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Bastian, Andreas A.; Warszawik, Eliza M.; Panduru, Praveen; Arenz, Christoph; Herrmann, Andreas

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Supporting Information

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Regioselective Diazo-Transfer Reaction at the C3-Position of the 2-Desoxystreptamine Ring of Neamine Antibiotics

**Andreas A. Bastian,^[a] Eliza M. Warszawik,^[a] Praveen Panduru,^[a] Christoph Arenz,^[b] and
Andreas Herrmann*^[a]**

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Content:

General.....	SI 2
Materials.....	SI 2
General procedures.....	SI 3
Analytical data.....	SI 5
¹ H-NMR- and HSQC spectra.....	SI 8
High performance liquid chromatography.....	SI 16
High resolution mass spectroscopic data.....	SI 17
References.....	SI 21

General

¹H-NMR- and heteronuclear single-quantum correlation (HSQC) spectra and Attached Proton Test (APT) were recorded on a Varian Unity Inova (500 MHz for ¹H-NMR, ¹³C-NMR and HSQC) and Oxford AS400 (400 MHz for ¹H-NMR and 100.6 MHz and ¹³C-NMR) NMR spectrometer at 25 °C. High resolution mass spectrometry (HRMS) was carried out on a LTQ ORBITRAP XL instrument (Thermo Scientific) employing electron impact ionization in positive ion mode (EI+). Chromatographic separations were carried out on a *Shimadzu* VP series HPLC modular system (DGU-14A3 Online Vacuum-Degasser, two LC-20 AT pumps, SIL-20A auto sampler, CTP-20 A column oven, RID-10 refractive detector, FRC-10 A fraction collector and Shimadzu LCsolution software). HPLC purification was performed with a Waters Spherisorb ODS-2 C₁₈ analytical (250 x 4.6 mm) and semi-preparative column (250 x 10mm) (spherical particles of 5 µm and 80 Å pore size) using isocratic elution at 40 °C. A pH-meter (Hanna Instruments pH 209) equipped with a glass combination electrode was used for pH adjustments of the reaction buffers.

Materials

All chemicals and reagents were purchased from commercial suppliers and used without further purification, unless otherwise noted. Neomycin B sulfate salt (VETRANAL[®]), paromomycin sulfate salt (98 %), ribostamycin sulfate salt, apramycin sulfate salt, amikacin sulfate salt, sulfonyl chloride (97 %), sodium azide (95 %), acetonitrile (99.8 %), imidazole (99 %) and methanolic 3N HCl solution were purchased from *Sigma Aldrich* and used as received. For HPLC purification heptafluorobutyric acid (HFBA) (*Fluka*, puriss. p.a., for ion chromatography) and acetone (*Sigma-Aldrich*, HPLC grade) were used. Ultrapure water (specific resistance > 18.4 MΩ cm) was obtained by Milli-Q water purification system (*Sartorius*[®]). Neamine hydrochloride was synthesized according to known procedure.¹

General procedures

Synthesis of diazo-transfer reagent Imidazole-1-sulfonyl azide hydrochloride **7**.²

Sulfonyl chloride (1.6 mL, 20 mmol) was added dropwise to an ice-cooled suspension of sodium azide (1.3g, 20 mmol) in acetonitrile (20 mL) and the mixture was stirred overnight at room temperature. Then imidazole (2.6g, 38 mmol) was added portion-wise to the ice-cooled mixture and the resulting slurry was stirred for additional 3h at room temperature. The reaction mixture was diluted with ethyl acetate (40 mL), washed twice with water (40 mL) and then twice with sat. aqueous NaHCO₃ solution (40mL), dried over MgSO₄ and filtered. The filtrate was cooled in an ice-bath and a 3 M HCl methanolic solution (10 mL) was added dropwise to precipitate the product. Finally, the filter cake was washed three times with ethyl acetate (10 mL) to obtain **7** as colorless hydrochloride salt. Yield: 1.9 g (9.1 mmol, 45% yield). ¹H-NMR (400 MHz, D₂O, 25 °C, TMS): δ = 9.53 ppm (s, 1H, H-2), 8.07 ppm (s, 1H, H-5), 7.67 ppm (s, 1H, H-4). ¹³C-NMR (100.6 MHz, D₂O, 25 °C, TMS): δ = 137.6 ppm, 122.6 ppm, 120.18 ppm. HRMS (EI+) (*m/z*): found 174.0078 [M-Cl]⁺, calc. 174.0080 [M-Cl]⁺.

