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Involvement of Two Distinct *N*-Acetylglucosaminyltransferases and a Dual-Function Deacetylase in Neomycin Biosynthesis

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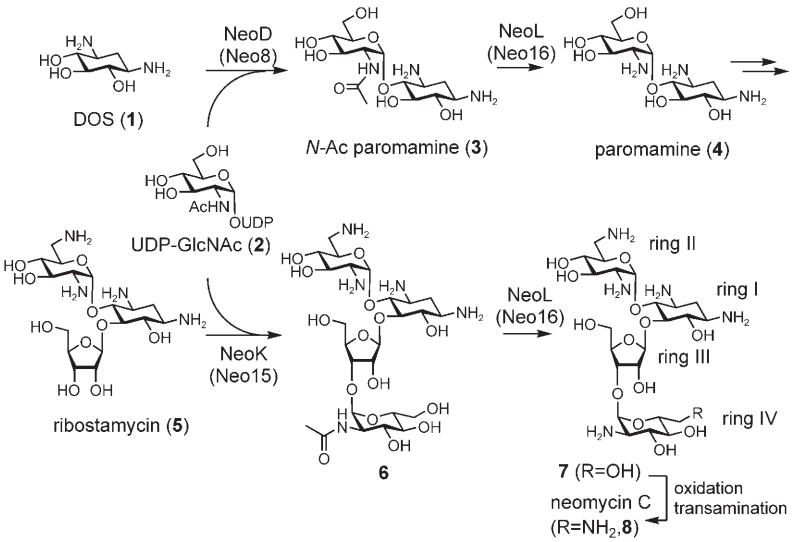
Neomycin is one of the clinically important 2-deoxystreptamine (DOS)-containing aminoglycoside antibiotics, which specifically interact with bacterial rRNA and inhibit protein synthesis.[1] These compounds have attracted attention through their potential as anti-HIV[2] and antiplasmid[3] agents owing to their unique nucleotide recognition ability. Structural diversification of aminoglycosides is therefore a promising approach for the generation of novel bioactive compounds. Glycoside diversification by the use of glycosyltransferases is an attractive way to carry this out.[4,5] However, in the biosynthetic pathway of aminoglycosides,[6] only a phosphoribosyltransferase has been characterized, even though many putative glycosyltransferase genes have been identified in the biosynthetic gene clusters.[7]

The only readily apparent glycosyltransferase gene in the neomycin biosynthetic gene cluster is *neoD* (*neo8*).[8,9] The deduced product (NeoD) belongs to the GT4 family, members of which catalyze retaining glycosyl transfer reactions with NDP-sugars.[10] Comparative genetics has revealed that *neoD*-homologous genes are conserved among all the reported gene clusters of DOS-containing aminoglycosides, and are thus proposed to be involved in the formation of a common biosynthetic intermediate, paromamine (**4**, Scheme 1).[9] Recently, 2′-*N*-acetylparomamine (**3**) was proposed to be a biosynthetic intermediate according to the deacetylase activity toward **3** of BtrD, an enzyme encoded in the butirosin biosynthetic gene cluster.[11] Thus, NeoD was presumed to catalyze *N*-acetylglucosaminylation of DOS. To confirm the function of NeoD, the enzymatic activity of the recombinant NeoD protein was investigated.

NeoD was co-expressed with molecular chaperone GroES and GroEL in *E. coli* to increase the amount of soluble protein (Figure 1 A).[12] Because NeoD showed low binding affinity for any kind of resin, even Ni affinity resin when expressed as a His-tagged protein, the cell-free extract of *E. coli* expressing NeoD was used for enzymatic assays. After incubating recombinant NeoD with UDP-GlcNAc and DOS, enzyme reaction products were treated with 2,4-dinitrofluorobenzene (DNFB), and the derivatives were analyzed by HPLC. As a result, about 80% of DOS was consumed, and a new product was observed at a retention time of 18 min (Figure 2 A). This peak was not observed in a control reaction with the cell-free extract of *E. coli* harboring an empty plasmid (Figure 2 B). The new peak showed *m/z* 696.4 by LC–ESIMS analysis, indicating that bis-(2,4-dinitrophenyl)-**3** ([*M*−H]$^-$ 696.3) was produced. The NeoD reaction product was further isolated from a large-scale enzyme reaction (4 mL) and its structure was confirmed by NMR and FABMS to be **3** (Supporting Information). Another possible glycosyl donor, UDP-glucose, and other possible gly-



