Application of Azides in Chemoselective Amidation Reactions

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Application of Azides in Chemoselective Amidation Reactions

Toepassing van azides in chemoselectieve amideringsreacties

(met een samenvatting in het Nederlands)

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List of abbreviations

Amino acids

Ala	A	L-alanine
Arg	R	L-arginine
Asn	N	L-asparagine
Asp	D	L-aspartic acid
Cys	C	L-cysteine
Gln	Q	L-glutamine
Glu	E	L-glutamic acid
Gly	G	glycine
His	H	L-histidine
Leu	L	L-leucine
Lys	K	L-lysine
Phe	F	L-phenylalanine
Pro	P	L-proline
Ser	S	L-serine
Thr	T	L-threonine
Trp	W	L-tryptophan
Tyr	Y	L-tyrosine
Val	V	L-Valine

General

aa	amino acid
ADF	Amsterdam density functional
APT	attached proton test
Aux	auxiliary
Boc	*t*-Butyloxycarbonyl
BOP	Benzotriazole-1-yl-oxy-tris(dimethylamino)phos-phonium hexafluorophosphate
br	broad
calcd.	calculated
Cbz	benzyloxycarbonyl
δ	chemical shift
d	doublet
dd	double doublet
DABCO	1,4-diazabicyclo[2.2.2]octane
DCCI	*N*,*N'*-dicyclohexyl-carbodiimide
DCM	dichloromethane
DCU	*N*,*N'*-dicyclohexylurea
DFT	density functional theory
D*i*PEA	*N*,*N*-diisopropylethylamine
DMA	dimethylacetamide
DMAP	dimethylaminopyridine
DMF	*N*,*N'*-dimethylformamide
DMSO	dimethylsulfoxide
EDCI	1-ethyl-3-(3'-dimethyl-aminopropyl)carbodiimide
ELSD	evaporative light scattering detector
EPL	expressed protein ligation
ESMS	electrospray ionization mass spectrometry
equiv	equivalent(s)
Fmoc	9*H*-fluorenylmethyl-oxycarbonyl
GFP	green fluorescent protein
h	hour
HBTU	*O*-Benzotriazole-*N*,*N*,*N'*,*N'*-tetramethyl-uronium-hexafluoro-phosphate
HOBt	*N*-Hydroxybenzotriazole

HPLC	high performance liquid chromatography
HSAc	thioacetic acid
HSQC	heteronuclear single quantum coherence
IRC	intrinsic reaction coordinate
IR	infra red
LCMS	liquid chromatography mass spectrometry
LMWGs	low molecular weight gelators
LMOGs	low molecular mass organogelators
m	multiplet
MALDI-TOF	matrix assisted laser desorption ionization time-of-flight
mp	melting point
MS	mass spectrometry
MTBE	methyl tertiary butyl ether
NCL	native chemical ligation
NHS	*N*-hydroxysuccinimide
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
O.D.	optical density
PCM	plausible continuum model
q	quartet
R_f	retention factor
RNA	ribo nucleic acid
R_t	retention time
rt	room temperature
s	singlet
SCL	safety catch linker
SPPS	solid phase peptide synthesis
t	triplet
tandem crDA	cycloaddition-retro-Diels-Alder
TBTA	*t*-butyl-2,2,2-trichloroacetimidate
TDM	4,4'-methylene bis(*N*,*N'*-dimethylaniline)
TEM	transmission electron microscopy
TFA	trifluoroacetic acid
THF	tetrahydofuran
T*i*S	triisopropylsilane
TLC	thin layer chromatography
TMOB	2,4,6-trimethoxybenzyl
TMS	tetramethylsilane
TOCSY	total correlation spectroscopy
tRNA	transfer RNA
UV	ultra violet
v	volume

1

General Introduction

1.1 Bioconjugation as a tool in chemical biology

Selective and bioorthogonal conjugation methods that allow for the site-selective modification of biomolecules are of significant interest and have found widespread use in chemical biology. These methods enable the synthesis of complex bioconjugates such as (poly)peptides and proteins,[1-5] the immobilization of compounds leading to micro arrays for high throughput experiments and the introduction of biophysical markers (e.g. affinity tags and fluorophores). In this chapter an overview is given of the chemical methods available for the chemoselective conjugation of biomolecules and peptides and proteins in particular.

1.2 Chemical peptide/protein synthesis

The modification of the structure of a protein offers great possibilities for studying its biological function. Particularly, the labeling of proteins by methods that feature the use of the genetically encoded green fluorescent protein[6] has been very useful. Although powerful, biological methods for the synthesis of proteins and peptides are generally limited by the use of natural amino acid residues. For instance, incorporation of non-proteinogenic or unnatural amino acids, peptide backbone modifications, post-translational modifications and site-selective introduction of reporter groups cannot be realized, solely by recombinant DNA methodologies.[7]

In contrast, chemical methods[3] may offer an increased flexibility since they allow the incorporation of unnatural building blocks that enable virtually unlimited structural modifications. Generally, the chemical synthesis of peptides is performed by the sequential coupling of amino acids on a solid support.[8-11] Today, solid phase peptide synthesis (SPPS) is a reliable tool for the routine preparation of peptides up to ~50 amino acids long. Beyond this size limit, however, the accumulation of byproducts in the repetitive coupling cycles dramatically reduces the overall yield and purity of the desired product.

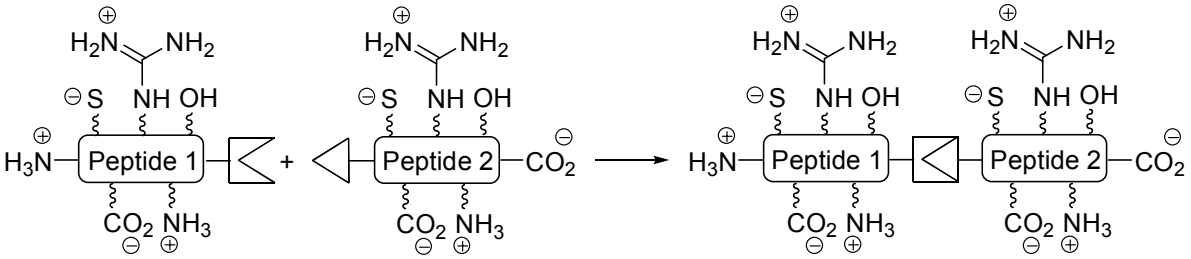
This limitation can partially be overcome by the convergent assembly of polypeptides from moderately sized peptide segments which can be obtained by standard SPPS methods. Usually, these segments are purified and characterized before coupling. Although the size of molecules that can be prepared by this fragment condensation is greatly increased,[12] the often poor solubility and reactivity of (partially) *protected* peptide segments restricts the application of this method.

Ideally, the coupling reactions should be performed without the need of amino acid side chain protection, in aqueous solvents to ensure a good solubility of the *unprotected* peptide segments. However, in the absence of side chain protection the regioselective construction of an amide linkage is not straightforward due to interference by functional groups, present

in the peptide segments. This challenge led to the development of alternative methods for the coupling of unprotected peptide segments by using chemoselective ligation reactions.[13]

1.3 Chemoselective ligation

Chemoselective ligation refers to the selective covalent coupling of unprotected, highly functionalized biomolecules that contain mutually and uniquely reactive functional groups in an aqueous environment.[14,15] The underlying concept that characterizes chemoselective ligation is bioorthogonality. This means that the pair of mutually reactive groups must exclusively undergo the ligation reaction without interference by of other functional groups present in the biomolecules to be coupled or present in the environment of the ligation.



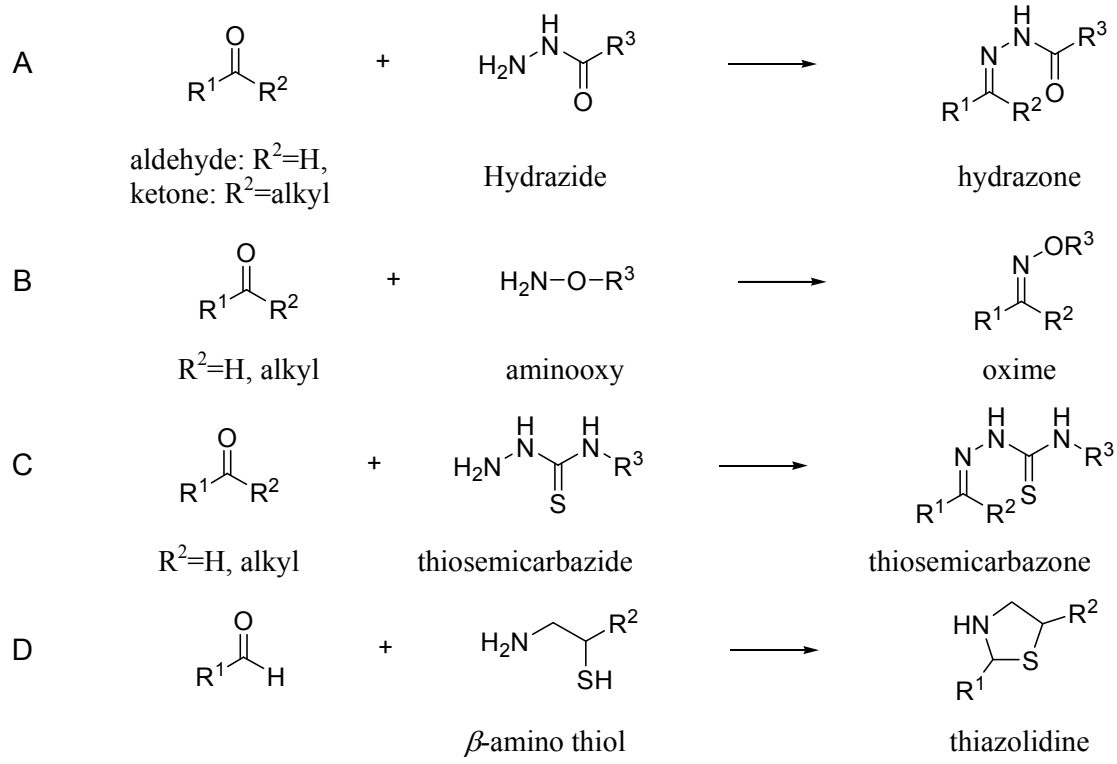
Scheme 1.1 Schematic representation of chemoselective peptide ligation.

Depending on the functional groups present, different degrees of bioorthogonality exist. For example, disulfide exchange can be considered as a chemoselective ligation reaction, since it tolerates water and can be carried out under mild, biologically-compatible conditions. However, many thiol-containing molecules exist *in vivo* so the reaction is not completely bioorthogonal. Nevertheless, it can still be employed in controlled systems that avoid cross-reactivity with other thiols.

1.4 Chemoselective ligation strategies

Methods for chemoselective ligation involve reactions capable of the covalent coupling of two reactive partners in the presence of other potentially reactive functionalities. Those reactions are still quite rare and hence there remains a constant interest in the development of new chemoselective ligation methods. The ideal chemoselective ligation reaction combines good bioorthogonality with a high reactivity. Thus, the reaction should be a) highly selective; b) fast, high yielding and thus clean; c) independent of an excess of potentially hard to remove reagents; d) efficient in water without the use of co-solvents and e) irreversible under physiological conditions.[16]

Over the years, several chemoselective ligation methods have been introduced and successfully used for the site-selective conjugation of unprotected biomolecules in rapid and highly efficient coupling reactions. An overview is given in Scheme 1.2 – Scheme 1.4.[3,4,7,14,16,17]

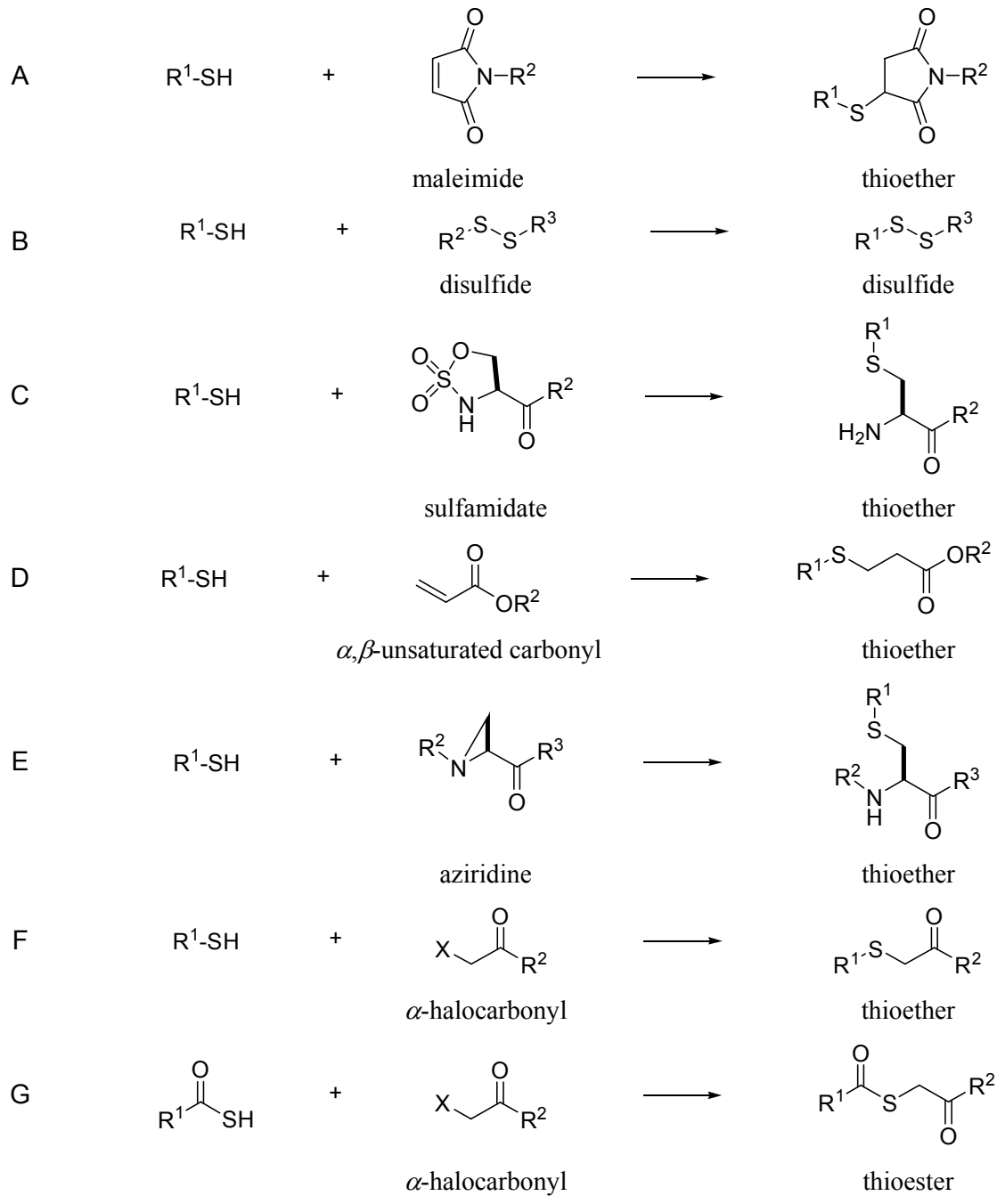


Scheme 1.2 Chemoselective ligation reactions featuring aldehyde and ketone electrophiles.

Several electrophile-nucleophile pairs can react selectively with each other under physiological conditions to form hydrazones, oximes, thiosemicarbazones and thiazolidines starting from aldehydes and ketones (Scheme 1.2). Even though water is produced in these condensation reactions, the products are of sufficient thermodynamic stability to enable couplings under (slightly acidic) aqueous conditions. Recently, Dirksen *et al.* reported that the generally modest reaction rates in oxime and hydrazone formation can be improved significantly by the addition of aniline as nucleophilic catalyst.[18,19]

Ligation reactions that involve the reaction of thiols with various electrophiles (Scheme 1.3) are not completely bioorthogonal as usually many thiol groups exist in a biological environment. In addition, the undesired oxidation of thiols to their disulfide-linked homodimers can compete with the desired ligation reaction. Nonetheless, thiolates are excellent nucleophiles that can react under aqueous conditions at near-neutal pH. Moreover, since other nucleophiles, such as amines, are protonated under these conditions, good

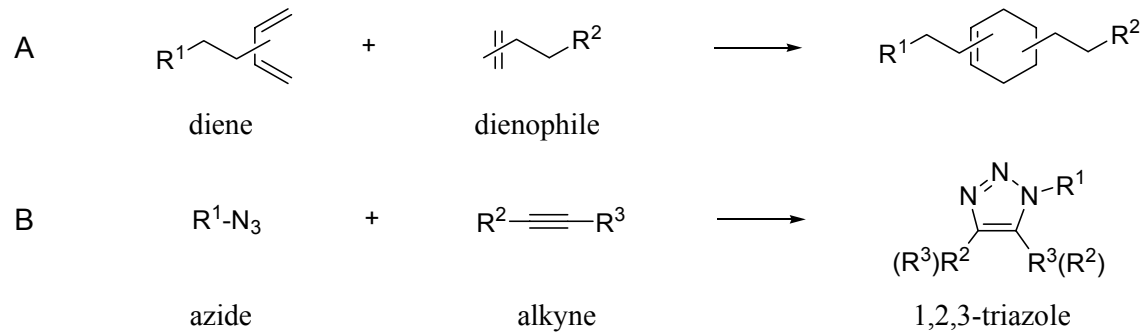
selectivities can still be obtained in thiol-based ligations. In fact, thiols have been used extensively in chemoselective ligation reactions.



Scheme 1.3 Chemoselective ligation reactions featuring thiol nucleophiles.

In another chemoselective ligation strategy featuring a sulfur nucleophile, thiocarboxylates rather than thiolates are reacted with an α-halocarbonyl compound to form a thio*ester* instead of a thio*ether* coupling product (Scheme 1.3F and G). At first glance this reaction might seem incompatible with the presence of thiols which are also capable of reacting with

α-halocarbonyls. However, chemoselectivity can still be achieved by using the difference in pK_a between the thiocarboxylate (pK_a ~3) and the sulfhydryl (pK_a ~8) group. For the reason that, at slightly acidic conditions (pH 5-6), the thiocarboxylate anion is the better nucleophile it reacts considerably faster than the neutral sulfhydryl group.[14]



Scheme 1.4 Chemoselective ligation reactions featuring cycloadditions.

Cycloaddition reactions, such as the Diels-Alder reaction[20] (A, Scheme 1.4) and the Huisgen 1,3-dipolar cycloaddition between azides and alkynes[21] (B), form ideal candidates for chemoselective ligation reactions since they usually take place without any byproduct formation.[22] Moreover, the reagents feature functional groups that are uncommon in biological systems, which generally implies a good bioorthogonality. Although, when maleimides are used as good dienophiles in the Diels-Alder cycloaddition, this method is, of course, not compatible with thiols.[23,24]

The inertness of azides in physiological settings combined with the high stability of the triazole adduct makes the Huisgen 1,3-dipolar cycloaddition between azides and alkynes in principle very interesting as chemoselective ligation method. However, despite the great functional group compatibility, its biocompatibility is limited since elevated temperatures are needed to obtain reasonable reaction rates. Furthermore, the complete lack of regioselectivity prevents precise control over the product that is formed, as typically a 1:1 mixture of the 1,4- and 1,5-substituted triazole derivative is generated.

The discovery, independently reported by Meldal and Sharpless,[25,26] that the rate of the reaction between azides and alkynes can be dramatically increased by the addition of catalytic amounts of Cu(I) salts as well as the exclusive formation of the 1,4-substituted regioisomer, has led to an enormous renewed interest in this cycloaddition reaction for chemoselective ligation purposes. It was found that the copper catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition is efficient in various solvents (including water) at low temperatures, has a high functional group tolerance and affords the 1,2,3-triazole product with minimal work-up and purification. In addition, the triazole products readily associate

with biological targets, through hydrogen bonding and dipole interactions,[27] interestingly, it can also function as an amide bond isostere.[28] The highly attractive properties of this reaction and its resulting product have led to numerous applications in diverse areas of chemistry and biology.[29-31] Moreover, the remarkable scope of this powerful copper-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition has become the paradigm of "click chemistry". A term that was introduced by Sharpless to indicate a set of reliable, nearly perfect reactions, originally designed to accelerate the process of lead discovery and optimization.[27,29]

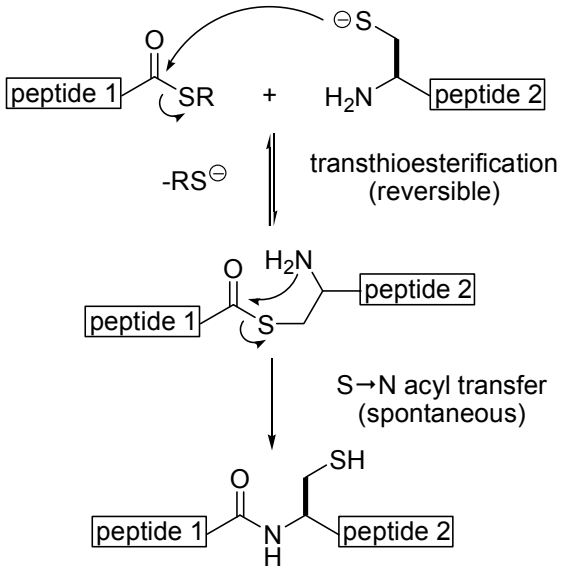
Although many chemoselective ligation reactions, such as the copper-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition, fall under the umbrella of click chemistry, it should be noted that chemoselective ligation and click chemistry are not mutually inconclusive. Since chemoselective ligation emerged from "biological chemistry", it embodied the need to form a stable covalent bond under physiological conditions (aqueous media, narrow pH range, high functional group tolerance, low temperature). Click chemistry on the other hand, refers to a class of selective covalent-bond forming reactions that has a large thermodynamic driving force and must: a) provide high yields; b) employ benign, easily removable solvents (or no solvent) and c) tolerate the presence of air and ideally water. It does not necessarily suggest the need to carry out reactions in a particular (biological) environment.[16]

1.5 Chemoselective amidation reactions for peptide ligation

The repertoire of chemoselective ligation reactions has now been recognized as a valuable tool for the conjugation of especially biomolecules including (poly)peptides. However, by application of the chemoselective ligation strategies described above, an "unnatural" backbone structure is introduced in the polypeptide ligation products. Although many examples of functional (poly)peptide conjugates obtained with these methods have been described in the literature, a chemoselective ligation reaction that features the "natural" amide bond in the product would be a significant addition to the available methodology.[5,32]

Currently, the most effective chemoselective ligation method for the chemical synthesis of polypeptides and proteins is the *Native Chemical Ligation* (NCL), as was developed by Kent and co-workers.[33,34] This method for the coupling of two unprotected peptides in an aqueous solution is based on the reaction between a thioester and the side chain of a cysteine residue – as reported for the first time by Wieland *et al*. already in 1956.[35] In NCL, the thiolate functionality of an *N*-terminal cysteine residue of one peptide attacks the *C*-terminal thioester functionality of a second peptide to effect transthioesterification. After spontaneous *S*→*N* acyl transfer, a native amide linkage is formed at the site of ligation

(Scheme 1.5). The high potential of the NCL strategy on the chemical synthesis of (modified) proteins is demonstrated by many examples in the literature.[36-38]



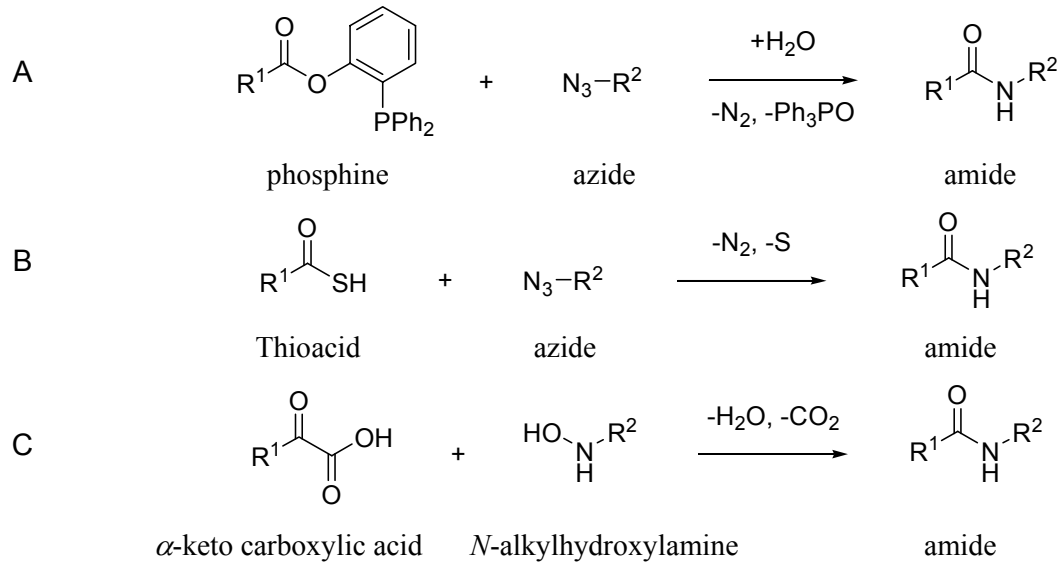
Scheme 1.5 Native Chemical Ligation.

Despite several existing methods for the preparation of thioesters (see also chapter 6), the inherent limitations of SPPS often make the generation of thioesters the bottleneck in NCL. As an alternative to purely chemical methods, the *Expressed Protein Ligation* (EPL)[39] strategy was developed. In EPL a bacterial expression system, based on the intein mediated self splicing mechanism of precursor proteins, is employed to generate peptide thioesters for reaction with *N*-terminal cysteine-bearing peptides. The *N*-terminal cysteine-bearing peptide can either be synthesized chemically or produced using recombinant techniques. This combination of chemical and recombinant DNA methodologies enables the semi-synthesis of large and diverse polypeptides and proteins.[37]

In spite of the widespread use of NCL and EPL as well as several approaches to broaden their applicability,[7,40,41] the requirement for an *N*-terminal cysteine (mimic) at the ligation site is sometimes an obstacle. In this respect, other chemoselective amidation reactions have been described in the literature (Scheme 1.6) that might be used to overcome this requirement since they are, in principle, independent of the amino acid residues at the site of ligation.

For example, in a "traceless" version of the so-called Staudinger ligation (Scheme 1.6A), originally developed for the modification of cell-surface glycans,[42] the reaction between an azide and a phosphine ester is used for the formation of an amide bond. In other examples (Scheme 1.6) an amide bond is introduced by reaction of a thioacid with an azide[43] (B) or

the decarboxylative condensation of an *N*-alkylhydroxylamine and an α-keto carboxylic acid[44] (C). These reactions may provide a basis for novel residue-independent chemoselective amide ligation methods as an extension or alternatives for NCL. At the start of the research described in this thesis, the application of these amidation reactions for chemoselective ligation of unprotected peptides was still unexplored.



Scheme 1.6 Chemoselective amidation reactions.

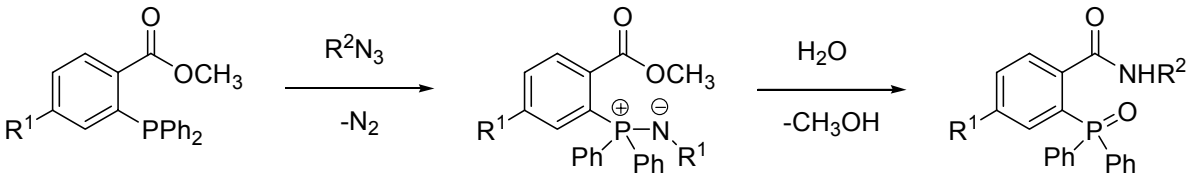
1.6 In vivo compatible ligations

Besides the chemical synthesis of polypeptides and proteins, chemoselective ligation reactions find also application in the site-selective modification of biomolecules to enable their imaging by various methods. In particular, the introduction of biophysical markers and reporter groups by chemoselective ligation methods offers great opportunities for the study of biological function. For example, the labeling of proteins with fluorophores and affinity labels has greatly facilitated their study both *in vitro* and *in vivo*.[45]

At present, the most prominent methods for *in vivo* protein labeling feature recombinant DNA techniques[46] to introduce epitope tags such as fluorescent protein fusions.[6] These biological methods are restricted to the use of proteinogenic amino acids for the modification of exclusively proteins. Furthermore, the often large size of the label (e.g. GFP ~25 kDa) can seriously influence structure and function of the resulting fusion protein. Alternatively, methods based on a two-step bioorthogonal chemical reporter strategy[47] may offer more flexibility. As these chemical methods are not dependent on genetic encoding, also small non-proteinogenic molecules can be used for the labeling of proteins or other

biomolecules such as glycans and lipids. In the chemical reporter strategy, a bioorthogonal functionality (the chemical reporter) is incorporated into the target biomolecule for subsequent conjugation to a suitably functionalized biophysical tag. For *in vivo* applications, these biomolecule-modifications should take place in their natural environment, i.e. cells or tissue. Thus, the reaction should be rapid, selective and efficient at physiological pH and temperatures. Moreover, the reagents must be non-toxic.

Its small size and stability under physiological conditions makes the azide moiety probably the most versatile bioorthogonal chemical reporter group available today. Incorporation of azide moieties into the target biomolecule is mostly achieved via a chemically modified metabolic precursor[48,49] or, in case of proteins, using their enzymatic activity for tagging with active site directed probes.[50-52]



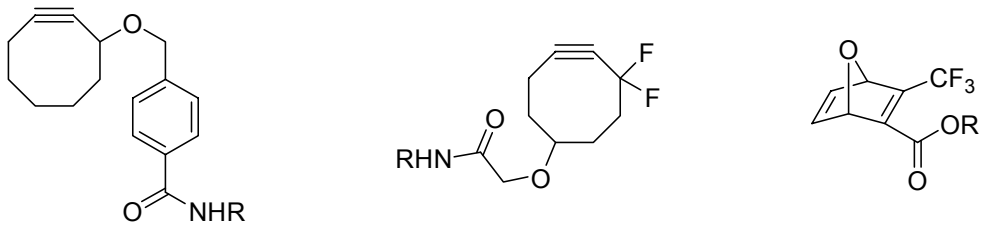
Scheme 1.7 The Staudinger ligation.

In a tremendous effort, Bertozzi and coworkers have employed a modified Staudinger reaction, which occurs between a phosphine and an azide to produce an iminophosphorane, for the modification of cell-surface glycans.[42] In this case, a specifically engineered triarylphosphine was used to trap the iminophosphorane by an intramolecular acylation reaction to form a stable amide bond after hydrolysis (Scheme 1.7). As both reactive partners are abiotic and chemically orthogonal to native cellular components and their reaction is efficient in water at room temperature, the Staudinger reaction meets many criteria required of a chemoselective ligation method in a cellular environment. Indeed, azides, incorporated within cell surface glycoconjugates by metabolism of a synthetic azidosugar, could be reacted with a biotinylated triarylphosphine to form stable cell-surface adducts suitable for fluorescent avidin staining. Although the Staudinger ligation method is highly biocompatible, even allowing for cell surface and live organism applications, it suffers from sluggish reaction kinetics and competing oxidation of the phosphine reagent. Therefore, this method is unsuitable for detection of low-abundance species or to visualize biological processes that occur faster than the timescale of the chemical reaction.

In contrast, the copper-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition (click chemistry) allows for high sensitivity detection of azides. The method has been successfully utilized for activity-based protein profiling in cell lysates and visualization of biomolecules

in fixed cells, where the unreacted probe can be readily washed out before imaging. However, the toxicity of the copper catalyst forms a serious limitation to *in vivo* applications of click chemistry.

Alternatively, alkynes can be activated toward 1,3-dipolar cycloaddition with azides by use of ring strain. For instance, cyclooctynes have been used for the bioorthogonal labeling of azides, moreover, the cyclooctyne probe (**1**, Figure 1.1) demonstrated no cellular toxicity.[53] Nevertheless, the sensitivity of this method did not exceed that of the Staudinger ligation method. Therefore, there remains great interest in new azide ligation reactions that combine high sensitivity and biocompatibility.[54]



1 **2** **3**

Figure 1.1 Reagents for ring strain promoted cycloaddition with azides.

Recently, the rate of the strain-promoted azide/alkyne Huisgen 1,3-dipolar cycloaddition reaction could be significantly improved by the introduction of electron withdrawing substituents to the cyclooctyne ring. The resulting difluorinated cyclooctyne (**2**) reagent demonstrated similar sensitivity to the copper-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition for azide detection, both *in vitro* and on cultured cells. In this way, good bio-compatibility (as in the Staudinger ligation) could be combined with a high sensitivity similar to the copper-catalyzed click reaction.[55,56]

The reaction kinetics obtained *in vitro* for **2** are also superior to another newly reported triazole forming reaction that features the combination of ring strain and electron withdrawing fluorous substituents to promote a cycloaddition–retro-Diels–Alder reaction (tandem crDA) between azides and trifluoromethyl-substituted oxanorbornadiene derivatives (**3**).[57] Moreover, in the latter method a relatively toxic furan side product is formed, which could be disadvantageous for *in vivo* and *in vitro* applications.

In principle, **2** should provide an excellent tool for *in vivo* labeling of azides, however, its efficiency was reduced compared to the Staudinger ligation. This difference in reaction efficiency *in vivo* versus *in vitro* for **2**, reveal solubility and bioavailability as important determinants for *in vivo* efficacy.[55]

Thus, the *in vivo* environment adds yet another criterium to the list of requirements for the ideal chemoselective ligation method. Next to selectivity, bioorthogonality, rapid reaction and non-toxic reagents, also the bio-availability of the reagents is crucial for *in vivo* applications. Therefore, there remains a need for development of next-generation reagents for azide ligation reactions that meet all mentioned criteria. Ultimately, in order to simultaneously monitor multiple biomolecules *in vivo*, a series of chemoselective ligation reactions that are fully orthogonal to one another is necessary.[58]

1.7 Aim and outline of the thesis

From the aforementioned paragraphs it is evident that chemoselective ligation reactions are of great interest to the fields of chemistry and biology. By the site-selective conjugation of biomolecules, complex bioconjugates can be obtained that may find application in the study of biological functions. In particular, convergent polypeptide synthesis is highly dependent on methods for the chemoselective ligation of peptide segments. The most sophisticated methods (NCL/EPL) facilitate the aqueous coupling of unprotected peptide segments via a native amide bond. A restriction of these methods, however, is the need for a cysteine (mimic) at the site of ligation. To date, a general applicable method for the chemical synthesis of (poly)peptides featuring a chemoselective amidation reaction that is independent of the amino acid residue at the ligation site, is not yet available and would be highly desirable.

On the other hand, chemoselective ligation reactions can also be used for the imaging of biomolecules, for example, via a two-step bioorthogonal chemical reporter strategy. In this context, the azide moiety is the most versatile bioorthogonal chemical reporter group available for introduction into biomolecules and subsequent conjugation to a suitably functionalized biophysical tag. Although several methods have been used and new methods to extend their applicability to *in vivo* studies are being pursued, there remains a need for alternative methods for azide-based chemoselective ligation. Moreover, the high complexity of biomolecules combined with the wide range of applications, generates a continuing demand for the development of new chemoselective ligation reactions as a tool for bioconjugation.

Obviously, *in vivo* applications have the highest requirements for the chemoselective ligation method to be used. Nonetheless, chemoselective amidation reactions that do not meet the exact conditions needed for application in a certain biological environment, are still of high significance. In case these reactions feature the right properties, they could form a valuable addition to the arsenal of click reactions with potential applications ranging

from bioconjugation to the efficient synthesis of libraries supporting the process of lead finding.
In this thesis, azide-based chemoselective amidation reactions will be evaluated as tool for the synthesis and modification of peptides.

Outline of the thesis

Chapter 2 evaluates the application of a traceless version of the so-called Staudinger ligation on the chemoselective ligation of peptide segments. First, the synthesis of *N*-terminal peptidyl azides and *C*-terminal peptide *o*-(diphenylphosphine)phenyl esters is described. Subsequently, their reaction is monitored by mass spectrometric analysis in order to explain the observed intermediates and gain a better insight into the mechanism of the Staudinger ligation for optimization of the amidation reaction. Finally, the optimized reaction conditions are used for the chemoselective coupling of peptides containing amino acids other than glycine at the ligation site.
In **chapter 3** factors concerning substituents at phosphorus, the removable auxiliary, solvent and amino acid residues at the ligation site are investigated for their influence on the Staudinger ligation via both experimental and computational methods. The results obtained in this chapter are used to get an insight into the bottle-necks of the chemoselective ligation of peptide segments by the Staudinger ligation.
In **chapter 4** the scope and limitations of the reaction between thio acids and azides as a new amide-forming chemoselective ligation reaction is explored. Therefore, a series of unprotected functionalized peptide thio acids and azidopeptides is synthesized and used in this amidation reaction. Furthermore, optimization of the thio acid/azide amidation reaction by the addition of $RuCl_3$ and PPh_3 to respectively activate the thio acid- and azide moiety is investigated.
Chapter 5 reports on the convenient synthesis of protected β-substituted aminoethane sulfonyl azides starting from amino acids. The resulting sulfonyl azides form stable building blocks that can be incorporated into peptides, but are also highly activated toward reaction with (peptide) thio acids. The properties of this thio acid/sulfonyl azide amidation reaction are investigated for compatibility with chemoselective ligation strategies.
In **chapter 6** an efficient loading strategy for the *N*-acyl sulfonamide linker is developed based on a resin-bound sulfonyl azide/thio acid amidation reaction. The activation of the linker towards nucleophilic cleavage is optimized using a microwave-assisted *N*-alkylation of the *N*-peptidyl sulfonamide moiety. Subsequent treatment with suitably functionalized nucleophiles provides access to *C*-terminally modified peptides which may be applied in chemoselective (bio)conjugation or ligation methods.

In **chapter 7** the spontaneous and irreversible coupling of thio acids and sulfonyl azides is used for the fast and efficient synthesis of a focussed series of peptide-based *N*-acyl sulfonamides with interesting physicochemical properties.
The properties of the thio acid/sulfonyl azide amidation reaction, as described in the previous chapters, resemble those of the "ideal click reaction". Additionally, the *N*-acyl sulfonamide moiety renders special properties to the coupling product since it functions as a carboxylic acid isostere. In **chapter 8**, the special properties of the thio acid/sulfonyl azide amidation reaction are employed for the chemoselective ligation of peptide segments and to obtain peptide dendrimers and proline dipeptide based organocatalysts. The synthetic approaches in this chapter give an outlook toward application of the thio acid/sulfonyl azide amidation reaction on the chemoselective conjugation of peptides.

1.8 References and notes

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2

Chemoselective Coupling of Peptide Segments Using the Staudinger Ligation

In this chapter, the first Staudinger ligations which yield tetra- and pentapeptides starting from azido peptides and *C*-terminal peptide *o*-(diphenylphosphine)phenyl esters are reported. Mass spectrometric analysis of the reaction mixture provided a better insight into the mechanism of the Staudinger ligation and was used to explain the observed intermediates and to optimize the ligation reaction. As a result, the optimized reaction conditions enabled the chemoselective coupling of peptides containing amino acids other than glycine at the ligation site.

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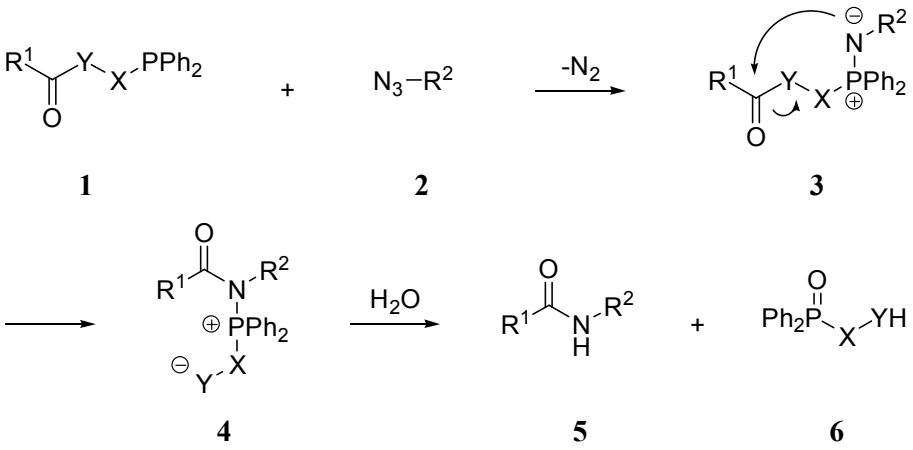
2.1 Introduction

Chemoselective ligation reactions are now established tools in chemical biology to obtain complex biomolecules and in particular polypeptides.[1,2] Currently, the most effective chemoselective ligation method for the chemical synthesis of polypeptides is the *Native Chemical Ligation* (NCL), as has been developed by Kent and co-workers.[3,4] Although several approaches have been used to broaden the applicability of NCL,[5-9] the method remains largely restricted in its use by the requirement of a *N*-terminal cysteine residue as cysteine comprises only 1.7% of all amino acid residues in proteins.[10] This restriction can partially be overcome by the use of cysteine-mimicking auxiliaries.[11-14] However, to be efficient, a glycine residue has to be present at the ligation site. This means that, although glycine is the most common amino acid in proteins (7.2% of all residues),[10] these methods are not sequence-independent and more general methods for the chemoselective ligation of peptides remain desirable.



Scheme 2.1 The traceless Staudinger ligation. Bertozzi: X = *o*-Ph, Y = O; Raines X = CH_2, Y = S.

Recently, a novel chemoselective ligation reaction has been described, based on a modified Staudinger reaction which was developed earlier for the modification of cell-surface glycans.[15] Here the highly selective reaction between an azide and a phosphine yields an iminophosphorane intermediate which can be trapped by an intramolecular acylation reaction to form a stable amide bond after hydrolysis (Chapter 1). Application in peptide synthesis would be highly interesting since this so-called *Staudinger ligation* method should be independent of the nature of the amino acid side chain at the ligation site. The proof of principle for application on peptide synthesis was independently shown by the groups of Raines[16,17] and Bertozzi[18] by using a *traceless* Staudinger ligation (Scheme 2.1).

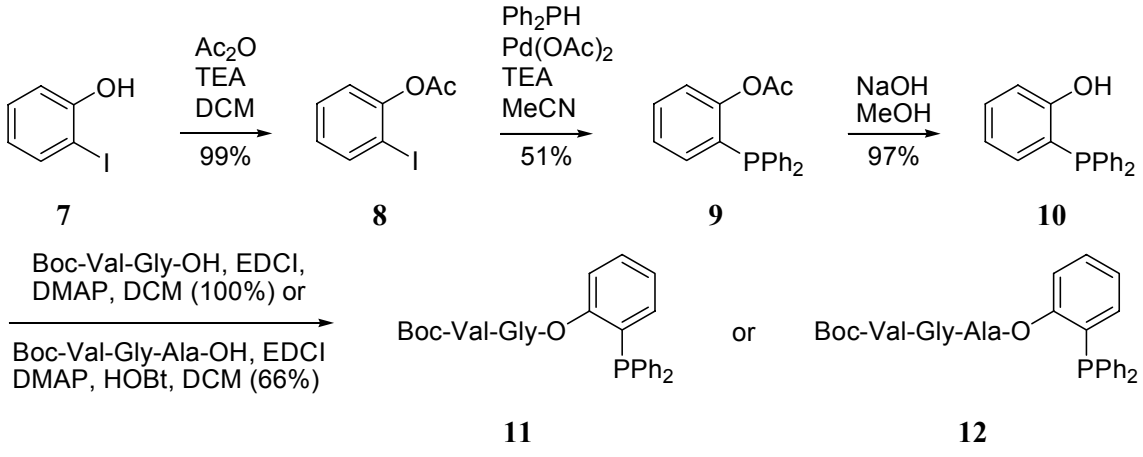
In this method a cleavable ester-linkage was introduced to the auxiliary which made it possible that no residual atoms of the phosphine remained in the amide product. However, in this work the method has only been used for acetyl transfer to an azido acid or for the synthesis of *di*peptides in which at least one glycine residue was present. To explore the possibilities of the Staudinger ligation for the total synthesis of polypeptides/proteins, in this chapter, the ligation of *peptide segments* was studied.

Although thioesters are for obvious reasons popular in chemical ligation strategies, we envisioned (oxy)esters as promising alternatives. In fact, protein synthesis in Nature involves the use of (oxy)esters of ribose in aa-tRNA substrates.[19] Thus, by proper tuning of the reactivity of an ester and/or the reaction conditions, it should be possible to use ultimately (oxy)esters in chemical ligation strategies for efficient synthesis of proteins. In addition, thioesters are not always synthetically easy accessible. Thus, it was decided to use (oxy)esters e.g. the *o*-(diphenylphosphine)phenol auxiliary (X = *o*-Ph, Y = O, Scheme 2.1),[18] to transfer a peptidyl segment to an azido peptide in a Staudinger ligation.

2.2 Results and Discussion

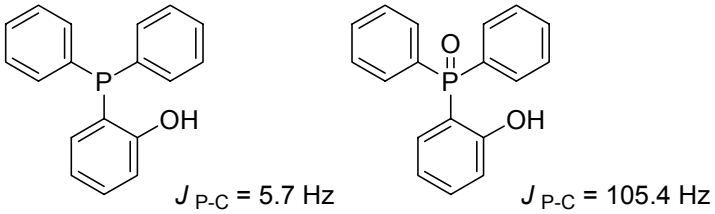
In order to explore the possibilities of the Staudinger ligation for the total synthesis of proteins, a series of peptide *o*-(diphenylphosphine)phenyl esters and azido peptides varying in side chain functionalities and amino acid residues at the ligation site were synthesized.

2.2.1 Synthesis of peptide o-(diphenylphosphine)phenyl esters



Scheme 2.2 Synthesis of phosphine esters **11** and **12**.

The *o*-(diphenylphosphino)phenol auxiliary **10** (Scheme 2.2) was first synthesized by a Pd-mediated reaction of diphenylphosphine with 2-iodophenol as described by Herd *et al.*[20,21] However, following this procedure **10** was isolated in low yield (~10%) caused by oxidation of the phosphine product. Presumably this oxidation takes place during workup when the reaction mixture, containing the Pd catalyst, is exposed to air. Alternatively, oxidation could be reduced by treating compound **7** with acetic anhydride prior to the reaction with Ph_2PH. This procedure afforded compound **10** in an improved overall yield of 49%. Oxidation of the phosphine products was detected via the coupling constants in the ^{13}C-NMR spectrum: the coupling constant for phosphine **10** is significantly smaller than the coupling constant for phosphine oxide **13** (Figure 2.1).

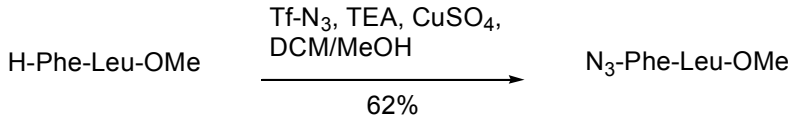


10 **13**

Figure 2.1 Coupling constants for phosphine **10** and phosphine oxide **13**.

Subsequently, **10** was coupled to Boc-Val-Gly-OH and Boc-Val-Gly-Ala-OH by carbodiimide-mediated esterification yielding phosphine esters **11** and **12**, respectively.

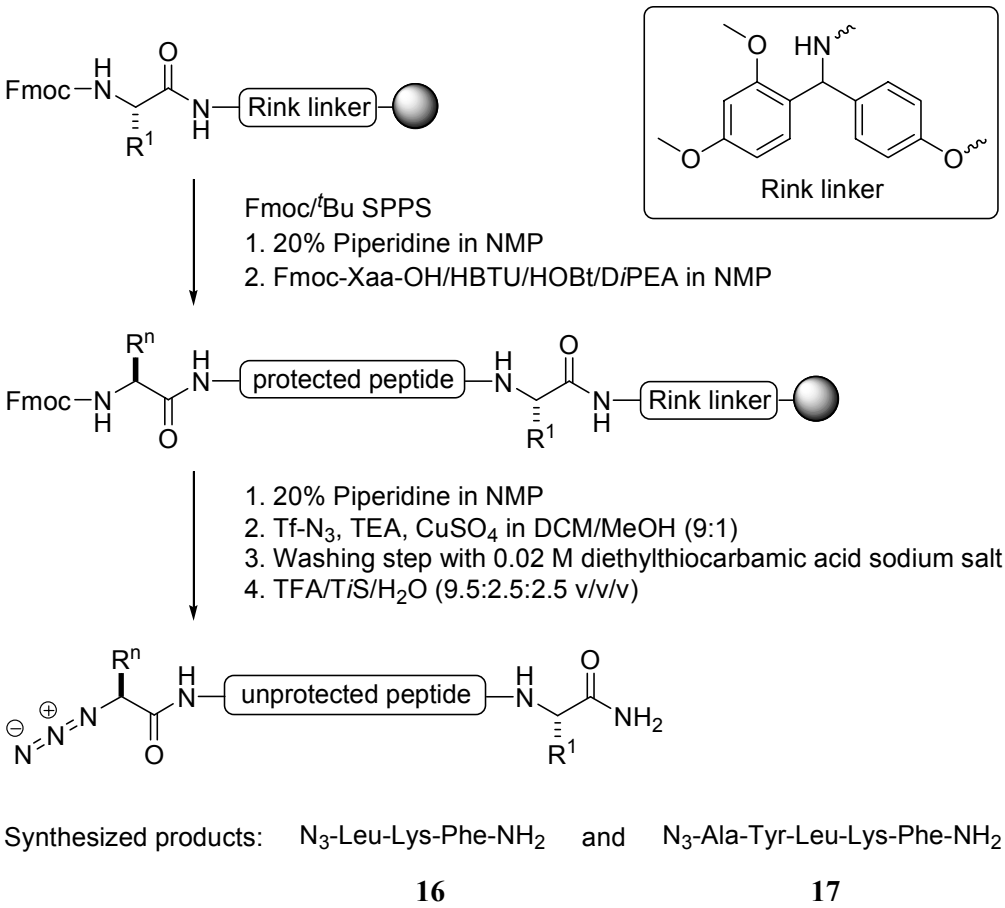
2.2.2 *Synthesis of azido peptides*



14 **15**

Scheme 2.3 Synthesis of azido peptide **15** in solution.

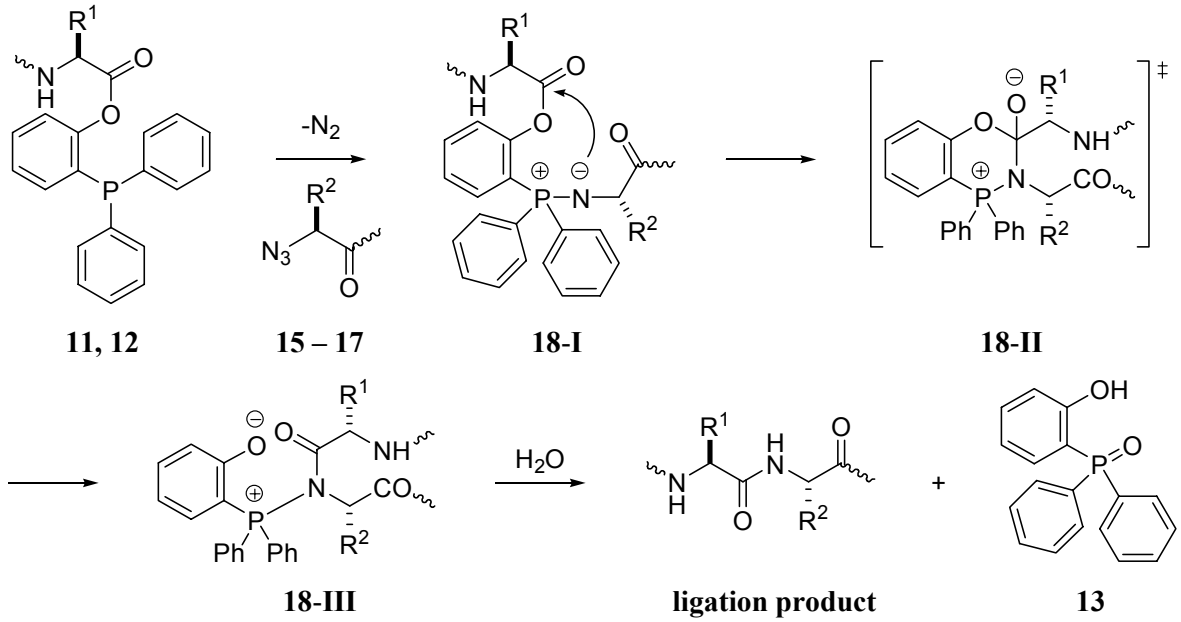
Then, the synthesis of the azido peptides was performd via a diazotransfer using triflic azide as diazo donor. Dipeptide **15** was synthesized in solution following the literature procedures of Wong *et al.*[22] and Pelletier *et al.*[23] (Scheme 2.3). The synthesis of azido peptides **16** and **17** was performed using a modified procedure for diazotransfer on the solid support, as was developed in our group (Scheme 2.4).[24]



Scheme 2.4 Solid phase synthesis of azido peptides **16** and **17**.

