Synthesis, antibacterial action, and ribosome inhibition of deoxyspectinomycins

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Abstract
Spectinomycin, an aminocyclitol antibiotic, is subject to inactivation by aminoglycoside modifying enzymes (AMEs) through adenylylation or phosphorylation of the 6-hydroxy group position. In this study, the effects of deoxygenation of the 2- and 6-hydroxy group positions on the spectinomycin actinamine ring are probed to evaluate their relationship to ribosomal binding and the antimicrobial activities of spectinomycin, semisynthetic aminomethyl spectinomycins (amSPCs), and spectinamides. To generate these analogs, an improved synthesis of 6-deoxyspectinomycin was developed using the Barton deoxygenation reaction. 6-Dehydrospectinamide was also synthesized from spectinamide 4 to evaluate the H-bond acceptor character on the C-6 position. All the synthesized analogs were tested for antibacterial activity against a panel of Gram (+) and Gram (−) pathogens, plus Mycobacterium tuberculosis. The molecular contribution of the 2- and 6-hydroxy group and the aryl functionalities of all analogs were examined by measuring inhibition of ribosomal translation and molecular dynamics experiments with MM/GBSA analysis. The results of this work indicate that the 6-hydroxy group, which is the primary target of AMEs, is a required motif for antimicrobial activity in current analogs. Removal of the 6-hydroxy group could be partially rescued by offsetting ribosomal binding contributions made by the aryl side chains found in the spectinamide and amSPCs. This study builds on the knowledge of the structure–activity relationships of spectinomycin analogs and is being used to aid the design of next-generation spectinomycins.

Introduction
Spectinomycin, an aminocyclitol antibiotic [1, 2], is an inhibitor of bacterial protein synthesis (IC50 = 0.4 μg ml−1) with moderate broad-spectrum activity against both Gram (+) and Gram (−) bacteria (Fig. 1a). Spectinomycin binds to the interface between the minor groove of helix-34 of the 16S ribosomal RNA and the RpsE protein to inhibit protein translation [3]. Clinically, spectinomycin has been widely used to treat Neisseria gonorrhoeae infections [4] and is well tolerated at a high dosage, lacking the ototoxicity commonly associated with aminoglycoside antibiotics [5]. However, spectinomycin is subject to native efflux [6, 7], target modification [8], and enzymatic inactivation of the drug [8, 9]. Therefore, to overcome innate resistance mechanisms, we developed semisynthetic analogs of spectinomycin called spectinamides [10] and aminomethyl spectinomycins (amSPCs) [11]. The spectinamides have excellent narrow spectrum antitubercular activity, and the amSPCs have broad-spectrum activity against the common respiratory tract, sexually transmitted and biothreat bacterial pathogens (Fig. 1b, c) [12–14]. These analogs have improved bacterial accumulation, with the spectinamides overcoming efflux by the Rv1258c transporter found in Mycobacterium tuberculosis which transports spectinomycin [10]. While these analogs overcome the resistance from efflux pumps, they are still subject to modification by aminoglycoside modifying enzymes (AMEs) in non-mycobacterial pathogens, which limits their development for broader indications, particularly for the treatment of...
Gram-negative infections [15, 16]. One strategy to alleviate AME resistance is to remove the sites of inactivation found on aminoglycosides [17]. Spectinomycin is susceptible to inactivation by nucleotidyltransferases ANT(9) [18] and the bifunctional enzyme ANT(3,9), as well as phosphorylation by the APH(9) enzyme, which all modify the 6-hydroxy group position of spectinomycin [15, 16, 19]. Previously, several deoxyspectinomycins were synthesized by researchers at Hoffmann-La Roche Inc. and were found to be microbiologically inactive [20–22]. However, it was unknown if the inactivity is due to lack of ribosomal binding or poor accumulation. As part of our systematic approach to develop the structure–activity relationship (SAR) around the spectinomycin ring, we chose to reinvestigate the antimicrobial properties of deoxyspectinomycin analogs and to evaluate their ability to inhibit bacterial ribosomes, which has not been previously reported.

In this study, we examine whether modifications to the 2- and 6-hydroxy groups in spectinomycin, spectinamides, and amSPCs (Fig. 2) can be tolerated, how modification or removal of these groups affects antibacterial potency, ribosome inhibition, and cellular accumulation, and finally, computationally examine the relative binding contributions of these motifs in the ribosome binding site. We report an improved synthesis of 6-deoxyspectinomycin using Barton’s radical deoxygenation reaction as the key step [23] and demonstrate an efficient synthesis of 6- and 2-deoxyspectinamides and 6-deoxyaminomethyl spectinomycin (6-deoxy-amSPC) analogs [21]. 6-epi-chlorospectinomycins and 6-dehydrospectinamide were also synthesized to examine hydrogen bonding acceptor character to the ribosome (Fig. 3). The antimicrobial activity and cellular accumulation of the analogs were determined, and the energetic contribution of key binding interactions was examined computationally. Results from these studies are combined to provide a holistic picture of the value of the 2- and 6-hydroxy groups to spectinomycin binding and antimicrobial activity.

**Material and methods**

**Chemicals, reagents, and instrumental**

Spectinomycin dihydrochloride pentahydrate was purchased from Waterstone Technology (catalog number 81249, CAS number 22189-32-8, 95% in purity). All solvents used for chromatography and liquid chromatography were purchased from Aldrich. Flash column chromatography silica
cartridges were obtained from Biotage Inc. Reactions were monitored by thin-layer chromatography on pre-coated Merck 60 F254 silica gel plates and visualized using UV light (254 nm). IR (neat) spectra were obtained using a Nicolet-iS5 FT-IR instrument. A Biotage FLASH column chromatography system was used to purify mixtures. 1H NMR (500 MHz, D2O) spectra were recorded on a Varian INOVA-500 spectrometer or on a Bruker 400 MHz NMR spectrometer. Chemical shifts (δ) are reported in parts per million relative to the residual solvent peak or internal standard (tetramethylsilane), and coupling constants (J) are reported in hertz (Hz). High-resolution mass spectra were recorded on a Waters Xevo G2 QTOF LC–MS using ESI. Purity of the products was confirmed by UPLC/MS (the Waters Xevo G2 QTOF LC–MS). Purity of the final compounds is given below, and data for intermediates are provided in Supplementary information.

