Supporting Information

Identification and characterization of novel inhibitors of mPTPB, an essential virulent phosphatase from *Mycobacterium tuberculosis*

Lan Chen[†#], Bo Zhou[#], Sheng Zhang[#], Li Wu[#†], Yuehong Wang[‖], Scott G. Franzblau[‖], and Zhong-Yin Zhang[#*]

[#]Department of Biochemistry and Molecular Biology, and [†]Chemical Genomics Core Facility, Indiana University School of Medicine, 635 Barnhill Drive, Indianapolis, Indiana 46202; [‖]Institute of Tuberculosis Research, University of Illinois at Chicago, Illinois 60612

Experimental Section

Materials. Recombinant mouse IFN-γ was purchased from PeproTech INC. (Rocky Hill, New Jersey). Anti-ERK1/2, anti-phospho-ERK1/2 antibodies were purchased from Cell Signaling (Beverly, MA). *p*-Nitrophenyl phosphate (*p*NPP) was purchased from Fluke Co. Dimethylformamide (DMF), benz[*cd*]indol-2(1H)-one, 4-butylaniline, 4-(dimethylamino)pyridine, triethylamine and chlorosulfonic acid were from Aldrich. The 7500-member library was from ChemDiv (San Diego). Methanol (HPLC grade), acetonitrile (HPLC grade), ammonium acetate, trifluoroacetic acid (TFA) dichloromethane and ethyl acetate were from Fisher Scientific

Instrumentation. HPLC purification was carried out on a Waters Breeze HPLC system equipped with a Waters Atlantis dC18 column (19 mm × 100 mm). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Advance II 500-MHz NMR spectrometer. Analytical HPLC analysis was carried out on a Waters Breeze HPLC system equipped with a Waters Symmetry C18 column (4.6 mm × 150 mm). Mass data were recorded using an Agilent 6130 Quadrupole LC/MS detector.

*Synthesis of Compound **1***. To 15 mL of chlorosulfonic acid, 1.69 g (10 mmole) of benz[*cd*]indol-2(1H)-one was added slowly with vigorous stirring at 25 ^{o}C. The solution was stirred for one hour at 25 ^{o}C. The mixture was then poured into 100 mL of ice-water mixture to quench the reaction. The product was extracted with dichloromethane (40 mL each time, 3 times). The organic phase was combined and washed with water (50 mL each time, 2 times), followed by sodium chloride solution (saturated, 50 mL). The dichloromethane solution was then dried over Na_2SO_4 (5 g) for 15 minutes. The crude 2-oxo-1,2-dihydrobenzo[*cd*]indole-6-sulfonyl chloride (1.2 g, 45% yield) was obtained by evaporating the dichloromethane under vacuum, and used for the next step without further purification. 26.7 mg (0.1 mmole) of 2-oxo-1,2-dihydrobenzo[*cd*]indole-6-sulfonyl chloride was mixed with 29.8 mg (0.2 mmole) of 4-

butylaniline, 2 mg 4-(dimethylamino)pyridine, and 30.3 mg triethylamine (0.3 mmole) in 5 mL dry DMF. The resulted solution was stirred for 2 hours and then poured into a mixture of ethyl acetate (50 mL) and water (100 mL). The organic phase was separated, and the aqueous phase was washed with ethyl acetate (50 mL each time, 3 times). The organic phase was combined, and washed with water (50 mL), 10% HCl (50 mL), water (50 mL), sodium bicarbonate (saturated, 50 mL), water (50 mL), 10% HCl (50 mL), water (50 mL), and sodium chloride (saturated, 50 mL) sequentially. The crude compound **1** was obtained by evaporating the ethyl acetate under vacuum. The pure compound **1** (22 mg, 58% yield) was obtained after HPLC purification (gradient from 40% MeOH in water containing 0.1% trifluoroacetic acid to 90% MeOH in water containing 0.1% trifluoroacetic acid). ^{1}H NMR (500 Hz, CD3OD): δ = 8.60 (d, J = 8.4 Hz, 1 H), 8.08-8.05 (m, 2 H), 7.83-7.79 (m, 1 H), 6.96-6.90 (m, 3 H), 6.87 (d, J = 8.5 Hz, 2 H), 2.45 (t, J = 7.6 Hz, 2 H), 1.50-1.43 (m, 2 H), 1.28 – 1.20 (m, 2 H), 0.87 (t, J = 7.3 Hz, 3 H). ^{13}C NMR (125 Hz, DMSO-d_6): δ = 168.73, 142.96, 138.24, 135.04, 133.63, 130.64, 129.27, 128.93, 127.61, 126.94, 126.05, 124.95, 124.50, 120.12, 104.64, 34.14, 33.14, 21.75, 13.72. Mass calculated for compound **1** $C_{21}H_{20}N_2O_3S$ $[M+H]^+$, 381.13, found $[M+H]^+$ 381.1. RP-HPLC: t_R = 19.37 min (mobile phase: gradient from 40% MeOH in water with 0.1% TFA to 90% MeOH in water with 0.1% TFA, over 25 min), purity > 99% (UV, λ = 360 nm). t_R= 14.21 min (mobile phase: gradient from 40% CH_3CN in water with 20 mM NH_4COCH_3 to 90% CH_3CN in water with 20 mM NH_4COCH_3, over 25 min), purity > 99% (UV, λ = 360 nm).

*Chemical Charactrization of **16***. Compound **16** were purchased from Chemdiv (www.chemdiv.com), and were subjected to NMR, MS, and analytical HPLC analysis for chemical data and purity information. The ^{1}H NMR spectrum is consistent with the spectrum provided by ChemDiv and the MS data is consistent with the molecular formula. These chemical data confirmed that the purchased compound has the correct chemical structure, as described by ChemDiv. The analytical HPLC analysis shows that the ordered compound is more than 95%

