

Supporting information

Active site-directed proteomic probes for adenylation domains in nonribosomal peptide synthetases

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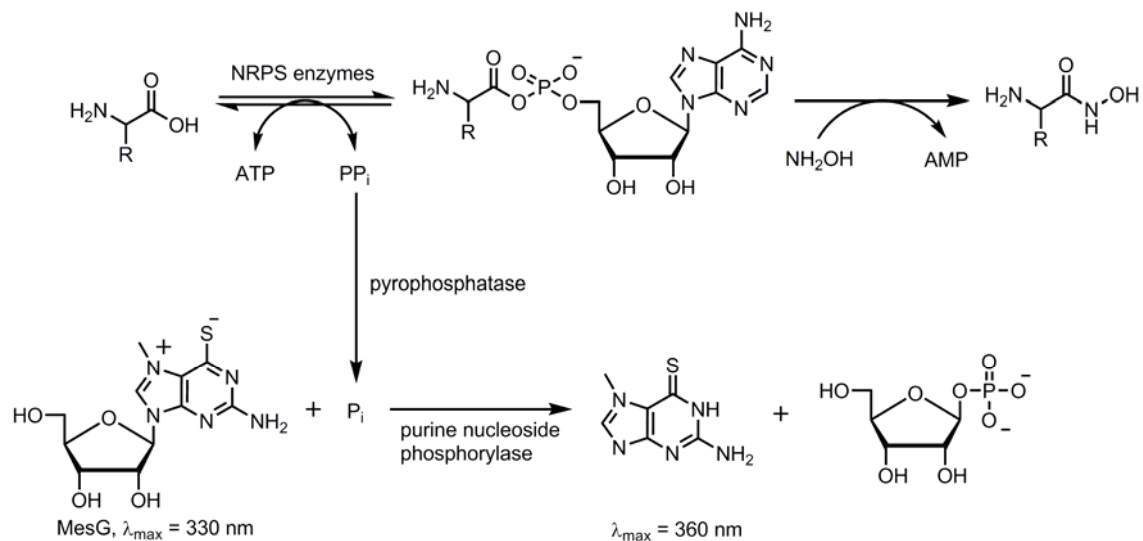


Figure S1. Schematic summary of the hydroxamate formation assay.¹ In the absence of the native acceptor, the tightly bound aminoacyl-adenylate can be released with hydroxylamine, forming the aminoacyl hydroxamate and releasing AMP. Product formation can then be monitored by coupling the release of pyrophosphate (PP_i) to the cleavage of the ultraviolet indicator MesG in a continuous format.

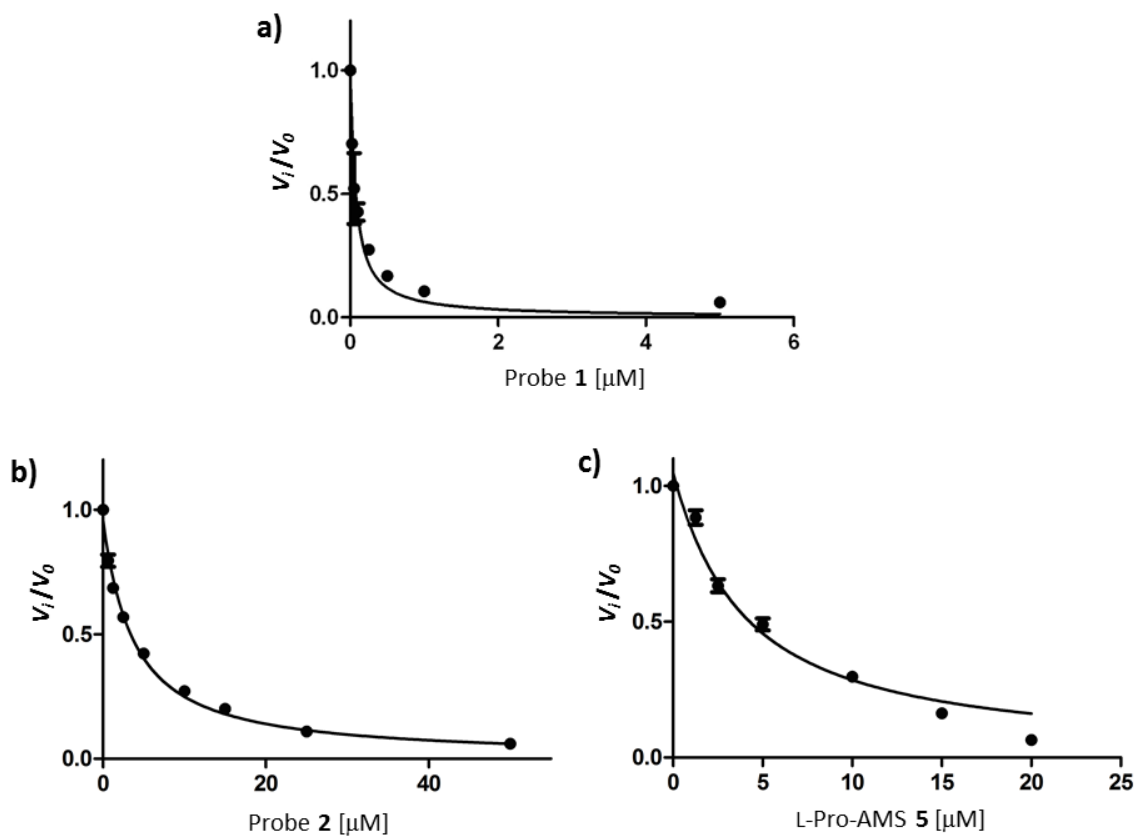


Figure S2. Inhibitory activities of recombinant NRPS enzymes by L-Phe-AMS-BPyne 1, L-Pro-AMS-BPyne 2, and L-Pro-AMS 5. (a) Inhibition of *holo*-GrsA by **1**. The reactions contained 20 nM GrsA, 1 mM L-Phe, standard assay buffer [20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM MgCl_2 , 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase, 0.04 U inorganic pyrophosphatase, and 0.2 mM MesG] and 0.0025% Igepal CA-630. (b) Inhibition of *holo*-TycB1 by **2**. The reactions contained 800 nM TycB1, 1 mM L-Pro, and the standard assay buffer. (c) Inhibition of *holo*-TycB1 by **5**. The reactions contained 800 nM TycB1, 1 mM L-Pro, and the standard assay buffer.

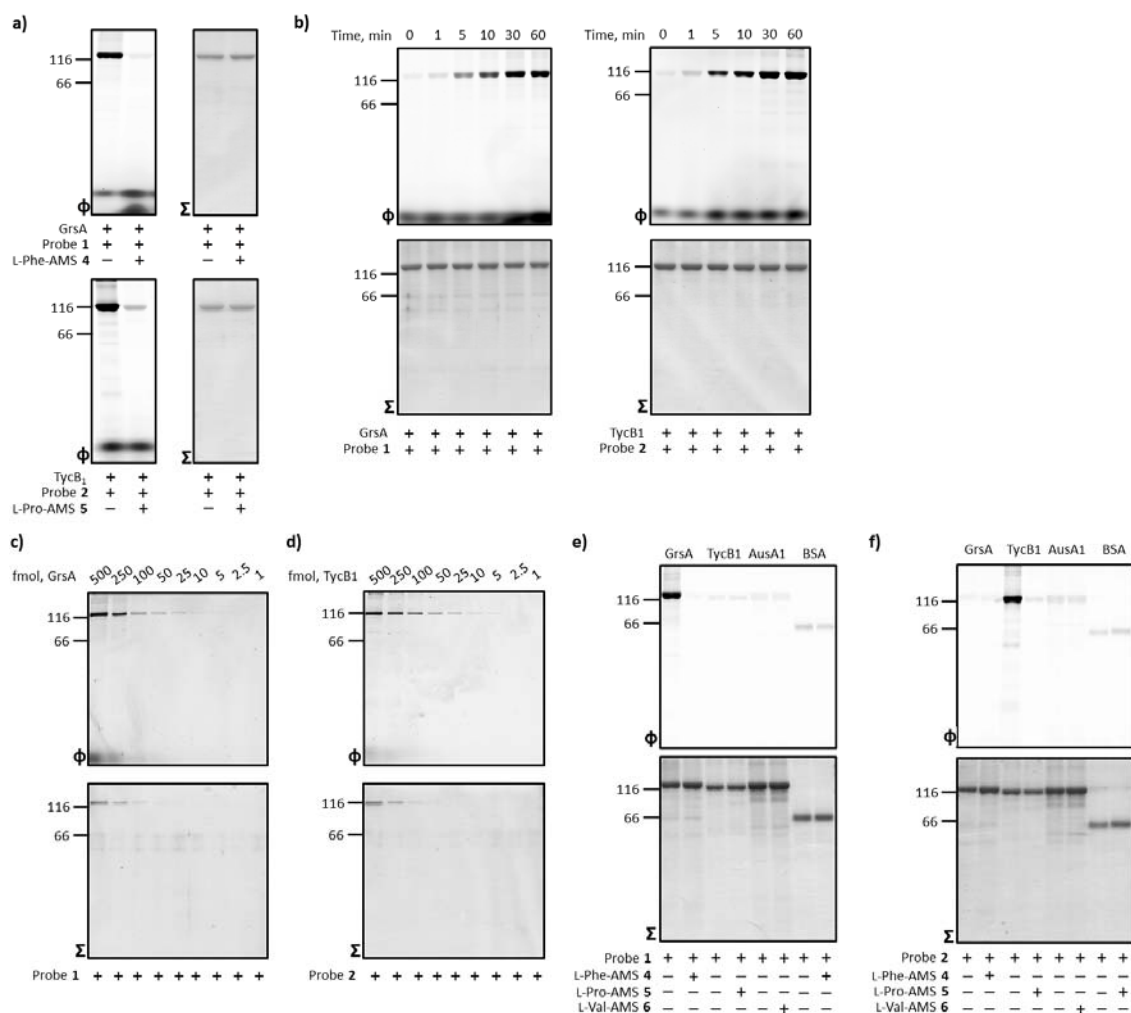


Figure S3. Full images of SDS-PAGE gels from Figure 3. Labeling of recombinant *holo*-GrsA and *holo*-TycB1 with probes 1 and 2. (a) Labeling of GrsA and TycB1 and competitive inhibition studies with excess inhibitors 4 and 5. GrsA (1 μ M) and TycB1 (1 μ M) were individually pre-incubated in either the absence or presence of 100 μ M of inhibitors 4 and 5 and treated with 1 μ M of the individual probes 1 and 2. (b) Ultraviolet photolysis time course studies of the labeling of GrsA with probe 1 (left) and TycB1 with probe 2 (right). SDS-PAGE analysis denoting the labeling of 1 μ M of GrsA and TycB1 with 1 μ M probes 1 and 2, respectively. (c, d) Limit of detection of GrsA and TycB1 labeling. GrsA (1–500 fmol) and TycB1 (1–500 fmol) were individually incubated with probes 1 and 2. (e, f) Labeling specificity of probes 1 and 2. GrsA (1 μ M), TycB1 (1 μ M), AusA1 (1 μ M), and BSA (1 μ M) were individually treated with 1 μ M of probes 1 and 2 in either the absence or presence of 100 μ M of the inhibitors 4, 5, and 6. For each panel, the top image (Φ) depicts the fluorescence observed with $\lambda_{\text{ex}} = 532$ nm and $\lambda_{\text{em}} = 580$ nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain) or a silver staining method. a) Full image for gel in Fig. 3a. b) Full image for gel in Fig. 3b. c) Full image for gel in Fig. 3c. d) Full image for gel in Fig. 3d. e) Full image for gel in Fig. 3e. f) Full image for gel in Fig. 3f.

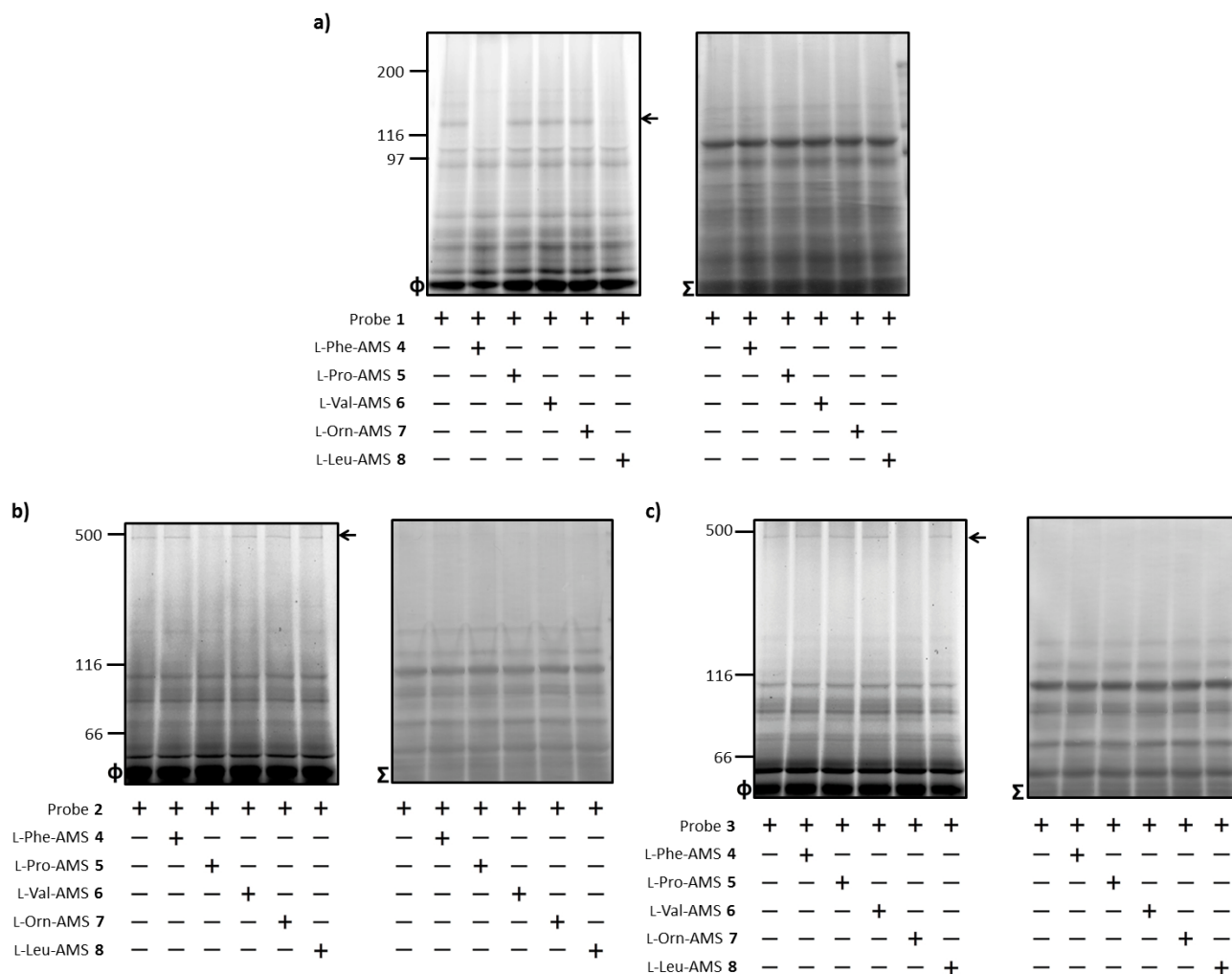


Figure S4. Full images of SDS-PAGE gels from Figure 4d. Proteomic applications of active site-directed proteomic probes **1–3** for A domains. (a) Individual labeling of A domains and profiling of A domain functions using a combination of probes **1–3** and inhibitors **4–8**. In order to investigate GrsA labeling, the *A. migulanus* ATCC 9999 lysate (1.5 mg/mL) was pre-incubated with individual members of inhibitors **4–8** (100 μ M) before the addition of 1 μ M of probe **1**. (b, c) To evaluate the labeling of GrsB, the *A. migulanus* DSM 5759 lysate (1.5 mg/mL) was individually treated with 100 μ M of inhibitors **4–8** before the addition of individual members of 1 μ M probes **2** and **3**. For each panel, the top image (Φ) depicts the fluorescence observed with $\lambda_{\text{ex}} = 532$ nm and $\lambda_{\text{em}} = 580$ nm and the bottom (Σ) denotes total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain). a) Full image for the top gel in Fig. 4d. b) Full image for the middle gel in Fig. 4d. c) Full image for the bottom gel in Fig. 4d.

Database : NCBItr 20140707 (45360603 sequences; 16206973370 residues)
 Taxonomy : Firmicutes (gram-positive bacteria) (670881 sequences)

Protein hits : [gi|68845342](#) RefName: Full-Gramicidin S synthase 2; AltName: Full-Gramicidin S synthase II; Includes: RefName: Full-ATP-dependent proline adenylase; Short-ProA; AltName: Full-Proline activase; Includes: RefName: Full-ATP-dependent valine adenylase; Short-VaA; A
[gi|68845358](#) RefName: Full-Gramicidin S synthase 2; AltName: Full-Gramicidin S synthase II; Includes: RefName: Full-ATP-dependent proline adenylase; Short-ProA; AltName: Full-Proline activase; Includes: RefName: Full-ATP-dependent valine adenylase; Short-VaA; A
[gi|645381984](#) DNA-directed RNA polymerase, beta' subunit [Aneurimibacillus aneurimilyticus]

1. [gi|68845342](#) Mass: 508991 Score: 6884 Matches: 229(41) Sequences: 179(36) emPAL 1.57

RefName: Full-Gramicidin S synthase 2; AltName: Full-Gramicidin S synthase II; Includes: RefName: Full-ATP-dependent proline adenylase; Short-ProA; AltName: Full-Proline activase; Includes: RefName: Full-ATP-dependent valine adenylase; Short-VaA; A

Query	Observed	My(e)pt	Mj(c)ic	ppm	Miss	Score	Expect	Rank	Unique	Peptide
32	301.1996	630.3847	600.3846	0.07	0	22	24	1	1	R.VELLK.L
263	331.1979	660.3812	660.3806	0.90	0	21	46	1	1	R.NSSLK.G
400	339.7004	677.3861	677.3861	0.10	0	26	12	1	1	K.VGFTVR.Q
406	340.6902	679.3658	679.3653	0.74	0	23	14	1	1	R.FGSTR.I
549	350.1767	698.3388	698.3388	0.10	0	21	15	1	1	R.YDVR.T
590	358.2384	714.4641	714.4640	0.26	0	31	1.8	1	1	K.ATLLVAK.I
682	365.2348	728.4550	728.4545	0.71	0	36	1.3	1	1	R.LVLTGR.H
687	367.6948	733.3751	733.3759	-1.07	0	24	25	1	1	R.EYVAPR.N
782	379.7116	757.4087	757.4082	0.57	0	24	14	1	1	K.DLGNLR.C
783	379.7242	757.4339	757.4334	0.72	0	24	31	1	1	R.GLEAAK.K
799	380.2219	758.4292	758.4286	0.70	0	26	19	1	1	K.EATVARE.E
835	384.7525	767.4904	767.4905	-0.08	0	21	3	1	1	K.LPAGVAK.K
868	387.2476	772.4806	772.4807	-0.13	0	27	7.6	1	1	K.ATLVR.M
997	400.7351	798.4556	799.4552	0.51	0	28	7.5	1	1	K.DVGLGR.V
1308	424.2374	846.4602	846.4600	0.27	0	32	4.3	1	1	R.LDTPVRL.L
1362	438.2350	874.4553	874.4549	0.55	0	22	43	1	1	R.TTFLHEK.I
1388	448.7278	895.4411	895.4399	1.33	0	42	0.36	1	1	U K.DGTLTYR.E
1407	451.7450	901.4755	901.4756	-0.15	0	20	30	1	1	K.DVLDLAEK.L
1462	463.7327	937.4508	937.4505	0.37	0	32	3.7	1	1	K.TNWFQK.V
1506	317.8466	950.5180	950.5185	-0.50	1	(25)	18	2	1	R.TRLESAFK.R
1507	478.2668	950.5191	950.5185	0.61	1	34	2	3	1	R.TRLESAFK.R
1529	485.2721	968.5296	968.5291	0.58	1	(27)	6.1	1	1	K.GLAELKRL + Gln-pro-Glu (N-term Q)
1531	485.8058	969.5971	969.5971	-0.02	0	50	0.032	1	1	K.VLTGQLR.I
1557	492.7722	983.5298	983.5287	1.04	0	20	30	1	1	K.AVENPQV.L
1558	329.5259	985.9558	985.9556	0.21	1	34	2.5	1	1	K.GLAELKRL
1561	497.2596	992.5047	992.5039	0.74	0	35	2.3	1	1	K.KNTNIVQVR.E
1860	504.7601	1007.5057	1007.4923	13.3	0	35	2.5	1	1	R.EGAEQYK.Y
1874	506.2585	1010.4985	1010.4855	12.9	0	26	15	1	1	K.KLMDGLYR.V + Oxidation (M)
2015	522.8076	1043.6006	1043.5863	13.7	0	34	2.8	1	1	R.LLGGDVEK.Q
2163	533.2894	1064.5642	1064.5502	13.2	0	46	0.19	1	1	K.LSYGELNAK.A
2207	535.2777	1068.5409	1068.5274	12.6	0	34	1.8	1	1	R.IGYMLDGVRL + Oxidation (M)
2306	358.8419	1073.5039	1073.5043	-0.33	0	24	14	1	1	K.DFTWHR.L
2413	538.7996	1077.5946	1077.5706	12.9	0	29	7.7	1	1	K.DGSEFLVKT
2506	545.3019	1088.5893	1088.5794	12.8	0	34	3.3	1	1	K.EFDVPLV.L
2541	365.1625	1092.4657	1092.4608	-0.12	0	27	4.8	1	1	K.EHVGMVYR.L + Oxidation (M)
2581	367.2025	1098.5858	1098.5866	0.24	0	41	0.31	1	1	K.AMAVISQVHK.E + Oxidation (M)
2634	368.8807	1106.6202	1106.6196	0.54	2	35	1.4	2	1	R.TRLESAFK.R
2637	370.2090	1107.8931	1107.8904	0.66	1	(38)	1.1	1	1	K.LFTDKTVER.M
2638	554.8106	1107.6065	1107.5924	12.8	1	41	0.43	1	1	K.LFTDKTVER.M
2847	370.5293	1108.5661	1108.5666	-0.44	0	36	1.8	1	1	K.TPDHAAVGVK.D
2853	566.8470	1131.6794	1131.6952	12.5	0	41	0.15	1	1	K.QLGGTFVVK-
2904	571.7689	1141.5232	1141.5074	13.8	0	44	0.14	1	1	R.SDGMVYGR.V + Oxidation (M)
2914	572.8349	1143.6552	1143.6400	13.0	0	37	1.4	1	1	K.RPVPVQLQK.D
3002	578.8401	1155.6656	1155.6499	13.6	0	60	0.0043	1	1	R.SLVGGDALSPLK.H
3019	581.3365	1160.6584	1160.6441	12.3	0	60	0.0046	1	1	K.EPLTLGQYK.D
3022	582.2900	1162.9654	1162.9506	12.7	0	41	0.52	1	1	K.EYDNPQK.A
3033	389.2602	1164.7588	1164.7594	-0.50	1	28	0.21	1	1	K.KGPLGVVLE.K
3034	583.3942	1164.7739	1164.7594	12.4	1	(27)	0.17	1	1	K.KGPLGVVLE.K
3075	393.1947	1176.9623	1176.9531	-0.71	0	31	4.8	1	1	R.AMTMSQVHK.E + 2 Oxidation (M)
3076	589.2976	1176.9807	1176.9531	14.9	0	(27)	1.6	1	1	R.AMTMSQVHK.E + 2 Oxidation (M)
3217	397.8669	1190.8787	1190.8779	0.72	1	36	2.1	1	1	K.DISSLDEHR.E
3218	596.3032	1190.8918	1190.8779	11.7	1	(33)	4.1	1	1	K.DISSLDEHR.E
3270	398.8951	1193.6634	1193.6629	0.45	1	21	29	1	1	K.SLVNRHEAR.T
3282	600.7966	1199.8787	1199.8645	11.9	0	44	0.17	1	1	R.FLHAEFDR.S + Oxidation (M)
3284	401.5472	1201.6199	1201.6190	0.73	1	45	0.29	1	1	K.EAVDKKADK.G
3285	601.8256	1201.6366	1201.6190	14.6	1	(26)	24	1	1	K.EAVDKKADK.G
3279	603.3321	1204.6497	1204.6340	13.1	0	38	1.2	1	1	R.DVEGLFVLR.K
3325	605.8016	1209.5886	1209.5733	12.6	0	22	40	1	1	K.ESRIMMER.S + 2 Oxidation (M)
3426	407.8812	1220.5617	1220.5608	0.76	1	22	32	1	1	K.KEHVGMVYR.L + Oxidation (M)
3443	408.5387	1222.5944	1222.5942	0.14	1	43	0.37	1	1	R.SRLNEDTK.D
3484	614.7664	1227.5182	1227.5012	13.8	0	22	13	1	1	R.ENPQVMTRE.E + 2 Oxidation (M)
3489	614.8321	1227.5497	1227.5346	12.3	1	62	0.0036	1	1	K.EEEAKDPVAK.K
3492	615.8346	1229.6346	1229.6193	12.4	0	38	1.3	1	1	K.FVPPFAPGER.M
3494	615.8385	1229.6624	1229.6445	14.6	0	48	0.13	1	1	K.FVPPFAPGER.M
3533	619.3359	1236.6873	1236.6424	12.1	0	58	0.011	1	1	K.LDPAFLYAK.K + Oxidation (M)
3550	415.2272	1242.6998	1242.6908	-0.82	0	(33)	3.2	1	1	K.LAELHGYK.D
3556	622.3456	1242.6766	1242.6608	12.7	0	37	1.3	1	1	K.LAELHGYK.D
3590	627.8134	1253.6121	1253.5962	12.7	0	45	0.23	1	1	K.QMLRFNDTK.I + Oxidation (M)
3608	630.8486	1259.6826	1259.6663	13.0	0	41	0.74	1	1	K.FVPPFAPGER.M
3615	421.5639	1261.6700	1261.6707	-0.56	0	(28)	13	1	1	R.TPHEFLLEV.Q
3617	631.8501	1261.6856	1261.6707	11.9	0	31	6.7	1	1	R.TPHEFLLEV.Q
3630	422.5544	1264.6412	1264.6412	0.05	0	61	0.0078	1	1	K.TPDHAAVDER.E
3832	633.3367	1264.6589	1264.6412	14.0	0	(49)	0.11	1	1	K.TPDHAAVDER.E
3847	635.8618	1269.7091	1269.6928	12.8	0	80	4.8e-005	1	1	R.ELGEEAQLR.K
3831	660.8461	1319.6776	1319.6605	12.7	0	26	22	1	1	K.YVVPTELEK.L
3837	441.5698	1321.6876	1321.6877	-0.07	1	(25)	30	1	1	R.EKLSYQELNAK.A
3840	661.6599	1321.7053	1321.6877	13.3	1	51	0.065	1	1	R.EKLSYQELNAK.A
3969	446.8969	1337.6689	1337.6683	0.47	1	27	16	1	1	K.KESRIMMER.S + 2 Oxidation (M)
3996	670.8959	1339.7773	1339.7599	13.0	0	43	0.15	1	1	R.EPEGETLVK.H
4015	450.2418	1347.7036	1347.7034	0.17	0	44	0.41	1	1	R.GYLNKPELTADK.F
4016	674.8672	1347.7198	1347.7034	12.2	0	(28)	14	2	1	R.GYLNKPELTADK.F
4030	676.8613	1351.7080	1351.6884	14.5	1	20	69	1	1	K.DAYRLDTPVRL.L
4043	452.9171	1355.7294	1355.7296	-0.14	2	37	1.2	1	1	K.EEEAKDPVAK.K
4064	454.5854	1360.7345	1360.7351	-0.40	1	42	0.46	1	1	R.DVEGLFVLR.E
4065	681.3833	1360.7520	1360.7351	12.5	1	(40)	0.81	1	1	R.DVEGLFVLR.E
4122	688.8716	1375.7287	1375.7095	13.9	0	37	2.3	1	1	R.GYLNRPDLTABK.F
4128	690.3536	1378.6927	1378.6728	14.4	0	(54)	0.038	1	1	R.GYLNGLTABK.F
4131	690.8448	1379.6791	1379.6568	13.3	0	72	0.00062	1	1	R.GYLNGLTABK.F + Deamidated (NQ)
4141	461.5703	1381.6891	1381.6911	-1.47	1	24	32	1	1	K.QMLRFNDTK.I + Oxidation (M)
4165	693.3489	1384.6833	1384.6656	12.8	0	45	0.21	1	1	K.AINSTVYMDR.LY + Oxidation (M)
4166	693.8421	1385.6696	1385.6497	14.4	0	(45)	0.21	1	1	K.AINSTVYMDR.LY + Oxidation (M); Deamidated (NQ)
4160	463.2790	1386.8151	1386.8156	-0.34	0	(40)	0.28	1	1	R.SVEIVGILGILK.A + Oxidation (M)
4161	694.4244	1386.8343	1386.8156	13.5	0	55	0.0093	1	1	R.SVEIVGILGILK.A + Oxidation (M)
4165	695.4091	1388.8037	1388.7850	13.5	0	73	0.00017	1	1	K.MVGLFNTLPLR.I + Oxidation (M)
4170	696.3595	1390.7044	1390.6881	11.7	0	31	8.1	1	1	K.GHLFEPNDTK.T
4189	698.8337	1395.6529	1395.6340	13.6	0	66	0.0017	1	1	R.GYMNQPALTEK.F + Oxidation (M)
4195	466.9365	1397.7876	1397.7878	-0.10	1	34	1.8	1	1	R.ELGEEAQLR.H
4216	704.3520	1406.7695	1406.7513	12.9	0	39	0.74	1	1	R.SLDMVGMGLVLA + 2 Oxidation (M)
4223	706.8906	1411.7666	1411.7493	12.2	0	37	1.1	1	1	K.ALPEPTIGLMAR.E + Oxidation (M)
4228	471.5415	1412.8028	1412.8027	0.03	0	49	0.07	1	1	K.KPLGLEAVQPK.Q
4236	708.8799	1415.7373	1415.7197	12.4	0	52	0.069	1	1	R.WLPGNIFLGR.A
4237	708.9174	1								

