Supporting Information

Toward Overcoming *Staphylococcus aureus* Aminoglycoside Resistance Mechanisms with a Functionally Designed Neomycin Analog

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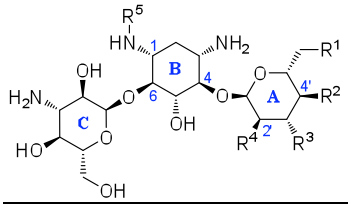
Figure S1. Structures of aminoglycosides in the kanamycin family.[1]



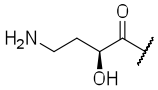
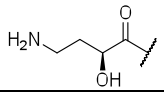
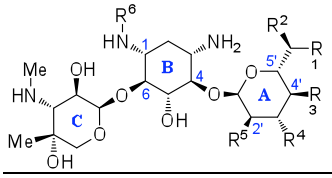
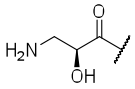
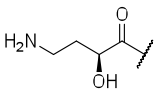
Name	R^1	R^2	R^3	R^4	R^5
Kanamycin A	NH_2	OH	OH	OH	H
Kanamycin B	NH_2	OH	OH	NH_2	H
Kanamycin C	OH	OH	OH	NH_2	H
Tobramycin	NH_2	OH	H	NH_2	H
Amikacin	NH_2	OH	OH	OH	
Dibekacin	NH_2	H	H	NH_2	H
Arbekacin	NH_2	H	H	NH_2	

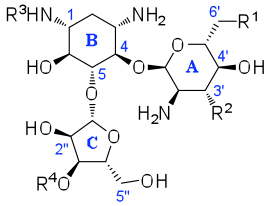
Figure S2. Structures of aminoglycosides in the kanamycin family.[1]

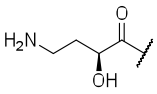
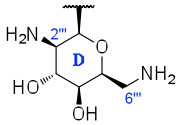
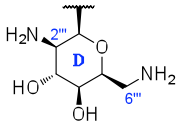
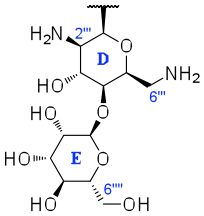
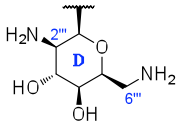


Name	R^1	R^2	R^3	R^4	R^5	R^6
Gentamicin C_1	NHMe	Me	H	H	NH_2	H
Gentamicin C_{1a}	NH_2	H	H	H	NH_2	H
Gentamicin C_2	NH_2	Me	H	H	NH_2	H
Gentamicin C_{2a}	Me	NH_2	H	H	NH_2	H
Gentamicin B	NH_2	H	OH	OH	OH	H
Sisomicin	NH_2	H	*	H	NH_2	H
Antibiotic G-52	NHMe	H	*	H	NH_2	H
Verdamycin	NH_2	Me	*	H	NH_2	H
Netilmicin	NH_2	H	*	H	NH_2	Et
Isepamicin	NH_2	H	OH	OH	OH	
ACHN-490	$NH(CH_2)_2OH$	H	*	H	NH_2	

* Unsaturated between positions C4' and C5'.

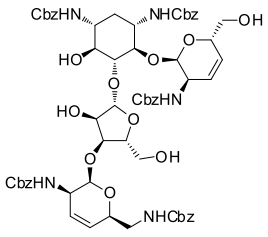
Figure S3. Structures of aminoglycosides in the neomycin family.[1]



Name	R^1	R^2	R^3	R^4
Ribostamycin	NH_2	OH	H	H
Butirosin	NH_2	OH		H
Paromomycin	OH	OH	H	
Neomycin B	NH_2	OH	H	
Lividomycin A	OH	H	H	
Lividomycin B	OH	H	H	

General Procedures

All reactions were carried out under an inert atmosphere of argon with dry solvents, using anhydrous conditions unless otherwise stated. Dry dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from a solvent delivery system with activated alumina columns. Methanol (MeOH) was distilled from CaH_2 under argon. Reagents were purchased at the highest commercial quality and used without further purification. Flash chromatography was performed with silica gel from SilicaFlash P60, particle size 40-63 μm, 230-400 mesh and distilled hexanes, ethyl acetate (EtOAc) or DCM. Free-base deprotected aminoglycosides were purified with homogeneous solvent systems consisting of $CHCl_3$/MeOH/$NH_4OH_{(aq)}$ in ratios ranging from 2:3:0.5 to 2:3:2, freshly prepared with 28% ammonia liquor before use. Yields refer to chromatographically and spectroscopically homogeneous material. Low temperature experiments conducted for longer than 3 h used a Cryocool apparatus with an acetone bath. Reactions were monitored by direct-injection low resolution mass spectrometry (LRMS) and thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica precoated plates (60F-254), visualized under UV light and developed with acidified ammonium molybdate/cerium sulfate and heat. NMR spectra were recorded on Bruker ARX-400, AV-400, AV-500 or AV-700 instruments and are calibrated using residual undeuterated solvent as an internal reference. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Low resolution mass spectra were recorded on a Thermo Finnigan Surveyor MSQ and high resolution mass spectra (HRMS) were recorded on an Agilent Technologies LC-MSD TOF mass spectrometer by electrospray ionization in positive mode, and either protonated molecular ions $[M+H]^+$ or sodium adducts $[M+Na]^+$ were used for empirical formula confirmation, unless otherwise stated. Optical rotations were recorded in a 1 dm cell at ambient temperature, on a Perkin-Elmer 343 polarimeter. Analytical HPLC was performed in Achaogen Inc. using mobile phases with 0.1% HFBA, column Sunfire C18, 3x50mm, 2.5 μm, flow of 0.5 mL/min at 40 ºC and Chemiluminescent Nitrogen Detection (CLND), water/MeOH gradient 25 to 75% in 20 min.



3

3',4',3''',4'''-Didehydro-3',4',3''',4'''-tetradeoxy-1,3,2',6',2''',6'''-penta-*N*-Cbz-paromomycin (3).