pure, using two different eluting solvent systems. Thus it is suitable for further biological evaluation. ^{1}H NMR (500 Hz, $CDCl_3$): δ = 7.68-7.63 (m, 1 H), 7.62-7.58 (m, 1 H), 7.37-7.35 (m, 1 H), 7.28 – 7.25 (m, no integration value due to the solvent peak), 7.02-6.99 (m, 1 H), 6.98-6.95 (m, 1 H), 6.81-6.69 (m, 4 H), 6.33-6.31 (m, 1 H), 6.26-6.24 (m, 1 H), 4.60-4.48 (m, 2 H), 4.44-4.38 (m, 1 H), 3.76 (s, 3 H), 3.66 (d, *J* = 6.3 Hz, 1 H), 3.07-3.03 (m, 4 H), 2.69-2.59 (m, 4 H), 1.19 (d, *J* = 6.4 Hz, 3 H). ^{13}C NMR (125 Hz, $CDCl_3$): δ = 159.49, 158.78, 153.78, 149.79, 145.56, 142.63, 139.09, 127.08, 126.69, 125.28, 118.13, 114.37, 110.48, 108.12, 55.55, 50.76, 50.66, 46.60, 36.60, 19.11. MS (ESI) cald for [$C_{25}H_{30}N_4O_4S$ + H] 483.2, found 483.2. RP-HPLC analysis was carried out on an Agilent 1200 HPLC system equipped with an Agilent XDB-C18 column (4.6 mm × 150 mm): tR = 16.54 min (mobile phase: gradient from 100% water with 0.1% TFA to 100% methanol, over 25 min), purity = 96.9% (UV, λ = 254 nm). tR= 13.78 min (mobile phase: gradient from 30% CH_3CN in water with 20 mM NH_4COCH_3 to 100% CH_3CN, over 25 min), purity = 97.9% (UV, λ = 254 nm).

*Chemical Characterization of **17***. Compound **17** were purchased from ChemDiv (www.chemdiv.com), and were subjected to NMR, MS, and analytical HPLC analysis for chemical data and purity information. The ^{1}H NMR spectrum is consistent with the spectrum provided by ChemDiv and the MS data is consistent with the molecular formula. These chemical data confirmed that the purchased compound has the correct chemical structure, as described by ChemDiv. The analytical HPLC analysis shows that the ordered compound is more than 95% pure, using two different eluting solvent systems. Thus it is suitable for further biological evaluation. ^{1}H NMR (500 Hz, $CDCl_3$): δ = 8.56-8.54 (m, 2 H), 7.78-7.73 (m, 1 H), 7.62-7.59 (m, 2 H), 7.30-7.25 (m, no integration value due to the solvent peak), 7.04-7.01 (m, 1 H), 6.97-6.90 (m, 3 H), 6.84-6.81 (m, 2 H), 4.62-4.52 (m, 2 H), 4.46-4.41, (m, 1 H), 3.67 (d, *J* = 6.0 Hz, 1 H), 3.09-3.06 (m, 4 H), 2.70-2.60 (m, 4 H), 1.20 (d, *J* = 6.7 Hz, 3 H). ^{13}C NMR (125 Hz, $CDCl_3$): δ = 159.84, 158.72, 158.10, 156.20, 149.34, 147.83, 138.94, 135.60, 132.51, 127.17, 126.72, 125.36, 117.73, 117.67, 115.56, 115.38, 68.93, 50.58, 50.28, 45.63, 41.25, 19.10. MS

(ESI) cald for [$C_{25}H_{28}FN_5O_2S$ + H] 482.2, found 482.2. RP-HPLC analysis was carried out on an Agilent 1200 HPLC system equipped with an Agilent XDB-C18 column (4.6 mm × 150 mm): tR = 13.57 min (mobile phase: gradient from 100% water with 0.1% TFA to 100% MeOH, over 25 min), purity = 95.8% (UV, λ = 254 nm). tR= 12.40 min (mobile phase: gradient from 40% CH_3CN in water with 20 mM NH_4COCH_3 to 100% CH_3CN in water, over 25 min), purity = 97.8% (UV, λ = 254 nm).

mPTPB expression and purification. The full-length mPTPB with a N-terminal His_6-tag was expressed and purified as described previously (Zhou et al., 2010). Protein concentration was determined using the Bradford dye-binding assay (Bio-Rad) diluted according to the manufacturer's recommendations with bovine serum albumin as standard. The purified mPTPB were made to 30% glycerol and stored at -20°C.

High-throughput screening for mPTPB inhibition. A library of 7,500 compounds was purchased from ChemDiv, Inc. (San Diego, CA) in 10 mM dimethylsulfoxide (DMSO) stock. These compounds were transferred into 384-well polyspropylene plates (Abgene, Epsom, UK) and diluted in 50 mM 3,3-dimethylglutarate buffer, pH = 7.0 with an ionic strength 0.15 M adjusted by addition of NaCl. The commonly used small molecule PTP substrate *p*-nitrophenyl phosphate (*p*NPP) (Liang et al., 2003) was adapted to perform the high-throughput screening. The initial screen of this 7,500 compounds library was performed in 384-well polystyrene assay plates (Nunc, Rochester, NY) and was carried out in a total reaction volume of 50 μL in a solution consisting of 50 mM 3,3-dimethylglutarate, 1 mM EDTA at pH 7.0, 2 mM *p*NPP substrate, 10 μM library compound, and 20 nM mPTPB. Addition of reagents was done by a Tecan Genesis 150 Workstation (Durham, NC) with a 96-channel pipetting head. The reaction was initiated by addition of enzyme and allowed to proceed for 3.5 min at room temperature. Product formation was determined by reading absorbance at 405 nm on a SpectraMax 384 Plus spectrometer (Molecular Devices, Silicon Valley, CA). In each plate, the last column was free of compound and the average slope was used as the control reaction rate of no-inhibition. The

percent inhibition of each well was calculated by comparing its reaction rate with the no-inhibition control.

Inhibition Study. Compounds exhibiting more than 50% of inhibitory activity against mPTPB were selected for IC_{50} measurement. The reaction was started by the addition of 5 μL of the enzyme to 195 μL of reaction mixture containing 2.5 mM (the K_m value) of *p*NPP and various concentrations of the inhibitor. The reaction was quenched after 5 min by the addition of 50 μl of 5N NaOH, and then 200 μL of reaction mixture was transferred to a 96-well plate. The absorbance at 405 nm was detected by a Spectra MAX340 microplate spectrophotometer (Molecular Devices). IC_{50} values were calculated by fitting the absorbance at 405nm *versus* inhibitor concentration to the following equation:

$$A_I/A_0=IC_{50}/(IC_{50}+[I])$$

where A_I is the absorbance at 405 nm of the sample in the presence of inhibitor; A_0 is the the absorbance at 405 nm in the absence of inhibitor; and [I] is the concentration of the inhibitor.