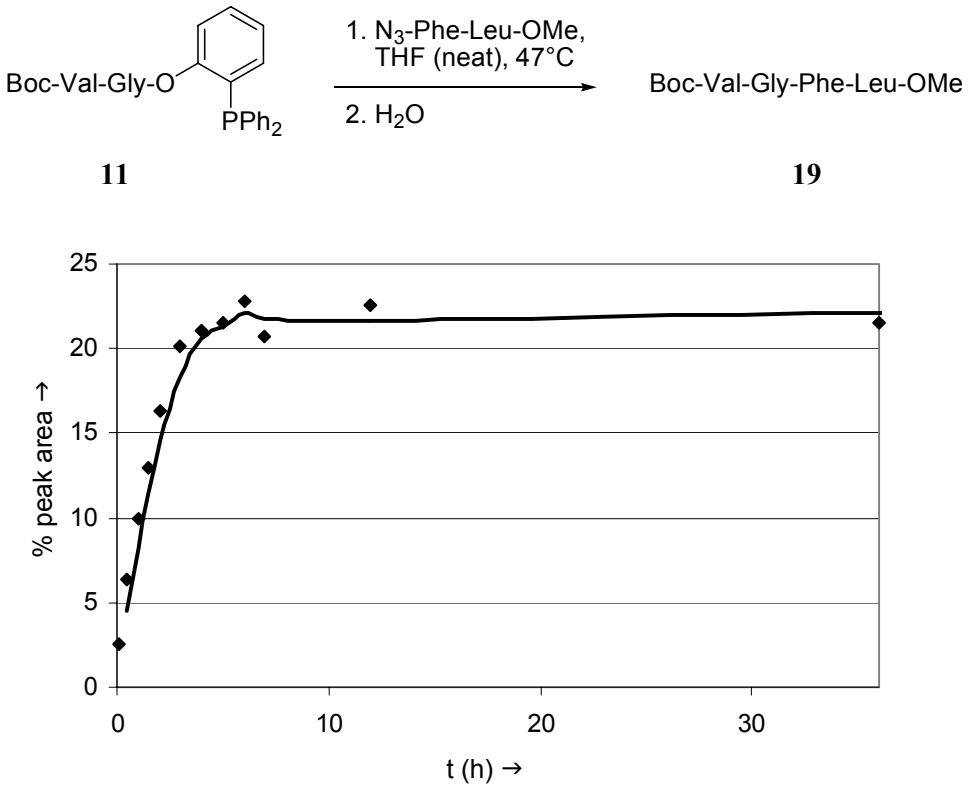
2.2.3 Ligation reactions

Ligation of phosphine **11** and azide **15** was carried out at 50 mM in THF/H_2O 3:1 v/v as was originally described by Bertozzi and coworkers[15,18] (entry 1, Table 2.1). The reaction was monitored by TLC and MS.[25] It was found that the iminophosphorane intermediate (**18-I**, Scheme 2.5) was formed almost instantaneously. An intramolecular *O*→*N*-acyl shift may result in the formation of amidophosphonium salt **18-III** via transition state **18-II**. However, **18-I**, **II** and **III** have identical molecular mass and therefore it is impossible to distinguish between these intermediates. Despite its presumed rapid hydrolysis, **18** was found to be very stable and the ligation product Boc-Val-Gly-Phe-Leu-OMe (**19**, Scheme 2.6) was formed in minimal amounts while by-products resulting from e.g. the hydrolysis of the ester bond and oxidation of the phosphine were found as major products instead.



Scheme 2.5 Putative mechanism for the Staudinger ligation using the *o*-(diphenylphosphino)phenol auxiliary.

In an attempt to reduce the side-product formation and/or to increase the concentration of possible intermediate **18**, it was decided to run the Staudinger ligation with only one equivalent or in the absence of water. After hydrolysis, the equilibrium mixture should result in the preferred formation of ligation product **19**. Indeed, TLC and ESMS analysis showed only the formation of **18**, and ligation product **19** was isolated in approx 25% yield. Apparently, the absence of excess water favoured the intramolecular acyl migration. The next step in the optimization process was to increase the temperature, according to Bertozzi *et al.*, 47°C should be ideal.[26] Running the Staudinger ligation at this temperature resulted in the formation of **19** in an increased yield of 36%. When this reaction was repeated and monitored by HPLC, it was found that formation of the ligation product reached a plateau after approx 5 h (Scheme 2.6).



Scheme 2.6 HPLC analysis Staudinger ligation of **11** with **15** to form **19**.

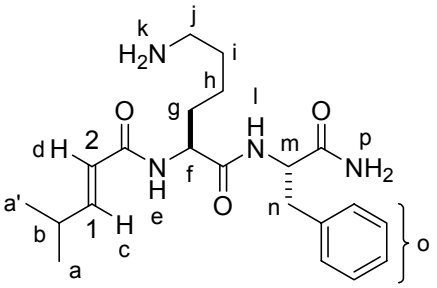
The results for the optimization of the Staudinger ligation of **11** with **15** to form **19** are summarized in Table 2.1.

Table 2.1 Optimization Staudinger ligation of **11** with **15** to form **19**.

Entry	Solvent	T	t	% Yield
1	THF/H_2O (3:1)	rt	7 d	ESMS
2	THF/1 equiv H_2O	rt	5 d	24
3	THF (neat)	rt	5 d	26
4	THF (neat)	47°C	16 h	36

The optimized reaction conditions were used for the ligation of peptides **12** and **15** in which a glycine residue was absent at the ligation site. The ligated peptide Boc-Val-Gly-Ala-Phe-Leu-OMe (**20**) was obtained in a satisfactory yield of 32% (entry 2, Table 2.2).

Since one of the major goals of chemoselective ligation strategies of peptides is the ligation of *unprotected* peptides, azido peptide **16** has been used as a model of an unprotected peptide. This model peptide was also chosen to study any non-specific aminolysis of the phosphine by the nucleophilic ε-amino group of the lysine residue. Ligation of **11** and **16** resulted in formation of Boc-Val-Gly-Leu-Lys-Phe-NH_2 (**21**) in a somewhat disappointing yield of 6%. 2D NMR studies (TOCSY) showed the presence of an amide-NH – Leu-αCH correlation, however, an amide-NH – Lys-εCH_2 correlation was also found, hence a non-specific aminolysis could not be ruled out. This means that **21** contained at least two ligation products with equal molecular mass and HPLC retention times. Although (oxy)esters are less sensitive towards nucleophilic attack than the intrinsically reactive thioesters, non-specific aminolysis, was most likely observed which was in contrast to the results of Raines *et al.*[16]



22

Position	δ_C	δ_H	$J_{H^1\text{-}H^2}$
1	150.0	6.66 (dd)	15.5 Hz
2	129.9	5.88 (d)	15.5 Hz

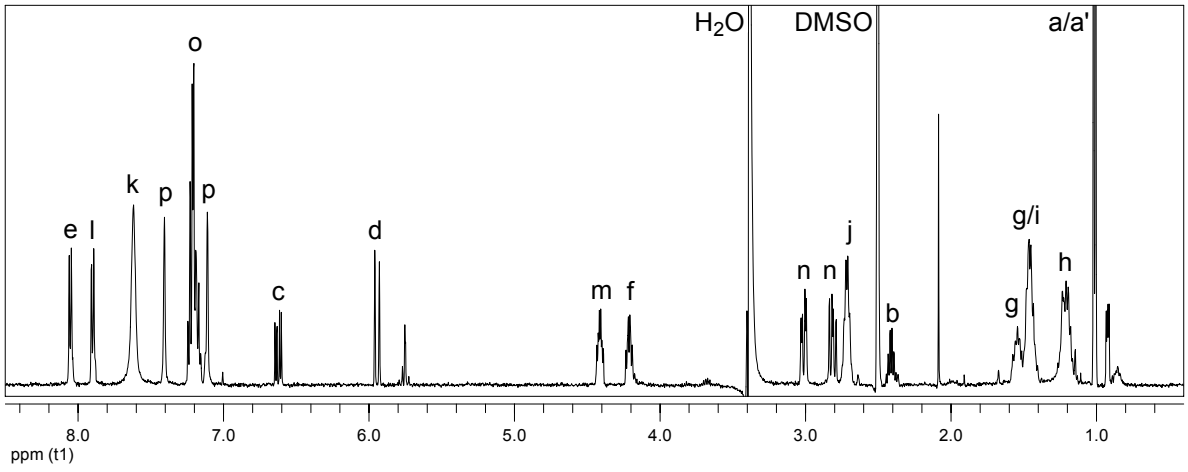


Figure 2.2 ^{1}H NMR spectrum and assignments of compound **22** (500 Mhz, DMSO, δ in ppm).

Besides ligation product **21**, the formation of a side product with m/z 389 was also detected in the reaction mixture. After 5 days of reaction time the compound was isolated as the major product in 31% yield by preparative HPLC and its identity was investigated by NMR

spectroscopy. ^{1}H NMR analysis showed the characteristic patterns for Leu, Lys and Phe residues, aditionally a *C*-terminal amide moiety could be identified. The molecular mass of 388 amu however, was 17 amu lower than that of tripeptide H-Leu-Lys-Phe-OMe. Combination of these findings led to **22** (Figure 2.2) as the proposed structure for the isolated side product. This hypothesis was confirmed by a HSQC measurement as the signals at 6.65 ppm and 5.96 ppm in the ^{1}H NMR spectrum could be assigned to the vinylic protons of desamino tripeptide **22**. The coupling constants revealed that **22** possessed the *E* configuration. The exact origin of this side product remains unclear but presumably it originates from a proton abstraction at the β-position of leucine by the basic nitrogen of the iminophosphorane intermediate, followed by elimination of a R_3P=NH fragment.
Next, ligation of peptides **12** and **16** resulted in the formation of Boc-Val-Gly-Ala-Leu-Lys-Phe-NH_2 (**23**) which could only be detected by ESMS. It should be mentioned, however, that the formation of the intermediate **18-I/III** resulting from **12** and **16** was rapid and unambiguously detected by ESMS and was stable for at least 2 h in aqueous THF. The apparent stability of said intermediates may have resulted in a slow product formation and a low yield.

Table 2.2 Results for the Staudinger ligation of unprotected peptide segments. The ligation site is located between the amino acid residues in italics.

Entry	Phosphine	Azide	Product	Sequence	% Yield
1	**11**	**15**	**19**	Boc-Val-*Gly-Phe*-Leu-OMe	36
2	**12**	**15**	**20**	Boc-Val-Gly-*Ala-Phe*-Leu-OMe	32
3	**11**	**16**	**21**	Boc-Val-*Gly-Leu*-Lys-Phe-NH_2	6
4	**12**	**16**	**22**	Boc-Val-Gly-*Ala-Leu*-Lys-Phe-NH_2	ESMS
5	**11**	**17**	**24**	Boc-Val-*Gly-Ala*-Tyr-Leu-Lys-Phe-NH_2	ESMS
6	**12**	**17**	**25**	Boc-Val-Gly-*Ala-Ala*-Tyr- Leu-Lys-Phe-NH_2	no rxn

Similarly, ligation of peptides **11** and **17** resulted in the formation of Boc-Val-Gly-Ala-Tyr-Leu-Lys-Phe-NH_2 (**24**) which could only be detected by ESMS. Finally, the ligation of **12** and **17** did not yield any detectable amount of the ligation product Boc-Val-Gly-Ala-Ala-Tyr-Leu-Lys-Phe-NH_2 (**25**). The results for all peptide ligations using the Staudinger ligation are summarized in Table 2.2.

2.3 Conclusions

In conclusion, it was shown that azido peptides and *C*-terminal peptide *o*-(diphenylphosphine)phenyl (oxy)esters are suitable synthons for the synthesis of, up to now, pentapeptides featuring the Staudinger ligation. Moreover, it was also shown that chemical ligation of peptides is, in principle, independent of the presence of glycine residues at the ligation site. However, increasing complexity of the peptide segments caused a decrease in the yield of their ligation, probably due to incomplete reactions caused by competing side reactions. These side reactions likely take place when the intramolecular acyl migration is slowed down by, for instance, steric congestion in the iminophosphorane intermediate. Therefore, although the proof of principle for a sequence independent Staudinger ligation has been delivered, further optimization of the conditions is needed for application to the chemical ligation of large peptide segments.

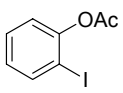
2.4 Experimental section

General information

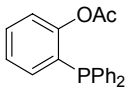
Analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90 Å, 5 μm, 250 × 4.6 mm), using a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at 1 mL/min, UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000). Preparative HPLC was performed on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 10 μm, 250 × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 11.5 mL/min. Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. ^{1}H NMR spectra were recorded on a Varian G-300 (300.1 MHz) or a Varian INOVA-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm) or or DMSO-d_6 (2.50 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) or a Varian INOVA-500 (125 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm) or DMSO-d_6 (39.5 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F^{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[27]

Chemicals and reagents

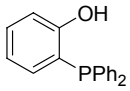
Unless stated otherwise, all chemicals were obtained from commercial sources and used as such. Peptide grade dichloromethane (DCM), tetrahydrofuran (THF), ethyl acetate (EtOAc), hexane, chloroform, HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Biosolve. Trifluoromethanesulfonic acid anhydride (triflic anhydride) was obtained from Fluka. 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and all amino acids were purchased from GI Biochem Shanghai. 4-(*N*,*N*-dimethylamino)pyridine (DMAP), *N*,*N'*-diisopropylethylamine (D*i*PEA), palladium(II) acetate and copper(II) sulfate were obtained from Acros Organics, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) and 2-iodophenol were purchased from Aldrich, acetic acid (HOAc) was obtained from Merck KGaA. 1-Hydroxybenzotriazole (HOBt) was purchased from Advanced Chemtech. DCM and DMSO were dried using molsieves 4Å and MeOH was dried using molsieves 3Å.



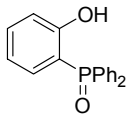
2-Iodophenyl acetate (8): Acetic anhydride (2.65 mL, 28.02 mmol) was dissolved in DCM (10 mL) and added drop wise to a solution of 2-iodophenol (**7**) (5.00 g, 22.73 mmol) and TEA (3.95 mL, 28.41 mmol) in dry DCM (25 mL). After stirring for 16 h the reaction mixture was concentrated *in vacuo*. The residue was dissolved in Et_2O (250 mL) and this solution was subsequently washed with H_2O (100 mL), 1N $KHSO_4$ (3 × 100 mL) and brine (100 mL), dried ($MgSO_4$) and evaporated to dryness. The product (5.90 g, 99%) was obtained as a clear oil. R_f: 0.70 (DCM); ^{1}H NMR ($CDCl_3$): δ = 7.84 (m, 1H), 7.36 (m, 1H), 7.11 (m, 1H), 6.98 (m, 1H), 2.37 (s, 1H).



***o*-(diphenylphosphine)phenyl acetate (9):** TEA (2.77 mL, 19.85 mmol), $Pd(OAc)_2$ (catalytic amount) and Ph_2PH (3.14 ml, 18.05 mmol) were successively added under a nitrogen atmosphere to a solution of 2-iodophenyl acetate (**8**) (4.73 g, 18.05 mmol) in dry and deoxygenated MeCN (50 mL). The reaction mixture was heated under reflux for 24 h followed by stirring at rt for 18 h. After filtration the solvent was removed *in vacuo*. The product (2.93 g, 51%) was isolated by flash column chromatography (EtOAc/hexane 2:8 v/v) as a clear oil that crystallized overnight. R_f: 0.63 (EtOAc/Hexane 2:8 v/v); ^{1}H NMR ($CDCl_3$): δ = 7.35 (m, 11H), 7.13 (m, 2H), 6.85 (m, 1H), 1.97 (s, 3H); ^{13}C NMR ($CDCl_3$): δ = 168.9, 152.8/152.5 ($J_{P\text{-}C}$ = 17.8 Hz), 135.5/135.4 ($J_{P\text{-}C}$ = 9.2 Hz), 134.1/133.8 ($J_{P\text{-}C}$ = 20.6 Hz), 133.7, 130.4/130.2 ($J_{P\text{-}C}$ = 14.9 Hz), 129.9, 129.0, 128.6/128.5 ($J_{P\text{-}C}$ = 8.0 Hz), 126.1, 122.6, 20.5; elemental analysis calcd (%) for $C_{20}H_{17}O_2P$: C 74.99, H 5.35, found: C 74.68, H 5.42.



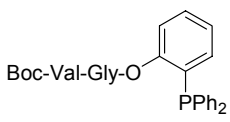
***o*-(diphenylphosphine)phenol (10):** 1.47 g (4.59 mmol) of *o*-(diphenylphosphine)phenyl acetate (**9**) was dissolved in dry and deoxygenated MeOH (35 mL). Subsequently NaOH (0.38 g, 9.18 mmol) was added under a nitrogen atmosphere. After stirring for 45 min the reaction was quenched with 1N $KHSO_4$ (9.7 mL). The reaction mixture was concentrated to approx 10 mL before 100 mL of EtOAc was added. Next, the EtOAc layer was washed with 1N $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), and dried (Na_2SO_4). The product (1.24 g, 97%) was obtained as a white solid. R_f: 0.54 (EtOAc/Hexane 2:8 v/v); ^{1}H NMR ($CDCl_3$): δ = 7.36 – 7.23 (br m), 7.00 – 6.84 (br m, total 14H), 6.19 (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 159.2/158.9 ($J_{P\text{-}C}$ = 18.3 Hz), 135.0/134.9 ($J_{P\text{-}C}$ = 5.7 Hz), 134.7 (2 lines, $J_{P\text{-}C}$ = 3.4 Hz), 133.5/133.2 ($J_{P\text{-}C}$ = 18.3 Hz), 131.6, 129.0/128.7 ($J_{P\text{-}C}$ = 19.5 Hz), 128.6, 121.1 (2 lines, $J_{P\text{-}C}$ = 2.3 Hz), 121.0/120.9 ($J_{P\text{-}C}$ = 5.7 Hz), 115.5 (2 lines, $J_{P\text{-}C}$ = 2.3 Hz). elemental analysis calcd (%) for $C_{18}H_{15}OP$: C 77.69, H 5.43, P 11.13, found: C 77.60, H 5.53, P 11.20.



***o*-(diphenylphosphine oxide)phenol (13):** 0.10 g (0.36 mmol) of *o*-(diphenylphosphine)phenol (**10**) was dissolved in MeOH. Subsequently 2% H_2O_2 (8 mL) was added. After stirring for 3 d, a portion of **13** was isolated by filtration as a white powder (53 mg, 50%). R_f: 0.36 (DCM/MeOH 96:4 v/v); ^{1}H NMR ($CDCl_3$): δ = 7.71 – 7.03 (br m), 7.00 – 6.85 (br m, total 14H), 3.61 (s, 1H); ^{13}C NMR ($CDCl_3$): δ = 162.5, 134.5/134.4 ($J_{P\text{-}C}$ = 2.3 Hz), 132.5/132.5 ($J_{P\text{-}C}$ = 3.4 Hz), 132.3/132.2 ($J_{P\text{-}C}$ = 10.3 Hz), 131.8/131.6 ($J_{P\text{-}C}$ = 11.5 Hz), 131.7/130.4 ($J_{P\text{-}C}$ = 105.4 Hz), 128.7/128.5 ($J_{P\text{-}C}$ = 12.6 Hz), 119.3/119.2 ($J_{P\text{-}C}$ = 12.6 Hz), 118.0/117.9 ($J_{P\text{-}C}$ = 6.9 Hz), 112.2/110.8 ($J_{P\text{-}C}$ = 105.4 Hz).

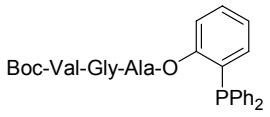
General procedure for the synthesis peptide *o*-(diphenylphosphine)phenyl esters 11 and 12:

The peptide carboxylic acid was dissolved in dry and deoxygenated DCM (20 mL) and the solution was cooled on ice. Subsequently *o*-(diphenylphosphine)phenol, DMAP and EDCI were added under a nitrogen atmosphere. After stirring for 16 h the reaction mixture was concentrated *in vacuo*. The residue was dissolved in EtOAc (250 mL) and this solution was subsequently washed with 1N $KHSO_4$ (2 × 100 mL) and brine (2 × 100 mL), dried (Na_2SO_4) and evaporated to dryness. The product was isolated by column chromatography.



Boc-Val-Gly-*o*-(diphenylphosphine)phenyl ester (11): The reaction was carried out following the general procedure using 0.67 g of Boc-Val-Gly-OH (2.43 mmol), 0.68 g of **10** (2.43 mmol), 30 mg of DMAP

(0.24 mmol, 0.1 equiv) and 0.49 g of EDCI (2.55 mmol, 1.05 equiv). After flash column chromatography (DCM/MeOH 98:2 v/v) the product (1.32 g) was obtained as a white foam in quantitative yield. R_f: 0.42 (DCM/MeOH 98:2 v/v); R_t: 22.45; $[\alpha]_D$: –27.0° c = 0.24 MeOH; ^{1}H NMR ($CDCl_3$): δ = 7.41 – 6.87 (broad m, 14H), 6.24 (s, 1H), 5.08 (d, J = 8.8 Hz, 1H,), 4.05 (m, 3H,), 2.18 (1H), 1.45 (s, 9H), 0.96 (dd, J = 9.9 Hz, J = 19.8 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ = 171.7, 167.7, 155.2, 152.2/152.0 ($J_{P\text{-}C}$ = 16.0 Hz), 135.2/135.1 ($J_{P\text{-}C}$ = 8.0 Hz), 134.1/133.8 ($J_{P\text{-}C}$ = 20.6 Hz) 130.3/130.1 ($J_{P\text{-}C}$ = 14.9 Hz), 130.0, 129.2, 128.7/128.6 ($J_{P\text{-}C}$ = 6.9 Hz), 126.6, 122.4, 80.0, 59.6, 40.9, 30.8, 28.3, 19.2, 17.4; ESMS calcd for $C_{30}H_{35}N_2O_5P$: 534.23, found: 479.40 $[(M\text{-}C_4H_8)+H]^+$, 535.45 $[M+H]^+$, 557.40 $[M+Na]^+$; elemental analysis calcd (%) for $C_{30}H_{35}N_2O_5P$: C 67.40, H 6.60, N 5.24, P 5.79; found: C 67.26, H 6.48, N 5.18, P 5.91.



Boc-Val-Gly-Ala-*o*-(diphenylphosphine)phenyl ester (12): The reaction was carried out following the general procedure using 0.71 g of Boc-Val-Gly-Ala-OH (2.06 mmol), 0.57 g of **10** (2.06 mmol), 0.35 g of HOBt (2.27 mmol, 1.10 equiv), 25 mg of DMAP (0.21 mmol, 0.1 equiv) and 0.42 g of EDCI (2.16 mmol, 1.05 equiv). After flash column chromatography (DCM/MeOH 96:4 v/v) the product (1.32 g) was obtained as a white foam in 66% yield. R_f: 0.35 (DCM/MeOH 96:4 v/v); R_t: 19.13 min; $[\alpha]_D$: –37.7° c = 0.24 MeOH; ^{1}H NMR ($CDCl_3$): δ = 7.40 – 7.19 (br m, 14H), 6.79 (m, 1H), 6.70 (d, J = 6.6 Hz, 1H), 5.14 (d, J = 8.0 Hz, 1H), 4.65 (m, 1H), 4.01 (m, 3H), 2.17 (m, 1H), 1.43 (s, 9H), 1.31 (d, J = 7.4 Hz, 3H), 0.98 (dd, J = 6.9 Hz, J = 12.6 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ = 172.1, 170.4, 168.2, 155.9, 152.6/152.3 ($J_{P\text{-}C}$ = 17.2 Hz), 135.4/135.2 ($J_{P\text{-}C}$ = 10.3 Hz), 134.0/133.8 ($J_{P\text{-}C}$ = 19.5 Hz), 134.0/133.7 ($J_{P\text{-}C}$ = 20.6 Hz), 130.1, 129.8/129.6 ($J_{P\text{-}C}$ = 14.9 Hz), 129.1, 128.9/128.7 ($J_{P\text{-}C}$ = 6.9), 126.5, 122.2, 80.0, 60.2, 48.4, 42.7, 30.7, 29.7, 28.3, 19.3, 17.5; ESMS calcd for $C_{33}H_{40}N_3O_6P$: 605.27, found: 606.50 $[M+H]^+$; elemental analysis calcd (%) for $C_{33}H_{40}N_3O_6P$: C 65.44, H 6.66; N 6.94, P 5.11, found: C 65.50, H 6.12, N 6.05, P 4.61.

N_3-Phe-Leu-OMe (15): Boc-Phe-OH (6.32 g, 23.8 mmol) and HCl.H-Leu-OMe (3.46 g, 23.8 mmol) were dissolved in DCM (150 mL) in the presence of D*i*PEA (9.16 mL, 52.4 mmol, 2.2 equiv). Subsequently, BOP (10.5 g, 23.8 mmol) was added and the obtained reaction mixture was stirred for 3 h. The solvent was removed *in vacuo* and the residue was redissolvend in EtOAc (100 mL) and subsequently washed with 1N $KHSO_4$ (3 × 100 mL), brine (100 mL), 5% $NaHCO_3$ (3 × 100 mL) and brine (3 × 100 mL). The EtOAc layer was dried (Na_2SO_4) and subsequently concentrated, yielding a white solid (Boc-Phe-Leu-OMe), which was purified by recrystallization from isopropanol/hexane. Yield: 8.40 g (90%). R_f:

0.81 (MeOH/DCM 4:96 v/v); mp: 99-102 °C; $[\alpha]_D$: –24.4° c = 0.25 MeOH; ^{1}H NMR ($CDCl_3$): δ = 7.32 (m, 5H), 6.37 (d, *J* = 8.2 Hz, 1H), 5.05 (s, 1H), 4.61 (m, 1H), 4.38 (m, 1H), 3.69 (s, 3H), 3.08 (d, *J* = 6.6 Hz, 2H), 1.62 (m, 3H), 1.41 (s, 9H), 0.90 (m, 6H); ^{13}C NMR ($CDCl_3$): δ = 172.8, 171.0, 155.3, 136., 129.3, 128.5, 126.8, 80.0, 55.5, 52.1, 50.6, 41.4, 38.0, 28.1, 24.5, 22.6, 21.8. Boc-Phe-Leu-OMe (1.14 g, 2.9 mmol) was dissolved in DCM/TFA (30 mL, 1:1 v/v) and after stirring for 1 h the reaction mixture was evaporated *in vacuo* and the residue was coevaporated with DCM (3 times) to remove any residual TFA. The resulting oil (TFA.H-Phe-Leu-OMe) was dissolved in DCM (10 mL) and neutralized by TEA followed by the addition of 1 equiv TEA. To this solution was added $CuSO_4$ (catalytic amount) in MeOH (3 mL) followed by a solution of triflic azide[22,23] (6 mmol, 2.1 equiv) in DCM (15 mL). The resulting reaction mixture was stirred overnight. Subsequently, the solvent was removed under reduced pressure and the residue was redissolved in EtOAc (100 mL) and subsequently washed with 1N $KHSO_4$ (3 × 100 mL), brine (100 mL), 5% $NaHCO_3$ (3 × 100 mL) and brine (3 × 100 mL). The EtOAc layer was dried (Na_2SO_4) and subsequently concentrated to yield a yellowish oil. The product (0.57 g, 62%) was isolated by flash column chromatography (DCM/MeOH 96:4 v/v) as a clear oil which slowly crystallized into a white solid. R_f: 0.77 (EtOAc); R_t: 22.47 min; mp: 45-46°C; $[\alpha]_D$: –4.8° c = 0.25 MeOH; ^{1}H NMR ($CDCl_3$): δ = 7.29 (m, 5H), 6.61 (d, *J* = 8.5 Hz, 1H), 4.59 (m, 1H), 4.29 (dd, *J* = 4.1 Hz, *J* = 7.7 Hz, 1H), 3.73 (s, 3H), 3.35 (dd, *J* = 4.1 Hz, *J* = 14.0, 2H), 1.57 (m, 3H), 0.89 (dd, *J* = 1.4 Hz, *J* = 6.6 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ = 173.0, 168.6, 135.7, 129.5, 129.3, 128.6, 127.2, 65.1, 52.5, 50.6, 41.1, 38.2, 24.5, 22.7, 21.7; elemental analysis calcd (%) for $C_{16}H_{22}N_4O_3$: C 60.86, H 6.97, N 17.60; found: C 60.74, H 6.88, N 17.68.

General procedure for the solid phase synthesis of azido peptides 16 and 17:[24]

The peptide sequences Fmoc-Leu-Lys(Boc)-Phe-NH-Rink Amide Argogel and Fmoc-Ala-Tyr(^{t}Bu)-Leu-Lys(Boc)-Phe-NH-Rink Amide Argogel were synthesized manually using standard Fmoc/^{t}Bu SPPS protocols with HBTU/HOBt and D*i*PEA as coupling reagents. Triflic anhydride (1.01 mL, 6 mmol, 1 equiv) was added portionwise to a solution of NaN_3 (1.95 g, 30 mmol, 5 equiv) in H_2O/DCM 1:1 v/v (20 mL) and stirred during 2 h at room temperature. After this period of stirring, the aqueous phase was extracted with DCM (2 × 2.5 mL) and the combined DCM phase was subsequently washed with 5% $NaHCO_3$ (once 5 mL) and dried ($MgSO_4$). The obtained solution of triflic azide was used directly in the diazo transfer reaction. After Fmoc removal by treatment of the peptide-resin (0.25 mmol) with 20% piperidine in NMP, an excess of triflyl azide (6 mmol, 24 equiv) in DCM (15 mL) in the presence of a catalytic amount of $CuSO_4$ (3 mg, 12 mmol, 0.05 equiv) in MeOH

(2 mL) were added. The reaction mixture was swirled for at least 16 h at room temperature. The completeness of the diazo transfer reaction could be followed with the Kaiser test[28]; colorless resin beads implied that the conversion of the amino group into the azido functionality was completed. The resin was subsequently washed with NMP (3 × 2 min), 0.5% D*i*PEA in NMP (3 × 2 min), 0.02 M diethyldithiocarbamic acid sodium salt in NMP (3 × 10 min), NMP (5 × 5 min) and DCM (3 × 3 min). The product was detached from the resin and deprotected by treatment with TFA/H_2O/T*i*S 95:2.5:2.5 v/v/v for 3 h followed by precipitation with MTBE/hexane 1:1 v/v at –20°C and finally lyophilized from *t*-butanol/H_2O 1:1 v/v.

N_3-Leu-Lys-Phe-NH_2 (16): R_t: 15.07 min; ESMS calcd for $C_{21}H_{33}N_7O_3$: 431.26, found: 432.35 $[M+H]^+$.

N_3-Ala-Tyr-Leu-Lys-Phe-NH_2 (17): R_t: 15.07 min; ESMS calcd for $C_{33}H_{47}N_9O_6$: 665.36, found: 666.50 $[M+H]^+$.

General Procedure for the Staudinger Ligations: An equimolar mixture of the phosphine ester (**11** or **12**) and azido peptide (**15, 16** or **17**) was dissolved in dry and oxygen-free THF or DMSO at a final concentration of 50 mM and stirred at 47ºC under a nitrogen atmosphere for 16 h – 5 days. Subsequently, an excess of H_2O was added and stirring was continued for 2 h. The reaction mixtures yielding the ligation products Boc-Val-Gly-Phe-Leu-OMe and Boc-Val-Gly-Ala-Phe-Leu-OMe were evaporated to dryness and redissolved in EtOAc (20 mL). Subsequently, the EtOAc layer was washed with 1N $KHSO_4$ (15 mL), brine (15 mL), 5% $NaHCO_3$ (15 mL) and brine (15 mL), dried (Na_2SO_4) and evaporated *in vacuo*. The residue was purified by column chromatography. The other ligation reaction mixtures (yielding **21** – **25**) were diluted with cold MTBE/hexane (1:1 v/v) to precipitate the peptides. The peptides were collected by centrifugation and the pellet was resuspended in MTBE/hexane and centrifuged again. The obtained pellet was dissolved in *t*-butanol/H_2O (1:1 v/v) and lyophilized.

Boc-Val-Gly-Phe-Leu-OMe (19): 11 (100 mg, 0.19 mmol) and **15** (60 mg, 0.19 mmol) were dissolved in THF (3.75 mL) and stirred for 16 h as described in the general ligation procedure. The tetrapeptide Boc-Val-Gly-Phe-Leu-OMe (**19**) was obtained in 36% yield (37 mg) after purification by flash column chromatography (EtOAc). R_f: 0.53 (EtOAc); R_t: 17.22 min; ^{1}H NMR ($CDCl_3$): δ = 7.32 (broad s, 1H), 7.27 (m, 7H), 5.41 (d, J = 8.5 Hz, 1H), 4.88 (m, 1H), 4.57 (m, 1H), 4.13-3.83 (br m, 3H), 3.70 (s, 3H), 3.15 (m, 2H), 2.08 (m,

1H), 1.65 (m, 3H), 1.44 (s, 9H), 0.95 (m, 12H); ^{13}C NMR ($CDCl_3$): δ = 173.0, 172.4, 171.0, 168.7, 156.0, 136.4, 129.3, 128.3, 126.7, 79.5, 59.3, 54.3, 52.2, 50.7, 42.6, 40.9, 38.7, 31.4, 28.3, 24.7, 22.7, 21.8, 19.1, 17.8; ESMS calcd for $C_{28}H_{44}N_4O_7$: 548.69, found: 549.45 $[M+H]^+$.

Boc-Val-Gly-Ala-Phe-Leu-OMe (20): 12 (100 mg, 0.17 mmol) and **15** (53 mg, 0.17 mmol) were dissolved in THF (3.3 mL) and stirred for 16 h as described in the general ligation procedure. The pentapeptide Boc-Val-Gly-Ala-Phe-Leu-OMe (**20**) was obtained in 32% yield (33 mg) after purification by flash column chromatography (gradient elution: 0-2% MeOH in DCM). R_f: 0.51 (EtOAc); R_t: 17.13 min; ^{1}H NMR ($CDCl_3$): δ = 7.70 – 6.91 (m, 9H), 5.35 (d, J = 7.7 Hz, 1H), 4.94 (m, 1H), 4.63 (m, 2H), 4.16 (m, 3H), 3.29 (m, 2H), 2.18 (m, 1H), 1.74 – 1.49 (m, 3H), 1.46 (d, J = 9.1 Hz, 3H), 1.43 (s, 9H), 0.92 (m, 12H); ^{13}C NMR ($CDCl_3$): δ = 173.0, 172.6, 172.3, 170.8, 168.4, 155.9, 136.6, 129.6, 128.0, 126.4, 79.1, 59.6, 53.9, 52.1, 50.7, 42.9, 41.0, 39.4, 31.6, 28.4, 24.8, 22.7, 22.2, 20.0, 19.2, 18.2; ESMS calcd for $C_{31}H_{48}N_5O_8$: 619.76, found: 621.50 $[M+H]^+$.

Boc-Val-Gly-Leu-Lys-Phe-NH$_2$ (21): 11 (25 mg, 46 μmol) and **16** (20 mg, 46 μmol) were dissolved in THF (920 μL) and stirred for 5 days as described in the general ligation procedure. The pentapeptide Boc-Val-Gly-Leu-Lys-Phe-NH_2 (**21**) was isolated in 6% yield (2 mg) by preparative HPLC. R_t: 15.37 min; ESMS calcd for $C_{33}H_{55}N_7O_7$: 661.42, found: 662.80 $[M+H]^+$. **21** contained at least two ligation products with equal molecular mass and HPLC retention time. The NH – Leu-αCH correlation was found at 8.2/4.4 ppm and the NH – Lys-εCH_2 correlation was found at 7.9/3.3 ppm. **21** was dissolved in 450 μL H_2O/D_2O 9/1 v/v at pH 3.72 at 278.1 K and a spectrum was recorded on a Varian INOVA-500 NMR spectrometer using a TOCSY pulse-sequence. Based on amide NH integration signals the NH – Leu-αCH ligation product was estimated to be present in 23-33% of the isolated peptides.

Desamino side product (22): The peptide was isolated in 31% yield (6 mg) by preparative HPLC. R_t: 13.93 min; ^{1}H NMR (499.9 MHz, DMSO d_6/H_2O): δ = 8.06 (d, J = 7.5 Hz, 1H), 7.91 (d, J = 8.1 Hz, 1H), 7.61 (br s, 3H), 7.41 (s, 1H), 7.2 (m, 5H), 7.11 (s, 1H), 6.65 (dd, J = 15.5 Hz, J = 6.3 Hz, 1H), 5.96 (d, J = 15.5 Hz, 1H), 4.41 (m, 1H), 4.21 (m, 1H), 3.00 (m, 1H), 2.82 (m, 1H), 2.71 (m, 2H), 2.41 (m, 1H), 1.54 (m, 1H), 1.47 (m, 3H), 1.21 (m, 2H), 1.02 (d, J = 6.8 Hz, 6H); ESMS calcd for $C_{21}H_{32}N_4O_3$: 388.25, found: 389.40 $[M+H]^+$.

Boc-Val-Gly-Ala-Leu-Lys-Phe-NH_2 (23): 12 (30 mg, 50 µmol) and **16** (22 mg, 50 µmol) were dissolved in THF (1 mL) and stirred for 7 days as described in the general ligation procedure. ESMS calcd for $C_{36}H_{60}N_8O_8$: 732.45, found: 733.80 $[M+H]^+$.

Boc-Val-Gly-Ala-Tyr-Leu-Lys-Phe-NH_2 (24): 11 (7 mg, 14 µmol) and **17** (9 mg, 14 µmol) were dissolved in DMSO (300 µL) and stirred for 7 days as described in the general ligation procedure. ESMS calcd for $C_{45}H_{69}N_9O_{10}$: 895.52, found: 897.50 $[M+H]^+$.

Attempted synthesis of Boc-Val-Gly-Ala-Ala-Tyr-Leu-Lys-Phe-NH_2 (25): 12 (10 mg, 17 µmol) and **17** (11 mg, 17 µmol) were dissolved in DMSO (340 µL) and stirred for 7 days as described in the general ligation procedure. The product could not be identified by ESMS.

2.5 References and notes

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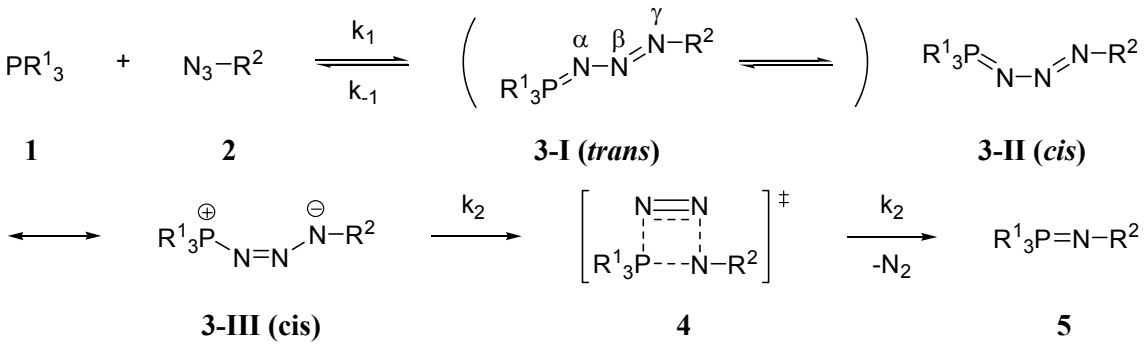
3

Mechanistic Investigation of the Staudinger Ligation with Experimental and Computational Methods

Recently, the Staudinger reaction gained renewed attention as a novel chemoselective ligation method. The application on peptide synthesis is, however, hampered by a poor efficiency for the coupling of more complex peptide segments, as was described in the previous chapter. To obtain a better insight into the limiting factors, the mechanism of the Staudinger ligation, is investigated by experimental and computational methods. It was found that the reaction outcome was mostly influenced by the amino acid residues at the ligation site. Steric congestion in the iminophosphorane intermediate can slow down the intramolecular acyl transfer in such a way that competing side reactions can take place and decrease the overall yield of the desired amide bond forming reaction. The research presented in this chapter supports the results obtained in chapter 2 with quantitative data.

3.1 Introduction

The reaction of a tertiary phosphine with an organic azide to yield an iminophosphorane is known as the Staudinger reaction,[1] and has proved to be a very useful reaction in synthetic organic chemistry.[2,3] Recently, the reaction gained renewed attention as a novel chemoselective ligation method.[4] Applications of this method on metabolic cell engineering,[5-13] peptide ligation,[14-21] the synthesis of bioconjugates[22-28] and immobilization for the coating of microarrays[13,29-31] have been described. The application on peptide synthesis however, is hampered by a poor efficiency for the coupling of more complex peptide segments as was shown in the previous chapter. The purpose of the research described in this chapter was to obtain better insight into the limiting steps of the Staudinger ligation.



Scheme 3.1 Mechanism of the classical Staudinger reaction.

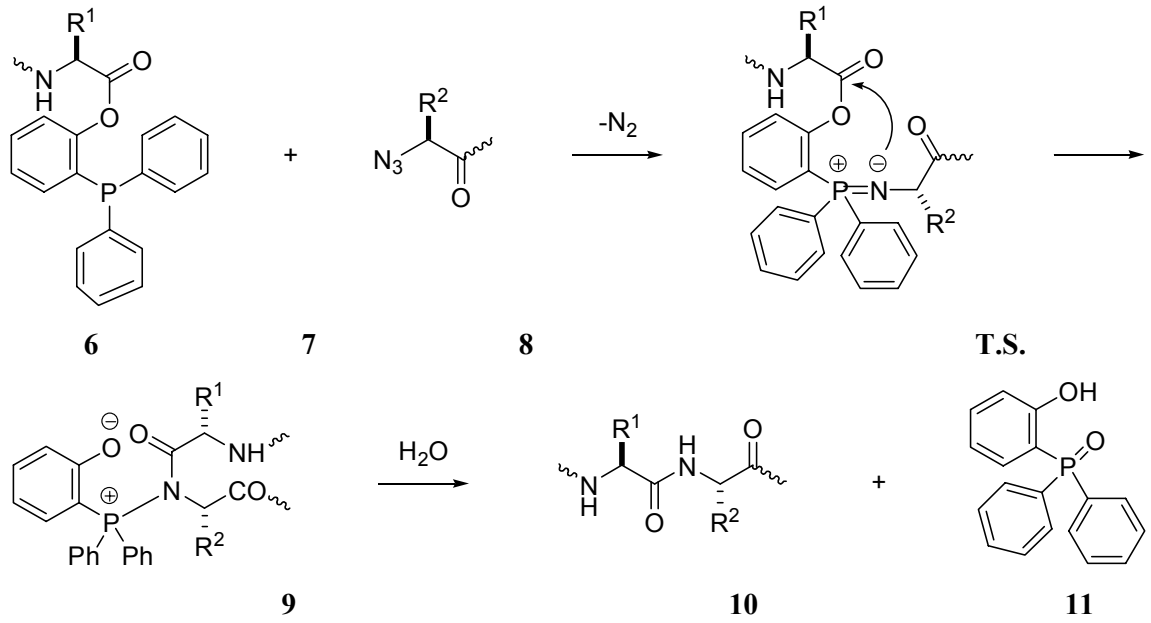
The mechanism of the classical Staudinger reaction (Scheme 3.1) has been thoroughly investigated by experimental[2,3] as well as computational methods.[32-34] The imination is a two-step process which starts with the electrophilic addition of the azide (**2**) to the phosphine (**1**) to form intermediate phosphazides **3-I**/**II**. The addition is generally not hindered by bulky substituents at phosphorus, since its trigonal-pyramidal geometry results in a good accessibility of the lone pair. Instead, the reaction is influenced by electronic effects, for example, donating groups in the phosphine and acceptor groups in the azide increase the reaction rate of this step.

In the literature, the isolation of some phosphazides has been described and their structure was determined experimentally. Most of them had the *trans* geometry (**3-I**), with respect to the central N^{α}-N^{β} linkage which exhibits, partially double bond character. However, it is assumed that formation of iminophosphorane **5** goes via the *cis* geometry (**3-III**) through a four-membered ring transition state **4**. The fast decomposition of the *cis* isomer might explain why most isolated phosphazides were identified as the *trans* isomer.

The stability of phosphazide **3** determines the kinetic order of the overall reaction and is influenced by electronic (delocalisation of the positive charge at phosphorus) and steric factors (bulky substituents at phosphorus). The influence of substituents at phosphorus on the decomposition rate of the phosphazide is thus a combination of their inductive, mesomeric and steric characteristics. Donating substituents (mesomeric and inductive) enhance the stability of the phosphazide and reduce its decomposition rate. As mentioned, bulky substituents do not affect the rate of formation of phosphazide **3** but can seriously decrease the rate of formation of iminophosphorane **5** by steric congestion.

The overall reaction can be first or second order depending on the relation between k_1 and k_2, whether the unimolecular decomposition (k_2) or the bimolecular formation (k_1) of the phosphazide is the rate-determing step. In addition, polar solvents facilitate the overall reaction by stabilizing all transition states and consequently decrease the activation energy. Usually, the Staudinger reaction proceeds smoothly without any side products, hence giving rise to almost quantitative yields.

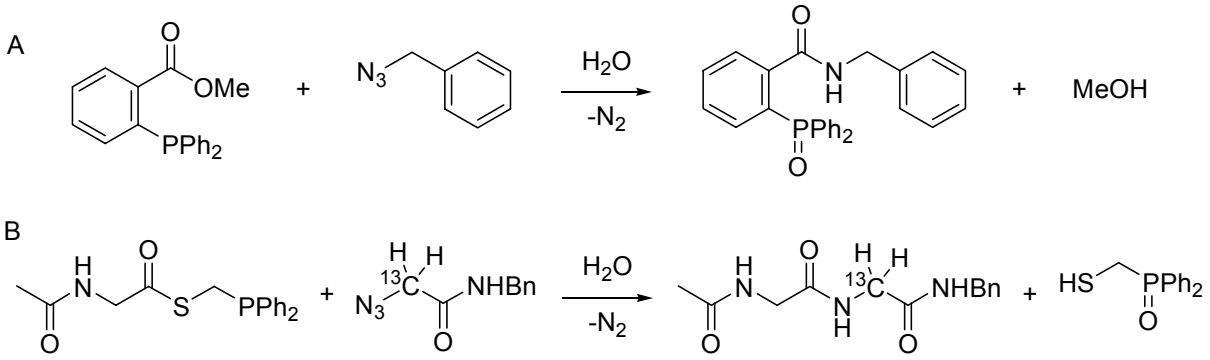
The so-called *Staudinger ligation* (Scheme 3.2) is a special application of the classical Staudinger reaction in which the intermediate iminophosphorane (**8**) is trapped in an intramolecular acylation reaction to form after hydrolysis a stable amide bond (**10**).



Scheme 3.2 Putative mechanism for the Staudinger ligation of peptide segments using the *o*-(diphenylphosphino)phenol auxiliary as was described in chapter 2.

Recently, the groups of Bertozzi[35] and Raines[36] have investigated the reaction mechanism of this Staudinger ligation by NMR-spectroscopy. Both found that the reaction was overall

second-order ($k \approx 0.01\ M^{-1}s^{-1}$), which implicated that for their model systems the rate-determing step was the formation of the initial phosphazide intermediate.
These findings, however, were not consistent with our results for the chemoselective coupling of *peptide segments* featuring the Staudinger ligation.[17] In this case, mass spectrometric analysis of the reaction mixture revealed that formation of the iminophosphorane intermediate (**8**) seemed fast compared to the intramolecular acyl transfer (chapter 2).



Scheme 3.3 Model systems used by Bertozzi (A) and Raines (B) respectively.

One clear difference between the method described in this thesis and the model systems of Bertozzi and Raines (Scheme 3.3) respectively, is the type of phosphine that was used. In fact, a variety of phosphines have been described in the literature that were evaluated for their use in the Staudinger ligation (Figure 3.1). The first generation phosphine **12**, which was developed by Saxon and Bertozzi, was so efficient that it could be coupled to azide-functionalized surfaces of living cells in an aqueous medium.[5] Subsequently, a second generation of phosphines was developed that enabled the *traceless* Staudinger ligation, in which no residual atoms of the phosphine moiety remained in the amide product. Therefore, a cleavable ester-linkage was introduced between the phosphine group and the electrophilic acyl component.
Bertozzi tested phosphines **12** – **16** in the ligation with an azidonucleoside in wet THF.[37] The behavior of phosphine **14** is similar to that of **12**, since the intramolecular reaction proceeds via a five-membered ring transition state and an alkoxy anion functions as leaving group. However, in case of **14** only iminophosphorane hydrolysis-side products could be isolated. In contrast, when phosphine **13** was used, the desired ligation product was obtained in high yield. It was speculated that due to the flexibility of the methylene bridge, **14** was less efficient to facilitate the intramolecular acyl transfer and as a result of this, the hydrolysis pathway predominated. Therefore the rigidity of phosphine **13** appeared to be

essential although in this case the acyl transfer proceeds via a six-membered ring transition state. The application of phosphine **15** also resulted in formation of the ligation product, however, in this case prolonged reaction times were required. In an attempt to increase the reaction rate, **16** was prepared as a potentially more efficient analogue of **15**, unfortunately, **16** was found to be prone to premature oxidation and therefore impractical to use.

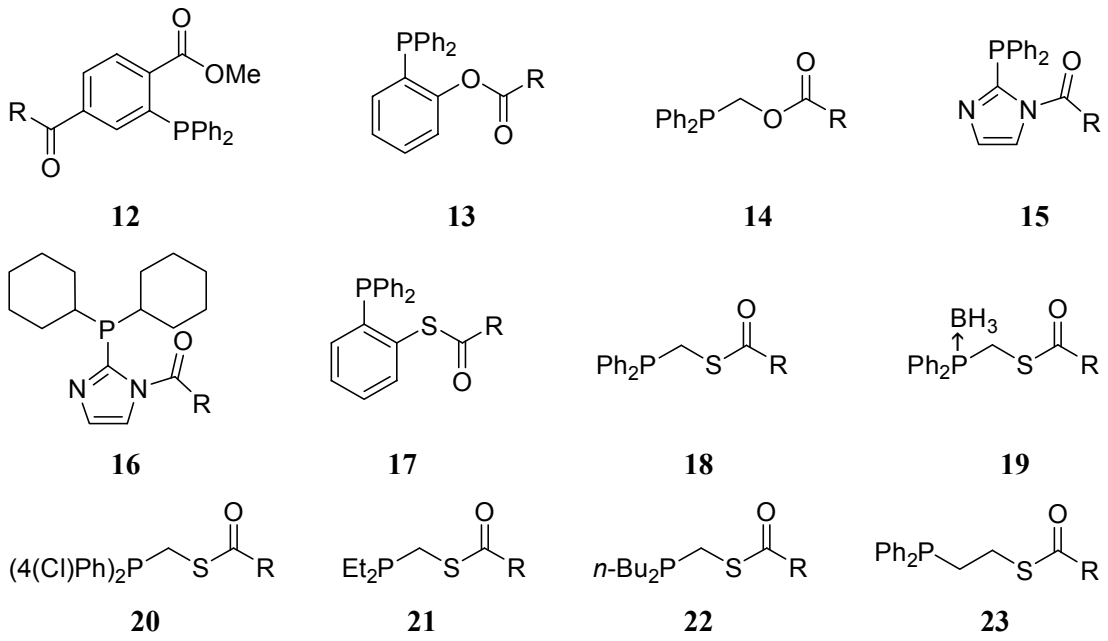


Figure 3.1 Phosphines described in literature for their use in the Staudinger ligation.

Phosphines **13** and **15** were used by Bianchi and Bernardi in the stereoselective synthesis of glycosyl amides starting from glycosyl azides.[25,26] It turned out that best results were obtained with **13** under optimized conditions in which aprotic polar solvents like DMF or DMA at elevated temperatures varying from 40 – 70°C were applied.

Raines and co-workers investigated the application of phosphines **17** and **18** for the coupling of amino acids in THF/H_2O (3:1) as the solvent.[14,15] Although both phosphines enabled the coupling reaction, surprisingly, this time the more flexible phosphinothioester **18** gave far better yields than the more rigid **17**. The reason for the opposite reactivity of phosphinothioester **17** and **18** compared to their oxo analogues **13** and **14**, remains unclear. The excellent qualities of phosphinothioester **18** were demonstrated by the coupling of a *C*-terminal dipeptidyl phosphinothioester to an α-azido peptide bearing an *N*-terminal glycine residue, in the chemical assembly of RNAse A.[37] Noteworthy is the observation that the Staudinger ligation with **18** is less efficient when no glycine is present at the ligation site.[19]

Phosphines **13**, **18** and the analogous **20** were evaluated by Mulard and co-workers for the preparation of carbohydrate-protein conjugates by the coupling of glutaric acid with an

azide functionalized glucoside in potassium phosphate buffer (0.2 M, pH 8.3).[28] It was found that phosphine **13** led predominately to hydrolysis of the iminophosphorane intermediate. This was most likely caused by the increased steric hindrance in the acyl transfer intermediate compared to the intermediate involving an acetyl group in the research of Bertozzi *et al*.[38] In contrast to **13**, phosphine **18** was rather sensitive to oxidation. In an attempt to decrease the sensitivity toward oxidation, phosphine derivative **20** was synthesized. Unfortunately, also **20** led to an incomplete reaction due to premature oxidation of the phosphine. Finally, the best results in terms of stability toward oxidation and efficient acyl transfer were obtained with borane protected phosphine **19** which was used in an one-pot deprotection and Staudinger ligation procedure.

The same protected phosphine **19** has also been used by van Maarseveen and co-workers for the synthesis of lactams via an intramolecular Staudinger ligation.[18]

Kiessling *et al*. studied the influence of substituents at phosphorus on stereoselective *N*-glycosylations.[27] Therefore, phosphine **18** and analogous structures **21** and **22** were evaluated for the coupling of aspartic acid with glycosyl azides. In all cases the product was formed in a moderate yield (20% – 55%) when DMF was used as solvent. However, when reactions were conducted in THF no product was formed in case of electron-deficient glycosyl azides. In one specific example only phosphine **21** yielded the anticipated product which was ascribed to a combination of an increased electron density at phosphorus compared to **18** and a reduced steric congestion at phosphorus compared to **22**.