Compound **2**,[2] 1,3,2'',2''',6'''-penta-*N*-Cbz-paromomycin, (1.0 g, 0.778 mmol) was dried by evaporation with toluene three times, and the residue was dissolved in dry CH_2Cl_2 (50 mL). The mixture was cooled to 0 ºC and treated with 2,6-lutidine (0.540 mL, 4.67 mmol), followed by dropwise TBSOTf (0.390 mL, 1.71 mmol). The mixture was stirred vigorously for 45 min and quenched with 15 mL of water. The volatiles were evaporated under vacuum to a slurry, which was dissolved with EtOAc and washed sequentially with 2 M HCl, sat. $NaHCO_3$ and sat. NaCl. The organic layer was dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0 to 5% MeOH/DCM, in 0.5% increments) yielded 0.813 g of 6',5'''-bis-*O*-*tert*-butyldimethylsilyl-1,3,2'',2''',6'''-penta-*N*-Cbz-paromomycin **S1** (0.535 mmol, 69%), as a white amorphous solid.

$[\alpha]_D$ 18.51° (*c* 0.5, MeOH)

HRMS (ESI) calcd. for $C_{75}H_{103}N_5O_{24}Si_2$, $[M+Na]^+$ = 1536.64237 found: 1536.64485 (1.61 ppm).

^{1}H NMR (400 MHz, CD_3OD), δ 7.22 (m, 25H), 5.46 (bs, 1H), 5.04-4.85 (m, 13H), 4.02-3.24 (m, 22H), 1.91 (m, 1H), 1.29 (m, 1H), 0.79 (m, 18H), -0.06 (m, 12H);

^{13}C NMR (101 MHz, CD_3OD) 157.67-156.92 (5C), 136.54-136.21 (5C), 127.81-126.67 (25 C), 109.20 (1C), 99.40 (1C), 96.99 (1C), 86.82 (1C), 82.68 (1C), 78.55 (1C), 76.62 (1C), 74.52 (1C), 74.12 (1C), 72.46 (1C), 72.20 (1C), 71.83 (1C), 69.66 (1C), 67.08 (1C), 66.22-65.98 (5C), 65.85 (1C), 65.70 (1C), 62.50 (1C), 62.07 (1C), 55.25 (1C), 52.50 (1C), 50.62 (1C), 50.34 (1C), 49.94 (1C), 40.70 (1C), 33.67 (1C), 24.90 (6 C), 17.64 (2C), -6.65 (2C).

Intermediate **S1**, 6',5'''-bis-*O*-*tert*-butyldimethylsilyl-1,3,2'',2''',6'''-penta-*N*-Cbz-paromomycin, (0.300 g, 0.198 mmol) dried by evaporation with toluene three times, and the residue was dissolved in toluene (10 mL) and MeCN (2.6 mL). The mixture was treated with imidazole (0.121 g, 1.783 mmol), triphenylphosphine (0.623 g, 2.378 mmol) and triiodoimidazole (0.423g, 0.950 mmol), and heated to reflux for 80 min. The mixture was cooled,

diluted with EtOAc, and the organic layer was washed sequentially with 5% sodium thiosulfate, 0.5 M HCl, sat. $NaHCO_3$ and sat. NaCl. The organic layer was dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0% to 3% MeOH/CH_2Cl_2, in 0.5% increments) yielded 0.175 g of 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-6',5'''-bis-*O*-*tert*-butyldimethylsilyl-1,3,2'',2''',6'''-penta-*N*-Cbz-paromomycin **S2** (0.121 mmol, 61%), as a white solid. NB: Iodoimidazole byproducts may co-elute with the product, and in such cases, the residue was diluted in EtOAc, washed sequentially with 0.5 M HCl, sat. $NaHCO_3$, sat. NaCl, and then dried over Na_2SO_4.

$[\alpha]_D$ 19.41° (*c* 1.0, MeOH)

HRMS (ESI) calcd. for $C_{75}H_{99}N_5O_{20}Si_2$, $[M+Na]^+$ = 1468.63103 found: 1468.63141 (0.26 ppm).

^1H NMR (400 MHz, CD_3OD), δ 7.22 (m, 25H), 5.84 (m, 2H), 5.51 (m, 2H) 5.04-4.85 (m, 12H), 4.02-3.24 (m, 23H), 1.91 (m, 1H), 1.29 (m, 2H), 0.79 (m, 18H), -0.06 (m, 12H).

^{13}C NMR (101 MHz, CD_3OD) 157.67-156.92 (5C), 136.54-136.21 (5C), 129.93 (1C) 127.81-126.67 (26 C), 124.64 (2C), 108.49 (1C), 99.08 (1C), 95.23 (1C), 84.94 (1C), 82.36 (1C), 77.58 (1C), 74.23 (1C), 73.90 (1C), 73.41 (1C), 69.41 (1C), 66.30-65.71 (5C), 64.82 (1C), 62.88 (1C), 51.00 (1C), 50.00 (1C), 46.95 (1C), 43.73 (1C), 29.00 (1C), 28.72 (1C), 24.81 (1C), 24.72 (6 C), 17.47 (2C), -6.65 (2C).

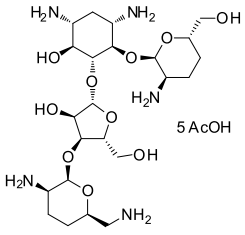
Intermediate **S2**, 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-6',5'''-bis-*O*-*tert*-butyldimethylsilyl-1,3,2'',2''',6'''-penta-*N*-Cbz-paromomycin (0.200 g, 0.138 mmol) was dissolved in dry pyridine (3 mL) and cooled to 0 ºC. The mixture was treated dropwise with 9 mL of HF-pyridine complex (70%) and stirred overnight at 0 ºC, when 10 mL of aq. NH_4Cl was added to the reaction mixture and diluted with EtOAc (250 mL). The organic layer was washed sequentially with 2 M HCl two times, sat. $NaHCO_3$ and sat. NaCl. The organic layer was dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0 to 5% MeOH/CH_2Cl_2, in 0.5% increments) yielded 0.135 g of title compound 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-1,3,2',6',2''',6'''-penta-*N*-Cbz-paromomycin **3** (0.111 mmol, 80%), as a white amorphous solid.

$[\alpha]_D$ 8.32° (*c* 0.5, MeOH)

HRMS (ESI) calcd. for $C_{74}H_{82}FN_9O_{21}$, $[M+Na]^+$ = 1240.46179, found 1240.45846 (-2.68 ppm).

^{1}H NMR (400 MHz, CD_3OD), δ 7.34 (m, 25H), 5.83 (bs, 2H), 5.61 (bs, 1H), 5.50 (bs, 1H), 5.16-5.06 (m, 13H), 4.77 (s, 2H), 4.28 (m, 6H), 4.00 (s, 1H), 3.73-3.33 (m, 13H), 2.03 (m, 2H); 1.44-1.31 (m, 1H).

