Supporting Information

Structure-Activity Relationship Studies of NPI-0052, A Novel Marine Derived 20S Proteasome Inhibitor

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General:

Analytical methods. ^{1}H NMR spectra were collected using a 500 MHz Bruker Avance NMR spectrometer using an inverse probe equipped with x,y,z-gradients, except for ^{13}C NMR spectra, which were acquired with a broad-band observe probe. Mass spectra were acquired using a Micromass Q-Tof2 mass spectrometer with electrospray ionization (ESI). HRESI spectra were referenced using a polyethylene glycol polymer mixture, which was co-injected during acquisition as an internal accurate mass standard, unless noted otherwise. Analytical HPLC data were acquired on an Agilent HP1100 HPLC equipped with an Agilent PDA detector, an evaporative light scattering detector (ELSD, Sedere), and in most instances, with an 1100 series MSD Agilent mass spectrometer. Semi-preparative HPLC was performed on a Gilson HPLC equipped with a Gilson 215 fraction collector and ELSD (Sedere).

Chemical reagents. HPLC solvents were obtained from Fisher Scientific and VWR. Deuterated solvents were obtained from Cambridge Isotope Laboratories, Inc. Omuralide was obtained from Calbiochem. All other chemical reagents were obtained from Sigma-Aldrich, except for DMSO, which was obtained from EM Sciences Corp (Catalog # MX 14566) and Fisher Scientific.

*Cell culture and reagents***.** The human multiple myeloma cell line RPMI 8226 was obtained from ATCC (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1mM sodium pyruvate. The HEK293 NF-κB/luciferase reporter cell line is a derivative of the human embryonic kidney cell line (ATCC; CRL-1573) and carries a luciferase reporter gene under the regulation of 5X NF-κB binding sites. The reporter cell line was grown in DMEM medium supplemented with 10% heat inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES and 250 μg/ml G418. All cells were grown at 37°C, 5% CO_2 and 95% humidified air.

Biological Assays:

Cell viability assays. The assays were performed essentially as described previously (Mitchell et al., 2004). Briefly, RPMI 8226 cells were plated at a density of $2x10^4$ cells/well in 96 well plates, treated with serial diluted compounds and incubated for 48 hours. Cell viability was assessed using resazurin, a dye that is reduced by metabolically active cells and therefore an indicator of cellular proliferation. The IC_{50} values (the drug concentration at which 50% of the maximal observed growth inhibition is established) were determined using a standard sigmoidal dose response curve fitting algorithm (XLfit 3.0, ID Business Solutions Ltd).

In vitro purified rabbit muscle 20S proteasome activity assays. The chymotrypsin-like activity of the 20S proteasome was determined essentially as described previously (Stein et al 1996). Briefly, serial diluted compounds were added in duplicate to 1μg/ml purified rabbit 20S proteasome in assay buffer containing 20 mM HEPES, pH7.3, 0.5 mM EDTA, 0.05% Triton X-100 and 0.035% SDS and pre-incubated for 5 min at 37°C. Reactions were initiated by the addition of the Suc-LLVY-AMC peptide substrate at a final

concentration of 20 μM. Fluorescence of the cleaved peptide substrate was measured at λ_{ex}=390 nm and λ_{em}=460 nm using a Fluoroskan Ascent 96-well microplate reader (Thermo Electron, Waltham, MA). The IC_{50} values (the drug concentration at which 50% of the maximal relative fluorescence is inhibited) were calculated by Prism (GraphPad Software) using a sigmoidal dose-response, variable slope model. The caspase-like activity of the 20S proteasome was determined as described above except that Z-LLE-AMC was used as the peptide substrate. For the evaluation of the trypsin-like activity, the SDS was omitted from the assay buffer and Boc-LRR-AMC was used as the peptide substrate.

Determination of NF-κB mediated luciferase activity. The HEK293 NF-κB/luciferase cells were seeded at a density of $1.5x10^4$ cells/well in Corning 3917 white opaque-bottom tissue culture plates and incubated overnight. Phenol-red free DMEM was used instead of DMEM and the G418 was omitted from the supplements. Serial diluted compounds were added to the cells and incubation continued for one hour. Cells were then stimulated with recombinant human TNF-α (10ng/ml) and incubation continued for an additional 6 hours. Steady Lite HTS luciferase reagent (Packard Bioscience) was added and after 10 min at room temperature the luciferase activity was measured using a Fusion microplate fluorometer (Packard Bioscience). The IC_{50} values (the drug concentration that resulted in a 50% reduction of luciferase activity) were calculated in Prism (GraphPad Software) using a sigmoidal dose response, variable slope model.

Cell-based 20S proteasome activity assay. Serial diluted compounds were added to $2.5x10^5$/ml RPMI 8226 cells and incubated for 1 hr at 37°C, 5% CO_2 and 95% humidified air. RPMI 8226 cells treated with DMSO at a final concentration of 0.1% served as the vehicle control. Following the 1 hr incubation, cells were pelleted by centrifugation at 2,000 rpm for 10 sec at room temperature and washed 3X with ice-cold 1X Dulbecco's Phosphate-Buffered Saline (DPBS, Mediatech, Herndon, VA). DPBS washed cells were lysed on ice for 15 min in lysis buffer (20 mM HEPES, 0.5 mM EDTA, 0.05% Triton X-100, pH 7.3) supplemented with protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Cell debris was pelleted by centrifugation at 14,000 rpm for 10 min, 4°C and cell lysates were transferred to new tubes. Protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL). To measure the chymotrypsin-like activity of the 20S proteasomes in RPMI 8226 cell lysates, the same procedure was followed as described in the section " *in vitro* purified rabbit muscle 20S proteasome activity assays ", with the exception that 20 mM HEPES, 0.5 mM EDTA, pH 8.0 and 0.03% SDS was used as the assay buffer. Results are presented as the percent inhibition of the 20S proteasome chymotrypsin-like activity relative to the DMSO control.

Analog Preparation and Analysis:

*Preparation of NPI-2055 **(4)** from **1***

NPI-0052 (**1**, 30 mg, 96μmol) was dissolved in 1:1 acetonitrile/water (4 ml) in a conical flask to which was added 5% HCl (1 ml) and a magnetic stir bar. The reaction mixture was stirred at 40ºC for about 4 hours and then allowed to stand at room temperature. The reaction was monitored occasionally by mass spectrometry, which indicated that approximately 90% of the NPI-0052 (**1**) was converted to NPI-2055 (**4**) after 6 weeks. The reaction mixture (without any prior workup) was chromatographed by reversed-phase HPLC using an ACE 5μ C18 column (150 x 22 mm ID) with a solvent gradient of 20% to 80% CH_3CN/Water over 13 min, 80 to 100% CH_3CN in 1 min, then 5 min at 100% CH_3CN, at a flow rate of 14.5 ml/min. NPI-2055 (**4**; 24.8 mg, ~88% purity) eluted at about 6 min. The sample containing **4** was redissolved in acetonitrile to a concentration of 4 mg/ml, and 350 μl aliquots were injected onto a reverse-phase HPLC column of dimensions described above. A ten minute method consisting of an isocratic solvent system of 25% CH_3CN / 75% water at flow rate of 14.5 ml/min was used. Compound **4** (6.9 mg) eluted after three minutes in the above method with a purity of 95.5% (0052TF14) and an overall yield of 23% from starting material. NPI-2055 (**4**): HRMS (ESI), *m/z* 296.1484 $[M+H]^+$, Δ_{calc}= -4.6 ppm, $C_{15}H_{22}NO_5$; ^{1}H NMR (DMSO-d_6) δ 1.22-1.35 (brm, 2H), 1.39 (s, 3H), 1.66 (br m, 2H), 1.86 (m, 2H), 1.94 (m, 1H), 2.04 (m, 1H), 2.17 (m, 1H), 2.65 (d, 1H, *J* = 7.6 Hz), 3.40 (m, 1H), 3.69 (m, 2H), 5.63(d, 1H, *J* = 9.5 Hz), 5.81 (d, 1H, *J* = 10.2 Hz), 6.91 (br s, 1H).

