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Synthesis, broad spectrum antibacterial activity, and X-ray co-crystal structure of the decoding bacterial ribosomal A-site with 4′-deoxy-4′-fluoro neomycin analogs†

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This study reports the synthesis, antibacterial evaluation and nature of fluorine–rRNA contacts revealed by an X-ray co-crystal structure of a series of 4′-deoxy-4′-fluoro B-neomycin analogs. 4′-Deoxyfluorination improves the inhibition profile towards resistant enzymes and renders equally potent antibiotics compared to the parent neomycin B. The 4′-deoxy-4′-fluoro-4′-*epi* neomycin analogs showed a preferential inhibition over the 4′-deoxy-4′-fluoro neomycin counterpart against the strains of *P. aeruginosa* carrying a chromosomal APH(3′)-IIb enzyme, known to inactivate the parent aminoglycoside. To the best of our knowledge, this is the first example of a neighboring-group aminoglycoside-modifying enzyme evasion by fluorine substitution. A unique F-G1491 stacking was observed in a co-crystal structure of 4′-deoxy-4′-fluoro-4′-*epi* neomycin with a bacterial ribosomal RNA A-site.

Introduction

Aminoglycosides (AGs) are clinically important and structurally diverse pseudo-saccharides decorated with multiple ammonium groups, which originate from natural sources and semi-synthesis in the laboratory (Fig. 1).[1] As part of the chemical warfare of soil bacteria, AGs evolved to bind to the highly conserved rRNA helix H44, known as the A-site, at the heart of the decoding center of the bacterial 30S ribosomal subunit.[2–4] The binding of an AG causes interference with the accuracy of mRNA–tRNA discrimination and leads to chaotic protein synthesis,[3,5] which results in a broad-spectrum bactericidal effect that is unique to AGs among other ribosome-targeted antibiotics.[6]

Clinically used AGs of the 4,6-disubstituted class (Fig. 1), such as gentamicin (**1**), amikacin (**2**), and tobramycin (**3**),[1] have been useful in controlling nosocomial pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa*, which are particularly difficult to treat in patients with cystic fibrosis,[7] immunodeficiency,[8] and burn wounds.[9] The 4,5-disubstituted class of AGs (Fig. 1) includes, among others, the natural products neomycin (**4**), used in topical ointments,[10] and paromomycin (**5**) that has an antibacterial and antiparasitic activity and is currently used for the treatment of leishmaniasis and dysenteric amoebiosis.[11]

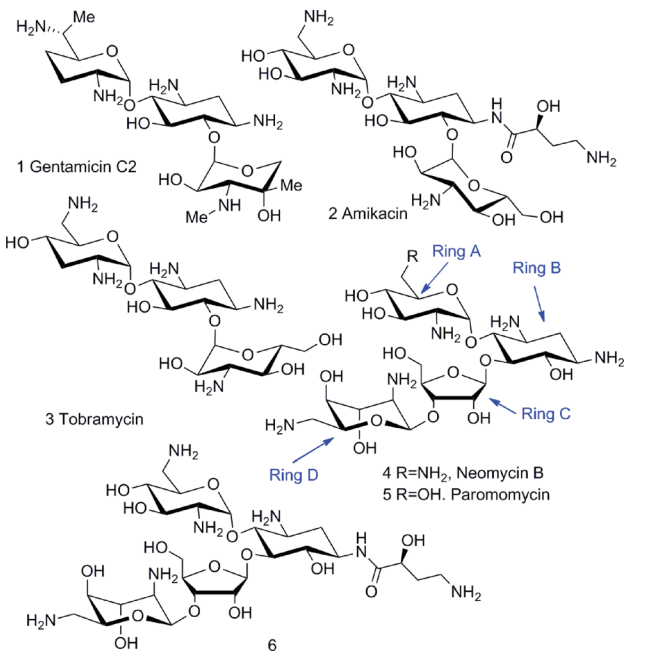

Fig. 1 Representative AG antibiotics of 4,5- and 4,6-disubstituted subclasses, and N1-substituted analogues.

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The evolutionary pressure due to the medical and veterinary use of antibiotics over the last half-century has led to the global emergence of aminoglycoside-modifying enzymes (AMEs) that dangerously undermine the effectiveness of clinically deployed antibiotics. These enzymes modify AG functional groups, turning them inactive, and include: *N*-acetyltransferases (AACs), *O*-phospho-transferases (APHs) and *O*-adenyltransferases (ANTs) that are the prevalent mode of resistance in clinical isolates.[1,12–15]

Multiple AMEs are responsible for the inactivation of AGs that bear 3′-OH and 4′-OH groups on ring A, such as neomycin (**4**) and paromomycin (**5**). Therefore, these AGs cannot be reliably used to control dangerous human pathogens, such as *S. aureus* and *P. aeruginosa*, due to the inactivation by the prevalent APH(3′) and ANT(4′) enzymes that target the ring A hydroxyl groups (Fig. 1).[12–14,16] The action of the APH(3′) enzyme can be averted by AGs that lack the 3′-OH group, such as tobramycin (**3**) and gentamycin (**1**), or by AGs that bear a N1-(*S*)-hydroxyaminobutyric acyl (HABA) group substituent,[17] such as amikacin (**2**) (ref. 18 and 19) or N1-HABA neomycin (**6**) (ref. 20) (Fig. 1).

The development of AG analogs, inspired by gentamicin (**1**) and tobramycin (**3**), has resulted in a number of potent deoxygenated analogs with improved antibiotic activity.[21] The removal of the 3′-OH and 4′-OH groups, however, presents a conundrum, because these functional groups also modulate the pK_a of the nearby ammonium groups, which correlates with nephrotoxicity trends in the AG series.[22,23] The prospect of replacing the ring A hydroxyl groups with fluorine atoms is a potential solution,[24–26] but poses a significant synthetic challenge.[27–30] Herein, we report the application of fluorination methodologies developed in our laboratory on the complex tetrasaccharide structure of neomycin (**4**). We show that an axial 4′-fluorine atom guards the neighboring 3′-OH group against the *P. aeruginosa* chromosomal APH(3′) enzyme, while it also averts inactivation by the ANT(4′) enzymes. Furthermore, we characterize an advanced 4′-fluorinated N1-HABA neomycin analog by determining its X-ray co-crystal structure with the bacterial ribosome A-site, thus gaining insight into its intricate binding interactions.

Results and discussion

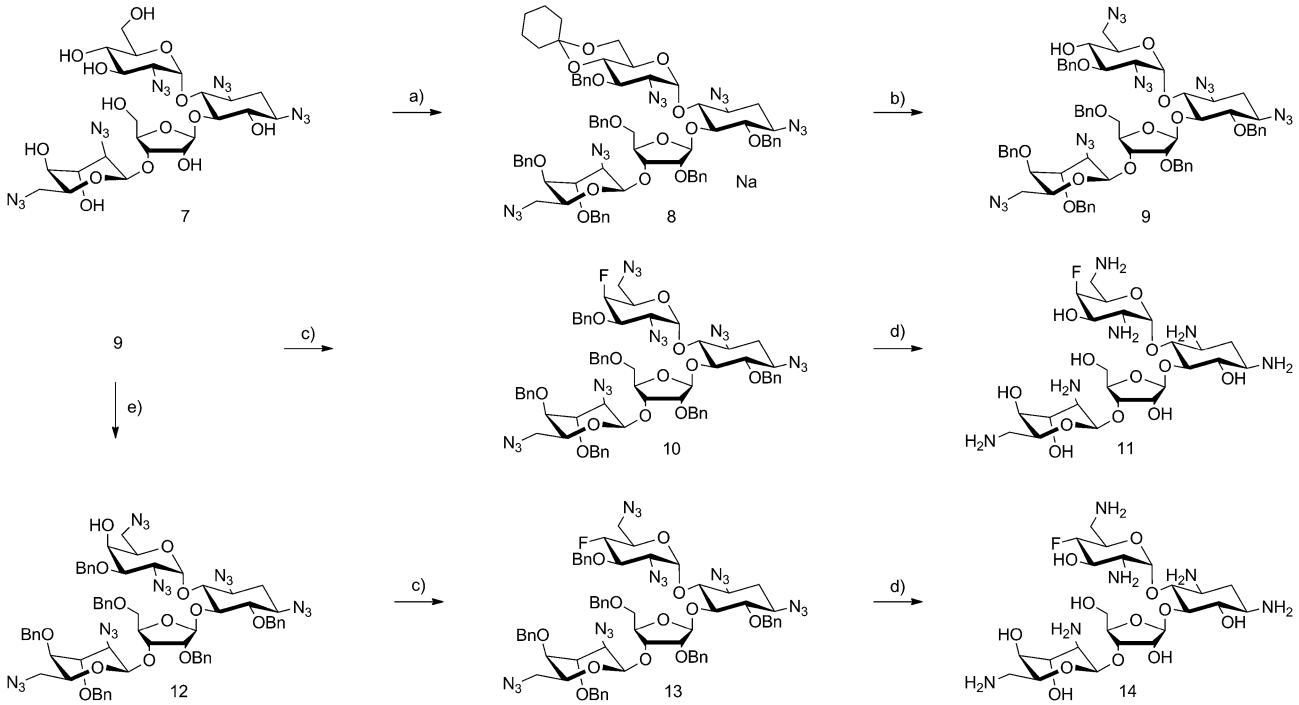
We adapted a synthetic route we have previously developed for the deoxyfluorination of the isolated ring A–B pseudodisaccharide (neamine)[31] towards the full neomycin scaffold, having both equatorial and axial 4′-fluorine substituents: 4′-deoxy-4′-fluoro-4′-*epi* neomycin (**11**) and 4′-deoxy-4′-fluoro neomycin (**14**), respectively (Scheme 1). We required a suitably elaborated intermediate, **9**, with an isolated 4′-OH group, featuring azides and benzyl ethers as fluorination-compatible protecting groups (Scheme 1). The synthetic effort began by the temporary protection of ring A in **7** (ref. 32), using 1,1-dimethoxycyclohexane in the presence of camphorsulfonic acid (CSA),[33] followed by the per-benzylation of the remaining hydroxyl groups to afford intermediate **8** in good overall yield. The cleavage of the cyclic ketal under acidic conditions led to the ring A diol,[32] which was selectively tosylated on its primary 6′-OH group and treated with NaN_3 to produce the neomycin analog, **9**, with a free 4′-OH group in acceptable yield (Scheme 1).

