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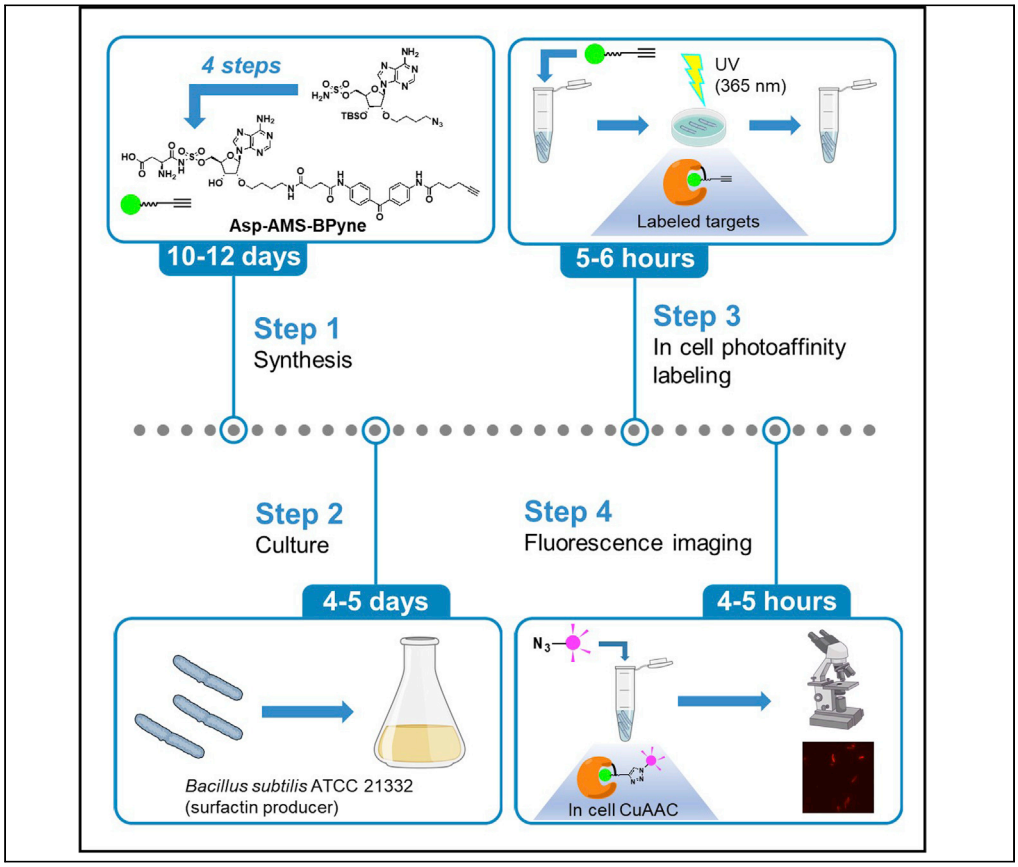
Activity-based protein profiling of a surfactin-producing nonribosomal peptide synthetase in *Bacillus subtilis*



We present an *in vitro* and in-cell activity-based protein profiling (ABPP) protocol for endogenous nonribosomal peptide synthetases (NRPSs). This protocol enables the fluorescence labeling and imaging of an endogenous SrfAB-NRPS with high selectivity and sensitivity in the surfactin producer *Bacillus subtilis*. While we optimized this protocol for use with *B. subtilis*, the protocol can be applied to *Aneurinibacillus migulanus* and *Escherichia coli*.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A protocol to aid an *in vitro* and in-cell ABPP of endogenous NRPSs

Applies to proteome labeling, in-cell labeling, and imaging of endogenous NRPSs

Steps can be optimized for use in the gram-positive bacterium *Bacillus subtilis*

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Activity-based protein profiling of a surfactin-producing nonribosomal peptide synthetase in *Bacillus subtilis*

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SUMMARY

We present an *in vitro* and in-cell activity-based protein profiling (ABPP) protocol for endogenous nonribosomal peptide synthetases (NRPSs). This protocol enables the fluorescence labeling and imaging of an endogenous SrfAB-NRPS with high selectivity and sensitivity in the surfactin producer *Bacillus subtilis*. While we optimized this protocol for use with *B. subtilis*, the protocol can be applied to *Aneurinibacillus migulanus* and *Escherichia coli*.

For complete details on the use and execution of this protocol, please refer to Ishikawa et al. (2022).

BEFORE YOU BEGIN

Peptide-based natural products include a wide range of important drugs, such as the antibiotic daptomycin, antitumor bleomycin, and immunosuppressant cyclosporine (Süssmuth and Mainz, 2017). These peptide-based natural products are biosynthesized by large, multienzyme protein complexes known as nonribosomal peptide synthetases (NRPSs). The typical NRPS module comprises three catalytic domains: adenylation (A), thiolation (T, also known as the peptidyl carrier protein), and condensation (C) (Hur et al., 2012). The A-domain activates the carboxy group of an amino acid to form an aminoacyl adenylate (aminoacyl-AMP) intermediate and transfers the aminoacyl-AMP to the thiol group of the 4′-phosphopantetheine prosthetic group of the T domain, which is generally carries substrates, intermediates, and products as its cargo. The aminoacyl-T thioester thus formed undergoes nucleophilic attack by the amino group of another aminoacyl-T thioester to form an amide bond in the C domain. Microbial synthesis of natural products depends on many regulatory mechanisms and the orchestrated regulation of biomachineries including transcription of relevant genes into mRNAs, translation of mRNAs into proteins, and post-translational protein modifications in the native producer. Nevertheless, much of our current knowledge on NRPSs is based on studies in which these proteins are expressed in heterologous hosts and analyzed in a simple system. To address these problems, we developed an in-cell activity-based protein profiling (ABPP) system for endogenous NRPSs and applied it to the study of their enzymatic activities in bacteria (Figure 1). For fluorescence labeling and imaging of a surfactin-producing nonribosomal peptide synthetase (SrfAB-NRPS) in *Bacillus subtilis* ATCC 21332, we used a photoaffinity-labeling (PAL)-based ABPP probe (**Asp-AMS-BPyne**) that contains the synthetic ligand 5′-*O*-(*N*-aspartyl)sulfamoyladenosine (**Asp-AMS**) highly specific toward the Asp-activating A-domains of NRPSs, a benzophenone (BP) photo-crosslinker, and a clickable alkyne (yne) (Figure 2). Importantly, the **Asp-AMS** was connected to the functional linker at the 2′-OH group of the adenosine skeleton. Modification at the 2′-OH of the adenosine skeleton preserves the binding affinity of the A-domains within NRPSs (Konno et al., 2015; Ishikawa et al., 2015). The incorporation of a BP-photoreactive functionality allows cross-linking of protein targets of **Asp-AMS-BPyne** by forming a covalent bond with adjacent amino acid residues. Furthermore, the

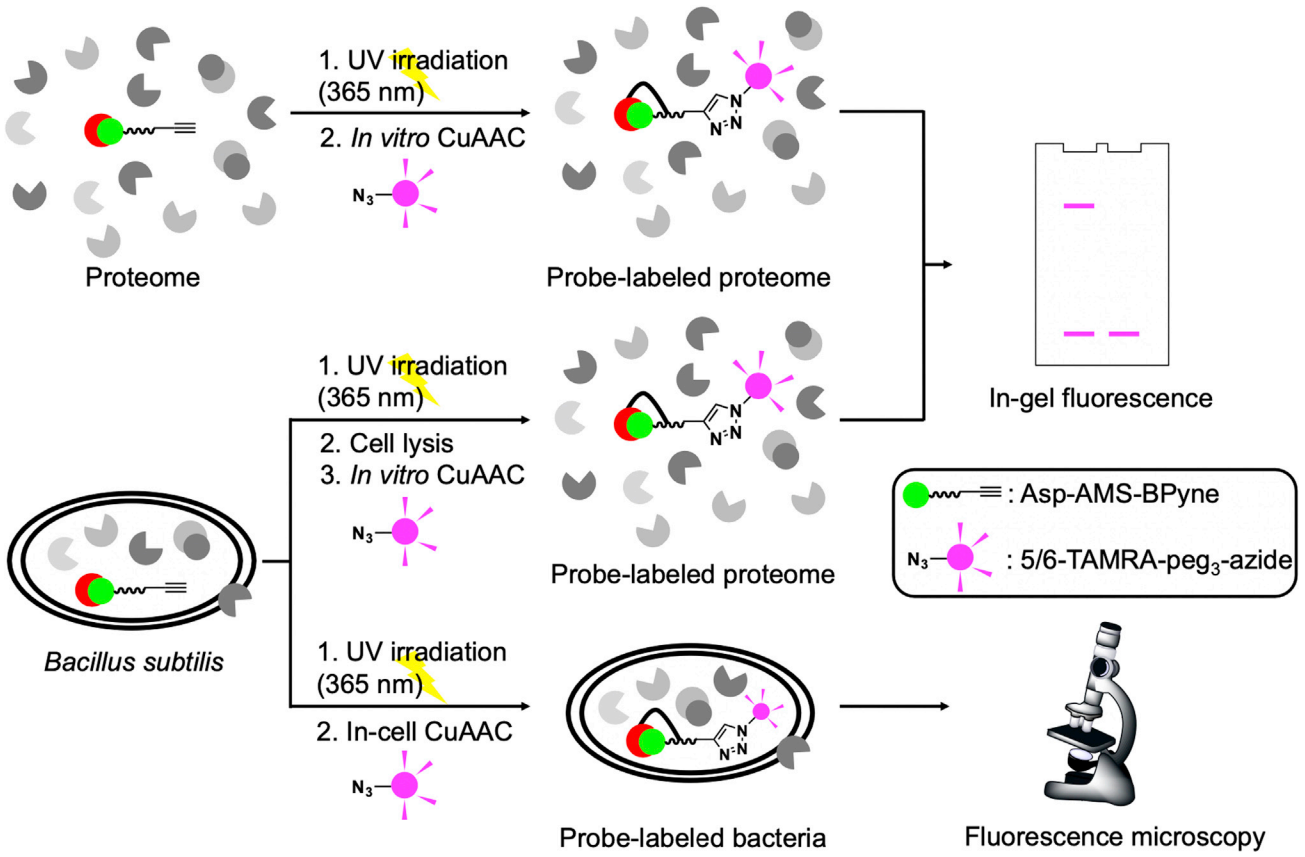

Figure 1. Schematic illustration of fluorescence labeling and imaging of an endogenous SrfAB-NRPS

For *in vitro* labeling of an endogenous SrfAB-NRPS, **Asp-AMS-BPyne** was added to bacterial proteomes. The samples were then irradiated with UV light (365 nm), reacted with the 5/6-TAMRA-peg$_3$-azide dye under Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAc), and visualized by SDS-PAGE coupled with in-gel fluorescence imaging. For in-cell labeling studies, *Bacillus subtilis* ATCC 21332 was cultured, collected, and incubated with **Asp-AMS-BPyne**. After washing, the bacterial cells were UV-irradiated, lysed, treated with the 5/6-TAMRA-peg$_3$-azide dye, and analyzed by SDS-PAGE. For fluorescence cell imaging, after photoaffinity crosslinking in living bacterial cells using **Asp-AMS-BPyne**, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, reacted with the 5/6-TAMRA-peg$_3$-azide dye under live-cell CuAAC conditions, and visualized by fluorescence microscopy. Reprinted with permission from Ishikawa et al. (2022).

attachment of an alkyne functionality enables the use of copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) to conjugate a reporter group for downstream analysis. **Asp-AMS-BPyne** (Ishikawa et al., 2022) and **Asp-AMS** (Kasai et al., 2015) are not commercially available and require moderate synthetic expertise. For complete details on both compounds, please refer to Ishikawa et al. (2022).

Note: Unless otherwise specified, all reagents were purchased from chemical suppliers and used without further purification.

Preparing reaction apparatus for moisture-sensitive reactions

Timing: 1 h

This section outlines how to set up a reaction apparatus for chemical synthesis, which is necessary to perform the reaction in a water-free environment under an inert atmosphere.

1. Prepare a reaction apparatus containing a two-neck round-bottom flask, a stirring bar, a rubber septum, and a balloon for Ar gas.
2. Dry the apparatus using a heating gun in *vacuo*.

