Supporting Information

Characterization of the Bifunctional Aminoglycoside-Modifying Enzyme ANT(3″)-Ii/AAC(6′)-IId from *Serratia marcescens*

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Structure assignment of the enzymic products. The ^{13}C APT (Attached Proton Test) spectrum of the acetylated kanamycin A revealed a total of 20 carbon resonances of which one corresponded to a methyl carbon (21.8 ppm), three to methylene carbons (34.6, 40.0, and 60.1 ppm), fifteen to methine carbons (48-102 ppm) and one to non-proton bearing carbon (174.3 ppm). The ^{1}H spectrum of this compound showed two one proton doublets at δ 5.05 and 5.24, which were assigned to the anomeric protons of 3-amino-3-deoxyglucose and 6-amino-6-deoxyglucose rings, respectively. Signals of the remaining protons of these moieties were assigned by analyzing the corresponding COSY and TOCSY spectra. The methylene CH_2-2 proton signals of the 2-deoxystreptamine moiety were identified in the COSY spectrum at δ 1.27 and 2.02. They showed a mutual cross-peak and both exhibited cross-peaks to the methine CH-1 and CH-3 proton signals at δ 2.87 and 2.98, respectively. Following the interpretation of the TOCSY spectrum, the methine CH-4, CH-5, and CH-6 proton signals of this moiety were assigned. By analyzing the gHMQC spectrum, the signals of all carbons with directly attached protons were unambiguously assigned. In the gHMBC spectrum, the resonance of the anomeric proton of the 6-amino-6-deoxyglucose moiety showed a cross-peak with the carbon signal at

δ 88.99 corresponding to the C-4 carbon of the 2-deoxystreptamine moiety. Simultaneously, the signal (δ 86.9) of the C-6 carbon of this moiety exhibited a cross-peak with the anomeric proton signal of the 3-amino-3-deoxyglucose (δ 5.05). The acetyl group attached at 6′-aminomethyl position was easily identified in the gHMBC spectrum as the resonances of the CH_3-8′ and CH_2-6′ protons displayed a cross-peak with the carbonyl C-7′ signal. The structure was assigned to 6′-*N*-acetyl-kanamycin A (**5**), and the various spectroscopic data were in good agreement with previously published data (*1*).

Both the ^{1}H and $^{13}C[^{1}H]$ NMR spectra of the adenylated spectinomycin exhibited rather broad signals probably because of some salt content. The linewidths of the proton and carbon signals were about 6 Hz and 6-16 Hz, respectively. Moreover, the sample contained a small but detectable amount of unknown impurities. The assignment started from one-proton doublet (J=5.4 Hz) at δ 6.03, which was attributed to the anomeric ribose proton of the AMP moiety, analysis of the COSY spectrum furnished signal assignments for all remaining ribose protons. The gHMQC analysis then permitted assignment of the corresponding carbon signals. The adenosine proton and carbon signals were identified based on their chemical shifts. (*2*) The proton and carbon signals of the spectinomycin moiety were assigned as follows. In the COSY spectrum one-proton triplet (J = 9.8 Hz) at δ 4.11 showed two cross-peaks at δ 3.02 and 4.01, which indicates that the signals belong to the following protons H-5a, H-6 and H-9a, respectively. The H-9a proton signal correlated with the signal at δ 4.30, which also displayed a cross-peak at δ 2.93. The former was therefore assigned to the H-9 proton and the latter to the H-8 proton. The methyl and H-2 proton signals at δ 1.02 and 3.56, respectively, were then identified based upon their mutual correlation. Assignment of the corresponding carbon signals was accomplished by the gHMQC analysis. The APT data and chemical shift values were then used for identification of the C-3, C-4, C-4a, C-7, and C-10a carbon signals. The resonances of the C-7 and C-10a carbons showed cross-peaks with the proton signals at δ 4.47 and 4.57, respectively, in the

gHMQC spectrum. Therefore, the former was assigned to the H-7 proton and the latter to the H-10a proton. Neither COSY nor gHMQC analyses furnished assignment of the spectinomycin methylene CH_2-3 proton signals.

