, *J* = 7.5 Hz, 2H), 1.94 (m, 1H), 1.66 (m, 4H), 1.45 (m, 61H). ^{13}C NMR (75 MHz, CD_3OD, δ): 176.05, 159.08, 158.87, 158.49, 158.19, 158.15, 157.87, 111.09, 100.52, 99.14, 87.03, 82.71, 81.54, 80.74, 80.67, 80.58, 80.38, 80.22, 79.93, 75.63, 75.29, 74.47, 73.28, 72.86, 71.58, 68.86, 56.85, 53.60, 52.33, 51.45, 42.57, 41.75, 40.14, 36.94, 35.92, 35.56, 32.83, 29.65, 29.01, 28.88, 28.85, 28.77, 27.40, 26.52. HRMS (ESI): calculated for $C_{61}H_{109}N_{10}O_{25}S$ $[M + H]^+$ 1413.7286, found 1413.7264.

Compound **23** was prepared according to a reference method.[11]

NF. A similar procedure as the synthesis of **KOF** was employed for the reaction between **23** (10 mg, 7.3 μmol) and compound **20** (4.0 mg, 7.3 μmol), leading to the Boc protected **NF** as a yellow solid (12 mg, 90%). ^{1}H NMR (300 MHz, MeOD, δ) 8.48 (d, *J* = 7.2 Hz, 1H), 8.42 (d, *J* = 8.4 Hz, 1H), 8.33 (d, *J* = 8.4 Hz, 1H),

7.70 (s, 1H), 7.65 (t, *J* = 8.1 Hz, 1H), 6.82 (d, *J* = 8.7 Hz, 1H), 5.37 (s, 1H), 5.15 (s, 1H), 4.93 (s, 1H), 4.24 (m, 4H), 4.12 (m, 3H), 3.90 (br, 2H), 3.76 (br, 2H), 3.59 (m, 8H), 3.48 (m, 4H), 3.39 (m, 4H), 3.31 (m, 1H), 2.90 (m, 2H), 2.74 (m, 4H), 2.22 (m, 4H), 1.95 (m, 3H), 1.85 (m, 2H), 1.63 (m, 6H), 1.45 (m, 54H), 1.28 (m, 5H), 0.96 (t, *J* = 7.5 Hz, 3H). HRMS (ESI): calculated for $C_{85}H_{135}N_{13}O_{28}S$ $[M + Na]^+$ 1840.9158, found 1840.9204. The Boc was then removed by TFA to give **NF** as a yellow solid (11 mg, 82%). ^{1}H NMR (300 MHz, D_2O, δ) 7.98 (d, *J* = 7.5 Hz, 1H), 7.82 (d, *J* = 8.1 Hz, 1H), 7.79 (d, *J* = 8.7 Hz, 1H), 7.30 (t, *J* = 8.1 Hz, 1H), 7.28 (s, 1H), 6.40 (d, *J* = 8.7 Hz, 1H), 6.04 (d, *J* = 3.9 Hz, 1H), 5.39 (s, 1H), 5.28 (s, 1H), 4.39 (s, 2H), 4.31 (m, 2H), 4.23 (m, 1H), 4.01 (m, 2H), 3.90 (m, 3H), 3.77 (m, 3H), 3.65 (m, 3H), 3.55 (m, 5H), 3.49-3.29 (m, 12H), 3.12 (m, 1H), 2.71 (m, 1H), 2.49 (m, 3H), 2.14 (m, 4H), 1.91 (m, 1H), 1.78 (m, 2H), 1.42 (m, 4H), 1.39 (m, 5H), 0.92 (t, *J* = 7.2 Hz, 3H). HRMS (ESI): calculated for $C_{55}H_{87}N_{13}O_{16}S$ $[M + 2H]^{2+}$ 1219.6271, found 1219.6224.

Synthesis of bi-functional inhibitors

Compound **25**

To a stirred solution of 2-(carboxymethyl)-5-hydroxybenzoic acid (**24**) (1.00 g) in MeOH (50 mL) was added sulfuric acid (200 µL) dropwise. The mixture was refluxed for 4 h. The solvents were removed by vacuum and the residue was partitioned between water (50 mL) and ethyl acetate (50 mL). The organic phase was washed with water (2 X 50 mL) and dried over Na_2SO_4. The solid was removed by filtration. The filtrate was vacuumed to remove the solvent. The crude product was purified by chromatography (Hexane/Ethyl Acetate: 1/1) to give **25** as a yellow oil (0.98 g, 86%). ^{1}H NMR (300 MHz, DMSO): δ (ppm) 9.76 (s, 1H), 7.31 (d, *J* = 2.7 Hz, 1H), 7.16 (d, *J* = 8.4 Hz, 1H), 6.92 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 3.84 (s, 2H), 3.75 (s, 3H), 3.57 (s, 3H).

Compound **26A**

To a stirred solution of **25** (200 mg, 0.89 mmol) in DMF (5 mL) was added K_2CO_3 (370 mg) and propargyl bromide (400 mg, 80% in toluene, 2.68 mmol). The reaction was stirred overnight at 70 ^{o}C. Water (50 mL) was added. The mixture was extracted with ethyl acetate (2 X 50 mL). The combined organic phase was washed with brine (3 X 50 mL) and dried over Na_2SO_4. The solid was filtered off. The filtrate was vacuumed to remove the solvent. The residue was further purified by chromatography (Hexane/Ethyl Acetate: 5/1) to give **26A** as a yellow oil (0.195 g, yield 83%). ^{1}H NMR (300 MHz, MeOD): δ (ppm) 7.59 (d, *J* = 2.7 Hz, 1H), 7.24 (d, *J* = 8.4 Hz, 1H), 7.15 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 4.77 (d, *J* = 2.4 Hz, 2H), 3.94 (s, 2H), 3.84 (s, 3H), 3.66 (s, 3H), 2.97 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (300 MHz, MeOD): δ (ppm) 174.2, 168.6, 158.1, 134.6, 131.8, 130.1, 120.0, 118.1, 79.4, 77.2, 56.8, 52.5, 52.3, 40.3. HRMS (ESI) m/z $[M + Na]^+$ 285.0731, calculated for $C_{14}H_{14}O_5Na$ 285.0739.

General method for compound **26B-D**

To a stirred solution of **25** (1.1 eq) in THF was added appropriate alcohol (1 eq) and triphenylphosphine (1.1 eq). The mixture was cooled to 5 ^{o}C with ice bath. Diisopropyl azodicarboxylate (1.1 eq) was then added dropwise to maintain the temperature below 5 ^{o}C. The reaction was allowed to warm to room temperature and stirred overnight. The solvents were removed by vacuum. The resulting residue was dissolved in ethyl acetate (50 mL) and washed with saturated sodium carbonate solution (3 X 50 mL). The organic phase was then dried, concentrated and purified by chromatography (hexane/ethyl acetate). The crude product (containing diisopropyl-1, 2-hydrazinedicarboxylate) was used for next step reaction without further purification and characterization.

General method for compound 2A-C

To a solution of **26** (1 eq) in methanol (5 mL) was added water (5 mL) and $LiOH{\cdot}H_2O$ (4 eq). The reaction was stirred overnight at room temperature. The solvents were removed by vacuum. The residue was

dissolved in water (10 mL). Concentrated HCl (2 mL) was added to adjust the pH to 1, resulting in white precipitation. The solid was collected by filtration and dried to give the product as a white solid.

Compound **2A**

290 mg of **26A** was used to produce 187 mg of **2A** (white solid, yield 72%). ^{1}H NMR (300 MHz, MeOD): δ (ppm) 7.63 (d, *J* = 2.7 Hz, 1H), 7.23 (d, *J* = 8.4 Hz, 1H), 7.14 (dd, *J* = 8.4 Hz, 3.0 Hz, 1H), 4.77 (d, *J* = 2.4 Hz, 2H), 3.95 (s, 2H), 2.96 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, MeOD): δ (ppm) 175.8, 170.0, 157.9, 134.5, 132.4, 130.7, 119.8, 118.4, 79.5, 77.1, 56.8, 40.4. HRMS (ESI) m/z $[M - H]^-$ 233.0451, calculated for $C_{12}H_9O_5$ 233.0450.

Compound **2B**

340 mg of **26B** was used to produce 111 mg of **2B** (off white solid, 40% total yield from **25**). ^{1}H NMR (300 MHz, MeOD): δ (ppm) 7.58 (d, *J* = 2.7 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 7.10 (dd, *J* = 8.7 Hz, 2.7 Hz, 1H), 4.25 (d, *J* = 2.4 Hz, 2H), 4.18 (m, 2H), 3.94 (s, 2H), 3.89 (m, 2H), 2.88 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, MeOD): δ (ppm) 175.9, 170.2, 159.1, 134.6, 132.5, 130.2, 119.6, 117.9, 80.4, 76.1, 69.3, 68.6, 59.2, 40.4. HRMS (ESI) m/z $[M - H]^-$ 277.0705, calculated for $C_{14}H_{13}O_6$ 277.0712.

Compound **2C**

330 mg of **26C** was used to produce 160 mg of **2C** (white solid, 50% total yield from **25**). ^{1}H NMR (300 MHz, MeOD): δ (ppm) 7.58 (d, *J* = 3.0 Hz, 1H), 7.21 (d, *J* = 8.4 Hz, 1H), 7.10 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 4.19 (m, 4H), 3.94 (s, 2H), 3.86 (m, 2H), 3.70 (m, 4H), 2.84 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, MeOD): δ (ppm) 175.9, 170.2, 159.2, 134.6, 132.4, 130.1, 119.6, 118.0, 80.5, 75.9, 71.5, 70.7, 70.1, 68.8, 59.0, 40.4. HRMS (ESI) m/z $[M - H]^-$ 321.0969, calculated for $C_{16}H_{17}O_7$ 321.0974.

Compound **2D**

To a solution of **26D** (330 mg) in methanol (5 mL) was added $LiOH \cdot H_2O$ (233 mg) and water (3 mL). The reaction was stirred overnight at room temperature. The solvents were removed by vacuum. The residue was dissolved in water (10 mL). Concentrated HCl (2 mL) was added to adjust the pH to 1. The mixture was then extracted with ethyl acetate (2 X 10 mL). The combined organic phase was washed with brine (2 X 10 mL), dried and concentrated to give **2D** as colorless oil (244 mg, 67% total yield from **25**). ^{1}H NMR (300 MHz, MeOD): δ (ppm) 7.58 (d, *J* = 2.7 Hz, 1H), 7.22 (d, *J* = 8.4 Hz, 1H), 7.10 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 4.17 (m, 4H), 3.94 (s, 2H), 3.86 (m, 2H), 3.71 (m, 2H), 3.65 (m, 6H), 2.83 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, MeOD): δ (ppm) 175.9, 170.1, 159.2, 134.6, 132.4, 130.1, 119.6, 118.0, 80.6, 75.9, 71.7, 71.5, 71.4, 70.8, 70.1, 68.8, 59.0, 40.4. HRMS (ESI) m/z $[M - H]^-$ 365.1232, calculated for $C_{18}H_{21}O_8$ 365.1236.

General method for Compound 3

A solution of **2** (1 eq) and *O*-(4-Methoxybenzyl)-hydroxylamine (1.2 eq) in toluene (180 mL) was refluxed using a Dean-Stark apparatus overnight. The solvent was removed by vacuum and the residue was purified by column chromatography.

Compound **3A**

2A (187 mg) was used to give **3A** (174 mg) as a white solid. Elution solvent: Hexane/Ethyl acetate 2/1; yield 62%. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.76 (d, *J* = 2.1 Hz, 1H), 7.52 (d, *J* = 8.7 Hz, 2H), 7.22 (m, 2H), 6.90 (d, *J* = 8.7 Hz, 2H), 5.08 (s, 2H), 4.77 (d, *J* = 2.1 Hz, 2H), 4.07 (s, 2H), 3.81 (s, 3H), 2.56 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 166.0, 161.5, 160.4, 157.2, 131.8, 128.9, 126.4, 126.2, 122.9, 114.0, 112.8, 78.1, 77.8, 76.4, 56.3, 55.4, 37.0. HRMS (ESI) m/z $[M + Na]^+$ 374.1008, calculated for $C_{20}H_{17}NNaO_5$ 374.1004.

