Electronic Supplementary Material (ESI) for Chemical Science

Supporting Information

Structural and Kinetic Study of Self-Assembling Macrocyclic Dimer Natural Product Aminoglycoside *66-40C* and Unnatural Variants

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General Procedures

All reactions were carried out under an inert atmosphere of nitrogen or argon with dry solvents, using anhydrous conditions unless otherwise stated. Dry dichloromethane (DCM) and tetrahydrofuran (THF) were obtained from a solvent delivery system with activated alumina columns. Methanol (MeOH) was distilled from CaH_2 under argon. Reagents were purchased at the highest commercial quality and used without further purification. Flash chromatography was performed with silica gel from SilicaFlash P60, particle size 40-63 μm, 230-400 mesh and distilled hexanes, ethyl acetate (EtOAc) or DCM. Free amines were purified with DCM or $CHCl_3$ applying gradients of ammoniacal MeOH (referring to a 1:9 solution which was prepared fresh with 28% $NH_4OH_{(aq)}$ liquor before use). Deprotected aminoglycosides were purified with homogeneous solvent systems consisting of $CHCl_3$/MeOH/$NH_4OH_{(aq)}$ in ratios ranging from 2:3:0.5 to 2:3:2.5. Yields refer to chromatographically and spectroscopically homogeneous material. Reactions were monitored by direct-injection low resolution mass spectrometry (LRMS) and thin layer chromatography (TLC) carried out on 0.25 mm E. Merck silica precoated plates (60F-254), visualized under UV light and developed with acidified ammonium molybdate/cerium sulphate and heat. NMR spectra were recorded on Bruker ARX-400, AV-400 or AV-700 instruments and are calibrated using residual undeuterated solvent as an internal reference. The following abbreviations are used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet. Low resolution mass spectra (LRMS) were recorded on a Thermo Finnigan Surveyor MSQ and high resolution mass spectra (HRMS) were recorded on an Agilent Technologies LC-MSD TOF mass spectrometer by electrospray ionization in positive mode. Either protonated molecular ions $[M+H]^+$ or sodium adducts $[M+Na]^+$ were used for empirical formula confirmation. Optical rotations were recorded in a 1 dm cell at ambient temperature, on a Perkin-Elmer 343 polarimeter. Analytical HPLC was performed in Achaogen Inc., San Francisco, CA, using mobile phases with 0.25 M NH_4OH, column X-Bridge C18, 2.1x50mm, 2.5 μm, flow of 0.5 mL/min at RT, UV monitoring (220 and 274 nm), water/MeCN gradient 25 to 95% in 20 min.

General procedures for NMR kinetic assays

The uncluttered H-NMR signals for the monomer 6'-aldehyde and 4,5-olefin and the dimer 6'-imine were used to follow the progress of self-assembly in real time.[1] Stock buffer solutions prepared by freeze-drying the buffer salt twice with excess D_2O, to a powder that was then dissolved 120 mM in D_2O (1.2X) and taken to the desired pD with NaOD in D_2O and/or TFA as required. The 1.2X buffer solutions prepared were MES pH 5.0, MES pH 5.5, MES pH 6.0, MES pH 6.5, HEPES pH 7.0, HEPES pH 7.5, HEPES pH 8.0, Bicine pH 9.0 and bicarbonate pH 10 (Figure 2).

The standardized procedure for a kinetic assay run begun by ice-cooling 500 μL of buffer in an NMR tube, followed by the rapid addition of a 100 μL D_2O solution of 6'-aldehydo sisomicin TFA salt **2** (5 mg, 5.45 μmol, 7.8 mM final concentration and 100 mM buffer). The tube was mixed by inversion four times and H-NMR acquisition started swiftly (t = 0). A Bruker AV400 magnet with a BBO probe was used for all kinetic experiments, programmed for iterative acquisitions every 10 minutes (8 min dummy scans and then 18 recorded scans, 2 min) for 5 to 8 hours. Two-fold higher and lower dilutions of salt **2** were assayed in buffer MES 6.5 under the same conditions (Figure S1).

In variations of these experiments, 1, 2 or 4 equivalents of sisomicin TFA salt **1** were dissolved in 500 μL 1.2X buffer (120 mM MES pH 6.5), then chilled to 0 ºC prior to addition of 6'-aldehydo sisomicin TFA salt **2** (5 mg, 5.45 μmol in 100 μL D_2O) and ^{1}H-NMR acquisition (Figure 3). For the cross-over experiment between 6'-acroleyl sisamine TFA salt **25** and 6'-aldehydo sisomicin TFA salt **2** (Figure 4), both compounds were combined in 10X concentration (5.45 μmol **2** in 50 μL and D_2O) and added to 500 μL buffer (120 mM MES pH 6.5). Separate control runs are shown in Figure S3.

General procedures for NMR integration and non-linear regression analysis

The collection of H-NMR spectra from kinetic experiments were analyzed using TopSpin version 2.6, applying exponential Fourier transformation and manual phase correction in small batches of 5 to 10 spectra (*multiefp* command). Integration was performed by hand, one peak at a time down the time series, setting the 6'-imine signal of *66-40C* as the reference integral of 1 unit (see attached spectra). The integration data was converted to a percentage accounting for aldehyde and imine species (plus undetermined

imine intermediates if present). An error of 2% was assumed for the integrals and carried over for non-linear fitting analysis. NB: the 6'-aldehydo sisomicin monomer **2** does not hydrate significantly under the buffered assay conditions, and only the signals originating from the aldehyde congener were taken into consideration.

The integration data was processed with OriginPro 8 software suite. Appearance of the imine product, as the percentage of total species, was plotted against time for each kinetic experiment. The half-life was calculated by applying a non-linear curve fit to exponential Equation 1, where y_o is the offset, A is the amplitude/initial value and t is the lifetime constant. The later parameter was related to half-life through Equation 2.

$$y = y_0 + Ae^{-x/t} \qquad \text{Eq. 1}$$

$$t_{1/2} = \frac{\ln 2}{\lambda} = \tau \ln 2. \qquad \text{Eq. 2}$$

Similarly, the rate was obtained by fitting Equation 3, where y_o is the offset, A is the initial value and R_o is the rate.

$$y = y_0 + Ae^{R_0 x} \qquad \text{Eq. 3}$$

First-order exponential equations were found acceptably fitting models for self-assembly of aminoglycoside *66-40C* **3** as well as for consumption of the 6'-aldehydo sisomicin monomer **2** up to 75% conversion (Table S1). The trends observed on residual analysis plots clearly indicate this model is imperfect and there are unaccounted variables, which were assigned to transient accumulation of unproductive imine species (Figure 2) and the concentration dependence observed in dilution experiments (Figure S1). Sequential ^{1}H-NMR spectra with raw integration data, non-linear regression reports and residual analysis plots are attached.

Table S1. Non-linear regression parameters for Equation 1 for buffer experiments (Figure 2).

pH	**5**	**5.5**	**6**	**6.5**	**7**	**7.5**	**8**	**9**	**10**
Number of Points	18	23	36	34	19	13	14	10	7
Degrees of Freedom	15	20	33	31	16	10	11	7	4
Reduced Chi-Sqr	0.061	0.485	0.637	0.850	1.652	0.950	0.969	1.217	0.508
Residual Sum of Squares	0.917	9.701	21.018	26.349	26.424	9.497	10.655	8.519	2.031
Adj. R-Square	0.999	0.996	0.994	0.991	0.983	0.985	0.984	0.957	0.990
y_o (SE)	0.73	0.81	0.88	0.92	0.86	0.81	0.84	0.77	0.84
	(0.007)	(0.007)	(0.005)	(0.005)	(0.010)	(0.010)	(0.008)	(0.013)	(0.014)
A (SE)	-0.72	-0.77	-0.75	-0.77	-0.69	-0.52	-0.53	-0.32	-0.40
	(0.006)	(0.011)	(0.011)	(0.014)	(0.022)	(0.019)	(0.019)	(0.023)	(0.018)
t (SE)	158.6	110.9	67.6	49.1	33.7	24.0	21.2	18.7	17.4
	(3.73)	(3.96)	(2.15)	(1.76)	(2.44)	(2.05)	(1.69)	(3.25)	(2.03)

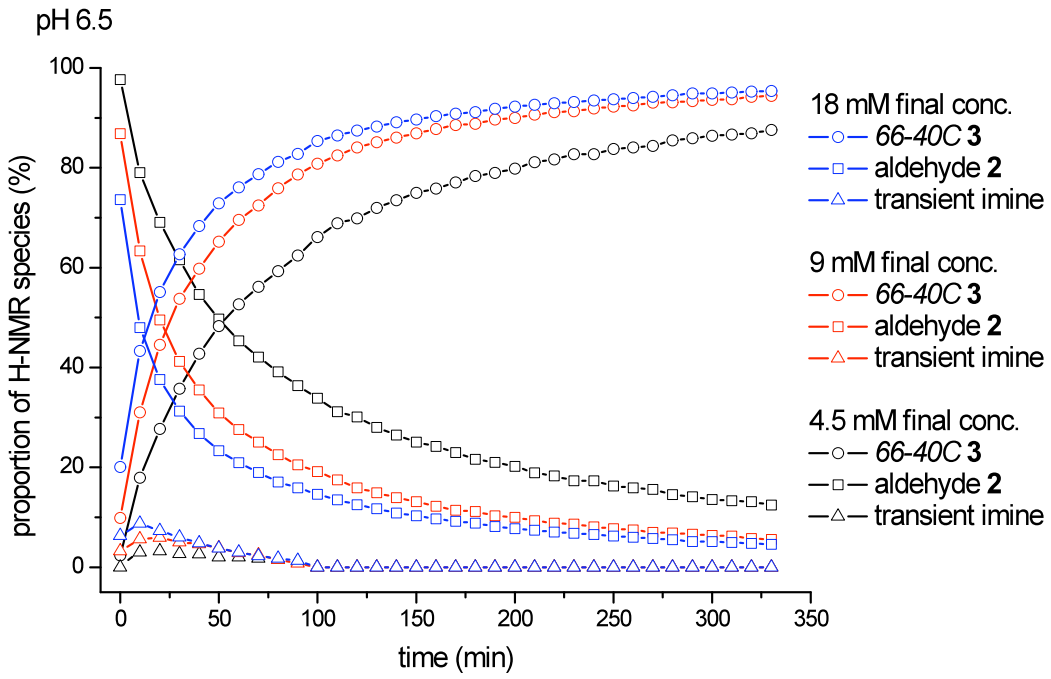


Fig S1 Comparison of evolution of H-NMR signals for identical experiments using increasing final concentration of aldehyde **2** in 100 mM MES buffer at pH 6.5.

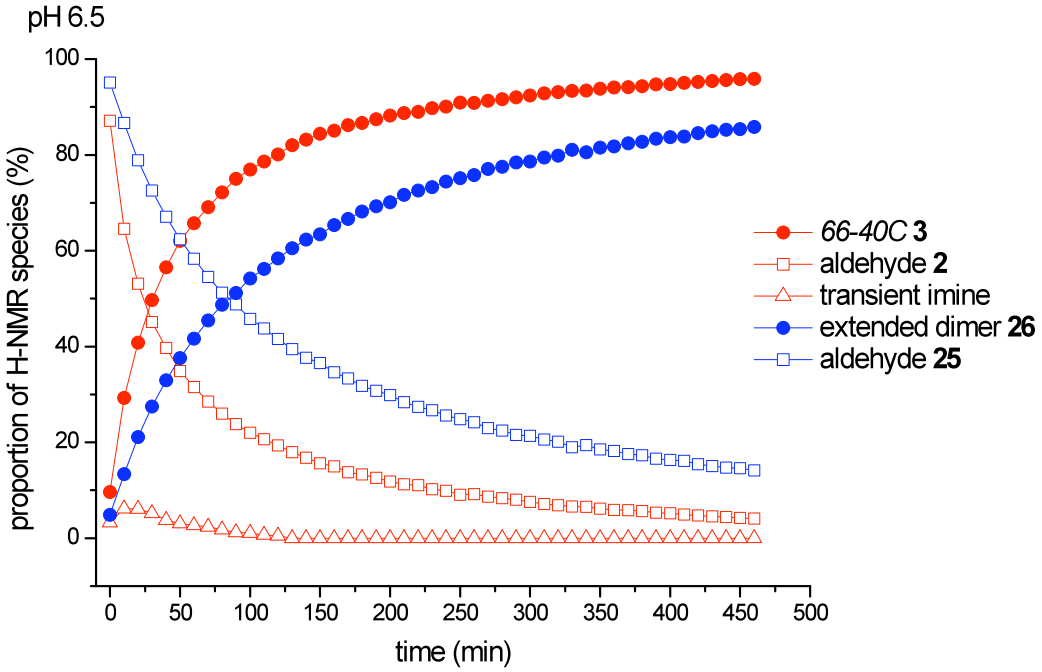


Fig S2. Comparison of evolution of H-NMR signals for identical experiments with aldehyde **2** and aldehyde **25** (9 mM final concentration, at pH 6.5 in MES buffer 100 mM).

Expanded X-ray Structure Figures for Compounds 18, 21 and 26

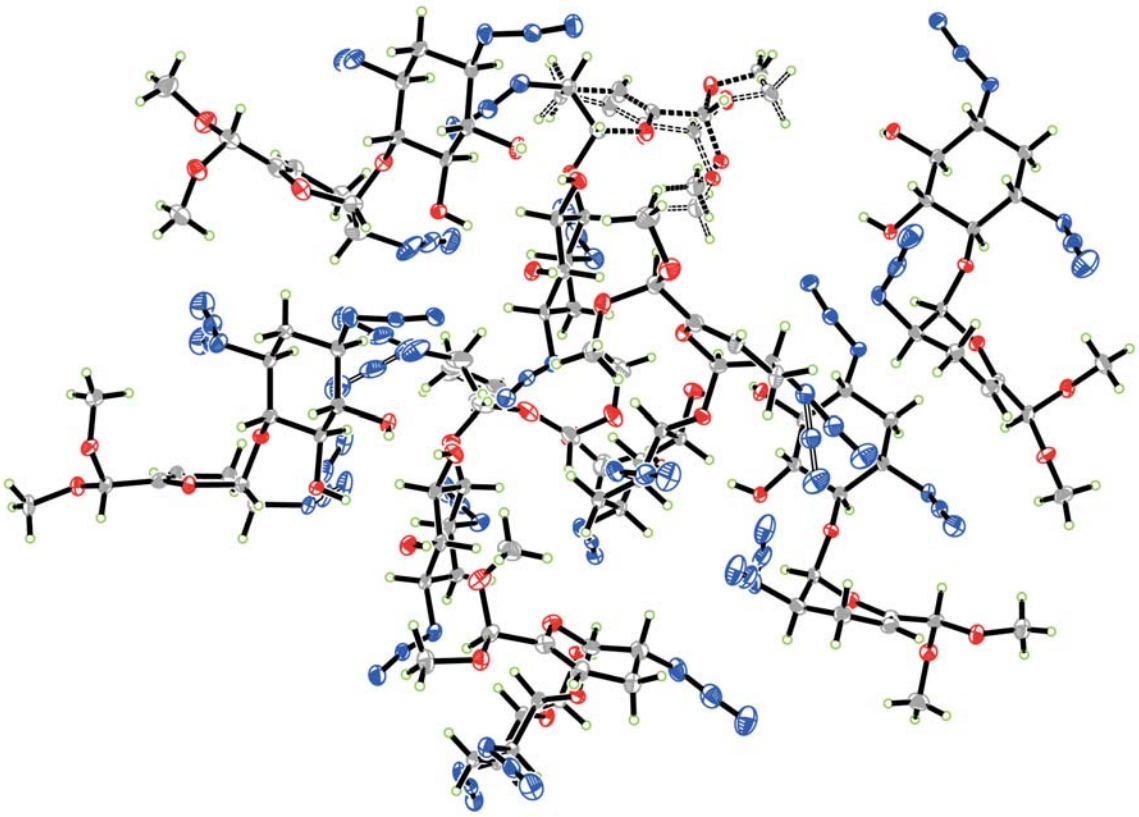


Fig S3. ORTEP representation at 30% probability level for the asymmetric unit in the crystal structure of compound **18**. For details see the crystallographic report attached.