*Preparation of NPI-2070 (**10**) from **1***

A sample of NPI-0052 (**1**, 250 mg; 0.799 mmol) was added to an acetone solution of sodium iodide (1.5 g; 10 mmol in 10 ml) and the resulting mixture stirred for 6 days. The solution was then filtered through a 0.45 micron syringe filter and injected directly on a normal phase silica HPLC column (Phenomenex Luna 10u Silica, 250 mm x 21.2 mm) in 0.95 ml aliquots. The HPLC conditions for the separation of **10** from unreacted **1** employed an isocratic HPLC method consisting of 24% EtOAc and 76% hexane, in which the majority of **10** eluted 2.5 minutes before **1**. Equivalent fractions from each of 10 injections were pooled to yield 35 mg (11% yield) of NPI-2070 (**10**): HRMS (ESI), *m/z* 406.0513 $[M+H]^+$, Δ_{calc}= -0.5 ppm, $C_{15}H_{21}NO_4I$; ^{1}H NMR (DMSO-d_6) δ 1.22 (m, 1H), 1.40 (br m, 1H), 1.69 (m, 1H), 1.73 (s, 3H), 1.82 (m, 1H), 1.91 (br m, 2H), 2.05 (m, 2H), 2.27 (br m, 1H), 2.57 (br t, 1H, *J* = 6.9 Hz), 3.51 (m, 2H), 3.67 (t, 1H, *J* = 8.6 Hz), 5.51 (d, 1H, *J* = 8.2 Hz), 5.72 (br d, 1H, *J* = 10.4 Hz), 5.80 (d, 1H, *J* = 10.4 Hz), 9.09 (s, 1H, NH).

*Preparation of NPI-2078 (**11**) from **10***

A sample of NaN_3 (80 mg; 1.23 mmol) was dissolved in DMSO (1 ml) and transferred to a vial containing NPI-2070 (**10,** 6.2 mg; 15 μmol) which was contaminated with approximately 10% of **1**. The solution was incubated at room temperature for 1 hr prior to purification on C18 HPLC (ACE 5μ C18-HL, 150 mm X 21 mm ID) using a solvent gradient of 10% acetonitrile/90% H_2O to 90% acetonitrile/10% H_2O over 17 minutes. Using this method, the desired azido derivative (**11**) co-eluted with contaminant **1** at 12.5 minutes (4.2 mg, 87% yield). A 2.4 mg portion of compound **11** was further purified using additional C18 HPLC chromatography (ACE 5μ C18-HL, 150 mm X 21 mm ID) using an isocratic solvent gradient consisting of 35% acetonitrile / 65% H_20. Under these

conditions compound **11** eluted after 20 minutes, while compound **1** eluted after 21.5 minutes. The resulting sample consisted of 1.1 mg compound **11**. HRMS data were acquired with negative ionization using **1** as an internal standard. NPI-2078 (**11**): HRMS (ESI), *m/z* 319.1406 $[M-H]^-$, Δ_{calc}= 0.0 ppm, $C_{15}H_{19}N_4O_4$; ^{1}H NMR (DMSO-d_6) δ 1.22 (m, 1H), 1.40 (br m, 1H), 1.69 (m, 1H), 1.73 (s, 3H), 1.76-1.85 (m, 3H), 1.91 (br m, 2H), 2.28 (br m, 1H), 2.56 (t, 1H, *J* = 6.9 Hz), 3.61 (t, 2H, *J* = 7.3 Hz); 3.67 (t, 1H, *J* = 8.6 Hz), 5.52 (d, 1H, *J* = 7.9 Hz), 5.71 (br d, 1H, *J* = 10.7 Hz), 5.80 (d, 1H, *J* = 10.7 Hz), 9.06 (s, 1H, NH).

*Preparation of NPI-2077(**12**) from **10***

Acetone (7.5 ml) was vigorously mixed with 5 N NaOH (3 ml) and the resulting mixture evaporated to a minimum volume *in vacuo*. A sample of 100 µl of this solution was mixed with NPI-2070 (**10,** 6.2 mg; 15 µmol) in acetone (1 ml) and the resulting biphasic mixture vortexed for 2 minutes. The reaction solution was immediately subjected to preparative C18 HPLC using conditions using the following conditions. A linear gradient of 10% acetonitrile/90% water to 90% acetonitrile/ 10% water over 17 minutes using an Ace 5 µ C18 HPLC column of dimensions 22 mm id by 150 mm length. Compound **12** eluted at 9.1 minutes under these conditions to yield 0.55 mg (9%) compound. NPI-2077 (**12**): ESMS, *m/z* 296.1 (M+H); ^{1}H NMR (DMSO-d_6) δ 1.23 (m, 1H), 1.40 (br m, 1H), 1.59-1.69 (m, 2H), 1.71 (s, 3H), 1.73-1.77 (m, 1H), 1.80 (br m, 1H),1.91 (br m, 2H), 2.28 (br m, 1H), 2.57 (t, 1H, *J* = 6.7 Hz), 3.59 (m, 2H); 3.66 (t, 1H, *J* = 8.5 Hz), 4.63 (t, 1H, *J* = 5.3 Hz; C-13(OH)), 5.49 (d, 1H, *J* = 7.9 Hz; C-5(OH)), 5.71 (br d, 1H, *J* = 10.4 Hz), 5.80 (d, 1H, *J* = 10.4 Hz), 8.95 (s, 1H); ^{13}C NMR (DMSO-d_6) δ 19.5, 20.9, 24.5, 25.2, 27.7, 37.6, 44.7, 58.3 (C-13), 69.1, 78.6, 85.9, 127.7, 128.5, 168.8, 176.1.

*Preparation of NPI-2080 (**13**) from **10***

A portion of CuI (100 mg; 0.53 mmol) was placed in a 25 ml pear bottom flask and flushed with Ar gas for 30 minutes and a continuous stream of Ar gas was flushed through the flask during the course of the reaction. The vessel was cooled to -78 ºC prior to addition of dry THF (5 ml) followed by the immediate dropwise addition of a solution of methyllithium in dry ether (5.0 ml, 8 mmol) with vigorous stirring. A solution of NPI-2070 (**10,** 12 mg; 30 µmol) in dry THF (1 ml) was added slowly to the clear lithium dimethylcuprate solution and the resulting mixture stirred at -78 ºC for 1 hr. The reaction was quenched by washing the THF solution through a plug of silica gel (1 cm diameter by 2 cm length) along with further washing using a solution of 50% EtOAc / 50% hexanes (50 ml). The combined silica plug washes were dried *in vacuo* and subjected to further C18 HPLC purification in 2 injections (ACE 5µ C18-HL, 150 mm X 21 mm ID) using an isocratic solvent gradient consisting of 35% acetonitrile / 65% water. Compound **13** eluted under these conditions at 23.5 minutes and yielded 2.4 mg material (27% isolated yield). NPI-2080 (**13**): HRMS (ESI), *m/z* 294.1696 $[M+H]^+$, Δ_{calc}= -3.2 ppm, $C_{16}H_{24}NO_4$; ^{1}H NMR (DMSO-d_6) δ 0.90 (t, 3H, *J* = 6.9 Hz), 1.22 (m, 1H), 1.40 (br m, 1H), 1.43-1.61, m, 4H), 1.69 (m, 1H), 1.73 (s, 3H), 1.80 (m, 1H), 1.91 (br m, 2H), 2.28 (br m, 1H), 2.42 (br t, 1H, *J* = 6.6 Hz), 3.65 (t, 1H, *J* = 8.6 Hz), 5.50 (d, 1H, *J* = 7.9 Hz), 5.71 (br d, 1H, *J* = 10.4 Hz), 5.80 (d, 1H, *J* = 10.4 Hz), 8.92 (s, 1H, N<u>H</u>); ^{13}C NMR in DMSO-d_6: δ 13.9, 19.9, 20.3, 20.9, 24.5, 25.3, 26.8, 37.6, 47.4, 69.1, 78.5, 86.0, 127.6, 128.4, 168.8, 175.8.

