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Biosynthesis of Butirosin: Transfer and Deprotection of the Unique Amino Acid Side Chain

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SUMMARY

Butirosin, an aminoglycoside antibiotic produced by *Bacillus circulans*, bears the unique (*S*)-4-amino-2-hydroxybutyrate (AHBA) side chain, which protects the antibiotic from several common resistance mechanisms. The AHBA side chain is advantageously incorporated into clinically valuable antibiotics such as amikacin and arbekacin by synthetic methods. Therefore, it is of significant interest to explore the biosynthetic origins of this useful moiety. We report here that the AHBA side chain of butirosin is transferred from the acyl carrier protein (ACP) BtrI to the parent aminoglycoside ribostamycin as a γ-glutamylated dipeptide by the ACP:aminoglycoside acyltransferase BtrH. The protective γ-glutamyl group is then cleaved by BtrG via an uncommon γ-glutamyl cyclotransferase mechanism. The application of this pathway to the in vitro enzymatic production of novel AHBA-bearing aminoglycosides is explored with encouraging implications for the preparation of unnatural antibiotics via directed biosynthesis.

INTRODUCTION

The aminoglycosides, a diverse family of bactericidal compounds that includes kanamycin, neomycin, and gentamicin, among others, exhibit a diversity of established and potential medicinal activities. Aminoglycosides were among the first antibiotics in clinical use, and they remain valuable agents for the treatment of difficult infections, especially those caused by Gram-negative bacteria such as *Mycobacterium tuberculosis* and methicillin-resistant *Staphylococcus aureus* (MRSA) [1, 2]. Recent investigations have revealed the potential utility of aminoglycosides as antiviral agents, with demonstrated activity against viral and catalytic RNAs including human immunodeficiency virus (HIV) [3, 4], the hepatitis delta virus (HΔV) ribozyme, the hammerhead ribozyme, and the group I intron [5]. Furthermore, aminoglycosides may induce plasmid incompatibility in bacteria, raising the exciting prospect of drug-induced reversal of plasmid-mediated antibiotic resistance in some clinical pathogens [6]. The bactericidal activity of this family of drugs derives from their strong affinity for the rRNA of the bacterial ribosome, which leads to the disruption of protein synthesis by interfering with codon fidelity and translocation [7, 8]. Likewise, the antiviral and antiplasmid activities of aminoglycosides arise from the affinity of these polycations for nucleic acid strands.

As is the case with all classes of clinical antibiotics, aminoglycosides face the problem of resistant pathogens [9, 10]. Although mutation of the ribosomal rRNA has been shown to effect aminoglycoside resistance in some cases, clinical resistance is most commonly conferred by the expression of at least one enzyme that modifies the aminoglycoside [11] by *O*-phosphorylation, *O*-nucleotidylylation, or *N*-acylation—all modifications which weaken the affinity of the drug for RNA. Semisynthetic *N*-substituted aminoglycoside derivatives such as amikacin [12] and arbekacin [13], however, are largely unaffected by a number of common resistance enzymes, and synthetic efforts have focused on the use of substrate-specific protection schemes for the stereospecific synthesis of aminoglycoside derivatives [14–16]. Engineered biosynthesis by genetic manipulation of aminoglycoside-producing organisms presents an attractive alternative to synthetic methods for the production of novel antibiotic compounds. For this strategy to be successful, intimate knowledge of the biosynthetic pathways leading to these natural products is required [17].

The strategy of exploring acyl-aminoglycoside derivatives to sidestep resistance enzymes was initially inspired by the discovery of the butirosins from *Bacillus circulans* [18]. Butirosins A and B are naturally occurring derivatives of xylostasin and ribostamycin, respectively, which bear an (*S*)-4-amino-2-hydroxybutyrate (AHBA) substituent at the C-1 amine of the central 2-deoxystreptamine (2-DOS) ring. The butirosins exhibit improved antibiotic properties over their parent molecules and retain bactericidal activity toward many aminoglycoside-resistant strains. The gene cluster encoding the biosynthetic pathway for butirosin in *B. circulans* has been sequenced [19, 20] (accession numbers AB097196 and AJ847918), and comparison to the neomycin cluster from *Streptomyces*