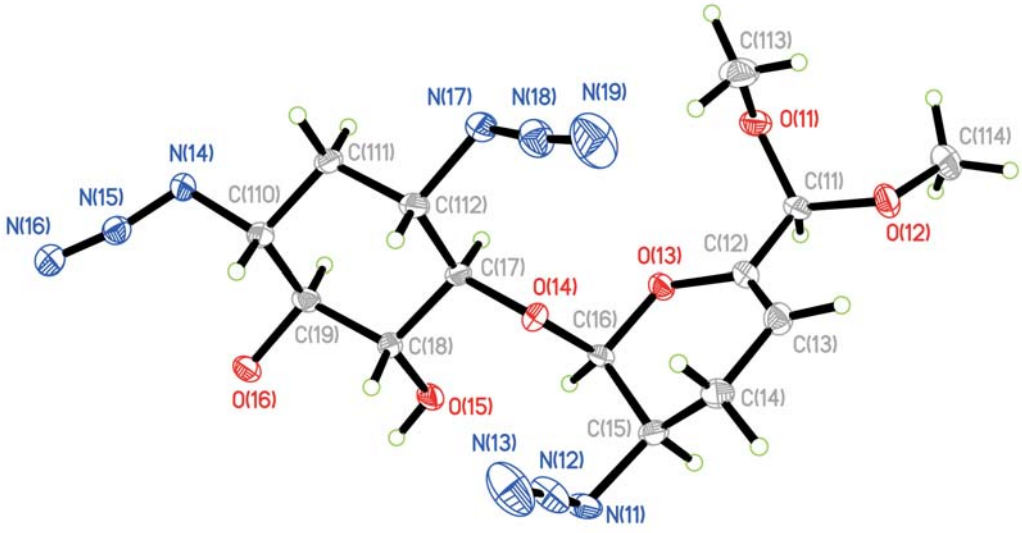


Fig S4. ORTEP representation at 30% probability level for molecule #1 in the crystal structure of compound **18**. For details see the crystallographic report file.

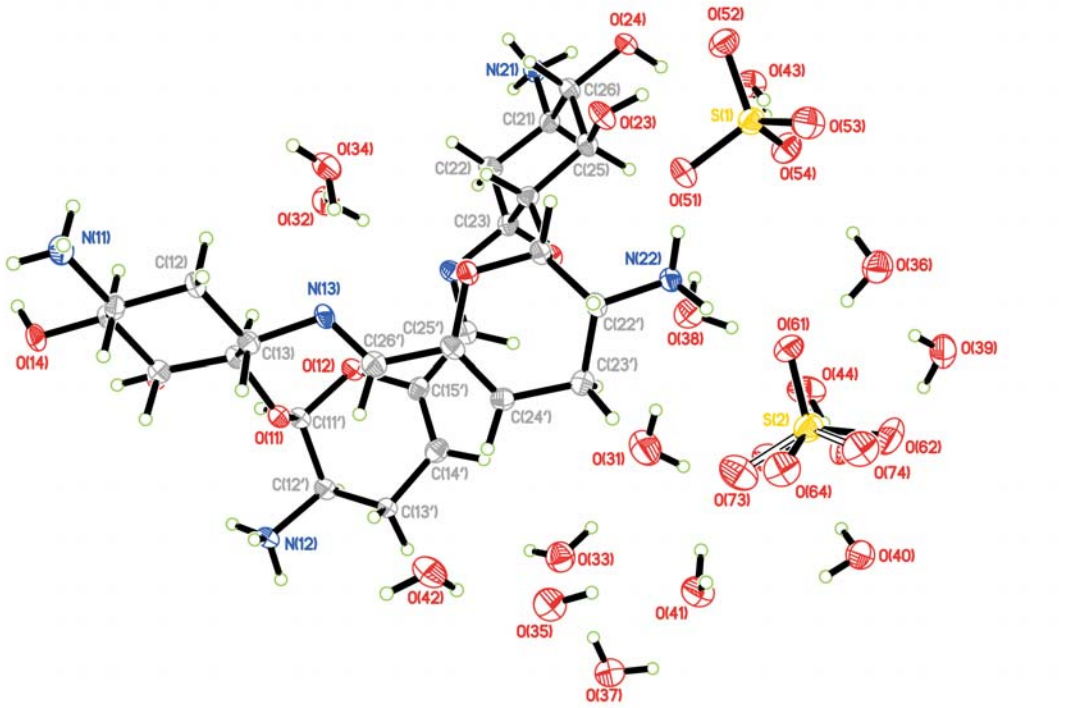


Fig S5. ORTEP representation at 30% probability level for the asymmetric unit in the orthorhombic crystal structure of compound **21**. For details see the crystallographic report file.

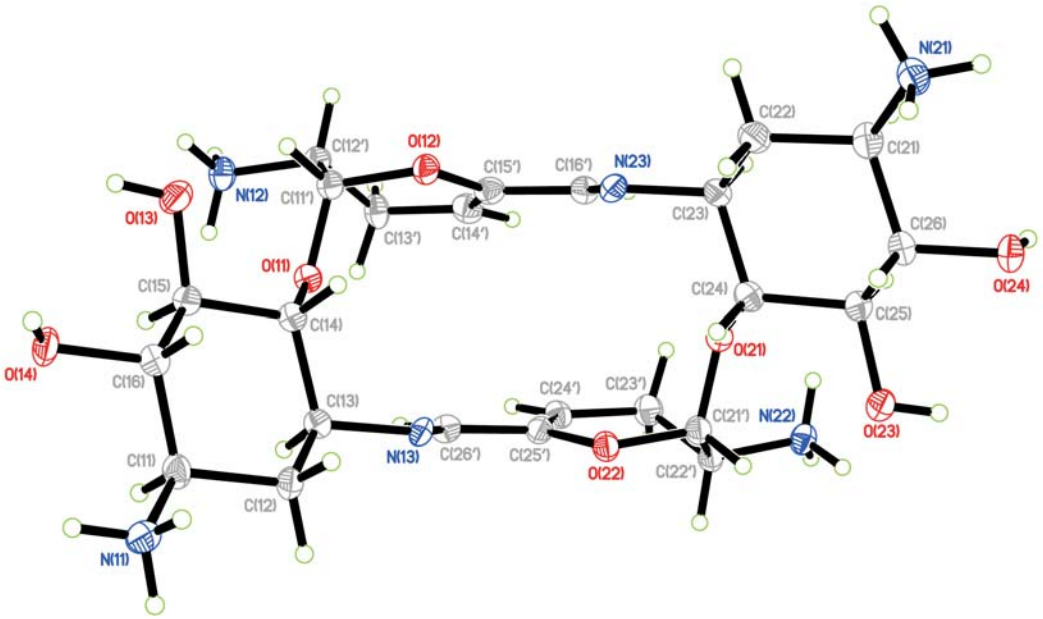


Fig S6. Top-view ORTEP representation at 30% probability level for the structure of compound **21** obtained from the orthorhombic crystal. For details see the crystallographic report file.

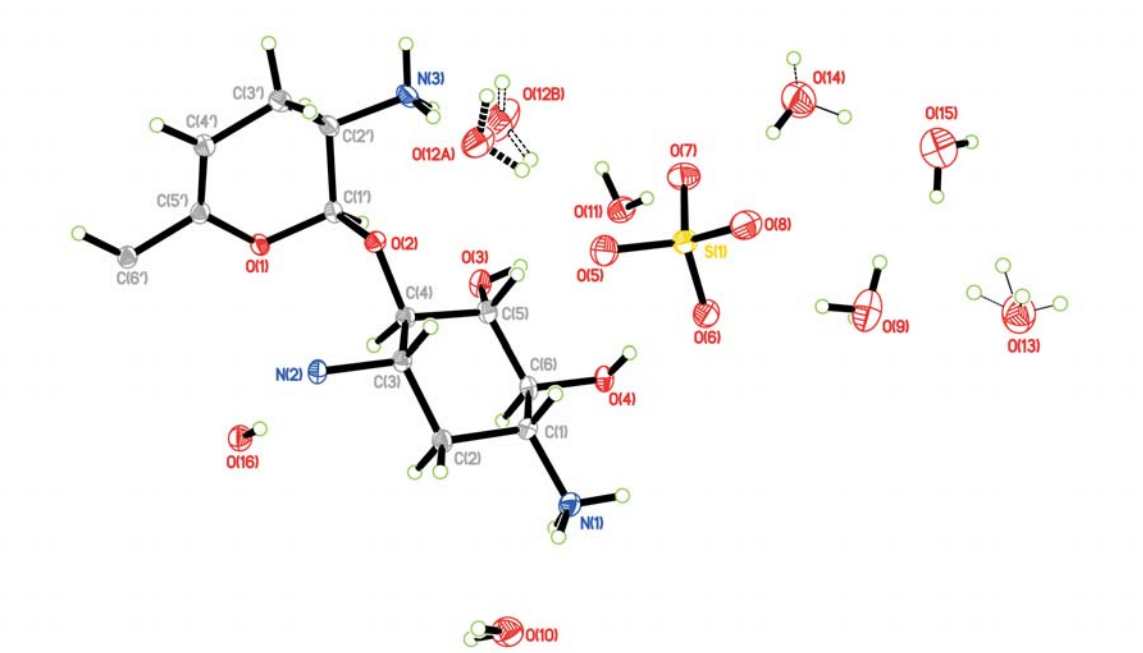


Fig S7. ORTEP representation at 30% probability level for the asymmetric unit in the monoclinic crystal structure of compound **21**. For details see the crystallographic report file.

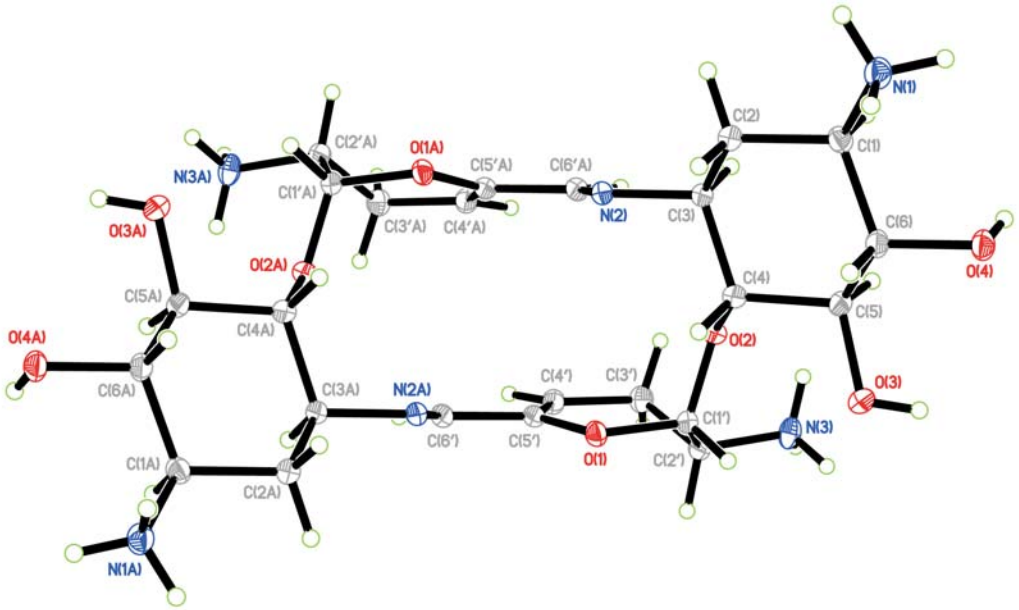


Fig S8. Top-view ORTEP representation at 30% probability level for the structure of compound **21** obtained by C_2-symmetry operation from the monoclinic crystal. For details see the crystallographic report file.

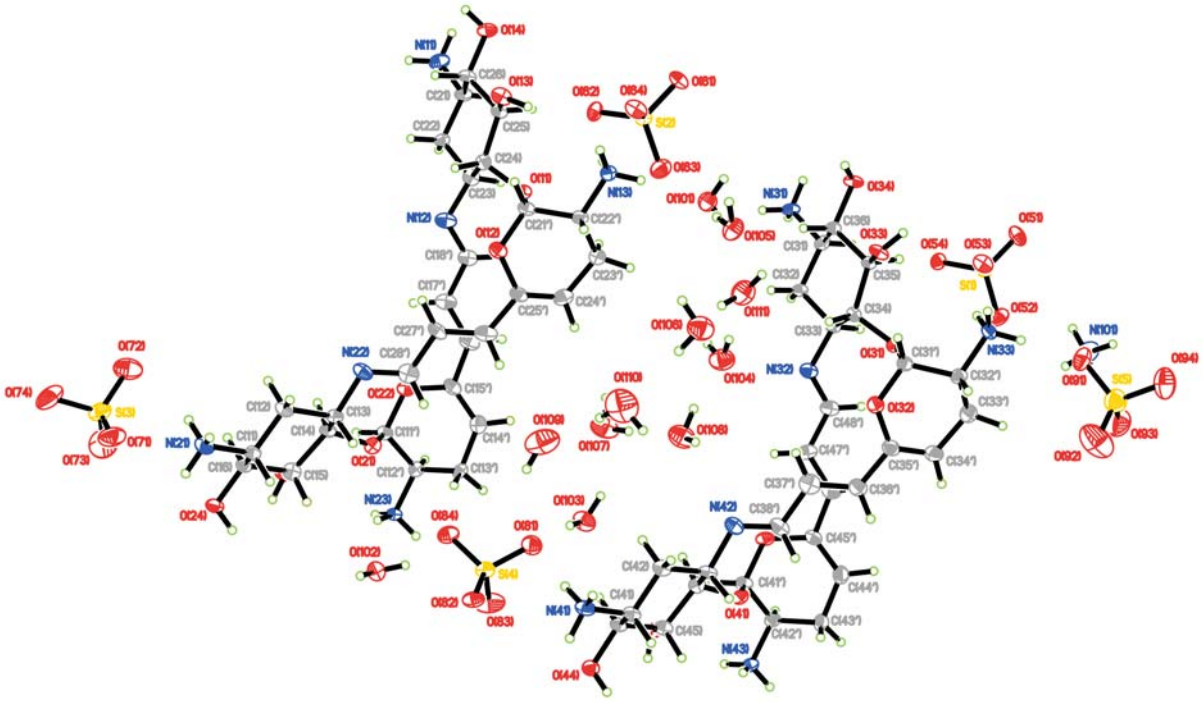


Fig S9. ORTEP representation at 30% probability level for the asymmetric unit in the crystal structure of compound **26**. One NH_4^+ ion, one molecule of EtOH and several water molecules required squeezing-out from the final model due to their high disorder. For details see the crystallographic report file.