*Preparation of NPI-2056 (**14**) from **1***

NPI-0052 (**1,** 10 mg; 32 μmol) was dissolved in acetone (5 mL) in a scintillation vial (20 mL) to which was added 10% (w/w) Pd/C (1-2mg) and a magnetic stir bar. The reaction mixture was stirred in a hydrogen atmosphere at room temperature for about 15 hours. The reaction mixture was filtered through a 3 cc silica column and washed with acetone. The filtrate was filtered again through a 0.2 μm syringe filter to remove any traces of catalyst. The solvent was evaporated from the filtrate under reduced pressure to yield **14** as a pure white powder (100%). NPI-2056 (**14**): HRMS (ESI), *m/z* 316.1305 $[M+H]^+$, Δ_{calc}= -3.5 ppm, $C_{15}H_{23}NO_4Cl$; ^{1}H NMR (DMSO-d_6) δ 0.89-1.00 (m, 2H), 1.13-1.27 (br m, 3H), 1.48 (m, 1H), 1.54-1.57 (m, 3H), 1.73 (s, 3H), 1.74-1.87 (br m, 2H), 1.93-2.06 (m, 2H), 2.64 (t, 1H, *J* = 7.0 Hz), 3.67 (t, 1H, *J*=8.2 Hz), 3.84-3.94 (m, 2H), 5.25 (d, 1H, *J* = 7.9 Hz, OH), 9.03 (s, 1H, NH).

*Preparation of NPI-2060 (**15**) and NPI-2061(**16**) from **1***

NPI-0052 (**1**, 101 mg; 0.323 mmol) was dissolved in CH_2Cl_2 (30 mL) in a 100 ml of round bottom flask to which was added 79 mg (0.460 mmol) of mCPBA and a magnetic stir bar. The reaction mixture was stirred at room temperature for about 18 hours. The reaction mixture was poured onto a 20 cc silica flash column and eluted with 120 ml of CH_2Cl_2, 75 ml of 1:1 EtOAc/Hex and finally with 40 ml of 100% EtOAc. The 1:1 EtOAc/Hex fractions yield a mixture of diastereomers of epoxy derivatives **15** and **16** (9:1), which were separated by normal phase HPLC using a Phenomenex Luna 10u Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compounds **15** (major product) and **16** (minor product) eluted at 21.5 and 19 min, respectively. Compound **16** was further chromatographed on a 3cc silica flash column to remove traces of mCPBA reagent. The final quantities obtained were 76.8 mg (72% yield) of **15** and 7 mg (6.5% yield) of **16**. NPI-2060 (**15**): HRMS (ESI), *m/z* 330.1099 $[M+H]^+$, Δ_{calc}= -2.9 ppm, $C_{15}H_{21}NO_5Cl$; ^{1}H NMR (DMSO-d_6) δ 0.99 (m, 1H), 1.11 (br m, 1H), 1.40 (m, 1H), 1.47 (m, 1H), 1.75 (s, 3H), 1.86 (br m, 1H), 1.95-2.05 (m, 3H), 2.65 (t, 1H, *J* = 7.0 Hz), 3.17 (t, 1H, *J* = 4.4 Hz), 3.23 (brs, 1H), 3.85-3.95 (m, 3H), 5.71 (d, 1H, *J* = 8.2 Hz, OH), 9.17 (s, 1H, NH). NPI-2061 (**16**): HRMS (ESI), *m/z* 330.1105 $[M+H]^+$, Δ_{calc}= -0.9 ppm, $C_{15}H_{21}NO_5Cl$; ^{1}H NMR (DMSO-d_6) δ 0.86 (m, 1H), 1.16 (br m, 1H), 1.32 (m, 1H), 1.56-1.65 (m, 2H), 1.75 (s, 3H), 1.87-2.08 (m, 4H), 2.65 (t, 1H, *J* = 7.0 Hz), 3.08 (br s, 1H), 3.15 (brd, 1H, *J* = 4.0 Hz), 3.84-3.96 (m, 3H), 5.73 (d, 1H, *J* = 8.2 Hz, OH), 9.12 (s, 1H, NH).

*Preparation of NPI-2064 (**17**) from **15***

NPI-2060 (**15,** 3.3 mg; 10 μmol) was dissolved in acetonitrile (0.5 ml) in a 1 dram vial to which was added 5% HCl (500 μl) and a magnetic stir bar. The reaction mixture was stirred at room temperature for about an hour. The reaction was monitored by mass spectrometry. The reaction mixture was directly injected on normal phase HPLC to obtain **17** as a pure compound without any work up. The HPLC conditions used for the purification were as follows: Phenomenex Luna 10u Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc in 1 min, then 5 min at 100% EtOAc at a flow rate of 14.5 ml/min. An ELSD was used

to monitor the purification process. Compound **17** eluted at about 18 min (2.2 mg, 60% yield). NPI-2065 (**17**): HRMS (ESI), *m/z* 366.0875 $[M+H]^+$, Δ_{calc}= 0.0 ppm, $C_{15}H_{22}NO_5Cl_2$; ^{1}H NMR (DMSO-d_6) δ 1.20 (m, 1H), 1.40 (br m, 1H), 1.56-1.70 (m, 3H), 1.72 (s, 3H), 1.93-2.10 (m, 4H), 2.62 (t, 1H, *J* = 7.0 Hz), 3.84-3.98 (m, 4H), 4.25 (brdd, 1H, *J* = 2.8, 6.0 Hz), 5.14 (d, 1H, *J* = 4.4 Hz, OH), 5.20 (d, 1H, *J* = 8.8 Hz, OH), 9.21 (s, 1H, NH). The structure was determined by 2D-NMR spectral analysis. The stereochemistry of NPI-2064 was determined based on coupling constants observed in the cyclohexane ring in 1:1 C_6D_6/DMSO-d_6. ^{1}H NMR (C_6D_6+DMSO-d_6;1:1) δ 1.35 (m, 1H, 10-Heq), 1.40 (m, 1H, 11-Ha), 1.56 (br d, 1H, J=14 Hz, 9-Heq), 1.62 (s, 3H), 1.74 (br tt, J=2.8, 12.5 Hz, 10-Ha), 1.78 (br m, 1H, 11-Heq), 1.91 (ddd, 1H, *J*= 7.2, 14.5 Hz), 2.02 (ddd, 1H, *J*=7.2, 14.5 Hz, 12-H), 2.09 (br tt, 1H, *J*=3.5, 13.5 Hz, 9-Ha), 2.16 (br tt, 1H, *J*=2.8,12 Hz, 6-Ha), 2.61 (t, 1H, *J* = 7.0 Hz), 3.71 (dt, 1H, *J*=7.2, 10.7 Hz, 13-H), 3.79 (dt, 1H, *J*=7.2, 10.7 Hz, 13-H), 4.09 (dd, 1H, *J*=8.8, 9.4 Hz, 5-H), 4.13 (br s, 1H, m, 7-Heq), 4.29 (br dd, 1H, *J* = 2.8, 6.0 Hz, 8-Heq), 5.22 (d, 1H, *J* = 4.4 Hz, 7-OH), 5.24 (d, 1H, *J* = 8.8 Hz, 5-OH), 9.36 (s, 1H, NH)

