Novel tools for protein analysis and modification

– From chemical probes to new ligation methods –

Dissertation

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1. Gutachter: Prof. Dr. Bernhard Westermann

2. Gutachter: Prof. Dr. Norbert Sewald

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"There is no sadder sight in the world than to see a beautiful theory killed by a brutal fact."

Thomas H. Huxley (1825–95), br. Biologist

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Summary and Outlook

In recent years, the facile modification of a diverse set of biomolecules under near physiological conditions enabled biological oriented scientist to track these molecules in living systems, to improve the stability of therapeutic proteins, to map protein-protein interactions and to study the three-dimensional structure of proteins under native cellular environments, among others. The classical approach relies on the application of reactive groups which are able to selectively react with a certain amino acid residue. A library of reagents has been already developed able to target almost every natural amino acid. The most commonly used reagents are equipped with either lysine-reactive groups (*e.g. N*-hydroxysuccinimide ester) or cysteine-reactive groups (*e.g.* maleimide).

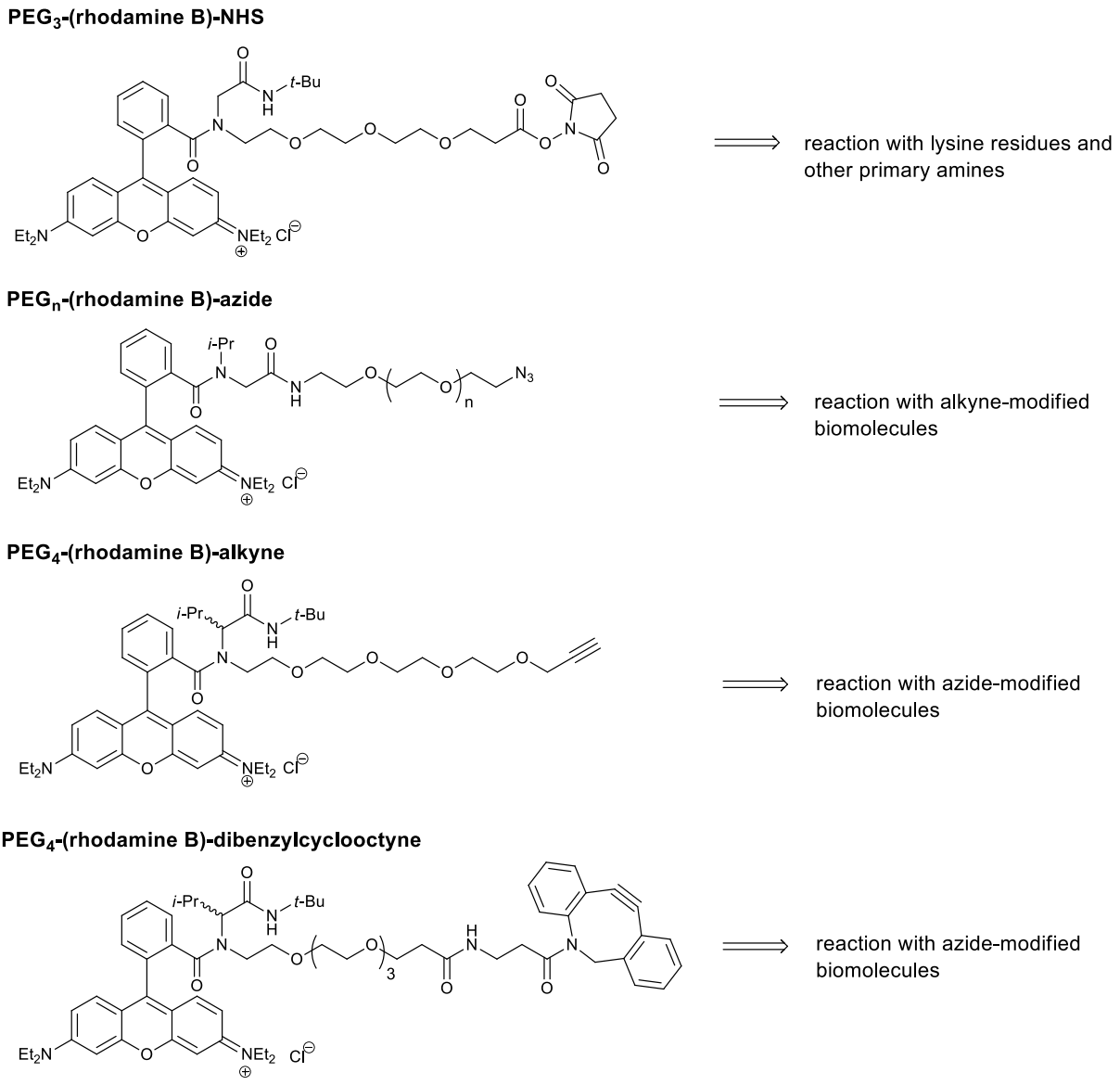


Fig. S1 MCR-derived fluorescent-tags for the chemical modification of various biomolecules.

Based on the current interest of our group to synthesise tools for the modification of biomolecules, in this work a lysine-reactive rhodamine-tag was prepared and successfully tested in the covalent labelling of purified bovine serum albumin (BSA) and a crude barley extract. For this purpose, a fast, modular and easy to carry out multicomponent reaction (MCR) approach was established to synthesise pH-independent fluorescent probes based on the application of the well-known Ugi-4-component reaction (Ugi-4CR). Moreover, using this MCR-approach a library of rhodamine-based fluorescent-tags equipped with terminal alkynes and strained cyclooctynes as well as azides, different glycol spacer lengths or different rhodamine dyes was prepared (Fig. S1). These "clickable" fluorescent-tags are of high value for various applications, such as activity-based protein profiling and the covalent modification of biomolecules previously modified with a complementary click-handle, *e.g.* by single or multisite mutations (chapter 2.2).

This MCR-approach can be used to modify biotin as well. Utilising the Ugi-4CR a biotin-containing affinity-probe equipped with two amine-reactive *N*-hydroxysuccinimide esters for the covalent modification of two free lysine residues within a protein or between a protein and its binding partner was prepared (Fig. S2). The reactivity of this novel trifunctional cross-linking reagent was evaluated using purified cytochrome *c* and a calmodulin/skMLCK peptide complex. Subsequent tryptic digest, affinity enrichment and MS-analysis identified five cross-links within cytochrome *c* and three cross-links between calmodulin and the skMLCK peptide (chapter 2.3).

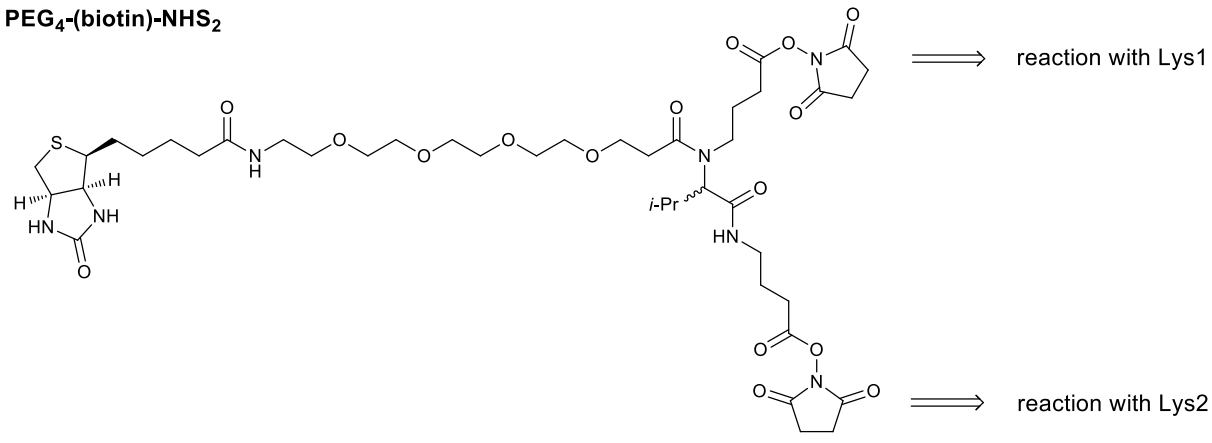


Fig. S2 MCR-derived, amine-reactive biotin-containing trifunctional cross-linking reagent for the chemical modification of two free lysine residues being in close spatial proximity within a protein or between a protein and its binding partner.

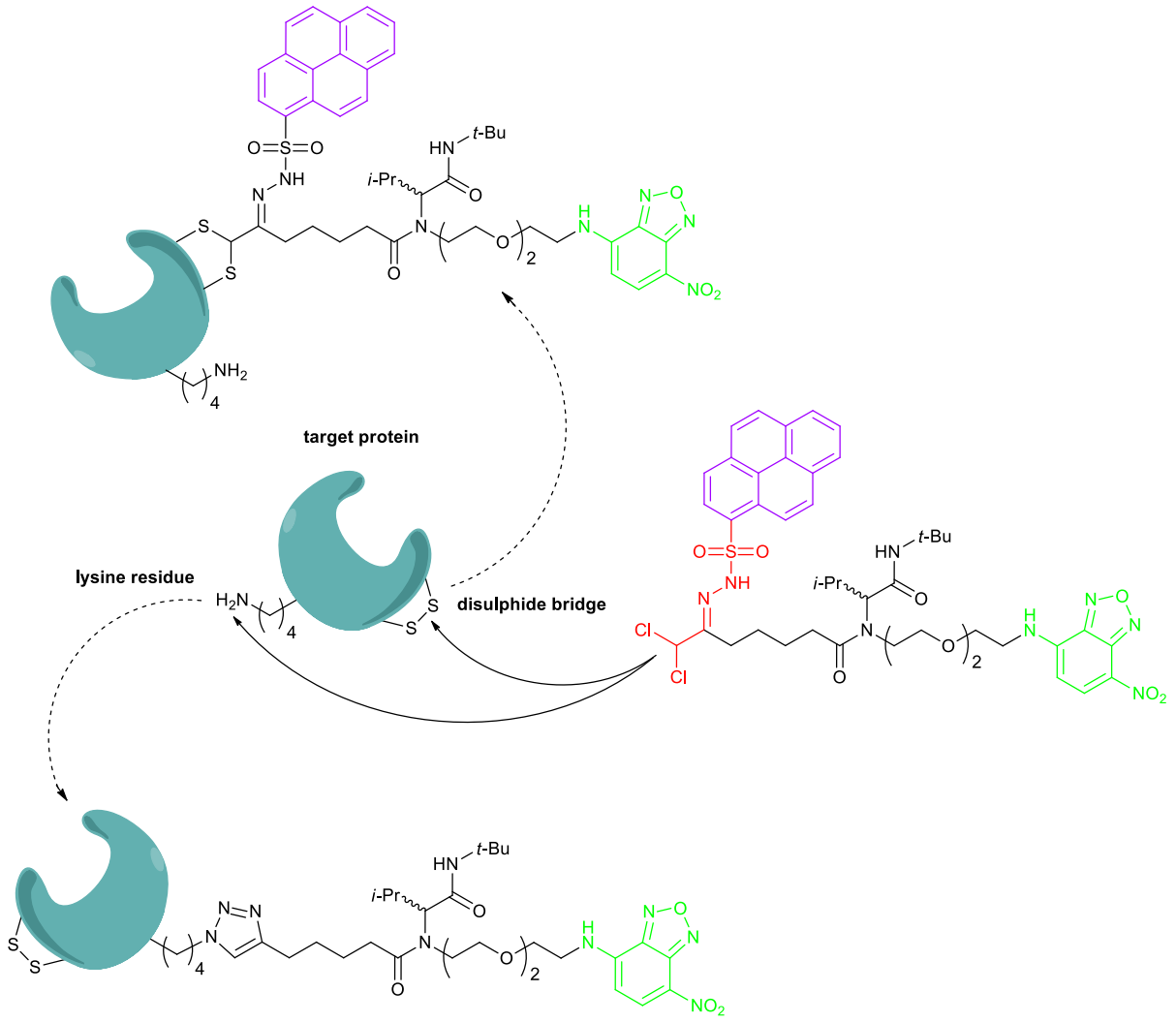


Fig. S3 Fluorescent NBD-dichloropyrenesulphonohydrazones: A class of novel fluorescent-tags enabling the modification of lysine residues as well as disulphide bonds?

The modification of lysine residues under near physiological conditions, in general, is highly dependent on the application of specifically activated esters, such as *N*-hydroxy-succinimide ester, which tend to rapidly hydrolyse in aqueous systems. Therefore, the design of novel amine-reactive labelling reagents with an increased stability towards hydrolysis was intended. In the present thesis, the focus was set on the Sakai triazole formation reaction, a reaction between primary amines and *α*,*α*-dichlorotosylhydrazones leading to the regioselective formation of 4-substituted 1,2,3-triazoles, which was initially introduced in the 1980s. However, this approach has found only infrequent use. Hence, the scope and limitations of the Sakai reaction were studied first utilising different primary amines and *α*,*α*-dichlorotosylhydrazones. Based on these findings, a plausible mechanism of this transformation has been proposed (chapter 3.2). Afterwards the reaction conditions were optimised in respect to the demands of a biological sample (*i.e.*

aqueous buffered solutions and neutral pH). In an initial proof of concept, purified BSA and lysozyme were successfully modified with a suitable fluorescent *α,α*-dichloro-tosylhydrazone. Furthermore, investigations concerning the applicability of specifically designed *α,α*-dichlorosulphonohydrazones as novel disulphide-bridging reagents were conducted (Fig. S3). However, further improvements concerning the probe design have to be performed as a consequence of fluorescence quenching events (chapter 3.3).

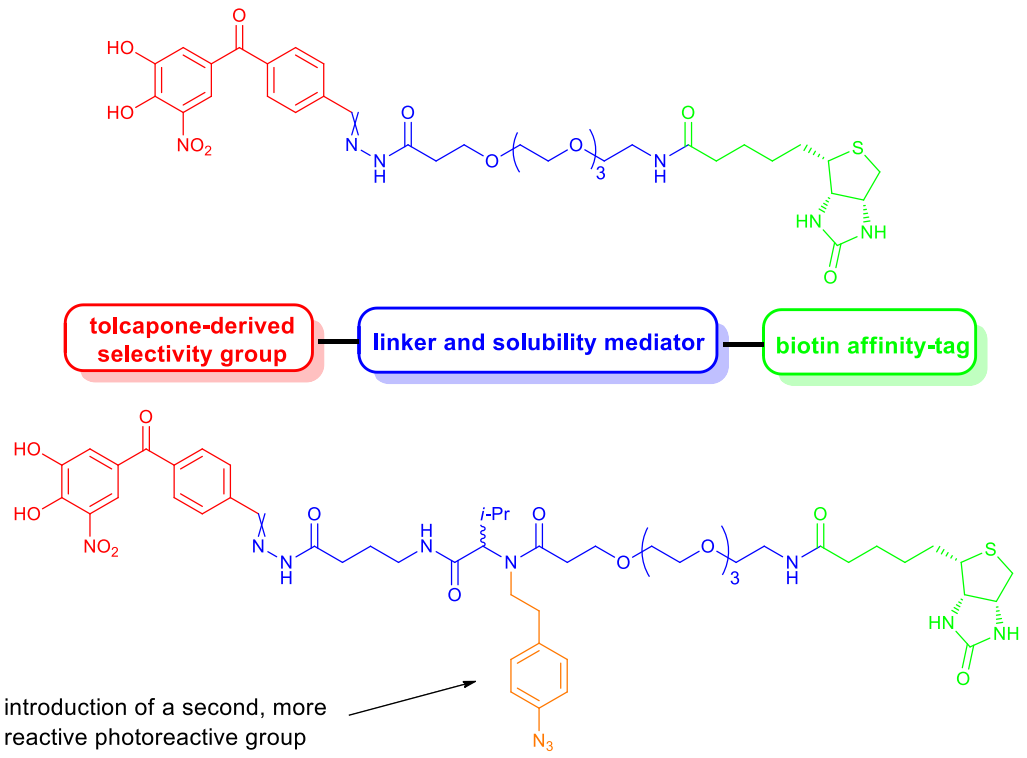


Fig. S4 Design of multifunctional small-molecular chemical probes putatively targeting cation-dependent PI-OMTs from *A. thaliana* to enable identification of the target proteins after subsequent photo-cross-linking and affinity-enrichment.

For the selective identification, enrichment or purifications of proteins of a common protein family, Cravatt and co-workers, among others, introduced the concept of activity-based protein profiling (ABPP). This methodology relies on the application of small molecular probes which are equipped with a targeting unit (*selectivity group*) to direct the probe to a specific protein class, *e.g.* inhibitors, substrates, cofactors, natural products and hormones, *etc.* After subsequent covalent attachment, a reporter-tag (fluorophore or biotin) enables the detection or purification of the covalently labelled proteins. In cooperation with the group of Thomas Vogt (IPB Halle, Dept. of Cell and Metabolic Biology) the synthesis of chemical probes specifically targeting cation-dependent plant

O-methyltransferases (Pl-OMTs) from *A. thaliana* was intended. The design of these probes was based on the modification of tolcapone (Fig. S4), a well-known inhibitor of the animal catechol *O*-methyltransferase, which shares great similarities with cation-dependent Pl-OMTs concerning substrate binding and catalysis mechanism. Despite selective binding of cation-dependent Pl-OMTs was observed (exemplified *via* binding studies using the tapetum specific cation-dependent Pl-OMT AtTSM1 and the cation-independent Pl-OMT AtOMT1), the selective enrichment of these enzymes could not be detected, though (chapter 4.2).

Moreover, the first "clickable" and photoreactive indole-3-acetic acid, the major auxin in higher plants, was synthesised (Fig. S5). Currently, investigations regarding the biological activity of the obtained probe in comparison to natural indole-3-acetic acid (IAA) are performed in the lab of Luz Irina A. Calderón Villalobos (IPB Halle, Dept. of Molecular Signal Processing). Given that the probe causes a similar phenotype then natural IAA, the *in vivo* application would contribute greatly to the understanding of auxin perception during plant development (chapter 4.3).

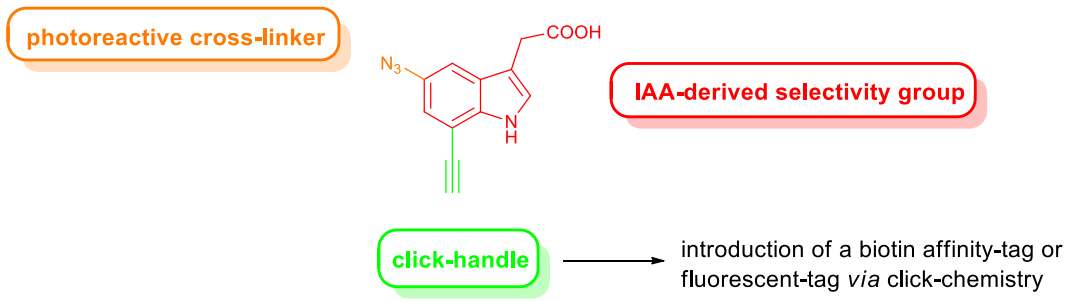


Fig. S5 First "clickable" and photoreactive IAA-analogue for the identification of IAA-receptors *in planta*.

Zusammenfassung und Ausblick

Die Modifikation von Biomolekülen unter physiologischen Bedingungen ermöglichte es biologisch orientierten Wissenschaftlern in den letzten Jahren die Funktion dieser Moleküle in einer Vielzahl von Organismen zu studieren. Weiterhin gelang es damit die Stabilität von therapeutisch bedeutenden Proteinen zu verbessern und Protein-Protein Wechselwirkungen bzw. die Struktur von Proteinen unter nativen Bedingungen zu untersuchen. Der herkömmliche Ansatz beruht dabei auf der Verwendung von reaktiven Gruppen, die selektiv mit einer bestimmten Aminosäureseitenkette reagieren. Zu diesem Zweck existiert bereits eine umfangreiche Bibliothek an Reagenzien. Dabei finden am häufigsten jedoch entweder Lysin-reaktive Gruppen (z. B. *N*-Hydroxysuccinimidester) oder Cystein-reaktive Gruppen (z. B. Maleimid) Verwendung.

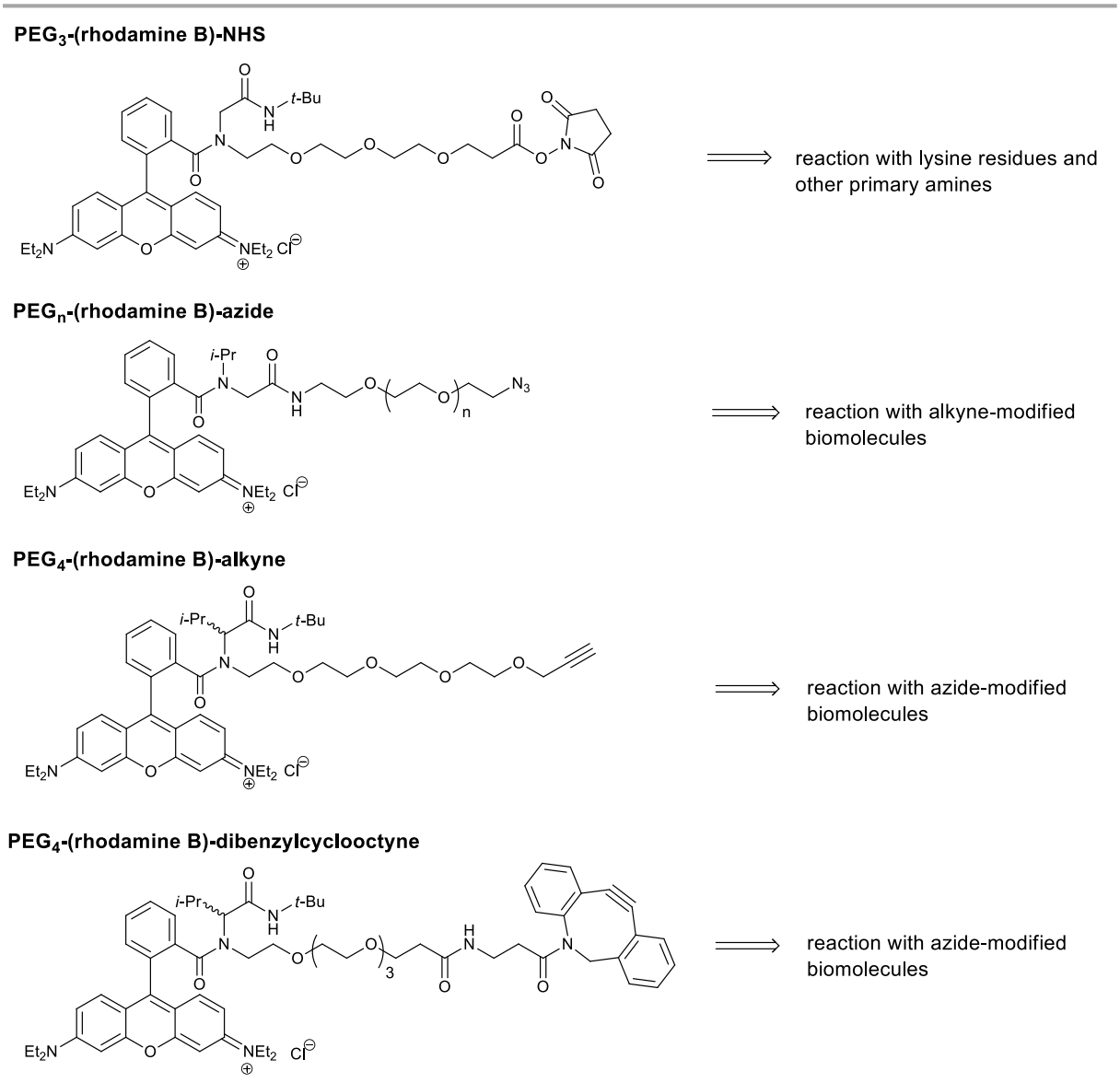


Abb. Z1 MCR-abgeleitete Fluoreszenzlabel für die chemische Modifikation von verschiedenen Biomolekülen.

Basierend auf solchen Aminosäureseitenketten-spezifischen Reagenzien wurde im Verlauf dieser Arbeit ein Lysin-reaktives Rhodamin-Label hergestellt und erfolgreich zur Fluoreszenzmarkierung von Rinderalbumin (BSA) und einem Proteinrohextrakt aus Gerste eingesetzt (Kapitel 2.2). Zu dem Zweck der Synthese solcher pH-Wert unabhängigen Fluoreszenzsonden wurde ein schneller, modularer und einfach durchzuführender Syntheseansatz basierend auf der Verwendung von Multi-komponentenreaktionen (MCR), speziell der bereits gut untersuchten Ugi-4-Komponentenreaktion (Ugi-4CR), entwickelt. Weiterhin wurde unter Anwendung dieses MCR-Ansatzes eine kleine Sunstanzbibliothek synthetisiert, welche unterschiedliche Rhodamin-Label beinhaltet. Diese sind entweder mit einem terminalen Alkin, einem gespannten Cylcooktin oder mit einem Azid funktionalisiert. Abgesehen von der Verwendung von unterschiedlich langen Ethylenglykolketten wurden auch Derivate synthetisiert, die andere Rhodamin-Derivate, z. B. Rhodamin 19P oder Rhodamin 101, beinhalten (Abb. Z1). Die auf diesem Wege erhaltenen "klickbaren" Fluoreszenzlabel können daraufhin auf vielfältige Weise eingesetzt werden, z. B. im aktivitätsbasierten Protein-Profiling oder zur Fluoreszenzmarkierung von Proteinen, die mit einem komplementären Klick-Anker versehen sind (u. a. durch Einführen von unnatürlichen Aminosäuren mittels Mutation).

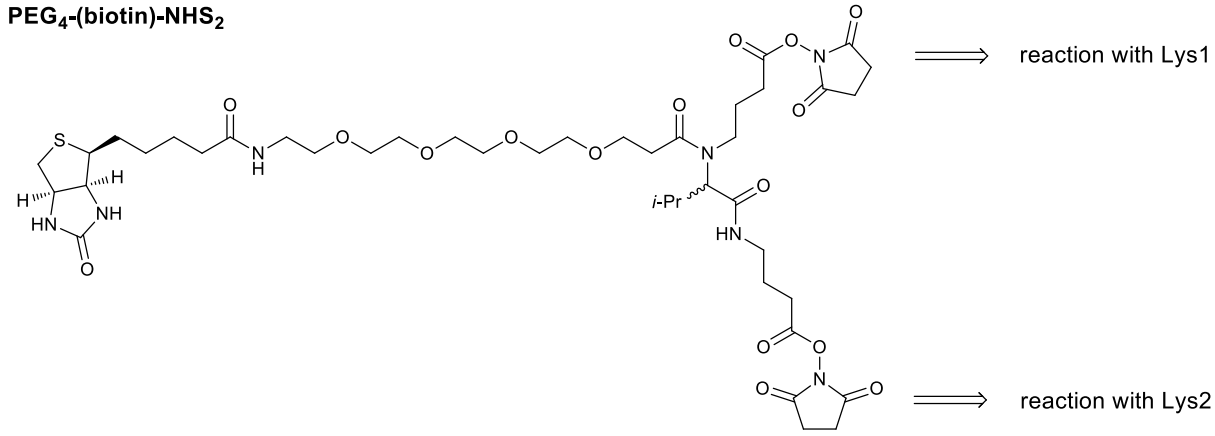


Abb. Z2 MCR-abgeleitete, Amin-reaktive trifunktionelle Vernetzungsreagenz zur chemischen Modifikation von zwei freien Lysin-Seitenketten innerhalb eines Proteins bzw. zwischen einem Protein und einem interagierenden Protein/Peptid.

Dieser MCR-Ansatz kann weiterhin zur Modifikation von Biotin eingesetzt werden. Unter Verwendung der Ugi-4CR konnte eine Affinitätssonde mit zwei Amin-reaktiven *N*-Hydroxysuccinimidestern bereitgestellt werden, die die kovalente Modifizierung von

zwei freien, räumlich benachbarten Lysin-Seitenketten innerhalb eines Proteins bzw. zwischen einem Protein und einem interagierenden Protein/Peptid ermöglicht (Abb. Z2). Die Reaktivität dieser neuartigen trifunktionellen Vernetzungsreagenz wurde anhand von reinem Cytochrom *c* und einem Calmodulin/skMLCK-Peptidkomplex ermittelt. Nach erfolgtem tryptischen Verdau, Affinitätsanreicherung und MS-Analyse konnten fünf Vernetzungen innerhalb von Cytochrom *c* und drei Vernetzungen zwischen Calmodulin und dem skMLCK-Peptid identifiziert werden (Kapitel 2.3).

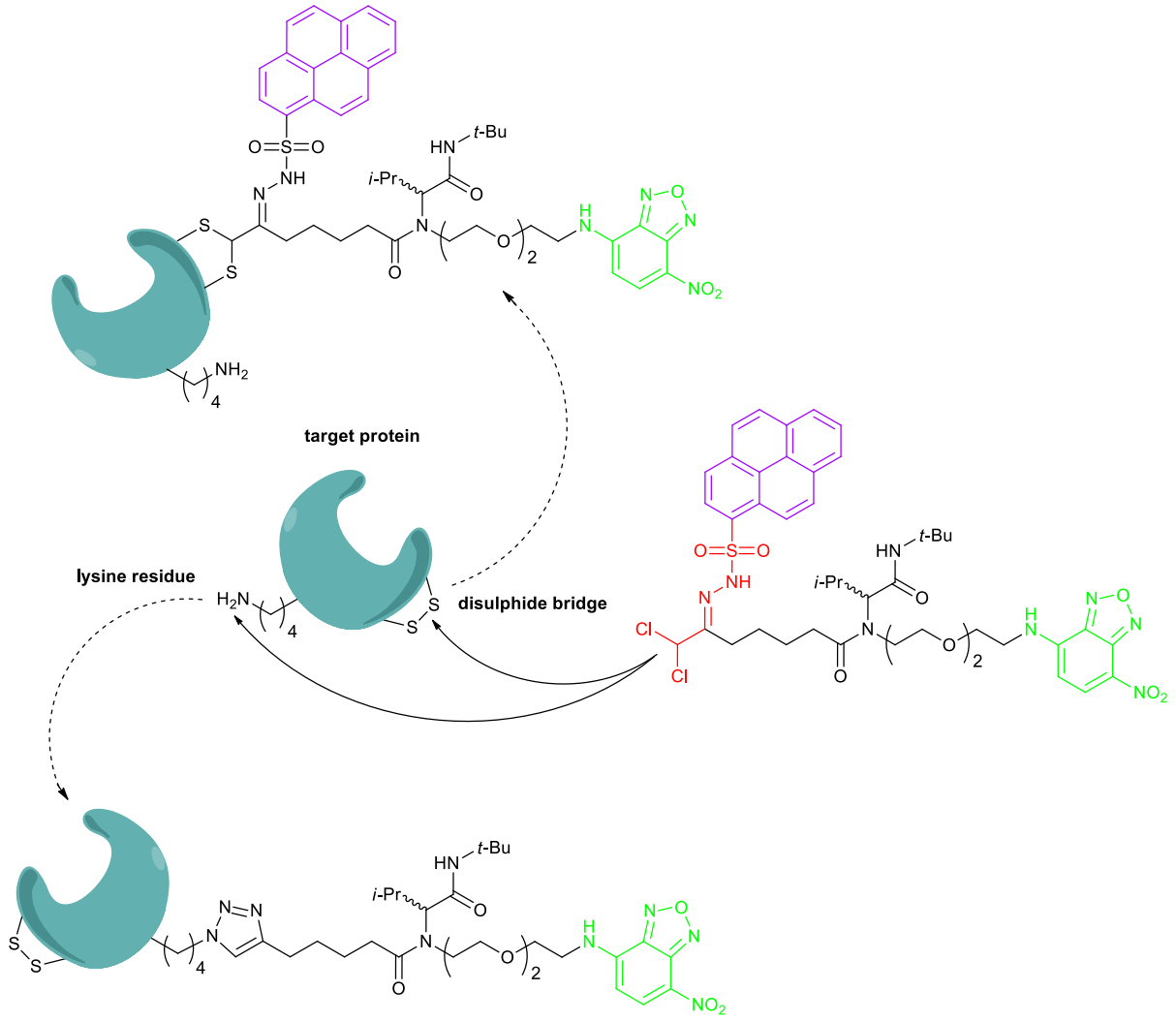


Abb. Z3 NBD-Dichloropyrensulfonohydrazon: Eine Klasse von neuartigen Fluoreszenzlabeln zur Modifizierung von Lysin-Seitenketten und Disulfidbrücken?

Die Modifizierung von Lysin-Seitenketten unter annähernd physiologischen Bedingungen beruht in aller Regel auf der Verwendung von Aktivestern, z. B. *N*-Hydroxy-succinimidestern, welche in wässrigen Medien einer schnellen Hydrolyse unterliegen. Demzufolge war es das Ziel im Rahmen dieser Arbeit neuartige Amin-reaktive Reagenzien mit erhöhter Hydrolysestabilität zu entwickeln. Dabei wurde der Fokus auf die Sakai

Reaktion gelegt, eine Reaktion zwischen primären Aminen und *α*,*α*-Dichlorotosylhydrazonen, wobei regioselektiv 4-substituierte 1,2,3-Triazole gebildet werden. Diese Art der Triazolbildung wurde bereits Mitte der 1980er Jahre erstmalig beschrieben, fand jedoch bislang nur wenig Verwendung. Daher wurde zunächst der Anwendungsbereich der Sakai Reaktion unter Verwendung verschiedenster primärer Amine und *α*,*α*-Dichlorotosylhydrazonen untersucht. Basierend auf den Ergebnissen dieser Untersuchungen wurde ein möglicher Mechanismus der Sakai Reaktion postuliert (Kapitel 3.2). Daraufhin wurden die Reaktionsbedingungen dahingehend optimiert, dass die Sakai Reaktion auch in Gegenwart einer biologischen Probe ablaufen kann (d. h. wässrige Pufferlösungen und pH ~ 7). Schließlich gelang es mithilfe eines geeigneten fluoreszierenden *α*,*α*-Dichlorotosylhydrazons sowohl BSA als auch Lysozym erfolgreich mit einem Fluoreszenzfarbstoff zu modifizieren. Weiterhin wurde die Anwendbarkeit von speziell synthetisierten *α*,*α*-Dichlorosulfonohydrazonen als neuartige Disulfid-verknüpfende Reagenzien untersucht (Abb. Z3). Aufgrund der auftretenden Fluoreszenzlöschung bedarf es hier jedoch noch weiteren Verbesserungen im Aufbau des Fluoreszenzlabels (Kapitel 3.3).

Zur Identifizierung, selektiven Anreicherung und Aufreinigung von Proteinen einzelner Proteinfamilie wurde das Konzept des aktivitätsbasierten Protein-Profilings (ABPP) entwickelt. Diese Methode beruht auf der Verwendung niedermolekularer chemischen Sonden, welche mit einer Selektiveinheit ausgestattet sind, die für die selektive Bindung einer einzelnen Enzymklasse zuständig ist (z. B. Inhibitoren, Substrate, Cofaktoren, *etc.*). Nach der Ausbildung einer kovalenten Bindung zwischen der Sonde und dem Zielprotein ermöglicht die Reportereinheit (z. B. Biotin, Fluorophor) die Detektion oder Aufreinigung des auf diese Weise modifizierten Proteins. In Zusammenarbeit mit der Gruppe von Thomas Vogt (IPB Halle, Abt. Stoffwechsel- und Zellbiologie) sollte eine geeignete Sonde zur Anreicherung von kationen-abhängigen pflanzlichen *O*-Methyltransferasen (Pl-OMTs) aus *A. thaliana* entwickelt werden. Das Design dieser Sonde basiert auf der Modifikation von Tolcapon (Abb. Z4), einem bekannten Inhibitor der tierischen Catechol-*O*-Methyltransferase, welche große Übereinstimmungen bezüglich Substratbindung und Katalysemechanismus zu kationen-abhängigen Pl-OMTs aufweist. Obwohl es gelang eine selektive Bindung kationen-abhängiger Pl-OMTs am Beispiel der Tapetum-spezifischen kationen-abhängigen Pl-OMT AtTSM1 im Vergleich zur kationen-unabhängigen Pl-OMT AtOMT1 zu demonstrieren, konnten keine kationen-abhängigen Pl-OMTs aus Knospenextrakten angereichert werden (Kapitel 4.2).

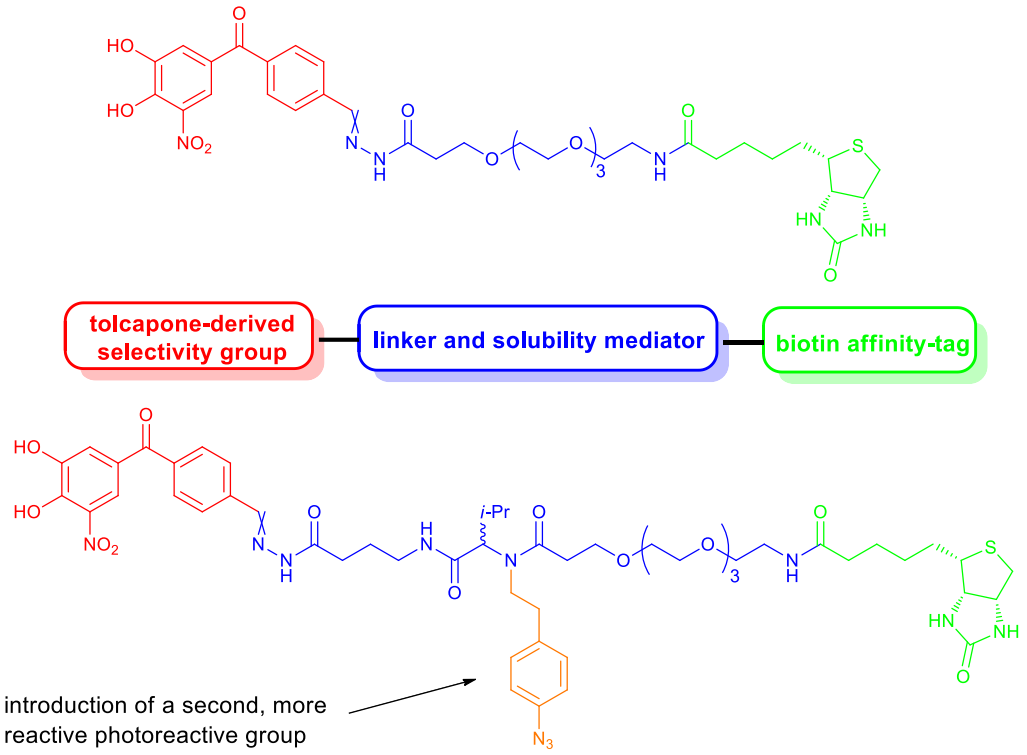


Abb. Z4 Aufbau von multifunktionellen chemischen Sonden zur potentiellen Anreicherung von kationenabhängigen Pl-OMTs aus Knospenextrakten von *A. thaliana*.

Des Weiteren wurde im Verlauf dieser Arbeiten das erste "klickbare" und zugleich photoreaktive Indol-3-essigsäure-Derivat synthetisiert (Abb. Z5). Untersuchungen bezüglich der biologischen Aktivität dieser Sonde im Vergleich zu natürlicher Indol-3-essigsäure (IAA), welches den überwiegenden Anteil an Auxin in Pflanzen ausmacht, werden zurzeit in der Gruppe von Luz Irina A. Calderón Villalobos (IPB Halle, Abt. Molekulare Signalverarbeitung) durchgeführt. Unter der Voraussetzung, dass die synthetische Sonde die Ausbildung eines ähnlichen Phänotyps begünstigt wie natürliches IAA, würde die *in vivo* Anwendung der hier beschriebenen Sonde umfassend zum Verständnis der Auxinperzeption während der pflanzlichen Entwicklung beitragen.

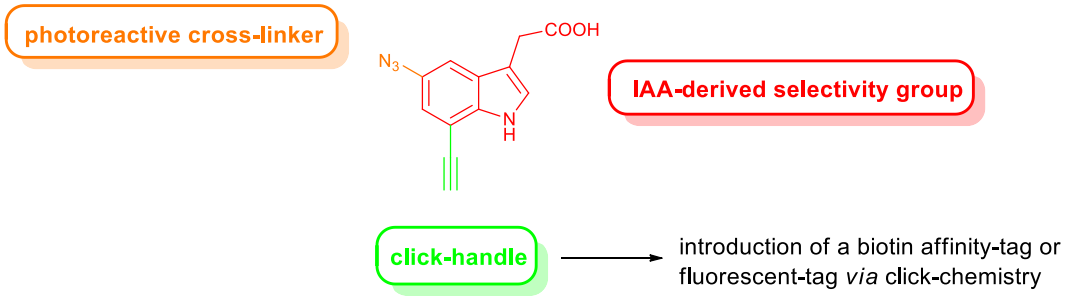


Abb. Z5 Aufbau eines "klickbaren" und photoreaktiven IAA-Derivates zur Identifizierung von IAA-Rezeptoren in Pflanzen

Table of Contents

List of Abbreviations

1D, 2D or 3D	one, two or three dimensional
26S	26S proteasome
$[\alpha]_D^T$	specific optical rotation
ABPP	activity-based protein profiling
ABP	activity-based probe
ABP1	auxin-binding protein 1
abs	absorption
AcOH	acetic acid
AFB1–5	auxin signalling F-Box proteins 1–5
Ala	alanine
approx.	approximately
ASK1	arabidopsis SKP1-like protein
Atl	autolysin
AtOMT1	*A. thaliana O*-methyltransferase 1
ARFs	auxin response factors (transcriptional activators)
AtTSM1	*A. thaliana* tapetum specific *O*-methyltransferase 1
Aux/IAA	auxin/indole-3-acetic acid transcriptional repressor
BARAC	biaryl-azacyclooctynone
BCN	bicyclo[6.1.0]nonyne
Bn	benzyl
Boc	*tert*-butoxycarbonyl
Boc_2O	di-*tert*-butyl dicarbonate
BODIPY	boron-dipyrromethene fluorescent dyes
Bu_4NOAc	tetrabutylammonium acetate
BSA	bovine serum albumin

BR-buffer	Britton-Robinson buffer
BRI1	brassinosteroid insensitive 1
br.	broad signal (NMR)
calcd	calculated
CaM	calmodulin
CuAAC	copper-catalysed azide-alkyne cycloaddition
CUL1	cullin 1
CoA	Coenzyme A
COI1	coronatine insensitive 1
CMC	1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide tolylsulfonate
Col-0	*A. thaliana* ecotype Columbia
COMT	catechol-*O*-methyltransferase
conc.	concentrated
CyHex	cyclohexyl
Cys	cysteine
d	doublet (NMR)
DCDMH	1,3-dichloro-5,5-dimethylhydantoin
DDQ	2,3-dichloro-5,6-dicyano-*p*-benzoquinone
Dept.	department
DES	deep eutectic solvent
DIBO	dibenzocyclooctynes
DIFO	difluorinated cyclooctynes
DiPEA	diisopropylethylamine
DMAP	4-dimethylaminopyridine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
E2	ubiquitin conjugating enzyme
EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
em	emission
equiv.	equivalent(s)
ESI-MS	electron-spray ionisation mass spectrometry
Et	ethyl
EtOAc	ethyl acetate
EtOH	ethanol
ex	excitation
Fig.	figure
FT-ESI	fourier transform electron spray ionisation mass spectrometry
GABA	*γ*-amino butyric acid
GLD	globoid cell leukodystrophy
GSTO1	glutathione *S*-transferase omega 1
h	hour(s)
HOMO	highest occupied molecular orbital
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectrometry
h*ν*	energy of a photon (h = Planck`s constant; *ν* = frequency)
IAA	indole-3-acetic acid
IC	internal conversion
IC_{50}	concentration of a compound needed to inhibit a given enzyme activity by half
IGFS	in-gel fluorescence scanning

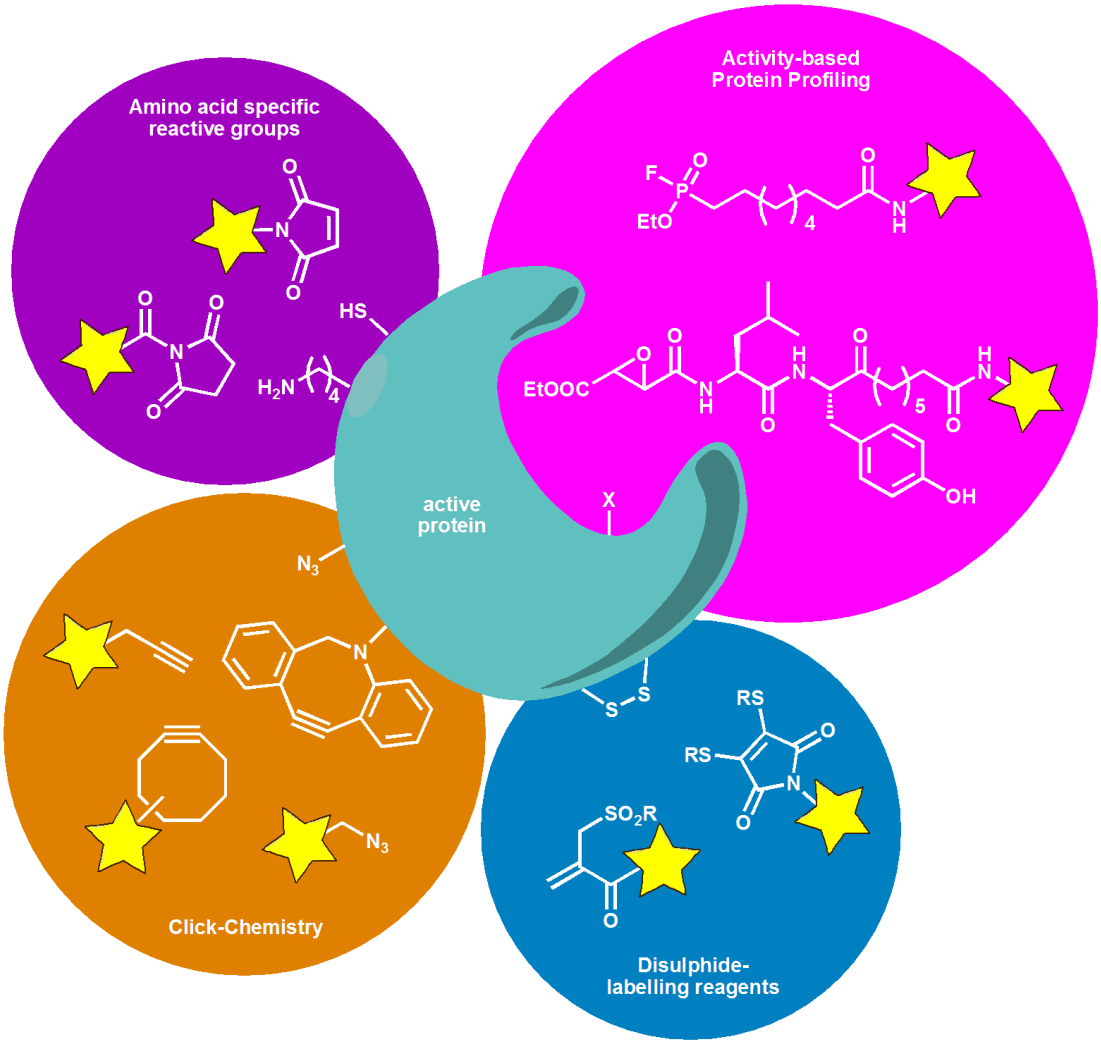
IPB	Leibniz Institute of Plant Biochemistry
i-Pr	*iso*-propyl
ISC	intersystem crossing
J	coupling constant (NMR)
K_d	dissociation constant
K_i	enzyme inhibitor constant
K_M	Michaelis constant
KOAc	potassium acetate
Kpi	potassium phosphate buffer
LC/MS	liquid chromatography coupled with mass spectrometry
LC/MS/MS	liquid chromatography coupled with tandem mass spectrometry
LDA	lithium diisopropylamide
LUMO	lowest unoccupied molecular orbital
LTQ	linear trap quadrupole
Lys	lysine
m	multiplet (NMR)
$[M]^+$, $[M-H]^-$, $[M+X]^+$, *etc.*	molecule-ions obtained upon electron-spray ionisation
MALDI-TOF-MS	matrix-assisted laser desorption ionisation-time of flight-mass spectrometry
MCR	multicomponent reaction
Me	methyl
MeCN	acetonitrile
MeI	methyl iodide
MeOH	methanol
min	minute(s)
MMPs	matrix metalloproteases

M.p.	melting point
MRSA	methicillin-resistant *Staphylococcus aureus*
MS	mass spectrometry
MsCl	methanesulphonyl chloride
MS/MS	tandem mass spectrometry
MTases	methyltransferases
MudPIT	multidimensional protein identification technology
μW	microwave
MW	molecular weight
m/z	mass to charge ratio
NBD-Cl	4-chloro-7-nitrobenzofurazan
NHS	*N*-hydroxysuccinimide
NMR	nuclear magnetic resonance spectroscopy
NR	non-radiative relaxation
Nu	nucleophile
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDB	protein database
PEG	polyethylene glycol
Ph	phenyl
PHD2	prolyl hydroxylase domain-containing protein 2
Pl-OMT	plant *O*-methyltransferases
PTPs	protein tyrosine phosphatases
pTsNHNH$_2$	*para*-toluenesulphonyl hydrazide
PS-1 and PS-2	presenilin 1 and presenilin 2
q	quartet (NMR)

QSY7	non-emitting quencher dye
quant.	quantitative
quint.	quintet (NMR)
RBX	ring-box protein 1
RET	resonance energy transfer
R_F	retardation factor
RhoB, Rho 19P and Rho101	rhodamine B, *etc.*
RP18	reverse phase 18
RBBP9	retinoblastoma-binding protein-9
RuAAC	ruthenium-catalysed azide-alkyne cycloaddition
RUB	ubiquitin-like proteins
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
r.t.	room temperature
[S]	substrate concentration
S_0 ... S_n	singlet ground or excited state
s	singlet (NMR)
SAE	sialic acid 9-*O*-acetylesterase
SAH	*S*-adenosyl-*L*-homocysteine
SAM	*S*-adenosyl-*L*-methionine
S. aureus	*Staphylococcus aureus*
SCF	SKP1–Cullin–F-Box
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec.	secondary
SEQUEST	tandem mass spectrometry data analysing tool for protein identification
skMLCK	skeletal muscle myosin light chain kinase

SKP1	S-phase kinase-associated protein 1
SPAAC	strain-promoted azide-alkyne cycloaddition
SPANC	strain-promoted alkyne nitrone cycloaddition
SPANOC	strain-promoted alkyne nitrile oxide cycloaddition
T_1	first triplet excited state
t	triplet (NMR)
TBAF	tetrabutylammonium flouride
t-Bu	*tert*-butyl
t-BuOH	*tert*-butanol
TCEP	tris(2-carboxyethyl)phosphine
tert	tertiary
THF	tetrahydrofuran
THL	tetrahydrolipstatin
TIR1	transport inhibitor resistant 1
TLC	thin layer chromatography
TMS	tetramethylsilane
TMSCl	trimethylsilyl chloride
TMSCN	trimethylsilyl cyanide
Ts	toluenesulphonyl
TsH	toluenesulphonic acid
Ubi	ubiquitin
Ugi-4CR	Ugi-4-component reaction
UV/Vis	ultraviolet/visible
VPEs	vacuolar processing enzymes
v/v	volume fraction

1 Introduction
Chemical Approaches in Proteomic Research



Abstract

Facing the demands of covalently modifying biomolecules under physiological conditions, *e.g.* to study the distribution of a certain target in cells or tissues or to elucidate protein-protein and protein-small molecule interactions, *etc.*, chemical challenges arise from the task to develop suitable reagent systems which react selectively with the target of interest in its natural environment. The forthcoming chapter is intended to introduce the basic concepts and techniques of modifying biomolecules. Furthermore, the scope and limitations of the presented methods will be discussed.

1.1 The incorporation of chemical approaches into proteomic research

Proteins, the functional units of life, are involved in almost all known biological processes in flora and fauna. Hence, the investigation of the *proteome* comprising the complete set of expressed proteins by an organism contributes to a better understanding of complex biological processes in various cells and tissues. A better insight concerning protein-protein interactions and the effect of post-translational modifications (*e.g.* phosphorylations, glycosylations) on protein activity, among others, has been achieved using proteomic techniques.

In the late 1990s, the overall aim of proteomic research was based on the quantitative measurement of all expressed proteins of an organism to study the mechanism of gene expression. However, other potential useful applications of this highly interdisciplinary field of science including aspects of biology, chemistry and bioinformatics emerged over the last few years. Due to the fact that in contrast to the quite static genome the composition of the proteome is highly dependent on the developmental stage of an organism and other environmental factors (Fig. 1.1), the application of specific proteomic techniques might be beneficial for the development of new biomarkers for early diagnoses of diseases or to discover new target-proteins associated with a specific physiological state (*e.g.* healthy *vs.* pathogenic). Another main task of proteomic research evolved from recent progress in decoding whole genome sequences of until now more than six hundred organisms which led to the desperate need of high-throughput methods for assigning the function of all predicted proteins.[1]

Fig. 1.1 Caterpillar (left) and butterfly (right) of the species *Orgyia antiqua L.* (Reproduced from F. Lottspeich, *Angew. Chem. Int. Ed.* **1999**, *38*, 2476-2492 with permission of John Wiley and Sons)

To fulfil the aforementioned scope of duties, different approaches have been developed for the comprehensive analysis of the proteome. The most common approach applied is two-dimensional gel electrophoresis (2D-PAGE). This technique involves the separation of proteins of a whole cell lysate followed by the analysis of the separated protein spots

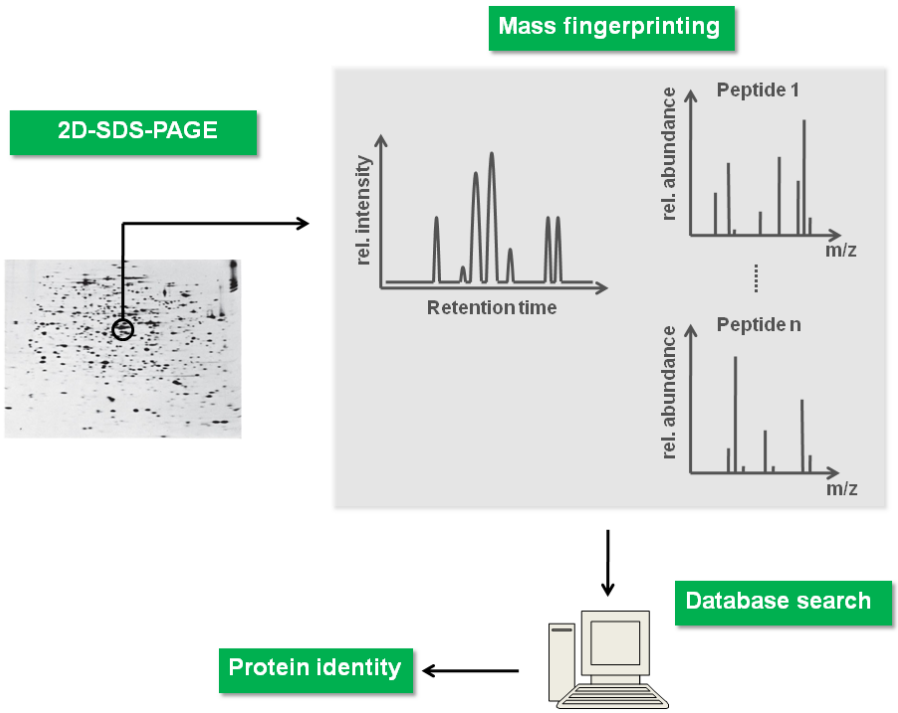


Fig. 1.2 General work-flow of proteome analysis based on 2D-SDS-PAGE. All proteins of a sample are separated first according to their isoelectric point and second according to theirs mass (2D-SDS-PAGE). Afterwards the separated protein spots are cut out of the gel, tryptically digested and the obtained peptides are analysed by LC/MS or LC/MS/MS. The MS and MS/MS spectra, the so-called *peptide-fingerprint*, are matched against protein sequence databases which provide information regarding the identity of the protein.

by mass spectrometric techniques (Fig. 1.2).[1b,2] The analysis of the proteome by 2D-PAGE, however, suffers from some significant drawbacks, *e.g.* low resolution (in general only about 1000 different proteins can be separated by 2D-PAGE) and the inability of analysing low-abundant as well as membrane-bound proteins, among others.[3]

Consequently, approaches to effectively reduce the complexity of a protein mixture are of high demand. Here, *chemical proteomics* mostly referred to as *activity-based protein profiling* (ABPP), first introduced by Cravatt and co-workers, comes into play. Based on the specific interaction of small molecular multifunctional probes with a protein, a specific *subproteome* is isolated which represents a fraction of the whole proteome containing proteins with a common function. In general, such *activity-based probes* (ABPs) should meet the following criteria: (i) they bind to a wide range of catalytically active enzymes which share a common function; (ii) binding occurs according to the catalytic mechanism of the target enzyme; (iii) they display minimal cross-reactivity towards other classes of enzymes; and (iv) these probes possess a reporter-tag which enables the rapid detection or isolation of the target enzymes. The most commonly used tags are either biotin, allowing the fast isolation of the target proteins by avidin/streptavidin-affinity-

purification, or fluorescent labels (*e.g.* xanthene dyes) which enable the detection of the labelled proteins, previously separated by SDS-PAGE, *via* fluorescence scanning. Moreover, radioactive labels, such as iodine-125, have been applied successfully. Besides the aforementioned affinity- or fluorescent tags, ABPs are composed of two (or three) additional functional units (Fig. 1.3): a *selectivity group* for specific target recognition (*e.g.* reversible/irreversible inhibitor); a *reactive group* for the covalent attachment of the (reversible) probe to the protein (*e.g.* photoreactive group); and a *linker* unit connecting both the warhead, consisting of selectivity and reactive group, and the reporter-tag with each other which minimises steric repulsions between the binding region of the protein and the reporter-unit of the whole chemical probe. The linker unit might serve additionally as solubility mediator (*e.g.* by introducing various ethylene glycol units). The reactive group, however, can be omitted when the selectivity group offers the possibility for the covalent binding to the active site of a protein (*e.g.* suicide inhibitor).[4]

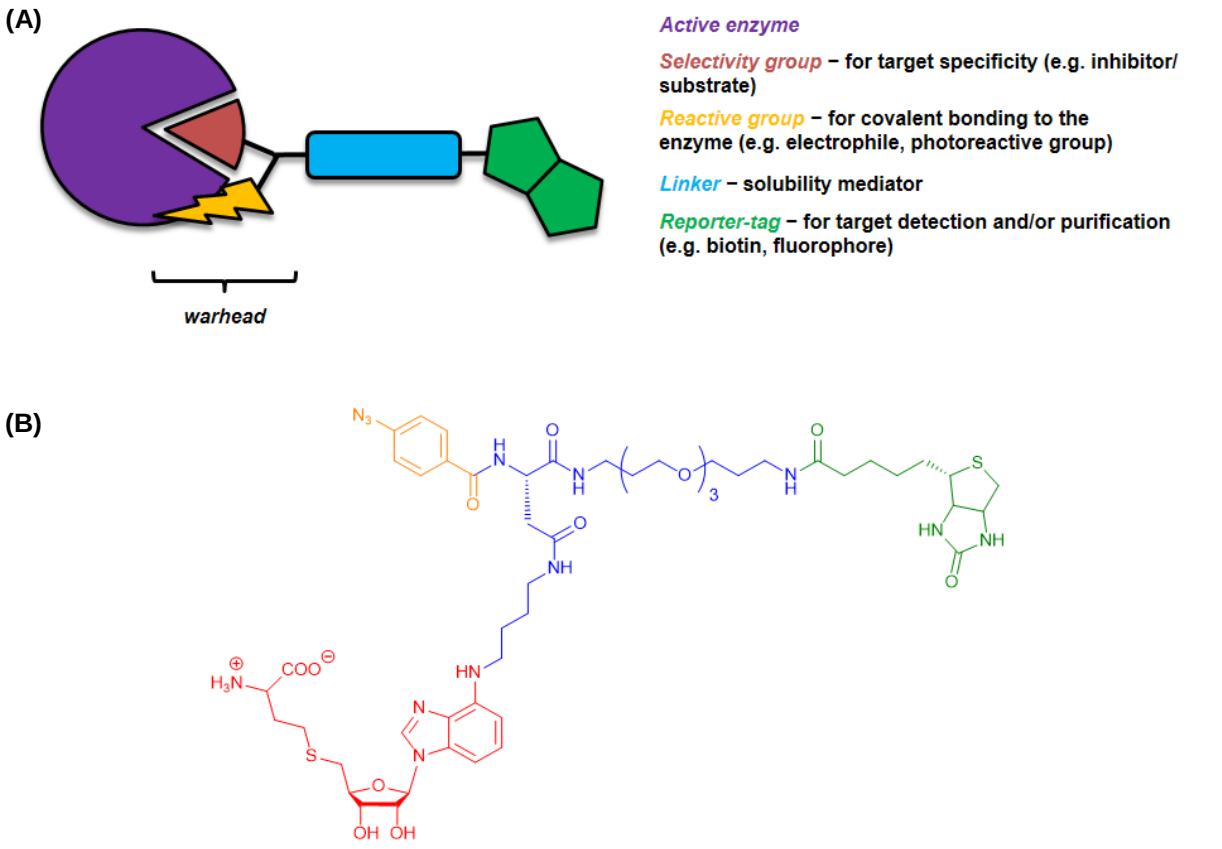


Fig. 1.3 Chemical probes for proteomic research. (A) General build-up of an activity-based probe (ABP); **(B)** Example of an ABP for the selective labelling of SAM-dependent proteins, *e.g. O*-methyltransferases (according to Dalhoff *et al.*, *ChemBioChem* **2010**, *11*, 256–265).

Over the last years, several methods have emerged to characterise probe labelled proteomes during ABPP experiments. As a consequence of its robustness, simplicity and

high-throughput the most commonly used method is *in-gel fluorescence scanning* (IGFS).[5] In the course of IGFS, a crude protein mixture is treated with a fluorescent ABP. The labelled proteins are separated by 1D-SDS-PAGE and subsequently analysed by fluorescence scanning (Fig. 1.4). However, the number of proteins targeted by an ABP exceeds in most cases the number of proteins which can be resolved by 1D-SDS-PAGE. Furthermore, this method is unable to detect low-abundant probe-labelled proteins. For the purpose of identifying these low-abundant targets, corresponding biotinylated probes were consequently developed. After avidin affinity-enrichment, SDS-PAGE separation of the biotinylated proteins and in-gel tryptic digestion, the resulting peptide mixture is analysed by LC/MS/MS and the targets of the ABP are identified using a suitable search algorithm (*e.g.* SEQUEST).[4b]

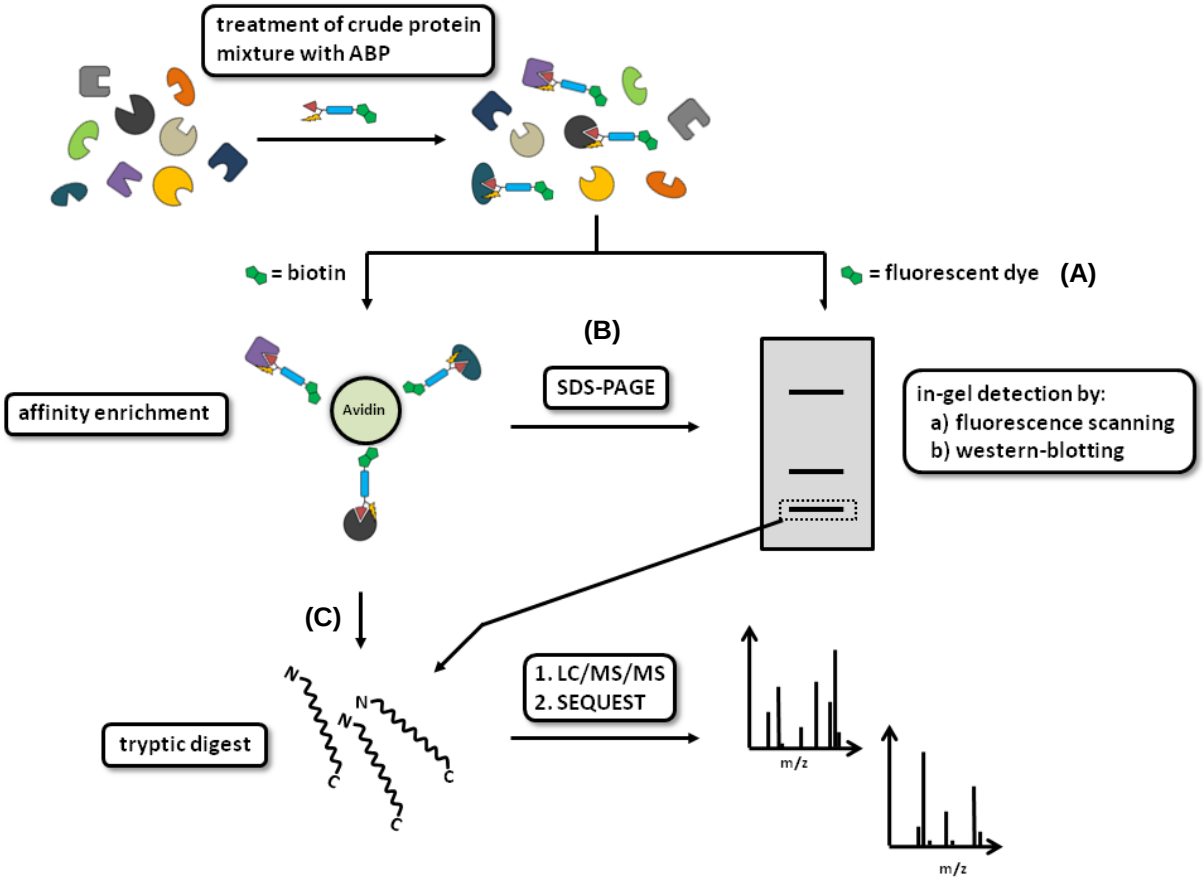


Fig. 1.4 Analytical platforms for the detection and identification of targets of ABPP-probes. A protein mixture is treated with a suitable APB equipped either with a fluorescent dye or a biotin affinity-label. The probe labelled proteins are further analysed by in-gel fluorescence scanning **(A)**. For the identification of low-abundant targets of an ABP, affinity enrichment is performed first, followed by either separation of the labelled proteins by SDS-PAGE, subsequent in-gel tryptic digestion and identification by LC/MS/MS **(B)**; or on-bead tryptic digestion and identification by multidimensional LC/MS/MS **(C)**.

To overcome the previously stated limitations of IGFS, gel-free approaches were developed to assign probe-targets. Most of the described methods are based on a LC/MS strategy to identify the targets of an ABP without the need of pre-fractionation of the labelled proteins by 1D-SDS-PAGE. Here, the probe-labelled proteins are separated from the unmodified fraction of the proteome by affinity enrichment using avidin-conjugated beads followed by on-bead tryptic digestion (Fig. 1.4). The obtained peptide mixture is further analysed by multidimensional LC/MS/MS to identify the targets of an ABPP-probe (ABPP-MudPIT). In comparison to classical gel-based approaches, ABPP-MudPit exhibits an enhanced resolution and sensitivity making this method feasible to analyse highly complex proteomic samples.[6]

The *in vivo* application of these chemical probes, however, is rather limited due to their high molecular mass resulting in a decreased cellular uptake and distribution. Moreover, bulky reporter-units might hamper an effective binding of the whole chemical probe in the binding region of the target enzyme. Therefore, the groups of Cravatt and Overkleeft independently developed a "tag-free" approach. In this case, a probe head (warhead) consisting of the selectivity (and reactive) group and a reporter-tag are required each carrying a bioorthogonal functional group. Conjugation of both parts, probe head and reporter-tag, can be achieved following the covalent attachment of the probe head to the protein. Here, the copper-assisted [3+2] Huisgen azide-alkyne cycloaddition (CuAAC) and the Staudinger ligation have been generally applied (Fig. 1.5).[7,8]

The *in-vivo* application of tag-free ABPs additionally enables the characterisation of the proteome in a native cellular environment. So far, several enzymes have been identified which could be labelled with an ABP only in living cells but not in cell homogenates.[4c,7c] This fact might be attributed to the susceptibility of many biological processes, *e.g.* to cell lysis.

Over the last decade, ABPs have been used successfully to characterise a broad spectrum of different enzyme classes, such as predominantly hydrolases (*e.g.* serine and cysteine hydrolases) but also kinases, glycosidases as well as oxidoreductases, among others. Furthermore, the application of ABPs has been shown to be suitable for: (i) the labelling of various receptor proteins, *e.g.* for plant hormones like jasmonate and brassinosteroids; (ii) the identification of the binding site/s of a certain substrate of biological interest; and (iii) drug development, especially for the identification of lead structures and drug off-targets (*Toxicoproteomics*).

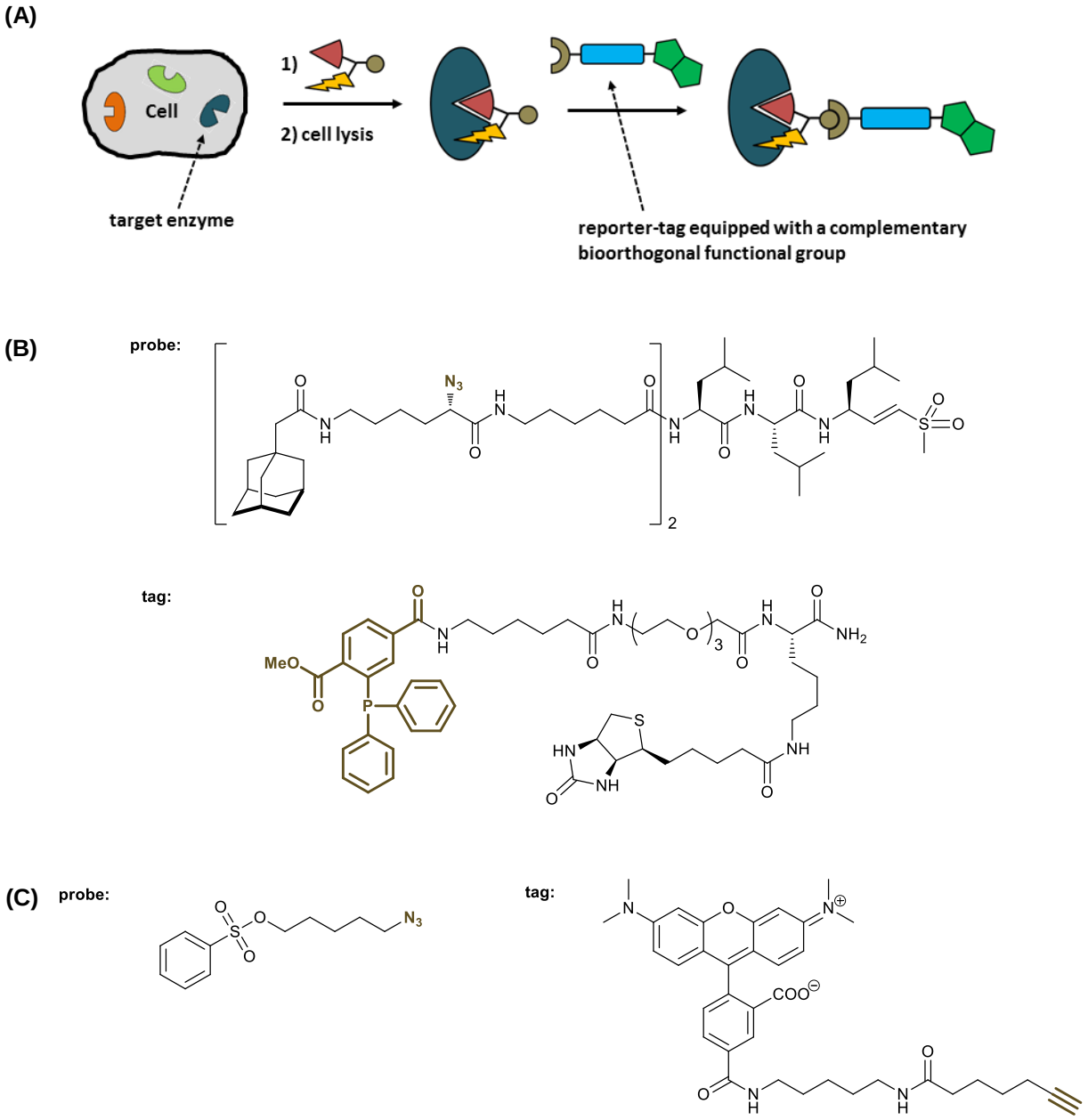


Fig. 1.5 A "tag-free approach" for ABPP based on the application of bioorthogonal reactions. (A) Living cells are treated first with the probe head consisting of the selectivity (and the reactive) group additionally equipped with a bioorthogonal handle. After subsequent cell lysis, a suitable reporter-tag (*e.g.* biotin, fluorophore) is added and conjugation of the probe labelled proteins and reporter-tag is achieved either *via* CuAAC or Staudinger ligation. The probe labelled proteins can be further analysed by the previously described methods. Examples of tag-free ABPs and corresponding reporter-tags: **(B)** azide-modified probe head targeting the immunoproteasome and corresponding triarylphosphine-modified biotin affinity-tag (according to Overkleeft *et al.*, *Angew. Chem. Int. Ed.* **2003**, *42*, 3626–3629); and **(C)** azide-modified phenyl sulphonate ester targeting *e.g.* glutathione-*S*-transferases, aldehyde dehydrogenases and enoyl CoA hydratases, among others, and the corresponding alkyne-modified rhodamine-tag (according to Cravatt *et al.*, *J. Am. Chem. Soc.* **2003**, *125,* 4686–4687).

Besides ABPP, specifically designed multifunctional chemical probes have found widespread applications, such as the chemical cross-linking of proteins to study either the three-dimensional structure of proteins or protein-protein interactions as well as the

covalent modification of post-translational modifications. In the following sections, the basic principles and considerations of designing a suitable chemical probe and their applications in proteomic research will be summarised. First, however, more general methods for the covalent modification of biomolecules (*e.g.* proteins, virus particles, antibodies, *etc.*) based on the application of small molecular chemical tags equipped either with amino acid specific or photo-activatable reactive groups as well as bioorthogonal functional handles will be discussed in more detail.

1.2 Chemical strategies to covalently modify proteins and other biomolecules

So far, the incorporation of small functional entities into biomolecules, such as proteins, lipids, antibodies, *etc.*, has found widespread applications in the field of chemical biology, *e.g.* the *in vivo* imaging of proteins,[9] PEGylation to enhance the stability of therapeutic proteins[10] and the attachment of cytotoxines to cancer-targeting agents (*e.g.* monoclonal antibodies).[11] For this purpose, chemical approaches were developed, whereby most of them rely on the chemoselective modification of a specific amino acid residue. Moreover, specifically designed photo-activatable reagents have been widely applied for the covalent modification of biomolecules to study diverse biomolecular interactions (*e.g.* protein-protein-interactions, the mechanism of cell surface adhesion, interactions between binding domain and a ligand, *etc.*).[12] As a consequence of the amount of competing reactivities in a complex biological sample, though, chemical approaches were consequently developed which are based on the application of bioorthogonal reactions. These reagents are able to react with ultimate chemoselectivity and high reaction rates under physiological conditions in the presence of a vast amount of different functionalities found *in vivo*.[13] In the following paragraphs the previously outlined approaches for the covalent modification of biomolecules will be discussed in more detail with respect to their specific strengths and limitations.

