Development of linking methods of alcohols and thiols with vinyl azides for bio-application

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**NANYANG
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**Development of Linking Methods of Alcohols and Thiols with
Vinyl Azides for Bio-application**

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

2019

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SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES

A thesis submitted to the Nanyang Technological University in partial fulfilment of the requirement for the degree of Doctor of Philosophy

2019

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Chiba Shunsuke

Authorship Attribution Statement

This thesis contains material from 3 papers published in the following peer-reviewed journals where I was the first or third author.

Chapter 1 is published as H. Hayashi, A. Kaga, and S. Chiba. Application of Vinyl Azides in Chemical Synthesis: A Recent Update. *J. Org. Chem.* **82**, 11981-11989 (2017). DOI: 10.1021/acs.joc.7b02455.

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- Prof. Chiba, Dr. Kaga and the author prepared the manuscript drafts.
- The manuscript was revised by Prof. Chiba, Dr. Kaga and the author
- References were searched by Dr. Kaga and the author

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The contributions of the co-authors are as follows:

- Prof. Chiba and Dr. Wang provided the initial project direction and edited the manuscript drafts.
- The manuscript was prepared and revised by Prof. Chiba, Assoc. Prof. Xing, Dr. Wang, Dr. Hu and the author.
- Dr. Wang and the author designed the study and performed all the experimental work at the School of Physical and Mathematical Science.
- All microscopy, including sample preparation, was conducted by Dr. Wang and the author in the CBC-05-11 for analysis, characterization and testing.
- Dr. Hu assisted in the collection of bioimaging data.
- Dr. Li and Dr. Ganguly assisted in the interpretation of the X-ray absorption spectroscopy data.

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The contributions of the co-authors are as follows:

- Prof. Chiba, Assoc. Prof. Xing, Dr. Ariyasu and the author provided the initial project direction and edited the manuscript drafts.
- Dr. Ariyasu and the author prepared the manuscript drafts. The manuscript was revised by Prof. Chiba, Assoc. Prof. Xing Dr. Ariyasu and the author
- Dr. Ariyasu and the author co-designed the study and performed all the laboratory work at the School of Physical and Mathematical Science.
- All microscopy, including sample preparation, was conducted by Dr. Ariyasu and the author in the CBC-05-11 for analysis, characterization and testing.
- Dr. Ariyasu and the author collected the SDS-PAGE and ELISA data.

Chapter 4 is experimental procedures of chapter 2 and 3.

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Abstract

This thesis provides new strategies to introduce azido group into biological molecules through linking vinyl azides and 2-azidoacrylates onto alcohols and thiols. Furthermore, the azido linked products were used for further biological applications.

The recent update and applications of vinyl azides in chemical synthesis were described in chapter 1 of the thesis. The unique reactivities of vinyl azides are introduced, such as vinyl azides as radical acceptors, nucleophiles and electrophiles.

Chapter 2 of the thesis presented the new strategy to specifically introduce azido group onto the hydroxyl group. The linking started from an enamine-type nucleophilic attack of vinyl azides to F^+ and Br^+ followed by trapping the iminodiazonium ion using the primary or secondary hydroxyl group of the molecules including natural product derivatives such as sugars and steroids. The dyes were attached to the afforded azido linked testosterone product for bioimaging applications.

Chapter 3 of the thesis describes the dual functionalization of cysteine containing peptide and protein through thiol-Michael addition of 2-azidoacrylates with sulfhydryl group. 2-azidoacrylates bearing the various functionalities such as fluorescent dyes were conjugated successfully with cysteine containing peptides and bovine serum albumin (BSA) to anchoring azido group in physiological conditions. The resulting conjugated peptides and BSA were used

to perform a second functionalization through successive azide–alkyne cycloaddition to install another functionality. Therefore, dual functionalized peptides and BSA are obtained.

Chapter 4 of the thesis is the experimental section of chapter 2 and 3.

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List of abbreviations

δ	chemical shift (ppm)
$^{\circ}\text{C}$	degree centigrade
AA	amino acid
Ac	acetyl
Ar	aryl (substituted aromatic ring) or argon atmosphere
Bn	benzyl
Boc	<i>tert</i> -butyloxycarbonyl
brs	broad singlet
Bu	butyl
Bz	benzoyl
Calcd	calculated
cat.	catalytic
CuAAC	copper(I)-catalyzed azide alkyne cycloaddition
cm^{-1}	wave number
Cys	cysteine
d	doublet
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
dd	doublet of doublets
ddd	doublet of doublet of doublets
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPA	diphenylphosphoryl azide
dr	diastereomeric ratio

dt	doublet of triplets
equiv.	equivalent
ESIHRMS	Electrospray Ionization High Resolution Mass Spectrometry
Et	ethyl
EWG	electron withdrawing group
Fmoc	fluorenylmethyloxycarbonyl
FTIR	Fourier Transform Infrared Spectroscopy
g	gram
h	hour
HPLC	High-Performance Liquid Chromatography
Hz	hertz
Ile	isoleucine
<i>J</i>	coupling constants
k	reaction rate
kcal	kilocalorie
Leu	leucine
LDA	lithium diisopropylamide
LG	leaving group
Lys	lysine
M	concentration (N, mol/dm ⁻³)
M ⁺	parent ion peak (mass spectrum)
m	multiplet
Me	methyl
mg	milligram
MHz	megahertz

min	minutes
mL	milliliters
mmol	millimole
MOM	methoxymethyl
mp	melting point
Ms	methanesulfonyl
NBS	<i>N</i> -bromosuccinimide
NCS	<i>N</i> -chlorosuccinimide
NMR	nuclear magnetic resonance
Nu	nucleophile
OTf	trifluoromethanesulfonate
PD	product
Ph	phenyl
PMB	<i>para</i> -methoxybenzyl
ppm	parts per million
Pr	propyl
q	quartet
s	singlet
SEM	2-(trimethylsilyl)ethoxymethyl
Ser	serine
sep	septet
S _N Ar	nucleophilic aromatic substitution
SPAAC	strain-promoted alkyne-azide cycloadditions
SPPS	solid-phase peptide synthesis
t	triplet

TBAF	tetrabutylammonium fluoride
TBAT	tetrabutylammonium difluorotriphenylsilicate
TBDPS	<i>tert</i> -butyldiphenylsilyl
TBS	<i>tert</i> -butyldimethylsilyl
Tf	trifluoromethanesulfonyl
TFA	trifluoroacetic acid
THF	tetrahydrofuran
THP	tetrahydropyran
TLC	thin layer chromatography
TMEDA	<i>N,N,N',N'</i> -tetramethylethylenediamine
TMS	trimethylsilyl
Ts	<i>para</i> -toluenesulfonyl
Tt	triplet of triplets
Trt	trityl

Chapter 1: General introduction on the reactivity of vinyl azides

Organic azides possess rich chemical reactivity for the synthesis of nitrogen-containing molecules.¹ The use of organic azide for 1,3-dipolar cycloaddition with alkyne² is one of the most frequently used methods for various applications in biology,^{3,4} medicine,⁵ and material chemistry.⁶ Among organic azides, vinyl azides **1-1** present unique chemical reactivities. One of the common reactivities of vinyl azides is the denitrogenative formation of highly strained 3-membered ring azaheterocycles, *2H*-azirines **1-3** through transient vinyl nitrene intermediates **1-2** under thermal treatment, photoirradiation or Lewis acid catalysis (Scheme 1).⁷

Scheme 1 Conversion of vinyl azides into *2H*-azirines

Highly strained and reactive *2H*-azirines can be further utilized for various molecular transformations⁸ of which the most widely used is the synthesis of indoles through the thermal treatment of azido cinnamates through intramolecular aromatic C–H amination (Scheme 2).⁹ This transformation can be mediated by Lewis acid catalysts¹⁰ or by photosensitization under milder reaction conditions.¹¹

Scheme 2 Indole synthesis from azido cinnamates

New synthetic methodologies have recently emerged by leveraging vinyl azides either as a radical acceptor or as an enamine-type nucleophile. This chapter highlights recent methodologies of using vinyl azides in chemical synthesis.¹²

1.1. Reactivity as a radical acceptor

The use of vinyl azide as radical acceptor was first reported by Suzuki's group in 1975.¹³ Trialkylboranes reacted with α -azidostyrene (**1-7**) under aerobic reaction conditions to afford alkyl phenyl ketones **1-8** through the aqueous acid hydrolysis of iminoborane intermediates. In this process, alkyl radicals generated from trialkylboranes under aerobic conditions add onto α -azidostyrene to form iminyl radicals **1-13**¹⁴ through denitrogenation, which react with trialkylboranes to form iminoboranes **1-14** with the release of alkyl radicals that maintain the radical chain. This process is featured by the capability of vinyl azides in new C–C bond formation and the generation of iminyl radicals with organic free radicals (Scheme 1.1-1).

Radical chain mechanism

Scheme 1.1-1 Generation of iminyl radicals from vinyl azide and further transformations

The author's group exploited the Mn(III)-mediated reactions of vinyl azide with 1,3-dicarbonyl compounds **1-15** for the synthesis of various nitrogen-heterocycles **1-21** (Scheme 1.1-2),¹⁵ which are initiated by the addition of α - or β - carbonyl radicals, oxidatively generated from 1,3-dicarbonyl compounds or cyclopropanols, respectively, to vinyl azides **1-7** to form the corresponding iminyl radicals **1-17**. Subsequent reduction of the iminyl radicals with the transient Mn(II) species provides iminyl Mn(III) species **1-18** that cyclize with the carbonyl group to construct 5 membered ring heterocycles **1-19** with the release of the Mn(III) complex.

Scheme 1.1-2 Mn(III)-catalyzed reactions of vinyl azides with 1,3-dicarbonyl compounds or cyclopropanols

For example, substituted *N*-*H* pyrroles can be synthesized by the Mn(III)-catalyzed reactions of vinyl azides with 1,3-dicarbonyl compounds, such as β -keto esters **1-22**, 1,3-diketones **1-25**, and β -keto acids **1-27**. (Scheme 1.1-3).

β -keto esters

1,3-diketone

β -keto acids

Scheme 1.1-3 Mn(III)-catalyzed synthesis of pyrroles from vinyl azides and 1,3-dicarbonyl compounds

In addition, cyclopropanols **1-30**, which act as source of β -carbonyl radicals **1-32**, react with vinyl azide to form iminyl radicals. Subsequent intramolecular cyclization constructs 6-membered ring azaheterocycles **1-33**. For example, the $\text{Mn}(\text{acac})_3$ -catalyzed reaction of vinyl azides with monocyclic cyclopropanols followed by aerobic and acidic treatments provides substituted pyridines via the addition of β -carbonyl radicals generated from cyclopropanols onto vinyl azides (Scheme 1.1-4).¹⁶

Example of pyridines

Scheme 1.1-4 Mn(III)-catalyzed synthesis of substituted pyridine from vinyl azides and cyclopropanols

The Nevado's group recently demonstrated the Ag-catalyzed synthesis of polycyclic ketones using α -azidostyrenes (**1-7**) and aliphatic carboxylic acids **1-38** and **1-40**.¹⁷ In this reaction, C-radicals generated from aliphatic carboxylic acid under the Minisci-type oxidation reaction conditions add to vinyl azides to form iminyl radicals, which in turn induce 1,5-*H* radical shift to generate other the C-radicals. Subsequent radical cyclization of the C-radicals onto the benzene ring completes the process to form the final polycyclic ketones products **1-39** and **1-41** (Scheme 1.1-5).

Scheme 1.1-5 Ag-catalyzed reactions of vinyl azides and aliphatic carboxylic acids

The author's group reported on the radical trifluoromethylation of vinyl azides using TMSCF_3 in the presence of $\text{PhI}(\text{OAc})_2$ and CsF .¹⁸ The reaction is initiated by the generation of CF_3 -radical through the oxidation of TMSCF_3 by $\text{PhI}(\text{OAc})_2$. The resulting CF_3 -radical adds onto vinyl azides **1-17** to form $\alpha\text{-CF}_3$ iminyl radicals **1-47**, which immediately dimerize to produce $\alpha\text{-CF}_3$ azines. The hydrolysis of the resulting azines under acidic conditions affords α -trifluoromethyl ketones **1-46** (Scheme 1.1-6).

Scheme 1.1-6 Formation of α -CF₃-azines through trifluoromethylation of vinyl azides

The transient α -CF₃ iminyl radicals **1-51** can be used for the formation of a N–C bond with a tethered unsaturated bond. This concept was explored in the construction of trifluoroethyl-substituted aza-polycyclic aromatic hydrocarbons **1-50** (aza-PAHs) using readily available α -(biaryl-2-yl)vinyl azides **1-49** (Scheme 1.1-7).¹⁹

Proposed mechanism

Examples of aza-PAHs

Scheme 1.1-7 Synthesis of aza-PAHs through the radical trifluoromethylation of α -(biaryl-2-yl)vinyl azides

In addition to the use of TMSCF_3 and $\text{PhI}(\text{OAc})_2$ for the oxidative generation of the CF_3 -radical, the Studer's group utilized the Togni reagent **1-57** for the reductive generation of the CF_3 -radical and subsequent trifluoromethylation of vinyl azides **1-49** and **1-58**.²⁰ In this case, tetrabutylammonium iodide acted as the electron-donating radical initiator to generate the

CF₃-radical, which adds onto α -(biaryl-2-yl)vinyl azides and 2-azido-*N*-phenylacrylamides to construct trifluoroethylphenanthridines **1-50** and quinoxalinones **1-59**, respectively (Scheme 1.1-8).

The Studer's method: synthesis of aza-PAHs using Togni's reagent

Scheme 1.1-8 Synthesis of aza-PAHs using electron catalyst and α -(biaryl-2-yl)vinyl azides

The Yu's group employed the Umemoto's reagent as a CF₃-radical source to synthesize trifluoroethylphenanthridines **1-50** under visible light photoredox catalysis (Scheme 1.1-9).^{21,22}

The Yu's method: synthesis of aza-PAHs using photoredox catalysis

Scheme 1.1-9 Synthesis of aza-PAHs through photoredox catalyst and α -(biaryl-2-yl)vinyl azides

1.2. Reactivity as an emamine-type nucleophile

Based on chemical structure of vinyl azides, they are expected to have similar nucleophilic reactivity as enamines. Enamines **1-60** undergo nucleophilic attack on electrophile (E⁺) (the Stork-enamine reactions)²³ to provide iminium ions **1-61** (Scheme 1.2-1a). Meanwhile, the reaction of vinyl azides **1-17** with E⁺ should afford the corresponding iminodiazonium ions **1-62** (Scheme 1.2-1b), the reactive intermediates observed in the Schmidt reaction.²⁴ In the Schmidt reaction, the iminodiazonium ions **1-65** undergo 1,2-substituent migration with the elimination of dinitrogen to provide nitrilium ions **1-66**, which are hydrolyzed to the corresponding amides **1-67** (Scheme 1.2-1c).

(a) reaction of emamine (Stork)

(b) reaction of vinyl azide

(c) Schmidt reaction of ketone

Scheme 1.2-1 Nucleophilic reactivity of enamines and vinyl azides

In 1970, the Hassner's group reported the Brønsted acid-mediated formation of amides from 2-azido-3-phenylpropene (**1-67**) (Scheme 1.2-2).^{25, 26} The reaction resulted in the formation of a mixture of *N*-benzylacetamide (**1-68**) and *N*-methylphenylacetamide (**1-69**) in 20% and 40% yields, respectively, along with phenylacetone (**1-70**) in 20% yield that was formed through the hydrolysis of the iminodiazonium ion intermediate (**1-71.1** and **1-71.2**) prior to the 1,2-substituent migration. Iminodiazonium ion intermediates are supposed to stay in equilibrium between their *E*- and *Z*-isomers **1.71.1** and **1.71.2**, and the substituent *anti* to the $N-N_2^+$ bond should undergo 1,2-migration.²⁷

Reaction mechanism

Scheme 1.2-2 Brønsted acid-mediated hydrolysis of vinyl azides

In 2014, the author's group exploited C-electrophiles for the nucleophilic attack of vinyl azides that resulted in the formation of a new C–C bond while affording amide products.²⁸ The processes are capable of affording single amide product with predictable migration selectivity of the substituents.

For example, vinyl azides **1-7** undergo nucleophilic attack on *N*-Ts-imines **1-73** in the presence of $\text{BF}_3 \cdot \text{OEt}_2$ and H_2O , providing β -*N*-Ts-amino amides **1-74** (Scheme 1.2-3). Accordingly, in the reactions between α -azidostyrene (**1-7**) and *N*-Ts-imines **1-73**, *N*-phenyl-amides **1-74** were obtained via 1,2-migration of the phenyl group, which has higher migratory aptitude than the alkyl group.

Scheme 1.2-3 Reaction of vinyl azide with *N*-Ts-imines

The reaction of 1-azidocyclooctenes (**1-77**) with *N*-Ts-imine (**1-73**) resulted in 9-membered ring lactam **1-78** through the hydrolysis of the cyclic nitrilium ion intermediate **1-80**, which is formed via the exclusive migration of the tertiary alkyl group from the iminodiazonium ion **1-79** (Scheme 1.2-4a). Interestingly, the use of 1-azidocyclononenes (**1-81**) provided bicyclic dihydroimidazole **1-82** through the intramolecular cyclization of the 10-membered ring nitrilium ion **1-84** with a tethered Ts-amide moiety (1.2-4b).

a) reaction of 1-azidocyclooctene (8 membered-ring)

b) reaction of 1-azidocyclononene (9 membered-ring)

Scheme 1.2-4 BF_3 -mediated reaction of cyclic vinyl azides with N -Ts-imines

Carbocations generated from the activated alcohols can be trapped by the nucleophilic attack of vinyl azides.^{26,29} For this purpose, triflimide (Trf_2NH) has been found to be an efficient catalyst (Scheme 1.2-5).^{30,31}

Scheme 1.2-5 Tf₂NH-catalyzed synthesis of amide from vinyl azides and activated alcohols

Iminodiazonium ions **1-93** can also serve as the π -polar electrophile for the construction of cyclic structures. For example, the reactions between vinyl azides and *N*-alkenylaldimines **1-91** allow the synthesis of highly substituted pyridines after DDQ oxidation (Scheme 1.2-6).³². The process is mediated by the intramolecular C–C bond formation between the iminodiazonium ion **1-93** and the enamine moieties **1-94** in the intermediates formed by the nucleophilic attack of vinyl azides.

Selected examples

Scheme 1.2-6 Synthesis of pyridine from vinyl azides and *N*-alkenylimines

The conjugate addition of enamines **1-60** to α,β -unsaturated carbonyl compounds can afford aminocyclobutanes **1-99** via the intramolecular C–C bond formation between the resulting iminium ion and enolate **1-98** (Scheme 1.2-7a).²¹ Vinyl azides **1-17** have similar reactivity as enamines; therefore, the reaction of vinyl azides with conjugate electrophiles is expected to provide azidocyclobutane **1-101** intermediates through cyclization onto iminodiazonium ions **1-100** (Scheme 1.2-7b).

a) conjugate addition of enamines (Stork)

b) conjugate addition of vinyl azide

Scheme 1.2-7 Conjugate addition of enamines and vinyl azides

The author's group discovered the synthesis of 1-pyrroline derivatives from Lewis acid-mediated reactions of vinyl azides with α,β -unsaturated esters via the denitrogenative ring expansion of azidocyclobutane intermediates where the structure of the conjugate electrophiles uniquely changes the substitution pattern of the 1-pyrroline skeletons derived from vinyl azides (Scheme 1.2-8).³³ For example, 3',4'-dihydrospiro[indoline-3,2'-pyrrol]-2-ones (**1-102**) can be afforded in diastereoselective fashion from the reactions of vinyl azides with 3-alkylidene-2-oxoindolines (**1-103**) (Scheme 1.2-8a). The diastereoselective formation of azidocyclobutane intermediates (**1-105**) is followed by the denitrogenative ring expansion of azidocyclobutanes through the migration of the quaternary carbon from the 2-oxoindoline core to the nitrogen atom. Meanwhile, 4,5-dihydro-3*H*-pyrroles **1-108** can be afforded from reactions with dimethyl 2-alkylidenemalonates **1-106** (Scheme 1.2-8b). The process involves cleavage of the C=C bond of vinyl azides. In this case, ring expansion occurs via the migration of the secondary carbon (marked in red) predominantly over that of the quaternary carbon, which is electronically deactivated by two methoxycarbonyl groups.

a) reaction with 3-alkylidene-2-oxindolines

b) reaction with 2-alkylidenemalonates

Scheme 1.2-8 Construction of 1-pyrroline through the conjugate addition of vinyl azides

Donor–acceptor cyclopropanes³⁴ were used as three-carbon electrophiles to carry out the reaction with vinyl azides.^{35,36} The author's group recently discovered the method to synthesize highly functionalized azidocyclopentanes **1-112** in diastereoselective fashion by treating vinyl azides and donor–acceptor cyclopropanes **1-111** in the presence of $\text{Sc}(\text{OTf})_3$ as a Lewis acid catalyst. In this reaction, the nucleophilic attack of vinyl azides **1-17** on

cyclopropanes **1-111** likely occurs in an S_N2 manner to form iminodiazonium ions **1-114** having a γ -carbanion. Intramolecular cyclization subsequently occurs to afford the final products **1-112** (Scheme 1.2-9).

Scheme 1.2-9 Reaction of vinyl azides with donor–acceptor cyclopropanes

The López's group reported the Cu(I)-mediated synthesis of 3-azidocyclopentenones **1-115** from vinyl diazo compounds and vinyl azides (Scheme 1.2-10).³⁷ This process is initiated by the formation of vinyl carbene-Cu complexes, which are attacked by vinyl azides to construct the first C–C bond. The resulting iminodiazonium ion moiety **1-117** is subsequently attacked by the transient vinyl copper species to construct cyclopentene scaffolds **1-118**. Allylic rearrangement affords the final products **1-116**.

Scheme 1.2-10 Reaction of vinyl azides with vinyl diazo compounds

The Cui's group discovered a unique reactivity of 3-azido-2-methyl-3-buten-2-ol.³⁸ The corresponding iminodiazonium ion intermediate **1-122** formed by the conjugate addition of **1-120** onto *p*-quinone methide (**1-119**) undergoes fragmentation to release dinitrogen and acetone to provide nitrile.

Scheme 1.2-11. Synthesis of nitrile products from 3-azido-2-methyl-3-buten-2-ols

1.3. Reactivity of 2-azidoacrylates as a conjugate electrophile

A specific type of vinyl azides, 2-azidoacrylates, can serve as an electrophile for 1,4-addition of stabilized carbon nucleophiles.

In 2010, the Yu's group reported the construction of pyrrolo[1,2- α]pyrazine **1-128** by the reaction of 2-formylpyrrole **1-126** and azido cinnamate **1-127** in the presence of Cs_2CO_3 at 50 °C (Scheme 1.3-2).³⁹ This formal [3+3]-annulation is initiated by the conjugate addition of the pyrrole nitrogen onto azido cinnamate **1-127** that induces simultaneous denitrogenation to form iminyl anion **1-130**. Subsequent intramolecular dehydrative cyclization leads to the final product **1-128**.

Scheme 1.3-1 Synthesis of pyrrolo[1,2- α]pyrazines from 1*H*-2-pyrrolecarbaldehyde and azido vinylidene

In 2011, the author's group demonstrated the use of 2-azidoacrylates as a Michael acceptor of active methine carbon nucleophiles in the presence of organic diamine catalysts.⁴⁰ The reactions proceed under mild reaction conditions to afford α -azidoesters with a newly formed C–C bond in good yields.

Scheme 1.3-2 Diamine-catalyzed conjugate addition to 2-azidoacrylate

1.4. Thesis perspective

As described in previous sections, various synthetic methodologies using vinyl azides have been developed mainly for the synthesis of N-containing molecules by taking advantage of their versatile chemical reactivity. As such, the author wondered if vinyl azides, owing to their reactivity as the enamine-type nucleophile and the conjugate electrophile, can work as a new chemical linker to prepare useful conjugates for applications in biology, medicine, and material science. In the Ph.D. study, the author aimed to develop conceptually novel strategies for the chemical linking of alcohols and thiols found in various biological active compounds with vinyl azides. Based on the results, the following linking protocols can be developed using vinyl azides.

1.4.1 Linking alcohols with vinyl azides

The author's group recently utilized vinyl azides as an enamine-type nucleophile for the synthesis of amides and nitrogen heterocycles³⁰⁻³⁴ where the transient iminodiazonium ion intermediates undergo Schmidt-type 1,2-substituent migration or act as the π -polar electrophile for the intramolecular C–C bond formation. Accordingly, the author demonstrated the capturing of iminodiazonium ions generated through electrophilic fluorination or bromination of vinyl azides by external alcohols in an intermolecular manner. The resulting linking products,

α -alkoxy- β -fluoroalkylazides, can be used for the synthesis of nitrogen-containing molecules and the bioimaging application of the cell surface. These results are discussed in Chapter 2.

Scheme 1.4.1 Linking vinyl azide with biological active molecules and bioimaging applications

1.4.2 Site-specific dual functionalization of cysteine residue in peptides and proteins with 2-azidoacrylates

Having demonstrated that 2-azidoacrylates can serve as the conjugate electrophile with active methine nucleophiles (Scheme 1.3-2), the author became interested in using 2-azidoacrylates as conjugate linkers for the thiols. In Chapter 3, the author described the use of functionalized 2-azidoacrylates as the Michael acceptor for cysteine residue of peptides and bovine serum albumin (BSA), a native protein containing one free cysteine residue. Installation of azido moiety and functionalized cysteine containing peptides and proteins can be achieved by the conjugation of the sulfhydryl group of the cysteine residue to 2-azidoacrylates bearing various functionalities, such as fluorescent dyes under physiological aqueous buffer conditions. Subsequent azide-alkyne cycloaddition introduces the second functionality, thus affording dual-functionalized peptide- and protein-based materials.⁴¹

Scheme 1.4.2 Dual functionalization of protein using 2-azidoacrylates as sulfhydryl-specific linker

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Chapter 2 Linking of alcohols with vinyl azides

2.1 Introduction

Organic molecules possessing azido functionalities (organic azides) are recognized as synthons with high synthetic potential for constructing nitrogen-containing compounds, especially nitrogen heterocycles.¹ Organic azides show the unique nature as 1,3-dipole, and they are thus commonly used in [3+2]-cycloaddition with various unsaturated compounds such as alkynes, alkenes, and nitriles. Among these reactions, cycloaddition between an azide and an alkyne² to construct a 1,2,3-triazole is often applied in medicinal chemistry³, chemical biology,⁴ and material science.⁵ In the medicinal and biological applications, the alkyne-azide [3+2]-cycloaddition process can be utilized in bioimaging, drug delivery, and bioconjugation.⁶ Therefore, it is essential to install azido functional groups at specific locations in organic molecules of interests.

Hydroxyl groups (alcohols) are often found in various biologically active molecules.⁷ One of the most common methods of converting alcohols into azides is by substituting the pre-activated hydroxyl group with an azido anion.^{8,9} Typically, alcohols are activated with tosylation or mesylation, which is followed by the addition of azido salts for the S_N2 substitution to obtain the desired organic azides (Scheme 2.1-1).

Scheme 2.1-1 Activation of alcohol and S_N2 azidation

On the other hand, several azidation reagents have been developed for direct transformation of alcohols to azides. In 2012, the Kitamura's group reported use of 2-azido-1,3-dimethylimidazolium ion salts (ADMP) for direct substitution of free hydroxyl groups for the synthesis of organic azides (Scheme 2.1-2).^{9a}

Scheme 2.1-2 Direct conversion of aliphatic alcohol to an azide using ADMP

In 2000, the Hu's group reported a one-pot synthesis of organic azides from alcohols using in-situ generated bis(2,4-dichlorophenyl)-phosphoryl 4-(dimethylamino)pyridinium azide (**2-A**) from 4-dimethylaminopyridine (DMAP) and NaN_3 (Scheme 2.2-3).^{9b}

Scheme 2.1-3 One-pot conversion of aliphatic alcohols to azides

The above transformations of alcohols into organic azides involve interconversion of the C-OH bond into the C-N₃ bond. On the other hand, the protocol to install the azide unit onto the alcohols would be an alternative strategy to use ubiquitous alcohols for further applications. In this regard, the Romo's group demonstrated use of functionalized α -diazo esters for installation of chemical probes onto hydroxyl group-containing bioactive molecules through Rh(II)-catalyzed carbene insertion into the O-H bond (Scheme 2.1-4).¹⁰ As an example of the bio-applications, thus-formed alkyne-installed compounds can be further modified by the click reaction.