The inhibition constants (K_i) for the inhibitor for mPTPB was determined at pH 7.0 and 25°C. The mode of inhibition and K_i value were determined in the following manner. At various fixed concentrations of inhibitor (0-3 K_i), the initial rate at a series of *p*NPP concentrations was measured by following the production of *p*-nitrophenol as describe above, ranging from 0.2- to 5-fold the apparent K_m values. The data were fitted to appropriate equations using SigmaPlot-Enzyme Kinetics to obtain the inhibition constant and to assess the mode of inhibition.

For selectivity studies, the PTPs, including mPTPA, PTP1B, TC-PTP, SHP2, FAP1, Lyp, VHX, VHR, LMWPTP, Cdc14, LAR, PTPα, and CD45 were expressed and purified from *E.coli*. The inhibition assay for these PTPs were performed under the same conditions as mPTPB except using a different *p*NPP concentration corresponding to the K_m of the PTP studied.

Cell culture and transfection. Raw264.7 mouse macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS (Invitrogen), penicillin (50 units/mL), and streptomycin (50 µg/mL) under a humidified atmosphere containing 5% CO_2 at 37°C. mPTPB and mPTPB/C160S were subcloned into the pcN-HA expression vector. Raw 264.7 cells were seeded at 40% confluency in antibiotic-free medium and grown overnight, HA-tagged mPTPB, mPTPB/C160S, or pcN-HA empty vector were transfected into cells by electroporation at 800 microfarads and 280 V. 24hr after transfection, 0.5 mg/ml G418 was added to the culture medium. Stable clones were picked after 2 weeks of selection.

Immunoblotting. Cell were grown to 80% confluency, washed with ice-cold phosphate buffered saline, and lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EGTA, 150 mM NaCl, 10% Glycerol, 10 mM sodium phosphate, 10 mM sodium fluoride, 1mM sodium vanadate, 1 mM benzamidine, 1% Triton X-100, 10 µg/mL leupeptin, 5 µg/mL aprotinin, 1 mM PMSF) on ice for 30 min. Cell lysate is then cleared by centrifuging at 13,000 rpm for 15 min. The phosphorylation of ERK1/2 was detected by western blotting using pERK1/2 antibody as previously described (Zhou et al., 2010).

Macrophage assay. Inhibition of growth of *M. tuberculosis* Erdman (ATCC 35801) in a macrophage cell culture was assessed as previously described (Falzari et al., 2005). J774A.1 cells were grown to confluency in 75 cm^2 cell culture flasks in DMEM medium containing 10% FBS. Using a cell scraper the cells were detached, centrifuged at 200 x g for 5-min. at room temperature and the pellet suspended to a final concentration of 1-3 $x10^5$ cells/ml. One ml aliquots of cell suspension were distributed into 24-well plates (Falcon Multiwell 24 well) containing 13mm cover slips (Nalge Nunc International) and the plates incubated at 37˚C in a 5% CO_2 incubator for overnight. Frozen bacterial cultures were thawed, sonicated for 15 seconds, diluted to a final concentration of 1-3 $x10^5$ CFU/ml with DMEM and 1 ml of the dilution

dispensed to each well of a new 24-well plate. J774.1 cells on cover slips were transferred to the 24-well plates containing *M. tuberculosis* Erdman and the plates incubated at 37˚C for 1 hour to allow for phagocytosis. Cover slips were rinsed with HBSS to remove the extracellular bacteria and the cover slips transferred to new 24-well plates with 1 ml of fresh media in each well. Cultures were incubated at 37 ˚C under 5% CO_2 for 16 hours, then transferred those of cover slips to 1 ml per well fresh media containing the test compounds at 10 μM and amikacin (to prevent growth of any extracellular bacilli) at 20 mg/ml. Interferon-γ (Sigma, 087k1288) was added at 50 U/mL. All experimental conditions were set up in triplicate. At T_0 (for untreated controls) and after 7 days incubation medium was removed and macrophages lysed with 200 μl of 0.25% SDS. After 10 min. incubation at 37˚C, 200 μl of fresh media was added. The contents of the wells were transferred to a microtube, sonicated (Branson Ultrasonics model 1510, Danbury, CT) for 15 s and 1:1, 1:10, 1:100, and 1:1000 dilutions were plated on 7H11 (Difco) agar plates. Colonies were counted after incubation at 37˚C for 2-3 weeks.

MIC measurements. Minimum inhibitory concentrations (MIC) against replicating cultures of *M. tuberculosis* were determined using the microplate Alamar Blue assay (MABA) following a 7 day incubation in Middlebrook 7H12 medium as previously described (Falzari et al., 2005).

Cytotoxicity measurements. Cytotoxicity for the J774A.1 cell line was determined following 72 hours exposure (Falzari et al., 2005). Viability was assessed on the basis of cellular conversion of MTS into a soluble formazan product using the Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay.

References:

Falzari, K.; Zhu, Z.; Pan, D.; Liu, H.; Hongmanee, P.; Franzblau, S. G. In vitro and in vivo activities of macrolide derivatives against Mycobacterium tuberculosis. *Antimicrob. Agents Chemother*. **2005**, *49*, 1447-1454.

Liang, F.; Huang, Z.; Lee, S.-Y.; Liang, J.; Ivanov, M. I.; Alonso, A.; Bliska, J. B.; Lawrence, D. S.; Mustelin, T.; Zhang, Z.-Y. Aurintricarboxylic acid blocks *in vitro* and *in vivo* activity of YopH, an essential virulent factor of *Yersinia pestis*, the agent of plague. *J. Biol. Chem*. **2003**, *278*, 41734-41741.

Zhou, B.; He, Y.; Zhang, X.; Xu, J.; Luo, Y.; Wang, Y.; Franzblau, S. G.; Yang, Z.; Chan, R. J.; Liu, Y.; Zheng, J.; Zhang, Z.-Y. Targeting mycobacterium protein tyrosine phosphatase B for anti-tuberculosis agents" *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 4573-4578.