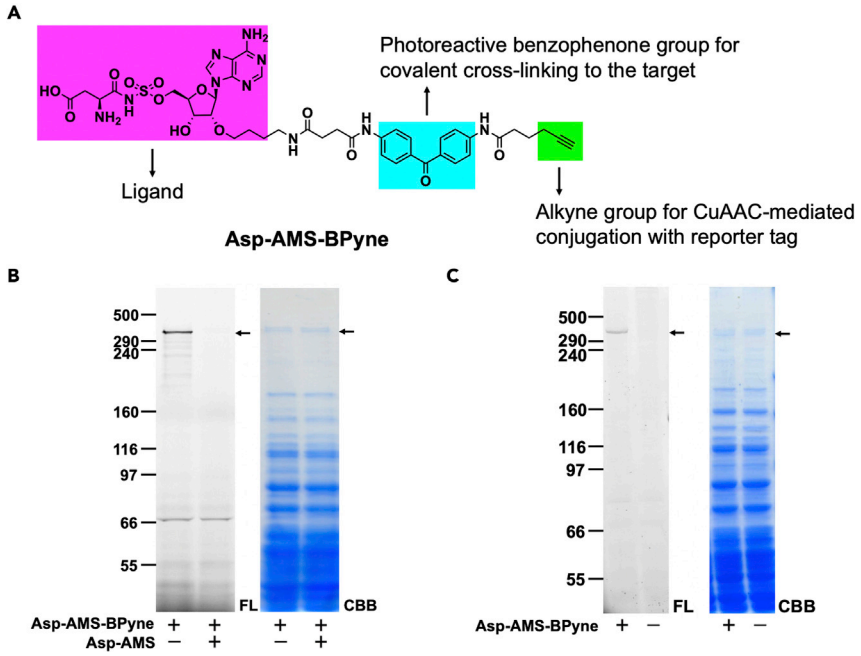

Figure 2. Characterization of the PAL-based ABPP probe Asp-AMS-BPyne

(A) Design of PAL-based ABPP probe **Asp-AMS-BPyne**. The probe consists of **Asp-AMS** for tight binding to the Asp-activating A-domains of NRPSs, a photoreactive benzophenone (BP) for covalent cross-linking to the targets, and a terminal alkyne (yne) for CuAAC-mediated conjugation with reporter tag. Chemical modifications at the 2′-OH group of the ribose sugar were tolerated.

(B) Labeling of endogenous SrfAB-NRPS in the cellular lysate of *B. subtilis* ATCC 21332. The *B. subtilis* ATCC 21332 lysate (2.0 mg/mL) was treated with 1 μM **Asp-AMS-BPyne** in either the absence or presence of **Asp-AMS** (100 μM).

(C) In-cell labeling of endogenous SrfAB-NRPS in *B. subtilis* ATCC 21332. *B. subtilis* ATCC 21332 cells were cultured (OD_{600} = 1.54) and treated with 10 μM **Asp-AMS-BPyne** or DMSO (vehicle). The arrows in (B) and (C) point to the endogenous SrfAB-NRPS. The gels were visualized by in-gel fluorescence (FL) and Coomassie brilliant blue (CBB) staining. Gel data with permission from Ishikawa et al. (2022).

3. After cooling to room temperature (20°C–25°C), fill the balloon and apparatus with Ar gas.

Preparing Asp-AMS-BPyne and Asp-AMS

Timing: 1 h

4. Preparation of stock solutions of **Asp-AMS-BPyne** and **Asp-AMS**.
   a. Prepare stock solutions of 100 μM and 10 mM **Asp-AMS-BPyne** in DMSO.
   b. Prepare stock solutions of 10 mM **Asp-AMS** in DMSO.
   c. Make 10–50 μL aliquots of the stock solution.
   d. Store the stock solutions at −80°C for long-term use (1–24 months).

Note: The compounds are readily soluble in DMSO at these concentrations and dissolved by pipetting up and down. The aliquots can be refrozen after thawing.

Preparing exponentially growing cells for *in vitro* and in cell labeling studies

Timing: 3 days

This section outlines the preparation of bacterial cells for *in vitro* and in cell labeling studies.

5. Streak out *Bacillus subtilis* ATCC 21332 from glycerol stock (−80°C) on nutrient agar plates on a clean bench and incubate at 37°C for 16–24 h.
6. Prepare a pre-culture by inoculating a single colony from the previously prepared nutrient agar plates in 5 mL cation-adjusted Mueller-Hinton II broth in an aerated and sterile test tube (35 mL) on a clean bench.
7. Grow at 37°C with 200 rpm shaking for 16–24 h.
8. Inoculate the appropriate volume of *B. subtilis* ATCC 21332 pre-cultures to provide a starting OD_{600} = 0.020–0.060 in 250 mL cation-adjusted Mueller-Hinton II broth in a 1000-mL baffled flask on a clean bench.
9. Grow at 30°C with 250 rpm shaking until the culture reaches the indicated OD_{600} values (OD_{600} = 0.71–1.56).

Note: We have chosen this condition as the bacterium grows slowly at 30°C and displays a steady growth curve. However, growing the bacterium at 37°C will not hinder the experiment.

Preparing exponentially growing cells for fluorescence imaging studies

Timing: 4 days

This section outlines the preparation of bacterial cells for live-cell imaging. For imaging of the surfactin-NRPSs, iron-enriched minimal salt medium is preferred over cation-adjusted Mueller-Hinton II broth. This medium is described to be highly effective for surfactin production (Wei et al., 2004; Yeh et al., 2005), enabling optimal surfactin yields of up to 3,600 mg/L in this medium (Wei et al., 2004; Yeh et al., 2005). The increased levels of surfactin should correlate with the expression levels of surfactin-NRPSs, which results in successful imaging with a high signal-to-noise ratio.

10. Streak out *B. subtilis* ATCC 21332 from glycerol stock (−80°C) on nutrient agar plates on a clean bench and incubate at 37°C for 16–24 h.
11. Prepare a pre-culture by inoculating a single colony from the previously prepared nutrient agar plates in 5 mL iron-enriched minimal salt medium (for seed culture) supplemented with 2.5 μL of 8 mM EDTA·2Na·$2H_2O$ and 8 mM $FeSO_4 \cdot 7H_2O$ mixed solution in an aerated and sterile test tube (35 mL) on a clean bench.
12. Grown at 30°C with 200 rpm shaking for 24–48 h.
13. Inoculate the appropriate volume of *B. subtilis* ATCC 21332 pre-cultures to provide a starting OD_{600} = 0.040–0.060 in 250 mL iron-enriched minimal salt medium (for large-scale cultivation) in a 1000-mL baffled flask on a clean bench.
14. Grow at 30°C with 200 rpm shaking until the culture reaches the indicated OD_{600} values (OD_{600} = 0.92).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Bacillus subtilis	ATCC	ATCC 21332
Chemicals, peptides, and recombinant proteins		
Compound **4**	Ishikawa and Kakeya, 2014	N/A
Compound **5**	Ishikawa et al. (2022)	N/A
Compound **6**	Ishikawa et al. (2022)	N/A
Compound **7**	Ishikawa et al. (2022)	N/A
Compound **8**	Konno et al. (2015)	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Asp-AMS-BPyne	Ishikawa et al. (2022)	N/A
Compound **10**	Ishikawa and Kakeya, 2014	N/A
Compound **11**	Kasai et al. (2015)	N/A
Asp-AMS	Kasai et al. (2015)	N/A
SiliaFlash Irregular Silica Gels F60, 40–60 μm, 60 Å	SiliCycle	Cat# R10030B
TLC Silica gel 60 F_{254} 25 Glass plates 20 × 20 cm	Merck	Cat# 100390
Boc-Asp(O*t*Bu)-OSu	FUJIFILM Wako	Cat# 4003402.0001
Cesium carbonate	Nacalai Tesque	Cat# 07805-02
Palladium 10% on carbon (wetted with *ca.* 55% water)	Tokyo Chemical Industry	Cat# P1785
Ar gas	Iwatani Fine Gas Co., Ltd.	N/A
H_2 gas	Iwatani Fine Gas Co., Ltd.	N/A
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride	Nacalai Tesque	Cat# 15022-44
1-Hydroxybenzotriazole monohydrate	Tokyo Chemical Industry	Cat# H0468
Citric acid, anhydrous	Nacalai Tesque	Cat# 09109-85
Sodium hydrogen carbonate	Nacalai Tesque	Cat# 31212-12
Sodium chloride	Nacalai Tesque	Cat# 31320-76
Sodium sulfate	Nacalai Tesque	Cat# 31916-86
Celite(R)545RVS	Nacalai Tesque	Cat# 08034-85
Methanol D4 "100%" for NMR studies	Eurisotop	Cat# D048B
Dimethylsulfoxide D6 for NMR studies	Eurisotop	Cat# D034B
Ethyl acetate	Nacalai Tesque	Cat# 14623-04
Methanol	Nacalai Tesque	Cat# 21915-64
Chloroform	Nacalai Tesque	Cat# 08401-23
N,*N*-Dimethylformamide, super dehydrated	FUJIFILM Wako	Cat# 049-32363
Dimethyl sulfoxide	Nacalai Tesque	Cat# 08904-85
Milli-Q water	Merck	Direct-Q UV3
Trifluoroacetic acid (TFA)	Nacalai Tesque	Cat# 34833-05
Nutrient Broth	BD	Cat# 234000
Mueller Hinton II Broth (Cation-Adjusted)	BD	Cat# 212322
Iron-enriched minimal salt medium	Ishikawa et al. (2022)	Wei et al. (2004) Yeh et al. (2005)
Agar powder	Nacalai Tesque	Cat# 01028-85
D-(+)-glucose	Nacalai Tesque	Cat# 13057-35
Ammonium nitrate	Nacalai Tesque	Cat# 02524-45
Disodium hydrogen phosphate	Nacalai Tesque	Cat# 31738-55
Potassium dihydrogen phosphate	Nacalai Tesque	Cat# 28736-75
Calcium chloride dihydrate	Nacalai Tesque	Cat# 08895-15
Magnesium sulfate heptahydrate	Nacalai Tesque	Cat# 06296-25
Ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid disodium salt dihydrate	Nacalai Tesque	Cat# 15111-45
Iron(II) sulfate heptahydrate	Nacalai Tesque	Cat# 19532-15
Glycerol	Nacalai Tesque	Cat# 17045-65
Tris(hydroxymethyl)aminomethane	Nacalai Tesque	Cat# 35401-25
Magnesium chloride hexahydrate	Nacalai Tesque	Cat# 20909-55
Tris(2-carboxyethyl)phosphine HCl (TCEP)	Nacalai Tesque	Cat# 07277-16
Lysozyme, from Egg White	FUJIFILM Wako	Cat# 127-06724
Recombinant DNase I (RNase-free)	Takara	Cat# 2270A
Protease inhibitor cocktail for general use (100×)	Nacalai Tesque	Cat# 04080-11
Tissue culture plate, 96 well, flat bottom with low evaporation lid	Falcon	Cat# 353072
5/6-TAMRA-peg$_3$-azide	Jena Bioscience	Cat# CLK-AZ109-1
Bovine albumin	Nacalai Tesque	Cat# 01281-97
Tris[(1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl]amine (TBTA)	Tokyo Chemical Industry	Cat# T2993
Copper(II) sulfate pentahydrate	Nacalai Tesque	Cat# 09605-75
Sodium dodecyl sulfate (SDS)	Nacalai Tesque	Cat# 08933-05
Ammonium persulfate	Nacalai Tesque	Cat# 02627-34
N,*N*,*N*′,*N*′-Tetramethylethylenediamine	Nacalai Tesque	Cat# 33401-72

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Dithiothreitol (DTT)	Nacalai Tesque	Cat# 14128-62
40 (w/v)%-Acrylamide/bis mixed solution (29:1)	Nacalai Tesque	Cat# 06141-45
Wide range gel preparation buffer (4×) for PAGE	Nacalai Tesque	Cat# 07831-94
Coomassie brilliant blue G-250	Nacalai Tesque	Cat# 09408-52
Bromophenol blue (BPB)	Nacalai Tesque	Cat# 05808-61
Molecular weight ladder	Thermo Fisher Scientific	Cat# LC5688
Molecular weight ladder	Cytiva	Cat# RPN851E
Protein assay CBB solution (5×)	Nacalai Tesque	Cat# 29449-15
Dulbecco's phosphate-buffered saline (PBS)	Nissui Pharmaceutical	Cat# 05913
Triton X-100	Nacalai Tesque	Cat# 12967-45
4% Paraformaldehyde phosphate buffer solution	FUJIFILM Wako	Cat# 163-20145
Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA)	Sigma-Aldrich	Cat# 762342
Aminoguanidine hydrochloride	Tokyo Chemical Industry	Cat# A1129
L-Ascorbic acid sodium salt	Nacalai Tesque	Cat# 11692-52
Ethanol (99.5)	Nacalai Tesque	Cat# 08948-54
Ethanol (99.5)	Nacalai Tesque	Cat# 14712-34
Phosphoric acid	Nacalai Tesque	Cat# 27617-94
Ammonium sulfate	Nacalai Tesque	Cat# 02633-15
Acetic acid	Nacalai Tesque	Cat# 08885-45
Glycine	Nacalai Tesque	Cat# 17141-95
Software and algorithms		
ChemDraw Professional 19.1	PerkinElmer	https://www.perkinelmer.com/category/chemdraw
Other		
NMR spectrometer (ECA-500)	JEOL	N/A
LCMS-IT-TOF	Shimadzu	N/A
Personal incubator	AS ONE	N/A
BioShaker (BR-43FL)	TAITEC	N/A
High speed refrigerated micro centrifuge (MX-307)	TOMY	N/A
Multiscan FC	Thermo Fisher Scientific	N/A
UVP crosslinker	Analytik Jena	N/A
Typhoon 9410 gel and blot imager	GE Healthcare	N/A
All-in-One fluorescence microscope BZ-X 700/710	KEYENCE	N/A

MATERIALS AND EQUIPMENT

80% (v/v) TFA solution, 2 mL; make fresh before use.