Scheme 1. Sequential glycosylation and deacetylation steps in neomycin biosynthesis.

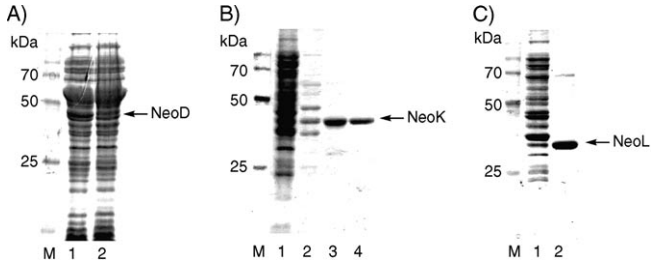


Figure 1. SDS-PAGE of enzymes used. A) Lane 1: cell-free extract of *E. coli* BL21(DE3) harboring pREP4-groESL and pET21neoD; lane 2: cell-free extract of *E. coli* BL21(DE3) harboring pREP4-groESL and empty pET21a vector. B) Lane 1: cell-free extract of *E. coli* BL21(DE3) expressing NeoK; lane 2: NeoK after DEAE Sepharose column chromatography; lane 3: NeoK after chromatography with Superdex Hi-Load 200; lane 4: NeoK after MonoQ chromatography. C) Lane 1: cell-free extract of *E. coli* BL21(DE3) expressing NeoL; lane 2: NeoL purified with TALON resin. Lanes M: molecular weight markers.

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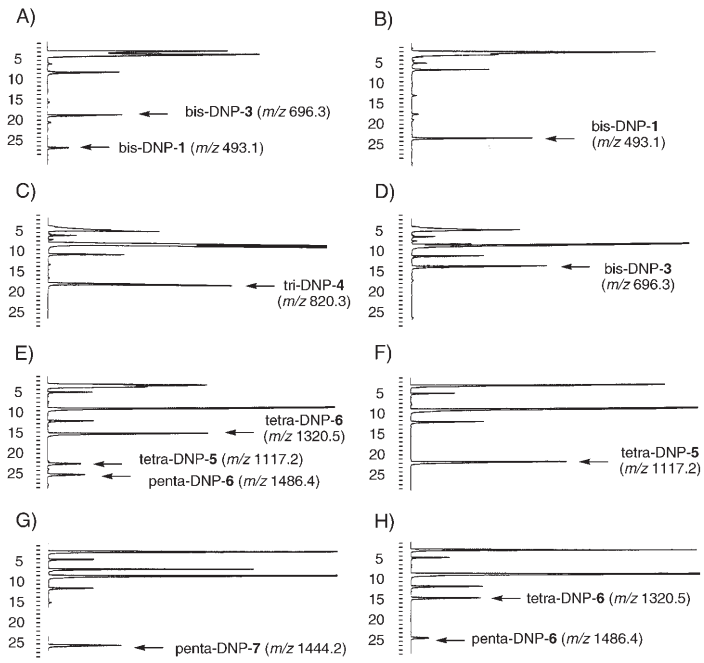

Figure 2. HPLC results of the DNP-derivatized enzyme reaction products; elution was monitored at 350 nm. A) NeoD reaction with UDP-GlcNAc (**2**) and DOS (**1**); B) as in (A), but without NeoD; C) NeoL reaction with *N*-acetylparomamine (**3**); D) as in (C), but without NeoL; E) NeoK reaction with UDP-GlcNAc (**2**) and **5**; F) as in (E), but without NeoK; G) NeoL reaction with **6;** H) as in (G), but without NeoL. See Experimental Section for HPLC conditions.

cosyl acceptors, paromamine, neamine, and ribostamycin were not accepted. These results clearly show that NeoD catalyzes the glycosylation of DOS with UDP-GlcNAc to give **3** in neomycin biosynthesis.