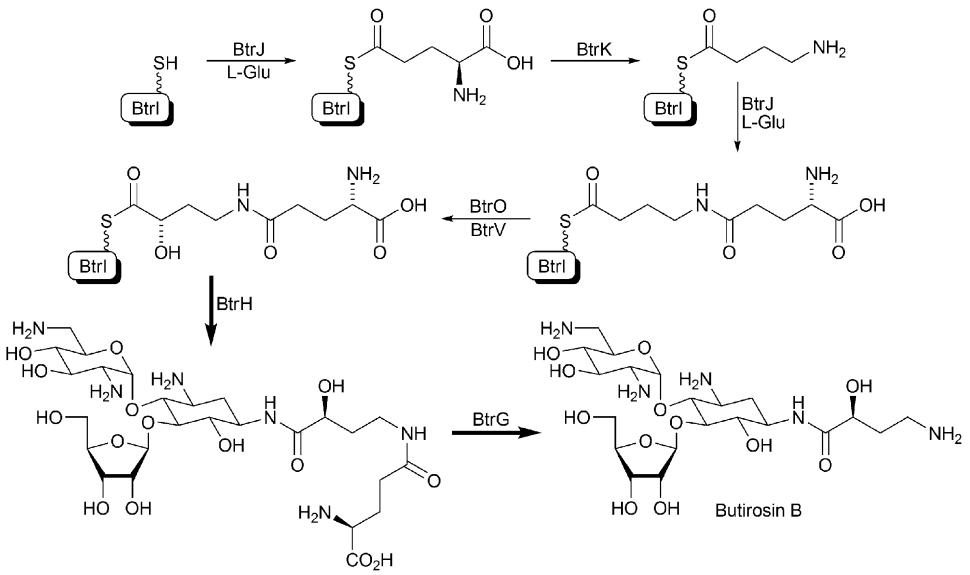

Figure 1. Biosynthetic Pathway for the AHBA Side Chain in Butirosin
Bold arrows indicate steps elucidated in this paper.

fradiae [21] revealed a set of seven genes putatively responsible for the biosynthesis and incorporation of the AHBA side chain. We recently reported on the biosynthesis of the AHBA side chain by five of these enzymes via an acyl carrier protein (ACP)-mediated pathway involving unusual protective biochemistry via γ-L-glutamylation of a potentially nucleophilic primary amine on the ACP-bound intermediate (Figure 1) [20]. Two *B. circulans* genes, *btrG* and *btrH*, were proposed to encode proteins responsible for the transfer of the AHBA side chain to the aminoglycoside and the removal of the γ-L-Glu protective group.

RESULTS AND DISCUSSION

Sequence Analysis of BtrG and BtrH

BtrG (156 amino acids, 18.1 kDa) is the archetype of the eponymous BtrG family of proteins, which contain the functionally uncharacterized COG2105 domain architecture. The nearest homologs to BtrG are found in other *Bacillus* species. Structural homologs of BtrG have been characterized by X-ray crystallography [22, 23] and NMR (PDB: 1XHS); however, no homolog has been assigned a proposed function, nor does the observed fold hint at any specific catalytic activity. The monomer mass of N-terminally His_6-tagged BtrG is 20,093 Da by LC-ESI-MS (calculated at 20,093.38). The deduced amino acid sequence of BtrH (302 amino acids, 35.9 kDa) exhibits weak similarity [24] (25% identity, 39% similarity over 266 amino acids) only to the uncharacterized hypothetical protein product of the gene *api78* from an identified pathogenicity island in *Yersinia pseudotuberculosis* [25]. The N-terminally His_6-tagged BtrH exhibits a monomer mass of 37,916 Da (calculated at 37,916.91) by LC-ESI-MS.

Functional Characterization of BtrG and BtrH

In order to facilitate the functional characterization of these two enzymes, it was desirable to synthetically prepare the acyl-S-BtrI substrate for the acyl transfer reaction (Figure 2). γ-L-Glu-AHBA-CoA was synthesized from appropriately protected L-glutamate by *p*-nitrophenylchloroformate-activated ligation to AHBA [26]. The product dipeptide was ligated via its *N*-hydroxysuccinamoyl ester to coenzyme A [27], and deprotection in TFA yielded the desired CoA. γ-L-Glu-AHBA-CoA was loaded onto apo-BtrI by the broad-spectrum phosphopantetheinyl transferase Sfp from *Bacillus subtilis* [28], yielding directly γ-L-Glu-AHBA-S-BtrI, identical to the species obtained by incubation of holo-BtrI with BtrJ, BtrK, BtrO, and BtrV in the presence of L-glutamate, ATP, FMN, and NADH [20].

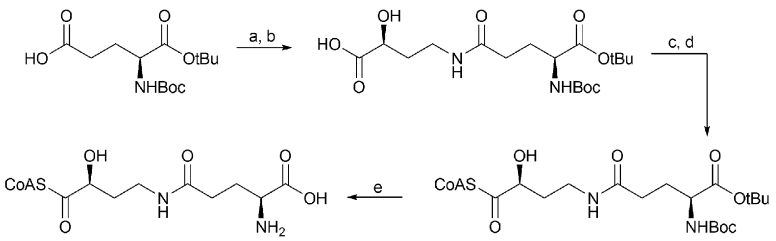

Figure 2. Synthesis of γ-L-Glu-AHBA-CoA
(a) *p*-nitrophenylchloroformate, Et_3N, DMAP; (b) AHBA, Et_3N; (c) DCC, *N*-hydroxysuccinimide; (d) coenzyme A, Li_2CO_3; (e) trifluoroacetic acid.

