SELECTIVE INHIBITION OF BACTERIAL AND HUMAN TOPOISOMERASES BY N-ARYLACYL O-SULFONATED AMINOGLYCOSIDE DERIVATIVES

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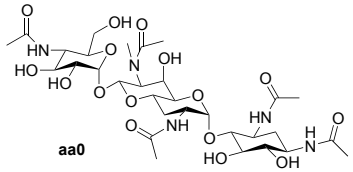
Table of Contents for Supporting Information

I. Synthesis of *N*-arylacyl *O*-sulfonated aminoglycosides

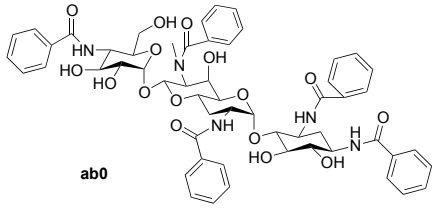
General Methods: Neomycin sulfate, apramycin sulfate, and pyridine sulfur trioxide complex were from Sigma Aldrich (St. Louis, MO, USA); kanamycin sulfate was from Bristol Laboratories Inc. (Syracuse, NY, USA). *N*-(benzyloxycarbonyloxy)succinimide, benzoyl chloride, and chlorosulfonic acid were purchased from Acros Organics (Morris Plains, NJ, USA). *N*-(phenylacetyl) succinamide was synthesized as previously reported.[1] Phenylacetyl chloride was purchased from TCI (Portland, OR, USA). Cation exchange chromatography used Amberlite IR 120 resin, Sigma Aldrich. 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 mwco 500, was purchased from Spectrum Laboratories (Rancho Dominguez, CA). A Fisher Accumet AB15 pH meter was used for all pH determinations.

A. Synthesis of *N*-arylacyl aminoglycosides

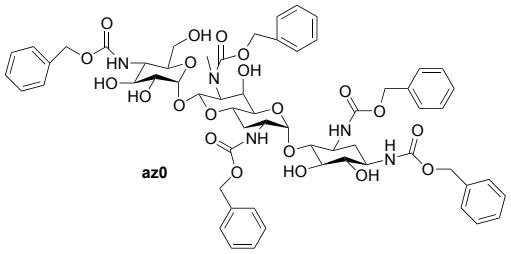
N-arylacylation of aminoglycosides was monitored using analytical HPLC: 20 µL of the reaction mixture was diluted in 80 µL ACN and 40 µL water; 100 µL of this solution was injected and eluted with a gradient of 10-95% ACN in water (0.1% TFA) over 40 min at 1 mL/min.[2] After complete acylation of amine groups, ~6-8 hours, the reaction was diluted with ice cold 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 ice 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 off 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. MS and HPLC confirmed complete *N*-acylated aminoglycoside product.



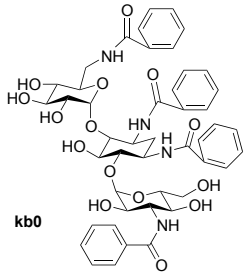
Penta *N*-acetyl-apramycin (aa0): Apramycin (0.3 mmol) was suspended in 8 mL H_2O saturated with $NaHCO_3$ and stirred to completely dissolve. A solution of *N*-acetyl succinamide (283.6 mg: 1.2 mol/mol amine group) in DMF (4 mL) was added fraction-wise (0.8 mL/90 min) to the apramycin. The clear solution was stirred at room temperature during which time a white precipitate formed; the product was further precipitated by adding additional ice-cold water (4 mL). The precipitate was collected by centrifugation at 4 °C and repeatedly washed with ice cold water (7 × 10 mL) and ethyl acetate (4 × 20 mL). The precipitate was dissolved in 10-12 mL of room temperature water, separated on a BioGel P2 column, eluted with water, and collected in 1 mL fractions. Fractions were spotted on silica plates and stained with p. anisaldehyde; fractions containing product were pooled and lyophilized to dryness, giving a white solid (**aa0**), 68% yield. ESI-LRMS calcd for $C_{31}H_{51}N_5O_{16}$ $[M + Na]^+$ *m/z* = 772.32, found 772.31.



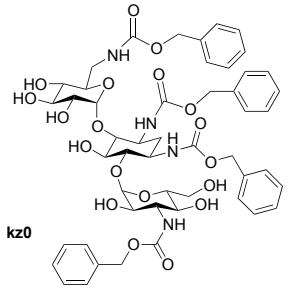
Penta *N*-benzoyl-apramycin (ab0): Apramycin (0.3 mmol) was dissolved in 9:1 MeOH:H_2O (10 mL) with Na_2CO_3 (382 mg) and stirred at 0 °C for 15 minutes. Iced benzoyl chloride (420 µL, 2.43 mol/mol amine group) was added dropwise to the solution. The reacting mixture was stirred for 0 °C, during which time a white precipitate formed. After 6 hours, the product was further precipitated by storing at 4 °C overnight. Organic solvent was removed under vacuum; the precipitate was collected by centrifugation and repeatedly washed with aq $NaHCO_3$ (5 × 10 mL) and ice-cold water (5 × 10 mL). 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 product (white solid, **ab0**) was lyophilized to dryness, 14% yield. ESI-LRMS calcd for $C_{56}H_{61}N_5O_{16}$ $[M + Na]^+$ *m/z* = 1082.40, found 1082.32.



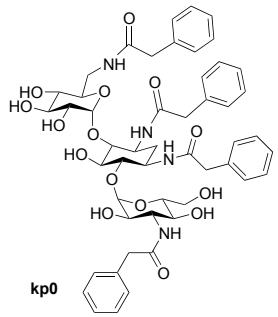
Penta *N*-benzyloxycarbonyl-apramycin (az0): A solution of *N*-(benzyloxycarbonyloxy) succinamide (456 mg, 1.2 mol/mol amine group) in DMF (4.5 mL) was added fraction-wise (0.54 mL/hr) to apramycin (0.3 mmol) dissolved in aq $NaHCO_3$ (10 mL). The reaction was stirred at room temperature, during which time a white precipitate formed. After 10 hours, the product was further precipitated by addition of ice-cold water (5 mL). The precipitate was collected by centrifugation at 4 °C, dissolved in 2:1 ACN:H_2O, separated on semi-preparative HPLC, 60% ACN (0.1%TFA), and collected in a peak eluting at 18 min. Organic solvent was removed by vacuum. The white solid (**az0**) was lyophilized to dryness, 28% yield. ESI-LRMS calcd for $C_{61}H_{71}N_5O_{21}$ $[M + Na]^+$ *m/z* = 1232.45, found 1232.36.



