Supporting Information

A chemical proteomic probe for detecting native carrier protein motifs in nonribosomal peptide synthetases

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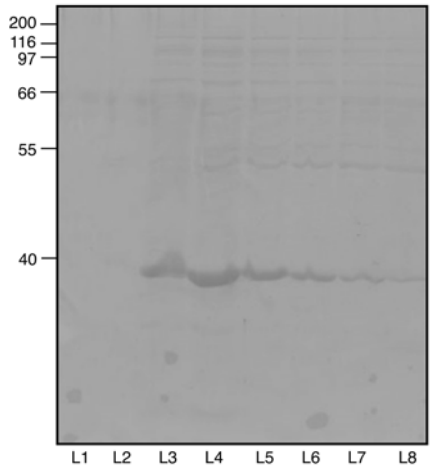


Figure S1. Full gel displaying the purification of native Sfp. Gel lanes depict fractions taken during anion exchange chromatography and as follows: L1 = 10 mM NaCl wash, L2 = 50 mM NaCl wash, L3–L8 = 100 mM NaCl wash. The target protein was collected and used from L3–L6. The gel was stained with Coomassie (Colloidal Coomassie Blue Stain).

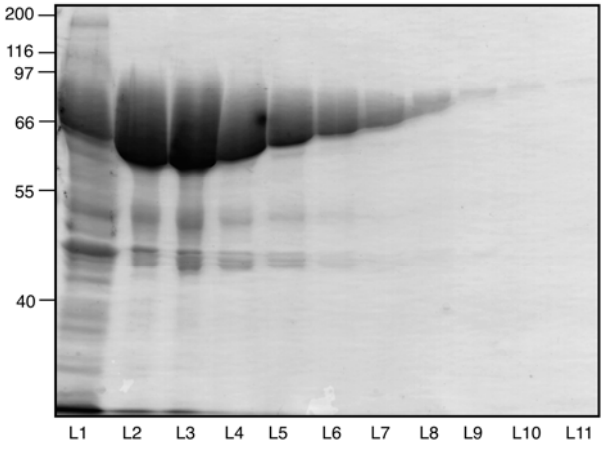


Figure S2. Full gel displaying the purification of CoaA (PanK)-MBP. Gel lanes depict fractions taken during amylose affinity chromatography and as follows: L1 = flow through and L2–L11 = 10 mM maltose wash. The target protein was collected and used from L2–L8. The gel was stained with Coomassie (Colloidal Coomassie Blue Stain).

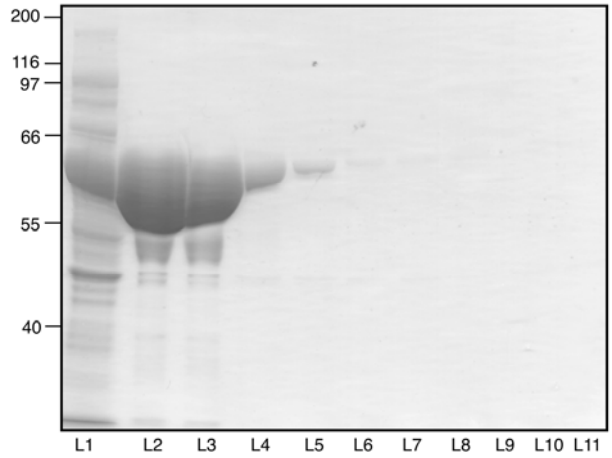


Figure S3. Full gel displaying the purification of CoaD (PPAT)-MBP. Gel lanes depict fractions taken during amylose affinity chromatography and as follows: L1 = flow through, L2–L11 = 10 mM maltose wash. The target protein was collected and used from L2–L5. The gel was stained with Coomassie (Colloidal Coomassie Blue Stain).

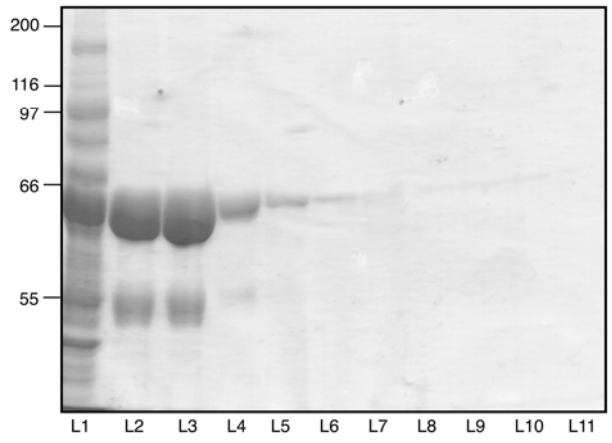


Figure S4. Full gel displaying the purification of CoaE (DPCK)-MBP. Gel lanes depict fractions taken during amylose affinity chromatography and as follows: L1 = flow through, L2–L11 = 10 mM maltose wash. The target protein was collected and used from L2–L6. The gel was stained with Coomassie (Colloidal Coomassie Blue Stain).

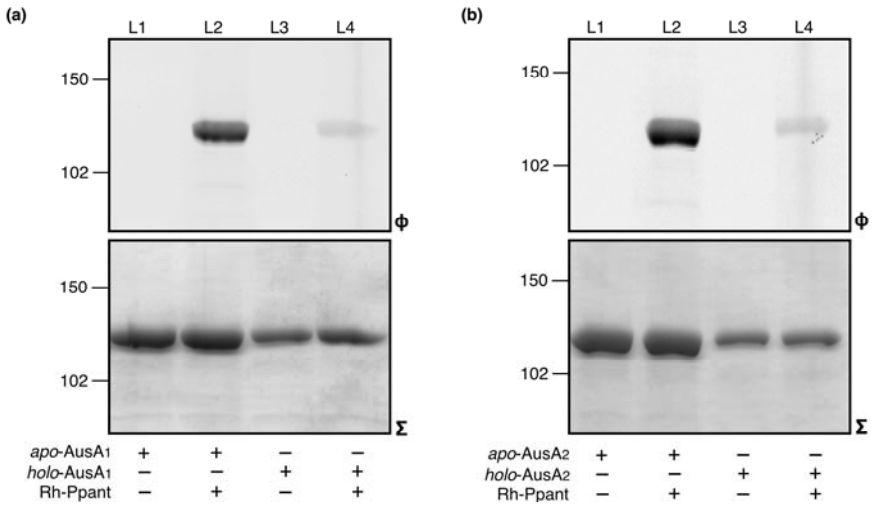


Figure S5. Verification of the high conversion from *apo*- to *holo*-CPs of AusA1 and AusA2 with the rhodamine (Rh) pantetheine analog S12 by a one-pot chemoenzymatic method. (a) Attachment of Rh-Ppant analog to *apo*- and *holo*-AusA1 using CoaA (PanK), CoaD (PPAT), CoaE (DPCK), and Sfp. Reagents and enzymes were added to 50-µL reaction mixtures in sequential order as follows. Final reaction concentrations: 50 mM potassium phosphate (pH 7.0), 8 mM ATP, 15 mM $MgCl_2$, 0.002 µg/µL CoaA-MBP, 0.01 µg/µL CoaD-MBP, 0.02 µg/µL CoaE-MBP, 0.01 µg/µL Sfp (native), 0.04 µg/µL *apo*- or *holo*-AusA1 (350 nM), and 500 µM Rh-Ppant analog. (b) Attachment of Rh-Ppant analog **S12** to *apo*- and *holo*-AusA2 using CoaA, CoaD, CoaE, and Sfp. Reagents and enzymes were added to 50-µL reaction mixtures in sequential order as follows. Final reaction concentrations: 50 mM potassium phosphate (pH 7.0), 8 mM ATP, 15 mM $MgCl_2$, 0.002 µg/µL CoaA-MBP, 0.01 µg/µL CoaD-MBP, 0.02 µg/µL CoaE-MBP, 0.01 µg/µL Sfp (native), 0.04 µg/µL *apo*- or *holo*-AusA2 (350 nM), and 500 µM Rh-Ppant analog **S12**. In negative controls, the Rh-Ppant analog **S12** was replaced with DMSO. These reaction mixtures were incubated for 1 h at 37 °C. For each panel, the image (Φ) denotes the fluorescence observed with λ_{ex} = 532 nm and λ_{ex} = 580 nm and the image (Σ) depicts total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

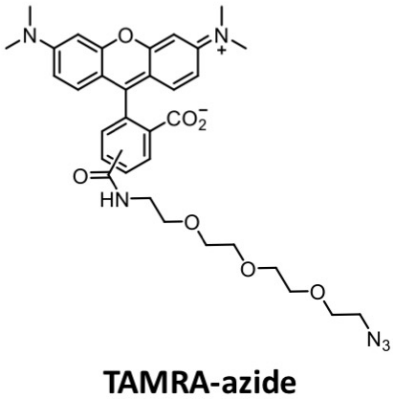


Figure S6. A Structure of TAMRA-azide detection reagent used in this study.

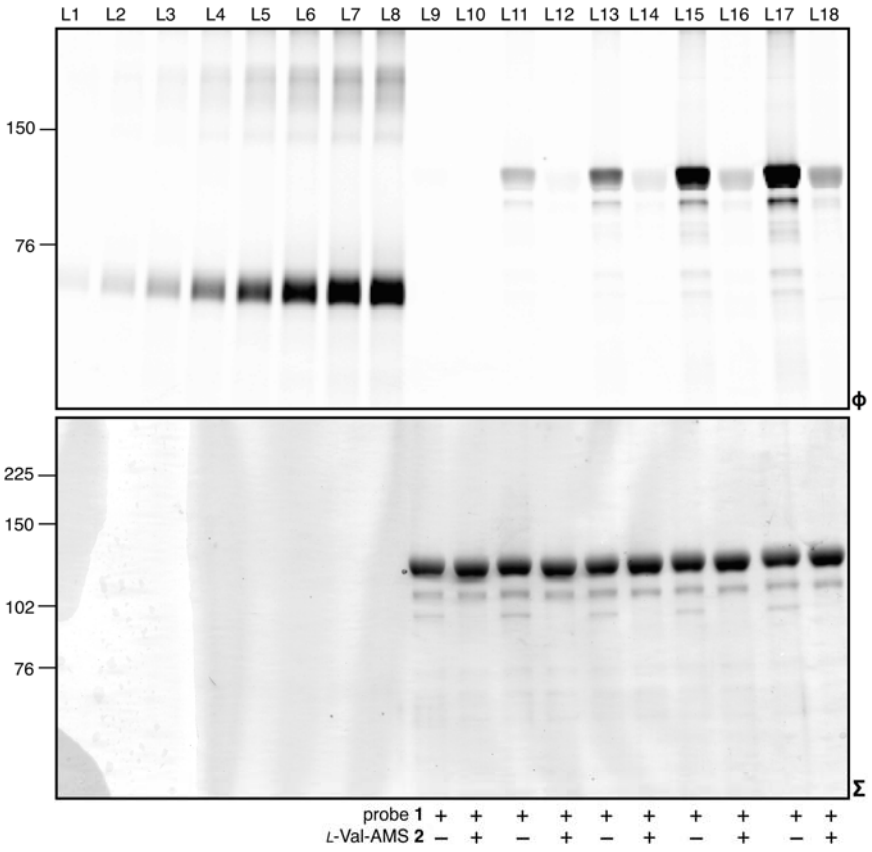
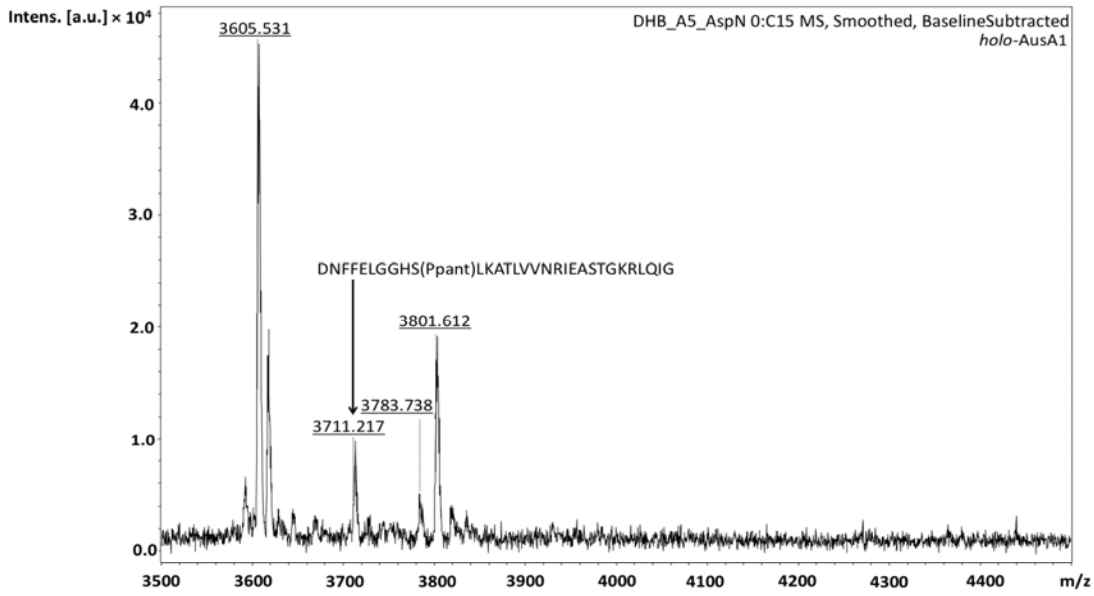
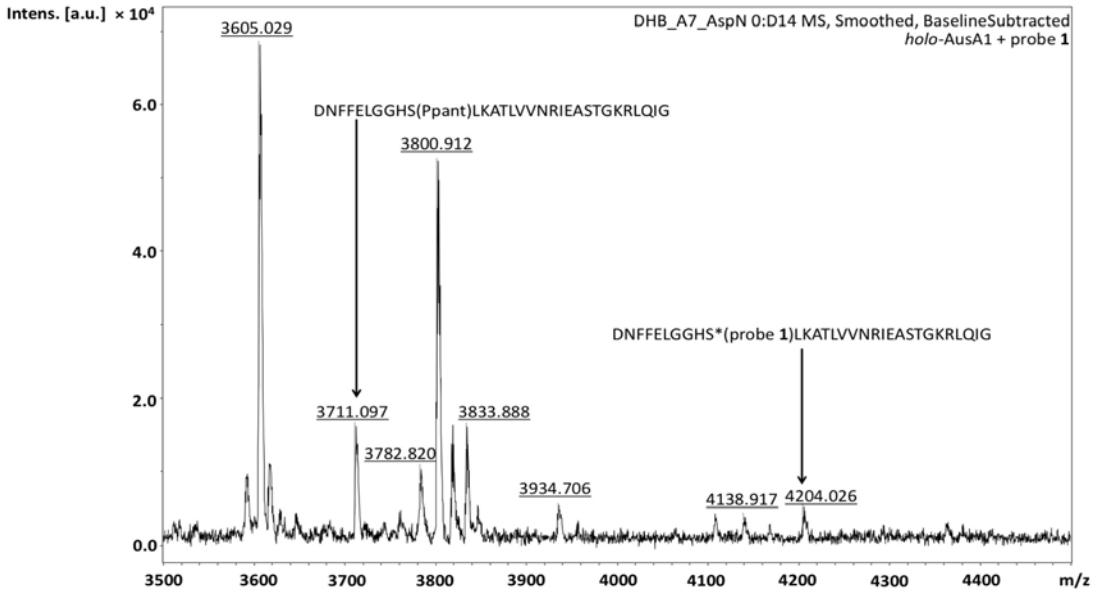


Figure S7. Measurements of labeled recombinant *holo*-AusA1. Fluorescent and Coomassie stained SDS-PAGE gel of Rh-BSA, a standard for fluorescent intensity and recombinant *holo*-AusA1 with probe **1**. Recombinant *holo*-AusA1 (1 μM) was reacted with probe **1** (1−100 μM) in either the absence or presence of 1 mM *L*-Val-AMS **2** for 12 h at 25 °C. Gel lanes are as follows: L1 = 50 nM Rh-BSA, L2 = 100 nM Rh-BSA, L3 = 200 nM Rh-BSA, L4 = 400 nM

Rh-BSA, L5 = 600 nM Rh-BSA, L6 = 800 nM Rh-BSA, L7 = 1000 nM Rh-BSA, L8 = 1200 nM Rh-BSA, L9 = 1 μM *holo*-AusA1 and 1 μM probe **1**, L10 = 1 μM *holo*-AusA1, 1 μM probe **1**, and 1 mM *L*-Val-AMS **2**, L11 = 1 μM *holo*-AusA1 and 10 μM probe **1**, L12 = 1 μM *holo*-AusA1, 10 μM probe **1**, and 1 mM *L*-Val-AMS **2**, L13 = 1 μM *holo*-AusA1 and 20 μM probe **1**, L14 = 1 μM *holo*-AusA1, 20 μM probe **1**, and 1 mM *L*-Val-AMS **2**, L15 = 1 μM *holo*-AusA1 and 50 μM probe **1**, L16 = 1 μM *holo*-AusA1, 50 μM probe **1**, and 1 mM *L*-Val-AMS **2**, L17 = 1 μM *holo*-AusA1 and 100 μM probe **1**, L18 = 1 μM *holo*-AusA1, 100 μM probe **1**, and 1 mM *L*-Val-AMS **2**. The image (Φ) denotes the fluorescence observed with λ_{ex} = 532 nm and λ_{ex} = 580 nm and the image (Σ) depicts total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain).