Procedures for regioselective azide introduction in aminoglycoside antibiotics using imidazole-1-sulfonyl azide hydrochloride.

Scheme S1: Regioselective transformation of neomycin B **3** in C3 position of 2-deoxystreptamine (2-DOS) ring.

a) Transformation of neomycin B **3** in 10 mM sodium phosphate buffer at pH = 7 (μ mol range)

After neomycin B sulfate (22 μ mol) was dissolved in 10 mM sodium phosphate buffer (15 mL, pH 7.4), a 48 mM aqueous solution of diazo-transfer reagent **7.HCl** (3.7 mL, 16 equiv.), which was adjusted to pH 8.0 by adding aq. 2M NaOH solution, was added into the solution of the antibiotic and the reaction mixture (pH = 7) was stirred for 18h at room temperature. The reaction was quenched by adding aqueous 7 wt % ethylamine solution (1.8 mL). After incubating at room temperature for 30 min the reaction mixture was freeze dried. Then the crude mixture was dissolved in water (3 mL) and each 60 μ L-fraction was purified by HPLC using a Waters Spherisorb ODS-2C₁₈ analytic column (water/acetone 1:0.9 containing 12.1mM HFBA) at a flow rate of 1 mL/min at 40°C to afford the antibiotic derivatives **8** as heptafluorobutyric acid salt.

b) Transformation of neomycin B **3** in a non buffered aqueous solution at pH 6.6 (μmol range)

Into a solution of aminoglycoside antibiotic (22 μmol) in water (15 mL) a solution of imidazole-1-sulfonyl azide hydrochloride **7.HCl** (74 mg, 0.35 mmol, 16 equiv.) in water (2.5 mL) was added and the reaction was started by adjusting a pH of 6.6 using aq. 2M NaOH solution. The reaction mixture was stirred for 40 hours and washed twice with dichloromethane to remove the excess of diazo-transfer reagent. After removing of dichloromethane residues under reduced pressure each 100 μL -fraction was purified by HPLC using a Waters Spherisorb ODS-2C₁₈ semi-preparative column (water/acetone 1:0.9 containing 12.1mM HFBA) at a flow rate of 4.75 ml/min at 40°C to afford the mono-azido antibiotic derivatives as heptafluorobutyric acid salt for mass spectrometry and NMR analysis.

c) Synthesis of 3-C-azido neomycin B **8** in gram scale

A solution of neomycin B sulfate (5.0 g, 5.5 mmol) in water (250mL) was combined with a solution of imidazole-1-sulfonyl azide hydrochloride (9.22 g, 44 mmol, 8 eq.) in water (250mL). A pH = 6.6 of the reaction mixture was adjusted by addition of aq. 2M NaOH solution (approx. 17 - 18 mL) and stirred at room temperature for 40 hours before washed three times with dichloromethane (300 mL). The aqueous solution was concentrated under reduced pressure to remove dichloromethane residues and freeze dried. The obtained crude mixture was purified by column chromatography using the upper layer of a biphasic chloroform/methanol/aq.17% ammonia 2:1:1 mixture. Finally the product was dissolved in water and filtered over celite to obtain **8** (2.9 g, 4.52 mmol, 82 % yield).

Scheme S2: Regioselective transformation in C3 position of 2-desoxystreptamine (2-DOS) ring in neamine antibiotics.

d) Synthesis of Antibiotic Derivatives **9-13** in aqueous solution at pH 6.6, at mmol scale and 9-fold higher concentrations.

A solution of antibiotic sulfate salt (0.22 mmol) in water (10 mL) was combined with a solution of imidazole-1-sulfonyl azide hydrochloride (737 mg, 3.52 mmol, 16 eq.) in water (10 mL). A pH value of 6.6 of the reaction mixture was adjusted by addition of aq. 2M NaOH solution and stirred at room temperature for 40 hours before washed twice with dichloromethane (20 mL). The aqueous solution was concentrated under reduced pressure to remove dichloromethane residues and freeze dried. The obtained crude mixture was purified by column chromatography using homogeneous dichloromethane/methanol/aq. 25% ammonia (from 2:3:1 to 2:3:2 v/v/v) mixture. After evaporation of the solvent the residue was resolved in water (3 ml) and traces of silica were removed by filtration through 0.45 μm syringe filters. Lyophilization yielded the 3-C-azido aminoglycoside antibiotic.

