Supporting Information

Functional profiling of adenylation domains in nonribosomal peptide synthetases by competitive activity-based protein profiling

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[#]These authors contributed equally to this work.

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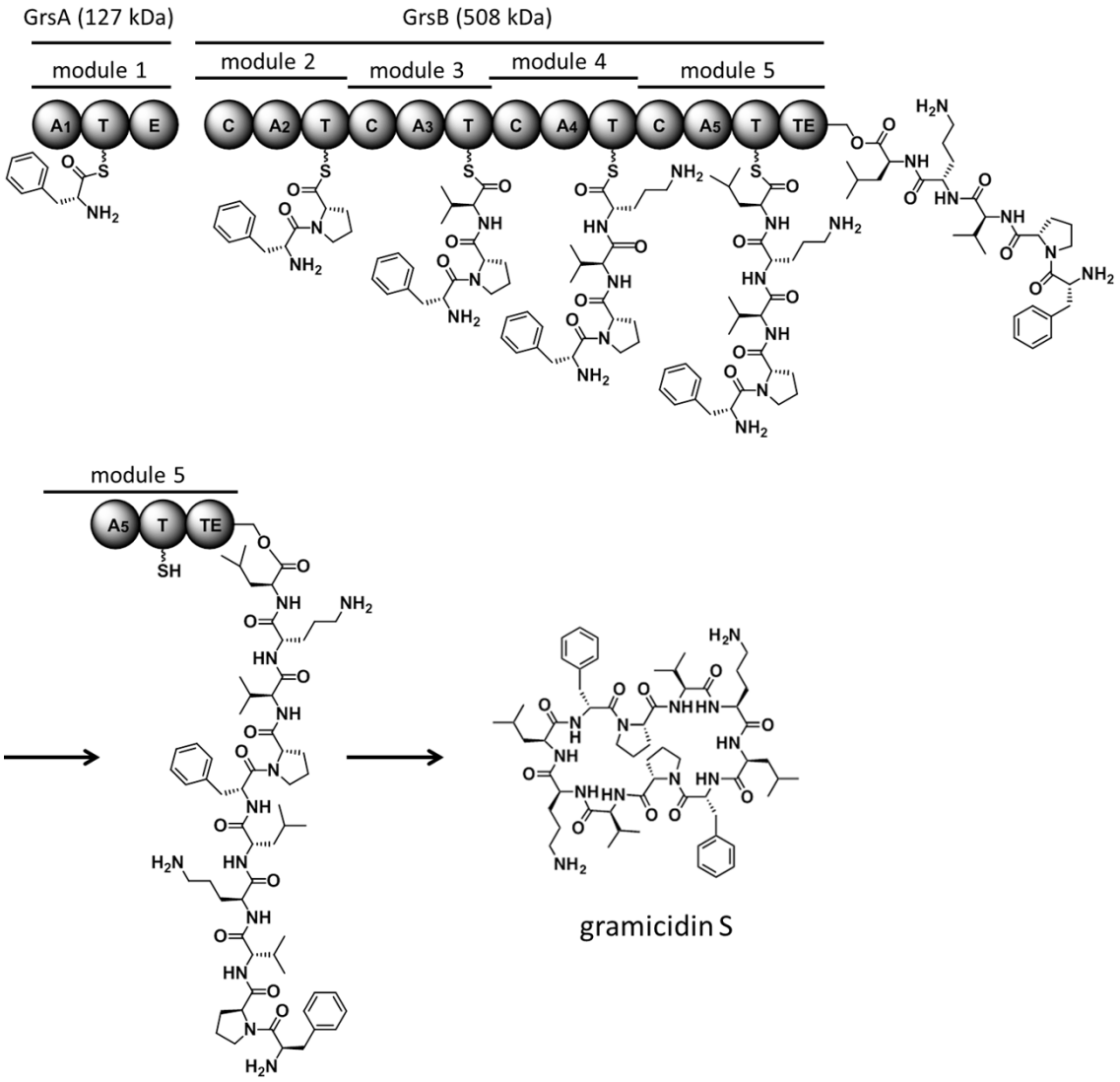


Figure S1. Nonribosomal peptide synthesis of the antibiotic gramicidin S. Modules are comprised of thiolation (T), adenylation (A) [A1: L-Phe; A2: L-Pro; A3: L-Val; A4: L-Orn; A5: L-Leu specific A-domains], epimerization (E), condensation (C), and thioesterase (TE) domains.

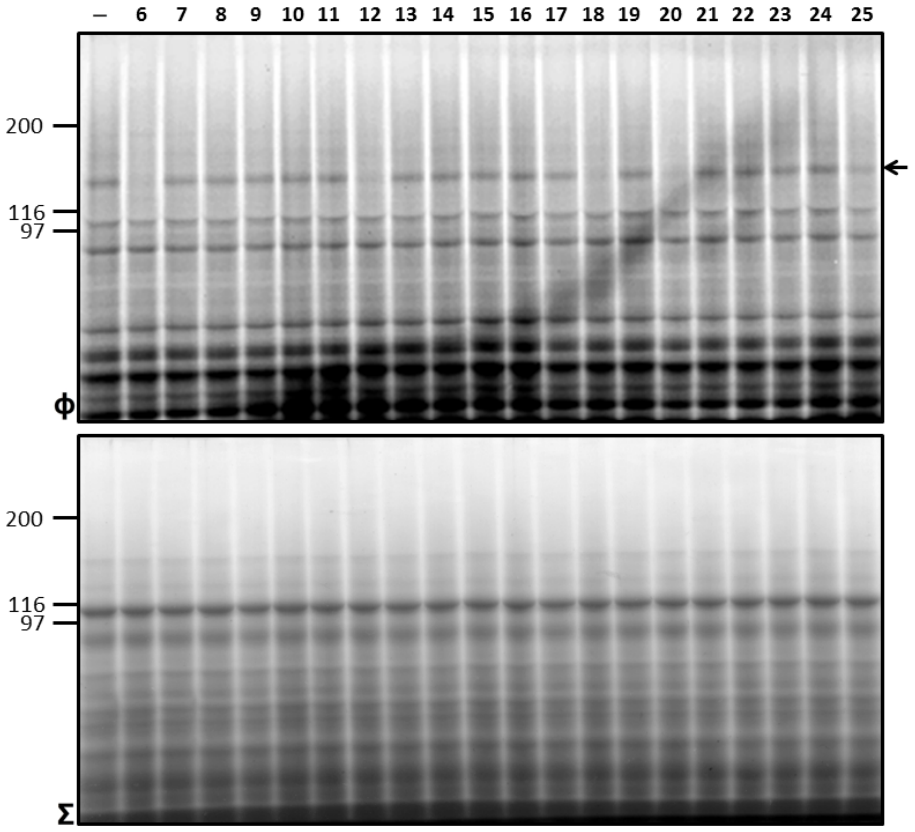


Figure S2. Full images of SDS-PAGE gels from Figure 3a. Competitive ABPP of **6**–**25** towards the A-domain of endogenous GrsA. Assessment of the inhibition potency (100 μM compound) in the *A. migulanus* ATCC 9999 proteome with probe **1** (1 μM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

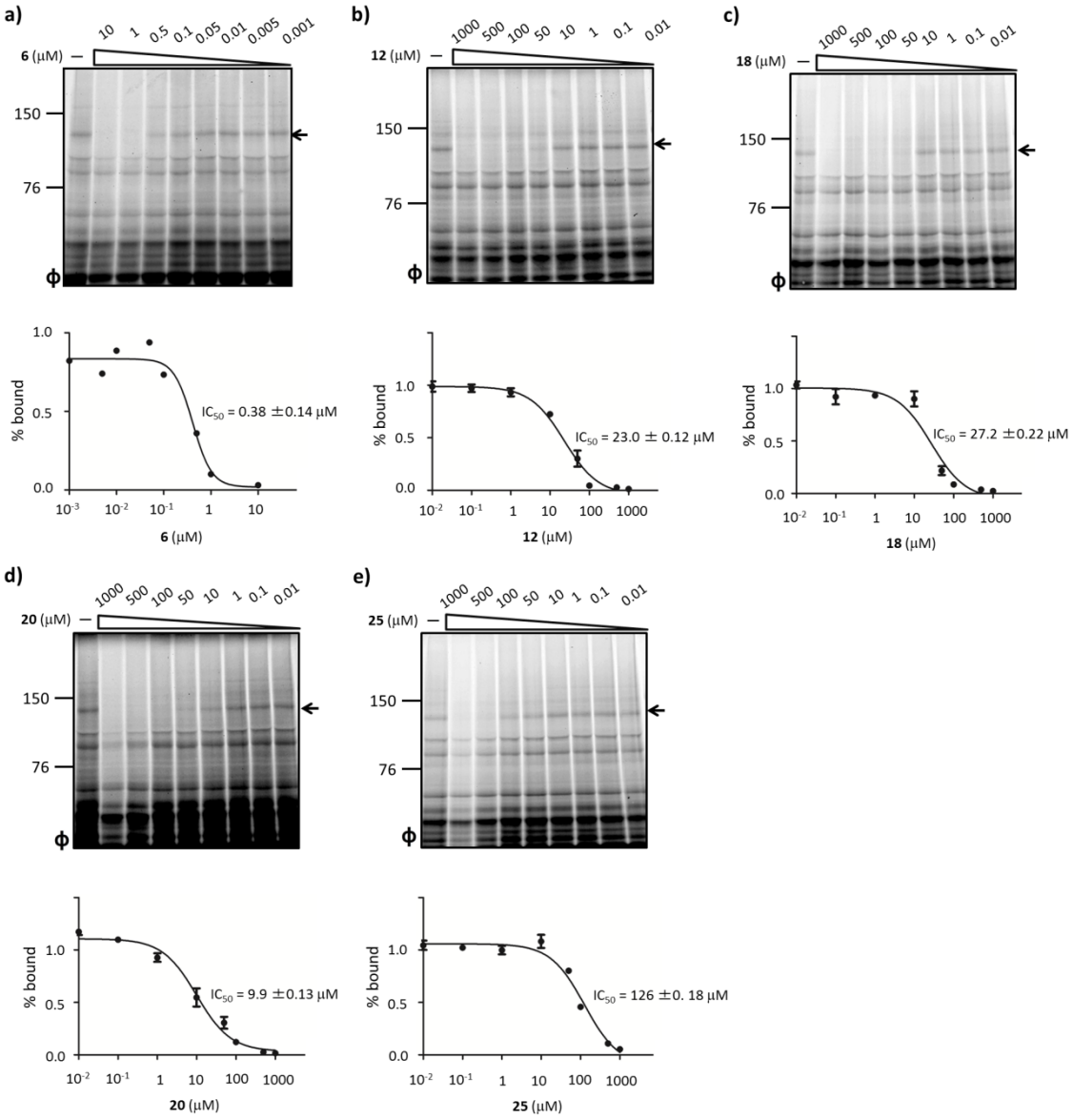


Figure S3. Full images of SDS-PAGE gels from Figure 3b. Competitive ABPP of **6**–**25** towards the A-domain of endogenous GrsA. Dose−response competitive ABPP experiments to assess the selectivity of (a) **6**, (b) **12**, (c) **18**, (d) **20**, and (e) **25** towards the A-domain of GrsA in the *A. migulanus* ATCC 9999 proteome with probe **1** (1 μM). The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

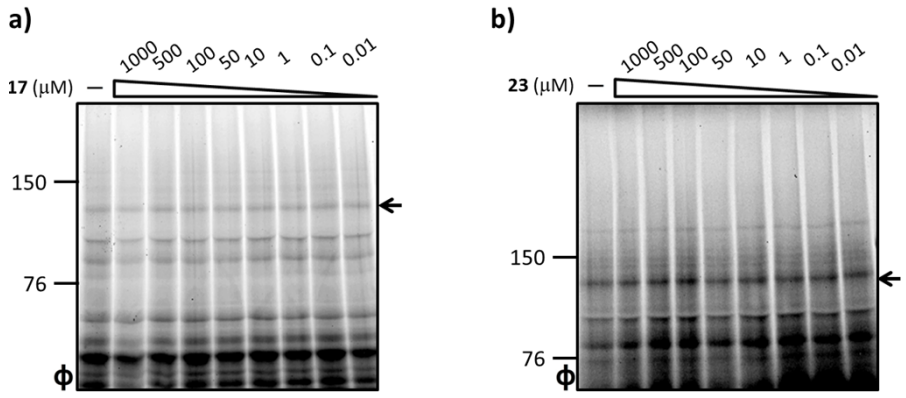


Figure S4. Competitive ABPP of 17 and 23 towards the A-domain of endogenous GrsA. Dose–response competitive ABPP experiments to assess the selectivity of (a) **17** and (b) **23** towards the A-domain of GrsA in the *A. migulanus* ATCC 9999 proteome with probe **1**. The *A. migulanus* ATCC 9999 lysate (2.0 mg/mL) was pre-incubated with inhibitors **17** and **23** (10 nM to 1 mM) before the addition of 1 μM of probe **1**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

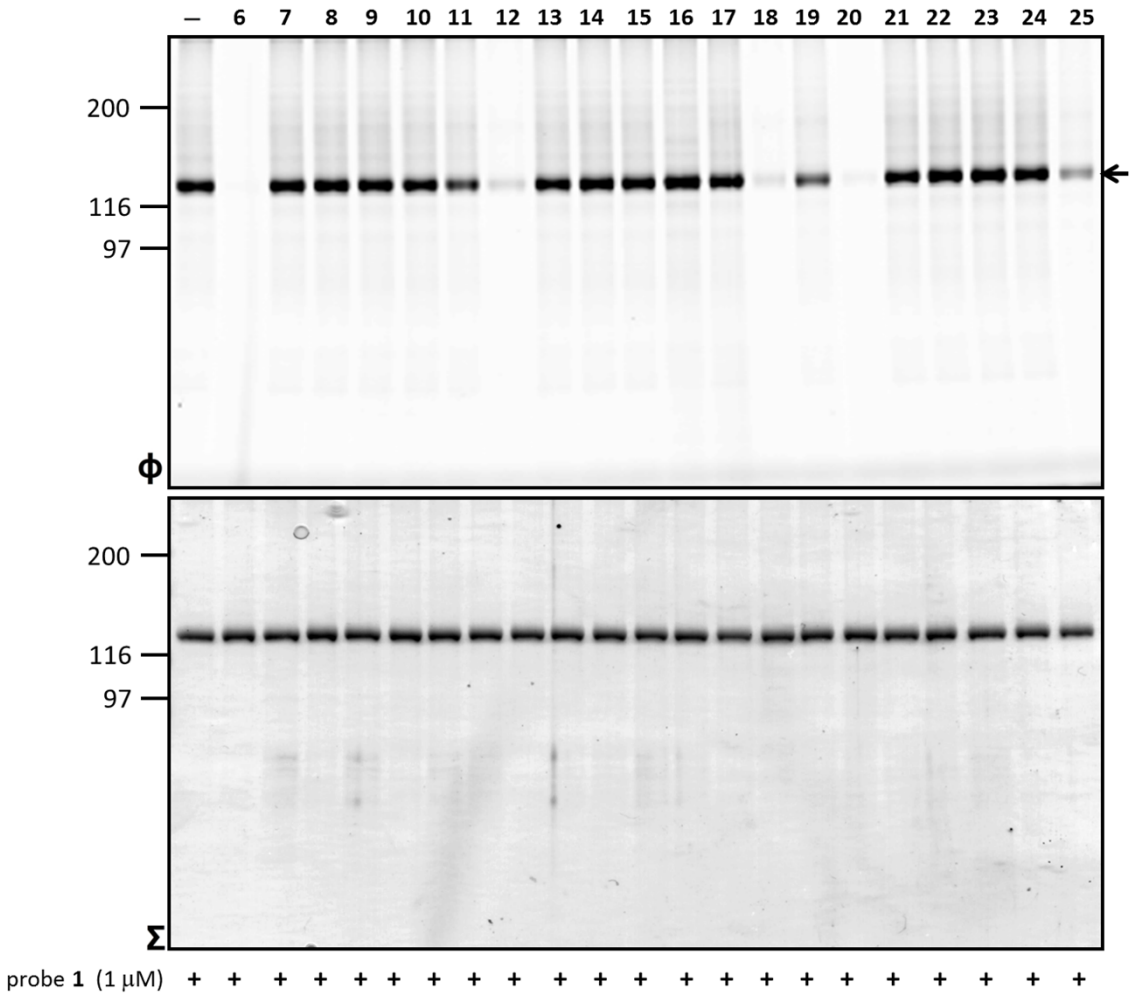


Figure S5. Competitive ABPP of 6–25 towards the A-domain of recombinant GrsA. Assessment of the inhibition potency (100 µM compound) in the A-domain of GrsA (1 µM) with probe **1** (1 µM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

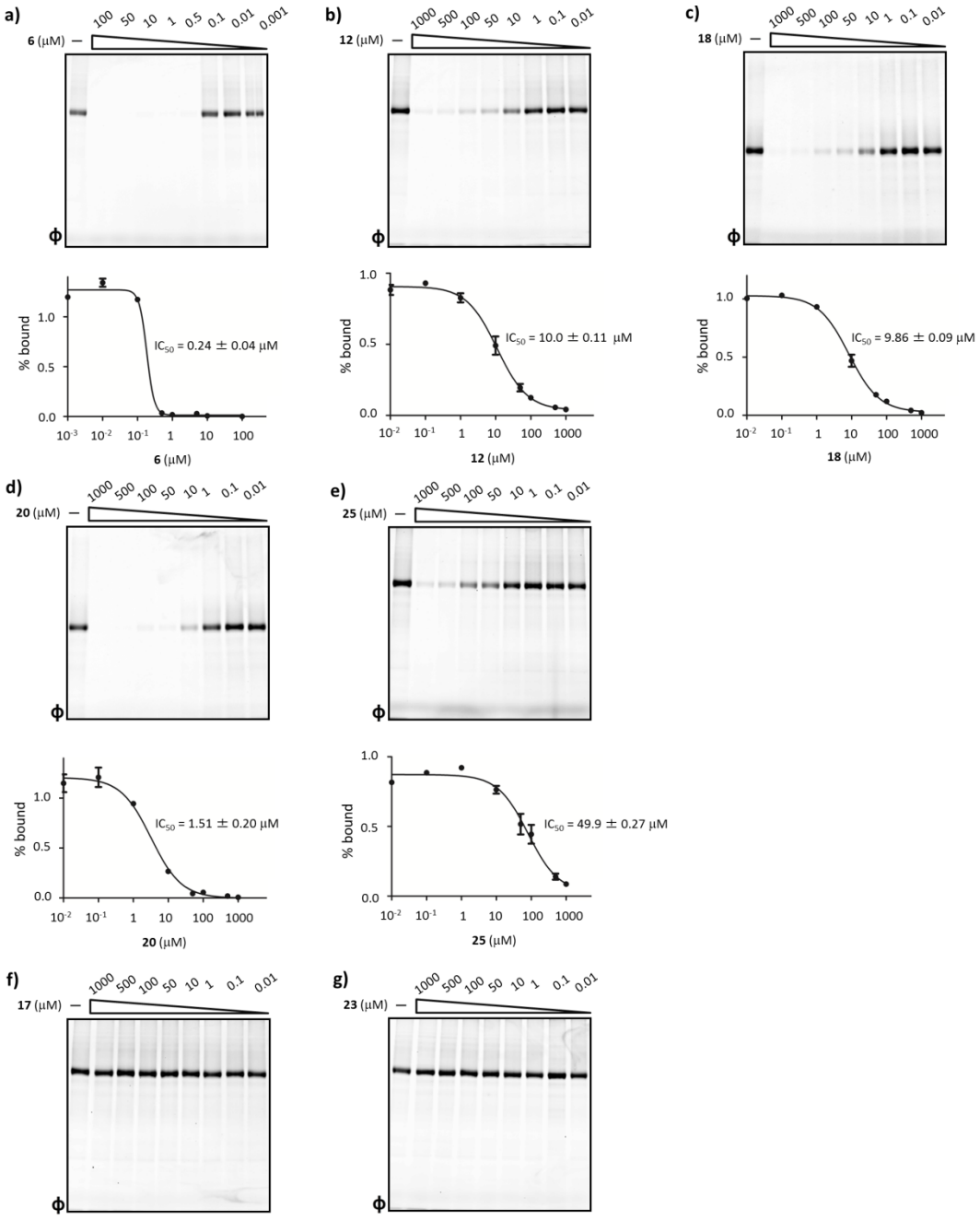


Figure S6. Competitive ABPP of 6, 12, 18, 20, 25, 17, and 23 towards the A-domain of recombinant GrsA. Dose–response competitive ABPP experiments to assess the selectivity of (a) **6**, (b) **12**, (c) **18**, (d) **20**, (e) **25**, (f) **17**, and (g) **23** towards the A-domain of GrsA with probe **1**. Recombinant GrsA (1 μM) was pre-incubated with inhibitors **6** (1 nM to 100 μM), **12** (10 nM to 1 mM), **18** (10 nM to 1 mM), **20** (10 nM to 1 mM), **25** (10 nM to 1 mM), **17** (10 nM to 1 mM), and **23** (10 nM to 1 mM) before the addition of 1 μM of probe **1**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

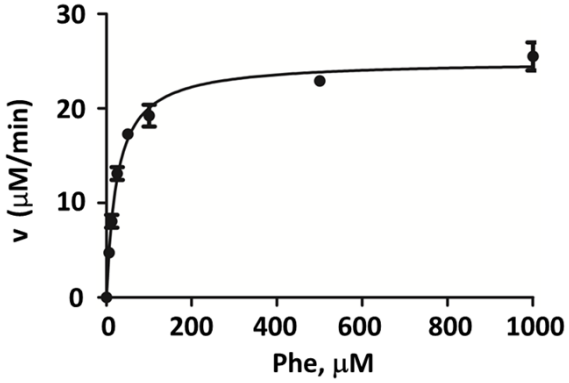


Figure S7. Steady-state kinetics of GrsA with L-Phe. Each reaction contained 50 nM *holo*-GrsA, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 6.25–1000 μM L-Phe.

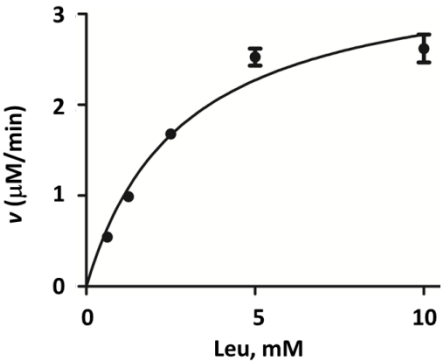


Figure S8. Steady-state kinetics of GrsA with L-Leu. Each reaction contained 700 nM *holo*-GrsA, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 0.625–10 mM L-Leu.

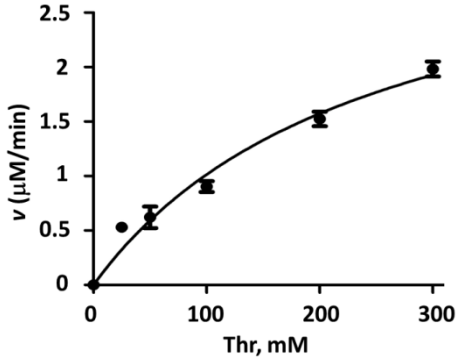


Figure S9. Steady-state kinetics of GrsA with L-Thr. Each reaction contained 1.4 μM *holo*-GrsA, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 25–300 mM L-Thr.

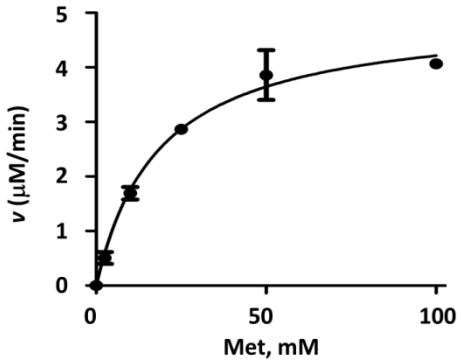


Figure S10. Steady-state kinetics of GrsA with L-Met. Each reaction contained 140 nM *holo*-GrsA, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 2.5–100 mM L-Met.

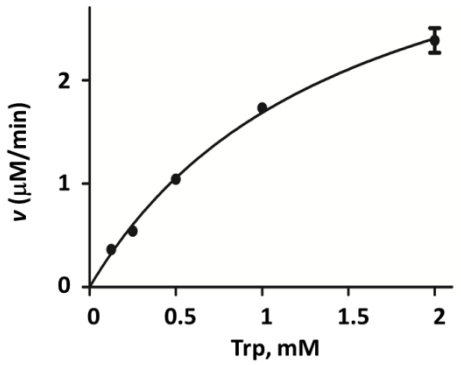


Figure S11. Steady-state kinetics of GrsA with L-Trp. Each reaction contained 700 nM *holo*-GrsA, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 0.125–2.0 mM L-Trp.

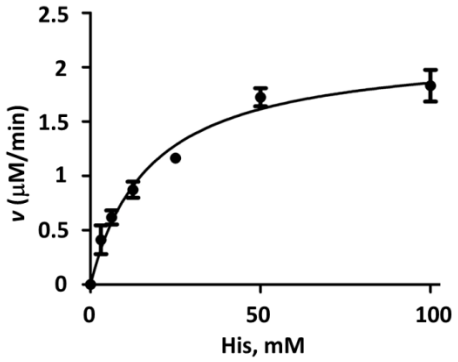


Figure S12. Steady-state kinetics of GrsA with L-His. Each reaction contained 1.4 μM *holo*-GrsA, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 3.125–100 mM L-His.

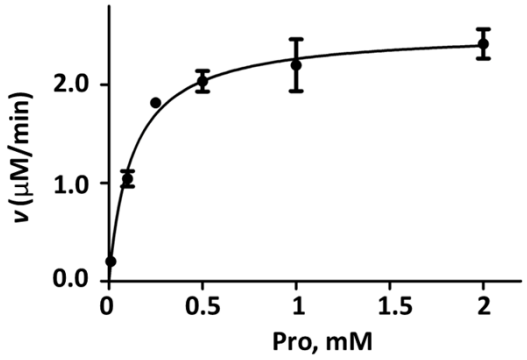


Figure S13. Steady-state kinetics of TycB1 with L-Pro. Each reaction contained 400 nM *holo*-TycB1, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 10–2000 μM L-Pro. Velocities were fit to the Michaelis-Menten equation.

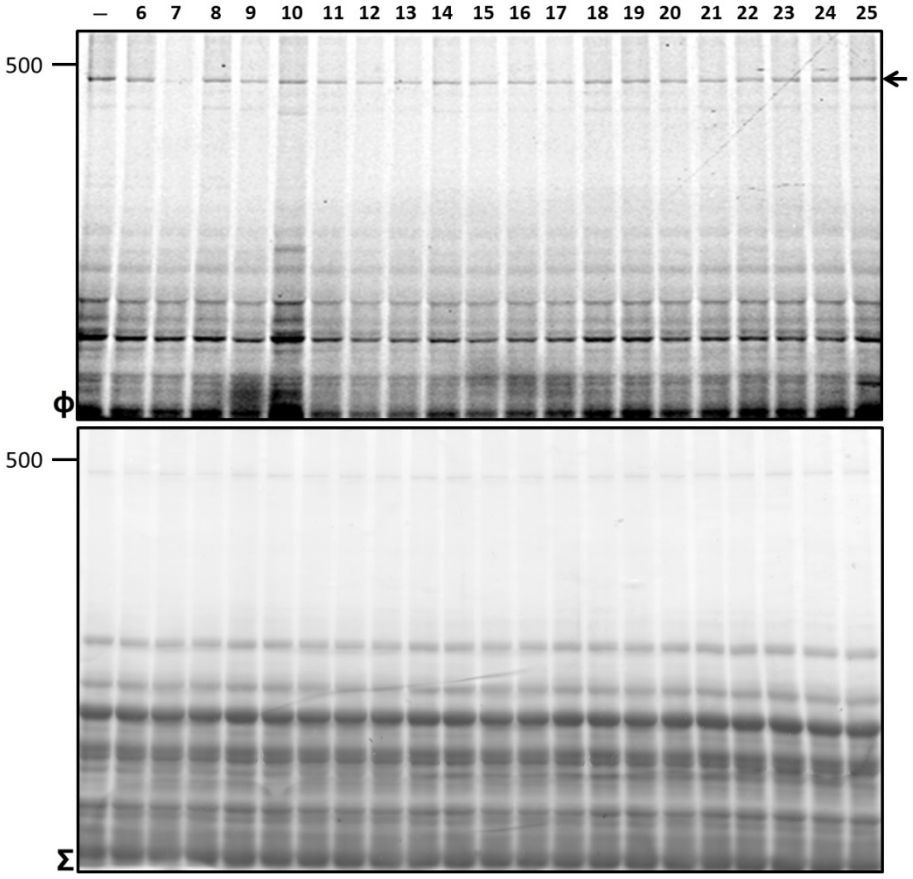


Figure S14. Full images of SDS-PAGE gels from Figure 4a. Competitive ABPP of **6**–**25** toward the A-domains of endogenous GrsB in the *A. migulanus* ATCC 5759 proteome. Assessment of the inhibition potency (100 μM compound) towards the Pro-activating domain of GrsB with probe **2** (1 μM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

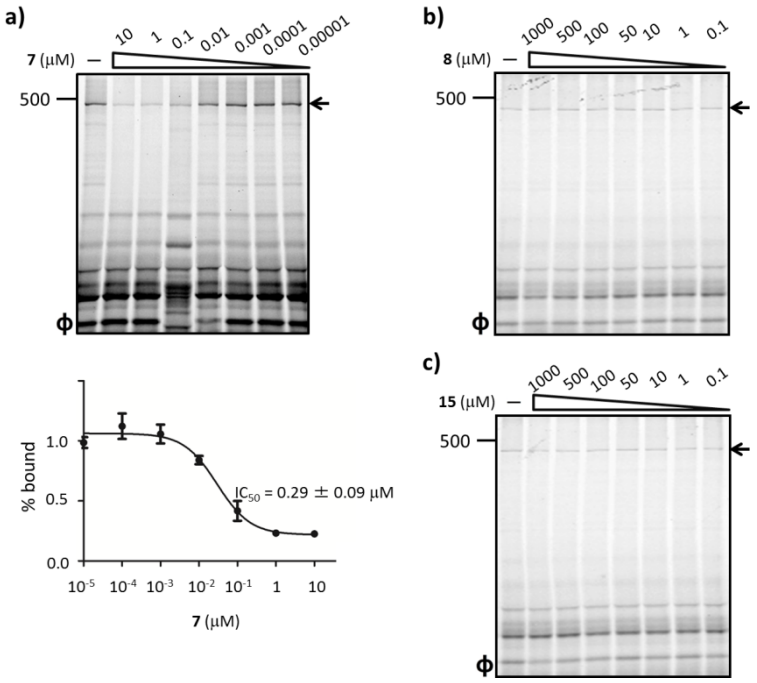


Figure S15. Dose–response competitive ABPP of 7, 8, and 15 towards the Pro-activating domain of endogenous GrsB. The *A. migulanus* DSM 5759 lysate (2.0 mg/mL) was pre-incubated with **7** (0.01 nM to 10 μM), **8** (0.1 to 1000 μM), and **15** (0.1 to 1000 μM) before the addition of 1 μM of probe **2**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

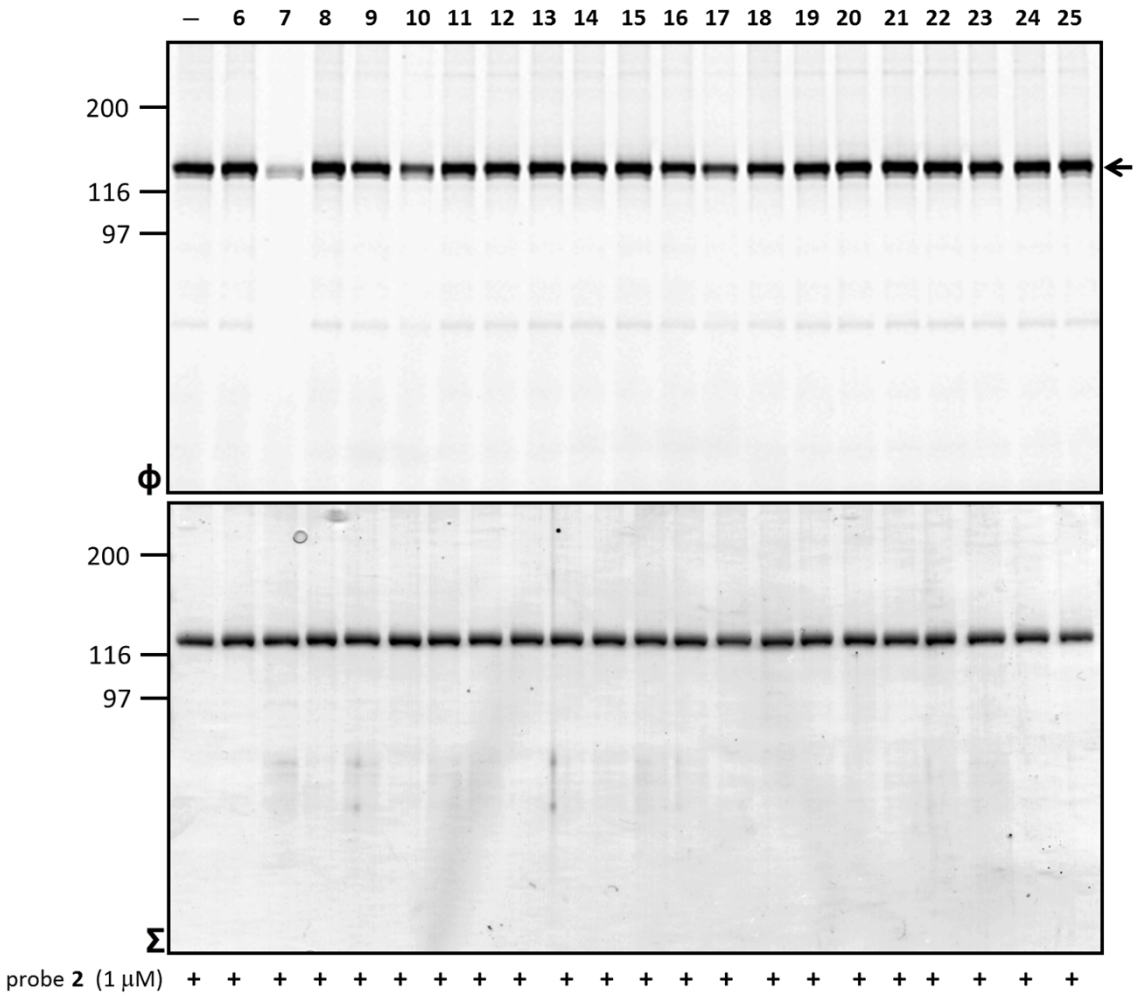


Figure S16. Competitive ABPP of 6–25 toward the A-domains of recombinant TycB1. Assessment of the inhibition potency (100 μM compound) towards the Pro-activating domain of TycB1 (1 μM) with probe **2** (1 μM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

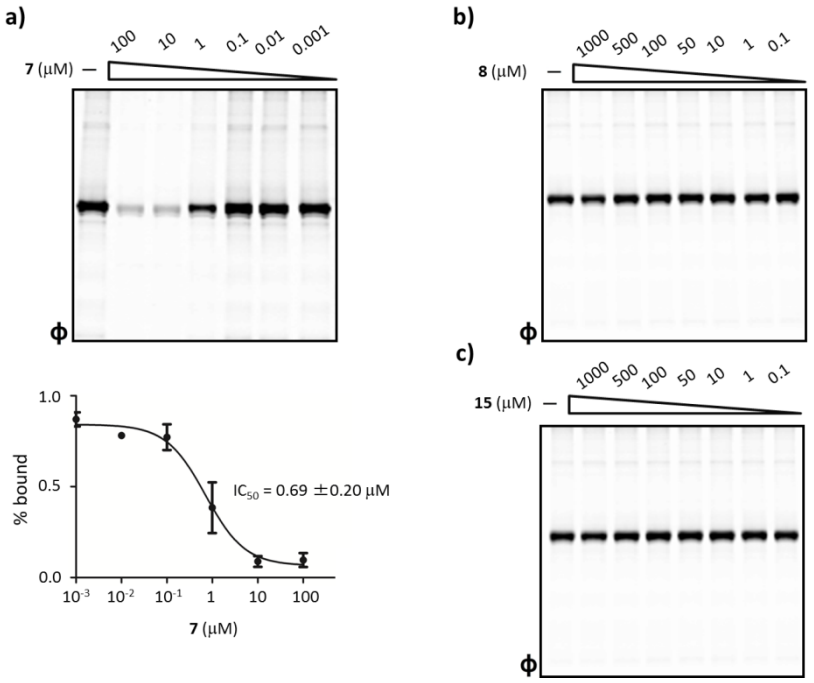


Figure S17. Competitive ABPP of 7, 8, and 15 towards the Pro-activating domain of recombinant TycB1. Dose–response competitive ABPP experiments to assess the selectivity of (a) **7**, (b) **8**, and (c) **15** towards the A-domain of TycB1 with probe **2**. Recombinant TycB1 (1 μM) was pre-incubated with inhibitors **7** (1 nM to 100 μM), **8** (0.1 μM to 1000 μM), and **15** (0.1 μM to 1000 μM) before the addition of 1 μM of probe **2**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

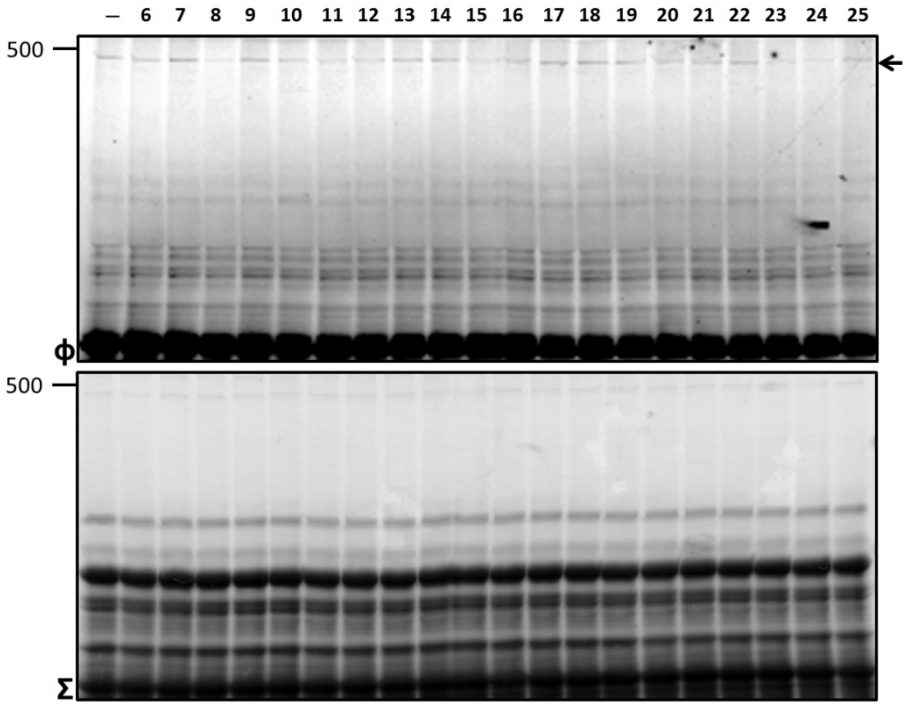


Figure S18. Full images of SDS-PAGE gels from Figure 4b. Competitive ABPP of **6**–**25** toward the A-domains of endogenous GrsB in the *A. migulanus* ATCC 5759 proteome. Assessment of the inhibition potency (100 μM compound) towards the Orn-activating domain of GrsB with probe **3** (1 μM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

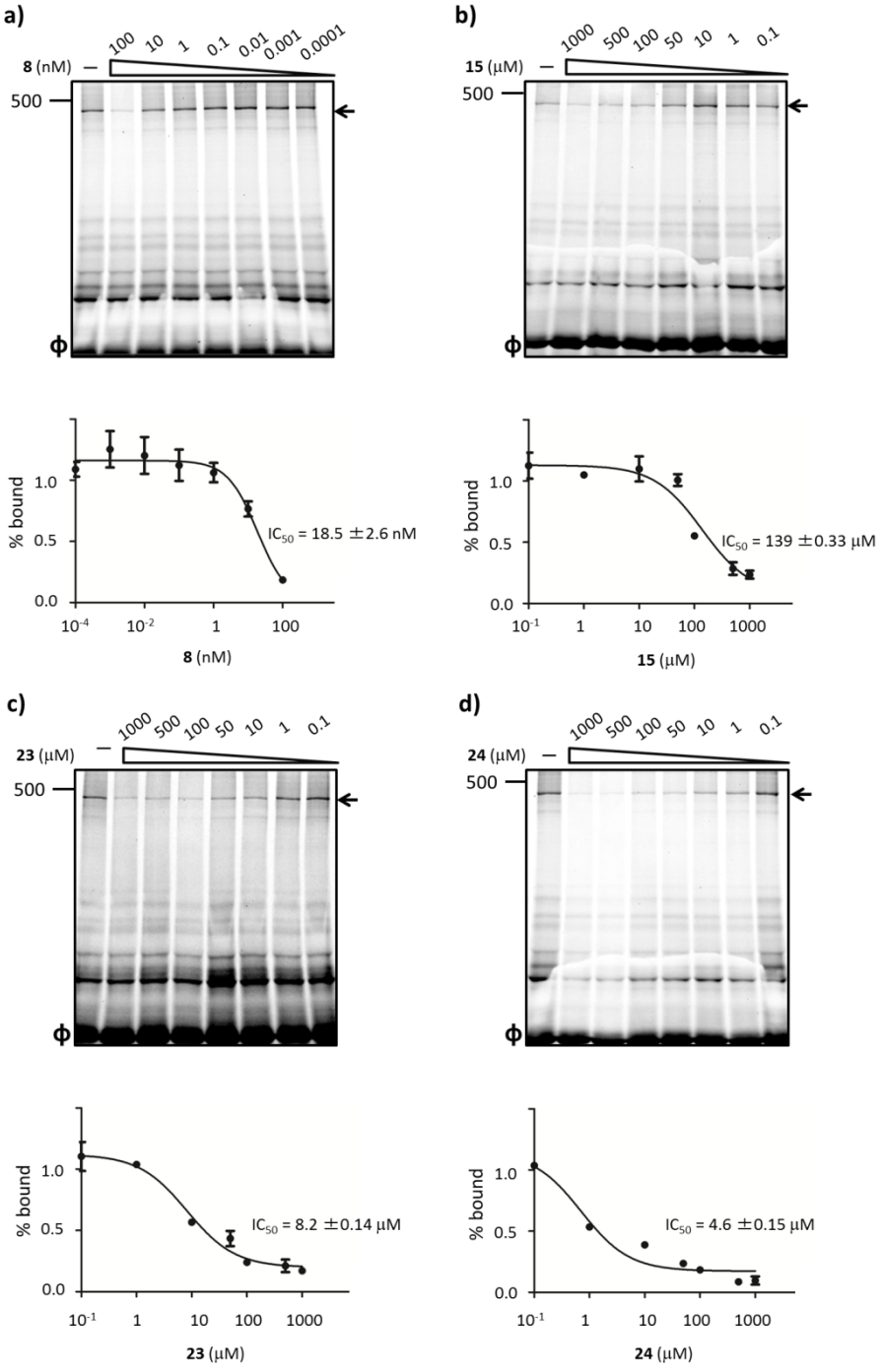


Figure S19. Dose–response competitive ABPP of 8, 15, 23, and 24 towards the Orn-activating domain of endogenous GrsB. The *A. migulanus* DSM 5759 lysate (2.0 mg/mL) was pre-incubated with (a) **8**, (b) **15**, (c) **23**, and (d) **24** at concentrations ranging from 0.1 pM to 1 mM (**8**: 0.1 pM to 100 nM; **15**: 100 nM to 1 mM; **23**: 100 nM to 1 mM; **24**: 100 nM to 1 mM) before the addition of 1 μM of probe **3**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

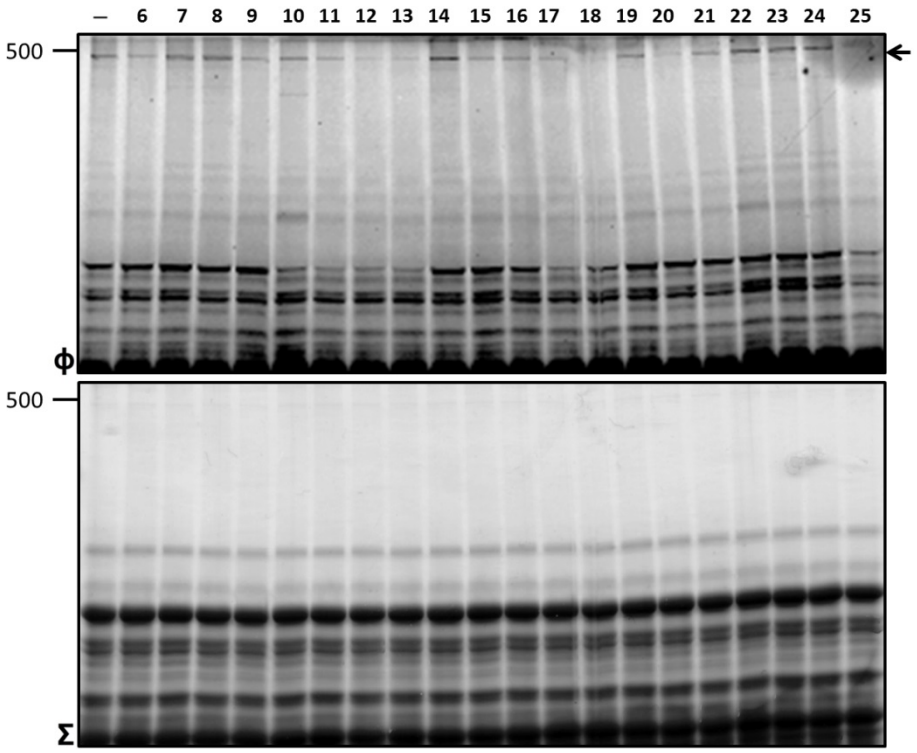


Figure S20. Full images of SDS-PAGE gels from Figure 4c. Competitive ABPP of **6**–**25** toward the A-domains of endogenous GrsB in the *A. migulanus* ATCC 5759 proteome. Assessment of the inhibition potency (100 μM compound) towards the Val-activating domain of GrsB with probe **4** (1 μM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

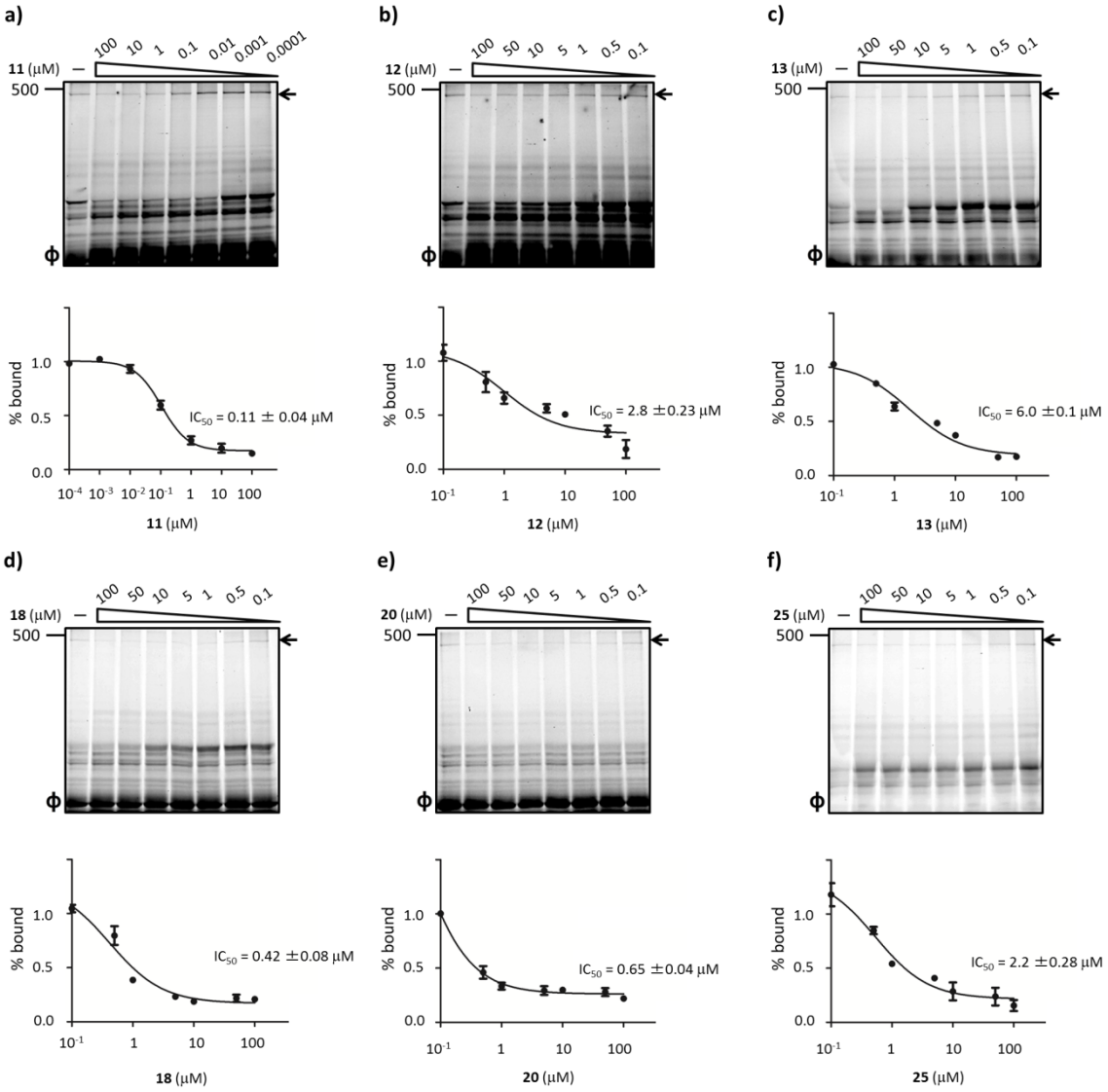


Figure S21. Dose–response competitive ABPP of 11, 12, 13, 18, 20, and 25 towards the Val-activating domain of endogenous GrsB. The *A. migulanus* DSM 5759 lysate (2.0 mg/mL) was pre-incubated with (a) **11**, (b) **12**, (c) **13**, (d) **18**, (e) **20**, and (f) **25** at concentrations ranging from 0.1 nM to 100 μM (**11**: 0.1 nM to 100 μM; **12**: 100 nM to 100 μM; **13**: 100 nM to 100 μM; **20**: 100 nM to 100 μM; **25**: 100 nM to 100 μM) before the addition of 1 μM of probe **4**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

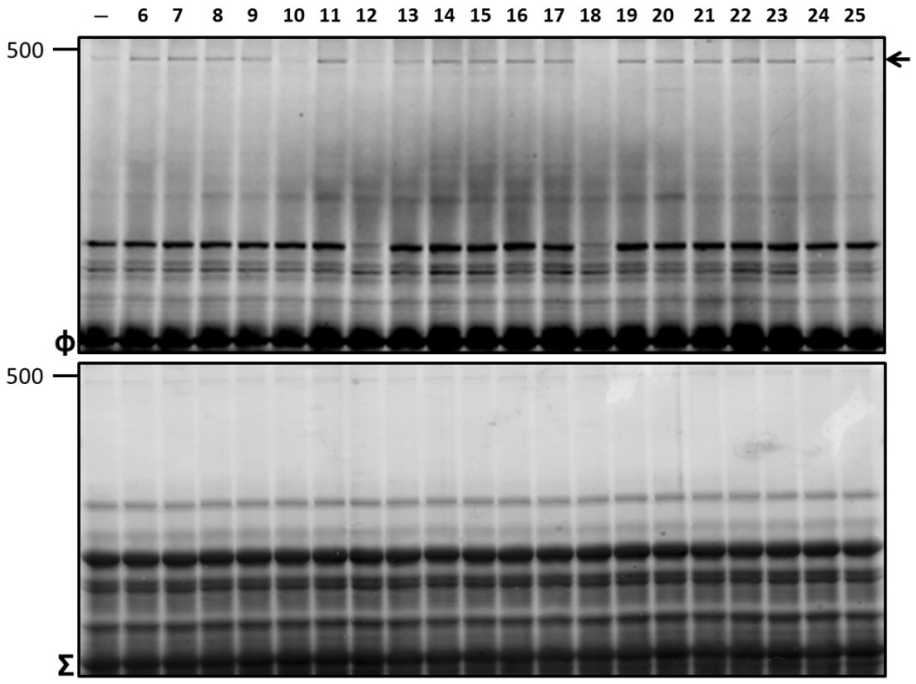


Figure S22. Full images of SDS-PAGE gels from Figure 4d. Competitive ABPP of **6**–**25** toward the A-domains of endogenous GrsB in the *A. migulanus* ATCC 5759 proteome. Assessment of the inhibition potency (100 μM compound) towards the Leu-activating domain of GrsB with probe **5** (1 μM). The top image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

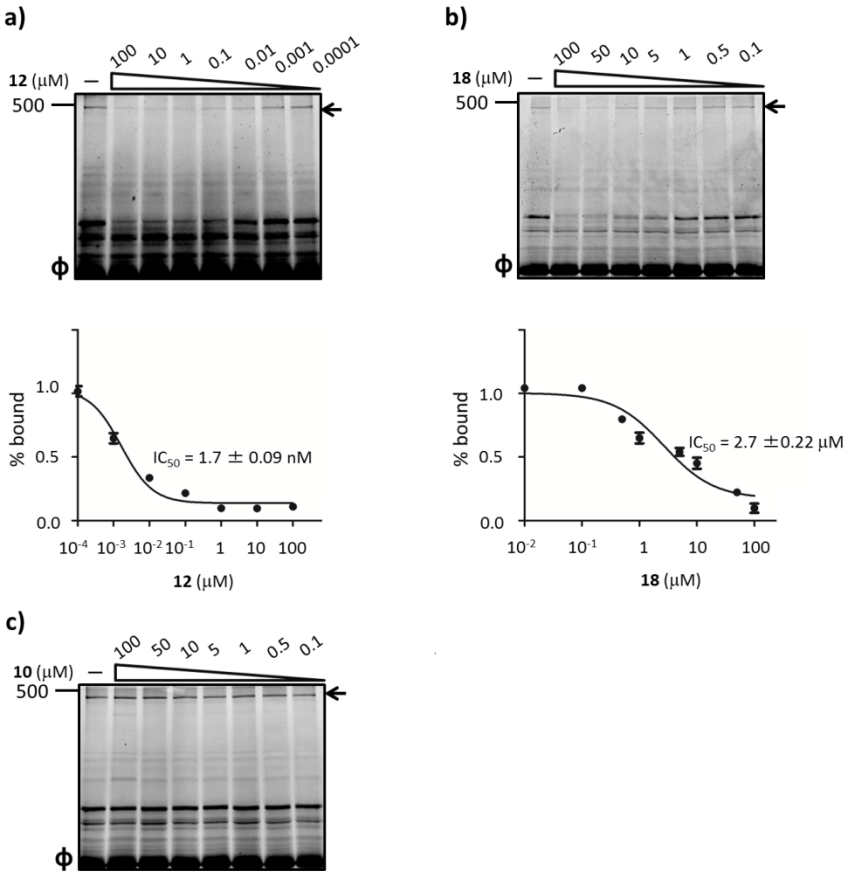


Figure S23. Dose–response competitive ABPP of 10, 12, and 18 toward the Leu-activating domain of endogenous GrsB. The *A. migulanus* DSM 5759 lysate (2.0 mg/mL) was pre-incubated with (a) **12**, (b) **18**, and (c) **10** at concentrations ranging from 0.1 nM to 1 mM (**12**: 0.1 nM to 100 μM; **18**: 100 nM to 100 μM; **10**: 100 nM to 100 μM) before the addition of 1 μM of probe **5**. The image (Φ) depicts the fluorescence observed with λ_{ex} = 532 nm and λ_{em} = 580 nm.

Table S1. Catalytic parameters of the adenylation reaction catalyzed by the A-domain of GrsA.[a]

substrate	k_{cat} [min^{-1}]	K_m [mM]	k_{cat}/K_m [mM^{-1} min^{-1}]
L-Phe	500 ± 12	0.0248 ± 0.0023	20161 ± 5217
L-Leu	5.09 ± 0.40	2.85 ± 0.57	1.79 ± 0.71
L-Thr	2.51 ± 0.39	246 ± 69	0.011 ± 0.0007
L-Met	35.8 ± 2.1	18.5 ± 3.3	1.92 ± 0.17
L-Trp	5.98 ± 0.43	1.49 ± 0.19	4.02 ± 2.2
L-Lys	n.d.[b]	n.d.[b]	n.d.[b]
L-His	1.56 ± 0.09	17.5 ± 3.0	0.089 ± 0.0037

[a] Catalytic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[1] [b] not detected.

Table S2. Catalytic parameters of the adenylation reaction catalyzed by the A-domain of TycB1.[a]

substrate	k_{cat} [min^{-1}]	K_m [μM]	k_{cat}/K_m [mM^{-1} min^{-1}]
L-Pro	6.36 ± 0.28	125 ± 24	50.8 ± 11.6

[a] Catalytic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[1] Comprehensive analysis of relative substrate specificities of the homologous A-domain within the first module of TycB (TycB1) have revealed that it exhibited strict substrate specificities and activated exclusively the cognate substrate L-Pro within the 10 amino acid substrates tested.[2]

Table S3. Dose−response competitive ABPP experiments to assess the selectivity of **6**, **12**, **18**, **20**, and **25** towards the A-domains of endogenous and recombinant GrsA.

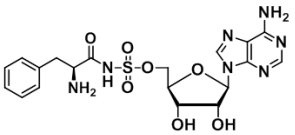
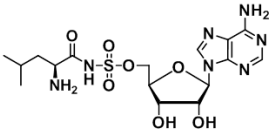
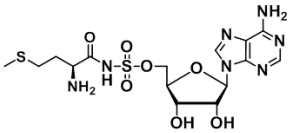
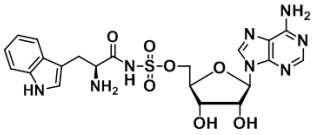
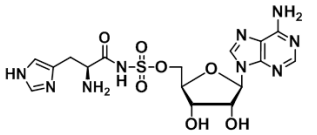
cpd	structure	IC_{50} (μM)	
		endogeous GrsA	recombinant GrsA
6		0.38 ± 0.14	0.24 ± 0.04
12		23.0 ± 0.12	10.0 ± 0.11
18		27.2 ± 0.22	9.86 ± 0.09
20		9.90 ± 0.13	1.51 ± 0.20
25		126 ± 0.18	49.9 ± 0.27

Table S4. Dose−response competitive ABPP experiments to assess the selectivity of **7** towards the Pro-activating domains of endogenous GrsB and recombinant TycB1.

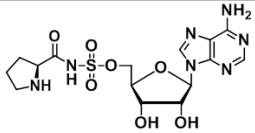
cpd	structure	IC_{50} (μM)	
		endogeous GrsB	recombinant TycB1
7		0.29 ± 0.09	0.69 ± 0.20

Table S5. Dose−response competitive ABPP experiments to assess the selectivity of **8**, **15**, **23**, and **24** towards the Orn-activating domain of endogenous GrsB.

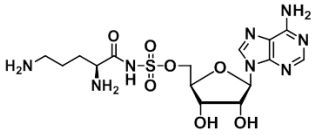
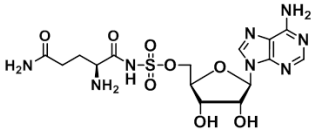
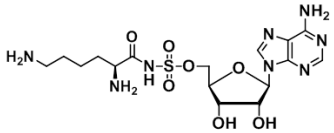
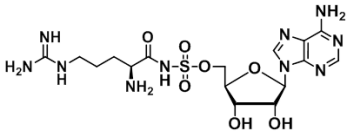
cpd	structure	IC_{50}
		endogeous GrsB
8		18.5 ± 2.6 (nM)
15		139 ± 0.33 (μM)
23		8.2 ± 0.14 (μM)
24		4.6 ± 0.15 (μM)

Table S6. Dose−response competitive ABPP experiments to assess the selectivity of **11**,**12**, **13**, **18**, **20**, and **25** towards the Val-activating domain of endogenous GrsB.

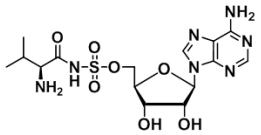
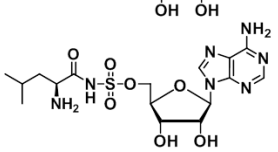
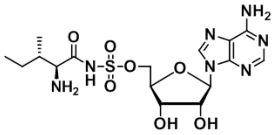
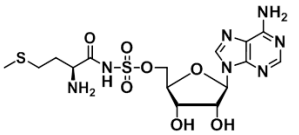
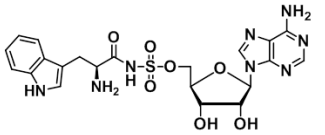
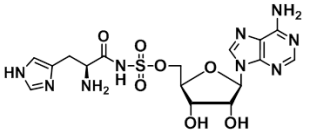
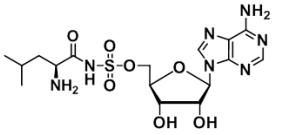
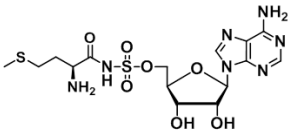
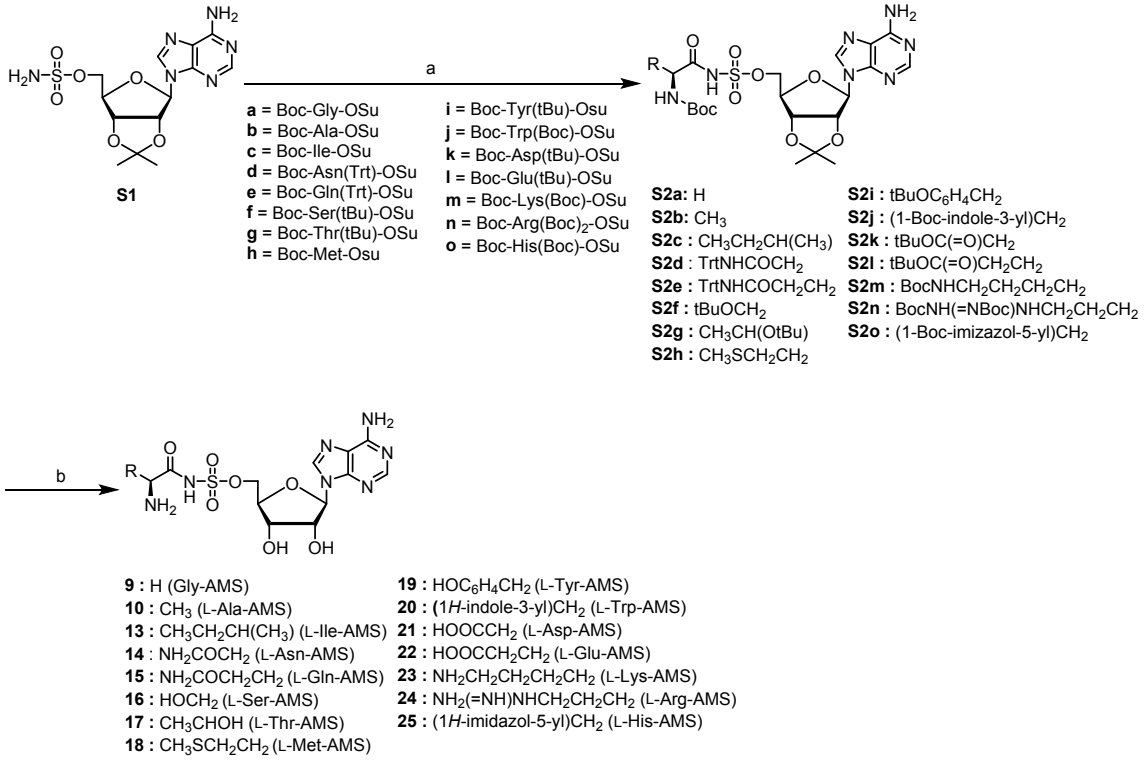
cpd	structure	IC_{50} (μM)
		endogeous GrsB
11		0.11 ± 0.04
12		2.8 ± 0.23
13		6.0 ± 0.10
18		0.42 ± 0.08
20		0.65 ± 0.04
25		2.2 ± 0.28

Table S7. Dose−response competitive ABPP experiments to assess the selectivity of **12** and **18** towards the Leu-activating domain of endogenous GrsB.

cpd	structure	IC_{50}
		endogeous GrsB
12		1.7 ± 0.09 nM
18		2.7 ± 0.22 μM

Chemical Synthetic Procedures



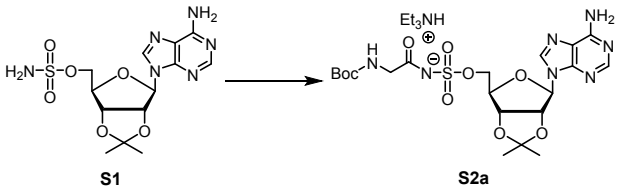
Scheme S1. Synthetic route to sulfamoyloxy-linked aminoacyl-AMP analogues. *Reagents and conditions*: [a] *N*-hydroxysuccinimide esters **a**-**o**, Cs_2CO_3, DMF, rt: 92% (**S2a**); 53% (**S2b**); 90% (**S2c**); 88% (**S2d**); 77% (**S2e**); 65% (**S2f**); 63% (**S2g**); 75% (**S2h**); 98% (**S2i**); 57% (**S2j**); 66% (**S2k**); 76% (**S2l**); 98% (**S2m**); 78% (**S2n**); 59% (**S2o**); [b] 80% aqueous TFA or a mixture of 90:5:5 (v/v) of TFA, H_2O, and TIS, rt: 52% (**9**); 77% (**10**); 70% (**13**); 70% (**14**); 40% (**15**); 23% (**16**); 49% (**17**); 57% (**18**); 74% (**19**); 90% (**20**); 83% (**21**); 98% (**22**); 61% (**23**); 70% (**24**); 74% (**25**).

General Synthetic Methods: All commercial reagents were used as provided unless otherwise indicated. 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1**[3] (Schemes S1) is known compounds. These compounds were prepared according to published literature procedures. All reactions were carried out under an atmosphere of nitrogen in dry solvents with oven-dried glassware and constant magnetic stirring unless otherwise noted. High performance liquid chromatography (HPLC) was performed on a Prominence CBM-20A (Shimadzu) system equipped with a Prominence SPD-20A UV/VIS detector (Shimadzu). ^{1}H-NMR spectra were recorded at 500 MHz. ^{13}C-NMR spectra were recorded at 125 MHz on JEOL NMR

spectrometers and standardized to the NMR solvent signal as reported by Gottlieb.[4] Multiplicities are given as s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, ddd = doublet of triplets, br = broad signal, m = multiplet using integration and coupling constant in Hertz. TLC analysis was performed using Silica Gel 60 F254 plates (Merck) and visualization was accomplished with ultraviolet light (λ = 254 nm) and/or the appropriate stain [phosphomolybdic acid, iodine, ninhydrin, and potassium permanganate]. Silica gel chromatography was carried out with SiliaFlash F60 230-400 mesh (Silicycle), according to the method of Still.[5] Mass spectral data were obtained using a LCMS-IT-TOF mass spectrometer (Shimadzu).

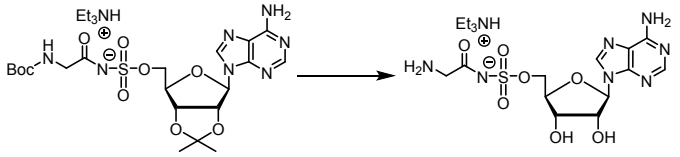
Chemical Synthesis of Gly-AMS 9

5′-*O*-[*N*-(*N*-Boc-glycyl)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2a)



Boc-Gly-OSu (53 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (90:10:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S2a** as a white solid (96 mg, 92%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.39 (s, 1H), 8.32 (s, 1H), 8.16 (s, 1H), 7.35 (br, 2H), 6.34 (t, J = 5.7 Hz), 6.16 (d, J = 2.9 Hz), 5.36 (dd, J = 6.3, 2.9 Hz, 1H), 5.01 (dd, J = 5.7 , 2.3 Hz, 1H), 4.39−4.36 (m, 1H), 3.99 (dddd, J = 22.9, 22.9, 22.9, 5.2 Hz, 2H), 3.40 (t, J = 4.9 Hz, 2H), 2.88 (q, J = 7.4 Hz, 12H, Et_3N-CH_2), 1.54 (s, 3H), 1.35 (s, 9H), 1.32 (s, 3H), 1.09 (t, J = 7.4 Hz, 18H, Et_3N-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.8, 156.1, 155.5, 152.8, 149.0, 139.5, 118.8, 113.1, 89.2, 83.8, 83.4, 81.6, 79.2, 77.5, 67.0, 45.6, 28.2, 27.0, 25.2, 9.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{20}H_{29}N_7O_9S$, 544.1820; found, 544.1792.

5′-*O*-(*N*-Glycyl)sulfamoyladenosine triethylammonium salt (9)

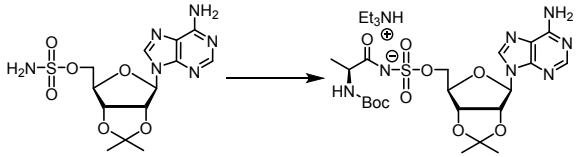


S2a **9**

Compound **S2a** (44 mg, 0.082 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 10 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (50:50:1 $CHCl_3$/MeOH/Et_3N) to afford compound **9** as a white solid (17 mg, 52%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.39 (s, 1H), 8.15 (s, 1H), 7.30 (br, 2H), 5.92 (d, J = 6.3 Hz, 1H), 4.60 (t, J = 5.5 Hz, 1H), 4.18−4.14 (m, 2H), 4.11−4.05 (m, 2H), 3.31 (s, 2H), 2.94 (q, J = 7.5 Hz, 1.3H, Et_3N-*CH*$_2$), 1.12 (t, J = 7.5 Hz, 2H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, DMSO-d_6) δ 169.8, 156.1, 152.7, 149.6, 139.4, 118.9, 87.0, 82.4, 73.5, 70.7, 67.6, 45.6, 42.7, 9.1. HRMS (ESI+): $[M+H]^+$ calcd for $C_{12}H_{18}N_7O_7S$, 404.0983; found, 404.0955.

Chemical Synthesis of L-Ala-AMS 10

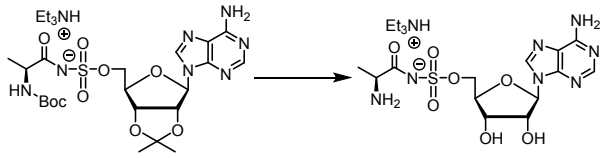
5′-*O*-[*N*-(*N*-Boc-*L*-alanyl)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2b)



S1 **S2b**

Boc-Ala-OSu (56 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S2b** as a white solid (39 mg, 53%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.46 (s, 1H), 8.22 (s, 1H), 6.23 (d, J = 3.4 Hz, 1H), 5.36 (q, J = 2.9 Hz, 1H), 5.15−5.08 (m, 1H), 4.55−4.50 (m, 1H), 4.26−4.18 (m, 2H), 4.03−3.90 (m, 1H), 3.18 (q, J = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 1.60 (s, 3H), 1.45−1.35 (m, 12H), 1.32−1.22 (m, 12H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 181.4, 157.4, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.8, 85.7, 85.6, 83.3, 80.0, 69.7, 53.7, 47.8, 28.8, 27.5, 25.6, 19.8, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{21}H_{30}N_7O_9S$, 556.1826; found, 556.1833.

5′-*O*-*N*-(*L*-Alanyl)sulfamoyladenosine triethylammonium salt (10)

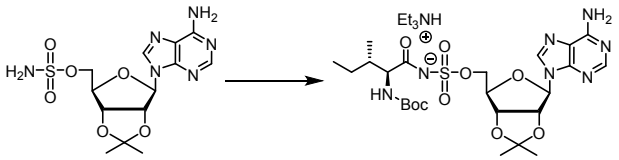


S2b 10

Compound **S2b** (40 mg, 0.061 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 $CHCl_3$/MeOH/Et_3N to 100:1 MeOH/Et_3N) to afford compound **10** as a white solid (21 mg, 77%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.14 (s, 1H), 7.27 (br, 2H), 5.91 (d, J = 5.7 Hz, 1H), 4.60 (t, J = 5.2 Hz, 1H), 4.18−4.12 (m, 2H), 4.11−4.03 (m, 2H), 3.48 (dd, J = 7.5, 6.9 Hz, 1H), 2.73 (q, J = 7.5 Hz, 2H, Et_3N-CH_2), 1.30 (d, J = 7.5 Hz, 1H), 1.03 (t, J = 7.5 Hz, 3H, Et_3N-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.2, 156.0, 152.7, 149.6, 139.4, 118.9, 87.0, 82.5, 73.4, 70.7, 67.6, 50.7, 45.7, 17.2, 10.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{13}H_{18}N_7O_7S$, 416.0988; found, 416.0983.

Chemical Synthesis of L-Ile-AMS 13

5′-*O*-[*N*-(*N*-Boc-*L*-isoleucyl)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2c)

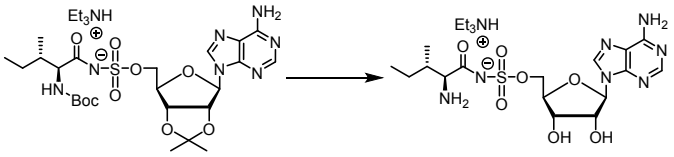


S1 S2c

Boc-Ile-OSu (64 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (90:10:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S2c** as a white solid (69 mg, 90%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.40 (s, 1H), 8.15 (s, 1H), 7.35 (br, 2H), 6.16 (d, J = 2.9 Hz, 1H), 5.84 (d, J = 8.0 Hz, 1H), 5.34 (dd, J = 5.8, 2.9 Hz, 1H), 5.00 (dd, J = 5.7, 1.7 Hz, 1H), 4.40−4.37 (m, 1H), 4.05−3.98 (m, 2H), 3.66 (dd, J = 8.6, 5.2 Hz, 1H), 2.81 (q, J = 7.5 Hz, 18H, Et_3N-CH_2), 1.75−1.66 (m, 1H), 1.54 (s, 3H), 1.35 (s, 9H), 1.31 (s, 3H), 1.06

(t, J = 7.5 Hz, 27H, Et_3N-CH_3), 0.83−0.76 (m, 8H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.8, 156.1, 155.1, 152.8, 149.0, 139.5, 118.9, 113.2, 89.2, 83.7, 83.5, 81.7, 77.5, 67.2, 60.5, 48.6, 39.2, 28.2, 27.1, 25.2, 24.3, 15.7, 11.7, 9.8. HRMS (ESI+): $[M+H]^+$ calcd for $C_{24}H_{38}N_7O_9S$, 600.2446; found, 600.2417.

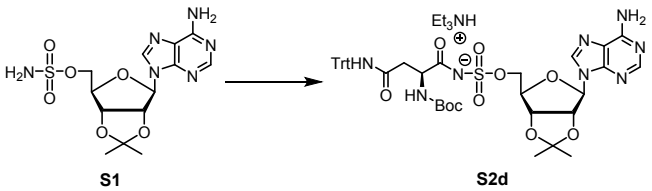
5′-*O*-*N*-(*L*-Isoleucyl)sulfamoyladenosine triethylammonium salt (13)



Compound **S2c** (30 mg, 0.050 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 4 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (50:50:1 $CHCl_3$/MeOH/Et_3N) to afford compound **13** as a colorless oil (16 mg, 70%). ^1H NMR (500 MHz, CD_3OD) δ 8.55 (s, 1H), 8.21 (s, 1H), 6.09 (d, J = 5.2 Hz, 1H), 4.64 (t, J = 5.2 Hz, 1H), 4.41−4.28 (m, 3H), 3.62−3.55 (m, 2H), 3.18 (q, J = 7.5 Hz, 36H, Et_3N-CH_2), 1.64−1.55 (m, 1H), 1.32 (t, J = 7.5 Hz, 54H, Et_3N-CH_3), 1.04−0.92 (m, 8H). ^{13}C NMR (125 MHz, CD_3OD) δ 175.0, 173.1, 150.6, 141.6, 137.6, 120.1, 89.5, 84.3, 79.5, 76.2, 69.0, 61.3, 47.4, 38.1, 25.7, 15.5, 12.2, 9.1. HRMS (ESI+): $[M+H]^+$ calcd for $C_{16}H_{26}N_7O_7S$, 460.1614; found, 460.1604.

Chemical Synthesis of L-Asn-AMS 14

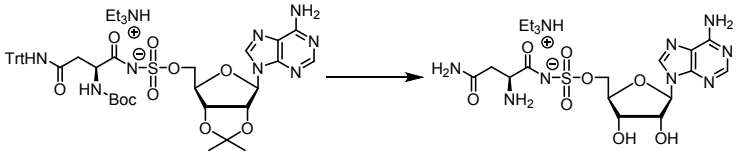
5′-*O*-[*N*-(*N*-Boc-*L*-asparaginyl(Trt))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2d)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (182 mg, 0.95 mmol) and *N*-hydroxysuccinimide (173 mg, 1.56 mmol) were added to a solution of Boc-Asn(Trt)-OH (300 mg, 0.63 mmol) in DMF (6 mL). After 3 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. Boc-Asn(Trt)-OSu (155 mg, 0.27 mmol) and cesium

carbonate (176 mg, 0.54 mmol) were added to a solution of 5′-O-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (70 mg, 0.18 mmol) in DMF (2 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 90:10:1 EtOAc/MeOH/Et_3N) to afford compound **S2d** as a white solid (150 mg, 88%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.42 (s, 1H), 8.21 (s, 1H), 7.27−7.14 (m, 15H), 6.22 (d, *J* = 2.9 Hz, 1H), 5.32−5.27 (m, 1H), 5.09−5.05 (m, 1H), 4.52−4.48 (m, 1H), 4.35−4.28 (m, 1H), 4.23−4.16 (m, 2H), 3.12 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 2.90−2.81 (m, 1H), 2.70−2.62 (m, 1H), 1.59 (s, 3H), 1.41 (s, 9H), 1.33 (s, 3H), 1.22 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 179.4, 172.2, 157.6, 157.3, 154.0, 150.5,146.0, 141.4, 130.1, 128.7, 127.7, 120.1, 115.2, 91.8, 85.7, 85.6, 83.2, 80.3, 71.6, 69.6, 55.7, 47.8, 41.3, 28.8, 27.5, 25.6, 9.2. HRMS (ESI$^-$): $[M-H]^-$ calcd for $C_{41}H_{45}N_8O_{10}S$, 841.2979; found, 841.2958.

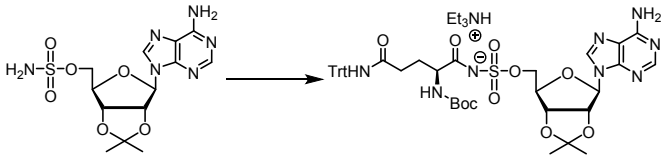
5′-*O*-*N*-(*L*-Asparaginyl)sulfamoyladenosine triethylammonium salt (14)



Compound **S2d** (100 mg, 0.12 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 2 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (90:10:1 to 85:15:1 $CHCl_3$/MeOH/Et_3N) to afford compound **14** as a white solid (39 mg, 66%). ^{1}H NMR (500 MHz, DMSO-*d*$_6$) δ 8.34 (s, 1H), 8.14 (s, 1H), 7.60 (br, 1H), 7.29 (br, 2H), 7.13 (br, 1H), 5.91 (d, *J* = 5.7 Hz, 1H), 4.58 (t, *J* = 5.2 Hz, 1H), 4.19–4.04 (m, 4H), 3.70 (dd, *J* = 9.2, 3.4 Hz, 1H), 2.81–2.72 (m, 1H, overlapping with Et_3N-*CH*$_2$), 2.45 (dd, *J* = 16.6, 9.2 Hz, 1H), 1.04 (t, *J* = 6.9 Hz, 1.5H, Et_3N-*CH*$_3$). ^{13}C NMR (500 MHz, DMSO-*d*$_6$) δ 171.9, 156.0, 152.7, 149.6, 139.4, 118.9, 87.0, 82.4, 73.6, 70.7, 67.5, 51.8, 45.7, 35.5, 10.0. HRMS (ESI−): $[M-H]^-$ calcd for $C_{14}H_{19}N_8O_8S$, 459.1052; found, 459.0755.

Chemical Synthesis of L-Gln-AMS 15

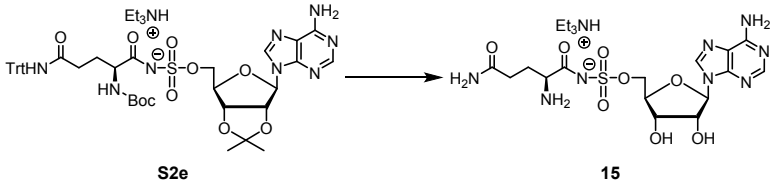
5′-*O*-[*N*-(*N*-Boc-*L*-glutaminyl(Trt))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2e)



S1 S2e

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (240 mg, 1.20 mmol) and *N*-hydroxysuccinimide (140 mg, 1.20 mmol) were added to a solution of Boc-Gln(Trt)-OH (400 mg, 0.82 mmol) in CH_2Cl_2 (5 mL). The solution was stirred at room temperature for 5 h. The reaction mixture was evaporated to dryness. The residue was purified by flash chromatography (1:4 to 2:1 EtOAc/hexane) to afford Boc-Gln(Trt)-OSu as a white solid (410 mg, 86%). Boc-Gln(Trt)-OSu (110 mg, 0.19 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 90:10:1 EtOAc/MeOH/Et_3N) to afford compound **S2e** as a colorless oil (99 mg, 77%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.48 (s, 1H), 8.21 (s, 1H), 7.30−7.15 (m, 15H), 6.21 (d, *J* = 3.5 Hz, 1H), 5.32 (dd, *J* = 6.0, 3.5 Hz, 1H), 5.09 (dd, *J* = 6.0, 1.5 Hz, 1H), 4.50 (br, 1H), 4.23 (d, *J* = 4.0 Hz, 2H), 4.05 (dd, *J* = 6.0, 5.0 Hz, 1H), 3.06 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 2.41−2.24 (m, 2H), 2.10−1.94 (m, 1H), 1.94−1.79 (m, 1H), 1.61 (s, 3H), 1.41 (s, 9H), 1.36 (s. 3H), 1.20 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 180.0, 174.7, 157.6, 157.3, 154.0, 150.5, 146.0, 141.4, 130.0, 128.7, 127.7, 120.1, 115.2, 91.6, 85.6, 85.4, 83.1, 80.1, 71.5, 69.7, 57.5, 47.7, 34.3, 30.8, 28.8, 27.5, 25.6, 9.4. HRMS (ESI−): $[M-H]^-$ calcd for $C_{42}H_{47}N_8O_{10}S$, 855.3141; found, 855.3157.

5′-*O*-*N*-(*L*-Glutaminyl)sulfamoyladenosine triethylammonium salt (15)



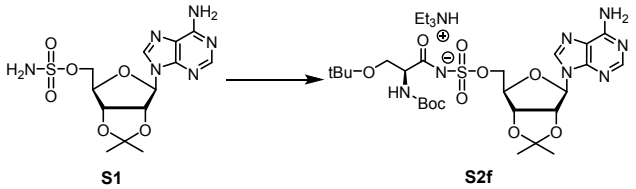
S2e 15

Compound **S2e** (35 mg, 0.035 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 30 min, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 $CHCl_3$/MeOH/Et_3N to MeOH) to afford compound **15** as a white solid (7.5 mg, 40%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.51 (s, 1H), 8.20 (s, 1H), 6.09 (d, *J* = 5.0 Hz, 1H), 4.63 (t, *J* =

5.0 Hz, 1H), 4.41−4.27 (m, 4H), 3.68 (t, *J* = 6.0 Hz, 1H), 3.14 (q, *J* = 7.5 Hz, 6H, Et_3N-CH_2), 2.47 (t, *J* = 7.0 Hz, 2H), 2.18−2.03 (m, 2H), 1.29 (t, *J* = 7.5 Hz, 9H, Et_3N-CH_3). ^{13}C NMR (500 MHz, CD_3OD) δ 181.5, 180.5, 157.3, 153.9, 150.8, 141.1, 120.2, 89.3, 84.4, 76.1, 72.2, 69.0, 60.6, 47.9, 30.9, 26.9, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{15}H_{21}N_8O_8S$, 473.1209; found, 473.1208.

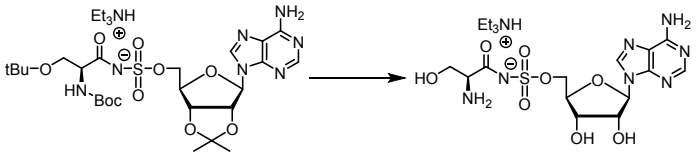
Chemical Synthesis of L-Ser-AMS 16

5′-*O*-[*N*-(*N*-Boc-*L*-seryl(tBu))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2f)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (160 mg, 0.81 mmol), *N*-hydroxysuccinimide (94 mg, 0.81 mmol), DIEA (0.35 mL, 2.0 mmol), and DMAP (8.3 mg, 0.068 mmol) were added to a solution of Boc-Ser(tBu)-OH·DCHA (300 mg, 0.68 mmol) in CH_2Cl_2 (5 mL). The solution was stirred at room temperature for 3 h. The reaction mixture was washed with a 0.1 M HCl solution, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (1:9 to 1:2 EtOAc/hexane) to afford Boc-Ser(tBu)-OSu as a white solid (147 mg, 60%). Boc-Ser(tBu)-OSu (70 mg, 0.19 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 80:20:1 EtOAc/MeOH/Et_3N) to afford compound **S2f** as a colorless oil (61 mg, 65%). ^1H NMR (500 MHz, CD_3OD): δ 8.49 (s, 1H), 8.22 (s, 1H), 6.24, (d, *J* = 3.5 Hz, 1H), 5.37 (dd, *J* = 5.5, 3.5 Hz, 1H), 5.12 (d, *J* = 4.5 Hz 1H), 4.54 (dd, *J* = 6.0, 4.0 Hz, 1H), 4.27−4.19 (m, 2H), 4.07 (t, *J* = 3.5 Hz, 1H), 3.76−3.68 (m, 1H), 3.63 (dd, *J* = 9.5, 3.5 Hz, 1H), 3.18 (q, *J* = 7.5 Hz, 6H, Et_3N-CH_2), 1.61 (s, 3H), 1.42 (s, 9H), 1.39 (s, 3H), 1.28 (t, *J* = 7.5 Hz, 9H, Et_3N-CH_3), 1.12 (s, 9H). ^{13}C NMR (125 MHz, CD_3OD): δ 178.5, 157.5, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.9, 85.7, 85.5, 83.4, 80.2, 74.3, 69.7, 64.2, 58.6, 47.8, 28.8, 27.8, 27.5, 25.6, 9.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_{25}H_{40}N_7O_{10}S$, 630.2552; found, 630.2552.

5′-*O*-*N*-(*L*-Seryl)sulfamoyladenosine triethylammonium salt (16)

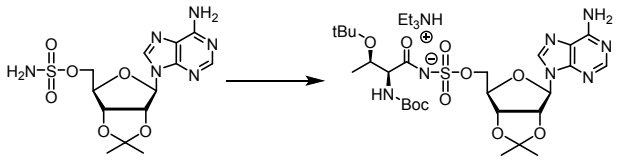


S2f 16

Compound **S2f** (27.4 mg, 0.038 mmol) was dissolved in a 90:5:5 (v/v) mixture of TFA, H_2O, and TIS at room temperature. After 30 min, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (80:20:1 to 50:50:1 $CHCl_3$/MeOH/Et_3N) to afford compound **16** as a white solid (4.7 mg, 23%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.50 (s, 1H), 8.21 (s, 1H), 6.09 (d, *J* = 5.0 Hz, 1H), 4.62 (t, *J* = 5.0 Hz, 1H), 4.43−4.26 (m, 3H), 3.97 (dd, *J* = 11.5, 4.0 Hz, 1H), 3.93−3.78 (m, 2H), 3.75−3.66 (m, 1H), 3.19 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 1.30 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (500 MHz, DMSO-*d*$_6$) δ 170.5, 156.0, 152.7, 149.6, 139.4, 118.9, 87.0, 82.5, 73.5, 70.7, 67.5, 60.8, 57.4, 45.5, 9.9. HRMS (ESI+): $[M+H]^+$ calcd for $C_{13}H_{20}N_7O_8S$, 434.1089; found, 434.1086.

Chemical Synthesis of L-Thr-AMS 17

5′-*O*-[*N*-(*N*-Boc-*L*-threonyl(tBu))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2g)

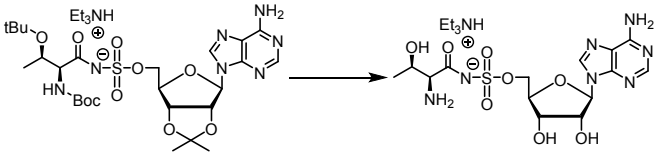


S1 S2f

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (170 mg, 0.87 mmol), *N*-hydroxysuccinimide (100 mg, 0.87 mmol), DIEA (0.38 mL, 2.2 mmol), and DMAP (8.9 mg, 0.073 mmol) were added to a solution of Boc-Thr(tBu)-OH (200 mg, 0.73 mmol) in CH_2Cl_2 (5 mL). The solution was stirred at room temperature for 2 h. The reaction mixture was washed with a 0.1 M HCl solution, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (1:5 to 1:2 EtOAc/hexane) to afford Boc-thr(tBu)-OSu as a white solid (220 mg, 82%). Boc-Thr(tBu)-OSu (72 mg, 0.19 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The

solution was stirred at room temperature for 1 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 80:20:1 EtOAc/MeOH/Et_3N) to afford compound **S2g** as a white solid (62 mg, 63%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.49 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 3.5 Hz, 1H), 5.37 (dd, *J* = 6.0, 3.5 Hz, 1H), 5.10 (dd, *J* = 6.0, 2.0 Hz, 1H), 4.53 (dd, *J* = 6.0, 3.5 Hz, 1H), 4.26 (dd, *J* = 11.0, 4.0 Hz, 1H), 4.22 (dd, *J* = 11.0, 3.0 Hz, 1H), 4.18 (dd, *J* = 6.0, 2.5 Hz, 1H), 3.88 (dd, *J* = 7.5, 2.5 Hz, 1H), 3.11 (q, *J* = 7.5 Hz, 6H, Et_3N-CH_2), 1.61 (s, 3H), 1.42 (s, 9H), 1.38 (s, 3H), 1.25 (t, *J* = 7.5 Hz, 9H, Et_3N-CH_3), 1.16 (s, 9H). ^{13}C NMR (125 MHz, CD_3OD): δ 179.2, 158.1, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.9, 85.7, 85.6, 83.4, 80.2, 74.9, 69.7, 63.4, 47.8, 29.0, 28.8, 27.5, 25.7, 21.9, 9.4. HRMS (ESI+): $[M+H]^+$ calcd for $C_{26}H_{40}N_7O_{10}S$, 642.2563; found, 642.2561.

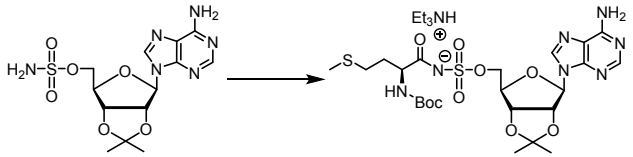
5′-*O*-*N*-(*L*-Threonyl)sulfamoyladenosine triethylammonium salt (17)



Compound **S2g** (41 mg, 0.054 mmol) was dissolved in a 90:5:5 (v/v) mixture of TFA, H_2O, and TIS at room temperature. After 30 min, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (90:10:1 to 60:40:1 $CHCl_3$/MeOH/Et_3N) to afford compound **17** as a white solid (13 mg, 49%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.51 (s, 1H), 8.19 (s, 1H), 6.08 (d, *J* = 4.5 Hz, 1H), 4.61 (t, *J* = 4.5 Hz, 1H), 4.45−4.27 (m, 4H), 4.19 (t, *J* = 6.0 Hz, 1H), 3.45 (d, *J* = 4.5 Hz, 1H), 3.13 (q, *J* =7.5 Hz, 6H, Et_3N-CH_2), 1.31 (d, *J* = 6.5 Hz, 3H), 1.28 (t, *J* = 7.5 Hz, 9H, Et_3N-CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ 174.6, 157.2, 153.9, 150.7, 141.2, 120.1, 89.5, 84.2, 76.2, 71.9, 68.9, 67.6, 62.9, 47.8, 21.0, 9.4. HRMS (ESI+): $[M+H]^+$ calcd for $C_{14}H_{22}N_7O_8S$, 448.1245; found, 448.1220.

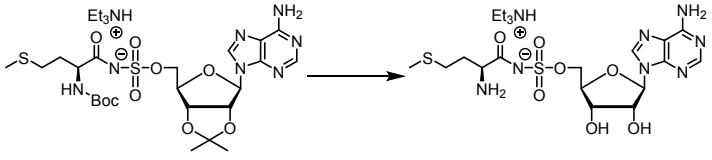
Chemical Synthesis of L-Met-AMS 18

5′-*O*-[*N*-(*N*-Boc-*L*-methionyl)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2h)



Boc-Met-OSu (68 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (94:6:1 EtOAc/MeOH/Et_3N) to afford compound **S2h** as a white solid (80 mg, 75%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 2.9 Hz, 1H), 5.38−5.33 (m, 1H), 5.14−5.09 (m, 1H), 4.56−4.52 (m, 1H), 4.27−4.22 (m, 2H), 4.11−4.00 (m, 1H), 3.19 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 2.53−2.45 (m, 2H), 2.08−2.00 (m, 4H), 1.90−1.80 (m, 1H), 1.61 (s, 3H), 1.44 (s, 9H), 1.39 (s, 3H), 1.29 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 180.2, 157.6, 157.3, 154.0, 150.5, 141.4, 120.1, 115.3, 91.8, 85.7, 85.6, 83.3, 80.1, 69.7, 57.4, 47.9, 34.3, 31.2, 28.8, 27.5, 25.6, 15.3, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{23}H_{34}N_7O_9S_2$, 616.1859; found, 616.1861.

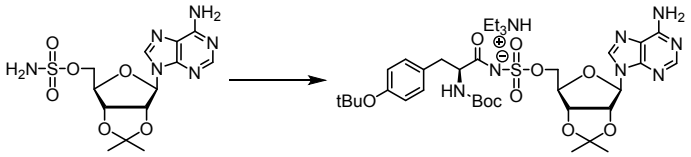
5′-*O*-*N*-(*L*-Methionyl)sulfamoyladenosine triethylammonium salt (18)



Compound **S2h** (40 mg, 0.061 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (75:25:1 $CHCl_3$/MeOH/Et_3N) to afford compound **18** as a white solid (12 mg, 57%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.14 (s, 1H), 7.26 (br, 2H), 5.90 (d, *J* = 5.7 Hz, 1H), 4.59 (t, *J* = 5.2 Hz, 1H), 4.28−4.23 (m, 2H), 4.11−4.04 (m, 2H), 5.52 (dd, *J* = 6.9, 5.2 Hz, 1H), 2.64 (q, *J* = 7.5 Hz, 1.5H, Et_3N-*CH*$_2$), 2.58−2.52 (m, 1H), 2.05−1.98 (m, 4H), 1.91−1.82 (m, 1H), 1.10 (t, *J* = 7.5 Hz, 2.3H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.2, 156.0, 152.7, 149.5, 139.4, 118.9, 87.1, 82.4, 73.4, 82.4, 73.4, 70.7, 67.6, 53.8, 45.7, 30.9, 29.0, 14.3, 10.6. HRMS (ESI−): $[M-H]^-$ calcd for $C_{15}H_{22}N_7O_7S_2$, 476.1022; found, 476.1027.

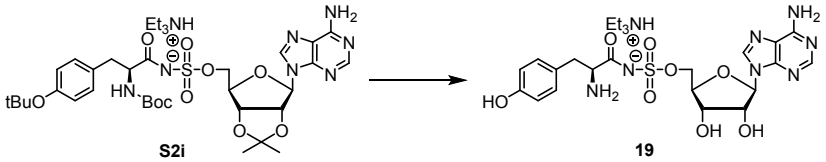
Chemical Synthesis of L-Tyr-AMS 19

5′-*O*-[*N*-(*N*-Boc-L-tyrosyl(tBu))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2i)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (127 mg, 0.66 mmol) and *N*-hydroxysuccinimide (76 mg, 0.66 mmol) were added to a solution of Boc-Tyr(tBu)-OH (100 mg, 0.30 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness to afford Boc-Tyr(tBu)-OSu as a colorless oil (120 mg, 92%). Boc-Tyr(tBu)-OSu (120 mg, 0.28 mmol) and cesium carbonate (293 mg, 0.90 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (58 mg, 0.15 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (90:10:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S2i** as a colorless oil (104 mg, 98%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.46 (s, 1H), 8.22 (s, 1H), 7.13 (d, *J* = 8.0 Hz, 2H), 6.87 (d, *J* = 8.0 Hz, 2H), 6.24 (d, *J* = 2.8 Hz, 1H), 5.36 (dd, *J* = 5.7, 2.9 Hz, 1H), 5.11 (dddd, *J* = 7.3, 7,3, 7.3, 1.8 Hz, 1H), 4.57–4.49 (m, 1H), 4.24–4.15 (m, 2H), 3.45–3.32 (m, 1H), 3.16 (q, *J* = 7.5 Hz, 18H, Et_3N-*CH*$_2$), 2.89–2.70 (m, 2H), 1.61 (s, 3H), 1.38 (s, 9H), 1.35 (s, 3H), 1.30 (s, 9H), 1.27 (t, *J* = 7.5, 27H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 178.7, 157.3, 154.8, 154.0, 150.5, 141.4, 134.9, 134.4, 131.0, 124.9, 120.1, 115.2, 91.8, 85.7, 83.2, 79.9, 79.3, 69.7, 58.3, 47.6, 39.2, 29.2, 28.8, 27.5, 25.6, 9.16. HRMS (ESI–): $[M–H]^-$ calcd for $C_{31}H_{42}N_7O_{10}S$, 704.2714; found, 704.2722.

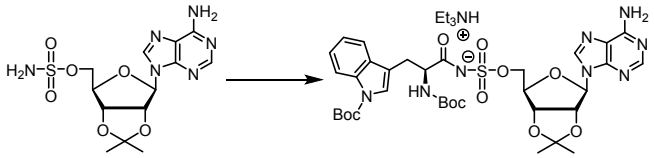
5′-*O*-(*N*-Tyrosyl)sulfamoyladenosine triethylammonium salt (19)



Compound **S2i** (58 mg, 0.082 mmol) was dissolved in a mixture of 90:5:5 (v/v) of TFA, H_2O, and TIS at room temperature. After 8 h, the flask was placed on the rotary evaporator, and the TFA and H_2O were removed at reduced pressure. The residue was purified by HPLC [COSMISIL $5C_{18}$-PAQ: C-18 reverse-phase column, ϕ 10 mm × 250 mm, acetonitrile/aqueous TFA (0.1%, 10:90), 3.0 mL/min, 220 nm, t_R: 12.5 min] to afford compound **19** as a yellow oil (31 mg, 74%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.60 (s, 1H), 8.39 (s, 1H), 7.09 (d, *J* = 8.0 Hz, 2H), 6.70 (d, *J* = 8.0 Hz, 2H), 6.14 (d, *J* = 4.6 Hz, 1H), 4.63 (t, *J* = 4.9 Hz, 1H), 4.44–4.35 (m, 3H), 4.34–4.29 (m, 1H), 3.94 (t, *J* = 6.3 Hz, 1H), 3.25–3.16 (m, 1H, overlapping with Et_3N-*CH*$_2$), 3.08–2.96 (m, 1H). NMR data were in agreement with published data.[6] HRMS (ESI–): $[M–H]^-$ calcd for $C_{19}H_{22}N_7O_8S$, 508.1251; found, 508.1280.

Chemical Synthesis of L-Trp-AMS 20

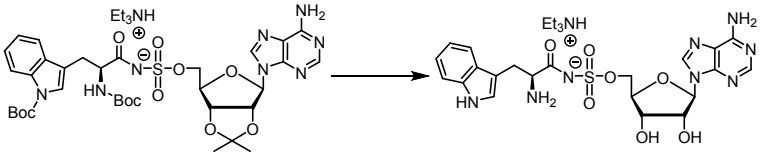
5′-*O*-[*N*-(*N*-Boc-*L*-tryptophanyl(Boc))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2j)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (230 mg, 1.2 mmol) and *N*-hydroxysuccinimide (138 mg, 1.2 mmol) were added to a solution of Boc-Trp(Boc)-OH (405 mg, 1.0 mmol) in DMF (10 mL). After 6 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. Boc-Trp(Boc)-OSu (98 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 91:9:1 EtOAc/MeOH/Et_3N) to afford compound **S2j** as a white solid (65 mg, 57%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.45 (s, 1H), 8.20 (s, 1H), 8.09−8.00 (m, 1H), 7.72−7.59 (m, 1H), 7.52−7.43 (m, 1H), 7.28−7.13 (m, 2H), 6.21 (d, *J* = 3.4 Hz, 1H), 5.36−5.27 (m, 1H), 5.12−5.00 (m, 1H), 4.55−4.45 (m, 1H), 4.37−4.11 (m, 3H), 3.28−3.21 (m, 1H), 3.12 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 3.04−2.95 (m, 1H), 1.64 (s, 9H), 1.59 (s, 3H), 1.40−1.29 (m, 12H), 1.23 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 179.6, 157.3, 154.0, 151.1, 150.5, 141.4, 136.7, 132.4, 125.2, 125.1, 123.5, 120.5, 120.1, 118.1, 115.9, 115.2, 91.8, 85.7, 85.5,

84.5, 83.2, 80.1, 69.7, 58.4, 47.8, 29.6, 28.7, 28.4, 28.2, 27.5, 25.5, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{34}H_{43}N_8O_{11}S$, 771.2772; found, 771.2781.

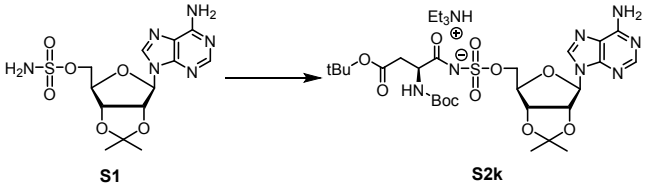
5′-*O*-*N*-(*L*-Tryptophanyl)sulfamoyladenosine triethylammonium salt (20)



Compound **S2j** (40 mg, 0.046 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 $CHCl_3$/MeOH/Et_3N) to afford compound **20** as a white solid (25 mg, 90%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 10.9 (br, 1H), 8.40 (s, 1H), 8.13 (s, 1H), 7.56 (d, J = 8.0 Hz, 1H), 7.34 (d, J = 8.6 Hz, 1H), 7.26 (br, 2H), 7.21 (d, J = 2.3 Hz, 1H), 7.07 (dd, J = 8.0, 6.9 Hz, 1H), 6.98 (t, J = 7.5 Hz, 1H), 5.92 (d, J = 5.7 Hz, 1H), 4.64−4.59 (m, 1H), 4.20−4.15 (m, 2H), 4.14−4.05 (m, 2H), 3.67 (dd, J = 8.6, 4.6 Hz, 1H), 3.32 (dd, J = 14.9, 4.0 Hz, 1H), 3.03 (dd, J = 14.9, 8.6 Hz, 1H), 2.57 (q, J = 7.5 Hz, 3H, Et_3N-CH_2), 0.97 (d, J = 7.5 Hz, 4.5H, Et_3N-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 172.7, 156.0, 152.7, 149.6, 139.4, 136.3, 127.2, 124.5, 121.0, 118.9, 118.5, 118.3, 111.4, 108.2, 87.1, 82.5, 79.2, 73.5, 70.7, 67.5, 55.7, 45.7, 27.5, 10.9. HRMS (ESI−): $[M-H]^-$ calcd for $C_{21}H_{23}N_8O_7S$, 531.1410; found, 531.1413.

Chemical Synthesis of L-Asp-AMS 21

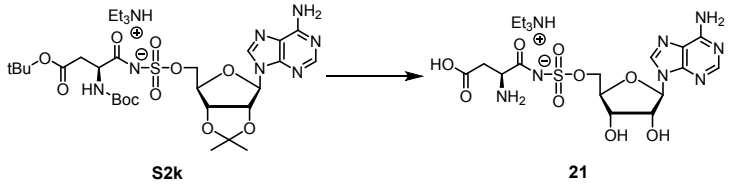
5′-*O*-[*N*-(*N*-Boc-*L*-aspartyl (tBu))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2k)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (298 mg, 1.56 mmol) and *N*-hydroxysuccinimide (180 mg, 1.56 mmol) were added to a solution of Boc-Asp(OtBu)-OH (300 mg, 1.04 mmol) in DMF (10 mL). After 22 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried

over Na_2SO_4 and evaporated to dryness. Boc-Asp(OtBu)-OSu (75 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The solution was stirred at room temperature for 3 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (94:6:1 EtOAc/MeOH/Et_3N) to afford compound **S2k** as a white solid (50 mg, 66%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.46 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 2.9 Hz, 1H), 5.38−5.34 (m, 1H), 5.14−5.10 (m, 1H), 4.55−4.52 (m, 1H), 4.33−4.28 (m, 1H), 4.25−4.20 (m, 2H), 3.18 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 2.75 (dd, *J* = 15.5, 5.2 Hz, 1H), 2.56 (dd, *J* = 15.5, 7.5 Hz, 1H), 1.61 (s, 3H), 1.44−1.37 (m, 21H), 1.28 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 178.9, 172.5, 157.4, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.9, 85.7, 85.6, 83.3, 81.9, 80.2, 69.6, 55.0, 47.8, 40.2, 28.8, 28.4, 27.5, 25.6, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{26}H_{38}N_7O_{11}S$, 656.2350; found, 656.2346.

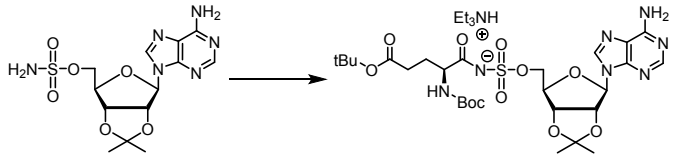
5′-*O*-*N*-(*L*-Aspartyl)sulfamoyladenosine triethylammonium salt (21)



Compound **S2k** (40 mg, 0.053 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 $CHCl_3$/MeOH/Et_3N) to afford compound **21** as a white solid (26 mg, 83%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.39 (s, 1H), 8.14 (s, 1H), 7.27 (br, 2H), 5.90 (d, *J* = 6.3 Hz, 1H), 4.60−4.55 (m, 1H), 4.18−4.00 (m, 4H), 3.58−3.54 (m, 1H), 2.86 (q, *J* = 7.5 Hz, 8H, Et_3N-*CH*$_2$), 2.55 (dd, *J* = 16.6, 4.0 Hz, 1H), 2.36 (dd, *J* = 16.6, 8.6 Hz, 1H), 1.08 (t, *J* = 7.5 Hz, 12H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, DMSO-d_6) δ 173.5, 172.3, 156.0, 152.7, 149.6, 139.4, 118.8, 87.0, 82.4, 73.5, 70.6, 67.5, 52.7, 45.4, 36.3, 9.4. HRMS (ESI−): $[M-H]^-$ calcd for $C_{14}H_{18}N_7O_9S$, 460.0887; found, 460.0882.

Chemical Synthesis of L-Glu-AMS 22

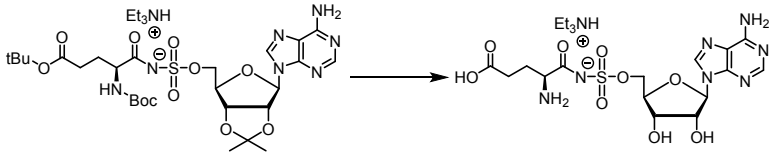
5′-*O*-[*N*-(*N*-Boc-*L*-glutamyl(tBu))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2l)



S1 S2l

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (380 mg, 1.98 mmol) and *N*-hydroxysuccinimide (228 mg, 1.98 mmol) were added to a solution of Boc-Glu(OtBu)-OH (500 mg, 1.65 mmol) in DMF (16 mL). After 24 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. Boc-Glu(OtBu)-OSu (78 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The solution was stirred at room temperature for 3 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 91:9:1 EtOAc/MeOH/Et_3N) to afford compound **S2l** as a white solid (76 mg, 76%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.46 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 2.9 Hz, 1H), 5.37−5.33 (m, 1H), 5.12−5.08 (m, 1H), 4.56−4.51 (m, 1H), 4.27−4.21 (m, 2H), 4.04−3.97 (m, 1H), 3.18 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 2.34−3.22 (m, 2H), 2.11−2.01 (m, 1H), 1.89−1.80 (m, 1H), 1.61 (s, 3H), 1.46−1.35 (m, 21H), 1.28 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 180.0, 174.3, 157.5, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.8, 85.7, 85.5, 83.3, 81.4, 80.1, 69.7, 57.4, 47.8, 32.8, 29.8, 28.8, 28.3, 27.5, 25.6, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{27}H_{40}N_7O_{11}S$, 670.2507; found, 670.2509.

5′-*O*-*N*-(*L*-Glutamyl)sulfamoyladenosine triethylammonium salt (22)



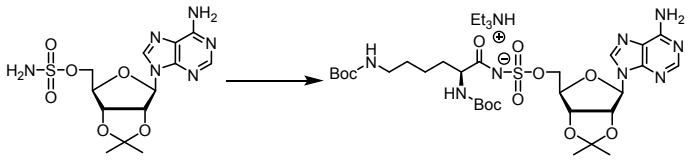
S2l 22

Compound **S2l** (40 mg, 0.052 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 $CHCl_3$/MeOH/Et_3N to 100:1 MeOH/Et_3N) to afford compound **22** as a white solid (32 mg, 98%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.14 (s, 1H), 7.26 (br, 2H), 5.90 (d, *J* = 5.7 Hz, 1H), 4.60−4.57 (m, 1H), 4.17−4.04 (m, 4H), 3.44 (dd, *J* = 6.8, 4.6 Hz, 1H), 2.70 (q, *J*

= 7.5 Hz, 9H, Et_3N-CH_2), 2.39−2.21 (m, 2H), 2.00−1.91 (m, 1H), 1.85−1.77 (m, 1H), 1.02 (d, *J* = 7.5 Hz, 15H, Et_3N-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 175.2, 172.6, 156.0, 152.6, 149.6, 139.3, 118.9, 87.0, 82.5, 73.5, 70.7, 67.5, 54.7, 45.5, 32.1, 27.1, 10.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{15}H_{20}N_7O_9S$, 474.1043.; found, 474.1045.

Chemical Synthesis of L-Lys-AMS 23

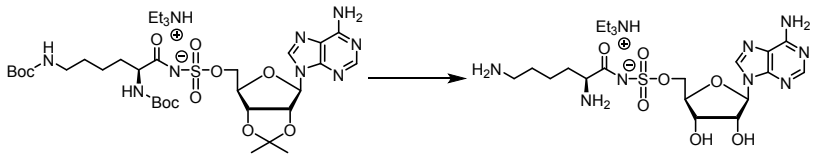
5′-*O*-[*N*-(*N*-Boc-*L*-lysyl(ε-Boc))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2m)



S1 S2m

Boc-Lys(Boc)-OSu (127 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (90:10:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S2m** as a white solid (106 mg, 98%). ^1H NMR (500 MHz, CD_3OD) δ 8.51 (s, 1H), 8.21 (s, 1H), 6.23 (d, *J* = 3.4 Hz, 1H), 5.34 (dd, *J* = 5.7, 3.4 Hz, 1H), 5.10 (d, *J* = 4.6 Hz, 1H), 4.56 (dd, *J* = 5.2, 3.5 Hz, 1H), 4.27−4.20 (m, 2H), 4.01−3.95 (m, 1H), 3.14 (q, *J* = 7.5 Hz, 12H, Et_3N-CH_2), 3.04−2.99 (m, 2H), 1.82−1.72 (m, 2H), 1.61 (s, 3H), 1.49−1.42 (m, 22H), 1.39 (s, 3H), 1.28 (t, *J* = 7.5 Hz, 18H, Et_3N-CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 180.9, 158.4, 157.6, 157.6, 157.3, 154.0, 150.4, 120.1, 115.2, 91.7, 85.7, 85.6, 83.2, 80.0, 79.7, 69.7, 58.0, 47.7, 41.1, 34.3, 34.2, 34.1, 30.6, 28.8, 27.5, 25.6, 24.1, 9.32. HRMS (ESI+): $[M+H]^+$ calcd for $C_{29}H_{47}N_8O_{11}S$, 715.3080; found, 715.3039.

5′-*O*-(*N*-*L*-lysyl)sulfamoyladenosine triethylammonium salt (23)



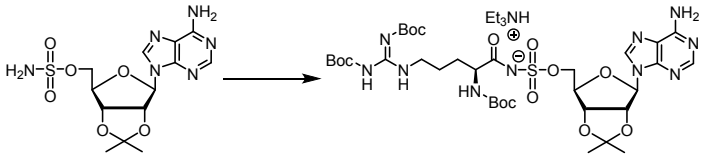
S2m 23

Compound **S2m** (50 mg, 0.070 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at

room temperature. After 10 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by HPLC [COSMISIL $5C_{18}$-PAQ: C-18 reverse-phase column, ϕ 10 mm × 250 mm, aqueous TFA (0.01%), 3.0 mL/min, 220 nm, t_R: 9.5 min] to afford compound **23** as a colorless oil (20 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.63 (s, 1H), 8.41 (s, 1H), 6.14 (d, *J* = 4.6 Hz, 1H), 4.64 (t, *J* = 4.9 Hz, 1H), 4.47−4.39 (m, 3H), 4.33 (dd, *J* = 7.5, 2.9 Hz, 1H), 3.78 (t, *J* = 5.7 Hz, 1H), 3.21 (q, *J* = 7.5 Hz, 3H, Et_3N-*CH*$_2$), 2.94 (t, *J* = 7.8 Hz, 2H), 2.00−1.86 (m, 2H), 1.74−1.68 (m, 2H), 1.58−1.49 (m, 2H), 1.31 (t, *J* = 7.5 Hz, 4.5H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD) δ 174.1, 152.3, 150.1, 146.2, 143.6, 120.2, 90.2, 84.2, 76.2, 71.8, 70.0, 56.0, 47.8, 40.3, 31.7, 28.0, 22.8, 9.1. HRMS (ESI+): $[M+H]^+$ calcd for $C_{14}H_{22}N_8O_7S$, 475.1718; found, 475.1691.

Chemical Synthesis of L-Arg-AMS 24

5′-*O*-[*N*-(*N*-Boc-*L*-arginyl (Boc)$_2$)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2n)

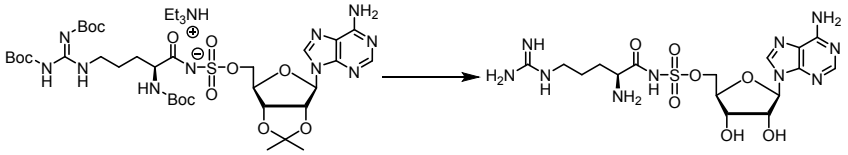


S1 **S2n**

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (150 mg, 0.76 mmol) and *N*-hydroxysuccinimide (87 mg, 0.76 mmol) were added to a solution of Boc-Arg(Boc)$_2$-OH (300 mg, 0.63 mmol) in THF (6 mL) at room temperature. After 15 h, the solvent was evaporated in vacuo. The residue was purified by flash chromatography (9:1 to 3:1 EtOAc/hexane) to afford Boc-Arg(Boc)$_2$-OSu as a white solid (250 mg, 68%). Boc-Arg(Boc)$_2$-OSu (110 mg, 0.19 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (2 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 90:10:1 EtOAc/MeOH/Et_3N) to afford compound **S2n** as a white solid (99 mg, 78%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.48 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 3.0 Hz, 1H), 5.33 (dd, *J* = 6.0, 3.0 Hz, 1H), 5.09 (dd, *J* = 5.0, 3.0 Hz, 1H), 4.52 (dd, *J* = 6.0, 3.5 Hz, 1H), 4.23 (d, *J* = 3.5 Hz, 2H), 4.04−3.94 (m, 1H), 3.94−3.76 (m, 3H), 3.17 (q, *J* = 7.5 Hz, 6 H, Et_3N-*CH*$_2$), 1.78 (br, 1H), 1.63 (br, 3H), 1.61 (s, 3H), 1.54 (s, 3H), 1.51 (s, 9H), 1.46 (s, 9H), 1.44−1.36 (m, 9H), 1.27 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD): δ 180.4, 157.5, 157.3, 156.14, 156.11, 154.0, 150.5, 141.4, 120.1, 115.3, 91.7, 85.8, 85.5, 85.1, 85.0, 83.2, 80.0, 79.9, 69.7, 57.9, 47.7, 45.8, 31.7, 28.8, 28.7, 28.3,

27.3, 26.3, 26.2, 25.6, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{34}H_{53}N_{10}O_{13}S$, 841.3520; found, 841.3521.

5′-*O*-*N*-(*L*-arginyl)sulfamoyladenosine (24)

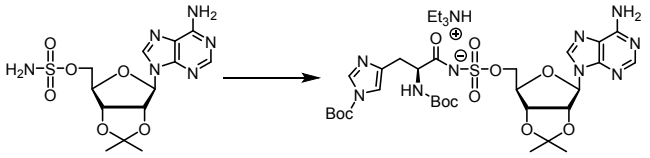


S2n **24**

Compound **S2n** (40 mg, 0.10 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 2 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (50:50:1 $CHCl_3$/MeOH/Et_3N to 100:1 MeOH/Et_3N) to afford compound **24** as a white solid (14 mg, 70%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.52 (s, 1H), 8.20 (s, 1H), 6.09 (d, J = 2.5 Hz, 1H), 4.64 (t, J = 5.0 Hz, 1H), 4.40 (t, J = 4.5 Hz, 1H), 4.36 (dd, J = 11.0, 3.0 Hz, 1H), 4.34−4.27 (m, 2H), 3.34−3.32 (m, 1H), 3.16 (t, J = 6.5 Hz, 2H), 1.82−1.70 (m, 1H), 1.70−1.60 (m, 3H). ^{13}C NMR (500 MHz, DMSO-d_6) δ 178.6, 156.8, 156.0, 152.6, 149.6, 139.4, 118.8, 86.8, 82.7, 73.7, 70.7, 67.2, 56.1, 40.6, 31.8, 25.2. HRMS (ESI–): $[M-H]^-$ calcd for $C_{16}H_{25}N_{10}O_7S$, 501.1634; found, 501.1639.

Chemical Synthesis of L-His-AMS 25

5′-*O*-[*N*-(*N*-Boc-*L*-histidyl(1-Boc))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (S2o)

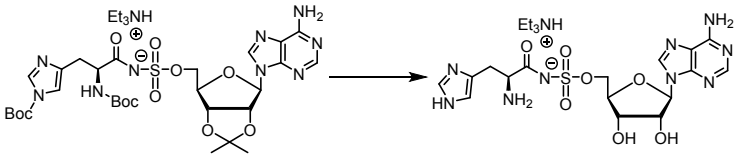


S1 **S2o**

Boc-His(Boc)-OSu (88 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **S1** (50 mg, 0.13 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 83:17:1 EtOAc/MeOH/Et_3N) to afford compound **S2o** as a white solid (55 mg, 59%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H),

8.21 (s, 1H), 8.03 (s, 1H), 6.23 (d, J = 3.4 Hz, 1H), 5.36−5.31 (m, 1H), 5.11−5.07 (m, 1H), 4.55−4.51 (m, 1H), 4.30−4.19 (m, 3H), 3.18 (q, J = 7.5 Hz, 6H, Et_3N-CH_2), 3.10−3.01 (m, 1H), 2.93−2.85 (m, 1H), 1.61 (s, 3H), 1.58 (s, 9H), 1.39−1.36 (m, 12H), 1.27 (t, J = 7.5 Hz, 9H, Et_3N-CH_3). ^{13}C NMR (125 MHz, CD_3OD) δ 179.5, 157.4, 157.3, 154.0, 150.5, 148.2, 141.4, 140.7, 137.8, 120.1, 116.0, 115.2, 91.8, 86.8, 85.7, 85.5, 83.3, 80.1, 69.7, 57.6, 47.8, 32.8, 28.8, 28.6, 28.0, 27.5, 25.6, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{29}H_{40}N_9O_{11}S$, 722.2568; found, 722.2571.

5′-*O*-*N*-(*L*-histidyl)sulfamoyladenosine triethylammonium salt (25)



Compound **S2o** (40 mg, 0.061 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 $CHCl_3$/MeOH/Et_3N to 100:1 MeOH/Et_3N) to afford compound **25** as a white solid (17 mg, 74%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.37 (s, 1H), 8.13 (s, 1H), 7.61 (s, 1H), 7.27 (br, 2H), 6.93 (s, 1H), 5.91 (d, J = 5.7 Hz, 1H), 4.62−4.57 (m, 1H), 4.19−4.14 (m, 1H), 4.13−4.07 (m, 2H), 4.04−4.00 (m, 1H), 3.63 (dd, J = 9.2, 3.4 Hz, 1H), 3.11 (dd, J = 14.9, 3.4 Hz, 1H), 2.81 (dd, J = 14.9, 9.2 Hz, 1H), 2.66 (q, J = 7.5 Hz, 4H, Et_3N-CH_2), 1.01 (t, J = 7.5 Hz, 6H, Et_3N-CH_3). ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.9, 156.0, 152.7, 149.6, 139.4, 135.0, 118.9, 87.0, 82.5, 73.5, 70.7, 67.4, 55.6, 45.7, 28.7, 10.6. HRMS (ESI−): $[M-H]^-$ calcd for $C_{16}H_{21}N_9O_7S$, 482.1206; found, 482.1212.

Chemical Biology Procedures

Protein Expression and Materials: Recombinant proteins *holo*-GrsA and *holo*-TycB1 were expressed and purified as described previously.[7,8,9,10] Recombinant *holo*-GrsA and *holo*-TycB1 were overproduced and isolated as C-terminal His-tagged constructs using the *E. coli* overexpression strain, BL21 (DE3), kindly provided by Prof. Mohamed A. Marahiel at Philipps-Universität Marburg, Germany.

Hydroxamate-MesG Assay[1]

Standard assay conditions: Reactions contained *holo*-GrsA (140–1400 nM) to maintain initial

velocity conditions, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase (Sigma–Aldrich, N8264), 0.04 U inorganic pyrophosphatase (Sigma–Aldrich, I1643), 0.2 mM MesG (Berry & Associates) and varying concentrations of substrates. The reactions (100 µL) were run in 96-well half-area plates (Corning, 3881) and the cleavage of MesG was monitored at A_{355} on an EnVision Multilabel Reader (PerkinElmer). Working stocks of hydroxylamine were prepared fresh by combining 500 µL of 4 M hydroxylamine, 250 µL of water and 250 µL of 7 M NaOH on ice.
Determination of kinetic parameters: Steady-state kinetic parameters of the substrates were determined for *holo*-GrsA using standard assay conditions as described above. GrsA was used at 700 nM with L-Leu (0.625–10 mM), 1.4 µM with L-Thr (25–300 nM), 140 nM with L-Met (2.5–100 mM), 700 nM with L-Trp (0.125–2.0 mM), and 1.4 µM with L-His (3.125–100 mM). TycB1 was used at 400 nM with L-Pro (10–2000 µM). In all experiments, the total DMSO concentration was kept at or below 2.0%. Initial velocities were fit to the Michaelis-Menten equation using Prism 5 (GraphPad Software).

Bacterial strains: *A. migulanus* ATCC 9999 and DSM 5759 were obtained from the American Type Culture Collection (ATCC) and Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), respectively.

Cultivation media: YPG media comprises yeast extract (50 g/L), Bacto Peptone (50 g/L), and glucose (5 g/L).[11]

Preparation of Cellular Lysates for Proteomic Labeling Experiments: *A. migulanus* ATCC 9999 and DSM 5756 were maintained on nutrient agar. Single colonies were used to inoculate YPG medium and cultures were shaken for 24 h at 37 °C. The seed culture (2 mL) was transferred to YPG media (250 mL) and the resulting mixture was incubated at 37 °C. Growth was routinely monitored at A_{660} on a U-2910 spectrophotometer (Hitachi). The cells were harvested by centrifugation and stored in the freezer until used. The frozen cell pellets were resuspended in Tris pH 8.0 (20 mM), $MgCl_2$ (1 mM), TCEP (1 mM), NP-40 (0.05%), and a protease inhibitor cocktail. Because of the lability of the synthetase during mechanical cell disruption processes,[12] a gentle treatment of cells with lysozyme (0.2 mg/mL) was used to release intracellular protein. The cell suspension was incubated at 0 °C for 30 min. The mixture was then incubated at 30 °C for 30 min. The solution was centrifuged for 5 min at 15,000 rpm and the pellets were discarded. The total protein concentration was quantitated by the method of Bradford.[13]

Competitive ABPP of the A-domain of endogenous GrsA in a complex proteome: *A. migulanus* ATCC 9999 proteome (2.0 mg/mL) was individually treated with inhibitors **6**–**25** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, 0.2 mg/mL lysozyme and the protease inhibitor cocktail. These samples were incubated for 10 min at room temperature and subsequently treated with probe **1** (1 μM from a 100 μM stock in DMSO). In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice. To initiate the click reaction, rhodamine (Rh)-azide, TCEP, TBTA ligand, and $CuSO_4$ were added to provide final concentrations of 100 μM, 1 mM, 100 μM, and 1 mM, respectively. After 1 h at room temperature, 5× SDS-loading buffer (strong reducing) was added and the samples were heated at 95 °C for 5 min. Samples were separated by 1D SDS-PAGE and fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare). IC_{50} values for the A-domain of GrsA were determined from dose–response curves from two trials at each inhibitor concentration (L-Phe-AMS **6**: 1 nM to 10 μM; L-Leu-AMS **12**: 10 nM to 1 mM; L-Met-AMS **18**: 10 nM to 1 mM; L-Trp-AMS **20**: 10 nM to 1 mM; L-His-AMS **25**: 10 nM to 1 mM; L-Thr-AMS **17**: 10 nM to 1 mM; L-Lys-AMS **23**: 10 nM to 1 mM) using Prism 5 (GraphPad Software).

Competitive ABPP of the A-domain of recombinant GrsA: Recombinant GrsA (1 μM) were individually treated with inhibitors **6**–**25** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40. These samples were incubated for 10 min at room temperature and subsequently treated with probe **1** (1 μM from a 100 μM stock in DMSO). In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice and reacted with Rh-azide for 1 h at room temperature. Reactions were treated with 5× SDS-loading buffer (strong reducing) and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare). IC_{50} values for the A-domain of GrsA were determined from dose–response curves from two trials at each inhibitor concentration (L-Phe-AMS **6**: 1 nM to 100 μM; L-Leu-AMS **12**: 10 nM to 1 mM; L-Met-AMS **18**: 10 nM to 1 mM; L-Trp-AMS **20**: 10 nM to 1 mM; L-His-AMS **25**: 10 nM to 1 mM; L-Thr-AMS **17**: 10 nM to 1 mM; L-Lys-AMS **23**: 10 nM to 1 mM) using Prism 5 (GraphPad Software).

Competitive ABPP of the A-domains of endogenous GrsB in a complex proteome: *A. migulanus* DSM 5759 proteome (2.0 mg/mL) was individually treated with inhibitors **6**–**25** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40, 0.2 mg/mL lysozyme, and the protease inhibitor cocktail. These samples were incubated

for 10 min at room temperature and subsequently treated with individual members of probes **2**–**5** (1 μM from a 100 μM stock in DMSO). In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 5 min on ice and reacted with Rh-azide for 1 h at room temperature. Reactions were treated with 5× SDS-loading buffer (strong reducing) and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare). IC_{50} values for the L-Pro activating domain of GrsB were determined from dose–response curves from two trials at various inhibitor concentrations (L-Pro-AMS **7**: 0.01 nM to 10 μM; L-Orn-AMS **8**: 100 nM to 1 mM; L-Gln-AMS **15**: 100 nM to 1 mM). IC_{50} values for the L-Orn activating domain of GrsB were determined from dose–response curves from two trials at each inhibitor concentration (L-Orn-AMS **8**: 0.1 pM to 100 nM; L-Gln-AMS **15**: 100 nM to 1 mM; L-Lys-AMS **23**: 100 nM to 1 mM; L-Arg-AMS **24**: 100 nM to 1 mM). IC_{50} values for the L-Val activating domain of GrsB were estimated from dose–response curves from two trials at each inhibitor concentration (L-Val-AMS **11**: 0.1 nM to 100 μM; L-Leu-AMS **12**: 100 nM to 100 μM; L-Ile-AMS **13**: 100 nM to 100 μM; L-Met-AMS **18**: 100 nM to 100 μM; L-Trp-AMS **20**: 100 nM to 100 μM; L-His-AMS **25**: 100 nM to 100 μM). IC_{50} values for the L-Leu activating domain of GrsB were estimated from dose–response curves from two trials at each inhibitor concentration (L-Leu-AMS **12**: 0.1 nM to 100 μM; L-Met-AMS **18**: 100 nM to 100 μM).

Competitive ABPP of the A-domain of recombinant TycB1: Recombinant TycB1 (1 μM) were individually treated with inhibitors **6**–**25** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40. These samples were incubated for 10 min at room temperature and subsequently treated with probe **2** (1 μM from a 100 μM stock in DMSO). In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice and reacted with Rh-azide for 1 h at room temperature. Reactions were treated with 5× SDS-loading buffer (strong reducing) and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare). IC_{50} values for the A-domain of TycB1 were determined from dose–response curves from two trials at each inhibitor concentration (L-Pro-AMS **7**: 1 nM to 100 μM; L-Orn-AMS **8**: 100 nM to 1 mM; L-Gln-AMS **15**: 100 nM to 1 mM) using Prism 5 (GraphPad Software).

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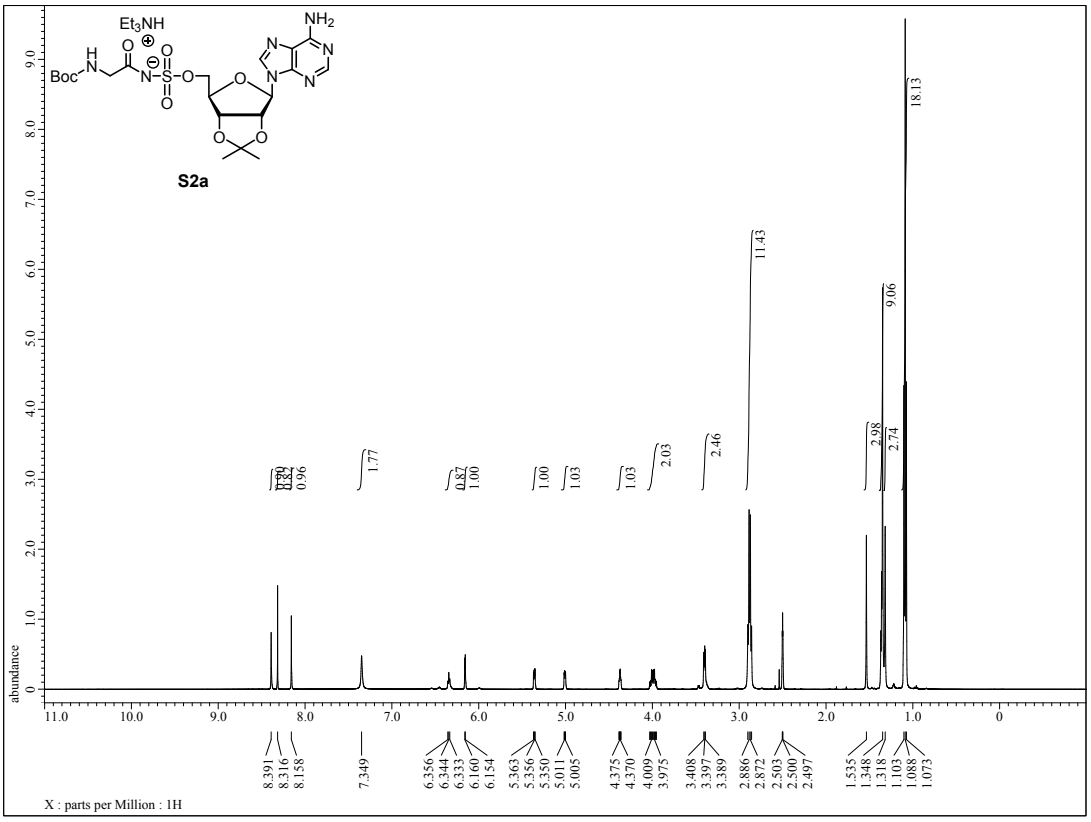
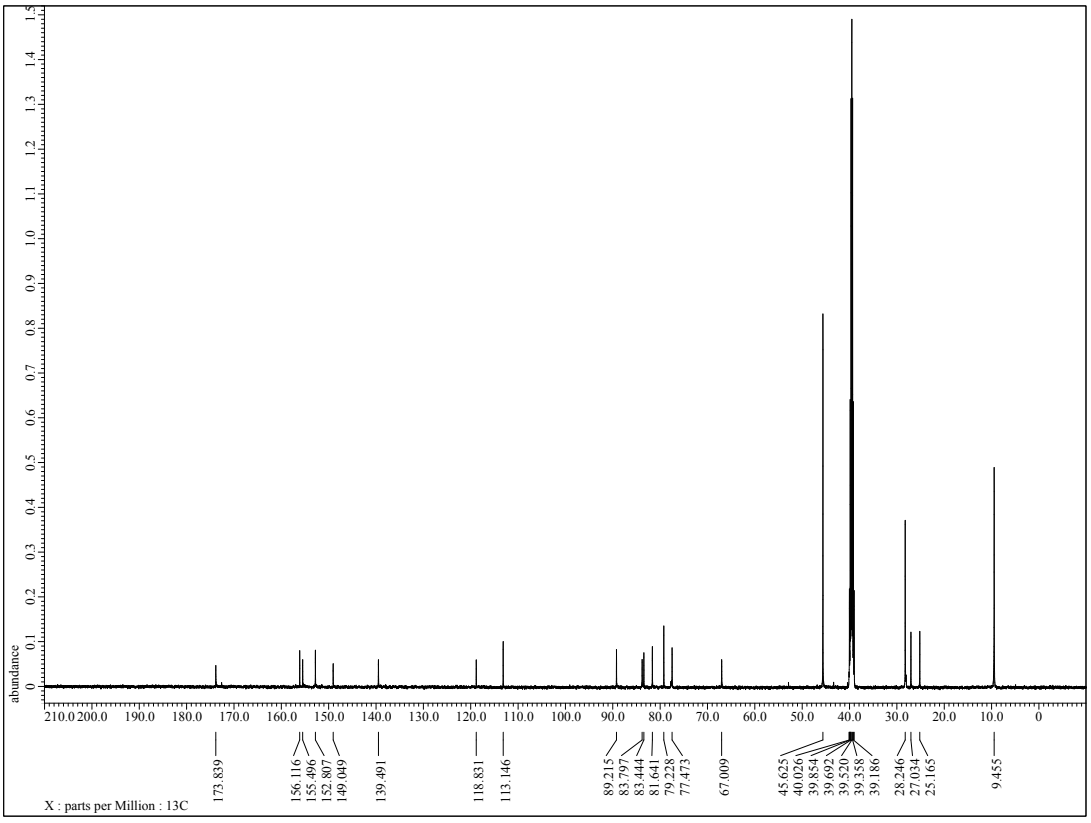
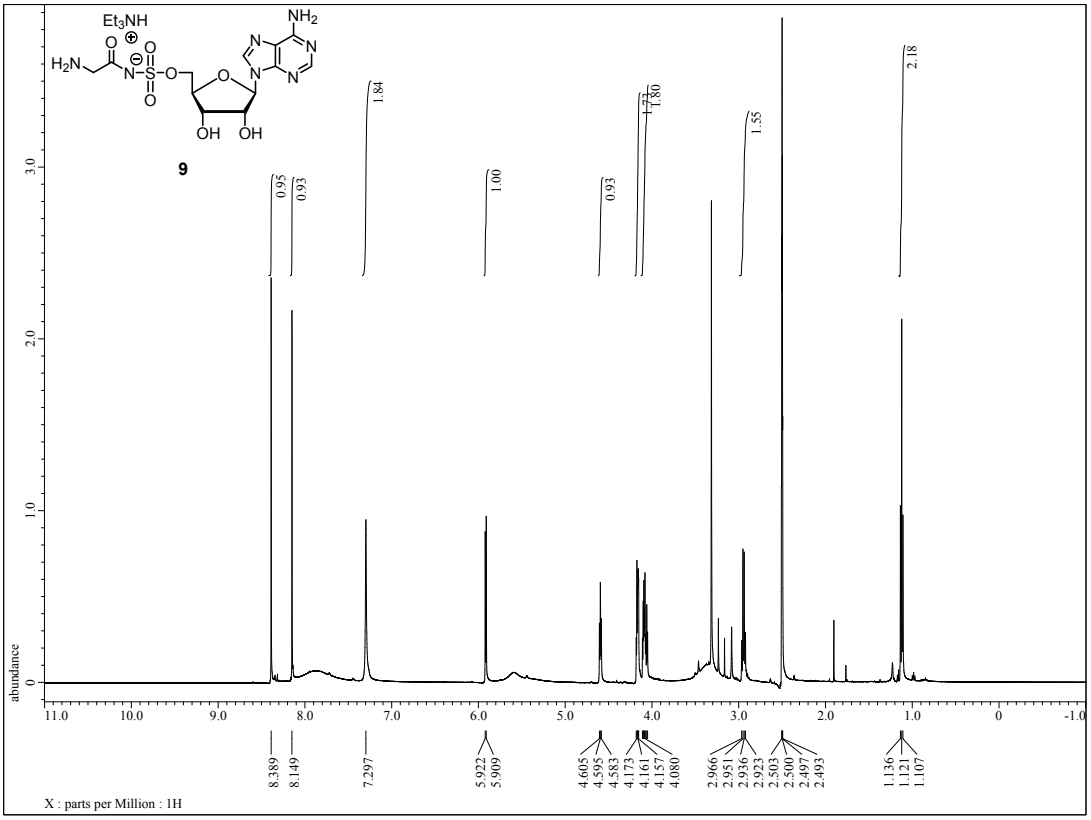
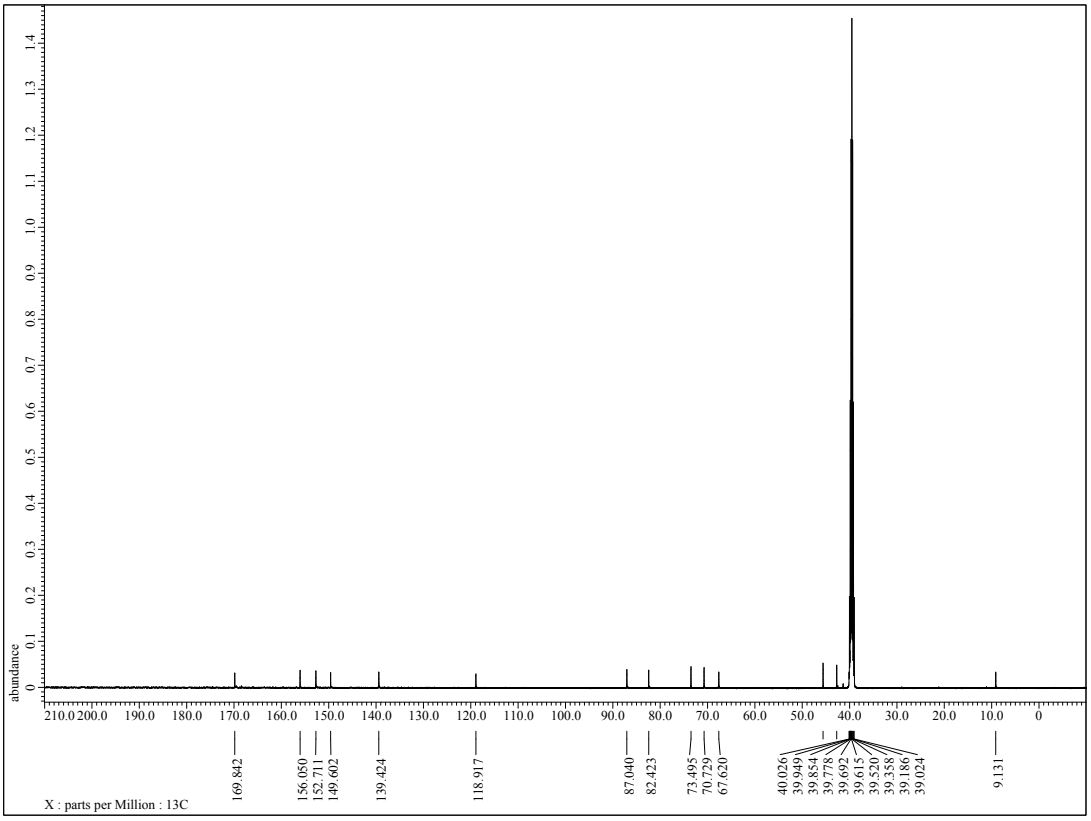
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2a**


Et3NH
NH2
Boc
S2a
abundance
X : parts per Million : 1H

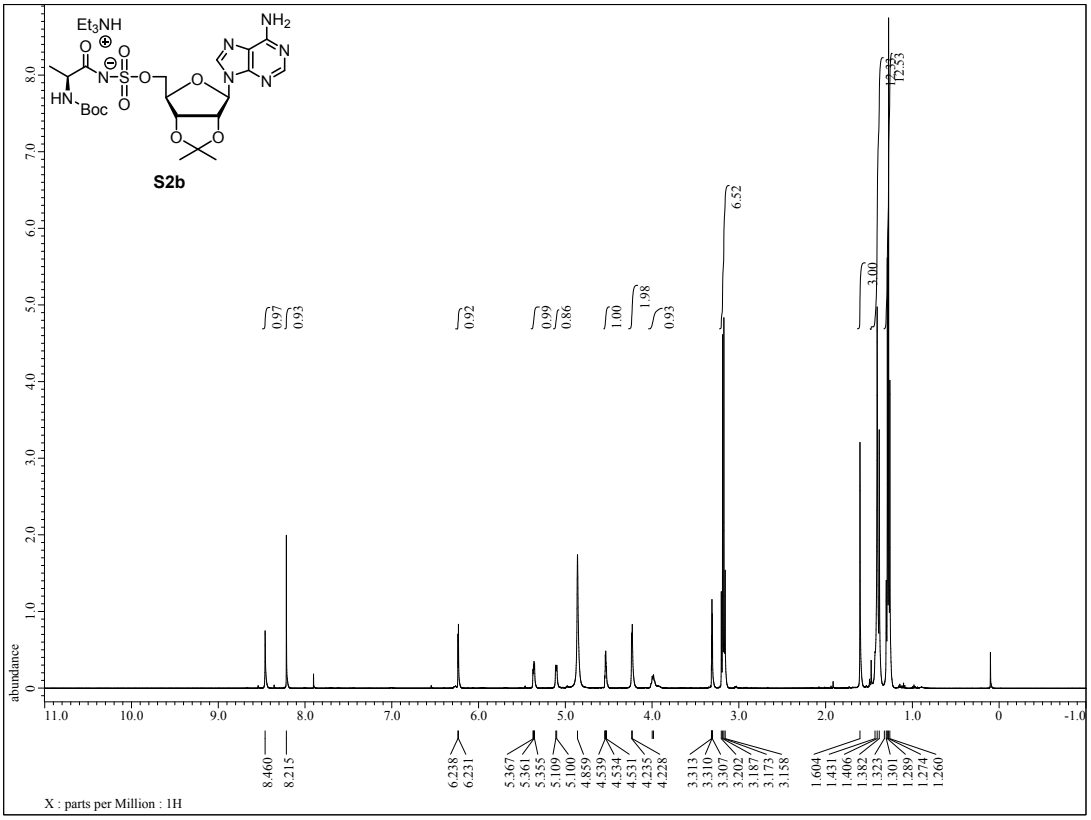

abundance
X : parts per Million : 13C

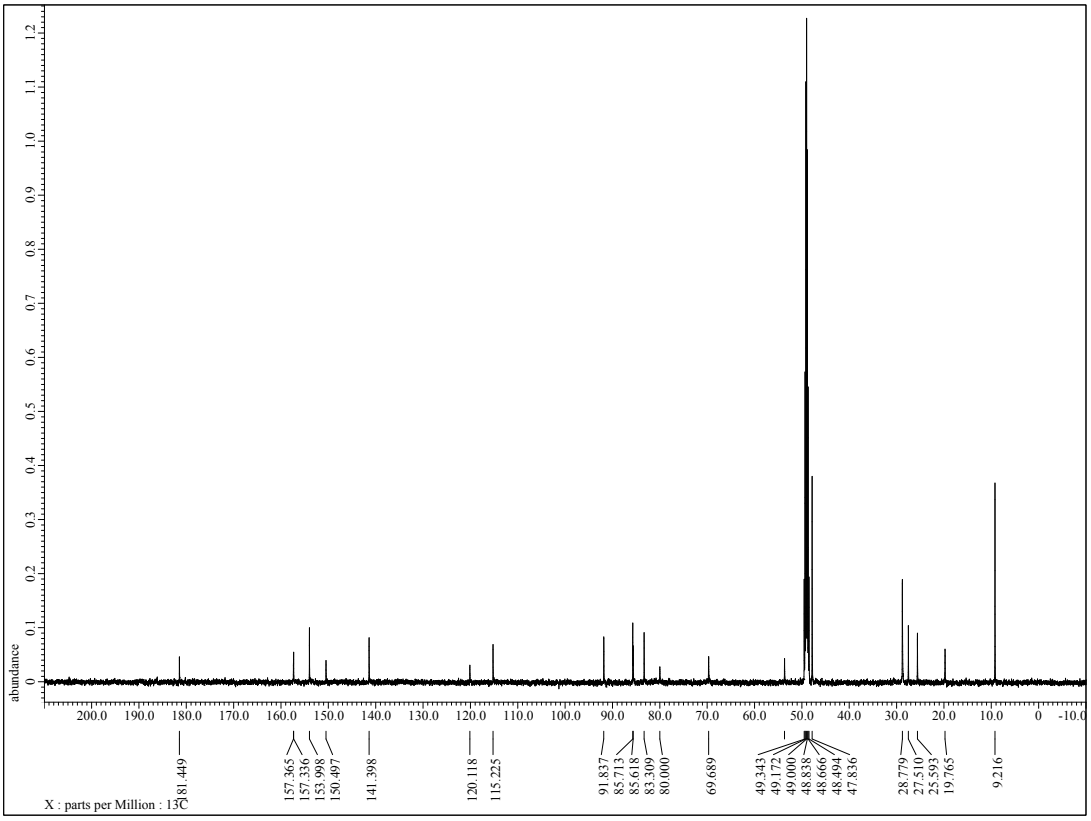
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **9**



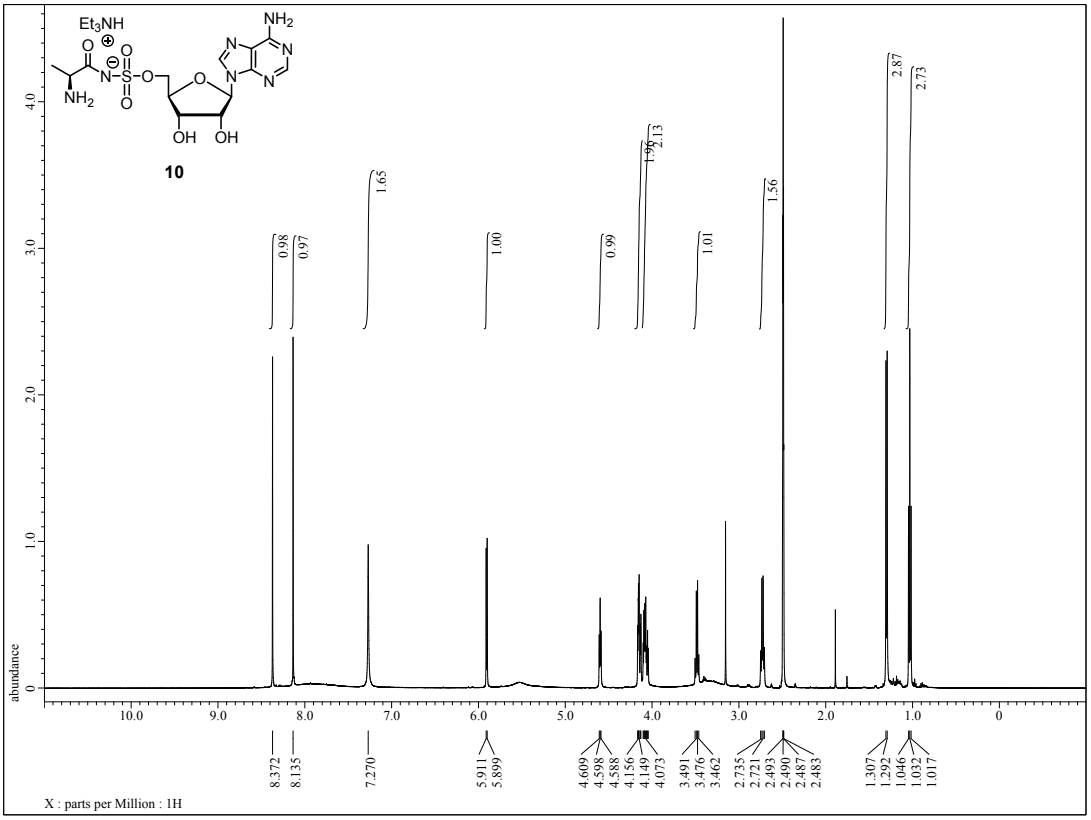


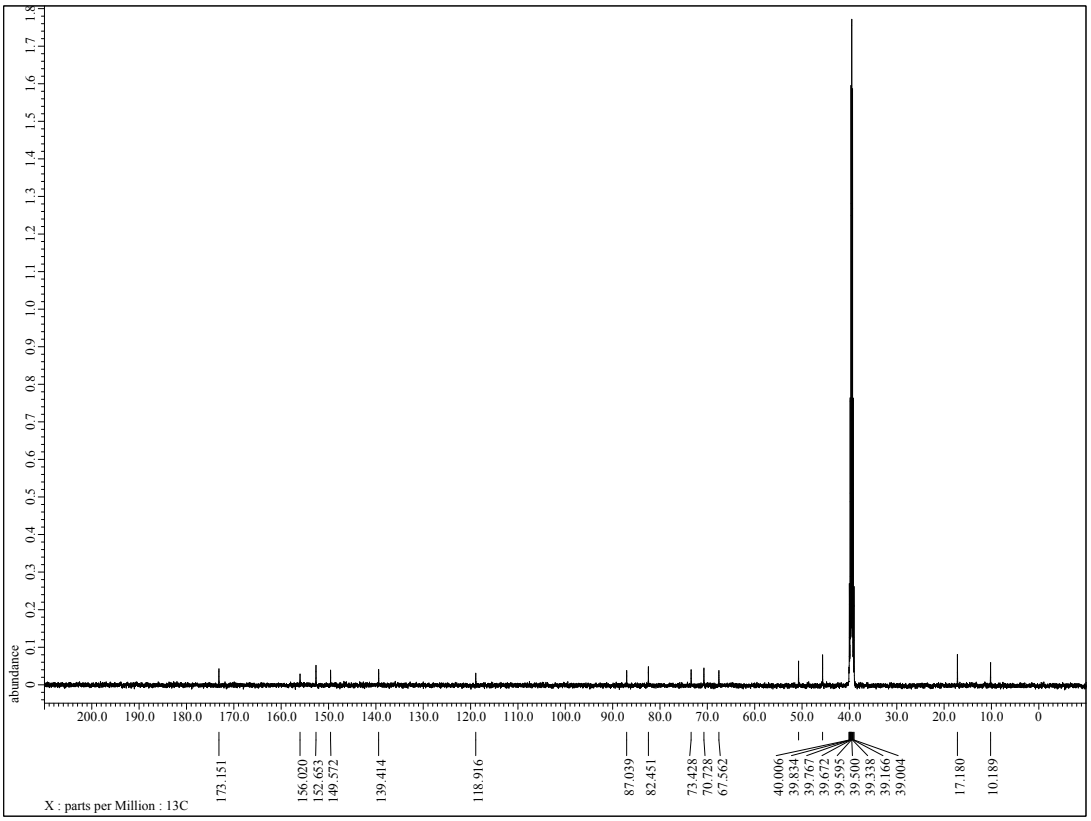
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2b**



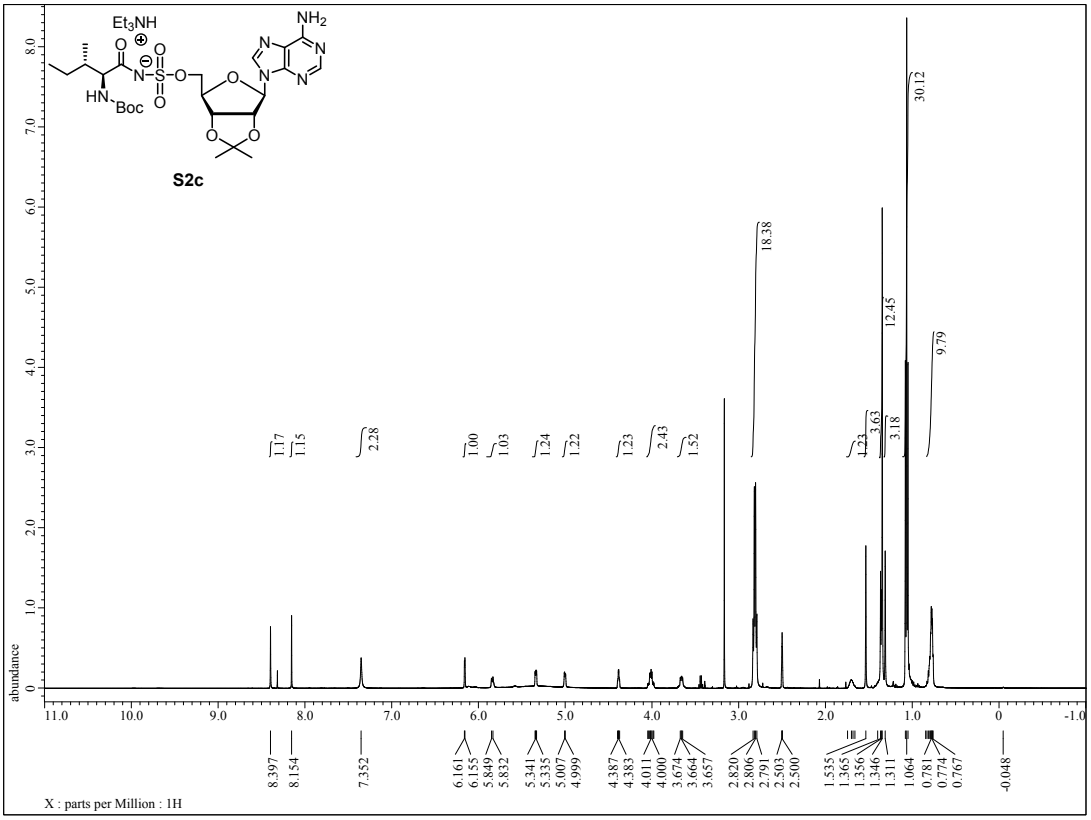


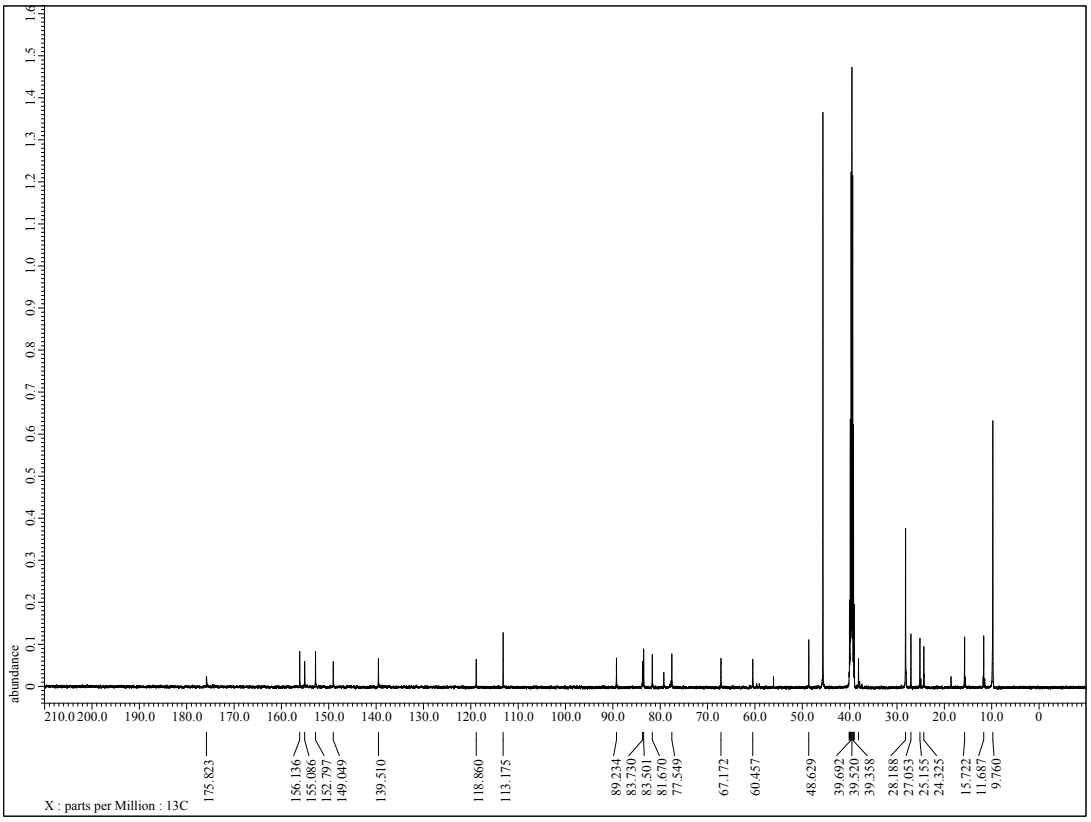
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **10**



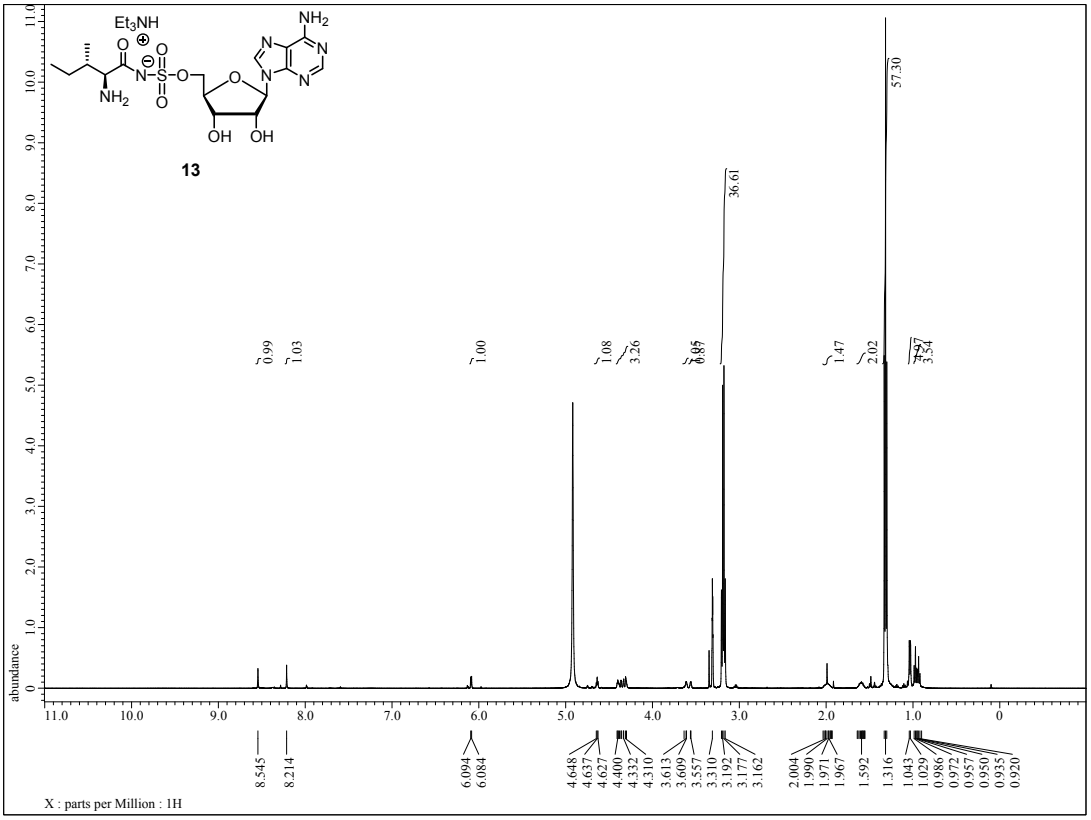


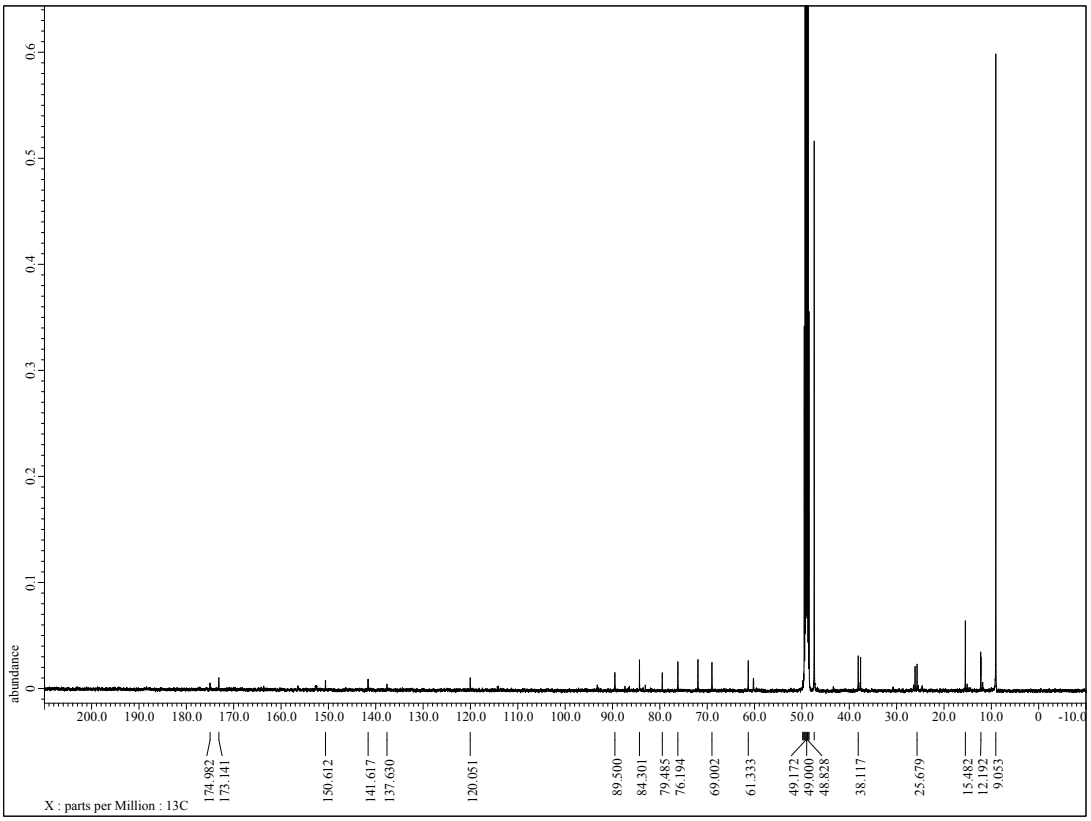
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2c**





^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **13**





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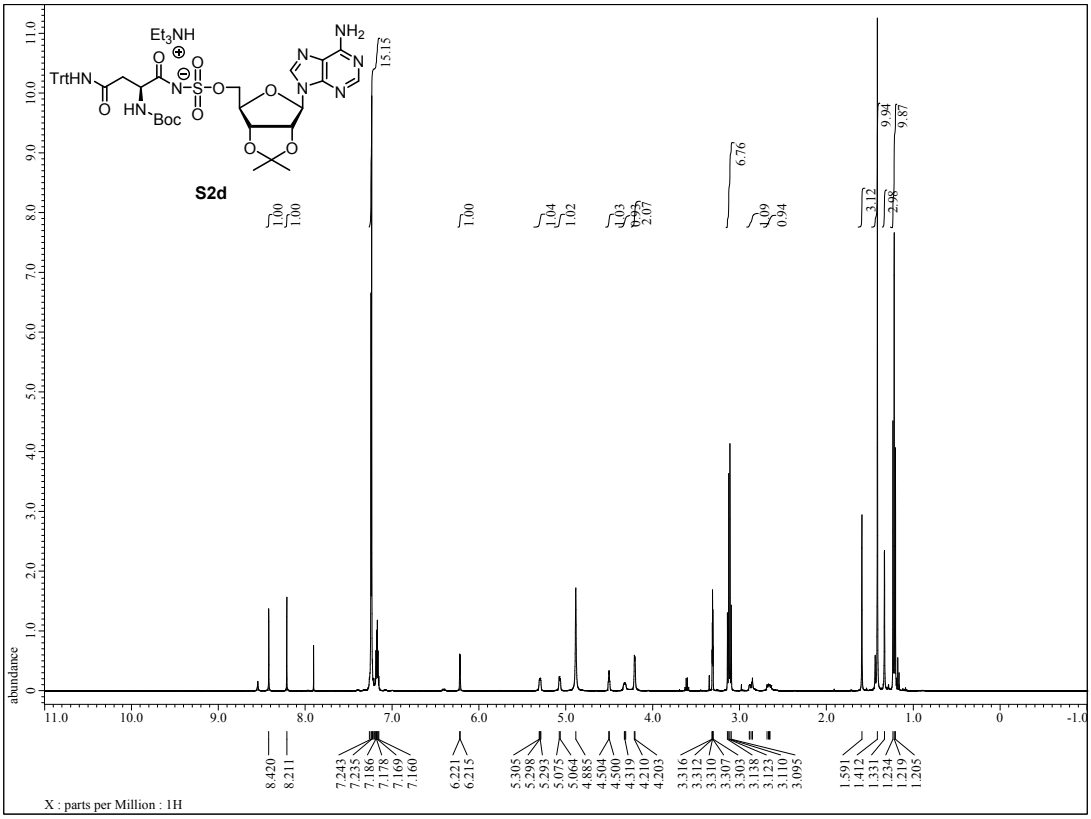
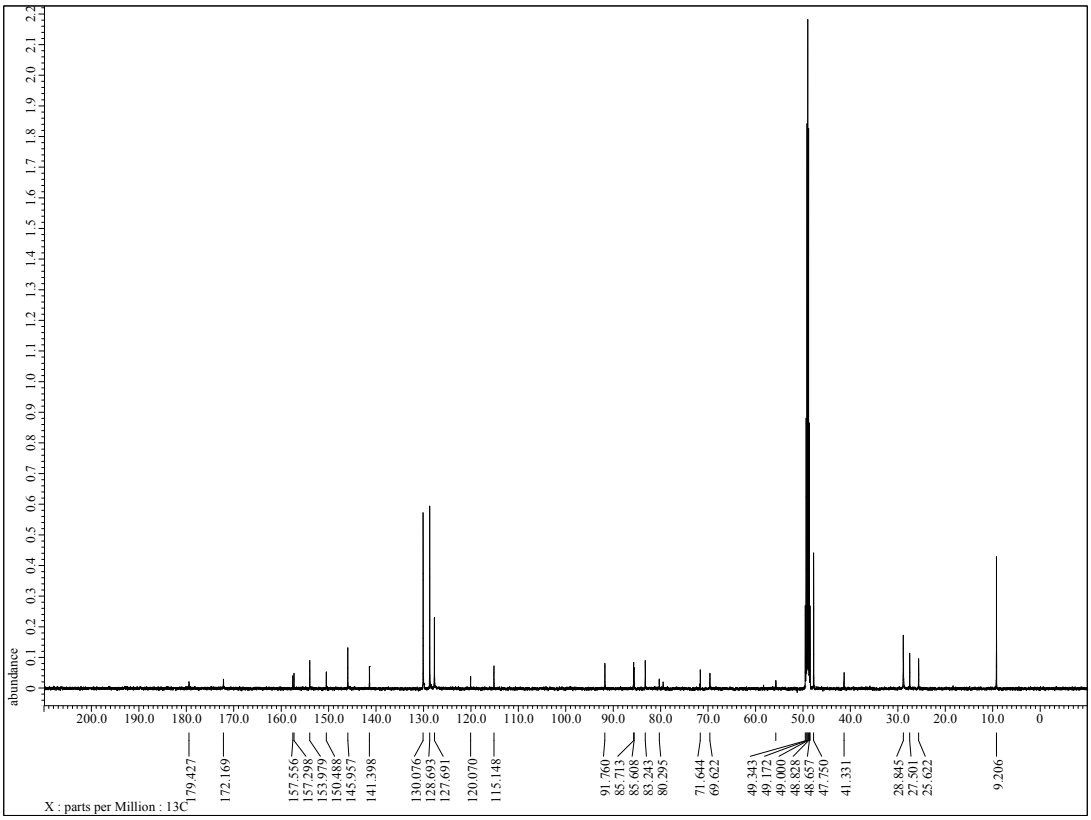
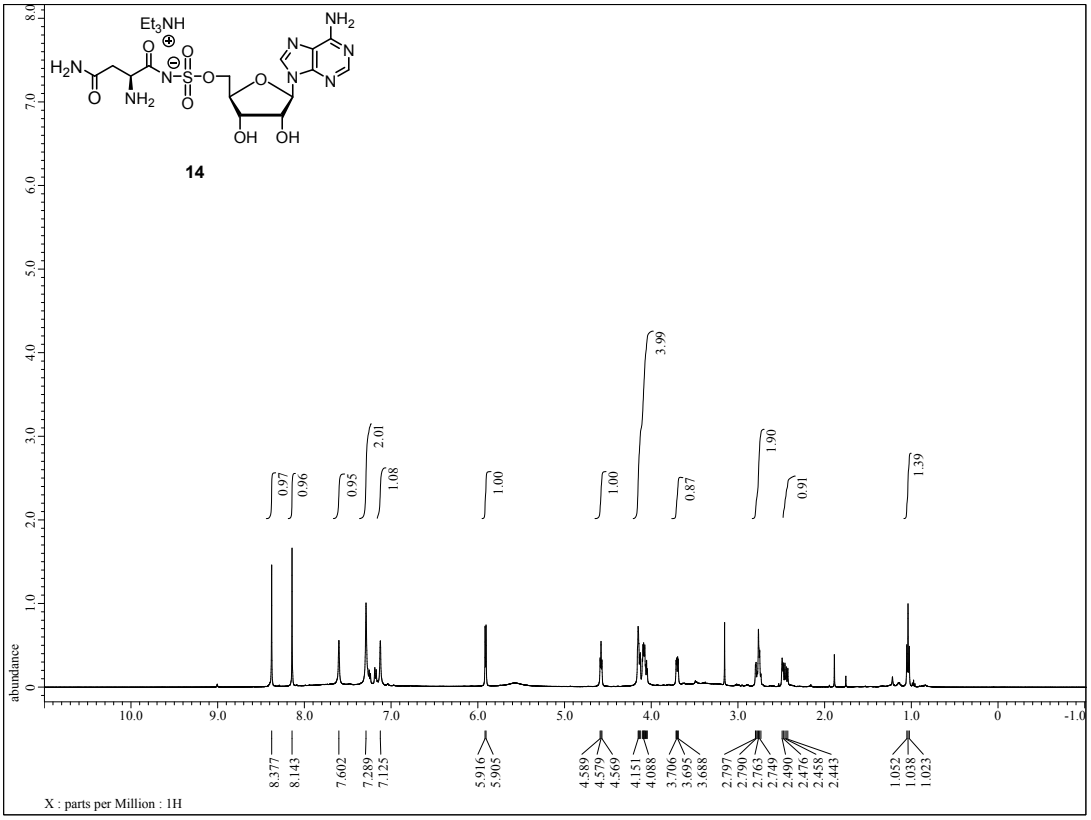
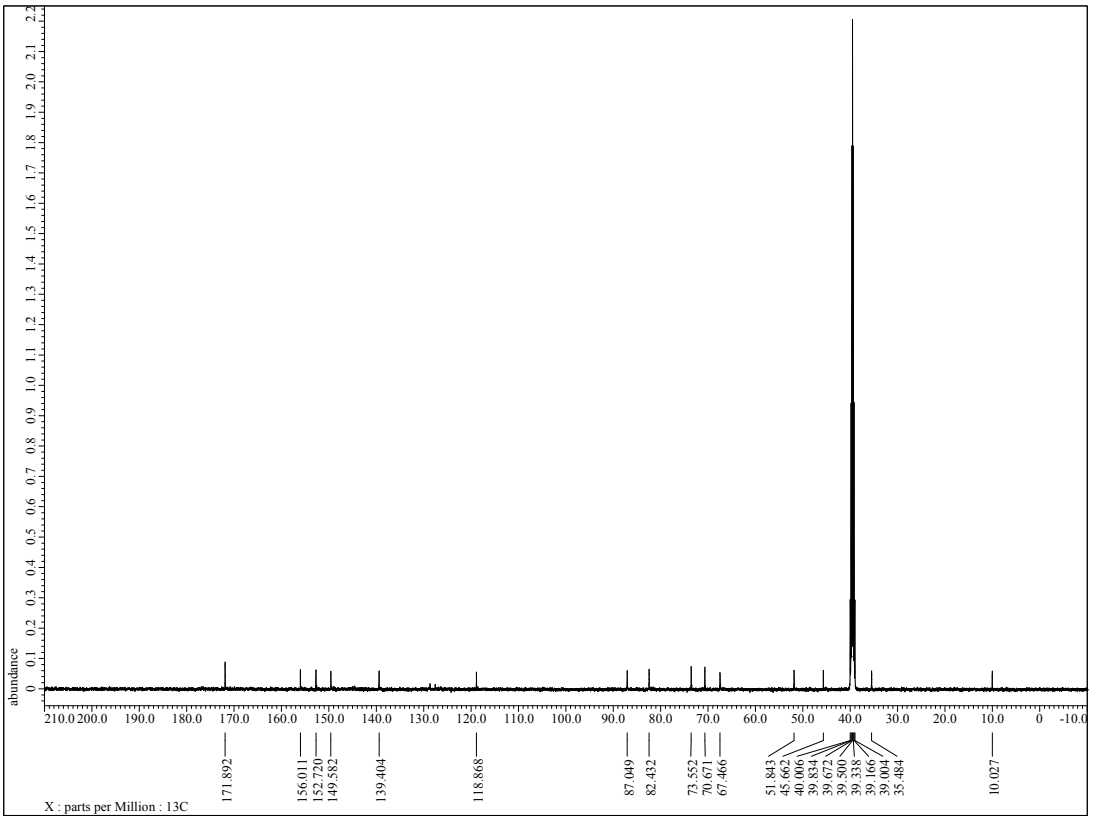


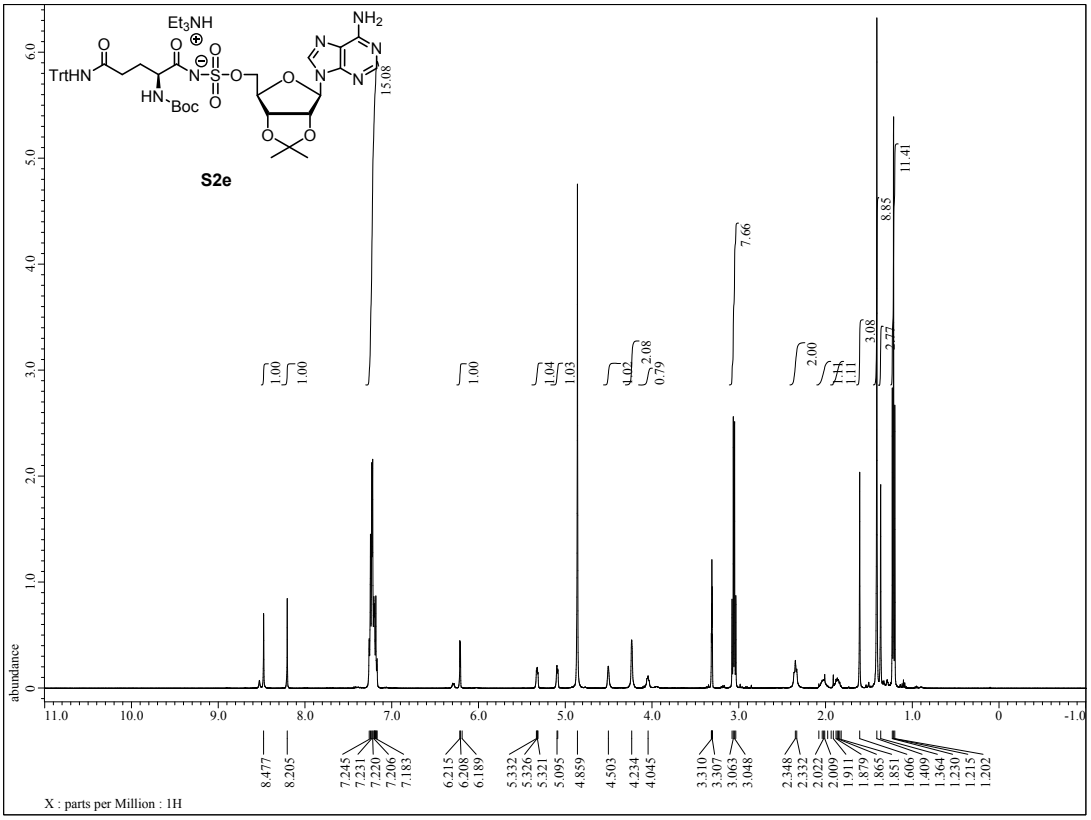


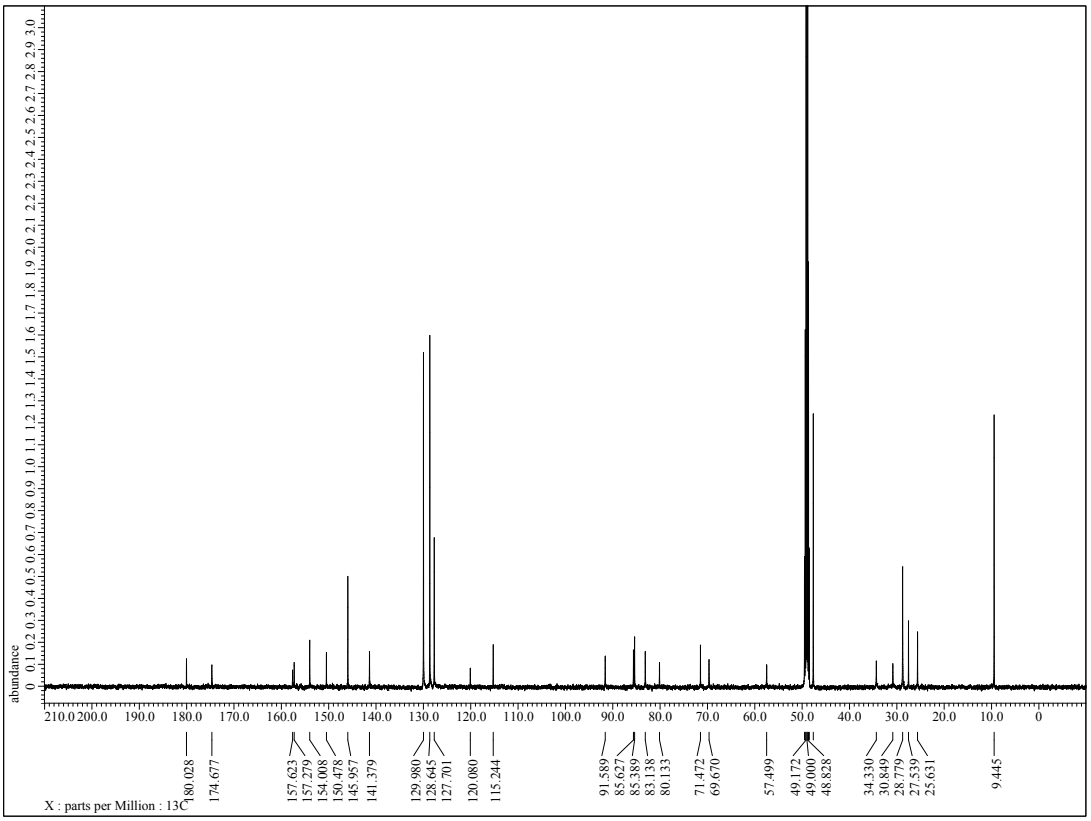
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **14**



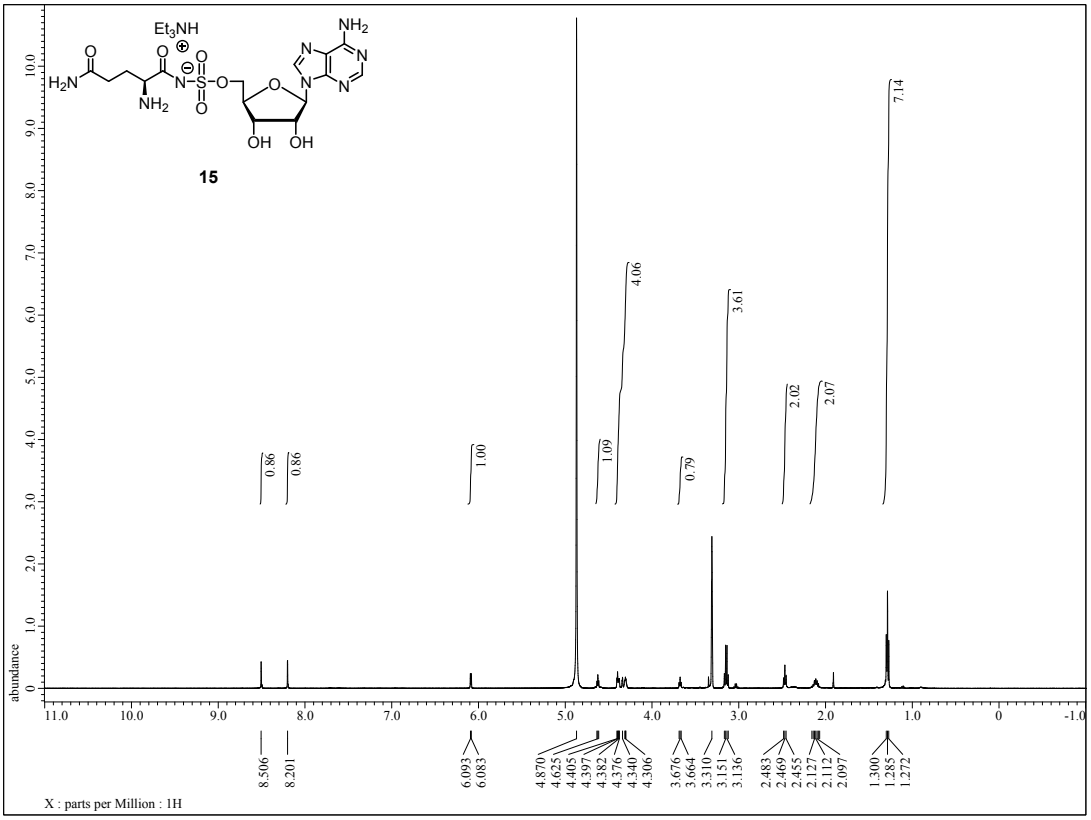


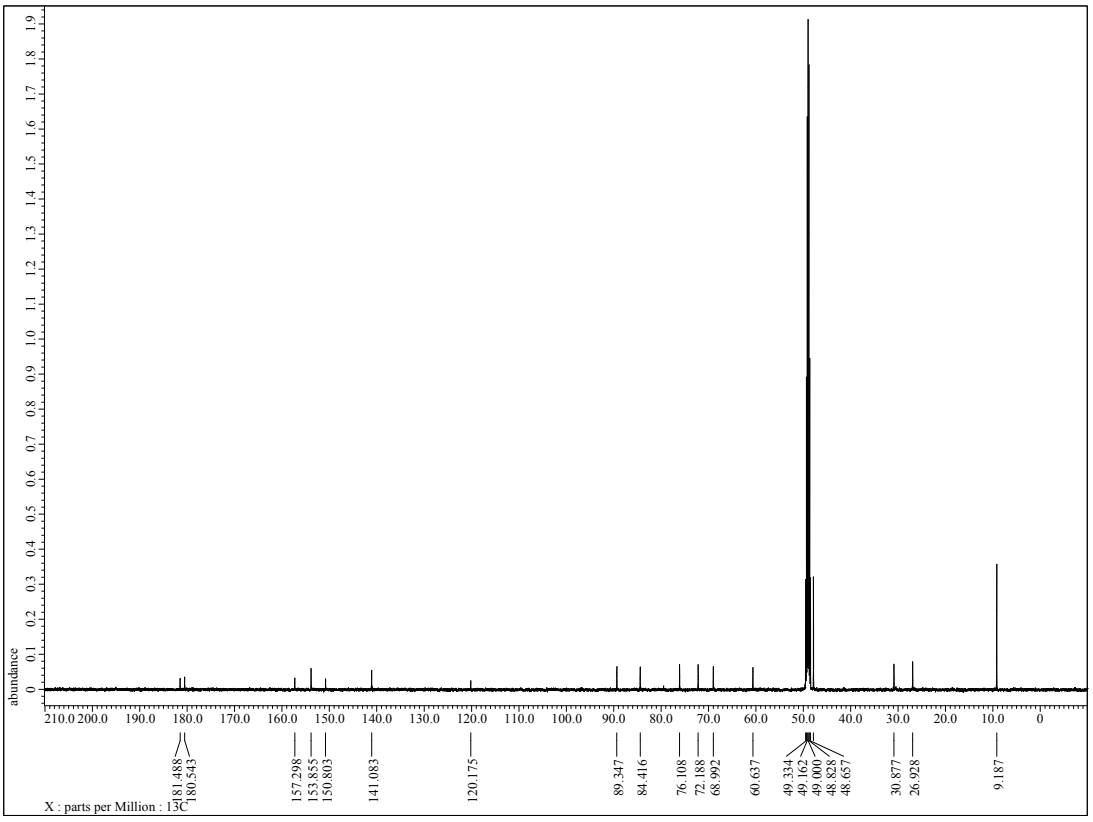
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2e**



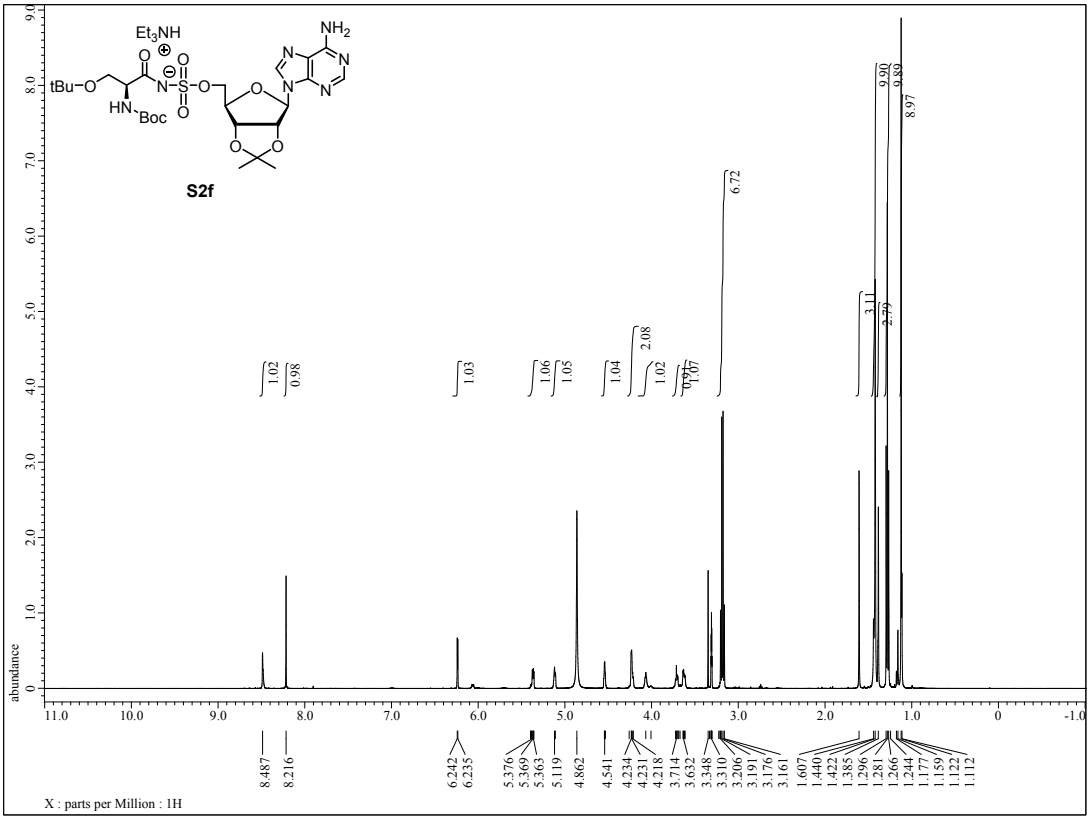


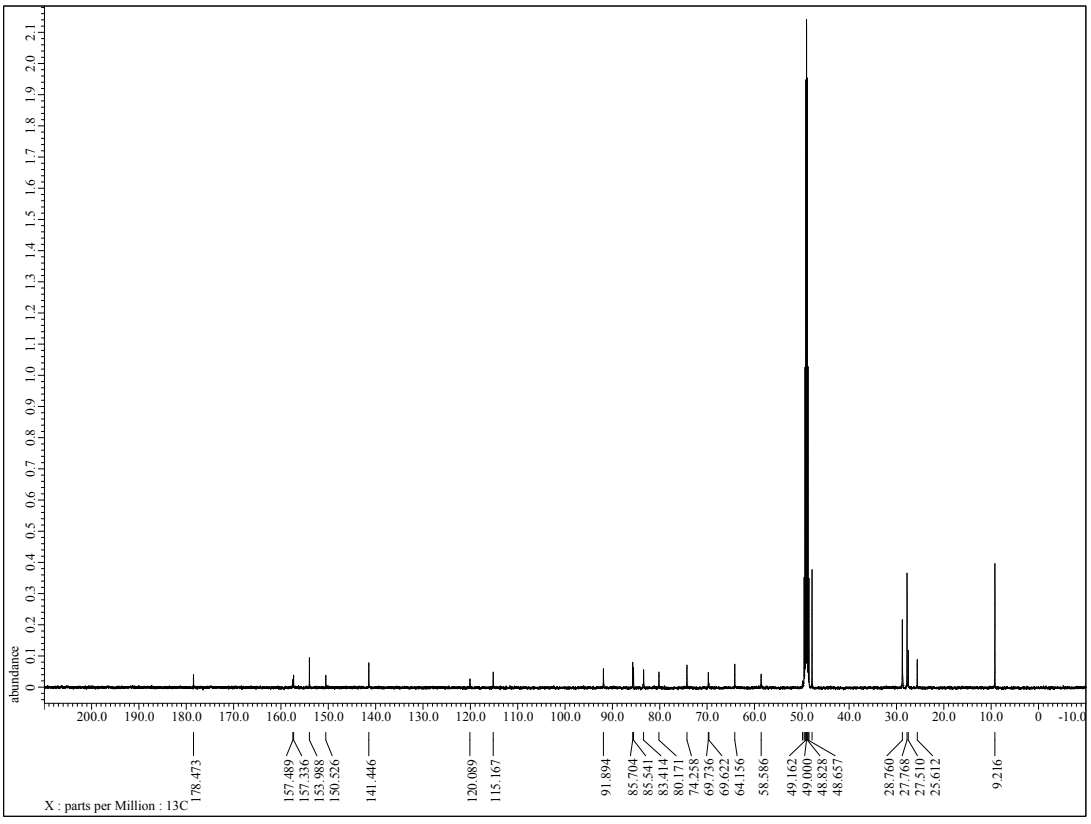
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **15**



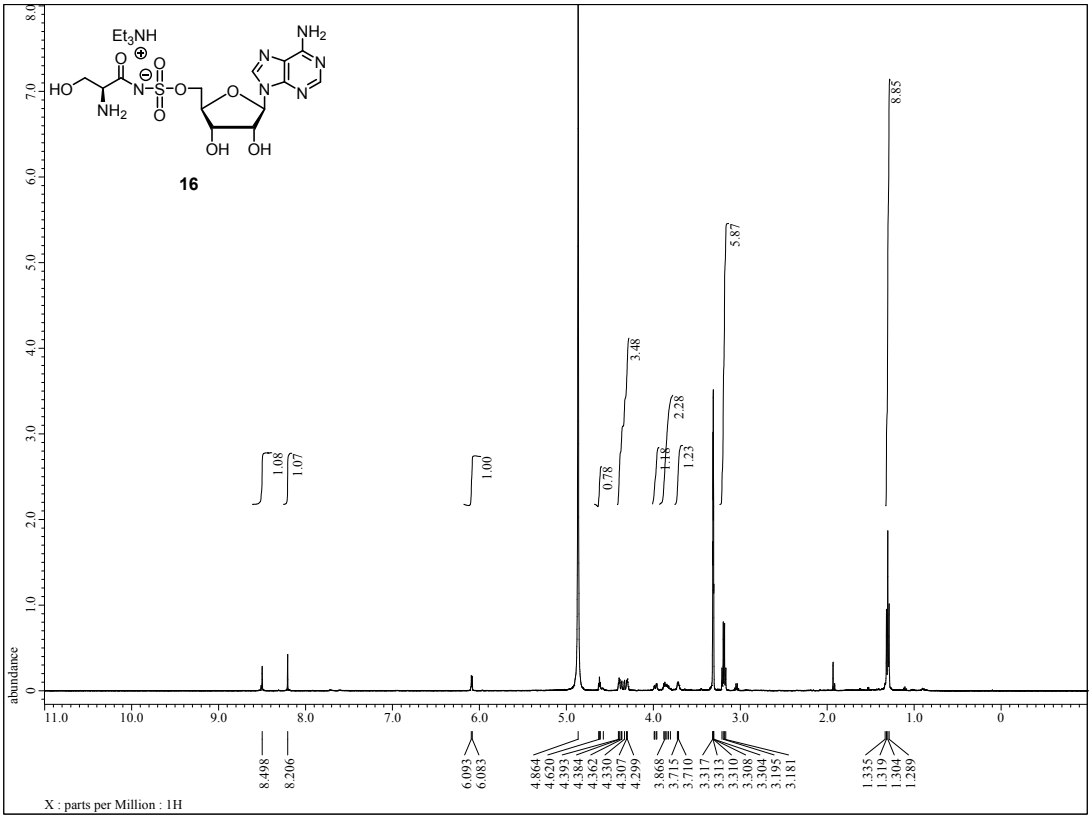


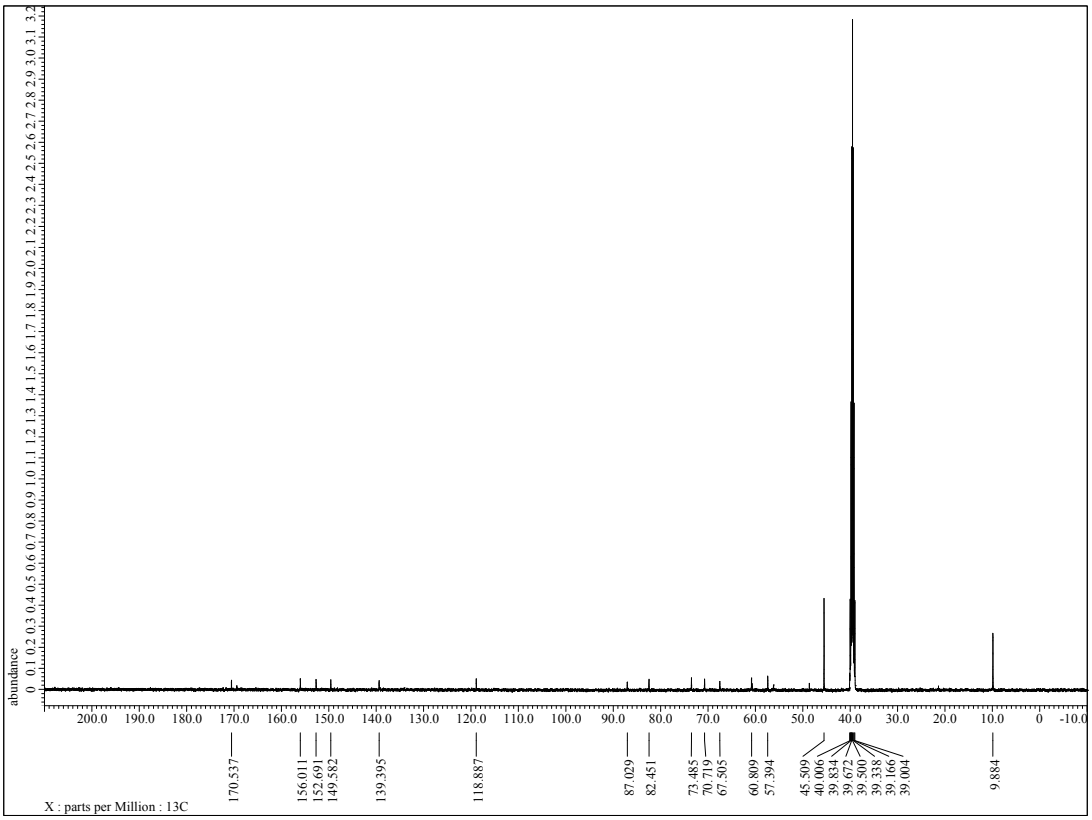
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2f**



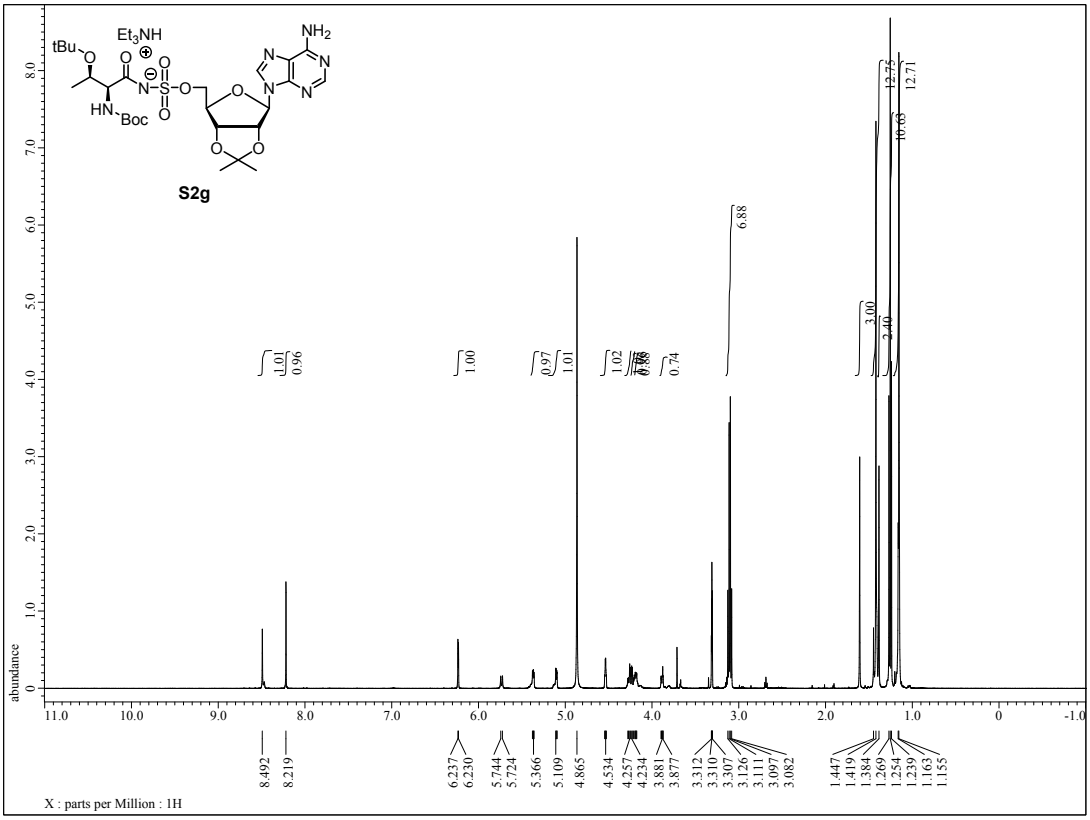


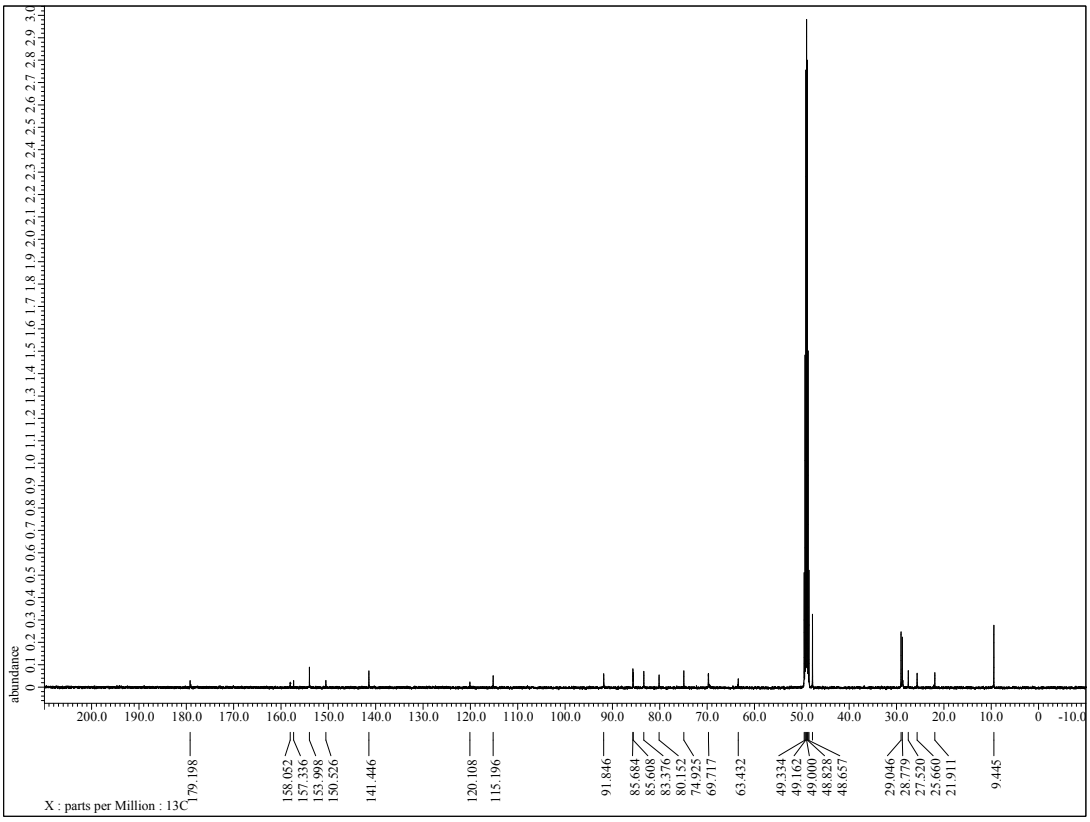
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **16**



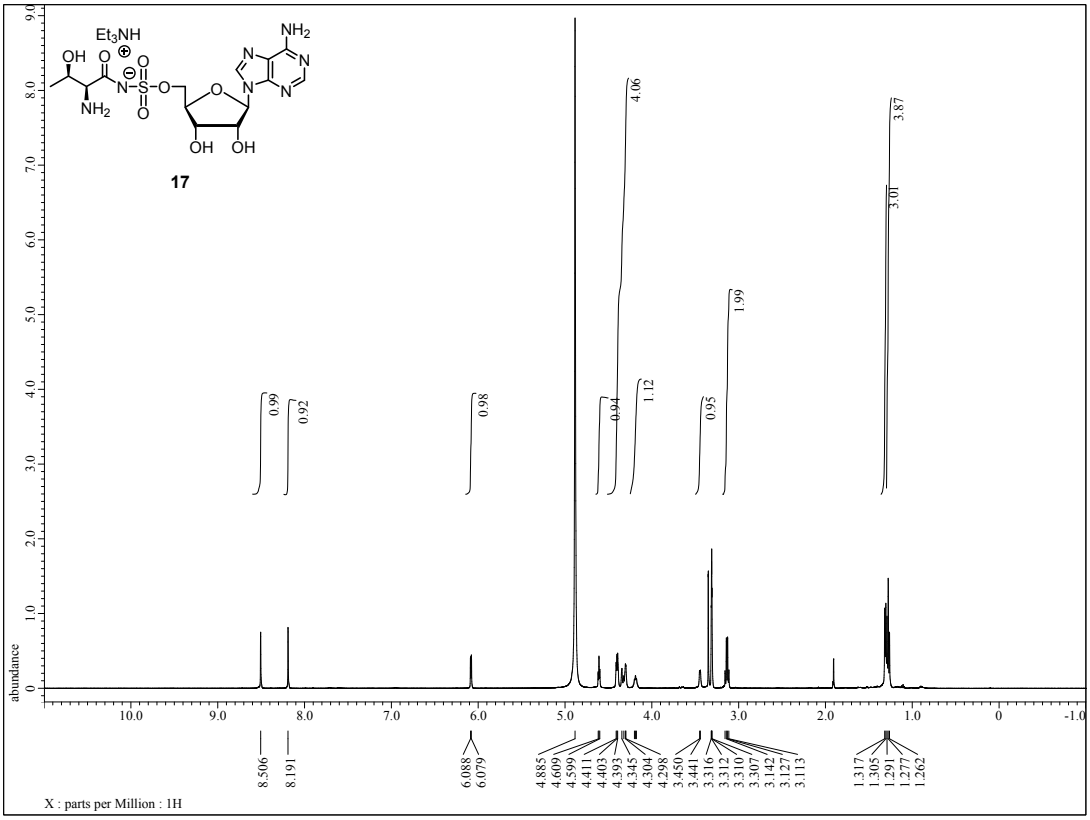


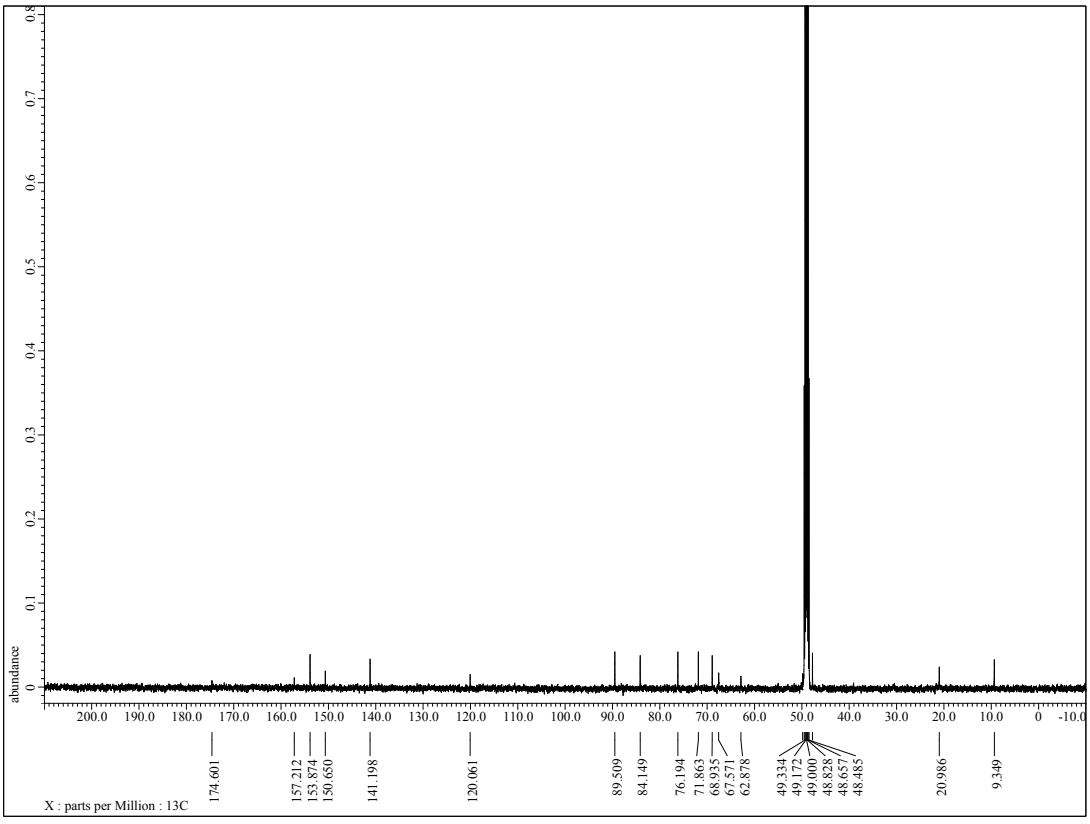
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2g**



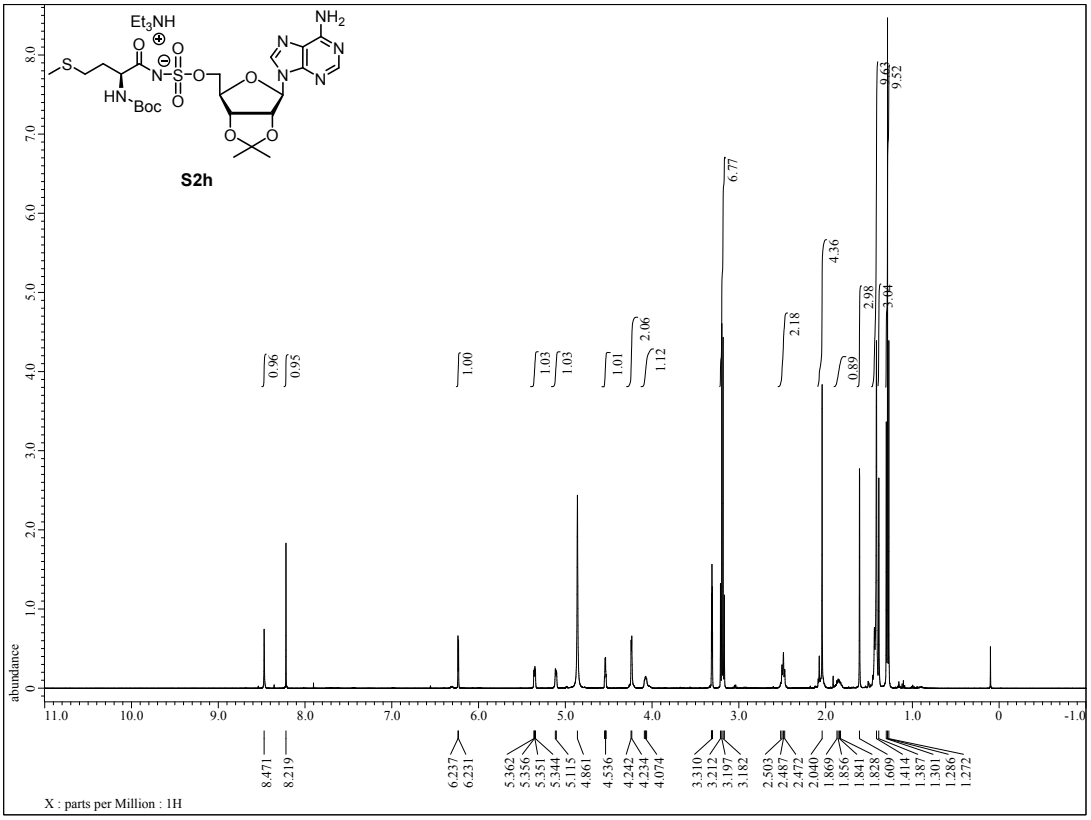


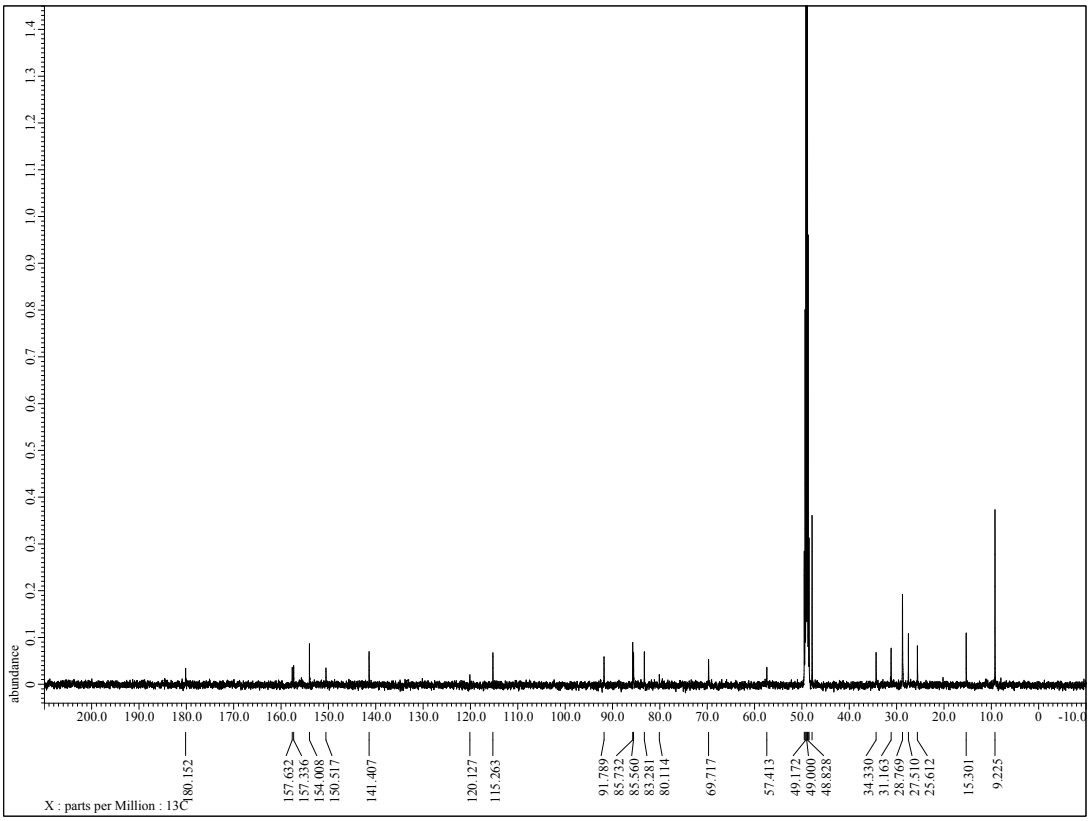
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **17**



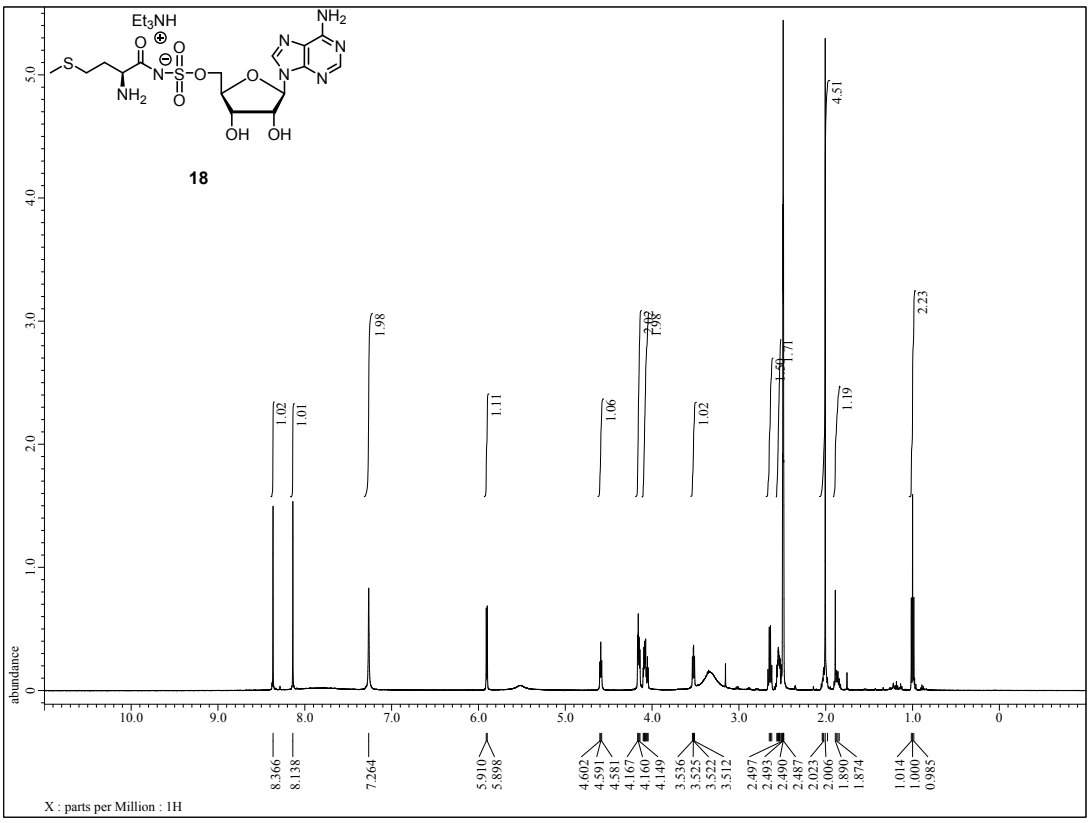


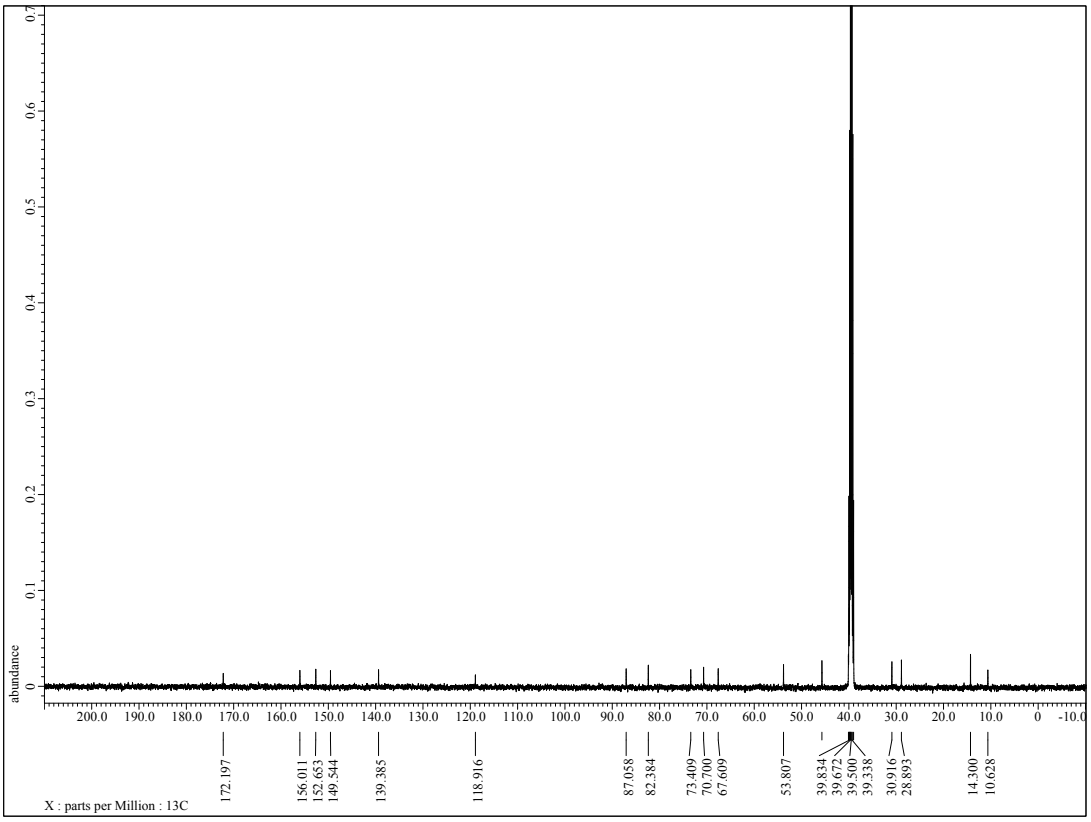
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2h**



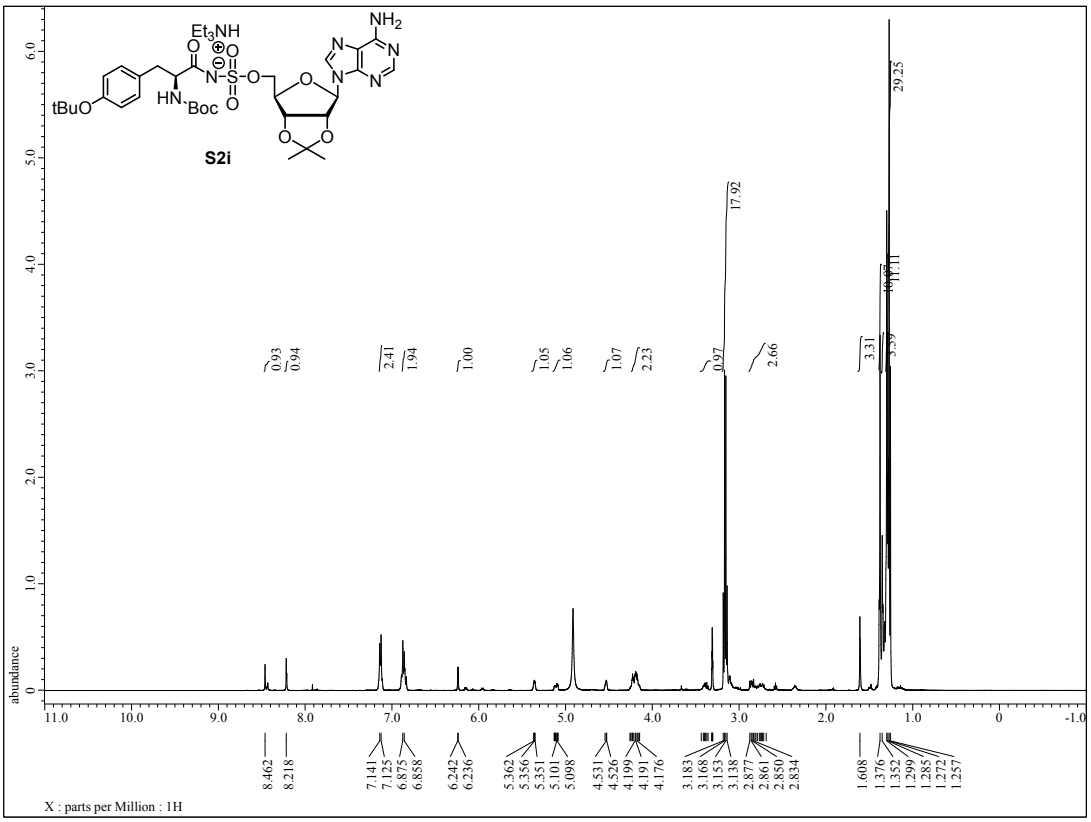


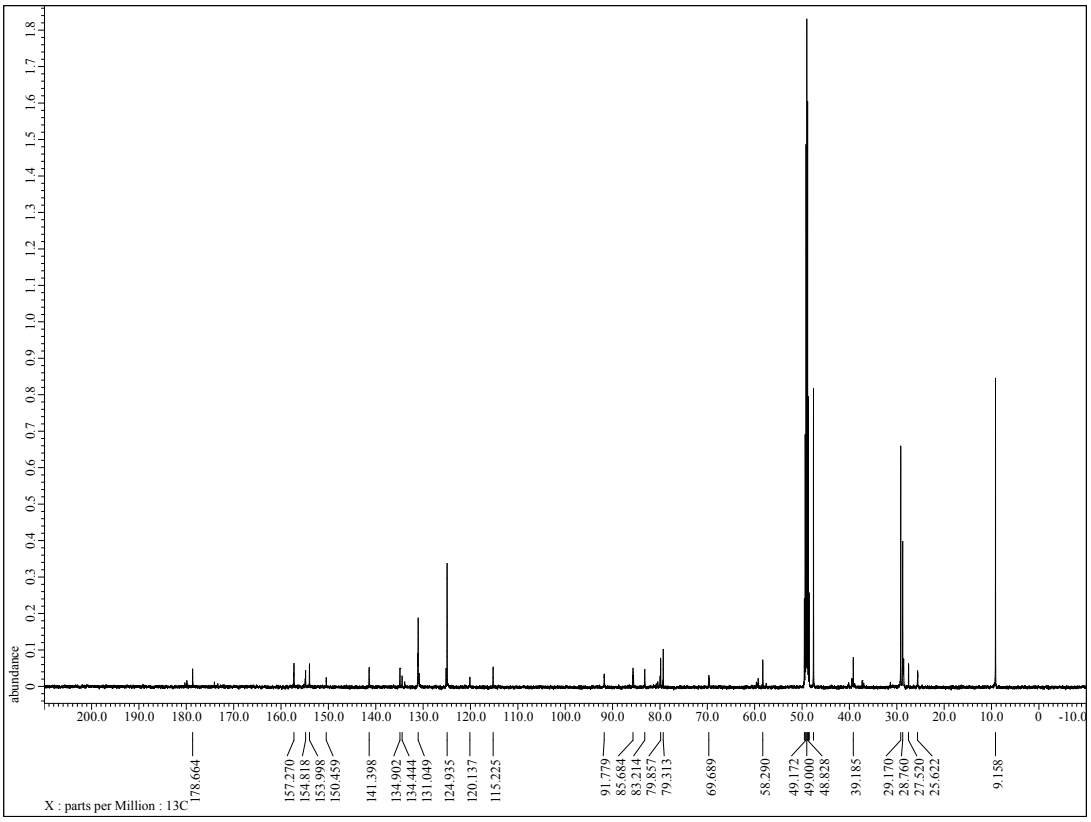
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **18**



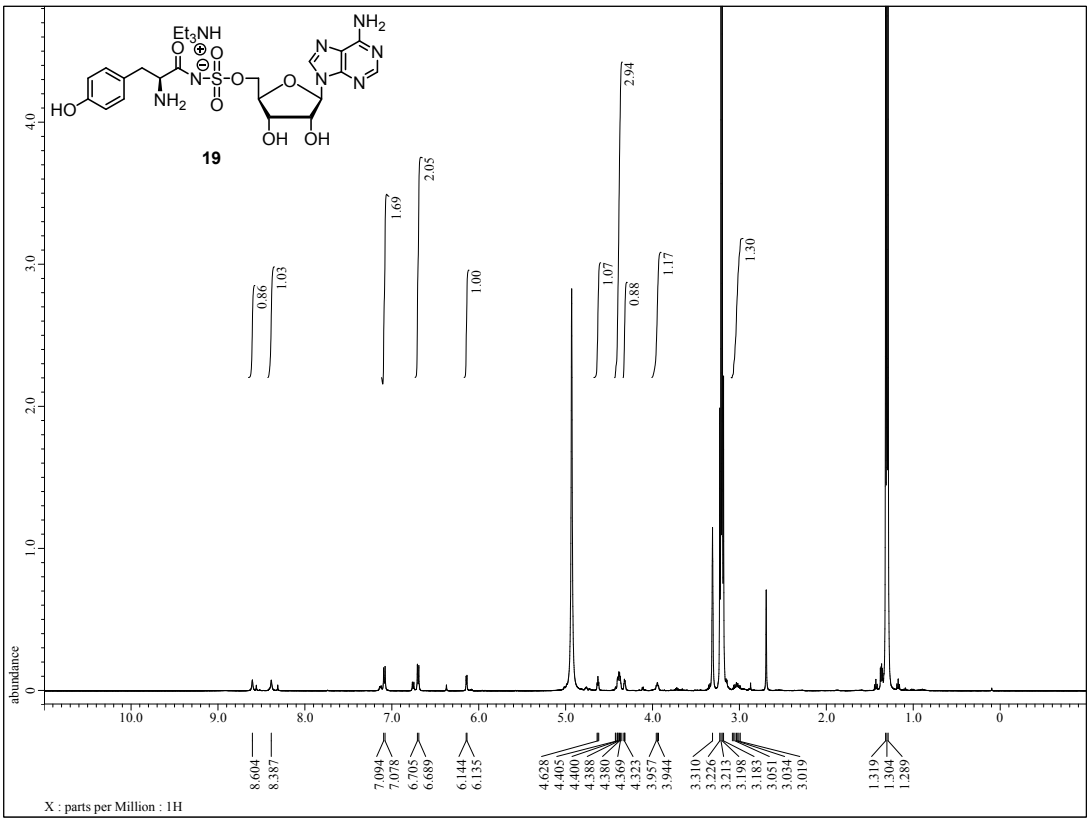


^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2i** in CD_3OD

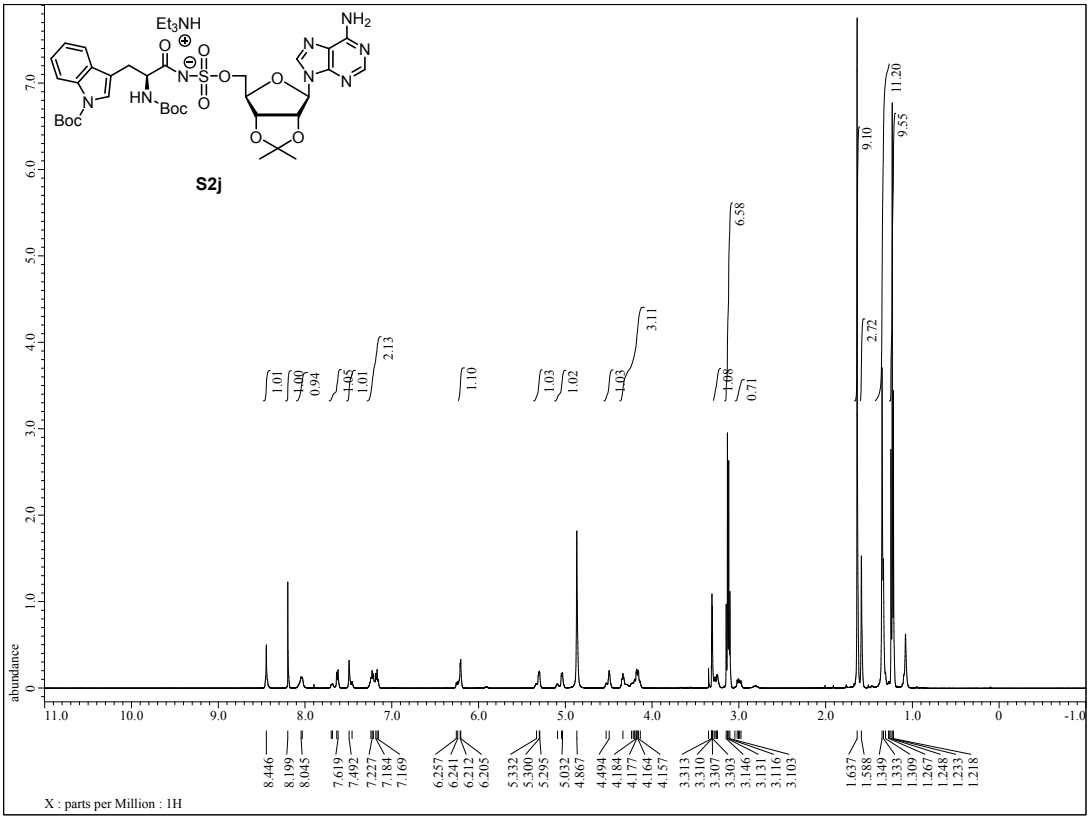


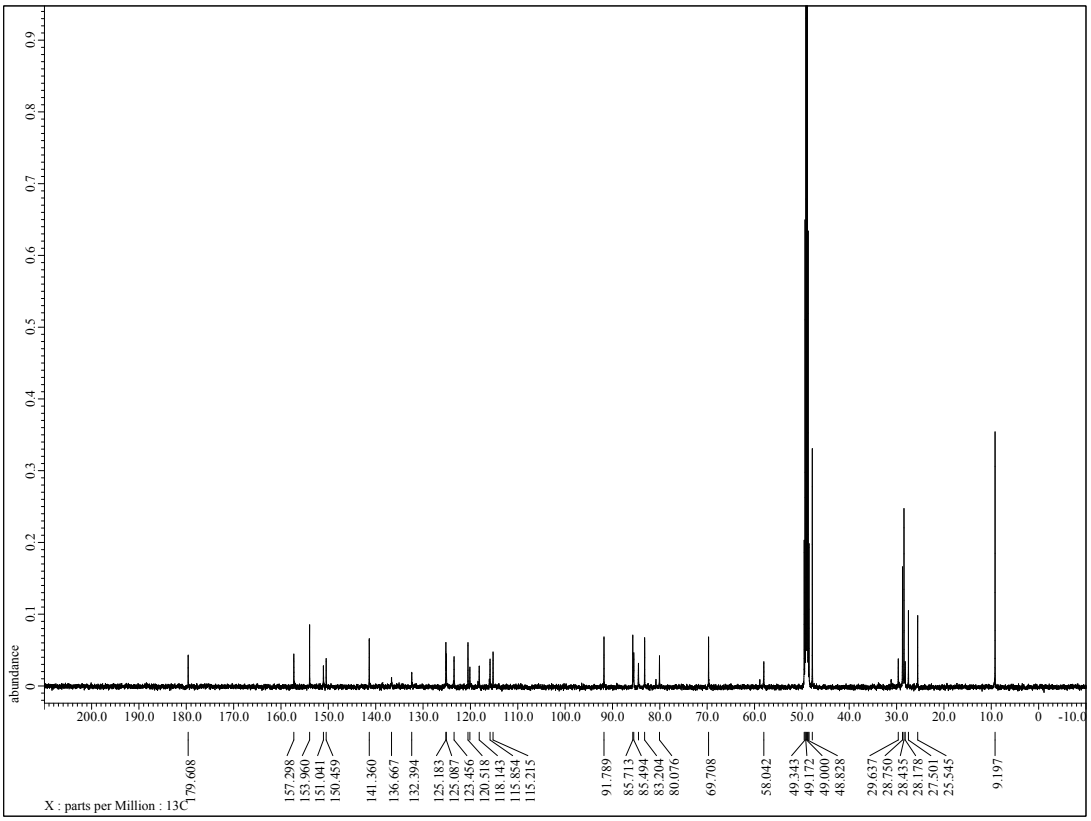


^{1}H-NMR (500 MHz) spectrum of **19** in CD_3OD

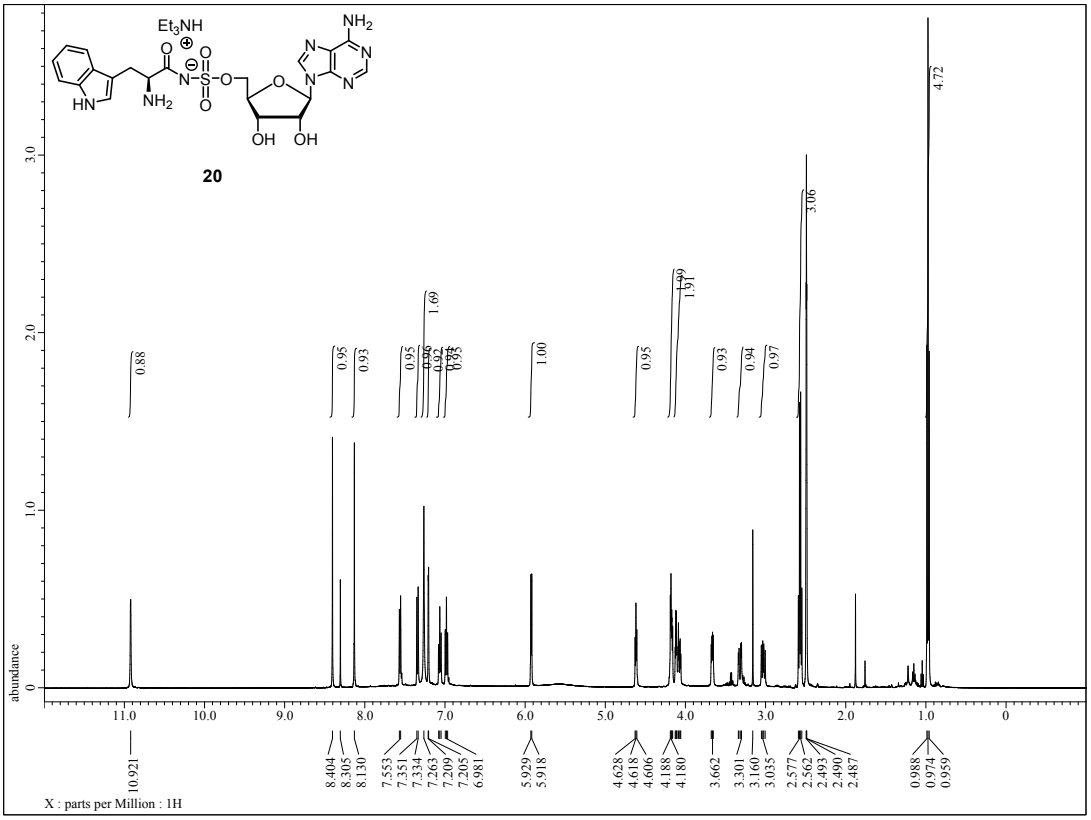


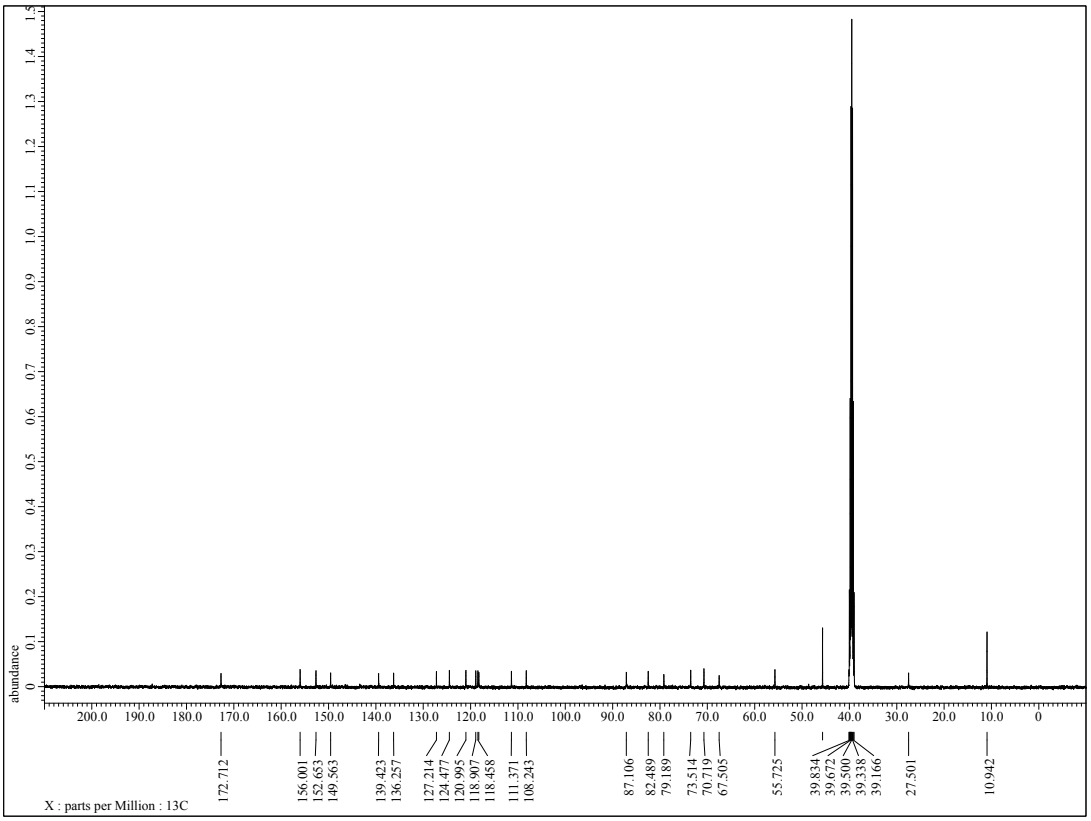
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2j**



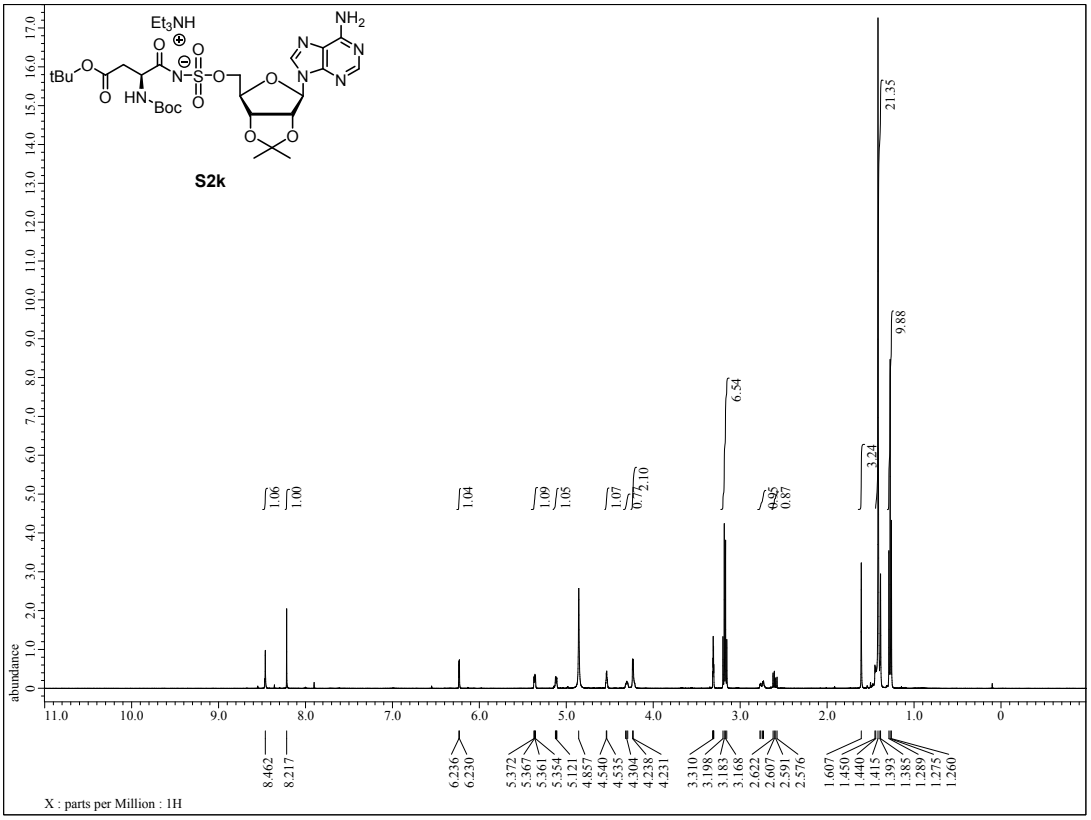


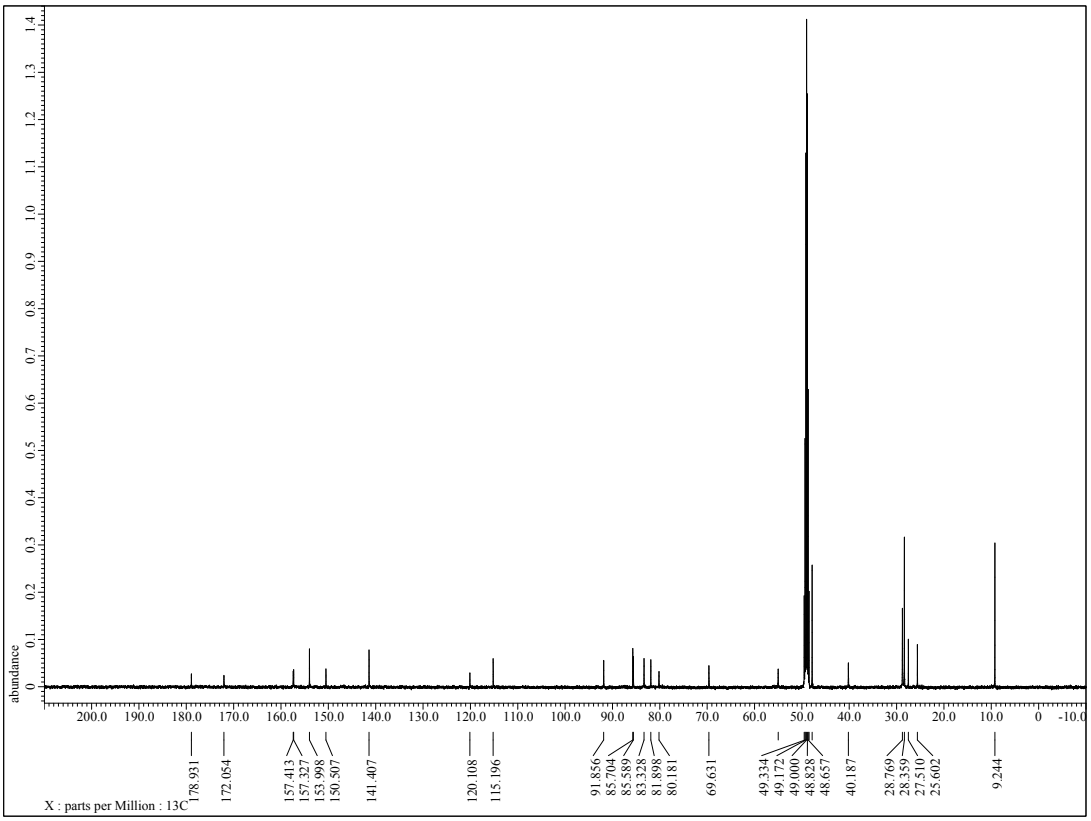
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **20**



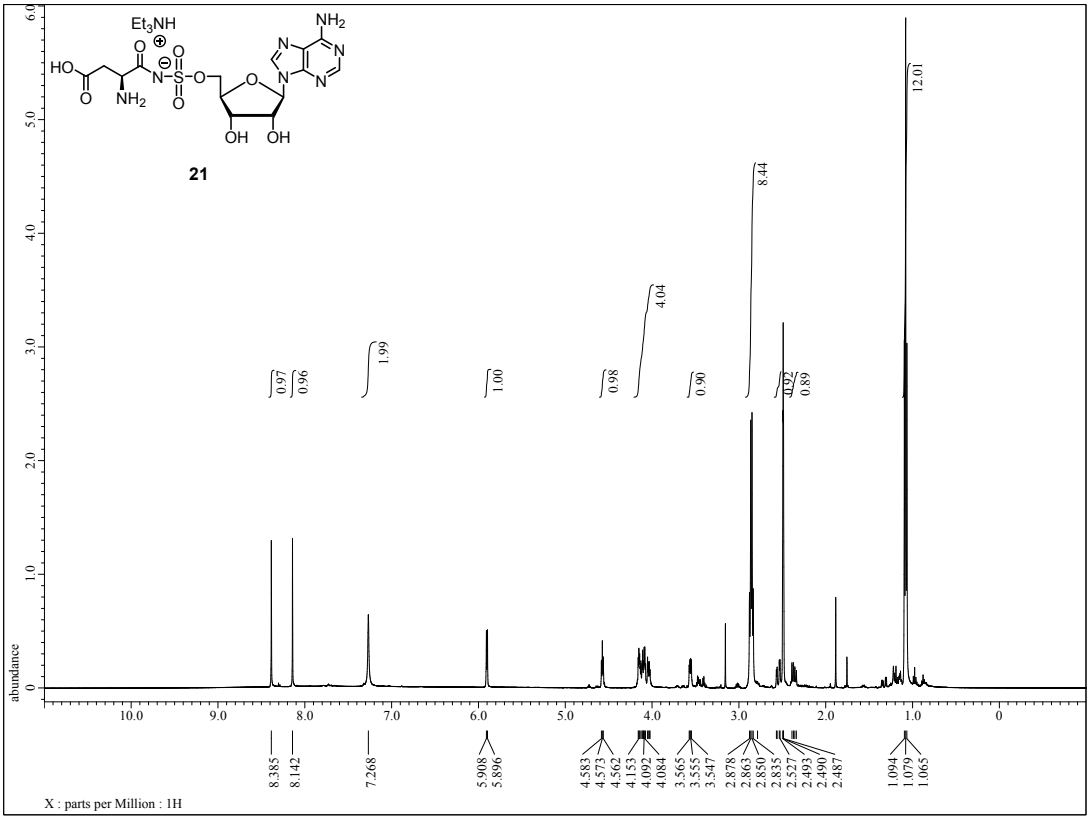


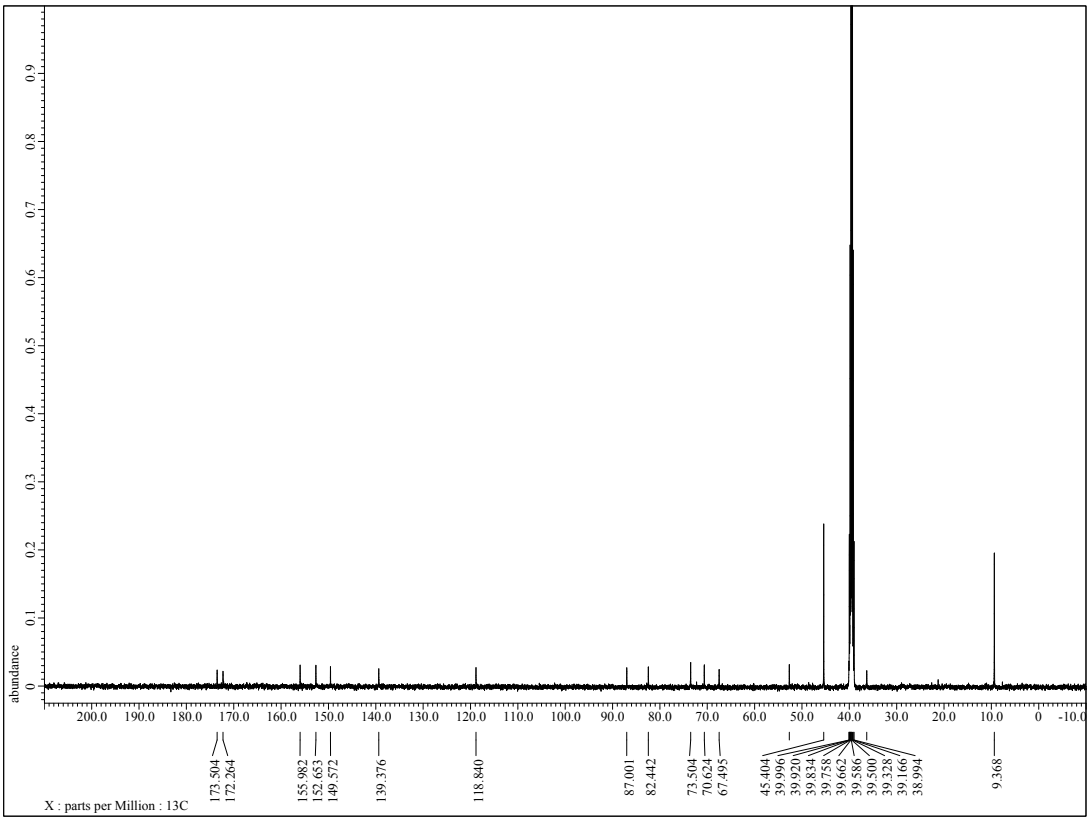
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2k**



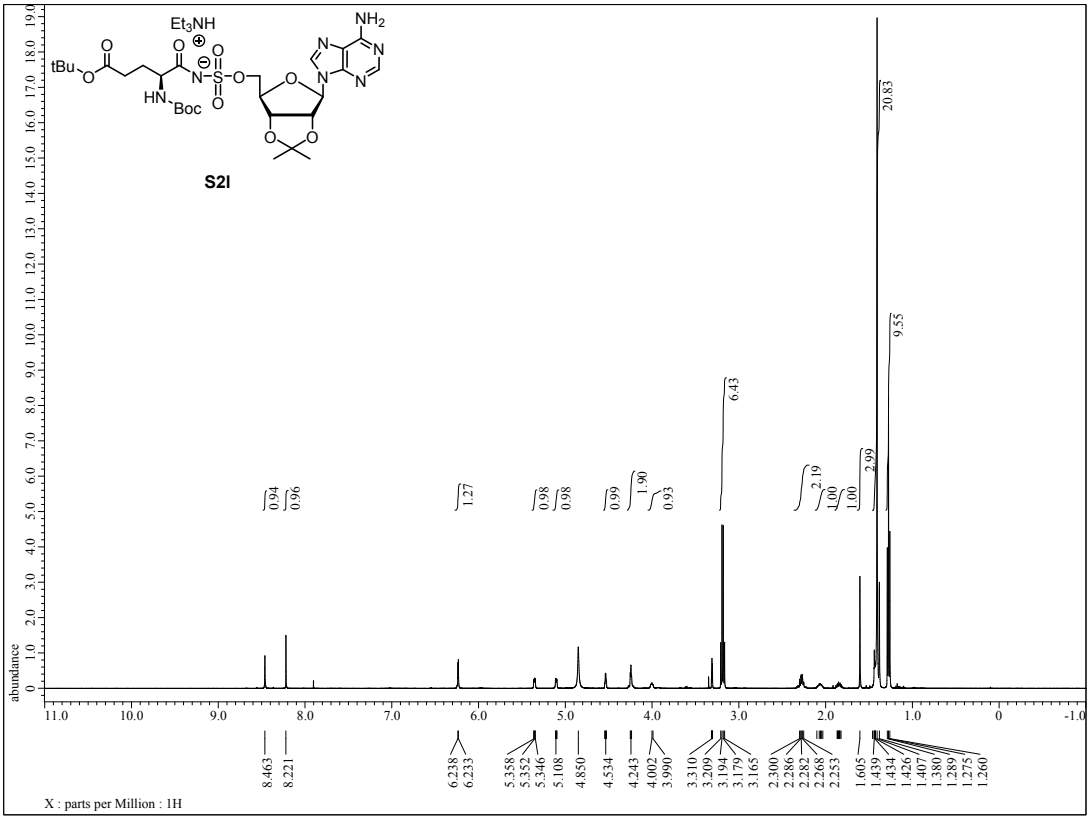


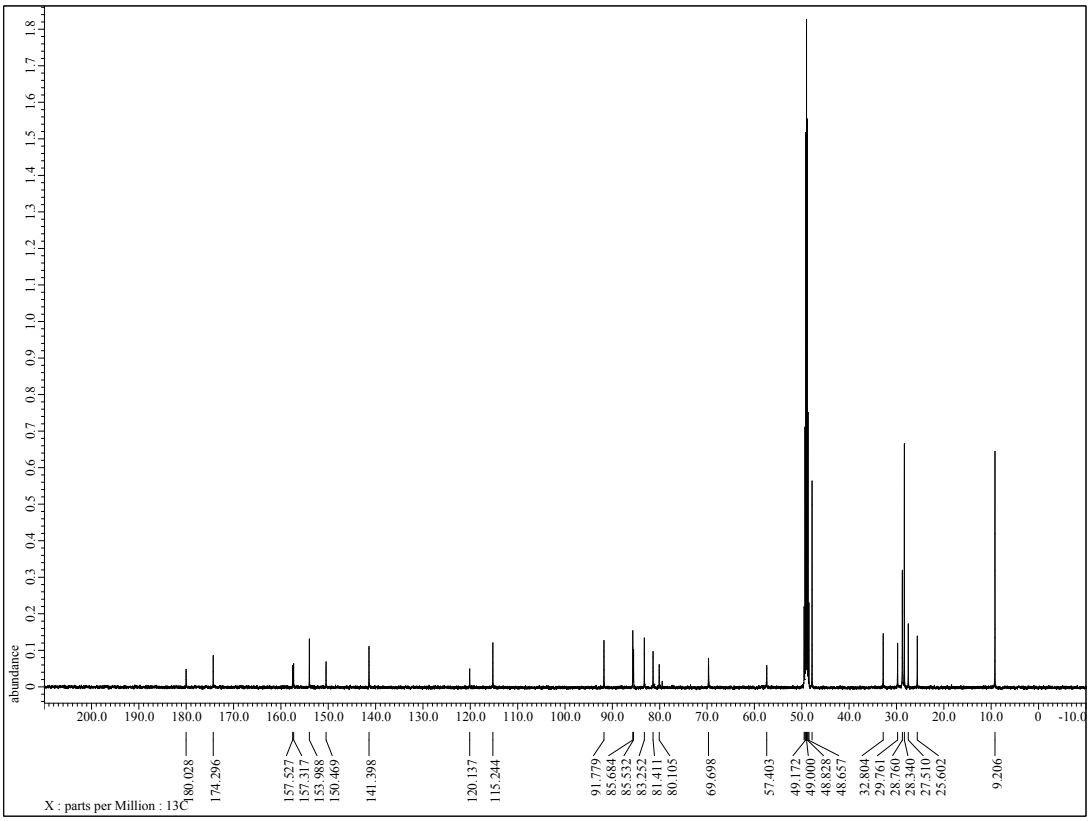
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **21**



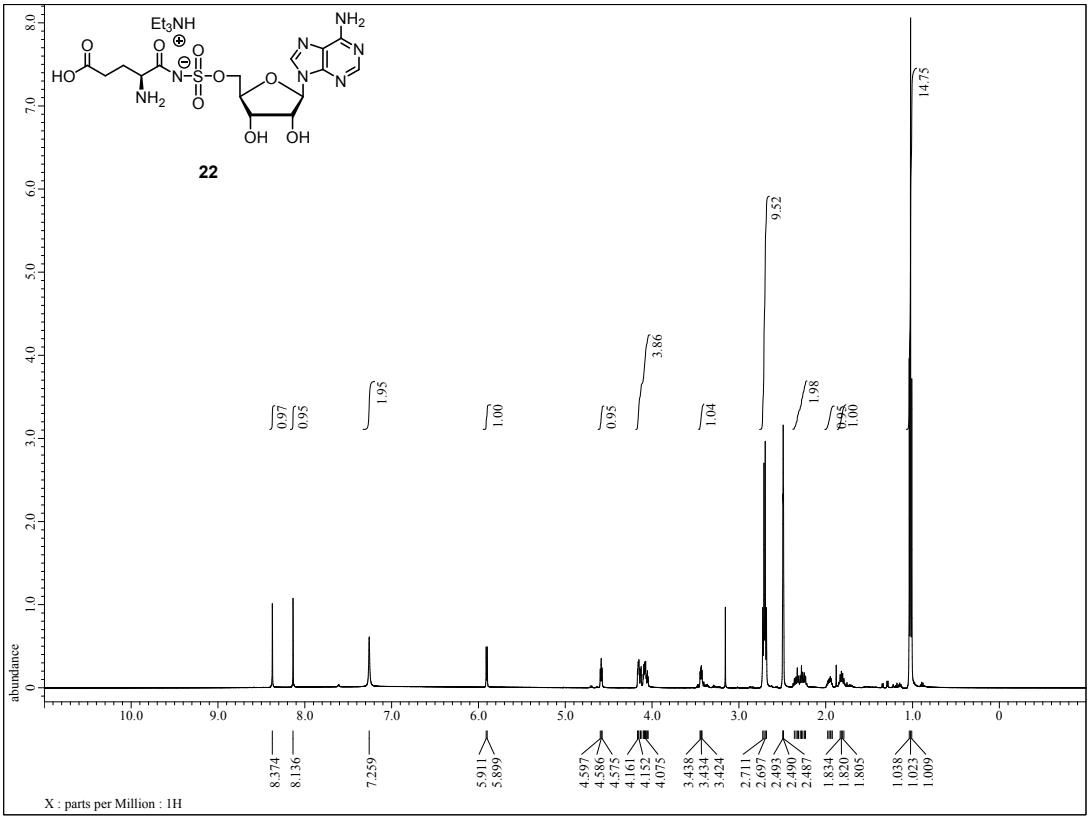


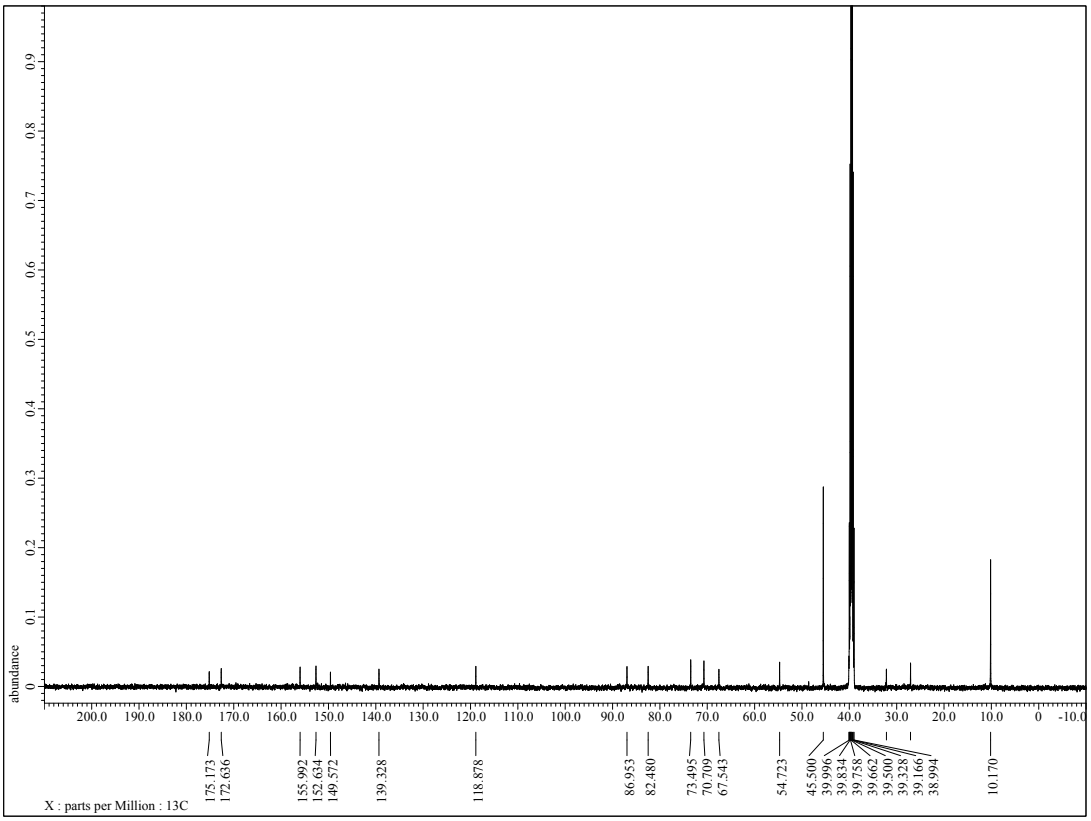
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2l**



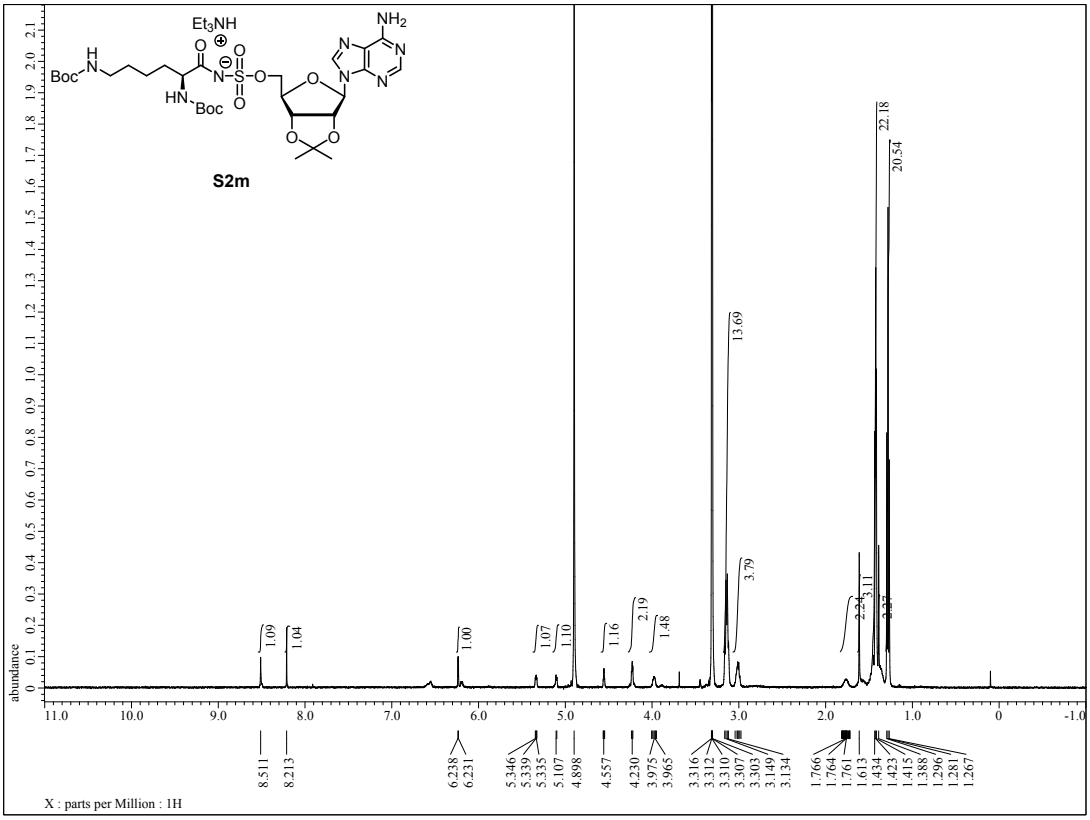


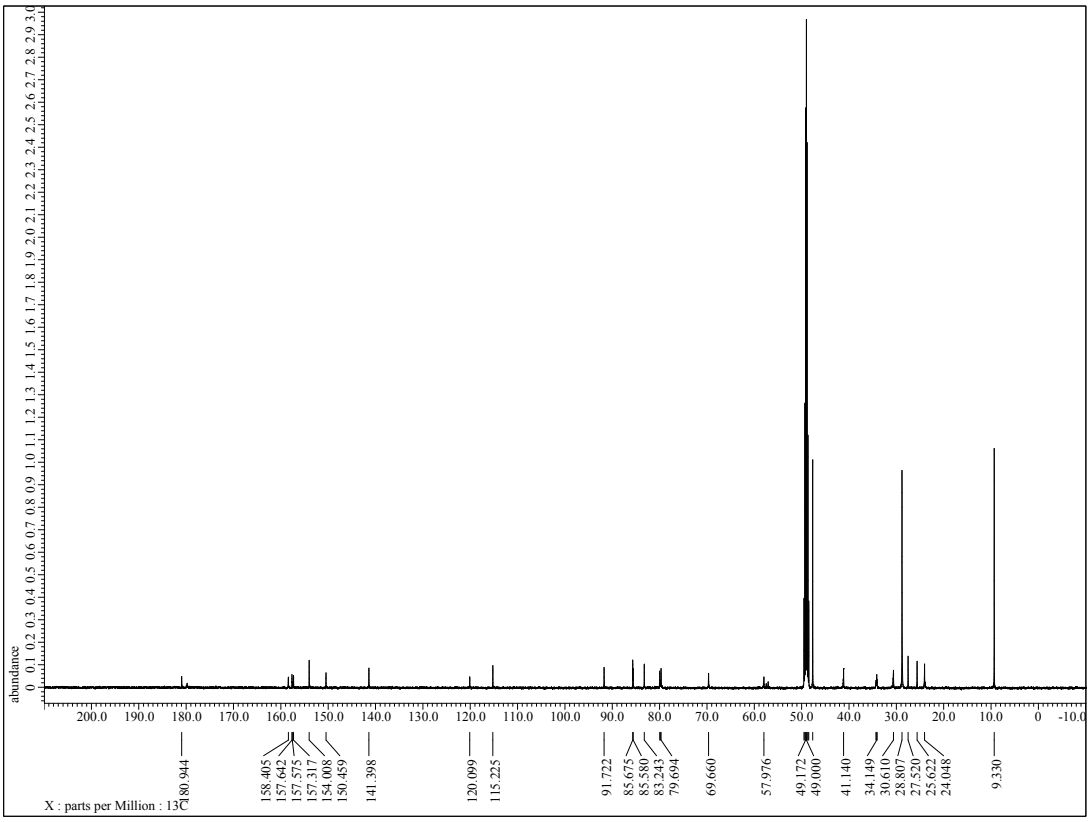
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **22**



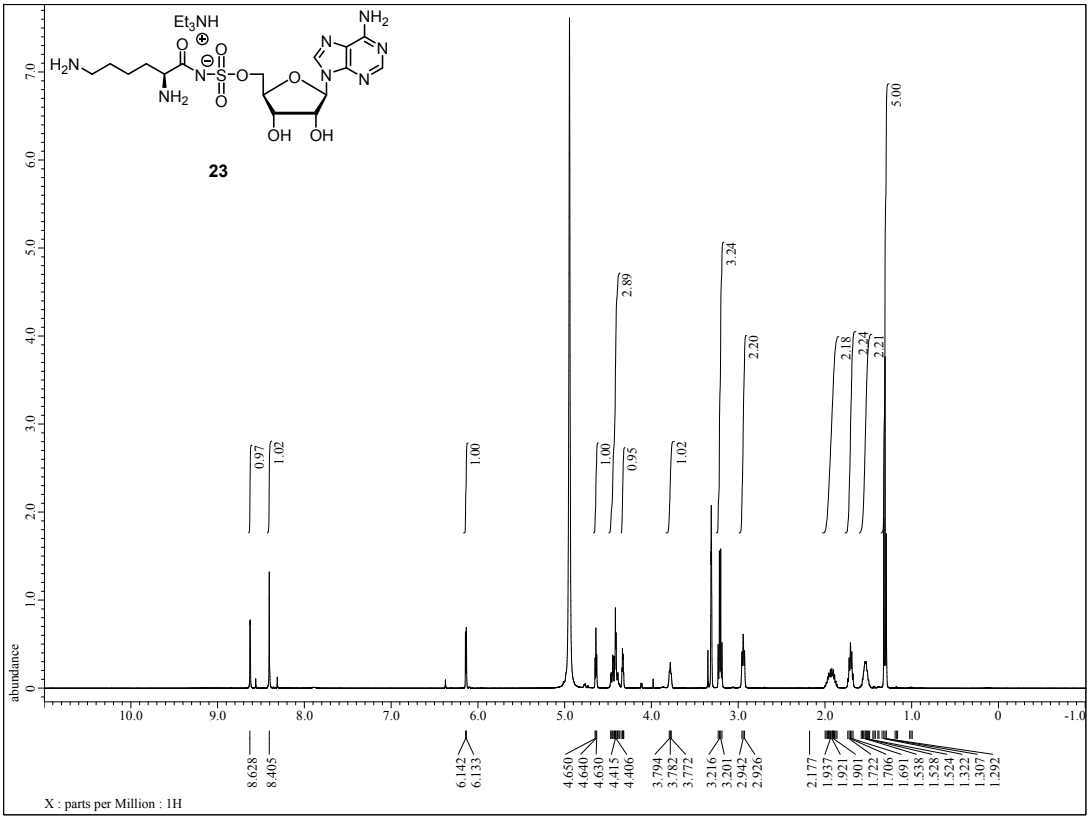


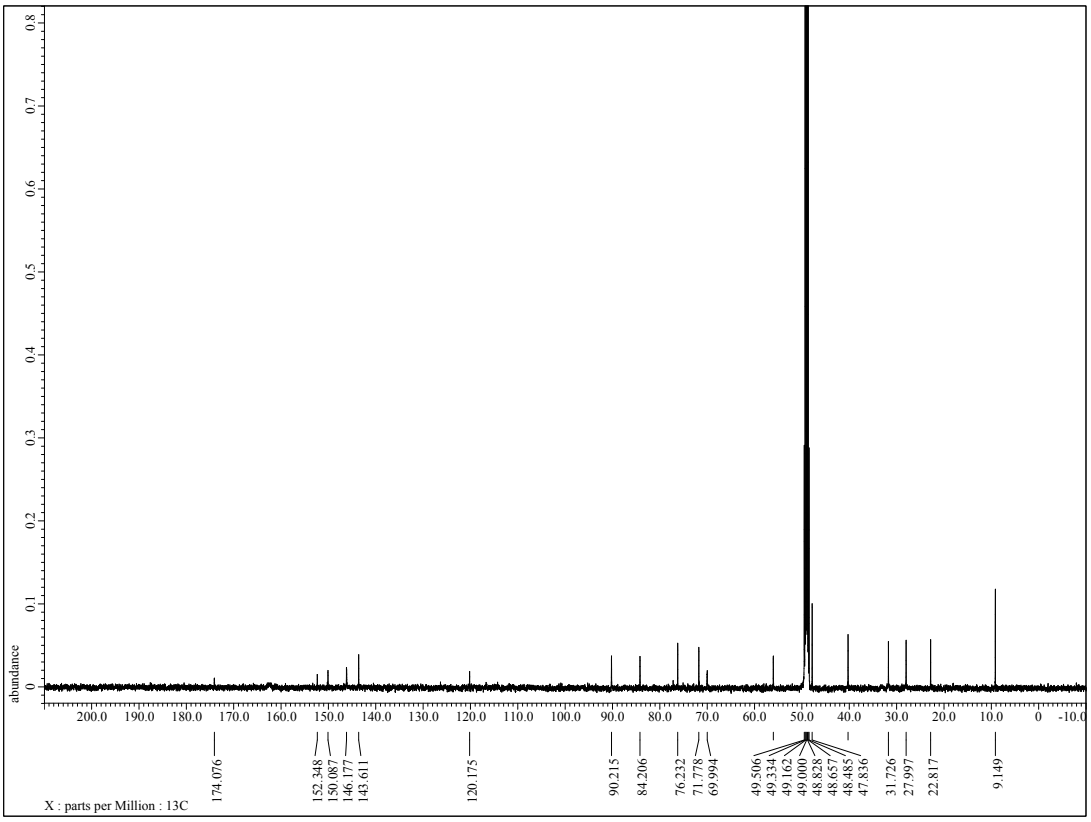
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2m**



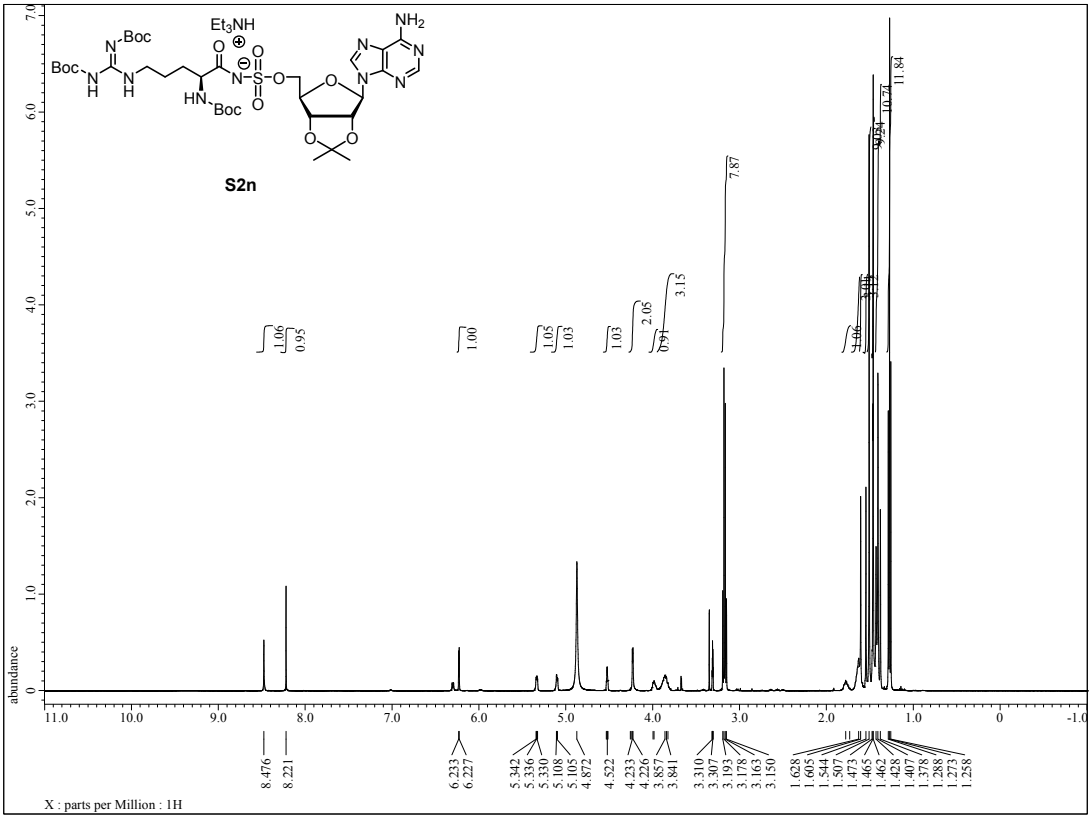


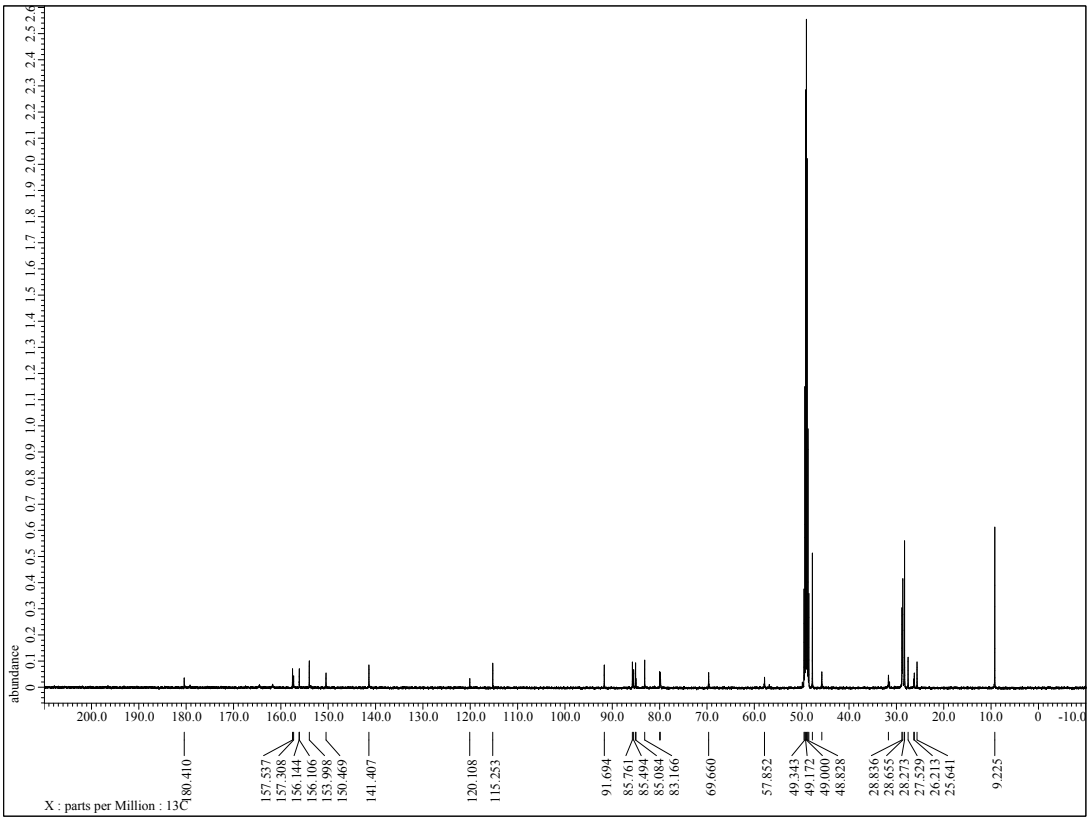
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **23**



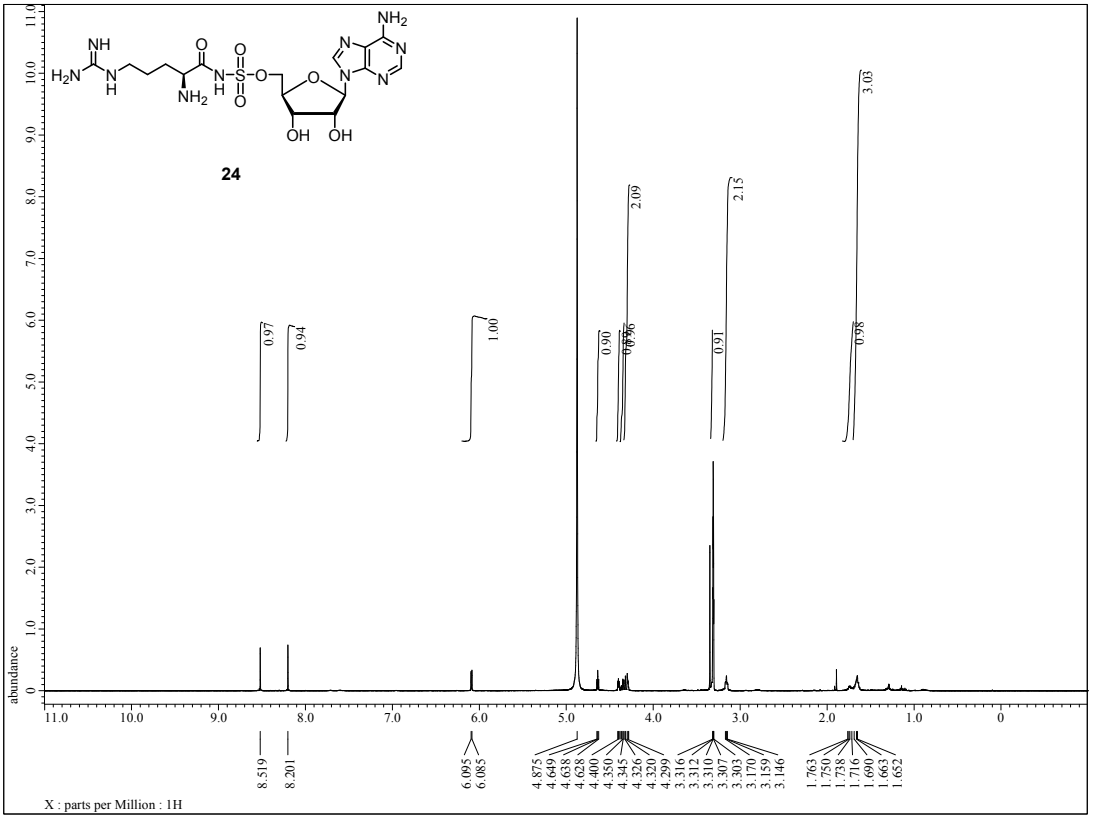


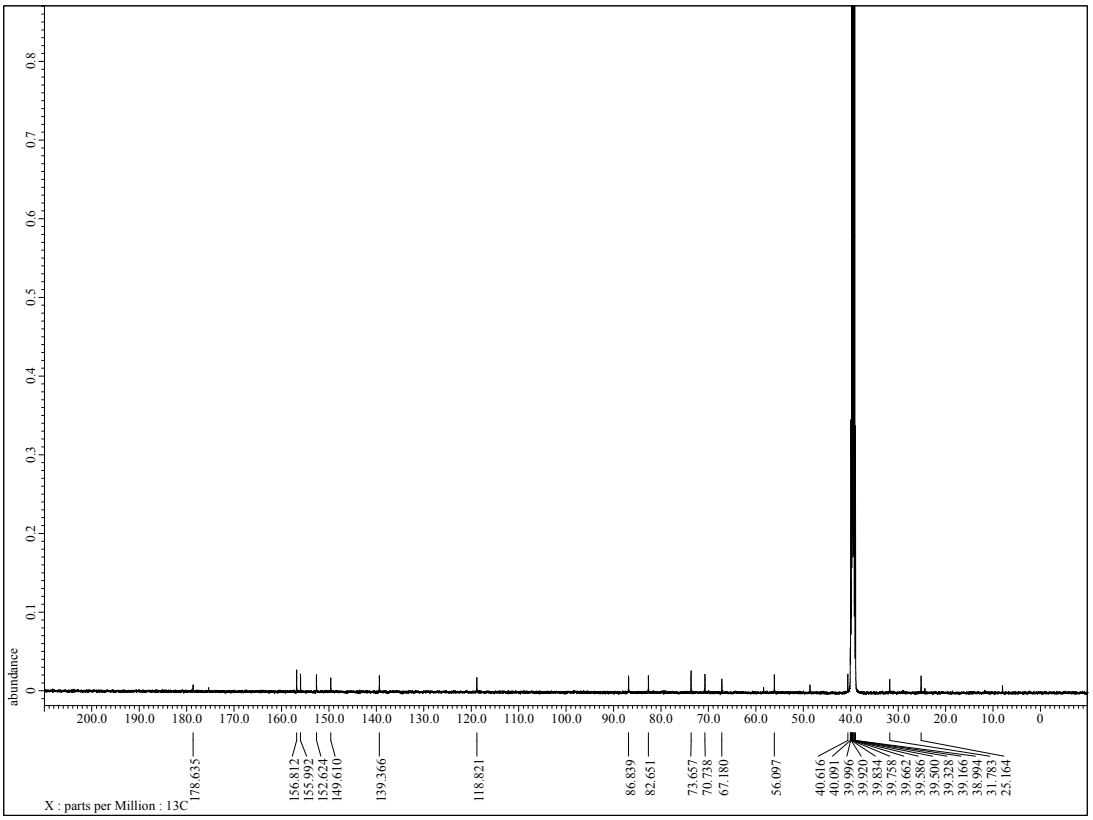
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2n**



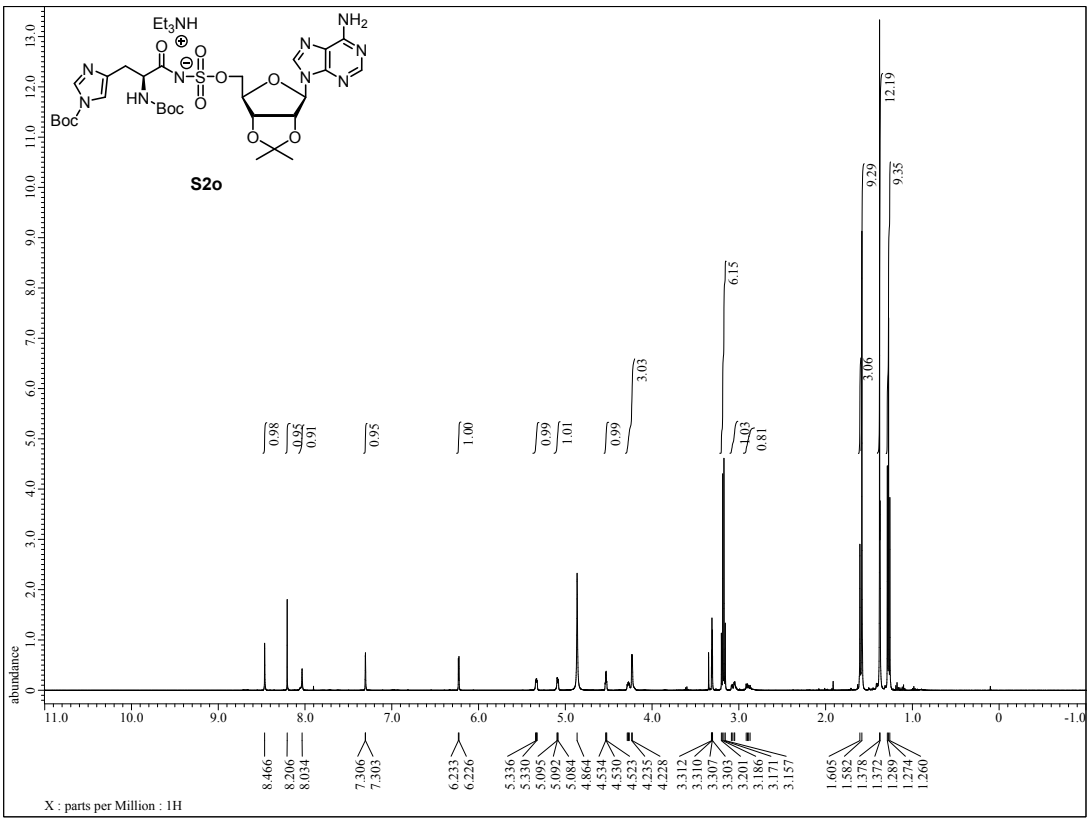


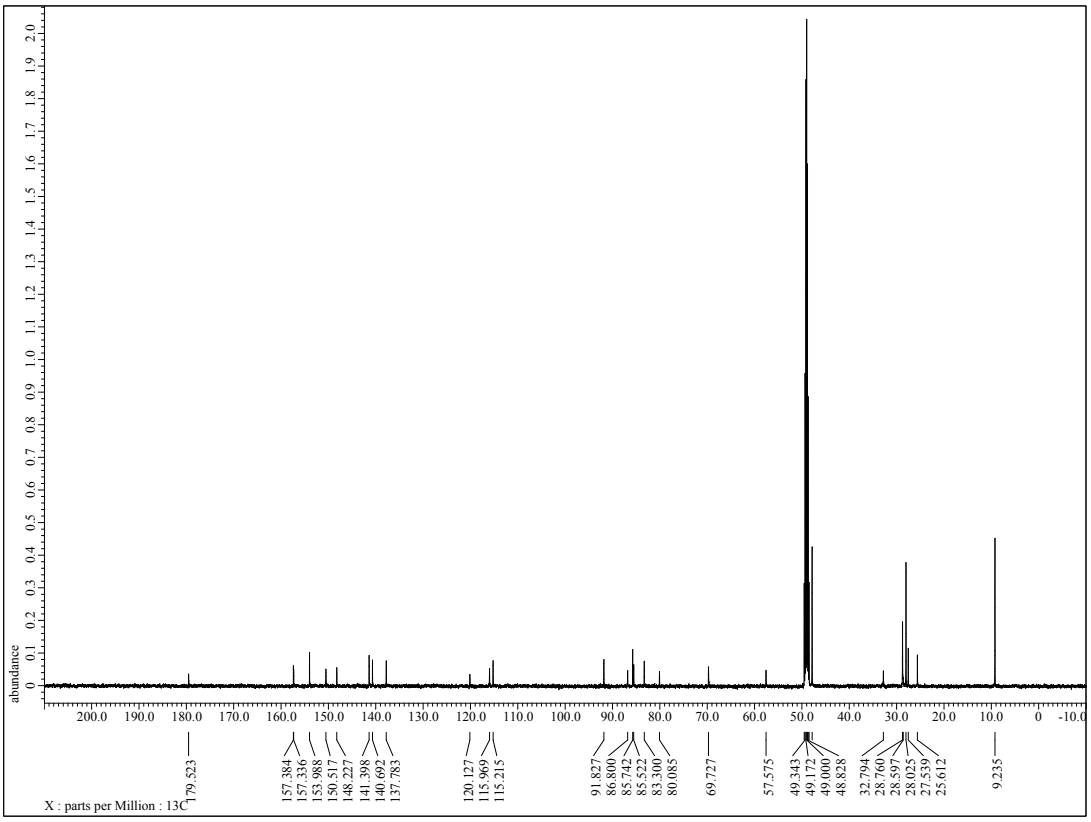
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **24**





^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2o**





^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **25**