ペプチド性天然化合物合成酵素アデニレーションドメインに対する選択的ラベル化技術の開発に関する研究

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略号一覧

A	Adenylation
aaRS	Aminoacyl-transfer RNA synthetase
ABPP	Activity-based protein profiling
Ac	Acetyl
A. migulanus	*Aneurinibacillus migulanus*
AMP	Adenosine monophosphate
AMS	Adenosine monosulfamate
aq	Aqueous
ATCC	American type culture collection
ATP	Adenosine triphosphate
$AusA_1$	Aureusimine synthetase A module 1
Boc	*tertiary*-Butoxycarbonyl
BPyne	Benzophenone-alkyne
br	Broad signal
BSA	Bovine serum albumin
C	Condensation
calcd	Calculated
CBB	Coomassie brilliant blue
CP	Carrier protein
d	Doublet
dd	Doublet of doublets
ddd	Doublet of triplets
DEBS	6-Deoxyerythronolide B synthase
DIAD	Diisopropyl azodicarboxylate
DIEA	*N,N*-Diisopropylethylamine
DMAP	*N,N*-Dimethylaminopyridine
DMF	*N,N*-Dimethylformamide
DMSO	Dimethyl sulfoxide
DSM	Deutsche Sammlung von Mikroorganismen
E	Epimerization
EDC･HCl	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
eq	Equivalent
ESI	Electrospray ionization

Et	Ethyl
FL	Fluorescence
GrsA	Gramicidin S synthetase A
GrsB	Gramicidin S synthetase B
GS	Gramicidin S
h	Hour
HOBt	1-Hydroxybenzotriazole
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
Hz	Heltz
J	Coupling constant
kDa	Kilodalton
LAH	Lithium aluminum hydride
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
L-Leu	L-Leucine
liq	Liquid
L-Lys	L-Lysine
L-Orn	L-Ornithine
L-Phe	L-Phenylalanine
L-Pro	L-Proline
L-Val	L-Valine
m	Multiplet
Me	Methyl
MesG	2-Amino-6-mercapto-7-methylpurine riboside
mRNA	Messenger ribonucleic acid
NBYS	Nutrient broth and yeast extract supplemented with salt
NHS	*N*-Hydroxysuccinimide
NRP	Nonribosomal peptide
NRPS	Nonribosomal peptide synthetase
OSu	Succinimidyl ester
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
P_i	Phosphate
pK_a	Acid dissociation constant
PP_i	Pyrophosphate

Quant	Quantification
RP	Reverse phase
rt	Room temperature
s	Singlet
sat.	Saturated
SDS	Sodium dodecyl sulfate
t	Triplet
TAMRA	Tetramethylrhodamine
TBAI	Tetrabutylammonium iodide
TBS	*tertiary*-Butyldimethylsilyl
^{t}Bu	*tertiary*-Butyl
TE	Thioesterase
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
tRNA	Transfer ribonucleic acid
TsOH	*para*-Toluenesulfonic acid
$TycB_1$	Tyrocidine synthetase B module 1
UV	Ultra violet
YP	Yeast extract and peptone
YPG	Yeast extract, peptone, and glucose

緒言

微生物が産生する多くの有用なペプチド性天然化合物は非リボソーム性ペプチド（NRP）に分類される。NRP にはシクロスポリン（免疫抑制剤）やバンコマイシン（抗生物質）に代表される医薬資源だけでなく、結核菌の病原性発現に必須とされるマイコバクチン（シデロフォア）、天然由来の界面活性剤であるサーファクチンなどがあり、多様な化学構造により非常に幅広い生物活性を示す（**Figure A**）[1a,b]。このような NRP は非リボソーム性ペプチド合成酵素（NRPS）と呼ばれる酵素群によって合成される[2]。

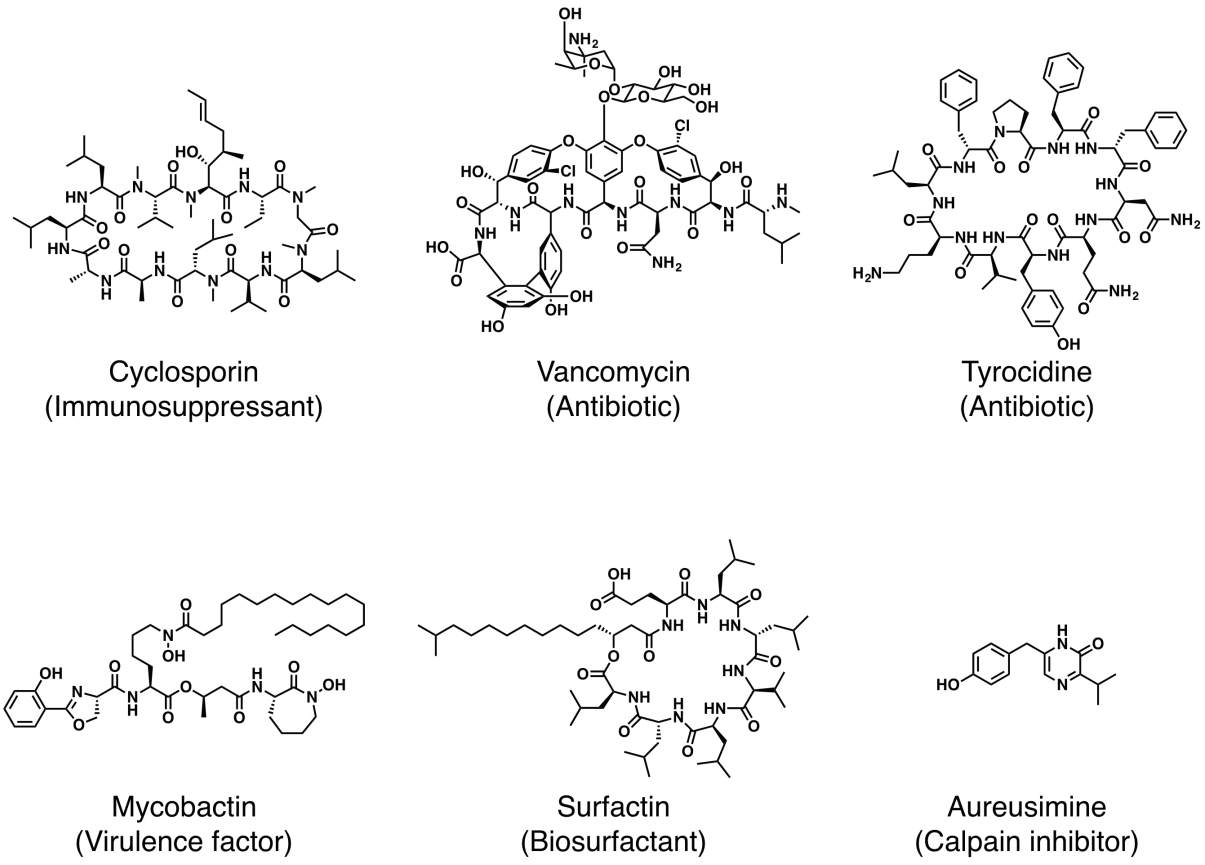


Figure A. Structures and biological activities of nonribosomal peptide natural products.

NRPS は一つのタンパク質上に複数の機能領域が存在し、多くの場合、200 kDa を超える巨大タンパク質である。NRPS において、1 つのビルディングブロックを導入する単位はモジュールと呼ばれ、モジュールはさらにそれぞれの機能を担うドメインに細分化される。例えば、抗生物質グラミシジン S は 1 つのモジュールからなる GrsA と 4 つのモジュールからなる GrsB によって合成される（**Figure B**）[3a,b]。

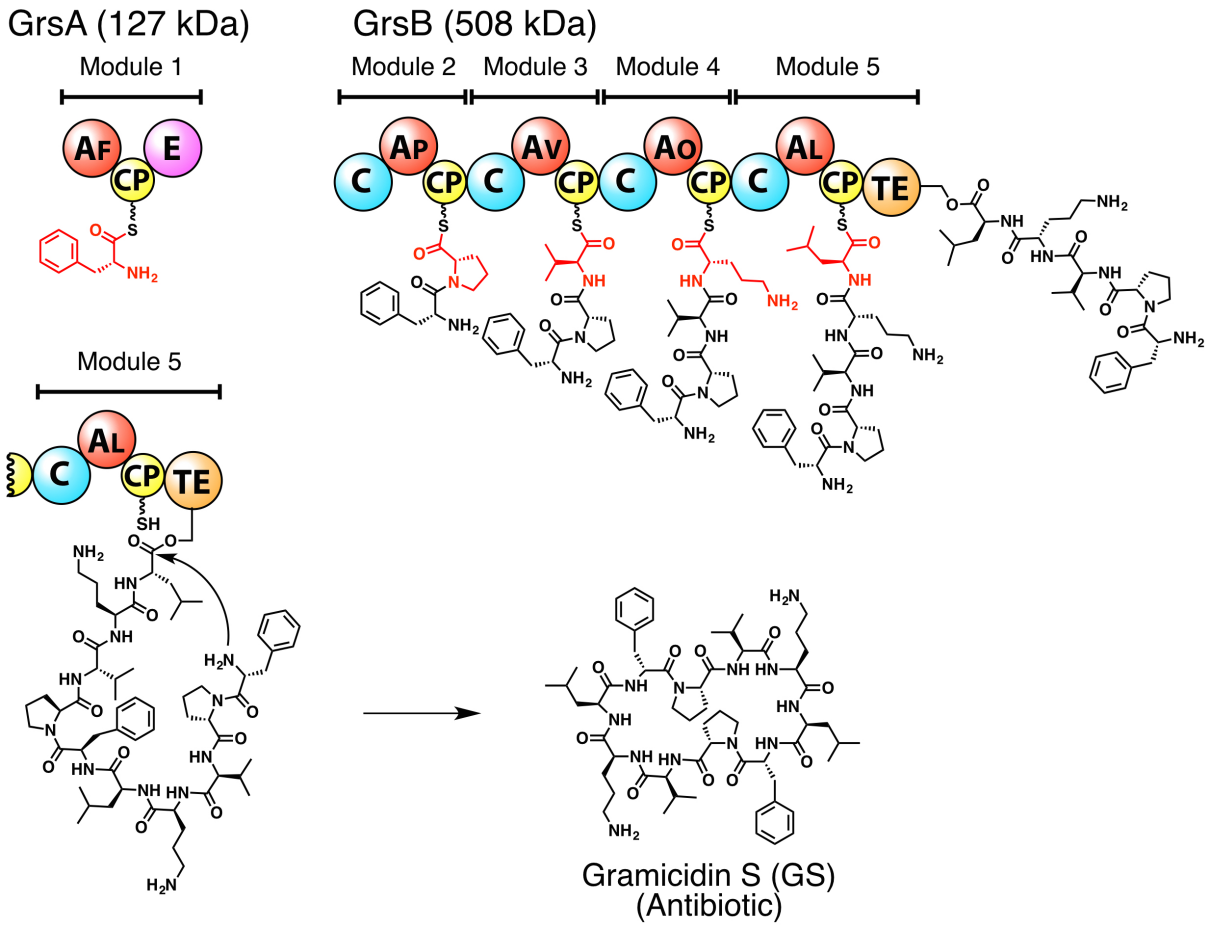


Figure B. Nonribosomal peptide synthesis of the antibiotic gramicidin S. Modules are comprised of adenylation (A) [AF, L-Phe; AP, L-Pro; AV, L-Val; AO, L-Orn; AL, L-Leu specific A-domains], carrier protein (CP), epimerization (E), condensation (C), and thioesterase (TE) domains.

NRPS モジュールの基本構成単位はアデニレーション（A）ドメイン、キャリアープロテイン（CP）、コンデンセーション（C）ドメインの 3 つのドメインからなる [4]。A ドメインはビルディングブロックとなる基質の選択と活性化を担っている。A ドメ

インの基質としては、天然アミノ酸だけでなく非天然アミノ酸やα-ヒドロキシ酸、アリール酸などがあり、その基質特異性は非常に厳密である（**Figure Ca**）。CP では保存されたセリン残基が翻訳後修飾により 4'-ホスホパンテテイン化されている。A ドメインで活性化された基質は 4'-ホスホパンテテイン末端のチオール基から求核攻撃を受け、チオエステル結合を介して CP 上に担持される（**Figure Cb**）。C ドメインは CP に担持された上流と下流の基質を縮合し、ペプチド結合を形成する役割を担う（**Figure Cc**）。その他の修飾酵素ドメインとして、L-アミノ酸を D-アミノ酸へと異性化するエピメラーゼ（E）ドメインや N-メチル化を担うメチルトランスフェラーゼドメイン、NRPS からペプチド鎖を加水分解もしくは環状ペプチド形成反応を触媒するチオエステラーゼ（TE）ドメインなどが存在し、NRP の特異な構造多様性を生み出している。

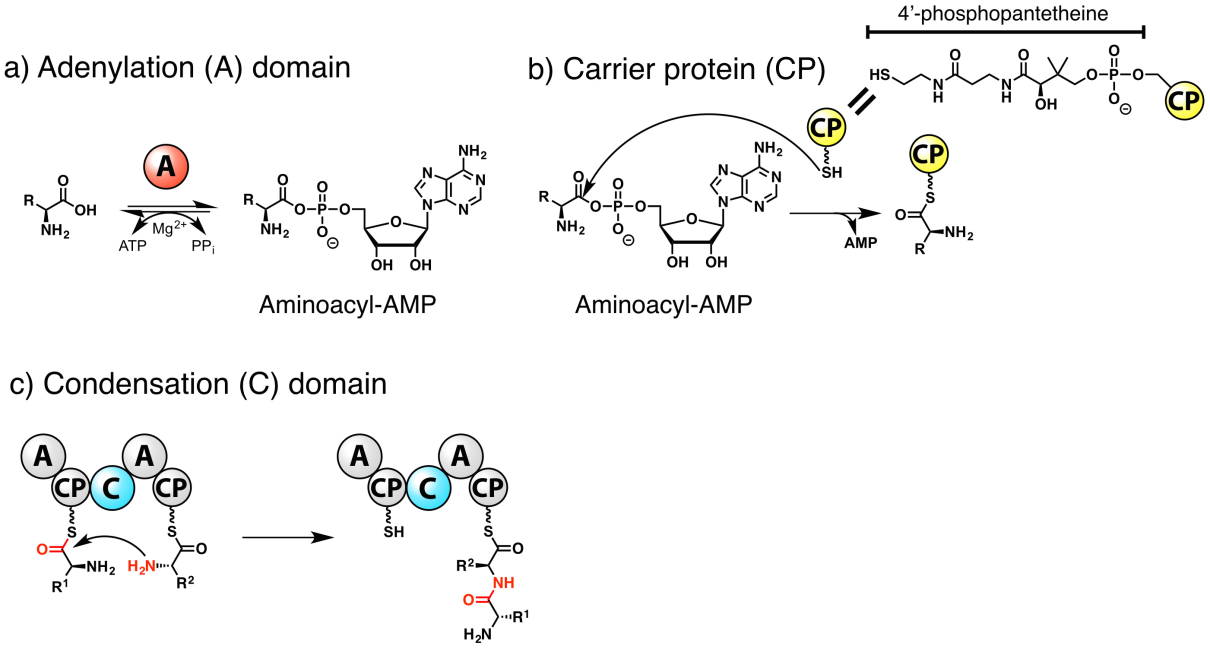


Figure C. Catalytic domains in NRPSs. a) Adenylation reaction catalyzed by the A-domains. b) Nucleophilic attack by the terminus thiol group of the 4'-phosphopantetheine arm of a downstream CP. c) Amide bond formation catalyzed by the C-domains.

NRPS に限らず生合成酵素に関する研究は、生合成酵素群をコードしている遺伝子クラスターの同定や、異種宿主発現による組み換えタンパク質の機能、立体構造評価を中心に行われてきた [5a-c]。この遺伝子工学を基にした研究はポストゲノム時代を迎

えさらに精力的に行われるようになり、これまでに数多くの生合成酵素が機能評価され、新規生合成経路の発見や酵素改変による天然化合物類縁体の創出などが報告されている[6a,b]。

しかしながら、NRPS に関するプロテオームレベルでの知見は数例しか報告されていない[7a,b]。そのため、NRPS に関するプロテオームレベルでのタンパク質間相互作用、転写調節、活性制御機構及び局在などはほとんど明らかになっていない。これは、プロテオーム中の NRPS を検出･評価する汎用性の高い方法がないことに起因する。これまでに報告されている生合成酵素の検出法を紹介する。Caffrey らはエリスロマイシン合成酵素である DEBS2 及び 3 に対する抗体を作製し、生産菌プロテオーム中の DEBS2、3 をそれぞれ選択的に検出することに成功している[8]。抗体はプロテオーム中の標的タンパク質を検出する強力な手段であるが、高い特異性のため汎用性に乏しく、研究対象とする全ての NRPS に対する抗体を作製することは多大な労力とコストを必要とする。その他の方法として、放射性同位体標識されたアミノ酸を用いる方法がある[9a,b]。これは A ドメインによって選択されたアミノ酸が CP に担持されることを利用しているが、不安定なチオエステル結合を介するため容易に加水分解反応を受ける。また、放射性同位体を扱うため作業も煩雑になる。さらに、この手法はプロテオームには適用できず、精製系でのみ用いられてきた。

一方、哺乳類細胞では低分子ラベル化剤を介したプロテオミクス研究が精力的に行われてきた。これまでに標的タンパク質の同定[10]や相互作用タンパク質の検出[11]、活性プロファイリング[12]など、遺伝学では解析困難な内在性タンパク質に関する様々な生命現象が明らかにされている。そこで、NRPS と特異的に結合する低分子ツールを開発することができれば、微生物プロテオーム中の NRPS を選択的にラベル化し、NRPS に関するプロテオームレベルでの数多くの現象を解析可能になると考え、合成分子プローブの開発研究に着手した（**Figure D**）。

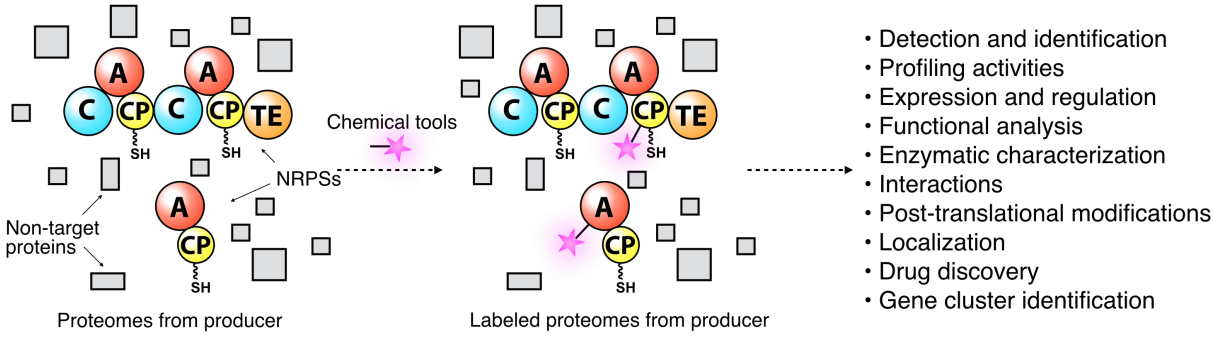


Figure D. Proteomics for natural product biosynthesis.

第 1 章　アデニレーションドメインを標的とした活性部位指向型プローブの設計･合成及び機能評価

第 1 節　はじめに

合成分子プローブによる標的タンパク質のラベル化を行うためには、リガンドと標的タンパク質の特異的結合が鍵となる。そこで、NRPS の基本構成単位であり、NRP のビルディングブロックとなる基質を決定するアデニレーション（A）ドメインに着目した。A ドメインの厳密な基質特異性を利用することができれば、A ドメインを指標に様々な NRPS を選択的にラベル化可能になると考えた。

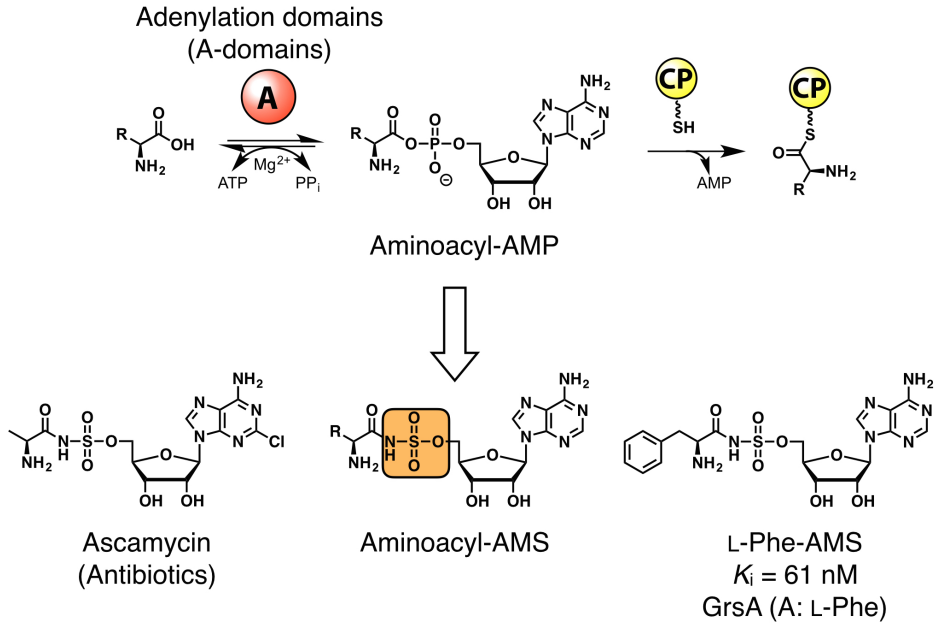


Figure 1-1. Adenylation reaction in NRPS and structures of inhibitors.

第 2 節　アデニレーションドメインに対する活性部位指向型プローブの設計

まず、A ドメインと特異的に結合するリガンドについて検討を行った。A ドメインで選択されたアミノ酸は Mg^{2+}存在下、ATP と反応して高反応性の中間体であるアミノアシル-AMP を生成する（**Figure 1-1**）。この反応は、tRNA にアミノ酸を担持する反応を触媒するアミノアシル tRNA 合成酵素（aaRS）と同様の触媒メカニズムである[13a,b]。この aaRS の阻害剤として、*Streptomyces* 属から単離されたアスカマイシンが報告されている[14a-c]。アミノアシル-AMS はアミノアシル-AMP の不安定なリン酸エステル部をスルファモイル基へと変換した安定な生物学的等価体である[15]。そのため、

aaRS の X 線結晶構造を得る際のリガンドとして用いられてきた [16a-d]。そこで、aaRS と同様の反応を触媒する A ドメインにおいてもアミノアシル-AMS をリガンドとして利用できると考えた。実際、L-Phe-AMS は L-Phe に基質特異性のある A ドメインを有するグラミシジン S 合成酵素 GrsA に高い阻害活性（K_i = 61 nM）を示すことが報告されている [17]。そこで、アミノアシル-AMS をリガンド部としてプローブの設計を行った。

次に、リンカーの導入部位を検討することにした。リンカーの導入部位としては、リガンドの結合活性への影響を最小限に抑えるために、標的タンパク質との結合に関与していなく、かつ溶媒側に配向している部位が望ましい。A ドメインはこれまでにいくつかの X 線結晶構造が明らかになっている [18a-c]。なかでも GrsA の A ドメインは基質である L-Phe と AMP との共結晶構造が得られている（PDB ID: 1AMU）[19]。この共結晶構造について分子モデリングソフト（Discovery Studio, ver. 3.1）を用いて精査したところ、AMP のアデノシン 2'位のヒドロキシ基 がタンパク質の外側に配向していることが示唆された（**Figure 1-2a**）。そこで、リンカーの導入部位はアデノシン 2'位のヒドロキシ基に決定した（**Figure 1-2b**）。

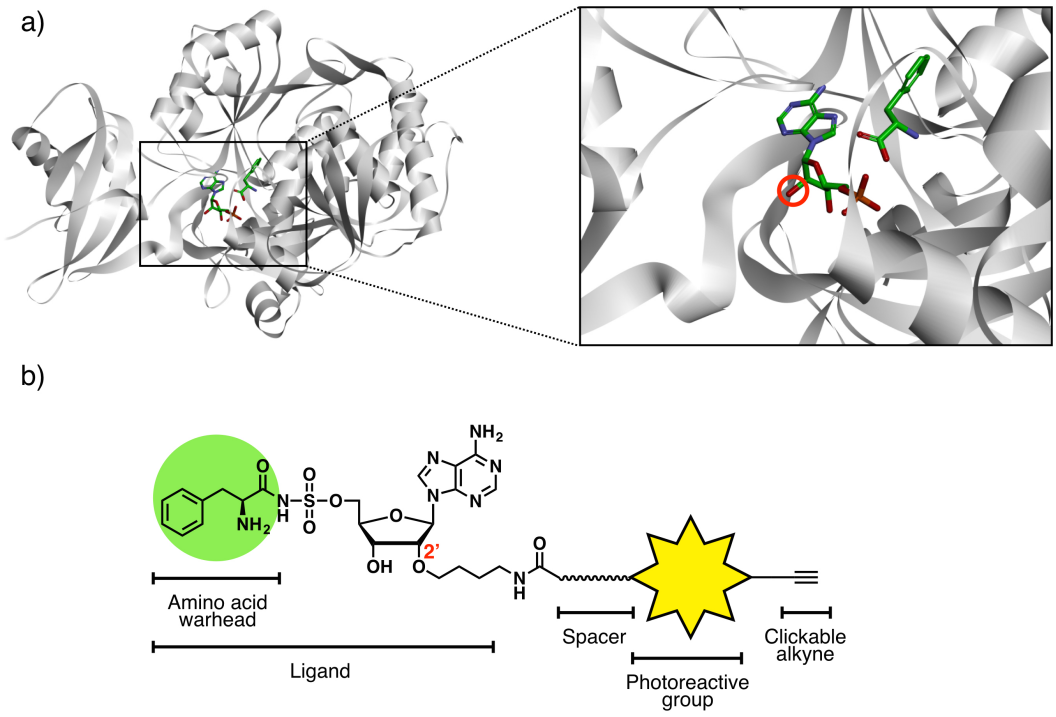


Figure 1-2. a) Crystal structure of the A-domain of GrsA (gray). The ligands L-Phe and AMP have been shown as stick structure, with the following color code: nitrogen, blue; oxygen, red; phosphate, orange; carbon, green. Modified from PDB code 1AMU using Discovery Studio ver. 3.1. b) Design of active site-directed proteomic probes for A-domains.

アミノアシル-AMS リガンドは A ドメインと共有結合を形成しない。そこで、リガンドが結合した後、A ドメインと共有結合を形成するために、光反応性官能基をリンカー部に導入することにした。光反応性官能基としては、主にアリールアジド、ベンゾフェノン、ジアジリンの三種類が知られている（**Figure 1-3**）[20a-d]。アリールアジドは 300 nm 以下の UV 照射によりナイトレンを生成し、近傍のタンパク質と共有結合を形成するが、一般的に 300 nm 以下の UV 照射はタンパク質に対する損傷が大きいとされている。一方、ベンゾフェノンとジアジリンは 360 nm 付近の UV 照射によりそれぞれ活性種を生成し、近傍のタンパク質と共有結合を形成する。ベンゾフェノンは光照射によりカルボニル部分が励起されてビラジカルベンゾフェノンとなる。この励起は可逆的であるため、長時間の照射により高いラベル化効率が期待できるが、ベンゾフェノン自体が比較的大きい分子のため、リガンドとタンパク質の結合に影響を与えることがしばしば問題となる。ジアジリンでは光照射により窒素分子が脱離して高反応性のカルベンを形成する。この励起は他の分子種と比較して迅速に生じることから、短時間照射でのラベル化が期待できる。また、ジアジリンは近傍にタンパク質が存在しない場合は水分子と反応するため、非特異的なラベル化を抑えることができる。NRPS は微生物にとって必須ではない二次代謝産物合成酵素であるため、その存在量は少ないことが予想された。一方で、NRPS のような巨大 NRPS は不安定であることが多い[21]。そこで、光反応性官能基には、高いラベル化効率を期待できるベンゾフェノンと、短時間の光照射で速やかにラベル化が可能なジアジリンを選択した。また、光反応性官能基の位置はラベル化効率に大きく影響を与えることが予想されたため、リガンドと光反応性官能基の間に性質や長さの異なる様々なスペーサー分子を導入することにした（**Figure 1-2b**）。

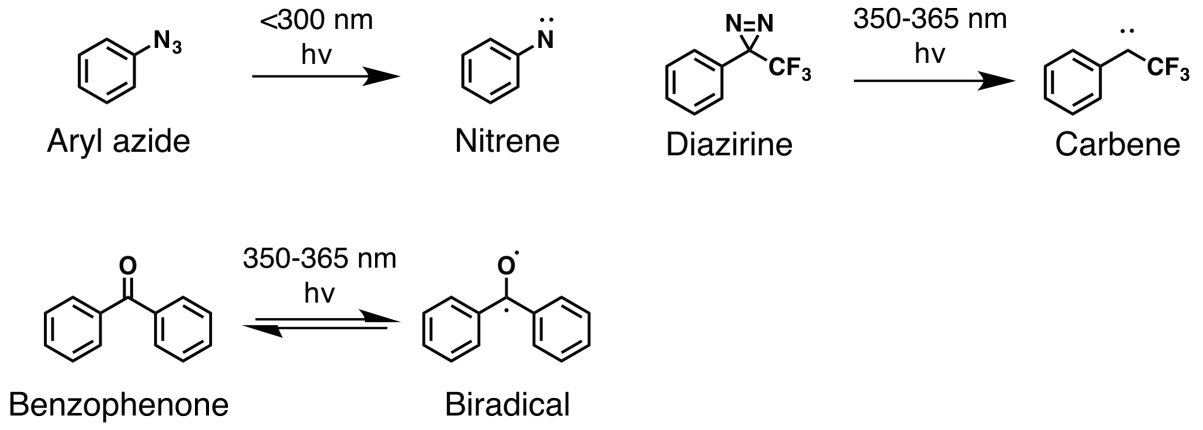


Figure 1-3. Schematic summary of photo-crosslinking functionalities.

タグ分子として用いられる蛍光分子などは、多くの場合、分子量が比較的大きくリガンドとタンパク質の結合に影響を与えたり、蛍光分子由来の非特異的吸着を生じることがある。Cravatt らは、アルキンとアジドを用いた Huisgen 環化付加反応（クリックケミストリー）が細胞抽出液中でも効率的かつ迅速に進行することを報告している[22a,b]。このようにタグ分子を後から導入することで、タグ分子による影響を最小限にすることに成功した。また、このような戦略を採用することで、様々な蛍光分子や精製のためのビオチンタグなどあらゆる機能性分子を用途に応じて導入可能になる。そこで、光反応後に Huisgen 環化付加反応（クリックケミストリー）を利用して様々なタグ分子を導入可能にするため、リンカーの末端部にアルキン体を導入することにした。合成分子プローブの機能評価は大腸菌組み換え GrsA を用いて行うこととし、リガンド部に L-Phe-AMS を有するプローブ **1–10** を設計した（**Figure 1-4**）。

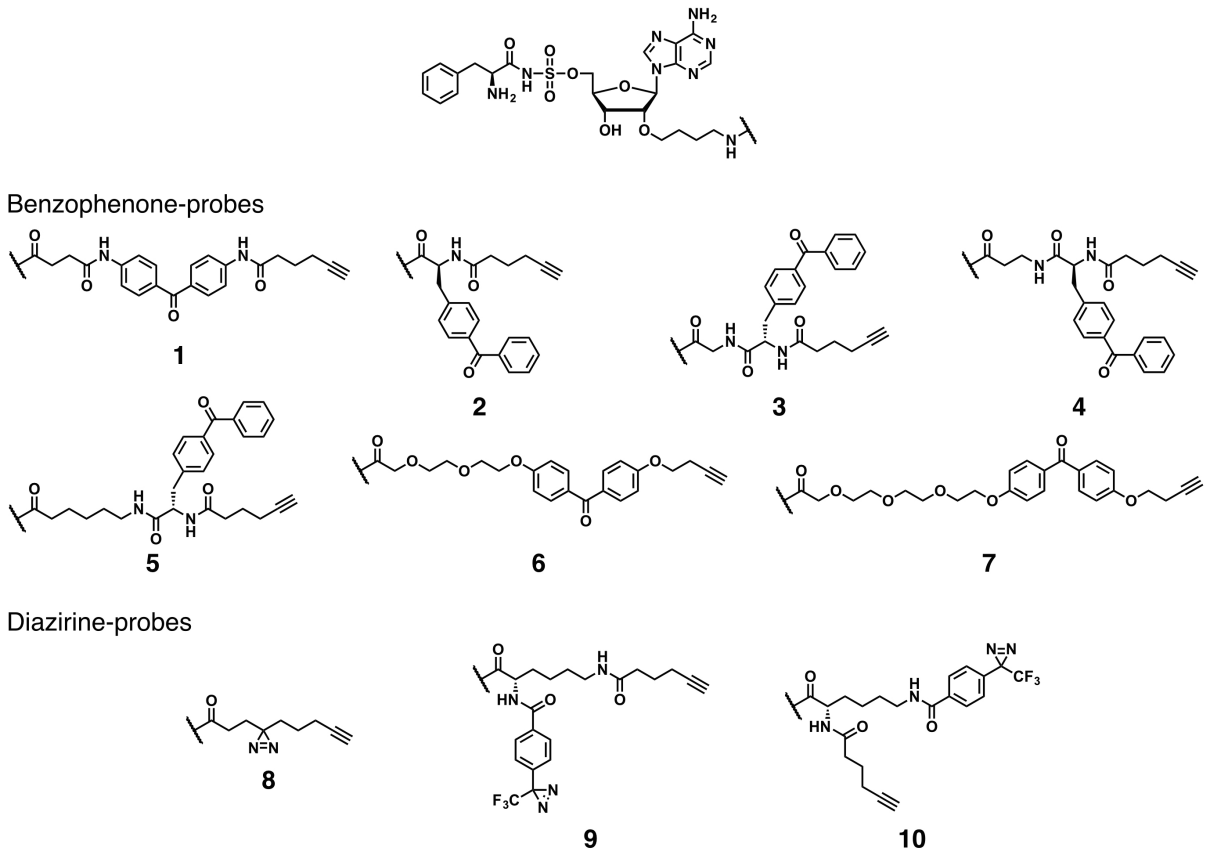
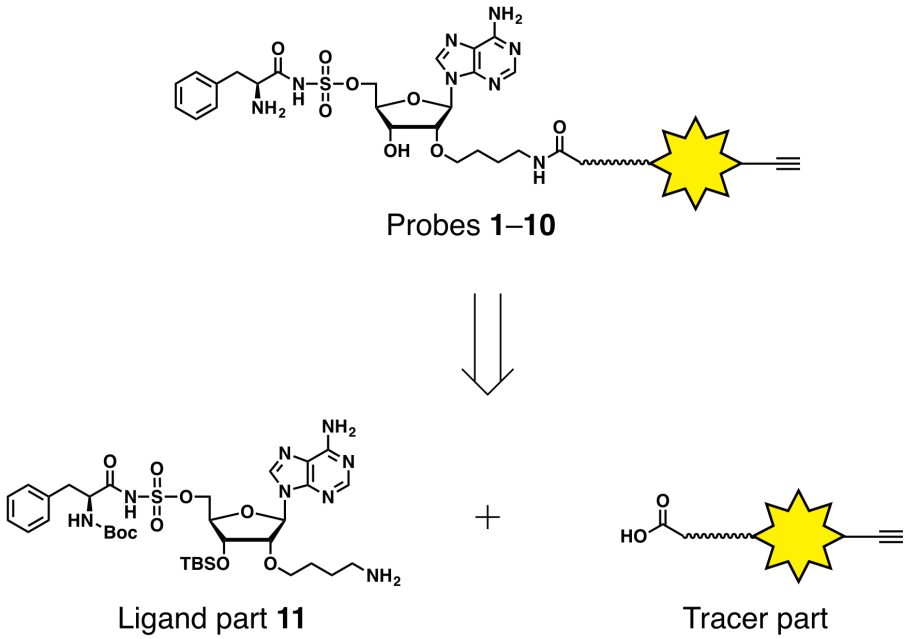


Figure 1-4. Structures of probes **1–10**.

第 3 節　活性部位指向型プローブ 1–10 の合成

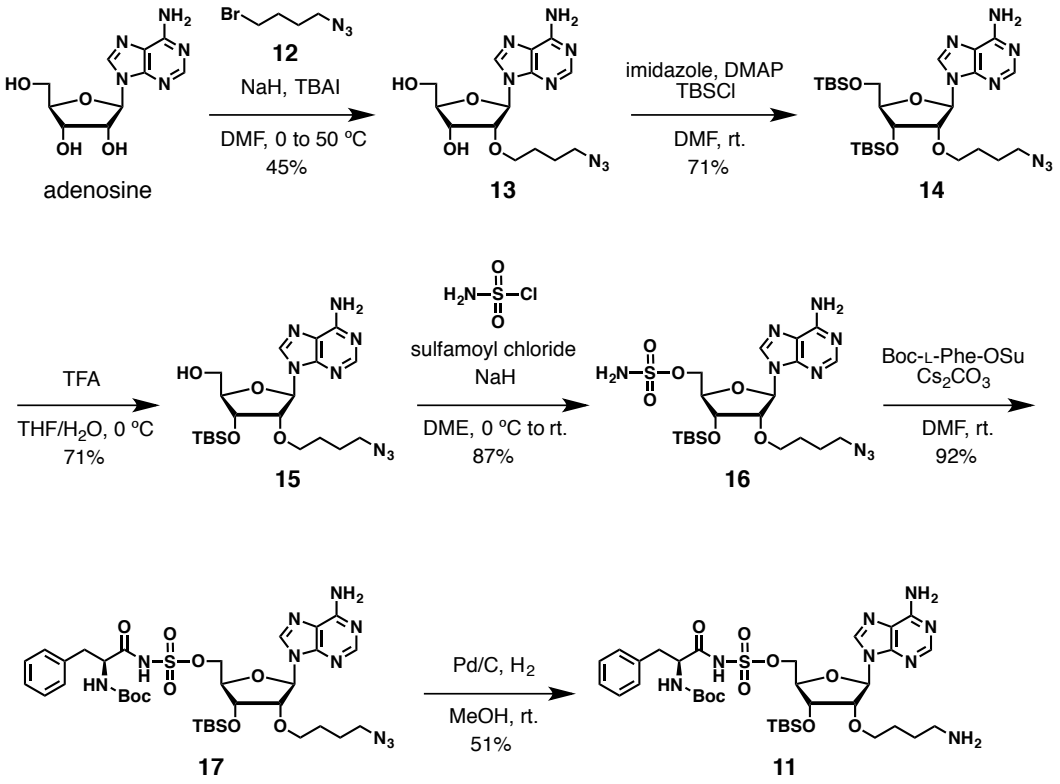
プローブ **1–10** はアデノシン 2’位のヒドロキシ基をアルキル化した共通骨格であるリガンド部と、スペーサーを介した様々な光反応性官能基とアルキンを含むトレーサー部からなる。そこで、共通骨格 **11** とトレーサー部をそれぞれ合成し、最後に縮合反応を用いてプローブ **1–10** を合成することにした（**Scheme 1**）。



Scheme 1. Synthetic strategy for probes **1–10**.

【リガンド部 **11** の合成】

リガンド部 **11** の合成は文献に従い行った（**Scheme 2**）[23]。アデノシンを出発原料として、別途合成した化合物 **12** と反応させることで、2'位がアルキル化された化合物 **13** を得た。アデノシン 2'位に対する *O*-アルキル化の高い選択性は、アデノシン 2'位のヒドロキシ基の pK_a が約 12 程度と一般的なアルコールと比較して低いことに起因すると考えられる。アデノシン 2'位のヒドロキシ基は、水溶液中でアデニン 3 位の窒素原子の孤立電子対と水素結合を形成することが明らかになっており、3'位と比較して pK_a が低下している [24a,b]。これにより、2'位のヒドロキシ基が優先して脱プロトン化され、選択性が生じると考えられる。続いて、3'位と 5'位のヒドロキシ基を TBS 基で保護した後、氷冷化で TFA に付して 5'位の TBS 基を選択的に脱保護し、スルファモイル化を行うことで化合物 **16** へと導いた。最後に Boc-L-Phe-OSu を縮合した後、アジド基を接触還元によりアミンへと導き、リガンド部 **11** を得た。

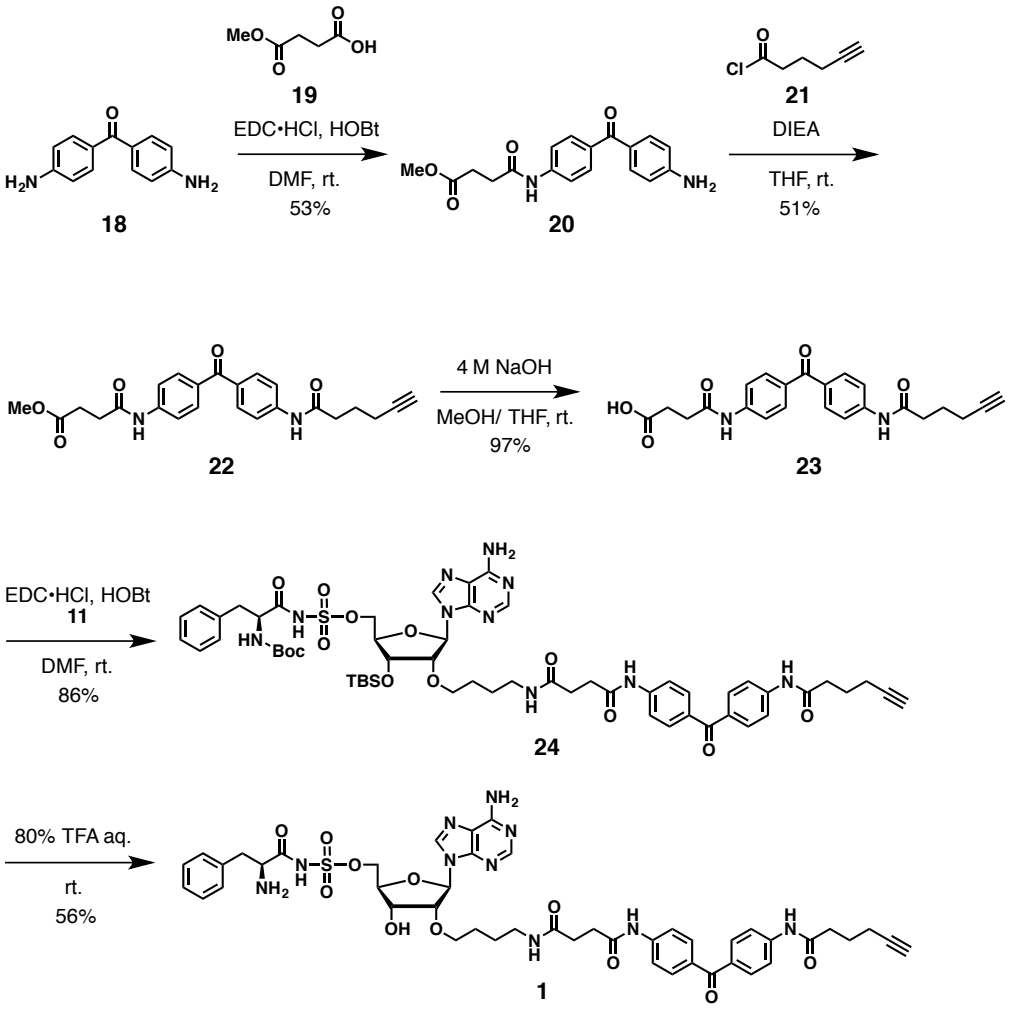


Scheme 2. Synthetic route to compound **11**.