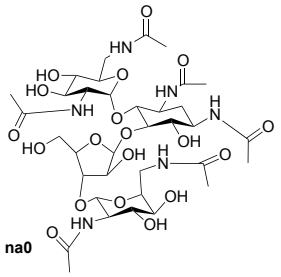
Tetra *N*-benzoyl-kanamycin A (kb0): Kanamycin A (100 mg) was dissolved in 9:1 MeOH:H_2O (10 mL) with Na_2CO_3 (200 mg) and stirred at 0 °C for 15 minutes. Cold benzoyl chloride (240 µL, 3 mol/mol amine group) was added dropwise to the solution. The mixture was stirred at 0 °C, during which time a white precipitate formed, and after 5.5 hours the product was further precipitated by addition of 1 mL ice-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 ice-cold water (5 × 10 mL). The precipitate was re-dissolved 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 white solid (**kb0**) was lyophilized to dryness, 68% yield. ESI-LRMS calcd for $C_{46}H_{52}N_4O_{15}$ $[M + Na]^+$ *m/z* = 923.33, found 923.32.



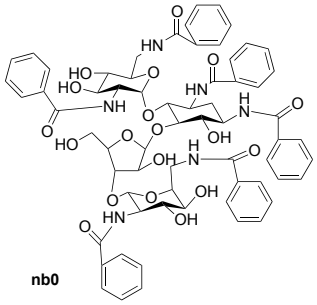
Tetra *N*-benzyloxycarbonyl-kanamycin A (kz0): A solution of *N*-(benzyloxycarbonyloxy) succinamide (1.3 mmol) in DMF (7.2 mL) was added fraction-wise (0.6 mL/90 min) to kanamycin A (0.258 mmol) dissolved in aq $NaHCO_3$ (9 mL). The reaction was stirred at room temperature, during which time a white precipitate formed, and after 11 hours, the product was further precipitated by addition of ice-cold water (5 mL). The precipitate was collected by centrifugation at 4 °C and repeatedly washed with ice-cold water (7 × 10 mL). The precipitate was collected by filtering through a glass-fritted funnel. Product was lyophilized to dryness, giving a white solid (**kz0**), 66% yield. ESI-LRMS calcd for $C_{50}H_{60}N_4O_{19}$ $[M + Na]^+$ *m/z* = 1043.38, found 1043.58.



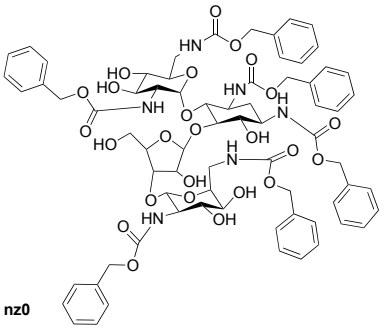
Tetra *N*-phenylacetyl-kanamycin A (kp0): A solution of *N*-(phenylacetyl) succinamide (359.5 mg, 2 mol/mol amine group) in DMF (6.5 mL) was added fraction-wise (1.25 mL/hr) to kanamycin A (0.207 mmol) dissolved in aq $NaHCO_3$ (6.7 mL). The reaction was stirred at room temperature, during which time a white precipitate formed, and after 8.5 hours the product was further precipitated by addition of ice-cold water (5 mL). The precipitate was collected by centrifugation at 4 °C and repeatedly washed with ice-cold water (7 × 10 mL). The precipitate was lyophilized to dryness to give a white solid (**kp0**), 81% yield. ESI-LRMS calcd for $C_{50}H_{60}N_4O_{15}$ $[M + Na]^+$ *m/z* = 979.40, found 979.49.



Hexa *N*-acetyl-neomycin B (na0): Neomycin B (0.3 mmol) was dissolved in aq $NaHCO_3$ (13 mL) with 1.25 mL H_2O and stirred at 0 °C. Acetic anhydride was added in 1 mol equivalents (28.4 μL) every 30 minutes, and the reaction was followed by spotting the solution on silica plates and staining with ninhydrin to detect free amines. After no unreacted product was detected (13 eq of acetic anhydride), the pH of the solution was adjusted to 7 with HCl, and the aqueous solution was separated on a BioGel P-2 column (41.7 cm × 1.5 cm), eluted with water. Product was detected by spotting fractions on silica plates and staining with Hanessian's stain. Fractions containing product were pooled, and further purified on a cation exchange column to remove unreacted material (as described in I. A.). Product was eluted with water and was lyophilized to dryness to give a white solid (**na0**), 54% yield. ESI-LRMS calcd for $C_{35}H_{58}N_6O_{19}$ $[M + Na]^+$ *m/z* = 889.37, found 889.35.

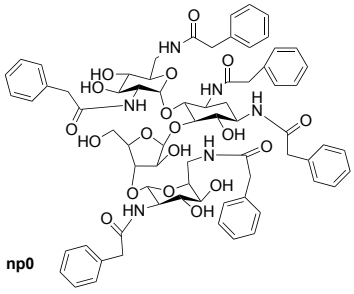


Hexa *N*-benzoyl-neomycin B (nb0): Neomycin B (0.25 mmol) was dissolved in 9:1 MeOH:H_2O (16 mL) with Na_2CO_3 (454 mg) and stirred at 0 °C for 15 minutes. Iced benzoyl chloride (420.8 μL, 2.43 mol/mol amine group) was added dropwise to the solution and the mixture was stirred at 0 °C, during which time a white precipitate formed, and after 2 hours, the product was further precipitated by addition of 10 mL ice-cold water. Organic solvent was removed under vacuum and the precipitate was collected by filtration through a glass-fritted funnel. The residue was washed with aq $NaHCO_3$ (10 mL) and ice-cold H_2O (10 mL) and collected by centrifugation at 4 °C and decanting. The precipitate was re-dissolved 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 white solid (**nb0**) was lyophilized to dryness, 63% yield. ESI-LRMS calcd for $C_{65}H_{70}N_6O_{19}$ $[M + Na]^+$ *m/z* = 1261.46, found 1261.47.



nz0

Hexa *N*-benzyloxycarbonyl-neomycin B (nz0): 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) and stirred at room temperature, during which time a white precipitate formed. After 7 hours, the mixture was further precipitated by addition of 10 mL ice-cold water. The precipitate was collected by centrifugation at 4 °C and repeatedly washed with ice-cold water (7 × 10 mL). Unreacted starting material was removed by dissolving the precipitate in room-temperature water and eluting the solvent through a cation exchange column (as described in I. A.). Product was purified by semi-preparative HPLC and lyophilized to dryness to give a white solid (**nz0**), 34% yield. ESI-LRMS calcd for $C_{71}H_{82}N_6O_{25}$ $[M + Na]^+$ *m/z* = 1441.52, found 1441.62.



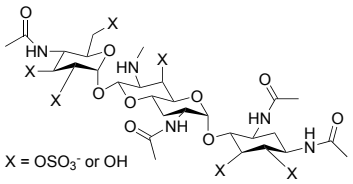
np0

Hexa *N*-phenylacetyl-neomycin B (np0): 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, during which time a white precipitate formed. After 6 hours, the product was further precipitated by addition of ice cold water (4.5 mL). The precipitate was collected by centrifugation at 4 °C and repeatedly washed with cold water (7 × 10 mL). 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 to give a white solid (**np0**), 24% yield. ESI-LRMS calcd for $C_{71}H_{82}N_6O_{19}$ $[M + Na]^+$ *m/z* = 1345.52, found 1345.49.