Selected example

fumagillol 63%

yohimbine 65%

Scheme 2.1-4 Linking alcohol with alkyne

The author's group recently disclosed a new chemical reactivity of vinyl azide as an enamine type nucleophile. For example, vinyl azides undergo nucleophilic attack to carbon electrophile (E⁺: for example *N*-Ts aldimines) in the presence of acid activators, forming iminodiazonium ion **2-B** with a newly formed C-C bond.^{11a} Subsequent Schmidt-type 1,2-

50

Scheme 2.1-6 Reaction of vinyl azide with *N*-alkenylimines

2.2 Working hypothesis and the aim of the project

Based on the chemical reactivity of the iminodiazonium ions, the author conceived that a linking method of alcohols with vinyl azides could be achieved through electrophilic halogenation of vinyl azides **2-1** in the presence of alcohols **2-2**, in which the initially formed α -halo-iminodiazonium ions **2-G** could be trapped by external alcohols **2-2** (Scheme 2.2). Neighboring group participation of the installed halogen atom (**X**) to the C=N bond might result in stabilization of iminodiazonium ions **2-G** through halonium ions **2-H**, preventing formation of nitrilium ions by migration of the substituent R. In this chapter, the author describes the development of this concept with the fluoro- or bromo-alkoxylation of vinyl azides. Alcohols

are uniquely connected to haloalkylazide moieties in the resulting unprecedented amination-type structure. Concise transformation of the products, α -alkoxy- β -haloalkylazides to halomethyl nitrogen-heterocycles was demonstrated using thermal intramolecular [3+2]-azide-alkene/alkyne cycloaddition. Moreover, tagging of different types of fluorescent probes to testosterone-linked azide was implemented by the click reactions. The as-prepared fluorescent testosterone derivatives having cyanine probes exhibited unique properties for rapid imaging of cell membranes in real time.

Scheme 2.2 Linking alcohols with vinyl azides through haloalkoxylation

2.3 Results and discussion

2.3.1 Preparation of vinyl azide 2-1

The vinyl azide **2-1a** was synthesized through the Hassner's method¹² from styrene. Regarding **2-1b**¹³, **2-1c**¹⁴, **2-1d**¹⁵, **2-1e**¹², **2-1f**¹⁵, **2-1g**¹⁴, **2-1h**¹⁶ and **2-1i**¹², they were known compounds and were prepared according to the reported procedures (Scheme 2.3.1).

Scheme 2.3.1 Synthesis of vinyl azides **2-1a** to **2-1i**

2.3.2 Optimization

Vinyl azide (**2-1a**) and cinnamyl alcohol (**2-2a**) were used as model substrates for the optimization of the reaction conditions (Table 2.3.2.1 and Table 2.3.2.2). For the formation of α -alkoxy- β -fluoroalkyl azides, four types of F^+ ion sources were screened (Table 2.3.2.1). Although almost no formation of desired linking product **2-3aa** was observed in the reactions with 1-fluoro-2,4,6-trimethylpyridinium tetrafluoroborate (entry 1), 1-fluoropyridinium tetrafluoroborate (entry 2), and *N*-fluorobenzenesulfonimide (entry 3), the reaction with Selectfluor^{17,18} afforded **2-3aa** in 80% yield within 6 h (entry 4).

Table 2.3.2.1 Reactions with F^+ sources ^a

<p> $\text{N}=\text{N}^+-\text{N}^-$ (Ph-CH=CH-) 2-1a (1.2 equiv) + 2-2a $\xrightarrow[\text{MeCN conditions}]{[F^+] (1.2 \text{ equiv})}$ 2-3aa </p>			
Entry	$[F^+]$ source	Condition	2-3aa yield [%] ^b
1		23 °C 24 h, then 40 °C, 28 h	0 (2-1a was decomposed)
2		23 °C 24 h, then 40 °C, 30 h	0 (2-1a was decomposed)
3		23 °C 21 h	9 (2-1a was decomposed)
4		0-23 °C 6 h	80

^a The reaction were preformed in 0.3-0.4 mmol scale under N_2 atmosphere; ^b Isolated yield

Several Br⁺ ion sources were next examined for the formation of α -alkoxy- β -bromoalkyl azides (Table 2.3.1.2). The reaction with *N*-bromosuccinimide (NBS) gave the corresponding alcohol-linked product **2-4aa** in 74% yield along with the succinimide adduct **2-15** in 13% yield (entry 1). Changing the reaction solvent from MeCN to CH₂Cl₂ did not significantly improve the reaction efficiency (entry 2). Use of *N*-bromophthalimide afforded **2-4aa** in 73% yield along with α -bromoacetophenone (**2-16**) in 36% yield (entry 3). The reaction with 2,4,4,6-tetrabromocyclohexa-2,5-dienone (TBCO)¹⁹ delivered **2-4aa** in 82% yield as a sole product. Other Br⁺ ion sources did not provide the desired product **2-4aa** at all (entries 5-8).

Table 2.3.2.2 Reactions with Br⁺ sources ^a

<p> <chem>N=[N+]N=Cc1ccccc1</chem> (2-1a, 1.5 equiv) + <chem>Ph-CH=CH-CH2OH</chem> (2-2a) $\xrightarrow[\text{conditions}]{[\text{Br}^+] (1.5 \text{ equiv})}$ <chem>Ph-CH=CH-CH2-O-C(Ph)(Br)-N=[N+]N</chem> (2-4aa) + Others </p>				
Entry	[Br ⁺] source	Condition	2-4aa yield [%] ^b	Others
1	<p>(NBS)</p>	0-23 °C, MeCN 0.5 h	74% (7%) ^c	<p>2-15 13 % ^d</p>
2	NBS	0-23 °C, CH ₂ Cl ₂ 0.5 h	79% (8%)	2-15 38 % ^d
3		0-23 °C, CH ₂ Cl ₂ 1 h	73%	<p>2-16 36% ^d</p>
4		0-23 °C, CH ₂ Cl ₂ 0.5 h	82	-
5		0-23 °C, MeCN 15 min	0% (Complex mixture)	-
6		0-23 °C, MeCN 1 h	0% (Complex mixture)	-
7		0-23 °C, MeCN 0.5 h	0% (Complex mixture)	-
8	<p>SbCl₅Br</p>	0-23 °C, MeCN 0.5 h	0% (Complex mixture)	-

^a The reaction were preformed in 0.3-0.4 mmol scale under N₂ atmosphere; ^b Isolated yield; ^c Recovery yield of **2-2a** calculated by ¹H NMR. ^d ¹H NMR yield based on vinyl azide **2-1a**.

2.3.3. Scope of vinyl azide **2-1**

With the optimized reaction conditions in hand, we next investigated the scope of vinyl azides **2-1** for both fluoro- and bromo-alkoxylation reactions using cinnamyl alcohol (**2-2a**). As depicted in Table 2.3.3, a range of α -arylvinyl azides **2-1b-2-1f** could be converted to the corresponding β -fluoroalkyl azides **2-3ba-2-3fa** and β -bromoalkyl azides **2-4ba-2-4fa** in good yields (entries 2-6). α -Alkyl-substituted vinyl azide **2-1g** was also viable for both the transformations (entry 7). Cyclic vinyl azide **2-1h** could be used to produce cyclic β -fluoroalkyl azide **2-3ha** as a mixture of two diastereomers in good yield (entry 8). The reaction of vinyl azide **2-1i** bearing an allyl ether unit proceeded chemoselectively to link **2-1a** with the vinyl azide moiety keeping the allyl ether part intact (entry 9).

Table 2.3.3 Substrate scope of vinyl azides, **2-1**^a

Condition A 2-1 (1.2 equiv)			Condition B 2-1 (1.5 equiv)		
Entry	vinyl azides 2-1	Product 2-3 (X = F) 2-4 (X = Br) yield [%] ^b	Entry	vinyl azides 2-1	Product 2-3 (X = F) 2-4 (X = Br) yield [%] ^b
1	2-1a (R = Ph)	2-3aa 80% 2-4aa 82%	7		 2-3ga 57% 2-4ga 64%
2	2-1b (R = 4-Me-C ₆ H ₄)	2-3ba 86% 2-4ba 80% ^c	8		 2-3ha 74% (dr = 1:1) ^e
3	2-1c (R = 4-Cl-C ₆ H ₄)	2-3ca 74% 2-4ca 77% ^c	9		 2-3ia 60% (dr = 1.2:1) ^e
4	2-1d (R = 4-CO ₂ Me-C ₆ H ₄)	2-3da 55% 2-4da 87%			
5	2-1e (R = 3-Br-C ₆ H ₄)	2-3ea 73% ^{c,d}			
6	2-1f (R = 2-naphthyl)	2-3fa 79% 2-4fa 97%			

^a Condition **A**: The reactions were carried out by treatment of cinnamyl alcohol (**2-2a**) and vinyl azides **2-1** (1.2 equiv) with Selectfluor (1.2 equiv) in MeCN (0.1 M) at 0 °C to room temperature under a N₂ atmosphere; Condition **B**: the reactions were carried out by treatment of cinnamyl alcohol (**2-2a**) and vinyl azides **2-1** (1.5 equiv) with TBCO (1.5 equiv) in CH₂Cl₂ (0.1 M) at 0 °C under a N₂ atmosphere. ^b Yields of isolated products. ^c NMR yields using 1,1,2,2-tetrachloroethane as an internal standard. ^d Due to instability of **2-3ea**, the crude residue including **2-3ea** was directly treated under thermal [3+2]-cycloaddition conditions to form **2-8ea**. ^e Diastereoselectivity (dr) was determined by ¹⁹F NMR.

2.3.4 Scope of alcohols

Next, the compatibility of these protocols was examined by linking vinyl azide (**2-1a**) with a wide range of alcohols (**2-2**; Table 2.3.4). Both alkenyl (**2-2b** with aryl-substituted alkene and **2-2c** with terminal alkene) and alkynyl (**2-2d-2-2f** with both terminal and internal alkynes) alcohols reacted smoothly, providing the corresponding β -haloalkylazides **2-3** and **2-4** in good yields. Other functional groups such as *N*-tosylindolyl (for **2-2g**), phenylsulfonyl (for **2-2h**), diethyl phosphoryl (for **2-2i**), and pinacolboryl (for **2-2j**) moieties were all compatible under the present reaction conditions. In the reactions of diol **2-2k** having primary and secondary hydroxyl groups, the primary one was preferentially linked (for **2-3ak** and **2-4ak**). For linking of 3,5-dimethylphenol (**2l**), the bromination protocol with TBCO only worked to give **2-4al**, while the reaction was sluggish due to over-bromination. We next investigated to tag natural products and their derivatives having hydroxyl group(s) in their structure.²⁰ Glucose derivative **2-2m**²¹ and *rac*-menthol (**2-2n**) were readily employed for the present linking protocol. It was found that nopol (**2-2o**) and nerol (**2-2p**) having reactive alkenes (strained and/or electron-rich) are not applicable for the linking protocol with Selectfluor, while that with TBCO delivered the corresponding azides **2-4ao** and **2-4ap** in good yields. Steroids such as androsterone (**2-2q**) and testosterone (**2-2r**) could be anchored well with both protocols, while the bromination method was only amenable to linking of cholesterol (**2-2s**) and betulin (**2-2t**) having an electron-rich alkene. In the reaction of betulin (**2t**), its primary hydroxyl group was selectively linked, while the secondary one next to the quaternary carbon was kept intact due to the steric hindrance.

Table 2.3.4 Substrate scope of alcohols ^{a, b}

^a Unless otherwise noted, the reactions were carried out by treatment of alcohols **2-2** and vinyl azide **2-1a** (1.2–2.2 equiv) with Selectfluor (1.2–2.2 equiv) in MeCN (conditions A) or TBCO (1.2–2.2 equiv) in CH₂Cl₂ (conditions B) at 0 °C to 23 °C under a N₂ atmosphere.; ^b Isolated yields and diastereoselectivity (dr) determined by ¹H and ¹⁹F NMR measurements were recorded above.; ^c Isolated yields of the linking product of the secondary hydroxyl group.; ^d The reaction was conducted in MeCN/CH₂Cl₂ (5:1).; ^e The reaction was conducted in CH₂Cl₂/THF (1:1).

2.3.5 Chemical transformation of the linked products

To validate the utility of the present protocol, further chemical transformation of the linked products was performed by intramolecular 1,3-dipolar cycloaddition reaction of the azide moiety with the alkene or alkyne tether (Scheme 2.3.5). Alkenyl alcohol-linked azide under thermal reaction conditions produced a unique nitrogen heterocycle, 2-(halomethyl)-2,5-dihydrooxazoles (**2-5aa** and **2-6aa**), and 2-(fluoromethyl)-5,6-dihydro-2*H*-1,3-oxazine (**2-7ab**) in high yields. Furthermore, intramolecular 1,3-dipolar cycloaddition reaction between azide and alkyne (for **2-3ae** and **2-3af**) also afforded the corresponding bicyclic triazole products **2-8ae** and **2-9af**.

^a Two-step yield from cinnamyl alcohol **2-2a**

Scheme 2.3.5 Intramolecular azide-alkene/alkyne cycloaddition

2.3.6 Bioimaging

The author next explored the potential applicability of the present linking protocol in bioimaging. As indispensable important cell components, steroids have been found to maintain cell membrane's integrity and fluidity as well as to secure the membrane-associated biological activities in living cells. It has been reported that several dye-labeled steroid analogues can be used for specific membrane staining.^{22,23} Thus, testosterone-linked azide **2-3ar** was chosen, and the click reactions of **2-3ar** and alkynes tethered to fluorescent probes such as *N*-propargyl dansylamide and azadibenzocyclooctyne-cyanine dyes (DBCO-Cy3 and DBCO-Cy5) were implemented to prepare **2-10-2-12** (see Section 2.3.6.1 and 2.3.6.2). It is noteworthy that the resulting triazole-linked aminal-type moiety was fairly stable under physiologically relevant serum conditions (pH 7.4) and in aqueous phosphate buffer solution (pH 7.6–3.0) (see Section 2.3.6.3). Moreover, MTT assay showed that there was little cell viability impaired during the process of cell staining, indicating less toxicity and suitable bio-compatibility of the triazole-linked aminal-type moiety in living cell applications (see Section 2.3.6.4).

2.3.6.1 Cu-catalyzed click reaction of 2-3ar

Dansyl-linked testosterone **2-10** was prepared through Cu-catalyzed click reaction. The conditions of Cu-catalyzed click reaction screened were shown in Table 2.3.6.1-1. Among the three conditions screened, using CuTc as the catalyst and *N,N*-diisopropylethylamine as the base provided **2-10** in the highest yield, 93% (entry 3).

Table 2.3.6.1-1 Optimization of Cu-catalyzed click reaction conditions

Entry	Cu cat. (mol %)	Additive (equiv)	Conditions	2-10 isolated yield [%]
1	CuSO ₄ ·5H ₂ O (20 mol%)	Sodium ascorbate (0.6 equiv)	H ₂ O/tBuOH/THF (1:1:1.5) 23 °C, 6.5 h	85
2	CuTc (20 mol%)	DIPEA (2.0 equiv)	DMF, 60 °C, 0.5 h	82
3	CuTc (10 mol%)	DIPEA (1.5 equiv)	DMF, 23 °C, 6.5 h	93

2.3.6.2 Cu-free azide-alkyne cycloaddition of 2-3ar (Synthesis of 2-11 and 2-11)

The synthesis of **2-11** and **2-12** was achieved by mixing DBCO-Cy3 and testosterone-linked azide **2-3ar** in DMSO at 23 °C for 24 h. The products were purified by High-Performance Liquid Chromatography (HPLC).

Scheme 2.3.6.2-1 Synthesis of **2-11** from DBCO-Cy3 and testosterone-linked azide **2-3ar**

Scheme 2.3.6.2-2 Synthesis of **2-12** from DBCO-Cy5 and testosterone-linked azide **2-3ar**

2.3.6.3 Stability test of **2-10**

The stability test of **2-10** performed under various physiological conditions. Dansyl-linked testosterone **2-10** showed high stability under various kinds of aqueous conditions (Table 2.3.6.3, entries 1–6). However, **2-10** was not stable in the solution containing the strong acid HCl at 23°C. The compound **2-10** was fragmented into testosterone (**2-2r**) and 2-fluoro-1-phenylethane-1-one (Table 2.3.6.2, entry 7). Therefore, it is worth noting that triazole-linked aminal-type moiety **2-10** is reasonably stable under physiologically relevant serum conditions.

Table 2.3.6.3 Stability check of **2-10**

Entry	Conditions	Recovery of 2-10 [%] ^a	Yield of 2-2r [%] ^b	Yield of F [%] ^b
1	pH = 7.4 PBS/CH ₃ CN (1:1), 37 °C, 24 h	86	0	0
2	pH = 7.4 FBS /CH ₃ CN (1:1), 37 °C, 24 h	80	0	0
3	pH = 7.4 10% FBS in PBS/CH ₃ CN (1:1), 37 °C, 24 h	79	0	0
4	pH = 7.4 10% FBS in Culture Medium/CH ₃ CN (1:1), 37 °C, 24 h	80	0	0
5	pH = 6.6 phosphate buffer / CH ₃ CN (1:1), 37 °C, 24 h	98	0	0
6	pH = 3.0 phosphate buffer/ CH ₃ CN (1:1), 37 °C, 24 h	88	4	4
7	1N aq. HCl (2 equiv), THF, 23 °C, 18 h (0.05 M)	16 ^b	46	50

^a Yield was determined by ¹⁹F NMR using fluorobenzene as an internal standard. ^b Yield was determined by ¹H NMR using 1,1,2,2-tetrachloroethane as an internal standard.

2.3.6.4 Cytotoxicity test of 2-10

Furthermore, MTT assay was used to confirm that the cell viability damage was minimal during the process of cell-staining (Figure 2.3.6.3). This experiment also indicated cytotoxicity and biocompatibility of triazole-linked aminal-type moiety for application in the living cell.

Figure 2.3.6.4 Cytotoxicity for different concentrations of **2-10** at the human embryonic kidney 293 (HEK293) cell line

2.3.6.5 Fluorescence imaging of human embryonic kidney 293

Human embryonic kidney 293 (HEK293) cells were then incubated with **2-10-2-12** (2.0 μ M). The capability of these dye-linked testosterone **2-10-2-12** as a membrane staining probe was examined by confocal microscopy. As shown in Figure 2.3.6.4, while fluorescence of dansyl probe **2-10** was found inside the cells rather than on the membrane surface probably due to the rapid internalization of dansyl probe **2-10** into the cells (Figure 2.3.6.4 a), the strong fluorescence signals of **2-11** and **2-12** were found on the cellular membrane (Figures 2.3.6.4 c and 2.3.6.4 d). These observations are most likely attributed to the difference of the charge properties between dansyl-linked testosterone **2-10** and cyanines-linked ones **2-11** and **2-12**. The fluorescence signals from dansyl probe **2-10** and organelle specific dyes (BODIPY TR ceramide, one commonly used Golgi apparatus tracker) were overlapped (Figure 2.3.6.4 b) and it indicated more prominent localization in Golgi apparatus than other major organelles. Further investigation is undergoing to account for such interesting difference between dansyl probe **2-10** and cyanines-linked probes **2-11** and **2-12**.

Cells incubation with (a) dansyl **2-10** (2 μ M) for 5 min, $\lambda_{ex} = 405$ nm; (b) fluorescence signals overlap of dansyl **2-10** with BODIPY TR ceramide for the Golgi apparatus staining; (c) Cy-3 **2-11** (2.0 μ M) for 2 min, $\lambda_{ex} = 514$ nm; and (d) Cy-5 **2-12** (2.0 μ M) for 2 min, $\lambda_{ex} = 633$ nm. The cell nucleus staining (c) and (d) by Hoechst 33258 ($\lambda_{ex} = 405$ nm) was used as control. Scale bar in all images = 20 μ m.

Figure 2.3.6.5 Dye-linked testosterone **2-10-2-12** and fluorescence imaging of live HEK293 cells

2.4 Conclusion

In conclusion, the linking of alcohols with vinyl azides was demonstrated with a wide range of alcohols through electrophilic fluorination or bromination of vinyl azides. The linked products, α -alkoxy- β -haloalkyl azides could be used for construction of unique nitrogen-containing heterocycles as well as for bioimaging of the cell surface.

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Chapter 3: Site-specific Dual Functionalization of Cysteine Residue in Peptides and Proteins with 2-Azidoacrylates

3.1 Introduction

The chemical modification of natural proteins could open many possibilities for biological and medical applications, such as probing protein dynamics *in vitro* and *in vivo*,¹ investigating protein structure² in biological systems and therapeutic efficacy³, developing artificial enzymes or protein-based materials,⁴ and constructing protein–drug conjugation.⁵ However, there are several synthetic challenges to overcome in the chemical modification of proteins, such as the installation of a chemical function of interest in a regioselective fashion.⁶ In addition, the modification reaction must possess fast reaction rate under physiological conditions as it is often conducted under high dilution reaction conditions.⁷

Among the most common 20 amino acids, cysteine is one of the most popular targets for protein modification because of the high nucleophilicity of its sulfhydryl group⁸ and its low natural abundance. In addition, adapting site-directed mutagenesis could easily introduce the sulfhydryl group into a protein. Therefore, various methods for cysteine-targeted protein modification have thus far been developed by many scientists. This section summarizes commonly used cysteine-specific linkers for the chemical functionalization of protein.

3.1.1 α -Haloalkyl reagents

One of the most common methods for cysteine-specific protein modification is substitution with bromoalkyl and iodoalkyl substrates (Scheme 3.1.1.1). However, the chemoselectivity of this method is lower as the sulfhydryl group of cysteine will compete with other nucleophilic side chains such as the amino group of the lysine residue and the imidazole group of the histidine residue.

Scheme 3.1.1.1 Examples of reagents used for cysteine-specific protein modification through nucleophilic substitution

Davis's group described the use of *para*-iodobenzyl bromide as the linker for modifying natural protein containing the cysteine residue (Scheme 3.1.1.2).⁹ The benzyl bromide reacts with the sulfhydryl group in the protein to form the corresponding thioether-linked *para*-iodophenyl moiety, which can be used for further modification by the Suzuki–Miyaura coupling to link another functional group at ambient temperature.

Scheme 3.1.1.2 Suzuki-Miyaura cross-coupling on a protein surface

Moreover, the Allemann's group demonstrated protein glycosylation using iodoacetamides as the linker (Scheme 3.1.1.3).¹⁰ As a result, they observed an increase in the stability of the modified proteins.

Scheme 3.1.1.3 Glycosylation of EcDHFR

Random acylation of the amino group in RNase with folic acid decreases its catalytic activity. This problem was overcome by the Raines' group (Scheme 3.1.1.4).¹¹ They attached the bromoacetamido group onto folic acid to promote specific alkylation to cysteine in RNase, resulting in prevention from random acylation with other functional groups. Therefore, the pendant folate moiety achieved therapeutic purpose without decreasing the enzymatic activity of the RNase.

Scheme 3.1.1.4 Cysteine-specific folate conjugation with the RNase

3.1.2 Perfluoroaromatic molecules

The Pentelute's group reported using nucleophilic aromatic substitution (S_NAr) of perfluoroaromatic molecules with the cysteine residue in peptides and proteins for their chemical modification (Scheme 3.1.2).¹² Perfluoroaromatic molecules can staple peptides or proteins having two cysteine residues, to increase structural stability of the tri-helical affibody. This stapling of the proteins by the authors enhanced binding ability, cell permeability, and overall stability. Furthermore, enhancement of the affibody's stability was achieved when the protein residue was stapled. However, conjugation yields of perfluoroaromatic molecules and cysteine were lower due to the poor solubility and reactivity of these molecules in aqueous solvents.

Scheme 3.1.2 S_NAr reaction between perfluoroaromatic molecules and cysteine residue in peptide and protein, and stapling of proteins

3.1.3 Maleimides

Maleimides act as a Michael acceptor in the reaction with the cysteine residues, to form thiosuccinimides.¹³ Originally, maleimides were used as blocking reagents for the sulfhydryl group within proteins for analytical purposes and for mapping the cysteine residue in proteins.¹⁴ Recently, increasing attention has been given to the use of maleimides in the chemical

modification of proteins. To date, the maleimide-cysteine conjugation has become the most reliable tool for the cysteine-targeted chemical modification of proteins. There are several advantages of using maleimides for chemical conjugation with the cysteine residue, such as high selectivity towards the sulfhydryl group with very fast reaction rate¹⁵. Furthermore, the reaction can also be conducted at ambient temperature in aqueous media in the absence of any additional chemical reagents. However, conjugated succinimide thioethers sometimes undergo retro-Michael reaction to release free maleimide in the solution (Scheme 3.1.3.1).

Scheme 3.1.3.1 Ring opening and retro-Michael addition maleimide-thiol conjugates

In 2009, Baker and Caddick's group reported the use of dibromomaleimide to show its bioorthogonality and reactivity towards the chemical modification of cysteine-containing peptides and proteins (Scheme 3.1.3.2).¹⁶ The coupling between dibromomaleimide and cysteine forms a thiol-maleimide bond by the addition-elimination sequence. The remaining second bromide can be further modified through the addition of another thiol. In most cases, only one equivalent of dibromomaleimide is required to modify the proteins under mild reaction conditions and with a shorter reaction time. Furthermore, the thiol-maleimide bond can be cleaved by adding tris(2-carboxyethyl)phosphine (TCEP) or thiol in large excess amounts.

Scheme 3.1.3.2 Modification of protein with dibromomaleimide

3.1.4 Allenamide derivatives

The team of Loh and Xing recently discovered that allenamides selectively react with the cysteine residue in peptides and proteins (Scheme 3.1.4).¹⁷ Interestingly, the resulting conjugates, vinyl sulfides show excellent chemical stability, as there is no sign of hydrolysis or polymerization.

Scheme 3.1.4 Protein modification with allenamide derivatives

3.1.5 β -Acyl acrylamides

The Bernarde's group reported the chemical modification of natural protein using β -acyl acrylamides (Scheme 3.1.5).¹⁸ Rapid thiol-Michael addition of the cysteine residue onto β -acyl acrylamides could be recorded under biocompatible reaction conditions with the excellent chemoselectivity towards the sulfhydryl group. It should be noted that the resulting product is very stable, not undergoing the retro thiol-Michael reaction. This protocol was used for bioimaging applications with the β -acyl acrylamide having the probe.

Scheme 3.1.5 Modification of protein with β -acyl acrylamide reagents

3.1.6 Other linkers

3.1.6.1 Vinyl sulfone

In 1988, Masri and Friedman discovered a Michael addition between the cysteine residue in bovine serum albumin (BSA) and aliphatic vinyl sulfones (Scheme 3.1.6.1).¹⁹ In addition to the conjugation with the cysteine residue, vinyl sulfone was found to react with histidine and lysine residue under lower pH conditions. These side reactions with lysine and histidine can be prevented by lowering the equivalents of vinyl sulfones. The advantages of using vinyl sulfone is that the conjugated thioether bond is more stable than the thiol-maleimide bond and the conjugation can be achieved with applicable ambient and catalyst-free conditions.

Scheme 3.1.6.1 Modification of BSA using vinyl sulfone

3.1.6.2 Thiol-yne reaction

The Che's group discovered that cysteine-containing peptide and BSA can be modified using electron-deficient alkynes (alkynoic amide, ester, or alkynones) (Scheme 3.1.6.2).²⁰ This modification can be implemented in aqueous medium to form vinyl sulfide as the conjugation product. They also demonstrated thiol-assisted cleavage of the resulting vinyl sulfide linkage in peptide to recover the original cysteine-containing peptide.

Scheme 3.1.6.2 Reactions between cysteine residues and electron-deficient alkynes

3.1.6.3 Disulfide exchange

Two cysteine residues located at appropriate positions within one protein can be oxidized to form an intramolecular disulfide bond. This reaction is a popular choice in protein modification and plays a key role in protein folding and structural integrity (Scheme 3.1.6.3).²¹

Inspired by intramolecular disulfide bond formation, crossed disulfide bond formation has been investigated as a chemical protein modification method. However, this method has several limitations, such as slow reaction rate and increased chance of forming protein dimers. In addition, the effectiveness of modification depends on the accessibility of the cysteine residues, so it is difficult to control. Therefore, the use of activated reagents is required to activate cysteine thiols in proteins for disulfide bond formation.²²

Scheme 3.1.6.3 Protein modification with disulfide exchange

3.1.7 Dual modification of peptides and proteins

Recently, dual functionalization of proteins has increased in demand because advanced peptide probes and protein-based therapeutics frequently require multiple functions based on conjugation with photofluorescent dyes or targeting moieties such as biotin and drug molecules. There are two main methods for the synthesis of dually functionalized peptides and proteins.²³ The first method is the modification of two different amino acid residues in the protein. This modification is often conducted by utilizing the difference between the accessibility or the reactivity of amino acid residues.²⁴ The C- and N-terminus of proteins are also frequently functionalized with different chemical functions of interest.²⁵ In addition, insertion of unnatural amino acids onto the targeted proteins allows for installation of the specific functional group.²⁶

The Caddick's group reported on the dual functionalization of the designed ankyrin repeat proteins (DARPin)s utilizing the accessibility of two different cysteine (Scheme 3.1.7.1).^{20b} Two cysteines were introduced at two different positions in the DARPin, which resulted in a large difference in their sulfhydryl nucleophilicity. In this way, α -bromoalkyl reagents and maleimide were used to modify the more accessible and less accessible sulfhydryl group, respectively.