^{13}C NMR (101 MHz, CD_3OD) 157.67-156.66 (5C), 136.57-136.42 (5C), 129,71 (1C), 127.81–126.64 (25C), 126.32 (1C), 125.02 (1C), 124.48 (1C), 107.66 (1C), 98.30 (1C), 94.72 (1C), 84.73 (1C), 81.46 (1C), 76.53 (1C), 76.25 (1C), 73.89 (1C), 76.25 (1C), 73.89 (1C), 69.33 (1C), 66.06 (1C), 65.76 (5C), 63.48 (1C), 61.54 (1C), 60.86 (1C), 51.16 (1C), 49.45 (1C), 43.67 (1C), 33.54 (1C).



4

3',4',3''',4'''-Tetradeoxy-paromomycin (4).

Intermediate **3**, 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-1,3,2',2''',6'''-penta-*N*-Cbz-paromomycin (20 mg, 16.4 μmol) was dissolved in 4:1 AcOH/water (5 mL), treated with 20% $Pd(OH)_2/C$ (20 mg) and stirred under a hydrogen atmosphere using a balloon for 4 h. The suspension was filtered through Celite and freeze-dried. The resulting residue was dissolved in $CHCl_3$/MeOH/$NH_4OH_{(aq.)}$ (2:3:1) and purified by column chromatography using the same solvent system (10 to 30% $NH_4OH_{(aq.)}$). The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. For characterization purposes, the aminoglycoside solution was treated with excess AcOH (50 μL) and freeze-dried to provide 8.3 mg of the penta-acetate salt of 3',4',3''',4'''-tetradeoxy-paromomycin **4** (97.4 μmol, 59%).

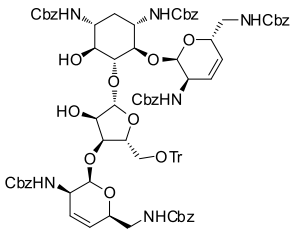
$[\alpha]_D$ 21.6° (*c* 0.15, H_2O).

HRMS (ESI) calcd. for $C_{23}H_{45}N_5O_{10}$, $[M+H]^+$ = 552.32316, found 552.32392 (1.38 ppm).

^{1}H NMR (400 MHz; D_2O): δ 5.50 (s, 1H), 5.24 (s, 1H), 4.90 (s, 1H), 4.38-4.35 (m, 1H), 4.25-4.23 (m, 1H), 4.07-4.03 (m, 1H), 3.92-3.71 (m, 6H), 3.65-3.31 (m, 8H), 3.25-3.14 (m, 2H), 3.05-2.99 (m, 1H), 2.35-2.30 (m, 1H), 2.05-1.96 (m, 4H), 1,83 (s, AcOH), 1.73-1.56 (m, 3H), 1.45-1.35 (m, 2H).

^{13}C NMR (101 MHz; D_2O): δ 180.80 (AcOH), 109.80 (1C), 97.22 (1C), 94.80 (1C), 84.12 (1C), 80.78 (1C), 77.55 (1C), 74.14 (1C), 72.83 (1C), 72.18 (1C), 72.11 (1C), 70.56 (1C), 62.78 (1C), 59.80 (1C), 49.43 (1C), 48.64 (1C), 48.54 (1C), 47.28 (1C), 42.10 (1C), 28.46 (1C), 23.56 (1C), 23.40 (1C), 22.74 (AcOH), 20.76 (1C), 20.49 (1C).

LC/CLND, water/MeOH gradient 25 to 75% in 20 min, R_t = 12.09 min, 96.5% purity.



6

3',4',3''',4'''-Didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-1,3,2',6',2''',6''',-hexa-*N*-Cbz-neomycin (6).

Compound **5**,[2] 1,3,6',2'',2''',6'''-hexa-*N*-Cbz-neomycin (1.45 g, 1.02 mmol) was dissolved in pyridine (19 mL), treated with added DMAP (6.1 mg, 0.05 mmol) and triphenylmethylene chloride (1.42 g, 5.111 mmol), and stirred with heating to 70 ºC overnight. The volatiles were evaporated and the crude residue was dissolved in CH_2Cl_2, washed sequentially with sat. $NaHCO_3$ and sat. NaCl. The organic layer was dried over Na_2SO_4 and concentrated to a residue. Purified by column chromatography (0 to 4% MeOH/CH_2Cl_2, with 0.7% increments) yielded 1.30 g of 5''-*O*-trityl-1,3,2',6',2''',6'''-hexa-*N*-Cbz-neomycin **S3** (0.782 mmol, 77%), as a white solid.

$[\alpha]_D$ 21.6° (*c* 0.51, $CHCl_3$).

HRMS (ESI) calcd. for $C_{90}H_{96}N_6O_{25}$, [M+Na]+ = 1683.62936, found 1683.63173 (1.41 ppm).

^{1}H-NMR (400 MHz, $(CD_3)_2SO$): δ 8.23-7.89 (m, 44H), 7.69-7.67 (m, 1H), 7.24-7.19 (m, 1H), 6.93-6.91 (m, 1H), 6.37-6.33 (m, 1H), 6.15-6.08 (m, 2H), 5.99-5.62 (m, 16H), 5.49-5.46 (m, 1H), 5.00-4.92 (m, 2H), 4.75-4.73 (m, 1H), 4.64-4.46 (m, 4H), 4.43-4.26 (m, 4H), 4.20-3.83 (m, 9H), 2.58-2.53 (m, 2H), 2.21-2.16 (m, 1H).

^{13}C-NMR (101 MHz, $(CD_3)_2SO$) δ 157.38-156.64 (6C), 144.73 (3C), 138.25-137.81 (6C), 129.52-127.69 (45C), 110.14 (1C), 98.75 (1C), 98.20 (1C), 86.88 (1C), 85.91 (1C), 81.15 (1C), 79.60 (1C), 78.04 (1C), 77.96 (1C), 74.15 (1C), 73.12 (1C), 72.67 (1C), 72.25 (1C), 71.94 (1C), 71.39 (1C), 70.64 (1C), 67.40 (1C), 66.34-65.86 (6C), 64.60 (1C), 60.69 (1C), 56.49 (1C), 43.21 (1C), 35.57 (1C), 21.70 (1C), 15.01 (1C).

Intermediate **S3**, 5''-*O*-trityl-1,3,2',6',2''',6'''-hexa-*N*-Cbz-neomycin (1.30 g, 0.783 mmol) was dissolved in toluene (43 mL) and MeCN (10.75 mL). The solution was treated with imidazole (0.320 g, 4.697 mmol), triphenylphosphine (2.46 g, 9.394 mmol) and triiodoimidazole (1.68 g, 3.758 mmol), and heated to reflux for 80 min. The mixture was cooled, diluted with EtOAc and washed sequentially with 5% sodium thiosulfate, 0.5 M HCl, sat. $NaHCO_3$ and sat.