Amino acid residue specific labelling techniques

The application of side chain selective reagents, which react under specific conditions with a certain proteogenic amino acid residue in a predictable fashion, have been used routinely to attach small molecular probes (*e.g.* biotin, fluorophore) to proteins and for the immobilisation of proteins on different matrices (*e.g.* polymers and microarray chips). In general, the most commonly targeted functional groups are the thiol group of cysteine

and the ε-amine group of lysine. The selective modification of amino acid residues of the remaining 18 proteogenic amino acids has only been marginally explored yet.[14]

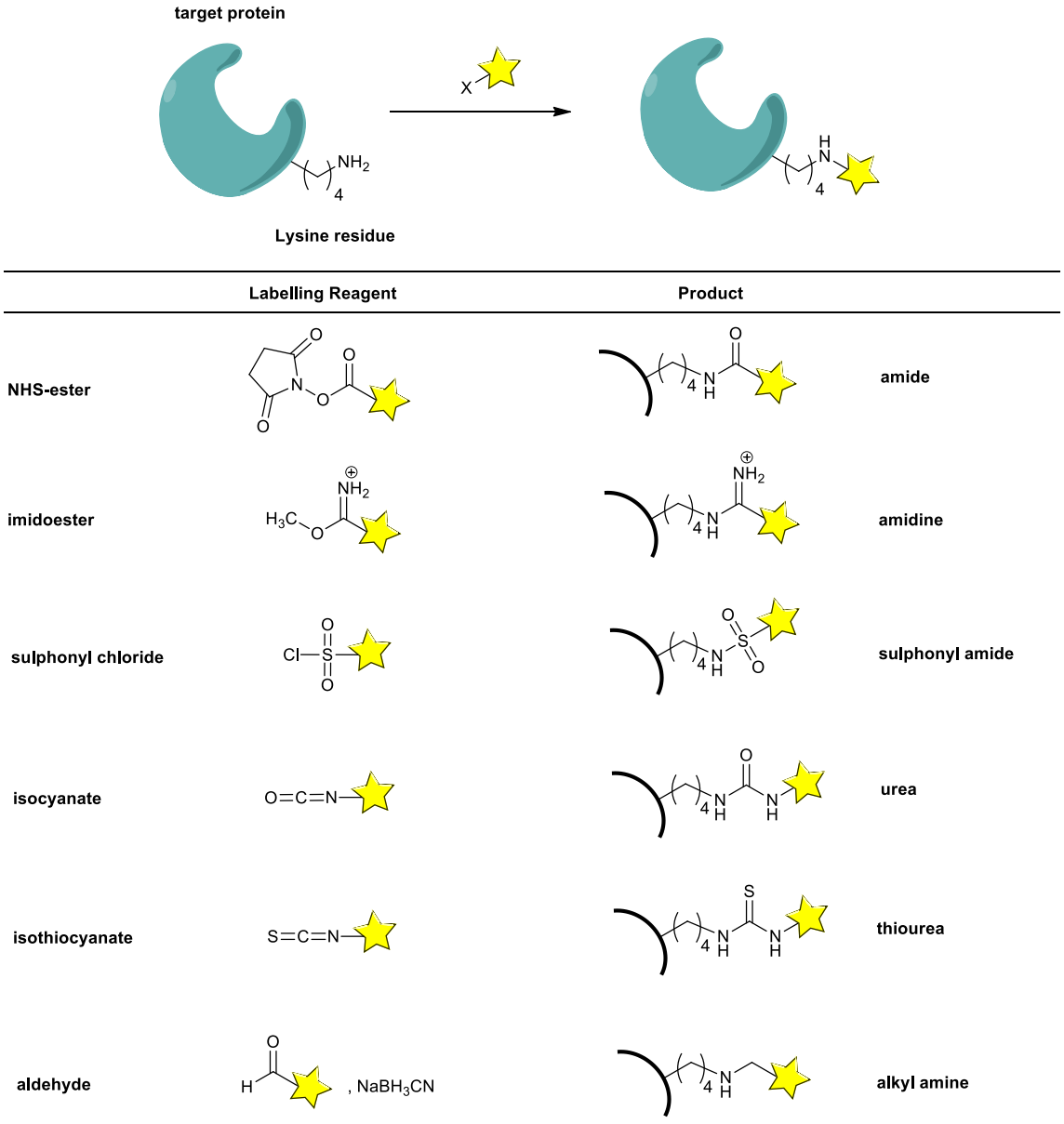


Fig. 1.6 Classic bioconjugation reactions for the covalent modification of lysine residues. The covalent modification of lysine residues can be achieved *via* amide, amidine, sulphonyl amide, urea, thiourea or alkyl amine formation using activated species, such as NHS-esters, imidoesters, sulphonyl chlorides, isocyanates and isothiocyanate, as well as carbonyl compounds in combination with a reducing agent.

Amine-reactive reagents. A variety of useful reagents targeting selectively the free ε-amine group of lysine and the *N*-terminus have been developed so far. Here, highly reactive acylating reagents, predominantly *N*-hydroxysuccinimide esters (NHS-ester) and its more water soluble analogues (sulpho-*N*-hydroxysuccinimide esters), are widely applied. Upon nucleophilic attack of a primary or secondary amine, a NHS group is

released leading to the formation of a stable amide or imide bond (Fig. 1.6). Reactive NHS-esters exhibit high reaction rates, however, they are highly susceptible to rapid hydrolysis under physiological conditions with half-lives in the order of minutes (pH > 7, 25–37 °C).[15] In general, under carefully controlled reaction conditions (pH, reaction time, reagent excess), side-reactions of NHS-esters with other amino acids usually cannot be observed in a significant amount, although they have been reported in the literature.[16] Other activated species, such as sulphonyl chlorides, imidoesters, isocyanates or isothiocyantes affording upon the reaction with free lysine residues sulphonyl amides, amidines, ureas or thioureas, have been used as well for the covalent modification of biomolecules. In addition, reductive amination has been frequently applied modifying free amine residues of biomolecules with aldehyde containing compounds.[17]

Developments towards the selective modification of amines continued in recent years aiming at increasing the selectivity (*e.g.* selective modification of the *N*-terminus) or the stability of the labelling reagents under physiological conditions. Most of these methods rely on transition metal catalysis which might hamper their applicability *in vivo*, though. For example, reductive amination for the coupling of free lysine residues with aldehydes using sodium cyanoborohydride requires, in general, acidic conditions. The application of an iridium catalyst, as described by McFarland and Francis, enables this reaction to be performed in high yields under physiological pH by transfer hydrogenation.[18] Another highly sophisticated method for the selective modification of the *N*-terminal α-amino group was described recently by Wong and Che which is based on the application of functionalised ketenes. They observed excellent *N*-terminal selectivity while modifying insulin, lysozyme and ribonuclease A utilising ketenes, which could not be achieved using NHS-esters.[19]

Thiol-reactive reagents. Highly selective methods for the modification of the thiol group of cysteine are also well established in chemical biology. As a consequence of the relatively low abundance of cysteine (< 2%) and the possible involvement of the thiol groups in disulphide bond formation, targeting cysteine is often used for single-site modification.[14]

The most commonly used technique to modify the free thiol group of cysteins relies on the Michael addition using α,β-unsaturated carbonyl compounds (*e.g.* maleimides) (Fig. 1.7). Formation of a thioether linkage between thiols and maleimides is well known to be highly specific in the pH-range between 6.5 and 7.5 (*e.g.* at pH 7 the reaction of maleimides with thiols proceeds 1,000 times faster compared to the reaction with amines). At higher

pH, however, hydrolysis of the maleimide group might occur leading to the formation of an open-chain maleamic form being nonreactive towards thiols.[17] Other well-established methods for the modification of cysteine residues are based on disulphide exchange reactions or alkylation reactions with alkyl halides (*e.g.* iodoacetamides).

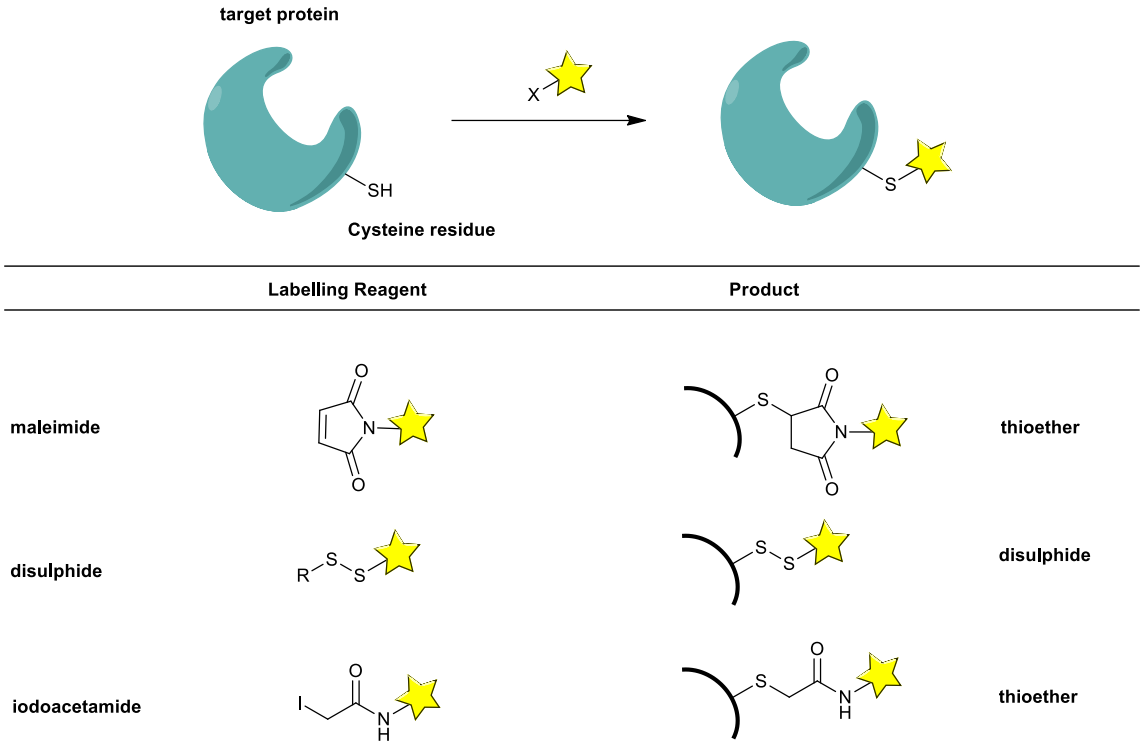


Fig. 1.7 Classic bioconjugation reactions for the covalent modification of cysteine residues. The covalent modification of cysteine residues can be achieved *via* disulphide exchange, alkylation or Michael addition leading to the formation of a disulphide or thioether linkage, respectively.

The relatively low natural abundance of cysteine in proteins, which are mostly tied up in disulphide bonding, can be circumvented by introducing an additional cysteine to the target protein. However, their incorporation by single site mutagenesis may lead to dimerisation of the protein or disulphide scrambling. Hence, an alternative approach based on the reduction of native, solvent accessible disulphide bonds, being present in most proteins, was developed. The reduction of these native disulphide bonds, though, may lead to the unfolding of the protein, aggregation of proteins or disulphide scrambling.[20] Consequently, reagents were developed to rebridge the reduced cysteine residues. By mimicking the role of a native disulphide bond, the structure and function of a protein is retained. For this purpose, specifically designed dibromo maleimides,[21] dithio maleimides[22] and *α,β*-unsaturated *β'*-monosulphone reagents[23] were developed, which are able to rebridge *in situ* reduced cysteine residues of peptides and proteins (Fig. 1.8).

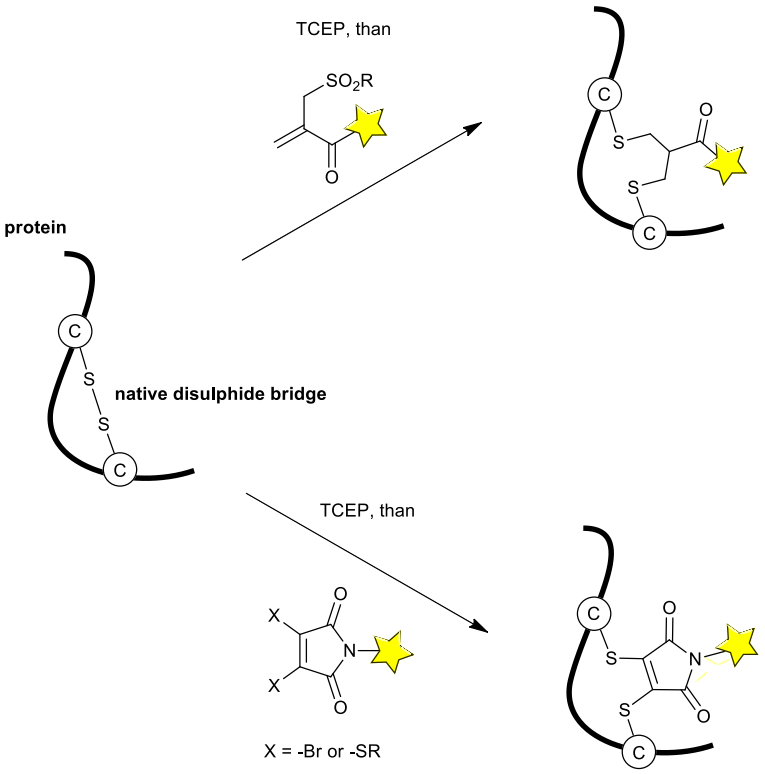


Fig. 1.8 Disulphide bridging of *in situ* reduced native disulphide bonds. Solvent accessible native disulphide bonds are first reduced *in situ* with tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and immediately treated with a suitable rebridging agent (*e.g.* dibromo- or dithio maleimides, *α,β*-unsaturated *β'*-monosulphone reagents) to maintain the structure and function of the target protein.

Photoreactive groups for the covalent modification of biomolecules

The application of photoreactive cross-linkers is a valuable tool in chemical biology to study even transient interactions between various biomolecules. Upon exposure to UV-light, highly reactive intermediates, predominantly carbenes from diazirines, radicals from benzophenones and nitrenes from aryl azides, are formed being capable of reacting with a wide range of functional groups in all kinds of biomolecules. The ideal photoreactive agent should be: (i) sufficiently stable under lab-light conditions; (ii) highly reactive upon irradiation; (iii) capable of reacting at wavelengths which cause no photolytic damage of the biological sample; and (iv) leading only to the formation of stable and unique products, enabling the isolation, purification and subsequent mass spectrometric analysis of the labelled proteins.[24] For a comprehensive mass spectrometric analysis of the obtained products, however, a broad knowledge of the photochemical properties of these photoreactive agents is of high demand. This will be the issue of the following paragraphs.

Aryl azides. Probably as consequence of their high chemical stability in the dark and fast synthetic accessibility, the most frequently used photoreactive agents are aryl azides. However, the obtained cross-linking yields, in general, are less than 30 %. Moreover, aryl azides are known for their rather confusing photochemical properties, which depend strongly on the nature of the substituents on the aromatic ring.[25] Photochemical studies performed by the groups of Schuster and Platz elucidated the mechanism of this photochemical reaction from excitation of the aryl azide to the covalent bond forming event between the reactive species and the target protein (Fig. 1.9).

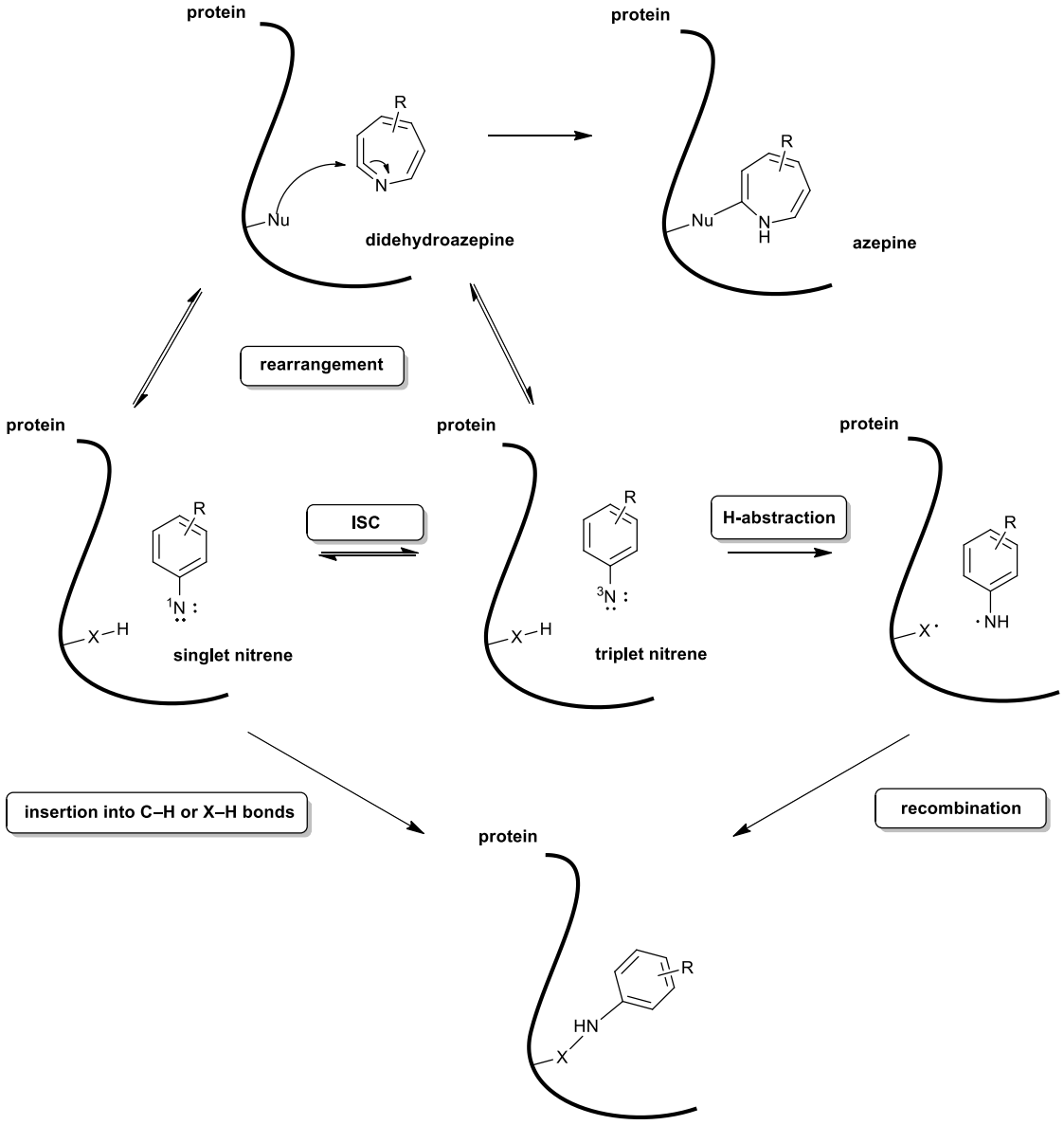


Fig. 1.9 Proposed mechanism of the addition of aryl nitrenes to biological targets. Singlet nitrenes and triplet nitrenes, respectively, are formed upon UV irradiation. These activated species are able to covalently modify the binding region *via* insertion into various C−H or X−H bonds (singlet nitrene) or radical chemistry (triplet nitrene). Moreover, both species are able to rearrange to didehydroazepines which react with a wide range of nucleophiles in the binding site.

Upon irradiation, the aryl azide is promoted to a singlet excited state which either is able to subsequently eliminate molecular nitrogen affording a singlet nitrene or undergoes intersystem crossing (ISC) to a triplet azide being capable of eliminating molecular nitrogen affording a triplet nitrene. The aforementioned singlet nitrene is capable of inserting into carbon–hydrogen bonds and heteroatom–hydrogen bonds, respectively, as well as to rearrange to the corresponding didehydroazepines which are able to react with a variety of nucleophiles. On the other hand, the triplet nitrene, which can be formed additionally by intersystem crossing from a singlet nitrene, is able to covalently modify the binding site *via* radical chemistry.[26]

As outlined previously, the overall cross-linking yields while utilising aryl azides are rather low, which might be a consequence of: (i) the instability of the azepine linkage during subsequent tryptic digestion; (ii) short excitation wavelength (< 280 nm) leading to photolytic damages of the biological sample; and (iii) the azido moiety which is prone to reduction under physiological conditions, consequently reducing the photoreactivity of the chemical probe.[24c] In general, irradiation of aryl azides predominantly results in the formation of didehydroazepines and triplet nitrene reactive species, making predictions of the course of the labelling event more or less impossible. These limitations, however, can be overcome by changing the substituents on the aromatic ring. For example, the application of perfluorinated aryl azide leads to the exclusive formation of a singlet nitrene which exhibits a reduced tendency to rearrange to didehydroazepine. Consequently, the overall cross-linking yields and the predictability of the course of the reaction are improved, hence simplifying mass spectrometric analysis. The application of these perfluorinated aryl azides additionally offers the possibility of photolytic activation at wavelengths around 350 nm which prevents any photolytic damage of the biological sample.[27]

Aryl diazirines. The potential application of aryl diazirines as photoactivatable agents for the covalent modification of proteins was first suggested by Knowles and Smith.[28] This class of compounds has proven to be remarkable stable to a variety of chemical conditions. Furthermore, photolysis can be achieved at wavelengths around 360 nm causing no photolytic damage to the biological sample and leading to the formation of a highly reactive carbene. However, photolysis might lead to the formation of diazo-isomers which are highly alkylating agents presumably responsible for undesired dark reactions. This observed photolytic behaviour makes the prediction of the outcome of the

photochemical reaction more difficult. This problem can be solved by utilising 3-trifluoromethyl-3-aryl diazirines in the photolabelling event. As a consequence of the strong electron-withdrawing effect of the trifluoromethyl group, the diazo-isomer is highly stabilised and can be considered as nonreactive under standard labelling conditions.[24a,29]

Upon irradiation, aryl diazirines are capable of either undergoing fragmentation to the corresponding carbene or rearrange to a linear diazo-isomer, which can act as a carbene precursor as well (Fig. 1.10). The formed singlet carbene is able to insert into a variety of carbon–hydrogen and heteroatom–hydrogen bonds (*e.g.* O–H, N–H), respectively.[30]

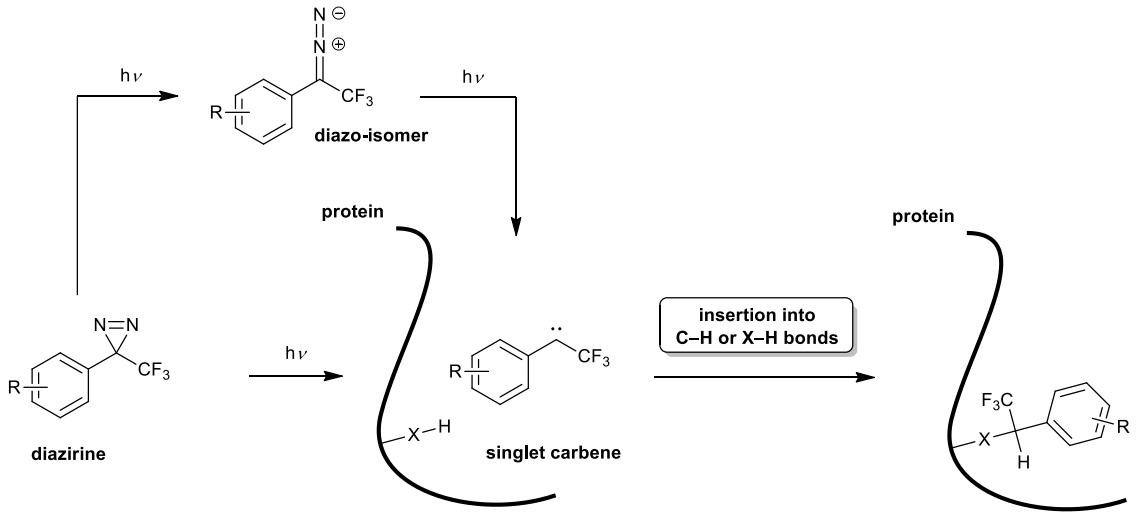


Fig. 1.10 Photolysis of 3-trifluoromethyl-3-aryl diazirines and addition of the formed reactive intermediates to biological targets. Upon photolysis, aryl diazirines either undergo fragmentation to the corresponding singlet carbene or rearrange to the diazo isomer which might be responsible for undesired dark reactions. The formed diazo isomer can act as carbene precursor as well. Covalent modification of the binding region of the protein proceeds *via* an insertion mechanism.

Benzophenones. Upon irradiation of benzophenone derivatives with wavelengths > 300 nm, diradicals are formed as the reactive species which are capable of abstracting a hydrogen atom rather unspecifically from an amino acid present in the binding region. A variety of unwanted radical reactions, however, might reduce the cross-linking yields, *e.g.* intramolecular hydrogen abstraction or the formation of long-lived charged intermediates *via* single electron transfer reactions. On the contrary to aryl azides and diazirines, benzophenones exhibit a variety of chemical and biochemical advantages making them highly suitable for the application in living systems: (i) enhanced chemical stability; (ii) high stability under lab-light conditions; (iii) excitation at wavelengths

> 350 nm avoiding protein-damaging conditions; and (iv) no observed reactivity towards solvent molecules (*e.g.* water).[24c,d]

The mechanism of benzophenone photochemistry is yet well understood (Fig. 1.11). In the initial step, one electron from a nonbonding *n*-orbital on oxygen is promoted to an antibonding π^*-orbital of the carbonyl group upon irradiation resulting in the formation of a triplet diradical. Consequently, the electron-deficient *n*-orbital of oxygen becomes highly electrophilic. To complete the half-filled *n*-orbital, hydrogen abstraction occurs upon interaction with a neighbouring weak C–H σ-bond. Finally, a new C–C bond is generated as a consequence of the rapid recombination of the formed ketyl- and alkyl radicals. Moreover, with no abstractable hydrogen atoms present, the excited triplet state of benzophenone is capable of readily relax in the ground state. In contrast to other photoreactive groups, which are activated in a photo-dissociative mode (*i.e.* the activation is irreversible), benzphenone is able to relax electronically, *i.e.* many excitation-relaxation cycles occur until a suitable hydrogen donor is found in the binding site, which enhances the possibility of the covalent attachment of the photoreactive probe. [24d]

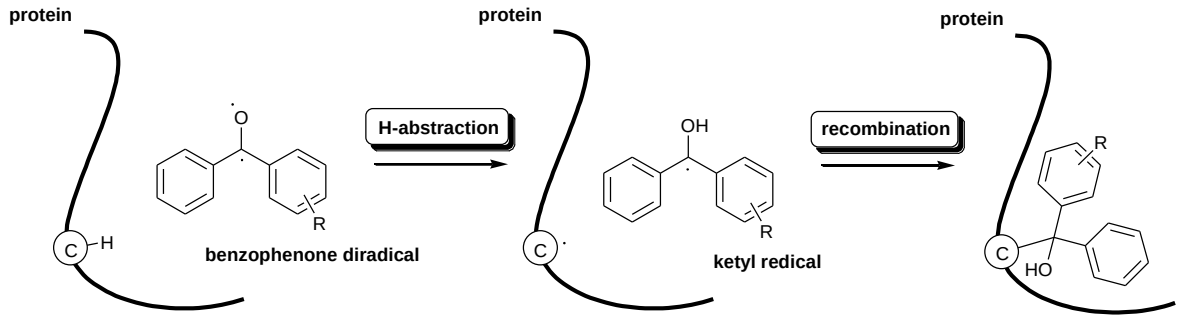


Fig. 1.11 Addition of benzophenone derived diradicals to biological targets. H-abstraction occurs as a consequence of the interaction of the benzophenone diradical, formed upon UV-irradiation, with a neighbouring weak C–H σ-bond resulting in the formation of an alkyl- and ketyl radical. After the subsequent combination of both radicals, a new C–C bond is generated.

Modifying biomolecules using bioorthogonal chemistry

In a bioorthogonal reaction, two compounds present in a complex biological sample react with each other in an ultimate chemoselective fashion with no detectable cross-reactivity towards other functional groups present. A true bioorthogonal reaction should meet the following criteria: (i) high reaction rates under physiological conditions; (ii) inert to other functional entities present in living systems; (iii) quantitative yield of the coupling reaction; (iv) no need of additional promoter species; and (v) no formation of toxic byproducts.[13,14]

The most prominent bioorthogonal reactions are based on the application of azides which have significant advantages over other functional groups, such as aldehydes and ketones. For example, azides are totally absent in biological systems and exhibit an orthogonal reactivity to the majority of biological functional groups. Furthermore, the azide group is small resulting in a minimal perturbation of the modified substrates.[14]

As a consequence of being completely absent in any biomolecule, methods to selectively incorporate azides into biomolecules of interest were developed.[31] However, the incorporation of azido-modified amino acids into proteins by single site or multisite mutation strategies is quite laborious. An alternative strategy was consequently developed. The group of van Hest, *e.g.*, was able to incorporate an azide-moiety into proteins residue specifically at free lysine residues or the *N*-terminus *via* an aqueous diazo transfer reaction. Moreover, changing the pH of the reaction mixture resulted in the incorporation of a single azide-moiety site specifically at the most reactive amine (presumably at the *N*-terminal amine).[32]

With the progress of incorporating small bioorthogonal handles into biomolecules, several reactions and suitable reagents which are capable of reacting with azides under physiological conditions have been developed over the last decade. The most commonly used reactions are: (i) the Staudinger ligation, utilising substituted phosphine reagents; (ii) the copper-catalysed [3+2] azide-alkyne cycloaddition (CuAAC); and (iii) the strain-promoted azide-alkyne cycloaddition (SPAAC) using highly constrained cyclooctyne reagents (Fig. 1.12); which will be discussed in the following paragraphs.[13,14,33]

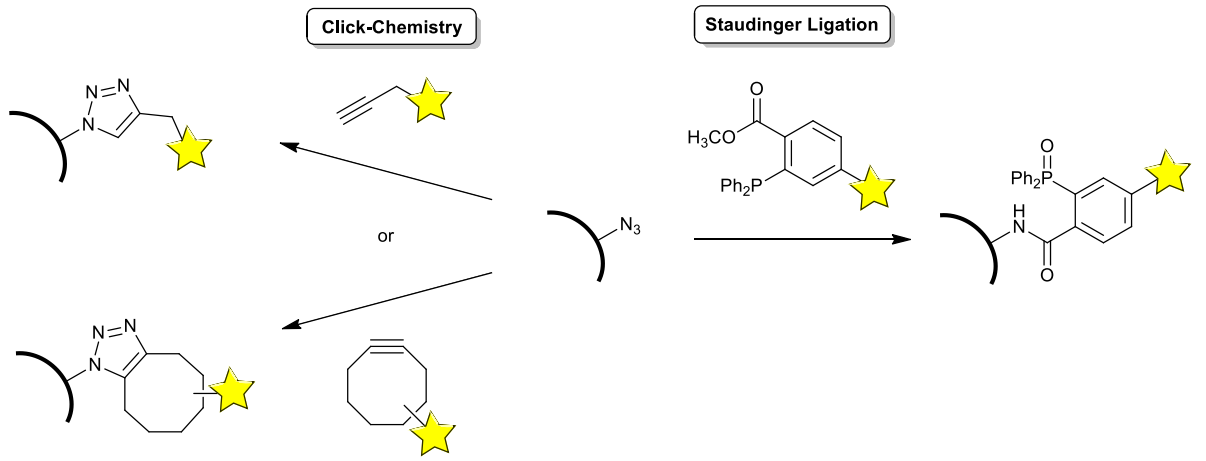


Fig. 1.12 Bioorthogonal reactions for the covalent modification of biomolecules. An azide moiety, previously incorporated into the target molecule, is capable of reacting either with substituted triaryl phosphines (Staudinger ligation) or with a suitable alkyne species in a [3+2] azide-alkyne cycloaddition (click-reaction) to form a covalent linkage between the target molecule and a reporter (*e.g.* fluorescent-tag).

Staudinger ligation. The reaction of azides and triphenylphosphines, whereby aza-ylide intermediates are formed under very mild conditions, was first reported by Hermann Staudinger. These intermediates can be trapped by a variety of different electrophiles, such as aldehydes and ketones, leading to the formation of imines (aza-Wittig reaction) or afford upon hydrolysis the corresponding phosphine oxide and an amine (Fig. 1.13A).[34] The latter observation was the starting point for the development of a bioorthogonal ligation method for the covalent modification of biomolecules equipped with an azide moiety. For this purpose, Bertozzi and co-workers developed suitable phosphine reagents able to intramolecular trap the formed aza-ylide intermediate creating a covalent linkage between the biomolecule of interest and the phosphine scaffold (Fig. 1.13B).[35]

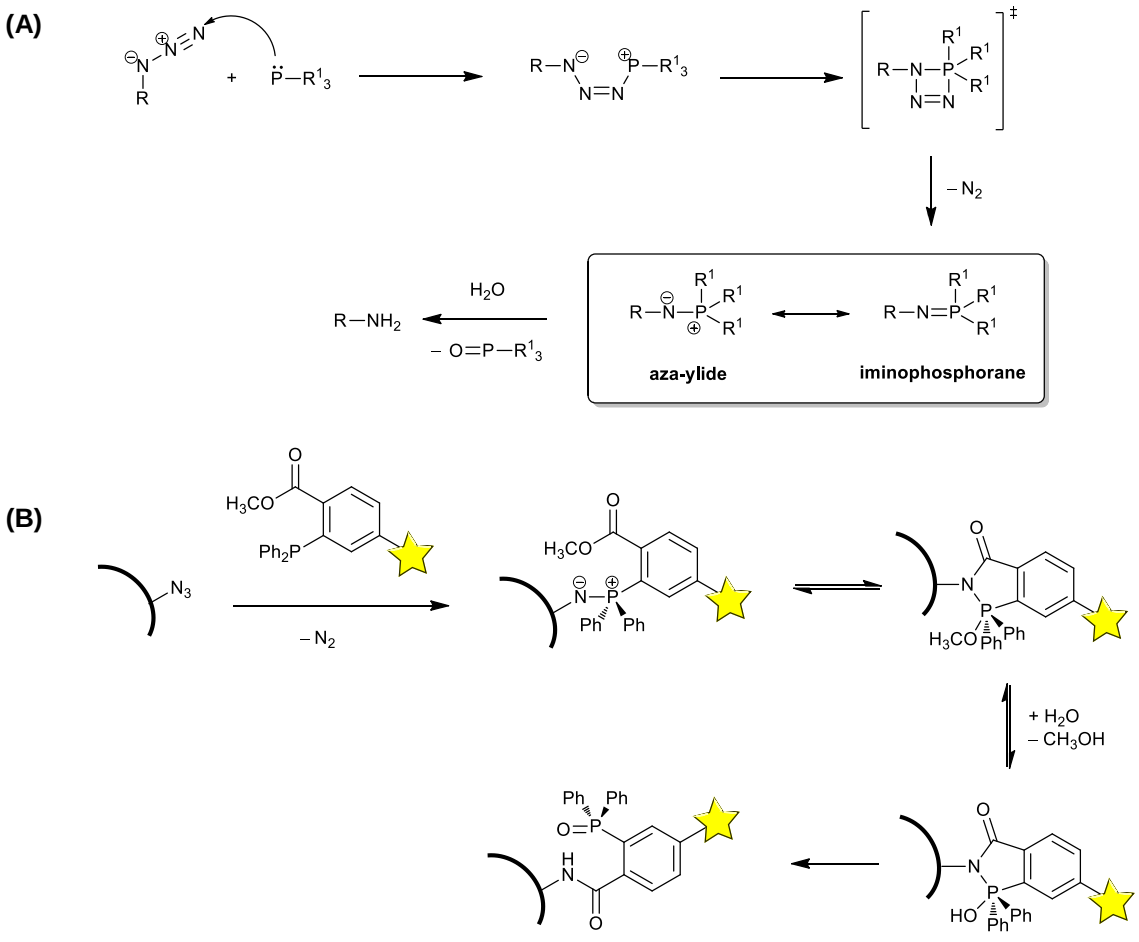


Fig. 1.13 Mechanism of the classical Staudinger reaction and the Staudinger ligation. (A) The mechanism of the classical Staudinger reaction involves nucleophilic attack of the phosphorous atom onto the nitrogen of the azide affording a phosphazide intermediate, followed by intramolecular cyclisation and subsequent elimination of molecular nitrogen. Hydrolysis of the formed aza-ylide results in the formation of a primary amine and phosphine oxide. **(B)** In the Staudinger ligation, on the other hand, phosphine reagents equipped with an intramolecular electrophilic trap are used instead of triphenylphosphine. The formed highly nucleophilic aza-ylide is trapped with an electrophilic ester leading to the formation of a covalent amide bond *via* a putative pentacoordinated phosphine intermediate. The cyclised intermediate rapidly hydrolyses affording stable amide-linked products.

The reaction described by Bertozzi is applicable under physiological conditions and exhibits no apparent toxic effect. Moreover, the used phosphine reagent does not react with any other functional group present in a biological sample in a substantial amount, hence the Staudinger ligation can be considered as being truly bioorthogonal.

The main disadvantage of the Staudinger ligation, however, is the tendency of the phosphine reagent to undergo oxidation by air or enzymes leaving the chemical probe unattached to the target molecule. Hydrolysis of the aza-ylide intermediate to phosphine oxide and the amine might diminish the overall labelling yield as well. In addition, the Staudinger ligation exhibits relatively slow reaction kinetics, hence limiting its applicability to visualise fast biological processes on a minute time-scale. Due to these limitations, bioorthogonal reagents enabling a much faster reaction with azides and additionally being more stable under physiological conditions regarding air oxidation and hydrolysis are of particular interest to the chemical biology community.[14,36] Nevertheless, the Staudinger ligation has found widespread applications in the field of chemical biology, *e.g.* for the modification of glycans on living cells,[35] site specific labelling of proteins,[37] lipid labelling[38] and DNA labelling,[39] among others.

Copper-assisted azide alkyne cycloaddition reaction. In 2001, Sharpless introduced the concept of *click-chemistry* which does not represent a single reaction, but rather aims at mimicking nature. Thus, reactions which can be considered as click-reactions should meet the following criteria: (i) very high chemical yields; (ii) high stereoselectivity; (iii) simple to perform; (iv) easy removable and inoffensive by-products; (v) readily available starting materials; and (vi) high atom economy. These required characteristics can be achieved, in general, as click-reactions exhibit a high thermodynamic driving force (> 20 kcal/mol). Reactions, such as addition reactions onto carbon-carbon multiple bonds (*e.g.* epoxidation, dihydroxylation), "non-aldol" type carbonyl reactions (*e.g.* formation of oxime ethers and hydrazones) and nucleophilic ring-opening reactions of strained heterocycles (*e.g.* epoxides, aziridines), among others, meet most of the aforementioned characteristics, hence they can be considered as click-reactions.[40] Another intriguing example of a click-reaction is the well-known azide-alkyne 1,3-dipolar cycloaddition, which was introduced by Huisgen in the early 1960s (Fig. 1.14A).[41]

In contrast to the previously described Staudinger ligation, in which the azide serves as a soft electrophile in the reaction with soft nucleophiles, azides can act additionally as 1,3-dipoles which can undergo cycloaddition reactions with dipolarophiles, *e.g.* alkynes,

leading to the formation of triazoles. The formation of triazoles from azides and alkynes is thermodynamically highly favoured (ca. –61 kcal/mol),[42] however, the reaction requires elevated temperatures or pressures. Moreover, in most cases a mixture of the 1,4- and 1,5-regioisomers is obtained, consequently, this reaction was ignored for years despite its great potential. Only the observation that catalytic amounts of copper(I) salts can enhance the reaction rate of the azide-alkyne cycloaddition reaction by $\sim 10^6$-fold affording exclusively the 1,4-regioisomer finally brought this reaction into focus again.[43] Since this pioneering work by Sharpless and Meldal, the copper-catalysed azide-alkyne cycloaddition (CuAAC) has become the quintessential representative of the click-chemistry concept as proposed by Sharpless and is often referred simply as "click-reaction". The elusive mechanism of the CuAAC was revealed recently by Fokin and collaborators by *in situ* reaction calorimetry and metal isotope crossover (Fig. 1.14B).[44]

Upon the initial formation of the σ-bound copper acetylide and subsequent recruitment of a second π-bound copper atom, the catalytically active complex is formed. Afterwards the organic azide coordinates reversibly to the π-bound copper complex. Subsequent nucleophilic attack by the β-carbon of the acetylide onto the nitrogen of the azide forms the first covalent C–N bond. Formation of the second covalent C–N bond as a consequence of the nuclophilic attack by the nitrogen of the azide onto the α-carbon results in ring closure affording a copper triazolide species. Finally, the catalyst is released upon proteolysis leading to the formation of 1,4-substituted 1,2,3-triazoles. The regioselectivity of the azide alkyne cycloaddition reaction can also be inverted affording exclusively the formation of the 1,5-regioisomer by the addition of a ruthenium catalyst (Fig. 1.14A).[45] However, the ruthenium catalysed azide-alkyne cycloaddition (RuAAC) has found only infrequent use.

In addition to its very high reaction rates, CuAAC proceeds under physiological conditions in the presence of a myriad of different functionalities present in a biological sample as a consequence of its supreme chemoselectivity. Therefore, CuAAC has found widespread applications not just only in organic synthesis, combinatorial chemistry, polymer and material science, but also in chemical biology as a highly versatile bioorthogonal ligation method.[46]

Compared to the Staudinger ligation the apparent advantage of CuAAC for the labelling of biomolecules is the increased reaction rate (25 times faster than the reaction of azides with phosphines in cell lysats),[13a] which allows the monitoring of rapid biological processes. Due to the strong dependency on the presence of a copper catalyst, the *in vivo*

application of the CuAAC methodology, however, is rather limited as a consequence of the toxicity of the copper(I) species.[47] This apparent toxicity is based on the formation of reactive oxygen species which can damage various biomolecules, such as proteins, lipids, DNA, *etc*. Consequently, methods relying on the application of highly strained cyclo-octynes rather than metal catalysis were developed by the group of Bertozzi. This reaction is mostly referred to as strain-promoted azide alkyne cycloaddition (SPAAC).

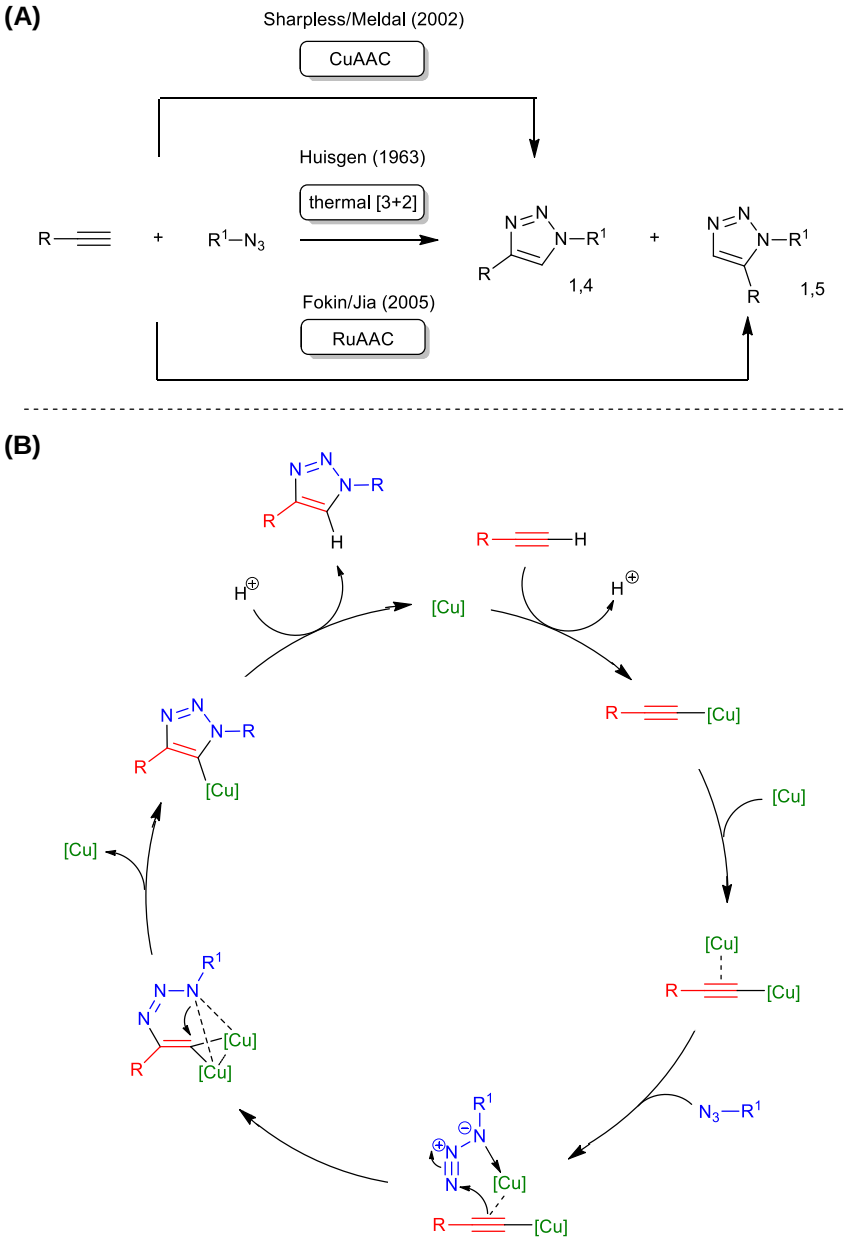


Fig. 1.14 Synthesis of triazoles starting from terminal alkynes and azides in a 1,3-dipolar [3+2] cycloaddition. (A) Substituted 1,2,3-triazoles can be obtained either *via* a thermal [3+2] cycloaddition affording a mixture of the 1,4- and 1,5-regioisomer or *via* a metal catalysed version of the [3+2] cycloaddition affording regioselectively the 1,4-regioisomer (CuAAC) or 1,5-regioisomer (RuAAC). **(B)** The proposed mechanism of the CuAAC involves: (i) the formation of a copper acetylide; (ii) recruitment of a second π-bound copper atom; (iii) coordination of the azide to the π-bound copper complex; (iv) nucleophilic attack by the θ-carbon of the acetylide onto the organic azide; and (v) cyclisation upon nucleophilic attack onto the α-carbon of the acetylide.

Strain-promoted azide alkyne cycloaddition reaction. The first observation regarding the ring strain-promoted cycloaddition reaction of organic azides by Alder and Stein dates back to early 1930s. They found that organic azides react with dicyclopentadiene considerably faster when compared to cyclopentadiene.[48] Research regarding the cycloaddition reaction between organic azides and strained alkenes and alkynes continued the following decades. In 1953, Blomquist and Liu reported the first synthesis of cyclooctyne, the smallest stable cycloalkyne, by oxidative decomposition of cyclooctane-1,2-dione dihydrazone and recognised the potential of highly strained alkynes in undergoing cycloaddition reaction by adding phenylazide.[49] The product of this explosive reaction was later on identified by Krebs and Wittig as cyclooctatriazole,[50] which was already prepared by Alder and Stein by heating a mixture consisting of *N*-cyclooctylidenebenzenamine and phenylazide.[51] A reason for this enhanced reactivity of cyclooctynes in a [3+2] cycloaddition derives from the severe distortion from the ideal 180° bond angle for sp-hybridised carbon. Upon the reaction with a suitable 1,3-dipole, most of the ring-strain is released resulting in a drastic rate acceleration (Fig. 1.15). In contrast to CuAAC, however, the [3+2] cycloaddition between strained cyclooctynes and organic azides results in the formation of a mixture of the 1,4- and 1,5-regioisomers. The lack in regioselectivity may hamper future applications of SPAAC in the fields of drug design and the design of peptidomimetics, among others.

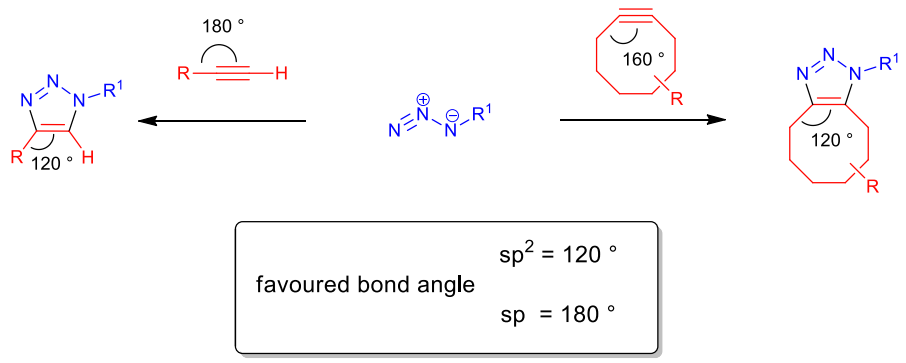


Fig. 1.15 Reaction of organic azides with either terminal alkynes or cyclooctynes – A comparison of bond angles.

Based on these initial findings, the lab of Bertozzi designed a cyclooctyne derivative equipped with a biotin handle and evaluated the potential of SPAAC as bioorthogonal ligation method using azide modified cell-surface glycans. Besides effective labelling, no apparent toxic effect on living cells could be detected.[52] However, the Staudinger ligation as well as CuAAC proved to be more efficient in the labelling reaction when compared to

the application of the strained cyclooctyne probe as a consequence of the comparable low reaction rates of SPAAC.

To improve the kinetic characteristics of the SPAAC methodology for a broader applicability in various *in vivo* and *in vitro* labelling experiments, various groups aimed at increasing the ring strain of the cyclooctyne either *via* the addition of a cyclopropyl ring opposite to the alkyne (*e.g.* bicyclo[6.1.0]nonyne, abbreviated BCN)[53] or the incorporation of annulated aromatic ring systems (*e.g.* dibenzocyclooctynes, DIBO).[54] Moreover, the introduction of σ-electron withdrawing groups in near proximity to the alkyne moiety (*e.g.* difluorinated cyclooctynes, DIFO)[55] proved to be highly successful in increasing the reaction rate of the cycloaddition reaction as a consequence of reducing the energy gap between the alkyne`s lowest unoccupied molecular orbital (LUMO) and the highest occupied molecular orbital (HOMO) of the azide. The incorporation of π-electron withdrawing groups (*e.g.* carbonyls), however, would result in the formation of a Michael acceptor system and enhances the probability of competing side reactions with other nucleophiles compromising the bioorthogonality of the reagent.[13b] One of the most reactive cycloocytne derivatives known so far, biaryl-azacyclooctynone (BARAC), exhibits reaction rates comparable to a moderate CuAAC system (~ 1 M^{-1} s^{-1}) combining ring strain as well as electron with-drawing groups (Fig. 1.16).[56] Further optimisations of the kinetic parameters of SPAAC, though, are of high demand for the chemical biology community, *e.g.* to facilitate the modification of low-abundant proteins and the visualisation of very rapid biological processes.

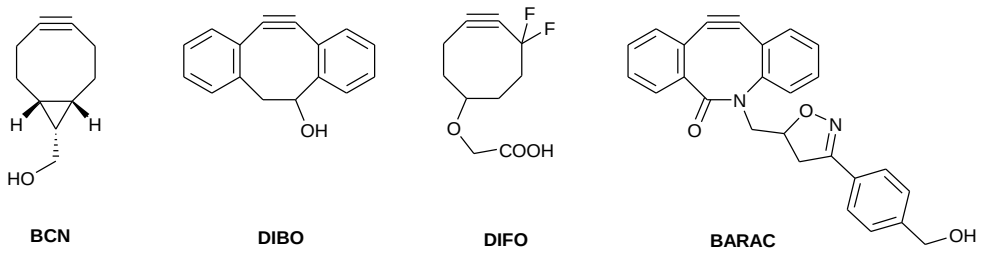


Fig. 1.16 Strained cyclooctynes for metal-free triazole formation (SPAAC).

Since the initial report of the cyclooctyne-azide cycloaddition reaction by Bertozzi and co-workers, the SPAAC methodology has found a broad range of applications in the field of chemical biology, such as covalent protein and cell-surface modification, lipid labelling, and chemical proteomics, among others, which has been the topic of several excellent reviews.[13,33,57]

Besides SPAAC, other metal-free click-reactions relying on the cycloaddition of either strained cyclooctynes with nitrones (SPANC)[58] and nitrile oxides (SPANOC),[59] or alkenes with azides,[60] nitrile oxides,[61] tetrazoles[62] and tetrazines[63] have gained considerable attention for bioorthogonal ligation purposes. At the same time, the ability of incorporating cyclooctynes and other small molecular bioorthogonal handles into biomolecules of interest by genetic approaches has made tremendous progress. These combined efforts will most probably, if not certainly, pave the way for the development of homogenous protein conjugates which might be of high value, *e.g.* for the design of novel biopharmaceuticals, among other putative applications.

1.3 Chemical cross-linking strategies to study protein-small molecule and protein-protein interactions

As outlined in chapter 1.1, the concept of applying small-molecular probes as tools to study protein-protein as well as protein-small molecule interactions has found widespread applications in the field of chemical biology. Especially activity-based protein profiling (ABPP) has evolved as a valuable tool as a consequence of its ability to label and enrich a variety of functionally different enzyme classes. In general, specifically designed activity-based probes (ABP) can be viewed either as chemical antibodies, which report on protein expression, or at the same time as probes, which enable the detection of active enzymes in a natural environment. [4k] Over the last years, the targets of ABPs have changed from relatively simple enzymes, such as serine and cysteine proteases, to more difficult targets, *e.g.* low abundant proteins, enzymes without nucleophilic amino acid in the active side and enzymes exhibiting very high substrate specificity.[4k] Therefore, one should always be aware of the substrate specificity and the catalytic mechanism of the target enzyme to be able to design a highly specific ABP. Hence, the development of an ABP starts, in most cases, with the design of suitable irreversible or reversible inhibitors. Afterwards these inhibitors are further modified chemically to meet the demands of a fully functional ABP.

Besides ABPP, chemical cross-linking in combination with mass spectrometry has emerged as a valuable tool in chemical biology to elucidate the three-dimensional structure of a protein of interest or to identify protein-protein interactions in a cellular environment. Here, specifically designed cross-linking reagents are required, which are equipped with two instead of one reactive group, enabling the formation of a covalent

bond between functional groups of amino acids within a protein (protein conformation) or between different interaction partners (protein-protein interactions). The basic considerations when designing either an ABP or a chemical cross-linking reagent will be discussed in the following paragraphs. Moreover, the versatility of the presented approaches to address completely different issues in the field of chemical biology will be illustrated with recent examples from the literature.

Design and application of activity-based probes

The incorporation of small-molecular tight binding inhibitors into multifunctional chemical probes, which is a prerequisite to generate highly specific ABPs, is often challenging because of potentially sensitive functional groups within the warhead. In addition, physiochemical properties of the ABP, such as solubility in an aqueous environment, stability under physiological conditions and cell permeability, have to be considered when applying the final probe *in vivo*. Therefore, the synthesis of a novel ABP is often accompanied by trial and error as a consequence of: (i) a loss in activity after assembling the whole probe; (ii) a limited cellular uptake and distribution of the probe; (iii) steric repulsions between the warhead and the reporter resulting in a less effective binding; and (iv) unspecific binding events with other biomolecules in the sample; among others. To access libraries of putative APBs to facilitate probe design, modular synthetic approaches (*e.g.* solid phase peptide synthesis, multicomponent reactions) have been utilised nowadays as a consequence of the modular nature of an ABP (warhead, linker, reporter-tag) as outlined in chapter 1.1.

In general, ABPs can be generated directly from already well-established irreversible or reversible binding inhibitors, which contribute to the target specificity of the whole probe (selectivity group). Despite known inhibitors, enzyme cofactor analogues (*e.g. S*-adenosyl-*L*-homocysteine) and natural products (*e.g.* β-lactone and β-lactame antibiotics) have been incorporated in such multifunctional molecular probes as well. Due to this tuneable target specificity, the application of ABPs enables to target a wide repertoire of active enzymes, ideally one highly specific probe for each active enzyme present in a biological sample comparable to the expansive application of specific antibodies. Besides the prefractionation of enzymes according to their function, ABPP has been applied for *in vivo* imaging, the identification of drug off-targets (*Toxicoproteomics*), inhibitor screening and the assignment of enzyme activities to proteins with previously unknown function, *etc.*

Localisation and quantification of enzyme activity in natural environments. The application of ABPs as a non-invasive optical imaging technique enables to sensitively and continuously monitor enzyme activity *in vivo* using fluorescence microscopy. For this purpose, fluorogenic probes with longer wavelengths than the biological auto-fluorescence background of approximately 480–500 nm are commonly applied. For the rapid real-time monitoring of enzyme activity in living organisms, ABPs can be further equipped with an additional fluorescence quencher moiety. Upon successful binding of the ABP to the target enzyme, the quencher moiety is cleaved off and the appearance of a fluorescence signal can be detected. In contrast to these quenched ABPs, the corresponding non-quenched APBs, however, which are not covalently attached to the target enzyme, must clear from the system before a specific signal can be detected *in vivo* (Fig. 1.17).[64]

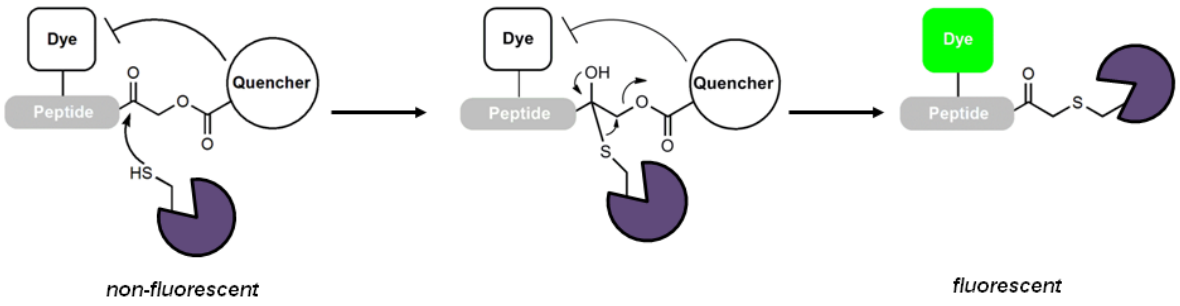


Fig. 1.17 Quenched activity-based probes for the *in vivo* imaging of cysteine proteases. Upon nucleophilic attack of an acyloxymethyl ketone electrophile by a cysteine protease, a transient tetrahedral intermediate is formed. Subsequent loss of the quenching moiety results in the formation of a fluorescently labelled cysteine protease.

These "smart probes" have been used successfully by the Bogyo lab to visualise cathepsin activities in living cells.[65] Here, an ABP containing an acyloxymethyl ketone reactive group, engaging a nucleophilic cysteine residue in the active side of the target protein, and a BODIPY fluorophore was additionally equipped with a quencher moiety (QSY7). Upon nucleophilic displacement of the quencher moiety during protease labelling, the appearance of a fluorescence signal was observed consequently. This phenomenon was proven first by the authors *in vitro* using cathepsin B and L. The applicability *in vivo* was subsequently evaluated using cultured monolayers of the murine fibroblast cell line NIH-3T3. The obtained results were in good agreement with the results obtained when applying immunofluorescent staining, another commonly used imaging technique. As cathepsin B and L are highly expressed in various tumours, a similar quenched cathepsin probe was utilised for the non-invasive imaging of subcutaneously

grafted tumours in mice.[66] Other quenched or non-quenched ABPs have been used for the *in vivo* imaging of caspase activity during apoptosis,[67] the monitoring of legumain activity in various tissues, which is associated with tumourigenesis,[68] and as tool to visualise tumour-associated macrophages,[69] among others.

Identification of drug off-targets. The identification of protein targets of drugs and drug candidates using APBs may contribute to the understanding of a drug`s mode of action. Moreover, insights into drug side effects and the toxicity of the compound can be obtained by identifying so-called off-targets (protein targets which interact with a drug but do not contribute to the desired drug mode of action). This might help to develop more selective drug candidates with reduced toxic side effects.[70] Based on this strategy, Fischer *et al.* were able to assign off-targets of the anti-Parkinson drugs entacapone and tolcapone, which both target the catechol-*O*-methyltransferase (COMT), a common target in Parkinson therapy.[71] Previous studies revealed that tolcapone exhibits a significant higher hepatotoxic effect compared to entacapone, although they share the same pharmacological active motif, as a consequence of a decoupling effect on the mitochondrial membrane potential affecting oxidative phosphorylation.[72] Indeed, the application of a suitable tolcapone and entacapone ABP revealed that only tolcapone additionally binds to mitochondrial membrane proteins associated with oxidative phosphorylation and *β*-oxidation.

Using ABPP, the group of Yao was able to identify off-targets of the approved anti-obessity drug orlistat (tetrahydrolipstatin, THL), which has been shown recently to inhibit also cell proliferation in various cancers cell lines.[73] In their study, an alkyne handle was introduced into THL for maintaining the native biological function and for the subsequent detection and purification of previously unknown cellular targets *via* CuAAC. In total, eight off-targets next to already validated targets could be identified. The authors suggested that the observed antitumor activity of orlistat might originate from the ability of inhibiting some of these newly established off-target proteins.

Furthermore, an intriguing example for the application of ABPs for the identification of drug off-targets has been reported by the Sieber lab.[74] In their study, bacterial cells of *S. aureus* were treated with a specifically designed photo-activatable vancomycin probe. Besides the expected D-Ala-D-Ala motif of nascent peptidoglycan resulting in the inhibition of cell wall biosynthesis, vancomycin was found to additionally bind to the Atl amidase domain. This leads to massive defects in cell morphology and induces

tolerance of *S. aureus* against low concentrations of vancomycin without the need of developing a genetic resistance. Based on these findings, the authors speculated that improved vancomycin inhibitors with a lower affinity to the Atl amidase may increase the antibacterial potential of vancomycin towards *S. aureus* and MRSA.

High-throughput inhibitor screening. In medicinal chemistry, the screening of large compound libraries is an important technique for the identification of novel drug candidates. The application of ABPP may facilitate this screening procedure to identify novel specific enzyme inhibitors. For this purpose, a library of compounds is evaluated regarding its ability to suppress binding of an ABP to a target enzyme (Fig. 1.18). In contrast to common approaches for inhibitor design, the application of ABPP offers the possibility to: (i) perform the screening in native proteomes; (ii) identify inhibitors of enzymes with so far unknown function; and (iii) simultaneously characterise the potency and selectivity of the tested inhibitors against various enzymes present in a native proteome.[4c,e] Up to now, both irreversible binding[75] as well as reversible binding inhibitors[76] have been identified using ABPP.

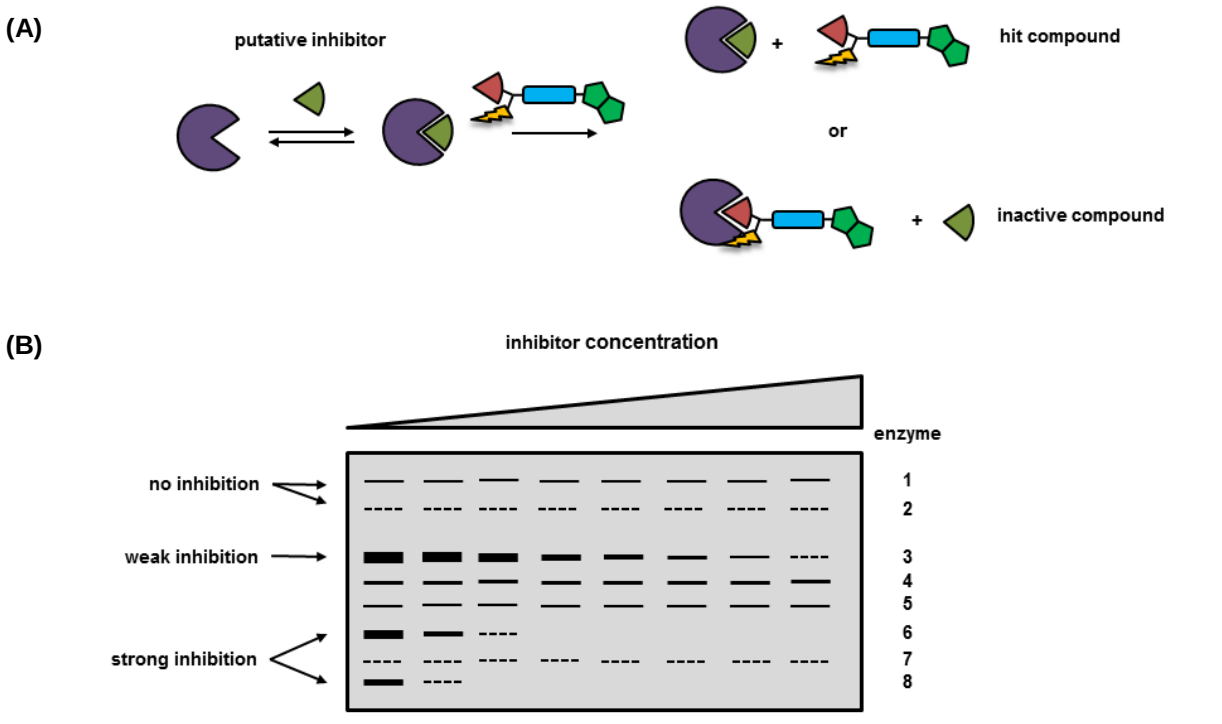


Fig. 1.18 ABPP-based inhibitor screening. (A) An enzyme mixture is treated with a test compound. After a certain time of incubation a broad spectrum ABP is added and a suitable inhibitor is identified according to its potential to suppress binding of the ABP in the active site of the enzyme. **(B)** A concentration-dependent application of putative inhibitors allows the determination of the relative potency against various targets in parallel.

This approach, however, is based on the analysis of probe labelled proteins *via* SDS-PAGE limiting its throughput to compound libraries of modest size (200–300 compounds). Cravatt and co-workers overcame this limitation by developing a fluorescence polarisation technology for ABPP-based high-throughput inhibitor screening.[77] Fluorescence polarisation can be used to study molecular interactions *via* excitation of a fluorophore with plane-polarised light. Consequently, the fluorophore emits light parallel to the plain unless rotation in the excited state occurs. If free in solution, small fluorophores (< 10 kDa) rotate quickly and emit depolarised light, hence resulting in a low fluorescence polarisation signal. When bound to a large molecule (*e.g.* a protein), rotation of the fluorophore is strongly hindered resulting in a high fluorescence polarisation signal.[78] Therefore, the authors suggested that binding of a fluorescent ABP to a target protein would result in an increased fluorescence polarisation signal. Based on this assumption, either reversible or irreversible inhibitors which suppress binding of the ABP to the enzyme should be detectable as a consequence of a significantly reduced fluorescence polarisation signal. With this method in hand, they were able to evaluate thousands of small molecules in parallel regarding their ability to inhibit two cancer related enzymes being poorly characterised at the present time, namely, the serine hydrolyses retinoblastoma-binding protein-9 (RBBP9) and the thioltransferase glutathione *S*-transferase omega 1 (GSTO1). In their study, the authors were able to identify the bioactive alkaloid emetine as a selective inhibitor of RBBP9, among others. Several electrophilic substances, such as omeprazole and rifampicin, were found to inhibit GSTO1.[77]

Functional characterisation of proteins with unknown function. With the fast progress in genome sequencing, scientists are able to predict all proteins expressed by prokaryotic as well as eukaryotic organisms. To understand the biological role of these predicted proteins, they have to be characterised according to their function. The functional annotation is performed, in general, based on sequence homology with already well-characterised proteins. However, this approach may fail as a consequence of an insufficient sequence homology between an uncharacterised enzyme and previously functionally annotated proteins in the protein database (PDB). Therefore, the application of ABPs has been studied to register the complete membership of enzyme superfamilies in proteomes.[4c] One important early example for the characterisation of a so far unknown enzyme function using ABPP was reported by Li and collaborators. They were able to

identify the integral membrane proteins presenilin 1 and 2 (PS-1 and PS-2), which have been linked to early-onset Alzheimer`s disease by genetic and biochemical approaches, as members of the aspartyl protease superfamily utilising a photoreactive, biotinylated *γ*-secretase ABP, although no apparent aspertyl protease motif is present in both enzymes.[79] The ABP used in their experiments contained a hydroxyethylene dipeptide isostere, which served as a transition state analogue, directing the probe to the active site of an aspartyl protease target.

Another notable example is the identification of sialic acid 9-*O*-acetylesterase (SAE) as a putative member of the serine hydrolase superfamily despite no evident sequence homology.[80] This functional annotation by Jessani and colleagues was based on the observation that SAE was effectively labelled with a fluorophosphonate ABP, typically highly specifically targeting serine hydrolases.

The emerging field of ABPP has proven to be a valuable tool in biochemistry, biology and medicinal chemistry. However, many enzyme families cannot be targeted by ABPP as a consequence of a limited accessibility to highly selective irreversible or reversible binding inhibitors. Consequently, future research will be directed most certainly towards the development of novel, highly selective warheads being able to target enzyme families involved in various important signalling pathways and gene regulation (*e.g.* kinases, transferases, hydrolases, *etc.*).

Design and application of chemical cross-linking reagents to study protein-protein interactions

Besides studying the physiological function of proteins using ABPP, chemical approaches to elucidate the interaction pattern of proteins in cellular environments as well as native protein conformations have found widespread applications in the field of chemical biology. In this regard, chemical cross-linking of functional groups of two spatial adjacent amino acids within a protein or a protein complex proved to be highly successful. Subsequent mass spectrometric analysis identifies the location of the formed cross-links which sheds light on the distance geometries of a protein or a protein complex structure. In general, for the structural analysis of proteins or the identification of stable interactions within a protein complex, high-resolution methods, such as X-ray crystallography or NMR spectroscopy, have been applied frequently. However, these methods require high amounts of purified (crystallised) proteins and elevated measuring time. The tryptic

digestion of cross-linked proteins and the subsequent identification of the corresponding cross-linked peptides by LC/MS/MS methods, on the other hand, exhibits several advantages compared to those high-resolution methods: (i) fast analysis; (ii) only femtomole amounts of protein generally required; (iii) proteins can be studied in their native environments; (iv) membrane proteins as well as proteins existing as mixtures (post-translational modifications) can be analysed; and (v) even weak, transient interactions can be detected. Moreover, a wide variety of chemical cross-linking reagents are commercially available by now being able to covalently modify certain functional groups of proteins, such as primary amines, thiol groups and carboxylic acids (cf. chapter 1.2), which offer the possibility to bridge a wide range of distances.[15,81] These reagents can be classified according to their two distinct reactive functionalities being responsible for the formation of a covalent bond, which can be either identical (*homobifunctional*) or different (*heterobifunctionl*). Both reactive functionalities are separated by a linker, whose length determines the maximum distance between two cross-linked amino acid residues within a protein or between a protein and its interaction partner which can be bridged by the cross-linker (Fig. 1.19). In addition, even cross-linking reagents which mediate the formation of a covalent bond containing no additional atoms between very close spatial adjacent residues are available (*zero-length cross-linker*).

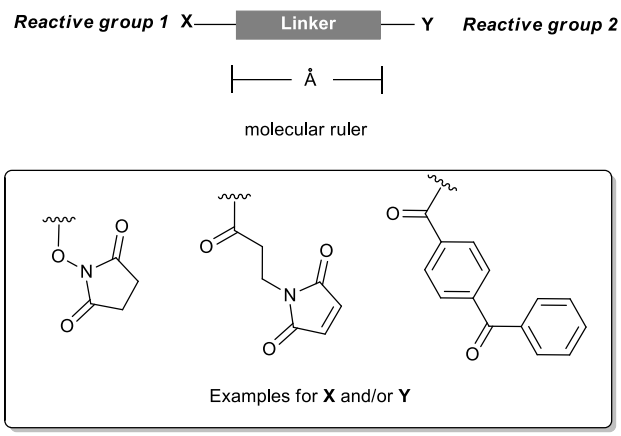


Fig. 1.19 Structure of homo- and heterobifunctional cross-linking reagents, respectively.

When utilising these bifunctional cross-linking reagents, mixtures of products are generally obtained as a consequence of the inaccessibility of a second cross-linkable residue or partial deactivation prior to cross-link formation (*e.g.* by hydrolysis). Overall, three different products of the cross-linking reaction between a protein/protein complex and a bifunctional cross-linking reagent can be obtained after tryptic digestion (Fig. 1.20): a single peptide which is modified by only one cross-linker function (*mono-link*); an intrapeptide link (*loop-link*); and an interpeptide link (*cross-link*). In the case of a mono-link only limited structural information is generated (solvent accessibility), whereas loop-links as well as cross-links provide a much broader knowledge about the spatial proximity of the covalently modified residues either within a single protein or between two closely

associated proteins.[15,17,81] In the following paragraphs, an overview about the scope and limitations of these conventional cross-linking reagents will be given.

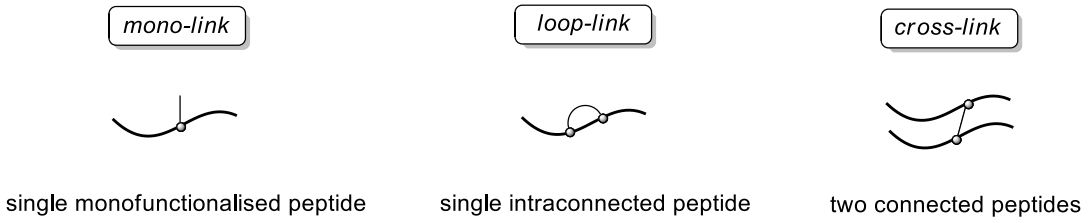


Fig. 1.20 Possible products of cross-linking reactions with bifunctional reagents.

Zero-length cross-linking reagents. As previously stated, zero-length cross-linking reagents mediate the formation of a covalent bond between amino acid residues in near spatial proximity without an intervening linker. The reagents described here, in general, initiate the formation of a covalent bond between: (i) primary amines and carboxylic acids leading to a peptide bond; (ii) primary amines and organic phosphates resulting in the formation of a phosphoramidate linkage; and (iii) primary amines and aldehydes leading to the formation of secondary or tertiary amines after subsequent reduction with, *e.g.* sodium cyanoborohydride.[17]

Carbodiimides are probably the most widely used zero-length cross-linkers. This class of reagents mediates the formation of either an amide linkage between a primary amine and a carboxylic acid or a phosphoramidate linkage between a primary amine and an organic phosphorous species. It has been shown that the application of carbodiimides is well suited to form conjugates between two protein molecules, between a protein and an interacting peptide, between oligonucleotides and proteins, or any combination of these with other small molecules in aqueous or non-aqueous environments.[17,82,83] The most frequently used carbodiimides for bioconjugation purposes are the water-soluble 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide tolylsulphonate (CMC). However, when working with carbodiimides, some undesired side-reactions might occur reducing the overall cross-linking yields. For example, it is well known that carbodiimides are able to form stable complexes with thiol groups. Reactions with phenols, alcohols and other nucleophiles have been reported as well as unwanted polymerisation reactions due to the high abundance of both amines and carboxylates in proteins. When working in an aqueous environment, hydrolysis of the *O*-acylisourea active intermediate is by far the most frequent side-reaction.[17]

Homobifunctional cross-linking reagents. As outlined previously, homobifunctional cross-linking reagents are equipped with two identical reactive groups, which are separated by a carbon chain spacer, thus enabling to cross-link only identical functional groups (*e.g.* amines, thiols). The specific length of the spacer represents the maximum distance between functional groups of proteins which can be bridged by the cross-linker. It should be noted that the average span of the cross-link is shorter than the calculated maximum distance for a given reagent.[84] As a consequence of the ease of linking two functional groups within a protein or between different proteins (or even other small molecules) utilising homobifunctional reagents, the amount of commercially available homobifunctional reagents, which possess almost all lengths or reactivities desired, increased dramatically over the last decades.

Despite the ease of creating protein conjugates, *etc.*, the application of these simple homobifunctional reagents, though, might lead to the formation of a multitude of poorly defined conjugates. In general, upon the initial reaction of the cross-linking reagent with a protein, an active intermediate is formed which can undergo several desired or undesired follow-up reactions: (i) formation of a cross-link with a second protein; (ii) intramolecular reaction with a neighbouring functional group forming a loop-link; (iii) hydrolysis leading to the deactivation of the activated protein resulting in the formation of a mono-link; or (iv) reaction with other cross-linking molecules leading to the formation of a mixture of different-sized oligomers. These shortcomings can be overcome by maintaining a protein concentration in the μM range limiting the formation of unwanted higher oligomers. Moreover, a two-step conjugation protocol was established to reduce the amount of different cross-linked products. In this particular case, a protein is first treated with a homobifunctional cross-linking reagent creating an activated protein. Excess reagents and by-products are removed afterwards and a second protein or another molecule to be conjugated is subsequently added resulting in the formation of the desired cross-link.[17,81]

Homobifunctional cross-linking reagents can be equipped with a variety of different reactive groups targeting specific amino acid residues, *e.g.* lysine, cysteine, thyrosine, histidine, *etc.* (for an overview cf. chapter 1.2). The most frequently applied homobifunctional cross-linking reagents, however, are equipped either with highly amine-reactive (*e.g.* activated NHS-ester) or thiol-reactive functional groups (*e.g.* maleimide). Furthermore, homobifunctional photoreactive cross-linkers equipped with two phenylazide moieties have been reported in the literature. This class of compounds

exhibits a high selectivity towards the reaction with primary amines compared to other functionalities. Further homobifunctional cross-linking reagents used for bioconjugation purposes include homobifunctional aldehydes (*e.g.* formaldehyde, glutaraldehyde), bis-epoxides, bis-hydrazides, bis-diazonium derivatives and bis-alkylhalides.[17]

These cross-linking reagents have been used predominantly for the structural elucidation of proteins, whereby the reagent acts as a molecular ruler to estimate the distances between the cross-linked amino acids, which allows drawing conclusions on both the tertiary and quaternary arrangement of the protein.[85] Other applications of these reagents involve the identification of protein-protein as well as protein-peptide interactions.[86]

Heterobifunctional cross-linking reagents. In contrast to the previously described homobifunctional cross-linking reagents, heterobifunctional cross-linkers incorporate two different reactive groups targeting different functional groups in biomolecules. Up to now, several heterobifunctional reagents have been described and are readily accessible by commercial vendors. In general, these reagents can be classified according to the basic cross-linking concepts which they combine, *i.e.* chemical or photoinduced cross-linking. The most commonly used reagents combine either two different residue specific, chemically activated reactive groups (*e.g.* NHS-ester targeting Lys-residues and maleimide targeting Cys-residues) or a single chemically as well as single photolytically activated reactive group (*e.g.* NHS-ester and a benzophenone moiety inserting into various C–H-bonds upon irradiation).[15,17,81]

The application of such heterobifunctional reagents offers the possibility of performing the cross-linking reaction in two or three steps, hence minimising the formation of undesired high-molecular aggregates. For example, a NHS-ester/maleimide hetero-bifunctional reagent will react first with free Lys-residues of a protein at its NHS-ester reactive function. The maleimide reactive group, however, does not react as a consequence of its higher stability in an aqueous environment enabling an intermediate purification step to remove excess reagent and other by-products. Afterwards the thiol-reactive maleimide reactive group is reacted with free Cys-residues of the protein. The application of heterobifunctionl cross-linkers containing a single photoreactive group exhibits an additional advantage as a consequence of the inability of the photoreactive group to react with other functional groups of a protein prior to activation with high intensity UV light. Consequently, the amount of unwanted side-products can be further

reduced. Furthermore, photochemical reagents enable the formation of cross-links between regions of proteins which could not be addressed previously using group-specific reagents making these compounds highly favourable to study protein-protein interaction. However, the efficiency of photoreactive reagents is comparably low as a consequence of the unsatisfying cross-linking yields which can be achieved (< 10%).[17,81]

In recent years, heterobifunctional cross-linking reagents have found numerous applications predominantly as tools either to study protein-protein as well as protein-peptide interactions,[87] to gain knowledge about a protein`s 3D-structure in solution *via* intramolecular cross-linking,[88] or for the preparation of hapten-conjugates[89] as well as enzyme immunoconjugates.[90]

Since more than thirty years, chemical cross-linking has been widely applied for the production of various protein conjugates. More recently, chemical cross-linking in combination with mass spectrometry has emerged as a powerful tool to identify the binding site of two interacting proteins in a protein complex as well as to assess a low-resolution protein structure by creating an ensemble of structurally defined interactions within a protein. For these purposes, a variety of different cross-linking reagents have been reported, which are both soluble under biological relevant conditions and stable under proteolytic conditions, to enable subsequent mass spectrometric analysis. However, progress in this field might by hampered as a consequence of the difficulties finding the ideal cross-linking conditions which are in agreement with the demands of the native protein and with the physicochemical properties of a multifunctional cross-linker.[15]

1.4 Objective of thesis

Although a variety of reagents have been already synthesised for the modification of various biomolecules, the synthesis of such multi-functionalised reagents involves multistep procedures. Based on the modular design of these reagents, the application of a more modular approach to synthesise such chemical probes was envisioned. This would allow changing systematically the properties of a given reagent to optimise the feasibility of a chemical probe in a specific labelling experiment. In this respect, the focus was set on the well-known Ugi four-component reaction (Ugi-4CR) which has not been further utilised in the synthesis of such multi-functionalised chemical probes at the beginning of the present study. This novel MCR-approach should enable the fast and efficient synthesis

of a library of chemical probes for the covalent modification of biomolecules. To prove this hypothesis, in the first part of this thesis, the scope and limitations of the Ugi-4CR in the synthesis of chemical probes will be explored. Therefore, first amino acid specific reactive groups (*e.g.* Lys-reactive NHS-ester) and various bioorthogonal handles (*e.g.* azide, alkyne) should be incorporated into rhodamine B utilising the Ugi-4CR. Furthermore, the Ugi-4CR-based synthesis of a biotin-containing trifunctional cross-linking reagent containing two amino-reactive NHS-ester moieties is intended which enables the subsequent purification and enrichment of cross-linked peptides, hence facilitating MS-detection (chapter 2).