Scheme 3.1.7.1 Dual modification of protein through the difference in the accessibility or the reactivity of the sulfhydryl group

The second method for dual modification of peptides and proteins is the utilization of a multifunctional linker to modify the single site of the targets. The protein modified by multifunctional linkers can be further functionalized. There are many available scaffolds that consist of bifunctional linkers; however, synthesis of the linkers is complicated and often faces

the problem that the modification succeeds at the peptide level but fails at the protein level.²⁷

This adds another layer of difficulty to the development of the dual-functional linker.

The Wagner and Distefano's group designed a diphosphate linker having alkynyl and formyl groups (triorthogonal reagent) that can be used to site-specifically incorporate a terminal alkyne and formyl group simultaneously into the protein (Scheme 3.1.7.2).²⁸ To illustrate the capabilities of dual functionalization, oxime ligation with the formyl group and the click reaction with the alkynyl group were demonstrated to simultaneously incorporate an azido-tetramethylrhodamine fluorophore and an aminooxy-PEG moiety.

Scheme 3.1.7.2 Dual modification of protein with dual-functionalized linker

3.2 Working hypothesis and aim for the project

In 2011, the author's group demonstrated the use of 2-azidoacrylates as a Michael acceptor of active methine carbon nucleophile in the presence of organic diamine catalysts, providing α -azidoesters in good yields (Scheme 3.2.1).²⁹

Scheme 3.2.1 2-Azidoacrylate as a Michael acceptor of active methine

With the knowledge that 2-azidoacrylate can act as a Michael acceptor, the author proposed the use of 2-azidoacrylate derivative as the Michael acceptor for the cysteine-selective conjugation. The resulting conjugates possess the azido group, which can be used for further functionalization through alkyne-azide cycloaddition.³⁰ This chapter provides the details of the design, development, and preliminary applications of the protocol using 2-azidoacrylates as cysteine specific linker (Scheme 3.2.2).

First functionalization

Second functionalization

Scheme 3.2.2 S-nucleophile as a Michael donor to modify a peptide and protein

3.3 Results and discussions

3.3.1 Preparation of 2-azidoacrylates **3-1** and of DBCO-FL **3-6**

3.3.1.1 Preparation of 2-azidoacrylates **3-1a**, **3-1b**, **3-1c**, and **3-V**

2-Azidoacrylate **3-1a**, **3-1b**, **3-1c** and **3-V** were synthesized from the corresponding acrylates. The acrylates were treated with a solution of bromine (Br_2) to synthesize the corresponding dibromides. Subsequently, NaN_3 was added to the solution of the crude dibromides to obtain a mixture of 2-azidoacrylates **3-1** and diazides. Finally, the crude mixture was treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to convert diazides into 2-azidoacrylates **3-1** to obtain them as the single product. The isolated yields of 2-azidoacrylates **3-1a**, **3-1b**, **3-1c**, and **3-V** are summarized in Scheme 3.3.1.1.

Scheme 3.3.1.1 Synthesis of 2-azidoacrylate **3-1**

3.3.1.2 Preparation of 2-azidoacrylates **3-1d** and **3-1e**

For the synthesis of **3-1d** and **3-1e** having a dye moiety, the acrylate **3-II** was first prepared using *tert*-butyl (4-hydroxybutyl)carbamate (**3-I**) and acryloyl chloride. The same protocol with Scheme 3.3.1.1 was then followed to convert **3-II** into 2-azidoacrylate **3-V**. Deprotection of the Boc protecting group from **3-V** was achieved with trifluoroacetic acid, which is followed by the addition of dansyl chloride with Et₃N to obtain **3-1d** and the addition of fluorescein isothiocyanate I (FITC) in the presence of Et₃N to obtain **3-1e**. The isolated yields of acrylate **3-II**, **3-1d**, and **3-1e** are summarized in Schemes 3.3.1.2-1, 3.3.1.2-2, and 3.3.1.2-3, respectively.

Scheme 3.3.1.2-1 Synthesis of acrylate **3-II** and **3-V**

Scheme 3.3.1.2-2 Synthesis of **3-1d**

Scheme 3.3.1.2-3 Synthesis of **3-1e**

3.3.1.3 Preparation of DBCO-FL **3-6**

The fluorescein-linked dibenzocyclooctyne (DBCO-FL) **3-6** was prepared using DBCO-amine and FITC with the presence of Et₃N. The isolated yield of DBCO-FL **3-6** is shown in Scheme 3.2.1.3.

Scheme 3.3.1.3 Synthesis of DBCO-FL **3-6**

3.3.2 Scope of 2-azidoacrylates **3-1**

The project began with the conjugation reactions of cysteine derivatives with four types of 2-azidoacrylates, having the ethyl ester (for **3-1a**), short polyethylene glycol (short-PEG) moiety (for **3-1b**), lipophilic dodecyl substituent (for **3-1c**), and fluorescent dansylamide moiety (for **3-1d**) (Scheme 3.3.2). To ensure that the reaction conditions are as close as possible to the physiological conditions, the conjugation reactions were performed in the sodium phosphate buffer (50 mM) at pH 7.4 and 23°C.

Scheme 3.2.2 Scope of 2-azidoacrylates **3-1**

3.3.3 Conjugation between cysteine and 2-azidoacrylate 3-1

2-Azidoacrylate **3-1a** and cysteine (**3-2**) were mixed (1:1 molar ratio) in the phosphate buffer and the mixture was stirred at 23 °C. The reaction was completed within 0.5 h to afford the conjugated product α -azido ester **3-3a** in 77% yield. A similar phenomenon was observed in the reaction of **3-1b** having short-PEG moiety with cysteine (**3-2**) to form desired product **3-3b** under the same reaction conditions with a slightly longer reaction time of 1 h. As for 2-azidoacrylates having hydrophobic substituents such as dodecyl (**3-1c**) and dansylamide (**3-1d**), the reactions were performed in the co-solvent system of phosphate buffer and THF (1:1) and the desired conjugates **3-3c** and **3-3d** were obtained in high yields even though longer reaction times were required.

Isolated yields were recorded above. ^a 50 mM phosphate buffer (pH = 7.4) was used.

^b The reaction was conducted in buffer/tetrahydrofuran (THF) (1:1).

Scheme 3.2.3 Reactions of 2-azidoacrylate **3-1** and cysteine methyl ester (**3-2**)

3.3.4 Conjugation between glutathione (3-4) and 2-azidoacrylate 3-1

Next, glutathione (GSH) (**3-4**) was chosen as an example of tri-peptides including a cysteine residue to examine the conjugation with 2-azidoacrylates. The reactions of **3-1a** and **3-1b** underwent smooth conjugation with GSH (**3-4**) in phosphate buffer at 23°C to provide **3-5a** and **3-5b** in high yields. However, the co-solvent system of phosphate buffer and THF was again required in the reactions of **3-1c** and **3-1d** having a hydrophobic moiety due to their poor solubility in phosphate buffer.

Isolated yields were recorded above, unless otherwise noted. ^a 50 mM phosphate buffer (pH = 7.4) was used. ^b ¹H NMR yields based on *tert*-butanol as the internal standard. ^c The reaction was conducted in buffer/tetrahydrofuran (THF) (1:1). ^d The reaction was conducted in buffer/THF (2:1).

Scheme 3.3.4 Reactions of GSH (**3-4**) and 2-azidoacrylates **3-1**

3.3.5 Conjugation between Cys-containing peptides 3-6a–3-6c and 2-azidoacrylate 3-1b

The peptides containing various reactive amino acids such as cysteine, serine, and lysine were prepared for the examination of the chemoselectivity in the present conjugation method. For this purpose, we prepared peptides having cysteine residue at the *N*-terminus (for **3-6a**), at the internal position (for **3-6b**), and at the *C*-terminus (for **3-6c**). The reactions of 2-azidoacrylate **3-1b** with cysteine-containing peptides **3-6a–3-6c** in the phosphate buffer were completed within 1.5 h to selectively form thiol-conjugate products **3-7a–3-7c**, the structures of which were confirmed by tandem mass spectrometry analyses. The reaction with an excess amount of **3-1b** (10 equiv) afforded the thiol-conjugated product **3-6b** as a sole product even after stirring for 24 h. These examinations proved that the present protocol with 2-azidoacrylates displays excellent chemoselectivity towards the sulfhydryl group (Scheme 3.3.5).

3-6a: H-Cys-Gly-Lys-Ser-Arg-Phe-OH

3-6b: H-Lys-Ser-Cys-Gly-Arg-Phe-OH

3-6c: H-Gly-Lys-Ser-Arg-Phe-Cys-OH

Scheme 3.3.5 Conjugation of Cys-containing peptides **3-6a–3-6c** with **3-1b**

3.3.6 Kinetic study

To determine the reactivity of 2-azidoacrylates towards the sulfhydryl group, the second-order initial rate constant of the conjugation between 2-azidoacrylate **3-1b** and GSH (**3-4**) was measured. The reason for choosing hydrophilic 2-azidoacrylate **3-1b** was to ensure the kinetic study conditions were as close as possible to the physiological conditions. The

reaction concentration was diluted to 1.0 mM to increase the reaction time and collect more data points using high-performance liquid chromatography (HPLC). It was observed that the reaction progress reached plateaus after 60% of 2-azidacrylate **3-1b** was consumed within 2 h. No side product was observed in the HPLC chromatogram. Therefore, the data were well-fitted to the second-order equation with an initial rate constant of $0.509 \pm 0.03 \text{ M}^{-1}\text{s}^{-1}$. This kinetic value is similar to that of a strain-promoted alkyne-azide cycloaddition (SPAAC),^{15, 31} which is often used in various bioorthogonal reactions.^{5d} Figure 3.3.6.2. shows a brief comparison of the bio-conjugation rates of several selected reagents with 2-azidacrylate **3-1b**.

Initial rate constant is $0.509 \pm 0.03 \text{ M}^{-1}\text{s}^{-1}$

Figure 3.3.6.1. Estimation of the rate constant in the conjugation reaction between **3-1b** and GSH

Figure 3.3.6.2 Rate constant of various chemoselective ligation reactions¹⁵

3.3.7 Dual functionalization of glutathione (3-4)

Based on the results regarding the chemoselectivity and the rate of the conjugation reaction with 2-azidacrylates, the author next became interested in the dual functionalization of GSH (**3-4**). First, the conjugation between 2-azidoacrylate **3-1b** and GSH (**3-4**) performed, followed by addition of fluorescein-linked dibenzocyclooctyne (DBCO) for the second functionalization. The azido moiety of **3-5b** selectively underwent strain-promoted alkyne-

azide cycloaddition (SPAAC) to link the fluorescein within 1 h, forming dual functionalized GSH derivative **3-7**.

First functionalization

Second functionalization

^a 50 mM phosphate buffer (pH = 7.4) was used. ^b Chemical conversion was determined by high-performance liquid chromatography. ^c One of the regioisomers is shown.

Scheme 3.3.7 Dual-functionalization of GSH (3-4**)**

3.3.8 Dual functionalization of bovine serum albumin (BSA) (3-10**)**

The author subsequently investigated dual functionalization of protein. The target protein to be modified was bovine serum albumin (BSA) (**3-10**), as it contains one free cysteine residue among 583 total amino acid residues (MW = 66.5kDa). For the second functionalization, the author utilized strain-promoted alkyne-azide cycloaddition (SPAAC). As

the first conjugation of BSA (**3-10**) with **3-1b** required lower reaction concentration (0.1 mM), the process was monitored by the Ellman test.³² Incubation of BSA (**3-10**) with 1 equivalent of 2-azidoacrylate **3-1b** was not able to reach full conversion within 24 h (Scheme 3.3.8.1). Therefore, 10, 20, and 50 equivalents of 2-azidoacrylate **3-1b** were added to BSA (**3-10**). Within 12 h of incubation with 10 equivalents of **3-1b**, 80% of BSA was conjugated, while the rate of conversion became sluggish after the reaction time of 12 h. Therefore, the optimized reaction conditions of the conjugation between BSA (**3-10**) and 2-azidoacrylate **3-1b** is set to the incubation of BSA with 10 equivalents of **3-1b** for 12 h.

Scheme 3.3.8.1 Optimization of modification of BSA with 2-azidoacrylate **1b**

The strain-promoted alkyne-azide cycloaddition (SPCCA) was then tested for the second modification of BSA. The conjugated BSA (**3-11**) underwent the click reaction with DBCO-FL **3-6**. To the reaction mixture were reach 10 equivalent of **3-1b**, followed by incubation for 12 h. The fluorescein emission intensities at 12 h and 24 h were approximately identical, which was observed from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SGS-PAGE). Therefore, using 2-azidoacrylate **3-1b** as the linker, PEGylation and installation of fluorescent dye into BSA were achieved. Very weak fluorescein emission was observed without installation of 2-azidoacrylate **3-1b** (Scheme 3.3.8.2, SDS-PAGE Lane 3). This fluorescein emission may be attributed to the thiol-yne reaction between cysteine residues from BSA and DBCO-FL **3-6**. In addition, trypsin was added to fragment BSA-PEG-FL **3-12**, the fragments of which were then analyzed by mass spectroscopy to reveal that **3-1b** and **3-6** were site-specifically installed at Cys58.

Scheme 3.3.9 Dual-color labelling of BSA (**3-10**) with **3-1e** and DBCO-Cy5 **3-14**

3.3.10 Dual functionalization of BSA (**3-10**) with fluorescein and biotin

The last example of BSA dual functionalization was examined by the use of the combination of fluorescein-linked 2-azidoacrylate **3-1e** and DBCO-biotin **3-16**. The formation of dual-functionalized BSA-FL-biotin **3-17** was confirmed by SDS-PAGE and enzyme-linked immunosorbent assay (ELISA). The SDS-PAGE lanes 2 and 3 show green fluorescence originating from fluorescein, proving that the conjugation between BSA and **3-1e** was successful (Scheme 3.3.10, SDS-PAGE). Confirmation of the incorporation between biotin and BAS-FL **3-13** was performed by ELISA. First, the control sample was prepared using

streptavidin-coated beans, anti-BSA antibody, and horseradish peroxidase (HRP)-conjugated secondary antibody. More significant HRP activity was observed from sample 4 compared to samples 1, 2, and 3 (Scheme 3.3.10, ELISA). This suggested that biotin moiety was successfully conjugated with BSA-FL **3-13**, while samples 1, 2, and 3 did not show significant HRP activity as there was no conjugation of biotin. With biotin's inert property towards fluorescence, specific incorporation of another reporter moiety such as fluorescent dye into biotinylated protein would be capable of tracing the target proteins.

Scheme 3.3.10 Dual functionalization of BSA (**3-10**) with **3-1e** and DBCO-biotin **3-16**

3.4 Conclusion

To summarize, the author developed site-specific dual functionalization at the side residue of cysteine in peptides and BSA using 2-azidoacrylates and discovered excellent chemoselectivity and a reasonable reaction kinetic rate of 2-azidoacrylates towards the cysteine residue included in peptides and protein. Alkyne-azide cycloaddition was performed with the resulting α -azidoester conjugates to introduce the second functionality into GSH (**4**) and BSA (**10**) to achieve site-specific dual functionalization.

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Chapter 4 Experimental Section

4.1 General Information

Reactions were carried out under an N₂ atmosphere unless otherwise stated. All organic solvents (acetone, MeCN, *t*-BuOH, CHCl₃, CH₂Cl₂, DMF, DMSO, MeOH, THF, Et₂O) were used as supplied (ACS or HPLC grade) unless otherwise stated. H₂O used for the reactions was obtained from the Millipore purification system. Thin layer chromatography (TLC) was performed on Merck TLC plates (0.25 mm) pre-coated with silica gel60 F254 and visualized by UV quenching and/or staining with KMnO₄ or ninhydrin solution and warming with a heat gun. Flash column chromatography was performed under a forced-flow of air using Merck silica gel 60 (40-60 μm particle size) with distilled solvents. ¹H NMR (300, 400 and 500 MHz) spectra were recorded on a Bruker Avance 300 400 and 500 spectrometers in CDCl₃, CD₃OD, DMSO-*d*₆, or D₂O [using CDCl₃ (for ¹H, δ = 7.26), CD₃OD (for ¹H, δ = 3.31), DMSO-*d*₆ (for ¹H, δ = 2.50) and D₂O (for ¹H, δ = 4.97) as the internal standard]. ¹³C NMR (100 MHz) spectra were recorded on a Bruker Avance 400 in CDCl₃, CD₃OD, DMSO-*d*₆ or D₂O [using CDCl₃ (for ¹³C, δ = 77.0), CD₃OD (for ¹³C, δ = 49.0) and DMSO-*d*₆ (for ¹³C, δ = 39.5) as internal standard]. All chemical shifts are reported in ppm (δ). The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublet, ddd = doublet of doublet of doublet, dt = doublet of triplet, sep = septet, m = multiplet, br = single broad. LCMS analysis was performed on ThermoFinnigan LCQ Fleet MS. High-resolution mass spectra were obtained with a Q-ToF Premier LC HR mass spectrometer (Waters). MALDI-TOF mass spectra of peptides were recorded in Spiral TOF mode by using a JMS S3000 (JEOL, Japan) equipped with a 345 nm laser. MALDI-MS/MS analyses were recorded in MALDI-TOF TOF mode using helium gas as collision gas. UV-visible spectra were taken with Cary 100 Bio UV-visible spectrophotometer, Agilent Technology, USA. Melting points were uncorrected and were recorded on a Buchi B-54 melting point apparatus.

Kinetic experiments were performed by high performance liquid chromatography (HPLC) on Shimadzu analytical and semi-preparative instruments with dual pumps, mixer and degasser, a variable wavelength UV detector and a SIL-10AP injector fitted with a 500 μL and 5000 μL sample loop. The mobile phases for the analytical and preparative HPLC were 50 mM of ammonia acetate in MilliporeS3 H_2O (Solvent A) and HPLC grade MeCN (Solvent B). The eluent was monitored simultaneously at 230 nm and 260 nm, unless otherwise stated. The column used for analytical is Shim-pack GIS C-18 5 μm (4.6 x 250 mm) and semi-preparative is Shim-pack GIS C-18 5 μm (10 x 250 mm). Flow rates for analytical and semi-preparative were 1 mL/min and 3 mL/min, respectively.

4.1.1 The safety issues for handling of azido compounds^{1,2}

4.1.1.1 Sodium azide (NaN_3)

Sodium azide is toxic (LD_{50} oral = 27 mg/kg for rats) and can be absorbed through skin. Appropriate gloves are necessary when using it. It decomposes explosively upon heating to above 275 $^\circ\text{C}$. Sodium azide is relatively safe especially in aqueous solution, *unless acidified to form HN_3* , which is volatile and highly toxic.

4.1.1.2 Organic azides

Organic azides are potentially explosive substances that can decompose with the slight input of energy from external sources (heat, light, pressure, etc). When designing the organic azides used for the project, we keep in mind the following equation. It is noted that this equation takes into account all nitrogen atoms in the organic azide, not just those in the azido group.

$$\frac{N_{\text{C}} + N_{\text{O}}}{N_{\text{N}}} \geq 3$$

N_{C} = Number of carbon atom
 N_{O} = Number of other atom about same size
 N_{N} = Number of nitrogen atom

All organic azides prepared in this work are satisfied with the equation above except for **1a** and they are enough stable to be stored under $-20\text{ }^{\circ}\text{C}$ at least for 6 months. We have never experienced a safety problem with these materials.

4.2 Linking alcohols with vinyl azides (Chapter 2)

4.2.1 Synthesis of vinyl azides **2-1a** (Section 2.3.1)

Scheme 4.2.1 Synthesis of vinyl azides **2-1a**

This procedure was slightly modified from the Hassner's method.³

To a suspension of NaN_3 (7.15 g, 110 mmol) in acetonitrile (30 mL) was added dropwise a solution of iodine monochloride (8.07 g, 49.7 mmol) in CH_2Cl_2 (60 mL) at $-20\text{ }^{\circ}\text{C}$, and the mixture was stirred at the same temperature. After 30 min, a solution of styrene (5.0 mL, 43.6 mmol) in CH_2Cl_2 (20 mL) was added slowly, and the mixture was stirred for 1 h. The reaction was quenched with saturated aqueous $\text{Na}_2\text{S}_2\text{O}_3$, and the organic materials were extracted two times with Et_2O . The combined extracts were washed with brine and dried over MgSO_4 . After evaporation of solvents, the resulting crude materials were used immediately for the next step without any further purification.

To a solution of the obtained compounds above in Et_2O (100 mL) was added $t\text{-BuOK}$ (5.92 g, 52.3 mmol) at $0\text{ }^{\circ}\text{C}$, and the mixture was stirred for 1.5 h at the same temperature. The reaction mixture was filtered through celite and the solvent was removed in vacuo. The resulting crude materials were purified by flash column chromatography (silica gel; hexane) to give vinyl azide **2-1a** (5.38 g, 37.1 mmol, 85% yield from styrene) as a pale yellow liquid. ^1H

NMR (400 MHz, CDCl₃) δ 4.98 (1H, d, J = 2.4 Hz), 5.45 (1H, d, J = 2.4 Hz), 7.37-7.40 (3H, m), 7.57-7.59 (2H, m); ¹³C NMR (100 MHz, CDCl₃) δ 98.0, 125.6, 128.5, 129.1, 134.3, 145.1. Vinyl azides **2-1b**⁴, **2-1c**⁵, **2-1d**², **2-1e**², **2-1f**⁶, **2-1g**⁴, **2-1h**⁷ and **2-1i**² were known compounds and prepared according to the reported procedures.

4.2.2 Synthesis of α -alkoxy- β -haloalkyl azides

4.2.2.1 Synthesis of α -alkoxy- β -fluoroalkyl azides **2-3** (Section 2.3.3 and 2.3.4)

Scheme 4.2.2.1 Synthesis of α -alkoxy- β -fluoroalkyl azides **2-3aa**

Typical procedure A

A solution of cinnamyl alcohol (**2-2a**) (55.3 mg, 0.412 mmol) and α -azidostyrene (**2-1a**) (71.8 mg, 0.495 mmol) in CH₃CN (2.0 mL) was added to a suspension of Selectfluor (175.2 mg, 0.495 mmol) in CH₃CN (2.0 mL) at 0 °C. The reaction mixture was gradually warmed to room temperature over for 6 h before it was quenched with water. The aqueous layer was extracted with EtOAc. The organic layers were washed with brine, dried over MgSO₄, and concentrated. Purification of the resulting crude product by flash column chromatography (silica gel, hexane:EtOAc = 95 : 5) afforded product **2-3aa** (98.6 mg, 0.332 mmol) in 80% yield as a pale yellow liquid.

(*E*)-(1-Azido-1-(cinnamyloxy)-2-fluoroethyl)benzene (2-3aa):

IR (NaCl) 3030, 2950, 2119, 1659, 1601, 1495, 1449, 1379, 1248 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 4.10 (1H, ddd, $J = 1.2, 6.0, 12.4$ Hz), 4.24 (1H, ddd, $J = 1.2, 6.0, 12.4$ Hz), 4.52 (1H, dd, $J = 9.6, 47.2$ Hz), 4.67 (1H, dd, $J = 9.6, 47.2$ Hz), 6.31 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.63 (1H, d, $J = 16.0$ Hz), 7.23-7.26 (1H, m), 7.32 (2H, dd, $J = 7.2, 8.0$ Hz), 7.38-7.45 (5H, m), 7.52-7.53 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 65.2, 87.0 (d, $J = 185.3$ Hz), 94.6 (d, $J = 18.2$ Hz), 124.7, 126.6, 126.9, 127.8, 128.5, 128.7, 129.4, 132.9, 135.2 (d, $J = 1.9$ Hz), 136.5; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.5 (1F, dd, $J = 47.2, 47.2$ Hz); ESIHRMS: Found: m/z 270.1292. Calcd for $\text{C}_{17}\text{H}_{17}\text{FNO}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 270.1294.

(*E*)-1-(1-Azido-1-(cinnamyloxy)-2-fluoroethyl)-4-methylbenzene (2-3ba):

Yield: 86% (113 mg, 0.36 mmol) from 0.42 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1b** and 1.2 equiv of Selectfluor for 2 h following typical procedure A; colorless liquid; IR (NaCl) 3053, 2986, 2116, 1659, 1612, 1578, 1510, 1497, 1449, 1381, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.37 (3H, s), 4.09 (1H, ddd $J = 1.2, 6.0, 12.4$ Hz), 4.22 (1H, ddd, $J = 1.2, 6.0, 12.4$ Hz), 4.50 (1H, dd, $J = 9.6, 47.2$ Hz), 4.65 (1H, dd, $J = 9.6, 47.2$ Hz), 6.31 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.63 (1H, d, $J = 16.0$ Hz), 7.22-7.26 (3H, m), 7.32 (2H, dd, $J = 7.2, 7.6$ Hz), 7.38-7.42 (4H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 21.1, 65.1, 87.1 (d, $J = 185.2$ Hz), 94.7 (d, $J = 18.1$ Hz), 124.8, 126.5, 126.8, 127.8, 128.5, 129.4, 132.2 (d, $J = 2.0$ Hz), 132.8, 136.5,

139.4; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.2 (1F, dd, $J = 47.2, 47.2$ Hz); ESIHRMS: Found: m/z 284.1453. Calcd for $\text{C}_{18}\text{H}_{19}\text{FNO}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 284.1451.

(*E*)-1-(1-Azido-1-(cinnamyloxy)-2-fluoroethyl)-4-chlorobenzene (2-3ca):

Yield: 74% (91.5 mg, 0.275 mmol) from 0.371 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1c** and 1.2 equiv of Selectfluor for 5 h by following typical procedure A; colorless liquid; IR(NaCl) 3026, 2951, 2120, 1659, 1597, 1491, 1449, 1400, 1248 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 4.07 (1H, ddd, $J = 1.2, 6.4, 12.4$ Hz), 4.23 (1H, ddd $J = 1.2, 6.0, 12.4$ Hz), 4.50 (1H, dd, $J = 9.6, 46.9$ Hz), 4.63 (1H, dd, $J = 9.6, 47.1$ Hz), 6.29 (1H, ddd, $J = 6.0, 6.4, 16.0$ Hz), 6.62 (1H, d, $J = 16.0$ Hz), 7.23-7.27 (1H, m), 7.32 (2H, dd, $J = 7.2, 7.6$ Hz), 7.38-7.41 (4H, m) 7.46-7.49 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 65.2, 86.5 (d, $J = 185.2$ Hz), 94.1 (d, $J = 18.6$ Hz), 124.4, 126.6, 127.9, 128.4, 128.6, 128.9, 133.1, 133.9 (d, $J = 1.5$ Hz), 135.6, 136.3; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.9 (1F, dd, $J = 46.9, 47.1$ Hz); ESIHRMS: Found: m/z 304.0909. Calcd for $\text{C}_{17}\text{H}_{16}\text{NOF}^{35}\text{Cl}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 304.0904.

(*E*)-Methyl 4-(1-azido-1-(cinnamyloxy)-2-fluoroethyl)benzoate (2-3da):

Yield: 55% (48.5 mg, 0.137 mmol) from 0.249 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1d** and 1.2 equiv of Selectfluor for 24 h following typical procedure A; colorless liquid; IR(NaCl) 3053, 2953, 2122, 1719, 1647, 1576, 1497, 1437, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400

MHz) δ 3.94 (3H, s), 4.08 (1H, ddd, $J = 1.2, 6.0, 12.0$ Hz), 4.25 (1H, ddd, $J = 1.2, 6.0, 12.0$ Hz), 4.53 (1H, dd, $J = 10.0, 46.8$ Hz), 4.66 (1H, dd, $J = 10.0, 47.0$ Hz), 6.31 (1H, ddd, $J = 6.0, 6.0, 15.6$ Hz), 6.63 (1H, d, $J = 15.6$ Hz), 7.26 (1H, t, $J = 7.6$ Hz), 7.32 (2H, dd, $J = 7.2, 7.6$ Hz), 7.39 (2H, d, $J = 7.2$ Hz), 7.63 (2H, d, $J = 8.4$ Hz), 8.09, (2H, d, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 52.3, 66.3, 86.4 (d, $J = 185.3$ Hz), 94.3 (d, $J = 18.7$ Hz), 124.3, 126.5, 127.1, 127.9, 128.5, 129.9, 131.2, 133.2, 136.3, 140.1, 166.4; ^{19}F NMR (CDCl_3 , 376 MHz) δ -223.4 (1F, dd, $J = 46.8, 47.0$ Hz); ESIHRMS: Found: m/z 328.1353. Calcd for $\text{C}_{19}\text{H}_{19}\text{NO}_3\text{F}$: $(\text{M}-\text{N}_2+\text{H})^+ 328.1349$.

(*E*)-1-(1-Azido-1-(cinnamyloxy)-2-fluoroethyl)-3-bromobenzene (2-3ea):

Yield: 73% (^1H NMR yield due to its instability) from 0.30 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1e** and 1.2 equiv of Selectfluor for 24 h following typical procedure A. Due to instability of **2-3ea**, the crude residue including **2-3ea** was directly treated under thermal [3+2]-cycloaddition conditions to form **2-5ea** (see section 5.2.5).

