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Supporting Information

Chemical and Structural Insights into the Regioversatility of the Aminoglycoside Acetyltransferase Eis

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SUPPORTING TABLES

Table S1. Mass analysis of aminoglycosides (AGs) acetylated by Eis.

AG		Calculated [M]$^+$	Observed [M+H]$^+$
AMK	Di[a]	669.31	670.35
KAN	Di	568.26	569.30
NET	Mono[b]	517.31	518.35
	Di	559.32	560.30
SIS	Di	531.29	532.35 (554.20 +Na)
TOB	Di	551.28	552.25 (574.20 +Na)

[a] Di indicates di-acetylation. [b] Mono indicates mono-acetylation.

Table S2. Proton chemical shifts determined for KAN and 3",6'-di-acetyl-KAN.[a]

Ring	H position	KAN	3",6'-di-acetyl-KAN	Δppm
II	1	3.61-3.54[b] (m)[c] [3.59][d]	3.63-3.45 (m) [3.53]	-0.06
	2_{ax}	2.02 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3} = 12.5$ Hz)	1.93 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3} = 12.8$ Hz)	-0.09
	2_{eq}	2.54 (ddd (app. dt), $J_{2eq,2ax} = 12.5$ Hz, $J_{2eq,1} = J_{2eq,3} = 4.0$ Hz)	2.54 (ddd (app. dt), $J_{2eq,2ax} = 12.8$ Hz, $J_{2eq,1} = J_{2eq,3} = 4.2$ Hz)	0.00
	3	3.61-3.54[b] (m) [3.57]	3.63-3.45 (m) [3.59]	0.02
	4	4.01-3.89[e] (m) [3.90]	3.94-3.66 (m) [3.85]	-0.05
	5	3.84-3.74 (m) [3.81]	3.94-3.66 (m) [3.73]	-0.08
	6	4.01-3.89[e] (m) [3.95]	3.94-3.66 (m) [3.90]	-0.05
I	1'	5.62 (d, $J_{1',2'} = 3.9$ Hz)	5.45 (d, $J_{1',2'} = 3.9$ Hz)	-0.17
	2'	3.64 (dd, $J_{1',2'} = 3.9$ Hz, $J_{2',3'} = 10.1$ Hz)	3.60 (dd, $J_{2',1'} = 3.9$ Hz, $J_{2',3'} = 9.8$ Hz)	-0.04
	3'	3.84-3.74 (m) [3.78]	3.94-3.66 (m) [3.72]	-0.06
	4'	3.35 (dd (app. t), $J_{4',3'} = J_{4',5'} = 9.5$ Hz)	3.51-3.43 (m) [3.31]	-0.04
	5'	4.01-3.89 (m) [3.96]	3.94-3.66 (m) [3.81]	-0.15
	$6'_a$	3.45 (dd, $J_{6'a,6'b} = 13.3$ Hz, $J_{6'a,5'} = 2.9$ Hz)	3.65-3.45 (m) [3.60]	**0.15**
	$6'_b$	3.14 (dd, $J_{6'b,6'a} = 13.3$ Hz, $J_{6'b,5'} = 8.7$ Hz)	3.65-3.45 (m) [3.52]	**0.38**
III	1"	5.14 (d, $J_{1'',2''} = 3.9$ Hz)	5.07 (d, $J_{1'',2''} = 3.5$ Hz)	-0.07
	2"	4.01-3.89 (m) [3.93]	3.69 (dd, $J_{1'',2''} = 3.5$ Hz)[†]	-0.24
	3"	3.50 (dd (app. t), $J_{3'',2''} = J_{3'',4''} = 10.5$ Hz)	4.11 (dd (app. t), $J_{3'',2''} = J_{3'',4''} = 10.3$ Hz)	**0.61**
	4"	3.69 (dd (app. t), $J_{4'',3''} = J_{4'',5''} = 10.5$ Hz)	3.49 (dd (app. t), $J_{4'',3''} = J_{4'',5''} = 10.3$ Hz)[%]	-0.20
	5"	4.01-3.89 (m) [3.93]	3.94-3.66 (m) [3.93]	-0.00
	$6''_a$	3.84-3.74 (m) [3.81]	3.94-3.66 (m) [3.80]	-0.01
	$6''_b$	3.84-3.74 (m) [3.78]	3.94-3.66 (m) [3.76]	-0.02
Acetyl	N$\underline{H}$-3"	×	8.14 (d, $J_{NH,3''} = 9.7$ Hz)	
	N$\underline{H}$-6'	×	8.06 (t, $J_{NH,6'} = 6.0$ Hz)	
	C$\underline{H}_3$C=O on 3"	×	2.04 (s)	
	C$\underline{H}_3$C=O on 6'	×	2.00 (s)	

[a] The chemical shift were established based on ^{1}H (400 MHz), zTOCSY, and gCOSY NMR. [b] Could be analogous position of the 2-deoxystreptamine (DOS) ring. [c] Multiplicity and *J* are given in (). [d] The numbers in [] were determined from gCOSY and/or zTOCSY. [e] Could be analogous position of the DOS ring. ×Indicates that the acetyl moiety is not present in the molecule. [†] Indicates partially obscured peak preventing determination of the second *J* value.

Table S3. Carbon chemical shifts determined for KAN and 3",6'-di-acetyl-KAN.[a]

Ring	C position	KAN[b]	3",6'-di-acetyl-KAN	Δppm
II	1	47.69	48.15	0.46
	2	27.36	27.50	0.14
	3	49.67	49.63	-0.04
	4	77.33	79.06	1.73
	5	83.70	83.45	-0.25
	6	72.72	72.95	0.23
I	1'	95.82	97.70	1.88
	2'	70.64	70.95	0.31
	3'	71.83	72.01	0.18
	4'	70.94	70.14	-0.95
	5'	68.56	71.08	2.52
	6'	40.34	39.44	**-0.90**
III	1"	100.40	100.84	0.44
	2"	68.06	69.99	1.93
	3"	54.77	53.85	**-0.92**
	4"	65.30	66.92	1.62
	5"	72.56	72.80	0.24
	6"	59.73	60.09	0.36
Acetyl	CH$_3\underline{C}$=O on 3"	×	175.20	
	CH$_3\underline{C}$=O on 6'	×	174.57	
	$\underline{C}$H$_3$C=O	×	22.0	
	$\underline{C}$H$_3$C=O	×	21.7	

[a] The chemical shift were established based on ^{13}C, gHSQC, and gHMBC NMR. ×Indicates that the acetyl moiety is not present in the molecule.

Table S4. Proton chemical shifts determined for AMK and 3",AHB-di-acetyl-AMK.[a]

Ring	H position	AMK	3",AHB-di-acetyl-AMK	Δppm
II	1	4.10-3.95 (m)[b] [4.06][c]	4.14-4.00 (m) [4.07]	0.01
	2_{ax}	1.80 (m)	1.87-1.70 (m) [1.80]	0.00
	2_{eq}	2.22-2.09 (m) [2.19]	2.23-2.17 (m) [2.20]	0.01
	3	3.53 (m)	3.65-3.40 (m) [3.56]	0.03
	4	3.90-3.80 (m) [3.85]	3.95-3.65 (m) [3.90]	0.05
	5	3.90-3.80 (m) [3.88]	3.95-3.65 (m) [3.90]	0.02
	6	3.90-3.80 (m) [3.83]	3.95-3.65 (m) [3.84]	0.01
	$C{=}OC\underline{H}(OH)CH_2CH_2NH_2$ (A1)	4.24 (dd, $J_{A1,A2a}$ = 2.8 Hz, $J_{A1,A2b}$ = 9.4 Hz)	[4.12] (dd, $J_{A1,A2a}$ = 2.8 Hz, $J_{A1,A2b}$ = 9.4 Hz)	-0.12
	$C{=}OCH(OH)C\underline{H}_2CH_2NH_2$ ($A2_a$)	1.93 (m)	1.87-1.70 (m) [1.74]	-0.19
	$C{=}OCH(OH)C\underline{H}_2CH_2NH_2$ ($A2_b$)	2.13 (m)	2.03-1.92 (m) [1.98]	-0.15
	$C{=}OCH(OH)CH_2C\underline{H}_2NH_2$ (A3)	3.18-3.10 (m) [3.15]	3.31-2.26 (m) [3.29]	**0.14**
I	1'	5.54 (d, $J_{1',2'}$ = 3.6 Hz)	5.57 (d, $J_{1',2'}$ = 3.7 Hz)	0.03
	2'	368-3.62 (m) [3.63]	3.95-3.65 (m) [3.66]	0.03
	3'	3.80-3.70 (m) [3.74]	3.95-3.65 (m) [3.78]	0.04
	4'	3.37-3.32 (m) [3.35]	3.35 (dd (app. t), $J_{4',3'} = J_{4',5'}$ = 9.5 Hz)	0.00
	5'	4.10-3.95 (m) [3.98]	4.14-4.00 (m) [4.02]	0.04
	$6'_a$	3.43-3.31 (m) [3.41]	3.65-3.40 (m) [3.45]	0.04
	$6'_b$	3.18-3.10 (m) [3.14]	3.14 (dd, J = 13.3 Hz, J = 8.7 Hz)	0.00
III	1"	5.14 (d, $J_{1",2"}$ = 3.3 Hz)	5.13 (d, $J_{1",2"}$ = 3.7 Hz)	-0.01
	2"	3.80-3.70 (m) [3.75]	3.65-3.40 (m) [3.51]	-0.24
	3"	3.37-3.32 (m) [3.36]	4.14-4.00 (m) [4.05]	**0.69**
	4"	368-3.62 (m) [3.66]	3.65-3.40 (m) [3.46]	-0.20
	5"	4.10-3.95 (m) [4.05]	4.14-4.00 (m) [4.08]	0.03
	$6"_a$	3.87-3.72 (m) [3.78]	3.95-3.65 (m) [3.78]	0.00
	$6"_b$	3.87-3.72 (m) [3.76]	3.95-3.65 (m) [3.72]	-0.04
Amide	$N\underline{H}$-1	7.62[‡]	8.52 (d, J = 9.8 Hz)*	
	$C{=}OCH(OH)(C\underline{H}_2)_2N\underline{H}$-$C{=}OCH_3$	×	8.00 (t, J = 6.2 Hz)*	
	$N\underline{H}$-3"	×	8.08 (d, J = 10.4 Hz)*	
Acetyl	$C\underline{H}_3C{=}O$ on AHB	×	1.97 (s)	
	$C\underline{H}_3C{=}O$ on 3"	×	2.03 (s)	

[a] The chemical shift were established based on ^{1}H (400 MHz), zTOCSY, and gCOSY NMR. [b] Multiplicity and J are given in (). [c] The numbers in [] were determined from gCOSY and zTOCSY. *Indicates that the values were determined from spectra taken in 9:1/H_2O:D_2O at 600 MHz. ×Indicates that the acetyl moiety is not present in the molecule. [‡] Detected in the zTOCSY (400 MHz).

Table S5. Proton chemical shifts determined for NET and 6'-acetyl-NET.[a]

Ring	H position	NET	6'-acetyl-NET	Δppm
II	1	3.26-3.17 (m)[b] [3.22][c]	3.60-3.35 (m) [3.38]	0.16
	2_{ax}	1.75 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3}$ = 12.4 Hz)	1.87 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3}$ = 12.0 Hz)	0.12
	2_{eq}	2.47 (ddd (app. dt), $J_{2eq,2ax}$ = 12.4, $J_{2eq,1} = J_{2eq,3}$ = 4.1 Hz)	2.64-2.55 (m) [2.60]	0.13
	3	3.40-3.31 (m) [3.34]	3.60-3.35 (m) [3.42]	0.08
	4	3.80-3.70 (m) [3.75]	3.92-3.70 (m) [3.89]	0.14
	5	3.80-3.70 (m) [3.75]	3.92-3.70 (m) [3.75]	=
	6	3.86 (dd (app. t), $J_{6,1} = J_{6,5}$ = 9.4 Hz)	3.92-3.70 (m) [3.89]	0.03
	7 ($NHC\underline{H}_2CH_3$)	3.10 (dq, $J_{7,7}$ = 12.1 Hz, J_{78} = 7.0 Hz) /	3.05 (dq, $J_{7,7}$ = 12.3 Hz, $J_{7,8}$ = 7.0 Hz) /	-0.05
		3.26-3.17 (m) [3.21]	3.28 (dq, $J_{7,7}$ = 12.3 Hz, $J_{7,8}$ = 7.0 Hz)	0.07
	8 ($NHCH_2C\underline{H}_3$)	1.29 (t, J = 7.0 Hz)	1.30 (t, J = 7.0 Hz)	0.01
I	1'	5.61 (bs)	5.50 (s)	-0.11
	2'	3.80-3.70 (m) [3.75]	3.86-3.78 (m) [3.83]	0.08
	$3'_{ax}$	2.60 (ddd (app. dt), $J_{3'ax,3'eq}$= 18.0 Hz)	2.64-2.55 (m) [2.57]	-0.03
	$3'_{eq}$	2.32 (ddd (app. dt), $J_{3'eq,3'ax}$ = 18.0 Hz)	2.30 (ddd (app. bd), $J_{3'eq,3'ax}$ = 18.4 Hz)	-0.02
	4'	5.18 (m (app. bdd))[^]	[4.87][£]	-0.31
	$6'_a$	3.66 (s)	3.92-3.70 (m) [3.76]	**0.10**
	$6'_b$	3.66 (s)	3.92-3.70 (m) [3.76]	**0.10**
III	1"	5.12 (d, $J_{1",2"}$ = 3.7 Hz)	5.14 (d, $J_{1",2"}$ = 3.5 Hz)	0.02
	2"	4.23 (dd, $J_{2",1"}$ = 3.7 Hz, $J_{2",3"}$ = 10.8 Hz)	4.29-4.23 (m) [4.26]	0.03
	3"	3.38 (d, $J_{3",2"}$ = 10.8 Hz)	3.60-3.35 (m) [3.42]	0.04
	$5"_{ax}$	3.49 (d, $J_{5"a,5"b}$ = 12.8 Hz)	3.60-3.35 (m) [3.50]	0.01
	$5"_{eq}$	4.02 (d, $J_{5"b,5"a}$ = 12.8 Hz)	[3.99]	-0.03
	$C\underline{H}_3$-4"	1.35 (s)	1.34 (s)	-0.01
	$NHC\underline{H}_3$	2.92 (s)	2.93 (s)	0.01
Acetyl	$N\underline{H}$-6'	×	8.32 (t, $J_{NH,6'}$ = 6.0 Hz)	
	$C\underline{H}_3C{=}O$ on 6'	×	2.02 (s)	

[a] The chemical shift were established based on ^{1}H (400 MHz), zTOCSY, and gCOSY NMR. [b] Multiplicity and J are given in (). [c] The numbers in [] were determined from gCOSY and zTOCSY. ×Indicates that acetyl moiety is not present in the molecule. [£] Partially obscured by H_2O peak in the spectra.

Table S6. Proton chemical shifts determined for NET and 2',6'-di-acetyl-NET.[a]

Ring	H position	NET	2',6'-di-acetyl-NET	Δppm
II	1	3.26-3.17 (m)[b] [3.22][c]	[3.13]	-0.09
	2_{ax}	1.75 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3}$ = 12.4 Hz)	[1.72]	-0.03
	2_{eq}	2.47 (ddd (app. dt), $J_{2eq,2ax}$ = 12.4, $J_{2eq,1} = J_{2eq,3}$ = 4.1 Hz)	[2.49]	0.02
	3	3.40-3.31 (m) [3.34]	[3.34]	=
	4	3.80-3.70 (m) [3.75]	[3.80-3.60][§]	X
	5	3.80-3.70 (m) [3.75]	[3.80-3.60][§]	X
	6	3.86 (dd (app. t), $J_{6,1} = J_{6,5}$ = 9.4 Hz)	[3.80-3.60][§]	X
	7	3.10 (dq, $J_{7,7}$ = 12.1 Hz, $J_{7,8}$ = 7.0 Hz) /	[2.97] /	-0.13 /
	(NHC$\underline{H}_2$CH$_3$)	3.26-3.17 (m) [3.21]	[3.21]	=
	8 (NHCH$_2$C$\underline{H}_3$)	1.29 (t, J = 7.0 Hz)	1.28 (t, J = 6.8 Hz)	-0.01
I	1'	5.61 (bs)	[5.32]	-0.29
	2'	3.80-3.70 (m) [3.75]	[4.20]	**0.45**
	$3'_{ax}$	2.60 (ddd (app. dt), $J_{3'ax,3'eq}$= 18.0 Hz)	[2.29]	-0.31
	$3'_{eq}$	2.32 (ddd (app. dt), $J_{3'eq,3'ax}$ = 18.0 Hz)	[2.11]	-0.21
	4'	5.18 (m (app. bdd))[^]	[4.87]	-0.31
	$6'_a$	3.66 (s)	[3.73]	**0.07**
	$6'_b$	3.66 (s)	[3.73]	**0.07**
III	1"	5.12 (d, $J_{1",2"}$ = 3.7 Hz)	5.14 (d, $J_{1",2"}$ = 3.5 Hz)	0.02
	2"	4.23 (dd, $J_{2",1"}$ = 3.7 Hz, $J_{2",3"}$ = 10.8 Hz)	[4.25]	0.02
	3"	3.38 (d, $J_{3",2"}$ = 10.8 Hz)	[3.37]	-0.01
	$5"_{ax}$	3.49 (d, $J_{5"a,5"b}$ = 12.8 Hz)	[3.48]	-0.01
	$5"_{eq}$	4.02 (d, $J_{5"b,5"a}$ = 12.8 Hz)	[4.00]	-0.02
	C$\underline{H}_3$-4"	1.35 (s)	1.33 (s)	-0.02
	NHC$\underline{H}_3$	2.92 (s)	2.92 (s)	=
Acetyl	N$\underline{H}$-2'	×	[8.06]	
	N$\underline{H}$-6'	×	[8.30]	
	C$\underline{H}_3$C=O on 2'	×	2.01 (s)	
	C$\underline{H}_3$C=O on 6'	×	2.01 (s)	

[a] The chemical shift were established based on ^{1}H (400 MHz), zTOCSY, and gCOSY NMR. [b] Multiplicity and J are given in (). [c] The numbers in [] were determined from gCOSY and zTOCSY. ×Indicates that the acetyl moiety is not present in the molecule. [§] Indicates that the peaks were not resolved due to interfering peaks from CoA.

Table S7. Proton chemical shifts determined for SIS and 6'-acetyl-SIS.[a]

Ring	H position	SIS	6'-acetyl-SIS	Δppm
II	1	3.41-3.27[b] (m)[c] [3.31][d]	3.50-3.30 (m) [3.36]	0.05
	2_{ax}	1.82 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3}$ = 12.4 Hz)	1.84 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3}$ = 12.5 Hz)	0.02
	2_{eq}	2.40-2.30 (m) [2.38]	2.64-2.44 (m) [2.49]	0.11
	3	3.41-3.27[b] (m) [3.36]	3.50-3.30 (m) [3.45]	0.09
	4	3.75-3.65[e] (m) [3.68]	3.89-3.64 (m) [3.72]	0.04
	5	3.75-3.65 (m) [3.70]	3.89-3.64 (m) [3.76]	0.06
	6	3.91[e] (dd (app. t), $J_{6,1} = J_{6,5}$ = 9.4 Hz)	3.89-3.64 (m) [3.85]	-0.06
I	1'	5.60 (s)	5.51[£]	-0.09
	2'	3.79 (bs)	3.89-3.64 (m) [3.81]	0.02
	$3'_{ax}$	2.40-2.30 (m) [2.34]	2.30 (dd (app. bd), $J_{3'ax,3'eq}$ = 17.8 Hz)	-0.04
	$3'_{eq}$	2.63 (dd (app. bd), $J_{3'eq,3'ax}$ = 18.2 Hz)	2.64-2.44 (m) [2.58]	-0.05
	4'	5.18 (bs)	4.89	-0.29
	$6'_a$	3.75-3.65 (m) [3.67]	3.89-3.64 (m) [3.81]	**0.14**
	$6'_b$	3.75-3.65 (m) [3.67]	3.89-3.64 (m) [3.81]	**0.14**
III	1"	5.15 (d, $J_{1",2"}$ = 3.6 Hz)	5.15	=
	2"	4.20 (dd, $J_{2",1"}$ = 3.6 Hz, dd, $J_{2",3"}$ = 10.8 Hz)	4.26-4.19 (m) [4.22]	0.02
	3"	3.40 (d, $J_{3",2"}$ = 10.8 Hz)	3.50-3.30 (m) [3.43]	0.03
	$5"_{ax}$	4.05 (dd, $J_{5"a,5"b}$ = 12.7 Hz)	4.02 (dd, $J_{5"a,5"b}$ = 12.9 Hz)	-0.03
	$5"_{eq}$	3.47 (dd, $J_{5"b,5"a}$ = 12.7 Hz)	3.50-3.30 (m) [3.48]	0.01
	C$\underline{H}_3$-4"	1.35 (s)	1.35 (s)	=
	NHC$\underline{H}_3$	2.92 (s)	2.92 (s)	=
Acetyl	N$\underline{H}$-6'	×	8.20 (t)	
	C$\underline{H}_3$C=O on 6'	×	2.03 (s)	

[a] The chemical shift were established based on ^{1}H (400 MHz), zTOCSY, and gCOSY NMR at pH 3. [b] Could be analogous position of the DOS ring. [c] Multiplicity and J are given in (). [d] The numbers in [] were determined from gCOSY. [e] Could be analogous position of the DOS ring. ×Indicates that the acetyl moiety is not present in the molecule. [£] Obscured due to interference resulting from solvent suppression.

Table S8. Proton chemical shifts determined for SIS and 6',2'-di-acetyl-SIS as well as 6',3 or 1-di-acetyl-SIS.[a]

Ring	H position	SIS	6',3 or 1- (Δppm)	6',2'- (Δppm)
II	1	3.62-3.54[b] (m)[c] [3.57][d]	[3.53[b]] (-0.04)	[3.45] (-0.12)
	2_{ax}	2.12 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3} = 12.6$ Hz)	[1.78] (-0.34)	[1.99] (-0.13)
	2_{eq}	2.58 (ddd (app. dt), $J_{2eq,2ax} = 12.6$ Hz, $J_{2eq,1} = J_{2eq,3} = 4.3$ Hz)	[2.25] (-0.33)	[2.59] (-0.01)
	3	3.62-3.54[b] (m) [3.57]	**[3.99[b]] (0.42)**	[3.58] (0.01)
	4	3.87[e] (dd (app. t), $J_{4,3} = J_{4,5} = 9.6$ Hz)	[3.83[e]] (-0.03)	[3.79] (-0.08)
	5	4.15 (dd (app. t), $J_{5,4} = J_{5,6} = 9.6$ Hz)	[4.12] (-0.03)	[4.04] (-0.11)
	6	3.80[e] (dd (app. t), $J_{6,1} = J_{6,5} = 9.6$ Hz)	[3.76[e]] (-0.04)	[3.80] (0.00)
I	1'	5.64 (s)	[5.54] (-0.10)	[5.39] (-0.25)
	2'	3.96 (d (app. bd), $J_{2',3'} = 4.3$ Hz)	[3.88] (-0.08)	**[4.26] (0.20)**
	$3'_{ax}$	2.75 (ddd (app. dt), $J_{3'ax,3'eq} = 18.9$ Hz)	[2.63] (-0.12)	[2.35] (-0.40)
	$3'_{eq}$	2.40 (ddd (app. bd), $J_{3'eq,3'ax} = 18.9$ Hz)	[2.33] (-0.07)	[2.15] (-0.25)
	4'	5.21 (t, $J_{4',3'} = 3.7$ Hz)	[4.90] (-0.31)	[4.92] (-0.29)
	$6'_{a}$	3.70 (d, $J_{6'a,6'b} = 14.0$ Hz)	**[3.83] (0.13)**	**[3.81] (0.11)**
	$6'_{b}$	3.75 (d, $J_{6'b,6'a} = 14.0$ Hz)	**[3.83] (0.08)**	**[3.81] (0.06)**
III	1"	5.16 (d, $J_{1'',2''} = 3.7$ Hz)		
	2"	4.24 (dd, $J_{2'',1''} = 3.7$ Hz, $J_{2'',3''} = 10.8$ Hz)		
	3"	3.62-3.48 (m) [3.51]		
	$5''_{a}$	4.03 (d, $J_{5''a,5''b} = 12.6$ Hz)		
	$5''_{b}$	3.51 (d, $J_{5''b,5''a} = 12.6$ Hz)		
	$C\underline{H}_3$-4"	1.37 (s)		
	$NHC\underline{H}_3$	2.95 (s)		
Acetyl	$N\underline{H}$-6'	×	8.35	8.27
	$N\underline{H}$-3/1 or $N\underline{H}$-2'	×	8.21 ($N\underline{H}$-3/1)	8.12 ($N\underline{H}$-2')
	$C\underline{H}_3C=O$ on X	×		
	$C\underline{H}_3C=O$ on 6'	×		

[a] The chemical shift were established based on ^1H (400 MHz), 2D-TOCSY and 2D-COSY NMR at pH 3. [b] Could be analogous position of the 2-deoxystreptamine (DOS) ring. [c] Multiplicity and *J* are given in (). [d] The numbers in [] were determined from gCOSY. [e] Could be analogous position of the DOS ring. ×Indicates that acetyl moiety is not present in the molecule.

Note: The chemical shifts of Ring III protons of the di-acetylated-SIS compounds were omitted because they could not be distinguished from one another and because Ring III does not contain potential sites of acetylation.

Table S9. Proton chemical shifts determined for TOB and 3",6'-di-acetyl-TOB.[a]

Ring	H position	TOB	3",6'-di-acetyl-TOB	Δppm
II	1	3.21-3.11[b] (m)[c] [3.18][d]	[3.38]	0.20
	2_{ax}	1.68 (ddd (app. q), $J_{2ax,2eq} = J_{2ax,1} = J_{2ax,3} = 12.7$ Hz)	[1.85]	0.17
	2_{eq}	2.29-2.21 (m) [2.27]	[2.49]	0.22
	3	3.43-3.31[c] (m) [3.34]	[3.52]	0.18
	4	3.72-3.58[e] (m) [3.63]	[3.73]	0.10
	5	3.80-3.75 (m) [3.77]	[3.79]	0.02
	6	3.72-3.58[e] (m) [3.70]	[3.79]	0.19
I	1'	5.68 (bs)	5.47 (bs)	-0.21
	2'	3.51-3.46 (m) [3.48]	[3.59]	0.11
	3'a	1.68 (ddd (app. q), $J_{3'a, 3'b} = J_{3'a, 2'} = J_{3'a, 4'} = 11.8$ Hz)	[1.95]	0.27
	3'b	2.29-2.21 (m) [2.23]	[2.26]	0.03
	4'	3.66-3.58 (m) [3.63]	[3.46]	-0.17
	5'	3.96-3.88 (m) [3.90]	[3.78]	-0.12
	$6'_{a}$	3.43-3.31 (m) [3.41]	[3.60]	**0.19**
	$6'_{b}$	3.43-3.31 (m) [3.41]	[3.60]	**0.19**
III	1"	5.11 (bs)	5.06 (bs)	-0.05
	2"	3.86-3.75 (m) [3.83]	(m) [3.69]	-0.14
	3"	3.43-3.31 (m) [3.36]	(m) [4.09]	**0.73**
	4"	3.66-3.58 (m) [3.60]	(m) [3.45]	-0.15
	5"	3.96-3.88 (m) [3.93]	(m) [3.91]	-0.02
	$6''_{a}$	(m) [3.79]	(m) [3.70]	-0.09
	$6''_{b}$	(m) [3.79]	(m) [3.70]	-0.09
Acetyl	$N\underline{H}$-3"	×	(m) [8.14]	
	$N\underline{H}$-6'	×	(m) [8.11]	
	$C\underline{H}_3C=O$ on 3"	×	2.03 (s)	
	$C\underline{H}_3C=O$ on 6'	×	1.98 (s)	

[a] The chemical shift were established based on ^1H (400 MHz), zTOCSY, and gCOSY NMR [b] Could be analogous position of the DOS ring. [c] Multiplicity and *J* are given in (). [d] The numbers in [] were determined from gCOSY and/or zTOCSY. [e] Could be analogous position of the DOS ring. ×Indicates that the acetyl moiety is not present in the molecule.

Table S10. Fraction of solvent ASA of TOB buried in the complex with an AME.

PDB accession code	% ASA buried of TOB
EisC204A-CoA-TOB	80
1M4D	73
3SG8	69
3VET	59
4EVY	53
4EBK	53

1M4D: AAC(2') from *M. tuberculosis* in complex with CoA and TOB [1].

3SG8: AG 2"-phosphotransferase type Iva in complex with TOB [2].

3VET: *O*-carbamoyltransferase TobZ in complex with TOB, carbamoyl phosphate, and ADP [3].

4EVY: AAC(6')-Ig from *Acinetobacter haemolyticus* in complex with TOB.

4EBK: ANT(4')-IIb in complex with TOB.

Table S11. Analysis of protein (AME) ASA buried in the interface with one TOB molecule.

PDB accession code	Total ASA buried (Å^2)	Non-polar ASA buried (Å^2)	Polar ASA buried (Å^2)	% non-polar ASA buried
EisC204A-CoA-TOB	401	229	172	57
1M4D	313	119	194	38
3SG8	352	138	214	39
3VET	283	128	155	45
4EVY	270	99	171	37
4EBK	301	89	207	30

1M4D: AAC(2') from *M. tuberculosis* in complex with CoA and TOB [1].

3SG8: AG 2"-phosphotransferase type Iva in complex with TOB [2].

3VET: *O*-carbamoyltransferase TobZ in complex with TOB, carbamoyl phosphate, and ADP [3].

4EVY: AAC(6')-Ig from *Acinetobacter haemolyticus* in complex with TOB.

4EBK: ANT(4')-IIb in complex with TOB.

SUPPORTING FIGURES

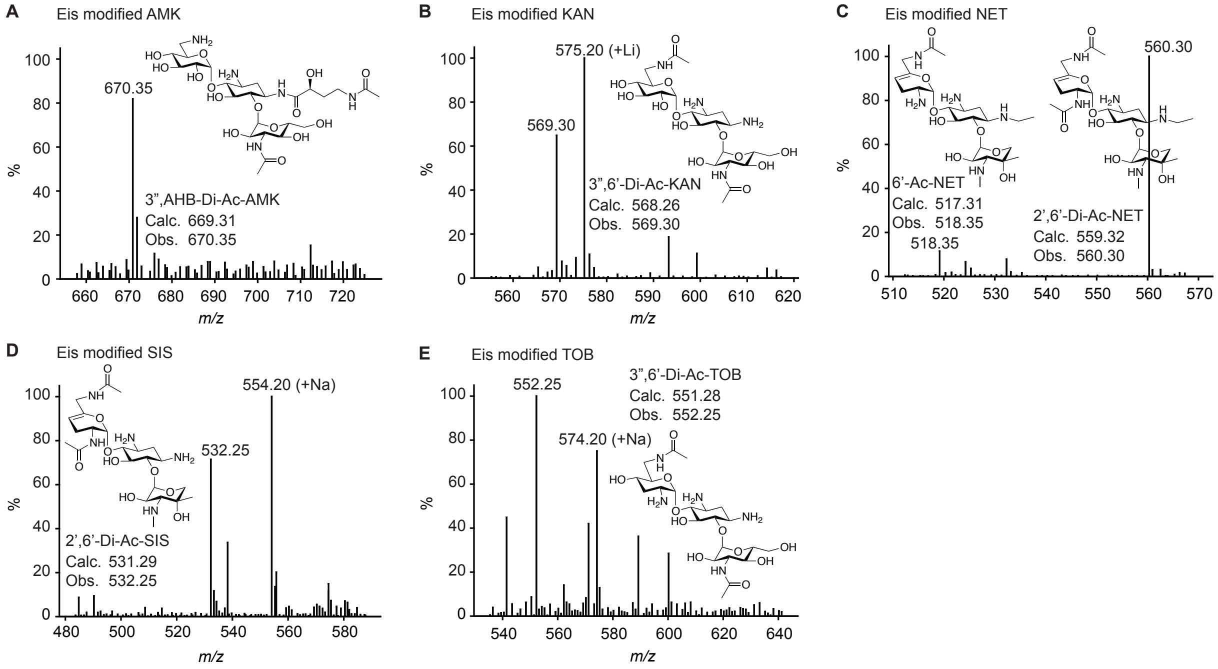

Figure S1. Mass spectra of Eis AG reaction products studied in this work.

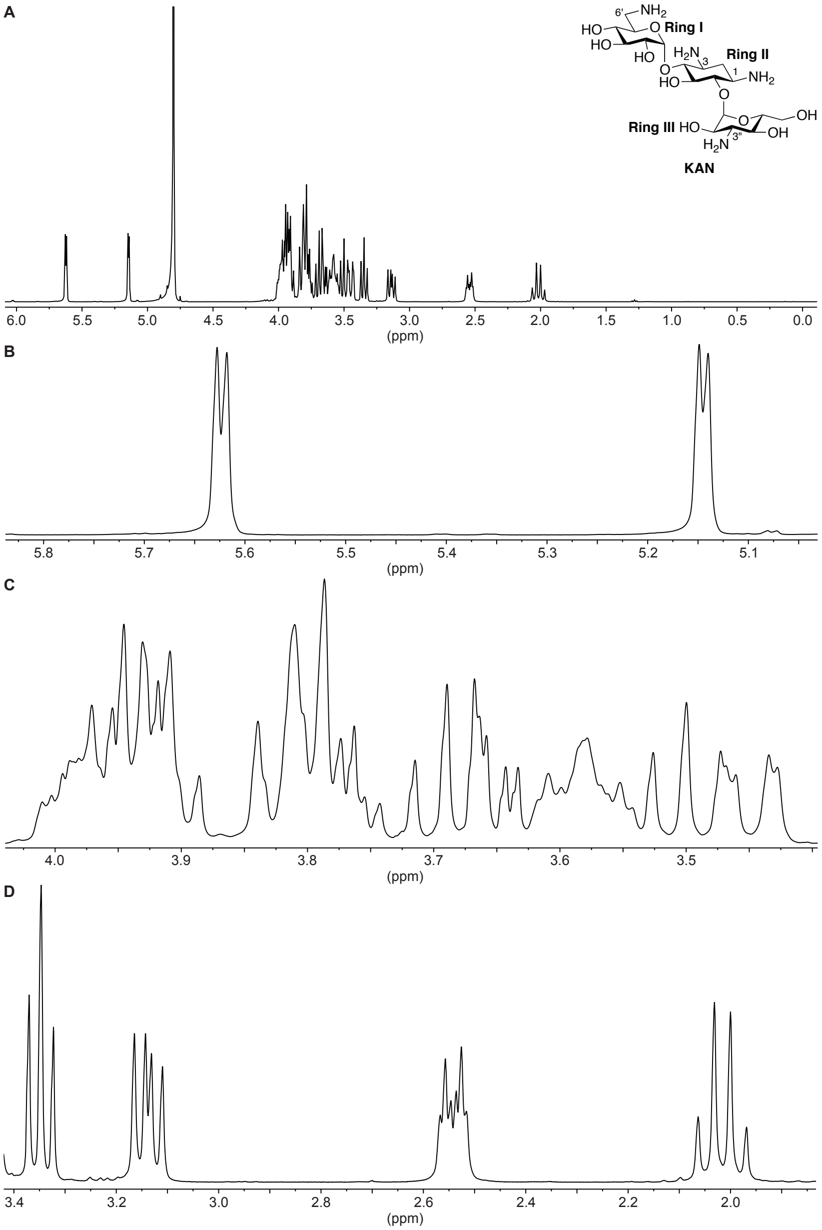

Figure S2. ^{1}H NMR of KAN in D_2O at pH 3 (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

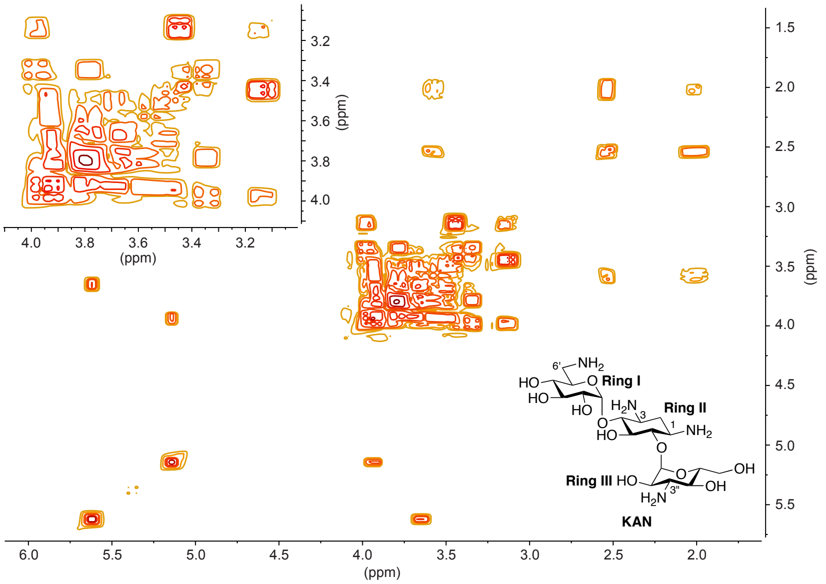

Figure S3. gCOSY of KAN in D_2O at pH 3 (400 MHz). The insert shows a portion of the spectrum more clearly.

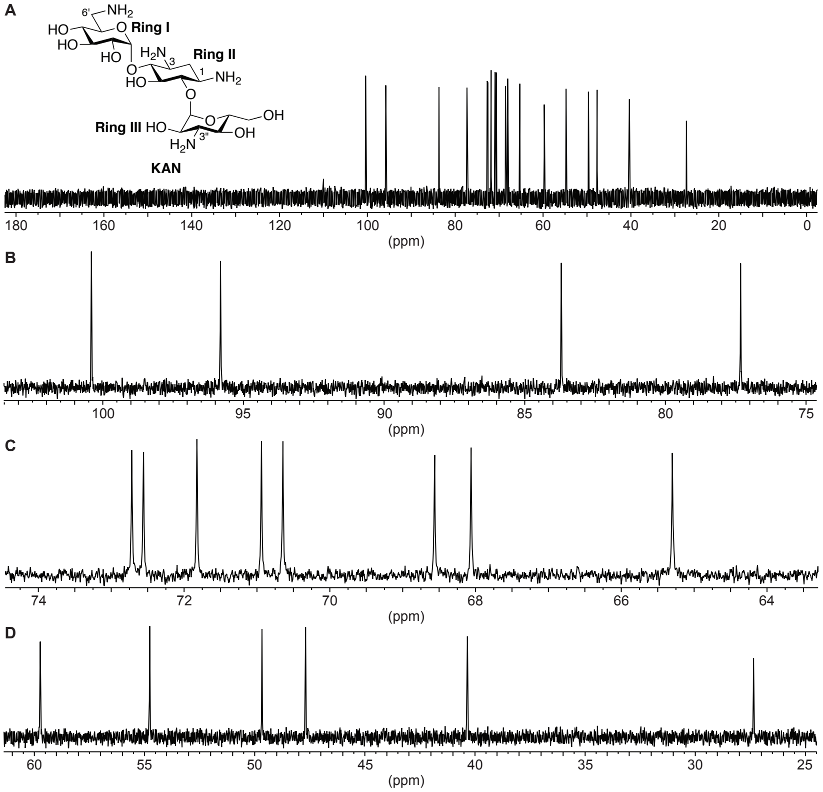

Figure S4. ^{13}C NMR of KAN in D_2O at pH 3 (100 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

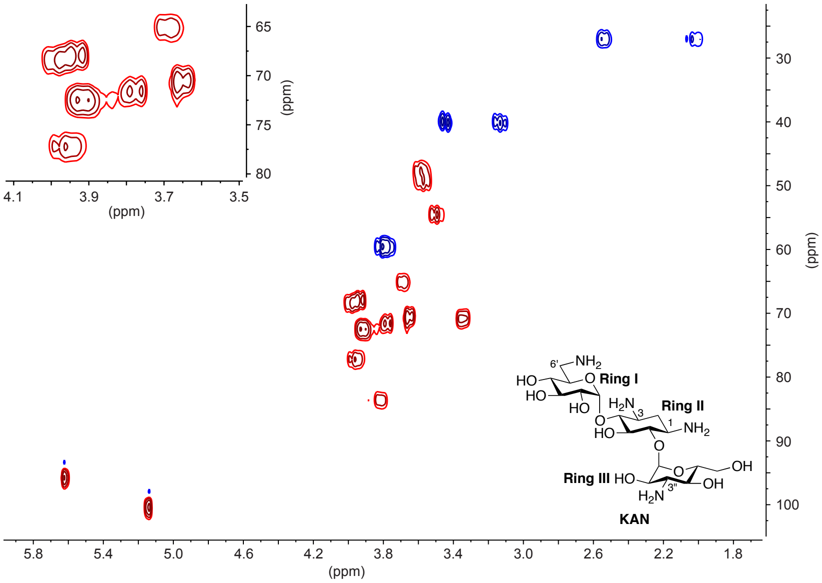

Figure S5. gHSQC of KAN in D_2O at pH 3 (400 MHz). The insert shows a portion of the spectrum more clearly (*Note*: CH_2 shown in blue, CH and CH_3 shown in red).

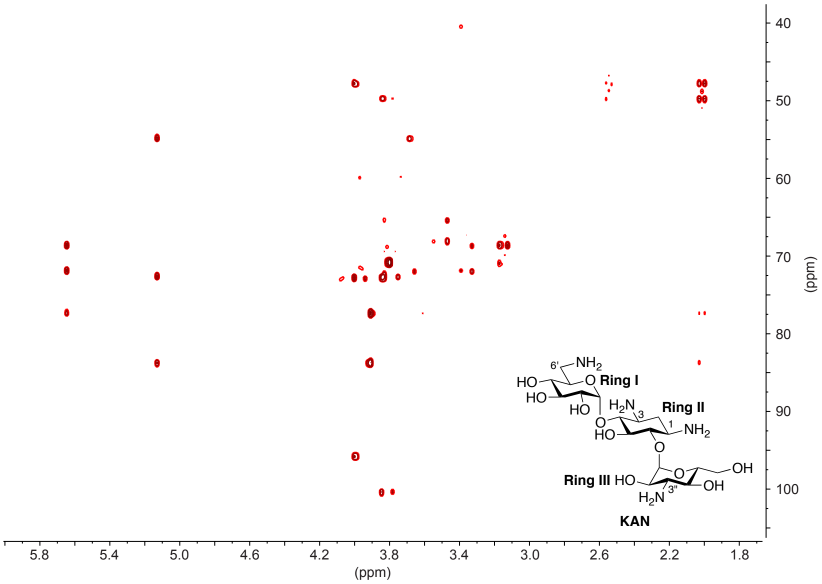

Figure S6. gHMBC of KAN in D_2O at pH 3 (100 MHz).

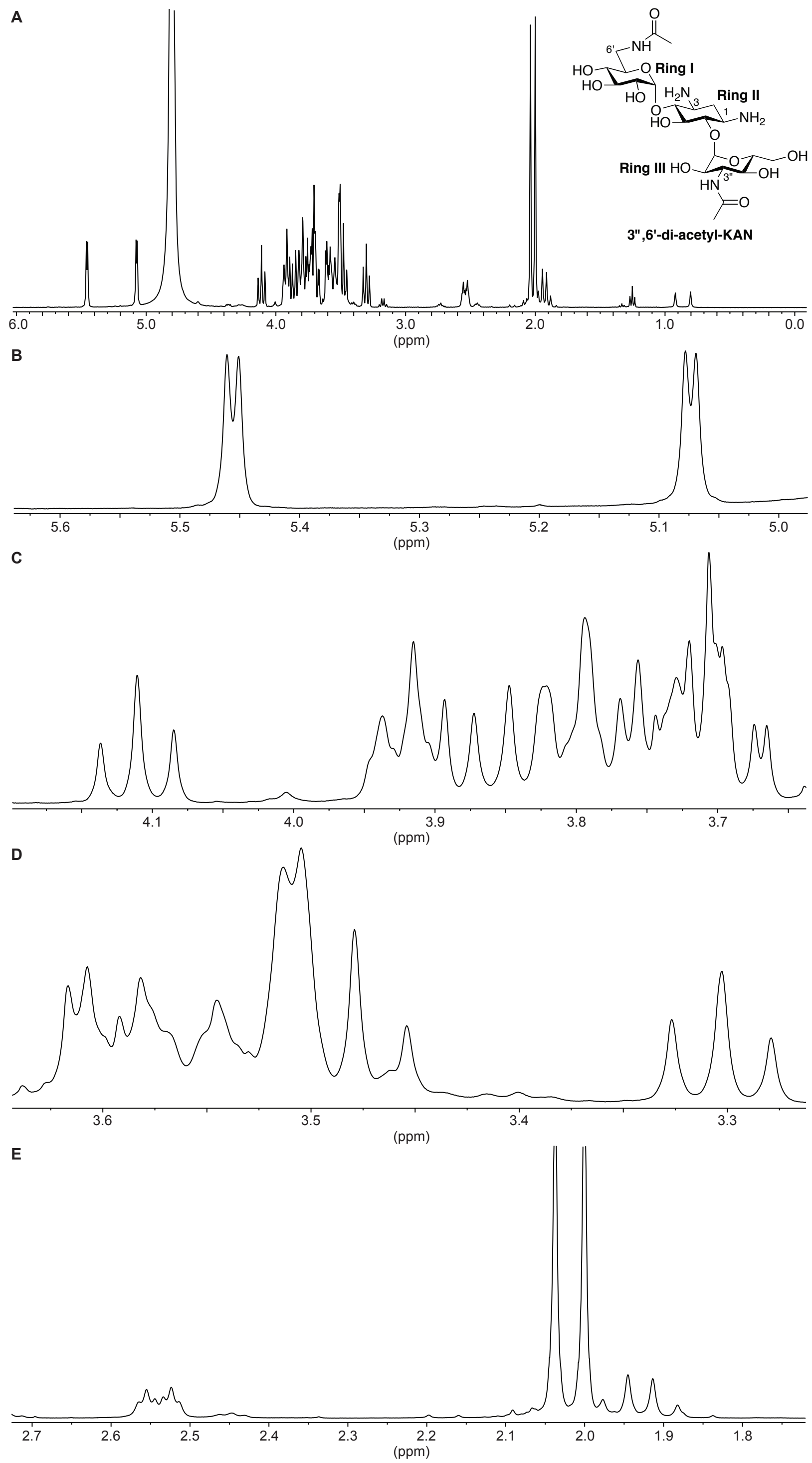

Figure S7. ^{1}H NMR of 3",6'-di-acetyl-KAN in D_2O at pH 3 (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

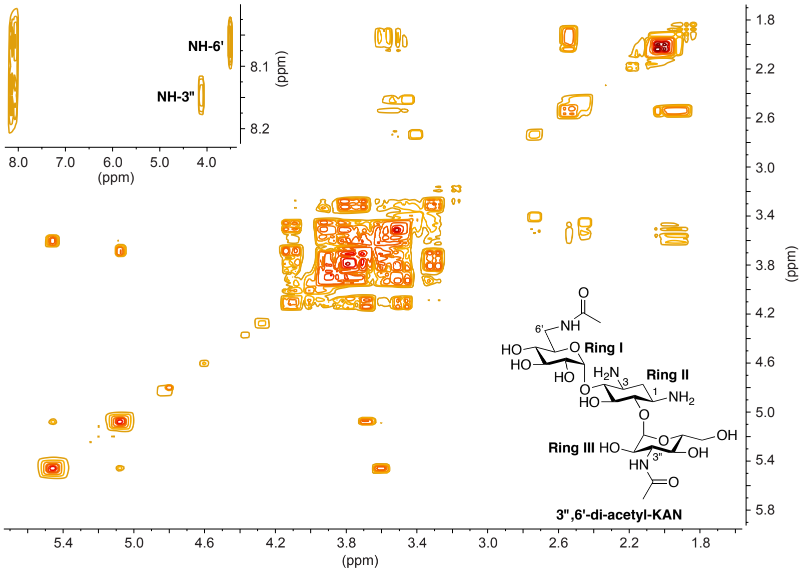

Figure S8. gCOSY of 3'',6'-di-acetyl-KAN in D_2O at pH 3 (400 MHz). The insert shows the amide protons coupling to the protons at the 3''- and 6'-positions.

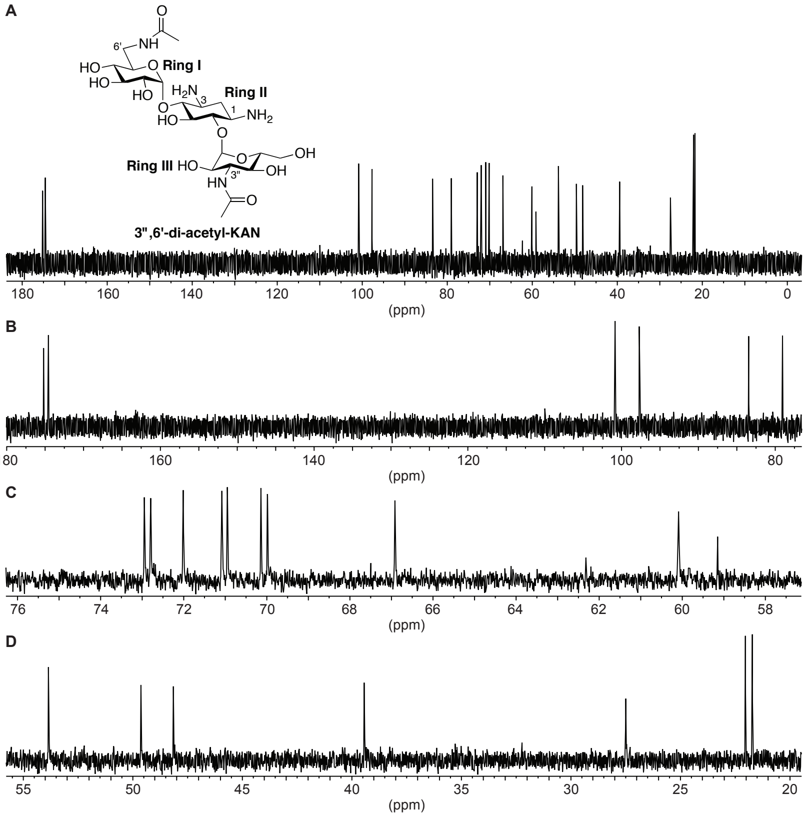

Figure S9. ^{13}C NMR of 3'',6'-di-acetyl-KAN in D_2O at pH 3 (100 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

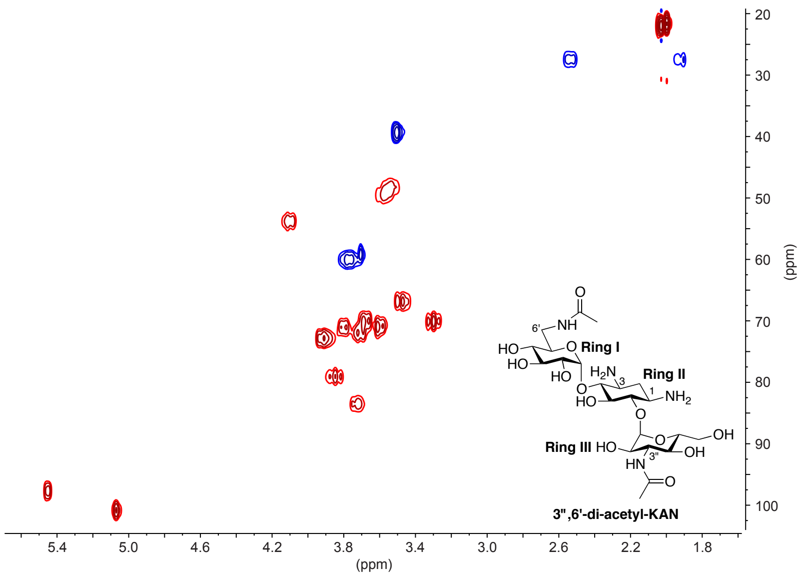

Figure S10. gHSQC of 3",6'-di-acetyl-KAN in D_2O at pH 3 (400 MHz).

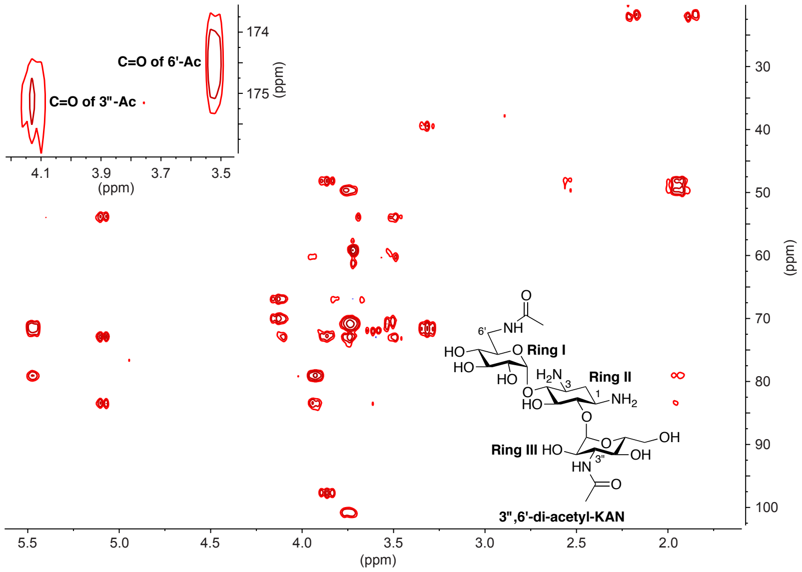

Figure S11. gHMBC of 3",6'-di-acetyl-KAN in D_2O at pH 3 (100 MHz). The insert shows the carbonyl carbons coupling to the protons at the 3"- and 6'-positions.

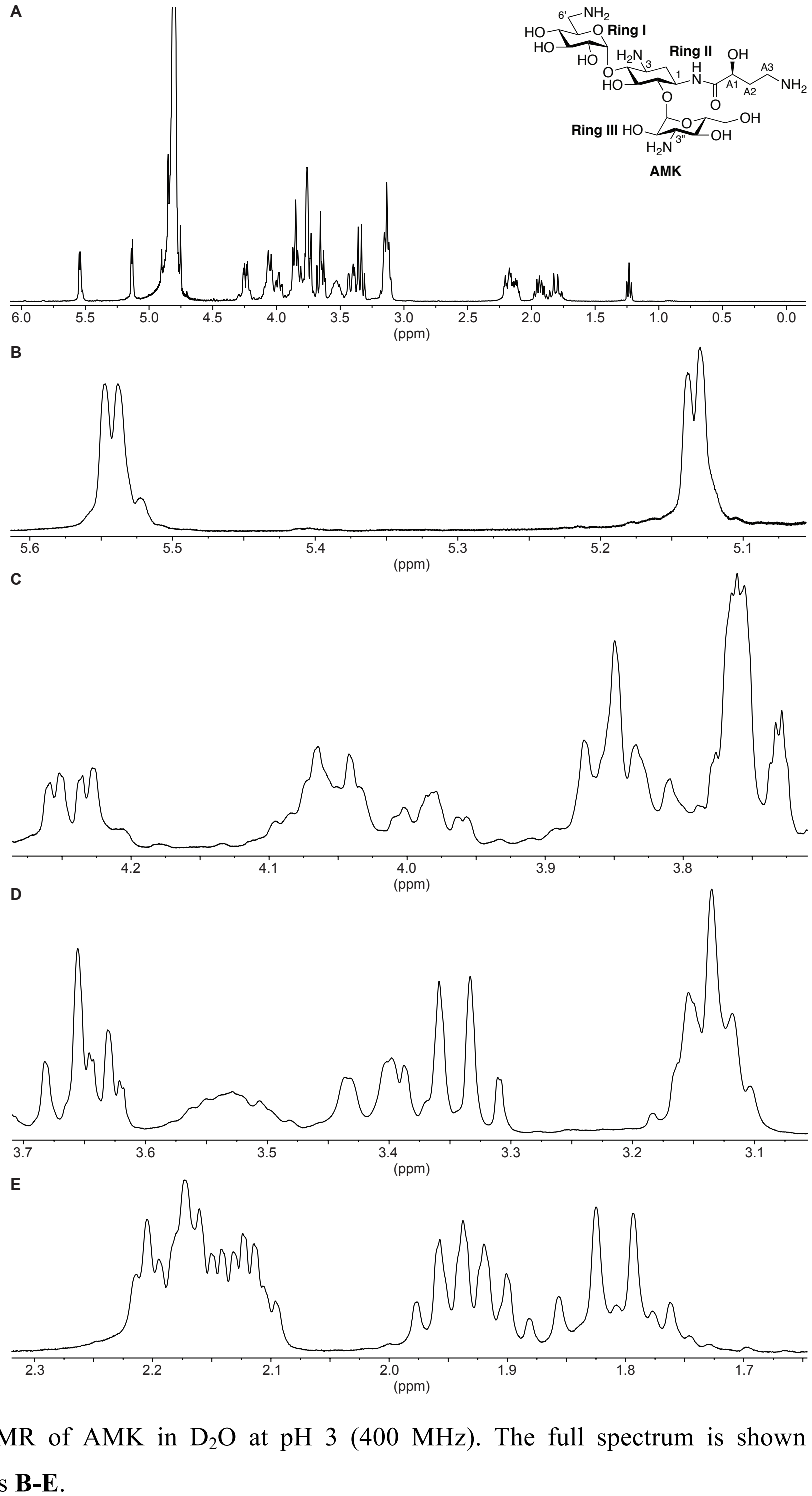

Figure S12. ^{1}H NMR of AMK in D_2O at pH 3 (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

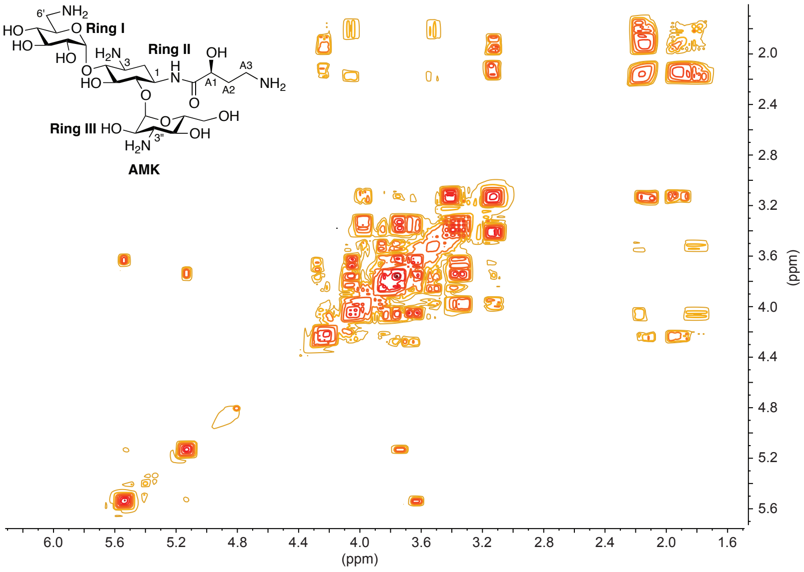

Figure S13. gCOSY of AMK in D_2O at pH 3 (400 MHz).

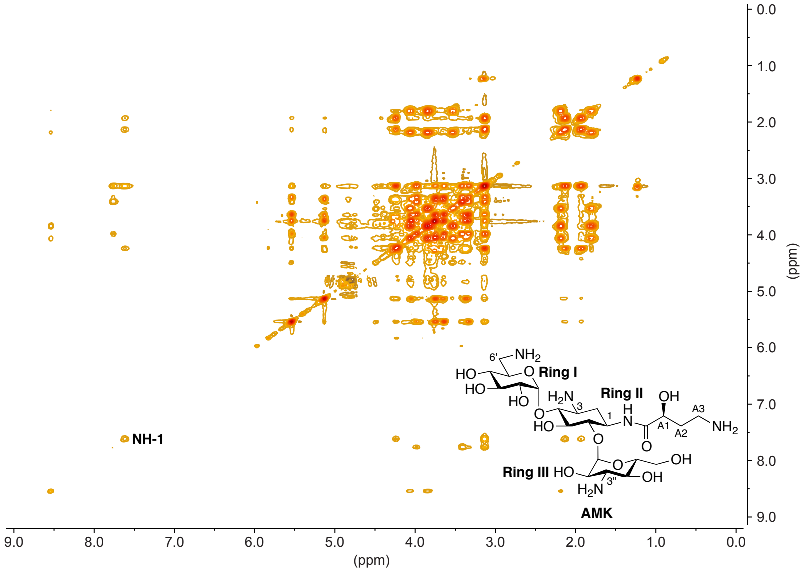

Figure S14. zTOCSY of AMK in D_2O at pH 3 (400 MHz).

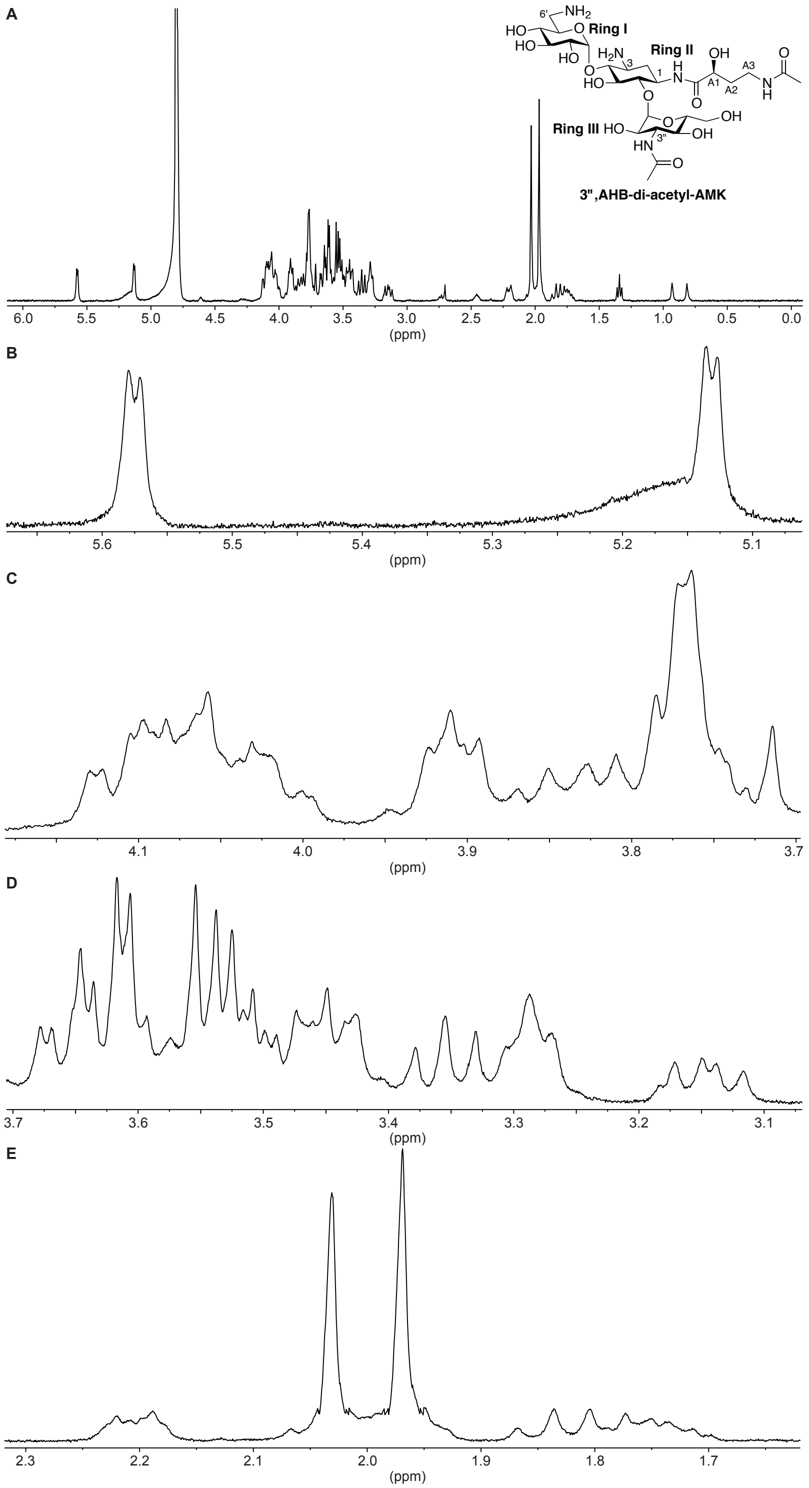

Figure S15. ^{1}H NMR of 3",AHB-di-acetyl-AMK in D_2O at pH 3 (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

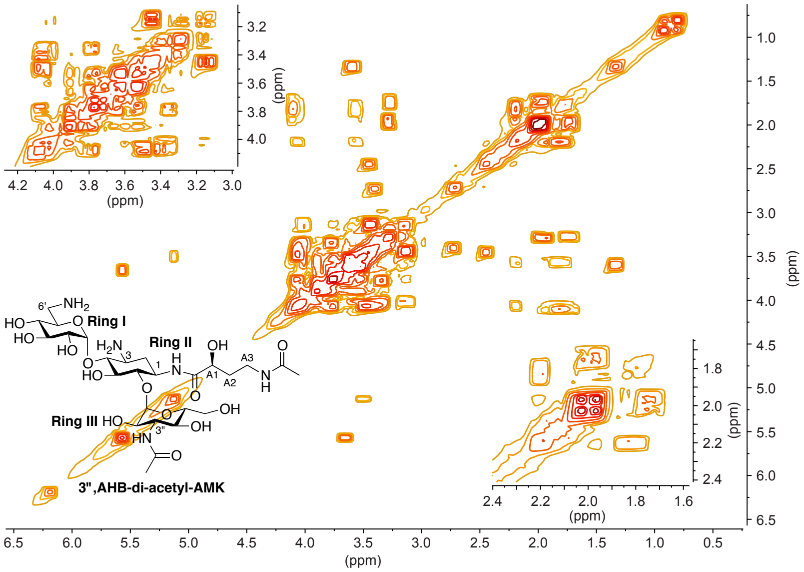

Figure S16. gCOSY of 3",AHB-di-acetyl-AMK in D_2O at pH 3 (400 MHz). The inserts show portions of the spectrum more clearly.

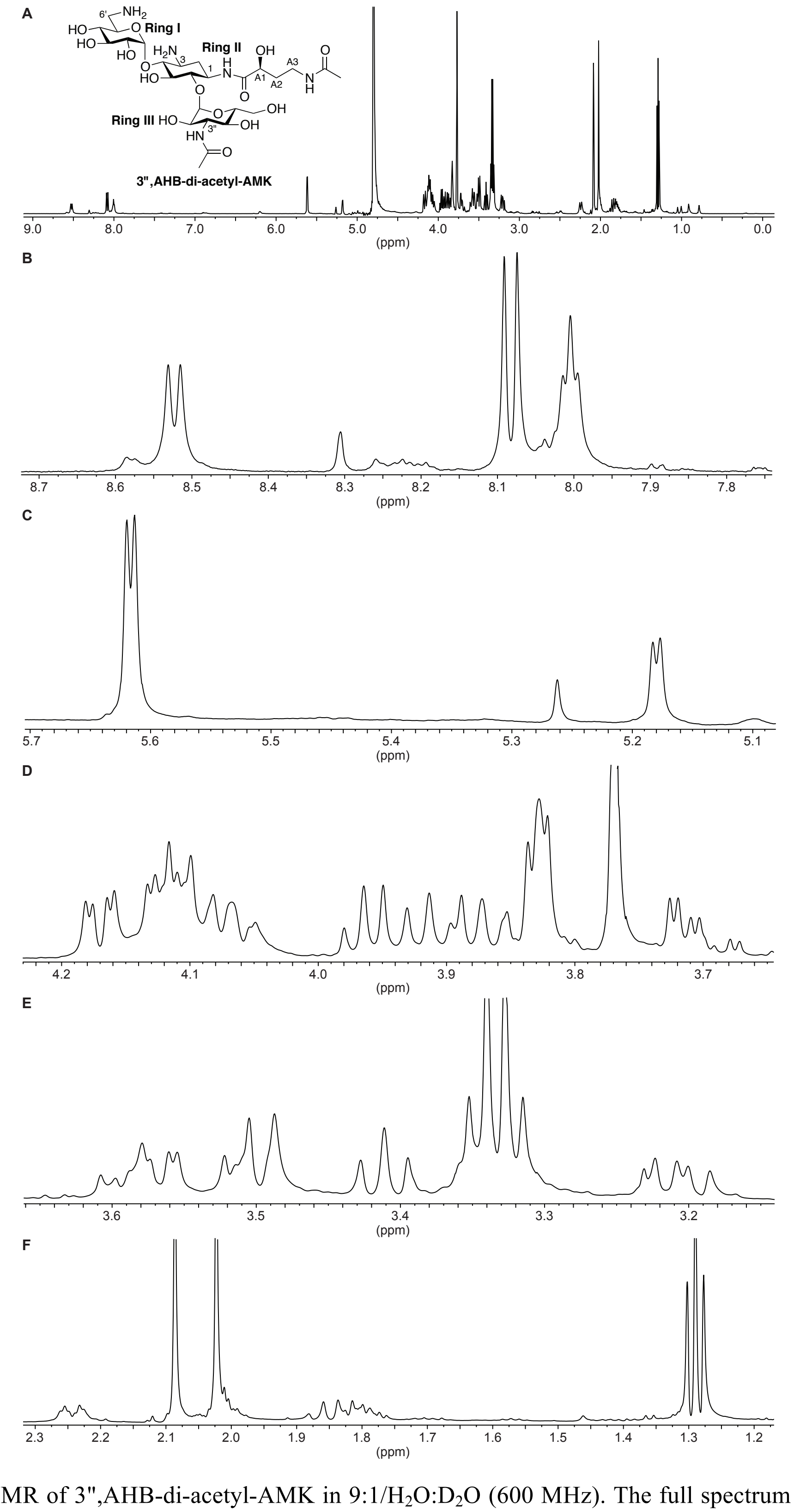

Figure S17. ^{1}H NMR of 3",AHB-di-acetyl-AMK in 9:1/H_2O:D_2O (600 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-F**.

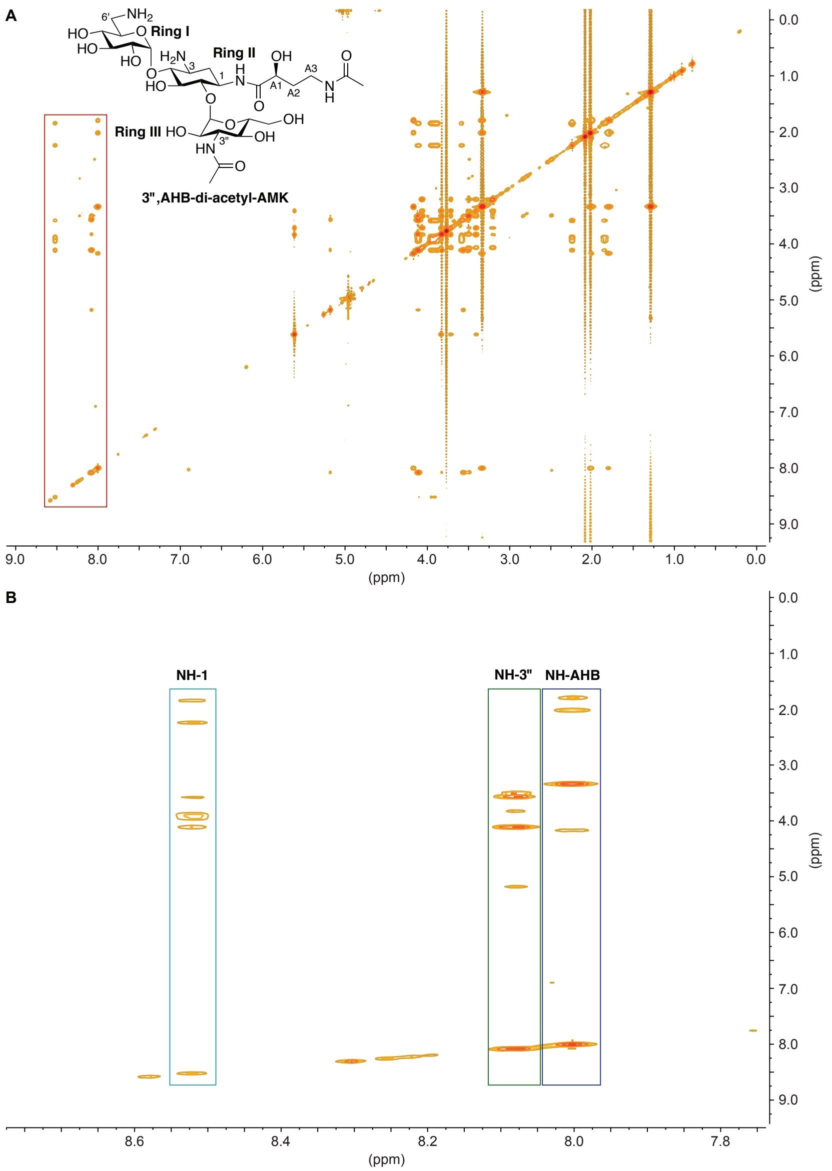

Figure S18. zTOCSY of 3",AHB-di-acetyl-AMK in 9:1/H_2O:D_2O (600 MHz). The full spectrum is shown in panel **A** and the expansion showing the amide protons coupling to the protons at the 1-, 3"-, and AHB-positions in panel **B**. A red box in panel **A** indicates the portion of the spectrum expanded and shown in panel **B**. In panel **B**, the coupling of the amide protons at the 1-, 3"-, and A3 of the AHB-positions are indicated by turquoise, green, and blue boxes, respectively.

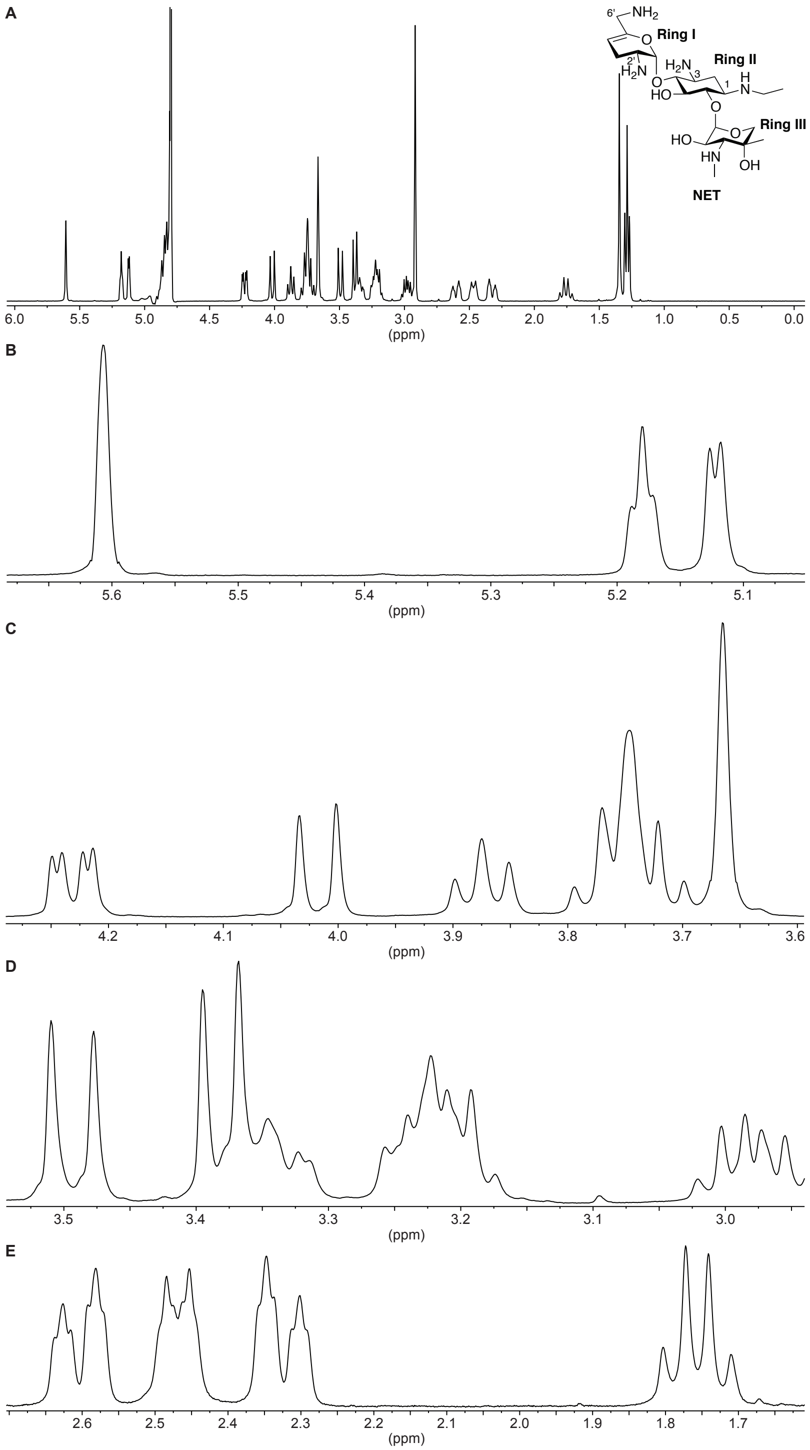

Figure S19. ^{1}H NMR of NET (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

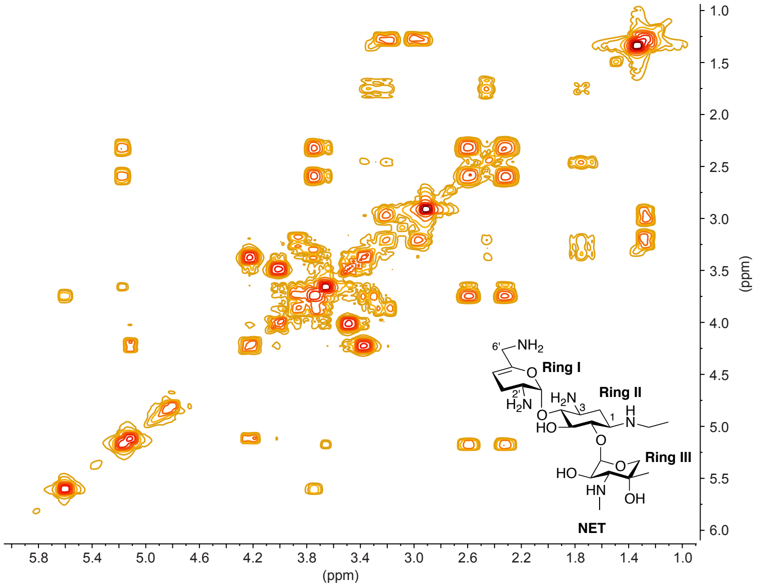

Figure S20. gCOSY of NET (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz).

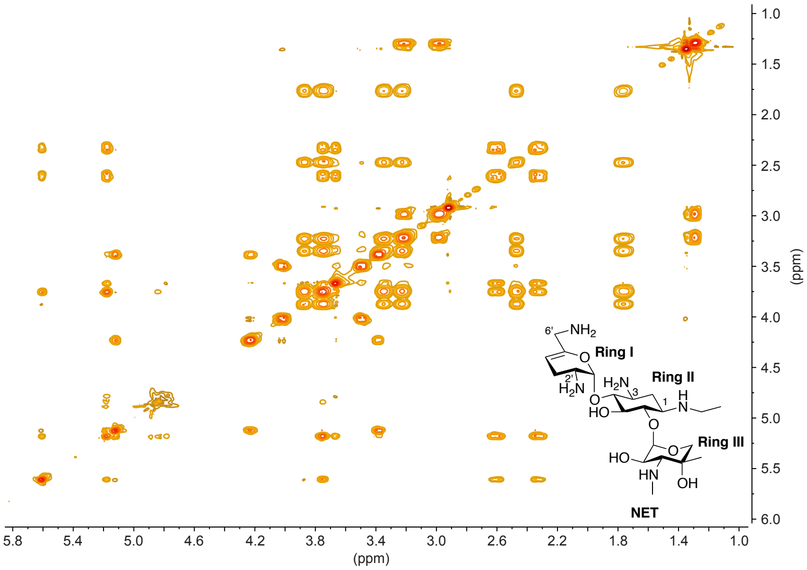

Figure S21. zTOCSY of NET (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz).

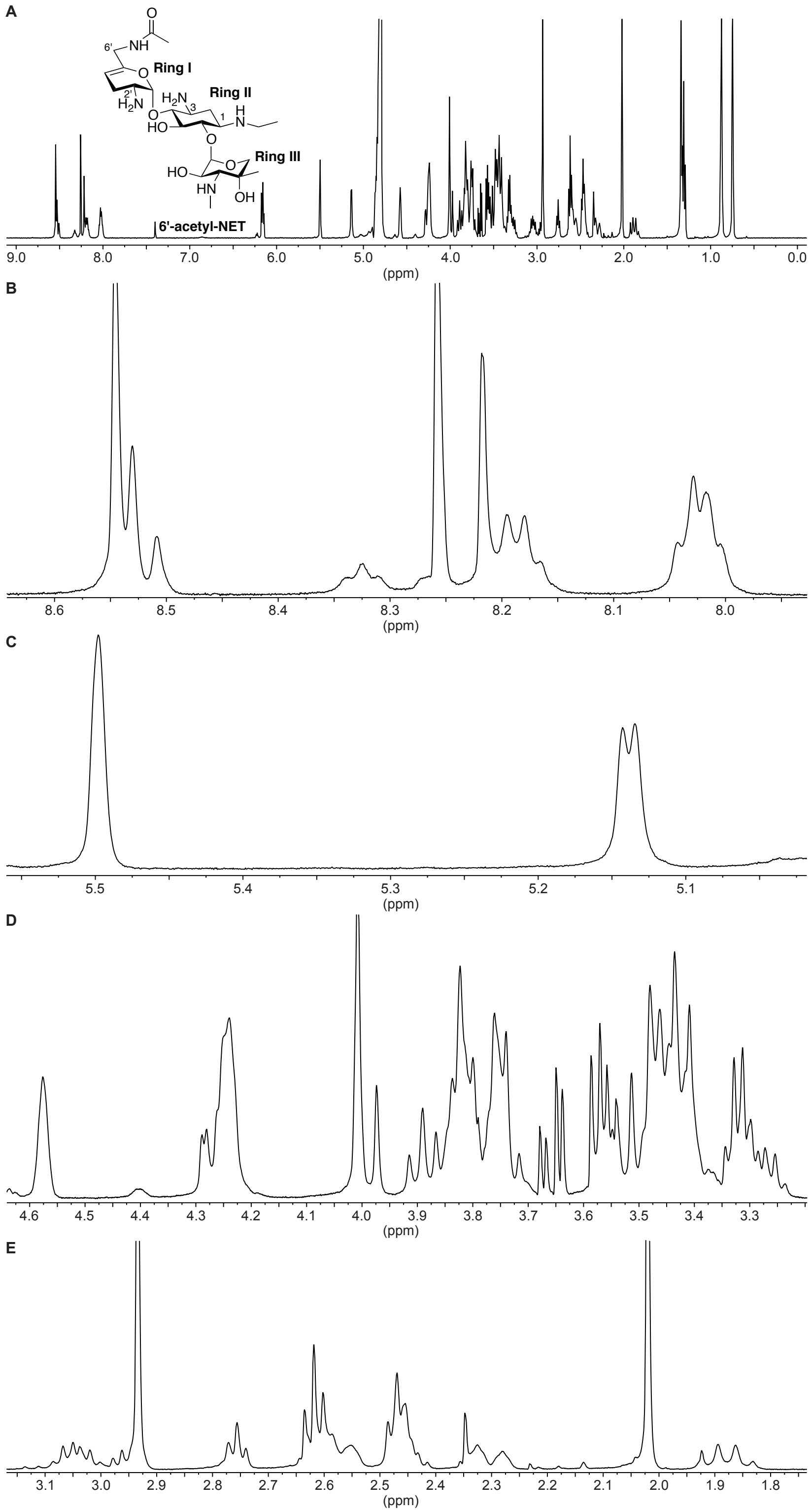

Figure S22. ^{1}H NMR of 6'-acetyl-NET Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

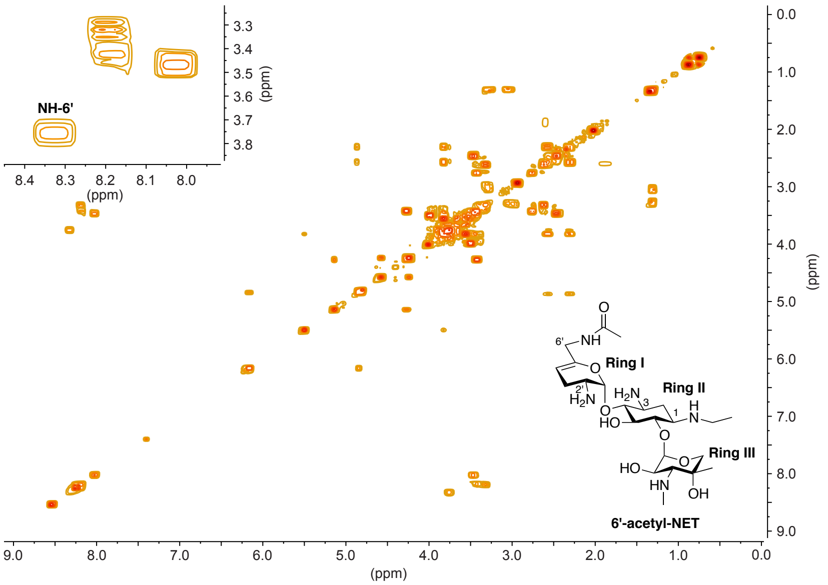

Figure S23. gCOSY of 6'-acetyl-NET Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The insert shows the amide protons coupling to the protons at the 6'-position.

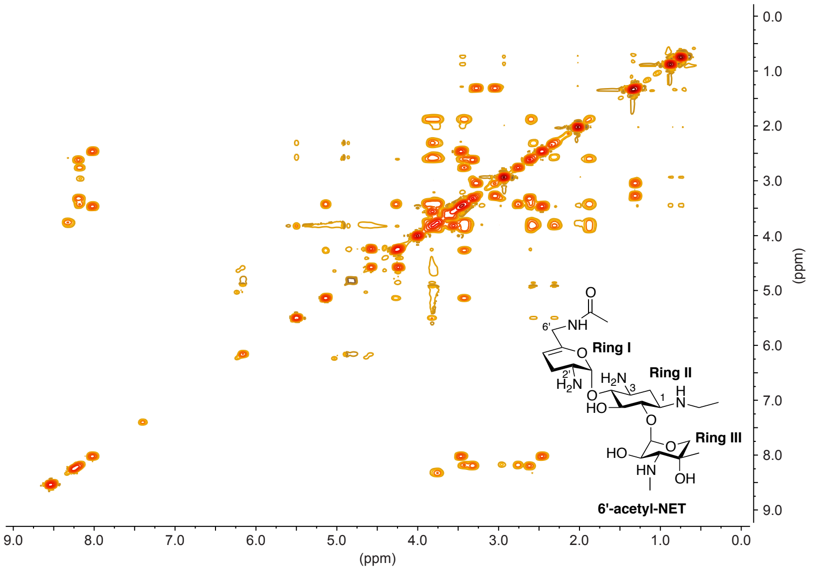

Figure S24. zTOCSY of 6'-acetyl-NET Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz).

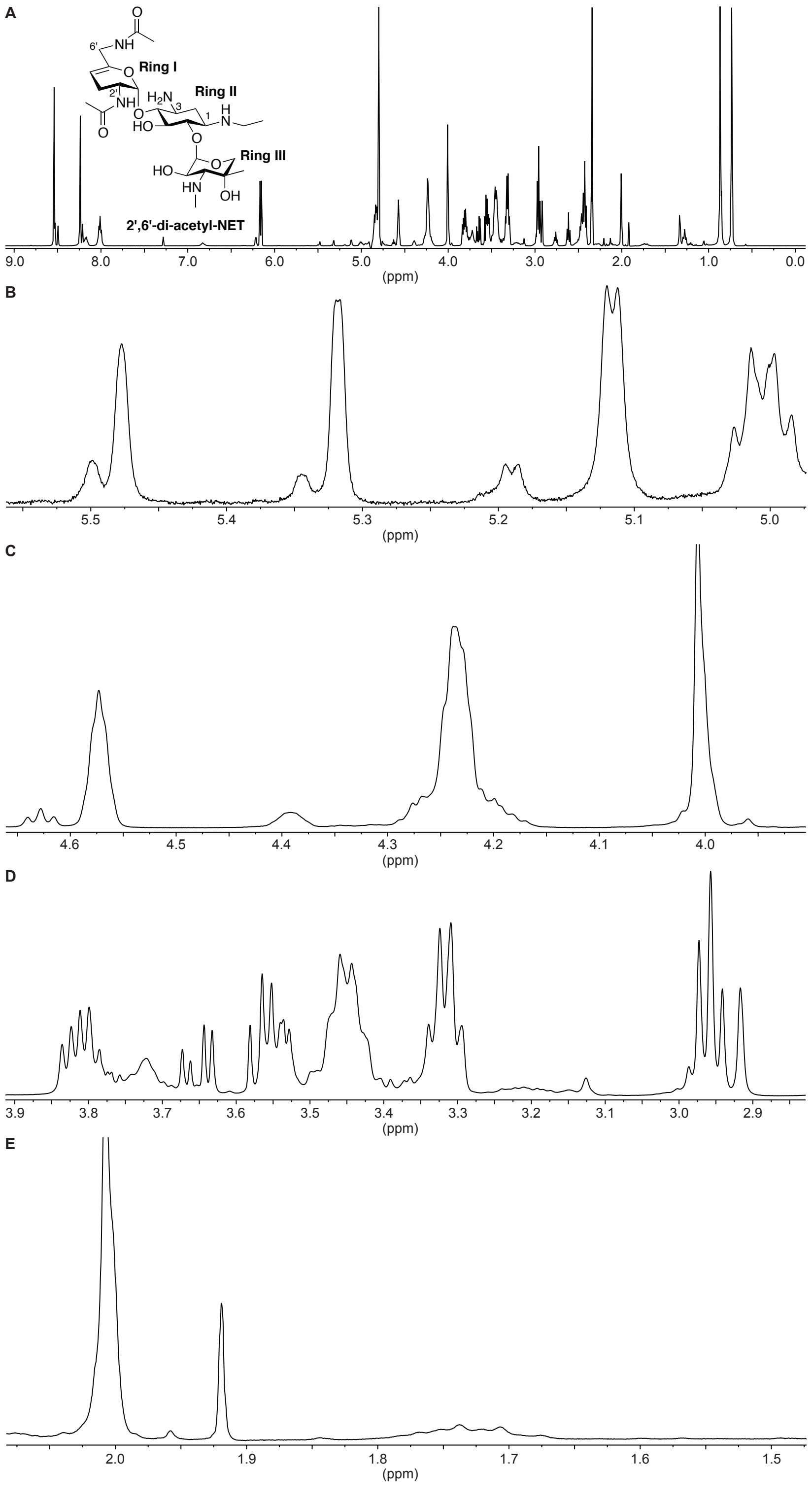

Figure S25. ^{1}H NMR of 2',6'-di-acetyl-NET Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

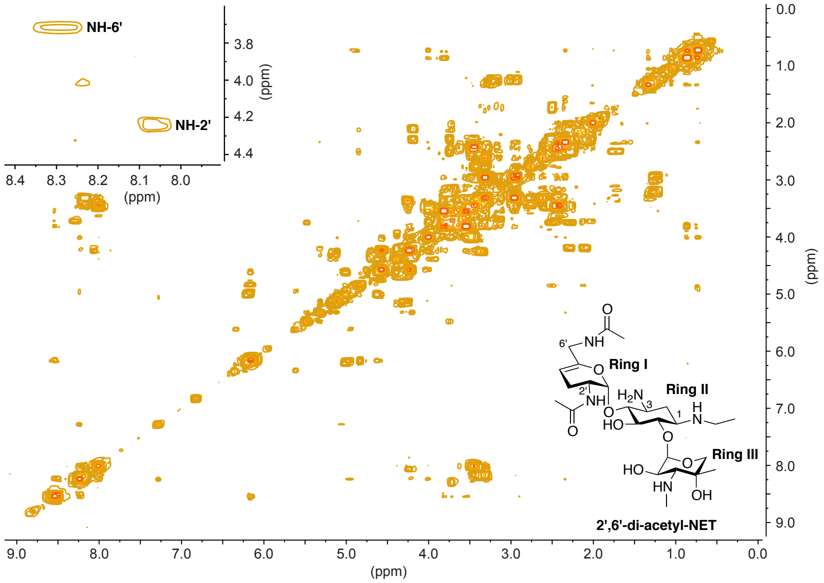

Figure S26. gCOSY of 2',6'-di-acetyl-NET Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 20 mM KH_2PO_4) (400 MHz). The insert shows the amide protons coupling to the protons at the 6'- and 2'-positions.

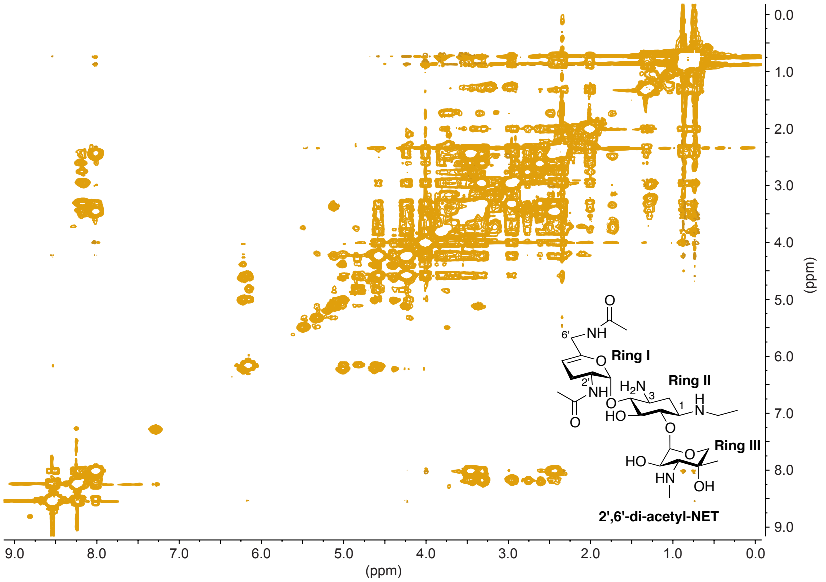

Figure S27. zTOCSY of 2',6'-di-acetyl-NET Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz).

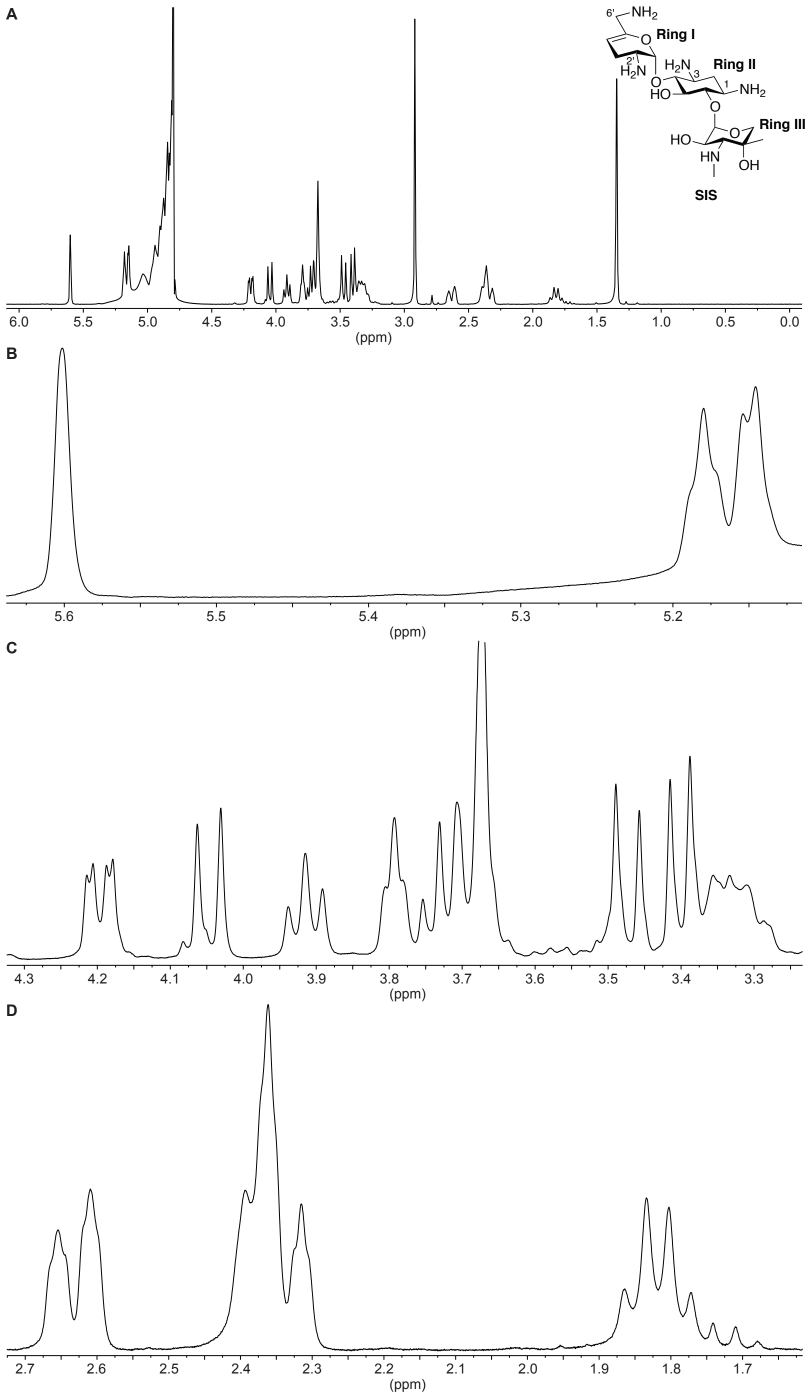

Figure S28. ^{1}H NMR of SIS (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

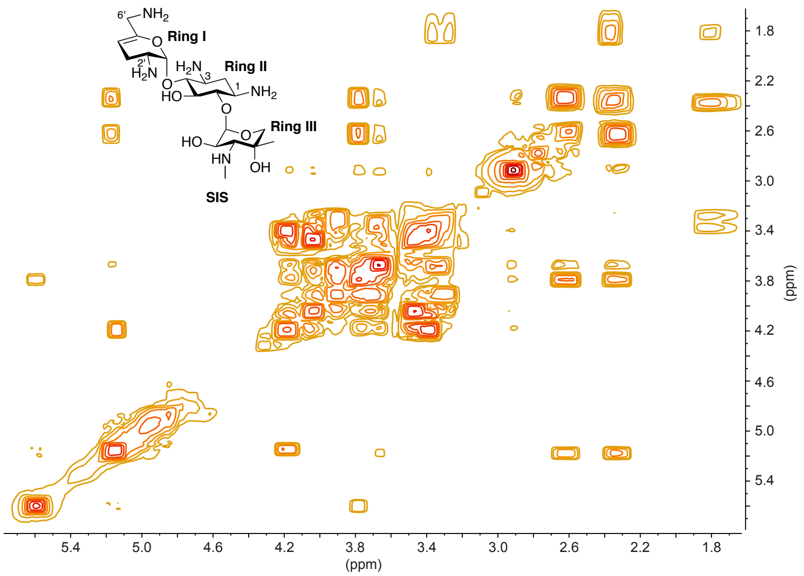

Figure S29. gCOSY of SIS (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz).

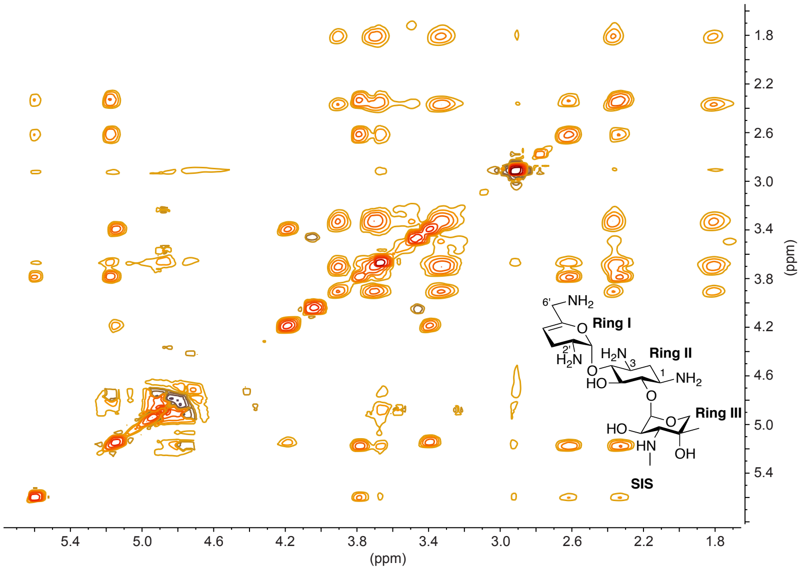

Figure S30. zTOCSY of SIS (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz).

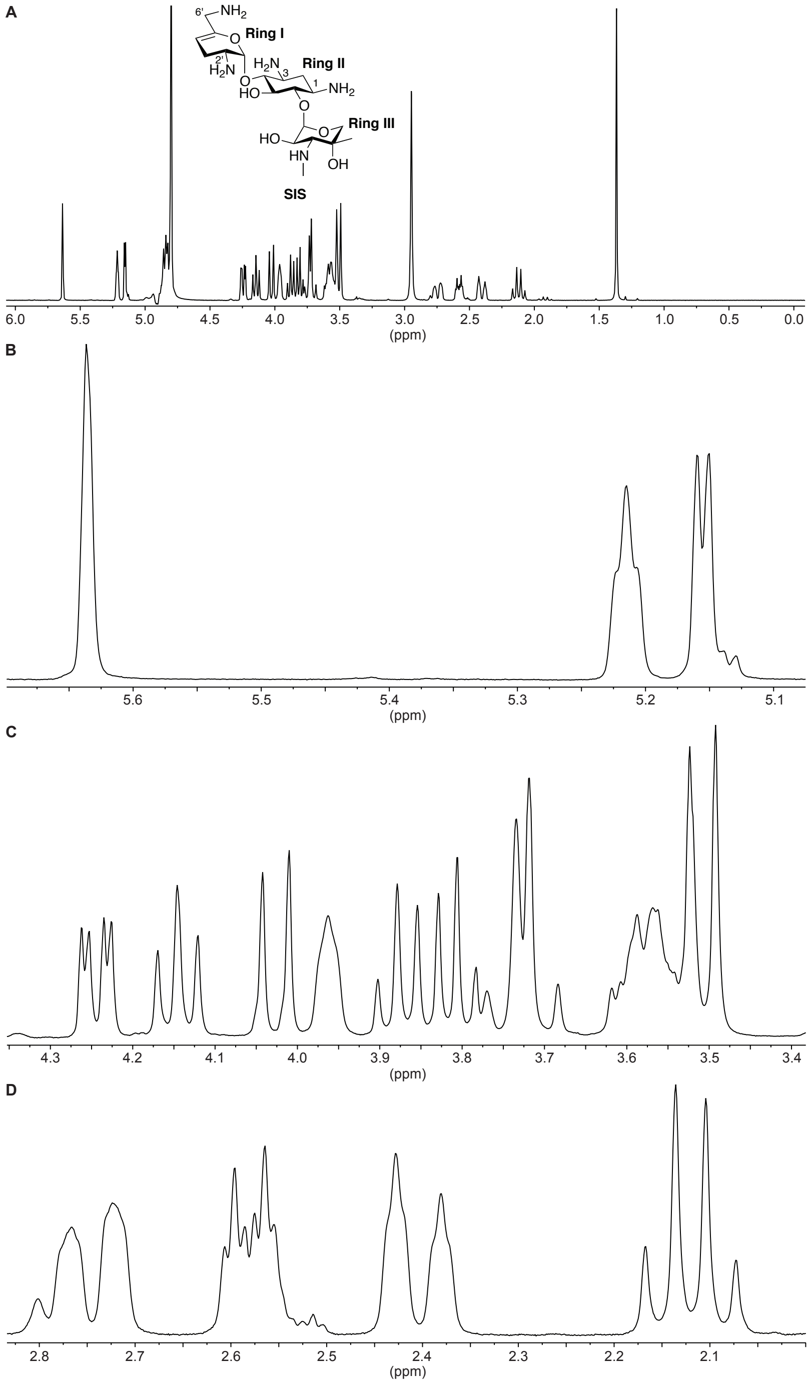

Figure S31. ^{1}H NMR of SIS (9:1/H_2O:D_2O, pH 3) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

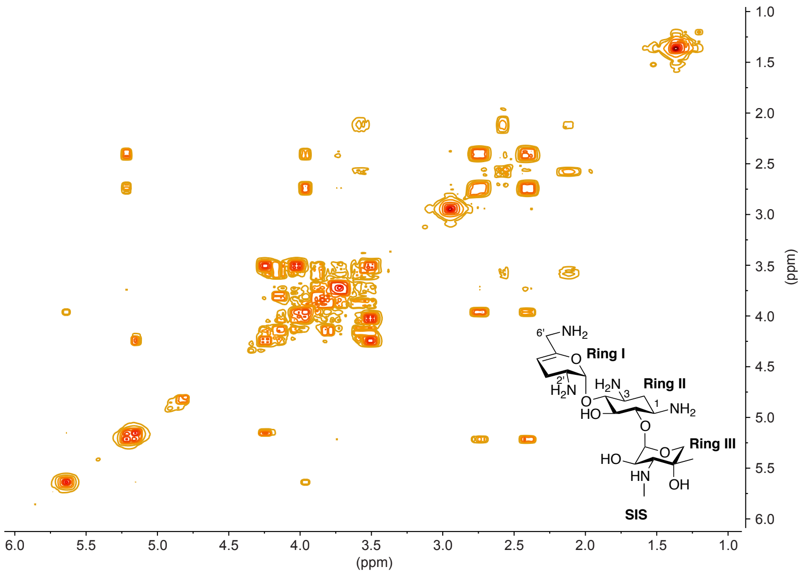

Figure S32. gCOSY of SIS (9:1/H_2O:D_2O, pH 3) (400 MHz).

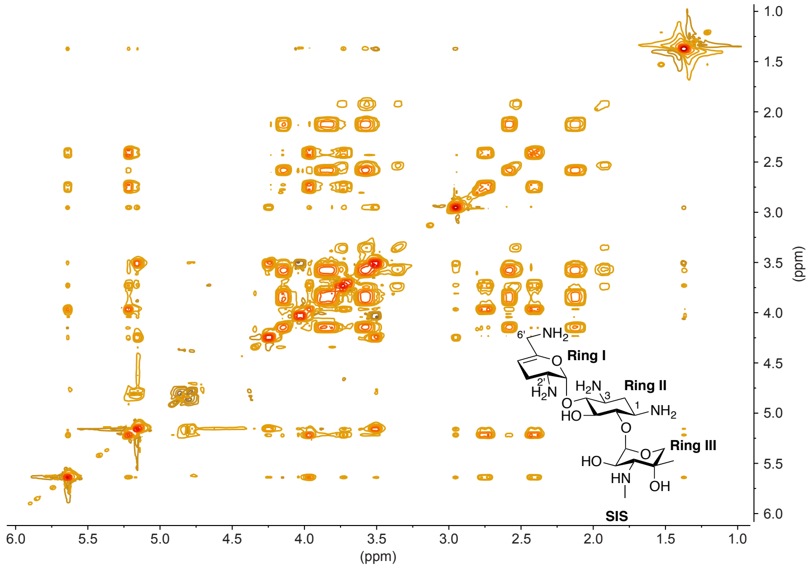

Figure S33. zTOCSY of SIS (9:1/H_2O:D_2O, pH 3) (400 MHz).

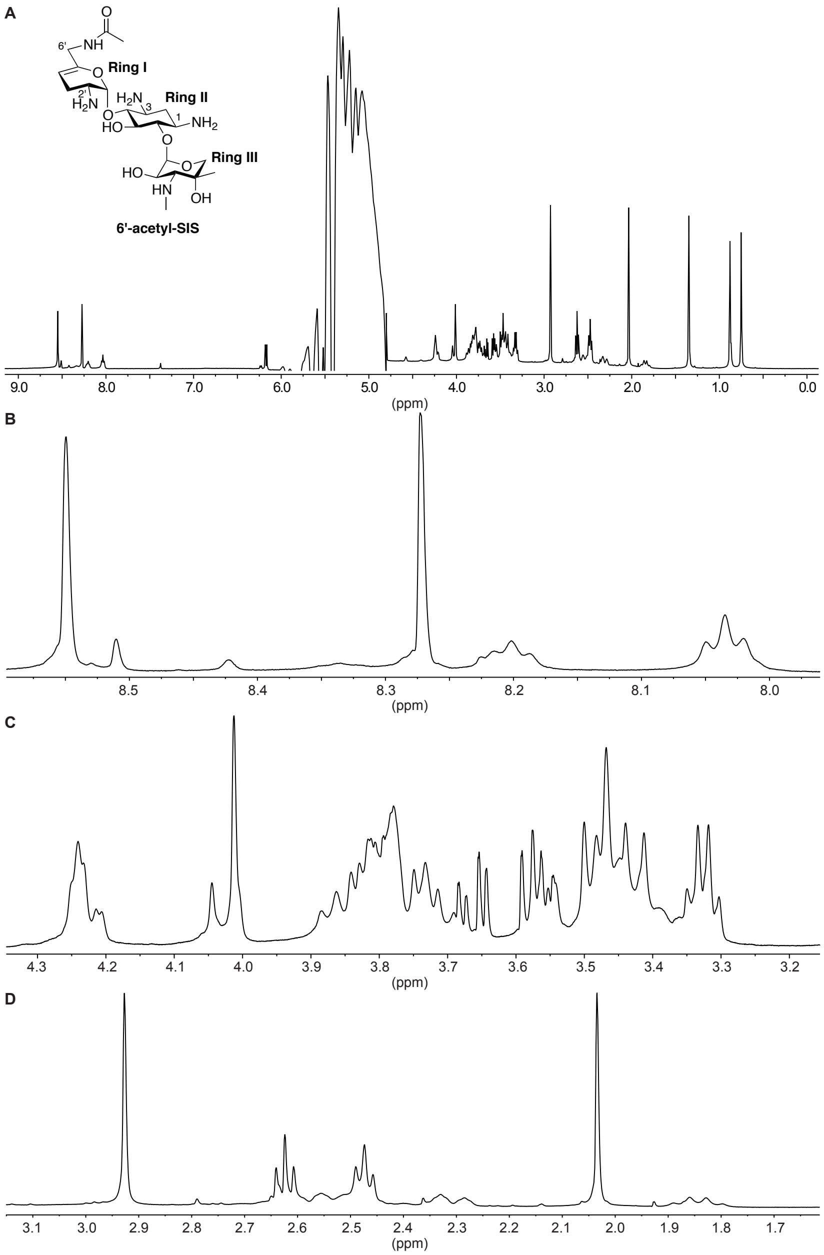

Figure S34. ^{1}H NMR of 6'-acetyl-SIS Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

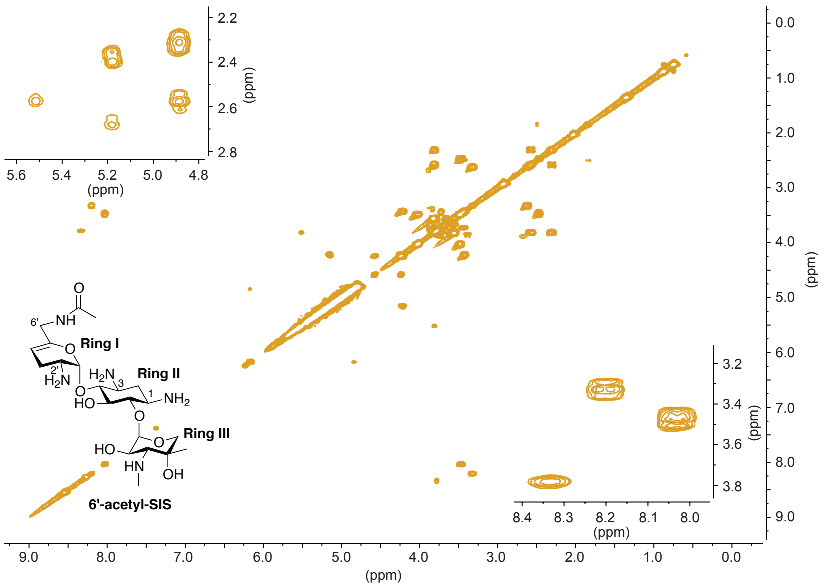

Figure S35. gCOSY of 6'-acetyl-SIS Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The inserts show part of the spectra that is obscured in the ^{1}H (top left) and the amide protons coupling to the proton at the 6'-position (bottom right).

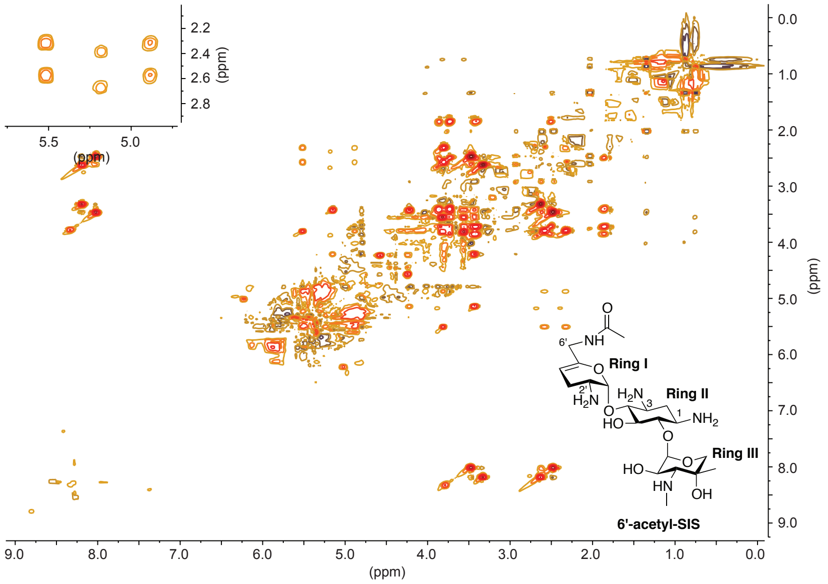

Figure S36. zTOCSY of 6'-acetyl-SIS Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (400 MHz). The inserts show part of the spectra that is obscured in the ^{1}H.

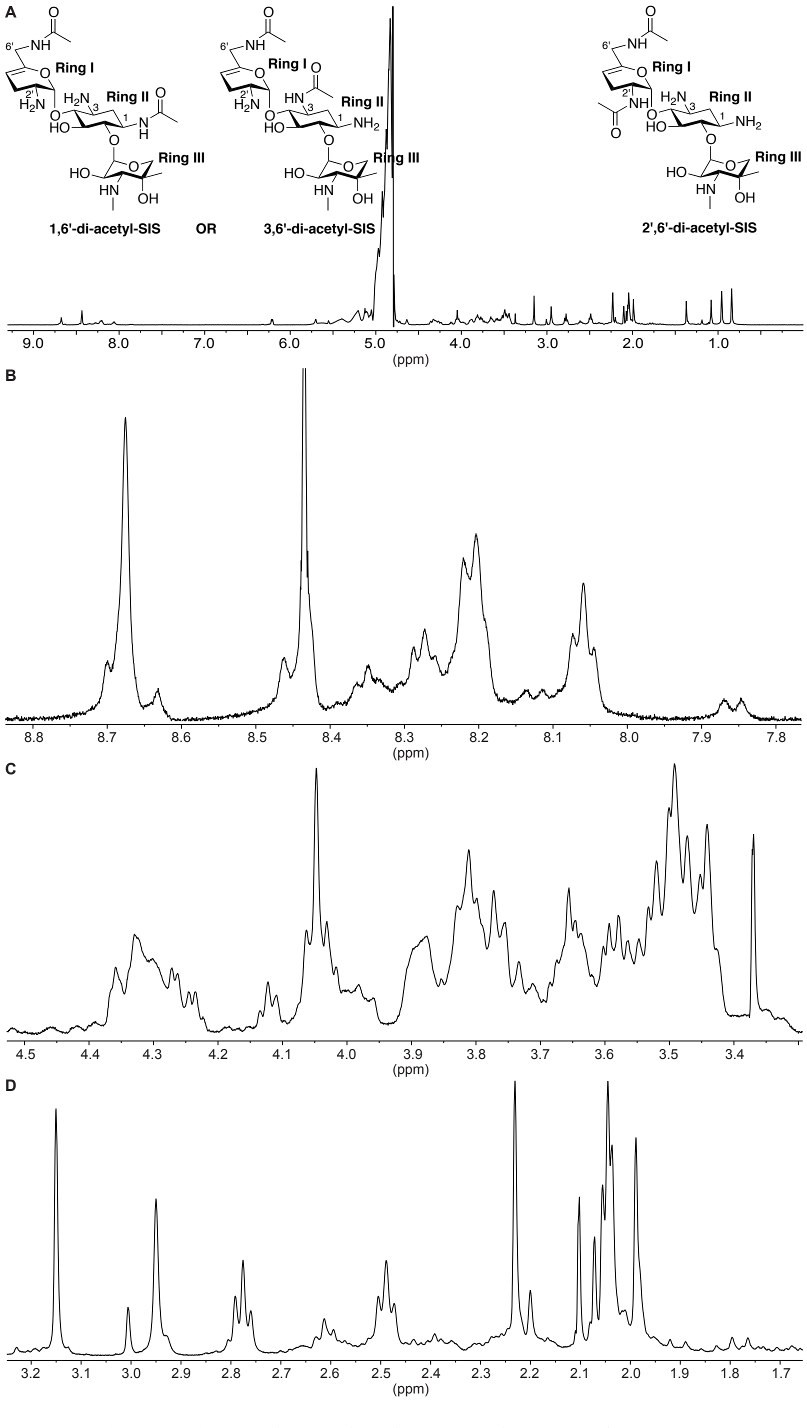

Figure S37. ^{1}H NMR of a mixture of two di-acetylated SIS products (9:1/H_2O:D_2O, pH 3) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

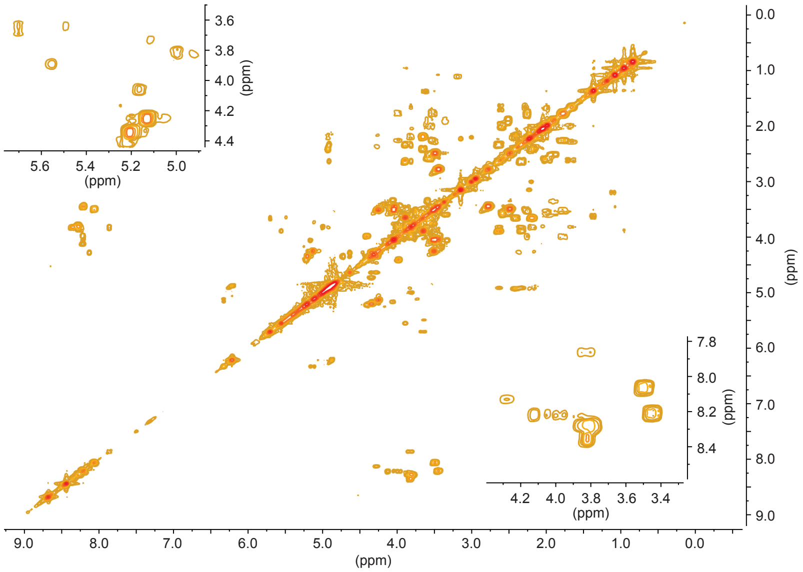

Figure S38. gCOSY of a mixture of two di-acetylated SIS products (9:1/H_2O:D_2O, pH 3) (400 MHz). The inserts show part of the spectra that is obscured in the ^{1}H (top left) and the amide protons coupling to the protons of the AG (bottom right).

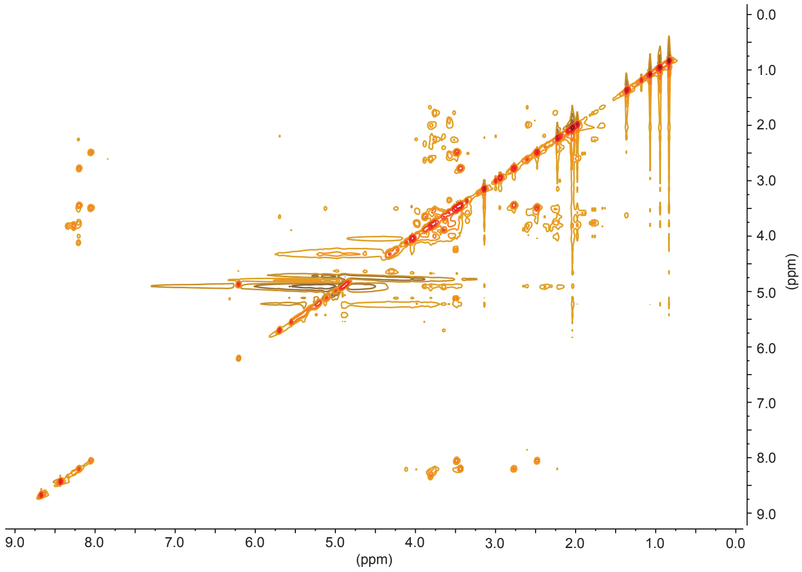

Figure S39. zTOCSY of a mixture of two di-acetylated SIS products (9:1/H_2O:D_2O, pH 3) (400 MHz).

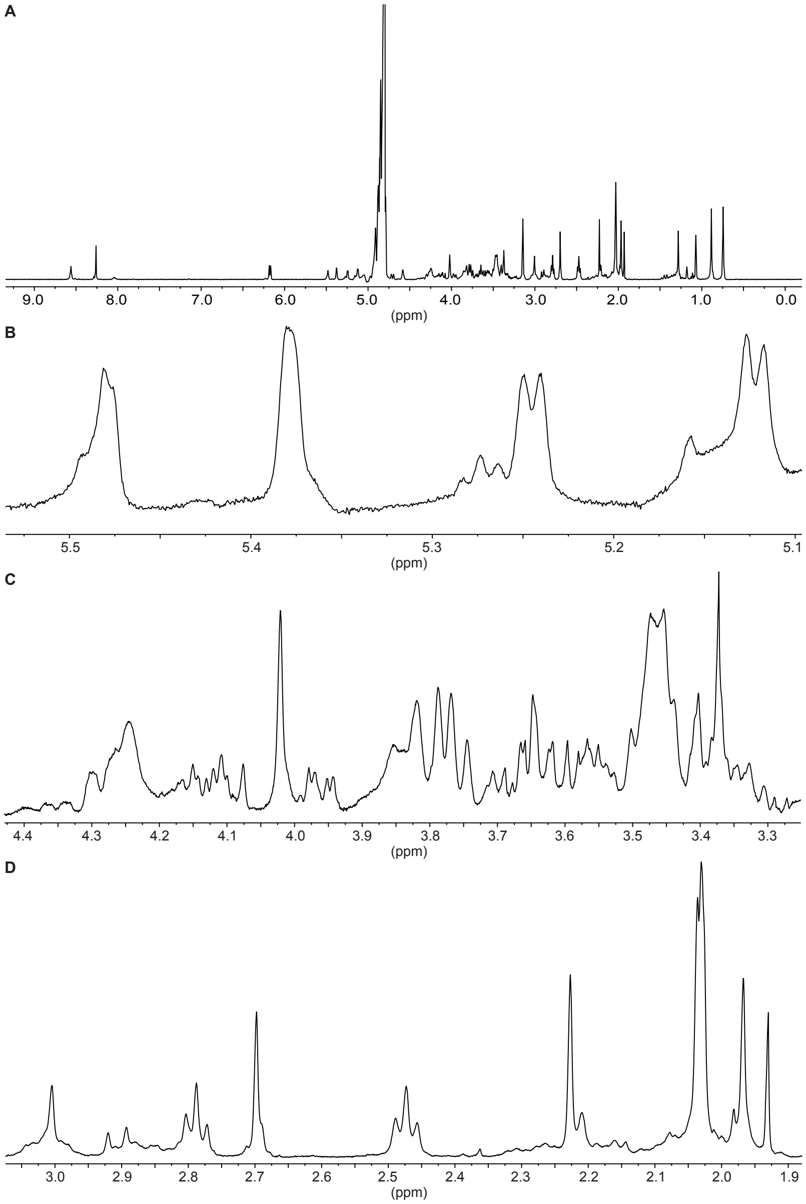

Figure S40. ^{1}H NMR of a mixture of two di-acetylated SIS products (9:1/H_2O:D_2O, pH 8) (400 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

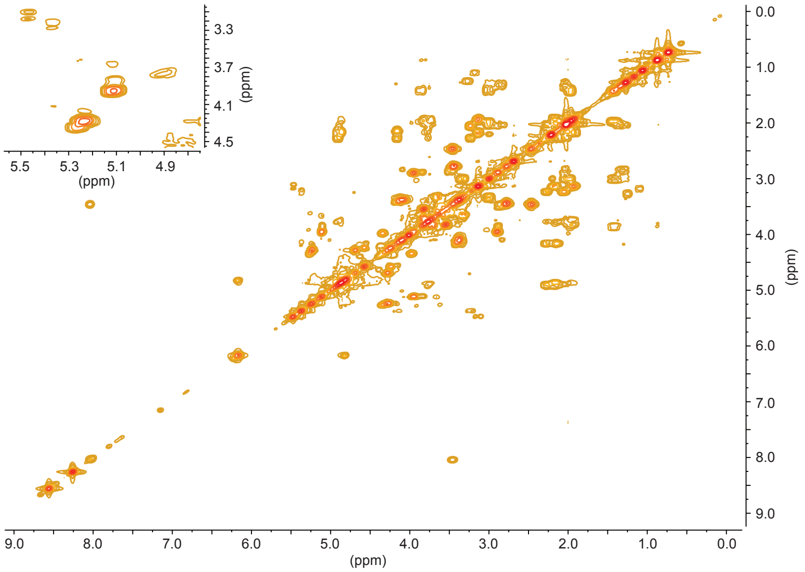

Figure S41. gCOSY of a mixture of two di-acetylated SIS products (9:1/H_2O:D_2O, pH 8) (400 MHz). The insert shows part of the spectra that is obscured in the ^{1}H.

