NOTE

Enzymatic preparation of neomycin C from ribostamycin

Fumitaka Kudo¹, Taiki Kawashima², Kenichi Yokoyama¹ and Tadashi Eguchi²

The Journal of Antibiotics (2009) **62**, 643–646; doi:10.1038/ja.2009.88; published online 28 August 2009

Keywords: aminoglycoside antibiotics; biosynthesis; dehydrogenase; enzymatic synthesis; neomycin C; neosamine C

The high regio- and stereospecificities of enzymes and their ability to catalyze reactions in the absence of organic solvents make enzymatic synthesis of antibiotics an attractive way to prepare complex natural products. Enzymatic total syntheses of several complex natural products have been recently achieved, which illustrates the power of this synthetic methodology.^{1,2} Combinations of biosynthetic enzymes and substrates, including unnatural types, can afford structurally diverse natural product-like compounds.³

In this study, we report the enzymatic synthesis of the aminoglycoside antibiotic neomycin C from ribostamycin by the use of four neomycin biosynthetic enzymes. So far, all nine enzymes for ribostamycin biosynthesis from D-glucose 6-phosphate have been characterized using the enzymes derived from the butirosin producer *Bacillus circulans* and the neomycin producer *Streptomyces fradiae*.⁴ Furthermore, two neomycin biosynthetic enzymes NeoF and NeoD were recently found to catalyze the glycosylation of ribostamycin using

Figure 1 Biosynthesis of neomycin C from ribostamycin.

¹Department of Chemistry, Tokyo Institute of Technology, Tokyo, Japan and ²Department of Chemistry and Materials Science, Tokyo Institute of Technology, Tokyo, Japan
 Correspondence: Professor T Eguchi, Department of Chemistry and Materials Science, Tokyo Institute of Technology, 2-12-2 O-okayama, Meguro-ku, Tokyo 152-8551, Japan.
 E-mail: eguchi@cms.titech.ac.jp

Received 25 June 2009; revised 1 August 2009; accepted 3 August 2009; published online 28 August 2009

UDP-*N*-acetylglucosamine (UDP-GlcNAc) as a sugar donor and the subsequent deacetylation to afford 6'''-deamino-6'''-hydroxyneomycin C (Figure 1).⁵ (As the biosynthetic gene cluster for neomycin was deposited with different symbols in DNA databases by multiple groups, we here use the butirosin biosynthetic *btr* gene-based code

names, which are systematically utilized by the Piepersberg *et al.*⁶ and also in our recent review.⁷) NeoB has also been reported to catalyze two transaminations: the conversion of neomycin C to 6'''-oxoneomycin C, which is a reverse reaction of the postulated *in vivo* biosynthetic reaction (Figure 1), and the conversion of 6'-oxoparo-