In the second part of the present thesis, the largely disregarded metal-free formation of triazoles from primary amines and *α*,*α*-dichlorotosylhydrazones (*Sakai reaction*) comes into focus of our studies. Although already described in 1986 by Sakai and colleagues, this mild and regioselective formation of 1,4-substituted triazoles has not been fully explored yet. First, a scope and limitation study should be performed enabling to draw conclusions regarding the mechanism of this reaction. Moreover, the applicability of the Sakai reaction as stable alternative to classical amine labelling agents (*e.g.* NHS-ester or carbodiimides) for the covalent modification of biomolecules (*e.g.* proteins or peptides) in aqueous buffered systems should be investigated (chapter 3).

In addition to the covalent modification of biomolecules using either amino acid residue specific or bioorthogonal labelling reagents (chapter 2 and 3), the synthesis and application of activity-based probes (ABPs) to covalently modify and enrich proteins with a common function is intended in the third and final part of this thesis. In a first subproject, the synthesis of a suitable ABP targeting cation-dependent plant *O*-methyl-transferases (Pl-OMT) should be accomplished. The probe design is based on the modification of already well-established nitro-catechol inhibitors of the human COMT (*i.e.* entacapone and tolcapone) as a consequence of high similarities between the human COMT and cation-dependent Pl-OMTs regarding substrate binding and the catalytic mechanism. Finally, the synthesis of a highly reactive "clickable photo-auxin" is envisioned. The incorporation of a photoactivatable group (here: phenylazide) and a clickable handle (here: terminal alkyne) into auxins (here: indole-3-acetic acid, IAA), important plant growth hormones, should enable to demonstrate auxin binding and to identify/isolate still unknown auxin receptors. This may contribute greatly to the understanding of auxin-mediated signalling pathways in plants.

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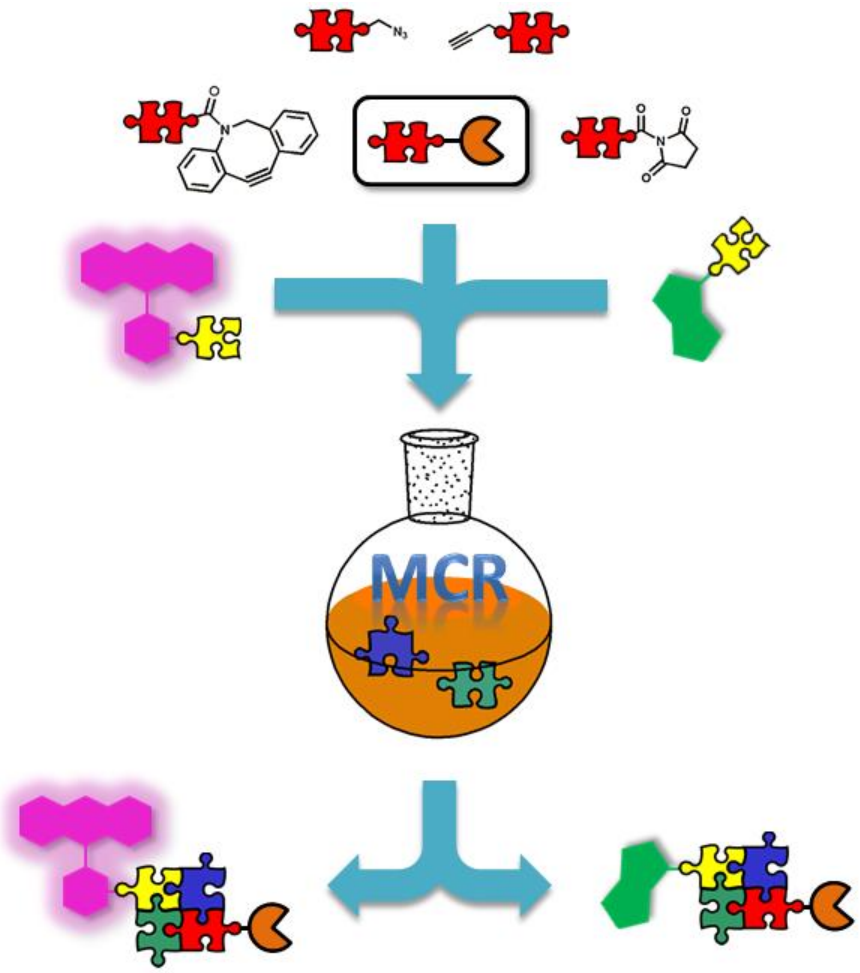
2 Fast and Efficient MCR-Based Synthesis of Functional Tools for Protein Modification



Abstract

In the forthcoming chapter, the application of multicomponent reactions, more specifically the Ugi four-component reaction (Ugi-4CR), as a novel synthetic strategy gaining access to structural diverse chemical probes used for proteomic studies will be introduced. Based on the application of the Ugi-4CR, a one-pot protocol for the synthesis of activated fluorescent labels equipped either with an azide, alkynyl or NHS-moiety will be presented. Moreover, their unique fluorescent properties and applicability, as exemplified by the labelling of the purified protein BSA, will be discussed. Furthermore, the synthesis and application of biotinylated, trifunctional chemical cross-linking reagents used for the covalent cross-linking of amino acid residues within a protein or between a protein and its binding partner to study, *e.g.* three dimensional protein structures will be presented.

2.1 Introduction

Based on the modular design of chemical probes used in proteomic studies, as already outlined comprehensively in chapter 1, the synthesis of such multifunctional molecular probes in a one-pot multicomponent reaction (MCR) was envisioned. In contrast to classical bimolecular reactions (compound A reacts with compound B to yield product C), MCRs combine three or more different components in one reaction vessel leading to the formation of a single, multi-substituted product. Moreover, MCRs involve the formation of multiple bonds in a single operation, ideally without isolating the intermediates, changing the reaction conditions or adding further reagents.[1] Therefore, MCRs cover by definition topics such as sustainability, atom- and eco-efficiency, high convergence (process efficiency) and reduction of the amount of intermediate steps or functional group manipulations, thus reducing time and energy, known as step efficiency (Fig. 2.1).[2]

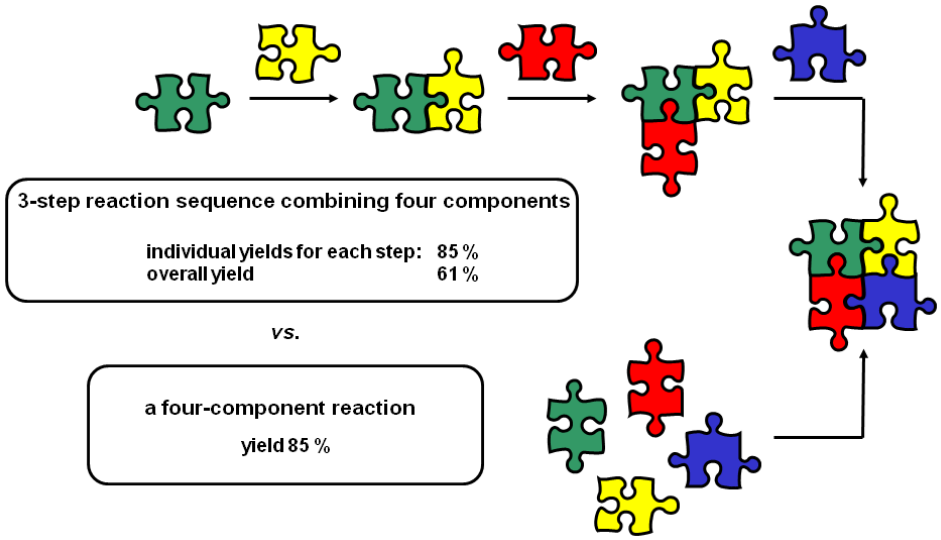


Fig. 2.1 Stepwise linear synthesis *vs*. multicomponent reactions. (Reproduced from Brauch *et al.*, *Chem. Soc. Rev.* **2013**, *42*, 4948–4962 with permission of The Royal Society of Chemistry)

For a fast, highly yielding and structural versatile synthesis of such multifunctional chemical probes, the Ugi-4-component reaction (Ugi-4CR) was chosen, currently the most versatile MCR with respect to the educt and product spectrum and applicability. So far, several excellent comprehensive reviews by Zhu, Orru and Dömling, among others, have appeared on the history, recent advances and stereoselectivity of the Ugi-4CR.[1,3,4]

The Ugi-4CR enables the synthesis of structural highly diverse peptoids by simply mixing one equivalent each of an amine, aldehyde, isocyanide and carboxylic acid in an appropriate protic solvent (*e.g.* methanol, trifluoroethanol) with water as the sole by-

product. Common synthetic approaches, however, exhibit a much higher synthetic effort. The initial step of the proposed mechanism of the Ugi-4CR involves imine formation (Fig. 2.2, **A**) followed by protonation of the imine resulting in the formation of a more reactive iminium-intermediate (**B**). Subsequent nucleophilic attack of the isocyanide affords a nitrilium intermediate (**C**) which reacts afterwards with the carboxylate (**D**). Finally, the formed adduct (*O*-acylimide) rearranges to yield a more stable product (*N*-acylimide) *via* a Mumm-type rearrangement (**E**).[5]

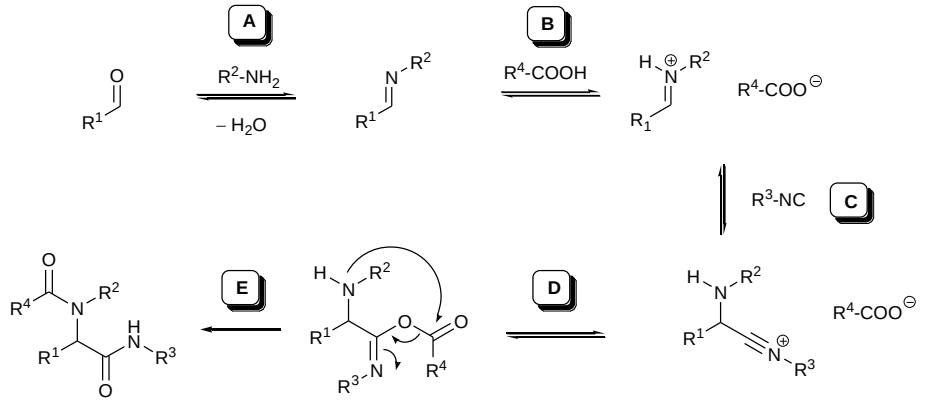


Fig. 2.2 Proposed mechanism of the Ugi-4-component reaction.

An advanced adoption of the Ugi-4CR as key synthetic step in the preparation of multifunctional chemical probes has significant advantages over other synthetic approaches: (i) easy to carry out reaction protocols; (ii) fast synthesis of structural diverse compound libraries by simply varying the starting materials, giving a fast access to the best suited probe for an individual experiment; (iii) no use of additional toxic and/or expensive coupling reagents; and (iv) high flexibility concerning availability or synthetic accessibility of different starting materials.[6]

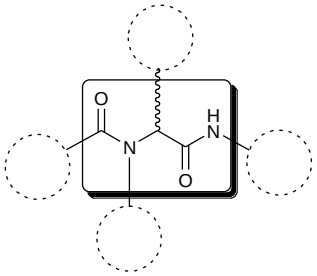


Fig. 2.3 General build-up of multi-functional chemical probes derived from the Ugi-4CR.

As its gets obvious from the nature of the Ugi-4CR, the implementation of this particular MCR enables the synthesis of multifunctional chemical probes with up to four different functional building blocks in a one-pot operation (Fig. 2.3). In 2012, Yao and co-workers made use of this unique feature while synthesising a small library of affinity-based probes (ABPs) targeting potential protein tyrosine phosphatases (PTPs).[7] In their study, they combined a phosphotyrosine mimic (aldehyde), a "click-handle" (carboxylic acid), a benzophenone derived photoreactive cross-linker (isocyanide) and a set of 25

different amine building blocks in a one-pot fashion to obtain a library consisting of 25 putative ABPs (Fig. 2.4). Furthermore, all 25 compounds were tested and most of them proved to be successful in targeting recombinant and endogenous PTPs. Moreover, they were able to assign other cellular targets (*e.g.* cathepsin D, prohibitin) which in the end might help to design more potent and specific ABPs for PTPs in the future.

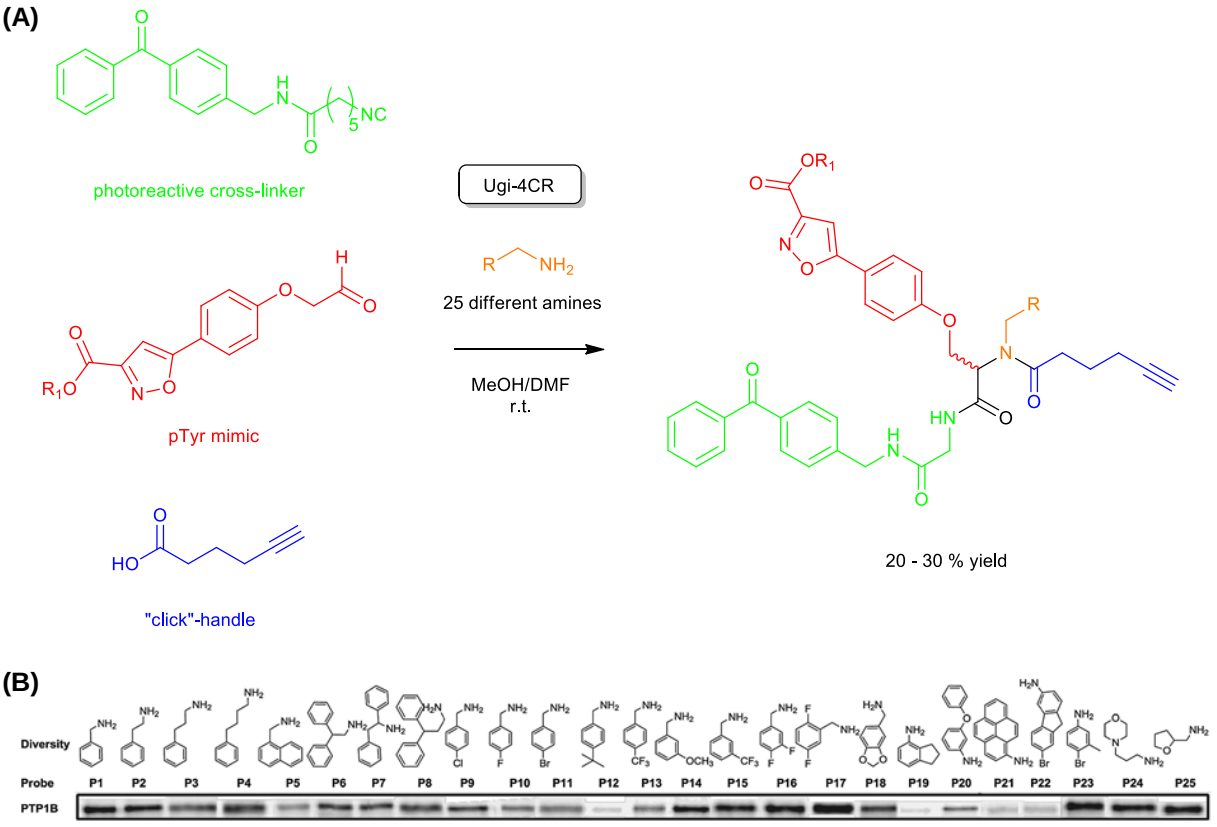


Fig. 2.4 Synthesis of a library of ABPs targeting protein tyrosine phosphatases (PTBs) utilising the Ugi-4CR. (A) In a combinatorial fashion, a small library of putative APBs was generated by combining a "click"-handle, a phosphotyrosine mimic, a benzophenone photoreactive cross-linker and 25 different primary amines in a one-pot Ugi-4CR. **(B)** Subsequent affinity-based profiling of recombinant PTP1B against the obtained 25 ABPs revealed successful binding of most probes to the target enzyme. For this purpose, after labelling of the recombinant protein with each probe, the mixture was subjected to a follow-up click-reaction with a fluorescent dye (rhodamine-N_3). Proteins were separated by SDS-PAGE and visualised by in-gel fluorescence scanning (Reproduced in part from Yao *et al.*, *Chem. Commun.* **2012**, *48*, 4453–4455 with permission of The Royal Society of Chemistry).

In a very recent study from 2013 utilising the Ugi-4CR in the synthesis of chemical probes, Schofield and colleagues created a library of APBs comprising a set of photoreactive chemical probes which are equipped either with a diazirine, aryl azide or benzophenone reactive group, putatively targeting the human hypoxia sensing enzyme prolyl hydroxylase domain-containing protein 2 (PHD2).[8] Afterwards the photo-crosslinking rates and efficiencies were compared. It was found that trifluoromethyl

diazirine and benzophenone, photoreactive groups which are thought of as being good cross-linking reagents, gave only poor cross-linking efficiencies. These findings suggest that the choice of the optimal photoreactive group depends strongly on the photochemical properties of the probe and the nature of the target enzyme, among other conditions. Therefore, experimental guided optimisation of ABP-design is a prerequisite for the effective detection and/or enrichment of a particular enzyme.

The simultaneous investigations by the research groups of Yao, Schofield and our group clearly confirm our assumption of the unification of MCRs and ABPP as being of high value for the chemical proteomics community speeding up the synthesis of libraries of putative ABPs. These libraries can be tested afterwards in fast screening routes to assess the best suited probe for an individual target.

In the following sections, further applications of the Ugi-4CR approach regarding the synthesis of chemical probes for the modification of proteins, more specifically pH-independent rhodamine fluorescent tags and biotinylated chemical cross-linking reagents for the study of protein-protein interactions, will be discussed.

2.2 Synthesis and application of pH-independent rhodamine-tags

The following results have been published previously in Organic and Biomolecular Chemistry entitled *"Fast and efficient MCR-based synthesis of clickable Rhodamine tags for protein profiling"* (Brauch *et al.*, *Org. Biomol. Chem.* **2012**, *10*, 958–965).

The ability of a substance to emit light of a certain wavelength after the absorption of energy (light or electromagnetic radiation) is called *photoluminescence*. This phenomenon can be further subdivided into *fluorescence* or *phosphorescence* depending on the nature of the electronically excited state. In general, upon the absorption of a photon (*excitation*), an electron of the fluorophore is promoted from the singlet ground state S_0 to higher vibrational energy levels of the first or second singlet excited state S_1 or S_2, respectively (Fig. 2.5). Immediately after excitation, relaxation to the lowest vibrational energy level of the first singlet excited state occurs without the emission of light (*internal conversion*). From this lowest singlet excited state an electron is able to relax to its ground state by various pathways, such as emission of a photon (fluorescence) and non-radiative relaxation either through bond rotation and vibration (heat) or molecular collision. Furthermore, relaxation in the ground state might occur through the interaction with a second molecule, such as molecular oxygen or another fluorophore (*fluorescence*

quenching). The singlet excited state additionally may undergo *intersystem crossing* to a triplet excited state T_1 which in turn is able to relax to the singlet ground state either by photon emission (phosphorescence) or non-radiative relaxation.[9]

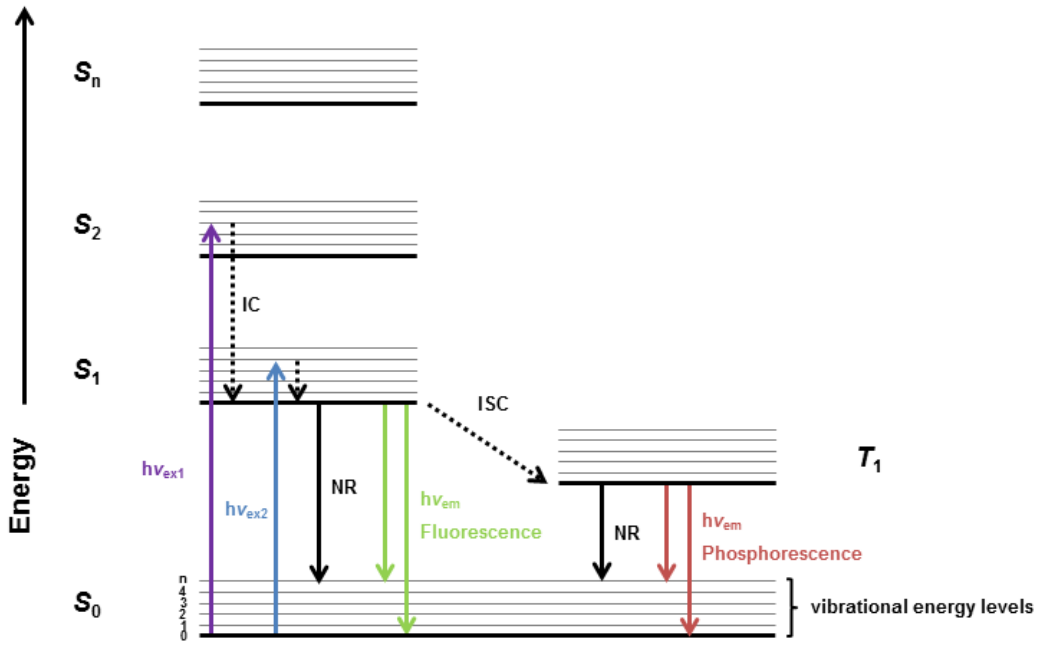


Fig. 2.5 Jablonski-Diagramm. Upon excitation of a fluorophore, an electron is promoted to an excited singlet state (either S_1 or S_2). Subsequently, relaxation to the lowest vibrational energy level of S_1 occurs on a picosecond timescale *via* internal conversion (IC) without the emission of light. The excess of vibrational energy is either converted into heat (absorbed by the surroundings) or is lost through collision with adjacent molecules. Relaxation to the ground state S_0 proceeds either by non-radiative relaxation (NR), light emission (fluorescence), or intersystem crossing (ISC) followed by NR respectively light emission (phosphorescence).

Synthesis of rhodamine-tags for the covalent modification of various biomolecules

The application of fluorescent-tags equipped with different functionalities (*e.g.* alkyne-, cyclooctyne-, azide- or other reactive moieties) to covalently modify biomolecules is of significant importance in the field of chemical biology, especially for the (*in vivo*) detection of low abundant proteins, otherwise difficult to analyse by standard blotting techniques. In general, these fluorescent labels exhibit most of the following characteristics: (i) emission-wavelengths higher than the auto-fluorescence of a biological sample (> 350 nm); (ii) high extinction coefficients and quantum yields; (iii) minimal photobleaching (loss of the ability to fluoresce as a consequence of structural damages); (iv) high solubility in relevant buffers, cell culture media and body fluids; (v) minimal spectral overlap with other fluorophores present in a sample (fluorescence quenching); and (vi) functional groups for site-specific labelling.[10]

In the present study, rhodamine B and its derivatives were chosen due to their significant advantages compared to other fluorescent dyes, *e.g.* high photostability, good membrane permeability, excellent photophysical properties and commercial availability. The major drawback while working with rhodamine-based fluorescent dyes, though, is the observed pH-sensitivity. Secondary amides of rhodamine tend to undergo intramolecular cyclisation leading to the leuko-form which results in the loss of fluorescence (Fig. 2.6). To avoid this, tertiary amides produced in multi-step sequences are generally applied.[11] For excellent reviews dealing with the topics of modifying rhodamine and the application of fluorescent dyes in the labelling of biomolecules refer to Beija *et al.*[12] and Gonçalves.[13]

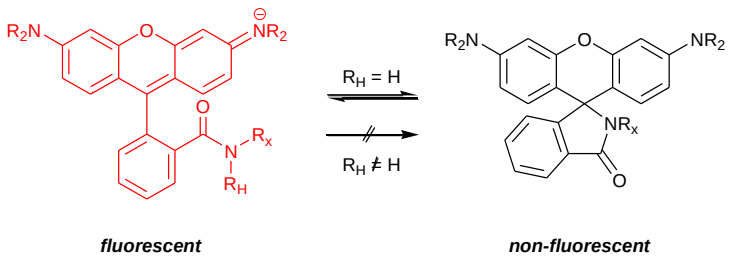


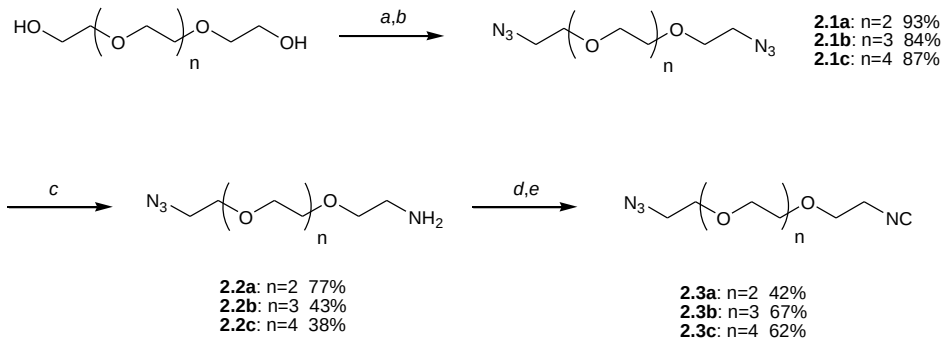
Fig. 2.6 Cyclisation of rhodamine dyes leading to a non-fluorescent leuko-form of rhodamine based fluorescent dyes. (Reproduced from Brauch *et al., Org. Biomol. Chem.* **2012**, *10*, 958–965 with permission of The Royal Society of Chemistry)

In the course of our studies concerning the applicability of MCRs, more specifically the Ugi-4CR, for the synthesis of chemical probes, it was intended to use rhodamine B and its derivatives as the carboxylic acid components in the Ugi-4CR. To the best of our knowledge, this approach was considered only once before (in this particular case RhoB was reacted with amine functionalities of proteins in an Ugi-4CR).[14] As a consequence of the formed tertiary amide bond, which is inherent to most Ugi-products, the pH-sensitivity of the fluorescent properties should be reduced.

The design of rhodamine probes was based on the interest of constructing chemical probes equipped either with residue specific reactive groups or with bioorthogonal functional handles allowing a rapid post-functionalisation *via* CuAAC (copper-assisted azide-alkyne cycloaddition) or SPAAC (strain-promoted azide-alkyne cycloaddition). To modify biomolecules using these bioorthogonal coupling methods, however, the target biomolecules have to be modified with a complementary reactive moiety prior to the labelling reaction (cf. chapter 1.2). Besides fluorescent labelling, these "clickable" fluorescent-tags have been frequently applied to detect and/or purify covalently bound

proteins, previously treated with the warhead of an activity-based probe (ABP) being additionally equipped with a complementary bioorthogonal handle (cf. chapter 1.1).

Concerning this study, most of the used Ugi-reactive building blocks were obtained from commercial sources. The PEGylated building blocks containing on the one hand an Ugi-reactive functional group (predominantly amine or isocyanide) and on the other hand an azide moiety, however, were synthesised starting from ethylene glycol derivatives having different chain lengths, hence affecting the water solubility of the resulting compounds. These bifunctional building blocks with chain lengths ranging from three to five ethylene glycol units were obtained in two synthetic steps affording amine **2.2a**–**c** in 33–71% overall yield or four steps yielding the corresponding isocyanides **2.3a–c** in 20–30% overall yield, respectively, starting from commercially available tetraethylene, pentethylene or hexaethylene glycol (Scheme 2.1).[6] Initially, the starting glycol precursors were converted into the corresponding diazide compounds **2.1a–c** followed by the selective reduction of just one azide-group with triphenylphosphine in an aqueous acid solution affording the mono-amines **2.2a–c** in moderate yields (Staudinger reduction). The corresponding isocyanides **2.3a–c** were obtained in an additional two-step sequence *via* formylation with ethyl formiate and dehydratisation using phosphorous oxychloride.

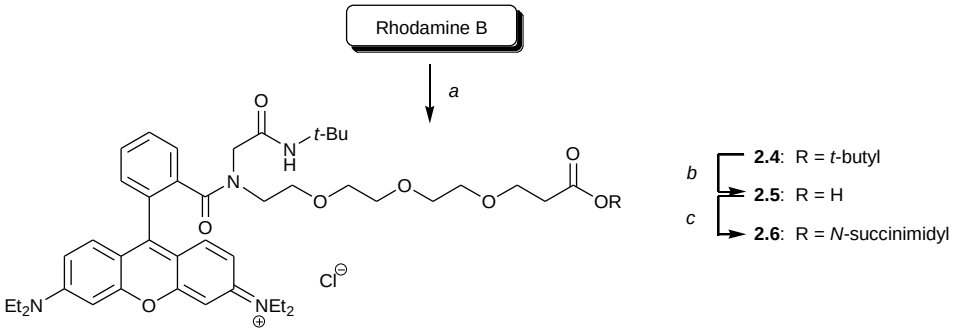


Scheme 2.1 Synthesis of Ugi-reactive, clickable PEGylated linkers/spacers. *Reagents and conditions*: (a) MsCl, Et_3N, THF, 0 °C for 1 h followed by r.t. for 3 h; (b) NaN_3, $NaHCO_3$, H_2O, 80 °C, 12 h; (c) H_3PO_4 (0.65 M), PPh_3/Et_2O, r.t., 24 h; (d) ethyl formiate, reflux, 3 h; (e) $POCl_3$, diisopropylamine, CH_2Cl_2, r.t., 2 h.

NHS-reactive rhodamine-tags. For the covalent modification of proteins in biological samples, side chain selective reagents are necessary which enable the site-specific incorporation of affinity- or fluorescent labels. To be of viable and versatile use for the chemical and biological community, these reagents should react under specific conditions with a limited number of side chains (*e.g.* free lysine and cysteine residues) or with a pre-targeted single side chain (*e.g.* unnatural azide- or alkyne-modified amino acids

incorporated into the protein of interest). For the chemical modification of biomolecules, the most commonly used reactive groups are amine selective reagents, such as activated *N*-hydroxysuccinimide esters, imidoesters or activated aryl fluorides and compounds which exhibit a high reactivity towards thiol groups, such as maleimids or *a*-halocarbonyl compounds (for a comprehensive overview cf. chapter 1.2 and references cited therein).[15]

A suitable peptoidic rhodamine-tag equipped with an activated NHS ester for the selective labelling of amine residues of a protein could be obtained in a three step reaction sequence starting from commercially available starting materials (**2.6**, Scheme 2.2). The Ugi-4CR was chosen as key synthetic step in the synthesis of **2.6** to generate the peptoidic framework as outlined previously, hence preventing the formation of the non-fluorescent leuko-form (Fig. 2.6). For this purpose, rhodamine B, *t*-butyl isocyanide, para-formaldehyde and *t*-butyl 12-amino-4,7,10-trioxadodecanoate were reacted in methanol under microwave heating to afford the *t*-buty ester protected precursor **2.4** in 63% yield (during the present study modifying rhodamine B and its derivatives, microwave heating was found to speed up the reaction rate of the Ugi-4CR tremendously, though leaving the overall yield of the reaction unaffected). Afterwards the ester was cleaved under acidic conditions (phosphoric acid) and the corresponding carboxylic acid **2.5** was treated with *N*-hydroxysuccinimide under standard coupling conditions (EDC, DMAP, dichloro-methane). After aqueous workup, the activated PEG_3-(rhodamine B)-NHS ester **2.6** could be isolated in 75% yield and sufficient purity.[6]



Scheme 2.2 Ugi-derived, amine-reactive NHS-rhodamine label. *Reagents and conditions*: (a) paraformaldehyde, *t*-butyl isocyanide, rhodamine B, *t*-butyl 12-amino-4,7,10-trioxadodecanoate, MeOH, µW (100 °C), 1 h (63%); (b) H_3PO_4 (85 v/v%), toluene, r.t., 3 h (65%); (c) *N*-hydroxysuccinimide, DMAP, EDC, CH_2Cl_2, r.t., 24 h (75%).

In a first proof of concept experiment, the applicability of the obtained amine-reactive rhodamine-tag for the functionalisation of proteins was evaluated by treating purified BSA (bovine serum albumin, MW ~ 66.8 kDa) and a crude extract of *A. thaliana* (Col-0) with PEG_3-(rhodamine B)-NHS ester **2.6**. To visualise the labelled proteins, the samples

were run on SDS-PAGE gels and subsequently analysed by means of fluorescence scanning and coomassie-blue staining (Fig. 2.7). As one might expect, compound **2.6** proved to be able to covalently modify purified BSA. Moreover, the application of fluorescence-tags exhibits a much higher sensitivity in detecting labelled proteins then commonly used staining techniques as one can conclude by the comparison with the coomassie-blue stained gel. In this particular case, protein bands in the stained gel are only barely visible in the lowest protein concentrations while being clearly visible when analysing the gel by fluorescence scanning. This fact is additionally confirmed by the labelling of a crude extract of *A. thaliana* with compound **2.6**. Here, protein bands became only visible in the coomassie-stained gel in the highest protein concentrations applied. The fluorescence scan, however, reveals several protein bands even in the lowest concentration tested (presumably mostly RuBisCO, MW ~ 52.7 kDa).

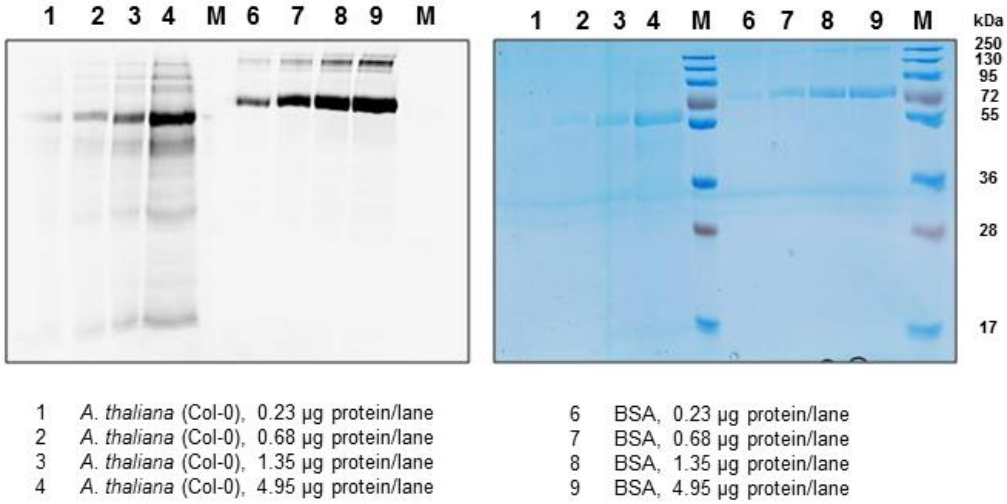


Fig. 2.7 SDS-PAGE gel of labelled BSA and a crude protein mixture from *A. thaliana* (Col-0). *Labelling conditions*: 50 µg protein in 10 µL buffer solution (8 M urea buffer and 30 mM Tris-buffer, pH = 8.5), 1 µL fluorescent-tag **2.6** (400 pmol·µL^{-1}, DMF), incubation for 30 min on ice. Marker: Prestained Protein Marker (Fermentas). Labelled proteins were first visualised by in-gel fluorescence scanning (left) followed by Coomassie-staining (right) (labelling experiments performed by Dr. Kai Naumann, IPB Halle). (Reproduced from Brauch *et al., Org. Biomol. Chem.* **2012**, *10*, 958–965 with permission of The Royal Society of Chemistry)

Due to a multitude of competing nucleophiles in a complex biological sample, the application of PEG$_3$-(rhodamine B)-NHS ester **2.6** for the site-selective modification of biomolecules is rather unsuitable. To overcome this lack in selectivity, bioorthogonal handles (*i.e.* azides, alkynes) have been incorporated into biomolecules of interest by genetic as well as chemical approaches.[16,17] Furthermore, particularly over the last decade, several reactions and suitable reagents to react with azides or alkynes under

physiological conditions have been developed. The most commonly used methodologies include: (i) the Staudinger ligation utilising substituted phosphine reagents; (ii) the copper-catalysed [3+2] azide-alkyne cycloaddition reaction (CuAAC); and (iii) the strain-promoted azide-alkyne cycloaddition reaction (SPAAC) using highly constrained cyclooctyne reagents (for general remarks concerning bioorthogonal ligation methods see chapter 1.2).[18]

Clickable rhodamine-tags. More than a decade ago, Sharpless and Meldal independently developed the copper catalysed azide alkyne cycloaddition reaction (CuAAC) leading to the regioselective formation of 1,4-substituted 1,2,3-triazoles (Fig 2.8).[19] Since that pioneering work, CuAAC has been frequently applied as tool for the covalent modification of proteins and various other biomolecules. However, due to the strong dependency on the presence of a copper catalyst, the *in vivo* application of the CuAAC methodology is rather limited as a consequence of the toxicity of copper(I). Consequently, methods relaying on the application of highly strained cyclooctynes were developed by the lab of Bertozzi enabling azides to react without the need of an additional promoter species.[20] This reaction is mostly referred to as strain-promoted azide alkyne cycloaddition reaction (SPAAC) and has found also numerous applications in the field of chemical biology.[21]

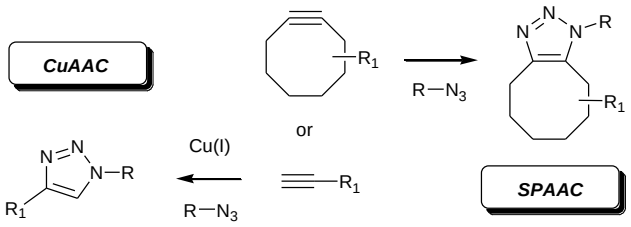
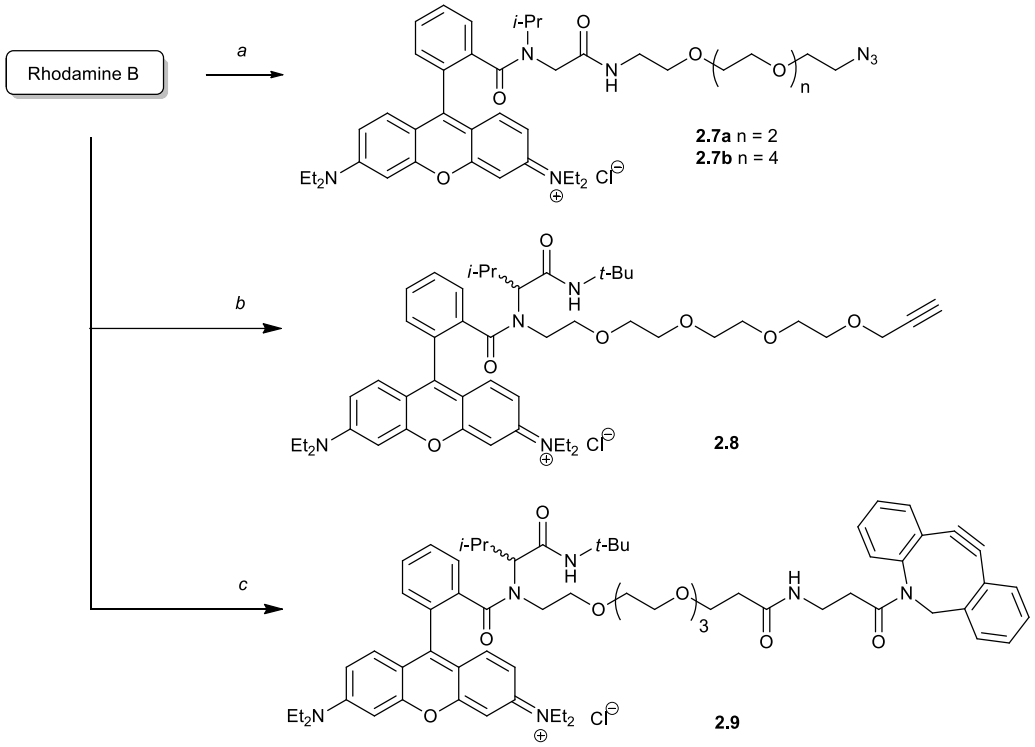


Fig. 2.8 Formation of 1,2,3-triazoles using either CuAAC or SPAAC. (Reproduced from Brauch *et al., Org. Biomol. Chem.* **2012**, *10*, 958–965 with permission of The Royal Society of Chemistry)

Again, the Ugi-4CR was utilised in the one-pot synthesis of clickable rhodamine derivatives equipped with either azide or alkyne functionalities (Scheme 2.3). The azide containing compounds PEG_n-(rhodamine B)-azide **2.7a** (n=3) and **2.7b** (n=5), which differ only in the number of ethylene glycol units, were prepared by mixing paraformaldehyde, *i*-propylamine, rhodamine B and N_3-PEG_3-isocyanide **2.3a** or N_3-PEG_5-isocyanide **2.3c** in methanol and subsequent microwave-heating at 100 °C for one hour. The corresponding PEG_n-(rhodamine B)-azides **2.7a** and **2.7b** could be isolated in 74% and 70% yield, respectively. As mentioned previously, while modifying rhodamine and its derivatives applying the Ugi-4CR, microwave-heating was found to reduce the reaction time, but no effect on the overall yield of the reaction could be detected, though. Under

standard reaction conditions generally applied in the Ugi-4CR (*i.e.* 2 hours imine formation, subsequent addition of isocyanide and carboxylic acid, 24 hours stirring at room temperature), the desired PEG_3-(rhodamine B)-azide **2.7a** could be isolated in 74% overall yield. The same yield was obtained by preforming the imine and subsequent heating of the reaction mixture for 1 hour in a microwave apparatus at 100 °C after the addition of isocyanide and carboxylic acid as mentioned above. A drastic decrease of the overall yield, though, could be observed by allowing the reaction to stir at room temperature for just 1 hour instead of 24 hours (yield: 32%). Moreover, the preformation of the imine seems essential for obtaining good overall yields in the Ugi-4CR, because a drastic decrease of the overall yield could be experienced when all four starting materials were added at once with subsequent microwave heating (yield: 36%).



Scheme 2.3 Ugi-derived clickable rhodamine-tags. *Reagents and conditions:* (a) paraformaldehyde, *i*-propyl-amine, N_3-PEG_3-NC **2.3a** (or N_3-PEG_5-NC **2.3c**), rhodamine B, MeOH, µW (100 °C), 1 h (74% for **2.7a** and 70% for **2.7b**); (b) *i*-butyraldehyde, acetylene-PEG_4-amine, *t*-butyl isocyanide, rhodamine B, µW (100 °C), 1 h (61%); (c) *i*-butyraldehyde, dibenzylcyclooctyne-PEG_4-amine, *t*-butyl isocyanide, rhodamine B, MeOH, r.t., 24 h (73%).

The alkyne- and cyclooctyne-modified rhodamine derivatives **2.8** and **2.9**, respectively, were generated by varying the starting materials of the Ugi-4CR. Instead of *i*-propylamine, the commercially available acetylene-PEG_4-amine (in case of **2.8**) or dibenzoaza-cyclooctyne-PEG_4-amine (in case of **2.9**) served as the amine building block and *t*-butyl

isocyanide was used as isocyanide component in the Ugi-4CR. Following the aforementioned protocol, both alkyne modified rhodamine derivatives could be isolated in satisfying yields (61% for **2.8** and 73% for **2.9**, respectively).

Fluorescent properties of Ugi-modified rhodamine dyes

Rhodamine B and its derivatives are well-known for their great photostability and their tuneable fluorescence characteristics. For example, their absorption and emission maximum, quantum yield, *etc.*, can be strongly affected by ring as well as nitrogen substitutions. As mentioned earlier, secondary amides of rhodamine tend to undergo rapid cyclisation affording a non-fluorescent spiro-form under basic condition (Fig. 2.6), which might limit their applicability in biological systems as a consequence of a decreased fluorescence intensity in certain intracellular environments.[22] To prove the proposed pH-insensitivity of the obtained Ugi-rhodamine dyes, PEG_5-(rhodamine B)-azide **2.7b** was dissolved exemplarily in a Britton-Robinson buffer varying in pH (Fig. 2.9). As expected, no disappearance of fluorescence could be observed under acidic as well as basic conditions; though, a shift in fluorescence intensity could be detected under acidic conditions due to the degree of protonation of the tertiary amines, hence altering its electron-donating properties.[23] Consequently, at neutral or basic conditions, respectively, this change in fluorescence intensity disappears.

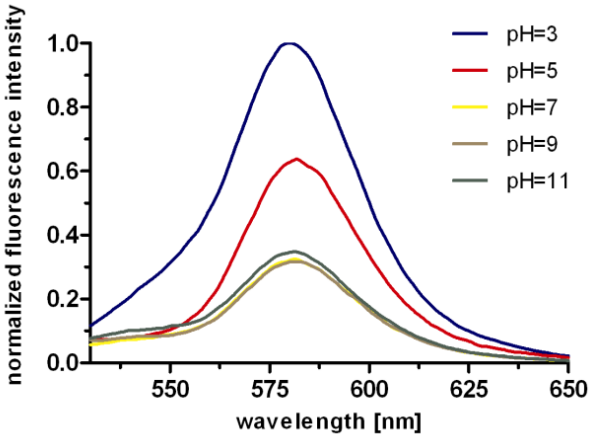
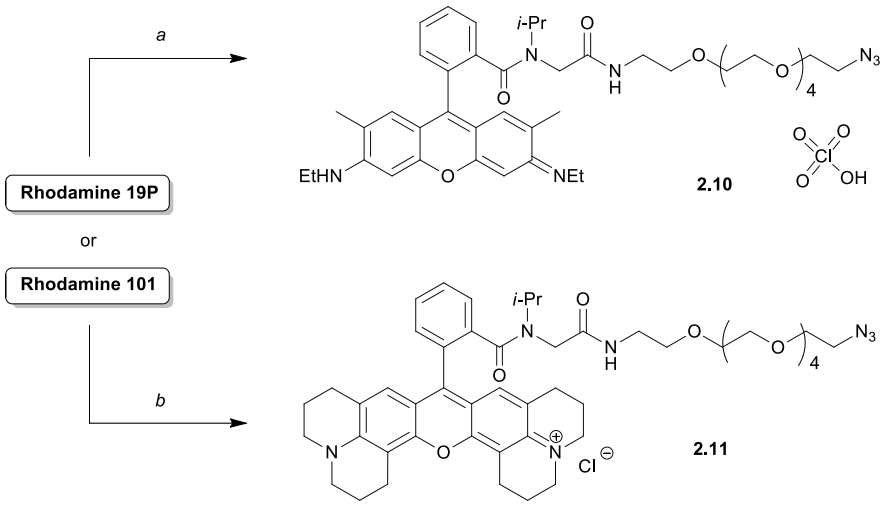


Fig. 2.9 Fluorescent intensity of 2.7b as a function of pH. Spectra were taken in a BR-buffer solution (10 mM) at an excitation wavelength of 510 nm (sample concentration 0.1 µM). (Reproduced from Brauch *et al., Org. Biomol. Chem.* **2012**, *10*, 958–965 with permission of The Royal Society of Chemistry)

Versatile applicability of the Ugi-4CR for the modification of different rhodamine dyes

To investigate whether or not the presented Ugi-protocol is suitable to modify other rhodamine dyes as well, two of the most commonly used dyes were chosen for further studies, *i.e.* rhodamine 19P and rhodamine 101. Each individual dye was reacted with paraformaldehyde, *i*-propylamine and N_3-PEG_5-isocyanide **2.3c** similar to the protocol

outlined for the synthesis of compound **2.7b** (Scheme 2.4). The corresponding PEG_5-(rhodamine 19P)-azide **2.10** and PEG_5-(rhodamine 101)-azide **2.11** could be isolated in good (81% for **2.10**) to unsatisfying yields (24% for **2.11**). This drastic decrease in the overall yield might be attributed to the inner salt form of rhodamine 101, hence making an additional protonation step necessary. Apart from the synthesis of compound **2.11**, it becomes obvious that the Ugi-4CR is well suited for the modification of a variety of rhodamine dyes equipped with a free carboxylic acid moiety (*e.g.* rhodamine 110, rhodamine 116, *etc.*) leading to a library of pH-insensitive fluorescent-tags containing either a bioorthogonal functional handle or an amine-reactive group.



Scheme 2.4 Modification of related rhodamine dyes. *Reagents and conditions*: (a) paraformaldehyde, *i*-propyl-amine, N_3-PEG_5-NC **2.3c**, rhodamine 19P, MeOH, µW (100 °C), 1 h (81%); (b) paraformaldehyde, *i*-propylamine N_3-PEG_5-NC **2.3c**, rhodamine 101, MeOH, µW (100 °C), 1 h (24%).

To estimate the effect of the chemical modification on the spectral properties of the used rhodamines dyes, the absorption and emission spectra of rhodamine B as well as of compounds **2.7b**, **2.10** and **2.11** were recorded in an aqueous buffered system (Fig. 2.10). From these spectra, the spectral properties corresponding to the different rhodamine dyes **2.7b**, **2.10** and **2.11** were determined (Table 2.1). Compared to unmodified rhodamine B only a slight bathochromic shift of the absorption and fluorescence maxima for compound **2.7b** was observed as a consequence of the chemical modification. The same bathochromic shift could be noticed for compounds **2.10** and **2.11** when compared to already published data.[24]

In this section of the present thesis, the focus was set exclusively on the modification of commercially available rhodamine fluorescent dyes by means of the Ugi-4CR. A small library comprising reactive, pH-independent rhodamine-based fluorescent-tags equipped either with an amine-reactive NHS ester or with bioorthogonal clickable handles was created using this novel MCR-approach. Additionally, the Ugi-4CR is well suited to synthesise affinity-tags which contain a biotin moiety instead of a fluorophore (enables the enrichment and purification of target proteins or peptides, respectively) and various bioorthogonal functional groups or amino acid residue specific reactive groups (which enable on the other hand the covalent attachment of the affinity-probe). This will be demonstrated in the following section.

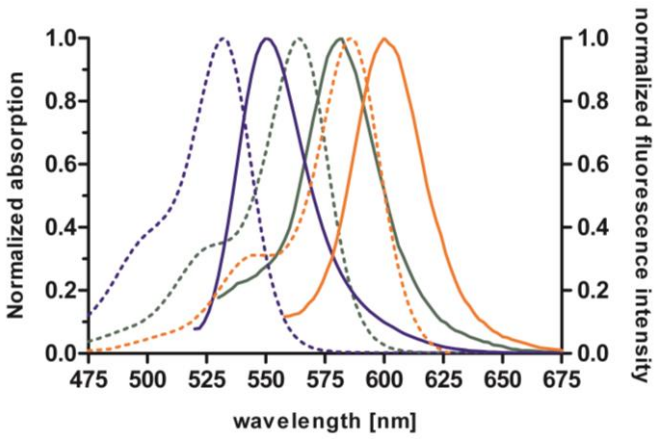


Fig. 2.10 Normalised absorption (dashed line) and fluorescence emission (solid line) spectra. Absorption and emission spectra of compounds **2.7b** (green), **2.10** (blue) and **2.11** (orange) were recorded in BR-buffer solution (10 mM, pH = 7, sample concentration 0.1 µM). (Reproduced from Brauch *et al., Org. Biomol. Chem.* **2012**, *10*, 958–965 with permission of The Royal Society of Chemistry)

Table 2.1 Spectral properties of commercially available and synthesised rhodamine dyes .

compound	λ_{abs}/nm	λ_{em}/nm	ε / M cm^{-1}
Rhodamine B	554	576	87,013
2.7b	564	582	67,093
2.10	532	550	71,751
2.11	586	600	66,823

[a]Sample concentration 0.1 µM in BR-buffer solution (10 mM)

2.3 Synthesis and application of biotin containing chemical cross-linking reagents to study protein-protein interactions

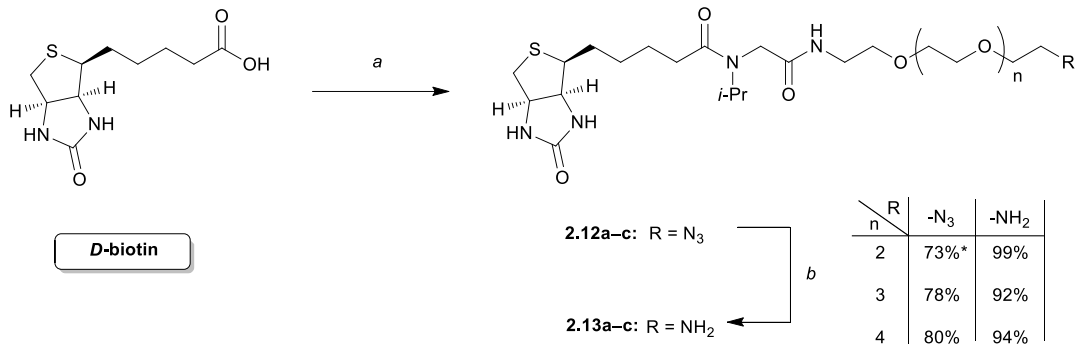
Parts of the following results have already been published in Analytical and Bioanalytical Chemistry entitled *"Multidimensional nano-HPLC coupled with tandem mass spectrometry for analyzing biotinylated proteins"* (Sproß, Brauch *et al.*, *Anal. Bioanal. Chem.* **2013**, *405*, 2163–2173).

The interaction of biotin (vitamin H) and avidin is well known as the strongest non-covalent interaction between a protein and a ligand ($K_d = 10^{15}\ M^{-1}$). Hence, the outstanding affinity of avidin (or streptavidin) for biotin has found widespread applications for the detection, enrichment and purification of proteins, virus particles and other small biomolecules.[15] Once a biotin-tag is attached to a biomolecule of interest, enrichment and/or purification using immobilised avidin or detection *via* western blotting techniques is enabled. For this purpose, selective biotin labels were developed equipped either with a variety of reactive groups which react selectively with a certain amino acid residue, *e.g.* lysine (NHS esters), cysteine (maleimide, iodoacetyl, pyridyl disulphide), glutamate or aspartate (amine). Moreover, bioorthogonal functional handles (*i.e.* azide, alkyne), which enable the site-selective modification of proteins or other biomolecules bearing a complementary bioorthogonal functional group, have been incorporated into biotin. These biotin-tags are prepared, in general, using standard multi-step peptide coupling conditions. However, in the present study this approach proved to be rather unsuccessful because only very low amounts of the pure desired products could be isolated (in most cases the formation of substantial amounts of inseparable side-products, mostly dicyclohexylurea or diisopropylurea, was observed). Consequently, the applicability of the Ugi-4CR for the modification of biotin as an alternative to standard peptide coupling methods was investigated.

Modification of biotin utilising the Ugi-4CR

First, the potential of the application of biotin in the Ugi-4CR was evaluated, but the results were inconclusive with yields varying from 40–80% under standard conditions (methanol, stirring at room temperature overnight). As a consequence of the already described promotive effect of microwave heating on the Ugi-4CR, the effect of the microwave concerning the modification of biotin in the Ugi-4CR was further explored. In a similar fashion as described for the modification of rhodamine B and its derivatives (see chapter 2.2), azide containing, PEGylated biotin derivatives (**2.12a–c**) were prepared starting from commercially available biotin, *i*-propylamine, paraformaldehyde and N_3-PEG_n-isocyanide **2.3a–c**. The corresponding peptoids PEG_n-(biotin)-azide **2.12a–c** could be isolated in good yields (73–80%). In contrast to the modification of rhodamine B, where no effect on the overall yield was detectable while applying microwave conditions, an impressive improvement of the chemical yield could be observed when using biotin (34% yield at room temperature, 73% yield under microwave heating). If

necessary, to regain the Ugi-reactive functionality, the azide can be reduced *via* hydrogenolysis with palladium on charcoal to the corresponding amines **2.13a–c** in almost quantitative yield (Scheme 2.5).



n \ R	-N3	-NH2
2	73%*	99%
3	78%	92%
4	80%	94%

Scheme 2.5 Synthesis of PEG_n-(biotin)-azide and PEG_n-(biotin)-amine. *Reagents and conditions*: (a) para-formaldehyde, *i*-propylamine, N_3-PEG_n-isocyanide **2.3a-c**, MeOH, µW (100 °C), 1 h (*34% yield while performing the reaction in MeOH at room temperature); (b) H_2 (1 atm), Pd/C, MeOH, r.t., overnight.

When applying this optimised MCR-protocol for the modification of biotin, a variety of different affinity-tags equipped either with side chain selective reactive groups or with bioorthogonal functional handles, similar to the previously described modification of rhodamine B and derivatives thereof, should be easily accessible as well. In the context of modifying biotin utilising the Ugi-4CR, however, the synthesis of chemical cross-linking-reagents to study protein-protein interactions or to draw conclusions on the three-dimensional structure of a protein was intended. The obtained cross-linking reagent were subsequently evaluated towards their potential to cross-link two amino acid residues in close spatial proximity within a protein or between a protein and an interacting peptide in the lab of Andrea Sinz (Department of Pharmacy, Martin-Luther University Halle-Wittenberg).

Synthesis and application of the homo-reactive, trifunctional cross-linker PEG_4-(biotin)-NHS_2

As stated in chapter 1.3, chemical cross-linking in combination with mass spectrometry is a valuable tool in chemical biology to study a proteins three-dimensional structure and to identify its binding partners. For this purpose, specifically designed reagents (*i.e.* homo- and heterobifunctional cross-linking reagents; cf. chapter 1.3) are required which enable the formation of a covalent bond between functional groups of amino acids either within a protein (to elucidate protein conformation) or between different interaction partners (to identify protein complexes). Afterwards the cross-linked proteins are digested

enzymatically and the corresponding cross-linked peptides are analysed by means of LC/MS/MS. However, subsequent MS analysis might be hampered as a consequence of the high complexity of a sample containing modified as well as unmodified peptides. In this case, enrichment of the cross-linked species by affinity purification is able to drastically reduce the complexity of a biological sample.[15,25] For the subsequent enrichment of the cross-linked peptides using immobilised avidin, trifunctional chemical cross-linking reagents were consequently developed, which are equipped with a biotin handle and a variety of different reactive functional groups, *e.g.* amine reactive NHS esters, thiol-reactive maleimides or photoreactive groups (Fig. 2.11).[15,25,26]

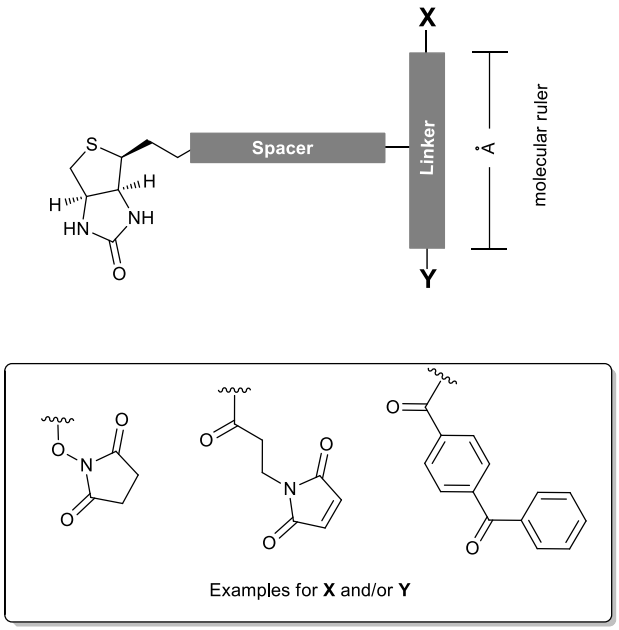
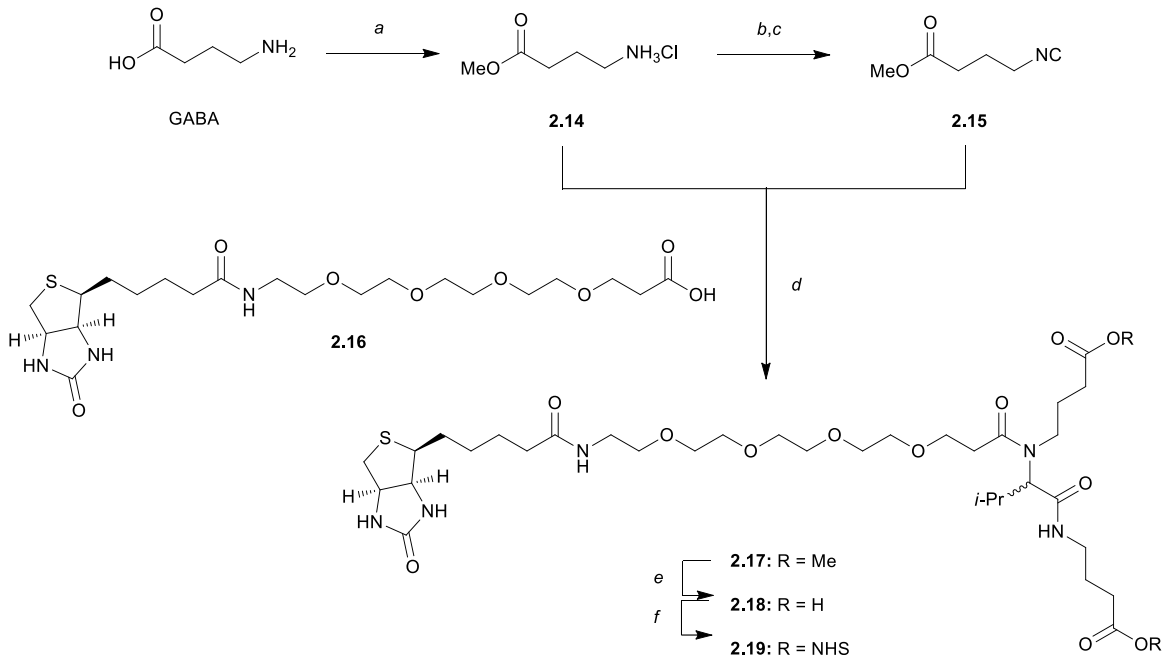


Fig. 2.11 Modular design of trifunctional chemical cross-linking reagents.

As a consequence of the modular design of these trifunctional cross-linking reagents, the application of a highly versatile one-pot multicomponent reaction, more specifically the Ugi-4CR, was envisioned to facilitate the synthesis of these reagents. In the course of the present study, the biotinylated, trifunctional cross-linker PEG_4-(biotin)-NHS_2 **2.18** was developed and evaluated towards its potential to cross-link proteins.

Synthesis of the biotinylated cross-linker PEG_4-(biotin)-NHS_2. Initially, the amine and isocyanide building blocks **2.14** and **2.15**, respectively, were prepared in a short reaction sequence starting from commercially available γ-amino butyric acid (GABA). To afford the methyl ester protected amine building block **2.14**, GABA was treated with thionyl chloride in methanol at room temperature (97% yield). Afterwards the methyl ester **2.14** was converted into the corresponding isocyanide **2.15** in a two-step sequence (formylation and subsequent dehydratisation with phosphorous oxychloride) in 70% overall yield (Scheme 2.6). Subsequent combination of all four building blocks (*i*-butyraldehyde, amine **2.14**, isocyanide **2.15** and the commercially available 15-[*D*(+)-biotinylamino]-4,7,10,13-tetraoxapentadecanoic acid **2.16**) in methanol in the presence of equimolar amounts of triethylamine led to the formation of the methyl ester protected precursor

PEG_4-(biotin)-$(COOMe)_2$ **2.17** in 51% yield. After alkaline cleavage of the methyl ester protective groups, the free dicarboxylic acid PEG_4-(biotin)-$(COOH)_2$ **2.18** was treated with *N*-hydroxysuccinimide under standard coupling conditions using EDC and DMAP affording the activated PEG_4-(biotin)-NHS_2 **2.19** in 87% yield and sufficient purity (Scheme 2.6).[27]



Scheme 2.6 Synthesis of the homo-reactive cross-linker PEG_4-(biotin)-NHS_2. *Reagents and conditions*: (a) thionyl chloride, MeOH, r.t., overnight (97%); (b) trimethyl orthoformiate, reflux, 4 h (99%); (c) diisopropylamine, $POCl_3$, CH_2Cl_2, r.t., 2 h (73%); (d) *i*-butyraldehyde, Et_3N, µW (100 °C), 1 h (51%); (e) LiOH·H_2O, THF/H_2O (1/1 v/v), r.t., 2.5 h (46%); (f) *N*-hydroxysuccinimide, DMAP, EDC, CH_2Cl_2, r.t., 24 h, 87%.

Application of PEG_4-(biotin)-NHS_2 for the enrichment of cross-linked peptides. To evaluate the potential of the biotinylated, trifunctional cross-linker PEG_4-(biotin)-NHS_2 **2.19**, cytochrome *c* was used in the cross-linking reaction (mass increase $C_{34}H_{55}N_5O_{10}S$ 725.3670 Da, spacer length ~ 16.5 Å). While monitoring the progress of the cross-linking reaction by MALDI-TOF-MS, an average incorporation of two cross-linker molecules per cytochrome *c* could be detected (Fig. 2.12A). Moreover, to identify the cross-linked amino acid residues within cytochrome *c*, the protein was digested with trypsin and the corresponding biotinylated, cross-linked peptides were enriched and subsequently analysed by 2D-nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS with a monomeric avidin monolithic affinity column in the first dimension and a RP18 column in the second dimension. Altogether, five cross-links within cytochrome *c* with C_α–C_α distances ranging

between 8.3 Å and 17.4 Å could be identified, which are in good agreement with the X-ray structure of unmodified cytochrome *c* (Table 2.2, Fig. 2.12B).[27]

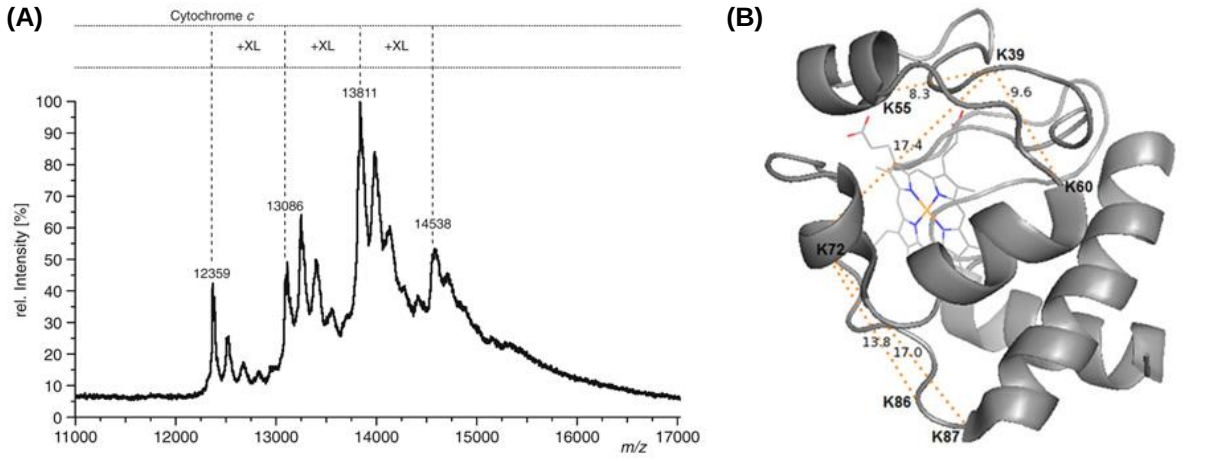


Fig. 2.12 Chemical cross-linking of cytochrome *c* with biotinylated, trifunctional cross-linking reagents. (A) MALDI-TOF mass spectra of cytochrome *c* cross-linked with cross-linker **2.19** after 15 min reaction time; and **(B)** X-ray structure of cytochrome *c* (pdb entry: 1HRC). Cross-links: orange dotted lines; the heme group is shown as sticks. Distances are given in Å (cross-linking experiments performed by Dr. Jens Sproß, MLU Halle). (Reproduced from Sproß *et al.*, *Anal. Bioanal. Chem.* **2013**, *405*, 2163−2173 with permission of Springer Science and Business Media)

Table 2.2 Intramolecular cross-linked peptides within cytochrome *c*, identified with 2D-nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS.

Calcd. $[M+H]^+$	*z*	Δ / ppm	Cyt *c* sequence A	Cyt *c* sequence B	C_α–C_α distance (Å)
3,383.790	4/5	0.8/0.1	61−73 [K72]	80−88 [K86/87]	13.8/17.0
3,875.996	4	1.5	40−55 [K53]	74−86 [K79]	11.6
4,532.306	3/4/5	1.3/1.7/1.2	56−73 [K60]	39−53 [K39]	9.6
4,646.306	4/5	1.3/0.5	56−72 [K72]	39−55 [K39]	17.4
4,774.399	4/5/6	-0.1/0.3/0.2	54−73 [K55/72]	39−53 [K39]	8.3/17.4

The trifunctional cross-linker PEG_4-(biotin)-NHS_2 **2.19** was additionally used to analyse a 20 kDa protein-peptide complex between the calcium-sensing protein calmodulin (CaM) and a small synthetic peptide having the same amino acid sequence as the CaM-binding domain of skeletal muscle myosin light chain kinase (skMLCK). Again, the progress of the cross-linking reaction was monitored by MALDI-TOF-MS (Fig. 2.13A). Besides the incorporation of the cross-linker into calmodulin, peptides corresponding to successfully cross-linked CaM/skMLCK could be detected as well. After affinity

enrichment and subsequent MS fragmentation using the aforementioned 2D-nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS system, three cross-links between CaM and the skMLCK peptide were identified with C_α–C_α distances (15.3 Å and 20.6 Å) which can be bridged by the cross-linker (Table 2.3, Fig. 2.13B).[27]

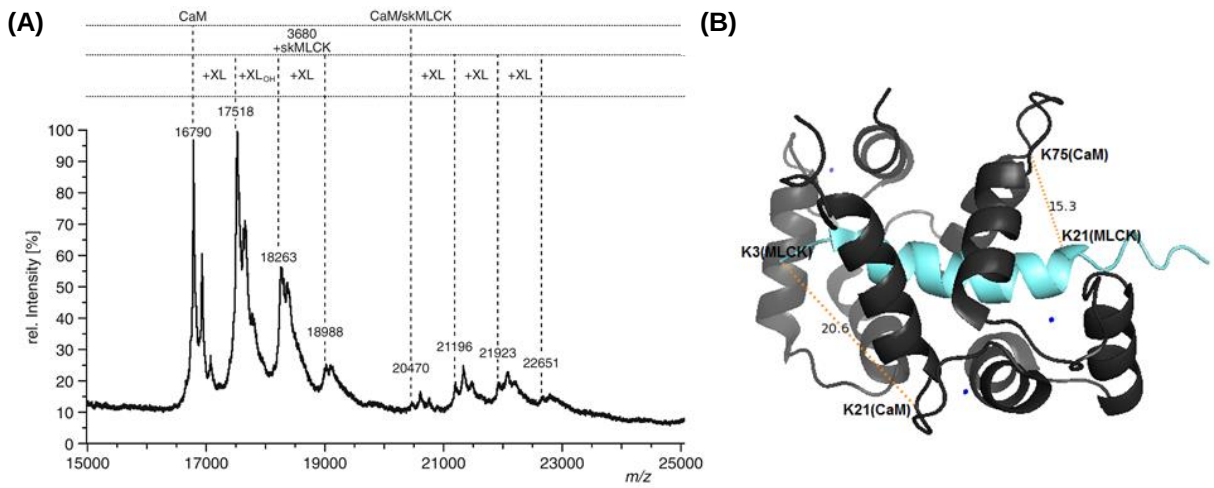


Fig. 2.13 Chemical cross-linking of a CaM/skMCLK peptide complex. (A) MALDI-TOF mass spectra of a cross-linked CaM/skMCLK peptide complex after 15 min reaction time; and **(B)** X-ray structure of a CaM/skMCLK peptide complex (pdb entry: 2BBM). Cross-links: orange dotted lines; calcium-ions are shown as blue spheres. Distances are given in Å (cross-linking experiments performed by Dr. Jens Sproß, MLU Halle). (Reproduced from Sproß *et al*., *Anal. Bioanal. Chem.* **2013**, *405*, 2163−2173 with permission of Springer Science and Business Media)

Table 2.3 Intermolecular cross-linked peptides between CaM and a small peptide representing the CaM-binding domain of skMLCK, identified with 2D-nano-HPLC/nano-ESI-LTQ-Orbitrap-MS/MS.

Calcd. $[M+H]^+$	*z*	Δ / ppm	CaM sequence	skMLCK sequence	C_α–C_α distance (Å)
2,380.290	4/5	3.8/3.3	75−77 [K75]	21−33 [K21]	15.3
3,438.742	4	2.0	75−86 [K75]	21−33 [K21]	15.3
3,870.092	5/6	4.9/4.0	14−21 [K21]	1−18 [K3]	20.6

2.4 Conclusions

With the growing impact of chemical proteomics, which enables the investigation of the quantitative/qualitative composition of the proteome and of small molecule-protein as well as protein-protein interactions, *etc.*, the demand of specifically designed small-molecular probes is of increasing importance in chemical biology. For this purpose, easy, fast and reliable techniques to synthesise such multi-functionalised chemical probes are highly appreciated. Based on the modular design of such probes, the application of

multicomponent reactions, more specifically the well-documented Ugi-4CR, was envisioned to synthesis libraries of such structural diverse chemical probes which enable the covalent modification of proteins with suitable fluorophores or affinity-tags. Moreover, specifically designed reagents can be used for chemical cross-linking purposes to study a protein`s three dimensional structure as well as to identify interacting proteins/peptides and their respective binding sites.

In the first part of the present study concerning the MCR-based synthesis of functional tools for the covalent modification of proteins, the applicability of the Ugi-4CR for the facile modification of rhodamine-based fluorophores was explored. The synthesis of an amine-reactive fluorescent probe equipped with an NHS ester (**2.6**) could be successfully accomplished, which reacts selectively with a protein`s free lysine residues and its *N*-terminus to produce a covalent linkage between the protein and the chemical probe. To demonstrate the reactivity of the obtained fluorescent-tag, purified protein BSA was treated with compound **2.6** under near physiological conditions. The results were compared to classical coomassie-blue staining afterwards.

In addition, "click-handles" such as azides (**2.7a/b**, **2.10**, **2.11**) and alkynes (**2.8**, **2.9**) could be swiftly incorporated utilising the Ugi-4CR. These ready-to-use fluorescent-tags are of high value for various *in vivo* or *in vitro* applications, *e.g.* activity-based protein profiling or the covalent labelling of azide or alkyne modified biomolecules either *via* CuAAC or SPAAC.[18,28] As a consequence of the tertiary amide bond, which is inherent to all Ugi-products, no loss of fluorescence was observed under basic conditions. This, however, has been previously described for rhodamine dyes with secondary peptide bonds.

The general applicability of this MCR-approach has been shown by modifying other rhodamine derivatives, *i.e.* rhodamine 19P and rhodamine 101.

Based on the Ugi-4CR, a protocol was developed which enables the fast modification of biotin without the need of toxic or expensive coupling reagents under very mild conditions. The optimised protocol was used to synthesise the biotinylated, trifunctional cross-linking reagent PEG_4-(biotin)-NHS_2 **2.19** equipped with two amine-reactive NHS esters for the covalent modification of free lysine residues within a protein or between a protein and its binding partner. The reactivity of the cross-linker was evaluated using either a single purified protein (cytochrome *c*) or a protein-peptide complex (CaM/skMCLK). After tryptic digestion and affinity enrichment, five cross-links within

cytochrome *c* and three cross-links between CaM and the skMLCK peptide could be identified by MS fragmentation.

Overall, the applicability of the Ugi-4CR for the preparation of small-molecular probes used in chemical biology could be successfully demonstrated. This fast, efficient, facile and highly diverse method for producing chemical probes can be performed even by inexperienced chemists or biologists, hence allowing a broad scientific audience to make use of this methodology.

2.5 Experimental section

General experimentel information

Unless otherwise stated all chemicals and solvents were obtained from commercial sources and were used without further purification. All ^{1}H and ^{13}C NMR spectra were recorded in CHLOROFORM-*d* (unless otherwise noted) on either a 300 MHz Varian MERCURY-VX 300 apparatus (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR, respectively) or on a 400 MHz Varian MERCURY-VX 400 apparatus (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, respectively). Chemical shifts are reported in δ values (ppm) with tetramethylsilane (TMS) as internal standard. Melting points were determined with a Leica DM LS2 hot stage microscope (without correction). ESI-MS spectra were obtained from an API-150EX spectrometer. HRMS spectra were recorded on an FT-ICR Bruker Apex III 70e mass spectrometer. Absorption spectra were recorded on a Jasco V-560 UV/VIS spectrophotometer. Fluorescence was measured on a Perkin Elmer Luminescence Spectrometer LS50B. Reactions under microwave heating were performed in an Emrys Microwave reactor (Biotage). Purification of the crude products by column chromatography was performed on silica gel 60 (230–400 mesh, 0.040–0.063 mm), Merck, Germany. TLC identification of products and reactants was performed on silica gel coated aluminium foil (silica gel 60 F_{254} with fluorescence indicator), Merck, Germany. Acetylene-PEG_4-amine was purchased from Jena Bioscience, dibenzylcyclooctyne-PEG_4-amine from Click Chemistry Tools and PEG_4-(biotin)-COOH from PolyPeptide.