*Preparation of NPI-2062 (**18**) from **1***

NPI-0052 (**1**, 30 mg; 96 μmol) was dissolved in CH_2Cl_2 (6 ml) in a scintillation vial (20 ml) to which Dess-Martin Periodinane (122 mg; 288 μmol) and a magnetic stir bar were added. The reaction mixture was stirred at room temperature for about 2 hours. The progress of the reaction was monitored by TLC (Hex:EtOAc, 6:4) and analytical HPLC. From the reaction mixture, the solvent volume was reduced to one third, absorbed on silica gel, poured on top of a 20 cc silica flash column and eluted in 20 ml fractions using a gradient of Hex/EtOAc from 10 to 100%. The fraction eluted with 30% EtOAc in Hex contained a mixture of rotamers of **18** in a ratio of 1.5:8.5. The mixture was further purified by normal phase HPLC using the Phenomenex Luna 10u Silica column (25 cm x 21.2 mm ID) with a solvent gradient of 25% to 80% EtOAc/Hex over 19 min, 80 to 100% EtOAc over 1 min, holding at 100% EtOAc for 5 min, at a flow rate of 14.5 ml/min. An ELSD was used to monitor the purification process. Compound **18** eluted at 13.0 and 13.2 mins as a mixture of rotamers with in a ratio of 1.5:8.5 (7 mg; 23%). Hydrogenation of the mixture of **18** yielded a single pure compound which confirmed that **18** exists as a mixture of rotamers. NPI-2062 (**18**): HRMS (ESI), *m/z* 312.1017 $[M+H]^+$, Δ_{calc}= 4.5 ppm, $C_{15}H_{19}NO_4Cl$; ^{1}H NMR (DMSO-d_6) δ 1.54 (s, 3H), 1.59 (m, 2H), 1.66-1.70 (m, 1H), 1.73-1.80 (m, 1H), 1.96 (m, 2H), 2.0-2.11 (m, 2H), 3.09 (t, 1H, *J* = 7.0 Hz), 3.63 (brs, 1H), 3.83-3.88 (m, 1H), 3.89-3.93 (m, 1H), 5.50 (dd, 1H, *J* = 2, 10 Hz), 5.92 (dd, 1H, *J* = 2.5, 10 Hz), 9.70 (s, 1H, NH); ^{13}C NMR (DMSO-d_6) δ 18.8 (q), 19.2 (t), 22.8 (t), 23.9 (t), 27.8 (t), 42.3 (t), 44.1 (d), 44.2 (d), 81.5 (s), 85.7 (s), 120.9 (d), 131.7 (d), 165.3 (s), 175.8 (s), 202.8 (s).

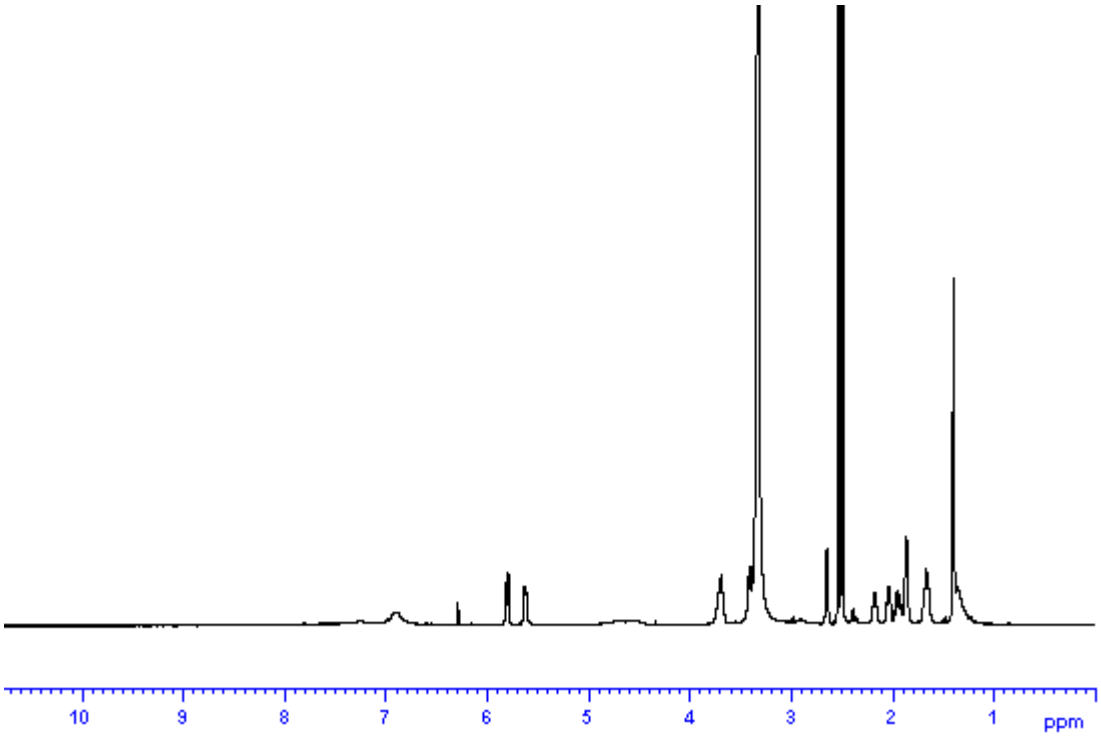
*Preparation of NPI-2076 (**19**) from **18***

The rotamer mixture of NPI-2062 (**18,** 5 mg; 16 μmol) was dissolved in dimethoxy ethane (monoglyme; 1.5 ml) in a scintillation vial (20 ml) to which water (15 μl (1% of the final solution concentration)) and a magnetic stir bar were added. The above solution was cooled to -78^{o}C on a dry ice-acetone bath, and a sodium borohydride solution (1.8 mg; 47 μmol of $NaBH_4$ in 0.5 ml of monoglyme (created to allow for slow addition)) was added drop-wise. The reaction mixture was stirred at -78^{o}C for about 14 minutes. The reaction mixture was acidified using 2 ml of 4% HCl solution in water and extracted with

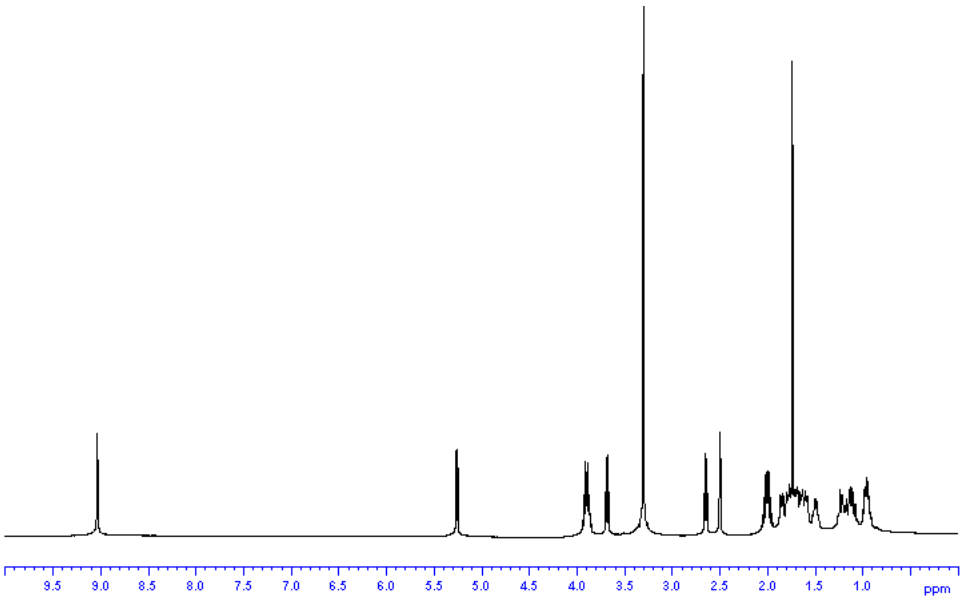
CH_2Cl_2. The organic layer was evaporated to yield mixture of **19** and **1** in a 9.5:0.5 ratio as a white solid, which was further purified by normal phase HPLC using a Phenomenex Luna 10u Silica column (25 cm x 21.2 mm ID). The mobile phase was 24% EtOAc/76% Hexane, which was held isocratic for 19 min, followed by a linear gradient of 24% to 100% EtOAc over 1 min, and held at 100% EtOAc for 3 min; the flow rate was 25 ml/min. An ELSD was used to monitor the purification process. Compound **19** (1.5 mg; 30%) eluted at 11.64 min as a pure compound. NPI-2076 (**19**): HRMS (ESI), *m/z* 314.1154 $[M+H]^+$, Δ_{calc}= -0.6 ppm, $C_{15}H_{21}NO_4Cl$; ^{1}H NMR (DMSO-d_6) δ 1.45 (m, 1H), 1.56 (m, 1H), 1.73 (s, 3H), 1.73 (m, 2H), 1.89 (brs, 2H), 1.95-2.01 (m, 2H), 2.29 (brs, 1H), 2.72 (t, 1H, *J* = 7.5 Hz), 3.82-3.85 (m, 1H), 3.88-3.92 (m, 2H), 5.34 (d, 1H, *J* = 6.0 Hz, OH), 5.43 (brd, 1H, *J* = 10 Hz), 5.75 (dd, 1H, *J* = 2.5, 10Hz), 9.03 (s, 1H, NH).