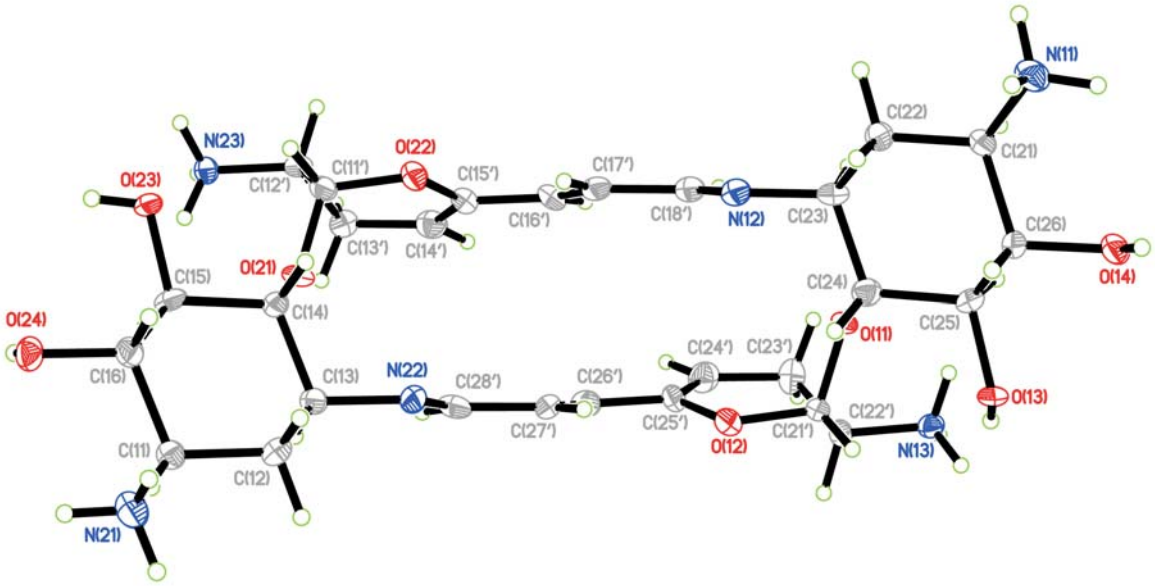
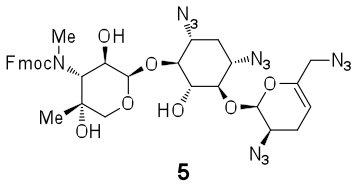


Fig S6. Top-view ORTEP representation at 30% probability level for the structure of molecule #1 from the crystal of compound **26**. For details see the crystallographic report file.



5

1,3,2',6'-Tetraazido-3''-*N*-Fmoc-sisomicin (5).

Sodium azide (7.6 g, 117 mmol) was dissolved in a minimum of water (25 mL) and an equal volume of DCM (25 mL) was added while stirring. The resulting suspension was cooled to 0 °C and a solution of Tf_2O (10 mL, 59.4 mmol) in DCM (10 mL) was added dropwise over 1 h with vigorous stirring. The mixture was then warmed to RT and stirred for 2 h, when sat. $NaHCO_3$ was added (50 mL). The organic layer was separated and the aqueous layer was extracted with DCM (10 mL, 2 times). The organic layers were combined and this freshly prepared TfN_3 solution was kept at 0 ºC until needed. Note: this DCM solution should be treated as a hazardous material, kept behind a shield and not dried by evaporation under any circumstances due to the potential explosiveness of TfN_3.[2,1,3] In a 500 mL round bottom flask, sisomicin sulphate **1** (3.0 g, 4.33 mmol), $NaHCO_3$ (7.6 g, 81 mmol) and $CuSO_4$ (119 mg, 0.75 mmol) were dissolved in H_2O (50 mL) and cooled to 0 ºC. The freshly prepared TfN_3 solution was added slowly at 0 ºC, followed by addition of MeOH (75 mL) and the ice bath was allowed to warm slowly to RT. After 4 h, the desired product was the major ion observed by LRMS, and the excess TfN_3 was quenched with *n*-butylamine (5.9 mL, 60 mmol). The solution was evaporated under vacuum and the residual aqueous mixture was extracted three times with DCM. The organic fraction was dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (3 → 5 → 7% of ammoniacal MeOH in DCM) yielded 1.60 g of 1,3,2',6'-tetraazido-sisomicin **4** (66%, 2.90 mmol), as a light yellow amorphous solid. R_f = 0.4, 20% ammoniacal MeOH/$CHCl_3$.[2,1]

Tetraazido intermediate **4** (500 mg, 0.907 mmol) was dissolved in MeCN (10 mL), added $NaHCO_3$ (230 mg, 2.7 mmol), *N*-(9-fluorenylmethoxycarbonyloxy) succinimide (450 mg, 1.35 mmol) and stirred overnight at RT, when the solution was diluted with EtOAc, washed with sat. NaCl, and the organic layer was evaporated to a residue under vacuum. Purification by column chromatography (1 → 2% MeOH in DCM) yielded 1,3,2',6'-tetraazido-3''-*N*-Fmoc-sisomicin **5** (661 mg, 94%, 0.854 mmol), as a light yellow amorphous solid.

R_f = 0.6, 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{27}H_{35}N_{13}O_9$, M + H^+ = 774.30665, found 774.30757 (0.59 ppm).

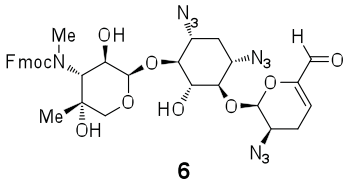
NMR analysis displayed two 3"-NMeFmoc rotamers, approx. 5:2 ratio in CD_3OD:

Major rotamer ^{1}H NMR (CD_3OD, 500 MHz) δ 7.82 – 7.76 (m, 2H), 7.66 – 7.60 (m, 2H), 7.42 – 7.35 (m, 2H), 7.35 – 7.28 (m, 2H), 5.88 (d, *J* = 2.40 Hz, 1H), 5.44 (d, *J* = 3.82 Hz, 1H), 4.99 – 4.94 (m, 1H), 4.49 (dd, *J* = 10.60, 6.80 Hz, 1H), 4.39 (dd, *J* = 17.03, 6.41 Hz, 1H), 4.30 – 4.22 (m, 1H), 4.29 (d, *J* = 11.38 Hz, 1H), 4.20 (d, *J* = 12.08 Hz, 1H), 4.15 (dd, *J* = 11.32, 3.93 Hz, 1H), 3.77 (d, *J* = 13.88 Hz, 1H), 3.73 – 3.54 (m, 4H), 3.49 – 3.30 (m, 3H), 3.23 (d, *J* = 12.13 Hz, 1H), 3.00 (s, 3H), 2.48 – 2.39 (m, 1H), 2.32 – 2.20 (m, 2H), 1.44 – 1.34 (m, 1H), 1.02 (s, 3H).

^{13}C NMR (CD_3OD, 125 MHz) δ 160.0, 147.1, 145.4, 145.2, 142.6487 142.6, 128.9, 128.8, 128.2 (2 C), 126.1, 126.0, 121.1, 121.0, 100.0, 99.3, 98.3, 81.0, 80.9, 76.0, 75.0, 70.5, 68.7, 65.9, 61.8, 61.5, 59.8, 56.0, 53.3, 48.5*, 33.9, 30.8, 22.2, 22.1. (*obtained from DEPT-135 spectrum).

Minor rotamer ^{1}H NMR (CD_3OD, 500 MHz) δ 7.82 – 7.76 (m, 2H), 7.70 – 7.60 (m, 2H), 7.43 – 7.35 (m, 2H), 7.34 – 7.28 (m, 2H), 5.90 (d, *J* = 2.38 Hz, 1H), 5.21 (d, *J* = 3.72 Hz, 1H), 4.99 – 4.94 (m, 1H), 4.59 (dd, *J* = 10.67, 5.60 Hz, 1H), 4.43 – 4.36 (m, 1H), 4.30 – 4.22 (m, 2H), 4.17 – 4.13 (m, 1H), 4.09 (d, *J* = 11.19 Hz, 1H), 3.92 (d, *J* = 12.00 Hz, 1H), 3.78 (d, *J* = 13.99 Hz, 1H), 3.73 – 3.53 (m, 3H), 3.49 – 3.30 (m, 3H), 3.22 (d, *J* = 11.99 Hz, 1H), 3.00 (s, 3H), 2.48 – 2.39 (m, 1H), 2.32 – 2.20 (m, 2H), 1.44 – 1.34 (m, 1H), 0.72 (s, 3H).

^{13}C NMR (CD_3OD, 125 MHz) δ 159.5, 147.2, 145.6, 145.2, 142.7, 142.6, 128.9 (2 C), 128.3, 126.3, 125.9, 121.1, 121.0, 100.2, 99.4, 98.2, 84.4, 81.3, 81.0, 75.8, 74.5, 70.8, 68.4, 65.7, 61.4, 61.0, 59.7, 56.2, 54.8, 53.3, 33.9, 31.0, 22.3, 21.9.



6

6'-Aldehydo-1,3,2'-triazido-3''-*N*-Fmoc-sisomicin (6)

Azide **5** (600 mg, 0.775 mmol) was dissolved in DCM (10 mL), treated with 3,4-dihydro-[2*H*]-pyran (210 µL, 2.33 mmol), SeO_2 (430 mg, 3.88 mmol),[2,1] and stirred

vigorously. After 36 h, the red solids were filtered and washed with DCM (20 mL). The filtrate and washings were washed with 2 M HCl and satd. $NaHCO_3$, dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by silica gel chromatography (1 → 2% MeOH in DCM) yielded 6'-aldehydo-1,3,2'-triazido-3"-*N*-Fmoc-sisomicin **6** (571 mg, 97%., 0.765 mmol), as a light yellow amorphous solid.

R_f = 0.6, 20:5:3, $CHCl_3$/EtOAc/MeOH, UV-active.

HRMS (ESI) calcd. for $C_{34}H_{38}N_{10}O_{10}$, M + H^+ = 747.28451, found 747.28392 (-0.39 ppm).

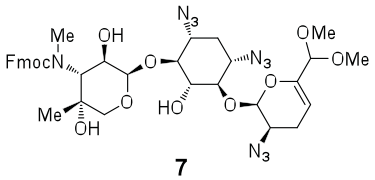
NMR analysis displayed two 3"-NMeFmoc rotamers, approx. 5:2 ratio in CD_3OD:

Major rotamer ^{1}H NMR (CD_3OD, 400 MHz) δ 9.18 (s, 1H), 7.82 – 7.75 (m, 2H), 7.70 – 7.58 (m, 2H), 7.43 – 7.34 (m, 2H), 7.33 – 7.25 (m, 2H), 6.21 – 6.15 (m, 1H), 5.95 (d, *J* = 2.43 Hz, 1H), 5.41 (d, *J* = 3.79 Hz, 1H), 4.48 (dd, *J* = 10.57, 6.79 Hz, 1H), 4.39 (dd, *J* = 10.56, 6.41 Hz, 1H), 4.26 (d, *J* = 11.31 Hz, 1H), 4.19 (d, *J* = 12.05 Hz, 1H), 4.12 (d, *J* = 12.54 Hz, 1H), 3.71 – 3.62 (m, 2H), 3.62 – 3.51 (m, 2H), 3.47 (ddd, *J* = 10.32, 7.28, 2.33 Hz, 1H), 3.43 – 3.30 (m, 2H), 3.20 (d, *J* = 12.10 Hz, 1H), 2.97 (s, 3H), 2.65 – 2.56 (m, 2H), 2.30 – 2.16 (m, 2H), 1.44 – 1.32 (m, 1H), 0.99 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 188.1, 156.0, 150.1, 145.5, 145.2, 142.7, 142.6, 128.9, 128.8, 128.2 (2 C), 126.1, 126.1, 122.7, 121.0 (2 C), 100.1, 98.0, 84.1, 81.0, 80.9, 76.0, 75.0, 70.5, 68.8, 65.9, 61.9, 61.4, 59.8, 55.5, 33.7, 30.8, 23.2, 22.2.

Minor rotamer ^{1}H NMR (CD_3OD, 400 MHz) δ 9.19 (s, 1H), 7.82 – 7.75 (m, 2H), 7.70 – 7.58 (m, 2H), 7.44 – 7.34 (m, 2H), 7.33 – 7.25 (m, 2H), 6.21 – 6.15 (m, 1H), 5.98 (d, *J* = 2.44 Hz, 1H), 5.21 (d, *J* = 3.52 Hz, 1H), 4.58 (dd, *J* = 10.49, 5.45 Hz, 1H), 4.36 (dd, *J* = 20.19, 11.24 Hz, 1H), 4.27 – 4.19 (m, 1H), 4.15 – 4.06 (m, 1H), 3.95 (d, *J* = 11.98 Hz, 1H), 3.71 – 3.62 (m, 2H), 3.62 – 3.43 (m, 2H), 3.43 – 3.30 (m, 2H), 3.19 (d, *J* = 12.02 Hz, 1H), 2.98 (s, 3H), 2.65 – 2.53 (m, 2H), 2.31 – 2.16 (m, 2H), 1.44 – 1.32 (m, 1H), 0.71 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 188.0, 159.5, 150.1, 145.6, 145.2, 142.8, 142.7, 128.9, 128.8, 128.4, 128.3, 126.3, 126.0, 122.9, 121.1, 121.0, 100.3, 97.8, 84.1, 81.4, 80.9, 75.8, 74.6, 70.8, 68.4, 65.7, 61.3, 61.1, 59.7, 55.7, 33.7, 31.0, 23.4, 22.0.



7

1,3,2'-Triazido-3''-*N*-Fmoc-6',6'-dimethoxy-sisomicin (7)

Aldehyde **6** (535 mg, 0.716 mmol) was dissolved in anhydrous MeOH (5 mL), treated with trimethylorthoformate (2 mL, 18.5 mmol) and TFA (115 μL, 1.5 mmol), and stirred at RT for 5 h, when no UV-active starting material was observed on TLC, the reaction was neutralized with Et_3N (230 μL, 1.65 mmol). The volatiles were removed under vacuum to a residue. Purification by ammoniacal silica gel chromatography to avoid acetal cleavage (1 → 2 % ammoniacal MeOH in DCM) yielded 1,3,2'-triazido-3''-*N*-Fmoc-6',6'-dimethoxy-sisomicin **7** (455 mg, 80%, 0.574 mmol), as a white amorphous solid.