Compound **3B**

2B (111 mg) was used to give **3B** (92 mg) as a white solid. Elution solvent: Hexane/Ethyl acetate 2/1; yield 58%. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.67 (d, *J* = 2.4 Hz, 1H), 7.51 (d, *J* = 8.7 Hz, 2H), 7.20 (m, 2H), 6.90 (d, *J* = 8.4 Hz, 2H), 5.07 (s, 2H), 4.28 (d, *J* = 2.1 Hz, 2H), 4.23 (t, *J* = 4.5 Hz, 2H), 4.05 (s, 2H), 3.93 (t, *J* = 4.5 Hz, 2H), 3.81 (s, 3H), 2.48 (t, *J* = 2.1 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 166.1, 161.6, 160.4, 158.5, 131.8, 128.8, 126.4, 126.2, 125.9, 123.0, 114.0, 112.0, 79.4, 78.2, 75.1, 68.1, 67.8, 58.8, 55.4, 37.1. HRMS (ESI) m/z $[M + Na]^+$ 418.1259, calculated for $C_{22}H_{21}NNaO_6$ 418.1267.

Compound **3C**

2C (160 mg) was used to give **3C** (141 mg) as a white solid. Elution solvent: Hexane/Ethyl acetate 1/1; yield 65%. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.67 (d, *J* = 2.4 Hz, 1H), 7.52 (d, *J* = 8.4 Hz, 2H), 7.21 (m, 2H), 6.90 (d, *J* = 8.7 Hz, 2H), 5.07 (s, 2H), 4.21 (m, 4H), 4.05 (s, 2H), 3.90 (t, *J* = 4.5 Hz, 2H), 3.81 (s, 3H), 3.75 (m, 4H), 2.44 (t, *J* = 2.4 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 166.1, 161.6, 160.4, 158.5, 131.8, 128.7, 126.3, 126.2, 125.7, 122.9, 113.9, 112.1, 79.7, 78.1, 74.8, 70.8, 69.7, 69.2, 68.0, 58.6, 55.4, 37.0. HRMS (ESI) m/z $[M + Na]^+$ 462.1531, calculated for $C_{24}H_{25}NNaO_7$ 462.1529.

Compound **3D**

2D (244 mg) was used to give **3D** (210 mg) as a white solid. Elution solvent: Hexane/Ethyl acetate 3/4; yield 65%. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 7.67 (d, *J* = 2.4 Hz, 1H), 7.53 (dd, *J* = 6.6 Hz, 2.1 Hz, 2H), 7.19 (m, 2H), 6.90 (dd, *J* = 6.6 Hz, 2.1 Hz, 2H), 5.07 (s, 2H), 4.21 (m, 4H), 4.05 (s, 2H), 3.90 (t, *J* = 4.8 Hz, 2H), 3.81 (s, 3H), 3.76 (m, 2H), 3.68 (m, 6H), 2.43 (t, *J* = 2.1 Hz, 1H). ^{13}C NMR (75 MHz, $CDCl_3$): δ (ppm) 166.1, 161.6, 160.4, 158.6, 131.8, 128.7, 126.3, 126.2, 125.7, 122.9, 113.9, 112.1, 79.8, 78.1, 74.7, 71.0, 70.8, 70.6, 69.7, 69.2, 68.0, 58.5, 55.4, 37.0. HRMS (ESI) m/z $[M + Na]^+$ 506.1773, calculated for $C_{26}H_{29}NNaO_8$ 506.1791.

Compound **4**

To a stirred solution of **3A** (27 mg, 0.077 mmol) in DCM (1.5 mL) was added TFA (1.5 mL). The reaction turned to dark red after 4 h. The solvents were removed by vacuum. The residue was washed with ether (3 X 1.5 mL) to give **4** as a white solid (15 mg, yield 81%). ^{1}H NMR (300 MHz, DMSO): δ (ppm) 10.39 (s, 1H), 7.57 (d, *J* = 2.4 Hz, 1H), 7.31 (m, 2H), 4.90 (d, *J* = 2.1 Hz, 2H), 4.18 (s, 2H), 3.60 (t, *J* = 2.1 Hz, 1H). ^{13}C NMR (75 MHz, DMSO): δ (ppm) 166.4, 161.5, 156.2, 129.1, 127.5, 126.0, 121.7, 111.9, 78.8, 78.7, 55.7, 36.2. HRMS (ESI) m/z $[M + H]^+$ 232.0616, calculated for $C_{12}H_{10}NO_4$ 232.0610.

General method for compound **7**

To a solution of **6** (1 eq) in DMSO/H_2O (400 μL/40 μL) was added **3** (1.2 eq), sodium ascorbate (0.4 eq) and $CuSO_4$ (0.2 eq). The reaction was stirred at room temperature for 1 h. Water (5 mL) was added and the mixture was extracted with ethyl acetate (3 X 10 mL). The combined organic phase was washed with brine (3 X 10 mL), dried and concentrated. The residue was purified by preparative thin layer chromatography (Prep-TLC), leading to a white solid. The solid was then dissolved in a mixture of TFA and DCM (1 mL/1 mL). After being stirred overnight, the reaction was vacuumed to remove the solvents. The residue was purified by HPLC to give the final product.

Compound **7A**

Solvent for Prep-TLC: 6% methanol in DCM. **6** (25 mg) was used to give **7A** (12 mg) as a white solid, yield 40%, keto form (100%), t_R 6.2 min (analytical HPLC method). ^{1}H NMR (300 MHz, D_2O): δ (ppm) 8.09 (s, 1H), 7.68 (d, *J* = 1.5 Hz, 1H), 7.36 (m, 2H), 6.04 (d, *J* = 3.6 Hz, 1H), 5.37 (d, *J* = 3.3 Hz, 1H), 5.33 (s, 2H), 5.27 (s, 1H), 4.42 (t, *J* = 6.0 Hz, 2H), 4.37 (d, *J* = 5.4 Hz, 1H), 4.33 (m, 3H), 4.21 (s, 1H), 4.10 (m, 1H), 4.00 (m, 1H), 3.92 (m, 2H), 3.82 (s, 1H), 3.75 (m, 2H), 3.58 (s, 2H), 3.48-3.55 (m, 4H), 3.38 (m, 3H), 3.27 (m, 3H), 3.09 (m, 1H), 2.75 (m, 1H), 2.62 (m, 2H), 2.49 (m, 1H), 2.11 (t, *J* = 7.2 Hz, 2H), 1.87 (m, 3H), 1.49 (m, 2H), 1.10 (m, 2H). HRMS (ESI) m/z $[M + H]^+$ 1044.4653, calculated for $C_{43}H_{70}N_{11}O_{17}S$ 1044.4672.

Compound **7B**

Solvent for Prep-TLC: 6% methanol in DCM. **6** (50 mg) was used to give **7B** (23 mg) as a white solid, yield 37%, keto form (100%), t_R 6.7 min (analytical HPLC method). ^{1}H NMR (300 MHz, D_2O): δ (ppm) 7.97 (s, 1H), 7.55 (d, *J* = 2.7 Hz, 1H), 7.31 (d, *J* = 8.4 Hz, 1H), 7.25 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 6.03 (d, *J* = 4.2 Hz, 1H), 5.37 (d, *J* = 3.0 Hz, 1H), 5.27 (s, 1H), 4.71 (m, 3H), 4.35 (m, 4H), 4.27 (m, 2H), 4.23 (m, 2H), 4.19 (m, 1H), 4.08 (m, 1H), 3.99 (m, 1H), 3.89 (m, 4H), 3.80 (s, 1H), 3.67 (m, 1H), 3.28-3.57 (m, 12H), 3.10 (m, 1H), 2.70 (m, 1H), 2.64 (m, 2H), 2.48 (m, 1H), 2.12 (t, *J* = 7.2 Hz, 2H), 1.82 (m, 3H), 1.49 (m, 2H), 1.13 (m, 2H). HRMS (ESI) m/z $[M + H]^+$ 1088.4969, calculated for $C_{45}H_{74}N_{11}O_{18}S$ 1088.4934.

Compound **7C**

Solvent for Prep-TLC: 6.5% methanol in DCM. **6** (40 mg) was used to give **7C** (11 mg) as a white solid, yield 21%, keto form (50%), t_R 7.0 min (analytical HPLC method). ^{1}H NMR (300 MHz, D_2O): δ (ppm) 7.95 (s, 1H), 7.76 (d, *J* = 2.7 Hz, 1H), 7.51 (d, *J* = 8.4 Hz, 1H), 7.35 (dd, *J* = 8.4 Hz, 2.7 Hz, 1H), 6.04 (d, *J* = 3.9 Hz, 1H), 5.39 (s, 1H), 5.29 (s, 1H), 4.63 (m, 2H), 4.32 (m, 8H), 4.21 (m, 2H), 4.12 (m, 1H), 4.06 (m, 1H), 3.96 (m, 4H), 3.81 (s, 1H), 3.72 (m, 6H), 3.56 (m, 5H), 3.27-3.47 (m, 6H), 3.08 (m, 1H), 2.75 (m, 1H), 2.66 (m, 2H), 2.48 (m, 1H), 2.14 (m, 2H), 1.86 (m, 3H), 1.51 (m, 2H), 1.16 (m, 2H). HRMS (ESI) m/z $[M + H]^+$ 1132.5217, calculated for $C_{47}H_{78}N_{11}O_{19}S$ 1132.5196.

Compound **7D**

Solvent for Prep-TLC: 7% methanol in DCM. **6** (40 mg) was used to give **7D** (9 mg) as a white solid, yield 18%, keto form (60%), t_R 7.2 min (analytical HPLC method). ^{1}H NMR (300 MHz, D_2O): δ (ppm) 7.94 (s, 1H), 7.76 (d, *J* = 3.0 Hz, 1H), 7.50 (d, *J* = 8.4 Hz, 1H), 7.36 (dd, *J* = 8.4 Hz, 3.0 Hz, 1H), 6.04 (d, *J* = 3.9 Hz, 1H), 5.39 (s, 1H), 5.29 (s, 1H), 4.61 (m, 2H), 4.31-4.41 (m, 8H), 4.22 (m, 2H), 4.09 (m, 1H), 4.00 (m, 1H), 3.90 (m, 4H), 3.81 (s, 1H), 3.74 (m, 4H), 3.66 (m, 6H), 3.58 (m, 2H), 3.52 (m, 1H), 3.48 (s, 2H), 3.29-3.43 (m,

6H), 3.11 (m, 1H), 2.75 (m, 1H), 2.67 (m, 2H), 2.49 (m, 1H), 2.13 (m, 2H), 1.84 (m, 3H), 1.50 (m, 2H), 1.16 (m, 2H). HRMS (ESI) m/z $[M + H]^+$ 1176.5500, calculated for $C_{49}H_{82}N_{11}O_{20}S$ 1176.5458.

Reference

(1) Huang, C.; Yu, Y.-T. In *Curr. Protoc. Mol. Biol.*; John Wiley & Sons, Inc.: Hoboken, New Jersey, **2013**.

(2) Kirk, S. R.; Luedtke, N. W.; Tor, Y. *J. Am. Chem. Soc.* **2000**, *122*, 980.

(3) Livak, K. J.; Schmittgen, T. D. *Methods* **2001**, *25*, 402.

(4) Wu, J. I.; Lessard, J.; Olave, I. A.; Qiu, Z.; Ghosh, A.; Graef, I. A.; Crabtree, G. R. *Neuron* **2007**, *56*, 94.

(5) Childs-Disney, J. L.; Disney, M. D. *ACS Chem. Biol.* **2016**, *11*, 375.