B. *O*-Sulfonation of *N*-arylacyl Aminoglycosides

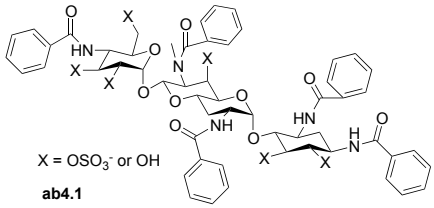
General techniques and chromatography: All glassware was oven-dried; all reactions were performed under argon gas. Two sulfonation methods were employed for sulfonation as previously described.[2] In one method, to a stirred solution of aminoglycoside (100 mg) dissolved in anhydrous DMF (1 mL) at 66 °C was added Pyr•SO_3 (3 equivalents per aminoglycoside hydroxyl) dissolved in DMF (1 mL) with anhydrous pyridine (1 mol equivalent).[3] The solution was stirred at 66 °C and monitored by analytical RPIP-HPLC as previously described.[2] Once no further sulfonation was detectable, after 12-20 hours, the solution was cooled to 4 °C with an ice bath. The solution was diluted by the addition of water (2 mL), and the resulting aqueous solution made alkaline with ice cold 10 M NaOH (added in 10 µL increments).

In the second method, $ClSO_3H$ was used to sulfonate the *N*-arylacyl aminoglycosides.[4, 5] To anhydrous pyridine (3 mL) stirring at room temperature, $ClSO_3H$ (8 equivalents per hydroxyl group) was added dropwise and the resulting clear solution was 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 the reverse phase ion pairing method (RPIP) previously described.[2] Once no further sulfonation was detectable, about 3-8 hours, the solution 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 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 by loading the aqueous product on to a preparative C18 column (4.6 mL bed volume) and purifying by RPIP as previously reported.[2]



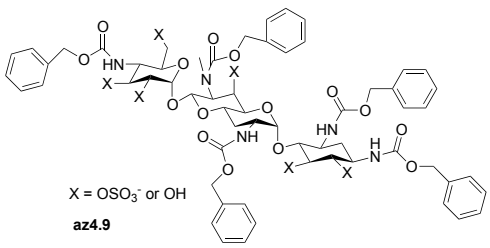
aa3-5

***O*-sulfonated Penta *N*-acetyl-neomycin (aa3-5)**: *N*-acetyl apramycin, **aa0**, (10 mg) was dissolved in anhydrous DMF (0.4 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (24 mg, 3 equivalents per hydroxyl group) dissolved in DMF (0.5 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C and monitored by analytical HPLC as described above. After 21 hours, no further sulfonation was detectable and the solution was cooled to 4 °C with an ice bath, diluted by addition of water (1 mL), and the resulting aqueous solution made alkaline by addition cold 10 M NaOH (in 10 µL increments). The resulting aqueous solution was extracted with diethyl ether (9 × 2 mL) to remove pyridine, condensed under vacuum to remove residual organic solvents, dialyzed in 100 MWCO tubing with frequent water changes (14 × 1 L over 2 days) to remove excess salt and lyophilized to dryness. LCMS analysis (as previously described[2]) showed a mixture of pentasulfated (**aa5**), tetrasulfated (**aa4**), and trisulfated (**aa3**) *N*-acetyl apramycin to give a product mixture of tri- through penta-sulfated derivatives of penta *N*-acetyl apramycin (**aa3-5**) as a white solid, 25% yield.



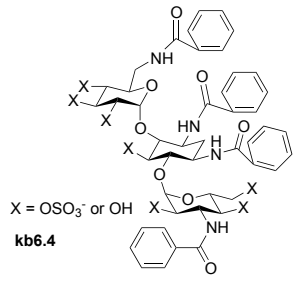
ab4.1

***O*-sulfonated Penta *N*-benzoyl-apramycin (ab4.1)**: $ClSO_3H$ (48 µL) was added dropwise to anhydrous pyridine (1 mL) stirring at room temperature, and the resulting solution was heated to 55 °C. *N*-bz apramycin, **ab0**, (15 mg) was added to the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 55 °C and reaction progress monitored by analytical HPLC as described above. After 10 hours, no further sulfonation was detectable, and the solution was cooled in an ice bath and adjusted to pH = 8 with slow addition of aqueous $NaHCO_3$ (5 mL). The resulting alkaline solution was transferred to a separatory funnel and extracted with DCM (11 × 25 mL) to remove pyridine, and the resulting aqueous layer was condensed on a rotary evaporator to remove residual organic solvent. Desalting and product purification were accomplished by loading the aqueous product on to a preparatory C18 column and eluting with RPIP buffers as previously described.[2] LCMS analysis (using the same RPIP method) showed the product to be a mixture of pentasulfated (**ab5**, 17%), tetrasulfated (**ab4**, 72%), and trisulfated (**ab3**, 11%) *N*-benzoyl apramycin to give *O*-sulfonated penta *N*-benzoyl apramycin with an average degree of sulfation of 4.1 sulfates per molecule (**ab4.1**) as a white solid, 29% yield**.**

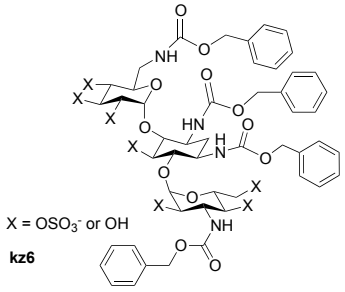


az4.9

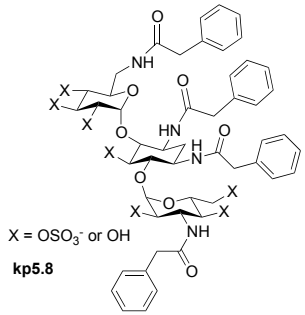
***O*-sulfonated Penta *N*-benzyloxycarbonyl-apramycin (az4.9)**: *N*-cbz apramycin, **az0**, (100 mg) was dissolved in anhydrous DMF (1 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (712 mg, 9 equivalents per hydroxyl group) dissolved in DMF (3 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C and monitored by analytical HPLC using previously the described RPIP method.[2] After 16 hours, no further sulfonation was detected, and the solution was cooled to 4 °C with an ice bath, diluted with water (5 mL), and the resulting aqueous solution made alkaline with ice cold 10 M NaOH (added in 10 µL increments). The resulting alkaline solution was extracted with diethyl ether (9 × 15 mL) to remove pyridine, and the aqueous layer was condensed under vacuum to remove residual organic solvents and dialyzed in 500 MWCO tubing with frequent water changes (14 × 1 L over 2 days) to remove salt. The product was lyophilized to dryness, and LCMS analysis showed the product to be a mixture of hexasulfated (**az6**, 18.4%), pentasulfated (**az5**, 55.5%), and tetrasulfated (**az4**, 26.1%) *N*-benzyloxycarbonyl apramycin to give *O*-sulfonated penta *N*-benzyloxycarbonyl-apramycin with an average degree of sulfation of 4.9 sulfates per molecule (**az4.9**) as a white solid, 61% yield.