For the introduction of fluorine at C-4′ of ring A of neomycin B (Fig. 1), we first explored the use of diethylaminosulfur trifluoride (DAST, for reviews see Hudlický[34]), as we have previously reported.[31] In practice, the well-established DAST reaction conditions produced a separable mixture of fluorinated products in reasonable yields: 26% for **13** and 33% for **10**. The formation of **13** can be explained by anchimeric assistance of the benzyl group, due to a non-concerted fluorine displacement *via* a carbocation intermediate.[31,35–37] In view of this, we pursued a milder fluorination approach. Thus, the treatment of **9** with triflic anhydride and pyridine in DCM at 0 °C, followed by the addition of tetrabutylammonium fluoride (TBAF), led to a clean S_N2 displacement, affording **10** in 65% yield as the only observable product (by crude NMR analysis). Subsequently, the reduction of the azido groups under Staudinger conditions and the cleavage of the benzyl protective groups by catalytic hydrogenation afforded 4′-deoxy-4′-fluoro-4′-*epi* neomycin (**11**) in 73% yield over two steps, which was then converted into the sulfate salt by treatment with ammonium sulfate (Scheme 1).

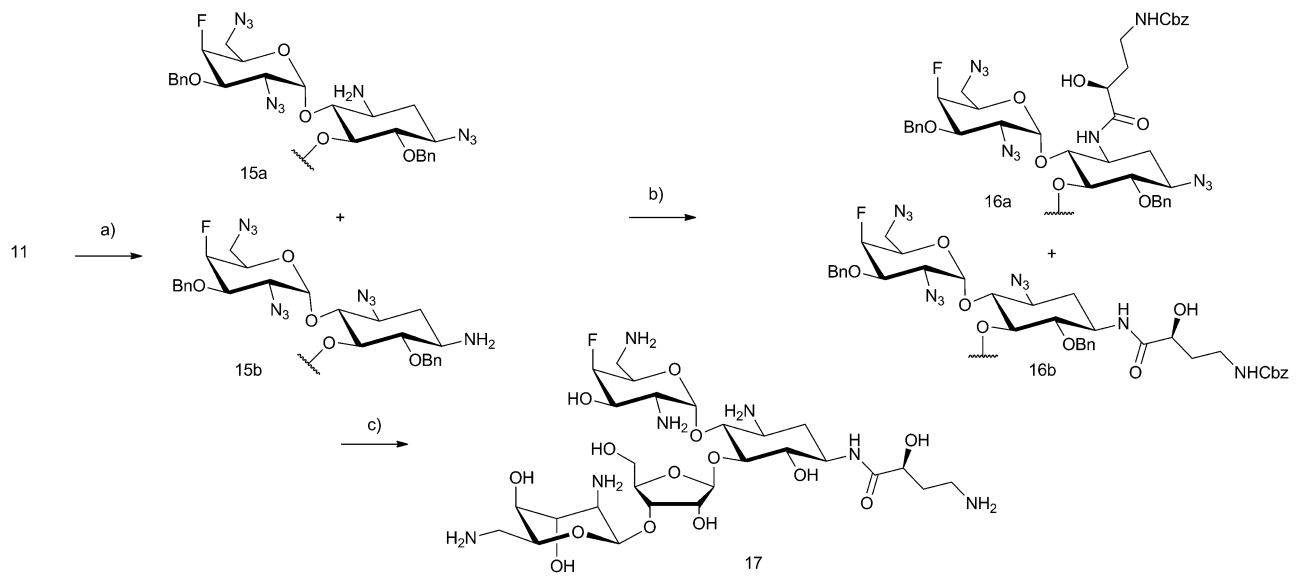
The synthesis of 4′-deoxy-4′-fluoro neomycin (**14**) was realized through an stereoselective oxidation–reduction sequence,[32] followed by the S_N2 displacement of the newly inverted alcohol at C4′. Thus, the treatment of **9** with Dess–Martin periodinane (DMP) in DCM, followed by the stereoselective reduction of the ketone intermediate with L-Selectride® in THF, afforded **12** (4′-*epi* **9**) in good yield (66%, two steps) (Scheme 1). The triflation of **12**, followed by treatment with TBAF, afforded **13** as the only observable product (by crude NMR analysis). As described above, the Staudinger reduction and hydrogenolysis of the benzyl groups afforded **14** in 88% yield over two steps, which was converted to sulfate salt.

Next, we sought to install an N1-HABA-substitutent on **11**, using a regioselective Staudinger reaction.[20] Thus, the treatment of **11** with 1.1 equivalents of PMe_3 afforded **15a** and **15b** as the two major products (Scheme 2), together with minor over-reduction products. We obtained improved regioselectivity by decreasing the equivalents of PMe_3 to 0.9, to afford an inseparable 1 : 0.8 mixture (by ^{1}H-NMR analysis of the crude mixture) of **15a** and **15b** in 68% yield based on the reacted starting material. The mixture was then treated with the *N*-hydroxysuccinimide ester of (*S*)-(−)-4-(benzyloxycarbonyl) amino-2-hydroxybutyric acid (Cbz-HABA-Su) and DIPEA in THF, producing a mixture of N1- and N3-acylated products, **16a** and **16b**, which were separable by thin layer chromatography. The intermediate **16b** was deprotected, as described above, to obtain the final analog **17**, which was then converted to the sulfate salt.

The structures of **16a** and **16b** were confirmed by 1D and 2D NMR experiments. The stereochemical assignment of the compound (**16a**) is based on the presence of a $^2J_{C\text{-}3,NH}$ correlation on its HMBC spectra, which was confirmed by a low field displacement of the ^{1}H-NMR chemical shift of H-3. Conversely, for compound **16b**, a $^2J_{C\text{-}1,NH}$ correlation is observed in its HMBC spectra and a low field chemical shift displacement of H-



Scheme 1 Reactants and conditions: (a) i. CSA, 1,1-dimethoxycyclohexane, DMF, 45 °C, 20 Torr, 1 h, 84% (based on the reacted starting material). ii. First: NaH, THF, 0 °C, 1 h; then: TBAI, BnBr, 24 h, room temperature, 65%, two steps. (b) First: 80% AcOH–THF, 60 °C, 18 h, 76%; then: TsCl, pyridine, 24 h, 81%; followed by: NaN_3, DMF, 50 °C, overnight, 49%, three steps. (c) First: Tf_2O, pyridine, 0 °C; then: TBAF, THF, 50 °C. For **10**: 65% (two steps); for **13**: 75% (two steps). (d) First: PMe_3, THF, NaOH 1 N 1 : 1, 50 °C, 30 min; then: 20% $Pd(OH)_2$/C, 80% AcOH, H_2 (60 psia), room temperature, 24 h, followed by: 50% AcOH, 60 psia, 24 h. For **11**: 73% (two steps); for **14**: 88% (two steps). (e) First: DMP, DCM, overnight, room temperature, 90%; then: L-Selectride®, THF, room temperature, overnight, 66%, two steps.



Scheme 2 Reagents and conditions: (a) first: 0.9 equivalents PMe_3, THF, −78 °C (30 min) to room temperature, 2 h; then 1 N NaOH, overnight (68% yield based on the reacted starting material (**15a**/**15b**) in a 1 : 0.8 mixture, by ^{1}H-NMR). (b) Cbz-HABA-Su, DIPEA, THF, room temperature, overnight, for 55% of **16a** and 32% of **16b** (isolated yields). (c) First: PMe_3, THF, NaOH, 50 °C, 30 min; then 20% $Pd(OH)_2$ on carbon, AcOH–H_2O 4 : 1, hydrogen, 60 psig, overnight, 97% (two steps).

1 is observed in its ^{1}H-NMR spectra (Fig. S1 and Table S1, ESI†). The NMR structural assignment made for **16b** was further confirmed by X-ray crystallography (see below).

According to the literature, the regioselective azide reduction depends on electronic and steric factors.[38,39] It is well established that the Staudinger reaction proceeds

through a triazaiminophosphadiene intermediate, formed after a nucleophilic attack of the phosphine on the azide moiety, followed by the rearrangement to the corresponding iminophosphorane with the expulsion of nitrogen.[40–42] It is expected, for Staudinger reactions where the formation of the triazaiminophosphadiene intermediate is the rate-determining step, that the electronic properties of the reacting azide will govern the regiochemical outcome of the reaction. The recordings of the natural abundance ^{15}N NMR 1D and 2D spectra for **10** (Fig. 2 and 3) suggest that our experimental observations of the regioselective Staudinger reductions correlate with the electron density at the corresponding α-nitrogen atoms.

Antibacterial activity and evasion of APH(3′)-IIb

To investigate the effect of 4′-fluorine substitutions on the antibiotic activity and on the AG resistance mechanisms, we screened our analogs, **11**, **14** and neomycin B (**4**), for their biological activity against a panel of susceptible and AG-resistant Gram-positive and Gram-negative bacterial strains (Fig. 4). The AG-resistant strains expressed ANT(4′)-I and -II, APH(3′)-IIb and -III, and AAC(6′)-I and -II.

The isomeric 4′-fluoro analogs, **11** and **14**, display a similar potency compared to the parent neomycin (**4**) against the susceptible isolates of *S. aureus*, *K. pnuemoniae* and *E. coli* (Fig. 4), suggesting that the bacterial rRNA targeted A-sites generally tolerate both axial and equatorial 4′-fluorine replacements.