General procedure A: Synthesis of PEGylated diazides from glycol precursors.

Under a nitrogen atmosphere, triethylamine (2.2 equiv.) in dry tetrahydrofuran (20 mL) was added to a solution containing glycol (n = 2, 3 or 4) and methanesulphonyl chloride (2.2 equiv.) in dry tetrahydrofuran (80 mL) at 0 °C. The resulting solution was stirred for 1 hour at 0 °C and for another 3 hours at room temperature. Next, distilled water (80 mL),

solid sodium hydrogen carbonate (0.54 equiv.) and sodium azide (2.2 equiv.) were added to the reaction mixture. The organic solvent was removed under reduced pressure and the remaining aqueous solution was heated at 80 °C during 12 hours. Afterwards the aqueous phase was extracted with dichloromethane (4x 200 mL) and the combined extracts were dried over sodium sulphate. The organic solvent was removed under reduced pressure to afford the diazido-PEGylated compounds **2.1a–c**, which were stored at −30 °C.

General procedure B: Staudinger reduction.

Under a nitrogen atmosphere, a suspension of the diazido-PEGylated compounds **2.1a–c** in 0.65 M aqueous phosphoric acid (100 mL) was cooled to 0 °C. Triphenylphosphine (0.86 equiv.) in dry diethyl ether (100 mL) was subsequently added dropwise. After complete addition, the reaction mixture was allowed to stir at room temperature for an additional 24 hours. The organic phase was separated and the aqueous phase was washed with diethyl ether (3x 100 mL). To the aqueous phase solid potassium hydroxide (3.5 equiv.) was added and traces of organic solvent were removed under reduced pressure. The remaining aqueous solution was placed at 0 °C for 24 hours, after which time a solid had formed which was removed by filtration. The filtrate was transferred in an aqueous 4 M sodium hydroxide solution by addition of solid sodium hydroxide and extracted with dichloromethane (16x 50 mL). The combined organic extracts were dried over sodium sulphate and the solvent was removed under reduced pressure. The residue was purified on silica to afford the mono-amine as a light yellow liquid, which was stored at −30 °C.

General procedure C: Conversion of an amine into its corresponding isocyanide.

A solution of the amines **2.2a–c** in ethyl formiate (20 mL) was refluxed for 3 hours, whereupon all volatiles were removed by rotary evaporation to yield the corresponding formamides as slightly red liquids. The liquid was dissolved in dry dichloromethane (60 mL) and placed under a nitrogen atmosphere. Diisopropylamine (3 equiv.) was added and the reaction mixture was cooled to 0 °C. After the dropwise addition of phosphorus oxychloride (1.2 equiv.), the reaction mixture was warmed to room temperature and allowed to stir for an additional 2 hours. The reaction was quenched with an aqueous sodium carbonate solution (6.5 g in 35 mL distilled water) and the resulting suspension was stirred for further 30 minutes followed by the addition of water. Afterwards the

organic layer was separated and the aqueous phase was extracted with dichloromethane (3x 40 mL). The combined organic extracts were dried over sodium sulphate and the organic solvent was evaporated *in vacuo*. The remaining residue was purified by column chromatography.

General Procedure D: Ugi-4CR

A solution containing aldehyde (1 equiv.) and primary amine (1 equiv.) in methanol was stirred at room temperature for 2 hours to preform the imine. After the addition of isocyanide (1 equiv.) and carboxylic acid (1 equiv.), the resulting mixture was allowed to stir at room temperature overnight. Finally, the solvent was evaporated and the residue was purified by column chromatography.

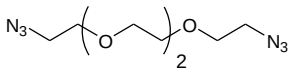
General Procedure E: Microwave-assisted Ugi-4CR

Imine formation was performed as described in the General Procedure above. Isocyanide (1 equiv.) and carboxylic acid (1 equiv.) were subsequently added and the resulting mixture was heated in a microwave apparatus (1 hour, 100 °C). After evaporation of all volatile material, the residue was purified by column chromatography.

General Procedure F: Azide reduction *via* hydrogenolysis

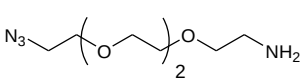
A solution containing the organic azide and 10 wt-% palladium on charcoal (10%) in methanol was stirred overnight at room temperature under hydrogen atmosphere. The reaction mixture was filtered through a pad of celite® and all volatiles were removed *in vacuo* to afford the corresponding amines in high purity and excellent yields.

1-Azido-2-{2-[2-(2-azidoethoxy)ethoxy]ethoxy}ethane (2.1a)



Starting from tetraethylene glycol (17.3 mL, 100 mmol), diazide **2.1a** was obtained according to the General Procedure A as a light yellow liquid (22.76 g, 93%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 3.32 (m, 4H), 3.61 (m, 12H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 50.4, 69.8, 70.4.

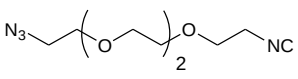
2-{2-[2-(2-Azidoethoxy)ethoxy]ethoxy}ethanamine (2.2a)



A suspension of **2.1a** (21.7 g, 88.8 mmol) in 0.65 M phosphoric acid (210 mL) was treated with triphenylphosphine as described in the General Procedure B. Purification by column chromatography afforded the desired amine **2.2a** as a yellow liquid (14.99 g, 77%). R_F = 0.64 (dichloromethane/methanol/

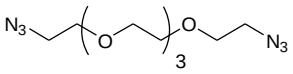
triethylamine 8/1/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 2.44 (s, 2H), 2.87 (t, *J* = 5.3 Hz, 2H), 3.61 (m, 2H), 3.53 (m, 2H), 3.67 (m, 10 H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 41.4, 50.4, 61.1, 69.8, 70.3, 70.4, 71.0, 72.4, 72.9; ESI-MS m/z: 219.2 $[M+H]^+$; HRMS (ESI) calcd for $C_8H_{19}N_4O_3$ 219.1452, found 219.1447.

1-Azido-2-{2-[2-(2-isocyanoethoxy)ethoxy]ethoxy} ethane (2.3a)



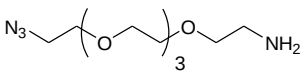
Amine **2.2a** (7.5 g, 34.4 mmol) was transferred into its corresponding isocyanide according to the General Procedure C. Purification by column chromatography afforded compound **2.3a** as a yellow liquid (3.25 g, 41%). R_F = 0.67 (hexane/ethyl acetate 1/4); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 3.40 (m, 2H), 3.58 (m, 2H), 3.66–3.74 (m, 12H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 41.6, 50.5, 68.5, 69.9, 70.5, 70.6, 70.7.

1,14-Diazido-3,6,9,12-tetraoxatetradecane (2.1b)



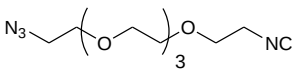
Starting from pentaethylene glycol (20.0 g, 83.9 mmol), diazide **2.1b** was obtained according to the General Procedure A as a slightly yellow liquid (20.29 g, 83%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 3.39 (m, 4H), 3.67 (m, 16H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 50.5, 69.9, 70.5, 70.6.

14-Azido-3,6,9,12-tetraoxatetradecan-1-amine (2.2b)



A suspension of **2.1b** (16.6 g, 57.6 mmol) in 0.65 M phosphoric acid (180 mL) was treated with triphenylphosphine as described in the General Procedure B. Purification by column chromatography afforded the desired amine **2.2b** as a yellow liquid (6.49 g, 43%). R_F = 0.58 (dichloromethane /methanol/ triethylamine 8/1/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 2.17 (s, 2H), 2.88 (t, *J* = 5.3 Hz, 2H), 3.40 (m, 2H), 3.53 (m, 2H), 3.68 (m, 14H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 41.6, 50.6, 70.0, 70.2, 70.5, 70.6, 70.7, 73.0; ESI-MS m/z: 263.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{10}H_{23}N_4O_4$ 263.1714, found 263.1713.

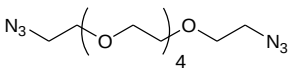
1-Azido-14-isocyano-3,6,9,12-tetraoxatetradecane (2.3b)



Amine **2.2b** (4.0 g, 15.3 mmol) was transferred into its corresponding isocyanide according to the General Procedure C to yield **2.3b** as a yellow liquid (2.78 g, 67%) after purification by column chromatography. R_F = 0.26 (petrol ether/ethyl acetate 1/4); ^{1}H NMR (300 MHz,

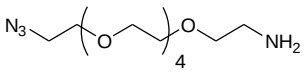
CHLOROFORM-*d*) *δ*: 3.39 (t, *J* = 5.3 Hz, 2H), 3.58 (m, 2H), 3.67 (m, 16H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 41.7, 50.6, 68.5, 69.9, 70.5, 70.6, 70.8.

1,17-Diazido-3,6,9,12,15-pentaoxaheptadecane (2.1c)



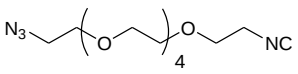
Starting from commercially available hexaethylene glycol (13 mL, 46.6 mmol), diazide **2.1c** was obtained according to the General procedure A as a slightly yellow liquid (14.35 g, 87%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 3.39 (t, *J* = 5.0 Hz, 4H), 3.67 (m, 20H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 50.6, 70.0, 70.5, 70.6.

17-Diazido-3,6,9,12,15-pentaoxaheptadecan-1-amine (2.2c)



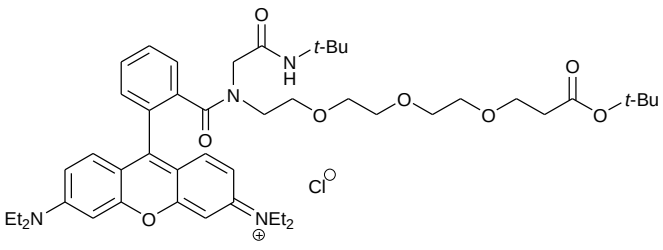
A suspension of **2.1c** (13.33 g, 40.1 mmol) in 0.65 M phosphoric acid (100 mL) was treated with triphenylphosphine as described in the General Procedure B. Purification by column chromatography afforded the desired amine **2.2c** as a light yellow liquid (4.61 g, 38%). R_F = 0.57 (dichloromethane /methanol/ triethylamine 8/1/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 1.92 (s, 2H), 2.88 (t, *J* = 5.3 Hz, 2H), 3.40 (t, *J* = 5.3 Hz, 2H), 3.53 (t, *J* = 5.3 Hz, 2H), 3.67 (m, 18H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 41.6, 50.6, 69.9, 70.2, 70.5, 70.6, 73.1; ESI-MS m/z: 307.0 $[M+H]^+$; HRMS (ESI) calcd for $C_{12}H_{27}N_4O_5$ 307.1976, found 307.1975.

1-Azido-17-isocyano-3,6,9,12,15-pentaoxaheptadecane (2.3c)



Amine **2.2c** (3.9 g, 12.7 mmol) was transferred into its corresponding isocyanide according to the General Procedure C to yield compound **2.3c** as a yellow liquid (2.38 g, 63%) after purification by column chromatography. R_F = 0.30 (hexane/ethyl acetate 1/4); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 3.39 (m, 2H), 3.58 (m, 2H), 3.66–3.74 (m, 20H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 41.7, 50.6, 68.6, 70.0, 70.5, 70.6, 70.8; ESI-MS m/z: 317.2 $[M+H]^+$, 339.2 M+Na]$^+$; HRMS (ESI) calcd for $C_{13}H_{24}N_4O_5Na$ 339.1639, found 339.1638.

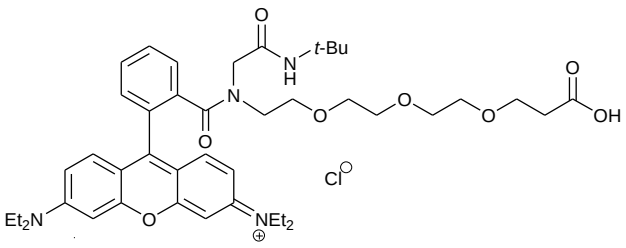
PEG$_3$-(rhodamine B)-COO^{t}Bu (2.4)



A mixture containing rhodamine B (481mg, 1.00 mmol), para-formaldehyde (30.0 mg, 1.00 mmol), *t*-butyl isocyanide (0.12 mL, 1.06 mmol) and *t*-butyl 12-amino-

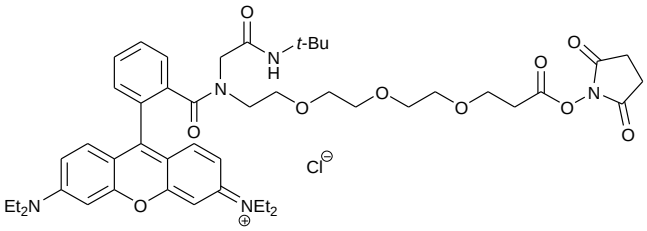
4,7,10-trioxadodecanoate (357 mg, 1.03 mmol) in methanol (3 mL) was reacted according to the General Procedure E to yield after purification by column chromatography compound **2.4** as a dark purple semi-solid (540 mg, 63%). R_F = 0.28 (dichloromethane/ methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.33 (t, *J* = 7.0 Hz, 12H), 1.41 (s, 9H), 1.44 (s, 9H), 2.49 (t, *J* = 6.7 Hz, 2H), 3.07 (t, *J* = 6.9 Hz, 2H), 3.28 (t, *J* = 5.1 Hz, 2H), 3.31–3.40 (m, 2H), 3.46 (t, *J* = 5.0 Hz, 2H), 3.50–3.64 (m, 10H), 3.65–3.73 (m, 4H), 4.36 (s, 2H), 6.67 (s, 2H), 7.27–7.31 (m, 1H), 7.34–7.45 (m, 3H), 7.55–7.60 (m, 2H), 7.87 (dd, *J* = 5.7, 3.1 Hz, 1H), 8.64 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 12.6, 28.0, 28.6, 36.2, 45.1, 46.0, 51.2, 54.0, 66.8, 67.0, 69.9, 70.1, 70.2, 70.3, 95.5, 113.9, 127.8, 129.1, 129.2, 129.8, 130.7, 132.4, 136.9, 155.6, 156.7, 157.6, 167.6, 169.2, 170.8; ESI-MS m/z: 815.6 $[M]^+$; HRMS (ESI) calcd for $C_{47}H_{67}O_8N_4$ 815.4953, found 815.4939.

PEG$_3$-(rhodamine B)-COOH (2.5)



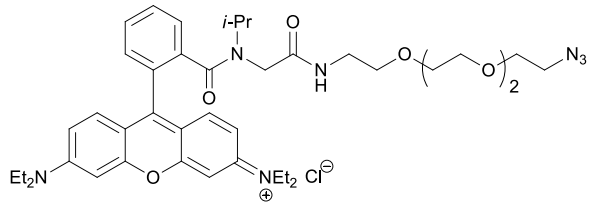
To a solution containing *t*-butyl ester **2.4** (101 mg, 0.12 mmol) in toluene (2 mL) was added phosphoric acid (85% v/v, 0.04 mL, 0.59 mmol) and the reaction mixture was stirred at room temperature for 3 hours. After completion of the reaction (indicated by TLC analysis), distilled water (20 mL) was added and the aqueous phase was washed with ethyl acetate (3x 20 mL). Afterwards the aqueous phase was saturated with solid sodium chloride and extracted with isopropanol/dichloromethane (2/1 v/v). The organic layer was dried (sodium sulphate) and the solvent was evaporated. The residue was taken up in a small volume of chloroform and filtered to remove residual inorganic salts. Evaporation of the filtrate afforded the free carboxylic acid as dark purple oil, which was used directly in the next step without further purification (62.3 mg, 65%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.33 (t, *J* = 6.9 Hz, 12H,), 1.40 (s, 9H), 2.66 (t, *J* = 6.0 Hz, 2H), 3.11 (t, *J* = 5.9 Hz , 2H), 3.18–3.24 (m, 2H), 3.37–3.50 (m, 4H), 3.52–3.81 (m, 14 H), 4.28 (s, 2H), 6.68 (s, 2H), 7.26–7.31 (m, 2H), 7.38 (d, *J* = 9.4 Hz , 2H), 7.59 (dd, *J* = 5.3, 3.5 Hz , 2H), 7.86 (dd, *J* = 5.7, 3.1 Hz , 1H), 7.95 (br. s., 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 12.7, 28.7, 35.6, 45.5, 46.0, 51.3, 53.8, 66.8, 68.4, 70.2, 70.3, 70.4, 70.7, 95.6, 114.0, 127.8, 129.4, 129.9, 130.7, 132.5, 136.4, 155.6, 156.9, 157.6, 167.9, 169.3, 173.4; ESI-MS m/z: 759.6 $[M]^+$; HRMS (ESI) calcd for $C_{43}H_{59}O_8N_4$ 759.4327, found 759.4314.

PEG$_3$-(rhodamine B)-NHS (2.6)



To an ice-cold solution containing carboxylic acid **2.5** (50.5 mg, 63.5 µmol), *N*-hydroxysuccinimide (11.2 mg, 97.3 µmol) and catalytic amounts of 4-dimethylaminopyridine in dry dichloromethane (5 mL) was added 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (19.4 mg, 101.2 µmol) and the ice bath was removed. Stirring was continued at room temperature overnight. The organic solvent was washed consecutively with 1 mL aqueous hydrochloric acid (1 M) and 2 mL distilled water. Finally, the organic phase was dried (sodium sulphate) and evaporated to yield **2.6** as dark purple semi-solid, which was stored under an inert-gas atmosphere (42.2 mg, 75%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.30–1.37 (m, 12H), 1.41 (s, 9H), 2.81–2.85 (m, 4H), 2.88 (t, J = 6.6 Hz, 2H), 3.08 (t, J = 6.9 Hz , 2H), 3.28 (t, J = 5.1 Hz, 2H), 3.35 (br. s., 2H), 3.43–3.48 (m, 2H), 3.49–3.70 (m, 14H), 3.82 (t, J = 6.4 Hz , 2H), 4.35 (s, 2H), 6.67 (s, 2H), 7.26–7.30 (m, 2H), 7.35–7.40 (m, 2H), 7.57 (dd, J = 5.7, 3.4 Hz , 2H), 7.87 (dd, J = 5.9, 2.9 Hz, 1H), 8.58 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 12.7, 25.4, 25.6, 28.7, 32.1, 45.2, 46.1, 51.3, 54.0, 65.7, 67.0, 69.9, 70.3, 70.4, 70.6, 95.5, 114.0, 127.9, 129.2, 129.9, 130.7, 132.5, 137.1, 155.7, 156.9, 157.7, 166.7, 167.7, 168.9, 169.3, 169.7; ESI-MS m/z: 856.5 $[M]^+$; HRMS (ESI) calcd for $C_{47}H_{62}O_{10}N_5$ 856.4491, found 856.4498.

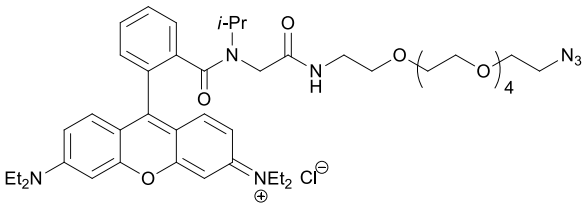
PEG$_3$-(rhodamine B)-azide (2.7a)



A mixture containing paraformaldehyde (12 mg, 0.40 mmol), *i*-propylamine (30 µL, 0.40 mmol), rhodamine B (192 mg, 0.40 mmol) and isocyanide **2.3a** (91.3 mg, 0.40 mmol) in methanol (2 mL) was reacted according to the General Procedure E to yield after purification by column chromatography azide **2.7a** as dark purple oil (230 mg, 74%). R_F = 0.26 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 0.66–0.99 (m, 6H), 1.33 (t, J = 7.0 Hz, 12H), 3.40 (t, J = 5.1 Hz, 2H), 3.44–3.81 (m, 22H), 4.11–4.24 (m, 1H), 4.29 (s, 2H), 6.65 (s, 2H), 7.29–7.36 (m, 4H), 7.53–7.58 (m, 2H), 8.03–8.05 (m, 1H), 9.05 (t, J = 5.6 Hz, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 12.7, 18.6, 38.9, 46.1, 47.0, 49.5, 50.7, 53.4, 69.4, 69.9, 70.2, 70.6, 95.6, 114.0, 128.1, 128.9, 129.0, 130.0,

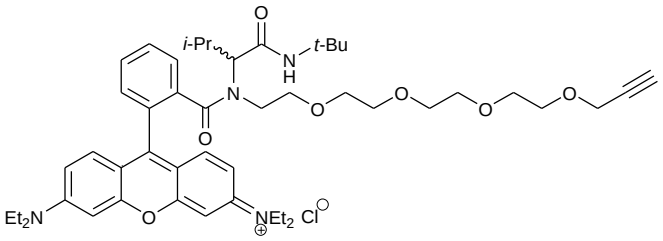
132.3, 137.9, 155.6, 157.4, 157.6, 169.3, 170.1; ESI-MS m/z: 742.6 $[M]^+$; HRMS (ESI) calcd for $C_{41}H_{56}O_6N_7$ 742.4287, found 742.4287.

PEG$_5$-(rhodamine B)-azide (2.7b).



A mixture containing paraformaldehyde (30 mg, 1.00 mmol), *i*-propylamine (86 µL, 1.00 mmol), isocyanide **2.3c** (325.5 mg, 1.03 mmol) and rhodamine B (481 mg, 1.00 mmol) in methanol (5 mL) was reacted according to the General Procedure E to yield after purification by column chromatography azide **2.7b** as dark purple oil (607 mg, 70%). R_F = 0.45 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 0.74–0.95 (m, 6H), 1.33 (t, *J* = 7.0 Hz, 12H), 3.40 (m, 2H), 3.53–3.69 (m, 30H), 4.19 (m, 1H), 4.26 (s, 2H), 6.65 (s, 2H), 7.25–7.31 (m, 1H), 7.31–7.38 (m, 3H), 7.54–7.60 (m, 2H), 8.01–8.06 (m, 1H), 8.99 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 12.6, 18.6, 30.9, 38.9, 46.1, 50.0, 50.6, 69.3, 69.9, 70.1, 70.5, 70.6, 95.6, 113.9, 128.1, 128.9, 129.0, 129.9, 130.7, 132.3, 155.6, 157.3, 157.6, 169.3, 170.1; ESI-MS m/z: 830.7 $[M]^+$; HRMS (ESI) calcd for $C_{45}H_{64}O_8N_7$ 830.4811, found 830.4806.

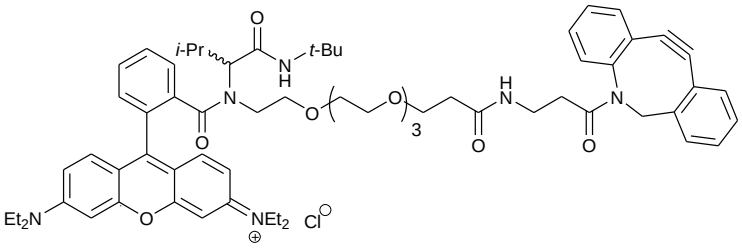
PEG$_4$-(rhodamine B)-acetylene (2.8)



A mixture containing acetylene-PEG$_4$-amine (100 mg, 0.43 mmol), *i*-butyraldehyde (39.6 µL, 0.43 mmol), rhodamine B (208 mg, 0.43 mmol) and *t*-butyl isocyanide (49.8 µL, 0.43 mmol) in methanol (3 mL) was reacted according to the General Procedure E to yield after purification by column chromatography alkyne **2.8** as purple oil (222 mg, 61%). R_F = 0.21 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) *δ*: 0.25–0.35 (m, 3H), 0.82 (d, *J* = 6.4 Hz, 3H), 1.17 (s, 9H), 1.28–1.41 (m, 12H), 2.17–2.35 (m, 1H), 2.45 (t, *J* = 2.3 Hz, 1H), 3.15–3.41 (m, 2H), 3.47–3.76 (m, 23H), 4.20 (d, *J* = 2.3 Hz, 2H), 6.75 (br. s., 1H), 6.79–6.83 (m, 2H), 6.90–7.01 (m, 2H), 7.26 (dd, *J* = 9.7, 7.3 Hz, 2H), 7.31–7.36 (m, 1 H), 7.63–7.77 (m, 3H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) *δ*: 12.6, 18.0, 19.7, 25.7, 28.3, 46.2, 50.6, 53.4, 58.3, 67.5, 69.0, 70.3, 70.4, 70.5, 74.5, 79.5, 96.1, 96.3, 113.4, 113.7, 114.2, 128.9, 129.7, 130.1, 132.5, 135.7, 155.1, 155.5, 155.6, 157.5, 157.6,

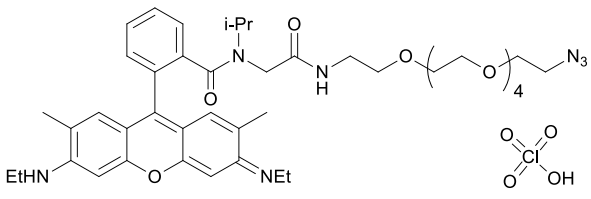
169.1, 171.0; ESI-MS m/z: 811.4 $[M]^+$; HRMS (ESI) calcd for $C_{48}H_{67}N_4O_7$ 811.5004, found 811.4983.

PEG$_4$-(rhodamine B)-dibenzylcyclooctyne (2.9)



A mixture containing *i*-butyraldehyde (3.65 mg, 0.05 mmol), rhodamine B (25.0 mg, 0.05 mmol), *t*-butyl isocyanide (4.20 mg, 0.05 mmol) and dibenzylcyclooctyne-PEG$_4$-amine (25.0 mg, 0.05 mmol) in methanol (10 mL) was reacted according to the General Procedure D to yield after purification by column chromatography cyclooctyne **2.9** as purple oil (42 mg, 73%). R_F = 0.30 (dichloromethane/ methanol 10/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.25–0.96 (m, 6H), 1.03–1.22 (m, 9H), 1.24–1.52 (m, 12H), 1.84–2.64 (m, 5H), 3.10–3.79 (m, 28H), 4.59–4.69 (m, 1H), 5.05–5.19 (m, 2H), 6.60–7.90 (m, 18H), 8.31 (br. s., 2H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 12.6, 17.4, 18.5, 19.7, 25.7, 28.3, 28.4, 34.7, 35.1, 36.7, 45.9, 46.0, 46.1, 51.2, 53.4, 55.4, 67.0, 67.6, 70.1, 70.3, 70.4, 80.1, 96.1, 96.2, 96.3, 113.5, 113.8, 114.1, 114.6, 125.4, 127.1, 127.7, 128.1, 128.2, 128.6, 129.0, 129.8, 130.1, 131.1, 131.2, 131.3, 132.0, 133.2, 133.7, 135.7, 148.0, 151.0, 155.3, 155.5, 155.7, 157.6, 157.7, 164.2, 167.5, 169.1, 170.8, 171.8; ESI-MS m/z: 1103.6 $[M]^+$; HRMS (ESI) calcd for $C_{66}H_{83}N_6O_9$ 1103.6216, found 1103.6187.

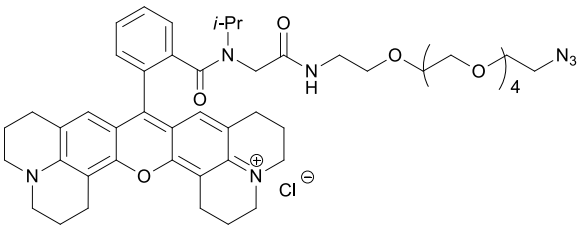
PEG$_5$-(rhodamine 19P)-azide (2.10)



A mixture containing paraformaldehyde (8.1 mg, 0.26 mmol), *i*-propylamine (21.9 µL, 0.27 mmol), rhodamine 19P (98.1 mg, 0.19 mmol) and isocyanide **2.3c** (64.5 mg, 0.20 mmol) in methanol (2 mL) was reacted according to the General Procedure E to yield after purification by column chromatography azide **2.10** as dark purple oil (140 mg, 81%). R_F = 0.25 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 0.67–1.04 (m, 6H), 1.39 (t, *J* = 7.2 Hz, 6H), 2.14–2.27 (m, 6H), 3.11–3.22 (m, 2H), 3.30–3.43 (m, 4H), 3.43–3.82 (m, 24H), 3.84–4.02 (m, 1H), 5.99 (br. s., 1H), 6.28 (t, *J* = 5.4 Hz, 1H), 6.35 (br. s, 1H), 6.66–6.75 (m, 2H), 6.91–7.01 (m, 2H), 7.35 (dd, *J* = 5.7, 2.8 Hz, 1H), 7.59–7.78 (m, 3H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 13.7,

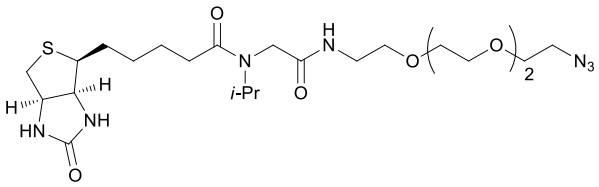
17.4, 20.7, 38.7, 39.1, 43.9, 50.6, 69.5, 69.9, 70.2, 70.4, 70.5, 70.6, 93.9, 114.0, 125.4, 127.1, 129.3, 129.7, 130.0, 130.7, 135.6, 154.5, 156.1, 157.3, 168.5, 169.0; ESI-MS m/z: 802.3 $[M]^+$; HRMS (ESI) calcd for $C_{43}H_{60}O_8N_7$ 802.4498, found 802.4478.

PEG$_5$-(rhodamine 101)-azide (2.11)



A mixture containing paraformaldehyde (13.2 mg, 0.42 mmol) and *i*-propylamine (36 µL, 0.42 mmol) in methanol (2 mL) was stirred at room temperature for 2 hours to preform the imine. To the formed imine was added a mixture containing isocyanide **2.3c** (134 mg, 0.43 mmol) and rhodamine 101 inner salt (206 mg, 0.42 mmol) in methanol (1 mL) previously acidified by the addition of conc. hydrochloric acid (34.6 µL). The resulting reaction mixture was heated in the microwave (1h at 100 °C). After evaporation of all volatile material, the residue was purified by column chromatography to yield azide **2.11** as purple oil (91 mg, 24%). R_F = 0.36 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 0.76–1.09 (m, 6H), 1.89–2.20 (m, 8H), 2.38–2.83 (m, 4H), 2.94–3.10 (m, 4H), 3.21–3.72 (m, 32H), 4.18 (s, 2H), 4.21–4.36 (m, 1H), 6.65–6.84 (m, 2H), 7.21–7.30 (m, 1H), 7.51–7.61 (m, 1H), 7.60–7.72 (m, 1H), 8.16–8.25 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 18.7, 19.5, 19.7, 20.4, 20.7, 38.8, 46.7, 48.3, 50.2, 50.5, 50.8, 51.0, 69.2, 69.8, 70.0, 70.4, 70.5, 104.7, 113.2, 123.2, 126.5, 126.9, 128.2, 128.8, 129.3, 129.4, 129.5, 131.0, 131.6, 135.8, 137.1, 150.9, 151.1, 151.8, 151.9, 152.8, 155.0, 168.6, 169.1, 169.9; ESI-MS m/z: 878.6 $[M]^+$; HRMS (ESI) calcd for $C_{49}H_{64}N_7O_8$ 878.4811, found 878.4812.

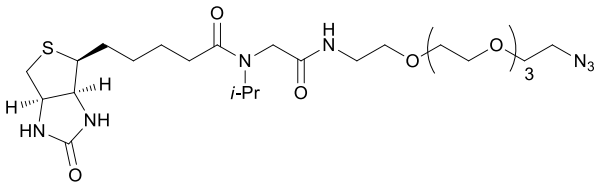
PEG$_3$-(biotin)-azide (2.12a)



A mixture containing paraformaldehyde (35.6 mg, 1.19 mmol), *i*-propylamine (0.10 mL, 1.17 mmol), biotin (244 mg, 1.00 mmol) and azido-PEG$_3$-isocyanide **2.3a** (231.0 mg, 1.01 mmol) in methanol (5 mL) was reacted according to the General Procedure E to yield after purification by column chromatography azide **2.12a** as pale yellow oil (394 mg, 73 %). R_F = 0.28 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.09 (m, 2H), 1.21 (dd, 4H, J = 6.5 Hz, 3.2 Hz), 1.48 (m, 2H), 1.70 (m, 4H), 2.25 (m, 1H), 2.45 (t, J = 7.6 Hz, 1H), 2.75 (m, 1H), 2.93 (m, 1H), 3.16 (m, 1H),

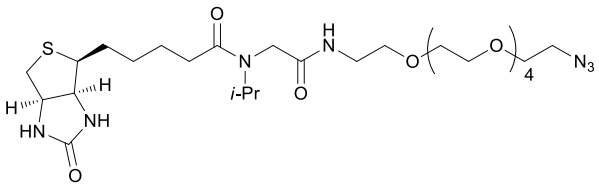
3.37–3.45 (m, 4H), 3.54 (m, 2H), 3.58–3.72 (m, 12H), 4.13 (m, 1H), 4.32 (m, 1H), 4.50 (m, 1H), 5.53 (m, 1H), 6.26 (m, 1H), 6.94 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 20.9, 25.0, 28.1, 28.2, 32.9, 39.1, 40.5, 44.7, 48.7, 50.6, 55.5, 60.1, 61.7, 69.5, 69.6, 70.0, 70.1, 70.5, 70.6, 163.7, 170.1, 173.5; ESI-MS m/z: 542.1 $[M-H]^-$, 544.5 $[M+H]^+$, 566.6 $[M+Na]^+$, 1109.6 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{23}H_{41}O_6N_7SNa$ 566.2731, found 566.2730.

PEG$_4$-(biotin)-azide (2.12b)



A mixture containing paraformaldehyde (76 mg, 2.40 mmol), *i*-propylamine (0.21 mg, 2.44 mmol), biotin (488 mg, 2.00 mmol) and azido-PEG$_4$-isocyanide **2.3b** (545 mg, 2.00 mmol) in methanol (5 mL) was reacted according to the General Procedure E to yield after purification by column chromatography azide **2.12b** as pale yellow oil (913 mg, 78%). R_F = 0.25 (ethyl acetate/methanol 7/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.09 (m, 2H), 1.21 (m, 4H), 1.37–1.56 (m, 2H), 1.55–1.84 (m, 4H), 2.25 (t, *J* = 7.5 Hz, 1H), 2.45 (t, *J* = 7.3 Hz, 1H), 2.69–2.82 (m, 1H), 2.84–2.97 (m, 1H), 3.09–3.24 (m, 1H), 3.36–3.45 (m, 4H), 3.50–3.58 (m, 2H), 3.58–3.71 (m, 14H), 3.83–3.94 (m, 2H), 4.08–4.22 (m, 1H), 4.32 (m, 1H), 4.50 (m, 1H), 5.45–5.58 (m, 1H), 6.24 (s, 1H), 6.81–7.03 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 20.9, 25.0, 28.2, 28.3, 32.9, 39.1, 40.5, 44.7, 48.7, 50.6, 55.5, 60.1, 61.7, 69.5, 69.6, 70.0, 70.1, 70.4, 70.5, 70.6, 163.6, 170.1, 173.5; ESI-MS m/z: 586.3 $[M-H]^-$, 588.4 $[M+H]^+$, 610.2 $[M+Na]^+$; HRMS (ESI) calcd for $C_{25}H_{45}O_7N_7SNa$ 610.2983, found 610.2994.

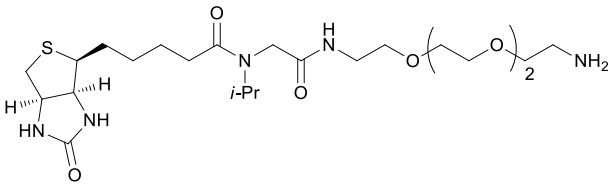
PEG$_5$-(biotin)-azide (2.12c)



A mixture containing paraformaldehyde (38.3 mg, 1.28 mmol), *i*-propylamine (0.11 mL, 1.28 mmol), biotin (244 mg, 1.00 mmol) and azido-PEG$_5$-isocyanide **2.3c** (319 mg, 1.00 mmol) in methanol (5 mL) was reacted according to the General Procedure E to yield after purification by column chromatography azide **2.12c** as pale yellow oil (507 mg, 80%). R_F = 0.27 (ethyl acetate/methanol 7/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.09 (d, 2H, *J* = 7.9 Hz), 1.21 (m, 4H), 1.48 (m, 2H), 1.71 (m, 4H), 2.21–2.37 (m, 2H), 2.46 (t, *J* = 7.3 Hz, 1H), 2.74 (m, 1H), 2.91 (m, 1H), 3.16 (m, 1H), 3.36–3.71 (m, 24H), 3.86 (m, 1H), 4.13 (m, 1H), 4.32 (m, 1H), 4.50 (m, 1H), 5.40 (m, 1H),

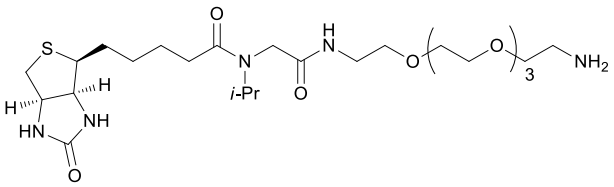
6.18 (m, 1H), 7.00 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 20.9, 25.0, 28.1, 28.2, 32.8, 39.1, 40.5, 44.7, 48.7, 50.6, 55.4, 60.1, 61.7, 69.7, 70.0, 70.1, 70.5, 70.6, 163.6, 170.0, 173.5; ESI-MS m/z: 631.1 $[M-H]^-$, 633.0 $[M+H]^+$, 654.8 $[M+Na]^+$; HRMS (ES) calcd for $C_{27}H_{50}O_8N_7S$ 632.3436, found 632.3438.

PEG$_3$-(biotin)-amine (2.13a)



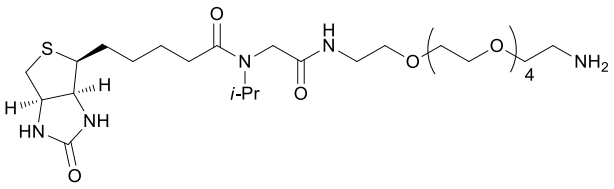
A mixture containing PEG$_3$-(biotin)-azide **2.12a** (725 mg, 1.33 mmol) and 75 mg Pd/C (10 wt-%) in methanol (10 mL) was reacted according to the General Procedure F to afford amine **2.13a** as pale yellow oil (688 mg, quant). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.09 (d, *J* = 6.7 Hz, 2H), 1.21 (dd, *J* = 6.5, 2.9 Hz, 4H), 1.49 (m, 2H), 1.61–2.03 (m, 6H), 2.23 (m, 1H), 2.45 (m, 1H), 2.73 (m, 1H), 2.89 (m, 2H), 2.93 (m, 1H), 3.15 (m, 1H), 3.40–3.77 (m, 16H), 4.13 (m, 1H), 4.33 (m, 1H), 4.50 (m, 1H), 5.17 (m, 1H), 6.12 (m, 1H), 7.21 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 21.0, 25.0, 28.1, 28.3, 32.8, 39.1, 40.6, 41.6, 44.7, 48.7, 55.4, 60.0, 61.7, 69.8, 70.1, 70.2, 70.5, 73.2, 73.3, 163.4, 170.0, 173.4; ESI-MS m/z: 516.6 $[M-H]^-$, 518.5 $[M+H]^+$, 540.4 $[M+Na]^+$; HRMS (ESI) calcd for $C_{23}H_{44}O_6N_5S$ 518.3007, found 518.3006.

PEG$_4$-(biotin)-amine (2.13b)



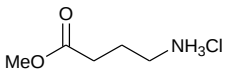
A mixture containing PEG$_4$-(biotin)-azide **2.12b** (440 mg, 0.75 mmol) and 49 mg Pd/C (10 wt-%) in methanol (10 mL) was reacted according to the General Procedure F to afford amine **2.13b** as pale yellow oil (385 mg, 92%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.09 (m, 2H), 1.21 (m, 4H), 1.47 (m, 2H), 1.71 (m, 4H), 1.89 (br. s., 3H), 2.25 (m, 1H), 2.46 (m, 1H), 2.74 (m, 1H), 2.83–2.95 (m, 2H), 3.16 (m, 1H), 3.38–3.45 (m, 2H), 3.49–3.58 (m, 4H), 3.58–3.71 (m, 12H), 3.83–3.94 (m, 2H), 4.05–4.22 (m, 1H), 4.32 (m, 1H), 4.50 (m, 1H), 5.51 (m, 1H), 6.26 (s, 1H), 7.24 (br. s., 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 20.9, 25.0, 28.1, 28.2, 32.8, 39.0, 40.5, 41.6, 44.6, 48.7, 50.6, 55.5, 60.0, 61.7, 69.6, 69.7, 70.1, 70.4, 70.5, 70.6, 73.1, 163.6, 170.0, 173.4; ESI-MS m/z: 562.2 $[M+H]^+$; HRMS (ESI) calcd for $C_{25}H_{48}O_7N_5S$ 562.3270, found 562.3268.

PEG$_5$-(biotin)-amine (2.13c)



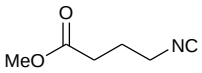
A mixture containing PEG$_5$-(biotin)-azide **2.12c** (484 mg, 0.80 mmol) and 51 mg Pd/C (10 wt-%) in methanol (10 mL) was reacted according to the General Procedure F to afford amine **2.13c** as pale yellow oil (453 mg, 94%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.09 (d, *J* = 6.7 Hz, 2H), 1.21 (m, 4H), 1.48 (m, 2H), 1.71 (m, 4H), 2.00 (m, 2H), 2.46 (t, *J* = 7.3 Hz, 1H), 2.74 (m, 1H), 2.83–2.95 (m, 3H), 3.16 (m, 1H), 3.38–3.71 (m, 24H), 3.86 (m, 2H), 4.14 (m, 1H), 4.32 (m, 1H), 4.50 (m, 1H), 5.47 (m, 1H), 6.24 (m, 1H), 7.12 (m, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 20.9, 25.0, 28.1, 28.2, 32.8, 39.1, 40.5, 41.6, 44.6, 48.7, 50.5, 55.5, 60.1, 61.7, 69.7, 70.1, 70.2, 70.5, 73.3, 163.6, 170.0, 173.4; ESI-MS m/z: 604.9 $[M-H]^-$, 606.6 $[M+H]^+$, 628.7 $[M+Na]^+$; HRMS (ESI) calcd for $C_{27}H_{52}O_8N_5S$ 606.3531, found 606.3527.

Methyl 4-aminobutyrate hydrochloride (2.14)



4-Aminobutyric acid (20.0 g, 194 mmol) was dissolved in methanol (320 mL). The mixture was cooled to 0 °C and thionyl chloride (43.5 mL, 600 mmol) was added dropwise. The cooling bath was removed and stirring was continued at room temperature overnight. After evaporation of all volatiles, diethyl ether (300 mL) was added and the solution was cooled on an ice-bath. The resulting precipitate was collected by filtration and washed thoroughly with diethyl ether to afford amine hydrochloride **2.14** as a white solid (29.0 g, 97%). M.p. 120–121 °C; ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 1.96 (quint., *J* = 7.4 Hz, 2H), 2.50 (t, *J* = 7.4 Hz, 2H), 3.00 (t, *J* = 7.4 Hz, 2H), 3.69 (s, 3H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 23.7, 31.4, 40.0, 52.3, 174.5; ESI-MS m/z: 118.1 $[M+H]^+$.

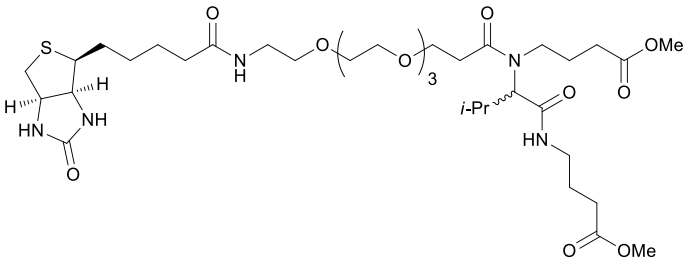
Methyl 4-isocyanobutanoate (2.15)



Amine hydrochloride **2.14** (15.0 g, 98.0 mmol) was dissolved in trimethyl orthoformiate (90.0 mL, 720 mmol) and heated under reflux for 4 hours. After completion of the reaction, all volatiles were removed *in vacuo* and the formamide was obtained as yellow oil which was dissolved in dry dichloromethane (300 mL) and cooled on an ice-bath. Diisopropylamine (37.0 mL, 267 mmol) was added followed by the dropwise addition of phosphorus oxychloride (9.80 mL, 107 mmol). Stirring of the mixture was continued for 2 hours at room temperature. Afterwards

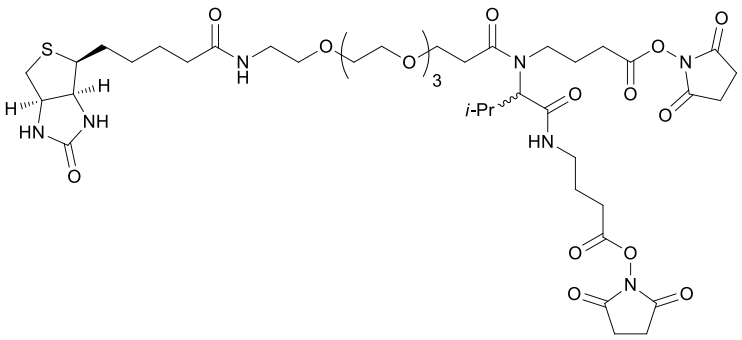
sodium carbonate (20.0 g in 100 mL distilled water) was added and the solution was stirred for additional 30 min. The solution was diluted with distilled water (100 mL) and the organic phase was separated. The aqueous layer was extracted with dichloromethane (3x 200 mL) and the combined organic extracts were dried (sodium sulphate), filtered and evaporated to dryness. Purification of the residue by column chromatography afforded isocyanide **2.15** as yellow oil (8.30 g, 73%). R_F = 0.88 (dichloromethane/ methanol 8/2); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.87 (m, 2H), 2.52 (t, J = 7.0 Hz, 2H), 3.51 (m, 2H), 3.70 (s, 3H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 24.1, 30.9, 40.6, 40.7, 40.8, 51.8, 156.3, 156.4, 156.5, 172.3.

PEG$_4$-(biotin)-(COOMe)$_2$ (2.17)



A mixture containing *i*-butyraldehyde (31.0 µL, 0.34 mmol), triethylamine (47.1 µL, 0.34 mmol), 15-[*D*(+)-biotinyl-amino]-4,7,10,13-tetraoxapentadecanoic acid **2.16** (141 mg, 0.29 mmol), isocyanide **2.15** (48.9 mg, 0.39 mmol) and amine hydrochloride **2.14** (51.9 mg, 0.34 mmol) in methanol (5 mL) was reacted according to the General Procedure E. After evaporation of all volatiles, the residue was dissolved in ethyl acetate, washed consecutively with distilled water and saturated aqueous sodium bicarbonate solution, dried (sodium sulphate), filtered and evaporated to dryness. Purification of the residue by column chromatography afforded compound **2.17** as colourless oil (116 mg, 51%). R_F = 0.17 (dichloromethane/methanol 9/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 0.76–0.98 (m, 6H), 1.47 (q, J = 7.1 Hz, 2H), 1.60–1.92 (m, 8H), 2.14 (br. s., 2H), 2.19–2.42 (m, 7H), 2.62–2.83 (m, 3H), 2.92 (dd, J = 12.8, 4.8 Hz, 1H), 3.11–3.39 (m, 4H), 3.39–3.47 (m, 3H), 3.57 (t, J = 4.8 Hz, 2H), 3.60–3.70 (m, 18H), 3.77–3.84 (m, 2H), 4.34 (m, 2H), 4.51 (dd, J = 7.2, 5.1 Hz, 1H), 5.19 (m, 1H), 6.34 (m, 1H), 6.85 (br. s., 1H); ^{13}C NMR (150 MHz, CHLOROFORM-*d*) δ: 18.9, 19.7, 24.6, 25.5, 26.6, 28.06, 28.14, 31.1, 31.3. 31.4, 33.8, 35.9, 38.5, 39.1, 40.5, 40.6, 50.8, 51.6, 51.7, 55.4, 60.1, 61.7, 61.8, 67.6, 69.9, 70.1, 70.3, 70.40, 70.44, 70.47, 70.49, 163.6, 170.9, 172.67, 172.74, 173.10, 173.5; ESI-MS m/z: 812.4 $[M+Na]^+$; HRMS (ESI) calcd for $C_{36}H_{63}N_5O_{12}SNa$ 812.4086, found 812.4078.

PEG$_4$-(biotin)-NHS$_2$ (2.19)



A solution containing PEG$_4$-(biotin)-(COOMe)$_2$ **2.16** (52 mg, 66 µmol) and lithium hydroxide monohydrate (14.3 mg, 341 µmol) dissolved in water/ tetrahydrofuran (1/1 v/v, 2 mL) was stirred at room temperature for 2.5 hours. The mixture was acidified by the addition of aqueous 1 M hydrochloric acid and the aqueous phase was extracted with *n*-butanol (2x 1 ml). Afterwards the combined organic extracts were dried (sodium sulphate), filtered and evaporated to dryness to afford the free carboxylic acid **2.18** as colourless oil (23.1 mg, 46%), which was used directly in the next step without further purification. ESI-MS *m/z*: 760.4 $[M-H]^-$, 762.4 $[M+H]^+$, 784.5 $[M+Na]^+$; HRMS (ESI) calcd for $C_{34}H_{58}N_5O_{12}S$ 760.3808, found 760.3809.

To an ice-cold solution containing *N*-hydroxysuccinimide (8.7 mg, 75.6 µmol), PEG$_4$-(biotin)-(COOH)$_2$ **2.18** (20.7 mg, 27.2 µmol) and catalytic amounts of 4-dimethylaminopyridine in dry dichloromethane (5 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (15.6 mg, 81.4 µmol). The mixture was allowed to warm to room temperature and stirring was continued overnight. After completion of the reaction was detected by ESI-MS, the organic phase was washed consecutively with 1 mL aqueous 1 M hydrochloric acid and 2 mL distilled water. Finally, the organic phase was dried (sodium sulphate), filtered and evaporated to dryness to afford **2.19** as colourless oil (22.6 mg, 87%), which was used directly for chemical cross-linking experiments without further purification. ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 0.77–0.99 (m, 6H), 1.40–1.53 (m, 2H), 1.56–2.06 (m, 8H), 2.14–2.43 (m, 5H), 2.49–2.79 (m, 7H), 2.80–2.87 (m, 8H), 2.87–2.96 (m, 1H), 3.11–3.36 (m, 4H), 3.38–3.49 (m, 3H), 3.49–3.59 (m, 2H), 3.59–3.68 (m, 12H), 3.79 (t, *J* = 6.2 Hz, 2H), 4.06–4.16 (m, 1H), 4.29–4.38 (m, 1H), 4.46–4.55 (m, 1H), 5.32–5.38 (m, 1H), 5.90–6.10 (m, 1H), 6.57–6.67 (m, 1H), 6.68–6.78 (m, 1H); ^{13}C-NMR (75 MHz, CHLOROFORM-*d*) δ: 18.7, 19.7, 24.1, 24.3, 25.4, 25.6, 26.8, 28.0, 28.2, 28.3, 33.7, 35.9, 38.0, 39.1, 40.5, 55.3, 60.0, 61.8, 67.6, 69.8, 70.1, 70.4, 70.5, 163.5, 168.0, 168.2, 169.1, 169.2, 169.3, 169.7, 170.9, 172.7, 173.0; ESI-MS *m/z*: 978.5 $[M+Na]^+$; HRMS (ESI) calcd for $C_{42}H_{65}N_7O_{16}SNa$ 978.4101, found 978.4099.

2.6 References

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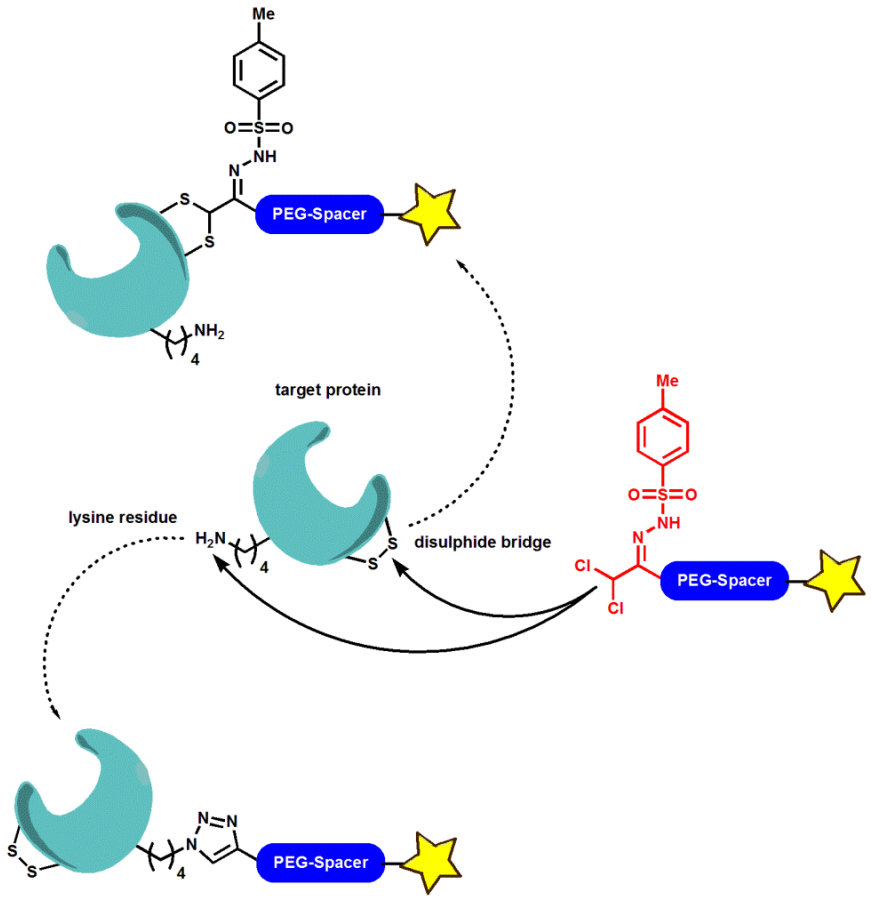
3 The Sakai Reaction – A Metal-Free Alternative Towards the Regioselective Formation of 1,2,3-Triazoles



Abstract

Traceless tosylhydrazone-based triazole formation (Sakai reaction) can be readily achieved by reacting primary amines with *α*,*α*-dichlorotosylhydrazones under ambient conditions. This fast and efficient alternative to the copper-assisted azide-alkyne cycloaddition (CuAAC) affords, exclusively, 1,4-substituted triazole "click-products" with good to excellent chemical yields. Moreover, the observed reactivity and chemoselectivity of this metal-free triazole formation methodology suggests that primary amines, inherent to most natural products and biomolecules, should be applicable in the Sakai reaction (*e.g.* for fluorescent labelling, biotinylation, PEGylation, *etc.*). Besides the synthesis of fluorescent *α*,*α*-dichlorotosylhydrazones, the optimisation of the reaction protocol to be compatible with the demands of a biological sample and the covalent modification of purified proteins as initial proof of concept will be presented. Furthermore, attempts utilising functionalised *α*,*α*-dichloro-sulphonohydrazones as novel disulphide-bridging agents will be discussed.

3.1 Introduction

Since the first preparation by Pechmann in the late 19th century,[1] 1,2,3-triazoles and derivatives thereof have evolved as one of the most important classes of heterocyclic compounds as a consequence of their widespread applications, *e.g.* in organic synthesis (as chiral ligands, catalysts, *etc.*) and in industry (as dyes, optical brightening agents, explosives and fluorescent whiteners, *etc.*). Moreover, triazoles exhibit a pronounced biological activity profile, hence making this class of compounds highly attractive for various medicinal and agrochemical applications.[2]

Due to their widespread applicability, a variety of synthetic methods leading to the formation of substituted triazoles have been developed in the last decades, which has been the focus of various reviews.[2,3] The most prominent approach leading to the formation of substituted 1,2,3-triazoles is based on the Huisgen azide alkyne [3+2] cycloaddition reaction, which has been initially reported in the early 1960s (Fig. 3.1).[4] In most cases, the formation of a mixture of the 1,4- and 1,5-regioisomer is generally observed, though. Consequently, methods were developed to selectively obtain one of the regioisomers (Fig. 3.1): either the 1,4-substituted (copper-catalysed azide-alkyne cycloaddition, short: CuAAC)[5] or the 1,5-substituted triazole (ruthenium-catalysed azide-alkyne cycloaddition, short: RuAAC).[6]

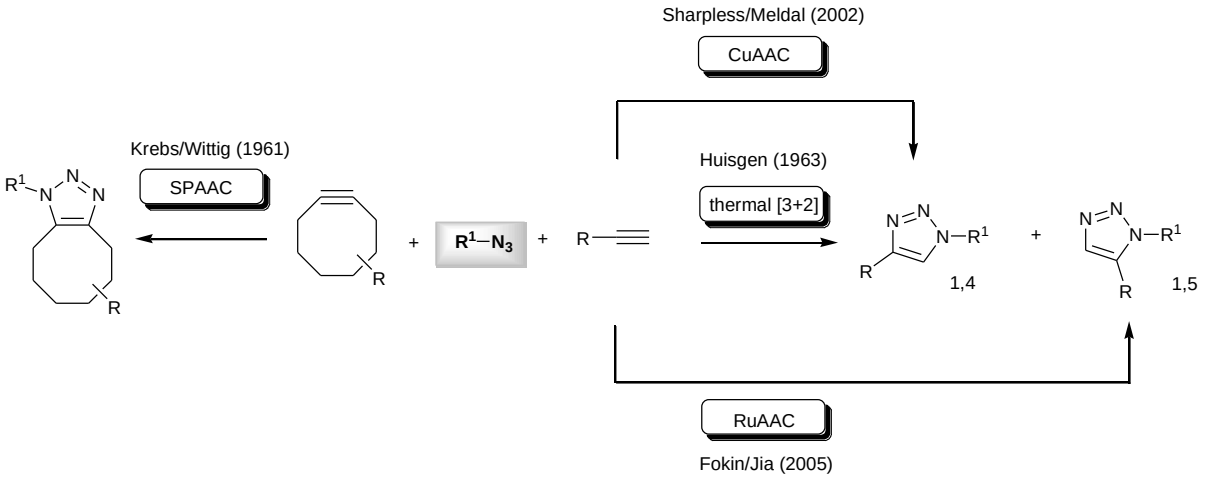


Fig. 3.1 Synthesis of 1,2,3-triazoles from organic azides and terminal alkynes or strained cyclooctynes. Mixtures of the 1,4- and 1,5-regioisomer can be obtained either by the thermal [3+2] azide alkyne cylcoaddition reaction or by the ring strain promoted azide alkyne cycloaddition reaction (SPAAC). The regioselective formation of both isomers can be achieved *via* transition metal catalysis: application of a copper-catalysts (CuAAC) leads to the formation of the 1,4-regioisomer; a ruthenium catalyst (RuAAC), on the other hand, affords the 1,5-regioisomer.

In recent years, especially CuAAC made an unprecedented impact in chemical biology because of its ultimate chemoselectivity and high reaction rates. For example, in bioorganic applications CuAAC has been frequently applied for the covalent labelling of various active enzymes, biomolecules and post-translational modifications.[7] However, due to the apparent toxicity of the copper(I) catalyst for various organisms, which derives presumably from the formation of reactive oxygen species,[8] the application of the CuAAC methodology is rather limited to *in vitro* applications. In addition to the observed toxicity, the possible formation of highly explosive copper azides in the course of the reaction limits the applicability of CuAAC on an industrial scale due to the requirement of severe safety precautions. Safer alternatives for the formation of 1,2,3-triazoles, which do not require the presence of a (toxic) metal catalyst, were consequently developed in recent years. In this regard, the strain-promoted azide alkyne cycloadditon reaction (SPAAC), which does not require the presence of an additional promoter species, has evolved as the method of choice for *in vivo* applications in chemical biology (Fig. 3.1).[7,9] The formation of a mixture containing both the 1,4- and the 1,5-regioisomer limits the application of the SPAAC methodology in medicinal chemistry, though. From the aforementioned disadvantages of the CuAAC and SPAAC methodology, one can conclude that a suitable triazole forming reaction should proceed with ultimate regioselectivity yielding either the 1,4- or 1,5-regioisomer, respectively, and without the demand of a toxic promoter species. Moreover, the reduction of the synthetic effort (both building blocks, azide as well as alkyne, have to be prepared prior to the reaction in multi-step sequences) would be worth striving for, *i.e.* one of the functionalities should be readily available, that is, inherent to most starting materials, so that it can be utilised in the triazole forming reaction without any further manipulation.

Almost thirty years ago, Sakai and collaborators described such a reaction leading to the formation of triazoles and thiadiazoles.[10] This so far underappreciated approach towards the metal-free formation of triazoles relies on the condensation of primary amines with *α*,*α*-dichlorotosylhydrazones resulting in the exclusive formation of the 1,4-regioisomer under ambient reaction conditions (Fig. 3.2). Sakai initially proposed a mechanism of the reaction which involves the intramolecular cyclisation of a diazo intermediate, formed in a Bamford-Stevens-type reaction from a tosylhydrazone in the presence of a suitable base. Observations by Hanselmann and colleagues[11] and our own group (unpublished results) suggest a slightly different mechanism, though (Fig. 3.2). The initial step of the reaction involves deprotonation of the *α*,*α*-dichlorotosylhydrazone

resulting in the formation of a vinyldiazine intermediate (**A**). Subsequent nucleophilic attack of a primary amine (**B**) followed by the elimination of hydrochloric acid affords an intermediate imine-tosylhydrazone species (**C**). After a putative 1,5-H shift (**D**), the stage is set for the final cyclisation step affording the 1,4-substituted 1,2,3-triazole (**E**).

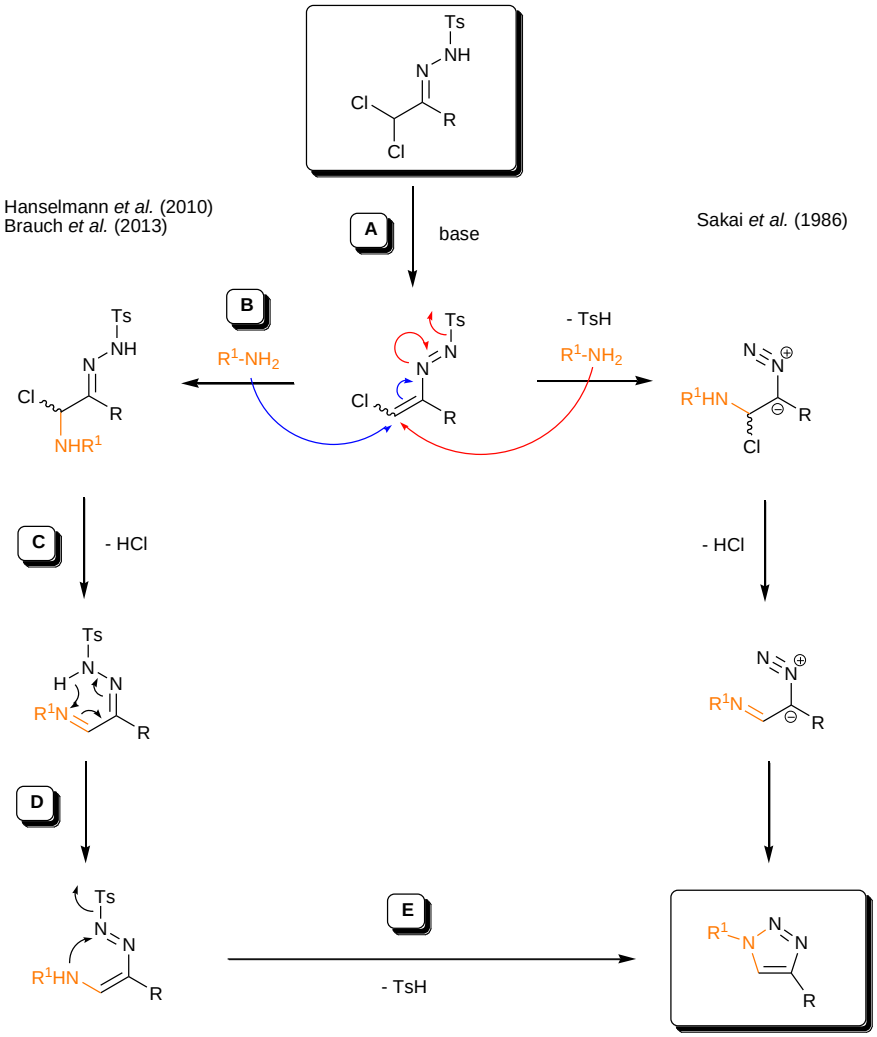


Fig. 3.2 Proposed mechanism of the Sakai triazole formation reaction. The mechanism initially proposed by Sakai and colleagues involves the formation of a diazo intermediate *via* a Bamford-Stevens-type reaction (right). Current investigations, however, suggest that the tosyl group is retained until the final cyclisation step of the reaction sequence (left).

In addition to the observed ultimate regioselectively, the mild reaction conditions applied and the reduced synthetic effort (only one building block of the Sakai reaction needs to be prepared: the *α*,*α*-dichlorotosylhydrazone; primary amines are easily accessible from chemical vendors), the reaction proceeds in a high chemoselective manner, hence a laborious protective group strategy seems to be unnecessary. Despite these advantages compared to other published methods leading to the formation of stable

1,2,3-triazole, the Sakai reaction has found only infrequent applications, though.[11,12] Consequently, the evident potential of the reaction has not been fully explored yet. Therefore, recent studies by our own group aim at further exploring the suitability of the Sakai triazole formation reaction as a metal-free alternative to already established methods. This will be presented in the following sections of the present thesis.

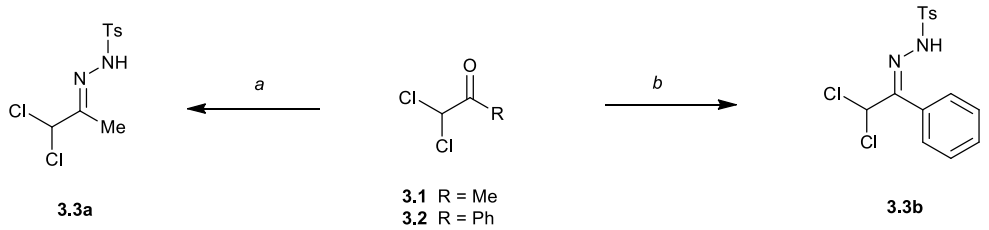
3.2 Scope and limitations of the Sakai triazole formation reaction

Parts of the following results have already been published in Angewandte Chemie entitled *"Traceless Tosylhydrazone-Based Triazole Formation: A Metal-Free Alternative to Strain-Promoted Azide–Alkyne Cycloaddition"* (van Berkel, Brauch *et al.*, *Angew. Chem. Int. Ed.* **2012**, *51*, 5343–5346; *Angew. Chem.* **2012**, *124*, 5437–5441).

To identify the scope and limitations of the Sakai reaction, various *α*,*α*-dichloro-tosylhydrazones were prepared first and subsequently reacted with a variety of primary amines (aromatic, aliphatic, electron-rich as well as electron-poor primary amines). Moreover, to estimate the chemoselectivity of this transformation, primary amines equipped with different functional groups, such as carboxylic acids, hydroxyl groups and acetal functionalities, were tested in the Sakai reaction. Finally, reactions of *α*,*α*-dichlorotosylhydrazones with other nucleophiles (*e.g.* thiols, hydrazines, hydroxyl-amines, *sec.* amines, *etc.*) were also investigated.

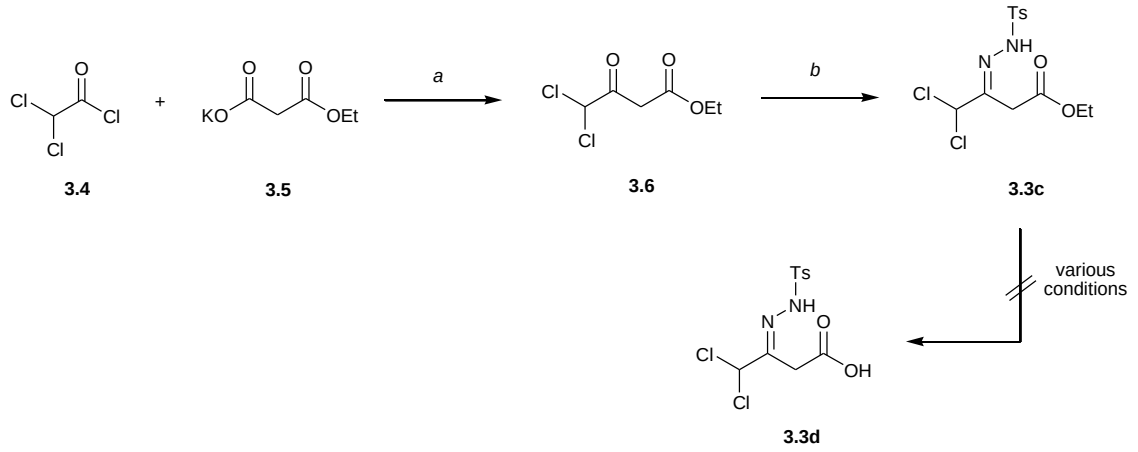
Synthesis of *α*,*α*-dichlorotosylhydrazones

In general, reactive *α*,*α*-dichlorotosylhydrazones can be generated by simply mixing *p*-toluenesulphonyl hydrazide and the corresponding *α*,*α*-dichloroketone precursors in an appropriate solvent. Starting from the commercially available dichloroacetone (**3.1**) and dichloroacetophenone (**3.2**), respectively, the corresponding *α*,*α*-dichlorotosyl-hydrazones **3.3a** and **3.3b** were obtained in 92% and 81% (Scheme 3.1).



Scheme 3.1 Synthesis of alky- or aryl-substituted *α*,*α*-dichlorotosylhydrazones. *Reagents and Conditions:* (a) *p*TsNHNH$_2$, AcOH, r.t., 4 h (92%); (b) *p*TsNHNH$_2$, THF, r.t., overnight (81%).

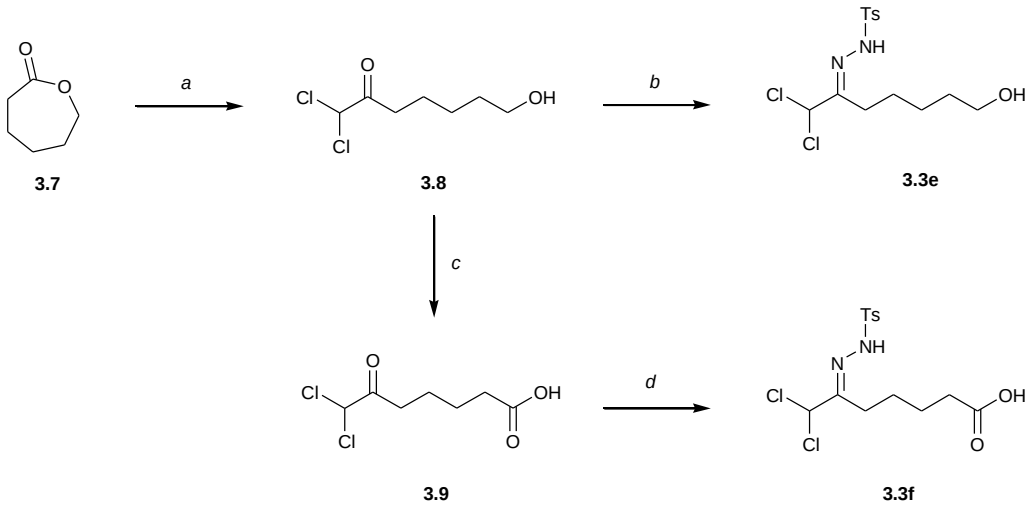
However, other *α*,*α*-dichloroketones needed to be prepared prior to the conversion to the corresponding *α*,*α*-dichlorotosylhydrazones. For this purpose, various methods have been reported leading to the formation of the desired *α*,*α*-dichloroketones in one- or multi-step procedures from different starting materials. For example, the *β*-keto ester **3.6** was prepared from commercially available dichloroacetyl chloride (**3.4**), a bulk chemical used for the production of agrochemicals (*e.g.* herbicides such as Benoxacor and Flurochloridone), and ethyl potassium malonate (**3.5**) in the presence of a base (triethylamine). Afterwards *p*-toluenesulphonyl hydrazide was added to a solution of the crude *β*-keto ester **3.6** resulting in the precipitation of the tosylhydrazone ethyl ester **3.3c** in 30% overall yield over two steps (Scheme 3.2).[11] Subsequent saponification of the ethyl ester moiety would lead to the formation of tosylhydrazone carboxylic acid **3.3d** which could be utilised in further functional group manipulations. Various attempts under acidic (no conversion) as well as mild basic conditions (decomposition) proved to be unsuccessful, though.



Scheme 3.2 Synthesis of the functionalised *α*,*α*-dichlorotosylhydrazone 3.3c. *Reagents and Conditions:* (a) $MgCl_2$, Et_3N, MeCN, r.t., overnight; (b) *p*$TsNHNH_2$, MeCN, r.t., overnight (30% over two steps).

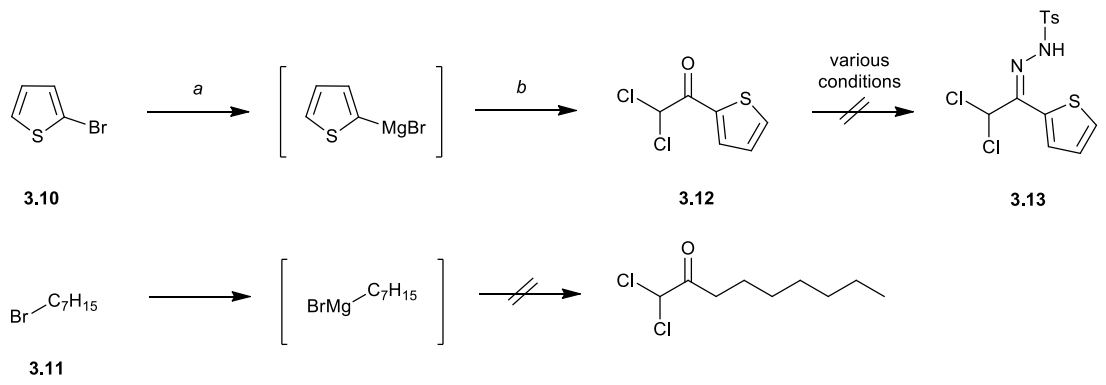
Barluenga and colleagues reported the rapid and versatile synthesis of *α*,*α*-dihalogeneoketones from readily available carboxylic acid esters using *in situ* generated dihalogenomethyllithium in 55–75% yield.[13] This approach was used for the synthesis of the functionalised tosylhydrazone **3.3e** and **3.3f**, respectively (Scheme 3.3).[14] Similar to the method described earlier by Barluenga, the carboxylic acid ester (here: *ε*-caprolactone **3.7**) was dissolved in dichloromethane/diethyl ether and treated with lithium diisopropylamide (LDA) affording the hydroxyl-functionalised *α*,*α*-dichloro-ketone **3.8** in 67% yield. Upon oxidation utilising sodium periodate and catalytic amounts

of ruthenium chloride monohydrate, the corresponding carboxylic acid **3.9** could be isolated in 94% yield. Finally, the functionalised *α,α*-dichloroketones **3.8** and **3.9** were dissolved in acetonitrile and *p*-toluenesulphonyl hydrazide was subsequently added leading to the formation of the functionalised *α,α*-dichlorotosylhydrazones **3.3e** (52%) and **3.3f** (40%).



Scheme 3.3 Synthesis of the functionalised *α,α*-dichlorotosylhydrazones 3.3e and 3.3f. *Reagents and Conditions:* (a) CH_2Cl_2, LDA, Et_2O, −78 °C, 20 min (67%); (b) *p*$TsNHNH_2$, MeCN, r.t., overnight (52%); (c) $NaIO_4$, $RuCl_3{\cdot}H_2O$, MeCN/CCl_4/H_2O (1/1/1.5), r.t., 3 h (94%); (d) *p*$TsNHNH_2$, MeCN, r.t., overnight (40%).