***O*-sulfonated Tetra *N*-benzoyl-kanamycin (kb6.4)**:. To a solution of *N*-bz kanamycin, **kb0** (10 mg), dissolved in anhydrous DMF (200 µL) and stirring at 70 °C was added Pyr•SO_3 (39 mg, 3 equivalents per hydroxyl group) dissolved in DMF (200 µL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The reaction was monitored by analytical HPLC, and after 22.5 hours no further sulfonation was detected. The mixture was cooled to 4 °C, diluted with water (2 mL), and made alkaline by addition of cold 10 M NaOH in10 µL increments. The resulting alkaline solution was extracted with diethyl ether (5 × 10 mL) to remove pyridine; the aqueous layer was condensed under vacuum to remove residual organic solvents and dialyzed in 100 MWCO tubing with frequent water changes (12 × 1 L over 1 day). The product was lyophilized to dryness, and LCMS analysis showed the product to be a mixture of heptasulfated (**kb7**, 47.5%) hexasulfated (**kb6**, 41.5%), and pentasulfated (**kb5**, 11%) *N*-benzoyl kanamycin to give *O*-sulfonated tetra *N*-benzoyl-kanamycin with an average degree of sulfation of 6.4 sulfates per molecule (**kb6.4**) as a white solid, 54% yield.

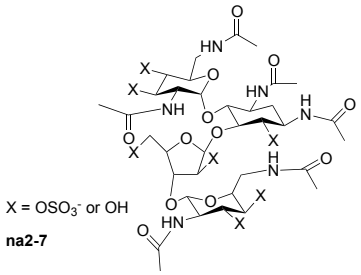


***O*-sulfonated Tetra *N*-benzyloxycarbonyl-kanamycin (kz6)**: To a solution of *N*-cbz kanamycin, **kz0** (120 mg), dissolved in anhydrous DMF (1 mL) and stirring at 66 °C was added Pyr•SO_3 (393 mg, 3 equivalents per hydroxyl group) dissolved in DMF (1.57 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C and monitored by analytical HPLC. After 20 hours, no further sulfonation was detected. The mixture was cooled to 4 °C, diluted with water (2 mL), and made alkaline by addition of cold 10 M NaOH in 10 µL increments. The resulting alkaline solution was extracted with diethyl ether (9 × 15 mL) to remove pyridine; the aqueous layer was precipitated with cold acetone (8 mL), centrifuged, and decanted. The supernatant was condensed under vacuum to remove residual organic solvent and dialyzed in 500 MWCO tubing with frequent water changes (9 × 1 L over 1 day). The product was lyophilized to dryness, and LCMS analysis showed the product to be a mixture of heptasulfated (**kz7**, 21.5%), four isoforms of hexasulfated (**kz6**, 8.5%, 38.7%, 27.1%, 12%) and four isoforms of pentasulfated (**kz5**, 0.8%, 0.5%, 6.4%) *N*-benzyloxycarbonyl kanamycin to give *O*-sulfonated tetra *N*-benzyloxycarbonyl-kanamycin with an average degree of sulfation of 6.0 sulfates per molecule (**kz6**) as a pale yellow solid.



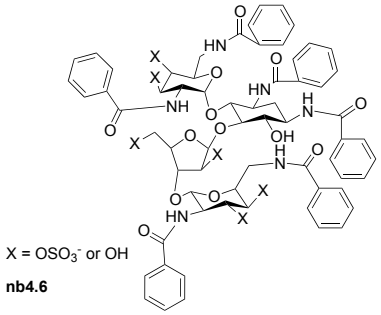
***O*-sulfonated Tetra *N*-phenylacetyl-kanamycin (kp5.8)**: *N*-pha kanamycin, **kp0**, (74 mg) was dissolved in anhydrous DMF (0.8 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (258 mg, 3 equivalents per hydroxyl group) dissolved in DMF (1.032 mL) with anhydrous pyridine (133 µL, 1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C and monitored by analytical HPLC. After 20 hours, no further sulfonation was detected and the mixture was cooled to 4 °C, diluted with water (2 mL), and made alkaline with cold 10 M NaOH added in 10 µL increments. The resulting alkaline solution was extracted with ether (6 × 15 mL) to remove pyridine; the aqueous layer was precipitated with cold acetone (8 mL), centrifuged and decanted. The supernatant was condensed under vacuum to remove organic solvent and

dialyzed in 500 MWCO tubing with frequent water changes (9 × 1 L over 1 day). The product was lyophilized to dryness, and LCMS analysis showed the product to be a mixture of heptasulfated (**kp7**, 15%), three isoforms of hexasulfated (**kp6**, 4.2%, 35.3%, 11.2%) and two isoforms of pentasulfated (**kp5**, 21.4%, 12.8%) *N*-phenylacetyl kanamycin to give *O*-sulfonated tetra *N*-phenylacetyl-kanamycin with an average degree of sulfation of 5.8 sulfates per molecule (**kp5.8**) as a white solid.



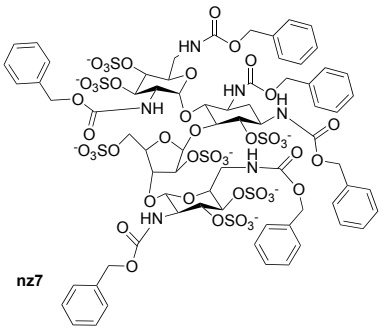
na2-7

***O*-sulfonated Hexa *N*-acetyl neomycin (na2-7)**: *N*-acetyl neomycin, **na0**, (40 mg) was dissolved in anhydrous DMF (0.4 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (102 mg, 3 equivalents per aminoglycoside hydroxyl) dissolved in DMF (0.5 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C overnight, cooled to 4 °C, diluted with water (2 mL), and made alkaline with cold 10 M NaOH (added in 10 µL increments). The resulting alkaline solution was extracted with diethyl ether (9 × 15 mL) to remove pyridine. The aqueous layer was condensed under vacuum to remove residual organic solvent and dialyzed in 100 MWCO tubing with frequent water changes (14 × 1 L over 2 days). The product was dried by lyophilizing. Analysis by direct inject ESI-MS showed product was a mixture of heptasulfated (**na7**), hexasulfated (**na6**), pentasulfated (**na5)**, tetrasulfated (**na4)**, trisulfated (**na3)**, and disulfated (**na2)** *N*-acetyl neomycin, give a product mixture of di- through hepta-sulfated derivatives of *N*-acetyl neomycin (**na2-7**) as a white solid, though quantification by separating products by degree of sulfation using RPIP-HPLC was not possible.



