Supplementary Information for:

N-Arylacyl *O*-sulfonated aminoglycosides as novel inhibitors of human neutrophil elastase, cathepsin G and proteinase 3

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List of Abbreviations:

ACN: acetonitrile

aq: aqueous

bz: benzoyl

CatG: cathepsin G

cbz: benzyloxycarbonyl

CE: capillary electrophoresis

$ClSO_3H$: chlorosulfonic acid

DCM: dichloromethane

DMF: *N, N*-dimethylformamide

DS: degree of sulfation

ESI: electrospray ionization

HNE: human neutrophil elastase

HPLC: high pressure/performance liquid chromatography

ITC: isothermal titration calorimetry

LC-MS: liquid chromatography mass spectrometry

LRMS: low resolution mass spectrometry

MeOH: methanol

MS: mass spectrometry

m/z: mass-to-charge ratio

mwco: molecular weight cut off

NHS: *N*-hydroxysuccinamide

NMR: nuclear magnetic resonance spectroscopy

NSP: neutrophil serine protease

pha: phenylacetyl

Pr3: proteinase 3

Pyr·SO_3: pyridine sulfur trioxide complex

RPIP: reversed-phase ion pairing

TEA: triethylamine

TFA: trifluoroacetic acid

TIC: total ion chromatogram

TLC: thin-layer chromatography

UV: ultaviolet

1. Quantification of concentration dependent inhibition of HNE, CatG and Pr3 mediated cell detachment.

To determine protease induced cell detachment, A549 cells were seeded at 2 x 10^4 cells per well in uncoated, tissue culture treated 96-well plates and grown for 18 hr in complete growth medium (RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 0.1% penicillin-streptomycin and 2mM GlutamaxTM). The growth medium was then removed and cells were cultured for 24 h in serum-free medium (RPMI-1640 medium supplemented with 0.1% penicillin-streptomycin). *N*-arylacyl *O*-sulfonated aminoglycosides at three concentrations (250, 2.5 and 0.025 μM) were incubated on ice for 30 min with enzyme (50 nM HNE, 250 nM CatG or 50 nM Pr3) before addition to cells. Cells were then incubated with compound and enzyme for 24 hr, after which supernatant was removed and cells stained with Hoescht 33342 nuclear dye. Cells in each well were imaged and counted using Operetta High Content Imaging System and Harmony Analysis Software (PerkinElmer Inc.).

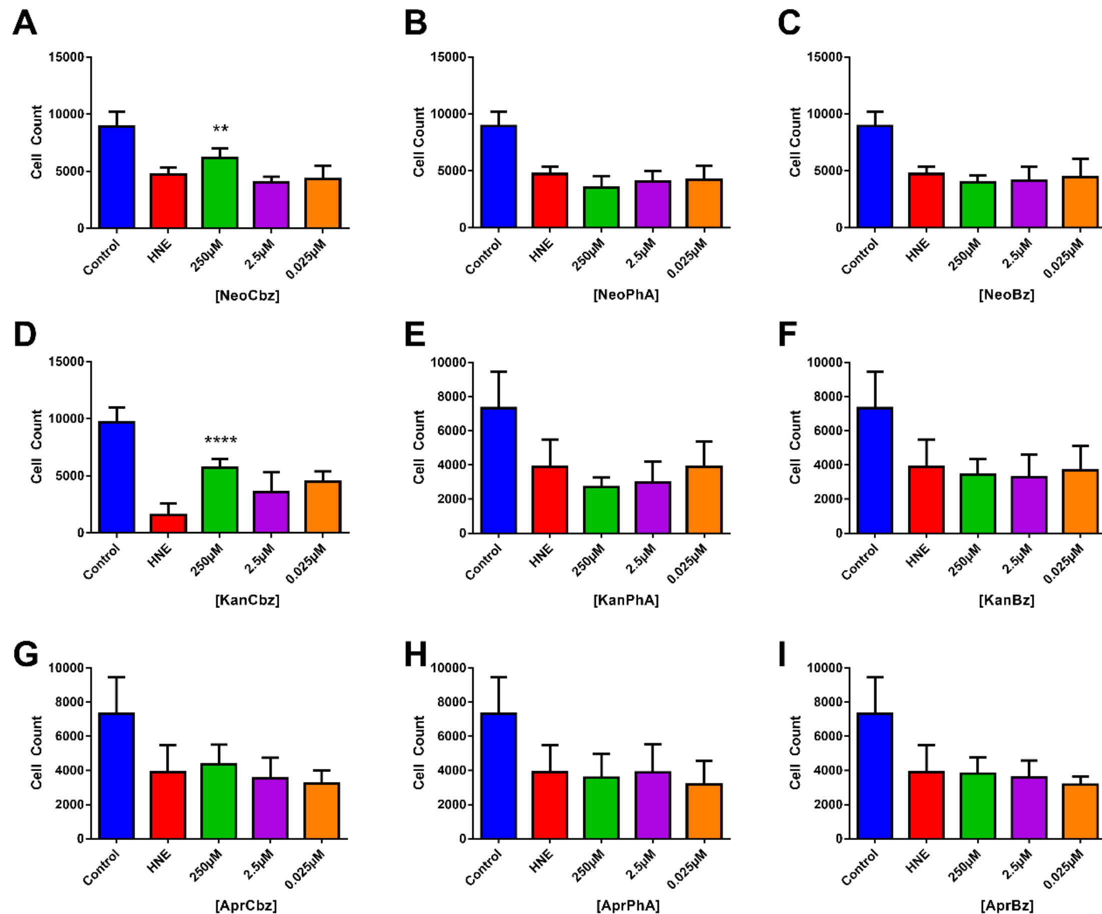


Figure S1: Quantification of concentration-dependent inhibition of HNE-mediated cell detachment by *N*-arylacyl *O*-sulfonated aminoglycosides. A549 lung epithelial cells were exposed to 50 nM HNE in the presence of decreasing concentrations of **NeoCbz** (A), **NeoPhA** (B), **NeoBz** (C), **KanCbz** (D), **KanPhA** (E), **KanBz** (F), **AprCbz** (G), **AprPhA** (H) and **AprBz** (I). After 24 h lung epithelial cell nuclei were stained with Hoeschst 33342 and cells were imaged and counted using the Operetta High Content Imaging System and Harmony Analysis Software respectively. At the highest concentration used (250 μM) both **NeoCbz** and **KanCbz** protected cells against protease mediated cell detachment. Data are presented as mean +SE, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$ as compared to protease treated cells (n=3 from 3 experiments each done in triplicate).

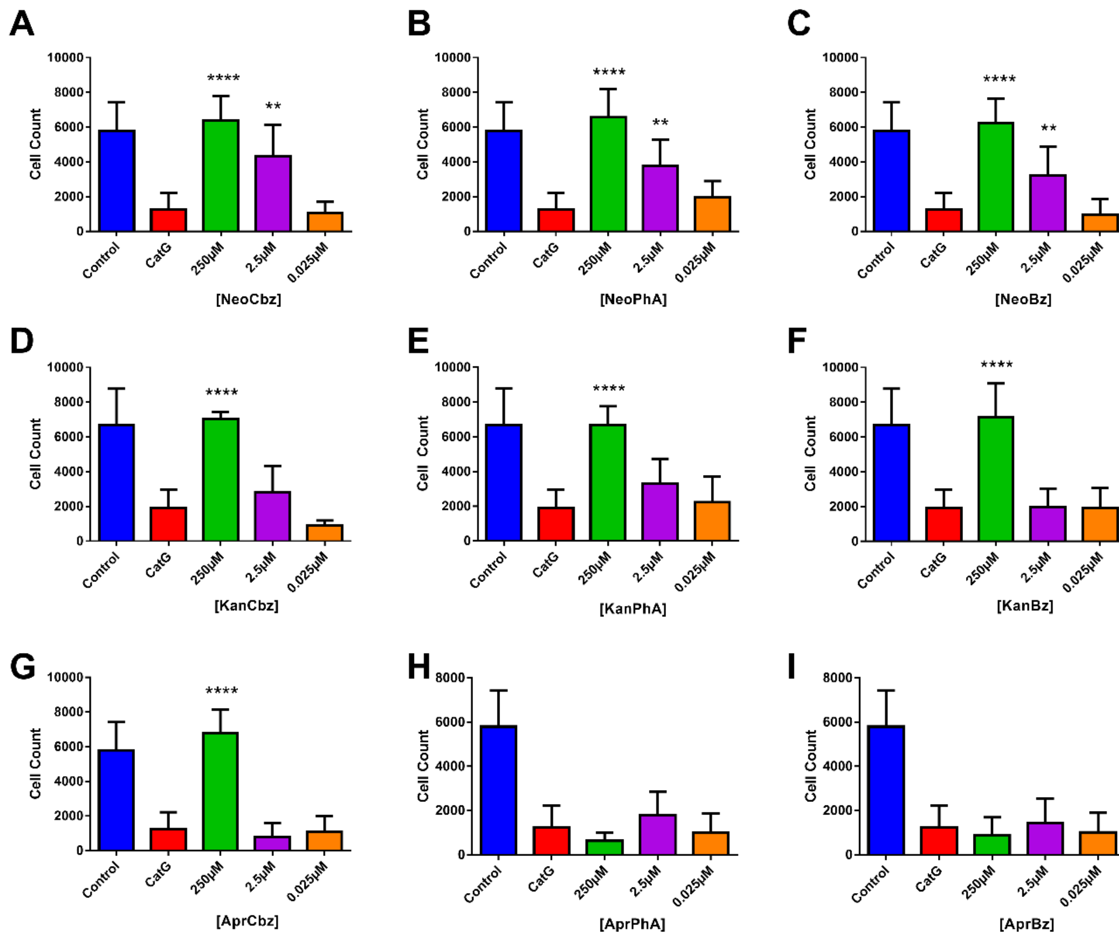


Figure S2: Quantification of concentration-dependent inhibition of CatG mediated cell detachment by *N*-arylacyl *O*-sulfonated aminoglycosides. A549 lung epithelial cells were exposed to 250 nM CatG in the presence of decreasing concentrations of **NeoCbz** (A), **NeoPhA** (B), **NeoBz** (C), **KanCbz** (D), **KanPhA** (E), **KanBz** (F), **AprCbz** (G), **AprPhA** (H) and **AprBz** (I). After 24 h lung epithelial cell nuclei were stained with Hoeschst 33342 and cells were imaged and counted using the Operetta High Content Imaging System and Harmony Analysis Software respectively. Neomycin core derivatives protected cells against CatG mediated cell detachment at both 250 and 2.5 µM. Kanamycin derivatives protected cells only at the highest concentration tested, while for the apramycin derivatives only **AprCbz** showed significant protection at the highest concentration tested. Data are presented as mean +SE, **p < 0.01, ***p < 0.001 ****p < 0.0001 as compared to protease treated cells (n=3 from 3 experiments each done in triplicate).

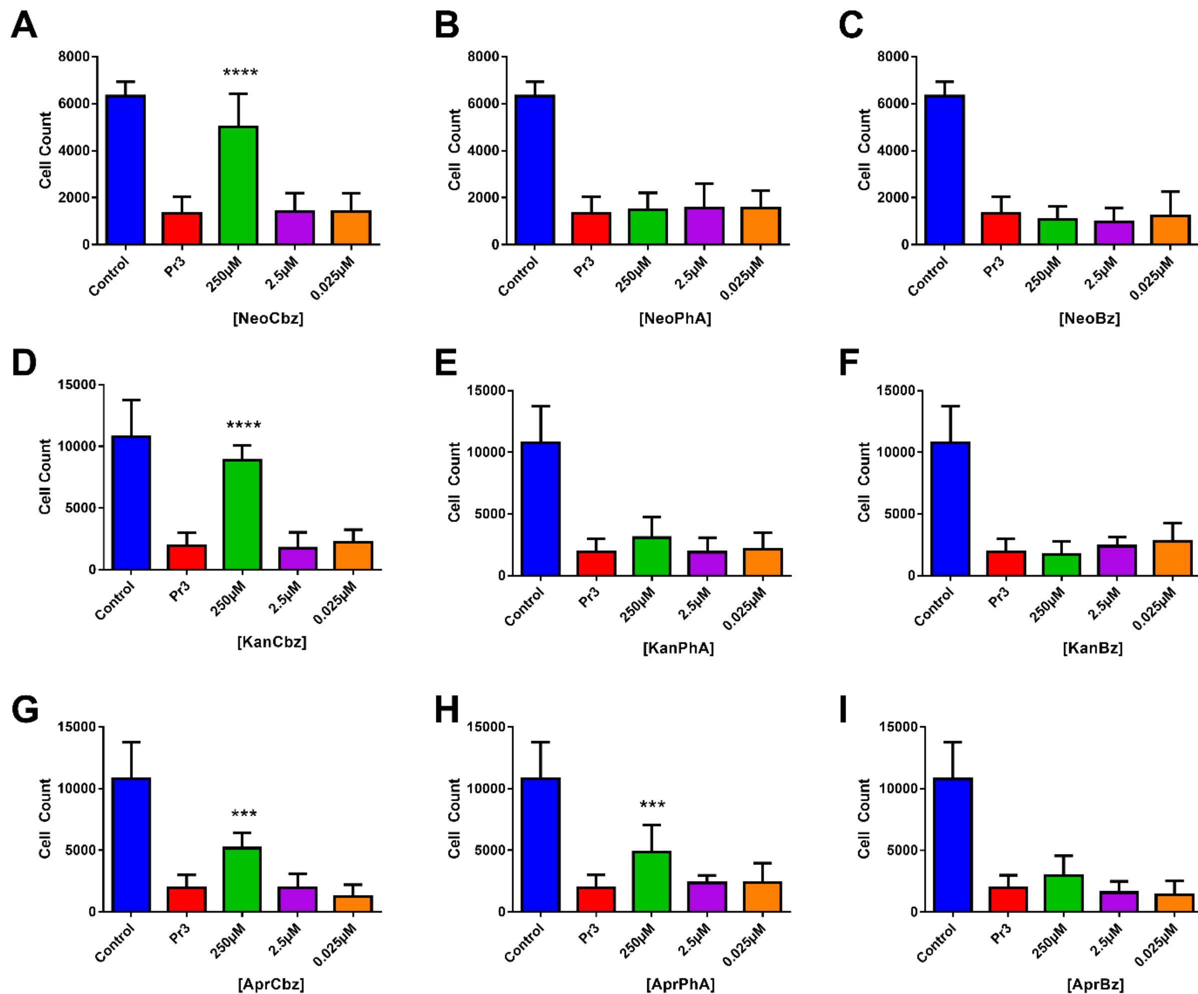
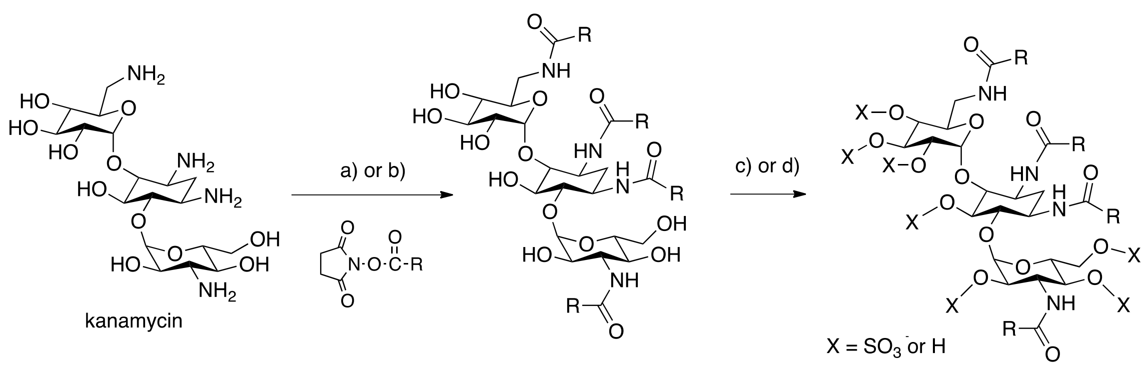