4245	473.9249	1418.7529	1418.7513	1.11	0	44	0.4	1	R.SPELVQVMSILKA + 2 Oxidation (M)
4257	357.9615	1427.7769	1427.7772	-0.20	1	(26)	20	1	K.KKELAEHLGQYK.D
4258	476.9331	1427.7775	1427.7772	0.20	1	39	0.96	1	K.KKELAEHLGQYK.D
4282	716.8448	1435.6750	1435.6554	13.6	0	59	0.008	1	R.WSPDGNVEFLGR.N + Oxidation (M)
4298	722.3475	1442.6805	1442.6676	8.82	0	35	2.1	1	K.YGGQETVFDLTR.D
4302	361.9463	1443.7559	1443.7569	-0.68	2	(44)	0.41	1	R.EAVVVDKADKGGK.Y
4306	462.2599	1443.7579	1443.7569	0.71	2	53	0.041	1	R.EAVVVDKADKGGK.Y
4307	722.8959	1443.7772	1443.7569	14.0	2	(39)	1.3	1	R.EAVVVDKADKGGK.Y
4342	730.8848	1459.7550	1459.7347	13.9	0	36	2.8	1	K.AGGAFFVDFPEYK.E
4344	487.6084	1459.8034	1459.8035	-0.07	0	(51)	0.059	1	R.TSFEINQKPVQK.I
4347	730.9181	1459.8216	1459.8035	12.4	0	58	0.0059	1	R.TSFEINQKPVQK.I
4348	487.9372	1460.7887	1460.7875	1.49	0	(25)	29	1	R.TSFEINQKPVQK.I + Deamidated (NQ)
4359	489.2826	1464.8259	1464.8262	-0.22	0	(30)	3.9	1	R.SLEMVGVFSILKA + Oxidation (M)
4361	733.4305	1464.8464	1464.8262	13.8	0	44	0.081	1	R.SLEMVGVFSILKA + Oxidation (M)
4371	735.4102	1468.8058	1468.7885	11.7	0	61	0.0045	1	K.SLPNLEGVNTNAY.Y
4373	491.2484	1470.7233	1470.7215	1.20	0	(43)	0.39	1	R.TSFHSINQVQVGR.V
4374	736.3789	1470.7433	1470.7215	14.8	0	(46)	0.2	1	R.TSFHSINQVQVGR.V
4377	491.5762	1471.7067	1471.7066	0.77	0	63	0.0034	1	R.TSFHSINQVQVGR.V + Deamidated (NQ)
4379	491.5964	1471.7673	1471.7671	0.13	0	27	19	1	K.THQFLTEQVEK.T
4382	492.2734	1473.7983	1473.7980	0.22	0	(24)	37	1	K.NVNLHFTFEK.T
4383	737.9195	1473.8165	1473.7980	12.6	0	25	22	1	K.NVNLHFTFEK.T
4410	498.2805	1491.8196	1491.8198	-0.09	0	24	23	1	K.LAEVHNLGVNK.I
4427	749.3801	1496.7456	1496.7260	13.1	0	(40)	0.8	1	R.TSFYSLNQEVPVGR.V
4428	749.8728	1497.7310	1497.7100	14.1	0	59	0.0096	1	R.TSFYSLNQEVPVGR.V + Deamidated (NQ)
4446	784.9390	1507.8554	1507.8358	13.0	0	(41)	0.27	1	K.GVQPDNVGLLVER.S
4447	503.6258	1507.8556	1507.8358	13.1	0	64	0.0015	1	K.GVQPDNVGLLVER.S
4497	381.4523	1521.7801	1521.7787	0.92	1	26	25	1	K.TPHVAIDEREK.L
4526	767.8821	1533.7497	1533.7380	14.2	0	32	5.7	1	K.GVQPNMSVQVMDR.S + 2 Oxidation (M)
4534	769.4031	1536.7916	1536.7719	12.8	0	(28)	14	1	K.GVTPNHPVAMTER.S + Oxidation (M)
4535	513.2717	1536.7934	1536.7719	14.0	0	35	3.3	1	K.GVTPNHPVAMTER.S + Oxidation (M)
4542	514.2955	1539.8648	1539.8443	13.3	1	53	0.021	1	R.KALPEPTGIQLMAR.E + Oxidation (M)
4543	770.9398	1539.8650	1539.8443	13.4	1	(25)	14	1	R.KALPEPTGIQLMAR.E + Oxidation (M)
4584	516.6028	1546.7867	1546.7867	12.9	0	22	73	1	K.IFHLFEEQVEK.T
4575	518.2587	1551.7942	1551.7338	13.2	1	66	0.002	1	K.LSDIDMLSEEEK.Q + Oxidation (M)
4576	776.8846	1551.7946	1551.7338	13.4	1	(40)	0.91	1	K.LSDIDMLSEEEK.Q + Oxidation (M)
4608	789.3890	1576.7634	1576.7403	14.7	0	49	0.13	1	K.ESEVMVVDNNGQK.A + Oxidation (M)
4613	526.9782	1577.9126	1577.8930	12.5	1	48	0.033	1	R.FAFDKVLTQGLLR.I
4614	789.9605	1577.9165	1577.8930	14.9	1	(29)	2.9	1	R.FAFDKVLTQGLLR.I
4658	796.4027	1594.7909	1594.7739	10.6	0	66	0.0025	1	K.AANNITTYEPAQRF.F
4659	532.6054	1594.7944	1594.7739	12.8	0	(53)	0.054	1	K.AANNITTYEPAQRF.F
4664	533.3085	1596.9038	1596.8835	12.7	1	44	0.17	1	R.KSLPNLEGVNTNAY.Y
4665	799.4597	1596.9049	1596.8835	12.4	1	(24)	16	1	R.KSLPNLEGVNTNAY.Y
4666	533.6420	1597.9043	1597.8828	13.5	1	27	9.5	1	K.LEEWKVLGLGR.V
4682	803.4597	1604.9048	1604.8814	14.6	0	24	18	1	R.ENIEVLFVFAFK.F
4750	824.4314	1646.8482	1646.8264	13.3	0	47	0.23	1	K.YSGQDQVYDPIAGR.S
4768	561.9894	1652.9463	1652.9250	12.9	0	43	0.18	1	K.TVEGLAQHFQVKA
4769	827.4809	1652.9472	1652.9250	13.5	0	(21)	28	1	K.TVEGLAQHFQVKA
4800	557.6613	1668.9621	1668.9403	13.1	1	37	0.74	1	K.EKKPLGLEAVGYK.Q
4806	838.4467	1674.8789	1674.8577	12.7	0	61	0.0094	1	K.YSGQEEVYDPIAGR.S
4818	843.4239	1684.8333	1684.8131	12.0	0	35	2.7	1	R.SGTAYNLQVQVMDGK.L + Oxidation (M)
4822	564.2823	1688.8250	1688.8032	12.9	0	60	0.011	1	K.EHLMNYDQSVTLPKK + Oxidation (M)
4823	845.9199	1688.8253	1688.8032	13.1	0	(59)	0.013	1	K.EHLMNYDQSVTLPKK + Oxidation (M)
4830	851.4553	1700.8961	1700.8733	13.4	0	34	4.4	1	K.ELGELVGNALPELR.I
4843	852.4337	1702.8529	1702.8315	12.6	0	26	26	1	K.AGGAFFVDFPEYK.E
4846	852.4634	1702.9123	1702.8890	13.7	0	36	2.6	1	K.YSGQEDVYDPIAGR.S
4847	852.8243	1703.8341	1703.8158	10.9	0	39	1.4	1	K.AGGAFFVDFPEYK.E
4867	572.9962	1715.9668	1715.9458	12.3	1	44	0.2	1	R.VYRIEGETLTKH
4900	868.4647	1734.9148	1734.8927	12.7	0	43	0.49	1	R.ELGEEISALEYEK.I
4906	584.6236	1750.8490	1750.8261	13.1	1	(20)	1.1e+002	1	K.VFQTEKEEENK.I
4907	876.4320	1750.8493	1750.8261	13.3	1	21	82	1	K.VFQTEKEEENK.I
4925	588.0098	1761.0079	1760.9825	14.2	1	44	0.13	1	K.RENIEVLFVFAFK.F
4926	882.4436	1762.8724	1762.8526	11.2	0	21	89	1	K.AGGAFFVDFPEYK.E
4928	589.0113	1764.0120	1763.9884	12.8	1	37	0.5	1	R.GVQPDNVGLLVER.S
4933	884.4574	1766.9022	1766.8839	9.28	1	61	0.0079	1	R.LNEDTDFKFLANK.Q
4934	589.9762	1766.9068	1766.8839	13.9	1	(26)	27	1	R.LNEDTDFKFLANK.Q
4953	557.6425	1789.9055	1789.8815	12.4	1	21	88	1	R.GVQPNMSVQVMDR.S + 2 Oxidation (M)
4957	886.9338	1791.8831	1791.8315	12.0	0	81	7.2e-005	1	R.VLYFDNATYATK.I
4982	449.4845	1793.9090	1793.9094	-0.22	1	22	76	1	R.EGVTPNHPVAMTER.S + Oxidation (M)
4976	602.3168	1803.9087	1803.9087	13.9	1	54	0.046	1	K.VRESVMVVDNNGQK.A + Oxidation (M)
4989	604.6679	1810.9817	1810.9577	13.2	0	50	0.084	1	K.HITAGEGLVYNEFK.R
5007	913.9525	1825.8903	1825.8669	12.8	0	29	13	2	R.NPLFDITFVLGNTDRK.S + Oxidation (M)
5020	920.9493	1839.8840	1839.8673	9.09	1	27	18	1	R.SDQSEYVYGRVDEQV.V + Oxidation (M)
5052	626.0102	1875.0087	1874.9850	12.7	2	47	0.2	1	K.ETGRDVEGLDFVVKR.E
5087	959.9955	1917.3764	1917.3513	13.1	0	35	4	1	R.GGYSVGLVDFVYVK.G
5091	642.9592	1925.8557	1925.8326	12.0	0	52	0.033	1	K.YNGYGAHNSMLGGGLER.N + Oxidation (M)
5092	643.2883	1926.8430	1926.8166	13.7	0	(26)	9.9	1	K.YNGYGAHNSMLGGGLER.N + Oxidation (M); Deamidated (NQ)
5101	484.9942	1935.9476	1935.9425	2.62	2	(42)	0.72	1	K.GVFEQTEKEEENK.I
5102	646.3296	1935.9689	1935.9425	12.6	2	52	0.081	1	K.GVFEQTEKEEENK.I
5128	977.9995	1953.9845	1953.9619	11.6	1	(27)	27	2	R.NPLFDITFVLGNTDRK.S + Oxidation (M)
5127	652.3359	1953.9858	1953.9619	12.3	1	51	0.1	1	R.NPLFDITFVLGNTDRK.S + Oxidation (M)
5137	656.7017	1967.0832	1967.0588	12.4	1	58	0.0085	1	K.HITAGEGLVYNEFK.R
5138	656.7021	1967.0846	1967.0588	14.1	1	(51)	0.048	1	K.HITAGEGLVYNEFK.R
5149	990.6215	1979.0284	1979.0000	13.4	0	29	16	1	K.SFEVEGITTFVYNSR.H
5154	663.0142	1986.0208	1985.9959	12.5	1	40	1.4	1	U K.TPHVAIVGWQDGLTYR.E
5182	664.6436	1990.9090	1990.8839	12.6	0	40	0.67	1	K.MFEPNTDITVYVLR.D + 3 Oxidation (M)
5172	665.6619	1993.9640	1993.9390	12.5	0	30	12	1	K.GVMEHSGYVYVMAWK.D + 2 Oxidation (M)
5187	503.5175	2010.0409	2010.0170	11.9	2	52	0.074	1	R.SRLNEDTDFKFLANK.Q
5188	671.0221	2010.0444	2010.0170	13.6	2	(50)	0.11	1	R.SRLNEDTDFKFLANK.Q
5195	505.5160	2018.0347	2018.0109	11.8	1	56	0.033	1	K.TPEHVAIVFEDEKVTYR.E
5196	673.6857	2018.0352	2018.0109	12.0	1	(44)	0.49	1	K.TPEHVAIVFEDEKVTYR.E
5209	677.6721	2029.9944	2029.9700	12.0	0	23	62	1	R.SYIMEDSGAALLTQGLK.L + 2 Oxidation (M)
5227	1025.0204	2048.0252	2047.9998	12.9	0	41	1.1	1	K.IYEVNQEVMVNVFK.H + 2 Oxidation (M)
5245	692.0351	2073.0835	2073.0565	13.0	0	63	0.0061	1	R.SHADVENIVQMFVNTLAK.N + Oxidation (M)
5246	1037.5508	2073.0870	2073.0565	14.7	0	(61)	0.0094	1	R.SHADVENIVQMFVNTLAK.N + Oxidation (M)
5282	702.3604	2104.0594	2104.0333	12.4	1	(36)	3.3	1	R.LSPMGEQMLFHALLDK.N + 2 Oxidation (M)
5283	527.0226	2104.0612	2104.0333	13.3	1	44	0.54	1	R.LSPMGEQMLFHALLDK.N + 2 Oxidation (M)
5288	703.3815	2107.1226	2107.0960	13.1	1	47	0.19	1	R.KSFEVEGITTFVYNSR.H
5294	704.7084	2111.1035	2111.0786	11.8	1	30	12	1	R.GYRELGEISALEYEK.I
5298	707.7203	2120.1390	2120.1153	11.2	0	42	0.52	1	R.LLFEAPTIGEISNYGAK.K
5301	1061.5708	2121.1270	2121.0993	13.1	0	(25)	31	1	R.LLFEAPTIGEISNYGAK.K + Deamidated (NQ)
5371	722.7258	2165.1555	2165.1256	13.8	0	40	1	1	U K.VLFEPTISALAGYADGGK.G + Deamidated (NQ)
5376	542.7793	2167.0722	2167.0408	14.5	1	23	66	1	R.YWKEHLMNYDQSVTLPKK + Oxidation (M)
5392	727.3632	2179.0678	2179.0402	12.7	0	66	0.0035	1	R.SHTDENIVQMFVNTLAMR.N + 2 Oxidation (M)
5615	784.3874	2360.1403	2360.1117	12.0	0	29	19	1	K.QTALHAYENPDTFFDVLVEK.L
5683	809.4171	2425.2295	2425.2060	9.69	2	26	42	1	R.DLSRNPFDITFVLGNTDRK.S + Oxidation (M)
5684	607.3164	2425.2363	2425.2060	12.5	2	(24)	66	1	R.DLSRNPFDITFVLGNTDRK.S + Oxidation (M)
5716	825.4072	2473.1997	2473.1721	11.2	0	65	0.0047	1	R.VHGNVLEQIYSESTEDQVER.I
5728	838.0582	2511.1527	2511.1224	12.1	0	69	0.0011	1	U K.IHEEVDFMYSQVSNSEQVEK.M + Oxidation (M)
5729	847.4349	2539.2828	2539.2482	13.6	0	33	8.2	1	K.ESYVAIQPVFEYYPVSSVQK.R
5764	852.1237	2553.3493	2553.3148	12.5	0	26	31	1	K.QNLDHMLVENYPLVEELQK.N + Oxidation (M)
5796	648.3552	2589.3918	2589.3591	13.6	1	(28)	16	1	R.GYLNKPELTADKFAVFPVGR.M
5797	864.1384	2589.3935	2589.3591	13.3	1	28	14	1	R.GYLNKPELTADKFAVFPVGR.M
5845	660.8363	2639.3161	2639.2798	13.8	0	22	1.2e+002	1	U R.YLFLDMRHHISQVSMQITK.E + 3 Oxidation

6104	836.4302	3341.6916	3341.6469	13.4	0	30	17	1	K.TVHQNVLFSQQHEYPFLYEIQNHTELK.Q
6116	1154.2594	3459.7564	3459.7095	13.6	0	27	41	1	K.VLQYTTCSFDVCYQEIFSTLLSGGQLYLR.K

2. [gi|58845359](#) Mass: 508362 Score: 6964 Matches: 226(41) Sequences: 174(36) emPAI 1.54
 RecName: Full=Gramicidin S synthase 2; AltName: Full=Gramicidin S synthase II; Includes: RecName: Full=ATP-dependent proline adenylyase; Short=ProA; AltName: Full=Proline activase; Includes: RecName: Full=ATP-dependent valine adenylyase; Short=ValA; A
3. [gi|545381984](#) Mass: 134825 Score: 250 Matches: 6(1) Sequences: 6(1) emPAI 0.13
 DNA-directed RNA polymerase, beta' subunit [Aneurinibacillus aneurinilyticus]

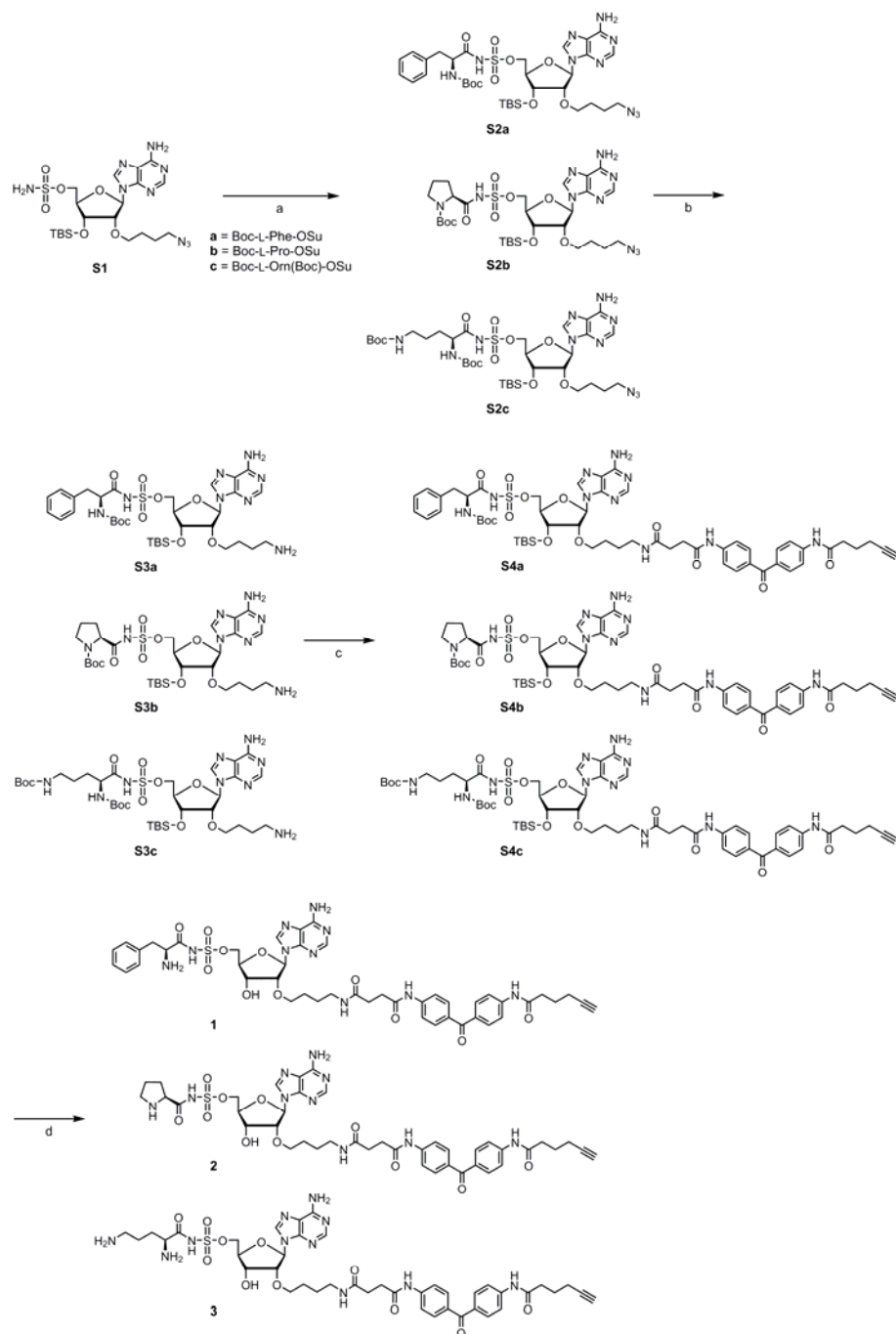
Figure S5. MASCOT report for MS/MS analysis of excised gel bands from *A. migulanus* DSM 5759 proteome.

1 MSTFK**KEHVQ** DMYRLSPMQE GMLFHALLDK DKN~~NAHLVQMS~~ IAIEGIVDVE
51 LLES~~LNILI~~ DR**YDVFR**TTF LHEKIKQPLQ VVLKERPVQL QFKDISSLDE
101 EKREQAIEQY KYQDGETVFD LTRDPLMRVA IFQTGKVNYQ MIWSFHILM
151 DGWCFNIIFN DLFNIYLSLK EKKPLQLEAV QPYKQFIKWL EKQDKQEALR
201 YWKEHLMNYD QSVTLPKKA AINNTTYEPA QFRFAFDKVL TQQLLRANQ
251 SQVTLNIVFQ TIWGIVLQKY NSTNHVVYGS VVSGRPSEIS GIEK**MVGLFI**
301 NTLPLRIQTQ KDQSFIELVK TVHQNVLFSQ QHEYFPLYEI QNHTELKQNL
351 IDHIMVIENY PLVEELQKNS IMQKVGFVTR DVK**MFEPTNY** DMTVMVLPDR
401 EISVRLDYNA AVYDIDFIKK IEGHMKEVAL CVANNPHVLV QDVPLLTQKE
451 KQHLLVELHD SITEYDPKTI HQLFTEQVEK TPEHVAVVFE DEKVTYRELH
501 ERSNQLARFL REKGVK**KESI** IGIMMERSVE MIVGILGILK AGGAFVPIDP
551 EYPKERIGYM LDSVRLVLTQ RHLKDKFAFT KETIVIEDPS ISHELTEEID
601 YINESEDLFY IYTS~~GTGK~~ PKGVMLEHKN IVNLLHFTFE KTNINFSDKV
651 LQYTTCSFDV CYQEIFSTLL SGGQLYLIRK ETQRDVEQLF DLVKRENIEV
701 LSPFVAFLEK IFNEREFINR FPTCVKHIIT AGEQLVVNNE FKRYLHEHNV
751 HLHNHYGPSE THVVTTYTIN PEAEIPELPP IGKPISTWI YILDQEQQLQ
801 PQGIVGELYI SGANVGR**GYL** NNQELTAEKF FADPFRPNER MYRTGDLAR**W**
851 LPDGNIEFLG RADHQVKIRG HRIELGEIEA QLLNCKGVKE AVVIDKADDK
901 GGKYLCAVVV MEVEVNDSEL REYLGKALPD YMIPSFVPL DQLPLTPNGK
951 IDR**KSLPNLE** GIVNTNAKYV VPTNELEEK AKIWEEVLGI SQIGIQDNFF
1001 SLGGHSLKAI TLISRMNKEC NVDIPLRLLF EAPTIQEISN YINGAKKESY
1051 VAIQPVPEQE YYPVSSVQKR MFILNEFDRS GTAYNLPGVM FLDGKLNRYRQ
1101 LEAAVKKLVE RHEALR**TSFH** SINGEPVQRV HQNVELQIAY SESTEDQVER
1151 IIAEFMQPFA LEVRPLLRVG LVKLEAERHL FIMDMHHIIS DGVSMQIMIQ
1201 EIADLYK**EKE** LPTLGIQYKD FTVWHNRLQ SDVIEKQEAY WLNVFTEEIP
1251 VLNLPTDYPR PTIQSFDGKR FTFSTGKQLM DDLYKVATET GTTLYMVLLA
1301 AYNVFLSK**YS** GQDDIVVGTG IAGRSHADVE NMLGMFVNTL AIR**SRLNED**
1351 TFKDFLANVK QTALHAYENP DYPFDLVEK LGIQR**DLSRN** PLFDTMFVLQ
1401 NTDRKSFEVE QITITPYVPN SRHSKFDLTL EVSEEQNEIL LCLEYCTKLF
1451 TDKTVERMAG HFLQILHAIV GNPTIISEI EILSEEEK**QH** ILFEFNDTKT
1501 TYPHMQTIQG LFEEQVEK**TP** DHVAVGWKQD TLYRELNERN ANQVARVLRQ
1551 KGVQPDNIVG LLVERSEML VGIMGILKAG GAYLPLDPEY PADRISYMIQ
1601 DCGVRIMLTQ QHLLSLVHDE FDCVILDEDS LYKGDSSNLA PVNQAGDLAY
1651 IMYTSGSTGK PKGVMVEHRN VIRLVK**NTNY** VQVREDDRII QTGAIGFDAL
1701 TFEVFGSLLH GAELYPVTKD VLLDAEKLHK FLQANQITIM WLTSPLFNQL
1751 SQGTEEMFAG LR**SLIVGGDA** LSPKHINNPK RKCPLNTMWN GYGPTENTTF
1801 STCFLIDK**EY** DDNIPIGKAI SNSTVYIMDR YGQLQPVGVP GELCVGGDGV
1851 AR**GYMNQPAL** TEEKFVPPNF APGERMYRTG DLAR**WLPDGT** IEYLGRIDQQ
1901 VKIR**GYRIEP** GEIETLLVKH KVKESVIMV VEDNNGQKAL CAYYVPEEEV
1951 TVSELREYIA KELPVYMVPA YFVQIEQMPL TQNGKVN**SA** LPKPDGEFGT
2001 ATEYVAPSSD IEMKLAIEIWH NVLGVNKIGV LDNFFELGGH SLRAMTMISQ
2051 VHKEFDVELP LKVLFETPTI SALAQYIADG QKGMYLAIQP VTPTDYYPVS
2101 SAQKR**MYILY** EFEGAGITYN VPNVMFIEGK LDYQRFEYAI KSLVNRHEAL
2151 RTSFYSLNGE PVQRVHQNVE LQIAYSEAKE DEIEQIVESF VQPFDEIAP
2201 LLRVGLVKLA SDR**YFLMDM** HHIISDGVSM QIITKEIADL YK**GKELAE**LH
2251 IQYKDFAVWQ NEWFQSDALE KQKTYWLNTF AEDIPVLNLS TDYPRPTIQS

2301 FEGDIVTFSFA GK**QLAEELKR** LAETGTTLY MLLLAAYNVL LHK**YSGQEEI**
 2351 **VVGTPIAGRS HADVENIVGM FVNTLALKNT** PIAVR**TFHEF LLEV**KQNALE
 2401 AFENQDYPFE NLIEKLQVRR DLSRNPLFDT MFSLSNIDEQ VEIGIEGLNF
 2451 SPYEMQYWIA KFDISFDILE KQDDIQFYFN YCTNLFKKET IERLATHFMH
 2501 ILQEIVINPE IKLCEINMLS EEEQQR**VLYD FNGT**DATYAT **NKIFHEL**FEE
 2551 **QVEKTPDHIA VIDEREKLSY QELNAKANQL** ARVLR**QKGVQ PNSMVGIMVD**
 2601 **RSLDMIVGML GVLKAGGAYV PIDIDYPQER** ISYMMEDSGA ALLLT**QK**KL
 2651 QQIAFSGDIL YLDQEEWLHE EASNLEPIAR PHYIAYIYT SGTTG**KPKGV**
 2701 **MIEHQSYVNV AMAWKDAYRL DTFPVR**LLQM ASF²AFADVS AGDFARALLT
 2751 GGQLIVCPNE VK**MDPASLYA IIKYDITIF** EATPALVIPL MEYIYEQKLD
 2801 ISQLQILIVG SDSCSMEDFK TLVSR**FGSTI RIVNSYGVTE** ACIDSSYYEQ
 2851 PLSSLHVTGT VPIGKPYANM KMYIMNQYLQ IQPVG**VIGEL CIGGAGVARG**
 2901 **YLNRPDLTAE KFVNPFPVPG EKLYRTGDLA** R**WMPDGNVEF LGR**NDHQVKI
 2951 RGIR**IELGEI EAQLRKHDSI KEATVIARE**D HMKEKYLCA**Y MVTEGEV**NVA
 3001 ELRAYLANDR AAMIPSYFVS LEAMPLTANG KIDKRSLPEP DGSISIGTEY
 3051 DRPRTMLEGK **LEEIWKDVLG LQRVGIHDDF** FTIGGHS**LKA MAVISQVHKE**
 3101 CQTEVPLR**VL FETPTIQGLA KYIETDTEQ** YMAIQPVSGQ DYYPVSSAQK
 3151 RMFIVNQFVG VGISYNMPSI MLIEGKLERT **RLESAFKRLI** ERHESL**RTSF**
 3201 **EIINGKPVQK IHEEVDFNMS YQVASNEQVE** KMIDEFIQPF DLSVAP**LLRV**
 3251 **ELLKLEEDRH VLIFDMHHII SDGISSNILM** **KELGELYQGN ALPELRIQYK**
 3301 DFAVWQNEWF QSEAFKKQEE YWVNVFADER PILDIP**TDYP RPMQQSFDGA**
 3351 QLTFGTG**KQL MDGLYRVATE** TGTTLYMVLL AAYNVLLSK**Y SGQEDIIVGT**
 3401 **PIVGRSHTDL ENIVGMFVNT LAMR**NKPEGE KTFKAFVSEI KQNALAAFEN
 3451 QDYPFEELIE KLEIQRDLSR NPLFD**TLFSL QNIGEE**SFEL AELTCKPFDL
 3501 VSKLEHAKFD LSLVAVV**FEE EIAFGLQYCT** KLYKEK**TVEQ LAQHFIQIVK**
 3551 **AIVENPDVKL SDIDMLSEEE KKQIMLEFND** TKIQY**TQNQT IQELFEEQVK**
 3601 KTPEHIAIVW EGQUALI**YHEL NIKANQLARV** LR**EKGVTPNH PVAIMTERSL**
 3651 **EMIVGIFSIL KAGGAYVPID P**AYPQ**ERIQY LLED**SGAALL LTQSHV**LNKL**
 3701 PVDIEWLDTL DEQNYVEDGT NLPFMNQSTD LAYI**YTS**GT TGKPKG**VMIE**
 3751 HQSIINCLQW RKEEYEF**PG DTALQVFSFA** FDGFVASLFA PILAGATSVL
 3801 PK**EEEAKDPV ALK**KLIASEE ITHYYGVPSL FSAILDVSS **KDLQNLRCVT**
 3851 LGGEK**LPAQI VKKIKEKNKE IEVNNEYGPT** ENSV**VTTIMR** DIQVEQ**EITI**
 3901 GRPLSNVDVY IVNCNHQLQP VGVV**GELCIG GQGLAR**GYLN **KPELTADK**VF
 3951 **VNPFVPGERM YKTGDLAKWR SDGMIEYVGR** VDEQ**VKVRGY RIELGEIESA**
 4001 **ILEYEKIKEA VVMVSEHTAS EQMLCAYIVG** EEDVLTDLR SYLAK**LLPSY**
 4051 **MIPNYFIQLD SIPLTPNGKV DRKALPEPQT** IGLMAREYVA PRNEIEAQLV
 4101 LIWQEV**LIGIE LIGITDNFFE LGGHSLKATL** LVAKIY**EQ IEMPLNVVFK**
 4151 HSTIMKIAEY ITHQES**NNV HQPILVNVEA DREALSLN**GE KQRK**NIELPI**
 4201 LLNEETDRNV FLFAPIGAQ**G VFYK**KLAEQI PTASLYG**DFD IEDDDRIQ**QY
 4251 IESMIQTQSD GQYVLIGYSS GGNLAF**EVAK EMERQGYSVS** DLV**LF**DVY**WK**
 4301 **GKVFEQTKEE EENIKIIME ELRENPGMFN** M**TREDFEL**YF ANEFVK**QSFT**
 4351 RKMRYMSFY TQLVNYGEVE ATIH**LQAEF EEEKIDENEK** ADEEEK**TYLE**
 4401 EKWNEKAWNK AAKRFV**KYNG YGAHSNMLGG DGLERNSSIL** KQILQ**GTFV**V
 4451 **K**

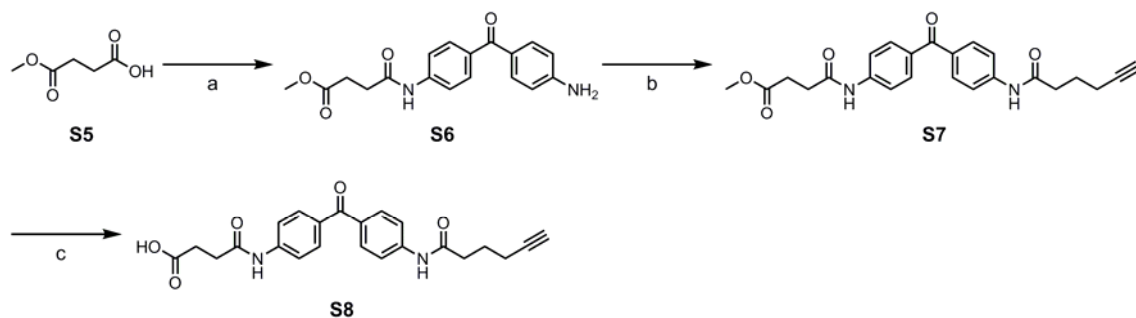
Figure S6. Compiled MS/MS data of gel excised fluorescent GrsB bands from *A. migulanus* DSM 5759 proteome. Peptides found are shown in red.