Figure 2 NMR spectra of neomycin C in D₂O synthesized with biosynthetic enzymes. (a) ¹H-NMR (500 MHz) and (b) ¹³C-NMR (125 MHz) were recorded on DRX500 (Bruker, Rheinstetten, Germany). Auto reference for the D₂O solvent was used for both spectra. Small amounts of Tris buffer and glycerol were contaminated from the purification process of the proteins. 2D-NMRs including ¹H-¹H COSY and TOCSY spectra (data not shown) were also recorded to determine the structure of neomycin C. The signals were assigned by comparison with those of 6'''-deamino-6'''-hydroxyneomycin C 5 and neomycin B.¹¹ ¹H NMR (500 MHz, D₂O): δ 1.39 (q, *J*=12.7 Hz, 1H, H-2ax), 2.06 (dt, *J*=12.7, 4.1 Hz, 1H, H-2eq), 2.86 (dd, *J*=3.6, 10.2 Hz, 1H, H-2'''), 2.98 (m, 3H, H-1, H-3, H-2'), 3.10 (dd, *J*=7.3, 13.5 Hz, 2H, H-6', H-6'''), 3.32 (m, 4H, H-4', H-6', H-4''', H-6'''), 3.44 (m, H-4 or H-6 with glycerol), 3.52 (m, H-4 or H-6 with glycerol), 3.57 (m, H-3''' with Tris), 3.64 (dd, *J*=5.0, 12.4 Hz, 1H, H-5''), 3.70 (m, H-5, H-3' with glycerol), 3.77 (ddd, *J*=3.1, 7.3, 9.7 Hz, 1H, H-5'''), 3.83 (dd, *J*=3.2, 12.4 Hz, 1H, H-5''), 3.93 (ddd, *J*=3.3, 7.3, 10.4 Hz, 1H, H-5'), 4.10 (m, 1H, H-4''), 4.24 (dd, *J*=4.7, 6.9 Hz, 1H, H-3''), 4.32 (dd, *J*=2.0, 4.7 Hz, 1H, H-2''), 5.07 (d, *J*=3.6 Hz, 1H, H-1'''), 5.33 (d, *J*=2.0 Hz, 1H, H-1''), 5.65 (d, *J*=3.7 Hz, 1H, H-1'); ¹³C NMR (125 MHz, D₂O): δ 32.7 (C2), 40.5 (C6' or C6'''), 40.6 (C6' or C6'''), 49.4 (C3), 50.5 (C1), 54.5 (C2' or C2'''), 54.6 (C2' or C2'''), 61.0 (C5''), 69.4 (C5' and C4'''), 71.0, 71.1, 71.2, 72.4 (C3' or C4' or C3''' or C5'''), 73.2 (C2''), 74.4 (C3''), 74.8 (C6), 79.9 (C4), 80.8 (C4''), 84.9 (C5), 97.0 (C1'), 98.1 (C1'') and 109.8 (C1''). Signals at 59.6 and 60.8 p.p.m. were derived from Tris and signals at 62.5 and 72.1 p.p.m. in ¹³C-NMR were derived from glycerol. The impurities are indicated as T for Tris and G for glycerol, respectively, in spectra.

amine to neamine using L-glutamate or L-glutamine as an amino donor.⁸ NeoQ was characterized as a flavin adenine dinucleotide-dependent dehydrogenase of paromamine to give 6'-oxoparomamine during neamine formation.⁸ No dehydrogenase for the oxidation of 6'''-deamino-6'''-hydroxyneomycin C has been identified as yet. As there is no other gene in the biosynthetic gene cluster that can be assigned for the oxidation of 6'''-deamino-6'''-hydroxyneomycin C, the repetitive use of NeoQ has been proposed.⁸ Characterization of the NeoQ function and reconstitution of the biosynthetic reactions between ribostamycin and neomycin C is important to elucidate the potential repetitive use of multiple enzymes in the neomycin biosynthetic pathway.

At first, we prepared 6'''-deamino-6'''-hydroxyneomycin C from ribostamycin by incubation with NeoF and NeoD according to earlier methods with minor modifications.⁵ Overexpressed NeoF protein was partially purified by anion exchange chromatography with DEAE Sepharose Fast Flow (GE Healthcare, Chalfont St Giles, UK) and the obtained crude enzyme (17 ml, 22 mg as crude enzyme) was directly used for the glycosylation of ribostamycin (56 mg) with UDP-GlcNAc • 2Na (86 mg) at 28 °C for 45 h. Without purification of the glycosylated product, the next deacetylation reaction was carried out by the addition of NeoD (0.3 mg, 0.25 ml) purified by TALON (Clontech, Mountain View, CA, USA) chromatography. After 24 h at 28 °C, the resulting enzymatic solution was heat-treated (100 °C, 2 min) and then applied to a Dowex AG1-X8 (Bio-Rad, Richmond, CA, USA, [OH⁻] form, 12 ml) column. The unadsorbed fraction was neutralized with acetic acid and then applied to an Amberlite CG50 (Rohm and Haas Co, Philadelphia, PA, USA, [NH₄⁺] form, 8 ml) column. A stepwise gradient of aqueous NH₄OH (0.04 M and then 2 M) eluted 6'''-deamino-6'''-hydroxyneomycin C, which was further passed through Dowex AG1-X8 ([SO₄²⁻] form, 8 ml) to obtain 6'''-deamino-6'''-hydroxyneomycin C sulfate (45 mg, 2 steps 45% yield).