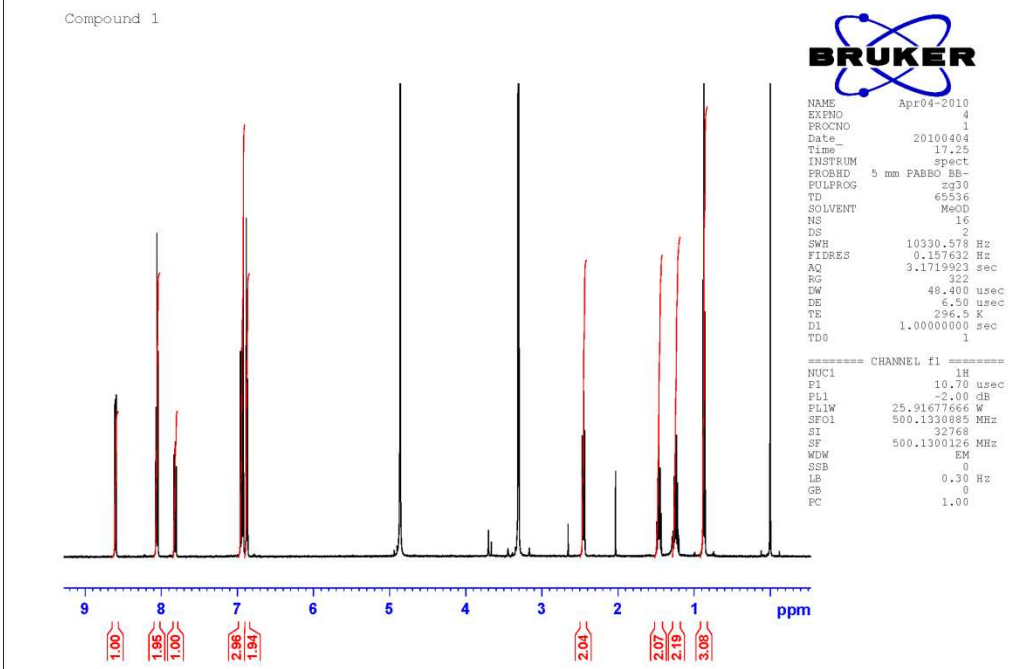


Figure **S1**. ^{1}H NMR spectrum of **1**.

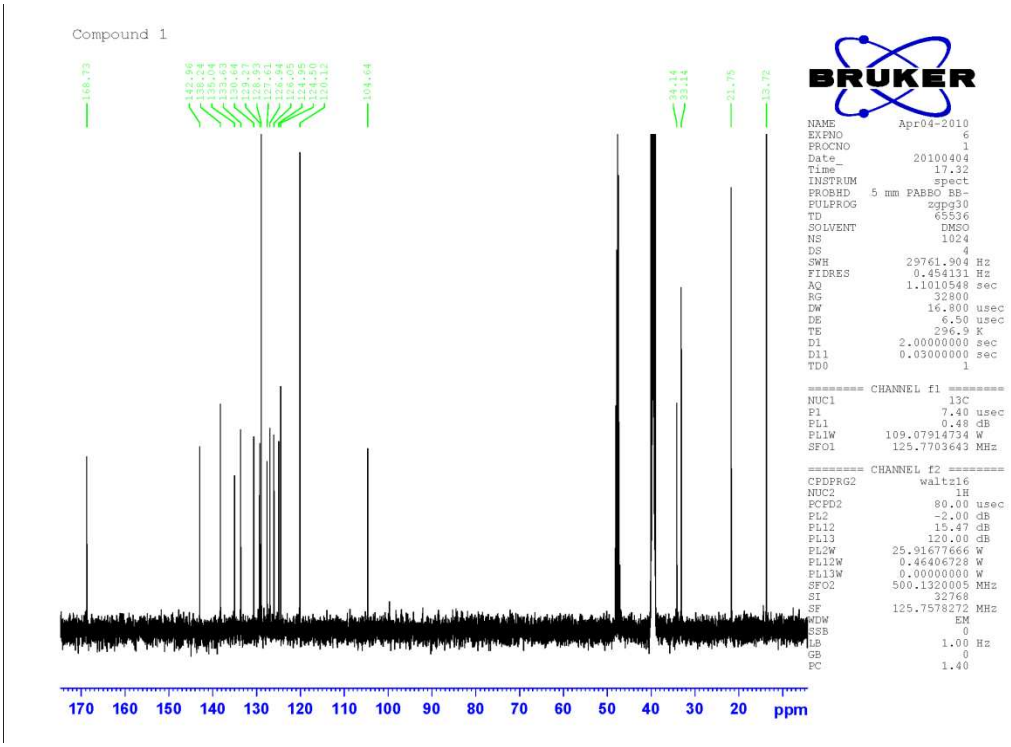


Figure **S2**. ^{13}C NMR spectrum of **1**.

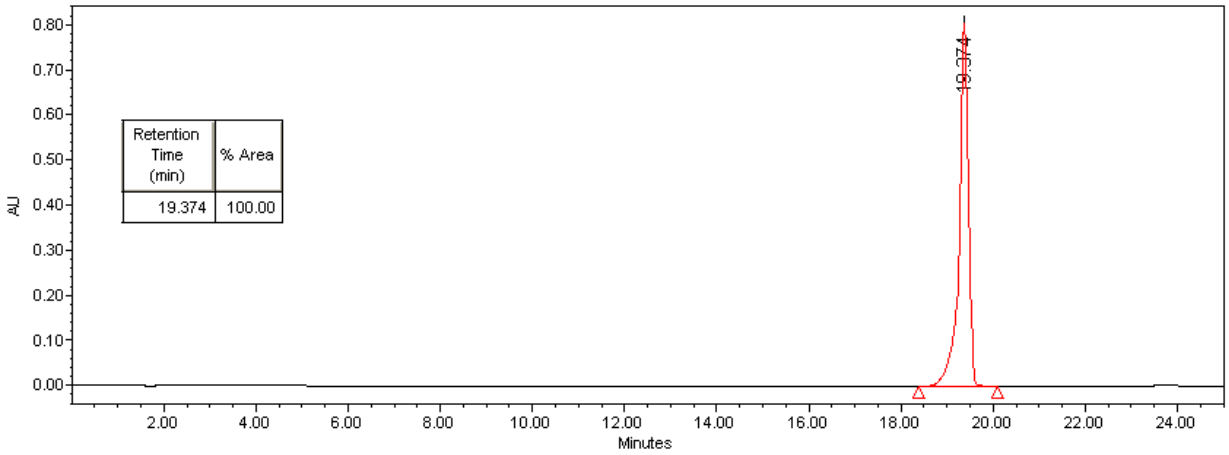


Figure **S3**. Purity analysis of **1** using analytical HPLC eluted with water/methanol .