Asp-AMS-BPyne stock solution		
Reagent	Final concentration	Amount
Asp-AMS-BPyne	10 mM	0.92 mg
DMSO	n/a	n/a
Total	**n/a**	100 μL
Aliquot; can store several months at −80°C.		

Asp-AMS-BPyne stock solution		
Reagent	Final concentration	Amount
Asp-AMS-BPyne (10 mM)	100 μM	10 μL
DMSO	n/a	90 μL
Total	**n/a**	100 μL
Aliquot; can store several months at −80°C.		

Asp-AMS stock solution		
Reagent	Final concentration	Amount
Asp-AMS	100 mM	4.6 mg
DMSO	n/a	n/a
Total	**n/a**	100 µL
Aliquot; can store several months at −80°C.		

Asp-AMS stock solution		
Reagent	Final concentration	Amount
Asp-AMS (100 mM)	10 mM	10 µL
DMSO	n/a	90 µL
Total	**n/a**	100 µL
Aliquot; can store several months at −80°C.		

Cation-adjusted Mueller-Hinton II broth		
Reagent	Final concentration	Amount
Mueller Hinton II Broth (Cation-Adjusted)	n/a	11 g
Milli-Q water	n/a	n/a
Total	**n/a**	**500 mL**
Autoclave the glass bottle; can store several months at room temperature (20°C–25°C).		

Cation-adjusted Mueller-Hinton II broth		
Reagent	Final concentration	Amount
Mueller Hinton II Broth (Cation-Adjusted)	n/a	5.5 g
Milli-Q water	n/a	n/a
Total	**n/a**	**250 mL**
Autoclave in 1000-mL baffled flask; can store several months at room temperature (20°C–25°C).		

NH_4NO_3 solution		
Reagent	Final concentration	Amount
NH_4NO_3	500 mM	40.0 g
Milli-Q water	n/a	n/a
Total	**n/a**	**1000 mL**
Autoclave in glass bottle; can store several months at room temperature (20°C–25°C).		

Na_2HPO_4 solution		
Reagent	Final concentration	Amount
Na_2HPO_4	300 mM	42.6 g
Milli-Q water	n/a	n/a
Total	**n/a**	**1000 mL**
Autoclave in glass bottle; can store several months at room temperature (20°C–25°C).		

KH_2PO_4 solution		
Reagent	Final concentration	Amount
KH_2PO_4	300 mM	40.8 g
Milli-Q water	n/a	n/a
Total	**n/a**	**1000 mL**
Autoclave in glass bottle; can store several months at room temperature (20°C–25°C).		

$CaCl_2$ solution		
Reagent	Final concentration	Amount
$CaCl_2 \cdot 2H_2O$	7 mM	103 mg
Milli-Q water	n/a	n/a
Total	**n/a**	**100 mL**
Autoclave in glass bottle; can store several months at room temperature (20°C–25°C).		

$MgSO_4$ solution		
Reagent	Final concentration	Amount
$MgSO_4 \cdot 7H_2O$	800 mM	19.7 g
Milli-Q water	n/a	n/a
Total	**n/a**	**100 mL**
Autoclave in glass bottle; can store several months at room temperature (20°C–25°C).		

EDTA and $FeSO_4$ mixed solution		
Reagent	Final concentration	Amount
$EDTA \cdot 2Na \cdot 2H_2O$	8 mM	3.0 g
$FeSO_4 \cdot 7H_2O$	8 mM	2.2 g
Milli-Q water	n/a	n/a
Total	**n/a**	**100 mL**
Autoclave in glass bottle; can store several months at 4°C.		

Note: The mixed solution dissolves easily. Furthermore, the pH does not need to be adjusted for the mixed solution to dissolve completely.

Glucose solution		
Reagent	Final concentration	Amount
D-(+)-glucose		40 g
Milli-Q water	n/a	n/a
Total	**n/a**	**698 mL**
Filter sterilize; can store several months at room temperature (20°C–25°C).		

Iron-enriched minimal salt medium for seed culture		
Reagent	Final concentration	Amount
Glucose solution (see above)	40 g/L	698 mL
NH_4NO_3 solution (500 mM)	50 mM	100 mL
Na_2HPO_4 solution (300 mM)	30 mM	100 mL
KH_2PO_4 solution(300 mM)	30 mM	100 mL
$CaCl_2$ solution (7 mM)	7 μM	1 mL
$MgSO_4$ solution (800 mM)	800 μM	1 mL
EDTA and $FeSO_4$ mixed solution (8 mM)	4 μM	500 μL
Total	**n/a**	**1000 mL**
Mix solution on a clean bench; can store several months at room temperature (20°C–25°C).		

Glucose solution		
Reagent	Final concentration	Amount
D-(+)-glucose		10 g
Milli-Q water	n/a	n/a
Total	**n/a**	**112 mL**
Filter sterilize; can store several months at room temperature (20°C–25°C).		

Iron-enriched minimal salt medium for large-scale cultivation		
Reagent	Final concentration	Amount
Glucose solution (see above)	40 g/L	112 mL
NH_4NO_3 solution (500 mM)	50 mM	25 mL
Na_2HPO_4 solution (300 mM)	30 mM	25 mL
KH_2PO_4 solution(300 mM)	30 mM	25 mL
$CaCl_2$ solution (7 mM)	7 μM	250 μL
$MgSO_4$ solution (800 mM)	800 μM	250 μL
EDTA and $FeSO_4$ mixed solution (8 mM)	2 mM	62.5 mL
Total	**n/a**	**250 mL**
Mix solution on a clean bench; can store several months at room temperature (20°C–25°C).		

Nutrient agar plates		
Reagent	Final concentration	Amount
Nutrient broth	n/a	8.0 g
Agar powder	1.5% (w/v)	15 g
Milli-Q water	n/a	n/a
Total	**n/a**	**1000 mL**
Can store several months at 4°C.		

Note: Autoclave the dissolved mixture and pour nutrient agar into each plate.

PBS buffer (-); can store several months at room temperature (20°C–25°C).

20 mM Tris-HCl buffer (pH 8.0); can store several months at room temperature (20°C–25°C).

2 M Tris-HCl buffer (pH 6.8); can store several months at room temperature (20°C–25°C).

1 M $MgCl_2$ solution, 10 mL; can store several months at room temperature (20°C–25°C).

1 M TCEP stock solution, 10 mL; can store several months at 4°C.

50 mM TCEP stock solution, 10 mL; aliquot and can store several months at −80°C.

T1 solution		
Reagent	Final concentration	Amount
$MgCl_2$ solution (1 M)	1 mM	50 μL
TCEP solution (1 M)	1 mM	50 μL
Tris-HCl buffer (pH8.0) (20 mM)	n/a	49.9 mL
Total	**n/a**	**50 mL**
Can store several months at 4°C.		

Protease inhibitor cocktail solution, 1 mL; aliquot and can store several months at −80°C.

Lysozyme solution		
Reagent	Final concentration	Amount
Lysozyme, from Egg White	10 mg/mL	10 mg
Tris-HCl buffer (pH8.0) (20 mM)	n/a	n/a
Total	**n/a**	**1 mL**
Make fresh before use.		

Protein assay CBB solution, 50 mL; can store several months at 4°C.

80% (v/v) Glycerol solution; can store several months at room temperature.

5/6-TAMRA-peg$_3$-azide stock solution		
Reagent	Final concentration	Amount
5/6-TAMRA-PEG$_3$-azide	20 mM	1 mg
DMSO	n/a	n/a
Total	**n/a**	**79.2 μL**
Aliquot; can store several months in the dark at −80°C.		

5/6-TAMRA-peg$_3$-azide stock solution		
Reagent	Final concentration	Amount
5/6-TAMRA-PEG$_3$-azide (20 mM)	5 mM	10 μL
DMSO	n/a	30 μL
Total	**n/a**	**40 μL**
Aliquot; can store several months in the dark at −80°C.		

TBTA stock solution		
Reagent	Final concentration	Amount
TBTA	20 mM	10.6 mg
DMSO	n/a	n/a
Total	**n/a**	**1 mL**
Aliquot; can store several months at −80°C.		

TBTA stock solution		
Reagent	Final concentration	Amount
TBTA (20 mM)	5 mM	250 μL
DMSO	n/a	750 μL
Total	**n/a**	**1 mL**
Aliquot; can store several months at −80°C.		

$CuSO_4$ stock solution		
Reagent	Final concentration	Amount
$CuSO_4 \cdot 5H_2O$	100 mM	25 mg
Milli-Q water	n/a	n/a
Total	**n/a**	**1 mL**
Aliquot; can store several months at −80°C.		

THPTA stock solution		
Reagent	Final concentration	Amount
THPTA	50 mM	2.2 mg
Milli-Q water	n/a	n/a
Total	**n/a**	**100 μL**
Can store several months at 4°C.		

$CuSO_4$ stock solution		
Reagent	Final concentration	Amount
$CuSO_4$ solution (100 mM)	20 mM	20 μL
Milli-Q water	n/a	80 μL
Total	**n/a**	**100 μL**
Can store several months at 4°C.		

Sodium ascorbate stock solution		
Reagent	Final concentration	Amount
Sodium ascorbate	100 mM	17.6 mg
Milli-Q water	n/a	n/a
Total	**n/a**	**1 mL**
Make fresh before use.		

Aminoguanidine stock solution, 100 mM, 1 mL; make fresh before use.

***In vitro* CuAAC click chemistry mix**		
Reagent	Final concentration	Amount
$CuSO_4$ (50 mM)	12.5 mM	10 μL
TBTA (5 mM)	1.25 mM	10 μL
TCEP (50 mM)	12.5 mM	10 μL
5/6-TAMRA-PEG_3-azide (5 mM)	1.25 mM	10 μL
Total	**n/a**	**40 μL**
Make fresh before use.		

⚠ CRITICAL: When preparing the CuAAC chemistry master mix, add reagents in the order listed in the table above to ensure proper copper reduction and formation of a complex between copper and ligand.

Note: Make enough master mix for at least one additional sample than necessary, to safeguard against volume loss during pipetting.

10% SDS solution; can store several months at room temperature (20°C–25°C).

10% (w/v) Ammonium persulfate solution, 10 mL; aliquot and can store several months at −30°C.

5× SDS sample loading buffer; can store several months at room temperature (20°C–25°C).

Note: Add dithiothreitol into the required volume of the buffer before use. Bromophenol blue is just used as an indicator for electrophoresis to determine the progress of the run.

3% SDS-PAGE gel (stacking gel); make fresh before use.

6% SDS-PAGE gel (separating gel); make fresh before use.

10× Running buffer; can store several months at room temperature (20°C–25°C) and dilute to 1× before use.

CBB fixing solution		
Reagent	Final concentration	Amount
Methanol	20% (v/v)	400 mL
Acetic acid	7.5% (v/v)	150 mL
Milli-Q water	n/a	1450 mL
Total	**n/a**	**2000 mL**
Can store several months at room temperature (20°C–25°C).		

CBB staining solution		
Reagent	Final concentration	Amount
Ammonium sulfate	10% (w/v)	100 g
Coomassie brilliant blue G-250	0.1% (w/v)	1 g
Phosphoric acid	3% (v/v)	30 mL
Ethanol (99.5)	20% (v/v)	200 mL
Milli-Q water	n/a	n/a
Total	**n/a**	**1000 mL**
Can store several months at room temperature (20°C–25°C).		

50% (v/v) Ethanol solution I; make fresh before use.

80% (v/v) Ethanol solution II; make fresh before use.

96% (v/v) Ethanol solution III; make fresh before use.

0.3% (v/v) Triton X-100 solution; make fresh before use.

STEP-BY-STEP METHOD DETAILS

Synthesis of compound 5

Timing: 2 days

This method outlines the preparation of compound **5** (Figure S1. Synthetic route to **Asp-AMS-BPyne**, related to steps 1–11).