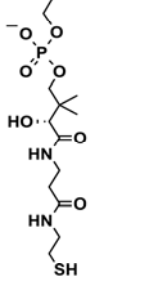
(a)





(b)

DNFFELGGHS(Ppant)LKATLVVNRIEASTGKRLQIG



DNFFELGGHS*(probe **1**)LKATLVVNRIEASTGKRLQIG

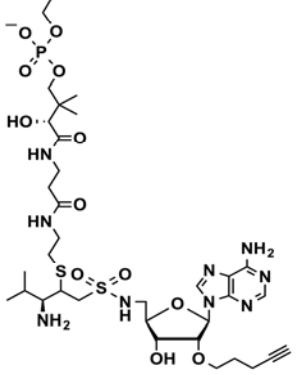


Figure S8. MALDI-TOF mass spectra of Asp-N digestion of modified AusA1. (a) The unmodified *holo*-AusA1 fragment with sequence DNFFELGGHS(Ppant)LKATLVVNRIEASTGKRLQIG (D932-G962 + Ppant): [D932-G962 + Ppant + H]$^+$ calcd for 3710.9088, found, 3711.097; modified peptide fragment with sequence DNFFELGGHS*LKATLVVNRIEASTGKRLQIG (D932-G962 + Ppant + **1**): [D932-G962 + Ppant + **1** + H]$^+$ calcd for 4204.1195, found, 4204.026. In addition, these unidentified peaks show background peaks. (b) Structures of the unmodified and mofified peptide fragments.

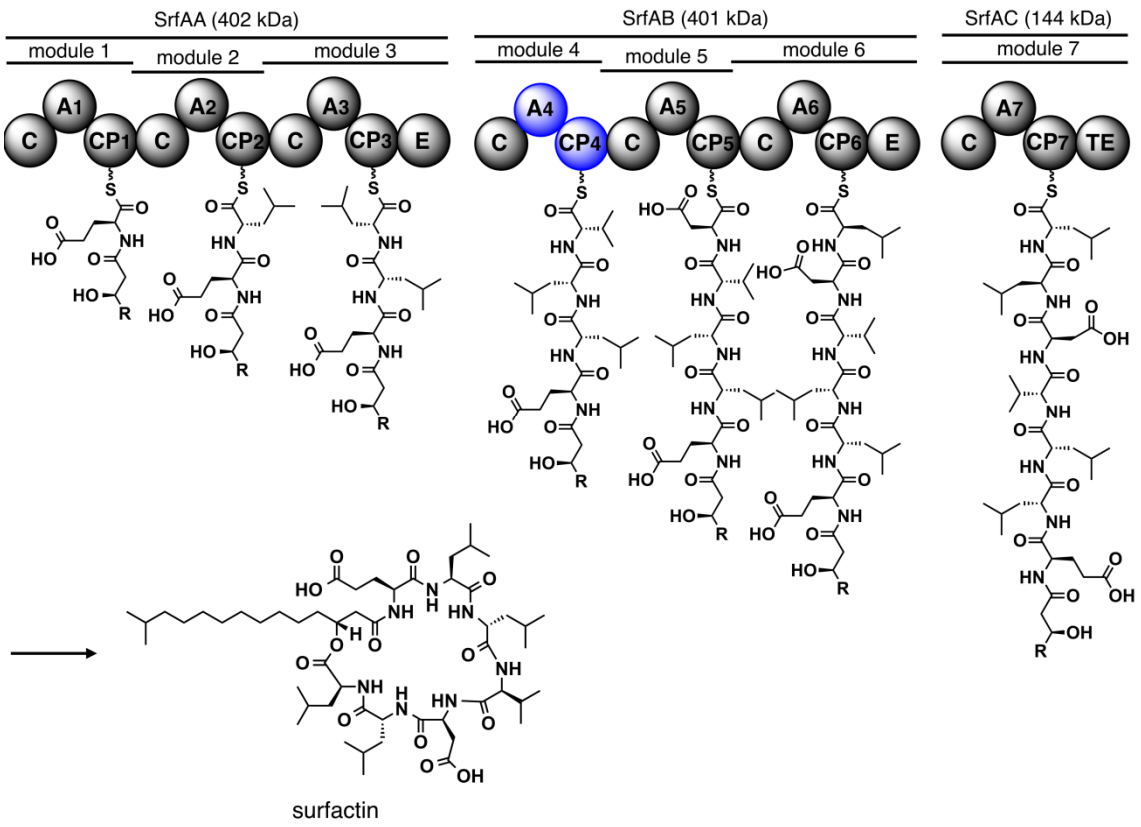


Figure S9. Nonribosomal synthesis of the surfactin. Modules are comprised of CP (CP1–CP7), A (A1–A7) [A1, *L*-Glu; A2, *L*-Leu; A3, *L*-Leu; A4, *L*-Val; A5, *L*-Asp; A6, *L*-Leu; A7, *L*-Leu specific A-domains], E, C, and TE domains. The target A and CP domains for probe **1** are colored blue.

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   1 MSKKSIQKVY ALTPMQEGML YHAMLDPHSS SYFTQLELGI HGAFDLEIFE
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 101 KEHIERYKQD VQRQGFNLAK DILFKVAVFR LAADQLYLVW SNHHIMMDGW
 151 SMGVLMKSLF QNYEALRAGR TPANGQGKPY SDYIKWLGKQ DNEEAESYWS
 201 ERLAGFEQPS VLPGRLPVKK DEYVNKEYSF TWDETLVARI QQTANLHQVT
 251 GPNLFQAVWG IVLSKYNFTD DVIFGTVVSG RPSEINGIET MAGLFINTIP
 301 VRVKVERDAA FADIFTAVQQ HAVEAERYDY VPLYEIQKRS ALDGNLLNHL
 351 VAFENYPLDQ ELENGSMEDR LGFSIKVESA FEQTSFDFNL IVYPGKTWTV
 401 KIKYNGAAFD SAFIERTAEH LTRMMEAAVD QPAAFVREYG LVGDEEQRQI
 451 VEVFNSTKAE LPEGMAVHQV FEEQAKRTPA STAVVYEGTK LTYRELNAAA
 501 NRLARKLVEH GLQKGETAAI MNDRSVETVV GMLAVLKAGA AYVPLDPALP
 551 GDRLRFMAED SSVRMVLIGN SYTGQAHQLQ VPVLTLDIGF EESEAADNLN
 601 LPSAPSDLAY IMYTSGSTGK PKGVMIEHKS ILRLVKNAGY VPVTEEDRMA
 651 QTGAVSFDAG TFEVFGALLN GAALYPVKKE TLLDAKQFAA FLREQSITTM
 701 WLTSPLFNQL AAKDAGMFGT LRHLIIGGDA LVPHIVSKVK QASPSLSLWN
 751 GYGPTENTTF STSFLIDREY GGSIPIGKPI GNSTAYIMDE QQCLQPIGAP
 801 GELCVGGIGV ARGYVNLPEL TEKQFLEDPF RPGERIYRTG DLARWLPDGN
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1051 QASYQQHYPV SPAQRRMYIL NQLGQANTSY NVPAVLLLEG EVDKDRLENA
1101 IQQLINRHEI LRTSFDMIDG EVVQTVHKNI SFQLEAAKGR EEDAEEIIKA
1151 FVQPFELNRA PLVRSKLVQL EEKRHLLLID MHHIITDGSS TGILIGDLAK
1201 IYQGADLELP QIHYKDYAVW HKEQTNYQKD EEYWLDVFKG ELPILDLPAD
1251 FERPAERSFA GERVMFGLDK QITAQIKSLM AETDTTMYMF LLAAFNVLLS
1301 KYASQDDIIV GSPTAGRTHP DLQGVPGMFV NTVALRTAPA GDKTFAQFLE
1351 EVKTASLQAF EHQSYPLEEL IEKLPLTRDT SRSPLFSVMF NMQNMEIPSL
1401 RLGDLKISSY SMLHHVAKFD LSLEAVEREE DIGLSFDYAT ALFKDETIRR
1451 WSRHFVNIIK AAAANPNVRL SDVDLLSSAE TAALLEERHM TQITEATFAA
1501 LFEKQAQQTP DHSAVKAGGN LLTYRELDEQ ANQLAHHLRA QGAGNEDIVA
1551 IVMDRSAEVM VSILGVMKAG AAFLPIDPDT PEERIRYSLE DSGAKFAVVN
1601 ERNMTAIGQY EGIIVSLDDG KWRNESKERP SSISGSRNLA YVIYTSGTTG
1651 KPKGVQIEHR NLTNYVSWFS EEAGLTENDK TVLLSSYAFD LGYTSMFPVL
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1851 ARGYLNRPQL TAERFVENPY SPGSLMYKTG DVVRRLSDGT LAFIGRADDQ
1901 VKIRGYRIEP KEIETVMLSL SGIQEAVVLA VSEGGLQELC AYYTSDQDIE
1951 KAELRYQLSL TLPSHMIPAF FVQVDAIPLT ANGKTDRNAL PKPNAAQSGG
2001 KALAAPETAL EESLCRIWQK TLGIEAIGID DNFFDLGGHS LKGMMLIANI
2051 QAELEKSVPL KALFEQPTVR QLAAYMEASA VSGGHQVLKP ADKQDMYPLS
2101 SAQKRMYVLN QLDRQTISYN MPSVLLMEGE LDISRLRDSL NQLVNRHESL
2151 RTSFMEANGE PVQRIIEKAE VDLHVFEAKE DEADQKIKEF IRPFDLNDAP
2201 LIRAALLRIE AKKHLLLLDM HHIIADGVSR GIFVKELALL YKGEQLPEPT
2251 LHYKDFAVWQ NEAEQKERMK EHEAYWMSVL SGELPELDLP LDYARPPVQS
2301 FKGDTIRFRT GSETAKAVEK LLAETGTTLH MVLHAVFHVF LSKISGQRDI
2351 VIGSVTAGRT NADVQDMPGM FVNTLALRME AKEQQTFAEL LELAKQTNLS
2401 ALEHQEYPFE DLVNQLDLPR DMSRNPLFNV MVTTENPDKE QLTLQNLSIS
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2651 PDSYTGDVIL LDGSRTILSL PLDENDEGNP ETAVTAENLA YMIYTSGTTG
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2801 RVLLTGGDKL KRAVKKPYTL VNNYGPTENT VVATSAEIHP EEGSLSIGRA
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2951 SEVQDAAVTA VKDKGGNTAI AAYVTPETAD IEALKSTLKE TLPDYMIPAF
3001 WVTLNELPVT ANGKVDRKAL PEPDIEAGSG EYKAPTTDME ELLAGIWQDV
3051 LGMSEVGVTD NFFSLGGDSI KGIQMASRLN QHGWKLEMKD LFQHPTIEEL
3101 TQYVERAEGK QADQGPVEGE VILTPIQRWF FEKNFTNKHH WNQSVMLHAK
3151 KGFDPERVEK TLQALIEHHD ALRMVYREEN GDIVQVYKPI GESKVSFEIV
3201 DLYGSDEEML RSQIKLLANK LQSSLDLRNG PLLKAEQYRT EAGDHLLIAV
3251 HHLVVDGVSW RILLEDFASG YMQAEKEESL VFPQKTNSFK DWAEELAAFS
3301 QSAHLLQQAE YWSQIAAEQV SPLPKDCETE QRIVKDTSSV LCELTAEDTK
3351 HLLTDVHQPY GTEINDILLS ALGLTMKEWT KGAKIGINLE GHGREDIIPN
3401 VNISRTVGWF TAQYPVVLDI SDADASAVIK TVKENLRRIP DKGVGYGILR
3451 YFTETAETKG FTPEISFNYL GQFDSEVKTD FFEPSAFDMG RQVSGESEAL
3501 YALSFSGMIR NGRFVLSCSY NEKEFERATV EEQMERFKEN LLMLIRHCTE
3551 KEDKEFTPSD FSAEDLEMDE MGDIFDMLEE NLK
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Figure S10. Compiled MS/MS data of gel excised fluorescent SrfAB (##) bands from the *B. subtilis* ATCC 21332 proteome. Amino acids of the identified peptides are colored either red or blue. The overlapped peptide sequences are colored green.