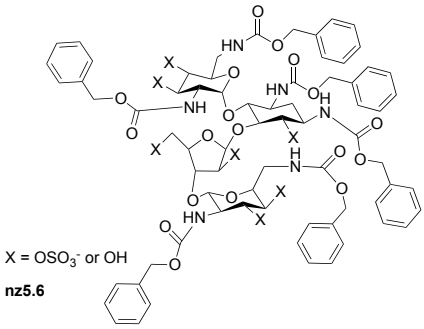
nb4.6

***O*-sulfonated Hexa *N*-benzoyl-neomycin (nb4.6)**: *N*-bz neomycin, **nb0**, (5 mg) was dissolved in anhydrous DMF (0.75 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (14.2 mg, 3 equivalents per hydroxyl group) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 68 °C and monitored by analytical HPLC. After for 3 hours, no further sulfonation was detected, and the solution was cooled to 4 °C, diluted with water (1 mL), and made alkaline with cold 10 M NaOH (added in 10 µL increments). The resulting alkaline solution was extracted with diethyl ether (11 × 1 mL) to remove pyridine. The resulting aqueous layer was condensed under vacuum to remove residual organic solvent and separated on a preparative C-18 column (4.6 mL bed volume) by eluting with 6 column volumes each: 100% H_2O; 20/80 MeOH/H_2O; 30/70 MeOH/H_2O; 50/50 MeOH/H_2O; 75/25 MeOH/H_2O. HPLC analysis showed the product was eluted with 20/80 MeOH/H_2O; the product was collected by removing organic solvent under vacuum and lyophilizing to dryness. LCMS analysis showed the product to be a mixture of hexasulfated (**nb6**, 14%), pentasulfated (**nb5**, 42.8%), tetrasulfated (**nb4**, 34.3%) and trisulfated (**nb3**, 8.9%) *N*-benzoyl neomycin to give *O*-sulfonated hexa *N*-benzoyl-neomycin with an average degree of sulfation of 4.6 sulfates per molecule (**nb4.6**) as a white solid, 65% yield.



nz7

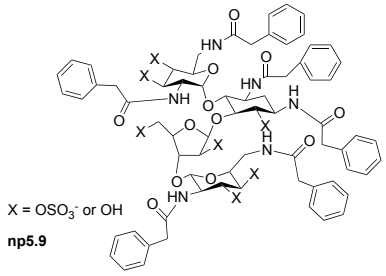
***O*-sulfonated Hexa *N*-benzyloxycarbonyl-neomycin (nz7)**: $ClSO_3H$ (134 µL) was added dropwise to anhydrous pyridine (2 mL) stirring at room temperature, and then the mixture was heated to 58 °C. *N*-cbz neomycin, **nz0**, (50 mg) in anhydrous pyridine (2 mL) was cannulated into the stirring $ClSO_3H$/pyridine solution. Heating was maintained at 58 °C and reaction progress monitored by analytical HPLC. After 6 hours, no further sulfonation was detected, and the mixture was cooled in an ice bath and adjusted to pH = 8 with slow addition of aqueous $NaHCO_3$ (7.5 mL) while stirring on ice. The resulting alkaline solution was transferred to a separatory funnel and extracted with DCM (10 × 15 mL) to remove pyridine. The resulting aqueous layer was 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 (4.6 mL bed volume) equilibrated and eluted in RPIP buffer.[2] LCMS analysis showed the product to be only hepta-*O*-sulfated hexa *N*-benzoyl neomycin (**nz7**) as a white solid, 95% yield.



X = OSO_3^- or OH

nz5.6

***O*-sulfonated Hexa *N*-benzyloxycarbonyl-neomycin (nz5.6)**: *N*-cbz neomycin, **nz0**, (60mg) was dissolved in anhydrous DMF (1 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (153 mg, 3 equivalents per hydroxyl group) dissolved in DMF (1 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C and monitored by analytical HPLC. After 21 hours, no further sulfonation was detected, and the solution was cooled to 4 °C, diluted with water (2 mL), and made alkaline with cold 10 M NaOH (added in 10 µL increments). The resulting alkaline solution was extracted with diethyl ether (9 × 10 mL) to remove pyridine. The aqueous layer was condensed under vacuum to remove residual organic solvents and dialyzed in 500 MWCO tubing with frequent water changes (14 × 1 L over 2 days). The product was lyophilized to dryness. LCMS analysis showed the product to be a mixture of hexasulfated (**nz6**, 57.7%) and pentasulfated (**nz5**, 42.3%) *N*-benzyloxycarbonyl neomycin to give *O*-sulfonated hexa *N*-benzyloxycarbonyl-neomycin with an average degree of sulfation of 5.6 sulfates per molecule (**nz5.6**) as a white solid, 67% yield.



X = OSO_3^- or OH

np5.9

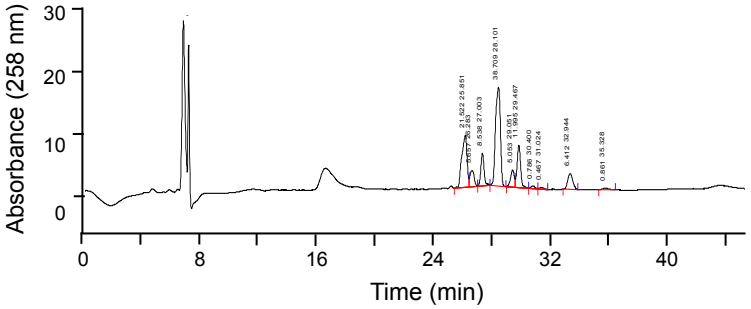
***O*-sulfonated Hexa *N*-phenylacetyl-neomycin (np5.9)**: *N*-pha neomycin, **np0**, (40mg) was dissolved in anhydrous DMF (0.4 mL) and stirred at 66 °C. To this solution was added Pyr•SO_3 (107 mg, 3 equivalents per hydroxyl group) dissolved in DMF (0.5 mL) with anhydrous pyridine (1 mol:mol Pyr•SO_3). The solution was stirred at 66 °C and monitored by analytical HPLC. After 21 hours, no further sulfonation was detected; the solution was cooled to 4 °C , diluted with water (2 mL), and made alkaline with cold 10 M NaOH (added in 10 µL increments). The resulting alkaline solution was extracted with diethyl ether (9 × 10 mL) to remove pyridine. The resulting aqueous layer was condensed under vacuum to remove residual organic solvent, dialyzed in 500 MWCO tubing with frequent water changes (14 × 1 L over 2 days), and

lyophilized to dryness. LCMS analysis showed the product to be a mixture of heptasulfated (**np7**, 3.6%) hexasulfated (**np6**, 82.1%), pentasulfated (**np5**, 14.3%) *N*-benzoyl neomycin to give *O*-sulfonated hexa *N*-benzoyl-neomycin with an average degree of sulfation of 5.9 sulfates per molecule (**np5.9**) as a white solid, 55% yield.