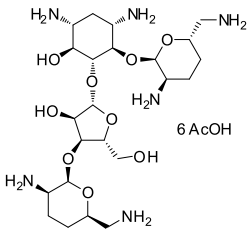
NaCl. The organic layer was dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0 to 2% MeOH/ CH_2Cl_2, with 0.5% increments) yielded 810 mg of title compound 3',4',3''',4'''-Didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-1,3,2',6',2''',6'''-hexa-*N*-Cbz-neomycin **6** (0.508 mmol, 65%), as a white solid.

$[\alpha]_D$ 4.9° (*c* 0.333, $CHCl_3$).

HRMS (ESI) calcd. for $C_{90}H_{92}N_6O_{21}$, $[M+Na]^+$ = 1474.5502, found 1474.5450 (4.30 ppm).

^{1}H-NMR (400 MHz, $(CD_3)_2SO$): δ 7.43-7.04 (45H, m), 6.64-6.61 (m, 1H), 5.77-5.72 (m, 2H), 5.51-5.47 (m, 1H), 5.32-5.25 (m, 3H), 5.18-4.96 (m, 14H), 4.85-4.77 (m, 2H), 4.58 (m, 1H), 4.25-4.18 (m, 2H), 4.12-3.98 (m, 5H), 3.59-3.43 (m, 5H), 3.25-3.08 (s, 5H), 3.04-2.98 (m, 1H), 2.88-2.83 (m, 1H), 1.82-1.77 (m, 1H), 1.36-1.32 (m, 1H).

^{13}C-NMR (101 MHz, $(CD_3)_2SO$): 156.84-155.62 (6 C), 143.74 (3C), 137.26-136.85 (6C), 129.97 (1C), 128.42-127.04 (46C), 125.67 (1C), 125.30 (1C), 108.15 (1C), 98.13 (1C), 96.38 (1C), 86.04 (1C), 82.54 (1C), 80.42 (1C), 80.05 (1C), 77.36 (1C), 74.12 (1C), 72.86 (1C), 68.07 (1C), 65.51-65.29 (6C), 64.45 (1C), 59.88 (1C), 51.16 (1C), 50.61 (1C), 47.68 (1C), 46.62 (1C), 44.11 (1C), 34.86 (1C), 20.89 (1C), 14.20 (1C).



7

3',4',3''',4'''-Tetradeoxy-neomycin (7).

Intermediate **6**, 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-1,3,2',6',2''',6'''-hexa-*N*-Cbz-neomycin (20 mg, 12.5 μmol) was dissolved in 1:4 MeOH/1 M HCl (5 mL), treated with 20% $Pd(OH)_2/C$ (20 mg) and stirred under a hydrogen atmosphere using a balloon for 4 h. The suspension was filtered through Celite and freeze-dried. The resulting residue was dissolved in $CHCl_3/MeOH/NH_4OH_{(aq.)}$ (2:3:1) and purified by column chromatography using the same solvent system (10 to 30% $NH_4OH_{(aq.)}$). The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. For characterization purposes, the aminoglycoside solution was treated with excess AcOH (50 μL) and freeze-dried to provide 8.3 mg of the hexa-acetate salt of 3',4',3''',4'''-tetradeoxy-neomycin **7** (8.9 μmol, 71%).

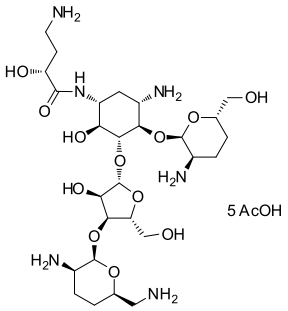
$[\alpha]_D$ 37.8° (*c* 0.315, H_2O).

HRMS (ESI) calcd. for $C_{23}H_{46}N_6O_9$, $[M+Na]^+$ = 573.32424, found 573.32185 (-4.17 ppm).

^1H NMR (400 MHz, D_2O) δ 5.75 (d, 3.51 Hz, 1H), 5.29 (d, 2.45 Hz, 1H), 4.92 (bs, 1H), 4.35 (m, 1H), 4.27 (m, 1H), 4.09 (m, 2H), 3.94-3.89 (m, 1H), 3.76 (m, 3H), 3.61 (m, 3H), 3.45-3.40 (m, 1H), 3.19-3.07 (m, 6H), 2.25 (m, 1H), 2.09-1.90 (m, 5H) 1.88 (s, AcOH), 1.66-1.59 (m, 2H) 1.59-1.40 (m, 2H).

^{13}C NMR (101 MHz, D_2O) δ 180.74 (AcOH), 109.95 (1C), 97.33 (1C), 93.84 (1C), 85.13 (1C), 81.02 (1C), 76.53 (1C), 74.39 (1C), 73.04 (1C), 72.56 (1C), 72.20 (1 C), 65.33 (1C), 60.06 (1C), 49.81 (1C), 48.46 (1C), 48.37 (1C), 47.30 (1C), 42.19 (1C), 42.11 (1C), 29.55 (1C), 25.8 (1C), 23.56 (1C), 22.69 (AcOH), 20.48 (1C), 20.16 (1C).

LC/CLND, water/MeOH gradient 25 to 75% in 30 min, R_t = 20.09 min, 98.7% purity.



9

1-*N*-((2''''*R*)-4''''-Amino-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-paromomycin (9).

Intermediate **3**, 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-1,3,2',2''',6'''-penta-*N*-Cbz-paromomycin (0.150 g, 0.136 mmol) was dissolved in DMF (15 mL), and treated with 2.0 M LiOH (1.5 mL) at room temperature for 24 h. The solution was neutralized with aq. NH_4Cl and diluted with EtOAc. The organic layer was partitioned, dried over Na_2SO_4 and concentrated to a residue which was used without further purification. The crude residue was dried by evaporation with toluene three times, dissolved in dry CH_2Cl_2 (5 mL) and cooled to 0 ^oC. The mixture was treated with (2*R*)-4-carboxybenzylamino-2-hydroxybutanoic acid (49 mg, 0.194 mol), DIPEA (0.241 mL, 0.369 mmol) and EDC (55 mg, 0.029 mmol). The mixture was warmed to room temperature while stirring for 12 h. The solution was added sat. NH_4Cl (5 mL) and the volatiles were evaporated under vacuum. The crude mixture was diluted with EtOAc, washed sequentially with 0.5 M HCl, sat. $NaHCO_3$, sat. NaCl, dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0 to 9% MeOH/CH_2Cl_2, with 1% increments) yielded 82 mg of 1-*N*-((2''''*R*)-4''''-carboxybenzylamino-2''''-hydroxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-3,2',2''',6'''-penta-*N*-Cbz-paromomycin **8** (62.1 μmol, 46% for 2 steps), as a white solid. HRMS (ESI) calcd. for $C_{67}H_{78}N_6O_{22}$, $[M+Na]^+$ = 1341.50588, found 1341.50614 (0.19 ppm).