(6) Fulmer, G. R.; Miller, A. J. M.; Sherden, N. H.; Gottlieb, H. E.; Nudelman, A.; Stoltz, B. M.; Bercaw, J. E.; Goldberg, K. I. *Organometallics* **2010**, *29*, 2176.

(7) Yang, Y.-Y.; Ascano, J. M.; Hang, H. C. *J. Am. Chem. Soc.* **2010**, *132*, 3640.

(8) Michael, K.; Wang, H.; Tor, Y. *Bioorg. Med. Chem.* **1999**, *7*, 1361.

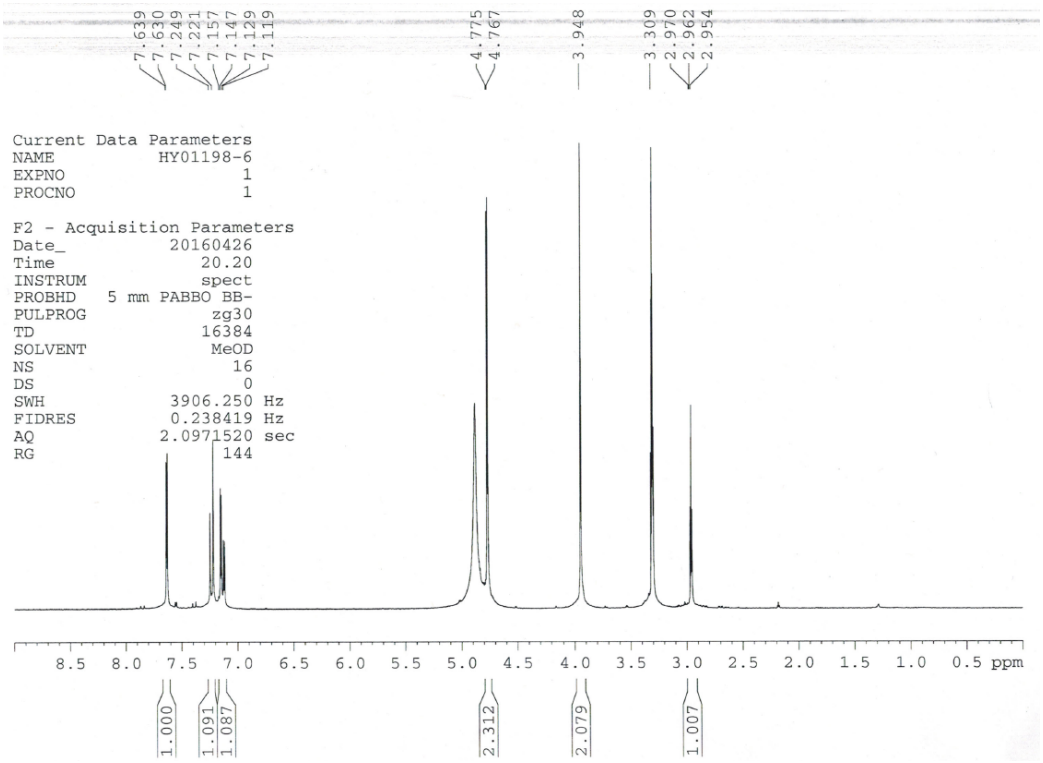
(9) (a) Gao, F.; Yan, X. X.; Baettig, O. M.; Berghuis, A. M.; Auclair, K. *Angew. Chem. Int. Ed.* **2005**, *44*, 6859; (b) Roestamadji, J.; Grapsas, I.; Mobashery, S. *J. Am. Chem. Soc.* **1995**, *117*, 11060.

(10) Liu, B.; Tian, H. *Chem. Commun.* **2005**, 3156.

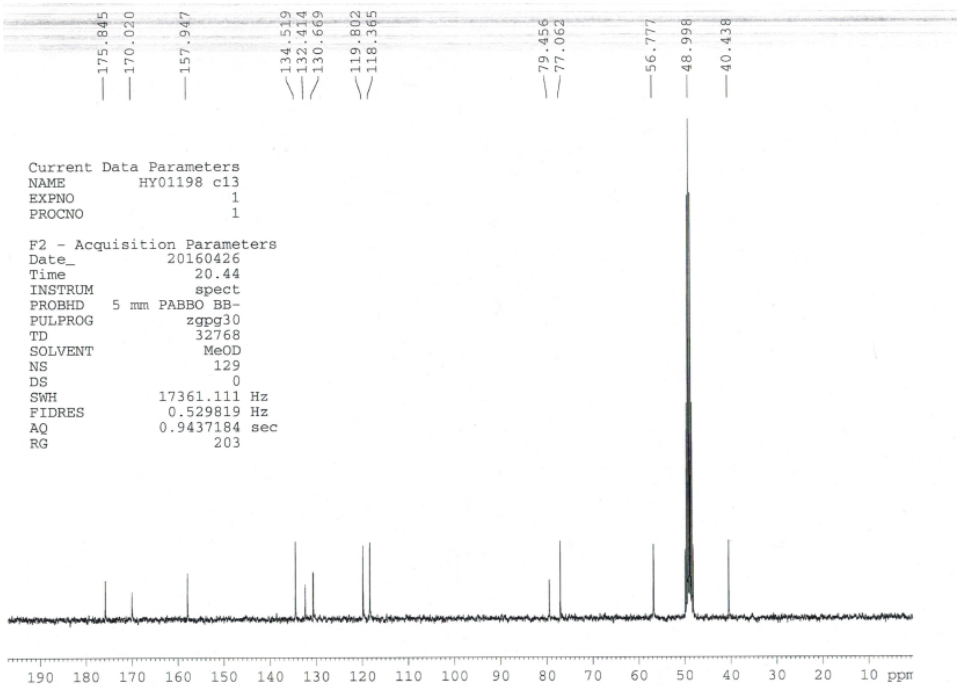
(11) Esko, J. D.; Tor, Y. Assisted enzyme replacement therapy. WO2011034951 A2, March 24, **2011**.

