Chemoenzymatic acylation of aminoglycoside antibiotics†

Nicholas M. Llewellyn* and Jonathan B. Spencer‡

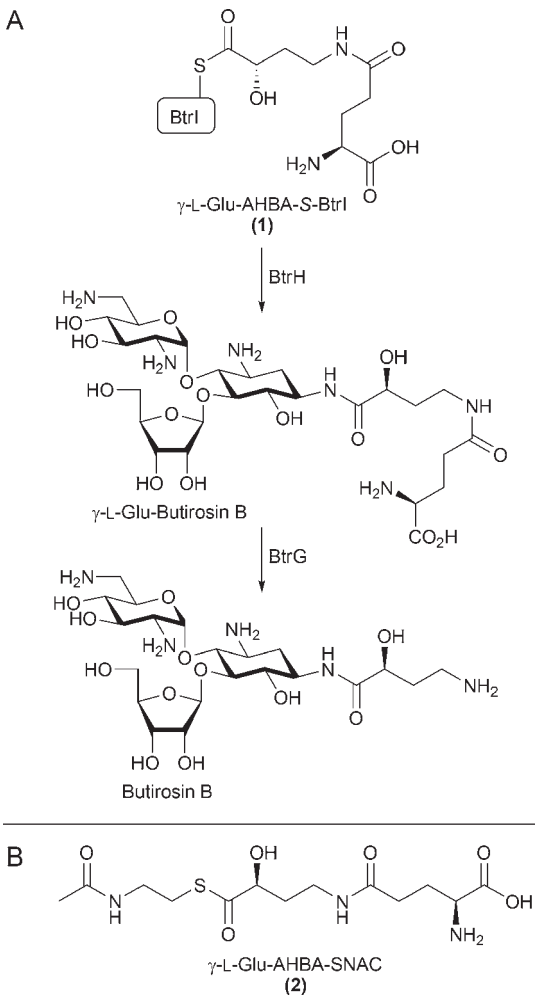
Received (in Cambridge, UK) 8th February 2008, Accepted 15th May 2008
First published as an Advance Article on the web 20th June 2008
DOI: 10.1039/b802248h

The chemoenzymatic installation of the clinically valuable (*S*)-4-amino-2-hydroxybutyryl side chain onto a number of 2-deoxystreptamine-containing aminoglycosides is described using the purified *Bacillus circulans* biosynthetic enzymes BtrH and BtrG in combination with a synthetic acyl-SNAC surrogate substrate.

The aminoglycosides are a diverse class of bactericidal antibiotic natural products of clinical importance.[1] Most of the significant aminoglycoside families, including the neomycin-, kanamycin-, and gentamicin-class aminoglycosides, consist of the core aminocyclitol 2-deoxystreptamine (2-DOS) decorated with various aminosugar substituents.[2] This family of antibiotics binds the A-site stem loop feature of the bacterial 16S rRNA,[3,4] thereby inhibiting protein synthesis *via* interference with codon fidelity,[5] translocation,[6] tmRNA aminoacylation,[7] and ribosome recycling.[8] Additionally, aminoglycosides and their derivatives exhibit a number of interesting activities toward other nucleic acid targets that may lead to the discovery of novel antiviral agents and genetic disorder therapies.[9–14]