The position at which the AMP moiety is attached to spectinomycin could only be deduced indirectly, as no splitting of the proton or carbon resonances due to a ^{31}P spin coupling was observed in the respective ^{1}H and $^{13}C[^{1}H]$ NMR spectra of the adenylated compound because of the aforementioned line broadening. However, the H-9 proton signal of the compound exhibited somewhat large (~0.56 ppm) downfield shift in comparison to its position in the ^{1}H spectrum of unmodified spectinomycin. (*3*) Moreover, this proton is likely in a diaxial position relatively to the H-9a proton as evident from the magnitude (~9.7 Hz) of the $^{3}J_{H\text{-}9,H\text{-}9a}$ coupling constant. We therefore reason that AMP is attached to spectinomycin at the equatorial position at C-9, as depicted in Figure 1. An additional point of interest is that the ^{13}C NMR spectrum showed a resonance at 93.3 ppm for a hydrated carbonyl at position C-4 of 9-*O*-(adenosine 5′-phosphoryl)-spectinomycin (**6**), consistent with the spectrum of the unmodificed spectinomycin (*3*) and the mass spectrum of **6**.

The individual fragments of the adenylated streptomycin molecule, streptidine, streptose, glucose and AMP moieties, were identified by analysis of 1D and 2D ^{1}H and ^{13}C NMR spectra, which provided unambiguous assignment of resonances corresponding to their hydrogen and carbon atoms. The proton and carbon resonances of AMP were assigned in a similar manner as described above for the adenylated streptomycin. The glucose CH_3-7″ and streptose CH_3-5′ methyl groups were easily identified in the gHMBC spectrum. Their proton resonances at δ 2.78 (CH_3-7″) and 1.15 (CH_3-5′) displayed cross-peaks with the methine carbon signals at δ 61.6 and 77.7, respectively, thus they were correspondingly assigned to the C-2″ and C-4′ carbons. Furthermore, the CH_3-5′ proton resonance correlated with the quaternary carbon signal (APT data) at δ 82.2. Consequently, the latter must belong

to the C-3′ carbon. The gHMBC analysis also revealed the correlation of the C-3′ resonance with two one-proton singlets at δ 4.97 and 5.13, which were assigned to the H-6′ and H-1′ protons, respectively. Having established the above assignments, the remaining proton and carbon resonances of the glucose and streptose moieties were determined by analyzing the COSY, gHMQC and gHMQCTOCSY spectra. Glycosidic linkage between these two moieties was then established based on the fact that the H-1″ resonance displayed a cross-peak with the C-2′ signal in the gHMBC spectrum.

Analysis of the gHMBC spectrum also allowed the carbon signal at δ 77.0 to be assigned to the streptidine C-4 carbon, as it showed a cross-peak with the streptose H-1′ proton signal (δ 5.13) due to a three bond glycosidic linkage. The methine CH-4 proton signal at δ 3.51 was then identified based on its correlation with the C-4 carbon signal in the HETCOR spectrum. Following the interpretation of the gHMBC spectrum, the H-3 proton signal and the C-1 and C-8 carbon signals were assigned. The proton signal at δ 3.56 exhibited cross-peaks with the C-4 carbon resonance and another three carbon signals at δ 58.70, 70.82, and 157.45, which is only possible when the signals belong to the H-3 proton, and the C-1, C-2, and C-8 carbons, respectively. The HETCOR analysis subsequently provided assignments of the C-3 carbon and H-1 and H-2 proton resonances. The carbon signals at δ 73.1 and 158.2, which showed cross-peaks with the proton H-1 signal in the gHMBC spectrum, were assigned to the C-5 and C-7 carbons, respectively. The correlation of the proton H-4 signal with the carbon signal at δ 71.5 permitted the latter to be assigned to the C-6 carbon. The corresponding proton signals were then identified using the HETCOR analysis. Similar reasoning as in the case of **6** enabled us to conclude that AMP moiety is attached to the glucose C-3″ carbon of streptomycin. The H-3″ resonance of the nucleotidylated streptomycin is shifted downfield by ~1 ppm in comparison to its non-nucleotidylated form (*4*).