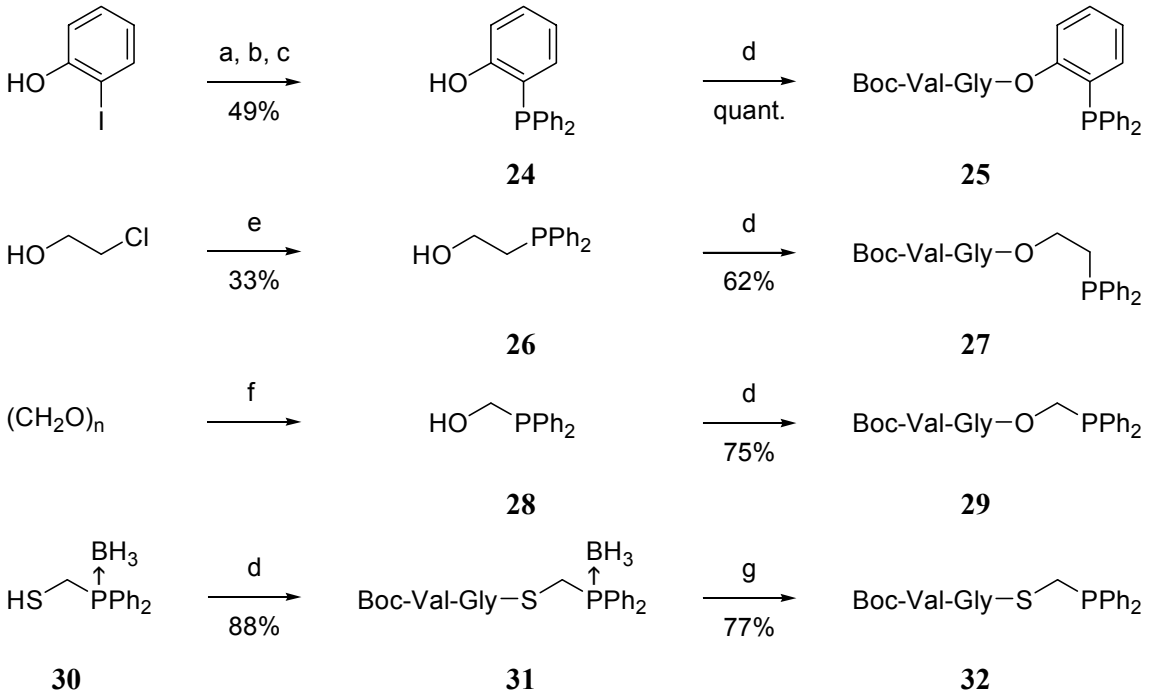
Phosphine **23** has been used by Han and Viola for coupling of Boc-glycine to a dipeptide mimic.[39] The reaction was reported to proceed quantitatively as was monitored by TLC analysis. It was proposed that an easy formation of the six-membered transition state resulted in an efficient reaction. This observation however, is in contrast to the previously described results of Raines *et al*.[14,15]

In summary, these literature examples show that the electronic and steric properties of the phosphine can dramatically influence the reaction outcome of the Staudinger ligation. However, the optimal structure for the phosphine for the coupling of peptide segments remains unclear. To gain better insight in the limiting steps of the Staudinger ligation of peptide segments, it was decided to address this issue by a structural optimization of the phosphine moiety via a combined experimental and computational approach. Moreover, a better understanding of the mechanism of the Staudinger ligation may also contribute to an increased overall efficiency.

3.2 Results and discussion

3.2.1 Experimental data

The experimental investigations were started by variation of the phosphine. Four different phosphine esters were synthesized to address the influence of the structure of the phosphine ester, also referred to as the auxiliary, on the efficiency of the traceless Staudinger ligation (Scheme 3.4).



Scheme 3.4 Synthesis of phosphine esters **25**, **27**, **29** and **32**. Conditions: a) Ac_2O (1.25 equiv), TEA (1.25 equiv), DCM, rt, 1.5 h; b) Ph_2PH (1 equiv), TEA (1.1 equiv), $Pd(OAc)_2$ (cat.), MeCN, N_2, rt 18 h; c) NaOH (2 equiv), MeOH, N_2, rt, 45 min; d) EDCI (1.05 equiv), DMAP (0.1 equiv), DCM, N_2, rt, 16 h; e) $Ph_2P^-K^+$ (1 equiv), THF, N_2, 16 h, 0°C; f) Ph_2PH (0.5 equiv), N_2, 120°C, 2 h; g) DABCO (2 equiv), toluene, 50°C, 16 h

Phosphine **24** has a rigid structure and reactions using this auxiliary proceed via a six-membered ring transition state. The synthesis of **25** and its subsequent reaction with N_3-Phe-Leu-OMe in a model Staudinger ligation has been described in the previous chapter. In the studies described in this chapter, **25** is used as a reference for comparison with phosphine esters **27**, **29**, **32** (Scheme 3.4), **42** and **44** (Scheme 3.7)**.**

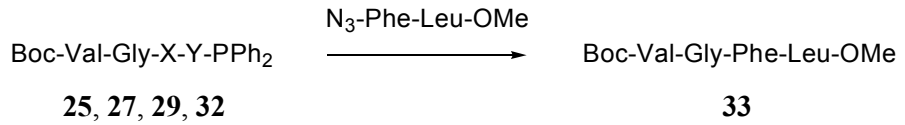
Phosphine ester **27** was obtained by the esterification of Boc-Val-Gly-OH with 2-(diphenylphosphino)ethanol **26** in the presence of EDCI and DMAP. Phosphine **26** on its turn was first synthesized from diphenyl phosphine potassium salt and 2-chloroethanol.[40]

The six-membered ring transition state in the reaction of **25**, resembles that of the reaction of **27**. However, in the latter case, the linkage between the phosphine and acyl donor group is more flexible.

The auxiliary of phosphine ester **29** is also flexible and should lead to a 5-membered ring transition state. It was obtained from esterification of Boc-Val-Gly-OH with **28**. Phosphinoalcohol **28** was synthesized from paraformaldehyde and diphenylphosphine.[41,42]

In phosphine ester **32** a flexible linkage is also present, in addition, it will lead to a 5 membered ring transition state. However, it differs from **29** since now a thioester linkage is present. The synthesis of **32** started with the esterification of Boc-Val-Gly-OH with **30**[43] to afford borane-protected phosphine ester **31** which could subsequently be deprotected by treatment with DABCO to yield phosphine ester **32**. In principle, the properties of a thiolate as a better leaving group than an alkoxide could be an advantage of phosphinothioester **32** compared to the phosphinooxy esters **25**, **27** and **29** in the traceless Staudinger ligation. On the other hand, the three phenyl substituents may render phosphine ester **25** more resistant to undesired oxidation compared to phosphine esters **27**, **29** and **32**.

Table 3.1 Staudinger ligation of phosphine esters **25**, **27**, **29** and **32**.

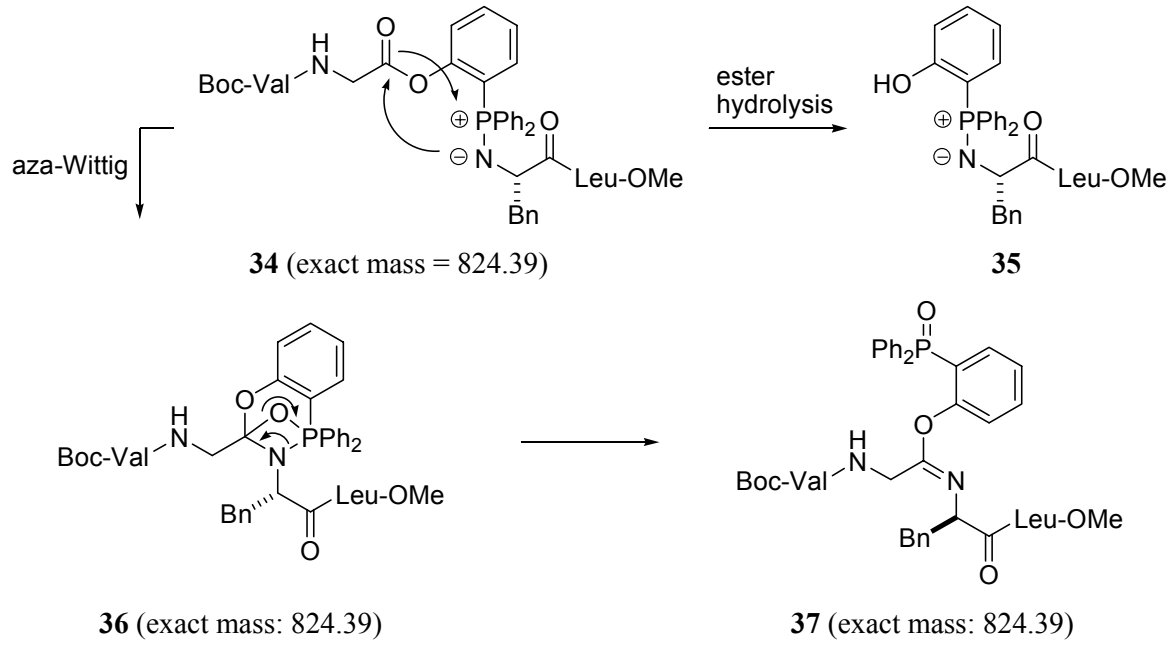


Entry	Phosphine	X	Y	% Yield (% Purity)[a]
1	**25**	O	*o*-Ph	28 (93)
2	**27**	O	C_2H_4	-
3	**29**	O	CH_2	26 (83)
4	**32**	S	CH_2	44 (32)

[a]Isolated yields, purities were determined by HPLC.

All phosphine esters were evaluated in a model Staudinger ligation of Boc-Val-Gly-Aux (Aux = phosphorus containing auxiliary) with N_3-Phe-Leu-OMe using the optimized conditions described in chapter 2. The reaction was monitored by ESMS and the results are summarized in table 3.1.

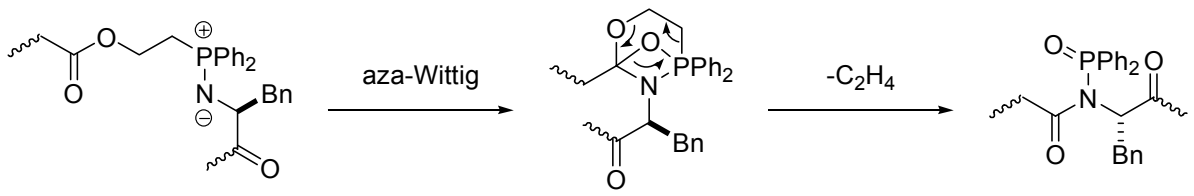
The best results were obtained with phosphine ester **25**. After 1 hour, the formation of tetrapeptide **33** was ascertained by ESMS analysis. Apparently, the reaction did not go to completion since the iminophosphorane intermediate, was still present after 48 h as was judged by ESMS. However, it should be mentioned that iminophosphorane intermediate **34** has an identical mass as the aza-Wittig adducts **36** and **37** (Scheme 3.5).[36] This might imply that the m/z-value 825 which was in first instance (chapter 2) assigned to **34**, could also indicate the occurrence of an aza-Wittig byproduct.



Scheme 3.5 Possible side products in the Staudinger ligation, as identified by MS.

Another byproduct that was identified by ESMS analysis, compound **35**, was assigned to originate from cleavage of the ester bond in intermediate **34**. This hydrolysis most likely occurred when water was added to the reaction mixture or by water during analysis by ESMS. The occurrence of these byproducts indicated a low reactivity of the iminophosphorane intermediate for subsequent intramolecular acyl transfer.

Surprisingly, no product formation could be detected using phosphine ester **27**, although after 1 h some intermediate iminophosphorane (**38**)/aza-Wittig (**39**) adducts were observed by MS analysis. Even after 48 h no product had been formed. Instead, two other compounds were observed which were assigned to oxidized **27** and byproduct **40**. The latter was probably formed from an aza-Wittig adduct that underwent a concurrent loss of an ethene molecule (Scheme 3.6).

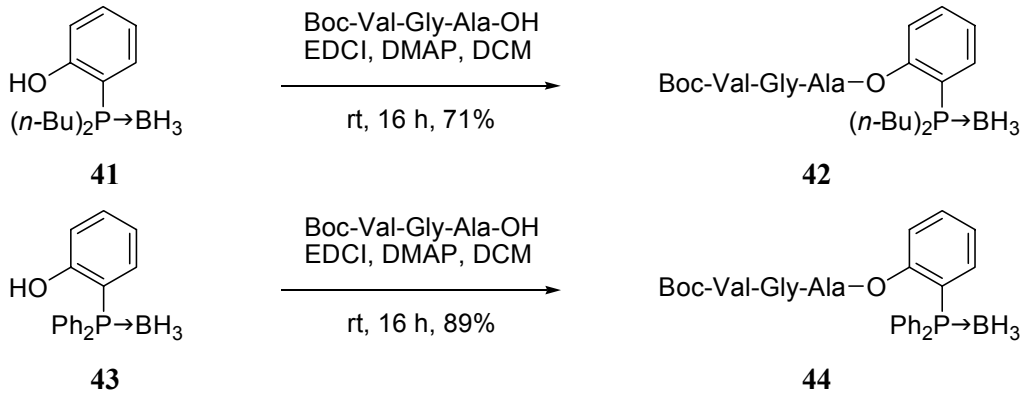


Scheme 3.6 Aza-Wittig reaction in case of phosphine ester **27**.

In case of **29**, the iminophosphorane intermediate/aza-Wittig adduct was detected within 1 h from the reaction mixture. Eventually, tetrapeptide **33** was isolated in a lower yield and purity than in case of **25**. The crude product was contaminated with a small amount of oxidized **29**.

When **32** was used, no product formation was detected within one hour after addition of the reagents. However, after 48 h the ligation product **33** and oxidized **32** could be identified from the reaction mixture. In first instance, **32** seemed to give the highest yield but the crude peptide turned out to be contaminated with a substantial amount of oxidized **32**.

In summary, the results from these experiments show that the best yields were obtained with phosphine ester **25**, even though the reaction was still incomplete after 48 h. Additionally, **25** seemed to be more resistant toward premature oxidation than phosphine esters **27**, **29** and **32**.



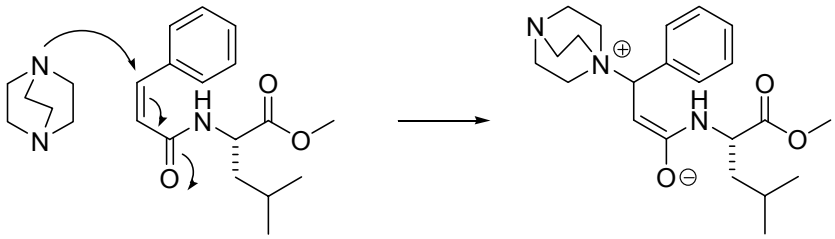
Scheme 3.7 Synthesis of phosphine esters **42** and **44.**

Next, the influence of the substituents at phosphorus on the efficiency of the Staudinger ligation was studied. As was mentioned in the previous paragraph, donating groups can stabilize the phosphazide intermediate and thereby enhance the rate of its formation. Coincidentally, the increased electron density on phosphorus renders the phosphine more

susceptible toward oxidation. In addition, bulky substituents might hamper the intramolecular acyl transfer by steric congestion.

It was decided to synthesize *di*-butyl substituted phosphine ester **42** (Scheme 3.7). To prevent premature oxidation of the phosphine, the borane-protected derivative was used which could be deprotected by DABCO followed by a Staudinger ligation in one pot. For comparison, phosphine ester **44** was reacted in a similar procedure. Both phosphines were synthesized by esterfication of the tripeptide Boc-Val-Gly-Ala-OH with borane protected 2-(*di*butylphosphino) phenol **41** and 2-(diphenylphosphino) phenol **43**,[43] in the same way as was previously described for the other phosphine esters.

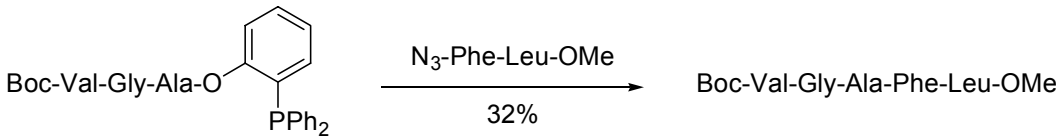
The borane protected phosphine esters **42** and **44** were reacted with an equimolar quantity of azidopeptide (N_3-Phe-Leu-OMe) in the presence of four equivalents of DABCO at 70°C in THF. HPLC analysis of the reaction mixture showed that both protected phosphines were liberated within 1 h. In the same time, formation of the iminophosphorane intermediate/ aza-Wittig adducts was confirmed by MS. However, formation of the pentapeptide ligation product was not observed within the following 48 h. Instead, two side products which form the major compounds of both reaction mixtures, were detected by ESMS analysis. The first compound originated from hydrolysis of the ester linkage in the iminophosphorane intermediate. The chemical structure of the second side product remained unclear but its molecular mass corresponded to a deamination side product (compound **22**, chapter 2), with an added molecule of DABCO analogous to a "Baylis-Hillman" intermediate, as depicted in Scheme 3.8.



22, Chapter 2 **45**

Scheme 3.8 Proposed structure of side product **45**.

Compared to the results obtained with Boc-Val-Gly-Ala-*o*-(diphenylphosphino)phenol (**46**, Scheme 3.9) as was described in the previous chapter, the conditions used here proved unsuitable for the reaction of **42** and **44** with N_3-Phe-Leu-OMe to yield ligation product **47**. Although its role is unclear, this might have been caused by the relatively large amount of DABCO present.

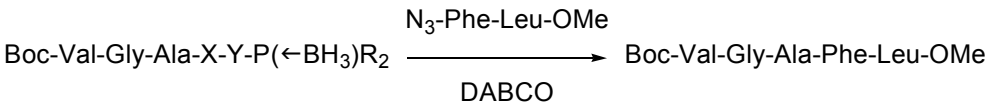


46 **47**

Scheme 3.9 Staudinger ligation of phosphine ester **46** with N_3-Phe-Leu-OMe to yield tetrapeptide **47**, as was described in the previous chapter.

Unfortunately, non of the described alternatives (Table 3.1 and Table 3.2) led to improvement of the efficiency of the Staudinger ligation compared to the isolated yields that were obtained with phosphine esters **25** and **46**. Moreover, only few intermediates could be identified with ESMS and HPLC analysis from the complex reaction mixture. Consequently a better insight into the rate-limiting step(s) of the Staudinger ligation was, unfortunately, not achieved experimentally. Therefore, it was decided to use a computational approach in attempts to rationalize the experimental results in this and the previous chapter.

Table 3.2 Staudinger ligation of phosphine esters **42** and **44**.



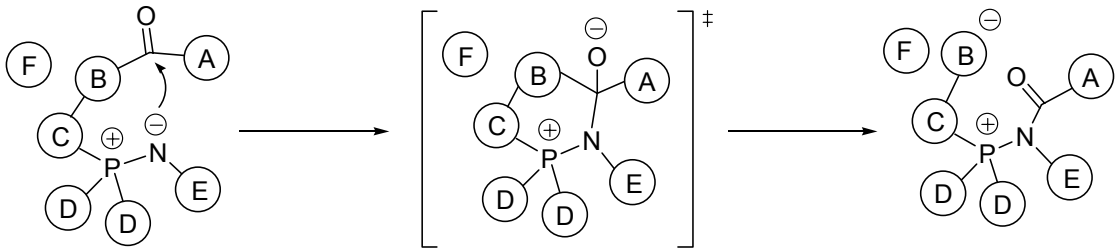
42, 44 **47**

Entry	Phosphine	X	Y	R	% Yield
1	**42**	O	*o*-Ph	*n*-Bu	complex mixture
2	**44**	O	*o*-Ph	Ph	complex mixture

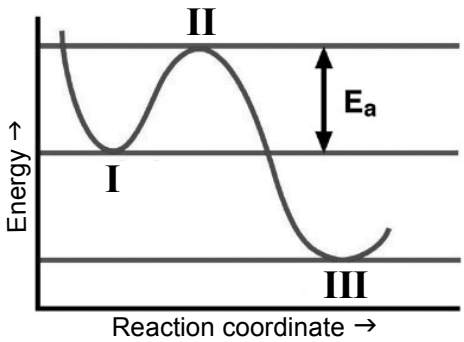
3.2.2 Computational investigations

In the literature, all factors concerning substituents at phosphorus (steric and electronic), the phosphorus containing auxiliary, solvent and amino acid residues at the ligation site, have been implicated in influencing the progress of the Staudinger ligation reaction. Experimental evaluation of these factors and combinations thereof, would need a tremendous amount of research effort. Therefore, it was decided to investigate the mechanism of the Staudinger ligation by computational methods to look for trends in

reactivity. The calculations focused on the intramolecular acyl transfer in which a new peptide bond is formed, since this reaction step is, in our opinion, the rate-determining step of the *hindered* Staudinger ligation (Scheme 3.10).

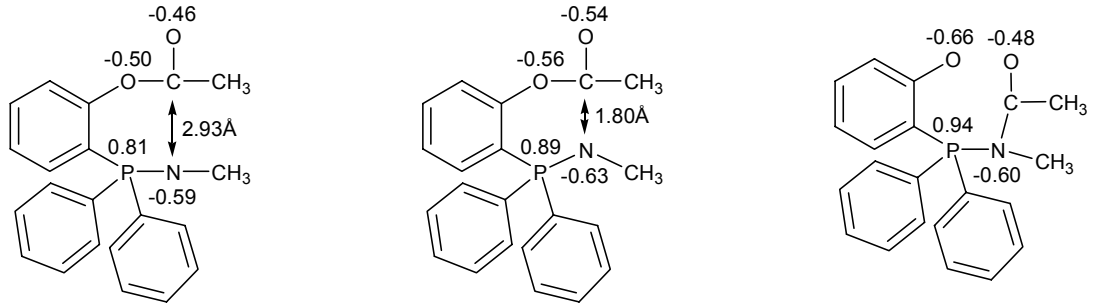


Iminophosphorane (I) **transition state (II)** **product (III)**



Scheme 3.10 Schematic representation of the intramolecular acyl transfer step of the Staudinger ligation reaction and factors which have been evaluated by computational methods. (A) = *N*-terminal residue at the ligation site; (B) = type of ester linkage (oxy or thio); (C) = auxiliary; (D) = substituents at phosphorus; (E) = *C*-terminal residue at the ligation site; (F) = Solvent polarity.

Computational methods have been employed to calculate the energy barrier (E_a) of the intramolecular acyl transfer. Therefore, a model structure was selected for the iminophosphorane that combined good accuracy with acceptable computer time (Figure 3.2).



iminophosphorane **transition state** **product**

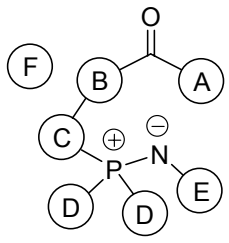
Figure 3.2 Schematic representation of the iminophosphorane start geometry, transition state and product in the *O*→*N*-acyl transfer reaction for model **48**, Mulliken charges and *N*-*C* distances (Å) are given.

In this model the *P-N* bond of the iminophosphorane had a single bond character in which a positive charge was positioned on phosphorus and a negative charge on nitrogen. This was in agreement with the proposed mechanism of the Staudinger ligation that features an intramolecular *O*→*N*-acyl shift which is induced by the nucleophilic nitrogen of the iminophosphorane intermediate.

Type of phosphorus containing auxiliary

In order to evaluate the type of linkage between the phosphine moiety and the acyl component, five phosphorus containing auxiliaries with different flexibility, type of ester linkage and ring-size in the transition state were modeled. For every iminophosphorane model, the energy barrier and distance between the reacting centres (*N* and *C*) at the transition state of the intramolecular acyl transfer reaction were calculated (Table 3.3).

Table 3.3 The calculated energy barriers as function of the auxiliary.



Entry	Model	A	B	C	D	E	F	E_a†	$d_{N\text{-}C}$‡
1	**48**	Me	O	*o*-Ph	Ph	Me	vac.	9.6	1.80
2	**49**	Me	O	C_2H_4	Ph	Me	vac.	-	-
3	**50**	Me	O	CH_2	Ph	Me	vac.	-	-
4	**51**	Me	S	*o*-Ph	Ph	Me	vac.	9.3	1.90
5	**52**	Me	S	CH_2	Ph	Me	vac.	9.2	1.76

†All energies (in kcal/mol) are corrected for the zero point vibrational energy and are calculated at 298 K and 1 atm. ‡The distance between the reacting centres (*N* and *C*) at the transition state, is measured in Å.

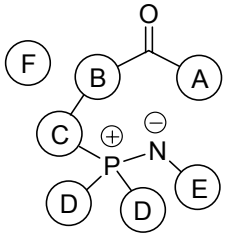
It was found that the transition state was reached at approx 1.80 Å, depending on the type of phosphorus containing auxiliary that was used. The acyl transfer reaction was a one step process for models **48**, **51** and **52**. In contrast, model **49** (entry 2, Table 3.3) did not

converge toward the expected product. Instead, it showed formation of the aza-Wittig intermediate, analogous to structure **39**. This is in good agreement with results obtained by experimental methods as described in the previous paragraph. The intramolecular acyl transfer reaction with model **50** displayed a different mechanism in which formaldehyde was released as a byproduct. As a consequence, no energy barrier for the acyl transfer reaction could be calculated for models **49** and **50**.

The intramolecular *O*→*N*-acyl shift reactions with models **48**, **51** and **52** were all exothermic and the corresponding calculated energy barriers were of the same magnitude. Within this series, the reaction with **48** was found to have the highest energy barrier, which might be explained by the better leaving group properties of the thiolate ion (**51** and **52**) compared to the alkoxide ion (**48**). Although the calculated energy differences were small, the obtained results were in contrast with literature data[36] and the experimental findings described in this and the previous chapter, since here the *o*-(diphenylphosphino)phenol auxiliary (**24**) was identified to be the most reactive. This disparity may arise from the simplification of the model that was used for the calculations compared to the more complex set of reactants that was used for the actual ligation reaction.

Besides the nucleophilicity of the iminophosphorane nitrogen, also the electrophilicity of the ester moiety of the auxiliary is important. Obviously, the phosphine ester needs to be susceptible toward intramolecular nucleophilic attack of the iminophosphorane nitrogen. However, at the same time it has to be stable toward intermolecular attack by other nucleophiles such as, for example, H_2O or the εNH_2-group of an unprotected lysine side chain. As a measure for the electrophilicity of the auxiliary, the atomic charge of the carbonyl carbon of the ester moiety was estimated by calculation of the Mulliken charges. It was found that the calculated positive charge for phosphino oxy-ester model **48** was significantly higher than for phosphino-thioester models **51** and **52** (Table 3.4). This result adds to an explanation of the susceptibility of the *o*-(diphenylphosphino)phenol esters toward hydrolysis and unselective aminolysis as was described in chapter 2.

Table 3.4 The calculated electron population as function of the auxiliary.



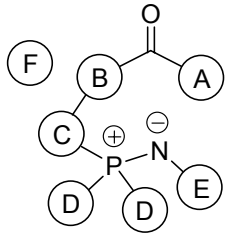
Entry	Model	A	B	C	D	E	F	E_a†	Q_P‡	Q_N‡	Q_C‡
1	**48**	Me	O	*o*-Ph	Ph	Me	vac.	9.6	0.892	-0.628	0.597
2	**51**	Me	S	*o*-Ph	Ph	Me	vac.	9.3	0.855	-0.616	0.328
3	**52**	Me	S	CH_2	Ph	Me	vac.	9.2	0.893	-0.595	0.324

†All energies (kcal/mol) are corrected for the zero point vibrational energy and are calculated at 298 K and 1 atm. ‡Mulliken charges (*e*).

Substituents at phosphorus

Next, the influence of the substituents at phosphorus was investigated. The models presented in Table 3.5 feature substituents which differ in their electronic and steric properties. Within this series a general trend could be observed since stronger electron donating properties correlated with lower energy barriers. This was in good agreement with the results of Bertozzi and co-workers,[35] who found that electron donating substituents at phosphorus accelerated the overall rate of the Staudinger ligation. In this respect, model **57** is a special case since its weakly donating alkyl substituents result in a barrier that is lower or comparable to the intramolecular acyl transfer reaction with model **55**, which bears strongly donating hydroxyl groups. Apparently, steric factors contribute to the outcome. In case of model **56** the barrier becomes higher when the phenyl substituents are changed for methyl groups, possibly for this auxiliary the more bulky phenyl groups have a positive effect by directing the iminophosphorane in a favored conformation for the intramolecular reaction.[14,15]

Table 3.5 Computational evaluation of the substituents at phosphorus.



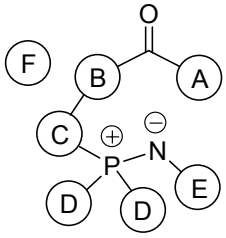
Entry	Model	A	B	C	D	E	F	E_a†	Q_P‡	Q_N‡	Q_C‡	$d_{N\text{-}C}$§
1	**48**	Me	O	*o*-Ph	Ph	Me	vac.	9.6	0.892	-0.628	0.597	1.80
2	**53**	Me	O	*o*-Ph	4$(NO)_2$Ph	Me	vac.	12.1	0.911	-0.617	0.587	1.74
3	**54**	Me	O	*o*-Ph	4(OMe)Ph	Me	vac.	9.2	0.886	-0.631	0.599	1.83
4	**55**	Me	O	*o*-Ph	4(OH)Ph	Me	vac.	9.0	0.881	-0.632	0.600	1.83
5	**56**	Me	O	*o*-Ph	4(NH_2)Ph	Me	vac.	7.6	0.869	-0.635	0.602	1.85
6	**57**	Me	O	*o*-Ph	Me	Me	vac.	8.7	0.890	-0.606	0.605	1.82
7	**51**	Me	S	*o*-Ph	Ph	Me	vac.	9.3	0.855	-0.616	0.328	1.90
8	**58**	Me	S	*o*-Ph	Me	Me	vac.	6.8	0.912	-0.617	0.303	1.94
9	**52**	Me	S	CH_2	Ph	Me	vac.	9.2	0.865	-0.601	0.387	1.76
10	**59**	Me	S	CH_2	Me	Me	vac.	10.9	0.882	-0.577	0.383	1.76

†All energies (kcal/mol) are corrected for the zero point vibrational energy and are calculated at 298 K and 1 atm. ‡Mulliken charges (*e*). §Distance (Å) between *N* and *C* at the transition state.

Solvent polarity

The next factor that was investigated was the solvent. Since the intramolecular acyl migration proceeds via charged intermediates, the stabilizing effect of polar solvents can promote the reaction progress. In those cases where solvent polarity was included in the calculations, it was found that for all models the energy barriers were indeed reduced as a function of a higher dielectric constant of the solvent while the reaction mechanism remained unchanged (Table 3.6). Also in this case the calculations were in good agreement with the results of Bertozzi and co-workers[35] who observed higher reaction rates in polar solvents than in non-polar solvents.

Table 3.6 Computational evaluation of the solvent



							F†		
							Vac.	THF	DMSO
Entry	Model	A	B	C	D	E	E_a‡	E_a‡	E_a‡
1	**48**	Me	O	*o*-Ph	Ph	Me	9.6	7.9	7.4
2	**51**	Me	S	*o*-Ph	Ph	Me	9.3	7.6	7.2
3	**52**	Me	S	CH_2	Ph	Me	9.2	6.8	6.1
4	**57**	Me	O	*o*-Ph	Me	Me	8.7	7.1	6.7
5	**58**	Me	S	*o*-Ph	Me	Me	6.8	5.1	4.7
6	**59**	Me	S	CH_2	Me	Me	10.9	8.0	7.3

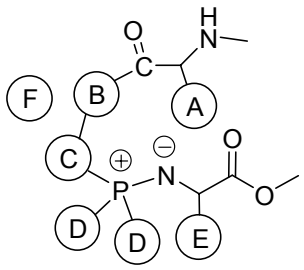
†The influence of solvent has been taken into account using the polarisable continuum model (PCM)[44,45] with THF (ε = 7.58) and DMSO (ε = 46.7). ‡All energies (in kcal/mol) are corrected for the zero point vibrational energy and are calculated at 298 K and 1 atm.

Amino acid residues at the ligation site

Thus far some trends in reactivity have been identified, however, the influence of the type of ester linkage (oxy or thio), auxiliary, substituents at phosphorus and solvent polarity were rather small on the outcome of the calculations. The obtained energy barriers were generally lower than 10 kcal/mol which implies that the reactions should proceed smoothly. The isolated yields for the model Staudinger ligation reactions described earlier in this chapter however, were poor to moderate. This means that the limiting factor in the Staudinger ligation reaction has, most likely, not yet been identified. Therefore, it was decided to investigate the influence of the amino acid identity at the ligation site.

Both the groups of Bertozzi[35] and Raines[36] have identified formation of the phosphazide (**3**, Scheme 3.1) as the rate-determing step in the Staudinger ligation, which means that the intramolecular acyl transfer proceeds relatively fast. These findings however, were based on unhindered reaction partners. Moreover, Raines and co-workers found that when non-glycyl ligations were performed, the reaction rate constant for product formation was two times lower than for ligations with a single glycine residue. Furthermore, the yield was reduced more than three times in case of the non-glycyl ligations. Side products were identified to originate from an aza-Wittig reaction and hydrolysis of the central *P-N* bond in the iminophosphorane intermediate.[36] Both side reactions may have arisen from a relatively slow intramolecular acyl transfer, most likely caused by steric congestion at the intramolecular reaction step.

Table 3.7 Computational evaluation of amino acid residues at the ligation site



Entry	Model	A	B	C	D	E	F	E_a†
1	**60**	H	O	*o*-Ph	Ph	H	vac.	7.1
2	**61**	H	O	*o*-Ph	Ph	*i*-Bu	vac.	16.1
3	**62**	*i*-Bu	O	*o*-Ph	Ph	H	vac.	10.1
4	**63**	*i*-Bu	O	*o*-Ph	Ph	*i*-Bu	vac.	22.6
5	**64**	H	S	CH_2	Ph	H	vac.	0.6
6	**65**	H	S	CH_2	Ph	*i*-Bu	vac.	17.4
7	**66**	*i*-Bu	S	CH_2	Ph	H	vac.	8.4
8	**67**	*i*-Bu	S	CH_2	Ph	*i*-Bu	vac.	21.4

†All energies (in kcal/mol) are corrected for the zero point vibrational energy and calculated at 298 K and 1 atm.

Using computational methods, the influence of an unhindered glycine residue (A = H, Table 3.7) was compared to that of a hindered leucine residue (A = *i*-Bu). In total, the energy barriers for eight models were calculated. Two different phosphorus-containing auxiliaries were used: *o*-(diphenylphosphino)phenol (B = O, C = *o*-Ph), first described by Bertozzi and co-workers and later used by us for the ligation of peptide segments,[17] as well as phosphinomethanethiol (B = S, C = CH_2), which was used by the group of Raines.
The results in Table 3.7 show significant differences for the amino acid residues used at the ligation site. Barriers for Gly – Gly ligations (entries 1 and 5) are much lower than for Leu – Leu ligations (entries 4 and 8) and ligations with one glycine residue at the ligation site are in between (entries 2, 3, 6 and 7). The lowest energy was calculated for a Gly – Gly ligation using the phosphinomethanethiol auxiliary (entry 5). These data show that from all investigated factors so far, the amino acid residue at the ligation site has the largest influence on the energy barrier of the intramolecular acyl transfer step in the Staudinger ligation. In the case of hindered Staudinger ligations, the energy barrier was increased in such a way that the intramolecular acyl migration becomes the rate-determing step, which is in good agreement with the experimental data described in this thesis and the literature.

3.3 Conclusions

In this chapter an explanation was sought for the low yields that were encountered in the Staudinger ligation of peptides with increasing complexity as was discussed in the previous chapter. It was hypothesized that the intramolecular acyl migration formed the rate-determing step, due to steric congestion of the peptide segments to be coupled.
In order to verify this hypothesis and find ways for improving the yield, the mechanism of the Staudinger ligation was investigated by experimental and computational methods. The investigations concentrated on the evaluation of factors concerning, the auxiliary, substituents at phosphorus, amino acid residues at the ligation site and solvent polarity on the intramolecular acyl migration step.
o-(Diphenylphosphino)phenol and phosphinomethanethiol were identified as preferred auxiliaries by experimental as well as computational investigations. Furthermore, donating substituents at phosphorus and polar solvents were found to be beneficial. Although these findings were in good agreement with literature data, computational investigations showed that the influence of the mentioned factors on the acyl migration step of the Staudinger ligation was rather limited. Instead, the type of amino acid residues at the ligation site was found to have a major influence on the energy barrier of the intramolecular acyl migration.
Steric congestion can slow down this reaction step in such a way that competing side reactions will take place leading to a decrease of the overall yield of the Staudinger ligation.

The research presented in this chapter supports the results obtained in chapter 2 with quantitative data.

3.4 Current developments of the Staudinger ligation: stat-of-the-art

As was concluded from the results in this and the previous chapter, steric congestion at the ligation site was identified as a major limitation of the traceless Staudinger ligation. In parallel to other auxiliary mediated ligations,[46] the diminished efficiency of hindered couplings was recognized by us as an intrinsic limitation of this method. Therefore, it was decided to shift our attention toward other methods for the chemoselective coupling of unprotected peptides that are independent of auxiliaries or side chain functionalities at the ligation site.

Research on the Staudinger ligation of peptide segments was continued by others, in particular the group of Raines. Their investigations focused on the phosphine auxiliary and initially, *o*-(diphenylphosphino)phenol and phosphinomethanethiol were identified as the most efficient in the unhindered Staudinger ligation.[36] In fact, *o*-(diphenylphosphino)phenol, which was also used in the research described in this thesis, had the highest reactivity but proved to be susceptible toward aminolysis, an observation which was previously described by us.[17] Generally, application of the phosphinomethanethiol auxiliary was only efficient for Staudinger ligations in which at least one glycine residue was present at the site of ligation. In the absence of a glycine residue at the ligation site, however, the reaction rates using phosphinomethanethiol were reduced significantly. Moreover, obtained yields were rather low due to the occurrence of an interfering aza-Wittig side reaction. Optimization involved variation of the substituents at phosphorus. In analogy to the investigations presented in this thesis, it was found that the electron density on phosphorus could be tuned via the attached substituents. Eventually, the yield of non-glycine couplings could be increased by the introduction of electron donating substituents at phosphorus, mainly to hinder the undesired aza-Wittig side.[47,48]

Recently, a water-soluble phosphine was developed that featured charged substituents at phosphorus. This phosphinothiol auxiliary could be integrated with expressed protein ligation (EPL) to yield a *C*-terminal protein phosphinothioester.[49] Subsequent employment in the Staudinger ligation could overcome the requirement for a cysteine residue at the ligation site as is the case for NCL/EPL.

However, given the many successful applications of NCL/EPL,[50,51] these methods remain unsurpassed for the convergent synthesis of proteins. Moreover, several approaches have been used to broaden their applicability.[52-54]

3.5 Experimental section

General information

Analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column using either method A (Alltech Adsorbosphere XL C8, 90 Å, 5 μm, 250 × 4.6 mm, in a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at 1 mL/min) or method B (Alltech Altima C8, 90Å, 5 μm, 250 × 4.6 mm in a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min), using a UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000). Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel 60F^{254} plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[55]

Chemicals and reagents

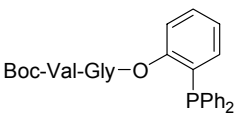
Unless stated otherwise, all chemicals were obtained from commercial sources and used as such. Peptide grade dichloromethane (DCM), tetrahydrofuran (THF), ethyl acetate (EtOAc), hexane**,** chloroform and HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Biosolve. 4-(*N*,*N*-dimethylamino)pyridine (DMAP) was obtained from Acros Organics, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) was purchased from Aldrich, acetic acid (HOAc) was obtained from Merck KGaA. 1-Hydroxybenzotriazole (HOBt) was purchased from Advanced Chemtech. DCM and toluene were dried using molsieves 4Å.

Calculations

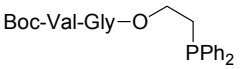
Calculations on models **48** – **59** were performed by employing density functional theory (DFT)[56] with the hybrid B3LYP exchange-correlation functional with the 6-31G** basis set using the Gaussian03[57] and Gamess-UK[58] programs. Calculations on models **60** – **67** were performed with a fixed *C-N* distance of 1.80 Å for the transition state, using the Amsterdam Density Functional (ADF)[59,60] package with the TZP Slater basis set. For carbon, nitrogen

and oxygen atoms orbitals 1s were frozen, for phospohorus 2p and deeper orbitals. All stationary points on models **48** – **59** were characterized either as minima (imino-phosphorane and product) or first-order saddle points (transition states). Reported energies include zero-point energy correction. To determine whether the transition states were related to desired reactants and products, intrinsic reaction coordinate (IRC) calculations were performed. The influence of solvent effects were taken into account using the polarisable continuum model (PCM).[44,45] Geometries characterising the minima and transition states on the potential energy surface were relocated in tetrahydrofuran (THF, ε = 7.58) and dimethylsulfoxide (DMSO, ε = 46.7).

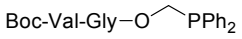
General procedure for the synthesis of phosphine esters 25, 27, 29, 32, 42, 44 and 46:
The peptide was dissolved in dry and deoxygenated DCM (20 mL) and the solution was cooled on ice. Subsequently the phosphine, DMAP and EDCI were added under a nitrogen atmosphere. After stirring for 16 h, the reaction mixture was evaporated *in vacuo*. The residue was dissolved in EtOAc (250 mL) and this solution was subsequently washed with 1N $KHSO_4$ (2 × 100 mL) and brine (2 × 100 mL), dried (Na_2SO_4) and evaporated to dryness. The product was isolated by flash column chromatography.



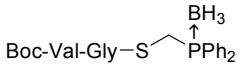
Boc-Val-Gly-*o*-(diphenylphosphine)phenyl ester (25): The synthetic procedure and analytical data for this compound have been described in chapter 2.



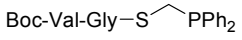
Boc-Val-Gly-2-(diphenylphosphino)ethyl ester (27): The reaction was carried out following the general procedure using 0.49 g of Boc-Val-Gly-OH (1.80 mmol), 0.42 g of **26** (1.80 mmol), 22 mg of DMAP (0.18 mmol, 0.1 equiv) and 0.38 g of EDCI (1.98 mmol, 1.1 equiv). After flash column chromatography (gradient elution; 0-2% MeOH in DCM) the product (0.56 g; 62%) was obtained as a white foam. R_f: 0.52 (DCM/MeOH 96:4 v/v); R_t: 18.25 min (method A); ^{1}H NMR ($CDCl_3$): δ = 7.45-7.38 (broad m), 7.35-7.32 (broad m, total 10H), 6.63 (broad s, 1H), 5.17 (m, 1H), 4.31 (m, 2H), 4.03-3.73 (broad m, 3H), 2.44 (m, 2H), 2.16 (m, 1H), 1.43 (s, 9H), 0.98 (dd, J = 6.9 Hz, J = 16.2 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ = 171.8, 169.4, 155.8, 137.4/137.2 ($J_{P\text{-}C}$ = 11.4 Hz), 132.7/132.4 ($J_{P\text{-}C}$: 19.5 Hz) 128.9, 128.6/128.5 ($J_{P\text{-}C}$: 21.8 Hz), 79.8, 63.2/62.9 ($J_{P\text{-}C}$: 25.2 Hz), 59.6, 40.9, 30.8, 28.2, 27.7/27.5 ($J_{P\text{-}C}$: 14.9 Hz), 19.2, 17.6; ESMS calcd For $C_{26}H_{35}N_2O_5P$ 486.23, found 509.35 $[M + Na]^+$, 524.75 $[M + K]^+$.



Boc-Val-Gly-(diphenylphosphino)methyl ester (29): The reaction was carried out following the general procedure using 0.63 g of Boc-Val-Gly-OH (2.31 mmol), 0.50 g of **28** (2.31 mmol), 28 mg of DMAP (0.23 mmol, 0.1 equiv) and 0.49 g of EDCI (2.54 mmol, 1.1 equiv). After flash column chromatography (gradient elution; 0-2% MeOH in DCM), the product (0.31 g; 75%) was obtained as a white foam. R_f: 0.48 (DCM/MeOH 96:4 v/v); R_t: 18.15 min (method A); ^{1}H NMR ($CDCl_3$): δ = 7.46-7.39 (broad m), 7.37-7.31 (broad m, total 10H), 6.56 (t, J = 4.9 Hz, 1H), 5.1 (d, J = 8.8 Hz, 1H), 4.91 (d, J = 5.8 Hz, 2H) 4.00-3.96 (broad m, 3H), 2.15 (m, 1H), 1.43 (s, 9H), 0.96 (dd, J = 6.9 Hz, J = 17.3 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ = 171.8, 169.5 (2 lines, $J_{P\text{-}C}$ = 3.4 Hz), 155.8, 134.7/134.6 ($J_{P\text{-}C}$ = 10.3 Hz), 133.1/132.9 ($J_{P\text{-}C}$ = 18.3 Hz) 129.2, 128.7/128.6 ($J_{P\text{-}C}$ = 6.9 Hz), 79.9, 64.5/64.3 ($J_{P\text{-}C}$ = 16.0 Hz), 59.6, 41.0, 30.8, 28.2, 19.2, 17.5; ESMS calcd For $C_25H_{33}N_2O_5P$ 472.21, found 495.40 $[M + Na]^+$, 511.40 $[M + K]^+$.

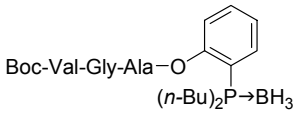


Boc-Val-Gly-borane-(diphenylphosphino)methanethiol ester (31): The reaction was carried out following the general procedure using 0.22 g of Boc-Val-Gly-OH (0.79 mmol), 0.20 g of **30** (0.79 mmol), 10 mg of DMAP (0.08 mmol, 0.1 equiv) and 0.18 g of EDCI (0.87 mmol, 1.1 equiv). After flash column chromatography (gradient elution; 0-4% MeOH in DCM) the product (0.35 g; 88%) was obtained as a white foam. R_f: 0.47 (DCM/MeOH 96:4 v/v); ^{1}H NMR ($CDCl_3$): δ = 7.77-7.61 (broad m), 7.58-7.35 (broad m, total 10H), 7.03 (broad s, 1H), 5.14 (d, J = 8.8 Hz, 1H), 4.19-3.96 (broad m, 3H) 3.70 (d, J = 7.1 Hz, 2H), 2.17 (m, 1H), 1.41 (s, 9H), 1.54-0.25 (broad m, 3H), 0.96 (dd, J = 6 .9 Hz, J = 19.5 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ = 194.8, 172.3, 155.9, 132.4/132.3 ($J_{P\text{-}C}$ = 10.3 Hz), 131.8, 128.9/128.8 ($J_{P\text{-}C}$ = 10.3 Hz), 127.6/126.9 ($J_{P\text{-}C}$ = 56.7 Hz), 80.1, 59.5, 48.6, 30.6, 28.2, 23.4/22.9 ($J_{P\text{-}C}$: 35.5 Hz), 19.3, 17.5.

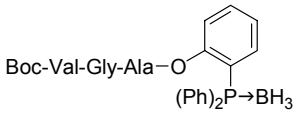


Boc-Val-Gly-(diphenylphosphino)methanethiol (32): DABCO (90 mg; 0.80 mmol; 2 equiv) was added under a nitrogen atmosphere to a solution of **31** (0.20 g; 0.40 mmol) in dry and deoxygenated toluene (40 mL) and the reaction mixture was heated to 50°C. After stirring for 3 h, the solvent was removed *in vacuo*. The residue was dissolved in EtOAc (50 mL) and this solution was subsequently washed with 1N $KHSO_4$ (2 × 20 mL) and brine (2 × 20 mL), dried (Na_2SO_4) and evaporated to dryness. The product (0.15 g; 77%) was isolated as a white foam by flash column chromatography (gradient elution; 0-4% MeOH in DCM). R_f: 0.55 (DCM/MeOH 96:4 v/v); R_t: 18.25 min (method A); ^{1}H NMR ($CDCl_3$): δ = 7.55-7.39 (broad m), 7.38-7.35 (broad m, total 10H), 7.00 (broad s, 1H), 5.19 (broad s, 1H), 4.26-3.94 (broad m, 3H) 3.51 (d, J = 3.6 Hz, 2H), 2.16 (m, 1H), 1.41 (s, 9H), 0.97 (dd, J = 6.6 Hz, 19.2 Hz, 6H); ^{13}C

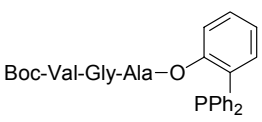
NMR ($CDCl_3$): δ = 195.9, 172.2, 155.9, 136.5/136.4 ($J_{P\text{-}C}$ = 13.7 Hz), 132.8/132.5 ($J_{P\text{-}C}$ = 19.5 Hz), 129.1, 128.6/128.5 ($J_{P\text{-}C}$ = 6.9 Hz), 79.9, 59.6, 48.8, 30.7, 28.2, 25.5/25.1 ($J_{P\text{-}C}$ = 24.1 Hz), 19.3, 17.6. ESMS calcd. for $C_{25}H_{33}N_2O_4PS$ 488.19, found 527.30 $[M + K]^+$.



Boc-Val-Gly-Ala-borane-*o*-(dibutylphosphino)phenol (42): The reaction was carried out following the general procedure using 0.49 g of Boc-Val-Gly-Ala-OH (1.18 mmol), 0.42 g of **41** (1.18 mmol), 14 mg of DMAP (0.12 mmol, 0.1 equiv) and 0.25 g of EDCI (1.30 mmol, 1.1 equiv). After flash column chromatography (EtOAc/hexanes 7:3 v/v) the product (0.49 g; 71%) was obtained as a white foam. R_f: 0.48 (EtOAc/hexanes 4:1 v/v); R_t: 21.53 min (method B); ^{1}H NMR ($CDCl_3$): δ = 7.87 (m, 1H), 7.53 (m, 1H), 7.30 (m, 2H), 7.15 (m, 1H), 6.79 (broad s, 1H), 5.05 (m, 1H), 4.80 (m, 1H), 4.10 (m, 1H), 3.92 (broad m, 3H), 2.22-0.25 (broad m, total 40H), 2.00 (m), 1.84 (m), 1.64 (m), 1.45 (m), 1.33 (m), 0.99 (m), 0.86 (m); ^{13}C NMR ($CDCl_3$): δ = 172.2, 170.9, 168.8, 156.3/156.1 ($J_{P\text{-}C}$: 20.1 Hz), 153.0, 136.2/135.9/135.8, 132.8, 126.3/126.2 ($J_{P\text{-}C}$ = 9.5 Hz) 122.8, 120.3/119.7 ($J_{P\text{-}C}$ = 46.1 Hz), 80.6/80.3, 60.7, 48.6, 30.4, 28.3, 25.0/24.9/24.3/24.124.0, 19.3, 18.0/17.7($J_{P\text{-}C}$: 15.6 Hz), 17.4/17.2($J_{P\text{-}C}$: 18.0 Hz), 13.5; ESMS calcd. For $C_{28}H_{49}BN_3O_7P$ 581.34, found 588.55 $[M - BH_3 + Na]^+$, 532.35 $[(M - BH_3 - C_4H_8) + Na]^+$, 488.20 $[(M - BH_3 - C_5H_8O_2) + Na]^+$.



Boc-Val-Gly-Ala-borane-*o*-(diphenylphosphino)phenol. (44): The reaction was carried out following the general procedure using 0.71 g of Boc-Val-Gly-Ala-OH (2.05 mmol), 0.60 g of **43** (2.05 mmol), 25 mg of DMAP (0.21 mmol, 0.1 equiv) and 0.43 g of EDCI (2.25 mmol, 1.1 equiv). After flash column chromatography (gradient elution; 0-4% MeOH in DCM) the product (1.13 g; 89%) was obtained as a white foam. R_f: 0.68 ($CHCl_3$/MeOH/HOAc 95:20:3); R_t: 20.67 min (method B); ^{1}H NMR ($CDCl_3$): δ = 7.66-7.43 (broad m), 7.35-7.19 (broad m), 7.09-6.75 (broad m, total 14H), 5.21 (m, 1H), 4.28 (m, 1H), 3.99-3.74 (broad m, 3H), 2.15 (m, 1H), 1.65-0.60 (broad m, total 21H), 1.43 (s), 1.28 (m), 0.98; ^{13}C NMR ($CDCl_3$): δ = 172.2, 169.7, 169.7, 168.3, 155.9, 152.3, 134.6, 133.9, 133.6, 133.0/132.9 ($J_{P\text{-}C}$: 11.0 Hz), 131.6, 129.0/128.9, 129.6, 128.2/127.5 ($J_{P\text{-}C}$ = 58.6 Hz), 126.2/126.0, 123.5, 121.5/120.7 ($J_{P\text{-}C}$ = 57.4 Hz), 79.9, 48.3, 42.6, 30.7, 28.2, 19.2, 17.9, 16.7; ESMS calcd. For $C_{32}H_{41}BN_3O_7P$ 621.28, found 627.95 $[(M - BH_3) + Na]^+$, 572.05 $[(M - BH_3 - C_4H_8) + Na]^+$, 528.25 $[(M - BH_3 - C_5H_8O_2) + Na]^+$.



Boc-Val-Gly-Ala-*o*-(diphenylphosphine)phenyl ester (46): The synthetic procedure and analytical data for this compound have been described in chapter 2.