The continuing proliferation of bacterial resistance to antibiotics has spurred the investigation of diverse and intriguing strategies to counter important resistance mechanisms.[15–17] One of the most successful strategies is the rational modification of existing drugs to improve their activity against resistant organisms. Aminoglycoside derivatives bearing the (*S*)-4-amino-2-hydroxybutyryl (AHBA) side chain are of particular importance for their improved activity against aminoglycoside-resistant organisms, including methicillin-resistant *Staphylococcus aureus* (MRSA).[18] The AHBA pharmacophore, originally observed in the natural product butirosin,[19] is semisynthetically incorporated into the aminoglycoside antibiotics kanamycin A and dibekacin to afford amikacin and arbekacin, respectively.[20,21] The regiospecific synthetic acylation of densely functionalized and structurally diverse aminoglycosides commonly requires multi-step, substrate-specific protection/deprotection schemes.[22–24] The obvious appeal of a general biocatalytic route to regiospecifically acylated aminoglycosides has, therefore, led our group to investigate the biosynthetic origins of the AHBA moiety in butirosin produced by *Bacillus circulans* NRRL B3312.[25,26] The ACP-mediated biosynthesis of AHBA from L-glutamate has been demonstrated to proceed with the unusual protection of a potentially labile ACP-bound γ-aminobutyryl intermediate as the *N*-γ-L-glutamyl amide prior to installation of the stereogenic C-2 hydroxyl. The γ-L-glutamylated side chain is then transferred onto the aminoglycoside substrate ribostamycin by the acyltransferase BtrH, and the resulting acyl-aminoglycoside species is cleaved by the γ-L-glutamyl cyclotransferase BtrG to reveal butirosin B (Scheme 1A).

Further examination of this biosynthetic machinery revealed BtrH to possess modest tolerance for close structural relatives of ribostamycin, including neamine, paromomycin, and neomycin, as non-native side chain acceptors;



Scheme 1 (A) Biosynthetic incorporation of the AHBA side chain in butirosin B. (B) Synthetic *N*-acetylcysteamine thioester used as a surrogate acyl donor for chemoenzymatic acylation.

Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, United Kingdom CB2 1EW. E-mail: nml30@cam.ac.uk; Fax: +44 (0)1223 336362; Tel: +44 (0)1223 336710
† Electronic supplementary information (ESI) available: Experimental details, synthetic methods, and LC-ESI-MS/MS spectra. See DOI: 10.1039/b802248h
‡ Deceased.