(*E*)-2-(1-Azido-1-(cinnamyloxy)-2-fluoroethyl)naphthalene (2-3fa):

Yield: 79% (107 mg, 0.308 mmol) from 0.391 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1f** and 1.2 equiv of Selectfluor for 6 h following typical procedure A.; pale yellow liquid;

IR(NaCl) 3059, 2951, 2118, 1659, 1601, 1506, 1497, 1449, 1354, 1246 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 4.12 (1H, dd, $J = 6.4, 12.4$ Hz), 4.26 (1H, dd, $J = 6.0, 12.4$ Hz), 4.60 (1H, dd, $J = 9.6, 47.0$ Hz), 4.77 (1H, dd, $J = 9.6, 47.2$ Hz), 6.34 (1H, ddd, $J = 6.0, 6.4, 15.6$ Hz), 6.63 (1H, d, $J = 15.6$ Hz), 7.27 (1H, t, $J = 7.2$ Hz), 7.32 (2H, dd, $J = 7.2, 7.6$ Hz), 7.39 (2H, d, $J = 7.6$ Hz), 7.52-7.56 (2H, m), 7.58 (1H, dd, $J = 1.2, 8.4$ Hz), 7.85-7.91 (3H, m) 8.07 (1H, s); ^{13}C NMR (CDCl_3 , 100 MHz) δ 65.3, 86.9 (d, $J = 185.2$ Hz), 94.8 (d, $J = 18.3$ Hz), 123.6, 124.7, 126.5, 126.6, 127.03, 127.2, 127.6, 127.9, 128.5, 128.6, 128.7, 132.5 (d, $J = 1.7$ Hz), 132.9, 133.0, 133.6, 136.5; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.9 (1F, dd, $J = 47.0, 47.2$ Hz); ESIHRMS: Found: m/z 324.1263. Calcd for $\text{C}_{21}\text{H}_{18}\text{FNNaO}$: ($\text{M}-\text{N}_2+\text{Na}$) $^+$ 342.1270.

(*E*)-(3-((2-Azido-1-fluoro-4-phenylbutan-2-yl)oxy)prop-1-en-1-yl)benzene (2-3ga):

Yield: 57% (81.2 mg, 0.25 mmol) from 0.44 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1g** and 1.2 equiv of Selectfluor for 9 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3026, 2959, 2112, 1655, 1603, 1497, 1454, 1375, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.14-2.19 (2H, m), 2.73-2.77 (2H, m), 4.33 (2H, dd, $J = 0.8, 6.0$ Hz), 4.44 (1H, dd, $J = 10.0, 46.8$ Hz), 4.51 (1H, dd, $J = 10.0, 46.8$ Hz), 6.29 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.65 (1H, d, $J = 16.0$ Hz), 7.19-7.26 (4H, m), 7.28-7.33 (4H, m) 7.39 (2H, d, $J = 7.6$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 29.6, 35.5, 64.0, 82.4 (d, $J = 178.6$ Hz), 93.3 (d, $J = 20.2$ Hz), 124.7, 126.2, 126.5, 127.9, 128.2, 128.5, 128.6, 132.8, 136.4, 140.7; ^{19}F NMR (CDCl_3 , 376 MHz) δ -228.4 (1F, dd, $J = 46.8, 46.8$ Hz); ESIHRMS: Found: m/z 298.1613. Calcd for $\text{C}_{19}\text{H}_{21}\text{FNO}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 298.1607.

1-(Cinnamyloxy)-2-fluoro-1-methyl-1,2,3,4-tetrahydronaphthalene (2-3ha):

dr = 1.1:1 (inseparable), the ratio was determined by ^{19}F NMR.

Yield: 74% (95.2 mg, 0.295 mmol) from 0.397 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1h** and 1.2 equiv of Selectfluor for 3.5 h following typical procedure A.; colorless liquid; IR (NaCl) 3026, 2940, 2112, 1659, 1601, 1489, 1454, 1378, 1302, 1246 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.15-2.47 (2Hx1+2Hx1.1, m), 2.79-2.91 (1Hx1+1Hx1.1, m), 2.99-3.14 (1Hx1+1Hx1.1, m), 4.06 (1Hx1, dd, $J = 1.2, 6.0$ Hz), 4.32-4.47 (1Hx1+2Hx1.1, m), 5.00 (1Hx1.1, ddd, $J = 2.4, 6.4, 49.2$ Hz), 5.11 (1Hx1, ddd, $J = 2.8, 6.4, 49.2$ Hz), 6.19 (1Hx1, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.35 (1Hx1.1, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.55 (1Hx1, d, $J = 16.0$ Hz), 6.68 (1Hx1.1, d, $J = 16.0$ Hz), 7.17-7.26 (2Hx1+2Hx1.1, m), 7.28-7.35 (5Hx1+5Hx1.1, m), 7.39-7.41 (1Hx1+1Hx1.1, m), 7.67 (1Hx1, dd, $J = 1.6, 7.6$ Hz), 7.74-7.76 (1Hx1.1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 24.0 (d, $J = 6.8$ Hz), 24.4 (d, $J = 19.8$ Hz), 24.7 (d, $J = 6.4$ Hz), 25.0 (d, $J = 20.1$ Hz), 64.1, 64.8 (d, $J = 2.3$ Hz), 88.8 (d, $J = 180.9$ Hz), 91.1 (d, $J = 179.9$ Hz), 91.4 (d, $J = 22.4$ Hz), 91.5 (d, $J = 21.3$ Hz), 125.0, 125.1, 126.15, 126.23, 126.3, 126.5, 126.6, 126.8, 127.8, 128.5, 128.9, 129.1, 129.2, 129.4, 131.2, 132.1, 132.5, 132.7, 135.5, 136.5, 136.6; ^{19}F NMR (CDCl_3 , 376 MHz) δ -191.5 (1Fx1, ddd, $J = 10.5, 33.8, 49.2$ Hz), -197.7 (1Fx1.1, ddd, $J = 8.6, 34.6, 49.2$ Hz); ESIHRMS: Found: m/z 296.1456. Calcd for $\text{C}_{19}\text{H}_{19}\text{FNO}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 296.1451.

(E)-(3-(allyloxy)-1-azido-1-(cinnamyloxy)-2-fluoropropyl)benzene (2-3ia):

dr = 1.2:1 (inseparable), the ratio was determined by ^{19}F NMR.

The ^1H and ^{13}C NMR spectra of two diastereomers are identical.

Yield: 47% (54.7 mg, 0.15 mmol) from 0.32 mmol of **2-2a** with 1.2 equiv of vinyl azide **2-1i** and 1.2 equiv of Selectfluor for 28 h following typical procedure A.; colorless liquid; IR (NaCl) 3075, 2945, 2118, 1641, 1493, 1449, 1389, 1248 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 3.37 (1H, ddd, J = 1.6, 11.6, 33.6 Hz), 3.45-3.53 (1H, m), 3.83-3.94 (2H, m), 4.03 (1H, dd, J = 6.4, 12.4 Hz), 4.18 (1H, ddd, J = 1.2, 6.0, 12.4 Hz), 5.01 (1H, ddd, J = 1.6, 7.6, 48.8 Hz), 5.12 (1H, dd, J = 1.2, 10.4 Hz), 5.17 (1H, dd, J = 1.2, 17.2 Hz), 5.78 (1H, tdd, J = 5.6, 10.4, 17.2 Hz), 6.30 (1H, ddd, J = 6.0, 6.0, 16.0 Hz), 6.61 (1H, d, J = 16.0 Hz), 7.22-7.26 (1H, m), 7.31 (2H, dd, J = 7.2, 7.6 Hz), 7.37-7.44 (5H, m), 7.46-7.49 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 65.1, 68.8 (d, J = 20.7 Hz), 72.3, 95.2 (d, J = 17.9 Hz), 96.8 (d, J = 189.3 Hz), 117.4, 124.6, 126.5, 127.0, 127.8, 128.5, 128.7, 129.5, 132.8, 134.0, 134.8, 136.5; ^{19}F NMR (CDCl_3 , 376 MHz) δ -193.5 (1F_x1+1F_x1.2, m); ESIHRMS: Found: m/z 340.1717. Calcd for $\text{C}_{21}\text{H}_{23}\text{FNO}_2$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 340.1713.

(E)-(1-Azido-2-fluoro-1-((4-phenylbut-3-en-1-yl)oxy)ethyl)benzene (2-3ab):

Yield: 63% (79.7 mg, 0.26 mmol) from 0.41 mmol of **2-2b** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of Selectfluor for 7 h following typical procedure A.; pale yellow solid, mp 42-

44°C; IR (KBr) 3075, 2945, 2118, 1641, 1493, 1448, 1389, 1248 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 2.53-2.58 (1Hx2, m), 3.51 (1H, ddd, *J* = 6.8, 6.8, 9.2 Hz), 3.66 (1H, ddd, *J* = 6.8, 6.8, 9.2 Hz), 4.49 (1H, dd, *J* = 10.0, 47.2 Hz), 4.63 (1H, dd, *J* = 10.0, 47.2 Hz), 6.21 (1H, ddd, *J* = 7.0, 7.2, 15.6 Hz), 6.47 (1H, d, *J* = 15.6 Hz), 7.18-7.24 (1H, m), 7.29 (2H, dd, *J* = 7.6, 7.6 Hz), 7.34 (2H, d, *J* = 7.6 Hz), 7.37-7.40 (3H, m), 7.48-7.50 (2H, m); ¹³C NMR (CDCl₃, 100 MHz) δ 33.3, 63.8, 86.8 (d, *J* = 185.7 Hz), 94.4 (d, *J* = 18.4 Hz), 126.0, 126.2, 126.9, 127.2, 128.5, 128.6, 129.3, 132.1, 135.3 (d, *J* = 1.3 Hz), 137.4; ¹⁹F NMR (CDCl₃, 376 MHz) δ -222.8 (1F, dd, *J* = 47.2, 47.2 Hz); ESIHRMS: Found: *m/z* 284.1449. Calcd for C₁₈H₁₉FNO: (M-N₂+H)⁺ 284.1451.

(1-Azido-2-fluoro-1-(pent-4-en-1-yloxy)ethyl)benzene (2-3ac):

Yield: 84% (313 mg, 1.26 mmol) from 1.50 mmol of **2-2c** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of Selectfluor for 6.5 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3026, 2951, 2116, 1653, 1597, 1491, 1449, 1383, 1248 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 1.73-1.80 (1Hx2, m), 2.15-2.21 (1Hx2, m), 3.40 (1H, ddd, *J* = 6.4, 6.4, 9.2 Hz), 3.40 (1H, ddd, *J* = 6.8, 6.8, 9.2 Hz), 4.47 (1H, dd, *J* = 9.6, 47.2 Hz), 4.60 (1H, dd, *J* = 9.6, 47.2 Hz), 4.97 (1H, d, *J* = 10.0 Hz), 5.04 (1H, d, *J* = 17.2 Hz), 5.76-5.86 (1H, m), 7.36-7.42 (3H, m), 7.47-7.49 (2H, m); ¹³C NMR (CDCl₃, 100 MHz) δ 28.9, 30.2, 63.4, 86.8 (d, *J* = 184.7 Hz), 94.3 (d, *J* = 18.2 Hz), 115.0, 126.8, 128.6, 129.3, 135.5, 137.8; ¹⁹F NMR (CDCl₃, 376 MHz) δ -222.9 (1F, dd, *J* = 47.2, 47.2 Hz); ESIHRMS: Found: *m/z* 222.1293. Calcd for C₁₃H₁₇FNO: (M-N₂+H)⁺ 222.1294.

(3-(1-Azido-2-fluoro-1-phenylethoxy)prop-1-yn-1-yl)trimethylsilane (2-3ad):

Yield: 83% (94.2 mg, 0.323 mmol) from 0.392 mmol of **2-2d** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of Selectfluor for 4 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3061, 2959, 2180, 2120, 1491, 1449, 1364, 1250 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.18 (9H, s), 4.11 (1H, d, $J = 16.0$ Hz), 4.18 (1H, d, $J = 16.0$ Hz), 4.50 (1H, dd, $J = 9.6, 46.9$ Hz), 4.66 (1H, dd, $J = 9.6, 47.4$ Hz), 7.40-7.43 (3H, m) 7.50-7.52 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ -0.3, 53.3, 86.7 (d, $J = 185.3$ Hz), 91.5, 94.9 (d, $J = 18.5$ Hz), 100.6, 127.0, 128.7, 129.6, 134.6; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.4 (1F, dd, $J = 46.9, 47.4$ Hz); ESIHRMS: Found: m/z 284.1213. Calcd for $\text{C}_{14}\text{H}_{19}\text{FNOSi}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 284.1220.

(1-Azido-2-fluoro-1-((3-phenylprop-2-yn-1-yl)oxy)ethyl)benzene (2-3ae):

Yield: 71% (85.6 mg, 0.29 mmol) from 0.41 mmol of **2-2e** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of Selectfluor for 18 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3061, 2922, 2118, 1599, 1490, 1441, 1371, 1263 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 4.34 (1H, d, $J = 15.6$ Hz), 4.41 (1H, d, $J = 15.6$ Hz), 4.53 (1H, dd, $J = 10.0, 47.2$ Hz), 4.70 (1H, dd, $J = 10.0, 47.2$ Hz), 7.27-7.32 (3H, m) 7.40-7.46 (5H, m) 7.53-7.56 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 53.4, 84.4, 86.3, 86.8 (d, $J = 185.6$ Hz), 94.9 (d, $J = 18.3$ Hz), 122.5, 127.0, 128.2, 128.5, 128.8, 129.6, 131.8, 134.6 (d, $J = 1.1$ Hz); ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.3 (1F, dd, $J = 47.2, 47.2$ Hz); ESIHRMS: Found: m/z 290.0965. Calcd for $\text{C}_{17}\text{H}_{14}\text{FNNaO}$: ($\text{M}-\text{N}_2+\text{Na}$) $^+$ 290.0957.

(1-Azido-2-fluoro-1-(prop-2-yn-1-yloxy)ethyl)benzene (2-3af):

Yield: 75% (55.8 mg, 0.24 mmol) from 0.32 mmol of **2-2f** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of Selectfluor for 6 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3063, 2955, 2120, 1493, 1449, 1389, 1248, 1148 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.00 (1H, dd, $J = 2.8, 2.8$ Hz), 2.53-2.57 (1Hx2, m), 3.53 (1H, ddd, $J = 7.2, 7.2, 9.2$ Hz), 3.67 (1H, ddd, $J = 7.2, 7.2, 9.2$ Hz), 4.49 (1H, dd, $J = 10.0, 47.0$ Hz), 4.63 (1H, dd, $J = 10.0, 47.0$ Hz), 7.38-7.44 (3H, m), 7.49-7.52 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 19.8, 62.3, 69.7, 80.6, 86.8 (d, $J = 185.1$ Hz), 94.3 (d, $J = 18.3$ Hz), 126.8, 128.7, 129.4, 135.0 (d, $J = 1.4$ Hz); ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.8 (1F, dd, $J = 47.0, 47.0$ Hz); ESIHRMS: Found: m/z 256.0865. Calcd for $\text{C}_{12}\text{H}_{12}\text{FN}_3\text{NaO}$: ($\text{M}+\text{Na}$) $^+$ 256.0862.

3-(2-(1-Azido-2-fluoro-1-phenylethoxy)ethyl)-1-tosyl-1H-indole (2-3ag):

Yield: 67% (73.6 mg, 0.154 mmol) from 0.229 mmol of **2-2g** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor for 3 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3053, 2986, 2122, 1597, 1495, 1449, 1422, 1371, 1265, 1175 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.29 (3H, s), 2.99-3.02 (1Hx2, m), 3.62 (1H, ddd, $J = 6.8, 6.8, 9.2$ Hz), 3.80 (1H, ddd, $J = 6.4, 6.4, 9.2$ Hz), 4.43 (1H, dd, $J = 9.6, 47.0$ Hz), 4.56 (1H, dd, $J = 9.6, 47.2$ Hz), 7.15-7.21 (3H, m), 7.24-7.32 (6H, m), 7.40 (2H, d, $J = 6.8$ Hz), 7.74 (2H, d, $J = 8.4$ Hz), 7.98 (1H, d, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 21.5, 25.5, 63.1, 86.9 (d, $J = 185.3$ Hz), 94.4 (d, $J = 18.2$ Hz), 113.6, 119.2, 119.4, 123.0, 123.7, 124.6, 126.7, 126.8, 128.6, 129.3, 129.8, 130.8, 134.9 (d, $J = 1.5$ Hz), 135.1, 135.3, 144.7; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.6 (1F, dd, $J =$

47.0, 47.2 Hz); ESIHRMS: Found: m/z 451.1490. Calcd for $C_{25}H_{24}FN_2O_3S$: $(M-N_2+H)^+$ 451.1492.

(1-Azido-2-fluoro-1-(2-(phenylsulfonyl)ethoxy)ethyl)benzene (2-3ah):

Yield: 72% (80.3 mg, 0.23 mmol) from 0.32 mmol of **2-2h** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor for 6 h (this compound is contaminated with a trace amount of inseparable impurity) following typical procedure A.; pale yellow liquid; IR (NaCl) 3063, 2986, 2122, 1597, 1449, 1393, 1321, 1246, 1144 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 3.45-3.51 (2H, m), 3.79-3.86 (1H, m), 3.89-3.95 (1H, m), 4.34 (1H, dd, $J = 10.0, 47.0$ Hz), 4.41 (1H, dd, $J = 10.0, 47.1$ Hz), 7.32-7.42 (5H, m), 7.55-7.60 (2H, m), 7.67 (1H, t, $J = 7.6$ Hz), 7.93-7.95 (2H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 56.2, 57.6, 86.2 (d, $J = 185.5$ Hz), 94.5 (d, $J = 18.5$ Hz), 126.7, 128.0, 128.7, 129.2, 129.3, 129.6, 133.8, 139.7; ^{19}F NMR ($CDCl_3$, 376 MHz) δ -222.8 (1F, dd, $J = 47.0, 47.1$ Hz); ESIHRMS: Found: m/z 372.0800. Calcd for $C_{16}H_{16}FN_3NaO_3S$: $(M+Na)^+$ 372.0794.

Diethyl (2-(1-azido-2-fluoro-1-phenylethoxy)ethyl)phosphonate (2-3ai):

Yield: 65% (94.2 mg, 0.284 mmol) from 0.437 mmol of **2-2i** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor for 18 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3063, 2984, 2122, 1495, 1450, 1393, 1250 cm^{-1} ; ^1H NMR (CDCl_3 , 400MHz) δ 1.34 (3H, t, $J = 7.2$ Hz), 1.37 (3H, t, $J = 7.2$ Hz), 3.63 (1H, dd, $J = 10.4, 13.2$ Hz), 3.80 (1H, dd, $J = 10.8, 13.2$ Hz), 4.15-4.26 (4H, m), 4.52 (1H, dd, $J = 10.0, 46.8$ Hz), 4.68 (1H, dd, $J = 10.0, 47.0$ Hz), 7.40-7.43 (3H, m), 7.48-7.50 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 16.4 (d, $J = 5.8$ Hz), 16.5 (d, $J = 5.8$ Hz), 57.9 (d, $J = 171$ Hz), 62.6 (d, $J = 6.4$ Hz), 62.6 (d, $J = 6.3$ Hz), 86.6 (d, $J = 185.7$ Hz), 95.3 (dd, $J = 14.6, 18.2$ Hz), 127.0, 128.9, 129.8, 133.7; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.7 (1F, dd, $J = 46.8, 47.0$ Hz); ESIHRMS: Found: m/z 354.1001. Calcd for $\text{C}_{13}\text{H}_{19}\text{FN}_3\text{NaO}_4\text{P}$: $(\text{M}+\text{Na})^+$ 354.0995.

2-(4-((1-Azido-2-fluoro-1-phenylethoxy)methyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2-3aj):

Yield: 71% (118 mg, 0.30 mmol) from 0.42 mmol of **2-2j** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of Selectfluor for 6 h following typical procedure A.; pale yellow solid, mp 60-62 $^{\circ}\text{C}$; IR (NaCl) 3053, 2980, 2120, 1518, 1449, 1398, 1360, 1267 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.36 (12H, s), 4.47 (1H, d, $J = 11.6$ Hz), 4.55 (1H, dd, $J = 10.0, 47.2$ Hz), 4.63 (1H,

d, $J = 11.6$ Hz), 4.70 (1H, dd, $J = 10.0, 47.3$ Hz), 7.38 (2H, d, $J = 7.6$ Hz), 7.41-7.46 (3H, m), 7.55-7.57 (2H, m), 7.82 (2H, d, $J = 7.6$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 24.8, 66.2, 83.8, 86.8 (d, $J = 184.8$ Hz), 94.7 (d, $J = 18.5$ Hz), 126.91, 126.94, 128.7, 129.5, 134.9, 135.2, 140.2; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.7 (1F, dd, $J = 47.2, 47.3$ Hz); ESIHRMS: Found: m/z 420.1877. Calcd for $\text{C}_{21}\text{H}_{25}\text{BFN}_3\text{NaO}_3$: $(\text{M}+\text{Na})^+$ 420.1871.

3-(1-Azido-2-fluoro-1-phenylethoxy)-1-phenylpropan-1-ol (2-3ak):

dr = 1:1 (inseparable), the ratio was determined by ^{19}F NMR.

Yield: 62% (84.8 mg, 0.268 mmol) from 0.434 mmol of **2-2k** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of Selectfluor for 3 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3362, 3062, 2954, 2118, 1602, 1493, 1450, 1393, 1246 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.03-2.14 (2Hx1+2Hx1, m), 2.40 (1H, s br), 2.45 (1H, s br), 3.51-3.80 (2Hx1+2Hx1, m), 4.51 (1H, dd, $J = 10.0, 47.2$ Hz), 4.52 (1H, dd, $J = 9.6, 47.2$ Hz), 4.62 (1H, dd, $J = 9.6, 47.2$ Hz), 4.63 (1H, dd, $J = 10.0, 47.2$ Hz), 4.95-4.98 (1Hx1+1Hx1, m), 7.27-7.31 (1Hx1+1Hx1, m), 7.34-7.41 (7Hx1+7Hx1, m), 7.48-7.50 (2Hx1+2Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 38.68, 38.72, 61.57, 61.63, 72.00, 72.06, 86.81 (d, $J = 185.2$ Hz), 94.34 (d, $J = 18.2$ Hz), 94.43 (d, $J = 18.2$ Hz), 125.72, 125.74, 126.76, 127.53, 127.54, 128.45, 128.66, 129.37, 129.39, 135.02, 144.09, 144.19; ^{19}F NMR (CDCl_3 , 376 MHz) δ -222.76 (1Fx1, dd, $J = 47.2, 47.2$ Hz), -222.79 (1Fx1, dd, $J = 47.2, 47.2$ Hz); ESIHRMS: Found: m/z 338.1286. Calcd for $\text{C}_{17}\text{H}_{18}\text{FN}_3\text{NaO}_2$: $(\text{M}+\text{Na})^+$ 338.1281.

3-(1-Azido-2-fluoro-1-phenylethoxy)-3-phenylpropan-1-ol (2-3ak')

Yield: 13% (17.8 mg, 0.056 mmol) from 0.434 mmol of **2-2k** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of Selectfluor; two diastereomers (1:1) could be separated by flash column chromatography (silica gel; hexane:EtOAc = 90:10).

Diastereomer 1: Yield: 6%; pale yellow liquid; IR (NaCl) 3361, 3062, 2955, 2118, 1602, 1493, 1450, 1367, 1244 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 1.63 (1H, s br), 2.03-2.06 (1H, m), 2.19-2.23 (1H, m), 3.58-3.62 (1H, m), 3.78-3.82 (1H, m), 4.65 (2H, d, *J* = 47.1 Hz), 4.99 (1H, dd, *J* = 6.8, 6.8 Hz), 7.18-7.26 (8H, m), 7.39 (2H, dd, *J* = 1.6, 8.0 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 41.0, 59.3, 74.6, 85.2 (d, *J* = 182.1 Hz), 94.9 (d, *J* = 19.5 Hz), 126.4, 126.9, 127.4, 128.26, 128.29, 129.2, 135.8, 142.6; ¹⁹F NMR (CDCl₃, 376 MHz) δ -223.4 (1F x 1, dd, *J* = 47.1, 47.1 Hz); ESIHRMS: Found: *m/z* 338.1283. Calcd for C₁₇H₁₈FN₃NaO₂: (M+Na)⁺ 338.1281.

Diastereomer 2: Yield: 7%; pale yellow liquid; IR (NaCl) 3362, 3062, 2955, 2118, 1602, 1493, 1450, 1365, 1251 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 1.46 (1H, s br), 1.90-1.99 (1H, m), 2.07-2.15 (1H, m), 3.48-3.54 (1H, m), 3.63-3.69 (1H, m), 4.43 (1H, dd, *J* = 10.0, 46.8 Hz), 4.55 (1H, dd, *J* = 10.0, 47.2 Hz), 4.82 (1H, dd, *J* = 6.8, 6.8 Hz), 7.28-7.32 (1H, m), 7.35-7.37 (4H, m), 7.44-7.47 (3H, m), 7.61 (2H, dd, *J* = 1.6, 8.0 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 41.0, 59.3, 74.8, 85.9 (d, *J* = 184.5 Hz), 95.0 (d, *J* = 18.6 Hz), 126.5, 127.0, 127.8, 128.5, 128.7, 129.5, 136.0, 142.4; ¹⁹F NMR (CDCl₃, 376 MHz) δ -221.8 (1F x 1, dd, *J* = 46.8, 47.2 Hz); ESIHRMS: Found: *m/z* 338.1284. Calcd for C₁₇H₁₈FN₃NaO₂: (M+Na)⁺ 338.1281.

(2*S*,3*R*,4*S*,5*R*,6*R*)-6-((1-azido-2-fluoro-1-phenylethoxy)methyl)tetrahydro-2*H*-pyran-2,3,4,5-tetraol tetraacetate (2-3am):

dr = 1.3:1 (inseparable), the ratio was determined by ^{19}F NMR.

Yield: 85% (134 mg, 0.262 mmol with trace amount of impurity) from 0.309 mmol of **2-2m** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor for 4 h following typical procedure A.; colorless liquid; IR (NaCl) 3061, 2943, 2120, 1768 (br), 1682, 1595, 1493, 1449, 1368 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.99 (3Hx1.3, s), 2.02-2.04 (6Hx1.3+9Hx1, m), 2.10 (3Hx1.3, s), 2.11 (3Hx1, s), 3.43 (1Hx1, dd, J = 4.4, 10.8 Hz), 3.43 (1Hx1.3, dd, J = 2.4, 10.8 Hz), 3.65-3.69 (1Hx1.3+1Hx1, m), 3.80-3.85 (2Hx1.3+2Hx1, m), 4.46 (1Hx1.3, dd, J = 9.6, 46.8 Hz), 4.48 (1Hx1, dd, J = 9.6, 46.8 Hz), 4.58 (1Hx1.3, dd, J = 9.6, 47.2 Hz), 4.65 (1Hx1, dd, J = 9.6, 47.2 Hz), 5.12-5.27 (3Hx1.3+3Hx1, m), 5.72-5.74 (1Hx1.3+1Hx1, m), 7.37-7.43 (4Hx1.3+4Hx1, m), 7.47-7.49 (1Hx1.3+1Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 20.52, 20.55, 20.58, 20.7, 62.1, 62.3, 68.2, 68.3, 70.2, 70.3, 72.97, 73.01, 73.16, 73.22, 86.4 (d, J = 185 Hz), 91.6, 94.1 (d, J = 18.8 Hz), 94.2 (d, J = 18.5 Hz), 126.85, 126.94, 128.6, 129.5, 168.9, 169.0, 169.2, 169.3, 169.4, 170.1, 170.2; ^{19}F NMR (CDCl_3 , 376 MHz) δ -223.00 (1Fx1, dd, J = 46.6, 47.4 Hz), -223.02 (1Fx1.3, dd, J = 47.2, 47.2 Hz); ESIHRMS: Found: m/z 534.1501. Calcd for $\text{C}_{22}\text{H}_{26}\text{FN}_3\text{NaO}_{10}$: ($\text{M}+\text{Na}$) $^+$ 534.1500.

(1-Azido-2-fluoro-1-(((1R,2S,5R)-2-isopropyl-5-methylcyclohexyl)oxy)ethyl)benzene (2-3an):

dr = 1.6:1 (inseparable), the ratio was determined by ^{19}F NMR.