R_f = 0.65, 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{36}H_{44}N_{10}O_{11}$, M + Na^+ = 815.30832, found 815.30968 (0.83 ppm).

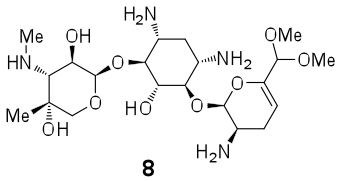
NMR analysis displayed two 3''-NMeFmoc rotamers, approx. 5:2 ratio in CD_3OD:

Major rotamer ^{1}H NMR (CD_3OD, 400 MHz) δ 7.82 – 7.75 (m, 2H), 7.70 – 7.58 (m, 2H), 7.43 – 7.33 (m, 2H), 7.33 – 7.25 (m, 2H), 5.83 (d, *J* = 2.28 Hz, 1H), 5.41 (d, *J* = 3.78 Hz, 1H), 5.17 – 5.12 (m, 1H), 4.72 (s, 1H), 4.47 (dd, *J* = 10.57, 6.79 Hz, 1H), 4.39 (dd, *J* = 9.63, 5.43 Hz, 1H), 4.26 (d, *J* = 11.28 Hz, 1H), 4.25 – 4.21 (m, 1H), 4.19 (d, *J* = 12.05 Hz, 1H), 4.11 (ddd, *J* = 9.46, 1.83, 1.37 Hz, 1H), 3.69 – 3.53 (m, 3H), 3.49 – 3.37 (m, 2H), 3.36 (s, 3H), 3.34 – 3.28 (m, 1H), 3.26 (s, 3H), 3.18 (d, *J* = 12.07 Hz, 1H), 2.96 (s, 3H), 2.47 – 2.37 (m, 1H), 2.33 – 2.18 (m, 2H), 1.46 – 1.32 (m, 1H), 0.98 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 160.0, 146.5, 145.5, 145.2, 142.7, 142.6, 128.8, 128.8, 128.2 (2 C), 126.1, 126.1, 121.0 (2 C), 101.8, 100.1, 98.9, 98.1, 81.0, 80.9, 76.1, 75.0, 70.5, 68.8, 65.9, 62.0, 61.6, 59.8, 56.3, 55.0, 52.2, 48.6, 33.9, 30.8, 22.2, 22.0.

Minor rotamer ^{1}H NMR (CD_3OD, 400 MHz) δ 7.81 – 7.75 (m, 2H), 7.70 – 7.58 (m, 2H), 7.43 – 7.33 (m, 2H), 7.33 – 7.25 (m, 2H), 5.87 (d, *J* = 2.26 Hz, 1H), 5.21 (d, *J* = 3.44 Hz, 1H), 5.18 – 5.12 (m, 1H), 4.73 – 4.70 (m, 1H), 4.57 (dd, *J* = 10.49, 5.64 Hz, 1H), 4.35 (dd, *J* = 10.37, 6.33 Hz, 1H), 4.26 – 4.19 (m, 1H), 4.14 – 4.06 (m, 2H), 3.95 (d, *J* = 11.98 Hz, 1H), 3.70 – 3.53 (m, 3H), 3.49 – 3.37 (m, 2H), 3.36 (s, 3H), 3.34 – 3.28 (m, 1H), 3.27 (s, 3H), 3.18 (d, *J* = 11.94 Hz, 1H), 2.97 (s, 3H), 2.47 – 2.36 (m, 1H), 2.33 – 2.18 (m, 2H), 1.46 – 1.32 (m, 1H), 0.71 (s, 3H).

^{13}C NMR (CD_3OD, 100 MHz) δ 159.5, 146.5, 145.6, 145.2, 142.8, 142.7, 128.9 (2 C), 128.4, 128.3, 126.3, 126.0, 121.1, 121.1, 101.8, 100.3, 99.1, 97.9, 84.1, 81.2, 75.9, 74.6, 70.8, 68.4, 65.7, 61.5, 61.2, 59.8, 56.5, 54.9, 52.4, 48.6, 33.9, 31.0, 22.1, 22.0.



8

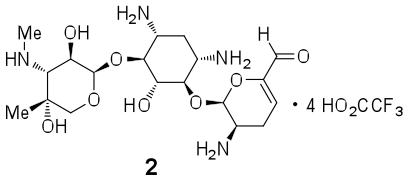
6',6'-Dimethoxy-sisomicin (8)

Acetal **7** (407 mg, 0.532 mmol) was dissolved in 15 mL MeOH, added piperidine (530 µL, 10 equiv., 5.3 mmol), $NH_4OH_{(aq)}$ (1 mL, 28% liquor) and PMe_3 (1.0 M in THF, 10.6 mL, 20 equiv., 10.6 mmol) upon which mild bubbling was observed. The reaction was stirred 10 h at RT, when LRMS analysis indicated completion of the deblocking reaction. The volatiles were removed under vacuum, leaving a residue which was redissolved in 20% ammoniacal MeOH in DCM for purification by column chromatography (20 → 40 % ammoniacal MeOH in DCM). The fractions containing aminoglycoside were pooled, evaporated under vacuum to a wet residue, which was redissolved in water, filtered through a 0.45 µm syringe filter and freeze-dried to yield 6',6'-dimethoxy-sisomicin **8** (233.6 mg, 89%, 0.474 mmol), as a white cotton-like solid. NMR and MS analyses were consistent with the previously reported acetate salt produced from the Birch reduction protocol using the analog 3'''-NCbz substrate.[1]

R_f = 0.2, 40% ammoniacal MeOH/$CHCl_3$.

^{1}H NMR (D_2O, 400 MHz) δ 5.31 (d, J = 2.2 Hz, 1H), 5.17 (dd, J = 5.1, 2.1 Hz, 1H), 5.07 (d, J = 4.0 Hz, 1H), 4.69 (s, 1H), 4.05 (d, J = 12.5 Hz, 1H), 3.81 (dd, J = 10.8, 3.9 Hz, 1H), 3.54 (t, J = 9.3 Hz, 1H), 3.44 – 3.36 (m, 7H), 3.31 (d, J = 12.5 Hz, 1H), 3.26 (t, J = 9.5 Hz, 1H), 3.09 (ddd, J = 10.3, 6.2, 2.2 Hz, 1H), 2.88 (ddd, J = 12.1, 9.8, 3.8 Hz, 1H), 2.72 (ddd, J = 12.4, 9.8, 4.2 Hz, 1H), 2.62 (d, J = 10.8 Hz, 1H), 2.53 (s, 3H), 2.25 (dt, J = 17.1, 5.7 Hz, 1H), 2.08 – 1.99 (m, 1H), 1.99 – 1.91 (m, 1H), 1.29 – 1.22 (m, 1H), 1.21 (s, 3H).

^{13}C NMR (D_2O, 100 MHz) δ 143.2, 102.1, 100.8, 100.1, 100.1, 86.2, 85.8, 73.9, 71.8, 68.7, 67.2, 62.9, 54.4, 54.3, 50.5, 49.0, 46.2, 36.3, 34.8, 23.7, 21.1.



2

6'-Aldehydo-sisomicin TFA salt (2).

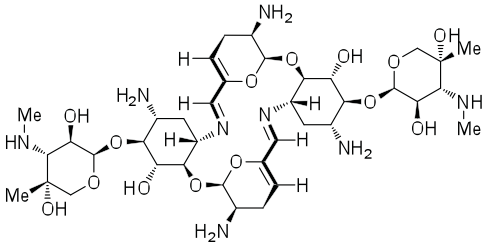
Acetal **8** (61 mg, 0.124 mmol) was dissolved in 1 mL H_2O, added TFA (96 µL, 1.2 mmol), stirred for 15 min and freeze-dried to yield 107.5 mg of 6'-aldehydo-sisomicin TFA salt **2** (0.119 mmol, 95%), as a white cotton-like solid.

HRMS (ESI) calcd. for $C_{19}H_{34}N_4O_8$, M + H^+ = 447.24494, found 447.24652 (3.54 ppm).

NB: In D_2O, this compound was observed as approx. 10:1 ratio of aldehyde and hydrate:[1,4]

^{1}H NMR (D_2O, 700 MHz) δ 9.07 (d, *J* = 0.52 Hz, 1H), 6.24 (t, *J* = 4.10, 4.10 Hz, 1H), 5.59 (d, *J* = 0.82 Hz, 1H), 4.96 (d, *J* = 3.77 Hz, 1H), 4.10 (dd, *J* = 10.89, 3.69 Hz, 1H), 3.94 (dd, *J* = 10.09, 9.43 Hz, 1H), 3.91 (ddd, *J* = 6.13, 4.37, 1.81 Hz, 1H), 3.88 (d, *J* = 12.85 Hz, 1H), 3.69 (dd, *J* = 9.33, 8.92 Hz, 1H), 3.64 (dd, *J* = 10.02, 9.25 Hz, 1H), 3.48 – 3.39 (m, 2H), 3.38 (d, *J* = 12.87 Hz, 1H), 3.37 (dd, *J* = 10.80, 1.17 Hz, 1H), 2.89 (ddd, *J* = 20.19, 5.80, 4.02 Hz, 1H), 2.80 (s, 3H), 2.59 (td, *J* = 20.39, 4.33, 4.33 Hz, 1H), 2.44 (td, *J* = 12.57, 4.26, 4.26 Hz, 1H), 1.84 – 1.77 (m, 1H), 1.23 (s, 3H).

^{13}C NMR (D_2O, 175 MHz) δ 188.3, 162.9 (TFA, q, *J* = 35.50, 35.50, 35.42 Hz), 148.6, 122.6, 116.2 (TFA, q, *J* = 291.56, 291.56, 291.51 Hz), 101.2, 97.0, 83.2, 79.5, 73.3, 69.8, 67.6, 66.2, 63.2, 49.6, 47.9, 45.8, 34.3, 27.4, 24.3, 20.7.



3

Aminoglycoside *66-40C* (3).[1]

Acetal intermediate **8** (28 mg, 56 µmol) was dissolved in 1 mL 0.5 N H_2SO_4 and stirred for 15 min to liberate aldehyde **2**. The solution was cooled to 0 ºC, and added dropwise sat. $Ba(OH)_2$ (10 mL of approx. 0.05 N solution, freshly prepared by heating 315 mg of the octahydrate salt in 20 mL H_2O at reflux for 20 min). The resulting white suspension of

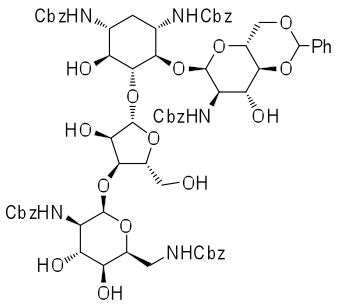
$BaSO_4$ was removed by filtration through a 0.45 μm syringe filter. The filtrate was freeze-dried to generate a white cotton-like solid. ^{1}H-NMR analysis in D_2O showed only signals belonging to dimer *66-40C* (**3**). The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.5) and purified by silica gel column chromatography using the same solvent system, increasing ammonia gradually to a 2:3:1 mixture. The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was dissolved in a minimum volume of water and freeze-dried. The dry residue obtained was redissolved in 1 mL of water, at which point insoluble traces of silica were generally observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. Finally, freeze-drying the filtrate yielded 24.3 mg of aminoglycoside *66-40C* **3** (quant., 28 μmol), as a light yellow cotton-like solid. NMR and MS analyses of the product were identical to the previously reported synthetic[1] and natural products.[4]

R_f = 0.3, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

HPLC purity was determined >90% under UV monitoring (220 and 274 nm).

^{1}H NMR (D_2O, 500 MHz) δ 7.50 (s, 2H), 5.62 (d, J = 2.6 Hz, 2H), 5.56 (dd, J = 5.8, 2.9 Hz, 2H), 5.19 (d, J = 3.9 Hz, 2H), 4.14 (dd, J = 10.9, 3.8 Hz, 2H), 4.12 (d, J = 12.9 Hz, 2H), 3.76 (dt, J = 21.2, 9.1 Hz, 4H), 3.59 (t, J = 9.5 Hz, 2H), 3.47 (d, J = 12.9 Hz, 2H), 3.29 (d, J = 10.8 Hz, 2H), 3.32 – 3.24 (m, 4H), 3.20 (ddd, J = 12.9, 10.1, 4.0 Hz, 2H), 2.87 (s, 6H), 2.48 – 2.32 (m, 4H), 2.08 (dt, J = 12.8, 4.2 Hz, 2H), 1.88 – 1.76 (m, 2H), 1.34 (s, 6H).

^{13}C NMR (D_2O, 125 MHz) δ 160.6, 145.3, 114.2, 100.4, 97.0, 85.4, 79.9, 75.4, 70.4, 67.5, 67.0, 65.4, 63.8, 50.4, 46.3, 35.2, 33.5, 22.6, 21.1.



10

4′,6′-*O*-Benzylidene-*per*-*N*-Cbz-paromomycin (10).

Paromomycin sulphate **9** (10 g) was dissolved in 20 mL of water and added through a dropper over 30 min to a rapidly stirring suspension of benzyl chloroformate (16 mL, 112 mmol) and Na_2CO_3 (24 g, 226 mmol) in 250 mL MeOH. After stirring overnight, the solution was diluted with 500 mL of DCM and the salts were filtered. The filtrate was evaporated to dryness, absorbed onto silica gel and purified by on a short column (4 → 6% MeOH, DCM) to yield 7.1 g of *per*-*N*-Cbz paromomycin (**S1**, 5.52 mmol).[5]

R_f = 0.3 in 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{63}H_{75}N_5O_{24}$, M + H^+ = 1286.4875, found 1286.4853 (-1.66 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.45 – 7.22 (m, 25H), 5.30 – 5.23 (m, 1H), 5.23 – 5.14 (m, 2H), 5.14 – 4.98 (m, 9H), 4.83 (s, 1H), 4.64 (s, 1H), 4.08 – 3.89 (m, 4H), 3.88 – 3.82 (m, 2H), 3.82 – 3.74 (m, 2H), 3.74 – 3.69 (m, 1H), 3.69 – 3.58 (m, 3H), 3.56 – 3.51 (m, 2H), 3.50 – 3.35 (m, 5H), 3.35 – 3.31 (m, 2H), 2.02 – 1.93 (m, 1H), 1.49 – 1.35 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 156.8 (5C), 136.5 - 136.2 (5C), 127.9 - 126.9 (25C), 108.8, 98.7, 98.4, 85.5, 81.9, 79.4, 77.7, 76.3, 74.1, 73.7, 73.1, 72.7, 70.9, 69.8, 67.3, 66.2 - 65.7 (5C), 61.3, 61.0, 56.0, 52.3, 50.8, 50.5, 40.8, 33.6.