General procedure for the Staudinger ligation of phosphine esters 25, 27, 29 and 32 with N_3-Phe-Leu-OMe to obtain tetrapeptide 33: Azidopeptide N_3-Phe-Leu-OMe (50 mg; 0.16 mmol) was dissolved in dry and deoxygenated THF (3.15 mL) and the solution was warmed to 47°C. Then, the phosphine ester (0.16 mmol; 1 equiv) was added under a nitrogen atmosphere. The reaction progress was monitored by MS analysis at t = 1, 16, and 48 h. After stirring for 48 h the reaction was quenched with 1 mL of H_2O. The reaction mixture was stirred for an additional 2 h before it was evaporated *in vacuo*. The residue was dissolved in EtOAc (10 mL) and this solution was subsequently washed with 1N $KHSO_4$ (2 × 2 mL) and brine (2 × 2 mL), dried (Na_2SO_4) and evaporated to dryness. The product was isolated by flash column chromatography (eluent: EtOAc) and compared to the analytical data for Boc-Val-Gly-Phe-Leu-OMe (compound **19**, chapter 2), the purity was assessed by analytical HPLC.

General procedure for the Staudinger ligation of phosphine esters 42 and 44 with N_3-Phe-Leu-OMe to obtain pentapeptide 47: Azidopeptide N_3-Phe-Leu-OMe (38.2 mg; 0.12 mmol) and DABCO (54 mg, 0.48 mmol, 4 equiv) were dissolved in dry and deoxygenated THF (2.5 mL) and the solution was heated to 70°C in a capped vial. Then, the phosphine ester (0.12 mmol; 1 equiv) was added under a nitrogen atmosphere. The reaction progress was monitored by ESMS and HPLC analysis at t = 1, 16 and 48 h. After stirring for 48 h the reaction was quenched with 1 mL of H_2O. The reaction mixture was stirred for an additional 2 h before it was evaporated *in vacuo*. Since no product formation could be detected, the reaction mixture was not further worked up.

Staudinger ligation of phosphine ester 46 with N_3-Phe-Leu-OMe to obtain pentapeptide 47: The synthetic procedure and analytical data for Boc-Val-Gly-Ala-Phe-Leu-OMe have been described in chapter 2.

3.6 References and notes

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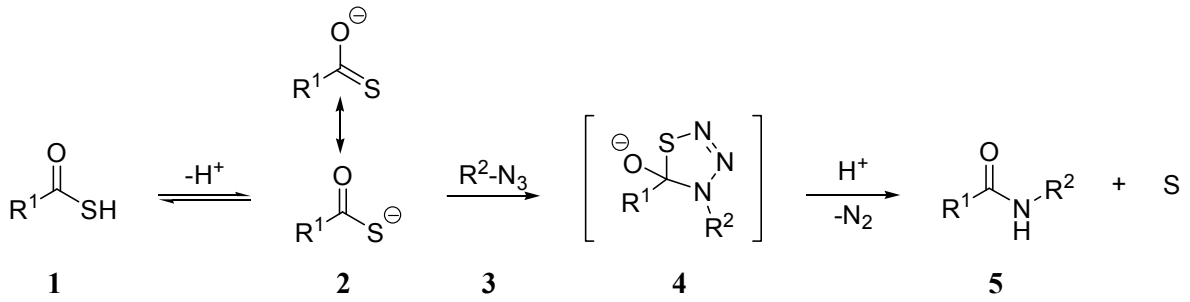
4

The Reaction of Peptide Thio Acids with Azidopeptides: Scope and Limitations

Recently, the reaction of thio acids with azides was reinvestigated and was found to proceed chemoselectively to yield amides. In this chapter, the scope and limitations of this thio acid/azide amidation reaction, as a potential novel chemoselective ligation reaction, were explored. Therefore, a series of unprotected functionalized peptide thio acids and azidopeptides were allowed to react. Optimization involved the addition of $RuCl_3$ and PPh_3, respectively to activate the thio acid and the azide. It was found that addition of PPh_3 did not lead to a more efficient reaction and although the addition of $RuCl_3$ as a catalyst did increase the efficiency, concurrently the chemoselectivity of the reaction was decreased.

4.1 Introduction

The chemoselective ligation of peptides requires the efficient coupling of two mutually and uniquely reactive functional groups under native (aqueous) conditions. Preferably, an amide bond is formed since this type of bond is ubiquitous in nature. However, chemoselective ligation methods that yield an amide bond are not very common. Two important examples of chemoselective amidation reactions have been discussed in this thesis: native chemical ligation (NCL)[1,2] and the so called Staudinger ligation.[3,4] Although their successful application as ligation methodology has been demonstrated in the literature, both methods remain restricted in their use by their dependence on the amino acid residues at the ligation site. The need for a cysteine residue (NCL) and occurrence of steric congestion at the site of ligation, largely determine the reaction outcome of these methods. Therefore, it is of considerable interest do develop more general chemoselective amidation reactions as novel chemoselective ligation methodology.



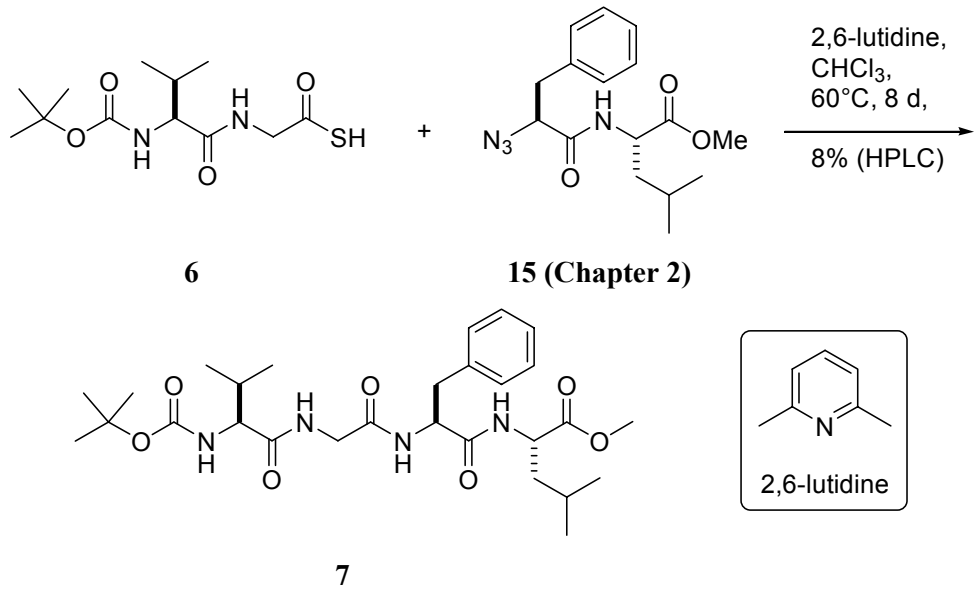
Scheme 4.1 Proposed mechanism for the thio acid/azide amidation reaction.

The amidation reaction of thio acids with azides is known from literature and at first it was assumed that the reaction takes place via the reduction of the azide to the corresponding amine prior to acylation by the thio acid.[5] However, the mechanism of the reaction of thio acids with (arylsulfonyl) azides was recently reinvestigated by Williams *et al.*[6-8] and it was found that the reaction proceeded via a thiatriazoline intermediate (**4**) rather than via conventional nucleophilic acyl substitution of the free amine (Scheme 4.1). This means that the amidation reaction is potentially chemoselective. Moreover, the reaction was found to proceed very efficiently in a variety of solvents giving rise to nitrogen and sulphur as the only byproducts. These observations enticed us to evaluate the application of this unconventional amidation reaction for the coupling of unprotected peptide segments and the results of these investigations are described in this chapter.

4.2 Results and discussion

The azides used in this research could be obtained from bromides via a simple nucleophilic substitution with NaN_3 or from the corresponding amine via a diazotransfer reaction using triflic azide as was described in chapter 2.[9-11]

For the synthesis of thio acids several methods exist, in some cases thio esters are used as a precursor and thus require a two step procedure to obtain the thio acid.[8,12] In the research presented here, thio acids were synthesized directly from the corresponding hydroxysuccinimide ester by treatment with NaHS as was essentially described by Goldstein and Gelb.[13]



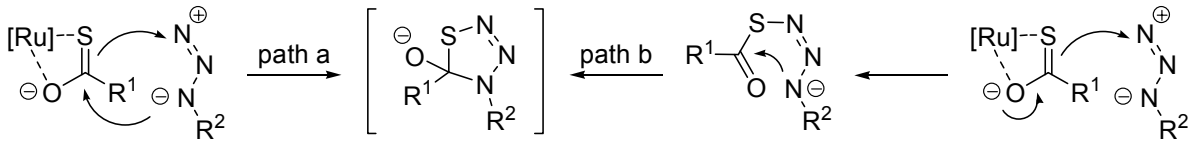
Scheme 4.2 Thio acid/azide amidation test reaction.

As model system, peptide thio acid **6** was reacted with N_3-Phe-Leu-OMe in the presence of 2,6-lutidine (4 equiv) as base at 60°C (Scheme 4.2). The reaction was monitored by HPLC and the formation of tetrapeptide **7** was found to be very slow. Disappointingly, **7** was formed in only 8% yield after eight days as was estimated from peak areas in the HPLC-chromatograms. Thereupon, several ways to improve the efficiency of this amidation reaction were investigated (*vide infra*).

4.2.1 Activation of the thio acid

In the course of these experiments, a paper by Wong *et al.* described the Ru(III)-promoted amide formation from thioacetic acid and glycosyl azides.[14] It was speculated that thiatriazoline formation can take place in two ways, either via a concerted 1,3-dipolar

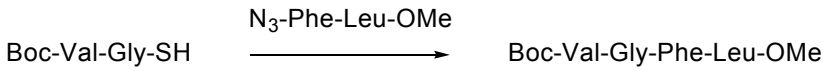
cycloaddition[15] (path a, Scheme 4.3) or via a two step cyclization reation (path b) similar to the diazotransfer reaction.[9,16]



Scheme 4.3 Proposed mechanism for the thiatriazoline formation in the prescence of ruthenium.

Since both types of reactions can be accelerated by metals, $RuCl_3$ was tried as a catalyst. Indeed, addition of $RuCl_3$ to the reaction mixture, as described in Scheme 4.2, was found to promote the amidation reaction. Presumably, the thio acid was activated via complexation of the thio carbonyl moiety to ruthenium as depicted in Scheme 4.3.[17] The model reaction of **6** and N_3-Phe-Leu-OMe was repeated, however, this time in the presence of 50 mol-% $RuCl_3$. Using these conditions, tetrapeptide **7** could be isolated in an improved yield of 24% after 72 h.

Table 4.1 Optimization of the solvent.



6 **7**

Entry[a]	Solvent	% Yield[b]	% Yield with $RuCl_3$[b]
1	$CHCl_3$	8	24
2	MeCN	4	41
3	MeCN/H_2O 3:1	8	9
4	acetone	1	23
5	MeOH	18	0[c]

[a]Conditions: thio acid (1 equiv), azide (1 equiv), $RuCl_3$ (0.5 equiv), 2,6-lutidine (4 equiv), 60°C, 36 h. [b]Isolated yields. [c]MeOH is reactive under these conditions and the corresponding methyl (oxy) ester of **6** was isolated.

The low solubility of $RuCl_3$ in $CHCl_3$ prompted us to optimize the solvent (Table 4.1). The highest coupling yield was achieved when MeCN was used as a solvent (entry 2). Under these conditions tetrapeptide **7** could be isolated in 41% yield after 36 h. In an attempt to increase the yield further, the reaction was run in a microwave reactor, unfortunately however, only degradation products were observed by TLC. It should be noted that when MeOH was used as solvent and in the absence of catalyst, tetrapeptide **7** was isolated in 18% yield. Conversely, in the presence of $RuCl_3$, MeOH was found to be reactive and resulted in a quantitative formation of the corresponding methyl ester of **6**.[18,19]

Table 4.2 Investigation of the chemoselectivity.

Entry[a]	Thio acid	Azide	Product	% Yield
1	Boc-Val-Gly-SH (**6**)	N_3-Phe-Leu-OMe (**15**, Ch2)	Boc-Val-Gly-Phe-Leu-OMe (**7**)	41[b]
2	Boc-Leu-SH (**8**)	N_3-Gly-OEt (**9**)	Boc-Leu-Gly-OEt (**10**)	13[b]
3	Boc-Leu-SH (**8**)	N_3-Gly-OH (**11**)	Boc-Leu-Gly-OH (**12**)	no rxn
4	Cbz-Gly-SH (**13**)	N_3-Gly-OEt (**9**)	Cbz-Gly-Gly-OEt (**14**)	22[b]
5	Cbz-Gly-SH (**13**)	N_3-Phe-Leu-OMe (**15**, Ch2)	Cbz-Gly-Phe-Leu-OMe (**15**)	24[b]
6	Boc-Leu-SH (**8**)	N_3-Leu-Lys-Phe-NH_2 (**16**, Ch2)	Boc-Leu-Leu-Lys-Phe-NH_2 (**16**)	11[c]
7	Boc-Val-Gly-SH (**6**)	N_3-Leu-Lys-Phe-NH_2 (**16**, Ch2)	Boc-Val-Gly-Leu-Lys-Phe-NH_2 (**17**)	3[c]

[a]Conditions: thio acid (1 equiv), azide (1 equiv), $RuCl_3$ (0.5 equiv), 2,6-lutidine (4 equiv), MeCN, 60°C, 36 h. [b]Isolated yields. [c]Yields were estimated from HPLC peak areas.

Next, the chemoselectivity of the reaction between a thio acid and an azide in the presence of a free amine-, a carboxylate- or a carboxamide functionality was investigated. Secondly, a set of different sterically demanding thio acids and azides was prepared to study the influence of the amino acid side chain on the coupling yield. The results are given in Table 4.2. Unfortunately, in all cases the isolated yields were rather low. More importantly, also the chemoselectivity was rather low as the presence of a free carboxylic acid moiety (entry 3) was found to be detrimental to the coupling reaction since no product could be isolated at all. The presence of a free amine (εNH_2 lysine side chain, entries 6 and 7) resulted in the formation of side products **18** and **19** and **20** respectively (Figure 4.1).

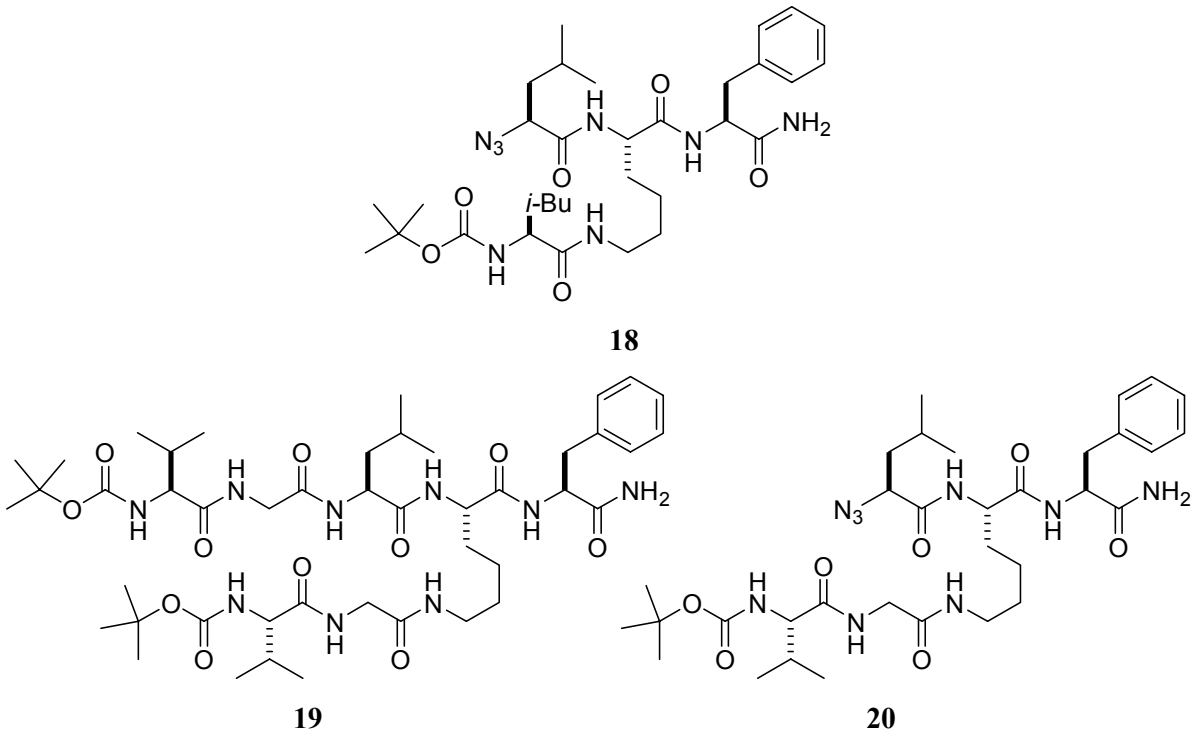


Figure 4.1 Structure of the isolated side products as identified by ESMS.

Side products **18**, **19** and **20** were identified by ESMS after their isolation from the crude reaction product by preparative HPLC, additionally, the chemical structure of **18** was confirmed by NMR spectroscopy. Furthermore, HPLC analysis of crude peptide **16** showed that under these conditions no selectivity could be observed for the amidation reaction of peptide thio acid **8** with N_3-Leu-Lys-Phe-NH_2, instead, side product **18** was formed as major product (Figure 4.2).

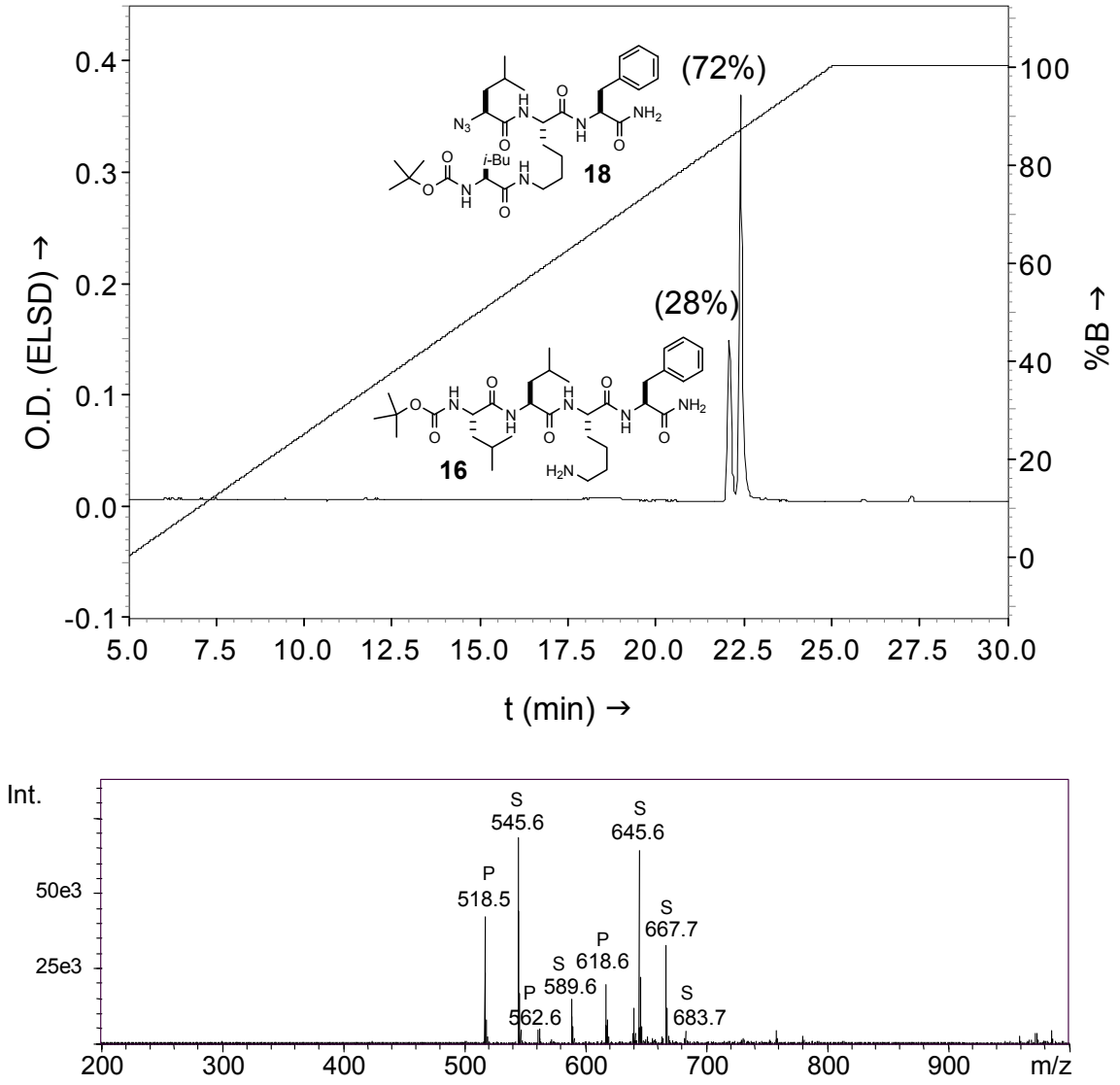
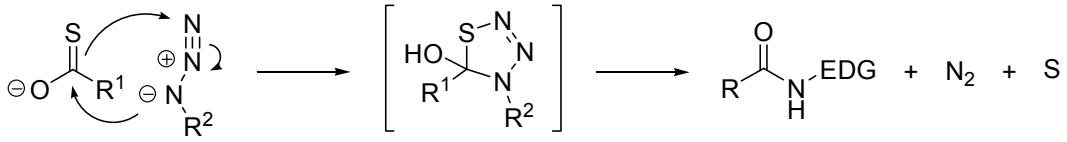


Figure 4.2 ESMS and HPLC analysis of the crude peptide **16**. ESMS calcd for product **16** (P) 618.41, found 518.5 $[(M–C_5H_8O_2)+H]^+$, 562.6 $[(M–C_4H_8)+H]^+$, 618.6 $[M+H]^+$; sideproduct **18** (S) 644.40, found 545.6 $[(M–C_5H_8O_2)+H]^+$, 589.6 $[(M–C_4H_8)+H]^+$, 645.6 $[M+H]^+$, 667.7 $[M+Na]^+$, $[M+K]^+$.

The yields of the described amide formation reactions were significantly lower than those reported in the literature,[6] even in the presence of $RuCl_3$.[14] However, an important difference is the nature of the thio acids and azides that have been used in this study. The examples mentioned in the literature make use of relatively unhindered thio acetic acid or thio benzoic acid. Obviously, the coupling of unprotected amino acids or peptide segments is far more challenging than simple acetyl/benzoyl transfer. Additionally, formation of the proposed thiatriazoline intermediate (**4**, Scheme 4.1) is favoured for highly polarized electron-deficient azides. The azidopeptides that have been used in this study, however, are

relatively electron rich since the amino acid side chain is electron donating, thereby decreasing the reactivity of the azide. This was in good agreement with recent results of Williams *et al.*[7] who studied the mechanism of the thio acid/azide amidation reaction. They found that the reaction of electron-rich azides and thio acids proceeds via a concerted 1,3-dipolar cycloaddition (Scheme 4.4) rather than via a two-step mechanism and requires extended heating of concentrated reaction mixtures to induce satisfactory reaction rates. In analogy, Wu and Hu found the reaction of selenocarboxylates, as more reactive thio acid congeners, with azides was only efficient in case of electron-deficient azides.[20,21] Apparently, electron rich azides are inefficient in the thio acid/azide amidation reaction.



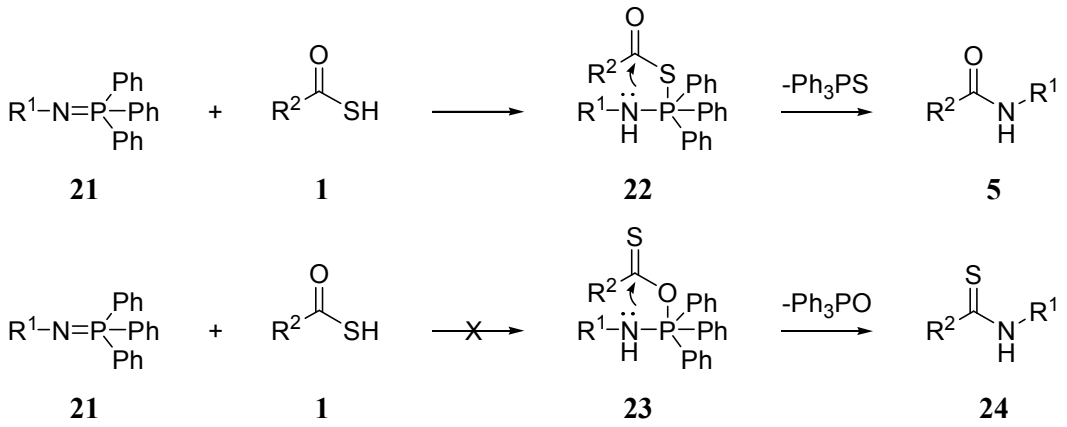
Scheme 4.4 Mechanism for the thio acid/azide amidation with electron-rich azides.

To compensate for the decreased azide reactivity, Ru(III) has been added to form a complex with the thio carbonyl moiety in order to increase the electrophilic character. Thus, this complex was presumably more susceptible towards nucleophiles e.g. amines and carboxylates, which might explain the low chemoselectivity (entires 3, 6 and 7, Table 4.2). These results prompted us to look for alternative ways of promoting the amidation reaction, involving activation of the azide.

4.2.2 Activation of the azide

Park *et al.*[22] have described a thio acid/azide amidation reaction in the presence of tertiary phosphines. Upon addition of a phosphine to an azide, a strongly nucleophilic iminophosphorane was formed which was subsequently acylated by the thio acid (Scheme 4.5). In contrast to standard methods in which activated carboxylic acids react with all free amines available to form an amide bond, in this case only the latent azide moiety reacts since it is converted into the active species by a highly selective Staudinger reaction. Moreover, the fact that the amide (**5**) was isolated as the sole product while no thioamide (**24**) was detected, could imply that the amidation reaction proceeded chemoselectively. It should be noted however, that the iminophosphorane intermediate is also known to be reactive toward other electroophiles, amongst other, carboxylic acids and esters in inter- and intramolecular reactions to yield amides thereby reducing the chemoselectivity.[3,23-28] However, if the prior-caption of the thio acid by the iminophosphorane is faster, this

*intra*molecular acyl migration may be favoured over *inter*molecular side reactions, leading to the chemoselective formation of an amide bond.



Scheme 4.5 Proposed mechanism for the thio acid/aza-ylide amidation reaction.

In an attempt to increase the yield of the thio acid/azide amidation model reaction of **6** and N_3-Phe-Leu-OMe, the triphenylphosphine mediated activation of the azide was investigated. Following the literature procedure of Park *et al.*, who optimized the reaction conditions by monitoring the reaction progress with NMR analysis, triphenylphosphine was added to N_3-Phe-Leu-OMe at 0°C in MeCN and the resulting mixture was stirred for 1 h. Next, the reaction mixture was heated to 65°C and stirred for an additional 40 h. Subsequently Boc-Val-Gly-SH (**6**) was added and the reaction mixture was stirred for 24 h at room temperature before it was worked up. Unfortunately, tetrapeptide **7** could be neither isolated nor detected by HPLC/ESMS. Presumably, also in this case, the increased complexity of dipeptide thio acid **6** compared to acetic acid as was used by Park *et al.* might explain the difference in the reaction outcome.

4.3 Conclusions

It was shown that a dipeptide thio acid and azidodipeptide will react to form a tetrapeptide albeit in a moderate yield. It was also shown that the addition of Ru(III) as catalyst to activate the thio acid, increased the yield however, simultaneously, decreased the chemoselectivity of the reaction. Activation of the azide toward the reaction with thio acids via a Staudinger reaction with triphenylphosphine to form the iminophosphorane intermediate did not lead to a more efficient reaction. Thus, the thio acid/azide amidation reaction is not a suitable alternative for the existing amide forming methods such as Native Chemical Ligation[1,2] and the Staudinger ligation,[3,4] in achieving the chemoselective ligation of peptides.

4.4 Experimental section

General information

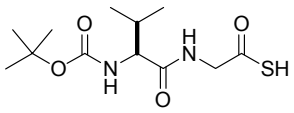
Analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 5 μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in MeCN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min, connected to a UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000). Preparative HPLC was performed on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 10 μm, 250 × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.085% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 11.5 mL/min. Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. IR spectra were recorded on a Biorad FTS6000 spectrometer. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel 60F^{254} plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[29] Specific optical rotations were measured using a Jasco P1010 polarimeter. Elemental analyses were done by Kolbe Mikroanalytisches Labor (Mülheim an der Ruhr, Germany).

Chemicals and reagents

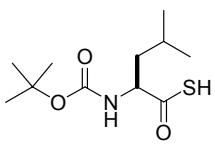
Unless stated otherwise, all chemicals were obtained from commercial sources and used as such. Peptide grade dichloromethane (DCM), tetrahydrofuran (THF), ethyl acetate (EtOAc), hexane, chloroform, acetone, HPLC grade acetonitrile and trifluoroacetic acid (TFA) were purchased from Biosolve. Trifluoromethanesulfonic acid anhydride (triflic anhydride) and dicyclohexyl carbodiimide (DCC) were obtained from Fluka. All amino acids were purchased from GI Biochem Shanghai. *N*,*N*′-Diisopropylethylamine (D*i*PEA), ruthenium(III)chloride ($RuCl_3$) and copper(II) sulfate were obtained from Acros Organics, *N*-hydroxysuccinimide (NHS) was purchased from Aldrich, acetic acid (HOAc) and *iso*propanol (*i*-PrOH) were obtained from Merck KGaA.

General procedure for the synthesis of thio acids 6, 8 and 13:[13]

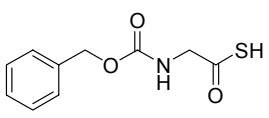
The corresponding amino/peptide acid (1 equiv) and NHS (1.10 equiv) were dissolved in MeCN to obtain a 0.10 M solution which was cooled on an ice bath. Next, DCC (1.05 equiv) was added to the cooled solution and the reaction mixture was allowed to warm up to rt and stirred for 16 h. After removal of the DCU side product by filtration, the filtrate was concentrated *in vacuo*. The HONSu-ester was isolated by crystallisation from EtOAc/hexane. Next, $NaHS.xH_2O$ (3 mmol, 2 equiv) was crushed and dissolved in 2-propanol (120 mL). After addition of the HONSu-ester (1.5 mmol, 1 equiv), the clear solution became opaque yellow. The progress of the reaction was monitored by TLC (eluent: EtOAc/hexane/HOAc 89.5:9.5:1.0 v/v/v). After 1 – 3 h of stirring, the reaction mixture was quenched[30] with 1N $KHSO_4$ (60 mL) and the reaction mixture was concentrated to a volume of 60 mL. The residue was extracted with EtOAc (1 × 100 mL) and subsequently washed with 1N $KHSO_4$ (2 × 50 mL) and brine (2 × 50 mL) and dried (Na_2SO_4). Removal of the solvent afforded the crude thio acid which was used without further purification.



Boc-Val-Gly-SH (6): The reaction was carried out following the general procedure using 1.0 g of Boc-Val-Gly-ONSu (2.8 mmol) and 315 mg of $NaHS.xH_2O$ (5.6 mmol). The product (743 mg, 91%) was obtained as a white solid. R_f: 0.19 (EtOAc/hexane/HOAc 9:1:0.1 v/v/v); R_t: 15.37 min; $[\alpha]_D$: −12.7° c = 0.1 DMF; ^{1}H NMR ($CDCl_3$): δ = 6.97 – 6.87 (broad s, 1H), 5.13 (d, *J* = 8.5 Hz, 1H), 4.23 (d, *J* = 5.6 Hz, 2H), 4.02 (m, 1H), 2.21 (m, 1H), 1.45 (s, 9H), 0.99 (m, 6H); ^{13}C NMR ($CDCl_3$): δ = 196.0, 172.8, 156.1, 80.0, 59.7, 50.3, 30.7, 28.2, 19.2, 17.9; ESMS calcd for $C_{12}H_{22}N_2O_4S$: 290.13, found: 601.50 $[(2M-2H)+Na]^+$; Anal. calcd (%) for $C_{12}H_{22}N_2O_5S$: C 49.63, H 7.64, N 9.65; found: C 49.78, H 7.57, N 9.53.

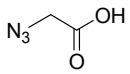


Boc-Leu-SH (8): The reaction was carried out following the general procedure using 200 mg of Boc-Leu-ONSu (0.6 mmol) and 68 mg of $NaHS.xH_2O$ (1.2 mmol). The product (147 mg, 98%) was obtained as a clear oil. R_f: 0.38 (EtOAc/hexane 9:1 v/v); R_t: 19.76 min; ^{1}H NMR ($CDCl_3$): δ = 4.91 (broad s, 1H), 4.29 (m, 1H,), 1.72 – 1.45 (broad m, 12H), 0.95 (m, 6H); ESMS calcd for $C_{11}H_{21}NO_3S$: 247.12, found: 515.35 $[(2M-2H)+Na]^+$.

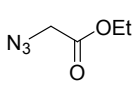


Cbz-Gly-SH (13): The reaction was carried out following the general procedure using 300 mg of Cbz-Gly-ONSu (1.0 mmol) and 112 mg of $NaHS.xH_2O$ (2.0 mmol). The product (172 mg, 74%) was

obtained as a clear oil. R_f: 0.38 (EtOAc/hexane/HOAc 9:1:0.1 v/v/v); R_t: 13.76 min; ^{1}H NMR ($CDCl_3$): δ = 7.32 (m, 5H), 5.86 (broad s, 1H), 5.12 (s, 2H), 4.00 (d, J = 6.0 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ = 196.8, 156.3, 135.7, 128.4, 128.2, 128.0, 67.2, 51.6; ESMS calcd for $C_{10}H_{11}NO_3S$: 225.05, found: 471.30 $[(2M-2H)+Na]^+$.



N_3-Gly-OH (11):[10] NaN_3 (1.30 g, 10 mmol) was partially dissolved in DMSO (56 mL) and stirred for 1.5 h. Next, ethyl bromoacetate (1.39 g, 10 mmol) was added. After the reaction mixture was stirred for 16 h, H_2O (200 ml) was added and the pH was adjusted to 2.5 with concentrated HCl. The product was extracted with EtOAc (3 × 100 ml) and the combined extracts were washed with brine (3 × 100 mL). The EtOAc-layer was dried ($MgSO_4$) and concentrated which afforded the product (0.70 g, 69%) as a clear oil. ^{1}H NMR ($CDCl_3$): δ = 10.72 (s, 1H), 3.98 (s, 2H); ^{13}C NMR ($CDCl_3$): δ = 173.3, 49.8; IR (KBr) ν = 2114 (s).



N_3-Gly-OEt (9):[10] NaN_3 (1.30 g, 10 mmol) was partially dissolved in DMSO (56 mL) and stirred for 1.5 h. Next, bromoacetic acid (1.11 mL, 10 mmol) was added. After the reaction mixture was stirred for 16 h, H_2O (200 ml) was added and th pH was adjusted to 2.5 with concentrated HCl. The product was extracted with EtOAc (3 × 100 ml) and the combined extracts were washed with brine (3× 100 ml). The EtOAc-layer was dried ($MgSO_4$) and concentrated which afforded the product (0.40 g, 31%) as a clear oil. R_t: 10.02 min; ^{1}H NMR ($CDCl_3$): δ = 4.25 (q, 2H), 3.88 (s, 2H), 1.32 (t, 3H); ^{13}C NMR ($CDCl_3$): δ = 168.2, 61.6, 50.1; IR (KBr) ν = 2114 (s).

General procedure for the reaction of azides and thio acids:
An equimolar mixture of the thio acid and azide was dissolved in dry and oxygen-free MeCN at a final concentration of 0.15 mM. Subsequently 2,6-lutidine (4 equiv) and $RuCl_3$ (0.5 equiv) were added and the reaction mixture was stirred at 60ºC under a nitrogen atmosphere for 36 h. Next the solvent was removed *in vacuo* and the residue was purified by column chromatography.

Boc-Val-Gly-Phe-Leu-OMe (7): The reaction was carried out following the general procedure using 87 mg of **6** (0.30 mmol), 96 mg of N_3-Phe-Leu-OMe (0.30 mmol), 140 µL of 2,6-lutidine (1.20 mmol), 31 mg of $RuCl_3$ (0.15 mmol) and 2 mL of MeCN. After flash column chromatography (eluent: EtOAc/hexane 9:1 v/v), the product (67 mg, 41%) was obtained as a white foam. The complete analytical data for Boc-Val-Gly-Phe-Leu-OMe has been described in chapter 2.

Boc-Leu-Gly-OEt (10): The reaction was carried out following the general procedure using 74 mg of **8** (0.30 mmol), 39 mg of **9** (0.30 mmol), 140 µL of 2,6-lutidine (1.20 mmol), 31 mg of $RuCl_3$ (0.15 mmol) and 2 mL of MeCN. After flash column chromatography (gradient elution: 0 – 2% MeOH in DCM), the product (12 mg, 13%) was obtained as a clear oil. R_f: 0.33 (DCM/MeOH 96:4 v/v); R_t: 15.24 min; ^{1}H NMR ($CDCl_3$): δ = 6.70 (s, 1H), 4.95 (d, *J* = 8.2 Hz, 2H) 4.25 (q, 2H) 4.04 (d, *J* = 5.2 Hz, 2H) 1.70 (m, 3H), 1.45 (s, 9H), 1.28 (t, 3H), 0.95 (m, 6H); ^{13}C NMR ($CDCl_3$): δ = 172.8, 169.7, 155.7, 80.2, 61.5, 52.9, 41.3, 28.3, 24.7, 22.9, 21.8, 14.1; ESMS calcd for $C_{15}H_{28}N_2O_5$: 316.20, found: 339.25 $[M+Na]^+$.

Cbz-Gly-Gly-OEt (14): The reaction was carried out following the general procedure using 68 mg of **13** (0.30 mmol), 39 mg of **9** (0.30 mmol), 140 µL of 2,6-lutidine (1.20 mmol), 31 mg of $RuCl_3$ (0.15 mmol) and 2 mL of MeCN. After flash column chromatography (eluent: EtOAc/hexane 9:1 v/v), the product (14 mg, 22%) was obtained as a clear oil. R_f: 0.39 (EtOAc/hexane 9:1 v/v); R_t: 13.24 min; ^{1}H NMR ($CDCl_3$): δ = 7.35 (broad s, 5H), 6.62 (s, 1H) 5.53 (s, 1H) 5.13 (s, 2H) 4.22 (q, 2H), 4.04 (d, *J* = 5.2 Hz, 2H), 3.94 (d, *J* = 5.8 Hz, 2H), 1.28 (t, 3H); ^{13}C NMR ($CDCl_3$): δ = 169.7, 169.2, 156.6, 136.0, 128.6, 128.3, 128.1, 67.3, 61.7, 44.4, 41.2, 14.1; ESMS calcd for $C_{14}H_{18}N_2O_5$: 294.12, found: 317.15 $[M+Na]^+$.

Cbz-Gly-Phe-Leu-OMe (15): The reaction was carried out following the general procedure using 68 mg of **13** (0.30 mmol), 96 mg of N_3-Phe-Leu-OMe (0.30 mmol), 140 µL of 2,6-lutidine (1.20 mmol), 31 mg of $RuCl_3$ (0.15 mmol) and 2 mL of MeCN. After flash column chromatography (eluent: EtOAc/hexane 8:2 v/v), the product (26 mg, 24%) was obtained as a clear oil. R_f: 0.55 (EtOAc/hexane 8:2 v/v); R_t: 16.83 min; ^{1}H NMR ($CDCl_3$): δ = 7.34-7.16 (broad m, 10H), 6.88 (d, *J* = 7.4 Hz, 2H) 6.54 (d, *J* = 7.4 Hz, 2H) 5.09 (s, 2H) 4.75 (m, 1H), 4.52 (m, 1H), 3.84 (d, *J* = 5.5 Hz, 2H), 3.68 (s, 3H), 3.06 (d, *J* = 6.3 Hz, 2H), 1.59-1.46 (broad m, 3H), 0.88 (m, 6H); ^{13}C NMR ($CDCl_3$): δ = 172.7, 170.4, 156.6, 136.2, 136.0, 129.3, 128.6, 128.5, 128.2, 128.1, 127.0, 67.2, 54.2, 52.3, 50.9, 44.4, 41.2, 38.2, 29.7, 24.7, 22.6, 21.9; ESMS calcd for $C_{26}H_{33}N_3O_6$: 483.24, found: 484.45 $[M+H]^+$, 506.45 $[M+Na]^+$.

Boc-Leu-Leu-Lys-Phe-NH_2 (16): The reaction was carried out following the general procedure using 37 mg of **8** (0.15 mmol), 65 mg of N_3-Leu-Lys-Phe-NH_2 (0.15 mmol), 70 µL of 2,6-lutidine (60 mmol), 16 mg of $RuCl_3$ (75 µmol) and 1 mL of MeCN. After flash column chromatography (eluent: EtOAc/hexane/HOAc 79.5:19.5:1.0 v/v/v), the crude

product (36.3 mg, 40%, purity: 28% by HPLC) was obtained as a clear sirup. From this crude product two small portions of pure **16** and pure **18** could be isolated by preparative HPLC and were analyzed by ESMS and NMR respectively. Crude product: R_f: 0.32 (EtOAc/hexane/HOAc 79.5:19.5:1.0 v/v/v). Product **16**: R_t: 17.08 min; ESMS calcd for $C_{32}H_{54}N_6O_6$: 618.41, found: 618.75 $[M+H]^+$. Side product **18**: R_t: 17.41 min; ESMS calcd for $C_{32}H_{52}N_8O_6$: 644.40, found: 645.65 $[M+H]^+$. ^1H NMR (500 MHz, DMSO-d_6): δ = 8.3 (d, J = 7.6 Hz, 1H), 7.97 (d, J = 8.2 Hz, 1H), 7.75 (s, 1H), 7.38 (s, 1H), 7.22 (m, 5H), 7.08 (s, 1H), 6.76 (d, J = 8.2 Hz, 1H), 4.43 (m, 1H) 4.22 (m, 1H) 3.90 (m, 1H) 3.76 (m, 1H), 2.98 (m, 3H), 2.82 (m, 2H), 1.60 – 1.19 (broad m, 21H).

Boc-Val-Gly-Leu-Lys-Phe-NH_2 (17): The reaction was carried out following the general procedure using 44 mg of **6** (0.15 mmol), 65 mg of N_3-Leu-Lys-Phe-NH_2 (0.15 mmol), 70 µL of 2,6-lutidine (60 mmol), 16 mg of $RuCl_3$ (75 µmol) and 1 mL of MeCN. After concentration of the reaction mixture, the residue was dissolved in EtOAc (50 mL) and washed with 5% $NaHCO_3$ (3 × 20 mL) and Brine (1 × 20 mL) and dried (Na_2SO4). The crude product (12 mg, 12%, purity 25% by HPLC was obtained as a white solid. From this crude product three compounds could be isolated by preparative HPLC and were analyzed by ESMS. Product **17**: R_t: 16.13 min; ESMS calcd for $C_{33}H_{55}N_7O_7$: 661.42, found: 661.65 $[M+H]^+$. Side product **19**: R_t: 16.78 min, ESMS calcd for $C_{45}H_{75}N_9O_{11}$: 918.13, found: 918.60 $[M+H]^+$. Side product **20**: R_t: 16.50 min, ESMS calcd for $C_{33}H_{53}N_9O_7$: 687.41, found: 688.60 $[M+H]^+$.

4.5 References and notes

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30. CAUTION: on acidification of the reaction mixture, highly toxic hydrogen sulfide evolves, this procedure should be performed in a well ventilated hood.

5

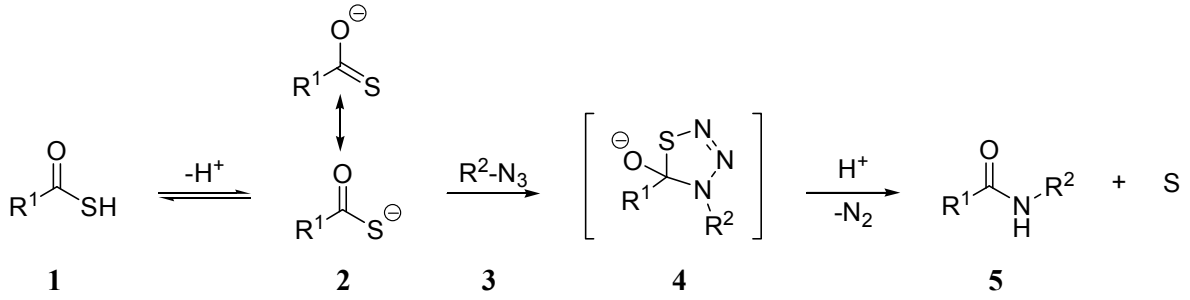
Highly Efficient Coupling of β-Substituted Aminoethane Sulfonyl Azides with Thio Acids, Toward a New Chemical Ligation Reaction

The convenient synthesis of protected β-substituted aminoethane sulfonyl azides starting from amino acids is reported. Their highly efficient coupling with thio acids was found to be chemoselective, fast, compatible with a variety of solvents (including water) and yielded nitrogen and sulphur as only non toxic side products. In case of peptide based thio acids and sulfonyl azides this reaction encompasses a new chemoselective ligation method for the conjugation of peptides.

Parts of this chapter have been published: Merkx, R., Brouwer, A.J., Rijkers, D.T.S., Liskamp, R.M.J., *Org. Lett.* **2005**, *7*(6), p. 1125; Brouwer, A.J., Merkx, R., Dabrowska, K., Rijkers, D.T.S., Liskamp, R.M.J., *Synthesis* **2006**, (3), p. 455.

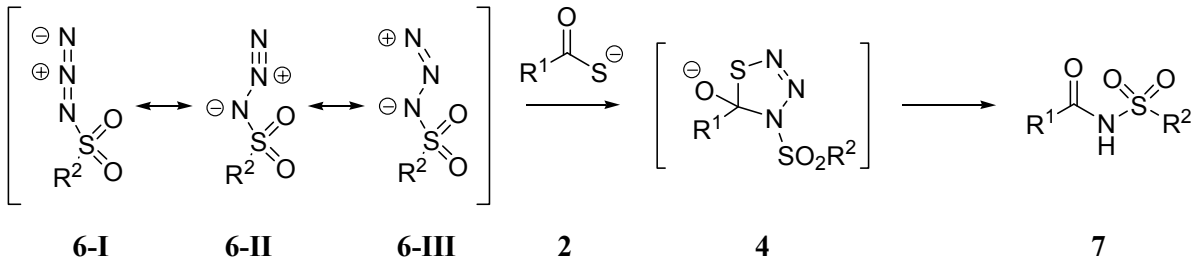
5.1 Introduction

Recently, the reaction of an azide with a thio acid to form an amide has been re-investigated by Williams *et al.*[1] They found that thio acids react chemoselectively with azides via the formation of a thiatriazoline intermediate (**4**, Scheme 5.1) rather than via reduction of the azide prior to acylation by the thio acid.



Scheme 5.1 Proposed reaction of an azide with a thio acid to form an amide.

In the previous chapter, the coupling of peptide thio acids with azido peptides to form a native amide bond was investigated. It was found that the reaction was rather sluggish and although the yield could be increased by the use of Ru(III) as a catalyst to activate the thio acid, simultaneously the selectivity of the reaction was diminished.
In this chapter the coupling of amino acid derived sulfonyl azides with (peptide) thio acids is investigated. The electron withdrawing sulfonyl moiety makes the azide highly polarized (**6-I – III**, Scheme 5.2) which should increase the reactivity of these electron-deficient *sulfonyl* azides toward the coupling with thio acids compared to relatively electron-rich *alkyl* azides.



Scheme 5.2 Proposed reaction of a sulfonyl azide a with a thio acid to form an *N*-acyl sulfonyl azide.

The application of peptide based thio acids and amino acid derived sulfonyl azides in this chemoselective amidation reaction would provide an entry toward large and complex peptide mimetic systems with minimal protecting group strategy.

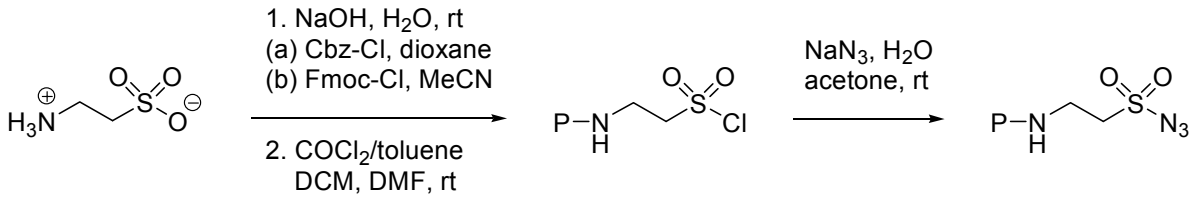
Additionally, the *N*-acyl sulfonamide linkage is very stable, being resistant to hydrolytic and basic cleavage,[2] furthermore it is a competent hydrogen bond donor and acceptor.[3] Moreover, the acyl sulfonamide moiety itself is non-immunogenic and is, in fact, a known pharmacophore.[4-24]

As mentioned in the previous chapter, (peptide) thio acids are readily available from the corresponding hydroxysuccinimide ester by treatment with NaHS following the literature procedure of Goldstein and Gelb.[25] Whereas, for synthetic applications, mostly aromatic sulfonyl azides are described in the literature, which have been mainly used in thermolysis reactions,[26-28] azidations,[29-31] diazo-transfer reactions,[32,33] and reactions with alkenes.[34] Only few papers describe reactions other than diazo-transfer reactions[32] with aliphatic sulfonyl azides. Since the amino acid-derived (aliphatic) sulfonyl azides, needed in this research, were not available, new synthetic procedures were developed.

5.2 Results and discussion

5.2.1 Synthesis of β-substituted aminoethane sulfonyl azides

Synthesis of sulfonyl azides in the literature is generally performed starting from sulfonyl chlorides in a reaction with sodium azide, also known as the Forstner-Fierz method.[35] An aqueous solution of acetone is mostly used as solvent, in which both the sulfonyl chloride and sodium azide dissolve. For the synthesis of amino acid derived sulfonyl azides the Fmoc- and Cbz-protected *β*-aminoethane sulfonyl chlorides earlier prepared in our group, were envisioned to be suitable starting materials.[36] Since the Cbz group is more stable under many reaction conditions, it was decided to start with the preparation of a Cbz-protected *β*-aminoethane sulfonyl azide derived from glycine (Scheme 5.3). For this purpose, taurine (**8**) was Cbz-protected and subsequently reacted with phosgene to give Cbz-protected tauryl sulfonyl chloride (**9a**).

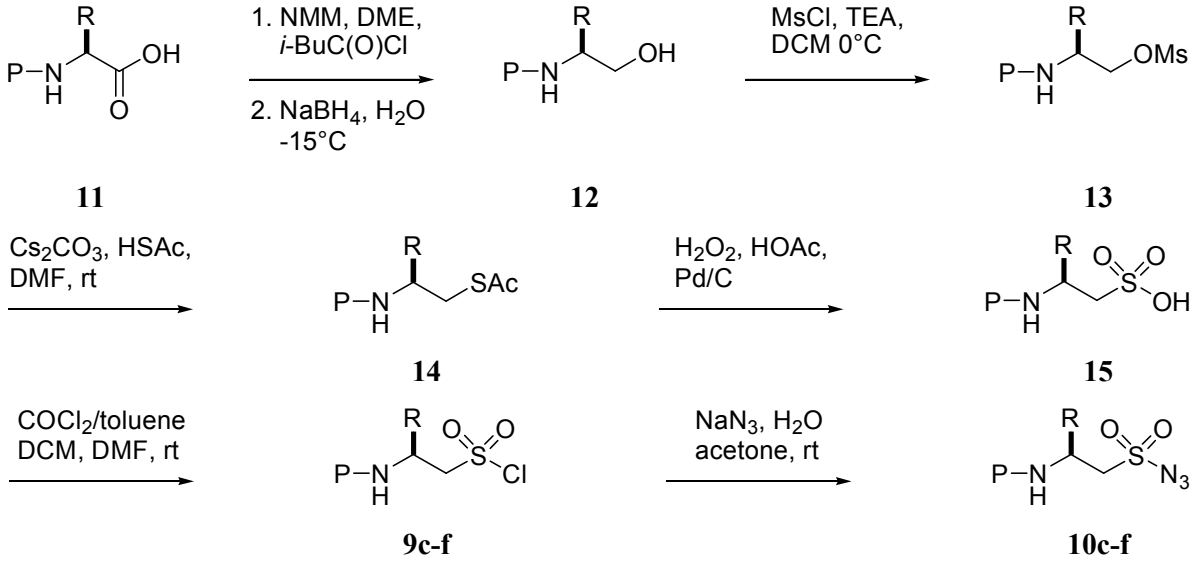


8 **9a,b** **10a,b**

Scheme 5.3 Synthesis of protected taurylsulfonyl azide. (a) P = Cbz, (b) P = Fmoc.

The first attempt for reaction of Cbz-protected taurylsulfonyl chloride with NaN_3 in an acetone – water mixture resulted in a fast and clean conversion to sulfonyl azide **9a** in 90% yield (77% from taurine). In contrast, the sulfonyl azide product **9a** could not be obtained by a diazotranfer reaction starting from the corresponding sulfonamide, most likely caused by deactivation of the amide by the electron-withdrawing sulfonyl moiety.