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   1 MEITFYPLTD AQKRIWYTEK FYPHTSISNL AGIGKLVSAD AIDYVLVEQA
  51 IQEFIRRNDA MRLRLRLDEN GEPVQYISEY RPVDIKHTDT TEDPNAIEFI
 101 SQWSREETKK PLPLYDCDLF RFSLFTIKEN EVWFYANVHH VISDGISMNI
 151 LGNAIMHIYL ELASGSETKE GISHSFIDHV LSEQEYAQSK RFEKDKAFWN
 201 KQFESVPELV SLKRNASAGG SLDAERFSKD VPEALHQQIL SFCEANKVSV
 251 LSVFQSLLAA YLYRVSGQND VVTGTFMGNR TNAKEKQMLG MFVSTVPLRT
 301 NIDGGQAFSE FVKDRMKDLM KTLRHQKYPY NLLINDLRET KSSLTKLFTV
 351 SLEYQVMQWQ KEEDLAFLTE PIFSGSGLND VSIHVKDRWD TGKLTIDFDY
 401 RTDLFSREEI NMICERMITM LENALTHPEH TIDELTLISD AEKEKLLARA
 451 GGKSVSYRKD MTIPELFQEK AELLSDHPAV VFEDRTLSYR TLHEQSARIA
 501 NVLKQKGVGP DSPVAVLIER SERMITAIMG ILKAGGAYVP IDPGFPAERI
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 601 LAYIIYTSGT TGRPKGVMIE HRQVHHLVES LQQTIYQSGS QTLRMALLAP
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1051 AVIEPAEKQE HYPLSLAQQR TYIVSQFEDA GVGYNMPAAA ILEGPLDIQK
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1151 IMASFVRTFD LAKAPLFRIG LLGLEENRHM LLFDMHHLIS DGVSIGIMLE
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1251 LQLLSDYPRP PVQSFEGDRV SIKLDAGVKD RLNRLAEQNG ATLYMVMLSA
1301 YYTLLSKYTG QDDIIVGTPS AGRNHSDTEG IIGMFVNTLA IRSEVKQNET
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1901 IDHQVKIRGQ RIELGEIEHQ LQTHDRVQES VVLAVDQGAG DKLLCAYYVG
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2001 NVSRGVSYVA PRNGTEQKVA DIWAQVLQAE QVGAYDHFFD IGGHSLAGMK
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2701 GTTGQPKGVM VEHHALVNLC FWHHDAFSMT AEDRSAKYAG FGFDASIWEM
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2851 IGRAIANTRV YILGEGNQVQ PEGVAGELCV AGRGLARGYL NREDETAKRF
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3001 IPAFWVTLNE LPVTANGKVD RKALPEPDIE AGSGEYKAPT TDMEELLAGI
3051 WQDVLGMSEV GVTDNFFSLG GDSIKGIQMA SRLNQHGWKL EMKDLFQHPT
3101 IEELTQYVER AEGKQADQGP VEGEVILTPI QRWFFEKNFT NKHHWNQSVM
3151 LHAKKGFDPE RVEKTLQALI EHHDALRMVY REGQEDVIQY NRGLEAASAQ
3201 LEVIQIEGQA ADYEDRIERE AERLQSSIDL QEGGLLKAGL FQAEDGDHLL
3251 LAIHHLVVDG VSWRILLEDF AAVYTQLEQG NEPVLPQKTH SFAEYAERLQ
3301 DFANSKAFLK EKEYWRQLEE QAVAAKLPKD RESGDQRMKH TKTIEFSLTA
3351 EETEQLTTKV HEAYHTEMND ILLTAFGLAM KEWTGQDRVS VHLEGHGREE
3401 IIEDLTISRT VGWFTSMYPM VLDMKHADDL GYQLKQMKED IRHVPNKGVG
3451 YGILRYLTAP EHKEDVAFSI QPDVSFNYLG QFDEMSDAGL FTRSELPSGQ
3501 SLSPETEKPN ALDVVGYIEN GKLTMSLAYH SLEFHEKTVQ TFSDSFKAHL
3551 LRIIEHCLSQ DGTELTPSDL GDDDLTLDEL DKLMEIF
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Figure S11. Compiled MS/MS data of gel excised SrfAA (#) bands from the *B. subtilis* ATCC 21332 proteome. Amino acids of the identified peptides are colored either red or blue. The overlapped peptide sequences are colored green.

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   1 MSKKSIQKVY ALTPMQEGML YHAMLDPHSS SYFTQLELGI HGAFDLEIFE
  51 KSVNELIRSY DILRTVFVHQ QLQKPRQVVL AERKTKVHYE DISHADENRQ
 101 KEHIERYKQD VQRQGFNLAK DILFKVAVFR LAADQLYLVW SNHHIMMDGW
 151 SMGVLMKSLF QNYEALRAGR TPANGQGKPY SDYIKWLGKQ DNEEAESYWS
 201 ERLAGFEQPS VLPGRLPVKK DEYVNKEYSF TWDETLVARI QQTANLHQVT
 251 GPNLFQAVWG IVLSKYNFTD DVIFGTVVSG RPSEINGIET MAGLFINTIP
 301 VRVKVERDAA FADIFTAVQQ HAVEAERYDY VPLYEIQKRS ALDGNLLNHL
 351 VAFENYPLDQ ELENGSMEDR LGFSIKVESA FEQTSFDFNL IVYPGKTWTV
 401 KIKYNGAAFD SAFIERTAEH LTRMMEAAVD QPAAFVREYG LVGDEEQRQI
 451 VEVFNSTKAE LPEGMAVHQV FEEQAKRTPA STAVVYEGTK LTYRELNAAA
 501 NRLARKLVEH GLQKGETAAI MNDRSVETVV GMLAVLKAGA AYVPLDPALP
 551 GDRLRFMAED SSVRMVLIGN SYTGQAHQLQ VPVLTLDIGF EESEAADNLN
 601 LPSAPSDLAY IMYTSGSTGK PKGVMIEHKS ILRLVKNAGY VPVTEEDRMA
 651 QTGAVSFDAG TFEVFGALLN GAALYPVKKE TLLDAKQFAA FLREQSITTM
 701 WLTSPLFNQL AAKDAGMFGT LRHLIIGGDA LVPHIVSKVK QASPSLSLWN
 751 GYGPTENTTF STSFLIDREY GGSIPIGKPI GNSTAYIMDE QQCLQPIGAP
 801 GELCVGGIGV ARGYVNLPEL TEKQFLEDPF RPGERIYRTG DLARWLPDGN
 851 IEFLGRIDNQ VKVRGFRIEL GEIETKLNMA EHVTEAAVII RKNKADENEI
 901 CAYFTADREV AVSELRKTLS QSLPDYMVPA HLIQMDSLPL TPNGKINKKE
 951 LPAPQSEAVQ PEYAAPKTES EKKLAEIWEG ILGVKAGVTD NFFMIGGHSL
1001 KAMMMTAKIQ EHFHKEVPIK VLFEKPTIQE LALYLEENES KEEQTFEPIR
1051 QASYQQHYPV SPAQRRMYIL NQLGQANTSY NVPAVLLLEG EVDKDRLENA
1101 IQQLINRHEI LRTSFDMIDG EVVQTVHKNI SFQLEAAKGR EEDAEEIIKA
1151 FVQPFELNRA PLVRSKLVQL EEKRHLLLID MHHIITDGSS TGILIGDLAK
1201 IYQGADLELP QIHYKDYAVW HKEQTNYQKD EEYWLDVFKG ELPILDLPAD
1251 FERPAERSFA GERVMFGLDK QITAQIKSLM AETDTTMYMF LLAAFNVLLS
1301 KYASQDDIIV GSPTAGRTHP DLQGVPGMFV NTVALRTAPA GDKTFAQFLE
1351 EVKTASLQAF EHQSYPLEEL IEKLPLTRDT SRSPLFSVMF NMQNMEIPSL
1401 RLGDLKISSY SMLHHVAKFD LSLEAVEREE DIGLSFDYAT ALFKDETIRR
1451 WSRHFVNIIK AAAANPNVRL SDVDLLSSAE TAALLEERHM TQITEATFAA
1501 LFEKQAQQTP DHSAVKAGGN LLTYRELDEQ ANQLAHHLRA QGAGNEDIVA
1551 IVMDRSAEVM VSILGVMKAG AAFLPIDPDT PEERIRYSLE DSGAKFAVVN
1601 ERNMTAIGQY EGIIVSLDDG KWRNESKERP SSISGSRNLA YVIYTSGTTG
1651 KPKGVQIEHR NLTNYVSWFS EEAGLTENDK TVLLSSYAFD LGYTSMFPVL
1701 LGGGELHIVQ KETYTAPDEI AHYIKEHGIT YIKLTPSLFH TIVNTASFAK
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1751 DANFESLRLI VLGGEKIIPT DVIAFRKMYG HTEFINHYGP TEATIGAIAG
1801 RVDLYEPDAF AKRPTIGRPI ANAGALVLNE ALKLVPPGAS GQLYITGQGL
1851 ARGYLNRPQL TAERFVENPY SPGSLMYKTG DVVRRLSDGT LAFIGRADDQ
1901 VKIRGYRIEP KEIETVMLSL SGIQEAVVLA VSEGGLQELC AYYTSDQDIE
1951 KAELRYQLSL TLPSHMIPAF FVQVDAIPLT ANGKTDRNAL PKPNAAQSGG
2001 KALAAPETAL EESLCRIWQK TLGIEAIGID DNFFDLGGHS LKGMMLIANI
2051 QAELEKSVPL KALFEQPTVR QLAAYMEASA VSGGHQVLKP ADKQDMYPLS
2101 SAQKRMYVLN QLDRQTISYN MPSVLLMEGE LDISRLRDSL NQLVNRHESL
2151 RTSFMEANGE PVQRIIEKAE VDLHVFEAKE DEADQKIKEF IRPFDLNDAP
2201 LIRAALLRIE AKKHLLLLDM HHIIADGVSR GIFVKELALL YKGEQLPEPT
2251 LHYKDFAVWQ NEAEQKERMK EHEAYWMSVL SGELPELDLP LDYARPPVQS
2301 FKGDTIRFRT GSETAKAVEK LLAETGTTLH MVLHAVFHVF LSKISGQRDI
2351 VIGSVTAGRT NADVQDMPGM FVNTLALRME AKEQQTFAEL LELAKQTNLS
2401 ALEHQEYPFE DLVNQLDLPR DMSRNPLFNV MVTTENPDKE QLTLQNLSIS
2451 PYEAHQGTSK FDLTLGGFTD ENGIGLQLEY ATDLFAKETA EKWSEYVLRL
2501 LKAVADNPNQ PLSSLLLVTE TEKQALLEAW KGKALPVPTD KTVHQLFEET
2551 VQRHKDRPAV TYNGQSWTYG ELNAKANRLA RILMDCGISP DDRVGVLTKP
2601 SLEMSAAVLG VLKAGAAFVP IDPDYPDQRI EYILQDSGAK LLLKQEGISV
2651 PDSYTGDVIL LDGSRTILSL PLDENDEGNP ETAVTAENLA YMIYTSGTTG
2701 QPKGVMVEHH ALVNLCFWHH DAFSMTAEDR SAKYAGFGFD ASIWEMFPTW
2751 TIGAELHVID EAIRLDIVRL NDYFETNGVT ITFLPTQLAE QFMELENTSL
2801 RVLLTGGDKL KRAVKKPYTL VNNYGPTENT VVATSAEIHP EEGSLSIGRA
2851 IANTRVYILG EGNQVQPEGV AGELCVAGRG LARGYLNRED ETAKRFVADP
2901 FVPGERMYRT GDLVKWVNGG IEYIGRIDQQ VKVRGYRIEL SEIEVQLAQL
2951 SEVQDAAVTA VKDKGGNTAI AAYVTPETAD IEALKSTLKE TLPDYMIPAF
3001 WVTLNELPVT ANGKVDRKAL PEPDIEAGSG EYKAPTTDME ELLAGIWQDV
3051 LGMSEVGVTD NFFSLGGDSI KGIQMASRLN QHGWKLEMKD LFQHPTIEEL
3101 TQYVERAEGK QADQGPVEGE VILTPIQRWF FEKNFTNKHH WNQSVMLHAK
3151 KGFDPERVEK TLQALIEHHD ALRMVYREEN GDIVQVYKPI GESKVSFEIV
3201 DLYGSDEEML RSQIKLLANK LQSSLDLRNG PLLKAEQYRT EAGDHLLIAV
3251 HHLVVDGVSW RILLEDFASG YMQAEKEESL VFPQKTNSFK DWAEELAAFS
3301 QSAHLLQQAE YWSQIAAEQV SPLPKDCETE QRIVKDTSSV LCELTAEDTK
3351 HLLTDVHQPY GTEINDILLS ALGLTMKEWT KGAKIGINLE GHGREDIIPN
3401 VNISRTVGWF TAQYPVVLDI SDADASAVIK TVKENLRRIP DKGVGYGILR
3451 YFTETAETKG FTPEISFNYL GQFDSEVKTD FFEPSAFDMG RQVSGESEAL
3501 YALSFSGMIR NGRFVLSCSY NEKEFERATV EEQMERFKEN LLMLIRHCTE
3551 KEDKEFTPSD FSAEDLEMDE MGDIFDMLEE NLK
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Figure S12. Compiled MS/MS data of gel excised fluorescent protein (###, SrfAB) bands from the *B. subtilis* ATCC 21332 proteome. Amino acids of the identified peptides are colored either red or blue. The overlapped peptide sequences are colored green.

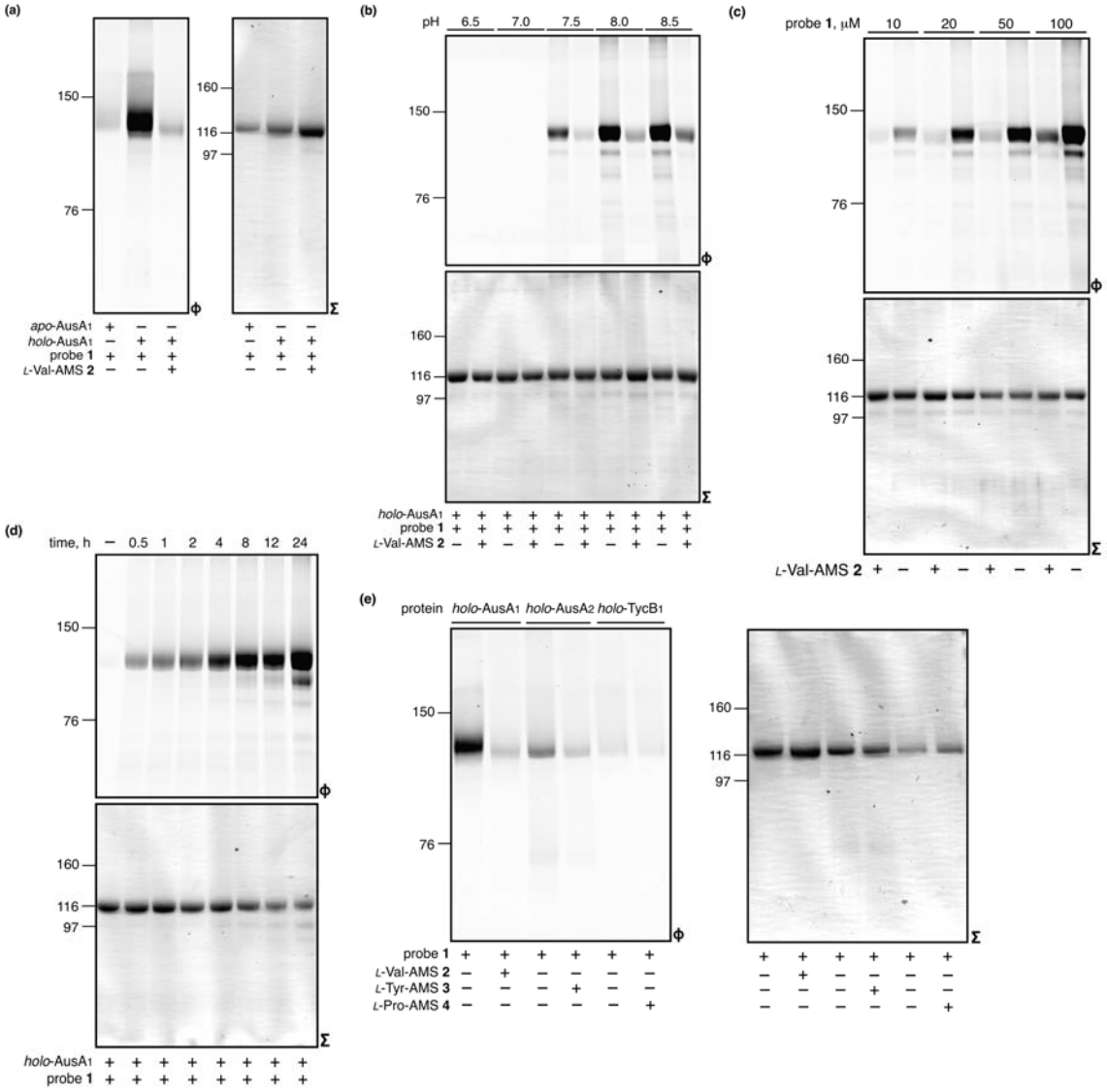


Figure S13. Full images of SDS-PAGE gels from Figure 3. Labeling of recombinant *holo*-AusA1 with probe **1**. (a) SDS-PAGE analysis depicting the labeling of 350 nM *apo*- and *holo*-AusA1 in 20 mM Tris (pH 8.0) for 12 h at 25 °C in either the absence, presence, of 1 mM *L*-Val-AMS **2**. (b) Determination of an optimal pH for the labeling of *holo*-AusA1. *holo*-AusA1 (350 nM) was incubated with 100 μM probe **1** in 20 mM Tris (pH 6.5–8.5) for 12 h at 25 °C in either the absence, or presence, of 1 mM **2**. (c) Concentration dependence of *in vitro* probe labeling of *holo*-AusA1. *holo*-AusA1 (350 nM) was reacted with 10–100 μM probe **1** for 12 h at

25 °C in either the absence, or presence, of 1 mM **2**. (d) Time-course study of the labeling of *holo*-AusA1 with probe **1**. SDS-PAGE analysis denoting the labeling of 350 nM *holo*-AusA1 in 20 mM Tris (pH 8.0) for 0.5–12 h at 25 °C with 100 μM probe **1**. (e) Labeling specificity of probe **1**. *holo*-AusA1 (350 nM), *holo*-AusA2 (350 nM), and *holo*-TycB1 (350 nM) were treated with 100 μM probe **1** in either the absence, or presence, of 1 mM of inhibitors **2**, **3**, and **4**. For each panel, the image (Φ) denotes the fluorescence observed with λ_{ex} = 532 nm and λ_{ex} = 580 nm and the image (Σ) depicts total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain). (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.

