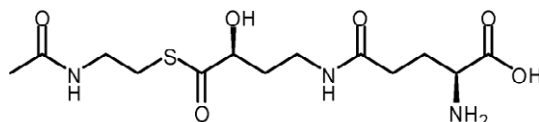


Experimental Details

Unless otherwise noted, all chemicals were purchased from Sigma-Aldrich Chemical Company and were used as received. 2-DOS and neamine were kindly provided by Dr. F. Huang. Paromamine was prepared by acid methanolysis of paromomycin according to the method of Dutcher and Donin.¹ The expression and purification of butirosin biosynthetic enzymes has been described elsewhere.²

Synthesis of γ -L-Glu-AHBA-SNAC (2)



N-Boc- α -*tert*-butyl- γ -L-Glu-AHBA (126 μ mol, synthesis described elsewhere)² was dissolved with gentle heating in 5 mL acetonitrile under nitrogen. A solution of *N,N'*-dicyclohexylcarbodiimide (132 μ mol) and 1-hydroxybenzotriazole hydrate (131 μ mol) in 5 mL acetonitrile was added slowly at room temperature, followed by neat *N*-acetylcysteamine (137 μ mol). The reaction was stirred for 1 hour at room temperature, then potassium carbonate (65 μ mol) was added and the reaction stirred a further 3 hours. Acetonitrile was removed by rotary evaporation, and the resulting residue was taken up in 10 mL ethyl acetate and extracted three times with 10 mL saturated aqueous sodium bicarbonate. The organic layer was dried over magnesium sulfate, filtered, and evaporated to a solid yellow residue. The dried residue was dissolved in 1 mL of trifluoroacetic acid:H₂O:triisopropylsilane (95:2.5:2.5) under nitrogen and stirred at room temperature for 1.5 hours. The product was precipitated by addition of 10 mL ice-cold diethyl ether and collected by centrifugation. The precipitate was taken up in H₂O and lyophilized to give 33 mg of **2** (94.4 μ mol, 75% over two steps).

HRMS (ESI) calculated for C₁₃H₂₄N₃O₆S ([M+H]⁺): 350.1386. Found: 350.1370 (-3.1 ppm). ¹H NMR (500 MHz, D₂O) δ : 1.72 (1H, m), 1.86 (3H, s), 1.92 (1H, m), 2.09 (2H, m), 2.36 (2H, m), 2.95 (2H, m), 3.24 (2H, m), 3.29 (2H, m), 3.88 (1H, t, *J* = 6.3 Hz), 4.22 (dd, *J*₁ = 3.9 Hz, *J*₂ = 9 Hz); ¹³C NMR (500 MHz, D₂O) δ : 21.7, 25.7, 27.2, 31.1, 33.0, 35.3, 38.4, 52.6, 74.9, 171.9, 174.1, 174.3, 207.4.

LC-ESI-MS/MS Analysis of Aminoglycosides

Aminoglycosides were analyzed by on-line LC-ESI-MS (Helium collision gas) using a Finnigan LCQ ion-trap mass spectrometer (Thermo Electron) coupled to an HP1100 HPLC system (Agilent). Aminoglycosides were separated on a 2.0 $\times$ 250 mm Luna 5 μ C18(2) column (Phenomenex) with the following gradient at a flow rate of 0.3 mL/min at 40 $^{\circ}$ C: 0 – 5 minutes, 10% B; 5 – 25 minutes, 10% – 50% B; 25 – 26 minutes, 50% – 10% B; 26 – 30 minutes, 10% B (buffer A: 0.1% pentafluoropropionic acid (PFPA) in H₂O; buffer B: 0.1% PFPA in

acetonitrile). Mass spectra were acquired between 300 and 1000 Da (except for the analysis of reactions involving 2-DOS, where mass spectra were acquired between 150 and 1000 Da). MS/MS analysis of peaks of interest was accomplished with relative fragmentation energy of 22% – 23.5%.

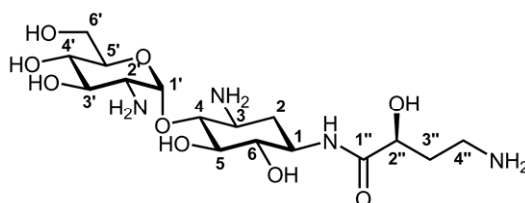
BtrH Activity Assay

The acyltransferase activity of BtrH with **2** as the acyl donor was assayed in a typical 50 μ L reaction containing 50 mM HEPES (pH 7.9), 5 mM **2**, 1 mM aminoglycoside, and ~2 nmol BtrH. The reaction was incubated at 30 $^{\circ}$ C for 6 to 24 hours. Proteins were precipitated by vortexing with 50 μ L chloroform and centrifugation. The clear aqueous layer was taken for LC-ESI-MS/MS analysis.

BtrG Activity Assay

The deglutamylating activity of BtrG was assayed in a typical 30 μ L reaction composed of 27 μ L of the BtrH reaction described above (after chloroform precipitation) and ~2 nmol BtrG. The reaction was incubated at 30 $^{\circ}$ C for 4 to 24 hours. Proteins were precipitated by vortexing with 30 μ L chloroform and centrifugation. The clear aqueous layer was taken for LC-ESI-MS/MS analysis.

Milligram-scale preparation of AHBA-paromamine



A 5 mL reaction containing 50 mM HEPES (pH 7.9), 5 mM **2**, 2 mM paromamine, and ~40 μ M BtrH was incubated at room temperature for 72 hours. BtrG was then added directly to a concentration of ~10 μ M, and the reaction was incubated at room temperature for a further 72 hours. Protein was removed by precipitation at 96 $^{\circ}$ C for 15 minutes followed by centrifugation at 13,000 rpm for 10 minutes. The cleared supernatant was twice vortexed vigorously with an equal volume of chloroform and centrifuged at 4,500 rpm for 10 minutes to remove residual protein. The aqueous layer was applied to a column of Dowex 50WX2 cation exchange resin (H^+ form, 400 mg dry weight), washed with 50 mL H_2O , and eluted with 25 mL of 3% aqueous NH_4OH solution. Fractions containing aminoglycoside were pooled and repeatedly reduced by rotary evaporation to remove ammonia. The residue was taken up in H_2O and applied to a column of Dowex 1X8 anion exchange resin (OH^- form, 3 g dry weight) to remove residual HEPES. The product was eluted from the column with water, and fractions containing aminoglycoside were pooled and lyophilized to afford 3.5 mg (8.2 μ mol, 82% yield) AHBA-paromamine as the free base. The 1H NMR signal for H-4 was unambiguously assigned by correlation with the anomeric proton H-1'

by NOESY, which allowed the assignment of all proton and carbon signals by COSY and HMQC experiments. The strong downfield shift of the signal for H-1 relative to H-3 confirms that the AHBA side chain is attached at the C-1 amine as anticipated.

HRMS (ESI) calculated for $C_{16}H_{32}N_4O_9Na$ ($[M+Na]^+$): 447.2061. Found: 447.2056 (-1.2 ppm). 1H NMR (500 MHz, D_2O) δ : 1.31 (1H, H-2(ax), ddd, $J_1 = J_2 = J_3 = 12.5$ Hz), 1.77 (1H, H-3'', m), 1.90 (2H, H-2(eq) and H-3'', m), 2.73 (1H, H-2', dd, $J_1 = 10.3$ Hz, $J_2 = 3.8$ Hz), 2.77 (2H, H-4'', t, $J = 7.2$ Hz), 2.82 (1H, H-3, m), 3.21 (1H, H-4, dd, $J_1 = J_2 = 9.3$ Hz), 3.32 (1H, H-4', dd, $J_1 = J_2 = 9.6$ Hz), 3.39 (1H, H-6, dd, $J_1 = J_2 = 9.8$ Hz), 3.51 (2H, H-5 and H-3', m), 3.68 (1H, H-6', dd, $J_1 = 12.6$ Hz, $J_2 = 5.9$ Hz), 3.77 (3H, H-1 and H-5' and H-6', m), 4.17 (1H, H-2'', dd, $J_1 = 8.0$ Hz, $J_2 = 4.1$ Hz), 5.17 (1H, H-1', d, $J = 3.8$ Hz); ^{13}C NMR (500 MHz, D_2O) δ : 33.9 (C-2), 34.7 (C-3'), 36.7 (C-4''), 49.2 (C-3), 49.4 (C-1), 55.2 (C-2'), 60.6 (C-6'), 69.8 (C-2''), 69.9 (C-4'), 72.8 (C-5'), 73.7 (C-3'), 74.0 (C-6), 76.0 (C-5), 86.9 (C-4), 101.0 (C-1'), 176.2 (C-2'').