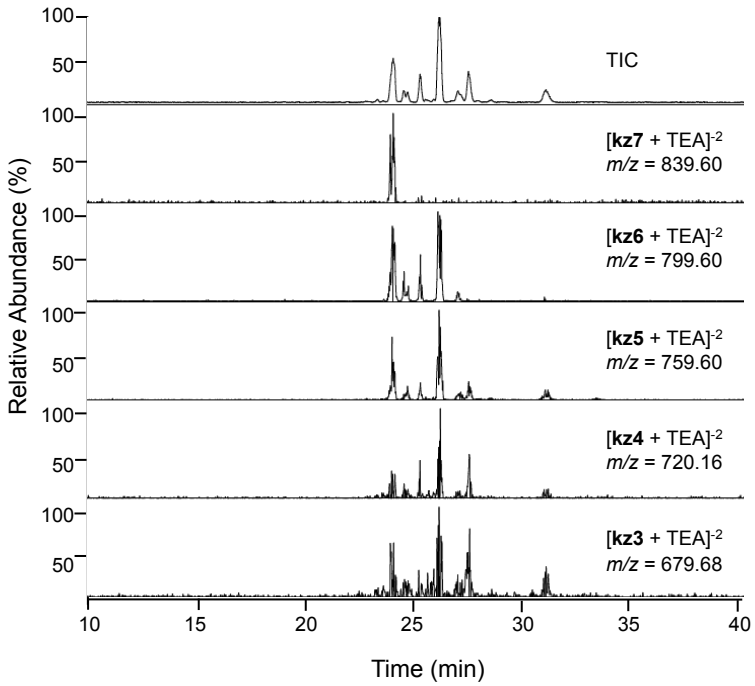
II. HPLC and LC-MS data for representative set of *O*-sulfated *N*-arylacyl aminoglycosides.

Reaction monitoring and separation of sulfation reaction products of *O*-sulfated *N*-arylacyl aminoglycosides was accomplished by RPIP methods developed for this class of compounds.[2] Compounds were detected and quantified by UV absorbance over 250 nm. Compounds were identified and characterized by LC-MS. Samples were separated by HPLC (Phenomenex Luna C18 100Å LC column, 4.6 mm × 250 mm) in 10 mM ammonium acetate aqueous buffer with pH adjusted to 8.3 with TEA in an ACN gradient of 10-30% over 20 min, then held at 30% for 18 minutes, followed by a 2 minute re-equilibration to 10% ACN. The HPLC was coupled to an LCQ Deca ESI-MS. Data were collected in the negative mode; source voltage = 3.00 kV, source current = 7.13 µA, capillary voltage = -46.00 V, capillary temperature = 203.00 °C, tube lens voltage = 25.00 V. Selected chromatograms are shown below.

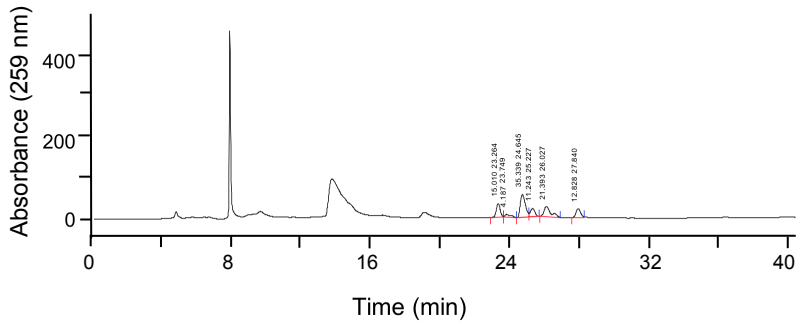
kz6, a mixture of kz7, kz6, and kz5, is separated and quantified by HPLC.



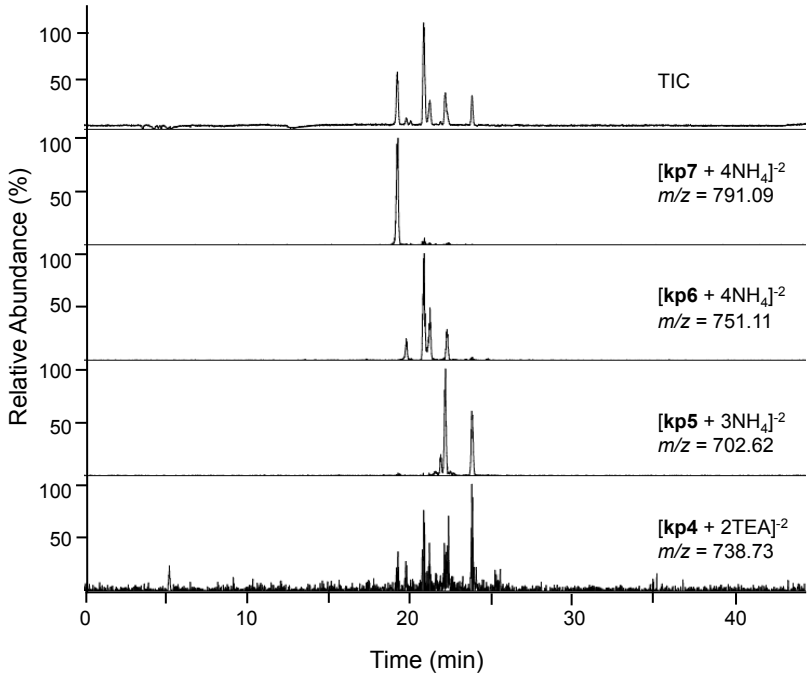
kz6, a mixture of kz7, kz6, and kz5, is separated and identified by LC-MS as previously described.[2] The total ion chromatogram (TIC) of negative ions is shown in the top panel; extracted ion chromatograms below indicate the presence of each detected degree of sulfation within the TIC. Persulfated *N*-cbz kanamycin (**kz7**) elutes at approximately 23 minutes followed by four isoforms of hexasulfated (**kz6**) and four isoforms of pentasulfated (**kz5**) *N*-cbz kanamycin. In-source fragmentation resulting in loss of sulfation produces peaks at the same time with lower degrees of sulfation including tetrasulfated (**kz4** and **kz3**) *N*-cbz kanamycin. All extracted ion chromatograms are shown for the triethylamine (TEA) salt form with a charge of -2.