次に各種トレーサー部の合成を行い、リガンド部 **11** との縮合反応と脱保護によるプローブの合成を行った。

【プローブ **1** の合成】

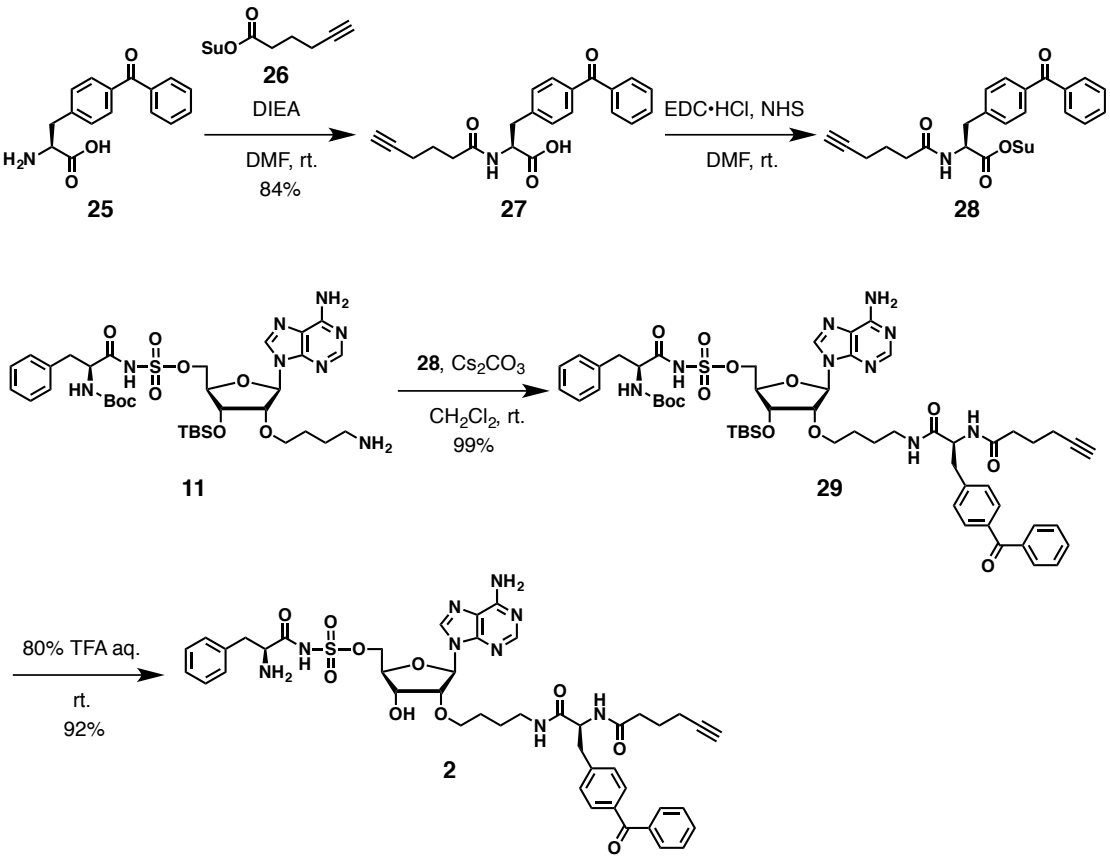
4, 4'-ジアミノベンゾフェノン **18** とコハク酸モノメチルエステル **19** を縮合して **20** とした後、5-ヘキシン酸塩化物 **21** と縮合することによりジアミド体 **22** へと導いた。次に、水酸化ナトリウムを用いてメチルエステルの加水分解を行い、トレーサー部となる化合物 **23** を得た。続いて、リガンド部 **11** とトレーサー部 **23** を EDC-HOBt 法により縮合して化合物 **24** とした後、酸性条件にて脱保護を行い、プローブ **1** を得た（**Scheme 3**）。



Scheme 3. Synthetic route to probe **1**.

【プローブ **2** の合成】

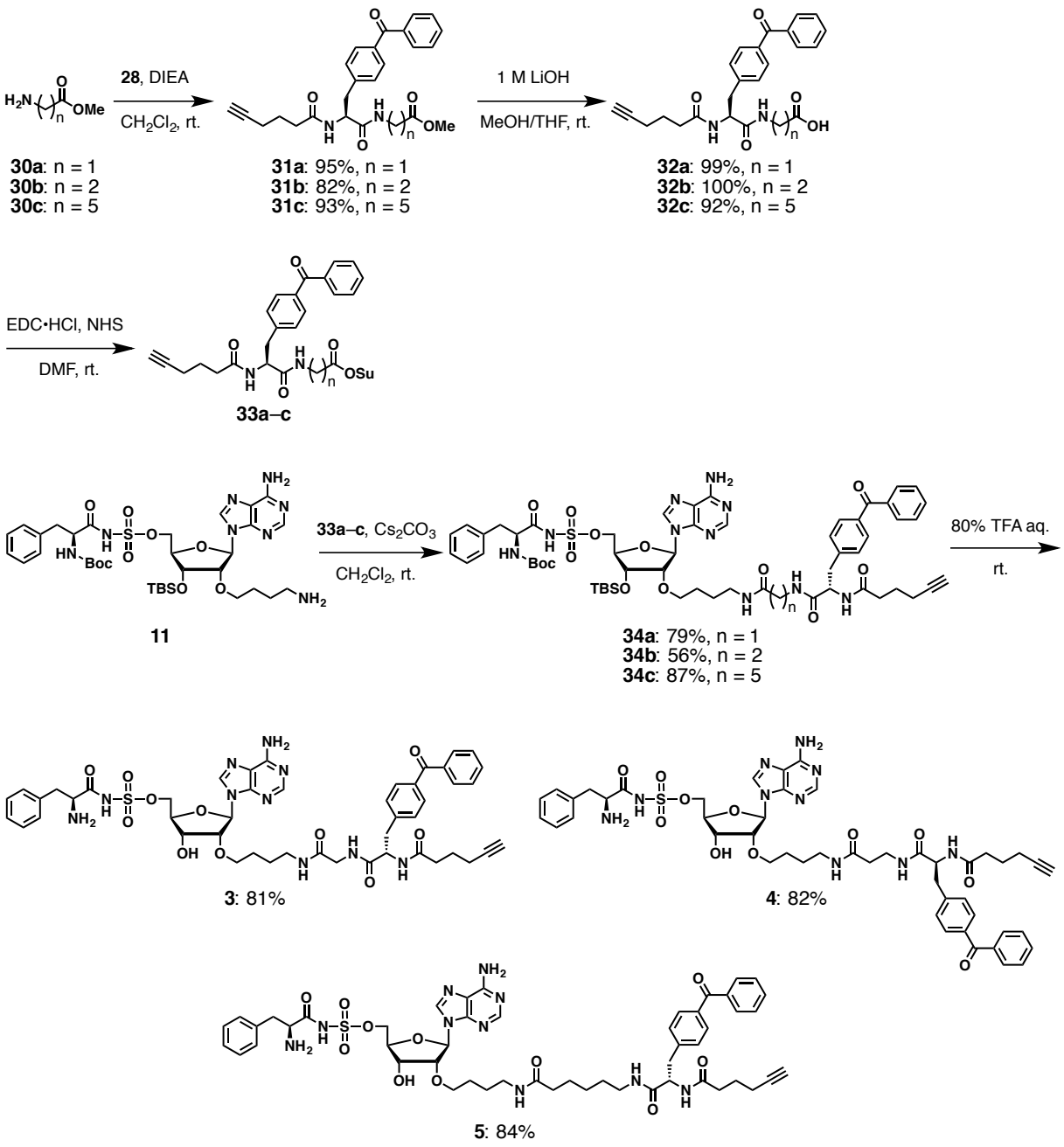
L-4-ベンゾイルフェニルアラニン **25** に対して、スクシンイミドエステル **26** を縮合してトレーサー部 **27** とした後、スクシンイミドエステル **28** へと導いた。続いて、リガンド部 **11** とスクシンイミドエステル **28** を縮合して化合物 **29** とした後、酸性条件にて脱保護を行い、プローブ **2** を得た（**Scheme 4**）。



Scheme 4. Synthetic route to probe **2**.

【プローブ **3–5** の合成】

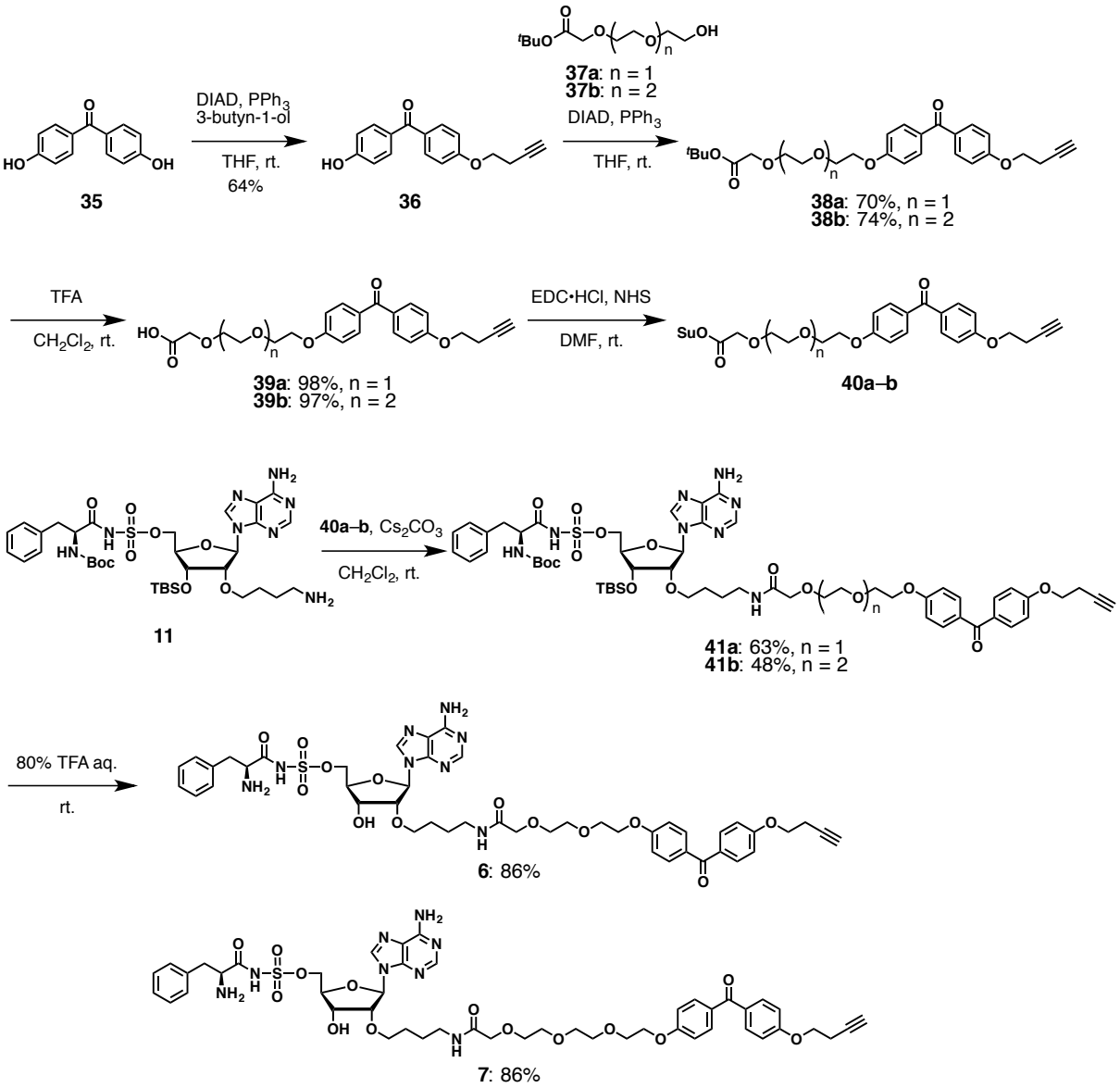
グリシンメチルエステル **30a**、β-アラニンメチルエステル **30b**、6-アミノヘキサン酸メチルエステル **30c** に対して、スクシンイミドエステル体 **28** を縮合して化合物 **31a–c** を得た。続いて、メチルエステルの加水分解とスクシンイミドエステル化を行い、化合物 **33a–c** を合成した。続いて、リガンド部 **11** と化合物 **33a–c** を縮合した後、酸性条件における脱保護によりプローブ **3–5** を得た（**Scheme 5**）。



Scheme 5. Synthetic route to probes **3**–**5**.

【プローブ **6**, **7** の合成】

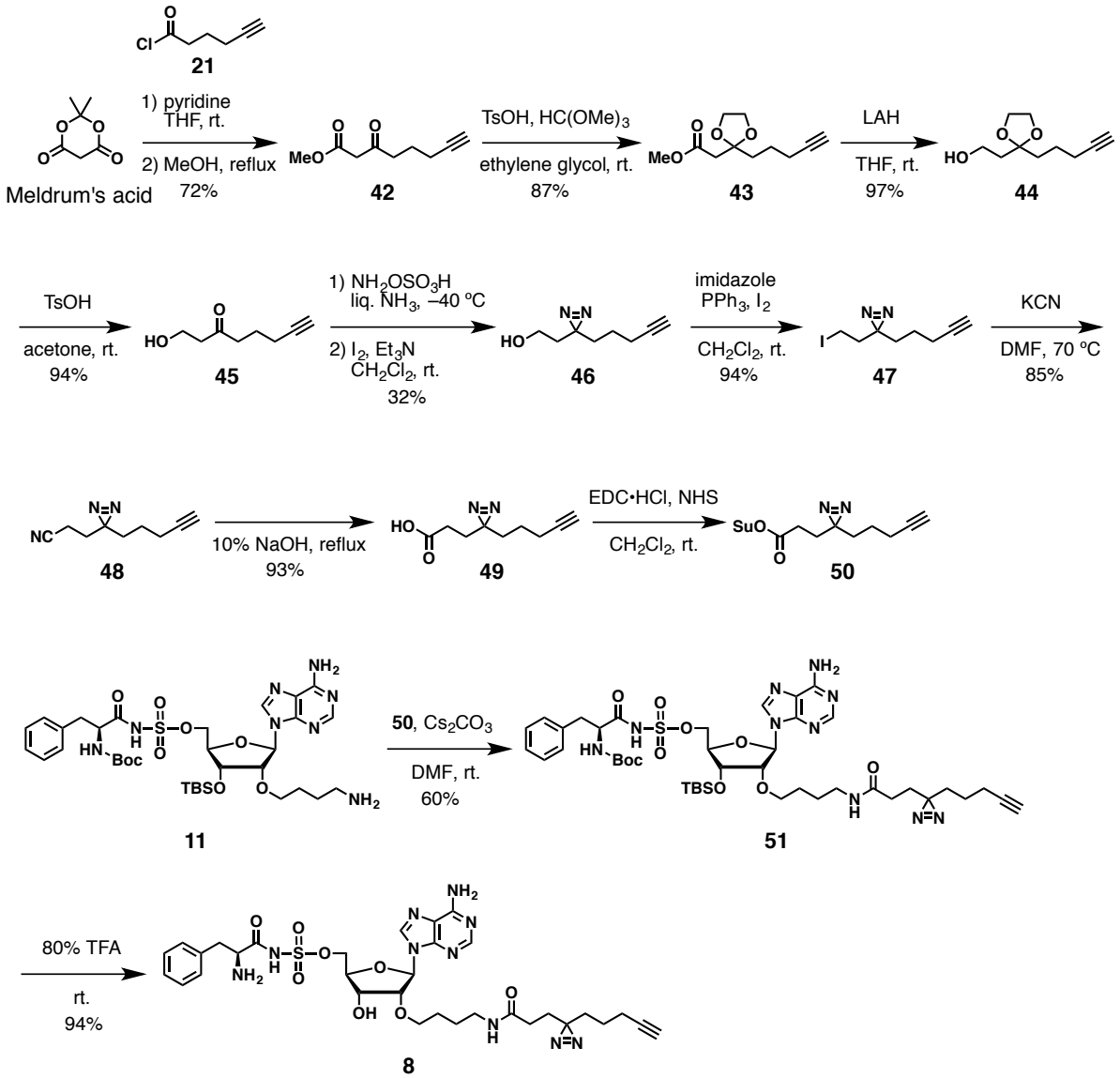
4, 4'-ジヒドロキシベンゾフェノン **35** に対して、光延反応によりモノエーテル体 **36** を得た後、再び光延反応を用いて別途合成したアルコール体 **37a**, **b** の導入を行い、ジエーテル体 **38a**, **b** を合成した。ジエーテル体 **38a**, **b** のエステルを酸処理によりカルボン酸とした後、NHS と縮合を行うことで活性エステル体 **40a**, **b** を得た。続いて、リガンド部 **11** と活性エステル体 **40a**, **b** を縮合後、酸性条件で脱保護することによりプローブ **6**, **7** を得た（**Scheme 6**）。



Scheme 6. Synthetic route to probes **6** and **7**.

【プローブ **8** の合成】

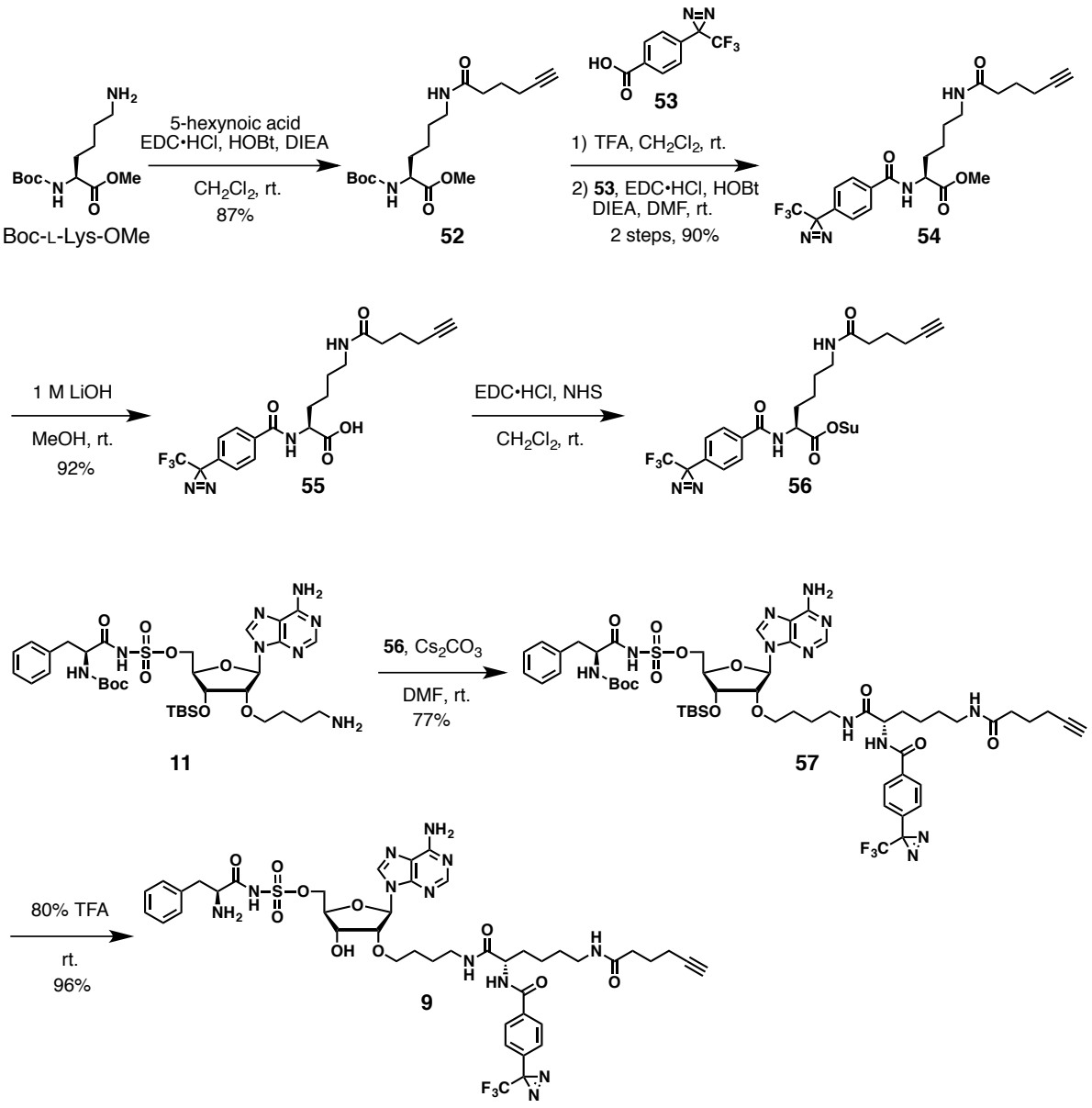
メルドラム酸を出発原料として、5-ヘキシン酸塩化物 **21** と反応させた後、MeOH 処理することで β-ケトメチルエステル体 **42** を得た。続いて、ケトンをアセタールで保護して化合物 **43** とした後、LAH によるエステルの還元とアセタールの脱保護によりケトアルコール体 **45** へと導いた。次に、ケトンを液体アンモニア中でヒドロキシルアミン-*O*-スルホン酸と反応させてジアジリジンを形成した後、ヨウ素を用いて酸化することでジアジリン体 **46** を得た。アルコールをヨウ素化した後、ニトリル基の導入と加水分解、続く NHS との縮合により活性エステル体 **50** を合成した。続いて、リガンド部 **11** と縮合した後、酸性条件で加水分解を行い、プローブ **8** を得た（**Scheme 7**）。



Scheme 7. Synthetic route to probe **8**.

【プローブ **9** の合成】

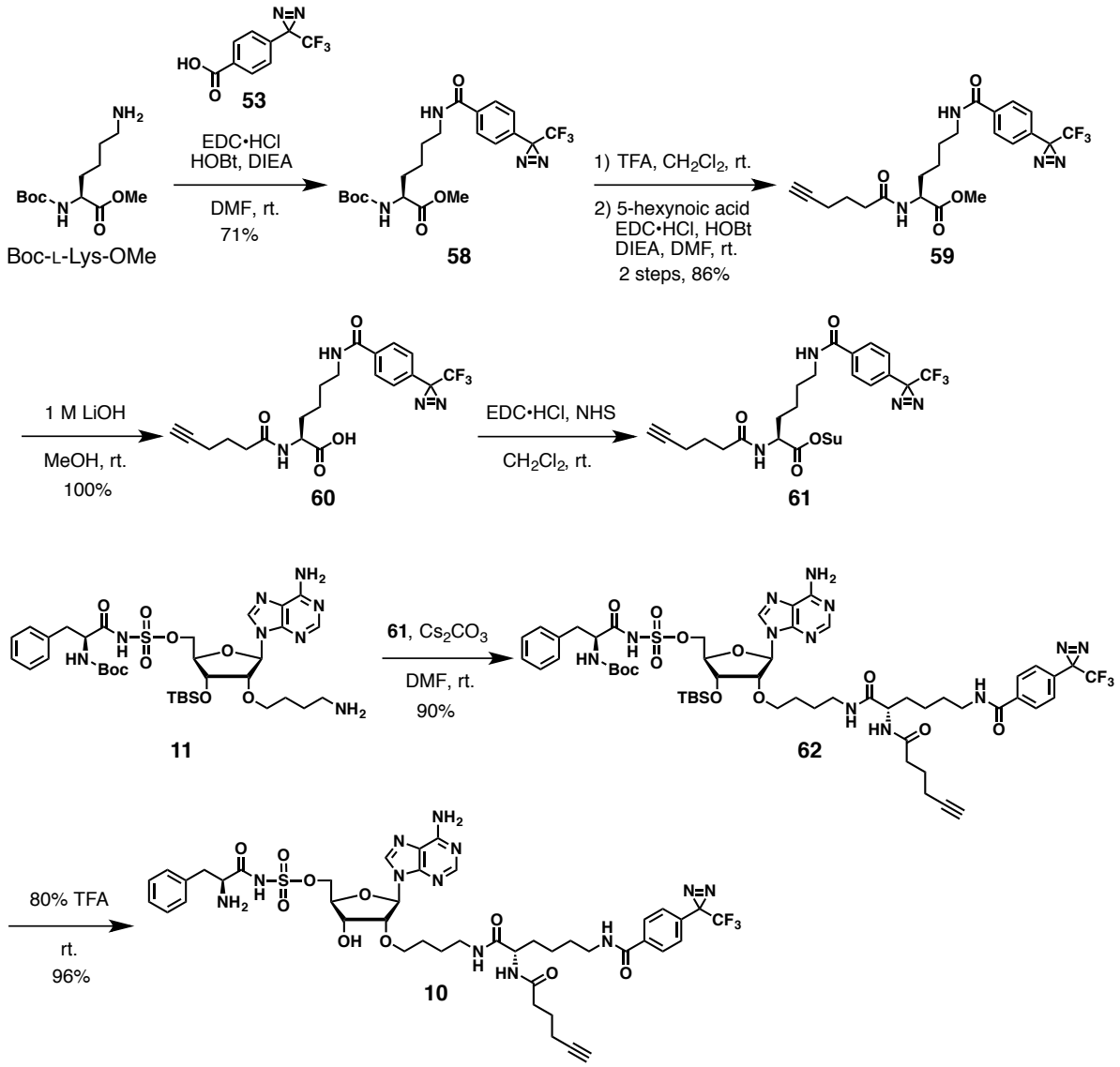
Boc-L-Lys-OMe を出発原料として、側鎖アミンと 5-ヘキシン酸を縮合して化合物 **52** を得た。酸性条件における脱保護と、カルボン酸 **53** の縮合によりジアミド体 **54** を合成した。メチルエステルの加水分解の後、活性エステル **56** へと導いた。続いて、リガンド部 **11** との縮合の後、脱保護によりプローブ **9** を得た（**Scheme 8**）。



Scheme 8. Synthetic route to probe **9**.

【プローブ **10** の合成】

原料である Boc- L-Lys-OMe の側鎖にカルボン酸 **53** を縮合し、化合物 **58** を得た。TFA による Boc 基の脱保護後、5-ヘキシン酸との縮合によりジアミド体 **59** へと導いた。メチルエステルの加水分解によりカルボン酸 **60** とした後、活性エステル体 **61** を合成した。続いて、リガンド部 **11** との縮合と、酸性条件による脱保護によりプローブ **10** を得た（**Scheme 9**）。



Scheme 9. Synthetic route to probe **10**.

第 4 節　活性部位指向型プローブの GrsA に対する阻害活性の評価

まず、大腸菌組み換え GrsA を用いて、アデノシン 2’位のヒドロキシ基の修飾がリガンドと A ドメインの結合に影響を与えるか検討することにした。合成したプローブの中で、構造的に大きいベンゾフェノン型のプローブ **1–7** について大腸菌組み換え GrsA の A ドメインに対する阻害活性を評価し、リガンドである L-Phe-AMS の阻害活性と比較した。

A ドメインにおける酵素活性評価には、Hydroxamate-MesG アッセイを用いた [25]。Hydroxamate-MesG アッセイの原理を **Figure 1-5** に示す。基質であるアミノ酸は A ドメイン存在下、ATP と反応してアミノアシル-AMP を生成する。この際生じるピロリン酸はピロホスファターゼによりモノリン酸へ変換される。MesG はプリンヌクレオシドホスホリラーゼ存在下、モノリン酸と反応して分解産物を生じる。この分解産物は 360 nm に極大吸収波長を示すことから、この吸光度変化を測定することで酵素活性を評価する方法である。また、中間体であるアミノアシル-AMP は A ドメインと高い親和性を示す。ヒドロキシルアミンは迅速に A ドメインの活性部位からアミノアシル-AMP を分解する役割を担っている。

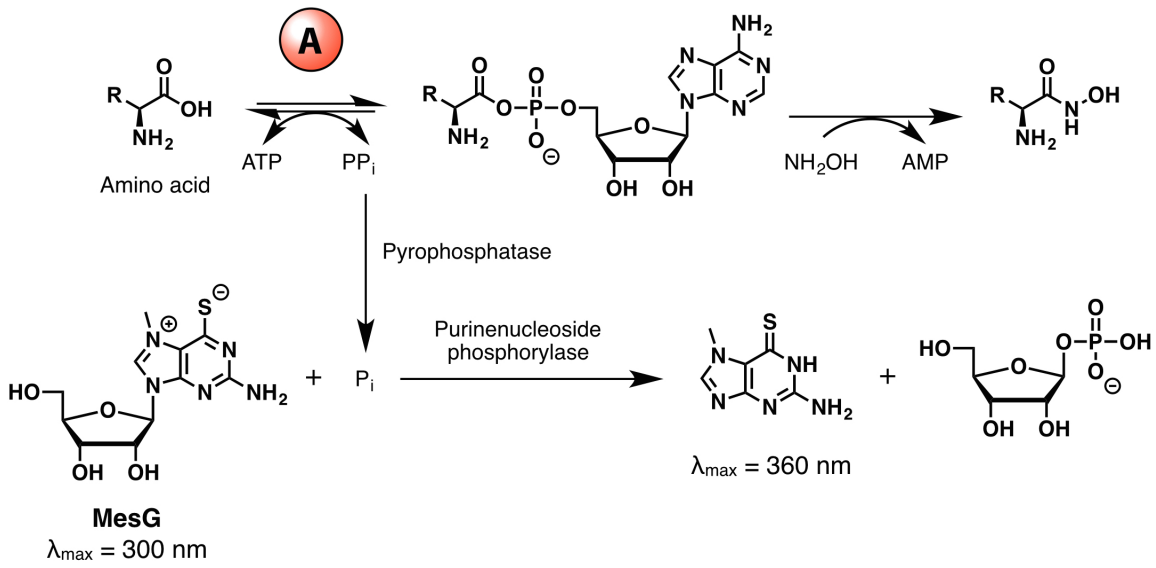
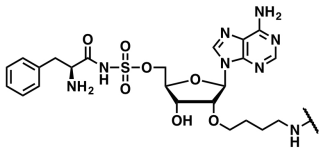
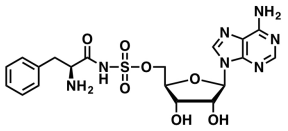
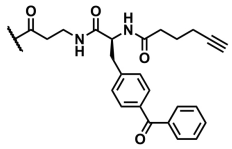
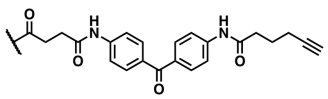
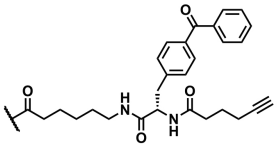
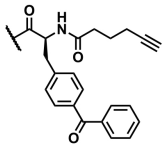
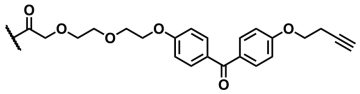
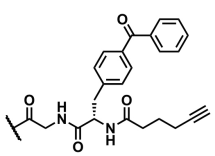
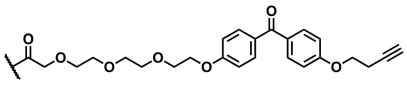


Figure 1-5. Schematic summary of the hydroxamate-MesG assay.

プローブ **1–7** の大腸菌組み換え GrsA に対する阻害活性を示す（**Table 1**）。まず、本アッセイ系を用いて L-Phe-AMS の阻害活性を測定したところ、K_i^{app} = 1.2 ± 0.14 nM であり文献値と良い一致を示した [23]。これに対して、プローブ **1–5** について同様に阻害活性を評価した結果、強い阻害活性（数 nM オーダー）を示すことが明らかとなった。一方で、PEG をスペーサーとして用いたプローブ **6**、**7** の阻害活性は、L-Phe-AMS と比較して 20 倍以上減弱した。これは、2'位の修飾による影響ではなく、PEG 分子を導入したことにより上昇したリンカー全体の自由度が A ドメインと結合した際ににある程度制限されたためと考察した [26]。すなわち、自由度の高い PEG リンカーを有するプローブ **6**、**7** では、プローブ **1–5** に比べて A ドメインとの結合時のエントロピー損失が大きくなり、阻害活性が減少したと考えられる。これらの結果から、アデノシン 2'位への修飾及び構造的に大きいベンゾフェノンの導入は、リガンドと A ドメインの結合にほとんど影響を示さないことが明らかとなった。

Table 1. Inhibitory activities of L-Phe-AMS and probes **1–7** toward recombinant GrsA.[a]



Compounds	Structures	K_i^{app} (nM)	Compounds	Structures	K_i^{app} (nM)
L-Phe-AMS		1.20 ± 0.14[b]	**4**		2.13 ± 0.36
1		1.43 ± 0.19	**5**		3.34 ± 0.52
2		6.87 ± 0.13	**6**		28.2 ± 4.2
3		0.64 ± 0.05	**7**		26.6 ± 4.6

[a]Kinetic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[25] Errors were given as the standard error of duplicate independent measurements.

[b]Value taken from ref. 23.

第 5 節　活性部位指向型プローブを用いた GrsA のラベル化実験

合成したプローブが A ドメインに対する阻害活性を維持することが明らかになったので、次に大腸菌組み換え GrsA を用いてラベル化実験を行った。実験の概要を **Figure 1-6** に示す。大腸菌組み換え GrsA に対してそれぞれのプローブを処理した後、365 nm の UV を照射した。その後、TAMRA-アジドと Cu(I) 存在下、Huisgen 環化付加反応に付して蛍光タグを導入した。SDS-PAGE でタンパク質を分離後、ゲルの蛍光を観察することでラベル化の有無を評価した。また、プローブによるラベル化が非特異的結合によるものではなく、リガンドが A ドメインに結合することにより特異的に生じることを示すために、過剰量の競合的阻害剤（L-Phe-AMS）を前処理後、プローブによるラベル化を行い、蛍光強度を比較した。

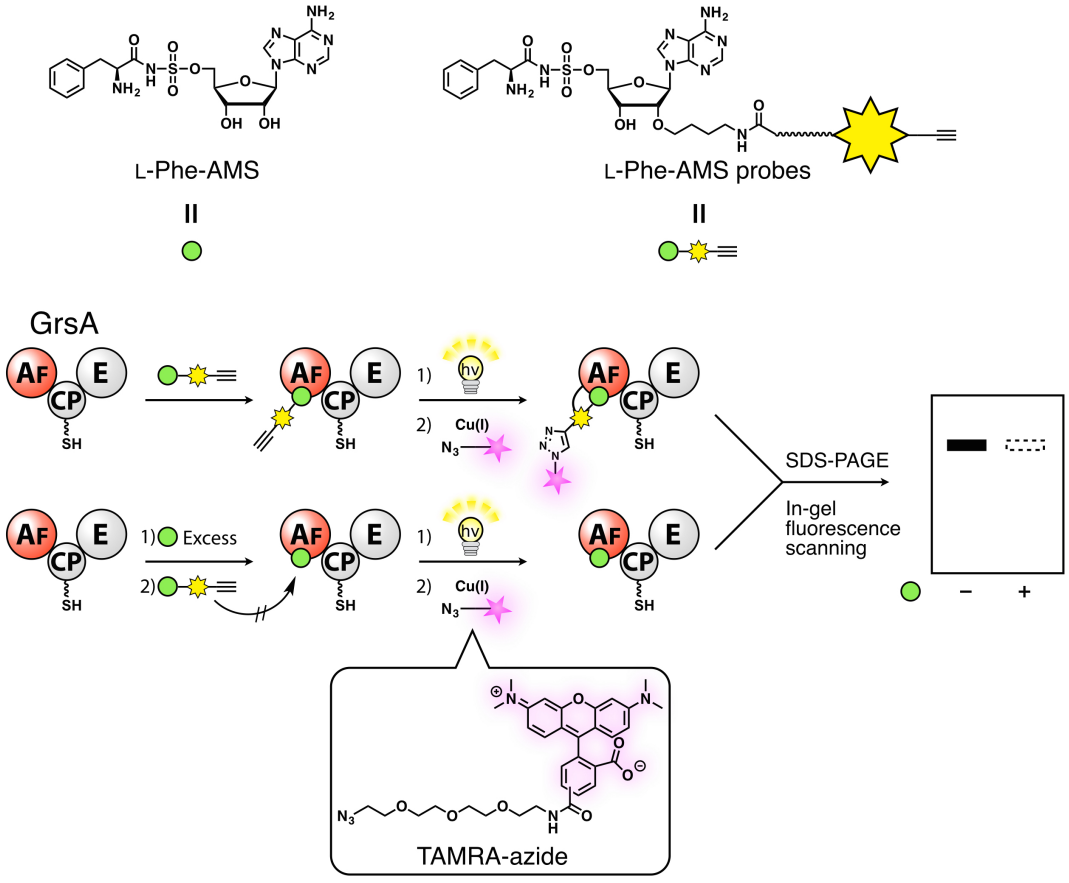


Figure 1-6. Schematic summary of the labeling of recombinant NRPSs by active site-directed proteomic probes for A-domains.

まず、ベンゾフェノン型プローブ **1–7** によるラベル化実験を行った。その結果、すべてのプローブで GrsA のラベル化が観察された（**Figure 1-7**）。また、このラベル化は L-Phe-AMS 存在下では消失することから、プローブによる GrsA のラベル化はリガンド部が A ドメインに結合することで進行することが明らかとなった。次に、ラベル化効率の算出を行った。TAMRA の蛍光強度に関する検量線は、市販の TAMRA-BSA を用いて作成した。大腸菌組み換え GrsA（1 μM）に対するプローブ **1–7**（1 μM）のラベル化効率を算出したところ、プローブ **1** が最も高いラベル化効率（43%）を示した。一方で、PEG 基を介したプローブ **6**、**7** のラベル化効率はそれぞれ 15、14%と低いことがわかった。これは、プローブ **6**、**7** の阻害活性がプローブ **1** より約 20 倍弱いこと、及び比較的自由度が高く長いスペーサーを導入したことにより、GrsA とベンゾフェノン部の接触回数が減少したことに起因すると考えられる。これらの結果から、ベンゾフェノン型プローブの中でプローブ **1** が最適な構造であると結論した。

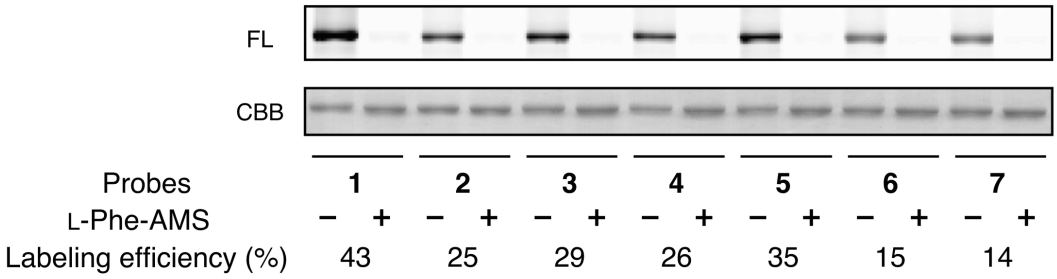


Figure 1-7. Labeling of recombinant GrsA with probes **1–7** and competitive inhibition study with excess L-Phe-AMS (100 eq.). The labeling efficiency was determined using a TAMRA-BSA conjugate as a standard for fluorescent intensity.

次に、ジアジリン型プローブ **8–10** によるラベル化実験を行った（**Figure 1-8a**）。プローブ **1** をポジティブコントロールとしてラベル化実験を行ったところ、プローブ **1–7** と同様に全てのプローブで GrsA のラベル化が観察され、これらのラベル化は L-Phe-AMS により消失した。このことから、ジアジリン型プローブ **8–10** も A ドメインに特異的に結合することにより、GrsA をラベル化していることが明らかになった。

これまでの実験は、UV 照射時間を 30 分に固定して行ってきた。しかし、光照射により生じる活性反応種が可逆性を示すベンゾフェノンと不可逆性かつ水とも反応するジアジリンでは、照射時間に対するラベル化量が異なるプロファイルを示すことが

予想される。そこで、UV 照射時間に対するラベル化量の経時変化を観察した。最も高いラベル化効率を示すベンゾフェノンプローブ **1**、アルキルジアジリンプローブ **8**、フェニルジアジリンプローブ **10** について経時変化を追跡したところ、プローブ **1** では時間依存的にラベル化量が増加した（**Figure 1-8b**）。一方で、ジアジリンプローブ **8**、**10** は 5 分以内の照射で迅速に GrsA のラベル化が進行し、その後ラベル化量の増加は観察されなかった。これは、5 分の UV 照射で全てのジアジリンがカルベンとなり、未反応のカルベンは水と反応したためと考えられる。また、プローブ **10** では 1 分程度の UV 照射で急速にラベル化が進行しており、プローブ **1** とは異なる特徴を有することが明らかとなった。

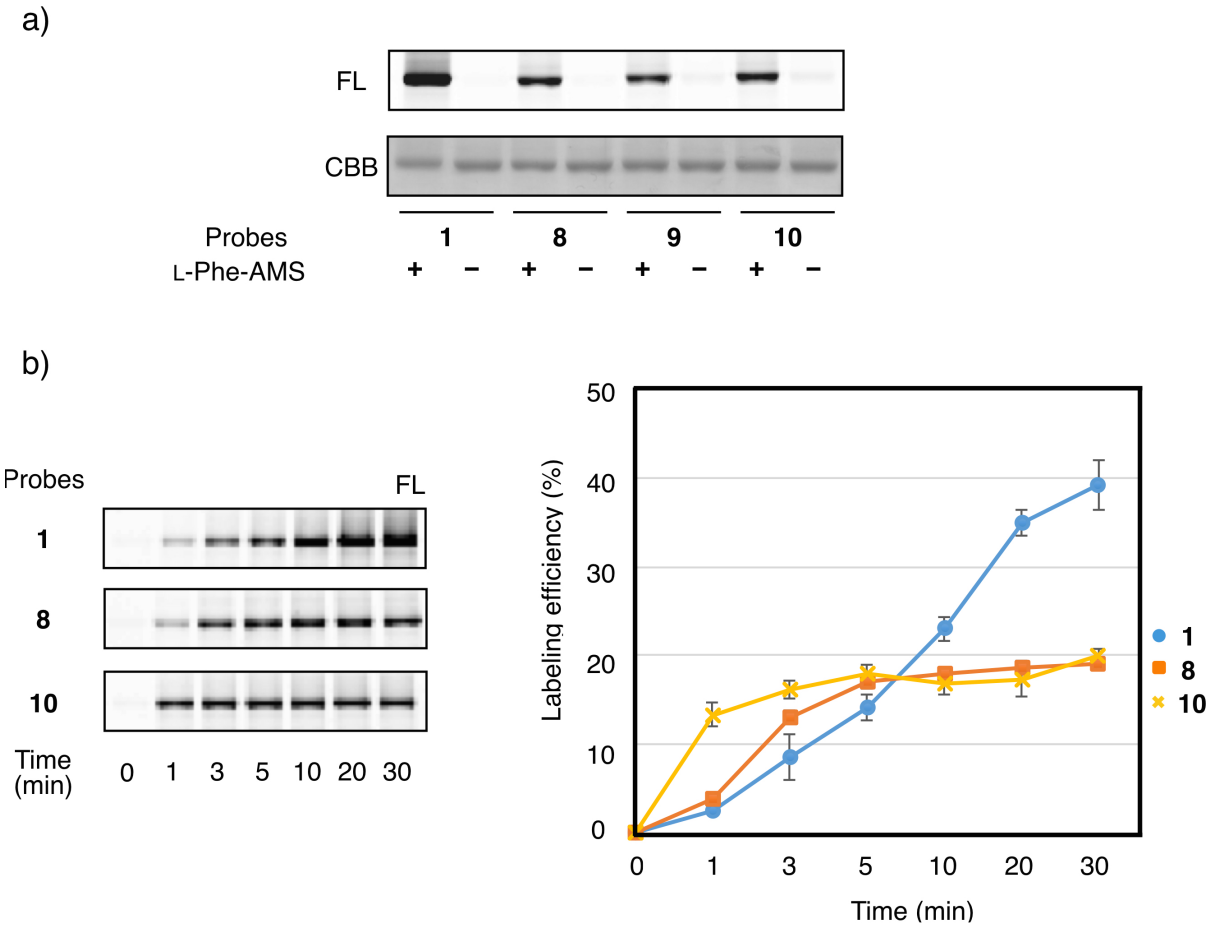


Figure 1-8. a) Labeling of recombinant GrsA with probes **1**, **8**, **9**, and **10** and competitive inhibition study with excess L-Phe-AMS (100 eq.). b) UV photolysis time course studies of probe **1**, **8**, and **10**.

