Regio- and Chemo-Selective N-6'-Derivatization of Aminoglycosides: Bisubstrate Inhibitors and Probes to Study Aminoglycoside 6’-N-Acetyltransferases**

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Abbreviations used: AcCoA, acetyl coenzyme A; ACN: acetonitrile; APCI, atmospheric pressure chemical ionization; CoA, coenzyme A; COSY, $^1$H-$^1$H Correlation spectroscopy; DCC: 1,3-dicyclohexylcarbodiimide; DCM, dichloromethane; DCU: 1,3-dicyclohexylcarbodiurea; DMAP, N,N-dimethylaminopyridine; DTDP, 4,4’-dithiodipyridine; DTT, 1,4-dithiothreitol; DIPEA (Hunig’s base), N,N-diisopropylethylamine; EDTA, ethylenediaminetetraacetic acid; ESI, electron spray ionization; EtOAc, ethyl acetate; HEPES, [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hex, hexane; HMBC, $^1$H-$^{13}$C heteronucleus multiple bonds correlation spectroscopy; HRMS, High resolution mass spectrometry; HSQC, $^1$H-$^{13}$C heteronucleus single quantum correlation spectroscopy; LB, Luria-Bertani media; TEA, triethylamine; THF, tetrahydrofuran; TLC, analytical thin layer chromatography; TOCSY, total correlation spectroscopy.

Materials. All commercial reagents were used without further purification unless otherwise specified. Anhydrous THF was prepared freshly by distillation over sodium. Acetonitrile and dichloromethane were distilled from calcium hydride under inert atmosphere. Bromoacetic acid, 1-bromopropionic acid, 1-bromobutanoic acid and 1-bromovaleric acid were recrystallized from hexane. Neamine
hydrochloride was prepared by methanolysis of neomycin B using the procedure reported before.\cite{1} Kanamycin A, ribostamycin, AcCoA, DTDP, EDTA and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). All aminoglycoside free bases were prepared by neutralization using the anionic exchange resin Amberlite IRA-400(OH) from Sigma-Aldrich.

**Instrumentation.** Melting points were not corrected. HRMS of compounds 1-3, 11a-c were analyzed by direct infusion electrospray ionization from a solution in 90:10 methanol:50 mM aqueous ammonium hydroxide at 2 µL/min in an IonSpec 7 Tesla FTICR instrument at a resolving power of approximately 80,000. Other HRMS samples were analyzed using a Kratos MS 25RFA mass spectrometer at a source temperature of 200°C and 70 eV. LRMS was performed using a Finnigan LCQDUO mass spectrometer with either ESI or APCI without fragmentation. Routine ¹H and ¹³C NMR spectra were recorded using Varian Mercury 400 or 300 or Unity 500 spectrometers. The chemical shifts (δ) were reported in parts per million (ppm) relative to the internal standard TMS (0 ppm). The peak patterns are indicated as follows: s, singlet; d, doublet; t, triplet; dt, doublet of triplet; ddd, doublet of doublet of doublet; td, triplet of doublet; m, multiplet; q, quartet; br s, broad singlet, etc. All ¹H NMR and correlation spectra including COSY, HMBC and HSQC of aminoglycoside coenzyme A bisubstrates and aminoglycosides were recorded with solutions of pD 4 unless otherwise stated. ¹H and ¹³C NMR assignments were confirmed by HSQC, HMQC and HMBC.

Bisubstrate analogs 1-3, 11a-c, and N-6’-neamine derivatives 8a and 8e-k were purified by reversed-phase HPLC using an Agilent Zorbax SB-CN column (4.6 × 250 mm, 5 µ) on an Agilent 1100 system with diode array UV detector. Samples were eluted at a flow rate of 3 ml/min, using the linear gradients shown in Table S1.

**Table S1.** Linear gradient profile for HPLC purification

<table>
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<tr>
<th>Time (min)</th>
<th>% A (0.05% TFA in H₂O)</th>
<th>% B (0.04% TFA in ACN)</th>
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<tr>
<td>0</td>
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</table>

**General procedure for the synthesis of 1-3.** To a solution of 7a (0.052 mmol) dissolved in deoxygenated ACN (1.5 ml) is added a solution of free base aminoglycoside (4, 5, or 6, 0.104 mmol) in
deoxygenated H₂O (1.5 ml). This mixture is next transferred into a solution of the sodium salt of CoA (18 mg, 0.026 mmol, in 3 ml TEA/H₂CO₃ buffer at pH 8.5 stirred for 10 minutes before use). The reaction is monitored by ESI-MS until disappearance of CoA (766.1 in negative mode). The mixture is then evaporated in vacuo to ~1 ml and acidified to pH 4 using TFA. The solution is diluted with water (4 ml) and purified by reversed phase HPLC. The desired product is collected and lyophilized to yield a white fluffy powder.

**General procedure for the synthesis of 7a-m.** N-hydroxy-5-norbornene-2,3-dicarboximide (3 mmol) and the desired carboxylic acid (3 mmol) are dissolved in DCM (20 ml). A few ml of THF is sometimes required to completely dissolve the carboxylic acid. DCC (1.05 eq.) is next added followed by a catalytic amount of DMAP (ca. 5 mg). Usually, a few minutes after addition of DCC, a white solid (DCU) precipitates out and the reaction is most often complete in 2 hours, as judged by TLC. DCU is filtered out and the filtrate is evaporated to dryness yielding the desired product as a pure solid (>95% purity from NMR). When higher purity is needed, the crude product is dissolved in 35:65 EtOAc:Hex (50 ml) and filtered again to obtain a product of >99% purity after evaporation.