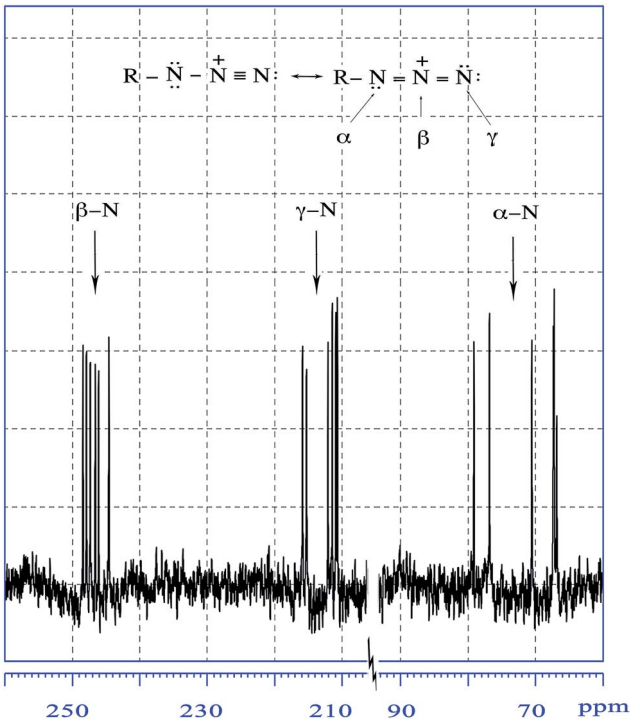


Fig. 2 Natural abundance ^{15}N NMR spectra of the chemical shifts of α-, β- and γ-nitrogen in the azide moieties for **10**. ^{15}N-NMR spectra for **9** were also obtained for comparison, see Table S2, ESI.†

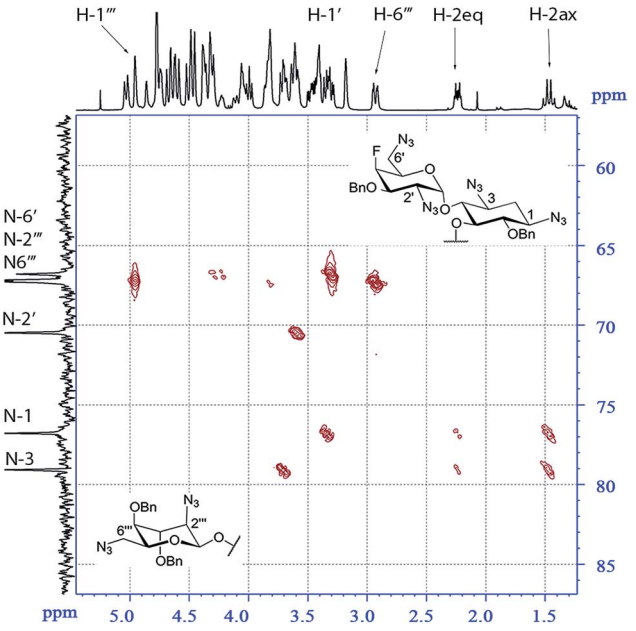


Fig. 3 The 2D ^{15}N-1H HMBC technique permitted the assignment of the α-^{15}N individual signals for α-N3, α-N1 and α-N2′ for compound **10**. A complete set of 1D and 2D spectra is available (ESI†).

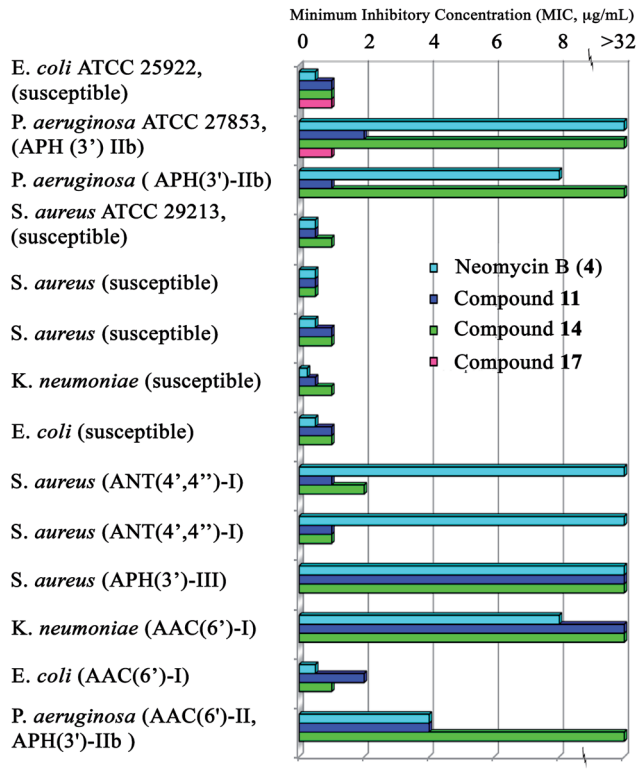


Fig. 4 The screening of antibacterial activity reveals that 4′-deoxy-4′-fluoro-4′-*epi* (**11**) and the N1-HABA analog (**17**) evade APH(3′)-II, compared to 4′deoxy-4′-fluoro (**14**) and the parent neomycin (**4**).

As we have hypothesized, the replacement of the 4′-OH group with fluorine led to the evasion of ANT(4′)-I and -II enzymes, which are widespread in the clinical isolates of *S. aureus*, and

are known to inactivate neomycin (**4**, MIC > 64 μg mL^{-1}) as well as amikacin (**2**, MIC > 32 μg mL^{-1}), gentamycin (**1**), tobramycin (**3**) and kanamycin.[43] The fluorinated analogs, **11** and **14**, displayed MIC values ranging from 1 to 2 μg mL^{-1}, which are equal to susceptible strains but more potent than neomycin B (**4**) (Fig. 4).

Remarkably, we also observed that the axially fluorinated analog, **11**, was not deactivated by the two *P. aeruginosa* strains expressing chromosomal APH(3′)-IIb (Fig. 4),[44] while the corresponding equatorial fluoro analog (D-gluco configuration), **14**, and parent neomycin, **4**, were ineffective against the *P. aeruginosa* strains (MIC > 16 μg mL^{-1}). For APH(3′)-Ia and APH(3′)-II (both from Gram-negative bacteria) the presence of a fluorine substituent at the position 4′ of ring A is expected to reduce the nucleophilicity of the 3′-OH group, this reduction being detrimental to the transfer of phosphate.[45] To our knowledge, this is the first example of a neighboring-group AME evasion by fluorine substitution. Similarly, these properties were corroborated for the analog, N1-HABA-4′-deoxy-4′-fluoro-4′-*epi* neomycin (**17**), against *P. aeruginosa* expressing the APH(3′)-IIb enzyme. The combination of an axial 4′-fluorine and N1-HABA modification, as in **17**, appears to give a more potent aminoglycoside compared to 4′deoxy-4′-fluoro-4′*epi* neomycin (**11**) alone (Fig. 4).

A-site RNA X-ray co-crystallization study

The N1-HABA-4′-deoxy-4′-fluoro-4′-*epi* neomycin analog, **17**, specifically binds to the A-site internal loop and forces the A1492 and A1493 residues to bulge out (Fig. 5a). The key characteristics and common interactions between the AGs and the bacterial A-site are highly conserved in the Bact/**17** complex (Fig. 5b and S2, ESI†).[40] Ring A, with a fluorine atom at 4′, is stacked on top of G1491 and forms a pseudo pair with A1408 through two hydrogen bonds, O5′…H–N6$_{A1408}$ and N6′-H…N1$_{A1408}$ (Fig. 6 and 7a).

The axial F4′ atom of **17** is accommodated by a fluorine–aryl interaction with G1491 (Fig. 5b, 6 and 7a) that partly replaces the stacking interaction of ring A observed in multiple AG–A-site complexes.[4,46] The F4′ atom points towards, and is in close contact with, the C_2, N_3 and C_4 atoms in G1491 (Fig. 5b, 6 and 7a). The distances from the F4′ atom to the C_2, N_3 and C_4 atoms are 2.7, 2.6 and 2.8 Å, respectively. These distances are quite short compared to the sums of the van der Waals radii of F (1.47 Å) with the C (1.7 Å) and N (1.55 Å) atoms (3.17 and 3.02 Å, respectively), and are similar to the distances from the H4′ atom to the C_2, N_3 and C_4 atoms (2.8, 2.7 and 2.7 Å) observed for the natural AG compounds (Fig. 7b), suggesting that there could be non-negligible forces between these atoms. Since the F atom is a neutral electron-rich atom, it can make interactions with electron-poor atoms. From the calculation of the electrostatic potential surfaces, the π clouds of protein aromatic systems, such as Phe, Tyr and Trp rings, are negatively charged, allowing the formation of cation–π interactions. On the contrary, nucleic acid bases, such as T, U and G, have electron-depleted π clouds resulting from the combined effects of their ring nitrogens and their amino/carbonyl substituents.[47] Therefore, it is reasonable that the axial F4′ atom in **17** can form a strong lone pair–π interaction with G1491. Similar fluorine–π stacking interactions have been observed in organic compounds, in which the F atoms are in close contact with the pyrimidine ring with a greater electron-deficient character.[48] For other types of selected deoxyfluoro sugar interactions see: Leumann and Dugovic,[49] Morales *et al.*,[50] Micouin, Tisné *et al.*[51] and Damha *et al.*[52] The stacking interaction between ring A with the axial F4′ atom in **17** and G1491, observed in this study, is the first example of fluorine–π stacking in the AG series. This fluorine atom appears to be accommodated without compromising the overall antibacterial potency (Fig. 4). The HABA group interacts with the upper side of the A-site helix and makes contact with G1497, C1496 and U1495 (Fig. 5b and S3†).

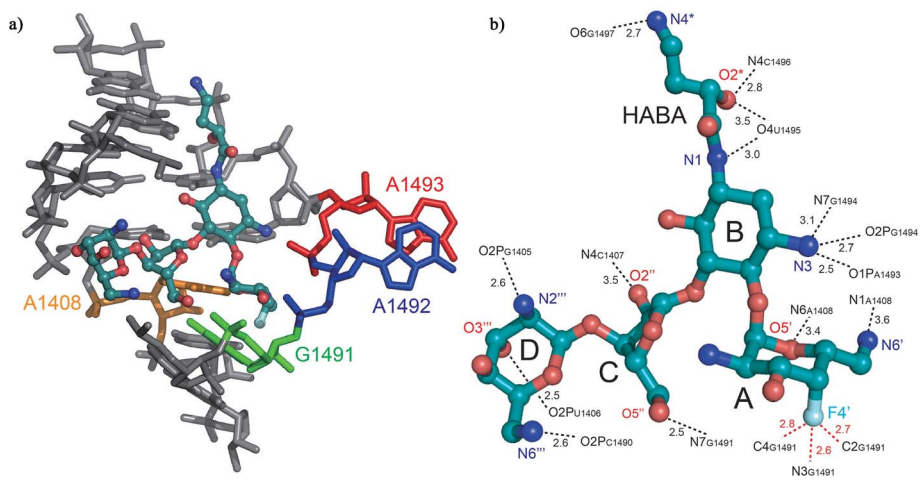


Fig. 5 (a) Binding of aminoglycoside **17** and its detailed molecular contact with the bacterial A-site internal loop. (b) The hydrogen bonds and fluorine–π interactions are represented by black and red dashed lines with distances in Å, respectively.