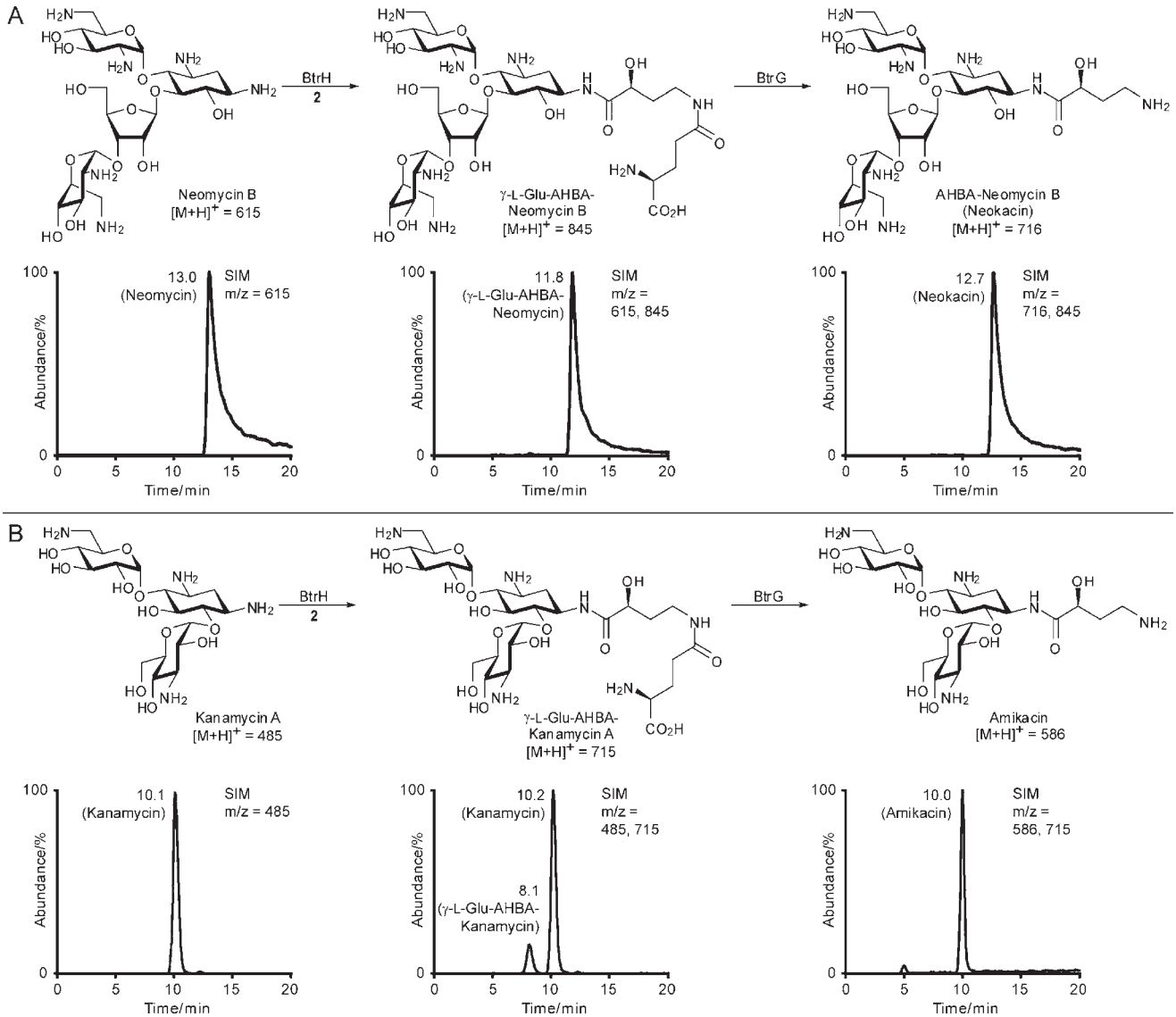


Fig. 1 (A) Chemoenzymatic conversion of neomycin to neokacin. Incubation of neomycin (left) with BtrH and **2** results in essentially complete conversion to γ-L-Glu-AHBA-neomycin (centre, no peak corresponding to neomycin observed). This intermediate is then quantitatively cleaved by incubation with BtrG to reveal neokacin (right, no peak corresponding to γ-L-Glu-AHBA-neomycin observed). (B) Chemoenzymatic conversion of kanamycin to amikacin. Incubation of kanamycin (left) with BtrH and **2** results in partial conversion (~15%) to γ-L-Glu-AHBA-kanamycin (centre). This intermediate is then quantitatively cleaved by incubation with BtrG to reveal amikacin (right, no peak corresponding to γ-L-Glu-AHBA-kanamycin observed, overlapping peak corresponding to unconverted kanamycin omitted). LC-ESI-MS traces with selective ion monitoring (SIM) for each relevant species are given below the corresponding structure. See ESI† for mass spectra and MS/MS fragmentation data for each species.

furthermore, BtrG exhibits good activity against unnatural γ-L-Glu-AHBA-aminoglycosides.[26] This finding encouraged us to investigate the application of this system to the production of a greater diversity of AHBA-aminoglycoside species. For this purpose, it was desirable to circumvent the ACP (BtrI) as the acyl donor, as reconstitution of the complete multi-enzyme biosynthetic pathway *in vitro* is inconvenient. Further, while the native acyl donor γ-L-Glu-AHBA-*S*-BtrI (**1**) may be prepared by loading synthetic γ-L-Glu-AHBA-CoA[26] onto *apo*-BtrI with the promiscuous phosphopantetheinyl transferase Sfp,[27] the spent *holo*-BtrI cannot be reloaded with another acyl group, and the small quantity of **1** that can reasonably be prepared limits the overall theoretical productivity of this non-catalytic approach. It is well established that synthetic *N*-acetylcysteamine thioesters are suitable surrogates for carrier protein-bound substrates;[28,29] therefore, we elected to pursue a chemoenzymatic strategy in which a synthetic *N*-acetylcysteamine thioester (SNAC) is substituted for **1** as the side chain donor. γ-L-Glu-AHBA-SNAC (**2**, Scheme 1B) was synthesized by reported strategies,[26,29] and the competency of this substrate as an acyl donor was tested by incubation with purified recombinant BtrH and ribostamycin. Satisfyingly, quantitative acylation of ribostamycin was observed by LC-ESI-MS/MS analysis of the reaction mixture. Further,