1. Weigh 50 mg (0.090 mmol) of compound **4** (Konno et al., 2015) in a 10 mL dried two-neck round-bottom flask containing a magnetic stirring bar under Ar.
2. Add 1 mL of dry *N*,*N*-dimethylformamide to the flask.
3. Add 46 mg (0.12 mmol) of Boc-Asp(O^{t}Bu)-OSu and 88 mg (0.27 mmol) of cesium carbonate to the flask.
4. Stir the solution at room temperature (20°C–25°C) for 1 h.
5. Filter the reaction mixture through a pad of Celite.
6. Dilute the filtrate with 10 mL of ethyl acetate.
7. Wash the organic layer with 5% aqueous citric acid (20 mL), saturated sodium bicarbonate (20 mL), and saturated sodium chloride (3 × 10 mL) using a separatory funnel.
8. Dry the organic layer with sodium sulfate and filter to remove the sodium sulfate.
9. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.
10. Purify the crude product using silica gel column chromatography (6/94 to 9/91 methanol/chloroform) to obtain compound **5** (64 mg, 0.077 mmol, yield 86%, R_f = 0.62 [methanol/chloroform/H_2O = 67/25/8]) as a colorless solid.

11. Characterize the product using ^{1}H and ^{13}C NMR spectroscopy and ESI-MS. (See the expected outcomes section).

Pause point: At this point, the product can be stored at a temperature between −20 and −80°C for several months.

Synthesis of compound 6

Timing: 3 days

This method outlines the preparation of compound **6** (Figure S1. Synthetic route to **Asp-AMS-BPyne**, related to steps 12–19).

12. Weigh 59 mg (0.071 mmol) of compound **5** in a 10 mL dried two-neck round-bottom flask containing a magnetic stirring bar under Ar.
13. Add 4 mL of methanol to the flask.
14. Add 6 mg of palladium 10% on carbon to the flask.
15. Stir the solution at room temperature (20°C–25°C) under a H_2 atmosphere for 18 h.
16. Filter the reaction mixture through a pad of Celite.
17. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.
18. Purify the crude product using silica gel column chromatography (methanol/chloroform = 14/86) to obtain compound **6** (40 mg, 0.050 mmol, yield 70%, R_f = 0.50 [methanol/chloroform/H_2O = 67/25/8]) as a white solid.
19. Characterize the product using ^{1}H and ^{13}C NMR spectroscopy and ESI-MS. (See the expected outcomes section).

Pause point: At this point, the product can be stored at a temperature between −20 and −80°C for several months.

Synthesis of compound 7

Timing: 2 days

This method outlines the preparation of compound **7** (Figure S1. Synthetic route to **Asp-AMS-BPyne**, related to steps 20–34).

20. Weigh 18 mg (0.044 mmol) of compound **6** in a 10 mL dried two-neck round-bottom flask containing a magnetic stirring bar under Ar.
21. Add 1 mL of dry *N*,*N*-dimethylformamide to the flask.
22. Add 6.7 mg (0.044 mmol) of 1-hydroxybenzotriazole and 7.7 mg (0.044 mmol) of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride to the flask.
23. After stirring at room temperature (20°C–25°C) for 10 min, add 30 mg (0.037 mmol) of compound **8** (Konno et al., 2015) to the flask.
24. Stir the solution at room temperature (20°C–25°C) for 3 h.
25. Extract the reaction mixture with ethyl acetate (3 × 100 mL) using a separatory funnel to obtain an organic layer.
26. Wash the organic layer with saturated sodium chloride (3 × 300 mL).
27. Dry the organic layer with sodium sulfate and filter to remove the sodium sulfate.
28. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.
29. Dilute the reaction mixture with 10 mL of ethyl acetate.

30. Wash the organic layer with 5% aqueous citric acid (20 mL), saturated sodium bicarbonate (20 mL), and saturated sodium chloride (3 × 10 mL) using a separatory funnel.
31. Dry the organic layer with sodium sulfate and filter to remove the sodium sulfate.
32. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.
33. Purify the crude product using silica gel column chromatography (9/91 to 17/83 methanol/chloroform) to obtain compound **7** (35 mg, 0.029 mmol, yield 79%, R_f = 0.53 [methanol/chloroform/H_2O = 67/25/8]) as a white solid.
34. Characterize the product using ^1H and ^{13}C NMR spectroscopy and ESI-MS. (See the expected outcomes section).

Pause point: At this point, the product can be stored at a temperature between −20 and −80°C for several months.

Synthesis of Asp-AMS-BPyne

Timing: 3 days

This method outlines the preparation of **Asp-AMS-BPyne** (Figure S1. Synthetic route to **Asp-AMS-BPyne**, related to steps 35–40).

35. Weigh 30 mg (0.025 mmol) of compound **7** in a 10 mL single-neck round-bottom flask containing a magnetic stirring bar.
36. Add 2 mL of a 4:1 (v/v) mixture of trifluoroacetic acid and H_2O to the flask.
37. Stir the solution at room temperature (20°C–25°C) for 10 h.
38. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.
39. Purify the crude product using silica gel column chromatography (20/80 to 33/67 methanol/chloroform) to obtain **Asp-AMS-BPyne** (20 mg, 0.022 mmol, yield 87%, R_f = 0.11 [methanol/chloroform/H_2O = 67/25/8]) as a white solid.
40. Characterize the product using ^1H and ^{13}C NMR spectroscopy and ESI-MS. (See the expected outcomes section).

Pause point: At this point, the product can be stored at a temperature between −20 and −80°C for several months.

Synthesis of compound 11

Timing: 2 days

This method outlines the preparation of compound **11** (Figure S2. Synthetic route to **Asp-AMS**, related to steps 41–48).

41. Weigh 50 mg (0.13 mmol) of compound **10** (Ishikawa and Kakeya, 2014) in a 10 mL single-neck round-bottom flask containing a magnetic stirring bar.
42. Add 2 mL of dry *N*,*N*-dimethylformamide to the flask.
43. Add 46 mg (0.12 mmol) of Boc-Asp(O^tBu)-OSu and 127 mg (0.39 mmol) of cesium carbonate to the flask.
44. Stir the solution at room temperature (20°C–25°C) for 3 h.
45. Filter the reaction mixture through a pad of Celite.
46. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.

47. Purify the crude product using silica gel column chromatography (6/94 methanol/chloroform) to obtain compound **11** (50 mg, 0.076 mmol, yield 58%, R_f = 0.51 [methanol/chloroform/H_2O = 67/25/8]) as a white solid.
48. Characterize the product using ^1H and ^{13}C NMR spectroscopy and ESI-MS. (See the expected outcomes section).

Pause point: At this point, the product can be stored at a temperature between −20 and −80°C for several months.

Synthesis of Asp-AMS

Timing: 3 days

This method outlines the preparation of **Asp-AMS** (Figure S2. Synthetic route to **Asp-AMS**, related to steps 49–54).

49. Weigh 40 mg (0.053 mmol) of compound **11** in a 10 mL single-neck round-bottom flask containing a magnetic stirring bar.
50. Add 2 mL of a 4:1 (v/v) mixture of trifluoroacetic acid and H_2O to the flask.
51. Stir the solution at room temperature (20°C–25°C) for 6 h.
52. Remove the volatile material using a rotary evaporator under reduced pressure to obtain a crude product.
53. Purify the crude product using silica gel column chromatography (33/67 methanol/chloroform to methanol) to obtain compound **Asp-AMS** (26 mg, 0.056 mmol, quant., R_f = 0.01 [methanol/chloroform/H_2O = 67/25/8]) as a white solid.
54. Characterize the product using ^1H and ^{13}C NMR spectroscopy and ESI-MS. (See the expected outcomes section).

Pause point: At this point, the product can be stored at a temperature between −20 and −80°C for several months.

Sample preparation for *in vitro* labeling studies

Timing: 2 h

This method outlines the preparation of samples for proteomics analysis.

55. Grow *B. subtilis* ATCC 21332 at 30°C with 250 rpm shaking in 250 mL cation-adjusted Mueller-Hinton II broth in a 1000-mL baffled flask.
56. Harvest the bacterial cells after 12 h (OD_{600} = 0.71) by centrifugation (10 min, 10,000 × g, 4°C).
57. Remove and discard the supernatant.
58. Wash twice with ice-cold phosphate-buffered saline (PBS) (-), resuspend in 10 mL of PBS (-), and aliquot in 1.5 mL Eppendorf tubes.
59. Centrifuge the solution (10 min, 10,000 × g, 4°C) to pellet the bacterial cells.
60. Remove the maximum possible volume of PBS using a pipette.
61. Store the pellets at −80°C until use.

Pause point: At this point, the pellets can be stored at −80°C for several months.

62. Resuspend the frozen cell pellets in cold T1 solution with protease inhibitor cocktail solution (200–500 μL).
63. Add a 10 mg/mL lysozyme solution to the resuspended cells to a final concentration of 0.2 mg/mL.

64. Incubate the mixture on ice for 30 min with gentle shaking (Shaker SR-1, AS ONE Corporation) (100 rpm).
65. Transfer the mixture to a medium size constant temperature incubator shaker (TAITEC Corporation).
66. Incubate at 37°C for 30 min with gentle shaking (100 rpm).

⚠ CRITICAL: Large NRPS proteins were found to be particularly sensitive to mechanical cell disruption processes, and it is therefore necessary to treat the bacterial cells gently to obtain the intracellular proteins (Augenstein et al., 1974).

67. Centrifuge the mixture for 10 min at 15,000 × g at 4°C and collect the supernatant.

⚠ CRITICAL: If viscosity is observed in bacterial cell lysates due to DNA content after step 66, add DNase I (1 U), incubate on ice for 5 min, centrifuge the mixture for 10 min at 15,000 × g at 4°C, and collect the supernatant.

68. Determine protein concentration using Bradford assay in a 96-well plate.
    a. Prepare 10 μL serial dilutions (2×) of sample in T1 solution and pipet 2 μL of each solution into separate wells.
    b. For BSA protein standard, prepare 10 μL serial dilutions (2×) with protein concentrations 2, 1, 0.5, 0.25, and 0.13 mg/mL in T1 solution and pipet 2 μL of each solution into separate wells.
    c. Add 198 μL of protein assay CBB solution and incubate for 10 min at room temperature (20°C–25°C).
    d. Read absorbance at 595 nm using a Multiscan FC plate reader.
69. Normalize protein concentration to 2.0–3.0 mg/mL.

Pause point: Lysate can be aliquoted and frozen at −80°C in 10% glycerol for at least 1 month.

In vitro labeling studies

Timing: 2–3 h

This method outlines the labeling of an endogenous SrfAB-NRPS *in vitro*.

70. For labeling studies, treat the protein extracts (45 μL, 2.0–3.0 mg/mL) with DMSO (0.5 μL) and **Asp-AMS-BPyne** (0.5 μL of 100 μM stock in DMSO, final concentration: 1 μM) for 10 min at room temperature (20°C–25°C).
71. For inhibition studies, treat the protein extracts (45 μL, 2.0–3.0 mg/mL) with **Asp-AMS** (0.5 μL of 10 mM stock in DMSO, final concentration: 100 μM) for 10 min at room temperature (20°C–25°C) and subsequently incubate the mixture with **Asp-AMS-BPyne** (0.5 μL of 100 μM stock in DMSO, final concentration: 1 μM) for 10 min at room temperature (20°C–25°C).

⚠ CRITICAL: A 96-well plate is used to obtain reproducible photo-labeling.

⚠ CRITICAL: The final DMSO concentration is kept at 2.2% (v/v).

72. Perform UV irradiation of the 96-well plate using a UVP Crosslinker (Analytik Jena) (8 W, 365 nm, on ice, Figure 3). Troubleshooting 1.

⚠ CRITICAL: To obtain reproducible, efficient, and specific photoaffinity labeling, the following distance and time of labeling are used: 1 cm distance from the top of a 96-well plate and 5 min.

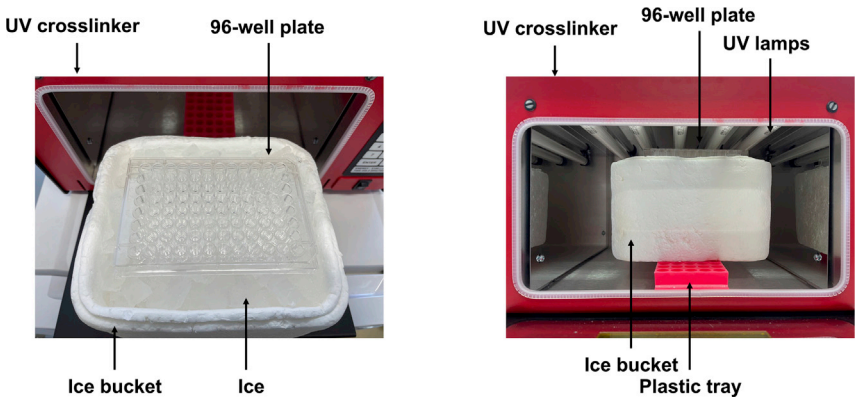

Figure 3. Apparatus for UV irradiation

CRITICAL: Wear proper personal protective equipment, such as UV goggles, lab gloves, and lab coat, when performing UV irradiation.