Figure S3: Quantification of concentration dependent inhibition of Pr3 mediated cell detachment by *N*-arylacyl *O*-sulfonated aminoglycosides. A549 lung epithelial cells were exposed to 50 nM Pr3 in the presence of decreasing concentrations of **NeoCbz** (A), **NeoPhA** (B), **NeoBz** (C), **KanCbz** (D), **KanPhA** (E), **KanBz** (F), **AprCbz** (G), **AprPhA** (H) and **AprBz** (I). After 24 h lung epithelial cell nuclei were stained with Hoeschst 33342 and cells were imaged and counted using the Operetta High Content Imaging System and Harmony Analysis Software respectively. **NeoCbz**, **KanCbz** and **AprCbz** all showed significant protection against Pr3 mediated cell detachment at 250 µM. Data are presented as mean +SE, **$p < 0.01$, ***$p < 0.001$ ****$p < 0.0001$ as compared to protease treated cells (n=3 from 3 experiments each done in triplicate).

2. Synthesis and Characterization of *N*-arylacyl *O*-sulfonated aminoglycosides

Synthesis of *O*-sulfonated *N*-arylacyl aminoglycoside derivatives, as shown with kanamycin (**Scheme S1**), was accomplished in two steps: 1) selective acylation of amine groups and 2) sulfonation of the *N*-arylacyl aminoglycoside to convert hydroxyl groups to sulfates. Using this methodology, apramycin, kanamycin, and neomycin were converted to their *N*-benzoyl (*N*-bz), *N*-carbobenzyloxy (*N*-cbz), and *N*-phenylacetyl (*N*-pha) derivatives by coupling with the corresponding *N*-(acyl)succinimide under alkaline conditions. Synthesis and of the per *N*-cbz derivatives of neomycin, kanamycin, and apramycin has been previously reported.[1, 2]

Neomycin sulfate, apramycin sulfate, and pyridine sulfur trioxide complex were from Sigma Aldrich (St. Louis, MO, USA); kanamycin sulfate was from Bristol Laboratories Inc. (Syracuse, NY, USA). *N*-(benzyloxycarbonyloxy)succinimide, benzoyl chloride, and chlorosulfonic acid were purchased from Acros Organics (Morris Plains, NJ, USA). *N*-(phenylacetyl) succinamide was synthesized as previously reported.[3] Phenylacetyl chloride was purchased from TCI (Portland, OR, USA). Cation exchange chromatography used Amberlite IR 120 resin, Sigma Aldrich. High performance liquid chromatography (HPLC)-grade acetonitrile (ACN) was from Fisher Scientific (Hampton, NH, USA). All other chemicals were purchased from Sigma. All water was filtered, deionized water from a Barnstead Nanopure Diamond system, Thermo Fisher Scientific (Hampton, NH, USA). Dialysis tubing, Spectra/Por Cellulose Ester membrane molecular weight cut off (MWCO) 500, was purchased from Spectrum Laboratories (Rancho Dominguez, CA). A Fisher Accumet AB15 pH meter was used for all pH determinations.



apramycin

neomycin

Core	R =	X=SO$_3^-$ 7	6	5	4	3
neomycin	CH$_3$	**na7**	**na6**	**na5**	**na4**	**na3**
apramycin		**--**	**ab6**	**ab5**	**ab4**	**ab3**
kanamycin		**kb7**	**kb6**	**kb5**	**kb4**	**kb3**
neomycin		**nb7**	**nb6**	**nb5**	**nb4**	**nb3**
apramycin		**--**	**az6**	**az5**	**az4**	**az3**
kanamycin		**kz7**	**kz6**	**kz5**	**kz4**	**kz3**
neomycin		**nz7**	**nz6**	**nz5**	**nz4**	**nz3**
apramycin		**--**	**ap6**	**ap5**	**ap4**	**ap3**
kanamycin		**kp7**	**kp6**	**kp5**	**kp4**	**kp3**
neomycin		**np7**	**np6**	**np5**	**np4**	**np3**

Scheme S1 Synthesis of *N*-arylacylated *O*-sulfonated aminoglycosides. Kanamycin is shown as an example. Per *N*-acylation was accomplished by either route a or b. Subsequent *O*-sulfonation by c or d gives desired product in varying degrees of sulfation. For each aminoglycoside, final products are noted by the core aminoglycoside (**n**, **k**, or **a**), the *N*-arylacyl group (**a**, acetyl; **b**, benzoyl; **z**, carboxybenzoyl; **p**, phenylacetyl) and the number of hydroxyl groups converted to sulfate groups (**3**-**7**). a) R-NHS, $NaHCO_3$, H_2O, 23 °C, 12-16 hr, 30-85%; b) R-COCl, $NaCO_3$, H_2O c) i: Pyr•SO_3, DMF, anhydrous pyr, 66 °C, 5-7 hr ii: H_2O, 10 mM NaOH, 4 °C, 45 65%; d) i: $ClSO_3H$, pyr, 57 °C, 4-6 hr, ii: H_2O, $NaHCO_3$, 35-95%.

2.1 N-acylation of aminoglycosides

For *N*-aryl aminoglyosides, free amines were converted into *N*-arylacyl moieties by either coupling free amines to NHS-activated esters as previously reported for introducing aryl groups onto free amine groups of *N*-desulfonated heparin or by reacting with acyl chlorides.[3] Initial

attempts to synthesize aryl amides were done using only acyl chlorides, but these reactions tended to produce over-acylated compounds having both *N*-acyl and *O*-acyl products, and hydrolysis of unwanted esters was cumbersome and decreased yield dramatically. All per-*N*-arylacylated aminoglycosides were analyzed by ESI-MS (electrospray ionization- mass spectroscopy). MS analysis was used to identify over or under-acylated products; these impurities were removed prior to sulfonation. All *N*-acylated aminoglycoside products were shown to be >95% pure by analytical HPLC after purification from reaction mixtures.

2.1.1 General N-acylation techniques and chromatography

Each reaction was monitored using analytical HPLC: 20 µL of the reaction mixture was diluted in 80 µL ACN and 40 µL water; 100 µL of this solution was injected and eluted with a gradient of 10-95% ACN in water (0.1% trifluoroacetic acid (TFA)) over 40 min at 1 mL/min. After complete acylation of amine groups, ~6-8 hours, the reaction was diluted with water (4 mL) to give additional white precipitate. The precipitate was collected by centrifugation (20 min, 4°C, 3500 rpm) and decanting followed by washing the solid with cold water (10 mL), centrifugation and decanting. This wash procedure was repeated seven times followed by lyophilization of a final suspension to give dry white solid. In reactions where the *N*-acylation was incomplete, as detected by analytical HPLC, under-reacted product was removed by dissolving the product in ACN:water (3:1) and passing the solution through a column of amberlite cation (H^+ form) exchange resin (4 mL bed volume). The per *N*-acylated material was eluted in ACN:water (3:1) (10 column volumes) and under-reacted product was retained on the column and subsequently removed by eluting with 1 M NaOH.

In some acylation reactions, over-acylated product (having at least one *O*-arylacyl) was detected by ESI-MS; the desired product was then purified using semi-preparative HPLC. To this end, the product mixture was dissolved in 2:1 ACN:water and replicate aliquots injected and separated. The per-*N*-arylacyl product was collected around 20 minutes, depending on the parent aminoglycoside and the aryl moiety, when eluted with 40-60% ACN in water with 0.1% TFA, 7 mL/min. Product purified with cation exchange or semi-prep chromatography was rotary evaporated to remove organic solvent and the resulting aqueous (aq) suspension lyophilized to give a white solid. Alternatively, over-acylated *N*-cbz aminoglycosides were subjected to ester hydrolysis by treatment with methoxide. Briefly, the over-acylated product was dissolved in *N, N*-dimethylformamide (DMF), to which was added 2 drops of 6% methoxide in methanol (MeOH) solution while stirring on ice for 2 hours; product was collected by ether precipitation. MS and HPLC confirmed complete removal of O-acyl groups.

2.1.2 Penta N-benzoyl-apramycin (N-bz Apr)

Apramycin (193.5 mg, 0.3 mmol) was dissolved in 9:1 MeOH:H_2O (10 mL) with Na_2CO_3 (382 mg) and stirred at 0 °C for 15 minutes. Iced benzoyl chloride (420 µL, 2.43 mol/mol NH_2) was added dropwise to the solution. The mixture was stirred for 6 hr at 0 °C. A white precipitate formed, and the mixture was further precipitated by storing at 4 °C overnight. Organic solvent was removed under vacuum and the precipitate was collected by centrifugation. Precipitate was washed with aq $NaHCO_3$ and iced H_2O. The precipitate was dissolved in 2:1 ACN:H_2O and purified by semi-preparative HPLC at 7 mL/min, 40% ACN (0.1% TFA). The product was collected from the peak eluting at 13 min. The precipitate was lyophilized to dryness (2 days),

14% yield. ESI-LRMS (electrospray ionization -low resolution mass spectroscopy) calcd for $C_{56}H_{61}N_5O_{16}$ $[M + Na]^+$ mass-to-charge ratio *(m/z)* = 1082.40, found 1082.32.

2.1.3. N-benzyloxycarbonyl-apramycin (N-cbz Apr)

A solution of *N*-(benzyloxycarbonyloxy) succinamide (456 mg, 1.2 mol/mol NH_2) in DMF (4.5 mL) was added fraction-wise (0.54 mL/hr) to apramycin (0.3 mmol) dissolved in aq $NaHCO_3$ (10 mL). The reaction was stirred at room temperature for 10 hours. A white precipitate formed at the end of the reaction, and the product was further precipitated by addition of 5 mL water. The precipitate was collected by centrifugation. The precipitate was dissolved in 2:1 ACN:H_2O and separated on semi-preparative HPLC (60% ACN, peak eluted at 18 min). Organic solvent was removed by vacuum. The precipitate was lyophilized to dryness (2 days), 28% yield. ESI-LRMS calcd for $C_{61}H_{71}N_5O_{21}$ $[M + Na]^+$ *m/z* = 1232.45, found 1232.36.

2.1.4 Penta N-phenylacetyl-apramycin (N-PhA Apr)

Apramycin (191.7 mg, 0.3 mmol) was dissolved in 9:1 MeOH:H_2O (15.75 mL) with Na_2CO_3 (400 mg) and stirred at 0 °C for 15 minutes. Iced phenylacetyl chloride (483 µL, 2.43 mol/mol NH_2) was added dropwise to the solution. The mixture was stirred for 2 hr at 0 °C. A white precipitate formed, and the mixture was further precipitated adding 10 mL cold water. Organic solvent was removed under vacuum and the precipitate was collected by filtration and washed with cold water (75 mL). Precipitate was dissolved in 10:0.8 dichloromethane (DCM):MeOH and separated by flash chromatography under N_2 on a silica column (5.5 g, 3 cm diameter) and eluted with 10:0.8 DCM:MeOH. The product was analyzed using thin-layer chromatography (TLC) (10:2:1:1 ethyl acetate: MeOH: H_2O; acetic acid) and visualized with Hanessian's stain.

The precipitate was lyophilized to dryness (2 days), 11% yield. ESI-LRMS calcd for $C_{61}H_{71}N_5O_{16}$ $[M + Na]^+$ m/z = 1152.48, found 1152.45.

2.1.5 Tetra N-benzoyl-kanamycin A (N-Bz Kan)

Kanamycin A (100 mg) was dissolved in 9:1 MeOH:H_2O (10 mL) with Na_2CO_3 (200 mg) and stirred at 0 °C for 15 minutes. Cold benzoyl chloride (240 µL, 3 mol/mol NH_2) was added dropwise to the solution. The mixture was stirred for 5.5 hr at 0 °C. A white precipitate formed, and the product was further precipitated by addition of 1 mL cold water. Organic solvent was removed under vacuum and the precipitate was collected by centrifugation. The precipitate was washed with aq $NaHCO_3$ (5 × 10 mL) and iced H_2O (5 × 10 mL). The precipitate was redissolved in 66% ACN and purified by semi-preparative HPLC at 7 mL/min, 37% ACN (0.1% TFA). The product was collected from the peak eluting at 20 min. The precipitate was lyophilized to dryness (2 days), 68% yield. ESI-LRMS calcd for $C_{46}H_{52}N_4O_{15}$ $[M + Na]^+$ m/z = 923.33, found 923.32.