After these promising results, it was investigated whether the Fmoc group would be stable under these reaction conditions. For this purpose the azide-substitution reaction was repeated with Fmoc-protected taurylsulfonyl chloride (**9b**). Unfortunately the yield of Fmoc-protected sulfonyl azide **10b** was only 49% due to Fmoc cleavage, which was clearly visible on TLC. During the reaction a white precipitate formed, which probably was sulfonyl chloride. To prevent Fmoc cleavage, it is very important that the azide nucleophile reacts directly with the sulfonyl chloride, which is difficult when the sulfonyl chloride is not completely dissolved. Thus, after addition of more acetone and inversion of the addition sequence (to ensure an excess of the sulfonyl chloride relative to the azide), Fmoc-protected sulfonyl azide **10b** was obtained in 90% yield (Table 5.1).



Scheme 5.4 Synthesis of *β*-substituted aminoethane sulfonyl azides. P = Cbz or Fmoc, R = *i*-Pr, Bn, CH_2O^tBu.

Now that both Fmoc- and Cbz-protected glycine-derived sulfonyl azides were successfully prepared, the next step was the synthesis of *β*-aminoethane sulfonyl azides with different side chains, including functionalized side chains. Fmoc-protected *β*-aminoethane sulfonyl chlorides **9c – f** were prepared following a procedure which was previously described by our group, starting from Fmoc- and Cbz-protected amino acids **11** (Scheme 5.4).[36] Fmoc-protected sulfonyl chlorides **9c,d** were smoothly converted to the corresponding sulfonyl

azides in good to excellent yields (79–99%) (Table 5.1). Cbz-phenylalanine-derived sulfonyl azide was also efficiently prepared in high yield (90%), following the procedure used for **10a**. For preparation of a functionalized sulfonyl chloride, **10f**, derived from Fmoc-Ser(^{t}Bu)-OH, was selected. Sulfonyl azide **9f** was prepared in 74% yield, using the described approach.

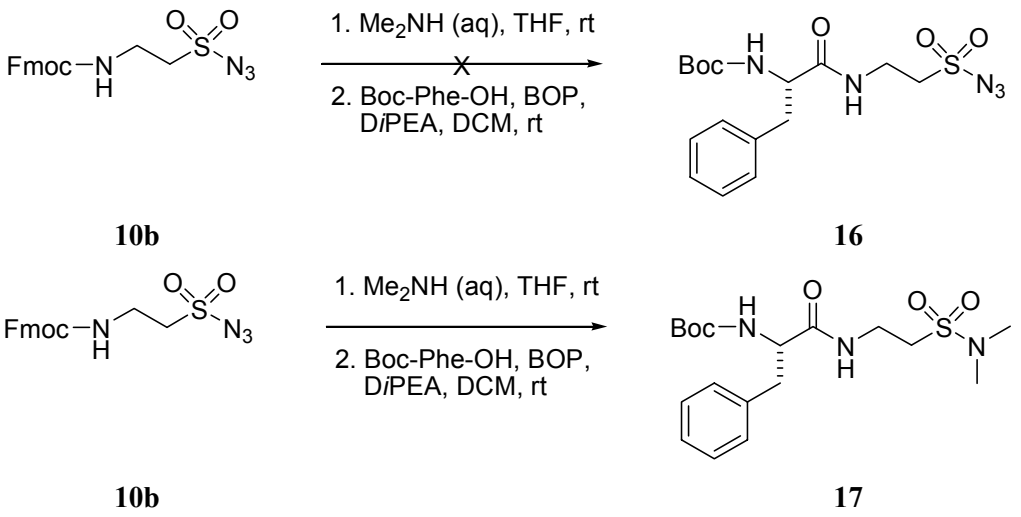
Table 5.1 Yields of Sulfonyl Azides **10a-f**.

Entry	Amino Acid	R	**10**	% Yield [a]
1	Cbz-Gly-OH	H	**10a**	90
2	Fmoc-Gly-OH	H	**10b**	92
3	Fmoc-Val-OH	*i*-Pr	**10c**	79
4	Fmoc-Phe-OH	Bn	**10d**	99
5	Cbz-Phe-OH	Bn	**10e**	90
6	Fmoc-Ser(^{t}Bu)-OH	CH_2O^tBu	**10f**	74

[a]Isolated yields, starting from the corresponding sulfonyl chlorides **9a-f**.

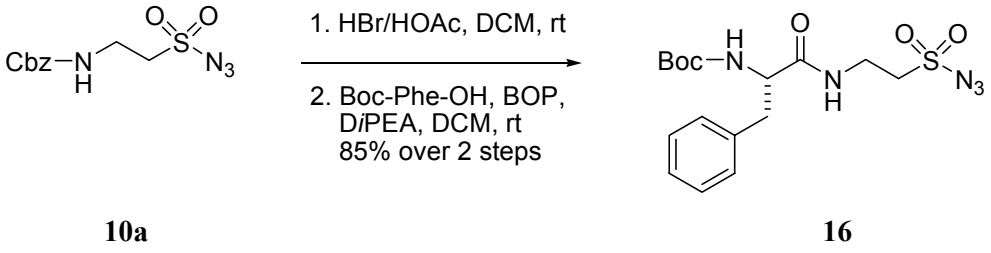
For use as a chemoselective ligation reaction *N*-terminal deprotected sulfonyl azides were desirable, to which amino acids or peptides can be coupled. For this purpose the *N*-terminus of Fmoc-tauryl sulfonyl azide **10b** was deprotected with a large excess of dimethylamine in THF (Scheme 5.5).

Boc-Phe-OH was directly coupled to the resulting free amine using BOP and D*i*PEA in dichloromethane. Instead of Fmoc-deprotection followed by coupling, the sulfonyl azide underwent a substitution reaction by dimethylamine leading to **17**, and no coupling product **16** retaining the sulfonyl azide moiety was found. In attempts to selectively remove the Fmoc group, different amounts of either dimethylamine or piperidine were used. In all reactions, both Fmoc cleavage and azide substitution were observed. At lower amounts of the amine, the Fmoc cleavage was slightly favored over the substitution reaction.



Scheme 5.5 Nucleophilic substitution at the sulfonyl azide.

Since Fmoc cleavage of sulfonyl azides was not possible, it was decided to cleave the Cbz group. For this purpose, sulfonyl azide **10a** was treated with HBr/HOAc, and the HBr-salt of the amine was transformed into the HCl-salt using an ion exchange resin (Scheme 5.6). Subsequently, Boc-Phe-OH was coupled using BOP to give peptido-aminoethane sulfonyl azide **16** in 85% yield, showing that the *N*-terminus of our sulfonyl azides can be used for coupling to amino acids and probably larger peptides. This example also shows that the sulfonyl azide moiety is stable under both strong acidic (HBr/HOAc) and basic non nucleophilic (D*i*PEA) conditions. Furthermore, under these coupling conditions no substitution products were found from nucleophilic attack of the free amine at the sulfonyl azide.



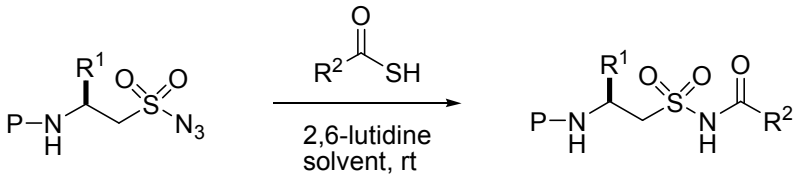
Scheme 5.6 Synthesis of peptidyl peptidosulfonyl azide.

The described method allowed for the efficient preparation of (peptide) aminoethane sulfonyl azides starting from different amino acids, including those containing functionalized side chains. The sulfonyl azides were obtained as stable amino acid mimetic building blocks that were evaluated for the coupling to (peptidyl) thio acids.

5.2.2 The coupling of β-substituted aminoethane sulfonyl azides with thio acids

First, the coupling of either Cbz- or Fmoc-protected *β*-aminoethane sulfonyl azides was investigated, which were derived from the amino acids glycine (entries 1 – 4), phenylalanine (entries 5 – 8), valine (entries 9 and 10) and (protected) serine (entries 11 and 12) (Table 5.2).

Table 5.2 Synthesis of *N*-Acyl sulfonamides .



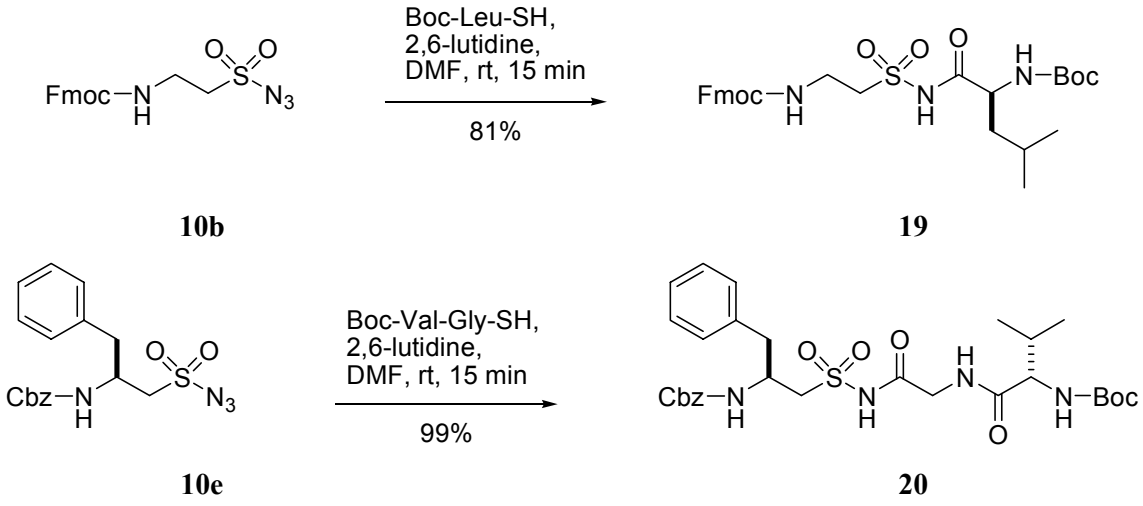
10 **18**

Entry[a]	P	R^1	R^2	**18**	% Yield [b]	Solvent
1	Fmoc	H	Me	**18a**	90	$CHCl_3$
2	Fmoc	H	Ph	**18b**	quant.	$CHCl_3$
3	Cbz	H	Me	**18c**	quant.	$CHCl_3$
4	Cbz	H	Ph	**18d**	quant.	$CHCl_3$
5	Fmoc	Bn	Me	**18e**	91	DMF
6	Fmoc	Bn	Ph	**18f**	87	DMF
7	Cbz	Bn	Me	**18g**	95	DMF
8	Cbz	Bn	Ph	**18h**	96	DMF
9	Fmoc	*i*-Pr	Me	**18i**	95	DMF
10	Fmoc	*i*-Pr	Ph	**18j**	96	DMF
11	Fmoc	CH_2O^tBu	Me	**18k**	93	DMF
12	Fmoc	CH_2O^tBu	Ph	**18l**	94	DMF

[a]Reaction conditions: sulfonyl azide (1 equiv), thio acid (1.3 equiv), 2,6-lutidine (1.3 equiv), solvent, rt, 15 min. [b]Isolated yields.

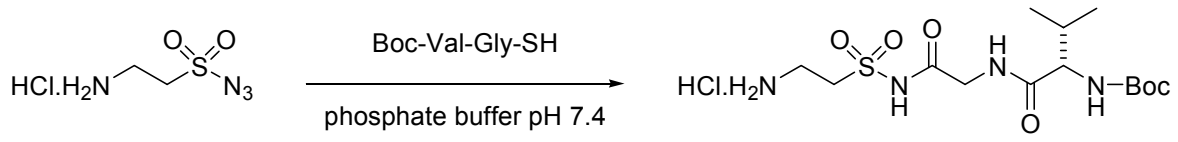
The sulfonyl azides reacted smoothly with thioacetic acid or thiobenzoic acid at room temperature: the reaction was complete in 15 min and the resulting acyl sulfonamides (**18a – l**) could be isolated in good to excellent yields. As a solvent, $CHCl_3$ was used in entries 1 – 4, because of the low solubility of the other sulfonyl azides, DMF as solvent was a good alternative for reactions in entries 5 – 12.

Then, as the next step towards the chemoselective ligation of amino acids and peptides, the thio acid[25] derived from Boc-leucine was used (Scheme 5.7). Its coupling to sulfonyl azide Fmoc-Gly-Ψ[CH_2SO_2]-N_3 (**10b**) proceeded very well and α-(amino) acyl sulfonamide **19** was obtained in 81% yield. Next, the reaction of the dipeptide thio acid Boc-Val-Gly-SH[25] with Cbz-Phe-Ψ[CH_2SO_2]-N_3 to obtain tripeptide mimic **20** was attempted (Scheme 5.7). Under identical conditions, the azide reacted smoothly with the dipeptide thio acid to give the orthogonally protected tripeptide mimic **20** containing a sulfonamide in 99% yield. Both amino groups can be selectively deprotected thereby allowing also a selective elongation of the peptide chain in either direction.



Scheme 5.7 Synthesis of peptidyl sulfonamides **19** and **20**.

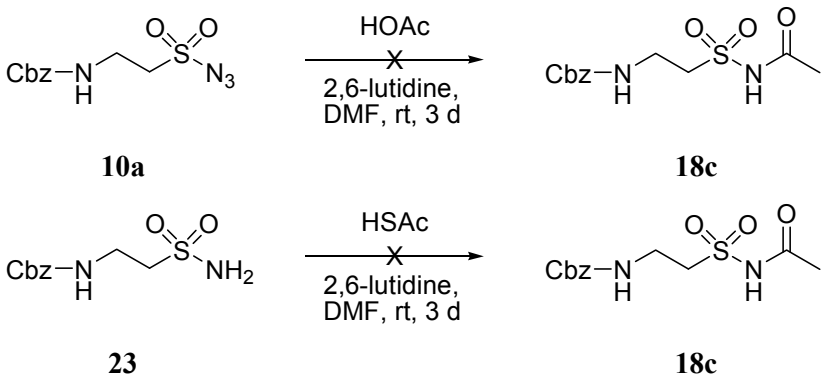
Furthermore, to investigate the potential of this reaction as a novel chemoselective (bio)conjugation or chemical ligation reaction, the reaction of entry 2 (Table 5.2) was also carried out in an aqueous system, e.g. THF/H_2O 2:1 v/v. Acyl sulfonamide **18b** could successfully be obtained in a quantitative yield. Moreover, the reaction of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (**21**) with Boc-Val-Gly-SH was tried in phosphate buffer (pH 7.4) without any additional base (Scheme 5.8). The reaction progress was followed by ESMS and HPLC and was found to be complete within 15 min after addition of the thio acid. These results demonstrate the efficiency of the thio acid/sulfonyl azide amidation reaction under native conditions.



21 **22**

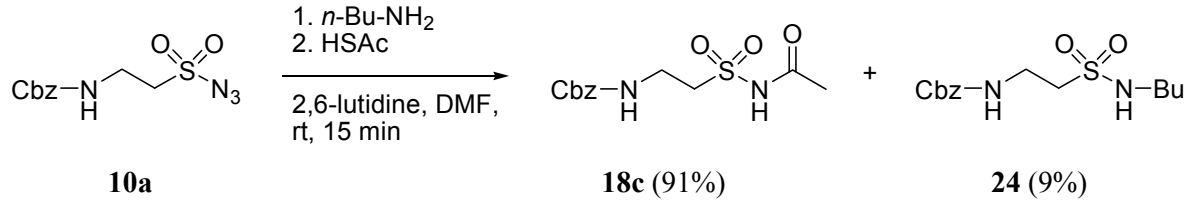
Scheme 5.8 Thio acid/sulfonyl azide amidation under native conditions.

Finally, the chemoselectivity of the reaction was investigated. It was found that neither Cbz-Gly-Ψ[CH_2SO_2]-NH_2 (**23**) nor Cbz-Gly-Ψ[CH_2SO_2]-N_3 (**10a**) reacted (after three days of stirring) with thioacetic acid and acetic acid, respectively to give acyl sulfonamide **18c**. These experiments underline the chemoselective character of the sulfonyl azide/thio acid reaction pair.



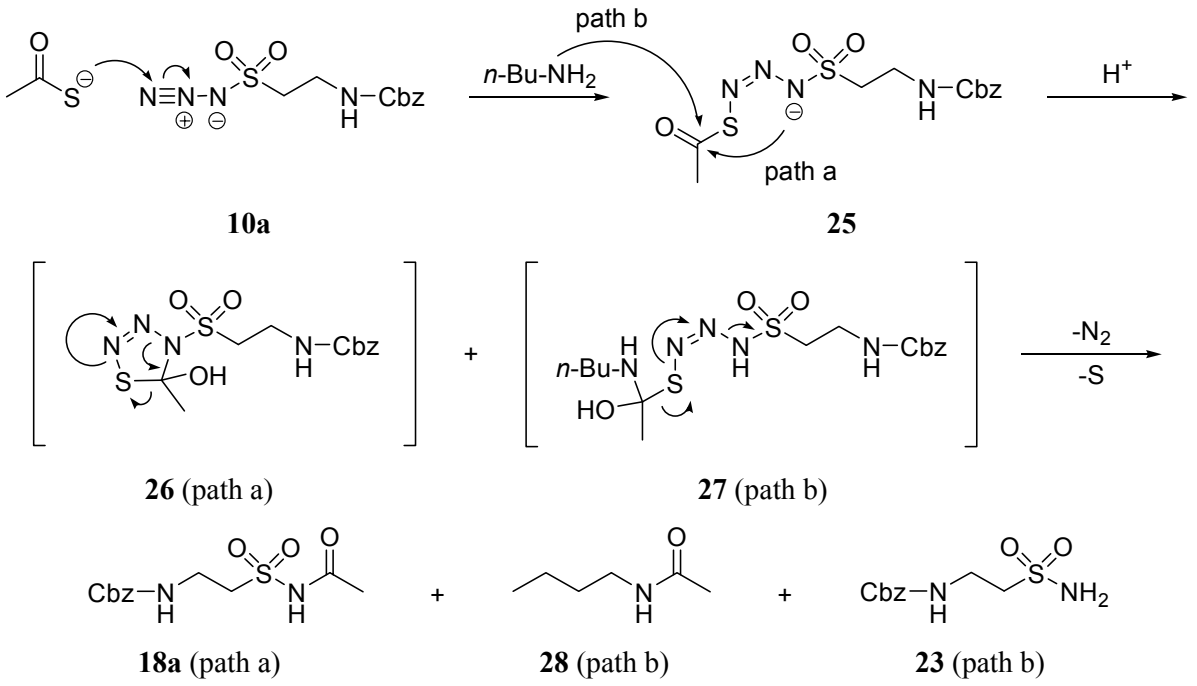
Scheme 5.9 Investigation on the chemoselectivity of the thio acid/sulfonyl azide amidation reaction.

To test the sensitivity of the sulfonyl azide moiety toward nucleophiles,[37] the reaction of entry 2 (Table 5.2) was repeated in the presence of butylamine as nucleophile. Therefore, equimolar quantities of butylamine and thioacetic acid were subsequently added to a mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (**10a**) and 2,6-lutidine in DMF. After a reaction time of 15 min, sulfonamide side product **24**, was isolated in 9% yield (Scheme 5.10). If butylamine is regarded as a lysine side chain mimic, it should be noted that this side reaction is unlikely to occur under native conditions (pH = 7.4) because the εNH_2 moiety will be protonated and thus unreactive under these conditions,[38] as already shown in Scheme 5.8.



Scheme 5.10 Investigation on the chemoselectivity of the thio acid/sulfonyl azide amidation reaction.

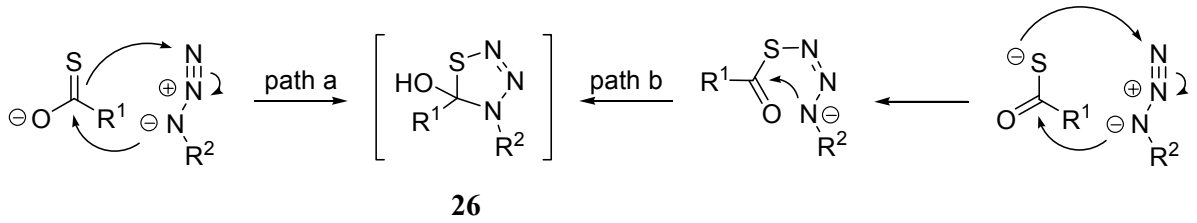
HPLC analysis also showed that, although *N*-acyl sulfonamide **18c** was formed as major product, the crude product contained two other compounds which could be identified as **28** and **23** on basis of their retention times compared to the corresponding chemically synthesized reference compounds.



Scheme 5.11 Proposed mechanism for the thio acid/sulfonyl azide amidation reaction.

These results provided further insight into the reaction mechanism since side products **28** and **23** are indicative for the formation of the thiatriazoline intermediate **26** via a two step cyclization reaction rather than via a concerted cycloaddition reaction (Scheme 5.11). In this mechanism, **10a** reacts first with thioacetic acid to form lineair intermediate **25** which subsequently intramolecularly rearranges to intermediate **26**. In the presence of butylamine, this amine nucleophile will compete *inter*molecularly with the *intra*molecular cyclization step to form sulfonamide **23** via intermediate **27**.

These findings were recently confirmed by Williams *et al*.[39] and can be used to explain the increased reactivity of electron-deficient azides compared to relatively electron-rich azides. Since electron-deficient (sulfonyl) azides are highly polarized, their reaction with thio acids proceeds via a two step cyclization reaction to form the thiatriazoline intermediate (**26**) (path a, Scheme 5.12). A mechanism that is in accordance with other ligation methods mentioned in this thesis (e.g. NCL and the Staudinger ligation)[40-43] that also make use of this prior capture concept.[44-46] Also the reaction rate (k_{obs} = 0.06 $M^{-1}s^{-1}$)[39] for the thio acid/sulfonyl azide amidation reaction was found to be comparable to that of the Staudinger ligation, however, for the latter only in case of electron rich azides.



Scheme 5.12 Mechanism for the formation of the thiatriazoline intermediate in case of electron-rich (path a) and electron-deficient (path b) azides.

In contrast, the reaction of relatively electron-rich (alkyl) azides with thio acids proceeds via a single step [3+2] cycloaddition mechanism to form the thiatriazoline intermediate (**26**). In this case, the reaction is rather sluggish (path a, Scheme 5.12) and requires elevated temperatures and/or activation by the addition of a catalyst to obtain satisfactory yields as was described in the previous chapter.

Conclusions

The application of the thio acid/sulfonyl azide amidation reaction on the chemoselective coupling of peptides was investigated. Therefore, new methods for the synthesis of amino acid-based sulfonyl azides were developed. Starting from amino acids, *β*-substituted aminoethane sulfonyl azides could be obtained as stable amino acid mimetic building blocks. However, in contrast to electron rich alkyl azides, the electron deficient sulfonyl azides are highly activated toward reaction with thio acids. The thio acid/sulfonyl azide amidation reaction is efficient and proceeds via a two step cyclization reaction rather than via a concerted 1,3-dipolar cycloaddition reaction in case of the sluggish reaction of relative electron-rich azides.

The thio acid/sulfonyl azide amidation reaction features some auspicious characteristics for application as novel chemical ligation or bioconjugation methodology. For instance, the

amidation reaction is chemoselective, highly efficient (also in aqueous solvents), it does not require the addition of any catalyst and non toxic nitrogen and sulfur are formed as the only side products. Additionally, the *N*-acyl sulfonamide product is highly stable and non immunogenic. Moreover, this chemoselective amidation strategy is easily implemented in the coupling of peptides.

5.3 Experimental section

General information

Analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 5 μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O) to 100% buffer B (0.1% TFA in MeCN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min, connected to a UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000). Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. IR spectra were recorded on a Biorad FTS6000 spectrometer. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel 60F^{254} plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[47] Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat (according to dr. Tottoli) and are uncorrected. Specific optical rotations were measured using a Jasco P1010 polarimeter. Elemental analyses were done by Kolbe Mikroanalytisches Labor (Mülheim an der Ruhr, Germany).

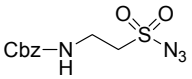
Chemicals and reagents

The coupling reagent 1H-benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) was obtained from Richelieu Biotechnologies Inc. N^{α}-9-Fluorenylmethyloxycarbonyl (Fmoc) amino acids were obtained from MultiSynTech. Peptide grade dichloromethane (DCM), *t*-butyl methylether (MTBE), *N*-methylpyrrolidone (NMP), *N*,*N*-dimethylformamide (DMF), acetronitrile, hexane, trifluoroacetic acid (TFA) and HPLC grade TFA were purchased from Biosolve. Succinic anhydride, piperidine, *N*,*N*-diisopropylethylamine (D*i*PEA), triethylamine (TEA), thioacetic acid (HSAc), thiobenzoic acid and triisopropylsilane (T*i*S) were obtained from Acros Organics, acetic acid (HOAc)

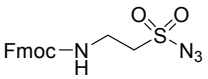
and isopropanol (*i*-PrOH) were purchased from Merck KGaA. Dowex® (2×8 Cl-form) and HBr (30% in AcOH) were obtained from Sigma Aldrich. Phosphate buffer was prepared by mixing solutions of Na_2HPO_4 (1.57 g in 250 mL H_2O) and NaH_2PO_4 (0.65 g in 250 mL H_2O) in such way (approx 1:1 v/v) until pH = 7.1.

General Procedure for the synthesis of sulfonyl azides 10a – f:

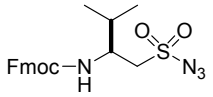
A solution of NaN_3 (195 mg, 3.00 mmol) in H_2O (10 mL) was added dropwise to a solution of the sulfonyl chloride **9a – f** (3 mmol) in acetone (15 mL) and DMF (15 mL). The reaction was stirred at rt for 1 h. After evaporation of acetone, H_2O (200 mL) was added and the product precipitated. The mixture was filtered and the residue was washed with 5% $NaHCO_3$ (10 mL) and H_2O (50 mL) and dried in vacuo. Sulfonyl azides **10a – f** were all obtained as white solids.



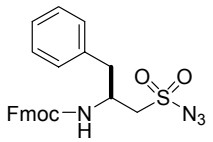
Cbz-Tau-N_3 (10a): The reaction was carried out following the general procedure using 4.69 g of sulfonyl chloride **9a** (16.9 mmol), NaN_3 (1.10 g, 16.9 mmol) and H_2O (90 mL) and acetone (90 mL) as solvents. The acetone solution of the sulfonyl chloride was added dropwise to the aqueous solution of NaN_3. Instead of precipitation, the reaction was worked up by evaporation of acetone, followed by re-dissolving in EtOAc (250 mL) and washing with 5% $NaHCO_3$ (100 mL), H_2O (100 mL), and brine (100 mL). Drying over Na_2SO_4 and evaporation of EtOAc afforded the sulfonyl azide. Yield: 4.3 g (90%); R_f: 0.63 (MeOH/DCM) 1:99 v/v); R_t: 15.50 min; mp: 78 °C; IR (KBr): 2150 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.35 (s, 5H), 5.36 (br s, 1H), 5.12 (s, 2H), 3.72 (m, 2H), 3.55 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.1, 135.9, 128.6, 128.4, 128.1, 67.2, 55.2, 35.7; ESMS calcd for $C_{10}H_{12}N_4O_4S$: 284.06, found: 285 $[M+H]^+$; Anal. calcd (%) for $C_{10}H_{12}N_4O_4S$: C, 42.25; H, 4.25; N, 19.71, found: C, 42.32; H, 4.21; N, 19.66.



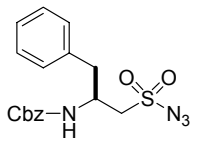
Fmoc-Gly-Ψ[CH_2SO_2]N_3 (10b): The reaction was carried out following the general procedure using 1.08 g of sulfonyl chloride **9b** (2.95 mmol) and NaN_3 (192 mg, 2.95 mmol) in the absence of DMF. Yield: 1.02 g (92%); R_f: 0.54 (MeOH/DCM 1:99 v/v); R_t: 17.64 min; mp: 118 °C. IR (KBr): 2141 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.29 – 7.78 (m, 8H), 5.33 (br s, 1H), 4.44 (d, 2H), 4.21 (t, 1H), 3.71 (m, 2H), 3.53 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.1, 143, 141.3, 127.8, 127.1, 124.9, 124.4, 120.0, 67.1, 55.2, 47.1, 35.7; ESMS calcd for $C_{17}H_{16}N_4O_4S$: 372.09, found: 373 $[M+H]^+$; Anal. calcd (%) for $C_{17}H_{16}N_4O_4S$: C, 54.83; H, 4.33; N, 15.04, found: C, 54.71; H, 4.25; N, 14.92.



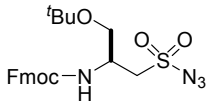
Fmoc-Val-Ψ[CH_2SO_2]-N_3 (10c): The reaction was carried out following the general procedure using 449 mg of sulfonyl chloride **9c** (1.1 mmol) and NaN_3 (72 mg, 1.1 mmol). Yield: 360 mg (79%); R_f: 0.54 (MeOH/DCM 1:99 v/v); R_t: 18.58 min; mp: 158 °C; IR (KBr): 2139 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 7.29 – 7.77 (m, 8H), 5.04 (d, 1H), 4.45 (m, 2H), 4.22 (t, 1H), 4.01 (m, 1H), 3.60 (dd, *J* = 14.7 Hz, *J* = 8.1 Hz, 1H), 3.49 (dd, *J* = 14.7 Hz, *J* = 3.7 Hz, 1H), 2.04 (m, 1H), 0.96 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.7, 143.7, 143.6, 141.3, 127.7, 127.1, 125.0, 119.0, 66.9, 57.0, 53.0, 47.2, 31.5, 19.2, 18.1; ESMS calcd for $C_{20}H_{22}N_4O_4S$: 414.14, found: 415 $[M + H]^+$; Anal. calcd (%) for $C_{20}H_{22}N_4O_4S$: C, 57.96; H, 5.35; N, 13.52, found: C, 57.77; H, 5.28; N, 13.39.



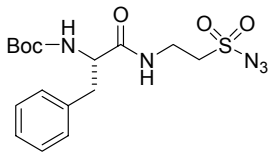
Fmoc-Phe-Ψ[CH_2SO_2]-N_3 (10d): The reaction was carried out following the general procedure using sulfonyl chloride **9d** (1.37, 3.0 mmol), NaN_3 (195 mmol), H_2O (30 mL), acetone (20 mL), and DMF (40 mL) as solvents. Yield: 1.37 g (99%); R_f: 0.56 (MeOH/DCM 1:99 v/v); R_t: 18.98 min; mp: 168 °C; IR (KBr): 2131 (s) cm^{-1}; ^1H NMR (300 MHz, THF-d_8,): δ 7.15 – 7.81 (m, 13H), 7.01 (d, 1H), 4.40 (m, 2H), 4.19 (m, 2H), 3.88 (dd, *J* = 14.7 Hz, *J* = 8.6 Hz, 1H), 3.71 (dd, *J* = 14.7 Hz, *J* = 3.9 Hz, 1H), 3.03 (dd, *J* = 13.6 Hz, *J* = 8.2 Hz, 1H), 2.95 (dd, *J* = 13.6 Hz, *J* = 5.8 Hz, 1H); ^{13}C NMR (75 MHz, THF-d_8): δ 156.4, 145.3, 142.3, 138.5, 130.3, 129.3, 128.3, 127.7, 127.4, 125.9, 120.6, 66.7, 58.4, 50.3, 48.3, 40.0; ESMS calcd for $C_{24}H_{22}N_4O_4S$: 462.14, found: 463 $[M+H]^+$; Anal. calcd (%) for $C_{24}H_{22}N_4O_4S$: C, 62.32; H, 4.79; N, 12.11, found: C, 62.40; H, 4.75; N, 12.03.



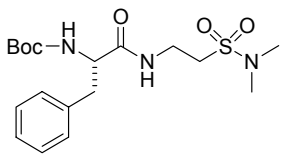
Cbz-Phe-Ψ[CH_2SO_2]-N_3 (10e): The reaction was carried out following the general procedure using 1.40 g of sulfonyl chloride **9e** (3.8 mmol), NaN_3 (247 mg, 3.8 mmol), H_2O (19 mL) and acetone (50 mL) as solvents. The reaction and workup were performed using the same procedure as for **10a**. Yield: 1.28 g (90%); R_f: 0.48 (MeOH/DCM 1:99 v/v); R_t: 17.64 min; mp: 141 °C; IR (KBr): 2137.57 (s) cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$): δ 5.23 (d, 1H), 7.15 – 7.38 (m, 10H), 5.09 (s, 2H), 4.32 (m, 1H), 3.71 (dd, *J* = 14.8 Hz, *J* = 7.7 Hz, 1H), 3.47 (dd, *J* = 14.8 Hz, *J* = 4.4 Hz, 1H), 3.02 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.4, 136.0, 135.9, 129.2, 128.9, 128.5, 128.2, 128.0, 127.3, 67.0, 57.2, 49.1, 39.3; ESMS calcd for $C_{17}H_{18}N_4O_4S$: 374.10, found: 375 $[M+H]^+$; Anal. calcd (%) for $C_{17}H_{18}N_4O_4S$: C, 54.53; H, 4.85; N, 14.96, found: C, 54.60; H, 4.78; N, 14.87.



Fmoc-Ser(O^{t}Bu)-Ψ[CH_2SO_2]-N_3 (10f): The reaction was carried out following the general procedure using 613 mg of sulfonyl chloride **9f** (1.62 mmol) and NaN_3 (105 mg, 1.62 mmol). Yield: 0.55 g (74%); R_f 0.73 (MeOH/DCM 1:99 v/v); R_t: 19.19 min; mp: 103 °C; IR (KBr): 2137 (s) cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.30 – 7.78 (m, 8H), 5.33 (br d, 1H), 4.35 – 4.51 (m, 3H), 4.23 (t, 1H), 3.64 (m, total 4H), 3.51 (m), 1.19 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 155.5, 143.6, 141.3, 127.8, 127.1, 125.0, 124.9, 120.0, 73.8, 67.0, 61.7, 55.9, 47.6, 47.1, 27.4; ESMS calcd for $C_{22}H_{26}N_4O_5S$: 458.16, found: 459 $[M+H]^+$; Anal. calcd (%) for $C_{22}H_{26}N_4O_5S$: C, 57.63; H, 5.72; N, 12.22, found: C, 57.49; H, 5.66; N, 12.06.



Boc-Phe-Gly-Ψ[CH_2SO_2]-N_3 (16): To a solution of sulfonyl azide **10a** (284 mg, 1.00 mmol) in DCM (10 mL) was added a solution of HBr in HOAc (33%, 6 mL). While stirring for 30 min at rt, a white precipitate formed. After removal of the solvents *in vacuo*, the residue was redissolved in H_2O (10 mL) and Dowex 2×8 (0.60 g, Cl-form) was added. Stirring for 5 min at rt, followed by filtration and concentration *in vacuo*, afforded the HCl-salt (188 mg) in quantitative yield. To the HCl-salt was subsequently added DCM (40 mL), Boc-Phe-OH (265 mg, 1.00 mmol), BOP (464 mg, 1.05 mmol), and D*i*PEA (385 mL, 2.21 mmol). The mixture was stirred overnight at rt. The pH was kept at approx 8 by addition of D*i*PEA during the reaction. After concentration, the residue was suspended in EtOAc (100 mL) and was washed with 1N $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), 5% $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL). Drying over Na_2SO_4, followed by column chromatography (EtOAc/hexane/HOAc 45.5:45.5:1 v/v/v), afforded **16** as a white solid. Yield: 336 mg (85%); R_f: 0.39 (EtOAc/hexane, 1:1 v/v); R_t: 16.50 min; mp: 72 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.18 – 7.35 (m, 5H), 6.63 (s, 1H), 5.01 (d, 1H), 4.35 (m, 1H), 3.72 (m, 2H), 3.30 – 3.51 (m, 4H), 3.06 (d, 2H), 1.40 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 172.0, 155.4, 136.3, 129.4, 129.2, 128.7, 128.3, 127.1, 80.5, 55.8, 54.6, 38.2, 33.8, 28.2; ESMS calcd for $C_{16}H_{23}N_5O_5S$: 397.14, found: 420.20 $[M + Na]^+$; Anal. calcd (%) for $C_{16}H_{23}N_5O_5S$: C, 48.35; H, 5.83; N, 17.62, found: C, 48.22; H, 5.69; N, 17.77.

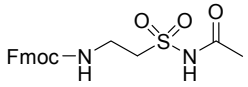


Boc-Phe-Gly-Ψ[CH_2SO_2]-N(Me)$_2$ (17): To a solution of sulfonyl azide **10b** (372 mg, 1.00 mmol) in THF (10 mL) was added 40% aqueous Me_2NH (5.1 mL). After stirring for 1 h at rt, the reaction mixture was concentrated *in vacuo* and coevaporated with toluene (3 × 20 mL). To the residue was added Boc-Phe-OH (265 mg, 1.00 mmol), BOP (443 mg, 1.00 mmol), DCM (6 mL), and D*i*PEA (0.36 mL, 2.2 mmol). After stirring for 1.5 h at rt,

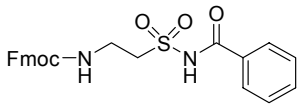
the mixture was concentrated and redissolved in EtOAc (20 mL). After washing with 1N $KHSO_4$ (1 × 10 mL), 1N NaOH (2 × 10 mL), H_2O (1 × 10 mL), and brine (1 × 10 mL), the organic layer was dried over Na_2SO_4 and concentrated. Column chromatography (EtOAc/hexanes 1:2 v/v) afforded **17** as a white solid. Yield: 141 mg (35%); R_f: 0.33 (EtOAc/hexanes 1:2 v/v); mp: 123 °C; ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.18 – 7.32 (m, 5H), 6.79 (s, 1H), 5.12 (s, 1H), 4.38 (m, 1H), 3.65 (m, 2H), 2.84 – 3.08 (m, 10H), 1.39 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.6, 155.2, 136.5, 129.2, 128.5, 126.9, 80.0, 55.7, 46.9, 38.3, 37.2, 33.6, 28.1; ESMS calcd for $C_{18}H_{29}N_3O_5S$: 399.18, found: 422.35 $[M + Na]^+$.

General Procedure for the synthesis of *N*-Acyl sulfonamides 18a – l:

To a mixture of sulfonyl azide **10** (1 equiv) and 2,6-lutidine (1.3 equiv) in 2.5 mL $CHCl_3$ or DMF, the thio acid (1.3 equiv) is added dropwise. After completion of the addition, the reaction mixture is allowed to stir for 15 min at room temperature. Then, the reaction mixture is evaporated to dryness and the residue is either crystallized from EtOAc/hexane or purified by column chromatography.

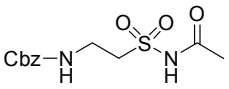


***N*-Acyl sulfonamide 18a:** The reaction was carried out following the general procedure, using 201 mg (0.54 mmol) of sulfonyl azide **10a**, 82 µL (0.70 mmol) of 2,6-lutidine and 50 µL (0.70 mmol) of thioacetic acid in $CHCl_3$. The product (188 mg, 90%) was isolated by column chromatography (EtOAc/hexane 4/1 v/v) as a white solid. R_f: 0.14 (EtOAc/hexane, 4:1 v/v). R_t: 16.01 min; mp: 161 °C. ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.70 (s, 1H), 7.91 (d, J = 7.4 Hz, 2H), 7.70 (d, J = 7.1 Hz, 2H), 7.49 – 7.31 (m, 5H), 4.35 (d, J = 6.6 Hz, 2H), 4.25 (t, J = 6.6 Hz, 1H), 3.50 (m, 2H), 3.39 (m, 2H), 1.98 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD): δ 170.6, 156.9, 143.9, 141.4, 127.9, 127.3, 125.2, 120.2, 67.1, 52.6, 47.2, 35.7, 29.9, 23.4; ESMS calcd for $C_{19}H_{20}N_2O_5S$: 388.11, found: 389.35 $[M + H]^+$, 411.15 $[M + Na]^+$, 387.30 $[M – H]^–$. Anal. calcd for $C_{19}H_{20}N_2O_5S$: C, 58.75; H, 5.19; N, 7.21, found: C, 58.69; H, 5.08; N, 7.11.

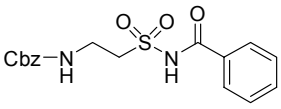


***N*-Acyl sulfonamide 18b:** The reaction was carried out following the general procedure, using 201 mg (0.54 mmol) of sulfonyl azide **10a**, 82 µL (0.70 mmol) of 2,6-lutidine and 82 µL (0.70 mmol) of thiobenzoic acid in $CHCl_3$. The product (243 mg, 100%) was isolated by column chromatography (EtOAc/hexane 4:1 v/v) as a white solid. R_f: 0.52 (EtOAc/hexane 4:1 v/v); R_t: 17.56; mp 176 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.08 (broad s, 1H), 7.95 – 7.92 (m, 4H), 7.89 (d, J = 7.4 Hz, 2H), 7.48 – 7.29 (m, 8H), 4.27 – 4.17 (m, 3H), 3.37 (broad m,

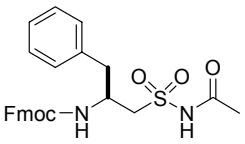
4H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 169.7, 156.2, 144.1, 141.0, 137.0, 131.4, 128.7, 128.1, 127.9, 127.4, 125.4, 120.4, 65.7, 51.6, 46.9, 36.1; ESMS calcd. for $C_{24}H_{22}N_2O_5S$: 450.12, found: 449.35 $[M - H]^-$. Anal calcd for $C_{24}H_{22}N_2O_5S$: C, 63.98; H, 4.92; N, 6.22, found: C, 64.11; H, 4.85; N, 6.12.



***N*-Acyl sulfonamide 18c:** The reaction was carried out following the general procedure, using 154 mg (0.54 mmol) of sulfonyl azide **10b**, 82 μL (0.70 mmol) of 2,6-lutidine and 50 μL (0.70 mmol) of thioacetic acid in $CHCl_3$. The product (162 mg, 100%) was isolated by column chromatography (EtOAc/hexane 4:1 v/v) as a white solid. R_f: 0.30 (EtOAc/hexane 4:1 v/v). R_t: 12.96; mp 149 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.71 (br s, 1H), 7.46 – 7.30 (m, 6H), 5.03 (s, 2H), 3.43 (m, 2H), 3.16 (m, 2H), 1.99 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.1, 156.2, 137.2, 128.6, 128.1, 65.8, 51.5, 35.3, 23.5; ESMS calcd for $C_{12}H_{16}N_2O_5S$: 300.08, found: 323.15 $[M + Na]^+$, 299.15 $[M - H]^-$. Anal. calcd for $C_{12}H_{16}N_2O_5S$: C, 47.99; H, 5.37; N, 9.33, found: C, 48.12; H, 5.38; N, 9.22.

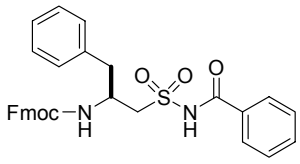


***N*-Acyl sulfonamide 18d:** The reaction was carried out following the general procedure, using 154 mg (0.54 mmol) of sulfonyl azide **10b**, 82 μL (0.70 mmol) of 2,6-lutidine and 82 μL (0.70 mmol) of thiobenzoic acid in $CHCl_3$. The product (196 mg, 100%) was isolated by column chromatography (EtOAc/hexane 4:1 v/v) as a white solid. R_f: 0.41 (EtOAc/hexane 4:1 v/v). R_t: 17.55; mp 108 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.12 (broad s, 1H), 7.94 (d, J = 7.1 Hz, 2H), 7.66 (m, 1H), 7.53 (m, 2H), 7.42 – 7.27 (m, 6H), 4.96 (s, 2H), 3.65 (m, 2H), 3.44 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$/CD_3OD): δ 167.5, 156.9, 136.2, 134.7, 133.7, 131.9, 129.3, 129.0, 128.7, 128.6, 128.4, 128.2, 67.2, 53.0, 35.9; ESMS calcd for $C_{17}H_{18}N_2O_5S$: 362.09, found: 363.15 $[M + H]^+$, 385.20 $[M + Na]^+$, 361.20 $[M - H]^-$. Anal. calcd for $C_{17}H_{18}N_2O_5S$: C, 56.34; H, 5.01; N, 7.73, found: C, 56.25; H, 4.93; N, 7.61.

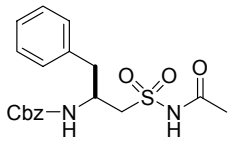


***N*-Acyl sulfonamide 18e:** The reaction was carried out following the general procedure, using 250 mg (0.54 mmol) of sulfonyl azide **10c**, 82 μL (0.70 mmol) of 2,6-lutidine and 50 μL (0.70 mmol) of thioacetic acid in DMF. The product (235 mg, 91%) was isolated by column chromatography (EtOAc/hexane 4:1 v/v) as a white solid. R_f: 0.52 (EtOAc/hexane 4:1 v/v); R_t; 17.53; mp 181 °C; $[\alpha]_D^{23}$ –31.2 (c = 0.1 DMF); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.71 (s, 1H), 7.90 (d, J = 7.4 Hz, 2H), 7.64 (m, 2H), 7.58 (d, J = 8.5 Hz, 1H), 7.44-7.17 (m, 9H), 4.23 (m, 2H), 4.16 (m, 2H), 3.59 (m, 1H), 3.47 (m, 1H), 2.83 (m, 2H) 1.90 (s,

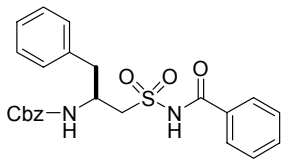
3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.3, 156.2, 143.5, 141.1, 136.0, 129.3, 128.6, 127.7, 127.0, 124.9, 124.8, 119.9, 54.8, 48.6, 46.9, 40.7, 29.6, 23.2; ESMS calcd for $C_{26}H_{26}N_2O_5S$: 478.16, found: 479.35 $[M + H]^+$, 501.30 $[M + Na]^+$, 477.35 $[M - H]^-$. Anal. calcd for $C_{26}H_{26}N_2O_5S$, C, 65.25; H, 5.48; N, 5.85, found: C, 65.06; H, 5.42; N, 5.69.



***N*-Acyl sulfonamide 18f:** The reaction was carried out following the general procedure, using 250 mg (0.54 mmol) of sulfonyl azide **10c**, 82 μL (0.70 mmol) of 2,6-lutidine and 82 μL (0.70 mmol) of thiobenzoic acid in DMF. The product (254 mg, 87%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.61 (EtOAc/hexane 4:1 v/v). R_t: 18.75. Mp 234 °C (decomp.). $[\alpha]_D^{23}$ –21.8 (c = 0.1 DMF); ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.18 (broad s, 1H), 7.91 (m, 4H), 7.58 – 7.19 (broad m, 15H), 4.19 – 4.03 (m, 4H), 3.82 (m, 1H), 3.63 (m, 1H), 2.85 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.8, 155.6, 144.3, 141.1, 138.1, 133.6, 132.1, 129.7, 129.0, 128.7, 128.1, 127.5, 126.8, 125.6, 120.6, 65.7, 56.2, 48.9, 47.0; ESMS calcd for $C_{31}H_{28}N_2O_5S$: 540.17, found: 541.55 $[M + H]^+$, 563.30 $[M + Na]^+$, 539.45 $[M - H]^-$. Anal. calcd for $C_{31}H_{28}N_2O_5S$: C, 68.87; H, 5.22; N, 5.18, found: C, 68.97; H, 5.16; N, 5.06.

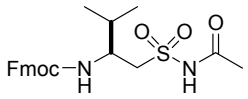


***N*-Acyl sulfonamide 18g:** The reaction was carried out following the general procedure, using 202 mg (0.54 mmol) of sulfonyl azide **10d**, 82 μL (0.70 mmol) of 2,6-lutidine and 50 μL (0.70 mmol) of thioacetic acid in DMF. The product (200 mg, 95%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.52 (EtOAc/hexane 4:1 v/v). R_t: 15.88; Mp: 178 °C. $[\alpha]_D^{23}$ –37.1 (c = 0.1 DMF). ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.73 (s, 1H), 7.54 (d, J = 8.8 Hz, 1H), 7.34-7.18 (m, 10H), 4.96 (m, 2H), 4.15 (m, 1H), 3.61 (m, 1H), 3.48 (m, 1H), 2.85 (m, 2H), 1.92 (s, 3H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.4, 155.9, 138.3, 137.8, 129.9, 129.0, 128.4, 128.1, 127.6, 127.1. ESMS calcd for $C_{19}H_{22}N_2O_5S$: 390.12, found: 391.35 $[M + H]^+$, 413.25 $[M + Na]^+$, 389.30 $[M - H]^-$. Anal. calcd for $C_{19}H_{22}N_2O_5S$: C, 58.45; H, 5.68; N, 7.17, found: C, 58.37; H, 5.67; N, 7.12.

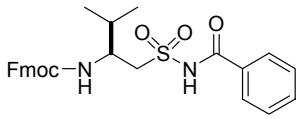


***N*-Acyl sulfonamide 18h:** The reaction was carried out following the general procedure, using 202 mg (0.54 mmol) of sulfonyl azide **10d**, 82 μL (0.70 mmol) of 2,6-lutidine and 82 μL (0.70 mmol) of thiobenzoic acid in DMF. The product (235 mg, 96%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.61 (EtOAc/hexane, 4:1 v/v). R_t: 17.43. Mp: 182 °C (decomp.). $[\alpha]_D^{23}$ –28.0 (c = 0.1 DMF); ^{1}H

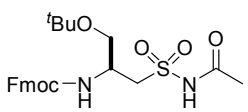
NMR (300 MHz, DMSO-d_6): δ 12.19 (br s, 1H), 7.92 (d, J = 7.1 Hz, 2H), 7.64 (m, 1H), 7.50 (m, 3H), 7.32-7.14 (m, 10H), 4.88 (dd, J = 12.9 Hz, J = 17.6 Hz, 2H), 4.21 (m, 1H), 3.85 (m, 1H), 3.62 (m, 1H), 2.92 (m, 1H), 2.82 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.6, 155.5, 137.9, 137.2, 133.4, 132.0, 129.4, 128.7, 128.5, 127.9, 127.6, 127.1, 126.6; ESMS calcd for $C_{24}H_{24}N_2O_5S$: 452.14, found: 453.30 $[M + H]^+$, 475.30 $[M + Na]^+$, 451.35 $[M - H]^-$. Anal. calcd for $C_{24}H_{24}N_2O_5S$: C, 63.70; H, 5.35; N, 6.19, found C, 63.66; H, 5.26; N, 6.08.



***N*-Acyl sulfonamide 18i:** The reaction was carried out following the general procedure, using 224 mg (0.54 mmol) of sulfonyl azide **10e**, 82 μL (0.70 mmol) of 2,6-lutidine and 50 μL (0.70 mmol) of thioacetic acid in DMF. The product (221 mg, 95%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.55 (EtOAc/hexane 4:1 v/v). R_t: 17.05. Mp: 200 °C. $[\alpha]_D^{22}$ +36.7 (c = 0.1 DMF); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.73 (s, 1H), 7.91 (d, J = 7.7 Hz, 2H), 7.4 (m, 2H), 7.53-7.30 (m, 5H), 4.32-4.22 (m, 3H), 3.87 (m, 1H), 3.49 (m, 2H), 1.94 (s, 3H), 1.78 (m, 1H), 0.82 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 170.0, 155.9, 144.2, 143.9, 140.9, 127.9, 127.3, 125.5, 120.4, 65.6, 53.7, 51.7, 47.0, 32.6, 23.4, 18.5, 17.8. ESMS calcd for $C_{22}H_{26}N_2O_5S$: 430.16, found: 431.25 $[M + H]^+$, 453.30 $[M + Na]^+$, 429.35 $[M - H]^-$; Anal. calcd for $C_{22}H_{26}N_2O_5S$: C, 61.38; H, 6.09; N, 6.51, found C, 61.46; H, 5.98; N, 6.39.

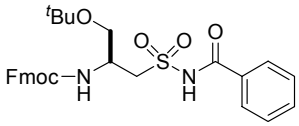


***N*-Acyl sulfonamide 18j:** The reaction was carried out following the general procedure, using 224 mg (0.54 mmol) of sulfonyl azide **10e**, 82 μL (0.70 mmol) of 2,6-lutidine and 82 μL (0.70 mmol) of thiobenzoic acid in DMF. The product (255 mg, 96%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.66 (EtOAc/hexane 4:1 v/v); R_t: 18.42. Mp: 231 °C. $[\alpha]_D^{22}$ +39.2 (c = 0.1 DMF). ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.20 (br s, 1H), 7.91 (m, 4H), 7.67 – 7.28 (m, 10H), 4.23 – 3.94 (m, 4H), 3.68 (m, 2H), 1.79 (m, 1H), 0.82 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 166.7, 155.8, 144.2, 143.8, 140.9, 133.3, 131.9, 128.7, 127.9, 127.31, 125.4, 120.3, 65.5, 54.2, 51.9, 46.9, 32.6, 18.6, 17.8; ESMS calcd for $C_{27}H_{28}N_2O_5S$: 492.17, found: 493.45 $[M + H]^+$, 515.40 $[M + Na]^+$, 491.25 $[M - H]^-$; Anal. calcd for $C_{27}H_{28}N_2O_5S$: C, 65.83; H, 5.73; N, 5.69, found C, 65.72; H, 5.27; N, 5.58.