### Chemical synthesis

**6-Deoxyspectinomycin dihydrochloride (5)**

To a solution of cbz protected 6-deoxyspectinomycin 16 [20] (0.080 g, 0.136 mmol) in 1 M HCl in MeOH (3 ml) was added Pd/C (30 mg) at room temperature and the reaction mixture was hydrogenated for 30 min under H2 atmosphere (H2 Balloon). Excess catalyst was filtered off and the evaporation, washing with acetone (5 ml) and EtOAc (5 ml) gave pure product 5 as a white solid. Yield: 0.060 g, 81%; 1H NMR (500 MHz, D2O) δ 4.76 (s, 1H), 4.57 (s, 1H), 4.18 (t, J = 10.5 Hz, 1H), 4.07 (td, J = 11.3, 4.0 Hz, 1H), 3.92 (q, J = 6.6 Hz, 1H), 3.40–3.32 (m, 2H), 3.28 (s, 3H), 2.77–2.74 (m, 1H), 2.71 (s, 3H), 1.89–1.72 (m, 3H), 1.17 (d, J = 6.2 Hz, 3H); 13C NMR (125 MHz, D2O) δ 93.8, 93.4, 92.0, 68.4, 67.8, 65.2, 61.1, 59.0, 56.0, 41.5, 30.6, 30.0, 25.5, 19.6. HRMS (ESI) m/z calcd for C14H23N2O6 [M + H]+, 317.1713; found, 317.1712.

**6-Deoxy-3′-deoxy-3′-dihydro-3′-(R)-(4-fluorobenzylaminomethyl)spectinomycin dihydrochloride (6)**

Compound 6 was synthesized analogously as 5 from 17, as a white solid. Yield: 0.017 g (95%); IRmax (neat): cm⁻¹ 3363, 2974, 1656, 1561, 1467, 1166, 1064; 1H NMR (500 MHz, D2O) δ 8.66 (d, J = 5.8 Hz, 1H, Ar-H), 8.47 (t, J = 8.0 Hz, 1H, Ar-H), 7.95–7.84 (m, 2H, Ar-H), 4.90 (s, 1H), 4.56 (s, 1H), 4.26 (t, J = 10.4 Hz, 1H), 4.17–3.96 (m, 5H), 3.37 (d, J = 11.6 Hz, 2H), 2.73 (s, 3H), 2.71 (s, 3H), 2.15 (dd, J = 10.6, 6.7 Hz, 1H), 1.83 (t, J = 13.5 Hz, 2H), 1.69 (d, J = 14.6 Hz, 1H), 1.18 (d, J = 6.0 Hz, 3H); 13C NMR (125 MHz, D2O) δ 169.0, 149.2, 146.9, 141.4, 128.3, 125.8, 93.2, 90.2, 60.8, 67.8, 65.3, 61.6, 58.7, 55.9, 52.3, 39.2, 34.1, 30.6, 30.5, 25.4, 19.8; HRMS (ESI) m/z calcd for C21H33N4O6 [M + H]+, 437.2400; found, 437.2393.

**6-Deoxy-3′-dihydro-3′-(R)-(pyridin-2-yl)acetylamino)spectinomycin trihydrochloride (7)**

Compound 7 (0.011 g, 70%) was synthesized analogously as 6 from 24, as a white solid. Yield: 0.011 g (70%); [α]D28 +0.072 (c 0.15, CH3OH); MP: 211.1–211.8; IRmax (neat): cm⁻¹ 3358, 2974, 1603, 1513, 1460, 1163, 1081; 1H NMR (500 MHz, D2O) δ 7.46–7.43 (m, 2H, Ar-H), 7.17–7.14 (m, 2H, Ar-H), 4.67 (s, 1H), 4.58 (s, 1H), 4.27 (d, J = 13.5 Hz, 1H), 4.20 (d, J = 13.6 Hz, 1H), 4.18–4.02 (m, 2H), 3.68 (dq, J = 12.3, 6.1 Hz, 1H), 3.47–3.26 (m, 3H), 3.13 (d, J = 13.7 Hz, 1H), 2.73 (s, 3H), 2.71 (s, 3H), 2.14 (dt, J = 8.8, 4.3 Hz, 1H), 1.88–1.63 (m, 3H), 1.14 (d, J = 5.9 Hz, 3H); 13C NMR (125 MHz, D2O) δ 163.2 (d, J = 247.0 Hz, C-F), 132.3, 132.2, 125.9, 116.3, 116.1, 93.4, 92.5, 72.2, 67.5, 67.4, 65.0, 61.2, 58.8, 55.9, 50.8, 48.9, 40.1, 30.5, 30.2, 25.4, 19.8; 19F NMR (D2O, 375 MHz) δ -112.0; HRMS (ESI) m/z calcd for C22H35FN3O6 [M + H]+, 456.2510; found, 456.2510.
6-Epi-chloro-6-deoxyspectinomycin dihydrochloride (8)