incubation of **2** with BtrH and a variety of non-native aminoglycoside acceptors led to acylation of all substrates tested, with varying levels of conversion. Paromamine, neamine, paromomycin, neomycin, and apramycin are all well tolerated as substrates for BtrH with essentially complete conversion observed (Fig. 1A and ESI†). 2-DOS is also accepted with moderate conversion (~70%) to the acylated species within the assay period (ESI†). Kanamycin and gentamicin C are acylated with modest conversion (~15% and ~10%, respectively) (Fig. 1B and ESI†). Notably, a number of these aminoglycoside substrates (2-DOS, paromamine, apramycin, kanamycin, and neomycin) had previously been tested with the native acyl donor **1** without any detectable side chain incorporation;[26] these new results demonstrate that BtrH possesses broader substrate tolerance than previously thought. Investigation into the reason for the improved substrate scope when using **2** as the acyl donor is ongoing. The effect may be due exclusively to the higher concentration of **2** that is reasonably achieved relative to the less abundantly available **1**; alternatively, possible interactions between BtrH and BtrI might impose a discriminatory conformational state on BtrH. All of the acyl-aminoglycosides produced by the action of BtrH are quantitatively and rapidly deglutamylated by incubation with BtrG, revealing the expected AHBA-aminoglycoside species (Fig. 1 and ESI†). However, **2** was also found to be a good substrate for BtrG; therefore, the *in vitro* construction of AHBA-aminoglycosides by this method must be accomplished by stepwise incubation, first with BtrH and **2**, then followed by BtrG. In order to test the scalability of this chemoenzymatic strategy for the preparation of AHBA-aminoglycosides and to confirm the expected regiochemistry of the acylation step, 3.2 mg of paromamine was incubated in a 5 mL reaction first with **2** and BtrH, then with BtrG. Following purification, 3.5 mg of AHBA-paromamine was isolated (82% yield), and NMR analysis of the product unambiguously confirmed that the AHBA side chain is indeed attached to the C-1 amine (ESI†).

In summary, the *in vitro* chemoenzymatic acylation of a wide range of 2-DOS-containing aminoglycoside substrates with the valuable AHBA pharmacophore has been demonstrated up to the milligram scale. The described method affords convenient access to a number of unnatural AHBA-aminoglycosides. Additional work is underway to further scale up the productive capacity of this chemoenzymatic system and to more thoroughly investigate the substrate promiscuity of these enzymes. It is anticipated that this method will provide an attractive alternative to synthetic approaches to interesting acyl-aminoglycoside species.