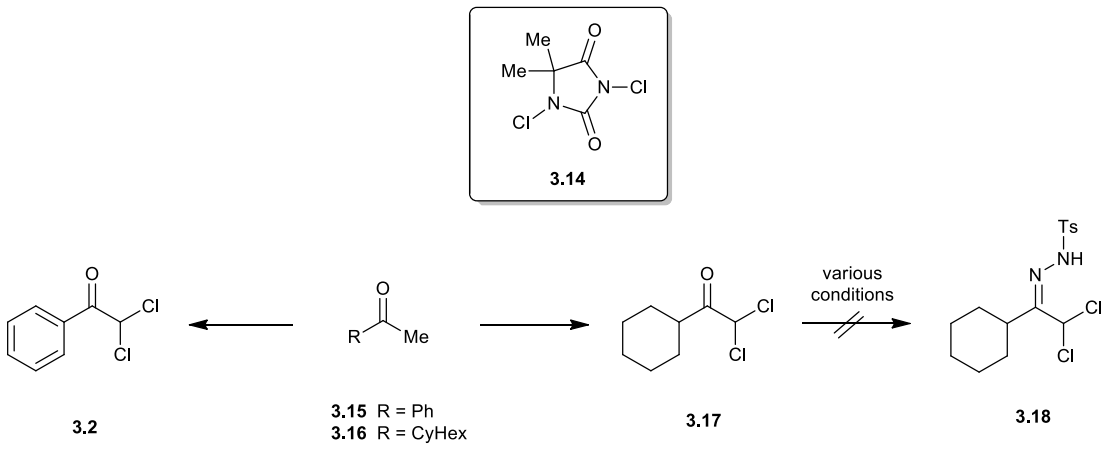
Another highly versatile method for the preparation of *α,α*-dichloroketones was reported by Föhlisch and Flogaus utilising a Grignard reaction and commercially available dichloroacetyl chloride **3.4**.[15] To test whether this method is suitable for the synthesis of different *α,α*-dichloroketones, 2-bromothiopene (**3.10**) as well as 1-bromoheptane (**3.11**) were converted into the corresponding Grignard reagents. However, reaction of 2-thiophene magnesium bromide with acetyl chloride **3.4** afforded ketone **3.12** in only



Scheme 3.4 Synthesis of *α,α*-dichloroketones according to Föhlisch and Flogaus. *Reagents and Conditions:* (a) Mg, THF, reflux, 2.5 h; (b) **3.4**, THF, -78 °C, 15 min (25%).

25% yield. The reaction of acetyl chloride **3.4** with 1-heptane magnesium bromide led to no product formation at all (Scheme 3.4). Consequently, further investigations regarding this approach were abandoned. Nevertheless, the isolated *α,α*-dichloroketone **3.12** was treated with *p*-toluenesulphonyl hydrazide under various conditions. Unfortunately, no formation of the desired tosylhydrazone **3.13** could be detected by TLC analysis (predominantly unreacted starting material).

A more recent approach leading to the formation of *α,α*-dichloroketones from aryl or enolisable ketones was reported by Zou and colleagues utilising 1,3-dichloro-5,5-dimethylhydantoin (DCDMH, **3.14**), a common disinfecting and bleaching agent used in industry. The chlorination reaction was performed in deep eutectic solvents (DES), formed by heating an equimolar mixture of choline chloride and *p*-toluenesulphonic acid monohydrate, which are thought to be versatile alternatives to ionic liquids.[16] Initially, acetophenone (**3.15**) was chosen as the starting material leading to the formation of the aforementioned dichloroacetophenone **3.2** in 66% yield (Scheme 3.5). Inspired by this findings, cyclohexyl metyl ketone (**3.16**) was utilised in this reaction affording the corresponding *α,α*-dichloroketone **3.17** in comparable low 28% yield, though. Again, various methods failed to produce the corresponding tosylhydrazone **3.18**.



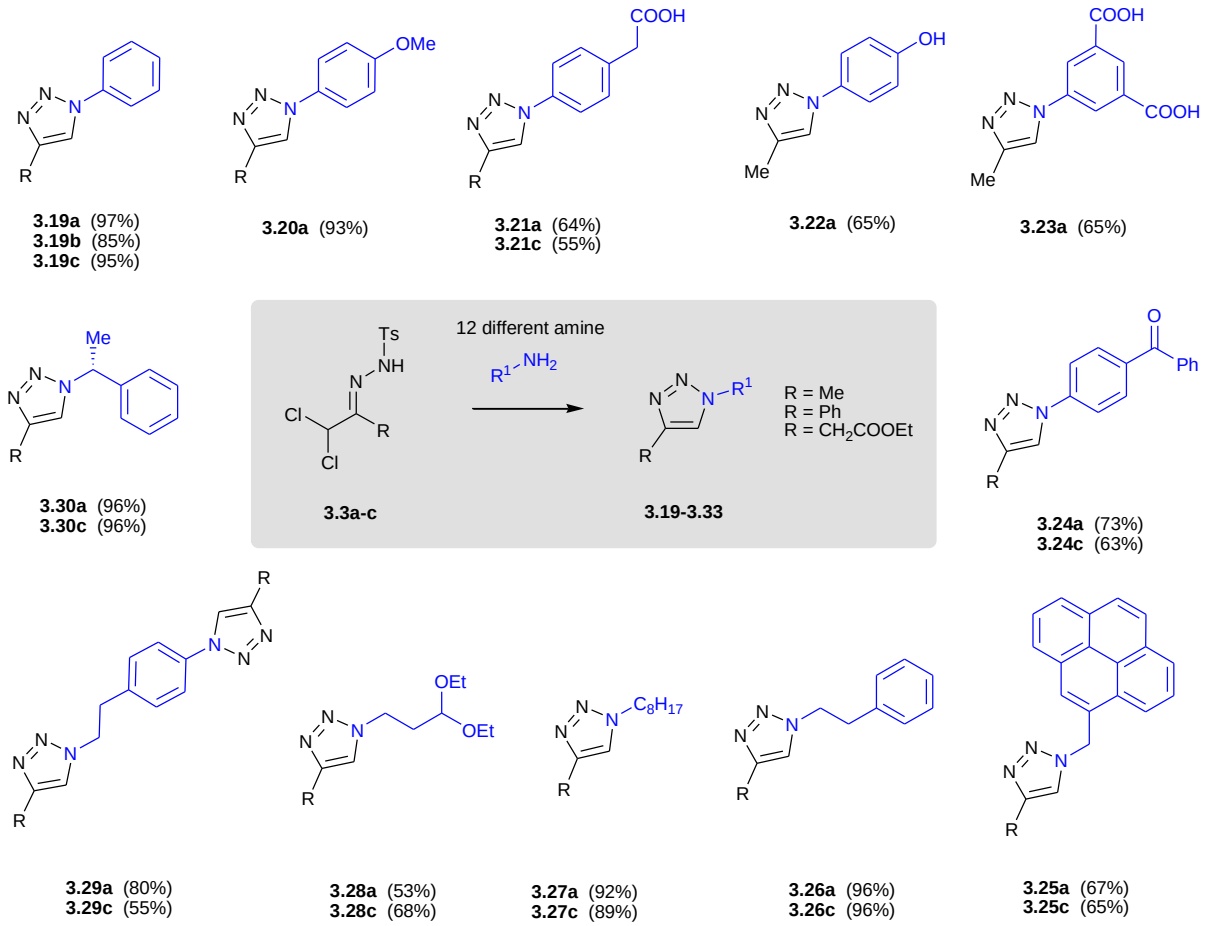
Scheme 3.5 Synthesis of *α,α*-dichloroketones according to Zou and co-workers. *Reagents and Conditions:* DCDMH **3.14**, MeCN, DES, r.t., 45 min (66% for **3.2** and 28% for **3.17**, respectively).

Despite the considerable low yields of the tested methods leading to the key intermediate *α,α*-dichloroketones, the limitation in creating libraries of different *α,α*-dichlorotosylhydrazones, hence enabling the synthesis of structural highly diverse triazole products, derives from the severe problems concerning tosylhydrazone formation. Therefore, before the Sakai reaction can be considered as a truly versatile

alternative to other triazole forming reactions, the substrate scope of the reaction regarding the applied *α*,*α*-dichlorotosylhydrazones needs to be further improved in future studies.

The Sakai reaction with primary amines

For the identification of the substrate scope regarding the amine component used in the Sakai reaction, various primary amines were reacted with the aforementioned *α*,*α*-dichlorotosylhydrazones **3.3a–c**. The reactions were run in ethanol/acetonitrile (3/2 v/v) in the presence of six equivalents of base (diisopropylethylamine) at room temperature. These simple reaction conditions afforded the corresponding triazole products (**3.19–3.30**) in good to excellent yields (Scheme 3.6).[14] At the beginning, different electron-rich as well as electron-poor aniline derivatives were tested in the Sakai reaction. The desired triazole products **3.19–3.24** could be isolated in good to excellent yields. However, the obtained results suggest that electron-withdrawing substituents on the aromatic ring system tend to decrease the overall yield of the reaction, which might be attributed to a reduced nucleophilicity of the aromatic amine moiety. This assumption is additionally confirmed by the complete inability of *p*-nitroaniline to react in the Sakai reaction. In addition, benzylic as well as aliphatic primary amines were tested towards their potential of participating in the Sakai reaction. Again, all products could be isolated in good to excellent yields (**3.25–3.30**). To test whether the Sakai reaction exhibits a certain degree of amine chemoselectivety, 4-aminophenylethylamine was reacted with tosylhydrazones **3.3a** and **3.3c**. In both cases, the bis-triazole products **3.29a** (80%) and **3.29c** (55%), respectively, were obtained in good yields. These findings suggest only a poor amine chemoselectivity regarding the reactivity of aromatic over aliphatic primary amines and *vice versa* in the Sakai reaction. Moreover, the Sakai reaction was found to tolerate a wide range of functional groups, such as carboxylic acids (**3.21a/c**, **3.23a**), phenolic hydroxyl groups (**3.22a**) as well as acetal functions (**3.28a/c**), leading to the corresponding triazole products in good yields. As a consequence of this observed chemoselectivity, a laborious protective group strategy seems to be unnecessary. Furthermore, it was found that the Sakai reaction proceeds without racemisation of the stereogenic center at the *α*-position of the amine moiety. For example, enantiomerically pure (*R*)-1-phenylethylamine was converted into its corresponding triazole products **3.30a** and **3.30c** utilising tosylhydrazones **3.3a** and **3.3c** in excellent yields (96%). Chiral HPLC analysis revealed that no racemisation occurred during the course of the reaction.

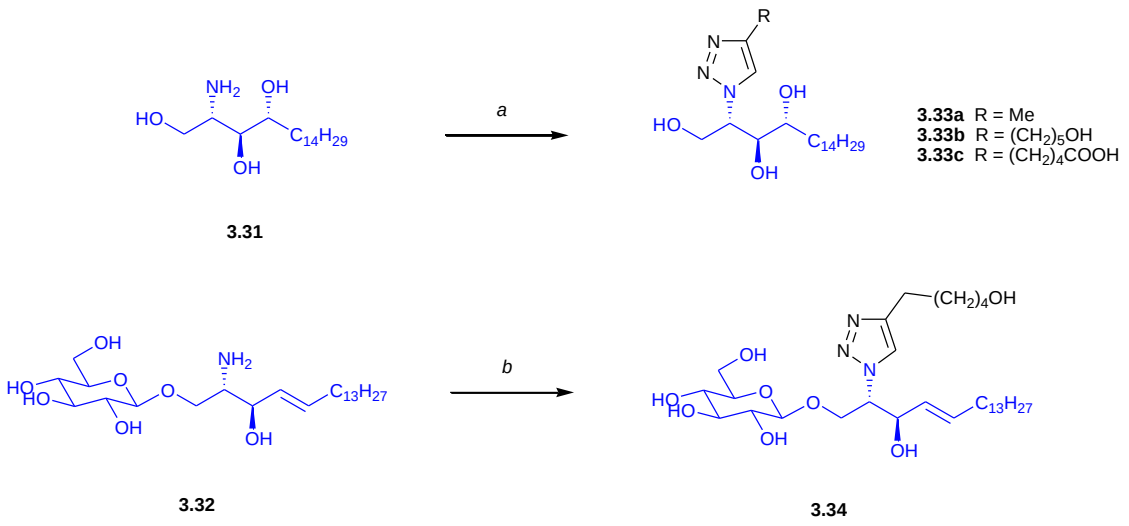


Scheme 3.6 Synthesis of primary amine-derived triazole products utilising the Sakai reaction. *Reagents and Conditions:* DiPEA (6 equiv.), EtOH/MeCN (3/2 v/v), r.t, 30 min up to 18 h.

Shifting towards more biologically relevant targets, phytosphingosine (**3.31**) and psychosine (**3.32**) were modified in the Sakai reaction. It is well known from literature that *N*-acylated phytoceramides as well as the analogous *N*-acylated ceramides exhibit a pronounced cytotoxic activity.[17] In addition, the corresponding triazole modified ceramides, which show an enhanced metabolic stability, have been synthesised recently and were successfully evaluated regarding their cytotoxic potential.[18] This fact clearly demonstrates the good amide-bond mimicry of 1,2,3-triazoles which results from the high structural and electronic similarities between 1,2,3-triazoles and native peptide bonds.[19] Based on these observations, unprotected phytosphingosine (**3.31**) was reacted in the Sakai reaction affording the triazole-modifed phytoceramides **3.33a–c** in good yields in a single operation (Scheme 3.7).

The modification of psychosine (**3.32**), a glycosphingolipid which is associated with globoid cell leukodystrophy (GLD), a genetic metabolic disorder that results from the absence of the enzyme galactosyl ceramide,[20] could also be easily accomplished utilising the Sakai reaction. The triazole-modified psychosine **3.34** was obtained in moderate 48%

yield utilising α,α-dichlorotosylhydrazone **3.3e** (Scheme 3.7). In this particular case, the application of the Sakai reaction might facilitate the synthesis of suitable chemical probes to identify the yet unknown targets of psychosine in the course of GLD.[21]



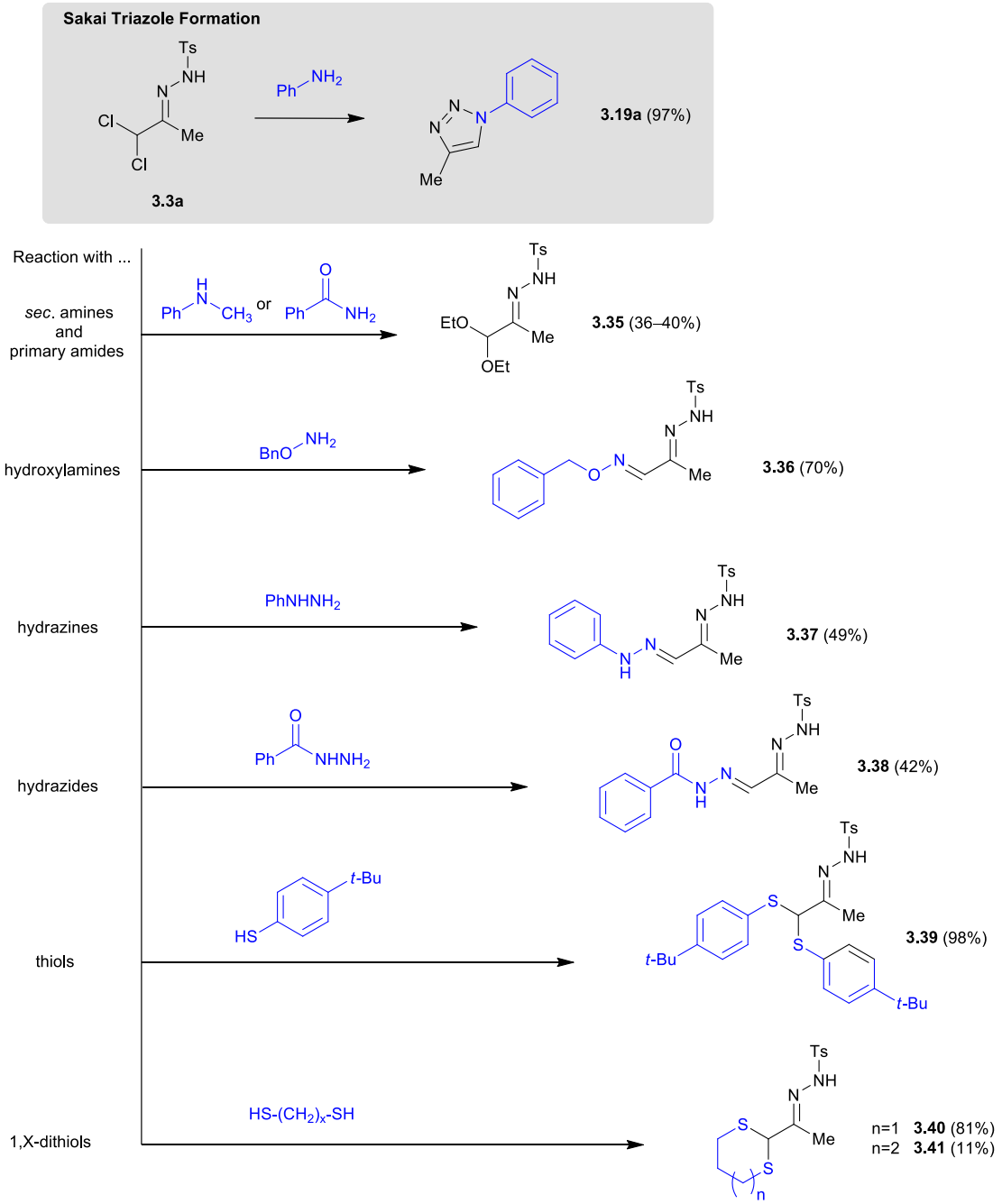
Scheme 3.7 Application of the Sakai reaction in the synthesis of triazole-modified phytoceramides and psychosine. *Reagents and Conditions:* (a) **3.3a,e,f**, DiPEA (6 equiv.), EtOH/MeCN (3/2 v/v), r.t., 2 h (77% for **3.33a**, 76% for **3.33b** respectively 89% for **3.33c**); (b) DiPEA (6 equiv.), EtOH/MeCN (3/2 v/v), r.t., 2.5 h (48%).

Again, no additional protection and deprotection steps were required for the previously outlined synthesis of triazole-modified phytosphingosine and psychosine. This pronounced chemoselectivity of the Sakai triazole formation reaction should enable the incorporation of a variety of modifications (*e.g.* biotin, fluorescent dyes) into complex natural products or even biomolecules, such as proteins and peptides.

Reaction between α,α-dichlorotosylhydrazones and other nucleophiles

In extensive scope and limitations studies, the chemoselective character of the Sakai reaction was investigated further (Scheme 3.8). For this purpose, various nitrogen-containing nucleophiles (*e.g.* hydrazines, hydroxylamines, hydrazides, *etc.*) and thiols were reacted with tosylhydrazone **3.3a** under slightly optimised reaction conditions (3 equiv. of potassium acetate were used as base instead of 6 equiv. DiPEA, see section 3.3). As previously expected, the reaction of secondary amines (here: *N*-methylaniline) with tosylhydrazone **3.3a** afforded no cyclised product. After purification of the reaction mixture by column chromatography, only the ethanol-adduct **3.35** could be isolated in 36% yield as a result of the nucleophilic attack of solvent molecules on hydrazone **3.3a**. Formation of the ethanol-adduct **3.35** was also observed when primary amides (here: benzamide) were used, which might be attributed presumably to the missing nucleophilic

character of the nitrogen atom. Moreover, *O*-benzyl-hydroxylamine, phenylhydrazine and benzhydrazide were used as alternative nitrogen nucleophiles in the Sakai reaction. Upon the reaction with tosylhydrazone **3.3a**, the formation of the non-cyclised bisimines **3.36** (70%), **3.37** (49%) and **3.38** (42%) in good to moderate yields was observed presumably as a consequence of the reduced ability of the nitrogen atom to replace the tosyl-group in the final cyclisation step. However, no triazole formation could be detected in each case. No other side products could be isolated either (besides traces of the ethanol-adduct

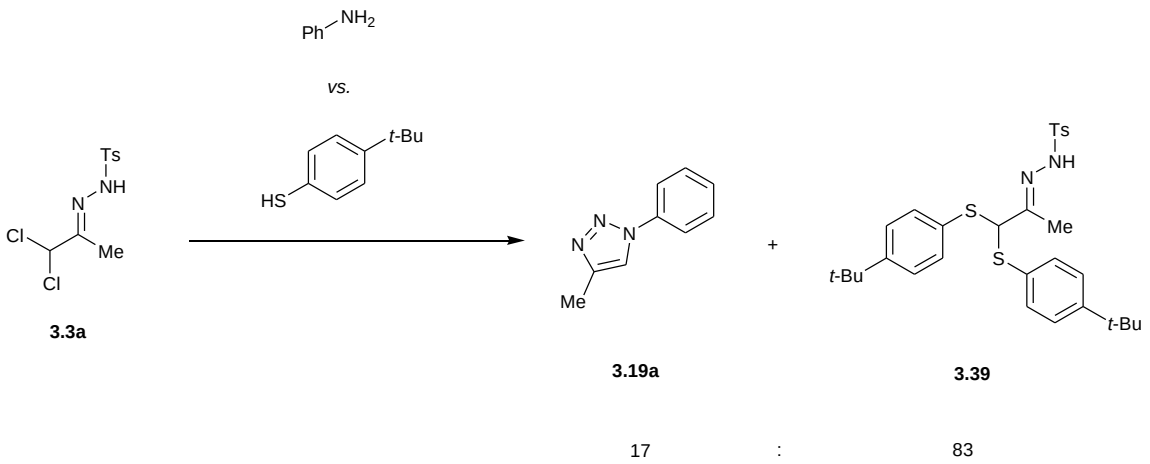


Scheme 3.8 Reaction of various nitrogen nucleophiles and thiols with *α,α*-dichlorotosylhydrazone 3.3a. *Reagents and Conditions:* KOAc (3 equiv.), EtOH/MeCN (3/2 v/v), r.t., overnight.

3.35). These findings confirm our hypothesis on the mechanism, *i.e.* the tosyl-group is retained until the final cyclisation step of the Sakai reaction (see section 3.1) and the formation of a diazo intermediate *via* a Bamford-Stevens-type reaction is most unlikely.

In addition, tosylhydrazone **3.3a** was reacted with 4-*tert*-butylthiophenol leading to the formation of the bis-substituted tosylhydrazone **3.39** in excellent yields. Moreover, 1,3-dithianes can be synthesised when dithiols instead are utilised in the reaction (*e.g.* **3.40** from 1,3-propanedithiol). The yields of the reaction are strongly dependent on the ring size of the formed cyclic products, though. For example, if 1,4-butanedithiol is used in the reaction instead of 1,3-propanedithiol, the yield of the reaction is strongly decreased from 81% to 11% which can be attributed to the formation of a less favoured seven-membered ring (dithiepane **3.41**).

Finally, under controlled conditions a competitive experiment was performed. Therfore, one equivalent of aniline and two equivalents of 4-*tert*-butylthiophenol were reacted with one equivalent of tosylhydrazone **3.3a** and the product distribution (triazole **3.19a** *vs*. bis-thioether **3.39**) was analysed. After purification of the reaction mixture, it turned out that the triazole product **3.19a** and the bis-thioether **3.39** were formed in a ratio 17 to 83 (Scheme 3.9). Hence, the observed chemoselectivity favouring the reaction with thiols requires to strictly avoid any free thiol species in the reaction mixture during the synthesis of triazoles from primary amines utilising the Sakai reaction.



Scheme 3.9 Competitive experiment. *Reagents and Conditions:* Aniline (1 equiv.), 4-*tert*-butylthiophenol (2 equiv.), KOAc (3 equiv.), EtOH/MeCN (3/2 v/v), r.t., overnight.

3.3 The Sakai reaction – A novel bioconjugation method?

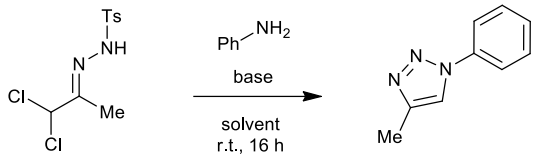
To extend the scope of the Sakai reaction further, its applicability as a novel bioconjugation method for the covalent modification of complex biological targets and surfaces should be explored. This methodology could be a viable alternative to classical amine coupling methods, such as the application of activated *N*-hydroxysuccinimide esters which exhibit a pronounced rate of hydrolysis in aqueous systems resulting in a decreased labelling yield (see chapter 1.2). For this purpose, the reaction had to be transferred into aqueous, buffered systems which are generally applied for bioconjugation purposes.

The applicability of the Sakai reaction for the modification of lysine residues might be affected, though, by the competing reaction with free cysteine residues. As a consequence of the relatively low natural abundance of cysteine (< 2%) in comparison to lysine (~ 6%) and the possible involvement of the thiol group in disulphide bond formation, however, this side reaction might be neglected.[22] In addition, besides modifying lysine residues, the ability of dithiolane formation upon the reaction between *α*,*α*-dichlorotosylhydrazones and dithiols suggests that these tosylhydrazones can be used as a novel class of disulphide bridging reagents apart from the well-established dibromo maleimides,[23] dithio maleimides[24] and *α*,*β*-unsaturated *β*′-mono-sulphone reagents[25] (see chapter 1.2). Therefore, methods to selectively distinguish between the reaction of *α*,*α*-dichloro-tosylhydrazones and primary amines (free lysine residues) or thiols (free cysteine or disulphide bonds), respectively, needed to be developed.

The Sakai reaction in water – Possible or not?

Initially, six equivalents diisopropylethylamine as base and an organic solvent mixture were used in the aforementioned scope and limitation studies. These conditions, however, are by far not compatible with highly complex biomolecules (*e.g.* proteins and peptides). The applicability of a water-soluble Lewis base instead of organic tertiary amines in the Sakai reaction was consequently explored. For this purpose, the triazole formation reaction between aniline and tosylhydrazone **3.3a** in the presence of potassium carbonate, potassium phosphate, potassium acetate and tetrabutylammonium acetate was investigated and compared to the already established protocol utilising diisopropyl-ethylamine (Table 3.1).

Table 3.1 The Sakai triazole formation reaction – Optimisation of the reaction conditions.



3.3a 3.19a

entry	solvent	base	isolated yield in %
1	EtOH/MeCN (3/2 v/v)	DiPEA (6 equiv.)	97
2	EtOH/MeCN (3/2 v/v)	K_2CO_3 (3 equiv.)	78
3	EtOH/MeCN (3/2 v/v)	K_3PO_4 (3 equiv.)	78
4	EtOH/MeCN (3/2 v/v)	KOAc (3 equiv.)	98
5	EtOH/MeCN (3/2 v/v)	Bu_4NOAc (3 equiv.)	98
6	H_2O/DMSO(95/5 v/v)	KOAc (3 equiv.)	58
7	H_2O/*t*-BuOH (1/2 v/v)	KOAc (3 equiv.)	88
8	PBS-buffer (100 mM, pH=7.4) /*t*-BuOH (3/2 v/v)		75

Already good results could be obtained with potassium carbonate and potassium phosphate (Table 3.1, entries 2–3) in comparison to diisopropylethylamine. However, potassium acetate and tetrabutylammonium acetate turned out to be the Lewis bases of choice (Table 3.1, entries 4–5) affording the triazole product **3.19a** in excellent yields. Besides the application of various water-soluble Lewis bases, the compatibility of the Sakai reaction with aqueous systems was investigated. In a first attempt, water containing five percent DMSO (highest tolerated concentration in biological systems), to dissolve the organic starting materials as well as the products, was used instead of an ethanol/ acetonitrile solvent-mix. The overall yield of the reaction drastically decreased to 58% (Table 1, entry 6), though. This might be attributed to solubility problems as a consequence of the immediate formation of a sticky precipitate, which sticks to the wall of the flask, upon the addition of tosylhydrazone **3.3a**. This solubility issue can be overcome by increasing the percentage of a water-miscible organic solvent used in the reaction mixture. For example, if a water/*t*-butanol solvent-mix (1/2 v/v) was used, the yield of the reaction increased to 88% (Table 3.1, entry 7). These conditions, however, are by far not compatible anymore with a biological sample. Moving towards biological more

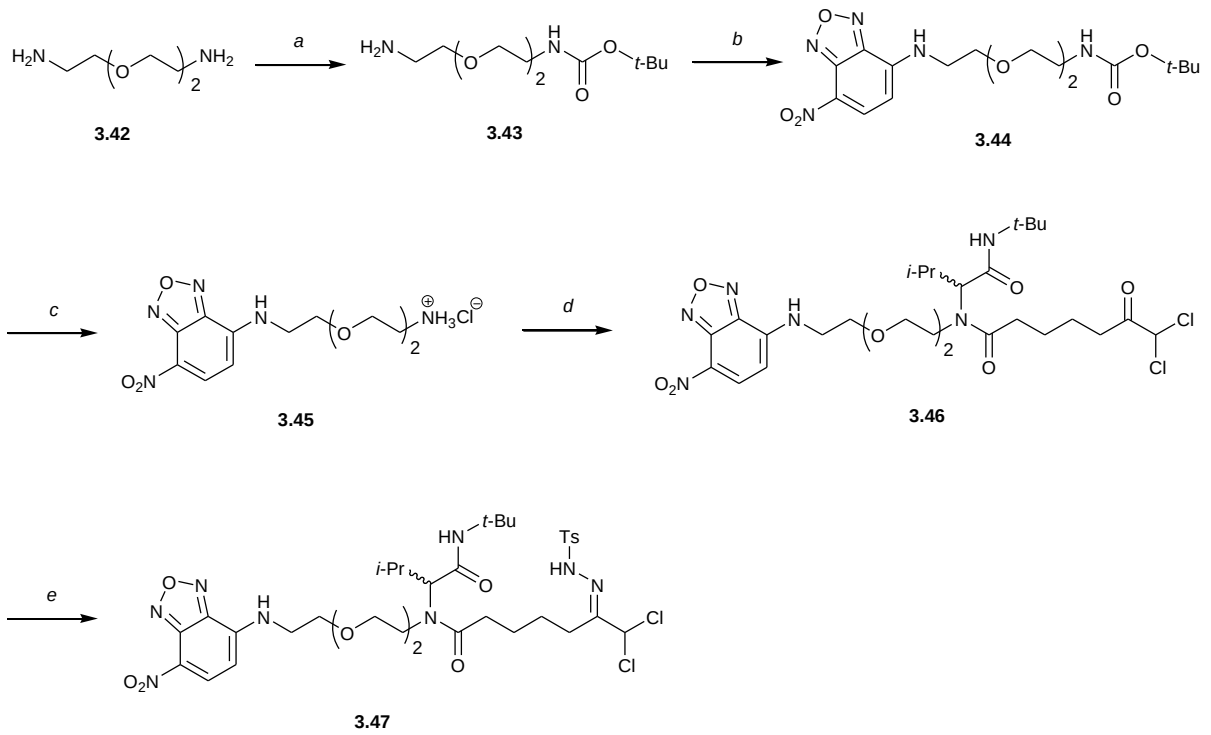
relevant systems, it was anticipated that as potassium phosphate was found to promote the Sakai-reaction, PBS-buffer (phosphate buffered saline), a common buffer solution used in biological research, might do as well. While using these optimised conditions, the desired triazole product **3.19a** could be isolated in 75% yield (Table 3.1, entry 8). Consequently, as a phosphate buffer was found to promote the Sakai reaction, it should be possible to run the Sakai reaction under near physiological conditions (without organic co-solvent) when using hydrophilic substrates like peptides or proteins, which in principle should enable the modification of biomolecules in their native environments.

Synthesis of functionalised *α*,*α*-dichlorotosylhydrazones

For a first proof of concept, the synthesis of a functionalised *α*,*α*-dichlorotosylhydrazones equipped with a fluorescent dye was envisioned which enables the rapid detection of labelled biomolecules *via* SDS-PAGE and subsequent fluorescent scanning. Initially, NBD (7-nitrobenzofurazan) was chosen as detection unit due to the ease of synthetic accessibility of suitable functionalised fluorescent dyes which can be utilised in the synthesis of fluorescent *α*,*α*-dichlorotosylhydrazones. However, it is well-known that the fluorescence properties of NBD-adducts are highly environment-sensitive, *e.g.* the fluorescence intensity decreases significantly in aqueous solutions.[26]

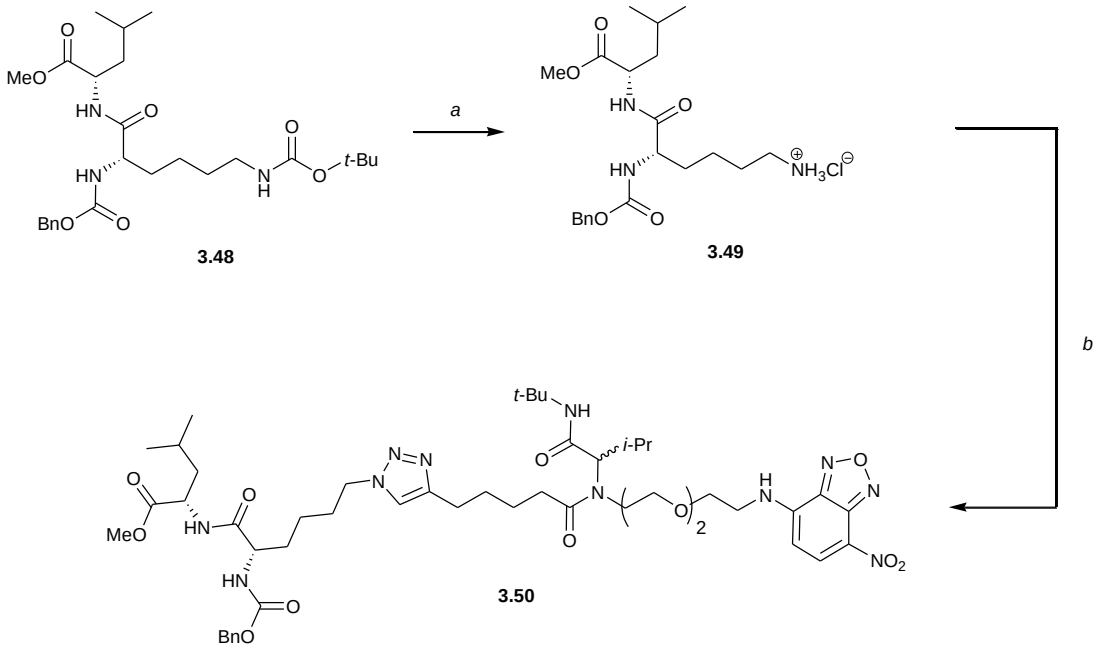
The synthesis of the fluorescent *α*,*α*-dichlorotosylhydrazone **3.47** equipped with a fluorescent NBD-moiety starts with the selective mono-Boc-protection of commercially available diamine 2,2'-(ethylenedioxy)-bis(ethylamine) **3.42** in 95% yield (Scheme 3.10). Afterwards amine **3.43** was treated with NBD-Cl in the presence of a weak base to generate the bright yellow fluorescent PEG_2-NBD-NHBoc **3.44** in 72% yield. After subsequent cleavage of the Boc-protective group under very mild conditions with trimethylsily chloride in dry methanol (*in situ* generation of gaseous hydrogen chloride)[27] in almost quantitative yield, the resulting amine hydrochloride PEG_2-NBD-NH_2·HCl **3.45** was reacted in a subsequent Ugi four-component reaction (Ugi-4CR) with *i*-butyraldehyde, *t*-butyl isocyanide and *α*,*α*-dichloroketone **3.9** to afford the fluorescent *α*,*α*-dichloroketone **3.46** in moderate 57% yield (not-optimised). For the coupling of both building blocks, the *α*,*α*-dichloroketone **3.9** and the fluorescent-tag **3.45**, the Ugi-4CR was chosen as coupling method instead of standard peptide coupling methods due to the very mild (slightly acidic) conditions which can be applied. Due to the strong inductive effect of the carbonyl group, the chlorine atom is highly susceptible to nucleophilic substitution under various (preferentially basic) conditions.[28] Finally, *α*,*α*-dichloroketone **3.46** was

treated with *p*-toluenesulphonyl hydrazide in dichloromethane to yield the amine-reactive fluorescent tosylhydrazone **3.47** in 70% yield and sufficient purity. The obtained amine-reactive fluorescent-tag exhibits a bright greenish yellow fluorescence with λ_{ex}= 462 nm and λ_{em}= 530 nm in an acetonitrile solution (see Fig. 3.6A).



Scheme 3.10 Synthesis of a fluorescent *α,α*-dichlorotosylhydrazone. *Reagents and Conditions*: (a) Boc_2O, dioxane, r.t., overnight (95%); (b) NBD-Cl, $NaHCO_3$, MeCN, r.t., overnight (72%); (c) TMSCl, MeOH, r.t., overnight (96%); (d) *i*-butyraldehyde, *t*-butyl isocyanide, dichloroketone **3.9**, Et_3N, MeOH, r.t., overnight (57%); (e) *p*$TsNHNH_2$, MeCN, r.t., overnight (70%).

To estimate the ability of the obtained dye-functionalised *α,α*-dichlorotosylhydrazone for the covalent modification of free lysine residues, tosylhydrazone **3.47** was reacted with the dipeptide Z-Lys-Leu-OMe·HCl (**3.49**), which was accessible from the Boc-protected, commercially available dipeptide Z-Lys(Boc)-Leu-OMe (**3.48**) *via* Boc-cleavage with trimethylsily chloride in dry methanol in almost quantitative yield (Scheme 3.11). The corresponding triazole-modified dipeptide equipped with a fluorescent NBD-moiety (**3.50**) could be isolated in moderate 54% yield. This clearly demonstrates the ability of *α,α*-dichlorotosylhydrazones to react with free lysine residues of peptides under weak basic conditions. Consequently, the Sakai reaction should be equally able to modify free lysine residues of proteins.



Scheme 3.11 Modification of a free lysine residue of a dipeptide utilising the Sakai reaction. *Reagents and Conditions:* (a) TMSCl, MeOH, r.t., overnight (98%); (b) NBD-tosylhydrazone **3.47**, KOAc (6 equiv.), EtOH/MeCN (3/2 v/v), r.t., overnight (54%).

The covalent modification of proteins utilising α,α-dichlorotosylhydrazones

As proof of concept, the obtained fluorescent-tag **3.47** was incubated with commercially available, purified bovine serum albumin (BSA, ~ 66.8 kDa) and lysozyme (~ 14.3 kDa) in an aqueous phosphate buffer under denaturing conditions (8 M urea). After incubation for 30 min in the dark, non-reacted tosylhydrazone **3.47** was quenched with a lysine solution. Afterwards effective labelling of BSA and lysozyme was established by running the samples on an SDS-PAGE gel and subsequent analysis by means of fluorescence

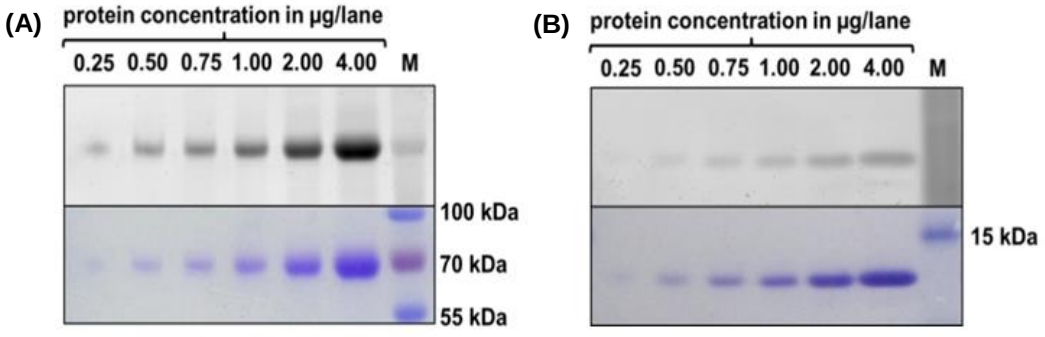


Fig. 3.3 SDS-PAGE gels of fluorescently labelled BSA (A) and lysozyme (B). *Labelling conditions*: 50 µg protein in 10 µL Kpi-buffer (200 mM, pH = 8.5) containing 8 M urea, 1 µL fluorescent-tag **3.48** solution (800 pmol·µL^{-1}), incubation for 30 min on ice. Marker: PageRuler™ prestained protein ladder (Thermo Scientific). Up: Proteins visualised by in-gel fluorescence-scanning; down: subsequent Coomassie-blue staining (performed by Dr. Felix Stehle, IPB Halle).

scanning (Fig. 3.3). In comparison to commonly used staining techniques (*e.g.* coomassie-blue staining), the application of this novel fluorescent-tag proved to be of similar sensitivity in detecting covalently labelled proteins.

In this initial labelling experiment, equimolar amounts of protein and fluorescent-tag **3.48** were used. As a consequence of the presence of about 59 lysine residues, in general, only one lysine residue of BSA should have been modified utilising the Sakai reaction. To proof this hypothesis, the labelled protein fraction was subjected to MALDI-TOF analysis to estimate the average degree of substitution. Despite the mild ionisation conditions applied, no modified proteins could be detected, though (only the BSA peak was visible in the MALDI-TOF MS spectrum). This might be attributed to unknown fragmentation events of the probe in the course of ionisation leading to a triazole modified species which cannot be differentiated from unmodified BSA in the MS spectra. To confirm triazole formation, lysozyme was used as model enzyme instead of BSA and treated with a 50fold excess of the fluorescent-tag **3.48**, which should enable, in theory, the modification of all six free lysine residues of lysozyme. Afterwards the labelled proteins were tryptically digested with trypsin and the obtained peptide mixture was analysed by LC/MS/MS.

From previous experiments in our laboratories, however, the preferential fragmentation of tertiary amide bonds of peptoids obtained in an Ugi-4CR under ESI-MS^2 conditions was established.[29] Therefore, NBD-modified dipeptide **3.50** was first subjected to FT-ESI-MS/MS analysis to identify the fragmentation pattern of NBD-tosylhydrazone **3.47** (Fig. 3.4). Under ESI-MS^2 conditions two fragment-ions can be observed with the most intense peak corresponding to the cleavage of the *t*-butyl amide bond and the less intense peak to the cleavage of the tertiary amide bond. Based on the observed fragmentation pattern, the expected mass shift of triazol-modified peptides can be calculated, which is essential for the identification of the covalently labelled lysine residues of lysozyme by comparative analysis of the tandem mass spectra of unmodified lysozyme. So far, tandem mass spectrometric analysis of tryptically digested, NBD-labelled lysozyme in the group of Wolfgang Hoehenwarter (Proteome Analytics Unit, IPB Halle) did not draw any conclusions on the labelling site(s), although covalent labelling was confirmed by SDS-PAGE and subsequent in-gel fluorescence scanning.

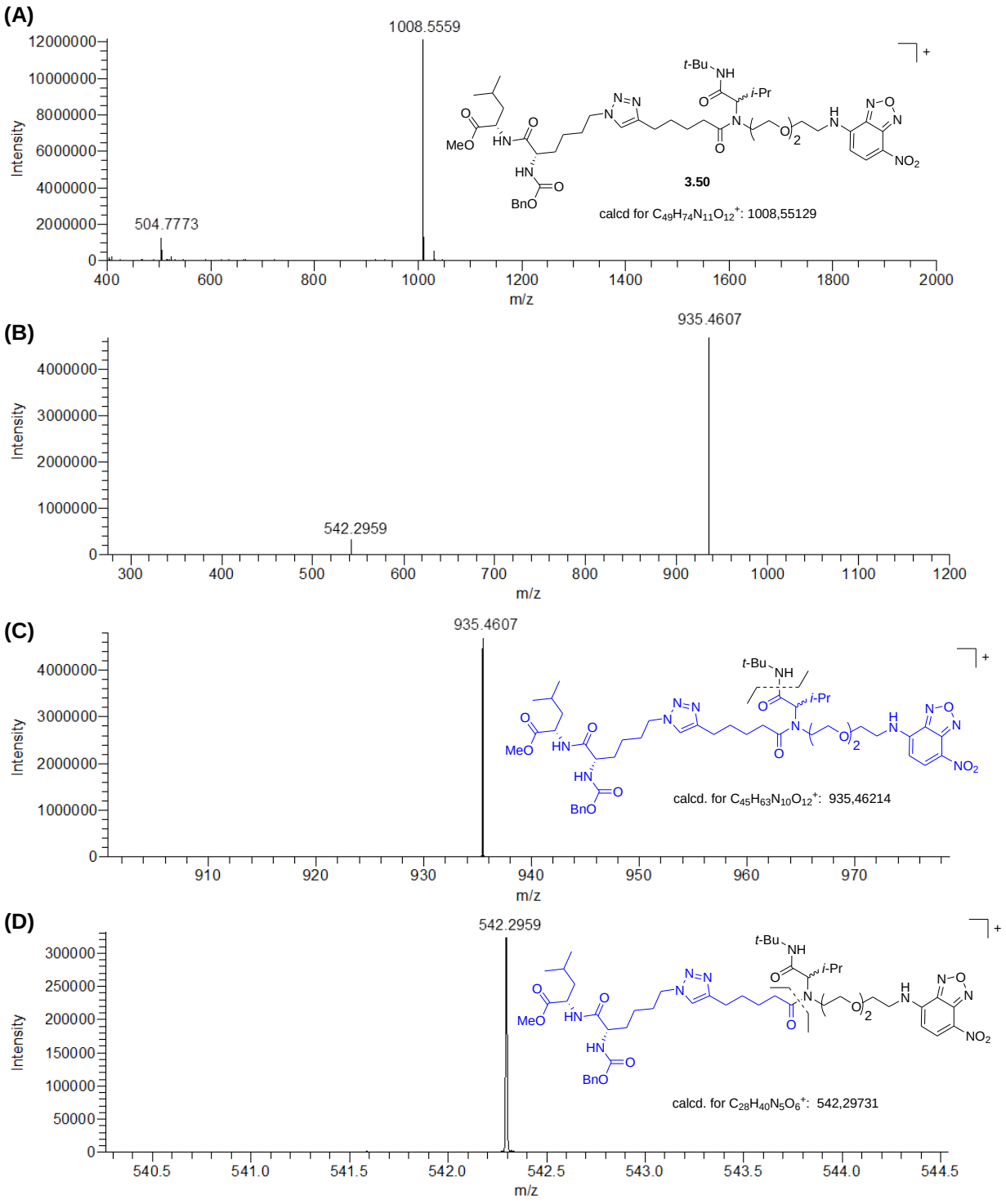
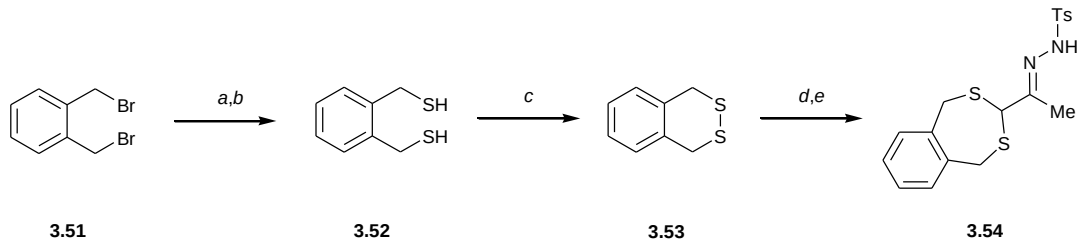


Fig. 3.4 Direct infusion FT-MS spectra of NBD-modified dipeptide 3.50. (A) FT-(+)-ESI-MS^1 spectra of compound **3.50**; **(B)** under (+)-ESI-MS^2 conditions the formation of two fragment ions can be observed; **(C)** and **(D)** small section of the FT-(+)-ESI-MS^2 spectra and proposed fragmentation site; putative structures of the formed fragment ions are displayed in blue (performed by Dr. Wolfgang Hoehenwarter, IPB Halle).

α,α-Dichlorotosylhydrazones – A class of novel disulphide-bridging agents?

As stated previously, *α,α*-dichlorotosylhydrazones predominantly undergo nucleophilic displacement in the presence of dithiols affording cyclised products suggesting that these

tosylhydrazones might be applicable as novel disulphide-bridging reagents. Therefore, in a first proof on concept, 1,4-dihydro-2,3-benzodithiine (**3.53**), which is accessible in three synthetic steps from *α,α'*-dibromoxylene (**3.51**) in 53% yield, was reacted with tosylhydrazone **3.3a** under weak basic conditions (Scheme 3.12). Prior to the addition of tosylhydrazone **3.3a**, the disulphide bond was reduced *in situ* with tris(2-carboxyethyl)-phosphine hydrochloride (TCEP·HCl), a common reducing agent for the cleavage of disulphide bonds.[30] As expected, the corresponding cyclised benzodithiepine **3.54** could be isolated after purification by column chromatography, however, in low yields (13%). As outlined previously, the low yield can be attributed to the formation of a less favoured seven-membered ring.



Scheme 3.12 *α,α*-Dichlorotosylhydrazones as disulphide-bridging agents. *Reagents and Conditions*: (a) thiourea, EtOH, reflux, 6 h; (b) NaOH, H_2O, reflux, overnight (90% over two steps); (c) $FeCl_3 \cdot 6H_2O$, MeOH/HOAc (1/1), 70 °C, 1 h (60%); (d) TCEP·HCl, *t*-BuOH/H_2O (2/1), r.t., 1.5 h; then (e) KOAc, tosylhydrazone **3.3a**, r.t., 16 h (13%).

The reaction with free lysine residues of proteins, however, cannot be neglected. Therefore, reagents are necessary which assist to differentiate between the reaction of *α,α*-dichlorotosylhydrazones with primary amines and disulphide moieties. In case of the Sakai reaction, one can take advantage of the fact that upon the reaction of primary amines with *α,α*-dichlorotosylhydrazones the tosyl-group is cleaved-off, whereas upon the reaction of dithiols the tosyl-group is retained in the final product. For example, the application of a suitable *α,α*-dichlorosulphonohydrazone equipped with two different fluorescent dyes should enable to differentiate between amine- or thiol-labelling *via* SDS-PAGE and subsequent fluorescence scanning (Fig. 3.5A). As a prerequisite, no fluorescence quenching should occur. In a hypothetical experiment a protein (or protein mixture) is treated under reducing conditions (addition of TCEP·HCl to cleave all solvent accessible disulphide bridges) with a *α,α*-dichlorosulphonohydrazone equipped with two different fluorescent dyes. After incubation, the reaction may be quenched by the addition of a lysine solution and the samples are loaded on a SDS-gel. After subsequent 1D-gel electrophoresis, the gels are analysed by means of fluorescence scanning using, *e.g.* a

Typhoon scanner, which is able to detect multiple fluorescent labels in the same experiment (*multiplexing*).[31] In general, three different possibilities are reasonable (Fig. 3.5B): (i) a fluorescence signal is visible in each channel, *i.e.* only a disulphide moiety of a protein has been modified; (ii) a fluorescent signal is obtained for each channel again, but the signal corresponding to Dye 2 exhibits an increased fluorescent intensity which indicates that lysine residues as well as disulphide bonds of the same protein have been modified in parallel; and (iii) only one fluorescent signal is obtained, *i.e.* the second fluorescent dye has been eliminated as a consequence of triazole formation upon the reaction with free lysine residues

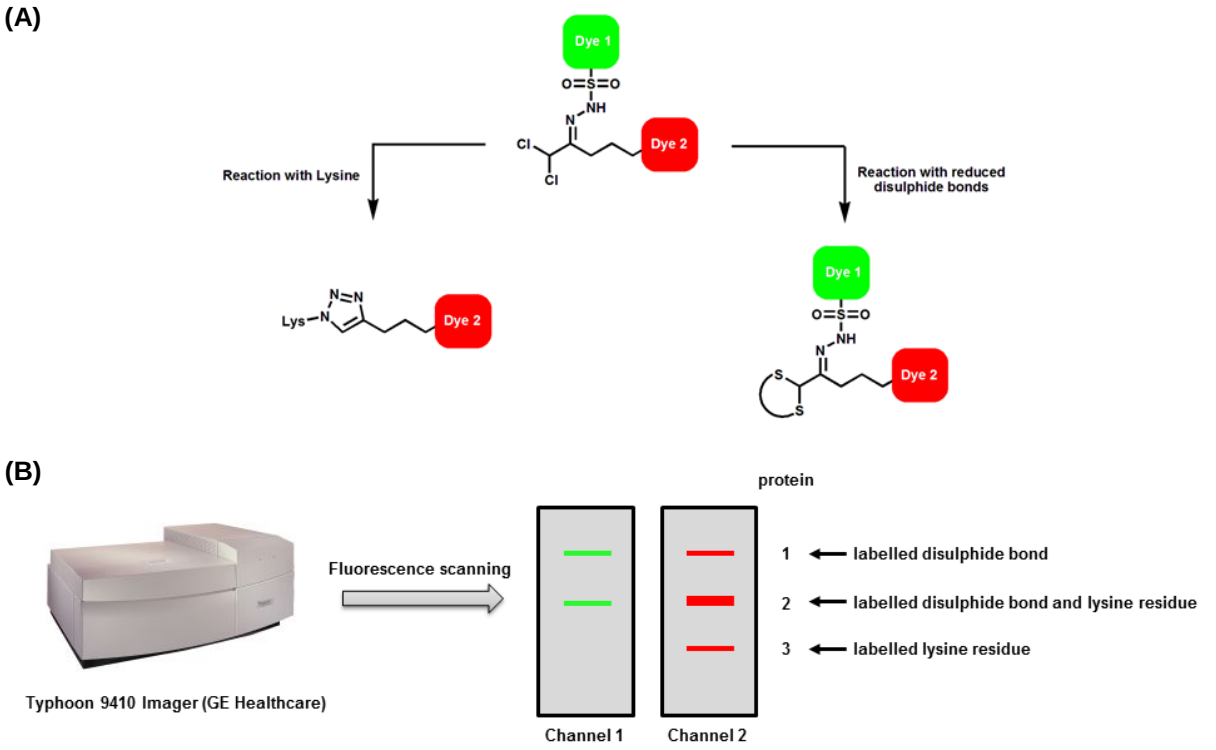
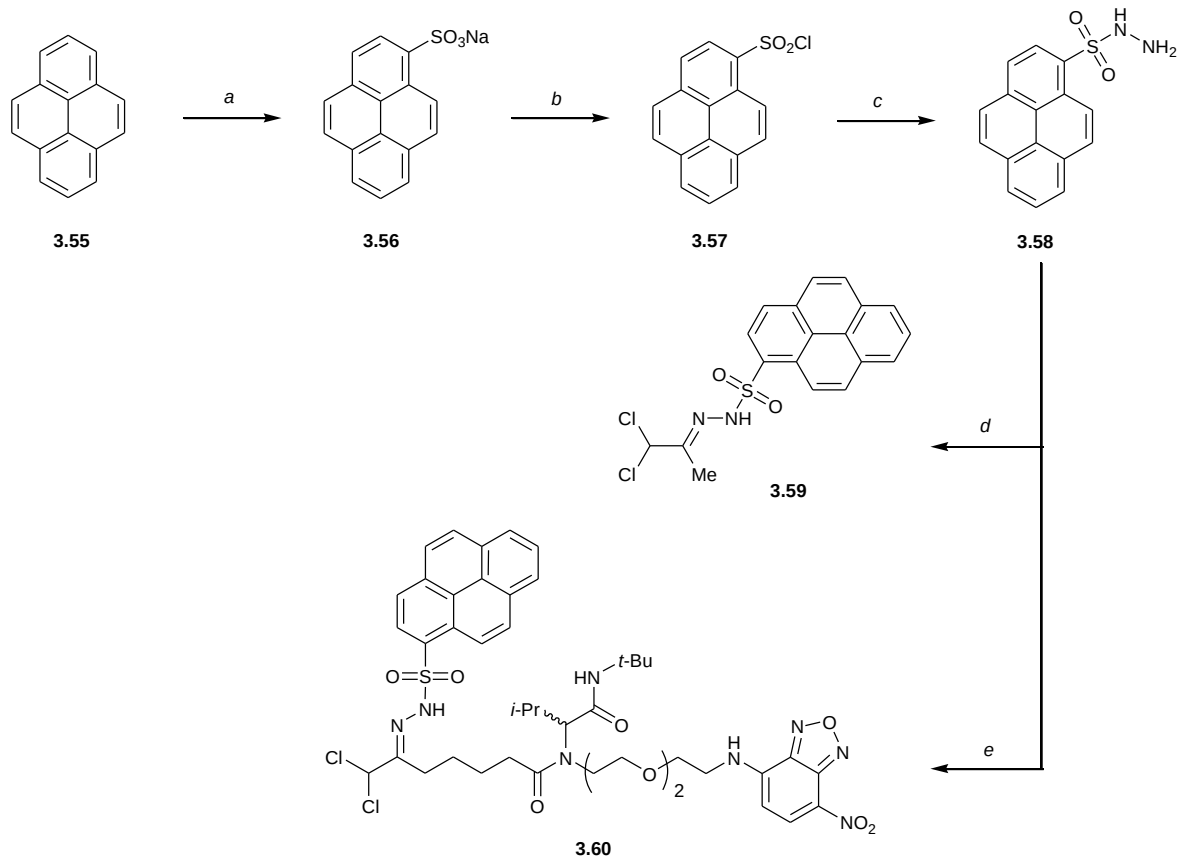


Fig. 3.5 Parallel detection of proteins previously labelled with fluorescent *α,α*-dichlorosulphonohydrazones either at reduced disulphide bonds or free lysine residues *via* fluorescence scanning.

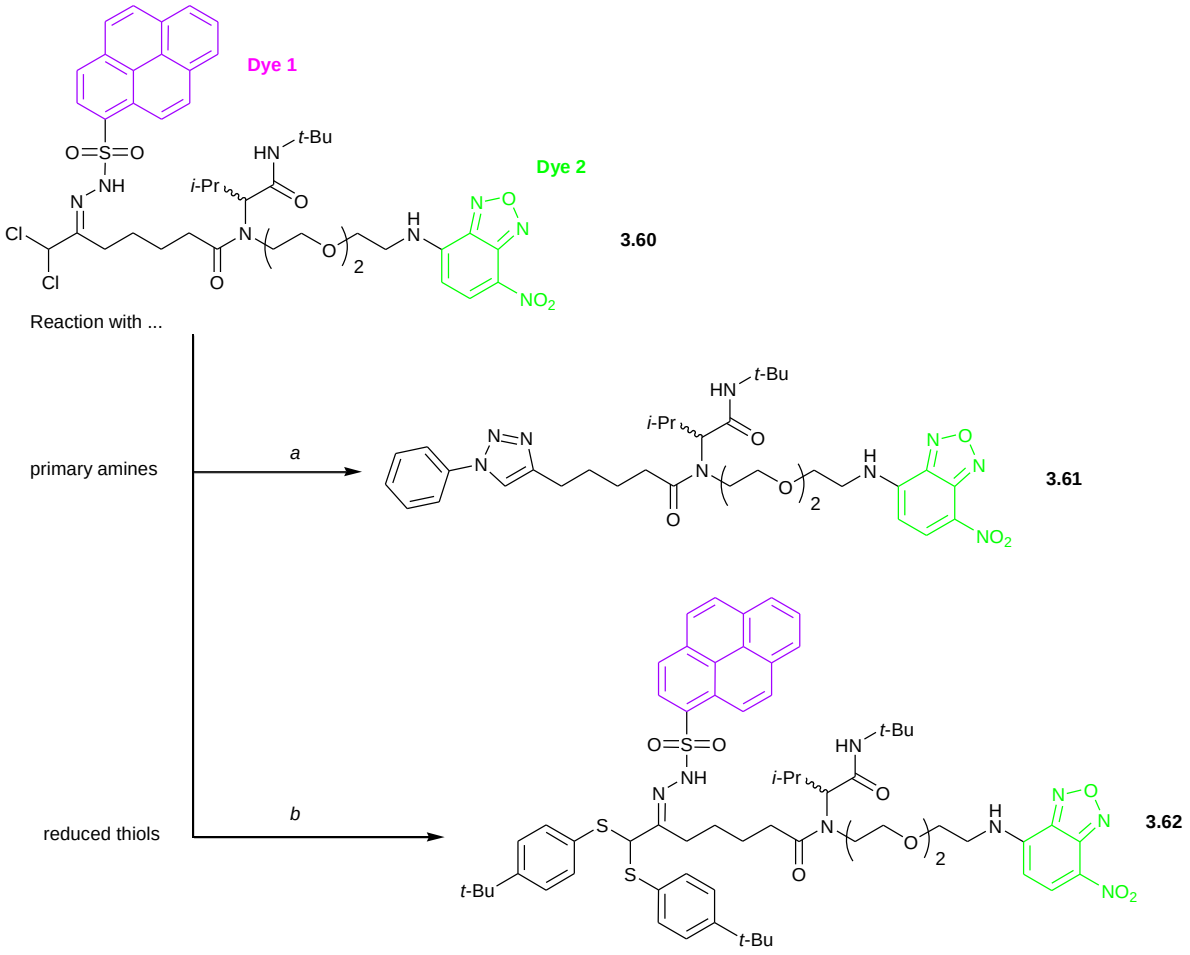
To test our hypothesis, a *α,α*-dichlorosulphonohydrazone was prepared which is equipped in addition to a fluorescent NBD-moiety with a fluorescent pyrene-1-sulphonyl moiety instead of a tosyl-group (Scheme 3.13). For this purpose, commercially available pyrene (**3.55**) was transferred into its corresponding pyrene-1-sulphonohydrazide (**3.58**) first. This fluorescent sulphonohydrazide can be reacted afterwards with *α,α*-dichloroketones affording the respective amine-reactive *α,α*-dichloropyrene-sulphonohydrazones (*e.g.* **3.59** and **3.60**, respectively) in four steps and 18% (**3.59**) or 15% (**3.60**) overall yield, respectively. First, pyrene was treated with chlorosulphonic

acid and the obtained sodium pyrene-1-sulphonate (**3.56**) was converted into the corresponding pyrene-1-sulphonyl chloride (**3.57**) with thionyl chloride in the presence of catalytic amounts of *N*,*N*-dimethylformamide. Afterwards sulphonyl chloride **3.57** was treated with hydrazine monohydrate and triethylamine to yield pure pyrene-1-sulphonohydrazide which was subsequently reacted with either 1,1-dichloroacetone **3.1** (affording hydrazone **3.59**) or NBD-dichloroketone **3.46** (affording hydrazone **3.60**). The obtained amine-reactive *α*,*α*-dichloropyrenesulphonohydrazones exhibit a bright violet fluorescence with λ_{abs} = 348 nm and λ_{em} = 390 nm (for **3.59**) and a bright greenish yellow fluorescence with λ_{abs1} = 348 nm, λ_{abs2} = 462 nm and λ_{em1} = 388 nm, λ_{em2} = 532 nm (for **3.60**) in an acetonitrile solution (Fig. 3.6B/C).



Scheme 3.13 Synthesis of fluorescent *α*,*α*-dichloropyrenesulphonohydrazones. *Reagents and Conditions*: (a) pyrene, chlorosulphonic acid, CH_2Cl_2, r.t., overnight (43%); (b) $SOCl_2$, DMF, r.t., overnight (84%); (c) $N_2H_4 \cdot H_2O$, Et_3N, MeCN, r.t., overnight (71%); (d) 1,1-dichloroacetone **3.1**, MeCN, r.t., overnight (71%); (e) NBD-dichloroketone **3.46**, THF, r.t. overnight (60%).

The obtained fluorescent *α*,*α*-dichloropyrenesulphonohydrazone **3.60** was reacted either with a primary amine or a thiol (Scheme 3.14) and the absorption and emission spectra of the obtained fluorescent triazole **3.61** (from aniline, 64% yield) and fluorescent bis-thioether **3.62** (from 4-*tert*-butylthiophenol, 34% yield) were recorded (Fig. 3.6D/E).



Scheme 3.14 Reaction of fluorescent PEG$_2$-NBD-α,α-dichloropyrenesulphonohydrazone 3.60 with primary amines and thiols. *Reagents and conditions*: (a) aniline, KOAc (6 equiv.), EtOH/MeCN (3/2 v/v), r.t., overnight (64%); (b) 4-*tert*-butylthiophenol, KOAc (6 equiv.), EtOH/MeCN (3/2 v/v-%), r.t., overnight (34%).

As one might expect, two strong absorption maxima appear in the UV/Vis-spectrum of the fluorescent NBD-dichloropyrenesulphonohydrazone **3.60** (Fig. 3.6C) which match the pyrene absorption maxima of methyl dichloropyrenesulphonohydrazone **3.59** (Fig. 3.6B) and the NBD absorption maxima of NBD-dichlorotosylhydrazone **3.47** (Fig. 3.6A). If hydrazone **3.60** was reacted with a primary amine, consequently, the pyrene absorption maxima disappeared leading to a UV/Vis-spectrum which is almost identical to NBD-dichlorotosylhydrazone **3.47** as a consequence of triazole ring formation (Fig. 3.6D). On the other hand, if thiols are used instead of primary amines, the pyrenesulphono-hydrazone moiety is retained in the final product and almost no change occurs in the UV/Vis-spectrum of the bis-thioether **3.62** compared to unmodified **3.60** (Fig. 3.6E). The previously outlined observations apply also to the corresponding emission spectra, *i.e.* disappearance of the pyrene emission maximum upon reaction with primary amines and presence of two emission maxima upon the reaction with thiols, respectively. However,

the fluorescence intensity of pyrene is strongly reduced in comparison to NBD despite its superior fluorescent properties, such as quantum yield and fluorescence lifetime,[32] which might be attributed to fluorescence quenching events.

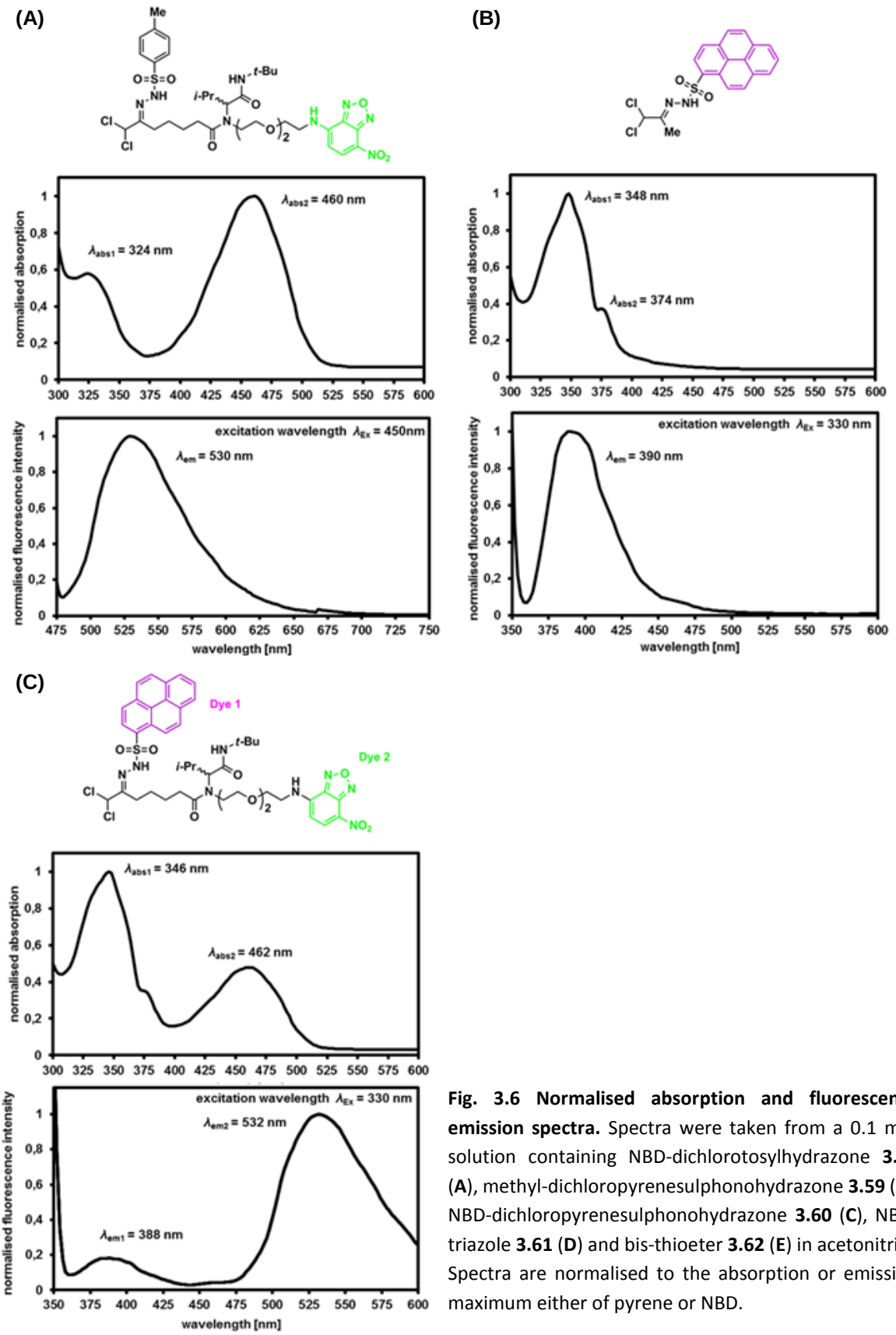


Fig. 3.6 Normalised absorption and fluorescence emission spectra. Spectra were taken from a 0.1 mM solution containing NBD-dichlorotosylhydrazone **3.48** (**A**), methyl-dichloropyrenesulphonohydrazone **3.59** (**B**), NBD-dichloropyrenesulphonohydrazone **3.60** (**C**), NBD-triazole **3.61** (**D**) and bis-thioeter **3.62** (**E**) in acetonitrile. Spectra are normalised to the absorption or emission maximum either of pyrene or NBD.

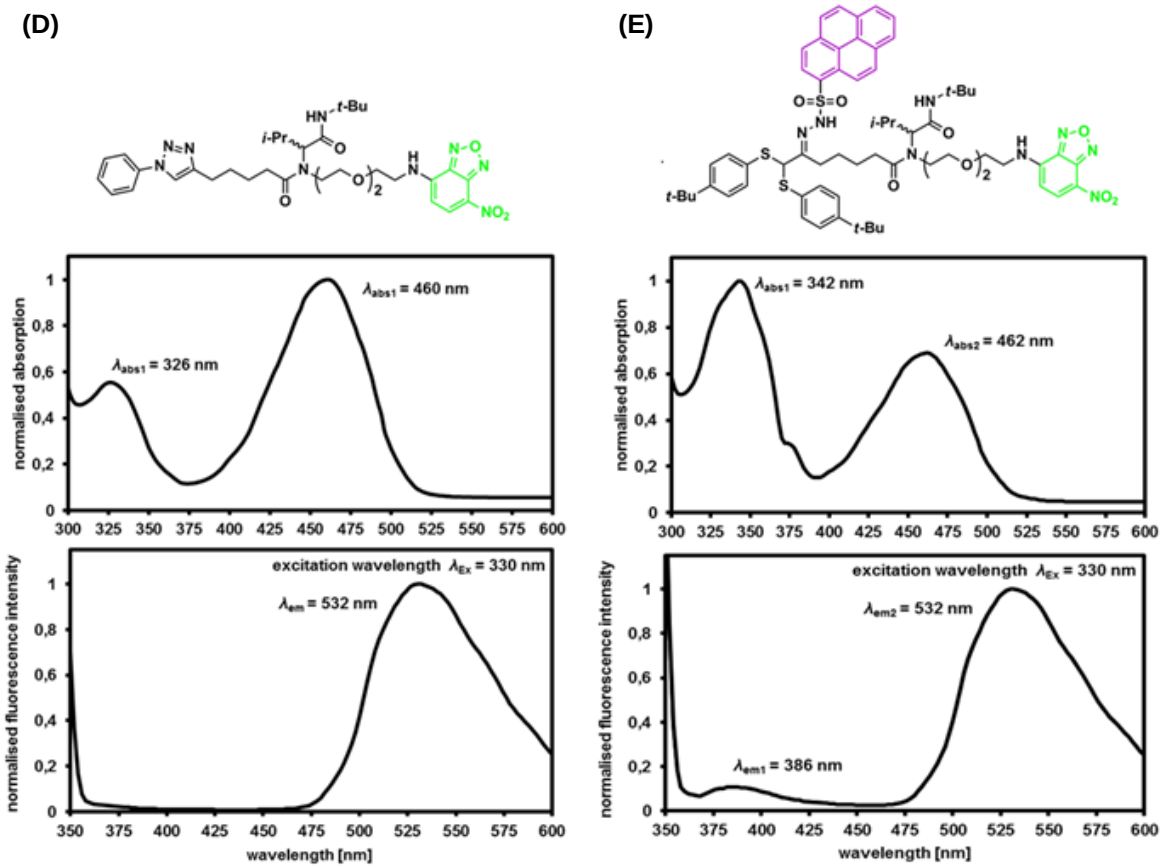


Fig. 3.6 (continued)

As a consequence of the partial overlap of the excitation spectrum of pyrene and absorption spectrum of NBD (Fig. 3.7), energy transfer from electrons in the pyrene excited state to electrons in the NBD ground state might have occured. Hence, this resonance energy transfer (RET), a non-radiative energy transfer between two dyes (a donor D and acceptor A) without the emission or absorption of photons, presumably plays a crucial role in the reduction of the fluorescence intensity of the donor fluorophore pyrene.[33] RET leads to a reduced fluorescence intensity of pyrene, which limits the applicability of NBD-dichloropyrene-

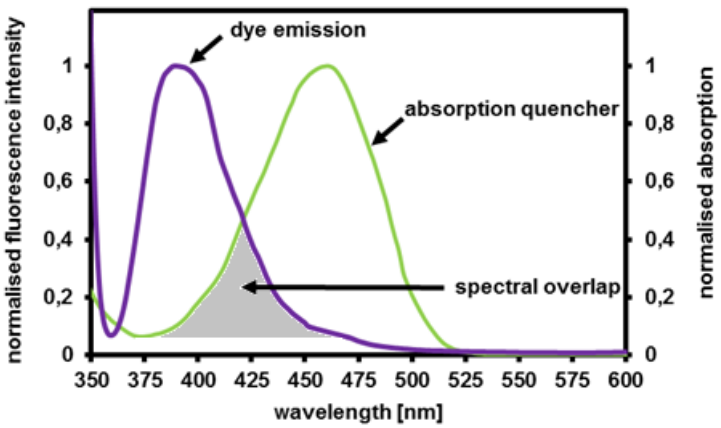


Fig. 3.7 Spectral overlap for resonance energy transfer (RET). Due to the partial spectral overlap of the emission spectrum of dichloro-pyrenesulphonohydrazone **3.59** (donor) and NBD-dichlorotosyl-hydrazone **3.48** (acceptor), fluorescence quenching occurs *via* a non-radiative energy transfer from the donor to the acceptor, hence reducing the fluorescence intensity of the donor dye.

sulphonohydrazone **3.60** in detecting fluorescently labelled disulphide bonds in the presence of fluorescently labelled lysine residues in a biological sample based on fluorescence scanning as proposed earlier. This limitation might be overcome by incorporating fluorescent dyes in these labelling reagents with non-overlapping emission and absorption spectra (*e.g.* near-infrared fluorescent dyes like Alexa Fluor 750 or Cy7 instead of NBD) or exchanging one of the fluorescent dyes, preferably NBD, against biotin. In this case, the combination of fluorescent scanning and western-blot analysis would enable the differentiation between disulphide-labelling and amine-labelling events. Moreover, such a labelling reagent would allow affinity enrichment of the labelled proteins prior to a gel-based analysis.

Of course, if one would be interested only in disulphide-labelling regardless of possible amine-labelling events, the *α*,*α*-dichloropyrenesulphonohydrazone **3.59** would be sufficient. In this case, only proteins with fluorescently labelled disulphide-bonds would be detectable upon fluorescence scanning. However, further investigations regarding the disulphide-bridging potential of *α*,*α*-dichloropyrenesulphonohydrazones need to be performed under near physiological conditions utilising biomolecules equipped with native disulphide bonds instead of small organic disulphides. However, if the application of a Typhoon scanner is intended, the pyrene moiety needs to be exchanged because pyrene and other fluorophores with excitation wavelengths < 450 nm cannot be analysed as a consequence of the installed laser light sources of commercial systems (457 nm, 488 nm argon ion laser; 532 nm Nd:YAG laser; and 633 nm HeNe laser).

3.4 Conclusions and future perspective

In this chapter, the scope and limitations of the Sakai reaction have been studied, an up to now rather unnoticed metal-free approach leading to the regioselective formation of 1,4-substituted 1,2,3-triazoles from primary amines and *α*,*α*-dichlorotosylhydrazones in the presence of a weak base. For this purpose, alkyl-, aryl- and functionlised *α*,*α*-dichloro-tosylhydrazones were prepared in one to three steps from commercially available bulk chemicals. These amine reactive reagents were reacted with various aromatic and aliphatic primary amines. All triazole products could be isolated in good to excellent yields after purification. Furthermore, as a consequence of the observed chemoselectivity of the Sakai reaction, no laborious protective group strategy was necessary to modify even highly complex natural products, such as phytosphingosine and psychosine. In addition, other amine nucleophiles (hydrazines, hydroxylamines, hydrazides) and thiols were

reacted with *α*,*α*-dichlorotosylhydrazones. From these results and previous observation by Hanselmann *et al.*, a plausible mechanism of the Sakai reaction, different from the initial mechanism proposed by Sakai and collaborators (formation of a Bamford-Stevens-type intermediate), was proposed. However, the synthesis of substituted *α*,*α*-dichlorotosylhydrazones from the corresponding *α*,*α*-dichloroketones, which are accessible by various published methods, though, often in quiet low yields, and *p*-toluenesulphonyl hydrazide represents the bottleneck of this approach.

Moreover, the applicability of the Sakai triazole formation reaction to modify biomolecules, such as proteins and peptides, *etc.*, was investigated. Therefore, the reaction conditions were optimised in respect to the demands of a biological sample. After various attempts, it was found that the reaction proceeds in an aqueous buffered solution in good yields. In a first proof of concept, purified BSA and lysozyme were covalently modified with a suitable designed fluorescent NBD-dichlorotosylhydrazone (**3.48**). Further studies should be directed towards the synthesis of amine-reactive probes with other functional reporter units instead of NBD as a consequence of its very low quantum yield in aqueous media ($\Phi < 0.01$).[26,31] For example, other fluorescent dyes with superior fluorescent properties or biotin for affinity enrichment of the covalently labelled biomolecules and detection by western-blot analysis could facilitate the analysis of the labelled proteins. Furthermore, studies to control the degree of substitution at the target protein by varying the pH of the reaction medium are worth considering (at neutral conditions the most nucleophilic amine, presumably the *N*-terminus, should participate in the Sakai reaction). In this respect, *α*,*α*-dichlorotosylhydrazones equipped with a PEG-chain would be required which enable to estimate the degree of substitution based on the observed mass shift in the SDS-gel. The synthesis of probes equipped with two amine reactive *α*,*α*-dichlorotosylhydrazone moieties for the chemical cross-linking of lysine residues (cf. chapter 1.3 and 2.3, respectively) to extend the scope of the Sakai reaction would also be imaginable.