Compound 8 was synthesized from cbz-spectinomycin [24]. Yield: 0.012 g (42%); 1H NMR (500 MHz, D2O) δ 4.93 (s, 1H), 4.75–4.73 (m, 1H); 4.66 (t, J = 10.6 Hz, 1H), 4.44 (dd, J = 10.5, 3.5 Hz, 1H), 4.05–3.99 (m, 1H), 3.93–3.81 (m, 1H), 3.54 (dd, J = 11.0, 3.3 Hz, 1H), 2.89 (s, 3H), 2.84–2.83 (m, 1H), 2.81 (s, 3H), 1.95–1.80 (m, 2H), 1.26 (d, J = 6.3 Hz, 3H); 13C NMR (125 MHz, D2O) δ 93.8, 93.4, 91.7, 68.6, 66.5, 63.0, 61.6, 58.8, 56.8, 54.4, 41.5, 30.5, 30.2, 19.5. HRMS (ESI) m/z calcd for C14H25N2O6 [M+H]⁺, as a white solid. Yield: 0.010 g (79%); IR νmax (neat): cm⁻¹: 3350, 2974, 1648, 1564, 1466, 1168, 1063; 1H NMR (500 MHz, D2O) δ 2.71 (s, 3H), 2.68 (1H), 3.25 (ddd, J = 14.1, 10.2, 4.3 Hz, 1H), 2.72 (s, 3H), 2.71 (s, 3H), 2.68–2.56 (m, 1H), 1.86–1.68 (m, 3H), 1.18 (d, J = 5.7 Hz, 3H); 13C NMR (125 MHz, D2O) δ 166.3, 146.5, 146.4, 144.4, 138.7, 125.7, 123.1, 90.3, 87.3, 67.4, 65.6, 65.3, 54.6, 51.4, 49.5, 36.5, 31.5, 27.4, 20.9, 17.1, 11.1; HRMS (ESI) m/z calcd for C21H33N4O6 [M+H]⁺, 437.2400; found, 437.2401.

2-Deoxyspectinomycin dihydrochloride (9)

Compound 9 [21] was synthesized analogously as 5 from 20, as a white solid. Yield: 0.010 g (80%); 1H NMR (500 MHz, D2O) δ 4.77 (s, 1H), 4.04 (t, J = 10.3 Hz, 1H), 3.96–3.88 (m, 2H), 3.74 (t, J = 10.2 Hz, 1H), 3.46 (t, J = 11.6 Hz, 1H), 3.22 (t, J = 10.2 Hz, 1H), 2.70 (s, 3H), 2.68 (3H), 2.63–2.57 (m, 1H), 1.81–1.68 (m, 3H), 1.15 (d, J = 6.1 Hz, 3H); 13C NMR (125 MHz, D2O) δ 93.5, 93.4, 91.6, 69.9, 68.4, 68.2, 68.0, 57.3, 54.3, 41.5, 30.1, 29.7, 23.3, 19.6; HRMS (ESI) m/z calcd for C14H24ClN2O6 [M+H]⁺, 317.1713; found, 317.1713.

2-Deoxy-3′-deoxy-3′-dihydro-3′(R)-(pyridin-2-yl)acetylamino)spectinomycin dihydrochloride (10)

Compound 10 was synthesized analogously as 6 from 21, as a white solid. Yield: 0.010 g (79%); IR νmax (neat): cm⁻¹: 3350, 2974, 1648, 1564, 1466, 1168, 1063; 1H NMR (500 MHz, D2O) δ 8.66 (d, J = 5.9 Hz, 1H), 8.48 (t, J = 8.0 Hz, 1H), 8.05–7.81 (m, 2H), 4.93 (s, 1H), 4.27–3.89 (m, 6H), 3.77 (q, J = 9.9, 9.2 Hz, 1H), 3.50 (td, J = 11.5, 4.1 Hz, 1H), 3.25 (ddd, J = 14.1, 10.2, 4.3 Hz, 1H), 2.72 (s, 3H), 2.71 (s, 3H), 2.68–2.56 (m, 1H), 1.86–1.68 (m, 3H), 1.18 (d, J = 5.7 Hz, 3H); 13C NMR (125 MHz, D2O) δ 166.3, 146.5, 146.4, 144.4, 138.7, 125.7, 123.1, 90.3, 87.3, 67.4, 65.6, 65.3, 54.6, 51.4, 49.5, 36.5, 31.5, 27.4, 20.9, 17.1, 11.1; HRMS (ESI) m/z calcd for C21H33N4O6 [M+H]⁺, 437.2400; found, 437.2401.

Molecular modeling

Mycobacterial RNA-RpsE protein complex generation

The molecular docking and molecular dynamics studies of spectinomycin and spectinomycin analogs were performed using a comparative model of the M. tuberculosis RNA-RpsE protein complex prepared using the Escherichia coli complex as a template. The Mycobacterial RpsE proteins (M. tuberculosis and Mycobacterium smegmatis) have a high degree of homology, with 89% sequence similarity. The differences in the RpsE protein sequences lie outside the spectinomycin binding site on the RpsE loop (Fig. 4). Therefore, the M. tuberculosis RpsE protein sequence was used to create a Mycobacterial RNA-RpsE protein complex for molecular modeling of both species. The E. coli RpsE protein structure (PDB ID: 4V56 (2QOU)) served as the structural template to generate the model, 55% homology [26]. The model was created using Schrödinger’s Prime program [27, 28] and was validated in PROCHECK [29] with 2.7% residues allowed and 0% disallowed (data not shown). A sequence alignment of RpsE proteins from the Mycobacteria and E. coli revealed two amino acid differences located in the RpsE protein loop in the spectinomycin binding site: V55T and R61I (Fig. 4). Despite variations in the RpsE protein, the rRNA sequence found in the spectinomycin binding site is highly conserved between Mycobacteria and E. coli. To reduce the computational cost of subsequent molecular docking and molecular simulations, the complex model was truncated to a 20 Å sphere around...
the spectinomycin binding site. The Mycobacterial RNA-RpsE complex was protonated at pH 7.0 using PROPKA and was energy minimized with the OPLS3 force field [30, 31]. Residues that are different in the RpsE protein loop between species are shown as sticks and are labeled. b The sequence alignment of the RpsE proteins [49, 50]. Black highlights represent identical residues, gray highlights represent similar residues, and the red boxes highlight the RpsE protein loop.