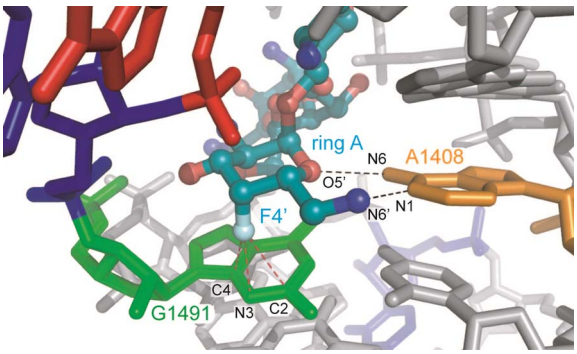


Fig. 6 Interactions of ring A of aminoglycoside **17** with the bacterial A-site RNA model. The F4′ atom is in close contact with the C2, N3 and C4 atoms of G1491.

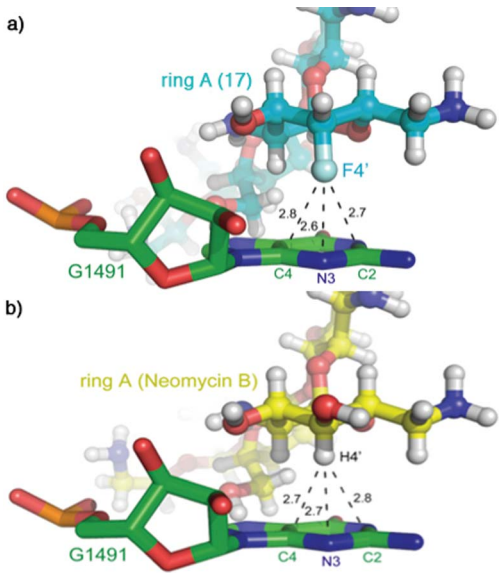


Fig. 7 Stacking interactions observed between ring A and G1491 (a) in the Bact/**17** complex and (b) stacking interactions between ring A and the Bact/neomycin (**4**) complex. The close contacts of F4′ or H4′ with G1491 are represented by dashed lines with distances in Å.

Conclusions

In conclusion, we have demonstrated that the inclusion of a fluorine atom at C4′ of neomycin maintains the antibiotic activity against susceptible bacterial strains. Interestingly, 4′-deoxy-4′-fluoro-4′-*epi* neomycin (**11**) showed a preferential inhibition over the 4′-fluoro neomycin counterpart against the strains of *P. aeruginosa* carrying the chromosomal APH(3′)-IIb enzyme, which is known to inactivate the parent aminoglycoside. This potency was further enhanced when an N1-HABA amide appendage, in combination with the 4′-deoxy-4′-fluoro-4′-*epi* modification, as in analog **17**, was tested. Thus, the introduction of a fluorine atom in selected AGs that are susceptible towards inactivating enzymes, may be an alternative to simple deoxygenation. A unique F-G1491 stacking was observed in a co-crystal structure of N1-HABA-4′-deoxy-4′-fluoro-4′-*epi* neomycin (**17**) with the ribosomal A-site. Such fluorine–RNA interactions can be used as A-site affinity probes[48,51] by ^{19}F-NMR for fully modified aminoglycosides with demonstrated antibacterial activity.

Further studies to probe the related interactions of fluorine-containing small molecules with nucleotides are in progress and will be reported in due course.

Experimental section

Antibacterial activity

The antibacterial activities of **11**, **14**, **17** and comparator antibiotics were determined according to the broth microdilution method recommended by the Clinical and Laboratory Standards Institute.[53] A screening panel composed of 6 Gram-positive strains including *S. aureus* ATCC 29213 and 5 clinical isolates. 8 Gram-negative strains, composed of 3 *P. aeruginosa* (ATCC 27853 and 2 clinical isolates), 3 *E. coli* (ATCC 25922 and 2 clinical isolates) and 2 *K. pneumoniae* clinical isolates from the Achaogen Strain Collection,[54] with diverse aminoglycoside resistance mechanisms were used to compare the activities of the fluorinated neomycin analogs. This panel included strains with key AMEs found in both Gram-negative bacteria and *Staphylococcus aureus*. AME genes and resistance mechanisms in the Achaogen Strain Collection have been confirmed by colony PCR.[54]

Crystallization, data collection and structure determination

The crystallization procedures used were essentially the same as published previously.[46] Two asymmetrical internal loops of the bacterial A-site were inserted between Watson–Crick base pairs in a sequence designed to fold as a double helix (Fig. S2, ESI†). The RNA oligomer was chemically synthesized by Dharmacon (Boulder, CO), purified by 20% denaturing polyacrylamide gel electrophoresis and desalted by reversed-phase chromatography. Crystals of the RNA in a complex with antibiotic **17** (Bact/**17**) were obtained under conditions shown in Table S3, ESI.† An X-ray dataset of Bact/**17** was collected at 100 K with synchrotron radiation at the structural biology beamlines BL-17A in the Photon Factory (Tsukuba, Japan). The dataset was processed with the program *CrystalClear* (Rigaku/MSC). The obtained intensity data were further converted to structure-factor amplitudes using *TRUNCATE* from the *CCP4* suite.[55] The statistics of the data collection and the crystal data are summarized in Table S4, ESI.† The initial phase of Bact/**17** was solved with the molecular replacement program, *AutoMR*, from the *Phenix* suite,[56,57] using the coordinate of the bacterial A-site in a complex with neomycin (PDB code: 2ET4).[4] The molecular structure was constructed and manipulated with the program *Coot*.[58] The atomic parameters of the structure were refined with the program, *CNS*, through a combination of simulated-annealing, crystallographic conjugate gradient minimization refinements and *B*-factor refinements.[59,60] The statistics of the structure refinement are summarized in Table S4, ESI.† The atomic coordinate of Bact/**17** has been deposited in the Protein Data Bank (PDB) with the ID code 4PDQ.

General methods

Standard ^{1}H, ^{13}C, ^{19}F, 1D or 2D spectra were recorded with a Bruker AVANCE™ II 400, AVANCE™ 400 RG, AVANCE™ 500 RG or AVANCE™ 700 RG NMR spectrometer. Natural abundance ^{15}N 1D and 2D spectra were recorded with Bruker AVANCE™ III HD NMR spectrometers with a Prodigy 55 CryoProbe™ at a concentration of 0.1 M in $CDCl_3$. Chemical shifts (ppm) were reported relative to internal TMS ($\delta = 0$) with $CDCl_3$ as the solvent or DSS ($\delta = 0$) with D_2O as the solvent for the proton spectra. The other spectra were calibrated according to the ratio Ξ of the resonance frequencies. The values used were: $\Xi = 0.25145020$ (TMS) and $\Xi = 0.25144953$ (DSS) for ^{13}C, $\Xi = 0.94094011$ for ^{19}F and $\Xi = 0.10136767$ for ^{15}N. The chemical shifts (in ppm) and coupling constants (in Hz) were obtained from the first-order analysis of the spectra. A minus sign (−) is used to label those signals that are negative in the ^{13}C spectra acquired in DEPT mode (θ_y 135°). Quaternary carbons are indicated by (q). Unless otherwise stated, the reactions were performed in flame or oven-dried glassware under an argon atmosphere using dry, deoxygenated solvents (passed over a column of activated alumina). Commercially available chemicals were used as received. All chemical products were purchased from Sigma-Aldrich (St. Louis, MO). Room temperature refers to 22–26 °C. All reactions were monitored by thin-layer chromatography (TLC), using pre-coated Silicycle (QC, Canada) silica gel (250 μm, 60 Å) plates with an F-254 indicator, and visualized by UV quenching, ceric molybdate, or ninhydrin staining. Flash column chromatography was performed on a ZEOprep 60 (40–63 μ) silica gel (Zeochem, Louisville, KY). IR spectra were recorded on a PerkinElmer Spectrum One ATR-FTIR system (Waltham, MA). Preparative HPLC was done using a Gilson preparative system (215 liquid handler, 322 dual pump and a 156 UV/vis detector). Optical rotations were measured using Perkin Elmer polarimeter model 343 (Waltham, MA). Samples were lyophilized using a Thermo Savant, Modulyo D Freeze Dryer (Sussex, UK).

Synthetic procedures

1,3,2′,2‴,6‴-Pentadeamino-1,3,2′,2‴,6‴-pentaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′,6′-*O*-cyclohexylidene paromomycin (8).[33] *Step 1*. A solution of 7 (ref. 32) (2.8 g, 3.76 mmol), CSA (170 mg, 0.75 mmol), and 1,1-dimethylcyclohexane (13.5 mL, 94 mmol) in 152 mL of anhydrous DMF was heated in a rotary evaporator at 45 °C and under reduced pressure (20 Torr) for 1 h. The cooled solution was neutralized with solid $NaHCO_3$ and the solvent removed under reduced pressure. The residue was purified by flash chromatography (gradient, 5–7% MeOH in DCM) affording unreacted 7 (1.11 g, 1.5 mmol, 40%) and 1,3,2′,2‴,6‴-pentadeamino-1,3,2′,2‴,6‴-pentaazido-4′,6′-*O*-cyclohexylidene paromomycin (1.57 g, 1.90 mmol, 84% based on the reacted starting material) as a white foam. HRMS (ESI-TOF) *m*/*z* $[M + H]^+$ calc. for $C_{29}H_{44}N_{15}NaO_{14}$: 848.3006; observed: 848.3013.