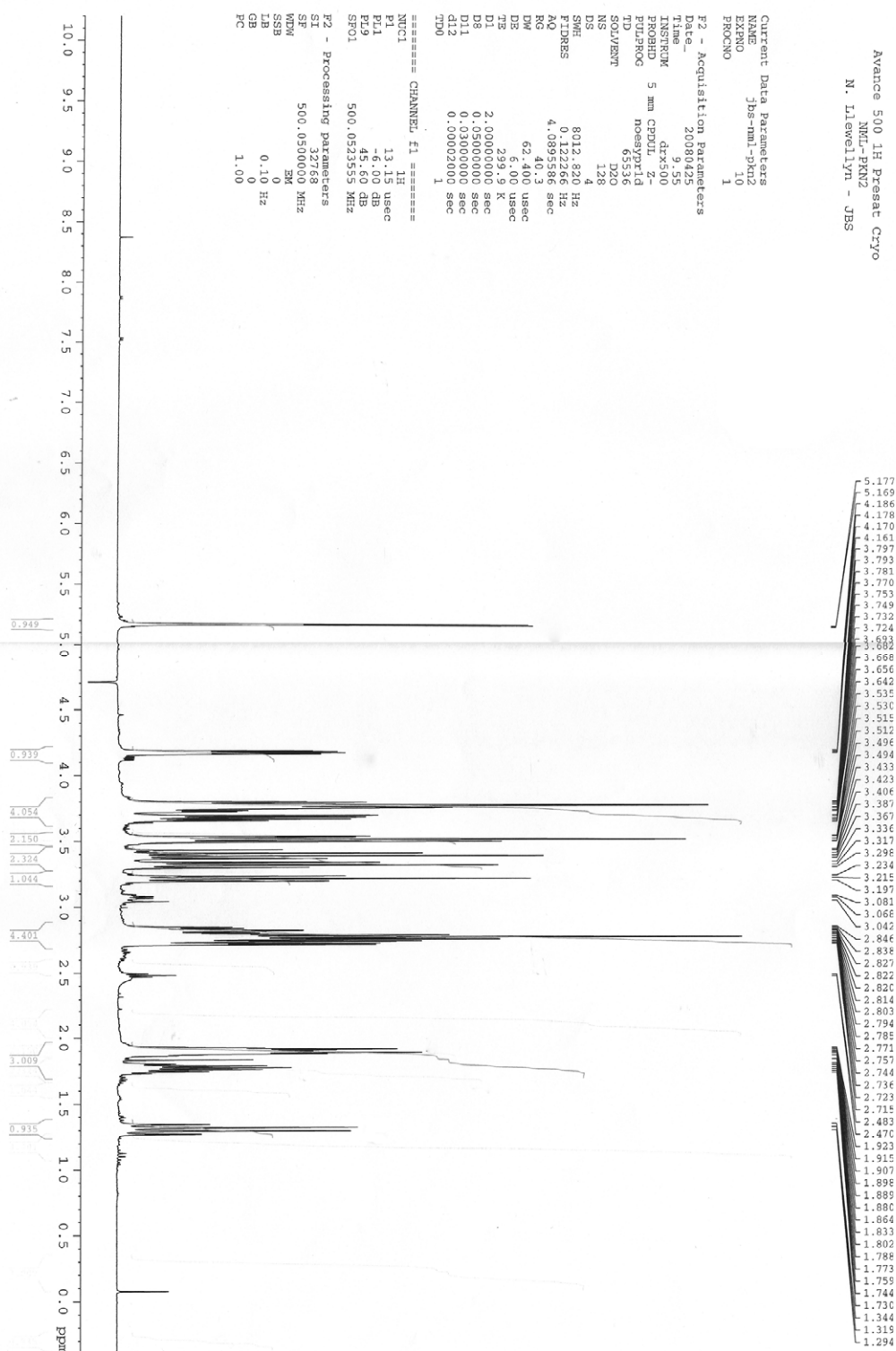


Fig. S1 ¹H NMR spectrum of AHBA-paromamine (water peak suppressed).

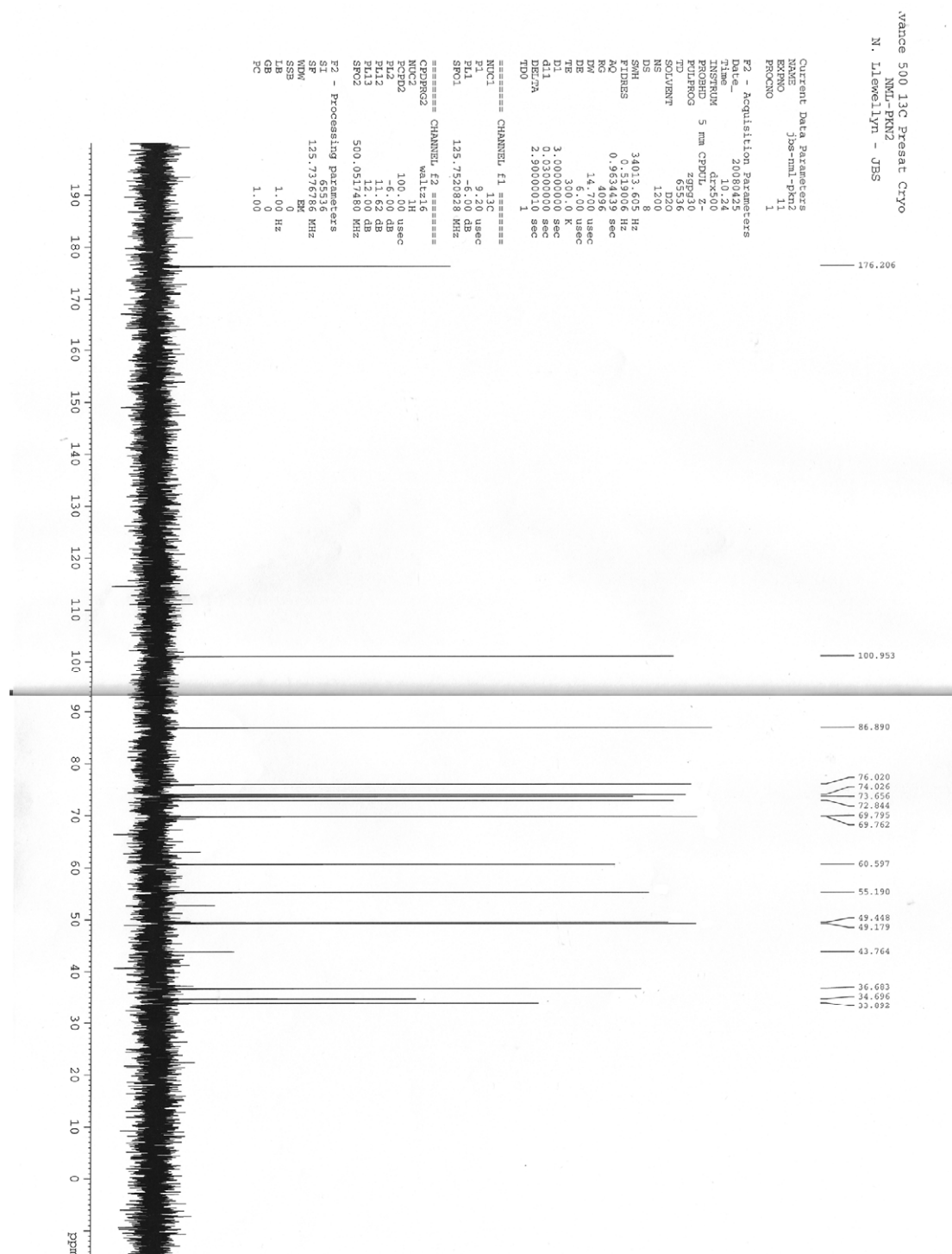


Fig. S2 ¹³C NMR spectrum of AHBA-paromamine.