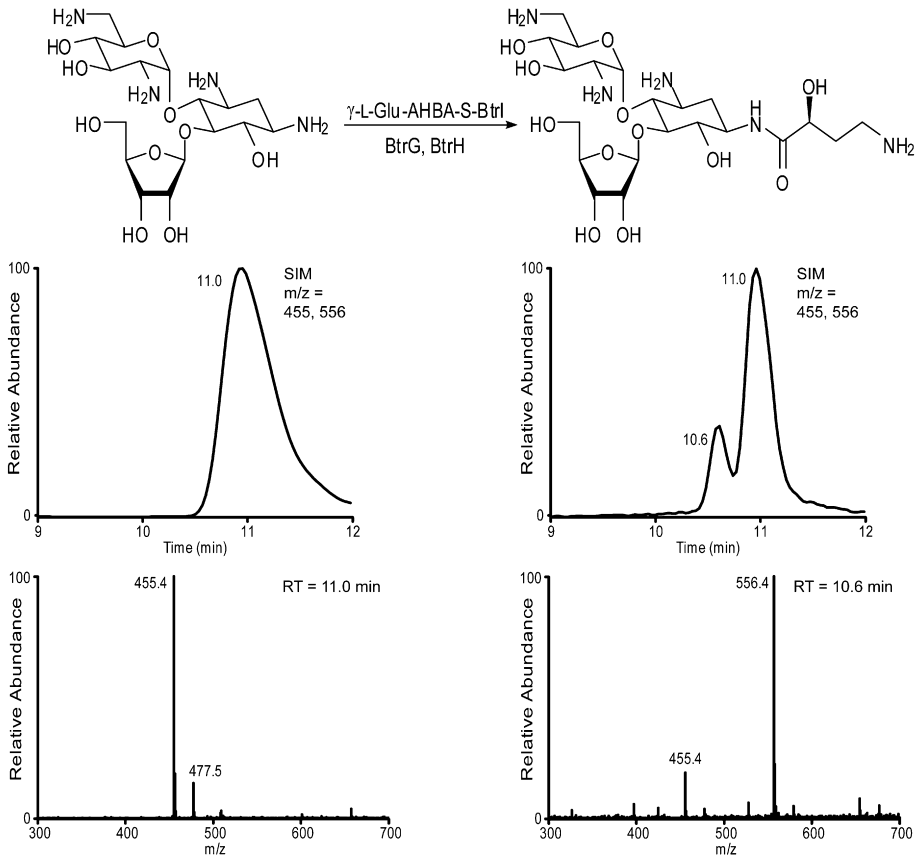

Figure 3. Production of Butirosin by BtrG and BtrH

Total ion current (top) and mass spectrum (bottom) traces showing conversion of ribostamycin to butirosin by BtrG and BtrH.

Incubation of γ-L-Glu-AHBA-S-BtrI with heterologously expressed BtrG and BtrH in the presence of ribostamycin yields authentic butirosin as judged by ion-pairing LC-ESI-MS/MS analysis (Figure 3; and see the Supplemental Data available with this article online), confirming the proposed roles of BtrG and BtrH as the last two enzymes in butirosin biosynthesis.

Incubation of BtrH with γ-L-Glu-AHBA-S-BtrI and ribostamycin yields a new product of mass 685.4 Da by ion-pairing LC-ESI-MS/MS (Figure 4). This mass and the observed mass fragmentations (Supplemental Data) are consistent with γ-L-Glu-butirosin ($[M+H]^+$ calculated at 685.32). This result assigns BtrH as the expected aminoglycoside 1-*N*-acyltransferase responsible for the ligation of the side chain in butirosin and demonstrates that acyl transfer precedes deglutamylation, consistent with the characterization of the γ-glutamyl moiety as a protective group. The aminoglycoside acetyltransferases (AACs) are obvious functional relatives of BtrH. Crystal structures determined for AAC-3 from *Serratia marcescens* [29] and AAC-6′ from *Enterococcus faecium* [30] reveal these two enzymes to share a common fold. These AACs also exhibit good sequence similarity (33% identity, 53% similarity over 71 residues) by pairwise alignment. However, BtrH exhibits no sequence similarity to any AACs and does not contain any known conserved domains [31]. This apparent lack of homology is rationalized by the fact that BtrH (1) utilizes a long dipeptide acyl donor substrate, whereas the AACs utilize a short acetyl substrate, and (2) transfers the acyl chain from an ACP, whereas the AACs utilize acetyl-CoA. Therefore, BtrH may represent a novel aminoglycoside acyltransferase.

