Supplementary Information

Peptide-sugar Ligation Catalyzed by Transpeptidase Sortase: A Facile Approach to Neoglycoconjugate Synthesis

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Cloning, expression, and purification of recombinant sortase

Primers (5' GATATACATATGCAAGCTAAACCTCAAATTCCG 3' and 5' GTGGTGCTCGAGTTTGACTTCTGTAGCTACAAAGAT 3') were used to PCR amplify the s*rtA* sequence corresponding to amino acids 60 to 204 from the genomic DNA of *Staphylococcus aureus*. The resulting amplicon was ligated into pGEM-T Easy vector, transformed into XL-I Blue competent cells and selected from LB agar plates containing 50 μg/ml ampicillin. The selected clones were verified by DNA sequencing. Plasmid DNA isolated from the positive clones was digested with NdeI and XhoI and ligated into pET23b vector for expressing a recombinant sortase with a C-terminal hexa-histidine tag. The ligation mixture was transformed into XL-I Blue competent cells and selected from LB agar plates containing 50 μg/ml ampicillin. Plasmid DNA isolated from the positive clones was used to transform *E. coli* BL21 (DE3) competent cells. The transformed clones were propagated in LB broth at 37^{o}C for 3 hours or till the OD_{600} reached 0.6 and the expression was induced by addition of IPTG to a final concentration of 0.2 mM, at 30 ^{o}C for 3 hours. The induced cells were pelleted, re-suspended in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM 2-mercaptoethanol, and lysed by sonication. The protein in the lysate supernatant was purified by Ni-NTA affinity chromatography. The purified protein was freed of excess imidazole using a desalting column. The identity of recombinant sortase was established by Electrospray (ES) mass spectrometry (MS). The experimental mass (17864.78 Da) was in agreement with the calculated value (17865 Da).

Synthesis of peptides

The peptides were synthesized by standard solid phase synthesis protocols using Fmoc chemistry on a semi-automated peptide synthesizer (Model 90, Advanced Chemtech). Wang resin pre-loaded with the desired amino acid was used as the starting material. The

coupling and deprotection was monitored at every step by the Kaiser test for free amines. Before each coupling step and on completion of the synthesis, N-terminal Fmoc group was removed using 20% piperidine (v/v in DMF). The peptides were cleaved from the resin and the side chains deprotected with a mixture containing trifluroacetic acid (TFA), ethanediol, phenol, thioanisole and water (80:5:5:5:5, v/v). The resin was removed by filtration and the crude peptides were precipitated using cold diethyl ether. The peptides were purified to ≥98% by RPHPLC (column C8: 10 mm X 250 mm, gradient: 4-72% B in 130 min, A; 0.1% TFA, B; acetonitrile containing 0.1% TFA, flow rate: 1 ml/min) lyophilized and stored at -70 ^{o}C. The chemical identity of the peptides was checked by mass spectrometry (Table 1).

Table 1. *ESI mass analyses of synthetic peptides used in sortase-mediated ligation reactions*

Sequence	Observed	Calculated
1. YALPETGK	877.55	877.99
2. YALPMTGK	879.53	880.07
3. RRRRRRRRRLPETGK	2049.01	2049.42
4. RRRRRRRRRLPMTGK	2051.28	2051.50
5. TRQARRNRRRRWRERQRGGGLPETGK	3234.24	3234.64

Sortase-catalyzed peptide ligation to aminosugars

6-deoxy-6-aminoglucose and 6-deoxy-6-aminomannose were obtained from Glycoteam, GmbH. Glucosamine and aminoglycoside antibiotics used in the study were procured from Sigma Chemical Company.

The ligation of various LPXTG containing peptides and sugar or aminoglycoside antibiotics was carried out in 0.3 M Tris-HCl buffer (pH 7.5) containing 150 mM NaCl, 5 mM $CaCl_2$, and 2 mM 2-mercaptoethanol. Each assay was set up in a 0.1 ml volume that contained 0.5 mM peptide, 2.5 mM sugar or antibiotics and 50 μM sortase. The reaction was allowed to proceed at 37 °C for 6 hours, quenched by addition of 20 -fold excess of 0.1% trifluoroacetic acid (TFA) and analyzed by RPHPLC using acetonitrile-water-TFA solvent system. The reaction products were characterized by ESMS and MALDI-TOF mass spectrometry.