The fermentation, isolation, and structure elucidation of NPI-0047 (**6**), NPI-2053 (**7**), NPI-2059 (**9**), NPI-2063 (**5**) and NPI-2065 (**8**) will be reported elsewhere.

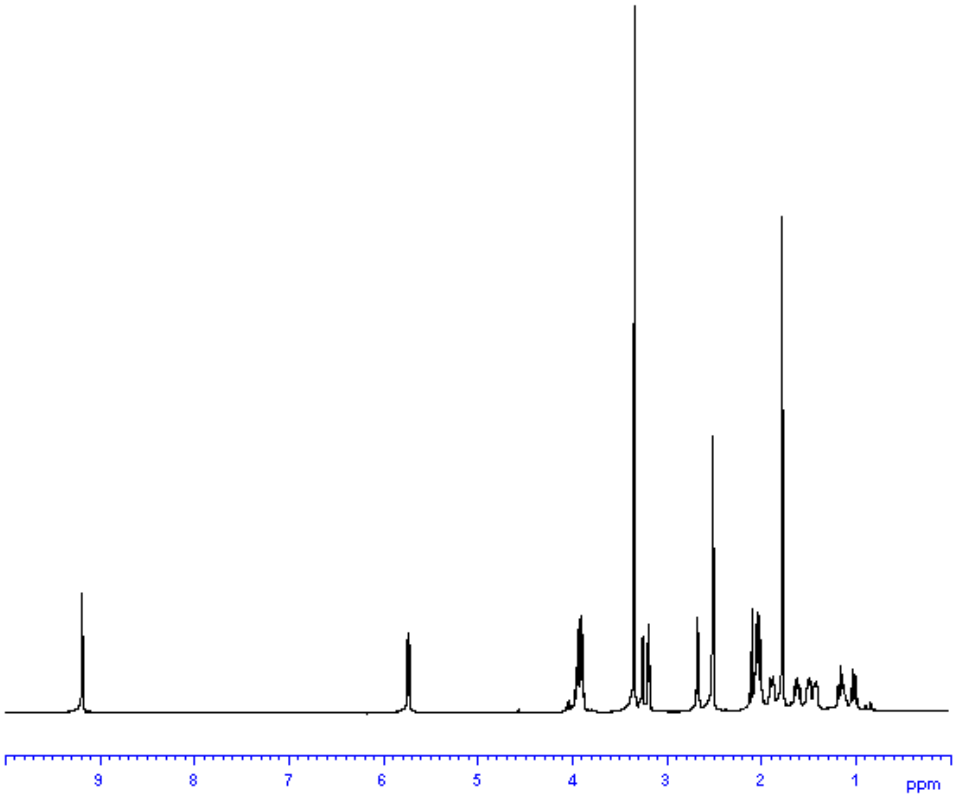
^{1}H NMR Spectra of Analogs 4, 10-19



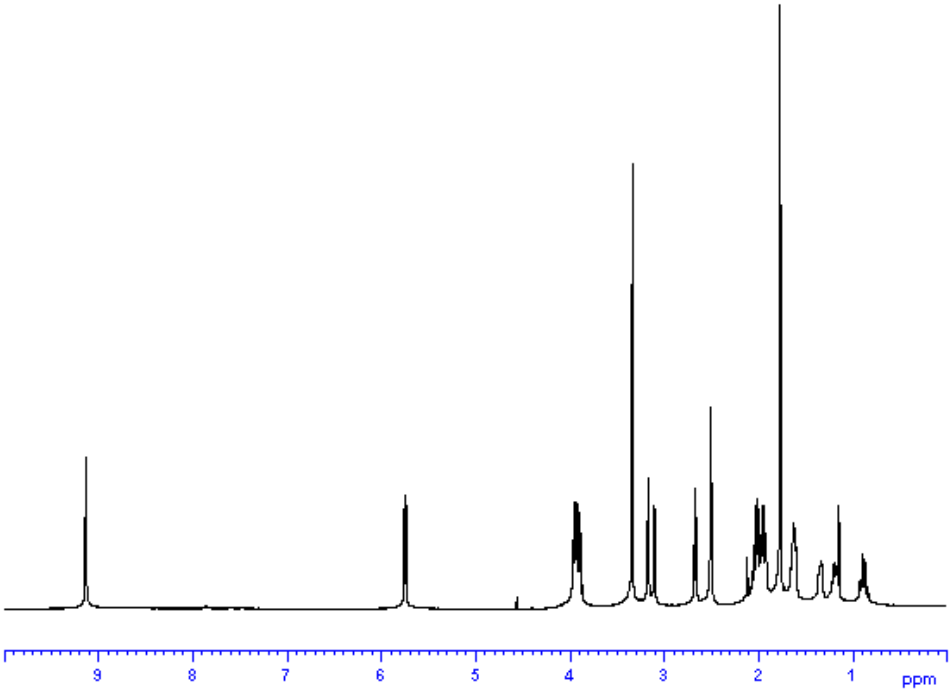
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2055 (4)



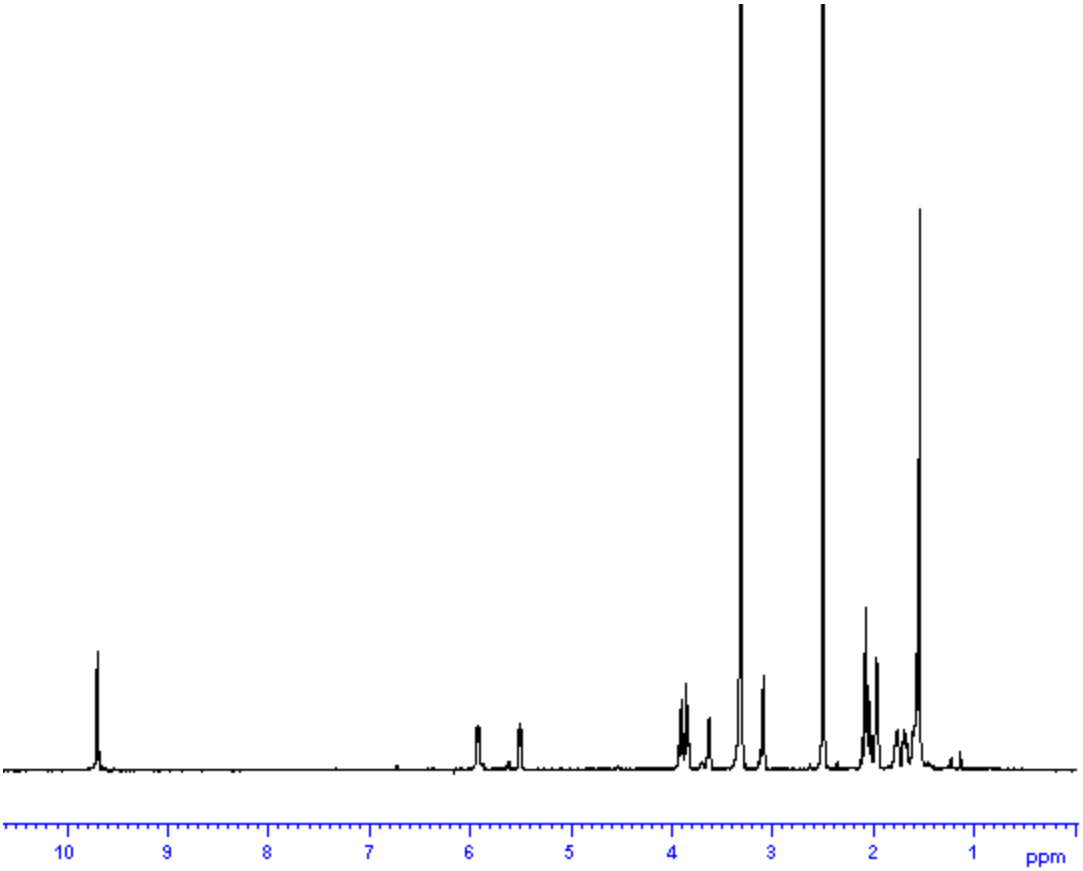
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2056 (14)



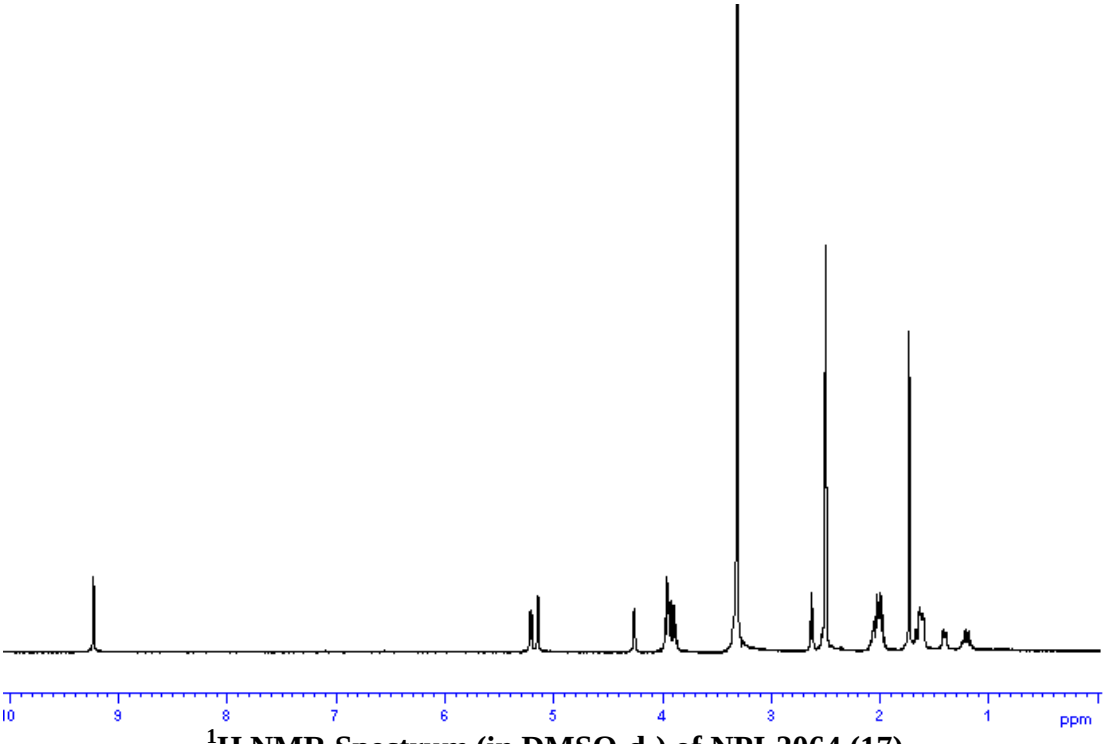
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2060 (15)



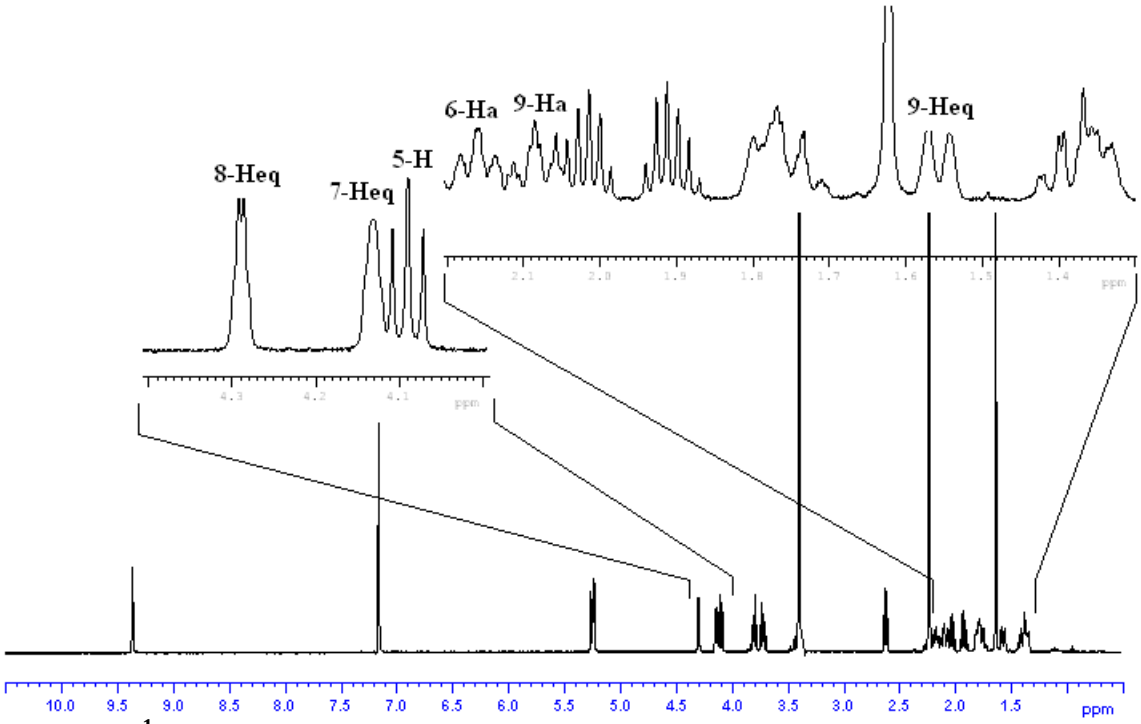
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2061 (16)



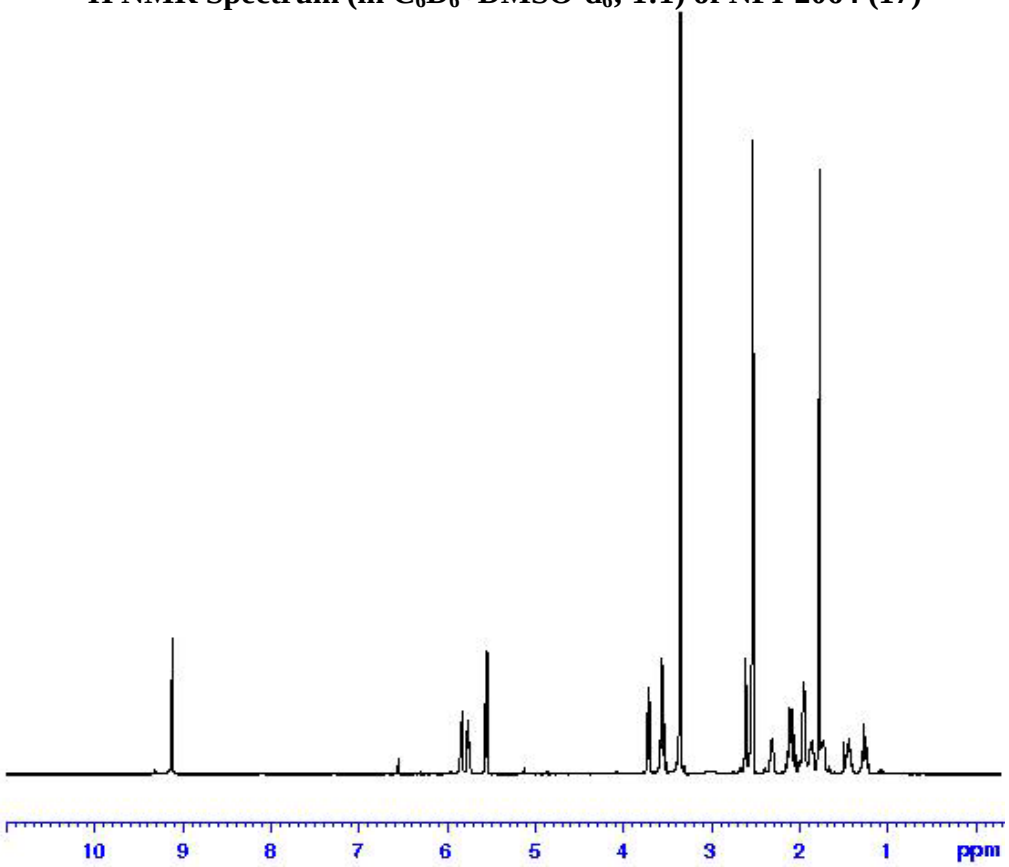
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2062 (18)



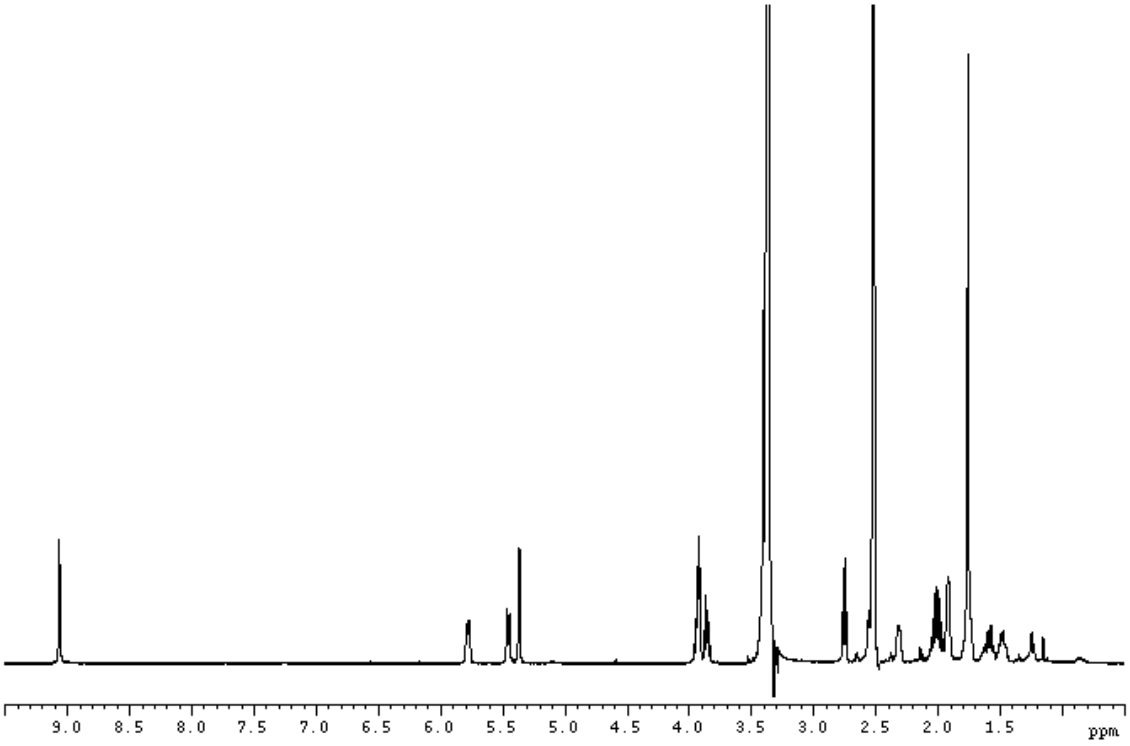
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2064 (17)



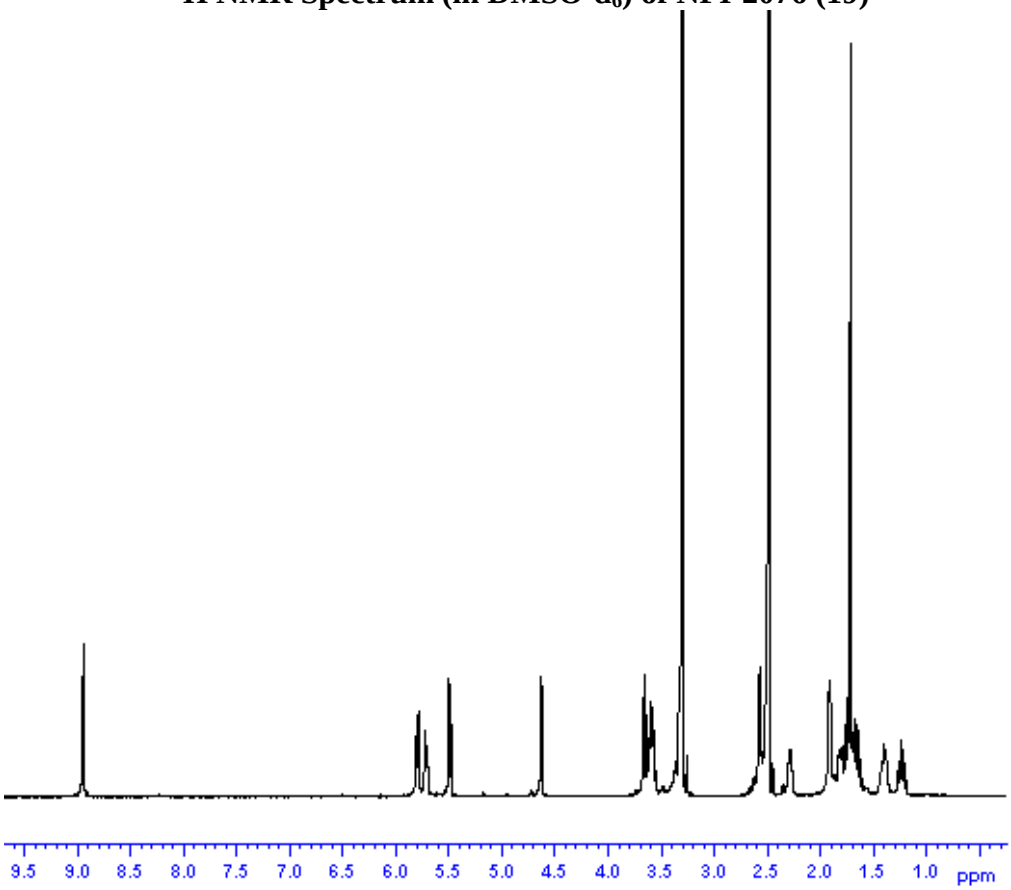
^{1}H NMR Spectrum (in C_6D_6+DMSO-d_6; 1:1) of NPI-2064 (17)