第 6 節　小括

本章では、A ドメインの厳密な基質特異性を利用して NRPS をラベル化することを目的とし、A ドメインに対する活性部位指向型プローブ **1–10** を設計･合成し、大腸菌組み換え GrsA を用いて機能評価を行った。

本プローブでは、A ドメインの反応中間体であるアミノアシル-AMP を模倣したアミノアシル-AMS をリガンド部として用いた。また、A ドメインと基質の X 線共結晶構造の情報からリンカー導入部としてアデノシン 2’位のヒドロキシ基を見出し、リンカー部に光反応性官能基と末端アルキンを導入したベンゾフェノン型プローブ **1–7** とジアジリン型プローブ **8–10** を設計した。各プローブはリガンド部とトレーサー部をそれぞれ構築した後、フラグメントカップリングを行うことで合成した。ベンゾフェノン型プローブ **1–7** の大腸菌組み換え GrsA に対する阻害活性から、アデノシン 2’位のヒドロキシ基への修飾は、リガンドと A ドメインの結合にほとんど影響を与えないことを明らかにした。また、競合的ラベル化実験から、本プローブ群が GrsA の A ドメインを特異的に認識してラベル化していることを示し、その中でプローブ **1** が最も高いラベル化効率を示すことを見出した。さらに、プローブ **10** はラベル化効率ではプローブ **1** に劣るものの、1 分程度の UV 照射により迅速に標的 NRPS をラベル化可能であることを明らかにした。プローブ **1** とプローブ **10** はそれぞれ異なる特徴を示すことから、NRPS の存在量や安定性に応じて使い分けることで、様々な状況に対応可能になると期待できる（**Figure 1-9**）。

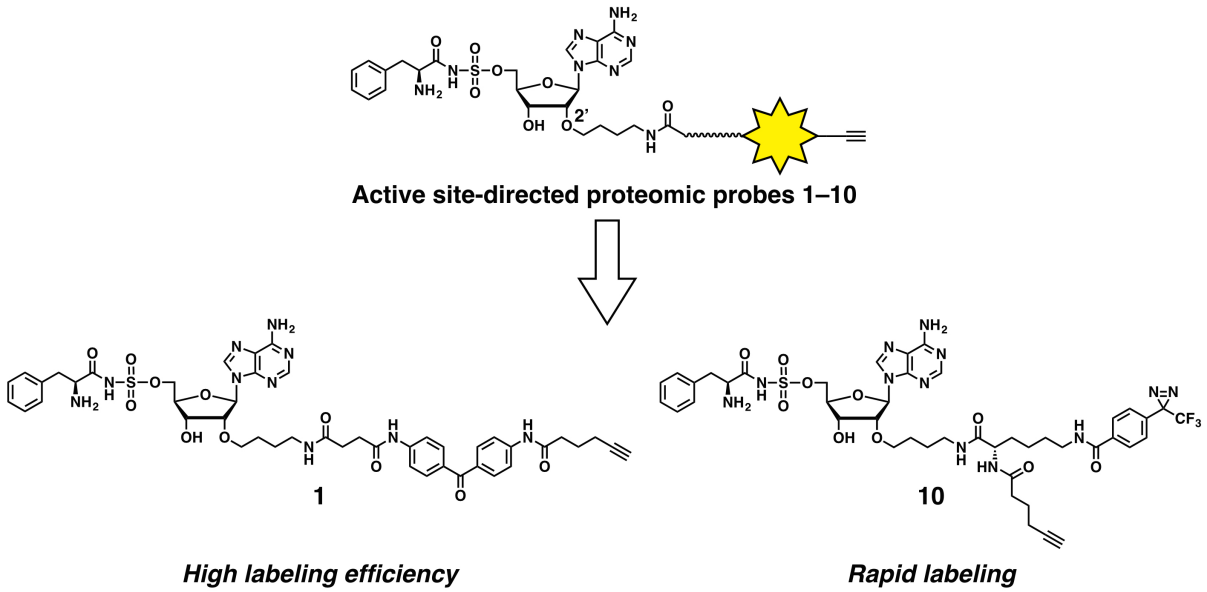


Figure 1-9. Active site-directed proteomic probes **1** and **10** for A-domains in NRPSs.

第２章　合成分子プローブを用いた内在性 NRPS の検出法の確立

第１節　はじめに

NRPS は一般的にマルチモジュール型であり、一つのタンパク質中に複数の A ドメインを含んでいる。例えば、抗生物質グラミシジン S（GS）は 1 モジュールからなる GrsA と 4 モジュールからなる GrsB によって合成される[3a,b]。GrsB には 4 つの A ドメインが存在し、それぞれ L-Pro、L-Val、L-Orn、L-Leu に基質特異性を有する。そのため、A ドメインを指標に内在性 NRPS をラベル化･検出する上で、プローブのリガンド部アミノ酸を置換することにより､各アミノ酸に基質特異性を有する A ドメインを選択的にラベル化することが重要な鍵となる。そこで、本章ではプローブ **1**（L-Phe-AMS-BPyne）を母骨格として、リガンド部アミノ酸を L-Pro 及び L-Orn に置換したプローブ L-Pro-AMS-BPyne、L-Orn-AMS-BPyne を新たに合成して、大腸菌組み換えタンパク質を用いた A ドメイン選択的ラベル化の評価を行った（**Figure 2-1a**）。また、内在性 NRPS として GrsA 及び GrsB を標的として、L-Phe-AMS-BPyne（**1**）、L-Pro-AMS-BPyne、L-Orn-AMS-BPyne を用いて GS 生産菌プロテオーム中に存在する内在性 NRPS のラベル化を試みた（**Figure 2-1b**）。また、GrsB の 4 つの A ドメインに対応する競合的阻害剤 L-Pro-AMS、L-Val-AMS、L-Orn-AMS、L-Leu-AMS をそれぞれ合成し、A ドメインのリガンド依存的ラベル化の評価を行った。

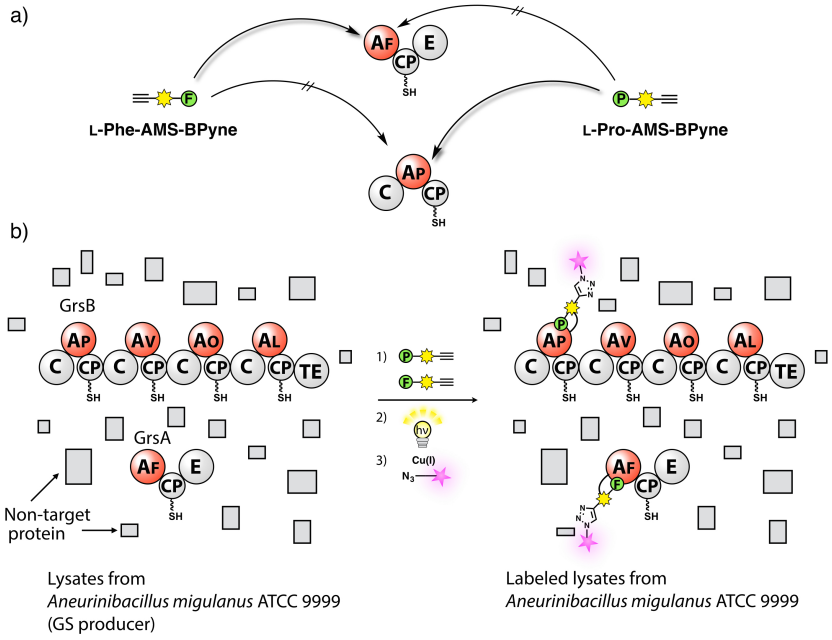
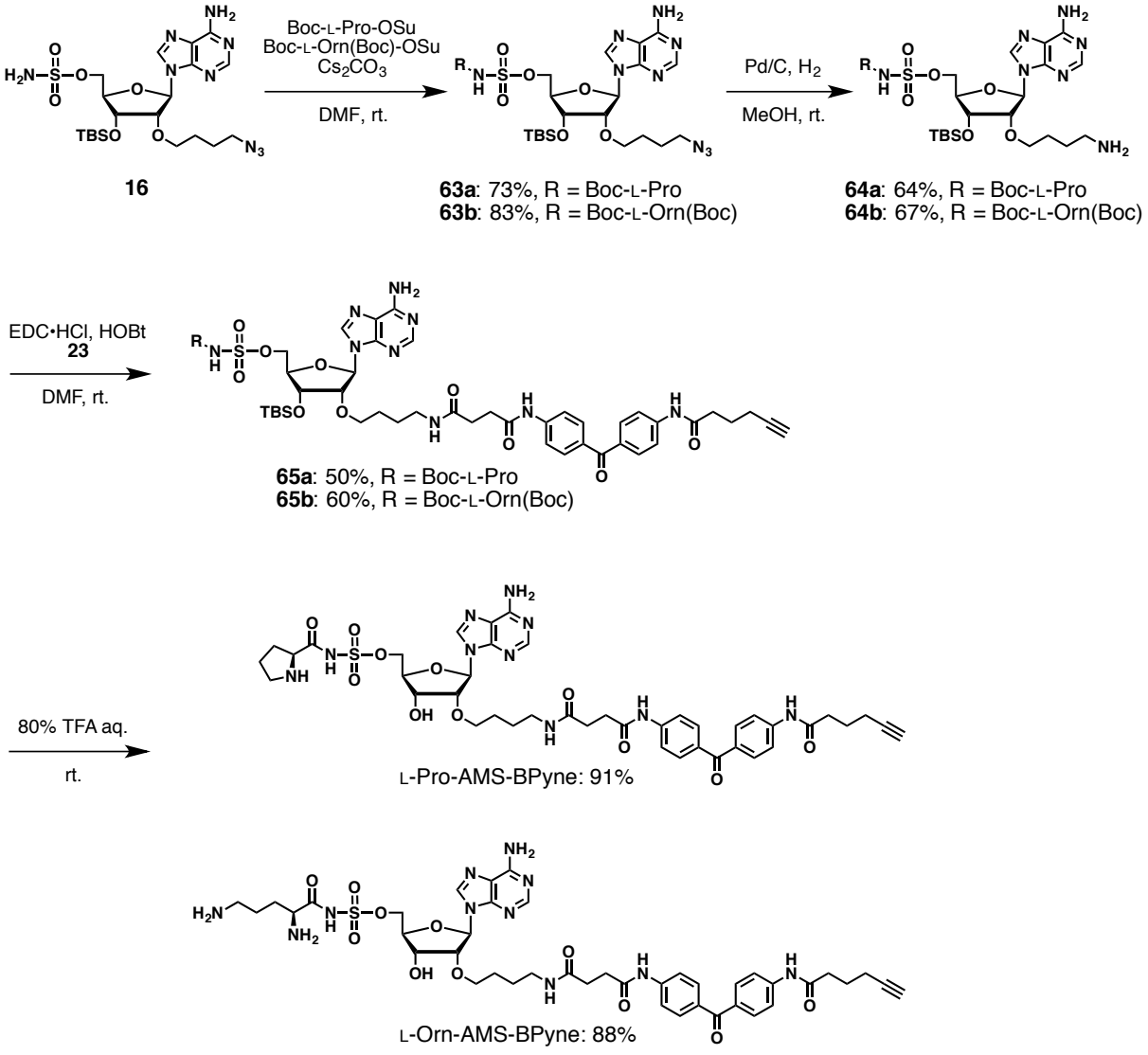


Figure 2-1. a) Selective labeling of the cognate A-domains by ligand-directed manner. b) Labeling of endogenous GrsA and GrsB proteins in natural product producer proteomes.

第 2 節　リガンド依存的ラベル化にむけた活性部位指向型プローブの合成

【L-Pro-AMS-BPyne、L-Orn-AMS-BPyne の合成】

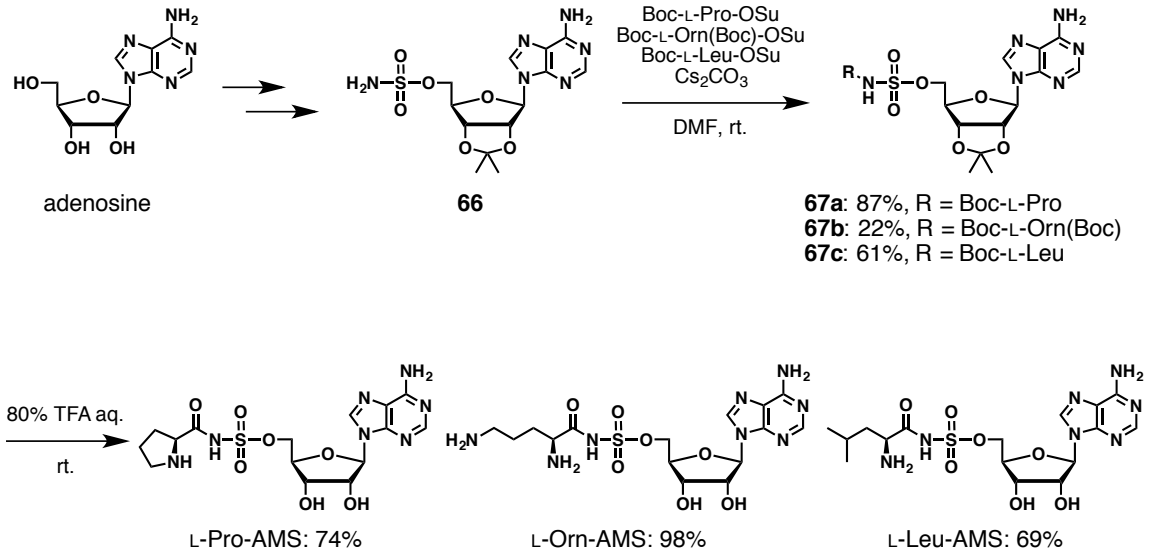
プローブの合成は L-Phe-AMS-BPyne（**1**）の合成法を活用した [23]。化合物 **16** を Boc-L-Pro-OSu もしくは Boc-L-Orn-OSu と反応させた後、接触還元に付してリガンド部 **64a**, **b** を得た。続いて、リガンド部 **64a**, **b** とトレーサー部 **23** を縮合した後、脱保護することにより L-Pro-AMS-BPyne、L-Orn-AMS-BPyne を合成した（**Scheme 10**）。



Scheme 10. Synthetic route to L-Pro- and L-Orn-AMS-BPyne.

【L-Pro-AMS、L-Orn-AMS、L-Leu-AMS の合成】

アデノシンを出発原料として、文献に従い 2’、3’位のアセトナイド化と 5’位のスルファモイル化により化合物 **66** を得た[27]。続いて、化合物 **66** に対して各種アミノ酸を導入後、酸性条件下で脱保護を行い L-Pro-AMS、L-Orn-AMS、L-Leu-AMS を合成した（**Scheme 11**）。また、L-Val-AMS は文献に従い合成した[28]。



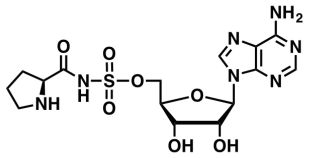
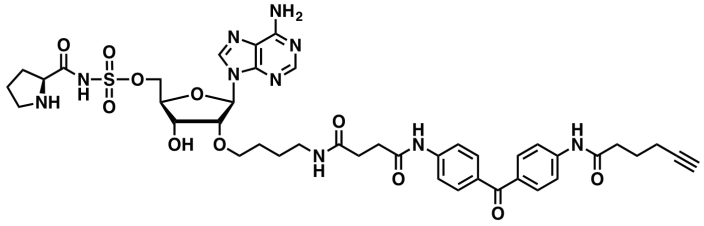
Scheme 11. Synthetic route to L-Pro-, L-Orn- and L-Leu-AMS.

第 3 節　$TycB_1$ に対する阻害活性の評価

まず、アデノシン 2'位のヒドロキシ基へのリンカー導入が他の A ドメインにおいても許容されるか否か検討することにした。そこで、チロシジン合成酵素の一つで L-Pro に基質特異性を有する $TycB_1$ を大腸菌組み換えタンパク質として調製し、L-Pro-AMS と L-Pro-AMS-BPyne の $TycB_1$ に対する阻害活性を比較した[29]。

Hydroxamate-MesG アッセイを用いて、$TycB_1$ に対する阻害活性を評価したところ、L-Pro-AMS の阻害活性は K_i^{app} = 431 ± 42 nM であり、L-Pro-AMS-BPyne の阻害活性は K_i^{app} = 327 ± 17 nM であった（**Table 2**）。L-Pro-AMS と L-Pro-AMS-BPyne で顕著な阻害活性の差は観察されなかったことから、アデノシン 2'位のヒドロキシ基への修飾は $TycB_1$ の A ドメイン（L-Pro）においても許容されることが明らかになった。

Table 2. Inhibitory activities of L-Pro-AMS and L-Pro-AMS-BPyne toward recombinant $TycB_1$.[a]

Compounds	Structures	K_i^{app} (nM)
L-Pro-AMS		431 ± 41
L-Pro-AMS-BPyne		327 ± 17

[a]Kinetic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[25] Errors were given as the standard error of duplicate independent measurements.

第 4 節　大腸菌組み換えタンパク質を用いたラベル化実験

内在性 NRPS のラベル化にむけて、プローブのラベル化に関する性質を詳細に検討することにした。まず、L-Pro-AMS-BPyne による $TycB_1$ のラベル化実験を行った（**Figure 2-2a**）。その結果、$TycB_1$ のラベル化が観察され、過剰量の L-Pro-AMS の前処理により消失した。このことから、L-Pro-AMS-BPyne は $TycB_1$ の A ドメインを特異的にラベル化することが明らかになった。次に L-Phe-AMS-BPyne と L-Pro-AMS-BPyne によるラベル化の最適な UV 照射時間について、組み換え GrsA 及び $TycB_1$ を用いて検討した（**Figure 2-2b**）。最大 60 分まで UV 照射を行った結果、両プローブとも 30 分以降ラベル化量に大きな変化はみられなかった。このことから、最適 UV 照射時間は 30 分と結論付けた。NRPS は微量タンパク質であることが予想されるため、次にプローブによる組み換えタンパク質の検出限界を調べた（**Figure 2-2c**）。その結果、L-Phe-AMS-BPyne による GrsA のラベル化では 10 fmol、L-Pro-AMS-BPyne による $TycB_1$ のラベル化では 5 fmol の NRPS まで検出可能であることが明らかになった。

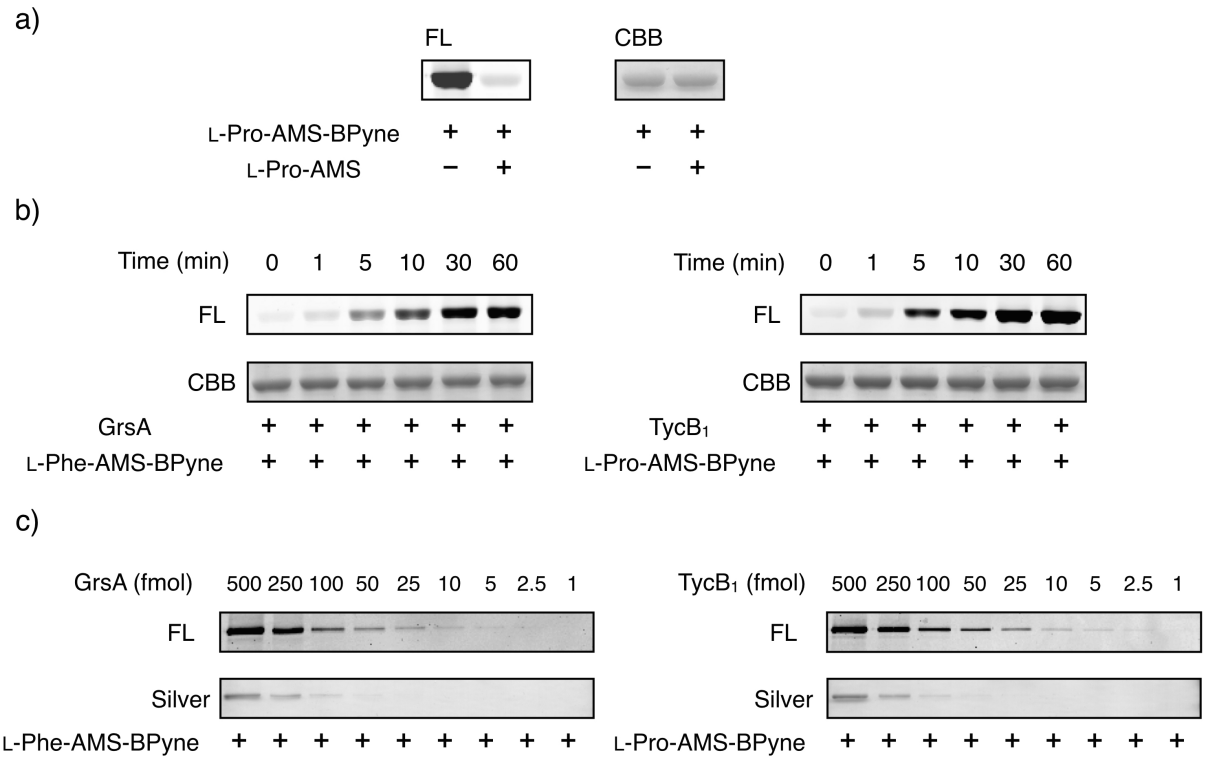


Figure 2-2. Labeling of recombinant GrsA and $TycB_1$ with L-Phe-AMS-BPyne and L-Pro-AMS-BPyne, respectively. a) Labeling of $TycB_1$ and competitive inhibition study with excess L-Pro-AMS. b) UV photolysis time course studies of the labeling of GrsA with (left) and $TycB_1$ (right). c) Limit of detection of GrsA (left) and $TycB_1$ (right).

続いて、プローブがリガンド部のアミノ酸に対応する基質特異性を有する A ドメインを選択的にラベル化可能か否か検証することにした。GrsA と $TycB_1$ に加えてアウレウシミン合成酵素の 1 モジュールで、L-Val に基質特異性を有する $AusA_1$ を大腸菌組み換えタンパク質として調製し、実験に用いた[28]。また、多くの低分子化合物が非特異的に結合することが知られている BSA を用いて、タンパク質との非特異的結合の有無についても同時に調べた。

まず、L-Phe-AMS-BPyne を用いて、A ドメイン選択性の評価を行った（**Figure 2-3**）。それぞれのタンパク質に対して L-Phe-AMS-BPyne を処理したところ、GrsA のみ選択的にラベル化されることがわかった。次に、L-Pro-AMS-BPyne を用いて同様の実験を行うと、$TycB_1$ のみが選択的にラベル化された。また、両プローブとも BSA との非特異的結合は観察されなかった。これらの結果から、両プローブはリガンド部アミノ酸に対応する基質特異性を有する A ドメインを高選択的にラベル化可能であることが明らかになった。

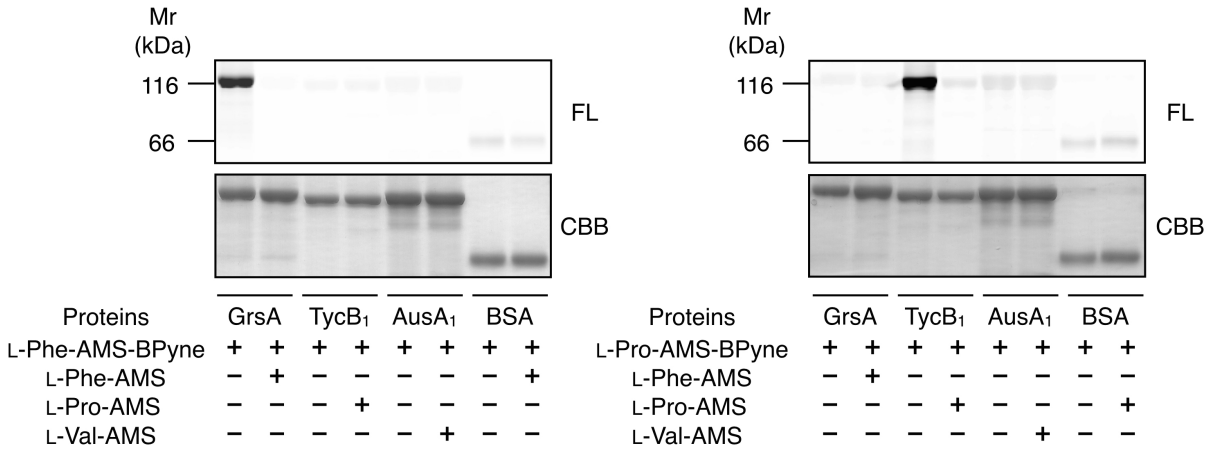


Figure 2-3. Labeling specificity of L-Phe-AMS-BPyne (left) and L-Pro-AMS-BPyne (right).

第 5 節　内在性 NRPS のラベル化･検出及び同定

プローブがリガンド部アミノ酸を置換することで高選択的に対応する A ドメインをラベル化することが判明したので、次に実際のペプチド性天然化合物生産菌プロテオーム中に存在する内在性 NRPS のラベル化を試みた。標的は GrsA（127 kDa）及び GrsB（508 kDa）とし、GS 生産菌（*Aneurinibacillus migulanus*）を GS 産生培地である YPG 培地を用いて培養した後、菌体をリゾチームで溶菌してプロテオームを作製した[30a,b]。

まず、L-Phe-AMS-BPyne を用いて *A. migulanus* ATCC 9999 株プロテオーム中の内在性 GrsA のラベル化を試みた。L-Phe-AMS-BPyne を処理した後、30 分の UV 照射と蛍光タグの付加を行い、SDS-PAGE 後のゲルの蛍光を観察した。その結果、分子量約 120 kDa の位置に組み換え GrsA と同程度の分子量のタンパク質が検出された（**Figure 2-4**）。また、L-Phe-AMS の前処理でこのタンパク質のラベル化が消失した。本プロテオームは内在性 GrsA を含んでいることが明らかになっていることから[23]、L-Phe-AMS-BPyne を用いることで内在性 GrsA をラベル化･検出可能であることがわかった。また、*A. migulanus* ATCC 9999 のプロテオーム中で特異的にラベル化されたバンドは GrsA のみであったことから、本プローブが非常に高い特異性を有していることが明らかになった。

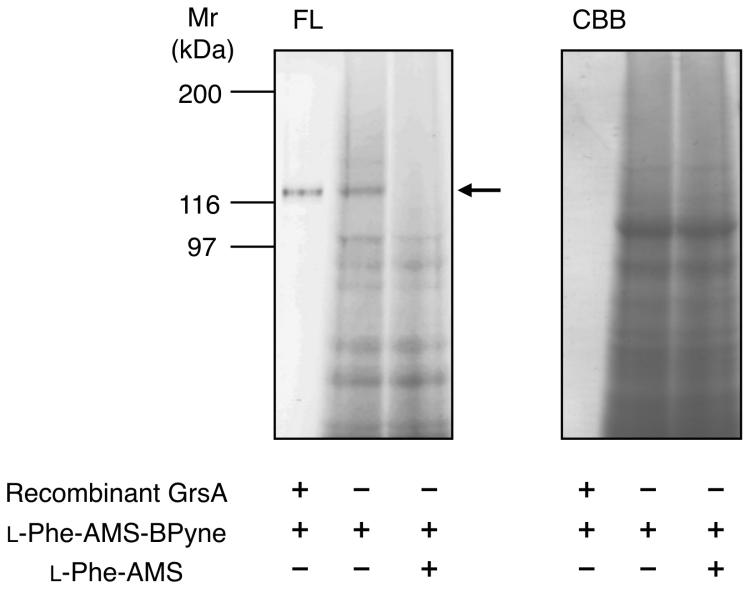


Figure 2-4. Labeling of endogenous GrsA in the *A. migulanus* ATCC 9999 cellular lysate by L-Phe-AMS-BPyne.

次に 4 つの A ドメインを有する内在性 GrsB のラベル化を試みた。内在性 GrsB のラベル化には GS の高生産性株である *A. migulanus* DSM 5759 を用いた[31]。*A. migulanus* ATCC 9999 と同様に YPG 培地で培養後、リゾチームを用いてプロテオームを作製した。まず、L-Pro-AMS-BPyne を用いてラベル化実験を行った。種々条件検討の結果、UV 照射時間を 5 分間にして SDS-PAGE で分離後のゲルの蛍光を観察すると、分子量約 500 kDa の位置にプローブでラベル化され、L-Pro-AMS で消失するバンドが確認された（**Figure 2-5a**）。続いて、L-Orn-AMS-BPyne を用いてラベル化実験を試みたところ、L-Pro-AMS-BPyne と同様に分子量約 500 kDa の位置にラベル化され、L-Orn-AMS の前処理により消失するバンドが観察された（**Figure 2-5b**）。これら 2 つのプローブを用いたラベル化実験から、このタンパク質は L-Pro 及び L-Orn に基質特異性を示す A ドメインを有する GrsB であることが強く示唆された。そこで、DSM 5759 のプロテオームを SDS-PAGE で分離後、銀染色により検出された分子量 500 kDa のバンドを切り出し、トリプシンで消化後に LC-MS/MS 解析を行ったところ、この高分子量タンパク質は GrsB と同定された。これらの結果から、本活性部位指向型プローブは A ドメインを指標に生産菌プロテオーム中から内在性 NRPS をラベル化･検出及び同定可能であることが示された。

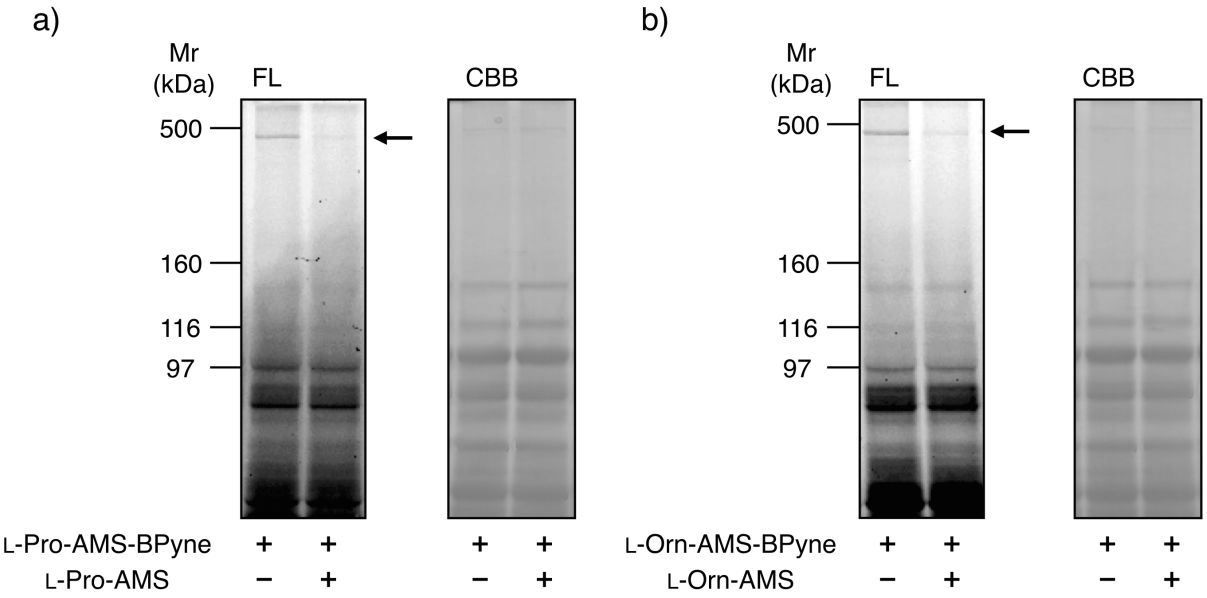


Figure 2-5. Labeling of endogenous GrsB in the *A. migulanus* DSM 5759 cellular lysate by L-Pro-AMS-BPyne and L-Orn-AMS-BPyne.

次に、5 種の阻害剤（L-Phe-AMS, L-Pro-AMS, L-Val-AMS, L-Orn-AMS, L-Leu-AMS）と本プローブを組み合わせて、A ドメインの基質許容性のプロファイリング及び GrsB の L-Pro や L-Orn に基質特異性を有する A ドメインを選択的にラベル化しているか否かを検証することにした。まず、L-Phe-AMS-BPyne と阻害剤を組み合わせて内在性 GrsA の A ドメインの機能的プロファイリングを試みた。*A. migulanus* ATCC 9999 のプロテオームに対して、それぞれの阻害剤（100 μM）と L-Phe-AMS-BPyne（1 μM）を用いてラベル化実験を行ったところ、L-Phe-AMS に加えて L-Leu-AMS の前処理でも L-Phe-AMS-BPyne のラベル化は消失した（**Figure 2-6**）。GrsA は L-Phe を本来の基質として取り込むが、弱いながら L-Leu に対する基質特異性が報告されている [32]。そのため、大過剰の L-Leu-AMS を用いたことでラベル化の消失が観察されたと考えられる。このことから、大過剰の L-Leu-AMS によるラベル化の消失は合理的な結果であり、阻害剤とプローブを組み合わせることで A ドメインの機能を評価可能であることが示唆された。

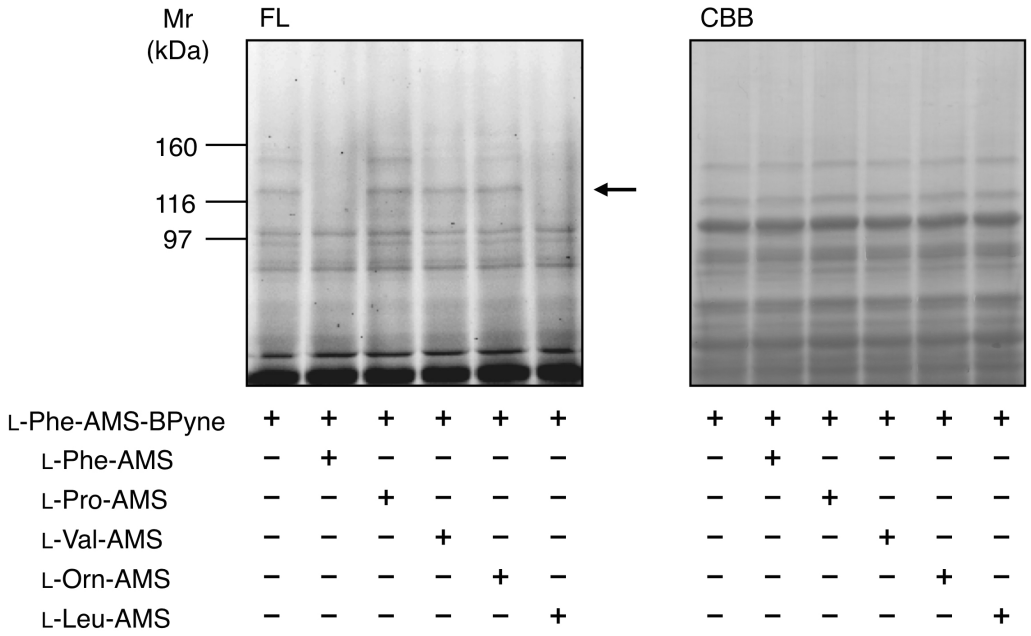


Figure 2-6. Profiling of A-domain function using a combination of L-Phe-AMS-BPyne and five inhibitors.

続いて、プローブが GrsB に存在する 4 つの A ドメインのうち標的である A ドメインをラベル化しているか評価するため、L-Pro-AMS-BPyne 及び L-Orn-AMS-BPyne と 5 種の阻害剤を組み合わせたラベル化実験を行った。その結果、L-Pro-AMS-BPyne による GrsB のラベル化は L-Pro-AMS によってのみ消失し、L-Orn-AMS-BPyne によるラベ

ル化は L-Orn-AMS の前処理によってのみ消失した（**Figure 2-7a**, **b**）。このことから、両プローブは GrsB の 4 つの A ドメインのうち、L-Pro と L-Orn に基質特異性を有する A ドメインとそれぞれ選択的に結合し、GrsB をラベル化していることが明らかになった。

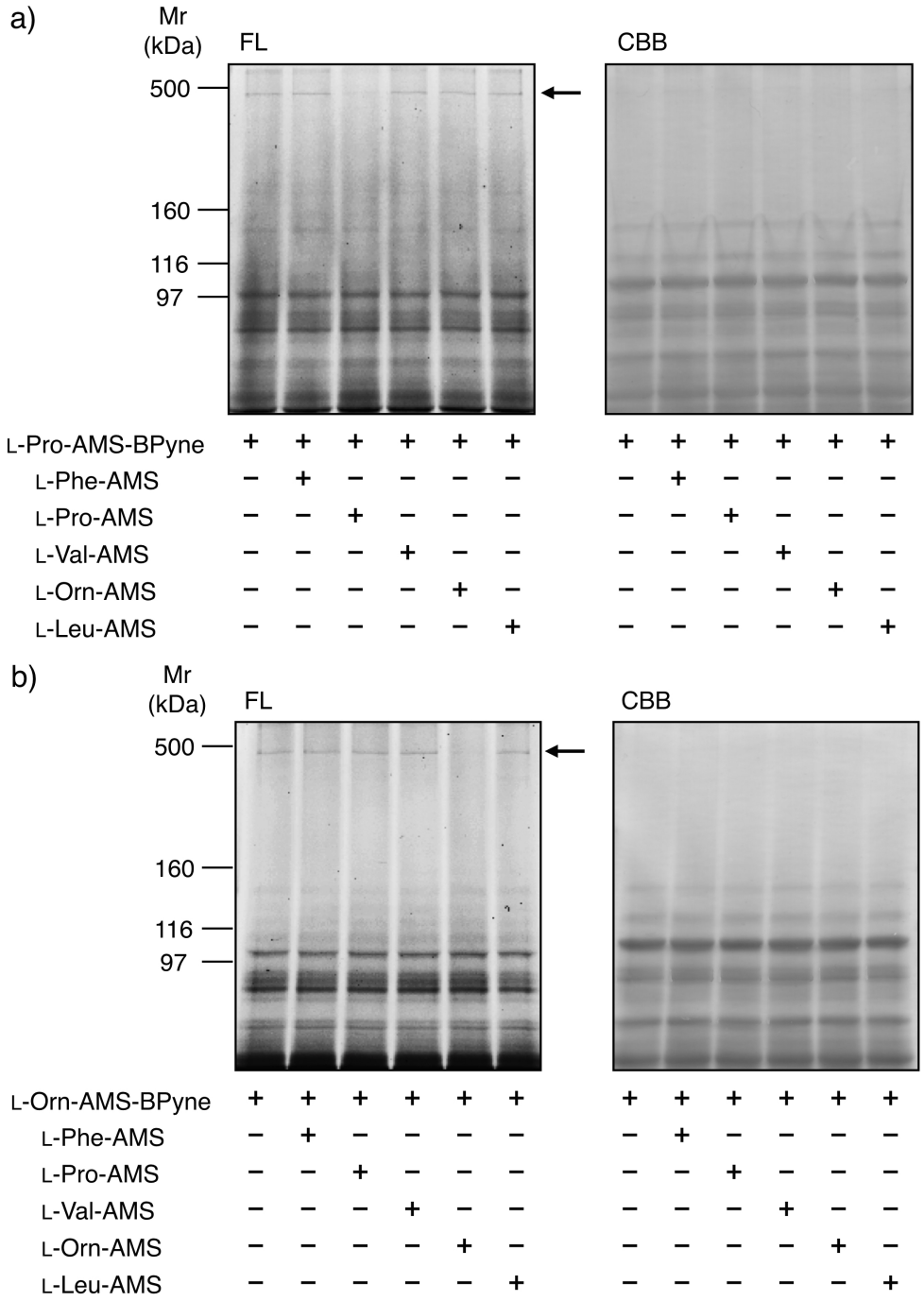


Figure 2-7. a), b) Individual labeling of A-domains and profiling of A-domain functions using a combination of L-Pro-AMS-BPyne, L-Orn-AMS-BPyne, and inhibitors.

第 6 節　小括

本章では、内在性 NRPS のプローブによるラベル化･検出及び同定を目的として、まず大腸菌組み換えタンパク質を用いた A ドメイン選択的ラベル化の評価を行った。リガンド部のアミノ酸が異なる L-Phe-AMS-BPyne（**1**）と L-Pro-AMS-BPyne を用いて、基質特異性の異なる GrsA（A: L-Phe）、$TycB_1$（A: L-Pro）、$AusA_1$（A: L-Val）及び BSA に対するラベル化実験を行い、本活性部位指向型プローブがリガンド部のアミノ酸を置換することで、対応する基質特異性を有する A ドメインを選択的にラベル化可能であることを明らかにした。

次に、微生物プロテオーム中に存在する内在性 NRPS のプローブによるラベル化実験を行った。内在性 GrsA 及び GrsB を標的として、GS 生産菌である *A. migulanus* ATCC 9999 及び DSM 5759 のプロテオームを調製して、L-Phe-AMS-BPyne、L-Pro-AMS-BPyne 及び L-Orn-AMS-BPyne によるラベル化を試みた。その結果、L-Phe-AMS-BPyne は内在性 GrsA を特異的にラベル化し、L-Pro-AMS-BPyne、L-Orn-AMS-BPyne は内在性 GrsB を特異的にラベル化した。プロテオーム中で特異的にラベル化されたタンパク質はこれらの NRPS のみであり、本活性部位指向型プローブが NRPS に高い特異性を有していることが示された。最後に、5 種の阻害剤（L-Phe-AMS, L-Pro-AMS, L-Val-AMS, L-Orn-AMS, L-Leu-AMS）とプローブを組み合わせて、内在性 GrsA の A ドメインの機能プロファイリングができる可能性を示した。また、同様の阻害実験を行い、L-Pro-AMS-BPyne と L-Orn-AMS-BPyne が内在性 GrsB の 4 つの A ドメインのうち、リガンド部アミノ酸に対応する基質特異性を有する A ドメインをそれぞれ選択的にラベル化できることを明らかにした。

第 3 章　合成分子プローブを利用した内在性 NRPS 活性のプロファイリング

第 1 節　はじめに

これまで微生物プロテオーム中の内在性 NRPS 活性の評価は標的 NRPS の精製条件を確立し、精製後の内在性 NRPS に対して放射性同位体を利用したアッセイによって行われてきた [30a,b]。このような煩雑な工程のため、内在性 NRPS 活性の直接的な評価はほとんど報告例がない。しかしながら、天然化合物生産菌の内在性 NRPS 活性の評価は、有用天然化合物生産量の増大や微生物による NRPS の転写調節･活性制御機構の理解に繋がる。

Activity-based protein profiling（ABPP）はプロテオーム中の標的タンパク質群活性を直接検出する方法であり [33a,b]、これまでに各種プロテアーゼ [34a-c] やキナーゼ [35]、ヒストンデアセチラーゼ [11,36]、ヒストンアセチルトランスフェラーゼ [37] など数多くのタンパク質群の活性プロファイルが報告されている。プロテオーム中の標的タンパク質群活性をプロファイリングすることで、病態モデルなどの環境変化における一部酵素の異常活性などが明らかにされてきた。遺伝学的に mRNA の量を測定することもあるが、プロテオーム中での酵素活性の強弱と mRNA 量は相関関係にないことから、既知タンパク質に対する ABPP は遺伝子レベルでは理解できないプロテオームレベルでのタンパク質の状態や変化を知る強力な手段となる。そこで、本研究で開発した A ドメインに対する活性部位指向型プローブを利用すれば、生産菌プロテオーム中の内在性 NRPS 活性を迅速かつ簡便に評価可能になると考えた。本章では、ABPP に基づいた内在性 NRPS 活性のプロファイリングを用いて、グラミシジン S 生産菌と非生産菌の識別、及びグラミシジン S 合成酵素活性の動態の追跡を行った（**Figure 3-1**）。

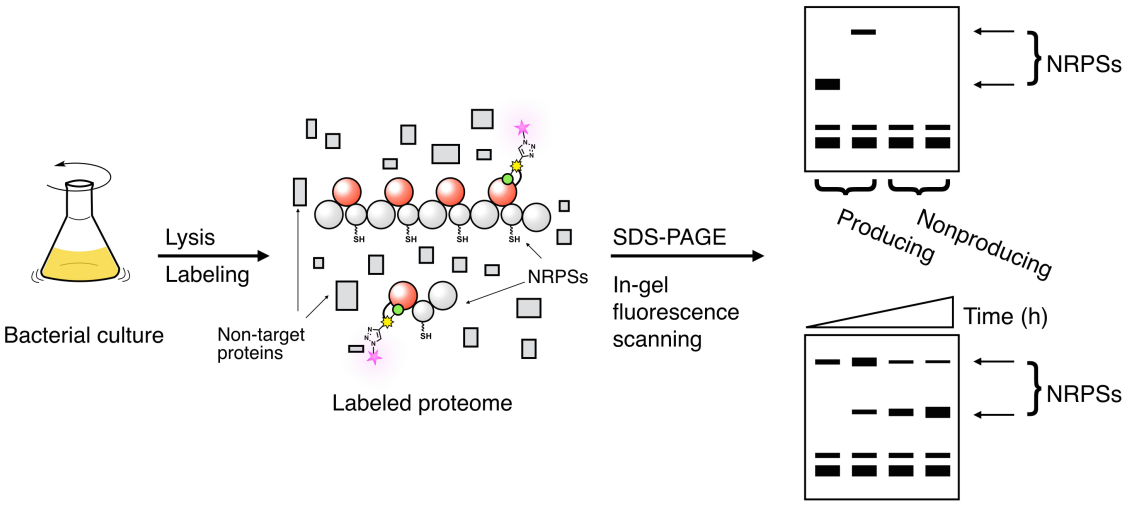


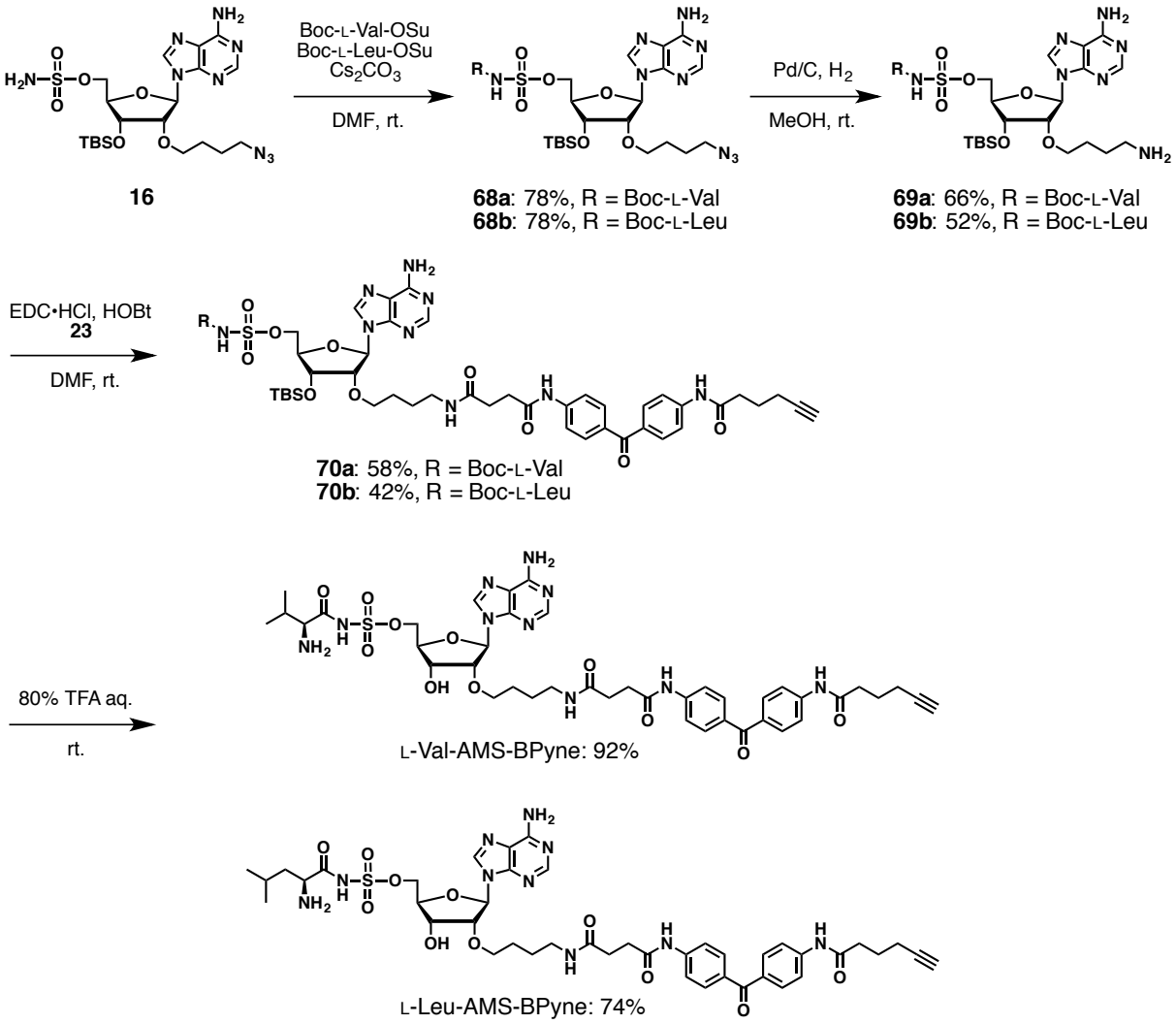
Figure 3-1. Functional proteomic analysis of NRPS activities using active site-directed proteomic probes for A-domains.