Yield: 82% (76.4 mg, 0.239 mmol) from 0.292 mmol of **2-2n** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor for 6 h following typical procedure A.; pale yellow solid; IR (NaCl) 3030, 2957, 2116, 1491, 1449, 1369, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.63 (3Hx1.6, d, $J = 6.8$ Hz), 0.81-0.99 (11Hx1+8Hx1.6, m), 1.02-1.43 (3Hx1+3Hx1.6, m), 1.57-1.71 (2Hx1+2Hx1.6, m), 1.90-1.95 (1Hx1, m), 2.18-2.28 (2Hx1.6, m), 2.35-2.43 (1Hx1, m), 3.34 (1Hx1.6, ddd, $J = 4.4, 10.0, 10.4$ Hz), 3.83 (1Hx, ddd, $J = 4.4, 10.4, 10.4$ Hz), 4.47-4.74 (2Hx1+2Hx1.6, m), 7.37-7.44 (3Hx1+3Hx1.6, m), 7.53-7.59 (2Hx1+2Hx1.6, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 15.7, 15.9, 21.4, 22.26, 22.34, 22.9, 23.0, 25.0, 25.3, 31.4, 31.5, 34.1, 34.2, 41.6, 42.5, 48.3, 48.6, 74.5, 75.1, 84.5 (d, $J = 182.6$ Hz), 86.0 (d, $J = 183.2$ Hz), 93.93 (d, $J = 19.6$ Hz), 93.94 (d, $J = 19.4$ Hz), 126.8, 127.2, 128.4, 128.5, 129.0, 129.3, 135.3, 138.4; ^{19}F NMR (CDCl_3 , 376 MHz) δ -221.9 (1Fx1.6, dd, $J = 47.0, 47.4$ Hz), -222.5 (1Fx1, dd, $J = 47.0, 47.4$ Hz); ESIHRMS: Found: m/z 314.1894. Calcd for $\text{C}_{18}\text{H}_{26}\text{FNNaO}$: ($\text{M}-\text{N}_2+\text{Na}$) $^+$ 314.1896.

(3*R*,5*S*,8*R*,10*S*,13*S*,14*S*)-3-(1-azido-2-fluoro-1-phenylethoxy)-10,13-dimethyltetradecahydro-1*H*-cyclopenta[*a*]phenanthren-17(2*H*)-one (2-3aq):

An inseparable diastereomeric mixture and the ratio could not be determined by NMR.

Yield: 82% (103 mg, 0.23 mmol) from 0.28 mmol of **2-2q** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor in CH₃CN-CH₂Cl₂ (5:1) solvent system for 2 h following typical procedure A.; colorless liquid; IR (NaCl) 3059, 2932, 2116, 1732, 1600, 1449, 1373, 1263cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 0.79 (3H, s), 0.87-0.91 (4H, m), 1.01-1.14 (1H, m), 1.18-1.35 (6H, m), 1.46-1.58 (6H, m), 1.68-1.74 (2H, m), 1.79-1.82 (3H, m), 1.92-1.98 (1H, m), 2.03-2.13 (1H, m), 2.40-2.47 (1H, m), 3.99-4.04 (1H, m), 4.45-4.69 (2H, m), 7.38-7.43 (3H, m), 7.52-7.56 (2H, m); ¹³C NMR (CDCl₃, 100 MHz) δ 11.3, 13.8, 20.1, 21.7, 27.8, 28.15, 28.2, 28.3, 30.8, 31.5, 32.9, 33.1, 34.6, 34.9, 35.0, 35.8, 35.9, 39.7, 40.0, 47.8, 51.5, 54.3, 54.4, 69.6, 85.7 (d, *J* = 183 Hz), 85.9 (d, *J* = 183 Hz), 126.9, 128.4, 129.1, 136.6, 136.8, 221.4, 221.5; ¹⁹F NMR (CDCl₃, 376 MHz) δ -222.78 (dd, *J* = 46.6, 47.4 Hz), -222.79 (dd, *J* = 46.6, 47.4 Hz) (Two peaks are overlapped and dr cannot be determined.); ESIHRMS: Found: *m/z*448.2616. Calcd for C₂₇H₃₆FNNaO₂: (M-N₂+Na)⁺448.2628.

(8*R*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(1-azido-2-fluoro-1-phenylethoxy)-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3(2*H*)-one (2-3ar):

dr = 1:1 (inseparable), the ratio was determined by ^{19}F NMR.

Yield: 81% (112 mg, 0.25 mmol) from 0.31 mmol of **2-2r** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of Selectfluor in $\text{CH}_3\text{CN}-\text{CH}_2\text{Cl}_2$ (5:1) solvent system for 5 h following typical procedure A.; pale yellow liquid; IR (NaCl) 3057, 2943, 2116, 1667, 1614, 1493, 1449, 1375, 1267 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.82-1.00 (6Hx1+6Hx1, m), 1.15-1.29 (4Hx1+4Hx1, m), 1.32-1.49 (2Hx1+2Hx1, m), 1.50-1.69 (4Hx1+4Hx1, m), 1.74-1.88 (2Hx1+2Hx1, m), 1.98-2.11 (2Hx1+2Hx1, m), 2.22-2.46 (4Hx1+4Hx1, m), 3.57 (1Hx1, dd, $J = 8.4, 8.4$ Hz), 3.67 (1Hx1, dd, $J = 8.0, 8.4$ Hz), 4.41-4.69 (2Hx1+2Hx1, m), 5.71 (1Hx1+1Hx1, s), 7.38-7.41 (3Hx1+3Hx1, m), 7.52-7.54 (2Hx1+2Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 11.7, 11.8, 15.2, 17.4, 20.5, 20.6, 23.4, 23.7, 29.1, 29.4, 31.4, 32.7, 33.9, 35.3, 35.4, 35.7, 36.3, 36.5, 38.5, 42.9, 43.0, 49.8, 50.1, 53.8, 53.8, 65.8, 82.6, 83.3, 85.3 (d, $J = 182.6$ Hz), 85.9 (d, $J = 182.6$ Hz), 94.3 (d, $J = 19.6$ Hz), 94.4 (d, $J = 19.2$ Hz), 123.8, 127.0, 127.1, 128.3, 128.4, 129.2, 136.4, 136.9, 171.0, 199.4; ^{19}F NMR (CDCl_3 , 376 MHz) δ -223.2 (1Fx1, dd, $J = 47.0, 47.0$ Hz), -223.5 (1Fx1, dd, $J = 46.6, 47.4$ Hz); ESIHRMS: Found: m/z 452.2705. Calcd for $\text{C}_{27}\text{H}_{35}\text{FN}_3\text{O}_2$: (M+H) $^+$ 452.2713.

4.2.2.2. Synthesis of α -alkoxy- β -bromoalkyl azides 2-4 (Section 2.3.3 and 2.3.4)

Scheme 4.2.2.2 Synthesis of α -alkoxy- β -bromoalkyl azides **2-4aa**

Typical procedure B

A solution of cinnamyl alcohol (**2-2a**) (40.3 mg, 0.300 mmol) and α -azidostyrene (**2-1a**) (65.4 mg, 0.450 mmol) in CH_2Cl_2 (2.0 mL) was added to a suspension of TBCO (190.3 mg, 0.450 mmol) in CH_2Cl_2 (1.0 mL) at 0 °C. After 30 min, the reaction was quenched with water. The aqueous layer was extracted with CH_2Cl_2 . The organic layers were washed with brine, dried over MgSO_4 , and concentrated. Purification of the resulting crude product by preparative thin layer chromatography (silica gel, hexane:EtOAc = 97:3) afforded **2-4aa** (88.1 mg, 0.246 mmol) in 82% yield as a pale yellow liquid.

(*E*)-(1-Azido-1-(cinnamyloxy)-2-bromoethyl)benzene (**2-4aa**):

IR (NaCl) 3028, 2930, 2118, 1659, 1598, 1495, 1449, 1377, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 3.76 (1H, d, J = 11.2 Hz), 3.82 (1H, d, J = 11.2 Hz), 4.10 (1H, dd, J = 6.0, 12.4 Hz), 4.23 (1H, dd, J = 6.0, 12.4 Hz), 6.30 (1H, ddd, J = 6.0, 6.0, 16.0 Hz), 6.64 (1H, d, J = 16.0 Hz), 7.24 (1H, t, J = 7.2 Hz), 7.32 (2H, dd, J = 7.2, 8.0 Hz), 7.39–7.45 (5H, m), 7.52–7.54 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 38.5, 65.6, 95.1, 124.5, 126.5, 126.8, 127.8, 128.52, 128.5,

129.3, 132.9, 136.4, 136.6; ESIHRMS: Found: m/z 352.0302. Calcd for $C_{17}H_{16}^{79}BrNNaO$: $(M-N_2+Na)^+$ 352.0313.

1-(1-Azido-2-bromo-1-phenylethyl)pyrrolidine-2,5-dione (2-15) (Table 2.3.2-2 entry 1):

Pale yellow liquid; IR 3062, 2986, 2129, 1721, 1497, 1443, 1412, 1335 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 2.81-2.86 (4H, m), 4.03 (1H, d, $J = 10.8$ Hz), 4.83 (1H, d, $J = 10.8$ Hz), 7.30-7.32 (2H, m), 7.38-7.46 (3H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 28.4, 39.0, 82.2, 125.4, 129.1, 129.7, 135.5, 176.0; ESIHRMS: Found: m/z 344.9975. Calcd for $C_{12}H_{11}^{79}BrN_4NaO_2$: $(M+Na)^+$ 344.9963.

(E)-1-(1-Azido-2-bromo-1-(cinnamyloxy)ethyl)-4-methylbenzene (2-4ba):

Yield: 80% (1H NMR yield) from 0.35 mmol of **2-2a** with 1.5 equiv of vinyl azide **2-1b** and 1.5 equiv of TBCO for 1.5 h following typical procedure B.; pure product could be partially obtained by preparative thin layer chromatography (silica gel, hexane:EtOAc = 97 : 3); pale yellow liquid; IR (NaCl) 3026, 2922, 2116, 1658, 1611, 1578, 1510, 1495, 1449, 1377, 1271 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 2.37 (3H, s), 3.74 (1H, d, $J = 11.2$ Hz), 3.80 (1H, d, $J = 11.2$ Hz), 4.09 (1H, dd, $J = 6.0, 12.4$ Hz), 4.22 (1H, dd, $J = 6.0, 12.4$ Hz), 6.29 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.64 (1H, d, $J = 16.0$ Hz), 7.21-7.26 (3H, m), 7.31 (2H, dd, $J = 7.6, 7.6$ Hz), 7.38-7.42 (4H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.2, 38.7, 65.5, 95.1, 124.6, 126.5, 126.7,

127.8, 128.5, 129.2, 132.8, 133.6, 136.5, 139.2; ESIHRMS: Found: m/z 344.0645. Calcd for $C_{18}H_{19}^{79}BrNO$: $(M-N_2+H)^+$ 344.0650.

(*E*)-1-(1-Azido-2-bromo-1-(cinnamyloxy)ethyl)-4-chlorobenzene (2-4ca):

Yield: 77% (1H NMR yield) from 0.37 mmol of **2-2a** with 1.5 equiv of vinyl azide **2-1c** and 1.5 equiv of TBCO for 2 h following typical procedure B.; pure product could be partially obtained by preparative thin layer chromatography (silica gel, hexane:EtOAc = 97 : 3); pale yellow liquid; IR (NaCl) 3026, 2964, 2118, 1654, 1597, 1578, 1489, 1449, 1377, 1269 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 3.72 (1H, d, J = 11.2 Hz), 3.77 (1H, d, J = 11.2 Hz), 4.08 (1H, dd, J = 6.0, 12.4 Hz), 4.23 (1H, dd, J = 6.0, 12.4 Hz), 6.28 (1H, ddd, J = 6.0, 6.0, 15.6 Hz), 6.64 (1H, d, J = 15.6 Hz), 7.24-7.27 (1H, m), 7.32 (2H, dd, J = 7.6, 7.6 Hz), 7.38-7.42 (4H, m), 7.47 (2H, d, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 37.9, 65.6, 94.7, 124.3, 126.5, 127.9, 128.4, 128.6, 128.7, 133.1, 135.3, 135.4, 136.3; ESIHRMS: Found: m/z 364.0109. Calcd for $C_{17}H_{16}^{79}BrClNO$: $(M-N_2+H)^+$ 364.0104.

Methyl 4-(1-azido-2-bromo-1-(cinnamyloxy)ethyl)benzoate (2-4da):

Yield: 87% (111 mg, 0.27 mmol) from 0.31 mmol of **2-2a** with 1.5 equiv of vinyl azide **2-1d** and 1.5 equiv of TBCO for 1 h; pale yellow liquid following typical procedure B.; IR (NaCl) 3055, 2951, 2120, 1722, 1610, 1578, 1497, 1435, 1377, 1280 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz)

δ 3.76 (1H, d, $J = 11.2$ Hz), 3.81 (1H, d, $J = 11.2$ Hz), 3.93 (3H, s), 4.09 (1H, ddd, $J = 1.2, 6.0, 12.0$ Hz), 4.25 (1H, ddd, $J = 1.2, 6.0, 12.0$ Hz), 6.30 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.64 (1H, d, $J = 16.0$ Hz), 7.23-7.26 (1H, m), 7.33 (2H, dd, $J = 7.2, 7.6$ Hz), 7.39 (2H, d, $J = 7.2$ Hz), 7.62 (2H, d, $J = 8.4$ Hz), 8.09 (2H, d, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 37.7, 52.3, 65.7, 94.9, 124.2, 126.6, 127.1, 128.0, 128.6, 129.8, 131.0, 133.2, 136.3, 141.5, 166.4; ESIHRMS: Found: m/z 388.0553. Calcd for $\text{C}_{19}\text{H}_{19}^{79}\text{BrNO}_3$: (M- N_2 +H) $^+$ 388.0548.

(*E*)-2-(1-Azido-2-bromo-1-(cinnamyloxy)ethyl)naphthalene (2-4fa):

Yield: 97% (118 mg, 0.29 mmol) from 0.30 mmol of **2-2a** with 1.5 equiv of vinyl azide **2-1f** and 1.5 equiv of TBCO for 50 min following typical procedure B.; pale yellow liquid; IR (NaCl) 3053, 2986, 2118, 1659, 1599, 1506, 1497, 1449, 1379, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 3.85 (1H, d, $J = 11.2$ Hz), 3.92 (1H, d, $J = 11.2$ Hz), 4.11 (1H, ddd, $J = 0.8, 6.0, 12.4$ Hz), 4.27 (1H, ddd, $J = 1.2, 6.0, 12.4$ Hz), 6.32 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.65 (1H, d, $J = 16.0$ Hz), 7.23-7.26 (1H, m), 7.32 (2H, dd, $J = 7.2, 7.6$ Hz), 7.39 (2H, d, $J = 7.6$ Hz), 7.52-7.58 (3H, m), 7.85-7.92 (3H, m), 8.08 (1H, s); ^{13}C NMR (CDCl_3 , 100 MHz) δ 38.3, 65.8, 95.3, 123.4, 124.6, 126.6 (overlapped), 127.0, 127.3, 127.6, 127.9, 128.55, 128.58, 128.61, 132.8, 133.0, 133.5, 133.8, 136.4; ESIHRMS: Found: m/z 380.0656. Calcd for $\text{C}_{21}\text{H}_{19}^{79}\text{BrNO}$: (M- N_2 +H) $^+$ 380.0650.

(E)-(3-((2-Azido-1-bromo-4-phenylbutan-2-yl)oxy)prop-1-en-1-yl)benzene (2-4ga):

Yield: 64% (^1H NMR yield stated in notebook) from 0.39 mmol of **2-2a** with 1.5 equiv of vinyl azide **2-1g** and 1.5 equiv of TBCO for 23 h following typical procedure B.; colorless liquid; IR (NaCl) 3051, 2982, 2112, 1654, 1602, 1578, 1495, 1452, 1377, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.23-2.28 (2H, m), 2.67-2.71 (2H, m), 3.52 (1H, d, $J = 11.2$ Hz), 3.58 (1H, d, $J = 11.2$ Hz), 4.30-4.31 (1Hx2, m), 6.27 (1H, ddd, $J = 6.0, 6.0, 16.0$ Hz), 6.65 (1H, d, $J = 16.0$ Hz), 7.20-7.32 (8H, m), 7.38 (2H, d, $J = 7.2$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 29.7, 33.6, 36.7, 64.1, 94.2, 124.6, 126.3, 126.6, 127.9, 128.3, 128.57, 128.58, 132.9, 136.4, 140.5; ESIHRMS: Found: m/z 358.0814. Calcd for $\text{C}_{19}\text{H}_{21}^{79}\text{BrNO}$: ($\text{M}-\text{N}_2+\text{H}$) $^+$ 358.0807.

(E)-(1-Azido-2-bromo-1-((4-phenylbut-3-en-1-yl)oxy)ethyl)benzene (2-4ab):

Yield: 80% (88.9 mg, 0.24 mmol) from 0.30 mmol of **2-2b** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 40 min following typical procedure B.; pale yellow liquid; IR (NaCl) 3053, 2986, 2118, 1641, 1599, 1492, 1449, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.56-2.61 (2H, m), 3.55 (1H, ddd, $J = 6.8, 6.7, 8.8$ Hz), 3.70 (1H, ddd, $J = 6.8, 6.7, 8.8$ Hz), 3.74 (1H, d, $J = 11.2$ Hz), 3.78 (1H, d, $J = 11.2$ Hz), 6.24 (1H, ddd, $J = 6.8, 6.8, 16.0$ Hz), 6.50 (1H, d, $J = 16.0$ Hz), 7.20-7.24 (1H, m), 7.31 (2H, dd, $J = 7.6, 8.0$ Hz), 7.36-7.43 (5H, m), 7.52-7.53 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 33.3, 38.3, 64.0, 94.9, 126.0, 126.2, 126.8, 127.1,

128.4, 128.5, 129.2, 132.2, 136.8, 137.4; ESIHRMS: Found: m/z 366.0465. Calcd for $C_{18}H_{18}^{79}BrNNaO$: (M-N₂+Na)⁺ 366.0469.

(1-Azido-2-bromo-1-(pent-4-en-1-yloxy)ethyl)benzene (2-4ac):

Yield: 84% (106.7 mg, 0.344 mmol) from 0.410 mmol of **2-2c** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 0.5 h following typical procedure B.; pale yellow liquid; IR (NaCl) 3053, 2943, 2118, 1639, 1492, 1449, 1265 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 1.74-1.81 (1Hx2, m), 2.18-2.23 (1Hx2, m), 3.45 (1H, ddd, 6.4, 6.4, 8.8 Hz), 3.58 (1H, m, J = 6.4, 6.4, 8.8 Hz), 3.72 (1H, d, J = 11.2 Hz), 3.76 (1H, d, J = 11.2 Hz), 5.00 (1H, d, J = 10.4 Hz), 5.07 (1H, dd, J = 1.6, 16.8 Hz), 5.79-5.89 (1H, m), 7.38-7.45 (3H, m), 7.49-7.51 (2H, m); ¹³C NMR (CDCl₃, 100 MHz) δ 28.8, 30.2, 38.3, 63.7, 94.7, 115.0, 126.7, 128.4, 129.1, 136.9, 137.8; ESIHRMS: Found: m/z 304.0321. Calcd for $C_{13}H_{16}^{79}BrNNaO$: (M-N₂+Na)⁺ 304.0313.

(3-(1-Azido-2-bromo-1-phenylethoxy)prop-1-yn-1-yl)trimethylsilane (2-4ad):

Yield: 67% (68.4 mg, 0.195 mmol) from 0.293 mmol of **2-2d** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 1 h following typical procedure B.; pale yellow liquid; IR (NaCl) 3053, 2963, 2181, 2118, 1492, 1449, 1366, 1265 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 0.19 (9H, s), 3.73 (1H, d, J = 11.6 Hz), 3.83 (1H, d, J = 11.6 Hz), 4.10 (1H, d, J = 15.6 Hz), 4.18 (1H, d, J = 15.6 Hz), 7.38-7.45 (3H, m), 7.49-7.52 (2H, m); ¹³C NMR (CDCl₃, 100 MHz) δ -0.3, 37.8,

53.9, 91.6, 95.6, 100.5, 127.0, 128.5, 129.4, 135.9; ESIHRMS: Found: m/z 346.0247. Calcd for $C_{14}H_{18}^{79}BrNNaOSi$: $(M-N_2+Na)^+$ 346.0239.

(1-Azido-2-bromo-1-((3-phenylprop-2-yn-1-yl)oxy)ethyl)benzene (2-4ae):

Yield: 76% (108 mg, 0.303 mmol) from 0.400 mmol of **2-2e** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 1 h following typical procedure B.; pale yellow liquid; IR (NaCl) 3053, 2983, 2120, 1491, 1443, 1371, 1265 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 3.79 (1H, d, J = 11.2 Hz), 3.98 (1H, d, J = 11.2 Hz), 4.34 (1H, d, J = 15.6 Hz), 4.43 (1H, d, J = 15.6 Hz), 7.31-7.33 (3H, m), 7.42-7.47 (5H, m), 7.55-7.57 (2H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 37.9, 54.0, 84.3, 86.4, 95.6, 122.4, 127.0, 128.2, 128.6 (overlapped), 129.5, 131.8, 135.9; ESIHRMS: Found: m/z 350.0154. Calcd for $C_{17}H_{14}^{79}BrNNaO$: $(M-N_2+Na)^+$ 350.0156.

(1-Azido-2-bromo-1-(but-3-yn-1-yloxy)ethyl)benzene (2-4af):

Yield: 92% (Recorded 92% in notebook) (89.4 mg, 0.304 mmol) from 0.332 mmol of **2-2a** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 40 min following typical procedure B.; pale yellow liquid; IR (NaCl) 3030, 2953, 2118, 1492, 1449, 1387, 1273 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 2.01 (1H, dd, J = 2.6, 2.6 Hz), 2.55 (1Hx2, m), 3.55 (1H, ddd, J = 6.8, 6.8, 9.2 Hz), 3.68 (1H, ddd, J = 6.8, 6.8, 9.2 Hz), 3.72 (1H, d, J = 11.2 Hz), 3.79 (1H, d, J = 11.2 Hz), 7.39-7.44 (3H, m), 7.50-7.52 (2H, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 19.8, 38.0, 62.6, 69.8, 80.6, 94.8, 126.8, 128.5, 129.3, 136.4; ESIHRMS: Found: m/z 287.9990. Calcd for $C_{12}H_{12}^{79}BrNNaO$: $(M-N_2+Na)^+$ 288.0000.

3-(2-(1-Azido-2-bromo-1-phenylethoxy)ethyl)-1-tosyl-1H-indole (2-4ag):

Yield: 98% (142.6 mg, 0.264 mmol) from 0.269 mmol of **2-2g** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 40 min following typical procedure B.; thick viscous brown liquid; IR (NaCl) 3053, 2938, 2118, 1597, 1495, 1449, 1371, 1265, 1172 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.29 (3H, s), 3.01-3.04 (1Hx2, m), 3.62-3.71 (3H, m), 3.78-3.84 (1H, m), 7.15-7.22 (3H, m), 7.28-7.32 (6H, m), 7.42 (1H, d, $J = 8.0$ Hz), 7.45 (1H, s), 7.75 (2H, d, $J = 8.4$ Hz), 7.99 (1H, d, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 21.5, 25.4, 38.4, 63.5, 94.7, 113.7, 119.2, 119.4, 123.0, 123.8, 124.6, 126.6, 126.8, 128.4, 129.2, 129.8, 130.8, 135.1, 135.3, 136.4, 144.8; ESIHRMS: Found: m/z 561.0576. Calcd for $\text{C}_{25}\text{H}_{23}^{79}\text{BrN}_4\text{NaO}_3\text{S}$: $(\text{M}+\text{Na})^+$ 561.0572.

(1-Azido-2-bromo-1-(2-(phenylsulfonyl)ethoxy)ethyl)benzene (2-4ah):

Yield: 70% (88.5 mg, 0.216 mmol) from 0.309 mmol of **2-2h** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 45 min following typical procedure B.; thick viscous brown liquid, IR (NaCl) 3055, 2928, 2118, 1495, 1449, 1391, 1321, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 3.46-3.50 (2H, m), 3.52 (1H, d, $J = 11.2$ Hz), 3.56 (1H, d, $J = 11.2$ Hz), 3.81-3.86 (1H, m), 3.88-3.94 (1H, m), 7.27-7.30 (2H, m), 7.36-7.37 (3H, m), 7.57 (2H, dd, $J = 7.6, 8.0$ Hz), 7.65-7.68 (1H, m, $J = 7.6$ Hz), 7.96 (2H, d, $J = 8.0$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 37.2, 56.2, 57.9, 95.1, 126.6, 128.1, 128.6, 129.3, 129.5, 133.8, 135.6, 139.8; ESIHRMS: Found: m/z 431.9997. Calcd for $\text{C}_{16}\text{H}_{16}^{79}\text{BrN}_3\text{NaO}_3\text{S}$: $(\text{M}+\text{Na})^+$ 431.9993.

Diethyl ((1-azido-2-bromo-1-phenylethoxy)methyl)phosphonate (2-4ai):

Yield: 41% (54.3 mg, 0.138 mmol) from 0.336 mmol of **2-2i** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 3 h following typical procedure B.; thick viscous brown liquid; IR (NaCl) 3053, 2984, 2120, 1492, 1449, 1392, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.35 (3H, t, $J = 7.2$ Hz), 1.37 (3H, t, $J = 7.2$ Hz), 3.63 (1H, dd, $J = 11.2, 12.8$ Hz), 3.73 (1H, d, $J = 11.2$ Hz), 3.78-3.84 (2H, m), 4.18-4.24 (4H, m), 7.40-7.43 (3H, m), 7.48-7.50 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 16.4 (d, $J = 5.8$ Hz), 16.5 (d, $J = 5.8$ Hz), 37.4, 58.4 (d, $J = 170.6$ Hz), 62.6 (d, $J = 6.3$ Hz), 62.9 (d, $J = 6.5$ Hz), 95.9 (d, $J = 14.7$ Hz), 126.9, 128.7, 129.6, 135.2; ESIHRMS: Found: m/z 414.0189. Calcd for $\text{C}_{13}\text{H}_{19}^{79}\text{BrN}_3\text{NaO}_4\text{P}$: $(\text{M}+\text{Na})^+$ 414.0194.

2-(4-((1-Azido-2-bromo-1-phenylethoxy)methyl)phenyl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2-4aj):

Yield: 70% (106 mg, 0.231 mmol) from 0.331 mmol of **2-2j** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 0.5 h following typical procedure B.; brown liquid; IR (NaCl) 3051, 2980, 2118, 1518, 1433, 1360, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.36 (12H, s), 3.80 (1H, d, $J = 11.2$ Hz), 3.86 (1H, d, $J = 11.2$ Hz), 4.48 (1H, d, $J = 11.6$ Hz), 4.64 (1H, dd, $J = 11.6$ Hz), 7.38-7.46 (5H, m), 7.55-7.57 (2H, m), 7.82 (2H, d, $J = 7.6$ Hz); ^{13}C NMR (CDCl_3 , 100 MHz) δ 24.8, 38.2, 66.6, 83.8, 95.2, 126.86, 126.88, 128.5, 129.3, 134.9, 136.5, 140.0; ESIHRMS: Found: m/z 480.1087. Calcd for $\text{C}_{21}\text{H}_{25}\text{B}^{79}\text{BrN}_3\text{NaO}_3$: $(\text{M}+\text{Na})^+$ 480.1070.

3-(1-Azido-2-bromo-1-phenylethoxy)-1-phenylpropan-1-ol (2-4ak):

dr = 1:1 (inseparable), the ratio was determined by ^1H NMR.

Yield: 73% (96.1 mg, 0.255 mmol) from 0.351 mmol of **2-2k** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of TBCO for 0.5 h following typical procedure B.; colorless liquid; IR (NaCl) 3421, 3059, 2954, 2116, 1492, 1449, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.08-2.13 (2Hx1+2Hx1, m), 2.51-2.53 (1Hx1+1Hx1, m), 3.56 (1Hx1, td, $J = 5.6, 9.6$ Hz), 3.67-3.69 (1Hx1+1Hx1, m), 3.75-3.80 (3Hx1+2Hx1, m), 4.99-5.01 (1Hx1+1Hx1, m), 7.27-7.31 (1Hx1+1Hx1, m), 7.35-7.43 (7Hx1+7Hx1, m), 7.48-7.50 (2Hx1+2Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 38.53, 38.60, 38.62, 38.64, 61.86, 62.04, 72.00, 72.17, 94.59, 94.61, 125.73, 125.75, 126.64, 126.67, 127.5, 128.45, 128.54, 129.26, 129.29, 136.5, 144.06, 144.12; ESIHRMS: Found: m/z 398.0495. Calcd for $\text{C}_{17}\text{H}_{18}^{79}\text{BrN}_3\text{NaO}_2$: ($\text{M}+\text{Na}$) $^+$ 398.0480.

3-(1-Azido-2-bromo-1-phenylethoxy)-3-phenylpropan-1-ol (2-4ak')

Yield: 1.4% (1.8 mg, 0.00478 mmol) from 0.351 mmol of **2-2k** with 1.2 equiv of vinyl azide **2-1a** and 1.2 equiv of TBCO; two diastereomers (1:1) could be separated by flash column chromatography (silica gel; hexane : EtOAc = 80 : 20).

Data for diastereomer 1: IR (NaCl) 3408, 3030, 2955, 2116, 1490, 1449, 1269 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.59 (1H, s br), 2.01-2.09 (1H, m), 2.21-2.27 (1H, m), 3.57-3.61 (1H, m), 3.75-3.87 (3H, m), 4.92 (1H, dd, $J = 6.4, 6.8$ Hz), 7.18-7.27 (8H, m), 7.37-7.39 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 37.1, 41.1, 59.2, 75.0, 95.7, 126.4, 126.9, 127.5, 128.2, 128.4, 129.1,

137.0, 142.4; ESIHRMS: Found: m/z 398.0486. Calcd for $C_{17}H_{18}^{79}BrN_3NaO_2$: $(M+Na)^+$ 398.0480.