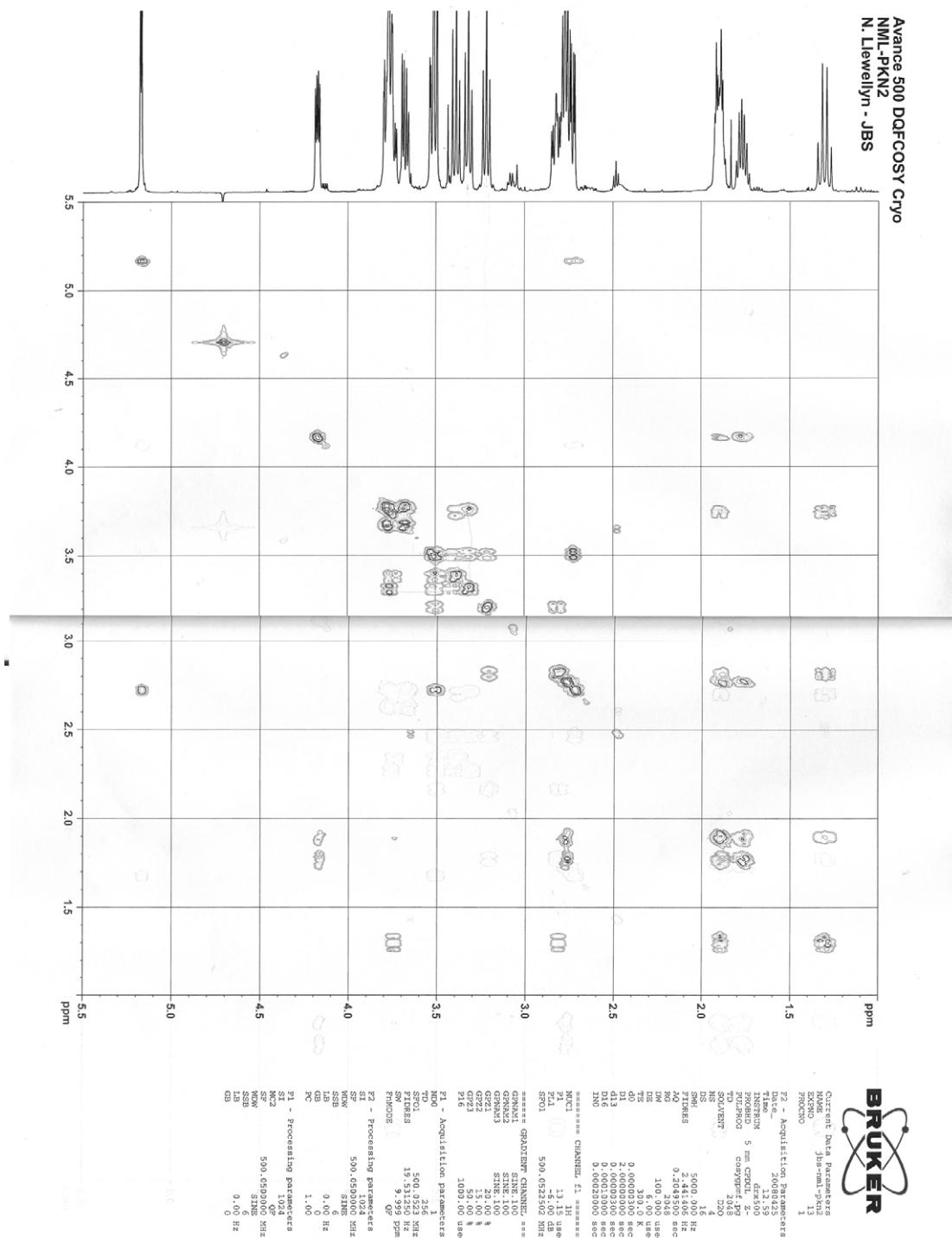
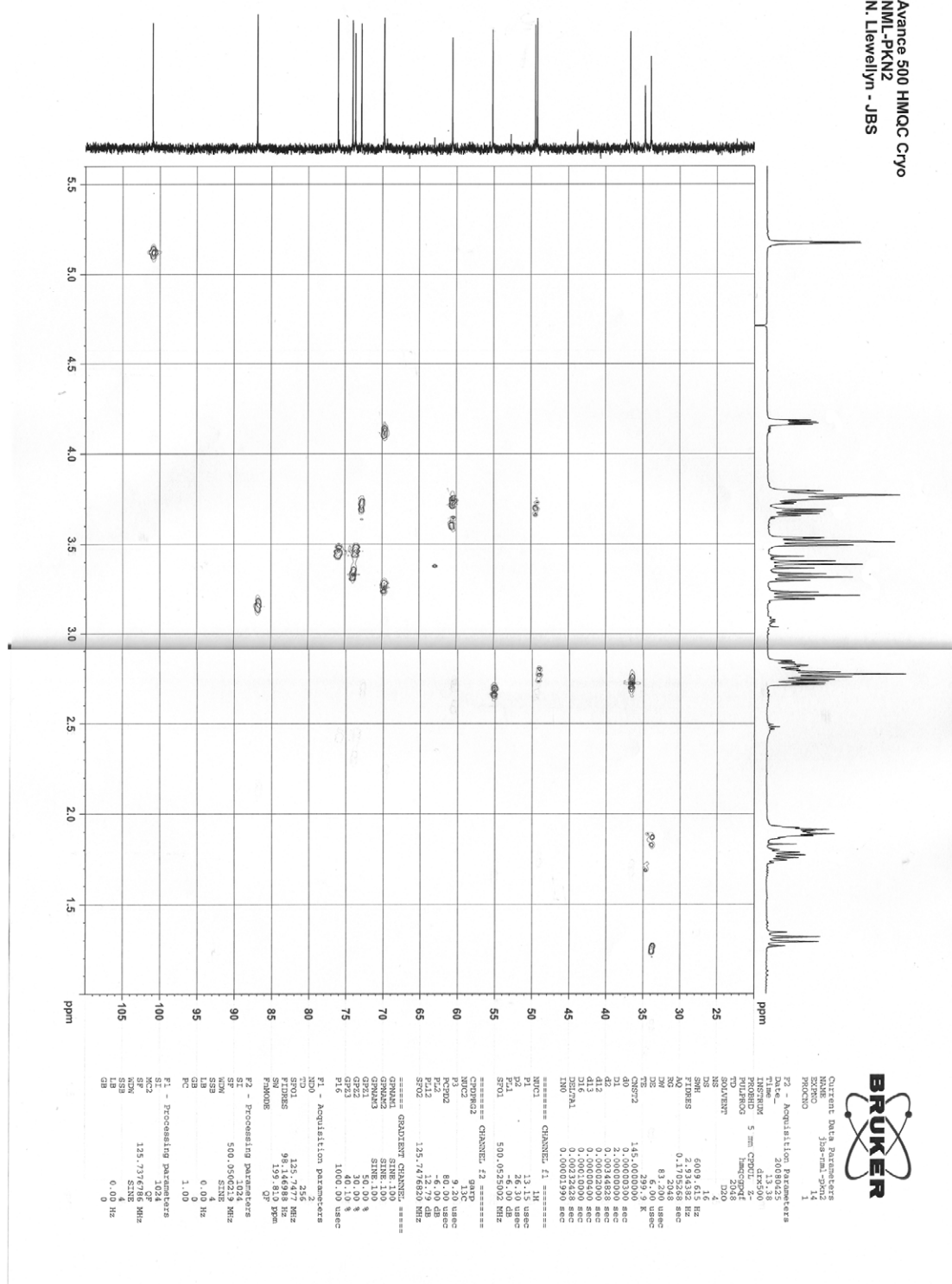
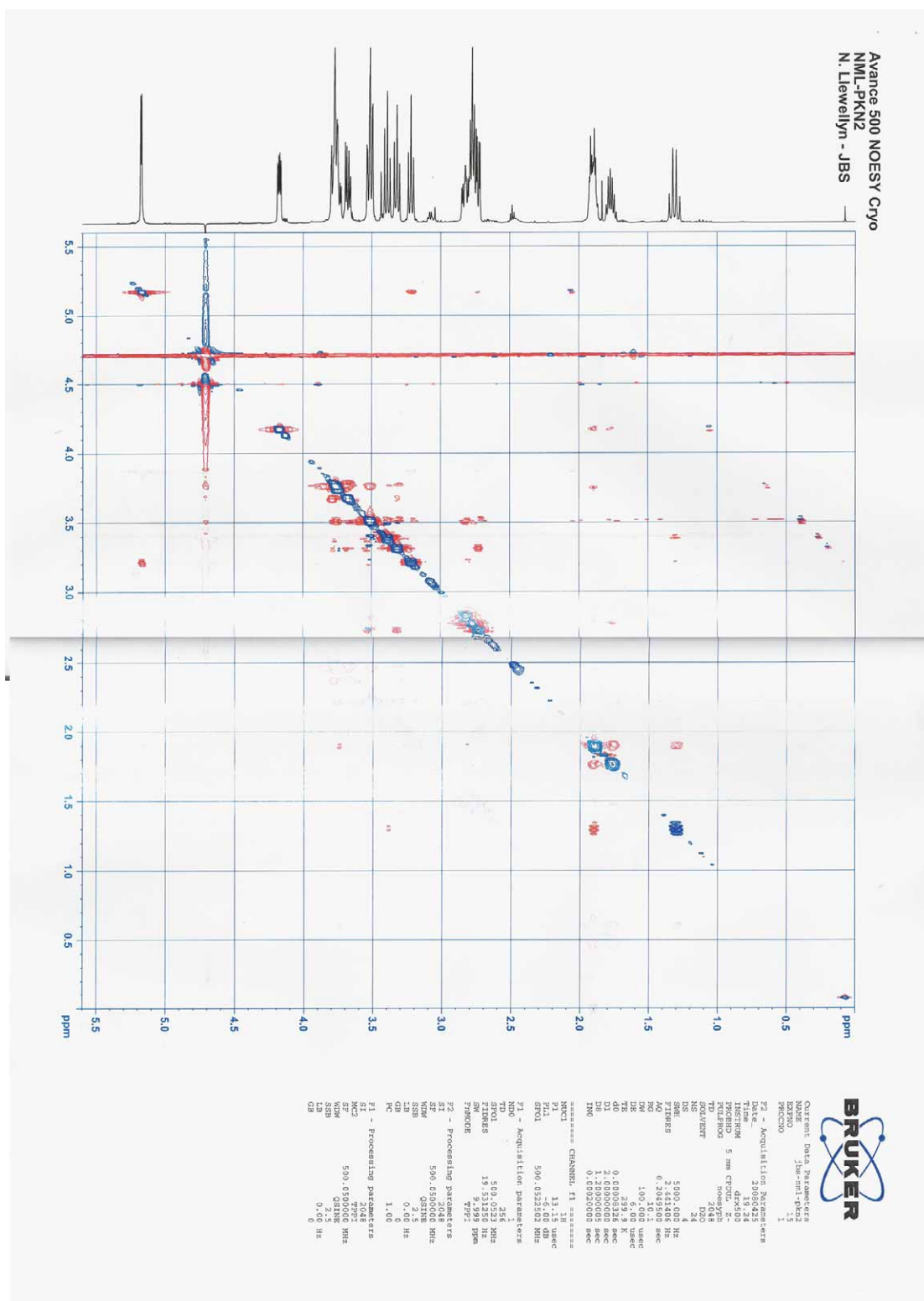


Fig. S3 COSY NMR spectrum of AHBA-paromamine (water peak suppressed).