Finally, as a consequence of the pronounced reactivity of *α*,*α*-dichlorotosylhydrazones with thiols, dithiols and *in situ* reduced disulphide bonds, investigations regarding the applicability of these reagents as novel disulphide-bridging agents for the modification of proteins were conducted. For this purpose, a suitable hydrazone equipped with two different fluorescent dyes to discriminate between disulphide- and amine-labelling events in a biological sample was prepared. NBD-dichloropyrenesulphonohydrazone **3.60** exhibited the expected reactivity, however, fluorescence analysis of the obtained

products revealed a reduced pyrene fluorescence intensity due to fluorescence quenching events (resonance energy transfer as a consequence of the partial overlap of the pyrene emission and NBD excitation spectrum). Therefore, further optimisation of the probe design concerning the nature of the used fluorescent dyes needs to be performed to turn this method into a viable tool to modify disulphide bonds comparable to already established methods utilising dibromo respectively dithio maleimides, *etc*.

3.5 Experimental section

General experimental information

Unless otherwise stated all chemicals and solvents were obtained from commercial sources and were used without further purification. All ^{1}H and ^{13}C NMR spectra were recorded in CHLOROFORM-*d* (unless otherwise noted) on either a 300 MHz Varian MERCURY-VX 300 apparatus (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR, respectively) or on a 400 MHz Varian MERCURY-VX 400 apparatus (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, respectively). Chemical shifts are reported in δ values (ppm) with tetramethylsilane (TMS) as internal standard. Melting points were determined with a Leica DM LS2 hot stage microscope (without correction). ESI-MS spectra were obtained from an API-150EX spectrometer. HRMS spectra were recorded on an FT-ICR Bruker Apex III 70e mass spectrometer. Purification by column chromatography was performed on silica gel 60 (230–400 mesh, 0.040–0.063 mm), Merck, Germany. TLC identification of products and reactants was performed on silica gel coated aluminum foil (silica gel 60 F254 with fluorescence indicator), Merck, Germany. Chiral HPLC measurements were performed using a diode array detector and a Chiracell OB-H column. Eluent was hexane/*i*-propanol**.** Optical rotation was determined using a Jasco DIP-1000 Digital Polarimeter. Fluorescence and absorption spectra were recorded on a Molecular Devices SpectraMax M5 apparatus.

Protein Labelling and analysis

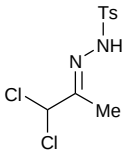
For *in vitro* protein labelling, 50 µg bovine serum albumin (Sigma Aldrich) or 50 µg lysozyme (Sigma Aldrich) were dissolved in 10 µL 200 mM potassium phosphate buffer (pH 8.5) containing 8 M Urea. The conjugation reaction was started by the addition of 1 µL NBD-dichlorotosylhydrazone **3.48** (800 pmol μL^{-1}, DMSO). The samples were incubated on ice in a dark environment for 30 min and the labelling reaction was stopped by the

addition of 1 µL lysine solution (10 mM, pH 9.0) followed by incubation for additional 10 minutes in the dark. For analysing the labelling reaction, a 10% SDS-PAGE Lammli-buffer was added and the samples were denatured for 5 min at 95 °C. After the subsequent washing of the gel (three times in water for 1–2 minutes), the labelled proteins were visualised by in-gel fluorescence scanning using a Typhoon 9410 Imager (GE Healthcare). Finally, the gel was stained with Coomassie-blue.

General procedure: Sakai Reaction

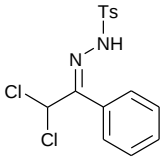
To a cooled solution (0 °C) of primary amine in ethanol (12 mL) was added diisopropylethylamine (6 equiv.) or potassium acetate (3 equiv.), respectively. The solution was stirred for 10 minutes, whereupon *α*,*α*-dichlorotosylhydrazone **3.3a–e** (1.3 equiv.) dissolved in acetonitrile (8 mL) was added dropwise to the cooled solution. Stirring was continued at room temperature until completion of the reaction (checked by TLC). After completion of the reaction was detected, all volatiles were removed under reduced pressure and the residue was purified by column chromatography or recrystallisation.

***N*'-(2,2-Dichloro-1-methylethylidene)-4-methylbenzenesulphonohydrazide (3.3a)**



To a suspension containing *p*-toluenesulphonyl hydrazide (6.7 g, 36 mmol) in propionic acid (30 mL) was added 1,1-dichloroacetone **3.1** (5.0 g, 39 mmol) and the resulting mixture was stirred at room temperature for 4 hours. The precipitate was collected by filtration, washed with cyclohexane and dried under reduced pressure to yield tosylhydrazone **3.3a** as a white solid (9.79 g, 92%). M.p. 152–155 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.84 (s, 3H), 2.39 (s, 3H), 7.44 (d, J = 7.9 Hz, 2H), 7.81 (d, J = 8.2 Hz, 2H), 9.19 (s, 1H), 11.89 (br. s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 9.8, 21.3, 127.6, 130.1, 135.8, 144.4, 151.6, 191.2.

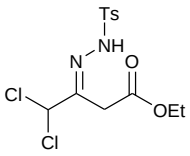
***N*'-(2,2-Dichloro-1-phenylethylidene)-4-methylbenzenesulphonohydrazide (3.3b)**



To a solution containing 2,2-dichloroacetophenone **3.2** (1.50 mL, 10.2 mmol) in dry tetrahydrofuran (10mL) was added dropwise *p*-toluene-sulphonyl hydrazide (1.64 g, 8.81 mL) in 10 mL dry tetrahydrofuran over a period of 15 minutes. The resulting mixture was allowed to stir overnight at room temperature. Cyclohexane (100 mL) was subsequently added and tetrahydrofuran was removed by rotary evaporation. The resulting precipitate was

collected by filtration, washed with cyclohexane and dried under reduced pressure to yield tosylhydrazone **3.3b** as a white solid (2.57 g, 81%). M.p. 121–122 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.39 (s, 3H), 7.23–7.29 (m, 2H), 7.43 (d, J = 8.3 Hz, 2H), 7.47–7.51 (m, 3H), 7.80 (d, J = 8.3 Hz, 2H), 9.40 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 21.1, 127.7, 128.6, 128.9, 129.8, 130.0, 135.4, 144.2, 150.6, 190.4.

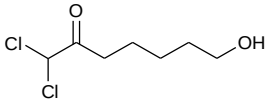
Ethyl-4,4-dichloro-3-{[(4-methylphenyl)sulphonyl]hydrazono}butanoate (3.3c)



To a suspension containing ethyl potassium malonate **3.5** (9.48 g, 55.6 mmol) in acetonitrile (200 mL) were consecutively added triethylamine (11.6 mL, 83.2 mmol) and magnesium chloride (6.44 g, 66.8 mmol) at 0 °C. After stirring at room temperature for 2 hours, the suspension was cooled to 0 °C and dichloroacethyl chloride **3.4** (6.12 g, 41.6 mmol) was slowly added. Afterwards the reaction mixture was warmed to room temperature and stirring was continued overnight. Ethyl acetate was added (120 mL) and the mixture was acidified with 2 M aqueous hydrochloric acid. The organic phase was separated, washed twice with 2 M aqueous hydrochloric acid (80 mL), dried (sodium sulphate) and all volatiles were evaporated to yield ethyl 4,4-dichloro-3-oxobutanoate **3.6** as a brownish oil which was used directly in the next step without further purification.

p-Toluenesulphonyl hydrazide (8.37 g, 44.9 mmol) was added portionwise over a period of 1 hour to a solution containing dichloroketone **3.6** (9.79 g, 41.6 mmol) in acetonitrile (56 mL). Stirring of the resulting solution was continued for 18 hours at room temperature. The reaction mixture was filtered and the filtrate was evaporated to dryness. Afterwards the residue was taken up in acetonitrile (8 mL) and stored at –30 °C for 3 days. Finally, the resulting precipitate was collected by filtration, washed with a minimum amount of ice-cold acetonitrile and dried under reduced pressure to yield tosylhydrazone **3.3c** as a pale yellow solid (4.52 g, 30%). M.p. 85–90 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.11 (t, J = 7.0 Hz, 3H), 2.40 (s, 3H), 3.56 (s, 2H), 4.03 (q, J = 7.0 Hz), 7.45 (d, J = 7.9 Hz, 2H), 7.80 (d, J = 8.5 Hz, 2H), 9.23 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 14.0, 21.2, 29.7, 60.9, 127.4, 130.0, 135.7, 144.3, 146.4, 167.1, 190.6.

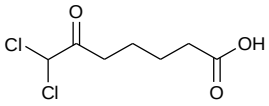
1,1-Dichloro-7-hydroxyheptan-2-one (3.8)



To a solution containing ε-caprolactone **3.7** (5.0 g, 43.8 mmol) and dichloromethane (5.59 mL, 87.6 mmol) in dry diethyl ether was slowly added lithium diisopropylamide (1.5 M in tetrahydrofuran, 58.4 mL, 87.6 mmol)

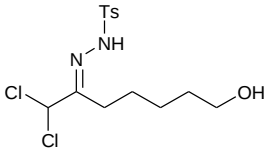
at −78 °C. Stirring was continued for 20 min, whereupon 2 M aqueous hydrochloric acid was added (80 mL). The organic phase was separated and the aqueous layer was extracted with diethyl ether (3x 50 mL). Afterwards the combined organic extracts were dried (sodium sulphate), evaporated to dryness and the residue was purified by column chromatography to afford **3.8** as yellow oil (5.84 g, 67%). R_F = 0.46 (hexane/ethyl acetate 2/3); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 1.38−1.47 (m, 2H), 1.56−1.65 (m, 2H), 1.67−1.75 (m, 2H), 2.85 (t, J = 7.2 Hz, 2H), 3.63−3.68 (m, 2H), 5.83 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 23.4, 25.0, 32.2, 34.8, 62.5, 69.8, 197.2; ESI-MS m/z: 197.0 $[M-H]^-$; HRMS (ESI) calcd for $C_7H_{12}O_2Cl_2Na$ 221.0107, found 221.0105.

7,7-Dichloro-6-oxoheptanoic acid (3.9)



A mixture containing alcohol **3.8** (1.0 g, 5.02 mmol), sodium periodate (4.26 g, 19.9 mmol) and ruthenium chloride monohydrate (58 mg, 0.28 mmol) in acetonitrile/ tetrachloromethane/water (390 mL, 1/1/1.5) was stirred for 3 hours at room temperature. Dichloromethane (150 mL) was added and the organic phase was separated. The remaining aqueous layer was extracted twice with dichloromethane (100 mL) and the combined organic extracts were dried (sodium sulphate) and evaporated to dryness. Purification of the residue by column chromatography afforded carboxylic acid **3.9** as pale yellow oil (1.01 g, 94%). R_F = 0.83 (ethyl acetate/acetic acid 98/2); ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 1.58−1.72 (m, 4H), 2.32 (t, J = 7.2 Hz, 2H), 2.83 (t, J = 7.0 Hz, 2H), 6.35 (s, 1H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 24.2, 25.3, 34.5, 36.7, 71.3, 177.2, 198.1; ESI-MS m/z: 211.5 $[M-H]^-$, 235.3 $[M+Na]^+$; HRMS (ESI) calcd for $C_7H_9O_3Cl_2$ 210.9934, found 210.9931.

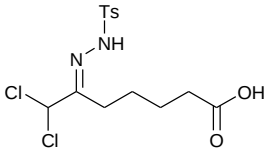
***N*'-[1-(Dichloromethyl)-6-hydroxyhexylidene]-4-methylbenzenesulphono-hydrazide (3.3e)**



To a solution containing **3.8** (2.0 g, 10.1 mmol) in acetonitrile (14 mL) was added *p*-toluenesulphonyl hydrazide (2.08 g, 10.9 mmol) portionwise over a period of 1 hour. Stirring was continued for 18 hours at room temperature. All volatiles were removed under reduced pressure and the residue was taken up in acetonitrile (3 mL) and stored at −30 °C overnight. The resulting precipitate was collected by filtration, washed with a minimum amount of ice-cold acetonitrile and dried *in vacuo* to afford tosylhydrazone **3.3e** as a white

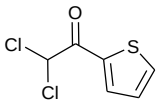
solid (1.92 g, 52%). M.p. 133–135 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.19–1.41 (m, 8H), 2.34 (s, 3H), 3.31 (t, J = 6.4 Hz, 2H), 7.39 (d, J = 8.2 Hz, 2H), 7.75 (d, J = 8.2 Hz, 2H), 9.12 (s, 1H), 12.07 (br. s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 27.2, 17.2, 24.5, 25.6, 32.3, 31.1, 127.4, 130.0, 135.8, 144.2, 154.1, 191.3.

7,7-Dichloro-6-{[(4-methylphenyl)sulphonyl]hydrazono}heptanoic acid (3.3f)



To a solution containing **3.9** (1.01 g, 4.74 mmol) in acetonitrile (7 mL) was added *p*-toluenesulphonyl hydrazide (0.91 g, 4.74 mmol) portionwise over a period of 1 hour. Stirring was continued for 18 hours at room temperature. The resulting suspension was filtered and the filter cake was washed with a small amount of ice-cold acetonitrile. After storing the filtrate for 1 day at –30 °C, the additional precipitate was collected by filtration. The combined precipitates were dried *in vacuo* to afford tosylhydrazone **3.3f** as a white solid (0.73 g, 40%). M.p. 119–120 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 1.21–1.31 (m, 2H), 1.39–1.49 (m, 2H), 2.19 (t, J = 7.3 Hz, 2H), 2.35–2.42 (m, 5H), 7.44 (d, J = 8.2 Hz, 2H), 7.78 (d, J = 8.5 Hz, 2H), 9.16 (s, 1H), 12.11 (br. s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 21.2, 22.6, 24.1, 24.4, 33.4, 127.3, 130.0, 135.7, 144.2, 153.7, 174.3, 191.2.

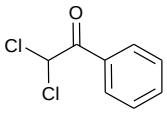
2,2-Dichloro-1-(thiophen-2-yl)ethanone (3.12)



To a suspension containing magnesium (4.86 g, 200 mmol) in dry tetrahydrofuran (10 mL) was added dropwise under reflux 2-bromo-thiophen (6.0 mL, 60.7 mmol) in dry tetrahydrofuran (90 mL). The reaction mixture was heated for further 2.5 hours at reflux. Afterwards the oil bath was removed and the mixture was allowed to cool to room temperature. The freashly prepared Grignard solution was subsequently added dropwise to a cooled solution (–78 °C) containing dichloroacethyl chloride **3.4** (6.70 mL, 67.5 mmol) in dry tetrahydrofuran (35 mL). Stirring was continued for 15 minutes at –78 °C. The reaction was quenched by the addition of saturated aqueous ammonium chloride solution and the aqueous layer was extracted with diethyl ether (4x 60 mL). Subsequently, the combined organic extracts were washed with saturated sodium bicarbonate solution (2x 60 mL), dried (sodium sulphate) and all volatiles were removed by rotary evaporation. Finally, the residue was purified by column chromatography to yield dichloroketone **3.12** as yellow oil (3.01 g, 25%). R_F = 0.31 (hexane/ethyl acetate 95/5); ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 6.49

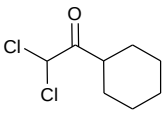
(s, 1H), 7.18–7.25 (m, 1H), 7.81 (d, *J* = 4.8 Hz, 1H), 8.01 (d, *J* = 3.9 Hz, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) *δ*: 68.0, 128.6, 134.9, 136.4, 137.1, 179.7.

2,2-Dichloro-1-phenylethanone (3.2)



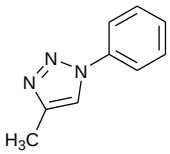
Choline chloride (2.79 g, 19.6 mmol) and *p*-toluenesulphonic acid monohydrate (3.80 g, 19.7 mmol) were heated at 100 °C on an oil bath for 40 minutes under inert gas atmosphere. The formed eutectic solvent was allowed to cool to room temperature followed by the addition of acetophenone **3.15** (1.16 mL, 9.92 mmol), acetonitrile (0.5 mL) and 1,3-dichloro-5,5-dimethylhydantoin **3.14** (2.20 g, 11.2 mmol). Stirring was continued for 45 minutes at room temperature. Afterwards methyl *t*-butyl ether (30 mL) was added to extract the product and the organic phase was separated from the flask carefully. The organic phase was washed with distilled water (2x 30 mL), dried (sodium sulphate) and all volatiles were removed *in vacuo*. Finally, the residue was purified by column chromatography to yield dichloroketone **3.2** as colourless oil (1.23 g, 66%). R_F = 0.22 (hexane/ethyl acetate 98/2); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) *δ*: 6.68 (s, 1H), 7.48–7.57 (m, 2H), 7.60–7.72 (m, 1H), 8.04–8.16 (m, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) *δ*: 67.8, 128.9, 129.7, 131.3, 134.5, 185.9.

2,2-Dichloro-1-cyclohexylethanone (3.17)



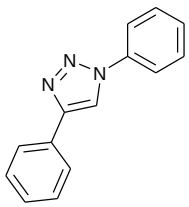
Choline chloride (5.72 g, 40.1 mmol) and *p*-toluenesulphonic acid monohydrate (7.72 g, 40.0 mmol) were heated at 100 °C on an oil bath for 40 minutes under inert-gas atmosphere. The formed eutectic solvent was allowed to cool to room temperature followed by the addition of cyclohexyl metyl ketone **3.16** (2.80 mL, 20.0 mmol), acetonitrile (1.0 mL) and 1,3-dichloro-5,5-dimethylhydantoin **3.14** (4.50 g, 22.4 mmol). Stirring was continued for 45 minutes at room temperature. Afterwards methyl *t*-butyl ether (50 mL) was added to extract the product and the organic phase was separated from the flask carefully. The organic phase was washed with distilled water (2x 50 mL), dried (sodium sulphate) and all volatiles were removed *in vacuo*. Finally, the residue was purified by column chromatography to yield dichloroketone **3.17** as colourless oil (1.08 g, 28%). R_F = 0.29 (hexane/ethyl acetate 98/2); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) *δ*: 1.18–1.41 (m, 3H), 1.49 (qd, *J* = 12.2, 2.9 Hz, 2H), 1.64–1.75 (m, 1H), 1.76–1.86 (m, 2H), 1.86–1.97 (m, 2H), 3.00 (tt, *J* = 11.4, 3.3 Hz, 1H), 5.89 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) *δ*: 25.3, 25.4, 29.9, 44.9, 69.3, 199.5.

4-Methyl-1-phenyl-1*H*-1,2,3-triazole (3.19a)



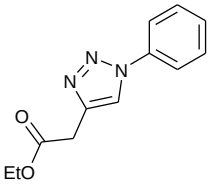
The title compound was prepared according to the General Procedure using aniline (0.12 mL, 1.30 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.19a** as a white solid (201 mg, 97%). M.p. 79–80 °C; R_F = 0.57 (hexane/ethyl acetate 3/2); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 2.44 (d, J = 0.9 Hz, 3H), 7.37–7.46 (m, 1H), 7.48–7.54 (m, 2H), 7.68–7.74 (m, 3H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 10.6, 119.3, 120.4, 128.4, 129.7, 137.2, 144.1; ESI-MS m/z: 160.1 $[M+H]^+$, 181.9 $[M+Na]^+$; HRMS (ESI) calcd for $C_{10}H_{10}N_3Na$ 182.0694, found 182.0690.

1,4-Diphenyl-1*H*-1,2,3-triazole (3.19b)



The title compound was prepared according to the General Procedure using aniline (0.12 mL, 1.30 mmol), tosylhydrazone **3.3b** (0.61 g, 1.70 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.19b** as an off-white solid (315 mg, 84%). M.p. 180–181 °C; R_F = 0.48 (hexane/ethyl acetate 3/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 7.33–7.41 (m, 1H), 7.41–7.50 (m, 3H), 7.51–7.58 (m, 2H), 7.75–7.82 (m, 2H), 7.88–7.95 (m, 2H), 8.20 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 117.6, 120.5, 125.8, 128.4, 128.7, 128.9, 129.7, 130.2, 137.1, 148.4; ESI-MS m/z: 220.0 $[M+H]^+$, 244.3 $[M+Na]^+$, 465.1 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{12}N_3$ 222.1028, found 222.1025.

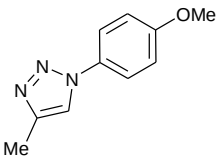
Ethyl (1-phenyl-1*H*-1,2,3-triazol-4-yl)acetate (3.19c)



The title compound was prepared according to the General Procedure using aniline (0.06 mL, 0.63 mmol), tosylhydrazone **3.3c** (300 mg, 0.82 mmol) and diisopropylethylamine (0.65 mL, 3.78 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.19c** as yellow oil (138 mg, 95%). R_F = 0.77 (hexane/ethyl acetate 2/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.30 (t, 3H), 3.92 (s,

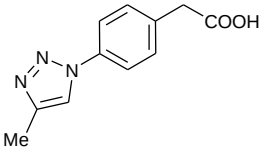
2H), 4.22 (q, 2H), 7.40–7.76 (m, 5H), 8.08 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 14.1, 31.8, 61.2, 120.5, 120.8, 128.7, 129.3, 129.7, 137.0, 141.3, 170.1; ESI-MS m/z: 231.5 $[M+H]^+$, 254.2 $[M+Na]^+$, 485.6 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{12}H_{13}N_3O_2Na$ 254.0905, found 254.0898.

1-(4-Methoxyphenyl)-4-methyl-1*H*-1,2,3-triazole (3.20a)



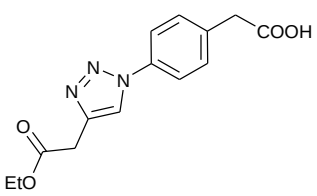
The title compound was prepared according to the General Procedure using anisidine (160 mg, 1.30 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 2.5 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.20a** as a pale yellow solid (228 mg, 93%). M.p. 87–88 °C; R_F = 0.52 (hexane/ethyl acetate 2/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 2.42 (d, *J* = 0.6 Hz, 3H), 3.85 (s, 3H), 7.00 (d, *J* = 9.1 Hz, 2H), 7.59 (d, *J* = 9.1 Hz, 2H), 7.54 (d, *J* = 0.6 Hz, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 10.8, 55.5, 114.6, 119.5, 121.9, 130.6, 143.8, 159.5; ESI-MS m/z: 190.2 $[M+H]^+$, 212.1 $[M+Na]^+$; HRMS (ESI) calcd for $C_{10}H_{11}N_3ONa$ 212.0800, found 212.0792.

[4-(4-Methyl-1*H*-1,2,3-triazol-1-yl)phenyl]acetic acid (3.21a)



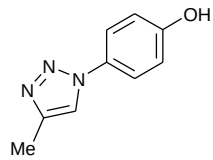
The title compound was prepared according to the General Procedure using 4-aminophenylacetic acid (76.5 mg, 0.51 mmol), tosylhydrazone **3.3a** (196 mg, 0.66 mmol) and diisopropyl-ethylamine (0.52 mL, 3.04 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was taken up in ethyl acetate and the organic phase was extracted with distilled water (3x 20 mL). Afterwards the combined aqueous extracts were acidified with aqueous 1 M hydrochloric acid and extracted with ethyl acetate (3x 10 mL). The combined organic extracts were dried (sodium sulphate) followed by the evaporation of all volatile material. The residue was recrystallised from water to yield triazole **3.21a** as an off-white solid (51 mg, 46%). M.p. 129–131 °C; ^{1}H NMR (300 MHz, METHANOL-d_4) δ: 2.40 (s, 3H), 3.71 (s, 2H), 7.49 (d, *J* = 8.5 Hz, 2H), 7.77 (d, *J* = 8.5 Hz, 2H), 8.29 (s, 1H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ: 10.3, 41.2, 121.4, 122.1, 132.0, 137.2, 137.4, 145.0, 174.8; ESI-MS m/z: 216.3 $[M-H]^-$, 218.4 $[M+H]^+$, 240.2 $[M+Na]^+$, 433.0 $[2M-H]^-$, 457.2 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{11}H_{12}N_3O_2$ 218.0924, found 218.0928.

{4-[4-(2-Ethoxy-2-oxoethyl)-1*H*-1,2,3-triazol-1-yl]phenyl} acetic acid (3.21c)



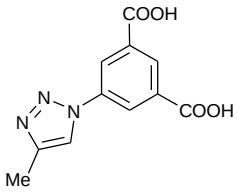
The title compound was prepared according to the General Procedure using 4-aminophenylacetic acid (78.5 mg, 0.52 mmol), tosylhydrazone **3.3c** (240 mg, 0.65 mmol) and diisopropylethylamine (0.51 mL, 3.00 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The resulting residue was taken up in ethyl acetate and the organic phase was extracted with distilled water (3x 20 mL). Afterwards the combined aqueous extracts were acidified with 1 M aqueous hydrochloric acid and extracted with ethyl acetate (3x 10 mL). The combined organic extracts were dried (sodium sulphate) followed by the evaporation of all volatile material. The residue was recrystallised from water to yield triazole **3.21c** as a yellow solid (83 mg, 53%). M.p. 142–143 °C; ^{1}H NMR (300 MHz, METHANOL-d_4) δ: 1.28 (t, J = 7.3 Hz, 3H), 3.70 (s, 2H), 3.88 (s, 2H), 4.20 (q, J = 7.0 Hz, 2H), 7.49 (d, J = 8.5 Hz, 2H), 7.78 (d, J = 8.5 Hz, 2H), 8.42 (s, 1H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ: 14.5, 32.2, 62.4, 121.4, 123.1, 132.0, 137.2, 137.3, 142.8, 171.7, 174.9; ESI-MS m/z: 288.2 $[M-H]^-$, 290.0 $[M+H]^+$, 312.1 $[M+Na]^+$, 577.8 $[2M-H]^-$, 601.3 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{13}H_{14}N_3O_2$ 244.1092, found 244.1092.

4-(4-Methyl-1*H*-1,2,3-triazole-1-yl)phenol (3.22a)



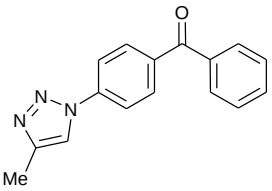
The title compound was prepared according to the General Procedure using 4-aminophenol (142 mg, 1.28 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was taken up in distilled water (20 mL) and the aqueous phase was acidified with 1 M aqueous hydrochloric acid. Afterwards the water phase was extracted with dichloromethane (3x 20 mL) and the combined organic extracts were dried (sodium sulphate) followed by the evaporation of all volatile material. Finally, the residue was purified by column chromatography to yield triazole **3.22a** as an off-white solid (95.1 mg, 65%). M.p. 147–148 °C; R_F = 0.29 (hexane/ethyl acetate 1/1); ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 2.38 (d, J = 0.9 Hz, 3H), 6.84–7.01 (m, 2H), 7.49–7.66 (m, 2H), 8.10 (d, J = 0.9 Hz, 1H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 10.4, 117.1, 122.0, 123.3, 130.8, 144.7, 159.5; ESI-MS m/z: 173.8 $[M-H]^-$, 175.8 $[M+H]^+$, 198.1 $[M+Na]^+$; HRMS (ESI) calcd for $C_9H_8N_3O$ 174.0673, found 174.0671.

5-(4-Methyl-1*H*-1,2,3-triazol-1-yl)benzene-1,3-dicarboxylic acid (3.23a)



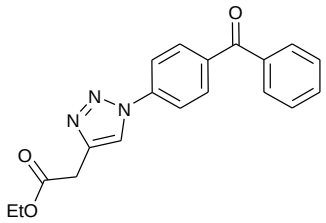
The title compound was prepared according to the General Procedure using 5-aminoisophthalic acid (235 mg, 1.30 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 3 hours, whereupon all volatiles were removed under reduced pressure. The residue was taken up in ethyl acetate and the organic phase was extracted with distilled water (3x 20 mL). Afterwards the combined aqueous extracts were acidified with conc. aqueous hydrochloric acid. The precipitate was collected by filtration, washed consecutively with distilled water and ethyl acetate and dried under reduced pressure to yield triazole **3.23a** as a white solid (210 mg, 65%). M.p. > 300 °C (decomposition); ^{1}H NMR (300 MHz, DMSO-d_6) δ: 2.37 (s, 3H), 8.05 (s, 1H), 8.59 (s, 1H), 8.60 (s, 1H), 8.82 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 10.6, 121.0, 123.9, 129.1, 133.1, 137.3, 143.7, 165.7; ESI-MS m/z: 246.1 $[M-H]^-$, 248.4 $[M+H]^+$, 493.6 $[M-H]^-$, 495.2 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{11}H_{10}N_3O_4$ 248.0671, found 248.0666.

[4-(4-Methyl-1*H*-1,2,3-triazol-1-yl)phenyl](phenyl)methanone (3.24a)



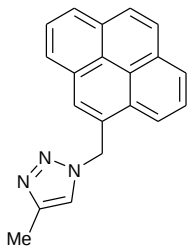
The title compound was prepared according to the General Procedure using 4-aminobenzophenone (50.1 mg, 0.25 mmol), tosylhydrazone **3.3a** (96.1 mg, 0.33 mmol) and diisopropyl-ethylamine (0.26 mL, 1.52 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.24a** as light yellow oil (47.4 mg, 73%). R_F = 0.50 (hexane/ethyl acetate 1/1), ^{1}H NMR (300 MHz, CHLOROFORM-d) δ: 2.47 (s, 3H), 7.48–7.56 (m, 2H), 7.60–7.67 (m, 1H), 7.80–7.90 (m, 4H), 7.95–8.00 (m, 2H), 8.18 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-d) δ: 10.9, 119.1, 119.6, 128.5, 130.0, 131.7, 132.8, 137.1, 137.2, 139.8, 144.6, 195.2; ESI-MS m/z: 263.8 $[M+H]^+$, 286.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{16}H_{14}N_3O$ 264.1131, found 264.1132.

Ethyl {1-[4-(phenylcarbonyl)phenyl]-1*H*-1,2,3-triazol-4-yl}acetate (3.24c)



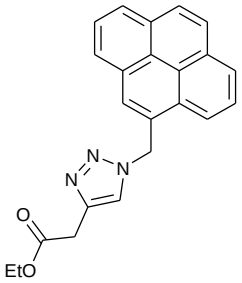
The title compound was prepared according to the General Procedure using 4-aminobenzophenone (99.4 mg, 0.50 mmol), tosylhydrazone **3.3c** (240 mg, 0.65 mmol) and diisopropylethylamine (0.51 mL, 3.00 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.24c** as yellow oil (106 mg, 63%). R_F = 0.53 (hexane/ethyl acetate 1/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.32 (t, J = 7.2 Hz, 3H), 3.94 (s, 2H), 4.24 (q, J = 7.2 Hz, 2H), 7.49–7.56 (m, 2H), 7.60–7.68 (m, 1H), 7.80–7.85 (m, 2H), 7.87–7.94 (m, 2H), 7.96–8.03 (m, 2H), 8.18 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 14.2, 31.8, 61.4, 113.6, 119.8, 120.7, 128.0, 128.5, 129.5, 130.0, 131.4, 131.7, 132.8, 132.9, 137.4, 139.6, 141.8, 170.1, 195.2; ESI-MS m/z: 336.2 $[M+H]^+$, 358.4 $[M+Na]^+$, 693.4 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{19}H_{17}N_3O_3Na$ 358.1162, found 358.1158.

1-(8,10-Dihydropyren-4-ylmethyl)-4-methyl-1H-1,2,3-triazole (3.25a)



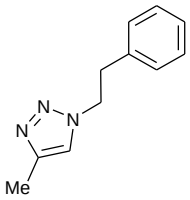
The title compound was prepared according to the General Procedure using 1-pyrenemethylamine hydrochloride (37.5 mg, 0.14 mmol), tosylhydrazone **3.3a** (53.5 mg, 0.18 mmol) and diisopropylethylamine (143 µL, 0.84 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.25a** as pale yellow oil (28 mg, 67%). R_F = 0.56 (ethyl acetate); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 2.20 (d, J = 0.6 Hz, 3H), 6.12 (s, 2H), 6.98 (s, 1H), 7.86 (d, J = 7.8 Hz, 1H), 8.00–8.20 (m, 8H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 10.8, 52.2, 121.0, 121.9, 124.3, 124.8, 124.9, 125.7, 125.8, 126.3, 127.0, 127.1, 127.5, 128.1, 128.9, 129.1, 130.5, 131.0, 131.9, 143.6; ESI-MS m/z: 298.1 $[M+H]^+$, 320.0 $[M+Na]^+$, 595.2 $[2M+H]^+$, 616.8 $[2M+Na]^+$, 914.9 $[3M+Na]^+$; HRMS (ESI) calcd for $C_{20}H_{15}N_3Na$ 320.1158, found 308.1157.

Ethyl [1-(8,10-dihydropyren-4-ylmethyl)-1*H*-1,2,3-triazol-4-yl]acetate (3.25c)



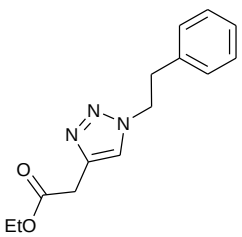
The title compound was prepared according to the General Procedure using 1-pyrenemethylamine hydrochloride (53.6 mg, 0.20 mmol), tosylhydrazone **3.3c** (96.0 mg, 0.26 mmol) and diisopropylethylamine (203 µL, 1.20 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.25c** as a pale yellow semi-solid (48 mg, 65%). R_F = 0.62 (hexane/ethyl acetate 3/7); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.14 (t, *J* = 6.8 Hz, 3H), 3.70 (s, 2H), 4.06 (q, *J* = 6.8 Hz, 2H), 6.14 (s, 2H), 7.34 (s, 1H), 7.86 (d, *J* = 7.6 Hz, 1H), 7.97–8.18 (m, 8H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 14.0, 31.8, 52.3, 61.0, 121.8, 122.4, 124.3, 124.8, 124.9, 125.7, 125.8, 126.2, 126.7, 127.1, 127.5, 128.1, 128.9, 129.1, 130.4, 131.0, 131.9, 140.9, 170.1; ESI-MS m/z: 370.1 $[M+H]^+$, 392.0 $[M+Na]^+$, 761.4 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{23}H_{19}N_3O_2Na$ 392.1369, found 392.1365.

4-Methyl-1-(2-phenylethyl)-1*H*-1,2,3-triazole (3.26a)



The title compound was prepared according to the General Procedure using 2-phenylethylamine (0.16 mL, 1.30 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 2 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.26a** as colourless oil (234 mg, 96%). R_F = 0.29 (petroleum ether/ethyl acetate 2/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 2.30 (d, *J* = 0.6 Hz, 3H), 3.18 (t, *J* = 7.3 Hz, 2H), 4.53 (t, *J* = 7.2 Hz, 2H), 7.05 (s, 1H), 7.10–7.12 (m, 2H), 7.25–7.32 (m, 3H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 10.7, 36.7, 51.3, 121.3, 126.9, 128.5, 128.6, 137.1, 142.9; ESI-MS m/z: 188.5 $[M+H]^+$, 210.1 $[M+Na]^+$, 397.2 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{11}H_{14}N_3$ 188.1182, found 188.1185.

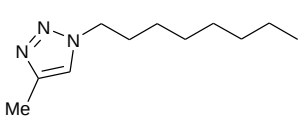
Ethyl [1-(2-phenylethyl)-1*H*-1,2,3-triazol-4-yl]acetate (3.26c)



The title compound was prepared according to the General Procedure using 2-phenylethylamine (0.08 mL, 0.63 mmol), tosylhydrazone **3.3c** (300 mg, 0.82 mmol) and diisopropylethylamine (0.65 mL, 3.78 mmol). Stirring was continued at room temperature for 2 hours, whereupon all volatiles were removed under reduced pressure. The

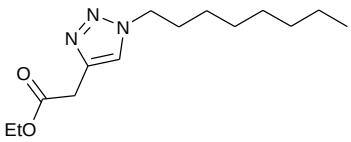
residue was purified by column chromatography to yield triazole **3.26c** as yellow oil (156 mg, 96%). R_F = 0.54 (hexane/ethyl acetate 2/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.27 (t, *J* = 7.0 Hz, 3H), 3.21 (t, *J* = 7.3 Hz, 2H), 3.78 (s, 3H), 4.17 (q, *J* = 7.0 Hz, 2H), 4.57 (t, *J* = 7.3 Hz, 2H), 7.10–7.33 (m, 5H), 7.38 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 14.1, 31.8, 36.7, 51.6, 61.1, 122.7, 127.0, 128.6, 128.7, 136.9, 140.3, 170.2; ESI-MS m/z: 260.1 $[M+H]^+$, 282.5 $[M+Na]^+$, 541.4 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{17}N_3O_2Na$ 282.1218, found 282.1210.

4-Methyl-1-octyl-1*H*-1,2,3-triazole (3.27a)



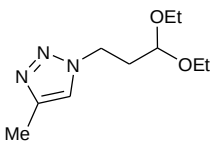
The title compound was prepared according to the General Procedure using octylamine (47 µL, 0.28 mmol), tosylhydrazone **3.3a** (83.2 mg, 0.28 mmol) and diisopropylethylamine (291 µL, 1.70 mmol). Stirring was continued at room temperature for 18 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.27a** as colourless oil (45 mg, 81%). R_F = 0.45 (petroleum ether/ethyl acetate 2/3); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 0.87 (t, *J* = 7.0 Hz, 3H), 1.26–1.31 (m, 10H), 1.87 (br. t, *J* = 7.0 Hz, 2H), 2.35 (d, *J* = 0.6 Hz, 3H), 4.30 (t, *J* = 7.0 Hz, 2H), 7.26 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 10.7, 13.9, 22.5, 26.4, 28.8, 28.9, 30.3, 31.6, 50.0, 120.8, 143.1; ESI-MS m/z: 196.3 $[M+H]^+$, 218.3 $[M+Na]^+$, 413.7 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{11}H_{22}N_3$ 196.1814, found 196.1808.

Ethyl (1-octyl-1*H*-1,2,3-triazol-4-yl)acetate (3.27c)



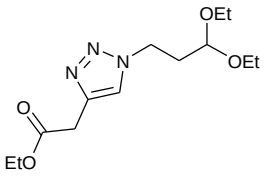
The title compound was prepared according to the General Procedure using octylamine (47 µL, 0.28 mmol), tosylhydrazone **3.3c** (100 mg, 0.28 mmol) and diisopropylethylamine (291 µL, 1.70 mmol). Stirring was continued at room temperature for 18 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.27c** as colourless oil (61 mg, 85 %). R_F = 0.33 (hexane/ethyl acetate 2/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.87 (t, *J* = 7.0 Hz, 3H), 1.26–1.32 (m, 13H), 1.90 (br. t, *J* = 7.0 Hz, 2H), 3.83 (s, 2H), 4.19 (q, *J* = 7.0 Hz, 2H), 4,34 (t, *J* = 7.4 Hz, 2H), 7.60 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 13.9, 14.0, 22.5, 26.3, 28.8, 28.9, 30.2, 31.6, 31.8, 50.2, 61.0, 122.2, 140.4, 170.3; ESI-MS m/z: 267.9 $[M+H]^+$, 289.6 $[M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{26}N_3O_2$ 268.2025, found 268.2019.

1-(3,3-Diethoxypropyl)-4-methyl-1*H*-1,2,3-triazole (3.28a)



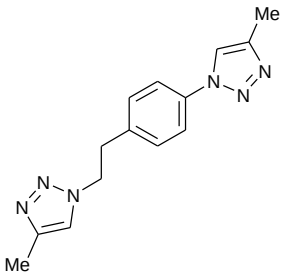
The title compound was prepared according to the General Procedure using 1-amino-3,3-diethoxypropane (31.9 µL, 0.20 mmol), tosylhydrazone **3.3a** (76.0 mg, 0.26 mmol) and diisopropylethylamine (203 µL, 1.20 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.28a** as colourless oil (23 mg, 54%). R_F = 0.37 (hexane/ethyl acetate 3/7); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.21 (t, *J* = 7.2 Hz, 6H), 2.20 (dt, *J* = 5.6, 7.2 Hz, 2H), 2.34 (d, *J* = 0.8 Hz, 3H), 3.49 (dq, *J* = 7.2, 9.6 Hz, 2H), 3.66 (dq, *J* = 7.2, 9.6 Hz, 2H), 4.41 (t, *J* = 6.8 Hz, 2H), 4.47 (t, *J* = 5.6 Hz, 1H), 7.30 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 10.7, 15.2, 34.3, 46.0, 62.0, 100.2, 121.4, 143.2; ESI-MS m/z: 214.1 $[M+H]^+$, 235.8 $[M+Na]^+$, 449.0 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{10}H_{19}N_3O_2Na$ 236.1369, found 236.1366.

Ethyl [1-(3,3-diethoxypropyl)-1*H*-1,2,3-triazol-4-yl]acetate (3.28c)



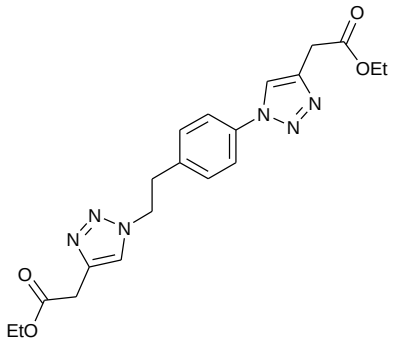
The title compound was prepared according to the General Procedure using 1-amino-3,3-diethoxypropane (31.9 µL, 0.20 mmol), tosylhydrazone **3.3c** (96.0 mg, 0.26 mmol) and diisopropylethylamine (203 µL, 1.20 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.28c** as colourless oil (39 mg, 68%). R_F = 0.59 (hexane/ethyl acetate 3/7); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.21 (t, *J* = 6.8 Hz, 6H), 1.28 (t, *J* = 7.2 Hz, 3H), 2.23 (dt, *J* = 5.6, 7.2 Hz, 2H), 3.50 (dq, *J* = 7.2, 9.2 Hz, 2H), 3.66 (dq, *J* = 7.2, 9.2 Hz, 2H), 3.82 (s, 2H), 4.19 (q, *J* = 7.2 Hz, 2H), 4.43–4.50 (m, 3H), 7.62 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 14.1, 15.2, 31.8, 34.3, 46.1, 61.1, 62.0, 100.2, 122.7, 140.4, 170.2; ESI-MS m/z: 286.3 $[M+H]^+$, 308.6 $[M+Na]^+$; HRMS (ESI) calcd for $C_{13}H_{23}N_3O_4Na$ 308.1581, found 308.1585.

4-Methyl-1-{4-[2-(4-methyl-1*H*-1,2,3-triazol-1-yl)ethyl]phenyl}-1*H*-1,2,3-triazole (3.29a)



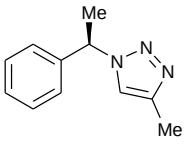
The title compound was prepared according to the General Procedure using 2-(4-aminophenyl)ethylamine (0.08 mL, 0.63 mmol), tosylhydrazone **3.3a** (0.48 g, 1.63 mmol) and diisopropylethylamine (0.65 mL, 3.78 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.29a** as an off-white solid (136 mg, 80%). M.p. 181–182 °C; R_F = 0.24 (ethyl acetate); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.20 (s, 3H), 2.32 (s, 3H), 3.22 (t, J = 7.0 Hz, 2H), 4.60 (t, J = 7.2 Hz, 2H), 7.34–7.42 (m, 2H), 7.74–7.76 (m, 1H), 7.76–7.79 (m, 2H), 8.49 (d, J = 0.9 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 10.5, 35.1, 49.9, 119.7, 120.4, 122.2, 130.0, 135.4, 138.1, 141.7, 143.1; ESI-MS m/z: 269.3 $[M+H]^+$, 291.2 $[M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{16}N_6Na$ 291.1329, found 291.1330.

Ethyl [1-(4-{2-[4-(2-ethoxy-2-oxoethyl)-1*H*-1,2,3-triazol-1-yl]ethyl}phenyl)-1*H*-1,2,3-triazol-4-yl]-acetate (3.29c)



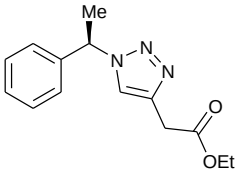
The title compound was prepared according to the General Procedure using 2-(4-aminophenyl)ethylamine (0.08 mL, 0.63 mmol), tosylhydrazone **3.3c** (900 mg, 2.46 mmol) and diisopropylethylamine (0.65 mL, 3.78 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.29c** as yellow oil (142 mg, 55%). R_F = 0.42 (ethyl acetate); ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 1.26 (t, J = 7.0 Hz, 3H), 1.31 (t, J = 7.0 Hz, 3H), 3.30 (t, J = 7.0 Hz, 2H), 3.79 (s, 2H), 3.91 (s, 2H), 4.16 (q, J = 7.0 Hz, 2H), 4.22 (q, J = 7.0 Hz, 2H), 4.62 (t, J = 7.0 Hz, 2H), 7.24 (d, J = 8.6 Hz, 2H), 7.42 (s, 1H), 7.66 (d, J = 8.6 Hz, 2H), 8.05 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-d) δ: 14.07, 14.10, 31.7, 31.8, 36.1, 51.2, 61.1, 61.2, 120.7, 122.9, 129.9, 136.0, 137.7, 140.4, 141.3, 170.1, 170.2; ESI-MS m/z: 413.4 $[M+H]^+$, 434.8 $[M+Na]^+$; HRMS (ESI) calcd for $C_{20}H_{24}N_6O_4Na$ 435.1757, found 435.1755.

4-Methyl-1-[(1*R*)-1-phenylethyl]-1*H*-1,2,3-triazole (3.30a)



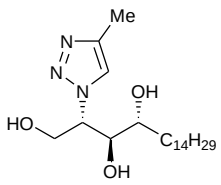
The title compound was prepared according to the General Procedure using (*R*)-1-phenylethylamine (0.17 mL, 1.30 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 3 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.30a** as colourless oil (233 mg, 96%). R_F = 0.41 (hexane/ethyl acetate 2/3); $[\alpha]_D^{20}$ −28.1 (c = 0.98, dichloromethane); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 1.95 (d, *J* = 7.0 Hz, 3H), 2.31 (d, *J* = 0.8 Hz, 3H), 5.77 (q, *J* = 7.0 Hz, 1H), 7.18 (d, *J* = 0.7 Hz, 1H), 7.24–7.38 (m, 5H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 10.8, 21.2, 59.8, 119.8, 126.4, 128.3, 128.9, 140.1, 143.3; ESI-MS m/z: 210.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{11}H_{13}N_3Na$ 210.1007, found 210.0999.

Ethyl {1-[(1*R*)-1-phenylethyl]-1*H*-1,2,3-triazol-4-yl}acetate (3.30c)



The title compound was prepared according to the General Procedure using (*R*)-1-phenylethylamine (0.08 mL, 0.63 mmol), tosylhydrazone **3.3c** (300 mg, 0.82 mmol) and diisopropylethylamine (0.65 mL, 3.78 mmol). Stirring was continued at room temperature for 2.5 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.30c** as yellow oil (156 mg, 96%). R_F = 0.65 (hexane/ethyl acetate 2/3); $[\alpha]_D^{17}$ −115.0 (c = 1.01, dichloromethane); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 1.25 (t, *J* = 7.0 Hz, 3H), 1.98 (d, *J* = 7.0 Hz, 3H), 3.80 (s, 2H), 4.16 (q, *J* = 7.0 Hz, 2H), 5.80 (q, *J* = 7.0 Hz, 1H), 7.25–7.36 (m, 5H), 7.51 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 14.1, 21.3, 31.9, 60.2, 61.1, 121.3, 126.4, 128.4, 128.9, 139.9, 140.5, 170.3; ESI-MS m/z: 260.1 $[M+H]^+$, 282.3 $[M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{17}N_3O_2Na$ 282.1218, found 282.1210.

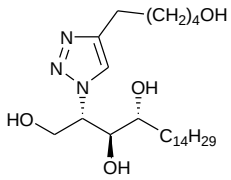
(2*S*,3*S*,4*R*)-2-(4-Methyl-1*H*-1,2,3-triazol-1-yl)octadecane-1,3,4-triol (3.33a)



The title compound was prepared according to the General Procedure using phytosphingosine **3.31** (413 mg, 1.30 mmol), tosylhydrazone **3.3a** (500 mg, 1.69 mmol) and diisopropylethylamine (1.33 mL, 7.80 mmol). Stirring was continued at room temperature for 2 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.33a** as a white solid (383 mg, 77%).

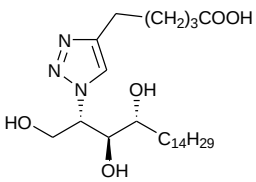
M.p. 100–101 °C; R_F = 0.43 (ethyl acetate); $[\alpha]_D^{20}$ −49.9 (c = 1.0, methanol); ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 0.90 (t, J = 7.0 Hz, 3H), 1.29 (br. s, 24H), 1.45–1.70 (m, 2H), 2.32 (s, 3H), 3.30–3.35 (m, 2H), 3.74 (dd, J = 7.0, 4.7 Hz, 1H), 4.11 (d, J = 6.2 Hz, 2H), 4.91–4.96 (m, 1H), 7.81 (s, 1H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 10.6, 14.5, 23.8, 26.7, 30.5, 30.76, 30.79, 30.83, 34.0, 61.2, 65.8, 72.9, 76.5, 123.32, 143.7; ESI-MS m/z: 382.5 $[M-H]^-$, 384.3 $[M+H]^+$, 406.7 $[M+Na]^+$, 766.5 $[2M-H]^-$, 767.6 $[2M+H]^+$, 789.6 $[2M+Na]^+$, 1172.8 $[3M+Na]^+$; HRMS (ESI) calcd for $C_{21}H_{41}N_3O_3Na$ 406.3046, found 406.3036.

(2*S*,3*S*,4*R*)-2-[4-(5-Hydroxypentyl)-1*H*-1,2,3-triazol-1-yl]octadecane-1,3,4-triol (3.33b)



The title compound was prepared according to the General Procedure using phytosphingosine **3.33b** (244 mg, 0.77 mmol), tosylhydrazone **3.3e** (367 mg, 1.00 mmol) and diisopropylethylamine (0.79 mL, 4.62 mmol). Stirring was continued at room temperature for 2.5 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.33b** as a white solid (267 mg, 76%). M.p. 80–81 °C; R_F = 0.26 (ethyl acetate/methanol 95/5); $[\alpha]_D^{19}$ −38.9 (c = 1.0, methanol); ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 0.89 (t, J = 7.0 Hz, 3H), 1.28 (br. s, 26H), 1.40–1.47 (m, 2H), 1.52–1.71 (m, 2H), 1.66–1.75 (m, 2H), 2.72 (t, J = 7.6 Hz, 2H), 3.30–3.34 (m, 2H), 3.55 (t, J = 6.4 Hz, 2H), 3.75 (dd, J = 6.8, 4.9 Hz, 1H), 4.12 (d, J = 6.2 Hz, 2H), 4.89–4.96 (m, 1H), 7.83 (s, 1H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 14.5, 23.8, 26.4, 26.5, 26.7, 30.4, 30.5, 30.74, 30.76, 30.78, 30.81, 33.1, 33.3, 33.8, 61.3, 62.8, 65.8, 73.0, 76.4, 123.0, 148.7; ESI-MS m/z: 453.6 $[M-H]^-$, 456.2 $[M+H]^+$, 478.4 $[M+Na]^+$, 933.5 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{25}H_{50}N_3O_4$ 456.3796, found 456.3794.

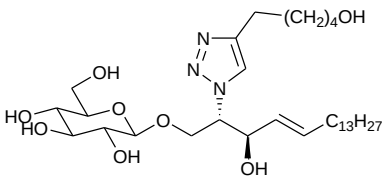
5-{1-[(1*S*,2*S*,3*R*)-2,3-Dihydroxy-1-(hydroxymethyl)heptadecyl]-1*H*-1,2,3-triazol-4-yl}pentanoic acid (3.33c)



The title compound was prepared according to the General Procedure using phytosphingosine **3.31** (96 mg, 0.30 mmol), tosylhydrazone **3.3f** (150 mg, 0.39 mmol) and diisopropyl-ethylamine (0.31 mL, 1.82 mmol). Stirring was continued at room temperature for 2 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.33c** as a white

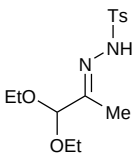
solid (127 mg, 89%). M.p. 68–70 °C; R_F = 0.38 (ethyl acetate/methanol 95/5 + 0.2% acetic acid); $[\alpha]_D^{21}$ −6.3 (c = 0.99, methanol); ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 0.89 (t, J = 7.0 Hz, 3H), 1.28 (br. s, 26H), 1.50–1.77 (m, 4H), 2.32 (t, J = 7.2 Hz, 2H), 2.72 (t, J = 7.0Hz, 2H), 3.29–3.35 (m, 2H), 3.75 (dd, J = 6.8, 4.9 Hz, 1H), 4.12 (d, J = 6.6 Hz, 2H), 4.92–4.95 (m, 1H), 7.84 (s, 1H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 14.5, 20.8, 23.7, 25.5, 26.1, 26.7, 30.0, 30.5, 30.7, 30.76, 30.77, 30.81, 33.1, 33.8, 34.6, 61.3, 65.8, 73.0, 76.4, 123.0, 148.4, 177.4; ESI-MS m/z: 468.3 $[M-H]^-$, 470.5 $[M+H]^+$, 492.4 $[M+Na]^+$, 937.7 $[2M-H]^-$, 939.8 $[2M+H]^+$, 961.9 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{25}H_{48}N_3O_5$ 470.3588, found 470.3590.

(2*S*,3*R*,4*E*)-3-Hydroxy-2-[4-(5-hydroxypentyl)-1*H*-1,2,3-triazol-1-yl]octadec-4-en-1-yl hexopyranoside (3.34)



The title compound was prepared according to the General Procedure using psychosine **3.32** (5 mg, 11 µmol), tosylhydrazone **3.3e** (5.3 mg, 14 µmol) and diisopropylethylamine (11.3 µL, 66 µmol). Stirring was continued at room temperature for 2.5 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by preparative thin layer chromatography (ethyl acetate/ethanol/water 90/10/6) to yield triazole **3.34** as colourless oil (3.2 mg, 48%). R_F = 0.16 (ethyl acetate/ethanol/water 90/10/6); $[\alpha]_D^{24}$ +1.8 (c = 0.036, chloroform); ^{1}H NMR (300 MHz, PYRIDINE-d_5) δ: 0.83–0.92 (m, 3H), 1.13–1.38 (m, 24H), 1.53–1.64 (m, 2H), 1.68–1.84 (m, 4H), 1.91 (br. s., 2H), 2.74–2.84 (m, 2H), 3.84 (t, J = 6.4 Hz, 2H), 4.09 (t, J = 5.9 Hz, 1H), 4.18 (dd, J = 9.4, 3.2 Hz, 1H), 4.44–4.55 (m, 5H), 4.58–4.62 (m, 1H), 4.89 (d, J = 7.6 Hz, 2H), 5.70–5.79 (m, 1H), 8.27 (s, 1H); ^{13}C NMR (150 MHz, CHLOROFORM-d) δ: 14.1, 22.7, 25.0, 25.2, 28.5, 29.2, 29.36, 29.38. 29.6, 29.69, 29.72, 29.76, 29.78, 31.9, 32.3, 61.0, 62.0, 65.3, 68.8, 70.9, 71.5, 73.2, 74.7, 103.5, 122.4, 127.4, 135.3, 147.5; ESI-MS m/z: 598.5 $[M-H]^-$, 600.1 $[M+H]^+$, 622.2 $[M+Na]^+$; HRMS (ESI) calcd for $C_{31}H_{57}N_3O_8Na$ 622.4038, found 622.4046.

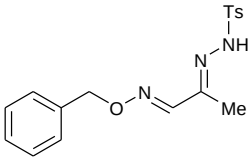
N'-(1,1-Diethoxypropan-2-ylidene)-4-methylbenzenesulphonohydrazide (3.35)



No triazole formation could be observed upon the reaction of *N*-methylamine (0.14 mL, 1.29 mmol) and benzamide (158 mg, 1.29 mmol) with tosylhydrazone **3.3a** (0.50 g, 1.69 mmol) in the presence of potassium acetate (0.38 g, 3.87 mmol) in an ethanol/acetonitrile solvent-mix according to the General Procedure. However, evaporation of the reaction mixture and purification of the residue

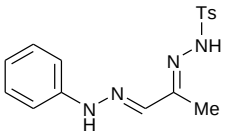
by column chromatography afforded the title compound **3.35** (ethanol adduct) as a yellow semi-solid (191 mg, 36%). R_F = 0.21 (hexane/ethyl acetate 3/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 1.08–1.19 (m, 6H), 1.74 (s, 3H), 2.42 (s, 3H), 3.25–3.42 (m, 2H), 3.43–3.62 (m, 2H), 4.63 (s, 1H), 7.27–7.33 (m, 2H), 7.49 (s, 1H), 7.81–7.86 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 9.6, 15.0, 21.6, 63.1, 103.7, 128.0, 129.5, 135.3, 144.2, 153.8; ESI-MS m/z: 313.3 $[M-H]^-$, 337.0 $[M+Na]^+$, 651.2 $[2M+Na]^+$.

N'-{1-[(Benzyloxy)imino]propan-2-ylidene}-4-methylbenzenesulphonohydrazide (3.36)



Upon the reaction of *O*-benzylhydroxylamine hydrochloride (209 mg, 1.31 mmol) with tosylhydrazone **3.3a** (0.53 g, 1.79 mmol) in the presence of potassium acetate (0.51 g, 5.12 mmol) according to the General Procedure, no cyclised triazole product was obtained. However, evaporation of the reaction mixture and purification of the residue by column chromatography afforded the title compound **3.36** (non-cyclised bisimine) as an off-white solid (316 mg, 70%). M.p. 127–128 °C; R_F = 0.22 (hexane/ethyl acetate 3/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 1.93 (s, 3H), 2.42 (s, 3H), 5.12 (s, 2H), 7.27–7.35 (m, 7H), 7.69 (s, 1H), 7.80–7.86 (m, 2H), 8.12 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 10.7, 21.6, 76.8, 128.0, 128.1, 128.3, 128.4, 129.7, 134.9, 136.9, 144.5, 149.1, 149.3; ESI-MS m/z: 344.1 $[M-H]^-$, 346.5 $[M+H]^+$, 368.2 $[M+Na]^+$; HRMS (ESI) calcd for $C_{17}H_{19}N_3O_3SNa$ 368.1039, found 368.1038.

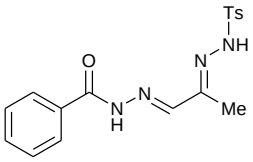
4-Methyl-*N*'-[1-(2-phenylhydrazinylidene)propan-2-ylidene]benzenesulphonohydrazide (3.37)



Upon the reaction of phenylhydrazine hydrochloride (195 mg, 1.34 mmol) with tosylhydrazone **3.3a** (0.51 g, 1.73 mmol) in the presence of potassium acetate (0.54 g, 5.50 mmol) according to the General Procedure, no cyclised triazole product was obtained. However, evaporation of the reaction mixture and purification of the residue by column chromatography afforded the title compound **3.37** (non-cyclised bisimine) as yellow oil (215 mg, 49%). R_F = 0.30 (hexane/ethyl acetate 2/1); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.04 (s, 3H), 2.38 (s, 3H), 6.78 (t, J = 7.2 Hz, 1H), 6.99 (d, J = 7.5 Hz, 2H), 7.17–7.24 (m, 2H), 7.35 (s, 1H), 7.40 (d, J = 7.9 Hz, 2H), 7.76 (d, J = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 11.4, 21.0, 112.2, 119.6,

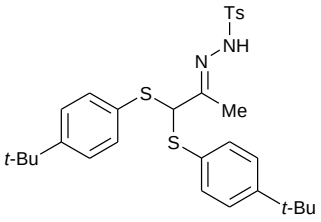
127.5, 129.2, 129.5, 136.1, 136.3, 143.4, 144.3, 153.6; ESI-MS m/z: 329.1 $[M-H]^-$, 331.5 $[M+H]^+$, 353.1 $[M+Na]^+$; HRMS (ESI) calcd for $C_{16}H_{19}N_4O_2S$ 331.1223, found 331.1225.

N'-(2-{2-[(4-Methylphenyl)sulphonyl]hydrazinylidene}propylidene)benzohydrazide (3.38)



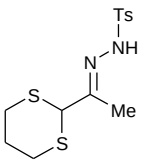
Upon the reaction of benzhydrazide (188 mg, 1.32 mmol) with tosylhydrazone **3.3a** (0.50 g, 1.69 mmol) in the presence of potassium acetate (0.39 g, 3.97 mmol) according to the General Procedure, no cyclised triazole product was obtained. However, evaporation of the reaction mixture and purification of the residue by column chromatography afforded the title compound **3.38** (non-cyclised bisimine) as an light yellow solid (200 mg, 42%). M.p. 163–164 °C; R_F = 0.36 (hexane/ethyl acetate 2/3); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.04 (s, 3H), 2.39 (s, 3H), 7.42 (d, J = 8.3 Hz, 2H), 7.47–7.63 (m, 3H), 7.77 (d, J = 7.9 Hz, 2H), 7.87 (d, J = 7.5 Hz, 2H), 7.92 (s, 1H), 11.00 (s, 1H), 11.82 (s, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 11.3, 21.0, 127.4, 127.6, 128.5, 129.6, 131.9, 133.0, 136.0, 143.6, 147.5, 152.0, 163.1; ESI-MS m/z: 357.4 $[M-H]^-$, 359.4 $[M+H]^+$, 381.1 $[M+Na]^+$, 715.5 $[2M-H]^-$, 739.3 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{17}H_{18}N_4O_3SNa$ 381.0992, found 381.0993.

N'-{1,1-Bis[(4-*t*-butylphenyl)sulfanyl]propan-2-ylidene}-4-methylbenzenesulphonohydrazide (3.39)



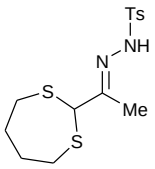
The title compound was prepared according to the General Procedure using 4-*tert*-butylthiophenol (0.23 mL, 1.29 mmol), tosylhydrazone **3.3a** (0.50 g, 1.69 mmol) and potassium acetate (0.38 g, 3.87 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield bis-thioether **3.39** as yellow oil (0.38 g, 98%). R_F = 0.42 (hexane/ethyl acetate 3/1); ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 1.24–1.30 (m, 18H), 1.86 (s, 3H), 2.45 (s, 3H), 5.06 (s, 1H), 7.15–7.23 (m, 8H), 7.31 (d, J = 7.9 Hz, 2H), 7.65 (s, 1H), 7.81 (d, J = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-d) δ: 12.6, 21.7, 31.2, 34.5, 62.0, 125.9, 128.0, 129.5, 130.0, 131.8, 135.6, 143.9, 151.1, 153.8; ESI-MS m/z: 553.4 $[M-H]^-$, 577.3 $[M+Na]^+$, 1131.4 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{30}H_{38}N_2O_2S_3Na$ 577.1988, found 577.1986.

N'-[1-(1,3-Dithian-2-yl)ethylidene]-4-methylbenzenesulphonohydrazide (3.40)



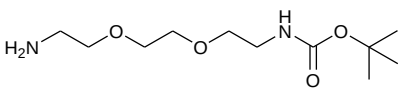
The title compound was prepared according to the General Procedure using 1,3-propanedithiol (0.13 mL, 1.28 mmol), tosylhydrazone **3.3a** (0.50 g, 1.69 mmol) and potassium acetate (0.38 g, 3.87 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield dithiane **3.40** as a white solid (342 mg, 81%). M.p. 145–146 °C; R_F = 0.27 (hexane/ethyl acetate 2/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 1.89–1.92 (m, 3H), 1.99–2.11 (m, 2H), 2.43 (s, 3H), 2.74 (ddd, *J* = 14.1, 9.5, 2.6 Hz, 2H), 2.91–3.01 (m, 2H), 4.52 (s, 1H), 7.32 (d, *J* = 7.9 Hz, 2H), 7.59 (s, 1H), 7.85–7.91 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 14.4, 21.6, 25.3, 28.7, 51.0, 128.1, 129.6, 135.2, 144.2, 153.0; ESI-MS m/z: 329.3 $[M-H]^-$, 331.5 $[M+H]^+$, 353.1 $[M+Na]^+$, 686.3 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{13}H_{19}N_2O_2S_3$ 331.0603, found 331.0601.

N'-[1-(1,3-Dithiepan-2-yl)ethylidene]-4-methylbenzenesulphonohydrazide (3.41)



The title compound was prepared according to the General Procedure using 1,4-butanedithiol (0.16 mL, 1.32 mmol), tosylhydrazone **3.3a** (0.50 g, 1.69 mmol) and potassium acetate (0.39 g, 3.97 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield dithiepane **3.41** as yellow oil (50 mg, 11%). R_F = 0.40 (hexane/ethyl acetate 2/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 1.85–2.05 (m, 7H), 2.42 (s, 3H), 2.63–2.73 (m, 2H), 3.00 (dd, *J* = 14.3, 6.4 Hz, 2H), 4.86 (s, 1H), 7.31 (d, *J* = 8.3 Hz, 2H), 7.82–7.88 (m, 2H), 7.96 (s, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 13.2, 21.5, 31.2, 31.6, 56.9, 127.9, 129.5, 135.2, 144.0, 154.0; ESI-MS m/z: 343.3 $[M-H]^-$, 345.2 $[M+H]^+$, 366.7 $[M+Na]^+$, 711.1 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{14}H_{20}N_2O_2S_3Na$ 367.0579, found 367.0578.

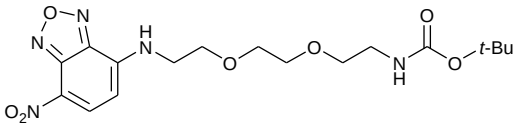
tert-Butyl {2-[2-(2-aminoethoxy)ethoxy]ethyl}carbamate (3.43)



To a solution containing 2,2'-(ethylenedioxy)-bis(ethylamine) **3.42** (50 mL, 341 mmol) in dry dioxane (150 mL) was added dropwise di-*tert*-butyl dicarbonate (14.9 g, 68.3 mmol) in dry dioxane (60 mL) and the resulting mixture was allowed to stir overnight at room temperature. The reaction mixture was filtered and the filtrate was evaporated to dryness. Afterwards the residue was taken up in distilled water (300 mL) and extracted

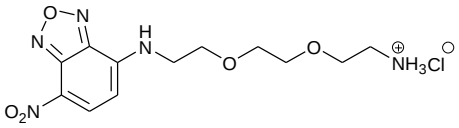
with dichloromethane (3x 250 mL). Finally, the combined organic extracts were dried (sodium sulphate) and all volatiles were removed under reduced pressure to yield the title compound **3.43** as light yellow oil (16.1 g, 95%). ^{1}H NMR (300 MHz, CHLOROFORM) δ: 1.37 (br. s., 2H), 1.41–1.49 (m, 9H), 2.84–2.92 (m, 2H), 3.32 (q, J = 5.3 Hz, 2H), 3.48–3.58 (m, 4H), 3.59–3.66 (m, 4H), 5.21 (br. s., 1H); ^{13}C NMR (75 MHz, CHLOROFORM-d) δ: 28.3, 40.2, 41.7, 67.0, 70.1, 73.4, 79.0, 155.9; ESI-MS m/z: 249.3 $[M+H]^+$; HRMS (ESI) calcd for $C_{11}H_{25}N_2O_4$ 249.1809, found 249.1809.

PEG$_2$-NBD-NHBoc (3.44)



To a suspension containing amine **3.43** (5.04 g, 20.3 mmol) and sodium bicarbonate (3.41 g, 40.6 mmol) in acetonitrile (100 mL) was added dropwise 4-chloro-7-nitrobenzofurazan (6.08 g, 30.5 mmol) in acetonitrile (100 mL) over a period of 1.5 hours. The resulting reaction mixture was allowed to stir overnight at room temperature. Afterwards the insoluble inorganic salts were removed by filtration and the filtrate was evaporated to dryness. Finally, the remaining residue was purified by column chromatography to afford **3.44** as a brown solid (6.05 g, 72%). M.p. 85–86 °C; R_F = 0.58 (dichloromethane/methanol 95/5); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.32–1.40 (m, 9H), 2.99–3.06 (m, 2H), 3.32–3.38 (m, 2H), 3.47–3.53 (m, 2H), 3.54–3.60 (m, 2H), 3.61–3.75 (m, 4H), 6.45–6.51 (m, 1H), 6.70–6.80 (m, 1H), 8.47–8.56 (m, 1H), 9.42–9.54 (m, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 28.2, 43.3, 67.9, 69.2, 69.5, 69.8, 77.6, 99.5, 137.9, 155.5; ESI-MS m/z: 410.4 $[M-H]^-$, 434.1 $[M+Na]^+$, 845.6 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{17}H_{25}N_5O_7Na$ 434.1646, found 434.1647.

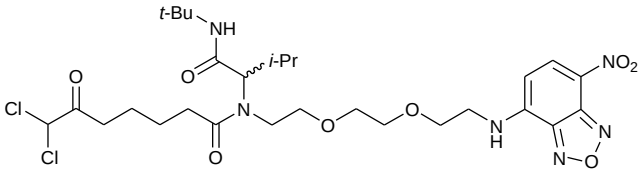
PEG$_2$-NBD-NH$_2$ hydrochloride (3.45)



Compound **3.44** (4.48 g, 10.9 mmol) was dissolved in dry methanol (60 mL) and trimethylsilyl chloride (7 mL, 54.1 mmol) was subsequently added dropwise *via* syringe. Stirring was continued overnight at room temperature. After completion of the reaction (checked by TLC), all volatiles were removed under reduced pressure. The residue was suspended in diethyl ether (15 mL), filtered and the solid was washed with several portions of diethyl ether. Afterwards the remaining solid was dried under reduced pressure to afford amine hydrochloride **3.45** as a brown solid (3.65 g, 96%). M.p. 192–193 °C; ^{1}H NMR (400 MHz, WATER-d_2) δ: 3.14–3.27 (m, 2H), 3.68–3.87 (m, 8H),

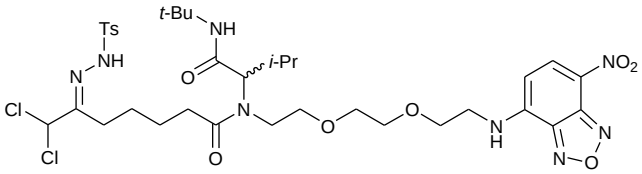
3.89–3.97 (m, 2H), 6.41 (d, *J* = 9.2 Hz, 1H), 8.51 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 38.4, 43.3, 66.6, 67.9, 69.5, 69.7, 99.5, 120.8, 137.9, 144.1, 144.4, 145.3; ESI-MS m/z: 310.3 $[M-2H]^-$, 312.4 $[M]^+$; HRMS (ESI) calcd for $C_{12}H_{18}N_5O_5$ 312.1303, found 312.1303.

PEG$_2$-NBD-α,α-dichloroketone (3.46)



A solution containing *i*-butyraldehyde (0.23 g, 2.47 mmol), amine hydrochloride **3.45** (699 mg, 2.01 mmol) and triethylamine (0.28 mL, 1.99 mmol) in methanol (5 mL) was stirred at room temperature for 2 hours to preform the imine. After the addition of carboxylic acid **3.9** (512 mg, 2.40 mmol) and *t*-butyl isocyanide (0.28 mL, 2.43 mmol), the resulting mixture was allowed to stir at room temperature overnight. Afterwards all volatiles were evaporated and the residue was purified by column chromatography to afford dichloroketone **3.46** as red brown oil (751 mg, 57%). R_F = 0.35 (hexane/ethyl acetate 3/7); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.82 (d, *J* = 6.6 Hz, 3H), 0.97 (d, *J* = 6.6 Hz, 3H), 1.25–1.37 (m, 9H), 1.69 (dt, *J* = 6.6, 3.3 Hz, 4H), 2.40–2.60 (m, 3H), 2.78–2.93 (m, 2H), 3.41–3.79 (m, 10H), 3.81–3.91 (m, 2H), 4.06 (br. s., 1H), 5.84 (s, 1H), 6.15–6.31 (m, 1H), 7.23 (br. s., 1H), 8.48 (d, *J* = 8.8 Hz, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 18.7, 19.7, 23.4, 24.6, 26.5, 28.6, 33.3, 34.8, 43.7, 50.9, 68.1, 69.3, 69.8, 70.3, 70.8, 98.5, 123.7, 136.4, 144.0, 144.3, 170.4, 174.8, 197.0; ESI-MS m/z: 659.4 $[M-H]^-$, 661.4 $[M+H]^+$, 683.4 $[M+Na]^+$; HRMS (ESI) calcd for $C_{28}H_{42}N_6O_8NaCl_2$ 683.2333, found 683.2331.

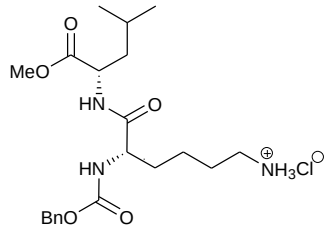
PEG$_2$-NBD-α,α-dichlorotosylhydrazone (3.47)



Dichloroketone **3.46** (240 mg, 0.36 mmol) was dissolved in dry dichloromethane (3 mL) and *p*-toluenesulphonyl hydrazide (108 mg, 0.58 mmol) was added in one portion. Stirring was continued at room temperature overnight. After completion of the reaction (checked by TLC analysis), the reaction mixture was diluted with dichloromethane (20 mL). Afterwards the reaction mixture was washed consecutively with 15 mL each of distilled water, aqueous 1 M hydrochloric acid and brine. The organic layer was dried (sodium sulphate), filtered and evaporated to

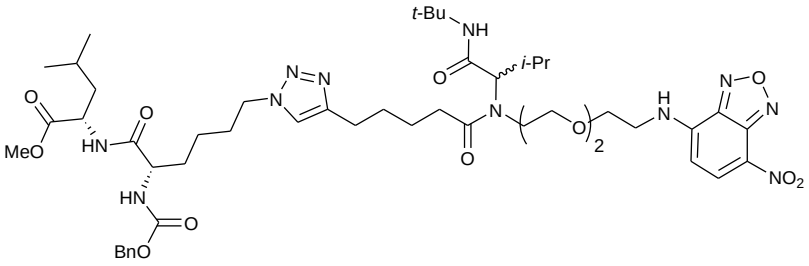
dryness to afford dichlorotosylhydrazone **3.47** as an orange solid (210 mg, 70%). M.p. 74–75 °C; ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.77–0.89 (m, 3H), 0.97–1.07 (m, 3H), 1.27–1.40 (m, 9H), 1.53–1.85 (m, 4H), 2.19–2.86 (m, 8H), 3.49–3.91 (m, 12H), 6.14–6.27 (m, 2H), 7.23–7.30 (m, 2H), 7.75–7.82 (m, 2H), 8.49 (d, *J* = 8.3 Hz, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 18.8, 19.5, 21.6, 23.1, 24.1, 24.5, 27.0, 28.5, 31.3, 43.6, 51.3, 68.1, 69.6, 70.5, 70.7, 72.9, 77.2, 98.8, 127.4, 129.6, 136.0, 136.3, 144.0, 144.3, 151.0, 169.8, 176.2.

Z-Lys-Leu-OMe hydrochloride (3.49)



To a solution containing dipeptide Z-Lys(Boc)-Leu-OMe **3.48** (1.0 g, 1.97 mmol) in dry methanol (20 mL) was added trimethylsilyl chloride (1.30 mL, 10 mmol) *via* syringe. Stirring was continued at room temperature overnight. After completion of the reaction (checked by TLC), all volatiles were removed under reduced pressure to afford dipeptide **3.49** as a white solid (0.86 g, 98%). M.p. 27–28 °C; ^{1}H NMR (400 MHz, WATER-d_2) δ: 0.71–1.02 (m, 6H), 1.35–1.88 (m, 9H), 2.99 (t, *J* = 7.5 Hz, 2H), 3.73 (m, 3H), 4.04–4.20 (m, 1H), 4.29–4.51 (m, 1H), 5.02–5.22 (m, 2H), 7.26–7.56 (m, 5H); ^{13}C NMR (100 MHz, WATER-d_2) δ: 21.5, 23.0, 23.1, 25.4, 27.3, 31.6, 40.1, 40.2, 52.4, 53.9, 55.8, 68.1, 128.6, 129.4, 129.8, 137.4, 158.7, 175.7, 175.9; ESI-MS m/z: 408.3 $[M]^+$; HRMS (ESI) calcd for $C_{21}H_{34}N_3O_5$ 408.2493, found 408.2488.

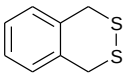
Methyl-*N*-[(benzyloxy)carbonyl]-6-[4-(5-{[1-(*tert*-butylamino)-3-methyl-1-oxo-butan-2-yl][2-(2-{2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethoxy}ethoxy)-ethyl]amino}-5-oxo-pentyl)-1*H*-1,2,3-triazol-1-yl]-*L*-norleucyl-*L*-leucinate (3.50)



The title compound was prepared according to the General Procedure using dipeptide **3.49** (31.9 mg, 71.9 µmol), NBD-tosyl-hydrazone **3.47** (79.2 mg, 95.4 µmol) and potassium acetate (45.2 mg, 0.46 mmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield triazole **3.50** as an orange semi-solid (epimeric mixture, 39 mg, 54%). R_F = 0.27 (dichloromethane/methanol 95/5); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.75–0.82

(m, 3H), 0.83–1.00 (m, 9H), 1.20–1.45 (m, 13H), 1.48–1.76 (m, 10H), 1.78–1.94 (m, 3H), 2.26–2.56 (m, 3H), 2.63–2.84 (m, 2H), 3.46–3.66 (m, 9H), 3.67–3.81 (m, 4H), 3.83–3.90 (m, 2H), 4.14–4.45 (m, 3H), 4.52–4.62 (m, 1H), 5.08 (s, 2H), 5.41–5.48 (m, 1H), 6.20–6.28 (m, 1H), 7.28–7.36 (m, 5H), 8.44–8.52 (m, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 18.8, 19.6, 21.7, 21.8, 22.6, 22.8, 24.6, 24.78, 24.80, 25.16, 25.23, 26.8, 28.5, 28.6, 28.8, 28.9, 29.8, 32.3, 33.3, 41.2, 50.75, 50.81, 50.9, 52.28, 52.31, 53.4, 67.0, 68.3, 69.4, 70.4, 70.7, 127.9, 128.0, 128.2. 128.5, 136.1, 136.5, 144.4, 173.2, 175.3; ESI-MS m/z: 1006.8 $[M-H]^-$, 1008.7 $[M+H]^+$, 1030.4 $[M+Na]^+$; HRMS (ESI) calcd for $C_{49}H_{73}N_{11}O_{12}Na$ 1030.5332, found 1030.5289.