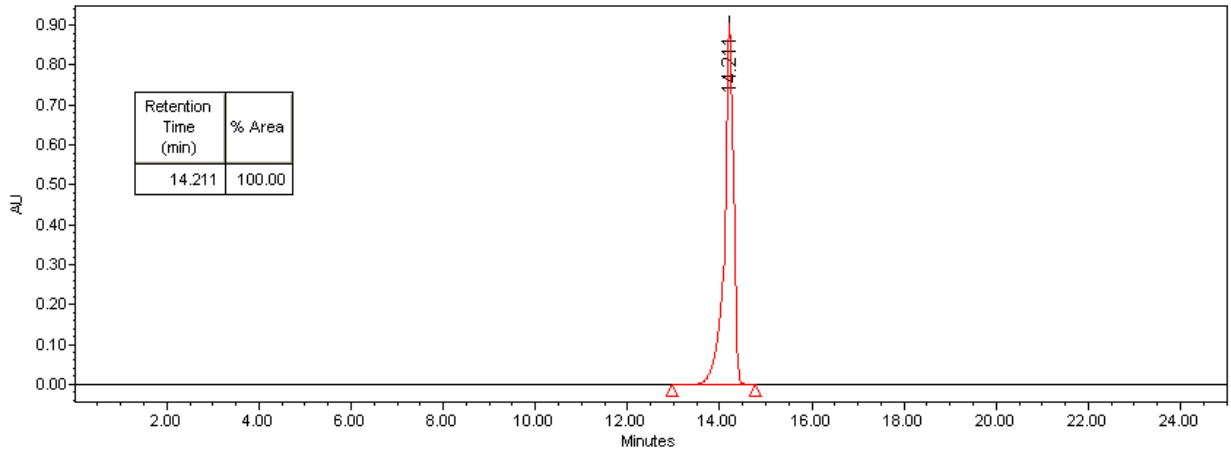


Figure **S4**. Purity analysis of **1** using analytical HPLC eluted with water/Acetonitrile.

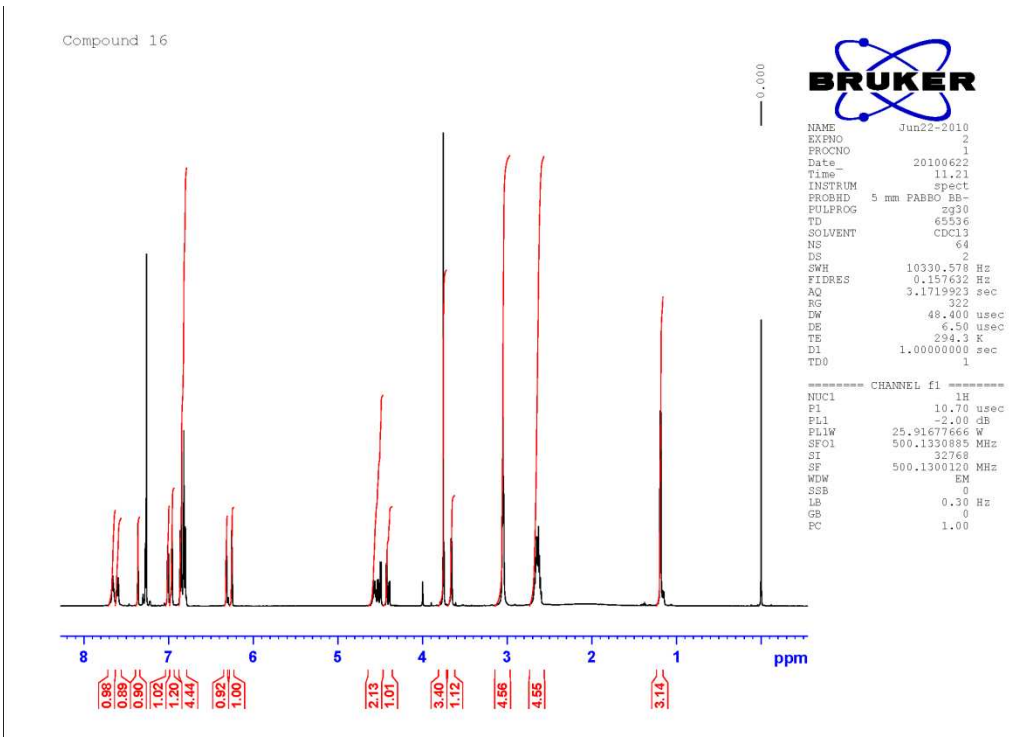


Figure **S5**. ^{1}H NMR spectrum of **16**.

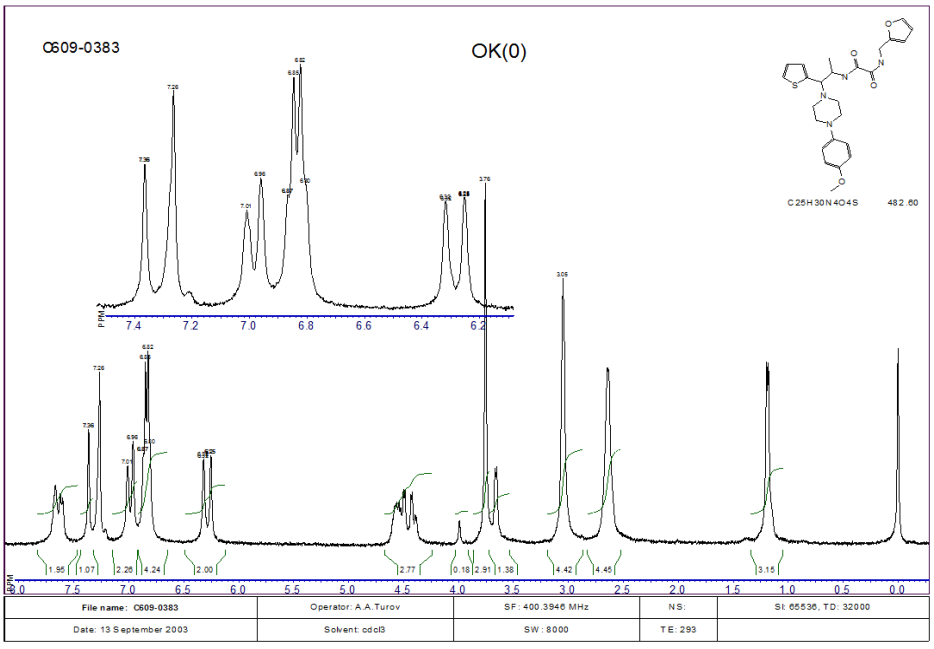


Figure **S6**. ^{1}H NMR spectrum of **16**, provided by ChemDiv.