Data for diastereomer 2: IR (NaCl) 3421, 3053, 2986, 2122, 1492, 1449, 1422, 1265 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 1.34 (1H, s br), 1.88-1.96 (1H, m), 2.01-2.11 (1H, m), 3.46-3.49 (1H, m), 3.60-3.64 (1H, m), 3.66 (1H, d, $J = 11.2$ Hz), 3.75 (1H, d, $J = 11.2$ Hz), 4.73 (1H, dd, $J = 6.8, 6.8$ Hz), 7.30-7.39 (5H, m), 7.43-7.49 (3H, m), 7.57 (2H, dd, $J = 1.6, 8.0$ Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 37.6, 40.9, 59.2, 75.5, 96.0, 126.5, 127.1, 127.8, 128.5, 128.6, 129.4, 136.9, 142.4; ESIHRMS: Found: m/z 398.0481. Calcd for $C_{17}H_{18}^{79}BrN_3NaO_2$: $(M+Na)^+$ 398.0480.

1-(1-Azido-2-bromo-1-phenylethoxy)-3,5-dimethylbenzene (2-4al):

Yield: 30% (44.2 mg, 0.128 mmol) from 0.421 mmol of **2-2l** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 6 h following typical procedure B. This product was contaminated with a trace amount of inseparable impurity; white solid, mp 94-98 °C; IR (NaCl) 3053, 2986, 2120, 1591, 1448, 1421, 1265 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 2.22 (6H, s), 3.82 (1H, d, $J = 11.2$ Hz), 3.86 (1H, d, $J = 11.2$ Hz), 6.67 (3H, s), 7.38-7.42 (3H, m), 7.54 (2H, dd, $J = 1.6, 8.0$ Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.3, 39.3, 94.8, 117.5, 125.1, 126.7, 128.4, 129.2, 136.8, 139.0, 153.3; ESIHRMS: Found: m/z 3318.0499. Calcd for $C_{16}H_{17}^{79}BrNO$: $(M-N_2+H)^+$ 318.0494.

(2*S*,3*R*,4*S*,5*R*,6*R*)-6-((1-azido-2-bromo-1-phenylethoxy)methyl)tetrahydro-2*H*-pyran-2,3,4,5-tetrayl tetraacetate (2-4am):

dr = 1:1 (inseparable), the ratio was determined by ^1H NMR.

Yield: 73% (128 mg, 0.22 mmol) from 0.30 mmol of **2-2m** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of TBCO for 0.5 h following typical procedure B.; brown liquid; IR (NaCl) 3053, 2986, 2122, 1759, 1449, 1421, 1367, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.99 (3Hx1, s), 2.01 (3Hx2+3Hx1, s), 2.02 (3Hx1+3Hx1, s), 2.08 (3Hx1+3Hx1, s), 3.41 (1H, dd, $J = 4.4, 10.8$ Hz), 3.58-3.71 (3Hx1+3Hx1, m), 3.77-3.82 (1Hx1+2Hx1, m), 5.13-5.24 (3Hx1+3Hx1, m), 5.71 (1Hx1+1Hx1, d, $J = 8.0$ Hz), 5.72 (1Hx1, d, $J = 8.0$ Hz), 7.35-7.41 (4Hx1+4Hx1, m), 7.45-7.48 (1Hx1+1Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 20.52, 20.56, 20.59, 20.62, 20.73, 37.37, 37.62, 62.24, 62.47, 68.07, 68.13, 70.18, 70.23, 72.99, 73.01, 73.08, 73.20, 91.55, 91.61, 94.81, 94.84, 126.78, 126.89, 128.43, 129.28, 135.92, 136.16, 168.97, 169.17, 169.38, 170.10, 170.17; ESIHRMS: Found: m/z 594.0695. Calcd for $\text{C}_{22}\text{H}_{26}^{79}\text{BrN}_3\text{NaO}_{10}$: ($\text{M}+\text{Na}$) $^+$ 594.0699.

(1-Azido-2-bromo-1-(((1*R*,2*S*,5*R*)-2-isopropyl-5-methylcyclohexyl)oxy)ethyl)benzene (2-4an):

An inseparable diastereomeric mixture and the ratio could not be determined by NMR.

Yield: 54% (70.1 mg, 0.184 mmol) from 0.340 mmol of **2-2n** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of TBCO for 1 h following typical procedure B.; white solid, mp 63-66 $^{\circ}\text{C}$;

IR (NaCl) 3059, 2954, 2116, 1492, 1449, 1369, 1273 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.58 (3H, d, $J = 7.2$ Hz), 0.83-0.92 (8H, m), 1.05-1.14 (2H, m), 1.29-1.31 (1H, m), 1.55-1.60 (2H, m), 2.20-2.24 (2H, m), 3.26 (1H, ddd, $J = 4.4, 10.4, 10.4$ Hz), 3.82 (1H, d, $J = 11.2$ Hz), 3.88 (1H, d, $J = 11.2$ Hz), 7.39-7.44 (3H, m), 7.49-7.52 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 16.0, 21.4, 22.3, 22.9, 25.1, 31.3, 34.0, 38.3, 41.5, 48.3, 75.7, 95.0, 127.1, 128.4, 129.2, 135.9; ESIHRMS: Found: m/z 374.1094. Calcd for $\text{C}_{18}\text{H}_{26}^{79}\text{BrNNaO}$: $(\text{M}-\text{N}_2+\text{Na})^+$ 374.1095.

(1*R*,5*S*)-2-(2-(1-azido-2-bromo-1-phenylethoxy)ethyl)-6,6-dimethylbicyclo[3.1.1]hept-2-ene (2-4ao):

dr = 1:1 (inseparable), the ratio was determined by ^1H NMR.

Yield: 70% (82.5 mg, 0.21 mmol) from 0.30 mmol of **2-2o** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 1 h following typical procedure B.; colorless liquid; IR (NaCl) 3061, 2916, 2116, 1655, 1467, 1449, 1381, 1269 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.82 (3Hx1, s), 0.83 (3Hx1, s), 1.17 (1Hx1+1Hx1, dd, $J = 2.0, 8.4$ Hz), 1.26 (3Hx1, s), 1.28 (3Hx1, s), 2.02-2.08 (2Hx1+2Hx1, m), 2.21-2.37 (5Hx1+5Hx1, m), 3.43-3.47 (1Hx1+1Hx1, m), 3.55-3.59 (1Hx1+1Hx1, m), 3.68-3.757 (2Hx1+2Hx1, m), 5.29(1Hx1+1Hx1, m), 7.38-7.43 (3Hx1+3Hx1, m), 7.48-7.50 (2Hx1+2Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 21.13, 26.28, 26.30, 31.35, 31.67, 31.69, 37.06, 37.08, 37.99, 38.28, 38.30, 40.72, 45.85, 62.92, 62.95, 94.98, 118.33, 118.36, 126.76, 128.39, 129.10, 136.96, 136.99, 144.43, 144.47; ESIHRMS: Found: m/z 384.0936. Calcd for $\text{C}_{19}\text{H}_{24}^{79}\text{BrNNaO}$: $(\text{M}-\text{N}_2+\text{Na})^+$ 384.0939.

(Z)-(1-Azido-2-bromo-1-((3,7-dimethylocta-2,6-dien-1-yl)oxy)ethyl)benzene (2-4ap):

Yield: 83% (104.2 mg, 0.275 mmol) from 0.333 mmol of **2-2p** with 1.5 equiv of vinyl azide **2-1a** and 1.5 equiv of TBCO for 0.5 h following typical procedure B.; pale yellow liquid; IR (NaCl) 3053, 2916, 2114, 1668, 1492, 1449, 1377, 1265 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 1.56 (3H, s), 1.65 (3H, s), 1.78 (3H, s), 2.01-2.04 (4H, m), 3.71 (1H, d, $J = 11.2$ Hz), 3.78 (1H, d, $J = 11.2$ Hz), 3.90 (1H, dd, $J = 7.2, 11.2$ Hz), 4.07 (1H, dd, $J = 6.8, 11.2$ Hz), 5.05 (1H, m), 5.42 (1H, dd, 1H, dd, $J = 6.8, 7.2$ Hz), 7.38-7.43 (3H, m), 7.49-7.52 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 17.6, 23.5, 25.6, 26.6, 32.2, 38.5, 61.3, 95.0, 120.3, 123.6, 126.8, 128.4, 129.1, 132.0, 136.8, 141.6; ESIHRMS: Found: m/z 372.0949. Calcd for $\text{C}_{18}\text{H}_{24}^{79}\text{BrNNaO}$: ($\text{M-N}_2+\text{Na}$) $^+$ 372.0939.

(3R,8R,9S,10S,13S,14S)-3-(1-azido-2-bromo-1-phenylethoxy)-10,13-dimethyltetradecahydro-1H-cyclopenta[a]phenanthren-17(2H)-one (2-4aq):

An inseparable diastereomeric mixture and the ratio could not be determined by NMR.

Yield: 85% (139.3 mg, 0.271 mmol) from 0.320 mmol of **2-2q** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of TBCO for 0.5 h following typical procedure B.; pale yellow liquid; IR (NaCl) 2931, 2114, 1736, 1492, 1449, 1371, 1269 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.79 (3H, s), 0.87-0.91 (4H, m), 1.03-1.13 (1H, m), 1.18-1.35 (6H, m), 1.42-1.55 (6H, m), 1.67-1.74 (2H, m), 1.79-1.82 (3H, m), 1.89-1.98 (1H, m), 2.03-2.12 (1H, m), 2.40-2.47 (1H, m), 3.72-3.80

(2H, m), 3.97-4.00 (1H, m), 7.36-7.42 (3H, m), 7.50-7.53 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 11.35, 13.79, 20.06, 21.72, 27.93, 28.06, 28.19, 28.23, 30.78, 30.82, 31.52, 31.54, 32.92, 33.07, 34.60, 34.74, 35.00, 35.82, 35.89, 35.90, 37.55, 37.68, 39.74, 40.00, 47.78, 51.44, 51.46, 54.37, 54.41, 70.25, 96.01, 96.03, 126.82, 126.84, 128.24, 128.99, 129.01, 137.55, 137.79, 221.30, 221.35; ESIHRMS: Found: m/z 536.1898. Calcd for $\text{C}_{27}\text{H}_{36}^{79}\text{BrN}_3\text{NaO}_2$: $(\text{M}+\text{Na})^+$ 536.1889.

(8*R*,9*S*,10*R*,13*S*,14*S*,17*S*)-17-(1-azido-2-bromo-1-phenylethoxy)-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydro-1*H*-cyclopenta[*a*]phenanthren-3(2*H*)-one (2-4ar):

dr = 1:1 (inseparable), the ratio was determined by ^1H NMR.

Yield: 90% (152 mg, 0.297 mmol) from 0.329 mmol of **2-2r** with 2.2 equiv of vinyl azide **1a** and 2.2 equiv of TBCO for 0.5 h following typical procedure B.; pale yellow liquid; IR (NaCl) 3057, 2941, 2116, 1672, 1614, 1492, 1449, 1375, 1269 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 0.82-1.00 (6Hx1+6Hx1, m), 1.18 (3Hx1, s), 1.20 (3Hx1, s), 1.24-1.46 (2Hx1+2Hx1, m), 1.50-1.69 (6Hx1+6Hx1, m), 1.77-1.86 (2Hx1+2Hx1, m), 1.98-2.14 (2Hx1+2Hx1, m), 2.23-2.46 (4Hx1+4Hx1, m), 3.53 (1Hx1, dd, J = 8.0, 8.4 Hz), 3.62 (1Hx1, dd, J = 7.6, 8.8 Hz), 3.67-3.78 (2Hx1+2Hx1, m), 5.71 (1Hx1+1Hx1, s), 7.38-7.41 (3Hx1+3Hx1, m), 7.49-7.52 (2Hx1+2Hx1, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 11.76, 17.31, 20.45, 20.58, 23.39, 23.63, 29.13, 29.14, 31.34, 32.63, 33.83, 33.85, 35.24, 35.31, 35.59, 35.61, 36.43, 36.57, 36.70, 37.66, 38.48, 38.50, 43.02, 49.65, 49.85, 53.66, 53.75, 83.24, 83.72, 95.16, 95.33, 109.86, 123.75, 123.77, 126.90,

126.95, 128.10, 128.12, 128.97, 129.01, 137.21, 137.71, 170.90, 170.92, 199.25, 199.28;

ESIHRMS: Found: m/z 512.1924. Calcd for $C_{27}H_{35}^{79}BrN_3O_2$: $(M+H)^+$ 512.1913.

(3*R*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-3-(1-azido-2-bromo-1-phenylethoxy)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthrene (2-4as):

dr = 1:1 (inseparable), the ratio was determined by 1H NMR.

Yield: 85% (85.5 mg, 0.144 mmol) from 0.169 mmol of **2-2s** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of TBCO for 1 h following typical procedure B.; white solid, mp 106-108 °C; IR (NaCl) 3030, 2935, 2114, 1686, 1492, 1449, 1381, 1271 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 0.67 (3Hx1+3Hx1, s), 0.85-0.92 (10Hx1+10Hx1, m), 0.96-1.16 (12Hx1+12Hx1, m), 1.24-1.56 (11Hx1+11Hx1, m), 1.72-2.01 (5Hx1+5Hx1, m), 2.07-2.11 (1Hx1, m), 2.34-2.40 (1Hx1, m), 2.45-2.50 (1Hx1+1Hx1, m), 3.49-3.55 (1Hx1+1Hx1, m), 3.72 (1Hx1+1Hx1, d, J = 11.2 Hz), 3.78 (1Hx1+1Hx1, d, J = 11.2 Hz), 5.17 (1Hx1, d, J = 4.8 Hz), 5.35 (1Hx1, d, J = 4.8 Hz), 7.38-7.41 (3Hx1+3Hx1, m), 7.51-7.54 (2Hx1+2Hx1, m); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 11.84, 18.71, 19.28, 21.00, 22.55, 22.81, 23.80, 24.26, 28.00, 28.20, 29.69, 29.75, 31.81, 31.86, 31.92, 35.75, 36.18, 36.43, 36.47, 37.22, 37.26, 37.84, 37.90, 39.51, 39.73, 40.17, 40.39, 42.30, 50.02, 50.07, 56.11, 56.72, 56.74, 75.36, 95.52, 95.59, 121.90, 122.16, 126.94, 128.23, 128.25, 129.07, 129.10, 137.49, 140.43, 140.47; ESIHRMS: Found: m/z 632.3188. Calcd for $C_{35}H_{52}^{79}BrN_3NaO$: $(M+Na)^+$ 632.3191.

(1*R*,3*aS*,5*aR*,5*bR*,7*aR*,9*S*,11*aR*,11*bR*,13*aR*,13*bR*)-3*a*-((1-azido-2-bromo-1-phenylethoxy)methyl)-5*a*,5*b*,8,8,11*a*-pentamethyl-1-(prop-1-en-2-yl)icosahydro-1*H*-cyclopenta[*a*]chrysen-9-ol (2-4at):

dr = 1:1 (inseparable), the ratio was determined by ^1H NMR.

Yield: 81% (220.7 mg, 0.331 mmol) from 0.407 mmol of **2-2s** with 2.2 equiv of vinyl azide **2-1a** and 2.2 equiv of TBCO in THF-CH₂Cl₂ (1:1) solvent system for 1 h following typical procedure B.; pale yellow solid, mp 102-104 °C; IR (NaCl) 3067, 2941, 2114, 1639, 1449, 1388, 1265 cm⁻¹; ^1H NMR (CDCl₃, 400 MHz) δ 0.64-0.82 (7Hx1+7Hx1, m), 0.95 -1.13 (11Hx1+11Hx1, m), 1.26-1.67 (19Hx1+19Hx1, m), 1.82-1.87 (1Hx1+1Hx1, m), 2.05-2.20 (2Hx1+2Hx1, m), 2.27-2.43 (1Hx1+1Hx1, m), 3.07-3.12 (2Hx1+1Hx1, m), 3.27 (1Hx1, d, J = 8.8 Hz), 3.47 (1Hx1, d, J = 8.8 Hz), 3.70-3.85 (2Hx1+3Hx1, m), 4.57 (1Hx1+1Hx1, s), 5.64 (1Hx1, d, J = 2.0 Hz), 5.67 (1Hx1, d, J = 2.0 Hz), 7.38-7.43 (3Hx1+3Hx1, m), 7.49-7.51 (2Hx1+2Hx1, m); ^{13}C NMR (CDCl₃, 100 MHz) δ 14.75, 14.83, 15.34, 15.65, 15.96, 16.04, 16.08, 18.26, 19.09, 19.19, 20.70, 20.78, 25.12, 25.18, 27.02, 27.18, 27.36, 27.96, 29.72, 29.86, 30.00, 34.16, 34.85, 34.89, 37.08, 37.12, 37.40, 37.56, 38.21, 38.63, 38.69, 38.81, 38.83, 40.77, 40.93, 42.50, 42.69, 47.12, 47.14, 47.72, 48.80, 48.90, 50.27, 50.37, 55.22, 55.28, 61.32, 61.76, 78.89, 78.93, 94.56, 94.80, 109.60, 109.66, 126.68, 126.69, 128.42, 128.46, 129.10, 129.14, 137.03, 150.32, 150.34; ESIHRMS: Found: m/z 690.3457. Calcd for C₃₅H₅₂⁸¹BrN₃NaO: (M+H)⁺ 690.3433.

4.2.2 Intramolecular Azide-Alkene/Alkyne Cycloaddition (Section 2.3.5)

Intramolecular 1,3-dipolar azide-alkene cycloaddition

Synthesis of 2-5aa from α -alkoxy- β -fluoroalkyl azides 2-3aa

Scheme 4.2.2-1 Synthesis of 2-(fluoromethyl)-2,5-dihydrooxazole **2-5aa**

Typical procedure C

A solution of **2-3aa** (55.0 mg, 0.185 mmol) in CH₃CN (2.0 mL) was heated at reflux for 2 h. The solvent was removed and the residue was purified by flash column chromatography (silica gel, hexane:EtOAc = 95 : 5) afforded product **2-5aa** (39.9 mg, 0.148 mmol) in 80% yield as a white solid. Recrystallization from acetone/hexane gave a colorless crystal.

Synthesis of 2-5aa by 2-step reactions from vinyl azides 2-1a

Scheme 4.2.2-2 2-step synthesis of 2-(fluoromethyl)-2,5-dihydrooxazole **2-5aa** from **2-1a**

Typical procedure D

A solution of cinnamyl alcohol (**2-2a**) (55.5 mg, 0.414 mmol) and α -azido styrene (**2-1a**) (72.0 mg, 0.496 mmol) in CH₃CN (2.0 mL) was added to a suspension of Selectfluor (175.8

mg, 0.496mmol) in CH₃CN (2.0 mL) at 0 °C. The reaction mixture was gradually warmed to room temperature over for 6 h before it was quenched with water. The aqueous layer was extracted with EtOAc. The organic layers were washed with brine, dried over MgSO₄, and concentrated in vacuo. The crude mixture was dissolved in CH₃CN (4 mL) and heated at 100 °C for 2 h. The solvent was removed and the resulting residue was purified by flash column chromatography (silica gel, hexane:EtOAc = 95:5) afforded product **2-5aa** (78.5 mg, 0.291 mmol) in 70% yield as a white solid.

4-Benzyl-2-(fluoromethyl)-2-phenyl-2,5-dihydrooxazole (2-5aa):

Colorless crystal (CCDC 1045921); mp 86-88 °C; IR (KBr) 3030, 2949, 1659, 1603, 1560, 1489, 1447, 1368, 1260 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 3.76 (1H, d, *J* = 15.2 Hz), 3.87 (1H, d, *J* = 15.2 Hz), 4.47 (1H, dd, *J* = 10.0, 47.6 Hz), 4.49 (1H, d, *J* = 14.4 Hz), 4.67 (1H, dd, *J* = 10.0, 47.1 Hz), 4.68 (1H, d, *J* = 14.4 Hz), 7.23-7.26 (3H, m), 7.28-7.40 (5H, m), 7.60 (2H, d, *J* = 6.8 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 37.2, 76.3, 86.2 (d, *J* = 183.1 Hz), 111.7 (d, *J* = 17.6 Hz), 126.1, 127.1, 128.3, 128.5, 128.81, 128.83, 135.0, 138.6 (d, *J* = 2.7 Hz), 173.0; ¹⁹F NMR (CDCl₃, 376 MHz) δ -224.5 (1F, dd, *J* = 47.1, 47.6 Hz); ESIHRMS: Found: *m/z* 270.1293. Calcd for C₁₇H₁₇NOF: (M+H)⁺ 270.1294.

4-Benzyl-2-(3-bromophenyl)-2-(fluoromethyl)-2,5-dihydrooxazole (2-5ea) (Table 2.3.3 entry 5):

Yield: 83% (85.4 mg, 0.25 mmol) from 0.30 mmol of **2-2a** and 0.36 mmol of vinyl azide **2-1e** (Table S3) in 2 steps following typical procedure D. ; pale yellow solid, mp 49-51 °C; IR (KBr) 3030, 2945, 1665, 1595, 1568, 1497, 1472, 1454, 1416, 1366, 1256 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 3.77 (1H, d, *J* = 15.2 Hz), 3.87 (1H, d, *J* = 15.2 Hz), 4.44 (1H, dd, *J* = 10.0, 47.4 Hz), 4.50 (1H, d, *J* = 14.4 Hz), 4.63 (1H, dd, *J* = 10.0, 47.2 Hz), 4.67 (1H, d, *J* = 14.4 Hz), 7.22-7.34 (6H, m), 7.47 (1H, d, *J* = 8.8 Hz), 7.53 (1H, d, *J* = 7.6 Hz), 7.77 (1H, dd, *J* = 1.6, 2.0 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 37.2, 76.5, 86.0 (d, *J* = 183.6 Hz), 111.1 (d, *J* = 18.1 Hz), 122.5, 124.9, 127.2, 128.8, 128.9, 129.4, 129.9, 131.6, 134.8, 140.9 (d, *J* = 3.4 Hz), 173.5; ¹⁹F NMR (CDCl₃, 376 MHz) δ -224.9 (1F, dd, *J* = 47.4, 47.2 Hz); ESIHRMS: Found: *m/z* 348.0402. Calcd for C₁₇H₁₆NOF⁷⁹Br: (M+H)⁺ 348.0399.

4-Benzyl-2-(bromomethyl)-2-phenyl-2,5-dihydrooxazole (2-6aa):

Yield: 92% (72.6 mg, 0.22 mmol) from 0.24 mmol of **2-4aa** at 80 °C in toluene for 2 h following typical procedure C.; white solid, mp 86-87 °C; IR (NaCl) 3051, 2984, 1659, 1602, 1494, 1452, 1361, 1263 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 3.73 (1H, d, *J* = 15.6 Hz), 3.77 (1H, d, *J* = 11.2 Hz), 3.90 (1H, d, *J* = 15.6 Hz), 3.94 (1H, d, *J* = 11.2 Hz), 4.45 (1H, d, *J* = 14.0 Hz), 4.70 (1H, d, *J* = 14.0 Hz), 7.26-7.36 (8H, m), 7.59 (2H, d, *J* = 7.2 Hz); ¹³C NMR (CDCl₃,

100 MHz) δ 37.1, 41.8, 76.7, 110.8, 125.8, 127.1, 128.3, 128.4, 128.8, 129.0, 134.9, 140.6, 172.8; ESIHRMS: Found: m/z 330.0501. Calcd for $C_{17}H_{17}^{79}BrNO$: $(M+H)^+$ 330.0494.

4-Benzyl-2-(fluoromethyl)-2-phenyl-5,6-dihydro-2H-1,3-oxazine (2-7ab):

Yield: 87% (32 mg, 0.113 mmol) from 0.130 mmol of **2-3ab** under reflux in toluene for 3.5 h following typical procedure C. (the crude residue was purified by flash column chromatography with Florisil); pale yellow liquid; IR (NaCl) 3028, 2949, 1667, 1601, 1495, 1449, 1354, 1279 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 1.93 (1H, dd, $J = 4.0, 17.6$ Hz), 2.37 (1H, dd, $J = 7.2, 10.8, 17.6$ Hz), 3.52 (1H, ddd, $J = 4.0, 10.8, 11.2$ Hz), 3.68 (2H, s), 3.88 (1H, dd, $J = 7.2, 11.2$ Hz), 4.50 (1H, dd, $J = 9.2, 47.6$ Hz), 4.52 (1H, dd, $J = 9.2, 47.5$ Hz), 7.22-7.40 (8H, m), 7.63 (2H, d, $J = 7.6$ Hz), ^{13}C NMR ($CDCl_3$, 100 MHz) δ 27.5, 47.8, 57.5, 87.9 (d, $J = 184.3$ Hz), 89.1 (d, $J = 17.4$ Hz), 126.8, 127.4, 128.3, 128.3, 128.7, 129.1, 136.6, 139.8, 168.9; ^{19}F NMR ($CDCl_3$, 376 MHz) δ -223.7 (1F, dd, $J = 47.5, 47.6$ Hz); ESIHRMS: Found: m/z 284.1456. Calcd for $C_{18}H_{19}NOF$: $(M+H)^+$ 284.1451.

6-(Fluoromethyl)-3,6-diphenyl-4,6-dihydrooxazolo[3,4-c][1,2,3]triazole (2-8ae):

Yield: 65% (30.8 mg, 0.104 mmol) from 0.159 mmol of **2-3ae** under reflux in toluene for 19 h following typical procedure C.; pale yellow solid, mp 71-72 $^{\circ}C$; IR (KBr) 3061, 2953, 1587, 1493, 1470, 1450, 1362, 1265 cm^{-1} ; 1H NMR ($CDCl_3$, 400 MHz) δ 4.76 (1H, dd, $J = 10.8, 46.9$

Hz), 5.07 (1H, dd, $J = 10.8, 46.7$ Hz), 5.36 (1H, d, $J = 12.0$ Hz), 5.50 (1H, d, $J = 12.0$ Hz), 7.33 (1H, t, $J = 7.6$ Hz), 7.41-7.48 (5H, m), 7.66-7.68 (2H, m), 7.82-7.84 (2H, m); ^{13}C NMR (CDCl_3 , 100 MHz) δ 65.1, 84.4 (d, $J = 188.4$ Hz), 96.5 (d, $J = 17.4$ Hz), 125.6, 125.8, 128.1, 128.9, 129.0, 129.9, 130.2, 133.7 (d, $J = 2.8$ Hz), 135.3, 138.5; ^{19}F NMR (CDCl_3 , 376 MHz) δ -224.1 (dd, $J = 46.9, 46.7$ Hz); ESIHRMS: Found: m/z 296.1201. Calcd for $\text{C}_{17}\text{H}_{15}\text{N}_3\text{OF}$: $(\text{M}+\text{H})^+$ 296.1199.

7-(Fluoromethyl)-7-phenyl-5,7-dihydro-4H-[1,2,3]triazolo[1,5-c][1,3]oxazine (2-9af):

Yield: 78% (73 mg, 0.31 mmol) from 0.40 mmol of **2-3af** under reflux in toluene for 16 h following typical procedure C.; pale yellow solid, mp 100-102 °C; IR (KBr) 3061, 2959, 1553, 1493, 1449, 1423, 1294, 1246 cm^{-1} ; ^1H NMR (CDCl_3 , 400 MHz) δ 2.86 (1H, dd, $J = 4.0, 16.4$ Hz), 3.20-3.29 (1H, m), 3.79 (1H, ddd, $J = 4.0, 11.6, 12.0$ Hz), 4.20 (1H, dd, $J = 6.8, 11.6$ Hz), 4.85 (1H, dd, $J = 10.4, 47.2$ Hz), 5.18 (1H, dd, $J = 10.4, 46.4$ Hz), 7.40 (5H, m), 7.62 (1H, s); ^{13}C NMR (CDCl_3 , 100 MHz) δ 21.1, 58.7, 84.6 (d, $J = 190$ Hz), 93.4 (d, $J = 16.9$ Hz), 126.5, 128.9, 130.0, 131.0, 131.7, 135.1; ^{19}F NMR (CDCl_3 , 376 MHz) δ -223.6 (1F, dd, $J = 47.0, 47.0$ Hz); ESIHRMS: Found: m/z 310.1348. Calcd for $\text{C}_{18}\text{H}_{17}\text{N}_3\text{OF}$: $(\text{M}+\text{H})^+$ 310.1356.