Intermediate **8**, 1-*N*-((2''''*R*)-4''''-carboxybenzylamino-2''''-hydroxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-3,2',2''',6'''-penta-*N*-Cbz-paromomycin (14.5 mg, 11 μmol) was dissolved in 4:1 AcOH/water (5 mL), treated with 20% $Pd(OH)_2/C$ (20 mg) and stirred under a hydrogen atmosphere using a balloon for 4 h. The suspension was filtered through Celite and freeze-dried. The resulting residue was dissolved in $CHCl_3$/MeOH/$NH_4OH_{(aq.)}$ (2:3:1) and purified by column chromatography using the same solvent system (10 to 30% $NH_4OH_{(aq.)}$). The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was freeze-dried. The dry residue obtained was

redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. For characterization purposes, the aminoglycoside solution was treated with excess AcOH (50 μL) and freeze-dried to provide 8.3 mg of the penta-acetate salt of 1-*N*-((2""*R*)-4""-amino-2""-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-paromomycin **9** (8.7 μmol, 79%), as a white solid.

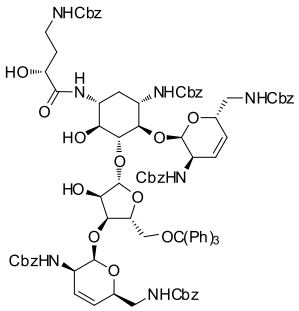
$[\alpha]_D$ 26.1° (*c* 0.5, H_2O).

HRMS (ESI) calcd. for $C_{27}H_{52}N_6O_{12}$, $[M+Na]^+$ = 675.35354, found 675.35179 (-2.59 ppm).

^{1}H NMR (500 MHz, D_2O) δ 5.56-5.53 (m, 1H), 5.28-5.27 (m, 1H), 4.95-4.92 (m, 1H), 4.43-4.40 (m, 1H), 4.36 (s, 1H), 4.29-4.23 (m, 2H), 4.09-4.06 (m, 1H), 3.95-3.75 (m, 5H), 3.68-3.49 (m, 4H), 3.48-3.34 (m, 2H) 3.21-3.18 (m, 1H), 3.11-3.04 (m, 2H), 2.81-2.77 (m, 1H), 2.70-2.64 (m, 1H), 2.15-2.00 (m, 3H), 1.95-1.87 (m, 3H), 1.82-1.81 (m, AcOH), 1.78-1.57 (m, 4H), 1.44-1.35 (m, 2H).

^{13}C NMR (126 MHz, D_2O) δ 181.28 (AcOH), 175.62 (1C), 110.27 (1C), 97.61 (1C), 95.29 (1C), 85.42 (1C), 81.02 (1C), 78.32 (1C), 74.52 (1C), 73.63 (1C), 72.63 (1C), 70.86 (1C), 69.54 (1C), 63.32 (1C), 60.16 (1C), 49.51 (1C), 49.04 (1C), 48.74 (1C), 42.57 (1C), 36.66 (1C), 35.84 (1C), 30.93 (1C), 30.63 (1C), 23.97 (2C), 23.22 (AcOH), 21.24 (1C), 20.89 (1C), 20.28 (1C).

LC/CLND, water/MeOH gradient 30 to 75% in 30 min, R_t = 15.47 min, 95.0% purity.



10

1-*N*-((2'''''*R*)-4'''''-Carboxybenzylamino-2'''''-hydroxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-3,2',6',2''',6'''-penta-*N*-Cbz-neomycin (10).

Intermediate **6**, 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-1,3,2',6',2''',6'''-hexa-*N*-Cbz-neomycin (0.394 g, 0.247 mmol) was dissolved in DMF (15 mL), and treated with 2.0 M LiOH (1.5 mL) at room temperature for 24 h. The solution was neutralized with sat. NH_4Cl and diluted with EtOAc. The organic layer was partitioned, dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0 to 4.5% MeOH/CH_2Cl_2, with 0.75% increments) yielded 0.216 g of 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-3,2',6',2''',6'''-penta-*N*-Cbz-neomycin **S4** (1.48 mmol, 60%), as a white solid.

$[\alpha]_D$ 4.9° (*c* 0.333, $CHCl_3$).

HRMS (ESI) calcd. for $C_{82}H_{86}N_6O_{19}$, $[M+H]^+$ = 1459.60213, found 1459.60205 (-0.05 ppm).

^{1}H-NMR (400 MHz, $(CD_3)_2SO$): δ 7.43-7.04 (m, 40H), 6.64-6.61 (m, 1H), 5.74 (m, 2H), 5.50 (m, 1H), 5.30 (m, 2H), 5.17-4.96 (m, 13H), 4.60 (m, 1H), 4.26-4.21 (m, 2H), 4.07-4.03 (m, 5H), 3.54-2.96 (m, 12H), 1.80-1.62 (m, 2H), 1.21-1.11 (m, 1H).

^{13}C-NMR (101 MHz, $(CD_3)_2SO$): 156.84-155.62 (5C), 143.74 (3C), 137.26-136.85 (5C), 129.97 (1C), 128.42-127.04 (41C), 125.67 (1C), 125.30 (1C), 107.75 (1C), 98.08 (1C), 96.36 (1C), 86.21 (1C), 86.04 (1C), 82.16 (1C), 80.84 (1C), 80.03 (1C), 77.91 (1C), 77.93 (1C), 72.86 (1C), 68.04 (1C), 65.58-65.32 (5C), 64.44 (1C), 51.13 (1C), 51.06 (1C), 47.68 (1C), 46.62 (1C), 44.11 (1C), 44.06 (1C), 36.75 (1C).