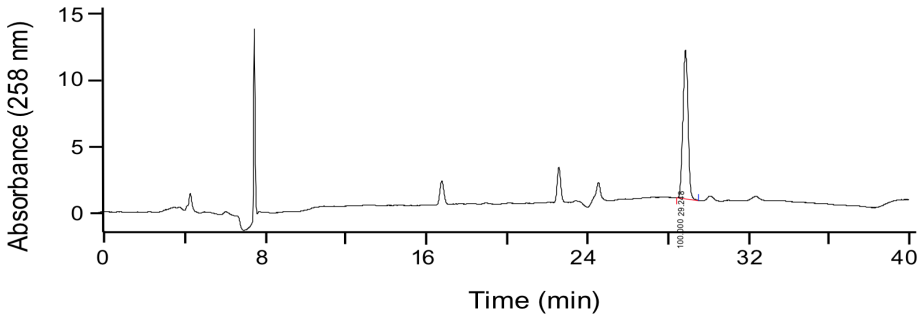
kp5.8, a mixture of kp7, kp6, and kp5, is separated and quantified by HPLC.



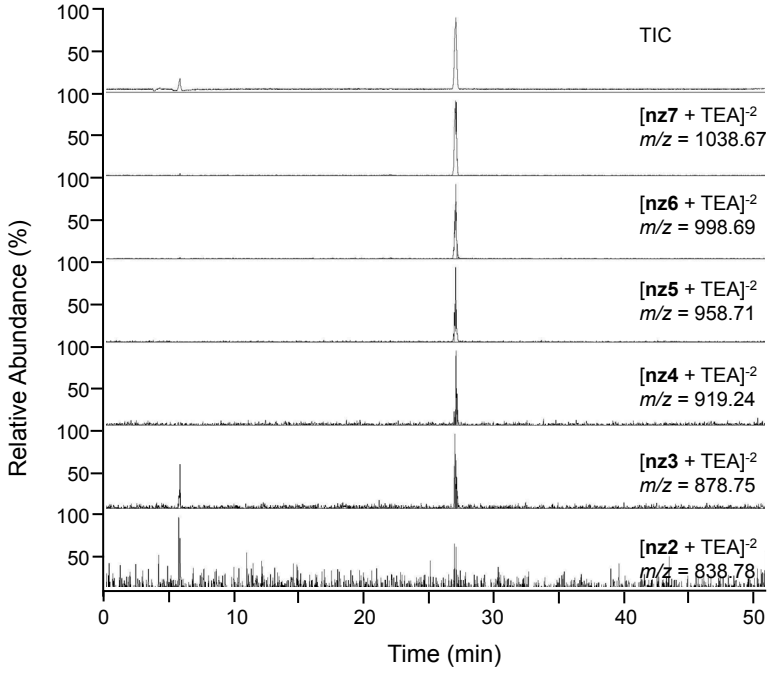
kp5.8, a mixture of kp7, kp6, and kp5, is separated and identified by LCMS LC-MS as previously described.[2] The total ion chromatogram (TIC) of negative ions is shown in the top panel; extracted ion chromatograms below indicate the presence of each detected degree of sulfation within the TIC. Persulfated *N*-pha kanamycin (**kp7**) elutes at approximately 19 minutes followed by hexasulfated (**kp6**) and pentasulfated (**kp5**) *N*-pha- kanamycin. In-source fragmentation resulting in loss of sulfation produces peaks at the same time that contain tetrasulfated (**kp4**) *N*-pha kanamycin. Extracted ion chromatograms were generated for either an ammonium (NH_4) or TEA salt form, depending on the most abundant ionization for a particular sulfation state, with a charge of -2.



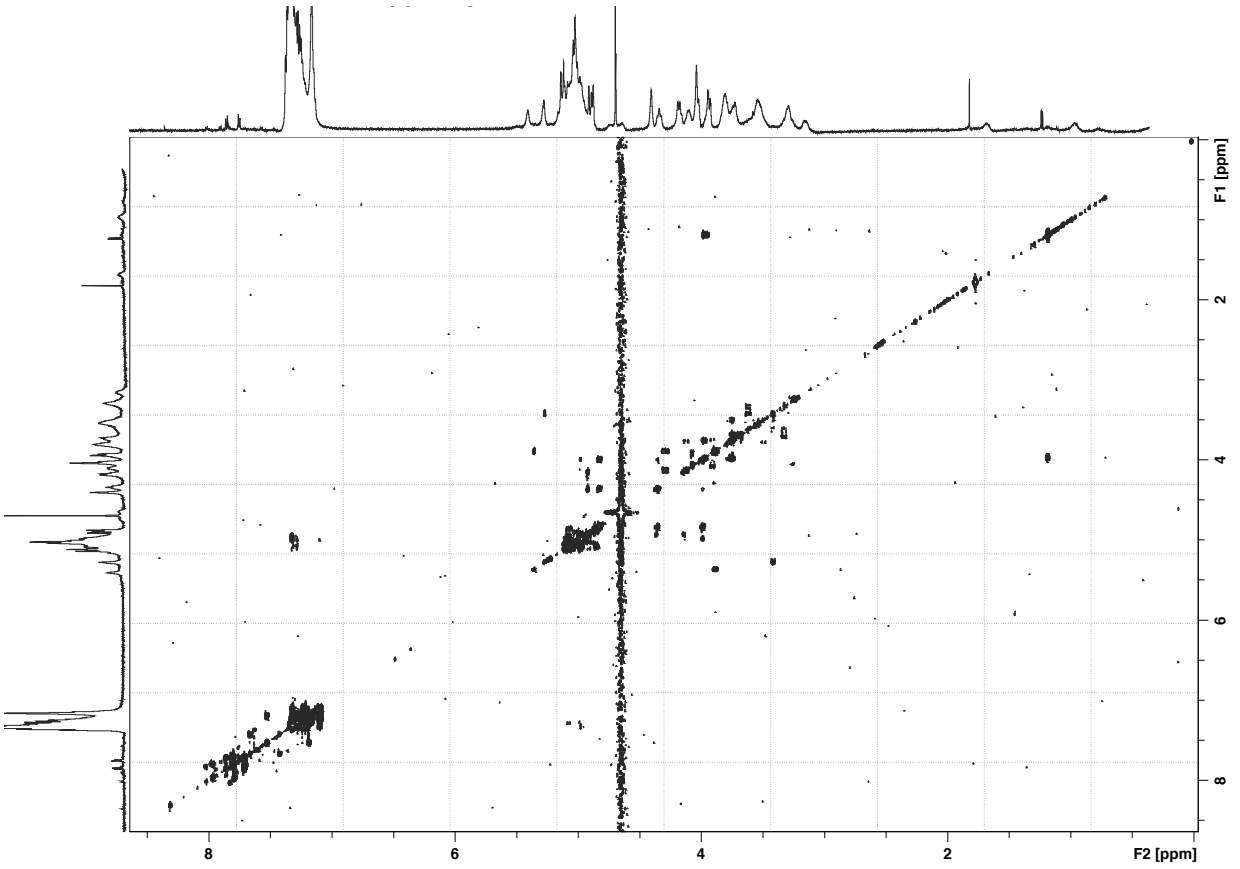
nz7 is eluted on HPLC.



nz7 is identified persulfated *N*-cbz neomycin by LCMS with previously described RPIP method.[2]