7



8

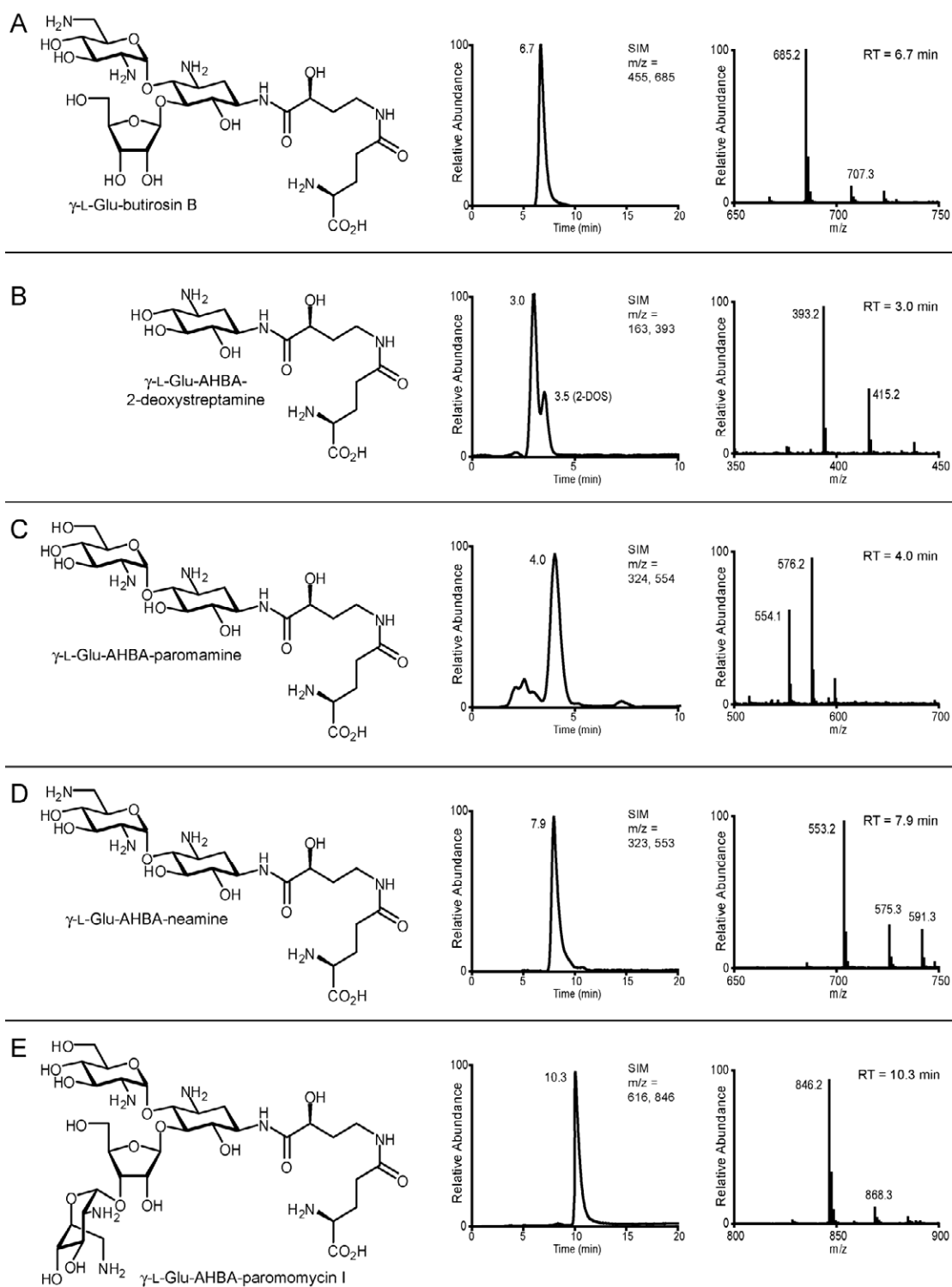


Fig. S6 BtrH-catalyzed transfer of the acyl chain from γ -L-Glu-AHBA-SNAC to A. ribostamycin; B. 2-deoxystreptamine; C. paromamine; D. neamine; E. paromomycin. Each panel includes the total ion current with selective ion monitoring (SIM) for parent aminoglycoside and γ -L-Glu-AHBA-aminoglycoside masses (left trace) and mass spectrum (right trace) of the γ -L-Glu-AHBA-aminoglycoside product. Continued overleaf.

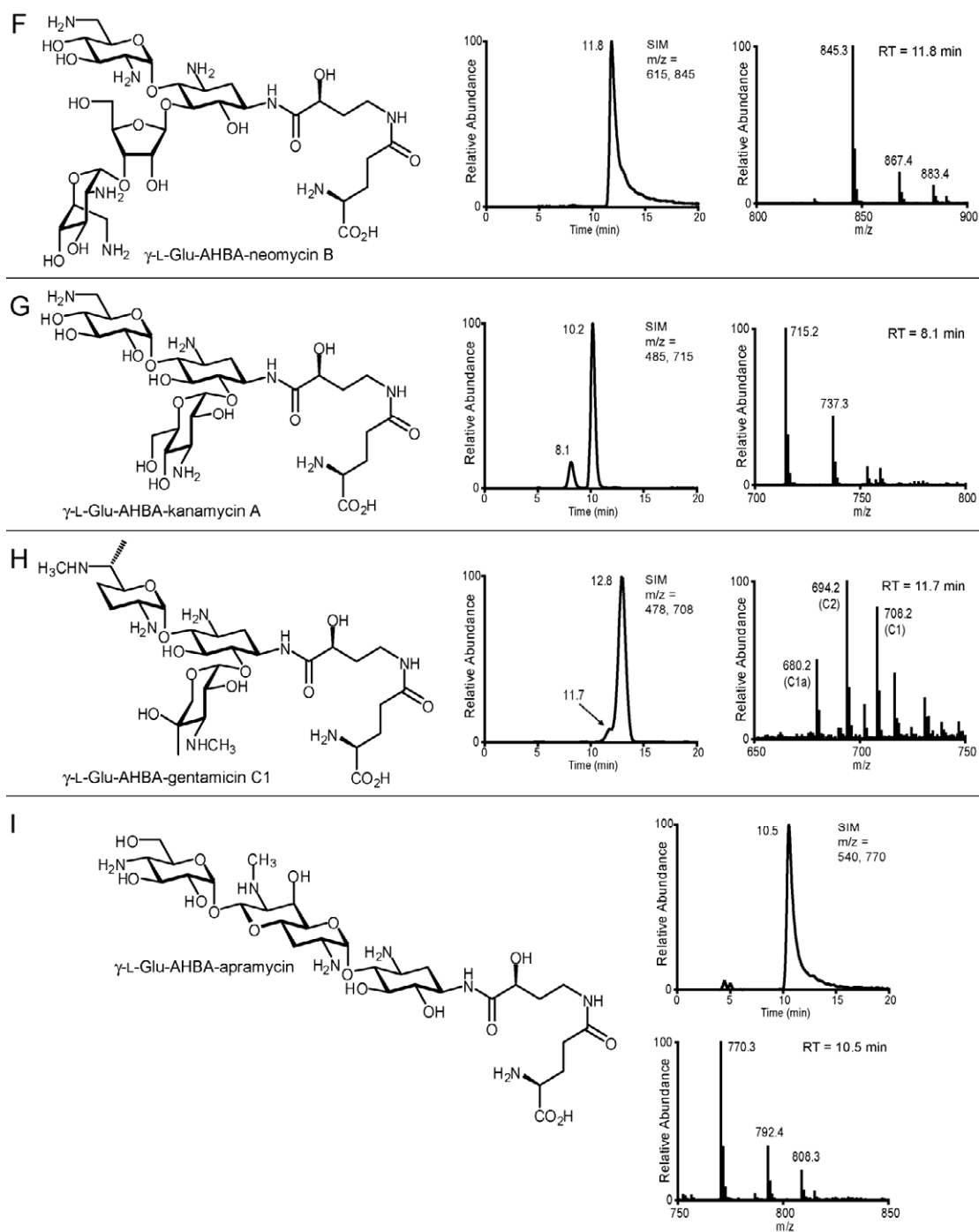


Fig. S6 Continued from previous page. BtrH-catalyzed transfer of the acyl chain from γ -L-Glu-AHBA-SNAC to F. neomycin; G. kanamycin; H. gentamicin (mixture of gentamicins C1, C2, and C1a); I. apramycin. Each panel includes the total ion current with selective ion monitoring (SIM) for parent aminoglycoside and γ -L-Glu-AHBA-aminoglycoside masses (left trace) and mass spectrum (right trace) of the γ -L-Glu-AHBA-aminoglycoside product (except for apramycin, in which the total ion current is the top trace and the mass spectrum is the bottom trace).