Glide standard precision molecular docking

All conformations generated from LigPrep were docked using Schrödinger’s Glide program [33–35]. Ligand sampling was flexible, and docked conformers were required to match at least one hydrogen bonding interaction from the constraints generated in the receptor grid. To soften the potential for nonpolar parts of the ligands, van der Waals (VDW) radii were scaled using a scaling factor of 0.80 for ligand atoms with a partial charge < 0.15 (default Glide settings). Post-docking minimization was performed on all poses generated by Glide using the OPLS3 force field prior to the assignment of Glide docking scores. Ligand poses with the highest ranked Glide scores were used to evaluate binding. The highest ranked analogs, 1 (spectinomycin), 2, 5, 6, 9, and 10, were advanced to molecular dynamics simulations.

Molecular dynamics

All molecular dynamic simulations were performed using AMBER18 [36] as previously described [25]. Briefly, the ff14SB and OL3 force field was used for the RNA and protein parameterization and the general Amber force field was used for parameterization of the ligand [37–39]. Restrained electrostatic potential charges within the ANTECHAMBER module in AMBER were applied to the positively charged spectinomycin, 2, 5, 6, 9, and 10 analogs. Sodium ions were applied to neutralize the charge of
the system. The complex was solvated in a TIP3P octahedron box with a 10 Å boundary. An initial energy minimization of the solvated complex was performed with the complex fixed for 1000 steps, followed by a second step of energy minimization without restraints for 2500 steps. The minimized complex was equilibrated to 300 K over 250,000 steps for 500 ps with weak restraints applied to the complex (Fig. S1). The equilibrated structure was simulated for 20 ns with the NPT ensemble at 300 K with terminal residues harmonically restrained (Fig. S1). The energetic contributions during the MD simulations were calculated by the MM/GBSA [40] method using 1000 frames from the last 2 ns of the MD ensemble. Lastly, the free energy contributions to ligand binding were decomposed for analysis using a per-residue basis [40].

**Ribosomal inhibition**

Purified 70S *M. smegmatis* bacterial ribosomes were used in translation reactions. Firefly luciferase mRNA was produced in vitro using T7 RNA polymerase. Translation reactions were carried out as previously described [11]. The IC₅₀ values represent the drug concentration that inhibits luciferase activity by 50%.

**Bacterial strains and growth conditions**

Strains were routinely grown with Mueller–Hinton broth at 37 °C with shaking at 225 rpm. Bacterial strains were obtained through ATCC or BEI Resources or from academic laboratories. The strains used were the following: *Staphylococcus aureus* ATCC 29213, *S. aureus* NRS70, *Enterococcus faecalis* ATCC 33186, *E. faecium* ATCC 19434, *Streptococcus pyogenes* ATCC 700294, *Streptococcus pneumoniae* R6, *Acinetobacter baumannii* ATCC 19606, *Pseudomonas aeruginosa* ATCC 15692, *Klebsiella pneumoniae* ATCC 700603, *Proteus mirabilis* ATCC 25933, *Stenotrophomonas maltophilia* ATCC 13637, *Enterobacter cloacae* ATCC 13047, *Staphylococcus epidermidis* ATCC 14990, *E. coli* K12, and *E. coli* JW5503 (*Δ*tolC). The *M. tuberculosis* H37Rv strain was grown in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose complex, 0.05% (w/v) Tween 80 at pH 7.4, (7H9/ADG pH 7.4) with shaking at 225 rpm at 37 °C.

**Antibacterial susceptibility testing**

Minimum inhibitory concentrations are determined following CLSI broth microdilution guidelines [41]. Briefly, experimental compounds (in DMSO) and control spectinomycin (Sigma Aldrich, DMSO) are serially diluted, twofold, across a 96-well round bottom plate in Mueller–Hinton II broth or 7H9/ADG pH 7.4 for *M. tuberculosis* H37Rv. Equal volume of bacterial strains of $5 \times 10^5$ CFU ml⁻¹ was added to each well to give a final drug concentration starting at 200 µg ml⁻¹. Plates were incubated for 18 h at 37 °C except *M. tuberculosis* H37Rv, which was incubated for 7 days at 37 °C. The MIC for all strains were determined as the lowest concentration to visually inhibit bacterial growth and are reported as the consensus of three independent experiments.

**Whole-cell accumulation assay**

Methods for this assay have been optimized from previously published studies [42, 43]. *E. coli* (BW25113 for WT or JW5503 for ΔtolC) is grown at 37 °C to mid-log phase (OD600 of 0.6–0.7) in Mueller–Hinton broth. The bacteria are pelleted and washed twice with PBS before being resuspended in 3.5 ml of PBS per 100 ml of cells cultured. Cells are allowed to equilibrate for 10 min at 37 °C prior to drugging. 1 ml of *E. coli* is incubated with a final concentration of 50 µM of compound (in DMSO) shaking at 37 °C for 10 min. 800 µl of treated cells is layered over supercooled (−78 °C) silicone oil (9:1 AR20 and high-temperature silicone oil, Sigma Aldrich) and pelleted to remove cells from free compound. For lysis, the pellets were resuspended in 200 µl of HPLC grade water and subjected to three freeze–thaw cycles using liquid nitrogen and a 65 °C water bath. Cell debris was separated from lysate by centrifugation, and 150 µl of extract is recovered. The pellet is resuspended in the remaining 50 µl of water, and once thoroughly resuspended, 100 µl of 5% TCA was added. The cells are once again pelleted, and lysates are combined. Lysates are pelleted at high speed for 10 min and then filtered through a 0.22 µm filter before injection for LC–MS/MS.