The $^{13}C[^{1}H]$ spectrum of 3″-O-(adenosine 5′-phosphoryl)-streptomycin (**7**) contained six additional unassigned resonances at δ 23.5, 59.0, 62.4, 70.8, 71.4, and 74.7. The signal at δ 23.5 was very broad (~62 Hz) and did not show up in the APT spectrum. The signal at δ 62.4 belonged to quaternary carbon (APT data). All other signals belonged to methine carbons and their corresponding proton chemical shifts (HETCOR data) are 3.42, 3.45, 3.40, and 3.42, respectively. ^{1}H and ^{13}C chemical shifts of the modified aminoglycosides **5**, **6**, and **7** are presented in Table S1.

Table S1. ^{1}H and ^{13}C chemical shifts of the modified aminoglycosides **5**, **6**, and **7**.

5			**6**			**7**		
H/C	^{1}H	^{13}C	H/C	^{1}H	^{13}C	H/C	^{1}H	^{13}C
				ppm				
1	2.87	49.12	2	3.56	67.92	1	3.39	58.70
2	2.02-1.27	34.64	3	1.99	40.76	2	3.45	70.67
3	2.98	50.32	4[a]		93.29	3	3.53	58.22
4	3.31	86.94	4a		91.44	4	3.51	76.99
5	3.66	74.06	5a	4.11	66.63	5	3.48	73.09
6	3.31	87.99	6	3.02	58.86	6	3.33	70.82
1′	5.24	100.30	7	4.47	61.22	7	3.54	157.45
2′	3.60	71.72	8	2.93	62.22	8	3.40	158.15
3′	3.69	72.58	9	4.30	73.01	1′	5.13	105.65
4′	3.28	70.87	9a	4.01	69.79	2′	4.33	83.56
5′	3.83	71.07	10a	4.57	93.15	3′		82.15
6′	3.63-3.38	39.99	2-CH_3	1.02	19.38	4′	4.35	77.65
7′		174.28	6-NCH_3	2.50	30.59	5′	1.15	12.27
8′	2.00	21.75	8-NCH_3	2.52	31.97	6′	4.97	89.06
1″	5.05	99.89	1′	6.03	87.24	1″	5.53	93.75
2″	3.54	71.30	2′	4.68	74.32	2″	3.31	61.61
3″	3.06	54.16	3′	4.48	70.17	3″	4.31	74.42
4″	3.37	68.73	4′	4.32	83.54	4″	3.59	72.25
5″	3.93	71.96	5′	4.21	65.23	5″	3.38	68.28
6″	3.79	60.05	2″	8.11	152.56	6″	3.61-3.75	60.29
			4″		148.53	7″	2.78	32.65
			5″		118.25	1′ (Amp)	6.01	87.32
			6″		155.19	2′ (Amp)	4.71	73.74
			8″	8.37	139.58	3′ (Amp)	4.42	70.03
						4′ (Amp)	4.34	83.32
						5′ (Amp)	4.18	65.79
						2″ (Amp)	7.98	152.85
						4″ (Amp)		148.88
						5″ (Amp)		118.62
						6″ (Amp)		155.42
						8″ (Amp)	8.21	139.78

[a] The carbonyl carbon of spectinomycin was hydrated during modification and purification.

The graphs of inhibition patterns for ANT(3″)-Ii/ AAC(6′)-IId.