The biosynthesis of neomycin requires another hexose transfer step for the formation of the second neosamine moiety, ring IV. The inability of NeoD to catalyze the glycosylation of ribostamycin indicated the presence of another glycosyltransferase. The putative biosynthetic gene clusters for structurally related aminoglycosides, paromomycin (*par*, AJ628955) and lividomycin (*liv*, AJ748832), have been identified from the producing microorganisms. Comparison of these gene clusters with that of neomycin revealed two conserved genes, *neoK* (*neo15*) and *neoH* (*neo12*), indicating their involvement in the biosynthesis of the common ring IV structure. NeoH showed moderate sequence homology to radical SAM (*S*-adenosyl-L-methionine) proteins and is thus presumed to catalyze epimerization or oxidation to modify the ring IV. In contrast, NeoK showed low similarities (<20%) to the C-terminal 150 amino acids of uncharacterized glycosyltransferases in the GT4 family. Thus, we investigated the function of NeoK as a potential glycosyltransferase involved in the attachment of ring IV.

The NeoK protein expressed in *E. coli* was purified to homogeneity by DEAE Sepharose column chromatography, gel filtration, and MonoQ column chromatography until it became a single band in SDS-PAGE (Figure 1B). Purified NeoK was then incubated with ribostamycin and UDP-GlcNAc. LC–ESIMS analysis of the 2,4-dinitrophenyl (DNP) derivative of the enzyme reaction products showed the generation of a new peak with *m/z* 1320.5 and *m/z* 1486.4 corresponding to the tetra- and penta-DNP-2‴-*N*-acetyl-6‴-deamino-6‴-hydroxyneomycin C (**6**, Figure 2E). These peaks were not observed in the reaction without NeoK, ribostamycin, or UDP-GlcNAc, and also in the reaction with the cell-free extract of *E. coli* harboring the empty plasmid. The penta-DNP **6** might be derived through a DNP derivatization of the primary alcohol of ring IV. To further confirm the function of NeoK, the reaction product was isolated from the large-scale NeoK reaction using 10 mg ribostamycin and excess UDP-GlcNAc (15 mg), and the structure was determined by NMR and FABMS. From ^{1}H NMR, the presence of the GlcNAc moiety was confirmed by observation of a singlet acetamide methyl proton signal at 2.2 ppm (3H) and a characteristic double doublet for H2‴ at 3.8 ppm. The signal for the anomeric proton of the GlcNAc moiety had a coupling constant of $J=3.6$ Hz, suggesting an α-glycosidic linkage. The substituted position was confirmed by long-range coupling between H1‴ (4.85 ppm) and C3″ (84.5 ppm) observed in the HMBC spectrum. These results unambiguously showed that the NeoK reaction product was **6** and prove that NeoK is a glycosyltransferase that catalyzes the retaining glycosylation of ribostamycin with UDP-GlcNAc as a sugar donor to generate **6**. Enzyme assays in the presence of various metals revealed NeoK to use divalent metal cations such as Mg^{2+} and Mn^{2+}, whereas Zn^{2+} and Cu^{2+} completely inhibited the reaction, possibly through metal exchange or allosteric effects (Figure 3).