Intermediate **S1** (7.1 g, 5.52 mmol) was dried by evaporation three times from toluene, dissolved in freshly distilled benzaldehyde (70 mL), cooled to 0 °C, treated with formic acid (10 mL) and stirred with cooling for 24 h, when the *mono*-benzylidene product dominated, as judged by TLC (20:5:3, $CHCl_3$, EtOAc, MeOH, R_f = 0.7) and LRMS (M+H^+ = 1374.5). The reaction mixture was poured over stirring sat. $NaHCO_3$, which was extracted with EtOAc and dried over Na_2SO_4. The volatiles were evaporated under vacuum and the resulting solution of benzaldehyde was added dropwise to stirring hexanes. The resulting white gummy precipitate was collected and purified by column chromatography

(2 → 4% MeOH, DCM) to yield 5.3 g of the title compound 4′,6′-*O*-Benzylidene-*per*-*N*-Cbz-paromomycin **10** (70%, 38.6 mmol), an amorphous solid.

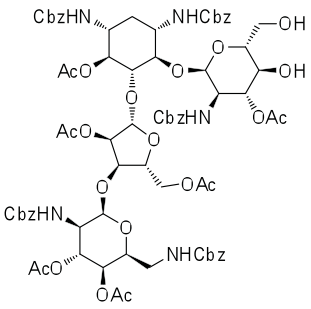
R_f = 0.5 in 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{70}H_{79}N_5O_{24}$, M + H^+ = 1374.5188, found 1374.5187 (-0.04 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.54 – 7.05 (m, 30H), 5.48 (s, 1H), 5.27 (s, 1H), 5.16 – 5.08 (m, 2H), 5.08 – 4.90 (m, 8H), 4.79 (s, 1H), 4.58 (s, 1H), 4.22 (dd, *J* = 8.67, 4.05 Hz, 1H), 4.04 – 3.78 (m, 7H), 3.79 – 3.63 (m, 2H), 3.63 – 3.50 (m, 3H), 3.50 – 3.37 (m, 5H), 3.37 – 3.28 (m, 3H), 3.28 – 3.24 (m, 1H), 1.91 (d, *J* = 10.96 Hz, 1H), 1.45 – 1.27 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 157.4 - 156.6 (5C), 137.5 - 136.2 (5C), 128.1 - 126.9 (27C), 125.8 (4C), 108.9, 101.1, 98.9, 98.7, 85.8, 82.0, 81.3, 79.6, 76.6, 74.1, 73.8, 72.7, 69.8, 68.2, 68.1, 67.4, 66.3 - 65.8 (5C), 63.2, 61.7, 56.5, 52.3, 50.9, 50.2, 40.8, 33.6.

NB: The benzylidene anomer configuration has not been determined.



11

6,3′,2″,5″,3‴,4‴-Hexa-*O*-acetyl-4′,6′-*O*-benzylidene-*per*-*N*-Cbz-paromomycin (11).

A solution of 4′,6′-benzyldene intermediate **10** (1.0 g, 0.723 mmol) in dry pyridine (20 mL) was treated with acetic anhydride (690 μL, 7.3 mmol) and DMAP (9 mg, 0.07 mmol), heated to 60 ºC for 16 h, and quenched with MeOH (approx. 5 mL). The crude reaction mixture was added dropwise into a stirring hexanes/pentane 1:1 mixture (200 mL). The solution was decanted and the crude precipitate was dissolved in EtOAc, washed successively with 2 N HCl, sat. $NaHCO_3$ and dried over Na_2SO_4. The organic fraction was evaporated under vacuum to a residue, which was purified by column chromatography (20 → 40% EtOAc/DCM) to yield 6,3′,2″,5″,3‴,4‴-hexa-*O*-acetyl-4′,6′-benzylidene-*per*-*N*-Cbz-paromomycin **S2** (1.10 g, 0.676 mmol, 93%), a white amorphous solid.

R_f = 0.5, 40% EtOAc/DCM.

HRMS (ESI) calculated for $C_{82}H_{91}N_5O_{30}$, M + Na^+ = 1648.56411, found: 1648.56404 (-0.02 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.42 – 7.11 (m, 30H), 5.74 (d, *J* = 3.64 Hz, 1H), 5.53 (s, 1H), 5.30 – 5.15 (m, 4H), 5.15 – 4.91 (m, 10H), 4.88 – 4.85 (m, 1H), 4.82 – 4.76 (m, 1H), 4.74 – 4.71 (m, 1H), 4.67 (s, 1H), 4.33 – 4.24 (m, 2H), 4.24 – 4.12 (m, 2H), 4.06 – 3.90 (m, 4H), 3.88 – 3.82 (m, 1H), 3.77 (d, *J* = 7.22 Hz, 1H), 3.74 – 3.59 (m, 5H), 3.35 – 3.29 (m, 1H), 3.23 (dd, *J* = 13.87, 7.03 Hz, 1H), 2.08 (s, 3H), 2.03 (s, 3H), 1.99 (s, 3H), 1.94 (s, 3H), 1.90 (s, 3H), 1.83 (s, 3H), 1.73 – 1.61 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 172.9, 172.2 - 172.1 (3 C), 171.2, 170.3, 158.8 - 158.0 (5 C), 139.0, 138.5 - 138.0 (5 C), 129.9 - 128.8 (28 C), 127.4 (2 C), 109.1, 102.5, 99.4, 98.9, 84.6, 80.7, 80.6, 79.5, 77.6, 76.2, 76.0, 73.8, 72.0, 70.3, 69.7, 68.1, 67.8, 67.6 (2 C), 67.5, 64.5, 63.6, 55.4, 51.4, 51.0, 50.9, 50.8, 41.8, 34.3, 21.4, 21.2, 20.9 (2 C), 20.7 (2 C).

Intermediate **S2** (1.05 g, 0.646 mmol) was dissolved in a minimum volume of DCM, which was then treated with 80% AcOH (20 mL) and heated to 60 °C, forming a suspension. After stirring 10 h, the suspension was neutralized with sat. $NaHCO_3$, extracted three times with EtOAc and dried over Na_2SO_4. The organic layer was evaporated under vacuum to a residue, which was purified by column chromatography (1 → 2% MeOH/DCM) to yield 6,3′,2″,5″,3‴, 4‴-hexa-*O*-acetyl-*per*-*N*-Cbz-paromomycin **11** (0.73 g, 0.474 mmol, 73%), a white amorphous solid.

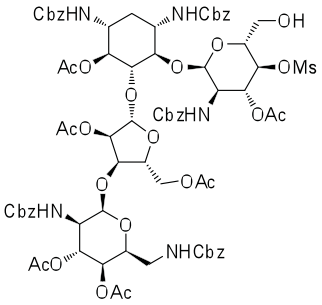
R_f = 0.6 in 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{75}H_{87}N_5O_{30}$, M + Na^+ = 1560.53281, found 1560.5348 (0.64 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.43 – 7.19 (m, 25H), 5.61 (d, *J* = 2.34 Hz, 1H), 5.24 – 4.97 (m, 11H), 4.97 – 4.91 (m, 2H), 4.81 – 4.74 (m, 1H), 4.74 – 4.69 (m, 1H), 4.64 (s, 1H), 4.57 (s, 2H), 4.25 – 4.19 (m, 1H), 4.19 – 4.08 (m, 2H), 4.00 (dd, *J* = 6.58, 5.98 Hz, 1H), 3.95 – 3.72 (m, 6H), 3.72 – 3.58 (m, 4H), 3.51 (dd, *J* = 10.18, 8.76 Hz, 1H), 3.31 (dd, *J* = 14.01, 6.28 Hz, 1H), 3.22 (dd, *J* = 13.74, 6.86 Hz, 1H), 2.07 (s, 3H), 2.03 (s, 3H), 1.98 (s, 3H), 1.94 (s, 3H), 1.91 (s, 3H), 1.84 (s, 3H), 1.68 – 1.56 (m, 1H). NB trace DCM, 5.45 ppm.

^{13}C NMR (CD_3OD, 100 MHz) δ 173.1, 172.6, 172.2, 172.1, 171.2, 170.3, 158.8 - 158.1 (5 C), 138.4 - 138.1 (5 C), 129.6 - 128.8 (25 C), 108.8, 99.4, 98.5, 83.9, 80.7, 79.5, 77.4, 76.4,

75.8, 75.5, 74.0, 73.8, 70.2, 69.7, 68.2, 67.7 - 67.4 (5 C), 63.9, 62.6, 55.2, 55.1, 51.5, 50.9, 41.7, 34.5, 21.4, 21.2, 21.1, 20.8, 20.7 (2 C). NB traces DCM: 54.9 ppm, MeOH: 50.8 ppm.



12

6,3′,2″,5″,3‴,4‴-Hexa-*O*-acetyl-4′-*O*-mesyl-*per*-*N*-Cbz-paromomycin (12).

Compound **11** (685 mg, 0.445 mmol), was dried by evaporating three times with toluene, dissolved in pyridine (10 mL), cooled to 0 °C and treated with TBSOTf (120 µL, 0.52 mmol). After 2 h the reaction was quenched with MeOH (1 mL), diluted with EtOAc (25 mL) and washed successively with 2 N HCl and sat. $NaHCO_3$, and dried over Na_2SO_4. The solvents were evaporated under vacuum to a residue, which was purified by column chromatography (30→40% EtOAc/DCM) to yield 618 mg of 6,3′,2″,5″,3‴,4‴-Hexa-*O*-acetyl-6′-*tert*-butyldimethylsilyl-*per*-*N*-Cbz-paromomycin (**S3**) (84%, 0.374 mmol), as a white amorphous solid.

R_f = 0.5, 30% EtOAc in DCM.

HRMS (ESI) calcd. for $C_{81}H_{101}N_5O_{30}Si$, M + Na^+ = 1674.61928, found 1674.61958 (0.09 ppm).

^{1}H NMR (CD_3OD, 500 MHz) δ 7.46 – 7.18 (m, 25H), 5.60 (s, 1H), 5.21 (d, *J* = 1.69 Hz, 1H), 5.10 (m, 10H), 4.97 (d, *J* = 3.18 Hz, 1H), 4.94 (dd, *J* = 7.71, 3.16 Hz, 2H), 4.82 – 4.76 (m, 1H), 4.75 – 4.71 (m, 1H), 4.65 (s, 1H), 4.56 (s, 2H), 4.23 – 4.10 (m, 2H), 4.02 (dd, *J* = 6.62, 5.69 Hz, 1H), 3.93 (t, *J* = 8.50, 8.50 Hz, 1H), 3.88 – 3.77 (m, 5H), 3.77 – 3.74 (m, 1H), 3.73 – 3.59 (m, 4H), 3.33 (dd, *J* = 13.83, 6.19 Hz, 1H), 3.23 (dd, *J* = 13.84, 7.04 Hz, 1H), 2.10 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.95 (s, 3H), 1.94 (s, 3H), 1.85 (s, 3H), 1.71 – 1.58 (m, 1H), 0.88 (s, 9H), 0.02 (s, 3H), 0.03 (s, 3H).

^{13}C NMR (CD_3OD, 125 MHz) δ 172.9, 172.7, 172.2, 172.1, 171.2, 170.3, 158.8 - 158.1 (5 C), 138.4 - 138.0 (5 C), 129.6 - 128.8 (25 C), 108.7, 99.4, 98.9, 84.2, 80.5, 79.6, 77.6, 76.3,

76.0, 75.8, 73.8, 73.7, 70.3, 69.1, 68.2, 67.7, 67.6, 67.6, 67.5, 63.7, 63.3, 55.2, 55.1, 51.6, 50.9, 50.8, 41.7, 34.5, 26.6 (3 C), 21.4, 21.4, 21.1, 20.9, 20.7 (2 C), 19.4, -5.3 (2 C).

Compound **S3** (575 mg, 0.348 mmol), was dissolved in anhydrous DCM (15 mL), cooled to 0 ºC and followed by addition of pyridine (85 µL, 1.05 mmol) and mesyl chloride (55 µL, 0.71 mmol). After 3 h the reaction was quenched with MeOH (1 mL) and washed successively with 2 N HCl and sat. $NaHCO_3$, and dried over Na_2SO_4. The solvents were evaporated under vacuum to a residue, which was purified by column chromatography (10 → 20% EtOAc/DCM) to yield 467 mg of 6,3′,2″,5″,3‴,4‴-Hexa-*O*-acetyl-4′-*O*-mesyl-6′-*tert*-butyldimethylsilyl-*per*-*N*-Cbz-paromomycin **S4** (78%, 0.27 mmol), as a white amorphous solid.

R_f = 0.6, 30% EtOAc in DCM.

HRMS (ESI) calcd. for $C_{82}H_{103}N_5O_{32}SSi$, M + H^+ = 1730.61489, found 1730.60765 (-2.09 ppm).

^{1}H NMR (CD_3OD, 500 MHz) δ 7.43 – 7.22 (m, 25H), 5.74 (d, *J* = 3.10 Hz, 1H), 5.26 – 5.15 (m, 4H), 5.15 – 5.01 (m, 6H), 4.98 – 4.96 (m, 1H), 4.96 – 4.91 (m, 2H), 4.81 – 4.76 (m, 1H), 4.74 – 4.71 (m, 1H), 4.67 (s, 1H), 4.58 – 4.55 (m, 3H), 4.22 – 4.12 (m, 3H), 4.06 – 3.98 (m, 2H), 3.98 – 3.86 (m, 3H), 3.86 – 3.81 (m, 1H), 3.80 – 3.64 (m, 4H), 3.60 (dd, *J* = 9.90, 8.78 Hz, 1H), 3.33 (dd, *J* = 14.08, 6.14 Hz, 1H), 3.24 (dd, *J* = 13.91, 7.06 Hz, 1H), 2.84 (s, 3H), 2.10 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), 1.93 (s, 3H), 1.93 (s, 3H), 1.87 (s, 3H), 1.72 – 1.61 (m, 1H), 0.89 (s, 9H), 0.05 (s, 3H), 0.04 (s, 3H).

^{13}C NMR (CD_3OD, 125 MHz) δ 172.7, 172.2 - 172.1 (3 C), 171.2, 170.3, 158.8 - 158.1 (5 C), 138.4 - 138.0 (5 C), 129.6 - 128.8 (25 C), 109.0, 99.4, 98.1, 84.2, 80.7, 79.2, 77.3, 76.5, 76.4, 75.7, 73.9, 72.6, 71.1, 70.3, 68.1, 67.7, 67.7, 67.6, 67.5, 63.7, 62.4, 61.6, 55.2, 51.4, 51.0, 50.8, 41.8, 38.9, 34.4, 26.5 (3 C), 21.5, 21.4, 21.0, 20.9, 20.7 (2 C), 19.2, -5.3 (2 C).