The efficient production of γ-L-Glu-butirosin by BtrH in the absence of BtrG demonstrates that BtrG is responsible for the deprotection of the γ-L-Glu-AHBA side chain following transfer onto ribostamycin. This order of operations is further supported by the observation that incubation of γ-L-Glu-AHBA-S-BtrI with BtrG alone does not lead to generation of AHBA-S-BtrI (data not shown). Functionally, BtrG resembles PuuD, the γ-glutamyl-γ-aminobutyrate hydrolase involved in the putrescine utilization pathway of *E. coli* K12 [32, 33]. However, BtrG bears no sequence similarity to PuuD, suggesting that it might employ a different mechanism for cleavage of the γ-L-Glu protective group. While PuuD is thought to employ a water molecule as nucleophile in a hydrolytic mechanism, it was proposed that BtrG might instead act as a cyclotransferase, cleaving the amide bond via transamidation using the α-amine of γ-L-Glu to yield butirosin and pyroglutamate as products. This hypothesis was tested by

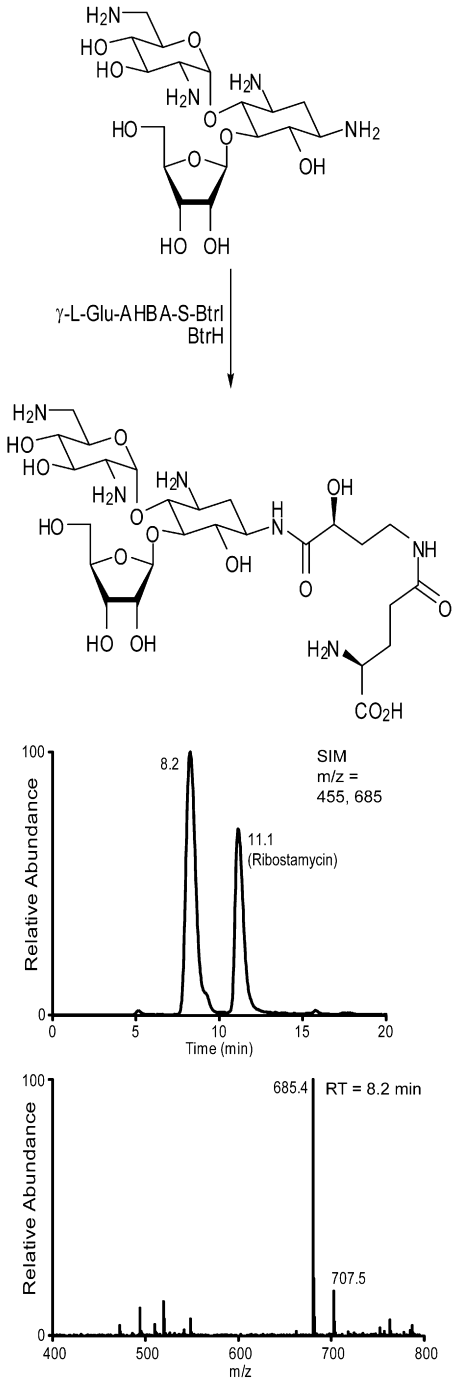

Figure 4. Acyltransferase Activity of BtrH

Total ion current and mass spectrum traces showing conversion of ribostamycin to γ-L-Glu-butirosin by BtrH.

ion-pairing LC-ESI-MS/MS using selective reaction monitoring (SRM) for the neutral deformylation of pyroglutamate (m/z 130 → 84) [34]. Assays of BtrG with BtrH, γ-L-Glu-AHBA-S-BtrI, and ribostamycin showed a peak under SRM at the same retention time as a pyroglutamate standard (Figure 5), while no such peak was seen in control experiments lacking BtrI, BtrH, or ribostamycin, or in standards of L-glutamate. BtrG is therefore assigned as a novel γ-glutamyl cyclotransferase (GGC).

This activity is comparable to the GGC activity observed in the γ-glutamyl cycle related to glutathione metabolism and amino acid transport [35, 36]. In this cycle, γ-glutamylated α-amino acids are digested to give pyroglutamate and the liberated amino acid. Interestingly, while the activity of this GGC has been extensively characterized in both tissue extracts and purified enzyme preparations (~25 kDa) [36, 37], neither gene nor peptide sequence for this enzyme is known to date (though an observed genetic polymorphism has isolated the GGC gene in mice to a region on chromosome 6 [38]). Further, a functionally similar γ-glutamylamine cyclotransferase (GGAC) enzyme has been identified in rabbit kidney which is separable from GGC and which exhibits differing substrate specificity [39]. It is hoped that the functional characterization of BtrG might lead to the positive identification of the GGC and GGAC genes. While these results are consistent with the proposed cyclizing transamidation of the substrate directly, an alternative mechanism involving initial formation of a γ-L-glutamyl-BtrG intermediate followed by pyroglutamate release cannot be excluded, and studies into the reaction mechanism of BtrG are ongoing.