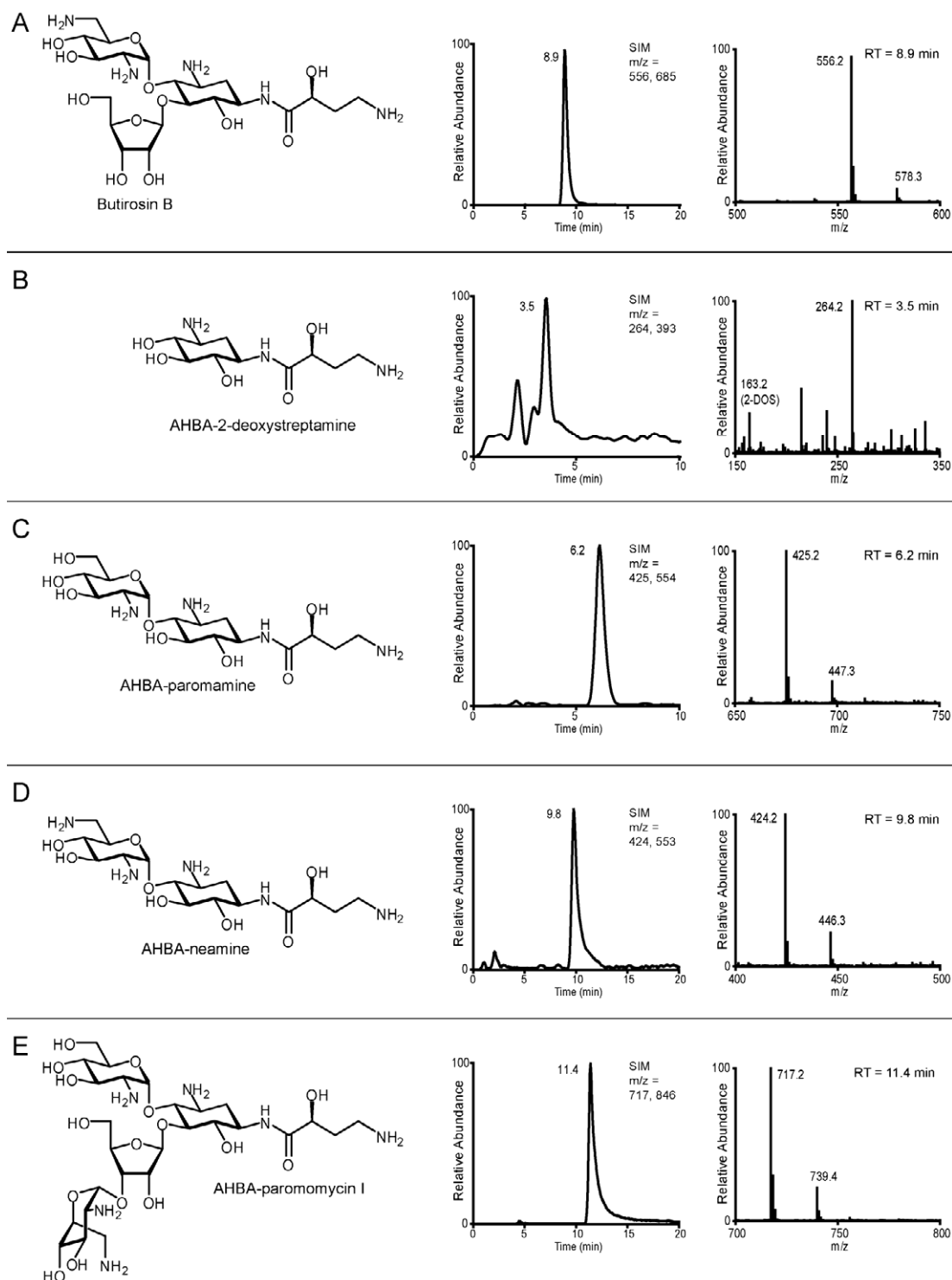


Fig. S7 BtrG-catalyzed deglutamylation of γ -L-Glu-AHBA-aminoglycosides generated by BtrH-mediated acyl transfer from γ -L-Glu-AHBA-SNAC. Incubation of γ -L-Glu-AHBA-aminoglycoside with BtrG yields A. butirosin B; B. AHBA-2-DOS; C. AHBA-paromamine; D. AHBA-neamine; E. AHBA-paromomycin. Each panel includes the total ion current with selective ion monitoring (SIM) for γ -L-Glu-AHBA-aminoglycoside and AHBA-aminoglycoside product masses (left trace) and mass spectrum (right trace) of the AHBA-aminoglycoside product. Continued overleaf.