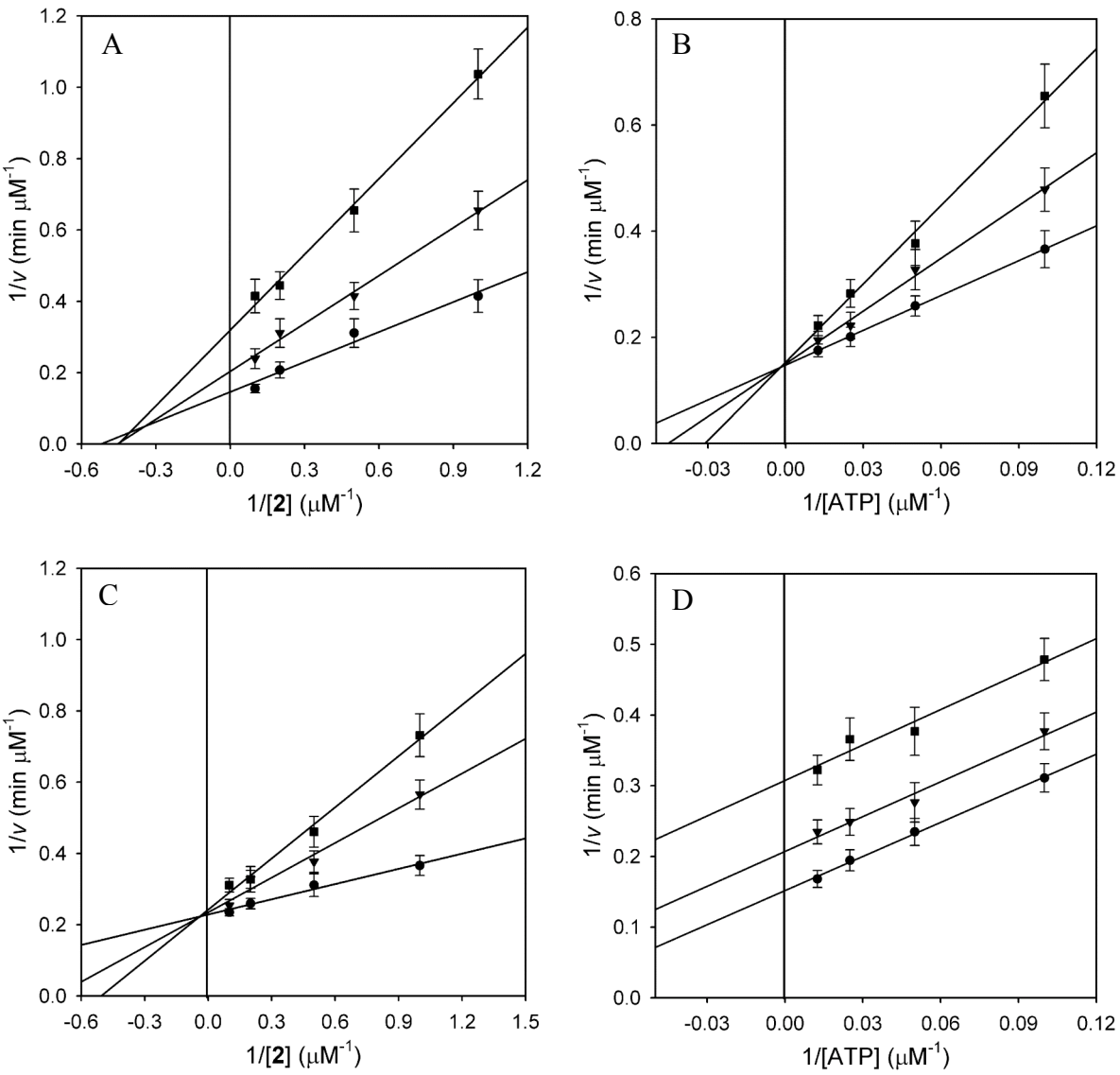

Figure S1. Inhibition patterns of dead-end inhibitors for ANT(3")-Ii domain. A, noncompetitive (mixed) inhibition of AMP-CPP (●, 20 μM; ▼, 40 μM; ■, 80 μM) with respect to spectinomycin (**2**) (1 – 10 μM). B, competitive inhibition of AMP-CPP (●, 10 μM; ▼, 20 μM; ■, 40 μM) with respect to ATP (10 – 80 μM). C, competitive inhibition of kanamycin A (**1**) (●, 5 μM; ▼, 10 μM; ■, 20 μM) against **2** (1 – 10 μM). D, uncompetitive inhibition of **1**(●, 5 μM; ▼, 10 μM; ■, 20 μM) for ATP (10 – 80 μM).