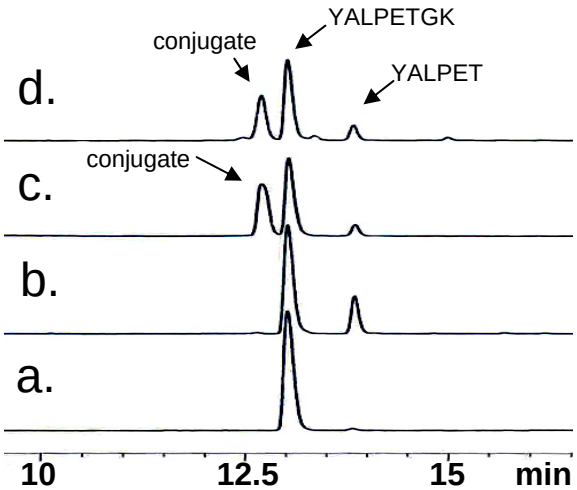
Table 2. *Characterization (mass spectrometry and yield) of sortase-catalyzed ligation products formed from LPXTG containing peptides and aminosugar nucleophiles*

Peptides	Aminosugars	Conjugate mass (Da)		
		Exp	Calc	% Yield
YALPETGK	6-deoxy-6-aminoglucose	853.41	853.41	41.7
YALPETGK	6-deoxy-6-aminomannose	853.43	853.41	33.7
YALPMTGK	6-deoxy-6-aminoglucose	855.62	855.41	54.3
YALPETGK	Kanamycin A	1158.64	1158.56	42.4
YALPETGK	Kanamycin B	1157.65	1157.58	38.6
YALPETGK	Tobramycin	1141.66	1141.58	73.5
YALPETGK	Ribostamycin	1128.62	1128.56	21.7
YALPETGK	Neomycin	1288.72	1288.63	29.1
YALPETGK	Paromomycin	1289.73	1289.62	17.7
YALPMTGK	Kanamycin A	1160.76	1160.56	36.1
YALPMTGK	Kanamycin B	1159.64	1159.57	21.7
YALPMTGK	Tobramycin	1143.66	1143.58	58.8
YALPMTGK	Ribostamycin	1130.61	1130.55	22.1
YALPMTGK	Neomycin	1290.73	1290.63	28.2
RRRRRRRRRLPMTGK	Tobramycin	2315.80	2315.79	62.8
RRRRRRRRRLPMTGK	Ribostamycin	2302.90	2302.74	35.9
RRRRRRRRRLPMTGK	Paromomycin	2463.17	2463.91	21.1
RRRRRRRRRLPETGK	Neomycin	2460.03	2460.83	43.7
TRQARRNRRRRWRERQRGGGLPETGK	Tobramycin	3498.30	3498.39	78.3
TRQARRNRRRRWRERQRGGGLPETGK	Neomycin	3646.55	3646.05	49.5

Reaction conditions: peptide, 0.5 mM; aminosugar/antibiotics, 2.5 mM; sortase, 50 μM; 37 C, pH 7.5. The reaction was carried out for 6 hr. The product yield is calculated based on HPLC peak areas.

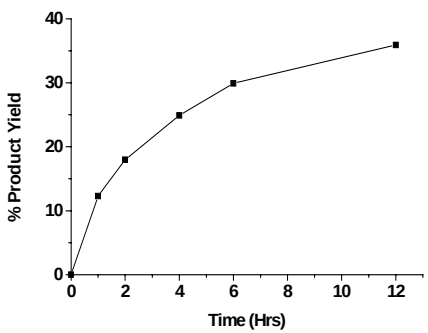
Supplementary Fig.1

A. *Sortase-catalyzed ligation of model YALPETGK peptide to aminohexoses*

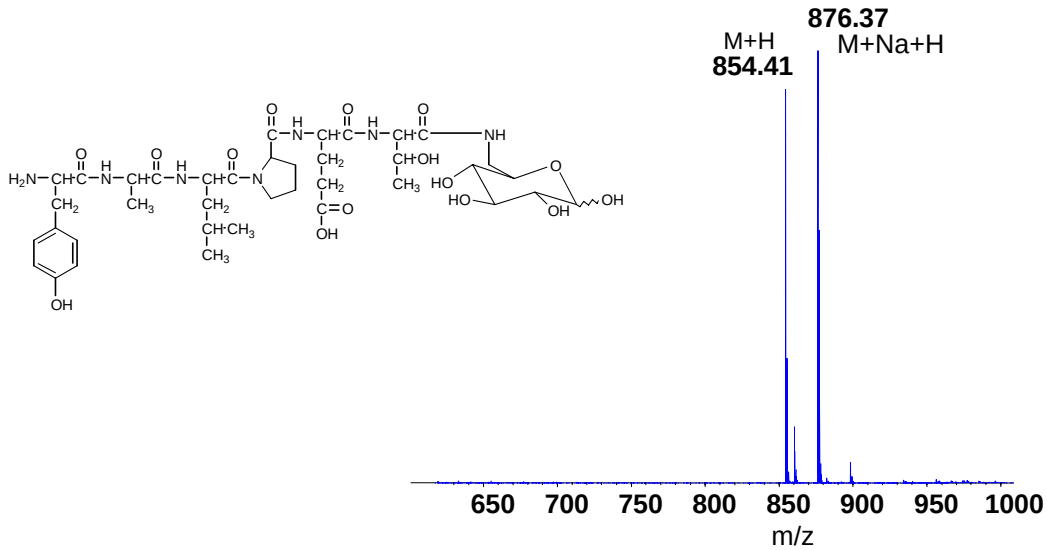


a) 0 h control; (b) 6 h reaction with glucosamine (2-deoxy-2-aminoglucose); (c) 6 h reaction with 6-deoxy-6-aminoglucose; and (d) 6 h reaction with 6-deoxy-6-aminomannose. Note the absence of peptide-sugar ligation in the case of glucosamine.[Reverse-phase HPLC (C18, 250 X 4.6 mm, Vydac), gradient : 4-72% acetonitrile containing 0.1% TFA in 30 min at a flow rate of 1 ml/min. Elution profile monitored at 210 nm]