**General procedure for the synthesis of 8a, 8e-m.** One of the NBD esters 7a, 7e-m (0.058 mmol) and neamine free base (0.12 mmol) are dissolved in H₂O:ACN 1:1 (8 ml). The solution is ultrasonicated for 30 × 2 seconds and stirred for 30 minutes. The reaction mixture is concentrated in vacuo to ~1 ml, diluted in water (30 ml), acidified to pH = 2 using TFA and washed with diethyl ether. The aqueous layer is concentrated to 4 ml and the product is purified by reversed phase HPLC.

**General procedure for the synthesis of 11a-c.** To a solution of 7b-d (0.052 mmol) dissolved in deoxygenated acetone (1.5 ml) is added a solution of free base neamine (4, 0.104 mmol) dissolved in deoxygenated H₂O (1.5 ml). This mixture is next transferred into a solution of the sodium salt of CoA (18 mg, 0.026 mmol, in 3 ml acetone:H₂O 2:3 containing 0.3 % w/v DTT and stirred for 10 minutes before use) before addition of DIPEA (0.52 mmol). The reaction is monitored by ESI-MS for disappearance of CoA (766.1 in negative mode). The reaction mixture is then evaporated in vacuum to ~1 ml. The residue is diluted in H₂O (30 ml) and acidified to pH 4 using TFA. The solution is washed with diethyl ether and the aqueous phase is lyophilized to afford the crude product (white fluffy powder). The crude product is dissolved in H₂O (4 ml) and purified by reversed phase HPLC. The desired product is collected and lyophilized to yield a white fluffy powder.
Neamine-CoA bisubstrate analog 1:

HPLC chromatogram of purification:

Yield: 83%. ¹H NMR (D₂O, 500 MHz, presaturated): δ 8.48 (s, 1H), 8.26 (s, 1H), 6.05 (d, J = 4.5 Hz, 1H), 5.57 (d, J = 3.5 Hz, 1H), 4.44 (br s, 1H), 4.11 (m, 2H), 3.86 (s, 1H), 3.81 (t, J = 9.2 Hz, 1H), 3.76-3.67 (m, 4H), 3.48-3.38 (m, 6H), 3.38-3.28 (m, 4H), 3.25-3.18 (m, 5H), 2.53 (t, J = 6.5 Hz, 2H), 2.35 (m, 1H), 2.30 (t, J = 6.0 Hz, 2H), 1.80 (q, J = 12.5 Hz, 1H), 0.76 (s, 3H), 0.66 (s, 3H); ¹³C NMR (D₂O, 125 MHz, by HSQC and HMBC): δ 177.9, 174.9, 173.7, 150.2, 149.1, 144.8, 142.3, 118.7, 97.1, 88.0, 83.9, 79.8, 79.7, 75.8, 75.1, 74.0, 73.0, 72.8, 72.0, 71.9, 71.8, 69.6, 66.0, 54.0, 50.2, 48.9, 40.0, 38.7, 35.9, 35.8, 34.8, 31.6, 28.3, 21.2, 18.8; HRMS for C₃₅H₆₂N₁₁O₂₃P₃S (M+H), calcd. 1130.3034, found 1130.3035.
Kanamycin A-CoA bisubstrate analog 2:

HPLC chromatogram of purification:

Yield: 72%. ¹H NMR (D₂O, 500 MHz, presaturated): δ 8.49 (s, 1H), 8.27 (s, 1H), 6.06 (d, J = 4.5 Hz, 1H), 5.32 (d, J = 4.5 Hz, 1H), 4.96 (d, J = 3.5 Hz, 1H ), 4.44 (br s, 1H), 4.10 (m, 2H), 3.86 (s, 1H), 3.78-3.61 (m, 9H), 3.58-3.52 (m, 3H), 3.46-3.40 (m, 4H), 3.36-3.28 (m, 5H), 3.18 (m, 2H), 3.14 (m, 4H), 2.53 (t, J = 6.5 Hz, 2H), 2.40 (m, 1H), 2.30 (t, J = 6.5 Hz, 2H), 1.74 (q, J = 12.5 Hz, 1H), 0.77 (s, 3H), 0.66 (s, 3H); ¹³C NMR (D₂O, 125 MHz, by HSQC and HMBC): δ 174.8, 174.0, 173.2, 150.4, 149.5, 144.6, 142.0, 118.5, 100.8, 98.1, 87.5, 83.8, 83.6, 79.4, 74.4, 74.0, 73.6, 72.8, 72.2, 72.1, 72.0, 71.3, 71.2, 70.6, 68.0, 65.5, 65.3, 55.0, 50.0, 48.6, 46.7, 40.0, 39.9, 38.4, 38.3, 38.5, 37.7, 31.8, 28.4, 22.0, 20.0; HRMS for C₄₁H₇₂N₁₁O₂₈P₃S (M+H) calcd. 1291.6264, found 1291.6267.
Ribostamycin-CoA bisubstrate analog 3:

HPLC chromatogram of purification:

Yield: 67%. $^1$H NMR (D$_2$O, 400 MHz, presaturated): $\delta$ 8.51 (s, 1H), 8.29 (s, 1H), 6.09 (d, $J = 4.2$, 1H), 5.74 (d, $J = 4.0$, 1H), 5.20 (s, 1H), 4.46 (br s, 1H), 4.14 (m, 2H), 4.08 (d, $J = 6.0$ Hz, 1H), 4.03 (t, $J = 7.2$ Hz, 1H), 3.96 (t, $J = 9.6$ Hz, 1H), 3.89 (s, 2H), 3.82-3.71 (m, 4H), 3.67 (dt, $J = 9.6$, 4.0 Hz, 1H), 3.59 (t, $J = 9.2$ Hz, 1H), 3.54-3.48 (m, 4H), 3.36 (m, 4H), 3.28-3.18 (m, 8H), 2.56 (t, $J = 6.5$ Hz, 2H), 2.39 (m, 1H), 2.34 (t, $J = 6.0$ Hz, 1H), 1.87 (q, $J = 12.0$ Hz, 1H), 0.81 (s, 3H), 0.71 (s, 3H); $^{13}$C NMR (D$_2$O, 125 MHz, by HSQC and HMBC): $\delta$ 175.0, 174.0, 173.0, 151.0, 149.4, 142.8, 139.6, 118.6, 110.4, 96.8, 87.8, 84.3, 83.6, 82.2, 76.0, 75.6, 74.5, 74.0, 72.5, 72.0, 71.9, 71.5, 70.3, 69.0, 68.5, 65.2, 61.8, 54.0, 50.0, 49.0, 39.9, 39.6, 38.5, 35.8, 35.6, 34.7, 31.2, 28.5, 21.8, 18.6; HRMS for C$_{40}$H$_{70}$N$_{11}$O$_{28}$P$_{3}$S (M+H) calcd. 1261.3440, found: 1262.3455.
Neamine (4)

The $^1$H NMR spectrum of neamine is strongly dependent on the pH/pD. In general, the free base produces a cleaner $^1$H NMR spectrum than the hydrochloride salt. Enhanced solvation of the salt form yields much shorter relaxation time and more overlapping chemical shifts. Our assignment for the free base neamine is fully consistent with a previous report.$^{[2]}$

![Neamine molecule](image)

**Neamine free base:** $^1$H NMR (D$_2$O, 400 MHz, presaturated): $\delta$ 5.15 (d, $J = 4.0$ Hz, H1’), 3.63 (br t, $J \sim 8$ Hz, H5’), 3.42 (t, $J = 9.2$ Hz, H3’), 3.36 (m, H5), 3.16 (t, $J = 9.2$ Hz, H4’), 3.13 (t, $J = 9.2$ Hz, H4), 3.00 (t, $J = 9.6$ Hz, H6), 2.89 (dd, $J = 13.4, 2.6$ Hz, H6’), 2.72-2.52 (m, H3, H6’, H2’, H1), 1.83 (td, $J = 12.4, 4.0$ Hz, H2eq), 1.06 (q, $J = 12.4$ Hz, H2ax). $^{13}$C NMR (D$_2$O, 75 MHz) $\delta$ 101.09 (C1’), 87.21 (C4), 77.75 (C6), 76.34 (C5), 73.74 (C3’), 72.79 (C5’), 71.61 (C4’), 55.54 (C2’), 50.18 (C1), 49.63 (C3), 41.60 (C6’), 35.79 (C2).

**Neamine HCl salt:** $^1$H NMR (D$_2$O, 400 MHz, presaturated): $\delta$ 5.85 (d, $J = 3.5$ Hz, H1’), 3.95 (m, H6), 3.90 (m, H4), 3.87 (m, H5), 3.62 (t, $J = 5.6$ Hz, H4’), 3.50 (m, H3’) 3.47 (m, H3), 3.45 (m, H5), 3.42 (m, H6’), 3.39 (m, H2’), 3.28 (m, H1), 3.22 (m, H6’), 2.37 (td, $J = 13.2, 4.0$ Hz, H2eq), 1.76 (q, $J = 13.2$ Hz, H2ax). $^{13}$C NMR (D$_2$O, 75 MHz) $\delta$ 96.21 (C1’), 77.89 (C5’), 75.37 (C4’), 72.69 (C3’), 70.84 (C5), 69.40 (C6), 68.39 (C4), 53.71 (C2’), 49.90 (C1), 48.62 (C3), 40.32 (C6’), 28.52 (C2).
Kanamycin A (5)

Kanamycin A sulfate: The $^1$H NMR of kanamycin A free base (pD = 10) was reported before.\textsuperscript{[2]} Here the NMR of its sulfate salt (pD = 4) was analyzed. $^1$H NMR (D$_2$O, 500 MHz, pD = 4, presaturated): $\delta$ 5.35 (d, $J = 3.5$ Hz, H1’), 4.89 (d, $J = 3.5$ Hz, H1”), 3.81 (dt, $J = 9.5$, 3.5 Hz, H5”), 3.74 (dt, $J = 9.5$, 3.5 Hz, H5’), 3.60 (br s, H6”), 3.57-3.54 (m, H3’, H5), 3.51 (dd, $J = 10.5$, 3.5 Hz, H2’), 3.43 (dd, $J = 10.5$, 3.5 Hz, H2”), 3.35-3.29 (m, H4, H4”), 3.26 (t, $J = 9.5$ Hz, H4’), 3.20 (m, H4), 3.16 (m, H6’), 3.03 (t, $J = 10.5$ Hz, H3”), 3.02-2.89 (m, H1, H3, H6”), 1.95 (br d, $J = 12.5$ Hz, H2eq), 1.27 (q, $J = 12.5$ Hz, H2ax); $^{13}$C NMR (D$_2$O, 125 MHz, by HSQC): $\delta$ 100.5 (C1”), 97.5 (C1’), 86.4 (C5’), 82.8 (C5”), 73.6 (C5), 72.3 (C2”), 72.0 (C4’), 71.3 (C4), 71.0 (C3’), 70.2 (C4”), 69.0 (C2”), 67.4 (C6), 60.0 (C6”), 54.6 (C3”), 50.5 (C1), 48.7 (C3), 40.8 (C6’), 33.1 (C2).