Notes and references

1 D. P. Arya, *Aminoglycoside antibiotics: from chemical biology to drug discovery*, Wiley-Interscience, Hoboken, NJ, 2007.
2 N. M. Llewellyn and J. B. Spencer, *Nat. Prod. Rep.*, 2006, **23**, 864–874.
3 D. Moazed and H. F. Noller, *Nature*, 1987, **327**, 389–394.
4 A. P. Carter, W. M. Clemons, D. E. Brodersen, R. J. Morgan-Warren, B. T. Wimberly and V. Ramakrishnan, *Nature*, 2000, **407**, 340–348.
5 R. Karimi and M. Ehrenberg, *Eur. J. Biochem.*, 1994, **226**, 355–360.
6 R. Karimi and M. Ehrenberg, *EMBO J.*, 1996, **15**, 1149–1154.
7 S. Corvaisier, V. Bordeau and B. Felden, *J. Biol. Chem.*, 2003, **278**, 14788–14797.
8 M. A. Borovinskaya, R. D. Pai, W. Zhang, B. S. Schuwirth, J. M. Holton, G. Hirokawa, H. Kaji, A. Kaji and J. H. Cate, *Nat. Struct. Mol. Biol.*, 2007, **14**, 727–732.
9 J. B. Tok, L. J. Dunn and R. C. Des Jean, *Bioorg. Med. Chem. Lett.*, 2001, **11**, 1127–1131.
10 A. Litovchick, A. Lapidot, M. Eisenstein, A. Kalinkovich and G. Borkow, *Biochemistry*, 2001, **40**, 15612–15623.
11 R. Schroeder, C. Waldsich and H. Wank, *EMBO J.*, 2000, **19**, 1–9.
12 M. Howard, R. A. Frizzell and D. M. Bedwell, *Nat. Med.*, 1996, **2**, 467–469.
13 R. Kellermayer, *Eur. J. Med. Genet.*, 2006, **49**, 445–450.
14 M. Hainrichson, I. Nudelman and T. Baasov, *Org. Biomol. Chem.*, 2008, **6**, 227–239.
15 J. R. Thomas, J. C. Denap, M. L. Wong and P. J. Hergenrother, *Biochemistry*, 2005, **44**, 6800–6808.
16 R. Chait, A. Craney and R. Kishony, *Nature*, 2007, **446** 668–671.
17 M. Latorre, P. Penalver, J. Revuelta, J. L. Asensio, E. Garcia-Junceda and A. Bastida, *Chem. Commun.*, 2007, 2829–2831.
18 N. Tsuchizaki, K. Ishino, F. Saito, J. Ishikawa, M. Nakajima and K. Hotta, *J. Antibiot.*, 2006, **59**, 229–233.
19 P. W. K. Woo, H. W. Dion and Q. R. Bartz, *Tetrahedron Lett.*, 1971, **12**, 2625–2628.
20 H. Kawaguchi, T. Naito, S. Nakagawa and K. I. Fujisawa, *J. Antibiot.*, 1972, **25**, 695–708.
21 S. Kondo, K. Iinuma, H. Yamamoto, K. Maeda and H. Umezawa, *J. Antibiot.*, 1973, **26**, 412–415.
22 T. L. Nagabhushan, A. B. Cooper, W. N. Turner, H. Tsai, S. McCombie, A. K. Mallams, D. Rane, J. J. Wright, P. Reichert, D. L. Boxler and J. Weinstein, *J. Am. Chem. Soc.*, 1978, **100**, 5253–5254.
23 T. Tsuchiya, Y. Takagi and S. Umezawa, *Tetrahedron Lett.*, 1979, **20**, 4951–4954.
24 J. Li, H. N. Chen, H. Chang, J. Wang and C. W. Chang, *Org. Lett.*, 2005, **7**, 3061–3064.
25 Y. Li, N. M. Llewellyn, R. Giri, F. Huang and J. B. Spencer, *Chem. Biol.*, 2005, **12**, 665–675.
26 N. M. Llewellyn, Y. Li and J. B. Spencer, *Chem. Biol.*, 2007, **14**, 379–386.
27 L. E. Quadri, P. H. Weinreb, M. Lei, M. M. Nakano, P. Zuber and C. T. Walsh, *Biochemistry*, 1998, **37**, 1585–1595.
28 I. E. Holzbaur, R. C. Harris, M. Bycroft, J. Cortes, C. Bisang, J. Staunton, B. A. Rudd and P. F. Leadlay, *Chem. Biol.*, 1999, **6**, 189–195.
29 D. E. Ehmann, J. W. Trauger, T. Stachelhaus and C. T. Walsh, *Chem. Biol.*, 2000, **7**, 765–772.