III. COSY NMR Spectrum of nz7



IV. Topoisomerase Inhibition Assays

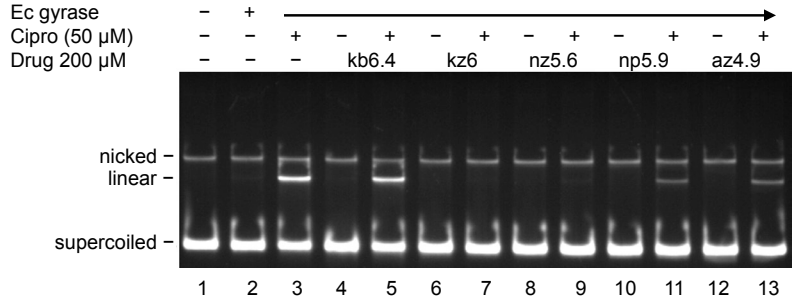
Gyrase supercoiling assay: Experiments measured the ability of *N*-arylacyl *O*-sulfonated aminoglycosides to inhibit gyrase supercoiling of relaxed DNA. Reaction mixtures (20 µL) contained 50 mM Tris (pH 8 at 23 °C), 10 mM $MgCl_2$, 50 µg/mL BSA, 1 mM ATP, 100 mM K-Glu, 5 µg/mL tRNA, 10 mM DTT, 15 µg/mL relaxed form I DNA, and 10 fmol/µL gyrase. Drug was added at 200 µM in 50 % DMSO. Reaction mixtures were incubated at 37 °C for 15 minutes, then 1 µL 0.5M EDTA was added to the reaction. Incubation was continued at 37 °C another 5 minutes before reactions were put on ice and dye mix added to each reaction. Reaction mixes were centrifuged then separated on a vertical 1.2 % TEA-agarose gel (20 V for 15 hr). DNA was quantified by Eagle Eye II.

Topoisomerase IV relaxation assay: This assay evaluated the compounds' abilities to disrupt the topoisomerase IV relaxation of supercoiled plasmid DNA. Reaction mixtures (20 µL) were run in 50 mM Tris (pH 8 at 23 °C), 10 mM $MgCl_2$, 50 µg/mL BSA, 1 mM ATP, 100 mM K-Glu, 10 mM DTT, 15 µg/mL negatively supercoiled pBR322 DNA, and 200 fmol/µL topoisomerase IV. Drug (200 µM in 50% DMSO) or 50% DMSO was added to the mixture. Reaction mixtures were incubated at 37 °C for 15 minutes. Then, 1 µL 0.5 M EDTA was added to each reaction, incubation continued another 5 minutes, then reactions were put on ice and 5.5 µL of 5 × dye mix was added. Plasmid DNA products were separated by electrophoresis through vertical 1.2% TAE agarose gels (20 V for 15 hr) and quantified by Stratagene Eagle Eye II.

Topoisomerase IV decatenation Assay: This assay evaluated the compounds' abilities to disrupt the topoisomerase IV decatenation of kinetoplast DNA. Reaction mixtures (20 µL) were run in 50 mM Tris (pH 8 at 23 °C), 10 mM $MgCl_2$, 50 µg/mL BSA, 1 mM ATP, 100 mM K-Glu, 10 mM DTT, 15 µg/mL kDNA, and 50 fmol/µL topoisomerase IV. Drug (200 µM in 50% DMSO) or 50% DMSO was added to the mixture. Reaction mixtures were incubated at 37 °C for 15 minutes. Then, 1 µL 0.5 M EDTA was added to each reaction, incubation continued another 5 minutes, then reactions were put on ice and 5.5 µL of 5 × dye mix was added. Plasmid DNA products were separated by electrophoresis through vertical 1.2% TAE agarose gels (20 V for 15 hr) and quantified by Stratagene Eagle Eye II.

Ciprofloxacin-induced cleavage of DNA with topoisomerase IV assay: Ciprofloxacin, a fluoroquinolone, is a topoisomerase poison that interrupts the religating activity of topoisomerase, halting the topoisomerase-DNA complex in the broken-strand form. Addition of certain *N*-arylacyl *O*-sulfonated aminoglycosides appears to block the ciprofloxacin-mediated DNA cleavage. Reaction mix (20 µL) contained 50 mM Tris (pH 8, 23 °C), 10 mM $MgCl_2$, 10 mM DTT, 50 µg/mL BSA, 1 mM ATP, 5 µg/mL tRNA, 15 µg/mL pBR322 DNA, 100 fmol/µL topoisomerase IV and 50 µM ciprofloxacin. Drug was added at 200 µM in 50% DMSO. The reaction was incubated at 37 °C for 10 minutes. At this point, 2.5 µL 10% SDS was added to the reaction, which then continued to incubate at 37 °C for 10 min. Then 2 µL 0.5 M EDTA was added to each reaction; reactions were incubated another 15 minutes before extracting with 20 µL phenol. The reaction was centrifuged, and the supernatant was removed, mixed with 4.5 µL 5 × DNA dye and the reaction mixture was separated on a vertical 1.2% TAE agarose gel with 0.5 µg/µL ethidium bromide (20 V for 12 hr). The reaction products were quantified with Eagle Eye II.

Blocking ciprofloxacin-induced cleavage of DNA. DNA cleavage reaction mixture contained *E. coli* gyrase (50 fmol), ciprofloxacin (50 µM), supercoiled DNA, and test compound (200 µM). Enzyme, cipro, and test compound were absent (−) in lane 1, enzyme was present (+) and cipro and test compound were absent in lane 2. Note that in the absence of ciprofloxacin, incubation of *N*-arylacyl *O*-sulfonated aminoglycosides with gyrase does not produce linear DNA.



V. Discussion of cell-based cytotoxicity

Compound nz7 and other partially sulfonated compounds were screened in triplicate with rat PC6-3 cells to look for evidence of cytotoxicity by standard MTT and LDH assays. None of the compounds showed cytotoxicity with this cell line at concentrations at 200 µM. There was no significant difference in mitochondrial activity or lactate dehydrogenase activity between untreated and treated cells. While this result is not definitive for determining that these compounds do not enter human cells, it suggests these compounds either do not enter these cells or if they do enter the cells they are not highly cytotoxic. Similarly, compounds showed no inhibition of bacterial growth in standard assays with E. coli for the determination of minimum inhibitory concentration (MIC). Assays were done in parallel with ciprofloxacin, which is a potent inhibitor of E. coli growth, and none of the test compounds used from this study showed a decrease in optical density (decrease in cell growth) when tested at concentrations up to 200 µM. This lack of a MIC with a single Gram-negative organism does not preclude the possibility that compounds from this work could be active with other types of bacteria. However, we believe identifying additional, minimally charged *N*-arylacyl *O*-sulfonated amino sugar derivatives that have a greater potency in the *in vitro* inhibition of gyrase and/or topoisomerase IV is necessary before expanding antimicrobial screening.

VI. Supporting Information References

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