2.1.6 Tetra N-benzyloxycarbonyl-kanamycin A (N-cbz Kan)

A solution of *N*-(benzyloxycarbonyloxy) succinamide (1.3 mmol) in DMF (7.2 mL) was added fraction-wise (0.6 mL/90 min) to kanamycin A (125 mg, 0.258 mmol) dissolved in aq $NaHCO_3$ (9 mL). The reaction was stirred at room temperature for 11 hours. A white precipitate formed at the end of the reaction, and the product was further precipitated by addition of water (5 mL). The precipitate was repeatedly washed with cold water (7 × 10 mL), centrifuged and decanted. Precipitate was collected by filtering through a glass fritted funnel. Product was lyophilized to

dryness (2 days), giving a white solid, 66% yield. ESI-LRMS calcd for $C_{50}H_{60}N_4O_{19}$ $[M + Na]^+$ m/z = 1043.38, found 1043.58.

2.1.7 Tetra N-phenylacetyl-kanamycin A (N-PhA Kan)

A solution of *N*-(phenylacetyl) succinamide (359.5 mg, 2 mol/mol NH_2) in DMF (6.5 mL) was added fraction-wise (1.25 mL/hr) to kanamycin A (0.207 mmol) dissolved in aq $NaHCO_3$ (6.7 mL). The reaction was stirred at room temperature over 8.5 hours. A white precipitate formed at the end of the reaction, and the product was further precipitated by addition of cold water (5 mL). The precipitate was collected by centrifugation and repeated washings with cold water (7 × 10 mL). The precipitate was lyophilized to dryness (2 days), 81% yield. ESI-LRMS calcd for $C_{50}H_{60}N_4O_{15}$ $[M + Na]^+$ m/z = 979.40, found 979.49.

2.1.8 Hexa N-benzoyl-neomycin B (N-Bz Neo)

Neomycin B (227.2 mg, 0.25 mmol) was dissolved in 9:1 MeOH:H_2O (16 mL) with Na_2CO_3 (454 mg) and stirred at 0 °C for 15 minutes. Iced benzoyl chloride (420.8 μL, 2.43 mol/mol NH_2) was added dropwise to the solution. The mixture was stirred for 2 hr at 0 °C. A white precipitate formed, and the product was further precipitated by addition of 10 mL cold water. Organic solvent was removed under vacuum and the precipitate was collected by filtration through a fritted funnel. The residue was washed with aq $NaHCO_3$ (10 mL) and cold H_2O (10 mL). The residue was redissolved in 40% ACN and purified by semi-preparative HPLC at 7 mL/min, 40% ACN (0.1% TFA). The product was collected from the peak eluting at 20 min. The precipitate was lyophilized to dryness (2 days), 63% yield. ESI-LRMS calcd for $C_{65}H_{70}N_6O_{19}$ $[M + Na]^+$ m/z = 1261.46, found 1261.47.

2.1.9 Hexa *N*-benzyloxycarbonyl-neomycin B (*N-Cbz Neo*)

A solution of *N*-(benzyloxycarbonyloxy) succinamide (3.6 mmol) in DMF (4.5 mL) was added fraction-wise (0.9 mL/hr) to neomycin B (0.5 mmol) dissolved in aq $NaHCO_3$ (17 mL) at room temperature over 7 hours. A white precipitate formed at the end of the reaction, and the mixture was further precipitated by addition of 10 mL water. The precipitate was collected by centrifugation and repeated washings with cold water and yielded a mixture of products of varying degrees of *N*-acylation. Compounds that were not per-*N*-acylated were removed by passing the precipitate over a cation exchange column. Per-*N*-acylated, *O*-acylated compounds were removed by semi-preparative HPLC. The combined fractions were evaporated and lyophilized to dryness, 34% yield. ESI-LRMS calcd for $C_{71}H_{82}N_6O_{25}$ $[M + Na]^+$ $m/z = 1441.52$, found 1441.62.

2.1.10 Hexa *N*-phenylacetyl-neomycin B (*N-PhA* Neo)

A solution of *N*-(phenylacetyl) succinamide (1.81 mmol) in DMF (4.5 mL) was added fraction-wise (0.9 mL/hr) to neomycin B (0.25 mmol) dissolved in aq $NaHCO_3$ (8 mL). The reaction was stirred at room temperature over 6 hours. A white precipitate formed at the end of the reaction, and the product was further precipitated by addition of 4.5 mL water. The precipitate was repeatedly collected by centrifugation and washed with cold water (7 × 10 mL). The precipitate was analyzed on HPLC and found to be a mixture of per *N*-acylated and over-acylated neomycin. The precipitate was dissolved in 2:1 ACN:water and purified by semi-preparative HPLC (52% ACN, 0.1% TFA, peak at 20 min). The precipitate was lyophilized to dryness (2 days), 24% yield. ESI-LRMS calcd for $C_{71}H_{82}N_6O_{19}$ $[M + Na]^+$ $m/z = 1345.52$, found 1345.49.

2.2 O-sulfonation of N-arylacyl aminoglycosides

The per-*N*-arylacyl aminoglycosides were sulfated with either pyridine sulfur trioxide complex (Pyr•SO_3) or chlorosulfonic acid ($ClSO_3H$) (Scheme S1). In using the two different sulfonation reagents under different reaction conditions, varied results were obtained in attempts to generate per-sulfated products. For example, when Pyr•SO_3 was used to *O*-sulfonate *N*-cbz kanamycin, the resulting product was a mixture of the per-sulfated derivative **KanCbz**, degree of sulfation (DS) = 7, and under-sulfated derivatives: six hydroxyls sulfonated (**KanCbz** DS = 6) and five hydroxyls sulfonated (**KanCbz** DS = 5). When $ClSO_3H$ was used to *O*-sulfonate *N*-cbz neomycin, the reaction produced *N*-cbz per-*O*-sulfonated neomycin (**NeoCbz** DS = 7). In two separate reactions, both Pyr•SO_3 and $ClSO_3H$ were used to *O*-sulfonate *N*-cbz apramycin. *O*-sulfonation with Pyr•SO_3 produced a mixture of products containing *N*-cbz per-*O*-sulfonated apramycin (**AprCbz** DS = 6) as well as under-sulfated products with either five sulfates (**AprCbz** DS = 5) or four sulfates (**AprCbz** DS = 4) per molecule. *O*-sulfonation of *N*-cbz apramycin with $ClSO_3H$ produced **AprCbz** DS = 6. Thus by using the liquid chromatography (LC) and liquid chromatography mass spectrometry (LC-MS) methods developed in this work, it is shown here that $ClSO_3H$ is the superior reagent to achieve complete, per-sulfation of these glycoconjugates. The completed panel of compounds is described in Table S1.

Table S1. Completed panel of *N*-arylacylated *O*-sulfonated aminoglycosides

Compound	Sulfating Reagent	% Yield[a]	mol wt ($SO_3^-NH_4^+$)	avg deg sulfation	% Per-sulfated	Na_2SO_4 (mM)[b]
N-bz *O*-SO_3^- apramycin	$ClSO_3H$	78	1642.67	6	100	< 0.32
N-cbz *O*-SO_3^- apramycin	$ClSO_3H$	30	1792.80	6	100	0.33
N-pha *O*-SO_3^- apramycin	$ClSO_3H$	33	1712.80	6	100	< 0.32
N-bz *O*-SO_3^- kanamycin	$ClSO_3H$	34	1397.07	5.1	29	0.78
N-cbz *O*-SO_3^- kanamycin	Pyr•SO_3	47	1605.53	6.0	22	< 0.32
N-pha *O*-SO_3^- kanamycin	Pyr•SO_3	50	1520.95	5.8	15	< 0.32
N-bz *O*-SO_3^- neomycin	$ClSO_3H$	54	1918.94	7	100	0.33
N-cbz *O*-SO_3^- neomycin	$ClSO_3H$	95	2099.09	7	100	< 0.32
N-pha *O*-SO_3^- neomycin	$ClSO_3H$	82	2003.10	6.8	91	< 0.32

[a]Percent yield was calculated from theoretical mass of 100% persulfated product

[b]Na_2SO_4 concentration for a 1mg/mL solution of *N*-arylacyl *O*-sulfonated aminoglycoside (500-750 mM) in H_2O

< 0.32 mM: below limit of detection; quantified by CE

nd: not determined

2.2.1 General techniques and chromatography

All glassware was oven-dried; all reactions were performed under argon gas for anhydrous reactions. Two sulfonation methods were employed here. In one method, aminoglycoside (100 mg) was dissolved in anhydrous DMF (1 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (3 equivalents per aminoglycoside hydroxyl) dissolved in DMF (1 mL) with anhydrous pyridine (1 mol equivalent).[4] The reaction was stirred at 66 °C for 12-20 hours and monitored by analytical HPLC by extracting 10 µL of the reaction mixture and adding 20 µL aq $NaHCO_3$, 20 µL ACN and 60 µL H_2O and vortexed to dissolve. 100 µL of this solution was injected on HPLC and separated by gradient elution in reversed-phase ion pairing (RPIP) buffers A and B: 0-100% buffer B over 20 min at 0.5 mL/min. Buffer A consisted of 9:1 water:ACN with 10 mm

ammonium acetate and pH adjusted to 8.3 with triethylamine (TEA). Buffer B consisted of 7:3 water: ACN with 10 mm ammonium acetate and pH adjusted to 8.3 with TEA. Once reaction progression, as monitored by HPLC, was deemed to be complete, the reaction was cooled to 4°C with an ice bath. Residual Pyr•SO_3 was quenched by the addition of water (2 mL), and the resulting aqueous solution made alkaline with cold 10 M NaOH (added in 10 μL increments).

In the second method, $ClSO_3H$ was used to sulfonate the *N*-arylacyl aminoglycosides.[5, 6] With this method, $ClSO_3H$ (8 equivalents per hydroxyl) was added drop-wise to anhydrous pyridine (3 mL) stirring at room temperature in a 50 mL round bottom flask and the mixture then heated to 57 °C. Separately, the *N*-arylacyl aminoglycoside (50 mg) was dissolved in anhydrous pyridine (2 mL) and evaporated for azeotropic removal of residual water; this procedure was repeated 2-3 times before dissolving the *N*-arylacyl aminoglycoside in anhydrous pyridine (2 mL) and cannulating the resulting solution into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 57 °C and reaction progress monitored by analytical HPLC with RPIP buffers as described above. Upon reaction completion, about 3-8 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with addition of aq $NaHCO_3$. The resulting aqueous solution was transferred to a separatory funnel and extracted with DCM (7-10 × 15 mL) to remove pyridine. The DCM layer was spotted each time on a TLC plate; removal of pyridine was considered complete when no ultraviolet (UV) absorbance was detected in the DCM layer. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting and product purification was accomplished using a benchtop C18 column.

The aqueous mixture of sulfonation reaction product(s) was loaded onto a column packed with C18 silica resin (1 × 5.8 cm, 6.5 mL bed volume). Non-volatile salts were first eluted with the ammonium acetate buffer (10 mM ammonium acetate adjusted to pH = 8.3 with TEA) under gravity flow. Eluent was collected in fractions (1 mL) and analyzed at 210 and 258 nm to detect elution of sodium sulfate and sugar, respectively. As determined with 210 nm detection, the sodium sulfate eluted within the first 100 mL. Then, sulfonated product was eluted by addition of 10% ACN. As determined with 258 nm detection, fractions containing sulfated aminoglycoside were pooled, and ACN was removed *en vacuo*. The remaining water and volatile salts were removed by lyophilization to give product as a white solid.

*2.2.2 O-sulfonated Penta N-benzoyl-apramycin (**AprBz**)*

$ClSO_3H$ (70 µL) was added drop-wise to anhydrous pyridine (2 mL) stirring at room temperature in a round bottom flask and the mixture then heated to 58 °C. *N*-bz apramycin (20 mg) in anhydrous pyridine (2.5 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. Upon reaction completion, 7 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aq $NaHCO_3$ (5 mL). The resulting aqueous solution was transferred to a separatory funnel and extracted with DCM (11 × 25 mL) to remove pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting and product purification were accomplished by loading the aqueous product on to a benchtop C18 column equilibrated in RPIP buffer. Final reaction products were analyzed on LC-MS or HPLC and co-injecting with known compounds characterized by LC-MS. Compound **AprBz** DS = 6, white solid, 78% yield**.** ^1H nuclear magnetic resonance spectroscopy (NMR) (600 MHz, D_2O):

δ 7.85–7.32 (m, 25H, Ph), 5.64-5.61 (m, 2H, $H1^B$, $H1^C$), 5.20-5.19 (m, 2H, $H3^C$ $H8^B$), 4.84 (s, 1H, $H6^B$), 4.65 (t, 1H, *J* = 9.11 Hz, $H5^A$), 4.51-4.35 (m, 5H, $H1^A$, $H2^C$, $H3^A$, $H5^C$, $H6^A$), 4.31 (m, 1H, $H2^B$), 4.15 (t, 1H, *J* = 9.6 Hz, $H4^A$), 4.09-4.04 (m, 2H, $H4^C$, $H6^C$), 3.73 (m, 1H, $H4^B$), 3.43 (d, 1H, *J* = 10.0 Hz, $H5^B$), 2.97 (m, 1H, $H7^B$), 2.41 (m, 1H, $H2a^A$), 2.24 (m, 1H, $H3a^B$), 1.84-1.80 (m, 2H, $H2b^A$, $H3b^B$)

*2.2.3 O-sulfonated Penta N-benzyloxycarbonyl-apramycin (**AprCbz**)*

$ClSO_3H$ (140 µL) was added drop-wise to anhydrous pyridine (3 mL) stirring at room temperature in a round bottom flask and the mixture then heated to 58 °C. *N*-cbz apramycin (50 mg) in anhydrous pyridine (1.5 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. Upon reaction completion, 6 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with addition of aq $NaHCO_3$ (10 mL). The resulting aqueous solution was transferred to a separatory funnel and extracted with DCM (7 × 25 mL) to remove pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting and product purification were accomplished by loading the aqueous product on to a benchtop C18 column. Final reaction products were analyzed on LC-MS or HPLC and co-injecting with known compounds characterized by LC-MS. Compound **AprCbz** DS = 6, white solid, 30% yield. ^1H NMR (600 MHz, D_2O) δ 7.28-7.10, (m, 25H, Ph), 5.69 (broad s, anomeric proton), 5.55 (broad s, anomeric proton), 5.28 (broad, s, anomeric proton).