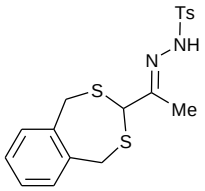
1,4-Dihydro-2,3-benzodithiine (3.53)



A solution containing *α*,*α*′-dibromoxylene **3.51** (5.29 g, 20.0 mmol) and thiourea (3.81 g, 50.0 mmol) in ethanol (50 mL) was heated under reflux for 6 hours. After cooling to room temperature, all volatiles were removed under reduced pressure and the remaining residue was taken up in distilled water (50 mL). To this suspension was subsequently added dropwise an aqueous sodium hydroxide solution (3.2 g, 80 mmol, dissolved in 10 ml distilled water) and the resulting mixture was refluxed overnight. Afterwards the reaction mixture was allowed to cool to room temperature, acidified by the addition of aqueous 2 M sulphuric acid and extracted with dichloromethane (2x 100 mL). The combined organic extracts were dried (sodium sulphate), filtered and evaporated to dryness to afford benzene-1,2-diyldimethanethiol **3.52** as a yellow liquid, which was used directly in the subsequent oxidation step without further purification.

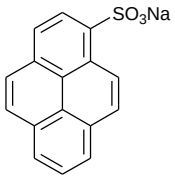
Crude dithiol **3.52** (3.06 g, 17.9 mmol) was dissolved in methanol/glacial acetic acid (80 mL, 1/1) and heated on an oil bath to 70 °C. To this mixture was added dropwise over 1 hour a solution containing iron(III) chloride hexahydrate (7.28 g, 26.9 mmol) in glacial acetic acid (10 mL). The oil bath was removed and the reaction mixture was allowed to cool to room temperature. Finally, methanol was removed by rotary evaporation and ice-water was added to the remaining solution. The resulting precipitate was removed by filtration, washed with water, dried under reduced pressure and recrystallised from aqueous methanol to afford the title compound **3.53** as an off-white solid (1.80 g, 53%). M.p. 72–73 °C; ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 4.07 (s, 4H), 7.05–7.11 (m, 2H), 7.14–7.20 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 34.5, 126.8, 130.1, 131.4, 132.8.

N'-[1-(1,5-Dihydro-2,4-benzodithiepin-3-yl)ethylidene]-4-methylbenzene-sulphonohydrazide (3.54)



1,4-Dihydro-2,3-benzodithiine **3.53** (88.9 mg, 0.54 mmol) was dissolved in *t*-butanol/water (10 mL, 1/1) and tris(2-carboxyethyl)phosphine hydrochloride (305 mg, 1.04 mmol) was added. Stirring was continued for 1.5 hours at room temperature. After the disapperance of the starting material (checked by TLC), the reaction mixture was cooled on an ice-water bath and potassium acetate (0.57 g, 5.81 mmol) was subsequently added. The solution was stirred for further 10 minutes, whereupon tosylhydrazone **3.3a** (0.20 g, 0.68 mmol) in *t*-butanol (5 mL) was added dropwise to the cooled solution. The resulting reaction mixture was stirred for 16 hours at room temperature. Afterwards all volatiles were removed under reduced pressure and the residue was purified by column chromatography to afford dithiepane **3.54** as a white solid (26.4 mg, 13%). M.p. 187–189 °C; R_F = 0.45 (hexane/ethyl acetate 3/2); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.91 (s, 3H), 2.34 (s, 3H), 3.95 (d, J = 14.9 Hz, 2H), 4.08–4.17 (m, 2H), 5.28 (s, 1H), 7.13–7.19 (m, 2H), 7.19–7.25 (m, 2H), 7.35 (d, J = 7.9 Hz, 2H), 7.73 (d, J = 8.3 Hz, 2H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 16.0, 21.0, 34.8, 126.7, 127.2, 127.5, 128.9, 129.4, 135.9, 140.4, 143.4, 157.7; ESI-MS m/z: 391.3 $[M-H]^-$, 415.0 $[M+Na]^+$, 807.3 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{18}H_{20}N_2O_2S_3Na$ 415.0579, found 415.0580.

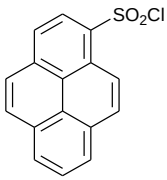
Sodium pyrene-1-sulphonate (3.56)



Chlorosulphonic acid (1.7 mL, 25.1 mmol) in dry dichloromethane (10 mL) was added dropwise to an ice-cold suspension containing pyrene **3.55** (5.0 g, 24.7 mmol) in dry dichloromethane (30 mL) under inert-gas atmosphere. Stirring was continued for 3 hours on an ice bath. Afterwards the ice-bath was removed and the reaction mixture was allowed to stir overnight at room temperature. The reaction mixture was poured on an ice-water bath (100 mL) and the organic phase was removed by rotary evaporation. Then, the remaining aqueous phase was filtered twice over a pad of celite® followed by the addition of solid sodium hydroxide (1.10 g, 27.5 mmol) and brine (20 mL). The resulting precipitate was filtered off, washed with distilled water and dried under reduced pressure to afford sodium pyrene-1-sulphonate **3.56** as a light yellow solid (3.20 g, 43%). M.p. > 300 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 8.06–8.12 (m, 1H), 8.18 (d, J = 3.9 Hz, 2H), 8.20–8.25 (m, 2H), 8.31 (dd, J = 7.7, 3.7 Hz, 2H), 8.52 (d, J = 7.9 Hz, 1H), 9.18 (d, J = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6)

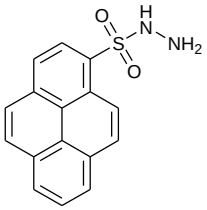
δ: 123.7, 123.6, 124.2, 124.8, 125.30, 125.33, 126.3, 126.7, 126.9, 127.3, 127.7, 130.1, 130.7, 131.3, 141.9; ESI-MS m/z: 280.6 $[M]^-$; HRMS (ESI) calcd for $C_{16}H_9O_3S$ 281.0279, found 281.0274.

Pyrene-1-sulphonyl chloride (3.57)



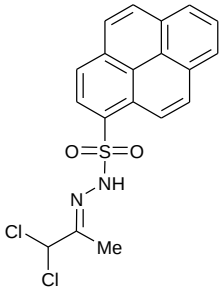
Under inert-gas atmosphere was added dropwise *N*,*N*-dimethylformamide (0.1 mL, 1.30 mmol) to a stirred solution containing sodium pyrene-1-sulphonate **3.56** (1.50 g, 4.93 mmol) in thionyl chloride (8 mL). Stirring was continued overnight at room temperature. Then, all volatiles were removed under reduced pressure and the residue was taken up in dichloromethane (200 mL). The resulting suspension was filtered and the filter cake was washed twice with dichloromethane (100 mL). Afterwards the filtrate was evaporated to dryness and the residue was taken up in a minimal amount dichloromethane and stored at –30 °C for 1 day. Finally, the resulting precipitate was collected by filtration, washed with cyclohexane and dried under reduced pressure to afford pyrene-1-sulphonyl chloride **3.57** as a yellow solid (1.25 g, 84%). M.p. 166–168 °C; ^{1}H NMR (400 MHz, DMSO-*d*$_6$) *δ*: 8.10–8.19 (m, 2H), 8.22 (d, *J* = 8.3 Hz, 1H), 8.29 (d, *J* = 8.8 Hz, 1H), 8.39 (dd, *J* = 13.6, 7.9 Hz, 2H), 8.44 (d, *J* = 9.2 Hz, 1H), 8.74 (d, *J* = 8.3 Hz, 1H), 9.10 (d, *J* = 9.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) *δ*: 122.4, 123.6, 123.7, 125.1, 126.3, 126.9, 127.4, 128.0, 128.1, 128.4, 130.0, 130.7, 131.5, 131.8, 135.6, 136.6.

Pyrene-1-sulphonohydrazide (3.58)



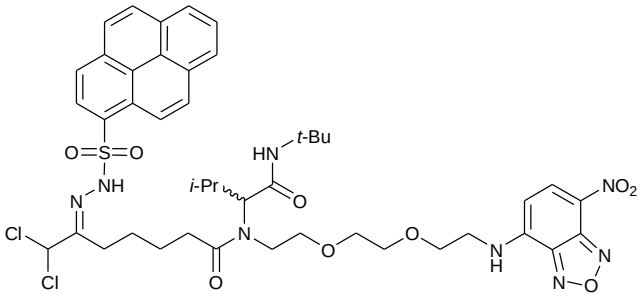
To a solution containing pyrene-1-sulphonyl chloride **3.57** (0.60 g, 2.00 mmol) in acetonitrile (15 mL) was consecutively added dropwise triethylamine (0.33 mL, 2.36 mmol) and hydrazine monohydrate (0.12 mL, 2.40 mmol). Stirring was continued overnight at room temperature, whereupon the reaction mixture was quenched by the addition of ice-water (40 mL). The resulting precipitate was filtered off, washed with distilled water and dried under reduced pressure to afford pyrene-1-sulphonohydrazide **3.58** as a yellow solid (0.42 g, 71%). M.p. 153–154 °C; ^{1}H NMR (400 MHz, DMSO-*d*$_6$) *δ*: 8.18–8.24 (m, 1H), 8.28–8.32 (m, 1H), 8.38–8.51 (m, 5H), 8.62 (d, *J* = 8.3 Hz, 1H), 9.03 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-*d*$_6$) *δ*: 123.2, 124.0, 124.1, 124.3, 126.8, 126.9, 127.0, 127.1, 127.9, 128.3, 129.2, 129.7, 130.0, 130.3, 130.5, 134.2; ESI-MS m/z: 294.9 $[M-H]^-$, 319.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{16}H_{11}N_2O_2S$ 295.0547, found 295.0546.

N'-(1,1-Dichloropropan-2-ylidene)pyrene-1-sulphonohydrazide (3.59)



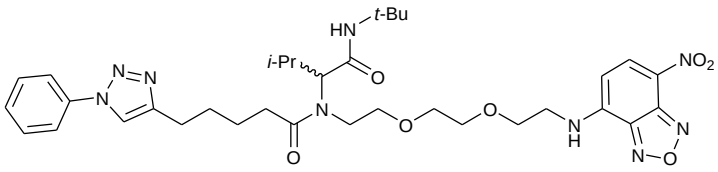
To a solution containing pyrene-1-sulphonohydrazide **3.58** (153 mg, 0.52 mmol) in acetonitrile (3 mL) was added 1,1-dichloroacetone **3.1** (70 µL, 0.71 mmol) and stirring was continued overnight at room temperature. After completion of the reaction has been detected (ESI-MS analysis), the reaction mixture was stored at −30 °C for 1 day. The resulting precipitate was collected by filtration, washed with cyclohexane and dried under reduced pressure to afford hydrazone **3.59** as a light yellow solid (150 mg, 71%). M.p. 163−164 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.87 (s, 3H), 8.24 (t, J = 7.7 Hz, 1H), 8.32 (d, J = 8.8 Hz, 1H), 8.41−8.46 (m, 1H), 8.47−8.56 (m, 4H), 8.75 (d, J = 8.3 Hz, 1H), 9.12 (s, 1H), 9.20 (d, J = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 9.6, 123.0, 123.4, 124.1, 124.4, 127.1, 127.2, 127.3, 127.4, 127.77, 127.83, 129.6, 130.0, 130.3, 130.5, 130.7, 134.8, 150.4, 190.7.

PEG$_2$-NBD-α,α-dichloropyrenesulphonohydrazone (3.60)



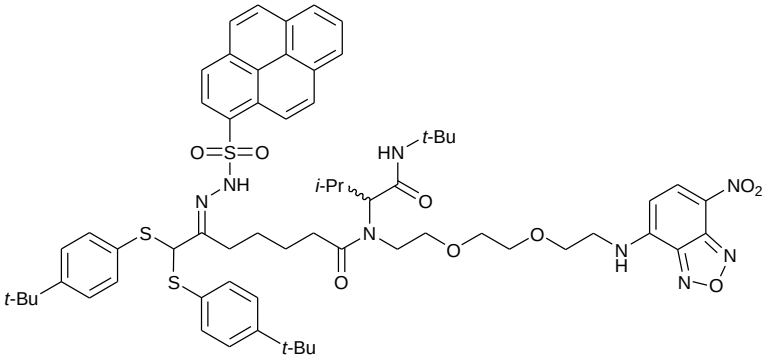
To a solution containing NBD-dichloroketone **3.46** (290 mg, 0.44 mmol) in dry tetrahydrofuran (5 mL) was added pyrene-1-sulphono-hydrazide **3.58** (143 mg, 0.48 mmol) and stirring was continued overnight at room temperature. After completion of the reaction has been detected (ESI-MS analysis), the reaction mixture was evaporated to dryness and the residue was taken up in a minimal amount of dichloromethane, filtered and the filtrate was concentrated under reduced pressure to afford hydrazone **3.60** as an orange solid (247 mg, 60%). M.p. 101−102 °C; ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 1.03−1.14 (m, 6H), 1.39 (s, 9H), 1.60−1.79 (m, 4H), 2.31−2.79 (m, 5H), 3.52−3.85 (m, 12H), 4.06 (br. s., 1H), 5.81−5.88 (m, 1H), 6.20 (s, 1H), 6.91−7.09 (m, 2H), 8.00−8.28 (m, 8H), 8.74 (d, J = 8.3 Hz, 1H), 9.22 (d, J = 9.6 Hz, 1H), 10.78 (br. s., 1H); ^{13}C NMR (100 MHz, CHLOROFORM-d) δ: 13.9, 19.2, 19.7, 24.3, 24.6, 25.6, 27.5, 27.7, 28.5, 31.9, 68.0, 70.6, 72.9, 77.2, 123.5, 123.7, 124.0, 124.7, 126.6, 126.7, 126.77, 126.81, 127.0, 127.1, 127.9, 128.3, 129.6, 129.9, 130.26, 130.33, 130.8, 135.1, 135.7, 143.6, 150.8.

_N_-_tert_-Butyl-_N_²-[2-(2-{2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethoxy}-ethoxy)ethyl]-_N_²-[5-(1-phenyl-1_H_-1,2,3-triazol-4-yl)pentanoyl]valinamide (3.61)



The title compound was prepared according to the General Procedure using aniline (18.6 µL, 200 µmol), hydrazone **3.60** (96 mg, 102 µmol) and potassium acetate (61 mg, 624 µmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to afford triazole **3.61** as orange oil (45 mg, 64%). R_F = 0.19 (hexane/ethyl acetate 1/4); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.76–0.84 (m, 3H), 0.96 (d, J = 6.6 Hz, 3H), 1.23–1.33 (m, 9H), 1.68–1.92 (m, 4H), 2.36–2.61 (m, 3H), 2.83 (t, J = 7.0 Hz, 2H), 3.48–3.79 (m, 10H), 3.79–3.90 (m, 2H), 4.05 (br. s., 1H), 6.15–6.28 (m, 1H), 7.37–7.56 (m, 3H), 7.70 (d, J = 7.9 Hz, 2H), 7.76 (s, 1H), 8.44 (d, J = 8.3 Hz, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 18.7, 19.7, 24.9, 25.4, 26.5, 28.6, 29.0, 33.4, 43.7, 50.9, 68.2, 69.3, 70.5, 70.6, 70.8, 77.2, 99.0, 119.0, 120.3, 123.8, 128.6, 129.7, 136.4, 137.1, 144.0, 144.3, 148.5, 170.4, 175.3; ESI-MS m/z: 692.6 $[M-H]^-$, 694.7 $[M+H]^+$, 716.6 $[M+Na]^+$; HRMS (ESI) calcd for $C_{34}H_{48}N_9O_7$ 694.3671, found 694.3679.

_N_-[1-(_tert_-Butylamino)-3-methyl-1-oxobutan-2-yl]-7,7-bis[(4-_tert_-butylphenyl)-sulfanyl]-_N_-[2-(2-{2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]ethoxy}ethoxy)-ethyl]-6-[2-(pyren-1-ylsulphonyl)-hydrazinylidene]heptanamide (3.62)



The title compound was prepared according to the General Procedure using 4-*tert*-butylthiophenol (45 µL, 253 µmol), hydrazone **3.60** (93 mg, 99 µmol) and potassium acetate (65 mg, 661 µmol). Stirring was continued at room temperature for 16 hours, whereupon all volatiles were removed under reduced pressure. The residue was purified by column chromatography to yield bis-thiether **3.62** as an orange solid (40 mg, 34%). M.p. 84–85 °C; R_F = 0.42 (hexane/ethyl acetate 3/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.90–0.96 (m, 3H), 1.00–1.10 (m, 12H), 1.12–1.18 (m, 9H), 1.30–1.38 (m, 9H), 1.57–1.86

(m, 4H), 2.29–2.59 (m, 3H), 2.63–2.83 (m, 2H), 3.46–3.93 (m, 13H), 4.97 (s, 1H), 6.00 (d, *J* = 8.8 Hz, 1H), 6.78–7.10 (m, 8H), 8.01–8.13 (m, 2H), 8.14–8.32 (m, 5H), 8.75 (d, *J* = 8.3 Hz, 1H), 9.30 (d, *J* = 9.6 Hz, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) *δ*: 18.9, 19.6, 23.2, 24.6, 26.6, 27.2, 28.5, 29.7, 31.0, 31.1, 31.8, 34.3, 34.4, 51.3, 62.1, 68.3, 69.8, 70.7, 77.2, 123.8, 123.9, 124.2, 124.9, 125.7, 125.8, 126.76, 126.79, 126.9, 127.0, 127.9, 128.6, 129.6, 130.1, 130.3, 130.4, 130.85, 130.92, 131.0, 131.2, 134.9, 136.0, 143.7, 144.0, 150.5, 150.6, 153.6, 169.9, 176.3; ESI-MS m/z: 1197.9 $[M-H]^-$, 1199.9 $[M+H]^+$, 1221.7 $[M+Na]^+$; HRMS (ESI) calcd for $C_{64}H_{77}N_8O_9S_3$ 1197.4981, found 1197.4980.

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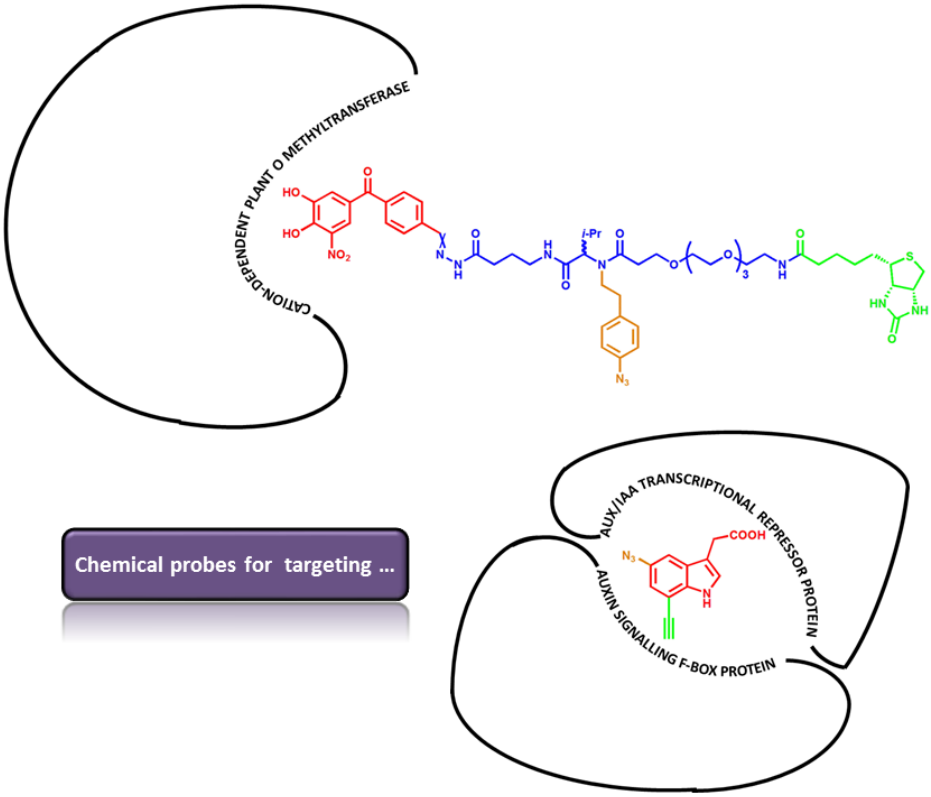
4 Synthesis of Chemical Probes for Activity-Based Protein Profiling in Plants



Abstract

Chemical probes that bind to the active site of an enzyme are useful tools for studying enzyme activities associated with discrete physiological states (healthy vs. pathogenic), for identifying novel inhibitors from diverse compound libraries and drug off-targets, for localising and quantifying protein activity in natural environments and for the functional characterisation of proteins with previously unknown function, among others. The biggest challenge when designing novel probes consists of identifying a suitable selectivity group to direct the probe to the target of interest. In general, these selectivity groups are derived from well-known tight binding inhibitors, (co-)substrates or from natural products. In the first part of the forthcoming chapter, the synthesis of a chemical probe that putatively targets cation-dependent plant-*O*-methyltransferases with high specificity will be presented. This probe is based on the modification of tolcapone, a well-known inhibitor of the cation-dependent animal catechol-*O*-methyltransferase which is an important target in the treatment of Parkinson`s disease. Moreover, the straightforward synthesis of the first clickable, photoreactive auxin analogue will be presented in the second part of the present chapter.

4.1 Introduction

Initially developed by Cravatt and co-workers to detect proteins with a common function, in recent years activity-based protein profiling (ABPP) evolved as a highly versatile methodology being important in various aspects of chemical biology (*e.g. in vivo* imaging) as well as in drug development (*e.g.* the identification of drug off-targets). The basic concept of ABPP relies on the covalent modification of a target protein with specifically designed small molecular chemical probes (activity-based probes = ABPs) in highly complex proteomes. In general, these probes require the presence of four different functional moieties (Fig. 4.1): (i) a *selectivity group* required for the (non-)covalent interaction with the target protein based on its catalytic mechanism (*e.g.* reversible or irreversible binding inhibitor, cofactor or substrate); (ii) a *reactive group* for the formation of a stable covalent bond between target protein and ABP (*e.g.* photoreactive groups); (iii) a *reporter-tag* enabling the detection and/or enrichment of the modified protein targets (*e.g.* fluorophores, biotin); and (iv) a *linker* unit incorporated to minimise steric clashes between the binding site of the protein and the reporter-tag and to enhance the water solubility of the whole chemical probe. Ultimately, the obtained covalently modified proteins can be identified by in-gel fluorescence scanning or avidin affinity enrichment followed by MS-analysis of the obtained protein stains or enriched proteins, respectively (cf. chapter 1).[1]

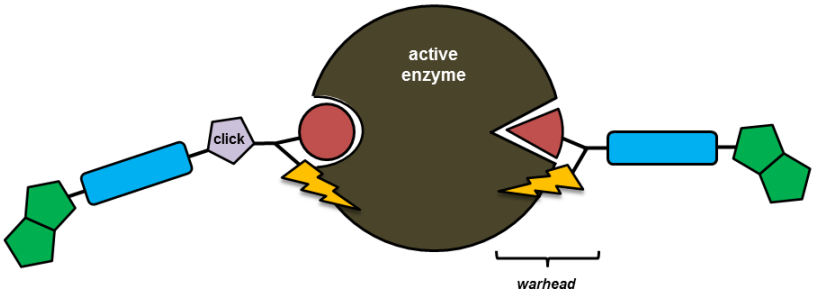


Fig. 4.1 General build-up of activity-based probes. ABPs are composed of up to four different functional building blocks: a **selectivity group** for target specificity, a **reactive group** for the covalent attachment of the probe to the target protein, a **reporter-tag** for target detection and/or purification and a **linker** to minimise steric clashes and/or to function as solubility mediator. To further reduce steric clashes and/or to increase the cellular uptake and distribution of the probe, which might be hampered due to bulky reporter-tags, the warhead and reporter-tag can be conjugated by bioorthogonal coupling methods (*e.g.* click-reaction) after covalent labelling of the target proteins was established.

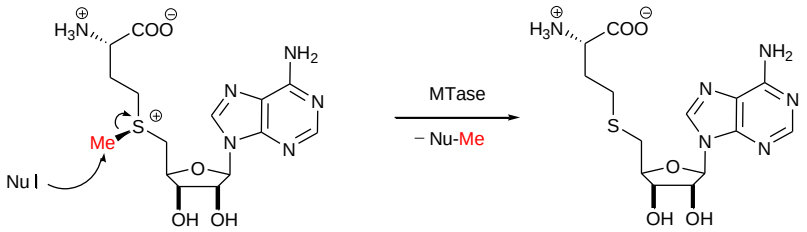
Despite its wide scope of applications, ABPP in plant science, however, is still in its early stages of development. In most cases, ABPs targeting cysteine proteases and serine

hydrolases have been used in first proof of concept studies demonstrating the utility of ABPP in plant science. The application of ABPs targeting plant specific proteins might facilitate mechanistic investigations regarding plant senescence, plant development and plant immunity. Moreover, ABP could contribute to the identification of plant pathogen-secreted inhibitors as well as yet unknown protein targets of commercial herbicides.[2] Recent examples of the application of ABPP in plant science include the identification of protein off-targets of common insecticides in plants (*e.g.* paraoxon and profenofos)[3] and the labelling and enrichment of matrix metalloproteases (MMPs) as well as vacuolar processing enzymes (VPEs). Both enzymes are involved in plant development and plant immunity, hence ABPP enables to investigate the role and regulation of MMPs and VPEs in plant defence upon pathogen infection.[4,5] Additionally, ABPs have proven to be useful to detect differential protein activities during plant development, which has been exemplified for plant natural product *O*-methyltransferases in *A. thaliana*.[6]

Designing ABPs, which target plant-specific proteins, is not a trivial task as it requires the selection of a suitable selectivity group directing the probe to a specific protein superfamily. These selectivity groups might be derived from already known inhibitors, (co-)substrates, natural products or even plant hormones. To illustrate the power of this approach, two examples of specifically designed ABPs, which have been prepared in the present study, targeting enzymes involved in plant natural product biosynthesis as well as regulation will be presented: (i) nitrocatechol affinity-probes to target cation-dependent plant *O*-methyltransferases form *A. thaliana*; and (ii) a "clickable", photoreactive indole-3-acetic acid analogue to identify auxin-binding proteins with a putative role in auxin-mediated signalling pathways. The design of these ABPs was based on the modification of the already established catechol-*O*-methyltransferase inhibitor tolcapone and the plant growth hormone indole-3-acetic acid (IAA), respectively.

4.2 Targeting cation-dependent plant *O*-methyltransferases with tolcapone-derived ABPs

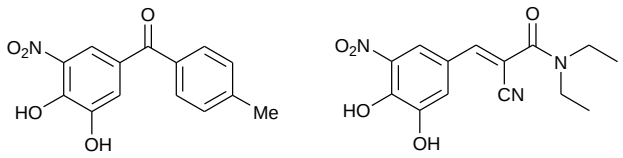
Plant *O*-methyltransferases (Pl-OMT) play a crucial role in various biosynthetic pathways including soluble phenylpropanoid, coumarin, flavonoid as well as lignin polymer biosynthesis.[7,8,9] In general, this class of enzymes catalyses the transfer of a methyl-group from a methyl-donor (*i.e. S*-adenosyl-*L*-methionine = SAM) to an oxygen nucleophile (Fig. 4.2).



***S*-Adenosyl-*L*-methionine (SAM)** **S-Adenosyl-*L*-homocysteine (SAH)**

Fig. 4.2 Methyl-group transfer from SAM to various acceptor nucleophiles catalysed by methyltransferases (MTases). As a consequence of the positively charged sulphur atom, the carbon-sulphur bond gets destabilised making this bond highly reactive towards a nucleophilic attack by nitrogen-, oxygen, sulphur- or carbon-nucleophiles.

Dalhoff *et al*. already developed a suitable ABP for the detection of Pl-OMTs based on the methyl group donor SAM. In this particular case, the more stable *S*-adenosyl-*L*-homocysteine (SAH) was incorporated into the chemical probe instead of the very unstable SAM.[10] However, for the selective identification of Pl-OMTs from *A. thaliana* this approach is limited due to the whole amount of known SAM-binding proteins in *A. thaliana* (~ 265 proteins). To our knowledge, only approximately thirty proteins of this pool belong to the class of Pl-OMTs, which can be additionally differentiated in cation-dependent and cation-independent Pl-OMTs, respectively. As a consequence of the known similarities between cation-dependent Pl-OMTs and the animal catechol-*O*-methyltransferase (COMT) concerning substrate binding and catalysis mechanism,[11] already well-known highly potent inhibitors of the animal COMT were chosen as the starting point for the development of a chemical probe targeting specifically cation-dependent Pl-OMTs. Currently, the most potent inhibitors of the animal COMT are tolcapone and entacapone (Fig. 4.3).[12] Both inhibitors are non-covalently bound in the active-site of the protein, hence an additional cross-linker is necessary to covalently attach the final probe to the protein for purification and detection purposes. By taking a closer look at the structure of tolcapone, the benzophenone core-structure attracts attention because benzophenone and its derivatives are well established photoreactive cross-linkers in chemical biology (cf. chapter 1.2).[13] Therefore, it was envisioned to use a tolcapone analogue as both putative inhibitor of cation-dependent Pl-OMTs and



tolcapone (Tasmar©) **entacapone (Comtan©)**

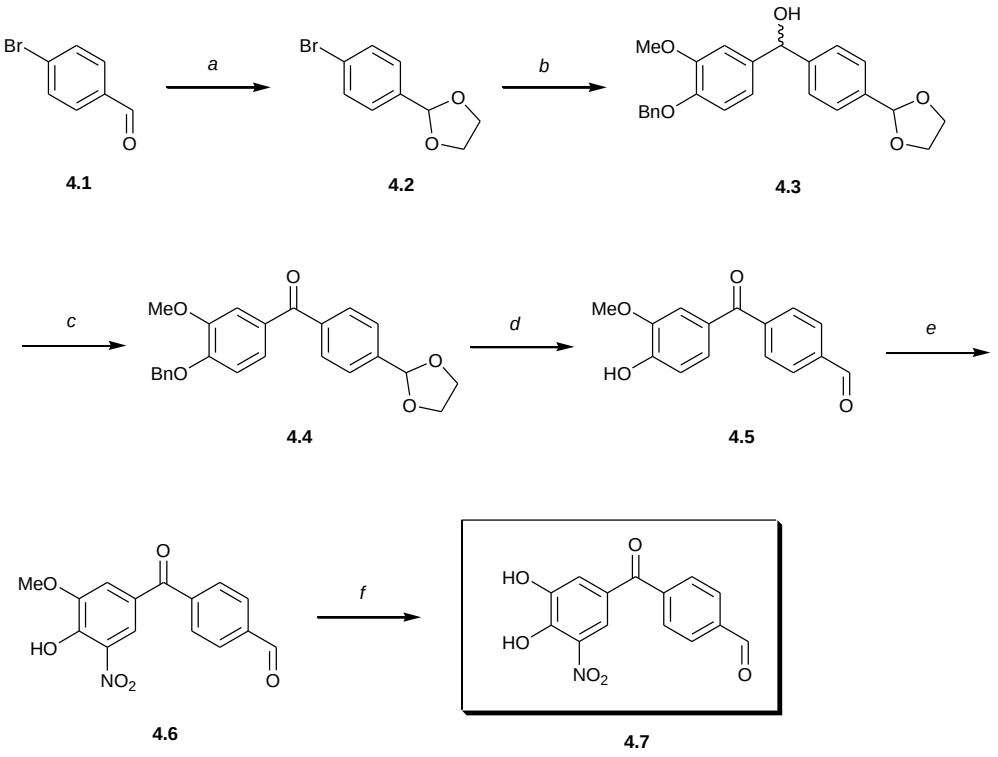
Fig. 4.3 Structure of the well-known animal COMT inhibitors tolcapone and entacapone used in the treatment of Parkinson`s disease.

photoreactive cross-linker for the covalent attachment of an additional reporter-tag (here: biotin) to the protein. A similar approach has been applied successfully by the Cravatt lab using a 30 member library consisting of derivatives of the two benzophenone scaffolds: 5-benzoyl indole and 7-benzoyl-benzo-1,4-diazepin-2,5-dione.[14] Each compound was additionally equipped with an alkyne handle to enable subsequent visualisation, enrichment and identification of the interacting proteins. Based on this approach, the authors were able to identify one compound that was able to inhibit hypoglycemic cancer cell proliferation in various human cancer cell lines with IC_{50} values ranging from 0.33 μM to 1.3 μM. Furthermore, the major cellular targets of this compound were determined *via* enrichment of the probe-labelled proteins with avidin, tryptic digestion and subsequent LC/MS/MS analysis of the peptide mixture.

As a consequence of the biological relevance of targeting COMT in the treatment of Parkinson`s disease, the synthesis of tolcapone and analogues thereof is well established.[15] The most crucial step in the synthesis of tolcapone probes, however, was the combination of both parts of the ABP: the tolcapone selectivity-group and a suitable biotin reporter-tag. Due to the severe problems purifying nitro-catechol compounds as previously experienced,[16] a simple method to combine both building blocks preferentially with water as the sole by-product was envisioned. For this purpose, the reaction between aldehydes and acylhydrazines to afford the corresponding acylhydrazones, in general, in high yields and purity was considered to combine both parts of the probe. The resulting acylhydrazones should be sufficiently stable under the conditions applied in the labelling reaction and during affinity enrichment using streptavidin beads.[17]

The aldehyde-modified building block tolcapone carbaldehyde **4.7**, which functions as both selectivity-and reactive group, could be obtained starting from commercially available 4-bromobenzaldehyde (**4.1**) in six steps and 28% overall yield (Scheme 4.1). Protection of the aldehyde moiety with ethylene glycol (**4.2**, 93%) and subsequent lithiation with *t*-butyl lithium followed by the addition of 4-benzyloxy-3-methoxy-benzaldehyde in dry THF at –78 °C afforded adduct **4.3** in 77% yield. The corresponding benzophenone **4.4** was obtained by Oppenauer oxidation[18] with sodium *t*-butoxide in refluxing cyclohexanone in 82% yield. Afterwards acidic conditions (excess 33% HBr in acetic acid) were applied to remove the benzyl-protective group. Simultaneously, the 1,3-dioxolane protective group is cleaved under these conditions and benzaldehyde **4.5** is obtained in 75% yield after recrystallisation. Using transfer hydrogenolysis conditions (ammonium formate, Pd/C, methanol, reflux) instead of acidic conditions to cleave the

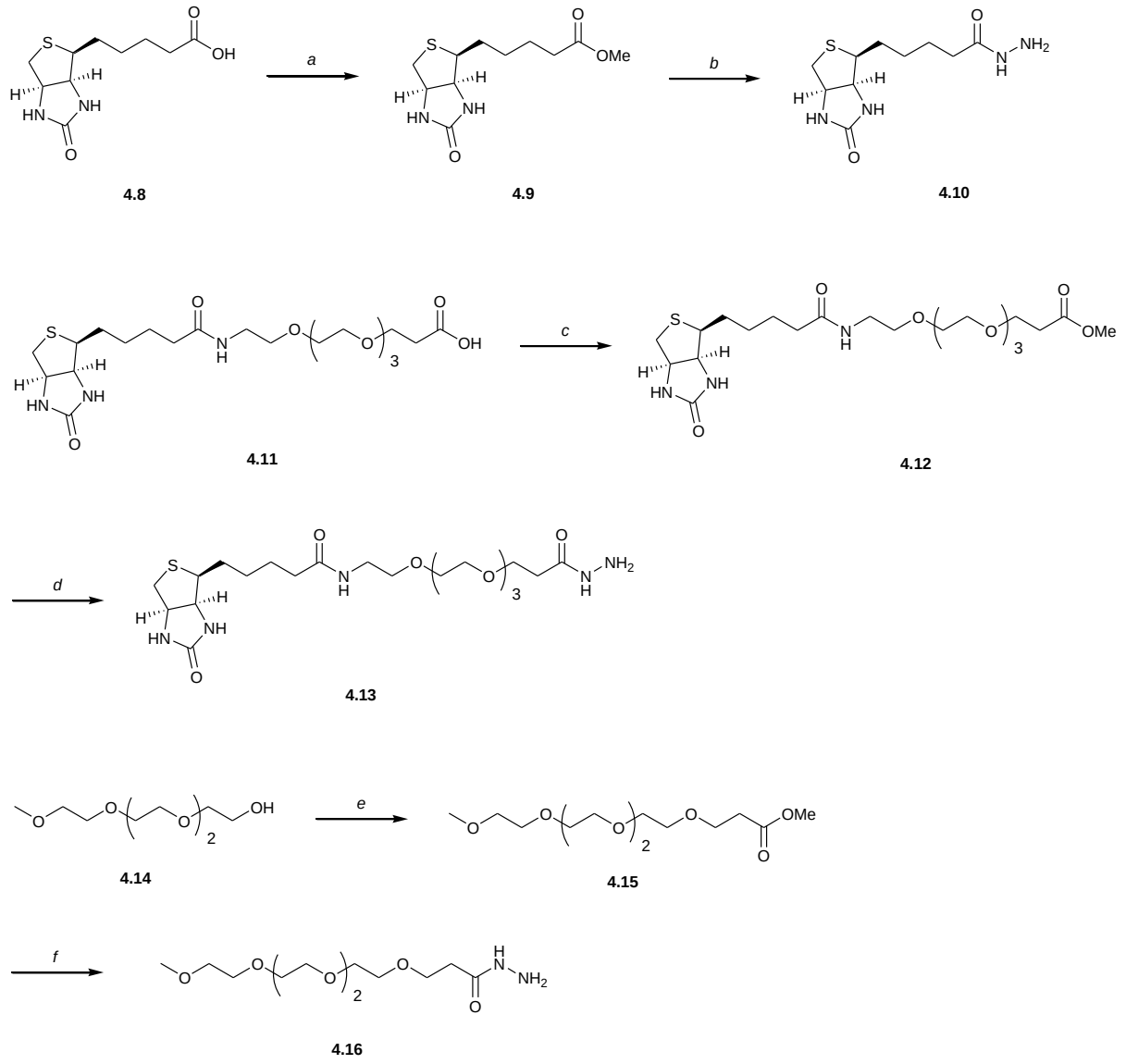
benzyl group, however, is rather unsuitable as a consequence of an observed additional reduction of the keto-function to the corresponding tertiary alcohol diminishing the chemical yield for this deprotection step from 75% (acidic conditions) to 33% (hydrogenolysis). Selective ortho-nitration was accomplished under mild conditions (65% HNO_3, glacial acetic acid) in good yields (**4.6**, 85%). Finally, deprotection of the methyl ether was performed using aluminium chloride and pyridine in refluxing ethyl acetate,[19] which furnished the aldehyde modified tolcapone analogue **4.7** in 75% yield.



Scheme 4.1 Synthesis of an aldehyde-modified tolcapone analogue. *Reagents and Conditions:* (a) glycol, *p*TsOH, toluene, reflux, overnight (93%); (b) *t*-BuLi, 4-benzyloxy-3-methoxy-benzaldehyde, THF, -78 °C to 0 °C, 2 h (77%); (c) sodium *t*-butoxide, cyclohexanone, reflux, 1 h (82%); (d) 33% HBr in AcOH, CH_2Cl_2, r.t., 2 h (75%); (e) conc. HNO_3 (65%), AcOH, r.t., 30 min. (85%); (f) $AlCl_3$, pyridine, EtOAc, reflux, 2 h (74%).

To enable affinity purification, biotin containing acylhydrazine reporter-tags were prepared in two steps from commercially available starting materials (Scheme 4.2): biotin hydrazide **4.10** from biotin (**4.8**) and PEG_4-biotin hydrazide **4.13** from PEG_4-biotin-COOH (**4.11**), respectively, to increase the water solubility of the resulting chemical probe.[20] First the starting carboxylic acids **4.8** and **4.11** were converted into the corresponding methyl esters **4.9** and **4.12** by simply stirring a methanolic solution containing the respective carboxylic acid in the presence of thionyl chloride. Upon treatment of the aforementioned methyl esters with hydrazine (8.8 equiv.) in methanol, biotin hydrazide

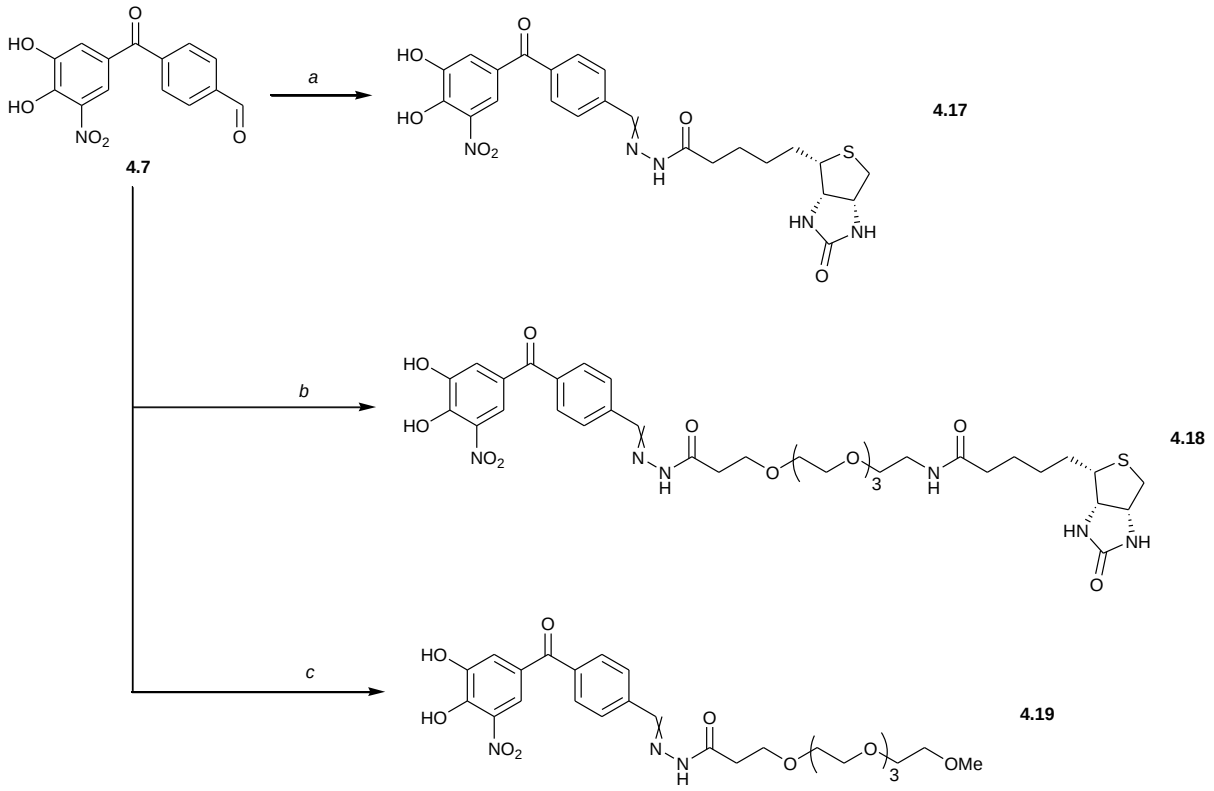
4.10 and PEG$_4$-biotin hydrazide **4.13** could be isolated in excellent yields. Moreover, the PEGylated acylhydrazine **4.16** was prepared from tetraethylene glycol monomethyl ether **4.14** and methyl acrylate in two steps and 25% overall yield (Scheme 4.2). The obtained acylhydrazine **4.16** was initially thought of acting as negative control to distinguish between specific labelling events within the active site of the protein (probe bound according to catalytic mechanism) and non-specific labelling events (chemical probe bound outside the active site).



Scheme 4.2 Acylhydrazine synthesis. *Reagents and conditions*: (a) $SOCl_2$, MeOH, 0 °C, 2.5 h (quant.); (b) N_2H_4, MeOH, r.t., overnight (74%); (c) $SOCl_2$, MeOH, 0 °C, 1 h (quant.); (d) N_2H_4, MeOH, r.t., overnight (quant.); (e) methyl acrylate, NaH, THF, r.t, overnight (32%); (f) N_2H_4, MeOH, r.t., overnight (77%).

The subsequent combination of both functional building blocks, tolcapone aldehyde **4.7** and acylhydrazine **4.10**, **4.13** and **4.16**, afforded the corresponding tolcapone-biotin-

acylhydrazone **4.17**, tolcapone-PEG$_4$-biotin-acylhydrazone **4.18** and tolcapone-PEG$_4$-acylhydrazone **4.19** as stereomeric mixtures in acceptable yields (25–70%) and high purity while leaving the keto-group unaffected (Scheme 4.3).



Scheme 4.3 Tolcapone-acylhydrazone formation. *Reagents and Conditions*: (a) AcOH, r.t., overnight (42%); (b) AcOH, r.t., overnight (25%); (c) THF/H_2O (1/1), r.t., overnight (70%).

Due to the very low solubility of tolcapone-biotin-acylhydrazone **4.17** in buffered aqueous solutions and in the presence of biologically compatible amounts of DMSO, further investigations regarding the enrichment of Pl-OMTs from crude cell lysates (flower buds) of *A. thaliana* were performed exclusively with the soluble tolcapone-PEG$_4$-biotin-acylhydrazone **4.18** and tolcapone-PEG$_4$-acylhydrazone **4.19**. These experiments were performed in close cooperation with the group Metabolite Profiling and Protein Biochemistry at the Leibniz Institute of Plant Biochemistry, Halle, Germany (Dr. Thomas Vogt and Dr. Lisette Wirsing). First, the potential of both compounds to inhibit the *A. thaliana* tapetum specific cation-dependent Pl-OMT AtTSM1 and the cation-independent Pl-OMT AtOMT1 was evaluated (Fig. 4.4). The obtained inhibition data clearly demonstrate that both probes inhibit only the cation-dependent AtTSM1, whereas no reduced enzyme activity dependent on probe concentration can be observed for the cation-independent AtOMT1. This fact can be attributed to the known similarities

between cation-dependent Pl-OMTs and the animal COMT concerning substrate binding and catalysis mechanism as stated previously. Therefore, the application of tolcapone-derived chemical probes should enable to specifically enrich cation-dependent Pl-OMTs from crude extracts of *A. thaliana*. However, tolcapone-PEG$_4$-biotin-acylhydrazone **4.18** is slightly more potent inhibiting AtTSM1 compared to tolcapone-PEG$_4$-acylhydrazone **4.19**, hence the latter is not suitable to function as a competition control, *i.e.* suppress binding of probe **4.18** in the active site of the enzyme to identify non-specific protein-probe interactions.

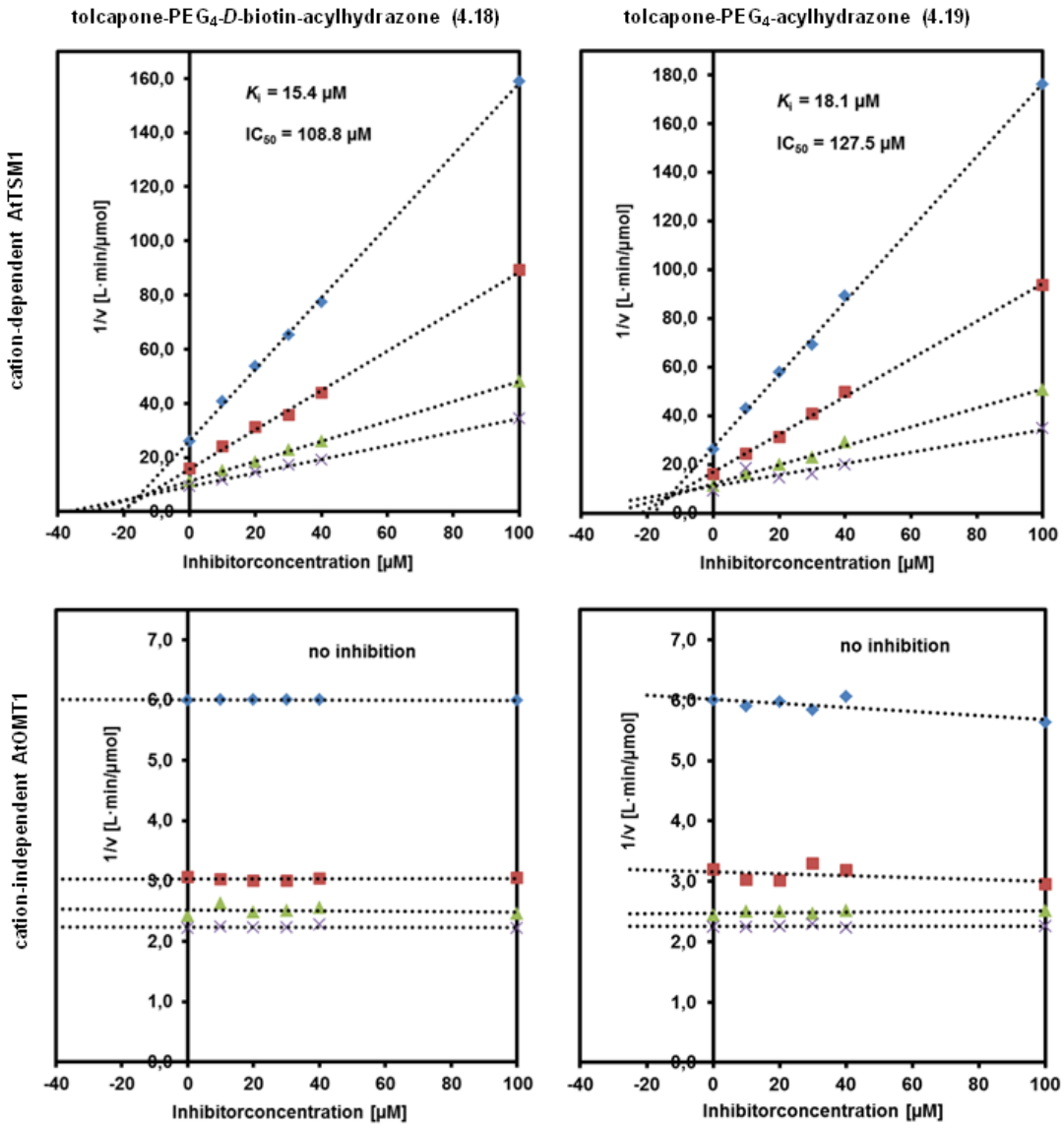


Fig. 4.4 Dixon plot analysis for the determination of K_i and IC_{50}-values. The reaction rates of the conversion of luteolin (substrate concentration: ◆5 µM, ■10 µM, ▲20 µM, ×30 µM) with the cation-dependent AtTSM1 and the cation-independent AtOMT1 were determined as a function of inhibitor concentrations (0, 10 µM, 20 µM, 30 µM, 40 µM, 100 µM). For competitive inhibitors the lines determined at different [S] intersect at $-K_i$. The IC_{50}-value can be calculated from $IC_{50} = [1+[S]/K_M)\cdot K_i$ with [S] = 100 µM luteolin and K_M = 16.4 µM (determination of K_i and IC_{50}-values performed by Dr. Lisette Wirsing, IPB Halle).

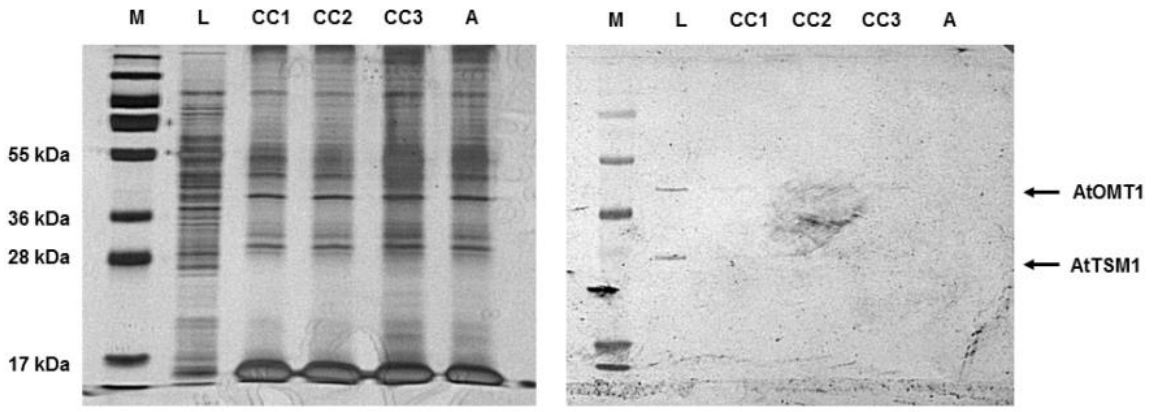
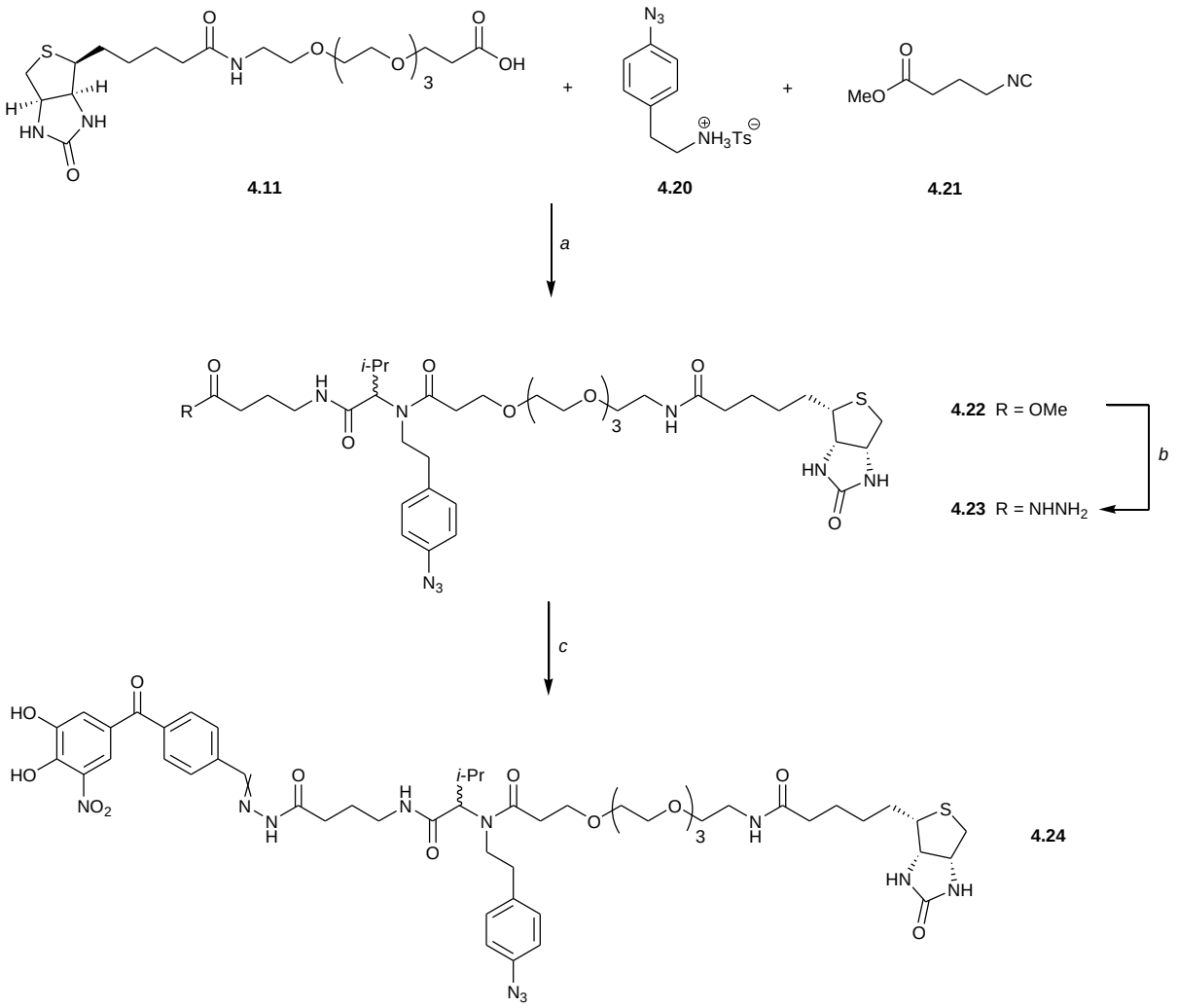


Fig. 4.5 Labelling of crude flower bud extracts of *A. thaliana* with tolcapone-PEG$_4$-biotin-acylhydrazone (4.18). Probe-labelled proteins were visualised either by silver staining (left) or western blot analysis with two enzyme-specific antibodies: cation-dependent AtTSM1 and cation-independent AtOMT1 (right); M = marker, L = crude flower bud extract; CC1 = competition control with **4.19**, heated; CC2 = competition control with **4.19**; CC3 competition control with 3,5-dinitrocatechol; A = assay without competitor (performed by Dr. Lisette Wirsing, IPB Halle).

Next, after selective binding of tolcapone-PEG$_4$-biotin-acylhydrazone **4.18** to cation-dependent Pl-OMTs was established, as exemplified with the enzyme AtTSM1, a crude flower bud extract of *A. thaliana* was used in the labelling reaction (in previous studies *A. thaliana* flower buds have been shown to contain high levels of AtTSM1[6]). Following affinity enrichment with magnetic avidin beads and several washing steps to remove non-covalently bound proteins, the probe-labelled proteins were separated by SDS-PAGE and subsequently visualised by silver staining and western blot analysis, respectively (Fig. 4.5). No differences in protein levels between competition controls 1 to 3 and the assay without competitor could be observed though, *i.e.* no reduction of proteome complexity or enrichment of single proteins. Furthermore, no protein bands corresponding to either the cation-dependent AtTSM1 or the cation-independent AtOMT1 could be detected in the western blot. This suggests two main problems accompanied with the design of the chemical probe which might reduce the labelling yield: (i) affinity of the probe is not sufficient to enrich Pl-OMTs from crude extracts; or (ii) a reduced photo-cross-linking efficiency as a consequence of the nitro-catechol motif.[21] As similar results were obtained using pig liver and kidney extracts where the same probes exhibited an approx. 100fold increased affinity (results not included), one can conclude that insufficient photo-cross-linking is responsible for the unsuccessful labelling and enrichment of Pl-OMTs.

Consequently, an additional phenyl azide moiety was incorporated in the probe design to enable sufficient photo-cross-linking. First, the commercially available PEG$_4$-biotin-

COOH (**4.11**) was reacted with *i*-butyraldehyde, 2-(4-azidophenyl)ethanaminium tosylate (**4.20**)[22] and methyl isocyanobutanoate (**4.21**) in a one-pot Ugi-4CR to furnish PEG_4-biotin-phenylazide-COOMe **4.22** in good yields (Scheme 4.4). Upon treatment of methyl ester **4.23** with hydrazine in methanol and subsequent addition of tolcapone aldehyde **4.7**, tolcapone-PEG_4-biotin-phenylazide-acylhydrazone (**4.24**) could be isolated as a stereomeric mixture in sufficient yield and purity.



Scheme 4.4 Synthesis of tolcapone-PEG_4-biotin-phenylazide-acylhydrazone (4.24). *Reagents and Conditions*: (a) *i*-butyraldehyde, triethylamine, MeOH, r.t., overnight (72%); (b) N_2H_4, MeOH, r.t., overnight (68%); (c) THF/H_2O (1/1), r.t., overnight (36%).

Despite the incorporation of a more reactive phenyl azide moiety, labelling of crude flower bud extracts with tolcapone-probe **4.24** gave similar results as obtained previously utilising tolcapone-probe **4.18** without additional photo-cross-linker. These findings suggest that the photo-cross-linking reaction is not sufficient for the covalent labelling and subsequent enrichment of cation-dependent Pl-OMTs from *A. thaliana*. This

lack in labelling efficiency might be attributed to the absence of a suitable amino acid residue in close spatial proximity to the formed reactive intermediate upon photolysis. Hence, further work in this field should be directed towards the optimisation of the distance between selectivity group and the photoreactive component to enable the covalent attachment of the chemical probe. Optimisation of the labelling conditions including sample preparation of plant extracts should be considered as well in further studies (*e.g.* reaction buffer, incubation time, irradiation conditions).

4.3 Clickable photo-auxin – Synthesis and biological activity as photo-labelling agent in ABPP

Plant hormones (also known as phytohormones) are an important class of signalling molecules which occur in very low concentrations in plants (in general 10^{-5} to 10^{-6} M) and promote changes in gene regulation. These chemical signals are essential key-players in all cellular processes, such as plant growth, fruit development and ripening, plant defence, plant longevity and plant death, *etc*. Examples of such small organic compounds include ethylene gas, tryptophane derivatives like auxins and jasmonates, brassinosteroids, strigolactone, abscisic acid, cytokinin and many more.[23]

To understand the effects of these plant hormones and to elucidate the mechanisms of hormone perception and signalling, it is imperative to identify their cellular targets. Recently, chemical probes equipped with the hormone of interest (or a hormone analogue) serving as the selectivity group, a photoreactive cross-linker and a biotin-tag have been applied to facilitate the identification of cellular protein targets and binding sites of various plant hormones.[24] For example, Kinoshita and co-workers used a biotin-tagged, photoreactive analogue of the brassinolide precursor castasterone targeting brassinosteroid insensitive 1 (BRI1), which is involved in brassinosteroid recognition and signalling, to identify a novel binding domain for plant steroid hormones (**4.25**, Fig. 4.6A).[25] A similar approach was used by Yan *et al.* to determine whether the isoleucine-conjugated jasmonate binds directly to the F-box protein coronatine insensitive 1 (COI1), which is well-known to be required for jasmonate associated plant fertility and defence. For this purpose, the potential of a biotin-tagged photoreactive coronatine, a mimic of the isoleucine-conjugated form of jasmonic acid, to bind COI1 was evaluated (**4.26**, Fig. 4.6B). Taken together with previous genetic evidence, photoaffinity labelling of COI1 with coronatine probe **4.26** proved that COI1 is truly a part of a co-receptor for jasmonate.[26]

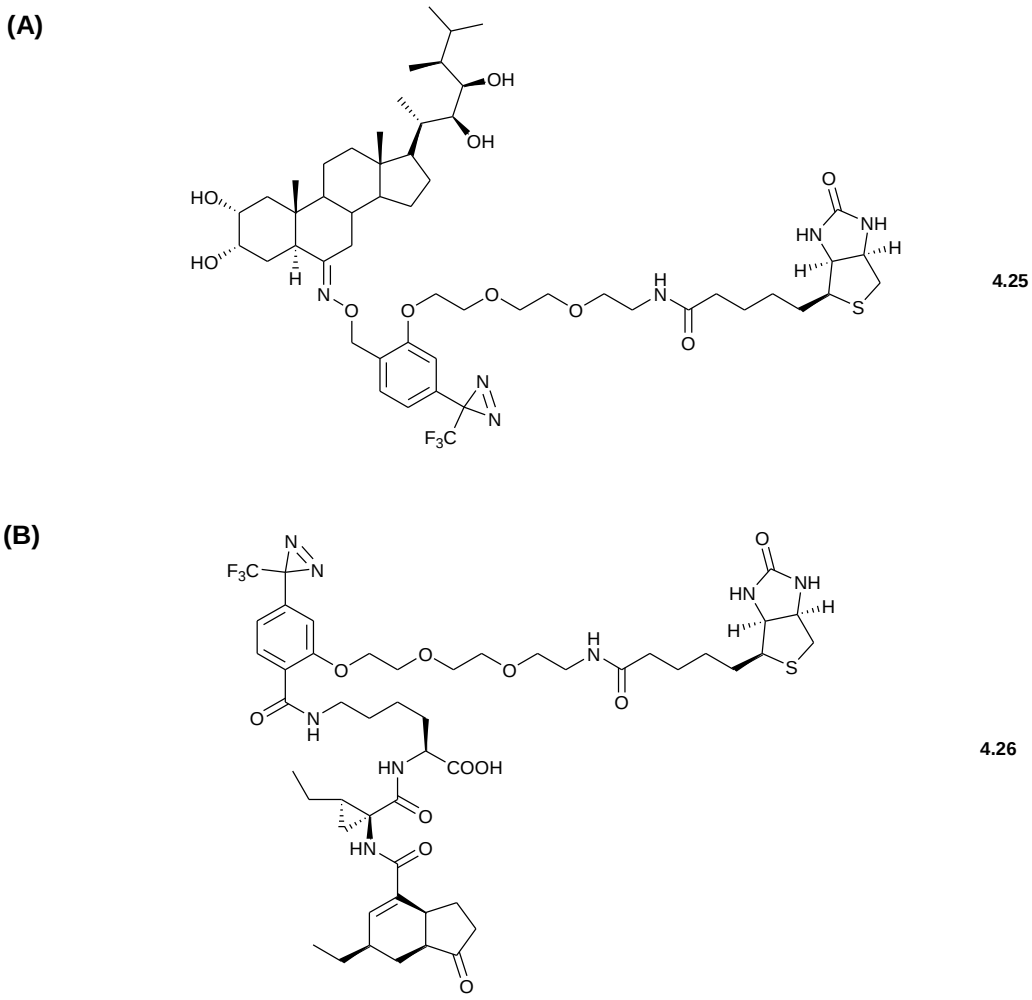


Fig. 4.6 Activity-based probes for the identification of plant hormone receptors. (**A**) Castasterone photoaffinity probe for the identification of a novel plant steroid hormone binding domain in BRI1 (according to T. Kinoshita *et al.*, *Nature* **2005**, *433*, 167-171); and (**B**) coronatine photoaffinity probe for the identification of COI1 as a co-receptor of jasmonate (according to Yan *et al.*, *Plant Cell* **2009**, *21*, 2220-2236).

Auxin is a class of small molecules involved predominantly in plant growth and development. The predominant auxin in higher plants is indole-3-acetic acid (IAA).[27] Although intense research has been carried out in the last decades, certain aspects of auxin biology and its mode of action are not fully disclosed yet. In respect of identifying novel auxin receptors in plants, biochemical tools were developed which involve photoaffinity labelling. This approach, pioneered by the group of Leonard in the early 1980s, utilises photoreactive analogues of auxins which bind irreversibly to auxin-sensitive sites upon irradiation with UV-light. For example, the Leonard lab was able to design the first photoreactive auxin analogues by incorporating a phenyl azide moiety (**4.27**, Fig. 4.7A). Moreover, they could demonstrate that plants, if grown in the dark,

exhibit almost the same phenotype when treated with the synthetic N_3-IAA compared to natural IAA.[28] Until today, these photoaffinity labels have been used, *e.g.* to map the auxin binding site of the auxin binding protein 1 (ABP1),[29] to localise IAA in cells for monitoring IAA transport in plants[30] and to identify novel putative auxin binding proteins,[31] among others. More recently, Hashimoto and colleagues reported the synthesis of a diazirinyl IAA with improved photochemical properties when compared to N_3-IAA (**4.28**, Fig. 4.7B). The application of diazirinyl IAA enables to perform photolysis at around 380 nm, whereas N_3-IAA requires more energy-rich UV light < 300 nm, which might cause photolytic damages of the biological sample.[32]

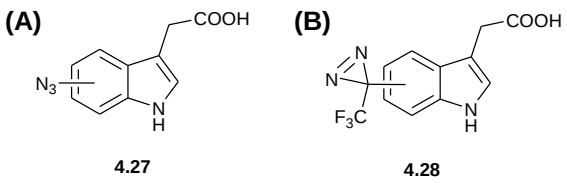


Fig. 4.7 Synthetic photoreactive analogues of IAA. (**A**) N_3-IAA, and (**B**) diazirinyl IAA.

Meanwhile, three independent and important players in auxin signalling have been identified using genetic and biochemical approaches. The best studied auxin co-receptor so far is the F-Box protein transport inhibitor resistant 1 (TIR1), which is a constituent of the SKP1–Cullin–F-Box (SCF)-type ubiquitin E3 ligase complex. SCF^{TIR1} promotes auxin dependent protein degradation of transcriptional repressors (Aux/IAA) by the 26S proteasome. Binding of IAA and analogues thereof to TIR1 is a prerequisite for the interaction with Aux/IAA proteins, which inhibit the activity of auxin response factor (ARFs) transcriptional activators at low auxin concentrations by forming heterodimers on the DNA. The recognition of Aux/IAA proteins by the SCF^{TIR1} complex in response to changes of the auxin level triggers the ubiquitination and the subsequent degradation of Aux/IAAs by the 26S proteasome, hence releasing ARFs and allowing activation of a transcriptional response (Fig. 4.8).[27,33]

Since both proteins, TIR1 and Aux/IAAs, are necessary for auxin perception, both constitute an auxin co-receptor system. Recent studies in *Arabidopsis* verified the existence of five homologues of TIR1 (AFB1-AFB5), which all bind auxin.[34] Moreover, 29 members of the Aux/IAA protein family have been identified.[35] From these findings, Calderón-Villalobos *et al.* suggested that a number of different TIR1/AFB–Aux/IAA co-receptor pairs with distinct auxin affinities may exist leading to a highly specific response at different auxin concentrations.[36] This concept might explain the huge variety of different auxin responses during plant growth and development. Some of these potential co-receptor pairs may exist only theoretically, though. The application of photoaffinity labelling techniques might be beneficial for the task of identifying the naturally occurring

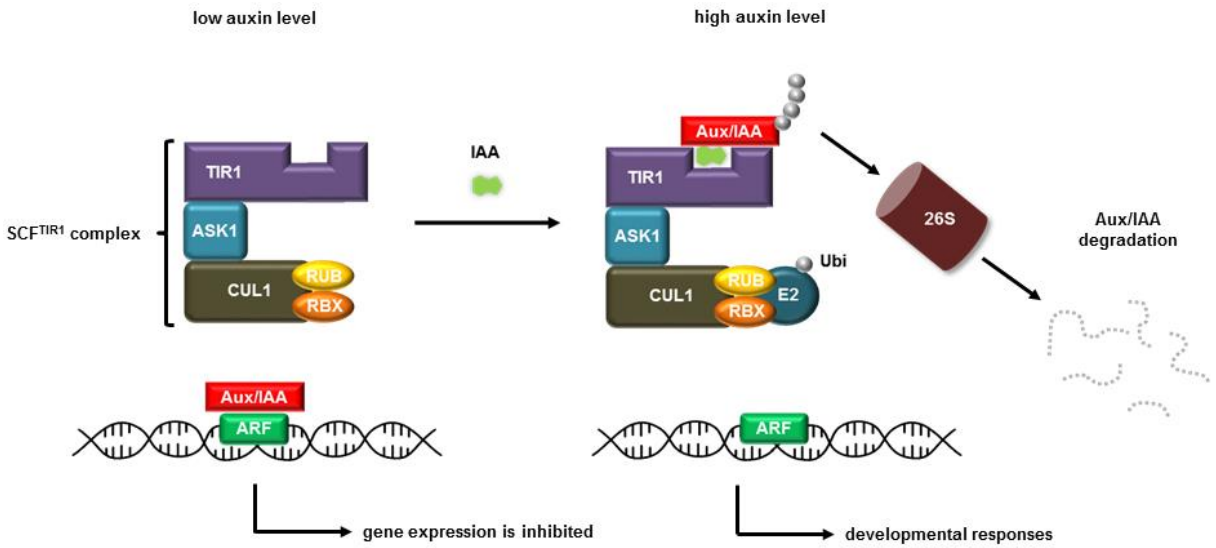
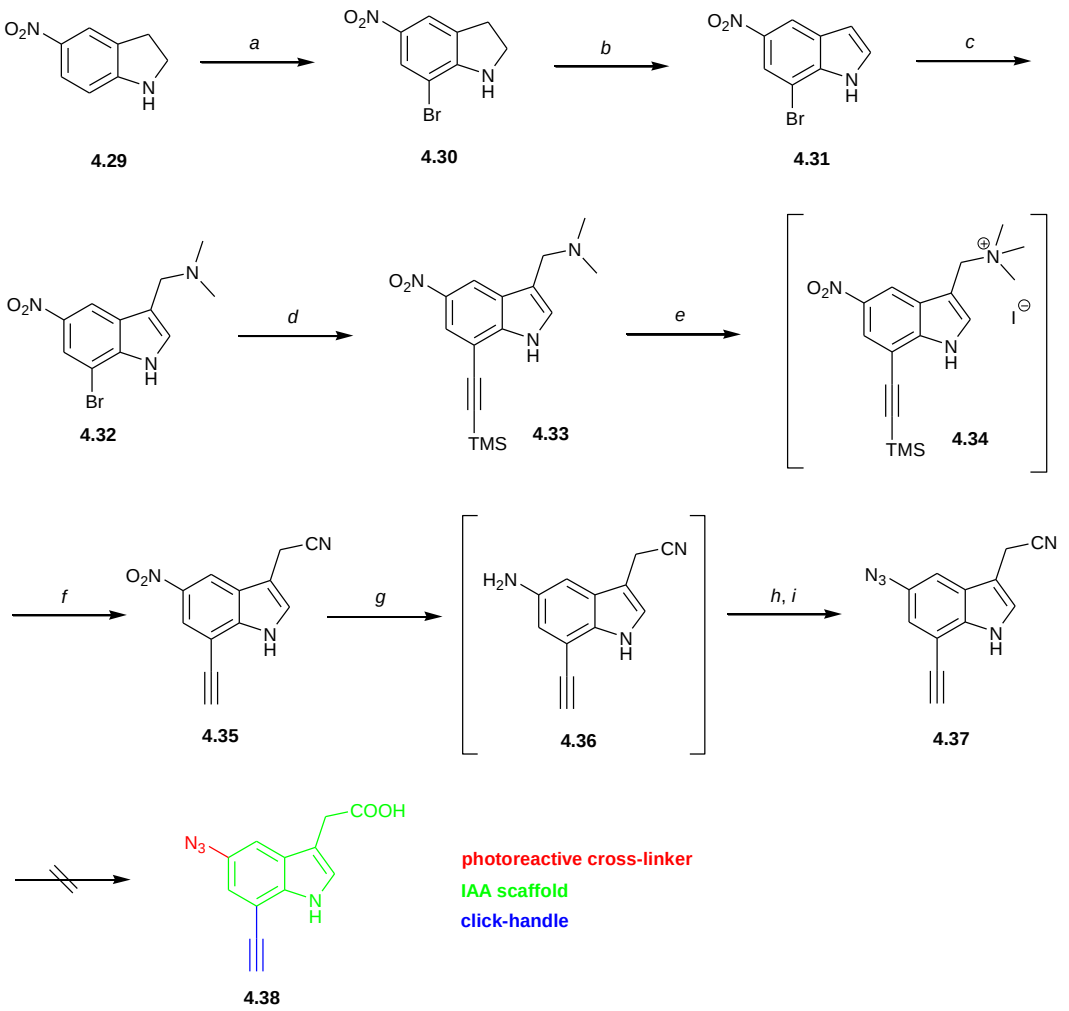


Fig. 4.8 Schematic representation of auxin signalling. At low auxin levels, ARF-dependent gene expression is inhibited due to the formation of ARF-Aux/IAA heterodimers. At high auxin levels, formation of a TIR1-Aux/IAA complex is promoted triggering ubiquitination and subsequent degradation of Aux/IAA resulting in an auxin mediated developmental response. (SCF^{TIR1} = SKP1–Cullin–F-Box-type ubiquitin E3 ligase multi-protein complex; ARF = auxin response factor; Aux/IAA = auxin/indole acetic acid proteins; TIR1 = transport inhibitor resistant 1; ASK1 = Arabidopsis SKP1-like protein; CUL1 = cullin 1; RUB = ubiquitin-like proteins; RBX = ring-box protein 1; E2 = ubiquitin conjugating enzyme; 26S = 26S proteasome, Ubi = ubiquitin)

co-receptor pairs *in planta*. Therefore, the synthesis and application of a photoreactive IAA analogue, which is additionally equipped with a biotin affinity-tag for the purification of the covalently labelled protein complexes after subsequent cell lysis, was intended and will be reported here. To enhance cellular uptake and distribution of the photoreactive IAA probe and more importantly to minimise steric repulsions to enable binding of both co-receptors, a terminal alkyne moiety was incorporated into N_3-IAA. Subsequent conjugation of both the covalently photo-cross-linked IAA protein complex and the biotin affinity-tag can be established *via* copper-catalysed azide-alkyne cycloaddition (CuAAC)[37] following the formation of the co-receptor pair and UV irradiation. After affinity enrichment and tryptic digestion of the labelled protein complex, the constituents can be identified by LC/MS/MS analysis.

A "clickable" photoreactive auxin (N_3-IAA alkyne **4.38**, Scheme 4.5) was synthesised starting from commercially available 5-nitroindoline (**4.29**) following an adopted protocol previously described by Leonard and colleagues.[28] First, 5-nitroindoline **4.29** was treated with bromine in glacial acetic acid which exclusively led to the formation of 7-bromo-5-nitroindoline **4.30** in excellent yield (90%).[38] After subsequent oxidation with 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (**4.31**, 96%), gramine **4.32** was obtained in a Mannich-type reaction using dimethylamine and formalin in almost quantitative yield,



Scheme 4.5 Synthesis of a clickable photo-auxin (Route A). *Reagents and Conditions*: (a) Br_2, AcOH, r.t., 1 h (90%); (b) DDQ, toluene, reflux, overnight (96%); (c) CH_2O, Me_2NH, AcOH, 70 °C, 4 h (96%); (d) TMS–C≡CH, $Pd(PPh_3)_4$, CuI, Et_3N, THF, reflux, 6 h (38%); (e) MeI, toluene/CH_2Cl_2 (2/1 v/v), r.t., overnight; (f) TMSCN, TBAF, THF, r.t., overnight (22%, 2 steps); (g) $Na_2S_2O_4$, EtOH/H_2O (1/1 v/v), 55 °C, 1 h; (h) $NaNO_2$, aq. AcOH, 0 °C, 15 min; (i) NaN_3, aq. AcOH, 0 °C, 1 h (19%, 3 steps).