Analytical Data

3-C-azido neomycin B (8). The title compound was prepared according to the general procedure

described above (c, 5.5 mmol scale). Derivative **8** was obtained as a white solid. For the measurement of regioselectivity and the characterization of the compound $^1\text{H-NMR}$, HSQC and APT spectra were recorded and electrospray ionization (ESI)-MS was employed. HPLC: $R_t = 7.6$ min, 86 % conversion. Yield: 2.9 g (4.52 mmol, 82 %). TLC: $R_f = 0.47$ (upper layer of $\text{CHCl}_3/\text{MeOH}/17\%\text{NH}_3$ 2:1:1 v/v/v). NMR analysis of antibiotic HFBA salt: $^1\text{H-NMR}$ (500 MHz, D_2O , 25 °C, TMS): δ (ppm) = 5.72 (d, $J = 4$ Hz, 1H, 1-H'), 5.33 (d, $J = 1.5$ Hz, 1H, 1-H''), 5.26 (s, 1H, 1-H'''), 4.40 (t, $J =$

5.5 Hz, 1H, 3-H'), 4.37 (dd, $J = 4.5$ Hz, $J = 2$ Hz, 1H, 2-H'), 4.28 (t, $J = 5$ Hz, 1H, 5-H'), 4.27 (m, 1H, 5-H'), 4.21 (m, 2H, 3-H'', 4-H'), 3.91-3.85 (m, 2H, 5-H'', 3-H'), 3.80-3.75 (m, 3H, 4-H'', 5-H, 3-H), 3.72-3.67 (m, 2H, 4-H, 5-H'), 3.58 (t, $J = 10$ Hz, 1H, 6-H), 3.56 (s(br), 1H, 2-H'''), 3.47-3.33 (m, 5H, 6-H', 6-H'', 6-H''', 4-H', 2-H'), 3.26 (dt, $J = 11.25$ Hz, $J = 3.5$ Hz, 1H, 1-H), 3.19 (dd, $J = 13.5$ Hz, $J = 8$ Hz, 1H, 6-H'), 2.47 (dt, $J = 13$ Hz, $J = 4$ Hz, 1H, 2-H_{eq}), 1.70 (dd, $J = 12.3$ Hz, 1H, 2-H_{ax}). $^{13}\text{C-NMR}$ (125.7 MHz, D_2O , 25 °C, TMS): δ (ppm) = 109.8 (C-1'), 94.5 (C-1''), 94.3 (C-1'), 84.9 (C-5), 80.5 (C-4'), 76.3 (C-4), 74.9 (C-3'), 72.8 (C-2'), 71.7 (C-6), 69.8 (C-4'), 69.1 (C-5'), 68.0 (C-3'), 67.7 (C-5'), 66.8 (C-3''), 66.7 (C-4'''), 59.9 (C-5'), 58.1 (C-3), 53.0 (C-2'), 50.2 (C-2'''), 49.2 (C-1), 39.7 (C-6'), 39.6 (C-6), 28.7 (C-2). $\text{M} = \text{C}_{23}\text{H}_{44}\text{N}_8\text{O}_{13}$; HRMS (EI+) (m/z): found 641.31 $[\text{M}+\text{H}]^+$, calc. 657.31 $[\text{M}+\text{H}]^+$.

3-C-azido neamine (9). The title compound was prepared according to the general procedure

described above (d, 0.22 mmol scale). Derivative **9** was obtained as a white solid. For the measurement of regioselectivity and the characterization of the compound $^1\text{H-NMR}$ and HSQC spectra were recorded and electrospray ionization (ESI)-MS was employed. HPLC: $R_t =$