Intermediate **S4**, 3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-3,2',6',2''',6'''-penta-*N*-Cbz-neomycin (0.220 g, 0.151 mmol) was dissolved in dry THF (5 mL), and treated with triethylamine (126 μL, 0.905 mmol). In a separate flask, (2*R*)-4-carboxybenzylamino-2-hydroxybutanoic acid (95 mg, 0.377 mmol) was dissolved in dry THF (10 mL), treated with DCC (78 mg, 0.377 mmol), *N*-hydroxysuccinimide (43 mg, 0.377 mmol) and stirred at room

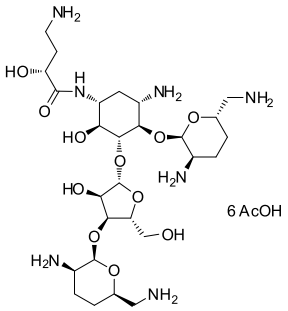
temperature for 30 min. The two mixtures were joined and stirred at room temperature for 2.5 h. The volatiles were evaporated under vacuum and the crude residue was diluted with EtOAc. The organic layer washed sequentially with 0.5 M HCl, sat. $NaHCO_3$, sat. NaCl, and dried over Na_2SO_4 and concentrated to a residue. Purification by column chromatography (0 to 3% MeOH/CH_2Cl_2, with 0.5% increments) yielded 0.210 g of the title compound 1-*N*-((2''''*R*)-4''''-carboxybenzylamino-2''''-hydroxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-3,2',6',2''',6'''-penta-*N*-Cbz-neomycin **10** (0.124 mmol, 82%), as a white solid.

$[\alpha]_D$ -3.1° (*c* 0.5, $CHCl_3$).

HRMS (ESI) calcd. for $C_{94}H_{99}N_7O_{23}$, $[M+Na]^+$ = 1716.66845, found 1716.66429 (-2.42 ppm).

^{1}H-NMR (400 MHz, $(CD_3)_2SO$): δ 7.64 (d, 7.6 Hz, 1H), 7.43-7.15 (m, 43H) 7.05 (m, 1H), 6.64 (d, 9.2 Hz, 1H), 5.77-5.71 (m, 2H), 5.53-5.48 (m, 2H), 5.30-5.25 (m, 2H), 5.18 (m, 2H), 5.08-4.94 (m, 12H), 4.81-4.78 (m, 3H), 4.58 (m, 1H), 4.25-3.85 (m, 8H), 3.63-3.42 (m, 5H), 3.25-2.83 (m, 8H), 1.85-1.76 (m, 2H), 1.60-1.57 (m, 1H), 1.39-1.35 (m, 1H).

^{13}C-NMR (101 MHz, $(CD_3)_2SO$) δ 173.68 (1C), 156.85-155.62 (6C), 143.73 (3C), 137.16 (6C), 129.99 (1C), 128.43-127.06 (46C), 125.68 (1C), 125.32 (1C), 108.10 (1C), 98.20 (1C), 96.40 (1C), 86.07 (1C), 82.86 (1C), 80.40 (1C), 80.01 (1C), 77.42 (1C), 73.58 (1C), 72.90 (1C), 69.29 (1C), 67.95 (1C), 65.58-65.24 (6C), 64.40 (1C), 55.04 (1C), 50.75 (1C), 49.13 (1C), 47.69 (1C), 46.61 (1C), 44.12 (1C), 44.06 (1C), 37.26 (1C), 34.76 (1C), 34.31 (1C).



1

1-*N*-((2''''*R*)-4''''-Amino-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin (1).

Intermediate **9**, 1-*N*-((2''''*R*)-4''''-carboxybenzylamino-2''''-hydroxybutanoyl)-3',4',3''',4'''-didehydro-3',4',3''',4'''-tetradeoxy-5''-*O*-trityl-3,2',6',2''',6'''-penta-*N*-Cbz-neomycin (34 mg, 19 μmol) was dissolved in 1:4 MeOH/1 M HCl (5 mL), treated with 20% $Pd(OH)_2/C$ (20 mg) and stirred under a hydrogen atmosphere using a balloon for 4 h. The suspension was filtered through Celite and freeze-dried. The resulting residue was dissolved in $CHCl_3/MeOH/NH_4OH_{(aq.)}$ (2:3:1) and purified by column chromatography using the same solvent system (10 to 30% $NH_4OH_{(aq.)}$). The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. For characterization purposes, the aminoglycoside solution was treated with excess AcOH (50 μL) and freeze-dried to provide 12.5 mg of the hexa-acetate salt of 1-*N*-((2''''*R*)-4''''-amino-2''''-hydroxybutanoyl)-3',4',3''',4'''-tetradeoxy-neomycin **1** (12.4 μmol, 65%), as a white solid.

$[\alpha]_D$ 42.1° (*c* 0.20, H_2O).

HRMS (ESI) calcd. for C27H53N7O11, $[M+Na]^+$ = 674.36975, found 674.36953 (-0.33 ppm).

^{1}H-NMR (400 MHz, D_2O): δ 5.72 (m, 1H), 5.28 (bs, 1H), 4.91 (s, 1H), 4.55 (m, 1H) 4.38 (m, 1H), 4.27 (m, 1H), 4.22-4.20 (m, 1H), 4.10-4.06 (m, 2H), 3.91 (m, 1H), 3.83-3.59 (m, 6H), 3.19-2.95 (m, 9H), 2.07-1.87 (m, 5H), 1.82 (AcOH), 1.62-1.41 (m, 6H).

^{13}C-NMR (101 MHz, D_2O): δ 180.09 (AcOH), 175.59 (1C), 110.10 (1C), 97.24 (1C), 93.92 (1C), 86.20 (1C), 80.66 (1C), 77.79 (1C), 74.17 (1C), 73.77 (1C), 72.99 (1C), 72.29 (1C), 69.14 (1C), 65.19 (1C), 60.00 (1C), 49.91 (1C), 48.84 (1C), 48.51 (1C), 47.29 (1C), 42.31 (1C), 42.18 (1C), 36.24 (1C), 31.98 (1C), 30.51 (1C), 25.38 (1C), 23.67 (1C), 22.86 (AcOH), 20.68 (1C), 20.51 (1C).

LC/CLND, water/MeOH gradient 25 to 75% in 20 min, R_t = 11.35 min, 99.6% purity.

References

1. a) Arya, D. P., *Aminoglycoside Antibiotics: From Chemical Biology to Drug Discovery.* 1 ed.; Wiley-Interscience: 2007. b) Diaz, L.; Delgado, A. *Curr. Med. Chem*. **2010**, 17, 2393-418. c) Walsh, C., *Antibiotics: Actions, Origins, Resistance.* ASM Press: Washington, D.C., 200314.
2. Hanessian, S.; Takamoto, T.; Masse, R.; Patil, G. *Can. J. Chem*. **1978**, 56, 1482..