Chemical Synthetic Procedures

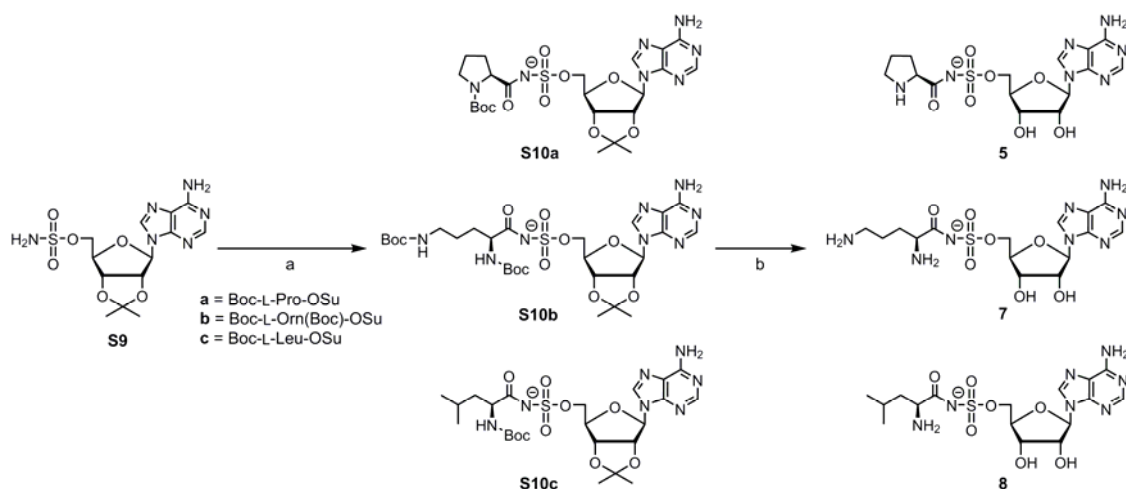


Scheme S1. Synthetic route to L-Phe-AMS-BPyne **1**, L-Pro-AMS-BPyne **2**, and L-Orn-AMS-BPyne **3**.

Reagents and conditions: [a] Cs₂CO₃, DMF, rt: 73% (**S2b**); 83% (**S2c**); [b] Pd/C, H₂, MeOH, rt: 64% (**S3b**); 67% (**S3c**); [c] **S8**, EDC, HOBT, DMF, rt: 86% (**S4a**); 50% (**S4b**); 60% (**S4c**); [d] 80% aqueous TFA, rt: 56% (**1**); 91% (**2**); 88% (**3**).



Scheme S2. Synthetic route to S8. Reagents and conditions: [a] 4, 4'-diaminobenzophenone, EDC, HOBT, DMF, rt, 53%; [b] 5-hexynoic chloride, DIEA, THF, rt, 51%; [c] 4 M NaOH aq., MeOH, THF, rt, 97%.



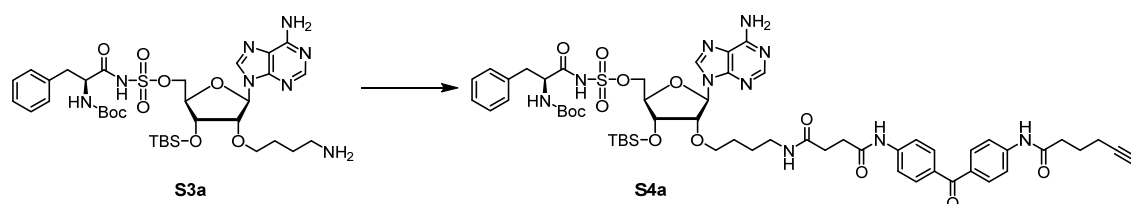
Scheme S3. Synthetic route to 5'-O-[N-(aminoacyl)sulfamoyl]adenosines 5, 7, and 8. Reagents and conditions: [a] *N*-hydroxysuccinimide esters a-c, Cs₂CO₃, DMF, rt: 87% (S10a); 22% (S10b); 61% (S10c); [b] 80% aqueous TFA, rt: 74% (5); 98% (7); 69% (8).

General Synthetic Methods: All commercial reagents were used as provided unless otherwise indicated. S1² (Scheme S1), S3a² (Scheme S1), S5³ (Scheme S2), and 5'-O-sulfamoyl-2',3'-isopropylideneadenosine S9⁴ (Scheme S3) 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). ¹H-NMR spectra were recorded at 500 MHz. ¹³C-NMR spectra were recorded at 125 MHz on JEOL NMR spectrometers and standardized to the NMR solvent signal as reported by Gottlieb.⁵ 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 ($\lambda = 254$ 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.⁶ Mass spectral data were obtained using a LCMS-IT-TOF mass spectrometer (Shimadzu).

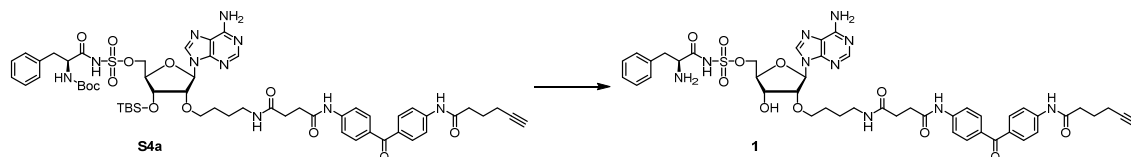
Chemical Synthesis of 1 Compound number in bold refers to the structures shown in Scheme S1.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-(4-(hex-5-ynamid o)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (S4a)



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (3.1 mg, 0.016 mmol) and 1-hydroxybenzotriazole (2.5 mg, 0.016 mmol) were added to a solution of compound **S8** (6.4 mg, 0.016 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **S3a** (10 mg, 0.013 mmol) was then added. After 12 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% NaHCO₃, and brine. The organic layer was dried over Na₂SO₄ and evaporated to dryness. The residue was purified by flash chromatography (6:1 CHCl₃/MeOH) to afford compound **S4a** as a white solid (13 mg, 86%).¹H NMR (500 MHz, CD₃OD): δ 8.48 (s, 1H), 8.19 (s, 1H), 7.75–7.70 (m, 8H), 7.23–7.17 (m, 4H), 7.15–7.09 (m, 1H), 6.14 (d, $J = 6.3$ Hz, 1H), 4.62–4.58 (m, 1H), 4.55–4.51 (m, 1H), 4.38–4.31 (m, 1H), 4.30–4.20 (m, 3H), 3.58–3.51 (m, 1H), 3.48–3.41 (m, 1H), 3.16 (dd, $J = 13.8, 4.6$ Hz, 1H), 3.06 (dd, $J = 6.9, 6.3$ Hz, 2H), 2.91–2.83 (m, 1H), 2.70 (dd, $J = 7.5, 6.9$ Hz, 2H), 2.58–2.51 (m, 4H), 2.31–2.26 (m, 3H), 1.94–1.86 (m, 2H), 1.54–1.37 (m, 4H), 1.33 (s, 9H), 0.94 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 196.5, 174.4, 174.0, 173.3, 157.4, 157.2, 153.9, 150.8, 144.3, 144.2, 141.3, 139.0, 134.0, 133.9, 132.3, 132.2, 130.6, 129.2, 127.4, 120.3, 120.0, 119.9, 87.5, 85.4, 84.1, 83.1, 80.2, 72.7, 71.5, 70.3, 69.6, 59.2, 40.0, 36.7, 33.2, 31.8, 28.8, 28.1, 26.9, 26.3, 25.5, 19.0, 18.6, –4.3, –4.5. (The ¹³C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): [M+H]⁺ calcd for C₅₇H₇₅N₁₀O₁₃SSi, 1167.5005; found, 1167.5004.

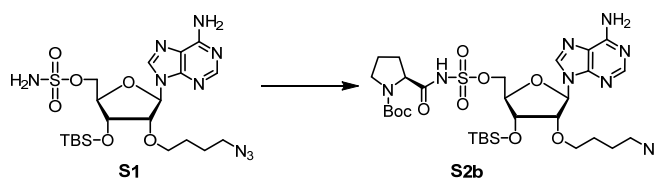
L-Phe-AMS-BPyne (1)



Compound **S4a** (12 mg, 0.010 mmol) was dissolved in a mixture of 4:1 (v/v) mixture of TFA and H₂O at room temperature. After 8 h, the flask was placed on the rotary evaporator and the TFA and H₂O were removed at reduced pressure. The residue was purified by flash chromatography (4:1 CHCl₃/MeOH) to afford compound **1** as a white solid (5.3 mg, 56%). ¹H NMR (500 MHz, CD₃OD): δ 8.50 (s, 1H), 8.19 (s, 1H), 7.76–7.71 (m, 8H), 7.31–7.26 (m, 4H), 7.21–7.15 (m, 1H), 6.16 (d, *J* = 4.6 Hz, 1H), 4.45 (dd, *J* = 4.6, 4.0 Hz, 1H), 4.41 (dd, *J* = 5.2, 4.6 Hz, 1H), 4.39–4.34 (m, 1H), 4.33–4.27 (m, 2H), 3.91–3.86 (m, 1H), 3.73–3.63 (m, 1H), 3.63–3.57 (m, 1H), 3.35–3.28 (m, 1H, overlapping with MeOH), 3.19–3.10 (m, 2H), 3.06–3.00 (m, 1H), 2.71 (dd, *J* = 7.5, 6.9 Hz, 2H), 2.59–2.52 (m, 4H), 2.32–2.26 (m, 3H), 1.94–1.87 (m, 2H), 1.66–1.44 (m, 4H). ¹³C NMR (125 MHz, CD₃OD): δ 196.6, 175.3, 174.5, 174.1, 173.3, 157.2, 154.0, 150.6, 144.3, 144.2, 141.1, 136.6, 134.0, 133.9, 132.3, 132.2, 130.6, 130.0, 128.4, 120.13, 120.07, 120.0, 87.8, 84.5, 84.1, 83.6, 71.6, 70.9, 70.3, 68.9, 58.3, 40.0, 38.7, 36.7, 33.2, 31.8, 27.8, 26.9, 25.5, 18.6. HRMS (ESI⁺): [M+H]⁺ calcd for C₄₆H₅₃N₁₀O₁₁S, 953.3616; found, 953.3616.

Chemical Synthesis of 2 Compound number in bold refers to the structures shown in Scheme S1.

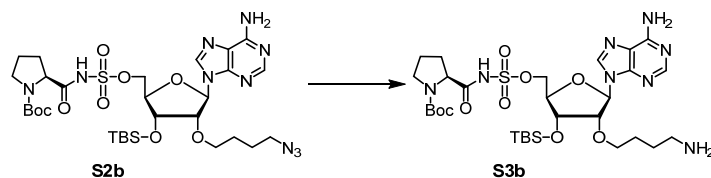
***tert*-Butyl (S)-2-((((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methoxy)sulfonyl)carbamoyl)pyrrolidine-1-carboxylate (**S2b**)**



Boc-Pro-OSu (52 mg, 0.17 mmol) and cesium carbonate (108 mg, 0.33 mmol) were added to a solution of compound **S1** (60 mg, 0.11 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 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 (10:1 to 5:1 CHCl₃/MeOH) to afford compound **S2b** as a white solid (61 mg, 73%). ¹H NMR (500 MHz, CD₃OD): δ 8.46 (s, 1H), 8.22 (s, 1H), 6.15 (d, *J* = 6.3 Hz, 1H), 4.67–4.60 (m, 1H), 4.58–4.50 (m, 1H), 4.47–4.41 (m, 1H), 4.40–4.32 (m, 1H), 4.30–4.25 (m, 1H), 4.21–4.09 (m, 1H), 3.63–3.55 (m, 1H), 3.52–3.42 (m, 2H), 3.41–3.33 (m, 1H), 3.19–3.11 (m, 2H), 2.27–2.14 (m, 1H), 2.02–1.70 (m, 3H), 1.60–1.46 (m, 4H), 1.46–1.39 (m, 9H), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s,

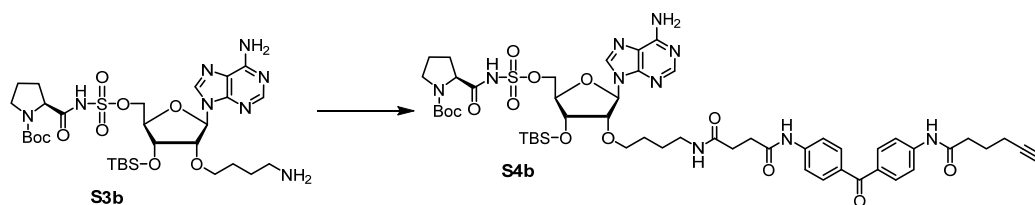
3H). ^{13}C NMR (125 MHz, CD_3OD): δ 178.7, 157.2, 156.1, 153.7, 150.7, 141.4, 120.3, 87.6, 85.2, 83.1, 81.4, 72.6, 71.3, 70.3, 63.0, 52.1, 47.7, 32.4, 28.7, 28.0, 26.6, 26.3, 24.5, 19.0, -4.4, -4.6. HRMS (ESI $^-$): $[\text{M}-\text{H}]^-$ calcd for $\text{C}_{30}\text{H}_{49}\text{N}_{10}\text{O}_9\text{SSi}$, 753.3179; found, 753.3176.

***tert*-Butyl (*S*)-2-((((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)methoxy)sulfonyl)carbamoyl)pyrrolidine-1-carboxylate (**S3b**)**



To a solution of **S2b** (50 mg, 0.066 mmol) in MeOH (2 mL) was added 10% Pd/C (5 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 24 h. The reaction mixture was filtered through a pad of Celite, which was further washed with MeOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 to 3:1 $\text{CHCl}_3/\text{MeOH}$) to afford compound **S3b** as a white solid (31 mg, 64%). ^1H NMR (500 MHz, CD_3OD): δ 8.54 (s, 1H), 8.20 (s, 1H), 6.17 (d, $J = 6.9$ Hz, 1H), 4.67–4.62 (m, 1H), 4.61–4.52 (m, 1H), 4.33–4.23 (m, 3H), 4.18–4.09 (m, 1H), 3.62–3.55 (m, 1H), 3.53–3.42 (m, 2H), 3.41–3.34 (m, 1H), 2.91–2.80 (m, 2H), 2.26–2.12 (m, 1H), 2.02–1.85 (m, 2H), 1.83–1.72 (m, 1H), 1.68–1.50 (m, 4H), 1.42 (s, 9H), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 181.6, 157.4, 156.4, 154.0, 150.8, 141.2, 120.1, 87.1, 86.0, 83.4, 81.0, 73.1, 70.8, 69.1, 64.0, 47.7, 40.5, 32.7, 28.8, 27.6, 26.3, 25.4, 24.6, 19.0, -4.4, -4.5. HRMS (ESI $^-$): $[\text{M}-\text{H}]^-$ calcd for $\text{C}_{30}\text{H}_{51}\text{N}_8\text{O}_9\text{SSi}$, 727.3274; found, 727.3271.

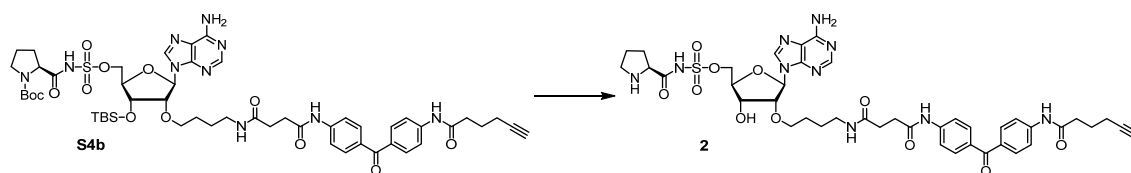
***tert*-Butyl (*S*)-2-((((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methoxy)sulfonyl)carbamoyl)pyrrolidine-1-carboxylate (**S4b**)**



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (8.0 mg, 0.041 mmol) and 1-hydroxybenzotriazole (6.3 mg, 0.041 mmol) were added to a solution of compound **S8** (17 mg, 0.041 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **S3b** (25 mg, 0.034

mmol) was then added. After 18 h, the reaction mixture was diluted with EtOAc. The combined organic layer was washed with 5% citric acid, 5% NaHCO₃, and brine. The organic layer was dried over Na₂SO₄ and evaporated to dryness. The residue was purified by flash chromatography (10:1 to 5:1 CHCl₃/MeOH) to afford compound **S4b** as a white solid (19 mg, 50%). ¹H NMR (500 MHz, CD₃OD): δ 8.47–8.38 (m, 1H), 8.20 (s, 1H), 7.76–7.68 (m, 8H), 6.13 (d, *J* = 5.7 Hz, 1H), 4.66–4.60 (m, 1H), 4.57–4.49 (m, 1H), 4.45–4.39 (m, 1H), 4.38–4.29 (m, 1H), 4.29–4.24 (m, 1H), 4.18–4.09 (m, 1H), 3.59–3.52 (m, 1H), 3.50–3.40 (m, 2H), 3.39–3.34 (m, 1H), 3.11–3.04 (m, 2H), 2.70 (t, *J* = 6.9 Hz, 2H), 2.59–2.51 (m, 4H), 2.32–2.26 (m, 3H), 2.25–2.12 (m, 1H), 2.04–1.82 (m, 4H), 1.82–1.70 (m, 1H), 1.58–1.38 (m, 13H), 0.94 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 196.5, 174.4, 174.0, 173.3, 157.2, 156.6, 153.9, 150.7, 144.3, 144.2, 141.4, 134.0, 133.9, 132.3, 132.2, 120.3, 120.0, 119.9, 87.6, 85.4, 84.1, 83.0, 81.2, 72.7, 71.5, 70.3, 69.9, 63.5, 47.7, 40.1, 36.7, 33.2, 31.8, 28.9, 28.7, 28.1, 26.9, 26.3, 25.5, 24.5, 19.0, 18.6, –4.4, –4.6. (The ¹³C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI[–]): [M–H][–] calcd for C₅₃H₇₁N₁₀O₁₃SSi, 1115.4698; found, 1115.4696.

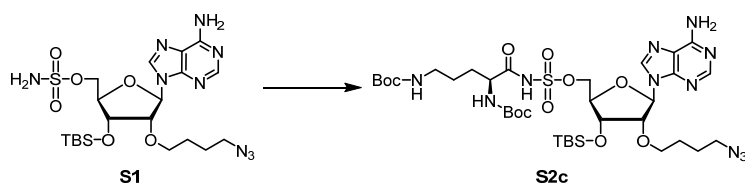
L-Pro-AMS-BPyne (**2**)



Compound **S4b** (19 mg, 0.017 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H₂O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H₂O were removed at reduced pressure. The residue was purified by flash chromatography (3:1 CHCl₃/MeOH) to afford compound **2** as a white solid (14 mg, 91%). ¹H NMR (500 MHz, CD₃OD): δ 8.50 (s, 1H), 8.19 (s, 1H), 7.78–7.69 (m, 8H), 6.15 (d, *J* = 5.2 Hz, 1H), 4.49 (t, *J* = 4.6 Hz, 1H), 4.42 (t, *J* = 5.2 Hz, 1H), 4.39–4.26 (m, 3H), 4.08 (dd, *J* = 8.6, 6.9 Hz, 1H), 3.69–3.62 (m, 1H), 3.61–3.54 (m, 1H), 3.39–3.34 (m, 1H), 3.28–3.22 (m, 1H), 3.16–3.10 (m, 2H), 2.70 (t, *J* = 6.9 Hz, 2H), 2.60–2.51 (m, 4H), 2.37–2.26 (m, 4H), 2.16–2.07 (m, 1H), 1.98–1.86 (m, 4H), 1.64–1.43 (m, 4H). ¹³C NMR (125 MHz, CD₃OD): δ 196.6, 174.9, 174.5, 174.1, 173.3, 157.3, 154.0, 150.7, 144.3, 144.2, 141.1, 134.0, 132.28, 132.25, 120.2, 120.1, 120.0, 87.7, 84.5, 84.1, 83.6, 71.6, 70.9, 70.4, 63.8, 47.3, 40.0, 36.7, 33.2, 31.8, 30.8, 37.8, 26.9, 25.5, 24.9, 18.6. HRMS (ESI⁺): [M+Na]⁺ calcd for C₄₂H₅₀N₁₀O₁₁SNa, 925.3279; found, 925.3251.

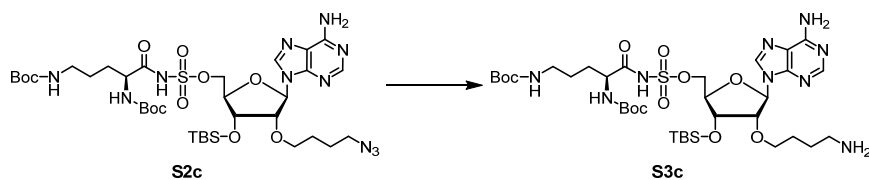
Chemical Synthesis of 3 Compound number in bold refers to the structures shown in Scheme S1.

((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 ((*S*)-2,5-bis((*tert*-butoxycarbonyl)amino)pentanoyl)sulfamate (S2c**)**



Boc-Orn(Boc)-OSu (73 mg, 0.17 mmol) and cesium carbonate (108 mg, 0.33 mmol) were added to a solution of compound **S1** (60 mg, 0.11 mmol) in DMF (1 mL). The solution was stirred at room temperature for 2 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 (10:1 to 5:1 CHCl₃/MeOH) to afford compound **S2c** as a white solid (80 mg, 83%). ¹H NMR (500 MHz, CD₃OD): δ 8.49 (s, 1H), 8.23 (s, 1H), 6.16 (d, *J* = 6.3 Hz, 1H), 4.63–4.58 (m, 1H), 4.57–4.50 (m, 1H), 4.48–4.40 (m, 1H), 4.39–4.32 (m, 1H), 4.32–4.26 (m, 1H), 4.07–3.92 (m, 1H), 3.66–3.57 (m, 1H), 3.51–3.44 (m, 1H), 3.19–3.12 (m, 2H), 3.09–2.99 (m, 2H), 1.87–1.73 (m, 1H), 1.66–1.47 (m, 7H), 1.41 (s, 18H), 0.96 (s, 9H), 0.174 (s, 3H), 0.169 (s, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 177.7, 158.5, 157.7, 157.0, 153.5, 150.6, 141.4, 120.3, 87.5, 85.2, 83.2, 80.4, 79.9, 72.6, 71.3, 70.2, 57.0, 52.1, 40.8, 31.1, 28.8, 28.0, 27.2, 26.6, 26.3, 19.0, –4.4, –4.6. HRMS (ESI⁺): [M+H]⁺ calcd for C₃₅H₆₂N₁₁O₁₁SSi, 872.4120; found, 872.4112.

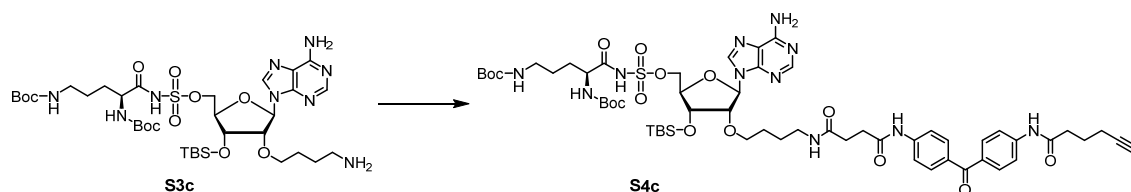
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*S*)-2,5-bis((*tert*-butoxycarbonyl)amino)pentanoyl)sulfamate (S3c**)**



To a solution of **S2c** (62 mg, 0.071 mmol) in MeOH (2 mL) was added 10% Pd/C (6 mg). The resulting suspension was hydrogenated under an atmosphere of H₂ at room temperature for 24 h. The reaction mixture was filtered through a pad of Celite, which was further washed with MeOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 CHCl₃/MeOH) to afford compound **S3c** as a white solid (40 mg, 67%). ¹H NMR (500 MHz, CD₃OD): δ 8.57 (s, 1H), 8.20 (s, 1H), 6.18 (d, *J* = 6.3 Hz, 1H), 4.63–4.51 (m, 2H), 4.32–4.20 (m, 3H), 4.05–3.93 (m, 1H), 3.62–3.55 (m, 1H), 3.53–3.45 (m, 1H), 3.07–3.00 (m, 2H), 2.91–2.83 (m, 2H), 1.88–1.79 (m, 1H), 1.69–1.48 (m, 7H), 1.47–1.38 (m, 18H), 0.96 (s, 9H), 0.17 (s, 3H), 0.16 (s, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 180.7, 158.4, 157.6, 157.4, 154.0, 150.8, 141.1, 120.1, 87.0, 86.0, 83.5, 80.1, 79.8, 73.0, 70.7, 69.1, 57.6, 41.0, 40.5, 32.0, 28.8, 27.6, 27.0, 26.3, 25.4, 19.0, –4.3, –4.5. HRMS (ESI⁺): [M+H]⁺ calcd for C₃₅H₆₄N₉O₁₁SSi,

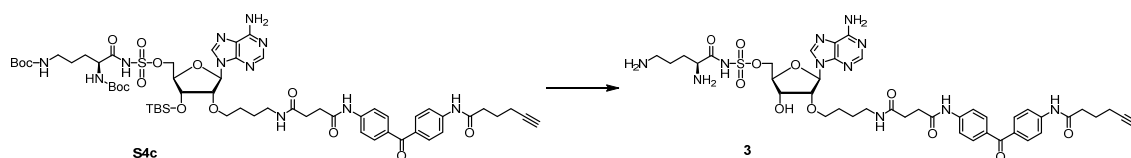
846.4215; found, 846.4201.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-((4-(4-(hex-5-ynamid o)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*S*)-2,5-bis((*tert*-butoxycarbonyl)amino)pentanoyl)sulfamate (S4c**))**



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (8.0 mg, 0.043 mmol) and 1-hydroxybenzotriazole (6.6 mg, 0.043 mmol) were added to a solution of compound **S8** (17 mg, 0.043 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **S3c** (30 mg, 0.035 mmol) was added. After 3 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% NaHCO₃, and brine. The organic layer was dried over Na₂SO₄ and evaporated to dryness. The residue was purified by flash chromatography (10:1 to 5:1 CHCl₃/MeOH) to afford compound **S4c** as a white solid (26 mg, 60%). ¹H NMR (500 MHz, CD₃OD): δ 8.46 (s, 1H), 8.20 (s, 1H), 7.76–7.70 (m, 8H), 6.14 (d, *J* = 5.7 Hz, 1H), 4.60 (dd, *J* = 4.6, 2.9 Hz, 1H), 4.51 (dd, *J* = 5.7, 5.2 Hz, 1H), 4.42–4.36 (m, 1H), 4.33–4.25 (m, 2H), 4.02–3.95 (m, 1H), 3.59–3.53 (m, 1H), 3.49–3.42 (m, 1H), 3.11–3.05 (m, 2H), 3.02 (t, *J* = 6.9 Hz, 2H), 2.71 (t, *J* = 6.9 Hz, 2H), 2.58–2.51 (m, 4H), 2.32–2.87 (m, 3H), 1.94–1.86 (m, 2H), 1.86–1.77 (m, 1H), 1.65–1.34 (m, 25H), 0.94 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 196.5, 174.4, 174.0, 173.3, 158.5, 157.8, 157.2, 153.9, 150.7, 144.3, 144.2, 141.2, 134.0, 133.9, 132.3, 132.2, 120.3, 120.0, 119.9, 87.6, 85.2, 84.1, 83.1, 80.3, 79.9, 72.6, 71.5, 70.4, 69.6, 57.7, 41.0, 40.0, 36.7, 33.2, 31.8, 28.8, 28.1, 27.2, 26.9, 26.3, 25.5, 19.0, 18.6, –4.4, –4.5. (The ¹³C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI⁺): [M+H]⁺ calcd for C₅₈H₈₄N₁₁O₁₅SSi, 1234.5638; found, 1234.5603.