2.2.4 *O*-sulfonated Penta *N*-phenylacetyl-apramycin (**AprPhA**)

$ClSO_3H$ (70.5 µL) was added drop-wise to anhydrous pyridine (2 mL) stirring at room temperature in a round bottom flask and the mixture then heated to 57 °C. *N*-pha apramycin (25 mg) in anhydrous pyridine (0.5 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 57 °C and reaction progress monitored by analytical HPLC. Upon reaction completion, 8 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aq $NaHCO_3$ (3.5 mL). Water (10 mL) was added to dissolve the precipitate that formed upon addition of $NaHCO_3$. The resulting aqueous solution was transferred to a separatory funnel and extracted with ether (8 × 20 mL) to remove most of the pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting and removal of remaining pyridine were accomplished by loading the aqueous product on to a benchtop C18 column equilibrated in RPIP buffer. Final reaction products were analyzed on LC-MS. Compound **AprPhA** DS = 6, white solid, 33% yield. ^1H NMR (600 MHz, D_2O) δ 7.38-7.22, (m, 25H, Ph), 5.67 (broad s, anomeric proton), 5.55 (broad s, anomeric proton), 5.32 (broad, s, anomeric proton).

*2.2.5 O-sulfonated Tetra N-benzoyl-kanamycin (**KanBz**)*

$ClSO_3H$ (200 µL) was added dropwise to anhydrous pyridine (2 mL) stirring at room temperature in a round bottom flask and the mixture then heated to 58 °C. *N*-bz kanamycin (50 mg) in anhydrous pyridine (2 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. After 8 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aq $NaHCO_3$ (10 mL). The resulting aqueous solution was transferred to a separatory funnel and

extracted with DCM (12 × 15 mL) to remove pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting was accomplished by loading the aqueous product on to a benchtop C18 column. Final reaction products were analyzed on LC-MS or HPLC and co-injecting with known compounds characterized by LC-MS. Product was a mixture of **KanBz** DS = 7 (29.3%), **KanBz** DS = 6 (1.2%), two isoforms of **KanBz** DS = 5 (16.8% and 3.9%), and **KanBz** DS = 4 (48.8%); product was a white solid, 34% yield.

*2.2.6 O-sulfonated Tetra N-benzyloxycarbonyl-kanamycin (**KanCbz**)*

N-cbz kanamycin (120 mg) was dissolved in anhydrous DMF (1 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (393 mg, 3 equivalents per aminoglycoside hydroxyl) dissolved in DMF (1.57 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3).[4] The reaction was stirred at 66 °C for 20 hours and monitored by analytical HPLC. The completed reaction was cooled to 4°C with an ice bath. Residual Pyr•SO_3 was quenched by the addition of water (2 mL), and the resulting aqueous solution made alkaline with cold 10 M NaOH (added in 10 µL increments). The mixture was extracted with diethyl ether (9 × 15 mL) to remove pyridine. The reaction was precipitated with cold acetone (8 mL), centrifuged, and decanted. By HPLC, product was found to be in the supernatant; the supernatant was condensed under vacuum to remove organic solvents and dialyzed in 500 mwco tubing with frequent water changes (9 × 1 L). The product was dried by lyophilizing (2 days). Product, pale yellow solid, was a mixture of **KanCbz** DS = 7 (21.5%) four isoforms of **KanCbz** DS = 6 (8.5%, 38.7%, 27.1%, 12%) and four isoforms of **KanCbz** DS = 5 (0.8%, 0.5%, 6.4%).

*2.2.7 O-sulfonated Tetra N-phenylacetyl-kanamycin (**KanPhA**)*

N-pha kanamycin (74 mg) was dissolved in anhydrous DMF (0.8 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (258 mg, 3 equivalents per aminoglycoside hydroxyl) dissolved in DMF (1.032 mL) with anhydrous pyridine (133 µL, 1 mol:mol Pyr•SO_3).[4] The reaction was stirred at 66 °C for 20 hours and monitored by analytical HPLC. The completed reaction was cooled to 4°C with an ice bath. Residual Pyr•SO_3 was quenched by the addition of water (2 mL), and the resulting aqueous solution made alkaline with cold 10 M NaOH (added in 10 µL increments). The mixture was extracted with ether (6 × 15 mL) to remove pyridine. The reaction was precipitated with cold acetone (8 mL), centrifuged and decanted; HPLC identified the product in the supernatant. The supernatant was condensed under vacuum to remove organic solvents and dialyzed in 500 mwco tubing with frequent water changes (9 × 1 L). Product was dried by lyophilizing (2 days). Product was a mixture of **KanPhA** DS = 7 (15%) three isoforms of **KanPhA** DS = 6 (4.2%, 35.3%, 11.2%) and two isoforms of **KanPhA** DS = 5 (21.4%, 12.8%).

2.2.8 *O*-sulfonated Hexa *N*-benzoyl-neomycin (**NeoBz**)

$ClSO_3H$ (150 µL) was added drop-wise to anhydrous pyridine (3 mL) stirring at room temperature in a round bottom flask and the mixture then heated to 58 °C. *N*-bz neomycin (50 mg) in anhydrous pyridine (1.5 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. Upon reaction completion, 6 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aq $NaHCO_3$ (7.5 mL). Water (10 mL) was added to dissolve the precipitate that formed upon addition of sodium bicarbonate, and the resulting aqueous solution was transferred

to a separatory funnel and extracted with DCM (9 × 15 mL) to remove pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting and product separation were accomplished by loading the aqueous product on to a benchtop C18 column. Final reaction products were analyzed on LC-MS or HPLC and co-injecting with known compounds characterized by LC-MS. Compound **NeoBz** DS = 7, white solid, 54% yield. ^{1}H NMR (600 MHz, D_2O): δ 7.73-7.33 (m, 30H, Ph), 5.65 (d, 1H *J* = 3.6 Hz, $H1^D$), 5.12 (d, 1H, *J* = 1.7 Hz, $H1^B$), 5.06 (s, 1H, $H2^C$), 5.03 (d, 1H, *J* = 4.2 Hz, $H1^C$), 4.99 (dd, 1H, *J* = 2.9 Hz, $H3^B$), 4.65 (dd, 1H, *J* = 4.2 Hz, *J* = 7.9 Hz, $H3^C$), 4.59 (dd, 1H, *J* = 10.3 Hz, 8.9 Hz, $H3^D$), 4.59-4.56 (m, 1H, $H4^B$), 4.50 (dd, 1H, *J* = 3.6 Hz, *J* = 10.3 Hz, $H2^D$), 4.45 (s, 1H, $H2^B$), 4.32-4.30 (m, 2H, $H4^D$, $H5^B$), 4.20 (m, 1H, $H3^A$), 4.08 (m, 1H, $H1^A$), 4.08-3.97 (m, 2H, $H5a^C$, $H6^A$), 3.91-3.90 (m, 2H, $H5^A$, $H5^D$), 3.79-3.72 (m, 4H, $H4^A$, $H4^C$, $H5b^C$, $H6a^B$), 3.62 (dd, 1H, *J* = 4.8 Hz, *J* = 14.3 Hz, $H6a^D$), 3.57 (dd, 1H, *J* = 3.6 Hz, *J* = 14.2 Hz, $H6b^B$) 3.35 (dd, 1H, 1H, *J* = 5.6 Hz, *J* = 14.3 Hz, $H6b^D$), 2.23 (m, 1H, $H2ax^A$), 1.25 (m, 1H, $H2eq^A$)

*2.2.9 O-sulfonated Hexa N-benzyloxycarbonyl-neomycin (**NeoCbz**)*

$ClSO_3H$ (134 μL) was added drop-wise to anhydrous pyridine (2 mL) stirring at room temperature in a round bottom flask, and then the mixture was heated to 58 °C. *N*-cbz neomycin (50 mg) in anhydrous pyridine (2 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. Upon reaction completion, 6 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aq $NaHCO_3$ (7.5 mL). The resulting aqueous solution was transferred to a separatory funnel and extracted with DCM (10 × 15 mL) to remove pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting and product

separation were accomplished by loading the aqueous product on to a benchtop C18 column equilibrated in RPIP buffer. Final reaction products were analyzed on LC-MS or HPLC and co-injecting with known compounds characterized by LC-MS. Compound **NeoCbz** DS = 7, white solid, 95% yield. ^{1}H NMR (600 MHz, D_2O): δ 7.38-7.24 (m, 30H, Ph), 5.41 (broad s, anomeric proton), 5.28 (broad s, anomeric proton,) 5.14-4.88 (m, 17H, anomeric proton, 6 × CH_2Ph, $H2^C$, $H3^B$, $H3^C$, $H3^D$).

*2.2.10 O-sulfonated Hexa N-phenylacetyl-neomycin (**NeoPhA**)*

$ClSO_3H$ (140 µL, 8 equivalents per hydroxyl) was added dropwise to anhydrous pyridine (3 mL) stirring at room temperature in a round bottom flask and the mixture then heated to 58 °C. *N*-pha neomycin (50 mg) in anhydrous pyridine (3.5 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. After 3 hr, the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aqueous $NaHCO_3$ (10 mL). The resulting aqueous solution was transferred to a separatory funnel and extracted with DCM (11 × 20 mL) to remove pyridine. The water layer was then condensed on a rotary evaporator to remove residual organic solvent. Desalting was accomplished by loading the aqueous product on to a C18 column. Final reaction products were analyzed on LC-MS or HPLC and co-injecting with known compounds characterized by LC-MS. Product was a mixture of **NeoPhA** DS = 7 (90%) and two isoforms of **NeoPhA** DS = 5 (9% and 1%); product was a white solid, 82% yield.

2.3 Characterization of N-arylacyl O-sulfonated aminoglycosides

HPLC separation and determination of degree of sulfation (DS) for product mixtures using LCMS was performed as previously described.[7] Briefly, HPLC separations for mass spectra analysis used the Phenomenex analytical column, described above, on either a Surveyor LC system or a Dionex UltiMate 3000. Column temperature was not controlled; sample tray was maintained at 4°C. An autosampler was used to inject *N*-aryl *O*-sulfonated aminoglycoside (20 μg); all samples were dissolved in water. The mass spectra were acquired on a Thermo LCQ Deca mass spectrometer with ESI ionization and quadrupole ion trap mass analyzer collecting centroid data in the negative mode. Mass range for samples with neomycin or apramycin cores was set from 600-2000 amu, and 500-2000 amu for kanamycin-core samples. Degree of sulfation (DS) was determined by peak integration of corresponding chromatograms with UV absorbance for peak detection/correlation to TIC chromatograms. Separations to determine UV absorbance were performed on a Shimadzu LC10AT VP series HPLC pump with PDA detector. Peak area was calculated by measuring absorbance at the wavelength of maximum absorbance at 254-258 nm for each compound. Each peak was assigned a sulfated state based on molecular mass of the corresponding peak in the total ion chromatogram (TIC). Percent area was calculated for each degree of sulfation in the UV chromatogram and used to assign an average degree of sulfation for the sample.

To tune the MS, 2 mg/mL *N*-carbobenzyloxy *O*-sulfonated neomycin in water was injected via T-flow with buffer A. The instrument was focused on the mass corresponding to the doubly charged persulfated ion $[M - 2H + TEA]^{-2}$ (m/z = 1038.7). Optimal ion detection was achieved

by setting the spray voltage to 3.00 kV, capillary temp at 203 °C, capillary voltage to -46 V, increasing the tube lens offset to 25 V and decreasing the sheath flow rate to 60 psi.