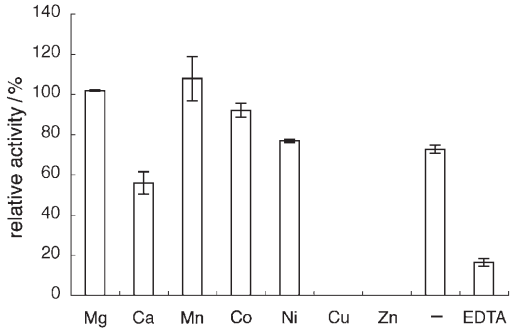

Figure 3. Metal requirement of NeoK. NeoK (1.5 μM) was incubated with UDP-GlcNAc (2.5 mM) and ribostamycin (2.5 mM) at 28 °C for 4 h in the absence (–) or presence of 2 mM divalent metal ions, or 10 mM EDTA as indicated.

NeoK and its homologous proteins do not belong to any of the known glycosyltransferase families on the CAZY website (http://www.cazy.org/index.html)[13] and therefore constitute a new glycosyltransferase family. According to the secondary structure prediction by 3D position-specific scoring matrix,[14] NeoK has moderate similarity in its secondary structure with GT-B-fold glycosyltransferases,[10] GtfD,[15] and MurG.[16] GT-B-fold glycosyltransferases generally have NDP-sugar binding sites mainly in their C-terminal domain.[17] Partial similarity in the C-terminal sequence of NeoK with GT4 glycosyltransferases, which also have a GT-B fold, may indicate a similar binding mode for the NDP-sugar. On the other hand, N-terminal do-

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mains of GT-B-fold glycosyltransferases are generally responsible for the binding of the sugar acceptor. The characteristic sequence of the N-terminal domain of NeoK could be associated with its recognition of the characteristic structure of ribostamycin (Supporting Information).

Functional characterization of NeoD and NeoK as *N*-acetylglucosaminyltransferases indicated the presence of two deacetylation steps. However, only one BtrD-homologous deacetylase, NeoL, was encoded in the *neo* gene cluster, indicating repetitive use of NeoL. Thus, NeoL was expressed in *E. coli* as an N-terminal His_6-tagged protein to confirm the deacetylation activity toward **3** and **6**. NeoL was purified with TALON cobalt affinity resin (Figure 1C) and incubated with **3** or **6**. As a result, both substrates were efficiently consumed, and a new peak with *m/z* 820.3 was generated by the reaction with **3** (Figure 2C) and a peak with *m/z* 1444.2 was generated by the reaction with **6** (Figure 2G), which correspond to the DNP derivatives of **4** and 6‴-deamino-6‴-hydroxyneomycin C (**7**), respectively. Furthermore, the reaction products were isolated and confirmed to be **4** and **7**, respectively, by NMR and FABMS. In both cases, the methyl proton signals of the acetamide group present in the NMR spectra of both substrates disappeared, and the signals for 2′-H or 2‴-H shifted ~1 ppm upfield from those of the substrates, showing that the acetamide groups were converted into amino groups. These results clearly prove that NeoL is a deacetylase that recognizes both **3** and **6** as substrates.

The substrate recognition mechanism of NeoL is an intriguing issue, as NeoL seems to recognize strictly the *N*-acetylglucosamine moiety of aminoglycosides. In the biosynthesis of neomycin, aminotransferases NeoB (Neo6) and NeoN (Neo18) were also reported to work repetitively to generate DOS and two neosamine C moieties, respectively.[9,18] On the other hand, NeoD and NeoK strictly recognize sugar acceptors in the glycosylation events. Accurate substrate recognition by these enzymes could be necessary for the construction of the characteristic framework of neomycins. Knowledge of these relaxed and strict substrate recognition properties toward similar substrates would be valuable for the future modulation of enzyme substrate specificities.

In conclusion, we have characterized two glycosyltransferases and a repetitively working deacetylase involved in neomycin biosynthesis. The combination of *N*-acetylglucosaminylation and subsequent deacetylation can also be observed in the biosynthesis of other secondary metabolites,[19] indicating its generality in the biosynthesis of glucosamine-containing secondary metabolites. Notably, NeoK was characterized as a member of a novel family of glycosyltransferase. This finding may indicate that many other glycosyltransferases remain to be discovered which could find potential use as tools for glycoside diversification.