第 2 節　活性部位指向型プローブの合成

GrsB の 4 つの A ドメインのうち、第 2 章では L-Pro と L-Orn の A ドメインに対するプローブを合成し、それぞれ特異的にラベル化可能であることを示した。そこで、本プローブ群の更なる汎用性を示すことを目的に、残りの 2 つの A ドメインである L-Val と L-Leu の A ドメインに対するプローブを合成した。

【L-Val-AMS-BPyne、L-Leu-AMS-BPyne の合成】

他のプローブと同様に、化合物 **16** に対して Boc-L-Val-OSu もしくは Boc-L-Leu-OSu を反応させた後、接触還元によりリガンド部 **69a**, **b** を得た。続いて、リガンド部 **69a**, **b** とスペーサー部 **23** を縮合した後、脱保護することにより L-Val-AMS-BPyne、L-Leu-AMS-BPyne をそれぞれ合成した（**Figure 12**）。

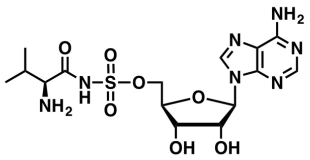
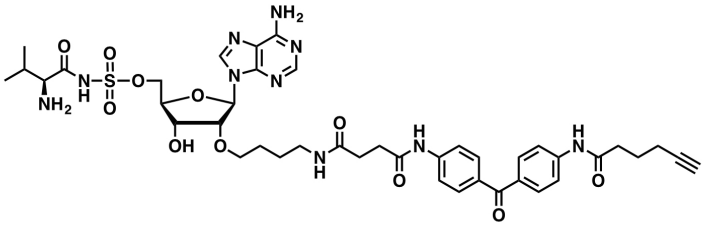


Scheme 12. Synthetic route to L-Val- and L-Leu-AMS-BPyne.

第 3 節　$AusA_1$ に対する阻害活性の評価及びラベル化実験

まず、大腸菌組み換え $AusA_1$ を用いて合成した L-Val-AMS-BPyne の阻害活性をリガンドにあたる L-Val-AMS と比較した。Hydroxamate-MesG アッセイにより阻害定数を求めたところ、L-Val-AMS の阻害活性は K_i^{app} = 212 ± 15 nM であり、L-Val-AMS-BPyne の阻害活性は K_i^{app} = 295 ± 16 nM であった（**Table 3**）。このことから、L-Val-AMS-BPyne の 2'位修飾もこれまでのプローブと同様にリガンドと A ドメインの結合にほとんど影響を与えないことが明らかになった。

Table 3. Inhibitory activities of L-Val-AMS and L-Val-AMS-BPyne toward recombinant $AusA_1$.[a]

Compounds	Structures	K_i^{app} (nM)
L-Val-AMS		212 ± 15
L-Val-AMS-BPyne		295 ± 16

[a]Kinetic parameters were determined by a coupled hydroxamate-MesG continuous spectrophotometric assay.[25] Errors were given as the standard error of duplicate independent measurements.

次に、$AusA_1$ を用いて L-Val-AMS-BPyne のラベル化能を詳細に調べた。まず、競合的ラベル化実験により、ラベル化が特異的に進行するか否かを評価したところ、プローブによる $AusA_1$ のラベル化は L-Val-AMS によって阻害された（**Figure 3-2a**）。このことから、L-Val-AMS-BPyne は $AusA_1$ の A ドメインに特異的に結合してラベル化することがわかった。次に、UV 照射時間によるラベル化量の変化を調べたところ、60 分の照射で最大に達した（**Figure 3-2b**）。また、60 分の時の $AusA_1$（1 μM）に対する L-Val-AMS-BPyne（1 μM）のラベル化効率を算出した結果、23.5 ± 1.4%であった。さらに、L-Val-AMS-BPyne（1 μM）での $AusA_1$ の検出限界は 25 fmol と見出された（**Figure**

3-2c)。最後に、A ドメイン選択性の評価を行った結果、L-Val-AMS-BPyne（1 μM）はこれまでのプローブと同様にリガンド依存的に対応する A ドメインを選択的にラベル化することが明らかになった（**Figure 3-2d**）。

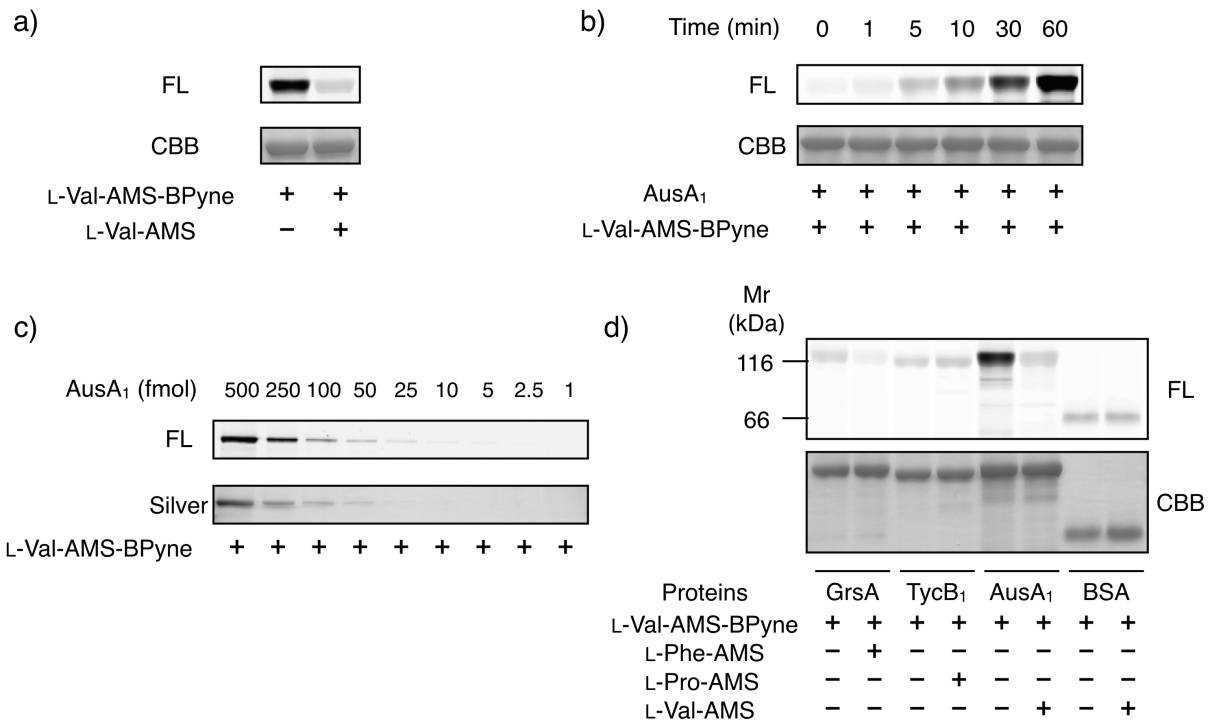


Figure 3-2. Labeling of recombinant $AusA_1$ with L-Val-AMS-BPyne. a) Labeling of $AusA_1$ and competitive inhibition study with excess L-Val-AMS. b) UV photolysis time course studies of the labeling of $AusA_1$ with L-Val-AMS-BPyne. c) Limit of detection of $AusA_1$. d) Labeling specificity of L-Val-AMS-BPyne.

第 4 節　グラミシジン S 生産菌を用いたラベル化実験

次に、L-Val-AMS-BPyne と L-Leu-AMS-BPyne を用いて、内在性 GrsB のラベル化を試みた。まず、*A. migulanus* DSM 5759 のプロテオーム中に L-Val-AMS-BPyne を処理したところ、選択的な GrsB のラベル化が確認された（**Figure 3-3a**）。また、L-Leu-AMS-BPyne を用いても同様に GrsB がラベル化されることがわかった（**Figure 3-3b**）。一方で、これら 2 つのプローブを用いた SDS-PAGE の結果では、GrsB の他に分子量約 100 kDa の位置に特異的にラベル化されるバンドが存在することがわかった。

そこで、TAMRA とビオチン双方を有するタグ分子（TAMRA-ビオチン-アジド）を用いて、これらバンドの精製を試みた。アビジンビーズを用いたプルダウンの後、SDS-PAGE でタンパク質を分離後、銀染色によりタンパク質の染色を行った。その結果、両プローブで約 100 kDa のタンパク質の取得に成功した（**Figure 3-3c**, **d**）。これらのバンドを切り出し、トリプシン消化後のペプチド断片を LC-MS/MS 解析した結果、これらのタンパク質はそれぞれバリン tRNA 合成酵素及びロイシン tRNA 合成酵素であることが明らかになった。第 1 章第 2 節で述べた通り、A ドメインと aaRS は同様の反応を触媒する。また、両プローブのリガンド部は aaRS の阻害剤として用いられてきたことから、GrsB に加えて aaRS の特異的ラベル化は合理的な結果と考えられる。

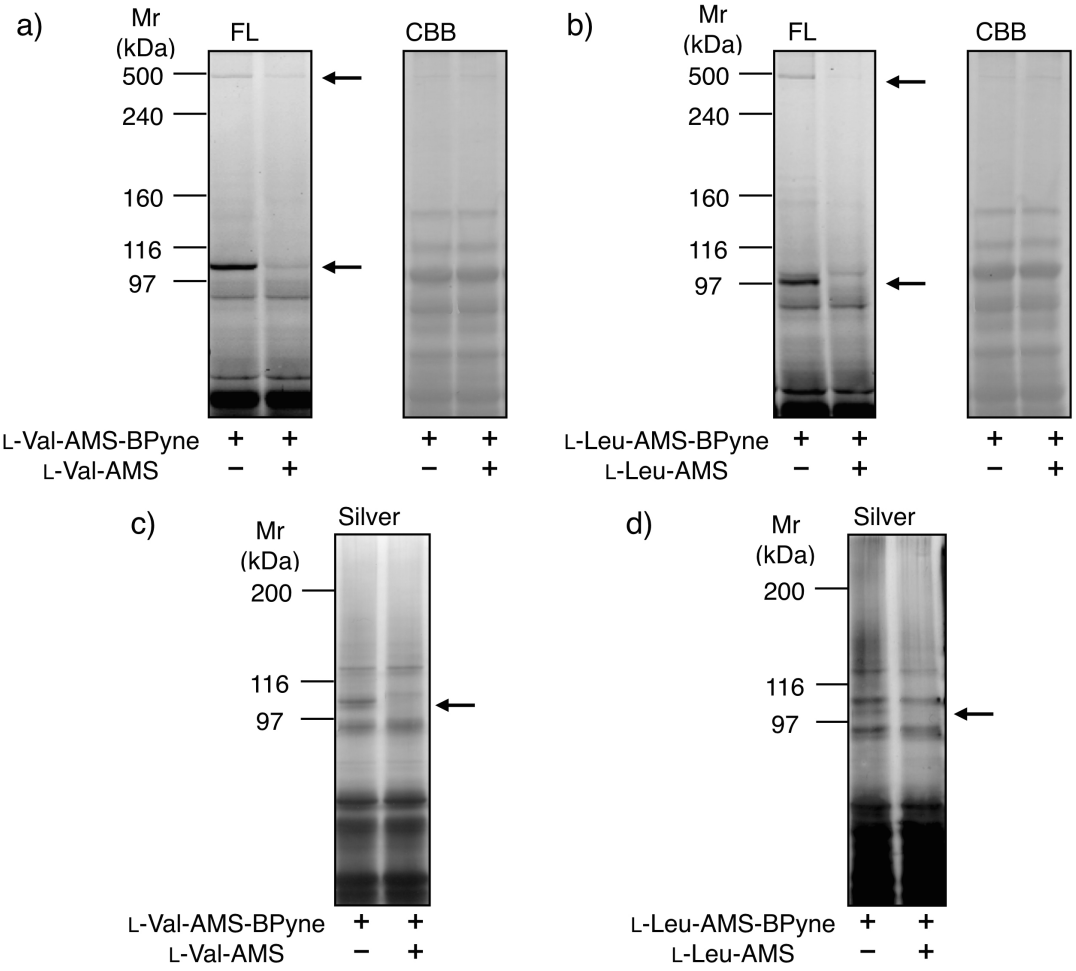


Figure 3-3. a), b) Labeling of endogenous GrsB in the *A. migulanus* DSM 5759 cellular lysate by L-Val-AMS-BPyne and L-Leu-AMS-BPyne. c) Pull-down assay of probe-binding protein using L-Val-AMS-BPyne and TAMRA-biotin-azide in the bacterial proteome. d) Pull-down assay of probe-binding protein using L-Leu-AMS-BPyne and TAMRA-biotin-azide in the bacterial proteome.

最後に、L-Val-AMS-BPyne と L-Leu-AMS-BPyne が GrsB の標的である A ドメインを認識し、ラベル化しているか否か阻害剤を用いて評価した。まず、5 種の阻害剤（L-Phe-AMS, L-Pro-AMS, L-Val-AMS, L-Orn-AMS, L-Leu-AMS）を前処理した後、L-Val-AMS-BPyne を用いて GrsB のラベル化を行うと、L-Val-AMS に加えて L-Leu-AMS でも阻害されることが判明した（**Figure 3-4**）。A ドメインの基質特異性は活性中心に位置する 10 個のアミノ酸残基によって決定され、これらのアミノ酸残基は「NRPS コード」として知られている[32]。そのため、現在では NRPS コードの相同性から A ドメインの基質特異性をある程度予想することが可能である。GrsB の L-Val に基質特異性を有する A ドメインは $AusA_1$ の A ドメインと非常に近い NRPS コードを有している。唯一の違いは 299 番目のアミノ酸が GrsB では L-Leu であるが $AusA_1$ では L-Ile である点である（**Table 4**）。$AusA_1$ については大腸菌組み換えタンパク質を用いた基質特異性の評価が行われており、L-Leu を含むいくつかのアミノ酸を基質として取り込むことが知られている[28]。このことから、GrsB の L-Val に基質特異性を有する A ドメインも同様の傾向を示すことが予想され、過剰量（100 eq.）の阻害剤を処理した結果、L-Leu-AMS でも L-Val-AMS-BPyne によるラベル化が阻害されたと考えられる。

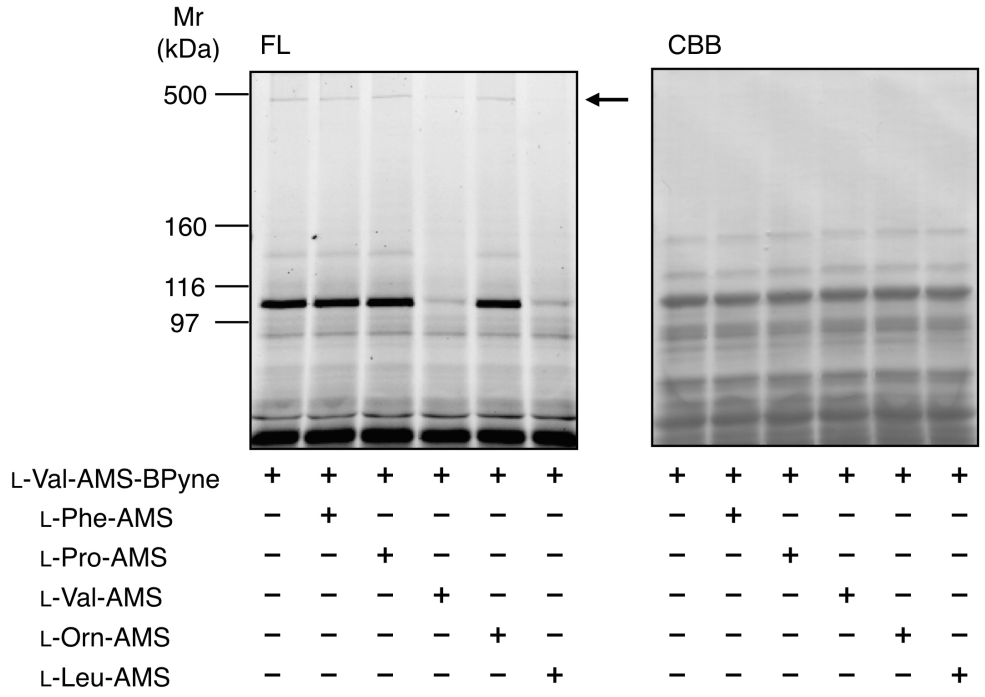


Figure 3-4. Individual labeling of A-domains and profiling of A-domain functions using a combination of L-Val-AMS-BPyne and inhibitors.

Table 4. Alignment of the module 3 A-domain of GrsB with the A domain of $AusA_1$.

Pos 1	Pos 2	Pos 3	Pos 4	Pos 5	Pos 6	Pos 7	Pos 8	Pos 9	Pos 10	
235	236	239	278	299	301	322	330	331	517	Phe A (GrsA) numbering
D	A	F	W	L	G	G	T	F	K	GrsB (Val)
D	A	F	W	I	G	G	T	F	K	$AusA_1$ (Val)

一方、L-Leu-AMS-BPyne と 5 種の阻害剤を用いた実験では、L-Leu-AMS を前処理した時のみラベル化が消失した（**Figure 3-5**）。このことから、L-Leu-AMS-BPyne は GrsB の L-Leu に基質特異性を有する A ドメインを選択的に認識し、ラベル化していることが明らかになった。これらの結果から、L-Val-AMS-BPyne と L-Leu-AMS-BPyne による内在性 GrsB のラベル化は、リガンド部アミノ酸に対応する基質特異性を有する A ドメインを選択的に認識してラベル化していることが明らかになった。

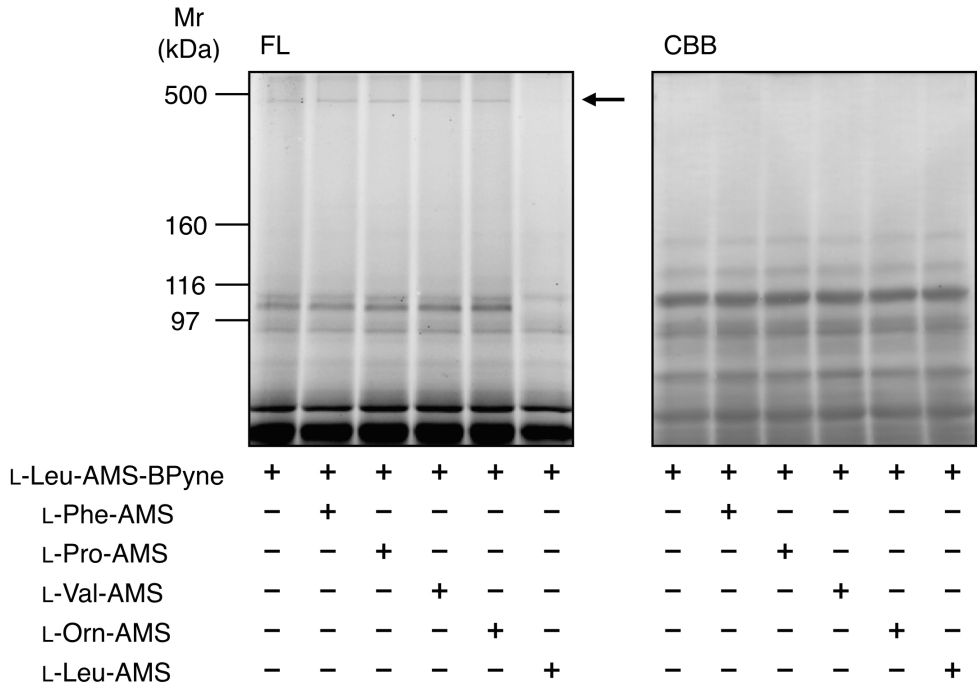


Figure 3-5. Individual labeling of A-domains and profiling of A-domain functions using a combination of L-Leu-AMS-BPyne and inhibitors.

第 5 節　NRPS 活性を指標としたグラミシジン S 生産菌及び生産能の識別

微生物による天然化合物の生産は、培地、温度、菌体数や培養時間など様々な環境変化の影響を受けてその生産量が大きく変化する。複雑な構造の有用天然化合物については化学合成による供給が困難なため、大量生産菌の発見や生産菌を用いた大量生産培養条件の確立は重要な課題である。しかし、天然化合物の粗精製は多くの場合煩雑であるため、その培養条件確立は容易ではない。このような背景のもと、本プローブを用いてプロテオーム中の NRPS 活性を検出し、それが生産菌の天然化合物生産能と相関すれば、簡便に生産条件の最適化を行うことが可能になると考えた。そこで、NRPS 活性を指標に生産菌や天然化合物生産能を識別可能か否か検証することにした。

グラミシジン S（GS）の生産菌として *A. migulanus* ATCC 9999 の他に *A. migulanus* DSM 2895、5668、5759 が知られている。しかし 2007 年、Berditsch らは GS 産生培地として知られている NBYS 培地や YP 培地において、これらの菌のうち ATCC 9999 と DSM 5759 は GS を産生するが、DSM 2895 と DSM 5668 は GS を産生しないことを報告している [31]。そこで、これら 4 種の菌のプロテオームを調製して、GrsA 及び GrsB 活性と GS 生産能の比較を行うことにした。本研究では GS 産生培地として用いられている酵母エキスとペプトンを主成分とする YPG 培地を用いて培養を行った [30a,b]。

それぞれの菌を 24 時間培養後、まず L-Phe-AMS-BPyne を用いて内在性 GrsA 活性を調べた。その結果、ATCC 9999 と DSM 5759 では GrsA 活性が検出された（**Figure 3-6a**）。一方で、DSM 2895 では明らかに GrsA 活性はなかったが、DSM 5668 のプロテオーム中からわずかに GrsA 活性が検出された。次に、L-Leu-AMS-BPyne を用いてそれぞれの菌の内在性 GrsB 活性の検出を試みた。その結果、ATCC 9999 と DSM 5759 に加えて、DSM 5668 でも GrsB 活性が存在することが明らかになった（**Figure 3-6b**）。そこで、YPG 培地で培養した DSM 5668 が実際に GS を産生しているか確認するため、RP-HPLC を用いて GS の定量を行った。その結果、ATCC 9999 と DSM 5759 に加えて DSM 5668 でも GS を産生していることが明らかになった（**Figure 3-6c**）。一方、DSM 2895 による GS の産生は、RP-HPLC において検出されなかった。これらの結果から、本プローブで検出した NRPS 活性と GS 生産能には相関がみられ、NRPS 活性を指標に生産菌の識別が可能なことが明らかになった。また、文献で報告されていた NBYS 培地や YP 培地ではグラミシジン S を産生しない DSM 5668 が YPG 培地では産生することを見出した。このことから、本プローブを用いて NRPS 活性を評価することで、培地成分の違いによる産生能の変化を検出することにも成功した。

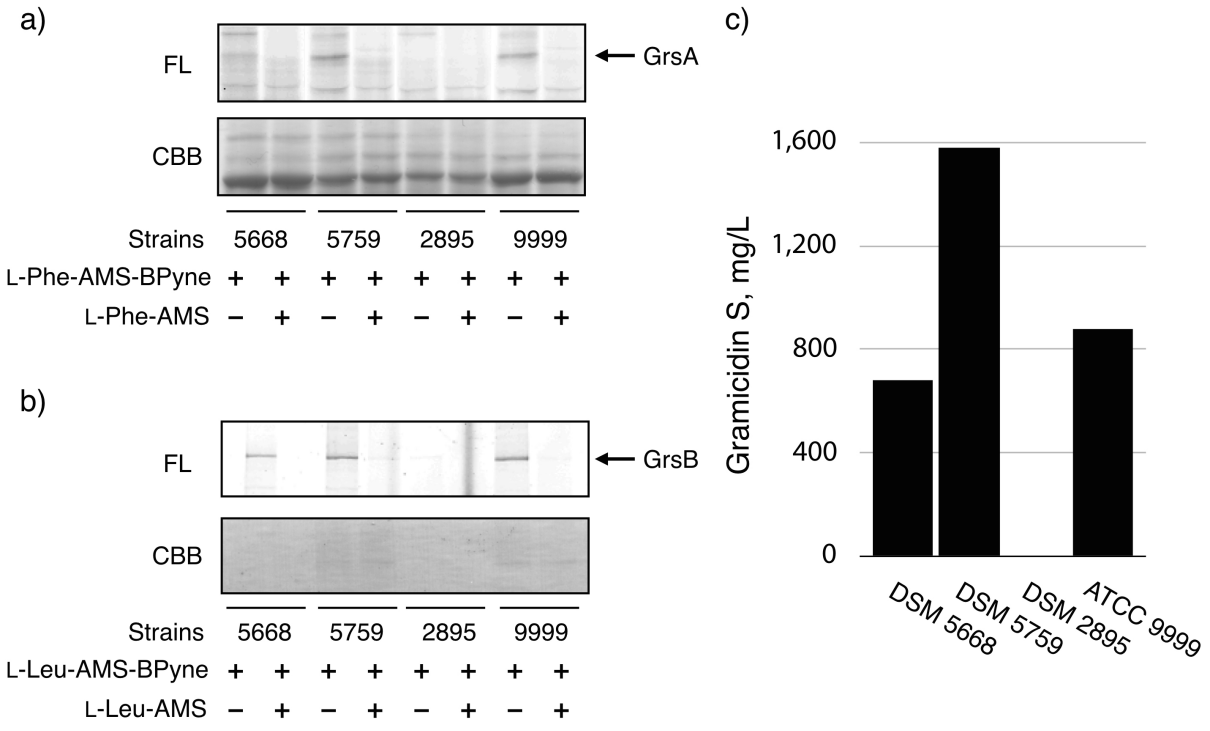


Figure 3-6. a) In-gel fluorescence analysis of the GrsA activity profiles obtained from reactions between the proteomes of *A. migulanus* strains and L-Phe-AMS-BPyne. b) In-gel fluorescence analysis of the GrsB activity profiles of the *A. migulanus* proteomes labeled by L-Leu-AMS-BPyne. c) Corresponding absolute (mg/L) production of gramicidin S. The cultures were cultivated in YPG medium. Gramicidin S yields were determined at 24 h after inoculation.

第 6 節　NRPS 活性の経時変化のモニタリング

次に、本プローブを用いることで、生産菌の培養期間における内在性 NRPS 活性の経時変化を追跡可能か否か検証した。*A. migulanus* DSM 5759 株を用いて培養を行い、12 時間から 24 時間まで 4 時間ごとに菌体を回収してプロテオームを調製し、内在性 GrsA 及び GrsB 活性がどのような挙動を示すか調べた。

まず、L-Phe-AMS-BPyne を用いて内在性 GrsA 活性の追跡を試みた。その結果、GrsA 活性は 16 時間の時から検出可能になり、その後、時間依存的に増大していくことが明らかになった（**Figure 3-7a**）。次に L-Leu-AMS-BPyne を用いて内在性 GrsB 活性の追跡を行ったところ、GrsB 活性は 12 時間の時点から検出された（**Figure 3-7b**）。その後 GrsB 活性は 16 時間で最大となり、20、24 時間では減少したことから GrsA 活性とは異なる活性プロファイルを示すことがわかった。

続いて、RP-HPLC を用いて GS 産生量の定量を行い、NRPS 活性との比較を行った。GS の産生は 12 時間の時点から検出され、16 時間から 20 時間の間で最も産生量が増大していることが明らかになった（**Figure 3-7c**）。このことから、内在性 GrsA 活性が増大し、GrsB 活性が最大となる 16 時間付近で生産能が最も高くなることが示唆された。一方で、*grsA* 及び *grsB* 遺伝子はポリシストロニックに遺伝子発現が制御されているにもかかわらず [3a]、プロテオームレベルでは異なる活性プロファイルを示した。これは、GrsB が分解もしくは不活性化されていることを示唆しているが、発現された NRPS の活性制御機構について現時点では明らかになっていない。GS は抗生物質として扱われるが、実際の生産菌は GS を菌体内に貯め込んでいるため [30a,b]、GS を産生する本来の目的は外敵の駆逐ではないと考えられる。また、GS に限らず、本来の役割が不明な NRP は数多く存在する。そのため、NRPS 活性が菌数や活動期、NRP の蓄積量などの因子によって制御･不活性化されているとすれば、内在性 NRPS 活性のプロファイリングは NRP の本来の役割を理解するための重要な指標となり得る。このように本プローブ群は、遺伝子レベルでは理解できない NRPS のプロテオーム中での挙動や制御プロセスを解析するための有用なツールとなることが期待できる。

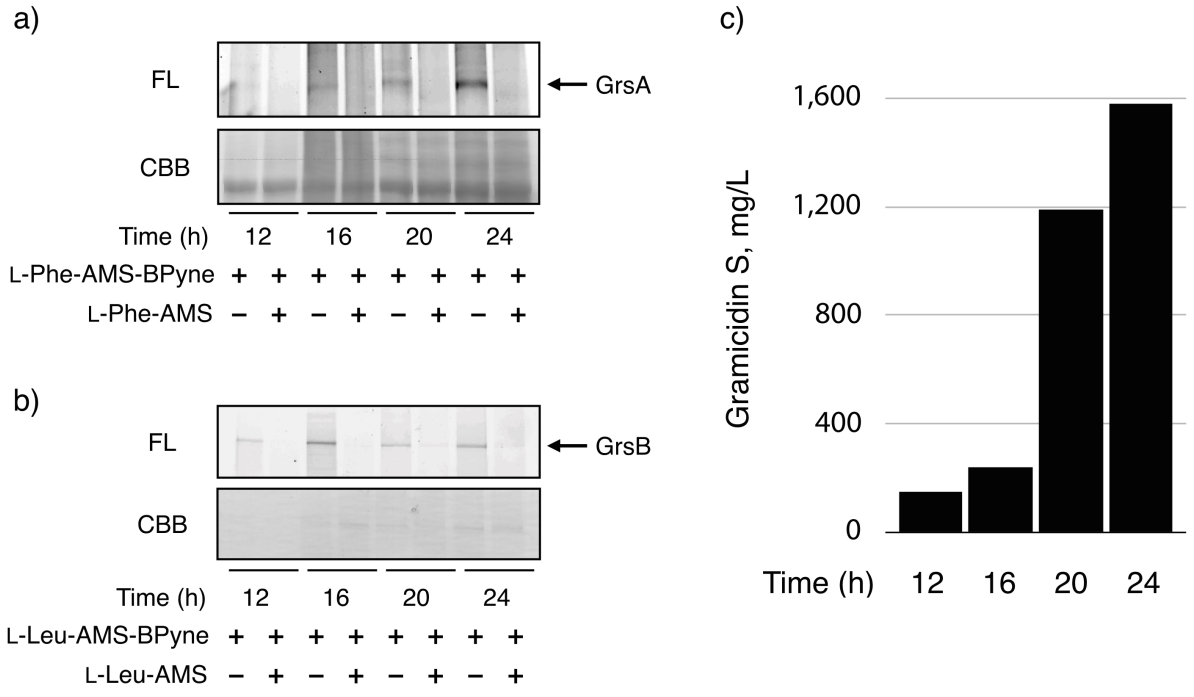


Figure 3-7. a) Visualizing the expression of the active GrsA in DSM 5759 proteomes by L-Phe-AMS-BPyne at the times indicated. b) Monitoring the GrsB activity in DSM 5759 proteomes by L-Leu-AMS-BPyne at the times indicated. c) Absolute yield (mg/L) of gramicidin S, monitored as a function of time for *A. migulanus* DSM 5759 in YPG medium.

第 7 節　小括

本章では、内在性 NRPS をラベル化･検出可能な A ドメインに対する活性部位指向型プローブを利用して内在性 NRPS 活性のプロファイリングを行い、グラミシジン S 生産菌の識別と NRPS 活性のモニタリングに応用した。

まず、グラミシジン S 生産菌の識別では、*A. migulanus* DSM 2895、5668、5759、及び ATCC 9999 の 4 種のグラミシジン S 生産菌を用いて、グラミシジン S 生産能とプロテオームレベルでの NRPS 活性に相関があるか否かを検証した。その結果、プローブで検出された内在性 NRPS 活性とグラミシジン S 産生能には相関がみられた。また、文献ではグラミシジン S を産生しないと報告されていた DSM 5668 が、YPG 培地ではグラミシジン S を産生することをプローブによる NRPS 活性の評価で明らかにした。このことから、本プローブ群を用いることで NRPS 活性を指標にペプチド性天然化合物生産能を簡便に評価することが可能になり、培養条件のスクリーニングなどへの応用が期待できる。

次に、*A. migulanus* DSM 5759 株の内在性 NRPS 活性の挙動について、プローブを用いてその動態のモニタリングを行った。その結果、内在性 GrsA 活性は 16 時間から時間依存的に増大する一方で、GrsB 活性は 16 時間を最大として減少していくことが明らかになった。これは、GrsB がプロテオーム中で分解や不活性化といった何らかの制御を受けていることを示唆しており、本プローブがプロテオームレベルの内在性 NRPS 活性の変化を解析するための有用なツールとなり得ることを見出した。

結語

本研究で著者は、プロテオームレベルで NRPS を検出･解析するための新たなツールとして、プロテオーム中の内在性 NRPS を迅速、簡便、高感度に検出可能な A ドメインを標的とした活性部位指向型プローブを開発した。本プローブはリガンド部のアミノ酸を置換することで対応する A ドメインをリガンド依存的にラベル化可能である。また、一つのタンパク質上に複数の A ドメインが存在するマルチモジュール型 NRPS においても、個々の A ドメインを選択的にラベル化可能であることを明らかにした。さらに、これらのプローブを用いて NRPS 活性のプロファイリングをすることで、ペプチド性天然化合物生産菌の識別や NRPS 活性のモニタリングに応用可能であることを見出した。

本プローブはリガンド部アミノ酸を置換することで、微生物が発現している様々な NRPS に対応可能である。そのため、リガンド部アミノ酸の異なるプローブライブラリーを構築することにより、微生物が発現している NRPS を網羅的に解析することが可能となる。多くの微生物は複数の生合成酵素遺伝子を有しており、未同定の NRP やその NRPS は数多く存在する [38]。プローブライブラリーを用いて、プロテオーム中の NRPS を網羅的に解析することで、微生物が発現している新規 NRPS の発見が期待できる。また、この網羅的探索技術を既存の遺伝子工学と組み合わせることにより、新規 NRPS の発見を起点に NRPS 遺伝子クラスターの同定、解析及び新規 NRP の発見に繋がる [39]。

また、臨床において薬剤耐性菌が問題視される結核菌や緑膿菌は、NRPS 産物を病原因子とするため、 NRPS やその関連タンパク質は創薬標的として注目されている [40a-d]。そのため、微生物の天然化合物産生過程をプロテオームレベルで詳細に理解することは極めて重要な課題である。本プローブは高い特異性を有するため、NRPS 間の相互作用や相互作用する他のタンパク質の同定、及び局在の観察などプロテオームレベルでの NRPS に関する様々な現象の解明が期待できる。これにより、病原細菌の二次代謝制御へ向けた創薬基盤が構築可能となる。

さらに、本プローブと A ドメインの競合的阻害剤を組み合わせた競合的 ABPP により、内在性 NRPS を用いた A ドメインの直接的機能評価及び基質候補物質の探索が可能になる [41]。これにより、遺伝子工学と組み合わせた方法 [42a,b] だけでなく、生産菌と内在性 NRPS をそのまま利用した新規 NRP 類縁体の創出が期待できる [43a,b]。

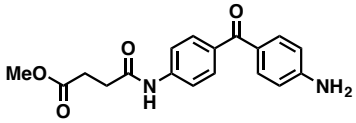
このように、本研究で開発した A ドメインに対する活性部位指向型プローブは、その高い特異性及び選択性から様々な研究への発展･応用が可能であり、その波及効果に期待する。

実験項

General Synthetic Methods: All commercial reagents were used as provided unless otherwise indicated. L-Phe-AMS,[23] compounds **11**,[23] **37a**,[44] **37b**,[45] **66**,[27] and L-Val-AMS[28] 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.[46] Multiplicities are given as s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, ddd = doublet of triplets, br = broad signal, m = multiplet using integration and coupling constant in Hertz. TLC analysis was performed using Silica Gel 60 F254 plates (Merck) and visualization was accomplished with ultraviolet light (λ = 254 nm) and/or the appropriate stain [phosphomolybdic acid, iodine, ninhydrin, and potassium permanganate]. Silica gel chromatography was carried out with SiliaFlash F60 230-400 mesh (Silicycle), according to the method of Still.[47] Mass spectral data were obtained using a LCMS-IT-TOF mass spectrometer (Shimadzu).

Synthesis of L-Phe-AMS-BPyne 1

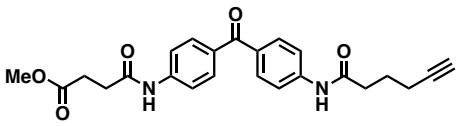
Methyl 4-((4-(4-aminobenzoyl)phenyl)amino)-4-oxobutanoate (20)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (905 mg, 4.72 mmol) and 1-hydroxybenzotriazole (723 mg, 4.72 mmol) were added to a solution of compound **19** (623 mg, 4.72 mmol) in DMF (20 mL). The solution was stirred at room temperature for 5 min and 4, 4′-diaminobenzophenone **18** (500 mg, 2.36 mmol) was added. After 12 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (2:1 EtOAc/hexane) to afford compound **20** as a white

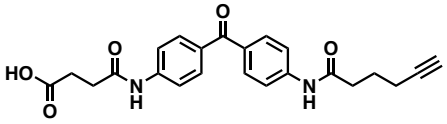
solid (410 mg, 53%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.26 (br, 1H), 7.69 (d, J = 8.6 Hz, 2H), 7.58 (d, J = 8.0 Hz, 2H), 7.49 (d, J = 8.6 Hz, 2H), 6.59 (d, J = 8.6 Hz, 2H), 6.06 (s, 2H), 3.59 (s, 3H), 2.68–2.59 (m, 4H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 192.4, 172.8, 170.3, 153.4, 141.9, 133.2, 132.4, 133.2, 132.4, 130.2, 124.2, 118.0, 112.5, 51.4, 31.0, 28.4. HRMS (ESI+): $[M+H]^+$ calcd for $C_{18}H_{19}N_2O_4$, 327.1345; found, 327.1326.

Methyl 4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanoate (22)



Oxalyl chloride (154 μL, 1.8 mmol) and DMF (20 μL) were added to a solution of 5-hexynoic acid (129 μL, 1.2 mmol) in benzene (10 mL). After 2 h, the flask was placed on the rotary evaporator and the DMF and benzene were removed at reduced pressure to afford 5-hexynoic chloride as a red oil. A solution of 5-hexynoyl chloride **21**, compound **20** (185 mg, 0.57 mmol), and DIEA (200 μL, 1.14 mmol) in THF (5 mL) was stirred at room temperature for 9 h. The reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (1:1 EtOAc/hexane) to afford compound **22** as a white solid (122 mg, 51%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.35 (br, 1H), 10.28 (br, 1H), 7.66–7.78 (m, 8H), 3.59 (s, 3H), 2.81 (t, J = 2.9 Hz, 1H), 2.69–2.59 (m, 4H), 2.45–2.48 (m, 2H), 2.23 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 1.81–1.73 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 193.3, 172.8, 171.2, 170.5, 143.0, 142.9, 131.7, 130.9, 130.8, 118.2, 118.1, 84.0, 71.7, 51.4, 35.2, 31.0, 28.4, 23.8, 17.3. HRMS (ESI+): $[M+H]^+$ calcd for $C_{24}H_{25}N_2O_5$, 421.1763; found, 421.1761.

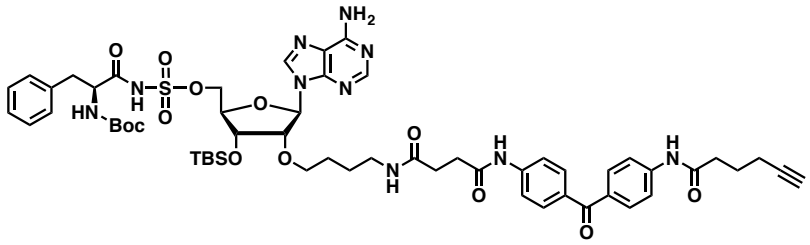
4-((4-(4-(Hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanoic acid (23)



To a solution of **22** (122 mg, 0.29 mmol) in a 3:1 (v/v) mixture of MeOH and THF (4 mL) was added 145 μL of a 4 M aqueous NaOH solution at room temperature. Stirring was

continued at room temperature for 12 h. The flask was then placed on a rotary evaporator and the MeOH and THF were removed at reduced pressure. The residue was diluted with H_2O and washed with EtOAc. The aqueous layer was acidified with citric acid monohydrate and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **23** as a pale yellow solid (114 mg, 97%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 10.33 (br, 1H), 10.29 (br, 1H), 7.75 (dd, J = 8.6, 3.4 Hz, 4H), 7.69 (d, J = 8.6 Hz, 4H), 2.80 (t, J = 2.9 Hz, 1H), 2.64−2.58 (m, 2H), 2.57−2.51 (m, 2H), 2.44−2.47 (m, 2H), 2.22 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 1.81−1.73 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6): δ 193.4, 173.8, 171.3, 170.8, 143.09, 143.06, 131.8, 131.7, 131.0, 130.9, 118.3, 118.2, 84.0, 71.7, 35.3, 31.3, 28.7, 23.8, 17.4. HRMS (ESI+): $[M+H]^+$ calcd for $C_{23}H_{23}N_2O_5$, 407.1607; found, 407.1604.