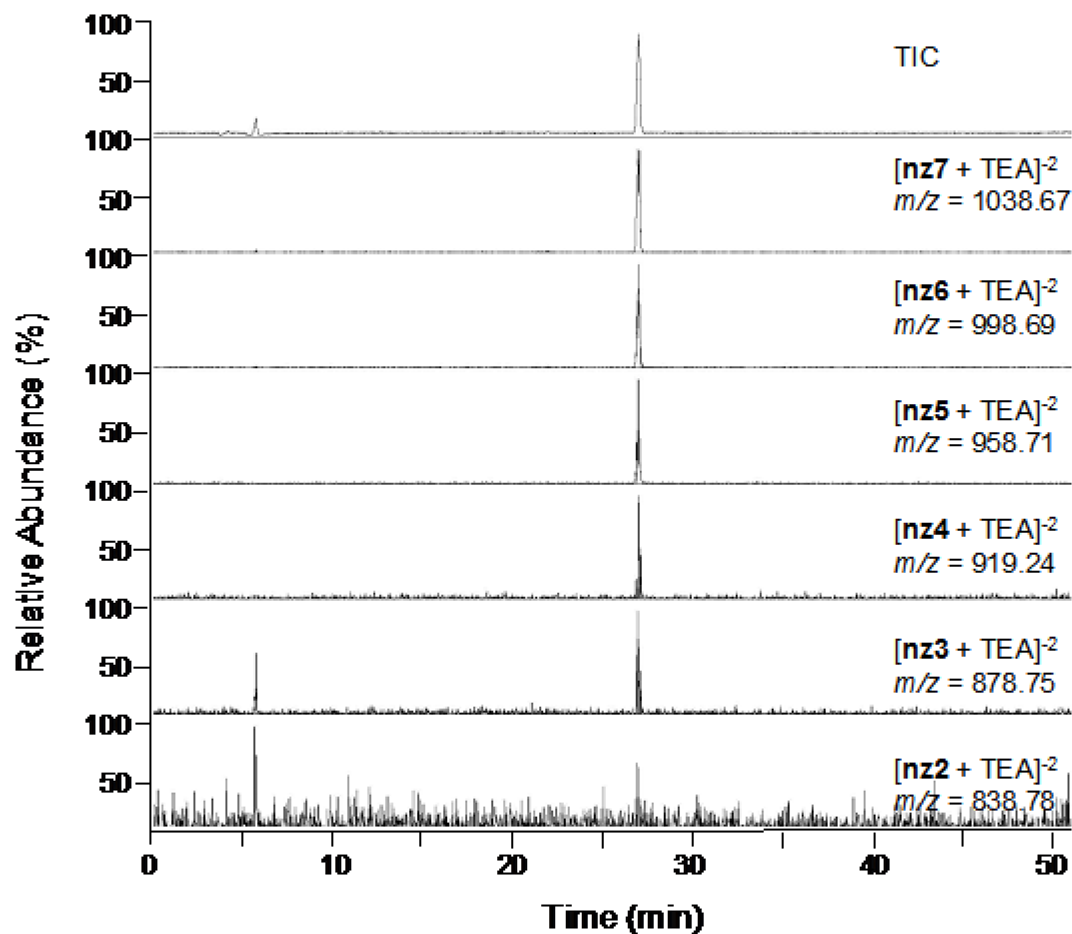


Figure S4: NeoCbz is identified as persulfated by LC-MS chromatograms

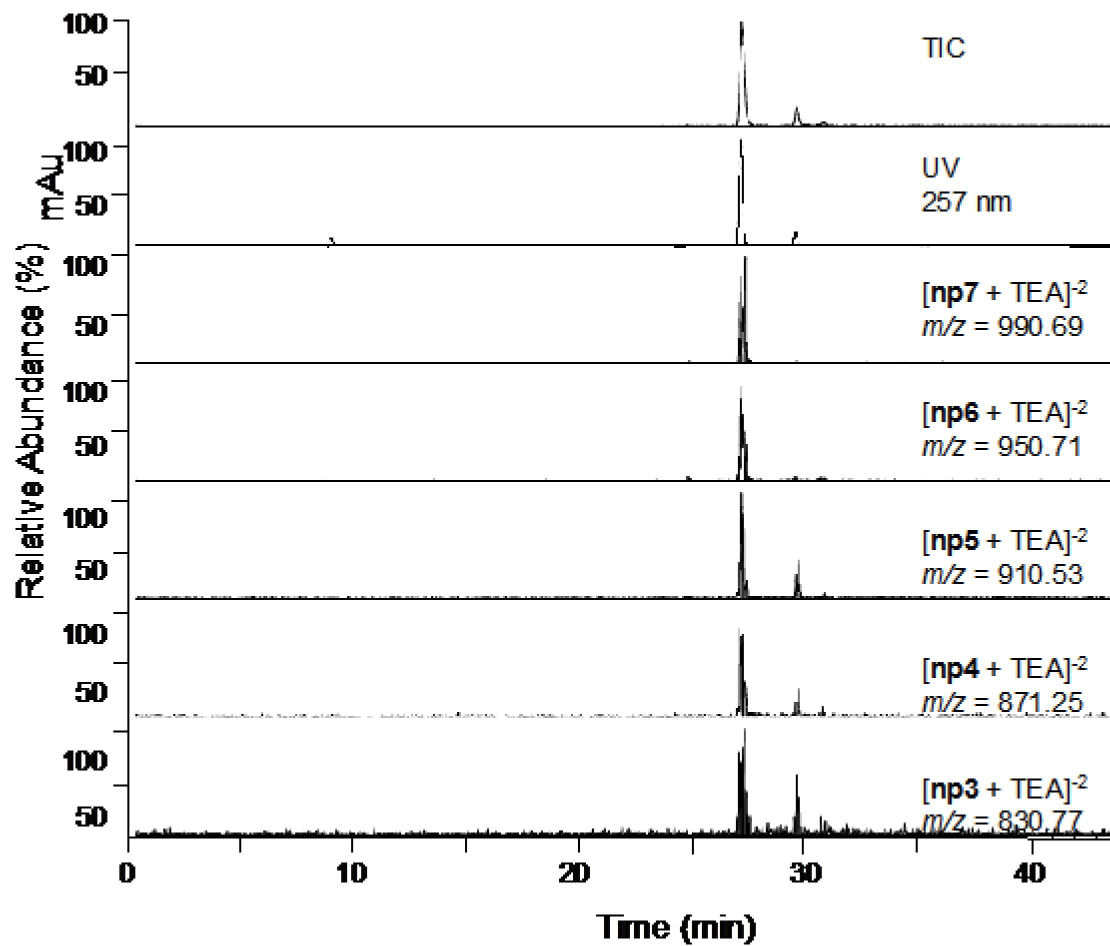


Figure S5: NeoPhA, a mixture of **NeoPhA** with degree of sulfation 7 and 5, is separated, quantified, and identified with LC-MS with an in-line UV detector. Average degree of sulfation is determined to be 6.8.

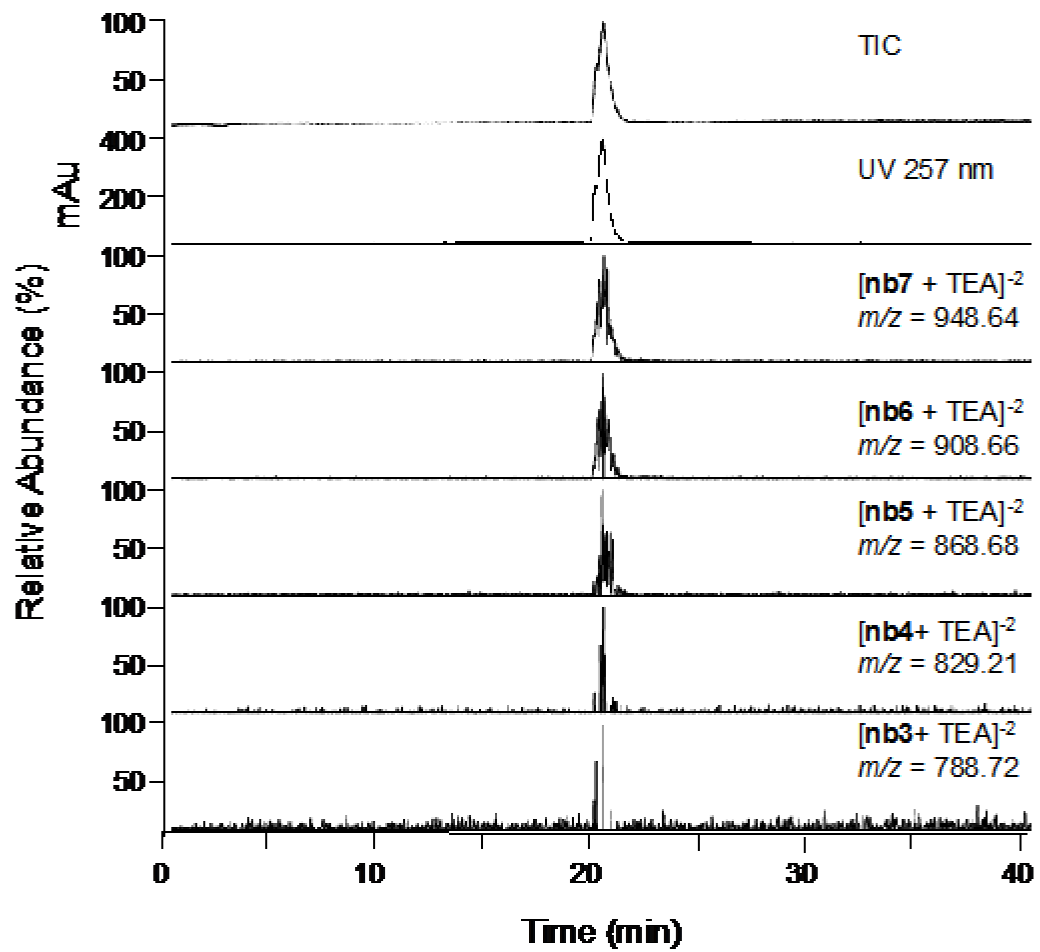


Figure S6: NeoBz is identified as persulfated by LC-MS chromatograms

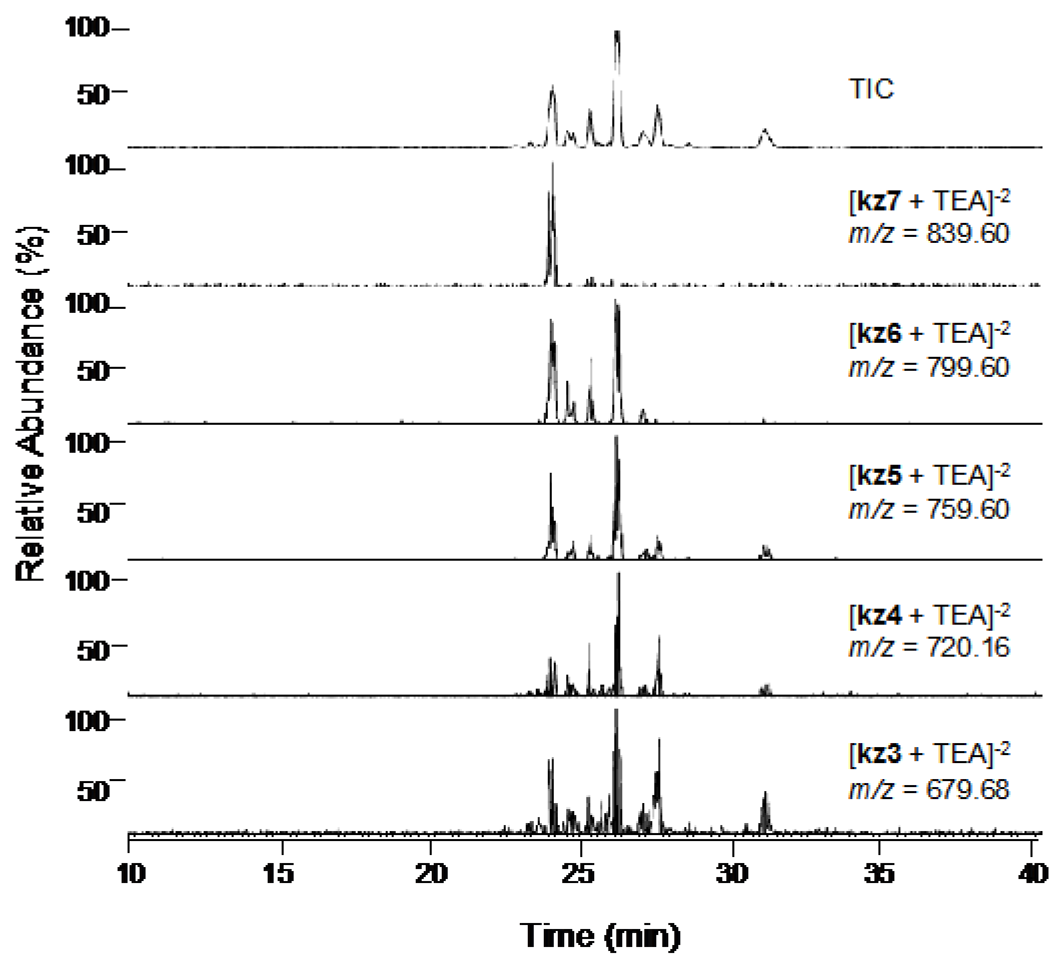


Figure S7: KanCbz a mixture of **KanCbz** with degree of sulfation 7, 6 and 5, is separated, quantified, and identified with LC-MS with an in-line UV detector. Average degree of sulfation is determined to be 6.0.

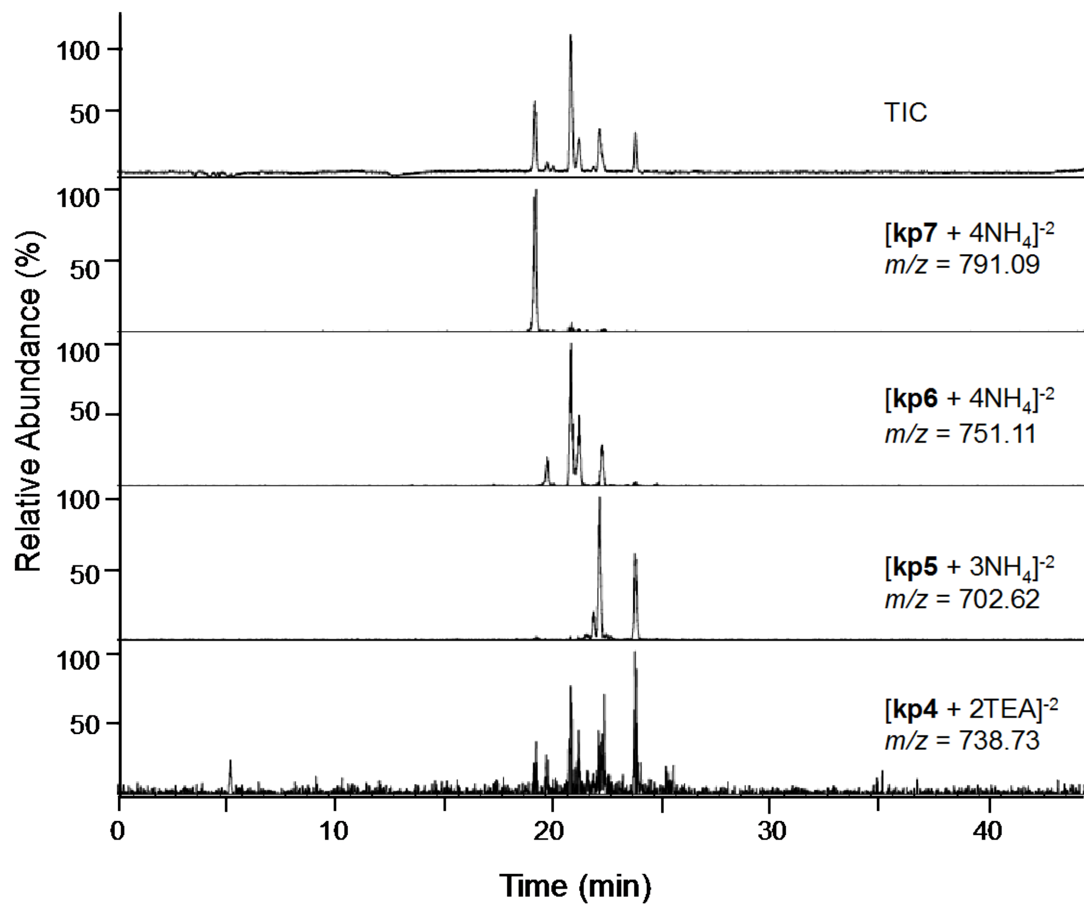


Figure S8: KanPhA, a mixture of **KanPhA** with degree of sulfation 7, 6 and 5, is separated, quantified, and identified with LC-MS with an in-line UV detector. Average degree of sulfation is determined to be 5.8.