4.2.3 The procedure for synthesis of 2-10 (Section 2.3.6.1)

A solution of **2-3ar** (77.8 mg, 0.172 mmol), alkyne (59.6 mg, 0.207 mmol), CuTc (3.3 mg, 0.017 mmol), and *N,N*-diisopropylethylamine (45 μ L, 0.259 mmol) in DMF (2.0 mL) was stirred at room temperature for 6.5 h. The reaction mixture was diluted with EtOAc and H₂O. The aqueous layer was extracted with EtOAc. The organic layers were washed with brine (3 times), dried over MgSO₄, and concentrated. The crude mixture was purified by flash column chromatography (silica gel, hexane:EtOAc = 50 : 50) afforded product **2-10** (118.8 mg, 0.161 mmol) as a mixture of two inseparable diastereomers (dr = 1 : 1, determined by ¹⁹F NMR) in 93% yield as a yellow solid; mp 150-152 °C; IR (KBr) 3055, 2943, 1661, 1612, 1587, 1574, 1450, 1354, 1265 cm⁻¹; ¹H NMR (CDCl₃, 400 MHz) δ 0.35-0.41 (1Hx1, m), 0.70-0.96 (6Hx1+6Hx1, m), 1.08-1.18 (4Hx1+4Hx1, m), 1.23-1.79 (7Hx1+7Hx1, m), 1.94-2.04 (3Hx1+2Hx1, m), 2.21-2.40 (4Hx1+4Hx1, m), 2.88 (6Hx1+6Hx1, s), 3.31 (1Hx1, dd, *J* = 7.6, 8.0 Hz), 3.68 (1Hx1, dd, *J* = 8.0, 8.0 Hz), 4.20-4.22 (2Hx1+2Hx1, m), 5.04-5.38 (2Hx1+2Hx1, m), 5.69-5.71 (2Hx1+2Hx1, s br), 7.15 (1Hx1+1Hx1, d, *J* = 7.6 Hz), 7.21-7.28 (3Hx1+3Hx1, m), 7.32-7.37 (3Hx1+3Hx1, m), 7.45-7.51 (2Hx1+2Hx1, m), 8.20 (1Hx1+1Hx1, dd, *J* = 7.6, 7.6 Hz), 8.25 (1Hx1+1Hx1, dd, *J* = 3.6, 8.8 Hz), 8.51 (1Hx1+1Hx1, d, *J* = 8.8 Hz); ¹³C NMR (CDCl₃, 100 MHz) δ 11.8, 11.9, 17.3, 20.2, 20.4, 23.4, 23.7, 28.0, 29.3, 31.3, 32.6, 33.8, 35.1,

35.2, 35.5, 35.6, 36.0, 36.1, 38.4, 38.5, 38.6, 42.9, 43.0, 45.3, 49.6, 49.7, 53.4, 53.7, 81.8 (d, J = 177.3 Hz), 82.6, 82.70, 82.73 (d, J = 184.4 Hz), 91.1 (d, J = 21.6 Hz), 91.6 (d, J = 22.0 Hz), 115.2, 118.5, 122.4, 122.6, 123.0, 123.78, 123.82, 126.3, 128.36, 128.43, 129.2, 129.3, 129.39, 129.45, 129.8, 130.6, 134.4, 134.6, 138.0, 138.1, 144.1, 144.4, 151.9, 170.96, 171.00, 199.4, 199.5; ^{19}F NMR (CDCl_3 , 376 MHz) δ -226.9 (1F $\times$ 1, dd, J = 46.6, 46.6 Hz), -227.9 (1F $\times$ 1, dd, J = 47.0, 47.0 Hz); ESIHRMS: Found: m/z 740.3633. Calcd for $\text{C}_{42}\text{H}_{51}\text{N}_5\text{O}_4\text{FS}$: ($\text{M}+\text{H}$) $^+$ 740.3646.

4.2.4. Synthesis of 2-11 and 2-12: Cu-free azide-alkyne cycloaddition of 2-3ar (Section 2.3.6.2)

Synthesis of Cy3-linked testosterone 2-11

A solution of compound DBCO-Cy3 (Sigma-Aldrich 777366) (100 μL , 2 mM in DMSO) and 2-3ar (100 μL , 20 mM in DMSO) was stirred in dark at room temperature 24 h. The reaction mixture was diluted with methanol. The crude product was purified by HPLC using a C-18 preparative column and linear 20 min gradient from 5% to 95% acetonitrile in

water with 50 mM of ammonium acetate, that could separate each of the regioisomers **2-11**.

The retention times were 15.3 min and 16.1 min, respectively.

HPLC Trace of **2-11**:

ESI-MS Found: m/z 715.95. Calcd for $C_{77}H_{86}FN_7O_{13}S_3$: (M)²⁻ 715.77. (15.3 min)

ESI-MS Found: m/z 715.87. Calcd for $C_{77}H_{86}FN_7O_{13}S_3$: (M)²⁻ 715.77. (16.1 min)

Isomer 1: 15.3 min

Isomer 2: 16.1 min

Synthesis of Cy5-linked testosterone 2-12

A solution of compound DBCO-Cy5 (Sigma-Aldrich 777374) (100 μ L, 2 mM in DMSO) and **2-3ar** (100 μ L, 20 mM in DMSO) was stirred in dark at room temperature 24 h. The reaction mixture was diluted with methanol. The crude product was purified by HPLC using a C-18 preparative column and linear 20 min gradient from 5% to 95% acetonitrile in water with 50 mM of ammonium acetate, that could separate each of the regioisomers **12**. The retention time of the desired products were 15.3 min and 16.0 min, respectively.

HPLC Trace of **2-12**:

ESI-MS Found: m/z 729.06. Calcd for $C_{79}H_{88}FN_7O_{13}S_3$: (M)²⁻ 728.78 (15.3 min)

ESI-MS Found: m/z 729.01. Calcd for $C_{79}H_{88}FN_7O_{13}S_3$: (M)²⁻ 728.78 (16.0 min)

Isomer 1: 15.3 min **Isomer 2: 16.0 min**

4.2.5. Stability test of 2-10 (Section 2.3.6.3)

Typical procedure in Phosphate-buffered saline (PBS) and Fetal Bovine Serum (FBS) buffer /CH₃CN system (Table 2.3.6.3 entries 1-2)

Compound **2-10** (25.5 mg, 0.033 mmol) was added to a mixture of pH 7.4 PBS or pH 7.4 of FBS (0.5 mL) and CH₃CN (0.5 mL) at 37 °C. After 24 hours, the reaction mixture was extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄ and concentrated. The crude mixture was analyzed by ¹H NMR and ¹⁹F NMR using 1,1,2,2-tetrachloroethane and fluorobenzene as internal standards, respectively.

Typical procedure in 10% of FBS in Culture Medium (CM) and 10% of FBS in PBS buffer /CH₃CN system (Table 2.3.6.3 entries 3-4)

Compound **2-10** (25.3 mg, 0.034 mmol) was added to a mixture of pH 7.4 10% of FBS in CM or pH 7.4 10% of FBS in PBS (0.5 mL) and CH₃CN (0.5 mL) at 37 °C. After 24 hours, the reaction mixture was extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄ and concentrated. The crude mixture was analyzed by ¹H NMR and ¹⁹F NMR using 1,1,2,2-tetrachloroethane and fluorobenzene as internal standards, respectively.

Typical procedure in phosphate buffer/CH₃CN system (Table 2.3.6.3 entries 5-6)

Compound **2-10** (25.1 mg, 0.034 mmol) was added to a mixture of pH 6.6 or pH 3.3 phosphate buffer (0.5 mL) and CH₃CN (0.5 mL) at 37 °C. After 24 hours, the reaction mixture was extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄ and concentrated. The crude mixture was analyzed by ¹H NMR and ¹⁹F NMR using 1,1,2,2-tetrachloroethane and fluorobenzene as internal standards, respectively

Typical procedure in HCl/THF system (Table 2.3.6.3 entry 7)

To a solution of compound **2-10** (62.0 mg, 0.085 mmol) in THF (1.7 mL) was added 1N HCl (171 µL, 0.171 mmol) at 23 °C. After 18 h, the reaction mixture was extracted with EtOAc. The organic layer was washed with brine, dried over MgSO₄ and concentrated. The crude mixture was analyzed by ¹H NMR using 1,1,2,2-tetrachloroethane as an internal standard.

4.2.6 Fluorescence spectra characterization

Dye-linked testosterone derivatives were dissolved in DMSO to prepare stock solutions (1 mM). The stock solution was diluted by PBS (pH = 7.4). The resulting solution was subject for spectra characterization (Figure S1). UV-VIS spectra were recorded on a SHIMAZU UV-1800 UV spectrophotometer. Fluorescence spectra were obtained on a SHIMAZU RF-5301PC spectrofluorophotometer.

Figure 5.2.9. UV-VIS and Fluorescence emission spectrum of a) dansyl-linked testosterone **2-10**; b) Cy3-linked testosterone **2-11**; c) Cy5-linked testosterone **2-12**.

4.2.7. General methods for cell culture and live cell imaging

Human embryonic kidney cells HEK293 were grown in Dulbecco's modified eagle medium (Gibico) supplemented with 10% fetal bovine serum (FBS, HyClone) and antibiotics (100 units/mL penicillin and 100 μ g/mL streptomycin) at 5% CO₂ and 37 °C.

4.2.8. Live Cell imaging of dye-linked testosterone **2-10-12** (Section 2.3.6.5)

HEK293 cells were seeded in 60 μ -dish (ibidiGmbH, Germany) in complete medium for 24 h incubation at 5% CO₂ and 37 °C. Then the medium was changed to fresh serum free medium in the presence of 2 μ M **2-10**, **2-11**, and **2-12**. Cell nuclei were stained with bisBenzimide H 33258 (Sigma). Mitochondria, lysosomes and Golgi apparatus were stained with MitoTracker Deep Red FM (Thermo Fisher), LysoTracker Deep Red (Thermo Fisher) and BODIPY TR Ceramide (Thermo Fisher) respectively. Staining of nuclei and these organelles were performed according to the manufacturers' protocols.⁸ The images were

acquired on confocal laser scanning microscope (LSM780, Carl Zeiss Inc., Germany). The laser setting were as follows: H33258 ($\lambda_{\text{ex}} = 405 \text{ nm}$); MitoTracker Deep Red FM ($\lambda_{\text{ex}} = 633 \text{ nm}$); LysoTracker Deep Red ($\lambda_{\text{ex}} = 633 \text{ nm}$); BODIPY TR Ceramide ($\lambda_{\text{ex}} = 561 \text{ nm}$); **2-10** (dansyl, $\lambda_{\text{ex}} = 405 \text{ nm}$); **2-11** (Cy3, $\lambda_{\text{ex}} = 514 \text{ nm}$) and **2-12** (Cy5, $\lambda_{\text{ex}} = 633 \text{ nm}$).

Figure 4.2.8-1 Confocal images of HEK293 cells stained with different dye-linked testosterone **2-10**. Scale bar in all images=20 μm.

Figure 4.2.8-2 Confocal images of HEK293 cells stained with different dyes. Scale bar in all images=20 μ m.

Figure 4.2.8-3 Confocal images of HEK293 cells stained with dansyl-linked testosterone (**2-10**) and different organelle specific trackers. Scale bar in the images (**a-i**) = 20 μm and that in (**j-l**) = 10 μm . For dansyl-linked testosterone **2-10**: **a**, **d**, **g**, and **j** (the zoom of **g**). For organelle trackers: **b** Mito-Tracker Deep Red FM (for mitochondria); **e** Lyso-Tracker Deep Red (for lysosomes); **h** BODIPY TR Ceramide (for Golgi apparatus) (**k** is the zoom of **h**). For merge of signal: **c** **2-10** and MitoTracker Deep Red FM; **f** **2-10** and LysoTracker Deep Red, **i** **2-10** and BODIPY TR Ceramide and (**l** is the zoom of **i**)

4.3 Site-specific Dual Functionalization of Cysteine Residue in Peptides and Proteins with 2-Azidoacrylates (Chapter 3)

4.3.1 Synthesis of 2-azidoacrylates 3-1

4.3.1.1 Typical procedure for synthesis of 3-1a, 3-1b, 3-1c and 3-V: (Section 3.3.1.1)

Synthesis of ethyl 2-azidoacrylate (3-1a)

Scheme 4.3.1.1 Synthesis of 3-1a

To an ice cold solution of ethyl acrylate (2.19 mL, 20.2 mmol) in CH_2Cl_2 (25 mL) was added dropwise a solution of bromine (1.24 mL, 24.2 mmol) in CH_2Cl_2 (20 mL). The reaction mixture was stirred for 3 h at $0\text{ }^\circ\text{C}$. After completion of the reaction, the mixture was quenched with $\text{Na}_2\text{S}_2\text{O}_3$, and the organic materials were extracted three times with CH_2Cl_2 . The combined extracts were washed with brine and dried over MgSO_4 . After evaporation of the solvent, the resulting crude materials including dibromide were used immediately for the next step without further purification.

To a solution of the crude compound including dibromide in *N,N*-dimethylformamide (DMF) (45 mL), NaN_3 (3.93 g, 60.6 mmol) was added portion-wise slowly at $0\text{ }^\circ\text{C}$. After the addition, the reaction mixture was stirred for 13 h at $23\text{ }^\circ\text{C}$ with monitoring the reaction progress by ^1H NMR. After completion of the reaction, the mixture was quenched with H_2O , and the organic materials were extracted three times with Et_2O . The combined extracts were washed two times with brine and dried over MgSO_4 . After removal of the volatile materials in

vacuo, the resulting crude mixture including **3-1a** and diazide were used for the next step without any further purification.

Based on ^1H NMR spectrum of the crude mixture of **3-1a** and diazide, the amount of diazide could be estimated. To a solution of the crude mixture obtained above in Et_2O (60 mL) was slowly added DBU (1,8-diazabicycloundec-7-ene) (2.46 mL, 15.7 mmol, 1.2 equiv to diazide) at $0\text{ }^\circ\text{C}$. After the mixture was stirred for 1.5 h at $0\text{ }^\circ\text{C}$, the mixture was quenched with H_2O , and the organic materials were extracted three times with Et_2O . The combined extracts were washed two times with brine and dried over MgSO_4 . After evaporation of the solvents, the resulting crude materials were purified by flash column chromatography (silica gel; hexane: EtOAc = 90:10) afforded ethyl 2-azidoacrylate (**3-1a**) (1.82 g, 12.9 mmol) in 64% yield over three steps as yellow liquid.

^1H NMR (400 MHz, CDCl_3) δ (ppm) 1.30 (3H, t, J = 7.1 Hz), 4.25 (2H, q, J = 7.1 Hz), 5.29 (1H, d, J = 1.3 Hz), 5.78 (2H, d, J = 1.2 Hz); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 13.9, 62.0, 110.6, 136.0, 162.0; ESIHRMS: Found: m/z 142.0612. Calcd for $\text{C}_5\text{H}_8\text{N}_3\text{O}_2$: $(\text{M}+\text{H})^+$ 142.0617.

2-azidoacrylates **3-1b** and **3-1c** were prepared from di(ethylene glycol) ethyl ether acrylate (CAS: 7238-17-8) and lauryl acrylate (CAS: 2156-97-0), respectively, according the same protocol.

2-(2-ethoxyethoxy)ethyl 2-azidoacrylate (**3-1b**)

Yield: 62% (2.28 g, 9.97 mmol) from 16.2 mmol of di(ethylene glycol) ethyl ether acrylate. ^1H NMR (400 MHz, CDCl_3) δ (ppm) 1.20 (3H, t, J = 7.0 Hz), 3.56 (2H, q, J = 7.0 Hz), 3.62-3.63 (2H, m), 3.67-3.70 (2H, m), 3.79-3.81 (2H, m), 4.41-4.43 (2H, m), 5.39 (1H, d, J = 1.4 Hz),

5.90 (1H, d, $J = 1.4$ Hz); ^{13}C NMR (100 MHz, CDCl_3) $\delta(\text{ppm})$ 15.1, 65.1, 66.7, 68.8, 69.8, 70.7, 111.3, 136.0, 162.0; ESIHRMS: Found: m/z 202.1085. Calcd for $\text{C}_9\text{H}_{16}\text{NO}_4$: $(\text{M}-\text{N}_2+\text{H})^+$ 202.1079.

Dodecyl 2-azidoacrylate (3-1c)

Yield: 65% (728 mg, 2.59 mmol) from 4.00 mmol of lauryl acrylate. ^1H NMR (400 MHz, CDCl_3) $\delta(\text{ppm})$ 0.86 (3H, t, $J = 7$ Hz), 1.30-1.37 (18H, br), 1.65-1.72 (2H, m), 4.21 (2H, t, $J = 6.7$ Hz), 5.32 (1H, d, $J = 1.3$ Hz), 5.81 (1H, d, $J = 1.2$ Hz); ^{13}C NMR (100 MHz, CDCl_3) $\delta(\text{ppm})$ 14.1, 22.7, 25.9, 28.5, 29.2, 29.3, 29.47, 29.54, 29.6 (overlap of two methylene carbons), 31.9, 66.3, 110.8, 136.3, 162.1; ESIHRMS: Found: m/z 282.2176. Calcd for $\text{C}_{15}\text{H}_{28}\text{N}_3\text{O}_2$: $(\text{M}+\text{H})^+$ 282.2182.

4-((*tert*-butoxycarbonyl)amino)butyl 2-azidoacrylate (3-V)

Yield: 57% (300 mg, 1.05 mmol) from 1.84 mmol of **3-II**. ^1H NMR (400 MHz, CDCl_3) $\delta(\text{ppm})$ 1.43 (9H, s), 1.58 (2H, dd, $J = 7.1, 7.3$ Hz), 1.74 (2H, dd, $J = 6.5, 6.8$ Hz), 3.13-3.18 (2H, m), 4.24 (2H, t, $J = 6.5$), 4.64 (1H, br), 5.34 (1H, d, $J = 1.4$ Hz), 5.83 (1H, d, $J = 1.4$ Hz), 5.90 (1H, d, $J = 1.4$ Hz); ^{13}C NMR (100 MHz, CDCl_3) $\delta(\text{ppm})$ 25.8, 26.7, 28.4, 40.1, 65.7, 79.3, 110.9, 136.2, 155.9, 162.0; ESIHRMS: Found: m/z 285.1547. Calcd for $\text{C}_{12}\text{H}_{21}\text{N}_4\text{O}_4$: $(\text{M}+\text{H})^+$ 285.1563.

4.3.1.2 Synthesis of 4-((*tert*-butoxycarbonyl)amino)butyl acrylate (**3-II**) (Section 3.3.1.2)

Scheme 4.3.1.2 Synthesis of **3-II**

To an ice cold solution of *tert*-butyl (4-hydroxybutyl)carbamate (**3-I**)⁹ (767 mg, 4.16 mmol) and Et₃N (1.74 mL, 12.4 mmol) in CH₂Cl₂ (20 mL) was added acryloyl chloride (751 mg, 8.34 mmol) dropwise at 0 °C. The reaction mixture was stirred for 1.5 h slowly warming up from 0 °C to 23 °C. The mixture was quenched with H₂O, and the organic materials were extracted once with CH₂Cl₂ and twice with EtOAc. The combined extracts were washed with brine and dried over MgSO₄. After evaporation of the solvents, the resulting crude materials were purified by flash column chromatography (silica gel; hexane:EtOAc = 80:20) afforded product **3-II** (686 mg, 2.82 mmol) in 68% yield as yellow liquid.

¹H NMR (400 MHz, CDCl₃) δ(ppm) 1.44 (9H, s), 1.58 (2H, dd, *J* = 7.1, 7.3 Hz), 1.70 (2H, dd, *J* = 6.5, 6.8 Hz), 3.15-3.17 (2H, m), 4.17 (2H, t, *J* = 6.5), 4.68 (1H, br), 5.82 (1H, dd, *J* = 1.4, 10.4 Hz), 6.11 (1H, dd, *J* = 10.4, 17.6 Hz), 6.40 (1H, d, *J* = 1.3, 17.8 Hz); ¹³C NMR (100 MHz, CDCl₃) δ(ppm) 25.9, 26.6, 28.3, 40.1, 64.1, 79.0, 128.4, 130.6, 155.9, 166.1; ESIHRMS: Found: *m/z* 266.1386. Calcd for C₁₂H₂₁NO₄Na: (M+Na)⁺ 266.1368.

4.3.1.3 4-((5-(dimethylamino)naphthalene)-1-sulfonamido)butyl 2-azidoacrylate (**3-1d**)

(Section 3.3.1.2)

Scheme 4.3.1.3 Synthesis of **3-1d**

To an ice cold solution of 4-((*tert*-butoxycarbonyl)amino)butyl 2-azidoacrylate (**3-V**) (273 mg, 0.961 mmol) CH_2Cl_2 (10 mL) was slowly added trifluoroacetic acid (TFA) (1 mL) and the reaction mixture was stirred for 2 h warming up slowly from 0 °C to 23 °C. The solvents were then removed under reduced pressure and the resulting crude mixture including amine **3-VI** was used for the next step without any purification

To an ice cold solution of the crude mixture including 4-aminobutyl 2-azidoacrylate (**3-VI**) obtained above and Et_3N (0.325 mL, 2.33 mmol) in CH_2Cl_2 (15 mL) was added dansyl chloride (251 mg, 0.932 mmol), and the reaction mixture was stirred for 19.5 h slowly warming up from 0 °C to 23 °C. The mixture was then quenched with H_2O , and the organic materials were extracted three times with CH_2Cl_2 . The combined extracts were washed with brine and dried over MgSO_4 . After evaporation of the solvents, the resulting crude materials were purified by flash column chromatography (silica gel; hexane:EtOAc = 80:20) afforded product **3-1d** (167 mg, 0.400 mmol) in 42% yield as fluorescence green oil.

^1H NMR (400 MHz, CDCl_3) δ (ppm) 1.48 (2H, dd, J = 6.7, 7.2 Hz), 1.60 (2H, dd, J = 6.6, 6.2 Hz), 2.88 (6H, s), 2.92-2.96 (2H, m) 4.05 (2H, t, J = 6.3 Hz), 5.12 (1H, t, J = 6.2), 5.29 (1H, d,

$J = 1.0$ Hz), 5.74 (1H, d, $J = 1.0$ Hz), 7.18 (1H, d, $J = 7.6$ Hz), 7.50-7.57 (1Hx1+1Hx1, m), 8.24 (1H, d, $J = 7.3$ Hz), 8.31 (1H, d, $J = 8.6$ Hz), 8.54 (1H, d, $J = 8.5$ Hz); ^{13}C NMR (100 MHz, CDCl_3) $\delta(\text{ppm})$ 25.4, 26.3, 42.8, 45.4, 65.3, 111.0, 115.2, 118.6, 123.2, 128.5, 129.6, 129.7, 129.9, 130.6, 134.6, 136.1, 152.1, 161.9; ESIHRMS: Found: m/z 418.1531. Calcd for $\text{C}_{19}\text{H}_{24}\text{N}_5\text{O}_4\text{S}$: $(\text{M}+\text{H})^+$ 418.1549.

4.3.1.4 Synthesis of 4-(3-(3',6'-dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9'-xanthen]-5-yl)thioureido)butyl 2-azidoacrylate (**3-1e**) (Section 3.3.1.2)

Scheme 4.3.1.4 Synthesis of **3-1e**

To an ice cold solution of 4-aminobutyl 2-azidoacrylate (**3-VI**) (prepared from 40 μmol of *N*-Boc **3-V** based on the procedure described in section 5.3.1-3) and Et_3N (5.7 μL , 40 μmol) in DMF (0.5 mL) was added fluorescein isothiocyanate isomer I (FITC) (15.5 mg, 40 μmol), and the reaction mixture was stirred for 3 h warming up slowly from 0 °C to 23 °C. The reaction mixture was directly applied for purification by flash column chromatography (Florisil®; $\text{CH}_2\text{Cl}_2:\text{MeOH} = 85:15$) to afford 2-azidoacrylate with the fluorescein moiety **3-1e** (22.3 mg, 39 μmol) in 97% yield as orange amorphous. Before usage for the protein modification, **3-1e**

^1H NMR (400 MHz, $\text{MeOD-}d_4$) δ (ppm) 2.26-2.30 (1H, m), 2.71-2.78 (1H, m), 3.60-3.64 (1H, m), 3.71-3.82 (1Hx1+1Hx1, m), 5.17 (1H, d, $J = 14.0$ Hz), 6.62-6.64 (4H, m), 7.09-7.16 (3H, m), 7.21-7.32 (4H, m), 7.45-7.56 (5H, m), 7.67 (1H, d, $J = 7.2$ Hz), 7.85 (1H, br); ^{13}C NMR (100 MHz, $\text{DMSO-}d_6$) δ (ppm) 34.0, 35.1, 36.8, 55.3, 55.4, 102.7, 108.5, 110.5, 113.8, 114.7, 114.8, 121.9, 122.9, 123.0, 124.9, 125.7, 127.3, 128.2, 128.3, 128.6, 128.8, 129.5, 129.6, 130.1, 132.8, 141.7, 148.8, 148.9, 151.7, 152.7, 169.0, 171.0, 180.6; ESIHRMS: Found: m/z 666.1696. Calcd for $\text{C}_{39}\text{H}_{28}\text{N}_3\text{O}_6\text{S}$: $(\text{M}+\text{H})^+$ 666.1699.

4.3.3 Conjugation between 2-azidoacrylates **3-1** and L-cysteine (**3-2**) (Section 3.3.3)

Scheme 4.3.3 Synthesis of **3-3a**

Typical procedure E

Ethyl 2-azidoacrylate (**3-1a**) (40.4 mg, 0.286 mmol) and L-cysteine methyl ester hydrochloride (**3-2**) (49.1 mg, 0.286 mmol) were mixed into pH 7.4, 50 mM of sodium phosphate buffer (10 mL) at 23 °C and the reaction mixture was stirred for 0.5 h. The mixture was then diluted with CH_2Cl_2 and the organic materials were extracted with CH_2Cl_2 four times. The combined organic extracts were dried over MgSO_4 and concentrated. The resulting crude products were purified by flash column chromatography (silica gel; $\text{EtOAc}:\text{Et}_3\text{N} = 100:2$) to afford conjugate **3-3a** (60.8 mg, 0.220 mmol) in 77% yield as pale yellow liquid.

Ethyl 3-(((*R*)-2-amino-3-methoxy-3-oxopropyl)thio)-2-azidopropanoate (**3-3a**)

dr = 1:1, the ratio was determined by ^{13}C NMR. The pairs of ^{13}C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

^1H NMR (400 MHz, CDCl_3) δ (ppm) 1.32 (3H, t, $J = 7.2$ Hz), 1.71 (2H, br), 2.85-2.92 (2H, m), 2.97-3.03 (2H, m), 3.67-3.70 (1H, m), 3.75 (3H, s), 4.09-4.13 (1H, m), 4.28 (2H, q, $J = 7.2$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 14.1, (33.94, 34.01), (37.87, 37.92), 52.3, (54.38, 54.42), 62.2, (62.39, 62.42), 168.8, 174.2; ESIHRMS: Found: m/z 277.0980. Calcd for $\text{C}_9\text{H}_{17}\text{N}_4\text{O}_4\text{S}$: ($\text{M}+\text{H}$) $^+$ 277.0971.

2-(2-ethoxyethoxy)ethyl azidopropanoate (**3-3b**)

3-(((*R*)-2-amino-3-methoxy-3-oxopropyl)thio)-2-

dr = 1:1, the ratio was determined by ^{13}C NMR. The pairs of ^{13}C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

Yield: 76%; Pale yellow oil (81.5 mg, 0.224 mmol) from 0.293 mmol of **3-1b** with 1.0 equiv of **3-2** for 1 h following typical procedure E; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 1.20 (3H, t, $J = 7.0$ Hz), 1.75 (2H, br), 2.84-2.92 (2H, m), 2.96-3.03 (2H, m), 3.51 (2H, q, $J = 7.0$ Hz), 3.75-3.65 (2H, m), 3.67-3.69 (1H, m), 3.75 (5H, br), 4.17 (1H, td, $J = 2.1, 5.4, 5.4$ Hz), 4.38 (2H, dd, $J = 4.7, 6.2$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 15.1, (33.93, 34.00), (37.84, 37.89),

52.2, (54.38, 54.42), (62.36, 62.37), 65.0, 66.6, 68.7, 69.7, 70.6, 168.8, 174.1; ESIHRMS: Found: m/z 365.1491. Calcd: for $C_{13}H_{25}N_4O_6$ S: $(M+H)^+$ 365.1495.

Dodecyl 3-(((*R*)-2-amino-3-methoxy-3-oxopropyl)thio)-2-azidopropanoate (3-3c)

dr = 1:1, the ratio was determined by ^{13}C NMR.

Yield: 86%; Pale yellow oil (105.6 mg, 0.253 mmol) from 0.294 mmol of **3-1c** with 1.0 equiv of **3-2** in 10 mL solution of buffer/THF (1:1) for 3 h following typical procedure E; 1H NMR (400 MHz, $CDCl_3$) δ (ppm) 0.86 (3H, t, J = 6.6 Hz), 1.25 (18H, br) 1.64-1.71 (4H, m), 2.84-2.91 (2H, m), 2.97-3.03 (2H, m), 3.68 (1H, t, J = 5.0 Hz), 3.75 (3H, s), 4.11 (1H, td, J = 2.0, 5.4, 5.4), 4.20 (2H, t, J = 6.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ (ppm) 14.1, 22.7, 25.8, 28.5, 29.2, 29.3, 29.47, 29.55, 29.62, 31.9, 34.0, 34.1, 37.99, 52.3, 54.5, 62.6, 66.4, 168.9 (overlap of two ester carbonyl groups); ESIHRMS: Found: m/z 417.2537. Calcd: for $C_{19}H_{37}N_4O_4$ S: $(M+H)^+$ 417.2536.