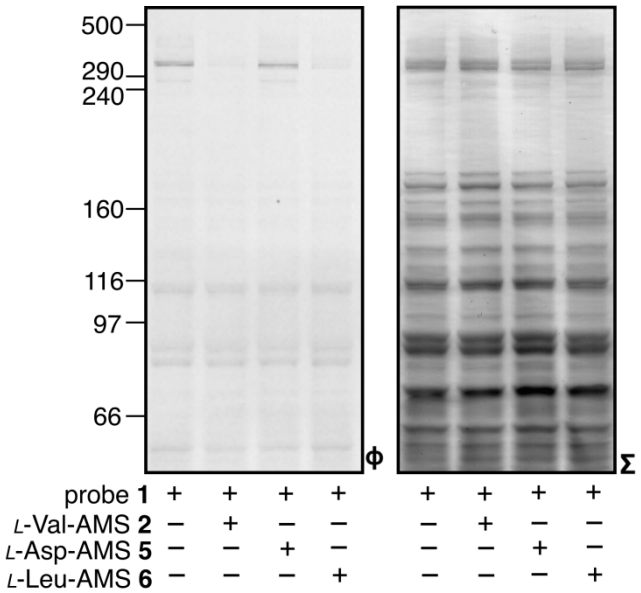
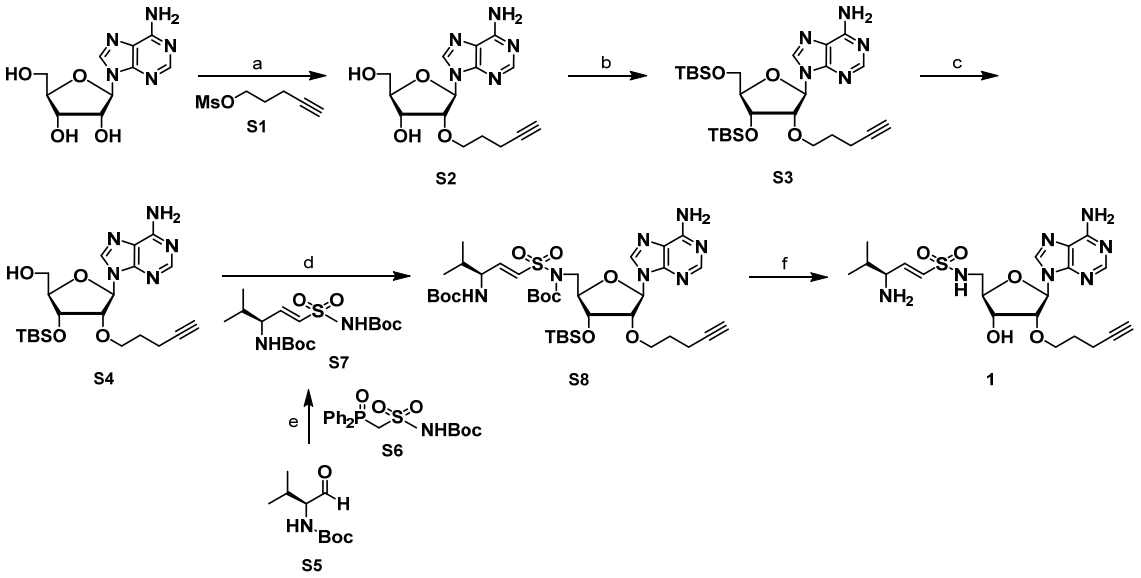
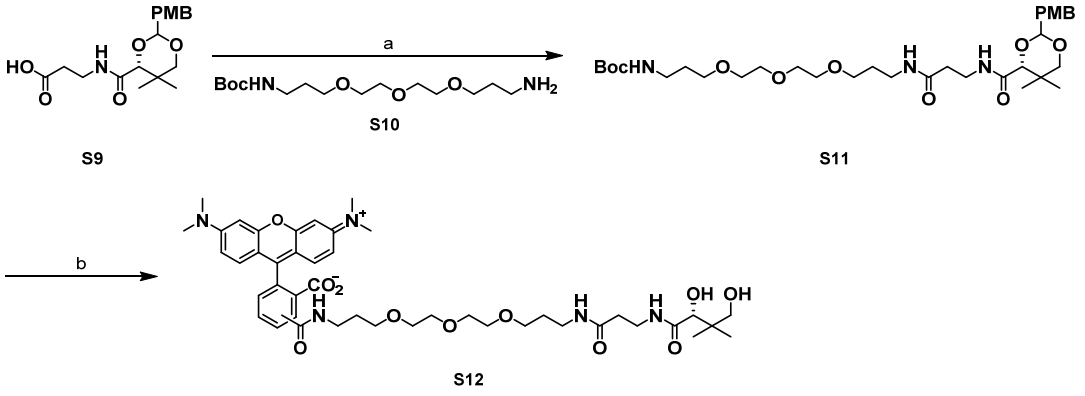


Figure S14. Full images of SDS-PAGE gels from Figure 4. Proteomic investigations using probe **1**. Investigation of the selective labeling of the CP4 domain of SrfAB using a combination of probe **1** and inhibitors **2**, **5**, and **6**. The *B. subtilis* ATCC 21332 proteome (2.0 mg/mL) was individually preincubated with inhibitors **2**, **5**, and **6** (10 μM) and reacted with 100 μM **1** for 2 h at 25 °C. For each panel, the image (Φ) denotes the fluorescence observed with λ_{ex} = 532 nm and λ_{ex} = 580 nm and the image (Σ) depicts total protein content by staining with Coomassie (Colloidal Coomassie Blue Stain). Full image for gel in Fig. 4b.

Chemical Synthetic Procedures



Scheme S1. Synthetic route to probe 1. *Reagents and conditions*: [a] NaH, **S1**, DMF, 7.1%; [b] TBSCl, imidazole, CH_2Cl_2, rt, 81%; [c] TCA, THF, H_2O, rt, 75%; [d] **S7**, DEAD, Ph_3P, THF, rt, 74%; [e] NaH, **S6**, DMF, CH_2Cl_2, rt, 33%; [f] 1) TFA, CH_2Cl_2, 0 °C; 2) TBAF, THF, rt, 98%, over two steps.



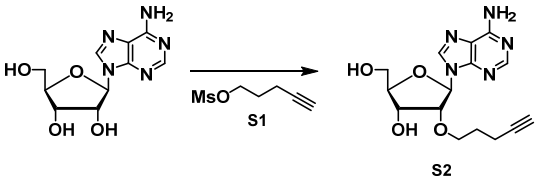
Scheme S2. Synthetic route to a rhodamine pantetheine analog S12. *Reagents and conditions*: [a] **S10**, EDC, HOBt, DMF, rt, 95%; [b] 1) TFA, CH_2Cl_2, rt; 2) Rh-OSu, DIEA, DMF, 18%, over two steps.

General Synthetic Methods: All commercial reagents were used as provided unless otherwise indicated. **S1**,[1] **S5**,[2] **S6**[3], **S9**,[4] **S10**[5], and

4-(3-chloro-5-(trifluoromethyl)pyridin-2-yl)-*N*-(4-methoxypyridin-2-yl)piperazine-1-carbothioa mide (ML267)[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, dd = doublet of doublets, dt = doublet of triplets, br = broad signal, m = multiplet using integration and coupling constant in Hertz. TLC analysis was performed using Silica Gel 60 F254 plates (Merck) and visualization was accomplished with ultraviolet light (λ = 254 nm) and/or the appropriate stain [phosphomolybdic acid, iodine, ninhydrin, and potassium permanganate]. Silica gel chromatography was carried out with SiliaFlash F60 230-400 mesh (Silicycle), according to the method of Still.[8] 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.

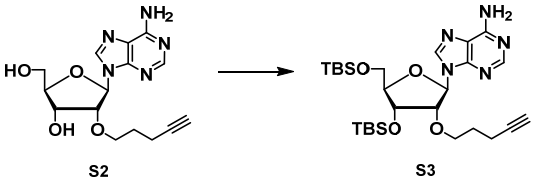
(2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-2-(hydroxymethyl)-4-(pent-4-yn-1-yloxy)tetrah ydrofuran-3-ol (S2)



NaH (2.2 g of a 60% suspension in mineral oil, 56.1 mmol) was added to a solution of adenosine (10 g, 37.4 mmol) in DMF (150 mL). The solution was stirred at room temperature for 1 h and compound **S1** (7.4 g, 45.6 mmol) was added. After 3 days, the reaction was quenched by the addition of H_2O and the DMF and H_2O were removed at reduced pressure. The residue was purified by flash chromatography ($CHCl_3$ to 19:1 $CHCl_3$/MeOH) and HPLC [COSMISIL C_{18}-AR-II: C-18 reverse-phase column, ϕ 10 mm × 250 mm, MeOH/H_2O (30:70), 8.0 mL/min, 210 nm, t_R: 34.0 min] to afford compound **S2** as a white solid (900 mg, 7.1%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.35 (s, 1H), 8.19 (s, 1H), 6.06 (d, *J* = 6.5 Hz, 1H), 4.54 (dd, *J* = 6.5, 5.0 Hz, 1H), 4.45 (dd, *J* = 5.0, 2.5 Hz, 1H), 4.17 (dd, *J* = 5.5, 2.5 Hz, 1H), 3.89 (dd, *J* =

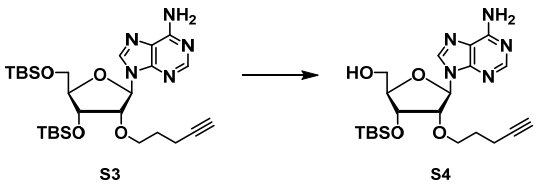
13.0, 3.0 Hz, 1H), 3.76 (dd, *J* = 13.0, 3.0 Hz, 1H), 3.73–3.68 (m, 1H), 3.57–3.49 (m, 1H), 2.21–2.09 (m, 2H), 2.07 (t, *J* = 3.0 Hz, 1H), 1.77–1.58 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD): δ 157.6, 153.6, 150.0, 141.9, 121.0, 89.4, 88.6, 84.1, 83.3, 71.2, 70.3, 69.7, 63.3, 29.6, 15.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{15}H_{20}N_5O_4$, 334.1510; found 334.1508.

9-((2*R*,3*R*,4*R*,5*R*)-4-((*tert*-Butyldimethylsilyl)oxy)-5-(((*tert*-butyldimethylsilyl)oxy)methyl)-3-(pent-4-yn-1-yloxy)tetrahydrofuran-2-yl)-9*H*-purin-6-amine (S3)



TBSCl (2.4 g, 15.9 mmol) and imidazole (2.2 g, 32.3 mmol) were added to a solution of compound **S2** (870 mg, 2.61 mmol) in CH_2Cl_2 (150 mL). The solution was stirred at room temperature. After 19 h, the reaction mixture was diluted with EtOAc. The mixture was washed with a 0.1 M aqueous HCl solution, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (1:1.5 EtOAc/hexane) to afford compound **S3** as a white solid (1.2 g, 81%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.35 (s, 1H), 8.23 (s, 1H), 6.14 (d, *J* = 4.1 Hz, 1H), 5.95 (s, 2H), 4.50 (t, *J* =4.9 Hz, 1H), 4.28 (t, *J* = 4.3 Hz, 1H), 4.12 (dt, *J* = 5.3, 2.9 Hz, 1H), 4.00 (dd, *J* = 11.5, 3.5 Hz), 3.78 (dd, *J* = 11.5, 2.3 Hz, 1H), 3.73–3.60 (m, 2H), 2.25 (dt, *J* = 7.0, 2.5 Hz, 2H), 1.87 (t, *J* = 2.6 Hz, 1H), 1.85–1.67 (m, 2H), 0.94 (s, 9H), 0.92 (s, 9H), 0.11 (s, 6H), 0.11 (s, 3H), 0.10 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.1, 152.8, 149.4, 138.9, 119.9, 86.8, 84.7, 83.3, 82.1, 69.9, 68.8, 68.7, 61.73, 28.5, 25.9, 25.6, 25.5, 18.3, 18.0, 14.9, –4.69, –5.01, –5.50, –5.57. HRMS (ESI+): $[M+H]^+$ calcd for $C_{27}H_{48}N_5O_4Si_2$, 562.3239; found, 562.3298.

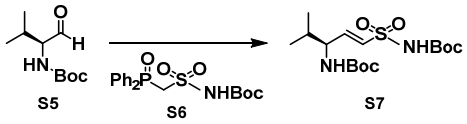
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(pent-4-yn-1-yloxy)tetrahydrofuran-2-yl)methanol (S4)



Compound **S3** (1.0 g, 1.8 mmol) was dissolved in a 4:1 (v/v) mixture of THF and H_2O at 0 °C.

Trichloroacetic acid (4.2 g, 26 mmol) was added. Stirring was continued at 0 °C for 2 h. The reaction was quenched by the addition of a 1 M aqueous NaOH solution. The resulting 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 (5:1 EtOAc/hexane) to afford compound **S4** as a white solid (600 mg, 75%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.33 (s, 1H), 7.93 (s, 1H), 6.03 (s, 2H), 5.89 (d, *J* = 7.5 Hz, 1H), 4.69 (dd, *J* = 7.8, 4.6 Hz 1H), 4.53 (d, *J* = 4.6 Hz, 1H), 4.19 (s, 1H), 3.95 (dd, *J* = 13.1, 1.7 Hz, 1H), 3.72 (d, *J* = 12.6 Hz, 1H), 3.56 (dt, *J* = 8.5, 4.6 Hz, 1H), 3.30–3.24 (m, 1H), 2.17–2.03 (m, 2H), 1.80 (t, *J* = 2.9 Hz, 1H), 1.71–1.51 (m, 2H), 0.95 (s, 9H), 0.14 (s, 3H), 0.13 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.4, 152.3, 148.5, 140.8, 121.2, 89.6, 89.5, 83.1, 81.0, 72.0, 69.1, 68.9, 62.9, 28.3, 25.7, 18.2, 14.8, −4.68, −4.72. HRMS (ESI+): $[M+H]^+$ calcd for $C_{21}H_{34}N_5O_4Si$, 448.2375; found, 448.2388.