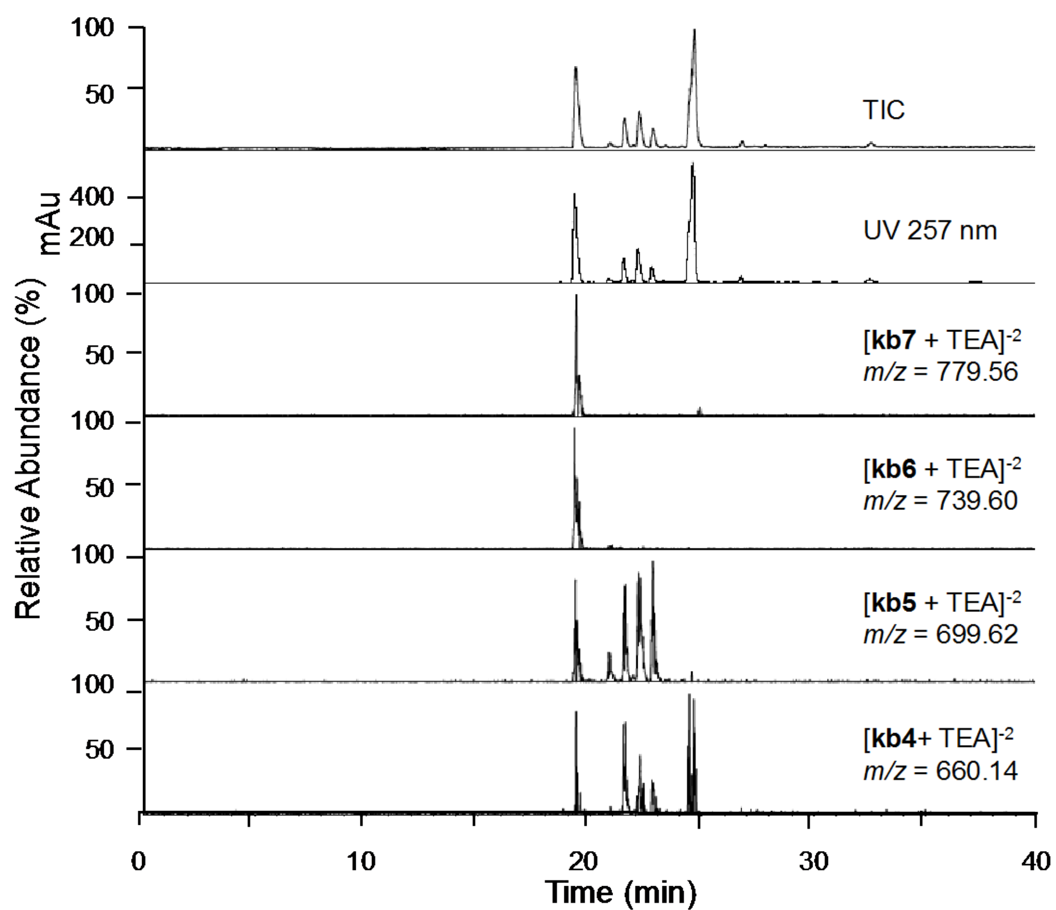


Figure S9: KanBz, a mixture of **KanBz** with degree of sulfation 7, 6 and 5, is separated, quantified, and identified with LC-MS with an in-line UV detector. Average degree of sulfation is determined to be 5.1.

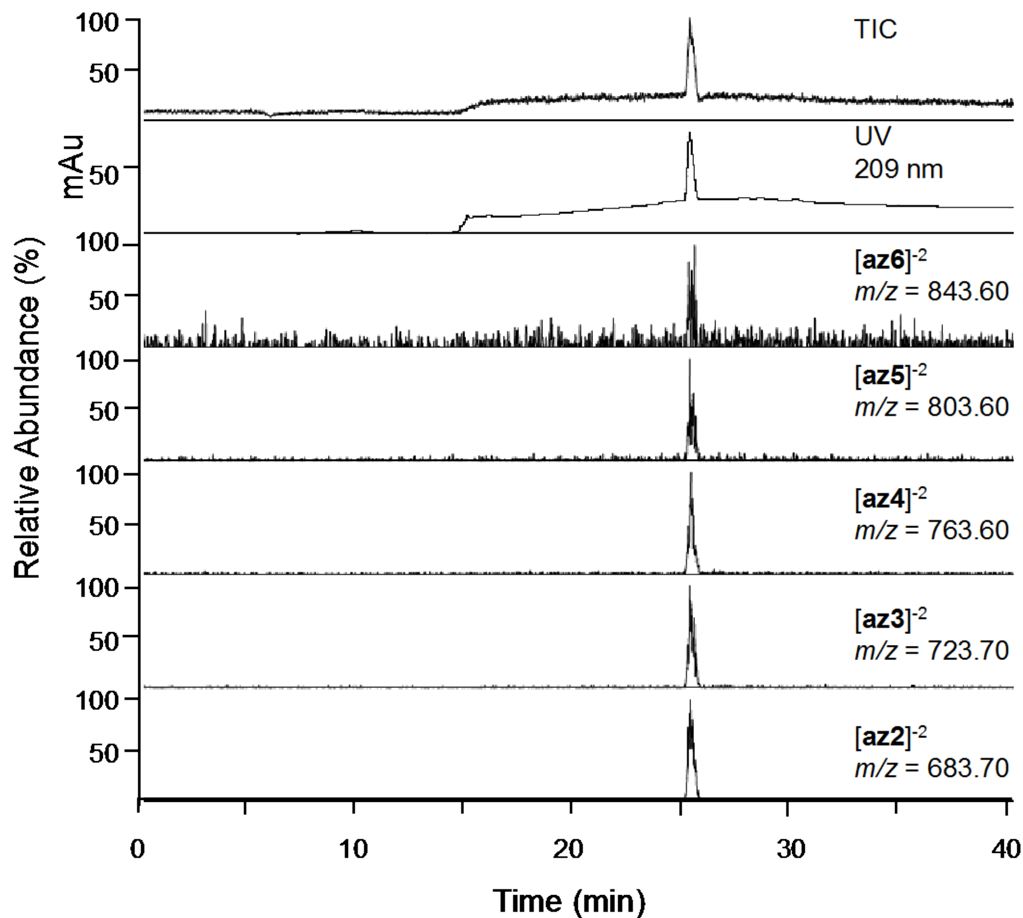


Figure S10: AprCbz is identified as persulfated by LC-MS chromatograms.

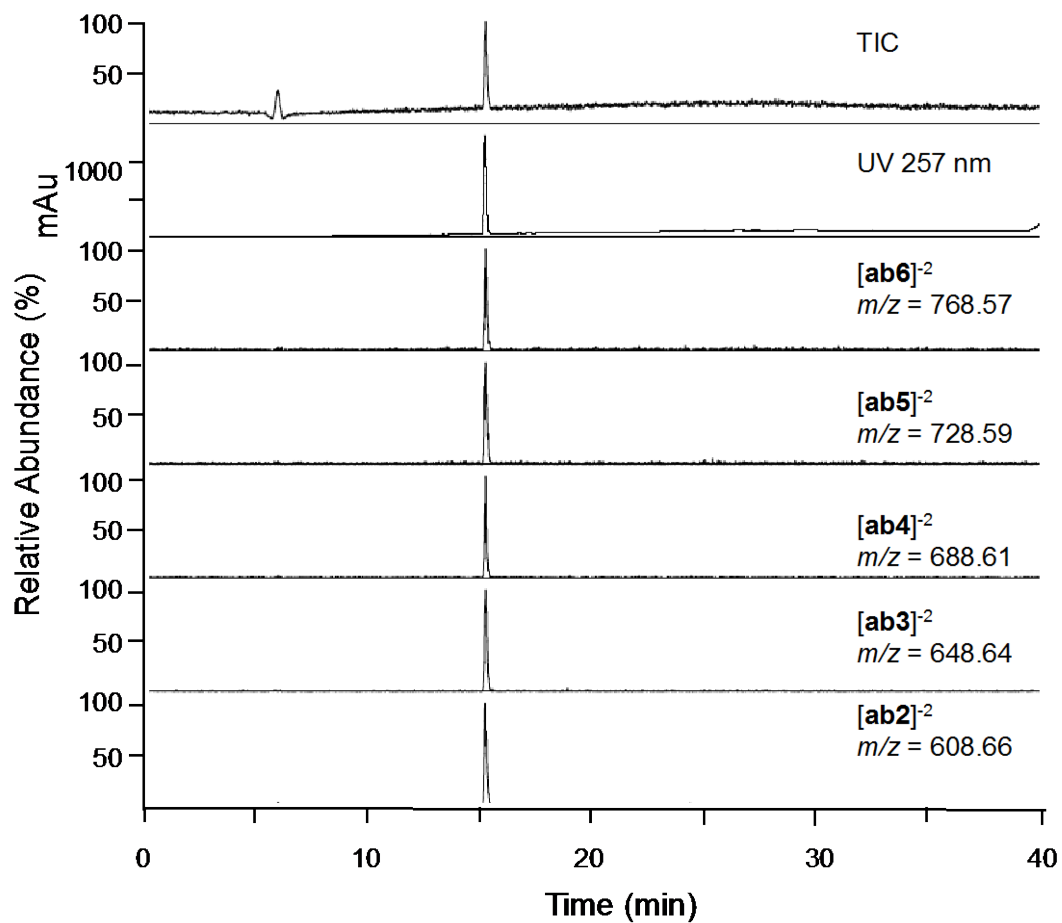


Figure S11: AprBz is identified as persulfated by LC-MS chromatograms.

^{1}H NMR (600 MHz, D_2O) for persulfated derivatives, where DS = single persulfated product. All samples were solubilized in D_2O and lyophilized prior to dissolving in D_2O for analysis.

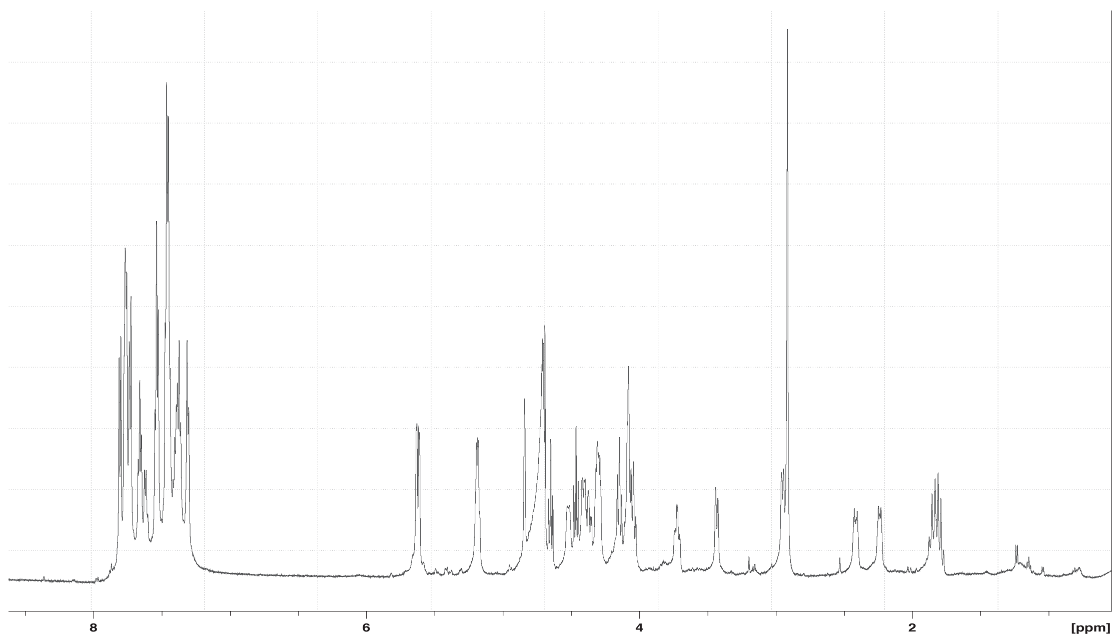


Figure S12 ^{1}H NMR of AprBz.