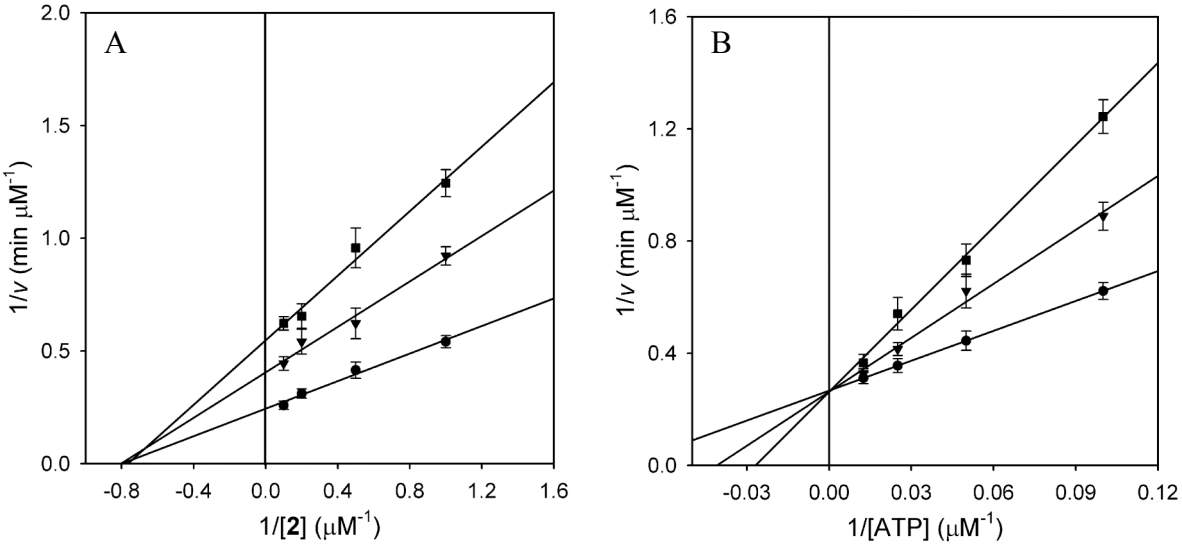

Figure S2. Inhibition patterns of the product for ANT(3")-Ii domain. A, noncompetitive inhibition of adenylated spectinomycin (**6**) (●, 50 μM; ▼, 100 μM; ■, 200 μM) with respect to **2** (1 – 10 μM). B, competitive inhibition of **6** (●, 100 μM; ▼, 200 μM; ■, 400 μM) with respect to ATP (10 – 80 μM).