NMR spectra

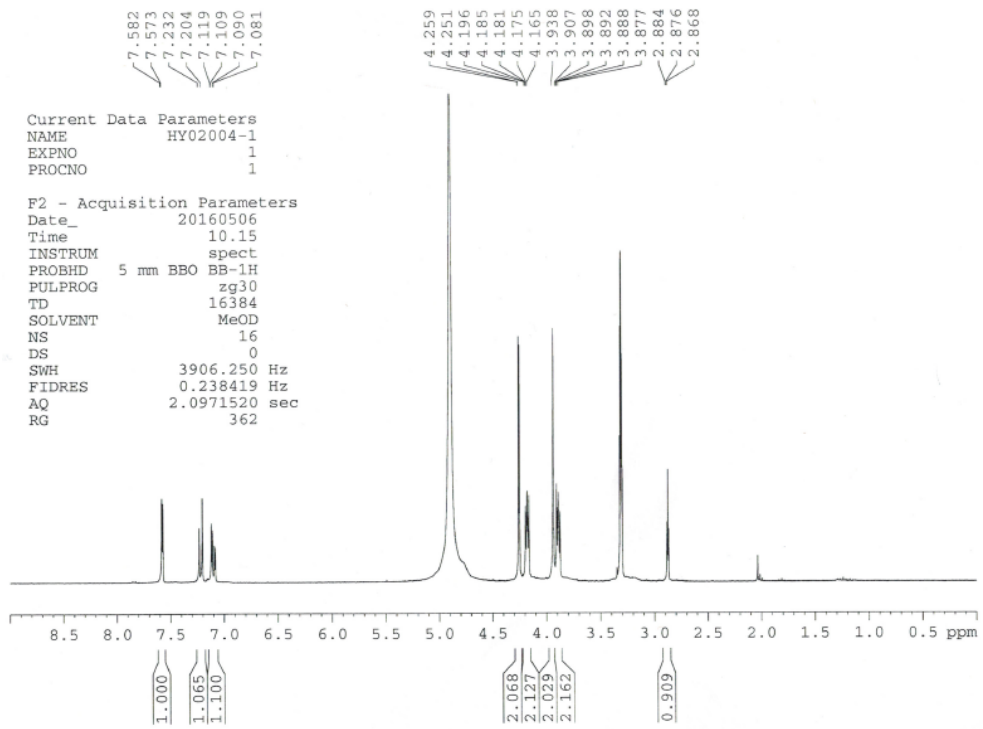
^{1}H NMR of **2A**



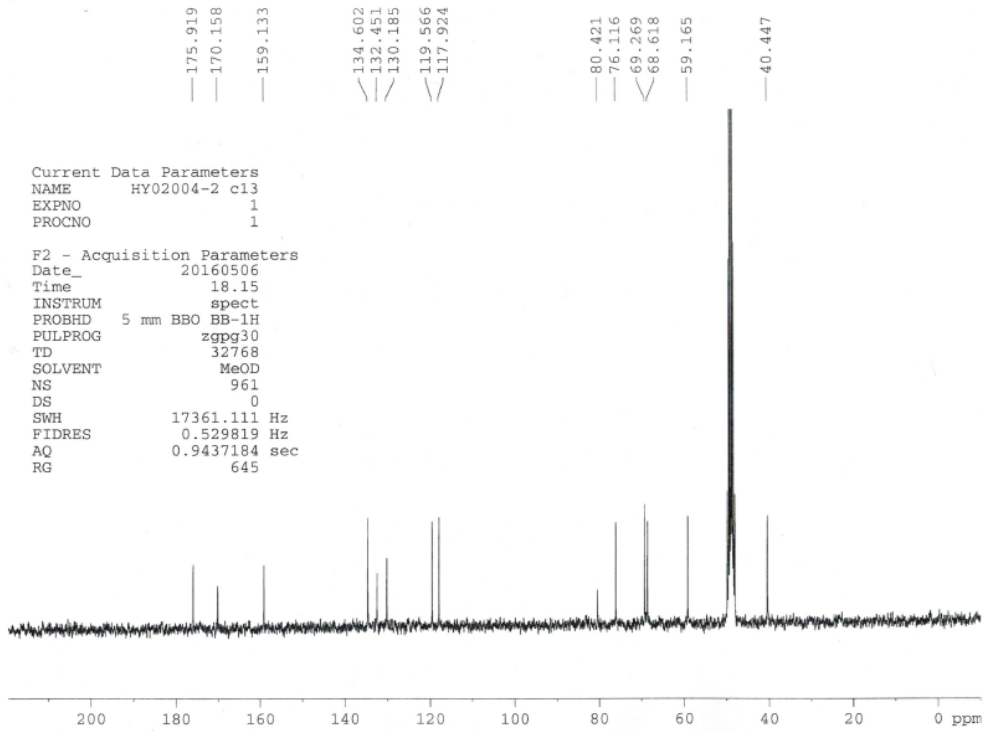
^{13}C NMR of **2A**



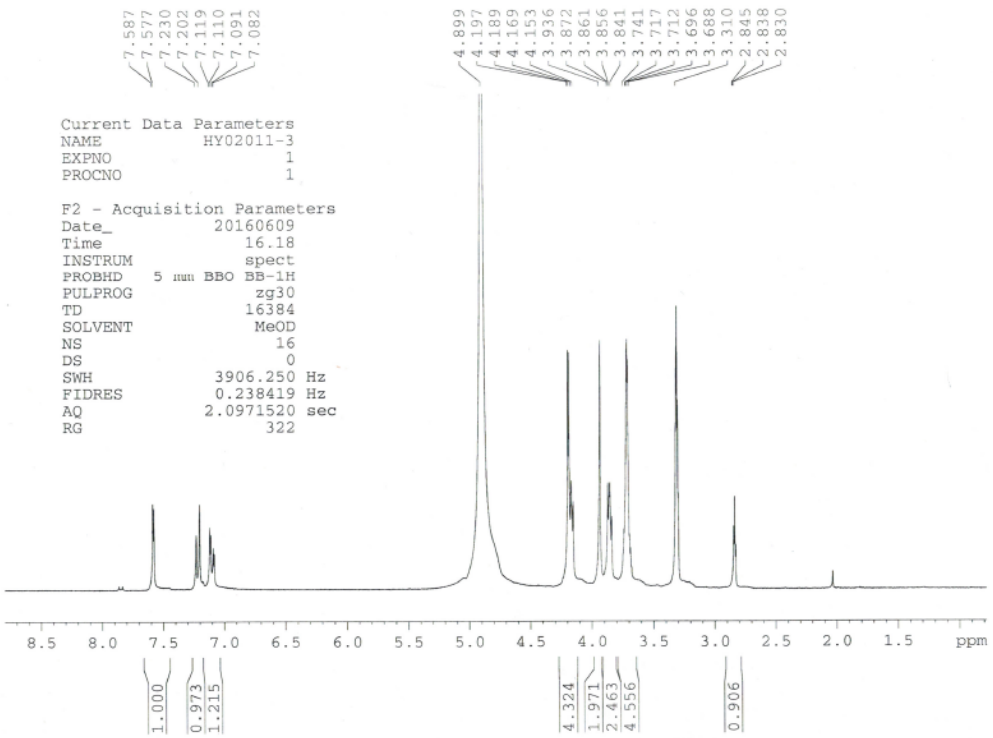
^{1}H NMR of **2B**



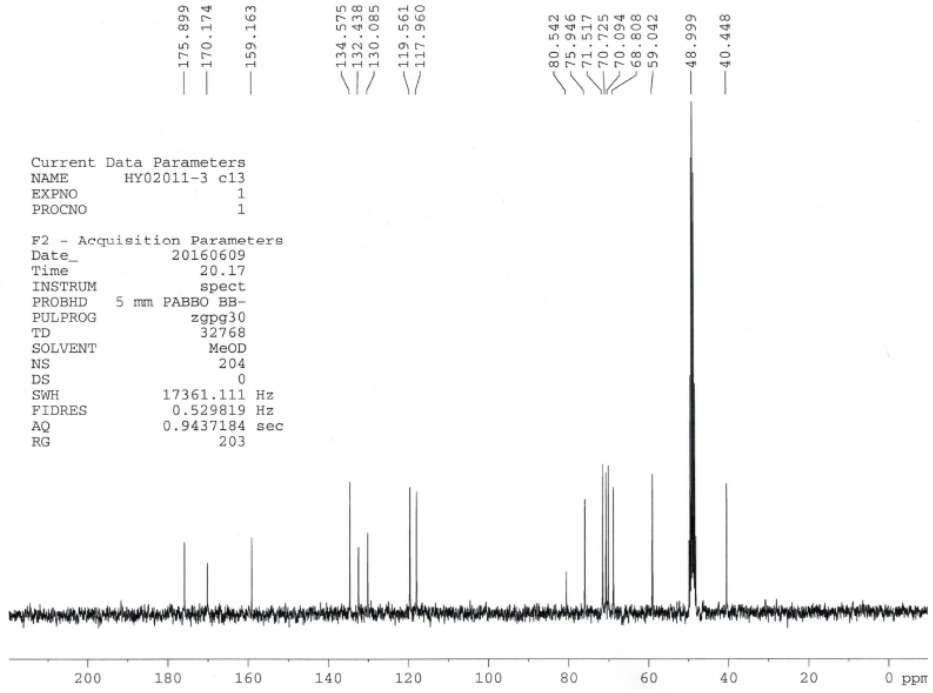
^{13}C NMR of **2B**



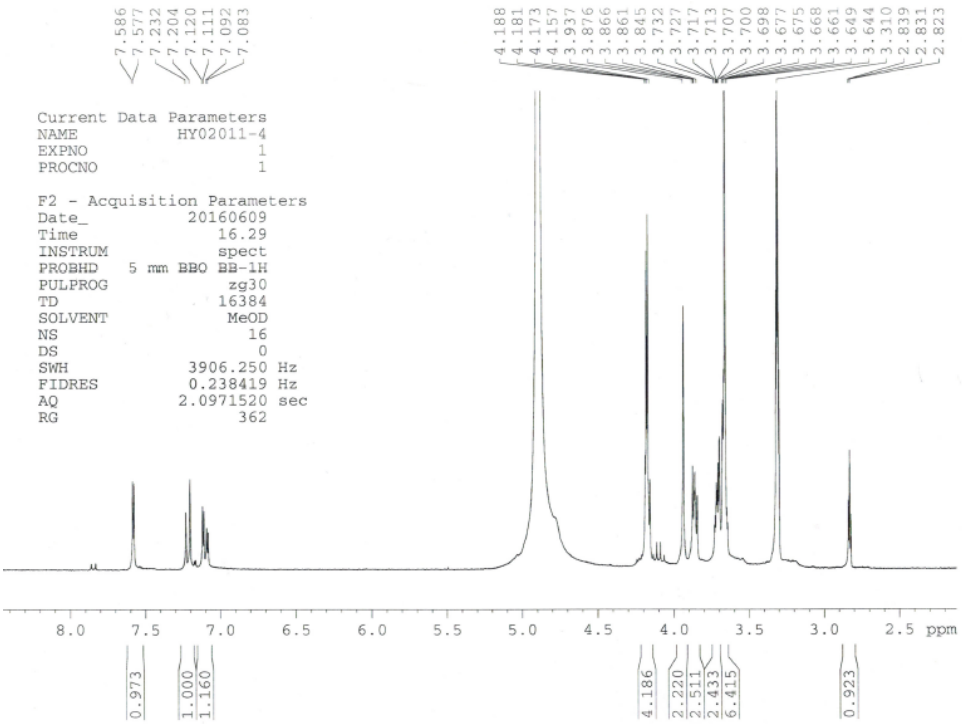
^{1}H NMR of **2C**



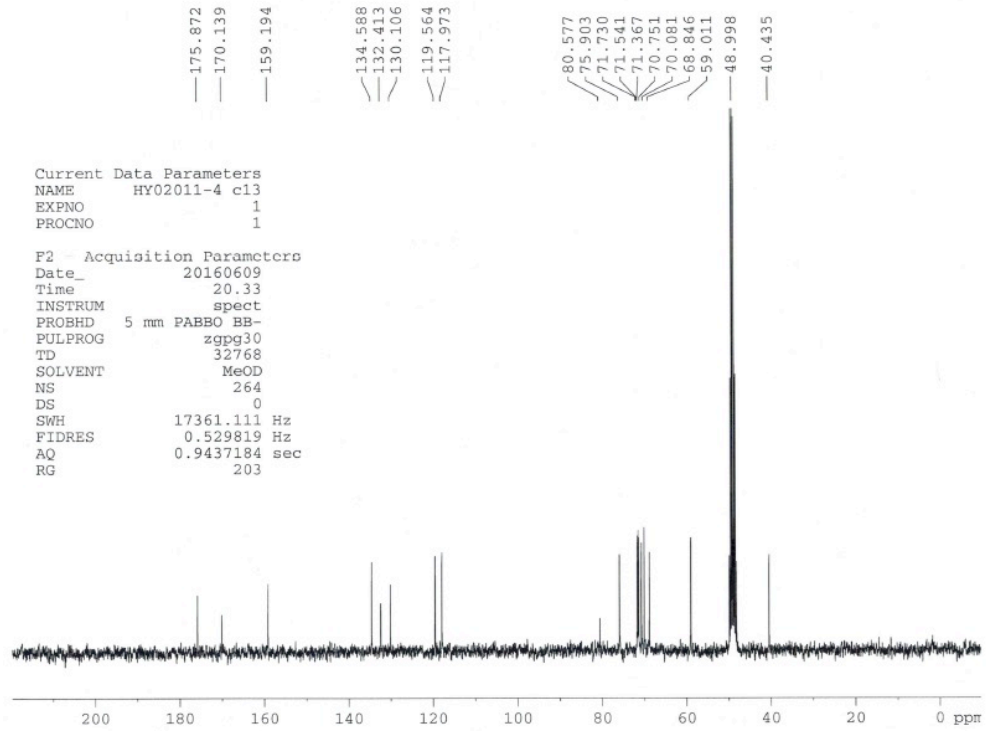
^{13}C NMR of **2C**



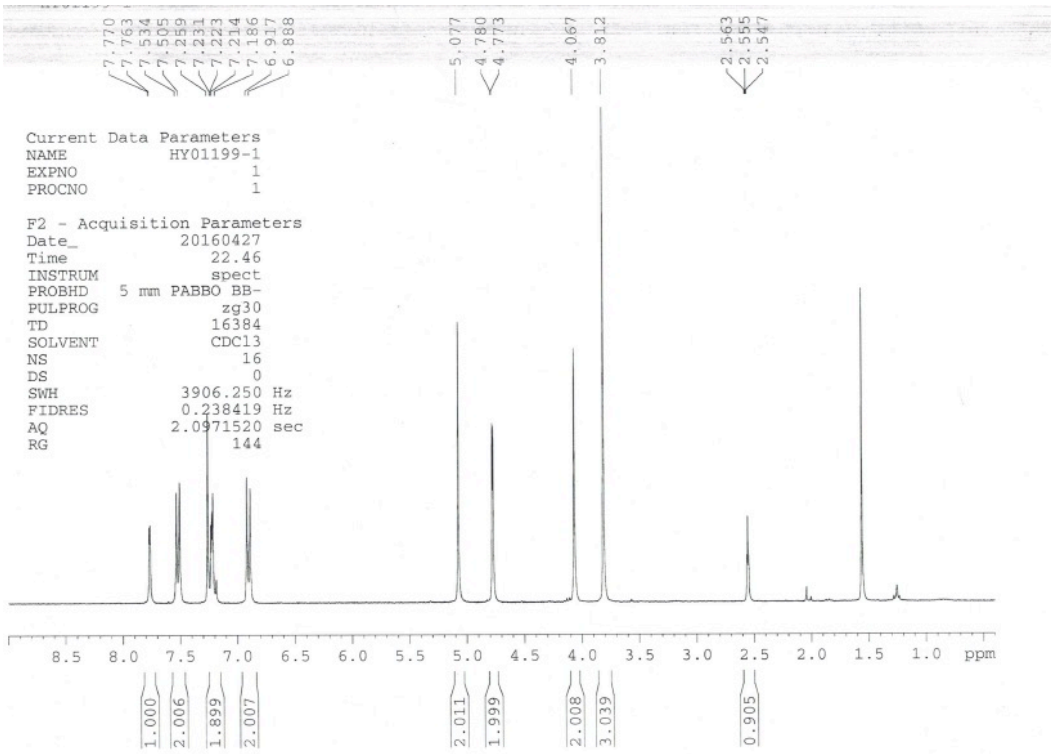
^{1}H NMR of **2D**



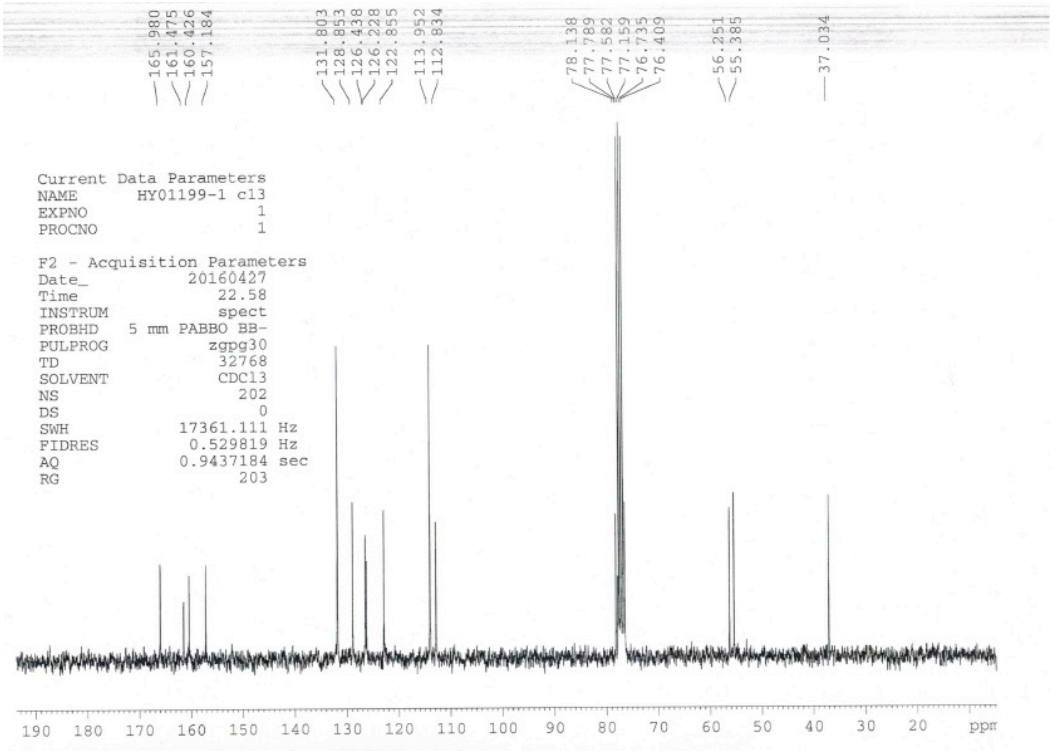
^{13}C NMR of **2D**