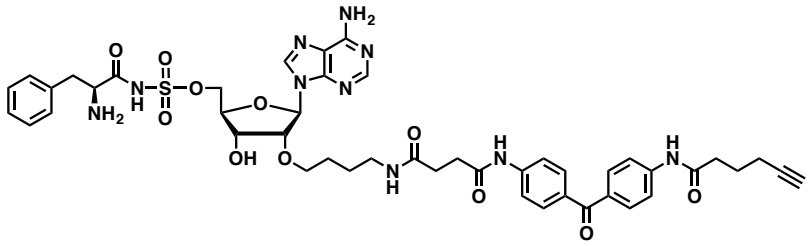
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (24)



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (3.1 mg, 0.016 mmol) and 1-hydroxybenzotriazole (2.5 mg, 0.016 mmol) were added to a solution of compound **23** (6.4 mg, 0.016 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **11** (10 mg, 0.013 mmol) was then added. After 12 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (6:1 $CHCl_3$/MeOH) to afford compound **24** as a white solid (13 mg, 86%).^{1}H NMR (500 MHz, CD_3OD): δ 8.48 (s, 1H), 8.19 (s, 1H), 7.75–7.70 (m, 8H), 7.23–7.17 (m, 4H), 7.15–7.09 (m, 1H), 6.14 (d, J = 6.3 Hz, 1H), 4.62–4.58 (m, 1H), 4.55–4.51 (m, 1H), 4.38–4.31 (m, 1H), 4.30–4.20 (m, 3H), 3.58–3.51 (m, 1H), 3.48–3.41 (m, 1H), 3.16 (dd, J = 13.8, 4.6 Hz, 1H), 3.06 (dd, J = 6.9, 6.3 Hz, 2H), 2.91–2.83 (m, 1H), 2.70 (dd, J = 7.5, 6.9 Hz, 2H), 2.58–2.51 (m, 4H), 2.31–2.26 (m, 3H), 1.94–1.86 (m, 2H), 1.54–1.37 (m, 4H), 1.33 (s, 9H), 0.94 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 196.5, 174.4,

174.0, 173.3, 157.4, 157.2, 153.9, 150.8, 144.3, 144.2, 141.3, 139.0, 134.0, 133.9, 132.3, 132.2, 130.6, 129.2, 127.4, 120.3, 120.0, 119.9, 87.5, 85.4, 84.1, 83.1, 80.2, 72.7, 71.5, 70.3, 69.6, 59.2, 40.0, 36.7, 33.2, 31.8, 28.8, 28.1, 26.9, 26.3, 25.5, 19.0, 18.6, –4.3, –4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+H]^+$ calcd for $C_{57}H_{75}N_{10}O_{13}SSi$, 1167.5005; found, 1167.5004.

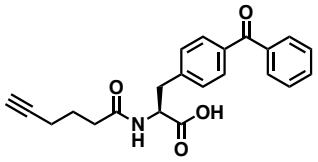
L-Phe-AMS-BPyne (1)



Compound **24** (12 mg, 0.010 mmol) was dissolved in a mixture of 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 8 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (4:1 $CHCl_3$/MeOH) to afford compound **1** as a white solid (5.3 mg, 56%). ^1H NMR (500 MHz, CD_3OD): δ 8.50 (s, 1H), 8.19 (s, 1H), 7.76–7.71 (m, 8H), 7.31–7.26 (m, 4H), 7.21–7.15 (m, 1H), 6.16 (d, J = 4.6 Hz, 1H), 4.45 (dd, J = 4.6, 4.0 Hz, 1H), 4.41 (dd, J = 5.2, 4.6 Hz, 1H), 4.39–4.34 (m, 1H), 4.33–4.27 (m, 2H), 3.91–3.86 (m, 1H), 3.73–3.63 (m, 1H), 3.63–3.57 (m, 1H), 3.35–3.28 (m, 1H, overlapping with MeOH), 3.19–3.10 (m, 2H), 3.06–3.00 (m, 1H), 2.71 (dd, J = 7.5, 6.9 Hz, 2H), 2.59–2.52 (m, 4H), 2.32–2.26 (m, 3H), 1.94–1.87 (m, 2H), 1.66–1.44 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD): δ 196.6, 175.3, 174.5, 174.1, 173.3, 157.2, 154.0, 150.6, 144.3, 144.2, 141.1, 136.6, 134.0, 133.9, 132.3, 132.2, 130.6, 130.0, 128.4, 120.13, 120.07, 120.0, 87.8, 84.5, 84.1, 83.6, 71.6, 70.9, 70.3, 68.9, 58.3, 40.0, 38.7, 36.7, 33.2, 31.8, 27.8, 26.9, 25.5, 18.6. HRMS (ESI+): $[M+H]^+$ calcd for $C_{46}H_{53}N_{10}O_{11}S$, 953.3616; found, 953.3616.

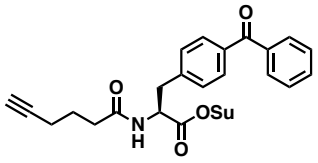
Synthesis of probe 2

(*S*)-3-(4-Benzoylphenyl)-2-(hex-5-ynamido)propanoic acid (27)



To a solution of L-4-benzoylphenylalanine **25** (450 mg, 1.67 mmol) and succinimidyl 5-hexynoate **26** (698 μL, 3.34 mmol) in DMF (10 mL) was added DIEA (583 μL, 3.34 mmol). The solution was stirred at room temperature for 1 h. The reaction mixture was diluted with EtOAc. The mixture was washed with H_2O and extracted with 5% $NaHCO_3$. The aqueous layer was acidified with solid citric acid monohydrate and extracted with $CHCl_3$. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **27** as a white solid (510 mg, 84%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76 (d, *J* = 6.9 Hz, 2H), 7.72 (d, *J* = 8.0 Hz, 2H), 7.58 (t, *J* = 7.5 Hz, 1H), 7.46 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.29 (d, *J* = 8.0 Hz, 2H), 6.36 (d, *J* = 8.0 Hz, 1H), 4.94 (dd, *J* = 6.3, 5.7 Hz, 1H), 3.33 (dd, *J* = 13.8, 5.7 Hz, 1H), 3.18 (dd, *J* = 13.8, 6.3 Hz, 1H), 2.35 (t, *J* = 6.9 Hz, 2H), 2.24−2.12 (m, 2H), 1.93 (t, *J* = 2.9 Hz, 1H), 1.84−1.76 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.9, 173.9, 173.3, 141.1, 137.5, 136.5, 132.8, 130.6, 130.2, 129.5, 128.5, 83.3, 69.6, 53.1, 37.5, 34.9, 24.1, 17.8. HRMS (ESI+): $[M+H]^+$ calcd for $C_{22}H_{22}NO_4$, 364.1549; found, 364.1545.

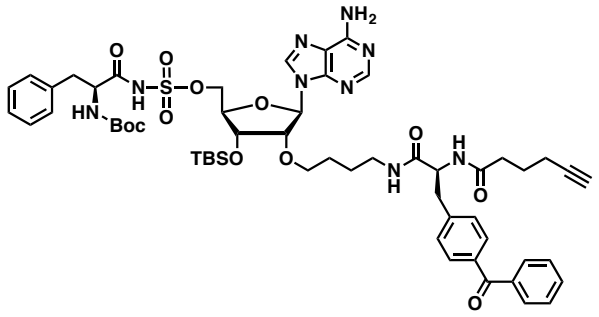
2,5-Dioxopyrrolidin-1-yl (*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanoate (28)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (318 mg, 1.66 mmol) and *N*-hydroxysuccinimide (191 mg, 1.66 mmol) were added to a solution of **27** (300 mg, 0.83 mmol) in DMF (5 mL). After 2 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 resulting white solid can be used in the next step

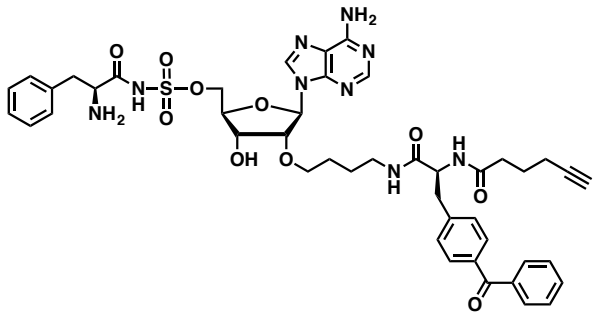
without further purification.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)butoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-Butoxycarbonyl)-*L*-phenylalanyl)sulfamate (29)



Compound **28** (42 mg, 0.09 mmol) and cesium carbonate (59 mg, 0.18 mmol) were added to a solution of **11** (50 mg, 0.06 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was concentrated under reduced pressure. The residue 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 (5:1 $CHCl_3$/MeOH) to afford compound **29** as a white solid (67 mg, 99%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.19 (s, 1H), 7.72 (d, *J* = 6.9 Hz, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.61 (t, *J* = 7.5 Hz, 1H), 7.49 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.38 (d, *J* = 8.6 Hz, 2H), 7.26−7.08 (m, 5H), 6.13 (d, *J* = 5.7 Hz, 1H), 4.64 (dd, *J* = 8.6, 6.3 Hz, 1H), 4.62−4.58 (m, 1H), 4.39−4.30 (m, 1H), 4.29−4.21 (m, 3H), 3.55−3.50 (m, 1H), 3.47−3.39 (m, 1H), 3.20−3.13 (m, 2H), 3.12−3.05 (m, 1H), 3.04−2.98 (m, 1H), 2.95 (dd, *J* = 13.2, 8.6 Hz, 1H), 2.88 (dd, *J* = 13.8, 8.6 Hz, 1H), 2.29 (t, *J* = 7.5 Hz, 2H), 2.19 (t, *J* = 2.9 Hz, 1H), 2.10−2.00 (m, 2H), 1.73−1.64 (m, 2H), 1.49−1.35 (m, 4H), 1.33 (s, 9H), 0.93 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.2, 175.0, 173.0, 157.4, 157.3, 153.9, 150.7, 144.1, 141.3, 139.0, 138.9, 137.2, 133.7, 131.3, 130.9, 130.6, 130.5, 130.3, 129.5, 129.2, 127.4, 120.3, 87.6, 85.2, 84.2, 83.0, 80.2, 72.7, 71.4, 70.3, 69.5, 59.3, 55.7, 40.0, 39.3, 35.6, 28.8, 28.0, 26.9, 26.3, 25.8, 19.0, 18.5, −4.3, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+H]^+$ calcd for $C_{56}H_{74}N_9O_{12}SSi$, 1124.4947; found, 1124.4956.

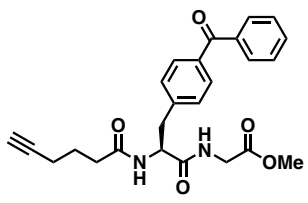
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (2)



Compound **29** (40 mg, 0.036 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 8 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (4:1 $CHCl_3$/MeOH) to afford compound **2** as a white solid (30 mg, 92%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.49 (s, 1H), 8.19 (s, 1H), 7.72 (d, *J* = 6.9 Hz, 2H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.61 (t, *J* = 7.5 Hz, 1H), 7.49 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.39 (d, *J* = 8.0 Hz, 2H), 7.30−7.25 (m, 4H), 7.19−7.14 (m, 1H), 6.14 (d, *J* = 4.6 Hz, 1H), 4.64 (dd, *J* = 8.6, 6.3 Hz, 1H), 4.45 (dd, *J* = 5.2, 4.6 Hz, 1H), 4.41−4.35 (m, 2H), 4.33−4.25 (m, 2H), 3.95−3.90 (m, 1H), 3.68−3.54 (m, 2H), 3.35−3.32 (m, 1H), 3.20−3.13 (m, 2H), 3.11−3.02 (m, 2H), 3.00−2.93 (m, 1H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.20 (t, *J* = 2.9 Hz, 1H), 2.09−2.02 (m, 2H), 1.73−1.65 (m, 2H), 1.53−1.39 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.2, 175.2, 175.1, 173.1, 156.9, 153.6, 150.5, 144.1, 141.2, 138.8, 137.2, 136.4, 133.7, 131.3, 130.9, 130.6, 130.5, 129.9, 129.5, 128.4, 120.2, 87.9, 84.24, 84.19, 83.5, 71.6, 70.7, 70.3, 68.9, 58.2, 55.8, 40.0, 39.1, 38.4, 35.6, 27.7, 26.8, 25.8, 18.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{45}H_{52}N_9O_{10}S$, 910.3558; found, 910.3553.

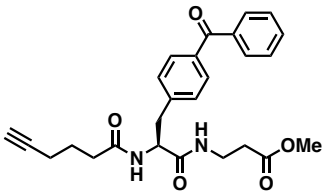
Syntheses of probes 3, 4, and 5

Methyl (*S*)-(3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanoyl)glycinate (31a)



Compound **28** (50 mg, 0.11 mmol) and DIEA (25 μL, 0.14 mmol) were added to a solution of methyl glycinate hydrochloride **30a** (12 mg, 0.095 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 4 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 (30:1 $CHCl_3$/MeOH) to afford compound **31a** as a white solid (39 mg, 95%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.74 (d, *J* = 8.6 Hz, 2H), 7.71 (d, *J* = 8.6 Hz, 2H), 7.56 (t, *J* = 7.5 Hz, 1H), 7.45 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 2H), 7.08 (t, *J* = 5.7 Hz, 1H), 6.66 (d, *J* = 8.0 Hz, 1H), 4.91 (q, *J* = 7.5 Hz, 1H), 3.98 (d, *J* = 5.7 Hz, 2H), 3.70 (s, 3H), 3.22 (dd, *J* = 13.8, 6.3 Hz, 1H), 3.11 (dd, *J* = 13.8, 7.5 Hz, 1H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.21−2.08 (m, 2H), 1.93 (t, *J* = 2.9 Hz, 1H), 1.81−1.72 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.4, 172.7, 171.4, 169.9, 141.7, 137.6, 136.3, 132.5, 130.5, 130.1, 129.4, 128.4, 83.4, 69.5, 53.8, 52.5, 41.2, 38.3, 34.8, 24.1, 17.8. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{25}H_{26}N_2O_5Na$, 457.1739; found, 457.1731.

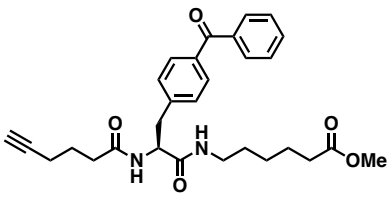
Methyl (*S*)-3-(3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)propanoate (31b)



Compound **28** (50 mg, 0.11 mmol) and DIEA (25 μL, 0.14 mmol) were added to a solution of methyl β-alaninate hydrochloride **30b** (13 mg, 0.093 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 10 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 (30:1 to 20:1 $CHCl_3$/MeOH) to afford compound **31b** as a white solid (35 mg, 82%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 8.6 Hz, 2H), 7.72 (d, *J* = 8.0 Hz, 2H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.46 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 6.67 (t, *J* = 5.7 Hz, 1H), 6.51 (d, *J* = 8.0 Hz, 1H), 4.72 (q, *J* = 7.5 Hz, 1H), 3.61 (s, 3H), 3.53−3.45 (m, 1H), 3.42−3.34 (m, 1H), 3.11 (d, *J* = 7.5 Hz, 2H), 2.50−2.42 (m, 1H), 2.42−2.34 (m, 1H), 2.33 (t, *J* = 7.5 Hz, 2H), 2.24−2.11 (m, 2H), 1.95 (t, *J* = 2.9 Hz, 1H), 1.84−1.76 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.3, 172.5, 172.3, 170.7, 141.7, 137.6, 136.3, 132.5, 130.5, 130.0, 129.3, 128.4, 83.4, 69.5, 54.2, 52.0, 38.8, 35.0, 34.9, 33.6, 24.1, 17.8. HRMS (ESI+): $[M+Na]^+$ calcd for

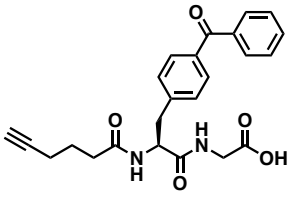
$C_{26}H_{28}N_2O_5Na$, 471.1896; found, 471.1883.

Methyl (*S*)-6-(3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)hexanoate (31c)



Compound **28** (50 mg, 0.11 mmol) and DIEA (25 μL, 0.14 mmol) were added to a solution of methyl 6-aminohexanate hydrochloride **30c** (17 mg, 0.094 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 10 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 (30:1 $CHCl_3$/MeOH) to afford compound **31c** as a white solid (43 mg, 93%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.76−7.68 (m, 4H), 7.56 (t, *J* = 7.5 Hz, 1H), 7.45 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 6.65 (d, *J* = 8.0 Hz, 1H), 6.53−6.48 (m, 1H), 4.79−4.72 (m, 1H), 3.62 (s, 3H), 3.24−3.02 (m, 4H), 2.32 (t, *J* = 7.5 Hz, 2H), 2.25 (t, *J* = 7.5 Hz, 2H), 2.21−2.10 (m, 2H), 1.60−1.52 (m, 2H), 1.44−1.35 (m, 2H), 1.27−1.19 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.3, 174.1, 172.4, 170.7, 141.9, 137.6, 136.3, 132.5, 130.5, 130.0, 129.4, 128.4, 83.3, 69.5, 54.3, 51.6, 39.3, 38.7, 34.9, 33.8, 29.0, 26.3, 24.4, 24.1, 17.8. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{29}H_{34}N_2O_5Na$, 513.2365; found, 513.2350.

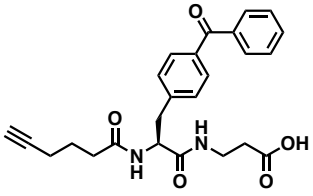
(*S*)-(3-(4-Benzoylphenyl)-2-(hex-5-ynamido)propanoyl)glycine (32a)



To a solution of **31a** (35 mg, 0.08 mmol) in a 1:1 (v/v) mixture of MeOH and THF (2 mL) was added 120 μL of a 1 M aqueous LiOH solution at 0 ºC. The mixture was stirred at room temperature for 12 h. The flask was then placed on a rotary evaporator and the MeOH and THF were removed at reduced pressure. The residue was diluted with 5% $NaHCO_3$ and washed with EtOAc. The aqueous layer was acidified with solid citric acid monohydrate and

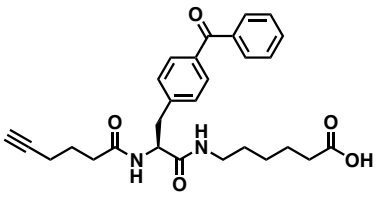
extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **32a** as a white solid (34 mg, 99%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.71 (d, *J* = 6.9 Hz, 2H), 7.67 (d, *J* = 8.0 Hz, 2H), 7.61−7.56 (br, 1H), 7.54 (t, *J* = 7.5 Hz, 1H), 7.42 (t, *J* = 7.5 Hz, 2H), 7.35−7.28 (m, 3H), 5.09−4.98 (m, 1H), 4.06−3.88 (m, 2H), 3.21 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.04 (dd, *J* = 13.8, 8.0 Hz, 1H), 2.28 (t, *J* = 7.5 Hz, 2H), 2.22−1.99 (m, 2H), 1.91 (t, *J* = 2.3 Hz, 1H), 1.76−1.64 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.9, 173.7, 172.2, 172.0, 141.7, 137.5, 136.2, 132.7, 130.5, 130.2, 129.5, 128.4, 83.3, 69.7, 53.9, 41.5, 38.4, 34.8, 24.2, 17.8. HRMS (ESI−): $[M-H]^-$ calcd for $C_{24}H_{23}N_2O_5$, 419.1612; found, 419.1613.

(*S*)-3-(3-(4-Benzoylphenyl)-2-(hex-5-ynamido)propanamido)propanoic acid (32b)



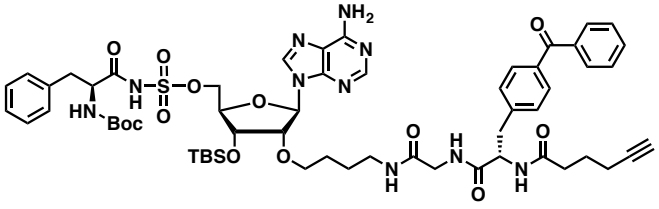
To a solution of **31b** (30 mg, 0.067 mmol) in a 1:1 (v/v) mixture of MeOH and THF (1 mL) was added 100 μL of a 1 M aqueous LiOH solution at 0 ºC. The mixture was stirred at room temperature for 9 h. The flask was then placed on a rotary evaporator and the MeOH and THF were removed at reduced pressure. The residue was diluted with 5% $NaHCO_3$ and washed with EtOAc. The aqueous layer was acidified with solid citric acid monohydrate and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **32b** as a white solid (34 mg, 100%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.75 (d, *J* = 7.5 Hz, 2H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.57 (t, *J* = 7.5 Hz, 1H), 7.45 (t, *J* = 7.5 Hz, 2H), 7.33−7.25 (m, 2H), 7.04 (d, *J* = 8.6 Hz, 1H), 4.99 (q, *J* = 7.5 Hz, 1H), 3.49−3.40 (m, 1H), 3.12−3.02 (m, 2H), 2.55−2.46 (m, 1H), 2.45−2.37 (m, 1H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.20−2.05 (m, 2H), 1.95 (t, *J* = 2.9 Hz, 1H), 1.80−1.69 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 196.9, 175.2, 172.9, 171.1, 141.6, 137.5, 136.3, 132.7, 130.5, 130.2, 129.3, 128.4, 83.3, 69.5, 54.0, 39.1, 34.8, 33.6, 24.1, 17.8. HRMS (ESI−): $[M-H]^-$ calcd for $C_{25}H_{25}N_2O_5$, 433.1769; found, 433.1750.

(*S*)-6-(3-(4-Benzoylphenyl)-2-(hex-5-ynamido)propanamido)hexanoic acid (32c)



To a solution of **31c** (36 mg, 0.073 mmol) in a 1:1 (v/v) mixture of MeOH and THF (1 mL) was added 110 μL of a 1 M aqueous LiOH solution at 0 ºC. The mixture was stirred at room temperature for 24 h. The flask was then placed on a rotary evaporator and the MeOH and THF were removed at reduced pressure. The residue was diluted with 5% $NaHCO_3$ and washed with EtOAc. The aqueous layer was acidified with solid citric acid monohydrate and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **32c** as a white solid (32 mg, 92%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 12.0 (br, 1H), 8.15 (d, J = 8.6 Hz, 1H), 7.98 (t, J = 5.7 Hz, 1H), 7.71−7.66 (m, 2H), 7.66−7.62 (m, 3H), 7.54 (dd, J = 8.0, 7.5 Hz, 2H), 7.40 (d, J = 8.6 Hz, 2H), 4.56−4.50 (m, 1H), 3.10−2.94 (m, 3H), 2.83 (dd, J = 13.8, 9.7 Hz, 1H), 2.73 (t, J = 2.9 Hz, 1H), 2.18−2.11 (m, 4H), 1.97 (ddd, J = 7.5, 7.5, 2.9 Hz, 2H), 1.60−1.50 (m, 2H), 1.49−1.42 (m, 2H), 1.37−1.29 (m, 2H), 1.23−1.15 (m, 2H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 195.5, 174.4, 171.3, 170.7, 143.5, 137.3, 135.0, 132.5, 129.5, 129.44, 129.38, 128.5, 84.1, 71.4, 53.7, 38.4, 38.0, 34.0, 33.6, 28.7, 25.9, 24.3, 24.2, 17.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{28}H_{31}N_2O_5$, 475.2233; found, 475.2230.

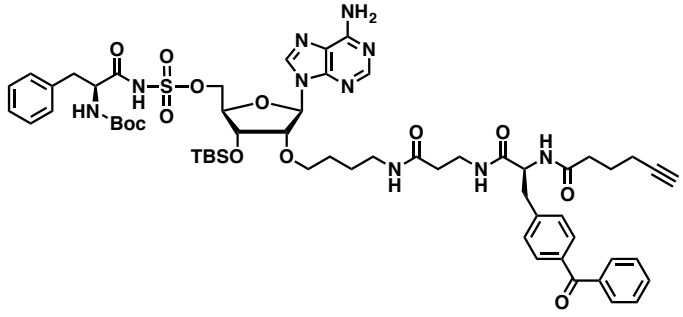
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(2-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)acetamido)butoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (34a)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (19 mg, 0.098 mmol) and *N*-hydroxysuccinimide (11 mg, 0.098 mmol) were added to a solution of **32a** (27 mg, 0.065 mmol) in DMF (1 mL). The solution was stirred at room temperature for 4 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 to afford compound **33a** as a white solid (34 mg). Compound **33a** and cesium carbonate (42 mg, 0.13 mmol) were added to a solution of compound **11** (33 mg, 0.043 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 1 h. The solvent was removed at reduced pressure. The residue was diluted with EtOAc. The resulting 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 (10:1 to 6:1 $CHCl_3$/MeOH) to afford compound **34a** as a white solid (40 mg, 79%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.51 (s, 1H), 8.20 (s, 1H), 7.73 (d, *J* = 7.5 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 2H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.50 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.38 (d, *J* = 8.6 Hz, 2H), 7.25−7.17 (m, 4H), 7.16−7.09 (m, 1H), 6.15 (d, *J* = 6.3 Hz, 1H), 4.64−4.58 (m, 2H), 4.56−4.51 (m, 1H), 4.38−4.31 (m, 1H), 4.30−4.22 (m, 2H), 3.88 (d, *J* = 17.2 Hz, 1H), 3.69 (d, *J* = 16.7 Hz, 1H), 3.58−3.53 (m, 1H), 3.49−3.42 (m, 1H), 3.26 (dd, *J* = 13.8, 6.3 Hz, 1H), 3.15 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.11−3.00 (m, 3H), 2.91−2.83 (m, 1H), 2.29 (t, *J* = 7.5 Hz, 2H), 2.19 (t, *J* = 2.9 Hz, 1H), 2.10−1.99 (m, 2H), 1.71−1.63 (m, 2H), 1.54−1.38 (m, 4H), 1.33 (s, 9H), 0.95 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.2, 175.6, 174.0, 171.2, 157.3, 157.1, 153.8, 150.8, 144.1, 141.4, 138.89, 138.85, 137.2, 133.7, 131.3, 130.9, 130.8, 130.6, 130.5, 129.5, 129.2, 127.4, 120.3, 87.5, 85.4, 84.2, 83.1, 80.2, 72.7, 71.4, 70.3, 69.8, 58.9, 56.3, 43.6, 40.1, 38.3, 35.4, 28.8, 28.0, 26.8, 26.3, 25.7, 19.0, 18.5, −4.3, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI−): $[M-H]^-$ calcd for $C_{58}H_{75}N_{10}O_{13}SSi$, 1179.5005; found, 1179.4998.

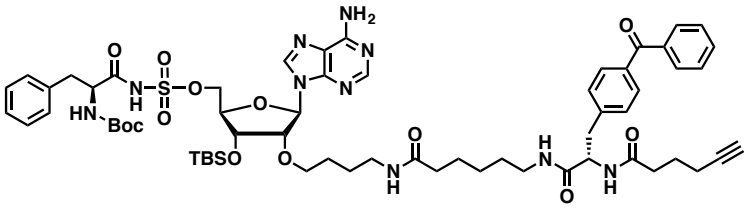
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(3-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)propanamido)butoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (34b)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (19 mg, 0.098 mmol) and

N-hydroxysuccinimide (11 mg, 0.098 mmol) were added to a solution of **32b** (29 mg, 0.067 mmol) in DMF (1 mL). The solution was stirred at room temperature for 4 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 to afford compound **33b** as a white solid (38 mg). Compound **33b** and cesium carbonate (44 mg, 0.135 mmol) were added to a solution of compound **11** (35 mg, 0.045 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 1 h. The residue was diluted with EtOAc. The resulting 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 (10:1 to 4:1 $CHCl_3$/MeOH) to afford compound **34b** as a white solid (30 mg, 56%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.18 (s, 1H), 7.88 (br, 1H), 7.73 (d, *J* = 6.9 Hz, 2H), 7.69 (d, *J* = 8.0 Hz, 2H), 7.61 (t, *J* = 7.5 Hz, 1H), 7.50 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.38 (d, *J* = 8.0 Hz, 2H), 7.25−7.17 (m, 4H), 7.16−7.08 (m, 1H), 6.14 (d, *J* = 6.3 Hz, 1H), 4.65 (dd, *J* = 9.7, 5.7 Hz, 1H), 4.62−4.57 (m, 1H), 4.55−4.49 (m, 1H), 4.38−4.31 (m, 1H), 4.29−4.22 (m, 3H), 3.58−3.51 (m, 1H), 3.48−3.36 (m, 3H), 3.22 (dd, *J* = 13.8, 5.7 Hz, 1H), 3.17 (dd, *J* = 13.8, 4.6 Hz, 1H), 3.08−3.00 (m, 2H), 2.99−2.92 (m, 1H), 2.91−2.83 (m, 1H), 2.35−2.25 (m, 4H), 2.20 (t, *J* = 2.9 Hz, 1H), 2.09−1.98 (m, 2H), 1.71−1.63 (m, 2H), 1.53−1.36 (m, 4H), 1.33 (s, 9H), 0.94 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.2, 175.2, 173.4, 173.3, 157.5, 157.3, 154.0, 150.7, 144.1, 141.2, 139.0, 138.9, 137.2, 133.7, 131.3, 131.0, 130.6, 130.5, 129.5, 129.2, 127.4, 120.3, 87.5, 85.3, 84.2, 83.1, 80.3, 72.7, 71.4, 70.3, 69.7, 59.3, 55.7, 40.1, 39.0, 37.0, 36.4, 35.5, 28.8, 28.1, 26.8, 26.3, 25.7, 19.0, 18.5, −4.3, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI−): $[M-H]^-$ calcd for $C_{59}H_{77}N_{10}O_{13}SSi$, 1193.5162; found, 1193.5163.

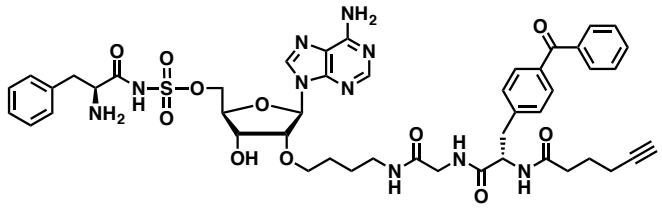
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(6-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)hexanamido)butoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (34c)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (18 mg, 0.094 mmol) and

N-hydroxysuccinimide (11 mg, 0.098 mmol) were added to a solution of **32c** (30 mg, 0.063 mmol) in DMF (1 mL). The solution was stirred at room temperature for 20 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 to afford compound **33c** as a white solid (39 mg). Compound **33c** and cesium carbonate (41 mg, 0.126 mmol) were added to a solution of compound **11** (33 mg, 0.042 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 3 h. The residue was diluted with EtOAc. The resulting 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 to 10:1 $CHCl_3$/MeOH) to afford compound **34c** as a white solid (45 mg, 87%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.51 (s, 1H), 8.19 (s, 1H), 7.73 (d, *J* = 6.9 Hz, 2H), 7.70 (d, *J* = 8.6 Hz, 2H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.51 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.22−7.17 (m, 4H), 7.16−7.10 (m, 1H), 6.15 (d, *J* = 6.3 Hz, 1H), 4.67 (dd, *J* = 8.6, 6.3 Hz, 1H), 4.62−4.58 (m, 1H), 4.55 (dd, *J* = 6.3, 5.2 Hz, 1H), 4.37−4.30 (m, 1H), 4.30−4.21 (m, 3H), 3.59−3.53 (m, 1H), 3.47−3.40 (m, 1H), 3.23−3.07 (m, 4H), 3.06−2.94 (m, 3H), 2.92−2.85 (m, 1H), 2.30 (t, *J* = 7.5 Hz, 2H), 2.21 (t, *J* = 2.9 Hz, 1H), 2.10 (t, *J* = 7.5 Hz, 2H), 2.08−2.03 (m, 2H), 1.73−1.66 (m, 2H), 1.58−1.37 (m, 8H), 1.34 (s, 9H), 1.31−1.20 (m, 2H), 0.95 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.2, 175.9, 175.1, 173.2, 157.3, 157.2, 153.8, 150.8, 144.2, 141.3, 138.9, 138.8, 137.2, 133.7, 130.9, 130.6, 130.5, 129.5, 129.2, 127.4, 120.2, 87.5, 85.4, 84.2, 83.1, 80.2, 72.7, 71.5, 70.3, 69.8, 58.9, 55.8, 40.3, 39.8, 39.2, 37.0, 35.6, 29.9, 28.8, 28.2, 27.4, 27.0, 26.6, 26.4, 25.8, 19.0, 18.5, −4.4, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI−): $[M-H]^-$ calcd for $C_{62}H_{83}N_{10}O_{13}SSi$, 1235.5631; found, 1235.5629.

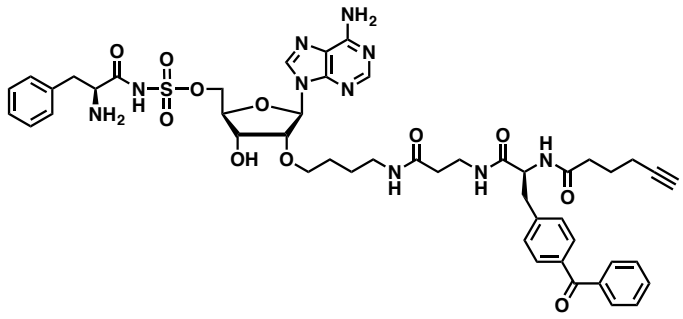
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(2-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)acetamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (3)



Compound **34a** (38 mg, 0.032 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and

H_2O were removed at reduced pressure. The residue was purified by flash chromatography (6:1 to 5:1 $CHCl_3$/MeOH) to afford compound **3** as a white solid (25 mg, 81%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.56 (s, 1H), 8.23 (s, 1H), 7.77−7.67 (m, 5H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.50 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.32−7.24 (m, 4H), 7.17 (t, *J* = 6.9 Hz, 1H), 6.17 (d, *J* = 4.6 Hz, 1H), 4.61 (dd, *J* = 9.2, 5.7 Hz, 1H), 4.46 (t, *J* = 4.6 Hz, 1H), 4.42−4.34 (m, 2H), 4.33−4.28 (m, 2H), 3.94 (dd, *J* = 8.0, 5.2 Hz, 1H), 3.87 (d, *J* = 17.2 Hz, 1H), 3.72−3.65 (m, 2H), 3.64−3.58 (m, 1H), 3.35−3.33 (m, 1H), 3.28−3.23 (m, 1H), 3.18−3.12 (m, 2H), 3.10−3.00 (m, 2H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.21 (t, *J* = 2.9 Hz, 1H), 2.08−2.01 (m, 2H), 1.72−1.64 (m, 2H), 1.62−1.45 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.3, 175.7, 175.0, 174.1, 171.4, 155.8, 151.9, 150.4, 144.0, 141.8, 138.9, 137.3, 136.4, 133.7, 131.3, 130.9, 130.6, 130.5, 130.1, 130.0, 129.5, 128.4, 120.1, 88.0, 84.5, 84.3, 83.6, 71.6, 70.8, 70.3, 68.9, 58.2, 56.3, 43.6, 40.2, 38.5, 38.3, 35.4, 27.7, 26.8, 25.7, 18.5. HRMS (ESI−): $[M-H]^-$ calcd for $C_{47}H_{53}N_{10}O_{11}S$, 965.3616; found, 965.3612.

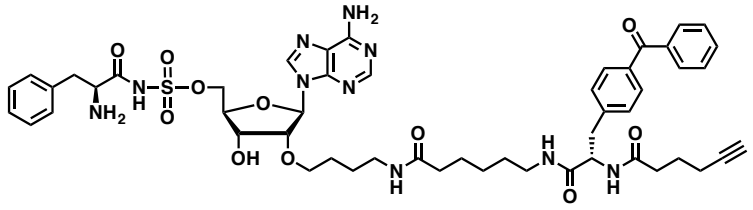
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(3-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)propanamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (4)



Compound **34b** (30 mg, 0.025 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 12 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (6:1 $CHCl_3$/MeOH) to afford compound **4** as a white solid (20 mg, 82%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.56 (s, 1H), 8.23 (s, 1H), 7.75−7.67 (m, 4H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.50 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 7.33−7.25 (m, 4H), 7.20−7.15 (m, 1H), 6.16 (d, *J* = 5.2 Hz, 1H), 4.65 (dd, *J* = 9.2, 5.7 Hz, 1H), 4.46 (t, *J* = 4.6 Hz, 1H), 4.41 (t, *J* = 5.2, 4.6 Hz, 1H), 4.39−4.34 (m, 1H), 4.33−4.27 (m, 2H), 3.93 (dd, *J* = 8.6, 5.2 Hz, 1H), 3.71−3.64 (m, 1H), 3.63−3.57 (m, 1H), 3.47−3.56 (m, 2H), 3.35−3.33 (m, 1H), 3.25−3.19 (m, 1H), 3.13−3.02 (m, 2H), 2.96 (dd, *J* = 13.8, 9.7 Hz, 1H), 2.35−2.26 (m, 4H), 2.21 (t, *J* = 2.9 Hz,

1H), 2.07−2.01 (m, 2H), 1.71−1.63 (m, 2H), 1.61−1.53 (m, 2H), 1.52−1.44 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.3, 175.2, 174.9, 173.4, 173.3, 155.9, 152.0, 150.4, 144.2, 141.7, 138.9, 137.2, 136.5, 133.7, 131.3, 131.0, 130.6, 130.5, 130.1, 130.0, 129.5, 128.5, 120.1, 87.9, 84.6, 84.2, 83.7, 71.6, 70.8, 70.3, 68.9, 58.2, 55.7, 40.0, 39.0, 38.5, 37.1, 36.4, 35.6, 27.9, 26.9, 25.8, 18.5. HRMS (ESI−): $[M-H]^-$ calcd for $C_{48}H_{55}N_{10}O_{11}S$, 979.3772; found, 979.3765.

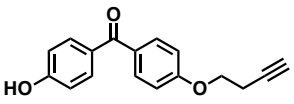
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(6-((*S*)-3-(4-benzoylphenyl)-2-(hex-5-ynamido)propanamido)hexanamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (5)



Compound **34c** (40 mg, 0.032 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (6:1 $CHCl_3$/MeOH) to afford compound **5** as a white solid (26 mg, 84%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.59 (s, 1H), 8.27 (s, 1H), 7.75−7.67 (m, 4H), 7.62 (t, *J* = 7.5 Hz, 1H), 7.51 (dd, *J* = 8.0, 7.5 Hz, 2H), 7.41 (d, *J* = 8.0 Hz, 2H), 7.33−7.24 (m, 4H), 7.12−7.16 (m, 1H), 6.18 (d, *J* = 4.6 Hz, 1H), 4.66 (dd, *J* = 9.2, 6.3 Hz, 1H), 4.46 (t, *J* = 4.6 Hz, 1H), 4.42−4.36 (m, 2H), 4.34−4.27 (m, 2H), 3.94 (dd, *J* = 8.6, 5.2 Hz, 1H), 3.73−3.64 (m, 1H), 3.65−3.58 (m, 1H), 3.35−3.32 (m, 1H), 3.23−3.15 (m, 2H), 3.14−3.03 (m, 4H), 3.01−2.95 (m, 1H), 2.31 (t, *J* = 7.5 Hz, 2H), 2.21 (t, *J* = 2.9 Hz, 1H), 2.12 (t, *J* = 7.5 Hz, 2H), 2.08−2.03 (m, 2H), 1.73−1.65 (m, 2H), 1.62−1.47 (m, 6H), 1.46−1.38 (m, 2H), 1.27−1.20 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 198.3, 176.0, 175.1, 174.9, 173.1, 154.8, 150.2, 144.2, 142.3, 138.9, 137.2, 136.4, 133.7, 131.3, 130.9, 130.6, 130.5, 130.1, 130.0, 129.5, 128.5, 120.1, 88.2, 84.5, 84.2, 83.7, 71.6, 70.7, 70.3, 68.9, 58.2, 55.8, 40.2, 40.0, 39.2, 38.5, 36.9, 35.6, 29.9, 27.8, 27.4, 27.0, 26.6, 25.8, 18.5. HRMS (ESI−): $[M-H]^-$ calcd for $C_{51}H_{61}N_{10}O_{11}S$, 1021.4242; found, 1021.4240.

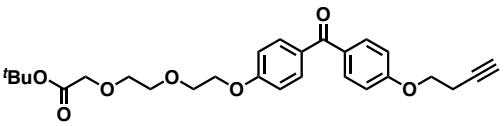
Syntheses of probes 6 and 7

(4-(But-3-yn-1-yloxy)phenyl)(4-hydroxyphenyl)methanone (36)



DIAD (1.05 mL, 2 mmol) was dropwise added to a solution of 4, 4′-dihydroxybenzophenone **35** (857 mg, 4 mmol), 3-butyn-1-ol (151 μL, 2 mmol) and PPh_3 (525 mg, 2 mmol) in THF (20 mL). The solution was stirred at room temperature for 6 h. The solvent was removed at reduced pressure. The residue was dissolved in 1 M aqueous NaOH and washed with EtOAc. The aqueous layer was acidified with 1 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flush chromatography (2:1 hexane/EtOAc) to afford compound **36** as a colorless oil (340 mg, 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78 (d, J = 8.6 Hz, 2H), 7.73 (d, J = 8.6 Hz, 2H), 6.97 (d, J = 8.6 Hz, 2H), 6.92 (d, J = 8.6 Hz, 2H), 4.17 (t, J = 6.9 Hz, 2H), 2.72 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 2.06 (t, J = 2.9 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 195.4, 162.0, 160.2, 132.8, 132.5, 131.0, 130.5, 115.4, 114.3, 80.2, 70.3, 66.3, 19.6. HRMS (ESI+): $[M+H]^+$ calcd for $C_{17}H_{15}O_3$, 267.1021; found, 267.1013.

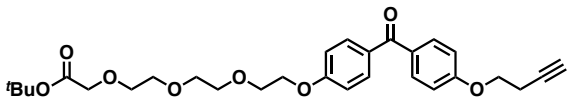
***tert*-Butyl 2-(2-(2-(4-(4-(but-3-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)acetate (38a)**



DIAD (1.16 mL, 2.2 mmol) was dropwise added to a solution of **36** (586 mg, 2.2 mmol), **37a** (440 mg, 2 mmol) and PPh_3 (577 mg, 2.2 mmol) in THF (20 mL). The solution was stirred at room temperature for 20 h. The solvent was removed at reduced pressure. The residue was purified by flush chromatography (4:1 to 2:1 hexane/EtOAc) to afford compound **38a** as a white solid (658 mg, 70%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78−7.73 (m, 4H), 6.98−6.93 (m, 4H), 4.21 (t, J = 4.6 Hz, 2H), 4.16 (t, J = 6.9 Hz, 2H), 4.02 (s, 2H), 3.89 (t, J = 4.6 Hz, 2H), 3.78−3.72 (m, 4H), 2.70 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 2.05 (t, J = 2.9 Hz, 1H), 1.46 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ194.5, 169.7, 162.2, 161.7, 132.29, 132.27, 131.2, 130.9, 114.2, 114.1, 81.7, 80.1, 71.0, 70.8, 70.3, 69.7, 69.2, 67.7, 66.2, 28.2, 19.6. HRMS (ESI+): $[M+Na]^+$

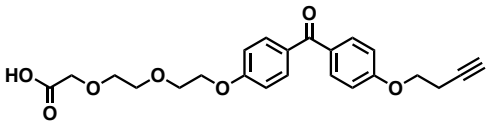
calcd for $C_{27}H_{32}O_7Na$, 491.2046; found, 491.2037.