3.9 min, 87 % conversion. Yield: 48 mg (0.14 mmol, 64 %). TLC: $R_f = 0.36$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}/25\%\text{NH}_4\text{OH}_{\text{aq}}$ 2:3:1 v/v/v). NMR analysis of antibiotic HFBA salt: $^1\text{H-NMR}$ of HFBA salt: $^1\text{H-NMR}$ (500 MHz, D_2O , 25 °C, TMS): δ (ppm) = 5.69 (d, $J = 3.5$ Hz, 1H, 1-H'), 4.23 (t, $J = 9.0$ Hz, 1H, 5-H'), 3.93 (t, $J = 10.0$ Hz, 1H, 3-H'), 3.80 (dt, $J = 10.8$ Hz, $J = 5.0$ Hz, 1H, 3-H), 3.68 (t, $J = 9.3$ Hz, 1H, 5-H), 3.63 (t, $J = 9.0$ Hz, 1H, 4-H), 3.53 (t, $J = 9.8$ Hz, 1H, 6-H), 3.50-3.46 (m, 2H, 4-H', 6-H'), 3.43 (dd, $J = 11.0$ Hz, $J = 3.5$ Hz, 1H, 2-H'), 3.32-3.24 (dt, 2H, 1-H, 6-H'), 2.51 (dt, $J = 13.0$ Hz, $J = 4.5$ Hz, 1H, 2-H_{eq}), 1.77 (dd, $J = 12.5$ Hz, 1H, 2-H_{ax}). $^{13}\text{C-NMR}$ (125.7 MHz, D_2O , 25 °C, TMS): δ (ppm) = 95.4 (C-1'), 78.8 (C-5), 74.8 (C-4), 72.2 (C-6), 70.3 (C-4'), 68.6 (C-3'), 68.2 (C-5'), 58.2 (C-3), 53.3 (C-2'), 49.4 (C-1), 39.7 (C-6'), 29.1 (C-2). $\text{C}_{12}\text{H}_{24}\text{N}_6\text{O}_6$; HRMS (EI+) (m/z): found 349.18 $[\text{M}+\text{H}]^+$, calc. 349.18 $[\text{M}+\text{H}]^+$.

3-C-azido amikacin (10). The title compound was prepared according to the general procedure described above (d, 0.22 mmol scale). Derivative **10** was

obtained as a white solid. For the measurement of regioselectivity and the characterization of the compound $^1\text{H-NMR}$ and HSQC spectra were recorded and electrospray ionization (ESI)-MS was employed. HPLC: $R_t = 3.5$ min, 37 % conversion. Yield: 39 mg (0.064 mmol, 29 %). TLC: $R_f = 0.20$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}/25\%\text{NH}_4\text{OH}_{\text{aq}}$

2:3:2 v/v/v). NMR analysis of antibiotic HFBA salt: $^1\text{H-NMR}$ (500 MHz, D_2O , 25 °C, TMS): δ (ppm) = 5.50 (d, $J = 4$ Hz, 1H, 1-H'), 5.14 (d, $J = 3.5$ Hz, 1H, 1-H''), 4.26 (dd, $J = 9$ Hz, $J = 3.5$ Hz, 1H, $\alpha\text{-H}_a$), 4.17 (dt, $J = 8.8$ Hz, $J = 3$ Hz, 1H, 5-H'), 4.09-4.04 (m, 2H, 1-H, 3-H'), 3.80 (s, 2H, 6-H''', 6-H'''), 3.78-3.71 (m, 4H, 6-H, 5-H, 5-H'', 2-H''), 3.69-3.66 (m, 2H, 4-H', 3-H), 3.62-3.55 (m, 2H, 2-H', 4-H), 3.44-3.35 (m, 3H, 6-H_a', 3-H'', 4-H''), 3.23-3.16 (m, 3H, 6-H_b', $\gamma\text{-H}_a$, $\gamma\text{-H}_b$), 2.22-2.13 (m, 2H, 2-H_{eq}, $\beta\text{-H}_a$), 1.95 (m, 1H, $\beta\text{-H}_b$), 1.66 (dd, $J = 12.7$ Hz, 1H, 2-H_{ax}). $^{13}\text{C-NMR}$ (125.7 MHz, D_2O , 25 °C, TMS): δ (ppm) = 175.6 (CO), 98.1 (C-1'), 97.2 (C-1''), 80.5 (C-4), 78.9 (C-6), 74.4 (C-5), 71.7 (C-5'), 71.1 (C-3'), 70.6 (C-2'), 70.1 (C-4'), 68.9 (C- α), 67.5 (C-5'), 67.5 (C-2'), 64.7 (C-4'), 59.0 (C-6'), 58.6 (C-3), 54.6 (C-3'), 48.2 (C-1), 39.7 (C-6'), 36.2 (C- γ), 31.1 (C-2), 30.1 (C- β). $M = \text{C}_{22}\text{H}_{41}\text{N}_7\text{O}_{13}$; HRMS (EI+) (m/z): found 612.28 $[\text{M}+\text{H}]^+$, calc. 612.28 $[\text{M}+\text{H}]^+$.