Step 2. To a solution of the aforementioned compound (906.9 mg, 1.1 mmol) in THF (11 mL), NaH (60% in mineral oil, 530 mg, 13.2 mmol) was added at 0 °C and the mixture was stirred for 1 h. TBAI (284 mg, 0.77 mmol) and BnBr (12 mL, 9.9 mmol) were added, the ice bath was removed and the mixture was stirred for 24 h at room temperature. Following the addition of MeOH (2 mL), the mixture was stirred for 30 min, then diluted with EtOAc, washed with water and brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The crude product was purified by flash chromatography (gradient elution: 1 : 9 to 1 : 4 EtOAc–hexanes) affording the title compound, **8**, (1.17 g, 0.86 mmol, 78%, 65%, two steps) as a light brown syrup. IR ν (cm^{-1}, film): 3039, 2940, 2105, 1500, 1458, 1369, 1335, 1266, 1217, 1129, 1198, 1074, 1042, 958, 930, 754, 700. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48–7.05 (m, 30H), 6.11 (d, $J = 3.9$ Hz, 1H), 5.65 (d, $J = 5.5$ Hz, 1H), 4.94 (m, 2H), 4.73 (m, 2H), 4.65–4.36 (m, 6H), 4.35–4.19 (m, 4H), 4.01–3.84 (m, 7H), 3.84–3.52 (m, 7H), 3.50–3.32 (m, 4H), 3.27 (t, $J = 9.3$ Hz, 1H), 3.14–3.09 (m, 1H), 3.02 (dd, $J = 10.1$, 3.9 Hz, 1H), 2.87 (dd, $J = 12.9$, 3.8 Hz, 1H), 2.28–2.11 (m, 2H), 1.79–1.27 (m, 12H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.4, 138.4, 138.0, 137.6, 137.1 and 137.0 (Ar*C*),128.7–127.39 (30 CH), 106.2, 99.7 (q), 98.7, 96.4, 84.2, 82.5, 82.2, 81.9, 76.8, 76.4, 75.6, 75.2, 75.1 (−), 74.8 (−), 74.6, 74.5, 73.3 (−), 72.9, 72.4 (−), 71.8 (−), 71.6, 70.4 (−), 64.0, 62.8, 61.9 (−), 60.3, 59.9, 57.4, 51.2 (−), 38.1 (−), 32.5 (−), 27.8 (−), 25.7 (−), 23.1 (−), 22.5 (−). HRMS (ESI-TOF) *m*/*z* $[M + Na]^+$ calc. for $C_{71}H_{79}N_{15}NaO_{14}$: 1388.5823; observed: 1388.5838. $[\alpha]_D^{25\ °C}$ + 78° (*c* 1.47 in CH_3Cl).

1,3,2′,6′,2‴,6‴-Hexadeamino-1,3,2′,6′,2‴,6‴-hexaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl neomycin (9). *Step 1*. A suspension of **8** (4.15 g, 3.02 mmol) in 80% AcOH–THF (25 : 1, 25 mL) was stirred for 18 h at 60 °C. The homogeneous solution obtained was concentrated under reduced pressure, the crude product was diluted with EtOAc, washed with aqueous bicarbonate solution, brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography (AcOEt–hexanes 2 : 3) affording 1,3,2′,2‴,6‴-pentadeamino-1,3,2′,2‴,6‴-pentaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl paromomycin (2.49 g, 2.46 mmol, 76%) as a white foam. The spectra recorded for the product were in agreement with the ones described by Vasella *et al.*[32]

Step 2. To a solution of the aforementioned compound (1.47 g, 1.14 mmol) and pyridine (0.46 mL, 5.7 mmol) in DCM (5.7 mL), TsCl (434 mg, 2.28 mmol) was added in one portion and the solution was stirred for 24 h at room temperature. The crude product was diluted with EtOAc, washed with 1 N HCl, water, brine and concentrated under reduced pressure. The product was purified by flash chromatography (EtOAc–hexanes 1 : 3) affording 1,3,2′,2‴,6‴-pentadeamino-1,3,2′,2‴,6‴-pentaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-6′-*O*-(*p*-toluenesulfonyl) paromomycin (1.33 g, 0.92 mmol, 81%) as a white foam. HRMS (ESI-TOF) *m*/*z* $[M + Na]^+$ calc. for $C_{72}H_{77}N_{15}NaO_{16}S$: 1462.5286; observed: 1462.5249.

Step 3. A suspension of NaN_3 (95 mg, 1.5 mmol) in DMF (2.9 mL), containing the above-mentioned compound (418 mg, 0.29 mmol), was stirred at 60 °C overnight. The crude product was diluted with EtOAc, washed three times with water, brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography (EtOAc–hexanes 1 : 4) affording 1,3,2′,6′,2‴,6‴-

hexadeamino-1,3,2′,6′,2‴,6‴-hexaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl neomycin (**9**) (303 mg, 0.231 mmol, 79%, 49%, three steps) as a white foam. IR ν (cm^{-1}, film): 3456, 3360, 3090, 2914, 2867, 2099, 1453, 1361, 1331, 1262, 1221, 1041, 740, 698. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53–7.12 (m, 30H), 6.25 (d, J = 3.6 Hz, 1H), 5.73 (d, J = 5.8 Hz, 1H), 5.04–4.93 (m, 3H), 4.76 (d, J = 10.6 Hz, 1H), 4.69–4.62 (m, 3H), 4.59–4.48 (m, 4H), 4.66 (dd, J = 12.65, Hz, 1H), 4.39–4.26 (m, 4H), 4.09 (ddd, J = 9.3, 6.4, 2.4 Hz, 1H), 4.05–3.97 (m, 2H), 3.93–3.78 (m, 4H), 3.76–3.66 (m, 2H), 3.61 (dd, J = 10.4, 3.1 Hz, 1H), 3.56–3.43 (m, 3H), 3.42–3.31 (m, 3H), 3.28 (dd, J = 9.9, 8.7 Hz, 1H), 3.17 (d, J = 2.4 Hz, 1H), 2.97–2.87 (m, 2H), 2.28 (dt, J = 13.2, 4.5 Hz, 1H), 1.46 (q, J = 12.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.3, 138.0, 138.0, 137.7, 137.1 and 137.0 (Ar*C*) 128.8–127.4 (30C), 106.2, 98.7, 95.9, 84.4, 82.6, 82.2, 81.8, 79.8, 75.7, 75.2 (−), 75.1, 75.0 (−), 74.5, 73.4 (−), 73.3 (−), 72.9, 72.5 (−), 71.8 (−), 71.6, 71.4, 71.1, 70.3 (−), 62.7, 60.5, 60.3, 57.4, 51.7 (−), 51.2 (−), 32.7 (−). ^{15}N NMR (41 MHz, $CDCl_3$) δ 66.7, 67.2 and 67.5 (α6′N, α2‴N and α6‴N), 71.5 (α2′N), 76.9 (α1N), 79.1 (α3N), 210.1, 210.7, 210.9, 211.4, 215.1 and 215.4 (6N, γN′s), 244.6, 246.4, 246.7, 247.7, 248.5 and 248.8 (6N, βN′s). HRMS (ESI-TOF) m/z $[M + Na]^+$ calc. for $C_{65}H_{70}N_{18}NaO_{13}$: 1333.52674; observed: 1333.52496. $[\alpha]_D^{25\ °C}$ 74° (c = 1.04 in $CHCl_3$).

Reaction of 9 with DAST

To a solution of **9** (100 mg, 0.076 mmol) in anhydrous DCM (1.0 mL), DAST (30 μL, 0.228 mmol) was added under argon and the mixture was stirred overnight at room temperature. The solution was diluted with DCM, washed with a saturated $NaHCO_3$ solution, dried over anhydrous $MgSO_4$, and concentrated under reduced pressure. The product was purified by flash chromatography (EtOAc–hexanes 1 : 4), affording a crude mixture of **13** and **10** (1.0/1.3, 90.9 mg, 0.068 mmol, 89%) that was separated by preparative HPLC (Prevail C18, 12 mL min^{-1}, 10% MeCN in water containing 0.1% HCOOH, several runs were required) affording **13** (26 mg, 0.02 mmol, 26%) and **10** (32.7 mg, 0.025 mmol, 33%) as white foams.

1,3,2′,6′,2‴,6‴-hexadeamino-1,3,2′,6′,2‴,6‴-hexaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-deoxy-4′-fluoro-4′-*epi* neomycin (10). *Step 1*. To a solution of **9** (100 mg, 0.076 mmol) and pyridine (0.19 mL) in DCM (3.8 mL), Tf_2O (0.13 mL, 0.76 mmol) was slowly added at 0 °C and the mixture stirred for 1 h at 0 °C. The reaction mixture was diluted with DCM, washed with icy aqueous $NaHCO_3$, icy 1 N HCl, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure affording crude triflate (101 mg, 0.07 mmol, 92%) — HRMS (ESI-TOF) m/z 1443.5 $[M + H]^+$ — that was used in the next step without further purification.

Step 2. A solution of the aforementioned triflate (101 mg, 0.071 mmol) and TBAF (1 N in THF, 0.21 mmol, 0.21 mL) in THF (1 mL) was heated at 50 °C for 3 h. The crude product was concentrated under reduced pressure. The residue was dissolved in EtOAc, washed twice with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc–hexanes 1 : 4) affording crude **10** (87 mg, 0.066 mmol, 95%) that was repurified by preparative HPLC (Prevail C18, 12 mL min^{-1}, isocratic elution, 10% MeCN in water containing 0.1% HCOOH) affording **10** (55 mg, 0.042 mmol, 65%, two steps) as a transparent glass. IR ν (cm^{-1}, film): 3360, 2924, 2867, 2101, 1497, 1454, 1364, 1332, 1260, 1123, 1086, 1040, 904, 727, 650. ^{1}H NMR (500 MHz, $CDCl_3$): see Table 1; additionally δ 7.44–7.06 (m, 30H), 4.96 (d, J = 10.6 Hz, H-Bn), 4.72 (s, 2H-Bn), 4.69 (d, J = 10.5 Hz, H-Bn), 4.62 (d, J = 12.0 Hz, H-Bn), 4.57 (d, J = 11.7 Hz, H-Bn), 4.54 (d, J = 11.7 Hz, H-Bn), 4.44 (d, J = 11.7 Hz, H-Bn), 4.41 (d, J = 11.8 Hz, H-Bn), 4.41 (d, J = 11.8 Hz, H-Bn), 4.31 (d, J = 12.0 Hz, H-Bn), 4.25 (d, J = 12.1 Hz, H-Bn).^{13}C NMR (126 MHz, $CDCl_3$) see Table 2; additionally: δ 138.1, 137.9, 137.6, 137.1, 137.0 and 136.9 (6 ArC), 128.73–127.34 (30C), 75.0 (−), 73.4 (2C both (−)), 72.4 (−), 72.1 (−), 71.7 (−). ^{15}N NMR (41 MHz, $CDCl_3$) δ = 66.7, 67.1 and 67.2 (α6′N, α2‴N and α6‴N), 70.4 (α2′N), 76.7 (α1N), 79.0 (α3N), 210.6, 210.8, 211.3, 211.9, 215.1 and 215.7 (6N, γN′s), 244.4, 246.0, 246.4, 247.2, 247.8 and 248.3 (6N, βN′s). ^{19}F NMR (282 MHz, $CDCl_3$) δ −219.0 (ddd, J = 50.7, 29.0, 27.6 Hz). HRMS (ESI-TOF) m/z $[M + Na]^+$ calc. for $C_{65}H_{69}FN_{18}NaO_{12}$: 1335.52241; observed: 1335.52248 $[(M + Na)^+]$. $[\alpha]_D^{25\ °C}$ + 40.6° (c = 0.91 in $CHCl_3$).