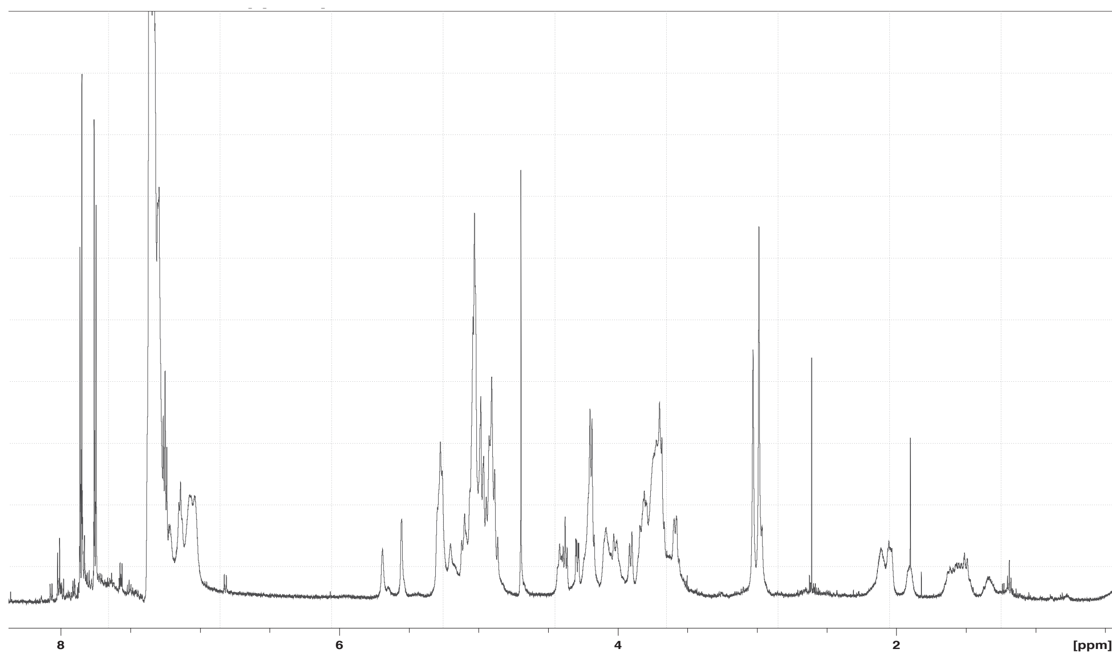


Figure S13 ^{1}H NMR of AprCbz.

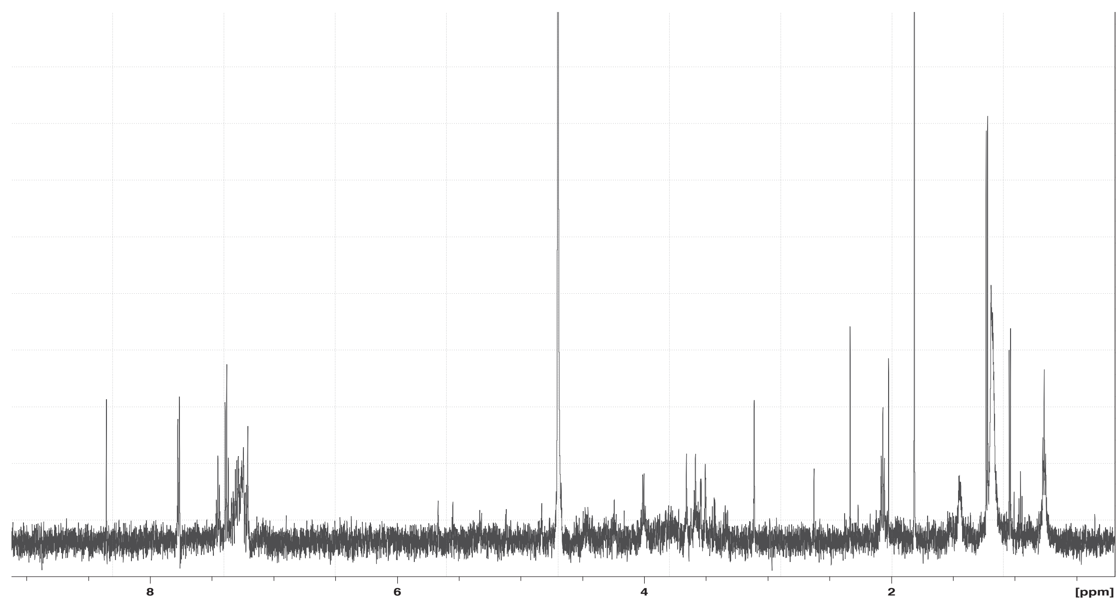


Figure S14 ^{1}H NMR of AprPhA.

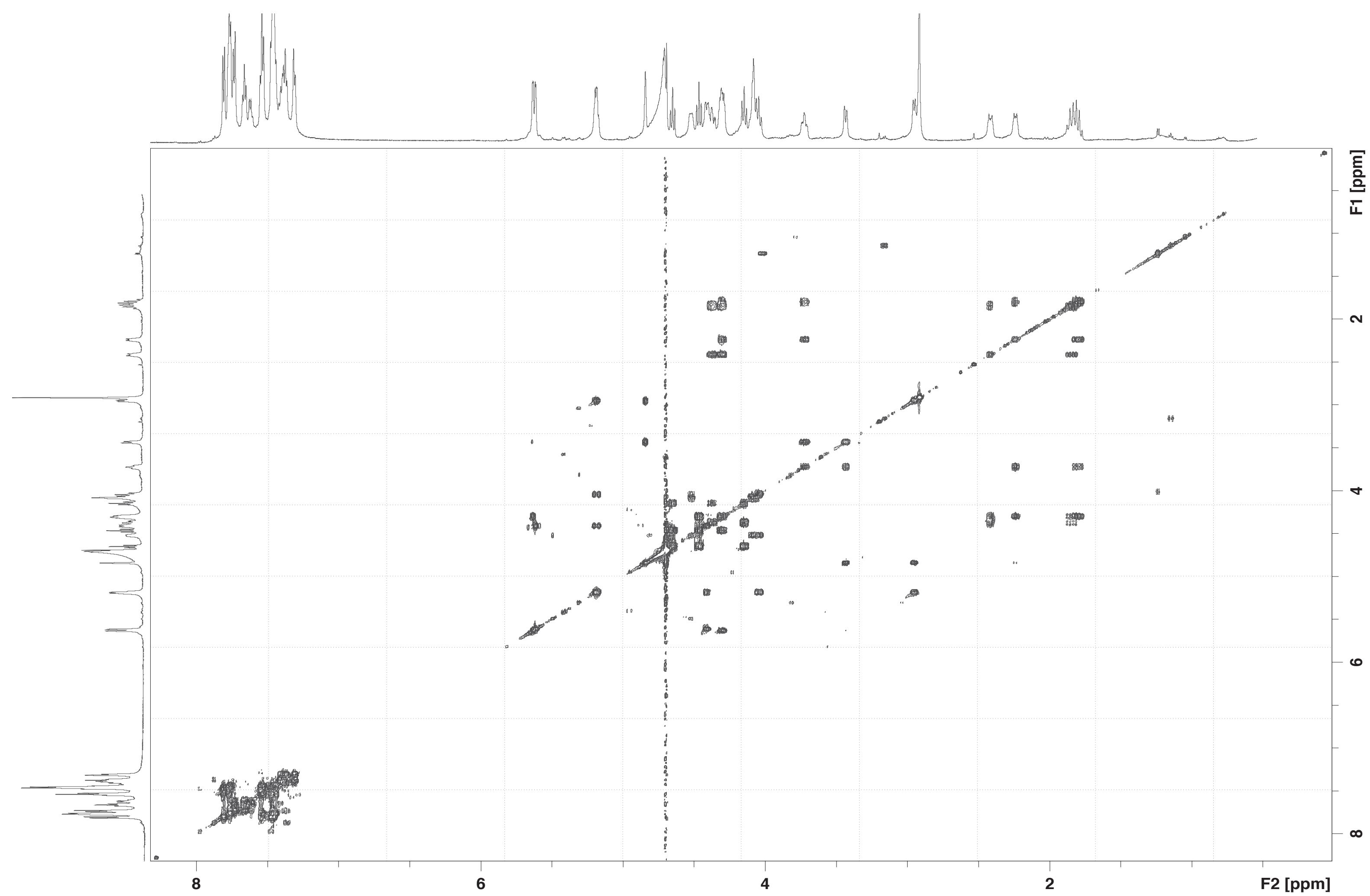


Figure S15 COSY NMR of AprBz.

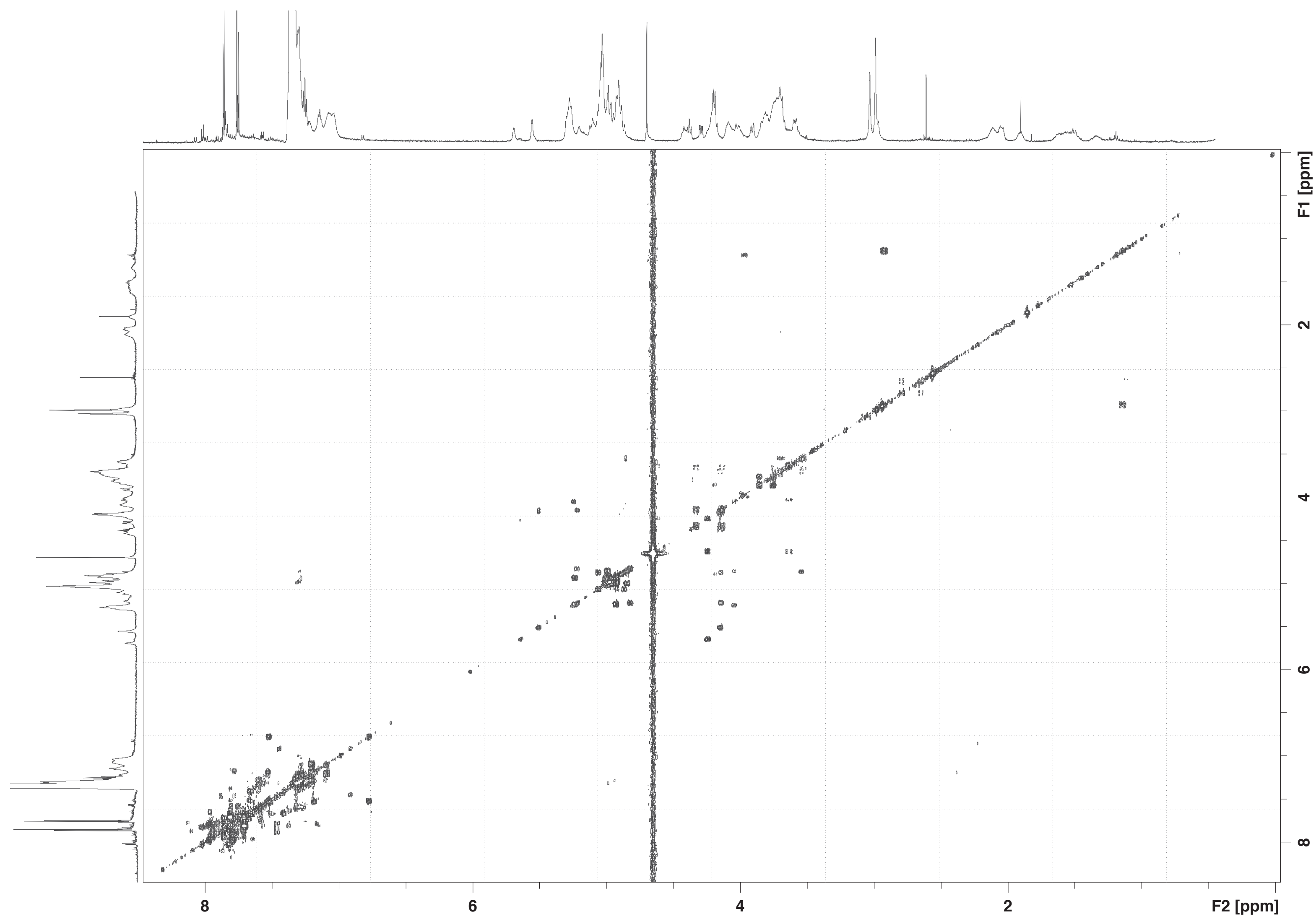


Figure S16 COSY NMR of AprCbz.

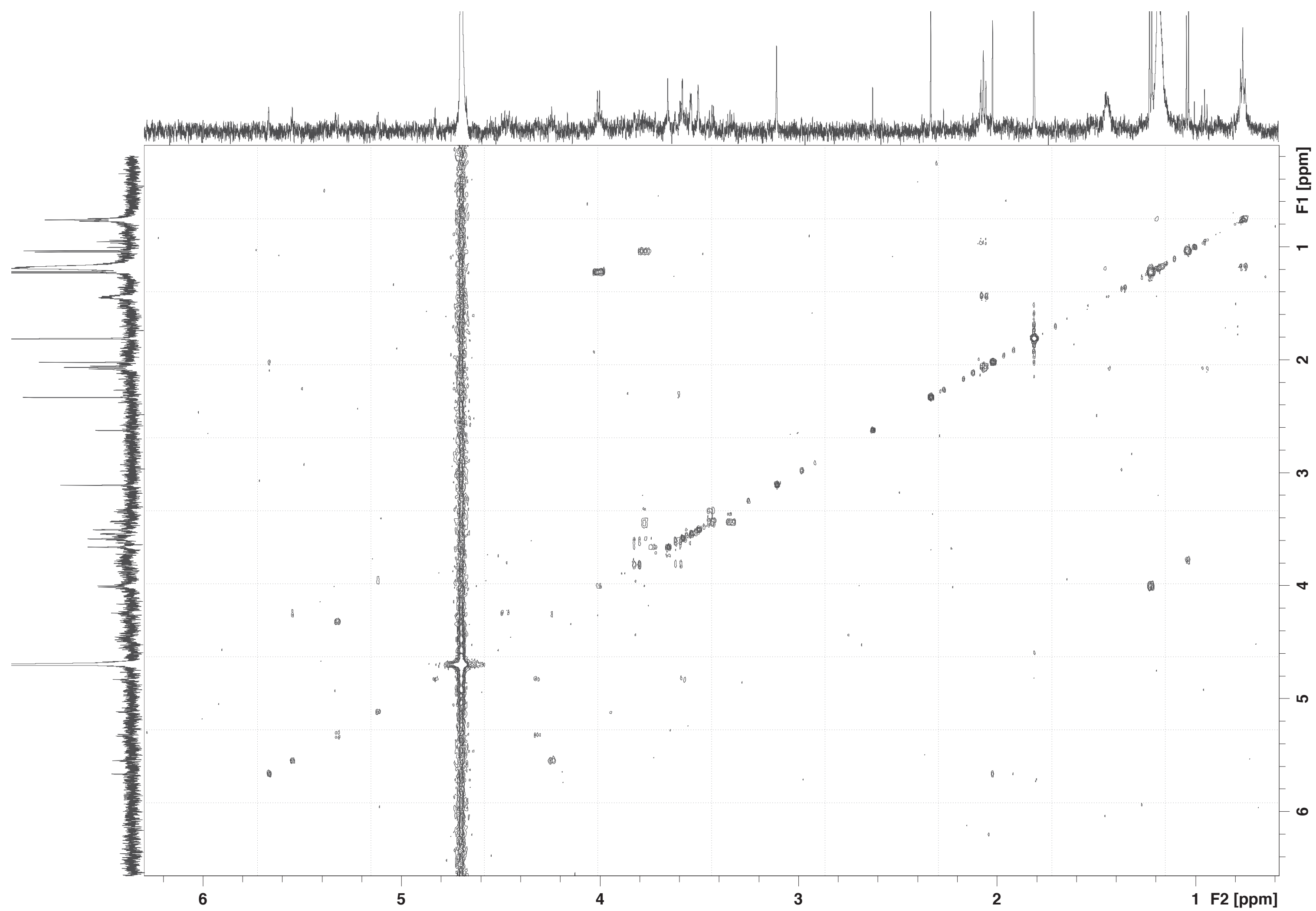


Figure S17 COSY NMR of AprPhA.