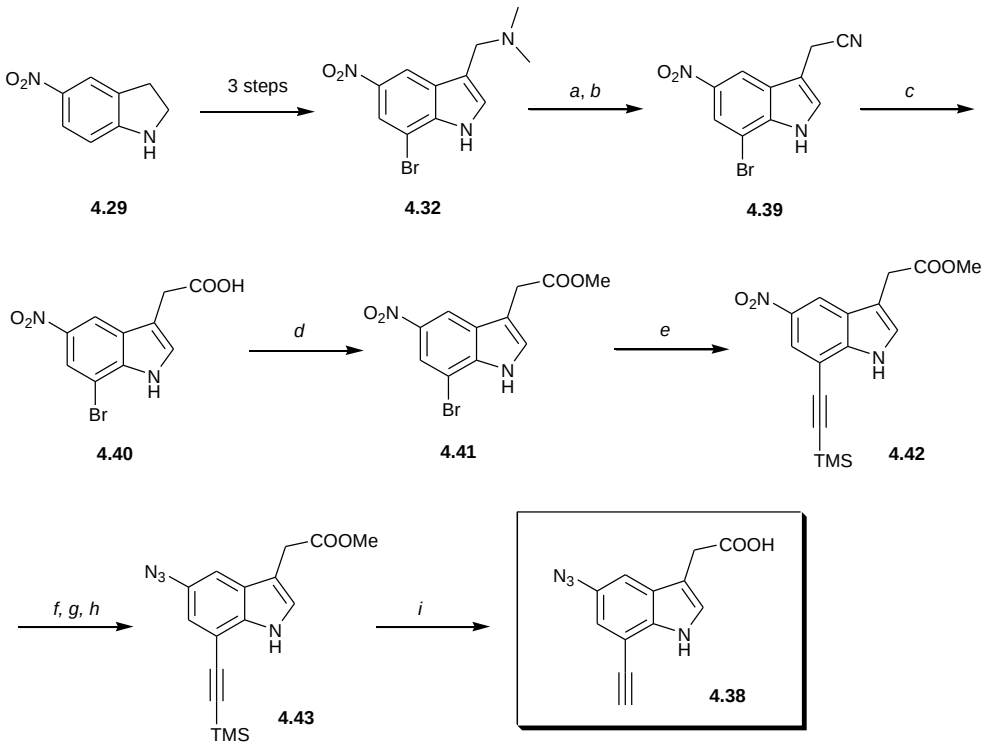
despite the presence of two strong electron-withdrawing substituents at the benzene ring. Afterwards the designated click-handle was introduced *via* Sonogoshira-coupling, a well-established palladium-catalysed cross-coupling reaction to form C–C-bonds between terminal alkynes and aryl or vinyl halides.[39] The reaction of gramine **4.32** with ethynyltrimetylsilane in the presence of catalytic amounts of copper(I)-iodide and tetrakis(triphenylphosphine)palladium(0) afforded trimetylsilyl-protected gramine **4.33**, however, in low yields (38%). Formation of the corresponding 7-ethynyl-5-nitro-indole-3-acetonitrile **4.35** can be achieved in poor 22% overall yield by methylation of **4.33** with a 2-fold excess methyl iodide (affording methiodide **4.34**) and subsequent nucleophilic displacement with trimethylsily cyanide in the presence of excess tetra-butylammonium fluoride (fluoride ion mediated elimination-addition reaction).[40] In

addition, the trimethylsilyl protective group was cleaved as well under these conditions. The low yield of the reaction might be attributed to an incomplete formation of the methiodide **4.34** as observed by ESI-MS analysis. NMR revealed the presence of an almost equimolar mixture of gramine **4.33** and methiodide **4.34**. Several optimisation attempts by changing the solvent system or the amount of methyl iodide failed leading always to a mixture containing the starting gramine **4.33** and the corresponding methiodide **4.34**. Despite this major drawback in the reaction sequence, 7-ethynyl-5-nitroindole-3-acetonitrile **4.35** was used in the subsequent steps towards the synthesis of N_3-IAA alkyne **4.38**. Next, the nitro-substituent had to be reduced to the amine **4.36**. Reduction of indole-3-acetonitrile **4.35** using catalytic hydrogenolysis is not applicable, however, due to the presence of a terminal alkyne undergoing reduction as well affording the corresponding alkane. Therefore, an alternative method was sought to selectively reduce the nitro-group. Sodium dithionite has been shown previously to effectively reduce 5-nitroindole to the corresponding 5-aminoindole in good yields.[41] After slight modifications of the published protocol, amine **4.36** was obtained. Due to the reported light and air sensitivity of **4.36**, the crude amine was used directly in the diazotization reaction with sodium nitrite in dilute acetic acid. Subsequent nucleophilic displacement with sodium azide afforded 5-azido-7-ethynyl-indole-3-acetonitrile **4.37** in 19% overall yield after purification by column chromatography. The low yield of the reaction most likely is attributed to partial decomposition of amine **4.36** during aqueous work-up. Finally, indole-3-acetonitrile **4.37** was subjected to acidic hydrolysis to furnish N_3-IAA alkyne **4.38.** However, upon addition of hydrochloric acid the reaction mixture turned brown immediately and no product could be isolated. No starting material could be recovered either. As a consequence of the decomposition of indole-3-acetonitrile **4.37** and the partially poor yields, a different reaction sequence leading to the formation of N_3-IAA alkyne **4.38** was designed (Scheme 4.6).

First, indole-3-acetonitrile **4.39** was synthesised *via* methylation with methyl iodide and subsequent elimination-addition reaction with trimethylsily cyanide in the presence of excess tetrabutylammonium fluoride as described earlier for the synthesis of **4.35**. The absence of the alkyne moiety leads to an impressive increase of the chemical yield for this transformation from 22% (for **4.35**) to 50% (for **4.39**). Subsequent acidic hydrolysis of indole-3-acetonitrile **4.39** in refluxing hydrochloric acid furnished indole-3-carboxylic acid **4.40** in 92% yield. The carboxylic acid **4.40** was protected with methyl iodide (**4.41**, 90%) and the corresponding methyl indole-3-acetate **4.41** was reacted with ethynyl-

trimetylsilane in a Sonogashira cross-coupling reaction to afford the trimetylsilyl-protected methyl indole-3-acetate **4.42** in excellent 78% yield when compared to 38% yield for the synthesis of trimetylsilyl-protected gramine **4.33**. The obtained trimetylsilyl-protected methyl indole-3-acetate **4.42** was reduced with sodium dithionite and consecutively treated with sodium nitrite and sodium azide to afford the corresponding methyl 5-azido-7-ethynyl-indole-3-acetate **4.43**, albeit in only poor 24% yield. In the last step of the reaction sequence, the methyl ester is cleaved under standard conditions (lithium hydroxide monohydrate, tetrahydrofuran/water), which simultaneously removed the trimetylsilyl protective group as well, affording N_3-IAA alkyne **4.38** in 78% yield. In summary, the first clickable and photoreactive auxin could be synthesised from readily available starting materials in nine steps and 5% overall yield. The reduction of the nitro-group seems to be the bottleneck of the whole reaction sequence, hence further optimisation attempts regarding this crucial step are needed.

Currently, investigations regarding the biological activity of synthetic N_3-IAA alkyne **4.38** in comparison to natural IAA are under way in the lab of Luz Irina A. Calderón-Villalobos (IPB Halle, Dept. of Molecular Signal Processing).



Scheme 4.6 Synthesis of a clickable photo-auxin (Route B). *Reagents and Conditions*: (a) MeI, toluene/CH_2Cl_2 (2/1 v/v), r.t., overnight; (b) TMSCN, TBAF, THF, r.t., overnight (50%, 2 steps); (c) conc. HCl, reflux, 6 h (92%); (d) MeI, $KHCO_3$, DMF, r.t., overnight (90%); (e) TMS−C≡CH, $Pd(PPh_3)_4$, CuI, Et_3N, THF, reflux, 6 h (78%); (f) $Na_2S_2O_4$, EtOH/H_2O (1/1 v/v), 55 °C, 1 h; (g) $NaNO_2$, AcOH (80%), 0 °C, 15 min; (h) NaN_3, AcOH (80%), 0 °C, 1 h (24%, 3 steps); (i) LiOH·H_2O, THF/H_2O (1/1 v/v), r.t., 3 h (78%).

4.4 Conclusions and future perspective

Specifically designed chemical probes equipped with a selectivity group, a reactive group and a reporter unit (ABPs = activity-based probes) have found widespread applications in recent years at the interface of chemical, biological as well as medicinal sciences. Herein, the synthesis of chemical probes to selectively enrich cation-dependent Pl-OMTs from *A. thaliana* was described. Starting from the well-known COMT inhibitor tolcapone, a suitable analogue (**4.7**) equipped with an additional functional handle (carbaldehyde moiety) for the attachment of a biotin affinity-tag was prepared in six steps and 28% overall yield. The combination of both parts of the chemical probe was performed *via* acylhydrazone formation between aldehyde **4.7** and PEG_4-biotin-acylhydrazine **4.13** to facilitate purification of the chemical probe **4.18**. After successful binding studies using purified AtTSM1, a tapetum specific cation-dependent Pl-OMT, crude *A. thaliana* flower bud extracts were treated with tolcapone-PEG_4-biotin-acylhydrazone **4.18**. Subsequent analysis by SDS-PAGE and western blotting using AtTSM1 specific antibodies revealed that this probe is not able to enrich proteins, though. The same results were obtained after the incorporation of an additional photo-cross-linker (tolcapone-PEG_4-biotin-phenylazide-acylhydrazone **4.24**). These results suggest that no suitable amino acid residue is in near spatial proximity to the reactive intermediate formed upon UV-irradiation, consequently making further modifications of the setup of the chemical probe necessary, *e.g.* distance between selectivity group and photo-cross-linker.

The synthesis of a photoreactive analogue of the phytohormone auxin was envisioned as well in the present thesis, which is additionally equipped with an alkyne handle to enable purification and subsequent identification of the cellular targets of the auxin analogue. The "clickable photo-auxin" N_3-IAA alkyne **4.38** was obtained in nine synthetic steps and 5% overall yield starting from commercially available 5-nitroindoline **4.29**. This probe should be a valuable tool to study the interaction of TIR1 and its paralogues (auxin signalling F-box proteins AFB1-5) with transcriptional repressors of the Aux/IAA family *in vivo*, for example, which will most certainly provide a deeper insight into auxin perception *in planta*. Currently, studies concerning the biological activity of the obtained N_3-IAA alkyne **4.38** in comparison to natural IAA are performed in yeast by the group of Luz Irina A. Calderón-Villalobos (IPB Halle, Dept. of Molecular Signal Processing).

4.5 Experimental section

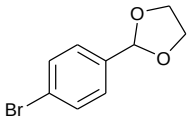
General experimental information

Unless otherwise stated all chemicals and solvents were obtained from commercial sources and were used without further purification. All ^{1}H and ^{13}C NMR spectra were recorded in CHLOROFORM-*d* (unless otherwise noted) on either a 300 MHz Varian MERCURY-VX 300 apparatus (300 MHz for ^{1}H NMR and 75 MHz for ^{13}C NMR, respectively) or on a 400 MHz Varian MERCURY-VX 400 apparatus (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR, respectively). Chemical shifts are reported in δ values (ppm) with tetramethylsilane (TMS) as internal standard. Melting points were determined with a Leica DM LS2 hot stage microscope (without correction). ESI-MS spectra were obtained from an API-150EX spectrometer. HRMS spectra were recorded on an FT-ICR Bruker Apex III 70e mass spectrometer. Purification by column chromatography was performed on silica gel 60 (230–400 mesh, 0.040–0.063 mm), Merck, Germany. TLC identification of products and reactants was performed on silica gel coated aluminum foil (silica gel 60 F254 with fluorescence indicator), Merck, Germany.

K_i-value determination

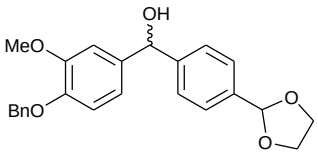
K_i-values were determined using four different substrate concentrations (luteolin: 5, 10, 20, 30 µM) at five different inhibitor concentrations (**4.18** or **4.19**: 10, 20, 30, 40, 100 µM) and without inhibitor. To evaluate the inhibitory potential of the tolcapone-derived chemical probes **4.18** and **4.19**, recombinant AtTSM1 (*A. thaliana* tapetum specific methyltransferase 1, At1g67990) and AtOMT1 (*A. thaliana O*-methyltransferase 1, At5g54160) were used. The reaction mixture (total volume 50 µL) contained: 10 µg recombinant protein, 200 µM *S*-adenosyl-*L*-methionine, 200 µM magnesium chloride (only in case of AtTSM1), 5–30 µM luteolin, 10–100 µM inhibitor, DMSO (5% v/v) and methanol (2% v/v) in phosphate-buffer (10 mM, pH = 7.5, 2% glycerin). The reaction was initiated by the addition of the substrate and incubated at 37 °C for 15 minutes, whereupon 20 µL trichloroacetic acid (10% v/v) dissolved in aqueous acetonitrile (50% v/v) was added to terminate the reaction. Substrate turnover was determined *via* HPLC (column: EC 100/4 NUCLEOSHELL RP 18; 2.7 µm) using an acetonitrile/phosphoric acid (5% v/v in water) gradient and a diode array detector (detection wavelength: 320–350 nm). Finally, Dixon plot analysis was performed to determine the K_i-values.

2-(4-Bromophenyl)-1,3-dioxolane (4.2)



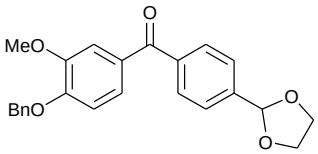
4-Bromobenzaldehyde **4.1** (31.6 g, 171 mmol), ethylene glycol (12.6 mL, 226 mmol) and *p*-toluenesulphonic acid monohydrate (337 mg, 1.77 mmol) were dissolved in toluene (300 mL) and heated under reflux overnight using a Dean-Stark trap. After cooling to room temperature, the reaction mixture was washed consecutively with saturated aqueous sodium bicarbonate solution (2x 200 mL), distilled water (2x 200 mL) and brine (2x 200 mL). Afterwards the organic layer was dried (sodium sulphate) and all volatiles were removed under reduced pressure. The residue was purified by vacuum distillation (3.2 mbar, ~ 110 °C) to yield **4.2** as a colourless liquid (36.4 g, 93%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 3.97–4.15 (m, 4H), 5.76 (s, 1H), 7.31–7.40 (m, 2H), 7.46–7.56 (m, 2H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 65.2, 103.0, 123.2, 128.1, 131.4, 136.9.

[4-(Benzyloxy)-3-methoxyphenyl][4-(1,3-dioxolan-2-yl)phenyl]methanol (4.3)



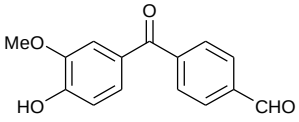
Under inert gas atmosphere was added dropwise *t*-butyl lithium (1.7 M in pentane, 20 mL, 34 mmol) to a cooled, stirred solution (–70 °C) containing phenyl bromide **4.2** (7.77 g, 33.9 mmol) in dry tetrahydrofuran (100 mL). The resulting yellow solution was stirred for further 2 hours, whereupon a solution of 4-benzyloxy-3-methoxy-benzaldehyde (6.21 g, 25.6 mmol) in dry tetrahydrofuran (20 mL) was added. Stirring was continued at −70 °C for 1 hour and for further 2 hours at 0 °C. Afterwards the reaction was quenched by the addition of ice and brine (100 mL). Following that, the organic layer was separated and the aqueous phase was extracted with diethyl ether. The combined organic layers were dried (sodium sulphate) and the organic solvent was evaporated. Finally, the remaining residue was purified by column chromatography to afford **4.3** as pale yellow oil (7.70 g, 77%). R_F = 0.46 (hexane/ethyl acetate 1/1); ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 2.35 (d, J = 2.6 Hz, 1H), 3.83 (s, 3H), 3.97–4.15 (m, 4H), 5.12 (s, 2H), 5.75 (d, J = 1.8 Hz, 1H), 5.78 (s, 1H), 6.72–6.83 (m, 2H), 6.91 (d, J = 1.8 Hz, 1H), 7.25–7.46 (m, 9H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 55.9, 65.3, 70.9, 75.7, 103.5, 110.2, 113.6, 118.9, 126.4, 126.5, 127.2, 127.8, 128.5, 136.8, 137.0, 137.1, 144.8, 147.6, 149.6; ESI-MS m/z: 393.3 $[M+H]^+$, 415.1 $[M+Na]^+$, 807.4 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{24}H_{24}O_5Na$ 415.1516, found 415.1515.

[4-(Benzyloxy)-3-methoxyphenyl][4-(1,3-dioxolan-2-yl)phenyl]methanone (4.4)



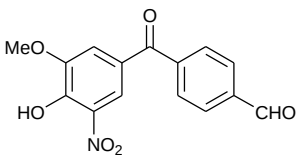
To a solution containing alcohol **4.3** (7.70 g, 19.6 mmol) in dry toluene (80 mL) was consecutively added sodium *t*-butoxide (2.82 g, 29.3 mmol) and cyclohexanone (12 mL, 116 mmol). The resulting mixture was heated under reflux for 1 hour. After cooling to 50 °C, distilled water (80 mL) was carefully added. Subsequent to the separation of the organic layer, the aqueous layer was extracted with ethyl acetate and the combined organic layers were washed with brine and dried afterwards (sodium sulphate). Finally, all volatiles were removed *in vacuo* and the remaining residue was purified by recrystallisation from aqueous ethanol to yield benzophenone **4.4** as a white solid (6.29 g, 82%). M.p. 141–142 °C; ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 3.94 (s, 3H), 4.02–4.20 (m, 4H), 5.24 (s, 2H), 5.88 (s, 1H), 6.89 (d, J = 8.5 Hz, 1H), 7.25–7.47 (m, 6H), 7.50 (d, J = 1.8 Hz, 1H), 7.58 (d, J = 8.2 Hz, 2H), 7.73–7.80 (m, 2H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 56.1, 65.4, 70.8, 76.6, 103.1, 111.8, 112.4, 125.3, 126.2, 127.2, 128.1, 128.7, 129.8, 130.3, 136.2, 138.9, 141.5, 149.4, 152.2, 195.1; ESI-MS m/z: 391.4 $[M+H]^+$, 413.4 $[M+Na]^+$, 803.3 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{24}H_{23}O_5$ 391.1540, found 391.1540.

4-[(4-Hydroxy-3-methoxyphenyl)carbonyl]benzaldehyde (4.5)



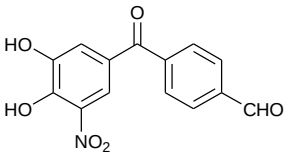
To an ice-cold solution containing benzophenone **4.4** (3.78 g, 9.68 mmol) in dry dichloromethane (50 mL) was added dropwise hydrobromic acid solution (33% in acetic acid, 17 mL, 97.1 mmol). The solution was allowed to warm to room temperature and stirring was continued for 2 hours. Afterwards the reaction mixture was poured on ice and the organic layer was separated. Following the extraction of the aqueous phase with dichloromethane (3x 50 mL), the combined organic extracts were washed with brine, dried (sodium sulphate) and the solvent was evaporated. Recrystallisation of the residue from dichloromethane/petroleum ether afforded **4.5** as an ochre-coloured solid (1.86 g, 75%). M.p. 171–172 °C; ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 3.98 (s, 3H), 6.18 (s, 1H), 6.96 (d, J = 8.5 Hz, 1H), 7.30 (dd, J = 8.2, 1.8 Hz, 1H), 7.53 (d, J = 2.1 Hz, 1H), 7.85–7.90 (m, 2H), 7.97–8.03 (m, 2H), 10.13 (s, 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 56.2, 111.5, 113.7, 126.5, 129.1, 129.4, 129.9, 138.1, 143.5, 146.8, 150.7, 191.7, 194.6; ESI-MS m/z: 255.0 $[M-H]^-$; HRMS (ESI) calcd for $C_{15}H_{11}O_4$ 255.0663, found 255.0657.

4-[(4-Hydroxy-3-methoxy-5-nitrophenyl)carbonyl]benzaldehyde (4.6)



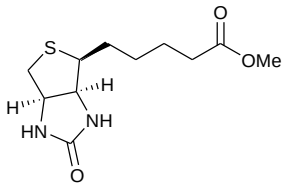
Aldehyde **4.5** (1.28 g, 5.0 mmol) was suspended in glacial acetic acid (12 mL) and concentrated nitric acid (65%, 0.38 mL, 5.49 mmol) was added. Stirring was continued for 30 minutes, whereupon the reaction mixture was poured on ice. The solid was filtered and dried under reduced pressure to afford **4.6** as a yellow solid (1.28 g, 85%). M.p. 189–190 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 3.97 (s, 3H), 7.64 (d, J = 1.8 Hz, 1H), 7.77 (d, J = 1.8 Hz, 1H), 7.94 (d, J = 8.2 Hz, 2H), 8.09 (d, J = 7.9 Hz, 2H), 10.15 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 56.8, 115.0, 120.0, 126.0, 129.6, 129.9, 136.5, 138.3, 141.6, 147.0, 149.7, 192.6, 193.0; ESI-MS m/z: 300.3 $[M-H]^-$; HRMS (ESI) calcd for $C_{15}H_{10}NO_6$ 300.0514, found 300.0509.

4-[(3,4-Dihydroxy-5-nitrophenyl)carbonyl]benzaldehyde (4.7)



To a suspension containing **4.6** (0.50 g, 1.66 mmol) in ethyl acetate (20 mL) was consecutively added aluminium chloride (266 mg, 1.99 mmol) and pyridine (0.54 mL, 6.69 mmol) under inert-gas atmosphere. The resulting orange suspension was heated under reflux for 2 hours. After cooling to 50 °C, an ice/conc. hydrochloric acid mixture (25 mL) was added and stirring was continued at 50 °C for 1 hour. Afterwards the reaction mixture was placed on an ice bath for 1 hour. The resulting precipitate was collected by filtration and dried in high vacuum to afford **4.7** as an ochre-coloured solid (351 mg, 74%). M.p. 178–179 °C; ^{1}H NMR (300 MHz, DMSO-d_6) δ: 7.52 (d, J = 2.1 Hz, 1H), 7.68 (d, J = 2.1 Hz, 1H), 7.91 (d, J = 8.2 Hz, 2H), 8.09 (d, J = 7.9 Hz, 2H), 10.14 (s, 1H); ^{13}C NMR (75 MHz, DMSO-d_6) δ: 118.4, 118.8, 126.1, 129.5, 129.8, 136.9, 138.2, 141.9, 146.4, 147.9, 192.7, 193.0; ESI-MS m/z: 286.2 $[M-H]^-$; HRMS (ESI) calcd for $C_{14}H_8NO_6$ 286.0357, found 286.0352.

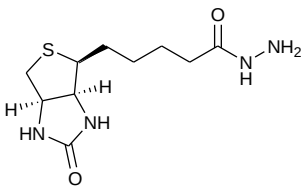
Biotin methyl ester (4.9)



To an ice-cold suspension containing biotin **4.8** (2.50 g, 10.2 mmol) in methanol (25 mL) was added dropwise *via* syringe thionyl chloride (2.4 mL, 33.1 mmol). Stirring was continued on an ice/water bath for 2.5 hours. After completion of the reaction (checked by ESI-MS analysis), all volatiles were removed under reduced pressure to afford methyl ester **4.9** as an off-white solid (2.88 g, quant.). M.p. 155–156 °C; ^{1}H NMR (300 MHz, METHANOL-d_4) δ: 1.37–1.85 (m, 6H), 2.35 (t, J = 7.0 Hz, 2H), 2.77 (d, J = 12.6 Hz, 1H), 3.00 (d, J = 11.7 Hz, 1H), 3.26–3.34 (m, 1H), 3.61–3.72 (m, 3H), 4.45 (br. s., 1H), 4.64

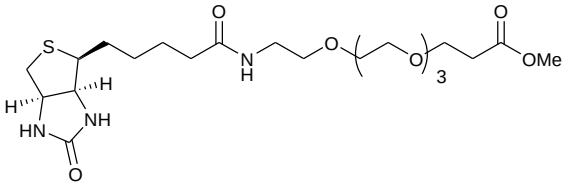
(br. s., 1H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ: 25.9, 29.4, 29.7, 34.5, 40.8, 52.1, 56.9, 62.8, 64.4, 175.8; ESI-MS m/z: 259.4 $[M+H]^+$, 281.2 $[M+Na]^+$, 517.5 $[2M+H]^+$; HRMS (ESI) calcd for $C_{11}H_{18}N_2O_3SNa$ 281.0930, found 281.0931.

Biotin hydrazide (4.10)



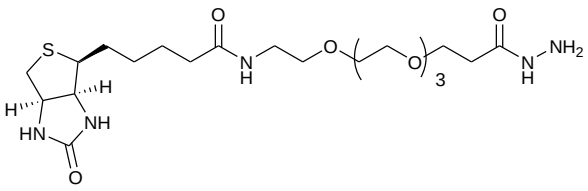
To a solution containing biotin methyl ester **4.9** (1.44 g, 5.57 mmol) in methanol (30 mL) was added hydrazine (1.5 mL, 47.8 mmol) while stirring. The resulting solution was stirred at room temperature overnight. Afterwards the solvent was evaporated and the residue was dissolved in distilled water (100 mL). Finally, the aqueous phase was washed with dichloromethane (3x 50 mL). Evaporation of the aqueous phase afforded the corresponding acylhydrazine **4.10** as a white solid (1.04 g, 74%). M.p. 198–199 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.22–1.38 (m, 2H), 1.39–1.67 (m, 4H), 2.02 (br. s., 2H), 2.58 (d, J = 12.5 Hz, 1H), 2.82 (dd, J = 12.5, 5.1 Hz, 1H), 3.06–3.13 (m, 1H), 4.10–4.16 (m, 1H), 4.30 (m, 1H), 6.38 (s, 1H), 6.44 (s, 1H), 8.97 (br. s., 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 25.1, 28.0, 28.2, 33.1, 39.9, 55.4, 59.2, 61.0, 162.7, 171.6; ESI-MS m/z: 281.3 $[M+Na]^+$; HRMS (ESI) calcd for $C_{10}H_{19}N_4O_2S$ 259.1223, found 259.1223.

PEG$_4$-biotin-methyl ester (4.12)



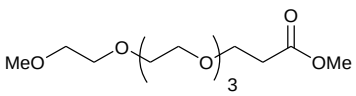
To an ice-cold solution containing PEG$_4$-biotin-COOH **4.11** (301 mg, 0.61 mmol) in methanol (3 mL) was added dropwise *via* syringe thionyl chloride (0.15 mL, 2.07 mmol). Stirring was continued on an ice/water bath for 1 hour. After completion of the reaction (checked by ESI-MS analysis), all volatiles were removed under reduced pressure to afford methyl ester **4.12** as a grey-coloured semi-solid (316 mg, quant.). ^{1}H NMR (300 MHz, CHLOROFORM-d) δ: 1.40–1.57 (m, 2H), 1.58–1.89 (m, 4H), 2.32–2.44 (m, 2H), 2.60 (t, J = 6.3 Hz, 2H), 2.82–3.00 (m, 2H), 3.14–3.27 (m, 1H), 3.42–3.51 (m, 2H), 3.56–3.68 (m, 14H), 3.69 (s, 3H), 3.76 (t, J = 6.4 Hz, 2H), 4.39–4.52 (m, 1H), 4.59–4.72 (m, 1H), 7.62 (br. s., 2H); ^{13}C NMR (75 MHz, CHLOROFORM-d) δ: 25.6, 27.8, 34.8, 35.0, 39.7, 40.2, 51.7, 55.4, 61.2, 62.5, 66.5, 69.3, 70.1, 70.3, 70.4, 70.5, 164.1, 172.0, 174.6; ESI-MS m/z: 506.2 $[M+H]^+$, 528.3 $[M+Na]^+$; HRMS (ESI) calcd for $C_{22}H_{39}N_3O_8SNa$ 528.2350, found 528.2346.

PEG$_4$-biotin-hydrazide (4.13)



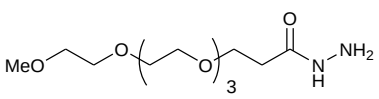
To a solution containing PEG$_4$-biotin-methyl ester **4.12** (291 mg, 0.58 mmol) in methanol (3 mL) was added hydrazine (0.16 mL, 5.09 mmol) while stirring. The resulting solution was stirred at room temperature overnight. Afterwards the solvent was evaporated and the residue was dissolved in distilled water (25 mL). Finally, the aqueous phase was washed with dichloromethane (3x 15 mL) and evaporation of the aqueous phase afforded the corresponding acylhydrazine **4.13** as a colourless semi-solid (296 mg, quant.). ^{1}H NMR (300 MHz, METHANOL-d_4) δ: 1.37–1.51 (m, 2H), 1.52–1.80 (m, 4H), 2.22 (t, J = 7.3 Hz, 2H), 2.41 (t, J = 6.2 Hz, 2H), 2.67–2.74 (m, 1H), 2.93 (dd, J = 12.9, 5.0 Hz, 1H), 3.16–3.25 (m, 1H), 3.28–3.33 (m, 3H), 3.33–3.39 (m, 4H), 3.54 (t, J = 5.4 Hz, 2 H), 3.59–3.67 (m, 12H), 3.72 (t, J = 6.2 Hz, 2H), 4.30 (dd, J = 7.8, 4.5 Hz, 1H), 4.49 (dd, J = 7.5, 4.5 Hz, 1H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ: 26.9, 29.5, 29.8, 35.8, 36.7, 40.4, 41.1, 57.0, 61.6, 63.4, 68.0, 70.6, 71.3, 71.4, 71.6, 166.0, 173.1, 176.2; ESI-MS m/z: 506.2 $[M+H]^+$, 528.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{21}H_{39}N_5O_7SNa$ 528.2462, found 528.2457.

Methyl 2,5,8,11,14-pentaoxaheptadecan-17-oate (4.15)



To a solution containing tetraethylene glycol monomethyl ether **4.14** (2.0 g, 9.41 mmol) in dry tetrahydrofuran (20 mL) were consecutively added sodium hydride (60%, 35 mg, 0.88 mmol) and methyl acrylate (1.71 mL, 18.8 mmol) under inert-gas atmosphere. Stirring was continued at room temperature overnight. Afterwards all volatiles were removed under reduced pressure and the residue was purified by column chromatography to afford PEG$_4$-methyl ester **4.15** as colourless oil (0.90 g, 32%). R_F = 0.22 (ethyl acetate); ^{1}H NMR (300 MHz, CHLOROFORM-d) δ: 2.61 (td, J = 6.5, 2.4 Hz, 2H), 3.38 (s, 3H), 3.52–3.57 (m, 2H), 3.60–3.79 (m, 19H); ^{13}C NMR (75 MHz, CHLOROFORM-d) δ: 34.8, 51.6, 59.0, 63.6, 66.5, 67.8, 69.0, 70.3, 70.4, 70.5, 70.6, 71.9, 171.9; ESI-MS m/z: 295.3 $[M+H]^+$, 317.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{13}H_{26}O_7Na$ 317.1570, found 317.1568.

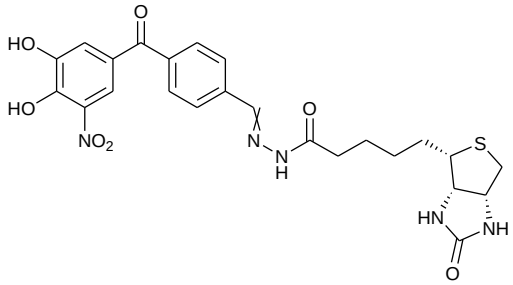
2,5,8,11,14-Pentaoxaheptadecane-17-hydrazide (4.16)



To a solution containing PEG$_4$-methyl ester **4.15** (0.85 g, 2.89 mmol) in methanol (15 mL) was added hydrazine (0.80 mL, 25.5 mmol) while stirring. The resulting solution was stirred at room

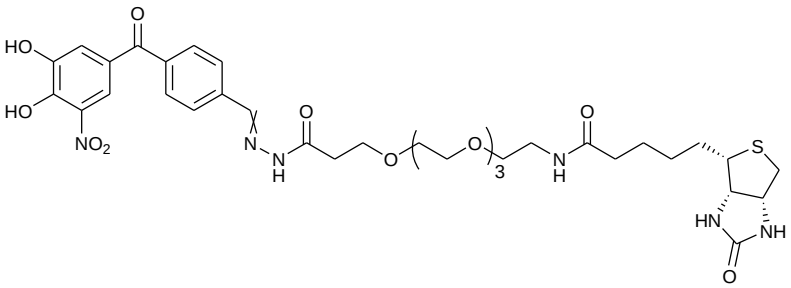
temperature overnight. Afterwards the solvent was evaporated and the residue was dissolved in distilled water (100 mL). Finally, the aqueous phase was washed with ethyl acetate (3x 50 mL) and evaporation of the aqueous phase afforded the corresponding acylhydrazine **4.16** as yellow oil (656 mg, 77%). ^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ: 2.47 (dt, *J* = 7.8, 5.8 Hz, 2H), 3.38 (s, 3H), 3.52–3.58 (m, 2H), 3.59–3.69 (m, 14H), 3.69–3.76 (m, 2H), 3.87 (br. s., 2H), 7.97 (br. s., 1H); ^{13}C NMR (75 MHz, CHLOROFORM-*d*) δ: 35.2, 58.9, 61.6, 66.7, 68.1, 70.10, 70.14, 70.4, 70.5, 71.8, 72.5, 171.8; ESI-MS m/z: 293.2 $[M-H]^-$, 295.2 $[M+H]^+$, 317.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{12}H_{26}N_2O_6Na$ 317.1683, found 317.1680.

Tolcapone-biotin-acylhydrazone (4.17)



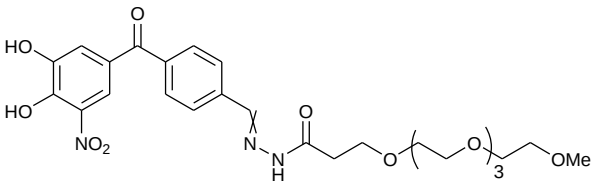
Aldehyde **4.7** (58.6 mg, 0.20 mmol) was dissolved in glacial acetic acid (3 mL) and acylhydrazine **4.10** (63.5 mg, 0.25 mmol) in glacial acetic acid (2 mL) was added dropwise. Stirring was continued at room temperature until completion of the reaction was detected (checked by ESI-MS analysis), whereupon the reaction mixture was filtered. Afterwards the filter cake was washed with several portions glacial acetic acid and distilled water. Finally, the remaining solid was dried under reduced pressure to afford the corresponding acylhydrazone **4.17** as a yellow solid (cis/trans isomeric mixture, 45.0 mg, 42%). M.p. 146–148 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 1.30–1.73 (m, 6H), 2.24 (t, *J* = 7.4 Hz, 1H), 2.58 (d, *J* = 12.1 Hz, 1H), 2.66 (t, *J* = 7.4 Hz, 1H), 2.82 (dd, *J* = 12.3, 5.7 Hz, 1H), 3.08–3.18 (m, 1H), 4.15 (d, *J* = 3.9 Hz, 1H), 4.25–4.36 (m, 1H), 6.37 (br. s., 1H), 6.46 (br. s., 1H), 7.49 (d, *J* = 2.0 Hz, $1H_{major}$), 7.52 (d, *J* = 2.3 Hz, $1H_{minor}$), 7.69 (d, *J* = 2.0 Hz, $1H_{major}$), 7.72 (d, *J* = 2.3 Hz, $1H_{minor}$), 7.75–8.11 (m, $4H_{major}$ + $4H_{minor}$), 8.25 (s, $1H_{minor}$), 8.86 (s, $1H_{major}$), 11.42 (s, $1H_{major}$), 11.54 (s, $1H_{minor}$); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 24.2, 28.1, 28.2, 28.3, 31.7, 55.4, 59.2, 61.0, 118.0, 118.2, 118.8, 126.3, 126.6, 126.9, 127.3, 128.5, 129.8, 129.9, 136.7, 137.0, 137.3, 137.5, 138.0, 138.1, 139.2, 141.2, 144.4, 146.0, 146.2, 147.8, 152.6, 161.1, 162.7, 174.5, 192.5, 192.6; ESI-MS m/z: 526.5$[M-H]^-$; HRMS (ESI) calcd for $C_{24}H_{24}N_5O_7S$ 526.1402, found 526.1394.

Tolcapone-PEG$_4$-biotin-acylhydrazone (4.18)



Aldehyde **4.7** (84 mg, 0.29 mmol) was dissolved in glacial acetic acid (10 mL) and acylhydrazine **4.13** (120 mg, 0.24 mmol) in glacial acetic acid (2 mL) was added dropwise. Stirring was continued at room temperature until completion of the reaction was detected (checked by ESI-MS analysis), whereupon the reaction mixture was poured on ice, filtered and the filtrate was extracted with ethyl acetate (3x 20 mL). The combined organic extracts were washed with brine, dried (sodium sulphate) and evaporated to dryness to afford acylhydrazone **4.18** as an orange semi-solid (cis/trans isomeric mixture, 45.0 mg, 25%). ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 1.36–1.47 (m, 2H), 1.49–1.77 (m, 4H), 2.19 (s, 2H), 2.60 (s, 1H), 2.70 (s, 1H), 2.86–2.95 (m, 1H), 3.05–3.11 (m, 1H), 3.13–3.22 (m, 1H), 3.50 (t, J = 5.7 Hz, 2H), 3.54–3.69 (m, 12H), 3.86 (d, J = 13.7 Hz, 2H), 4.24–4.33 (m, 1H), 4.43–4.51 (m, 1H), 7.55 (d, J = 2.0 Hz, 1H$_{minor}$ + 1H$_{major}$), 7.78–7.88 (m, 4H$_{minor}$ + 2H$_{major}$), 7.93 (d, J =2.0 Hz, 1H$_{major}$ + 1$_{minor}$), 7.96 (d, J = 8.6 Hz, 2H$_{major}$), 8.04 (br. s., 1H$_{minor}$), 8.21 (s, 1H$_{major}$); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 26.8, 29.5, 29.8, 30.8, 34.2, 36.5, 36.7, 40.3, 41.0, 57.0, 61.6, 63.4, 67.7, 68.0, 70.6, 71.2, 71.4, 71.5, 119.9, 120.0, 120.8, 128.0, 128.5, 128.7, 131.1, 131.2, 136.1, 136.2, 139.4, 139.5, 139.7, 139.9, 144.1, 147.9, 149.8,149.9, 166.1, 170.9, 175.7, 176.1, 194.9; ESI-MS m/z: 773.8 [M–H]$^-$, 797.3 [M+Na]$^+$; HRMS calcd for $C_{35}H_{46}N_6O_{12}SNa$ 797.2787, found 797.2790.

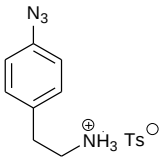
Tolcapone-PEG$_4$-acylhydrazone (4.19)



Aldehyde **4.7** (35 mg, 0.12 mmol) and acylhydrazine **4.16** (60 mg, 0.20 mmol) were dissolved in tetrahydrofuran/water (1/1 v/v, 5 mL) and the reaction mixture was stirred at room temperature overnight. Afterwards dichloromethane (20 mL) was added to the reaction mixture and the organic phase was washed consecutively with distilled water, aqueous 1M hydrochloric acid and brine. Finally, the organic phase was dried (sodium sulphate) and evaporated to dryness to afford the corresponding acylhydrazone **4.19** as yellow oil (cis/trans isomeric mixture, 49 mg, 70%). ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 2.55–2.62 (m, 2H), 3.32–3.33 (m, 2H), 3.47–3.53 (m, 2H),

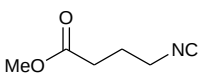
3.55–3.67 (m, 12H), 3.70–3.81 (m, 2H), 3.81–3.90 (m, 2H), 7.54–7.58 (m, $1H_{major}$ + $1H_{minor}$), 7.82 (s, $2H_{major}$ + $2H_{minor}$), 7.84–7.88 (m, $2H_{minor}$), 7.93 (d, J = 2.2 Hz, $1H_{major}$ + $1H_{minor}$), 7.94–7.99 (m, $2H_{major}$), 8.03 (br. s., $1H_{minor}$), 8.18–8.22 (m, $1H_{major}$); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 30.9, 34.0, 34.2, 36.3, 36.5, 59.1, 67.7, 68.0, 69.1, 69.4, 71.3, 71.4, 71.5, 72.9, 119.6, 121.3, 128.0, 128.9, 131.1, 131.2, 136.2, 139.4, 139.5, 139.7, 144.1, 147.8, 147.9, 148.7, 149.6, 170.9, 175.6, 194.8; ESI-MS m/z: 562.3 $[M-H]^-$, 564.5 $[M+H]^+$, 586.2 $[M+Na]^+$; HRMS (ESI) calcd for $C_{26}H_{32}N_3O_{11}$ 562.2042, found 532.2042.

2-(4-Azidophenyl)ethanaminium(4-methylphenyl)methansulphonate (4.20)



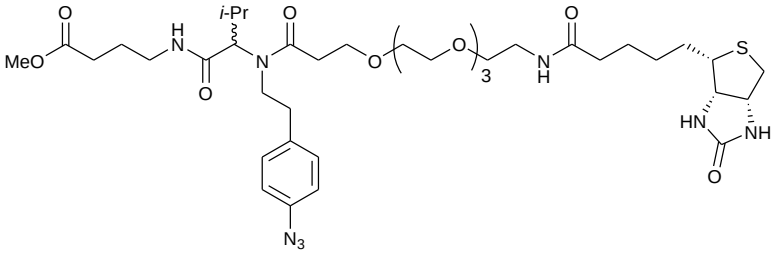
To an ice-cold solution containing 2-(4-aminophenyl)ethylamine (3.97 g, 29.1 mmol) and *p*-toluenesulphonic acid monohydrate (21.45 g, 113 mmol) in distilled water (325 mL) was added an aqueous sodium nitrite solution (2.36 g, 34.2 mmol in 16 mL distilled water). After completion of the addition, the reaction mixture was stirred for further 20 minutes followed by the addition of an aqueous sodium azide solution (5.75 g, 88.5 mmol in 22 mL distilled water). The formed precipitate was collected by filtration. The filtrate was placed on ice for 1 hour and the formed additional precipitate was filtered off again. The combined solids were dried under reduced pressure to afford phenyl azide **4.20** as an off-white solid (9.53 g, 98%). M.p. 175–176 °C; ^1H NMR (300 MHz, METHANOL-d_4) δ: 2.36 (*s*, 3H), 2.92 (t, J = 7.1 Hz, 2H), 3.13 (t, J = 7.5 Hz, 2H), 7.01 (d, J = 8.6 Hz, 2H), 7.22 (d, J = 8.6 Hz, 2H), 7.26 (d, J = 8.6 Hz, 2H), 7.69 (d, J = 8.3 Hz, 2H); ^{13}C NMR (75 MHz, METHANOL-d_4) δ: 21.3, 33.9, 41.9, 120.5, 126.9, 129.9, 131.4, 134.8, 140.4, 141.8, 143.4; ESI-MS m/z: 163.3 $[M]^+$; HRMS (ESI) calcd for $C_8H_{11}N_4$ 163.0978, found 163.0976.

Methyl 4-isocyanobutanoate (4.21)



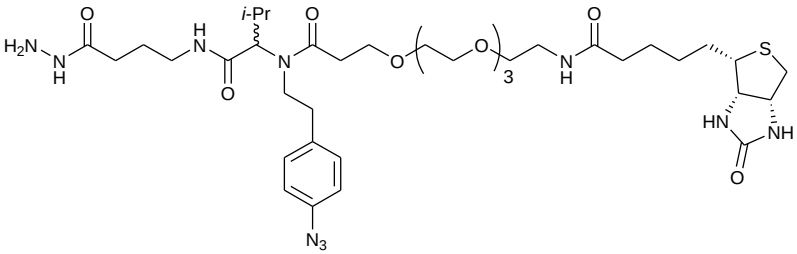
The synthesis of the methyl ester protected isocyanide **4.21** has already been described in the present thesis (cf. experimental section 2.5, page 81–82).

PEG$_4$-biotin-phenylazide-methyl ester (4.22)



A solution containing *i*-butyraldehyde (43 µL, 0.47 mmol), amine **4.20** (130 mg, 0.39 mmol) and triethylamine (54 µL, 0.39 mmol) in methanol (5 mL) was allowed to stir at room temperature for 3 hours to preform the imine. Afterwards isocyanide **4.21** (66 mg, 0.52 mmol) and PEG$_4$-biotin-COOH **4.11** (192 mg, 0.39 mmol) were added to the reaction mixture and stirring was continued at room temperature overnight. Finally, the solvent was evaporated and the residue was purified by column chromatography to afford methyl ester **4.22** as yellow oil (epimeric mixture, 234 mg, 72%). R_F = 0.32 (dichloromethane/methanol 9/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.75–0.88 (m, 3H), 0.90–1.04 (m, 3H), 1.38–1.53 (m, 2H), 1.59–1.94 (m, 6H), 2.14–2.29 (m, 3H), 2.30–2.50 (m, 3H), 2.53–2.99 (m, 6H), 3.12–3.73 (m, 22H), 3.75–3.86 (m, 2H), 4.33 (m, 2H), 4.46–4.55 (m, 1H), 5.30 (br. s., 1H), 6.39 (d, *J* = 6.1 Hz, 1H), 6.66 (d, *J* = 5.7 Hz, 1H), 6.91–6.99 (m, 2H), 7.17–7.25 (m, 2H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 18.7, 19.7, 24.6, 25.5, 26.6, 28.1, 28.2, 31.3, 33.5, 34.0, 35.2, 35.9, 38.6, 39.1, 40.5, 51.6, 55.4, 60.1, 61.7, 61.8, 67.6, 69.9, 70.1, 70.4, 70.5, 118.9, 119.2, 130.2, 130.4, 135.0, 138.4, 163.6, 163.7, 169.5, 170.8, 171.3, 172.5, 173.1, 173.4; ESI-MS m/z: 833.8 $[M-H]^-$, 835.6 $[M+H]^+$, 857.5 $[M+Na]^+$; HRMS (ESI) calcd for $C_{39}H_{62}N_8O_{10}SNa$ 857.4201, found 857.4201.

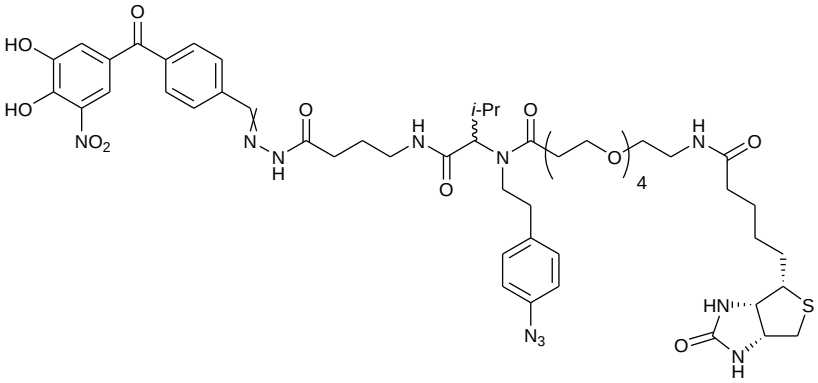
PEG$_4$-biotin-phenylazide-hydrazide (4.23)



Methyl ester **4.22** (205 mg, 0.25 mmol) was dissolved in methanol (3 mL) and hydrazine (0.07 mL, 2.23 mmol) was added while stirring. The reaction mixture was stirred overnight at room temperature until completion of the reaction was detected (checked by TLC-analysis), whereupon the reaction mixture was evaporated to dryness. The residue was taken up in water/dichloromethane (20 mL, 1/1 v/v), acidified with aqueous 1 M hydrochloric acid and the organic phase was separated. The aqueous phase was washed with dichloromethane (3x 10 mL), made strongly alkaline by the addition of aqueous 1 M

sodium hydroxide solution and extracted with dichloromethane (3x 10 mL). Finally, the combined organic extracts were dried (sodium sulphate) and the solvent was evaporated to afford acylhydrazine **4.24** as yellow oil (epimeric mixture, 139 mg, 68%). R_F = 0.24 (dichloromethane/methanol 9/1); ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.76–1.01 (m, 6H), 1.43–1.56 (m, 2H), 1.57–2.13 (m, 8H), 2.16–2.29 (m, 4H), 2.29–2.41 (m, 1H), 2.54–3.03 (m, 6H), 3.03–3.18 (m, 1H), 3.18–3.25 (m, 1H), 3.30–3.40 (m, 1H), 3.40–3.48 (m, 2H), 3.52–3.70 (m, 12H), 3.75–3.87 (m, 2H), 3.89–4.14 (m, 2H), 4.30–4.42 (m, 1H), 4.50–4.57 (m, 1H), 4.58–4.65 (m, 1H), 4.65–4.73 (m, 1H), 5.52 (br. s, 1H), 5.65 (br. s., 1H), 6.11–6.19 (m, 1H), 6.56 (br. s., 1H), 6.70–6.81 (m, 2H), 6.90–6.99 (m, 2H), 7.21–7.30 (m, 2H), 7.79–7.85 (m, 1H), 7.91–7.97 (m, 1H), 8.99 (br. s., 1H), 9.24 (br. s., 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 18.6, 19.5, 19.6, 25.2, 25.3, 25.4, 27.2, 27.3, 28.00, 28.04, 28.2, 28.3, 33.6, 33.8, 35.46, 35.51, 35.9, 36.0, 37.8, 38.0, 39.2, 40.7, 40.8, 53.4, 55.2, 55.4, 60.1, 60.2, 61.9, 62.2, 67.8, 69.9, 69.97, 70.03, 70.3, 70.37, 70.44, 70.5, 118.9, 119.2, 130.2, 130.4, 135.05, 135.09, 136.7, 137.8, 138.4, 164.1, 164.3, 169.7, 170.48, 170.53, 172.0, 172.2, 172.9, 173.0, 173.3, 173.4, 173.5, 173.6; ESI-MS m/z: 835.6 $[M+H]^+$, 857.7 $[M+Na]^+$; HRMS (ESI) calcd for $C_{38}H_{62}N_{10}O_9SNa$ 857.4314, found 857.4294.

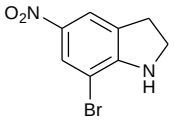
Tolcapone-PEG$_4$-biotin-phenylazide-acylhydrazone (4.24)



Aldehyde **4.7** (12.2 mg, 42.7 µmol) and acyl-hydrazine **4.23** (45.2 mg, 54.1 µmol) were dissolved in tetrahydrofuran/ water (1/1 v/v, 5 mL) and the reaction mixture was stirred at room temperature overnight. Afterwards dichloromethane (20 mL) was added to the reaction mixture and the organic phase was washed consecutively with distilled water, aqueous 1 M hydrochloric acid and brine. Finally, the organic phase was dried (sodium sulphate) and evaporation of the organic solvent afforded acylhydrazone **4.24** as yellow oil (stereomeric mixture, 17 mg, 36%). ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 0.72–1.05 (m, 6H), 1.32–1.49 (m, 2H), 1.50–1.78 (m, 4H), 1.87–2.04 (m, 2H), 2.19 (br. s., 2H), 2.27–2.45 (m, 2H), 2.52–3.02 (m, 7H), 3.17 (br. s., 1H), 3.25–3.41 (m, 6H), 3.44–4.01 (m, 17H), 4.29 (br. s., 1H), 4.48 (br. s., 1H), 4.60 (d, J = 11.0 Hz, 1H), 6.81–7.04 (m, 2H), 7.12–7.33 (m, 2H), 7.46–8.00 (m, 6H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 19.1, 19.8,

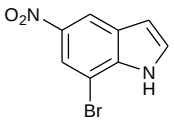
20.1, 20.7, 25.1, 25.3, 26.0, 26.8, 28.4, 29.1, 29.5, 29.8, 31.0, 32.7, 34.5, 35.0, 36.4, 36.7, 39.6, 40.3, 41.1, 46.2, 47.9, 57.0, 61.6, 63.3, 64.7, 67.5, 68.5, 68.8, 70.6, 71.2, 71.5, 119.8, 120.0, 120.2, 120.8, 128.0, 128.2, 128.3, 128.6, 131.1, 131.2, 131.4, 136.0, 136.8, 137.9, 139.3, 139.6, 139.7, 144.0, 147.6, 149.8, 166.1, 171.6, 171.9, 172.6, 173.7, 173.8, 174.8, 174.9, 175.2, 176.0, 177.0, 194.8; ESI-MS m/z: 1102.9 $[M-H]^-$, 1104.9 $[M+H]^+$, 1126.5 $[M+Na]^+$; HRMS (ESI) calcd for $C_{52}H_{68}N_{11}O_{14}S$ 1102.4673, found 1102.4665.

7-Bromo-5-nitro-2,3-dihydro-1*H*-indole (4.30)



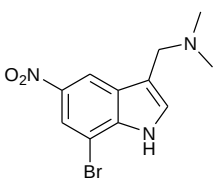
To a stirred solution containing 5-nitroindoline **4.29** (15 g, 91.4 mmol) in glacial acetic acid (150 mL) was added bromine (5.2 mL, 101 mmol) in glacial acetic acid (30 mL) over a period of 1 hour at room temperature. Afterwards the reaction mixture was poured on 600 mL ice-water containing 0.5 mL aqueous sodium bisulfite solution (40%). The precipitate was collected by filtration and recrystallised from ethanol-water to afford indoline **4.30** as ochre-coloured solid (20.1 g, 90%). M.p. 159–160 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.18 (t, J = 8.6 Hz, 2H), 3.71 (t, J = 9.2 Hz, 2H), 7.30 (s, 1H), 7.84 (d, J = 1.8 Hz, 1H), 8.07 (d, J = 2.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 28.5, 46.2, 97.5, 119.5, 128.0, 130.6, 137.0, 156.4; ESI-MS m/z: 240.9 $[M-H]^-$, 243.2 $[M-H]^-$; HRMS (ESI) calcd for $C_8H_6N_2O_2Br$ 240.9618, found 240.9613.

7-Bromo-5-nitro-1*H*-indole (4.31)



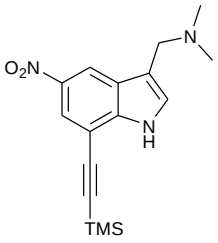
7-Bromo-5-nitro-2,3-dihydro-1*H*-indole **4.30** (10.0 g, 41.2 mmol) and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (10.5 g, 45.3 mmol) were suspended in toluene (400 mL) and heated under reflux overnight. Following the evaporation of all volatiles, the residue was purified by column chromatography to afford indole **4.31** as a yellow solid (9.5 g, 96%). R_F = 0.41 (petroleum ether/dichloromethane 1/1); M.p. 194–195 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 6.90 (d, J = 3.5 Hz, 1H), 7.68 (d, J = 3.5 Hz, 1H), 8.18 (d, J = 1.8 Hz, 1H), 8.62 (d, J = 2.2 Hz, 1H), 12.15 (br. s., 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 104.0, 105.6, 116.9, 118.5, 127.8, 130.3, 137.3, 141.0; ESI-MS m/z: 239.1 $[M-H]^-$, 241.1 $[M-H]^-$; HRMS (ESI) calcd for $C_8H_4N_2O_2Br$ 238.9462, found 238.9457.

1-(7-Bromo-5-nitro-1*H*-indol-3-yl)-*N*,*N*-dimethylmethanamine (4.32)



7-Bromo-5-nitro-1*H*-indole **4.31** (9.0 g, 37.3 mmol) was suspended in glacial acetic acid (20 mL) and a solution containing aqueous formalin (37%, 3.4 mL, 45.4 mmol) and aqueous dimethylamine (40%, 8.5 mL, 67.1 mmol) in glacial acetic acid (10 mL) was added dropwise with stirring at room temperature. The resulting mixture was heated on an oil bath at 70 °C. After completion of the reaction was detected (checked by TLC-analysis, approx. 4 hours), 1.1 mL each of aqueous formalin and aqueous dimethylamine was added and the reaction mixture was allowed to cool slowly to room temperature, poured on ice and made strongly alkaline by the addition of conc. aqueous ammonia solution. The resulting precipitate was collected by filtration, washed with water and dried under reduced pressure to afford gramine **4.32** as a yellow solid (10.6 g, 96%). M.p. 156–158 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 2.17 (s, 6H), 3.60 (s, 2H), 7.56 (s, 1H), 8.17 (d, J = 1.8 Hz, 1H), 8.61 (d, J = 1.8 Hz, 1H), 12.00 (br. s., 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 43.4, 52.4, 104.2, 116.0, 118.9, 127.5, 130.9, 137.7, 141.1; ESI-MS m/z: 296.1 $[M-H]^-$, 298.2 $[M-H]^-$, 298.3 $[M+H]^+$, 300.3 $[M+H]^+$; HRMS (ESI) calcd for $C_{11}H_{11}N_3O_2Br$ 296.0040, found 296.0038.

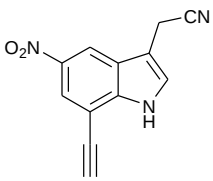
***N*,*N*-Dimethyl-1-{5-nitro-7-[(trimethylsilyl)ethynyl]-1*H*-indol-3-yl}methanamine (4.33)**



A solution containing gramine **4.32** (2.96 g, 9.93 mmol), ethynyltrimethylsilane (1.80 mL, 12.5 mmol), tetrakis(triphenylphosphine)-palladium (1.15 g, 1.00 mmol), copper(I)iodide (0.29 g, 1.52 mmol) and triethylamine (4.20 mL, 30 mmol) in dry tetrahydrofuran (60 mL) was heated under reflux for 6 hours under inert-gas atmosphere. Afterwards the reaction mixture was allowed to cool to room temperature, filtered and the filtrate was evaporated. The remaining residue was taken up in dichloromethane and the organic phase was consecutively washed with distilled water and brine. Next, the organic solvent was dried (sodium sulphate), filtered and all volatiles were removed under reduced pressure. Finally, purification by column chromatography afforded gramine **4.33** as an ochre-coloured solid (1.20 g, 38%). R_F = 0.25 (dichloromethane/ methanol 9/1); M.p. 195–196 °C; ^{1}H NMR (400 MHz, CHLOROFORM-d) δ: 0.32 (s, 9H), 2.33 (s, 6H), 3.71 (s, 2H), 7.40 (s, 1H), 8.24 (d, J = 2.2 Hz, 1H), 8.66 (d, J = 2.2 Hz, 1H), 8.73 (br. s., 1H); ^{13}C NMR (100 MHz, CHLOROFORM-d) δ: –0.1, 45.0, 54.1, 98.6, 101.1, 106.7,

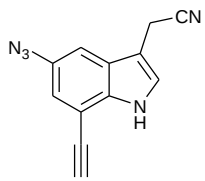
117.2, 121.2, 126.7, 139.7, 141.6; ESI-MS m/z: 314.5 $[M-H]^-$, 316.3 $[M+H]^+$, 631.2 $[2M+H]^+$; HRMS (ESI) calcd for $C_{16}H_{22}N_3O_2Si$ 316.1476, found 316.1476.

(7-Ethynyl-5-nitro-1*H*-indol-3-yl)acetonitrile (4.35)



To a solution containing gramine **4.33** (0.63 g, 2.0 mmol) in a toluene/ dichloromethane solvent-mix (30 mL, 2/1 v/v) was added dropwise methyl iodide (0.25 mL, 4.0 mmol) under inert-gas atmosphere. The resulting suspension was stirred at room temperature overnight, whereupon all volatiles were removed under reduced pressure. The remaining residue was taken up in dry tetrahydrofuran (30 mL) and to the resulting solution were consecutively added trimethylsilyl cyanide (0.38 mL, 2.98 mmol) and tetrabutyl-ammonium fluoride (1 M in tetrahydrofuran, 8.0 mL, 8.0 mmol). Stirring was continued at room temperature overnight. The reaction mixture was quenched by the addition of saturated aqueous sodium carbonate solution (50 mL) and ethyl acetate (50 mL). Next, the aqueous layer was separated and the organic phase was consecutively washed with each 50 mL distilled water and brine. Finally, the organic phase was dried (sodium sulphate), evaporated to dryness and the remaining residue was purified by column chromatography to afford indole-3-acetonitrile **4.35** as a yellow solid (101 mg, 22%). R_F = 0.40 (hexane/ethyl acetate 1/1); M.p. 223–224 °C (decomposition); ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.22 (s, 2H), 4.76 (s, 1H), 7.64 (s, 1H), 8.13 (d, J = 2.2 Hz, 1H), 8.73 (d, J = 2.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.0, 77.9, 86.8, 105.9, 108.1, 116.6, 119.1, 120.4, 125.6, 128.5, 139.3, 140.5; ESI-MS m/z: 223.9 $[M-H]^-$; HRMS (ESI) calcd for $C_{12}H_6N_3O_2$ 224.0466, found 224.0463.

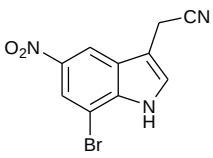
(5-Azido-7-ethynyl-1*H*-indol-3-yl)acetonitrile (4.37)



A solution containing indole-3-acetonitrile **4.35** (171 mg, 0.76 mmol) in ethanol (4 mL) was warmed to 55 °C on an oil bath. To this mixture was added sodium dithionite (667 mg, 3.83 mmol) in distilled water (4 mL) and the resulting mixture was stirred until completion of the reaction was detected (checked by TLC-analysis, approx. 1 hour). The reaction mixture was allowed to cool to room temperature, filtered and the organic solvent was removed under reduced pressure. Next, the remaining aqueous phase was made strongly alkaline by addition of aqueous 1 M sodium hydroxide solution and extracted with dichloromethane (3x 10 mL). Afterwards the combined organic extracts were washed with brine, dried

(sodium sulphate) and evaporated to dryness to afford the corresponding amine **4.36**. The crude amine **4.36** (47 mg, 0.24 mmol) was dissolved in 80% aqueous acetic acid (5 mL) and cooled on an ice-bath. A solution containing sodium nitrite (21 mg, 0.31 mmol) in distilled water (0.5 mL) was added and stirring was continued for 15 minutes, whereupon a solution containing sodium azide (50 mg, 0.77 mmol) in distilled water (0.5 mL) was added. The reaction mixture was protected from light and stirring was continued until completion of the reaction was detected (checked by TLC-analysis, approx. 1 hour). Next, the reaction was quenched by the addition of ice-water and the aqueous phase was extracted with dichloromethane (3x 10 mL). Finally, the combined organic extracts were dried (sodium sulphate), evaporated to dryness (room temperature) and the remaining residue was purified by column chromatography to afford phenyl azide **4.37** as an off-white solid (32 mg, 19%). R_F = 0.29 (hexane/ethyl acetate 2/1); M.p. 136−137 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.07 (s, 2H), 4.61 (s, 1H), 7.02 (d, J = 2.2 Hz, 1H), 7.43 (s, 1H), 7.47 (d, J = 2.2 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.1, 79.0, 85.8, 104.7, 106.6, 109.5, 117.1, 119.2, 126.5, 127.0, 131.0, 134.5; ESI-MS m/z: 220.0 $[M-H]^-$; HRMS (ESI) calcd for $C_{12}H_6N_5$ 220.0629, found 220.0624.

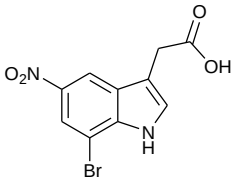
(7-Bromo-5-nitro-1*H*-indol-3-yl)acetonitrile (4.39)



To a solution containing gramine **4.32** (5.02 g, 16.8 mmol) in a toluene/dichloromethane solvent-mix (120 mL, 2/1 v/v) was added dropwise methyl iodide (2.1 mL, 33.7 mmol) under inert-gas atmosphere. The resulting suspension was stirred at room temperature overnight, whereupon all volatiles were removed under reduced pressure. The remaining residue was taken up in dry tetrahydrofuran (120 mL) and to the resulting solution were consecutively added trimetylsilyl cyanide (3.4 mL, 27.1 mmol) and tetrabutylammonium-fluoride (1 M solution in tetrahydrofuran, 50.0 mL, 50.0 mmol). Stirring was continued at room temperature overnight. The reaction mixture was quenched by the addition of saturated aqueous sodium carbonate solution (300 mL) and ethyl acetate (600 mL). Next, the aqueous layer was separated and the organic phase was consecutively washed with each 300 mL distilled water and brine. Finally, the organic phase was dried (sodium sulphate), evaporated to dryness and the remaining residue was purified by column chromatography to afford indole-3-acetonitrile **4.39** as a yellow solid (2.36 g, 50%). R_F = 0.30 (hexane/ethyl acetate 3/2); M.p. 247−249 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 4.22 (s, 2H), 7.69 (d, J = 2.2 Hz, 1H), 8.24 (d, J = 2.2 Hz, 1H), 8.72 (d, J = 1.8 Hz, 1H), 12.22

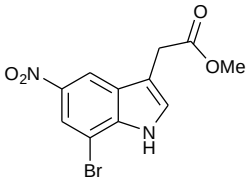
(br. s., 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 13.1, 104.4, 108.7, 115.2, 118.9, 119.2, 126.1, 129.0, 137.6, 141.1; ESI-MS m/z: 278.1 $[M-H]^-$, 280.0 $[M-H]^-$; HRMS (ESI) calcd for $C_{10}H_5N_3O_2Br$ 277.9571, found 277.9567.

(7-Bromo-5-nitro-1*H*-indol-3-yl)acetic acid (4.40)



Indole-3-acetonitrile **4.39** (2.35 g, 8.39 mmol) was suspended in conc. aqueous hydrochloric acid (200 mL) and heated under reflux for 6 hours. The reaction mixture was allowed to cool to room temperature and stirring was continued overnight, whereupon the suspension was filtered. Finally, the remaining solid was washed with several portions distilled water and dried under reduced pressure to afford indole-3-acetic acid **4.40** as a yellow solid (2.31 g, 92%). M.p. 225–226 °C; ^{1}H NMR (400 MHz, DMSO-d_6) δ: 3.81 (s, 2H), 7.58 (d, J = 2.7 Hz, 1H), 8.16–8.21 (m, 1H), 8.58 (d, J = 2.0 Hz, 1H), 12.01 (br. s., 1H); ^{13}C NMR (100 MHz, DMSO-d_6) δ: 30.5, 104.0, 112.7, 115.8, 118.5, 127.5, 128.9, 137.5, 140.8, 172.5; ESI-MS m/z: 297.0 $[M-H]^-$, 299.1$[M-H]^-$; HRMS (ESI) calcd for $C_{10}H_6N_2O_4Br$ 296.9516, found 296.9516.

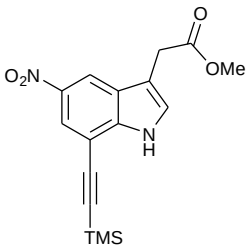
Methyl (7-bromo-5-nitro-1*H*-indol-3-yl)acetate (4.41)



A solution containing indole-3-acetic acid **4.40** (2.20 g, 7.36 mmol) in dry *N*,*N*-dimethylformamide (60 mL) was cooled on an ice-water bath, whereupon potassium bicarbonate (0.82 g, 8.19 mmol) and methyl iodide (0.51 mL, 8.11 mmol) were consecutively added. The ice-bath was removed and stirring was continued at room temperature until completion of the reaction was detected (checked by TLC-analysis). Then, diethyl ether (250 mL) was added to the reaction mixture and the organic phase was washed with each 200 mL distilled water, saturated aqueous sodium bisulfate solution, saturated aqueous sodium bicarbonate solution and brine. Next, the organic phase was dried (sodium sulphate) and evaporated to dryness. Finally, the remaining residue was purified by column chromatography to afford methyl indole-3-acetate **4.41** as a yellow solid (2.08 g, 90%). R_F = 0.32 (hexane/ethyl acetate 2/1); M.p. 182–183 °C; ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 3.75 (s, 3H), 3.82 (d, J = 0.9 Hz, 2H), 7.40–7.44 (m, 1H), 8.31 (d, J = 2.2 Hz, 1H), 8.55 (d, J = 1.3 Hz, 1H), 8.64 (br. s., 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 30.8, 52.3, 104.4, 112.7, 115.6, 120.1, 126.6, 127.0, 137.7, 142.3,

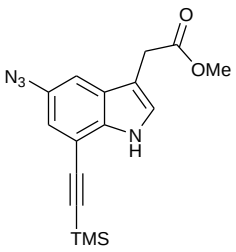
171.4; ESI-MS m/z: 311.2 $[M-H]^-$, 313.1 $[M-H]^-$, 335.0 $[M+Na]^+$, 337.0 $[M+Na]^+$; HRMS (ESI) calcd for $C_{11}H_8N_2O_4Br$ 310.9673, found 310.9671.

Methyl {5-nitro-7-[(trimethylsilyl)ethynyl]-1*H*-indol-3-yl}acetate (4.42)



A solution containing methyl indole-3-acetate **4.41** (2.05 g, 6.55 mmol), tetrakis(triphenylphosphine)palladium (0.74 g, 0.64 mmol), ethynyltrimethylsilane (1.13 mL, 7.83 mmol), copper(I)iodide (0.27 g, 1.42 mmol) and triethylamine (2.8 mL, 19.9 mmol) in dry tetrahydrofuran (100 mL) was heated under reflux for 6 hours under inert-gas atmosphere. Afterwards the reaction was allowed to cool to room temperature, filtered and the filtrate was evaporated. The remaining residue was taken up in dichloromethane (200 mL) and the organic phase was consecutively washed with distilled water (2x 100 mL) and brine (2x 100 mL). Next, the organic solvent was dried (sodium sulphate), filtered and all volatiles were removed under reduced pressure. Finally, purification by column chromatography afforded methyl indole-3-acetate **4.42** as a yellow solid (1.70 g, 78%). R_F = 0.25 (hexane/ethyl acetate 3/1); M.p. 147–148 °C; ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.32 (s, 9H), 3.74 (s, 3H), 3.82 (s, 2H), 7.39 (d, J = 2.2 Hz, 1H), 8.25 (d, J = 2.2 Hz, 1H), 8.55 (d, J = 2.2 Hz, 1H), 8.65 (br. s., 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: −0.1, 30.7, 52.2, 98.5, 101.2, 106.8, 112.0, 116.8, 121.2, 126.1, 126.3, 139.5, 141.7, 171.5; ESI-MS m/z: 329.1 $[M-H]^-$, 331.3 $[M+H]^+$, 352.9 $[M+Na]^+$; HRMS (ESI) calcd for $C_{16}H_{17}N_2O_4Si$ 329.0963, found 329.0960.

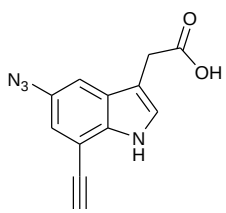
Methyl {5-azido-7-[(trimethylsilyl)ethynyl]-1*H*-indol-3-yl}acetate (4.43)



A solution containing methyl indole-3-acetate **4.42** (0.33 g, 1.0 mmol) in ethanol (15 mL) was warmed to 55 °C on an oil bath. Sodium dithionite (0.90 g, 5.17 mmol) in distilled water (15 mL) was added and the resulting mixture was stirred until completion of the reaction was detected (checked by TLC-analysis, approx. 1 hour). The reaction mixture was allowed to cool to room temperature, filtered and the organic solvent was removed under reduced pressure. Next, the remaining aqueous phase was made strongly alkaline by addition of aqueous 1 M sodium hydroxide solution and extracted with dichloromethane (3x 15 mL). Afterwards the combined organic extracts were washed with brine, dried (sodium sulphate) and evaporated to dryness to afford the corresponding amine. The crude amine was dissolved in 80% aqueous acetic acid (10 mL)

and cooled on an ice-bath. A solution containing sodium nitrite (84 mg, 1.22 mmol) in distilled water (1 mL) was added and stirring was continued for 15 minutes, whereupon a solution containing sodium azide (203 mg, 3.12 mmol) in distilled water (0.5 mL) was added. The reaction mixture was protected from light and stirring was continued until completion of the reaction was detected (approx. 1 hour). Next, the reaction was quenched by the addition of ice-water and the aqueous phase was extracted with dichloromethane (3x 15 mL). Finally, the combined organic extracts were dried (sodium sulphate), evaporated to dryness (room temperature) and the remaining residue was purified by column chromatography to afford phenyl azide **4.43** as a white solid (79 mg, 24%). R_F = 0.22 (hexane/diethyl ether 3/1); M.p. 113–114 °C; ^{1}H NMR (400 MHz, CHLOROFORM-*d*) δ: 0.30 (s, 9H), 3.71 (s, 3H), 3.73 (s, 2H), 7.03 (d, J = 2.2 Hz, 1H), 7.22–7.27 (m, 2H), 7.68–7.76 (m, 1H), 8.27–8.35 (m, 1H); ^{13}C NMR (100 MHz, CHLOROFORM-*d*) δ: 0.1, 31.0, 52.0, 99.8, 99.9, 107.4, 109.1, 109.9, 117.3, 124.7, 127.7, 132.2, 134.8, 172.0; ESI-MS m/z: 325.2 $[M–H]^–$, 348.9 $[M+Na]^+$, 674.5 $[2M+Na]^+$; HRMS (ESI) calcd for $C_{16}H_{17}N_4O_2Si$ 325.1126, found 325.1128.

(5-Azido-7-ethynyl-1*H*-indol-3-yl)acetic acid (4.38)



To a solution containing protected phenyl azide **4.43** (50 mg, 0.15 mmol) in tetrahydrofuran/water (10 mL, 1/1 v/v) was added lithium hydroxide monohydrate (35.8 mg, 0.85 mmol) and stirring was continued at room temperature until completion of the reaction was detected (checked by ESI-MS). Then, the reaction mixture was washed with dichloromethane (3x 10 mL). Afterwards the aqueous phase was acidified by the addition of aqueous 1 M hydrochloric acid and extracted with dichloromethane (3x 10 mL). Finally, the combined organic extracts were dried (sodium sulphate) and evaporated to dryness to afford **4.38** as an off-white solid (29 mg, 78%). M.p. 134–136 °C; ^{1}H NMR (400 MHz, METHANOL-d_4) δ: 3.71 (d, J = 0.9 Hz, 2H), 3.83 (s, 1H), 6.94 (d, J = 1.8 Hz, 1H), 7.28 (s, 1H), 7.29 (d, J = 2.2 Hz, 1H); ^{13}C NMR (100 MHz, METHANOL-d_4) δ: 31.8, 80.0, 83.3, 107.9, 109.9, 110.8, 117.8, 127.2, 129.6, 132.6, 136.6, 175.9; ESI-MS m/z: 239.2 $[M–H]^–$; HRMS (ESI) calcd for $C_{12}H_7N_4O_2$ 239.0575, found 239.0575.

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S. Brauch, S. S. van Berkel, F. Stehle, L. A. Wessjohann and B. Westermann
25th Irseer Naturstofftage, Irsee (Germany), February 20–22

2012 *Traceless Tosylhydrazone-Based Triazole Formation: Towards a Viable and Robust Alternative for the Covalent Labelling of Various Biomolecules*
S. Brauch, S. S. van Berkel and B. Westermann
18. Lecture Conference (ORCHEM 2012), Weimar (Germany), September 24–26

Traceless Tosylhydrazone-Based Triazole Formation: Towards a Viable and Robust Alternative for the Covalent Labelling of Various Biomolecules
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Curriculum Vitae

Personal Details

Name	Dipl. Chem. Sebastian Brauch
Date of Birth	May 17th, 1985
Place of Birth	Wolfen, Germany
Nationality	German

Education

06/2010 – 09/2013	PhD candidate Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany Thesis title: Novel tools for protein analysis and modification – From chemical probes to new ligation methods – Supervisor: Prof. Dr. B. Westermann
10/2005 – 05/2010	Chemistry studies at the Martin-Luther University Halle-Wittenberg, Germany
06/2004	Abitur (A-levels, school leaving examination)

Research Experience

10/2013 – present	**Research Assistant**, Post-doctoral fellow Radboud University, Nijmegen, The Netherlands Project: Polymersome nanoreactors for biphasic enzymatic transformations Supervisor: Prof. Dr. J. C. M. van Hest and Prof. Dr. F. P. J. T. Rutjes
06/2010 – 09/2013	**Research Assistant**, Doctoral level Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany Project: Synthesis of chemical labelling reagents for the covalent modification of various biomolecules Supervisor: Prof. Dr. B. Westermann

10/2009 – 05/2010 **Research Assistant**, Diploma level
Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany
Project: MCR-based synthesis of chemical probes for proteomic studies
Supervisor: Prof. Dr. B. Westermann

04/2009 – 07/2009 **Research Assistant**, internship
Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany
Project: Studies towards the sequential Ugi-Mumm/Ugi-Smiles coupling
Supervisor: Prof. Dr. B. Westermann

03/2008 – 03/2009 **Research Assistant**, internship
Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany
Project: Synthesis of photoreactive building blocks for proteomic studies
Supervisor: Prof. Dr. B. Westermann

Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur unter Verwendung der angegebenen Literatur und Hilfsmitteln angefertigt habe. Des Weiteren wurde die vorliegende Arbeit an keiner anderen wissenschaftlichen Einrichtung zur Erlangung eines akademischen Grades eingereicht.

Halle/Saale, den 23. September 2013

Sebastian Brauch