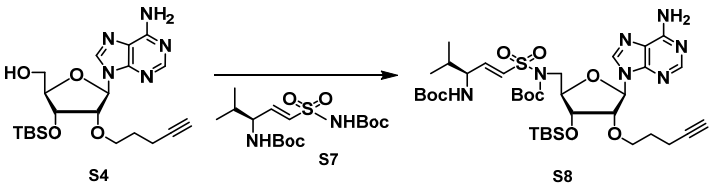
tert-Butyl (*S*,*E*)-(1-(*N*-(_tert_-butoxycarbonyl)sulfamoyl)-4-methylpent-1-en-3-yl)carbamate (S7)



LiHMDS (7.6 mL of a 1.3 M solution in THF, 9.88 mmol) was added to a solution of compound **S6** (1.5 g, 3.96 mmol) in DMF (5 mL). The solution was stirred at room temperature for 1 h and compound **S5** (500 mg, 2.48 mmol) was added. Stirring was continued at room temperature for 3 h. The reaction mixture was diluted with EtOAc. The mixture was washed with a 0.1 M aqueous HCl solution and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (1:4 EtOAc/hexane) to afford compound **S7** as a white solid (440 mg, 47%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.10 (br, 1H), 6.91 (dd, *J* = 14.9, 5.2 Hz, 2H), 6.56 (d, *J* = 14.9 Hz, 1H), 4.58 (d, *J* = 8.0 Hz, 1H), 4.28 (br, 1H), 1.98–1.82 (m, 1H), 1.48 (s, 9H), 1.45 (s, 9H), 0.97 (d, *J* = 6.9 Hz, 3H), 0.94 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 155.3, 149.8, 147.4, 127.9, 83.7, 80.0, 56.2, 41.1, 32.1, 28.3, 27.9, 18.8, 17.9, 14.2. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{16}H_{30}N_2O_6NaS$, 401.1717; found, 401.1798.

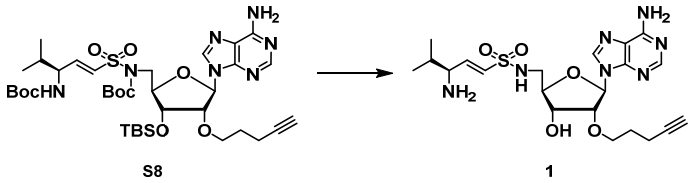
tert-Butyl (((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3-((_tert_-butyldimethylsilyl)oxy)-4-(pent-4-yn-1-yloxy)tetrahydrofuran-2-yl)methyl)(((*S*,*E*)-3-((_tert_-butoxycarbonyl)amino)-4-methylpent-1

-en-1-yl)sulfonyl)carbamate (S8)



Compound **S7** (49.2 mg, 0.13 mmol), PPh_3 (35.2 mg, 0.13 mmol), and DEAD (60.9 μL of a 2.2 M solution in toluene, 0.13 mmol) were added to a solution of compound **S4** (50 mg, 0.11 mmol) in THF (6 mL). The solution was stirred at room temperature. After 2 h, the reaction mixture was evaporated under reduced pressure. The residue was purified by flash chromatography (1:1 to 1:2 EtOAc/hexane and 99:1 to 97:3 $CHCl_3$/MeOH) to afford compound **S8** as a colorless solid (70.7 mg, 78%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.35 (s, 1H), 8.08 (s, 1H), 6.79 (dd, *J* = 15.0, 5.0 Hz, 1H), 6.62 (d, *J* = 15.5 Hz, 1H), 6.02 (d, *J* = 5.5 Hz, 1H), 5.72 (br, 2H), 4.75 (t, *J* = 4.5 Hz, 1H), 4.56 (br, 1H), 4.43 (br, 1H), 4.36–4.27 (m, 1H), 4.16 (dd, *J* = 15.0, 7.0 Hz, 1H), 3.85 (dd, *J* = 15.0, 7.0 Hz, 1H), 3.68–3.59 (m, 1H), 3.59–3.48 (m, 1H), 2.21–2.12 (m, 1H), 1.97–1.79 (m, 1H), 1.85 (t, *J* = 2.5 Hz, 1H), 1.79–1.62 (m, 2H), 1.48 (s, 9H), 1.41 (s, 9H), 0.94 (s, 9H), 0.92 (d, *J* = 7.0 Hz, 3H), 0.89 (d, *J* = 7.0 Hz, 3H), 0.13 (s, 6H). ^{13}C NMR (500 MHz, $CDCl_3$): δ 155.7, 155.2, 153.0, 151.1, 149.8, 147.1, 140.3, 128.4, 120.6, 87.5, 85.0, 83.5, 83.2, 80.2, 72.0, 69.1, 68.8, 56.2, 48.1, 32.3, 28.5, 28.4, 28.0, 25.8, 18.9, 18.2, 18.0, 15.0, –4.6, –4.7. HRMS (ESI+): $[M+H]^+$ calcd for $C_{37}H_{62}N_7O_9SSi$, 808.4094; found, 808.4034.

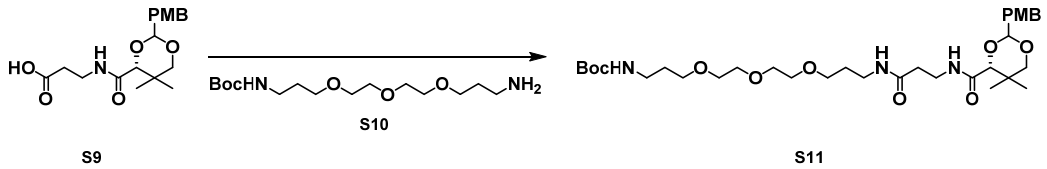
(*S*,*E*)-3-Amino-*N*-(((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3-hydroxy-4-(pent-4-yn-1-yl oxy)tetrahydrofuran-2-yl)methyl)-4-methylpent-1-ene-1-sulfonamide (1)



Compound **S8** (58.6 mg, 0.73 mmol) was dissolved in a 1:1 (v/v) mixture of CH_2Cl_2 and TFA at 0 °C. Stirring was continued at 0 °C for 12 h. The flask was placed on the rotary evaporator, and the CH_2Cl_2 and TFA were removed at reduced pressure. The residue was dissolved in THF (2 mL). The solution was stirred at room temperature and TBAF (380 μL of a 1 M TBAF solution in THF) was added. After 10 h, the reaction mixture was evaporated to dryness. The residue was purified by HPLC [Senshu pak: PEGASIL ODS SP 100 reverse-phase column, ϕ 20 mm × 250

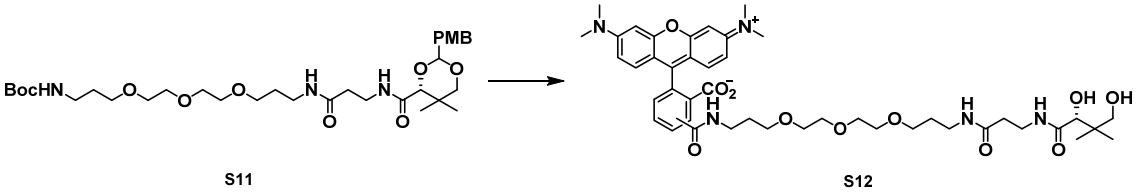
mm, acetonitrile/aqueous TFA (0.1%, 20:80), 8.0 mL/min, 210 nm, t_R: 14.5 min] to afford compound **1** as a colorless oil (37.4 mg, quant.). ^{1}H NMR (500 MHz, CD_3OD): δ 8.44 (s, 1H), 8.35 (s, 1H), 6.78 (d, *J* = 15.5 Hz, 1H), 6.61 (dd, *J* = 15.5, 8.0 Hz, 1H), 6.11 (d, *J* = 5.2 Hz, 1H), 4.54 (t, *J* = 5.4 Hz, 1H), 4.67 (dt, *J* = 4.7, 1.5 Hz, 1H), 4.21 (dd, *J* = 8.0, 4.0 Hz, 1H), 3.84–3.78 (m, 1H), 3.78–3.72 (m, 1H), 3.65–3.57 (m, 1H), 3.39 (dd, *J* = 6.0, 3.8 Hz, 1H), 3.37 (t, *J* = 3.4 Hz, 1H), 2.23–2.14 (m, 2H), 2.12 (t, *J* = 2.9 Hz, 1H), 2.08–2.02 (m, 1H), 1.81–1.72 (m, 1H), 1.72–1.62 (m, 1H), 1.05 (dd, *J* = 6.9, 2.9 Hz, 3H), 1.00 (t, *J* = 6.6 Hz, 3H). ^{13}C (125 MHz, CD_3OD): δ 153.2, 149.7, 147.0, 143.8, 173.1, 135.3, 121.0, 89.4, 85.6, 84.1, 82.9, 71.3, 70.5, 69.8, 57.9, 45.3, 32.2, 29.6, 18.8, 17.9, 15.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{21}H_{32}N_7O_5S$, 494.2180; found, 494.2242.

***tert*-Butyl (1-((4*R*)-2-(4-methoxybenzyl)-5,5-dimethyl-1,3-dioxan-4-yl)-1,5-dioxo-10,13,16-trioxa-2,6-diazanonadecan-19-yl)carbamate (S11)**



Compound **S10** (40.0 μL, 0.013 mmol), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (29.0 mg, 0.015 mmol), 1-hydroxybenzotriazole (23.0 mg, 0.015 mmol), and *N*,*N*-diisopropylethylamine (26.0 μL, 0.015 mmol) were added to a solution of compound **S9** (50.0 mg, 0.015 mmol) in DMF (20 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (20:1 $CHCl_3$/MeOH) to afford compound **S11** as a colorless oil (76 mg, 95%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.42 (d, *J* = 8.5 Hz, 2H), 7.01 (t, *J* = 6.0 Hz, 1H), 6.90 (d, *J* = 9.0 Hz, 2H), 6.54 (br, 1H), 5.44 (s, 1H), 5.04 (br, 1H), 4.05 (s, 1H), 3.80 (s, 3H), 3.71–3.45 (m, 16H), 3.32 (dd, *J* = 12.0, 6.0 Hz, 2H), 3.18 (br, 2H), 2.38 (t, *J* = 5.0 Hz, 2H), 31.78–1.66 (m, 4H), 1.41 (s, 9H), 1.09 (s, 3H), 1.08 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 171.0, 169.4, 160.3, 156.2, 130.3, 127.6, 113.8, 101.4, 84.0, 78.6, 70.6, 70.3, 70.2, 69.5, 55.4, 38.1, 35.9, 35.1, 33.2, 29.8, 28.9, 28.5, 22.0, 19.3. HRMS (ESI+): $[M+Na]^+$ calced for $C_{32}H_{53}N_3NaO_{10}$, 662.3623; found 662.3622.

Rh-pantetheine analog (S12)



Compound **S11** (30 mg, 0.044 mmol) was dissolved in a 1:1 (v/v) mixture of CH_2Cl_2 and TFA at room temperature. After 30 min, the flask was placed on the rotary evaporator and the CH_2Cl_2 and TFA were removed at reduced pressure to afford TFA salt. A solution of the crude salt, 5(6)-TAMRA-OSu (25.0 mg, 0.047 mmol) and *N*,*N*-diisopropylethylamine (16.0 µL, 0.092 mmol) in DMF (500 µL) was stirred at room temperature for 24 h. The reaction mixture was evaporated under reduced pressure. The residue was purified by HPLC [Senshu pak: PEGASIL ODS SP 100 reverse-phase column, ϕ 20 mm × 250 mm, acetonitrile/H_2O (35:65), 8.0 mL/min, 210 nm, t_R: 18.5 min] to afford compound **S12** as a pink solid (7.0 mg, 18% over two steps). ^{1}H NMR (500 MHz, D_2O): δ 8.48 (s, 1H), 8.08 (d, *J* = 7.0 Hz, 1H), 7.49 (d, *J* = 8.0 Hz, 1H), 7.08 (d, *J* = 10.0 Hz, 2H), 6.77 (d, *J* = 9.5 Hz, 2H), 6.25 (br, 2H), 3.90 (s, 1H), 3.76–3.10 (m, 20H), 2.38 (t, *J* = 6.0 Hz, 1H), 2.04 (s, 12H), 1.97 (t, *J* = 6.5 Hz, 2H), 1.70 (t, *J* = 6.0 Hz, 2H), 0.83 (s, 3H), 0.79 (s, 3H). HRMS (ESI+): $[M+Na]^+$ calced for $C_{44}H_{59}N_5NaO_{11}$, 856.4103; found 856.4119.

Chemical Biology Procedures

Protein expression and materials: Recombinant proteins *apo*-AusA1,[9] *apo*-AusA2,[10] and *holo*-TycB1[11,12,13] were expressed and purified as previously described. The *holo*-TycB1 expression construct was kindly provided by Prof. Mohamed A. Marahiel at Philipps-Universität Marburg, Germany. Recombinant *E. coli* CoA biosynthetic enzymes (pantothenate kinase (CoaA, PanK), phosphopantetheine-adenyltransferase (CoaD, PPAT), dephospho-CoA kinase (CoaE, DPCK)) were expressed and purified as MBP-fusions as described previously.[14] Recombinantly expressed Sfp (PPTase; *Bacillus subtilis*) was overexpressed in *E. coli* as the native protein (untagged) and purified as described previously.[14] The CoaA, CoaD, CoaE, and Sfp expression constructs were kindly provided by Prof. Michael D. Burkart at University of California, San Diego, USA.

Phosphopantetheinylation of *apo*-AusA1 and *apo*-AusA2: The *in vitro* phosphopantetheninylation of *apo*-AusA1 was conducted in a 1-mL reaction vessel containing 0.1 µg/µL *apo*-AusA1 or 0.6 µg/µL *apo*-AusA2, 0.008 µg/µL Sfp (native), 15 mM $MgCl_2$, 1 mM TCEP, 0.5 mM CoA, and 50 mM potassium phosphate (pH 7.0) at 37 °C for 12 h. The reaction mixtures were dialyzed against 20 mM Tris (pH 8.0), 1 mM $MgCl_2$, and 1 mM TCEP

using Amicon Ultra Centrifugal Filter Units, MWCO 50 kDa (Millipore). The *holo*-AusA1 and *holo*-AusA2 proteins were quantified by the method of Bradford.[15] After the addition of 10% glycerol (v/v) the proteins were stored at −80 °C.

Fluorescent labeling of *holo*-AusA1 and *holo*-AusA2 with CoaA, CoaD, CoaE, and Sfp and Rh-Ppant analog S12: To verify the high conversion of phosphopantetheinylation of *apo*-AusA1 and *apo*-AusA2, the one-pot reactions of *holo*-AusA1 and *holo*-AusA2 were carried out with the Rh-Ppant analog **S12**. One-pot reaction mixtures contained the following (final volume of 50 µL): 50 mM potassium phosphate buffer (pH 7.0), 8 mM ATP, 15 mM $MgCl_2$, 0.002µg/µL MBP-CoaA, 0.01 µg/µL MBP-CoaD, 0.01 µg/µL MBP-CoaE, 0.01 µg/µL Sfp (native), and 350 nM of *holo*-AusA1 or *holo*-AusA2. In all experiments, the total DMSO concentration was kept at 1.0%. The reactions were initiated by the addition of 500 µM Rh-Ppant analog **S12** and incubated at 37 °C for 1 h. 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 recombinant *apo*-AusA1 and *holo*-AusA1 by probe 1: Standard conditions for probe **1**-recombinant protein reactions were as follows: recombinant *apo*-AusA1 (350 nM) and *holo*-AusA1 (350 nM) were treated with probe **1** (100 µM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. The inhibition study was carried out by pre-incubation of *holo*-AusA1 (350 nM) with *L*-Val-AMS **2** (1 mM from a 100 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.0%. After 12 h at room temperature, these samples were subjected to the click reaction. Rh-azide, TCEP, TBTA ligand, and $CuSO_4$ were added to provide final concentrations of 100 µM, 1 mM, 100 µM, and 1 mM, respectively. After 1 h at room temperature, 5× SDS-loading buffer (strong reducing) was added and the samples were heated at 95 °C for 5 min. Samples were separated by 1D SDS-PAGE and fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Determination of an optimal pH for the labeling of *holo*-AusA1 by probe 1: Recombinant *holo*-AusA1 (350 nM) was incubated with probe **1** (100 µM from a 10 mM stock in DMSO) in 20 mM Tris–HCl, pH 6.5, 7.0, 7.5, 8.0, and 8.5, 1 mM $MgCl_2$, and 1 mM TCEP. For inhibition studies, *holo*-AusA1 (350 nM) was pre-incubated with *L*-Val-AMS **2** (1 mM from a 100 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.0%. After 12 h at room temperature, these samples were 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).