L-Orn-AMS-BPyne (3)

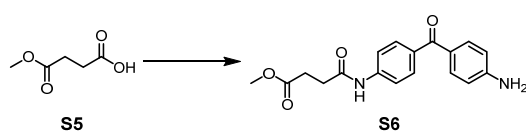


Compound **S4c** (20 mg, 0.016 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H₂O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H₂O were removed at reduced pressure. The residue was purified by flash chromatography (4:1 CHCl₃/MeOH) to afford compound

3 as a white solid (13 mg, 88%). ¹H NMR (500 MHz, CD₃OD): δ 8.65 (s, 1H), 8.38 (s, 1H), 7.74–7.70 (m, 8H), 6.20 (d, *J* = 4.0 Hz, 1H), 4.50 (t, *J* = 5.2 Hz, 1H), 4.46–4.41 (m, 1H), 4.41–4.34 (m, 2H), 4.32–4.28 (m, 1H), 3.77–3.73 (m, 1H), 3.72–3.61 (m, 2H), 3.20–3.13 (m, 2H), 3.03–2.94 (m, 2H), 2.72 (t, *J* = 6.9 Hz, 2H), 2.59–2.52 (m, 4H), 2.32–2.26 (m, 3H), 2.00–1.80 (m, 6H), 1.67–1.58 (m, 2H), 1.58–1.48 (m, 2H). ¹³C NMR (125 MHz, CD₃OD): δ 196.5, 174.53, 174.47, 174.1, 173.3, 152.3, 149.8, 146.3, 144.24, 144.22, 143.4, 134.0, 133.9, 132.2, 120.2, 120.1, 119.9, 88.6, 84.4, 84.1, 83.8, 71.7, 70.6, 70.4, 69.0, 55.9, 40.04, 39.99, 36.7, 33.1, 31.7, 29.4, 27.7, 27.0, 25.5, 24.3, 18.6. HRMS (ESI⁺): [M+Na]⁺ calcd for C₄₂H₅₃N₁₁O₁₁SNa, 942.3544; found, 942.3550.

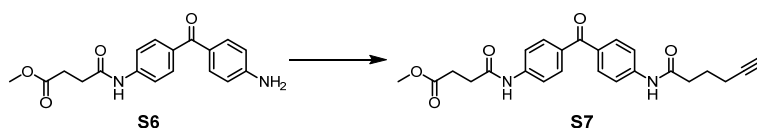
Chemical Synthesis of S8 Compound number in bold refers to the structures shown in Scheme S2.

Methyl 4-((4-(4-aminobenzoyl)phenyl)amino)-4-oxobutanoate (S6)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (905 mg, 4.72 mmol) and 1-hydroxybenzotriazole (723 mg, 4.72 mmol) were added to a solution of compound **S5** (623 mg, 4.72 mmol) in DMF (20 mL). The solution was stirred at room temperature for 5 min and 4, 4'-diaminobenzophenone (500 mg, 2.36 mmol) was added. After 12 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% NaHCO₃, and brine. The organic layer was dried over Na₂SO₄ and evaporated to dryness. The residue was purified by flash chromatography (2:1 EtOAc/hexane) to afford compound **S6** as a white solid (410 mg, 53%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 10.26 (br, 1H), 7.69 (d, *J* = 8.6 Hz, 2H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.49 (d, *J* = 8.6 Hz, 2H), 6.59 (d, *J* = 8.6 Hz, 2H), 6.06 (s, 2H), 3.59 (s, 3H), 2.68–2.59 (m, 4H). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 192.4, 172.8, 170.3, 153.4, 141.9, 133.2, 132.4, 133.2, 132.4, 130.2, 124.2, 118.0, 112.5, 51.4, 31.0, 28.4. HRMS (ESI⁺): [M+H]⁺ calcd for C₁₈H₁₉N₂O₄, 327.1345; found, 327.1326.

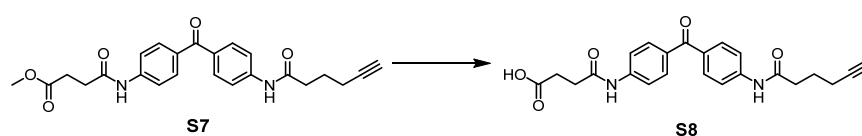
Methyl 4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanoate (S7)



Oxalyl chloride (154 μL, 1.8 mmol) and DMF (20 μL) were added to a solution of 5-hexynoic acid (129 μL, 1.2 mmol) in benzene (10 mL). After 2 h, the flask was placed on the rotary evaporator and the DMF and

benzene were removed at reduced pressure to afford 5-hexynoic chloride as a red oil. A solution of crude 5-hexynoyl chloride, compound **S6** (185 mg, 0.57 mmol), and DIEA (200 μ L, 1.14 mmol) in THF (5 mL) was stirred at room temperature for 9 h. The reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 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 white solid (122 mg, 51%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 10.35 (br, 1H), 10.28 (br, 1H), 7.66–7.78 (m, 8H), 3.59 (s, 3H), 2.81 (t, $J = 2.9$ Hz, 1H), 2.69–2.59 (m, 4H), 2.45–2.48 (m, 2H), 2.23 (ddd, $J = 6.9, 6.9, 2.9$ Hz, 2H), 1.81–1.73 (m, 2H). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$): δ 193.3, 172.8, 171.2, 170.5, 143.0, 142.9, 131.7, 130.9, 130.8, 118.2, 118.1, 84.0, 71.7, 51.4, 35.2, 31.0, 28.4, 23.8, 17.3. HRMS (ESI⁺): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{24}\text{H}_{25}\text{N}_2\text{O}_5$, 421.1763; found, 421.1761.

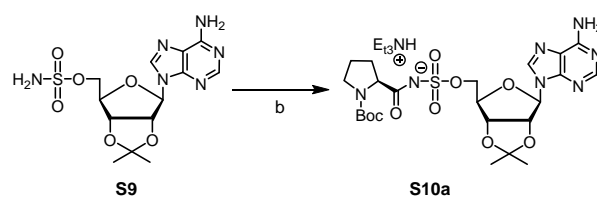
4-((4-(4-(Hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanoic acid (**S8**)



To a solution of **S7** (122 mg, 0.29 mmol) in 3:1 MeOH/THF (4 mL) was added 145 μ L of a 4 M aqueous NaOH solution at room temperature. Stirring was continued at room temperature for 12 h. The flask was then placed on a rotary evaporator and the MeOH and THF were removed at reduced pressure. The residue was diluted with H_2O and washed with EtOAc. The aqueous layer was acidified with citric acid monohydrate and back extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4 , and evaporated to dryness to afford compound **S8** as a pale yellow solid (114 mg, 97%). ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ 10.33 (br, 1H), 10.29 (br, 1H), 7.75 (dd, $J = 8.6, 3.4$ Hz, 4H), 7.69 (d, $J = 8.6$ Hz, 4H), 2.80 (t, $J = 2.9$ Hz, 1H), 2.64–2.58 (m, 2H), 2.57–2.51 (m, 2H), 2.44–2.47 (m, 2H), 2.22 (ddd, $J = 6.9, 6.9, 2.9$ Hz, 2H), 1.81–1.73 (m, 2H). ^{13}C NMR (125 MHz, $\text{DMSO-}d_6$): δ 193.4, 173.8, 171.3, 170.8, 143.09, 143.06, 131.8, 131.7, 131.0, 130.9, 118.3, 118.2, 84.0, 71.7, 35.3, 31.3, 28.7, 23.8, 17.4. HRMS (ESI⁺): $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{23}\text{H}_{23}\text{N}_2\text{O}_5$, 407.1607; found, 407.1604.

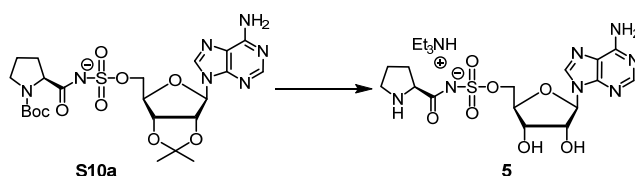
Chemical Synthesis of 5 Compound number in bold refers to the structures shown in Scheme S3.

5'-O-[N-(N-Boc-L-prolyl)sulfamoyl]-2',3'-O-isopropylideneadenosine triethylammonium salt (S10a**)**



Boc-Pro-OSu (63 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5'-*O*-sulfamoyl-2',3'-isopropylideneadenosine **S9** (50 mg, 0.13 mmol) in DMF (1 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 (91:9:1 to 86:14:1 EtOAc/MeOH/Et₃N) to afford compound **S10a** as a white solid (77 mg, 87%). ¹H NMR (500 MHz, CD₃OD): δ 8.48–8.42 (m, 1H), 8.21 (s, 1H), 6.23 (d, *J* = 3.4 Hz, 1H), 5.41–5.35 (m, 1H), 5.17–5.10 (m, 1H), 4.56–4.51 (m, 1H), 4.32–4.17 (m, 2H), 4.16–4.07 (m, 1H), 3.51–3.43 (m, 1H), 3.41–3.32 (m, 1H), 3.19 (q, *J* = 7.5 Hz, 6H, Et₃N-CH₂), 2.22–2.10 (m, 1H), 1.97–1.85 (m, 2H), 1.82–1.74 (m, 1H), 1.60 (s, 3H), 1.44–1.37 (m, 12H), 1.28 (t, *J* = 7.5 Hz, 9H, Et₃N-CH₃). ¹³C NMR (125 MHz, CD₃OD): δ 181.5, 157.3, 156.4, 154.0, 150.5, 141.4, 120.2, 115.3, 91.8, 85.8, 83.3, 81.0, 80.4, 69.5, 63.9, 47.9, 47.7, 32.6, 28.8, 27.5, 25.6, 24.5, 9.2. HRMS (ESI⁻): [M-H]⁻ calcd for C₂₃H₃₂N₇O₉S, 582.1981; found, 582.1981.

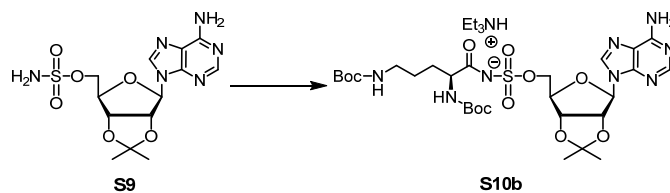
5'-*O*-[*N*-(*L*-Prolyl)sulfamoyl]adenosine triethylammonium salt (**5**)



Compound **S10a** (30 mg, 0.044 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H₂O at room temperature. After 2 h, the flask was placed on the rotary evaporator and the TFA and H₂O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 to 50:50:1 CHCl₃/MeOH/Et₃N) to afford compound **5** as a white solid (16 mg, 74%). ¹H NMR (500 MHz, DMSO-*d*₆): δ 8.35 (s, 1H), 8.14 (s, 1H), 7.27 (br, 2H), 5.90 (d, *J* = 5.7 Hz, 1H), 4.61–4.56 (m, 1H), 4.18–4.12 (m, 2H), 4.11–4.03 (m, 2H), 3.91–3.86 (m, 1H), 3.23–3.16 (m, 1H), 3.10–3.04 (m, 1H), 2.72 (q, *J* = 6.9 Hz, 2H, Et₃N-CH₂), 2.19–2.10 (m, 1H), 1.95–1.86 (m, 1H), 1.85–1.72 (m, 2H), 1.03 (t, *J* = 6.9 Hz, 3H, Et₃N-CH₃). ¹³C NMR (125 MHz, DMSO-*d*₆): δ 171.7, 156.0, 152.6, 149.6, 139.4, 118.9, 87.1, 82.4, 73.4, 70.7, 67.7, 61.9, 45.7, 45.3, 29.1, 23.4, 10.2. HRMS (ESI⁻): [M-H]⁻ calcd for C₁₅H₂₀N₇O₇S, 442.1145; found, 442.1146.

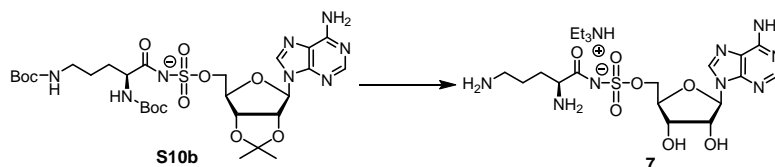
Chemical Synthesis of 7 Compound number in bold refers to the structures shown in Scheme S3.

5'-O-[N-(N-Boc-L-ornithinyl)(δ -Boc)sulfamoyl]-2',3'-O-isopropylideneadenosine triethylammonium salt (S10b)



Boc-Orn(Boc)-OSu (125 mg, 0.29 mmol) and cesium carbonate (254 mg, 0.78 mmol) were added to a solution of 5'-O-sulfamoyl-2',3'-isopropylideneadenosine **S9** (100 mg, 0.26 mmol) in DMF (3 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 83:17:1 CHCl₃/MeOH/Et₃N) to afford compound **S10b** as a white solid (45 mg, 22%). ¹H NMR (500 MHz, CD₃OD): δ 8.47 (s, 1H), 8.22 (s, 1H), 6.24 (d, J = 3.4 Hz, 1H), 5.35 (q, J = 2.9 Hz, 1H), 5.15–5.09 (m, 1H), 4.56–4.52 (m, 1H), 4.23 (d, J = 4.0 Hz, 2H), 4.01–3.94 (m, 1H), 3.19 (q, J = 7.5 Hz, 6H, Et₃N-CH₂), 3.10–2.98 (m, 2H), 1.86–1.73 (m, 1H), 1.61 (s, 3H), 1.60–1.49 (m, 3H), 1.47–1.40 (m, 18H), 1.39 (s, 3H), 1.28 (t, J = 7.5 Hz, 9H, Et₃N-CH₃). ¹³C NMR (125 MHz, CD₃OD): δ 180.7, 158.4, 157.6, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.8, 85.7, 85.6, 83.3, 80.0, 79.7, 69.7, 57.6, 47.8, 41.0, 31.8, 28.8, 27.5, 25.6, 9.2. HRMS (ESI⁺): [M+H]⁺ calcd for C₂₈H₄₅N₈O₁₁S, 701.2929; found, 701.2926.

5'-O-[N-(L-Ornithinyl)sulfamoyl]adenosine triethylammonium salt (7)

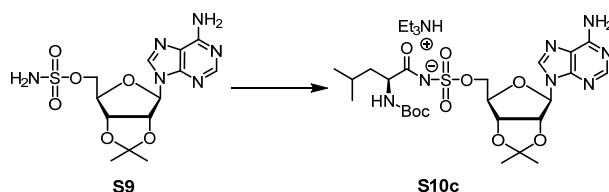


Compound **S10b** (40 mg, 0.047 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H₂O at room temperature. After 3 h, the flask was placed on the rotary evaporator, and the TFA and H₂O were removed at reduced pressure. The residue was purified by HPLC [COSMISIL 5C₁₈-PAQ: C-18 reverse-phase column, ϕ 10 mm \times 250 mm, aqueous TFA (0.01%), 4.0 mL/min, 210 nm, t_R : 9.8 min] to afford compound **7** as a colorless oil (26 mg, 98%). ¹H NMR (500 MHz, CD₃OD): δ 8.63 (s, 1H), 8.39 (s, 1H), 6.13 (d, J = 4.6 Hz, 1H), 4.63 (dd, J = 5.2, 4.6 Hz, 1H), 4.46–4.31 (m, 4H), 3.77 (dd, J = 6.3, 5.7 Hz, 1H), 3.20 (q, J = 7.5 Hz, 6H, Et₃N-CH₂), 3.03–2.97 (m, 2H), 2.05–1.78 (m, 4H), 1.31 (t, J = 7.5 Hz, 9H, Et₃N-CH₃). ¹³C NMR (125 MHz, CD₃OD): δ 174.6, 152.6, 150.1, 146.5, 143.5, 120.1, 90.2, 84.3, 76.3, 71.8, 69.5, 55.9, 47.8, 40.0, 29.3, 24.3,

9.2. HRMS (ESI⁺): [M+Na]⁺ calcd for C₁₅H₂₄N₈O₇SNa, 483.1386; found, 483.1381.

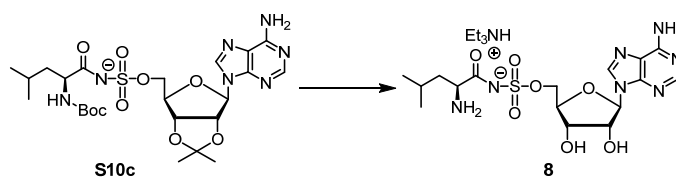
Chemical Synthesis of 8 Compound number in bold refers to the structures shown in Scheme S3.

5'-O-[N-(N-Boc-L-leucyl)sulfamoyl]-2',3'-O-isopropylideneadenosine triethylammonium salt (S10c)



Boc-Leu-OSu (193 mg, 0.59 mmol) and cesium carbonate (381 mg, 1.17 mmol) were added to a solution of 5'-O-sulfamoyl-2',3'-isopropylideneadenosine **S9** (150 mg, 0.39 mmol) in DMF (4 mL). The solution was stirred at room temperature for 4 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 (97:3:1 CHCl₃/MeOH/Et₃N) to afford compound **S10c** as a white solid (168 mg, 61%). ¹H NMR (500 MHz, CD₃OD): δ 8.47 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 3.4 Hz, 1H), 5.37–5.32 (m, 1H), 5.14–5.08 (m, 1H), 4.56–4.51 (m, 1H), 4.23 (d, *J* = 3.4 Hz, 2H), 4.09–4.00 (m, 1H), 3.19 (q, *J* = 7.5 Hz, 6H, Et₃N-CH₂), 1.77–1.65 (m, 1H), 1.61 (s, 3H), 1.60–1.50 (m, 1H), 1.48–1.34 (m, 13H), 1.29 (t, *J* = 7.5 Hz, 9H, Et₃N-CH₃), 0.92 (d, *J* = 1.7 Hz, 3H), 0.91 (d, *J* = 1.7 Hz, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 181.7, 157.7, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.8, 85.8, 85.6, 83.3, 79.9, 79.5, 69.7, 56.8, 47.9, 43.7, 28.8, 27.5, 26.1, 25.6, 23.7, 22.2, 9.2. HRMS (ESI⁺): [M+H]⁺ calcd for C₂₄H₃₈N₇O₉S, 600.2452; found, 600.2437.

5'-O-[N-(L-Leucyl)sulfamoyl]adenosine triethylammonium salt (8)



Compound **S10c** (70 mg, 0.10 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H₂O at room temperature. After 5 h, the flask was placed on the rotary evaporator, and the TFA and H₂O were removed at reduced pressure. The residue was purified by flash chromatography (83:17:1 to 67:33:1 CHCl₃/MeOH/Et₃N) to afford compound **8** as a white solid (35 mg, 69%). ¹H NMR (500 MHz, CD₃OD): δ 8.50 (s, 1H), 8.19 (s, 1H), 6.08 (d, *J* = 5.2 Hz, 1H), 4.66–4.61 (m, 1H), 4.44–4.36 (m, 2H), 4.36–4.28 (m, 2H), 3.70–3.65 (m, 1H), 3.11 (q, *J* = 7.5 Hz, 3H, Et₃N-CH₂), 1.84–1.73 (m, 2H), 1.64–1.53 (m, 1H), 1.26 (t, *J* = 7.5 Hz, 4.5H, Et₃N-CH₃), 0.96 (d, *J* = 6.3 Hz, 3H), 0.93 (d, *J* = 6.3 Hz, 3H). ¹³C NMR (125 MHz, CD₃OD): δ 176.8, 157.2, 153.9, 150.7, 141.2, 120.1, 89.5, 84.2, 76.1, 71.9, 69.1, 55.7, 47.7, 42.2, 25.7, 23.2, 22.2, 9.4. HRMS (ESI⁺):

[M+H]⁺ calcd for C₁₆H₂₆N₇O₇S, 460.1614; found, 460.1600.

Chemical Biology Procedures

Protein Expression and Materials: Recombinant proteins *holo*-GrsA and *holo*-TycB1 were expressed and purified as previously described.^{2,7,8,9} 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 *ausA1* (A₁-T₁) gene was PCR amplified genomic DNA from *S. aureus* ATCC 700699 using primers *ausA1* F (5'-GCCTCCATGACCATGGTTATGGGTAATTTGAGATTTCAAC-3') and *ausA1* R (5'-CCGAATTCGTCAGCACATAATCATCTTTAACTATAGCTTC-3'), and subsequently cloned into plitmus28-*ausA1*. Plasmid litmus28-*ausA1* was digested with *Nco*I and *Bam*HI, and the gene was subcloned into pET28b to produce pET28b-*ausA1*, an expression vector for *apo*-AusA1 with a hexahistidine appended to the C terminus. Sequencing revealed the expression plasmid to be error free. For expression and purification of *apo*-AusA1, pET28b-*ausA1* 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 A₆₀₀ of 0.6 at 37 °C, then induced with IPTG to a final concentration of 0.1 mM, and allowed to grow for 12 h at 18 °C. Cells were pelleted and resuspended in lysis buffer (20 mM Tris-HCl, pH 8.0 and 0.5% Triton-X). The cells were then lysed by sonication at 4 °C using an ultrasonic disruptor UD201 (Tomy Digital Biology Co., Ltd, Japan). The resulting cell lysate was centrifuged to remove cell debris and the supernatant was loaded onto a Ni Sepharose high-performance resin (GE Healthcare) and eluted with a gradient of imidazole (20–250 mM). 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 MgCl₂, and 1 mM TCEP). After the addition of 10% glycerol (v/v) the protein was stored at –80 °C.

Preparation of Lysates for Proteomic Labeling Experiments: *Aneurinibacillus migulanus* ATCC 9999 was cultured and the whole cell lysate was isolated as described previously.² The cellular lysate of *A. migulanus* ATCC 9999 has already been used to characterize an affinity probe for adenylation (A) domains in “Specific enrichment of nonribosomal peptide synthetase module by an affinity probe for adenylation domains. *Bioorg. Med. Chem. Lett.* **2014**, 24, 865-869.”

Aneurinibacillus migulanus DSM 5759 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). The strain was maintained on nutrient agar. Single colonies were used to inoculate a rich complex medium (YPG) and shaken for 24 h at 37 °C. The medium contained 50 g/L yeast extract, 50 g/L bacto-peptone, and 5 g/L glucose. The seed culture (2 mL) was transferred to 250 mL of YPG and the resulting mixture was incubated at 37 °C. Growth was routinely monitored at A₆₆₀ on a U-2910 spectrophotometer (Hitachi). The cells (A₆₆₀ = 15.0) were harvested by centrifugation and stored in the freezer until used. The frozen cell pellet was resuspended in 20 mM Tris (pH

8.0), 1 mM MgCl₂, 1 mM TCEP, 0.05% Igepal CA-630, and protease inhibitor cocktail. Because of the lability of the synthetase during mechanical cell disruption processes,¹¹ 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 pellet was discarded. The total protein concentration was quantitated by the method of Bradford.¹⁰

Hydroxamate-MesG Assay¹

Standard assay conditions: Reactions contained NRPS enzymes to maintain initial velocity conditions, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM MgCl₂, 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 1 mM substrates. The reactions (100 µL) were run in 96-well half-area plates (Corning, 3881) and the cleavage of MesG was monitored at A₃₅₅ 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*-GrsA, compound **1** was tested from 25 to 5,000 nM using L-Phe (1 mM) as the competing substrate. The enzyme was fixed at 20 nM. Compounds **2** and **5** were varied from 0.625 to 50 µM, and *holo*-TycB1 (800 nM) and L-Pro (1 mM) 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).

Labeling of Recombinant GrsA and TycB1 by the Active Site-Directed Proteomic Probes 1 and 2:

Standard conditions for probes **1** and **2**-recombinant protein reactions were as follows: recombinant GrsA (1 µM) and TycB1 (1 µM) were treated with probes **1** and **2** (1 µM from a 100 µM stock in DMSO) in assay buffer [20 mM Tris (pH 8.0), 1 mM MgCl₂, 1 mM TCEP, and 0.0025% Igepal CA-630], respectively. Inhibition studies were performed by pre-incubation of GrsA (1 µM) and TycB1 (1 µM) with L-Phe-AMS **4** and L-Pro-AMS **5** (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature, respectively. In all experiments 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 CuSO₄ 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).

Ultraviolet Photolysis Time Studies: Recombinant GrsA (1 µM) and TycB1 (1 µM) were treated with probes **1** and **2** (1 µM from a 100 µM stock in DMSO; final DMSO concentration of 2.2%) in assay buffer, respectively. After 10 min at room temperature, these samples were irradiated at 365 nm for the indicated time (0–60 min) on ice, reacted with Rh-azide, and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Limit of Detection of GrsA and TycB1 Labeling: Recombinant GrsA (1–500 fmol) and TycB1 (1–500 fmol) were treated with probes **1** and **2** (1 µM from a 100 µM stock in DMSO; final DMSO concentration of 2.2%) in assay buffer, respectively. 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).

Comparing the Labeling Property with GrsA, TycB1, AusA1, and BSA: For GrsA (A: L-Phe), TycB1 (A: L-Pro), AusA1 (A: L-Val), and BSA labeling experiments, probes **1** and **2** (1 μM) were individually added to a 46 μL reaction containing GrsA (1 μM), TycB1 (1 μM), AusA1 (1 μM), BSA (1 μM), and assay buffer. For inhibition studies, GrsA (1 μM), TycB1 (1 μM), and AusA1 (1 μM) were pre-incubated with L-Phe-AMS **4**, L-Pro-AMS **5**, and L-Val-AMS **6** (100 μM) for 10 min at room temperature, respectively. 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, reacted with Rh-azide for 1 h at room temperature, and separated by gel electrophoresis. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

GrsA Labeling of *A. migulanus* ATCC 9999 Proteomes: *Aneurinibacillus migulanus* proteome (1.4 mg/mL) was treated with probe **1** (1 μM from a 100 μM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM MgCl₂, 1 mM TCEP, 0.05% Igepal CA-630, 0.2 mg/mL lysozyme, and protease inhibitor cocktail. Inhibition studies were performed by pre-incubation of *A. migulanus* proteome (1.4 mg/mL) with L-Phe-AMS **4** (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature. 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, reacted with Rh-azide for 1 h at room temperature, and separated by gel electrophoresis. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

GrsB Labeling of *A. migulanus* DSM 5759 Proteomes: *Aneurinibacillus migulanus* proteome (1.5 mg/mL) was individually treated with probes **2** and **3** (1 μM from a 100 μM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM MgCl₂, 1 mM TCEP, 0.05% Igepal CA-630, 0.2 mg/mL lysozyme, and protease inhibitor cocktail. For inhibition studies, *A. migulanus* proteome (1.5 mg/mL) was individually pre-incubated with L-Pro-AMS **5** and L-Orn-AMS **7** (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature. 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 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).

Labeling of Individual A Domains and Profiling of A Domain Functions in Native Proteomic Environments: In order to investigate GrsA labeling, *A. migulanus* ATCC 9999 proteome (1.5 mg/mL) was individually treated with inhibitors **4–8** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM MgCl₂, 1 mM TCEP, 0.05% Igepal CA-630, 0.2 mg/mL lysozyme, and 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, the samples were exposed to ultraviolet light for 30 min on ice and treated with Rh-azide for 1 h at room temperature. To evaluate the labeling of GrsB, the *A. migulanus* DSM 5759 proteome (1.5 mg/mL) was individually treated with inhibitors **4–8** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM MgCl₂, 1 mM TCEP, 0.05% Igepal CA-630, 0.2 mg/mL lysozyme, and protease inhibitor cocktail. These samples were incubated for 10 min at room temperature and treated with individual members of probes **2** and **3** (1 μM from a 100 μM stock in DMSO). In all reactions, the DMSO concentrations were maintained at a level of 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).

Mass Spectroscopic Analysis, 1D SDS-PAGE, and In-gel Digestion: The proteins were separated by 1D SDS-PAGE by using 6% wide range gels (Nacalai tesque) and visualized using Sil-best stain one (Nacalai tesque). The bands were excised, destained using destaining solution in the Silver Stain MS Kit (Wako Pure Chemical Industries, Ltd), and subjected to in-gel digestion with TPCK-treated trypsin in the digestion buffer (10 mM Tris-HCl, pH 8.0 and 0.05% decyl glucoside) for 12 h at 37 °C.

