Discovery and Canine Preclinical Assessment of a Non-Toxic Procaspase-3-Activating Compound

Supplementary Data

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Materials and Methods

*Formulation of **PAC-1** and **S-PAC-1**.* To expedite the translational investigation of procaspase-3 activators for treating human cancer patients, the tolerability of **PAC-1** when administered intravenously (the most common drug delivery route for conventional anticancer agents) was assessed. To this end, a formulation procedure that reliably maintained high concentrations of **PAC-1** (a lipophilic drug) in aqueous solution was first optimized. The solubilizing agent allowing for the dissolution and maintenance of **PAC-1** in aqueous solution was 2-hydroxypropyl-β-cyclodextrin (HPβCD) (3). **PAC-1** is soluble at 20 mg/mL in an aqueous 200 mg/mL solution of HPβCD and is stable for at least 5 days when stored at 4 °C.

For all *in vivo* experiments, **PAC-1** and **S-PAC-1** were freshly reconstituted to a stock concentration of 15 mg/mL. Briefly, 200 mg/mL of solublizing agent (2-hydroxypropyl-β-cyclodextrin) was dissolved in 70% of the final volume of sterile water for injection. An appropriate mass of **PAC-1** or **S-PAC-1** was added to this solution and the pH adjusted to 1.5-2 at which **PAC-1** is fully soluble. After complete dissolution of **PAC-1** in the 2-hydroxypropyl-β-cyclodextrin solution, the pH is restored to 5-6. This solution is then sterile filtered and stored at 4 °C until use. The stability of this stock solution was followed by LC-MS over 5 days and exhibited no degradation products during this time.

For i.p. and IV injections of these drugs in mice, the drug was delivered directly from this stock concentration or was diluted in sterile water for injection to achieve lower doses. For administration to dogs in a continuous infusion, the drug stock was diluted 1:1 in a saline solution.

In Silico LogBB Predictions. LogBB predictions were carried out according to previously published methods (4). Briefly, equation S1 was used to determine the LogBB. ClogP was determined using ChemDraw Ultra 12. Polar surface area (PSA) was determined using the Daylight web-based software available at www.daylight.com/meetings/emug00/Ertl/tpsa.html.

$$\text{LogBB} = -0.0148\ (\text{PSA}) + 0.152\ (\text{ClogP}) + 0.139 \qquad \text{(S1)}$$

Inclusion criteria for client owned dogs enrolled in clinical trial. The inclusion criteria for eligible patients were the following: histologically or cytologically confirmed multicentric lymphoma, measurable tumor burden, favorable performance status, a life expectancy of >4 weeks, no previous chemotherapy within 3 weeks of study entry, and no significant co-morbid illness including renal or hepatic failure, history of congestive heart failure, or clinical coagulopathy. In addition, pet owners signed a written informed consent form prior to study entry according to university guidelines.

*Pharmacokinetics of **PAC-1** and **S-PAC-1** in mice.* C57/BL6 mice were treated with doses of **PAC-1** and **S-PAC-1** via tail vein injection or by i.p. injection. At specified time points, mice were sacrificed and blood was collected, centrifuged, and the serum was frozen at -80°C until analysis.

*Assessment of Serum concentrations of **PAC-1** and **S-PAC-1**.* Serum concentrations of **PAC-1** and **S-PAC-1** were determined by HPLC. The proteins in a 50 μL aliquot of serum are precipitated by the addition of 100 μL of acetonitrile and the sample is mixed by vortex and centrifuged to remove the proteins. The resulting supernatant is evaporated to dryness with a SpeedVac. The solid is then reconstituted in a mobile phase of 55% methanol with 1% acetic acid. The sample was then run on an Agilent 1100 HPLC with DAD and a Phenomenex Synergi 4u Polar-RP reverse phase column. Separation was achieved with a 15 minute isocratic separation at a flow rate of 0.8 ml/min and analytes were detected by absorbance at 285 nm. The resulting peaks from **PAC-1** or **S-PAC-1** were analyzed and quantitated by comparison to a calibration curve.

Evaluation of basal procaspase-3 levels in dogs with lymphoma. For *ex vivo* procaspase-3 measurement in spontaneously arising lymphoma cells, peripheral lymph nodes of canine lymphoma patients were aspirated using a 20 gauge needle. The cells were immediately flushed into a microcentrifuge tube containing 1 mL cold PBS and equally separated into 2 microcentrifuge tubes. One sample aliquot was

incubated with rabbit IgG antibody to serve as an isotype control, and the remaining sample aliquot was used for measurement of procaspase-3 concentration within the cells. All cells were then washed in 2 mL cold PBS. After centrifugation and aspiration, a cell pellet could be detected at the base of each microcentrifuge tube, and 2.5 mL lysis buffer was added to lyse existing red blood cells. Following centrifugation and removal of supernatant, cells were resuspended in 1 mL of fixation/permeabilization working solution (eBioscience, San Diego, CA) and allowed to incubate for 2 hours at 4°C. After permeabilization was complete, the cells were washed twice with permeabilization buffer. The cells were lightly vortexed and incubated with either 1:25 concentration of rabbit anti-mouse procaspase-3 antibody (Epitomics, Burlingame, CA) or rabbit IgG antibody (Santa Cruz Biotechnology, Santa Cruz, CA) as an isotype control. Cells were incubated at 4^{o}C in the dark for 30 minutes, and then washed again in 2 mL of permeabilization buffer. After centrifugation and aspiration, the cells were exposed to 1:10 dilution of secondary sheep anti-rabbit IgG:RPE antibody (AbD Serotec, Raleigh, NC) for 30 minutes at 4^{o}C, then analyzed by flow cytometry.

*Quantification of cleaved caspase-3 in response to **S-PAC-1** treatment.* For assessing the mechanistic activity of **S-PAC-1**, peripheral lymph nodes of canine lymphoma patients were aspirated using a 20 gauge needle before and after **S-PAC-1** constant rate infusion. Upon collection, malignant lymphoma cells were immediately flushed into a microcentrifuge tube containing 1 mL cold PBS. After centrifugation and aspiration, excess red blood cells were removed by the addition of red blood cell lysis buffer. Following centrifugation and removal of supernatant, cells were resuspended in 1 mL of fixation/permeabilization working solution (eBioscience, San Diego, CA) and allowed to incubate for 30 minutes at 4°C. After permeabilization was complete, the cells were washed twice with permeabilization buffer. The cells were lightly vortexed and incubated with rabbit anti-human cleaved caspase-3, Alexa Fluor 488 conjugated antibody (Cell Signaling Technology, Danvers, MA) for 30 minutes in the dark, then analyzed by flow cytometry.