Timing of Side Chain Transfer

With the functional assignments of BtrG and BtrH, the complete pathway for the biosynthesis and incorporation of the valuable AHBA side chain in butirosin has been fully elucidated. To verify the timing of side chain addition in this biosynthetic pathway, the activity of BtrH was assayed toward the major intermediates in the biosynthesis of the aminoglycoside scaffold. Of the biosynthetic precursors of ribostamycin, 2-DOS and paromamine are not substrates for BtrH. Neamine is acylated by BtrH, though less efficiently than ribostamycin (Supplemental Data), and no acylation of neamine is observed in a competitive assay with ribostamycin. This result is in keeping with the fact that AHBA-neamine has not been observed in cultures of *B. circulans* and has only been previously accessed by acid degradation of butirosin [40, 41]. Thus, ribostamycin is the preferred natural substrate for BtrH, supporting the original assignment of side chain addition as the final step in butirosin biosynthesis [20].

Formation of Alternative Aminoglycoside Products

In order to explore opportunities to apply this pathway to the preparation of new and potentially useful antibiotics, the tolerance of BtrG and BtrH for alternative aminoglycoside acceptors was investigated. In addition to ribostamycin, BtrH accepts the related 4,5-disubstituted aminoglycosides paromomycin and neomycin (Figure 6 and Supplemental Data), but not the 4,6-disubstituted aminoglycosides gentamicin C or kanamycin. Again, alternative substrates are processed with diminished efficiency relative to the natural substrate, ribostamycin. All γ-L-Glu-AHBA-aminoglycosides produced by BtrH are cleaved to the corresponding AHBA-aminoglycoside by BtrG,

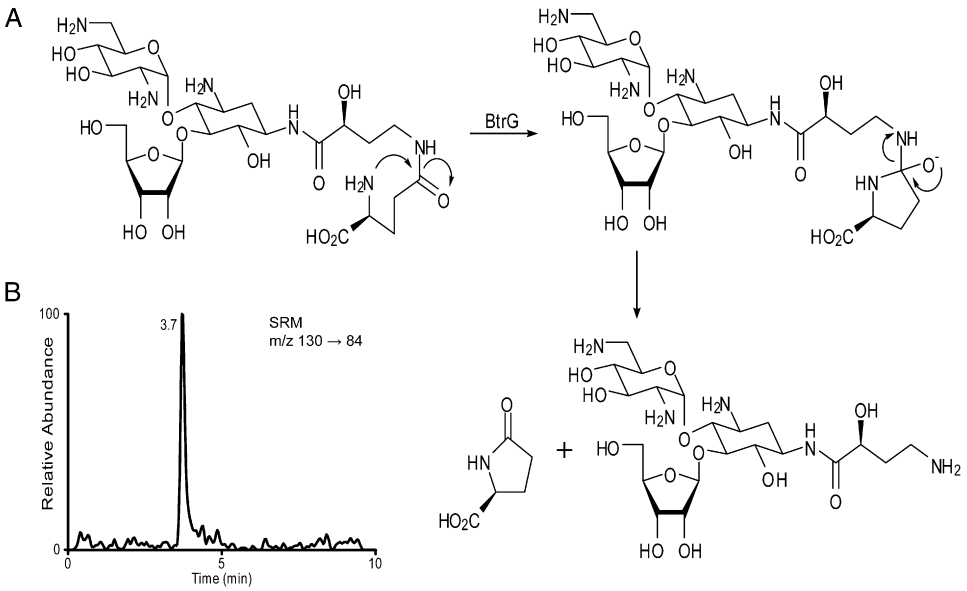

Figure 5. γ-Glutamyl Cyclotransferase Activity of BtrG
(A) Proposed mechanism of deglutamylation by BtrG.
(B) Selective reaction monitoring (SRM) trace demonstrating evolution of pyroglutamate in the course of deprotection.

indicating good substrate tolerance for this enzyme (Supplemental Data). While AHBA-paromomycin [42] and AHBA-neomycin [43] have been previously prepared by synthetic acylation of the parent natural product, these compounds were accessible only in low yield due to the difficulty of selectively acylating only the C-1 amine in the presence of several other nucleophilic groups. Biosynthetic preparation is an attractive alternative route toward these unnatural compounds, which are reported to have valuable antibiotic properties, particularly toward *Pseudomonas aeruginosa*. These results encourage attempts toward the engineered biosynthesis of both known and novel AHBA-bearing aminoglycosides.