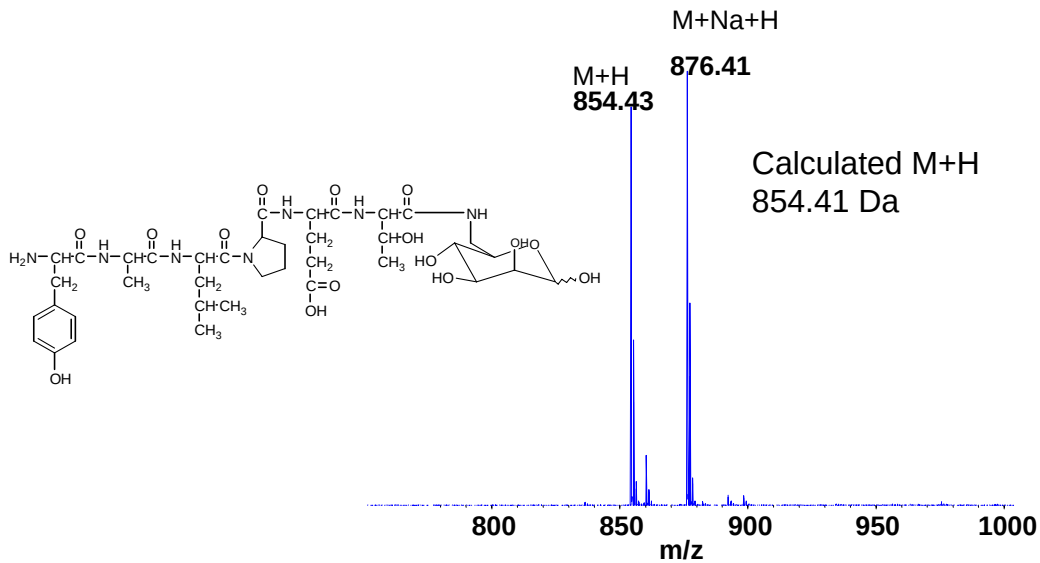
B. *Time course of sortase-catalyzed ligation of YALPETGK to 6-deoxy-6-aminomannose*



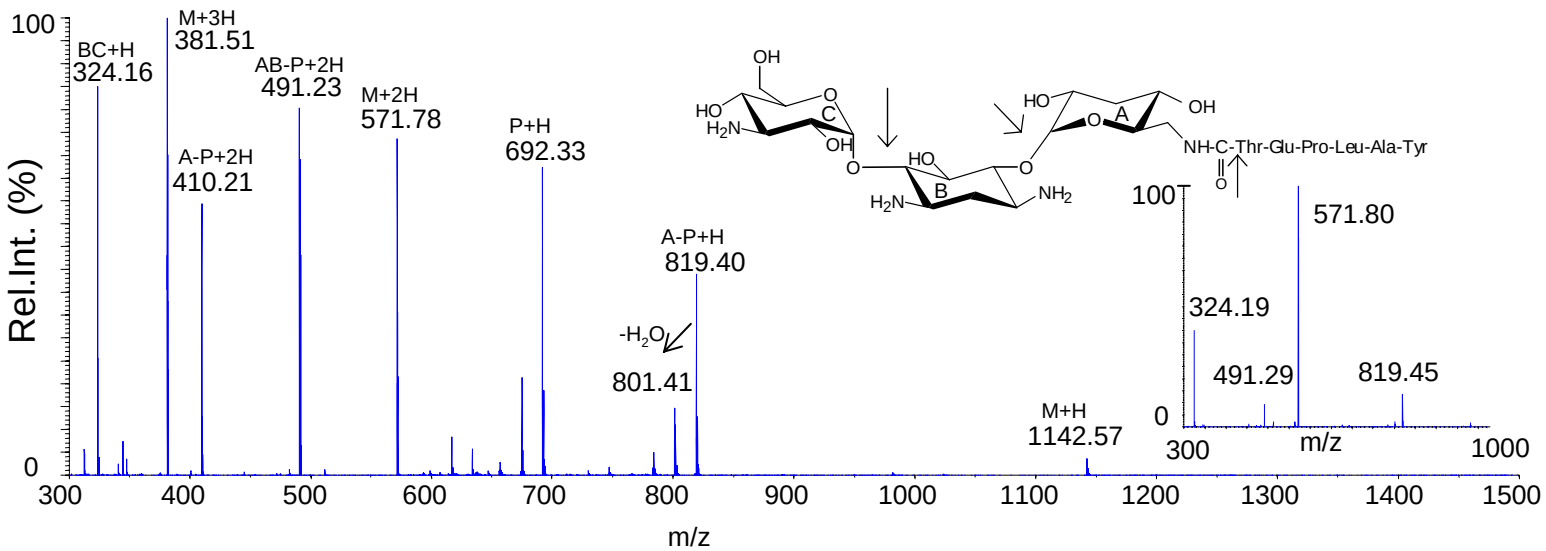
C. *ESMS of 6-aminoglucose-TEPLAY conjugate*



