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Supporting Information

Inhibition of Aminoglycoside-Deactivating Enzymes APH(3′)-IIIa and AAC(6′)-Ii by Amphiphilic Paromomycin O2′′-Ether Analogues

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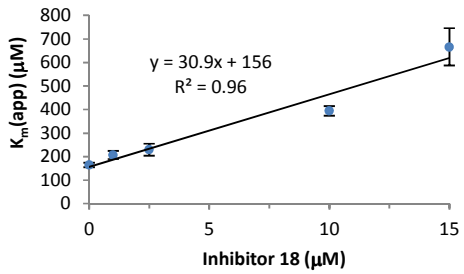
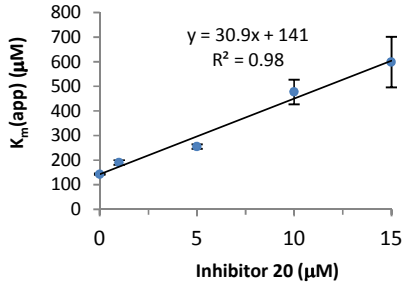
Supporting Information

a)



b)



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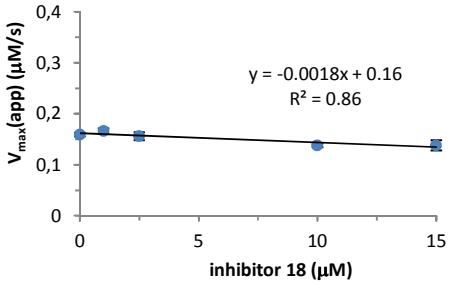

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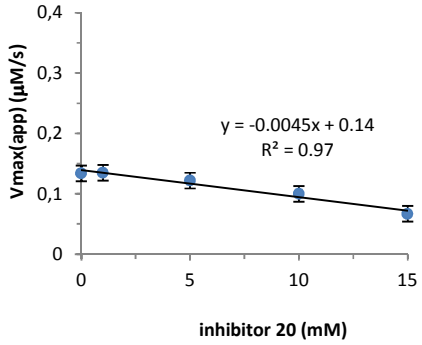


Figure S1. K_m(app) and V_{max}(app) of amikacin for APH(3′)-IIIa in presence of inhibitor **18** and **20**.

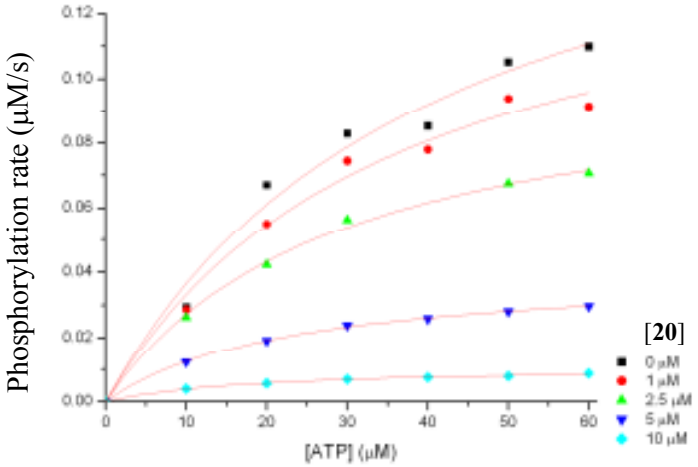
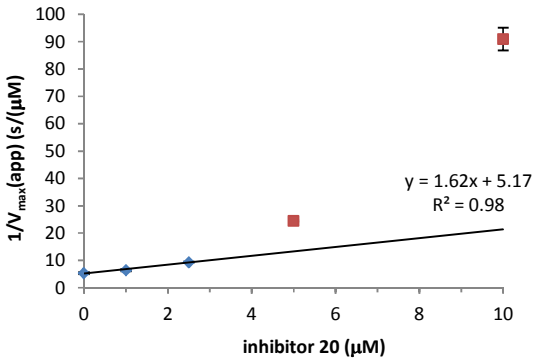


Figure S2. Inhibition of APH(3′)-IIIa catalyzed amikacin phosphorylation by analogue **20** as a function of ATP concentration.

a)



b)

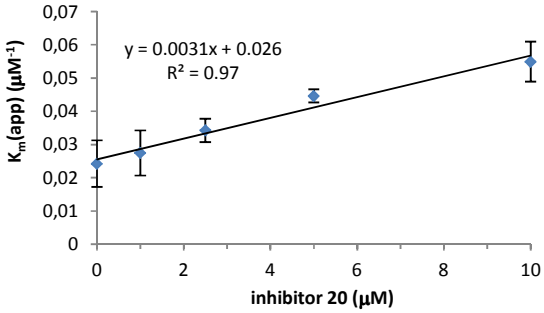


Figure S3. Effect of inhibitor **20** on K_m(app) of ATP and V_{max}(app) of APH(3′)-IIIa catalyzed phosphorylation of amikacin.

Experimental

Expression, purification and general protocol of APH(3′)-IIIa and AAC(6')-Ii.