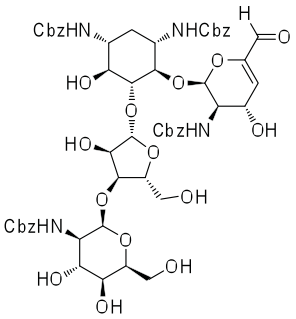
Compound **S4** (435 mg, 0.25 mmol) was dried by evaporating three times with toluene, dissolved in pyridine (5 mL), cooled to 0 °C and treated with 2.5 mL of HF·pyridine (70% in pyridine solution). After 2 hours at 0 °C, the solution was neutralized with sat. $NaHCO_3$ and extracted three times with EtOAc. The combined organic layers were washed successively with 2 N HCl, sat. $NaHCO_3$, and dried over Na_2SO_4. The solvents were evaporated under vacuum to a residue, which was purified by column chromatography (40

→ 50% EtOAc/DCM) to yield 282 mg of title compound 6,3′,2″,5″,3‴,4‴-hexa-*O*-acetyl-4′-*O*-mesyl-*per*-*N*-Cbz-paromomycin **12** (70%, 0.174 mmol), as a white amorphous solid. R_f = 0.3, 70% EtOAc in DCM.

HRMS (ESI) calcd. for $C_{76}H_{89}N_5O_{32}S$, M + H^+ = 1616.52098, found 1616.52841 (2.30 ppm).

^{1}H NMR (CD_3OD, 500 MHz) δ 7.41 – 7.23 (m, 25H), 5.80 (d, *J* = 3.24 Hz, 1H), 5.27 – 4.99 (m, 11H), 4.99 – 4.96 (m, 1H), 4.96 – 4.92 (m, 1H), 4.74 – 4.71 (m, 1H), 4.71 – 4.64 (m, 2H), 4.60 – 4.53 (m, 3H), 4.25 – 4.14 (m, 3H), 4.05 – 3.96 (m, 2H), 3.96 – 3.89 (m, 2H), 3.87 – 3.83 (m, 1H), 3.81 (d, *J* = 1.48 Hz, 1H), 3.79 – 3.75 (m, 1H), 3.75 – 3.66 (m, 2H), 3.66 – 3.58 (m, 2H), 3.35 – 3.28 (m, 1H), 3.24 (dd, *J* = 14.04, 7.12 Hz, 1H), 2.86 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.01 (s, 3H), 1.95 (s, 3H), 1.92 (s, 3H), 1.86 (s, 3H), 1.72 – 1.61 (m, 1H).

^{13}C NMR (CD_3OD, 125 MHz) δ 173.2, 172.2 - 172.1 (3 C), 171.3, 170.3, 158.9 - 158.0 (5 C), 138.4 - 138.1 (5 C), 129.6 - 128.9 (25 C), 109.0, 99.4, 97.6, 84.2, 80.8, 78.8, 77.4, 76.6, 76.4, 75.9, 73.8, 72.3, 71.3, 70.3, 68.1, 67.8, 67.6 (2 C), 67.5, 63.7, 61.6, 61.4, 55.1, 51.3, 51.1, 50.8, 41.7, 38.7, 34.3, 21.4, 21.2, 21.0, 20.8, 20.7 (2 C).



13

6'-Aldehydo-4',5'-dehydro-4'-deoxy-*per*-*N*-Cbz-paromomycin (13).

Alcohol **12** (256 mg, 0.158 mmol), was dried by evaporation three times from toluene, dissolved in dry DCM (5 mL) and Et_3N (1.1 mL), and cooled to 0 °C. In a separate flask, a DMSO (680 µL, 9.5 mmol) solution containing SO_3·pyridine complex (230 mg, 1.45 mmol) was prepared. The DMSO solution was added dropwise to the alcohol mixture, which was allowed to warm to RT overnight. After 12 h, only the UV-active product spot was observed on TLC. The reaction was quenched with water, diluted with EtOAc and washed successively with 2 N HCl and sat. $NaHCO_3$, dried over Na_2SO_4 and filtered. The

organic layer was evaporated to a residue that was purified by column chromatography (10 → 30% EtOAc/DCM) to yield 198 mg of 6'-aldehydo-6,3′,2″,5″,3‴,4‴-hexa-*O*-acetyl-4',5'-dehydro-4'-deoxy-*per*-*N*-Cbz-paromomycin **S5** (82%, 0.13 mmol), as an off-white amorphous solid.

R_f = 0.6, 50% EtOAc in DCM – UV active.

HRMS (ESI) calcd. for $C_{75}H_{83}N_5O_{29}$, M + Na^+ = 1540.50659, found 1540.50626 (-0.11 ppm).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.02 (s, 1H), 7.43 – 7.21 (m, 25H), 5.80 (s, 1H), 5.74 (s, 1H), 5.23 (s, 1H), 5.12 – 4.98 (m, 8H), 4.98 – 4.85 (m, 5H), 4.71 – 4.59 (m, 3H), 4.27 (dd, *J* = 5.23, 4.35 Hz, 1H), 4.20 – 4.11 (m, 1H), 4.11 – 3.98 (m, 3H), 3.98 – 3.85 (m, 2H), 3.69 – 3.62 (m, 1H), 3.62 – 3.49 (m, 3H), 3.23 (dd, *J* = 12.63, 6.64 Hz, 1H), 3.11 (dd, *J* = 12.53, 5.16 Hz, 1H), 2.08 (s, 3H), 2.00 (s, 3H), 2.00 (s, 3H), 1.98 (s, 3H), 1.90 (s, 3H), 1.84 (s, 3H), 1.71 – 1.61 (m, 2H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ 186.4, 170.1 - 168.4 (6 C), 156.0 - 155.3 (5 C), 148.1, 137.1 - 136.8 (5 C), 128.3 – 127.3 (25 C), 117.5, 106.8, 97.3, 96.9, 81.4, 78.8, 78.6, 75.6, 74.4, 73.7, 71.4, 68.2, 66.9, 65.8, 65.5 (2 C), 65.4 (2 C), 65.1 (2 C), 62.2, 50.6, 49.3, 48.9, 40.1, 32.8, 20.6, 20.5, 20.4, 20.4, 20.3, 20.0.

Sodium methoxide in MeOH was prepared by addition of a piece of sodium (~20 mg) to anhydrous MeOH (20 mL). The resulting alkaline solution was diluted with anhydrous MeOH to approx. pH 8 to 9 (Accutint pH paper roll). Aledhyde **S5** (173 mg, 0.114 mmol) was dissolved in 10 mL of freshly prepared solution of NaOMe in MeOH (15 mL) and stirred for 5 h. The solution was neutralized with a few drops of AcOH, and the solvents were evaporated under vacuum to a residue, which was purified by column chromatography (2 → 4 % MeOH in DCM), to yield 119 mg of the title compound 6'-aldehydo-4',5'-dehydro-4'-deoxy-*per*-*N*-Cbz-paromomycin **13** (82%, 0.94 mmol).

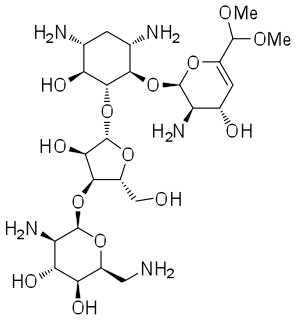
R_f = 0.6 in 20:5:3 $CHCl_3$/EtOAc/MeOH – UV active.

HRMS (ESI) calcd. for $C_{63}H_{71}N_5O_{23}$, M + Na^+ = 1288.44320, found 1288.44331 (0.04 ppm).

^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.02 (s, 1H), 7.47 – 7.11 (m, 25H), 6.21 (d, *J* = 9.47 Hz, 1H), 5.87 (s, 1H), 5.60 (s, 1H), 5.54 (s, 1H), 5.42 (d, *J* = 5.87 Hz, 1H), 5.36 (d, *J* = 3.96 Hz, 1H), 5.17 – 4.94 (m, 12H), 4.90 (d, *J* = 12.20 Hz, 1H), 4.84 (s, 1H), 4.76 (dd, *J* = 5.52,

5.08 Hz, 1H), 4.66 (s, 1H), 4.45 – 4.34 (m, 1H), 4.18 – 4.06 (m, 2H), 3.92 – 3.81 (m, 2H), 3.80 – 3.76 (m, 1H), 3.76 – 3.70 (m, 1H), 3.70 – 3.63 (m, 1H), 3.63 – 3.41 (m, 5H), 3.41 – 3.32 (m, 4H), 3.32 – 3.21 (m, 3H), 3.21 – 3.12 (m, 1H), 1.68 (d, J = 10.30 Hz, 1H), 1.49 – 1.33 (m, 1H).

^{13}C NMR (DMSO-d_6, 100 MHz) δ 186.8, 156.6 - 155.2 (5 C), 147.2, 137.1 - 136.8 (5 C), 128.3 - 127.2 (25 C), 124.5, 108.6, 98.2, 97.6, 85.4, 81.9, 78.0, 77.2, 73.6, 73.3, 72.6, 69.8, 67.3, 65.7, 65.4, 65.3, 65.1 (2 C), 62.4, 62.2, 54.0, 52.6, 51.1, 49.5, 41.1, 33.8.



14

4',5'-Dehydro-4'-deoxy-6',6'-dimethoxy-*per*-*N*-Cbz-paromomycin (14).

Aldehyde **13** (78 mg, 61.6 μmol) was dissolved in anhydrous MeOH (5 mL), treated with trimethylorthoformate (170 μL, 1.55 mmol) and TFA (9.5 μL, 0.123 mmol), and stirred at RT for 5 h, when no UV-active starting material was observed on TLC, the solution was neutralized with Et_3N (19 μL, 0.136 mmol). The volatiles were removed under vacuum to a residue, which was purified by ammoniacal silica gel chromatography to avoid acetal cleavage (4 → 6 % ammoniacal MeOH in DCM), to yield 4',5'-dehydro-4'-deoxy-6',6'-dimethoxy-*per*-*N*-Cbz-paromomycin **S6** (67.1 mg, 83%, 51 μmol).

R_f = 0.65 in 20:5:3 $CHCl_3$/EtOAc/MeOH.

HRMS (ESI) calcd. for $C_{65}H_{77}N_5O_{24}$, M + Na^+ = 1334.48507, found 1334.48620 (0.42 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 7.41 – 7.16 (m, 25H), 5.54 (s, 1H), 5.15 – 4.86 (m, 12H), 4.56 (s, 1H), 4.21 (d, J = 7.28 Hz, 1H), 4.17 – 4.08 (m, 2H), 3.99 – 3.86 (m, 3H), 3.85 – 3.79 (m, 2H), 3.72 (d, J = 11.58 Hz, 1H), 3.63 – 3.38 (m, 5H), 3.37 – 3.30 (m, 3H), 3.30 – 3.25 (m, 2H), 3.21 (s, 6H), 2.00 (d, J = 12.14 Hz, 1H), 1.40 – 1.28 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 159.3 - 158.4 (5 C), 147.3, 138.3 - 138.0 (5 C), 129.6 - 128.6 (25 C), 111.0, 104.9, 101.8, 100.3, 99.2, 86.7, 83.6, 79.5, 78.7, 76.0, 75.4, 74.6, 71.6, 69.3, 68.1 - 67.5 (5 C), 65.0, 63.6, 55.8, 54.9, 54.2, 53.0, 52.9, 51.4, 42.6, 35.3.

Approx. 7 to 10 mL of ammonia was condensed into a two-neck flask equipped with a cold finger condenser at -78 °C. A solution of acetal intermediate **S6** (51 mg, 38.8 μmol) in anhydrous THF (1 mL) was added to the ammonia solution, followed by a drop of *t*BuOH. Approx. 20 to 30 mg (~1 mmol) of sodium metal were added to the mixture, which was stirred vigorously at -78 °C until the reaction turned deep blue. After 5 min, LRMS analysis indicated complete removal of the protecting groups, and the reaction was quenched with excess AcOH (100 μL). The ammonia was slowly evaporated by bubbling argon at RT, to give a white residue of salts, which was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.5) and purified by column chromatography using the same solvent system, increasing the proportion of ammonia gradually to a 2:3:1 mixture. The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was dissolved in a minimum volume of water and freeze-dried. The dry residue obtained was redissolved in a minimum of water, at which point insoluble traces of silica were observed, and were removed by filtration of the solution through a 0.45 μm syringe filter. Finally, freeze-drying the filtrate yielded 22 mg of the title compound 4',5'-dehydro-4'-deoxy-6',6'-dimethoxy-paromomycin **14** (34.3 μmol, 88%), as a white cotton-like solid. For characterization purposes, the aminoglycoside was redissolved in a minimum volume of water, treated with AcOH (20 μL) and freeze-dried to provide the aminoglycoside acetate salt, as a yellow solid.

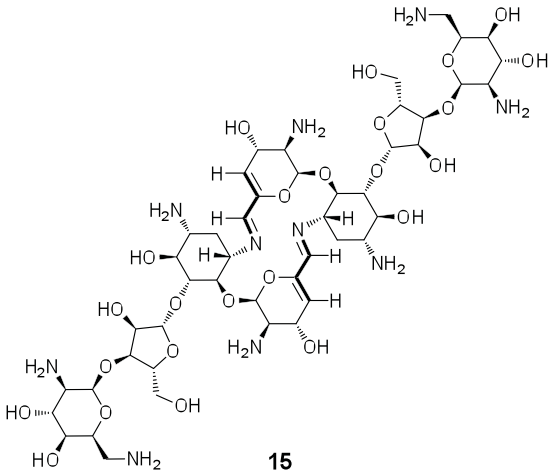
$[\alpha]^{22}_D$ +71.5º (*c* 0.30 in MeOH).

R_f = 0.2, 2:3:1 $CHCl_3$/MeOH/NH_4OH. HRMS (ESI) calcd. for $C_{25}H_{47}N_5O_{14}$, M + H^+ = 642.32020, found 642.31923 (-0.72 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 5.50 (d, *J* = 1.91 Hz, 1H), 5.31 (d, *J* = 3.91 Hz, 1H), 5.18 (s, 1H), 5.18 (s, 1H), 4.72 (s, 1H), 4.41 (dd, *J* = 6.33, 5.19 Hz, 1H), 4.31 (dd, *J* = 4.55, 4.08 Hz, 1H), 4.25 (dd, *J* = 4.82, 2.16 Hz, 1H), 4.21 – 4.18 (m, 1H), 4.12 (dd, *J* = 3.23, 2.79 Hz, 1H), 4.04 (ddd, *J* = 6.66, 4.51, 3.91 Hz, 1H), 3.97 (dd, *J* = 10.08, 9.32 Hz, 1H), 3.78 (dd, *J* = 12.38, 3.37 Hz, 1H), 3.72 (dd, *J* = 9.53, 9.03 Hz, 1H), 3.71 – 3.69 (m, 1H), 3.66 (dd, *J* = 12.41, 4.80 Hz, 1H), 3.64 – 3.61 (m, 1H), 3.58 (dd, *J* = 10.22, 9.51 Hz, 1H), 3.48 – 3.46

(m, 1H), 3.41 – 3.36 (m, 1H), 3.34 (s, 3H), 3.33 (s, 3H), 3.31 (dd, *J* = 7.02, 6.57 Hz, 1H), 3.24 (dd, *J* = 13.56, 3.67 Hz, 1H), 3.24 – 3.19 (m, 1H), 2.39 – 2.33 (m, 1H), 1.81 (s, 15H), 1.77 – 1.70 (m, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 180.9 (AcOD), 147.0, 109.7, 101.8, 101.3, 96.2, 95.3, 82.9, 81.1, 78.8, 75.4, 73.2, 71.9, 70.1, 67.6, 67.1, 61.8, 60.3, 55.0, 54.7, 51.7, 50.7, 49.6, 48.1, 40.3, 27.9, 22.9 (AcOD).