4′-Deoxy-4′-fluoro-4′-*epi* neomycin (11). *Step 1*. To a solution of **10** (50 mg, 0.038 mmol) in THF (2.5 mL), a NaOH solution (0.1 N, 0.63 mL) was added, followed by PMe_3 (1 M in THF, 1.14 mmol, 1.14 mL). The mixture was stirred for 30 min at 50 °C. The crude product was concentrated under reduced pressure. The aqueous phase was extracted with EtOAc. The organic phase was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography ($CHCl_3$–MeOH–NH_4OH 14 : 2 : 0.25) affording 6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′deoxy-4′-fluoro neomycin (40.1 mg, 0.035 mmol, 91%) — HRMS (ESI-TOF) m/z $[M + H]^+$ 1157.59487 — as a colorless syrup that was not characterized further.

Step 2. To a solution of the aforementioned compound in AcOH–water (4 : 1, 5 mL), $Pd(OH)_2$ (20% in C, 40 mg) was added and the mixture was stirred under hydrogen (60 psig) for 24 h at room temperature. Water (4 mL) was added and the suspension was stirred under hydrogen (60 psig) for a further 24 h at room temperature. The suspension was filtered through a syringe filter (25 mm, w/0.45 μm PTFE membrane) and concentrated under reduced pressure. The product was dissolved in water, neutralized with NH_4OH, added to an ion exchange column (Amberlite CG-50™ ammonium form) and eluted with 0.2 N ammonium hydroxide. The fractions containing the product were combined, concentrated under reduced pressure; the product was redissolved in water and lyophilized, affording the title compound (free base, 15.6 mg, 0.025 mmol, 73%, two steps) as an off-white foam. A portion of the product (9.8 mg, 0.016 mmol) and ammonium sulfate (6.3 mg, 0.048 mmol) were dissolved in a minimum volume of water (0.1 mL), MeOH (1 mL) was added under vigorous stirring and the precipitate was isolated by centrifugation, affording 4′-deoxy-4′-fluoro-4′-*epi* neomycin sulfate (**11**) (15.3 mg, 0.016 mmol) as a white amorphous solid that was characterized and tested for biological activity. IR ν (cm^{-1}): 2873, 2097, 1618, 1522, 1419, 1048, 792. ^{1}H NMR (500 MHz, D_2O): see Table 1. ^{13}C NMR (126 MHz, D_2O): see Table 2. ^{19}F NMR (376 MHz, D_2O) δ −219.9

Table 1 ^{1}H-NMR chemical shifts (ppm) and coupling constants (Hz) for the fluorinated azido derivatives

	13[a]	10[a]	14[b,c]	11c[c]	16b[d]	16a[d]	17[e]
H-1′	6.17	6.27	6.14	6.02	6.09	5.77	6.20
H-2′	2.90	3.51	3.49	3.66	3.60	3.54	3.70
H-3′	4.05	4.00	4.31	4.41	3.96	3.76	4.49
H-4′	4.11	4.72	4.44	5.02	4.75	4.60	5.04
H-5′	4.23	4.18	4.35	4.41	4.24	3.92	4.40
H-6a′	3.32	3.27	3.33	3.41	3.30	3.30	3.38
H-6b′	3.50	3.56	3.50	3.41	3.59	3.50	3.38
$J_{1',2'}$	3.8	3.6	3.3	3.9	1.8	3.6	4.1
$J_{2',3'}$	10.1	10.7	11.0	11.3	10.8		11.7
$J_{3',4'}$	8.0	2.4	9.1	2.4	2.4		
$J_{4',5'}$	9.8	0.0	9.1	—	0.0		
$J_{5',6a'}$	6.7	5.1	nd	nd	5.3	5.9	3.7
$J_{5',6b'}$	2.2	8.0	nd	nd	7.9	7.8	6.0
$J_{6a',6b'}$	13.8	12.8	nd	nd	12.7	12.6	
$J_{1',F}$	3.1		3.3	—			
$J_{2',F}$	1.0		1.4	—	11.2		
$J_{3',F}$	14.4	24.7	nd	28.6	27.1	26.3[f]	28.3
$J_{4',F}$	49.7	51.1	49.5	50.3	51.3	49.6[f]	50.1
$J_{5',F}$	nd	29.6	nd	30.0	29.8	28.7[f]	29.4[f]
$J_{6a',F}$	—		nd	—			
$J_{6b',F}$	2.2	1.0	nd	nd			
H-1	3.43	3.42	3.33	3.34	4.04	3.62	3.97
H-2$_{ax}$	1.43	1.44	1.88	1.91	1.53	1.46	1.92
H-2$_{eq.}$	2.25	2.24	2.40	2.41	2.30	2.17	2.20
H-3	3.45	3.46	3.40	3.41	3.61		3.53
H-4	3.64	3.64	4.04	4.07	3.76	3.75	4.20
H-5	3.95	3.94	3.94	3.96	4.03	4.03	3.96
H-6	3.30	3.27	3.73	3.74	3.45	3.40	3.70
$J_{1,2ax}$	12.2	12.5	12.6	12.8	12.0[f]	10.0	12.8
$J_{1,2eq.}$	4.6	4.5	4.8	4.6	4.7	4.7	
$J_{1,6}$	9.5	9.7	10.6	10.3	8.6	7.1	
$J_{2ax,2eq.}$	13.2	13.2	12.6	12.7	13.1	13.3	13.2
$J_{2ax,3}$	12.6	12.5	12.6	12.7	12.0	11.0	12.1
$J_{2eq.,3}$	4.6	4.5	3.8	4.0	4.7	4.7	4.4
$J_{3,4}$	9.9	9.5	9.8	8.7	8.6		10.1
$J_{4,5}$	8.6	8.6	8.9	9.6	8.0	6.1	9.3
$J_{5,6}$	9.1	9.8	9.2	9.4	8.0	7.3	
H-1″	5.67	5.67	5.44	5.45	5.5	5.46	5.45
H-2″	3.94	3.97	4.46	4.47	4.03	3.96	4.47
H-3″	4.26	4.24	4.56	4.56	4.35	4.35	4.56
H-4″	4.27	4.28	4.24	4.25	4.03	3.96	4.35
H-5a″	3.55	3.55	3.76	3.77	3.59	3.56	3.78
H-5b″	3.79	3.77	3.93	3.94	3.79	3.76	3.95
$J_{1'',2''}$	5.9	5.8	2.1	2.2	4.7	4.3	1.9
$J_{2'',3''}$	4.6	4.9	4.6	4.7	3.7		4.7
$J_{3'',4''}$	nd	2.4	7.0	6.9	3.7		6.9
$J_{4'',5a''}$	2.9	3.1	4.8	5.2	nd	3.8	4.7
$J_{4'',5b''}$	2.0	2.4	2.9	2.8	nd		
$J_{5a'',5b''}$	10.5	10.6	12.5	12.3	nd	11.3	12.4
H-1‴	4.92	4.88	5.31	5.31	4.92	4.90	5.34
H-2‴	3.35	3.33	3.60	3.60	3.40	3.42	3.61
H-3‴	3.76	3.74	4.27	4.24	3.76	3.76	4.27
H-4‴	3.12	3.12	3.84	3.84	3.15	3.15	3.85
H-5‴	3.78	3.75	4.34	4.35	3.81	3.81	4.35
H-6a‴	2.86	2.88	3.37	3.38	2.92	2.93	3.38
H-6b‴	3.65	3.63	3.42	3.44	3.67	3.67	3.43
$J_{1''',2'''}$	1.9	1.9	1.8	1.6	1.9	1.9	1.8
$J_{2''',3'''}$	2.8	2.4	3.1	nd	3.1	3.1	3.0
$J_{3''',4'''}$	2.8	1.9	3.0	nd	2.6	2.6	3.0
$J_{4''',5'''}$	1.9	1.9	1.3	nd	1.6	1.6	1.0
$J_{5''',6a'''}$	4.0	4.0	nd	6.9	3.1	6.9	3.8
$J_{5''',6b'''}$	8.9	8.5	nd	3.9	8.6	8.6	8.2
$J_{6a''',6b'''}$	12.9	12.9	nd	13.7	13.0	13.7	13.4

[a] 500 MHz, $CDCl_3$. [b] $W_{2,4} = 1.3$ Hz. [c] 500 MHz, D_2O. [d] 700 MHz, $CDCl_3$. [e] 700 MHz, $CDCl_3$. [f] Measured with 2D-COSY.