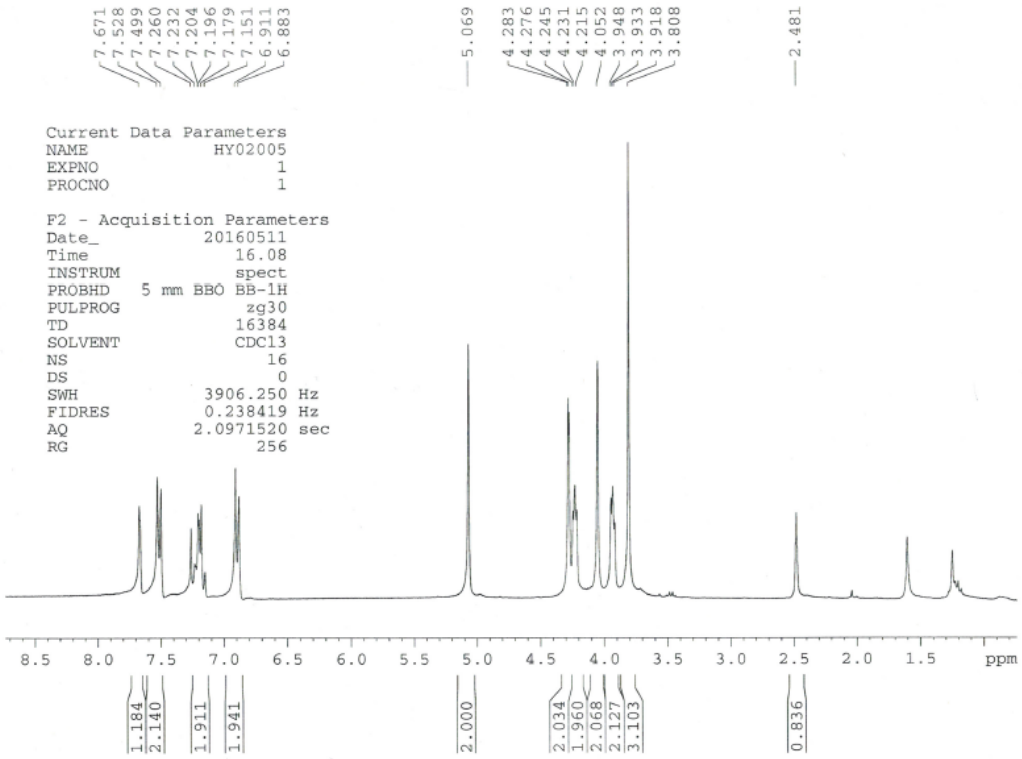
^{1}H NMR of **3A**



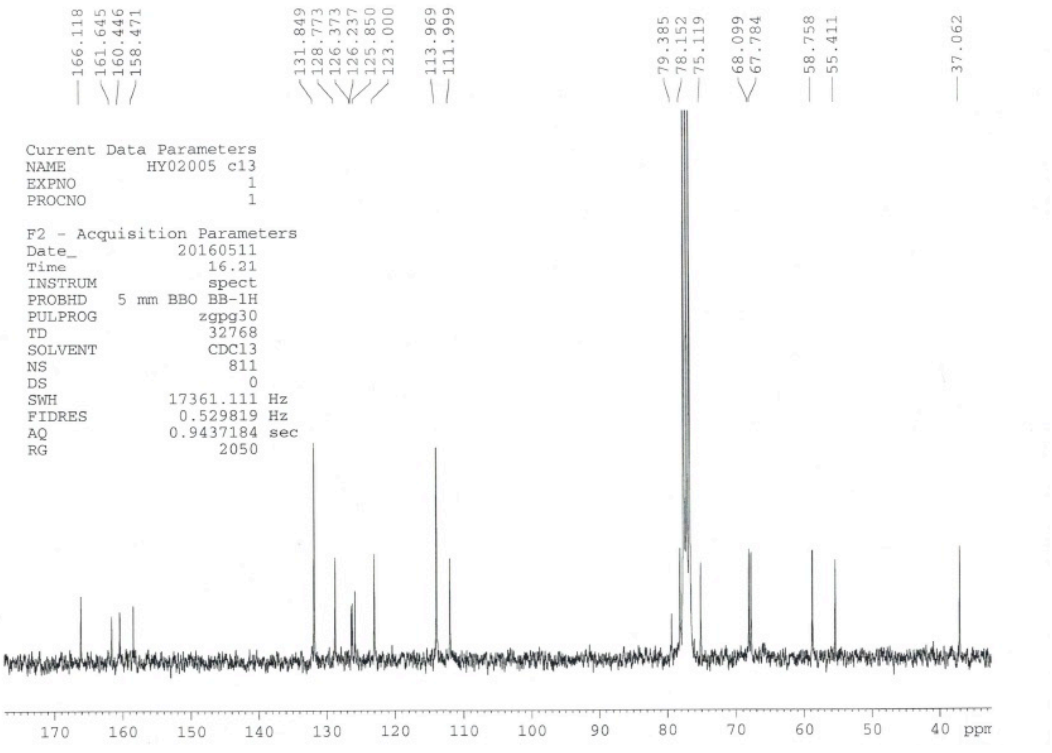
^{13}C NMR of **3A**



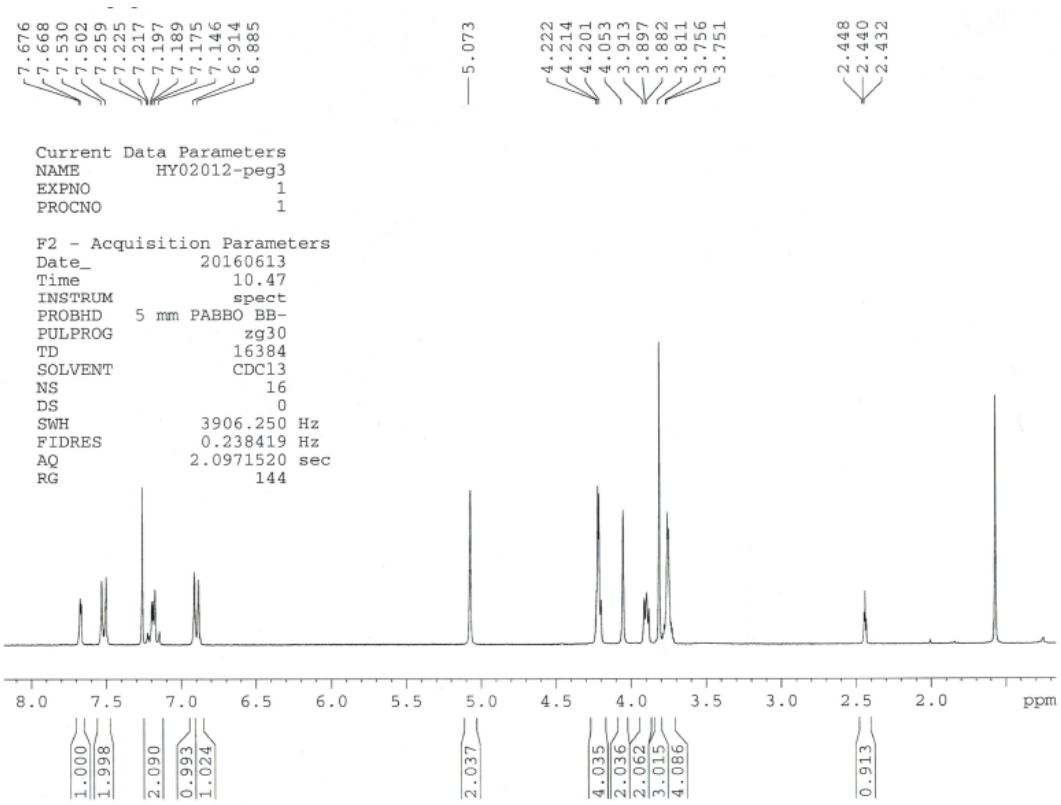
^{1}H NMR of **3B**



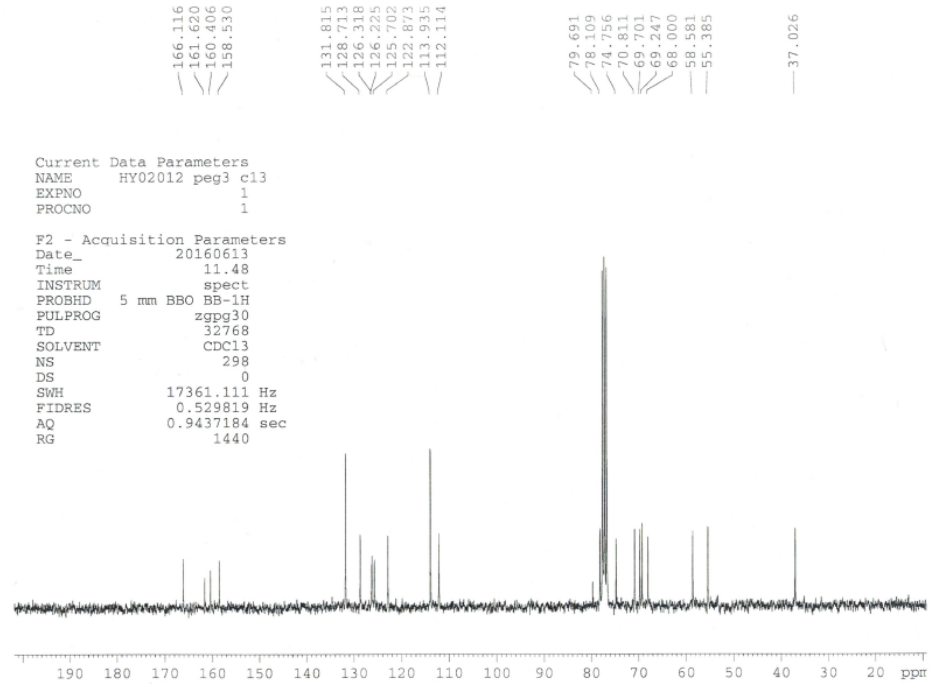
^{13}C NMR of **3B**



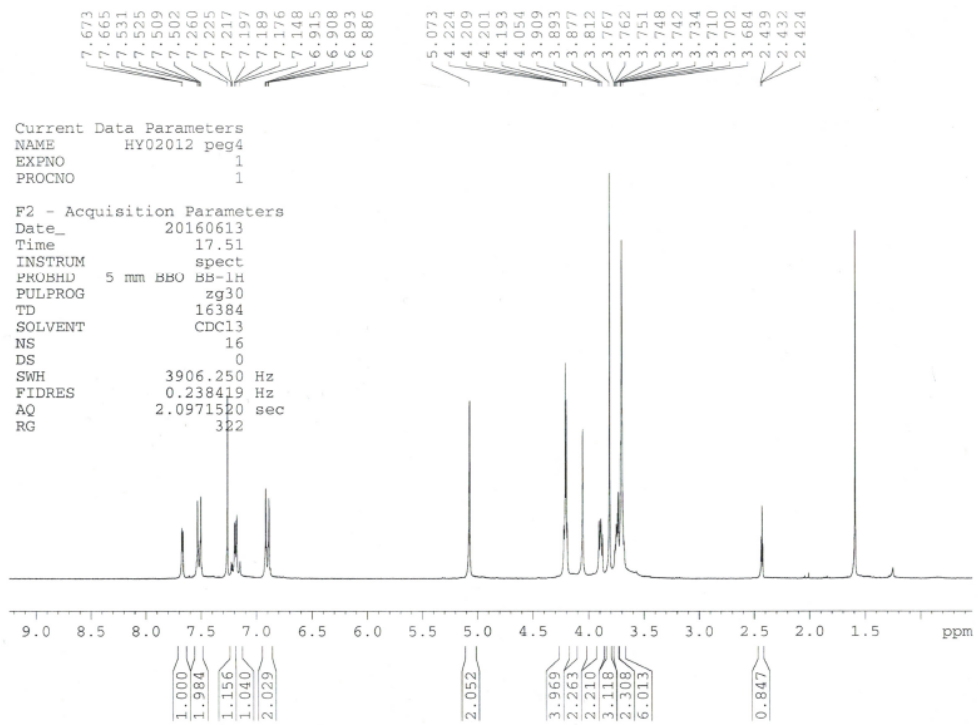
^{1}H NMR of **3C**



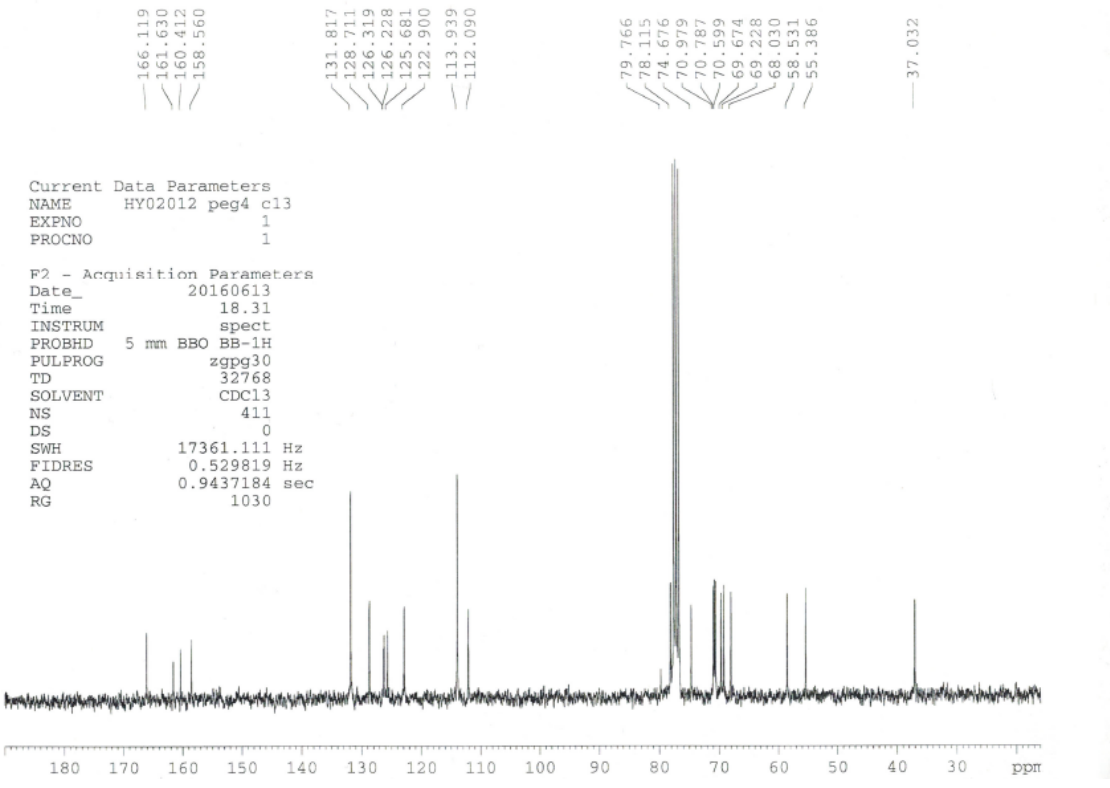
^{13}C NMR of **3C**



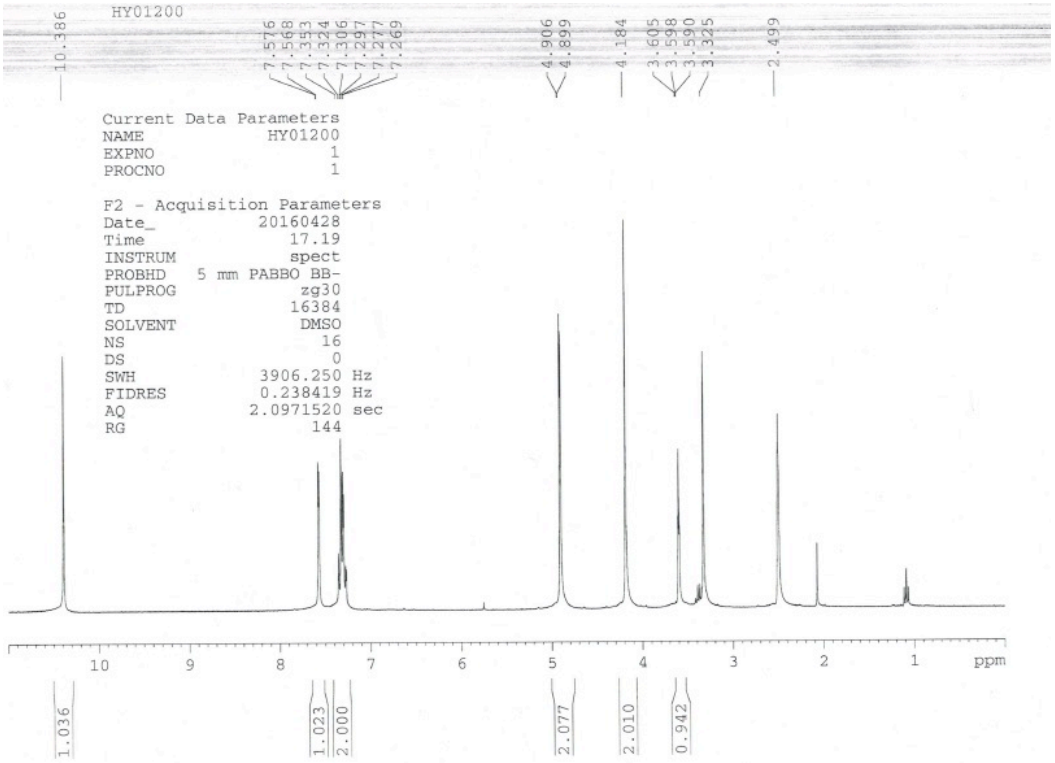
^{1}H NMR of **3D**



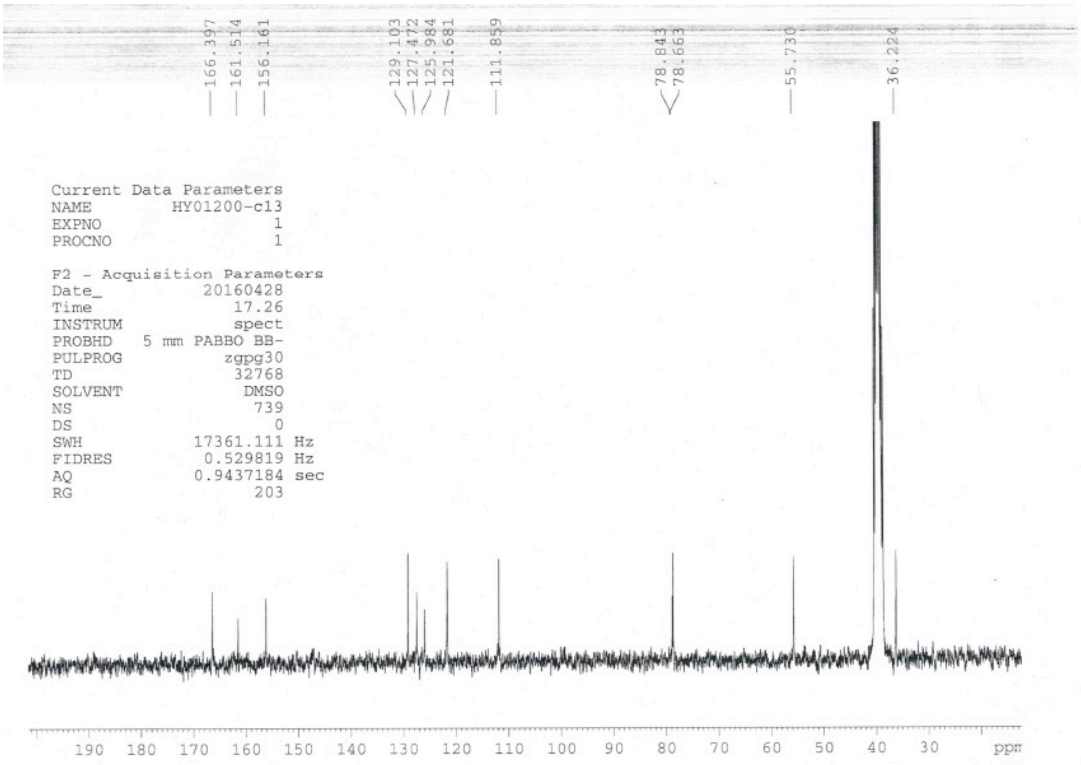
^{13}C NMR of **3D**