Next, the probable flavin adenine dinucleotide-dependent dehydrogenation of 6'''-deamino-6'''-hydroxyneomycin C catalyzed by NeoQ with the abovementioned substrate was investigated. When the following conditions for the enzyme reaction and derivatization were used, we observed a time-dependent decrease of the substrate by HPLC analysis (data not shown); NeoQ (0.35 mg ml⁻¹, purified with Ni-NTA His • Bind Resin (Novagen, Darmstadt, Germany)), substrate 2 mM, flavin adenine dinucleotide 0.3 mM in 20 mM Tris buffer (pH 8.0) containing 0.2 M KCl, at 28 °C, followed by derivatization with 2,4-dinitrofluorobenzene for UV detection of aminoglycosides. The result indicated that NeoQ also catalyzes the dehydrogenation of 6'''-deamino-6'''-hydroxy-neomycin C as well as paromamine to give the corresponding aldehyde. The absence of the product is presumably because of a decomposition of the aldehyde compound during the dinitrophenyl derivatization under basic and high temperature (60 °C) conditions. Furthermore, the addition of NeoB (0.6 mg ml⁻¹, purified by Ni-NTA His • Bind Resin chromatography), L-glutamine (Gln, 10 mM) and pyridoxal phosphate (0.2 mM) to the NeoQ reaction mixture followed by the abovementioned derivatization gave a new peak on the HPLC trace, which showed *m/z* 1609.5 corresponding to the MW [M-H]⁻ of the neomycin C dinitrophenyl derivative (data not shown). A large-scale NeoQ and NeoB reaction (6 mg of NeoQ, 11 mg of NeoB, 17 mg of 6'''-deamino-6'''-hydroxyneomycin C (1 mM), flavin adenine dinucleotide (1 mM), Gln (20 mM) and pyridoxal phosphate (0.4 mM) in the Tris-HCl buffer (pH 8.0) containing 0.2 M of KCl, total 20 ml at 28 °C for 44 h) gave a mixture of starting material and the new product, which was purified by the same procedure as that for the purification of 6'''-deamino-6'''-hydroxyneomycin C. As the preliminary analytical scale experiment suggested the inefficient conversion

of this reaction, the mixture was further treated under the same enzymatic conditions for a longer time (44 h) to ensure maximum consumption of the starting material and the resulting aminoglycosides were purified in an identical manner. As a result, 12 mg of the product was obtained and the structure of the main product was confirmed as neomycin C by ¹H-NMR, ¹³C-NMR (Figure 2) and HR-FAB-MS (positive, glycerol) *m/z* 637.3033 (M+Na)⁺, calcd for C₂₃H₄₆N₆O₁₃Na: 637.3020. Despite the use of newly prepared enzymes for both reactions, 10–20% of the starting material remained after the reaction, probably because of the reversibility of the transamination.⁸ However, the conversion of 6'''-deamino-6'''-hydroxyneomycin C to neomycin C using purified NeoQ and NeoB strongly suggests a repetitive use of NeoQ and NeoB in neomycin biosynthesis.

The only uncharacterized step in the neomycin B biosynthesis is now the epimerization at the C-5''' position of neomycin C. As the exact timing of the epimerization is currently unclear,⁹ any of the four biosynthetic intermediates in the pathway shown in Figure 1 may be the substrate for the epimerization. The enzymatic synthesis reported here can supply all the possible substrates needed to investigate the corresponding epimerization reaction. A comparison of aminoglycoside biosynthetic gene clusters indicated the involvement of NeoN, a putative radical SAM enzyme that is also encoded in the paromomycin and lividomycin biosynthetic clusters.^{5,7} As such an epimerization by a radical SAM enzyme is unprecedented¹⁰ and would be an important tool to synthesize structurally diverse aminoglycosides, the functional characterization of NeoN is important and is currently underway.