15

3-6'-*Bis*-imino-(4',5'-dehydro-3',4'-dideoxy-paromomycin) dimer (15)

The 4',5'-dehydro-3'-deoxy-paromomycin dimer **15** was generated under identical conditions as *aminoglycoside 66-40C* (**3**). Applying 0.5 N H_2SO_4 for 15 min, followed by sat. $Ba(OH)_2$, filtration and freeze-drying gave a fluffy cotton-like solid of crude dimer **15**. This residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:1) and purified by silica gel column chromatography using the same solvent system, increasing ammonia gradually to a 2:3:2.5 mixture. Evaporation, filtration (0.45 μm) and freeze-drying of the collected fractions yielded 12.3 mg of the title compound 3-6'-*bis*-imino-(4',5'-dehydro-3',4'-dideoxy-paromomycin) dimer **15** (62%, 10.6 μmol), as a light yellow cotton-like solid.

$[\alpha]^{22}_D$ +29.0º (*c* 0.20 in H_2O).

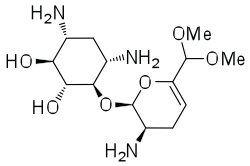
R_f = 0.2, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

HRMS (ESI) calcd. for $C_{46}H_{78}N_{10}O_{24}$, M + H^+ = 1155.52632, found 1155.52361 (-1.17 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 7.52 (s, 2H), 5.78 (d, *J* = 3.01 Hz, 2H), 5.56 (d, *J* = 1.99 Hz, 2H), 5.33 (s, 2H), 5.23 (d, *J* = 1.38 Hz, 2H), 4.59 (dd, *J* = 10.16, 1.73 Hz, 2H), 4.54 (dd, *J*

= 7.67, 4.50 Hz, 2H), 4.46 (d, *J* = 4.48 Hz, 2H), 4.27 (ddd, *J* = 6.07, 3.96, 1.14 Hz, 2H), 4.17 – 4.12 (m, 4H), 3.89 – 3.84 (m, 4H), 3.79 (t, *J* = 9.17, 9.17 Hz, 2H), 3.75 – 3.74 (m, 2H), 3.72 (dd, *J* = 12.41, 4.88 Hz, 2H), 3.67 (dd, *J* = 10.15, 9.45 Hz, 2H), 3.52 – 3.50 (m, 2H), 3.35 (dd, *J* = 13.65, 6.58 Hz, 2H), 3.33 – 3.29 (m, 6H), 3.24 (ddd, *J* = 12.77, 10.93, 3.80 Hz, 2H), 2.10 (ddd, *J* = 12.56, 4.39, 3.89 Hz, 2H), 1.89 – 1.82 (m, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 159.9, 145.2, 116.1, 110.4, 95.9, 94.8, 85.8, 80.5, 78.2, 74.7, 73.4, 72.8, 70.1, 67.6, 67.2, 65.0, 61.6, 59.9, 52.8, 50.7, 50.2, 40.3, 30.8.



19

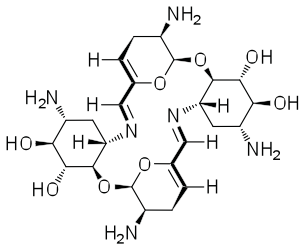
6',6'-Dimethoxy-sisamine (19)

Acetal intermediate 1,3,2'-triazido-6',6'-dimethoxy-sisamine (**18**)[1] (200 mg, 0.486 mmol) was dissolved in MeOH (10 mL), added $NH_4OH_{(aq)}$ (50 µL, 28% liquor) and PMe_3 (1.0 M in THF, 7.3 mL, 15 equiv., 7.3 mmol) upon which mild bubbling was observed. The reaction was stirred 10 h, when LRMS analysis indicated completion of the deblocking reaction. The volatiles were removed under vacuum, leaving a residue which was redissolved in 10% ammoniacal MeOH in DCM for purification by column chromatography (12 → 20 % ammoniacal MeOH in DCM). The fractions containing aminoglycoside were pooled, evaporated under vacuum to a wet residue, which was redissolved in water, filtered through a 0.45 µm syringe filter and freeze-dried to yield 117 mg of 6',6'-dimethoxy-sisomamine **8** (0.351 mmol, 72%), as a white cotton-like solid. NMR and MS analysis were consistent with the product previously reported using the Birch reduction protocol.[1]

R_f = 0.2, 30% ammoniacal MeOH/$CHCl_3$.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.30 (d, *J* = 2.1 Hz, 1H), 5.18 (dd, *J* = 5.1, 2.1 Hz, 1H), 4.69 (s, 1H), 3.48 – 3.36 (m, 1H), 3.40 (s, 6H), 3.17 (q, *J* = 9.3 Hz, 2H), 3.12 (ddd, *J* = 10.2, 6.3, 2.2 Hz, 1H), 2.73 (tdd, *J* = 11.1, 5.9, 4.5 Hz, 2H), 2.26 (dt, *J* = 17.2, 5.8 Hz, 1H), 2.09 – 1.95 (m, 2H), 1.30 – 1.16 (m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 143.2, 102.2, 100.8, 100.1, 85.7, 76.8, 75.4, 54.4, 54.3, 49.9, 49.0, 46.1, 34.6, 23.6.



21

3-6'-*Bis*-imino-sisamine dimer (21)[1] and crystallization procedure.

Sisamine dimer **21** was generated under identical conditions as aminoglycoside *66-40C* (**3**).[1] Using intermediate **19** (25 mg, 75.0 μmol), incubating with 1 mL 0.5 N H_2SO_4 for 15 min liberated sisamine 6'-aldehyde **20** (HRMS (ESI) calcd. for $C_{12}H_{22}N_3O_5$, $M + H^+$ = 288.15540, found 288.15604 (2.23 ppm)). Neutralization with sat. $Ba(OH)_2$, filtration and freeze-drying gave a cotton-like solid of crude dimer **21**. The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.5) and purified by silica gel column chromatography using the same solvent system, increasing ammonia liquor gradually to a 2:3:0.75 mixture. Evaporation, filtration (0.45 μm filter) and freeze-drying of the collected fractions yielded 18.3 mg of 3-6'-*bis*-imino-sisamine dimer **21** (90%, 34.0 μmol), as a light yellow cotton-like solid. NMR and MS analysis of the product were identical to those previously reported.[1]

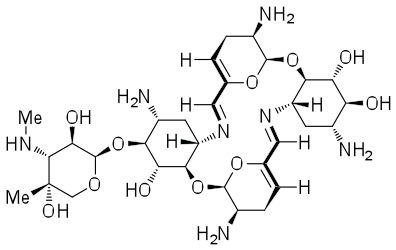
Crystallization conditions: sisamine dimer **21** (1 mg, 1.86 μmol) was dissolved in water (100 μL) in a 2 mL vial, then added excess $(NH_4)_2SO_4$ in solution (150 mM, 25 μL, 2 equiv., 3.7 mmol). The sample vials were incubated at RT within a closed tank of ethanol for 48 to 72 hours, leading to formation of long needles. Alternatively, using a carefully titrated amount of H_2SO_4 (74 mM solution, 25 μL, 1 equiv., 1.86 μmol) also led to successful crystallization under these conditions. Crystals were stored in their wet ethanolic crystallization liquor at 4 ºC until X-ray acquisition was performed. Elemental analysis of dry crystals of **21** indicated enrichment to approx. 1 equiv. of sulfur.

R_f = 0.2, 30% ammoniacal MeOH/$CHCl_3$.

^{1}H NMR (D_2O, 400 MHz) δ 7.45 (s, 2H), 5.57 – 5.44 (m, J = 8.6 Hz, 4H), 3.70 (t, J = 9.1 Hz, 2H), 3.62 – 3.52 (m, 2H), 3.45 – 3.32 (m, 2H), 3.21 (td, J = 11.4, 4.1 Hz, 2H), 3.09 –

2.99 (m, 2H), 2.92 (td, J = 12.2, 3.3 Hz, 2H), 2.37 – 2.15 (m, 4H), 2.10 – 1.96 (m, 2H), 1.81 – 1.64 (m, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 160.8, 145.2, 115.1, 98.4, 79.5, 76.9, 75.6, 65.5, 50.0, 46.2, 33.7, 23.8.



22

Cross-over product hetero-dimer 22.

Sisomicin 6'-dimethyl acetal **8** (20 mg, 40 μmol) and its sisamine analog **19** (13.5 mg, 40 μmol) were combined, and treated with 0.5 M HCl (1 mL) for 15 min. The solution was cooled to 0 ºC, and added dropwise sat. $Ba(OH)_2$ (10 mL of approx. 0.05 N solution) until pH 7 to 8. The resulting white suspension of $BaSO_4$ was removed by filtration through a 0.45 μm syringe filter. The filtrate was freeze-dried to generate a white cotton-like solid. The residue was dissolved in a freshly prepared solution of $CHCl_3$/MeOH/NH_4OH (2:3:1) and purified by preparative TLC (0.5 mm thick, E. Merck silica precoated plate) using the same solvent system. The middle UV-active band corresponding to cross-over product **22** was collected, and the silica was washed with $CHCl_3$/MeOH/NH_4OH (2:3:2, 100 mL). The filtrate was evaporated to a residue which was purified by silica gel column chromatography using same solvent system, increasing ammonia in the mixture gradually from 2:3:0.5 to 2:3:1 ratio. The fractions containing aminoglycoside were identified by TLC, collected and evaporated under vacuum to furnish a wet residue, which was dissolved in a minimum volume of water and freeze-dried. The dry residue obtained was redissolved in 1 mL of water, then filtered through a 0.45 μm syringe filter. Freeze-drying of the filtrate yielded 14 mg of cross-over heterodimer **22** (20 μmol, 50% theor.), as a light yellow cotton-like solid.

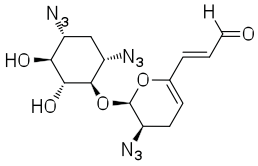
$[\alpha]^{22}_D$ +30.0º (c 0.19 in H_2O).

R_f = 0.4, 2:3:1 $CHCl_3$/MeOH/NH_4OH.

HRMS (ESI) calcd. for $C_{31}H_{51}N_7O_{11}$, M + H^+ = 698.37193, found 698.36943 (-3.58 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 7.51 – 7.49 (s, 2H), 5.56 – 5.51 (m, 4H), 5.19 (d, *J* = 4.0 Hz, 1H), 4.14 (d, *J* = 12.7 Hz, 1H), 4.00 (dd, *J* = 10.8, 3.9 Hz, 1H), 3.76 (dt, *J* = 17.7, 9.2 Hz, 2H), 3.71 (t, *J* = 9.3 Hz, 1H), 3.61 (t, *J* = 9.3 Hz, 1H), 3.47 (t, *J* = 9.6 Hz, 1H), 3.44 – 3.39 (m, 2H), 3.28 – 3.21 (m, 2H), 3.11 – 3.01 (m, 3H), 3.00 – 2.94 (m, 2H), 2.73 (s, 3H), 2.37 – 2.24 (m, 4H), 2.07 (dt, *J* = 12.9, 4.2 Hz, 1H), 1.99 (dt, *J* = 13.1, 4.3 Hz, 1H), 1.82 – 1.69 (m, 2H), 1.31 (s, 3H).

^{13}C NMR (D_2O, 175 MHz) δ 160.80, 160.67, 145.29 (2C), 113.1, 113.0, 101.1, 95.0, 94.9, 84.3, 79.7, 79.6, 76.1, 75.0, 72.9, 72.0, 70. 0, 67.8, 66.3, 65.0, 64.9, 63.3, 62.4, 50.3 (2C), 46.3 (2C), 34.5, 31.4 (2C), 20.9.



23

6'-Acrylaldehydo-1,3,2'-triazido-sisamine (23).

Triethylphosphonoacetate (60 µL, 0.3 mmol) was dissolved in anhydrous THF (5 mL), cooled to 0 ºC, treated with *n*BuLi (2.0 M in cyclohexane, 152 µL, 0.3 mmol), followed by aldehyde **17** (170 mg, 0.465 mmol), and stirred 15 min, when TLC indicated complete consumption of starting aldehyde (R_f = 0.6 *vs* 0.9, 2:1, EtOAc/hexanes). The reaction was quenched with sat. NH_4Cl (1 mL), diluted with EtOAc and washed with sat. NaCl. The organic fraction was dried over Na_2SO_4, filtered and evaporated to a residue. Purification by column chromatography (30 % EtOAc in hexanes) yielded 159 mg of (*E*)-methyl 6'-acrylate-1,3,2'-triazido-sisamine **S7** (0.365 mmol, 78%), as a white amorphous solid.

R_f = 0.9, 2:1, EtOAc/hexanes, UV-active.

HRMS (ESI) calcd. for $C_{16}H_{21}N_9O_6$, M + Na^+ = 458.1507, found 458.1510 (-0.65 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.00 (d, *J* = 15.4 Hz, 1H), 6.19 (d, *J* = 15.4 Hz, 1H), 5.69 (s, 1H), 5.31 (d, *J* = 2.6 Hz, 1H), 4.20 (q, *J* = 7.1 Hz, 2H), 3.73 – 3.62 (m, 1H), 3.62 – 3.54 (m, 3H), 3.46 – 3.24 (m, 4H), 2.59 (dd, *J* = 17.3, 11.4 Hz, 1H), 2.46 (dt, *J* = 17.5, 6.0 Hz, 1H), 2.21 (dt, *J* = 6.9, 3.8 Hz, 1H), 1.44 – 1.33 (m, 1H), 1.29 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 167.2, 146.2, 138.1, 117.9, 107.9, 96.8, 81.6, 76.3, 75.9, 60.7, 60.2, 59.9, 55.4, 32.6, 22.5, 14.4.

Ester **S7** (142 mg, 0.326 mmol) was dissolved in THF (10 mL), cooled to -15 ºC and treated with DIBAL (1.0 M in hexanes, 1.0 mL, 0.98 mmol) for 15 min. When no UV-active material was observed on TLC, the reaction was quenched by neutralization with a few drops of AcOH, diluted with EtOAc and washed twice with sat. Rochelle's salt solution and subsequently with sat. NaCl. The organic fraction was dried over Na_2SO_4, filtered and evaporated to a residue under vacuum. Purification by column chromatography (40 % EtOAc in hexanes), yielded 94.5 mg of 1,3,2'-triazido-6'-propenol-sisamine **S8** (68%, 0.186 mmol), as a white amorphous solid.