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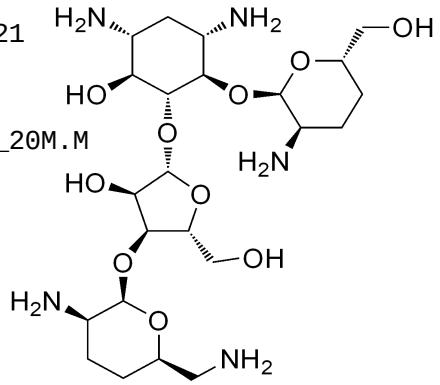
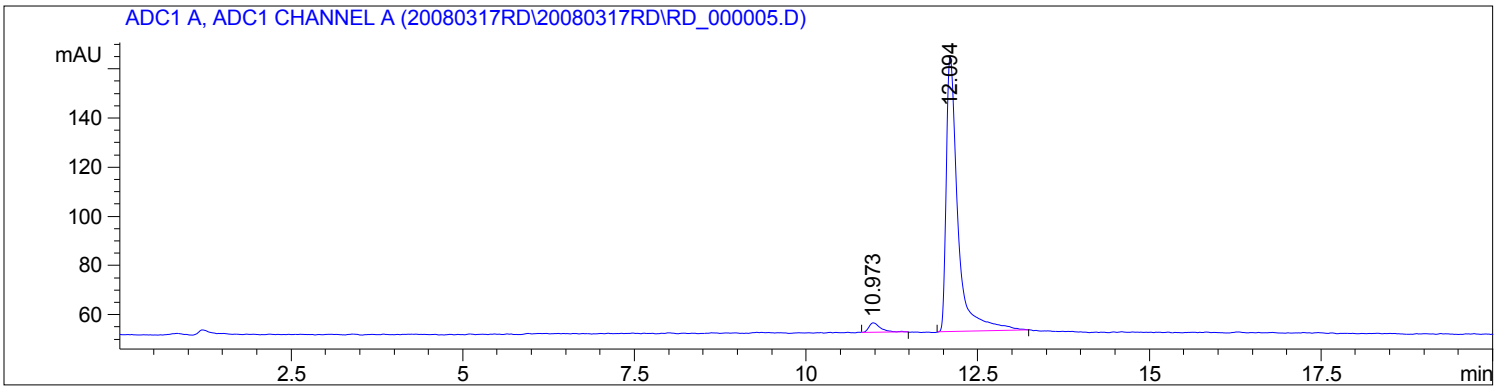
Sample Info : 200x dilution



Compound **4**



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Area Percent Report

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Use Multiplier & Dilution Factor with ISTDs

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Totals : 1374.75919 116.09214

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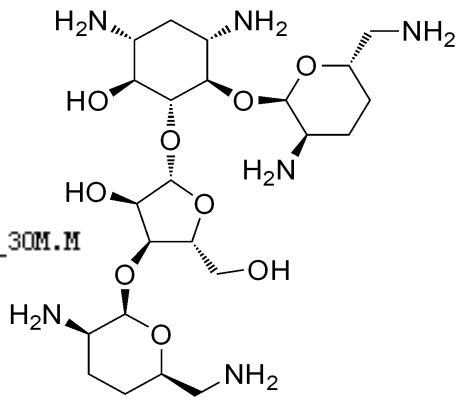

Compound **7**

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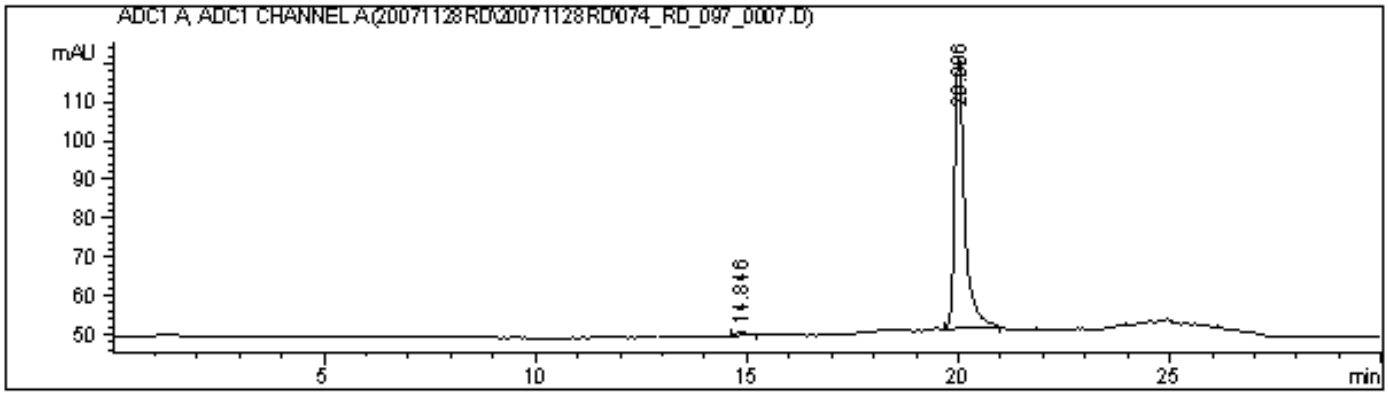


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                          Area Percent Report
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Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


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Peak RetTime Type  Width     Area      Height     Area
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Totals :                  1189.48227   71.54610

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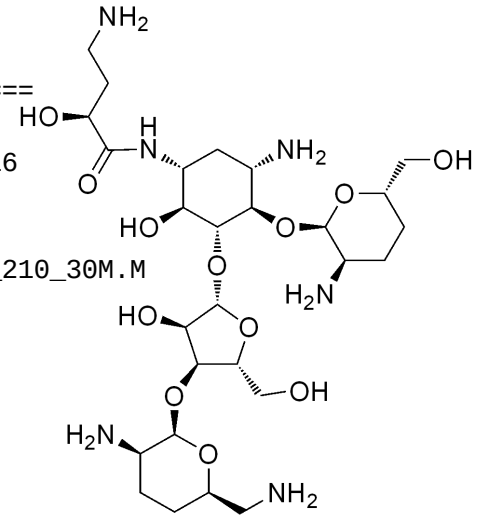