⚠ CRITICAL: Removing the 96-well plate lid will provide more efficient irradiation and, therefore, higher labeling efficiency.

73. Perform CuAAC chemistry reaction by adding 4 μL of *in vitro* CuAAC click chemistry mix into each well. Troubleshooting 2.

Note: $CuSO_4$, TBTA, TCEP, and 5/6-TAMRA-PEG_3-azide are added to provide final concentrations of 1 mM, 100 μM, 1 mM, and 100 μM, respectively.

74. Incubate each sample at room temperature (20°C–25°C) in the dark for 1 h.
75. Take 40 μL of each sample for SDS-PAGE and in-gel fluorescence analysis.
76. Add 10 μL of reducing 5× SDS-loading buffer to each sample, mix by pipetting up and down, and heat at 95°C for 5 min.

Pause point: Samples can be stored at −80°C for up to one week. In this case, perform the heating step again (95°C for 5 min) before loading on gel.

77. Place an SDS-PAGE (6%) gel in a clean plastic electrophoresis chamber and corresponding gel holder.
78. Prepare 1× SDS-PAGE running buffer (200 mL).
79. Fill the inner portion between the gel and the gel holder with the 1× running buffer.
80. Pour the remaining 1× running buffer into the outer chamber.
81. Load 20 μL of each sample on the gel.
82. Run the gel at 20 mA until the dye front migrates into the running gel (~10 min), and increase to 40 mA until the dye front reaches the bottom of the gel (~45 min).
83. Remove the gel from the apparatus and remove the spacers and glass plates.
84. Place the gel into a small tray.
85. Add ~100 mL Milli-Q water into the tray and wash the gel for 5 min with gentle shaking (50 rpm).
86. Scan the gel with a Typhoon 9410 Gel and Blot Imager (GE Healthcare) with 532 nm laser excitation and 580 nm emission using standard procedures (Figure 2B).
87. Pour off the Milli-Q water, add ~50 mL fixing solution into the tray, and fix the gel for 30 min with gentle shaking (50 rpm).
88. Pour off the fixing solution, add ~50 mL staining solution into the tray, and stain the gel with Colloidal Coomassie Blue Stain for > 6 h with gentle shaking (50 rpm).
89. Pour off the staining solution and add ~100 mL Milli-Q water into the tray.
90. Destain the gel with gentle shaking (50 rpm) until the gel is visibly destained (> 2 h).

In-cell labeling studies

Timing: 5–6 h

This method outlines the labeling of endogenous SrfAB-NRPS in living bacterial cells.

91. Grow *B. subtilis* ATCC 21332 at 30°C with 250 rpm shaking in 250 mL cation-adjusted Mueller-Hinton II broth in a 1000-mL baffled flask.
92. Harvest the bacterial cells (6 h, OD_{600} = 1.54) by centrifugation (10 min, 10,000 × g, 4°C).
93. Remove and discard the supernatant.
94. Wash twice with 1 mL of PBS at room temperature (20°C–25°C).
95. Transfer the bacterial cells to a 1.5 mL Eppendorf tube.
96. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
97. Add 400 μL of PBS to the bacterial cells and resuspend by pipetting.
98. Treat the bacterial cells with DMSO (0.4 μL) or **Asp-AMS-BPyne** (0.4 μL of 10 mM stock in DMSO, final concentration: 10 μM) for 60 min at room temperature (20°C–25°C).

⚠ CRITICAL: The final DMSO concentration is kept at 0.1%.

99. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
100. Remove and discard the supernatant.
101. Wash twice with 1 mL of PBS.
102. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
103. Add 100 μL of PBS to the bacterial cells and resuspend by pipetting.
104. Transfer the bacterial cells suspension to a 96-well plate.

⚠ CRITICAL: A 96-well plate is used for obtaining reproducible photo-labeling.

105. Perform UV irradiation of the 96-well plate using a UVP Crosslinker (Analytik Jena) (8 W, 365 nm, on ice, Figure 3). Troubleshooting 1.

⚠ CRITICAL: To obtain reproducible, efficient, and specific photoaffinity labeling, the following distance and time of labeling are used: 1 cm distance from the top of a 96-well plate and 5 min.

⚠ CRITICAL: Removing the 96-well plate lid will provide more efficient irradiation and therefore higher labeling efficiency.

106. Transfer the bacterial cells to a 1.5 mL Eppendorf tube.
107. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.

Pause point: At this point, the bacterial pellets can be stored at −80°C for up to one week.

108. Add 100 μL of cold T1 solution with protease inhibitor cocktail solution to the bacterial cell pellets and resuspend by pipetting.
109. Add 2 μL of 10 mg/mL lysozyme solution (final concentration: 0.2 mg/mL) to the resuspended cells.
110. Incubate the resulting mixture on ice for 30 min with gentle shaking (Shaker SR-1, AS ONE Corporation) (100 rpm).
111. Transfer the mixture to a medium size constant temperature incubator shaker (TAITEC Corporation). Incubate at 37°C for 30 min with gentle shaking (100 rpm).
112. Centrifuge the mixture for 10 min at 15,000 × g at 4°C and collect the supernatant.
113. Determine protein concentration using Bradford assay in a 96-well plate.

a. Prepare 4 μL of serial dilutions (2×) of the sample in T1 solution and pipet 2 μL of each solution into separate wells.
b. For BSA protein standard, prepare 10 μL serial dilutions (2×) with protein concentrations 2, 1, 0.5, 0.25, and 0.13 mg/mL in T1 solution. Pipet 2 μL of each solution into separate wells.
c. Add 198 μL of protein assay CBB solution and incubate for 10 min at room temperature (20°C–25°C).
d. Read absorbance at 595 nm using a Multiscan FC plate reader.

114. Normalize protein concentration to 2.0 mg/mL.
115. Transfer 46 μL of the resulting proteome samples to a 96-well plate.
116. Add 4 μL of *in vitro* CuAAC click chemistry mix into each well. Troubleshooting 2.

Note: $CuSO_4$, TBTA, TCEP, and 5/6-TAMRA-peg_3-azide are added to provide final concentrations of 1 mM, 100 μM, 1 mM, and 100 μM, respectively.

117. Incubate each sample at room temperature (20°C–25°C) in the dark for 1 h.
118. Take 40 μL of each sample for SDS-PAGE and in-gel fluorescence analysis.
119. Add 10 μL of reducing 5× SDS-loading buffer to each sample, mix by pipetting up and down, and heat at 95°C for 5 min.

Pause point: Samples can be stored at −80°C for up to one a week. In this case, perform the heating step again (95°C for 5 min) before loading on gel.

120. Place an SDS-PAGE (6%) gel in a clean plastic electrophoresis chamber and corresponding gel holder.
121. Prepare 1× SDS-PAGE running buffer (200 mL).
122. Fill the inner portion between the gel and the gel holder with the 1× running buffer. Pour the remaining 1× running buffer into the outer chamber.
123. Load 20 μL of each sample on the gel (6%).
124. Run the gel at 20 mA until the dye front migrates into the running gel (~10 min), and increase to 40 mA until the dye front reaches the bottom of the gel (~45 min).
125. Remove the gel from the apparatus and remove the spacers and glass plates.
126. Place the gel into a small tray.
127. Add ~100 mL Milli-Q water into the tray and wash the gel for 5 min with gentle shaking (50 rpm).
128. Scan the gel with a Typhoon 9410 Gel and Blot Imager (GE Healthcare) with 532 nm laser excitation and 580 nm emission using standard procedures (Figure 2C). expected outcomes.
129. Pour off the Milli-Q water, add ~50 mL fixing solution into the tray, and fix the gel for 30 min with gentle shaking (50 rpm).
130. Pour off the fixing solution, add ~50 mL staining solution into the tray, and stain the gel with Colloidal Coomassie Blue Stain for > 6 h with gentle shaking (50 rpm).
131. Pour off the staining solution and add ~100 mL Milli-Q water into the tray.
132. Destain the gel with gentle shaking (50 rpm) until the gel is visibly destained (> 2 h).

Fluorescence imaging studies in living bacterial cells

Timing: 5–6 h

This method outlines the fluorescence imaging of an endogenous SrfAB-NRPS in living bacterial cells.

133. Grow *B. subtilis* ATCC 21332 at 30°C with 200 rpm shaking in 250 mL iron-enriched minimal salt medium in a 1000-mL baffled flask.
134. Transfer the bacterial cells harvested from the culture (16 h, OD_{600} = 0.92) to a 1.5 mL Eppendorf tube.
135. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.

136. Remove and discard the supernatant.
137. Wash twice with 1 mL of PBS at room temperature (20°C–25°C).
138. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
139. Remove and discard the supernatant.
140. Add 1 mL of PBS to the bacterial cells and resuspend by pipetting.
141. Treat the bacterial cells with DMSO (1 μL) or **Asp-AMS-BPyne** (1 μL of 10 mM stock in DMSO, final concentration: 10 μM) for 60 min at room temperature (20°C–25°C).

⚠ CRITICAL: The final DMSO concentration is kept at 0.1% (v/v).

142. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
143. Remove and discard the supernatant.
144. Wash twice with 1 mL of PBS.
145. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
146. Remove and discard the supernatant.
147. Add 100 μL of PBS to the bacterial cells and resuspend by pipetting.
148. Transfer the bacterial cells to a 96-well plate.

⚠ CRITICAL: A 96-well plate is used for obtaining reproducible photo-labeling.

149. Perform UV irradiation for the 96-well plate using a UVP Crosslinker (Analytik Jena) (8 W, 365 nm, on ice, Figure 3). Troubleshooting 1.

⚠ CRITICAL: To obtain reproducible, efficient, and specific photoaffinity labeling, the following distance and time of labeling are used: 1 cm distance from the top of a 96-well plate and 5 min.

150. Transfer the bacterial cells to a 1.5 mL Eppendorf tube.
151. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
152. Add 400 μL of 4% paraformaldehyde in PBS to the bacterial cells and resuspend by pipetting.
153. After 10 min at room temperature (20°C–25°C), centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
154. Remove and discard the supernatant.
155. Wash twice with 1 mL of PBS.
156. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.

⏸ **Pause point:** The bacterial cells can be resuspended in ethanol solution I and stored at −30°C for up to 24 h. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells and wash twice with 1 mL of PBS prior to step 157.

157. Add 200 μL of triton-X 100 solution to the bacterial cells and resuspend by pipetting.
158. After 15 min at room temperature (20°C–25°C), centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
159. Remove and discard the supernatant.
160. Perform the in-cell CuAAC chemistry reaction. Troubleshooting 3 and 4.
    a. Add 1.25 μL of 20 mM $CuSO_4$ solution, 2.5 μL of 50 mM THPTA solution, and 0.3 μL of 20 mM 5/6-TAMRA-PEG_3-azide solution to a 1.5 mL Eppendorf tube (total volume: 4.05 μL).
    b. Incubate the mixture in the dark at room temperature (20°C–25°C) for 3 min.
    c. Add 12.5 μL of 100 mM sodium ascorbate, 12.5 μL of 100 mM aminoguanidine, and 221 μL of PBS to a 1.5 mL Eppendorf tube (total volume: 246 μL).
    d. Add the 151a solution to the 151c solution (total volume: 4.05 μL + 246 μL = 250.05 μL).
    e. Add 221 μL of the 151d solution to the bacterial cell pellet.

⚠ CRITICAL: When preparing the CuAAC chemistry master mix, add reagents in the order that is listed in step 160 to ensure proper copper reduction and formation of a complex between copper and ligand.

Note: $CuSO_4$, THPTA, 5/6-TAMRA-PEG_3-azide, sodium ascorbate, and aminoguanidine are added to provide final concentrations of 100 μM, 500 μM, 25 μM, 5 mM, and 5 mM, respectively.

Note: Make enough master mix for at least one additional sample than necessary, to safeguard against volume loss during pipetting.

161. Incubate each sample at room temperature (20°C–25°C) in the dark for 1 h.
162. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
163. Wash three times with 1 mL of PBS.
164. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
165. Wash once with 1 mL of ethanol solution I.
166. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
167. Wash once with 1 mL of ethanol solution II.
168. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.
169. Wash once with 1 mL of ethanol solution III. Troubleshooting 5.
170. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells.