Ribostamycin (6)

Ribostamycin sulfate: $^1$H NMR (D$_2$O, 500 MHz, pD = 4, presaturated): $\delta$ 5.80 (d, $J = 4.0$ Hz, H1’), 5.19 (br s, H1”), 4.06 (br d, H2”), 4.01 (t, $J = 7.0$ Hz, H3”), 3.86 (m, H4”, H5’), 3.81 (t, $J = 10.0$ Hz,
H4’), 3.74 (dd, J = 12.5, 2.0 Hz, H5’), 3.68 (m, H6, H5), 3.52 (dd, J = 12.5, 5.5 Hz, H5”), 3.47 (t, J = 7.5 Hz, H4), 3.30 (dd, J = 12.5, 3.0 Hz, H6’), 3.25 (t, J = 10.0 Hz, H3’), 3.21 (dd, J = 10.0, 4.0 Hz, H2’), 3.15-3.03 (m, H1, H3, H6’), 2.15 (td, J = 12.5, 4.0 Hz, H2eq), 1.56 (q, J = 12.5 Hz, H2ax); 13C NMR (D2O, 125 MHz, by HSQC): δ 110.8 (C1”), 95.3 (C1’), 85.7 (C4), 82.8 (C4”), 78.3 (C5’), 75.8 (C2”), 73.7 (C6), 71.8 (C3’), 69.2 (C3”), 69.0 (C4’), 68.8 (C5), 61.4 (C5”), 54.6 (C2”), 50.7 (C1), 49.1 (C3), 41.1 (C6’), 30.8 (C2).

7a (n = 1): TLC Rf = 0.47 in EtOAc/Hex (1/1). Yield: 99%, the crude product was recrystallized using hexane to give earth-red crystals, m.p. 90-92 °C. 1H NMR (CDCl3, 400 MHz): δ 6.20 (br s, 2H), 4.04 (s, 2H), 3.46 (br s, 2H), 3.34 (br s, 2H), 1.80 (d, J = 8.8 Hz, 1H), 1.54 (d, J = 8.8 Hz, 1H). 13C NMR (CDCl3, 75 MHz) δ 169.5, 135.0, 51.6, 45.1, 43.7, 21.9. HRMS for C11H10NO4 calcd. 298.98 (100) and 300.98 (97), found 299.00 (100) and 301.00 (97).

7b (n = 2): TLC Rf = 0.60 in EtOAc/Hex (1/1). Yield: >99%, the crude product was recrystallized using hexane to give yellowish crystals, m.p. 118-120 °C. 1H NMR (CDCl3, 400 MHz): δ 6.20 (br s, 2H), 3.58 (t, J = 7.2 Hz, 2H), 3.46 (br s, 2H), 3.34 (br s, 2H), 3.17 (t, J = 7.2 Hz, 2H), 1.80 (d, J = 8.8 Hz, 1H), 1.54 (d, J = 8.8 Hz, 1H); 13C NMR (CDCl3, 75 MHz) δ 169.8, 135.0, 51.6, 45.1, 43.6, 35.0, 24.0. HRMS for C12H12NO4, calcd. 312.98 (100) and 314.98 (97), found 313.00 (100) and 315.00 (97).

7c (n = 3): TLC Rf = 0.40 in EtOAc/Hex (1/2). Yield: 97%, the crude product was recrystallized using hexane to give white crystals, m.p. 65-66 °C. 1H NMR (CDCl3, 400 MHz): δ 6.19 (br s, 2H), 3.49 (t, J = 6.4 Hz, 2H), 3.45 (br s, 2H), 3.33 (br s, 2H), 2.76 (t, J = 6.8 Hz, 2H), 2.25 (tt, J = 6.8, 6.4 Hz, 2H), 1.79 (d, J = 8.8 Hz, 1H), 1.54 (d, J = 8.8 Hz, 1H); 13C NMR (CDCl3, 75 MHz) δ 170.0, 135.0, 51.6, 45.0, 43.6, 31.9, 29.8, 27.8. HRMS for C13H14NO4, calcd. 326.99 (100) and 328.99 (97), found 327.00 (100) and 329.00 (97).

7d (n = 4): TLC Rf = 0.36 in EtOAc/Hex (1/2). Yield: 95%, the crude product was recrystallized using hexane to give white crystals, m.p. 51-52 °C. 1H NMR (CDCl3, 400 MHz): δ 6.18 (t, J = 2.0 Hz, 2H), 3.44 (m, 4H), 3.30 (m, 2H), 2.57 (t, J = 7.2 Hz, 2H), 2.05-1.83 (m, 4H), 1.78 (td, J = 9.0, 2.0 Hz, 1H),
1.53 (d, J = 9.0 Hz, 1H); $^{13}\text{C NMR}$ (CDCl$_3$, 75 MHz) δ 170.1, 134.9, 51.6, 45.0, 43.5, 33.0, 31.7, 30.3, 23.5. HRMS for C$_{14}$H$_{16}$NO$_4$, calcd. 341.00 (100) and 343.00 (97), found 341.01 (100) and 343.01 (97).

![Chemical Structure](image)

$^7\text{e-m}$: TLC R$_f$ = 0.15 in EtOAc/Hex (1/1). Yield: 90%. $^1\text{H NMR}$ (CDCl$_3$, 400 MHz): δ 6.18 (br s, 2H), 3.45 (br s, 2H), 3.33 (br s, 2H), 2.25 (s, 3H), 1.79 (d, J = 8.8 Hz, 1H), 1.54 (d, J = 8.8 Hz, 1H); $^{13}\text{C NMR}$ (CDCl$_3$, 75 MHz) δ 169.5, 135.0, 51.6, 45.1, 43.7, 17.2. MS for C$_{11}$H$_{11}$NO$_4$(M+H) calculated 222.07, found 222.00.