Expression and purification of APH(3′)-IIIa[1] and AAC(6')-Ii[2] were achieved using previously published protocols. Phosphorylation kinetic parameters here measured by a standard protocol[1] with the following modifications: tris(hydroxymethyl)aminomethane (0.60 g, 5 mM), KCl (0.30 g, 4 mM) and $MgCl_2 \bullet 6H_2O$ (0.02 g, 100 μM) were dissolved in water (100 mL), the pH was adjusted to 7.5 using HCl 6.0 M, and the solution was cooled to 4 °C. A fraction (20 mL) of the solution was kept aside at 4 °C (solution A). To the remaining 80 mL, the reduced form of nicotinamide adenine dinucleotide (NADH, 34 mg, 50 μM), phosphoenolpyruvate (*PEP, 68 mg*, 400 μM*), the sodium salt of* adenosine-5'-triphosphate (ATP, 56 mg, 100 μM), a commercial cocktail of pyruvate kinase and lactate dehydrogenase (PK/LDH, Sigma-Aldich P0294, 200 μL) and the purified APH(3′)-IIIa (30 μL of a 1.6 mg/mL stock solution) were added and the solution was kept at 4 °C. A 900-μL aliquot of this solution was added to a cuvette and placed in a spectrophotometer (Varian Cary UV 100-Bio) regulated at 37 °C for 10 min to allow it to equilibrate. The phosphorylation reactions were initiated by the addition of a solution of an aminoglycoside, or a mixture of an aminoglycoside and an inhibitor, followed by a gentle agitation. The total volume of the added aminoglycoside solution was adjusted to 100 μL with the 20 mL of solution A described above (described above). The phosphorylation rates were determined by monitoring the decrease in absorbance at 340 nm. An extinction coefficient of 6220 $cm^{-1}M^{-1}$ was used for NADH.

Determination of initial phosphorylation rates (v_0) of APH(3′)-IIIa

The general protocol described above was followed, but using only 10 μL of purified APH(3′)-IIIa (1.6 mg/mL). This adjustment allowed phosphorylation rates to be measured over a more reasonable period of time (minutes). The concentrations of aminoglycosides were fixed at 50 μM for this assay.

Determination of inhibition constants for AAC(6')-Ii and APH(3′)-IIIa.

K_i for the inhibition of AAC(6')-Ii was determined using a known protocol.[2] For APH(3′)-IIIa, K_m(app) and V_{max}(app) of amikacin were determined in the presence of 0, 1, 2.5, 5, 10 and 15 μM of inhibitor. The concentration of amikacin was varied from $K_m/2$ to 3-fold K_m (K_m = 150 μM). Nonlinear regression was performed using Origin 6.0 Microcal software. K_i' was determined using the equation : $\frac{1}{V_{\max}(app)} = \frac{1}{V_{\max}} + \frac{[I]}{V_{\max} K_i'}$. K_i was determined using the following equation: $\frac{K_m(app)}{V_{\max}(app)} = \frac{K_m}{V_{\max}} + \frac{K_m[I]}{V_{\max} K_i}$.

Determination of IC_{50} values.

Amikacin concentration was fixed at 1.33-fold K_m (200 μM). Phosphorylation rates of amikacin were determined in presence of 0, 0.5, 1, 1.5, 2, 3, 5, 10, 15, 20 and 30 μM of inhibitors. Amikacin phosphorylation rates decreased with increasing inhibitor concentrations until IC_{50} values were obtained.

RNA Synthesis, Purification, and Crystallization

X-ray structure of analogue **18** bound in the A-site of rRNA was obtained according to a previously published protocol for RNA synthesis, purification, and crystallization.[3]

Crystal code	analogue **18**
Crystal data	
Space group	$P2_12_12_1$
Unit cell (Å)	a = 34.2
	b = 44.7
	c = 94.6
Z[a]	1
Data collection	
Beamline	ID29 of ESRF
Wavelength (Å)	0.9000
Resolution (Å)	40.4-2.6
of the outer shell (Å)	2.7-2.6
Observed reflections	33546
Unique reflections	5003
Completeness (%)	99.6
in the outer shell (%)	100.0
R_{merge}[b] (%)	5.5
in the outer shell (%)	35.2
Redundancy	6.7
in the outer shell	7.0
Refinement	
Resolution range (Å)	10-2.6
Used reflections	4848
R-factor[c] (%)	23.8
R_{free}[d]	26.7
Number of RNA atoms	900
Number of antibiotic molecules	2
Number of hexa-hydrated magnesium	0
Number of water molecules	38
R.m.s. deviation	
Bond length (Å)	0.006
Bond angles (°)	0.9
Improper angles (°)	1.3

[a]Number of dsRNA in the asymmetric unit.
[b]$R_{merge} = 100 \times \Sigma_{hklj}|I_{hklj} - \langle I_{hklj}\rangle| / \Sigma_{hklj}\langle I_{hklj}\rangle$.
[c]R-factor $= 100 \times \Sigma||F_o| - |F_c|| / \Sigma|F_o|$, where $|F_o|$ and $|F_c|$ are optimally scaled observed and calculated structure factor amplitudes, respectively.
[d]Calculated using a random set containing 10% of observations that were not included throughout refinement.

Table 1. Crystal data, statistics of data collection and structure refinement.

Accession Numbers

Crystal structure reported herein for the bacterial ribosomal decoding site complexed with paromomycin analogue **18** has been assigned the RCSB ID code rcsb065757 and PDB ID code 3S4P.

References

1) G. A. McKay, P. R. Thompson, G. D. Wright, *Biochemistry* **1994**, *33*, 6936-6944.
2) F. Gao, X. Yan, O. M. Baettig, A. M. Berghuis, K. Auclair, *Angew. Chem. Intl. Ed.* **2005**, *44,* 6859-6862.
3) Q. Vicens, E. Westhof, *Structure* **2001**, *9*, 647-658.