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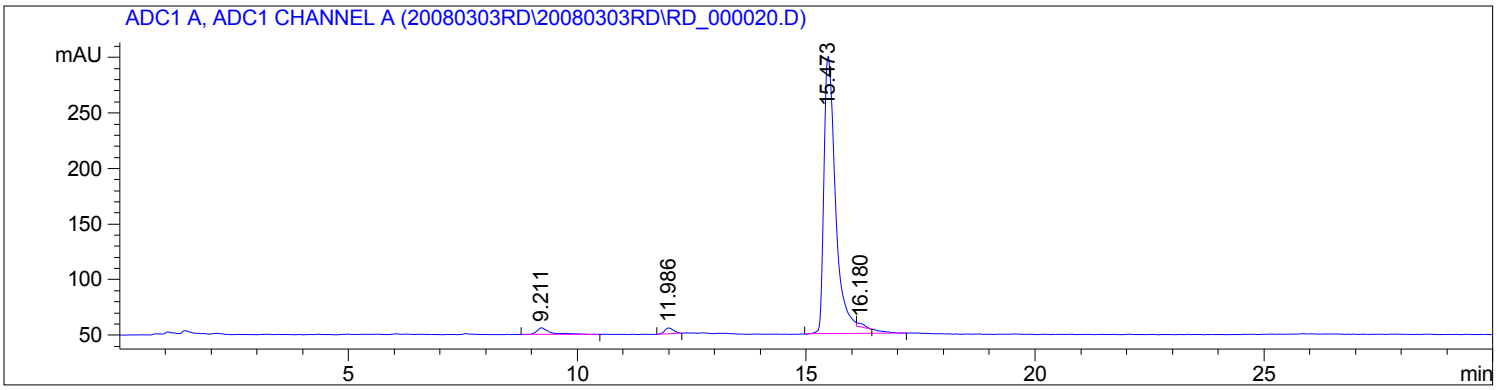
Method Info : Integration method.

Sample Info : 200x dilution

Compound **9**



==========

Area Percent Report

==========

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: ADC1 A, ADC1 CHANNEL A

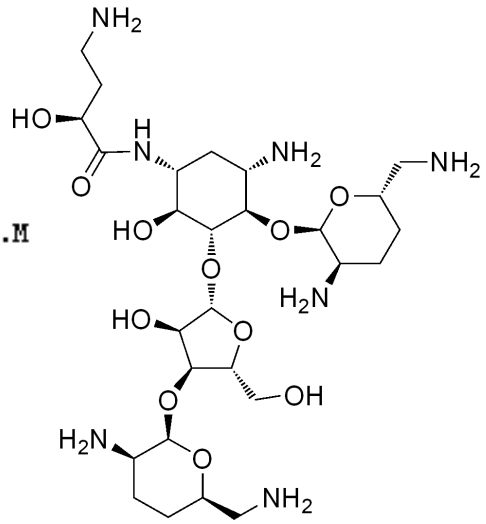
Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	9.211	BB	0.2892	130.25575	6.00235	2.6956
2	11.986	BB	0.2045	73.47517	5.34177	1.5206
3	15.473	BB R	0.2782	4591.18359	249.45851	95.0143
4	16.180	MM T	0.1952	37.18066	3.17505	0.7695

Totals : 4832.09518 263.97769

==========

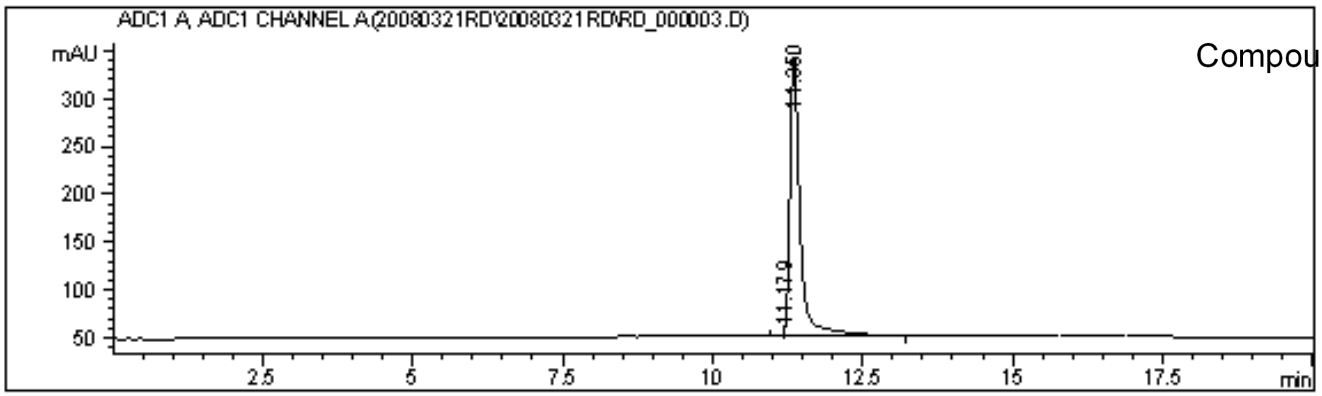
*** End of Report ***

Data File C:\CHEM32\1\DATA\20080321RD\20080321RD\RD_000003.D
Sample Name: DH-092-020



```
==========================================================================
Acq. Operator   : rd                              Seq. Line :   3
Acq. Instrument : Chemstation 4                   Location : Vial 11
Injection Date  : 3/21/2008 11:09:22 AM              Inj :   1
                                                Inj Volume : 20 µl
Acq. Method     : C:\Chem32\1\DATA\20080321RD\20080321RD\05-25-75B_20M.M
Last changed    : 2/21/2007 10:34:02 AM by rpd
Analysis Method : C:\CHEM32\1\METHODS\INT 30MIN.M
Last changed    : 3/24/2008 11:50:10 AM by rd
                  (modified after loading)
Method Info     : Integration method.

Sample Info     : 200x dilution
```



Compound **1**

```
==========================================================================
                        Area Percent Report
==========================================================================

Sorted By             :      Signal
Multiplier            :      1.0000
Dilution              :      1.0000
Use Multiplier & Dilution Factor with ISTDs


Signal 1: ADC1 A, ADC1 CHANNEL A

Peak RetTime Type  Width     Area      Height     Area
  #   [min]        [min]   [mAU*s]     [mAU]       %
----|-------|----|-------|----------|----------|--------|
   1  11.179 MF    0.0663   12.38994    3.11244   0.3586
   2  11.350 FM    0.1963 3442.45679  292.25522  99.6414

Totals :                  3454.84672  295.36765


==========================================================================
                        *** End of Report ***
```