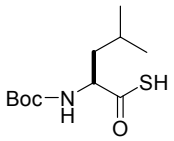


***N*-Acyl sulfonamide 18k:** The reaction was carried out following the general procedure, using 208 mg (0.54 mmol) of sulfonyl azide **10f**,

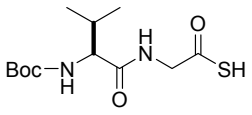
82 µL (0.70 mmol) of 2,6-lutidine and 50 µL (0.70 mmol) of thioacetic acid in DMF. The product (202 mg, 93%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.59 (EtOAc/hexane 4:1 v/v). R_t: 17.62; Mp: 96 °C. $[\alpha]_D^{22}$ +33.9 (c = 0.1 DMF); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.74 (s, 1H), 7.91 (d, J = 7.4 Hz, 2H), 7.71 (d, J = 7.4 Hz, 2H), 7.69 – 7.33(m, 5H), 4.38 – 4.20 (m, 3H), 3.97, (m, 1H), 3.61 – 3.28 (m, 4H), 1.98 (s, 3H), 1.10 (s, 9H). ^{13}C NMR (75 MHz, DMSO-d_6) δ 169.9, 155.7, 144.1, 144.0, 141.0, 127.9, 127.3, 125.4, 120.4, 73.1, 65.7, 62.7, 52.9, 47.6, 46.9, 27.4, 23.5; ESMS calcd for $C_{24}H_{30}N_2O_6S$: 474.18, found: 475.18 $[M + H]^+$, 497.35 $[M + Na]^+$, 473.45 $[M – H]^–$; Anal. calcd for $C_{24}H_{30}N_2O_6S$: C, 60.74; H, 6.37; N, 5.90, found C, 60.58; H, 6.33; N, 5.82.



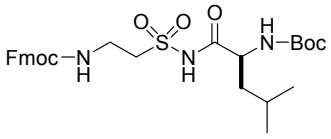
***N*-Acyl sulfonamide 18l:** The reaction was carried out following the general procedure, using 208 mg (0.54 mmol) of sulfonyl azide **10f**, 82 µL (0.70 mmol) of 2,6-lutidine and 82 µL (0.70 mmol) of thiobenzoic acid in DMF. The product (235 mg, 94%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.66 (EtOAc/hexane 4:1 v/v). R_t: 18.82; Mp: 157 °C. $[\alpha]_D^{22}$ +32.7 (c = 0.1 DMF). ^{1}H NMR (300 MHz, DMSO-d_6): δ 12.21 (s, 1H), 7.93 (m, 4H), 7.64 – 7.32 (m, 10H), 4.13 (m, 4H), 3.77 (m, 2H), 3.39 – 3.19 (m, 2H) 1.07 (s, 9H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 167.0, 156.1, 144.6, 144.3, 141.4, 133.8, 132.4, 129.2, 128.3, 127.8, 125.9, 120.8, 73.6, 66.1, 63.1, 53.8, 48.2, 47.3, 27.9; ESMS calcd for $C_{29}H_{32}N_2O_6S$: 536.20, found: 537.35 $[M + H]^+$, 559.40 $[M + Na]^+$, 535.45 $[M – H]^–$.



Boc-Leu-SH: The synthetic procedure and analytical data for this compound have been described in chapter 4.

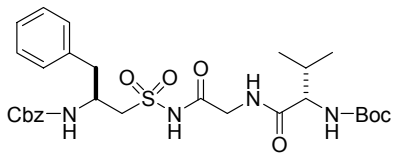


Boc-Val-Gly-SH: The synthetic procedure and analytical data for this compound have been described in chapter 4.

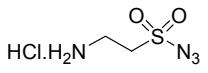


***N*-acyl sulfonamide 19:** The reaction was carried out following the general procedure using 223 mg of sulfonyl azide **10a** (0.60 mmol), Boc-Leu-SH (193 mg, 0.78 mmol, added as a solution in DMF) and 2,6-lutidine (91 µL, 0.78 mmol). The product (272 mg, 81%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.31 (EtOAc/hexane/HOAc 49.5:49.5:1 v/v/v); R_t: 20.15 min; ^{1}H NMR (300 MHz, DMSO-d_6): δ

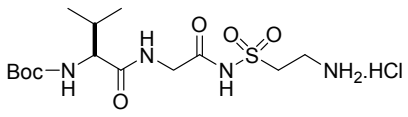
11.94 (s, 1H), 7.91 (d, *J* = 7.4 Hz, 2H), 7.69 (d, *J* = 7.4 Hz, 2H) 7.49 – 7.30 (m, 5H), 7.21 (d, *J* = 7.1 Hz, 1H), 4.34 (d, *J* = 7.1 Hz, 2H), 4.24 (m, 1H), 4.02 (m, 1H), 3.48 – 3.36 (m, 4H), 1.63 – 1.24 (m, 3H), 1.38 (s, 9H), 0.86 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 173.7, 156.1, 155.8, 144.0, 141.0, 127.9, 127.3, 125.4, 120.4, 53.4, 51.6, 46.9, 35.1, 31.2, 31.2, 28.4, 24.5, 23.2, 21.3; ESMS calcd for $C_{28}H_{37}N_3O_7S$: 559.24, found: 560.55 $[M + H]^+$, 582.40 $[M + Na]^+$, 558.50 $[M – H]^–$.



***N*-Acyl sulfonamide 20:** The reaction was carried out following the general procedure using 64 mg of sulfonyl azide **10d** (0.17 mmol), Boc-Val-Gly-SH (64 mg, 0.22 mmol, added as a solid) and 2,6-lutidine (26 μL, 0.22 mmol). The product (102 mg, 99%) was obtained by crystallization from EtOAc/hexane as a white solid. R_f: 0.48 (EtOAc/hexane/HOAc, 49.5:49.5:1 v/v/v); R_t: 17.08; Mp: 191 °C. $[\alpha]_D^{23}$ –29.9 (c = 0.1 DMF); ^{1}H NMR (300 MHz, DMSO-d_6): δ 11.89 (s, 1H), 8.16 (m, 1H), 7.53 (d, *J* = 9.1 Hz, 1H), 7.34 – 7.17 (m, 10H) 6.75 (d, *J* = 9.1 Hz, 1H), 4.98 (m, 2H), 4.18 (m, 1H), 3.84 (m, 3H), 3.59 (m, 2H), 2.90 (m, 1H), 2.78 (m, 1H), 1.99 (m, 1H), 1.38, (s, 9H), 0.86 (m, 6H); ^{13}C NMR (75 MHz, DMSO-d_6) δ 172.1, 169.2, 155.7, 155.4, 137.9, 137.3, 129.4, 128.5, 127.9, 127.6, 127.2, 126.6, 78.2, 65.3, 60.0, 59.7, 56.1, 48.4, 42.3, 30.6, 28.4, 19.4, 18.3; ESMS calcd for $C_{29}H_{40}N_4O_8S$: 604.26, found: 605.55 $[M + H]^+$, 627.50 $[M + Na]^+$; Anal. calcd for $C_{29}H_{40}N_4O_8S$: C, 57.60; H, 6.67; N, 9.27, found C, 57.52; H, 6.65; N, 9.21.

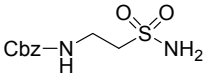


HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (**21**): HBr (30% in AcOH, 90 mL) was added dropwise to a solution of Sulfonyl azide **10a** (4.26 g, 15 mmol) in DCM (150 mL) and the reaction mixture was stirred for 30 min at room temperature. The solvent was removed *in vacuo* and the residue was co-evaporated with $CHCl_3$ (3×) before H_2O (150 mL) and Dowex® (2×8 Cl-form, 12 g) were added. The resulting mixture was stirred for 1 h at room temperature. After filtration, the solution was concentrated to dryness and the residue was co-evaporated with toluene (3×) and $CHCl_3$ (3×). The product (2.51 g, 90%) was obtained as a white solid. ^{1}H NMR (300 MHz, DMSO-d_6): δ 8.23 (br s, 3H), 4.10 (m, 2H), 3.29 (m, 3H).



***N*-Acyl sulfonamide 22:** A solution of Boc-Val-Gly-SH (29 mg, 0.10 mmol, 1 equiv) in phosphate buffer (1 mL) was added to a solution of **21** (28 mg, 0.15 mmol, 1.5 equiv) in phosphate buffer (pH = 7.4, 1 mL) and stirred for 15 min. The reaction mixture

was analyzed with ESMS and HPLC. R_t: 11.55 min; ESMS calcd for $C_{14}H_{28}N_4O_6S$: 380.17, found: 380.50 $[M + H]^+$, 401.95 $[M + Na]^+$.



sulfonamide 23: A mixture of sulfonyl azide **24** (57 mg, 0.20 mmol), PPh_3 (53 mg, 0.20 mmol), THF (3 mL), and H_2O (2 mL) was stirred at rt. After 2 min the sulfonyl azide was completely dissolved and the evolution of N_2 had stopped. The mixture was concentrated *in vacuo* and EtOAc (20 mL) was added. After washing with 1N $KHSO_4$ (10 mL) and brine (10 mL), the organic layer was dried (Na_2SO_4) and concentrated. Column chromatography (DCM:MeOH 4:96 v/v) afforded the product (50 mg, 97%) as a white solid. R_f: 0.21 (DCM:MeOH 95:5 v/v); mp 78 °C; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.13 (t, 2H), 3.38 (m, 3H), 5.03 (s, 2H), 6.93 (br s, 2H), 7.36 (m, 5H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 35.8, 35.9, 54.1, 65.7, 128.0, 128.1, 128.6, 137.2, 156.1, 156.2; ESMS calcd for $C_{10}H_{14}N_2O_4S$: 258.07, found: 281.15 $[M + Na]^+$, 322.15 $[M + CH_3CN + Na]^+$.

The reaction of 24 and thio acetic acid in the presence of *n*-Bu-NH_2: Thioacetic acid (56 µL, 0.78 mmol, 1.3 equiv) added dropwise to a mixture of **24** (171 mg, 0.6 mmol, 1 equiv), 2,6-lutidine (91 µL, 0.78 mmol, 1.3 equiv) and *n*-Bu-NH_2 (59 µL, 0.6 mmol, 1 equiv) in DMF (2 mL). After 15 min of stirring, the solvent was removed *in vacuo*. Side product **24** was isolated by flash column chromatography (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v) as a clear oil (17 mg, 9%). The remaining fractions were collected, concentrated and analyzed by HPLC (using a linear gradient of 100% buffer A (0.1% TFA in H_2O/MeCN 95:5 v/v) to 100% buffer B (0.1% TFA in MeCN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min). Side product **28** R_t: 9.17 min; side product **23** R_t: 12.25 min; compound **18a** R_t: 13.15 min.

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6

Resin-bound Sulfonyl Azides: Efficient Loading and Activation Strategy for the *N*-Acyl Sulfonamide Linker

The *N*-acyl sulfonamide linker, as originally introduced by Kenner, is stable toward acidic and basic conditions and thus compatible with both Boc and Fmoc SPPS methods. However, *N*-alkylation makes this linker sensitive toward nucleophilic displacement allowing for the *C*-terminal modification of resin bound peptides. Although the widespread application of the *N*-acyl sulfonamide linker in peptide synthesis, the loading and activation steps remain subject for improvement with respect to efficiency and degree of epimerization of the *C*-terminal amino acid. We addressed these issues by the introduction of a resin-bound sulfonyl azide/thio acid amidation as the loading step, followed by a microwave-assisted *N*-alkylation of the *N*-peptidyl sulfonamide moiety. Subsequent treatment with functionalized nucleophiles yielded *C*-terminally modified peptides which can be applied for chemoselective (bio)conjugation or ligation reactions.

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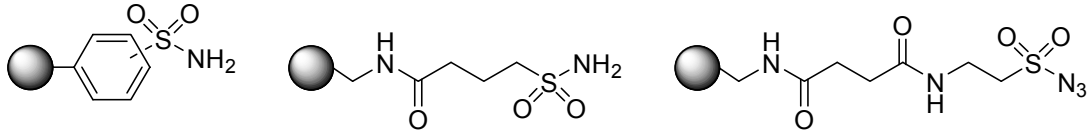
6.1 Introduction

In the previous chapter, the convenient synthesis of β-substituted aminoethane sulfonyl azides and their highly efficient and chemoselective reaction with thio acids derived from amino acids and peptides was described.[1,2] The resulting α-amino acyl sulfonamides find applications in medicinal chemistry,[3-23] chemoselective ligation and (bio)conjugation reactions[2,24-26] and as a linker for anchoring to the solid support.[27-38] In this chapter, the application of a resin-bound tauryl sulfonyl azide is described in an efficient loading and activation strategy for access of the *N*-acyl sulfonamide linker.

6.1.1 The N-acyl sulfonamide Linker

It is evident that a successful solid phase synthesis begins with choice of and adequate combination of the protecting group strategy and of the solid support. In particular the selection of a suitable linker[39-41] is a key consideration, since this largely dictates the chemistry which can be applied. Therefore, a linker that can combine inertness to a variety of reaction conditions with release of the product under mild conditions is highly desirable. As a possible answer to this demand, Kenner[42] introduced the "safety catch principle" to describe a strategy that allows the linker to remain stable until it is activated for cleavage by chemical modification. In this strategy, the growing peptide chain is connected to the solid support via an *N*-acyl sulfonamide linkage. Interestingly, the *N*-acyl sulfonamide moiety is stable toward acidic and basic conditions, making it compatible with Boc as well as Fmoc SPPS methods. However, *N*-alkylation makes this linker susceptible toward nucleophilic displacement.



A **B** **C**

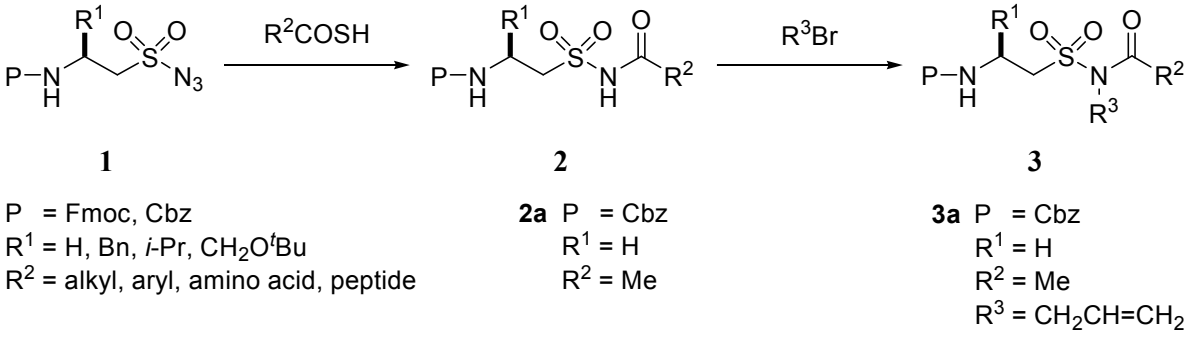
Figure 6.1 Original (**A**) and modified Kenner linker types (**B**), (**C**).

The original arenesulfonamide Kenner linker **A** (Figure 6.1) suffered from poor loading efficiency, racemization of the *C*-terminal amino acid in the loading step and a low reactivity of the activated linker after *N*-alkylation. Backes and Ellman[43,44] addressed these problems by the introduction of a more nucleophilic aliphatic sulfonamide linker **B** that could be loaded with minimal racemization by lowering the temperature. The reactivity of the linker was increased by alkylation with an electron-withdrawing haloacetonitrile rather

than methylation by diazomethane or trimethylsilyldiazomethane. Although these modifications allowed for the synthesis of *C*-terminally modified- and cyclic peptides,[27-36] the loading of the first amino acid onto the sulfonamide linker is still of major concern with respect to efficiency and degree of epimerization. Moreover, alkylation with haloacetonitriles is time-consuming but not always efficient as was demonstrated by Biancalana *et al.*[30] This means that the key steps of this linker remain subject to further improvement.[45-47] We have addressed these issues by using resin-bound sulfonyl azide **C** in a thio acid/sulfonylazide amidation reaction as the loading step, followed by a microwave-assisted *N*-alkylation of the peptidyl *N*-acyl sulfonamide by electrophilic halides. The results will be discussed in this chapter.

6.2 Results and Discussion

In chapter 5 the highly efficient and chemoselective coupling of *β*-substituted aminoethane sulfonyl azides **1** (Scheme 6.1) with amino acid- and peptide-based thio acids was described. The increased acidity of the resulting *N*-acyl sulfonamide product **2** allows for alkylation by suitable nucleophiles.



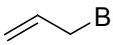
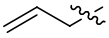
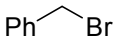
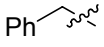
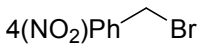
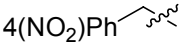
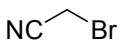
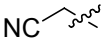
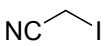
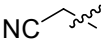
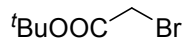
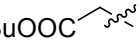
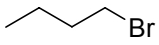
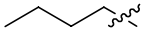
Scheme 6.1 General structure of the (*N*-alkylated) *N*-acyl sulfonamides.

In the literature, several alkylation methods have been reported as activation protocols for the *N*-acyl sulfonamide linker. Standard conditions, however, still rely on methylation by trimethylsilyldiazomethane to give an efficient alkylation,[29] while haloacetonitrile activation provides an enhanced reactivity toward nucleophilic displacement.[43] We envisioned alkylation by electrophilic bromides as a suitable alternative for the development of a new activation protocol that combines efficient alkylation properties with a high linker reactivity in a reliable and straightforward procedure as an significant improvement of the known literature procedures.

Based on experience in our group with sulfonamides,[48] the alkylation of *N*-acyl sulfonamides was in first instance investigated with Cbz-Gly-Ψ[CH_2SO_2]-NH-Ac (**2a**, Scheme 6.1) as a model compound. The reaction of **2a** with allylbromide (R^3 = $CH_2CH{=}CH_2$) in the presence of one equivalent of K_2CO_3 in DMF, however, yielded **3a** in only 17%. Increasing the amount of K_2CO_3 to 3 equivalents, in the presence of 2 equivalents of allylbromide also resulted in alkylation of the urethane NH, and the dialkylated product was isolated in 15% yield. Fortunately, when D*i*PEA (2 equivalents) was used, **2a** could be chemoselectively alkylated into **3a** with 75% yield. Further optimization of the reaction conditions showed that when the reaction was performed in a microwave reactor at an elevated temperature of 150°C, **3a** was formed in a yield of 81% after only 6 minutes.

To investigate the scope of this chemoselective alkylation protocol, we used a diverse set of bromides (Table 6.1). From these results it is apparent that alkylation with suitable alkyl bromides is very efficient and that reaction times can be reduced dramatically by the aid of microwave irradiation. Compared to the other bromides, alkylation by *n*-butylbromide (entry 7) and haloacetonitriles (entry 4 and 5) was less efficient.

Table 6.1 Solution phase alkylation of *N*-acyl sulfonamide **2a**

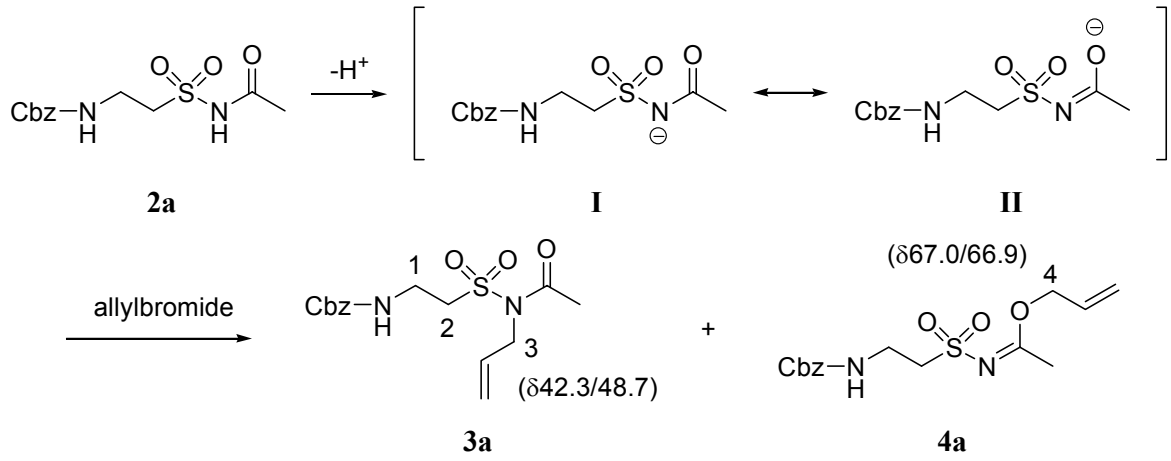
Entry	Electrophile	Yield (%) method A[a]	Yield (%) method B[b]	R^3	3
1		75	81		**3a**
2		nd	76		**3b**
3		75	76		**3c**
4		53	31		**3d**
5		55	27		**3d**
6		94	77		**3e**
7		nd	30		**3f**

[a]Reaction conditions method A: electrophile (1 equiv), D*i*PEA (2 equiv), DMF, rt, 16 h.
[b]Reaction conditions method B: electrophile (1 equiv), D*i*PEA (2 equiv), DMF, μW, 150°C, 6 min.

Unexpectedly, the conversion of **2a** resulted into two spots on TLC, NMR analysis of the crude product revealed the formation of a side product in 13% yield. When the experiment

was carried out on a larger scale, **3a** and the side product could be isolated by column chromatography and their identity was investigated by NMR, HPLC and MS analysis. Both compounds showed an identical mass but differed in retention time. Moreover, the ^{1}H- and ^{13}C-NMR spectra of both compounds were significantly different with respect to the CH_2 moieties (numbered 1, 2, 3 respectively, Scheme 6.2). The isomers could possibly originate from tautomerization of **2a**. It is known that many sulfonamides predominantly exist as an amide or as an imide tautomer, although the majority exists completely in the amide tautomer.[49] For *N*-acyl sulfonamide **2a**, NMR spectroscopy did not indicate the presence of two tautomeric forms. Additionally, the ^{1}H-NMR-spectrum showed signals that could be assigned to an acidic *N*-acyl sulfonamide proton which was an indication for the amide tautomer. Rather than tautomerization, alkylation of resonance structures **I** and **II** of deprotonated **2a** may lead to the formation of isomers **3a** and **4a** (Scheme 6.2). The chemical shifts found for carbon atoms 3 and 4 corresponded to calculated values[50] for proposed structures **3a** and **4a**.

Interestingly, the conversion of **2a** to **3a**, in the presence of excess (5 equiv) of allylbromide and excess (6 equiv) of D*i*PEA, proceeded without any detectable formation of **4a** and the product could be isolated in quantitative yield after reacting for 16 h at room temperature. Similarly, the complete conversion of **2a** to **3c** and **3e** using excess of reagents was achieved in a microwave reactor at an elevated temperature of 150°C within 6 minutes.

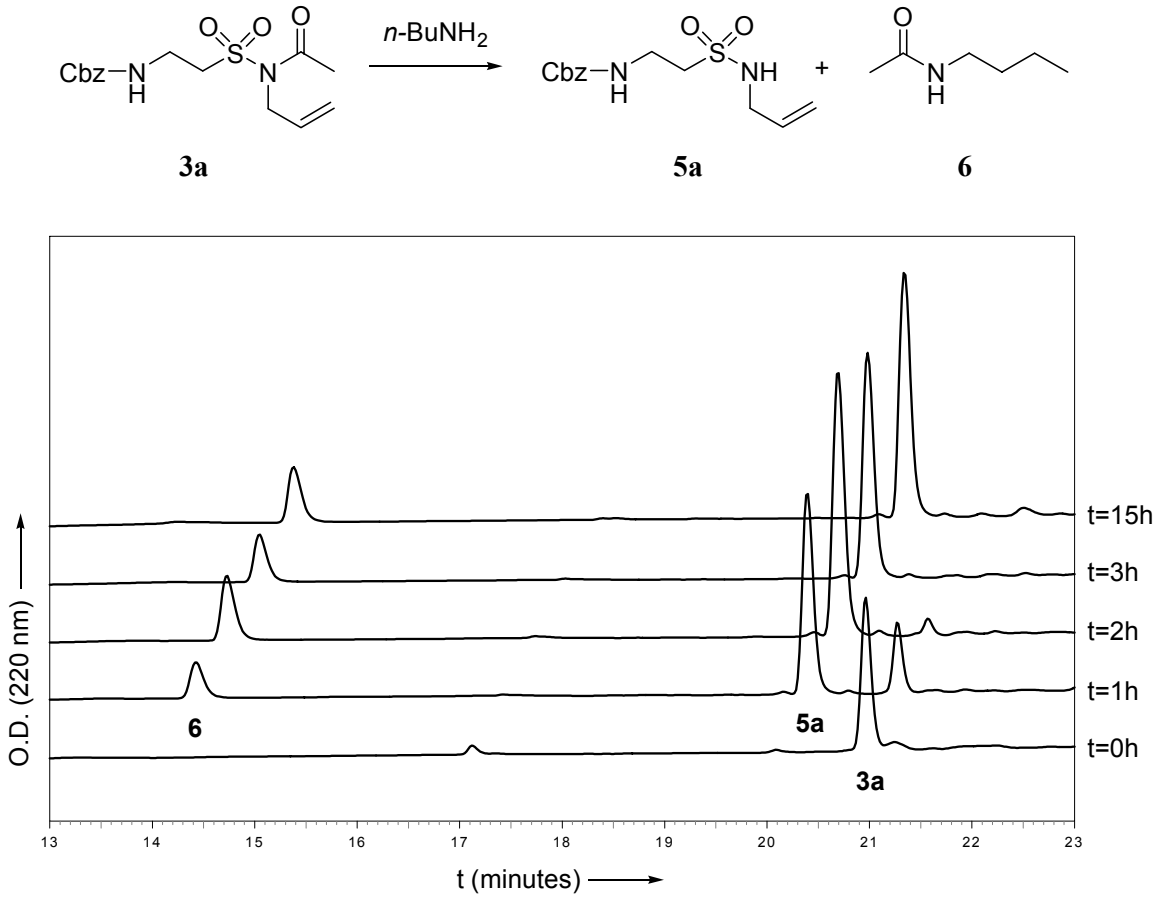


2a **I** **II**

3a **4a**

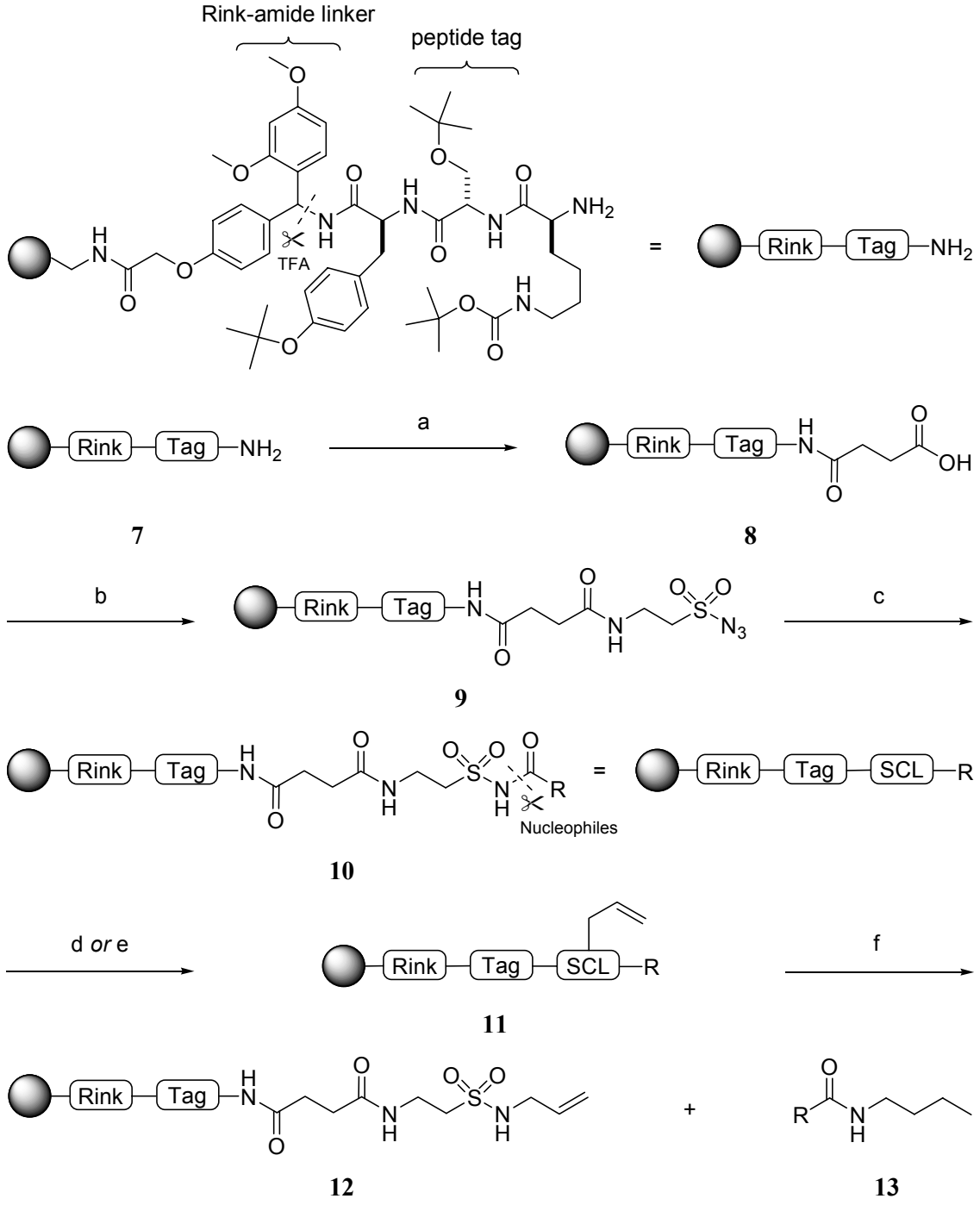
Scheme 6.2 Proposed structure of the two isomers, chemical shifts are in ppm (δcalcd./found).

Next, the susceptibility of **3a** toward nucleophilic displacement was evaluated by treatment with *n*-butylamine (4 equiv) in DMF (Scheme 6.3). The reaction was monitored by analytical HPLC and was found to be complete within 3 h. After their isolation and purification by preparative HPLC, the chemical structures of **5a** and **6** were confirmed by NMR-spectroscopy.



Scheme 6.3 Nucleophilic displacement test reaction.

After this, the applicability of the thio acid/sulfonylazide amidation reaction and subsequent activation and cleavage by nucleophiles of the resulting *N*-acyl sulfonamide linker on the solid support was investigated. In this approach, the *N*-acyl sulfonamide linker is formed by coupling amino thio acids to a resin-bound sulfonyl azide (**C,** Figure 6.1**)**. Synthesis of the sulfonyl azide linker H-Gly-Ψ[CH_2SO_2]-N_3 (**1**, P = H, R^1 = H, Scheme 6.1) could efficiently be performed on a multigram scale starting from taurine, following the method as was described in chapter 5.[1] As mentioned, the "safety-catch" *N*-acyl sulfonamide linker is stable toward acidic and basic conditions until alkylation increases the sensitivity toward nucleophiles. This inertness however, also requires a time-consuming two-step micro-cleavage for monitoring the solid phase reactions by MS. To overcome this problem we introduced a Rink-amide linker moiety as second cleavage site, to allow for orthogonal cleavage with TFA.[51] Additionally, a short peptide tag was introduced to enable simple isolation by precipitation from MTBE/hexane and subsequent identification by MS analysis of the cleavage products. The complete strategy is depicted in scheme 6.4.



Scheme 6.4 Proof of principle improved loading and activation strategy for the *N*-acyl sulfonamide linker. Reaction conditions: a) succinic anhydride (5 equiv), TEA (7.5 equiv), DCM, 16 h; b) HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (1.2 equiv), BOP (4 equiv), D*i*PEA (8 equiv); NMP, 16 h, c) RCOSH (3 equiv), 2,6-lutidine (20 equiv), $CHCl_3$, 1.5 h; d) allylbromide (1.1 equiv), D*i*PEA (2.1 equiv), rt, 16 h; e) allylbromide (1.1 equiv), D*i*PEA (2.1 equiv), DMF, μW, 150°C, 6 min; f) *n*-$BuNH_2$ (20 equiv), $CHCl_3$, 3.5 h.

As a proof of principle for the improved loading and activation strategy we started by building up a short peptide sequence (Lys-Ser-Tyr) on the Rink-amide resin by conventional Fmoc/^{t}Bu methods. The free *N*-terminus of lysine was treated with succinic anhydride and TEA to give carboxylic acid functionalized resin **8**. Attachment of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 was achieved via a BOP mediated coupling. All solid phase reactions were monitored by the Kaiser test[52] (free amines), and the Malachite green test[53] (free carboxylic acids) respectively. Furthermore, the identity and complete conversion to **8** respectively **9** was confirmed by mass analysis of the cleaved products (**8'** and **9'** Figure 6.2 A/B).

Next, sulfonyl azide resin **9** was reacted with 3 equiv of thioacetic acid (R = Me) in the presence of 3 equiv of 2,6-lutidine in chloroform. MS analysis revealed that even after 1.5 h no reaction had taken place. However, when the same resin was treated again with 3 equiv of thioacetic acid, in the presence of an excess of 2,6-lutidine (20 equiv), the conversion to **10** (R = Me) went to completion as was confirmed by mass analysis of the cleaved product (**10'**, Figure 6.2 C). In the same way the amino thio acid Boc-Val-SH and dipeptide thio acid Boc-Val-Gly-SH could successfully be coupled to the resin under these conditions.

Alkylation of the resulting *N*-acyl sulfonamide resin **10** (R = Me) with 1.1 equiv allylbromide and 2.1 equiv of D*i*PEA went almost to completion after 16 h at room temperature (**11'**, Figure 6.2 D). In analogy to solution phase experiments, the reaction time could be decreased tremendously by the use of microwave irradiation. MS analysis of the cleaved products showed that similar results could be obtained via a microwave-assisted alkylation in 6 minutes at 150°C (**11'**, Figure 6.2 E).

Finally, the alkylated *N*-acyl sulfonamide resin **11** was treated with an excess of *n*-butylamine (20 equiv) to evaluate its sensitivity toward nucleophiles. Cleavage of **11** was complete within 3 h, as became clear from mass analysis of the cleaved product (**12'**, Figure 6.2 F).

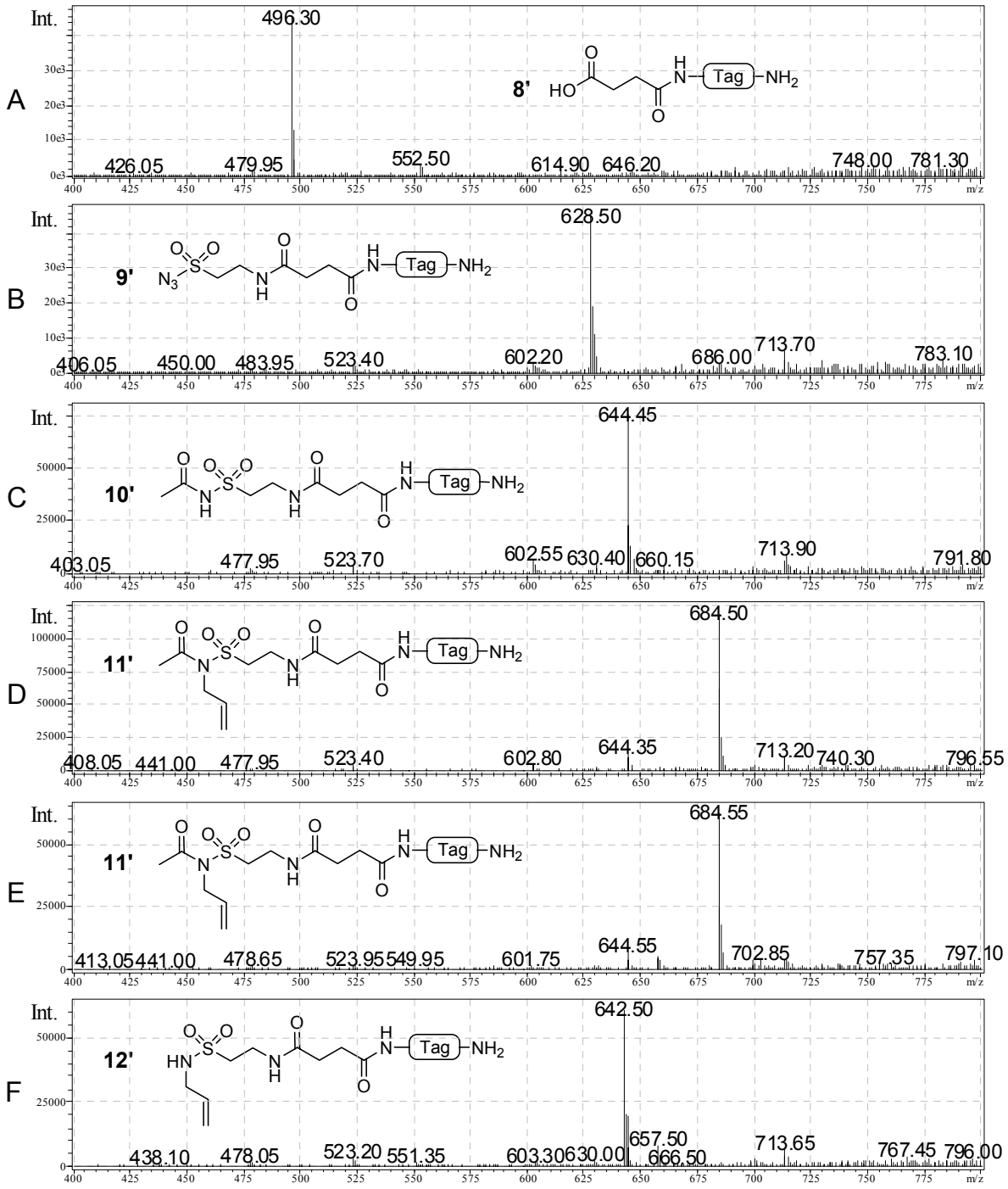
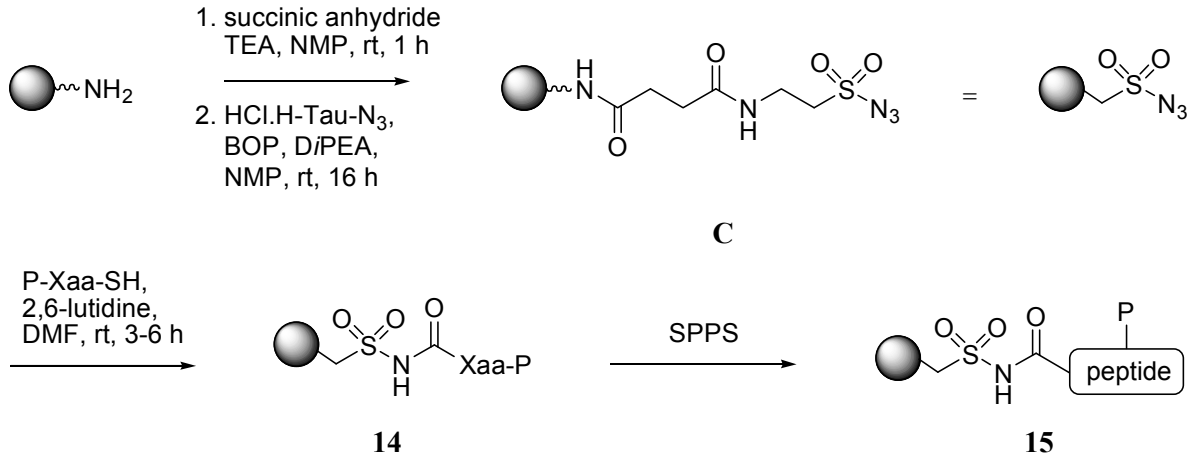


Figure 6.2 MS analysis *N*-acyl sulfonamide linker strategy. ESMS spectra and structure of cleaved products calcd./found $[M+H]^+$ for: (A) **8'** 495.23/496.30; (B) **9'** 627.24/628.50; (C) **10'** 643.26/644.45; (D) **11'** reaction conditions d 683.29/684.55; (E) **11'** reaction conditions e 683.29/684.55; (F) **12'** 641.28/642.50.

With these results in hand, the attention was turned to an application of this approach to peptide synthesis (Scheme 6.5). First, the loading efficiency of thioacids on the resin was determined by treatment of the resin-bound sulfonyl azide **C** with Boc-Phe-SH in the

presence of 2,6-lutidine as a base in DMF[54] for 3 h at room temperature. Under these mild reaction conditions, amino thio acids are configurationally stable as was shown earlier by Williams *et al.*[24] After removal of the Boc group with TFA, Fmoc-Gly-OH was coupled with BOP/D*i*PEA and the total yield was calculated via an Fmoc determination[55] and was found to be 73% over five reaction steps. This corresponded to a loading yield of 94% for the thio acid Boc-Phe-SH.



Scheme 6.5 Synthesis, loading and elongation of the *N*-acyl sulfonamide resin.

Boc-Phe-SH was synthesized from Boc-protected phenylalanine hydroxysuccinimide ester (Boc-Phe-ONSu) by treatment with NaHS essentially as was described by Goldstein and Gelb.[56] Although alternative methods for the synthesis of thioacids have been described in the literature,[57-59] these methods have, to the best of our knowledge, never been applied to the synthesis of Fmoc-protected amino thio acids. This can probably be assigned to the assumed sensitivity of the Fmoc-group toward the nucleophilic sulfhydryl ion. We found that NaHS in combination with Fmoc-protected amino acids to obtain thioacids did not result in premature cleavage of the Fmoc-group. In this protocol the protected amino acid of choice was preactivated with BOP/D*i*PEA and converted into the corresponding thio acid by the addition of NaHS. In this way, Fmoc-Ser(^{t}Bu)-OH was converted into the corresponding thio acid. Fmoc-Ser(^{t}Bu)-SH was used without further purification, other than removal of excess NaHS by a simple acid washing step, in the coupling reaction with resin-bound sulfonyl azide linker **C**. In this case a high loading efficiency of 85% was obtained after 6 h (entry 5, Table 6.2). Similarily, coupling of Boc-Phe-SH in the presence of excess (80 equiv) of 2,6-lutidine followed by TFA treatment and a BOP/D*i*PEA-mediated coupling of Fmoc-Gly-OH, resulted in an excellent loading yield of 94%. Thus, the BOP/D*i*PEA/NaHS approach enabled the direct synthesis of Boc- or Fmoc-protected

amino thio acids from commercially available reagents,[60] which were used in the loading of the sulfonyl azide resin.

Table 6.2 Optimization of the loading efficiency

Entry	P-Xaa-SH	P-Xaa-SH (equiv)	2,6-lutidine (equiv)	t (h)	loading yield (%)
1	Fmoc-Ser(^{t}Bu)-SH	4	20	3	81
2	Fmoc-Ser(^{t}Bu)-SH	8	20	3	83
3	Fmoc-Ser(^{t}Bu)-SH	4	20	16	83
4	Fmoc-Ser(^{t}Bu)-SH	4	80	3	83
5	Fmoc-Ser(^{t}Bu)-SH	4	80	6	85
6	Fmoc-Ser(^{t}Bu)-SH	4	80	16	85
7	Boc-Phe-SH	4	80	3	94
8	Boc-Phe-SH	4	80	6	94

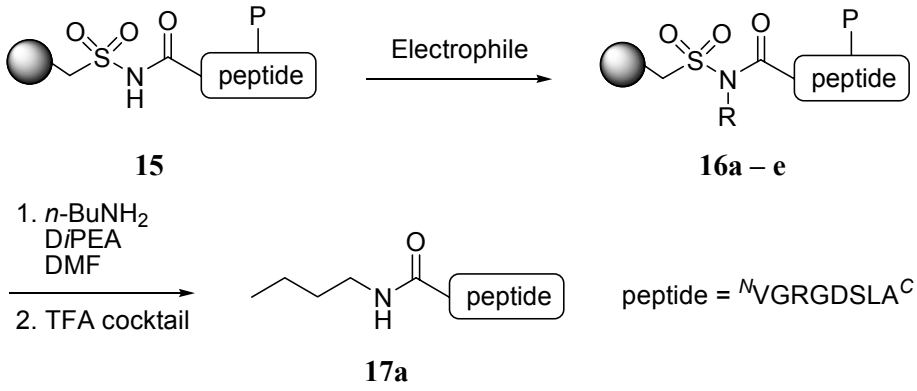
Reaction conditions: **C** (1 equiv), P-Xaa-SH, 2,6-lutidine, DMF, rt. Loading yields are determined by Fmoc determination.[55]

As a model peptide, an octapeptide containing the RGD sequence: Boc-Val-Gly-Arg(Pbf)-Gly-Asp(O^{t}Bu)-Ser(^{t}Bu)-Leu-Ala~ was synthesized. First, resin-bound sulfonyl azide **C** was treated with Boc-Ala-SH as described. Then, the Boc group was removed by acidolysis with TFA and the α-amino group was acylated with Fmoc-Leu-OH in the presence of BOP/D*i*PEA as the condensing agent. The remaining amino acid residues were coupled via conventional Fmoc/^{t}Bu solid phase peptide synthesis protocols and the final amino acid (valine) was introduced as the corresponding Boc derivative.

Next, we studied the microwave-assisted alkylation and subsequent cleavage by *n*-butylamine of the resin-bound *N*-peptidyl sulfonamide. Although alkylation using allylbromide proved efficient, the introduction of electronwithdrawing substituents might result in a better activation of the *N*-acyl sulfonamide linker. On basis of the alkylation reactions as were described in Table 6.1, three bromides with electron-withdrawing substituents were selected: 4-nitrobenzylbromide, *t*-butylbromoacetate and methylbromoacetate for evaluation of the activation and cleavage efficiency. Trimethylsilyl

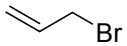
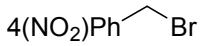
diazomethane and allylbromide were added to the series for comparison with standard procedures from the literature. The results are summarized in Table 6.3.

Table 6.3 Activation and cleavage efficiency.



15 **16a – e**

17a

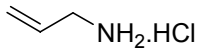
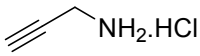
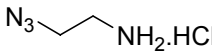
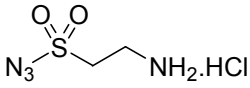
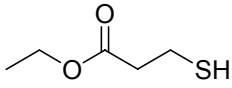
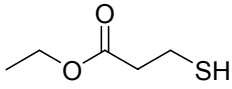
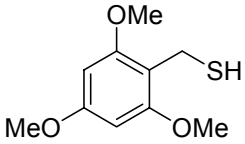
Entry	Electrophile	**16**	% Yield (% Purity)[a] of **17a**
1[b]		**16a**	52 (72)
2[b]		**16b**	68 (86)
3[b]		**16c**	48 (84)
4[c]		**16c**	37 (100)
5[b]		**16d**	66 (84)
6[d]	$TMSCHN_2$	**16e**	89 (74)

[a]Yields are based on the crude cleavage product; purities are assessed by analytical HPLC. [b]Reaction conditions: alkylbromide (20 equiv), D*i*PEA (10 equiv) in DMF, 6 min, 150°C μW; then *n*-$BuNH_2$ (10 equiv), D*i*PEA (10 equiv) in DMF, rt, 24 h. [c]Reaction conditions: alkylbromide (20 equiv), D*i*PEA (10 equiv) in DMF, 16 h, rt. [d]Reaction conditions: $TMSCHN_2$ (2M in hexane/THF, 350 equiv), 2 h, rt.[28]; then *n*-$BuNH_2$ (10 equiv), D*i*PEA (10 equiv) in DMF, rt, 24 h.

Alkylation with 4-nitrobenzylbromide (entry 2) followed by cleavage with *n*-butylamine proved to be the most efficient approach. The combination of isolated yield and purity of the *C*-terminally modified RGD peptide was comparable to alkylation with trimethylsilyl diazomethane and subsequent cleavage with *n*-butylamine (entry 6). Apparently, efficient alkylation and activation due to its electron withdrawing propensity make 4-

nitrobenzylbromide[46,61] a promising alternative for iodoacetonitrile, bromoacetonitrile or trimethylsilyl diazomethane.

Table 6.4 Synthesis of *C*-Terminally Modified RGD Peptides

Entry	Nucleophile	**17**	% Yield (% Purity)[a]
1[b]		**17b**	57 (82)
2[b]		**17c**	59 (81)
3[b]		**17d**	96 (83)
4[b]		**17e**	intractable material
5[c]		**17f**	46 (92)
6[d]		**17f**	52 (92)
7[c]		**17g**	intractable material
8[e]	D*i*PEA.H_2S	**17h**	33 (66)[f]

[a]Yields are based on the crude cleavage product; purities are assessed by analytical HPLC. [b]Reaction conditions: R-NH_2 (10 equiv), DiPEA (15 equiv) in DMF, rt, 24 h. [c]Reaction conditions: R-SH (50 equiv), DiPEA (10 equiv), Na-thiophenolate (0.5 equiv) in DMF, rt, 24 h. [d]Reaction conditions: R-SH (50 equiv), D*i*PEA (10 equiv) in DMF, rt, 24h. [e]Reaction conditions: H_2S.DiPEA in dioxane, rt, 24h. [f]Part of the crude peptide product was identified as the oxy acid originating from hydrolysis of the thio acid

To demonstrate the applicability of the described strategy, the RGD peptide was *C*-terminally modified with functional nucleophiles. As the RGD motif plays an important role in cell-cell adhesion processes,[62] introduction of functional groups in the nucleophile facilitates the chemoselective coupling of this peptide to, among others, fluorophores, affinity tags, proteins and dendrimer scaffolds.[63] Suitable nucleophiles were therefore selected (Table 6.4) to obtain *C*-terminally modified peptides which can be used in (ring-closing) metathesis or Diels-Alder reactions (entry 1),[64-70] Cu(I)-catalyzed click reactions

(entries 2 and 3),[71-73] Staudinger ligations (entry 3),[74,75] sulfonyl azide-based ligation reactions (entries 4, 7 and 8)[2,24-26] or native chemical ligations (entries 5 and 6).[76,77]

The activated peptidyl sulfonamide resin **16b** was treated with a suitable nucleophile with D*i*PEA as base in DMF for 24 h at room temperature. After this, the DMF solution was evaporated *in vacuo* and the residue was treated with a mixture of TFA and scavengers to remove all protecting groups. The TFA solution was diluted with MTBE/hexane and the precipitated peptide was collected by centrifugation and subsequently lyophilized. The yield was based on weight of the crude peptide, the purity and identity were assessed by HPLC and LCMS respectively (Table 6.4).

Among the nitrogen-based nucleophiles, allylamine (entry 1), propargylamine (entry 2) and 2-azidoethylamine (entry 3) resulted in good to excellent overall cleavage with high purities (>80%). However, nucleophilic displacement with tauryl sulfonyl azide (entry 4) resulted in a mixture of compounds which was difficult to characterize, possibly due to sensitivity of the sulfonyl azide moiety toward nucleophiles.[1]

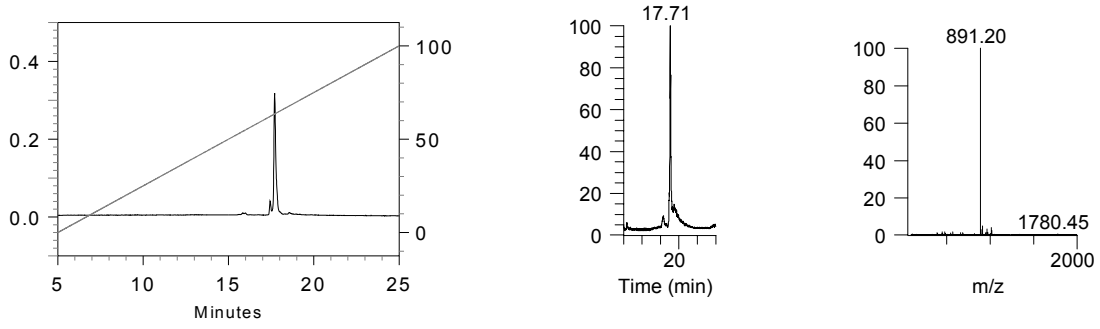


Figure 6.3 Analysis of **17f** (entry 6, Table 6.4): (A) HPLC (ELSD); (B) LCMS; (C) ESMS.

In the series of the sulfur-based nucleophiles, the peptide *C*-terminally modified with ethyl 3-mercaptopropionate was isolated in a fair to good yield with an excellent purity (entry 5). Interestingly, activation with the 4-nitrobenzyl moiety enabled the isolation of the peptide thio ester in the *absence* of thiophenolate as additive in a comparable yield and purity (entry 5, Table 6.4; Figure 6.3). This result was an indication that alkylation with 4-nitrophenyl bromide resulted in a linker which was more reactive than the methylated congener –as obtained with trimethylsilyl diazomethane– which requires thiophenolate as additive to obtain reasonable yields in the synthesis of thio esters. This was in line with the data reported by Link *et al.*[46] and Kiessling *et al.*[47], the latter also found that activation by allylation was efficient to obtain peptide thio esters without the addition of Na-thiophenolate. If we compare resin **16a** and **16b** (Table 1), it is clear that **16b** is more

susceptible toward nucleophilic displacement since a better yield and higher purity of the *C*-terminally modified peptide **17a** was obtained.
Unfortunately, treatment of the resin-bound activated peptidyl sulfonamide with 2,4,6-trimethoxybenzylmercaptan did not result in formation of the desired thio ester (entry 7). A peptide thioacid was isolated in a moderate yield of 33% after treatment of the resin with a mixture of H_2S/D*i*PEA (entry 8). This direct approach to the synthesis of peptide thioacids was a promising alternative to the two-step synthesis of entry 8 which was based on the procedure as described by Vetter.[78]

6.3 Conclusions

In conclusion, this chapter describes a convenient protocol for improving the loading efficiency of the first amino acid building block onto an *N*-acyl sulfonamide linker by reacting a resin-bound sulfonyl azide and a thio acid. These amino acid-based thio acids have been prepared by a preactivation with BOP/D*i*PEA in the presence of NaHS. The mild reaction conditions allow for the efficient synthesis of Boc- as well as Fmoc-protected thio acids starting from commercially available reagents. Moreover, the activation of the *N*-peptidyl sulfonamide resin has been optimized and relies on a microwave-assisted alkylation with 4-nitrobenzylbromide. This activated *N*-peptidyl sulfonamide linker is more active toward nucleophiles as compared to activation by methylation or allylation. In this way efficient alkylation could be combined with a high linker reactivity in a straightforward procedure. Thus, these results contribute to a better access toward *C*-terminally modified peptides with respect to overall yield, purity and diversity.