$^7\text{f}$: TLC R$_f$ = 0.33 in EtOAc/Hex (1/2). Yield: 91%. $^1\text{H NMR}$ (CDCl$_3$, 400 MHz): δ 6.19 (br s, 2H), 3.45 (br s, 2H), 3.32 (br s, 2H), 2.52 (t, J = 7.6 Hz, 2H), 1.77 (m, 3H), 1.54 (d, J = 8.8 Hz, 1H), 1.02 (m, 3H); $^{13}\text{C NMR}$ (CDCl$_3$, 75 MHz): δ 169.9, 134.9, 51.4, 45.1, 43.6, 31.2, 16.3, 13.6; MS for C$_{13}$H$_{15}$NO$_4$(M+H) calculated 250.10, found 250.00.

$^7\text{g}$: TLC R$_f$ = 0.60 in EtOAc/Hex (1/2). Yield: 90%. $^1\text{H NMR}$ (CDCl$_3$, 400 MHz): δ 6.18 (br s, 2H), 3.44 (br s, 2H), 3.32 (br s, 2H), 2.53 (t, J = 7.6 Hz, 2H), 1.75 (m, 3H), 1.52 (m, 3H), 1.25 (br s, 14H), 0.88 (t, J = 6.8 Hz, 3H); $^{13}\text{C NMR}$ (CDCl$_3$, 75 MHz): δ 169.9, 134.7, 51.5, 45.4, 43.7, 33.0, 29.6, 29.4, 29.3, 29.1, 29.0, 28.5, 28.5, 23.5, 22.2, 14.1. MS for C$_{21}$H$_{31}$NO$_4$(M+H) calculated 362.23, found 362.20.

$^7\text{h}$: TLC R$_f$ = 0.65 in EtOAc/Hex (1/1). Yield: 94%. $^1\text{H NMR}$ (CDCl$_3$, 400 MHz): δ 8.09 (d, J = 8.0 Hz, 2H), 7.65 (t, J = 8.0 Hz, 1H), 7.48 (t, J = 8.0 Hz, 2H), 6.28 (br s, 2H), 3.50 (br s, 2H), 3.40 (br s, 2H), 1.83 (d, J = 8.8 Hz, 1H), 1.58 (d, J = 8.8 Hz, 1H); $^{13}\text{C NMR}$ (CDCl$_3$, 75 MHz): δ 170.2, 134.9, 134.7, 130.7, 130.4, 128.9, 51.7, 45.2, 43.5; MS for C$_{16}$H$_{13}$NO$_4$(M+H) calculated 284.08, found 284.10.

$^7\text{i}$: TLC R$_f$ = 0.66 in EtOAc/Hex (1/1). Yield: 100%. $^1\text{H NMR}$ (CDCl$_3$, 400 MHz): δ 7.31 (m, 5H), 6.19 (br s, 2H), 3.87 (s, 2H), 3.44 (br s, 2H), 3.32 (br s, 2H), 1.80 (d, J = 8.8 Hz, 1H), 1.58 (d, J = 8.8 Hz, 1H).
Hz, 1H); $^1^3$C NMR (CDCl$_3$, 75 MHz): $\delta$ 170.0, 135.1, 135.0, 129.5, 129.0, 127.9, 51.5, 45.0, 43.4, 37.2; MS for C$_{17}$H$_{15}$NO$_4$ (M+H) calculated 298.10, found 298.00.

7j: TLC R$_f$ = 0.55 in EtOAc/Hex (1/1). Yield: 99%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.93 (d, $J$ = 8.0 Hz, 1H), 7.86 (d, $J$ = 8.0 Hz, 1H), 7.82 (d, $J$ = 8.0 Hz, 1H), 7.58 (t, $J$ = 8.0 Hz, 1H), 7.50 (t, $J$ = 8.0 Hz, 1H), 7.45 (m, 2H), 6.15 (br s, 2H), 4.30 (s, 2H), 3.41 (br s, 2H), 3.27 (br s, 2H), 1.74 (br s, 1H), 1.47 (d, $J$ = 8.8 Hz, 1H). $^{13}$C NMR (CDCl$_3$, 75 MHz): $\delta$ 170.0, 166.4, 150.2, 138.2, 134.9, 127.2, 125.4, 125.0, 124.8, 124.8, 123.8, 122.5, 113.3, 51.2, 44.7, 43.2, 37.8; MS for C$_{21}$H$_{17}$NO$_4$ (M+H) calcd. 348.12, found 348.10.

7k: TLC R$_f$ = 0.77 in EtOAc/Hex (1/1). Yield: 93%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 8.05 (d, $J$ = 7.6 Hz, 1H), 7.48 (t, $J$ = 8.0 Hz, 1H), 7.27 (t, $J$ = 8.0 Hz, 1H), 7.25 (d, $J$ = 7.6 Hz, 1H), 6.26 (br s, 2H), 3.49 (br s, 2H), 3.39 (br s, 2H), 2.59 (s, 3H), 1.82 (d, $J$ = 8.8 Hz, 1H), 1.56 (d, $J$ = 8.8, 1H); $^{13}$C NMR (CDCl$_3$, 75 MHz): $\delta$ 169.9, 136.2, 134.8, 133.3, 132.4, 130.7, 127.76, 125.8, 51.4, 44.9, 43.3, 21.3. MS for C$_{17}$H$_{15}$NO$_4$ (M+H) calcd. 298.10, found 298.0.

7l: TLC R$_f$ = 0.53 in EtOAc/Hex (1/1). Yield: 96%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 8.04 (d, $J$ = 7.6 Hz, 1H), 7.52 (m, 2H), 7.36 (m, 1H), 6.27 (br s, 2H), 3.50 (br s, 2H), 3.39 (br s, 2H), 1.82 (d, $J$ = 8.8 Hz, 1H), 1.57 (d, $J$ = 8.8 Hz, 1H); $^{13}$C NMR (CDCl$_3$, 75 MHz): $\delta$ 169.9, 136.5, 134.7, 130.1, 129.0, 127.5, 126.7, 51.2, 44.4, 43.1; MS for C$_{16}$H$_{12}$ClNO$_4$ (M+H) calcd. 318.05, found 318.00.