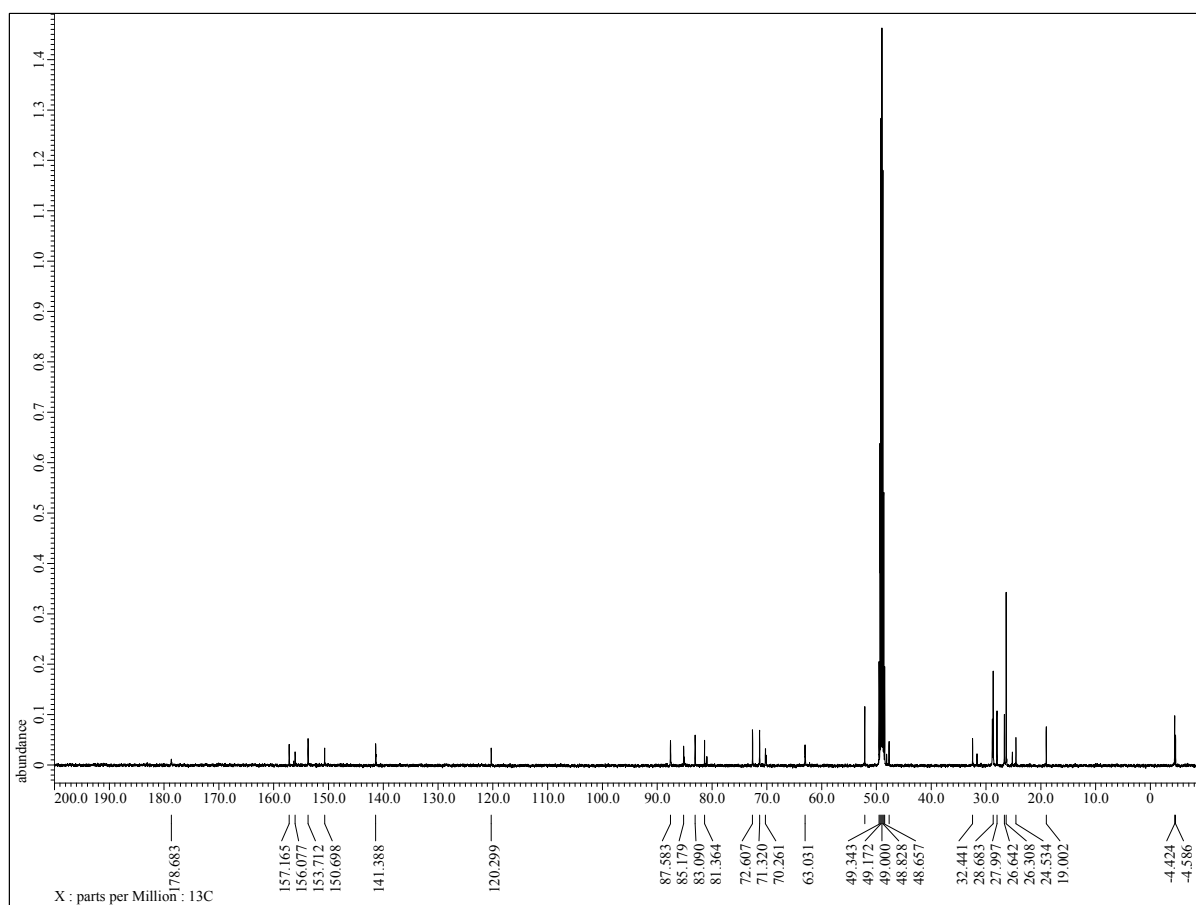
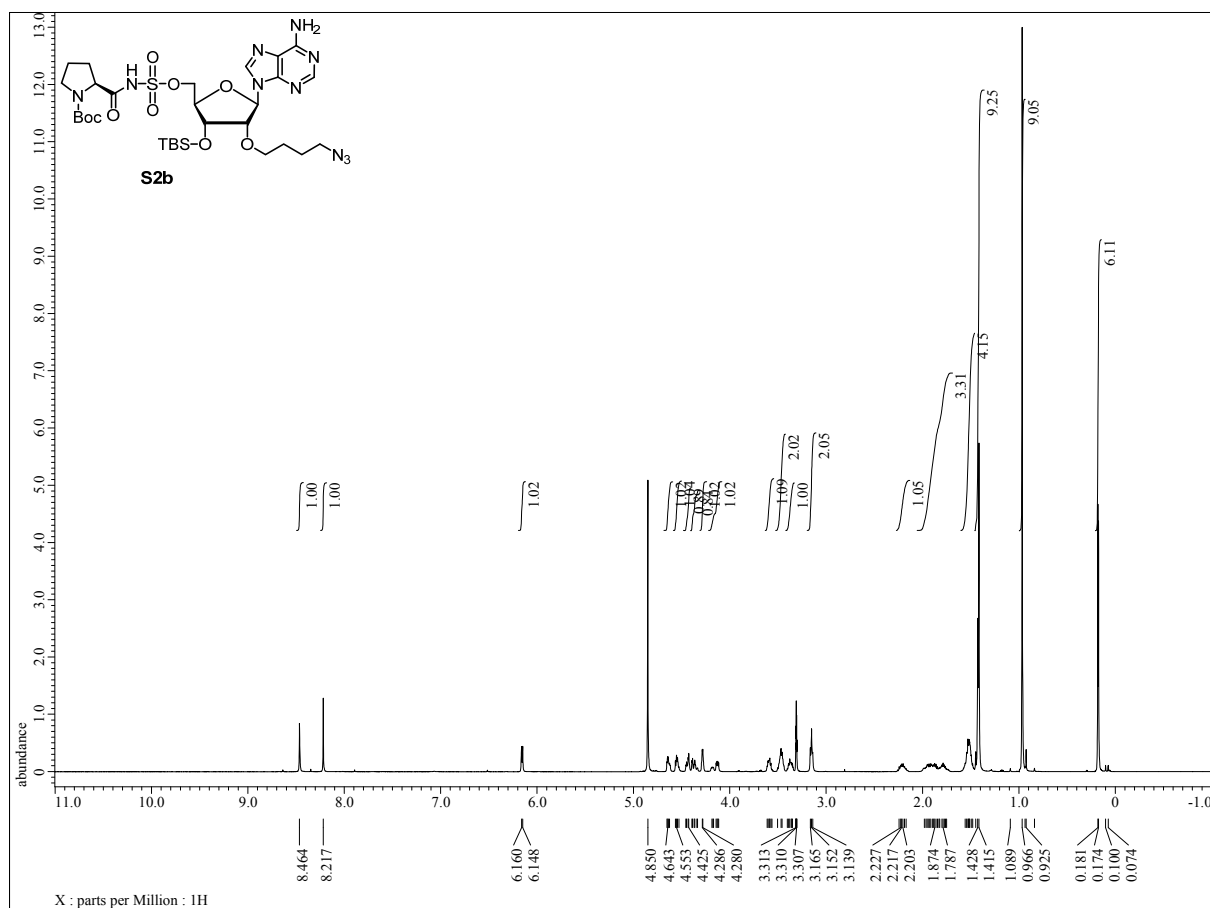
LC-MS/MS analysis: The digest mixture was separated using a nanoflow LC (Easy nLC, Thermo Fisher Scientific) on NTCC analytical column (C-18 reverse-phase column, ϕ 0.075 \times 100 mm, 3 μ m bead size, Nikkyo Technos Co., Ltd.). The buffer compositions were as follows. Buffer A was composed of 100% H₂O and 0.1% formic acid; buffer B was composed of 100% CH₃CN and 0.1% formic acid. Peptide were eluted from the C-18 column using a linear gradient of 35–100% buffer B over 10 min at a flow rate of 300 nL/min and subjected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) with a nanospray ion source using data-dependent TOP10 MS/MS method in the mass range of m/z = 300–500 and m/z = 500–1500. Peptide identifications were made using MS/MS Ions Search in MASCOT program v2.3 (Matrix Science Inc., Boston, MA).