Concentration dependence of the *in vitro* probe labeling of *holo*-AusA1: Recombinant

holo-AusA1 (350 nM) was treated with probe **1** (10–100 μM) in 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. For inhibition studies, *holo*-AusA1 (350 nM) was pre-incubated with *L*-Val-AMS **2** (1 mM from a 100 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.0%. These reaction mixtures were incubated for 12 h at room temperature. These samples were 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).

Time-course studies of the labeling of *holo*-AusA1 with probe 1: Recombinant *holo*-AusA1 (350 nM) was treated with probe **1** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. In all experiments, the total DMSO concentration was kept at 2.0%. These reaction mixtures were incubated for the indicated time (0–24 h) at room temperature. These samples were 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).

Comparison of the labeling properties of probe 1 with *holo*-AusA1, *holo*-AusA2, and *holo*-TycB1: For *holo*-AusA1 (A: *L*-Val), *holo*-AusA2 (A: *L*-Tyr), and *holo*-TycB1 (A: *L*-Pro) labeling experiments, probe **1** (100 μM from a 10 mM stock in DMSO) were individually added to a 46-μL reaction vessel containing either *holo*-AusA1 (350 nM), *holo*-AusA2 (350 nM), or *holo*-TycB1 (350 nM), and 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. For inhibition studies, *holo*-AusA1 (350 nM), *holo*-AusA2 (350 nM), and *holo*-TycB1 (350 nM) were pre-incubated with *L*-Val-AMS **2**, *L*-Tyr-AMS **3**, and *L*-Pro-AMS **4** (1 mM from a 100 mM stock in DMSO), respectively, for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.0%. After 12 h at room temperature, these samples were 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).

Measurements of the percentage of *holo*-AusA1 labeled by probe 1: Recombinant *holo*-AusA1 (1 μM) was treated with probe **1** (1, 10, 20, 50, and 100 μM) for 12 h at room temperature in 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. For inhibition studies, recombinant *holo*-AusA1 (1 μM) was pre-incubated with *L*-Val-AMS **2** (1 mM from a 100 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.0%. After 12 h at room temperature, these samples were 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). Protein labeling by probe **1** was quantitated by measuring integrated band intensities using ImageJ. A TAMRA-conjugated BSA

(50, 100, 200, 400, 600, 800, 1000, and 1200 nM) (Thermo Fisher Scientific) was used as a standard of fluorescence intensity.

Peptide mapping of the labeled *holo*-AusA1: Recombinant *holo*-AusA1 (350 nM) was reacted with probe **1** (50 μM) for 12 h at room temperature in 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. The proteins were subjected to SDS-PAGE and visualized using Coomassie Brilliant Blue (CBB) staining. The bands were excised, destained, and subjected to in-gel digestion with an endoproteinase Asp-N sequence grade (Roche). The digest mixture was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) using a UltrafleXtreme mass spectrometer (Bruker Daltonics) in a positive mode. α-Cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltomics) was used as a matrix. Selected MS ion peaks were applied to the tandem mass spectrometry using LIFT mode.

Preparation of lysates for proteomic labeling experiments: *Bacillus subtilis* (Ehrenberg) Cohn (ATCC 21332) was obtained from the American Type Culture Collection (ATCC). The seed culture from frozen stock was revived for 24 h and grown in a minimal salt (MS) medium with 4 μM of Fe^{2+} at 30 °C at 200 rpm.[16] The medium contained 4% glucose, 30 mM KH_2PO_4, 40 mM Na_2HPO_4, 50 mM NH_4NO_3, 800 μM $MgSO_4$, 4 μM $FeSO_4$, 7 μM $CaCl_2$, and 4 μM sodium EDTA.[16] The seed cultures (1 mL) was transferred to 200 mL of medium which contained 4% glucose, 30 mM KH_2PO_4, 40 mM Na_2HPO_4, 50 mM NH_4NO_3, 800 μM $MgSO_4$, 2 mM $FeSO_4$, 7 μM $CaCl_2$, and 4 μM sodium EDTA and the resulting mixture was incubated for 24 h at 30 °C at 200 rpm.[16] The cells 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 $MgCl_2$, 1 mM TCEP, and protease inhibitor cocktail. Because of the lability of the synthetase during mechanical cell disruption processes,[17] 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.[15]

SrfAB labeling of *B. subtilis* ATCC 21332 proteomes: *B. subtilis* ATCC 21332 proteome (2.0 mg/mL) was treated with probe **1** (100 μM from a 10 mM stock in DMSO) in 20 mM Tris (pH 8.0), 1 mM TCEP, and 1 mM $MgCl_2$. Inhibition studies were performed by pre-incubation of proteome (2.0 mg/mL) with *L*-Val-AMS **2** (1 mM from a 100 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.0%. These reaction mixtures were incubated for the indicated time (0–12 h) at room temperature. These samples were 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).

Selective labeling of the CP_4 domain of SrfAB in native proteomic environments: *B. subtilis* ATCC 21332 proteome (2.0 mg/mL) was individually treated with *L*-Val-AMS **2**, *L*-Asp-AMS **5**, and *L*-Leu-AMS **6** (10 μM from 1 mM and 500 μM stock in DMSO, respectively). These samples were incubated for 10 min at room temperature and subsequently reacted with probe **1** (100 μM from a 10 mM stock in DMSO). In all experiments, the total DMSO concentration was kept at 2.0%. After 2 h at room temperature, these mixtures were treated 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).

Mass spectroscopic analysis, 1D SDS-PAGE, and in-gel digestion: The proteins [SrfAA, SrfAB, and ### (hydrolyzed SrfAB)] were separated by 1D SDS-PAGE by using 6% wide range gels (Nacalai Tesque) and visualized using Sil-best stain one (Nacalai Tesque) or Coomassie Brilliant Blue (CBB) staining. The bands were excised, destained, and subjected to in-gel digestion with TPCK-treated bovine trypsin (Worthington Biochemical Corporation). The digest mixtures were separated using a nanoflow LC (Easy nLC, Thermo Fisher Scientific) on a NTCC analytical column (C-18 reverse-phase column, ϕ 0.075 × 100 mm, 3 μm bead size, Nikkyo Technos Co., Ltd.). Buffer compositions were as follows: buffer A was composed of 100% H_2O and 0.1% formic acid, buffer B was composed of 100% acetonitrile and 0.1% formic acid. Peptides were eluted from the C-18 column using a linear gradient of 0−35% buffer B over 10 min at a flow rate of 300 nL/min and subjected to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) in positive mode with a nanospray ion source using the data-dependent TOP10 MS/MS method. Peptide identifications were made using MS/MS ions search toward the NCBI-nr database using the MASCOT program v2.5 (Matrix Science Inc.). The taxonomy was selected as *B. subtilis*. In addition, the variable modifications were selected as acetyl (protein *N*-terminal), deamidated (NQ), Gln->pyro-Glu (*N*-terminal Q), oxidation (M), and propionamide (C).

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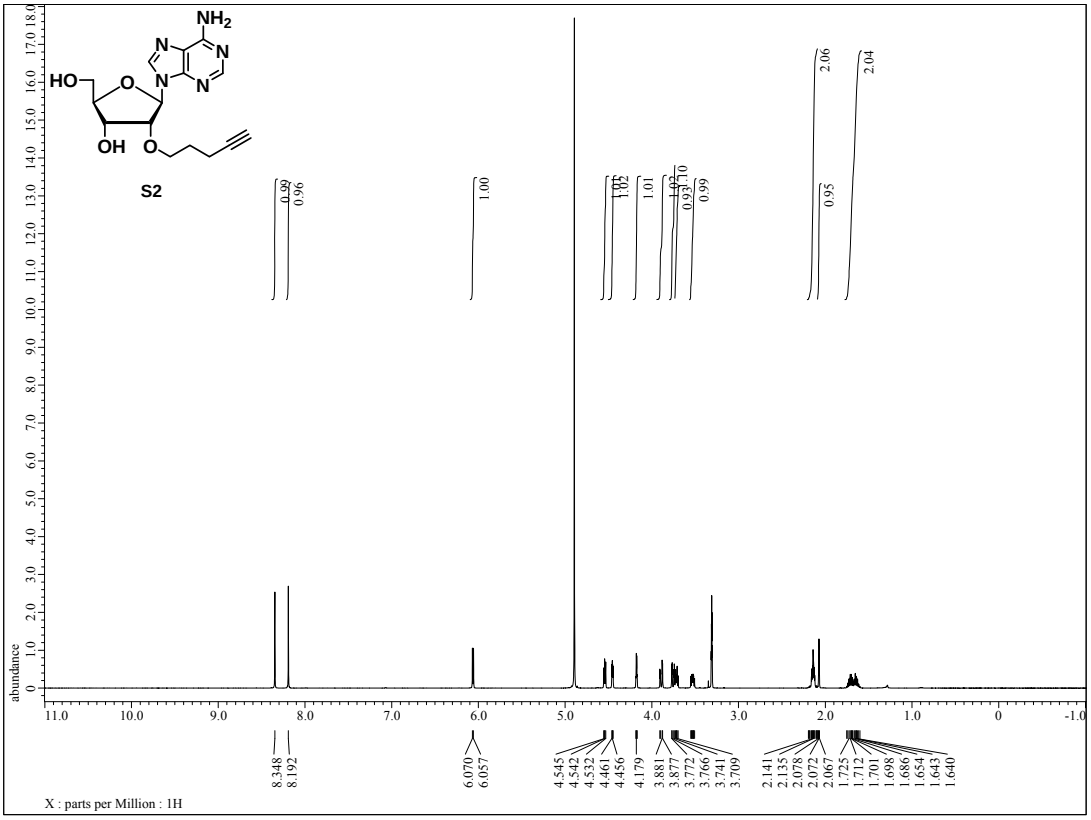
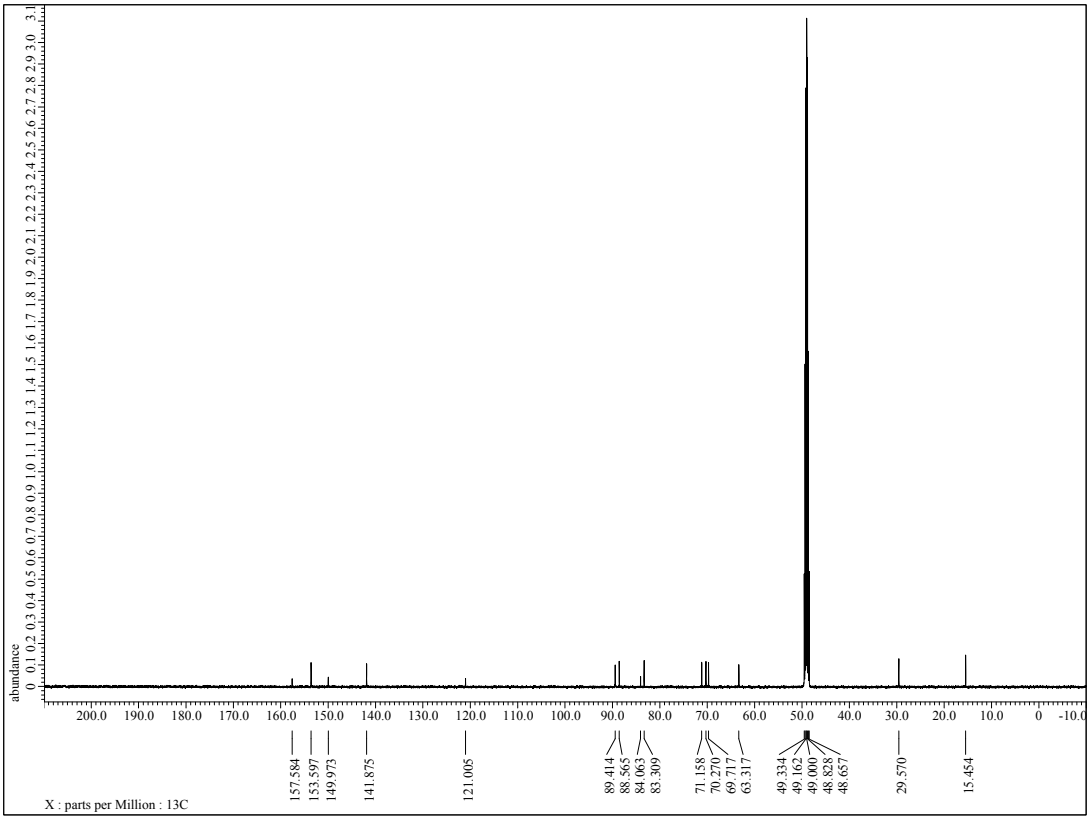
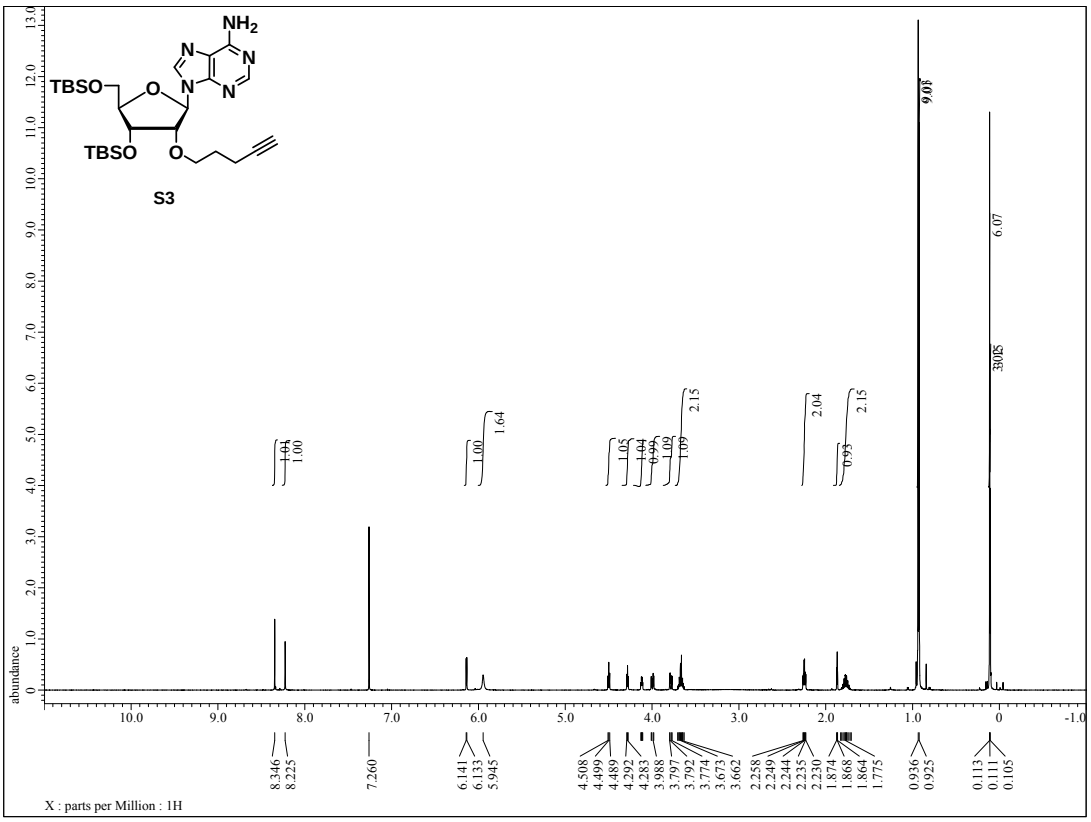
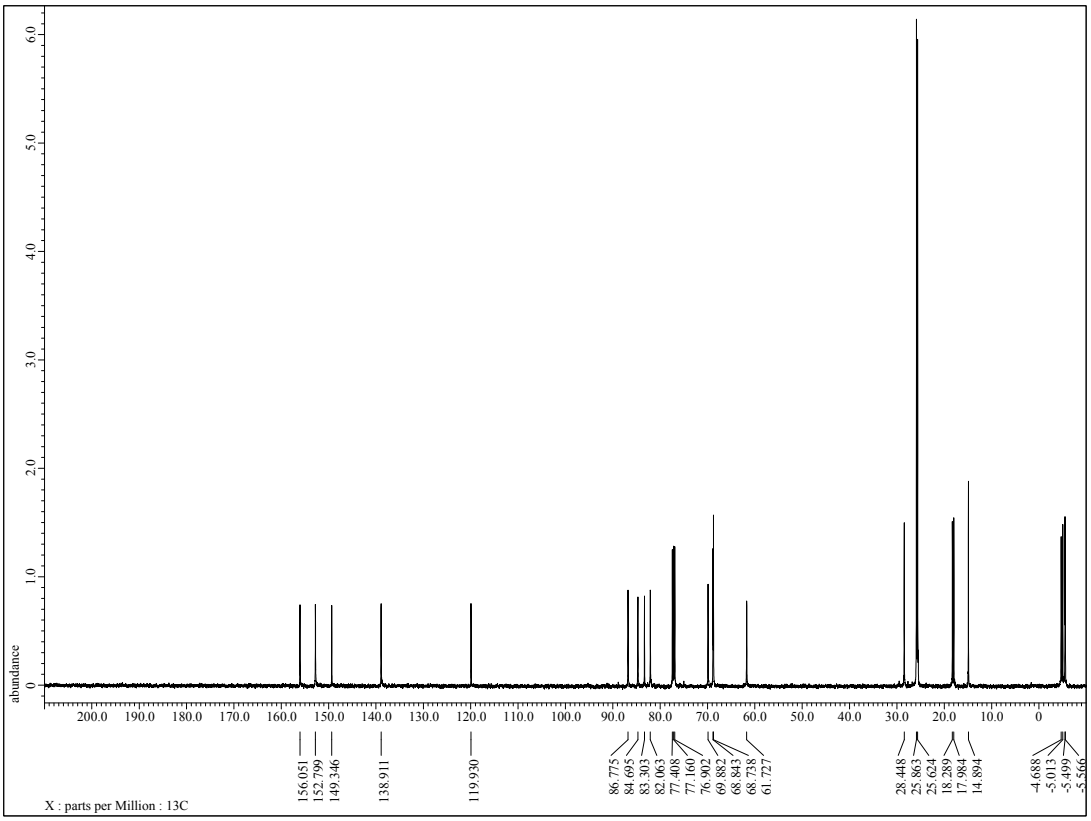
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S2** in CD_3OD