6.4 Experimental section

6.4.2 General information

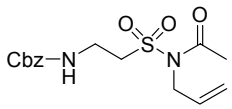
Analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (column A: Alltech Altima C8, 90Å, 5 μm, 250 × 4.6 mm or column B: Alltech Adsorbosphere XL C8, 90Å, 5 μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min, connected to a UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000). Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. Liquid chromatography electrospray ionization mass spectrometry was performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (Alltech Altima

XL C8, 90Å, 5μm, 250 × 4.6 mm), coupled to a Finnigan LCQ Deca XP MAX mass spectrometer operating in a positive ionization mode. Preparative HPLC was performed on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 10 μm, 250 × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 5 mL/min. Microwave-assisted syntheses were conducted in closed reaction vessels using a Biotage Initiator microwave reactor equipped with a temperature and pressure sensor for monitoring the reaction conditions. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) or a Varian INOVA-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) or a Varian INOVA-500 (125 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. *Rf* values were determined by thin layer chromatography (TLC) on Merck precoated silicagel 60F^{254} plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[79] Melting points were measured on a Büchi Schmelzpunktbestimmungsapparat (according to dr. Tottoli) and are uncorrected. Specific optical rotations were measured using a Jasco P1010 polarimeter. Elemental analyses were done by Kolbe Mikroanalytisches Labor (Mülheim an der Ruhr, Germany).

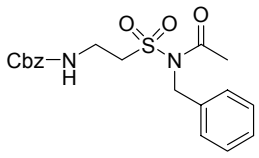
6.4.3 Chemicals and reagents

Tentagel™ S RAM resin functionalized with a 4-((2',4'-dimethoxyphenyl)aminomethyl)phenoxyacetamido moiety (Rink amide linker) and Tentagel™ S NH_2 amino methyl resin were obtained from Rapp Polymere GmbH. The coupling reagent 1H-benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) was obtained from Richelieu Biotechnologies Inc. N^{α}-9-Fluorenylmethyloxycarbonyl (Fmoc) amino acids were obtained from MultiSynTech. Peptide grade dichloromethane (DCM), *t*-butyl methylether (MTBE), *N*-methylpyrrolidone (NMP), *N*,*N*-dimethylformamide (DMF), acetronitrile, hexane, trifluoroacetic acid (TFA) and HPLC grade TFA were purchased from Biosolve. Succinic anhydride, piperidine, *N*,*N*-diisopropylethylamine (D*i*PEA), triethylamine (TEA) and triisopropylsilane (T*i*S) were obtained from Acros Organics, acetic acid (HOAc) and isopropanol (*i*PrOH) were purchased from Merck KGaA.

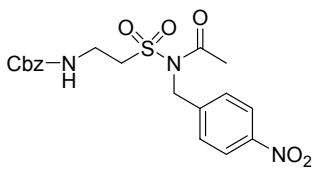
General procedure for the in solution alkylation of *N*-acyl sulfonamide 2a (Table 6.1):
Method A: To a solution of **2a** (1 equiv) and D*i*PEA, (2 equiv) in DMF, the electrophile (1 equiv) was added. The reaction mixture was stirred for 16 h at rt and subsequently concentrated to dryness. The product was isolated by column chromatography.
Method B: To a solution of **2a** (1 equiv) and D*i*PEA, (2 equiv) in DMF, the electrophile (1 equiv) was added. The reaction mixture was heated at 150°C for 6 min by microwave irradiation and subsequently concentrated to dryness. The product was isolated by column chromatography.



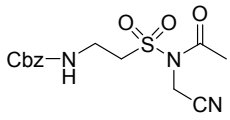
Compound 3a *Method A:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 26 µL allylbromide (0.30 mmol) in 5 mL DMF. Prior to column chromatography the crude product was dissolved in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL) and brine (1 × 50 mL). *Method B:* The reaction was carried out following the general procedure, using 45 mg of **2a** (0.15 mmol), 52 µL (0.30 mmol) of D*i*PEA and 13 µL allylbromide (0.15 mmol) in 5 mL DMF. Prior to column chromatography the crude product was dissolved in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL) and brine (1 × 50 mL) and dried (Na_2SO_4). The product (method A: 77 mg; 75 %; method B: 81% by NMR) was isolated by flash column chromatography (EtOAc/hexane 1:1 v/v) as a clear oil. R_f 0.27 (EtOAc/hexane, 1:1 v/v); R_t 16.95 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (m, 5H), 5.91-5.78 (m, 1H), 5.46 (m, 1H), 5.29 (m, 2H) 5.10 (s, 2H), 4.32 (d, J = 5.2 Hz, 2H), 3.65 (m, 4H), 2.28 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.5, 156.4, 136.4, 132.6, 128.8, 128.5, 128.4, 118.7, 67.3, 54.9, 49.0, 35.8, 24.4; ESMS calcd. for $C_{15}H_{20}N_2O_5S$ 340.11, found 363.40 $[M + Na]^+$.[2]



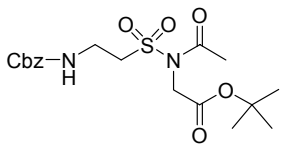
Compound 3b *Method B:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 36 µL benzylbromide (0.30 mmol) in 4 mL DMF. Prior to column chromatography the crude product was dissolved in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL) and brine (1 × 50 mL). The product (101 mg; 76%) was isolated by flash column chromatography (EtOAc/hexane 1:1 v/v) as a clear oil; R_f 0.23 (EtOAc/hexane, 1:1 v/v); R_t 17.12 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34 (m, 10H), 5.41 (m, 1H), 5.09 (s, 2H), 4.92 (s, 2H), 3.63-3.50 (m, 2H), 3.54-3.50 (m, 2H) 2.24 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 171.2, 156.1, 136.0, 128.9, 128.5, 128.2, 128.1, 128.0, 127.2, 67.0, 54.8, 49.6, 35.5, 24.6; ESMS calcd. for $C_{19}H_{22}N_2O_5S$ 390.12, found 413.25 $[M + Na]^+$.



Compound 3c *Method A:* The reaction was carried out following the general procedure, using 45 mg of **2a** (0.15 mmol), 52 µL (0.30 mmol) of D*i*PEA and 32 mg 4-nitrobenzylbromide (0.15 mmol) in 2 mL DMF. *Method B:* The reaction was carried out following the general procedure, using 45 mg of **2a** (0.15 mmol), 52 µL (0.30 mmol) of D*i*PEA and 32 mg 4-nitrobenzylbromide (0.15 mmol) in 2 mL DMF. The product (method A: 49 mg; 75 % by NMR; method B: 50 mg; 76%) was isolated by flash column chromatography (EtOAc/hexane, 1:1 v/v) as a white solid. R_f 0.35 (EtOAc/hexane, 1:1 v/v); R_t 17.47 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.21 (d, J = 8.3 Hz, 2H), 7.52 (d, J = 8.3 Hz, 2H), 7.35 (m, 5H), 5.30 (m, 1H), 5.11 (s, 2H), 4.98 (s, 2H), 3.71 (m, 2H), 3.60 (m, 2H), 2.33 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 156.1, 147.6, 143.5, 136.0, 128.6, 128.2, 124.0, 67.2, 54.7, 49.0, 35.6, 24.6; ESMS calcd. for $C_{19}H_{21}N_3O_7S$ 435.11, found 436.29 $[M+H]^+$, 458.68 $[M + Na]^+$.

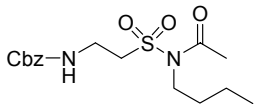


Compound 3d *Method A entry 4:* The reaction was carried out following the general procedure, using 45 mg of **2a** (0.15 mmol), 52 µL (0.30 mmol) of D*i*PEA and 11 µL bromoacetonitrile (0.15 mmol) in 2 mL DMF. *Method B entry 4:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 21 µL bromoacetonitrile (0.30 mmol) in 4 mL DMF. *Method A entry 5:* The reaction was carried out following the general procedure, using 45 mg of **2a** (0.15 mmol), 52 µL (0.30 mmol) of D*i*PEA and 11 µL iodoacetonitrile (0.30 mmol) in 2 mL DMF. *Method B entry 5:* The reaction was carried out following the general procedure, using 45 mg of **2a** (0.15 mmol), 52 µL (0.30 mmol) of D*i*PEA and 11 µL iodoacetonitrile (0.15 mmol) in 2 mL DMF. The product (*Method A entry 4:* 27 mg, 53%; *Method A entry 5:* 28 mg, 55%; *Method B entry 4:* 32 mg, 31%; *Method B entry 5:* 14 mg, 27%) was isolated by preparative HPLC as a white solid. R_f 0.60 (EtOAc/hexane/HOAc, 4:1:0.05 v/v/v); R_t.15.75 (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.36 (m, 5H), 5.39 (m, 1H), 5.11 (s, 2H), 4.60 (s, 2H), 3.74 (m, 2H), 3.66 (m, 2H), 2.45 (s, 3H); 13C NMR (75 MHz, $CDCl_3$): δ 169.1, 156.2, 135.9, 128.6, 128.4, 128.2, 114.8, 67.2, 54.7, 35.4, 32.9, 24.2; ESMS calcd. for $C_{14}H_{17}N_3O_5S$ 339,09, found 340.13 $[M+H]^+$, 362.50 $[M + Na]^+$.

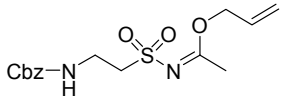


Compound 3e *Method A:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 44 µL *t*-butyl bromoacetate (0.30 mmol) in 4 mL DMF. Prior to column chromatography the crude

product was dissolved in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL) and brine (1 × 50 mL). *Method B:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 44 µL *t*-butyl bromoacetate (0.30 mmol) in 5 mL DMF. Prior to column chromatography the crude product was dissolved in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL) and brine (1 × 50 mL). The product (*Method A:* 77 mg; 94 %; *Method B:* 77% by NMR) was isolated by flash column chromatography (EtOAc/hexane, 1:1 v/v) as a clear oil. R_f 0.40 (EtOAc/hexane, 1:1 v/v); R_t 18.58 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.34 (m, 5H), 5.77 (m, 1H), 5.10 (s, 2H), 4.38 (s, 2H), 3.75 (m, 4H), 2.30 (m, 3H), 1.47 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 170.7, 168.1, 156.5, 136.5, 128.8, 128.4, 128.3, 83.7, 67.2, 55.1, 47.9, 35.8, 28.2, 24.3. ESMS calcd. for $C_{18}H_{26}N_2O_7S$ 414.15, found 437.25 $[M + Na]^+$.[2]



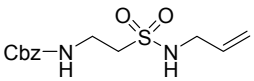
Compound 3f *Method B:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 32 µL butylbromide (0.30 mmol) in 4 mL DMF with a heating time of 3 x 5 min. The product (32 mg; 30 %) was isolated by flash column chromatography (EtOAc/hexane, 1:1 v/v) as a clear oil. R_f 0.39 (EtOAc/hexane, 1:1 v/v); R_t 16.83 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (m, 5H), 5.39 (m, 1H), 5.11 (s, 2H), 3.73-3.58 (m, 6H), 2.31 (s, 3H), 1.69-1.59 (m, 2H), 1.38-1.26 (m, 2H), 0.94 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.9, 156.1, 136.1, 128.5, 128.2, 67.0, 54.5, 46.9, 35.6, 32.3, 24.30, 19.9, 13.5; ESMS calcd. for $C_{16}H_{24}N_2O_5S$ 356.14, found 379.30 $[M + Na]^+$.



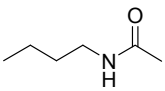
Compound 4a *Method B:* The reaction was carried out following the general procedure, using 90 mg of **2a** (0.30 mmol), 105 µL (0.60 mmol) of D*i*PEA and 26 µL allylbromide (0.30 mmol) in 5 mL DMF. Prior to column chromatography the crude product was dissolved in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL) and brine (1 × 50 mL). The product (18 mg; 18%) was isolated by flash column chromatography (EtOAc/hexane 1:1 v/v) as a clear oil. R_f 0.64 (EtOAc/hexane, 4:1 v/v); R_t 17.47 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (m, 5H), 5.95-5.85 (m, 1H), 5.56 (m, 1H), 5.36-5.28 (m, 2H) 5.10 (s, 2H), 4.62 (d, J = 5.8 Hz, 2H), 3.74 (m, 2H), 3.29 (m, 2H), 2.48 (s, 3H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 174.1, 156.1, 136.2, 130.5, 128.5, 128.2, 128.1, 119.7, 69.2, 66.9, 54.3, 35.9, 20.8; ESMS calcd. for $C_{15}H_{20}N_2O_5S$ 340.11, found 363.25 $[M + Na]^+$.

Nucleophilic displacement test reaction of alkylated *N*-acyl sulfonamide 3a

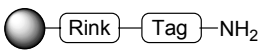
A solution of **3a** (64 mg; 0.19 mmol) and *n*-butylamine (74 µL; 0.76 mmol) in DMF (2 mL) was stirred for 16 h at rt. The reaction progress was monitored by HPLC analysis and samples were taken after 1, 2, 3 and 15 h. After removal of the solvent the products were isolated by preparative reverse phase HPLC.



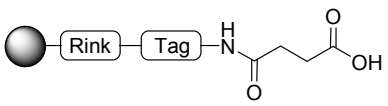
Compound 5a: The product (38 mg, 67%) was isolated as a white solid. R_t 16.35 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 7.35 (m, 5H), 5.83 (m, 1H), 5.43 (s, 1H), 5.30-5.20 (m, 2H), 5.11 (s, 2H) 4.62 (s, 1H), 3.73 (s, 2H), 3.67 (s, 2H), 3.22 (s, 2H).



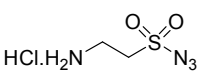
Compound 6: The product (16 mg, 75%) was isolated as a colorless oil. R_t 10.80 min (column B); ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.81 (s, 1H), 3.27 (m, 2H), 2.02 (s, 3H), 1.50 (m, 2H), 1.36 (m, 2H), 0.93 (m, 3H).



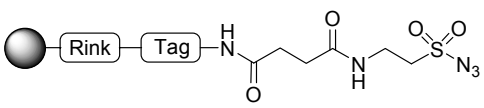
Compound 7: The peptide sequence H-Lys(Boc)-Ser(^{t}Bu)-Tyr(^{t}Bu)-NH-Rink Amide Tentagel™ was synthesized manually using standard Fmoc/^{t}Bu SPPS protocols with BOP and D*i*PEA as coupling reagents. The efficiency of each deprotection and coupling step was evaluated by a quantitative Kaiser test.[52]



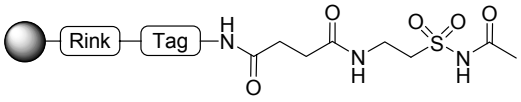
Compound 8: To resin bound-tripeptide **7** (~0.44 mmol) in DCM (10 mL), succinic anhydride (220 mg; 2.2 mmol) and TEA (457 µL; 3.3 mmol) were added and the obtained reaction mixture was gently shaken for 16 h. Completion of the reaction was confirmed by a negative Kaiser test[52] and a positive Malachite green test.[53] The resin was subsequently washed with DCM (10 mL; 3 × 2 min), *i*-PrOH (10 mL; 1 × 2 min) and DCM (10 mL; 3 × 2 min). For analysis purposes a small portion of resin **8** was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 1 mL) and shaken for 3h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 10 mL) to precipitate the **8'**. Subsequently **8'** was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. ESMS calcd. for $C_{22}H_{33}N_5O_8$ 495.23, found 496.30 $[M + H]^+$.



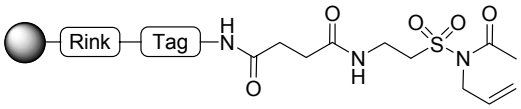
HCl.H-Gly-Ψ[CH_2SO_2]-N_3: The synthetic procedure and analytical data for this compound have been described in chapter 5.



Compound 9: To acid functionalized resin **8** (~0.44 mmol) in NMP (10 mL), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (99 mg; 0.53 mmol), BOP (778 mg; 1.76 mmol) and D*i*PEA (0.61 mL; 3.52 mmol) were added and the obtained reaction mixture was gently shaken for 16 h. The resin was subsequently washed with NMP (10 mL; 3 × 2 min), *i*-PrOH (10 mL; 1 × 2 min) and DCM (10 mL; 3 × 2 min). For analysis purposes a small portion of resin **9** was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 1 mL) and shaken for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 10 mL) to precipitate **9'**. Subsequently **9'** was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. ESMS calcd. for $C_{24}H_{37}N_9O_9S$ 627.24, found 628.50 $[M + H]^+$.

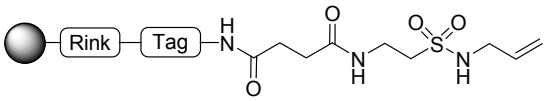


Compound 10: To the sulfonyl azide functionalized resin **9** (~0.22 mmol) in $CHCl_3$ (5 mL), 2,6-lutidine (1.54 mL; 13.2 mmol) and thioacetic acid (47 µL; 0.66 mmol) were added and the obtained reaction mixture was gently shaken for 1.5 h. The resin was subsequently washed with $CHCl_3$ (5 mL; 3 × 2 min), *i*-PrOH (5 mL; 1 × 2 min) and DCM (5 mL; 3 × 2 min). For analysis purposes a small portion of resin **10a** was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 1 mL) and stirred for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 10 mL) to precipitate **10'**. Subsequently **10'** was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. ESMS calcd. for $C_{26}H_{41}N_7O_{10}S$ 643.26, found 644.45 $[M + H]^+$.

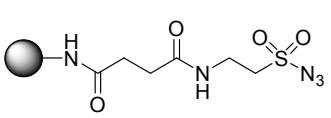


Compound 11, *conditions d:* To the *N*-acyl sulfonamide functionalized resin **10** (~83 µmol) in DMF (2 mL), D*i*PEA (30 µL; 0.17 mmol) and allylbromide (8 µL; 90.7 µmol) were added and the obtained reaction mixture was gently shaken for 16 h. The resin was subsequently washed with DMF (2 mL; 3 × 2 min), *i*-PrOH (2 mL; 1 × 2 min) and DCM (2 mL; 3 × 2 min). For analysis purposes a small portion of resin **11** was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 1 mL) and stirred for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 10 mL) to precipitate **11'**. Subsequently **11'** was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. ESMS calcd. for $C_{29}H_{45}N_7O_{10}S$ 683.29, found 684.50 $[M + H]^+$. *Conditions e:* To the *N*-acyl sulfonamide functionalized resin **10** (~82.5 µmol) in DMF (2 mL), D*i*PEA (30 µL; 0.17 mmol) and allylbromide (8 µL; 90.7 µmol) were added and the obtained reaction mixture was heated at 150°C for 6 min in a microwave reactor. The resin was subsequently washed with DMF (2 mL; 3 × 2 min), *i*-PrOH (2 mL; 1 × 2 min) and DCM (2 mL; 3 × 2

min). For analysis purposes a small portion of resin **11** was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 1 mL) and stirred for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 10 mL) to precipitate **11'**. Subsequently **11'** was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. ESMS calcd. for $C_{29}H_{45}N_7O_{10}S$ 683.29, found 684.55 $[M + H]^+$.



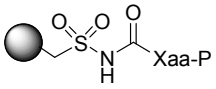
Compound 12: To the *N*-alkylated resin **11** (~13 µmol) in $CHCl_3$ (0.5 mL), *n*-butylamine (26 µL; 0.66 mmol) was added and the obtained reaction mixture was gently shaken for 3.5 h. The resin was subsequently washed with $CHCl_3$ (5 mL; 3 × 2 min), *i*-PrOH (5 mL; 1 × 2 min) and DCM (5 mL; 3 × 2 min). For analysis purposes a small portion of resin **12** was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 1 mL) and stirred for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 10 mL) to precipitate **12'**. Subsequently **12'** was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. ESMS calcd. for $C_{27}H_{43}N_7O_9S$ 641.28, found 642.50 $[M + H]^+$.



Resin-bound sulfonyl azide C: A portion of Tentagel™ S NH_2 amino methyl resin (3 g; ~0.8 mmol) was allowed to swell in NMP for 1 h. Subsequently, the resin was washed with 50% HOAc in NMP (10 mL; 2 × 10 min), NMP (10 mL; 2 × 2 min), *i*-PrOH (10 mL; 1 × 2 min), NMP (10 mL; 2 × 2 min), 20% D*i*PEA in NMP (10 mL; 1 × 2 min), NMP (10 mL; 3 × 2 min), *i*PrOH (10 mL; 1 × 2 min) and finally NMP (10 mL; 3 × 2 min). A solution of succinic anhydride (801 mg; 8 mmol) and D*i*PEA (1.4 mL; 8 mmol) in NMP (10 mL) was added to the resin and the resulting mixture was shaken for 1 h before the resin was filtered and washed with NMP (10 mL; 3 × 2 min), *i*-PrOH (10 mL; 1 × 2 min) and NMP (10 mL; 3 × 2 min). Completion of the reaction was confirmed by a negative Kaiser test[52] and a positive Malachite green test[53] for the acid functionalized resin. Then, a solution of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (597 mg; 3.2 mmol), BOP (1.4 g; 3.2 mmol) and D*i*PEA (1.1 mL; 6.4 mmol) in NMP (10 mL) was added to the resin, and the obtained reaction mixture was shaken for 16 h. Finally, the resin was filtered and washed with NMP (10 mL; 3 × 2 min), *i*-PrOH (10 mL; 1 × 2 min) and DCM (10 mL; 3 × 2 min) and dried under vacuum. The sulfonyl azide functionalized resin **C** was stored for further use.

General procedure for the synthesis of N^{α}-protected amino thioa cids P-Xaa-SH

An N^{α}-Fmoc- or N^{α}-Boc-protected amino acid (1 equiv; 0.20 mmol), BOP (1.1 equiv) and D*i*PEA (1.5 equiv) in *i*-PrOH (10 mL) was stirred for 10 min at room temperature before a solution of $NaHS.xH_2O$ (4 equiv) in *i*-PrOH (10 mL) was added. The progress of the reaction was monitored by TLC-analysis (eluent: EtOAc/hexane/HOAc, 9:1:0.1 v/v/v). After 15 min of stirring the reaction was quenched[80] with 1N $KHSO_4$ (20 mL) and the reaction mixture was concentrated to a volume of 20 mL. The residue was extracted with EtOAc (1 × 40 mL) and subsequently washed with 1N $KHSO_4$ (2 × 20 mL) and brine (1 × 20 mL) and dried (Na_2SO_4). After removal of the solvent, the crude reaction product was immediately used for coupling to the resin-bound sulfonyl azide **C**.

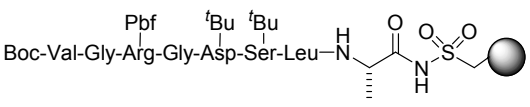


Compound 14: A portion of dried sulfonyl azide functionalized resin **C** (1 equiv; ~50 µmol) was allowed to swell in DMF (1 mL) for 1 h before the solvent was drained. To the swollen resin, 2,6-lutidine (80 equiv) and the N^{α}-protected amino thio acid (4 equiv) in DMF (800 µL) were added and the obtained reaction mixture was gently shaken for 3-6 h. The resin was subsequently washed with DMF (1mL; 3 × 2 min), *i*-PrOH (1mL; 1 × 2 min) and DCM (1mL; 4 × 2 min). In case of loading with an N^{α}-Fmoc-protected amino thio acid the resin was dried under high vacuum overnight before determination of the amino acid loading, in case of loading with an N^{α}-Boc-protected amino thio acid the Boc group was cleaved to enable coupling of the next amino acid residue.

General procedure for quantification of the *N*-acyl sulfonamide resin

A small portion of resin (~5 mg), loaded with Fmoc-protected amino acid/peptide was weighted into a 20 mL volumetric flask and a 20% piperidine in NMP solution (2 mL) was added. The solution was gently shaken for 30 min before methanol was added. The absorbance (*A*) was measured at λ=300 nm. With this value the loading (*f'*) of the resin, corrected for the added mass (Mw_{added}), was calculated using the formulas[55] listed below.

$$f' = \frac{1000 * f}{1000 - (f * \mathrm{Mw}_{\mathrm{added}})} \qquad f = \frac{1.28 * A * 2}{\#\,\mathrm{mg\ resin}}$$



Compound 15: A portion of *N*-acyl sulfonamide resin **14** (1 equiv; ~0.8 mmol) which was loaded with Boc-Ala-SH, was allowed to swell in DMF (10 mL) for 1 h before the solvent was drained. Then, the Boc

group was removed by acidolysis with a mixture of TFA/DCM/T*i*S/H_2O (5:5:0.1:0.1 v/v/v/v, 10 mL; 3 × 10 min) and the resin was washed with DCM (10 mL; 1 × 2 min), NMP (10 mL; 1 × 2 min), 20% D*i*PEA in NMP (10 mL; 1 × 2 min), NMP (10 mL; 1 × 2 min), *i*-PrOH (10 mL; 1 × 2 min) and NMP (10 mL; 3 × 2 min). Subsequently, the α-amino functionality was acylated with Fmoc-Leu-OH in the presence of BOP/D*i*PEA as the condensing agent. The remaining amino acid residues were coupled using standard Fmoc/^{t}Bu solid phase peptide synthesis protocols using BOP (4 equiv) and DIPEA (8 equiv) as coupling reagents. All amino acids (4 equiv) were Fmoc-protected except for the final amino acid residue (valine) which was introduced as the corresponding Boc derivative.

General procedure for the alkylation of *N*-peptidyl sulfonamide resin 15 by bromides

A portion of *N*-peptidyl sulfonamide resin **15** (1 equiv; 15 μmol) was transferred to a microwave vessel and swollen in DMF by gentle agitation (1 mL) for 1 h. To this suspension the electrophile (20 equiv) and D*i*PEA (10 equiv) were added. The vessel was capped and heated for 6 min at 150°C by microwave irradiation. The solvent was removed by filtration and the resin was washed with DMF (1 mL; 3 × 2 min), Et_2O (1 mL; 1 × 2 min) and DMF (1 mL; 3 × 2 min). The alkylated resin **16** was either used immediately or washed with DCM (1 mL; 3 × 2 min), dried and stored for further use.

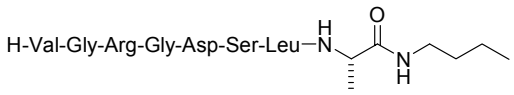
Procedure for alkylation of *N*-peptidyl sulfonamide resin 15 by $TMSCHN_2$[28]

A portion of peptide *N*-peptidyl sulfonamide resin **15** (150 mg; 22.5 μmol) was allowed to swell in THF (4 mL) for 1 h. To this suspension $TMSCHN_2$ (2M solution in Et_2O; 4 mL, ~350 equiv) was added. The resulting mixture was shaken for 2 h before the solvent was drained. The resin was subsequently washed with THF (10 mL; 5 × 2 min) and DMF (10 mL; 3 × 2 min). The alkylated resin **16e** was either used immediately or washed with DCM (1 mL; 3 × 2 min), dried and stored for further use.

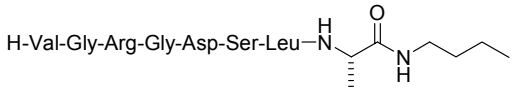
General procedure for cleavage of alkylated *N*-peptidyl resin 16 with amine nucleophiles

A portion of alkylated *N*-acyl sulfonamide resin 5 (1 equiv; ~15 μmol) was allowed to swell in DMF (0.8 mL) for 1 h. To this suspension the amine nucleophile (10 equiv) and D*i*PEA (10 equiv) were added. The mixture was shaken for 24 h at room temperature before the resin was filtered and washed with DMF (5 × 1 mL). The combined DMF washings were evaporated under high vacuum. The crude peptide was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 2 mL) and stirred for 3 h. The TFA

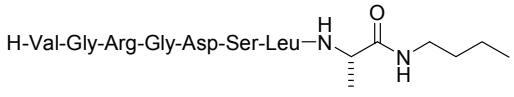
mixture was diluted with MTBE/hexane (1:1 v/v, 2 × 10 mL) and the crude peptide was collected by centrifugation. Subsequently, the product was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. The identity and purity were determined by ESMS, HPLC-MS and quantitative HPLC (column A).



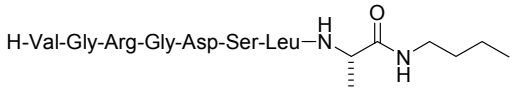
Compound 17a via 16a: Alkylation was carried out following the general procedure, using 100 mg (15 μmol) of *N*-peptidyl sulfonamide resin **15**, D*i*PEA (26 μL; 0.15 mmol) and allylbromide (26 μL; 0.30 mmol). The subsequent cleavage was carried out following the general procedure, using *n*-butylamine (15 μL; 0.15 mmol) and D*i*PEA (26 μL; 0.15 mmol). The product (7 mg, 52%) was obtained as a white solid. R_t 11.53 min (72% purity); ESMS calcd. for $C_{35}H_{64}N_{12}O_{11}$ 828.48, found 829.45 $[M+H]^+$.



Compound 17a via 16b: Alkylation was carried out following the general procedure, using 100 mg (15 μmol) of *N*-peptidyl sulfonamide resin **15**, D*i*PEA (26 μL; 0.15 mmol) and 4-nitrobenzylbromide (65 mg; 0.30 mmol). The subsequent cleavage was carried out following the general procedure, using *n*-butylamine (15 μL; 0.15 mmol) and D*i*PEA (26 μL; 0.15 mmol). The product (8 mg, 68%) was obtained as a white solid. R_t 11.51 min (86% purity); ESMS calcd. for $C_{35}H_{64}N_{12}O_{11}$ 828.48, found 829.30 $[M+H]^+$.

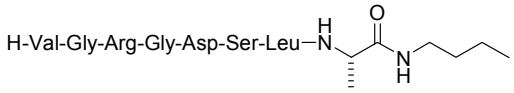


Compound 17a via 16c, entry 3: Alkylation was carried out following the general procedure, using 100 mg (15 μmol) of *N*-peptidyl sulfonamide resin **15**, D*i*PEA (26 μL; 0.15 mmol) and *t*-butyl bromoacetate (44 μL; 0.30 mmol). The subsequent cleavage was carried out following the general procedure, using *n*-butylamine (15 μL; 0.15 mmol) and D*i*PEA (26 μL; 0.15 mmol). The product (6 mg, 48%) was obtained as a white solid. R_t 11.51 min (84% purity); ESMS calcd. for $C_{35}H_{64}N_{12}O_{11}$ 828.48, found 829.25 $[M+H]^+$.

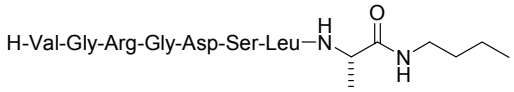


Compound 17a via 16c, entry 4: Alkylation was carried out following the general procedure using 100 mg (15 μmol) of *N*-peptidyl sulfonamide resin **15**, D*i*PEA (26 μL; 0.15 mmol) and *t*-butyl bromoacetate (44 μL; 0.30 mmol), only

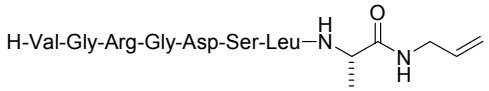
this time the reaction mixture was gently agitated for 16 h at rt. The subsequent cleavage was carried out following the general procedure, using *n*-butylamine (15 µL; 0.15 mmol) and D*i*PEA (26 µL; 0.15 mmol). The product (5 mg, 37%) was obtained as a white solid. R_t 11.52 min (100% purity); ESMS calcd. for $C_{35}H_{64}N_{12}O_{11}$ 828.48, found 829.50 $[M+H]^+$.



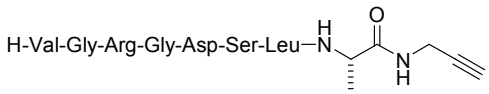
Compound 17a via 16d: Alkylation was carried out following the general procedure, using 100 mg (15 µmol) of *N*-peptidyl sulfonamide resin **15**, D*i*PEA (26 µL; 0.15 mmol) and methyl bromoacetate (28 µL; 0.30 mmol). The subsequent cleavage was carried out following the general procedure, using *n*-butylamine (15 µL; 0.15 mmol) and D*i*PEA (26 µL; 0.15 mmol). The product (8 mg, 66%) was obtained as a white solid. R_t 11.52 min (84% purity); ESMS calcd. for $C_{35}H_{64}N_{12}O_{11}$ 828.48, found 829.40 $[M+H]^+$.



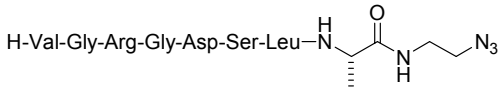
Compound 17a via 16e: Alkylation was carried out following the general procedure using 150 mg (22.5 µmol) of *N*-peptidyl sulfonamide resin **15** and $TMSCHN_2$ (~350 equiv). The subsequent cleavage was carried out following the general procedure, using *n*-butylamine (22 µL; 0.23 mmol) and D*i*PEA (39 µL; 0.23 mmol). The product (17 mg, 89%) was obtained as a white solid. R_t 11.58 min (74% purity); ESMS calcd. for $C_{35}H_{64}N_{12}O_{11}$ 828.48; found 829.50 $[M+H]^+$.



Compound 17b: The reaction was carried out following the general procedure, using 150 mg (22.5 µmol) of *N*-peptidyl sulfonamide resin **16b**, allylamine (17 µL; 225 µmol) and D*i*PEA (39 µL; 225 µmol). The product (11 mg; 57%) was obtained as a white solid. R_t 10.57 min (82% purity); ESMS calcd. for $C_{34}H_{60}N_{12}O_{11}$ 812.45, found 813.45 $[M+H]^+$.

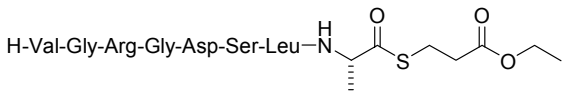


Compound 17c: The reaction was carried out following the general procedure, using 150 mg (22.5 µmol) of *N*-peptidyl sulfonamide resin **16b**, propargylamine hydrochloride (21 mg; 225 µmol) and D*i*PEA (39 µL, 225 µmol). The product (11 mg; 59%) was obtained as a white solid. R_t 10.43 min (81% purity); ESMS calcd. for $C_{34}H_{58}N_{12}O_{11}$ 810.43, found 811.40 $[M+H]^+$.

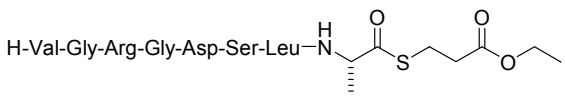


Compound 17d: The reaction was carried out following the general procedure, using 150 mg (22.5 µmol) of *N*-peptidyl sulfonamide resin **16b**, 2-azidoethylamine hydrochloride (28 mg; 225 µmol) and D*i*PEA (39 µL, 225 µmol). The product (18 mg, 96%) was obtained as a white solid. R_t 10.71 min (83% purity); ESMS calcd. for $C_{33}H_{59}N_{15}O_{11}$ 841.45, found 842.70 $[M+H]^+$.

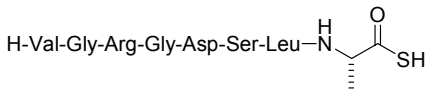
General procedure for cleavage of alkylated *N*-peptidyl resin 16b by thiol nucleophiles
A portion of alkylated *N*-peptidyl sulfonamide resin **16b** (1 equiv; 22.5 µmol) was allowed to swell in DMF (0.8 mL) for 1 h. To this suspension the thiol nucleophile (50 equiv), D*i*PEA (10 equiv) and sodium thiophenolate (in selected cases; 0.5 equiv) were added. The mixture was shaken for 24 h at room temperature before the resin was filtered and washed with DMF (5 × 1 mL). The combined DMF washings were evaporated under high vacuum. The crude peptide was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v, 2 mL) and stirred for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 2 × 10 mL) to precipitate the product. Subsequently, the crude peptide was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. The identity and purity were determined by ESMS, HPLC-MS and quantitative HPLC (column A).



Compound 17f, entry 5: The reaction was carried out following the general procedure, using 150 mg (22.5 µmol) *N*-peptidyl sulfonamide resin **16b**, ethyl 3-mercaptopropionate (147 µL; 1.13 mmol), D*i*PEA (39 µL, 225 µmol) and sodium thiophenolate (1.5 mg; 11.3 µmol). The product (9 mg; 46%) was obtained as a white solid. R_t 12.71 min (92% purity); ESMS calcd. for $C_{36}H_{63}N_{11}O_{13}S$ 889.43, found 890.35 $[M+H]^+$.



Compound 17f, entry 6: The reaction was carried out following the general procedure, using 150 mg (22.5 µmol) *N*-peptidyl sulfonamide resin **16b**, ethyl 3-mercaptopropionate (147 µL; 1.13 mmol) and D*i*PEA (39 µl; 225 µmol) without the addition of sodium thiophenolate. The product (11 mg; 52%) was obtained as a white solid. R_t 12.63 min (92% purity); ESMS calcd. for $C_{36}H_{63}N_{11}O_{13}S$ 889.43, found 889.85 $[M+H]^+$.



Compound 17h: A batch of alkylated *N*-acyl sulfonamide resin **16b** (150 mg; 22.5 µmol) was allowed to swell in DMF (0.8 mL) for 1 h. The resin was filtered and added to a solution of H_2S.D*i*PEA in dioxane (1 mL; 1.3 mmol). The mixture was shaken for 24 h at room temperature before the resin was filtered and washed with DMF (5 × 1 mL). The combined DMF washings were evaporated under high vacuum. The crude peptide was dissolved in a mixture of TFA, H_2O and T*i*S (9.5:0.25:0.25 v/v/v; 2 mL) and stirred for 3 h. The TFA mixture was diluted with MTBE/hexane (1:1 v/v, 2 × 10 mL) to precipitate the product. Subsequently the product was redissolved in CH_3CN/H_2O (1:1 v/v) and lyophilized. The product (6 mg; 33%) was obtained as a white solid. R_t 10.87 min (66% purity); ESMS calcd. for for $C_{31}H_{55}N_{11}O_{11}S$ 789.38, found 790.45 $[M+H]^+$ (thio acid); ESMS calcd. for $C_{31}H_{55}N_{11}O_{12}$ 773.40, found 773.02 (oxy acid).

6.5 References and Notes

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80. CAUTION: on acidification of the reaction mixture highly toxic hydrogen sulfide evolves, this procedure should be performed in a well ventilated hood

7

Synthesis and Evaluation of Peptide-based *N*-Acyl Sulfonamides as a New Class of Low Molecular Weight Organo/Hydrogelators

In this chapter the spontaneous and irreversible coupling of thio acids and sulfonyl azides was used for the fast and efficient synthesis of a focussed series of peptide-based *N*-acyl sulfonamides with interesting physicochemical properties. Starting from amino acid-derived thio acids and sulfonyl azides that differ in the ability to form non-covalent interactions, a peptide-based gelator could be obtained which was capable of forming a pH-responsive gel in aqueous solution at low concentration.

Manuscript in preparation.

7.1 Introduction

Reliable and efficient methods for the spontaneous and irreversible coupling of chemically diverse building blocks are of high interest for the generation of collections of compounds. They may, for instance, facilitate the process of structure based design and combinatorial chemistry in drug discovery. In this chapter the highly efficient thio acid/sulfonyl azide amidation reaction, which resembles the properties of the "ideal click reaction" is applied to the synthesis of a focussed series of peptide-based *N*-acyl sulfonamides with interesting physicochemical properties starting from amino acid derived building blocks.

7.1.1 Low molecular weight gelators

Over the last years low molecular weight gelators (LMWGs)[1] have received increased attention,[2,3] the reasons for this are twofold. On one hand, the supramolecular self-assembly of LMWGs into three-dimensional continuous networks in organic fluids or water, represents a powerful bottom-up fabrication approach for the synthesis of supramolecular devices and highly tunable nanomaterials. On the other hand, gels form a unique class of materials since they form stable structures that can retain their macroscopic shape, a property characteristic of the solid state of matter, even though they consist for the greater part of liquid. These special features make gels promising materials for wide applications in industrial fields such as cosmetics, foods, medical science and tissue engineering.[4]

Gels are highly complex systems and, in spite of the huge number of investigations, there is no simple definition of the gel state. In their paper, Weiss *et al.*[5] explain the concept as follows: "a gel has a continuous structure with macroscopic dimensions that is permanent on the time scale of an analytical experiment and is solid-like in its rheological behavior below a certain stress limit. It is viscoelastic and comprised of an organic liquid (in organogels) or water (in hydrogels) as the major component and a low concentration of an immobilizing agent, a gelator." As implied, any gel must develop a three dimensional network that permeates its volume and remains stable within specific ranges of concentration and temperature.

It is well known that many polymers, synthetic and natural, form gels. This quality largely stems from the tendency of long-chain macromolecules to give rise to networks capable of immobilization of the liquid component by surface tension. By contrast, LMWGs are clearly defined molecular structures and it is their unidirectional self-assembly that serves to build non-covalent networks of entangled fiber-like aggregates.

In the process of gel formation, the following general steps can be identified (Figure 7.1).[6] At elevated temperatures, the intermolecular interactions, which form the driving forces for aggregation, are overcome and the gelator molecules are completely dissolved (a). Upon cooling the gelator molecules self-assemble (b). Assembly is favored in one direction when it originates from intermolecular interactions that are directionally dependent. This anisotropy leads to the formation of fibers (c). Over time more fibers grow and interact with each other to form a 3D-network determining the gel structure (d). In case the gel is formed by non-covalent interaction, the described process of gel formation is thermoreversible.

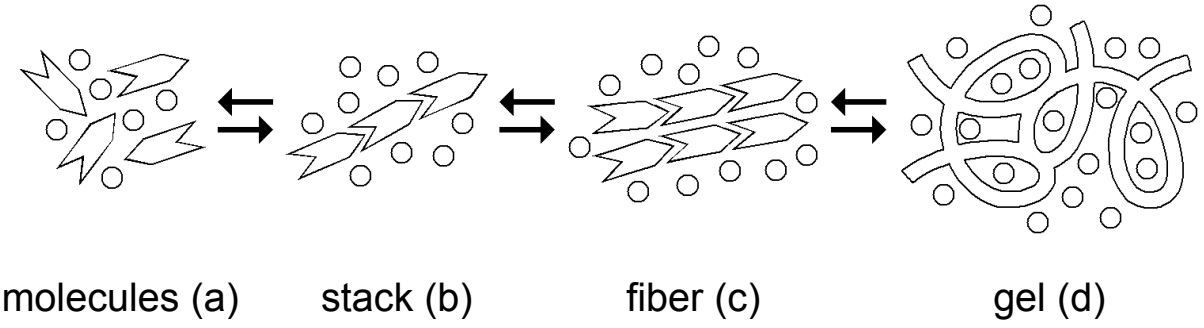


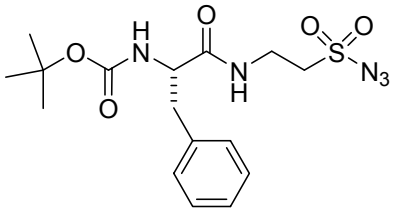
Figure 7.1 Schematic representation of the gel formation process (O = solvent).

The main forces responsible for the non-covalent intermolecular interactions between gelator molecules include hydrogen-bonding, polar- and van der Waals interactions and π–π stacking. Although numerous gelators have been described in the literature, featuring a large structural diversity, they can be classified into two categories according to the difference in the driving force for molecular aggregation: hydrogen-bond-based gelators and nonhydrogen-bond-based gelators. In the former group amide and urea moieties are frequently used to introduce hydrogen bonding within organogelators, in the latter group, typically cholesterol or fatty acid derivatives are introduced to obtain hydrogelators.

7.2 Results and discussion

A major attraction of the supramolecular approach toward gel-phase materials is the possibility to exquisitely control network properties and morphology by precise variations of the LMWG chemical structure. However, only few examples have been reported in which the gelating properties are the result of rational design rather than serendipity.[7]

With this respect, the discovery of the organogelating properties of peptido-aminoethane sulfonyl azide **1** was no exception. After its synthesis (chapter 5), the crude product was purified by crystallization from EtOAc/hexane. However, when hexane was added to a solution of **1** in EtOAc, the solution readily turned into a gel that stayed in position even after the flask was held upside down. The gelation occurred at a concentration of 6 mg/mL (16 mM). At lower concentrations **1** was still able to form a gel, only this time, not all liquid was trapped by the gelator and a lumpy mixture instead of a firm gel, was formed after addition of hexane.



1

Figure 7.2 Chemical structure of peptido-aminoethane sulfonyl azide **1**.

Most organogels are achieved through the ordered arrangement of gelators during the cooling of a mixture of low molecular mass organogelators (LMOGs) and organic solvents after heating to form a homogeneous solution.[2,8,9] However, a solvent-solvent gel preparation procedure distinguishes **1** from most other LMOGs. To our knowledge, hardly any examples of such a compound have been described in literature.[10]

In a typical example, **1** was dissolved in ethylacetate (2 mg in 20 μL), subsequently hexane (300 μL) was added upon which the clear solution immediately became viscous. The morphology of the obtained gel was investigated by transmission electron microscopy (TEM) and it turned out that the gel consisted of a network of intertwined fibers, characteristic for organogels.[7] More interestingly, also the formation of supramolecular assemblies such as helical ribbons and nanotubes[11,12] could be detected (Figure 7.3). Notably, the observed assemblies for **1** resembled the structures that previously have been described for amyloidogenic amylin(20-29)-derived peptides by our group.[13,14] In contrast, here the structures were obtained using a relatively simple dipeptide mimic instead of more complex decapeptides.

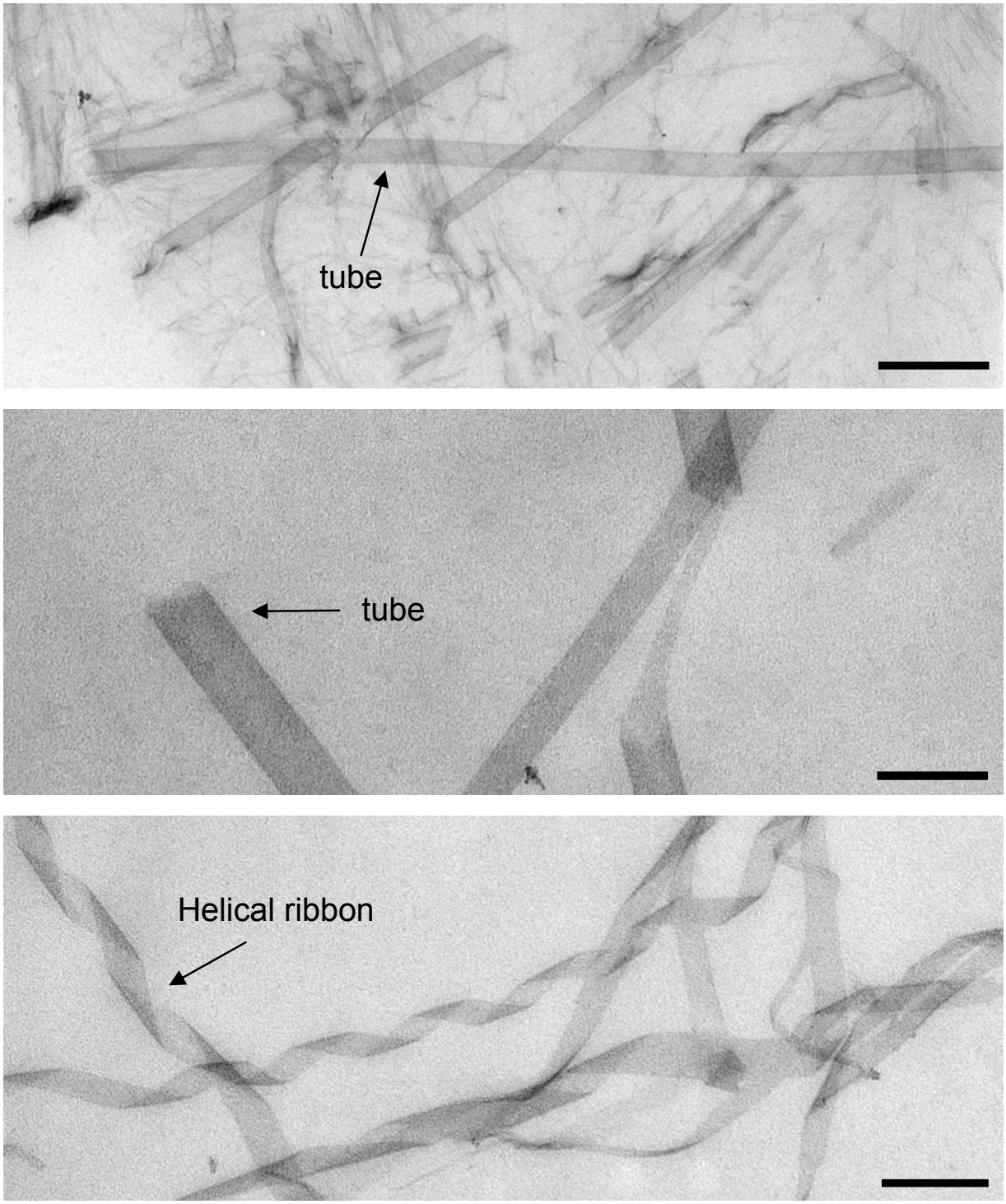
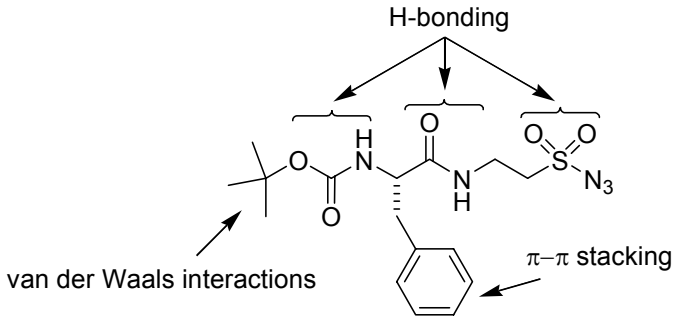


Figure 7.3 TEM images of gel of **1** in EtOAc/hexane. Top: network of intertwined fibers and tubes. Middle: tubes. Bottom: helical ribbons. Scale bar: 500 nm (top), 200 nm (middle and bottom).

The gelation process for **1** was found to be thermoreversible, which proved that the self-assembly originated from non-covalent interactions. Interestingly, the molecular chirality within the gelator monomer induced a supramolecular handedness of the helical ribbons. Ultimately the helical ribbons lead to the formation of nanotubes rather than fibers. These observations demonstrate the direct relationship between the chemical structure of the gelator molecule and the morphology of the resulting supramolecular assemblies.
Even though a better understanding of the mechanisms leading to gelation has made the development of new gelators feasible, detailed rational design of its macromolecular physiochemical properties remains a challenge. To gain a better insight into the process of gelation, the relationship between gelator structure and properties of the resulting gel can be studied via structural variation.



1

Figure 7.4 Chemical structure of peptido-aminoethane sulfonyl azide **1**. The arrows indicate positions for non-covalent interactions.

The chemical structure of **1** (Figure 7.4) displays several motifs capable of non-covalent interaction via, for instance, hydrogen bonding, van der Waals interactions and π–π stacking. Obviously, hydrogen-bonding is expected to play a predominant role in the molecular recognition in organic solvents.
When the physicochemical properties properties of analogs **2** and **3** (Figure 7.5) were investigated, it was found they also behaved as organogelators, but only under respectively basic and acidic conditions. Although capable of forming a gel in organic solvent, **2** and **3** were less potent gelators than **1** since, in contrast to the latter example, not all the liquid was captured at the same concentration. In contrast to sulfonyl azide **4**, compound **5** did not display any gelation in EtOAc/hexane mixtures. Therefore, it was assumed that next to hydrogen bonding, also π–π stacking played an important role in the self-assembly of these compounds.

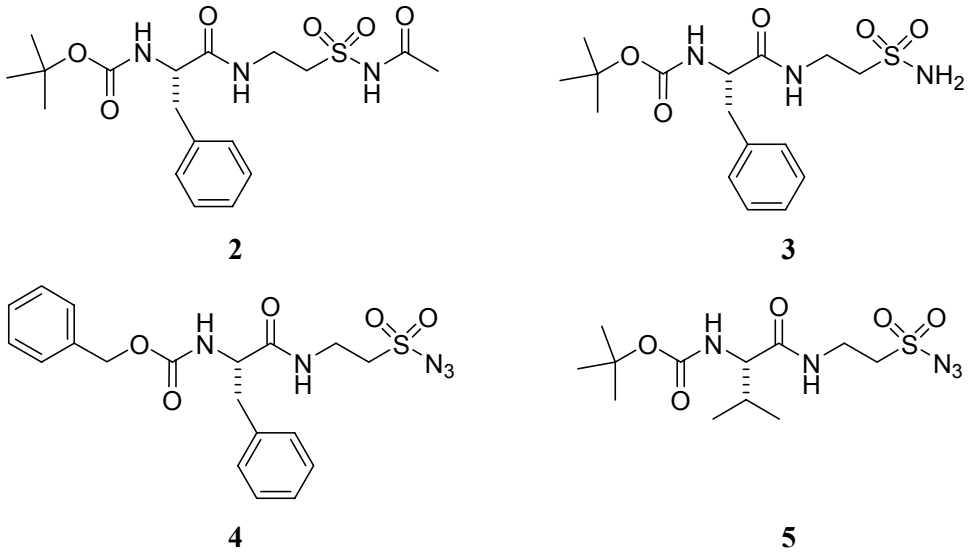


Figure 7.5 Structure of compounds **2** – **5**.