D. *ESMS of 6-aminomannose-TEPLAY conjugate*

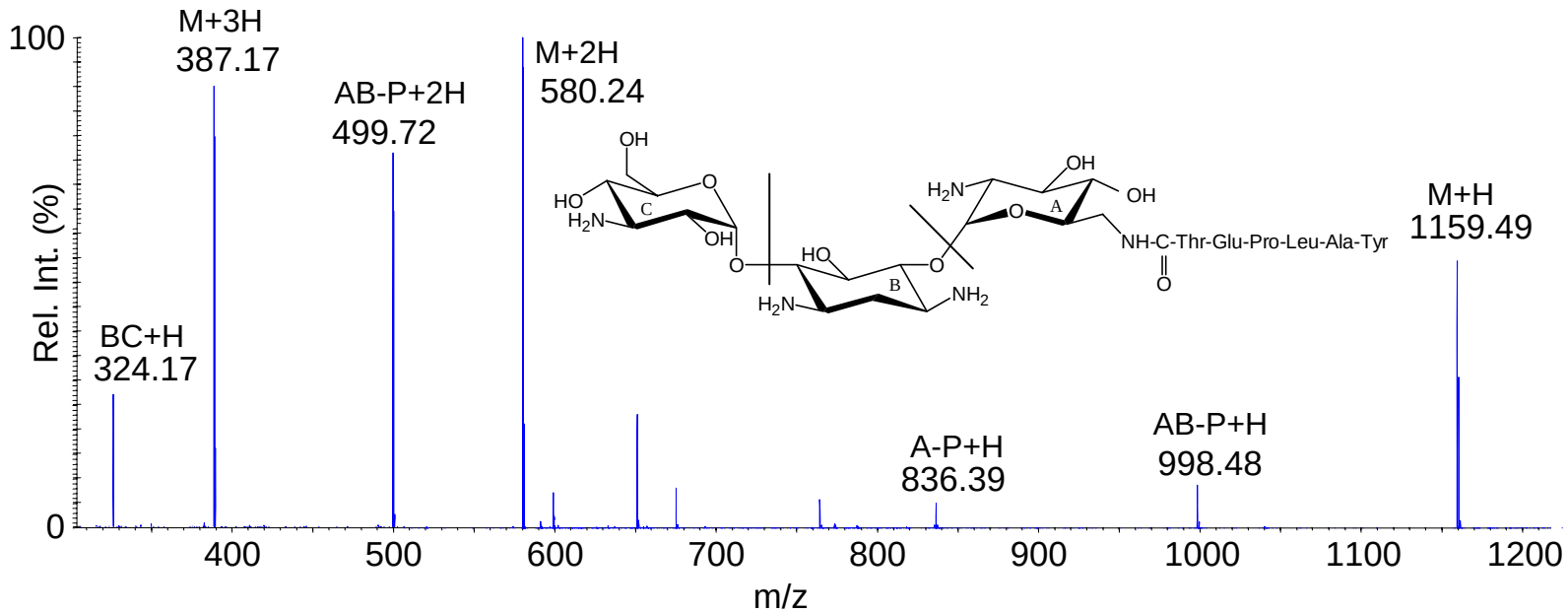


Supplementary Fig. 2. *ESI-TOF mass characterization of Tobramycin-TEPLAY conjugate*



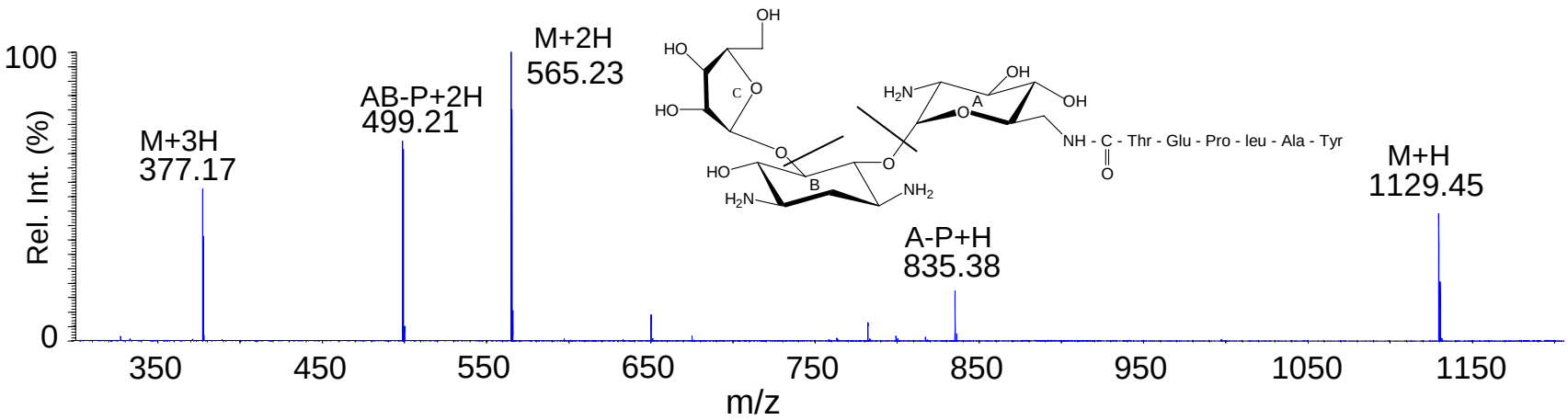
The three rings of the antibiotics structure are labeled as A, B and C, and the peptide portion is designated as P. The ions carry the labels based on the part of the structure from which they are derived. For example, m/z 819.40 labeled as A-P represents a structure in which ring A is attached to the peptide. Inset shows the MS/MS spectra of m/z 571.8.