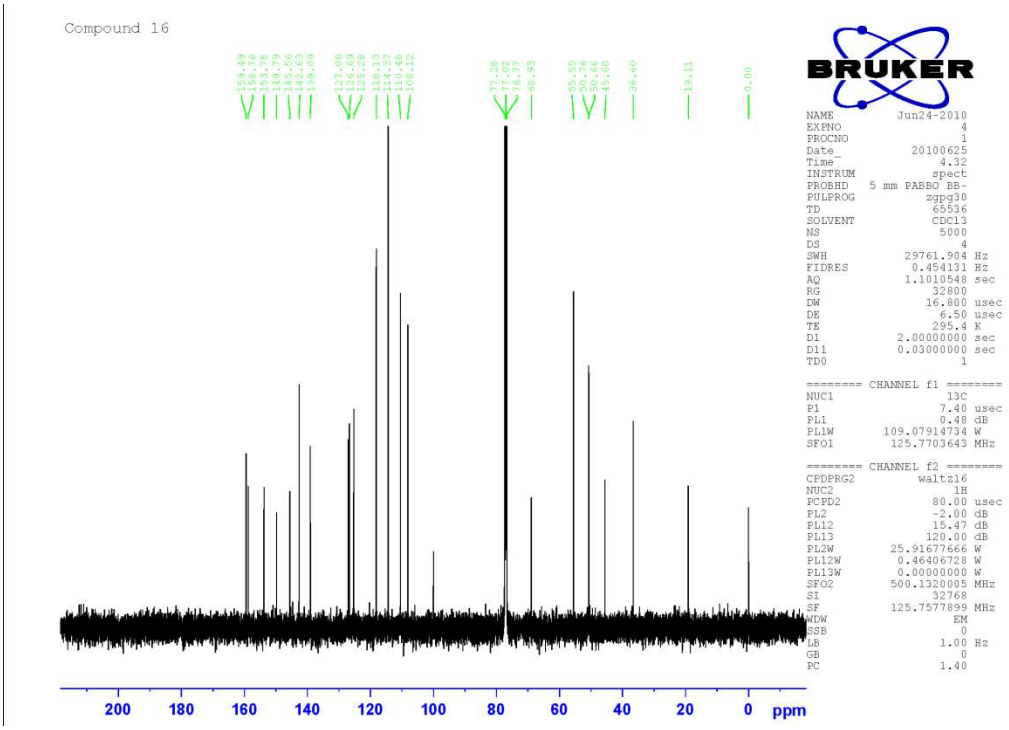


Figure **S7**. ^{13}C NMR spectrum of **16**.

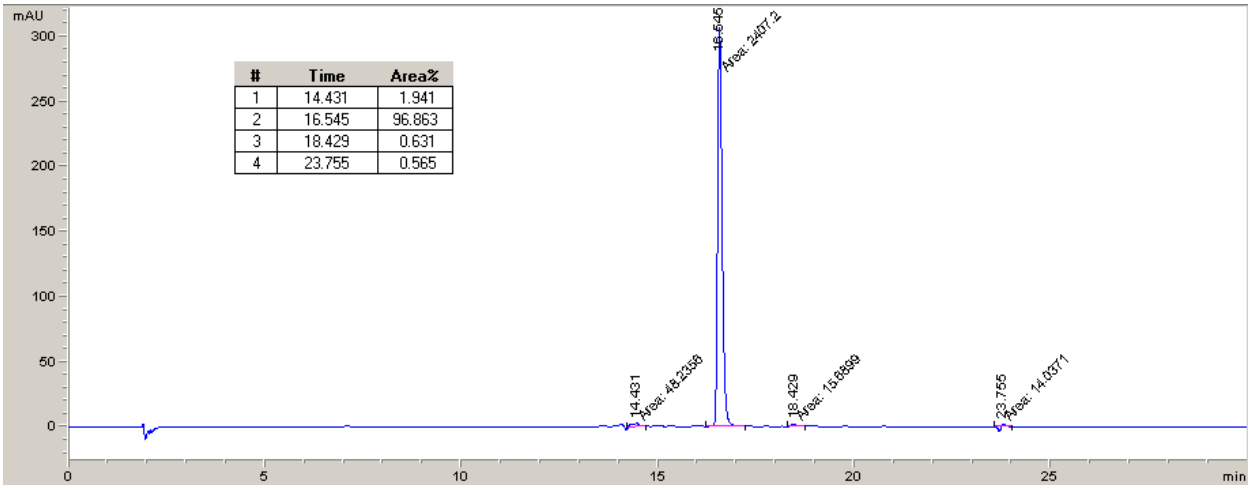


Figure **S8**. Purity analysis of **16** using analytical HPLC eluted with water/methanol.

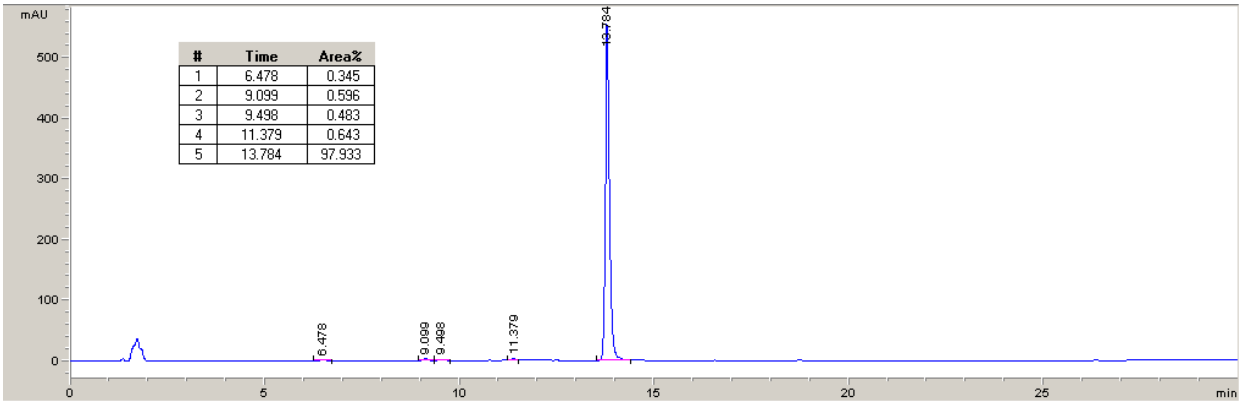


Figure **S9**. Purity analysis of **16** using analytical HPLC eluted with water/Acetonitrile.