⏸ **Pause point:** The bacterial cells can be resuspended in ethanol solution I and stored at −30°C for up to 24 h. Centrifuge (10 min, 10,000 × g, 20°C) to pellet the bacterial cells and wash twice with 1 mL of PBS prior to step 171.

171. Add 100 μL of PBS to the bacterial cell pellets and resuspend by pipetting.
172. Acquire images using a fluorescence microscope.

Note: Frame size: 960 × 720.

Note: Fluorescence filter: 560/40 nm excitation, 630/75 nm emission (BZ-X filter TexasRed).

Note: Objective lens: PlanApo_λ 100 × H 1.45/0.13 mm, silicone oil-immersion. Digital zoom: Off. Exposure time: 3 s. Gain: +6 dB. Electronic brightfield aperture: 0%. Electronic dimming: 40%. White balance: Off. Black balance: On. Pseudo-color：Red. Haze reduction: Off. Video capture: 8-bit monochrome. Binning: 2 × 2. Quick full-focusing: Off).

EXPECTED OUTCOMES

Asp-AMS-BPyne is synthesized in four steps from compound **4** (Figure S1. Synthetic route to **Asp-AMS-BPyne**). The characterization data are listed in Table 1. The corresponding NMR spectra are shown in Figures 4, 5, 6, and 7. **Asp-AMS** is synthesized in two steps from compound **10** (Figure S2. Synthetic route to **Asp-AMS**). The characterization data are listed in Table 1. The corresponding NMR spectra are shown in Figures 8 and 9.

LIMITATIONS

A major limitation of these protocols is that it is difficult to describe general protocols to study different cases based on the reference case of SrfAB-NRPS in the surfactin biosynthetic pathway from the gram-positive bacterium *B. subtilis*. In addition to the method described here, many optimization experiments are required for each study, including concentration dependence of probe labeling (*in vitro* and in-cell conditions), limit of detection of endogenous SrfAB-NRPS labeling (*in vitro* condition), probe specificity of endogenous SrfAB-NRPS labeling (*in vitro* condition),

Table 1. NMR and MS data

Compound	^{1}H NMR, δ [ppm]	^{13}C NMR, δ [ppm]	HRMS (ESI)
5	500 MHz, CD_3OD 8.53 (s, 1H), 8.23 (s, 1H), 6.16 (d, *J* = 6.3 Hz, 1H), 4.61 (dd, *J* = 4.6, 2.9 Hz, 1H), 4.54 (dd, *J* = 6.3, 5.2 Hz, 1H), 4.43–4.26 (m, 4H), 3.63–3.57 (m, 1H), 3.50–3.44 (m, 1H), 3.15 (t, *J* = 6.3 Hz, 2H), 2.81 (dd, *J* = 15.5, 5.2 Hz, 1H), 2.62 (dd, *J* = 15.5, 8.0 Hz, 1H), 1.60–1.48 (m, 4H), 1.47–1.38 (m, 18H), 0.96 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H)	125 MHz, CD_3OD 171.8, 157.4, 156.9, 153.4, 150.7, 141.4, 120.2, 87.4, 85.4, 83.3, 82.2, 80.5, 72.7, 71.3, 69.7, 54.5, 52.1, 39.7, 28.8, 28.3, 28.0, 26.6, 26.3, 19.0, –4.4, –4.5 The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.	Calcd for $C_{33}H_{55}N_{10}O_{11}SSi^-$ [M-H]$^-$, 827.3542; found, 827.3544
6	500 MHz, CD_3OD 8.57 (s, 1H), 8.21 (s, 1H), 6.18 (d, *J* = 6.9 Hz, 1H), 4.64–4.60 (m, 1H), 4.57 (dd, *J* = 10.9, 4.0 Hz, 1H), 4.35–4.22 (m, 4H), 3.63–3.55 (m, 1H), 3.53–3.46 (m, 1H), 2.88 (dd, *J* = 7.5, 6.9 Hz, 2H), 2.79 (dd, *J* = 15.5, 4.6 Hz, 1H), 2.62 (dd, *J* = 15.5, 8.0 Hz, 1H), 1.68–1.51 (m, 4H), 1.49–1.36 (m, 18H), 0.96 (s, 9H), 0.174 (s, 3H), 0.166 (s, 3H)	125 MHz, CD_3OD 179.0, 172.1, 157.4, 154.0, 150.9, 141.1, 120.1, 87.0, 86.0, 83.4, 82.0, 80.2, 73.2, 70.7, 69.1, 55.0, 40.5, 40.4, 28.8, 28.4, 27.5, 26.4, 25.3, 19.0, –4.3, –4.4	Calcd for $C_{33}H_{57}N_8O_{11}SSi^-$ [M-H]$^-$, 801.3637; found, 801.3641
7	500 MHz, CD_3OD 8.52 (s, 1H), 8.20 (s, 1H), 7.77–7.68 (m, 8H), 6.15 (d, *J* = 6.3 Hz, 1H), 4.60 (q, *J* = 2.9 Hz, 1H), 4.53 (dd, *J* = 6.3, 4.6 Hz, 1H), 4.40–4.32 (m, 2H), 4.31–4.24 (m, 2H), 3.59–3.53 (m, 1H), 3.48–3.42 (m, 1H), 3.10–3.03 (m, 2H), 2.80 (dd, *J* = 15.5, 4.6 Hz, 1H), 2.70 (t, *J* = 6.9 Hz, 2H), 2.62 (dd, *J* = 15.5, 8.0 Hz, 1H), 2.58–2.50 (m, 4H), 2.32–2.25 (m, 3H), 1.94–1.86 (m, 2H), 1.55–1.35 (m, 22H), 0.94 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H)	125 MHz, CD_3OD 196.5, 174.5, 174.0, 173.3, 172.0, 157.1, 153.7, 150.7, 144.3, 144.2, 141.3, 134.0, 133.8, 132.3, 132.2, 120.2, 120.0, 119.9, 87.4, 85.5, 84.1, 83.2, 82.1, 80.4, 72.7, 71.5, 70.4, 69.4, 54.8, 40.2, 40.1, 36.7, 33.2, 31.83, 28.8, 28.4, 28.1, 26.9, 26.3, 25.5, 19.0, 18.6, –4.4, –4.5 The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.	Calcd for $C_{56}H_{77}N_{10}O_{15}SSi^-$ [M-H]$^-$, 1189.5060; found, 1189.5067
Asp-AMS-BPyne	500 MHz, DMSO-d_6 10.31 (br, 2H), 8.40 (s, 1H), 8.15 (s, 1H), 7.80–7.66 (m, 8H), 7.29 (br, 2H), 6.02 (d, *J* = 5.7 Hz, 1H), 5.32 (br, 1H), 4.47 (t, *J* = 5.2 Hz, 1H), 4.38–4.28 (m, 1H), 4.20–4.03 (m, 3H), 3.74 (dd, *J* = 6.9, 4.0 Hz, 1H), 3.61–3.53 (m, 1H), 3.43–3.37 (m, 1H, overlapping with DMSO), 3.15 (br, 1H), 2.97 (dd, *J* = 12.0, 6.3 Hz, 2H), 2.90–2.80 (m, 2H), 2.66–2.54 (m, 3H), 2.46–2.44 (m, 2H, overlapping with DMSO), 2.38 (dd, *J* = 7.5, 6.9 Hz, 2H), 2.23 (ddd, *J* = 7.5, 7.5, 2.9 Hz, 2H), 1.82–1.73 (m, 2H), 1.51–1.40 (m, 2H), 1.39–1.30 (m, 2H)	125 MHz, DMSO-d_6 193.4, 171.7, 171.24, 171.22, 170.9, 156.0, 152.8, 149.4, 143.1, 143.0, 139.3, 131.7, 131.6, 130.9, 118.9, 118.2, 118.1, 85.3, 84.0, 82.9, 80.7, 71.7, 69.5, 69.2, 67.3, 51.4, 38.2, 35.5, 35.2, 31.9, 30.1, 26.6, 25.7, 23.8, 17.4	Calcd for $C_{41}H_{47}N_{10}O_{13}S^-$[M-H]$^-$, 919.3045; found, 919.3049
11	500 MHz, CD_3OD δ 8.46 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 2.9 Hz, 1H), 5.38–5.34 (m, 1H), 5.14–5.10 (m, 1H), 4.55–4.52 (m, 1H), 4.33–4.28 (m, 1H), 4.25–4.20 (m, 2H), 3.18 (q, *J* = 7.5 Hz, 6H, Et_3N-CH_2), 2.75 (dd, *J* = 15.5, 5.2 Hz, 1H), 2.56 (dd, *J* = 15.5, 7.5 Hz, 1H), 1.61 (s, 3H), 1.44–1.37 (m, 21H), 1.28 (t, *J* = 7.5 Hz, 9H, Et_3N-CH_3)	125 MHz, CD_3OD δ 178.9, 172.5, 157.4, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.9, 85.7, 85.6, 83.3, 81.9, 80.2, 69.6, 55.0, 47.8, 40.2, 28.8, 28.4, 27.5, 25.6, 9.2	Calcd for $C_{26}H_{38}N_7O_{11}S^-$ [M-H]$^-$, 656.2350; found, 656.2346
Asp-AMS	500 MHz, DMSO-d_6 δ 8.39 (s, 1H), 8.14 (s, 1H), 7.27 (br, 2H), 5.90 (d, *J* = 6.3 Hz, 1H), 4.60–4.55 (m, 1H), 4.18–4.00 (m, 4H), 3.58–3.54 (m, 1H), 2.86 (q, *J* = 7.5 Hz, 8H, Et_3N-CH_2), 2.55 (dd, *J* = 16.6, 4.0 Hz, 1H), 2.36 (dd, *J* = 16.6, 8.6 Hz, 1H), 1.08 (t, *J* = 7.5 Hz, 12H, Et_3N-CH_3)	125 MHz, DMSO-d_6 δ 173.5, 172.3, 156.0, 152.7, 149.6, 139.4, 118.8, 87.0, 82.4, 73.5, 70.6, 67.5, 52.7, 45.4, 36.3, 9.4	Calcd for $C_{14}H_{18}N_7O_9S^-$[M-H]$^-$, 460.0887; found, 460.0882

Note: ^{1}H-NMR spectra were recorded at 500 MHz. ^{13}C-NMR spectra were recorded at 125 MHz on a JEOL NMR spectrometer (ECA-500, JEOL Ltd.). Mass spectral data were obtained using a LCMS-IT-TOF mass spectrometer (Shimadzu Corporation).

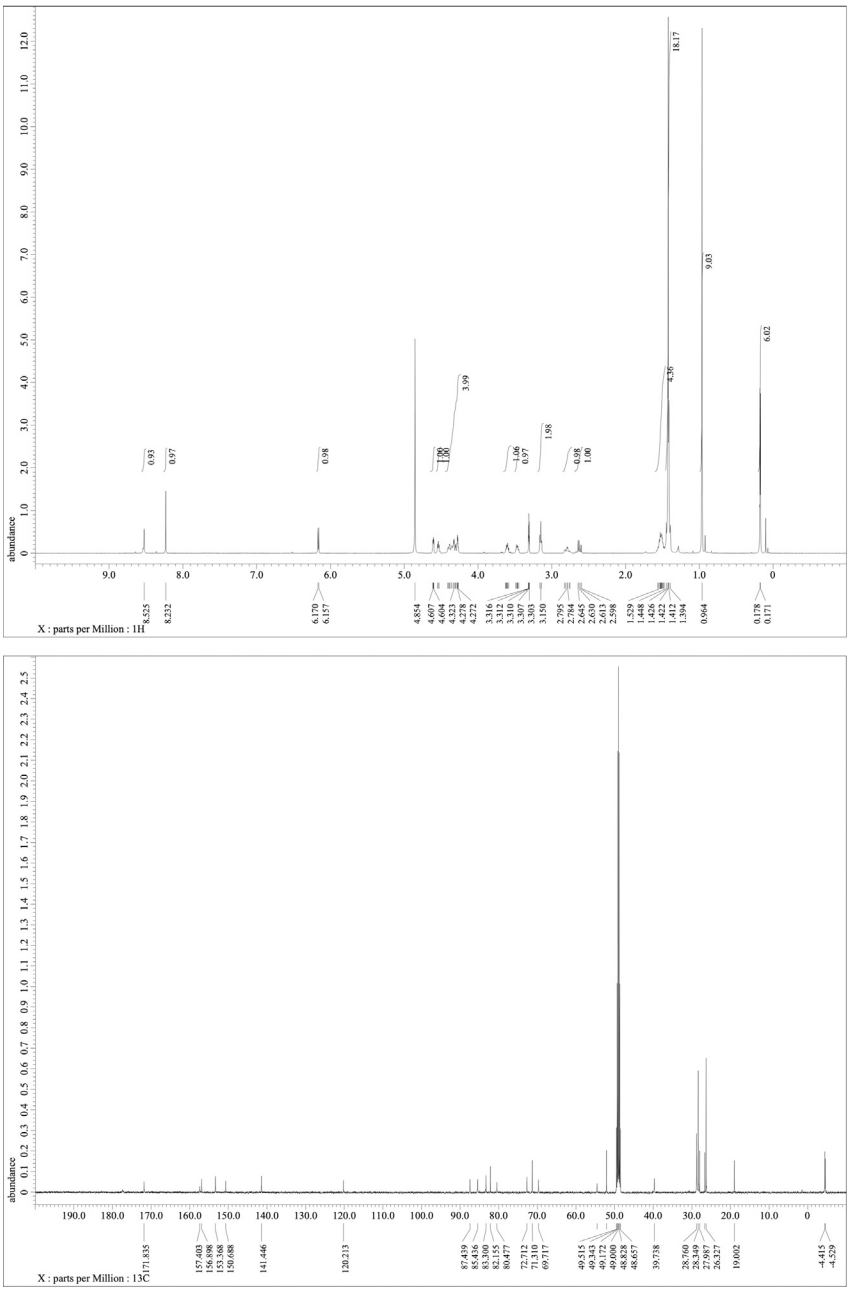

Figure 4. ^{1}H (500 MHz, upper) and ^{13}C (125 MHz, lower) spectra of 5 in CD_3OD

time-dependent cell permeability of the probe (in-cell condition), and imaging studies (in-cell condition). Furthermore, these optimization steps need to be performed for each probe used in the study.