Samples are analyzed with a tandem Waters Acquity M Class series UPLC system and Xevo G2 QTOF tandem MS/MS with Zspray. 100 nl of extract was separated using a Phenomenex Kinetex 2.6 µm XB-C18, 100 Å (300 µm × 150 mm) column with solvent A, 0.1% formic acid in water, and solvent B, 0.1% formic acid in acetonitrile. The inlet method for these samples utilized a flow rate of 8 µl min⁻¹ with the following gradient: 0–4 min, 99.9% solvent A and 0.1% solvent B; 4–5 min, 10% solvent A and 90% solvent B; 5–6 min, 99.9% solvent A and 0.1% solvent B. Tandem mass spectra were acquired with a cone voltage and collision energy optimized for each compound. High-resolution spectra were calibrated by co-infusion of 2 ng ml⁻¹ leucine enkephalin lockspray (Waters). Data were quantified using Waters MassLynx software where the AUC was determined by integrating the corresponding daughter peak of the parent compound. Concentrations of the unknown compounds were determined by the linear fit of the corresponding standards. Concentrations are reported as the average of three biological replicates.
Results and discussion

Synthesis

Adapting literature procedures, compound 14 [22, 44] was synthesized from spectinomycin via catalytic hydrogenation of ketogroup, cbz protection of amine, and diacetone protection diol. Deoxygenation at C-6 position in compound 14 was achieved efficiently by following Barton’s radical deoxygenation [23] method (Scheme 1). Thus, selective installation of imidazolyl thiocarbamate group on C-6-hydroxy group was carried out using 1,1′-thiocarbonyldimimidazole in the presence of catalytic amount of DMAP and treatment of thiocarbamate with tributyltin hydride and AIBN gave deoxygenated product 15 in good yields. An acetonide group was removed under 1 M HCl in methanol to give diol and selective oxidation of 4′-hydroxy group with IBX yielded cbz protected 6-deoxyspectinomycin 16. Lastly, compound 16 was converted to 6-deoxyspectinomycin [20] 5 by removal cbz group under catalytic hydrogenation (Scheme 1). To achieve 6-deoxyspectinamide, compound 16 underwent reductive amination reaction with ammonium nitrate and 2-picoline-borane complex to provide 4′R-amino-6-deoxyspectinomycin, which then coupled with 2-pyridyl acetic acid to give compound 17 [45, 46]. The newly generated stereochemistry on the 3′(R) position was assigned using coupling constant (J_3′R,4′ = 4.4 Hz; J_3′R,4′ = 2.3 Hz) [47] values between 3′ and 4′ hydrogens by synthesizing compound 16b (data available in Supplementary Scheme 1, compound 16b). Finally, catalytic hydrogenolysis of cbz product of 17 under Pd/C gave 6-deoxyspectinamide 6.

Attempts to deoxygenation of C-2-hydroxy group using the Barton imidazolylthiocarbamate intermediate failed presumably due to the steric hindrance of C-2-hydroxy group, we consequently followed literature procedure to make 2-deoxyspectinomycin. The 2-deoxyderivative 18 was achieved from spectinomycin using the protocol of Foley et al. (Scheme 2) [21]. Benzyl protection of 6-hydroxy group followed by removal of acetonide group yielded diol 19. Compound 9 was achieved by oxidation of compound 19 to give compound 20 and followed by cbz group deprotection. The 2-deoxyspectinamide 10 was synthesized from 20 via 21 following similar protocol [45] as used in 6.

6-Deoxy-amSPC 7 was synthesized from amSPC intermediate 22 [11]. First, the benzy1 amine group in 22 [11] was protected as tert-butyl carbamate derivative and then the
6-imidazolylthiocarbamate derivative 23 was synthesized using 1,1′-thiocarbonyldiimidazole. Deoxygenation at C-6 position was then achieved efficiently by following Barton’s radical deoxygenation [23] method as used in compound 15 to give deoxygenated product 24 in moderate yield. Finally, deoxy compound 7 was achieved by removing tert-butoxy carbonyl group using trifluoroacetic acid and benzyloxy carbonyl group deprotection under hydrogenolysis condition (Scheme 3). The 6-epi-chloro compound 8 was synthesized following literature protocol [24]. The synthesis of 6-dehydrospectinamide 11 was accomplished from cbz protected 25 via selective oxidation of C-6-hydroxy group by Pfitzner–Moffatt method and followed hydrogenolytic deprotection of cbz groups (Scheme 4).

Antibacterial susceptibility and ribosomal activity of deoxy analogs

The antibacterial susceptibilities of the synthesized deoxy analogs were tested against a panel of Gram (+) and Gram (−) pathogens, plus M. tuberculosis (abbreviated in Table 1, full data available in Supplementary Table 1). All deoxyspectinomycin analogs lacked MIC activity in line with previous reports [20]. Deoxyspectinamides 6 and 10 and deoxy-amSPC 7 had some limited MIC activity against M. tuberculosis and S. pneumoniae, weaker than their corresponding parent spectinamide 2 and amSPC 3, consistent with their reduced ribosomal binding affinities described subsequently.