***tert*-Butyl**
2-(2-(2-(2-(4-(4-(but-3-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)ethoxy)acetate (38b)



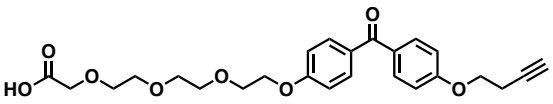
DIAD (0.36 mL, 0.68 mmol) was dropwise added to a solution of **36** (150 mg, 0.57 mmol), **37b** (180 mg, 0.68 mmol) and PPh_3 (178 mg, 0.68 mmol) in THF (5 mL). The solution was stirred at room temperature for 24 h. The solvent was removed at reduced pressure. The residue was purified by flush chromatography (1:1 hexane/EtOAc) to afford compound **38b** as a white solid (217 mg, 74%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78−7.73 (m, 4H), 6.98−6.93 (m, 4H), 4.20 (t, *J* = 5.2 Hz, 2H), 4.16 (t, *J* = 6.9 Hz, 2H), 4.01 (s, 2H), 3.88 (t, *J* = 5.2 Hz, 2H), 3.75−3.67 (m, 8H), 2.71 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 2.05 (t, *J* = 2.9 Hz, 1H), 1.46 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.5, 169.7, 162.2, 161.7, 132.31, 132.28, 131.2, 130.9, 114.2, 114.1, 81.7, 80.1, 71.0, 70.83, 70.76, 70.74, 70.3, 69.6, 69.1, 67.7, 66.2, 28.2, 19.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{29}H_{36}O_8Na$, 535.2488; found, 535.2309.

2-(2-(2-(4-(4-(But-3-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)acetic acid (39a)



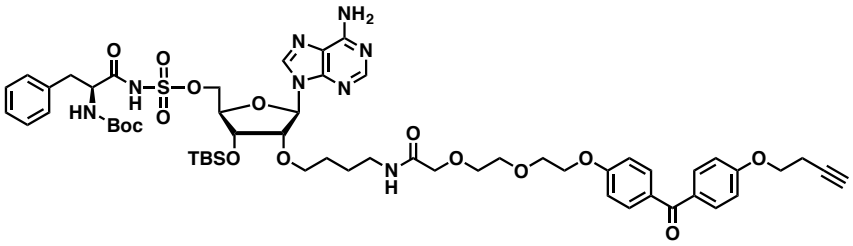
Compound **38a** (300 mg, 0.64 mmol) was dissolved in a 1:1 (v/v) mixture of TFA and CH_2Cl_2 (10 mL) at room temperature. After 12 h, the flask was placed on the rotary evaporator and the TFA and CH_2Cl_2 were removed at reduced pressure to afford compound **39a** as a white solid (258 mg, 98%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.20−8.01 (br, 2H), 7.76−7.71 (m, 4H), 6.97−6.91 (m, 4H), 4.20−4.11 (m, 6H), 3.91−3.86 (m, 2H), 3.77−3.73 (m, 4H), 2.72−2.66 (m, 2H), 2.05 (t, *J* = 2.9 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ195.0, 173.7, 162.1, 161.8, 132.40, 132.37, 130.8, 130.7, 114.12, 114.09, 80.1, 71.0, 70.6, 70.3, 69.6, 68.4, 67.4, 66.1, 19.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{23}H_{25}O_7$, 413.1600; found, 413.1595.

2-(2-(2-(2-(4-(4-(But-3-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)ethoxy)acetic acid (39b)



Compound **38b** (180 mg, 0.35 mmol) was dissolved in a 1:1 (v/v) mixture of TFA and CH_2Cl_2 (2 mL) and stirred at room temperature. After 18 h, the flask was placed on the rotary evaporator and the TFA and CH_2Cl_2 were removed at reduced pressure to afford compound **39b** as a white solid (155 mg, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.78−7.74 (m, 4H), 6.98−6.94 (m, 4H), 5.68 (br, 2H), 4.21 (t, *J* = 4.6 Hz, 2H), 4.16 (t, *J* = 6.9 Hz, 2H), 4.14 (s, 2H), 3.88 (t, *J* = 4.6 Hz, 2H), 3.76−3.68 (m, 8H), 2.71 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 2.06 (t, *J* = 2.9 Hz, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.8, 172.6, 162.1, 161.8, 132.40, 132.38, 131.0, 130.9, 114.2, 114.1, 80.2, 71.2, 70.7, 70.30, 70.27, 69.6, 68.8, 67.6, 66.2, 19.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{28}H_{29}O_8$, 457.1862; found, 457.1859.

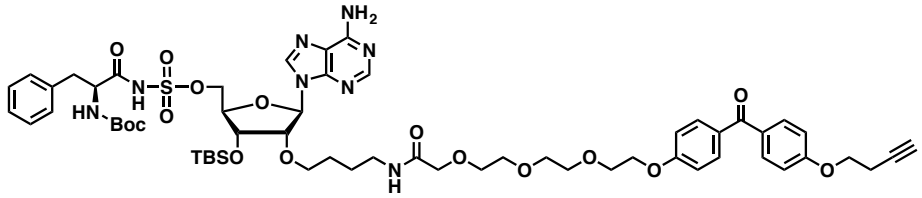
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(2-(2-(2-(4-(4-(but-3-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)acetamido)butoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (41a)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (35 mg, 0.18 mmol) and *N*-hydroxysuccinimide (21 mg, 0.18 mmol) were added to a solution of **39a** (37 mg, 0.09 mmol) in DMF (1 mL). The solution was stirred at room temperature for 24 h. The reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness to afford compound **40a** as a white solid (47 mg). Compound **40a** and cesium carbonate (59 mg, 0.18 mmol) were added to a solution of compound **11** (47 mg, 0.06 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 3 h. The solvent was removed at reduced pressure. The residue was diluted with EtOAc. The resulting 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 (10:1 to 8:1 $CHCl_3$/MeOH) to afford compound **41a** as a white solid (44 mg, 63%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.18 (s, 1H), 7.74−7.69 (m, 4H), 7.22−7.16 (m, 4H), 7.15−7.08 (m, 1H), 7.04−6.98 (m, 4H), 6.13 (d, *J* = 5.7 Hz, 1H), 4.61−4.57 (m, 1H), 4.50 (dd, *J* = 5.7, 4.6 Hz, 1H), 4.34 (dd, *J* = 11.5, 4.0 Hz, 1H), 4.29−4.22 (m, 3H), 4.21−4.14 (m, 3H), 3.95 (s, 2H), 3.87−3.83 (m, 2H), 3.74−3.70 (m, 2H), 3.68−3.65 (m, 2H), 3.55−3.49 (m, 1H), 3.43−3.37 (m, 1H), 3.18−3.12 (m, 1H), 3.10−3.04 (m, 2H), 2.91−2.84 (m, 1H), 2.68 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 2.35 (t, *J* = 2.9 Hz, 1H), 1.47−1.36 (m, 4H), 1.33 (s, 9H), 0.92 (s, 9H), 0.14 (s, 3H), 0.12 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.5, 172.5, 163.8, 163.6, 157.4, 157.2, 153.9, 150.8, 141.3, 139.0, 133.40, 133.37, 131.8, 130.6, 129.2, 127.4, 120.3, 115.3, 87.5, 85.3, 83.1, 81.3, 80.2, 79.5, 72.6, 72.0, 71.6, 71.5, 71.12, 71.10. 70.7, 69.5, 68.9, 67.6, 59.1, 39.9, 39.5, 28.8, 28.1, 27.1, 26.4, 20.1, 19.0, −4.3, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+H]^+$ calcd for $C_{57}H_{77}N_8O_{15}SSi$, 1173.4998; found, 1173.4998.

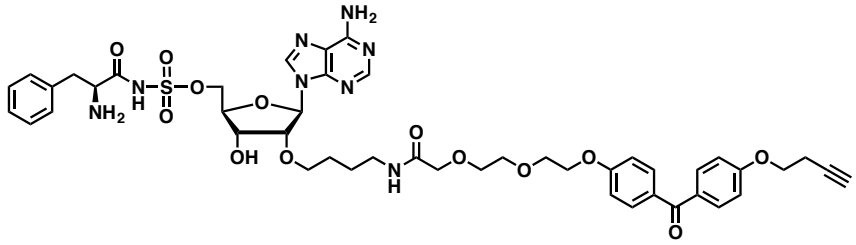
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-((1-(4-(4-(but-3-yn-1-yloxy)benzoyl)phenoxy)-11-oxo-3,6,9-trioxa-12-azahexadecan-16-yl)oxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (41b)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (35 mg, 0.18 mmol) and *N*-hydroxysuccinimide (21 mg, 0.18 mmol) were added to a solution of **39b** (41 mg, 0.09 mmol) in DMF (1 mL). The solution was stirred at room temperature for 9 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 to afford compound **40b** as a white solid (50 mg). Compound **40b** and cesium carbonate (59 mg, 0.18 mmol) were added to a solution of compound **11** (47 mg, 0.06 mmol) in CH_2Cl_2 (1 mL). The solvent was stirred at room temperature for 3 h. The solvent was removed at reduced pressure. The residue was diluted with EtOAc. The resulting 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 (10:1 $CHCl_3$/MeOH) to afford compound **41b** as a white solid (35 mg, 48%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.48 (s, 1H), 8.19 (s, 1H), 7.75−7.70 (m, 4H), 7.21−7.17 (m, 4H), 7.15−7.09 (m, 1H), 7.05−7.00 (m, 4H), 6.14 (d, *J* = 6.3 Hz, 1H), 4.61−4.58 (m, 1H), 4.53 (dd, *J* = 6.3, 4.6 Hz, 1H), 4.36−4.30 (m, 1H), 4.29−4.22 (m, 3H), 4.21−4.15 (m, 4H), 3.93 (s, 2H), 3.86−3.83 (m, 1H), 3.71−3.68 (m, 2H), 3.66−3.61 (m, 6H), 3.59−3.53 (m, 1H), 3.46−3.41 (m, 1H), 3.16 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.09 (dd, *J* = 6.9, 6.3 Hz, 2H), 2.88 (dd, *J* = 13.8, 8.0 Hz, 1H), 2.69 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 2.35 (t, *J* = 2.9 Hz, 1H), 1.52−1.37 (m, 4H), 1.33 (s, 9H), 0.94 (s, 9H), 0.15 (s, 3H), 0.14 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.5, 172.6, 163.9, 163.6, 157.4, 157.3, 154.0, 150.8, 141.3, 139.0, 133.4, 131.9, 131.8, 130.6, 129.2, 127.4, 120.3, 115.30, 115,27, 87.5, 85.4, 83.1, 81.3, 80.2, 72.7, 71.9, 71.7, 71.5, 71.3, 71.1, 71.08, 70.6, 69.6, 68.9, 67.6, 59.2, 40.0, 39.5, 28.8, 28.1, 27.0, 26.4, 20.1, 19.0, −4.3, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+H]^+$ calcd for $C_{59}H_{81}N_8O_{16}SSi$, 1217.5261; found, 1217.5262.

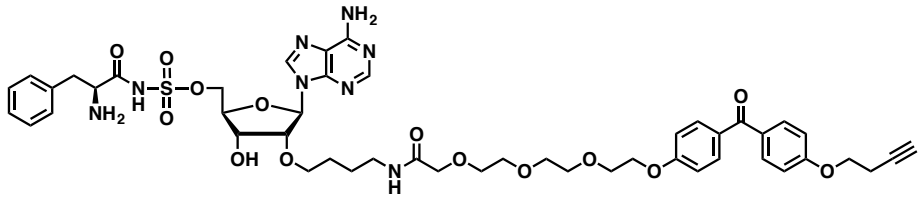
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-(2-(2-(2-(4-(4-(but-3-yn-1-yloxy)benzoyl)phenoxy)ethoxy)ethoxy)acetamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (6)



Compound **41a** (20 mg, 0.017 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (3:1 $CHCl_3$/MeOH) to afford compound **6** as a white solid (14 mg, 86%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.18 (s, 1H), 7.74−7.69 (m, 4H), 7.29−7.24 (m, 4H), 7.19−7.14 (m, 1H), 7.05−7.00 (m, 4H), 6.13 (d, *J* = 4.6 Hz, 1H), 4.42 (dd, *J* = 5.2, 4.6 Hz, 1H), 4.38−4.34 (m, 2H), 4.32−4.25 (m, 2H), 4.24−4.20 (m, 2H), 4.17 (t, *J* = 6.9 Hz, 2H), 3.97 (s, 2H), 3.92 (dd, *J* = 5.2, 4.6 Hz, 1H), 3.89−3.85 (m, 2H), 3.75−3.71 (m, 2H), 3.70−3.67 (m, 2H), 3.66−3.62 (m, 1H), 3.57−3.51 (m, 1H), 3.16−3.11 (m, 1H), 3.06 (dd, *J* = 14.3, 8.6 Hz, 1H), 2.72−2.66 (m, 2H), 2.35 (t, *J* = 2.9 Hz, 1H), 1.55−1.41 (m, 4H). ^{13}C NMR (125 MHz,

CD_3OD) δ 196.6, 175.2, 172.7, 163.8, 163.7, 157.1, 154.0, 150.6, 141.1, 136.4, 133.41, 133.36, 131.8, 130.6, 130.0, 128.5, 120.2, 115.28, 115.25, 87.9, 84.3, 83.6, 81.3, 72.0, 71.59, 71.56, 71.09, 71.06, 70.73, 70.69, 68.94, 68.91, 67.6, 58.2, 39.6, 38.5, 27.8, 27.0, 20.1. HRMS (ESI+): $[M+H]^+$ calcd for $C_{46}H_{55}N_8O_{13}S$, 959.3609; found, 959.3604.

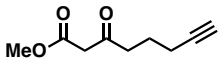
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-((1-(4-(4-(but-3-yn-1-yloxy)benzoyl)phenoxy)-11-oxo-3,6,9-trioxa-12-azahexadecan-16-yl)oxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (7)



Compound **41b** (18 mg, 0.015 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (10:1 to 4:1 $CHCl_3$/MeOH) to afford compound **7** as a white solid (13 mg, 86%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.51 (s, 1H), 8.18 (s, 1H), 7.75−7.70 (m, 4H), 7.31−7.25 (m, 4H), 7.20−7.15 (m, 1H), 7.05−7.00 (m, 4H), 6.15 (d, *J* = 5.2 Hz, 1H), 4.44 (t, *J* = 4.6 Hz, 1H), 4.41 (dd, *J* = 5.2, 4.6 Hz, 1H), 4.37−4.33 (m, 1H), 4.32−4.26 (m, 2H), 4.23−4.20 (m, 2H), 4.18 (t, *J* = 6.9 Hz, 2H), 3.93 (s, 2H), 3.90 (dd, *J* = 8.6, 4.6 Hz, 1H), 3.87−3.84 (m, 2H), 3.72−3.55 (m, 10H), 3.18−3.14 (m, 2H), 3.06−3.00 (m, 1H), 2.69 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 2.36 (t, *J* = 2.9 Hz, 1H), 1.61−1.45 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.6, 175.2, 172.6, 163.9, 163.7, 157.2, 154.0, 150.6, 141.1, 136.6, 133.4, 131.9, 131.7, 130.6, 130.0, 128.4, 120.1, 115.3, 115.2, 87.8, 84.5, 83.7, 81.3, 71.9, 71.7, 71.6, 71.5, 71.3, 71.1, 70.8, 70.6, 68.93, 68.88, 67.6, 58.3, 39.5, 38.6, 27.8, 27.0, 20.1. HRMS (ESI+): $[M+H]^+$ calcd for $C_{48}H_{59}N_8O_{14}S$, 1003.3871; found, 1003.3871.

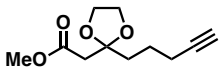
Synthesis of probe 8

Methyl 3-oxooct-7-ynoate (42)



Oxalyl chloride (4.8 mL, 55.5 mmol) and DMF (144 μL) were added to a solution of 5-hexynoic acid (4.0 mL, 37.0 mmol) in CH_2Cl_2 (90 mL) at 0 ºC. After 1 h at room temperature, the flask was placed on the rotary evaporator and the DMF and CH_2Cl_2 were removed at reduced pressure to afford 5-hexynoyl chloride as a red oil. To a solution of Meldrum′s acid (3.55 g, 24.7 mmol) in CH_2Cl_2 (50 mL) was added pyridine (5.0 mL, 61.8 mmol) at 0 ºC. The solution was stirred at 0 ºC for 15 min and a solution of 5-hexynoyl chloride **21** in CH_2Cl_2 (10 mL) was added. The solution was stirred at room temperature for 2 h. The reaction mixture was washed with 1 M aqueous HCl, H_2O, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was dissolved in MeOH (20 mL) at room temperature and the solution was refluxed for 3 h. The reaction mixture was evaporated at reduced pressure. The residue was purified by flash chromatography (9:1 hexane/EtOAc) to afford compound **42** as a colorless oil (3.0 g, 72%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.72 (s, 3H), 3.46 (s, 2H), 2.69 (t, J = 6.9 Hz, 2H), 2.22 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 1.95 (t, J = 2.9 Hz, 1H), 1.83−1.76 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 202.1, 167.6, 83.4, 69.3, 52.5, 49.2, 41.4, 22.0, 17.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_9H_{12}O_3Na$, 191.0684 ; found, 191.0861.

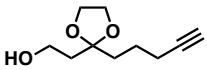
Methyl 2-(2-(pent-4-yn-1-yl)-1,3-dioxolan-2-yl)acetate (43)



$TsOH \cdot H_2O$ (3.4 g, 17.8 mmol) and $HC(OMe)_3$ (5.8 mL, 53.4 mmol) were added to a solution of compound **42** (3.0 g, 17.8 mmol) in ethylene glycol (3.0 mL, 53.4 mmol). The solution was stirred for 3 h at room temperature. The reaction mixture was diluted with sat. $NaHCO_3$ and extracted with Et_2O. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (8:1 hexane/EtOAc) to afford compound **43** as a colorless oil (3.3 g, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ

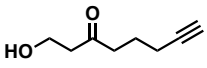
4.01−3.93 (m, 4H), 3.67 (s, 3H), 2.64 (s, 2H), 2.20 (ddd, J = 7.5, 7.5, 2.9 Hz, 2H), 1.95−1.89 (m, 3H), 1.66−1.59 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.0, 109.1, 84.2, 68.6, 65.3, 51.9, 42.7, 36.7, 22.6, 18.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{11}H_{17}O_4$, 213.1127; found, 213.1124.

2-(2-(Pent-4-yn-1-yl)-1,3-dioxolan-2-yl)ethan-1-ol (44)



Lithium aluminum hydride (806 mg, 21.2 mmol) was added to a solution of compound **43** (3.0 g, 14.1 mmol) in THF (40 mL) at 0 ºC. The solution was stirred for 30 min at room temperature. A saturated solution of Rochelle salt was slowly added. The resulting mixture was vigorously stirred at room temperature for 18 h. The solution was diluted with EtOAc and washed with H_2O and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (2:1 hexane/EtOAc) to afford compound **44** as a colorless oil (2.5 g, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.03−3.96 (m, 4H), 3.74 (t, J = 5.2 Hz, 2H), 2.53 (br, 1H), 2.20 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 1.95 (t, J = 2.9 Hz, 1H), 1.92 (t, J = 5.7 Hz, 2H), 1.79−1.74 (m, 2H), 1.63−1.56 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 112.0, 84.1, 68.8, 64.9, 58.9, 38.3, 36.0, 22.9, 18.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{10}H_{16}O_3Na$, 207.0997; found, 207.0992.

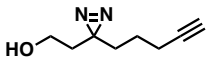
1-Hydroxyoct-7-yn-3-one (45)



TsOH·H_2O (640 mg, 3.4 mmol) was added to a solution of compound **44** (2.5 g, 13.6 mmol) in acetone (30 mL). The solution was stirred at room temperature for 20 min. The reaction mixture was diluted with sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (3:2 hexane/EtOAc) to afford compound **45** as a colorless oil (1.8 g, 94%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.85 (t, J = 5.2 Hz, 2H), 2.69 (t, J = 5.2 Hz, 2H), 2.60 (t, J = 7.5 Hz, 2H), 2.23 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 1.97 (t, J = 2.9 Hz, 1H), 1.84−1.77 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 211.1, 83.5, 69.4, 58.0, 44.6, 41.7, 22.1, 17.8. HRMS

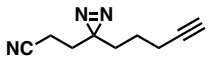
(ESI+): $[M+Na]^+$ calcd for $C_8H_{12}O_2Na$, 163.0735; found, 163.0733.

2-(3-(Pent-4-yn-1-yl)-3*H*-diazirin-3-yl)ethan-1-ol (46)



Anhydrous ammonia (10 mL) was condensed into a three-necked round-bottomed flask containing compound **45** (1.1 g, 7.85 mmol) at –78 ºC. The mixture was stirred at –40 ºC for 5 h. The solution was cooled down to –78 ºC in a dry ice/acetone bath, and a solution of hydroxylamine-*O*-sulfonic acid (1.77 g, 15.7 mmol) in anhydrous MeOH (10 mL) was added over a period of 30 min. The resulting mixture was stirred at –40 ºC for 1 h. The remaining ammonia was evaporated at room temperature. The resulting slurry was filtered and the filter cake was washed with MeOH. The filtrate was evaporated at reduced pressure. Et_3N (1.5 mL) was added to a solution of the diaziridine in CH_2Cl_2 (5 mL). A solution of I_2 (3.0 g, 11.8 mmol) in CH_2Cl_2 (5 mL) was slowly added until the appearance of a persistent orange-brown coloration. The solvent was removed at reduce pressure. The residue was purified by flash chromatography (5:1 to 3:1 hexane/EtOAc) to afford compound **46** as a colorless oil (388 mg, 32%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.47 (t, *J* = 6.3 Hz, 2H), 2.17 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.95 (t, *J* = 2.9 Hz, 1H), 1.67 (t, *J* = 6.3 Hz, 2H), 1.59−1.55 (m, 2H), 1.38−1.31 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 83.5, 69.1, 57.6, 35.7, 32.2, 26.9, 22.8, 18.0. HRMS (ESI+): $[M+H]^+$ calcd for $C_8H_{13}N_2O$, 153.1028; found, 153.1025.

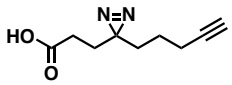
3-(3-(Pent-4-yn-1-yl)-3*H*-diazirin-3-yl)propanenitrile (48)



To a solution of imidazole (135mg, 1.98 mmol) and PPh_3 (191 mg, 0.73 mmol) in CH_2Cl_2 (4 mL) were added I_2 (201 mg, 0.79 mmol) at 0 ºC. The solution was stirred at 0 ºC for 5 min and a solution of compound **46** (100 mg, 0.66 mmol) in CH_2Cl_2 (2 mL) was added. The mixture was stirred at room temperature for 3 h. The reaction mixture was diluted with sat. $Na_2S_2O_3$ and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (30:1 hexane/EtOAc) to afford compound **47** as a colorless oil (163 mg, 94%). ^{1}H NMR (500 MHz,

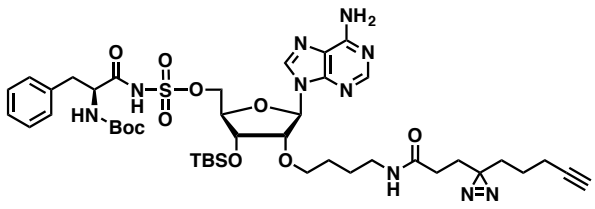
$CDCl_3$) δ 2.88 (t, *J* = 7.5 Hz, 2H), 2.17 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 2.06 (t, *J* = 7.5 Hz, 2H), 1.96 (t, *J* = 2.9 Hz, 1H), 1.60−1.55 (m, 2H), 1.36−1.29 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 83.3, 69.3, 37.8, 31.3, 28.9, 22.7, 18.0, −3.7. KCN (50 mg, 0.76 mmol) was added to a solution of compound **47** (100 mg, 0.38 mmol) at room temperature. The solution was stirred at 70 ºC for 2 h. The reaction mixture was diluted with H_2O and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness. The residue was purified by flash chromatography (5:1 hexane/EtOAc) to afford compound **48** as a colorless oil (52 mg, 85%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.20−2.14 (m, 4H), 1.97 (t, *J* = 2.9 Hz, 1H), 1.79 (t, *J* = 7.5 Hz, 2H), 1.65−1.59 (m, 2H), 1.36−1.29 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 118.5, 83.0, 69.5, 31.1, 29.7, 27.2, 22.6, 17.9, 12.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_9H_{12}N_3$, 184.0851; found, 184.0852.

3-(3-(Pent-4-yn-1-yl)-3*H*-diazirin-3-yl)propanoic acid (49)



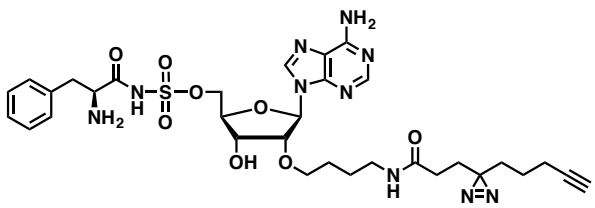
Compound **48** (50 mg, 0.31 mmol) was dissolved in 10% aqueous NaOH (5 mL). The solution was refluxed for 10 h. The reaction mixture was washed with EtOAc. The aqueous phase was acidified with 6 M aqueous HCl and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **49** as a colorless oil (52 mg, 93%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.19−2.14 (m, 4H), 1.95 (t, *J* = 2.9 Hz, 1H), 1.79−1.74 (m, 2H), 1.57−1.52 (m, 2H), 1.37−1.29 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 178.5, 83.3, 69.2, 31.6, 28.4, 28.0, 27.8, 22.7, 18.0. HRMS (ESI+): $[M+H]^+$ calcd for $C_9H_{11}N_2O_2$, 179.0821 ; found, 179.0831.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(3-(3-(pent-4-yn-1-yl)-3*H*-diazirin-3-yl)propanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (51)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (13.8 mg, 0.072 mmol) and *N*-hydroxysuccinimide (8.3 mg, 0.072 mmol) were added to a solution of **49** (9.0 mg, 0.048 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 12 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 to afford compound **50** as a white solid (13 mg). Compound **50** and cesium carbonate (31 mg, 0.096 mmol) were added to a solution of compound **11** (25 mg, 0.032 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The solvent was removed at reduced pressure. The residue was diluted with EtOAc. The resulting 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 (10:1 to 8:1 $CHCl_3$/MeOH) to afford compound **51** as a white solid (18 mg, 60%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.21 (s, 1H), 7.24−7.18 (m, 4H), 7.17−7.10 (m, 1H), 6.15 (d, *J* = 5.7 Hz, 1H), 4.63−4.59 (m, 1H), 4.56−4.52 (m, 1H), 4.38−4.31(m, 1H), 4.29−4.22 (m, 3H), 3.59−3.54 (m, 1H), 3.49−3.43 (m, 1H), 3.17 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.08−3.02 (m, 2H), 2.87 (dd, *J* = 13.8, 8.0 Hz, 1H), 2.21 (t, *J* = 2.9 Hz, 1H), 2.13 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.96 (t, *J* = 7.5 Hz, 2H), 1.66 (t, *J* = 7.5 Hz, 2H), 1.54−1.38 (m, 6H), 1.36−1.22 (m, 11H), 0.95 (s, 9H), 0.17 (s, 3H), 0.16 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 174.3, 174.2, 157.5, 157.3, 154.0, 150.7, 141.3, 139.0, 130.6, 129.2, 127.4, 120.3, 87.7, 85.2, 84.1, 83.0, 80.3, 72.6, 71.5, 70.2, 69.6, 59.3, 40.2, 40.1, 39.9, 32.5, 31.2, 30.0, 29.0, 28.8, 28.2, 26.9, 26.3, 24.0, 19.0, 18.5, −4.3, −4.5. HRMS (ESI–): $[M–H]^-$ calcd for $C_{43}H_{63}N_{10}O_{10}SSi$, 939.4209; found, 939.4214.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-hydroxy-4-(4-(3-(3-(pent-4-yn-1-yl)-3*H*-diazirin-3-yl)propanamido)butoxy)tetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (8)

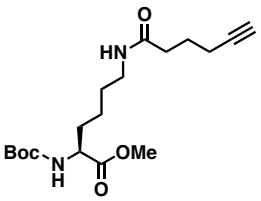


Compound **51** (18 mg, 0.019 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 9 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (5:1 to 4:1 $CHCl_3$/MeOH) to afford compound **8** as a white solid (13 mg, 94%). ^{1}H NMR

(500 MHz, CD_3OD) δ 8.59 (s, 1H), 8.27 (s, 1H), 7.31−7.26 (m, 4H), 7.22−7.17 (m, 1H), 6.19 (d, *J* = 5.2 Hz, 1H), 4.46 (t, *J* = 4.6 Hz, 1H), 4.42 (t, *J* = 4.6 Hz, 1H), 4.39−4.35 (m, 1H), 4.33−4.28 (m, 2H), 3.93 (dd, *J* = 8.0, 5.2 Hz, 1H), 3.74−3.68 (m, 1H), 3.65−3.59 (m, 1H), 3.35−3.32 (m, 1H), 3.14−3.09 (m, 2H), 3.06 (dd, *J* = 14.3, 8.0 Hz, 1H), 2.22 (t, *J* = 2.9 Hz, 1H), 2.13 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.97 (t, *J* = 7.5 Hz, 2H), 1.67 (t, *J* = 7.5 Hz, 2H), 1.62−1.46 (m, 6H), 1.32−1.25 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD) δ 174.9, 174.3, 155.3, 151.1, 150.3, 142.0, 136.4, 130.6, 130.0, 128.5, 120.1, 88.1, 84.6, 84.1, 83.7, 71.6, 70.7, 70.2, 68.9, 58.2, 40.0, 38.5, 32.5, 31.1, 30.0, 29.0, 27.9, 26.9, 24.0, 18.5. HRMS (ESI–): $[M–H]^-$ calcd for $C_{32}H_{41}N_{10}O_8S$, 725.2830; found, 725.2826.

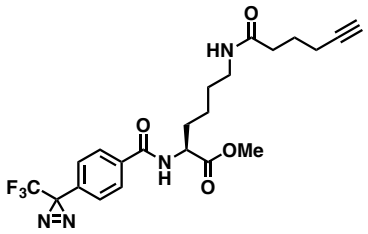
Synthesis of probe 9

Methyl N^2-(*tert*-butoxycarbonyl)-N^6-(hex-5-ynoyl)-*L*-lysinate (52)



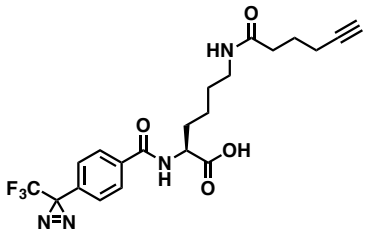
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (176 mg, 0.92 mmol) and 1-hydroxybenzotriazole (127 mg, 0.92 mmol) were added to a solution of 5-hexynoic acid (125 μL, 1.16 mmol) and DIEA (268 μL, 1.54 mmol) in CH_2Cl_2 (8 mL). The solution was stirred at room temperature for 5 min and Boc-Lys-OMe (201 mg, 0.77 mmol) was added. After 4 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 (40:1 $CHCl_3$/MeOH) to afford compound **52** as a white solid (240 mg, 87%). ^1H NMR (500 MHz, $CDCl_3$) δ 6.06 (br, 1H), 5.18 (d, *J* = 8.0 Hz, 1H), 4.13 (dd, *J* = 12.6, 7.5 Hz, 1H), 3.67 (s, 3H), 3.17 (q, *J* = 6.3 Hz, 2H), 2.24 (t, *J* = 7.5 Hz, 2H), 2.18 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.93 (t, *J* = 2.9 Hz, 1H), 1.83−1.68 (m, 3H), 1.64−1.54 (m, 1H), 1.52−1.43 (m, 2H), 1.37 (s, 9H), 1.34−1.27 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.3, 172.5, 155.6, 83.6, 80.0, 69.3, 53.2, 52.4, 39.2, 35.1, 32.5, 29.1, 28.4, 24.2, 22.6, 17.9. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{18}H_{30}N_2O_5Na$, 377.2047; found, 377.2045.

Methyl N^6-(hex-5-ynoyl)-N^2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoyl)-*L*-lysinate (54)



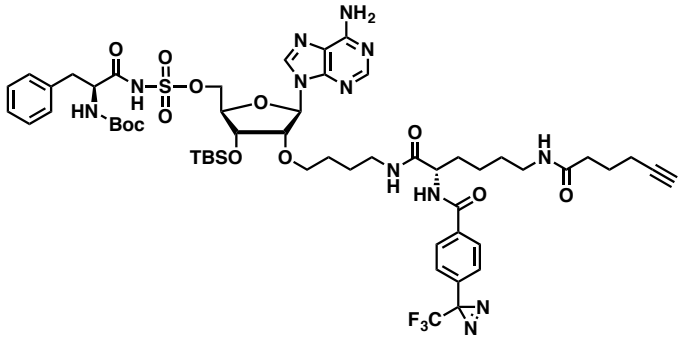
Compound **52** (107 mg, 0.3 mmol) was dissolved in a 1:1 (v/v) mixture of TFA and CH_2Cl_2 at room temperature. After 2 h, the flask was placed on the rotary evaporator and the TFA and CH_2Cl_2 were removed at reduced pressure to afford TFA salt as a white solid (122 mg, quant.). This material can be used in the next step without further purification. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (72.8 mg, 0.38 mmol) and 1-hydroxybenzotriazole (52.4 mg, 0.38 mmol) were added to a solution of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl] benzoic acid **53** (87 mg, 0.38 mmol) and DIEA (111 μL, 0.64 mmol) in CH_2Cl_2 (3 mL). The solution was stirred at room temperature for 5 min and the amine (122 mg, 0.3 mmol) was added. After 18 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 (1:2 hexane/EtOAc) to afford compound **54** as a white solid (127 mg, 90%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.89 (d, J =8.0 Hz, 2H), 7.29 (d, 6.9 Hz, 1H), 7.20 (d, *J* = 8.0 Hz, 2H), 6.11 (br, 1H), 4.70−4.63 (m, 1H), 3.73 (s, 3H), 3.29−3.22 (m, 1H), 3.21−3.14 (m, 1H), 2.29−2.19 (m, 2H), 2.14 (ddd, *J* = 6.9, 6.9, 2.3 Hz, 2H), 1.96−1.80 (m, 3H), 1.78−1.70 (m, 2H), 1.57−1.46 (m, 2H), 1.45−1.30 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.1, 172.9, 166.4, 134.8, 132.6, 127.9, 126.6, 123.0, 120.9, 83.4, 69.3, 52.8, 52.6, 38.5, 35.1, 31.3, 29.1, 24.3, 22.5, 17.9. HRMS (ESI+): $[M+H]^+$ calcd for $C_{22}H_{26}F_3N_4O_4$, 467.1906; found, 467.1908.

N^6-(Hex-5-ynoyl)-N^2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoyl)-*L*-lysine (55)



To a solution of **54** (72.8 mg, 0.16 mmol) in MeOH (1.5 mL) was added 234 μL of a 1 M aqueous LiOH solution at 0 °C. The mixture was stirred at room temperature for 24 h. The flask was then placed on a rotary evaporator and the MeOH were removed at reduced pressure. The residue was diluted with H_2O and washed with EtOAc. The aqueous layer was acidified with citric acid monohydrate and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **55** as a white solid (66 mg, 92%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.85 (d, J = 8.0 Hz, 1H), 7.76 (d, J = 8.0 Hz, 2H), 7.03 (d, J = 8.0 Hz, 2H), 6.25 (br, 1H), 4.86−4.82 (m, 1H), 3.46 (br, 1H), 3.19−3.10 (m, 1H), 2.31 (t, J = 7.5 Hz, 2H), 2.18−2.13 (m, 2H), 1.95 (t, J = 2.3, 1H), 1.94−1.85 (m, 2H), 1.80−1.72 (m, 2H), 1.58−1.40 (m, 3H), 1.39−1.28 (m, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.1, 174.5, 166.2, 134.2, 132.4, 127.7, 126.4, 125.2, 123.0, 120.8, 118.7, 83.1, 69.7, 52.6, 38.7, 35.2, 31.1, 29.1, 24.2, 22.2, 17.8. HRMS (ESI+): $[M+H]^+$ calcd for $C_{21}H_{24}F_3N_4O_4$, 453.1744; found, 453.1744.

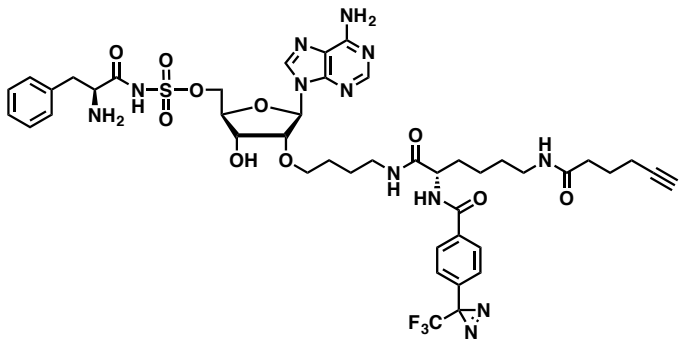
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-((*S*)-6-(hex-5-ynamido)-2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzamido)hexanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (57)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (13.8 mg, 0.072 mmol) and

N-hydroxysuccinimide (8.3 mg, 0.072 mmol) were added to a solution of **55** (22 mg, 0.048 mmol) in CH_2Cl_2 (1 mL). The solution was stirred at room temperature for 12 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 to afford compound **56** as a white solid (27 mg). Compound **56** and cesium carbonate (31 mg, 0.096 mmol) were added to a solution of compound **11** (25 mg, 0.032 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The solvent was removed at reduced pressure. The residue was diluted with EtOAc. The resulting 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 (15:1 to 10:1 $CHCl_3$/MeOH) to afford compound **57** as a white solid (30 mg, 77%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.20 (s, 1H), 8.07 (dd, *J* = 5.7, 5.2 Hz, 1H), 7.98 (dd, *J* = 5.7, 5.2 Hz, 1H), 7.95 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.23−7.16 (m, 4H), 7.15−7.09 (m, 1H), 6.14 (d, *J* = 5.7 Hz, 1H), 4.63−4.58 (m, 1H), 4.54 (dd, *J* = 9.7, 4.6 Hz, 1H), 4.45 (dd, *J* = 8.6, 5.2 Hz, 1H), 4.39−4.31 (m, 1H), 4.30−4.19 (m, 3H), 3.59−3.53 (m, 1H), 3.51−3.43 (m, 1H), 3.22−3.05 (m, 5H), 2.86 (dd, *J* = 13.2, 8.6 Hz, 1H), 2.29−2.22 (m, 3H), 2.15 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.90−1.70 (m, 4H), 1.59−1.36 (m, 8H), 1.33 (s, 9H), 0.93 (s, 9H), 0.15 (s, 3H), 0.13 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 180.4, 175.3, 174.2, 168.8, 157.5, 157.3, 154.0, 150.7, 141.3, 139.0, 136.7, 133.2, 130.6, 129.4, 129.2, 127.6, 127.4, 124.5, 122.3, 120.3, 87.7, 85.1, 84.1, 83.0, 80.3, 72.7, 71.5, 70.3, 69.6, 59.4, 55.5, 40.1, 40.0, 39.9, 35.9, 32.7, 30.0, 28.7, 28.1, 26.9, 26.3, 26.0, 24.4, 19.0, 18.6, −4.3, −4.5. HRMS (ESI–): $[M–H]^-$ calcd for $C_{55}H_{74}F_3N_{12}O_{12}SSi$, 1211.4991; found, 1211.4997.

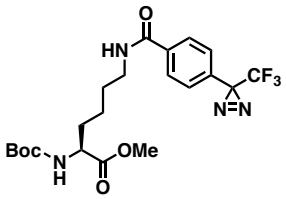
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-((*S*)-6-(hex-5-ynamido)-2-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzamido)hexanamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (9)



Compound **57** (28 mg, 0.023 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 5 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (5:1 to 4:1 $CHCl_3$/MeOH) to afford compound **9** as a white solid (22 mg, 96%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.56 (s, 1H), 8.29 (s, 1H), 7.95 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 8.0 Hz, 2H), 7.31−7.26 (m, 4H), 7.21−7.16 (m, 1H), 6.18 (dd, *J* = 4.6, 4.0 Hz, 1H), 4.49−4.43 (m, 2H), 4.42−4.35 (m, 2H), 4.34−4.27 (m, 2H), 3.94 (dd, *J* = 8.0, 5.2 Hz, 1H), 3.74−3.67 (m, 1H), 3.67−3.59 (m, 1H), 3.33−3.30 (m, 1H, overlapping with MeOH), 3.21−3.14 (m, 4H), 3.10−3.13 (m, 1H), 2.29−2.22 (m, 3H), 2.16 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.90−1.70 (m, 4H), 1.67−1.35 (m, 8H). ^{13}C NMR (125 MHz, CD_3OD) δ 175.4, 174.9, 174.3, 168.9, 154.9, 150.2, 142.2, 136.7, 136.4, 133.2, 130.6, 130.0, 129.4, 128.5, 127.6, 124.5, 122.3, 120.1, 119.3, 116.9, 88.2, 84.5, 84.1, 83.7, 71.6, 70.7, 70.3, 68.9, 58.2, 55.7, 40.0, 39.9, 38.5, 35.9, 32.6, 30.0, 27.8, 26.9, 26.0, 24.4, 18.6. HRMS (ESI–): $[M–H]^-$ calcd for $C_{44}H_{52}F_3N_{12}O_{10}S$, 997.3602; found, 997.3599.

Synthesis of probe 10

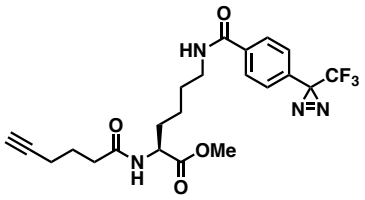
Methyl *N*^{2}-(*tert*-butoxycarbonyl)-*N*^{6}-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoyl)-*L*-lysinate (58)



1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (119 mg, 0.62 mmol) and 1-hydroxybenzotriazole (85.6 mg, 0.62 mmol) were added to a solution of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl] benzoic acid **53** (180 mg, 0.78 mmol), and DIEA (181 μL, 1.04 mmol) in CH_2Cl_2 (5 mL). The solution was stirred at room temperature for 5 min and Boc-Lys-OMe (136 mg, 0.52 mmol) was added. After 12 h, the reaction mixture was diluted with EtOAc. The resulting 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 (2:1 hexane/EtOAc) to afford compound **58** as a white solid (175 mg, 71%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.79 (d, *J* = 8.3 Hz, 2H), 7.19 (d, *J* = 8.0 Hz,

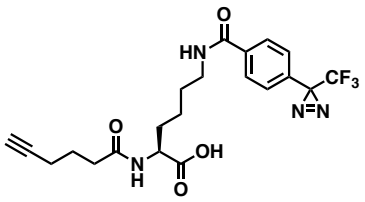
2H), 6.68 (br, 1H), 5.19 (d, J = 8.0 Hz, 1H), 4.29−4.23 (m, 1H), 3.70 (s, 3H), 3.42 (q, J = 6.3 Hz, 2H), 1.84−1.77 (m, 1H), 1.69−1.59 (m, 3H), 1.46−1.34 (m, 11H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 173.3, 166.7, 155.7, 135.8, 132.2, 127.6, 126.6, 125.3, 123.1, 120.9, 118.7, 80.1, 53.1, 52.4, 39.8, 32.6, 28.8, 28.3, 22.7. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{21}H_{27}F_3N_4O_5Na$, 495.1831; found, 495.1830.

Methyl N^2-(hex-5-ynoyl)-N^6-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoyl)-*L*-lysinate (59)



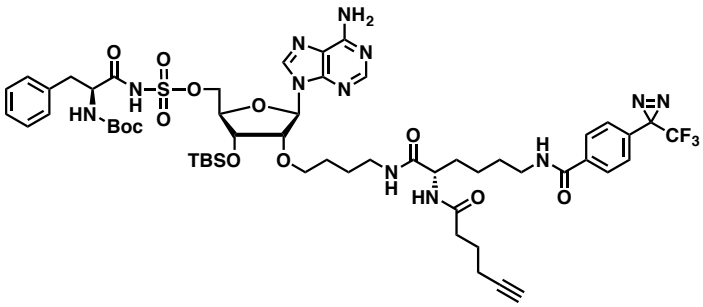
Compound **58** (110 mg, 0.23 mmol) was dissolved in a 1:1 (v/v) mixture of TFA and CH_2Cl_2 at room temperature. After 1 h, the flask was placed on the rotary evaporator and the TFA and CH_2Cl_2 were removed at reduced pressure to afford TFA salt as a white solid (80 mg, quant.). This material can be used in the next step without further purification. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (53 mg, 0.28 mmol) and 1-hydroxybenzotriazole (38 mg, 0.28 mmol) were added to a solution of 5-hexynoic acid (30 μL, 0.28 mmol) and DIEA (80 μL, 0.46 mmol) in CH_2Cl_2 (2 mL). The solution was stirred at room temperature for 5 min and the amine (80 mg, 0.23 mmol) was added. After 15 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 (1:2 hexane/EtOAc) to afford compound **59** as a white solid (93 mg, 86%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.83 (d, J = 8.6 Hz, 2H), 7.17 (d, J = 8.6 Hz, 2H), 7.00 (dd, J = 5.7, 5.2 Hz, 1H), 6.49 (d, J = 8.0 Hz, 1H), 4.57−4.49 (m, 1H), 3.69 (s, 3H), 3.45−3.33 (m, 2H), 2.36−2.25 (m, 2H), 2.15 (ddd, J = 6.9, 6.9, 2.9 Hz, 2H), 1.94 (t, J = 2.9 Hz, 1H), 1.88−1.54 (m, 6H), 1.42−1.33 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 172.9, 172.7, 166.7, 135.6, 132.2, 127.6, 126.5, 123.0, 120.9, 83.3, 69.4, 52.5, 51.7, 39.5, 34.8, 32.0, 28.5, 24.1, 22.5, 17.8. HRMS (ESI+): $[M+H]^+$ calcd for $C_{22}H_{26}F_3N_4O_4$, 467.1901; found, 467.1901.