Supporting Figures

Figure S1

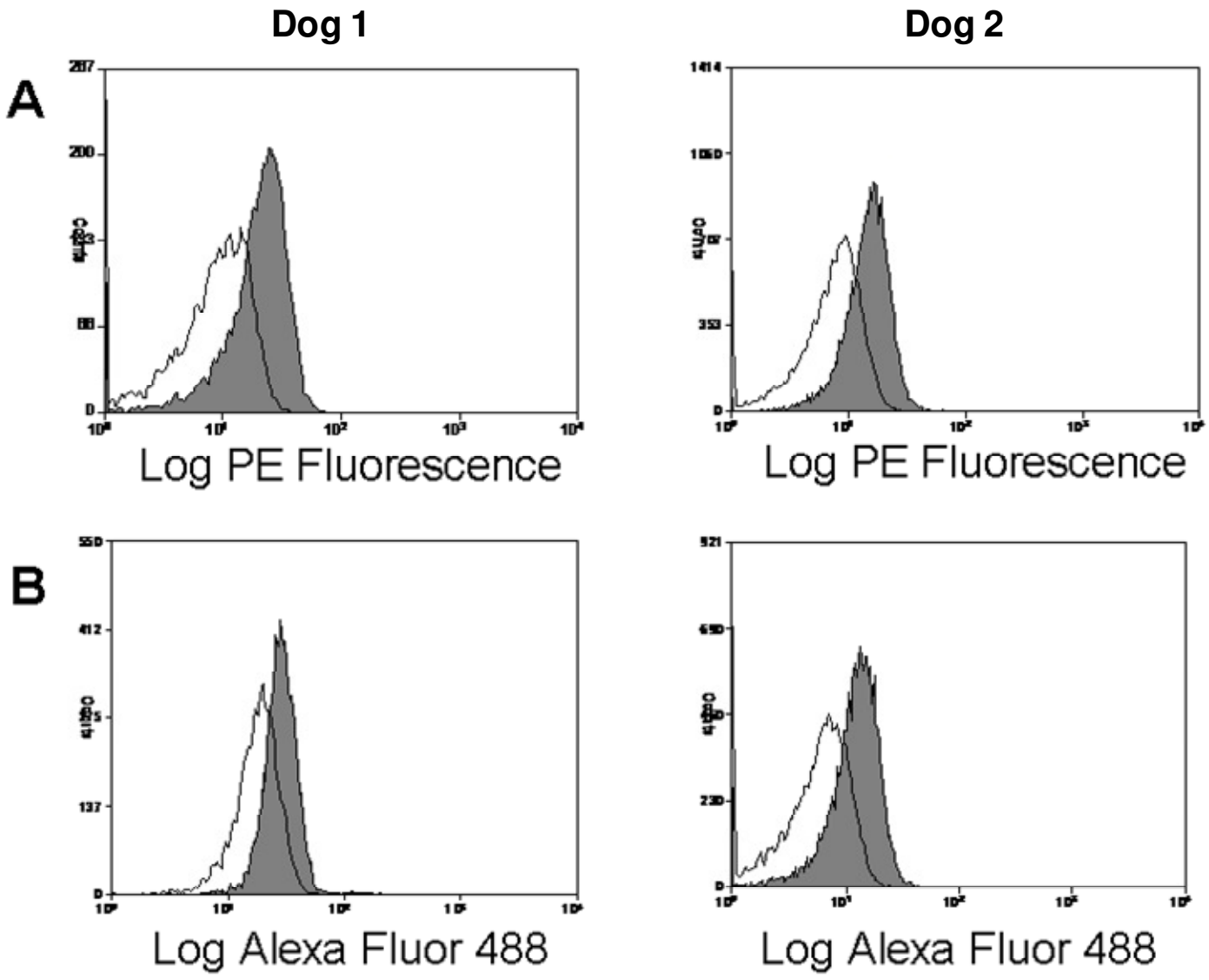


Figure S1. Target validation and modulation in dogs 1 and 2. A, The presence of procaspase-3 in lymph node aspirates from dogs 1 and 2 prior to drug administration was validated by staining with an anti-procaspase-3 antibody. Samples were analyzed by flow cytometry and procaspase-3 levels (gray shaded area) were compared to control samples that were stained with an isotype control (open area). B, Before treatment (open

area) and seven days after administration of **S-PAC-1** (shaded area), lymph node aspirates were collected and analyzed by flow cytometry using an antibody for cleaved caspase-3.

Supporting Tables

Dose (mg/kg)	Toxicity	
	PAC-1	**S-PAC-1**
10	-	ND
12.5	ND	-
20	+	ND
25	ND	-
30	++	ND
37.5	ND	-
50	+++	ND
350	ND	-

(-) no observable toxicity
(+) mild neurological symptoms
(++) overt neurotoxicity
(+++) severe neurotoxicity
ND, Not Determined
n=3 for each condition

Table S1. Observed neurotoxicity of **PAC-1** and **S-PAC-1** in C57/BL6 mice.

Table S2

A)

Parameter	Reference Range	Day 0	Day 7	Day 14	Day 21
WBC	6-17.0 x 10^3	8.5 ± 1.0	8.6 ± 1.8	8.9 ± 1.2	9.6 ± 1.9
Neutrophil	3-11.5 x 10^3	4.7 ± 1.1	5.7 ± 0.3	5.5 ± 0.7	5.7 ± 1.5
Platelet	2-9 x 10^5	2.0 ± 0.5	2.0 ± 0.7	2.2 ± 0.5	2.2 ± 0.8
Creatinine	0.5-1.6 mg/dl	0.9 ± 0.1	0.9 ± 0.0	0.8 ± 0.1	1.0 ± 0.2
ALT	17-87 U/L	34.7 ± 1.5	32.3 ± 1.2	35.7 ± 2.1	40.3 ± 6.5
BUN	7.0-31.0 mg/dl	14.8 ± 2.1	17.0 ± 0.4	14.4 ± 6.1	16.4 ± 3.8
Sodium	141-161 mEq/L	148.0 ± 3.0	147.3 ± 4.5	147.7 ± 2.1	149.3 ± 1.5
Potassium	3.9-5.7 mEq/L	4.9 ± 0.3	4.7 ± 0.3	5.1 ± 0.2	5.0 ± 0.3
Chloride	104-125 mEq/L	115.3 ± 2.3	112.7 ± 2.1	113.0 ± 1.7	112.3 ± 2.3
Calcium	7.9-11.5 mg/dl	10.4 ± 0.1	10.7 ± 0.4	10.8 ± 0.4	10.9 ± 0.4
Phosphorous	2.4-6.5 mg/dl	4.0 ± 0.5	4.0 ± 0.8	4.1 ± 0.5	3.9 ± 0.3
ALP	12-110 U/L	26.0 ± 4.6	29.7 ± 5.5	28.7 ± 4.7	27.7 ± 4.2
Bilirubin	0.08-0.5 mg/dl	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

B)

Parameter	Reference Range	Day 0	Day 7	Day 14	Day 21
WBC	6-17.0 x 10^3	8.6 ± 0.9	9.1 ± 1.4	9.7 ± 1.0	9.7 ± 0.8
Neutrophil	3-11.5 x 10^3	5.4 ± 0.8	6.1 ± 0.3	6.5 ± 0.7	6.1 ± 0.6
Platelet	2-9 x 10^5	2.0 ± 0.7	2.3 ± 0.3	2.2 ± 0.6	2.0 ± 0.6
Creatinine	0.5-1.6 mg/dl	0.8 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.9 ± 0.1
ALT	17-87 U/L	31.3 ± 1.7	31.5 ± 3.0	31.5± 4.2	32.0 ± 3.7
BUN	7.0-31.0 mg/dl	15.0 ± 3.7	15.5 ± 1.8	17.1 ± 4.6	18.4 ± 3.9
Sodium	141-161 mEq/L	147.8 ± 0.5	149.5 ± 3.1	148.3 ± 2.1	149.3 ± 1.7
Potassium	3.9-5.7 mEq/L	4.6 ± 0.2	4.7 ± 0.3	4.7 ± 0.2	4.8 ± 0.3
Chloride	104-125 mEq/L	113.8 ± 3.5	111.5 ± 2.6	111.5 ± 1.7	112.8 ± 2.4
Calcium	7.9-11.5 mg/dl	10.4 ± 0.1	10.6 ± 0.3	10.7 ± 0.4	10.6 ± 0.3
Phosphorous	2.4-6.5 mg/dl	3.6 ± 0.2	4.1 ± 0.7	4.3 ± 0.3	4.3 ± 0.2
ALP	12-110 U/L	24.0 ± 3.6	31.5 ± 6.2	27.3 ± 3.3	25.3 ± 3.3
Bilirubin	0.08-0.5 mg/dl	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0

C)

Parameter	Reference Range	Day 0	Day 7	Day 14	Day 21	Day 28
WBC	6-17.0 x 10^3	8.8 ± 3.3	11.1 ± 4.2	9.7 ± 3.1	11.0 ± 3.9	10.1 ± 2.4
Neutrophil	3-11.5 x 10^3	6.7 ± 0.6	8.7 ± 0.8	7.2 ± 0.5	8.8 ± 1.1	7.6 ± 0.4
Platelet	2-9 x 10^5	2.6 ± 0.5	2.6 ± 0.8	2.7 ± 0.7	2.7 ± 1.0	2.4 ± 1.0
Creatinine	0.5-1.6 mg/dl	1.2 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	1.6 ± 0.9
ALT	17-87 U/L	50.7 ± 19.9	46.3 ± 11.0	41.0 ± 13.2	43.0 ± 15.5	53.0 ± 30.5
BUN	7.0-31.0 mg/dl	17.2 ± 2.6	14.0 ± 1.6	14.6 ± 1.1	15.5 ± 2.8	24.5 ± 16.7
Sodium	141-161 mEq/L	148.7 ± 2.1	145.3 ± 2.1	147.0 ± 1.7	149.3 ± 1.2	149.7 ± 1.5
Potassium	3.9-5.7 mEq/L	4.4 ± 0.3	4.3 ± 0.2	4.4 ± 0.3	4.4 ± 0.4	4.0 ± 0.6
Chloride	104-125 mEq/L	117.0 ± 1.0	111.3 ± 3.1	113.3 ± 1.5	118.0 ± 1.7	113.7 ± 4.9
Calcium	7.9-11.5 mg/dl	11.3 ± 1.9	11.7 ± 2.1	11.4 ± 2.5	11.0 ± 1.1	12.0 ± 2.7
Phosphorous	2.4-6.5 mg/dl	3.6 ± 1.0	3.5 ± 0.3	3.2 ± 0.3	3.2 ± 0.6	3.8 ± 0.8
ALP	12-110 U/L	26.7 ± 11.2	37.3 ± 18.4	45.7 ± 16.5	45.0 ± 18.5	49.0 ± 20.7
Bilirubin	0.08-0.5 mg/dl	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1