The ability of the analogs to inhibit ribosomal translation was performed with M. smegmatis ribosomes in a cell-free translation assay [11] (Table 1). The 6- and 2-deoxyspectinomycins, 5 and 9, show poor ribosomal binding inhibition (Ribo IC50 > 60 μg ml−1) compared to spectinomycin 1 (Ribo IC50 0.36 μg ml−1). The molecular docking of 5 and 9 suggests that the hydrogen bonding interactions from the 6- and 2-hydroxy groups are crucial for ribosomal binding inhibition (Fig. S2A, B). Substitution of the hydroxy group at the 6-position on spectinomycin was tested to determine if the loss in activity could be rescued while avoiding AME activity. However, addition of a chlorine at the 6-position (8) maintained poor activity (Table 1) and disrupted the majority of hydrogen bonding interactions involved with spectinomycin binding (Fig. S2C). Therefore, the hydroxy group at the 6-position of spectinomycin forms hydrogen bonding interactions that are crucial to maintain ribosomal inhibition.

The 6-deoxyspectinamide 6 showed improved ribosomal inhibition compared to 5, (Ribo IC50 = 9.50 μg ml−1) suggesting the aryl side chain could partially rescue inhibitor activity. The aryl side chain extends from the spectinomycin binding site and interacts directly with the RpsE protein loop through VDW interactions (Fig. S3). Similarly, 6-deoxy-amSPC 7 had improved ribosomal inhibition (Ribo IC50 = 23.3 μg ml−1) from the contribution of the aryl side chain (Fig. S4). This feature may be used in part to compensate for the loss of hydrogen bonding interaction with the spectinomycin core and the bacterial ribosome, a phenomenon that could not be observed in 5 as it lacks the aryl side chain. 2-Deoxyspectinamide 10 also showed improved ribosomal inhibition (Ribo IC50 = 10.2 μg ml−1) compared to the 2-deoxyspectinomycin 9 (Ribo IC50 > 60 μg ml−1). From the molecular docking of 10, the aryl side chain is also shown to gain interactions with the RpsE protein backbone, providing further evidence that aryl side chain rescues ribosomal binding (Fig. S3).
Table 1  Antibacterial susceptibility (MIC, μg ml\(^{-1}\)) and ribosomal affinity IC\(_{50}\) (μg ml\(^{-1}\)) studies of synthesized compounds

| Compounds | \(M.\) tuberculosis H37Rv MIC (μg ml\(^{-1}\)) | \(S.\) pneumoniae R6 MIC (μg ml\(^{-1}\)) | \(E.\) coli K-12 MIC (μg ml\(^{-1}\)) | \(E.\) coli cellular accumulation (μM) | Ribosome translation inhibition IC\(_{50}\) (μg ml\(^{-1}\)) |
|-----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Spectinomycin (I) | 50 | 12.5 | 25 | 1.1 ± 0.1 | 0.36 |
| 2 | 1.6 | 12.5 | 200 | 5.4 ± 0.8 | 0.89 |
| 3 | 6.25 | 3.13 | 50 | 40 ± 1 | 0.74 |
| 4 | 1.6 | 12.5 | 100 | 5.9 ± 0.8 | 0.84 |
| 5 | >200 | >200 | >200 | 2.3 ± 0.4 | >60 |
| 6 | 50 | 25 | >200 | 10.0 ± 0.6 | 9.50 |
In the case of the 6-dehydrorospectinamide 11, we observed an increase in ribosomal binding affinity compared to other 6-deoxy analogs (Ribo IC\textsubscript{50} = 34.7 \textmu g ml\textsuperscript{-1}). The molecular docking studies of the parent compound, 4, and the 11 analog reveal the carbonyl at the 6-position docked with a similar predicted affinity (Fig. S5). The exact molecular entity of 11 is not known in solution state at this stage as it likely exists in equilibrium with its hydrated form as indicated by its \textsuperscript{13}C NMR (Fig. 3). Therefore, we also docked the 12 (gem-diol-11) (Fig. S5). This analog exhibited two hydrogen bond interactions with G1064 and G1193, but loses the hydrogen bonding interaction with the backbone of Arg60 and has a weaker docking score compared to 4 and 11 (Fig. S5). These results point to reason...
for the weak binding (Ribo IC₅₀ = 34.7 µg ml⁻¹) for 11 compared to 4 (Ribo IC₅₀ = 0.84 µg ml⁻¹) is due to 12 (gem-diol-11) being the dominant binding isoform.

**Cellular accumulation**

To examine if altered cellular accumulation contributes to reduced MIC activity of deoxyspectinomycin analogs, the concentration of all analogs within *E. coli* cells was measured and compared to their corresponding spectinomycin family parent (Fig. 5 and Table S3) [43]. These experiments indicated a substantial difference in the accumulation between the different families, spectinomycins (1, 5, 8, 9; range <0.4 to 1.2 µM), spectinamides (2, 4, 6, 10; range <5.4 to 10 µM), and amSPCs (3, 7; 40 and 47 µM, respectively). When examining matched pairs within families (1 vs 5 and 9; 2 vs 6 and 10; 3 vs 7), deoxygenation led to a slight increase in accumulation, with the most pronounced effect for 6-deoxy analogs. The increased accumulation is, however, modest and unlikely to impact MIC levels. The pattern of uptake was generally mirrored in the *E. coli* ΔtolC strain. However, these data suggest that the early spectinamide, 2, is subjected to efflux by tolC. The depletion of the efflux pump results in a 15-fold increase in intracellular accumulation, which matches the trends in susceptibility where knocking out the efflux pump reduces the MIC of 2 from 200 to 50 µg ml⁻¹. Overall, these results indicate that altered accumulation does not affect the antimicrobial potency among the deoxyspectinomycin analogs.