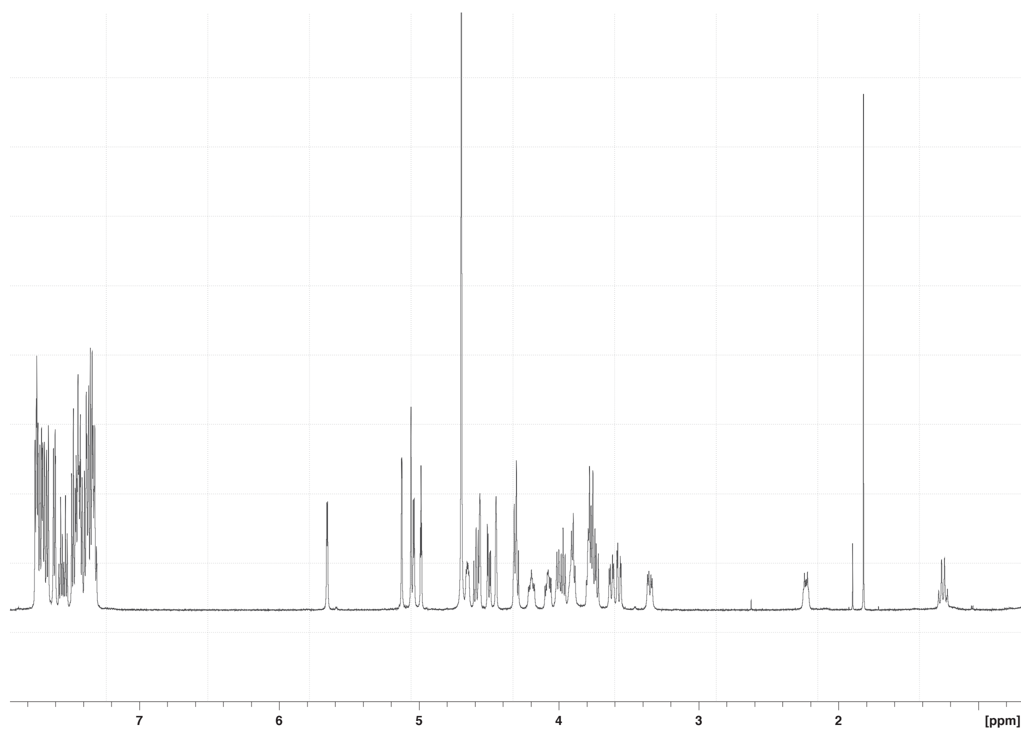


Figure S18 ^{1}H NMR of NeoBz.

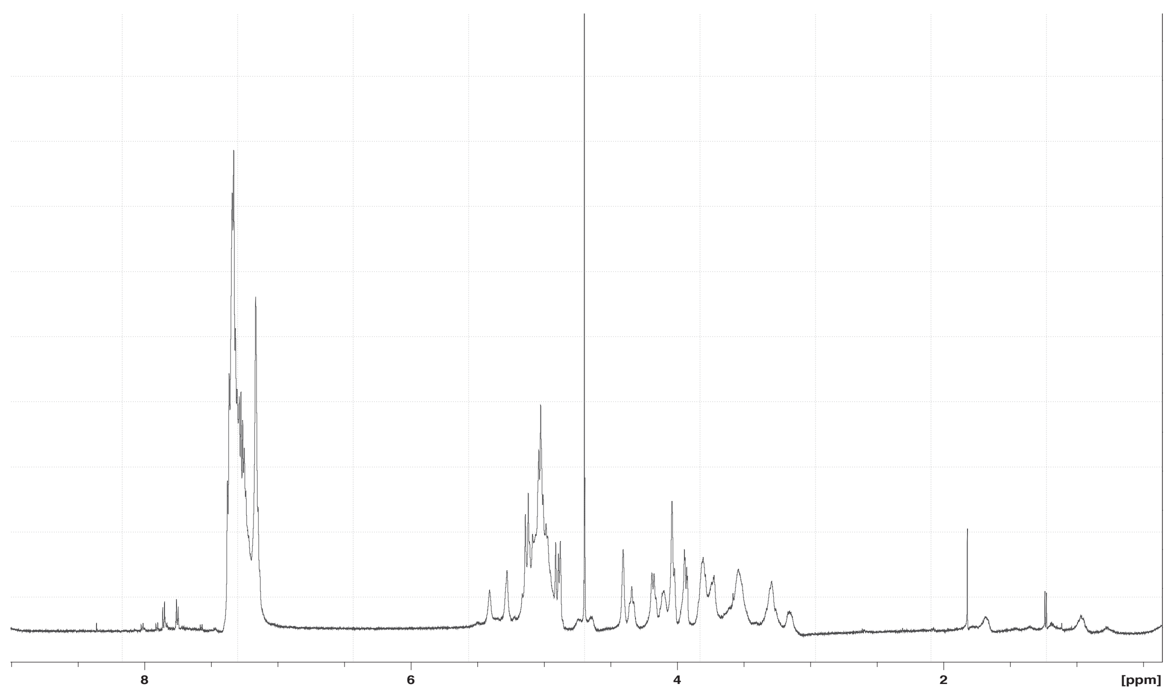


Figure S19 ^{1}H NMR of NeoCbz.

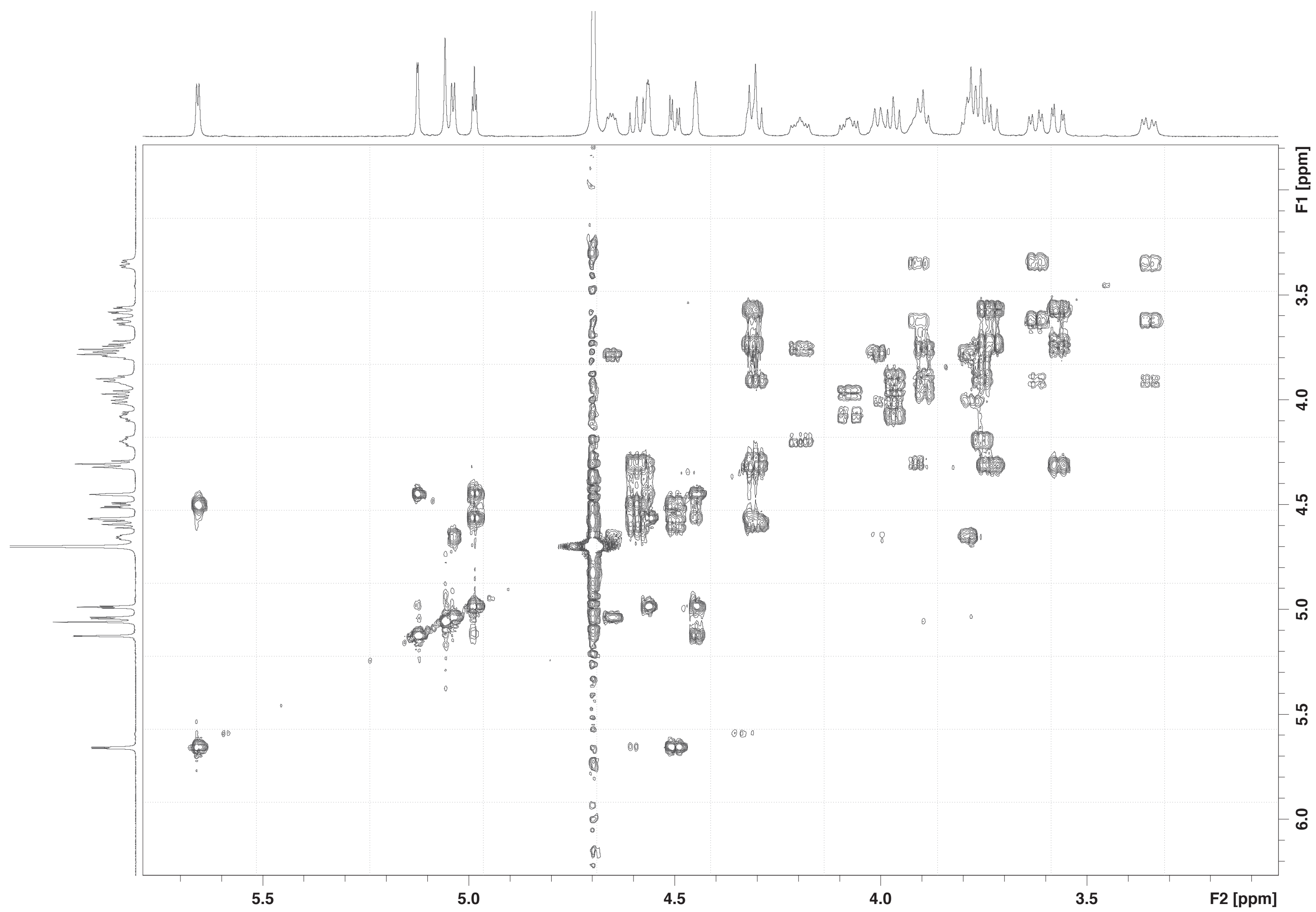


Figure S20 COSY NMR of NeoBz.

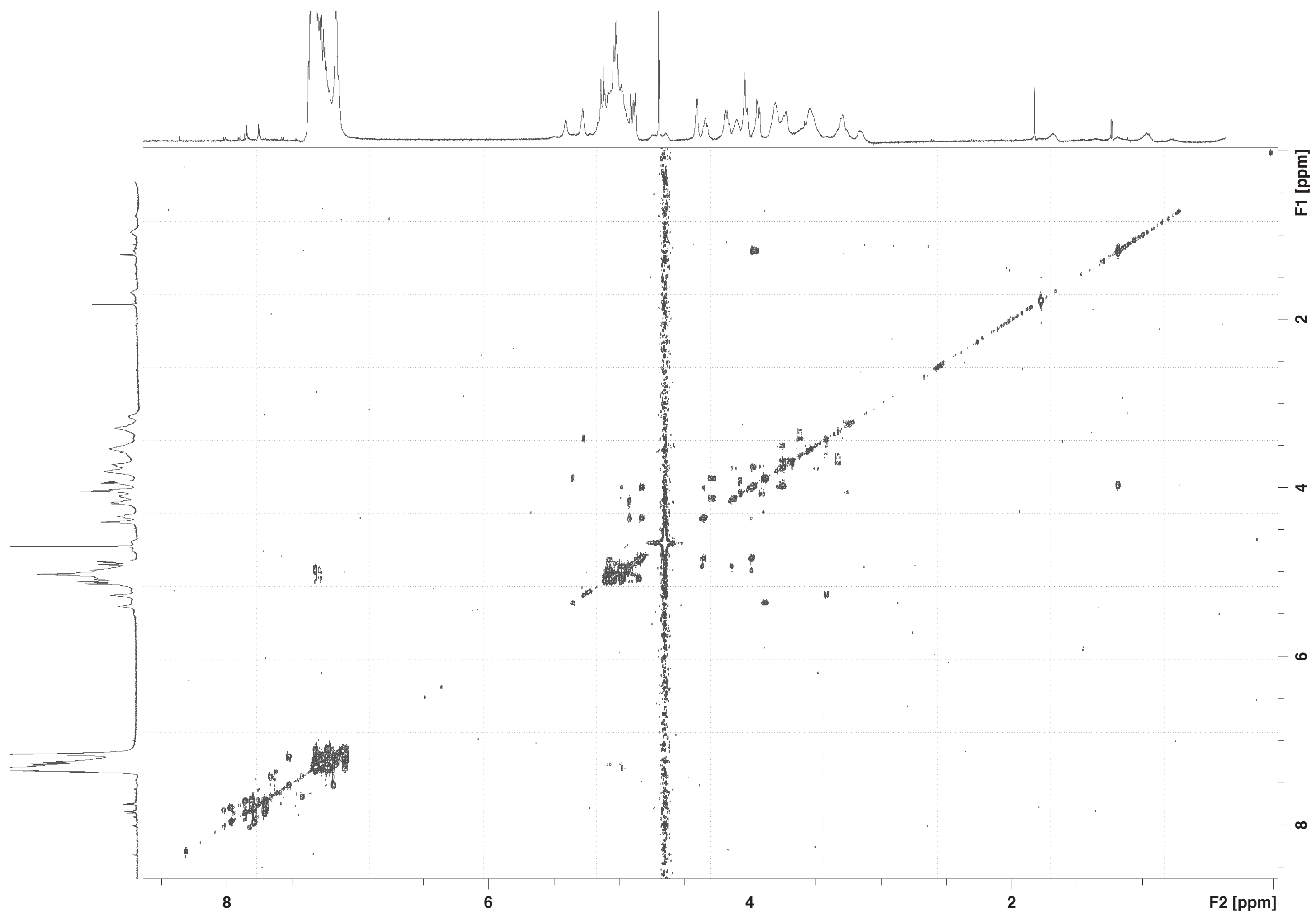


Figure S21 COSY NMR of NeoCbz.

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