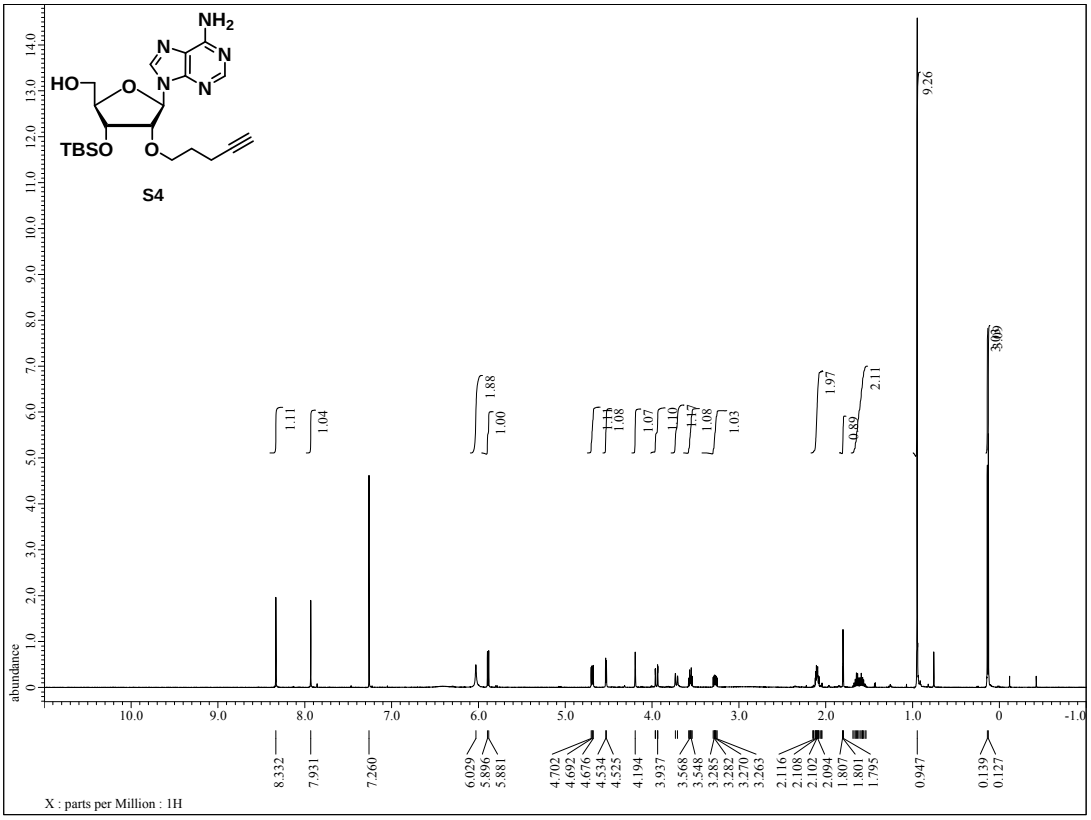


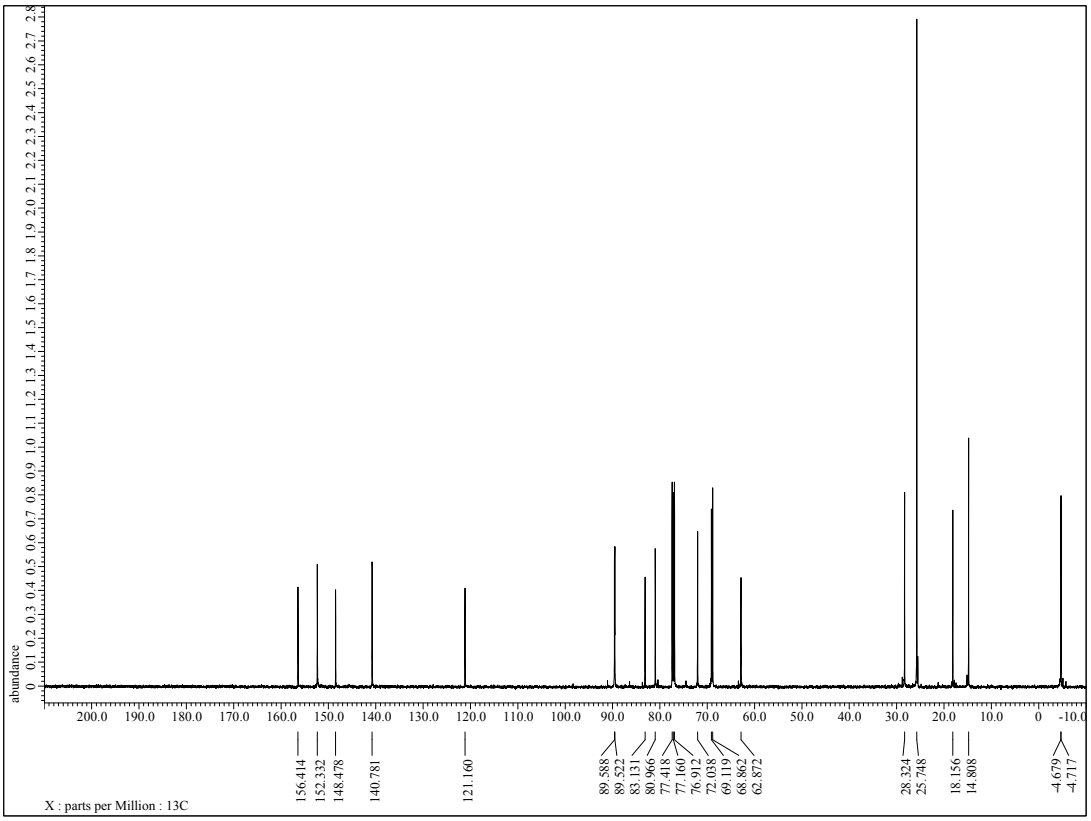
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S3** in $CDCl_3$



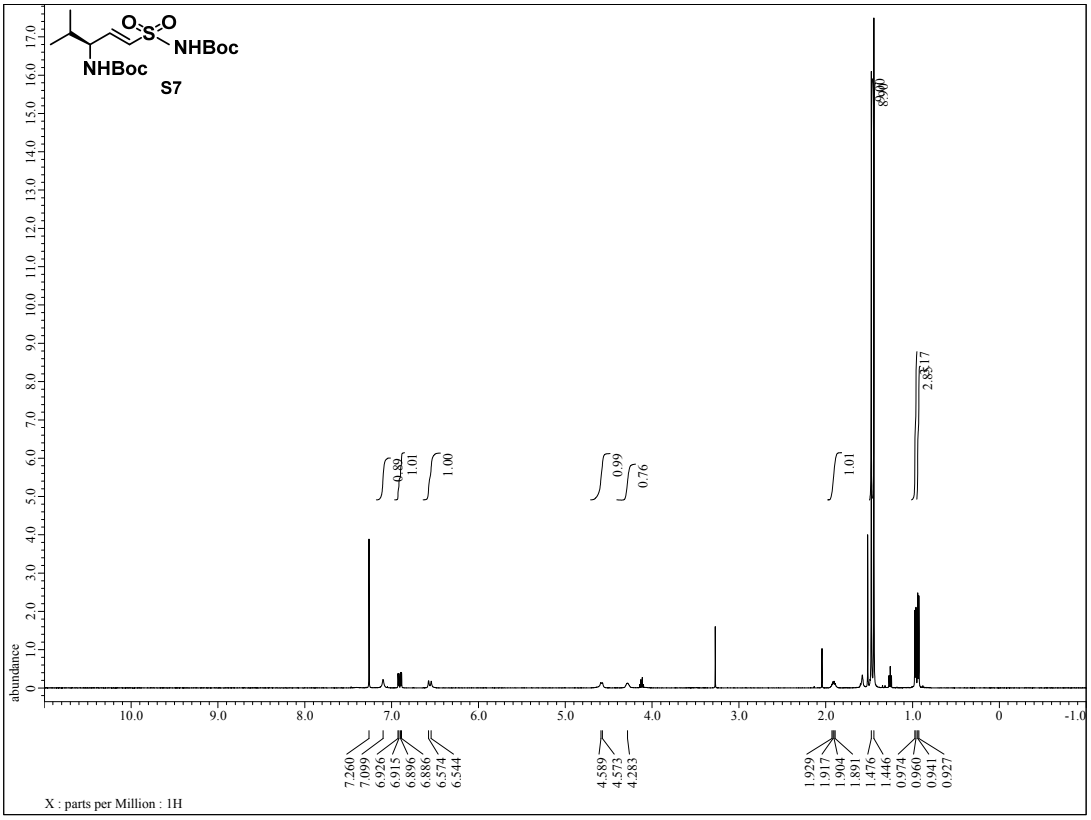


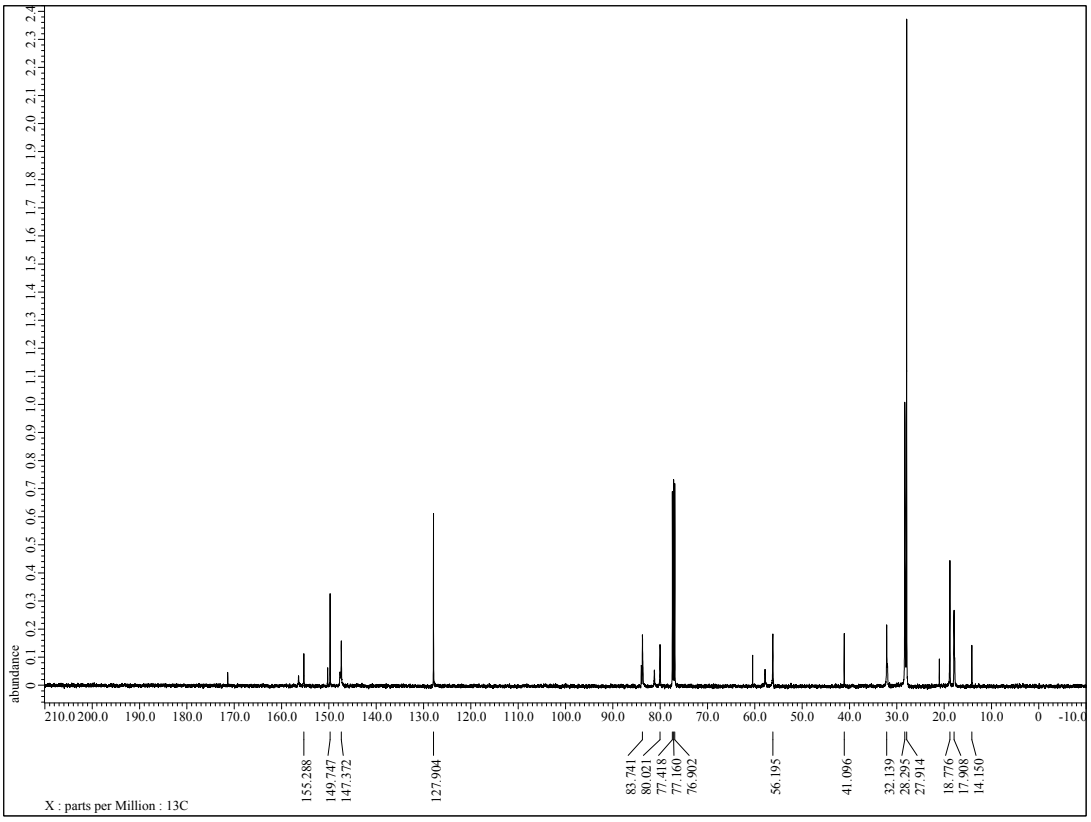
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S4** in $CDCl_3$