This protocol focuses on only one NRPS in the surfactin biosynthetic pathway for the gram-positive bacterium *B. subtilis* using one probe, **Asp-AMS-BPyne**. However, in our recent published study, we developed an **Asp-AMS-BPyne** derivative and confirmed the fluorescence labeling and imaging of an overproduced EntE-NRPS of the enterobactin biosynthetic pathway in the gram-negative bacterium *Escherichia coli* (Ishikawa et al., 2021).

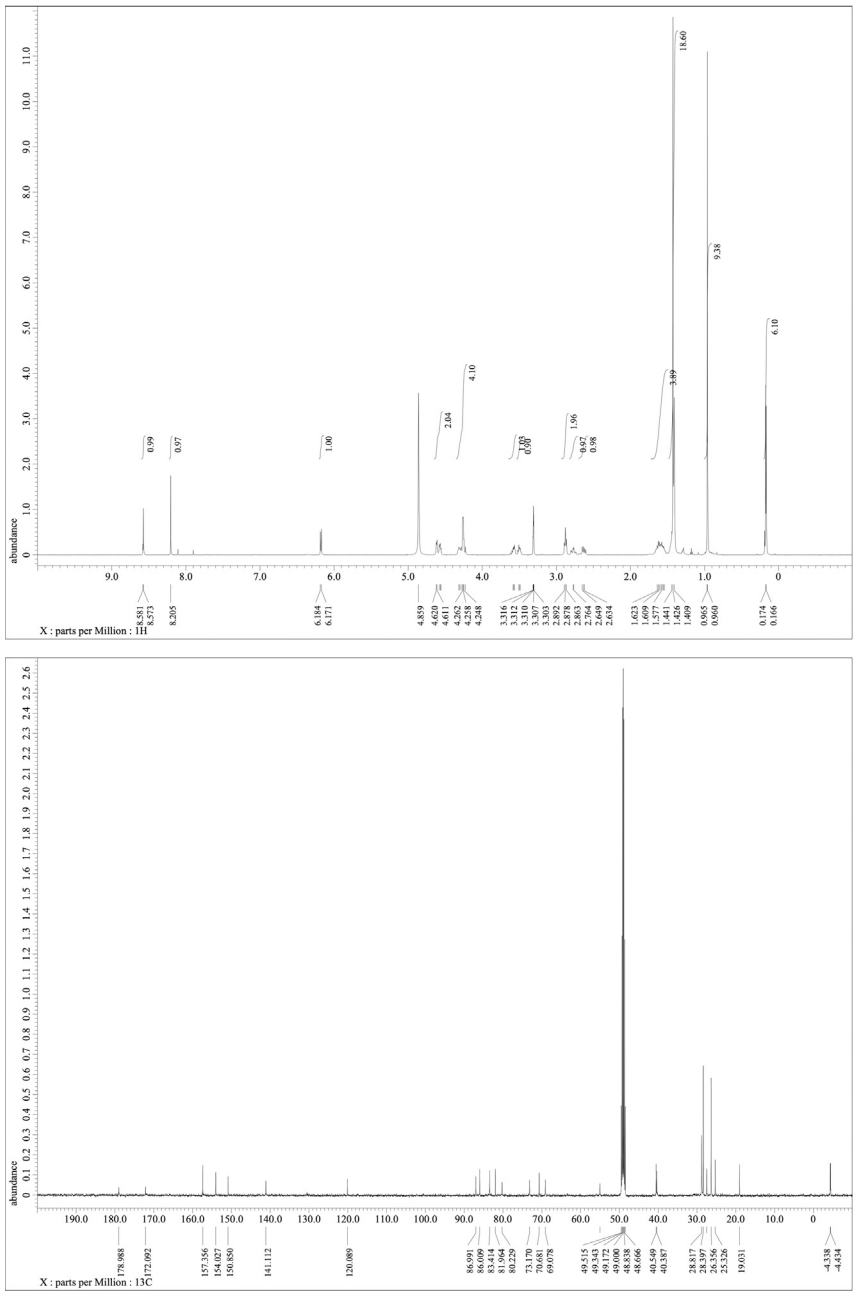

Figure 5. ^{1}H (500 MHz, upper) and ^{13}C NMR (125 MHz, lower) spectra of 6 in CD_3OD

The concentration of SrfAB-NRPS in the cytoplasm of *B. subtilis* is important for performing fluorescence imaging studies with a high signal-to-noise ratio. Our data (Ishikawa et al., 2022) indicated that **Asp-AMS-BPyne** detected SrfAB-NRPS in 1.4 μg of lysate protein. However, we could not quantify the concentration of SrfAB-NRPS in the cytoplasm of *B. subtilis*; therefore, the current protocol cannot provide the detection limit of **Asp-AMS-BPyne** in fluorescence imaging studies.

Another limitation is the non-specific photo cross-linking of proteins by the probe, particularly under *in vitro* conditions, thus requiring appropriate control experiments. Examples include the probe vs. probe plus inhibitor experiment during *in vitro* labeling and probe vs. no probe during in cell labeling and imaging. It is important to note that different probes (**Val-AMS-BPyne** and **Leu-AMS-BPyne**)

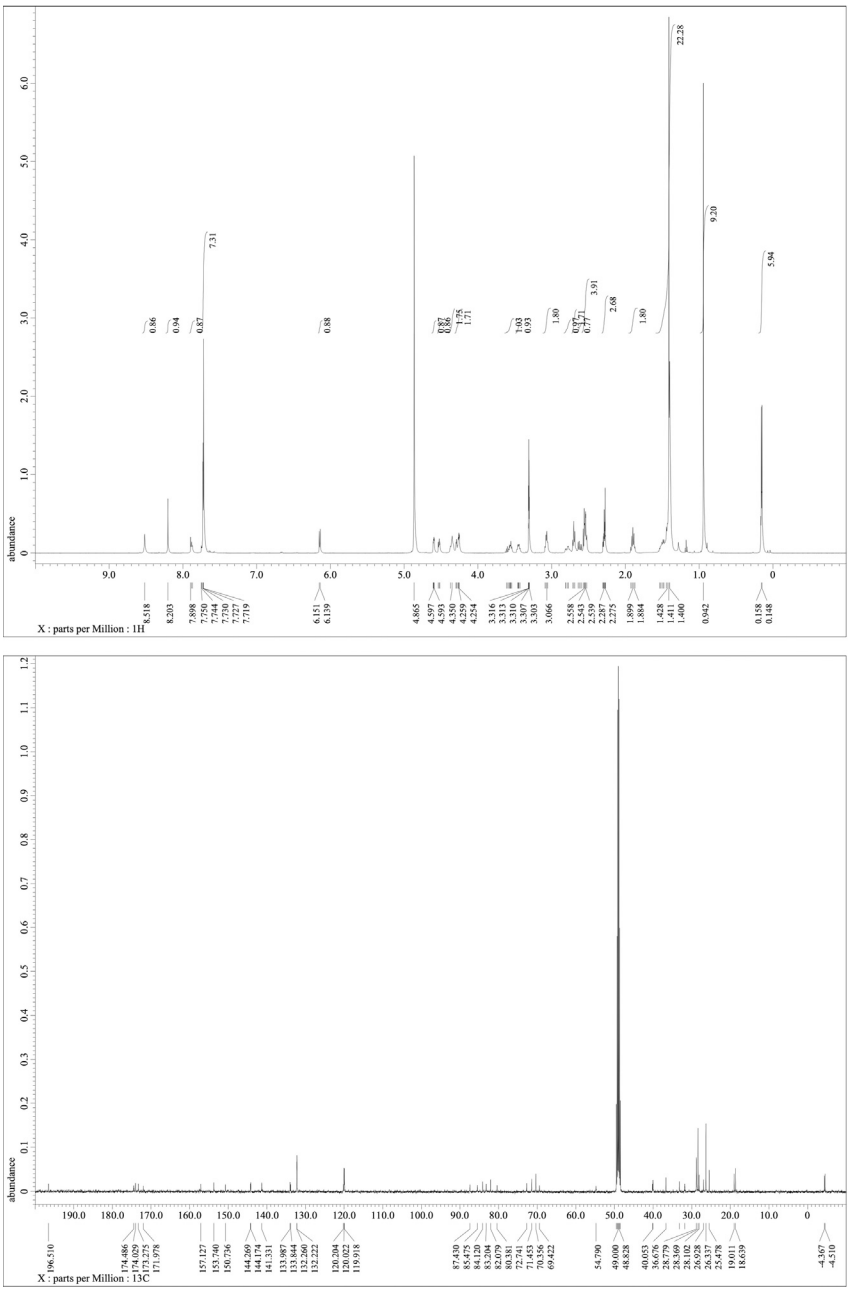

Figure 6. ^{1}H (500 MHz, upper) and ^{13}C NMR (125 MHz, lower) spectra of 7 in CD_3OD

specifically target not only the Val-activating A-domain and Leu-activating A-domain, respectively, but also valine-tRNA synthetase and leucyl-tRNA synthetase, respectively, *in vitro* (Ishikawa et al., 2015; Konno et al., 2017). Accordingly, the aminoacyl-AMS-BPyne scaffold occasionally recognizes a shared catalytic mechanism, the formation of an aminoacyl-adenylate monophosphate catalyzed by A-domains and tRNA synthetases.

TROUBLESHOOTING

Problem 1

Low photoaffinity labeling efficiency (steps 72, 105, and 149).

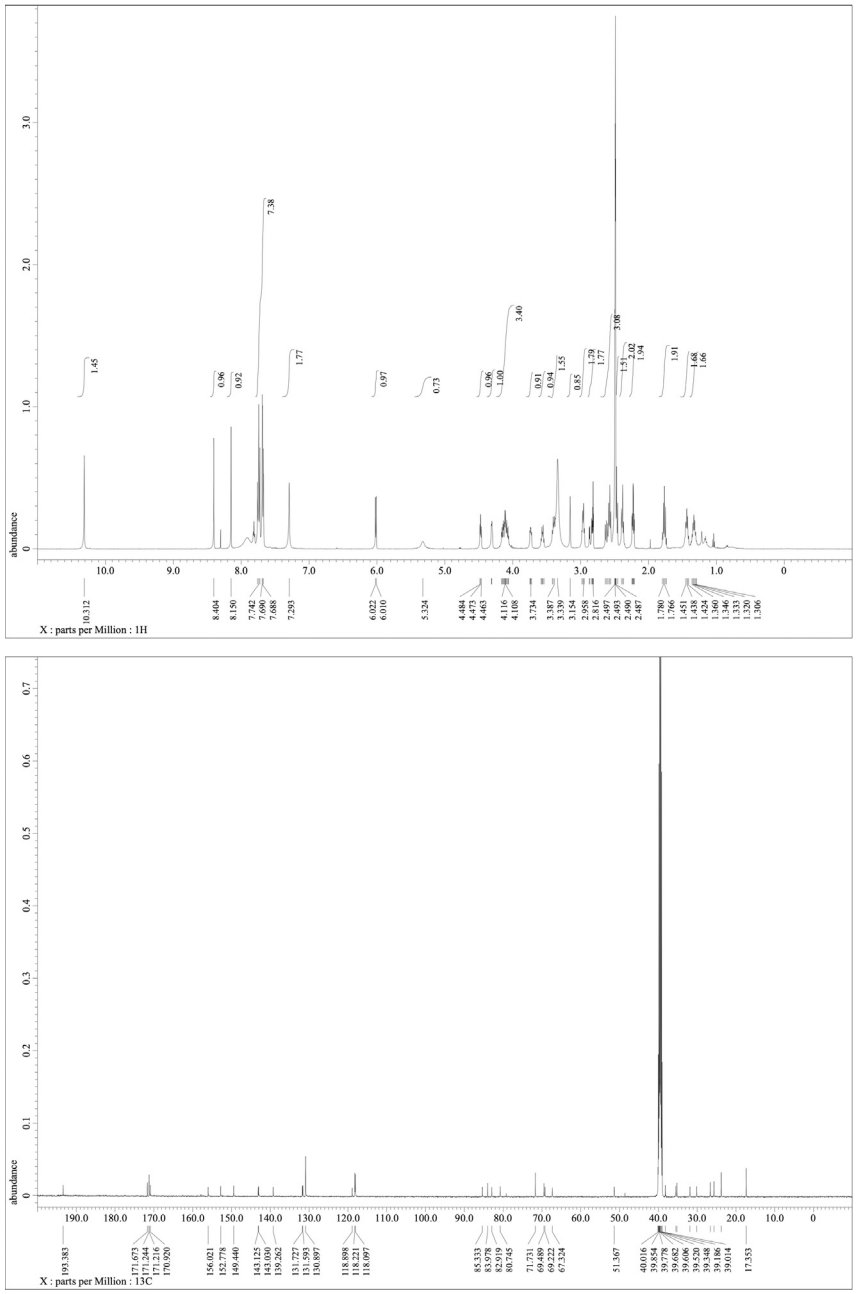

Figure 7. ^{1}H (500 MHz, upper) and ^{13}C NMR (125 MHz, lower) spectra of Asp-AMS-BPyne in DMSO-d_6