Supplementary Fig. 3. *ESI-TOF mass characterization of the Kanamycin B-TEPLAY conjugate*



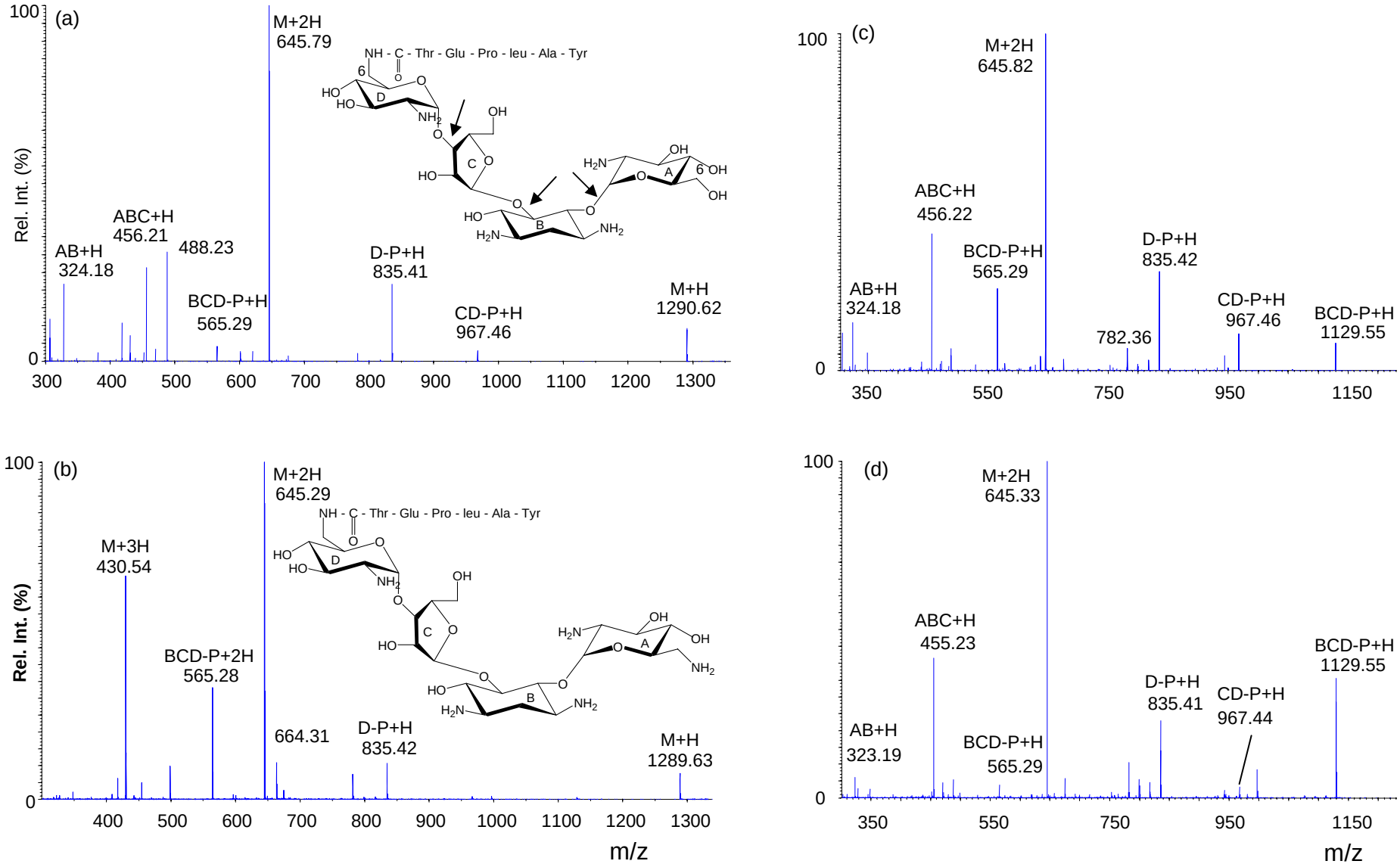
The ion identification codes are as described in the legend to Fig.2.