3-C-azido paromomycin (11). The title compound was prepared according to the general procedure

described above (d, 0.22 mmol). Derivative **11** was obtained as a white solid. For the measurement of regioselectivity and the characterization of the compound $^1\text{H-NMR}$ and HSQC and spectra were recorded and electrospray ionization (ESI)-MS was employed. HPLC: $R_t = 5.2$ min, 62 % conversion. Yield: 71 mg (0.11 mmol, 50 %). TLC: $R_f = 0.36$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}/25\%\text{NH}_4\text{OH}_{\text{aq}}$ 2:3:1.5 v/v/v). NMR analysis of antibiotic HFBA salt: $^1\text{H-NMR}$ (500 MHz, D_2O , 25 °C, TMS): δ (ppm) = 5.72 (d, $J = 3$

Hz, 1H, 1-H'), 5.33 (s, 1H, 1-H''), 5.26 (s, 1H, 1-H'''), 4.48 (t, $J = 5.5$ Hz, 1H, 3-H'), 4.38 (d, $J = 4$ Hz, 1H, 2-H'), 4.28 (m, 1H, 5-H'''), 4.22-4.16 (m, 2H, 3-H''', 4-H'), 4.02 (d, $J = 9.5$ Hz, 1H, 5-H'), 3.92-3.84 (m, 2H, 5-H_a'', 3-H'), 3.82-3.75 (m, 4H, 4-H''', 6-H_a', 6-H_b', 5-H), 3.73-3.63 (m, 3H, 5-H_b'', 4-H, 3-H), 3.57 (t, $J = 10$ Hz, 1H, 6-H), 3.56 (s(br), 1H, 2-H'''), 3.50 (t, $J = 9.75$ Hz, 1H, 4-H'), 3.44 (dd, $J = 17.5$ Hz, $J = 9.5$ Hz, 1H, 6-H_a''), 3.39-3.34 (m, 2H, 6-H_b'', 2-H'), 3.25 (dt, $J = 11.5$ Hz, $J = 3.5$ Hz, 1H, 1-H), 2.44 (dt, $J = 12.5$ Hz, $J = 4$ Hz, 1H, 2-H_{eq}), 1.67 (dd, $J = 12.15$ Hz, 1H, 2-H_{ax}). $^{13}\text{C-NMR}$ (125.7 MHz, D_2O , 25 °C, TMS): δ (ppm) = 110.3 (C-1'), 95.11 (C-1''), 94.9 (C-1'''), 85.3 (C-5), 80.67 (C-4'), 76.8 (C-4), 74.9 (C-3'), 73.1 (C-2'), 72.5 (C-5'), 72.2 (C-6), 69.9 (C-5'''), 69.1 (C-3'), 68.7 (C-4'), 67.3 (C-3'''), 67.0 (C-4'''), 60.0 (C-5'), 59.6 (C-6'), 58.4 (C-3), 53.8 (C-2'), 50.5 (C-2'''), 49.6 (C-1), 40.0 (C-6'''), 29.1 (C-2). $\text{C}_{23}\text{H}_{43}\text{N}_7\text{O}_{14}$; HRMS (EI+) (m/z): found 642.29 $[\text{M}+\text{H}]^+$, calc. 642.29 $[\text{M}+\text{H}]^+$.