N^2-(Hex-5-ynoyl)-N^6-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzoyl)-*L*-lysine (60)



To a solution of **59** (85 mg, 0.18 mmol) in MeOH (1.5 mL) was added 270 μL of a 1 M aqueous LiOH solution at 0 °C. The mixture was stirred at room temperature for 18 h. The flask was then placed on a rotary evaporator and the MeOH were removed at reduced pressure. The residue was diluted with H_2O and washed with EtOAc. The aqueous layer was acidified with citric acid monohydrate and extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated to dryness to afford compound **60** as a white solid (81 mg, 100%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.80 (d, J = 8.0 Hz, 2H), 7.42 (br, 1H), 7.15 (d, J = 8.0 Hz, 2H), 7.02 (d, J = 6.9 Hz, 1H), 4.52−4.45 (m, 1H), 3.46−3.30 (m, 2H), 2.32 (t, J = 6.9 Hz, 2H), 2.13 (ddd, J = 6.9, 6.9, 2.3 Hz, 2H), 1.95 (t, J = 2.3 Hz, 1H), 1.90−1.81 (m, 1H), 1.78−1.69 (m, 3H), 1.67−1.52 (m, 2H), 1.45−1.32 (m, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 174.6, 173.4, 167.4, 135.2, 132.5, 127.8, 126.5, 125.2, 123.0, 120.9, 118.7, 83.3, 69.6, 52.3, 39.8, 34.9, 31.5, 28.7, 24.3, 22.6, 17.9. HRMS (ESI+): $[M+H]^+$ calcd for $C_{21}H_{24}F_3N_4O_4$, 453.1750; found, 453.1752.

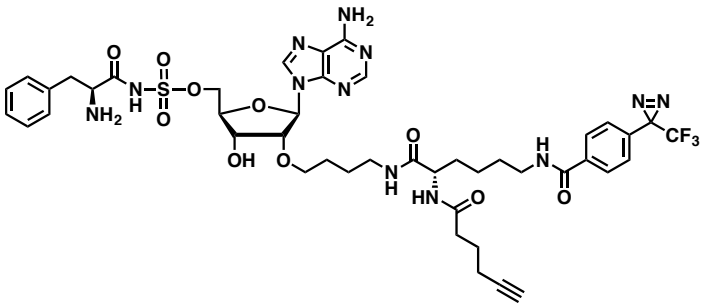
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-((*S*)-2-(hex-5-ynamido)-6-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzamido)hexanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-phenylalanyl)sulfamate (62)



1-Ethyl-3-(3-dimethylamonopropyl) carbodiimide hydrochloride (13.8 mg, 0.072 mmol) and *N*-hydroxysuccinimide (8.3 mg, 0.072 mmol) were added to a solution of **60** (22 mg, 0.048 mmol) in CH_2Cl_2 (1 mL). After 12 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 to afford compound **61** as a white solid (28 mg). Compound **61** and cesium carbonate (31 mg, 0.096 mmol) were added to a solution of compound **11** (25 mg, 0.032 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The solvent was removed at reduced pressure. The residue was diluted with EtOAc. The resulting 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 (15:1 to 10:1 $CHCl_3$/MeOH) to afford compound **62** as a white solid (35 mg, 90%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.47 (s, 1H), 8.20 (s, 1H), 7.93−7.86 (m, 3H), 7.32 (d, *J* = 8.0 Hz, 2H), 7.26−7.18 (m, 4H), 7.16−7.10 (m, 1H), 6.15 (d, *J* = 5.7 Hz, 1H), 4.64−4.60 (m, 1H), 4.54 (dd, *J* = 5.7, 4.6 Hz, 1H), 4.40−4.32 (m, 1H), 4.30−4.20 (m, 4H), 3.58−3.53 (m, 1H), 3.49−3.43 (m, 1H), 3.40−3.34 (m, 2H), 3.16 (dd, *J* = 13.8, 5.2 Hz, 1H), 3.10−3.34 (m, 2H), 2.87 (dd, *J* = 13.8, 8.6 Hz, 1H), 2.33 (t, *J* = 7.5 Hz, 2H), 2.23 (t, *J* = 2.9 Hz, 1H), 2.16 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.81−1.71 (m, 3H), 1.70−1.58 (m, 3H), 1.52−1.38 (m, 6H), 1.33 (s, 9H), 0.94 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 180.3, 175.4, 174.4, 168.7, 157.5, 157.3, 153.9, 150.7, 141.3, 139.0, 137.3, 133.0, 130.6, 129.2, 129.0, 127.6, 127.4, 120.3, 87.7, 85.1, 84.2, 82.9, 80.3, 72.6, 71.4, 70.3, 69.7, 59.3, 54.8, 40.7, 40.1, 39.9, 35.6, 32.9, 30.0, 28.8, 28.1, 26.9, 26.3, 25.8, 24.3, 19.0, 18.6, −4.3, −4.5. HRMS (ESI–): $[M–H]^-$ calcd for $C_{55}H_{74}F_3N_{12}O_{12}SSi$, 1211.4991; found, 1211.4989.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-((*S*)-2-(hex-5-ynamido)-6-(4-(3-(trifluoromethyl)-3*H*-diazirin-3-yl)benzamido)hexanamido)butoxy)-3-hydroxytetrahydrofuran-2-yl)methyl (*L*-phenylalanyl)sulfamate (10)

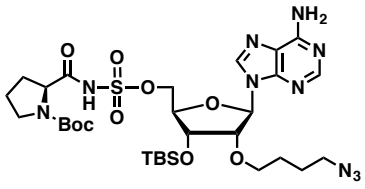


Compound **62** (33 mg, 0.027 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 5 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography

(5:1 to 4:1 $CHCl_3$/MeOH) to afford compound **10** as a white solid (26 mg, 96%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.50 (s, 1H), 8.20 (s, 1H), 7.89 (d, *J* = 8.6 Hz, 2H), 7.32 (d, *J* = 8.0 Hz, 2H), 7.30−7.24 (m, 4H), 7.18−7.14 (m, 1H), 6.16 (d, *J* = 4.6 Hz, 1H), 4.46 (t, *J* = 4.6 Hz, 1H), 4.41 (dd, *J* = 5.2, 4.6 Hz, 1H), 4.38 (dd, *J* = 10.9, 2.3 Hz, 1H), 4.34−4.27 (m, 2H), 4.23 (dd, *J* = 5.7, 5.2 Hz, 1H), 3.90 (dd, *J* = 8.0, 4.6 Hz, 1H), 3.70−3.65 (m, 1H), 3.64−3.58 (m, 1H), 3.37 (dd, *J* = 7.5, 6.9 Hz, 2H), 3.30−3.27 (m, 1H, overlapping with MeOH), 3.20−3.09 (m, 2H), 3.08−3.01 (m, 1H), 2.34 (t, *J* = 7.5 Hz, 2H), 2.24 (t, *J* = 2.9 Hz, 1H), 2.16 (ddd, *J* = 6.9, 6.9, 2.9 Hz, 2H), 1.83−1.71 (m, 3H), 1.71−1.35 (m, 9H). ^{13}C NMR (125 MHz, CD_3OD) δ 175.7, 175.4, 174.4, 168.8, 157.2, 154.0, 150.6, 141.1, 137.2, 136.6, 133.0, 130.6, 130.0, 129.0, 128.4, 127.6, 124.5, 122.3, 120.1, 119.2, 116.9, 87.8, 84.3, 84.1, 83.5, 71.6, 70.8, 70.3, 69.0, 58.3, 54.9, 40.7, 39.9, 38.7, 35.5, 32.7, 30.0, 27.8, 26.9, 25.8, 24.3, 18.6. HRMS (ESI–): $[M–H]^-$ calcd for $C_{44}H_{52}F_3N_{12}O_{10}S$, 997.3602; found, 997.3599.

Syntheses of L-Pro-AMS-BPyne and L-Orn-AMS-BPyne

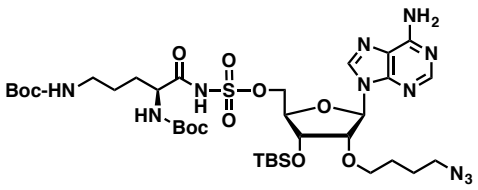
***tert*-Butyl (*S*)-2-(((((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methoxy)sulfonyl)carbamoyl)pyrrolidine-1-carboxylate (63a)**



Boc-Pro-OSu (52 mg, 0.17 mmol) and cesium carbonate (108 mg, 0.33 mmol) were added to a solution of compound **16** (60 mg, 0.11 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was then filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (10:1 to 5:1 $CHCl_3$/MeOH) to afford compound **63a** as a white solid (61 mg, 73%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.46 (s, 1H), 8.22 (s, 1H), 6.15 (d, *J* = 6.3 Hz, 1H), 4.67–4.60 (m, 1H), 4.58–4.50 (m, 1H), 4.47–4.41 (m, 1H), 4.40–4.32 (m, 1H), 4.30–4.25 (m, 1H), 4.21–4.09 (m, 1H), 3.63–3.55 (m, 1H), 3.52–3.42 (m, 2H), 3.41–3.33 (m, 1H), 3.19–3.11 (m, 2H), 2.27–2.14 (m, 1H), 2.02–1.70 (m, 3H), 1.60–1.46 (m, 4H), 1.46–1.39 (m, 9H), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 178.7, 157.2, 156.1,

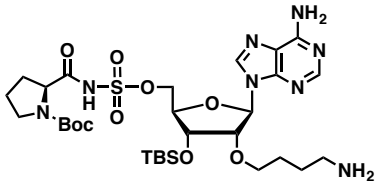
153.7, 150.7, 141.4, 120.3, 87.6, 85.2, 83.1, 81.4, 72.6, 71.3, 70.3, 63.0, 52.1, 47.7, 32.4, 28.7, 28.0, 26.6, 26.3, 24.5, 19.0, –4.4, –4.6. HRMS (ESI–): $[M–H]^-$ calcd for $C_{30}H_{49}N_{10}O_9SSi$, 753.3179; found, 753.3176.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Aamino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*S*)-2,5-bis((*tert*-butoxycarbonyl)amino)pentanoyl)sulfamate (63b)



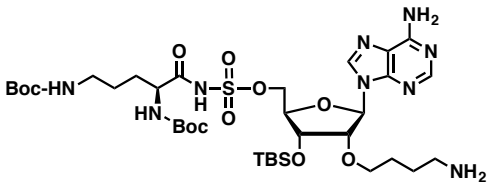
Boc-Orn(Boc)-OSu (73 mg, 0.17 mmol) and cesium carbonate (108 mg, 0.33 mmol) were added to a solution of compound **16** (60 mg, 0.11 mmol) in DMF (1 mL). The solution was stirred at room temperature for 2 h. The reaction mixture was then filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (10:1 to 5:1 $CHCl_3$/MeOH) to afford compound **63b** as a white solid (80 mg, 83%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.49 (s, 1H), 8.23 (s, 1H), 6.16 (d, J = 6.3 Hz, 1H), 4.63–4.58 (m, 1H), 4.57–4.50 (m, 1H), 4.48–4.40 (m, 1H), 4.39–4.32 (m, 1H), 4.32–4.26 (m, 1H), 4.07–3.92 (m, 1H), 3.66–3.57 (m, 1H), 3.51–3.44 (m, 1H), 3.19–3.12 (m, 2H), 3.09–2.99 (m, 2H), 1.87–1.73 (m, 1H), 1.66–1.47 (m, 7H), 1.41 (s, 18H), 0.96 (s, 9H), 0.174 (s, 3H), 0.169 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 177.7, 158.5, 157.7, 157.0, 153.5, 150.6, 141.4, 120.3, 87.5, 85.2, 83.2, 80.4, 79.9, 72.6, 71.3, 70.2, 57.0, 52.1, 40.8, 31.1, 28.8, 28.0, 27.2, 26.6, 26.3, 19.0, –4.4, –4.6. HRMS (ESI+): $[M+H]^+$ calcd for $C_{35}H_{62}N_{11}O_{11}SSi$, 872.4120; found, 872.4112.

***tert*-Butyl**
(*S*)-2-(((((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimet-hylsilyl)oxy)tetrahydrofuran-2-yl)methoxy)sulfonyl)carbamoyl)pyrrolidine-1-carboxylate (64a)



To a solution of **63a** (50 mg, 0.066 mmol) in MeOH (2 mL) was added 10% Pd/C (5 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 24 h. The reaction mixture was filtered through a pad of Celite, which was further washed with MeOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 to 3:1 $CHCl_3$/MeOH) to afford compound **64a** as a white solid (31 mg, 64%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.54 (s, 1H), 8.20 (s, 1H), 6.17 (d, *J* = 6.9 Hz, 1H), 4.67–4.62 (m, 1H), 4.61–4.52 (m, 1H), 4.33–4.23 (m, 3H), 4.18–4.09 (m, 1H), 3.62–3.55 (m, 1H), 3.53–3.42 (m, 2H), 3.41–3.34 (m, 1H), 2.91–2.80 (m, 2H), 2.26–2.12 (m, 1H), 2.02–1.85 (m, 2H), 1.83–1.72 (m, 1H), 1.68–1.50 (m, 4H), 1.42 (s, 9H), 0.97 (s, 9H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 181.6, 157.4, 156.4, 154.0, 150.8, 141.2, 120.1, 87.1, 86.0, 83.4, 81.0, 73.1, 70.8, 69.1, 64.0, 47.7, 40.5, 32.7, 28.8, 27.6, 26.3, 25.4, 24.6, 19.0, –4.4, –4.5. HRMS (ESI–): $[M–H]^-$ calcd for $C_{30}H_{51}N_8O_9SSi$, 727.3274; found, 727.3271.

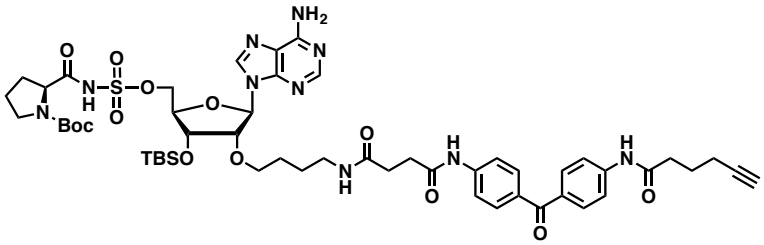
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl
((*S*)-2,5-bis((*tert*-butoxycarbonyl)amino)pentanoyl)sulfamate (64b)



To a solution of **63b** (62 mg, 0.071 mmol) in MeOH (2 mL) was added 10% Pd/C (6 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature

for 24 h. The reaction mixture was filtered through a pad of Celite, which was further washed with MeOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 $CHCl_3$/MeOH) to afford compound **64b** as a white solid (40 mg, 67%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.57 (s, 1H), 8.20 (s, 1H), 6.18 (d, J = 6.3 Hz, 1H), 4.63–4.51 (m, 2H), 4.32–4.20 (m, 3H), 4.05–3.93 (m, 1H), 3.62–3.55 (m, 1H), 3.53–3.45 (m, 1H), 3.07–3.00 (m, 2H), 2.91–2.83 (m, 2H), 1.88–1.79 (m, 1H), 1.69–1.48 (m, 7H), 1.47–1.38 (m, 18H), 0.96 (s, 9H), 0.17 (s, 3H), 0.16 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 180.7, 158.4, 157.6, 157.4, 154.0, 150.8, 141.1, 120.1, 87.0, 86.0, 83.5, 80.1, 79.8, 73.0, 70.7, 69.1, 57.6, 41.0, 40.5, 32.0, 28.8, 27.6, 27.0, 26.3, 25.4, 19.0, –4.3, –4.5. HRMS (ESI+): $[M+H]^+$ calcd for $C_{35}H_{64}N_9O_{11}SSi$, 846.4215; found, 846.4201.

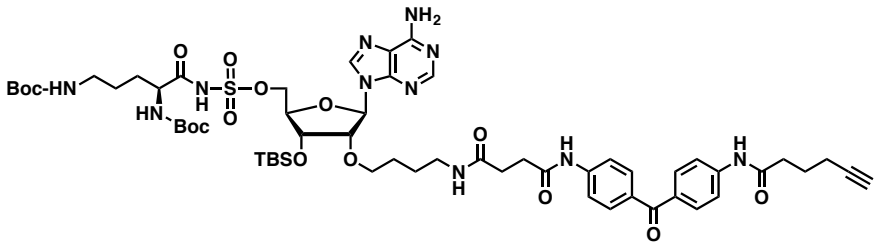
***tert*-Butyl (*S*)-2-(((((2*R*,3*R*,4*R*,5*R*)-5-(6-amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methoxy)sulfonyl)carbamoyl)pyrrolidine-1-carboxylate (65a)**



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (8.0 mg, 0.041 mmol) and 1-hydroxybenzotriazole (6.3 mg, 0.041 mmol) were added to a solution of compound **23** (17 mg, 0.041 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **64a** (25 mg, 0.034 mmol) was then added. After 18 h, the reaction mixture was diluted with EtOAc. The combined organic layer was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (10:1 to 5:1 $CHCl_3$/MeOH) to afford compound **65a** as a white solid (19 mg, 50%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.47−8.38 (m, 1H), 8.20 (s, 1H), 7.76−7.68 (m, 8H), 6.13 (d, J = 5.7 Hz, 1H), 4.66−4.60 (m, 1H), 4.57−4.49 (m, 1H), 4.45−4.39 (m, 1H), 4.38−4.29 (m, 1H), 4.29−4.24 (m, 1H), 4.18−4.09 (m, 1H), 3.59−3.52 (m, 1H), 3.50−3.40 (m, 2H), 3.39−3.34 (m, 1H), 3.11−3.04 (m, 2H), 2.70 (t, J = 6.9 Hz, 2H), 2.59−2.51 (m, 4H), 2.32−2.26 (m, 3H), 2.25−2.12 (m, 1H), 2.04−1.82 (m, 4H), 1.82−1.70 (m, 1H), 1.58−1.38 (m, 13H), 0.94 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz,

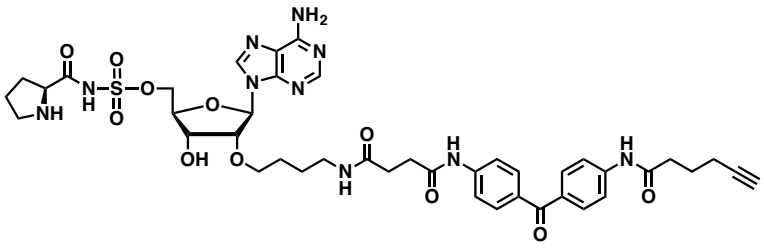
CD_3OD): δ 196.5, 174.4, 174.0, 173.3, 157.2, 156.6, 153.9, 150.7, 144.3, 144.2, 141.4, 134.0, 133.9, 132.3, 132.2, 120.3, 120.0, 119.9, 87.6, 85.4, 84.1, 83.0, 81.2, 72.7, 71.5, 70.3, 69.9, 63.5, 47.7, 40.1, 36.7, 33.2, 31.8, 28.9, 28.7, 28.1, 26.9, 26.3, 25.5, 24.5, 19.0, 18.6, −4.4, −4.6. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI−): $[M-H]^-$ calcd for $C_{53}H_{71}N_{10}O_{13}SSi$, 1115.4698; found, 1115.4696.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*S*)-2,5-bis((*tert*-butoxycarbonyl)amino)pentanoyl)sulfamate (65b)



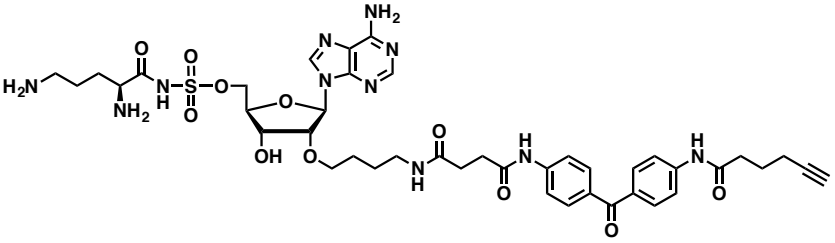
1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (8.0 mg, 0.043 mmol) and 1-hydroxybenzotriazole (6.6 mg, 0.043 mmol) were added to a solution of compound **23** (17 mg, 0.043 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **64b** (30 mg, 0.035 mmol) was added. After 3 h, the reaction mixture was diluted with EtOAc. The mixture was washed with 5% citric acid, 5% $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and evaporated to dryness. The residue was purified by flash chromatography (10:1 to 5:1 $CHCl_3$/MeOH) to afford compound **65b** as a white solid (26 mg, 60%). ^1H NMR (500 MHz, CD_3OD): δ 8.46 (s, 1H), 8.20 (s, 1H), 7.76−7.70 (m, 8H), 6.14 (d, J = 5.7 Hz, 1H), 4.60 (dd, J = 4.6, 2.9 Hz, 1H), 4.51 (dd, J = 5.7, 5.2 Hz, 1H), 4.42−4.36 (m, 1H), 4.33−4.25 (m, 2H), 4.02−3.95 (m, 1H), 3.59−3.53 (m, 1H), 3.49−3.42 (m, 1H), 3.11−3.05 (m, 2H), 3.02 (t, J = 6.9 Hz, 2H), 2.71 (t, J = 6.9 Hz, 2H), 2.58−2.51 (m, 4H), 2.32−2.87 (m, 3H), 1.94−1.86 (m, 2H), 1.86−1.77 (m, 1H), 1.65−1.34 (m, 25H), 0.94 (s, 9H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 196.5, 174.4, 174.0, 173.3, 158.5, 157.8, 157.2, 153.9, 150.7, 144.3, 144.2, 141.2, 134.0, 133.9, 132.3, 132.2, 120.3, 120.0, 119.9, 87.6, 85.2, 84.1, 83.1, 80.3, 79.9, 72.6, 71.5, 70.4, 69.6, 57.7, 41.0, 40.0, 36.7, 33.2, 31.8, 28.8, 28.1, 27.2, 26.9, 26.3, 25.5, 19.0, 18.6, −4.4, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+H]^+$ calcd for $C_{58}H_{84}N_{11}O_{15}SSi$, 1234.5638; found, 1234.5603.

L-Pro-AMS-BPyne



Compound **65a** (19 mg, 0.017 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (3:1 $CHCl_3$/MeOH) to afford L-Pro-AMS-BPyne as a white solid (14 mg, 91%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.50 (s, 1H), 8.19 (s, 1H), 7.78−7.69 (m, 8H), 6.15 (d, J = 5.2 Hz, 1H), 4.49 (t, J = 4.6 Hz, 1H), 4.42 (t, J = 5.2 Hz, 1H), 4.39−4.26 (m, 3H), 4.08 (dd, J = 8.6, 6.9 Hz, 1H), 3.69−3.62 (m, 1H), 3.61−3.54 (m, 1H), 3.39−3.34 (m, 1H), 3.28−3.22 (m, 1H), 3.16-3.10 (m, 2H), 2.70 (t, J = 6.9 Hz, 2H), 2.60–2.51 (m, 4H), 2.37−2.26 (m, 4H), 2.16−2.07 (m, 1H), 1.98−1.86 (m, 4H), 1.64−1.43 (m, 4H). ^{13}C NMR (125 MHz, CD_3OD): δ 196.6, 174.9, 174.5, 174.1, 173.3, 157.3, 154.0, 150.7, 144.3, 144.2, 141.1, 134.0, 132.28, 132.25, 120.2, 120.1, 120.0, 87.7, 84.5, 84.1, 83.6, 71.6, 70.9, 70.4, 63.8, 47.3, 40.0, 36.7, 33.2, 31.8, 30.8, 37.8, 26.9, 25.5, 24.9, 18.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{42}H_{50}N_{10}O_{11}SNa$, 925.3279; found, 925.3251.

L-Orn-AMS-BPyne

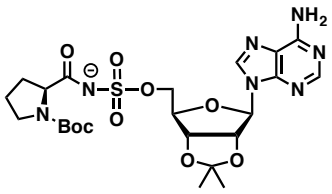


Compound **65b** (20 mg, 0.016 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (4:1 $CHCl_3$/MeOH) to afford L-Orn-AMS-BPyne as a white solid (13 mg, 88%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.65 (s, 1H), 8.38 (s, 1H), 7.74−7.70 (m, 8H), 6.20 (d, J = 4.0 Hz, 1H),

4.50 (t, J = 5.2 Hz, 1H), 4.46−4.41 (m, 1H), 4.41−4.34 (m, 2H), 4.32−4.28 (m, 1H), 3.77−3.73 (m, 1H), 3.72−3.61 (m, 2H), 3.20−3.13 (m, 2H), 3.03−2.94 (m, 2H), 2.72 (t, J = 6.9 Hz, 2H), 2.59−2.52 (m, 4H), 2.32−2.26 (m, 3H), 2.00−1.80 (m, 6H), 1.67−1.58 (m, 2H), 1.58−1.48 (m, 2H). ^{13}C NMR (125 MHz, CD_3OD): δ 196.5, 174.53, 174.47, 174.1, 173.3, 152.3, 149.8, 146.3, 144.24, 144.22, 143.4, 134.0, 133.9, 132.2, 120.2, 120.1, 119.9, 88.6, 84.4, 84.1, 83.8, 71.7, 70.6, 70.4, 69.0, 55.9, 40.04, 39.99, 36.7, 33.1, 31.7, 29.4, 27.7, 27.0, 25.5, 24.3, 18.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{42}H_{53}N_{11}O_{11}SNa$, 942.3544; found, 942.3550.

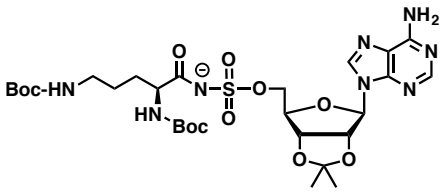
Syntheses of L-Pro-AMS, L-Orn-AMS and L-Leu-AMS

5′-*O*-[*N*-(*N*-Boc-L-prolyl)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (67a)



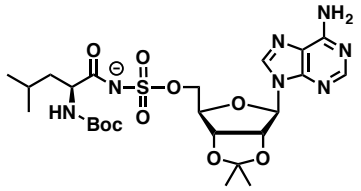
Boc-Pro-OSu (63 mg, 0.20 mmol) and cesium carbonate (127 mg, 0.39 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **66** (50 mg, 0.13 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (91:9:1 to 86:14:1 EtOAc/MeOH/Et_3N) to afford compound **67a** as a white solid (77 mg, 87%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.48−8.42 (m, 1H), 8.21 (s, 1H), 6.23 (d, J = 3.4 Hz, 1H), 5.41−5.35 (m, 1H), 5.17−5.10 (m, 1H), 4.56−4.51 (m, 1H), 4.32−4.17 (m, 2H), 4.16−4.07 (m, 1H), 3.51−3.43 (m, 1H), 3.41−3.32 (m, 1H), 3.19 (q, J = 7.5 Hz, 6H, Et_3N-CH_2), 2.22−2.10 (m, 1H), 1.97−1.85 (m, 2H), 1.82−1.74 (m, 1H), 1.60 (s, 3H), 1.44−1.37 (m, 12H), 1.28 (t, J = 7.5 Hz, 9H, Et_3N-CH_3). ^{13}C NMR (125 MHz, CD_3OD): δ 181.5, 157.3, 156.4, 154.0, 150.5, 141.4, 120.2, 115.3, 91.8, 85.8, 83.3, 81.0, 80.4, 69.5, 63.9, 47.9, 47.7, 32.6, 28.8, 27.5, 25.6, 24.5, 9.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{23}H_{32}N_7O_9S$, 582.1981; found, 582.1981.

5′-*O*-[*N*-(*N*-Boc-L-ornithinyl(δ-Boc))sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (67b)



Boc-Orn(Boc)-OSu (125 mg, 0.29 mmol) and cesium carbonate (254 mg, 0.78 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **66** (100 mg, 0.26 mmol) in DMF (3 mL). The solution was stirred at room temperature for 3 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (95:5:1 to 83:17:1 $CHCl_3$/MeOH/Et_3N) to afford compound **67b** as a white solid (45 mg, 22%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.47 (s, 1H), 8.22 (s, 1H), 6.24 (d, *J* = 3.4 Hz, 1H), 5.35 (q, *J* = 2.9 Hz, 1H), 5.15−5.09 (m, 1H), 4.56−4.52 (m, 1H), 4.23 (d, *J* = 4.0 Hz, 2H), 4.01−3.94 (m, 1H), 3.19 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 3.10−2.98 (m, 2H), 1.86−1.73 (m, 1H), 1.61 (s, 3H), 1.60−1.49 (m, 3H), 1.47−1.40 (m, 18H), 1.39 (s, 3H), 1.28 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, CD_3OD): δ 180.7, 158.4, 157.6, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.8, 85.7, 85.6, 83.3, 80.0, 79.7, 69.7, 57.6, 47.8, 41.0, 31.8, 28.8, 27.5, 25.6, 9.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_{28}H_{45}N_8O_{11}S$, 701.2929; found, 701.2926.

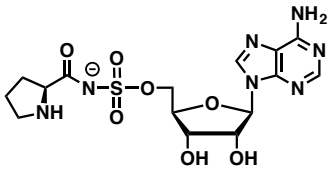
5′-*O*-[*N*-(*N*-Boc-L-leucyl)sulfamoyl]-2′,3′-*O*-isopropylideneadenosine triethylammonium salt (67c)



Boc-Leu-OSu (193 mg, 0.59 mmol) and cesium carbonate (381 mg, 1.17 mmol) were added to a solution of 5′-*O*-sulfamoyl-2′,3′-isopropylideneadenosine **66** (150 mg, 0.39 mmol) in DMF (4 mL). The solution was stirred at room temperature for 4 h. The reaction mixture was filtered through a pad of Celite. The filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (97:3:1 $CHCl_3$/MeOH/Et_3N) to afford

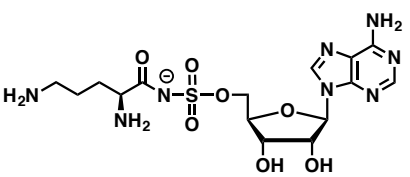
compound **67c** as a white solid (168 mg, 61%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.47 (s, 1H), 8.22 (s, 1H), 6.23 (d, *J* = 3.4 Hz, 1H), 5.37−5.32 (m, 1H), 5.14−5.08 (m, 1H), 4.56−4.51 (m, 1H), 4.23 (d, *J* = 3.4 Hz, 2H), 4.09−4.00 (m, 1H), 3.19 (q, *J* = 7.5 Hz, 6H, Et_3N-*CH*$_2$), 1.77−1.65 (m, 1H), 1.61 (s, 3H), 1.60−1.50 (m, 1H), 1.48−1.34 (m, 13H), 1.29 (t, *J* = 7.5 Hz, 9H, Et_3N-*CH*$_3$), 0.92 (d, *J* = 1.7 Hz, 3H), 0.91 (d, *J* = 1.7 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 181.7, 157.7, 157.3, 154.0, 150.5, 141.4, 120.1, 115.2, 91.8, 85.8, 85.6, 83.3, 79.9, 79.5, 69.7, 56.8, 47.9, 43.7, 28.8, 27.5, 26.1, 25.6, 23.7, 22.2, 9.2. HRMS (ESI+): $[M+H]^+$ calcd for $C_{24}H_{38}N_7O_9S$, 600.2452; found, 600.2437.

L-Pro-AMS triethylammonium salt



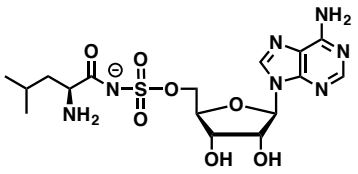
Compound **67a** (30 mg, 0.044 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 2 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (67:33:1 to 50:50:1 $CHCl_3$/MeOH/Et_3N) to afford L-Pro-AMS triethylammonium salt as a white solid (16 mg, 74%). ^{1}H NMR (500 MHz, DMSO-d_6): δ 8.35 (s, 1H), 8.14 (s, 1H), 7.27 (br, 2H), 5.90 (d, *J* = 5.7 Hz, 1H), 4.61−4.56 (m, 1H), 4.18−4.12 (m, 2H), 4.11−4.03 (m, 2H), 3.91−3.86 (m, 1H), 3.23−3.16 (m, 1H), 3.10−3.04 (m, 1H), 2.72 (q, *J* = 6.9 Hz, 2H, Et_3N-*CH*$_2$), 2.19−2.10 (m, 1H), 1.95−1.86 (m, 1H), 1.85−1.72 (m, 2H), 1.03 (t, *J* = 6.9 Hz, 3H, Et_3N-*CH*$_3$). ^{13}C NMR (125 MHz, DMSO-d_6): δ 171.7, 156.0, 152.6, 149.6, 139.4, 118.9, 87.1, 82.4, 73.4, 70.7, 67.7, 61.9, 45.7, 45.3, 29.1, 23.4, 10.2. HRMS (ESI−): $[M-H]^-$ calcd for $C_{15}H_{20}N_7O_7S$, 442.1145; found, 442.1146.

L-Orn-AMS triethylammonium salt



Compound **67b** (40 mg, 0.047 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 3 h, the flask was placed on the rotary evaporator, and the TFA and H_2O were removed at reduced pressure. The residue was purified by HPLC [COSMISIL 5C_{18}-PAQ: C-18 reverse-phase column, ϕ 10 mm × 250 mm, aqueous TFA (0.01%), 4.0 mL/min, 210 nm, t_R: 9.8 min] to afford L-Orn-AMS triethylammonium salt as a colorless oil (26 mg, 98%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.63 (s, 1H), 8.39 (s, 1H), 6.13 (d, J = 4.6 Hz, 1H), 4.63 (dd, J = 5.2, 4.6 Hz, 1H), 4.46−4.31 (m, 4H), 3.77 (dd, J = 6.3, 5.7 Hz, 1H), 3.20 (q, J = 7.5 Hz, 6H, Et_3N-*CH_2*), 3.03−2.97 (m, 2H), 2.05−1.78 (m, 4H), 1.31 (t, J = 7.5 Hz, 9H, Et_3N-*CH_3*). ^{13}C NMR (125 MHz, CD_3OD): δ 174.6, 152.6, 150.1, 146.5, 143.5, 120.1, 90.2, 84.3, 76.3, 71.8, 69.5, 55.9, 47.8, 40.0, 29.3, 24.3, 9.2. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{15}H_{24}N_8O_7SNa$, 483.1386; found, 483.1381.

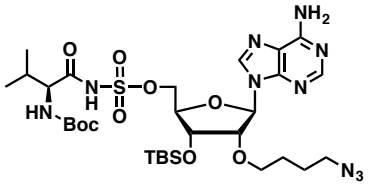
L-Leu-AMS triethylammonium salt



Compound **67c** (70 mg, 0.10 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 5 h, the flask was placed on the rotary evaporator, and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (83:17:1 to 67:33:1 $CHCl_3$/MeOH/Et_3N) to afford L-Leu-AMS triethylammonium salt as a white solid (35 mg, 69%). ^{1}H NMR (500 MHz, CD_3OD): δ 8.50 (s, 1H), 8.19 (s, 1H), 6.08 (d, J = 5.2 Hz, 1H), 4.66−4.61 (m, 1H), 4.44−4.36 (m, 2H), 4.36−4.28 (m, 2H), 3.70−3.65 (m, 1H), 3.11 (q, J = 7.5 Hz, 3H, Et_3N-*CH_2*), 1.84−1.73 (m, 2H), 1.64−1.53 (m, 1H), 1.26 (t, J = 7.5 Hz, 4.5H, Et_3N-*CH_3*), 0.96 (d, J = 6.3 Hz, 3H), 0.93 (d, J = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD): δ 176.8, 157.2, 153.9, 150.7, 141.2, 120.1, 89.5, 84.2, 76.1, 71.9, 69.1, 55.7, 47.7, 42.2, 25.7, 23.2, 22.2, 9.4. HRMS (ESI+): $[M+H]^+$ calcd for $C_{16}H_{26}N_7O_7S$, 460.1614; found, 460.1600.

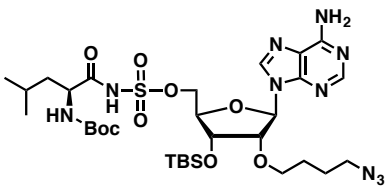
Syntheses of L-Val-AMS-BPyne and L-Leu-AMS-BPyne

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl) oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-valyl)sulfamate (68a)



Boc-Val-OSu (52 mg, 0.17 mmol) and cesium carbonate (108 mg, 0.33 mmol) were added to a solution of compound **16** (60 mg, 0.11 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was then filtered through a pad of Celite. The filtrate was concentrated under reduce pressure. The residue was purified by flash chromatography (10:1 to 5:1 $CHCl_3$/MeOH) to afford compound **68a** as a white solid (65 mg, 78%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.44 (s, 1H), 8.23 (s, 1H), 6.15 (d, *J* = 5.7 Hz, 1H), 4.65−4.59 (m, 1H), 4.55 (t, *J* = 5.2 Hz, 1H), 4.51 (dd, *J* = 10.9, 3.4 Hz, 1H), 4.39 (dd, *J* = 10.9, 3.4 Hz, 1H), 4.31−4.26 (m, 1H), 3.91 (d, *J* = 5.7 Hz, 1H), 3.64−3.55 (m, 1H), 3.52−3.46 (m, 1H), 3.20−3.11 (m, 2H), 2.13−2.01 (m, 1H), 1.62−1.48 (m, 4H), 1.41 (s, 9H), 0.98−0.94 (m, 12H), 0.90 (d, *J* = 6.9 Hz, 3H), 0.17 (s, 3H), 0.16 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 176.4, 157.9, 156.9, 153.3, 150.6, 141.5, 120.4, 87.8, 84.8, 82.9, 80.5, 72.5, 71.4, 70.8, 62.4, 52.1, 32.4, 28.8, 28.0, 26.6, 26.3, 19.9, 19.0, 18.2, −4.4, −4.6. HRMS (ESI−): $[M-H]^-$ calcd for $C_{30}H_{51}N_{10}O_9SSi$, 755.3336; found, 755.3336.

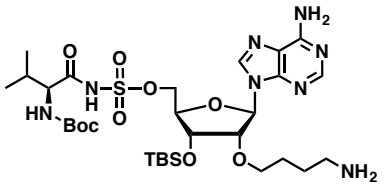
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-azidobutoxy)-3-((*tert*-butyldimethylsilyl) oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-leucyl)sulfamate (68b)



Boc-Leu-OSu (56 mg, 0.17 mmol) and cesium carbonate (108 mg, 0.33 mmol) were added to a solution of compound **16** (60 mg, 0.11 mmol) in DMF (1 mL). The solution was stirred at room temperature for 1 h. The reaction mixture was then filtered through a pad of Celite. The

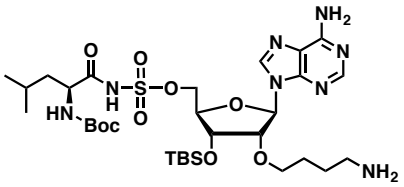
filtrate was concentrated under reduce pressure. The residue was purified by flash chromatography (10:1 $CHCl_3$/MeOH) to afford compound **68b** as a white solid (66 mg, 78%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.45 (s, 1H), 8.23 (s, 1H), 6.15 (d, *J* = 6.3 Hz, 1H), 4.63−4.60 (m, 1H), 4.57−4.53 (m, 1H), 4.48 (dd, *J* = 10.9, 3.4 Hz, 1H), 4.37 (dd, *J* = 10.9, 2.9 Hz, 1H), 4.30−4.26 (m, 1H), 4.10−4.05 (m, 1H), 3.63−3.57 (m, 1H), 3.51−3.45 (m, 1H), 3.18−3.13 (m, 2H), 1.76−1.65 (m, 1H), 1.60−1.47 (m, 6H), 1.41 (s, 9H), 0.96 (s, 9H), 0.94−0.90 (m, 6H), 0.18 (s, 3H), 0.17 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 157.8, 156.9, 153.4, 150.6, 141.4, 120.3, 87.7, 84.9, 82.9, 80.4, 72.5, 71.3, 70.6, 55.9, 52.1, 42.4, 28.8, 28.0, 26.6, 26.3, 23.6, 22.0, 19.0, −4.4, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+H]^+$ calcd for $C_{31}H_{55}N_{10}O_9SSi$, 771.3643; found, 771.3639.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-valyl)sulfamate (69a)



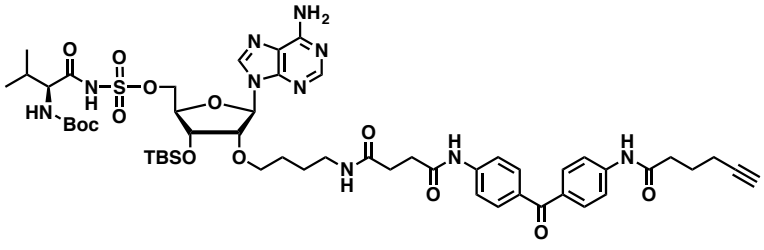
To a solution of **68a** (50 mg, 0.066 mmol) in MeOH (2 mL) was added 10% Pd/C (5 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 24 h. The reaction mixture was filtered through a pad of Celite, which was further washed with MeOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 $CHCl_3$/MeOH) to afford compound **69a** as a white solid (32 mg, 66%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.57 (s, 1H), 8.20 (s, 1H), 6.17 (d, *J* = 6.3 Hz, 1H), 4.64−4.58 (m, 1H), 4.57−4.52 (m, 1H), 4.33−4.20 (m, 3H), 3.95−3.88 (m, 1H), 3.62−3.54 (m, 1H), 3.52−3.46 (m, 1H), 2.90−2.83 (m, 2H), 2.19−2.07 (m, 1H), 1.68−1.50 (m, 4H), 1.41 (s, 9H), 1.01−0.91 (m, 12H), 0.87 (d, *J* = 6.9 Hz, 3H), 0.17 (s, 3H), 0.16 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 180.4, 157.8, 157.4, 154.0, 150.8, 141.1, 120.1, 87.1, 86.0, 83.5, 80.0, 73.1, 70.8, 69.0, 63.1, 40.5, 33.2, 28.8, 27.5, 26.3, 25.4, 20.2, 19.0, 18.0, −4.4, −4.5. HRMS (ESI−): $[M-H]^-$ calcd for $C_{30}H_{53}N_8O_9SSi$, 729.3431; found, 729.3431.

((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-4-(4-aminobutoxy)-3-((*tert*-butyldimethylsilyl)oxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-leucyl)sulfamate (69b)



To a solution of **68b** (50 mg, 0.065 mmol) in MeOH (2 mL) was added 10% Pd/C (5 mg). The resulting suspension was hydrogenated under an atmosphere of H_2 at room temperature for 24 h. The reaction mixture was filtered through a pad of Celite, which was further washed with MeOH (10 mL). The combined filtrate was concentrated under reduced pressure. The residue was purified by flash chromatography (4:1 to 3:1 $CHCl_3$/MeOH) to afford compound **69b** as a white solid (25 mg, 52%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.57 (s, 1H), 8.20 (s, 1H), 6.18 (d, *J* = 6.9 Hz, 1H), 4.63−4.53 (m, 2H), 4.31−4.18 (m, 3H), 4.09−3.98 (m, 1H), 3.62−3.54 (m, 1H), 3.52−3.45 (m, 1H), 2.87 (dd, *J* = 7.5, 6.9 Hz, 2H), 1.76−1.67 (m, 1H), 1.66−1.48 (m, 6H), 1.41 (s, 9H), 0.97 (s, 9H), 0.95−0.88 (m, 6H), 0.174 (s, 3H), 0.171 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 181.9, 157.7, 157.4, 154.0, 150.9, 141.1, 120.1, 86.9, 86.0, 83.4, 80.0, 73.2, 70.7, 69.1, 56.9, 43.9, 40.5, 28.8, 27.5, 26.3, 25.4, 23.8, 22.3, 19.0, −4.3, −4.4. HRMS (ESI+): $[M+H]^+$ calcd for $C_{31}H_{57}N_8O_9SSi$, 745.3738; found, 745.3734.