Supplementary Fig. 4. *ESI-TOF mass characterization of ribostamycin-TEPLAY conjugate*



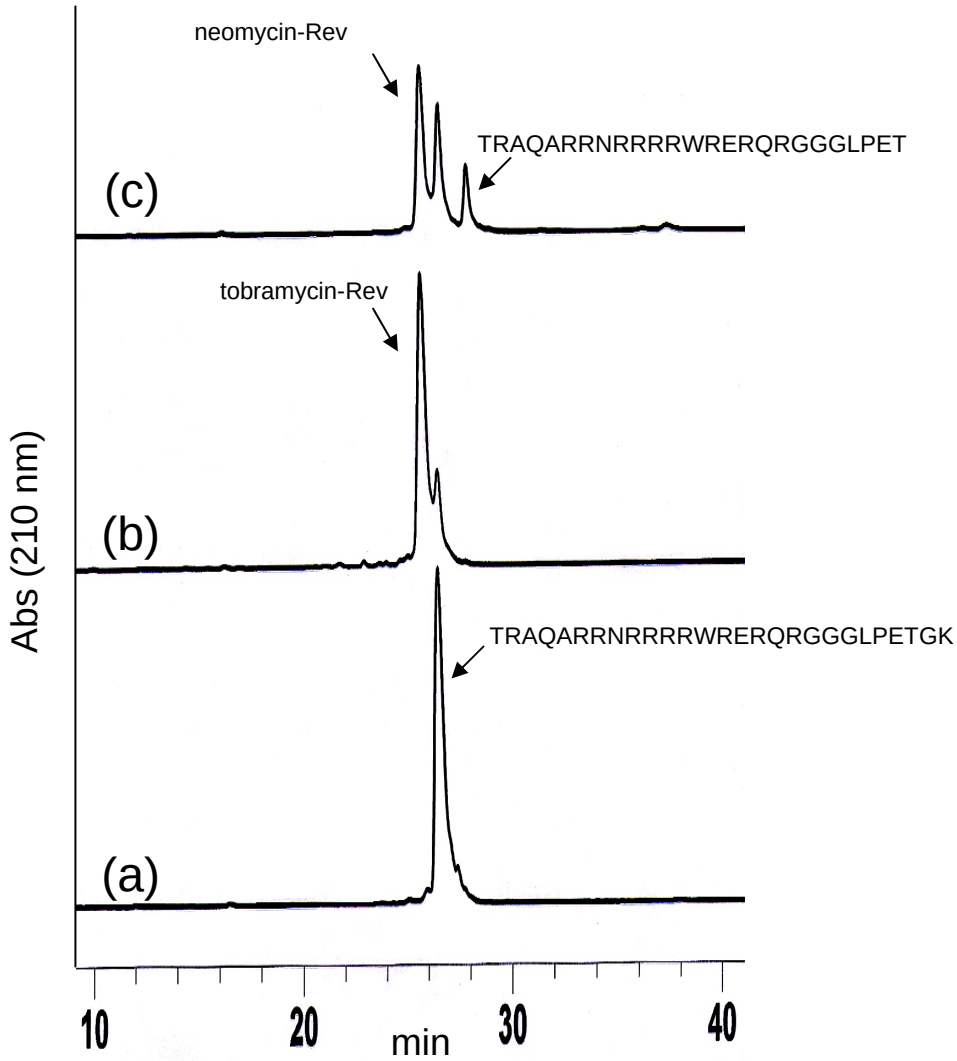
The ion identification codes are as described in the legends to Fig. 2.

Supplementary Fig. 5. *ESI-TOF mass characterization of paromomycin conjugate and neomycin conjugate*



(a), MS of paromomycin-TEPLAY conjugate; (b) MS of neomycin-TEPLAY conjugate; (c) MS/MS spectra of m/z 645.8 of paromomycin conjugate; (d) MS/MS spectra of m/z 645.3 of neomycin conjugate. The ion identification codes are similar as described in the legends to Fig 2.

Supplementary Fig.6. *Sortase-catalyzed conjugation of Rev peptide to tobramycin and neomycin*