7m: TLC R$_f$ = 0.49 in EtOAc/Hex (1/1). Yield: 97%. $^1$H NMR (CDCl$_3$, 400 MHz): $\delta$ 7.38 (d, $J$ = 8.4 Hz, 2H), 7.26 (t, $J$ = 8.4 Hz, 1H), 6.25 (br s, 2H), 3.50 (br s, 2H), 3.39 (br s, 2H), 1.81 (d, $J$ = 7.2 Hz, 1H), 1.57 (d, $J$ = 7.2, 1H); $^{13}$C NMR (CDCl$_3$, 75 MHz): $\delta$ 169.9, 134.8, 133.5, 129.6, 128.5, 127.8, 51.2, 44.4, 43.0; MS for C$_{16}$H$_{11}$Cl$_2$NO$_4$ (M+H) calcd. 351.02, found 351.00.
8a: Yield: 70%. ¹H NMR (D₂O, 400 MHz, pD 4.0, TFA salt, presaturated): 5.50 (d, J = 4.0 Hz, H1’), 3.92 (s, 2H), 3.70-3.61 (m, H3’, H4, H6), 3.43 (t, J = 8.8 Hz, H4’), 3.38-3.31 (m, H5’, H3, H6’), 3.21-3.16 (m, H5, H2’), 3.10 (td, J = 10, 3.8 Hz, H1), 2.31 (td, J = 8.8, 4.0 Hz, H2eq), 1.66 (q, J = 8.8 Hz, H2ax); ¹³C NMR (D₂O, 75 MHz): δ 169.6, 96.8 (C1’), 79.0 (C3’), 75.0 (C4’), 72.4 (C5’), 71.8 (C6), 70.2 (C5), 68.3 (C4), 54.0 (C2’), 50.7 (-COCH₂Br), 49.8 (C1), 48.2 (C3), 39.2 (C6’), 28.8 (C2); MS (ES) for C₁₄H₂₇BrN₄O₇ (M+H) calcd. 442.11, 444.10, found 442.20 (100%), 444.20 (98%).

8e: Yield: 60%. ¹H NMR (D₂O, 400 MHz, pD 4.0, TFA salt, presaturated): 5.50 (d, J = 4.0 Hz, H1’), 3.72-3.63 (m, H3’, H4, H6), 3.46 (t, J = 8.8 Hz, H4’), 3.40-3.32 (m, H5’, H3, H6’), 3.24-3.19 (m, H5, H2’), 3.13 (td, J = 10, 3.8 Hz, H1), 2.32 (td, J = 8.8, 4.0 Hz, H2eq), 1.82 (s, -COCH₃), 1.67 (q, J = 8.8 Hz, H2ax); ¹³C NMR (D₂O, 75 MHz): δ 169.6, 96.8 (C1’), 79.0 (C3’), 75.0 (C4’), 72.4 (C5’), 71.8 (C6), 70.2 (C5), 68.3 (C4), 54.0 (C2’), 50.7 (-COCH₂Br), 49.8 (C1), 48.2 (C3), 39.2 (C6’), 28.8 (C2), 21.9 (-COCH₃); MS (ES) for C₁₄H₂₈N₄O₇ (M+Na) calcd. 387.20, found 387.20.

8h: Yield: 80%. ¹H NMR (D₂O, 400 MHz, pD 4.0, TFA salt, presaturated): 7.59 (d, J = 8.0 Hz, ArH2, ArH6), 7.45 (t, J = 6.8 Hz, ArH4), 7.35 (t, J = 8.0Hz, ArH3 ArH5), 5.55 (d, J = 4.0 Hz, H1’), 3.81-3.70 (m, H3’, H6, H4), 3.62 (m, H6’), 3.49 (t, H4’), 3.40-3.30 (m, H5’, H5, H3), 3.26 (br d, J = 13.2 Hz, H2’), 3.13 (td, J = 10, 4.0 Hz, H1), 2.32 (td, J = 12.0, 4.0 Hz, H2eq), 1.67 (q, J = 12 Hz, H2ax); ¹³C NMR (D₂O, 75 MHz) δ 167.7, 135.4, 132.2, 128.7, 126.5, 97.0 (C1’), 79.0 (C3’), 75.2 (C4’), 72.8 (C5’), 72.0 (C6), 71.0 (C5), 69.0 (C4), 54.0 (C2’), 50.0 (C1), 48.6 (C3), 40.0 (C6’), 28.5 (C2); MS (ESI) for C₁₉H₃₀N₄O₇ (M+Na) calcd. 439.21, found 439.11.

8i: Yield: 75%. ¹H NMR (D₂O, 400 MHz, presaturated): δ 7.24-7.14 (m, 5H), 5.48 (d, J = 4.4, H1’), 3.73-3.65 (m, H3’, H4, H6), 3.47 (s, 2H, CO-CH₂-Ph), 3.45-3.40 (m, H6’, H4’), 3.40-3.30 (m, H5’, ArH2, ArH4), 7.35 (t, J = 8.0Hz, ArH3 ArH5), 5.55 (d, J = 4.0 Hz, H1’), 3.81-3.70 (m, H3’, H6, H4), 3.62 (m, H6’), 3.49 (t, H4’), 3.40-3.30 (m, H5’, H5, H3), 3.26 (br d, J = 13.2 Hz, H2’), 3.13 (td, J = 10, 4.0 Hz, H1), 2.32 (td, J = 12.0, 4.0 Hz, H2eq), 1.67 (q, J = 12 Hz, H2ax); ¹³C NMR (D₂O, 75 MHz) δ 167.7, 135.4, 132.2, 128.7, 126.5, 97.0 (C1’), 79.0 (C3’), 75.2 (C4’), 72.8 (C5’), 72.0 (C6), 71.0 (C5), 69.0 (C4), 54.0 (C2’), 50.0 (C1), 48.6 (C3), 40.0 (C6’), 28.5 (C2); MS (ESI) for C₁₉H₃₀N₄O₇ (M+Na) calcd. 439.21, found 439.11.
H6', H3), 3.17-3.07 (m, H5, H1, H2'), 2.32 (td, \( J = 12.0, 4.0 \) Hz, H2eq), 1.67 (q, \( J = 12.0 \) Hz, H2ax);