SIGNIFICANCE

The pathway for ACP-mediated biosynthesis and transfer of the valuable (*S*)-4-amino-2-hydroxybutyrate (AHBA) side chain in butirosin has been completely elucidated. This pathway includes unusual protection/deprotection chemistry in the form of the γ-L-glutamylation of a potentially labile ACP-bound intermediate, transfer of the protected, fully elaborated side chain to ribostamycin by the acyltransferase BtrH, and intramolecular cyclic deglutamylation of the product by the γ-glutamyl cyclotransferase BtrG. The transfer and deprotection of the AHBA side chain constitute the final steps in the biosynthesis of butirosin. The catalytic activity of BtrG is of particular interest as it is, to our knowledge, the first enzyme containing the COG2105 domain architecture to be functionally characterized and may lead to the discovery of interesting biosynthetic chemistry in other organisms. BtrG and BtrH are capable of acting on nonnative aminoglycoside substrates, raising the prospect for engineered biosynthesis of novel AHBA-bearing aminoglycosides.

EXPERIMENTAL PROCEDURES

Aminoglycosides were purchased from Sigma-Aldrich, with the exception of 2-DOS, paromamine, and neamine, which were prepared according to reported methods [44]. Acetonitrile (MeCN) for HPLC was purchased from Fisher. HPLC buffer additives trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), and pentadecafluorooctanoic acid (PDFOA) were purchased from Sigma-Aldrich. All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Cloning of *btrG* and *btrH* Genes

The genes *btrG* and *btrH* were amplified by PCR from genomic DNA of *Bacillus circulans* NR3312 using the following pairs of primers, respectively (restriction sites are underlined): Forward pG1 5′-CGTAAGTGAG TTTAAGCATATGATTAGCTGGACGAA-3′ (NdeI) and reverse pG2 5′-GCGATTTTCTGCACTCGAGAAATTTCTCGTCATAAC-3′ (XhoI); forward pH1 5′-AGAGGAGAAGCCATATGTGCCTCACTCGTTATGAC G-3′ (NdeI) and reverse pH2 5′-TCCTTCTGTATGGGATCCCGGTTTC CGCACAGCGGT-3′ (BamHI).

PCR reactions were carried out as follows using *Pfu* polymerase (Stratagene): 4 min at 94°C, then 1 min at 94°C, 1 min at 60°C, 2 min at 72°C for 25 cycles, final extension at 72°C for 10 min. The amplified genes were cloned into appropriate restriction sites of pET-28a(+) (Novagen) and transformed into *E. coli* BL21(DE3) (Novagen) for overexpression. All constructs were checked by DNA sequencing.

Overexpression and Purification of Recombinant BtrG and BtrH

One liter of culture in LB medium of *E. coli* BL21(DE3) (Novagen) harboring recombinant plasmid was grown at 37°C for 3 hr with kanamycin selection (50 μg/ml). Protein overexpression was induced by addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG), and