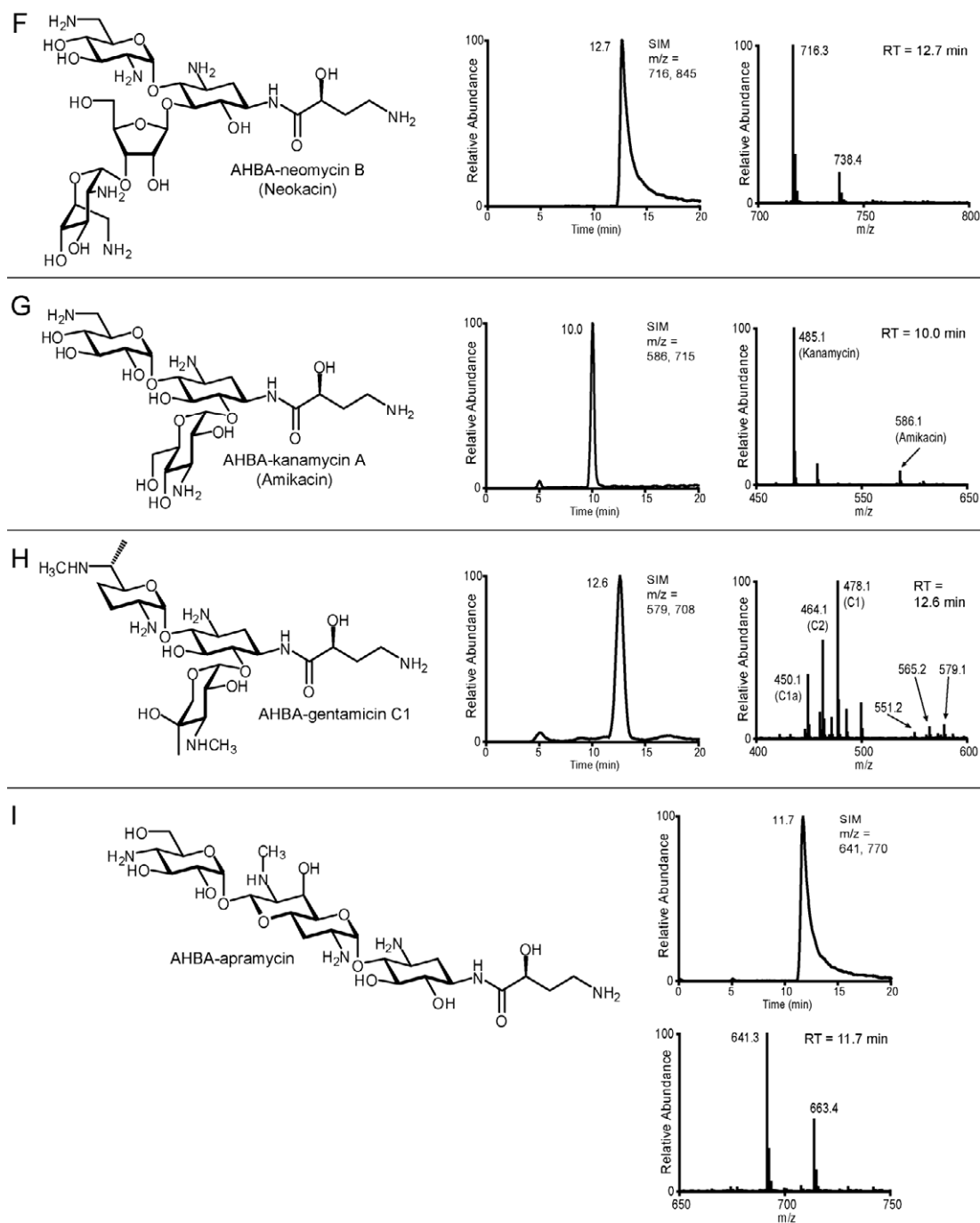


Fig. S7 Continued from previous page. BtrG-catalyzed deglutamylation of γ -L-Glu-AHBA-aminoglycosides generated by BtrH-mediated acyl transfer from γ -L-Glu-AHBA-SNAC. Incubation of γ -L-Glu-AHBA-aminoglycoside with BtrG yields F. neokacin; G. amikacin; H. AHBA-gentamicins; I. AHBA-apramycin. Each panel includes the total ion current with selective ion monitoring (SIM) for γ -L-Glu-AHBA-aminoglycoside and AHBA-aminoglycoside product masses (left trace) and mass spectrum (right trace) of the AHBA-aminoglycoside product (except for apramycin, in which the total ion current is the top trace and the mass spectrum is the bottom trace).

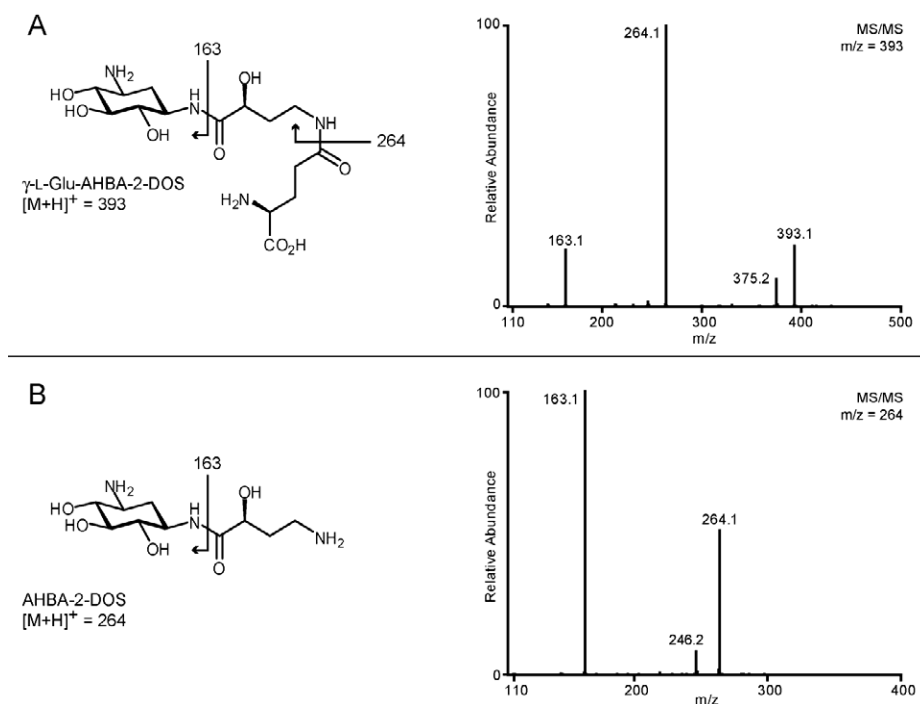


Fig. S8 Mass fragmentation spectra of A. γ -L-Glu-AHBA-2-DOS and B. AHBA-2-DOS.

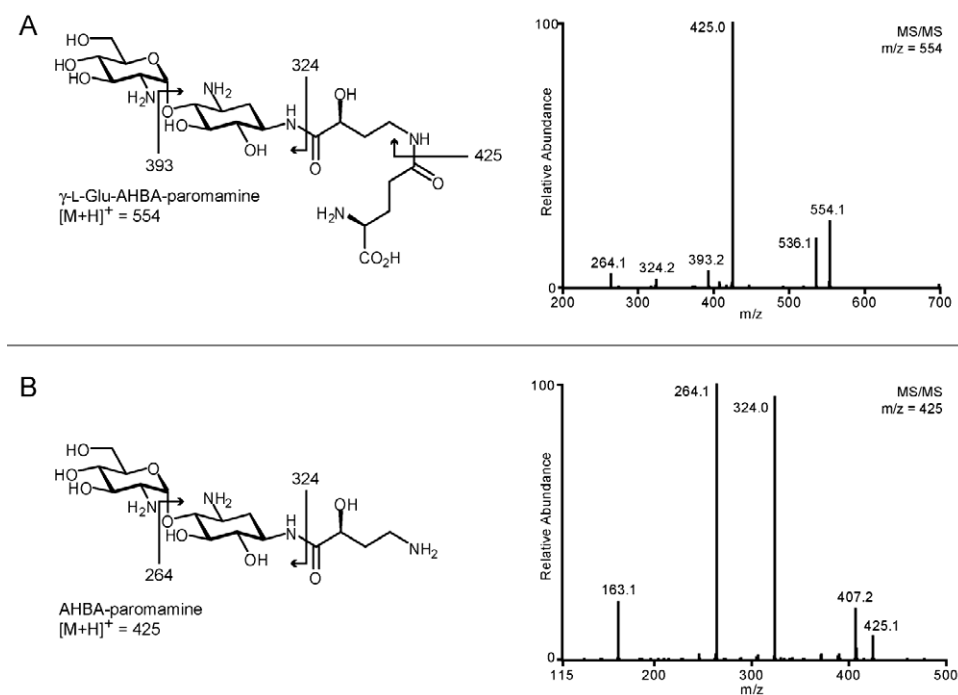


Fig. S9 Mass fragmentation spectra of A. γ -L-Glu-AHBA-paromamine and B. AHBA-paromamine.

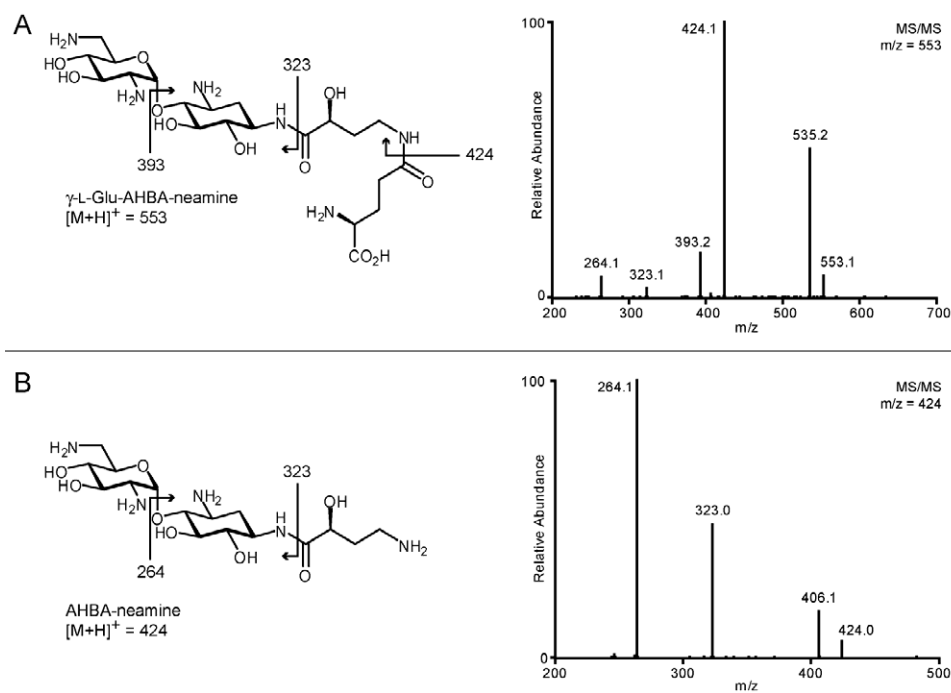


Fig. S10 Mass fragmentation spectra of A. γ -L-Glu-AHBA-neamine and B. AHBA-neamine.