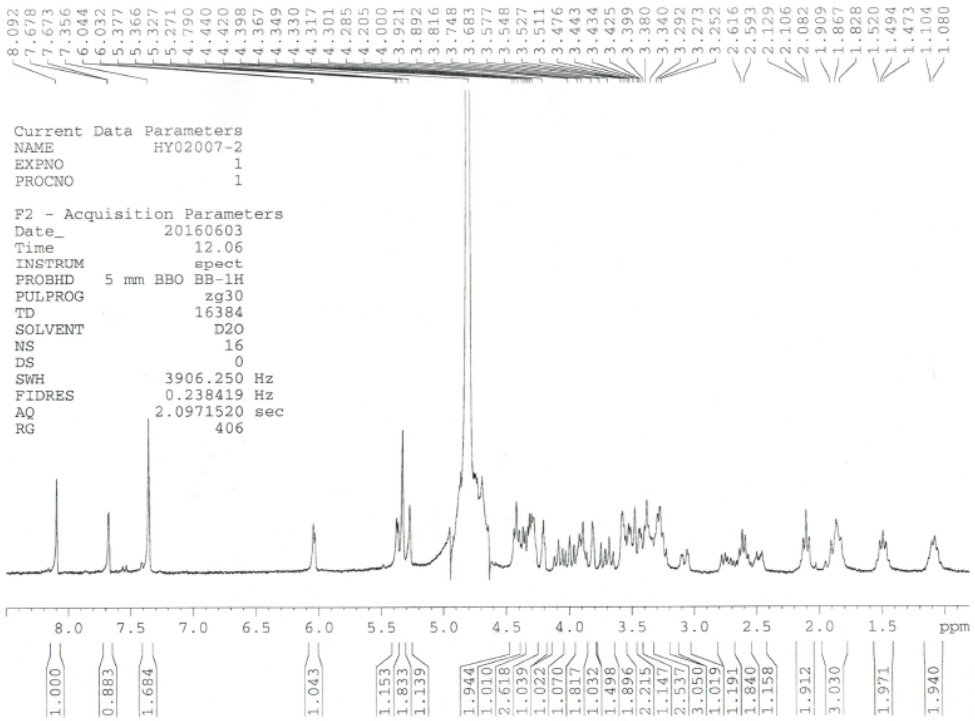
^{1}H NMR of **4**



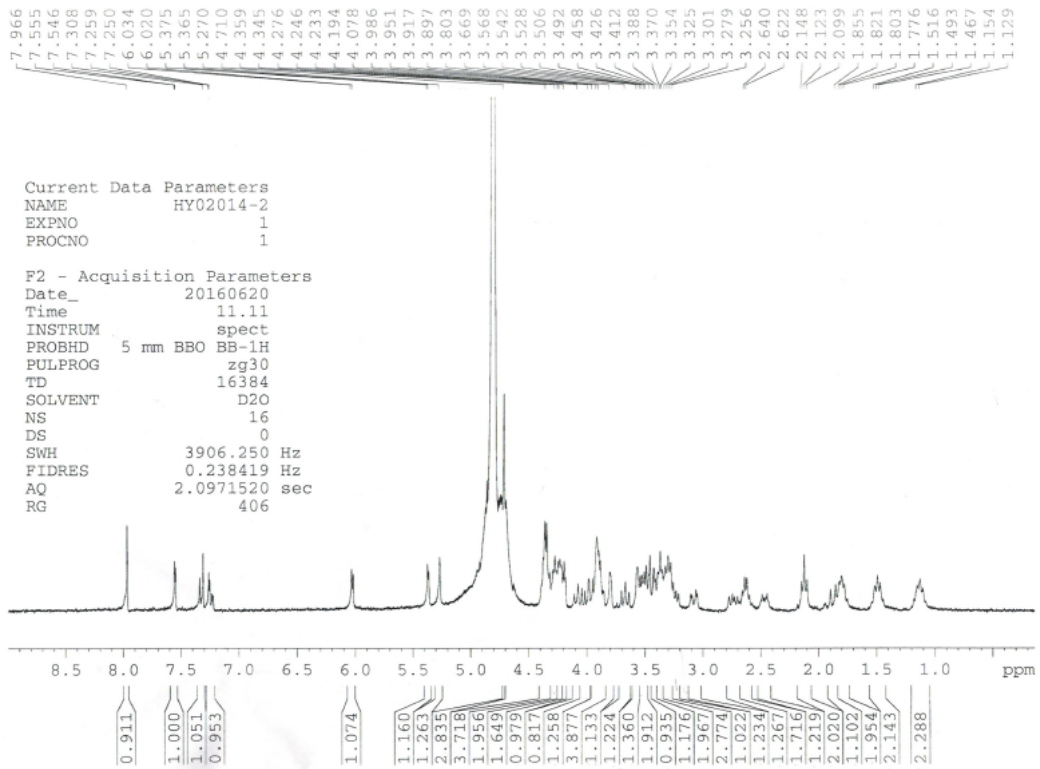
^{13}C NMR of **4**



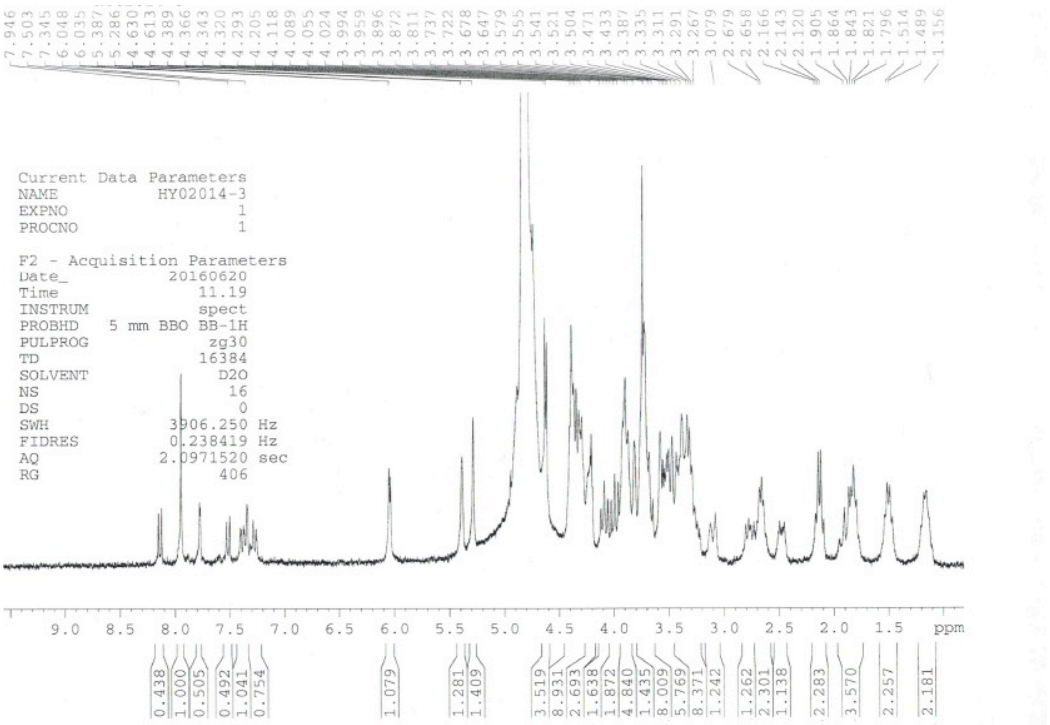
^{1}H NMR of **7A**



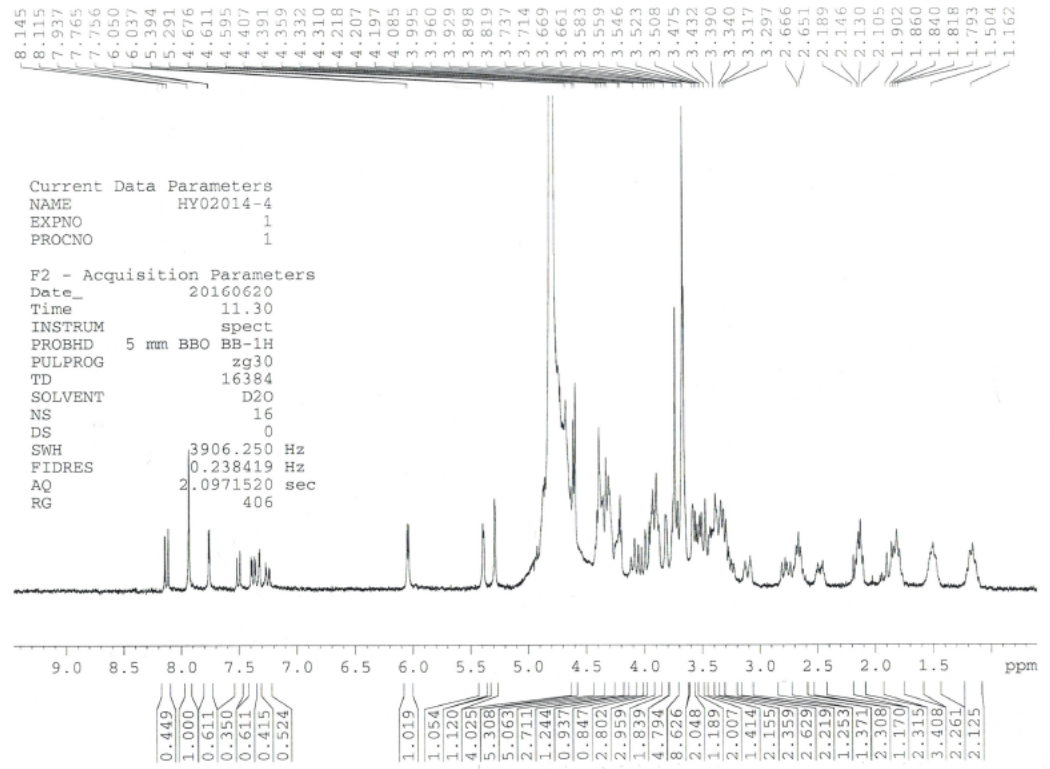
^{1}H NMR of **7B**



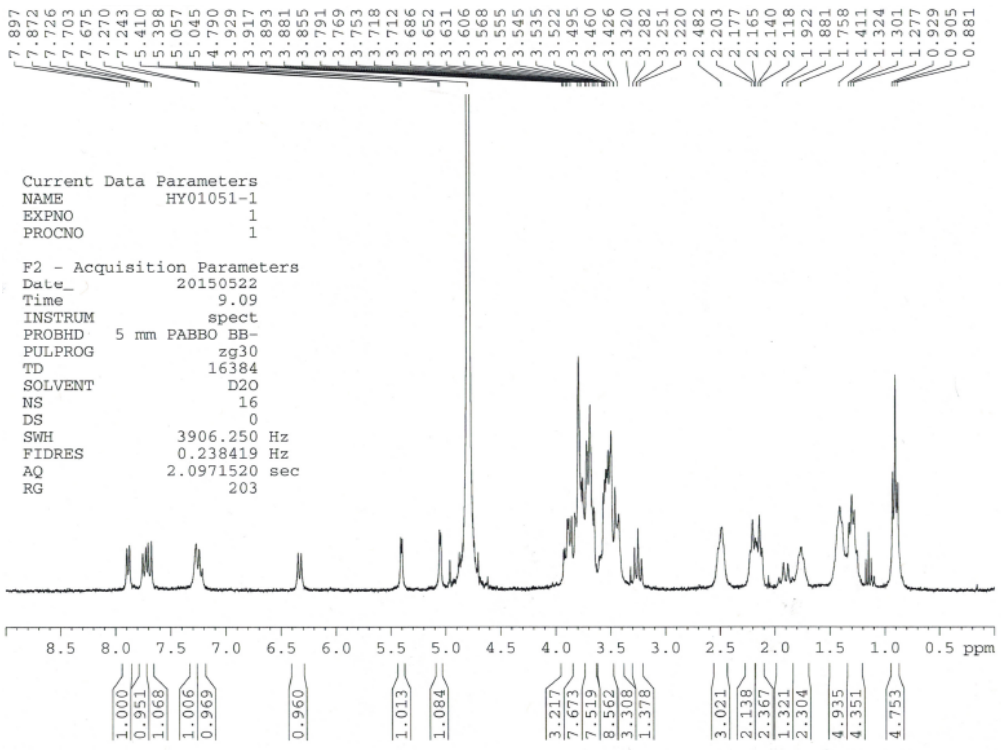
^{1}H NMR of **7C**



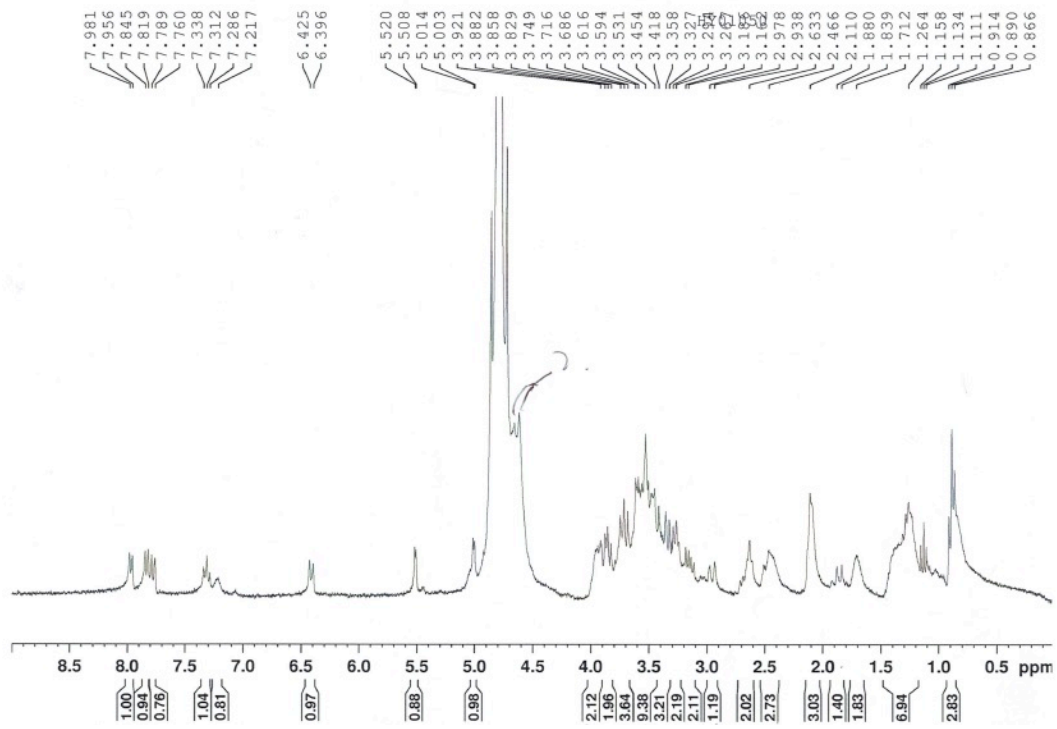
^{1}H NMR of **7D**



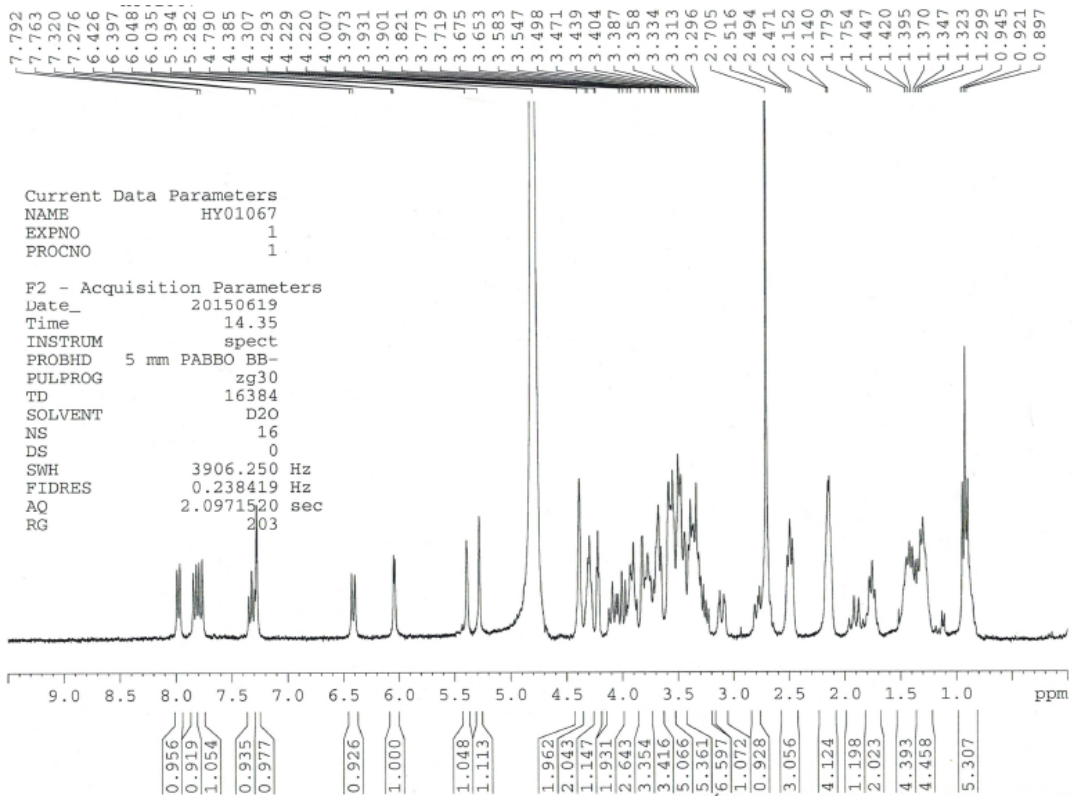
^{1}H NMR of **KNF**



^{1}H NMR of **KOF**

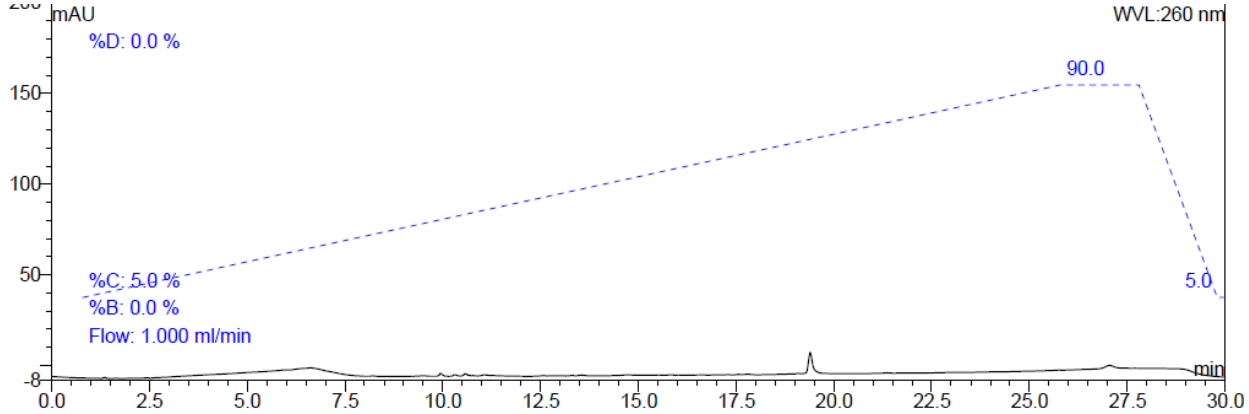