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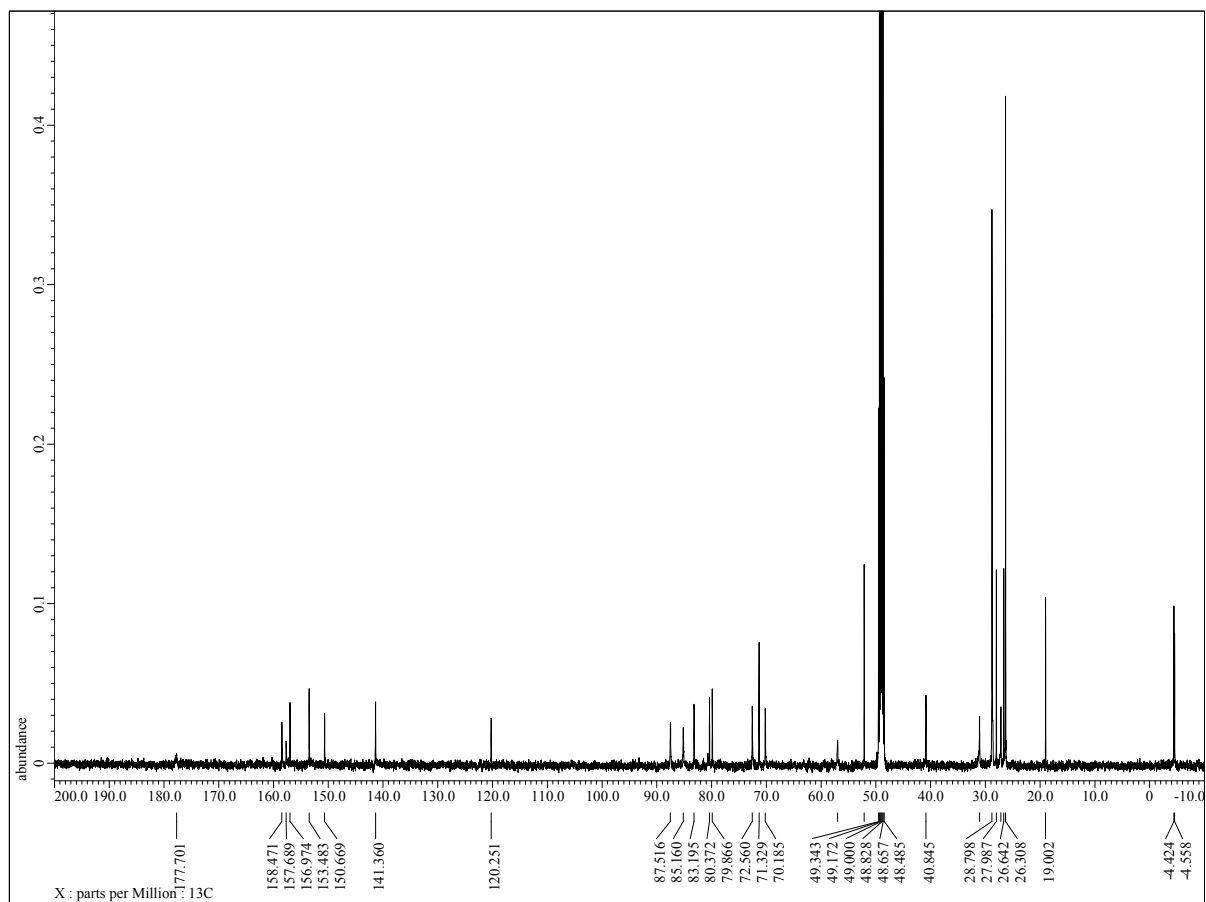
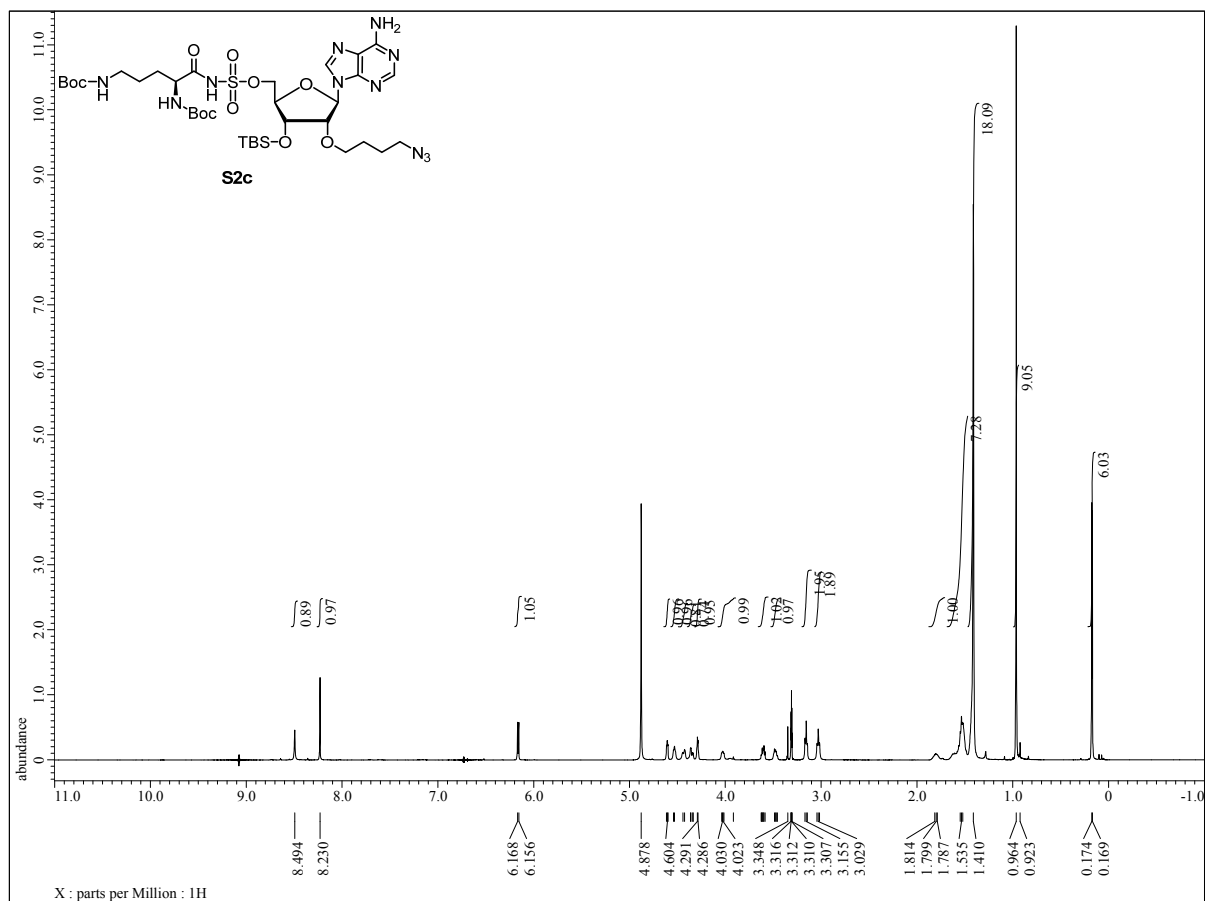
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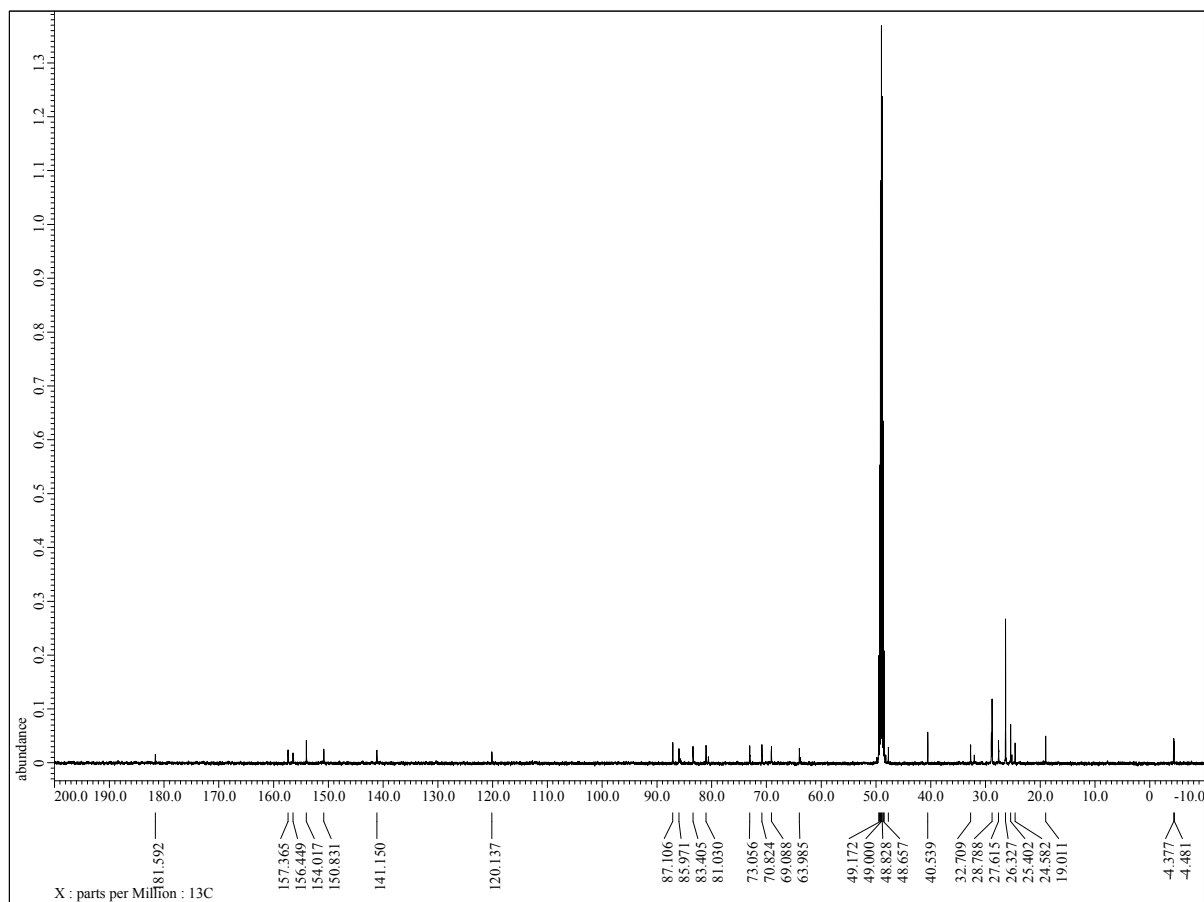
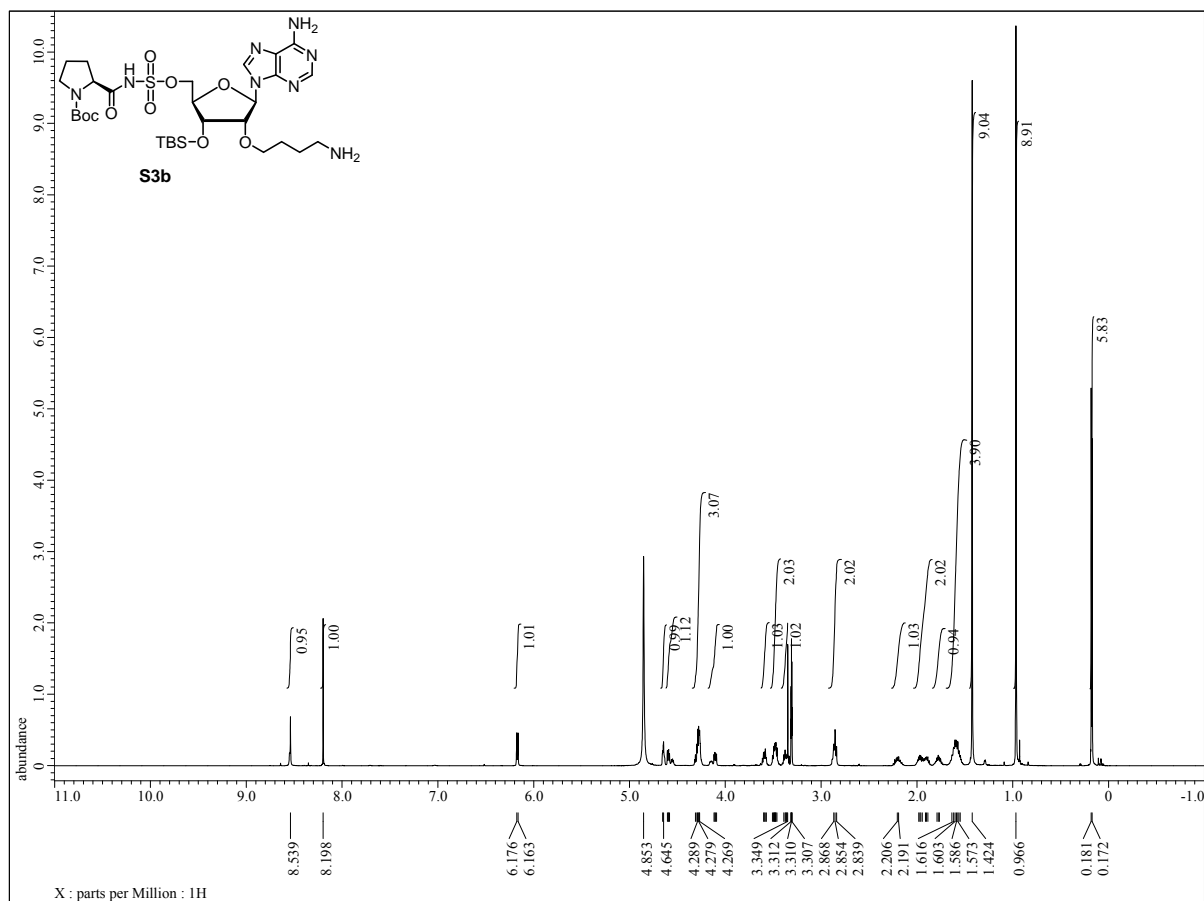
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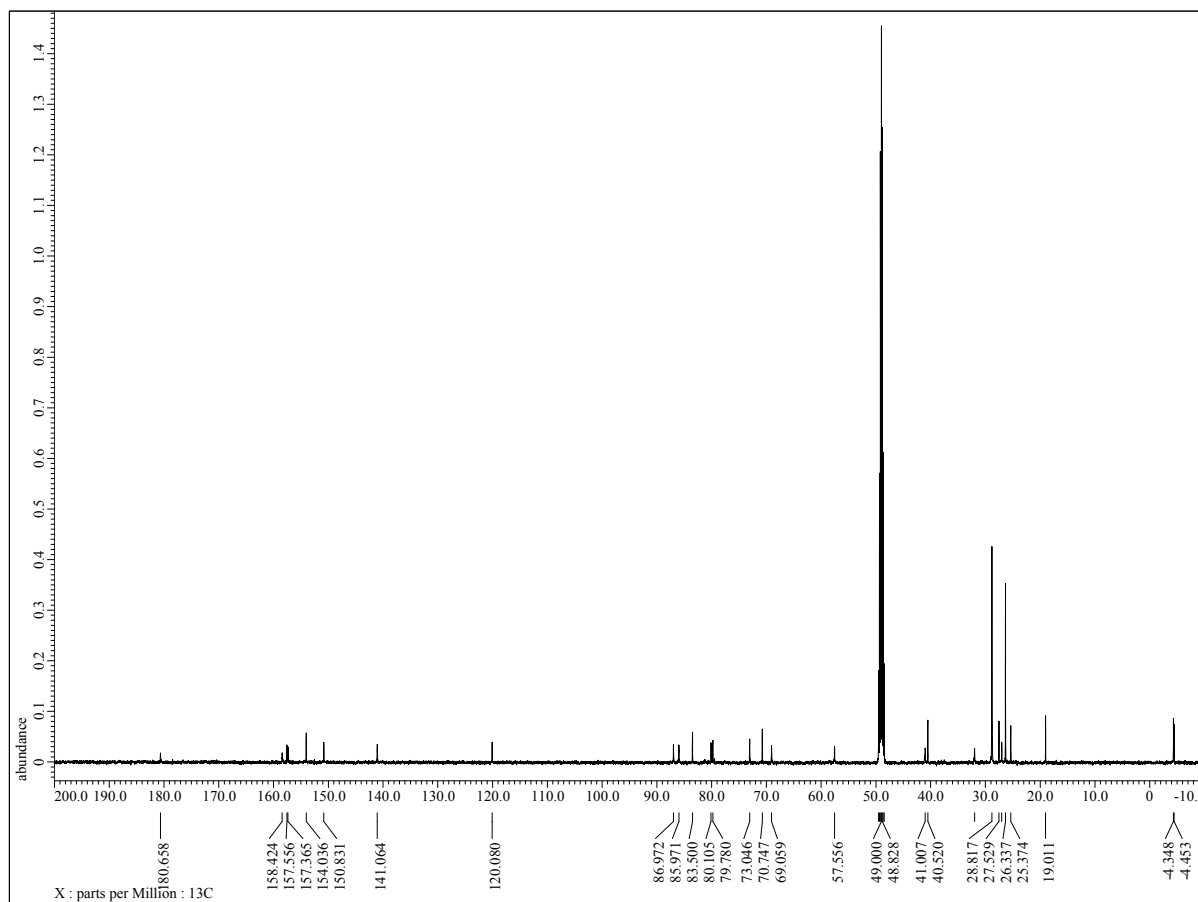
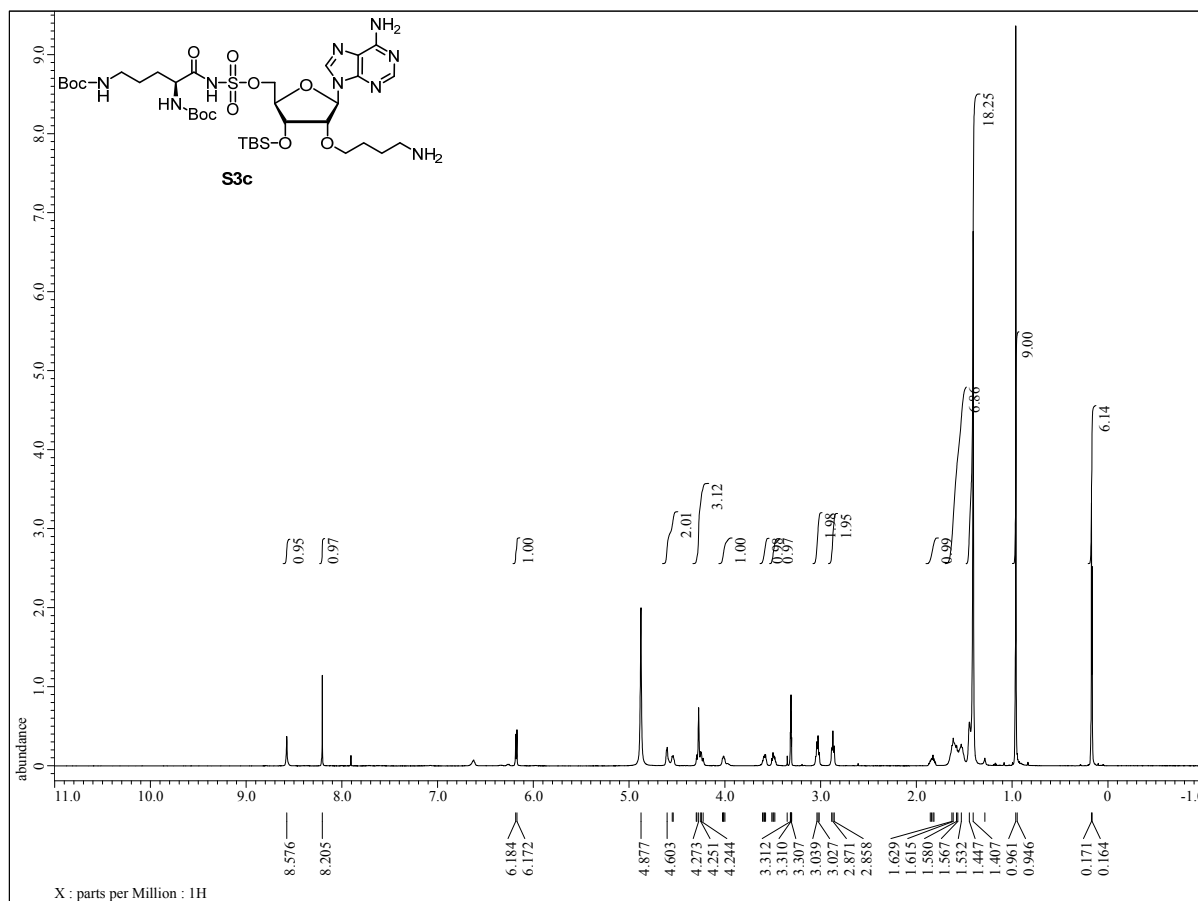
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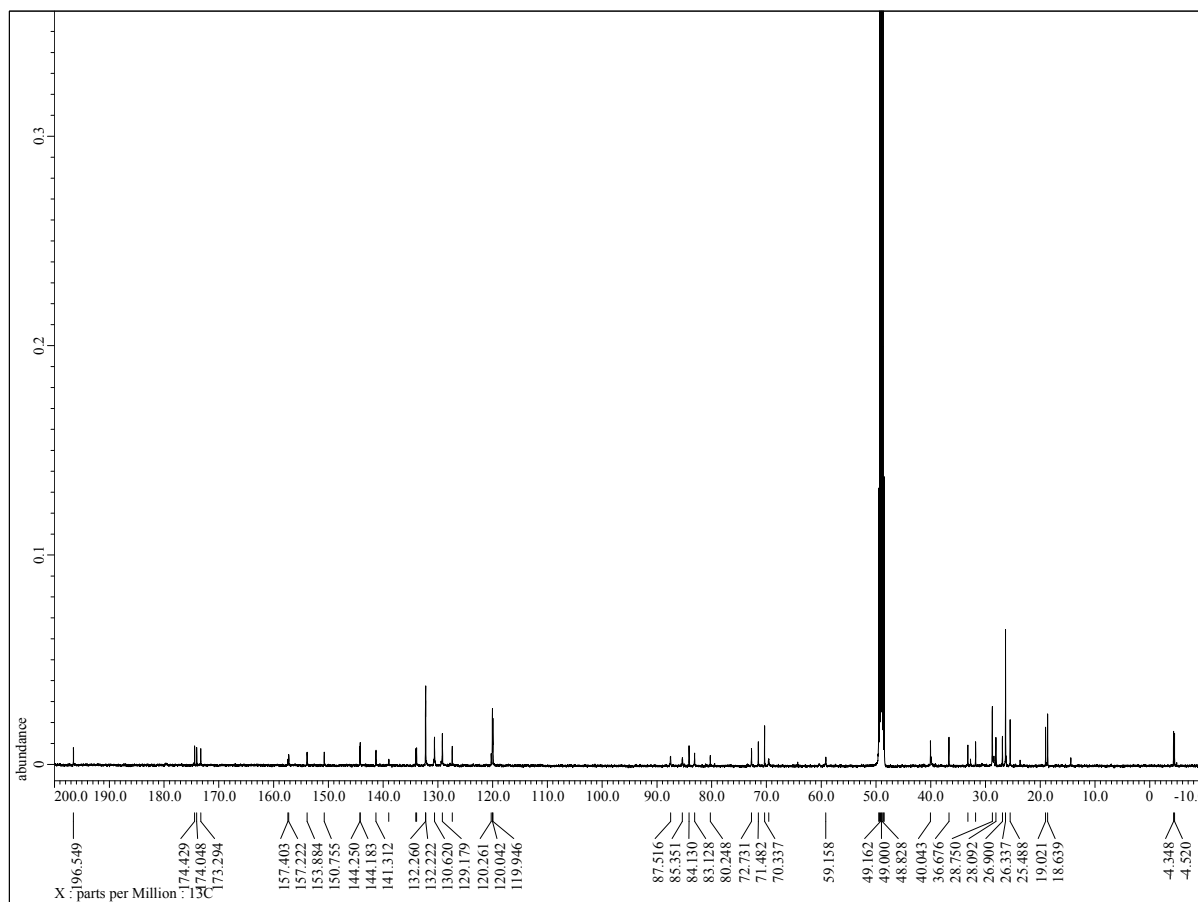
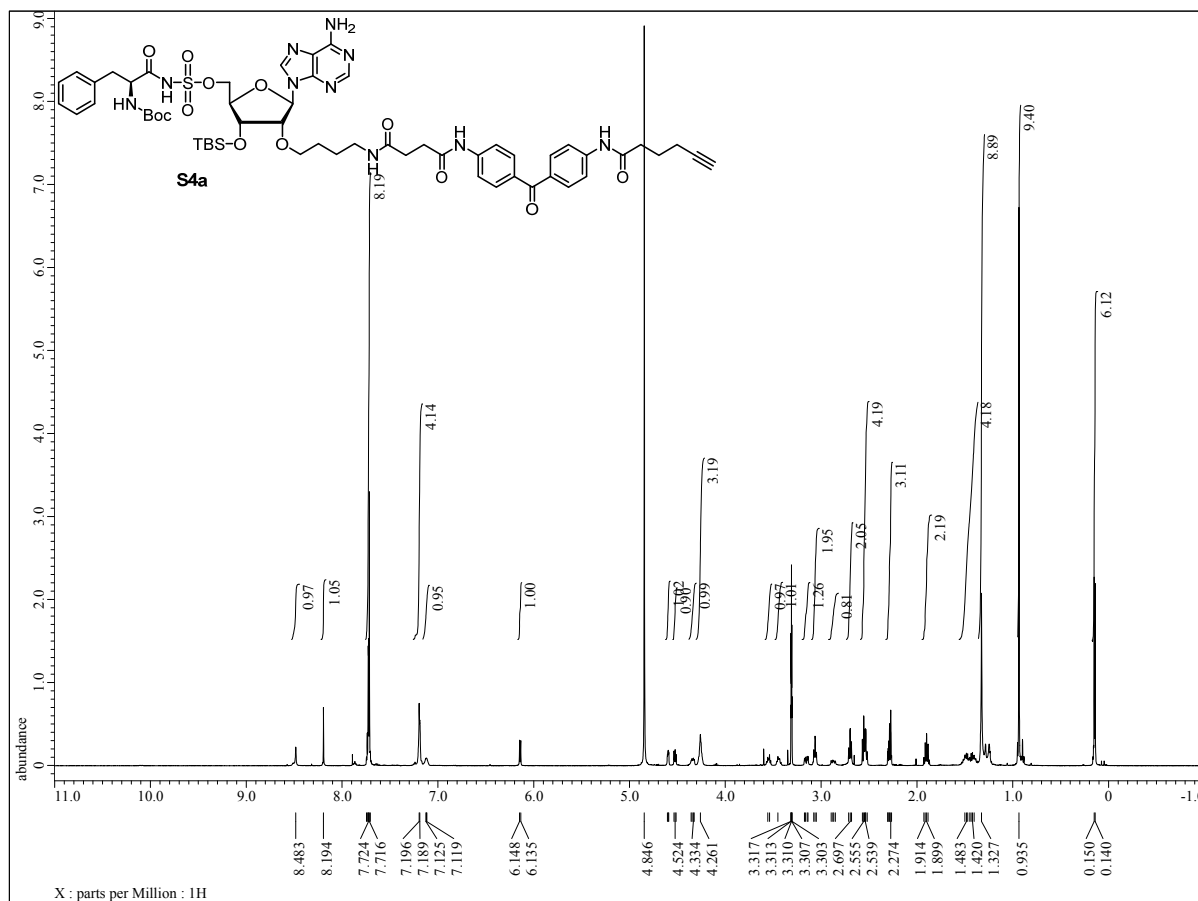
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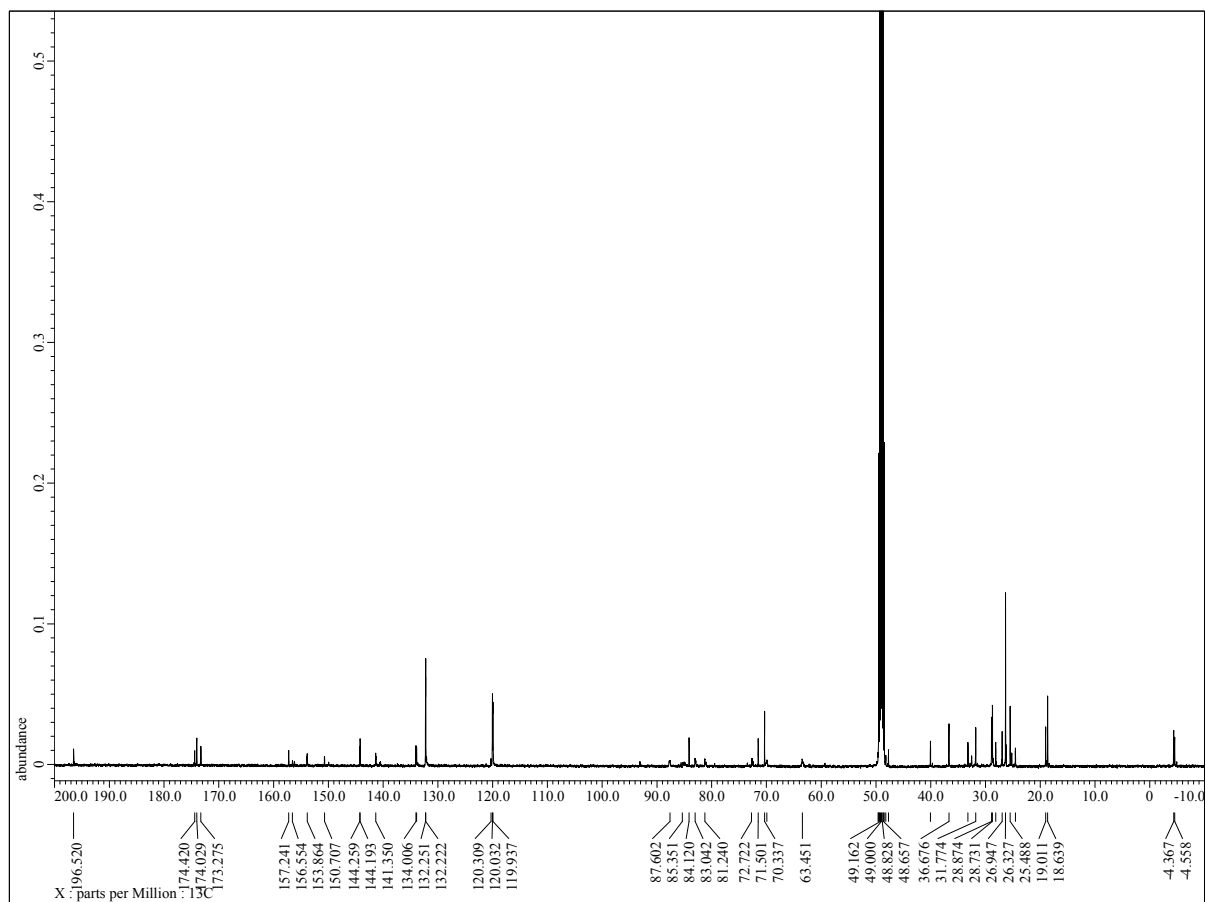
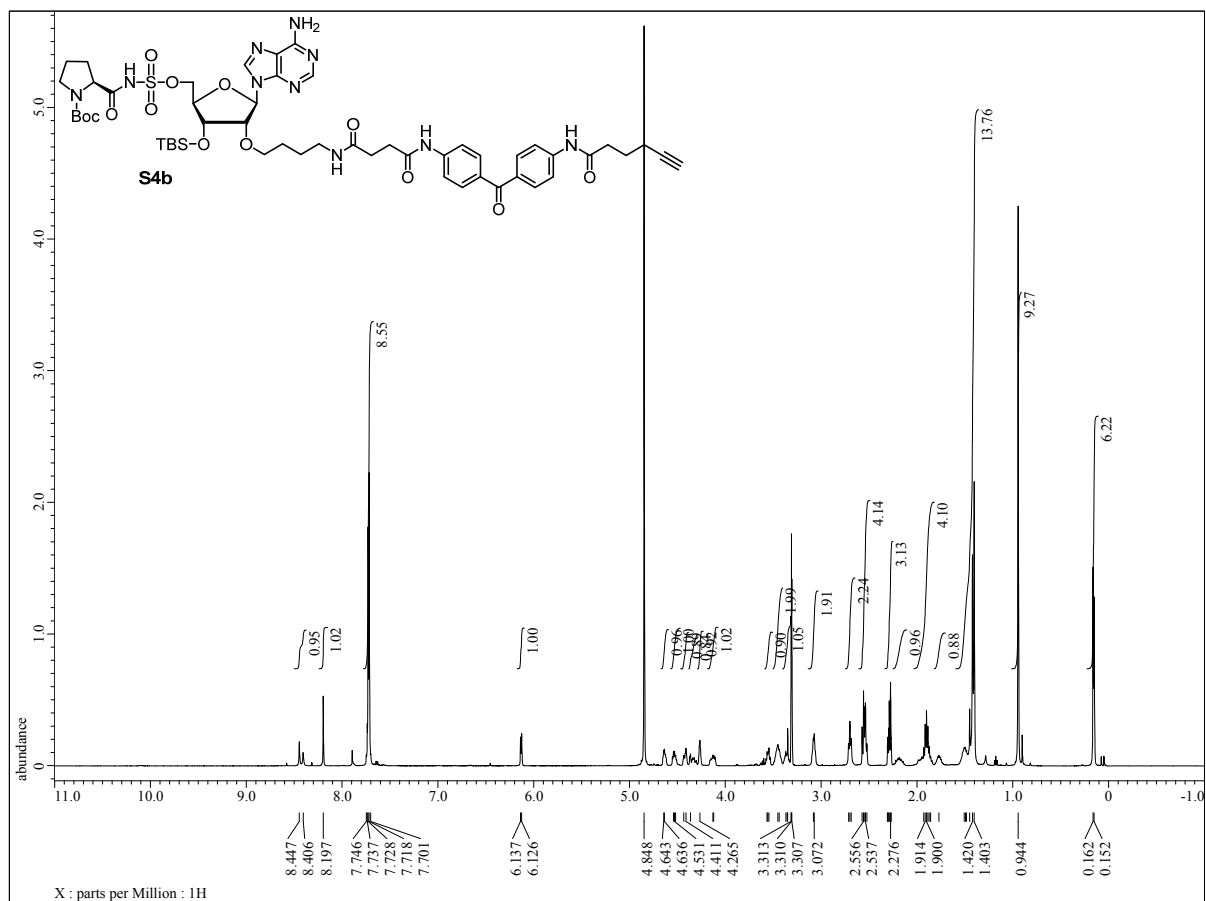
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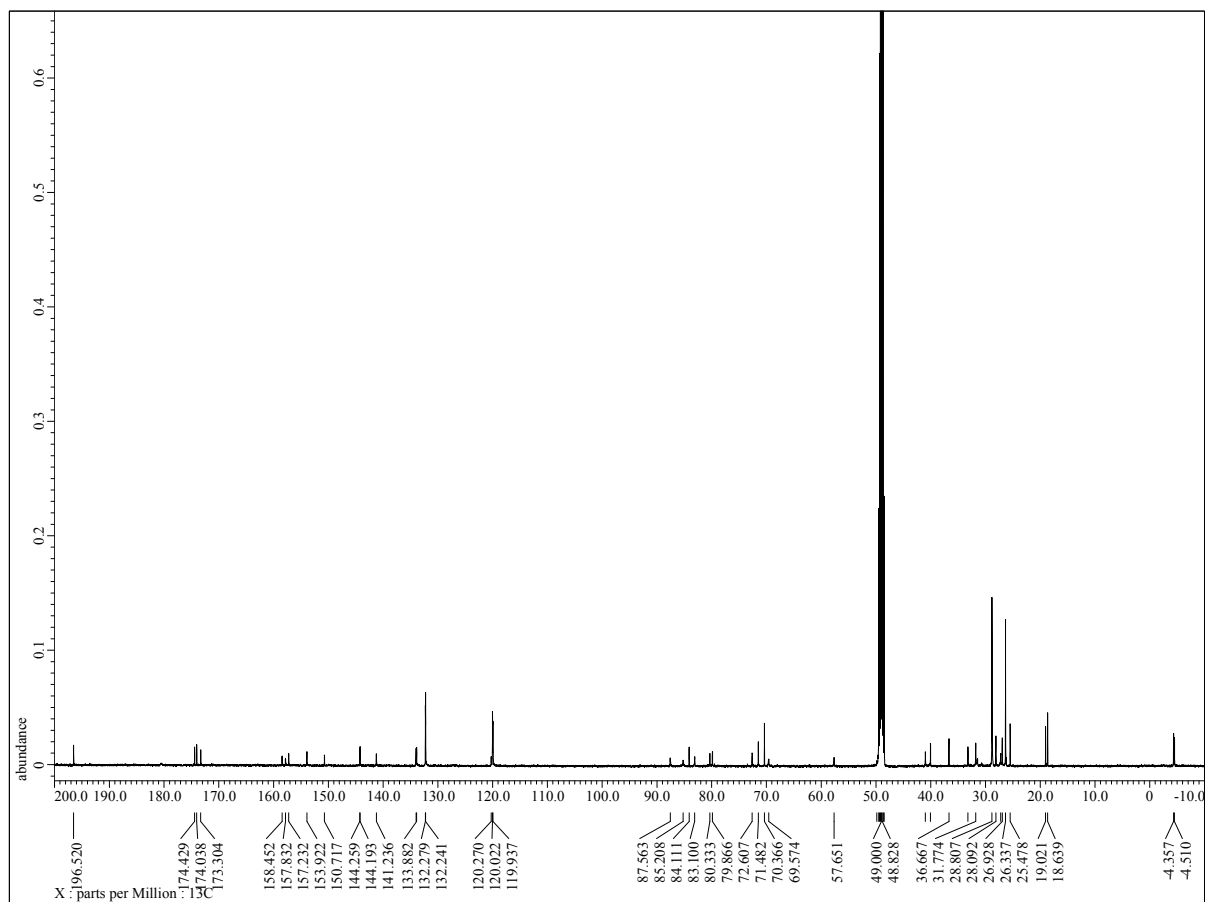
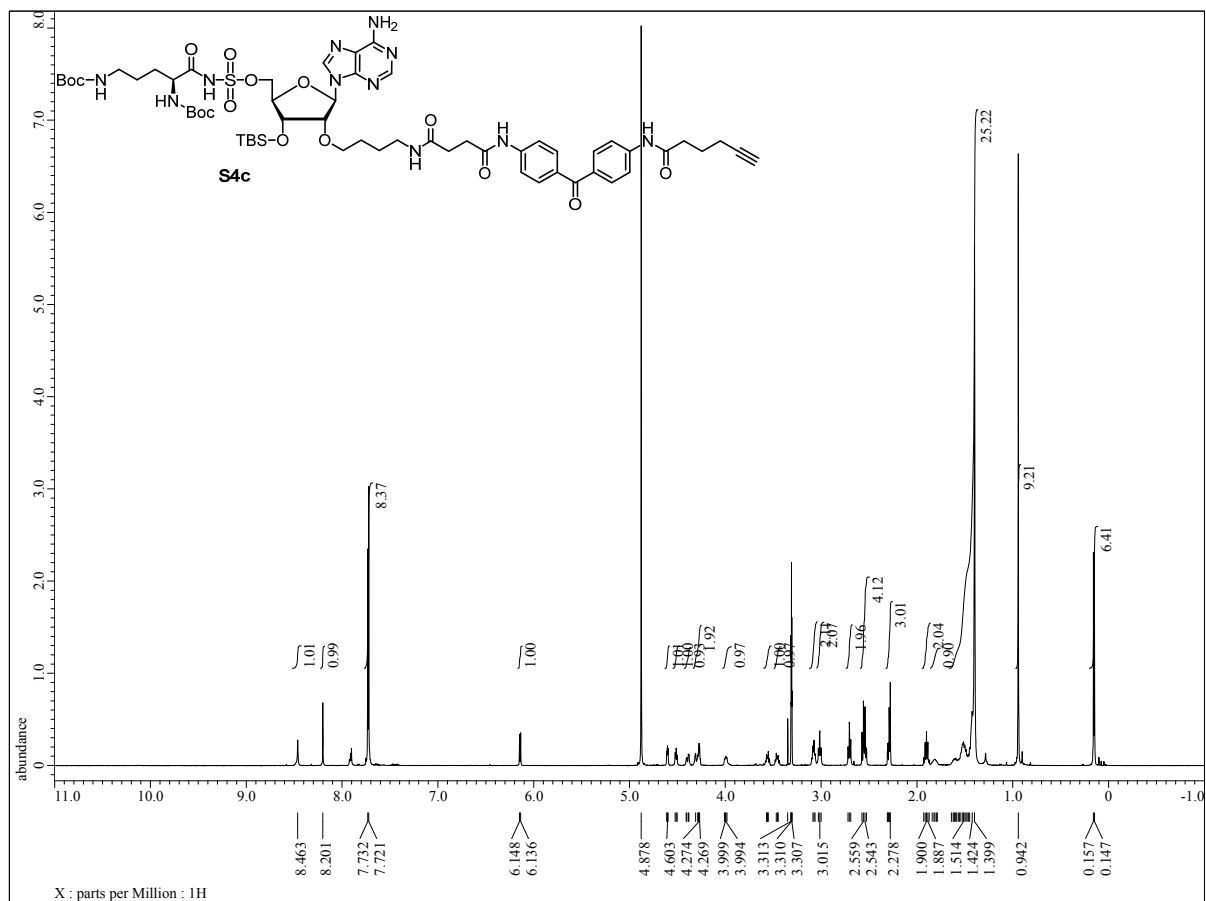
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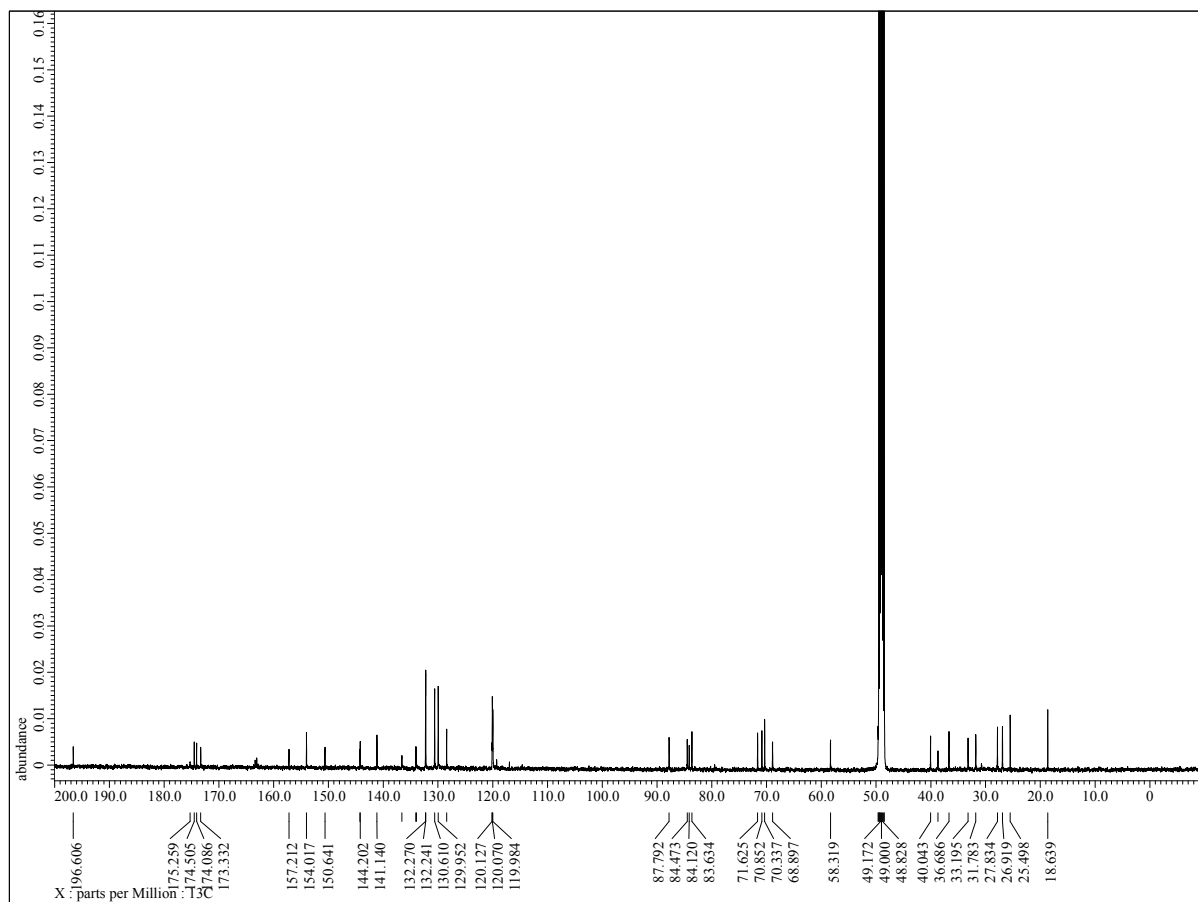