**Molecular dynamics simulations**

Spectinomycin binding is mediated through a complex pattern of hydrogen bond interactions between the rRNA nucleotides and the ligand [26]. A better understanding of the contributions of the 2- and 6-hydroxy group positions on the spectinomycin scaffold is crucial for the development of novel analogs that overcome inactivation by AMEs. We chose to examine these interactions using molecular dynamics simulations, rather than the rigid body docking used earlier in this paper. These are computationally intense experiments that account for dynamically changing interactions between the ligand, rRNA nucleotides, RpsE loop, and associated water molecules. Removing or modifying the 2- and 6-hydroxy groups on spectinomycin and analogs had large effect on the binding and antibacterial activity (Table 1). To examine the molecular basis of these effects, the docked conformations of spectinomycin 2, 5, 6, 9, and 10 analogs were subjected to a 20 ns molecular dynamic simulation (Figs. 6 and 7). The docking pose of spectinomycin into the *M. tuberculosis* rRNA/RpsE complex homology model was compared to the *E. coli* X-ray crystal structure (PDB ID: 2QOU [26]) to ensure the binding mode was not altered between species. The ligand RMSD between the two structures was 0.65 Å (data not shown). The final spectinomycin binding mode following a 20 ns MD simulation is shown (Fig. 6). Like the X-ray crystal structure, the nucleotides in the spectinomycin binding pocket participate in several key hydrogen bonding interactions. The 2-hydroxy group coordinates with a water molecule and interacts with A1191, while the 6-hydroxy group has one binding interaction with G1064.

In the simulation, 6-deoxyspectinomycin 5 lost the 6-hydroxy group-mediated hydrogen bonding interaction with the G1064 nucleotide (Fig. 6b) and resulted in a 1.65 Å RMSD shift in the pocket compared to spectinomycin (Fig. 6d). The free energy of binding for spectinomycin and analogs was calculated using MM/GBSA in Amber18 [40] (Table 2). The MM/GBSA method calculates binding free energies using molecular mechanics at low computational cost [40, 48]. Furthermore, GBSA is accurate and robust in ranking binding affinities for structure-based drug discovery. Removal of the 6-hydroxyl group resulted in a decrease of 8.6 kcal mol⁻¹ in binding energy compared to spectinomycin and is reflective of the poor IC₅₀. Removal of the 2-hydroxy in 9 results in the loss of two hydrogen bonding interactions in the pocket, i.e., a water molecule and the A1191 nucleotide (Fig. 6c). Despite the remaining hydrogen bonding partners, 9 produces a larger shift in the pocket with a RMSD of 2.81 Å (Fig. 6d). This shift results in a 6.3 kcal mol⁻¹ decrease in the binding energy compared to spectinomycin (Table 2). While both analogs had poor binding affinities, loss of the 2-hydroxy group was more favorable than the 6-hydroxy group for the spectinomycin core.

Interactions driving spectinomycin binding are also observed in the spectinamide 2-bound complex, with the addition of a hydrogen bond between the aryl chain on spectinamide and G1193 (Fig. 7a). The aryl chain also gains VDW interactions on the RpsE protein loop. Binding of 2 did not perturb the spectinomycin binding pocket, with a RMSD value of 0.75 Å (Fig. 7d). However, binding of 6, the corresponding 6-deoxy analog, shifted 2.47 Å from 2 in the pocket and reduced the calculated ΔG of binding by 16.0 kcal mol⁻¹ (Table 2). Despite the loss of one hydrogen...
bond interaction with G1064, 6 maintains VDW interaction RpsE protein loop (Fig. 7b), improving the IC50 compared to the 6-deoxyspectinomycin analog 5. Additionally, 6 gains a hydrogen bonding interaction between G1193 phosphate backbone and the spectinomycin scaffold because of the large shift in the binding pocket (Fig. 7b). In contrast, the 2-deoxyspectinamide analog 10 remains in the spectinomycin binding pocket with a RMSD value of 1.28 Å compared to 2 (Fig. 7d). Losing the A1191 hydrogen bond is alleviated through interactions from the aryl chain to G1193 and VDW interactions with Val56 on the RpsE protein loop (Fig. 7c). Binding of 10 reduced the ΔG of binding by 5.3 kcal mol⁻¹ compared to 2, suggesting the loss of the 2-hydroxy group is more favorable than the 6-hydroxy group as observed with deoxyspectinomycin analogs. However, despite loss of the hydroxy groups, both deoxyspectinamide analogs recover binding interactions and rescue activity via additional protein–ligand interactions on the RpsE protein loop.

The calculated ΔG of binding for the analogs correlates to the measured ribosomal IC50 data; however, the energetic contribution for each nucleotide is unknown from this analysis. Therefore, the free energy decomposition [40] of each nucleotide and amino acid in the binding pocket was compared between the analogs (Fig. 8). From the spectinomycin MD simulation, G1064, C1066, and G1068 have the strongest interaction in the binding pocket, with A1191, C1192, and G1193 being the weakest (Fig. 8 and Table S2). When the 6-hydroxy group is removed, the hydrogen bonding interaction from G1064 is lost and results in a 1.8 kcal mol⁻¹ decrease in free energy (Fig. 8). The loss of the 6-hydroxy group indirectly effected nucleotide binding interactions with 5, 0.82 kcal mol⁻¹ loss for C1066 and 0.45 kcal mol⁻¹ loss for G1068. In contrast, the removal of the 2-hydroxy group did not produce these large changes in the free energy of binding (Fig. 8). Despite the loss of A1191 hydrogen bonding interactions, the C1066 had the largest loss in free energy of 2.33 kcal mol⁻¹. This trend was observed for the deoxyspectinamidine analogs compared to the parent 2 compound. The 6-deoxy 6 analog had a decrease in G1064 free energy by 2.29 kcal mol⁻¹, as the hydrogen bond interaction is lost as observed for 5. Additionally, the binding free energy for C1192 decreased, 3.96 kcal mol⁻¹, which was not observed for 5. This change in C1192 binding energy may be a result of the large shift in the binding pocket. The aryl side chain increased binding

**Fig. 6** Snapshot from 20 ns molecular dynamic simulation of spectinomycin analogs. The binding mode of a spectinomycin, b 5, and c 9 from 20 ns MD simulations. The protein is shown as a gray ribbon with nucleotides and waters interacting with the ligand are labeled.
interactions with G1193, 1.13 kcal mol\(^{-1}\). The 2-deoxy analog 10 maintained similar binding free energies compared to the parent compound 2. This is not surprising, as 10 did not perturb the binding pocket, RMSD value of 1.28 Å. The loss of the A1191 binding interaction in the pocket resulted in a 1.16 kcal mol\(^{-1}\) decrease in energy, as well as indirect effect on C1066, 1.58 kcal mol\(^{-1}\). The aryl chains in the spectinamide series did increase binding interactions through the RpsE protein loop, as observed in the increase of Val56 free energy (Fig. 8).

From the decomposition of the free energy of binding, loss of the 2-hydroxy group in 9 and 10 did not greatly perturb the binding free energy of the nucleotides in the pocket except for a decrease in C1066 and A1191 (Fig. 8). However, loss of the 6-hydroxy group in 6 greatly impacted the free energy landscape of the binding pocket. Binding free energies decreased for G1064, A1191, and C1192 and increased with G1068 and G1193 (Fig. 8). This increase in energy is a result of shifting the spectinamide out of the pocket to maintain contacts between aryl side chain and the RpsE protein. This compensatory mechanism shown for both 6 and 10 suggests that the aryl side chain on these analogs promote favorable binding interactions, i.e., Val56 (Fig. 8). Structure-based drug discovery of the aryl chain on deoxyspectinamides to increase protein–ligand interactions may be a viable method to overcome the energetic penalties for removing the 2- or 6-hydroxy groups to avoid AME activity.

**Table 2** Binding free energy onto the Mycobacterial RNA/RpsE complex determined using the MM/GBSA method in Amber18

| Compound | \(E_{VDW}\) (kcal mol\(^{-1}\)) | \(E_{nonpolar}\) (kcal mol\(^{-1}\)) | \(\Delta G_{total}\) (kcal mol\(^{-1}\)) | Ribosomal IC\(_{50}\) (μg ml\(^{-1}\)) |
|----------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Spectinomycin | −38.9 ± 2.70 | −5.41 ± 0.05 | −78.5 ± 2.32 | 0.36 |
| 5 | −36.9 ± 2.60 | −5.21 ± 0.05 | −69.9 ± 2.92 | >60 |
| 9 | −39.3 ± 2.55 | −5.34 ± 0.05 | −72.2 ± 2.21 | >60 |
| 2 | −57.2 ± 2.98 | −7.29 ± 0.06 | −97.6 ± 2.78 | 0.89 |
| 6 | −49.8 ± 2.76 | −6.86 ± 0.05 | −81.6 ± 2.49 | 9.50 |
| 10 | −56.3 ± 2.91 | −7.34 ± 0.06 | −92.3 ± 2.27 | 10.2 |

\(^{a}\)Values represent an average over the 1000 MD frames ± standard deviation

\(^{b}\)Tested with *M. smegmatis*

**Fig. 7** Modeled binding modes of spectinamide analogs from 20 ns molecular dynamic simulation in AMBER18. The binding mode of a 2, b 6, and c 10 from 20 ns MD simulations. The protein is rendered as a gray ribbon with nucleotides and waters interacting with the ligand shown as sticks. The dashed lines represent hydrogen bonding interactions. d An overlay of spectinomycin (yellow), 2 (purple), 6 (orange), and 10 (green) from the 20 ns MD simulations.
This study sought to determine how removal or modification of the AME targeted motifs in spectinomycin, and the related spectinomycines and amSPCs, affected ribosomal potency, antibacterial activity, and cellular accumulation. We used an efficient method for the deoxygenation of spectinomycin enabling the synthesis of deoxyspectinomycines and amSPCs. The deoxyspectinomycin analogs had poor antibacterial activity and ribosomal binding affinity, as did the 6-dehydrospectinamides and the epi-chloro spectinomycin analogs. Molecular dynamic simulations followed by energy decomposition of the individual nucleotides involved in spectinomycin binding revealed the 6-hydroxy group has a larger effect on binding compared to the 2-hydroxy group consistent with in vitro protein translational inhibition data. These results experimentally validate our modeling approach, which allow for key binding site residues to be targeted in the design of future spectinomycin analogs.

Other important spectinomycin binding/structure–activity information can be gleaned from examining the testing data in this study, including an evaluation of the contribution the aryl side chains make to spectinamide and amSPC binding. In this case, the loss of binding affinity caused by deoxygenation of the spectinomycin ring affinity is demonstrated to be partially rescued by the additional interactions between the RpsE protein and the aryl side chains extending from the deoxyspectinamide and deoxy-amSPC analogs. Deoxygenation decreases the hydrophilicity of the spectinomycins, a goal of our program, as more lipophilic analogs are expected to have higher permeability in mammalian and bacteria cells. However, deoxygenation of the analogs in this study led to only small increases in cellular accumulation and was not found to affect the antimicrobial potency.

This study builds on the knowledge of the SAR of spectinomycin analogs and is being used to aid the design of next-generation spectinomycins. Analogs that maintain a non-AME modifiable hydrogen bond donor at the 6-position to interact with G1064 in combination with aryl side chains modifications are being explored in subsequent studies. Such analogs may be successful new class of antibiotics targeting the helix-34 ribosomal binding site, overcoming AME resistance, if their overall binding affinities can be increased and their cellular permeability optimized.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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