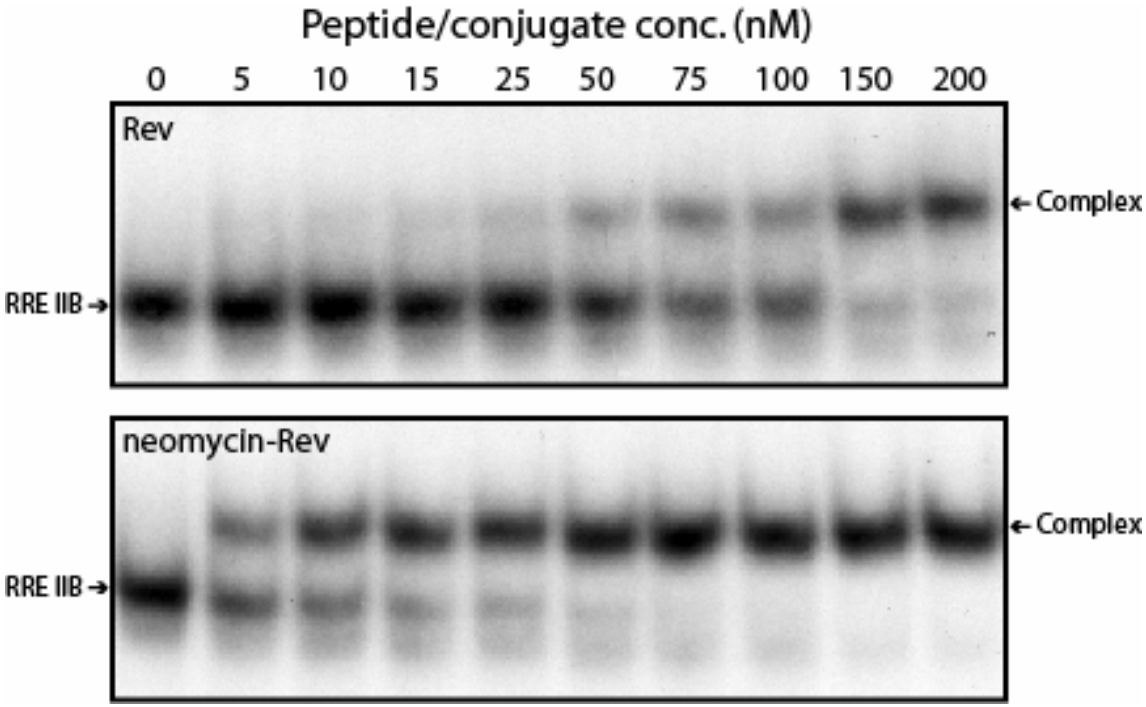


(a) 0 h control, (b) 6 h reaction with tobramycin, and (c) 6 h reaction with neomycin. The conjugate product peaks for tobramycin and neomycin respectively are labeled as tobramycin-Rev and neomycin-Rev.

Electrophoretic mobility gel shift assays

The ability of neomycin-Rev conjugate (TRQARRNRRRRWRERQRGGGLPET-neo) or Rev peptide (TRQARRNRRRRWRERQRGGGLPETGK) to bind to RRE RNA was evaluated using a 46-mer RRE IIB sequence (5' GGACCUGGUAUGGGCGCAGCGCAAGCUGACGGUACAGGCCAGGUCC 3'). This oligo has been previously used to characterize the RRE binding propensity of Rev peptide mimetics [NL Mills, MD Daugherty, AD Frankel, and RK Guy (2006) J Am Chem Soc 128: 3496-97] using gel shift assays. We followed the same experimental protocol as described by Mills *et al* cited above. Briefly, ^{32}P-labelled RNA [0.5 nM RRE IIB taken in 10 mM HEPES (pH7.5) containing 100 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT and 0.05 mg/ml *yeast* tRNA) was incubated with varying concentrations of Rev peptide or conjugate for 30 min at 4 °C. The samples were run on a 10% native PAGE with TBE buffer for 3 h at 225 V. Subsequent to autoradiography, the gel bands corresponding to bound and free RNA were excised and radioactivity associated with each band was determined using a liquid scintillation counter. The data was fitted assuming a single binding mode and binding affinity (K_d) was calculated using the Graphpad Prism Software. The K_d for neomycin-Rev conjugate and Rev peptide binding to RRE IIB RNA was found be 9.3 nM and 114.3 nM respectively. Our K_d of 114.3 nM for Rev binding to RRE IIB is consistent with those of Mills et al (see reference above) who reported a value of 100 nM for a similar Rev peptide.

Supplementary Fig. 7. *Electrophoretic mobility shift assay for neomycin-Rev conjugate binding to RRE RNA*



Representative gel-shift assays to show Rev and neomycin-Rev conjugate binding to RRE IIB RNA sequence

Sortase-catalyzed ligation of aminoglycosides to proteins

The Mrp protein (NP_372281) of *Staphylococcus aureus* that carries a sortase recognition pentapeptide sequence motif (LPNTG) near the C-terminus was cloned expressed and purified. Primers, 5' GCT AGC GTG CAA AAT TAT CGA AAA GTA AGT A 3' and 5' CTC GAG TGA TTC TTT TTC GTT TTT AGT ACG T 3', were used for PCR amplification of the gene encoding Mrp protein. The amplified PCR product was ligated into Nhe1-Xho sites of pET23b vector and transformed into BL21(DE3) cells for expressing recombinant Mrp with a C-terminal hexa-histidine tag. The expressed protein was purified by Ni-NTA affinity chromatography. The purified protein migrated as a single band on SDS-PAGE albeit showed a slightly higher molecular weight (Figure 8A). Nonetheless, MALDI-TOF mass (Fig. 8B) of the protein (23856 ± 20 Da) was found to be consistent with the amino acid composition (calculated, 23872).