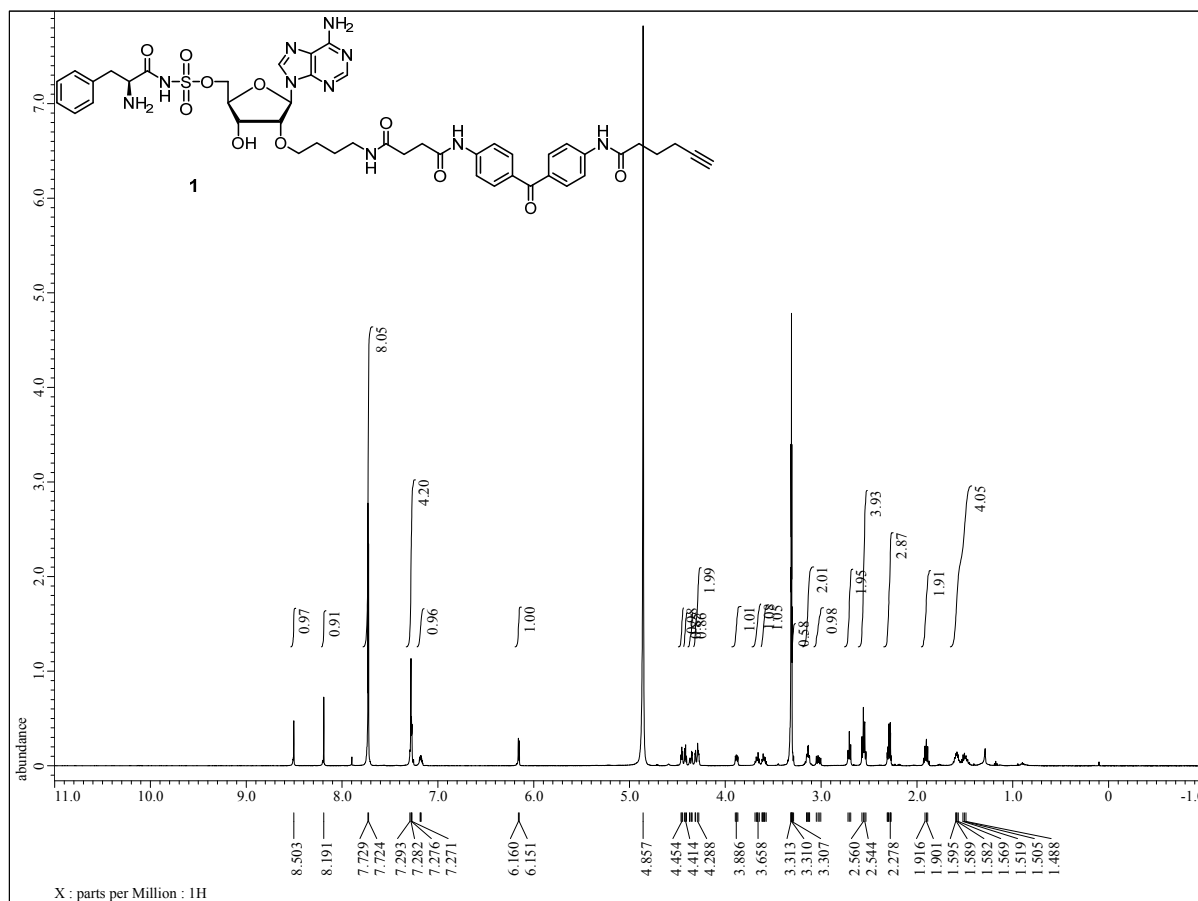
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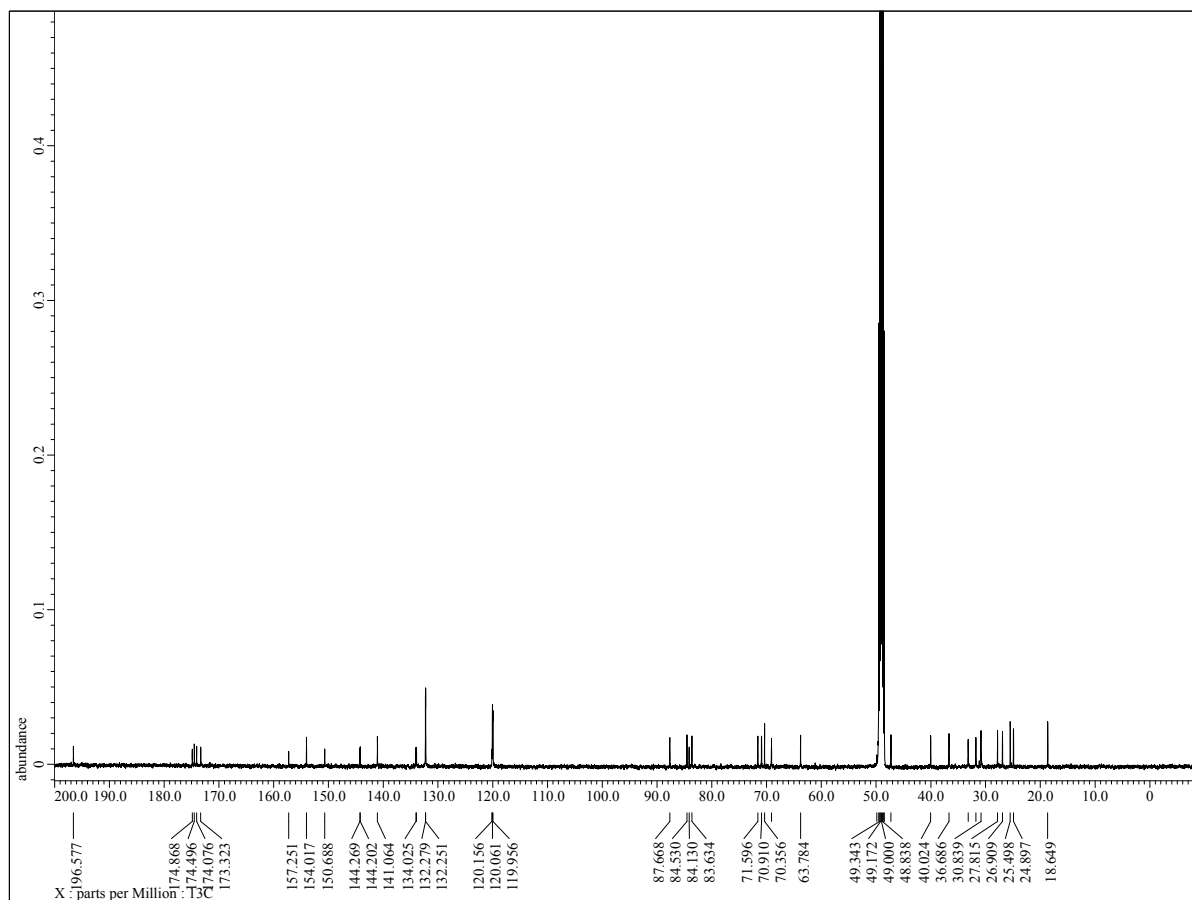
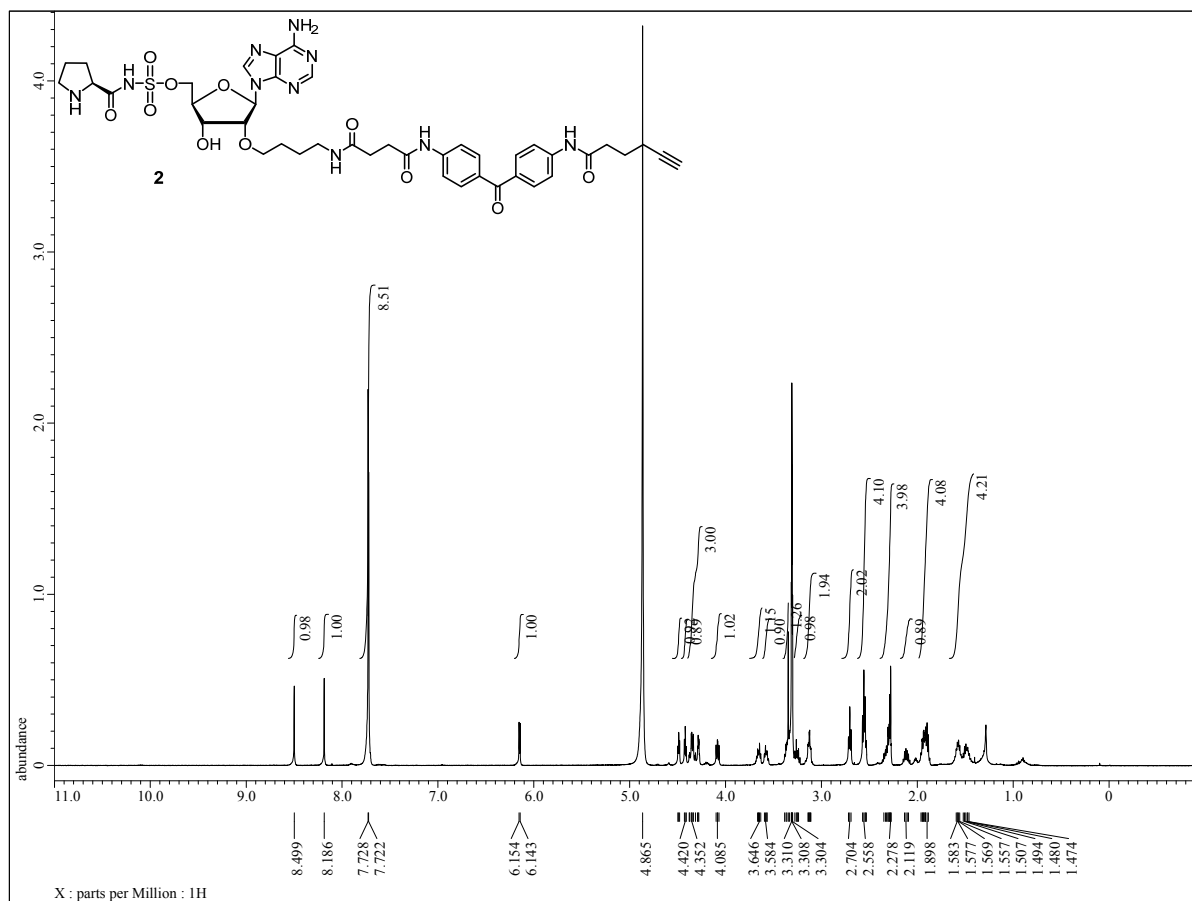
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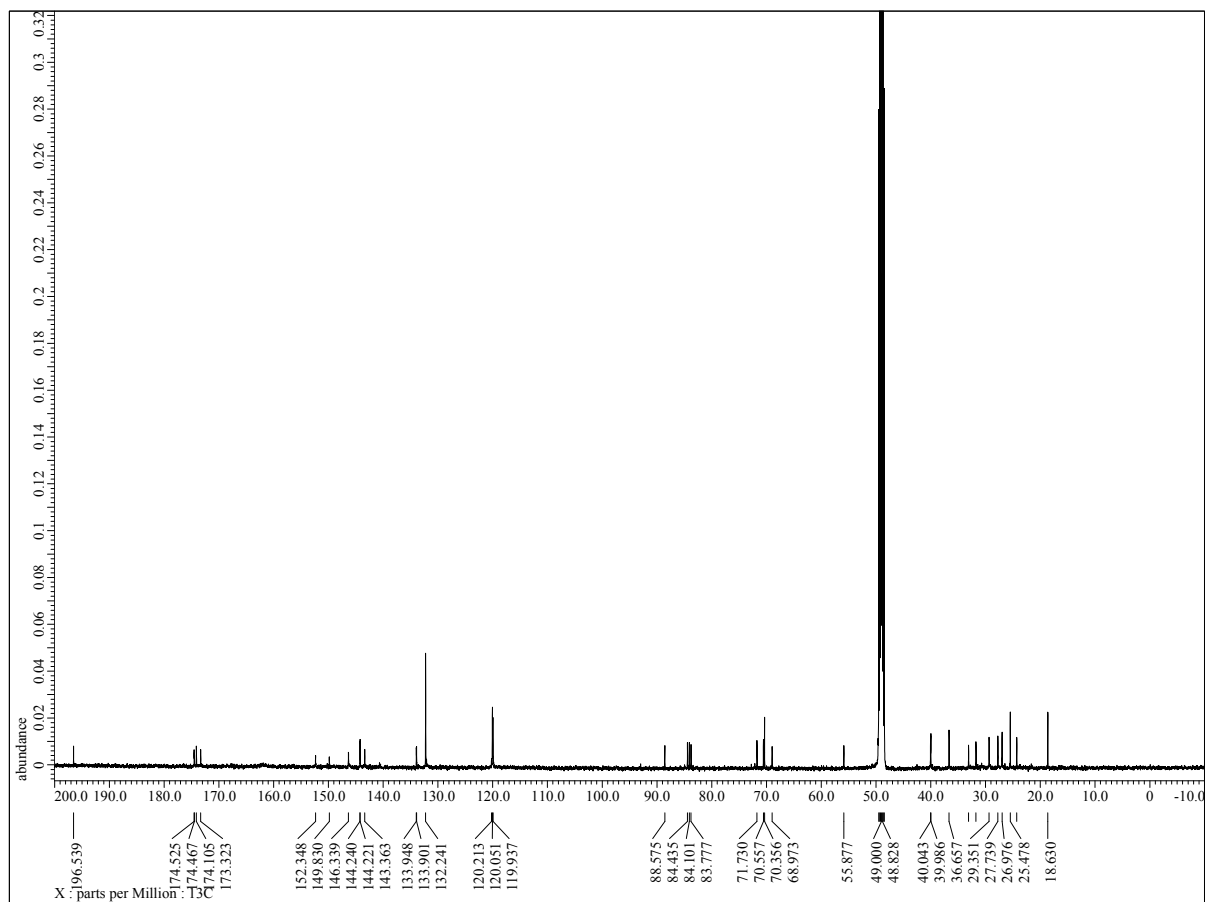
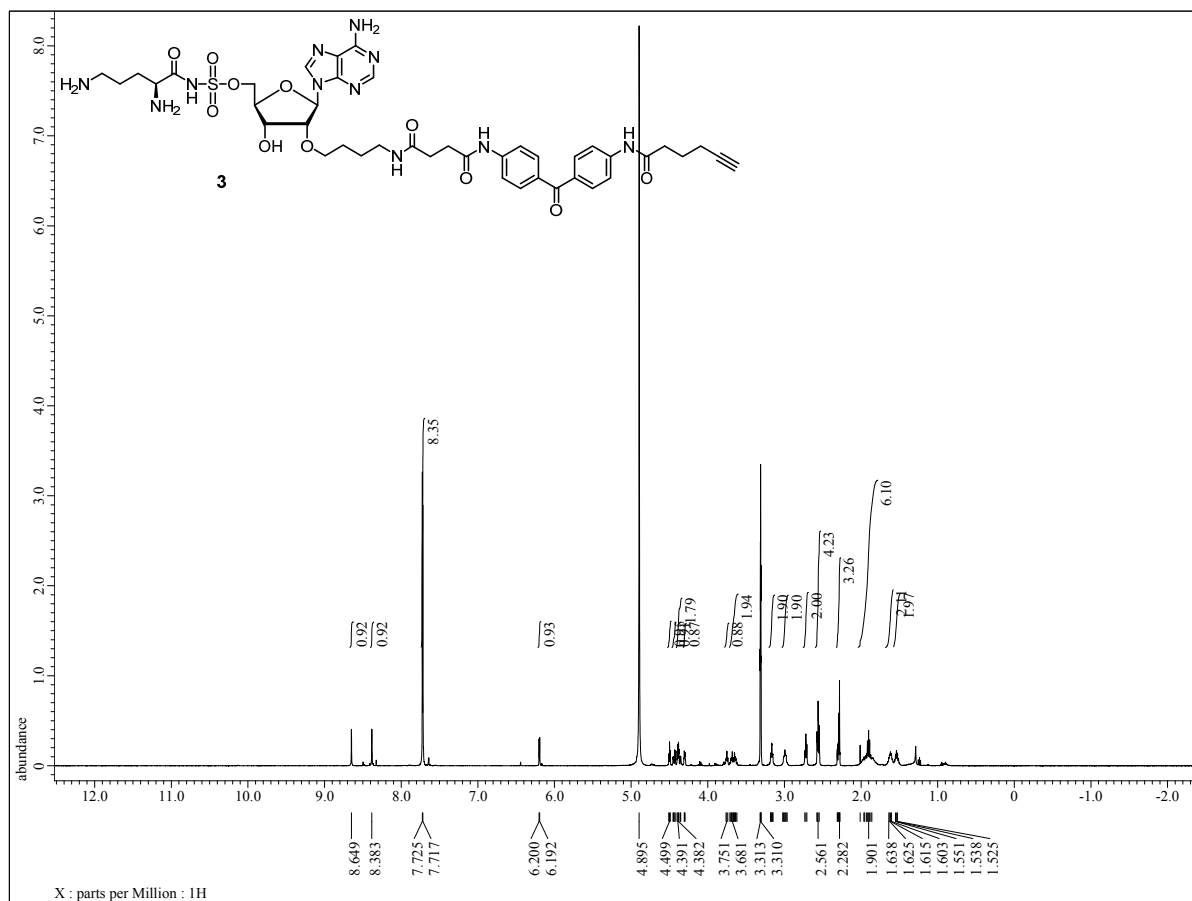
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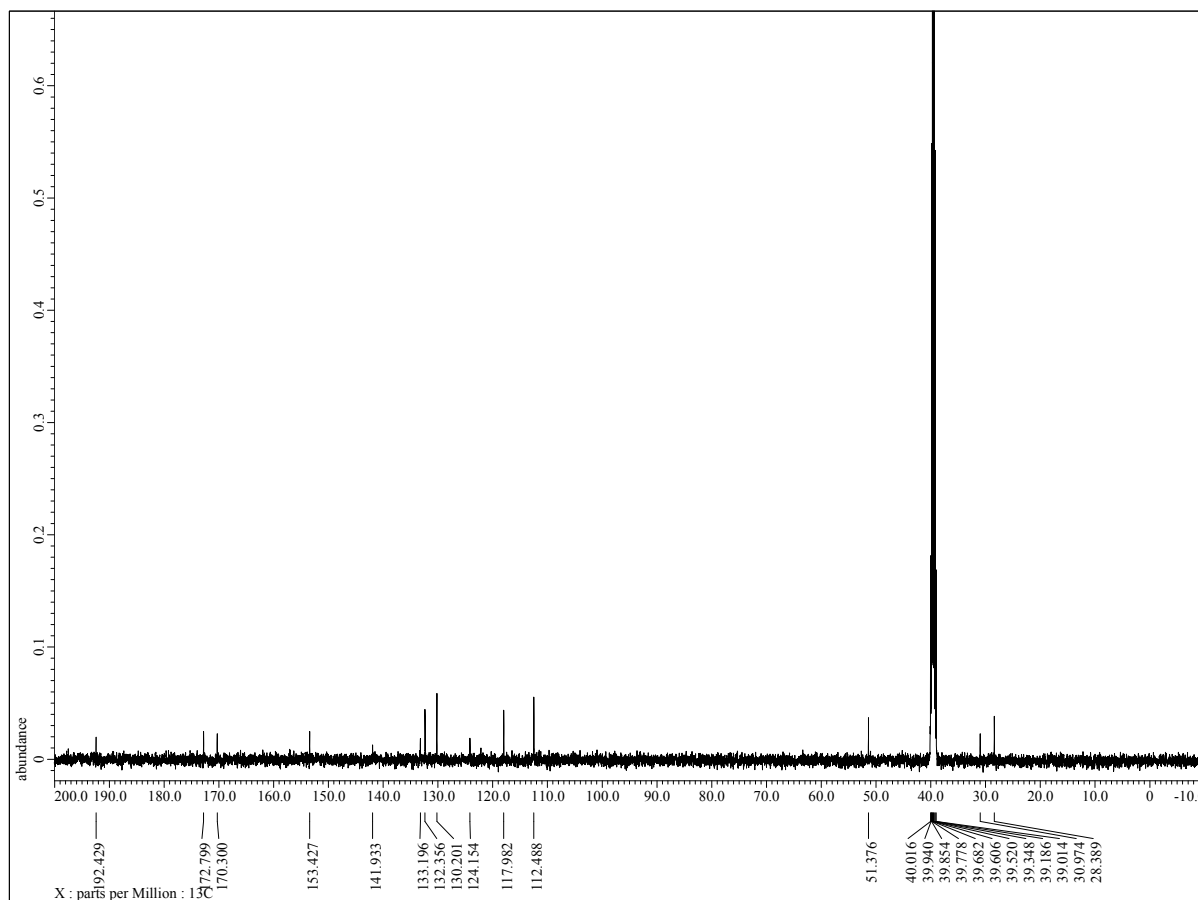
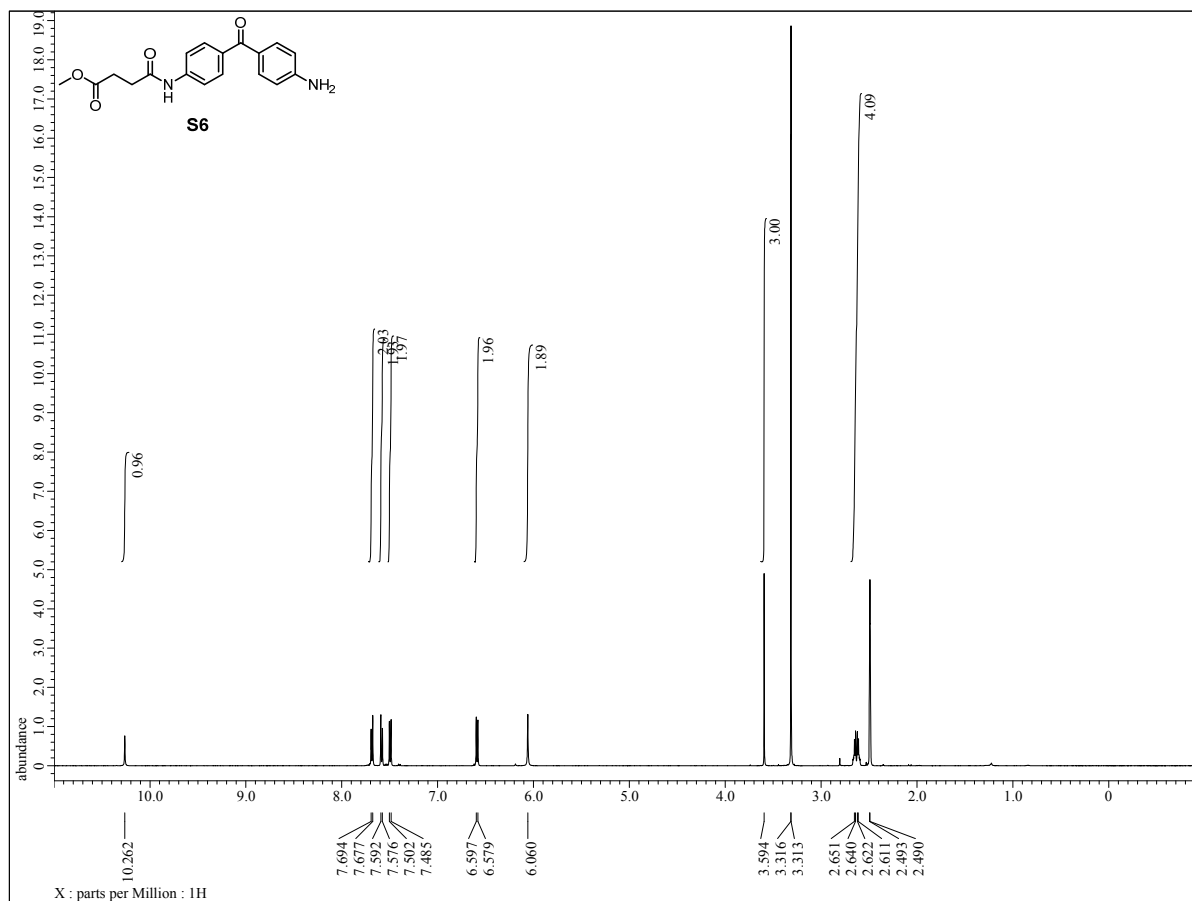
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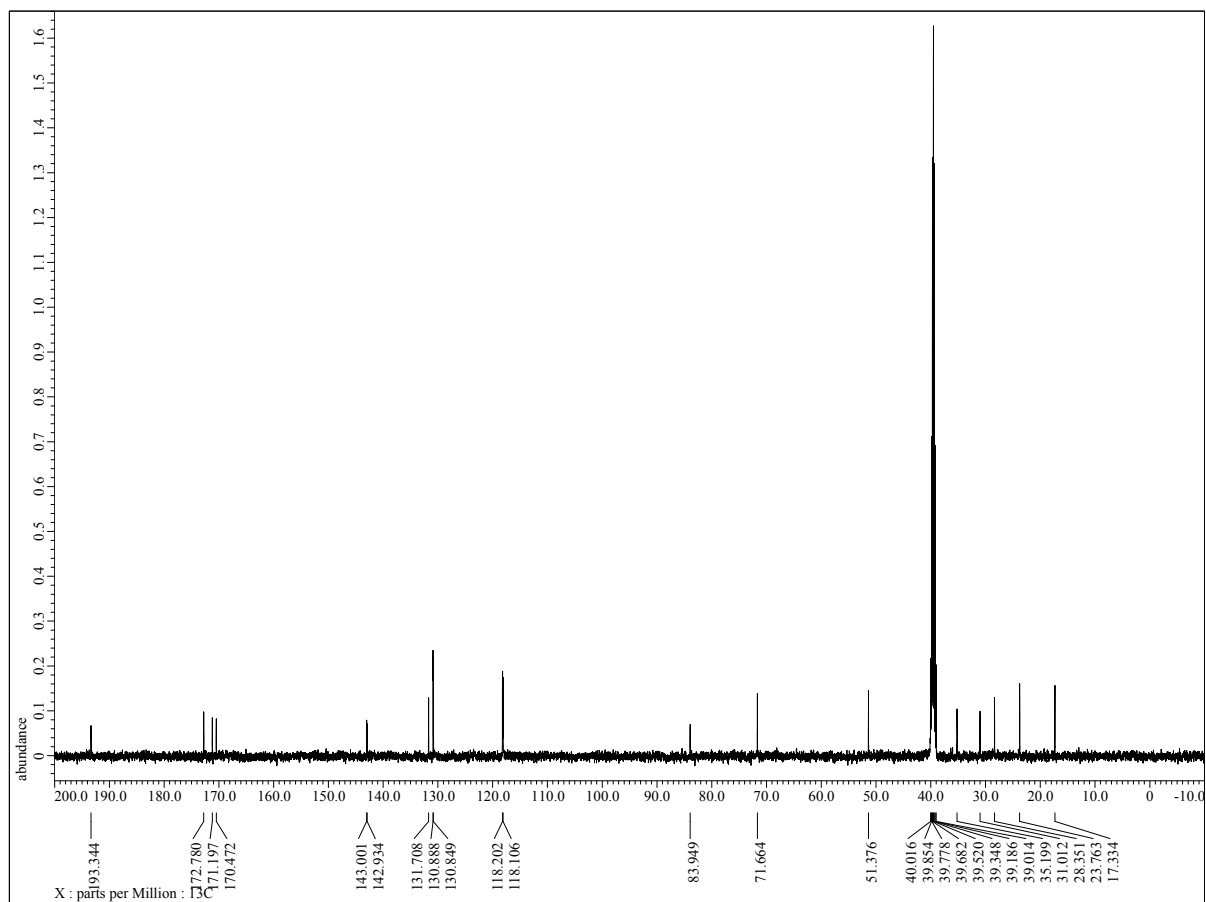
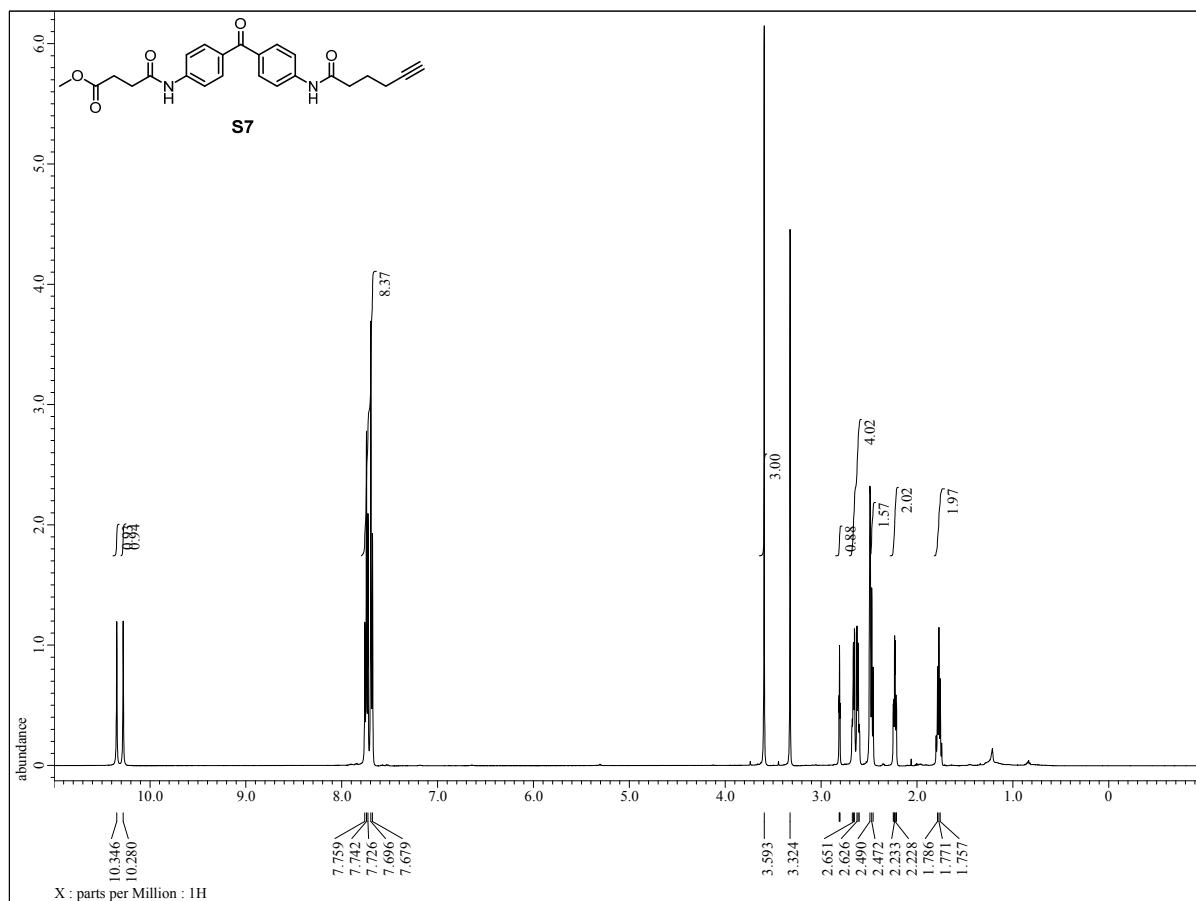
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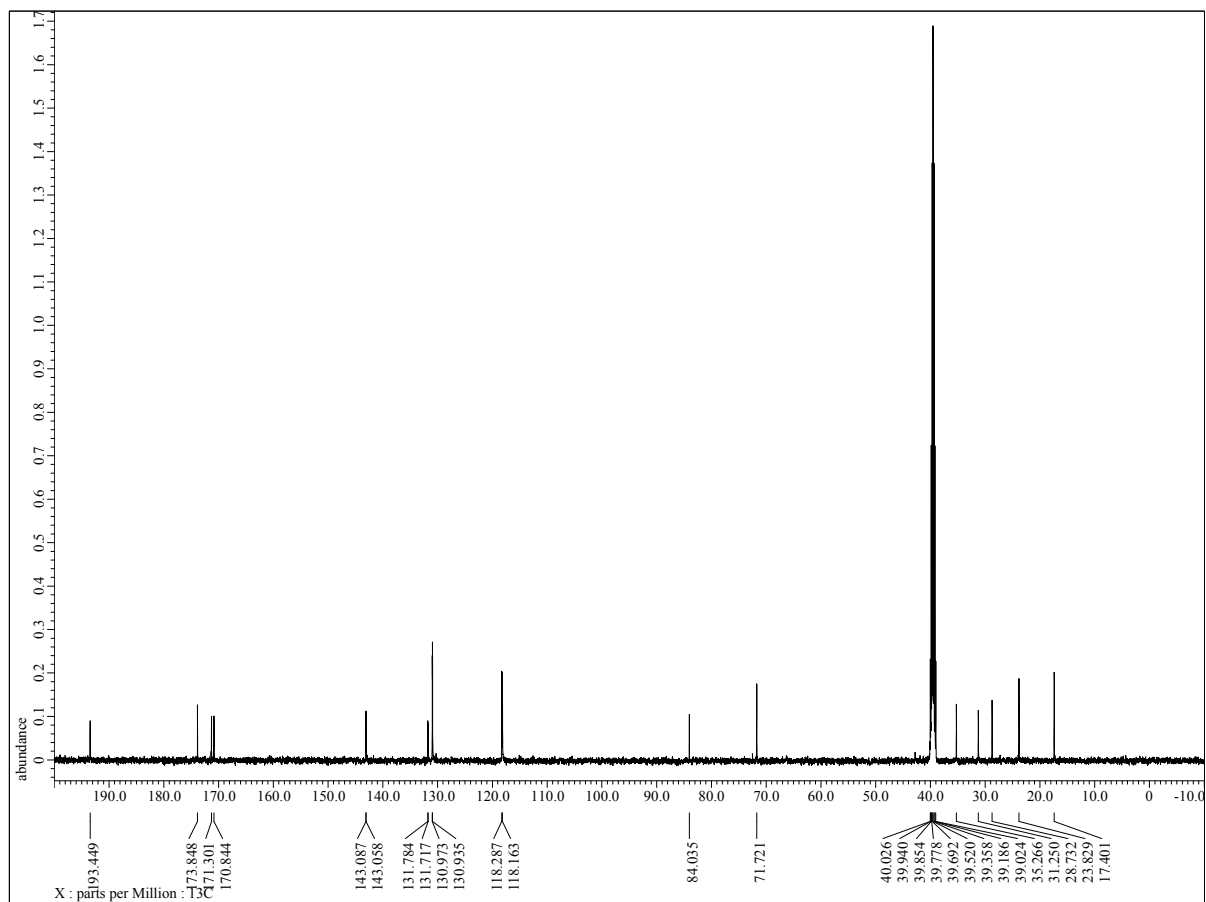
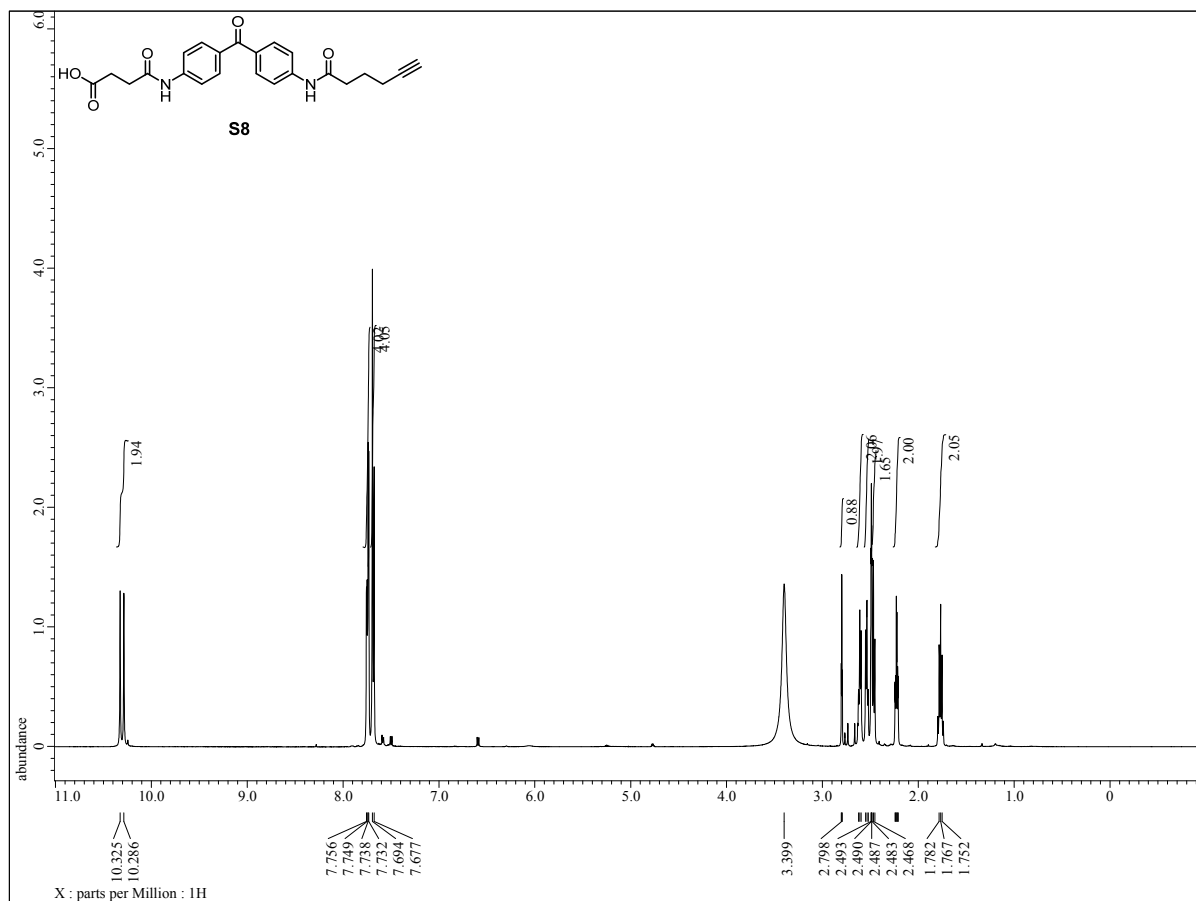
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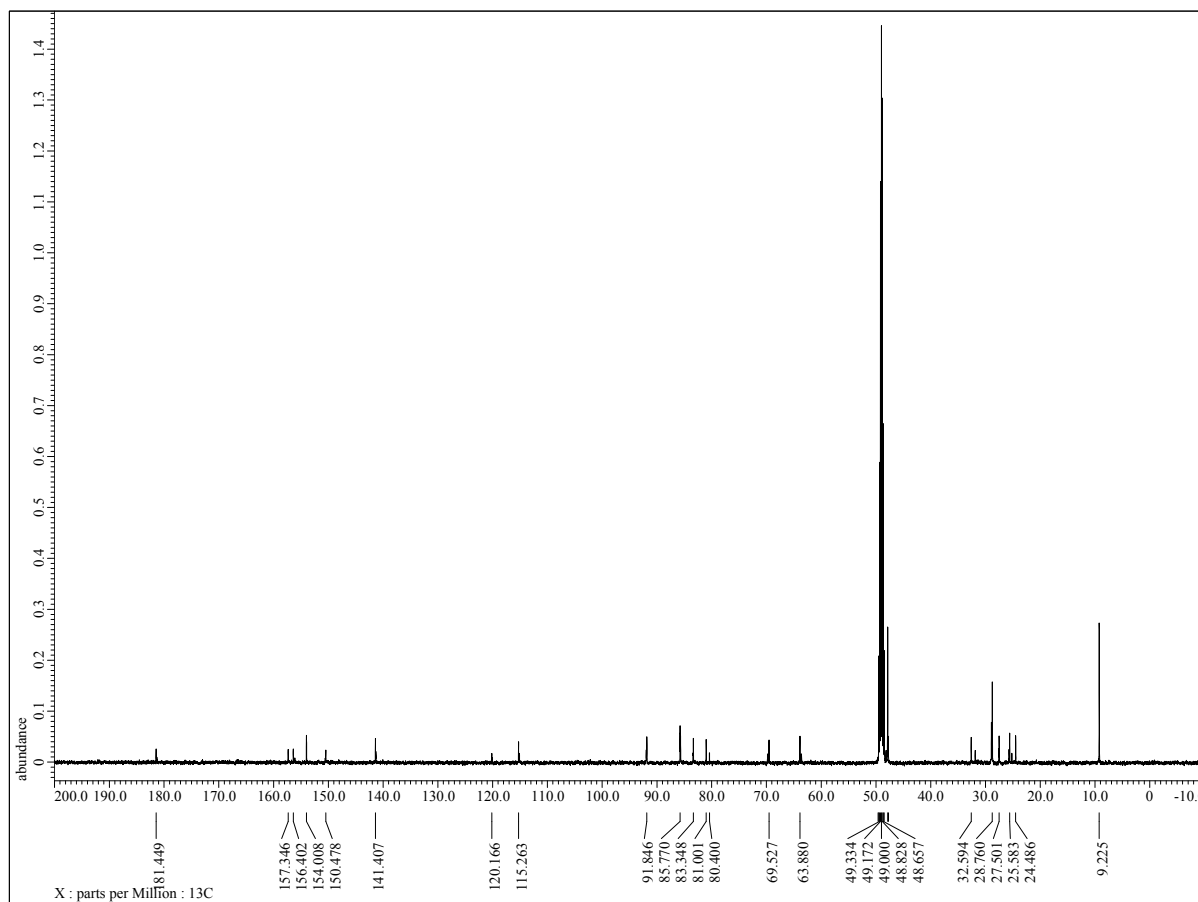
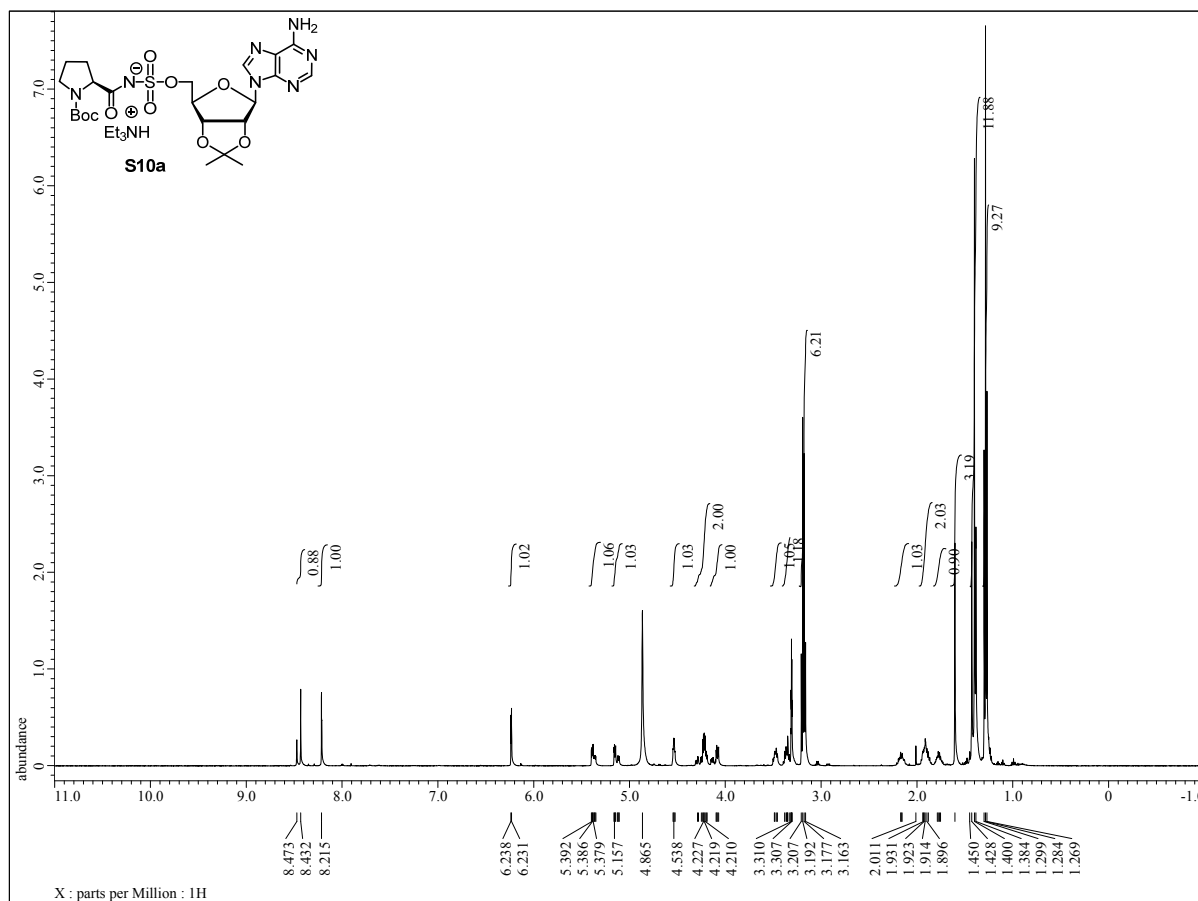