Table 2 ^{13}C-NMR chemical shifts (ppm) and C–F coupling constants (Hz) for the fluorinated azido derivatives

	10[a]	13[a]	14[b]	11[b]	16b[c]	16a[c]	17[b]
C-1′	95.7	95.2	95.3	95.6	95.9	96.2	95.5
C-2′	58.3	61.7	53.3	50.3	58.7	58.7	50.9
C-3′	73.8	76.9	66.9	66.8	74.1	74.0	63.7
C-4′	86.3	91.2	99.2	89.9	86.3	86.0	89.8
C-5′	68.8	68.9	70.5	64.2	68.9	68.7	66.9
C-6′	50.6	50.9	39.7	39.6	50.5	50.3	39.6
$^{4}J_{C\text{-}1',F}$							
$^{3}J_{C\text{-}2',F}$		1.0	8.2	3.7	2.5	2.6	4.3
$^{2}J_{C\text{-}3',F}$	18.2	17.4	20.2	17.8	18.2	17.9	17.9
$^{1}J_{C\text{-}4',F}$	185.7	184.4	181.5	180.2	185.3	185.9	180.8
$^{2}J_{C\text{-}5',F}$	18.2	24.4		18.0	18.2	18.4	17.9
$^{3}J_{C\text{-}6',F}$	4.9			5.9		5.4	
C-1	60.3	60.3		50.2	47.8	46.7	48.8
C-2	32.4	32.4	29.5	29.5	32.1	31.0	29.9
C-3	60.0	60.0	48.9	48.7	59.8	59.6	48.9
C-4	75.0	75.1	77.1	76.6	76.1	76.2	75.4
C-5	81.7	81.6	85.0	85.2	82.2	79.5	85.5
C-6	84.3	84.3	72.8	72.9	81.8	81.7	73.7
C-1″	106.1	106.0	110.2	110.3	106.4	105.9	110.3
C-2″	82.7	82.5	73.3	74.9	81.3	81.6	73.3
C-3″	75.7	75.5	74.9	74.6	75.6	75.2	74.6
C-4″	82.3	82.1	81.2	81.3	82.1	81.7	81.0
C-5″	70.1	70.1	60.2	60.3	70.3	70.2	60.0
C-1‴	98.6	98.6	95.3	95.3	98.5	98.4	95.0
C-2‴	57.3	57.2	50.9	50.9	57.5	57.5	50.9
C-3‴	72.9	72.8	67.8	67.8	72.8	74.2	67.7
C-4‴	71.5	71.4	67.3	67.3	71.5	71.5	67.2
C-5‴	74.3	74.4	70.4	70.5	74.3	74.3	70.0
C-6‴	51.0	51.1	40.7	40.5	51.1	51.1	40.4

[a] 126 MHz, $CDCl_3$. [b] 126 MHz, D_2O. [c] 176 MHz, $CDCl_3$.

(dt, $J = 49.9$, 29.3 Hz). HRMS (ESI-TOF) m/z $[M + H]^+$ calc. for $C_{23}H_{46}FN_6O_{12}$: 617.31577; found: 617.31515. $[\alpha]_D^{25}$ °C + 40° (c 0.72 in water).

1,3,2′,6′,2‴,6‴-Hexadeamino-1,3,2′,6′,2‴,6‴-hexaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-*epi* neomycin (12). *Step 1*. To a solution of **9** (197 mg, 0.150 mmol) in DCM (3.8 mL), DMP (194 mg, 0.458 mmol) was added in one portion and the mixture stirred overnight at room temperature. The crude product was diluted with DCM, washed with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc–hexanes 1 : 5), affording 1,3,2′,6′,2‴,6‴-hexadeamino-1,3,2′,6′,2‴,6‴-hexaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-deoxy-4′-oxo neomycin (178 mg, 0.136 mmol, 90%) — MS m/z 1309.5 $(M + H)^+$ — that was used in the next step without further characterization.

Step 2. To a solution of ketone (178 mg, 0.136 mmol) in THF (1.4 mL), L-Selectride® (1 N in THF, 0.2 mmol, 0.2 mL) was added and the solution was stirred at room temperature overnight. 10% AcOH was added and the solution was concentrated until dry. The product was suspended in EtOAc, washed with aqueous $NaHCO_3$, water and brine, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc–hexanes 1 : 5), affording the title compound (130.0 mg, 0.099 mmol, 66%, two steps) as a white foam. IR ν (cm^{-1}, film): 3863, 3767, 3742, 3362, 3039, 2870, 2519, 2348, 2101, 1500, 1458, 1366, 1334, 1260, 1216, 1028, 914, 736, 697. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46–7.06 (m, 30H), 6.24 (d, J = 3.7 Hz, 1H), 5.67 (d, J = 5.7 Hz, 1H), 4.94 (d, J = 10.6 Hz, 1H), 4.88 (d, J = 1.9 Hz, 1H), 4.72–4.51 (m, 6H), 4.48–4.37 (m, 3H), 4.35–4.20 (m, 5H), 4.17 (dd, J = 8.5, 4.6 Hz, 1H), 4.05–3.87 (m, 4H), 3.82–3.72 (m, 3H), 3.72–3.52 (m, 4H), 3.47–3.22 (m, 6H), 3.11 (t, J = 2.3 Hz, 1H), 2.88 (dd, J = 13.0, 4.0 Hz, 1H), 2.18 (dq, J = 13.3, 5.3, 4.6 Hz, 1H), 1.40 (q, J = 12.7 Hz, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 138.2, 137.9, 137.6, 137.1, 137.0 and 137.0, (6 ArC), 128.7–127.4 (30 ArCH), 106.1, 98.6, 95.8, 84.3, 82.5, 82.1, 81.6, 75.6, 75.5, 75.0 (−), 74.9, 74.3, 73.3 ((−), 2C), 72.9, 72.3 (−), 72.2 (−), 71.7 (−), 71.5, 70.2 (−), 69.3, 66.8, 60.3, 60.1, 58.5, 57.3, 51.4 (−), 51.0 (−), 32.4 (−). HRMS (ESI-TOF) m/z $[M + NH_4]^+$ calc. for $C_{65}H_{74}N_{19}O_{13}$: 1328.5708; found: 1328.5705. m/z $[M + Na]^+$ calc. for $C_{65}H_{70}N_{18}NaO_{13}$: 1333.5262; found: 1352.52589. $[\alpha]_D^{25\ °C}$ + 71.6° (c 1.3 in $CHCl_3$).

1,3,2′,6′,2‴,6‴-Hexaamino-1,3,2′,6′,2‴,6‴-hexaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′deoxy-4′-fluoro neomycin (13). *Step 1*. To a solution of **12** (177 mg, 0.135 mmol) and pyridine (0.45 mL) in DCM (4.5 mL), Tf_2O (0.23 mL, 1.35 mmol) was added at 0 °C and the mixture was stirred for 8 h at 4 °C. The reaction mixture was diluted with DCM, washed with icy aqueous $NaHCO_3$ and icy 1 N HCl, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure, affording crude triflate (195 mg, 0.135 mmol, 100%) — LRMS (ESI-TOF) m/z 1443.5 $[M + H]^+$ — that was used in the next step without further purification.

Step 2. A solution of the aforementioned triflate (135 mg, 0.135 mmol) and TBAF (1 N in THF, 0.41 mmol, 0.41 mL) in THF (1.9 mL) was heated at 50 °C for 30 minutes. The crude product was concentrated under reduced pressure. The residue was dissolved in EtOAc, washed twice with water, dried over anhydrous Na_2SO_4 and concentrated. The residue was purified by flash chromatography (EtOAc–hexanes 1 : 4), affording crude **13** (151 mg, 0.115 mmol, 85%) that was repurified by preparative HPLC (Prevail C18, 12 mL min^{-1}, 10% MeCN in water containing 0.1% HCOOH), affording the title compound (133 mg, 0.101 mmol, 75% two steps) as a transparent glass. IR ν (cm^{-1}, film): 3810, 3765, 3595, 3441, 3019, 2928, 2874, 2521, 2350, 2103, 1500, 1457, 1373, 1334, 1262, 1213, 1121, 1030, 960, 917, 822, 746, 698. ^{1}H NMR (500 MHz, $CDCl_3$): see Table 1; additionally δ 7.44–7.08 (m, 30H), 4.95 (d, J = 10.6 Hz, H-Bn), 4.83 (d, J = 11.1 Hz, H-Bn), 4.70–4.46 (m, 2H-Bn), 4.64–4.58 (m, 2H-Bn), 4.52–4.39 (m, 4H-Bn), 4.35–4.17 (m, H-5′, H-3″, H-4″, 2H-Bn). ^{13}C NMR (126 MHz, $CDCl_3$): see Table 2; additionally: δ 138.1 (q), 137.8 (q), 137.6 (2q), 136.9 (q), 136.9 (q), 129.3–126.5 (m, 30CH), 75.1 (−), 74.8 (−), 73.2 (2C (−)), 72.3 (−), 71.7 (−). ^{19}F NMR (282 MHz, $CDCl_3$) δ −195.0 (ddd, $J_{F,1'}$ = 3.6 Hz, $J_{F,4'}$ = 54.0 Hz, $J_{F,3'}$ = 12.0 Hz). HRMS (ESI-TOF) m/z $[M + Na]^+$ calc. for $C_{65}H_{69}FN_{18}NaO_{12}$: 1335.52186; found: 1335.52722. $[\alpha]_D^{25\ °C}$ + 78.4° (c = 0.64 in $CHCl_3$).

4′-Deoxy-4′-fluoro neomycin (14). *Step 1*. As described for **11** (Step1), **13** (77.5 mg, 0.059 mmol), PMe_3 (1 M in THF, 1.77 mmol, 1.77 mL), THF (3.9 mL) and a NaOH solution (0.1 N, 1.6 mL) were used. The mixture was stirred for 30 min at 50 °C. Purification by flash chromatography ($CHCl_3$–MeOH–NH_4OH 14 : 2 : 0.25) afforded perbenzylated **14** (64.5 mg, 0.056 mmol, 94%) — HRMS (ESI-TOF) m/z $[M + H]^+$ 1179.57714 — as a colorless syrup.