^{1}H NMR of **NF**

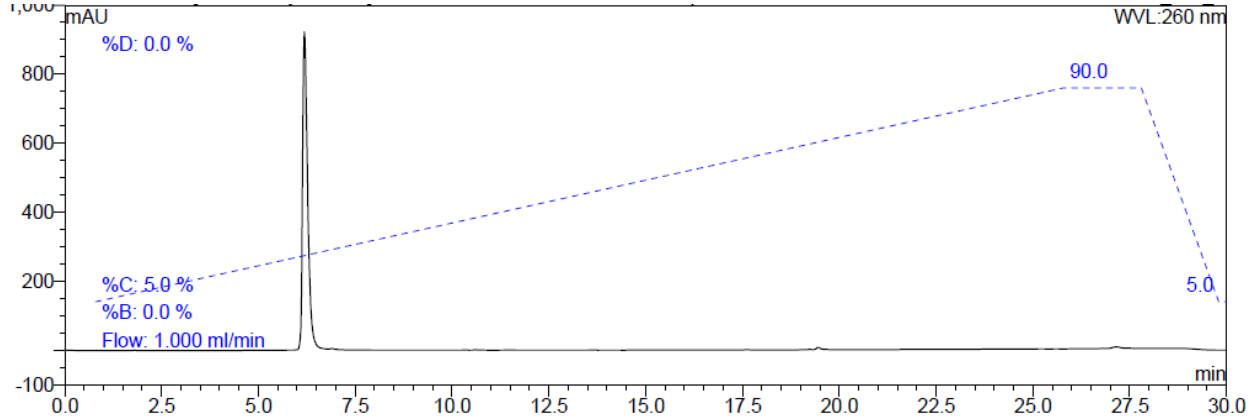

7.792
7.763
7.320
7.276
6.426
6.397
6.048
6.035
5.394
5.282
4.790
4.385
4.307
4.293
4.229
4.220
4.007
3.973
3.931
3.901
3.821
3.773
3.719
3.675
3.653
3.583
3.547
3.498
3.471
3.439
3.404
3.387
3.358
3.334
3.313
3.296
2.705
2.516
2.494
2.471
2.152
2.140
1.779
1.754
1.447
1.420
1.395
1.370
1.347
1.323
1.299
0.945
0.921
0.897
Current Data Parameters
NAME HY01067
EXPNO 1
PROCNO 1
F2 - Acquisition Parameters
Date_ 20150619
Time 14.35
INSTRUM spect