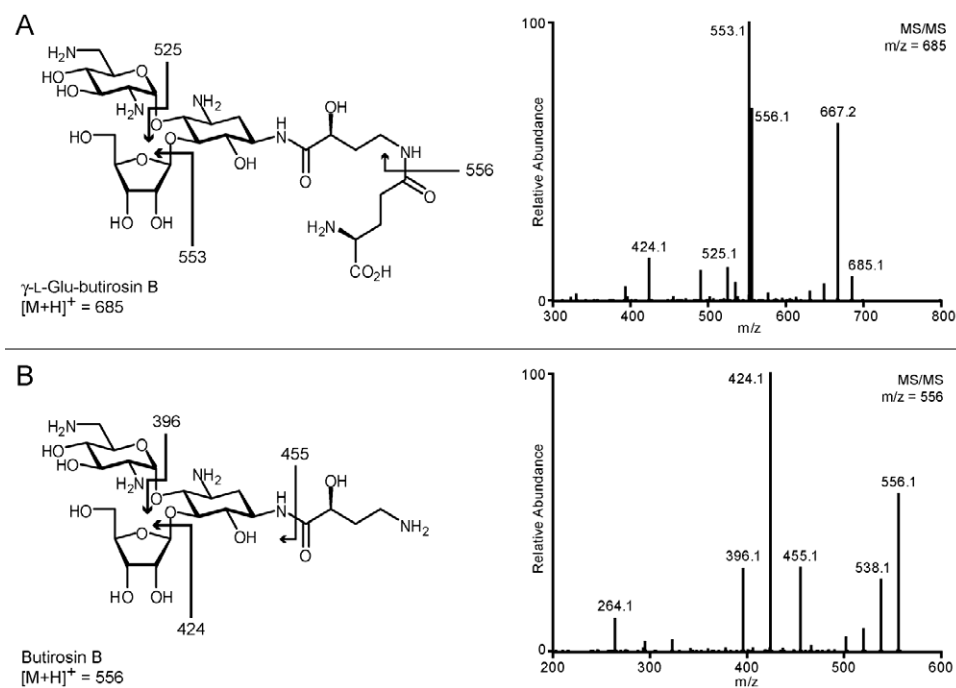


Fig. S11 Mass fragmentation spectra of A. γ -L-Glu-butirosin B and B. butirosin B.

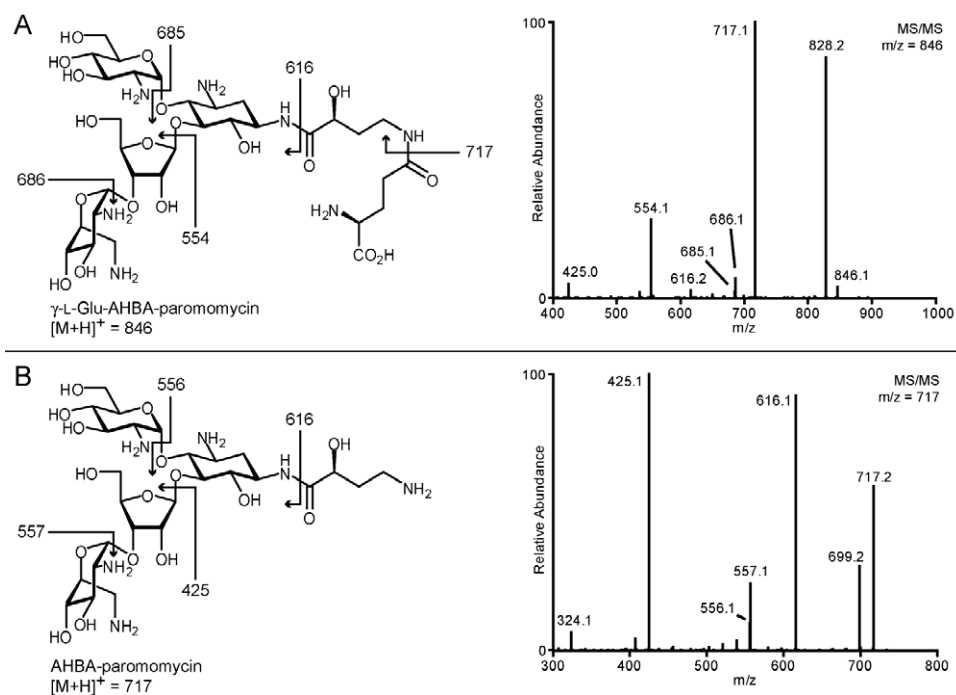


Fig. S12 Mass fragmentation spectra of A. γ -L-Glu-AHBA-paromomycin and B. AHBA-paromomycin.

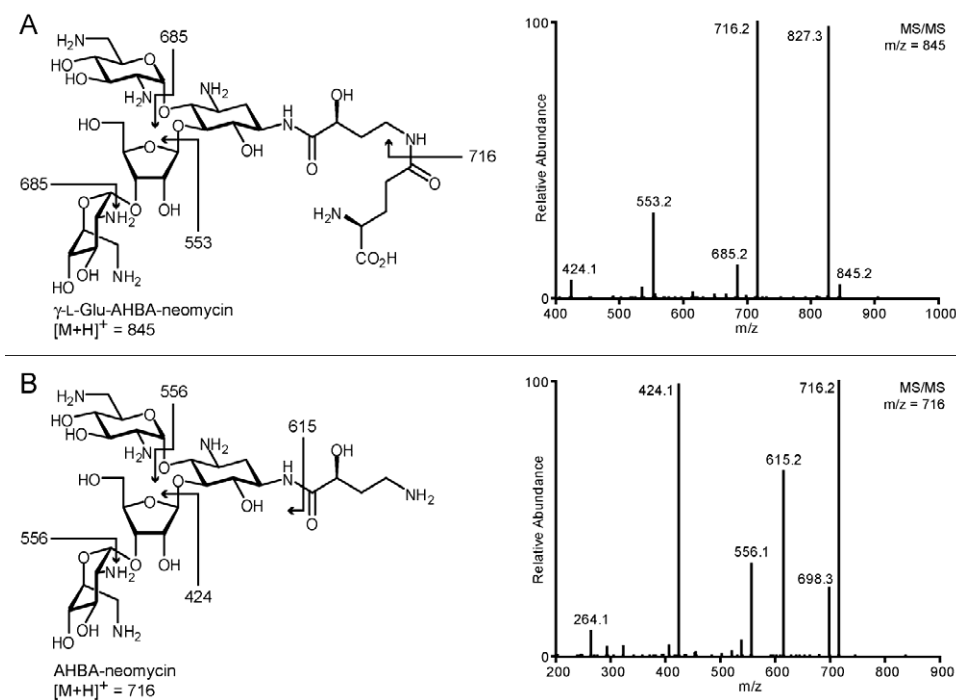


Fig. S13 Mass fragmentation spectra of A. γ -L-Glu-AHBA-neomycin and B. AHBA-neomycin.

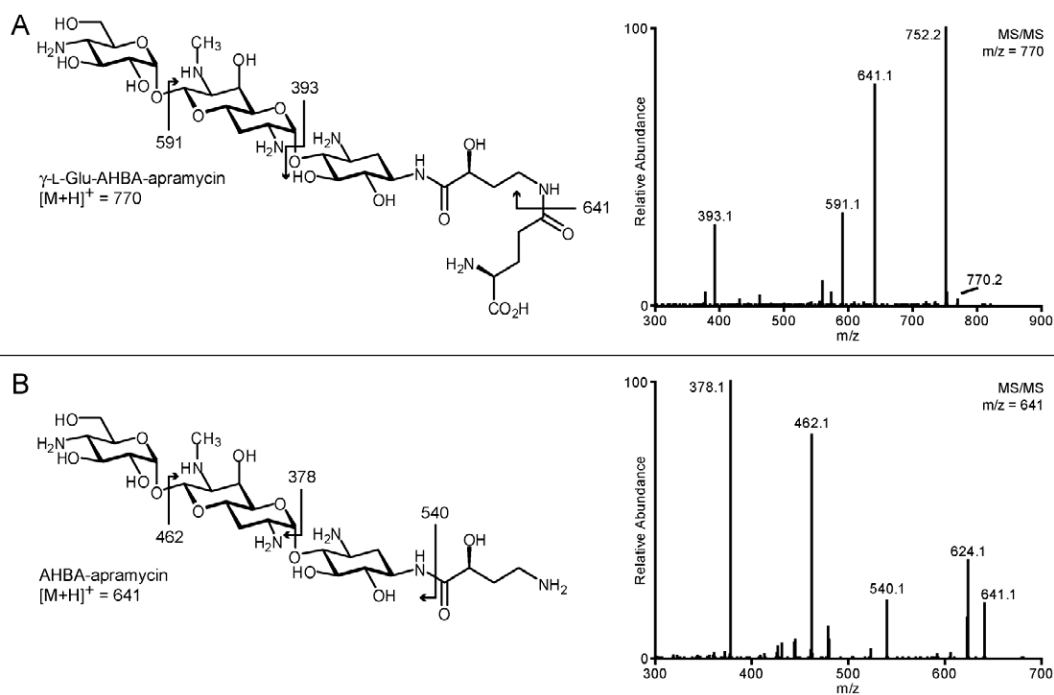


Fig. S14 Mass fragmentation spectra of A. γ -L-Glu-AHBA-apramycin and B. AHBA-apramycin.