Potential solution

Lowering the distance between the UV lamp and the 96-well plate may increase the efficiency of photoaffinity labeling. Increasing the UV irradiation time to over 5 min can increase the photoaffinity labeling *in vitro* (Ishikawa et al., 2022). However, the fluorescence band intensity of SrfAB was saturated at 5 min, with increased nonspecific photoaffinity labeling observed on longer incubation (20–60 min) (Ishikawa et al., 2022). A higher concentration of **Asp-AMS-BPyne** can also increase photoaffinity labeling. However, **Asp-AMS-BPyne** underwent increased nonspecific photoaffinity labeling at high concentrations (5–10 μM) (Ishikawa et al., 2022).

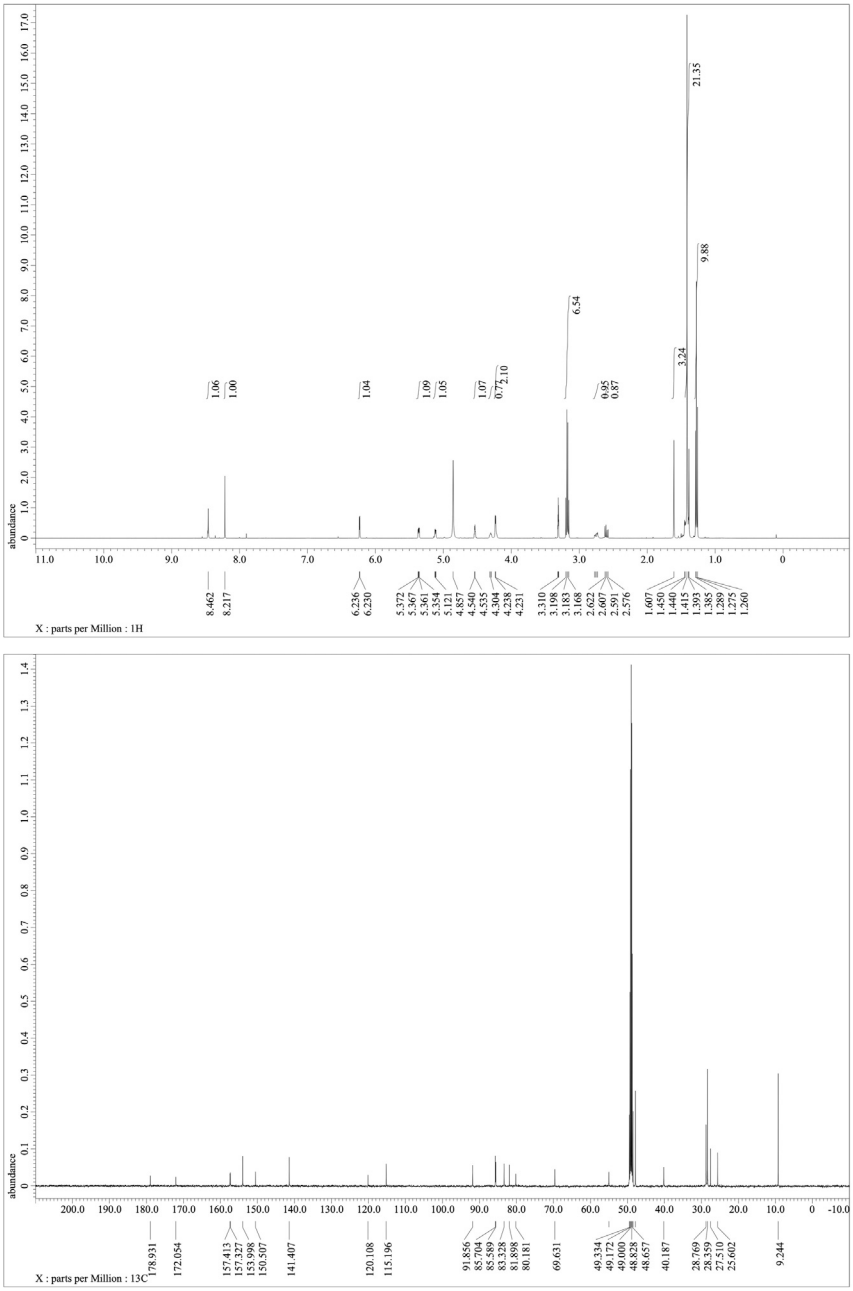

Figure 8. ^{1}H (500 MHz, upper) and ^{13}C NMR (125 MHz, lower) spectra of 11 in CD_3OD

Problem 2

Inefficient *in vitro* CuAAC (steps 73 and 116).

Potential solution

We prepared the CuAAC master mix just before use. When preparing the CuAAC master mix, add reagents in the order listed in the materials and equipment section.

Problem 3

Inefficient in cell CuAAC (step 160).

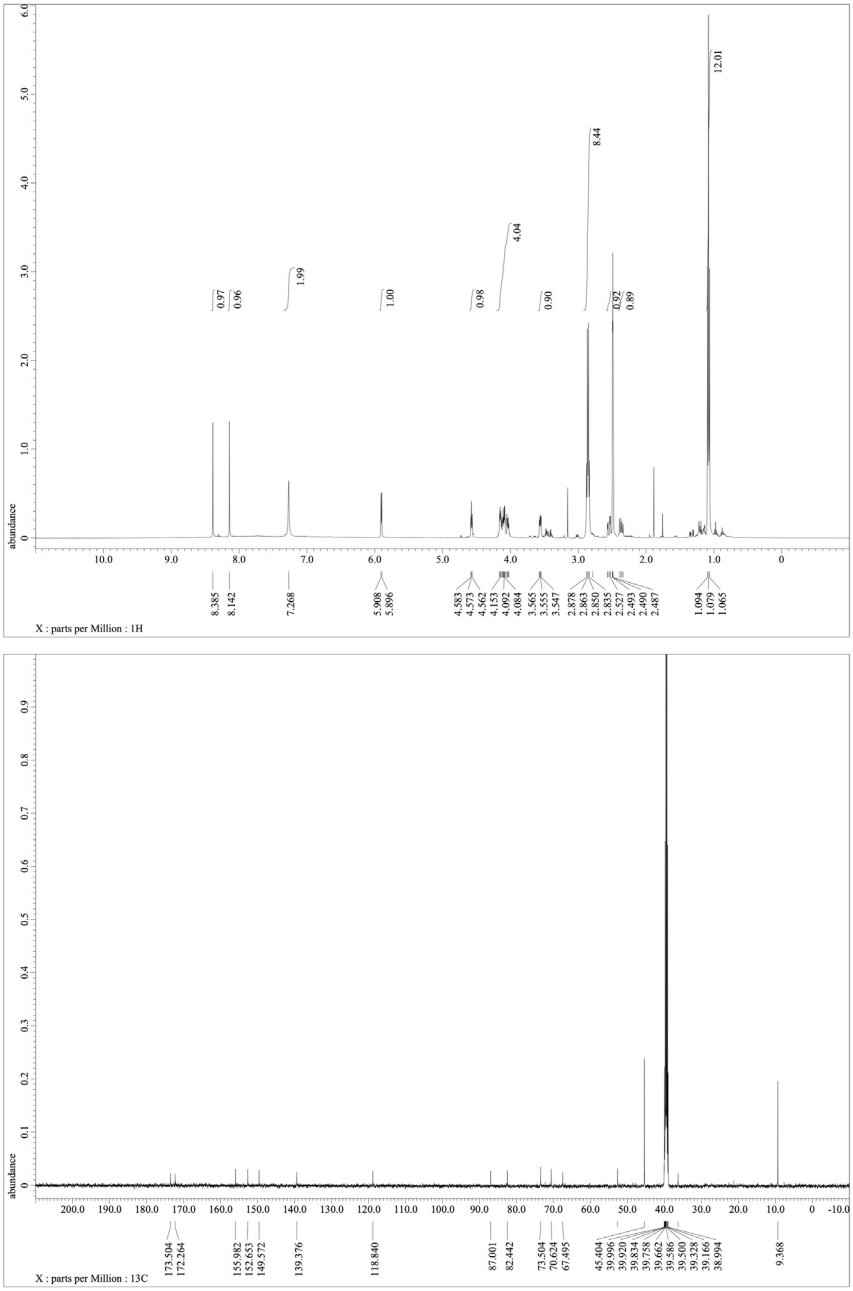

Figure 9. ^{1}H (500 MHz, upper) and ^{13}C (125 MHz, lower) spectra of Asp-AMS in DMSO-d_6

Potential solution

We used freshly prepared reagents for sodium ascorbate and aminoguanidine hydrochloride. In some cases, we also observed high background fluorescence signals from CuAAC-based fluorescence imaging using an azide-BDP-FL (Jena Bioscience, Cat# CLK-044-1). See problem 4. When preparing the CuAAC master mix, add reagents in the order described in step 160.

Problem 4

Selection of fluorescence dye azides (step 160).

Potential solution

In this study, we tested 5/6-TAMRA-PEG$_3$-azide (Jena Bioscience, Cat# CLK-AZ109-1, see key resources table), Cy$_3$-azide (Jena Bioscience, Cat# CLK-046-1), sulfo-Cy$_3$-azide (Jena Bioscience, Cat# CLK-AZ119-1), and azide-BDP-FL (Jena Bioscience, Cat# CLK-044-1) in fluorescence imaging studies (unpublished results). Of note, in the context of fluorescence microscopy, click chemistry based in cell imaging was successful using 5/6-TAMRA-PEG$_3$-azide (Jena Bioscience, Cat# CLK-AZ109-1, see key resources table), Cy$_3$-azide (Jena Bioscience, Cat# CLK-046-1), and sulfo-Cy$_3$-azide (Jena Bioscience, Cat# CLK-AZ119-1), with a high signal-to-noise ratio (unpublished data). In contrast, the use of azide-BDP-FL (Jena Bioscience, Cat# CLK-044-1) resulted in high-level background fluorescence (unpublished data). In our recently published study, we performed fluorescence labeling and imaging studies of an overproduced EntE-NRPS in the gram-negative bacterium *E. coli* (Ishikawa et al., 2021). These imaging studies were only successful using Cy$_3$-azide (Jena Bioscience, Cat# CLK-046-1) (unpublished data). These results suggest that the screening of fluorescence dye azides may be required in some cases.

Problem 5

Low signal-to-noise ratio in fluorescence imaging studies (step 169).

Potential solution

The low signal-to-noise ratio should be caused by residual 5/6-TAMRA-PEG$_3$-azide (Jena Bioscience, Cat# CLK-AZ109-1, see key resources table). Additional washes with ethanol solution III (1 mL) would result in increased signal-to-noise ratio in fluorescence imaging studies.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Fumihiro Ishikawa (ishikawa@phar.kindai.ac.jp).

Materials availability

All compounds described in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

This study did not generate any datasets or codes.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101462.

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AUTHOR CONTRIBUTIONS

F.I., R.O., C.U., and G.T. conceived the project and drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing financial interests.

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