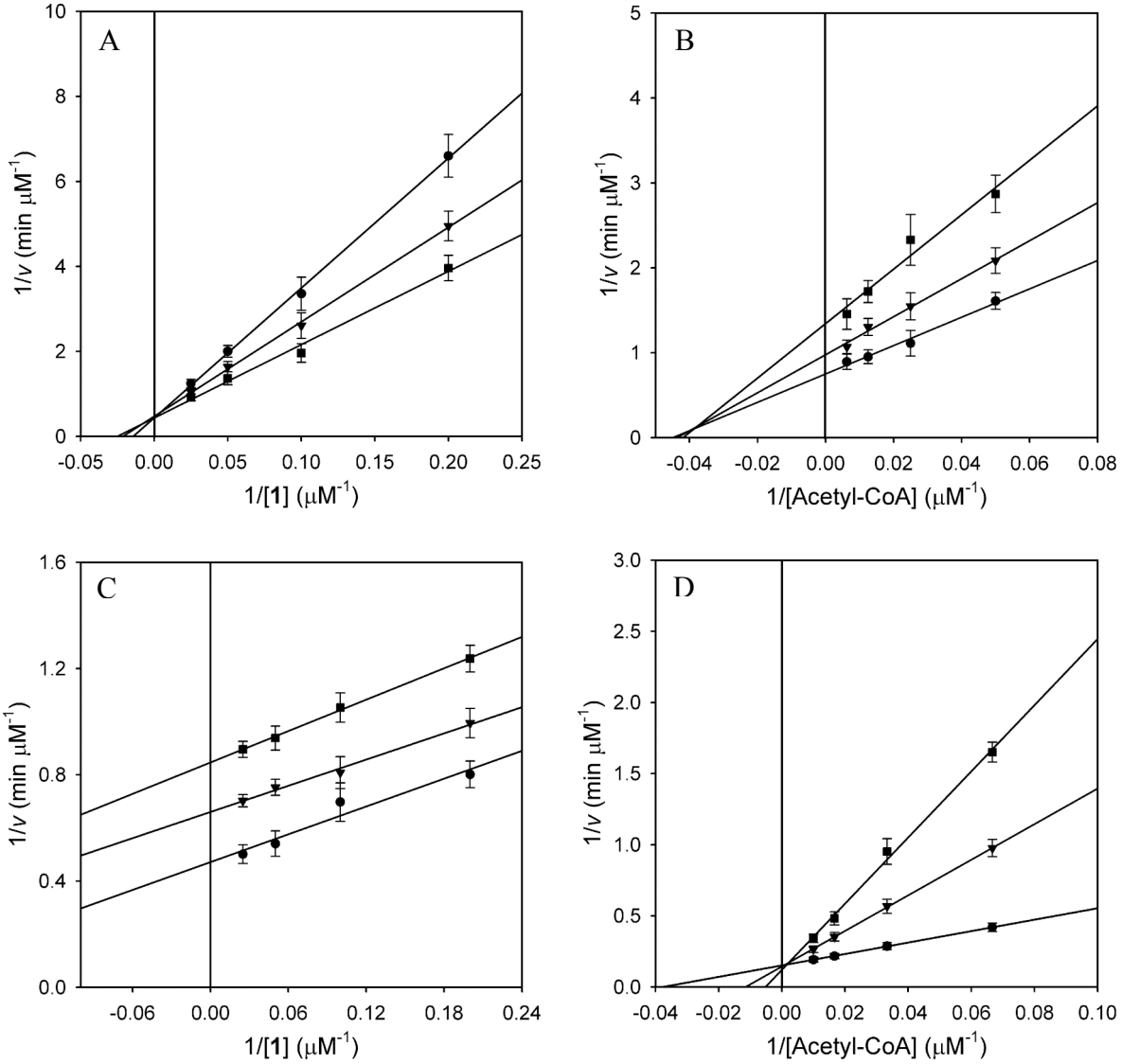

Figure S3. Inhibition patterns of dead-end inhibitors for AAC(6')-IId domain. A, competitive inhibition of paromomycin (**4**) (●, 50 μM; ▼, 100 μM; ■, 200 μM) with respect to **1** (5 – 40 μM). B, noncompetitive (mixed) inhibition of **4** (●, 50 μM; ▼, 100 μM; ■, 200 μM) with respect to acetyl-CoA (15 – 100 μM). C, uncompetitive inhibition of butyryl-CoA (●, 60 μM; ▼, 120 μM; ■, 240 μM) with respect to **1** (5 – 40 μM). D, competitive inhibition of butyryl-CoA (●, 50 μM; ▼, 200 μM; ■, 400 μM) with respect to acetyl-CoA (15 – 100 μM).