R_f = 0.3, 1:1 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{14}H_{19}N_9O_6$, M + H^+ = 394.1582, found 394.1584 (0.52 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.17 (td, *J* = 15.44, 5.43, 5.43 Hz, 1H), 6.03 (d, *J* = 15.57 Hz, 1H), 5.60 (d, *J* = 2.32 Hz, 1H), 4.91 (dd, *J* = 5.44, 2.41 Hz, 1H), 4.27 – 4.18 (m, 2H), 3.77 (s, 1H), 3.66 – 3.60 (m, 1H), 3.60 – 3.58 (m, 1H), 3.58 – 3.54 (m, 1H), 3.44 – 3.36 (m, 2H), 3.36 – 3.33 (m, 1H), 3.33 – 3.27 (m, 1H), 2.53 (dd, *J* = 16.18, 11.56 Hz, 1H), 2.39 (td, *J* = 16.82, 6.07, 6.07 Hz, 1H), 2.26 – 2.18 (m, 1H), 1.83 – 1.75 (m, 1H), 1.45 – 1.34 (m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz) δ 146.6, 128.1, 124.8, 99.6, 97.0, 82.1, 76.1, 75.6, 63.0, 60.0, 59.6, 56.0, 32.5, 21.9.

Alcohol **S8** (53.2 mg, 0.137 mmol) was dissolved in anhydrous DCM (5 mL), treated with MnO_2 (5 μ ~85%, 330 mg, 3.82 mmol) and stirred at RT for 1 hr. The reaction was filtered through Celite^{TM}, washing with 1% MeOH/EtOAc. The filtrate and washings were combined, and evaporated to a residue under vacuum. Purification by chromatography (20 → 30 % EtOAc in DCM) yielded 38.6 mg of the title compound 6'-acrylaldehydo-1,3,2'-triazido-sisamine **23** (73%, 98.6 μmol), as a white amorphous solid.

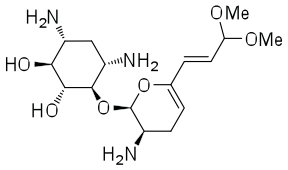
R_f = 0.5, 1:1 EtOAc/hexanes, UV-active.

HRMS (ESI) calcd. for $C_{14}H_{17}N_9O_6$, M + Na^+ = 414.1245, found 414.1243 (-0.56 ppm).

^{1}H NMR ($CDCl_3$, 400 MHz)) δ 9.60 (d, *J* = 7.96 Hz, 1H), 6.82 (d, *J* = 15.39 Hz, 1H), 6.46 (dd, *J* = 15.37, 7.94 Hz, 1H), 5.71 (d, *J* = 2.43 Hz, 1H), 5.46 (dd, *J* = 5.64, 3.01 Hz, 1H), 3.65 (ddd, *J* = 11.16, 6.67, 2.55 Hz, 1H), 3.60 (d, *J* = 3.35 Hz, 1H), 3.59 – 3.56 (m, 1H), 3.41 – 3.38 (m, 1H), 3.38 – 3.35 (m, 1H), 3.35 – 3.29 (m, 1H), 2.87 (s, 1H), 2.63 (ddd, *J* =

17.72, 11.29, 3.00 Hz, 1H), 2.54 (dd, *J* = 15.05, 9.07 Hz, 1H), 2.23 (td, *J* = 13.24, 4.27, 4.27 Hz, 1H), 1.61 (s, 1H), 1.45 – 1.34 (m, 1H).

^{13}C NMR ($CDCl_3$, 100 MHz)) δ 193.5, 146.3, 145.1, 127.7, 110.0, 96.8, 81.7, 76.1, 75.7, 60.1, 59.7, 55.2, 32.4, 22.7.



24

6',6'-Dimethoxypropenyl-sisamine (24).

Aldehyde **23** (47 mg, 0.120 mmol) was dissolved in anhydrous MeOH (5 mL), treated with trimethylorthoformate (380 µL, 3.47 mmol) and TFA (18.5 µL, 0.24 mmol), and stirred at RT for 3 h, when no UV-active starting material remained on TLC, the reaction was neutralized with Et_3N (37 µL, 0.265 mmol). The solvents were removed under vacuum to a residue and purified by chromatography on silica gel, which was neutralized with Et_3N (20 → 30 % EtOAc in hexanes with 1% Et_3N). The collected fractions were evaporated to a minimum volume, which was added dropwise to stirring pentane. The precipitate was collected and dried under vacuum, to yield 44 mg of 1,3,2'-triazido-6',6'-dimethoxypropenyl-sisamine **S9** (84%, 0.101 mmol), as a white amorphous solid.

R_f = 0.8, 1:1 EtOAc/hexanes.

HRMS (ESI) calcd. for $C_{16}H_{23}N_9O_6$, M + Na^+ = 460.1664, found 460.1666 (0.63 ppm).

^{1}H NMR (CD_3OD, 400 MHz) δ 6.12 (d, *J* = 15.64 Hz, 1H), 5.86 (dd, *J* = 15.71, 4.84 Hz, 1H), 5.82 (d, *J* = 2.16 Hz, 1H), 4.98 (dd, *J* = 4.99, 2.90 Hz, 1H), 4.83 (s, 1H), 3.58 (t, *J* = 9.46, 9.46 Hz, 1H), 3.49 (t, *J* = 9.20, 9.20 Hz, 1H), 3.42 (m, 2H), 3.34 (ddd, *J* = 12.30, 9.80, 4.47 Hz, 1H), 3.27 (s, 6H), 3.22 (dd, *J* = 9.67, 9.18 Hz, 1H), 2.47 (ddd, *J* = 16.69, 10.63, 2.36 Hz, 1H), 2.34 (td, *J* = 17.17, 5.76, 5.76 Hz, 1H), 2.08 (td, *J* = 12.98, 4.46, 4.46 Hz, 1H), 1.25 – 1.14 (m, 1H).

^{13}C NMR (CD_3OD, 100 MHz) δ 147.7 129.4, 125.9, 103.9, 103.2, 98.4, 81.9, 78.0, 77.8, 62.0, 61.8, 56.2, 53.0 (2C), 33.9, 23.2.

Dimethoxy acetal **S9** (30 mg, 68.6 µmol) was dissolved in MeOH (5 mL), treated with trimethylphosphine (1 M in THF, 1 mL, 1.0 mmol) and 28% ammonia liquor (50 µL), and

stirred overnight, when LRMS showed the molecular ion corresponding to the product indicating complete reduction of azide groups. The volatiles were evaporated under vacuum to a residue, which was purified by column chromatography (10 → 20% ammoniacal MeOH/$CHCl_3$) to yield 22.9 mg of the title compound 6',6'-dimethoxypropenyl-sisamine **24** (93%, 63.7 μmol), as a white cotton-like solid.

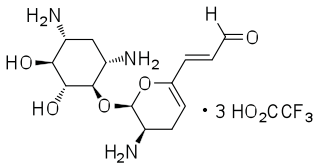
R_f = 0.6, 40% ammoniacal MeOH/$CHCl_3$.

$[\alpha]^{22}_D$ 0.95º (*c* 1.1 in MeOH).

HRMS (ESI) calcd. for $C_{16}H_{29}N_3O_6$, M + Na^+ = 382.1949, found 382.1948 (-0.16 ppm).

^{1}H NMR (CD_3OD, 700 MHz) δ 6.10 (dd, *J* = 15.70, 0.74 Hz, 1H), 5.81 (dd, *J* = 15.71, 4.89 Hz, 1H), 5.33 (d, *J* = 2.50 Hz, 1H), 4.99 (dd, *J* = 5.55, 2.70 Hz, 1H), 4.80 (d, *J* = 4.86 Hz, 1H), 3.31 – 3.28 (m, 2H), 3.25 (s, 3H), 3.25 (s, 3H), 3.06 – 3.03 (m, 1H), 3.01 (ddd, *J* = 10.99, 6.31, 2.51 Hz, 1H), 2.60 (ddd, *J* = 12.11, 10.02, 3.95 Hz, 1H), 2.56 (ddd, *J* = 12.62, 9.35, 4.24 Hz, 1H), 2.22 (td, *J* = 17.49, 5.93, 5.93 Hz, 1H), 2.10 (ddd, *J* = 17.50, 11.07, 2.31 Hz, 1H), 1.95 (td, *J* = 12.93, 4.18, 4.18 Hz, 1H), 1.24 – 1.18 (m, 1H).

^{13}C NMR (CD_3OD, 175 MHz) δ 147.0, 129.7, 125.6, 105.3, 104.0, 101.9, 88.1, 78.9, 77.9, 53.2, 53.2, 52.5, 51.6, 48.5, 36.8, 27.0.



25

6'-Acrylaldehydo-sisamine TFA salt (25).

Acetal **24** (7.4 mg, 20.6 μmol) was dissolved in 1 mL H_2O, added TFA (8 μL, 5 equiv. 0.1 mmol), stirred for 15 min and freeze-dried to yield 13.5 mg of 6'-acrylaldehydo-sisamine TFA salt **25** (quant.), as an light yellow amorphous solid.

HRMS (ESI) calcd. for $C_{14}H_{23}N_3O_5$, M + H^+ = 314.1710, found 314.17158 (1.69 ppm).

NB: In D_2O, this compound was observed as approx. 3:1 ratio of aldehyde and hydrate:

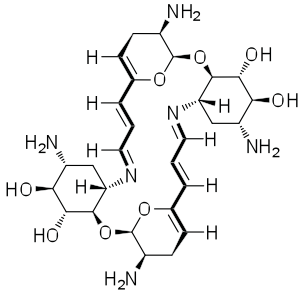
Major: ^{1}H NMR (D_2O, 700 MHz) δ 9.38 (d, *J* = 8.3 Hz, 1H), 7.06 (t, *J* = 9.2 Hz, 1H), 6.33 (dd, *J* = 15.5, 8.1 Hz, 1H), 5.68 (d, *J* = 1.8 Hz, 1H), 5.61 (t, *J* = 4.2 Hz, 1H), 3.88 (dd, *J* = 10.0, 9.6 Hz, 1H), 3.79 (td, *J* = 6.8, 1.9 Hz, 1H), 3.55 (t, *J* = 9.3 Hz, 1H), 3.46 (dd, *J* = 10.3, 9.5 Hz, 1H), 3.41 (ddd, *J* = 12.6, 10.4, 4.3 Hz, 1H), 3.21 (ddd, *J* = 12.4, 10.5, 4.3 Hz, 1H),

2.69 (dt, *J* = 19.4, 5.4 Hz, 1H), 2.46 (ddd, *J* = 19.5, 7.0, 3.8 Hz, 1H), 2.38 (dt, *J* = 12.5, 4.3 Hz, 1H), 1.83 – 1.76 (m, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 197.5, 162.9 (q, *J* = 35.6 Hz, TFA), 147.2, 145.9, 126.4, 116.2 (q, *J* = 291.5 Hz, TFA), 111.6, 96.3, 79.2, 74.6, 72.2, 49.5, 48.3, 46.1, 27.7, 23.1.

Minor: ^{1}H NMR (D_2O, 700 MHz) δ 8.35 (d, *J* = 10.5 Hz, 1H), 7.03 (d, *J* = 15.0 Hz, 1H), 6.42 (dd, *J* = 14.9, 10.4 Hz, 1H), 5.84 (dd, *J* = 5.9, 3.7 Hz, 1H), 5.63 – 5.59 (m, 1H), 4.02 – 3.94 (m, 2H), 3.62 (ddd, *J* = 9.1, 6.5, 2.2 Hz, 1H), 3.60 (t, *J* = 7.3 Hz, 1H), 3.55 – 3.50 (m, 1H), 3.27 – 3.20 (m, 1H), 2.64 – 2.55 (m, 2H), 2.50 – 2.42 (m, 1H), 1.95 – 1.88 (m, 1H).

^{13}C NMR (D_2O, 175 MHz) δ 171.7, 152.3, 145.3, 117.9, 117.5, 96.7, 82.1, 74.8, 71.9, 59.5, 49.3, 46.2, 28.2, 21.2.



26

3-6'-*Bis*-propenimino-sisamine dimer (26).

The expanded sisamine dimer (**26**) was generated under identical conditions as *aminoglycoside 66-40C* (**3**). Using acetal intermediate **24** (22.9 mg, 63.7 μmol), incubating with 1 mL 0.5 N H_2SO_4 for 15 min liberated sisamine 6'-acrylaldehyde **25**. The solution was neutralized with sat. $Ba(OH)_2$, filtered and lyophilized to give a fluffy cotton-like crude dimer **26**. The residue was dissolved in $CHCl_3$/MeOH/NH_4OH (2:3:0.25) and purified by silica gel column chromatography using the same solvent system, increasing ammonia liquor gradually to a 2:3:0.5 mixture. Evaporation, filtration (0.45 μm filter) and freeze-drying of the collected fractions yielded 18.7 mg of 3-6'-*bis*-propenimino-sisamine dimer **26** (97%, 63.3 μmol), as a light yellow cotton-like solid.

Crystallization conditions: the water/ethanol diffusion procedure employed for sisamine dimer **21** was identically applied to extended dimer **26** (1 mg, 1.69 μmol), dissolved in H_2O (100 μL) in a 2 mL vial, was added 2 equiv. $(NH_4)_2SO_4$ in soltion (150 mM, 23 μL, 3.4 μmol). The sample vials were incubated at room temperature within a closed tank of

ethanol for 48 to 72 hours, leading to formation of long needles. Crystals were stored in their wet ethanolic crystallization liquor at 4 ºC until X-ray acquisition was performed.

R_f = 0.8, 2:3:2 $CHCl_3$/MeOH/NH_4OH.

$[\alpha]^{22}_D$ -373.6º (*c* 0.4 in H_2O).

HRMS (ESI) calcd. for $C_{28}H_{42}N_6O_8$, M + Na^+ = 613.2956, found 613.2950 (-1.01 ppm).

^{1}H NMR (D_2O, 700 MHz) δ 7.75 (d, *J* = 9.37 Hz, 2H), 6.29 (d, *J* = 15.48 Hz, 2H), 6.16 (dd, *J* = 15.50, 9.38 Hz, 2H), 5.34 (d, *J* = 2.54 Hz, 2H), 5.21 (dd, *J* = 6.16, 2.78 Hz, 2H), 3.66 (dd, *J* = 9.44, 8.97 Hz, 2H), 3.56 (dd, *J* = 9.53, 9.04 Hz, 2H), 3.44 (dd, *J* = 10.11, 9.54 Hz, 2H), 3.29 – 3.26 (m, 2H), 3.25 (ddd, *J* = 11.78, 9.47, 4.90 Hz, 2H), 3.06 (ddd, *J* = 12.66, 10.26, 3.99 Hz, 2H), 2.36 (td, *J* = 17.63, 6.17, 6.17 Hz, 2H), 2.26 (ddd, *J* = 17.51, 11.52, 1.96 Hz, 2H), 2.02 (td, *J* = 12.93, 4.31, 4.31 Hz, 2H), 1.73 – 1.66 (m, 2H).

^{13}C NMR (D_2O, 175 MHz) δ 166.2, 145.7, 138.5, 125.1, 107.1, 99.3, 85.0, 76.1, 73.9, 65.8, 50.2, 46.5, 32.9, 23.1.

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