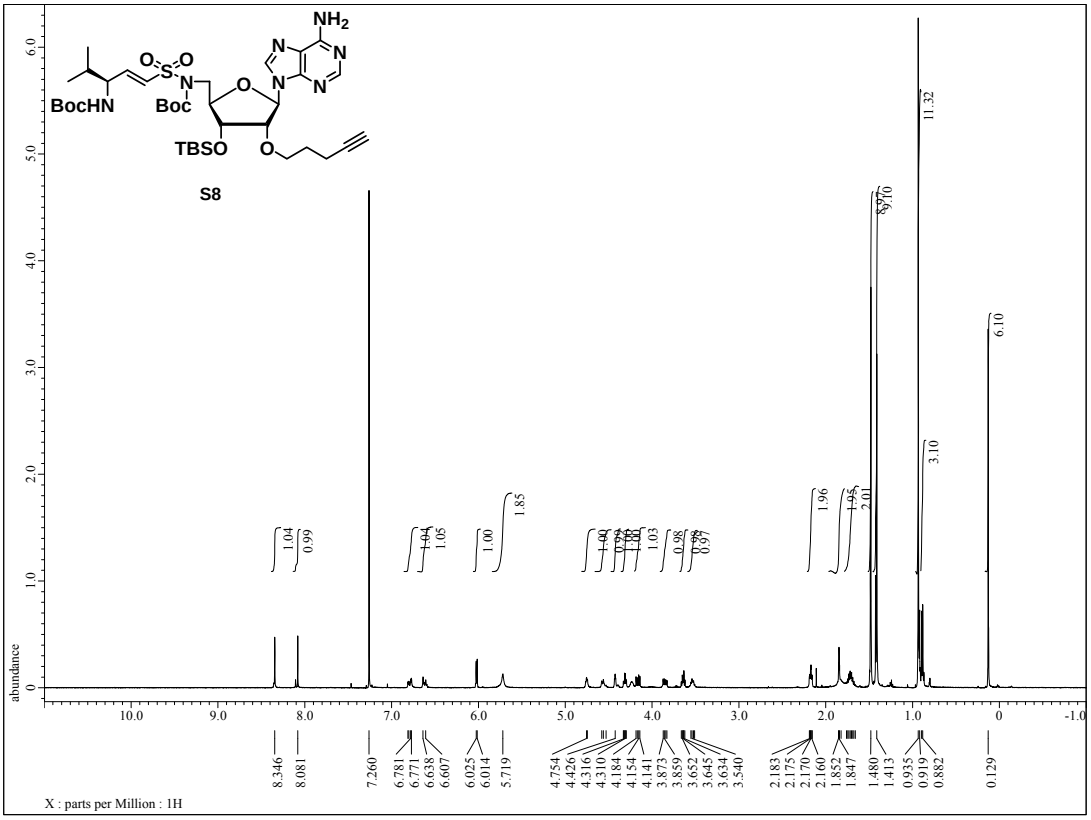


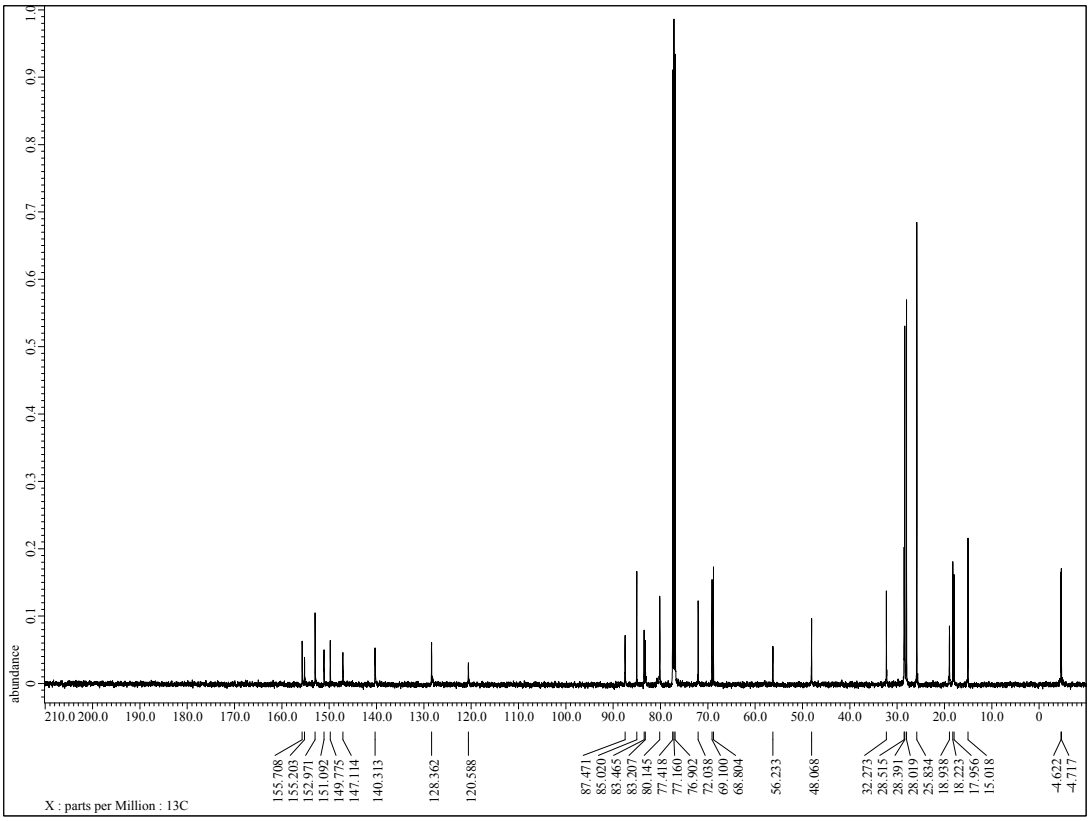
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S7** in $CDCl_3$



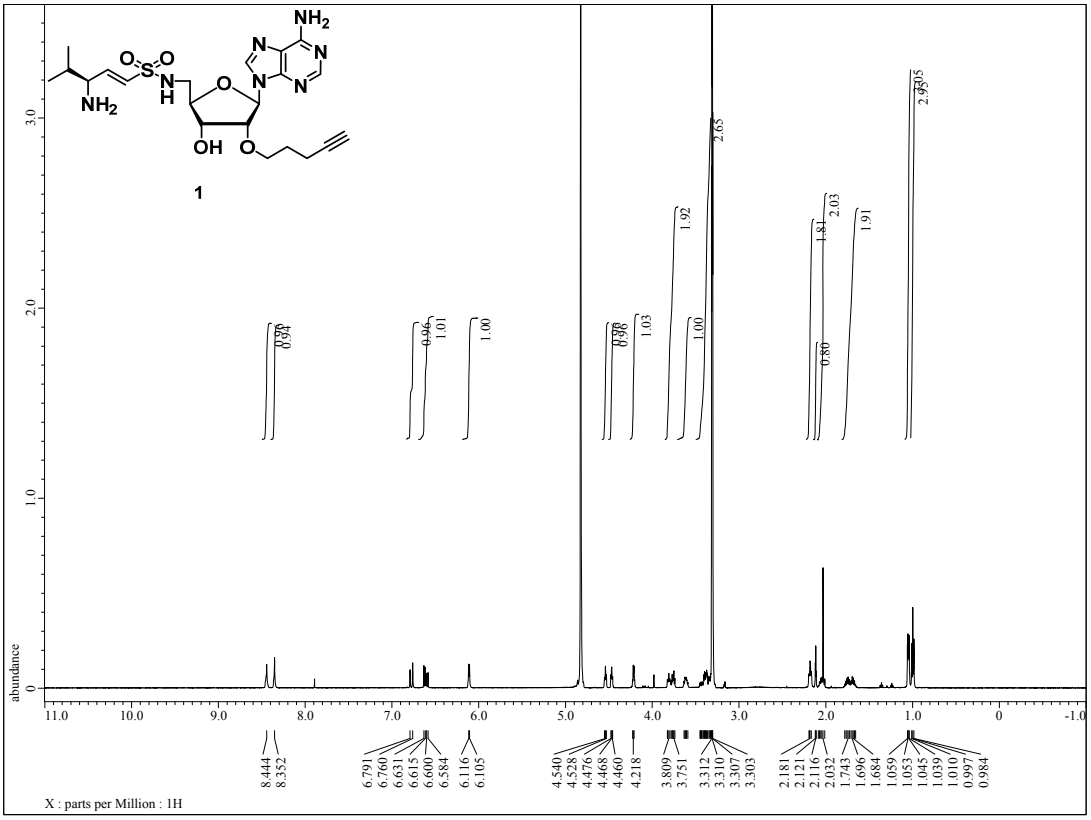


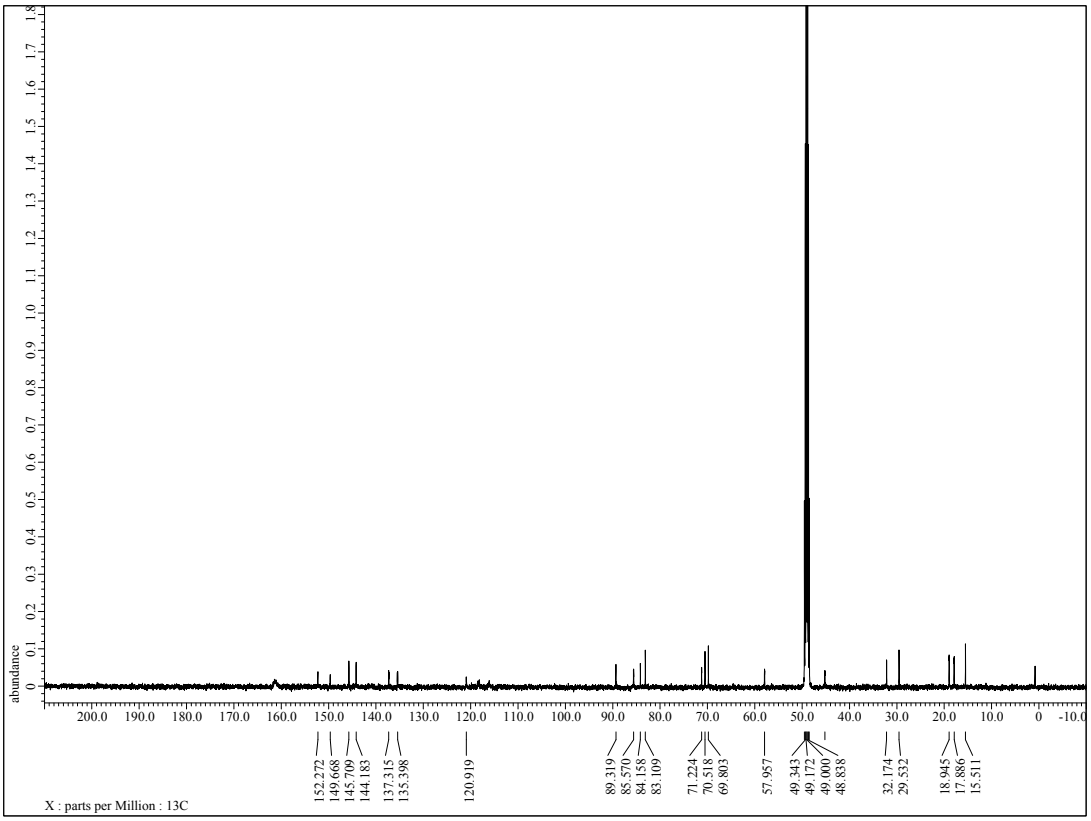
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S8** in $CDCl_3$



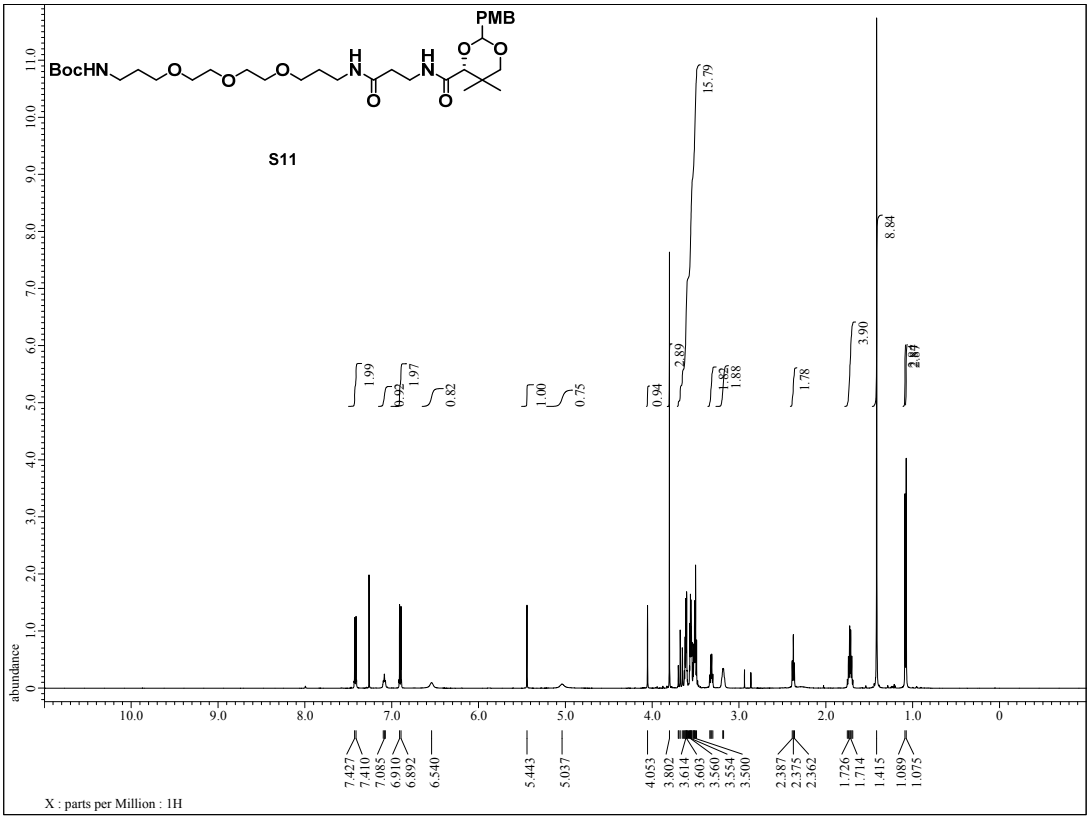


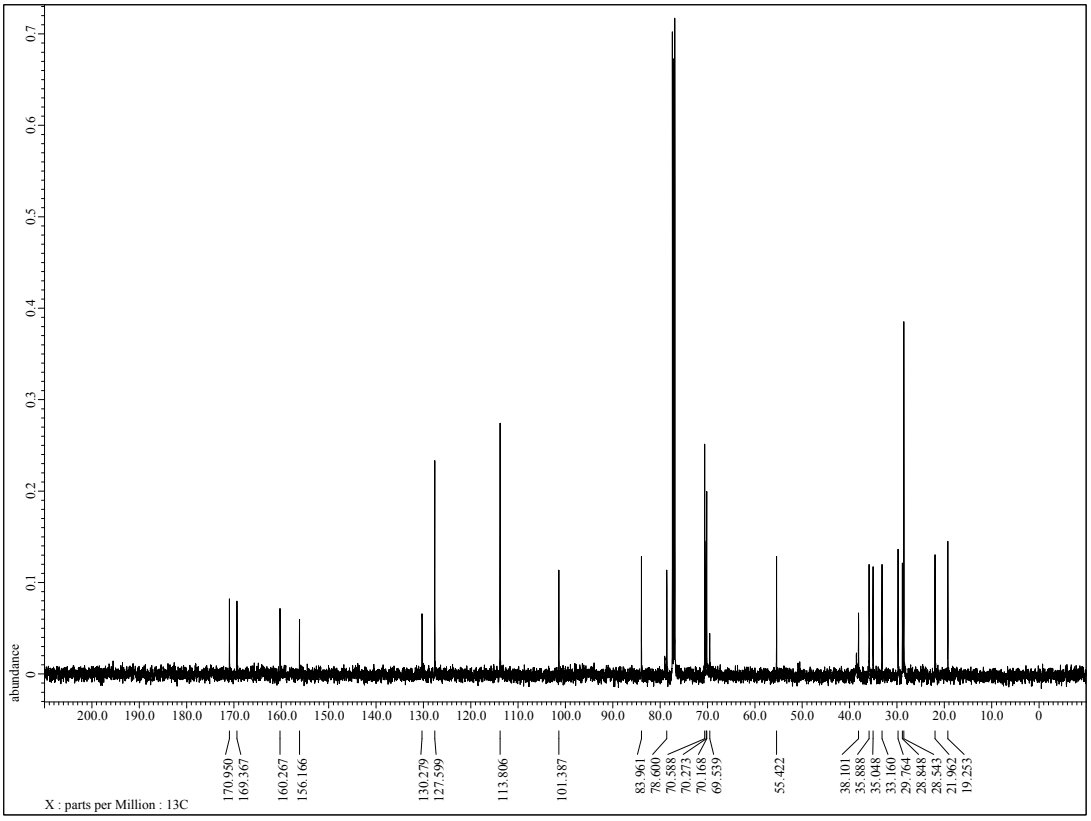
^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **1** in CD_3OD





^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) spectra of **S11** in $CDCl_3$





^{1}H-NMR (500 MHz) spectrum of **S12** in D_2O