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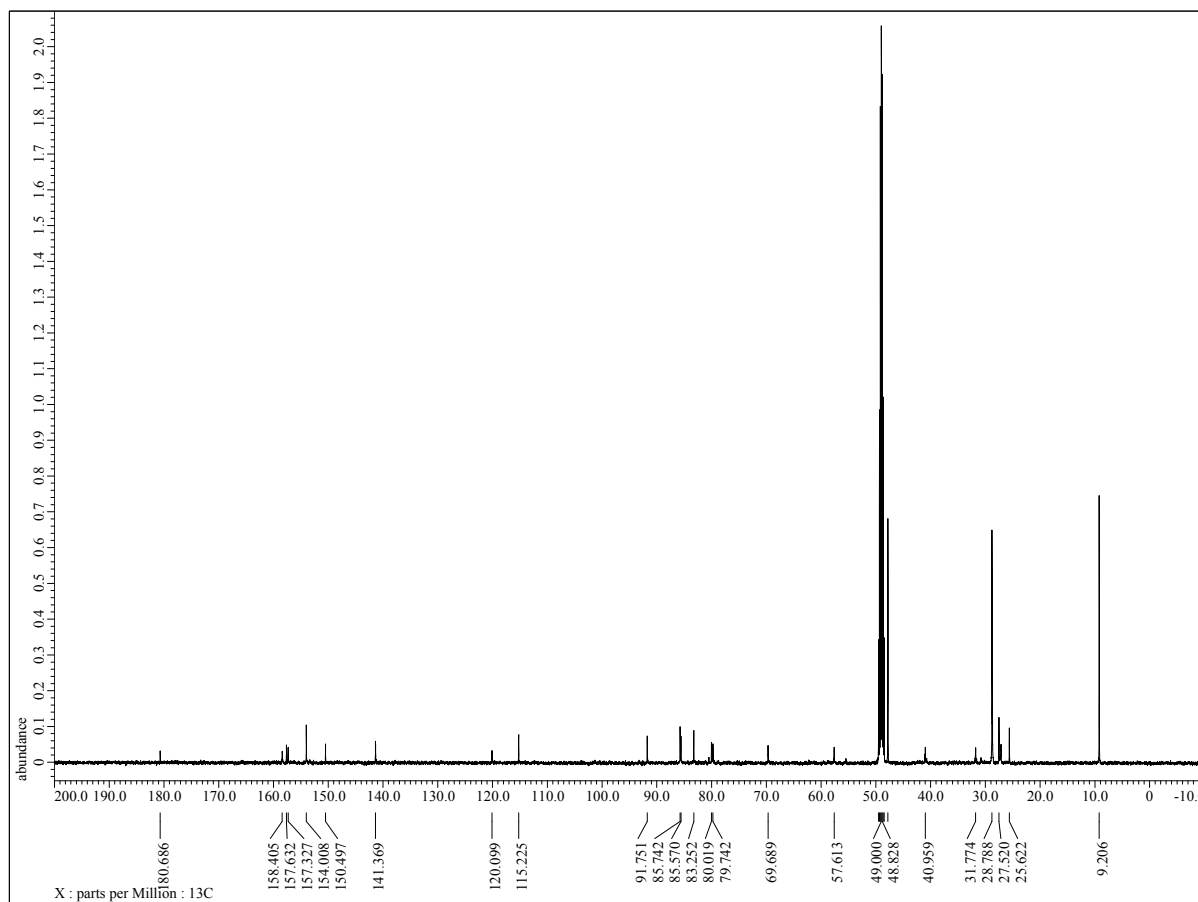
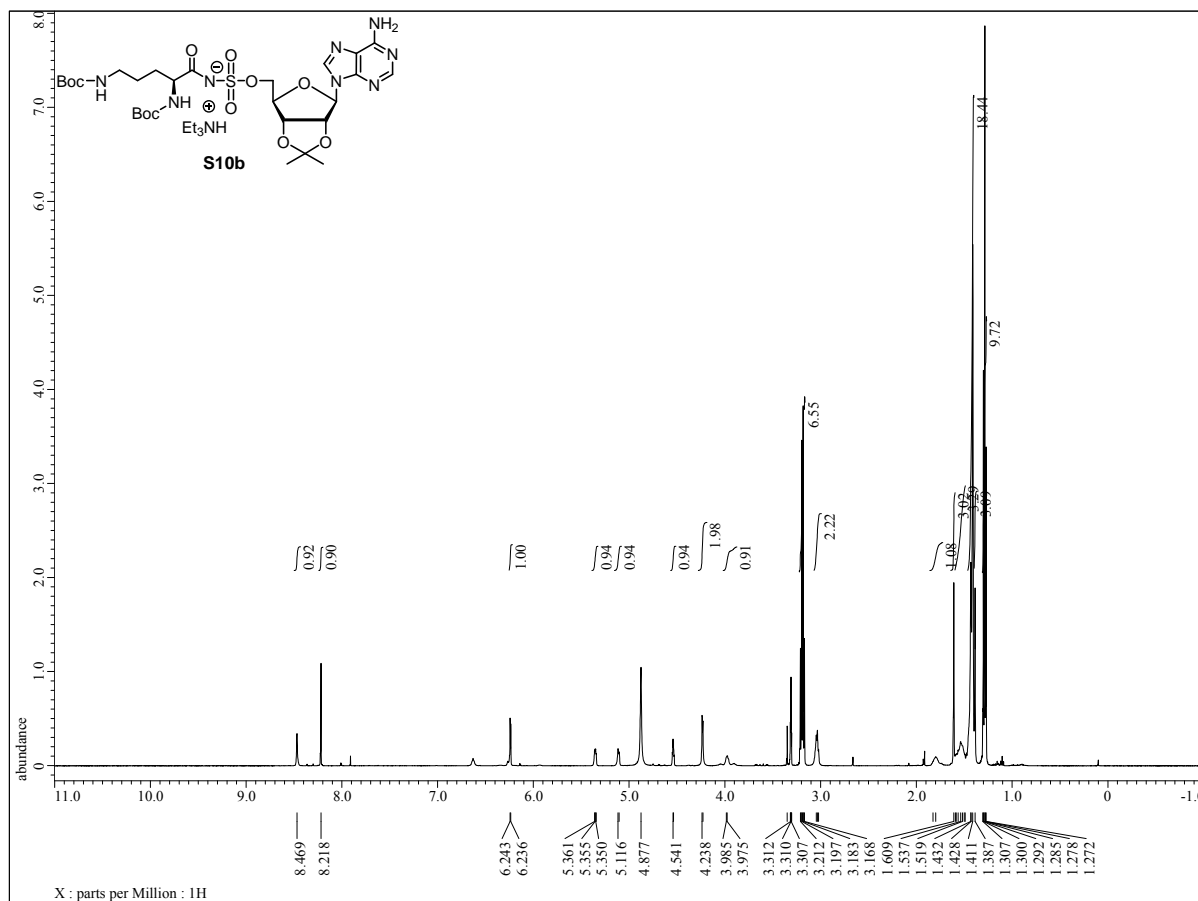
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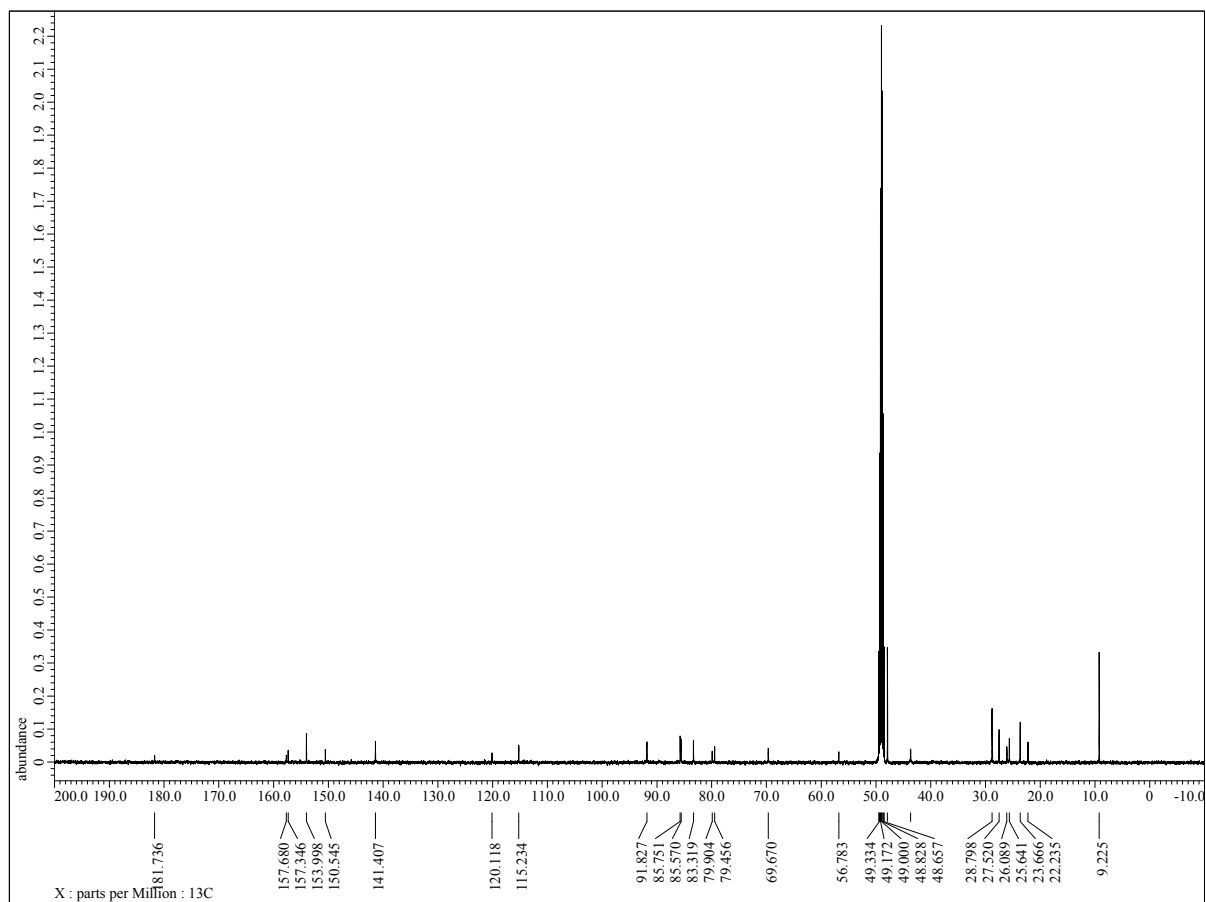
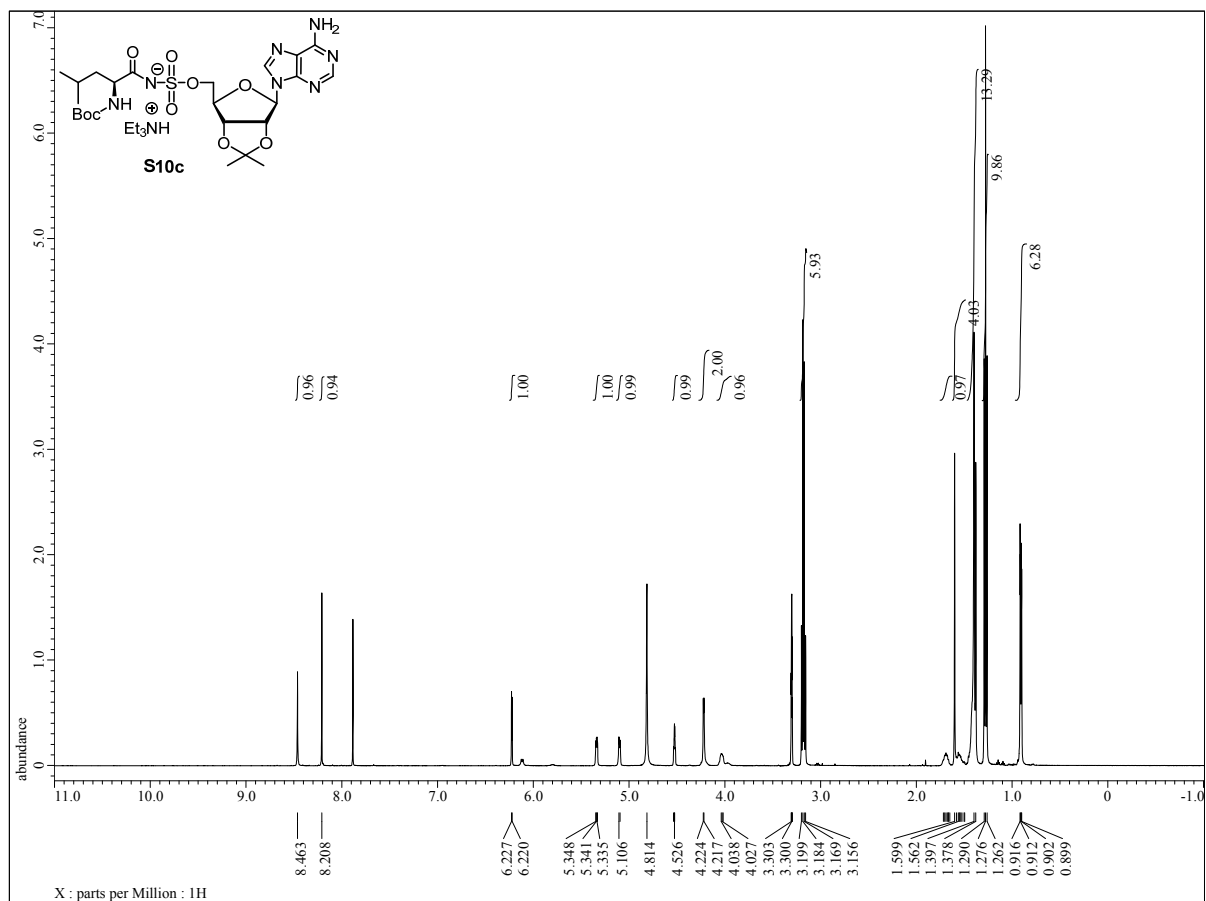
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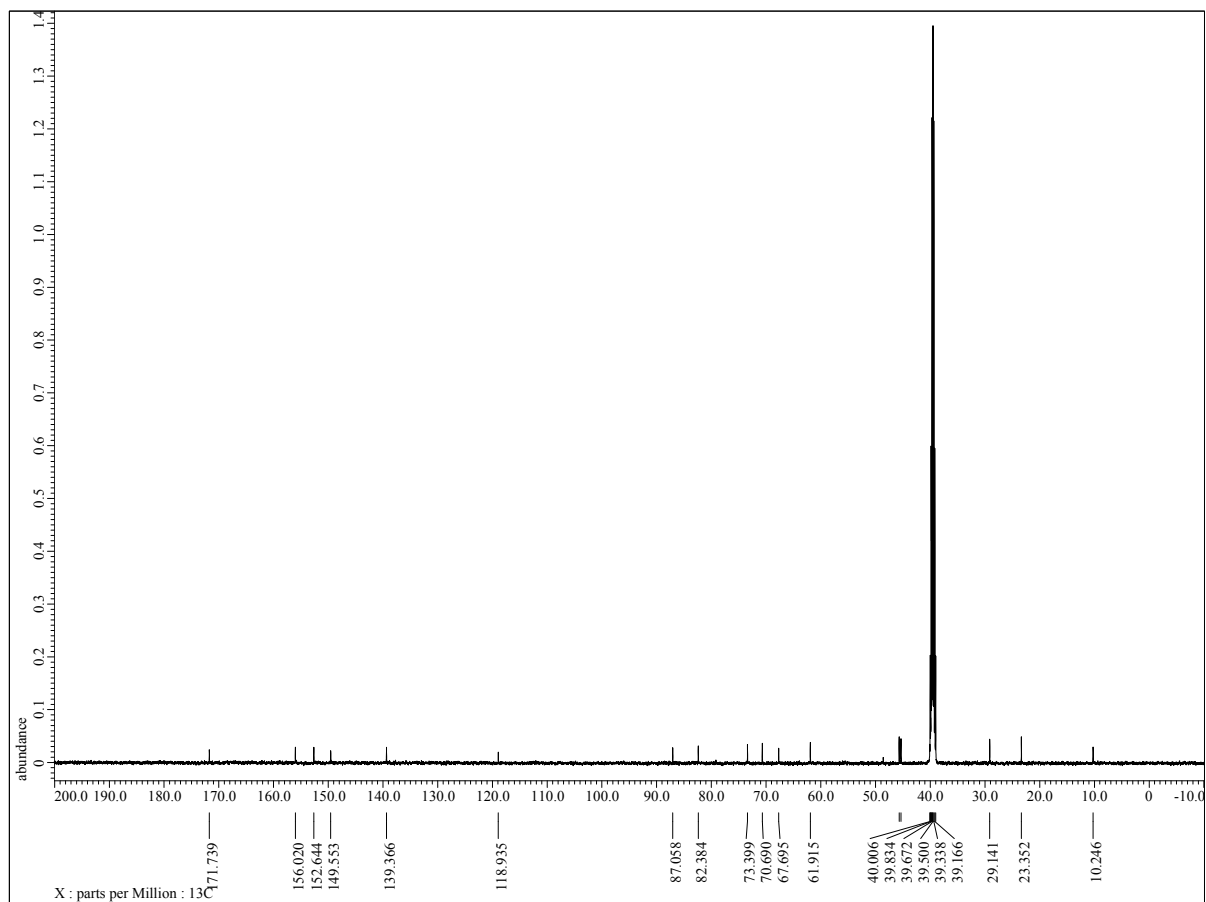
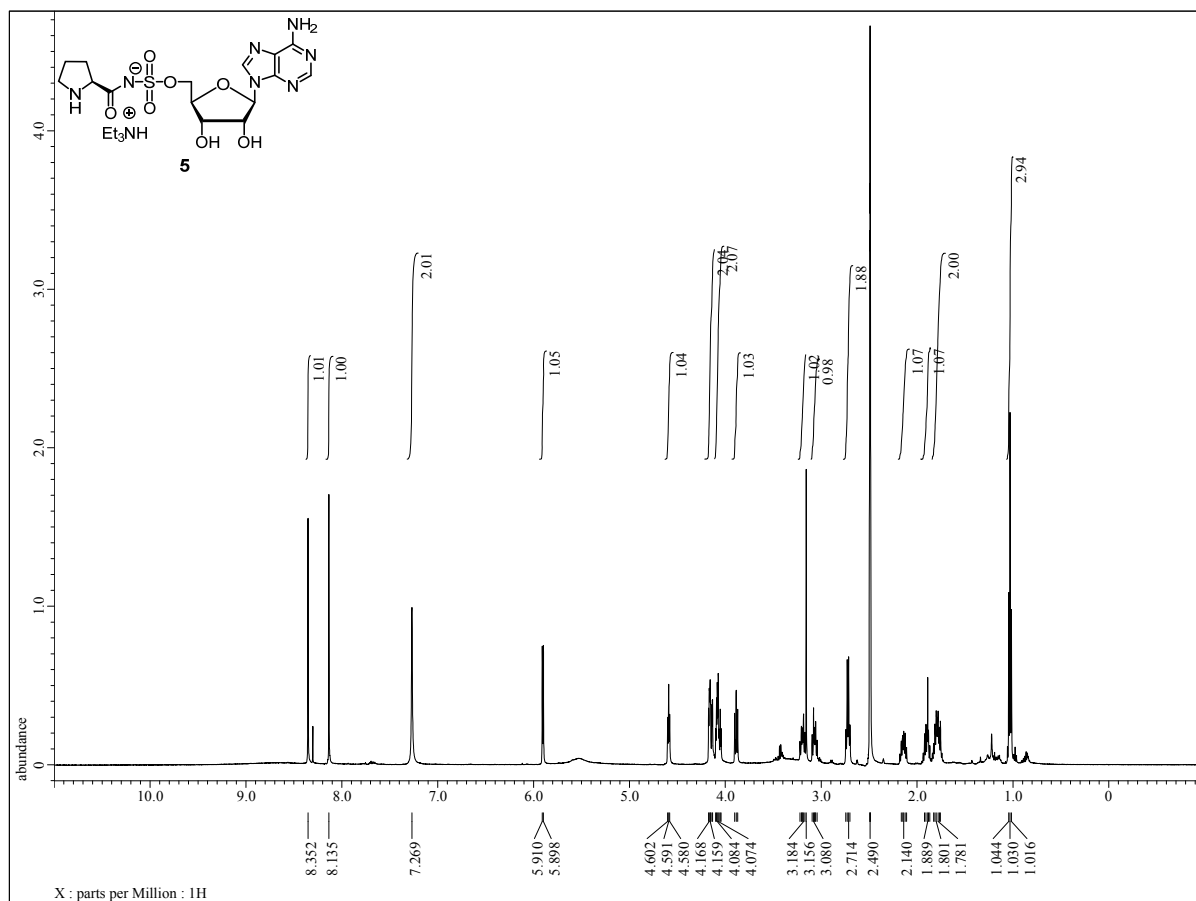
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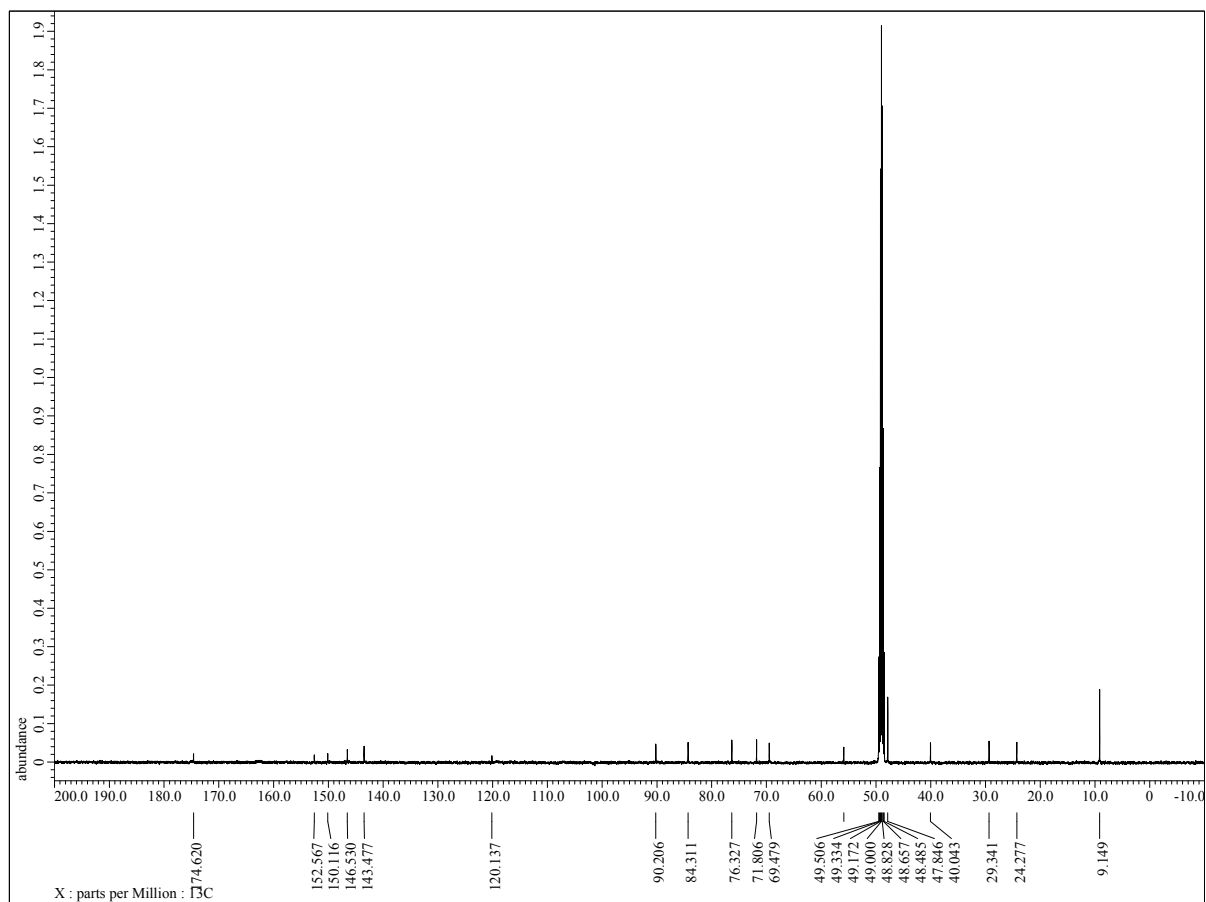
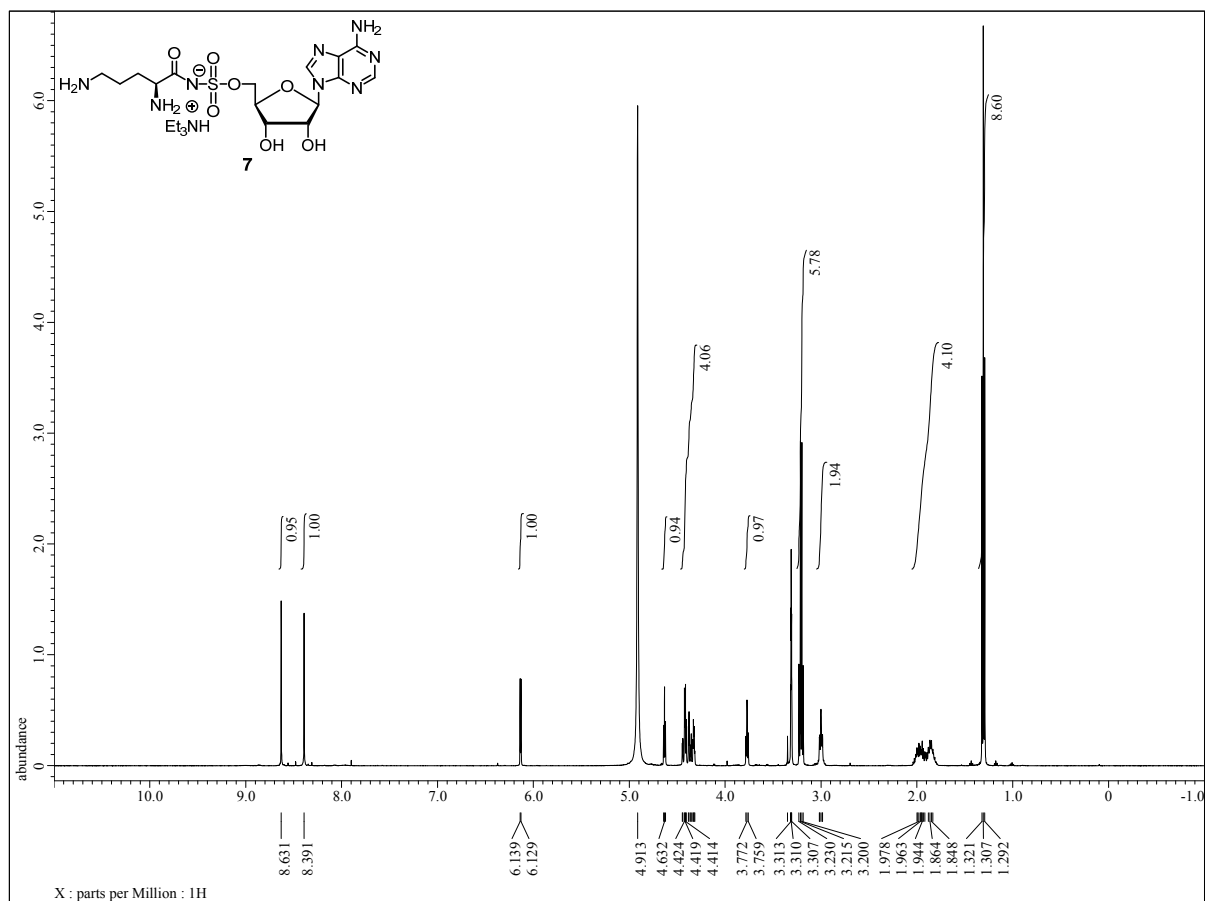
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^1H -NMR (500 MHz) and ^{13}C -NMR (125 MHz) spectra of **5** in $\text{DMSO-}d_6$



$^1\text{H-NMR}$ (500 MHz) and $^{13}\text{C-NMR}$ (125 MHz) spectra of **7** in CD_3OD



$^1\text{H-NMR}$ (500 MHz) and $^{13}\text{C-NMR}$ (125 MHz) spectra of **8** in CD_3OD