Experimental Section

Expression of NeoD, NeoK, and NeoL: The *neoD, neoK*, and *neoL* genes were amplified by PCR using cosmid cfra10[8] as a template to introduce an NdeI/HindIII restriction site into the *neoD* and *neoK* gene fragments and an NdeI/XhoI restriction site into the *neoL* gene fragment. After confirmation of the DNA sequences, each gene was introduced into corresponding overexpression vectors as follows: The *neoD* gene was introduced into the NdeI/HindIII site of the pET21a plasmid to obtain pET21neoD. The *neoK* gene was introduced into the NdeI/HindIII site of the pET30b plasmid to obtain pET30neoK. The *neoL* gene was introduced into the NdeI/XhoI site of pColdI plasmid (Takara) to obtain pColdneoL. The constructed plasmids were separately introduced into *E. coli* BL21-(DE3). In the case of NeoD, *E. coli* BL21(DE3) was transformed with pET21neoD and pREP4-groESL[12] for co-expression of NeoD with the molecular chaperone. The *E. coli* BL21(DE3) carrying the expression plasmids were grown at 37 °C until OD_{600} ~ 0.6–1.0. The expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.2 mM), and the culture was continued at 15 °C for 24 h. The cells were harvested by centrifugation and stored at −30 °C until use.

Enzyme preparation: The wet cells of the *E. coli* expressing NeoD, NeoK, or NeoL were suspended in a 10-fold excess of buffer A (HEPES-NaOH (pH 7.0, 50 mM), 10% glycerol) and disrupted by sonication. Cell-free extract was prepared by removing cell debris by centrifugation at 15000 rpm for 30 min at 4 °C. The cell-free extract was directly used for the NeoD reaction. NeoK and NeoL were further purified as follows.

NeoK was first purified with DEAE Sepharose Fast Flow (Amersham Biosciences) column chromatography by a linear gradient of NaCl (0–0.25 M) in buffer B (Tris-HCl (pH 8.0, 50 mM), 10% glycerol). The fractions containing NeoK were combined, concentrated, and loaded onto a Superdex Hi-load 200 gel filtration column (Pharmacia Biotech) equilibrated with buffer B containing NaCl (100 mM). The NeoK obtained was further applied to a MonoQ column (Pharmacia Biotech) equilibrated with buffer B and eluted by a linear gradient of 60→160 mM NaCl in buffer B. The purified NeoK was concentrated, and the buffer was exchanged to buffer A by passage through a 5-mL HiTrap desalting column (Pharmacia Biotech).

Cell-free extract containing NeoL was applied to TALON cobalt affinity resin, washed with imidazole (20 mM), and eluted by imidazole (200 mM) in buffer A. Because NeoL was strongly adsorbed to the membrane filter, the imidazole was removed by ammonium sulfate precipitation.

Enzyme assays: *Glycosyltransferase assays*: NeoD-containing cell-free *E. coli* extract (20 μL) or purified NeoK (9.3 μM) was incubated with a sugar acceptor (DOS, paromamine, neamine, or ribostamycin, 3 mM) and a sugar donor (UDP-Glc, UDP-GlcNAc, or UDP-glucosamine, 3 mM) in HEPES-NaOH (pH 7.0, 50 mM), 10% glycerol, $MgCl_2$ (2 mM) (final volume: 40 μL) at 28 °C for 12 h.

Deacetylase assays: NeoL (8.0 μM) was incubated with *N*-acetylparomamine (5 mM, **3**) or 2‴-*N*-acetyl-6‴-deamino-6‴-hydroxyneomycin C (5 mM, **6**) in HEPES-NaOH (pH 7.0, 50 mM), 10% glycerol, $MgCl_2$ (2 mM) (final volume: 40 μL). Compounds **3** and **6** were prepared by the use of NeoD and NeoK, respectively, as described below.