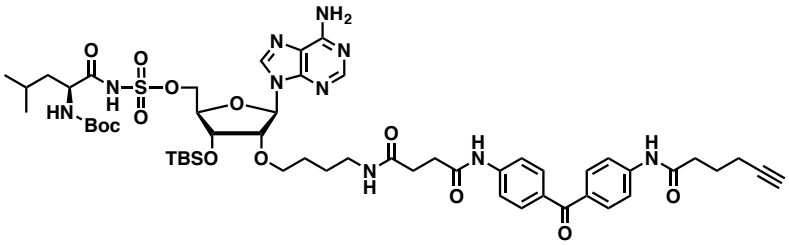
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-valyl)sulfamate (70a)



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (8.0 mg, 0.041 mmol) and 1-hydroxybenzotriazole (6.3 mg, 0.041 mmol) were added to a solution of compound **23** (17 mg, 0.041 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **69a** (25 mg, 0.034 mmol) was then added. After 12 h, the reaction mixture was diluted

with EtOAc. The combined organic layer 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 (5:1 $CHCl_3$/MeOH) to afford compound **70a** as a white solid (22 mg, 58%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.48 (s, 1H), 8.20 (s, 1H), 7.76−7.68 (m, 8H), 6.14 (d, *J* = 6.3 Hz, 1H), 4.62 (dd, *J* = 4.6, 2.9 Hz, 1H), 4.53 (dd, *J* = 6.3, 4.6 Hz, 1H), 4.39 (dd, *J* = 10.9, 3.4 Hz, 1H), 4.32–4.24 (m, 2H), 3.92–3.86 (m, 1H), 3.59−3.52 (m, 1H), 3.48−3.41 (m, 1H), 3.07 (dd, *J* = 6.9, 6.3 Hz, 2H), 2.70 (dd, *J* = 7.5, 6.9 Hz, 2H), 2.59–2.50 (m, 4H), 2.32−2.26 (m, 3H), 2.16−2.07 (m, 1H), 1.94−1.86 (m, 2H), 1.55−1.38 (m, 13H), 0.97−0.91 (m, 12H), 0.87 (d, *J* = 6.9 Hz, 3H), 0.154 (s, 3H), 0.146 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.5, 174.4, 174.0, 173.3, 158.0, 157.3, 153.9, 150.7, 144.3, 144.2, 141.3, 134.0, 133.9, 132.3, 132.2, 120.3, 120.0, 119.9, 87.6, 85.4, 84.1, 83.1, 80.2, 72.7, 71.5, 70.3, 69.5, 63.1, 40.1, 36.7, 33.2, 31.8, 28.8, 28.1, 26.9, 26.3, 25.5, 20.2, 19.0, 18.6, 18.1, −4.4, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI−): $[M-H]^-$ calcd for $C_{53}H_{73}N_{10}O_{13}SSi$, 1117.4854; found, 1117.4861.

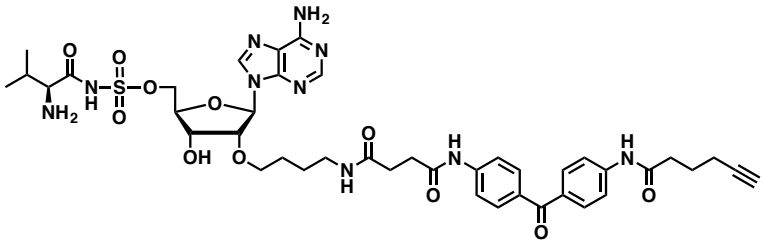
((2*R*,3*R*,4*R*,5*R*)-5-(6-Amino-9*H*-purin-9-yl)-3-((*tert*-butyldimethylsilyl)oxy)-4-(4-(4-((4-(4-(hex-5-ynamido)benzoyl)phenyl)amino)-4-oxobutanamido)butoxy)tetrahydrofuran-2-yl)methyl ((*tert*-butoxycarbonyl)-*L*-leucyl)sulfamate (70b)



1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (6.1 mg, 0.032 mmol) and 1-hydroxybenzotriazole (5.0 mg, 0.032 mmol) were added to a solution of compound **23** (13 mg, 0.032 mmol) in DMF (1 mL). The solution was stirred at room temperature for 10 min and **69b** (20 mg, 0.027 mmol) was then added. After 12 h, the reaction mixture was diluted with EtOAc. The combined organic layer 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 (10:1 to 5:1 $CHCl_3$/MeOH) to afford compound **70b** as a white solid (13 mg, 42%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.44 (s, 1H), 8.20 (s, 1H), 7.75−7.70 (m, 8H), 6.14 (d, *J* = 6.3 Hz, 1H), 4.63−4.59 (m, 1H), 4.53 (dd, *J* = 5.7, 4.6 Hz, 1H), 4.40 (dd, *J* = 10.9, 3.9 Hz, 1H), 4.33−4.24 (m, 2H), 4.07−4.01 (m, 1H), 3.58−3.53 (m,

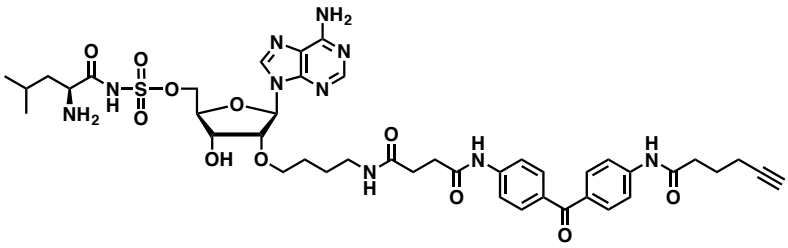
1H), 3.48−3.42 (m, 1H), 3.08 (dd, *J* = 12.0, 6.3 Hz, 2H), 2.70 (dd, *J* = 7.5, 6.9 Hz, 2H), 2.58−2.51 (m, 4H), 2.32−2.26 (m, 3H), 1.94−1.87 (m, 2H), 1.74−1.65 (m, 1H), 1.60−1.37 (m, 15H), 0.95 (s, 9H), 0,90 (d, *J* = 6.9 Hz, 6H), 0.16 (s, 3H), 0.15 (s, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.6, 174.4, 174.1, 173.3, 157.9, 157.3, 154.0, 150.8, 144.3, 144.2, 141.3, 134.0, 133.9, 132.3, 132.2, 120.3, 120.1, 120.0, 87.6, 85.2, 84.1, 83.0, 80.3, 72.7, 71.5, 70.3, 69.7, 58.3, 43.2, 40.0, 36.7, 33.2, 31.8, 28.8, 28.1, 26.9, 26.3, 25.5, 23.7, 22.1, 19.0, 18.6, −4.3, −4.5. (The ^{13}C signal of the sulfamoyloxy-linked carbonyl, around 180 ppm, was not observed.) HRMS (ESI+): $[M+Na]^+$ calcd for $C_{54}H_{76}N_{10}O_{13}SSiNa$, 1155.4981; found, 1155.4991.

L-Val-AMS-BPyne



Compound **70a** (20 mg, 0.018 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (3:1 $CHCl_3$/MeOH) to afford L-Val-AMS-BPyne as a white solid (15 mg, 92%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.54 (s, 1H), 8.19 (s, 1H), 7.76−7.69 (m, 8H), 6.16 (d, *J* = 5.2 Hz, 1H), 4.50 (t, *J* = 4.6 Hz, 1H), 4.43 (t, *J* = 5.2 Hz, 1H), 4.39 (dd, *J* = 11.5, 2.9 Hz, 1H), 4.32 (dd, *J* = 10.9, 2.9 Hz, 1H), 4.29 (dd, *J* = 6.9, 2.9 Hz, 1H), 3.69−3.63 (m, 1H), 3.61−3.55 (m, 1H), 3.51 (d, *J* = 4.6 Hz, 1H), 3.18−3.10 (m, 2H), 2.71 (dd, *J* = 7.5, 6.9 Hz, 2H), 2.59−2.52 (m, 4H), 2.34−2.26 (m, 4H), 1.94−1.87 (m, 2H), 1.63−1.44 (m, 4H), 1.05 (d, *J* = 6.9 Hz, 3H), 1.00 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.6, 175.0, 174.5, 174.1, 173.3, 157.1, 153.9, 150.6, 144.3, 144.2, 141.2, 134.0, 133.9, 132.28, 132.25, 120.1, 120.0, 119.9, 87.7, 84.6, 84.1, 83.7, 71.6, 70.9, 70.4, 68.8, 62.3, 40.0, 36.7, 33.2, 31.8, 31.3, 27.8, 26.9, 25.5, 19.2, 18.6, 17.6. HRMS (ESI−): $[M-H]^-$ calcd for $C_{42}H_{51}N_{10}O_{11}S$, 903.3465; found, 903.3458.

L-Leu-AMS-BPyne



Compound **70b** (13 mg, 0.011 mmol) was dissolved in a 4:1 (v/v) mixture of TFA and H_2O at room temperature. After 6 h, the flask was placed on the rotary evaporator and the TFA and H_2O were removed at reduced pressure. The residue was purified by flash chromatography (5:1 $CHCl_3$/MeOH) to afford L-Leu-AMS-BPyne as a white solid (7.5 mg, 74%). ^{1}H NMR (500 MHz, CD_3OD) δ 8.51 (s, 1H), 8.19 (s, 1H), 7.73 (d, *J* = 2.9 Hz, 8H), 6.16 (d, *J* = 5.2 Hz, 1H), 4.49 (dd, *J* = 4.6, 4.0 Hz, 1H), 4.43 (dd, *J* = 5.2, 4.6 Hz, 1H), 4.38 (dd, *J* = 10.9, 2.9 Hz, 1H), 4.35−4.26 (m, 2H), 3.70−3.62 (m, 2H), 3.62−3.55 (m, 1H), 3.16−3.10 (m, 2H), 2.71 (t, *J* = 6.9 Hz, 2H), 2.58−2.52 (m, 4H), 2.32−2.26 (m, 3H), 1.94−1.86 (m, 2H), 1.83−1.74 (m, 2H), 1.63−1.43 (m, 5H), 0.97 (d, *J* = 6.3 Hz, 3H), 0.93 (d, *J* = 6.3 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 196.6, 176.2, 174.5, 174.1, 173.3, 157.2, 154.0, 150.7, 144.3, 144.2, 141.1, 134.0, 133.9, 132.3, 132.2, 120.12, 120.07, 119.98, 87.7, 84.6, 84.1, 83.7, 71.6, 70.9, 70.3, 68.9, 55.6, 42.0, 40.0, 36.7, 33.2, 31.8, 27.8, 26.9, 25.7, 25.5, 23.2, 22.1, 18.6. HRMS (ESI+): $[M+Na]^+$ calcd for $C_{43}H_{54}N_{10}O_{11}SNa$, 941.3592; found, 941.3584.

Chemical Biology Procedures

Protein Expression and Materials: Recombinant proteins *holo*-GrsA and *holo*-$TycB_1$ were expressed and purified as previously described.[23,29,48,49] These proteins were overproduced and isolated as C-terminal His-tagged constructs using the *E. coli* overexpression strain, BL21 (DE3), kindly provided by Prof. Mohamed A. Marahiel at Philipps-Universität Marburg, Germany. The $AusA_1$ (A_1-T_1) gene was PCR amplified genomic DNA from *S. aureus* ATCC 700699 using primers $ausA_1$ F (5′-GCCTCCATGACCATGGTTATGGGTAATTTGAGATTTCAAC-3′) and $AusA_1$ R (5′-CCGAATTCGTCAGCACATAATCATCTTTAACTATAGCTTC-3′), and subsequently cloned into litmus28-*ausA1*. Plasmid litmus28-*ausA1* was digested with *NcoI* and *BamHI*, and the gene was subcloned into p28b to produce p28b-*ausA1*, an expression vector for

apo-AusA$_1$ with a hexahistidine appended to the C terminus. Sequencing revealed the expression plasmid to be error free. For expression and purification of *apo*-AusA$_1$, p28b-AusA$_1$ was transformed into *E. coli* BL21 (DE3) cells. Overnight cultures were used to inoculate 1 L of LB medium supplemented with 50 μg/mL kanamycin. Cultures were allowed to grow to an A_{600} of 0.6 at 37 °C, then induced with IPTG to a final concentration of 0.1 mM, and allowed to grow for 12 h at 18 °C. Cells were pelleted and resuspended in lysis buffer (20 mM Tris–HCl, pH 8.0 and 0.5% Triton-X). The cells were then lysed by sonication at 4 °C using an ultrasonic disruptor UD201 (Tomy Digital Biology Co., Ltd, Japan). The resulting cell lysate was centrifuged to remove cell debris and the supernatant was loaded onto a Ni Sepharose high-performance resin (GE Healthcare) and eluted with a gradient of imidazole (20–250 mM). Eluted proteins were visualized by SDS-PAGE with Coomassie Brilliant Blue stain and quantitated by the method of Bradford.[50] Fractions containing the recombinant proteins were pooled and dialyzed against assay buffer (20 mM Tris–HCl, pH 8.0, 1 mM $MgCl_2$, and 1 mM TCEP). After the addition of 10% glycerol (v/v) the protein was stored at −80 °C.

Hydroxamate-MesG Assay[25]

***Standard assay conditions*:** Reactions contained NRPS enzymes to maintain initial velocity conditions, 20 mM Tris (pH 8.0), 2.5 mM ATP, 1 mM $MgCl_2$, 1 mM TCEP, 150 mM hydroxylamine (pH 7.0), 0.1 U purine nucleoside phosphorylase (Sigma-Aldrich, N8264), 0.04 U inorganic pyrophosphatase (Sigma-Aldrich, I1643), 0.2 mM MesG (Berry & Associates), and 1 mM substrates. The reactions (100 μL) were run in 96-well half-area plates (Corning, 3881) and the cleavage of MesG was monitored at A_{355} on an EnVision Multilabel Reader (PerkinElmer). Working stocks of hydroxylamine were prepared fresh by combining 500 μL of 4 M hydroxylamine, 250 μL of water, and 250 μL of 7 M NaOH on ice.

***Determination of K_i^{app} values of inhibitors by the hydroxamate-MesG assay* (Table 1-3):** K_i^{app} determination was performed using standard assay conditions. For *holo*-GrsA, probes **1**–**7** were tested from 0.01 to 20 μM using L-Phe (1 mM) as the competing substrate. The enzyme was fixed at 20 nM. L-Pro-AMS-BPyne and L-Pro-AMS were varied from 0.625 to 50 μM, and *holo*-TycB$_1$ (800 nM) and L-Pro (1 mM) were held constant. L-Val-AMS-BPyne and L-Val-AMS were tested from 2.5 to 100 μM using L-Val (1 mM) as the competing substrate. The enzyme was fixed at 1.2 μM. In all experiments, the total DMSO concentration was kept at 2.0%. Initial velocities were fit to the Morrison equation using Prism 5 (GraphPad Software).

Labeling of recombinant GrsA by probes 1–10 (Figure 1-7, 1-8a): Standard conditions for probes **1**–**10**-GrsA reactions were follows: recombinant GrsA (1 μM) was treated with probes **1**–**10** (1 μM from 100 μM stock in DMSO) in standard buffer 1 [20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, and 0.0025% Igepal CA-630]. Inhibition studies were performed by preincubation of GrsA (1 μM) with L-Phe-AMS (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice. To initiate the click reaction, TAMRA-azide, TCEP, TBTA ligand, and $CuSO_4$ were added (to 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 ºC 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).

Measurements of GrsA labeling by probes 1–10 (Figure 1-7): The probes **1**–**10**-recombinant GrsA reactions were performed by incubating recombinant GrsA (1 μM) with probes **1**–**10** (1 μM from a 100 μM stock in DMSO; final DMSO concentration of 1.1%) for 10 min at room temperature in assay buffer 1, respectively. The sample was then irradiated at 365 nm for 30 min on ice. To initiate the click reaction, TAMRA-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 ºC for 5 min. The concentration of TAMRA-conjugated BSA (Invitrogen) was determined using the extinction coefficient of TAMRA (ε_{560} = 65,000 M^{-1} cm^{-1}). Samples (5 μL) were separated by 1D SDS-PAGE and fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager. Protein labeling by probes **1**–**10** was quantitated by measuring integrated band intensities using ImageJ.

UV photolysis time study (Figure 1-8b, 2-2b, and 3-2b): Recombinant GrsA (1 μM), $TycB_1$ (1 μM) and $AusA_1$ (1 μM) were treated with probes **1**, **8**, **10**, L-Pro-AMS-BPyne, and L-Val-AMS-BPyne (1 μM from a 100 μM stock in DMSO; final DMSO concentration of 1.1%) in assay buffer 1, respectively. After 10 min at room temperature, these samples were irradiated at 365 nm for the indicated time (0–60 min) on ice, reacted with TAMRA-azide, and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager.

Labeling of recombinant $TycB_1$ and $AusA_1$ by the L-Pro-AMS-BPyne and L-Val-AMS-BPyne (Figure 2-2a, 3-2a): Standard conditions for L-Pro-AMS-BPyne and L-Val-AMS-BPyne-recombinant protein reactions were as follows: recombinant $TycB_1$ (1 μM) and $AusA_1$ (1 μM) were treated with probes L-Pro-AMS-BPyne and L-Val-AMS-BPyne (1 μM from a 100 μM stock in DMSO) in assay buffer 1, respectively. Inhibition studies were performed by pre-incubation of $TycB_1$ (1 μM) and $AusA_1$ (1 μM) with L-Pro-AMS and L-Val-AMS (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature, respectively. In all experiments total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min ($TycB_1$) and 60 min ($AusA_1$) on ice, respectively. These samples were reacted with TAMRA-azide, and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager.

Limit of detection of GrsA, $TycB_1$, and $AusA_1$ labeling (Figure 2-2c, 3-2c): Recombinant GrsA (0.125–62.5 nM), $TycB_1$ (0.125–62.5 nM), and $AusA_1$ (0.125–62.5 nM) were treated with L-Phe-AMS-BPyne, L-Pro-AMS-BPyne, and L-Val-AMS-BPyne (1 μM from a 100 μM stock in DMSO; final DMSO concentration of 2.2%) in assay buffer 1, respectively. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min (GrsA, $TycB_1$) and 60 min ($AusA_1$) on ice, respectively, and reacted with TAMRA-azide for 1 h at room temperature. Reactions were treated with 5· SDS-loading buffer (strong reducing) and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Comparing the labeling property with GrsA, $TycB_1$, $AusA_1$, and BSA (Figure 2-3, 3-2d): For GrsA (A: L-Phe), $TycB_1$ (A: L-Pro), $AusA_1$ (A: L-Val), and BSA labeling experiments, L-Phe-AMS-BPyne, L-Pro-AMS-BPyne, and L-Val-AMS-BPyne (1 μM) were individually added to a 46 μL reaction containing GrsA (1 μM), $TycB_1$ (1 μM), $AusA_1$ (1 μM), BSA (1 μM), and assay buffer 1. For inhibition studies, GrsA (1 μM), $TycB_1$ (1 μM), and $AusA_1$ (1 μM) were pre-incubated with L-Phe-AMS, L-Pro-AMS, and L-Val-AMS (100 μM) for 10 min at room temperature, respectively. In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min (GrsA, $TycB_1$) and 60 min ($AusA_1$) on ice, respectively, and reacted with TAMRA-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).

Bacterial strains: *A. migulanus* ATCC 9999 was obtained from the American Type Culture Collection (ATCC). The same strain deposited as *A. migulanus* DSM 2895, along with two other gramicidin S producing strains, *A. migulanus* DSM 5759 and DSM 5668, were received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ).

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

Preparation of cellular lysates for proteomic labeling experiments: *A. migulanus* ATCC 9999, DSM 5756, DSM 5668, and DSM 2895 were maintained on nutrient agar. Single colonies were used to inoculate YPG medium and cultures were shaken for 24 h at 37 ºC. The seed culture (2 mL) was transferred to YPG media (250 mL) and the resulting mixture was incubated at 37 ºC. Growth was routinely monitored at A_{660} on a U-2910 spectrophotometer (Hitachi). The cells were harvested by centrifugation and stored in the freezer until used. The frozen cell pellets were resuspended in Tris pH 8.0 (20 mM), $MgCl_2$ (1 mM), TCEP (1 mM), Igepal CA-630 (0.05%), and a protease inhibitor cocktail. Because of the lability of the synthetase during mechanical cell disruption processes,[30a] a gentle treatment of cells with lysozyme (0.2 mg/mL) was used to release intracellular protein. The cell suspension was incubated at 0 ºC for 30 min. The mixture was then incubated at 30 ºC for 30 min. The solution was centrifuged for 5 min at 15,000 rpm and the pellets were discarded. The total protein concentration was quantitated by the method of Bradford.[50]

GrsA labeling of *A. migulanus* ATCC 9999 proteomes (Figure 2-4): *A. migulanus* proteome (1.4 mg/mL) was treated with L-Phe-AMS-BPyne (1 μM from a 100 μM stock in DMSO) in standard buffer 2 [20 mM Tris (pH 8.0), 1 mM $MgCl_2$, 1 mM TCEP, 0.05% Igepal CA-630, 0.2 mg/mL lysozyme, and protease inhibitor cocktail]. Inhibition studies were performed by pre-incubation of *A. migulanus* proteome (1.4 mg/mL) with L-Phe-AMS (100 μM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice, reacted with TAMRA-azide for 1 h at room temperature, and separated by gel electrophoresis. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

GrsB labeling of *A. migulanus* DSM 5759 proteomes (Figure 2-5a, b, 3-3a, and b): *A. migulanus* proteome (1.5 mg/mL) was individually treated with L-Pro-AMS-BPyne,

L-Val-AMS-BPyne, L-Orn-AMS-BPyne, and L-Leu-AMS-BPyne (1 µM from a 100 µM stock in DMSO) in assay buffer 2. For inhibition studies, *A. migulanus* proteome (1.5 mg/mL) was individually pre-incubated with L-Pro-AMS, L-Val-AMS, L-Orn-AMS, and L-Leu-AMS (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 5 min and reacted with TAMRA-azide for 1 h at room temperature. Reactions were treated with 5· SDS-loading buffer (strong reducing) and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager (GE Healthcare).

Labeling of individual A-domains in endogenous GrsA and GrsB (Figure 2-6, 2-7a, b, 3-4, and 3-5): For labeling of GrsA, *A. migulanus* ATCC 9999 proteome (1.5 mg/mL) was individually treated with L-Phe-AMS, L-Pro-AMS, L-Val-AMS, L-Orn-AMS, and L-Leu-AMS (100 µM from a 10 mM stock in DMSO) individually in assay buffer 2. These samples were incubated for 10 min at room temperature and subsequently treated with L-Phe-AMS-BPyne (1 µM from a 100 µM stock in DMSO). In all experiments, the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, the samples were exposed to ultraviolet light for 30 min on ice and treated with TAMRA-azide for 1 h at room temperature. For labeling of GrsA, the *A. migulanus* DSM 5759 proteome (1.5 mg/mL) was treated with L-Phe-AMS, L-Pro-AMS, L-Val-AMS, L-Orn-AMS, and L-Leu-AMS (100 µM from a 10 mM stock in DMSO) individually in assay buffer 2. These samples were incubated for 10 min at room temperature and treated with L-Pro-AMS-BPyne, L-Val-AMS-BPyne, L-Orn-AMS-BPyne, and L-Leu-AMS-BPyne individually (1 µM from a 100 µM stock in DMSO). In all reactions, the DMSO concentrations were maintained at a level of 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 5 min on ice and reacted with TAMRA-azide for 1 h at room temperature. Reactions were treated with 5× SDS-loading buffer and subjected to SDS-PAGE. Fluorescent gel visualization was performed using a Typhoon 9410 Gel and Blot Imager.

Enrichment of the cellular targets (Figure 3-3c, d): *A. migulanus* DSM 5759 proteomes were adjusted to a final protein concentration of 1.0–1.5 mg/mL and incubated with L-Val-AMS-BPyne and L-Leu-AMS-BPyne (10 µM from a 10 mM stock in DMSO) for 10 min at room temperature. Enrichments by L-Val-AMS-BPyne and L-Leu-AMS-BPyne used 1.4 and 2.0 mg of proteome as starting material, respectively. Control samples to correct for nonspecific cross-linking were preincubated with L-Val-AMS and L-Leu-AMS (100 µM from

a 100 mM stock in DMSO). In all experiments the total DMSO concentration was kept at 0.2%. Samples were split into 50 μL aliquots and photoactivated on ice for 5 min (365 nm). Cross-linked samples were then recombined and reacted with TAMRA-biotin-azide (Click Chemistry Tools). Samples were incubated for 1 h at room temperature. Ice-cold 4:1 MeOH/$CHCl_3$ (4 mL) was then added to the reaction mixture and mixed vigorously by vortexing. Water (3 mL) was subsequently added to the mixture and mixed by vortexing again. The biphasic solution was centrifuged (4000 × *g*, 15 min, 4 ºC), and protein precipitated at the interface. Liquid layers were removed, and the resulting precipitate was resuspended in ice-cold 4:1 MeOH/$CHCl_3$ (750 μL) and mixed vigorously by vortexing. Water (600 μL) was then added to the mixture and mixed by vortexing again. Samples were repelleted by centrifugation (9000 × *g*, 15 min, 4 ºC), resuspended in ice-cold MeOH (600 μL), sonicated on ice, and then repelleted by centrifuged (9000 × *g*, 15 min, 4 ºC). The resulting pellet was redissolved in 650 μL of PBS (2.5% SDS), sonicated at room temperature, and heated for 5 min at 60 ºC. Redissolved protein was added to 8.5 mL of PBS to give a final SDS concentration of 0.2%. Samples were then incubated with 120 μL of streptavidin-agarose resin (Novagen) and rotated for 1 h at room temperature. Bound samples were then washed sequentially with 1.0% SDS in H_2O (3 × 1 mL), 6 M urea in H_2O (3 × 1 mL), and PBS (3 × 1 mL). The bound proteins were treated with 2× SDS-loading buffer, and the samples were heated at 95 ºC for 5 min. The elute proteins were analyzed by SDS-PAGE and stained with a silver staining method.

Mass spectrometry: The proteins were separated by 1D SDS-PAGE and visualized using Sil-best stain one (Nacalai Tesque). The bands were excised, destained using destaining solution in the Silver Stain MS Kit (Wako Pure Chemical Industries, Ltd.), and subjected to in-gel digestion with TPCK-treated trypsin in the digestion buffer (10 mM Tris–HCl, pH 8.0, and 0.05% decyl glucoside) for 12 h at 37 ºC. The digest mixture was 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.). The buffer compositions were as follows: buffer A was composed of 100% H_2O and 0.1% formic acid, buffer B was composed of 100% CH_3CN and 0.1% formic acid. Peptides were eluted from the C-18 column using a linear gradient of 35−100% buffer B over 10 min at a flow rate of 300 nL/min and then analyzed using a Q-Exactive mass spectrometer (Thermo Fisher Scientific) with a nanospray ion source using the data-dependent TOP10 MS/MS method in the mass range of m/z = 300−500 and m/z = 500−1500. Peptide identifications were made using MS/MS Ions Search in the MASCOT program v2.3 (Matrix Science Inc., Boston, MA,

USA).

Profiling NRPS activities in natural product producing bacteria (Figure 3-6a, b): *A. migulanus* ATCC 9999, DSM 5759, DSM 5668, and DSM 2895 were cultured for 24 h and whole cell lysates were isolated as described in experimental section. For the labeling of endogenous GrsA, individual proteomes (2.0 mg/mL) were treated with L-Phe-AMS-BPyne (1 µM from a 100 µM stock in DMSO) in assay buffer 2. Inhibition studies were conducted by preincubation of individual proteomes (2.0 mg/mL) with L-Phe-AMS (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice, reacted with TAMRA-azide for 1 h at room temperature, and separated by gel electrophoresis. For labeling of endogenous GrsB, individual proteomes (2.0 mg/mL) were incubated with L-Leu-AMS-BPyne (1 µM from a 100 µM stock in DMSO) in assay buffer 2. Inhibition studies were performed by preincubation of individual proteomes (2.0 mg/mL) with L-Leu-AMS (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 5 min on ice, reacted with TAMRA-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.

Profiling the expression patterns of NRPS enzymes (Figure 3-7a, b): *A. migulanus* DSM 5759 was cultured for the indicated time (12, 16, 20, and 24 h) and whole cell lysates were isolated as described in experimental section. For monitoring the expression of endogenous GrsA, DSM 5759 proteomes (2.0 mg/mL) were treated with L-Phe-AMS-BPyne (1 µM from a 100 µM stock in DMSO) in assay buffer 2. Inhibition studies were performed by preincubation of DSM 5759 proteome (2.0 mg/mL) with L-Phe-AMS (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments the total DMSO concentration was kept at 2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 30 min on ice, reacted with TAMRA-azide for 1 h at room temperature, and separated by gel electrophoresis. For tracking the expression of endogenous GrsB, DSM 5759 proteome (2.0 mg/mL) was incubated with L-Leu-AMS-BPyne (1 µM from a 100 µM stock in DMSO) in assay buffer 2. For inhibition studies, DSM 5759 proteomes (2.0 mg/mL) were preincubated with L-Leu-AMS (100 µM from a 10 mM stock in DMSO) for 10 min at room temperature. In all experiments the total DMSO concentration was kept at

2.2%. After 10 min at room temperature, these samples were irradiated at 365 nm for 5 min on ice, reacted with TAMRA-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.

Production and extraction of gramicidin S: After the cultures had reached the indicated OD_{660} values, 1 mL of culture was removed from the flasks to evaluate the yield of gramicidin S. The cells were immediately centrifuged (7500 × *g*, 15 min, 4 ºC). The pellet was suspended in 1 mL of the pre-extraction solution (150 mM NaCl, 20 mM HCl) and incubated at 80 ºC for 15 min. The suspensions were diluted 1:1 with ethanol, and gramicidin S was extracted by stirring the suspension at room temperature for 1 h. The cell debris was removed by centrifugation, and the amount of gramicidin S in the extract was determined by HPLC.

Quantification of gramicidin S by HPLC (Figure 3-6c, 3-7c): The ethanol and H_2O were removed at reduced pressure, and the residue was dissolved in 1 mL of 50% ethanol. Analytical HPLC (COSMOSIL $5C_{18}$ AR-II reverse-phase column, ϕ 4.6 × 250 mm, 1.0 mL/min, 210 nm, t_R: 24.2 min) was performed on a Prominence CBM-20A (Shimadzu) system equipped with a Prominence SPD-20A UV/VIS detector (Shimadzu). Water (solvent A) and acetonitrile (solvent B), each containing 0.1% trifluoroacetic acid were used as solvents. A gradient was run from 10 to 100% solvent B in 5 to 25 min. For all samples, a constant injection volume of 10 µL was applied. The total area of the gramicidin S peak was used as a determination of concentration. Individual calibration curves were constructed for three concentration ranges as follows: 10 to 100 mg/L, 0.1 to 1 g/L, and 1 to 10 g/L. For calibration, the gramicidin S was extracted from the cell pellets of *A. migulanus* ATCC 9999 and isolated by preparative HPLC (COSMOSIL $5C_{18}$ AR-II reverse-phase column, ϕ 10 × 250 mm, acetonitrile/aqueous TFA (0.1%, 60:40) 3.0 mL/min, 220 nm, t_R: 12.5 min). The acetonitrile and trifluoroacetic acid were removed *in vacuo* and the water was removed by lyophilization to yield the cyclic peptide (10.6 mg) as a white solid. HRMS (ESI+): $[M+2H]^{2+}$ calcd for 571.3608; found, 571.3643.

参考文献

1. a) Walsh, C. T. *Science* **2004**, 303, 1805-1810. b) Clardy, J.; Fischbach, M. A.; Walsh, C. T. *Nat. Biotechnol.* **2006**, 24, 1541–1550.
2. Döhren, H. V.; Keller, U.; Vater, J; Zocher, R. *Chem. Rev.* **1997**, 97, 2675–2705.
3. a) Krätzschmar, J.; Krause, M.; Marahiel, M. A. *J. Bacteriol.* **1989**, 171, 5422–5429. b) Hoyer, K. M.; Mahlert, C.; Marahiel. M. A. *Chem. Biol.* **2007**, 14, 13–22.
4. Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev.* **1997**, 97, 2651–2673.
5. a) Letzel, A. C.; Pidot, S. J.; Hertweck, C. *Nat. Prod. Rep.* **2013**, 30, 392–428. b) Strieker, M.; Tanović, A.; Marahiel, M. A. *Curr. Opin. Struct. Biol.* **2010**, 20, 234–240. c) Sieber, S. A.; Marahiel, M. A. *Chem. Rev.* **2005**, 105, 715–738.
6. a) Davidsen, J. M.; Bartley, D. M.; Townsend, C. A. *J. Am. Chem. Soc.* **2013**, 135, 1749–1759. b) Evans, B. S.; Chen, Y.; Metcalf, W. W.; Zhao, H.; Kelleher, N. L. *Chem. Biol.* **2011**, 18, 601–607.
7. a) Straight, P. D.; Fischbach, M. A.; Walsh, C. T.; Rudner, D. Z.; Kolter, R. *Proc. Natl. Acad. Sci. USA* **2007**, 104, 305–310. b) Agathos, S. N.; Demain, A. L. *Ann. N.Y. Acad. Sci.* **1984**, 434, 44–47.
8. Caffrey, P.; Bevitt, D. J.; Staunton, J.; Leadlay, P. F. *FEBS Lett.* **1992**, 304, 225–228.
9. a) Lee, S. G.; Lipmann, F. *Methods Enzymol.* **1975**, 43, 585–602. b) Chen, H.; Hubbard, B. K.; O'Connor, S. E.; Walsh, C. T. *Chem. Biol.* **2002**, 9, 103–112.
10. Harding, M. W.; Galat, A.; Uehling, D. E.; Schreiber, S. L. *Nature* **1989**, 341, 758–760.
11. Salisbury, C. M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. USA* **2007**, 104, 1171–1176.
12. Cravatt, B. F.; Sorensen, E. *Curr. Opin. Chem. Biol.* **2000**, 4, 663–668.
13. a) Weber. T.; Marahiel, M. A. *Structure* **2001**, 9, 3–9. b) Linne, U.; Schäfer, A.; Stubbs, M. T.; Marahiel, M. A. *FEBS Lett.* **2007**, 581, 905–910.
14. a) Isono, K.; Uramoto, M.; Kusakabe, H.; Miyata, N.; Koyama, T., Ubukata, M.; Sethi, S. K.; McCloskey, J. A. *J. Antibiot.* **1984**, 37, 670–672. b) Osada, H.; Isono, K. *Antimicrob. Agents Chemother.* **1985**, 27, 230–233. c) Vondenhoff, G. H.; Aerschot, A. V. *Eur. J. Med. Chem.* **2011**, 46, 5227–5236.
15. Ueda, H.; Shoku, Y.; Hayashi, N.; Mitsunaga, J.; In, Y.; Doi, M.; Inoue, M.; Ishida, T. *Biochem. Biophys. Acta* **1991**, 1080, 126–134.
16. a) Nakama, T.; Nureki, O.; Yokoyama, S. *J. Biol. Chem.* **2001**, 276, 47387–47393. b) Kotik-Kogan, O.; Moor, N.; Tworowski, D.; Safro, M. *Structure* **2005**, 13, 1799–1807. c) Fukunaga, R.; Yokoyama, S. *J. Mol. Biol.* **2006**, 359, 901–912. d) Fukai, S.; Nureki, O.; Sekine, S.; Shimada, A.; Tao, J.; Vassylyev, D. G.; Yokoyama, S. *Cell* **2000**, 103, 793–

803.

17. Finking, R.; Neumüller, A.; Solsbacher, J.; Konz, D.; Kretzschmar, G.; Schweitzer, M.; Krumm, T.; Marahiel, M. A. *ChemBioChem* **2003**, 4, 903–906.
18. a) Tanovic, A.; Samel, S. A.; Essen, L. O.; Marahiel, M. A. *Science* **2008**, 321, 659–663. b) May, J. J.; Kessler, N.; Marahiel, M. A.; Stubbs, M. T. *Proc. Natl. Acad. Sci. USA* **2002**, 99, 12120–12125. c) Yonus, H.; Neumann, P.; Zimmermann, S.; May, J. J.; Marahiel, M. A.; Stubbs, M. T. *J. Biol. Chem.* **2008**, 283, 32484–32491.
19. Conti, E.; Stachelhaus, T.; Marahiel, M. A.; Brick, P. *EMBO J.* **1997**, 16, 4174–4183.
20. a) Hatanaka, Y.; Sadakane, Y. *Curr. Top. Med. Chem.* **2002**, 2, 271–288. b) Vodovozova, E. L. *Biochemistry (Moscow)* **2007**, 72, 1–20. c) Dubinsky, L.; Krom, B. P.; Meijler, M. M. *Bioorg. Med. Chem.* **2012**, 20, 554-570. d) Dormán, G.; Prestwich, G. D. *Biochemistry* **1994**, 33, 5661–5673.
21. Agathos, S. N.; Demain, A. L. *Appl. Microbiol. Biotechnol.* **1986**, 24, 319–322.
22. a) Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, 125, 4686–4687. b) Speers, A. E.; Cravatt, B. F. *Chem. Biol.* **2004**, 11, 535–546.
23. Ishikawa, F.; Kakeya, H. *Bioorg. Med. Chem. Lett.* **2014**, 24, 865–869.
24. a) Izatt, R. M.; Hansen, L. D.; Rytting, J. H.; Christensen, J. J. *J. Am. Chem. Soc.* **1965**, 87, 2760–2761. b) Yano, J.; Kan, L. S.; Ts'o, P. O. *Biochem. Biophys. Acta* **1980**, 629, 178–183.
25. Wilson, D. J.; Aldrich, C. C. *Anal. Biochem.* **2010**, 404, 56–63.
26. Velazquez-Campoy, A.; Luque, I.; Freire, E. *Thermochim. Acta* **2001**, 380, 217–227.
27. Neres, J.; Labellio, N. P.; Somu, R. V.; Boshoff, H. I.; Wilson, D. J.; Vannada, J.; Chen, L.; Barry, C. E., III; Bennett, E. M.; Aldrich, C. C. *J. Med. Chem.* **2008**, 51, 5349–5370.
28. Wilson, D. J.; Shi, C.; Teitelbaum, A. M.; Gulick, A. M.; Aldrich, C. C. *Biochemistry* **2013**, 52, 926–937.
29. Mootz, H. D.; Marahiel, M. A. *J. Bacteriol.* **1997**, 179, 6843–6850.
30. a) Augenstein, D. C.; Thrasher, K.; Sinskey, A. J.; Wang, D. I. *Biotechnol. Bioeng.* **1974**, 16, 1433–1447. b) Matteo, C. C.; Grade, M.; Tanaka, A.; Piret, J.; Demain, A. L. *Biotechnol. Bioeng.* **1975**, 17, 129–142.
31. Berditsch, M.; Afonin, S.; Ulrich, A. S. *Appl. Environ. Microbiol.* **2007**, 73, 6620–6628.
32. Stachelhaus, T.; Mootz, H. D.; Marahiel, M. A. *Chem. Biol.* **1999**, 6, 493–505.
33. a) Jessani, N.; Cravatt, B. F. *Curr. Opin. Chem. Biol.* **2004**, 8, 54–59. b) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, 77, 383–414.
34. a) Sanman, L. E.; Bogyo, M. *Annu. Rev. Biochem.* **2014**, 83, 249–273. b) Jessani, N.; Liu, Y.; Humphrey, M.; Cravatt, B. F. *Proc. Natl. Acad. Sci. USA* **2002**, 99, 10335–10340. c)

Jessani, N.; Niessen, S.; Wei, B. Q.; Nicolau, M.; Humphrey, M.; Ji, Y.; Han, W.; Noh, D. Y.; Yates, J. R., III; Jeffrey, S. S.; Cravatt, B. F. *Nat. Methods* **2005**, 2, 691–697.

35. Lanning, B. R.; Whitby, L. R.; Dix, M. M.; Douhan, J.; Gilbert, A. M.; Hett, E. C.; Johnson, T. O.; Joslyn, C.; Kath, J. C.; Niessen, S.; Roberts, L. R.; Schnute, M. E.; Wang, C.; Hulce, J. J.; Wei, B.; Whiteley, L. O.; Hayward, M. M.; Cravatt, B. F. *Nat. Chem. Biol.* **2014**, 10, 760–767.
36. Salisbury, C. M.; Cravatt, B. F. *J. Am. Chem. Soc.* **2008**, 130, 2184–2194.
37. Montgomery, D. C.; Sorum, A. W.; Meier, J. L. *J. Am. Chem. Soc.* **2014**, 136, 8669–8676.
38. Amaike, S.; Keller, N. P. *Annu. Rev. Phytopathol.* **2011**, 49, 107–133.
39. Bumpus, S. B.; Evans, B. S.; Thomas, P. M.; Ntai, I.; Kelleher, N. L. *Nat. Biotechnol.* **2009**, 27, 951–956.
40. a) Ferreras, J. A.; Ryu, J. S.; Di Lello, F.; Tan, D. S.; Quadri, L. E. *Nat. Chem. Biol.* **2005**, 1, 29–32. b) Duckworth, B. P.; Nelson, K. M.; Aldrich, C. C. *Curr. Top. Med. Chem.* **2012**, 12, 766–796. c) Imperi, F.; Massai, F.; Facchini, M.; Frangipani, E.; Visaggio, D.; Leoni, L.; Bragonzi, A.; Visca, P. *Proc. Natl. Acad. Sci. USA* **2013**, 110, 7458–7463. d) Foley, T. L.; Rai, G.; Yasgar, A.; Daniel, T.; Baker, H. L.; Attene-Ramos, M.; Kosa, N. M.; Leister, W.; Burkart, M. D.; Jadhav, A.; Simeonov, A.; Maloney, D. J. *J. Med. Chem.* **2014**, 57, 1063–1078.
41. Kasai, S.; Konno, S.; Ishikawa, F.; Kakeya, H. *Chem. Commun.* **2015**, 51, 15764–15767.
42. a) Eppelmann, K.; Stachelhaus, T.; Marahiel, M. A. *Biochemistry* **2002**, 41, 9718–9726. b) Kries, H.; Wachtel, R.; Pabst, A.; Wanner, B.; Niquille, D.; Hilvert, D. *Angew. Chem. Int. Ed.* **2014**, 53, 10105–10108.
43. a) Peypoux, F.; Michel, G. *Appl. Microbiol. Biotechnol.* **1992**, 36, 515–517. b) Bonmatin, J. M.; Labbé. H.; Grangemard, I.; Peypoux, F.; Maget-Dana, R.; Ptak, M.; Michel, G. *Lett. Pept. Sci.* **1995**, 2, 41–47.
44. Mills, A. D.; Yoo, C.; Butler, J. D.; Yang, B.; Verkman, A. S.; Kurth, M. J. *Bioorg. Med. Chem. Lett.* **2010**, 20, 87–91.
45. Pfeifer, J. R.; Reiβ, P.; Koert, U. *Angew. Chem. Int. Ed.* **2006**, 45, 501–504.
46. Gottlieb, H. E.; Kotlyar, V.; Nudelman, A. *J. Org. Chem.* **1997**, 62, 7512-7515.
47. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978**, 43, 2923-2925.
48. Stachelhaus, T.; Hüser, A.; Maraheil, M. A. *Chem. Biol.* **1996**, 3, 913-921.
49. Stachelhaus, T.; Mootz, H. D.; Bergendahl, V.; Marahiel, M. A. *J. Biol. Chem.* **1998**, 273, 22773-22781.
50. Bradford, M. M. *Anal. Biochem.* **1976**, 72, 248-254.

発表論文

1. “Active site-directed proteomic probes for adenylation domains in nonribosomal peptide synthetases”
   Sho Konno, Fumihiro Ishikawa, Takehiro Suzuki, Naoshi Dohmae, Michael D. Burkart, Hideaki Kakeya.
   Chemical Communications **2015**, 51, 2262–2265.

2. “Profiling nonribosomal peptide synthetase activities using chemical proteomic probes for adenylation domains”
   Fumihiro Ishikawa, Sho Konno, Takehiro Suzuki, Naoshi Dohmae, Hideaki Kakeya.
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