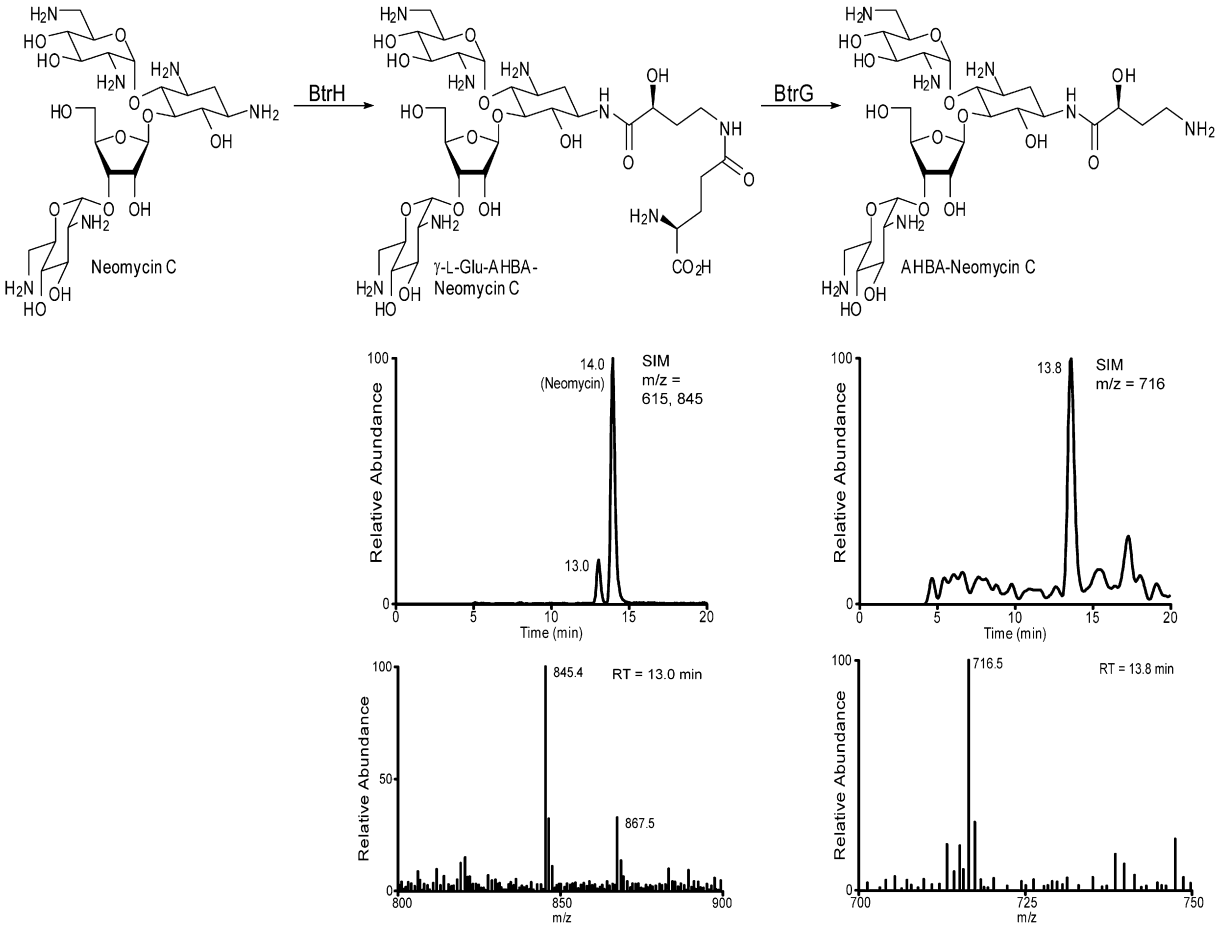

Figure 6. Acylation and Deprotection of the Unnatural Substrate Neomycin

LC-ESI-MS traces demonstrating the acylation of neomycin by BtrH and the deprotection of the intermediate by BtrG to give AHBA-neomycin.

cell growth continued at 16°C overnight. Ni^{2+}-NTA affinity chromatography purification was performed according to the manufacturer's protocols (Novagen). The harvested cells were resuspended in 30 ml binding buffer (20 mM Tris-HCl [pH 7.9], 0.5 M NaCl, 5 mM imidazole, 10% glycerol) and disrupted by sonication. N-terminally His_6-tagged proteins were purified in one step by Ni^{2+}-NTA affinity column and exchanged into storage buffer by ultrafiltration. The purified proteins were stored in 50 mM HEPES (pH 7.0) containing 50% glycerol at −20°C.

LC-ESI-MS Analysis of Proteins

LC-ESI-MS analysis of protein samples was performed on a Finnigan LCQ (Thermo Finnigan) coupled with an HP1100 HPLC system (Agilent). Samples were separated on a 2.0 × 250 mm Jupiter 5μ C4 column (Phenomenex) by the following gradient at a flow rate of 0.2 ml/min and column temperature of 40°C: 0–5 min 5% B, 5–6 min 5%–35% B, 6–31 min 35%–65% B, 31–36 min 65% B, 36–41 min 65%–95% B (buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in MeCN). Absorbance was monitored at 214 nm. Mass spectra were acquired from 200 to 2000 Da.

Synthesis of *N*-Boc-α-*tert*-Butyl-γ-L-Glu-AHBA Dipeptide

N-Boc-L-glutamic acid α-*tert*-butyl ester (0.25 mmol) was dissolved in 5 ml acetonitrile under nitrogen. The solution was cooled in an ice bath, and triethylamine (0.4 mmol), *p*-nitrophenylchloroformate (0.3 mmol) and 4-dimethylaminopyridine (0.02 mmol) were added. After 1 hr, (*S*)-4-amino-2-hydroxybutyric acid (1 mmol) and triethylamine (1.05 mmol) were dissolved in 5 ml H_2O and added dropwise to the reaction. The reaction stirred for 1 hr at room temperature, and then acetonitrile was removed by evaporation. The remaining solution was stirred vigorously with 20 ml ethyl acetate and acidified to pH ~2 with 1 M HCl. The organic phase was separated and the aqueous layer was washed twice with 20 ml ethyl acetate. Organic extracts were dried over magnesium sulfate, filtered, and dried by evaporation. The residue was taken up in 5 ml ethyl acetate, and the product was precipitated by addition of 20–30 PET ether. The precipitate was collected by centrifugation, dissolved in methanol, and evaporated to give 65 mg (0.16 mmol, 64%) of product as a fluffy white solid.