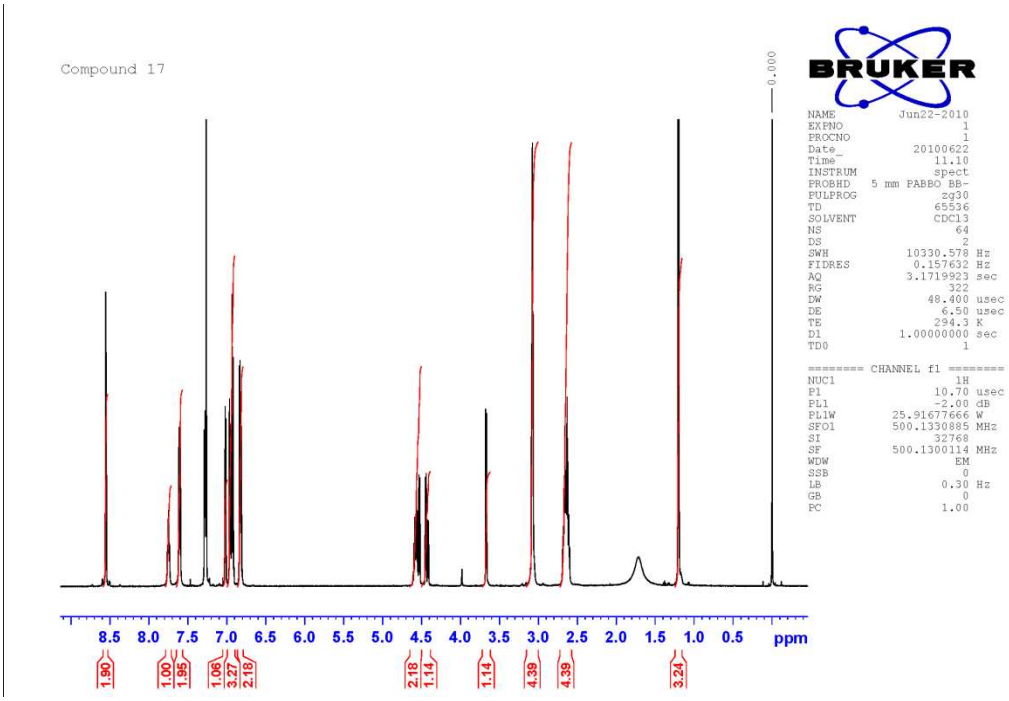


Figure **S10**. ^{1}H NMR spectrum of **17**.

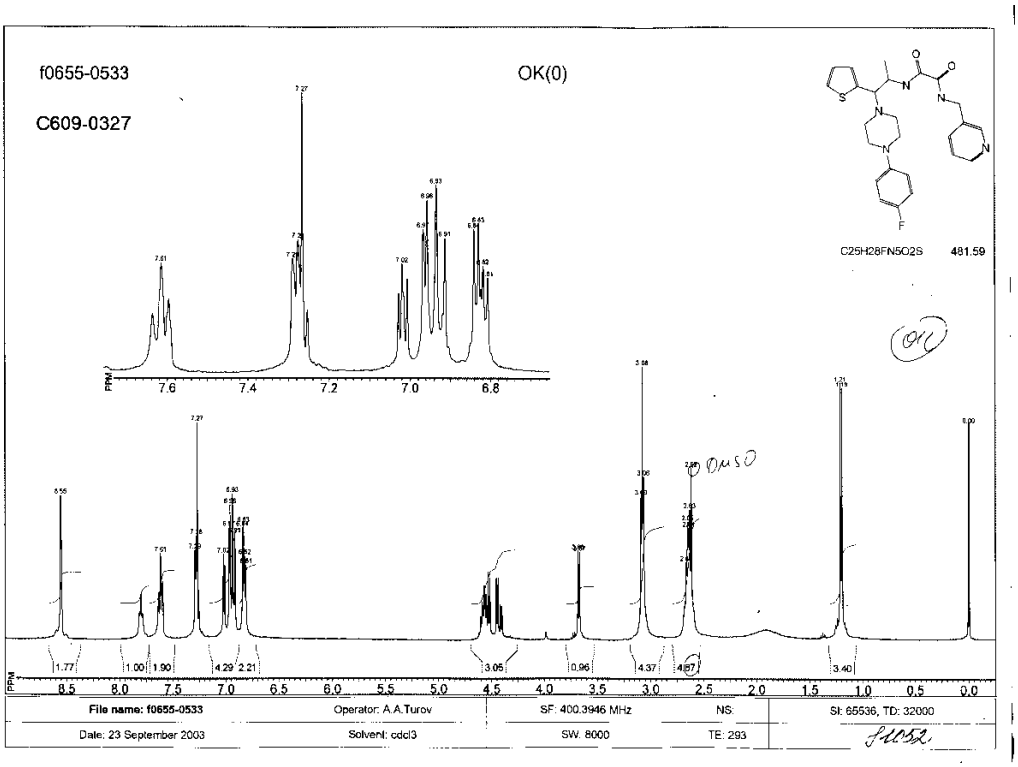


Figure **S11**. ^{1}H NMR spectrum of **17**, provided by ChemDiv.