3-C-azido ribostamycin (12). The title compound was prepared according to the general procedure described above (d, 0.22 mmol scale). Derivative **12** was obtained as a white solid. For the measurement of regioselectivity and the

characterization of the compound $^1\text{H-NMR}$ and HSQC spectra were recorded and electrospray ionization (ESI)-MS was employed. HPLC: HPLC: $R_t = 3.8$ min, 89 % conversion. Yield: 83 mg (0.17 mmol, 77 %). TLC: $R_f = 0.58$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}/25\%\text{NH}_4\text{OH}_{\text{aq}}$ 2:3:1.5 v/v/v). NMR analysis of

antibiotic HFBA salt: $^1\text{H-NMR}$ (500 MHz, D_2O , 25 °C, TMS): δ (ppm) = 5.77 (d, $J = 3.5$ Hz, 1H, 1-H'), 5.31 (d, $J = 1.5$ Hz, 1H, 1-H''), 4.28 (dt, $J = 9$ Hz, $J = 3$ Hz, 1H, 5-H'), 4.21 (dd, $J = 4.5$ Hz, $J = 3$ Hz, 1H, 2-H''), 4.15 (t, $J = 7.5$ Hz, $J = 4.5$ Hz, 1H, 3-H''), 4.05 (dt, $J = 6.5$ Hz, $J = 2.5$ Hz, 1H, 4-H''), 3.93-3.88 (m, 2H, 5-H_a'', 3-H'), 3.82-3.72 (m, 3H, 3-H, 5-H, 4-H), 3.65 (dd, $J = 12.5$ Hz, $J = 6.5$ Hz, 1H, 5-H_b''), 3.60 (t, $J = 9.8$ Hz, 1H, 6-H), 3.48-3.43 (m, 2H, 4-H', 6-H_a'), 3.39 (dd, $J = 10.5$ Hz, $J = 4.0$ Hz, 1H, 2-H'), 3.29 (dt, $J = 11.5$ Hz, $J = 4.0$ Hz, 1H, 1-H), 3.23 (dd, $J = 13.5$ Hz, $J = 7.5$ Hz, 1H 6-H_b'), 2.50 (dt, 12.5 Hz, $J = 4.0$ Hz, 1H, 2-H_{eq}), 1.73 (dd, $J = 12.5$ Hz, 1H, 2-H_{ax}). $^{13}\text{C-NMR}$ (125.7 MHz, D_2O , 25 °C, TMS): δ (ppm) = 110.6 (C-1'), 95.0 (C-1'), 85.5 (C-5), 82.1 (C-4'), 76.7 (C-4), 75.0 (C-2'), 72.3 (C-6), 70.3 (C-4'), 68.9 (C-3'), 68.8 (C-3'), 68.3 (C-5'), 60.9 (C-5'), 58.4 (C-3), 53.5 (C-2'), 49.5 (C-1), 39.9 (C-6'), 29.1 (C-2). $\text{C}_{17}\text{H}_{32}\text{N}_6\text{O}_{10}$; HRMS (EI+) (m/z): found 481.22 $[\text{M}+\text{H}]^+$, calc. 481.22 $[\text{M}+\text{H}]^+$.

3-C-azido apramycin (13). The title compound was prepared according to the general procedure

described above (d, 0.22 mmol scale). Derivative **13** was obtained as a white solid. For the measurement of regioselectivity and the characterization of the compound $^1\text{H-NMR}$ and HSQC spectra were recorded and electrospray ionization (ESI)-MS was employed. HPLC: $R_t = 4.8$ min, 79 % conversion. Yield: 88 mg (0.16 mmol, 71 %). TLC: $R_f =$

0.29 ($\text{CH}_2\text{Cl}_2/\text{MeOH}/25\%\text{NH}_4\text{OH}_{\text{aq}}$ 2:3:1 v/v/v). NMR analysis of antibiotic HFBA salt: $^1\text{H-NMR}$ (500 MHz, D_2O , 25 °C, TMS): δ (ppm) = 5.52 (d, $J = 4$ Hz, 1H, 1-H'), 5.51 (d, $J = 4$ Hz, 1H, 1-H'), 5.25 (d, $J = 8.5$ Hz, 1H, 8-H'), 4.54 (s, 1H, 6-H'), 3.04-3.93 (m, 4H, 3-H'', 5-H'', 4-H', 5-H'), 3.87 (dd, $J = 12.5$ Hz, $J = 3$ Hz, 1H, 6-H_a''), 3.81 (dd, $J = 12.5$ Hz, $J = 4.5$ Hz, 1H, 6-H_b''), 3.73 (dd, $J = 10$ Hz, $J = 4$ Hz, 1H, 2-H'), 3.68-3.62 (m, 3H, 4-H, 3-H, 2-H'), 3.60 (t, $J = 9$ Hz, 1H, 5-H), 3.51 (t, $J = 9.8$ Hz, 1H, 6-H), 3.40 (dd, $J = 8.5$ Hz, $J = 2.5$ Hz, 1H, 7-H'), 3.82 (t, $J = 10.3$ Hz, 1H, 4-H'), 3.26 (dt, $J = 11.5$ Hz, $J = 4$ Hz, 1H, 1-H), 2.82 (s, 3H, CH_3), 2.48 (dt, $J = 13$ Hz, $J = 4.3$ Hz, 1H, 2-H_{eq}), 2.39 (dt, $J = 11$ Hz, $J = 4.3$ Hz, 1H, 3-H_{eq}'), 2.04 (dd, $J = 12$ Hz, 1H, 3-H_{ax}'), 1.73 (dd, $J = 12.3$ Hz, 1H, 2-H_{ax}). $^{13}\text{C-NMR}$ (125.7 MHz, D_2O , 25 °C, TMS): δ (ppm) = 98.8 (C-1'), 97.6 (C-1''), 95.8 (C-8'), 83.2 (C-4), 77.9 (C-5), 75.1 (C-6), 73.0 (C-2'), 72.1 (C-5'), 71.9 (C-3'), 70.9 (C-5'), 68.6 (C-4'), 65.5 (C-6'), 62.8 (C-6''), 62.3 (C-7'), 61.1 (C-3), 54.6 (C-4'), 52.4 (C-1), 50.6 (C-2'), 32.4 (N-CH₃), 32.1 (C-2), 29.2 (C-3'). $\text{M} = \text{C}_{21}\text{H}_{39}\text{N}_7\text{O}_{11}$; HRMS (EI+) (m/z): found 566.27 $[\text{M}+\text{H}]^+$, calc. 566.28 $[\text{M}+\text{H}]^+$.

NMR-spectra of neomycin B and antibiotic derivatives 8-13.

Figure S3: ^1H -NMR (500 MHz, D_2O) spectrum of neomycin B x 6 HFBA **3**.

Figure S4: HSQC (500 MHz, D_2O) spectrum of neomycin B x 6 HFBA **3**.

Figure S5: ^1H -NMR (500 MHz, D_2O) spectrum of neomycin B **3** (**A**), 3-C-azido neomycin B x 5 HFBA **8** formed in buffered aqueous solution at pH 7 (**B**) and 3-C-azido neomycin B (free base) obtained in a non-buffered reaction at pH 6.6 (**C**). Since the neamine antibiotics are purified from the fermentation of the *actinomycete Streptomyces fradiae*, the commercially available neomycin B can contain up to 11 % of impurities. The largest impurity is neomycin C (dash arrows in spectrum **A**) which exhibits the $\text{CH}_2\text{-NH}_2$ group at ring IV in axial instead of equatorial position.³ Therefore, also neomycin C is transformed at ring I resulting in the corresponding azido-derivative (dash arrows in spectrum **C**). However, as shown in NMR-spectrum **C** beside the main product 3-C-azido neomycin B **8** (black arrow) also other regioisomers exhibiting a single azido group are formed (grey arrows). Comparing the integrals the regioselectivity of the diazo-transfer reaction was calculated (see manuscript, Table 1).

Figure S6: ^{13}C -NMR (125.7 MHz, D_2O) spectrum of 3-C-azido neomycin B **8** (free base).

Figure S7: HSQC (500 MHz, D_2O) spectrum of 3-C-azido neomycin B **8** (free base).

Figure S8: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido paromomycin x 4 HFBA **11**.

Figure S9: HSQC (500 MHz, D_2O) spectrum of 3-C-azido paromomycin x 4 HFBA **11**.

Figure S10: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido ribostamycin x 3 HFBA **12**.

Figure S11: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido ribostamycin x 3 HFBA **12**.

Figure S12: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido neamine x 3 HFBA **9**.

Figure S13: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido neamine x 3 HFBA **9**.

Figure S14: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido amikacin x 4 HFBA **10**.

Figure S15: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido amikacin x 4 HFBA **10**.

Figure S16: ^1H -NMR (500 MHz, D_2O) spectrum of 3-C-azido apramycin x 4 HFBA **13**.

Figure S17: HSQC (500 MHz, D_2O) spectrum of 3-C-azido apramycin x 4 HFBA **13**.

High performance liquid chromatography (HPLC)

Figure S18: HPLC analysis of reaction mixture of neomycin B **3** (NeoB) with 8 equiv. of diazo-transfer reagent **7.HCl** at 5.5 mmol (a) and 22 μ mol (b) range, and using 16 equiv. **7.HCl** at 22 μ mol range (c) after incubation for 40, 40 and 20 hours, respectively.

High resolution mass spectrometry analysis

Sample preparation (Fig. S17): (a) To a 4.8 mM solution of neomycin B sulphate in 10 mM sodium phosphate buffer (2.5 mL, pH 7.4) was added an aqueous 48 mM imidazole-1-sulfonyl hydrochloride **7.HCl** solution (2 mL). After addition of a 10 mM sodium phosphate buffer (4.91 mL, pH 7.4) and an aqueous Na₂CO₃ solution (0.59 mL, 10 mg/mL) the reaction was incubated for 18 hours at room temperature. (b) To a 4.8 mM solution of neomycin B sulphate in 10 mM sodium phosphate buffer (2.5 mL, pH 7.4) was an aqueous 48 mM imidazole-1-sulfonyl hydrochloride **7.HCl** solution (2 mL). After addition of 10 mM sodium phosphate buffer (4.41 mL, pH 7.4), aqueous Na₂CO₃ solution (0.59 mL, 10 mg/mL) and an aqueous CuSO₄ solution (0.5 mL, 1 mg/mL) the reaction was incubated for 18 hours at room temperature. (c) To a 4.8 mM solution of neomycin B sulphate in 10 mM sodium phosphate buffer (2.5 mL, pH 7.4) was added an aqueous 48 mM imidazole-1-sulfonyl hydrochloride **7.HCl** solution (2 mL). After addition of 10 mM sodium phosphate buffer (5.0 mL, pH 7.4) and an aqueous CuSO₄ solution (0.5 mL, 1 mg/mL) the reaction was incubated for 18 hours at room temperature. (d) To a 4.8 mM solution of neomycin B sulphate in 10 mM sodium phosphate buffer (2.5 mL, pH 7.4) was added an aqueous 48 mM imidazole-1-sulfonyl hydrochloride **7.HCl** solution (2 mL). After addition of 10 mM sodium phosphate buffer (5.5 mL, pH 7.4) the reaction was incubated for 18 hours at room temperature.

Figure S19: ESI-MS spectra of reaction mixture of neomycin B **3** (NeoB) with 8 equiv. of diazo-transfer reagent **7.HCl** after incubation for 18 hours at different pH values and performed in presence and absence of Cu(II) (i-vi = number of introduced azido groups).

Figure S20: ESI-MS spectrum of reaction mixture of neomycin B **3** (NeoB) with 8 equiv. diazo-transfer reagent **7.HCl** after incubation for 40 hours at room temperature (i-ii = number of introduced azido groups).

Figure S21: ESI-MS spectrum of reaction mixture of paromomycin **4** (Paro) with 16 equiv. diazo-transfer reagent **7.HCl** after incubation for 40 hours at room temperature (i-ii = number of introduced azido groups).

Figure S22: ESI-MS spectrum of reaction mixture of ribostamycin **5** (Ribo) with 16 equiv. diazo-transfer reagent **7.HCl** after incubation for 40 hours at room (i-ii = number of introduced azido groups).

Figure S23: ESI-MS spectrum of reaction mixture of neamine **1** (Neam) with 16 equiv. diazo-transfer reagent **7.HCl** after incubation for 40 hours at room temperature (i-ii = number of introduced azido groups).

Figure S24: ESI-MS spectrum of reaction mixture of amikacin **2** (Amik) with 16 equiv. diazo-transfer reagent **7.HCl** after incubation for 40 hours at room temperature (i-ii = number of introduced azido groups).

Figure S25: ESI-MS spectrum of reaction mixture of apramycin **6** (Apra) with 16 equiv. diazo-transfer reagent **7.HCl** after incubation for 40 hours at room temperature (I = number of introduced azido groups).

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