\( ^{13} \)C NMR (D$_2$O, 75 MHz) \( \delta \) 169.8, 135.1, 131.8, 130.0, 127.6, 97.2 (C1'), 79.4 (C3'), 75.6 (C4'), 72.8 (C5'), 72.0 (C6), 70.2 (C5), 69.1 (C4), 54.0 (C2'), 50.0 (C1), 48.9(C3), 42.6 (CH$_2$Ph), 38.0 (C6'), 28.4 (C2); MS (ESI) for C$_{20}$H$_{32}$N$_4$O$_7$ (M+H) calcd. 441.23, found 441.20.

8j: Yield: 85%. \(^1\)H NMR (D$_2$O, 400 MHz, presaturated): \( \delta \) 7.84 (t, \( J = 8.4 \) Hz, H5'', H8''), 7.79 (d, \( J = 8.0 \) Hz, H2''), 7.47(m, H6'', H7''), 7.40 (t, \( J = 8.0 \) Hz, H3''), 7.36 (d, \( J = 8.0 \) Hz, H4''), 5.33 (d, \( J = 4.0 \) Hz, H1'), 3.98 (s, CO-CH$_2$-Ar), 3.64-3.58 (m, H3', H4, H6), 3.48-3.35 (m, H4', H6', H5'), 3.32 (m, H3), 3.15 (dt, \( J = 11.0, 4.0 \) Hz, H1), 2.88 (t, \( J = 9.6 \) Hz, H5), 2.69 (dd, \( J = 11.2, 4.0 \) Hz, H2'), 2.33 (td, \( J = 12.4, 4.8 \) Hz, H2eq), 1.67 (q, \( J = 12.8 \) Hz, H2ax); MS (ESI) for C$_{24}$H$_{34}$N$_4$O$_7$ (M+Na) calcd. 513.24, found 513.20; \( ^{13} \)C NMR (D$_2$O, 100 MHz, presaturated): \( \delta \) 163.3 (CO), 133.8 (C10''), 131.8 (C9''), 131.1 (C1''), 129.1 (C4''), 128.9 (C2''), 128.5 (C8''), 126.9 (C3''), 126.4 (C6''), 126.2 (C5''), 123.7 (C7''), 96.7 (C1'), 79.0 (C3'), 75.2 (C4'), 72.7 (C5''), 71.9 (C6), 70.0 (C5), 68.6 (C4), 53.9 (C2'), 49.9 (C1), 48.7 (C3), 40.5 (CH$_2$), 38.9 (C6'), 28.6 (C2).
$^1$H NMR spectrum of 8j:

$^1$H-1H COSY spectrum of 8j:
$^1$H-$^{13}$C HSQC spectrum of 8j:

$^1$H-$^{13}$C HMBC spectrum of 8j:
Neamine-CoA bisubstrate 11a-c:

11a: HPLC chromatogram of crude sample during purification

Yield: 91%. $^1$H NMR (D$_2$O, 500 MHz, presaturated): $\delta$ 8.61 (s, 1H), 8.41 (s, 1H), 6.18 (d, $J = 5.0$ Hz, 1H), 5.69 (d, $J = 4.5$ Hz, 1H), 4.56 (br s, 1H), 4.24 (m, 2H), 3.97 (s, 1H), 3.91 (m, 1H), 3.87 (t, $J = 10.5$ Hz, 1H), 3.82-3.80 (m, 2H), 3.60-3.57 (m, 4H), 3.53-3.29 (m, 11H), 2.76 (t, $J = 6.5$ Hz, 2H), 2.62 (t, $J = 6.5$ Hz, 2H), 2.53 (t, $J = 6.5$ Hz, 2H), 2.48 (dt, $J = 12.0$, 4.0 Hz, 1H), 2.42 (t, $J = 6.5$ Hz, 2H), 1.80 (q, $J = 12.5$ Hz, 1H), 0.89 (s, 3H), 0.78 (s, 3H); $^{13}$C NMR (D$_2$O, 125 MHz by HSQC and HMBC): $\delta$ 177.7, 175.0, 173.5, 150.0, 149.1, 145.1, 142.5, 119.2, 96.8, 88.7, 83.9, 79.2, 78.6, 75.6, 74.7, 74.0, 73.0, 72.8, 72.0, 71.9, 70.8, 69.0, 65.8, 54.2, 50.0, 49.1, 39.9, 38.8, 36.0, 35.9, 35.8, 30.9, 28.4, 27.6, 22.0, 19.7; HRMS for C$_{36}$H$_{64}$N$_{11}$O$_{23}$P$_3$S (M+H) calcd. 1144.3183, found 1144.3188.
Yield: 52%. $^1$H NMR (D$_2$O, 500 MHz, presaturated): $\delta$ 8.60 (s, 1H), 8.38 (s, 1H), 6.18 (d, $J = 5.5$ Hz, 1H), 5.69 (d, $J = 4.0$ Hz, 1H), 4.57 (br s, 1H), 4.34 (m, 2H), 3.97 (s, 1H), 3.93 (m, 1H), 3.87 (t, $J = 9.0$ Hz, 1H), 3.84-3.77 (m, 2H), 3.61-3.55 (m, 5H), 3.52-3.43 (m, 4H), 3.38-3.28 (m, 6H), 2.59 (t, $J = 6.5$ Hz, 2H), 2.49 (m, 3H), 2.42 (t, $J = 6.5$ Hz, 2H), 2.33 (t, $J = 6.5$ Hz, 2H), 1.92 (q, $J = 12.5$ Hz, 1H), 1.79 (br t, $J = 7.0$ Hz, 2H), 0.88 (s, 3H), 0.77 (s, 3H); $^{13}$C NMR (D$_2$O, 125 MHz, by HSQC and HMBC): $\delta$ 176.6, 174.8, 174.1, 151.0, 149.5, 146.0, 142.0, 118.0, 96.7, 87.8, 83.4, 79.2, 75.0, 74.4, 73.5, 72.5, 72.2, 71.7, 71.5, 70.6, 68.5, 65.0, 54.0, 49.2, 48.5, 38.9, 37.8, 36.8, 35.5, 35.3, 34.8, 30.5, 30.2, 28.0, 25.4, 20.8, 17.6; HRMS for C$_{37}$H$_{66}$N$_{11}$O$_{23}$P$_3$S (M+H) calcd. 1158.3346, found 1158.3345.
1.50 (m, 2H), 1.51 (m, 2H), 0.88 (s, 3H), 0.77 (s, 3H); $^{13}$C NMR (D$_2$O, 125 MHz, by HSQC and HMBC): δ 178.0, 175.2, 173.6, 149.8, 148.8, 145.0, 142.4, 118.8, 97.0, 87.9, 83.6, 79.2, 75.0, 74.4, 73.5, 72.6, 72.3, 71.9, 71.6, 70.8, 68.8, 65.2, 53.8, 49.9, 48.4, 39.0, 38.8, 38.4, 35.5, 35.3, 35.0, 30.5, 30.2, 28.2, 27.8, 24.4, 21.0, 18.4; HRMS for C$_{38}$H$_{68}$N$_{11}$O$_{23}$P$_3$S (M+H) calcd. 1172.3496, found 1172.3501.