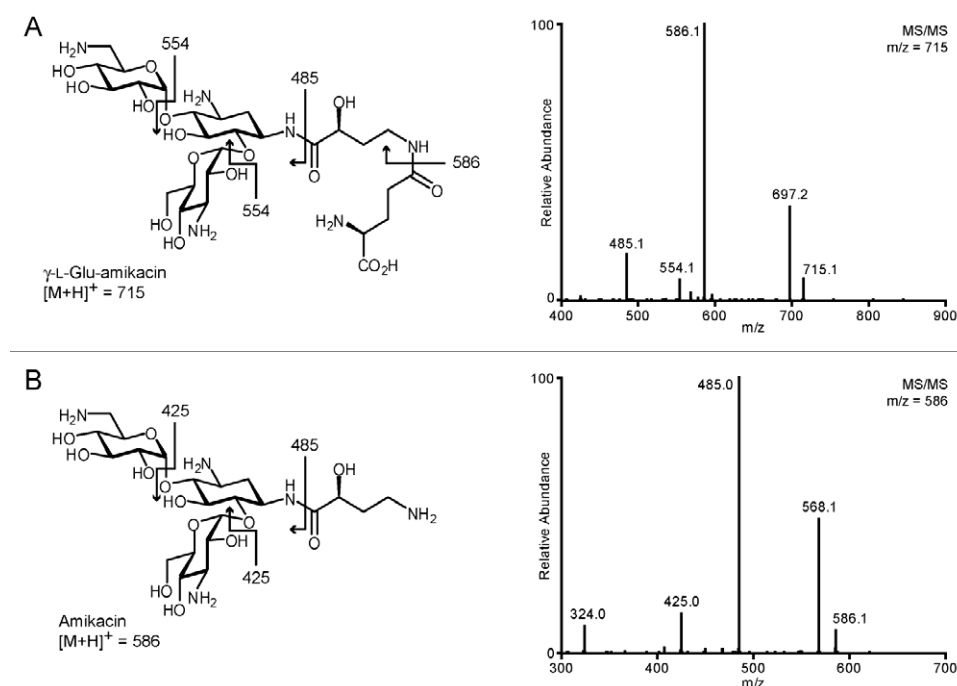


Fig. S15 Mass fragmentation spectra of A. γ -L-Glu-amikacin and B. amikacin.

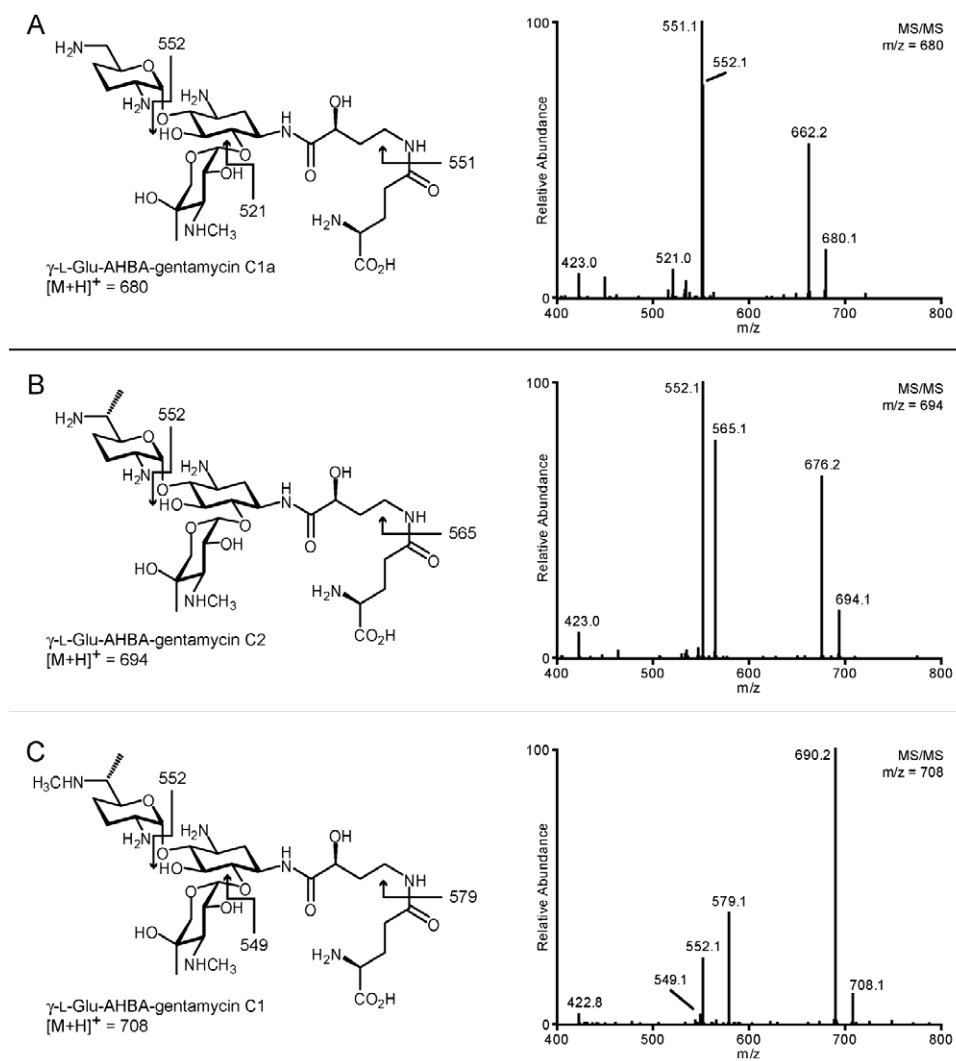


Fig. S16 Mass fragmentation spectra of A. γ -L-Glu-AHBA-gentamycin C1a; B. γ -L-Glu-AHBA-gentamycin C2; and C. γ -L-Glu-AHBA-gentamycin C1.

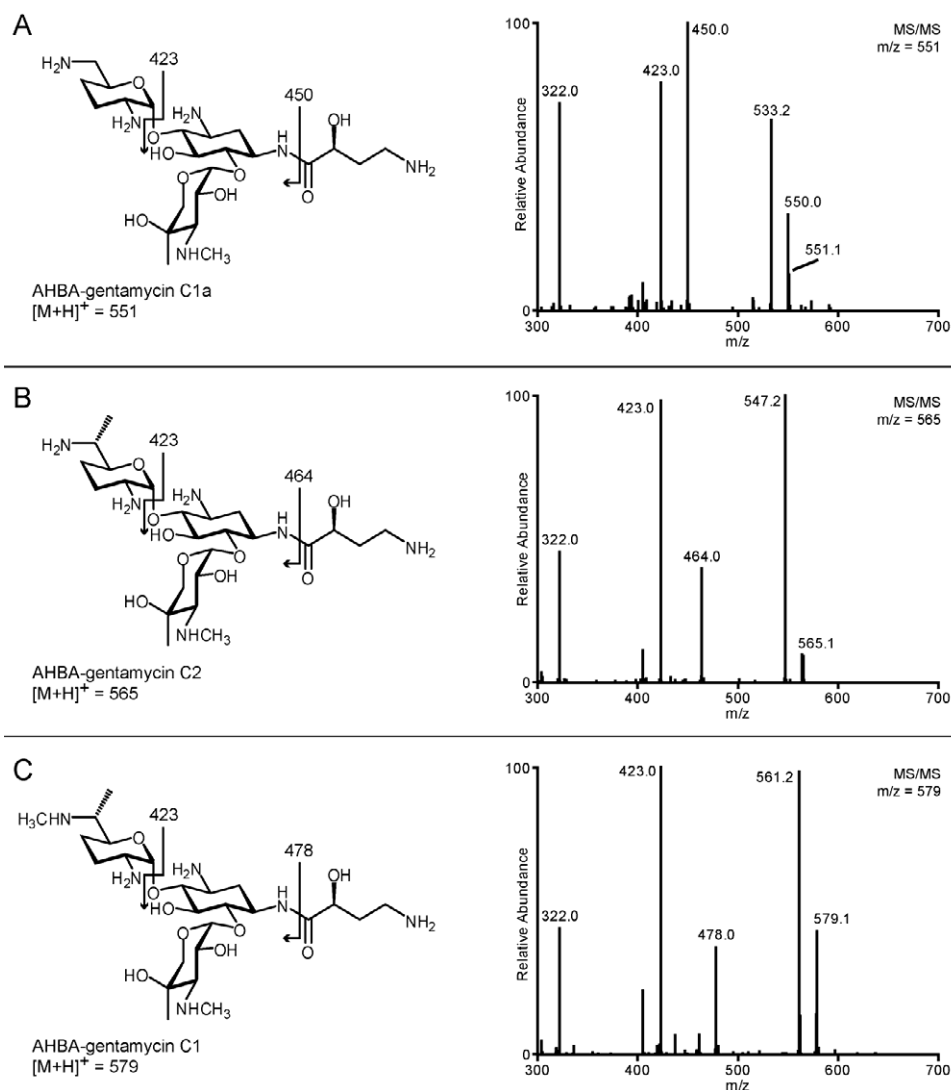


Fig. S17 Mass fragmentation spectra of A. AHBA-gentamicin C1a; B. AHBA-gentamicin C2; and C. AHBA-gentamicin C1.