Following all enzymatic reactions, ethanol (40 μL) was added to the reaction mixture, and the precipitant was removed by centrifugation. DNP derivatization of products was performed using published a method[8] with slight modifications. An aliquot of the supernatant (20 μL) was treated with 5% 2,4-dinitrofluorobenzene in methanol (20 μL), DMSO (10 μL), and 2 M NaOH (2 μL) at 60 °C for 1 h. The reaction mixture was extracted with ethyl acetate (500 μL). After removal of the solvent by a centrifugal evaporator, the resi-

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due was dissolved in methanol (100 μL) and analyzed by HPLC and LC–ESIMS.

HPLC analysis: HPLC was performed on an L-6250 Intelligent Pump instrument (Hitachi) equipped with an L-4000H UV detector (Hitachi) and a D-2500 Chromato-Integrator (Hitachi). An aliquot of the solution (5 μL) was injected into the HPLC system equipped with a Pegasil ODS column (4.6×250 mm, Senshu). The elution was made by a linear gradient of 60→80% aqueous methanol (30 min, 1 mL min^{-1}) to analyze **3**, and 50→60% aqueous acetonitrile (30 min, 1 mL min^{-1}) to analyze the other aminoglycosides. The elution was monitored at 350 nm.

LC–ESIMS analysis: LC–ESIMS analysis was performed on an LCQ mass spectrometer (Finnigan) coupled with Nanospace HPLC (Shiseido) equipped with a Nanospace SE-1 UV detector (Shiseido). An aliquot of the solution (2 μL) was injected into the HPLC system equipped with an RP-18 GP column (Kanto). For the analysis of **3**, the elution was made by 10% aqueous methanol for 10 min, followed by a linear gradient of 50→80% aqueous methanol for 35 min at a flow rate of 50 μL min^{-1}. For the analyses of the other aminoglycosides, the elution was made by 10% aqueous acetonitrile for 5 min, followed by a linear gradient of 50→80% aqueous acetonitrile for 40 min at a flow rate of 50 μL min^{-1}. The elution was monitored at 350 nm. The ESI mass spectrometer was operated in the negative-ion mode

Metal requirement of NeoK: NeoK (1.5 μM) was treated with divalent metals ($MgCl_2$, $CaCl_2$, $MnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, $ZnCl_2$, 2 mM) or EDTA (10 mM) at room temperature for 0.5 h. NeoK was then incubated with UDP-GlcNAc (2.5 mM) and ribostamycin (2.5 mM) at 28 °C for 4 h.

Isolation of *N*-acetylparomamine (3) by large-scale NeoD reaction: The cell-free extract of *E. coli* expressing NeoD (4 mL) was incubated with DOS (5 mM, 3 mg) and UDP-GlcNAc (8 mM, 20 mg) at 28 °C for 12 h. The reaction was quenched by the addition of ethanol (4 mL) and centrifuged at 15000 rpm for 20 min. The supernatant was then passed through a DOWEX AG1X8 (OH^- form) column and unbound compounds were collected. The collected solution was neutralized with AcOH and applied to Amberlite CG50 (NH_4^+ form). The column was washed with aqueous NH_4OH (50 mM) followed by elution with aqueous NH_4OH (1 M). Solvent was removed in vacuo by a rotary evaporator and the counteranion was exchanged to SO_4^{2-} by passing through a DOWEX AG1X8 (SO_4^{2-} form) column to obtain *N*-acetylparomamine sulfate (3.5 mg) as colorless powder. ^{1}H NMR (400 MHz, D_2O): δ=1.38 (q, J=12.7 Hz, 1H, H-2$_{ax}$), 1.90 (s, 3H, Ac), 2.09 (dt, J=4.2, 12.7 Hz, 1H, H-2$_{eq}$), 2.95 (m, 2H, H-1, H-3), 3.27 (t, J=9.3 Hz, 1H, H-6), 3.30 (t, J=9.4 Hz, 1H, H-4), 3.35 (t, J=9.3 Hz, 1H, H-4′), 3.43 (t, J=9.3 Hz, 1H, H-5), 3.59–3.64 (m, 2H, H-3′, H-6′), 3.71 (m, 1H, H-5′), 3.76 (dd, J=2.0, 12.0 Hz, 1H, H-6′), 3.83 (dd, J=3.8, 10.9 Hz, 1H, H-2′), 5.11 (d, J=3.7 Hz, 1H, H-1′); ^{13}C NMR (100 MHz, D_2O): δ=21.9, 31.5, 49.4, 50.2, 53.8, 60.6, 69.9, 70.8, 73.0, 74.0, 75.2, 84.9, 98.8, 174.3; HR FABMS: m/z [M+H]$^+$ 366.1888, calcd for $C_{14}H_{28}O_8N_3$: 366.1877.