**Expression and purification of AAC(6')-Ii.** AAC(6')-Ii was obtained using a protocol previously described elsewhere.$^{[3]}$ The *Escherichia coli* strain BL21 was transformed with a pET22b expression plasmid containing the AAC(6')-Ii gene. The bacteria were grown in Luria-Bertani (LB) media at 37°C containing ampicillin (100 µg/mL). Expression of the protein was induced using isopropyl-β-D-thiogalactoside (IPTG). After harvesting the cells by centrifugation and washing them with a 0.85% NaCl solution, the cells were lysed by sonication. AAC(6')-Ii was purified in a two-step process: first, the lysate was run through a Q-Sepharose ion exchange column (GE Healthsciences); second, the AAC(6')-Ii containing fractions were further purified using a Gentamicin agarose affinity column (BioRad).

**AAC6'-Ii inhibition assay:** Enzyme activity was monitored using a procedure described elsewhere.$^{[4]}$ Thus reaction mixtures in HEPES (1 mM, pH = 7.5) and containing 4,4'-dithiodipyridine (DTDP, 2 mM), aminoglycoside (200 µM), and AAC(6')-Ii (25 µg/mL) were prepared with varying concentrations of AcCoA. Reaction volumes were typically 400 µl. The assay mixtures were preincubated for 3 min at 37°C. The initial velocities $V$ in absence of inhibitor were fit to Equation S1, where $[S]$ is the concentration of AcCoA, $K_m$ is the Michaelis-Menten constant, and $V_m$ is the maximal velocity. The calculated $K_m$ for AcCoA is 9.56 µM.

$$\frac{[S]}{V}\approx\frac{[S]}{V_m}+\frac{K_m}{V_m}$$  \hspace{1cm} (S1)

The initial reaction velocity (steady-state) obtained at various concentrations of inhibitor were fit to Hanes-Wolff plot.$^{[5]}$ Equation S2 for competitive inhibition, Equation S3 for noncompetitive inhibition, or Equation S4 for uncompetitive inhibition, where $[I]$ is the concentration of inhibitor and $K_i$ is the inhibition constant.

$$\frac{[S]}{V} = \frac{[S]}{V_m}+(1+[I]/K_i)\frac{K_m}{V_m}$$  \hspace{1cm} (S2)
\[
\frac{[S]}{V} = \frac{1+([I]/K_i)}{V_m} + K_m/V_m \quad (S3)
\]

\[
\frac{[S]}{V} = \frac{(1+[I]/K_i)S}{V_m} + \frac{(1+[I]/K_i)K_m}{V_m} \quad (S4)
\]

All the experimental data fit Equation S2 much better than S3 or S4, which suggested competitive inhibition. However, these results imply that the bisubstrates are tight binding inhibitors (K_i is less than 1000 times the concentration of AAC(6')-II). Thus Equations S5, S6 and S7 were applied for tight binding competitive inhibition, tight binding noncompetitive inhibition, or tight binding uncompetitive inhibition respectively.[5]

\[
IC_{50} = K_i(1+([S]/K_m)) + [E]/2 \quad (S5)
\]

\[
IC_{50} = K_i + [E]/2 \quad (S6)
\]

\[
IC_{50} = K_i(1+K_m/[S]) + [E]/2 \quad (S7)
\]

The IC_{50} was determined from Equation S8 where \(v_i\) is the initial velocity in the presence of inhibitor at concentration [I] and \(v_0\) is the initial velocity in the absence of inhibitor.

\[
\frac{v_i}{v_0} = 1/(1+[I]/IC_{50}) \quad (S8)
\]

The experimental data fit Equation S5 much better than S6 or S7, confirming that the bisubstrates are tight binding competitive inhibitors.

References: