Supporting Information

Accurate detection of adenylation domain functions in nonribosomal peptide synthetases by an enzyme-linked immunosorbent assay system using active site-directed probes for adenylation domains

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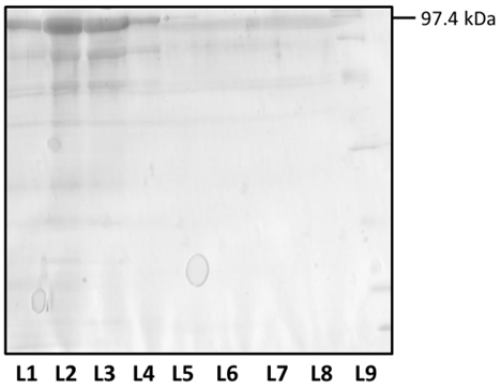


Figure S1. Full gel depicting the purification of recombinant AusA2. Gel lanes depict fractions taken during Ni-sepharose chromatography and are as follows: L1 = 20 mM imidazole wash, L2 = 40 mM imidazole wash, L3 = 80 mM imidazole wash, L4 = 100 mM imidazole wash, L5 = 150 mM imidazole wash, L6 = 200 mM imidazole wash, L7 = 250 mM imidazole wash, L8 = 500 mM imidazole wash, and L9 = SDS-PAGE standards, broad range (Bio-Rad Laboratories, Inc.). The target protein was collected and used from L1–L4. The gel was stained with Coomassie Brilliant Blue (CBB).

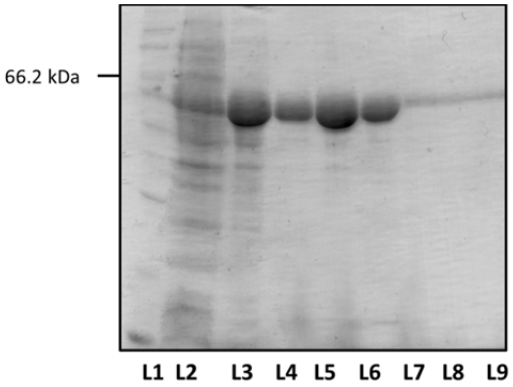


Figure S2. Full gel depicting the purification of recombinant EntE. Gel lanes depict fractions taken during Ni-sepharose chromatography and are as follows: L1 = SDS-PAGE standards, broad range (Bio-Rad Laboratories, Inc.), L2 = flow through, L3 = 5 mM imidazole wash, L4 = 10 mM imidazole wash, L5 = 20 mM imidazole wash, L6 = 40 mM imidazole wash, L7 = 80 mM imidazole wash, L8 = 100 mM imidazole wash, and L9 = 150 mM imidazole wash. The target protein was collected and used from L4–L6 and used in subsequent experiments. The gel was stained with Coomassie Brilliant Blue (CBB).

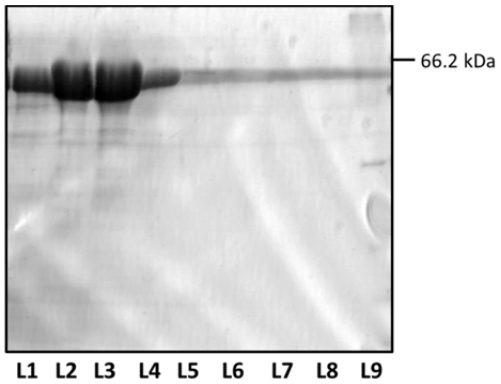


Figure S3. Full gel depicting the purification of recombinant EntE (S240C). Gel lanes depict fractions taken during Ni-sepharose chromatography and are as follows: L1 = 20 mM imidazole wash, L2 = 40 mM imidazole wash, L3 = 80 mM imidazole wash, L4 = 100 mM imidazole wash, L5 = 150 mM imidazole wash, L6 = 200 mM imidazole wash, L7 = 250 mM imidazole wash, L8 = 500 mM imidazole wash, and L9 = SDS-PAGE standards, broad range (Bio-Rad Laboratories, Inc.). The target protein was collected and used from L1–L5 and used in subsequent experiments. The gel was stained with Coomassie Brilliant Blue (CBB).

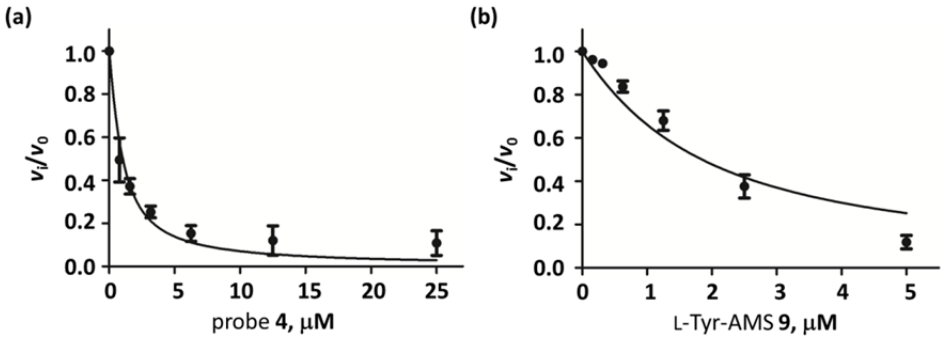


Figure S4. Inhibitory activities of recombinant AusA2 by L-Tyr-AMS-biotin 4 and L-Tyr-AMS 9. (a) Inhibition of *apo*-AusA2 by **4**. The reactions contained 635 nM *apo*-AusA2, 1 mM L-Tyr, standard assay buffer [20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, and 0.2 mM MesG]. (b) Inhibition of AusA2 by **9**. The reactions contained 635 nM *apo*-AusA2, 1 mM L-Tyr, and the standard assay buffer.

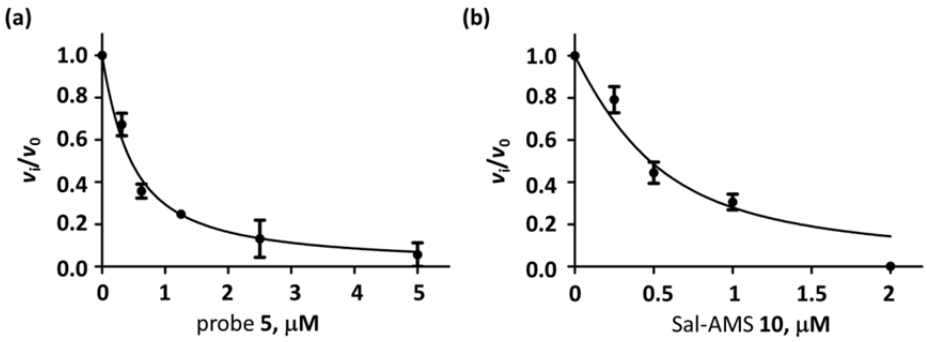


Figure S5. Inhibitory activities of recombinant EntE by DHB-AMS-biotin 5 and Sal-AMS 10. (a) Inhibition of EntE by **5**. The reactions contained 200 nM EntE, 50 μM DHB, standard assay buffer [20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, and 0.2 mM MesG]. (b) Inhibition of EntE by **10**. The reactions contained 400 nM EntE, 50 μM DHB, and the standard assay buffer.

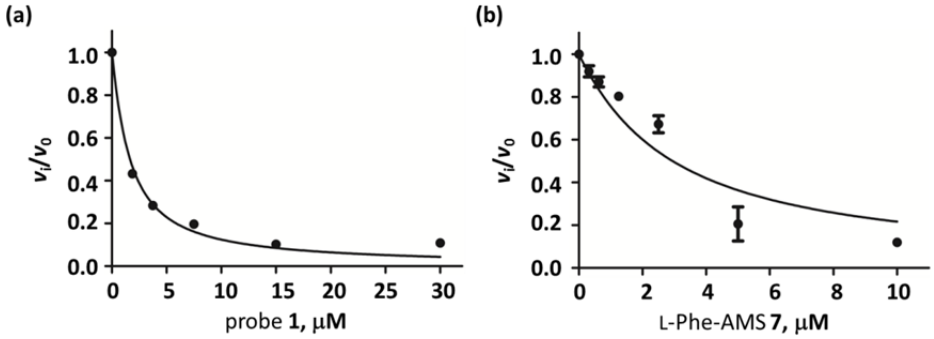


Figure S6. Inhibitory activities of recombinant AusA2 by L-Phe-AMS-biotin 1 and L-Phe-AMS 7. (a) Inhibition of *apo*-AusA2 by **1**. The reactions contained 635 nM *apo*-AusA2, 1 mM L-Tyr, standard assay buffer [20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, and 0.2 mM MesG]. (b) Inhibition of AusA2 by **7**. The reactions contained 635 nM *apo*-AusA2, 1 mM L-Tyr, and the standard assay buffer.

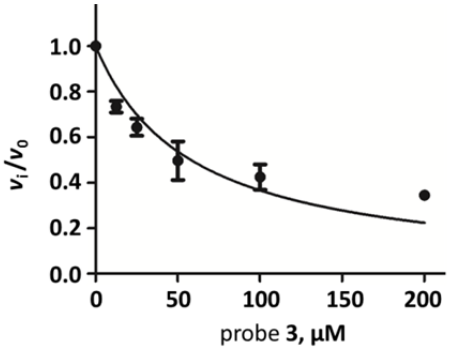


Figure S7. Inhibitory activity of recombinant TycB1 by L-Pro-AMS-biotin 3. Inhibition of *holo*-TycB1 by **3**. The reactions contained 400 nM *holo*-TycB1, 1 mM L-Pro, standard assay buffer [20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, and 0.2 mM MesG].

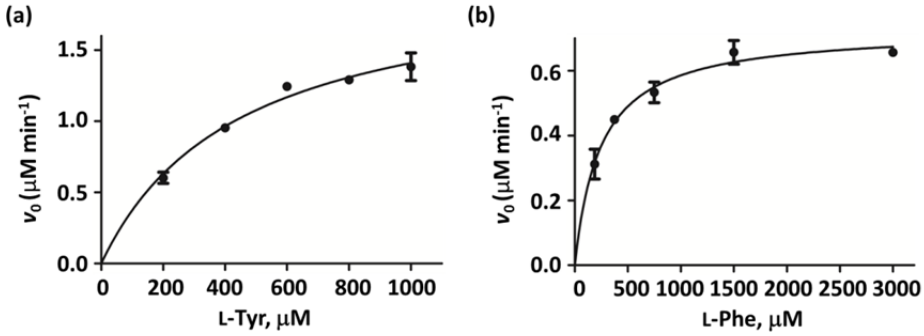


Figure S8. Steady-state kinetics of AusA2. (a) Each reaction contained 635 nM *apo*-AusA2, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 200–1000 μM L-Tyr. (b) The reactions contained 635 nM *apo*-AusA2 and 188–3000 μM L-Phe. Velocities were fit to the Michaelis-Menten equation.

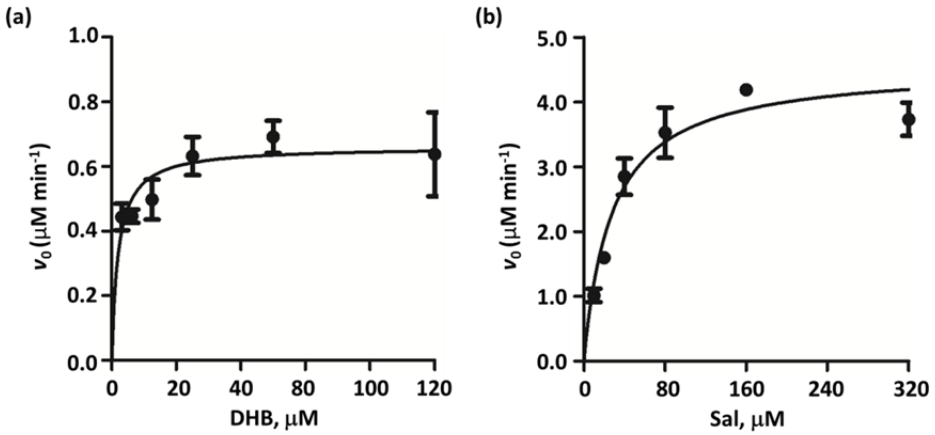


Figure S9. Steady-state kinetics of EntE. (a) Each reaction contained 250 nM EntE, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 3.125–120 μM DHB. (b) The reactions contained 250 nM EntE and 20–320 μM Sal. Velocities were fit to the Michaelis-Menten equation.

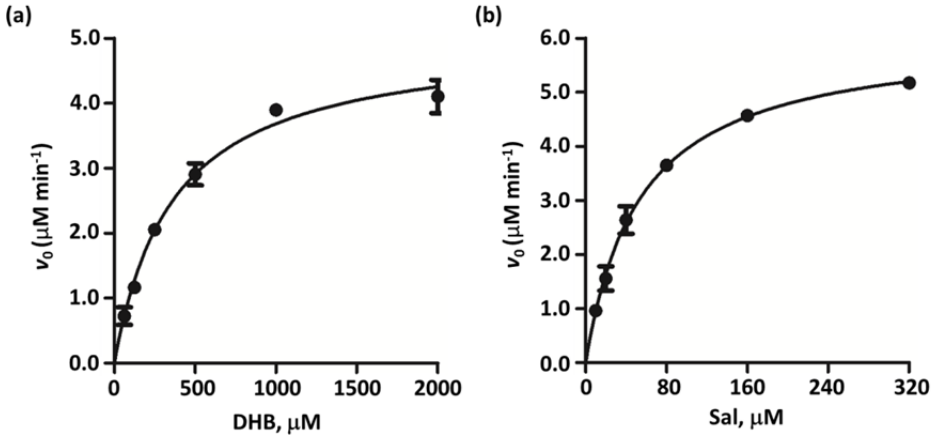


Figure S10. Steady-state kinetics of EntE (S240C). (a) Each reaction contained 500 nM EntE (S240C), 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, 0.2 mM MesG, and 62.5–2000 μM DHB. (b) The reactions contained 500 nM EntE (S240C) and 10–320 μM Sal. Velocities were fit to the Michaelis-Menten equation.

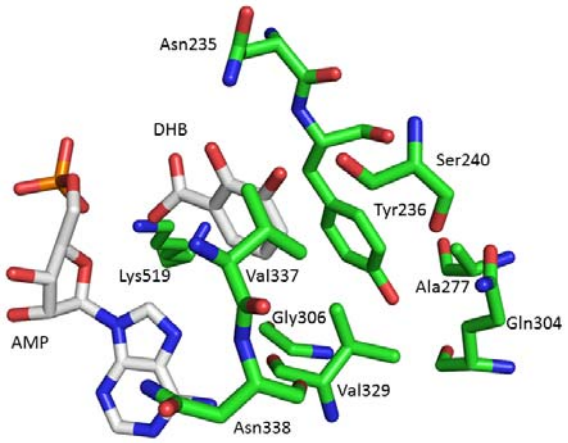


Figure S11. Close-up view of the residues in the active site of DhbE involved in the specificity conferring code.[1] The ligands DHB and AMP are shown as ball and stick models, with the following color code: nitrogen, blue; oxygen, red; phosphate, orange; carbon, white. Modified from PDB code 1MD9 using using PyMOL.

Table S1. Inhibition constants of probes **1**–**6** and the cognate competitors **7**–**10** of the probes.[a]

	$K_i^{app.}$ (nM)			
compounds	GrsA	TycB1	AusA2	EntE
probe **1**	34.0 ± 2.8[b]		413 ± 31	
probe **2**		6380 ± 880		
probe **3**				
probe **4**			220 ± 37	
probe **5**				13.6 ± 2.1
probe **6**				
L-Phe-AMS **7**	1.20 ± 0.14[b]		807 ± 138	
L-Pro-AMS **8**		431 ± 42[c]		
L-Tyr-AMS **9**			471 ± 69	
Sal-AMS **10**				10.7 ± 2.4

[a]Apparent K_i values were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[2] Errors were given as the standard deviation of multiple independent measurements.

[b]Ishikawa, F; Kakeya, H. *Bioorg. Med. Chem. Lett.* **2014**, 24, 865–869.

[c]Konno, S; Ishikawa, F.; Suzuki, T.; Dohmae, N.; Burkart, M. D.; Kakeya, H. *Chem. Commun.* **2015**, 51, 2262–2265.

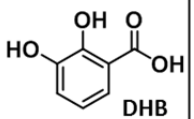
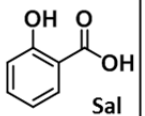
Table S2. Kinetic parameters of the A-domains of NRPS enzymes.[a]

protein	substrate	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m (mM^{-1} min^{-1})
GrsA	L-Phe[b]	500 ± 12	24.8 ± 2.3	20161 ± 5217
GrsA	(*S*)-β-Phe	67.2 ± 3.6	522 ± 74	129 ± 74.3
TycB1	L-Pro[b]	6.36 ± 0.28	125 ± 24	50.8 ± 11.6
AusA2	L-Phe	1.15 ± 0.047	244 ± 40	4.71 ± 1.18
AusA2	L-Tyr	3.21 ± 0.25	445 ± 83	7.21 ± 3.01
EntE	DHB	2.64 ± 0.17	2.02 ± 0.76	1311 ± 229
EntE	Sal	11.4 ± 0.91	27.6 ± 7.8	412 ± 117
EntE (S240C)	DHB	10.1 ± 0.42	364 ± 44	27.6 ± 9.6
EntE (S240C)	Sal	12.2 ± 0.33	54 ± 4.2	226 ± 7.7

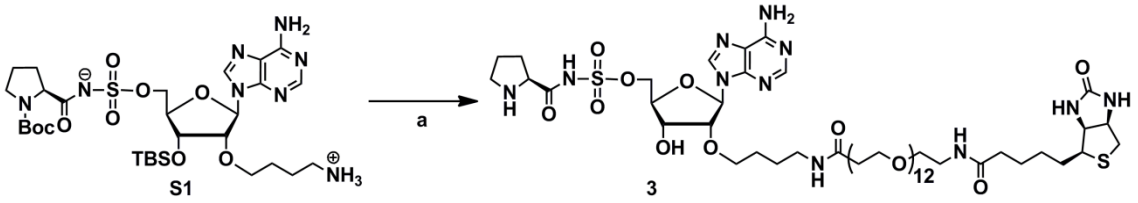
[a]Kinetic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[2] Errors were given as the standard deviation of multiple independent measurements.

[b]Kasai, S.; Konno, S.; Ishikawa, F.; Kakeya, H. *Chem. Commun.* (2015) DOI: 10.1039/C5CC04953A.

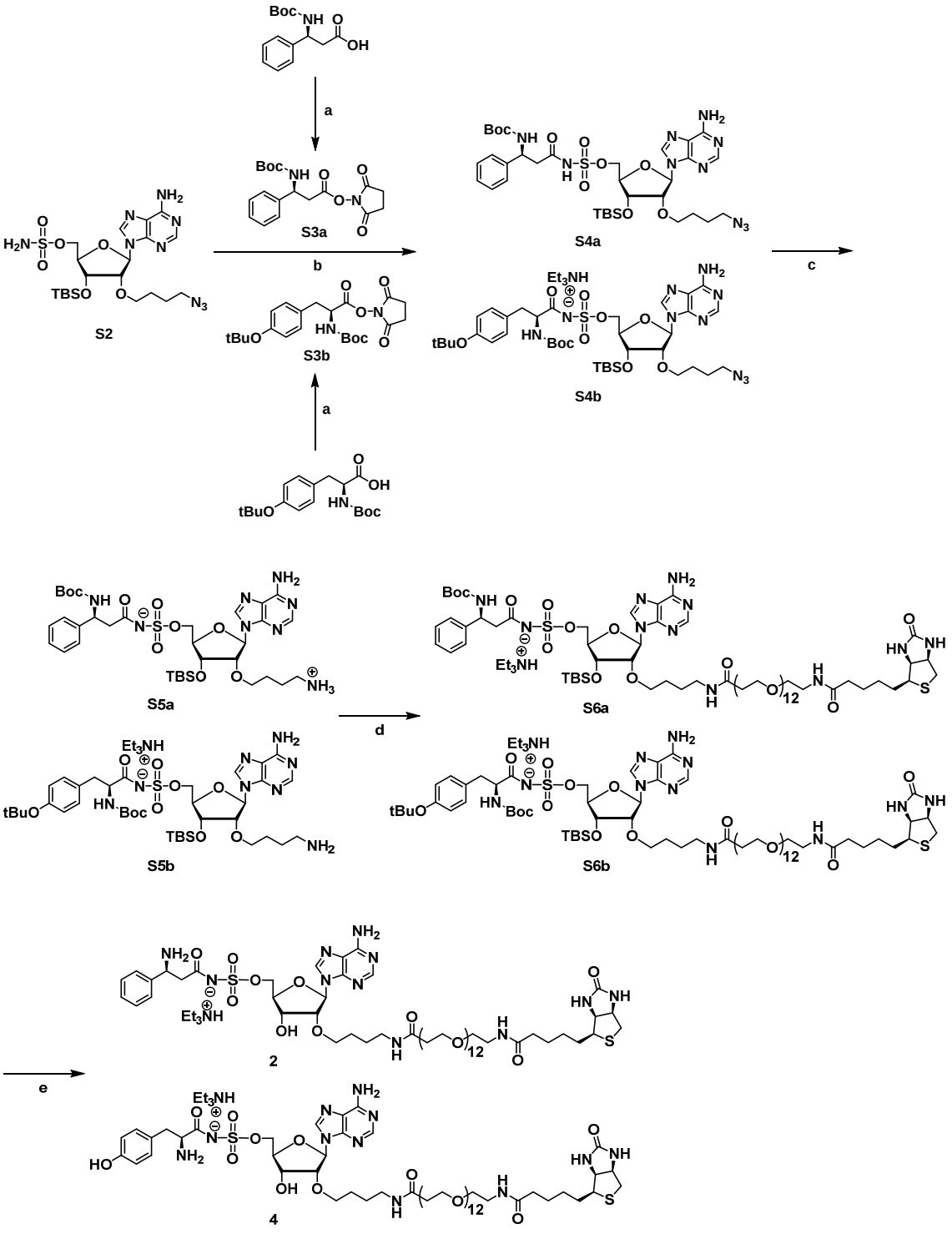
Table S3. Comparison of the 10-residue specificity codes for aryl acid adenylating enzymes.[1]

	substrate	235	236	240	277	306	308	331	339	340	520
EntE	 DHB	N	Y	S	A	Q	G	V	V	N	K
DhbE		N	Y	S	A	Q	G	V	V	N	K
BasE		N	F	S	S	Q	G	V	V	N	K
YbtE	 Sal	N	F	C	A	Q	G	V	L	C	K
MbtA		N	F	C	A	Q	G	V	L	N	K
PchD		N	F	C	A	Q	G	V	I	C	K

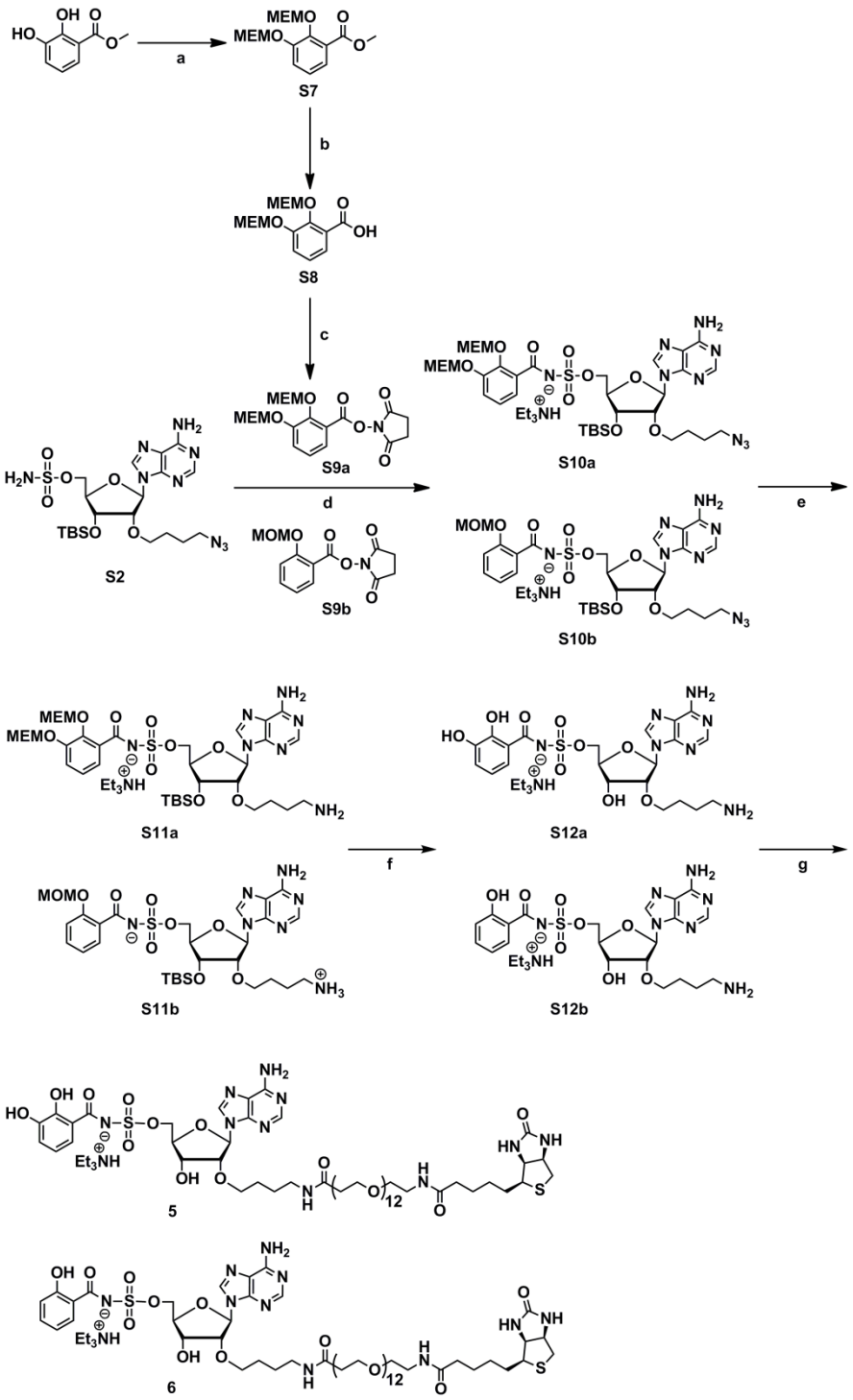
Chemical Synthetic Procedures



Scheme S1. Syntehtic route to L-Pro-AMS-biotin 3. *Reagents and conditions*: [a] 1) EZ-link NHS-Peg_{12}-Biotin, DIEA, DMF, rt; 2) 80% aqueous TFA, rt, 45%, over two steps.



Scheme S2. Synthetic route to (*S*)-β-Phe-AMS-biotin 2 and L-Tyr-AMS-biotin 4. *Reagents and conditions*: [a] EDC, NHS, CH_2Cl_2, rt; [b] Cs_2CO_3, DMF, rt; [c] Pd/C, EtOH, H_2, rt: 32% (**S5a**), over two steps; 37% (**S5b**), over two steps; [d] EZ-link NHS-Peg_{12}-Biotin, DIEA, DMF, rt: 96% (**S6a**); 92% (**S6b**); [e] 80% aqueous TFA, rt: 53% (**2**); a mixture of 90:5:5 (v/v) of TFA, H_2O, and TIS, rt, 75% (**4**).

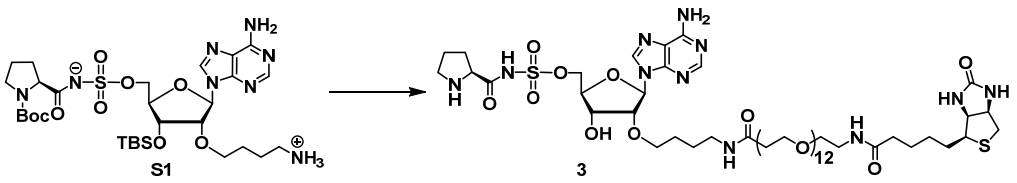


Scheme S3. Synthetic route to DHB-AMS-biotin 5 and Sal-AMS-biotin 6. *Reagents and conditions*: [a] NaH, MEMCl, THF, 0 °C→rt, 73%; [b] 1 M NaOH aq., MeOH, 70 °C, 47%; [c] NHS, EDC, CH_2Cl_2, rt; [d] Cs_2CO_3, DMF, rt: 79% (**S10a**); 70% (**S10b**); [e] Pd/C, H_2, EtOH, rt: 31% (**S11a**); 88% (**S11b**); [f] 80% aqueous TFA, rt: 61% (**S12a**); 43% (**S12b**); [g] EZ-link NHS-Peg_{12}-Biotin, DIEA, DMF, rt: 41% (**5**); 66% (**6**).

General Synthetic Methods: All commercial reagents were used as provided unless otherwise indicated. **S1**[3] (Scheme S1), **S2**[4] (Schemes S2 and S3), **S9b**[5] (Scheme S3), L-Phe-AMS-biotin **1**,[4] L-Phe-AMS **7**,[4] L-Pro-AMS **8**,[3] L-Tyr-AMS **9**,[5] and Sal-AMS **10**[6] are 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.[7] Multiplicities are given as s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, ddd = doublet of doublet of doublets, dddd = doublet of doublet of doublet of doublets, 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.[8] Mass spectral data were obtained using a LCMS-IT-TOF mass spectrometer (Shimadzu).

Chemical Synthesis of L-Pro-AMS-biotin 2. Compound number in bold refers to the structures shown in Scheme S1.

L-Pro-AMS-biotin (2)

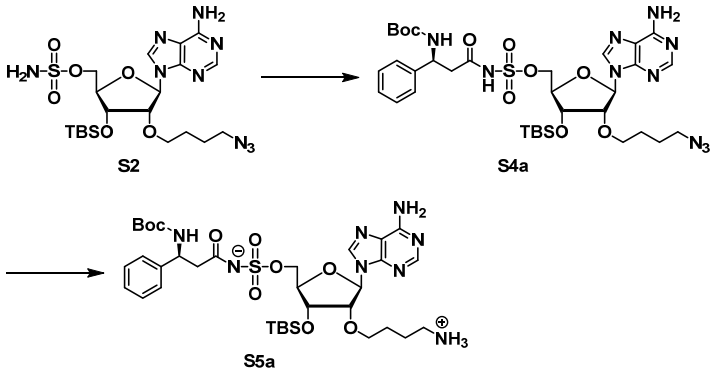


EZ-link NHS-Peg$_{12}$-Biotin (Thermo Fisher Scientific Inc.) (16 mg, 0.017 mmol) and DIEA (3 μL, 0.017 mmol) were added to a solution of compound **S1** (10 mg, 0.014 mmol) in DMF (2 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was concentrated under reduced pressure. HRMS (ESI–): $[M–H]^-$ calcd for $C_{67}H_{118}N_{11}O_{24}S_2Si$, 1552.7562; found, 1552.7785. The residue was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 12 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 (9:1 $CHCl_3$/MeOH to MeOH) to afford compound **3** as a colorless oil (8.4 mg, 45%, over two steps). ^{1}H NMR (500 MHz, CD_3OD): δ 8.52 (s, 1H), 8.22 (s, 1H), 6.18 (d, *J* = 5.1 Hz, 1H), 4.52–4.44 (m, 3H), 4.35 (dd, *J* = 6.6, 2.6 Hz, 1H), 4.33–4.28 (m, 2H), 3.75–3.68 (m, 2H), 3.66–3.58 (m, 51H), 3.54 (t, *J* = 5.5 Hz, 2H), 3.36 (t, *J* = 5.5 Hz, 2H), 3.24–3.18 (m, 1H), 3.13 (t, *J* = 6.9 Hz, 1H), 2.92 (dd, *J* = 12.9, 4.9 Hz, 1H), 2.71 (d, *J* = 12.6 Hz, 1H), 2.41 (t, *J* = 6.0 Hz, 2H), 2.20 (t, *J* = 7.5 Hz, 2H), 2.05–1.89 (m, 2H), 1.78–1.39 (m, 12H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.1, 174.7, 173.8, 166.1, 157.3, 154.1, 150.8, 141.1, 120.2, 87.6, 84.7, 83.6, 71.6, 71.51, 71.48, 71.4, 71.3, 71.2, 71.0, 70.6, 69.1, 68.3, 63.8, 63.4, 61.6, 57.0, 47.3, 41.1, 40.3, 40.0, 37.6, 36.8, 30.8, 29.8, 29.5, 27.9, 26.92, 26.85, 24.9. HRMS (ESI–): $[M–H]^-$ calcd for $C_{56}H_{96}N_{11}O_{22}S_2$, 1388.6173; found, 1388.6238.

Chemical Synthesis of (*S*)-β-Phe-AMS-biotin 2. Compound number in bold refers to the structures shown in Scheme S2.

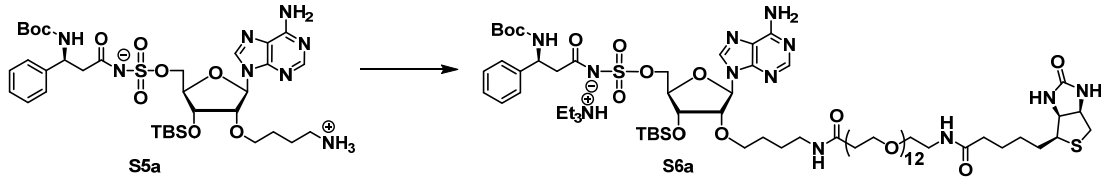
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*S*)-3-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoyl) sulfamate (S5a)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (109 mg, 0.57 mmol) and *N*-hydroxysuccinimide (66 mg, 0.57 mmol) were added to a solution of (*S*)-3-(Boc-amino)-3-phenylpropionic acid (100 mg, 0.38 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at room temperature for 13 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-(*S*)-β-Phe-OSu as a colorless oil (130 mg, 94%). A solution of **S2** (80 mg, 0.14 mmol), Boc-(*S*)-β-Phe-OSu (130 mg, 0.36 mmol), and cesium carbonate (176 mg, 0.38 mmol) in DMF (10 mL) was stirred at room temperature for 12 h. The reaction mixture was

then filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. HRMS (ESI+): $[M+H]^+$ calcd for $C_{34}H_{53}N_{10}O_9SSi$, 805.3487; found, 805.3438. To a solution of **S4a** in EtOH (10 mL) was added 10% Pd/C (40 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 14 h. The reaction mixture was filtered through a pad of Celite, which was further washed with EtOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (80:20:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S5a** as a white solid (35 mg, 32%, over two steps). ^{1}H NMR (500 MHz, CD_3OD): δ 8.59–8.54 (m, 1H), 8.20 (s, 1H), 7.36–7.10 (m, 5H), 6.17 (d, *J* = 6.9 Hz, 1H), 4.62–4.51 (m, 2H), 4.32–4.19 (m, 2H), 4.16–4.09 (m, 1H), 3.60–3.53 (m, 1H), 3.52–3.43 (m, 1H), 3.40–3.32 (m, 1H), 2.85 (t, *J* = 7.2 Hz, 2H), 2.70–2.50 (m, 2H), 1.69–1.50 (m, 4H), 1.44–1.34 (m, 9H), 0.97 (s, 9H), 0.18 (s 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 180.3, 179.3, 157.4, 154.0, 150.9, 141.1, 129.5, 129.3, 128.3, 127.9, 127.4, 127.3, 120.1, 86.9, 86.1, 83.5, 83.4, 73.1, 70.7, 69.0, 58.3, 47.1, 40.5, 28.8, 27.5, 26.3, 25.3, 19.0, –4.36, –4.45. HRMS (ESI–): $[M-H]^-$ calcd for $C_{34}H_{53}N_8O_9SSi$, 777.3425; found, 777.3408.

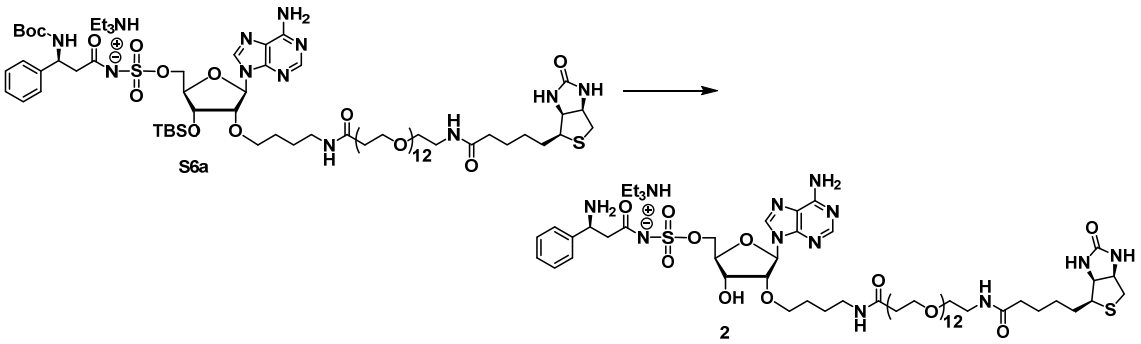
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-((6,46-dioxo-50-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-9,12,15,18,21,24,27,30,33,36,39,42-dodecaoxa-5,45-diazapentacontyl)oxy)tetrahydrofuran-2-yl)methyl ((*S*)-3-((*tert*-Butoxycarbonyl)amino)-3-phenylpropanoyl)sulfamate triethylammonium salt (S6a)



EZ-link NHS-Peg_{12}-Biotin (Thermo Fisher Scientific Inc.) (21 mg, 0.022 mmol) and DIEA (4.7 μL, 0.027 mmol) were added to a solution of compound **S5a** (14 mg, 0.018 mmol) in DMF (2 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (86:14:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S6a** as a colorless oil (28 mg, 96%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.57 (s, 1H), 8.21 (s, 1H), 7.36–7.20 (m, 5H), 6.16 (d, *J* = 6.9 Hz, 1H), 4.63–4.46 (m, 4H), 4.34–4.19 (m, 3H), 4.12 (dddd, *J* = 12.5, 12.5, 12.5, 1.8 Hz, 1H), 3.65–3.59 (m, 52H), 3.36 (t, *J* = 5.2 Hz, 2H), 3.20 (q, *J* = 7.3 Hz, 18H), 3.10–3.01 (m, 2H), 2.96–2.89 (m, 2H), 2.70 (d, *J* = 13.2 Hz, 1H), 2.39 (t, *J* = 6.1 Hz, 2H), 2.22 (t, *J* = 7.5 Hz, 2H), 1.80–1.33 (m,

19H), 1.30 (t, J = 7.3 Hz, 27H), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 180.0, 179.0, 176.1, 175.0, 173.8, 166.1, 157.3, 154.0, 150.9, 141.3, 129.5, 129.3, 128.1, 127.8, 127.5, 127.3, 120.2, 93.0, 87.1, 86.0, 83.4, 72.9, 71.5, 71.4, 71.30, 71.27, 70.6, 68.9, 68.3, 63.4, 61.6, 59.4, 57.0, 47.9, 46.9, 41.1, 40.4, 40.0, 37.7, 36.7, 29.8, 29.5, 28.8, 28.2, 26.9, 26.4, 26.3, 19.1, 9.21, –4.34, –4.45. HRMS (ESI–): $[M–H]^-$ calcd for $C_{71}H_{120}N_{11}O_{24}S_2Si$, 1602.7718; found, 1602.7710.

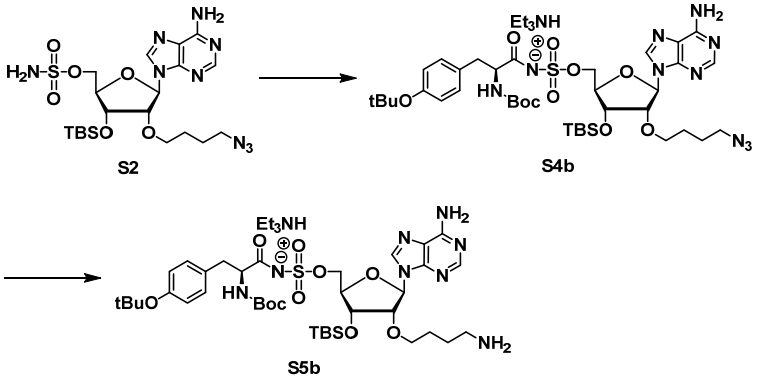
(*S*)-β-Phe-AMS-biotin triethylammonium salt (2)



Compound **S6a** (14 mg, 0.0088 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 12 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 (86:14:1 to 80:20:1 $CHCl_3$/MeOH/Et_3N) to afford compound **2** as a colorless oil (6.5 mg, 53%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.57 (s, 1H), 8.22 (s, 1H), 7.49–7.34 (m, 5H), 6.18 (d, J = 5.2 Hz, 1H), 4.70–4.63 (m, 1H), 4,53–4.41 (m, 3H), 4.36–4.22 (m, 4H), 3.70–3.52 (m, 52H), 3.36 (t, J = 5.2 Hz, 2H), 3.25–3.17 (m, 1H), 3.14 (q, J = 7.4 Hz, 4H, Et_3N-CH_2), 2.96–2.88 (m, 2H), 2.80–2.73 (m, 1H), 2.73–2.64 (m, 1H), 2.40 (t, J = 6.0 Hz, 2H), 2.22 (t, J = 7.2 Hz, 2H), 1.81–1.40 (m, 10H), 1.29 (t, J = 7.4 Hz, 6H, Et_3N-CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ 180.5, 177.8, 176.1, 173.9, 166.1, 157.3, 154.0, 150.8, 141.2, 130.24, 130.20, 128.4, 128.2, 120.4, 87.6, 84.8, 83.8, 71.5, 71.4, 71.30, 71.26, 71.0, 70.6, 68.9, 68.3, 63.4, 61.6, 57.0, 54.0, 47.8, 43.2, 41.1, 40.4, 40.0, 37.7, 36.7, 29.8, 29.5, 27.8, 26.9, 26.85, 9.39. HRMS (ESI–): $[M–H]^-$ calcd for $C_{60}H_{98}N_{11}O_{22}S_2$, 1388.6329; found, 1388.6135.

Chemical Synthesis of L-Tyr-AMS-biotin 4. Compound number in bold refers to the structures shown in Scheme S2.

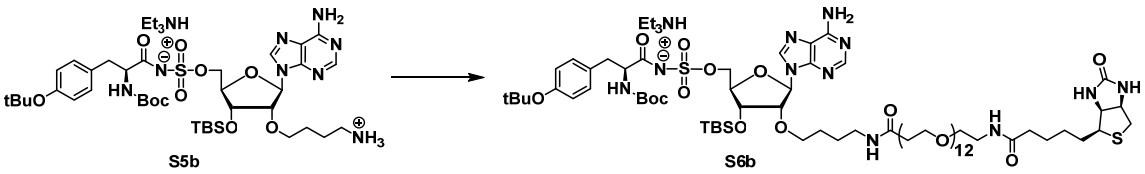
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*S*)-3-(4-(tert-butoxy)phenyl)-2-((*tert*-butoxycarbonyl)amino)propanoyl)sulfamate triethylammonium salt (S5b)



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 **S3b** as a colorless oil (97 mg, 75%). Boc-Tyr(tBu)-OSu (41 mg, 0.11 mmol) and cesium carbonate (90 mg, 0.27 mmol) were added to a solution of compound **S2** (50 mg, 0.090 mmol) in DMF (10 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was then filtered through a pad of Celite. The filtrate was concentrated under reduced pressure to afford **S4b** as a yellow oil. HRMS (ESI+): $[M+H]^+$ calcd for $C_{38}H_{61}N_{10}O_{10}SSi$, 877.4062; found, 877.4031. To a solution of **S4b** in EtOH (10 mL) was added 10% Pd/C (40 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite, which was further washed with EtOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (87.5:12.5:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S5b** as a white solid (28 mg, 37%, over two steps). ^{1}H NMR (500 MHz, CD_3OD): δ 8.54 (s, 1H), 8.20 (s, 1H), 7.13 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 2H), 6.18 (d, *J* = 6.9 Hz, 1H), 4.63–4.53 (m, 2H), 4.31–4.19 (m, 4H), 3.61–3.54 (m, 1H), 3.51–3.40 (m, 1H), 3.20–3.13 (m, 1H), 3.10 (q, *J* = 7.3 Hz, 4H, Et_3N-*CH*$_2$), 2.92–2.78 (m, 3H), 1.68–1.47 (m, 4H), 1.35 (s, 9H), 1.27 (s, 9H, overlapping with Et_3N-*CH*$_3$), 0.96 (s, 9H), 0.172 (s, 3H), 0.166 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 179.9, 157.4, 157.2, 154.9, 154.0, 150.9, 141.1, 134.3, 131.2, 125.0, 120.1, 86.9, 86.1, 83.5, 80.0, 79.3, 73.2, 70.7, 69.2, 59.1, 47.7, 40.5, 39.7, 29.2, 28.8, 27.5, 26.3, 25.5, 19.0, 9.48, –4.36, –4.45. HRMS (ESI+): $[M+H]^+$ calcd for $C_{38}H_{63}N_8O_{10}SSi$, 851.4157; found,

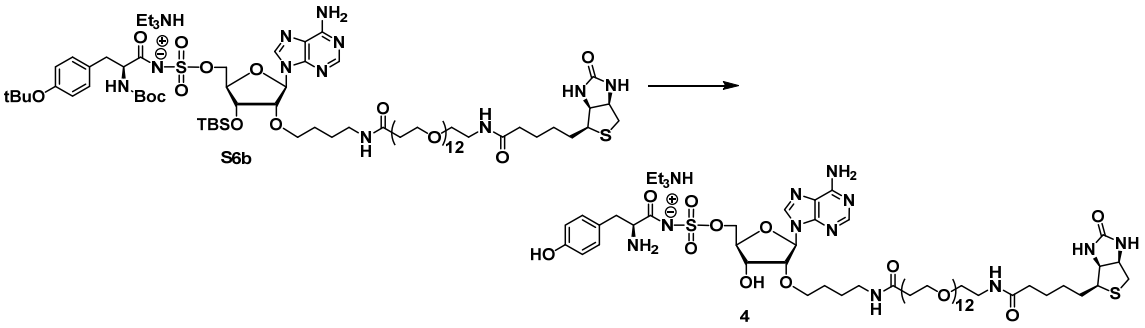
851.4056.

((2*S*,3*S*,4*S*,5*S*)-5-(6-Amino-9*H*-purin-9-yl)-4-((6,46-dioxo-50-((3a*S*,4*S*,6a*R*)-2-oxohexahydro-1*H*-thieno[3,4-d]imidazol-4-yl)-9,12,15,18,21,24,27,30,33,36,39,42-dodecaoxa-5,45-diazapentacontyl)oxy)-3-hydroxytetrahydrofuran-2-yl)methyl (L-tyrosyl)sulfamate triethylammonium salt (S6b)



EZ-link NHS-Peg$_{12}$-Bbiotin (Thermo Fisher Scientific Inc.) (21 mg, 0.022 mmol) and DIEA (4.7 μL, 0.027 mmol) were added to a solution of compound **S5b** (14 mg, 0.017 mmol) in DMF (2 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (86:14:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S6b** as a colorless oil (24 mg, 90%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.60 (s, 1H), 8.21 (s, 1H), 7.13 (d, *J* = 8.6 Hz, 2H), 6.83 (d, *J* = 8.6 Hz, 2H), 6.17 (d, *J* = 6.9 Hz, 1H), 4.64–4.52 (m, 2H), 4.49 (dd, *J* = 7.8, 4.9 Hz, 1H), 4.34–4.17 (m, 5H), 3.69–3.52 (m, 52H), 3.46–3.39 (m, 1H), 3.36 (t, *J* = 5.2 Hz, 2H), 3.19 (q, *J* = 7.2 Hz, 10H, Et_3N-*CH*$_2$), 3.05 (t, *J* = 6.6 Hz, 2H), 2.92 (dd, *J* = 12.6, 5.1 Hz, 1H), 2.70 (d, *J* = 12.6 Hz, 1H), 2.39 (t, *J* = 6.3 Hz, 2H), 2.22 (t, *J* = 7.2 Hz, 2H), 1.80–1.38 (m, 10H), 1.35 (s, 9H), 1.27 (s, 9H, overlapping with Et_3N-*CH*$_3$), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 179.6, 176.1, 175.3, 173.7, 166.1, 157.3, 157.1, 154.9, 154.0, 150.9, 141.3, 134.4, 131.2, 124.9, 120.1, 87.0, 86.1, 83.4, 79.9, 79.3, 73.0, 71.5, 71.4, 71.29, 71.26, 70.6, 69.1, 68.3, 63.4, 61.6, 59.0, 57.0, 47.8, 41.1, 40.4, 40.0, 39.7, 37.7, 36.7, 29.8, 29.5, 29.2, 28.8, 28.2, 26.9, 26.4, 26.3, 19.1, 9.24, –4.34, –4.46. HRMS (ESI–): $[M–H]^-$ calcd for $C_{75}H_{128}N_{11}O_{25}S_2Si$, 1674.8294; found, 1674.8287.

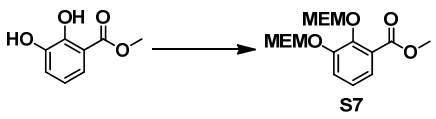
L-Tyr-AMS-biotin triethylammonium salt (4)



Compound **S6b** (13 mg, 0.0089 mmol) was dissolved in a 90:5:5 (v/v) mixture of TFA, H_2O, and TIS at room temperature. After 12 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 (80:20:1 $CHCl_3$/MeOH/Et_3N) to afford compound **4** as a colorless oil (9.4 mg, 75%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.55 (s, 1H), 8.21 (s, 1H), 7.13 (d, J = 8.6 Hz, 2H), 6.73 (d, J = 8.6 Hz, 2H), 6.18 (d, J = 4.6 Hz, 1H), 4.52–4.46 (m, 3H), 4.38–4.28 (m, 4H), 3.88–3.81 (m, 1H), 3.70–3.53 (m, 52H), 3.36 (t, J = 5.2 Hz, 2H), 3.22–3.11 (m, 3H, overlapping with Et_3N-CH_2), 2.94–2.89 (m, 1H), 2,71 (d, J = 12.6 Hz, 1H), 2.41 (t, J = 6.1 Hz, 2H), 2.20 (t, J = 7.2 Hz, 2H), 1.78–1.40 (m, 10H), 1.29 (t, J = 7.4 Hz, 10.5H, Et_3N-CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ 176.1, 175.6, 175.3, 173.9, 166.1, 158.0, 157.2, 154.0, 150.7, 141.2, 131.8, 127.0, 120.1, 116.7, 87.7, 84.7, 83.7, 71.5, 71.4, 71.32, 71.25, 71.0, 70.6, 68.9, 68.3, 63.4, 61.6, 58.5, 57.0, 47.8, 41.1, 40.4, 39.9, 37.9, 37.7, 36.7, 29.8, 29.5, 27.8, 26.9, 26.2, 9.31. HRMS (ESI–): $[M–H]^-$ calcd for $C_{60}H_{98}N_{11}O_{23}S_2$, 1404.6278; found, 1404.6089.

Chemical Synthesis of Dhb-AMS-biotin 5. Compound number in bold refers to the structures shown in Scheme S3.

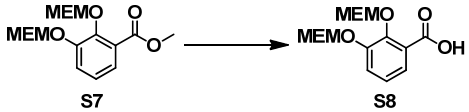
Methyl 2,3-bis((2-methoxyethoxy)methoxy)benzoate (S7)



To a solution of methyl 2,3-dihydroxybenzoate (1 g, 5.95 mmol) in THF (300 mL) was added NaH (950 mg of 60% NaH dispersion in mineral oil, 23.8 mmol). The solution was stirred at 0 °C for 1 h and MEMCl (2.02 mL, 17.9 mmol) was added. After 12 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 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 (1:1 EtOAc/hexane) to afford compound **S7** as a colorless oil (1.48 g, 73%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.41 (dd, *J* = 16.0, 3.4 Hz, 1H), 7.34 (dd, *J* = 16.0, 3.4 Hz, 1H), 7.07 (dd, *J* = 16.0, 16.0 Hz, 1H), 5.29 (s, 2H), 5.23 (s, 2H), 3.97–3.93 (m, 2H), 3.89 (s, 3H), 3.85–3.81 (m, 2H), 3.58–3.83 (m, 4H), 3.37 (s, 3H), 3.36 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.5, 151.0, 146.4, 126.7, 124.3, 124.2, 120.5, 98.6, 94.4, 71.8, 71.6, 69.1, 68.1, 59.2, 52.3. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{16}H_{24}NaO_8$, 367.1369; found, 367.1363.

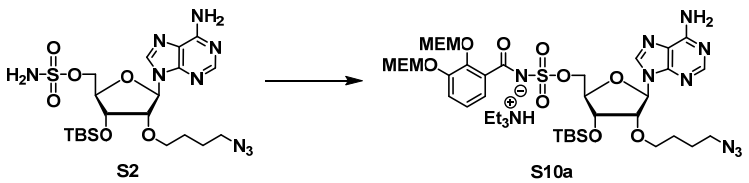
Methyl 2,3-bis((2-methoxyethoxy)methoxy)benzoic acid (S8)



S7 S8

To a solution of **S7** (716 mg, 2.08 mmol) in MeOH (10 mL) was added 8 mL of a 1 M NaOH solution at room temperature. Stirring was continued at 70 °C for 2 h. The flask was then placed on a rotary evaporator and the MeOH was removed at reduced pressure. The residue was diluted with H_2O and washed with Et_2O. The aqueous layer was acidified with a 1 M aqueous HCl and back extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford 2,3-bis((2-methoxyethoxy)methoxy)benzoic acid **S8** as a yellow oil (323 mg, 47%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.76 (dd, *J* = 16.0, 3.4 Hz, 1H), 7.43 (dd, *J* = 16.0, 3.4 Hz, 1H), 7.17 (dd, *J* = 16.0, 16.0 Hz, 1H), 5.45 (s, 2H), 5.32 (s, 2H), 3.92–3.87 (m, 2H), 3.86–3.82 (m, 2H), 3.59–3.52 (m, 4H), 3.37 (s, 3H), 3.35 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 166.6, 149.8, 146.0, 125.5, 125.0, 124.0, 121.4, 99.6, 94.5, 71.7, 71.6, 70.4, 68.3, 59.2. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{15}H_{22}NaO_8$, 353.1212; found, 353.1201.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl) oxy)tetrahydrofuran-2-yl)methyl (2,3-bis((2-methoxyethoxy)methoxy)benzoyl)sulfamate triethylammonium salt (S10a)

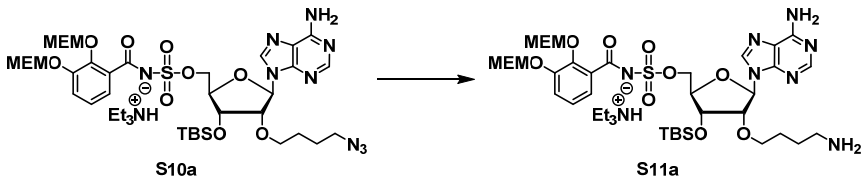


S2 S10a

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (109 mg, 0.57 mmol) and

N-hydroxysuccinimide (66 mg, 0.57 mmol) were added to a solution of **S8** (173 mg, 0.52 mmol) in CH_2Cl_2 (10 mL). The solution was stirred at room temperature for 14 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 *N*-hydroxysuccinimidyl 2,3-bis((2-methoxyethoxy)methoxy)benzoate **S9a** as a colorless oil (198 mg, 89%). A solution of **S2** (150 mg, 0.26 mmol), **S9a** (198 mg, 0.46 mmol), and cesium carbonate (254 mg, 0.78 mmol) in DMF (10 mL) was stirred at room temperature for 12 h. The reaction mixture was then 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 **S10a** as a colorless oil (160 mg, 79%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.60 (s, 1H), 8.21 (s, 1H), 7.19 (ddd, *J* = 8.6, 8.6, 1.3 Hz, 2H), 7.02 (dd, *J* = 7.8, 7.8 Hz, 1H), 6.18 (dd, *J* = 6.8 Hz, 1H), 5.28 (s, 2H), 5.25–5.20 (m, 2H), 4.68 (dd, *J* = 4.6, 1.8 Hz, 1H), 4.62 (dd, *J* = 6.9, 4.6 Hz, 1H), 4.40 (dddd, *J* = 11.3, 11.3, 11.3, 1.7 Hz, 2H), 4.32–4.29 (m, 1H), 3.99–3.93 (m, 2H), 3.83–3.80 (m, 2H), 3.58–3.52 (m, 6H), 3.34–3.29 (m, 6H, overlapping with MeOH), 3.17 (q, *J* = 7.2 Hz, 10H, Et_3N-*CH*$_2$), 3.11 (t, *J* = 6.6 Hz, 2H), 1.54–1.39 (m, 4H), 1.27 (t, *J* = 7.2 Hz, 15H, Et_3N-*CH*$_3$), 0.97 (s, 9H), 0.18 (s, 3H), 0.16 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 175.8, 157.4, 154.0, 151.7, 150.9, 145.1, 141.3, 137.2, 125.0, 123.4, 120.2, 118.7, 99.2, 95.5, 87.2, 86.1, 83.4, 73.0, 72.8, 71.2, 69.9, 69.3, 69.1, 68.8, 59.1, 59.0, 52.1, 47.8, 28.0, 26.6, 26.3, 19.0, 9.18, –4.42, –4.53. HRMS (ESI–): $[M–H]^-$ calcd for $C_{35}H_{54}N_9O_{13}SSi$, 868.3331; found, 868.3336.

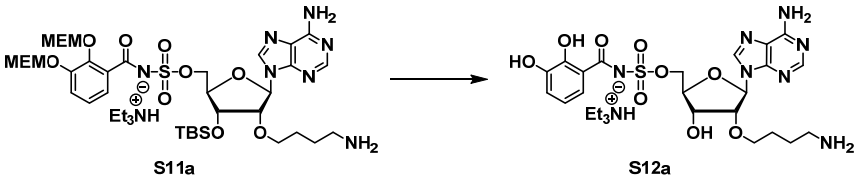
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl) oxy)tetrahydrofuran-2-yl)methyl (2,3-bis((2-methoxyethoxy)methoxy)benzoyl)sulfamate triethylammonium salt (S11a)



To a solution of **S10a** (128 mg, 0.16 mmol) in EtOH (10 mL) was added 10% Pd/C (80 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 12 h. The reaction mixture was filtered through a pad of Celite, which was further washed with EtOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (90:10:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S11a** as a colorless oil (42 mg, 31%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.62 (s, 1H), 8.20 (s, 1H), 7.20

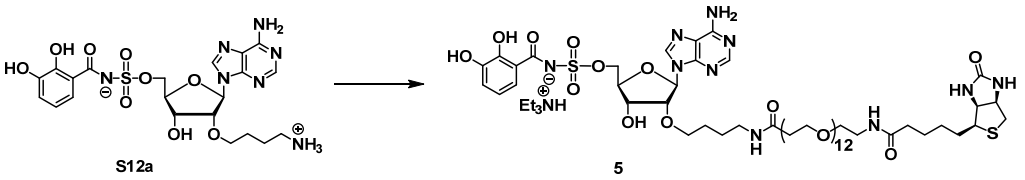
(d, *J* = 8.0 Hz, 2H), 7.03 (dd, *J* = 8.0, 8.0 Hz, 1H), 6.19 (d, *J* = 4.6 Hz, 1H), 5.28 (s, 2H), 5.21 (ddd, *J* = 5.7, 5.7, 5.7 Hz, 2H), 4.70–4.61 (m, 2H), 4.41–4.35 (m, 2H), 4.33–4.29 (m, 1H), 3.95 (t, *J* = 4.0 Hz, 2H), 3.82 (t, *J* = 4.6 Hz, 2H), 3.58–3.42 (m, 6H), 3.35–3.27 (m, 6H, overlapping with MeOH), 3.14 (q, *J* = 7.2 Hz, 2H, Et_3N-*CH*$_2$), 2.82 (t, *J* = 6.3 Hz, 2H), 1.67–1.44 (m, 4H), 1.27 (t, *J* = 7.2 Hz, 3H, Et_3N-*CH*$_3$), 0.95 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.0, 157.4, 154.0, 151.7, 150.9, 145.1, 141.1, 137.1, 125.1, 123.5, 120.1, 118.9, 99.2, 95.5, 87.0, 86.2, 83.2, 73.4, 73.0, 72.8, 70.6, 69.9, 69.3, 69.1, 59.1, 59.0, 47.7, 40.4, 27.4, 26.3, 25.2, 19.0, 9.30, –4.39, –4.49. HRMS (ESI–): $[M–H]^-$ calcd for $C_{35}H_{56}N_7O_{13}SSi$, 842.3426; found, 842.3428.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (2,3-dihydroxybenzoyl)sulfamate triethylammonium salt (S12a)



Compound **S11a** (22 mg, 0.026 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 (67:33:1 to 50:50:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S12a** as a white solid (8.5 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.57 (s, 1H), 8.18 (s, 1H), 7.45 (dd, *J* = 8.1, 1.1 Hz, 1H), 6.87 (dd, *J* = 8.0, 1.8 Hz, 1H), 6.63 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.19 (d, *J* = 6.3 Hz, 1H), 4.62 (t, *J* = 5.1 Hz, 1H), 4.53 (dd, *J* = 5.1, 2.3 Hz, 1H), 4.41–4.36 (m, 2H), 4.35–4.31 (m, 1H), 3.67–3.61 (m, 1H), 3.60–3.54 (m, 1H), 3.13 (q, *J* = 7.5 Hz, 7H, Et_3N-*CH*$_2$), 2.96–2.88 (m, 2H), 1.74–1.57 (m, 4H), 1.27 (t, *J* = 7.5 Hz, 10.5H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD): δ 175.3, 157.3, 154.0, 150.8, 150.7, 146.8, 140.9, 121.9, 120.8, 120.1, 119.4, 118.6, 87.3, 85.5, 83.6, 71.4, 71.0, 69.5, 47.8, 40.6, 27.4, 25.6, 9.29. HRMS (ESI–): $[M–H]^-$ calcd for $C_{21}H_{26}N_7O_9S$, 552.1513; found, 552.1512.

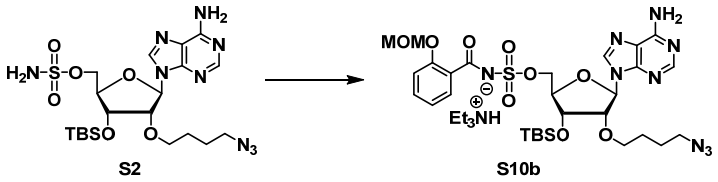
DHB-AMS-biotin triethylammonium salt (5)



EZ-link NHS-Peg$_{12}$-Biotin (Thermo Fisher Scientific Inc.) (25 mg, 0.027 mmol) and DIEA (5.2 μL, 0.030 mmol) were added to a solution of compound **S12a** (8.5 mg, 0.015 mmol) in DMF (2 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (83:17:1 $CHCl_3$/MeOH/Et_3N) to afford compound **5** as a colorless oil (9.5 mg, 41%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.58 (s, 1H), 8.19 (s, 1H), 7.46 (d, *J* = 7.5 Hz, 1H), 6.86 (d, *J* = 7.5 Hz, 1H), 6.63 (ddd, *J* = 7.8, 7.8, 2.6 Hz, 1H), 6.18 (d, *J* = 3.4 Hz, 1H), 4.64–4.59 (m, 1H),4.54–4.45 (m, 2H), 4.44–4.35 (m, 2H), 4.34–4.27 (m, 2H), 3.70–3.52 (m, 52H), 3.38–3.33 (m, 2H), 3.19 (q, *J* = 7.5 Hz, 18H, Et_3N-*CH*$_2$), 3.12–3.07 (m, 1H), 2.97–2.86 (m, 1H), 2.71 (br, 1H), 2.40 (t, *J* = 5.7 Hz, 2H), 2.22 (t, *J* = 6.0 Hz, 2H), 1.77–1.39 (m, 10H), 1.30 (t, *J* = 7.5 Hz, 27H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD): δ 176.1, 175.2, 173.9, 166.1, 157.3, 154.0, 150.9, 150.7, 146.9, 141.2, 121.9, 120.9, 120.1, 119.4, 118.6, 87.3, 85.3, 83.4, 71.5, 71.4, 71.28, 71.25, 70.6, 69.5, 68.3, 63.4, 61.6, 57.0, 47.8, 41.1, 40.4, 40.0, 37.7, 36.8, 29.8, 29.5, 27.9, 26.9, 26.3, 9.24. HRMS (ESI–): $[M–H]^-$ calcd for $C_{58}H_{93}N_{10}O_{24}S_2$, 1377.5806; found, 1377.5794.

Chemical Synthesis of Sal-AMS-biotin 6. Compound number in bold refers to the structures shown in Scheme S3.

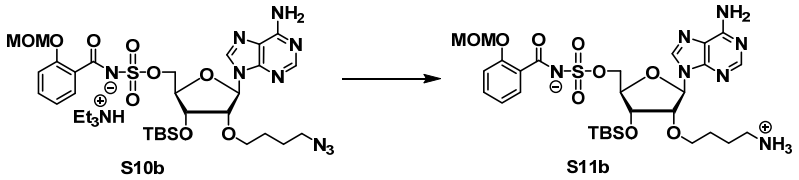
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl) oxy)tetrahydrofuran-2-yl)methyl (2-(methoxymethoxy)benzoyl)sulfamate triethylammonium salt (S10b)



A solution of **S2** (185 mg, 0.33 mmol), *N*-hydroxysuccinimidyl 2-methoxymethoxybenzoate **S9b** (140 mg, 0.50 mmol), and cesium carbonate (322 mg, 0.99 mmol) in DMF (10 mL) was stirred at room temperature for 14 h. The reaction mixture was then filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash

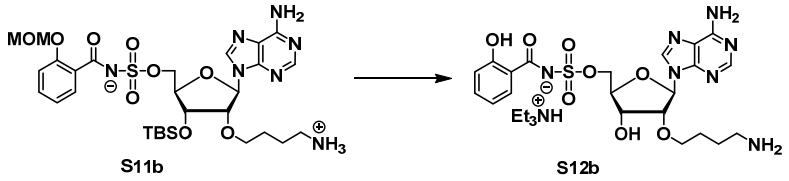
chromatography (90:10:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S10b** as a white solid (167 mg, 70%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.60 (s, 1H), 8.22 (s, 1H), 7.50 (dd, *J* = 14.9, 3.4 Hz, 1H), 7.31–7.26 (m, 1H), 7.14 (d, *J* = 8.0 Hz, 1H), 6.99 (ddd, *J* = 7.5, 7.5, 1.0 Hz, 1H), 6.18 (d, J = 6.9 Hz, 1H), 5.18 (s, 2H), 4.69 (dd, *J* = 4.0, 2.3 Hz, 1H), 4.63 (dd, *J* = 6.3, 5.2 Hz, 1H), 4.41 (dddd, *J* = 12.9, 12.9, 12.9, 1.7 Hz, 2H), 4.35–4.30 (m, 1H), 3.60–3.54 (m, 1H), 3.45 (s, 3H), 3.43–3.38 (m, 1H), 3.16 (q, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_2$), 3.11 (t, *J* = 6.3 Hz, 2H), 1.57–1.39 (m, 4H), 1.26 (t, *J* = 7.5 Hz, 13.5H, Et_3N-*CH*$_3$), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.3, 157.4, 155.8, 154.0, 150.8, 141.3, 132.4, 131.2, 130.0, 122.6, 120.2, 117.4, 96.5, 87.2, 86.0, 83.3, 73.0, 71.2, 69.3, 56.6, 52.1, 47.7, 27.9, 26.6, 26.3, 19.0, 9.14, –4.43, –4.54. HRMS (ESI–): $[M–H]^-$ calcd for $C_{29}H_{42}N_9O_9SSi$, 720.2595; found, 720.2595.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl) oxy)tetrahydrofuran-2-yl)methyl (2-(methoxymethoxy)benzoyl)sulfamate (S11b)



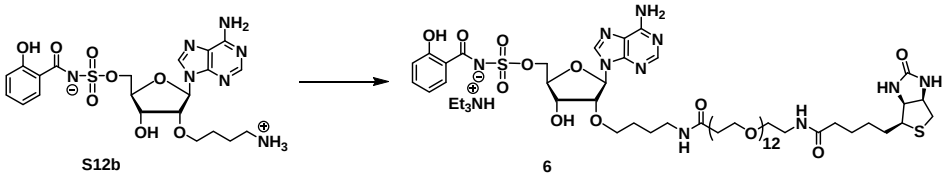
To a solution of **S10b** (111 mg, 0.15 mmol) in EtOH (5 mL) was added 10% Pd/C (45 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 16 h. The reaction mixture was filtered through a pad of Celite, which was further washed with EtOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (5:1 to 4:1 $CHCl_3$/MeOH) to afford compound **S11b** as a white solid (91 mg, 88%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.61 (s, 1H), 8.21 (s, 1H), 7.50 (dd, *J* = 14.9, 3.4 Hz, 1H), 7.31–7.28 (m, 1H), 7.13 (d, *J* = 8.0 Hz, 1H), 6.99 (t, *J* = 7.5 Hz, 1H), 6.19 (d, *J* = 5.7 Hz, 1H), 5.17 (s, 2H), 4.68–4.64 (m, 2H), 4.40 (d, *J* = 2.8 Hz, 2H), 4.30–4.33 (m, 1H), 3.59–3.53 (m, 1H), 3.51–3.46 (m, 1H), 3.44 (s, 3H), 2.83 (t, *J* = 7.2 Hz, 2H), 1.65–1.44 (m, 4H), 0.96 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.5, 157.4, 155.9, 154.0, 150.9, 141.3, 132.3, 131.3, 130.2, 122.7, 120.1, 117.5, 96.7, 87.0, 86.2, 83.3, 73.4, 70.6, 69.4, 56.7, 40.5, 27.4, 26.3, 25.3, 19.0, –4.40, –4.50. HRMS (ESI+): $[M+H]^+$ calcd for $C_{29}H_{46}N_7O_9SSi$, 696.2847; found, 696.2816.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (2-hydroxybenzoyl)sulfamate triethylammonium salt (S12b)



Compound **S11b** (30 mg, 0.043 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 (75:25:1 to 67:33:1 $CHCl_3$/MeOH/Et_3N) to afford compound **S12b** as a white solid (10 mg, 43%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.56 (d, *J* = 3.4 Hz, 1H), 8.17 (s, 1H), 7.95 (dd, *J* = 16.0, 3.5 Hz, 1H), 7.29 (ddd, *J* = 8.6, 8.6, 1.1 Hz, 1H), 6.81–6.76 (m, 2H), 6.19 (d, *J* = 6.3 Hz, 1H), 4.61 (dd, *J* = 16.6, 5.2 Hz, 1H), 4.54 (dd, *J* = 7.5, 2.3 Hz, 1H), 4.41–4.37 (m, 2H), 4.36–4.33 (m, 1H), 3.68–3.63 (m, 1H), 3.61–3.55 (m, 1H), 3.16 (q, *J* = 7.5 Hz, 4H, Et_3N-*CH*$_2$), 2.92 (t, *J* = 6.3 Hz, 2H), 1.75–1.57 (m, 4H), 1.29 (t, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD): δ 175.2, 162.1, 157.3, 154.0, 150.8, 140.9, 134.5, 131.4, 120.5, 120.1, 119.3, 117.9, 87.4, 85.4, 83.6, 71.4, 71.1, 69,5, 47.8, 40.6, 27.4, 25.6, 9.27. HRMS (ESI–): $[M–H]^-$ calcd for $C_{21}H_{26}N_7O_8S$, 536.1564; found, 536.1562.

Sal-AMS-biotin triethylammonium salt (6)



EZ-link NHS-Peg$_{12}$-Biotin (Thermo Fisher Scientific Inc.) (25 mg, 0.027 mmol) and DIEA (6.0 μL, 0.035 mmol) were added to a solution of compound **S12b** (10 mg, 0.019 mmol) in DMF (2 mL). The solution was stirred at room temperature for 12 h. The reaction mixture was concentrated under reduced pressure. The residue was purified by flash chromatography (80:20:1 $CHCl_3$/MeOH/Et_3N) to afford compound **6** as a colorless oil (17 mg, 66%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.57 (s, 1H), 8.19 (s, 1H), 7.96 (d, *J* = 7.5 Hz, 1H), 7.34–7.27 (m, 1H), 6.83–6.76 (m, 2H), 6.17 (d, *J* = 6.3 Hz, 1H), 4.61 (dd, *J* = 5.5, 5.5 Hz, 1H), 4.53 (dd, *J* = 3.7, 3.7 Hz, 1H), 4.49 (dd, *J* = 7.8, 4.9 Hz, 1H), 4.40 (dddd, *J* = 11.0, 11.0, 11.0, 1.6 Hz, 2H), 4.34–4.27 (m, 2H), 3.70–3.52 (m, 52H), 3.36 (t, *J* = 5.5 Hz, 2H), 3.24–3.16 (m, 1H), 3.13 (q, *J* = 7.2 Hz, 15H, Et_3N-*CH*$_2$), 2.92 (dd, *J* = 12.9, 4.9 Hz, 1H), 2.70 (d, *J* = 15.2 Hz, 1H), 2.40 (t, *J* = 6.1 Hz,

2H), 2.22 (t, J = 7.5 Hz, 2H), 1.78–1.38 (m, 10H), 1.27 (t, J = 7.2 Hz, 22.5H, Et_3N-CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ 176.1, 174.9, 173.8, 166.1, 162.1, 157.3, 154.0, 150.9, 141.2, 134.4, 131.4, 120.7, 120.1, 119.3, 117.9, 87.4, 85.2, 83.4, 71.5, 71.4, 71.31, 71.26, 70.6, 69.5, 68.3, 63.3, 61.6, 57.0, 47.8, 41.1, 40.4, 40.0, 37.7, 36.7, 29.8, 29.5, 27.9, 26.9, 26.2, 9.41. HRMS (ESI–): $[M–H]^-$ calcd for $C_{58}H_{93}N_{10}O_{23}S_2$, 1361.5856; found, 1361.5847.

Chemical Biology Procedures

Preparation of Overexpression Constructs. Recombinant proteins *holo*-GrsA, *apo*-TycA, and *holo*-TycB1 were expressed and purified as described previously.[4,9] These proteins 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. The *ausA2* (A2-T2-R) gene was PCR amplified genomic DNA from *Staphylococcus aureus* ATCC 700699 using primers ausA2 F (5′-GCCTCCACGACCATGGAACTTCTAAATTGGGTCAATAC-3′) and ausA2 R (5′-CCGAATTCGTCTTATTGAATATTGTTTTGATATATTGTGC-3′), and subsequently cloned into plitmus28-*ausA2*. Plasmid litmus28-*ausA2* was digested with *Nco*I and *Eco*RI, and the gene was subcloned into pET28b to produce pET28b-*ausA2*, an expression vector for *apo*-AusA2 with a 6×His-tag appended to the C-terminus. The *entE* gene was PCR amplified from pKK223-3 containing the *entE* gene, kindly provided by Prof. Christopher T. Walsh at Harvard University, USA, using primers entE F (5′-GCCTCCATGACCATGGGCATTCCATTCACC-3′) and entE R (5′-CCGAGAGTCCGAATTCGTGGCTGATGCGCG-3′), and subsequently cloned into plitmus28-*entE*. Plasmid litmus28-*entE* was digested with *Nco*I and *Eco*RI, and the gene was subcloned into pET28b to produce pET28b-*entE*, an expression vector for EntE with a 6×His-tag appended to the C-terminus. Sequencing revealed the expression plasmid to be error free.

Site-Directed Mutagenesis. Site-directed mutant EntE (S240C) was constructed from a pET28b-*entE* template PCR mutagenesis using primers EntE (S240C) F (5′-TACGCCATGAGTTGCCCAGGATCGCTGGGCGTC-3′) and EntE (S240C) R (5′-GACGCCCAGCGATCCTGGGCAACTCATGGCGTA-3′), and the PCR-amplified products were subsequently treated with *Dpn*I. The *Dpn*I-treated DNA was transformed into *E. coli* DH5α cells.

Protein Expression and Purification. For expression and purification of *apo*-AusA2, pET28b-*ausA2* was transformed into *E. coli* BL21 (DE3) cells. Overnight cultures were used to inoculate 1 L of LB medium supplemented with 50 μg/mL kanamycin. Cultures were allowed to grow to an A_{600} of 0.7 at 37 °C, then induced with IPTG to a final concentration of 0.3 mM, and allowed to grow for a further 18 h at 18 °C. For expression and purification of EntE and the

mutant EntE (S240C), pET28b-*entE* and pET-*entE (S240C)* were transformed into *E. coli* BL21 (DE3) cells. Overnight cultures were used to inoculate 1 L of LB medium supplemented with 50 μg/mL kanamycin. Cultures were allowed to grow to an A_{600} of 0.7 at 37 °C, then induced with IPTG to a final concentration of 0.1 mM, and allowed to grow for a further 3 h at 37 °C. Cells were pelleted and resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0, 0.5% Triton-X and protease inhibitor cocktail). The cells were then lysed by sonication at 4 °C using an ultrasonic disruptor UD201 (Tomy Digital Biology Co., Ltd, Japan). The resulting cell lysates were centrifuged to remove cell debris and the supernatants were loaded onto a Ni-NTA agarose column (Qiagen) and eluted with a gradient of imidazole. Eluted proteins were visualized by SDS-PAGE with Coomassie Brilliant Blue stain and quantitated by the method of Bradford.[10] Fractions containing the recombinant proteins were pooled and dialyzed against assay buffer (20 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$ and 1 mM TCEP). After the addition of 10% glycerol (v/v) the proteins were stored at −80 °C.

Preparation of Lysates for Proteomic Binding Experiments. Recombinant proteins *holo*-GrsA and *holo*-TycB1 were overproduced as described previously.[4,9] *E. coli* cell pellets containing overproduced GrsA were resuspended in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% NP-40 and protease inhibitor cocktail, and then lysed by sonication at 4 °C using an ultrasonic disruptor UD201. In contrast, *E. coli* cell pellets containing overexpressed TycB1 were resuspended in 50 mM HEPES (pH 8.0), 100 mM NaCl, 10 mM $MgCl_2$, 1 mM TCEP, 1 mM EDTA, 0.05% NP-40, and protease inhibitor cocktail and subsequently sonicated at 4 °C using an ultrasonic disruptor UD201. The lysates were centrifuged for 5 min at 15,000 rpm and the pellets were discarded. The protein concentrations were measured by the method of Bradford[10] and cell lysates were diluted to provide a final concentration of 1.0 mg/mL.

Hydroxamate-MesG Assay.[2]

Standard assay conditions: Reactions contained varying amounts of NRPS enzymes (200–635 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 K_i^{app} values of inhibitors by the hydroxamate-MesG assay: K_i^{app} determination was performed using standard assay conditions. For *holo*-TycB1, probe **3** was tested from 12.5 to 200 μM using L-Pro (1 mM) as the competing substrate. The enzyme was fixed at 400 nM.

For *apo*-AusA2, probe **4** and the probe's cognate competitor **9** were tested from 0.79 to 25 μM and 0.16 to 5 μM, respectively, using L-Tyr (1 mM) as the cognate substrate. The enzyme was fixed at 635 nM. Compounds **1** and **7** were varied from 1.9 to 30 μM and 0.31 to 10 μM, respectively, and *apo*-AusA2 (635 nM) and L-Tyr (1 mM) were held constant. For EntE, probe **5** was tested from 0.31 to 5 μM using DHB (50 μM) as the competing substrate. The enzyme was fixed at 200 nM. Compound **10** was varied from 0.25 to 2 μM and EntE (400 nM) and DHB (50 μM) were held constant. In all experiments, the total DMSO concentration was kept at 2.0%. Initial velocities were fit to the Morrison equation using Prism 5 (GraphPad Software).

Determination of kinetic parameters: Steady-state kinetic parameters of the substrates were determined for each enzyme using standard assay conditions as described above. The enzyme and substrate concentrations are listed here: GrsA was used at 50 nM with L-Phe (6.25–1000 μM) and 100 nM with (*S*)-β-Phe (100–2000 μM); TycB1 was used at 400 nM with L-Pro (10–2000 μM); AusA2 was used at 635 nM with L-Tyr (200–1000 μM) and L-Phe (188–3000 μM); EntE was used at 250 nM with DHB (3.13–100 μM) and Sal (20–320 μM); the mutant EntE (S240C) was used at 500 nM with DHB (62.5–2000 μM) and Sal (10–320 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).

ELISA protocol. Probes **1**, **2**, **4**, **5** and **6** were dissolved in immobilization buffer (PBST: PBS containing 0.05% Tween 20) to provide final concentrations of 3.5 μg/mL. In contrast, probe **3** was dissolved in immobilization buffer (PBS containing 0.0025% NP-40) to provide a final concentration of 3.5 μg/mL. Streptavidin High Binding Capacity Coated 96-well plates (Pierce) were treated with 100 μL of the probes for 1 h at room temperature, followed by extensive washing with 200 μL of PBST. Control wells were treated identically except no probes were added to the immobilization buffers. In Figure 3, wells were incubated with 100 μL of a serially diluted solution of GrsA (0.0781–5.0 μg/mL), TycA (0.156–10 μg/mL), AusA2 (0.313–20 μg/mL), and EntE (0.0391–2.5 μg/mL) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40 for 1 h at room temperature. Control wells were identically treated with 100 μL of GrsA (5.0 μg/mL), TycA (10 μg/mL), AusA2 (20 μg/mL), and EntE (5.0 μg/mL). In Figure 4, wells were treated with 100 μL of GrsA (5.0 μg/mL), TycA (10 μg/mL), AusA2 (20 μg/mL), and EntE (1.0 μg/mL) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40 for 1 h at room temperature. Control wells were identically treated with 100 μL of GrsA (5.0 μg/mL), TycA (10 μg/mL), AusA2 (20 μg/mL), EntE (1.0 μg/mL), and the binding buffer. In Figure 5, wells were incubated with 100 μL of a serially diluted cell lysate (0.0156–1.0 mg/mL) containing overproduced GrsA and TycB1 for 1 h at room temperature. Control wells were identically treated with 100 μL of cell lysates (1.0 mg/mL) containing overproduced GrsA and TycB1. In Figure 6, wells were treated with a serially diluted solution of GrsA (0.313–20

μg/mL) and TycA (0.313–20 μg/mL) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40 for 1 h at room temperature. Control wells were identically treated with 100 μL of GrsA (20 μg/mL) and TycA (20 μg/mL). In Figure 7, probe **5**-immobilized wells were incubated with 100 μL of a serially diluted EntE (0.0313–2.0 μg/mL) and the mutant EntE (S240C) (0.156–10 μg/mL) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40 for 1 h at room temperature. In contrast, probe **6**-immobilized wells were treated with 100 μL of a serially diluted EntE (0.313–20 μg/mL) and the mutant EntE (S240C) (0.313–20 μg/mL) in 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% NP-40 for 1 h at room temperature. Control wells were identically treated with 100 μL of EntE (2.0 μg/mL and 20 μg/mL) and the mutant EntE (S240C) (10 μg/mL and 20 μg/mL). After extensive washing with 200 μL of PBST, wells were treated with a solution of 100 μL of an anti-6×His, monoclonal antibody (9C11, Wako Pure Chemical Industries, Ltd.), 1 : 5000 in PBST, for 1 h at room temperature. After three washes with 200 μL of PBST, a solution of 100 μL of goat anti-mouse-HRP conjugate (Bio-Rad Laboratories, Inc.), 1 : 5000 in PBST was incubated for 1 h at room temperature, followed by three washes with 200 μL of PBST, and each well was then treated with 100 μL of 0.4 mg/mL *o*-phenylenediamine (OPD) in 0.05 M phosphate-citrate pH 5.0 containing 0.4 mg/mL urea hydrogen peroxide at room temperature. The yellow color was allowed to develop for approximately 5 min and the reaction was quenched by the addition of 50 μL of 1 M H_2SO_4. The absorbance at 492 nm was measured using an EnVision Multilabel Reader (PerkinElmer).

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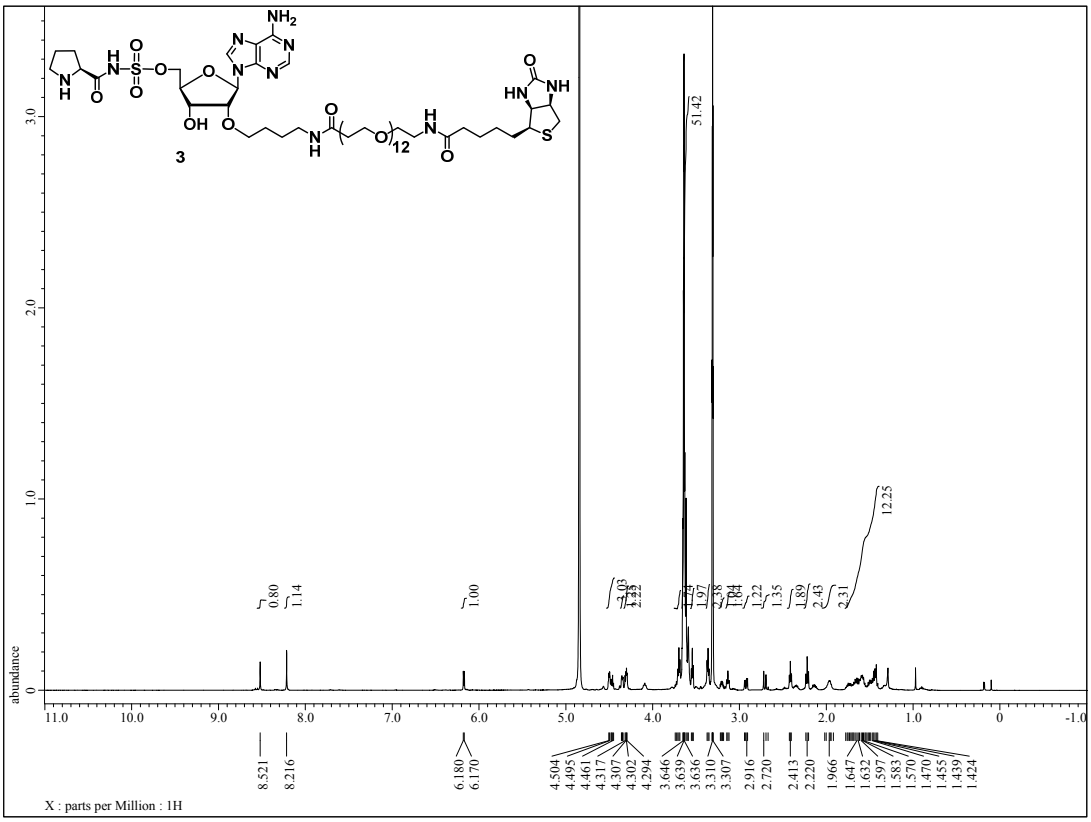
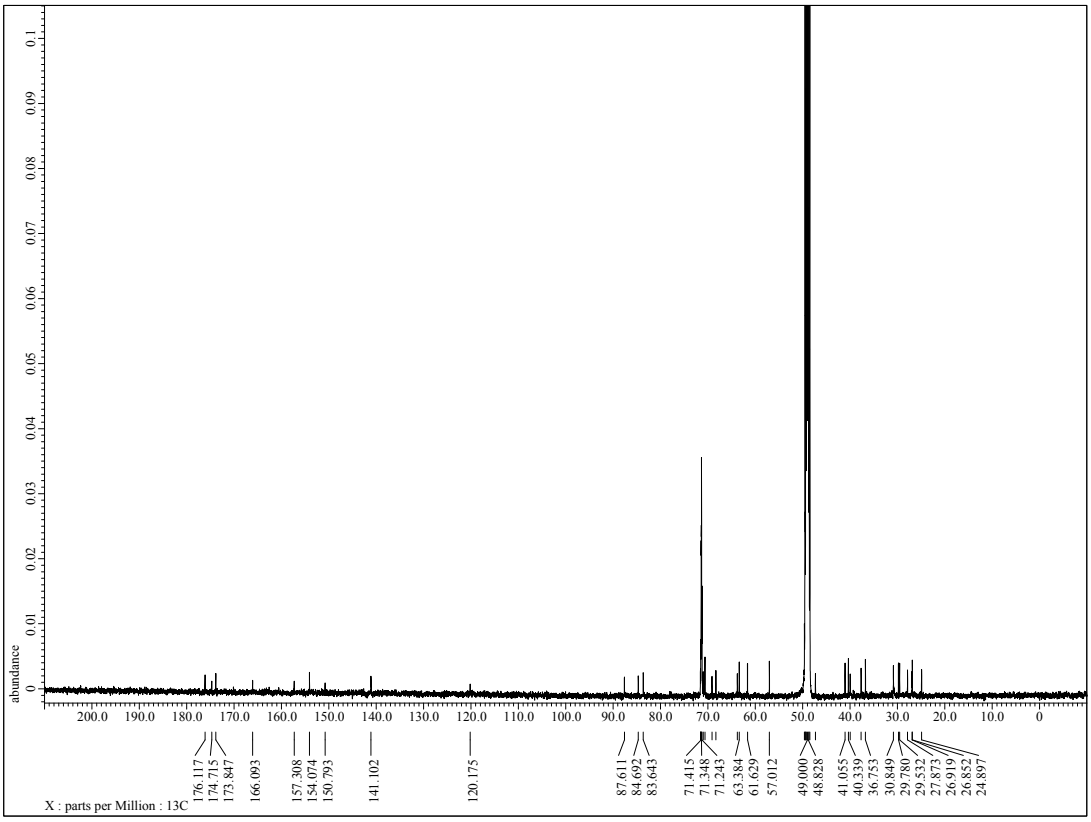
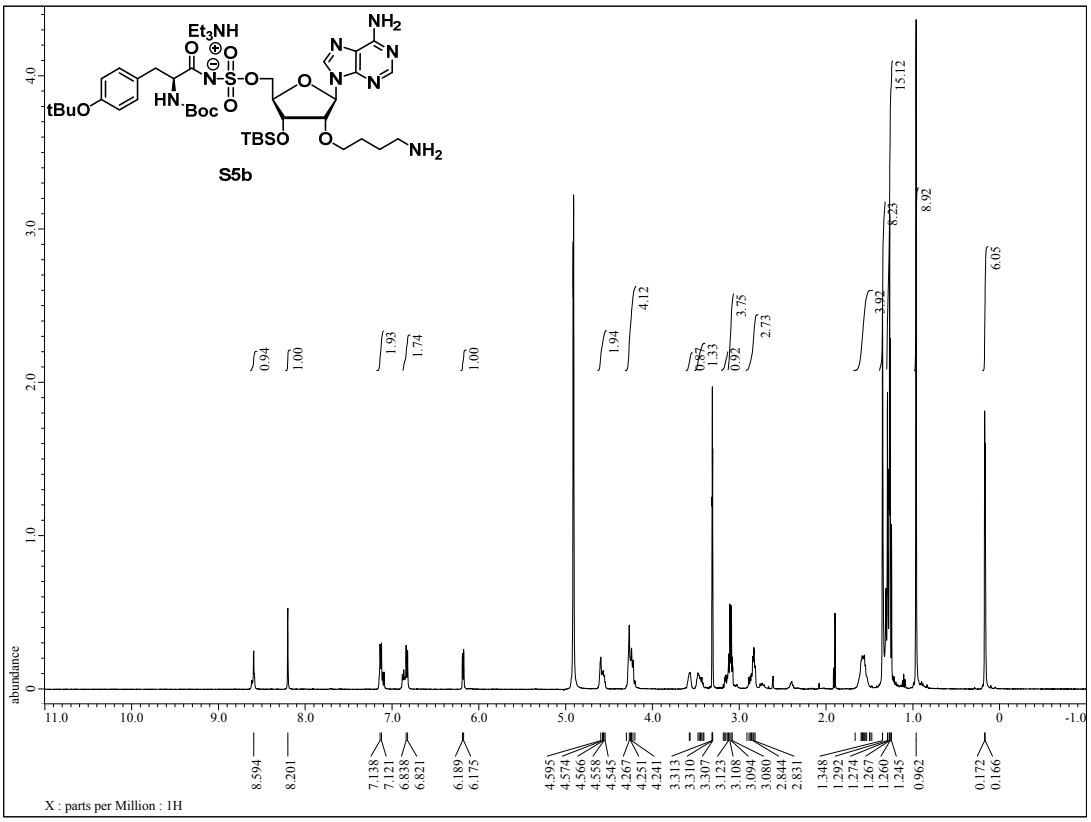
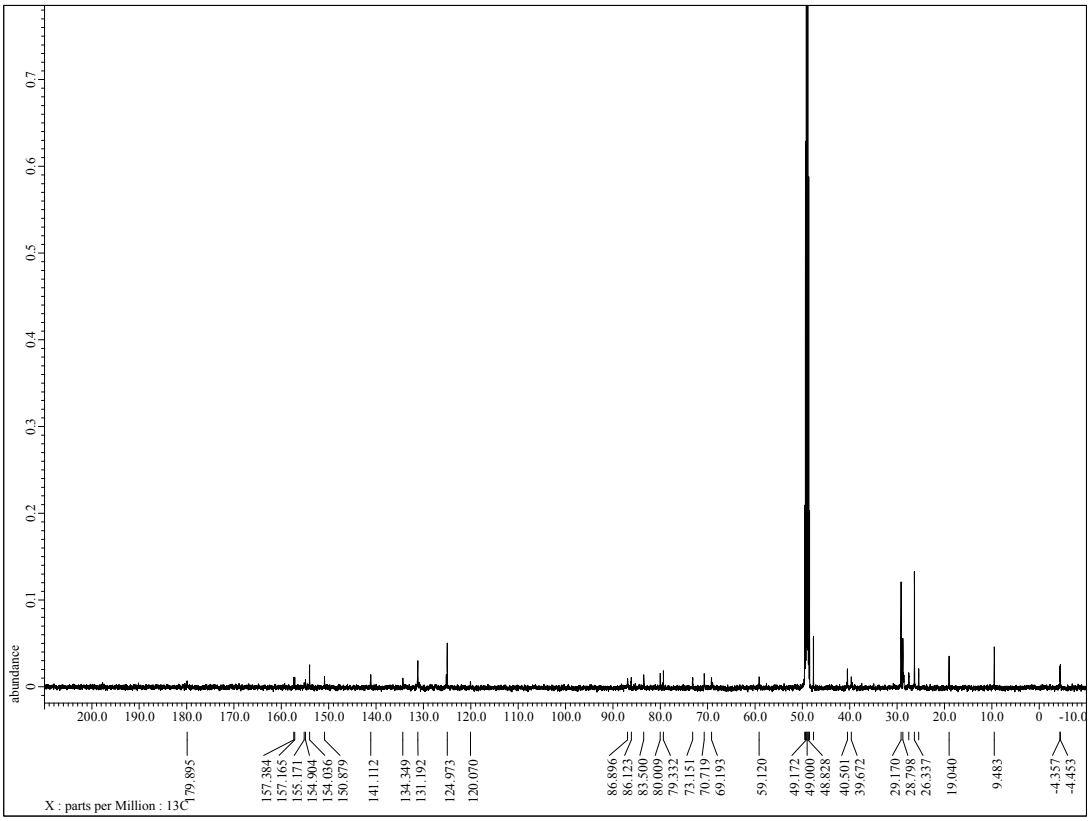
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **3** in CD_3OD





^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S5b** in CD_3OD





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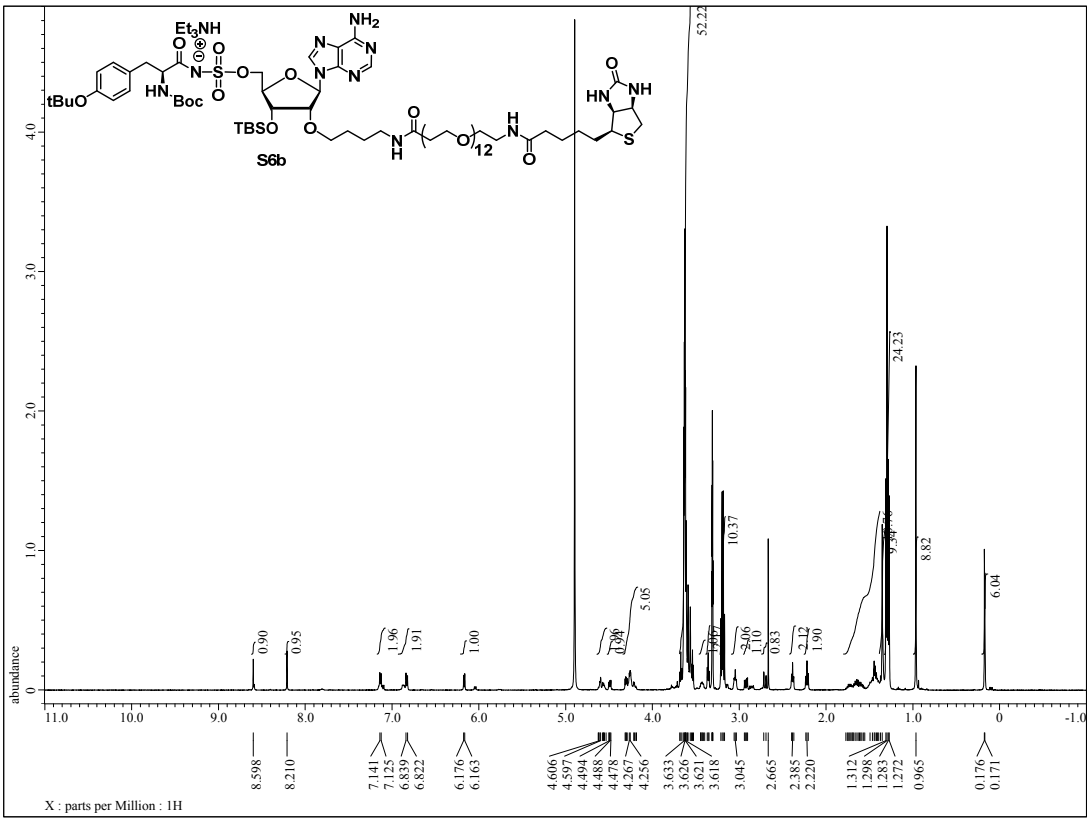

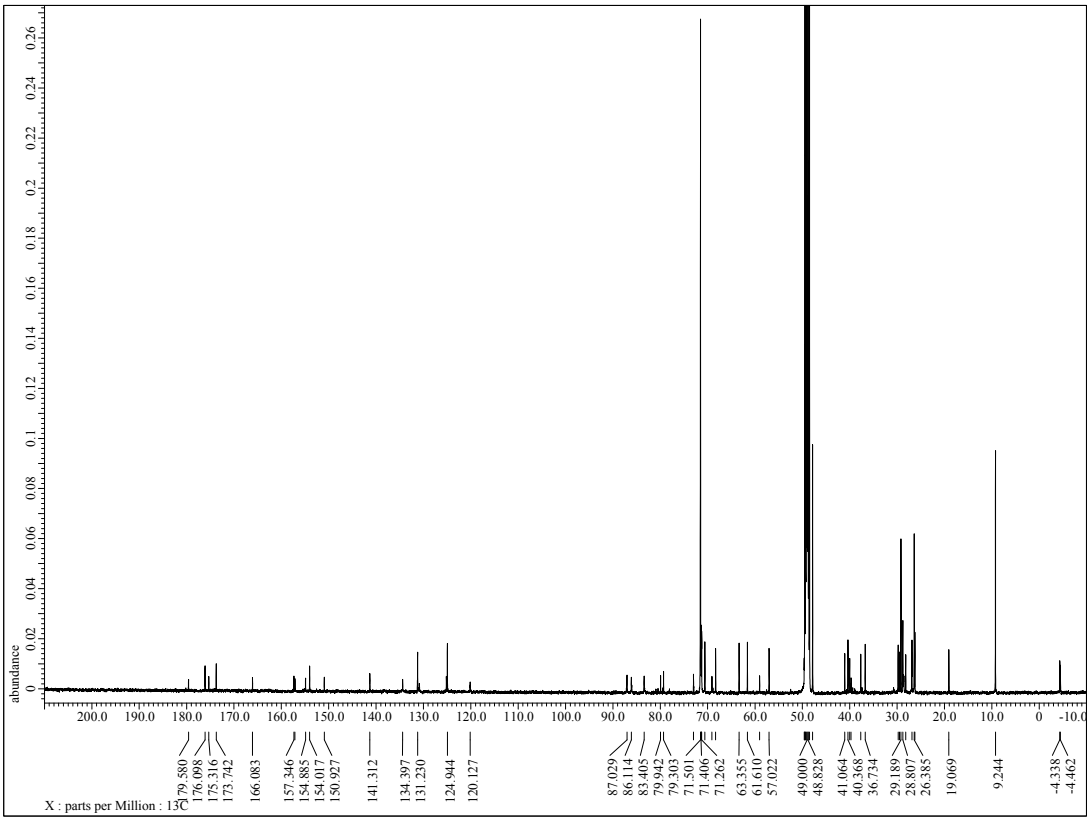

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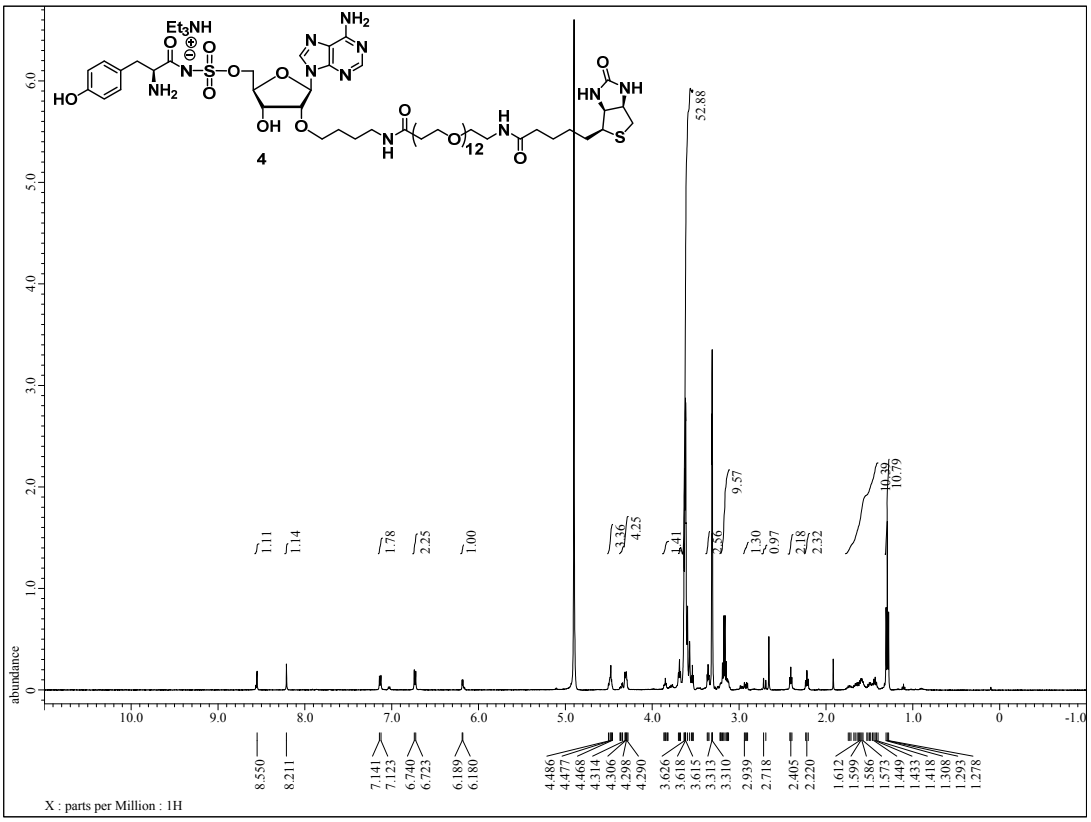

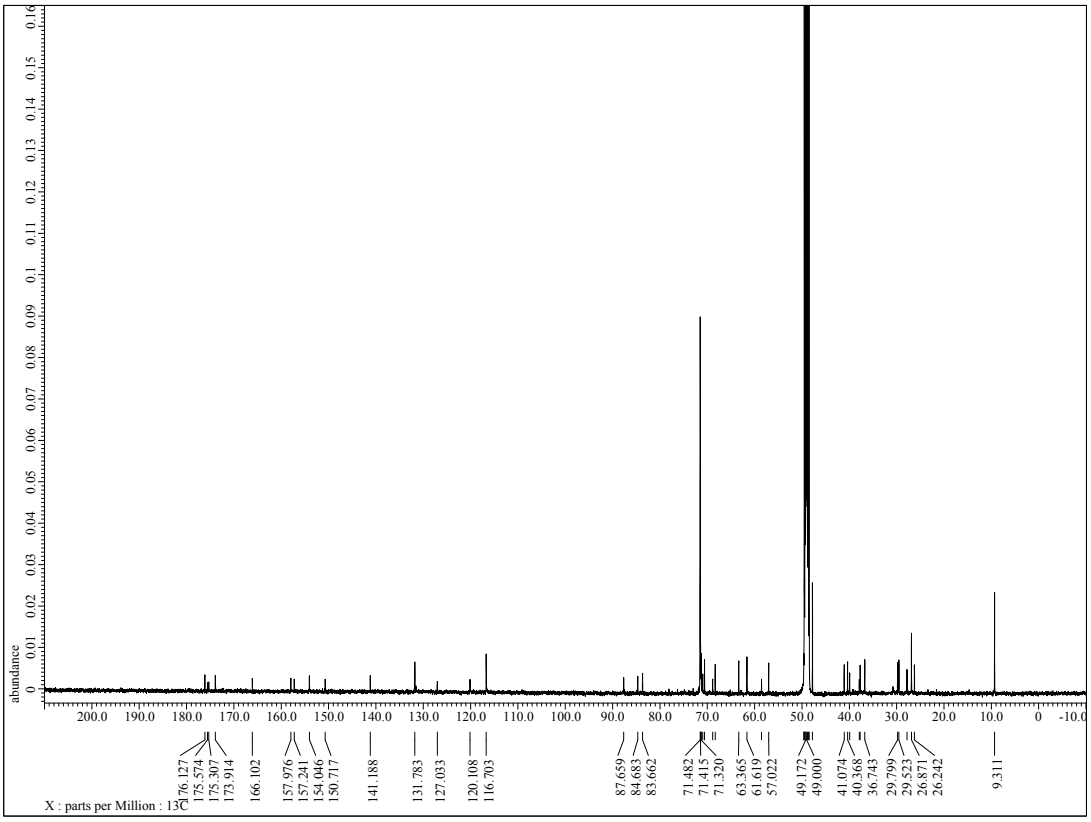

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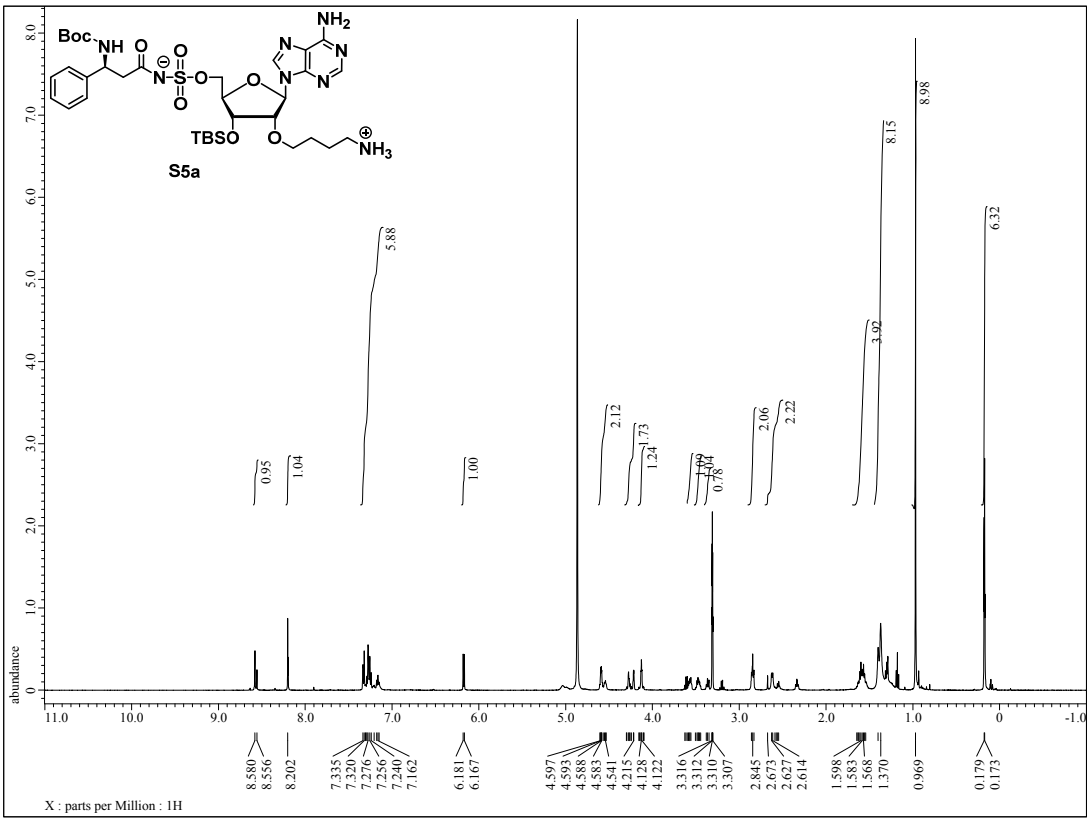

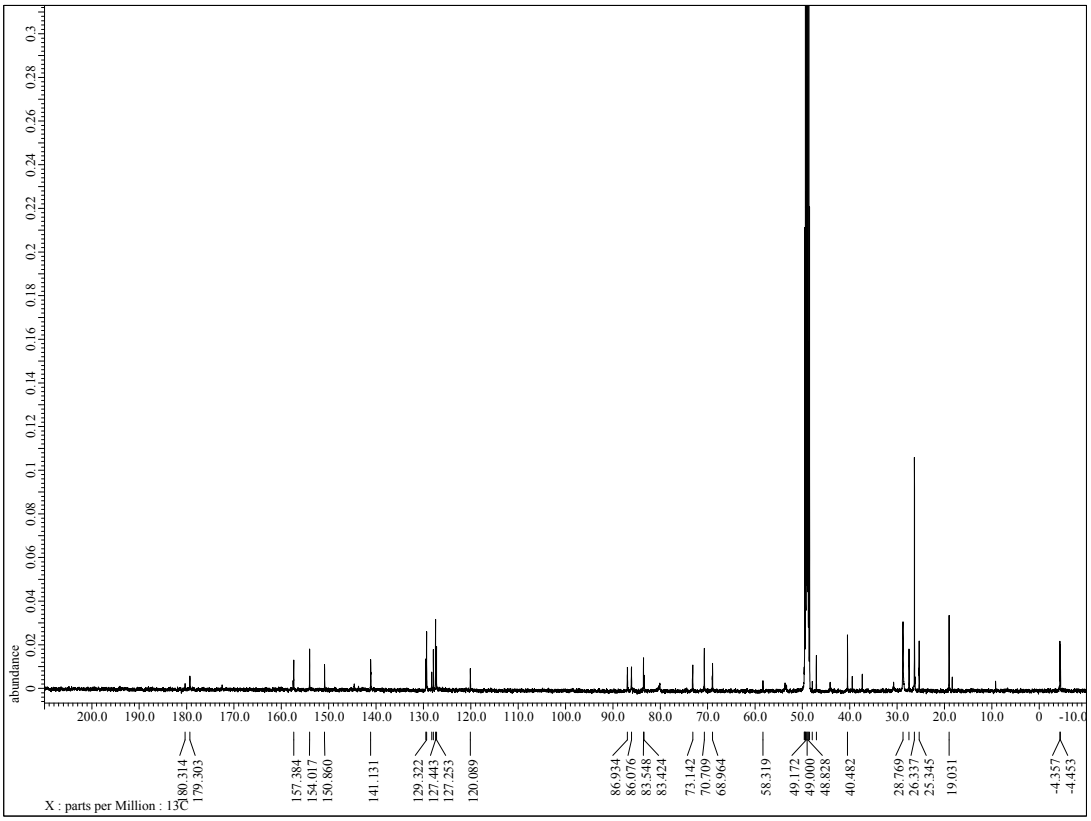

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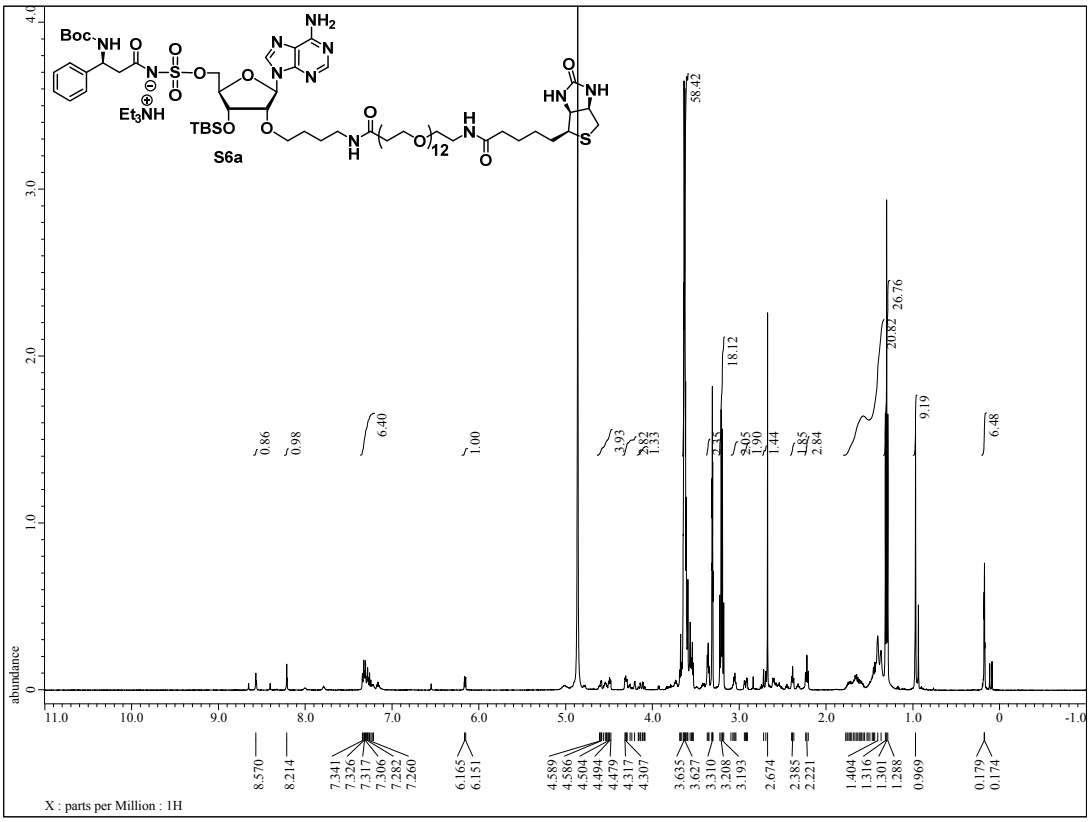

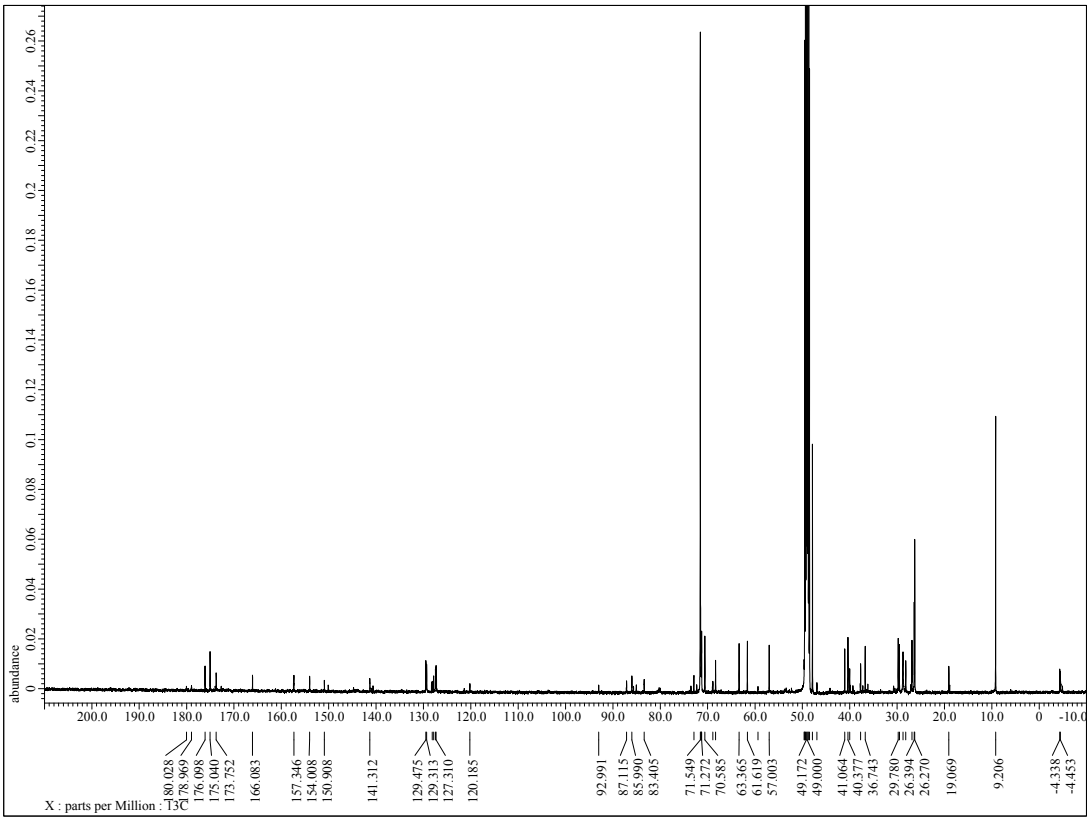

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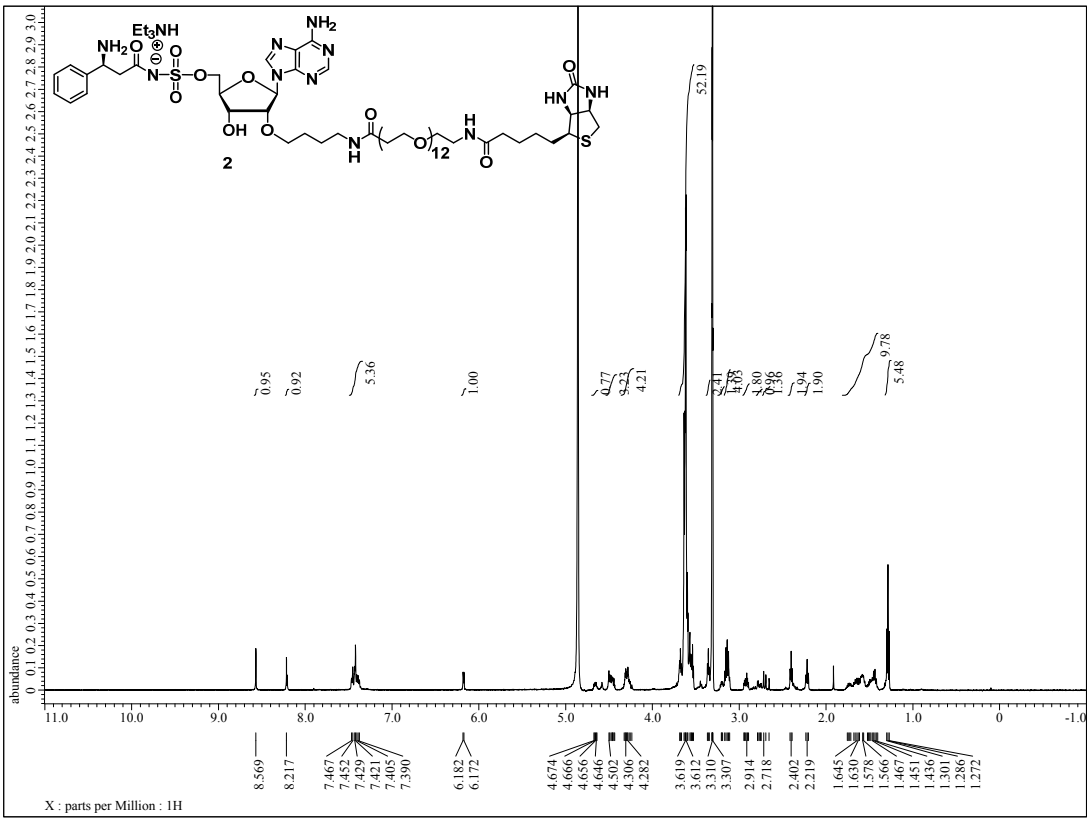

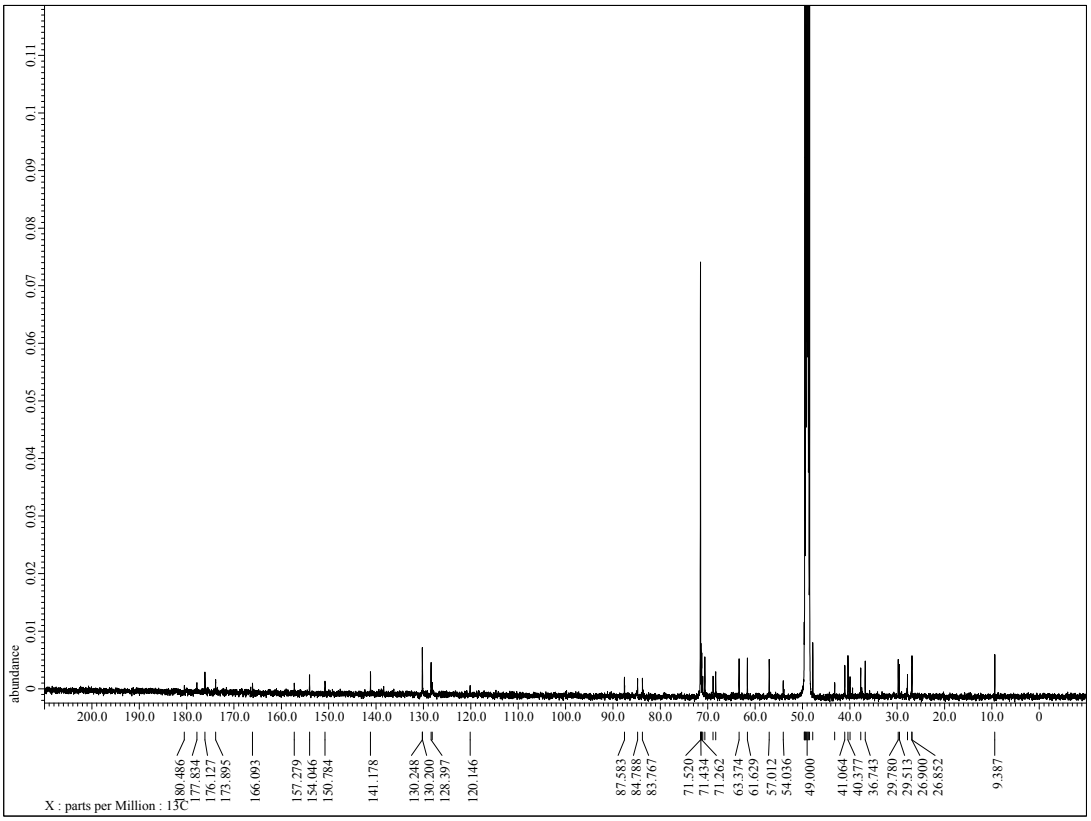

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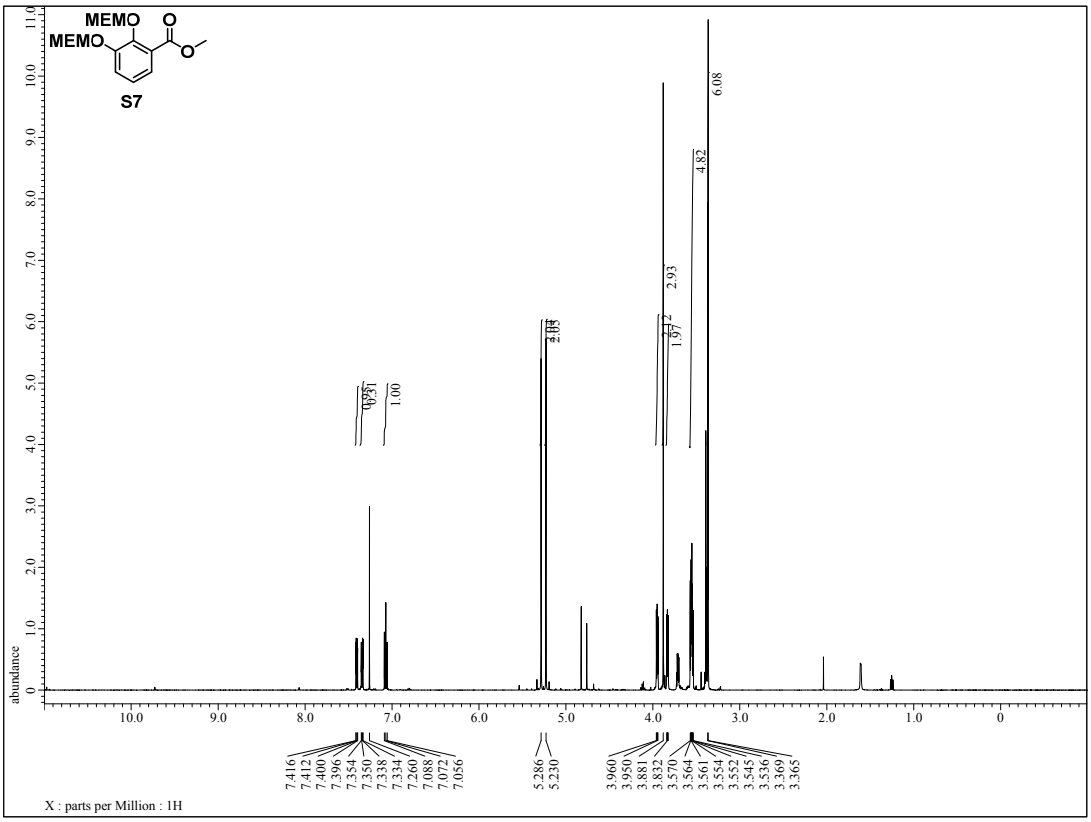

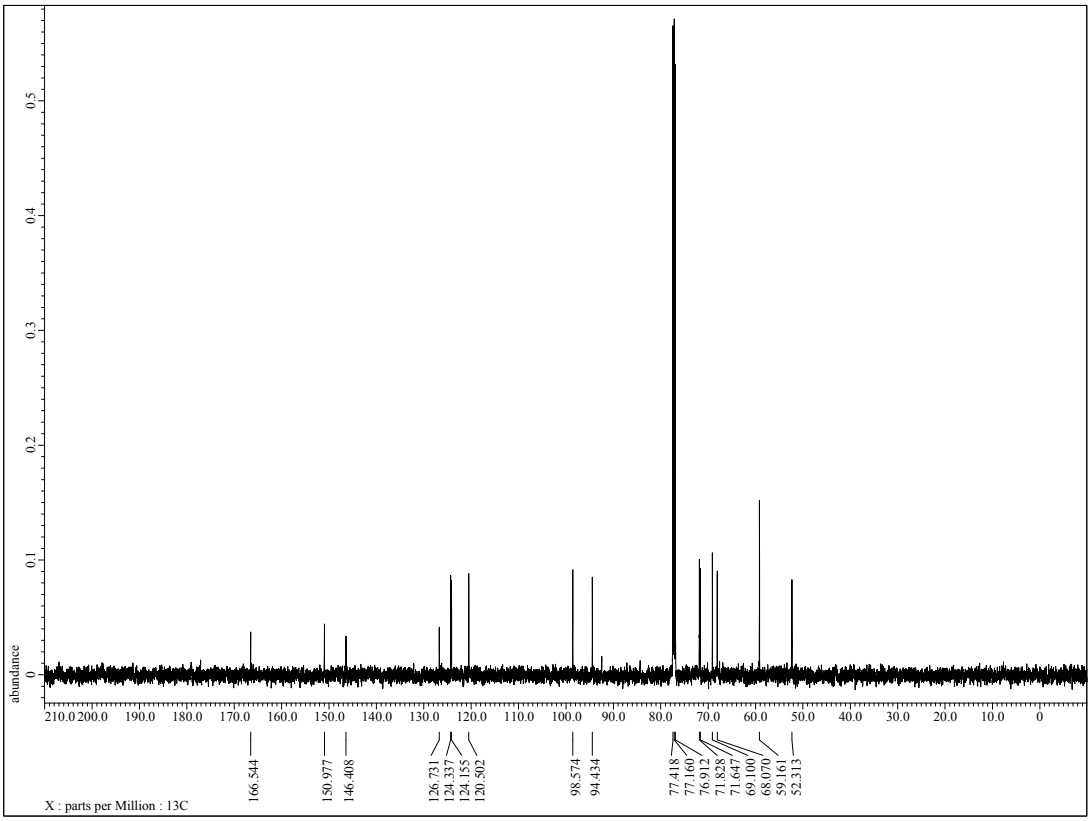

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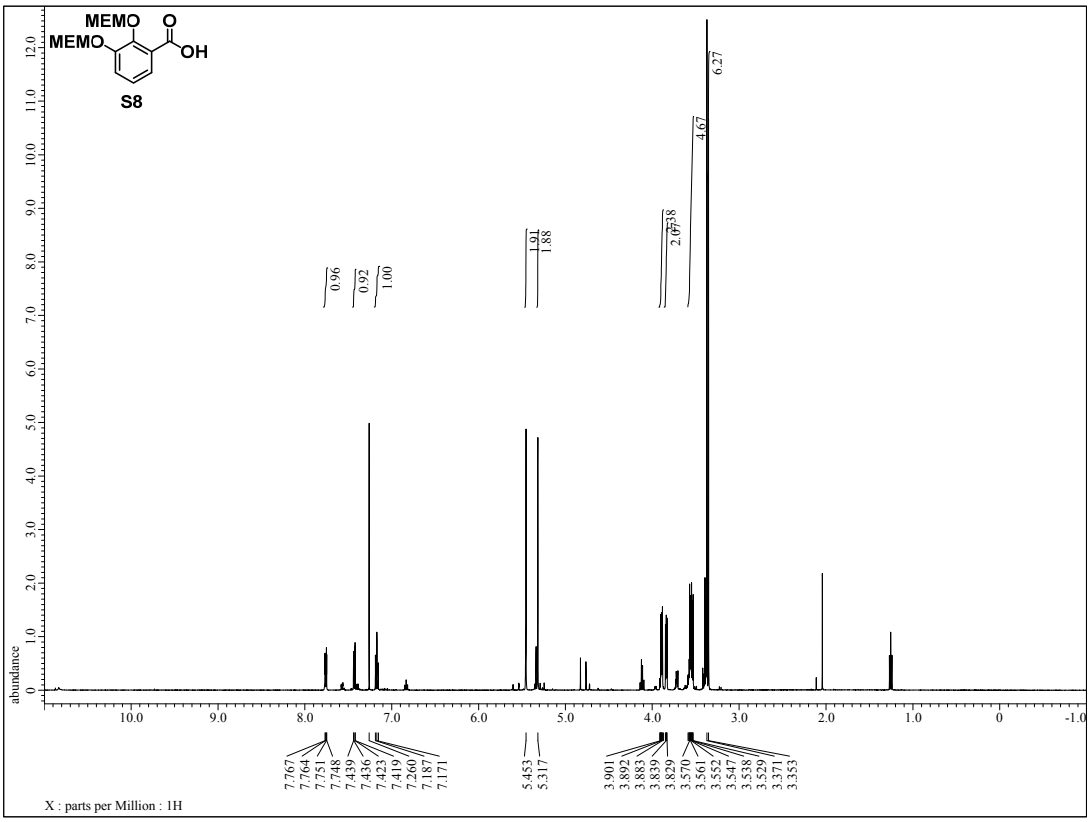

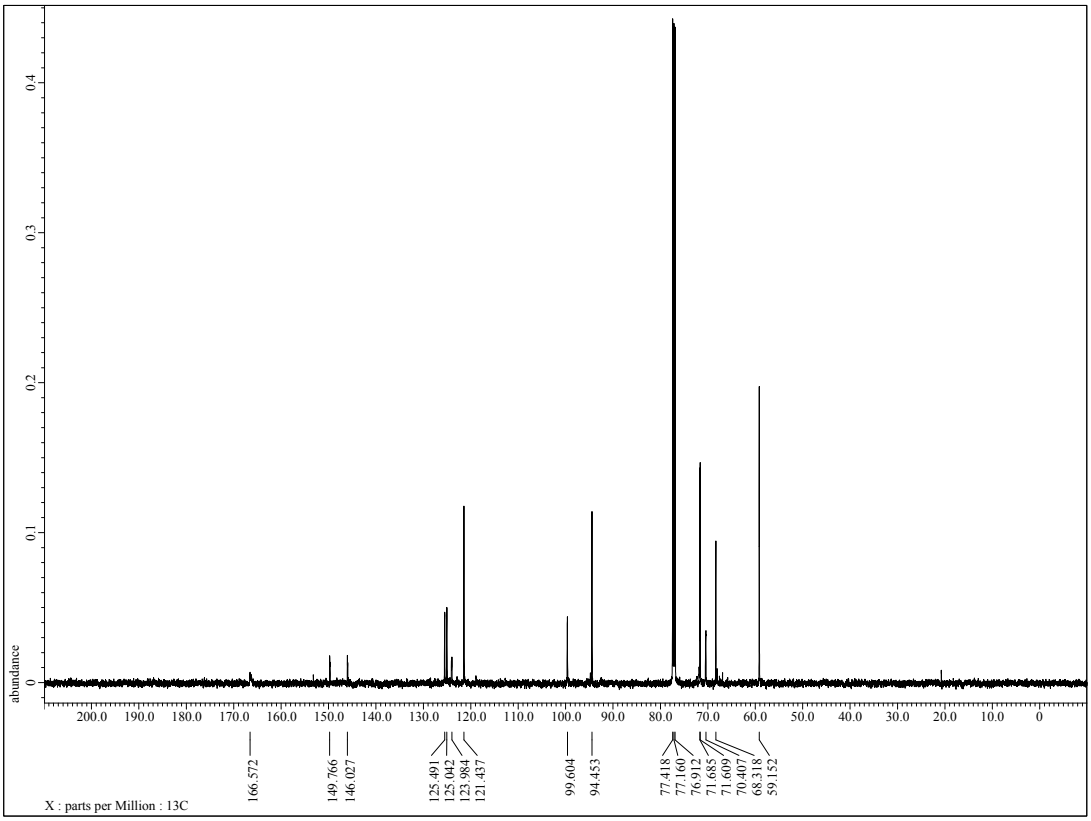

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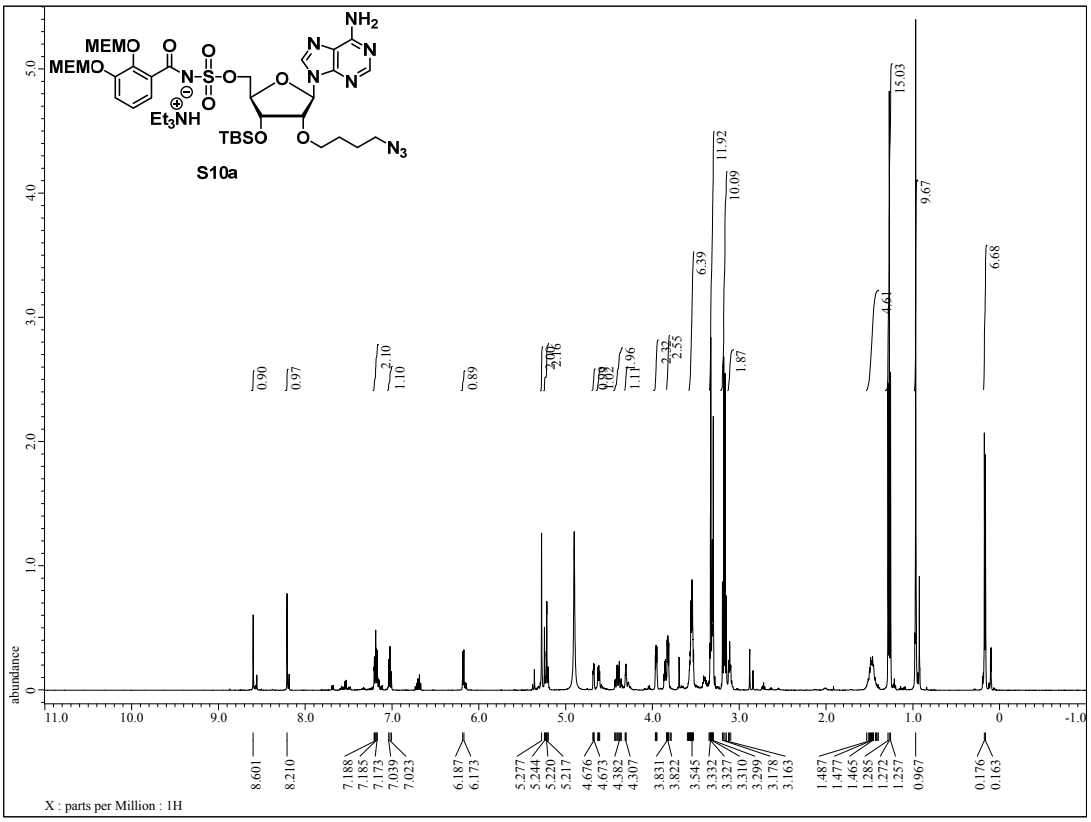

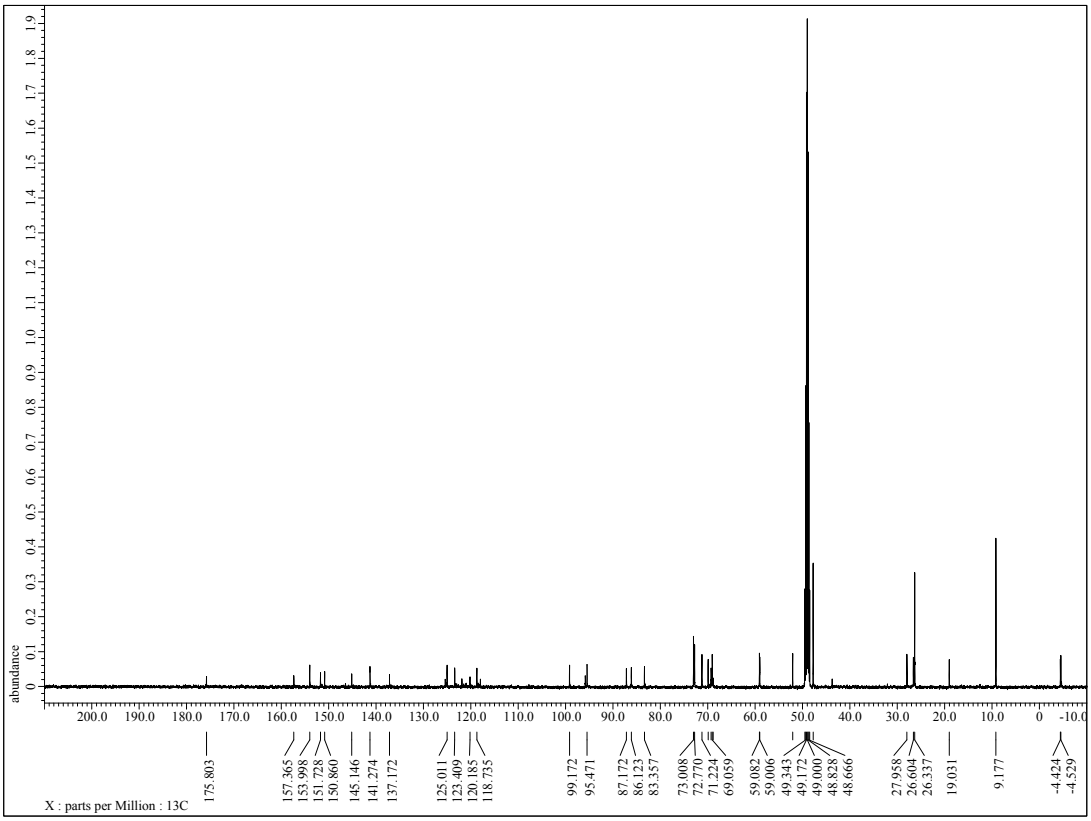

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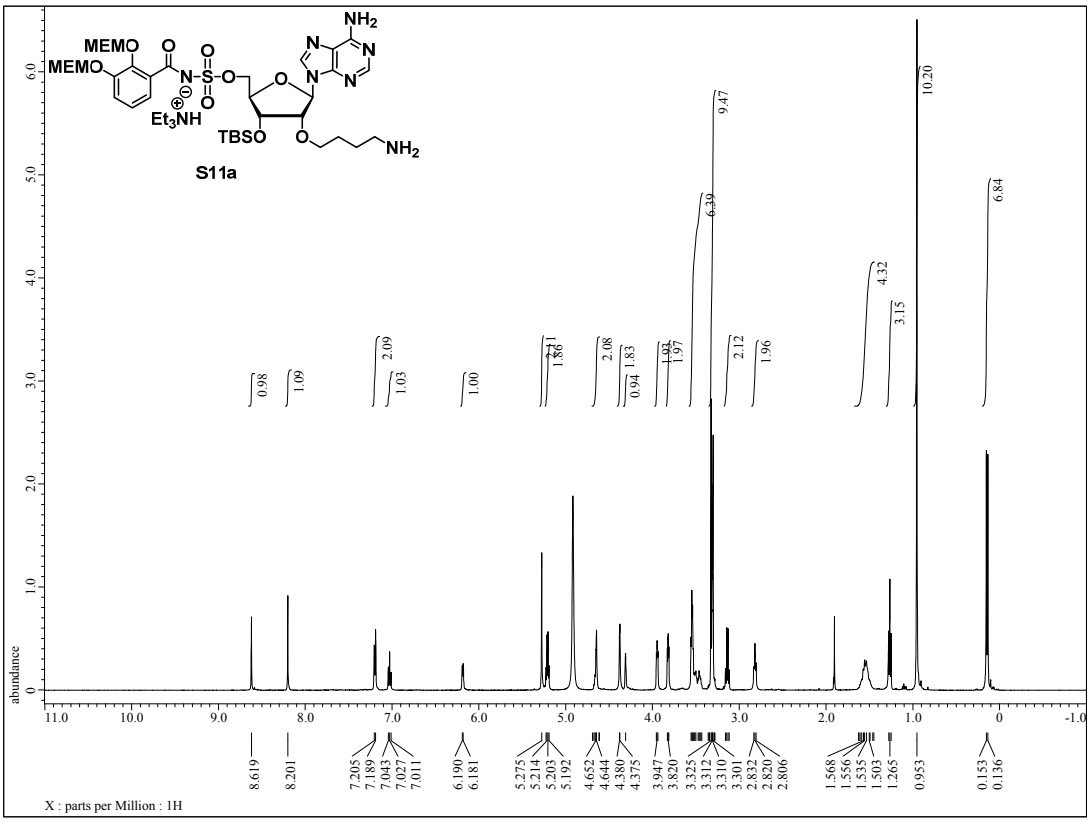

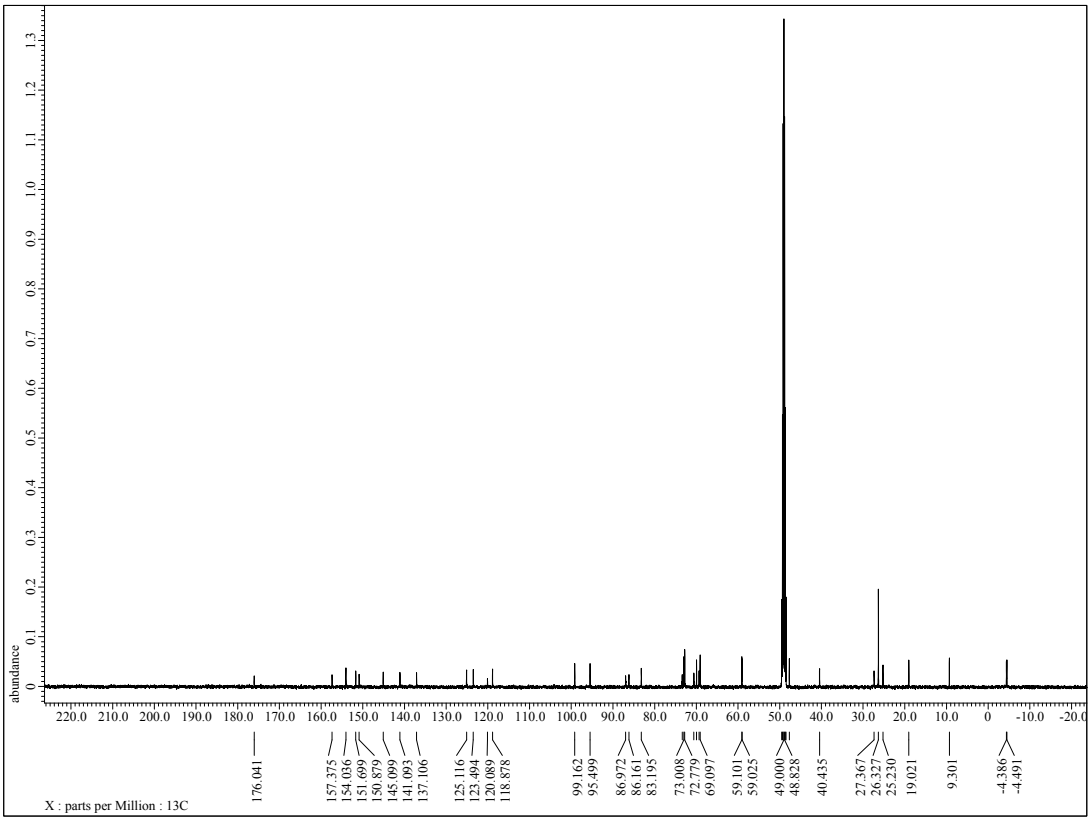

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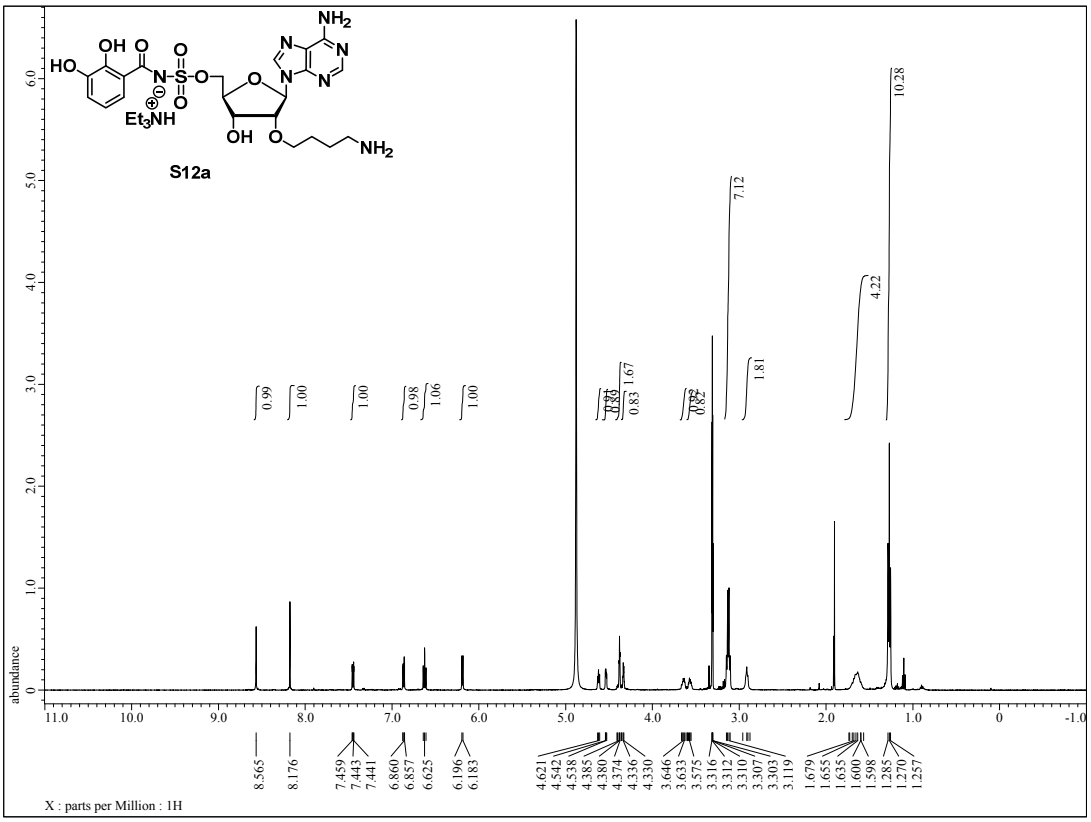

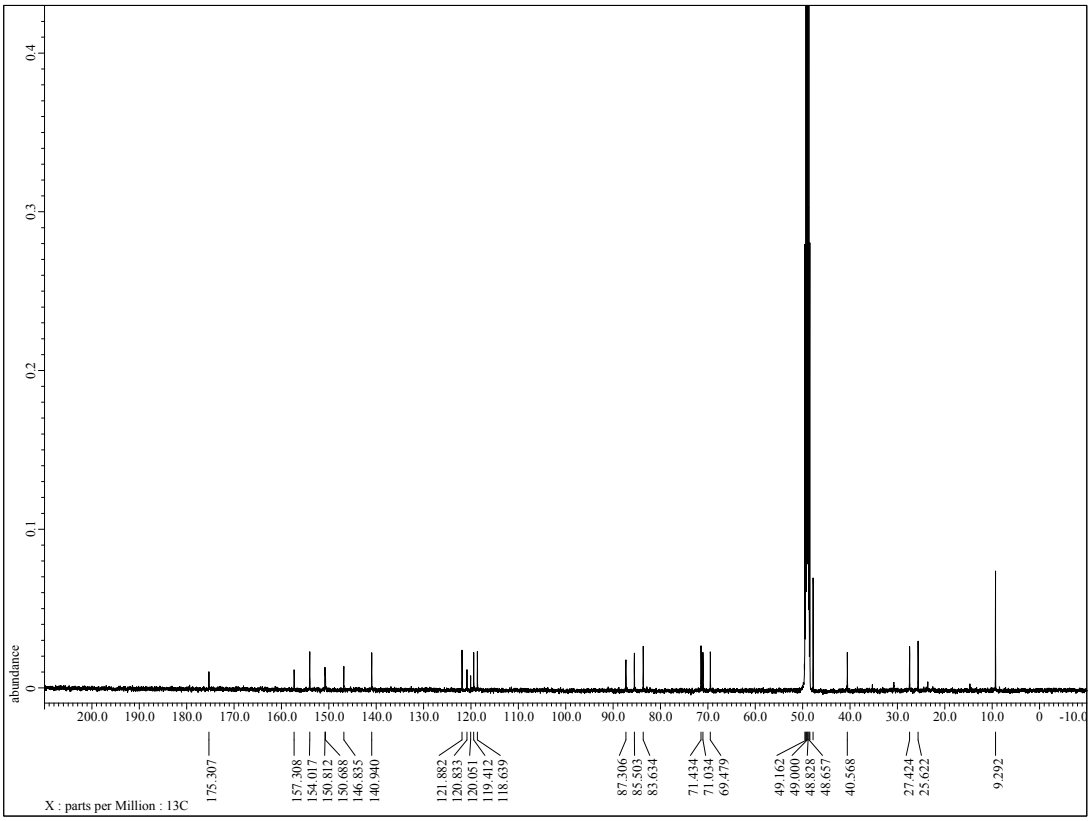

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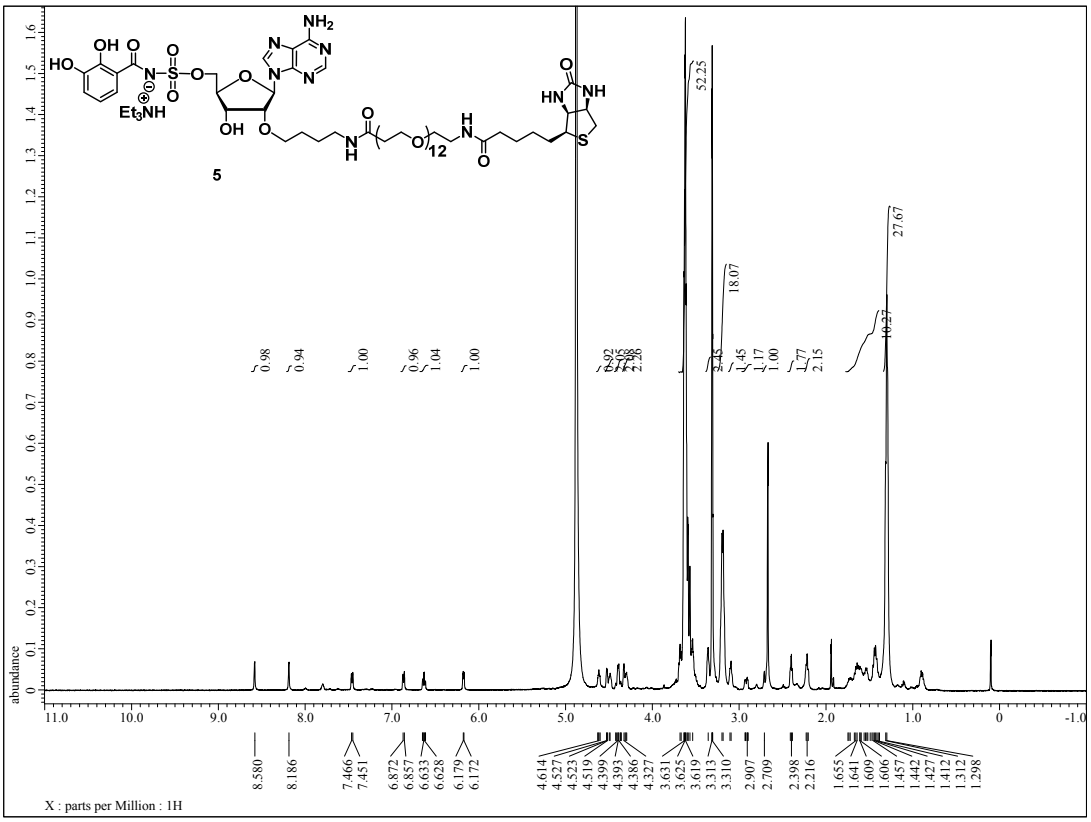

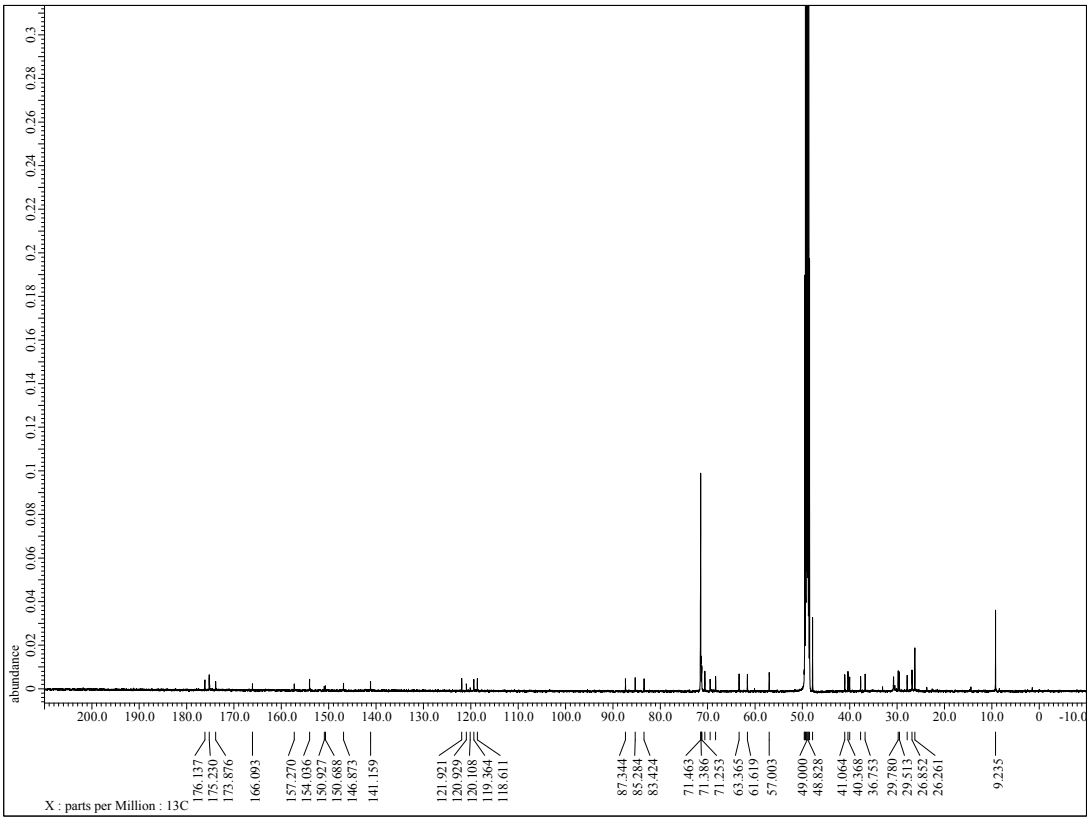

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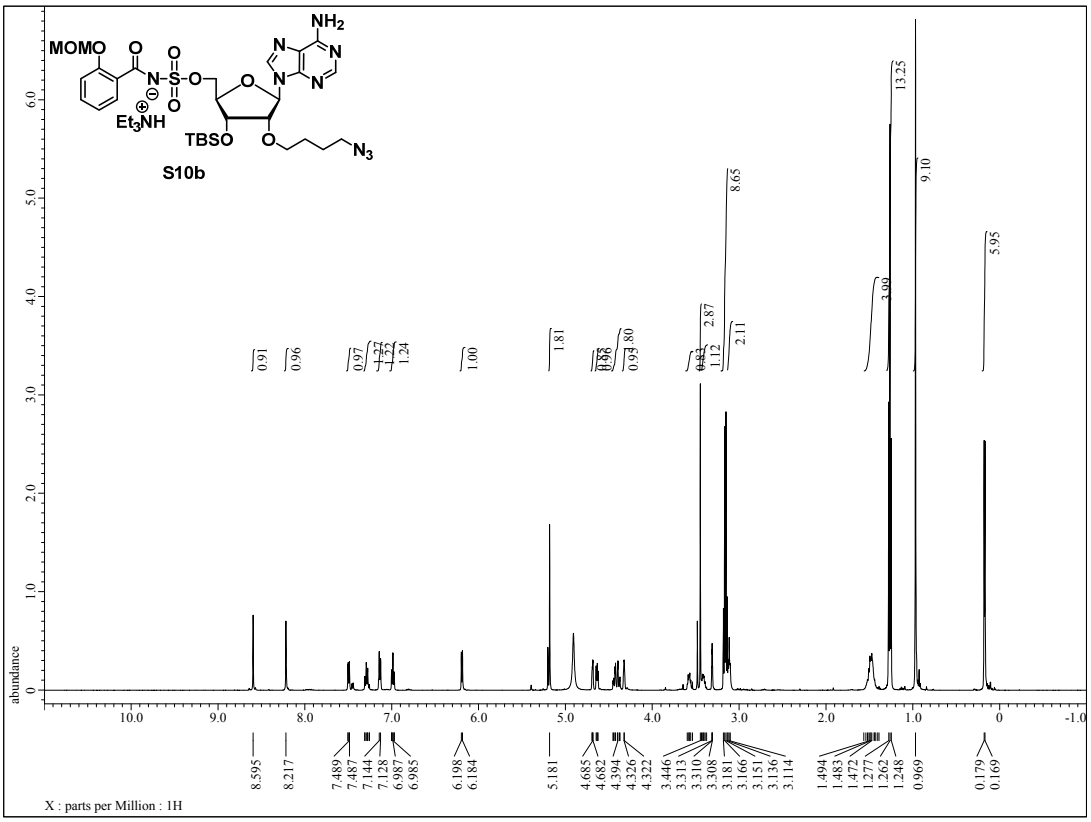

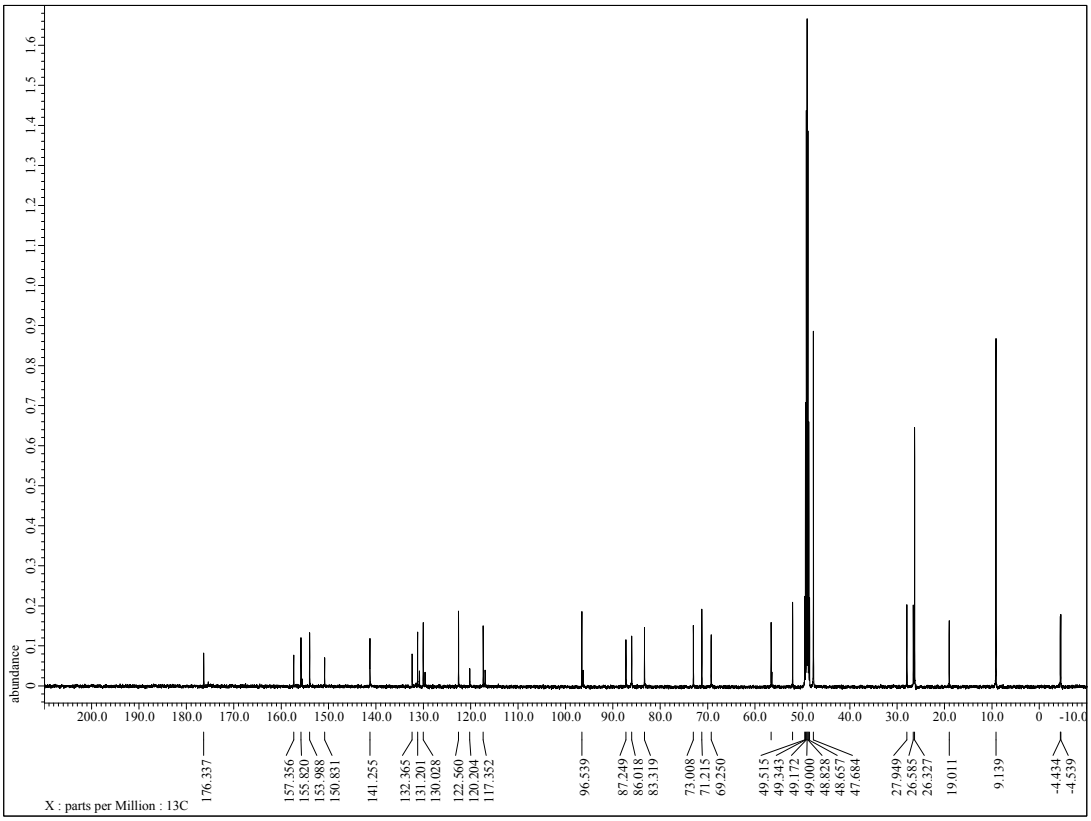

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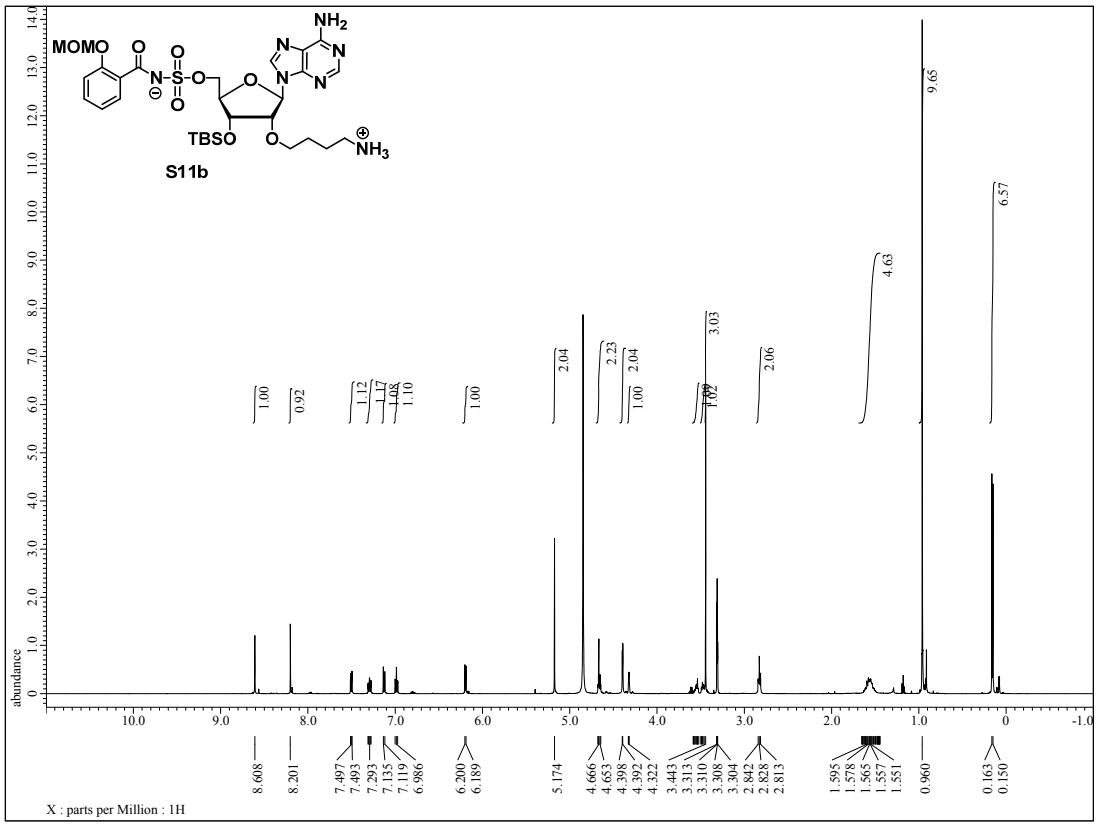

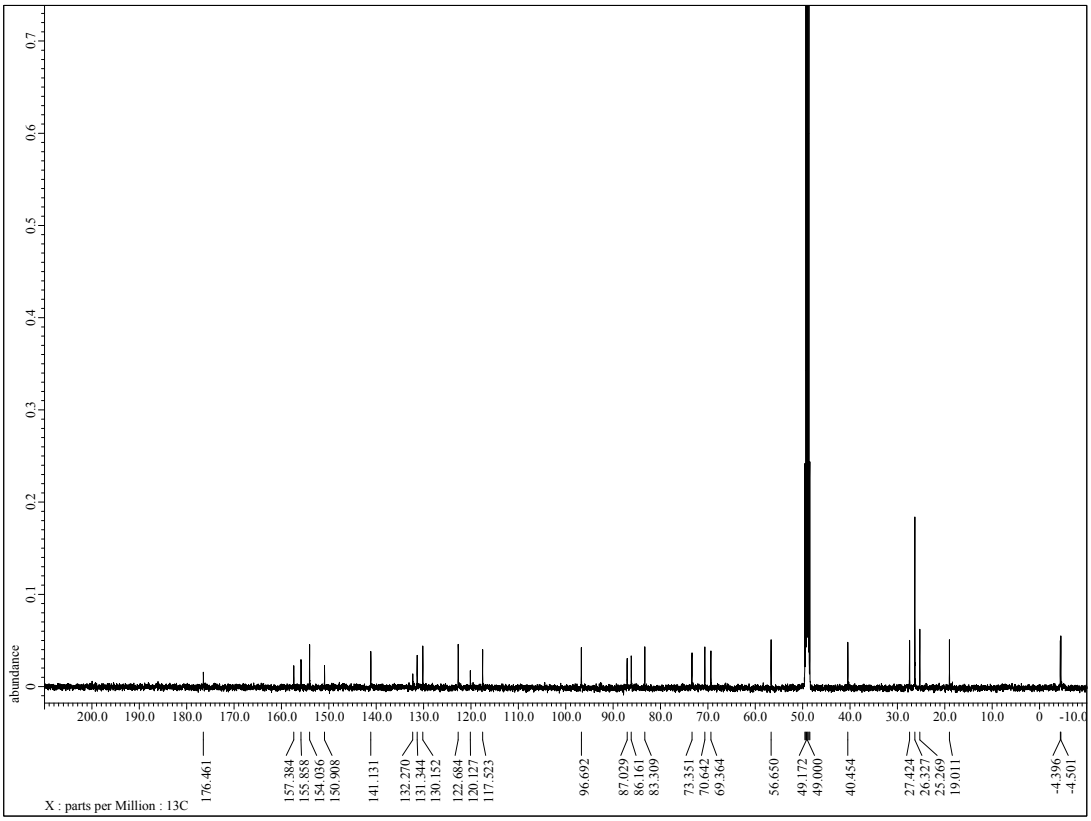

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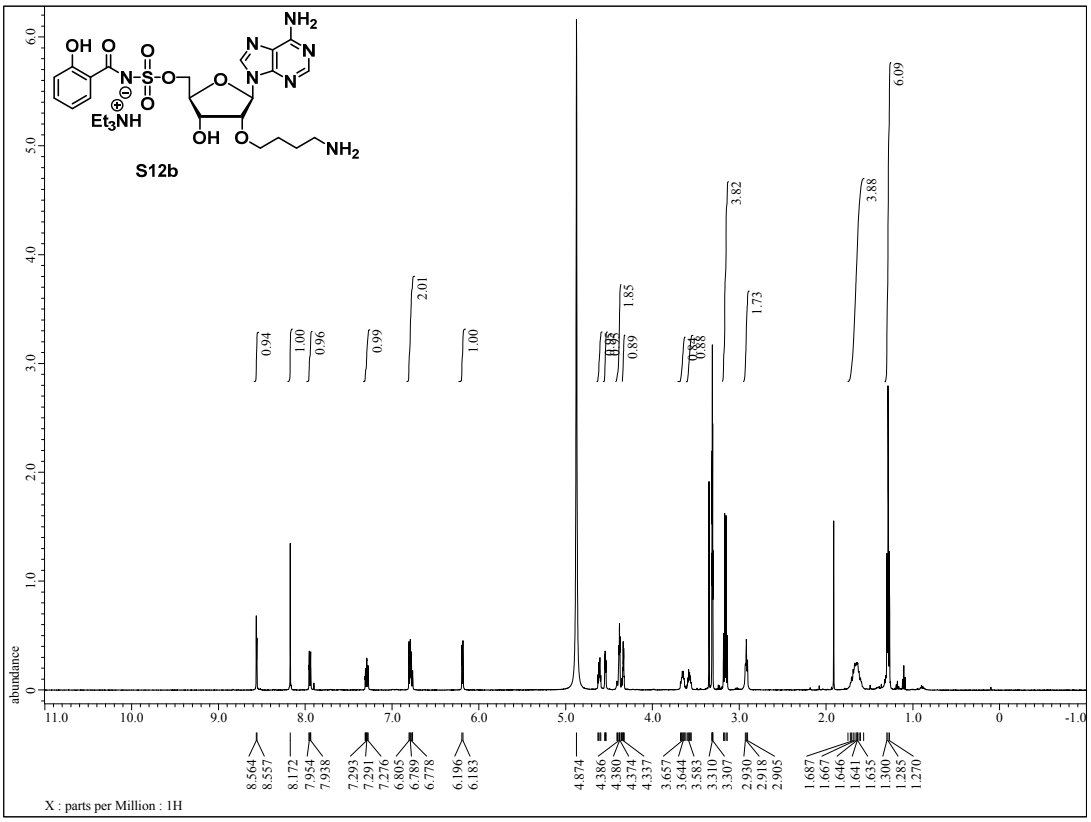

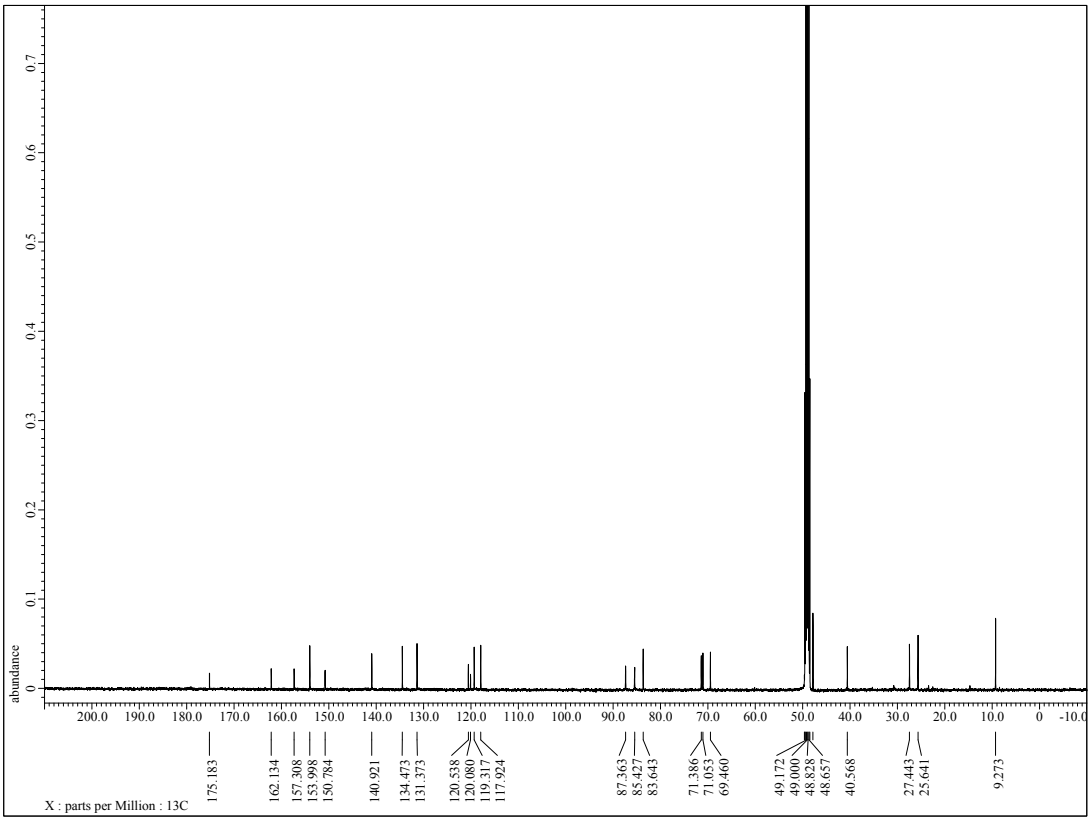

^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **6** in CD_3OD