Isolation of 2‴-*N*-acetyl-6‴-deamino-6‴-hydroxyneomycin C (6) by large-scale NeoK reaction. Purified NeoK (10 μM, 1.1 mg) was incubated with ribostamycin sulfate (5.1 mM, 10.0 mg) and UDP-GlcNAc (7.7 mM, 15.0 mg) in HEPES-NaOH (50 mM, pH 7.0, 3 mL), 10% glycerol, $MgCl_2$ (2 mM) at 28 °C for 12 h. The enzyme reaction product was purified by the same procedure used to isolate *N*-acetylparomamine described above. 2‴-*N*-acetyl-6‴-deamino-6‴-hydroxyneomycin C sulfate (9.3 mg) was obtained as colorless powder. ^{1}H NMR (500 MHz, D_2O): δ=1.24 (q, J=12.5 Hz, 1H, H-2$_{ax}$), 1.96 (m, 4H, Ac, H-2$_{eq}$), 2.82 (m, 2H, H-1, H-2′), 2.9 (ddd, J=4.1, 9.5, 12.3 Hz, 1H, H-3), 3.01 (dd, J=7.4, 13.5 Hz, 1H, H-6′), 3.22 (dd, J=3.0, 13.5 Hz, 1H, H-6′), 3.28 (t, J=9.5 Hz, 1H, H-6), 3.29 (t, J=9.5 Hz, 1H, H-4′), 3.43 (t, J=9.5 Hz, 1H, H-4), 3.44 (t, J=9.3 Hz, 1H, H-4‴), 3.58–3.89 (m, 10H, H-3′, H-5′, H-5″, H-2‴, H-3‴, H-5‴, H-6‴), 4.08 (dt, J=3.0, 6.2 Hz, 1H, H-4″), 4.15 (dd, J=5.0, 7.0 Hz, 1H, H-3″), 4.23 (dd, J=2.0, 4.8 Hz, 1H, H-2″), 4.93 (d, J=3.6 Hz, 1H, H-1‴), 5.30 (d, J=2.1 Hz, 1H, H-1″), 5.55 (d, J=3.7 Hz, 1H, H-1′); ^{13}C NMR (125 MHz, D_2O): δ=21.9, 34.4, 40.9, 49.8, 50.3, 53.5, 55.0, 60.4, 61.6, 69.8, 70.4, 70.6, 71.3, 72.2, 72.6, 73.1, 74.6, 76.5, 80.8, 81.1, 84.5, 96.9, 97.9, 109.3, 174.4; HR FABMS: m/z [M+H]$^+$ 658.3131, calcd for $C_{25}H_{48}O_{15}N_5$: 658.3147.