Step 2. As described for **11** (Step 2), perbenzylated **14** (63.5 mg, 0.056 mmol), $Pd(OH)_2$ (20% in C, 40 mg), AcOH–water (4 : 1, 5 mL) and hydrogen (60 psig) were used. The title compound (free base, 30.3 mg, 0.049 mmol, 88%) was obtained as a white foam. A portion of the product (7.1 mg, 0.0115 mmol) and ammonium sulfate (0.035 mmol, 4.6 mg) were dissolved in a minimum volume of water (0.1 mL), MeOH (1 mL) was added under vigorous stirring and the precipitate was isolated by centrifugation, affording 4′-deoxy-4′-fluoro-4′-*epi* neomycin sulfate, **14**, (11.1 mg, 0.0115 mmol) as a white amorphous solid that was characterized and tested for biological activity. IR ν (cm^{-1}): 2921, 1618, 1521, 1044. ^{1}H NMR (500 MHz, D_2O) see Table 1. ^{13}C NMR (126 MHz, D_2O): see Table 2. ^{19}F NMR (471 MHz, D_2O) δ −199.6 (dd, J = 50.1, 13.7 Hz). HRMS (ESI-TOF) m/z $[M + Na]^+$ calc. for $C_{23}H_{45}FN_6NaO_{12}$: 639.29772; found: 639.29717. HRMS (ESI-TOF) m/z $[M + H]^+$ calc. for $C_{23}H_{46}FN_6O_{12}$: 617.31577; found: 617.31293. $[\alpha]_D^{25\ °C}$ + 40.5° (c = 0.55 in water).

1,2′,6′,2‴,6‴-Pentadeamino-1,2′,6′,2‴,6‴-pentaazido-6, 3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-deoxy-4′-fluoro-4′-*epi* neomycin (15a) and 3,2′,6′,2‴,6‴-pentadeamino-3,2′,6′,2‴,6‴-pentaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-deoxy-4′-fluoro-4′-*epi* neomycin (15b). To a solution of **11** (27.6 mg, 0.021 mmol) in THF (1.1 mL), PMe_3 (1 N solution in THF, 0.019 mmol, 19 μL) was added under argon at −78 °C. The mixture was stirred for 30 min at −78 °C and then for 2 h at room temperature. A solution of NaOH (1 N, 300 μL) was added and the suspension was stirred vigorously at room temperature overnight. The crude product was diluted with EtOAc, washed with water, dried over anhydrous $MgSO_4$ and concentrated until dry under reduced pressure. The product was purified by flash chromatography (the gradient elution used was: EtOAc–hexanes 1 : 4, then 1% MeOH in DCM) affording the unreacted starting material (**11**) (11.8 mg, 0.009 mmol) and an inseparable mixture of **15a** and **15b** (10.4 mg, **15a** : **15b** = 1 : 0.8 by ^{1}H-NMR, 0.008 mmol, 68% based on the consumed starting material) — LRMS (ESI-TOF) m/z 1287.6 $[M + H]^+$ — that was used in the next step without further characterization.

3-*N*-[(*S*)-4-Benzyloxycarbonylamino-2-hydroxybutanoyl]-1,2′,6′,2‴,6‴-pentadeamino-1,2′,6′,2‴,6‴-pentaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-deoxy-4′-fluoro-4′-*epi* neomycin (16a) and 1-*N*-[(*S*)-4-benzyloxycarbonylamino-2-hydroxybutanoyl]-3,2′,6′,2‴,6‴-pentadeamino-3,2′,6′,2‴,6‴-pentaazido-6,3′,2″,5″,3‴,4‴-hexa-*O*-benzyl-4′-deoxy-4′-fluoro-4′-*epi* neomycin (16b). To a solution of **15a–b** (10.4 mg, 0.008 mmol) and (2*S*)-Cbz-HABA-Su (5.6 mg, 0.016 mmol) in THF (160 μL), DIPEA (4.1 μL) was added at room temperature and the mixture was stirred overnight. The

solution was concentrated under reduced pressure and the residue was purified by preparative TLC (5 mm, 1/2 plate, 2% MeOH in DCM), affording **16a** (6.7 mg, 0.0044 mmol, 55%) and **16b** (4 mg, 0.0026 mmol, 32%) as syrups.

For 16a. IR ν (cm^{-1}, film): 2101, 1709, 1540, 1525, 1254, 1089, 1077, 1056, 1045, 1028, 1017, 975, 773. ^{1}H NMR (700 MHz, $CDCl_3$) see Table 1; additionally: δ 7.37–7.19 (m, 35H), 7.07 (d, J = 9.1 Hz, CONH), 5.13 (t, J = 6.4 Hz, OCONH), 5.07 (d, J = 12.3 Hz, H-Bn), 5.01 (d, J = 12.2 Hz, H-Bn), 4.83 (d, J = 11.0 Hz, H-Bn), 4.69 (d, J = 11.1 Hz, H-Bn), 4.62 (d, J = 12.1 Hz, H-Bn), 4.60 (s, 2H-Bn), 4.58 (d, J = 12.0 Hz, H-Bn), 4.45 (d, J = 12.1 Hz, H-Bn), 4.43 (d, J = 12.1 Hz, H-Bn), 4.44 (d, J = 11.8 Hz, H-Bn), 4.43 (d, J = 11.8 Hz, H-Bn), 4.35 (d, J = 12.1 Hz, H-Bn), 4.26 (d, J = 12.1 Hz, H-Bn), 4.62 (m, 1H), 3.92 (m, 1H), 3.49 (s, 1H), 3.18 (m, 1H), 2.01 (m, 1H). ^{13}C NMR (176 MHz, $CDCl_3$): see Table 2; additionally: δ 172.7 (CO), 157.9 (CO), 157.9, 138.0, 137.9, 137.6, 137.2, 137.0 and 136.9 (7 ArC), 128.7–127.6 (35C), 67.1 (−), 71.7 (−), 72.0 (−), 72.4 (−), 72.9 (−), 73.3 (−), 73.8 (−), 69.3, 35.0 (−), 37.1 (−). ^{19}F NMR (282 MHz, $CDCl_3$) δ −218.6 (dt, J = 50.5, 28.3 Hz). HRMS (ESI-TOF) m/z $[M + H]^+$ calc. for $C_{77}H_{85}FN_{17}O_{16}$: 1522.63442; found: 1522.63387. $[\alpha]_D^{25\ °C}$ (c in $CHCl_3$).

For 16b. IR ν (cm^{-1}, film): 2932, 2103, 1711, 1535, 1454, 1219, 1082, 1062, 1049, 1028, 774, 699. ^{1}H NMR (700 MHz, $CDCl_3$) see Table 1; additionally: δ 7.49–7.11 (m, 35H), 7.01 (d, J = 8.5 Hz, CONH), 5.03 (t, J = 6.8 Hz, OCONH), 4.90 (d, J = 11.2 Hz, H-Bn), 4.72 (s, 2H-Bn), 4.64 (d, J = 12.1 Hz, H-Bn), 4.60 (d, J = 11.7 Hz, H-Bn), 4.58 (d, J = 11.7 Hz, H-Bn), 4.54 (d, J = 11.3 Hz, H-Bn), 4.48 (d, J = 11.7 Hz, H-Bn), 4.47 (d, J = 11.7 Hz, H-Bn), 4.43 (d, J = 11.9 Hz, H-Bn), 4.34 (d, J = 12.0 Hz, H-Bn), 4.27 (d, J = 12.2 Hz, H-Bn), 3.98 (m, 1H), 3.41 (m, 1H), 3.05 (s, 1H), 1.92 (m, 1H), 1.36 (m, 1H). ^{13}C NMR (176 MHz, $CDCl_3$) see Table 2; additionally: δ 173.0 (CO), 158.2 (CO), 138.2, 138.1, 137.7, 137.1, 137.0, 137.0 and 136.0 (7 ArC), 128.7–127.5 (35C), 73.8 (−), 73.3 (−), 73.1 (−), 72.4 (−), 72.1 (−), 71.7 (−), 68.5, 67.3 (−), 35.1 (−), 36.8 (−). ^{19}F NMR (282 MHz, $CDCl_3$) δ −218.6 (dt, J = 50.5, 28.3 Hz). HRMS (ESI-TOF) m/z $[M + H]^+$ calc. for $C_{77}H_{85}FN_{17}O_{16}$: 1522.63442; found: 1522.63387. $[\alpha]_D^{25\ °C}$ + 42.7° (c 1.1 in $CHCl_3$).

1-*N*-[(*S*)-4-Amino-2-hydroxybutanoyl]-4′-deoxy-4′-fluoro-4′-*epi* neomycin (17). *Step 1*. As described for **11**, **16b** (17.8 mg, 0.0117 mmol), PMe_3 (1 M in THF, 0.3 mmol, 0.3 mL), THF (0.8 mL) and a NaOH solution (0.1 N, 0.4 mL) were used. The mixture was stirred for 30 min at 50 °C. Purification by flash chromatography ($CHCl_3$–MeOH–NH_4OH 14 : 2 : 0.25) afforded perbenzylated **17** (10.4 mg, 0.0075 mmol, 64%) — LRMS (ESI-TOF) m/z $[M + Na]^+$ 1414.7 — as a colorless syrup.

Step 2. As described for **15** (Step 2), perbenzylated **17** (10.4 mg, 0.0075 mmol), $Pd(OH)_2$ (20% in C, 5 mg), AcOH–water (4 : 1, 5 mL) and hydrogen (60 psig) were used. The solid product obtained after concentrating the mixture was dissolved in water, neutralized with aqueous NH_4OH and lyophilized, affording **17** (acetate, 7.9 mg, 0.0073 mmol, 97%, two steps) as an off-white foam. The resulting acetate (8.1 mg, 0.0073 mmol) was dissolved in water (0.1 ml). Ammonium sulfate (2.9 mg, 0.022 mmol) was added and the solution was added drop wise to vigorously stirred methanol (0.5 ml). The solid precipitate formed was isolated by centrifugation, affording sulfate **17** (7.6 mg, 0.0072 mmol) that was characterized and tested for biological activity. IR ν (cm^{-1}): 3035, 1624, 1415, 1266, 1050. ^{1}H NMR (700 MHz, D_2O) see Table 1; additionally: δ ^{1}H NMR (700 MHz, D_2O) δ 4.33 (m, 1H), 3.16 (m, 2H), 2.17 (m, 1H), 2.03 (m, 1H). ^{13}C NMR (176 MHz, D_2O): see Table 2; additionally: δ 175.7 (NHCO), 70.4 (−), 39.6 (−), 30.9 (−). ^{19}F NMR (282 MHz, D_2O) δ −219.9 (ddd, J = 50.7, 31.0, 18.0 Hz). HRMS (ESI-TOF) m/z $[M + H]^+$ calc. for $C_{27}H_{53}FN_7O_{14}$: 718.3629; found: 718.36174. $[\alpha]_D^{25\ °C}$ + 12.9° (c 1.22 in water).

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