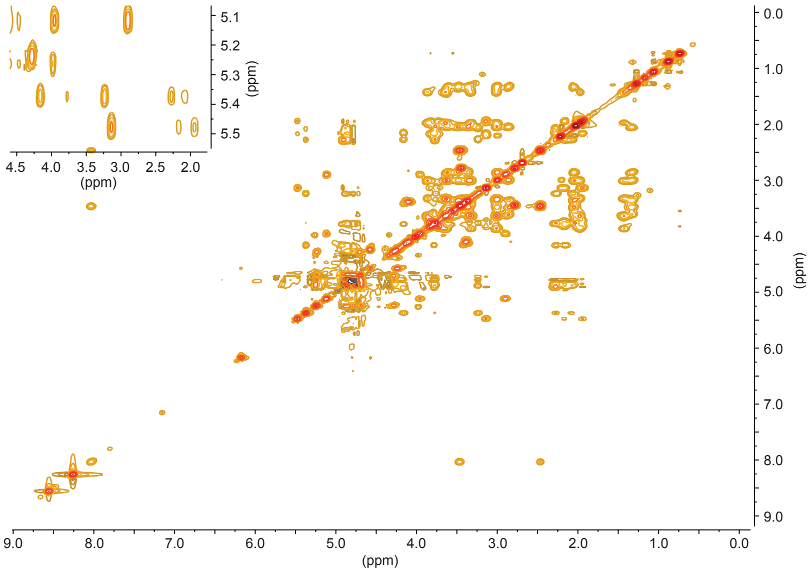

Figure S42. zTOCSY of a mixture of two di-acetylated SIS products (9:1/H_2O:D_2O, pH 8) (400 MHz). The insert shows part of the spectra that is obscured in the ^{1}H.

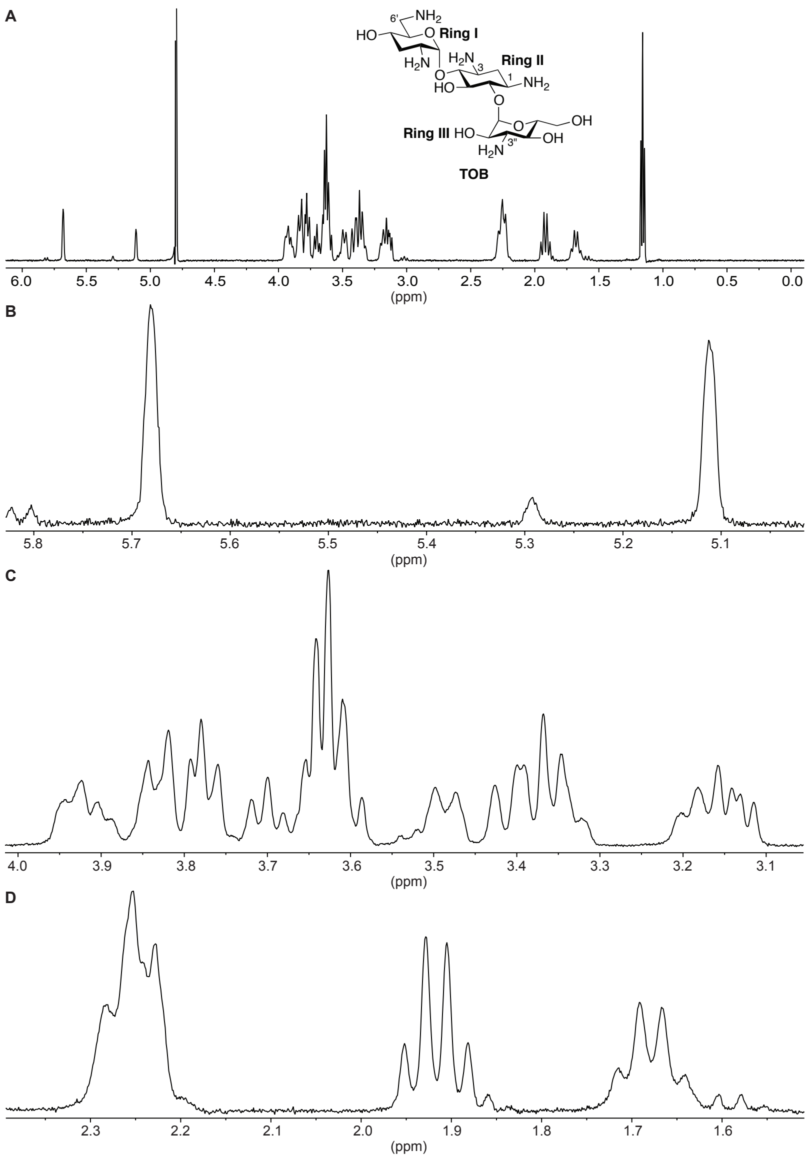

Figure S43. ^{1}H NMR of TOB (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (500 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-D**.

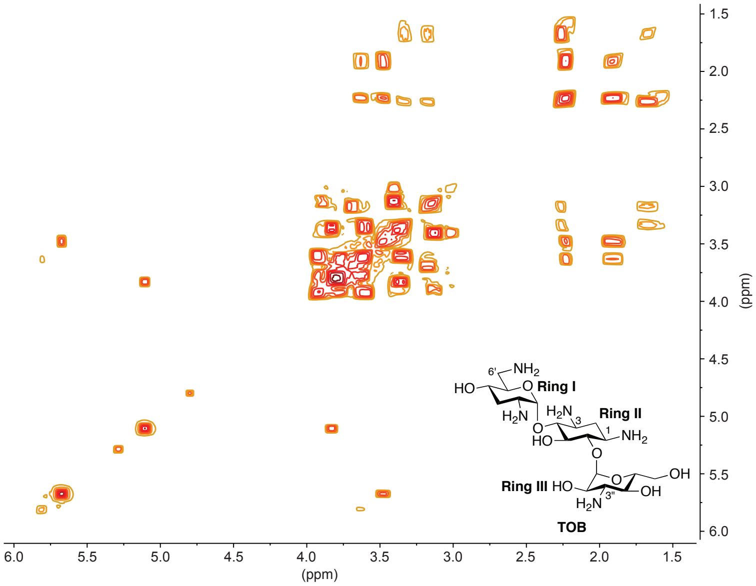

Figure S44. gCOSY of TOB (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (500 MHz).

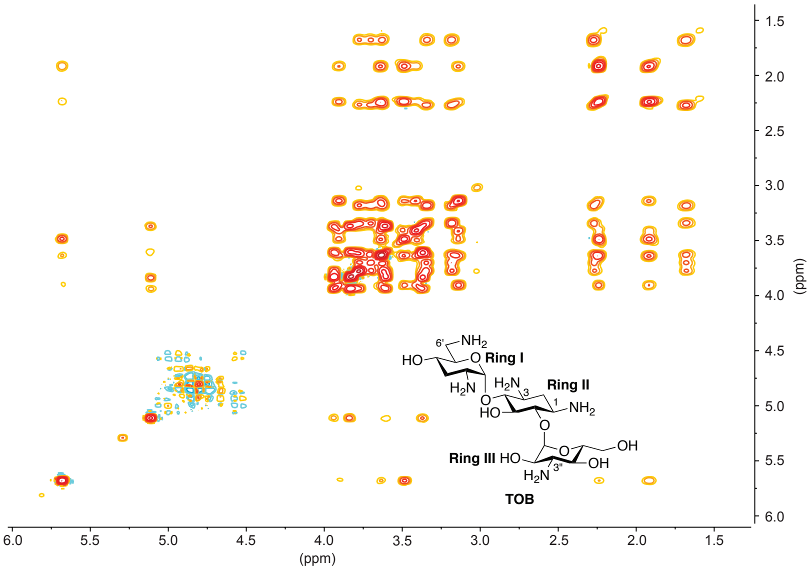

Figure S45. zTOCSY of TOB (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (500 MHz).

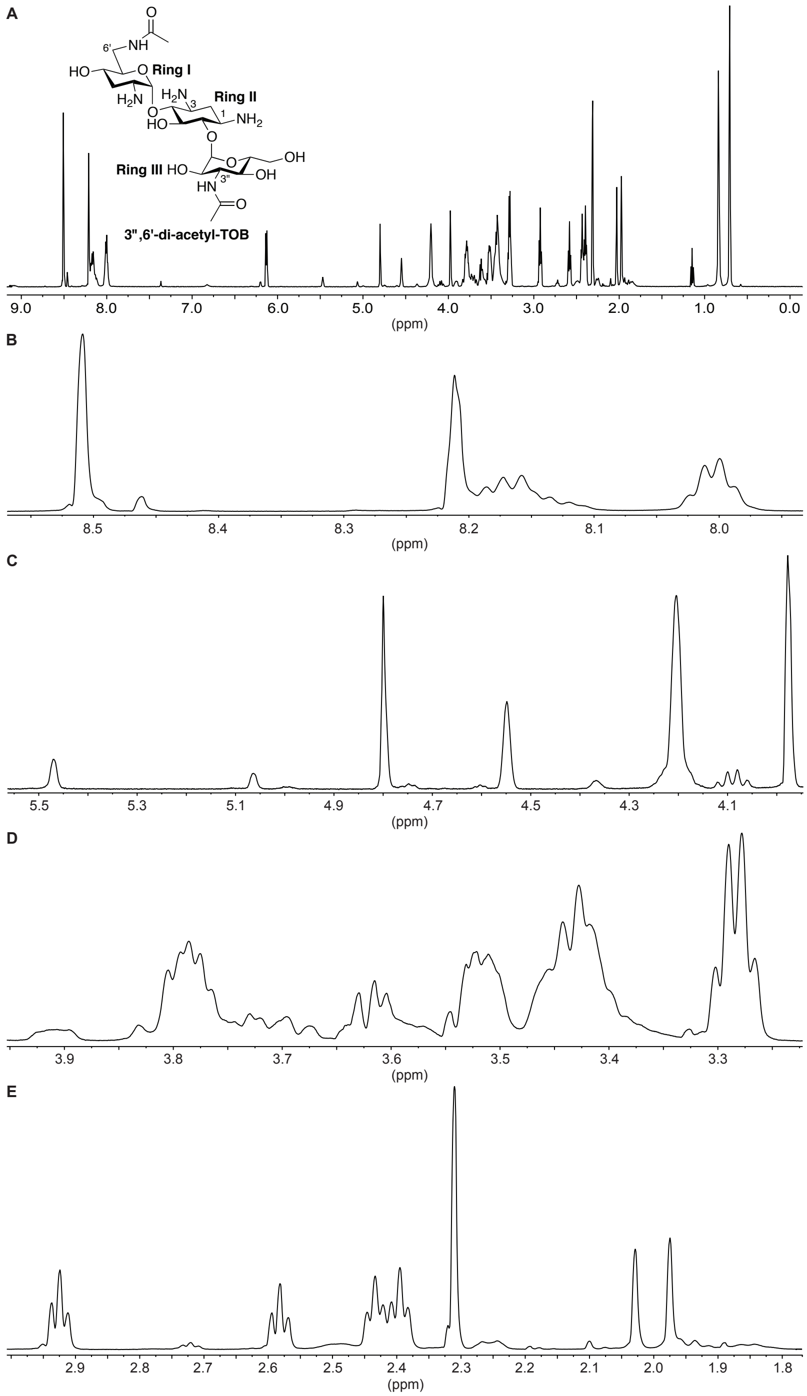

Figure S46. ^{1}H NMR of 3",6'-di-acetyl-TOB Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (500 MHz). The full spectrum is shown in panel **A** and the expansions in panels **B-E**.

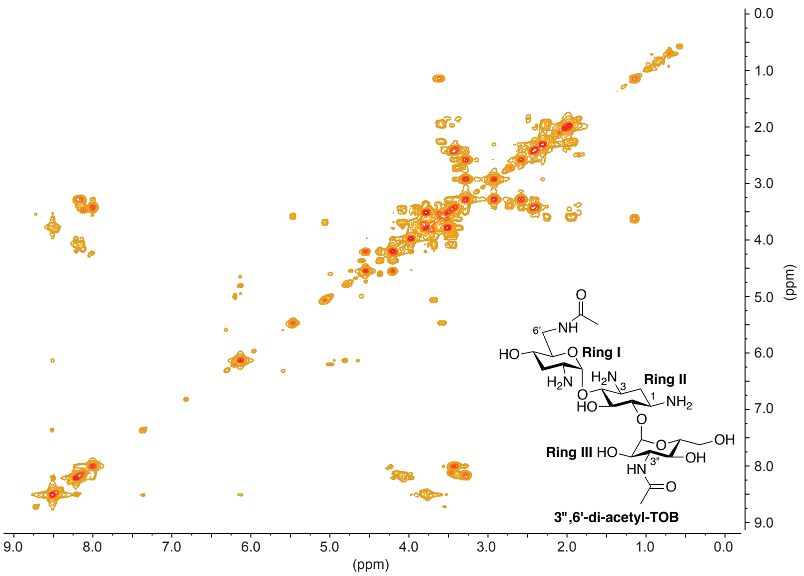

Figure S47. gCOSY of 3",6'-di-acetyl-TOB Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (500 MHz).

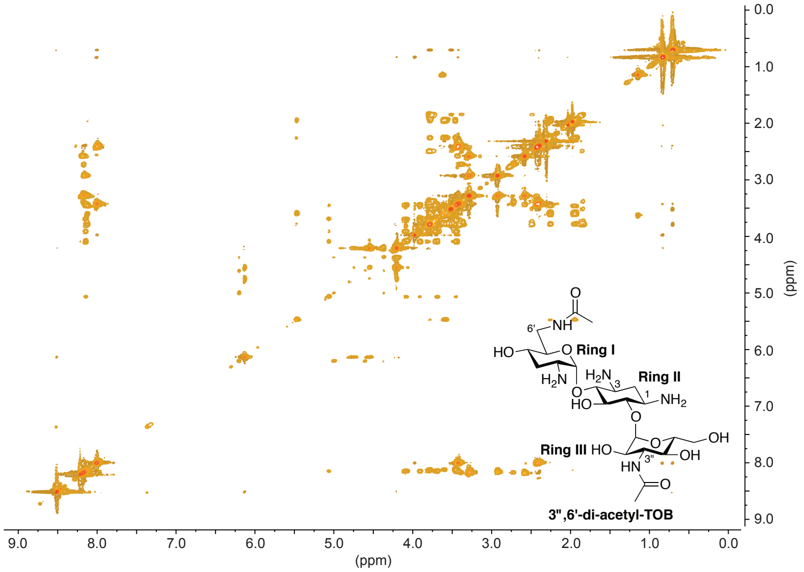

Figure S48. zTOCSY of 3",6'-di-acetyl-TOB Eis reaction mixture (9:1/H_2O:D_2O, pH 8, 25 mM KH_2PO_4) (500 MHz).

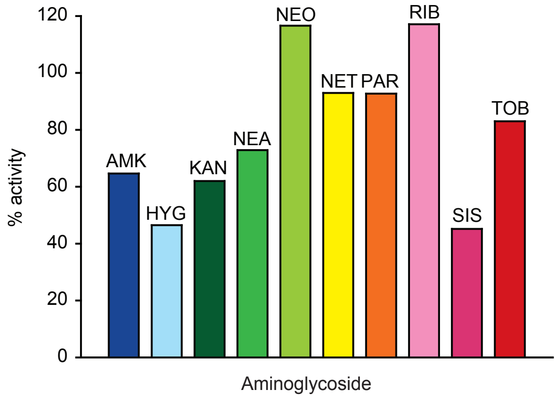

Figure S49. Relative activity towards the ten AGs tested for the EisC204A mutant. All activities are calculated relative to the activity of the wild-type Eis with the respective AGs.

[1] M. W. Vetting, S. S. Hegde, F. Javid-Majd, J. S. Blanchard, S. L. Roderick, *Nat Struct Biol* **2002**, *9*, 653-658.
[2] K. Shi, D. R. Houston, A. M. Berghuis, *Biochemistry* **2011**, *50*, 6237-6244.
[3] C. Parthier, S. Gorlich, F. Jaenecke, C. Breithaupt, U. Brauer, U. Fandrich, D. Clausnitzer, U. F. Wehmeier, C. Bottcher, D. Scheel, M. T. Stubbs, *Angew Chem Int Ed Engl* **2012**, *51*, 4046-4052.