D)

Parameter	Range	Day 0	Day 14	Day 28
WBC	6-17.0 x 10^3	14.3 ± 8.1	9.6 ± 4.2	14.1 ± 11.2
Neutrophil	3-11.5 x 10^3	10.3 ± 5.6	6.6 ± 2.2	9.8 ± 7.9
Platelet	2-9 x 10^5	2.7 ± 0.6	2.9 ± 0.7	2.8 ± 0.7
Creatinine	0.5-1.6 mg/dl	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.3
ALT	17-87 U/L	63.3 ± 34.4	69.7 ± 45.5	58.7 ± 32.6
BUN	7.0-31.0 mg/dl	15.3 ± 4.9	14.1 ± 3.3	13.5 ± 1.7
Sodium	141-161 mEq/L	148.0 ± 4.0	147.7 ± 0.6	148.7 ± 1.5
Potassium	3.9-5.7 mEq/L	4.5 ± 0.2	4.3 ± 0.2	4.5 ± 0.1
Chloride	104-125 mEq/L	116.0 ± 2.6	115.7 ± 2.9	116.0 ± 2.0
Calcium	7.9-11.5 mg/dl	10.0 ± 0.6	9.8 ± 0.3	10.1 ± 0.4
Phosphorous	2.4-6.5 mg/dl	4.2 ± 0.1	3.6 ± 0.3	3.2 ± 0.9
ALP	12-110 U/L	72.7 ± 63.6	46.0 ± 54.6	68.7 ± 57.9
Bilirubin	0.08-0.5 mg/dl	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

WBC-white blood cell, ALT- alanine transferase, BUN- blood urea nitrogen, ALP- alkaline phosphatase

Table S2. Select hematologic and non-hematologic parameters of A, healthy research dogs treated with single dose (25 mg/kg) of **S-PAC-1** (n=4); B, Healthy research dogs treated with a continuous rate infusion (see manuscript text) of **S-PAC-1** for 24 hours (n=3); C, Dogs with lymphoma treated with **S-PAC-1** in a 24 hour continuous rate infusion every week for 4 consecutive treatments (n=3); and D, Dogs with lymphoma treated with **S-PAC-1** in a 72 hour constant rate infusion every other week for 2 consecutive treatments (n=3). Day zero represents the pre-treatment values. Treatments began on day zero after blood collection and proceeded as described above. For all values the error represents the SEM.

Chemical Information

Materials and Methods

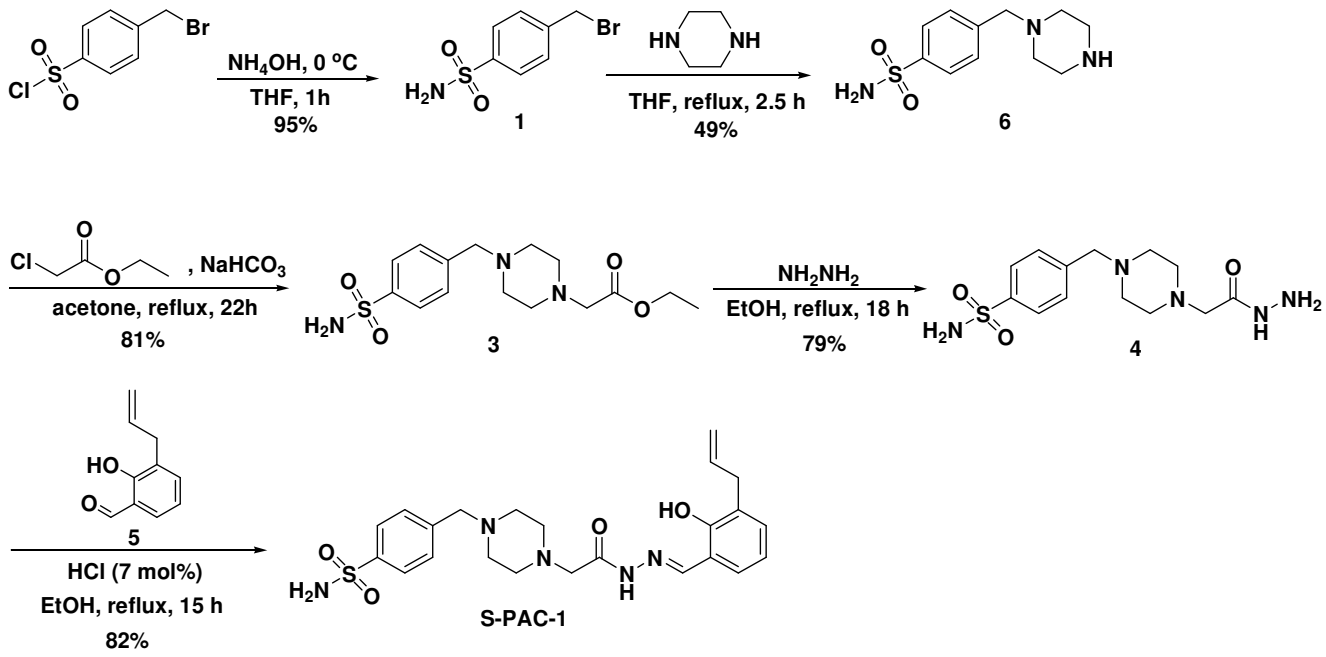
General—All reactions requiring anhydrous conditions were conducted under a positive atmosphere of nitrogen or argon in oven-dried glassware. Standard syringe techniques were used for anhydrous addition of liquids. Dry tetrahydrofuran was obtained by passing over activated alumina columns or molecular sieves in a commercial solvent purification system (Innovative Technologies). Unless otherwise noted, all starting materials, solvents, and reagents were acquired from commercial suppliers and used without further purification. Flash chromatography was performed using 230-400 mesh silica gel. Compound **1**, (9) **2,** (1) and **5** (10) were prepared according to the literature methods, in which **5** was synthesized with slight modification as shown below. **PAC-1** was synthesized as previously reported (1).

Compound Analysis—All NMR experiments were recorded either in D_2O (Sigma), CD_3OD (sigma) or Acetone-*d6* (Sigma) on a Varian Unity 400 MHz or 500 MHz spectrometer with residual undeuterated solvent as the internal reference. Chemical shift, δ (1, 11); coupling constants, *J* (Hz); multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet); and integration are reported. High-resolution mass spectral data was recorded on a Micromass Q-Tof Ultima hybrid quadrupole/time-of-flight ESI mass spectrometer at the University of Illinois Mass Spectrometry Laboratory. All melting points are uncorrected. LC-MS performed on a C18 column, 2.1x5 mm, mobile phase A is 0.1% TFA in H_2O, B is acetonitrile using a gradient system with constant 0% B over 0-2 min, then 0-50% B from 2-5 min, then 50-100% B over 5-7 min, constant 100% over 7-8 min, and from100-0% over 8-10.

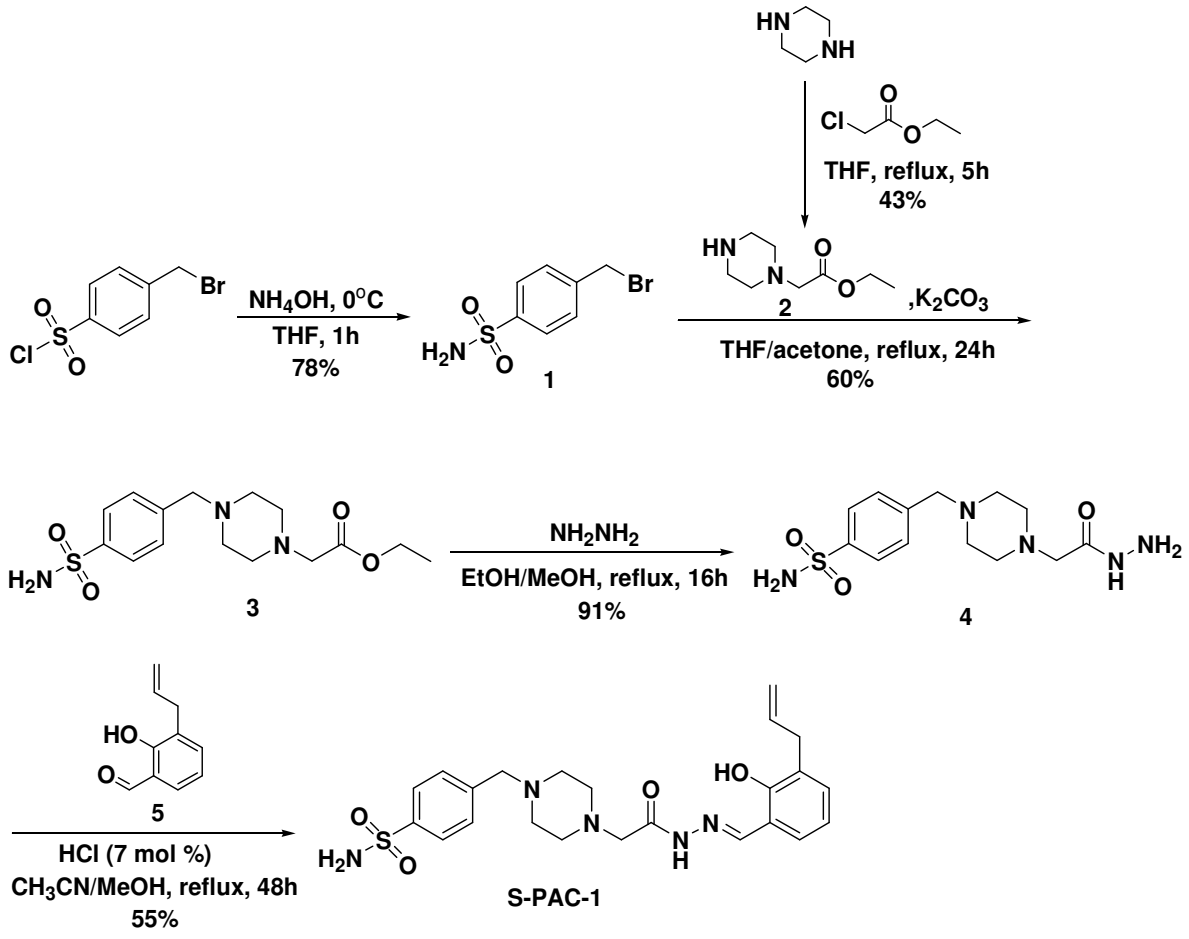
*Synthesis of **S-PAC-1***. The synthetic route to **S-PAC-1** is shown in scheme S1 and S2; this route is a modification of the synthetic route to **PAC-1** previously reported (1). Briefly, 4-(bromomethyl)benzenesulfonamide (**1**) is coupled with piperazino-ester **2** to provide intermediate **3** in good

yield. Compound **3** is then reacted with hydrazine to produce hydrazide **4,** which is condensed with aldehyde **5** to furnish **S-PAC-1**. Notably, the reaction sequence is highly scalable; indeed, the yields listed in Scheme S2 are for production of 40 grams of **S-PAC-1**. Critical to this scale-up was the development of facile purification protocols for each intermediate. Thus, compound **3** is purified via recrystallization from ethanol, hydrazide **4** is purified through recrystallization from methanol, and the final product is purified through silica gel chromatography and recrystallization from methanol. Over 160 g of **S-PAC-1** were successfully produced after conducting this synthesis four times on a 40 gram scale. Details of a milligram scale synthesis of **S-PAC-1** may also be found in the Scheme S1.

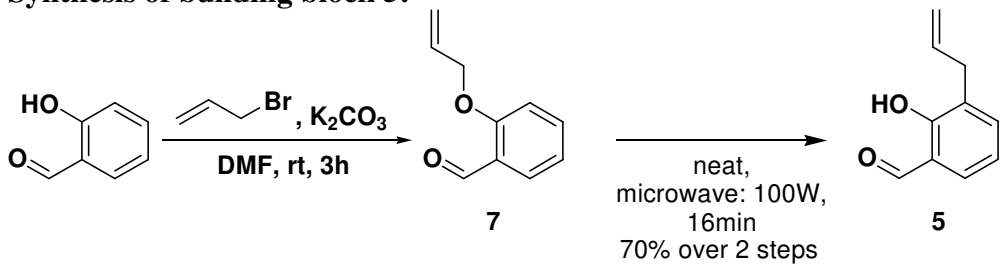
Scheme S1. Milligram-scale synthesis of **S-PAC-1.**



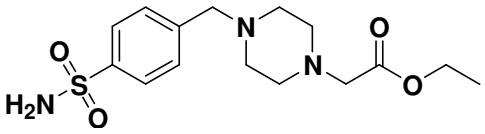
Scheme S2. Gram-scale synthesis of **S-PAC-1.**



Synthesis of building block 5:

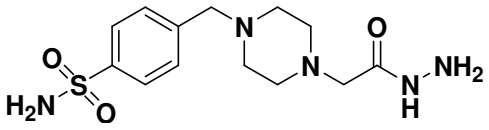


Ethyl 2-(4-(4-sulfamoylbenzyl)piperazin-1-yl)acetate (3)



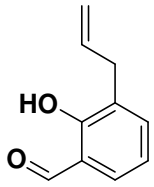
To a stirred mixture of **1** (108 g, 440.7 mmol, 1.4 equiv.) and K_2CO_3 (132.4 g, 958.2 mmol, 3 equiv.) in 3:2 THF/acetone (2192 mL in total) was added ethyl 2-(piperazin-1-yl)acetate, **2** (79.9 g, 319.4 mmol, 1 equiv.). The reaction was refluxed for 24 h monitoring by TLC. The solution was filtered and the solid was washed with acetone (80 mL). The filtrate was concentrated *in vacuo*, and then purified by recrystallization in ethanol to afforded **3** (57.4 g, 60%) as light yellow solid. On the milligram-scale, the crude product was purified by flash column chromatography on silica gel (1:4 MeOH/EtOAc). ^{1}H-NMR (500 MHz, CD_3OD): δ 7.86 (d, *J* = 8.0 Hz, 2H), 7.52 (d, *J* = 8.0Hz, 2H), 4.17 (q, *J* = 7.1Hz, 2H), 3.61 (s, 2H), 3.24 (s, 2H), 2.58 (broad d, *J* = 50.4Hz, 8H), 1.26 (t, J = 7.1Hz, 3H). ^{13}C NMR (126 MHz, CD_3OD): δ 170.4, 142.8, 142.4, 129.7, 126.0, 61.9, 60.6, 58.5, 52.5, 52.4, 13.3. HRMS (ESI): found: 342.1487 (M+1); calcd for $C_{15}H_{24}N_3O_4S$: 342.1488. m.p.: 153.0 – 154.5. IR (neat): 3319, 1739, 1160 cm^{-1}.

4-((4-(2-hydrazinyl-2-oxoethyl)piperazin-1-yl)methyl)benzenesulfonamide (4)



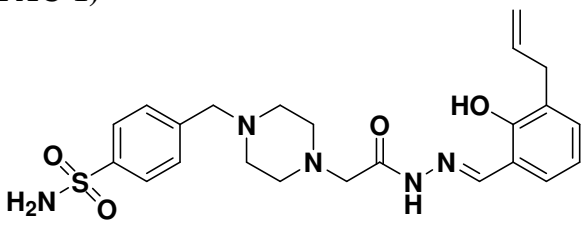
To a stirred solution of **3** (110.9 g, 324.8 mmol, 1 equiv.) in 2:1 ethanol/methanol (650 mL in total, 0.5 M) was added anhydrous hydrazine (30.6 mL, 974.5 mmol, 3 equiv.). The reaction was refluxed for 16 h monitoring by TLC (1:1 MeOH/EtOAc). The reaction mixture was concentrated *in vacuo*. The residue was dissolved in CH_2Cl_2 and washed with water (100 mL) and brine (100 mL). The aqueous layer was extracted with CH_2Cl_2 (3 x 80 mL) and EtOAc (80 mL). The combined organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo*. Purification with recrystallization in methanol gave **4** (96.3 g, 91%) as white solid. On the milligram-scale, the reaction was run in ethanol (0.06 M). ^{1}H NMR (500 MHz, CD_3OD): δ 7.85 (d, J = 8.2Hz, 2H), 7.51 (d, J = 8.2Hz, 2H), 3.61 (s, 2H), 3.04 (s, 2H), 2.54 (broad s, 8H). ^{13}C NMR (126 MHz, CD_3OD): δ 170.2, 142.8, 142.4, 129.7, 126.0, 61.9, 59.8, 53.0, 52.6. HRMS (ESI): found: 328.1436 (M+1); calcd for $C_{13}H_{22}N_5O_3S$: 328.1443. m.p.: 194.5 – 196.0. IR (neat): 3320, 1670, 1158 cm^{-1}.

3-Allyl-2-hydroxybenzaldehyde (5)



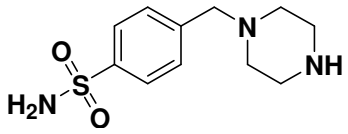
The synthesis of **5** was performed according to literature[5] with modification. To a stirred solution of salicylaldehyde (87.3 mL, 818.9 mmol, 1 equiv.) in DMF (150 mL), allyl bromide (85.1 mL, 982.6 mmol, 1.2 equiv.) and potassium carbonate (147.1 g, 1065 mmol, 1.3 equiv.) were added. The reaction mixture was stirred at room temperature for 3 hours. Water (700 mL) was added to the reaction mixture, and extracted with ethyl acetate (300 mL x 2). The organic fraction was washed with water (200 mL x 2), 0.1 M potassium hydroxide (200 mL x 2) and then brine (200 mL x 2), dried over $MgSO_4$ and filtered. Crude **7** was obtained after further drying under high vaccum, which was then microwaved at constant 100W for 16 min (max. temp. = 200 °C, CEM DISCOVER^{TM} system). Purification with flash column chromatography on silica gel (1:9 EtOAc/Hexanes) gave **5** (93.0 g, 70% over two steps) as light yellow oil.

(E)-4-((4-(2-(2-(3-allyl-2-hydroxybenzylidene)hydrazinyl)-2-oxoethyl)piperazin-1-yl)methyl)benzenesulfonamide (S-PAC-1)



To a stirred solution of **5** (28.8 g, 177.3 mmol, 1 equiv.) in 1:2 acetonitrile/methanol (1180 mL in total, 0.15 M), **4** (98.7 g. 301.4 mmol, 1.7 equiv.) and HCl (7 mol%) were added. The reaction was refluxed for 48 h monitoring by TLC. The solution was concentrated *in vacuo*. The crude product was purified by flash column chromatography on silica gel (1:4 MeOH/EtOAc), and followed by recrystallization in methanol to afford S-PAC-1 (45.9 g, 55%) as off-white solid. On the milligram-scale, the reaction was run in ethanol (0.02 M). ^{1}H NMR (500 MHz, $(CD_3)_2CO$): δ 11.86 (s, 1H), 10.79 (s, 1H), 8.51 (s, 1H), 7.85 (d, *J* = 8.2Hz, 2H), 7.53 (d, *J* = 8.2Hz, 2H), 7.19 (d, *J* = 7.6Hz, 2H), 6.86 (t, *J* = 7.6Hz, 1H), 6.56 (s, 2H), 6.02 (tdd, 1H, J=6.7Hz, J=10.1Hz, J=16.9Hz), 5.04 (m, 2H), 3.60 (s, 2H), 3.42 (d, *J* = 6.7Hz, 2H), 3.18 (s, 2H) 2.57 (broad d, *J* = 46.5Hz, 8H). ^{13}C NMR (126 MHz, $(CD_3)_2CO$): δ 165.7, 156.4, 150.0, 143.4, 143.2, 136.9, 131.8, 129.32, 129.30, 127.9, 126.2, 119.1, 117.8, 115.1, 62.0, 61.1, 53.7, 52.9, 33.8. HRMS (ESI): found: 472.2014 (M+1); calcd for $C_{23}H_{30}N_5O_4S$: 472.2019. m.p.: 108.5 – 111.0. IR (neat): 3227, 1684, 1606, 1157 cm^{-1}. Purity: >99.5% (LC-MS).

4-(piperazin-1-ylmethyl)benzenesulfonamide (**6**)



Anhydrous piperazine (860 mg, 10.0 mmol, 5 equiv.) was added to THF (10 mL), and the mixture was heated to reflux until the piperazine was fully dissolved. To the solution **1** (500 mg, 2.0 mmol, 1 equiv.) was added. The reaction mixture was refluxed for 2.5 h monitoring by TLC. The reaction mixture was neutralized with 1M KOH solution, and then concentrated *in vacuo*. Purification with flash column chromatography on silica gel (1:4 MeOH/EtOAc) afforded **6** (250 mg, 49%) as light yellow semi-solid. ^{1}H NMR (400 MHz, D_2O/$(CD_3)_2CO$): δ 7.84 (d, *J* = 8.4Hz, 2H), 7.51 (d, *J* = 8.4Hz, 2H), 3.69 (s, 2H), 3.42 (s, 2H), 3.22 (m, 4H), 2.74 (m, 4H), 1.87 (apparent s, 1H). ^{13}C NMR (126 MHz, $CDCl_3$): δ 140.93, 140.87, 131.0, 126.3, 61.1, 49.0, 43.13. HRMS (ESI): found: 256.1114 (M+1); calcd for $C_{11}H_{18}N_3O_2S$: 256.1120. IR (neat): 3142, 1158 cm^{-1}.

References

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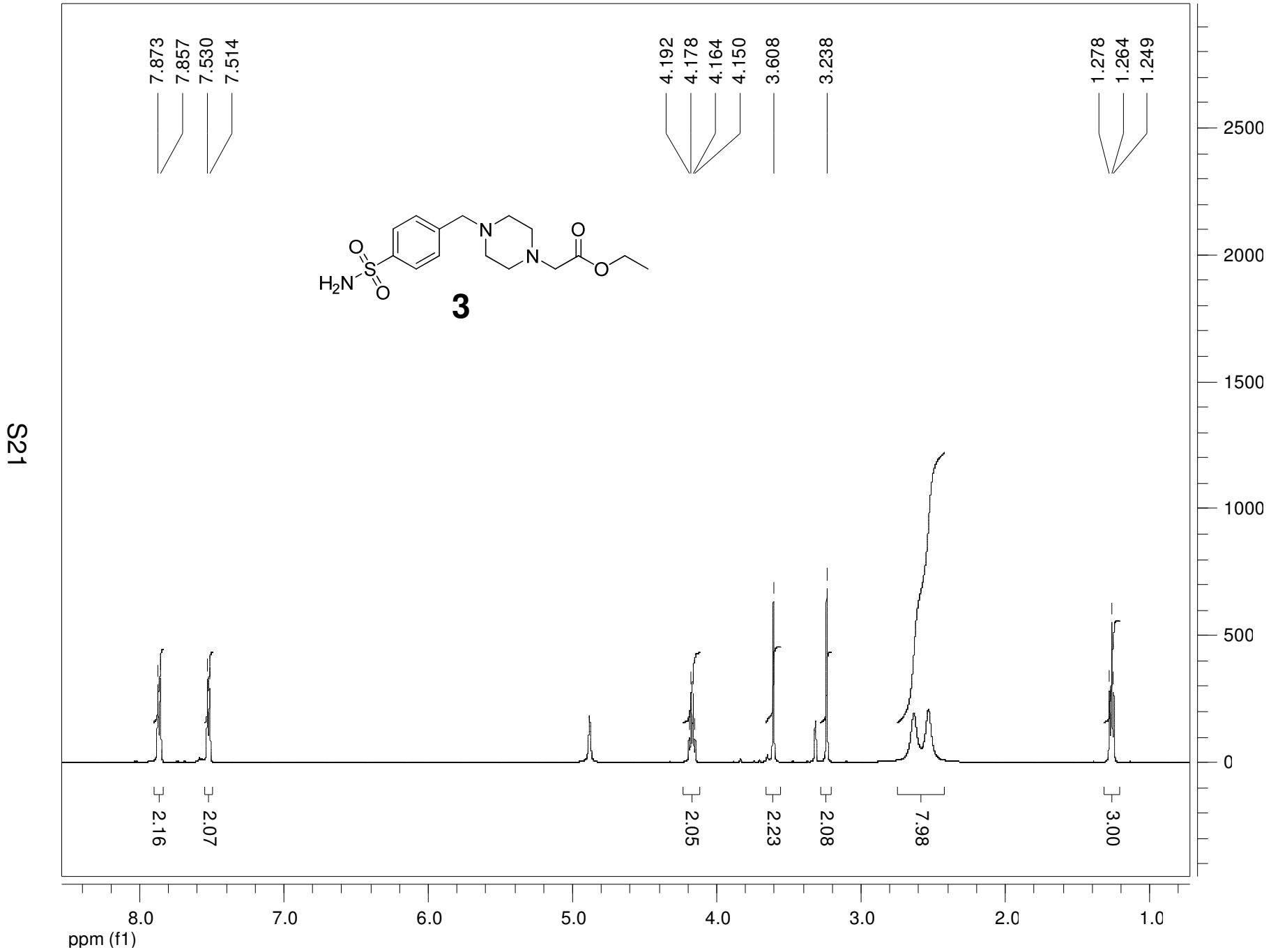
1H NMR, CD_3OD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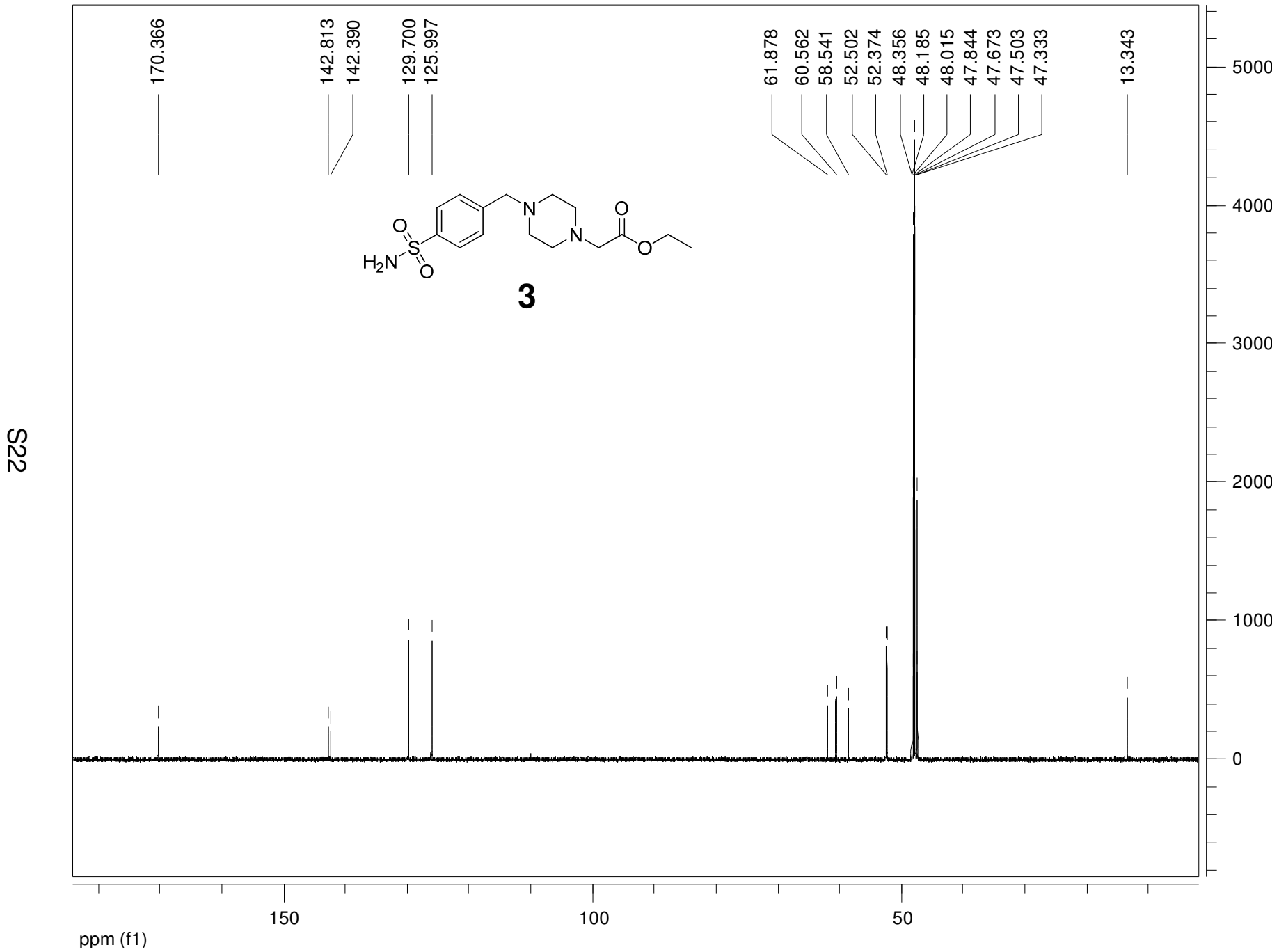
13C NMR, CD_3OD



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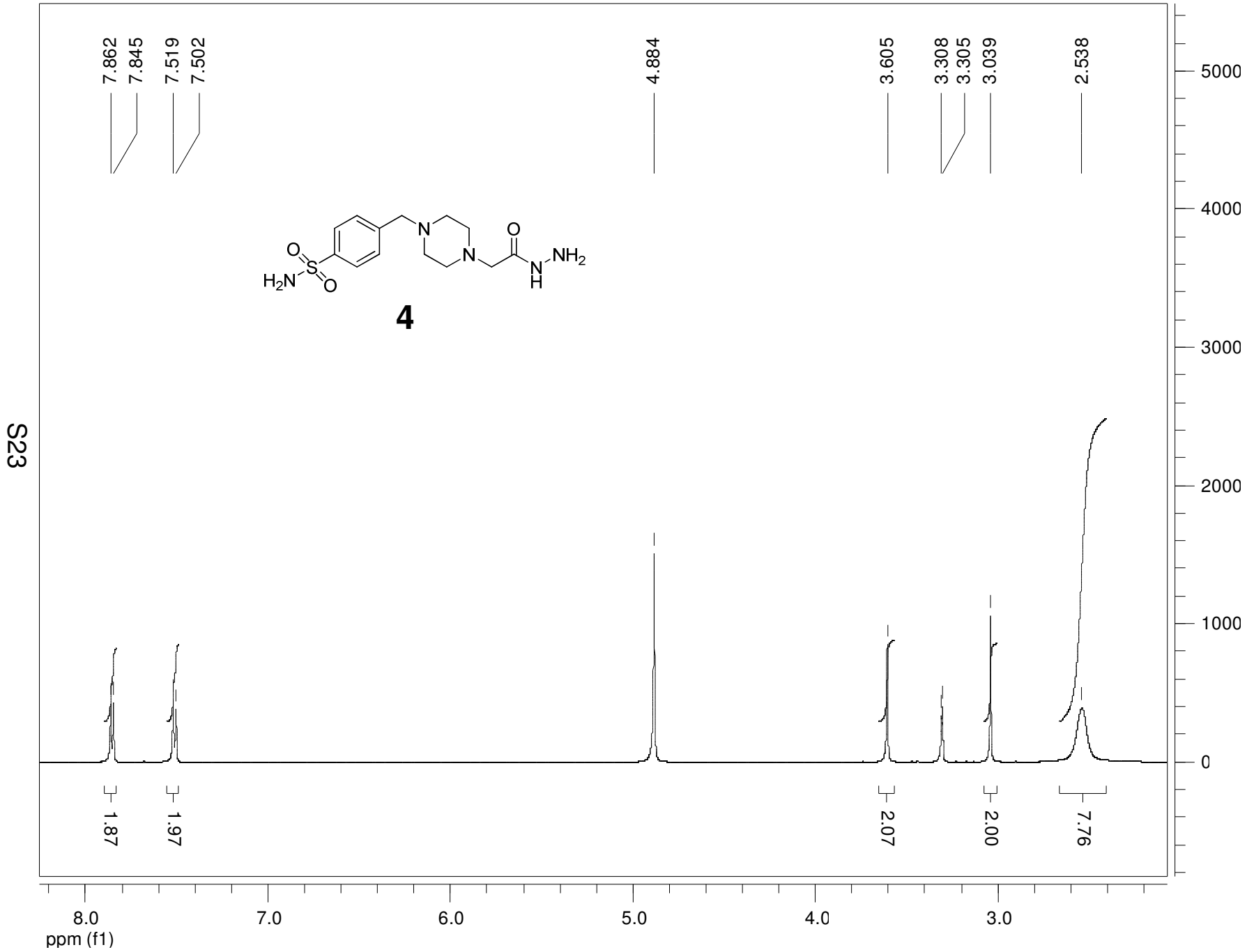

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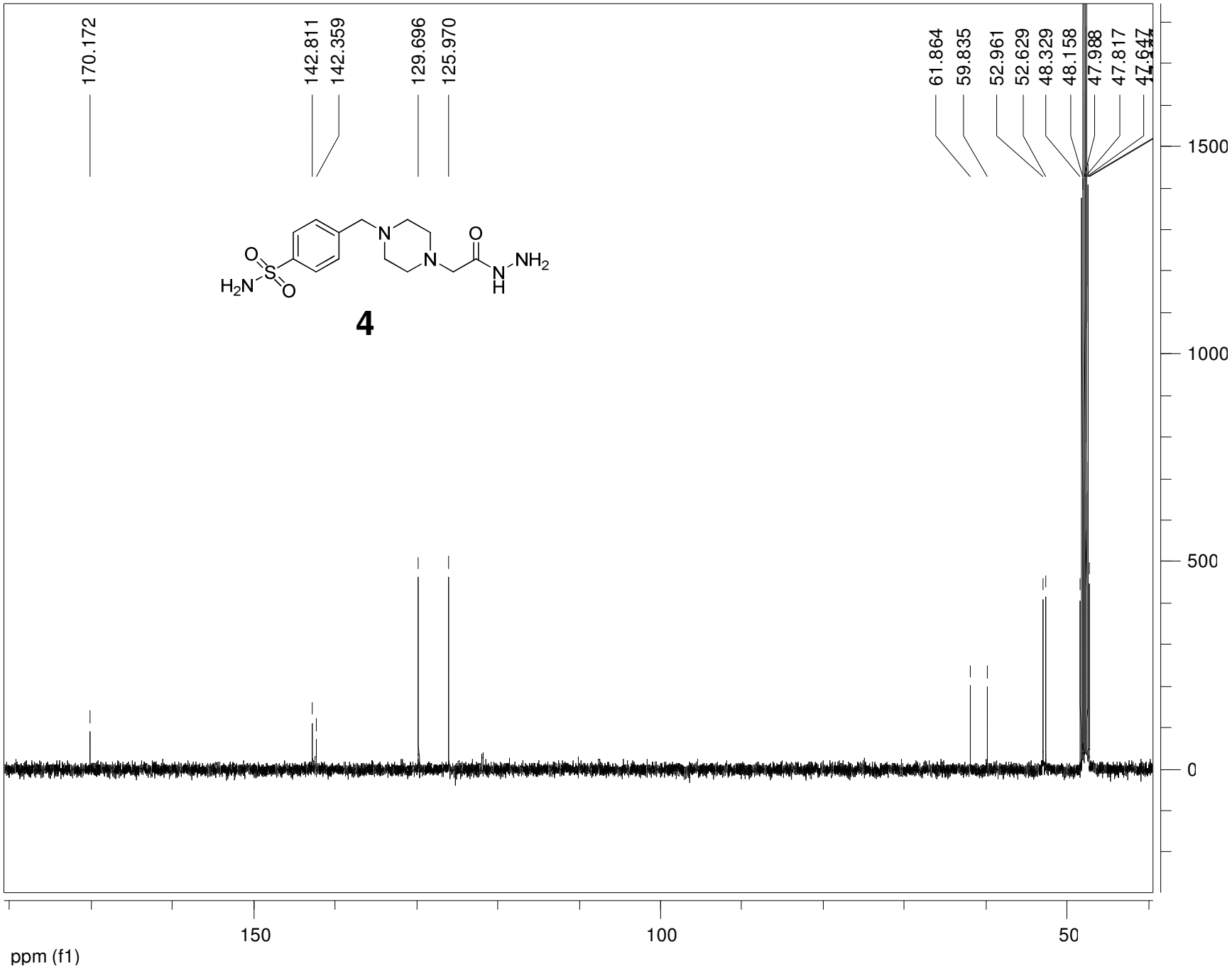
13C NMR, CD_3OD