Isolation of paromamine (4) by large-scale NeoL reaction: Purified NeoL (2.0 μM, 0.13 mg) was incubated with *N*-acetylparomamine sulfate (2.7 mM, 2.5 mg) in 2 mL 50 mM HEPES-NaOH (pH 7.0), 10% glycerol at 28 °C for 12 h. The enzyme reaction product was purified by the same procedure used to isolate *N*-acetylparomamine described above, to obtain paromamine sulfate (2.3 mg) as colorless powder. ^{1}H NMR (500 MHz, D_2O): δ=1.44 (q, J=12.5 Hz, 1H, H-2$_{ax}$), 2.14 (dt, J=4.1, 12.5 Hz, 1H, H-2$_{eq}$), 2.97–3.08 (m, 3H, H-1, H-3, H-2′), 3.35–3.41 (m, 3H, H-5, H-6, H-4′), 3.51 (t, J=9.3 Hz, 1H, H-4), 3.64–3.72 (m, 2H, H-3′, H-6′), 3.78–3.81 (m, 2H, H-5′, H-6′), 5.33 (d, J=4.0 Hz, 1H, H-1′); ^{13}C NMR (125 MHz, D_2O): δ=31.8, 49.0, 50.3, 54.7, 60.4, 69.6, 71.5, 73.1, 73.8, 75.2, 84.8, 98.9; HR FABMS: m/z [M+H]$^+$ 324.1783 calcd for $C_{12}H_{26}O_7N_3$: 324.1771.

Isolation of 6‴-deamino-6‴-hydroxyneomycin C (7) by large scale NeoL reaction. Purified NeoL (2.0 μM, 0.19 mg) was incubated with 2‴-*N*-acetyl-6‴-deamino-6‴-hydroxyneomycin C sulfate (5.0 mM, 14.0 mg) in HEPES-NaOH (50 mM, pH 7.0, 3 mL), 10% glycerol at 28 °C for 12 h. The enzyme reaction product was purified by the same procedure used to isolate *N*-acetylparomamine described above to obtain 6‴-deamino-6‴-hydroxyneomycin C sulfate (13.7 mg) as a colorless powder. ^{1}H NMR (500 MHz, D_2O): δ=1.33 (q, J=12.7 Hz, 1H, H-2$_{ax}$), 2.02 (dt, J=4.2, 12.7 Hz, 1H, H-2$_{eq}$), 2.8 (dd, J=3.6, 10.3 Hz, 1H, H-2‴), 2.91–2.96 (m, 3H, H-1, H-3, H-2′), 3.08 (dd, J=7.5, 13.5 Hz, 1H, H-6′), 3.28 (dd, J=3.4, 13.6 Hz, 1H, H-6′), 3.31 (t, J=9.5 Hz, 1H, H-4′), 3.35 (t, J=9.6 Hz, 1H, H-4‴), 3.38 (t, J=9.5 Hz, 1H, H-6), 3.49 (t, J=9.4 Hz, 1H, H-4), 3.56 (t, J=9.7 Hz, 1H, H-3‴), 3.59–3.72 (m, 5H, H-5, H-3′, H-5″, H-5‴, H-6‴), 3.78 (dd, J=2.2, 12.4 Hz, 1H, H-6‴), 3.82 (dd, J=2.8, 12.4 Hz, 1H, H-5″), 3.92 (ddd, J=3.3, 7.5, 9.8 Hz, 1H, H-5′), 4.09 (dt, J=2.8, 6.1 Hz, 1H, H-4″), 4.19 (dd, J=4.8, 6.9 Hz, 1H, H-3″), 4.29 (dd, J=2.0, 4.7 Hz, 1H, H-2″), 5.00 (d, J=4.6 Hz, 1H, H-1‴), 5.31 (d, J=1.9 Hz, 1H, H-1″), 5.63 (d, J=3.7 Hz, 1H, H-1′); ^{13}C NMR (125 MHz, D_2O): δ=33.1, 40.5, 49.4, 50.4, 54.61, 54.64, 60.4, 61.3, 69.4, 69.6, 71.1, 71.2, 72.9, 73.3, 74.8, 75.1, 80.0, 80.9, 84.8, 97.1, 98.4, 109.5; HR FABMS: m/z [M+H]$^+$ 616.3017, calcd for $C_{23}H_{46}O_{14}N_5$: 616.3041.

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Keywords: biosynthesis · deacetylases · glycosyltransferases · neomycin

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