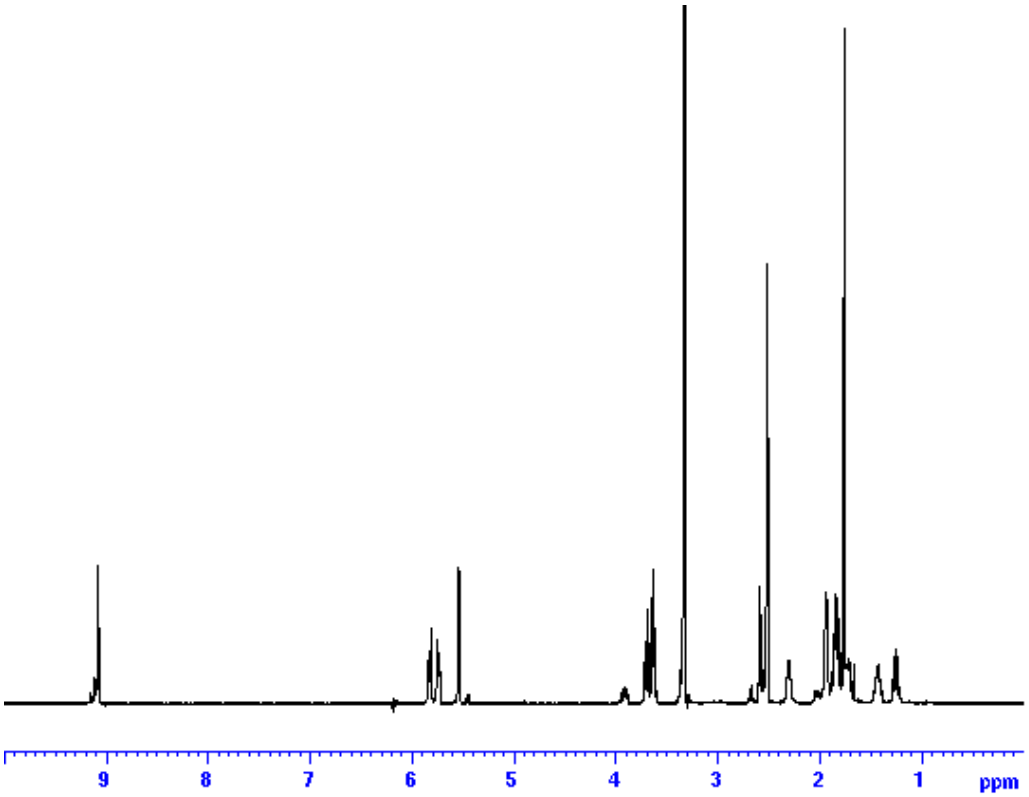
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2070 (10)



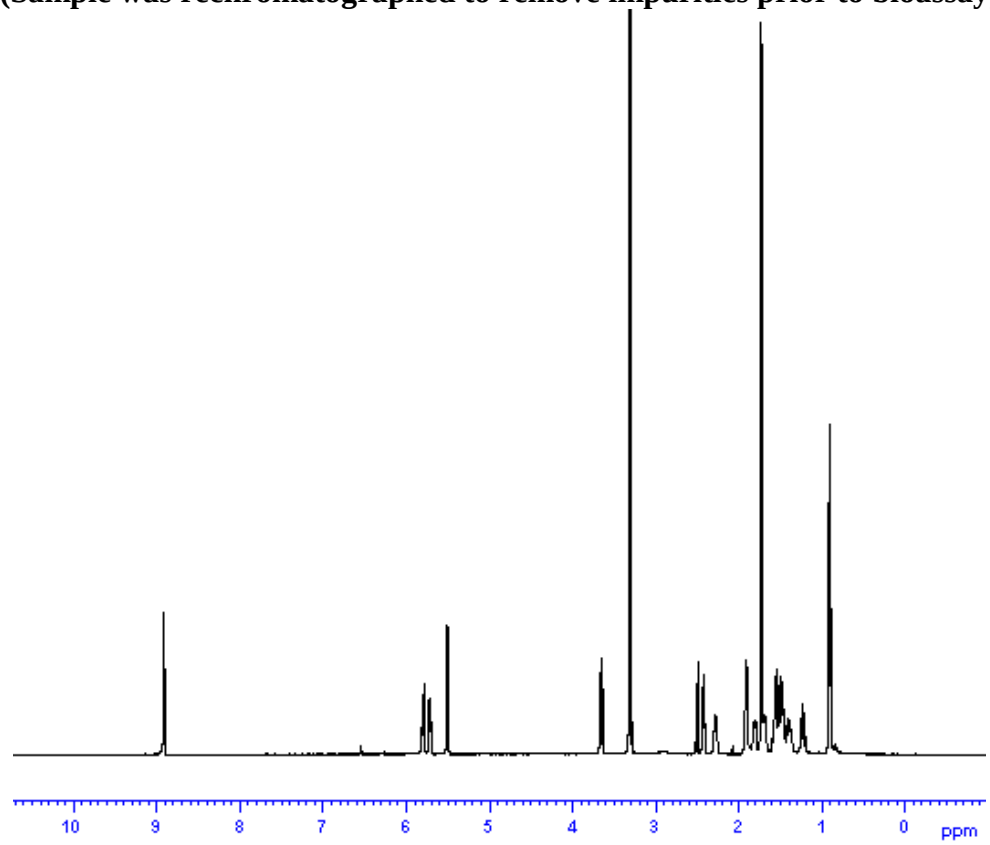
^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2076 (19)



^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2077 (12)



^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2078 (11)
(Sample was rechromatographed to remove impurities prior to bioassay)



^{1}H NMR Spectrum (in DMSO-d_6) of NPI-2080 (13)

Determination of Purity – HPLC Methods:

Purity was assessed using one or two of the following analytical HPLC methods (I-IV):

HPLC Method	**Method-I** (0052TF14)	**Method-II** (0052TFA2)	**Method-III** (0052TFA3)	**Method-IV** (CRD4_POS)
Mobile Phase	A2: Water w/ 0.01% TFA B2: CH_3CN w/ 0.01% TFA	A2: Water w/ 0.01% TFA B2: CH_3CN w/ 0.01% TFA	A2: Water w/ 0.01% TFA B2: CH_3CN w/ 0.01% TFA	A1: Water B1: CH_3CN
Gradient	0% B2 for 1 min; 0>35% B2 from 1-8 min., hold for 11 min.; 35>100% B2 from 19-27 min., hold for 9 min.; 100>0% B2 for 1 min	0% B2 for 1 min; 0>100% B2 from 1-16 min., hold for 9 min.; 100>0% B2 for 1 min; 4 min equilibration	0% B for 1 min; 0>35% B from 1-8 min., hold for 11 min.; 35>100% B from 19-27 min., hold for 9 min.; 100>0% B2 for 1 min	15% B for 1 min; 15>100% B from 1-16 min., hold for 9 min.; 100>15% B for 1 min; 4 min equilibration
Run Time	41 minutes	30 minutes	41 minutes	30 minutes
Flow Rate	1.5 mL/min	1.0 mL/min	1.0 mL/min	1.0 mL/min
Column Temp	35ºC	30ºC	30ºC	30ºC

Purity of NPI-0052 and its analogs by HPLC

Str #	**ID**	**Method-I (0052TF14) % Purity**		**Method-II (0052TFA2) % Purity**		**Method-III (0052TFA3) % Purity**		**Method-IV (CRD4_POS) % Purity**	
		UV 210 nm	**ELSD**	**UV 210 nm**	**ELSD**	**UV 210 nm**	**ELSD**	**UV 210 nm**	**ELSD**
1	NPI-0052	99	100	100	100				
6	NPI-0047	97	100	96	99				
7	NPI-2053	96	100	99	100				
4	NPI-2055	96	91	98	93				
14	NPI-2056	96	99	97	100				
9	NPI-2059			94	100	97	100		
15	NPI-2060			95	98			97	96
16	NPI-2061	94	100	97	99				
18	NPI-2062			95*	96*				
5	NPI-2063	99	100	97	99				
17	NPI-2064	97	100	95	99				
8	NPI-2065			95	100	99	100		
10	NPI-2070	98	100	98	100				
19	NPI-2076			97	100			96	100
12	NPI-2077			91	100				
11	NPI-2078	95	100	98	100				
13	NPI-2080			91	100				

***** Sum of two rotamers

References

Mitchell, S.S.; Nicholson, B.; Teisan. S.; Lam, K.S.; Potts, B.C.M. Aureoverticillactam, A Novel 22-Atom Macrocyclic Lactam from the Marine Actinomycete *Streptomyces aureoverticillatus*. *J. Nat. Prod.* **2004**, *67***,** 1400-1402.

Stein, R.L.; Melandri, F.; Dick, L. Kinetic Characterization of the Chymotryptic Activity of the 20S Proteasome. *Biochemistry* **1996**, *35***,** 3899-3908.