In conclusion, we showed the repetitive dehydrogenase activity of NeoQ at the C-6 position of the glucosamine moiety of paromamine and 6'''-deamino-6'''-hydroxyneomycin C, and the transamination activity of NeoB at the corresponding positions. The substrate recognition mechanism of the dehydrogenase, NeoQ, should be interesting, because the C-6' positions of paromomycin and lividomycin remain as hydroxy groups even though the corresponding enzymes in the biosynthesis of these antibiotics should work for the dehydrogenation at C-6'''. We also achieved the enzymatic synthesis of neomycin C from ribostamycin in a preparative scale. This methodology can be applied for enzymatic synthesis of the other aminoglycoside antibiotics. Thus, the functional characterization of many other biosynthetic enzymes for aminoglycosides such as kanamycin and gentamicin will pave the way for the creation of structurally diverse aminoglycosides, including unnatural types, in the future.

ACKNOWLEDGEMENTS

This work was supported in part by the Mitsubishi Chemical Corporation Fund, the Naito Foundation and KAKENHI 21710224 (FK).

- 1 Cheng, Q., Xiang, L., Izumikawa, M., Meluzzi, D. & Moore, B. S. Enzymatic total synthesis of enterocin polyketides. *Nat. Chem. Biol.* **3**, 557–558 (2007).
- 2 Sattely, E. S., Fischbach, M. A. & Walsh, C. T. Total biosynthesis: *In vitro* reconstitution of polyketide and nonribosomal peptide pathways. *Nat. Prod. Rep.* **25**, 757–793 (2008).
- 3 Kalaitzis, J. A., Cheng, Q., Thomas, P. M., Kelleher, N. L. & Moore, B. S. *In vitro* biosynthesis of unnatural enterocin and wailupemycin polyketides. *J. Nat. Prod.* **72**, 469–472 (2009).
- 4 Kudo, F. & Eguchi, T. Biosynthetic enzymes for the aminoglycosides butirosin and neomycin. *Methods Enzymol.* **459**, 493–519 (2009).
- 5 Yokoyama, K., Yamamoto, Y., Kudo, F. & Eguchi, T. Involvement of two distinct *N*-acetylglucosaminyltransferases and a dual-function deacetylase in neomycin biosynthesis. *ChemBiochem* **9**, 865–869 (2008).
- 6 Piepersberg, W., Aboshanab, K. M., Schmidt-Beissner, H. & Wehmeier, U. F. in *Aminoglycoside Antibiotics. The biochemistry and genetics of aminoglycoside producers* (eds Arya, D. P.) 15–118 (John Wiley & Sons, Inc., Hoboken, NJ, 2007).
- 7 Kudo, F. & Eguchi, T. Biosynthetic genes for aminoglycoside antibiotics. *J. Antibiot.* **62**, 471–481 (2009).

- 8 Huang, F. *et al.* Elaboration of neosamine rings in the biosynthesis of neomycin and butirosin. *ChemBiochem* **8**, 283–288 (2007).
- 9 Autissier, D., Barthelemy, P., Mazieres, N., Peyre, M. & Penasse, L. 6'''-Deamino-6'''-hydroxy derivatives, as intermediates in the biosynthesis of neomycin and paromomycin. *J. Antibiot.* **34**, 536–543 (1981).
- 10 Wang, S. C. & Frey, P. A. S-adenosylmethionine as an oxidant: the radical SAM superfamily. *Trends Biochem. Sci.* **32**, 101–110 (2007).
- 11 Reid, D. G. & Gajjar, K. A proton and carbon 13 nuclear magnetic resonance study of neomycin B and its interactions with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.* **262**, 7967–7972 (1987).