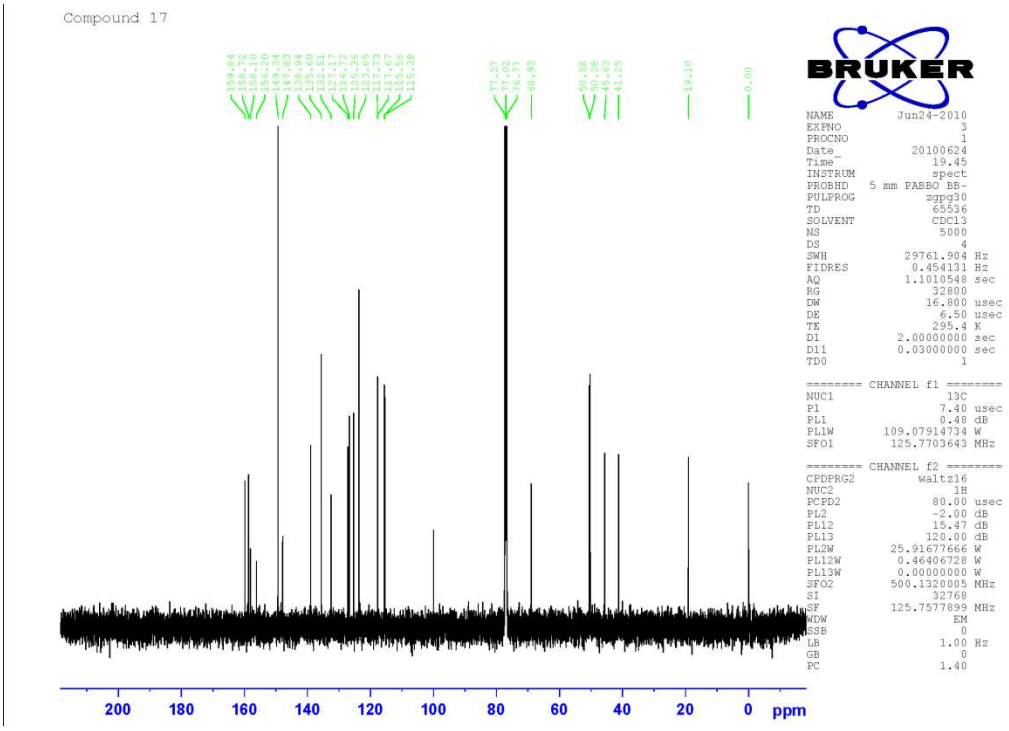
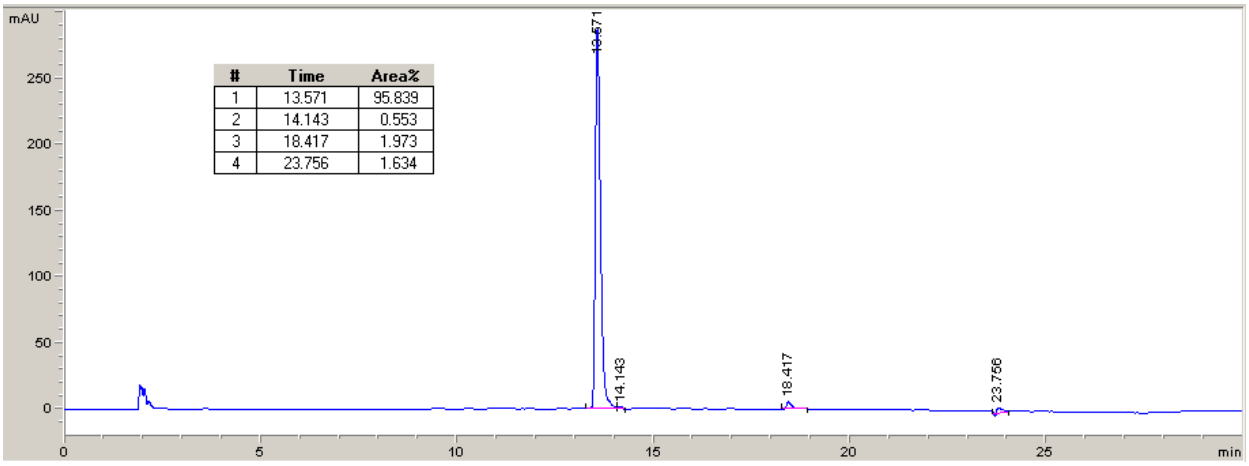
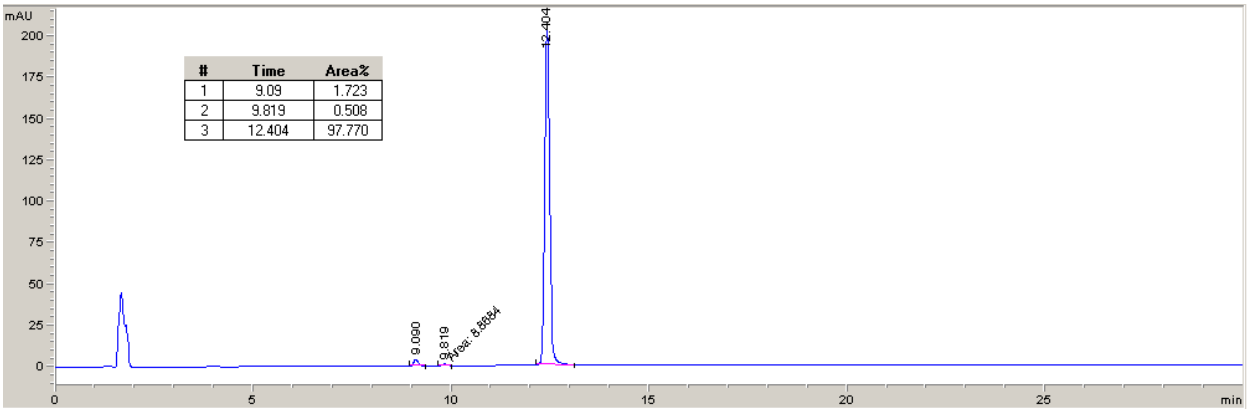


Figure **S12**. ^{13}C NMR spectrum of **17**.



#	Time	Area%
1	13.571	95.839
2	14.143	0.553
3	18.417	1.973
4	23.756	1.634

Figure **S13**. Purity analysis of **17** using analytical HPLC eluted with water/methanol.



#	Time	Area%
1	9.09	1.723
2	9.819	0.508
3	12.404	97.770

Figure **S14**. Purity analysis of **17** using analytical HPLC eluted with water/Acetonitrile.