4-((5-(dimethylamino)naphthalene)-1-sulfonamido)butyl 3-(((*R*)-2-amino-3-methoxy-3-oxopropyl)thio)-2-azidopropanoate (3-3d):

dr = 1:1, the ratio was determined by ^{13}C NMR. The pairs of ^{13}C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

Yield: 79%; Fluorescent green oil (131.8 mg, 0.239 mmol) from 0.304 mmol of **3-1d** with 1.0 equiv of **3-2** in 10 mL solution of buffer/THF (1:1) for 1.5 h following typical procedure E; ^1H NMR (400 MHz, CDCl_3) δ (ppm) 1.52 (2H, dd, $J = 6.9, 7.3$ Hz), 1.63 (2H, dd, $J = 6.3, 7.7$ Hz), 1.73 (2H, br), 2.85-3.01 (6Hx1+4Hx1+2Hx1, m), 3.68-3.71 (1H, m), 3.75 (3H, s), 4.06-4.13 (2H+1H, m), 5.03-5.11 (1H, m), 7.19 (1H, d, $J = 7.5$ Hz), 7.51-7.58 (1H+1H, m), 8.24 (1H, d, $J = 7.2$ Hz), 8.28 (1H, d, $J = 8.6$ Hz), 8.55 (1H, d, $J = 8.6$ Hz); ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) 25.3, 26.0, (33.86, 33.92), (37.74, 37.77), 42.5, 45.2, 52.2, 54.3, 62.2, 65.3, 115.1, 118.6, 123.0, 128.2, 129.3, 129.5, 129.7, 130.3, 134.7, 151.9, 168.7, 174.1; ESIHRMS Found: m/z 553.1904. Calcd: $\text{C}_{23}\text{H}_{33}\text{N}_6\text{O}_6\text{S}_2$: $(\text{M}+\text{H})^+$ 553.1903.

4.3.4 Conjugation between 2-azidoacrylates **3-1** and glutathione (GSH) (**3-4**) (Section 3.3.4)

Modification of glutathione with ethyl 2-azidoacrylates (**3-1a**)

Scheme 4.3.4 Synthesis of **3-5a**

Typical procedure F

Ethyl 2-azidoacrylate (**3-1a**) (41.2 mg, 0.291 mmol) and L-glutathione reduced (GSH) (**3-4**) (89.7 mg, 0.291 mmol) were mixed in pH 7.4, 50 mM of sodium phosphate buffer (10 mL) at 23 °C. After 1 h, reaction mixture was frozen at -80 °C and frozen reaction mixture was lyophilized, affording conjugate **3-5a** in 73% yield based on the ^1H NMR using *tert*-butanol as the internal standard.

***N*⁵-((2*R*)-3-((2-azido-3-ethoxy-3-oxopropyl)thio)-1-((carboxymethyl)amino)-1-oxopropan-2-yl)-*L*-glutamine (3-5a)**

dr = 1:1, the ratio was determined by ¹³C NMR. The pairs of ¹³C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

¹H NMR (400 MHz, D₂O) δ(ppm) 1.31 (3H, t, *J* = 7.1 Hz), 2.13-2.20 (2H, m), 2.53-2.59 (2H, m), 2.91-3.22 (4H, m), 3.77 (3H, m), 4.30 (2H, q, *J* = 7.1 Hz), 4.59-4.67 (2H, m); ¹³C NMR (100 MHz, D₂O) δ(ppm) 13.4, 26.3, 31.4, (33.31, 33.41), (33.77, 33.87), 43.4, (53.17, 53.23), 54.1, 62.3, 63.4, 170.9, 171.7, 174.0, 174.9, 176.2.; ESIHRMS: Found: *m/z* 449.1447. Calcd for C₁₅H₂₅N₆O₈S: (M+H)⁺ 449.1455.

(15*R*,20*S*)-20-amino-11-azido-15-((carboxymethyl)carbamoyl)-10,17-dioxo-3,6,9-trioxa-13-thia-16-azahenicosan-21-oic acid (3-5b)

dr = 1:1, the ratio was determined by ¹³C NMR. The pairs of ¹³C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

Yield: 89% (based on ¹H NMR using *tert*-butanol as internal standard) from 0.298 mmol of **3-1b** with 1.0 equiv of GSH in 10 mL of pH 7.4, 50 mM of sodium phosphate buffer for 1 h following typical procedure F; ¹H NMR (400 MHz, D₂O) δ(ppm) 1.09 (3H, t, *J* = 7.1 Hz), 2.04-

2.10 (2H, m), 2.38-2.51 (2H, m), 2.83-3.12 (4H, m), 3.49 (2H, q, $J = 7.1$ Hz), 3.55-3.57 (2H, m), 3.60-3.62 (2H, m), 3.67-3.73 (5H, m), 4.31-4.33 (2H, m), 4.50-4.53 (2H, m); ^{13}C NMR (100 MHz, D_2O) $\delta(\text{ppm})$ 14.2, 26.3, 31.5, (33.27, 33.38), (33.81, 33.94), 43.5, (53.19, 53.26), 54.2, 62.3, 65.3, 66.6, 68.3, 68.9, 69.7, 170.7, 171.7, 174.0, 174.9, 176.2.; ESIHRMS: Found: m/z 537.1984. Calcd for $\text{C}_{19}\text{H}_{33}\text{N}_6\text{O}_{10}\text{S}$: $(\text{M}+\text{H})^+$ 537.1979.

Typical procedure G

Dodecyl 2-azidoacrylate (**3-1c**) (103.8 mg, 0.369 mmol) and GSH (94.5 mg, 0.307 mmol) were added into pH 7.4, 50 mM of sodium phosphate buffer (15 mL) and THF (15 mL) at 23 °C. After 10 h, reaction mixture was frozen at -80 °C and frozen reaction mixture was lyophilized. MeOH was added into the lyophilized crude product, and it was centrifuged at 8000 rpm for 5 min. The supernatant was carefully decanted. This step was repeated 3 times. The decanted supernatant was combined and evaporated. After evaporation, Et_2O was added into the evaporated crude product, and white precipitate was formed. The precipitate was centrifuged at 8000 for 5 min. The supernatant (Et_2O) was carefully decanted. This step was repeated 3 times. The white solid were dried under vacuum to give 167.7 mg (0.285 mmol, 93% yield) of **3-5c**

***N*⁵-((2*R*)-3-((2-azido-3-(dodecyloxy)-3-oxopropyl)thio)-1-((carboxymethyl)amino)-1-oxopropan-2-yl)-*L*-glutamine (3-5c)**

dr = 1:1, the ratio was determined by ¹³C NMR. The pairs of ¹³C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

White solid, mp 205 °C; ¹H NMR (500 MHz, MeOD-*d*₄) δ(ppm) 0.90 (3H, t, *J* = 7.1 Hz), 1.29 (17H, br), 1.39 (2H, m), 1.69 (2H, m), 2.15 (2H, m), 2.55 (2H, m), 2.90 (3H, m), 3.16 (1H, m), 3.63 (1H, t, *J* = 6.2 Hz), 3.77 (2H, m), 4.21 (2H, t, *J* = 6.6 Hz), 4.32 (1H, m), 4.58 (1H, m); ¹³C NMR (100 MHz, MeOD-*d*₄ -30 °C) δ(ppm) 14.9, 24.2, 27.3, 28.1, 29.8, 30.7, 31.0, (31.08, 31.14), 31.2, 33.1, 33.5, 33.86, 33.90, 35.3, 44.3, 54.1, 55.4, 63.4, 63.5, 67.2, 170.9, 172.6, 174.5, 175.3, 176.7.; ESIHRMS: Found: *m/z* 589.3018. Calcd for C₂₅H₄₅N₆O₈S: (M+H)⁺ 589.3020.

***N*⁵-((2*R*)-3-((2-azido-3-(4-((5-(dimethylamino)naphthalene)-1-sulfonamido)butoxy)-3-oxopropyl)thio)-1-((carboxymethyl)amino)-1-oxopropan-2-yl)-*L*-glutamine (3-5d)**

dr = 1:1, the ratio was determined by ¹³C NMR. The pairs of ¹³C NMR signals originated from the diastereomers are shown in the parenthesis such as (XX, YY).

Yield: 88%; Fluorescent green solid (191.2 mg, 0.264 mmol, mp 184-185 °C) from 0.300 mmol of **3-1d** with 1.0 equiv. of GSH in 10 mL of pH 7.4, 50 mM of sodium phosphate buffer and 5 mL of THF for 12.5 h following typical procedure G; ¹H NMR (400 MHz, D₂O + MeOD-*d*₄) δ(ppm) 1.37-1.41 (2H, m), 1.47-1.52 (2H, m), 2.15-2.18 (2Hx1+1Hx1, m), 2.52-2.57 (2Hx1+1Hx1, m), 2.80-3.09 (6Hx1+4Hx1+1Hx1, m), 3.10-3.13 (1H, m), 3.66-3.80. (2Hx1+2Hx1, m), 3.93-3.97 (2H, m), 4.26-4.30 (1H, m), 4.55-4.59 (1H, m), 7.30 (1H, d, *J* = 7.5 Hz), 7.59-7.64 (1Hx1+1Hx1, m), 8.19 (1H, d, *J* = 7.3 Hz), 8.31 (1H, d, *J* = 8.7 Hz), 8.53 (1H, d, *J* = 8.6 Hz); ¹³C NMR (100 MHz, D₂O + MeOD-*d*₄) δ(ppm) 26.3, 26.8, (27.62, 27.76), (32.83, 32.99), (34.25, 34.33), (35.11, 35.20), 43.2, (44.53, 44.59), 45.9, (54.09, 54.44), (55.36, 55.44), 63.4, 66.7, 116.6, 120.3, 124.5, 129.4, 130.2, 130.6, 130.8, 131.2, 136.4, 152.9, 170.9, (172.14, 172.23), (174.33, 174.46), (175.51, 175.57), (176.24, 176.41); ESIHRMS: Found: *m/z* 725.2386. Calcd for C₂₉H₄₁N₈O₁₀S₂: (M+H)⁺ 725.2387.

4.3.5 Conjugation between 2-azidoacrylate 3-1b and Cys-containing peptides 3-6

4.3.5.1 Peptide synthesis CGKSRF (3-6a)

2-Chlorotrityl chloride resin (0.221 mmol, 220 mg) was swelled in CH_2Cl_2 for 20 min. Fmoc-Phe-OH (128 mg, 0.331 mmol, 1.5 equiv) and *N,N*-diisopropylethylamine (DIPEA) (0.115 mL, 0.662 mmol, 3.0 equiv) were dissolved in CH_2Cl_2 . The coupling reaction was carried for 3 h. The unreacted tertiary chlorides of the resin were capped using MeOH (2.0 mL) and DIPEA (0.2 mL) in CH_2Cl_2 (2.0 mL) for 20 min. The Fmoc group was removed using 20% piperidine in DMF. Fmoc-Arg(Pbf)-OH (445 mg, 0.662 mmol, 3.0 equiv) was coupled to the free amine on resin using 1-hydroxybenzotriazole (HOBt) (149 mg, 1.10 mmol, 5.0 equiv) and *N,N'*-diisopropylcarbodiimide (DIC) (0.171 mL, 1.10 mmol, 5.0 equiv) in DMF (4 mL) for 2 h.

Other amino acid residues were coupled in the same way using the following amount of the reagents: Fmoc-Ser(*t*-Bu)-OH (254 mg, 0.662 mmol, 3.0 equiv), HOBt (149 mg, 1.10 mmol, 5.0 equiv), DIC (0.171 mL, 1.10 mmol, 5.0 equiv) in DMF (4 mL); Fmoc-Lys(Boc)-OH (310 mg, 0.662 mmol, 3.0 equiv), HOBt (149 mg, 1.10 mmol, 5.0 equiv), DIC (0.171 mL, 1.10 mmol, 5.0 equiv) in DMF (4 mL); Fmoc-Gly-OH (199 mg, 0.662 mmol, 3.0 equiv), HOBt (149 mg, 1.10 mmol, 5.0 equiv), DIC (0.171 mL, 1.10 mmol, 5.0 equiv) in DMF (4 mL); Fmoc-Cys(Trt)-OH (387 mg, 0.662 mmol, 3.0 equiv), HOBt (149 mg, 1.10 mmol, 5.0 equiv), DIC (0.171 mL, 1.10 mmol, 5.0 equiv) in DMF (4 mL).

The peptide on-resin was treated with TFA/CH₂Cl₂ (5:95) to separate the peptide from the resin. The resin was filtered off and filtrates were evaporated under reduced pressure. The resulting protected-peptide was treated with TFA:triisopropylsilane:H₂O (95:2.5:2.5) for 3 h for deprotection of all the protecting groups attached on the amino acid side chains (Pbf on Arg, *t*-Bu on Ser, Boc on Lys, Trt on Cys), and then, volatile materials were removed under reduced pressure. The resulting crude peptide was obtained through precipitation out in cold Et₂O and centrifugation to obtain the crude peptide (88.3 mg, 58% yield) from 0.221 mmol of 2-chlorotrityl chloride resin; ESIHRMS: Found: *m/z* 697.3463. Calcd for C₂₉H₄₉N₁₀O₈S: (M+H)⁺ 697.3456

Peptides KSCGRF (**3-6b**) and GKSRFC (**3-6c**) were synthesized following the protocol described above. The purity of peptides was checked by LC-MS and HPLC. The peptides were used without further purification.

KSCGRF (3-6b)

Crude peptide (88.4 mg, 56% yield) from. 0.227 mmol of 2-chlorotrityl chloride resin;

ESIHRMS: Found: m/z 697.3438. Calcd for $C_{29}H_{49}N_{10}O_8S$: $(M+H)^+$ 697.3456.

GKSRFC (3-6c)

Crude peptide (49.2 mg, 35% yield) from. 0.200 mmol of 2-chlorotrityl chloride resin;

ESIHRMS: Found: m/z 697.3456. Calcd for $C_{29}H_{49}N_{10}O_8S$: $(M+H)^+$ 697.3456.

4.3.5.2 Conjugation of peptides using 2-azidoacrylate **3-1b** (Section 3.3.5)

General procedure: synthesis of **3-7a**

Scheme 4.3.5.2 Conjugation of **3-1b** with **3-6a**

Typical procedure H

A 10 mM solution of peptide CGKSRF (**3-6a**) in pH 7.4, 50 mM sodium phosphate buffer [solution (A)] and a 100 mM solution of 2-azidoacrylate **3-1b** in THF [solution (B)] were prepared. The solution (A) (1000 μ L) and the solution (B) (100 μ L) were mixed to make the ratio of **3-6a** to **3-1b** 1:1. The reaction mixtures were stirred for 1 h at 23 °C. The reaction was monitored by LC-MS and site selectivity was investigated by MALDI-MS/MS.

3-7b and **3-7c** were synthesized following the protocol described above.

4.3.5.3 MALDI-MS/MS analyses of modified peptides **3-7a–3-7c**

All peptide samples **3-7a–c** for MALDI-TOF MS analysis were co-crystallized by using α -cyano-4-hydroxycinnamic acid (CHCA) (Aldrich) solution (10 mg/mL MeOH). MALDI-TOF mass spectra of peptides were recorded in Spiral TOF mode by using a JMS S3000 (JEOL, Japan) equipped with a 345 nm laser. MALDI-MS/MS analyses were recorded in MALDI-TOF TOF mode using helium gas as collision gas.

Figure 4.3.5.3-A MALDI-MS/MS spectrum of **3-7a**.

Figure 4.3.5.3-B. MALDI-MS/MS spectrum of **3-7b**.

Figure 4.3.5.3-C. MALDI-MS/MS spectrum of **3-7c**.

4.3.6 Kinetic study in modification of the cysteine residue with 2-azidozcrilate **3-1b**

4.3.6.1 Calculation of second order rate constant

The present conjugation reactions were the bimolecular reaction between **A** and **B** that follow second-order reaction kinetics. Reaction rate could be presented as follows:

$$\text{Rate } (v) = -\frac{d[A]}{dt} = k[A][B] \text{ (eq 1)}$$

As the reaction was performed with 1:1 stoichiometry of the substrates, *eq 1* could be converted to *eq 2*:

$$[A] = [B]$$

$$v = -\frac{d[A]}{dt} = k[A]^2 \text{ (eq 2)}$$

By integrating, *eq 2* could be expressed as:

$$\frac{1}{[A]} = kt + C \text{ (C: Constant)}$$

If $[A]$ equals $[A]_0$ at $t = 0$, C equals $1/[A]_0$. Thus, the following equation is obtained (eq 3):

$$\frac{1}{[A]} = kt + \frac{1}{[A]_0} \text{ (eq 3)}$$

Eq 3 indicates that the second-order initial rate constant could be determined by plotting $\frac{1}{[A]}$ against time. The slope of the graph provides second-order initial rate constant.

4.3.6.2 Experimental protocol for conjugation between 3-1b and GSH (3-4) (Section 3.3.6)

To collect accurate kinetic data, the same experiment was performed twice.

Diluted solutions (2.0 mM) of **3-1b** and GSH (**3-4**) in 50 mM sodium phosphate buffer (pH 7.4) were prepared. 2.0 mL of the solution of **3-1b** and 2.0 mL of the solution of GSH (**3-4**) were sequentially mixed to make final reactant concentration of 1.0 mM. For analysis, a sample (20 μ L) was taken from the mixture and injected to analytical HPLC (Shimadzu C18 column with 50 mM of ammonia acetate in H₂O 50% and MeCN 50%).

Figure 4.3.6.2 Estimation of rate constant in the conjugation reaction between **3-1b** and **3-4**.

Figure 4.3.7 Conversion ratio of **3-7** estimated by HPLC.

4.3.8 Dual functionalization of BSA (3-10**) with 2-azidoacrylate **3-1b** and DBCO-FL **3-6** (Section 3.3.8)**

4.3.8.1. General procedure for the SDS-PAGE analysis

The solution of labeled BSA (**3-10**) (5.0 μL) was mixed with 2X loading buffer (5.0 μL) in a 1.5 mL Eppendorf tube. The samples were analyzed by SDS-PAGE by loading a sample (10 μL) in each lane of a 10% SDS-PAGE gel and running in a Mini-PROTEAN Tetra Cell (Bio-Rad, USA) at 10 mA at room temperature for 2 h. After SDS-PAGE separation, the band of labeled-BSA was visualized with a Typhoon trio (GE Healthcare, USA) and stained with Coomassie Brilliant Blue (CBB).

4.3.8.2 Optimization of the reaction conditions in conjugation of BSA with 3-1b

The reaction mixture at 1st conjugation of BSA (**3-10**) with **3-1b** was analyzed by using the Ellman test to quantify unreacted free sulfhydryl group in BSA at different reaction time points in the following way. The reactions of BSA (**3-10**) (0.10 mM, 500 μ L, 0.050 μ mol in 50 mM phosphate buffer (pH 7.4)) and **1b** were conducted in the four different stoichiometry ratios (**3-10:3-1b** = 1:1, 1:10, 1:20, and 1:50). From these reactions, a portion (50 μ L) of the reaction mixture was taken at different time points (0, 1, 3, 6, 12, and 24 h), which was added into 500 μ L of 100 mM phosphate buffer (pH 8.0) containing 0.20 mM DTNB [the Ellman's reagent, 5,5-dithio-bis-(2-nitrobenzoic acid)], and the mixture was stirred for 5 min. The absorbance at 400-500 nm of the test solutions was monitored by UV-visible spectrophotometer. Chemical conversion of the sulfhydryl group in BSA (**3-10**) was estimated by decrease of the free sulfhydryl group. As shown in below table, 85% chemical conversion was attained by 12 h of incubation with 10 equivalents of **3-1b**.

Scheme 4.3.8.2 Optimization of 1st conjugation between BSA **3-10** and **3-1b**

4.3.8.3. Optimization of the reaction conditions in SPAAC of DBCO-FL 3-6 (Section 3.3.8)

After incubation of BSA (**3-10**) (0.10 mM, 500 μ L, 0.050 μ mol in 50 mM phosphate buffer (pH 7.4)) with 10 equivalents of **3-1b** (10 mM, 50 μ L, 0.50 μ mol in 50 mM phosphate buffer (pH 7.4)) for 12 h at 23 $^{\circ}$ C to form BSA-PEG **3-11**, DBCO-FL **3-6** in DMSO (10 equiv) was added and the reaction mixture was incubated for different reaction time (1, 3, 6, 12, or 24 h). Each sample was analyzed with SDS-PAGE (Figure S6).

Figure 4.3.8.3. SDS-PAGE for optimization of SPAAC

4.3.8.4 Confirmation of BSA-PEG-FL 3-12 in SDS-PAGE

3-1b (10 mM, 10 μ L, 0.10 μ mol in 50 mM phosphate buffer (pH 7.4)) was mixed with BSA (**10**) (final concentration 0.10 mM, 100 μ L, 0.010 μ mol in 50 mM phosphate buffer (pH 7.4)) for 12 h at 23 $^{\circ}$ C. Once the reaction between **3-1b** and the free sulfhydryl group in BSA (**3-10**) was completed, 10 equivalents of DBCO-FL **3-6** (10 mM, 10 μ L, 0.10 μ mol in DMSO) were mixed for 12 h at 23 $^{\circ}$ C. At same time, for preparation of control samples, BSA (**3-10**)

were incubated with **3-1b** without further SPAAC of DBCO-FL **3-6** or incubated with DBCO-FL **3-6** in the absence of **3-1b**. Prepared samples were analyzed with SDS-PAGE.

4.3.8.5. Trypsin digestion and MALDI-MS analyses of BSA-PEG-FL **12**

The reaction mixture of BSA-PEG-FL **3-12** was purified with PD-10 desalting column (GE Healthcare, USA) to remove excess 2-azidoacrylate **3-1b** and DBCO-FL **3-6**. The purified BSA-PEG-FL **3-12** was reduced with 15 mM DTT (dithiothreitol) in phosphate buffer at 23 °C for 10 h. After reduction of disulfide bonds in BSA-PEG-FL **3-12**, the remaining DTT was removed by PD-10 column. The trypsin solution was added to the Eppendorf tube containing reduced BSA-PEG-FL **3-12** (BSA:trypsin = ca. 50:1 (w/w)). The reaction mixture was incubated at 37 °C for 12 h, and the trypsin-digested mixture was then lyophilized. The fluorescein-labeled fragment was purified by HPLC and analyzed by MALDI-MS (Figure 5.3.11-2).

Figure 4.3.8.5 (a) MALDI-MS spectrum of labeled BSA fragment purified by HPLC. (b) Proposed structure of fluorescein-labeled BSA fragment.

4.3.9 Dual functionalization of BSA (3-10) with 3-1e and DBCO-Cy5 3-14 (Scheme 3.3.9)

Scheme 4.3.9 Dual colour labelling of BSA (10)

2-azidoacrylate **3-1e** (10 mM, 1.0 μ L, 0.010 μ mol in DMSO) was mixed with BSA (**3-10**) (final concentration 0.10 mM, 10 μ L, 0.0010 μ mol in buffer) at 23 °C. After incubation for 12 h, 10 equivalent of DBCO-Cy5 **3-14** (10 mM, 1.0 μ L, 0.010 μ mol in DMSO) (Sigma-Aldrich, USA) was added and the mixture was stirred for 12 h at 23 °C. At same time, for preparation of control samples, BSA (**3-10**) was incubated with 2-azidoacrylate **3-1e** without further SPAAC with DBCO-Cy5 **3-14** or incubated with DBCO-Cy5 **3-14** in the absence of **3-1e**. The as-prepared samples were analyzed with SDS-PAGE.

4.3.10 Dual functionalization of BSA (3-10) with 3-1e and DBCO-biotin 3-16 (Section 3.3.10)

Scheme 4.3.10 Dual functionalization of BSA (10) with fluorescein and biotin

2-azidoacrylate **3-1e** (10 mM, 1.0 μ L, 0.010 μ mol in DMSO) was mixed with BSA (**3-10**) (final concentration 0.10 mM, 10 μ L, 0.0010 μ mol in buffer) at 23 °C. After incubation for 12 h, 10 equivalent of the probe molecules DBCO-biotin **3-16**¹⁰ (10 mM, 1.0 μ L, 0.010 μ mol in DMSO) was added and the mixture was stirred for 12 h at 23 °C. At same time, for preparation of control samples, BSA (**3-10**) was incubated with 2-azidoacrylate **3-1e** without further SPAAC of DBCO-biotin **3-16** or incubated with DBCO-biotin **3-16** in the absence of 2-azidoacrylate **3-1e**. The as-prepared samples were analyzed with SDS-PAGE.

4.3.11 ELISA analysis of BSA-FL-biotin 3-17 (Section 3.3.10 ELISA)

The solution of BSA-FL-biotin **17** or its control sample ([BSA] = ca 3 μ M) was added into the dispersion of streptavidin-agarose (10 μ L) and incubated at 23 °C for 1 h in phosphate-buffered saline (PBS) buffer. After washing the resin with PBST buffer (PBS containing 0.05% tween 20), anti-BSA antibody (mouse IgG) (Sigma-Aldrich, USA, 1/15000 dilution) was added to the resin in PBS buffer and incubated at 23 °C for 1 h. After washing the resin with PBST buffer, horseradish peroxidase (HRP)-conjugated anti mouse IgG (BioLegend, USA, 1/5000 dilution) was added to the resin in PBS buffer and incubated at 23 °C for 1 h. After washing the resin with PBST buffer, antibodies-immobilized resin was incubated in the citrate-phosphate buffer (pH 5.0) containing 4 mg/mL of *o*-phenylene diamine and 1/10000 (w/w) H₂O₂ at 23 °C for 6 h. The absorbance at 450 nm of each sample solutions was analyzed by UV-vis photometer (Cary 100 Bio UV-visible spectrophotometer, Agilent Technology, USA).

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5. Conclusion and perspective

In conclusion, this thesis presented the development of linking azido group onto hydroxyl group through fluoro- or bromo-alkoxylation of vinyl azide (Chapter 2) and sulfhydryl group through conjugate addition of 2-azidoacrylate and cysteine (Chapter 3). The linked products were demonstrated for further biological applications such as bioimaging (Chapter 2) and dual functionalization of peptides and protein (Chapter 3).

For further studies, modular and selective azido linking protocol onto other essential functional groups, such as amines and thiols could be developed. These unique linking protocols would widen applicability in medical and biological studies.(for Chapter 2).

Scheme 5.1 Selective linking protocol of thiols and amines with vinyl azides

2-azidoacrylates could be used for modification of other essential biomacromolecules which contain cysteine residue, such antibodies for drug tracking (for Chapter 3). Based on modification targets and combination, it is possible to perform widen the range of medicinal and biological applications.

Scheme 5.2 Modification of biomacromolecules for medicinal or biological applications

List of Publication

[1] Kaga, A.; Wu, X.; Lim, J. Y. J.; Hayashi, H.; Lu, Y.; Yeow, E. K. L.*; Chiba, S.*

“Degenerative Xanthate Transfer to Olefins under Visible Light Photocatalysis”

Beilstein J. Org. Chem. **2018**, *14*, 3047.

[2] Hayashi, H.; Kaga, A.; Wang, B.; Gagosz, F.*; Chiba, S.*

“Use of a benzyl ether as a traceless hydrogen donor in anti-Markovnikov hydrofunctionalization of alkenes with xanthates”

Chem. Commun. **2018**, *54*, 7535.

[3] Chan, H. L.; Lyu, L.; Aw, J.; Zhang, W.; Li, J.; Yang, H. -H.; Hayashi, H.; Chiba, S.*; Xing, B.*

“Unique Fluorescent Probe Molecule for Bacterial Surface Localization and Resistant Enzyme Imaging”

ACS Chem Bio. **2018**, *13*, 1890.

[4] Kaga, A.⁺; Hayashi, H.⁺; Hakamata, H.⁺; Oi, M.; Uchiyama, M.; Takita, R.*; Chiba, S.*

(⁺ equal contribution)

“Nucleophilic Amination of Methoxy Arenes by a Sodium Hydride-Iodide Composite”

Angew. Chem. Int. Ed. **2017**, *56*, 11807.

[5] Ariyasu, S.⁺; Hayashi, H.⁺; Xing, B.*; Chiba, S.* (⁺ equal contribution)

“Site-specific Dual Functionalization of Cysteine Residue in Peptides and Proteins with 2-Azidoacrylates”

Bioconjugate Chem. **2017**, 28, 897.

[6] Wang, Y.-F.; Hu, M.; Hayashi, H.; Xing, B.*; Chiba, S.*

“Linking of Alcohols with Vinyl Azides”

Org. Lett. **2016**, 18, 992.

Review

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“Application of vinyl azides in chemical synthesis a recent update”

J. Org. Chem., **2017**, 82, 11981.