PROBHD 5 mm PABBO BB-
PULPROG zg30
TD 16384
SOLVENT D2O
NS 16
DS 0
SWH 3906.250 Hz
FIDRES 0.238419 Hz
AQ 2.0971520 sec
RG 203
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 ppm
0.956
0.919
1.054
0.935
0.977
0.926
1.000
1.048
1.113
1.962
2.043
1.147
1.931
2.643
3.354
3.416
5.066
5.361
6.597
1.072
0.928
3.056
4.124
1.198
2.023
4.393
4.458
5.307

HPLC

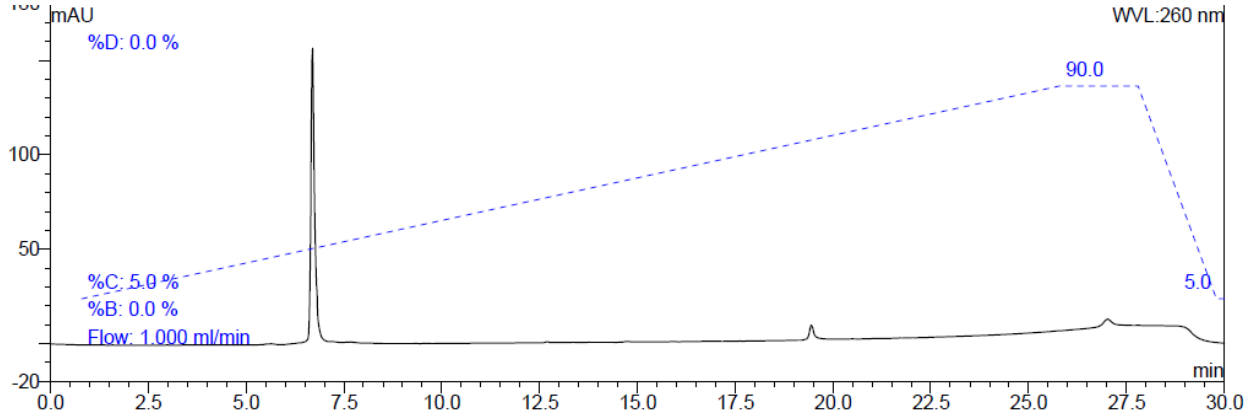
Background



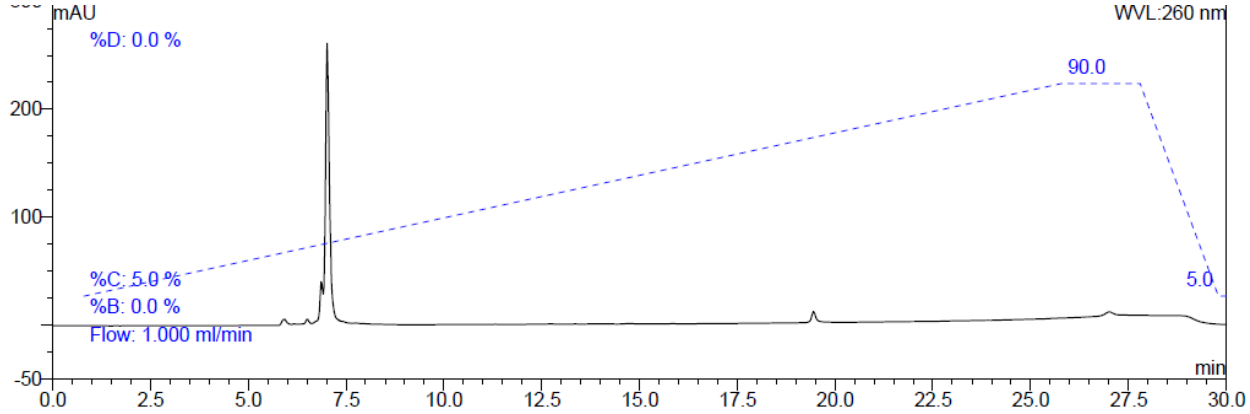
Compound **7A**



Compound **7B**



Compound **7C**



Compound **7D**