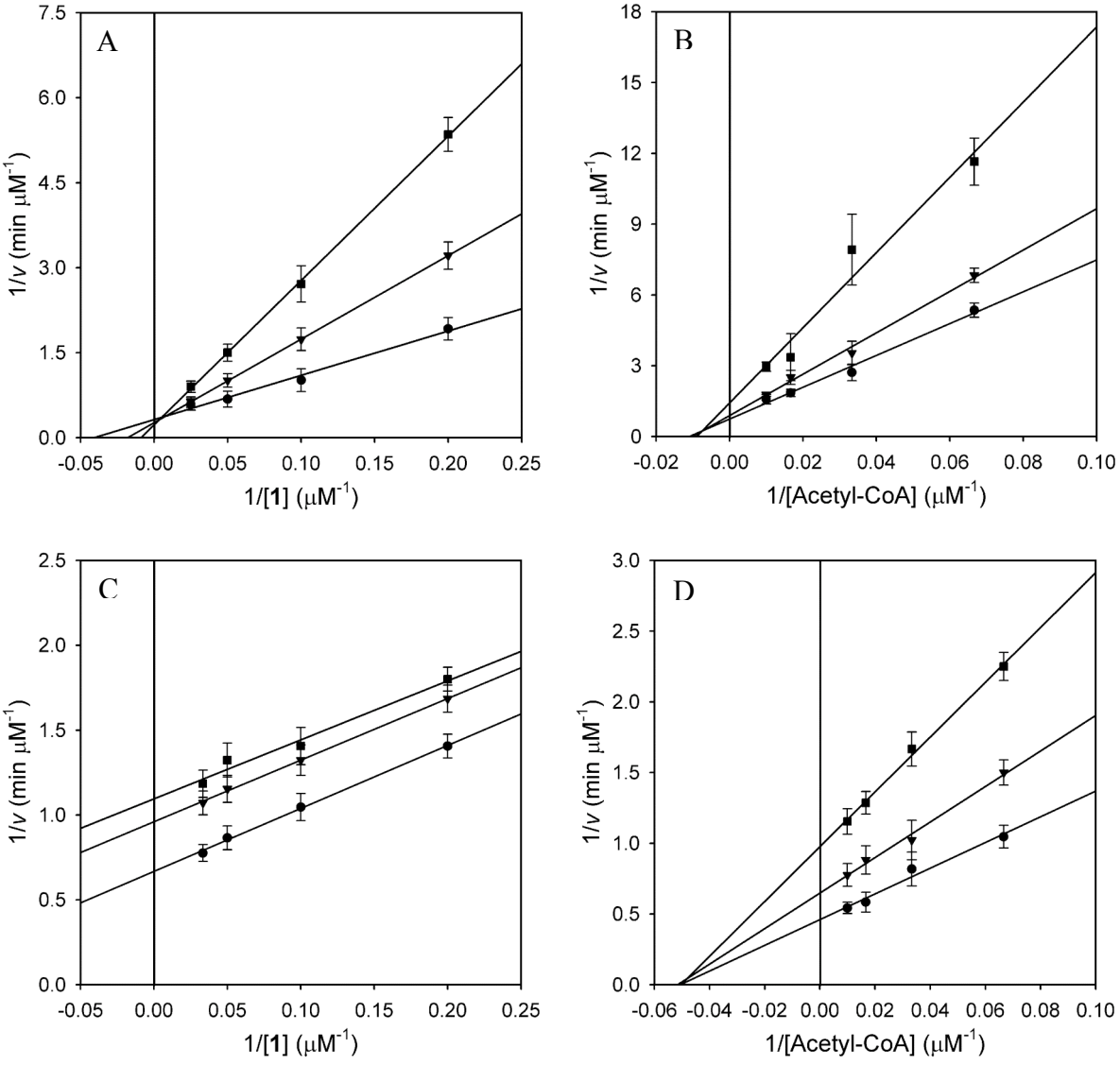

Figure S4. Inhibition patterns of the products for AAC(6')-IId domain. A, competitive inhibition of acetylated kanamycin A (**5**) (●, 20 μM; ▼, 80 μM; ■, 160 μM) with respect to **1** (5 – 40 μM). B, noncompetitive (mixed) inhibition of **5** (●, 300 μM; ▼, 600 μM; ■, 1000 μM) with respect to acetyl-CoA (15 – 100 μM). C, uncompetitive inhibition of CoASH (●, 20 μM; ▼, 40 μM; ■, 80 μM) with respect to **1** (5 – 30 μM). D, noncompetitive (mixed) inhibition of CoASH (●, 25 μM; ▼, 50 μM; ■, 100 μM)with respect to acetyl-CoA (15 – 100 μM).

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