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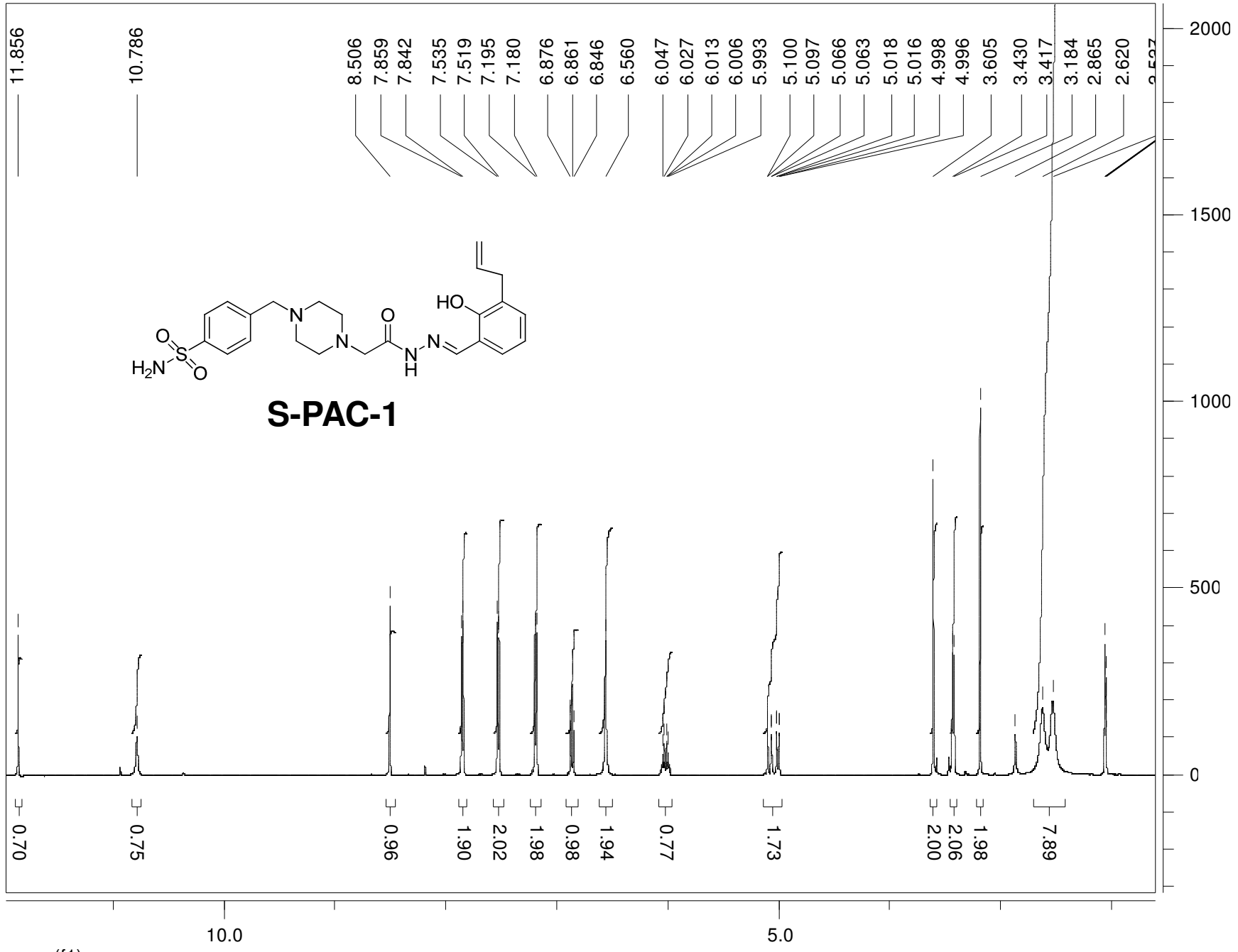
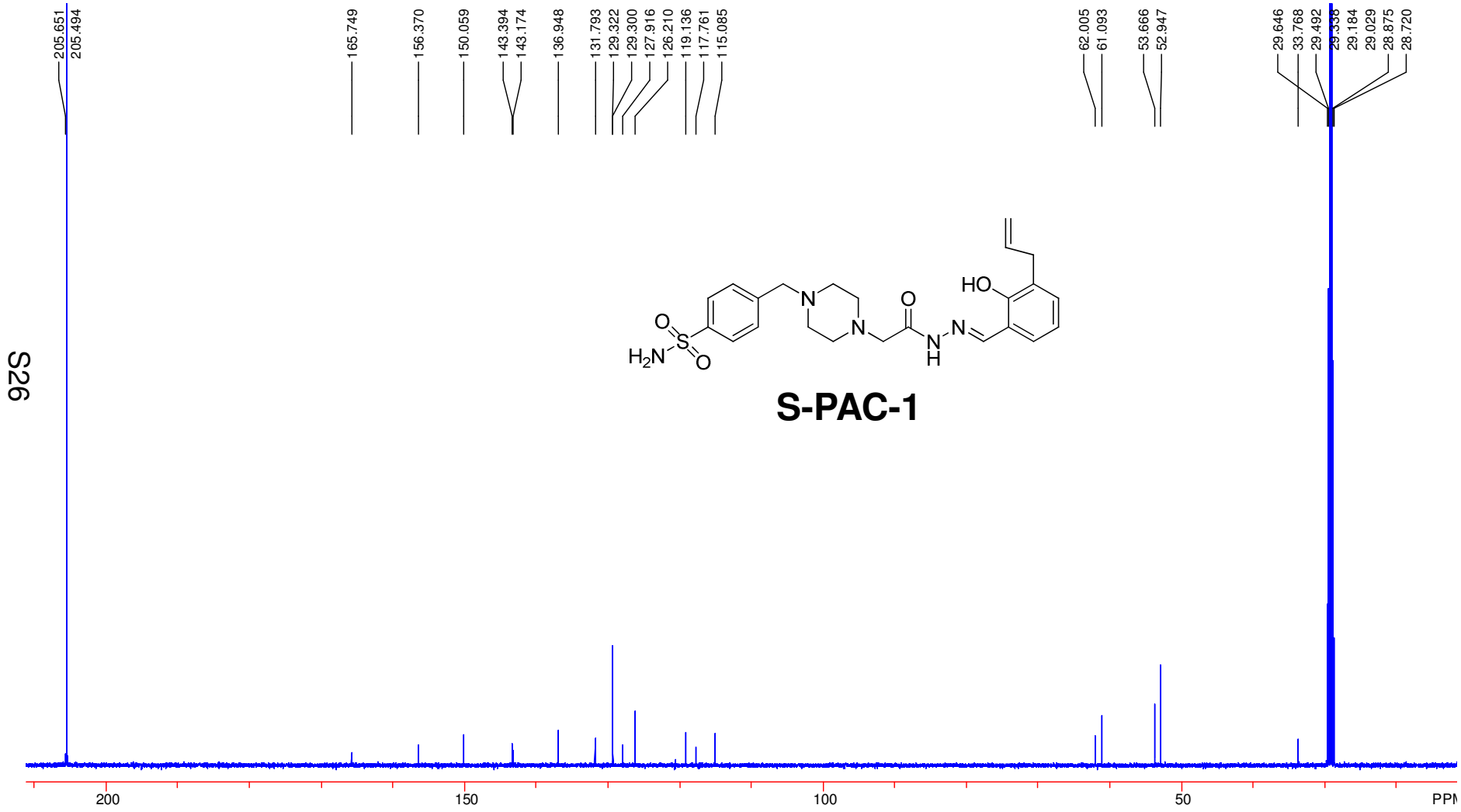
1H NMR, $(CD_3)_2CO$



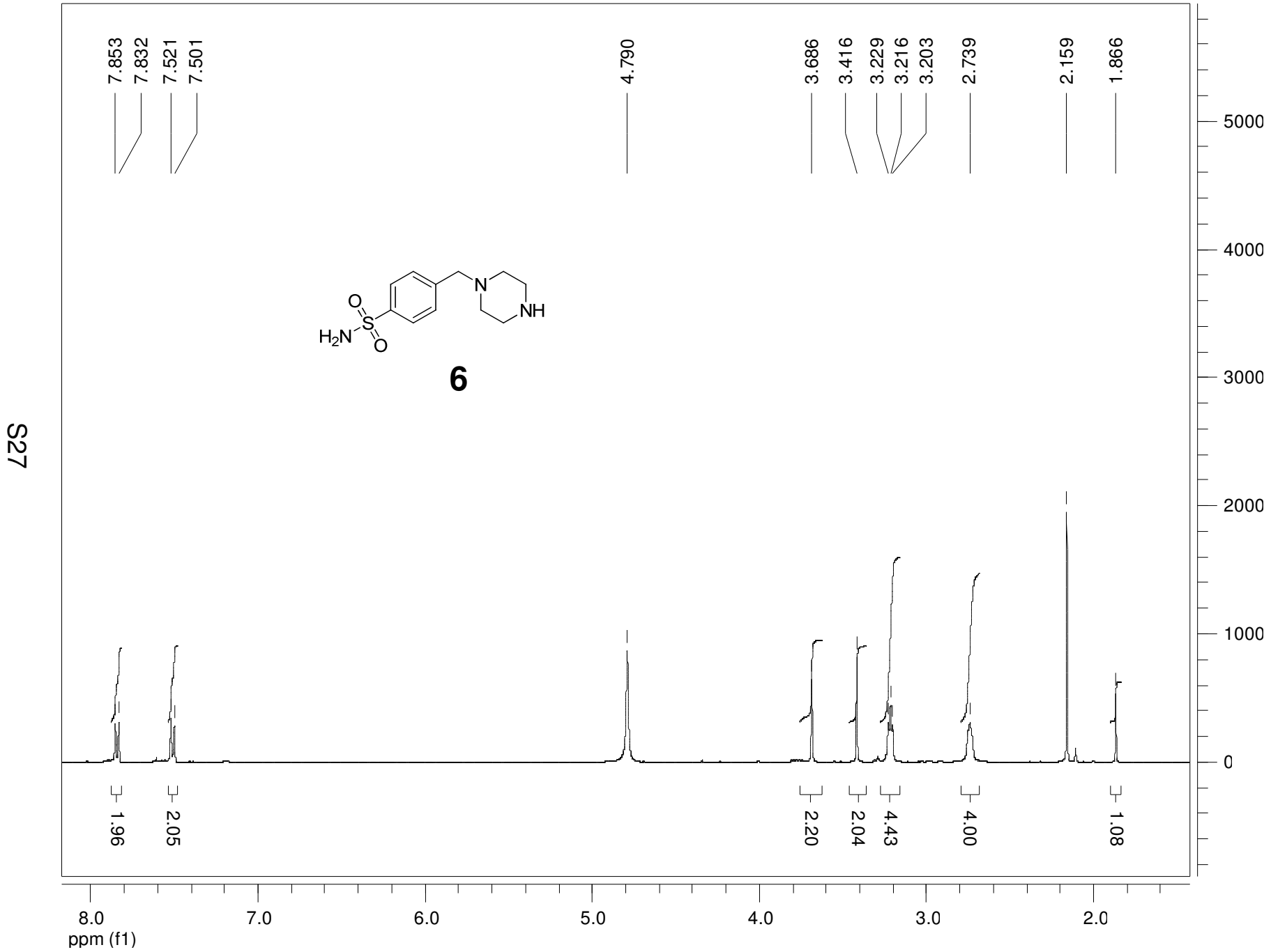
S-PAC-1

13C NMR, $(CD_3)_2CO$



S-PAC-1

1H NMR, $D_2O/(CD_3)_2CO$



13C NMR, $D_2O/(CD_3)_2CO$