Synthesis of γ-L-Glu-AHBA-CoA

N-Boc-α-*tert*-butyl-γ-L-Glu-AHBA dipeptide (13.5 μmol), *N*,*N*′-dicyclohexylcarbodiimide (17.0 μmol), and *N*-hydroxysuccinimide (26.0 μmol) were dissolved in 1.5 ml acetonitrile under nitrogen. The reaction was stirred overnight at room temperature, and then coenzyme A (20.4 μmol) was dissolved in 0.25 ml of 40 mM aqueous lithium carbonate and added dropwise to the reaction. After stirring at room temperature for 4 hr, the reaction mixture was subjected directly to preparative HPLC on a 21.2 × 250 mm Luna 10μ C18(2) column (Phenomenex) by the following gradient at a flow rate of 15 ml/min at room temperature: 0–5 min 5% B, 5–25 min 5%–40% B, 25–27 min 40%–95% B, 27–30 min 95% B (buffer A: 0.1% TFA in H_2O; buffer B: 0.1% TFA in MeCN). Absorbance was monitored at 254 nm and

fractions containing product were pooled. Acetonitrile was removed by evaporation, and water was removed by lyophilization.

The dried residue was dissolved in 1 ml of trifluoroacetic acid:H_2O: triisopropylsilane (95:2.5:2.5) under nitrogen and stirred at room temperature for 2 hr. The product was precipitated by addition of 10 ml ice-cold ether and collected by centrifugation. The precipitate was taken up in H_2O and lyophilized to give 3 mg (3.0 μmol, 22% over two steps) product.

4′-Phosphopantetheinylation of *apo*-BtrI

A reaction mixture (total volume 10 μL) containing 50 mM HEPES (pH 6.5), 1 mM γ-L-Glu-AHBA-CoA, 1 mM $MgCl_2$, 0.3 nmol Sfp, and 1.5 nmol *apo*-BtrI was incubated at 37°C for 30 min and analyzed directly by LC-ESI-MS.

Enzymatic Assays of BtrH

The acyltransferase activity of BtrH was assayed in a typical 30 μl reaction containing 50 mM HEPES (pH 6.5), 1 mM γ-L-Glu-AHBA-CoA, 1 mM aminoglycoside, 0.3 nmol BtrH, 0.3 nmol Sfp, and 1.5 nmol *apo*-BtrI. The reaction was incubated at 37°C for 1.5 hr. Proteins were precipitated by heating to 95°C for 5–10 min followed by vortexing with 30 μl chloroform and centrifugation. The clear aqueous layer was taken for LC-ESI-MS/MS analysis. Samples were separated on a 2.0 × 250 mm Luna 5μ C18(2) column (Phenomenex) by the following gradient at a flow rate of 0.3 ml/min and column temperature of 40°C: 0–20 min 10%–50% B, 20–21 min 50%–10% B, 21–25 min 10% B (buffer A: 0.1% PFPA in H_2O; buffer B: 0.1% PFPA in MeCN). Mass spectra were acquired from 300 to 1000 Da (except for assays with 2-DOS as aminoglycoside acceptor, in which case mass spectra were acquired from 150 to 1000 Da). MS/MS was carried out on target ions with 20% relative collision energy (helium as collision gas).

Enzymatic Assays of BtrG

The deglutamylating activity of BtrG was assayed in a typical 30 μl reaction as above, with the addition of 60–70 pmol BtrG.

Determination of Pyroglutamate Formation by BtrG

A typical 30 μl BtrG assay was incubated and worked up as described above, except samples were not heat-denatured prior to chloroform precipitation to avoid potential nonenzymatic cyclization of glutamate. Samples were separated on a 2.0 × 250 mm Luna 5μ C18(2) column (Phenomenex) by the following gradient at a flow rate of 0.25 ml/min and column temperature of 40°C: 0–4 min 5% B, 4–7 min 5%–45% B, 7–9 min 45% B, 9–10 min 45%–5% B (buffer A: 1 mM PDFOA and 0.05% TFA in H_2O; buffer B: 1 mM PDFOA and 0.05% TFA in MeCN). Mass spectra were acquired with selective reaction monitoring for the neutral deformylation of pyroglutamate (m/z 130 → 84).

Supplemental Data

Supplemental Data include four figures and are available at http://www.chembiol.com/cgi/content/full/14/4/379/DC1/.

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