About 40 μM Mrp protein was incubated with and 80 μM sortase at pH 7.5 (0.3 M Tris-HCl buffer containing 150 mM NaCl, 5 mM $CaCl_2$, and 2 mM 2-mercaptoethanol) in the presence or absence of tobramycin for 6 h (37 °C). The respective reaction mixture was analyzed by RPHPLC (Fig. 8C) and the identity of peaks obtained from each reaction was established by MALDI-TOF mass spectrometry (Fig. 9).

Supplementary Fig. 8. *Sortase-catalyzed conjugation of aminoglycoside to Mrp protein*

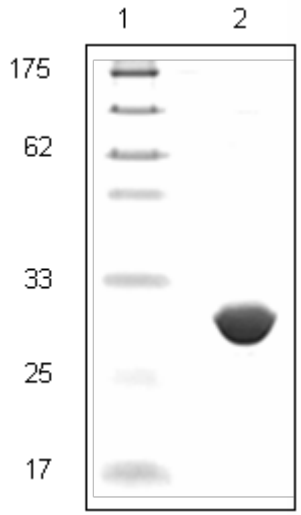


Fig. 8A. *SDS PAGE of Mrp protein*
Lane 1; protein standard,
Lane 2; purified Mrp-protein

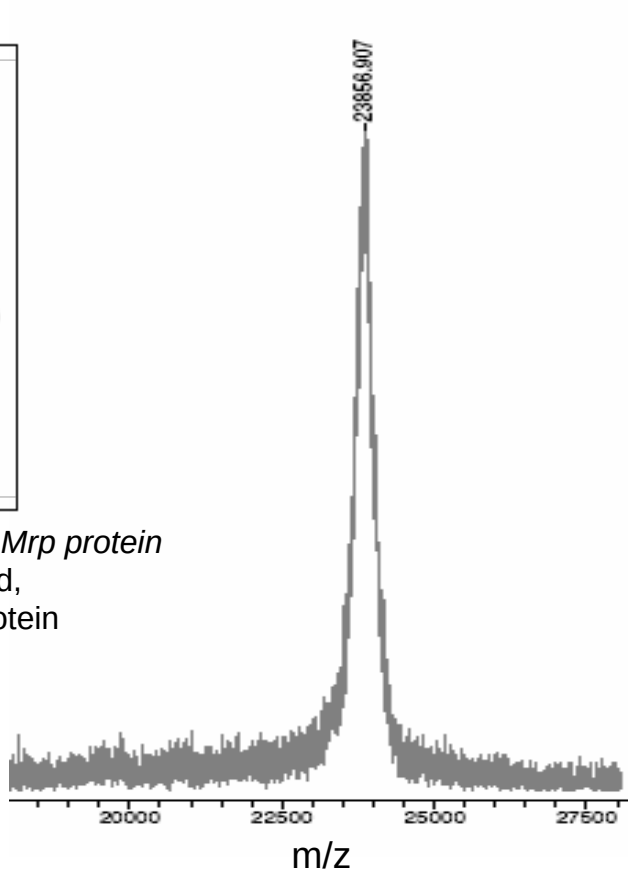


Fig. 8B *MALDI-TOF analysis of Mrp protein*

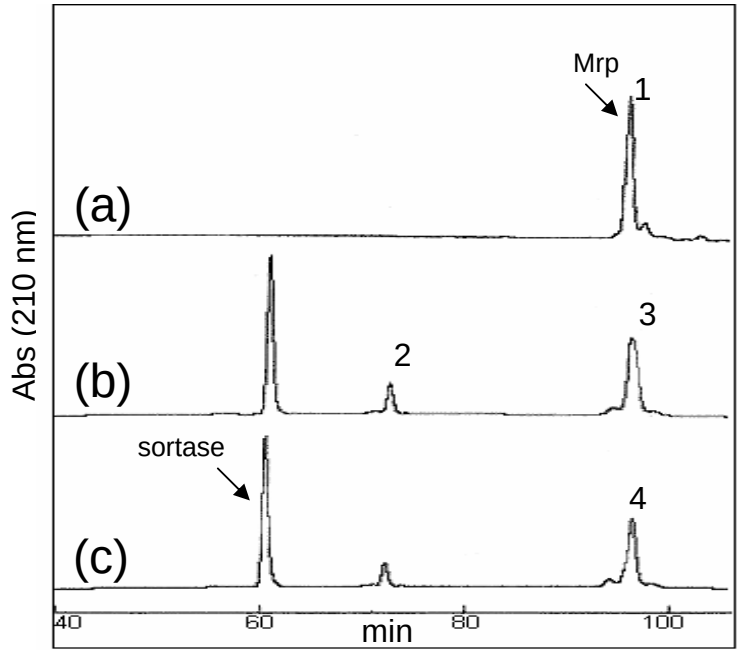


Fig 8C. *HPLC analysis of Tobramycin conjugation to Mrp protein.* (a) HPLC of Mrp alone. (b), Mrp treated with sortase in the presence of tobramycin. (c), Mrp treated with sortase in the absence of tobramycin. Peaks labelled as 2, 3, and 4 were collected, dried and subjected to MALDI-TOF mass analysis (Fig. 9).

Supplementary Fig. 9. MALDI-TOF analyses of reaction products isolated by HPLC (Fig. 8)