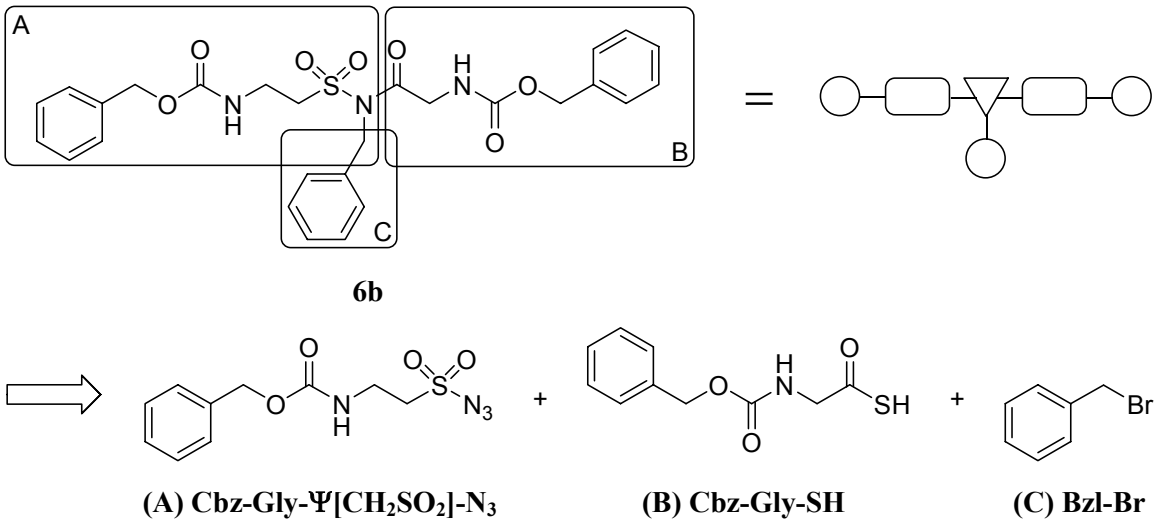
Remarkably, **4** was able to form a gel in both EtOAc/hexane (1:15 v/v) and DMSO/H_2O/HOAc (10:50:1 v/v/v) mixtures with a minimal gelation concentration of 1.0 mg/mL (2.3 mM) and 0.8 mg/mL (1.9 mM) respectively. The specific combination of non-covalent interactions made **4** act as a supergelator[15] in organic as well as aqueous solvents. The physicochemical properties of compounds **1** and **2** – **5** are summarized in Table 7.1.

Table 7.1 Physicochemical properties of compounds **1** and **2** – **5**.

Entry	Compound	Organogelation	Hydrogelation
1	**1**	Yes:[a] (<1 min), translucent	No:[e] turbid
2	**2**	Yes:[b] (<1 min), translucent	No:[e] turbid
3	**3**	Yes:[c] (<5 min), translucent	No:[e] turbid
4	**4**	Yes:[d] (<1 min), translucent	Yes:[f] (<1min), translucent
5	**5**	No:[a] turbid	No:[e] turbid

Conditions: [a]2 mg in 20 μL EtOAc and 300 μL hexane. [b]4.5 mg in 20 μL EtOAc, 20 μL D*i*PEA and 500 μL hexane. [c]2 mg in 20 μL EtOAc, 10 μL HOAc and 300 μL hexane. [d]0.5 mg in 20 μL EtOAc and 500 μL hexane. [e]2 mg in 50 μL DMSO, 20 μL HOAc and 500 μL H_2O. [f]0.5 mg in 100 μL DMSO, 10 μL HOAc and 500 μL H_2O.

Clearly, a small change in the chemical structure of a compound can result in a complete change of its self-assembly properties. Following this, it was attempted to design a peptide-derived *N*-acyl sulfonamide hydrogelator by increasing the susceptibility toward π–π stacking interactions. Therefore, a series of *N*-acyl sulfonamides featuring a variety in phenyl rings was synthesized.



Scheme 7.1 Chemical structure, schematic representation and retrosynthesis of compound **6b**.

Structural and chemical variation could easily be achieved by using amino acid derived starting materials.[16] Since the coupling of thio acids with sulfonylazides and, if desired, subsequent alkylation of the resulting *N*-acyl sulfonamide proceeded very efficiently, this method was well suited for the synthesis of a focussed series of potential hydrogelators. Using the thio acid/sulfonylazide amidation method,[17-20] a series of 7 compounds was synthesized and screened for their gelation properties (Scheme 7.1 and Figure 7.6).

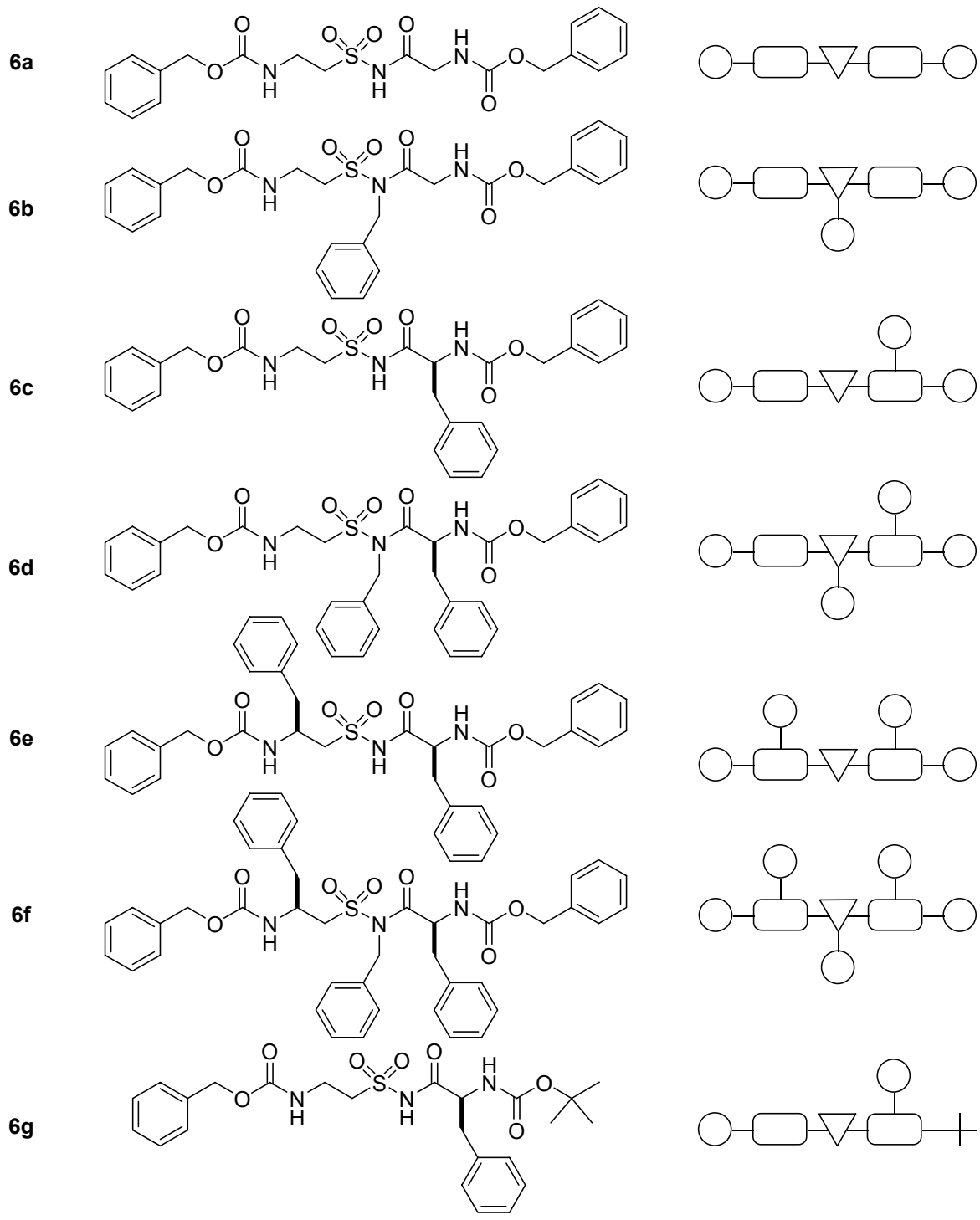


Figure 7.6 Series of peptide-based *N*-acyl sulfonamides **6a – g** screened for potential gelation properties.

The physicochemical properties of all *N*-acyl sulfonamide analogs, depicted in Figure 7.6, were investigated in both organic and aqueous solvents, the results of this screening are shown in Table 7.2. It was found that none of the screened compounds was able to gel EtOAc/hexane-mixtures, however, within this series, compound **6e** was identified as a hydrogelator (Table 7.2).

Table 7.2 Physicochemical properties of compounds **6a – g**.

Entry	**6**	A	B	C	Hydrogelation[a]
1	**6a**	Cbz-Gly-Ψ[CH_2SO_2]-N_3	Cbz-Gly-SH	-	No: turbid
2	**6b**	Cbz-Gly-Ψ[CH_2SO_2]-N_3	Cbz-Gly-SH	Bzl-Br	No: turbid
3	**6c**	Cbz-Gly-Ψ[CH_2SO_2]-N_3	Cbz-Phe-SH	-	No: turbid
4	**6d**	Cbz-Gly-Ψ[CH_2SO_2]-N_3	Cbz-Phe-SH	Bzl-Br	No: turbid
5	**6e**	Cbz-Phe-Ψ[CH_2SO_2]-N_3	Cbz-Phe-SH	-	Yes: (<1min), translucent
6	**6f**	Cbz-Phe-Ψ[CH_2SO_2]-N_3	Cbz-Phe-SH	Bzl-Br	No: turbid
7	**6g**	Cbz-Gly-Ψ[CH_2SO_2]-N_3	Boc-Phe-SH	-	No: turbid

[a]Conditions: 2 mg in 50 μL DMSO, 20 μL HOAc and 500 μL H_2O.

A concentration of 0.8 mM proved to be sufficient for the complete gelation of a DMSO/HOAc/H_2O (10:2:50 v/v/v) mixture. Apparently a delicate balance in aromatic groups was needed for gelation. With this respect, the ability of compounds **6a – c** and **6g** for π–π stacking interactions was apparently too low. Even though **6d** displays the same number of aromatic groups, it has a suboptimal configuration for gelation compared to **6e**, furthermore, the presumably essential *N*-acyl sulfonamide moiety is missing. *N*-benzyl acyl sulfonamide **6f** displays an additional aromatic group, however, like **6b** and **6d**, it lacks the acidic proton of the *N*-acyl sulfonamide moiety which might be important for hydrogen bonding and solubility. Moreover, the *N*-acyl sulfonamide moiety renders the gelation pH-responsive. Under acidic conditions, a mixture of **6e** in DMSO/HOAc/H_2O (10:2:50 v/v/v) at 5.6 mM readily formed a gel. Interestingly, when D*i*PEA was added to the gel, it changed into a clear solution, after re-addition of HOAc, however, the solution rapidly turned into a gel again.

7.3 Conclusions

The thio acid/sulfonyl azide amidation reaction yields an *N*-acyl sulfonamide moiety in the coupling product which is highly interesting since it acts as a carboxylic acid isostere and is, in fact, a known pharmacophore.[21] In this chapter, the spontaneous and irreversible coupling of thio acids and sulfonyl azides was used for the fast and efficient synthesis of a focussed series of peptide-based *N*-acyl sulfonamides with interesting physicochemical properties.

For example, peptide sulfonylazide Boc-Phe-Gly-Ψ[CH_2SO_2]-N_3 (**1**) was found to form a gel in EtOAc/hexane mixtures and the formation of supramolecular assemblies such as helical ribbons and nanotubes was shown. In this case the thio acid/sulfonyl azide amidation reaction enabled fast alteration of its physicochemical properties by structural variation. Starting from amino acid-derived thio acids and sulfonyl azides varying in phenyl groups, a peptide-based supergelator could be obtained which was capable of forming a pH-responsive gel in aqueous solution.

7.4 Experimental section

General information

Unless stated otherwise, all analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 5 μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min, connected to a UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000) unless stated otherwise. Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. Preparative HPLC was performed on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 10 μm, 250 × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 5 mL/min. Microwave-assisted syntheses were conducted in closed reaction vessels using a Biotage Initiator microwave reactor equipped with a temperature and pressure sensor for monitoring the reaction conditions. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) or a Varian INOVA-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75 MHz)

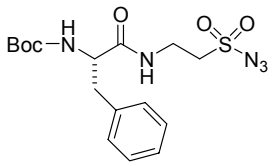
or a Varian INOVA-500 (125 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel $60F^{254}$ plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[22] Electron microscopy was performed using a Jeol 1200 EX transmission electron microscope operating at 60 kV.

Chemicals and reagents

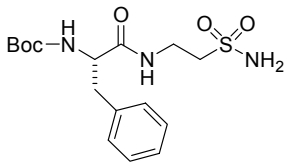
The coupling reagent 1H-benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) was obtained from Richelieu Biotechnologies Inc. *t*-Butyl oxycarbonyl (Boc) and carbobenzoxy (Cbz) amino acids were obtained from MultiSynTech. Peptide grade dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), acetonitrile, ethyl acetate (EtOAc) and hexane were purchased from Biosolve. *N*,*N*-Diisopropylethylamine (D*i*PEA), was obtained from Acros Organics, acetic acid (HOAc) and isopropanol (*i*-PrOH) were purchased from Merck KGaA. Pd/C and *p*-nitrobenzaldehyde were obtained from Fluka. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) was obtained from Aldrich.

Transmission electron microscopy

A small quantity of a gel of **1** in EtOAc/hexane was placed on a carbon coated copper grid. After 15 min, any excess of compound **1** was removed by washing the copper grid on a drop of demi-water (this was repeated 4 times) and the sample was stained with methylcellulose/ uranyl acetate and dried on air.

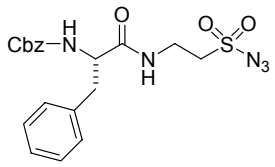


Boc-Phe-Gly-Ψ[CH_2SO_2]-N_3 (1): The synthetic procedure and analytical data for this compound have been described in chapter 5.

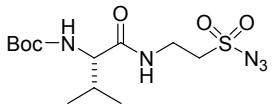


Sulfonamide 3: A mixture of **1** (255 mg, 0.64 mmol) and Pd/C (100 mg) in THF/H_2O (25 mL, 1.5:1 v/v) was allowed to stir for 16 h under a H_2 atmosphere. Then, the reaction mixture was filtered over Hyflo and the filtrate was evaporated to dryness and subsequently co-evaporated with toluene (3 ×) and $CHCl_3$ (3 ×). The product was obtained as a white solid in quantitative yield. R_f: 0.34 (EtOAc); R_t: 17.07 min (Alltech Adsorbosphere narrow bore C18, 90Å, 5 μm, 250 × 4.6 mm) using a linear gradient of

100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 0.4 mL/min); ^{1}H NMR ($CDCl_3$): δ 7.34 – 7.19 (m, 5H), 6.73 (br s, 1H), 5.11 (br s, 3H), 4.27 (m, 1H), 3.66 (br s, 2H), 3.26 (m, 2H), 3.06 (m, 2H), 1.39 (br s, 9H); ^{13}C NMR (DMSO-d_6): δ 171.7, 155.2, 138.1, 129.2, 128.0, 126.2, 78.0, 55.8, 53.6, 37.4, 28.1; Anal. calcd (%) for $C_{15}H_{21}N_5O_5S$: C, 51.74; H, 6.78; N, 11.31; found: C, 50.10; H, 6.68; N, 10.85.



Cbz-Phe-Gly-Ψ[CH_2SO_2]-N_3 (4): To a mixture of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (187 mg, 1.0 mmol) in DCM (20 mL) and D*i*PEA (193 μL, 1.1 mmol) was added a solution of Cbz-Phe-OH (300 mg, 1.0 mmol), BOP (464 mg, 1.05 mmol) and D*i*PEA (193 μL, 1.1 mmol) in DCM (20 mL). The reaction mixture was stirred overnight at rt. The pH was kept at approx. 8 by addition of D*i*PEA during the reaction. After concentration, the residue was taken up in EtOAc (100 mL) and the solution was washed with 1N $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), 5% $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL) and dried over Na_2SO_4. The product was isolated by column chromatography (EtOAc/hexane 2:3 v/v) to afford **4** (292 mg, 68%) as a white solid. R_f: 0.13 (EtOAc/hexane 2:3 v/v); R_t: 16.53 min; ^{1}H NMR ($CDCl_3$): δ 7.38 – 7.25 (m), 7.19 (m, total 10H), 6.42 (br s, 1H), 5.2 (br s, 1H), 5.08 (4.37, 1H), 3.69 (m, 2H), 3.41 (m, 1H), 3.29 (m, 1H), 3.09 (d, J = 6.9 Hz, 2H); ^{13}C NMR ($CDCl_3$): δ 171.6, 136.1, 135.9, 129.2, 128.8, 128.5, 128.3, 128.0, 127.25, 67.2, 56.3, 54.6, 38.1, 33.8; ESMS calcd for $C_{19}H_{21}N_5O_5S$ 431.13, found: 454.20 $[M+Na]^+$; Anal. calcd (%) for $C_{19}H_{21}N_5O_5S$: C, 52.89; H, 4.91; N, 16.23; found: C, 52.95; H, 5.86; N, 16.18.

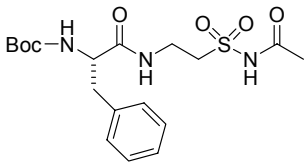


Boc-Val-Tau-N_3 (5): To a mixture of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (149 mg, 0.8 mmol) in DCM (20 mL) and D*i*PEA (154 μL, 1.1 mmol) was added a solution of Boc-Val-OH (174 mg, 0.8 mmol), BOP (371 mg, 1.05 mmol) and D*i*PEA (154 μL, 1.1 mmol) in DCM (20 mL). The reaction mixture was stirred overnight at rt. The pH was kept at approx. 8 by addition of D*i*PEA during the reaction. After concentration, the residue was taken up in EtOAc (100 mL) and the solution was washed with 1N $KHSO_4$ (3 × 50 mL), brine (1 × 50 mL), 5% $NaHCO_3$ (3 × 50 mL) and brine (1 × 50 mL) and dried over Na_2SO_4. The product was isolated by column chromatography (EtOAc/hexane 1:1 v/v) to afford **5** (216 mg, 77%) as a white solid. R_f: 0.32 (EtOAc/hexane 1:1 v/v); R_t: 14.81 min; ^{1}H NMR ($CDCl_3$): δ 6.78 (br s, 1H), 5.00 (d, J = 8.5 Hz, 1H), 3.94 (m, 1H), 3.79 (m, 2H), 3.55 (m, 2H), 2.20 (m, 1H), 1.45 (s, 9H), 0.98 (dd, J = 6.9 Hz, J = 10.5 Hz, 6H); ^{13}C NMR ($CDCl_3$): δ 172.5, 155.9, 80.1, 59.9,

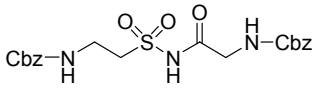
54.8, 34.0, 30.6, 28.3, 19.2, 17.6; Anal. calcd (%) for $C_{15}H_{21}N_5O_5S$: C, 41.25; H, 6.63; N, 20.04; found: C, 41.46; H, 5.40; N, 20.00.

General Procedure for the synthesis of *N*-acyl sulfonamides 2 and 6a,c,e,g

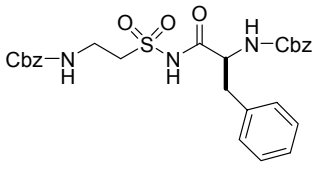
To a mixture of sulfonyl azide A (1 equiv) and 2,6-lutidine (1.3 equiv) in DMF, the thio acid B (1.3 equiv) was added dropwise. After completion of the addition, the reaction mixture was allowed to stir for 15 min at room temperature. Then, the reaction mixture was evaporated to dryness and the residue was either purified by crystallization or by column chromatography.



***N*-Acyl sulfonamide 2:** The reaction (0.2 mmol scale) was carried out following the general procedure. The product (69 mg, 83%) was obtained as a white solid by crystallization from EtOAc/hexane. R_f: 0.05 (EtOAc/hexane/AcOH 49.5:49.5:1 v/v/v); R_t: 24.94 min (Alltech Adsorbosphere XL C8, 90Å, 5μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/MeCN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 1 mL/min; ^{1}H NMR ($CDCl_3$): δ 9.64 (br s, 1H), 7.29 (m), 7.19 (m, total 5H), 6.69 (br s, 1H), 5.06 (br s, 1H), 4.31 (m, 1H), 3.83 – 3.43 (m, 4H), 3.08 (br s, 2H), 2.13 (s, 3H), 1.40 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 171.1, 170.0, 156.0, 136.2, 129.2, 128.7, 127.1, 81.1, 55.9, 51.5, 38.2, 34.2; ESMS calcd for $C_{18}H_{27}N_3O_6S$: 413.16; found: 435.90 $[M+Na]^+$; Anal. calcd (%) for $C_{18}H_{27}N_3O_6S$: C, 52.29; H, 6.58; N, 10.16; found: C, 52.34; H, 6.52; N, 10.08.

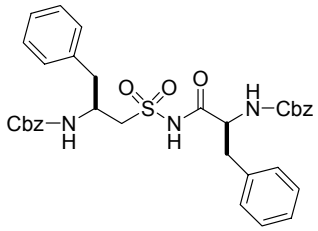


***N*-Acyl sulfonamide 6a:** The reaction (2.95 mmol scale) was carried out following the general procedure. The product (659 mg, 83%) was obtained as a white solid by crystallization from MeOH/hexane. R_f: 0.46 (EtOAc/hexane/AcOH 49.5:49.5:1 v/v/v); R_t: 15.76 min; ^{1}H NMR (DMSO-d_6): δ 11.90 (br s, 1H), 7.61 (br s, 1H), 7.45 (br s, 1H), 7.36 (br s, 10H), 5.04 (s, 2H), 5.03 (m, 2H), 3.78 (d, J = 5.8 Hz, 2H), 3.52 (m, 2H), 3.41 (m, 2H); ^{13}C NMR (DMSO-d_6): δ 169.8, 156.7, 156.2, 137.1, 128.6, 128.1, 128.0, 65.9, 65.8, 51.9, 43.9, 35.1; ESMS calcd for $C_{20}H_{23}N_3O_7S$: 449.13, found: 472.35 $[M+Na]^+$; Anal. calcd (%) for $C_{20}H_{23}N_3O_7S$: C, 53.44; H, 5.16; N, 9.35; found: C, 53.36; H, 5.21; N, 9.30.

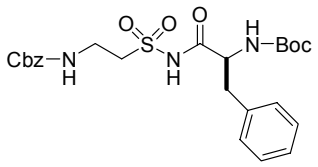


***N*-Acyl sulfonamide 6c:** The reaction (1.0 mmol scale) was carried out following the general procedure. The product (540 mg, 100%) was obtained as a white solid by flash column

chromatography (EtOAc/hexane/HOAc 39.5:59.5:1.0 v/v/v); R_f: 0.59 (EtOAc/hexane/AcOH 89.5:9.5:1.0 v/v/v); R_t: 17.46 min; ^{1}H NMR ($CDCl_3$): δ 9.80 (br s, 1H), 7.28 (m), 7.21 (m), 7.1 (m, total 15H), 5.62 (br s, 1H), 5.60 (br s, 1H), 5.04 (m, 2H), 5.00 (m, 2H), 4.55 (m, 1H), 3.51 (br s, 4H), 3.06 (m), 2.91 (m, total 2H); ^{13}C NMR ($CDCl_3$): δ 171.2, 156.4, 136.1, 135.6, 135.1, 129.4, 128.6, 128.1, 127.3, 67.6, 67.0, 56.5, 53.0, 37.6, 35.5; ESMS calcd for $C_{27}H_{29}N_3O_7S$: 539.17, found: 561.80 $[M+Na]^+$; Anal. calcd (%) for $C_{27}H_{29}N_3O_7S$: C, 60.10; H, 5.42; N, 7.79; found: C, 59.88; H, 5.48; N, 7.66.



***N*-Acyl sulfonamide 6e:** The reaction (1.75 mmol scale) was carried out following the general procedure. Unfortunately, the crude product was partially lost during attempted purification by flash column chromatography. Finally, the product (250 mg, 23%) was obtained as a white solid by preparative HPLC. R_f: 0.45 (EtOAc/hexane/AcOH 49.5:49.5:1.0 v/v/v); R_t: 18.57 min; ^{1}H NMR (500 MHz, DMSO-d_6): δ 12.21 (s, 1H), 7.28 (d, J = 7.3 Hz, 1H), 7.49 (d, J = 8.8 Hz, 1H), 7.29 (m, 20H), 4.96 (m, 4H), 4.32 (br s, 1H), 4.21 (br s, 1H), 3.63 (m, 1H), 3.52 (m, 1H), 3.03 (m, 1H), 2.90 (m, 1H) 2.77 (m, 2H); ^{13}C NMR (125 MHz, DMSO-d_6): δ 155.0 (3 lines), 137.3, 136.8, 136.6, 135.1, 129.4, 128.6, 128.1, 127.3, 67.6, 67.0, 56.5, 53.0, 37.6, 35.5; ESMS calcd for $C_{34}H_{35}N_3O_7S$: 629.22, found: 630.50 $[M+H]^+$; Anal. calcd (%) for $C_{34}H_{35}N_3O_7S$: C, 64.85; H, 5.60; N, 6.67; found: C, 64.96; H, 5.53; N, 6.63.

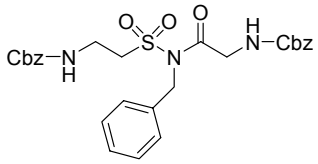


***N*-Acyl sulfonamide 6g:** The reaction (1.0 mmol scale) was carried out following the general procedure. The product (499 mg, 99%) was obtained as a white solid by crystallization from $CHCl_3$/hexane. R_f: 0.61 (EtOAc/hexane/AcOH 89.5:9.5:1 v/v/v); R_t: 17.18 min; ^{1}H NMR ($CDCl_3$): δ 9.19 (br s, 1H), 7.34 (m), 7.18 (m, total 10H), 5.53 (br s, 1H), 5.10 (s, 1H), 4.95 (m, 1H), 4.33 (m, 1H), 3.60 (m, 4H), 3.13 (m, 1H), 2.95 (m, 1H), 1.39 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 171.2, 156.3, 155.9, 136.15, 135.4, 129.3, 128.9, 128.5, 128.1, 127.3, 81.4, 67.0, 56.3, 53.2, 37.0, 35.5, 28.2; ESMS calcd for $C_{24}H_{31}N_3O_7S$: 505.19, found: 528.00 $[M+Na]^+$; Anal. calcd (%) for $C_{24}H_{31}N_3O_7S$: C, 57.01; H, 6.18; N, 8.31; found: C, 57.03; H, 6.24; N, 8.29.

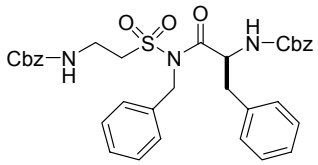
General Procedure for the synthesis of *N*-benzyl acyl sulfonamides 6b,d,f:

To a solution of the *N*-acyl sulfonamide (1.0 equiv) and D*i*PEA (2.0 equiv) in DMF, benzylbromide (1.0 equiv) was added. The reaction mixture was heated at 150°C for 6 min by microwave irradiation. After concentration, the residue was taken up in EtOAc and

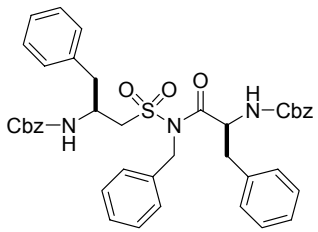
subsequently washed with 1N $KHSO_4$ (3 ×), brine (1 ×), 5% $NaHCO_3$ (3 ×) and brine (1 ×). Drying over Na_2SO_4 and removal of the solvents resulted in the crude alkylation product, which was purified by column chromatography.



***N*-Benzyl acyl sulfonamide 6b:** The reaction (0.7 mmol scale) was carried out following the general procedure. The product (310 mg, 82%) was isolated by flash column chromatography (EtOAc/hexane 1:1 v/v) as a clear oil that solidified upon standing. R_f: 0.38 (EtOAc/hexane 1:1 v/v); R_t: 17.85 min; ^{1}H NMR ($CDCl_3$): δ 7.33–7.24 (m, 15), 5.68 (br s, 1H), 5.52 (br s, 1H), 5.08 (s, 2H), 5.06 (s, 2H), 4.86 (s, 2H), 4.16 (m, 2H), 3.59 (br s, 2H), 3.51 (2H); ^{13}C NMR ($CDCl_3$): δ 170.5, 156.7, 156.1, 136.0 (2 lines), 135.4, 128.8, 128.5, 128.1, 128.0, 127.9, 127.6, 67.2, 66.9, 54.2, 49.2, 45.4, 35.5; ESMS calcd for $C_{27}H_{29}N_3O_7S$: 539.17, found: 562.4 $[M+Na]^+$; Anal. calcd (%) for $C_{27}H_{29}N_3O_7S$: C, 60.10; H, 5.42; N, 7.79; found: C, 57.63; H, 6.22; N, 7.35.



***N*-Benzyl acyl sulfonamide 6d:** The reaction (0.3 mmol scale) was carried out following the general procedure using 179 μL of benzylbromide (1.5 mmol). The product (181 mg, 100%) was isolated by flash column chromatography (EtOAc/hexane 3:7 v/v) as a clear oil. R_f: 0.22 (EtOAc/hexane 3:7 v/v); R_t: 19.13 min; ^{1}H NMR ($CDCl_3$): δ 7.35 – 7.18 (m), 6.95 (br s, total 20H), 5.73 (br s, 1H), 5.30 (m), 5.08 (m), 4.96 (m, total 8H), 3.80 (m, 1H), 3.62 (m, 2H), 3.53 (m, 1H), 3.07 (m, 1H), 2.61 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 173.9, 156.6, 156.2, 136.2, 136.0, 159.0, 135.2, 129.2, 128.4, 127.9, 67.2, 66.9, 55.9, 54.3, 49.8, 38.0, 35.6; ESMS calcd for $C_{34}H_{35}N_3O_7S$: 629.22, found: 651.75 $[M+Na]^+$; Anal. calcd (%) for $C_{34}H_{35}N_3O_7S$: C, 64.85; H, 5.60; N, 6.67; found: C, 63.65; H, 5.35; N, 6.24.



***N*-Benzyl acyl sulfonamide 6f:** The reaction (0.2 mmol scale) was carried out following the general procedure. The product (108 mg, 75%) was isolated by flash column chromatography (EtOAc/hexane 1:4 v/v) as a clear oil which solidified upon standing. R_f: 0.21 (EtOAc/hexane 1:4 v/v); R_t: 18.57 min; ^{1}H NMR ($CDCl_3$): δ 7.35 – 7.17 (m) 7.02 (br s, total 25H), 5.93 (br s, 1H), 5.28 (d, J = 6.3 Hz, 1H), 5.15–5.06 (m, 3H), 4.93 (m, 2H), 4.82 (s, 2H), 4.47 (br s, 1H), 3.91 (m, 1H), 3.64 (m, 1H), 3.18 (m, 1H), 3.08 (m, 1H), 2.85 (m, 1H), 2.56 (m, 1H); ^{13}C NMR ($CDCl_3$): δ 173.4, 157.0, 155.6, 136.4, 135.6, 135.4, 129.4,129.1, 128.7, 128.6,

128.4, 128.2, 128.1, 128.0, 127.7, 127.2, 127.0, 67.4, 66.7, 56.6, 50.2, 48.8, 40.9, 37.5; ESMS calcd for $C_{41}H_{41}N_3O_7S$: 719.27, found: 742.60 $[M+Na]^+$; Anal. calcd (%) for $C_{41}H_{41}N_3O_7S$: C, 68.41; H, 5.74; N, 5.84; found: C, 68.46; H, 5.70; N, 5.76.

7.5 References and Notes

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8

The Thio Acid/Sulfonyl Azide Amidation Reaction: Synthetic Approaches and Outlook Toward Bioconjugations, Peptide Dendrimers and Organocatalysts

The special properties of the thio acid/sulfonyl azide amidation reaction, as were described in the previous chapters, resemble those of the "ideal click reaction". Additionally, the *N*-acyl sulfonamide moiety renders special properties to the coupling product since it functions as a carboxylic acid isostere. This chapter describes the successful application of the thio acid/sulfonyl azide amidation reaction as a novel chemoselective ligation method to obtain peptide conjugates and discusses some future applications.

8.1 Introduction

The regioselective coupling of unprotected peptide segments requires the chemoselective reaction of two mutually reactive groups without the interference of other functional groups that are present in the peptide side chains. Such a reaction should be efficient under mild (aqueous) conditions giving rise to a stable and non-toxic product, preferably without the need for a catalyst which could be difficult to remove from the product.
The special characteristics of the thio acid/sulfonyl azide amidation reaction, as described in chapter five, could be applied as a novel chemoselective ligation or bioconjugation method for the site-selective coupling of unprotected peptides. Additionally, the resulting *N*-acyl sulfonamide is more than just a passive linker; it renders special properties to the coupling product since it functions as a carboxylic acid isostere (Chapters 5 – 7).
In this chapter, application of the thio acid/sulfonyl azide amidation reaction on the chemoselective coupling of peptides is investigated by three examples: the post-synthetic introduction of biophysical markers (such as a fluorophore and an affinity label) to a peptide sequence as well as the ligation to an additional peptide segment, the attachment of peptides to a multivalent scaffold to obtain peptide dendrimers and the synthesis of peptide-based *N*-acyl sulfonamides as potential organocatalysts.

8.2 The thio acid/sulfonyl azide amidation reaction as novel methodology for the chemoselective ligation of peptides

8.2.1 Introduction

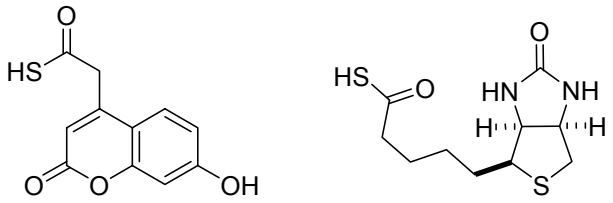
The modification of biomolecules offers great possibilities for studying their biological function. For instance, the labeling of proteins with fluorophores, affinity labels or other chemically or optically active species has proven to be an immensely important tool for research in the life sciences. In this regard, biological methods that feature the use of genetically encoded green fluorescent protein[1] have been particularly useful. Although powerful, biological probes are generally limited by the use of natural amino acid residues and by their often large size. In contrast, chemical methods offer increased flexibility since they allow the use of non-natural building blocks for the introduction of, among others, (small molecule) biophysical markers or reporter groups.
Traditionally, chemical bioconjugation methods rely on modification of the existing functionality of a protein by the use of appropriate chemical reagents.[2,3] Typical examples are alkylation of the thiol side chain of cysteine by reaction with α-halo carbonyls and the reaction of the ε-amino group of lysine with succinimide or esters. However, these methods

offer no regioselectivity since they can not discriminate between two similar functionalities at different positions in the protein sequence. Additionally, both the thiol side chain of cysteine and the ε-amino group of lysine can react as nucleophiles which can give rise to side-product formation. Thus, these methods generally are not suitable to obtain homogeneous products and consequently alteration of the biological function by a change in the molecular structure, can not be excluded.

In contrast, other chemoselective ligation or bioconjugation methods allow for the site-selective modification of peptides, proteins and other biomolecules to obtain homogeneous conjugates. In particular, successful applications of aldehyde-assisted ligations, native chemical ligation, Staudinger ligation and the copper-catalyzed 1,3-dipolar cycloaddition (click chemistry) have recently been described in the literature (Chapter 1). Nevertheless, the complexity of biomolecules such as peptides and proteins combined with their wide application areas, generate a continuing demand for the development of new methods as alternative for the above mentioned examples. Ideally, these methods should combine bioorthogonality with an efficient and fast reaction to form a stable product under mild conditions. For instance, the copper-catalyzed 1,3-dipolar cycloaddition (click chemistry) has proven to be a very powerful method for the efficient conjugation of peptide derivatives in both organic and aqueous solvents. However, *in vitro* and *in vivo* applications are generally limited because a copper catalyst is needed, which may be cytotoxic. In this respect, the thio acid/sulfonyl azide amidation reaction, could offer an advantage over the copper-catalyzed 1,3-dipolar cycloaddition between azides and alkynes since it is independent of any toxic catalyst. In this paragraph the chemoselective ligation of peptides using the thio acid/sulfonyl azide amidation reaction is investigated by the site-selective introduction of biophysical (fluorescent and affinity) labels to peptides as well as the ligation to an additional peptide segment.

8.2.2 Results and discussion

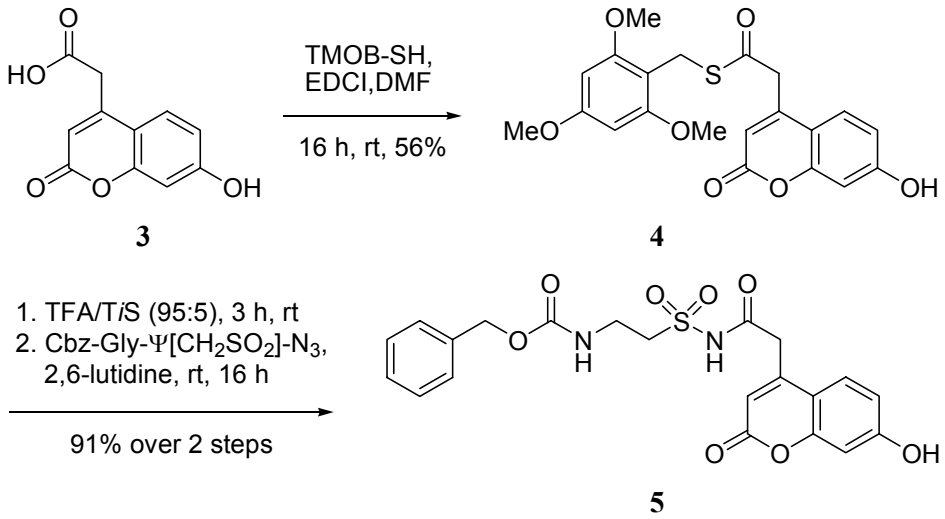
As a proof of concept, two biophysical labels were chosen to synthesize (one fluorescent and one affinity label, derived from coumarin and biotin respectively). Both biophysical labels feature a thio acid moiety for reaction with sulfonyl azide functionalized peptides (Figure 8.1).



1 **2**

Figure 8.1 Chemical structure of biophysical thio acid labels **1** and **2**.

Coumarin thio acid derivative **1** was synthesized from the trimethoxybenzyl (TMOB) thio ester precursor. This TMOB-thio ester **4** was readily prepared from the corresponding carboxylic acid **3** and TMOB-thiol[4] by EDCI-mediated esterification (Scheme 8.1). Next, treatment with TFA released coumarin thio acid derivative **1** which was immediately reacted with Cbz-Gly-Ψ[CH_2SO_2]-N_3 in the presence of 2,6-lutidine as a base. The reaction proceeded very smoothly to give *N*-acyl sulfonamide **5** in excellent yield (91%) and purity.

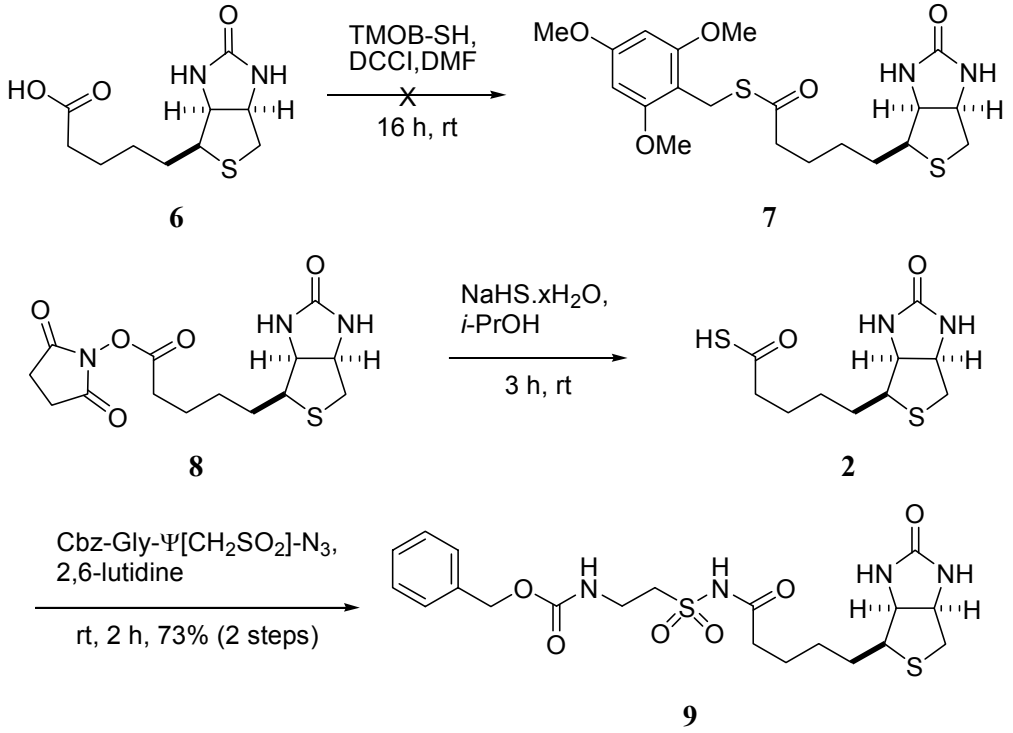


3 **4** **5**

Scheme 8.1 Synthesis of coumarin thio acid derivative **1** and subsequent reaction with a sulfonylazide functionalized glycine derivative.

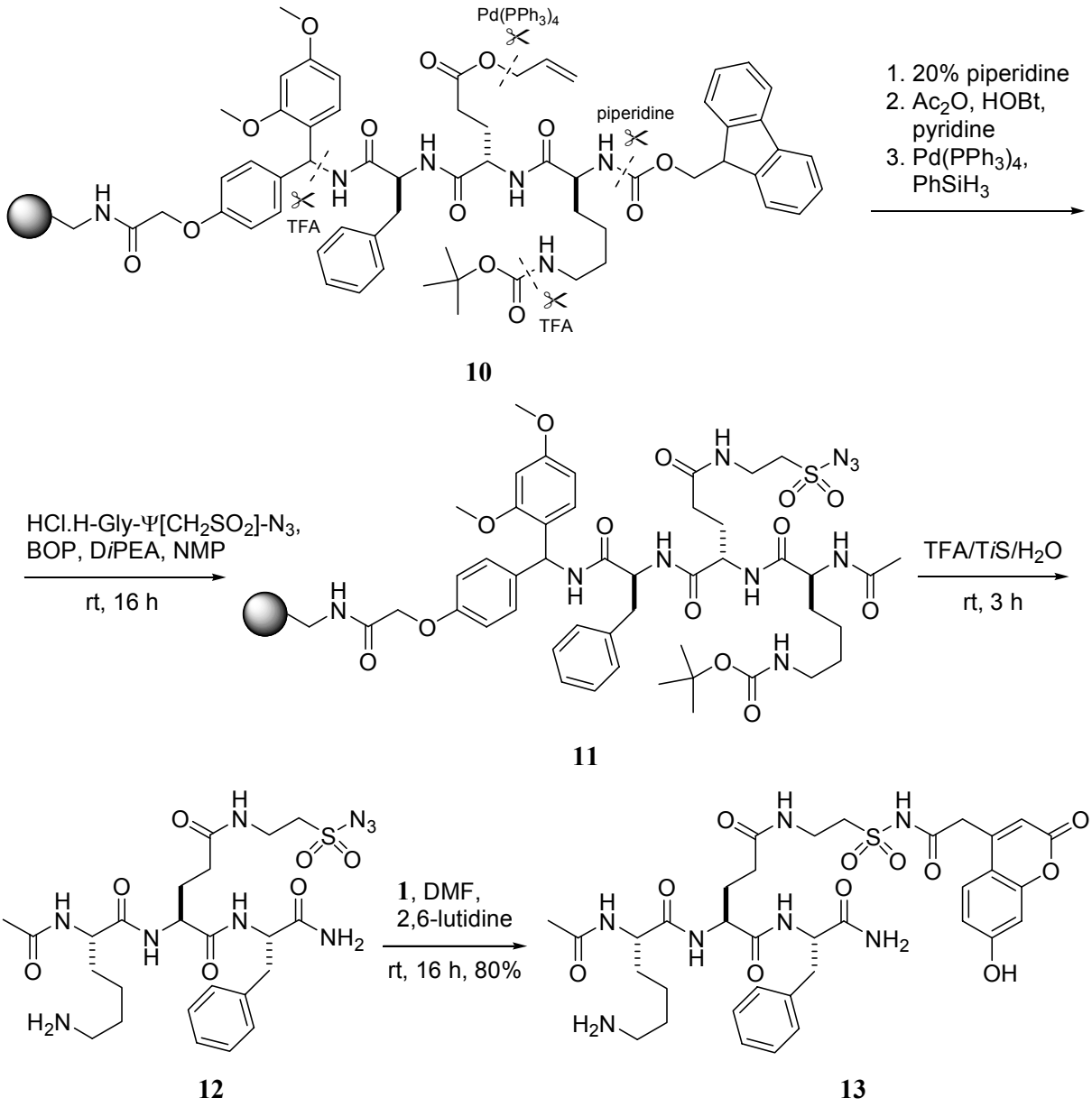
Similarly, it was tried to synthesize biotin thio acid derivative **2** via the corresponding TMOB-thio ester. However, in this case the carbodiimide-mediated esterification using DCCI, did not yield the expected thio ester product, instead, the *N*-acylurea side product was isolated as the major product. This was an indication for the low reactivity of the DCCI activated ester toward the trimethoxybenzyl thiol nucleophile. Fortunately, **2** could

alternatively be prepared via reaction of the corresponding biotin succinimide ester (**8**)[5] by treatment with NaHS (chapter 4).[6] Subsequently, *N*-acyl sulfonamide **9** could be obtained by the straightforward reaction with Cbz-Gly-Ψ[CH_2SO_2]-N_3 in the presence of 2,6-lutidine as base (Scheme 8.2).



Scheme 8.2 Synthesis of biotin thio acid derivative **2** and subsequent reaction with a sulfonylazide functionalized glycine derivative to give **9**.

The sulfonyl azide functionality was envisioned to be straightforwardly introduced into peptides via the HCl.H-Gly-Ψ[CH_2SO_2]-N_3 building block, using standard peptide coupling methods as was described in chapter 5. Subsequently, these sulfonyl azide functionalized peptides can be reacted chemoselectively with functional thio acids to form the *N*-acyl sulfonamide ligation product. To investigate this strategy, a sulfonyl azide-functionalized tripeptide was synthesized for reaction with a biophysical label that featured the thio acid moiety. The choice of amino acid sequence for this model peptide was based on UV-visibility for HPLC analysis (Phe), presence of a carboxylic acid handle for attachment of the HCl.H-Gly-Ψ[CH_2SO_2]-N_3 building block (Glu) and a positively chargeable amine function in the side chain for ESMS analysis (Lys).



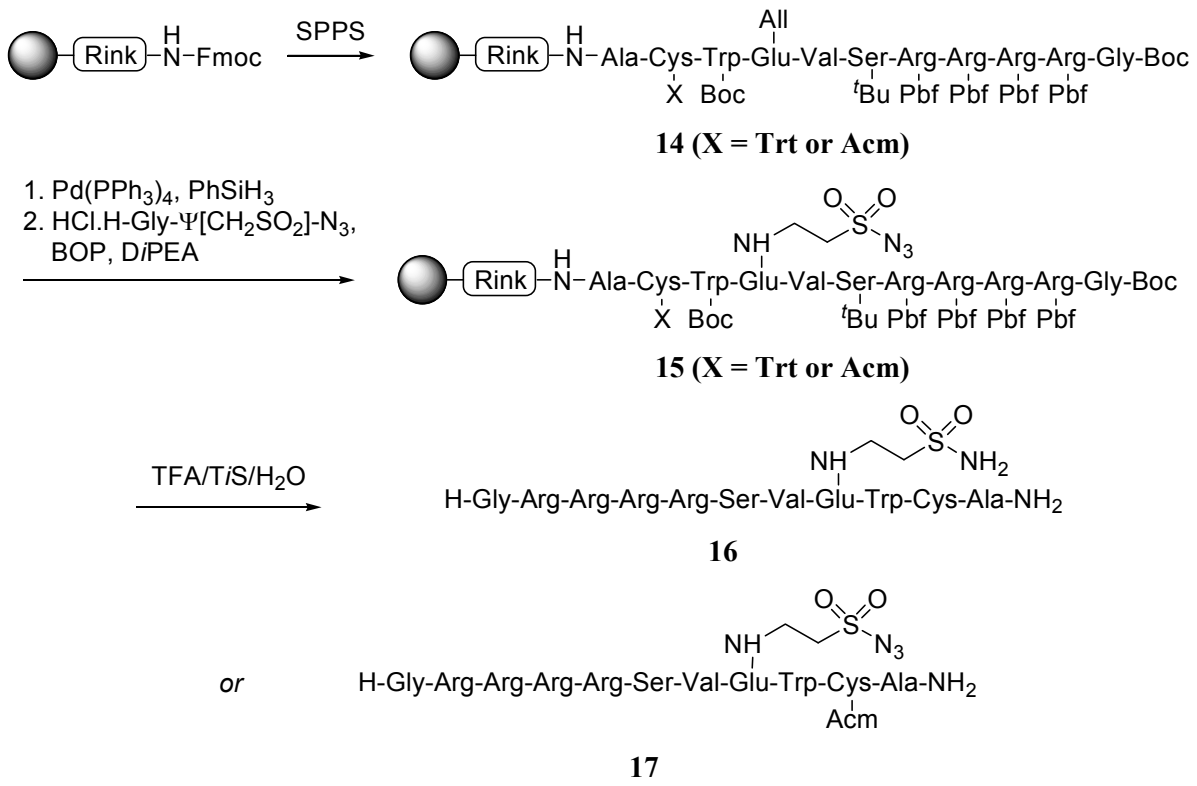
Scheme 8.3 Solid phase synthesis of sulfonylazide functionalized tripeptide **12** and subsequent reaction with coumarin thio acid derivative **1**.

The synthesis of resin-bound tripeptide **10** (Scheme 8.3) was performed using conventional Fmoc SPPS methods. Attachment of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 was achieved via a BOP-mediated coupling after selective deprotection of the glutamic acid side chain. For this purpose, orthogonally protected Fmoc-Glu(OAll)-OH was used since the allyl protecting group can be removed selectively on the solid support using $Pd(PPh_3)_4$ and $PhSiH_3$.[7] All solid phase reactions were monitored by the Kaiser test[8] (free amines) and the Malachite green test[9] (free carboxylic acids) respectively.

Finally, sulfonyl azide-functionalized peptide **11** was treated with a mixture of TFA and scavengers to simultaneously cleave the peptide from the resin and remove the Boc protecting group from the lysine side chain. The TFA solution was diluted with MTBE/hexane and the precipitated peptide was collected by centrifugation. The crude peptide was purified by preparative HPLC and lyophilized to give **12** as a white solid, the purity and identity were assessed by HPLC and ESMS respectively.

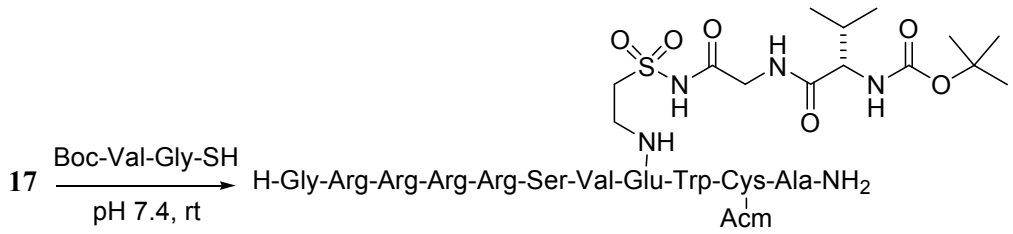
Subsequently, **12** was reacted with coumarin thio acid derivative **1** under mild conditions that consisted of DMF as a solvent and 2,6-lutidine as a base at room temperature. As expected, the reaction proceeded smoothly and the *N*-acyl sulfonamide ligation product (**13**) was islolated in a good yield of 80%. This example clearly demonstrated the successful solid phase synthesis of a sulfonyl azide functionalized peptide and subsequent coupling to a fluorescent thio acid label. Moreover, these results direct towards application of the thio acid/sulfonyl azide amidation reaction as a new chemoselective ligation or bioconjugation method for the site-selective modification of peptides and, in principle, other biomolecules.

In one of the projects in our group, the properties of human lactoferrin (hLF) derived peptides are studied. Although their antimicrobial activity has been described,[10] the biological mode of action remains not completely understood. The introduction of a biophysical label to these peptides might help to shed more light on this. As a target, hLF (1-11) corresponding to the first eleven residues of hLF (^{N}GRRRRSVEWCA^{C}) was chosen. Replacement of Gl**n** (Q) for Gl**u** (E) at position 8 provided a carboxylic acid handle for the introduction of a sulfonylazide functionality by the attachment of a HCl.H-Gly-$\Psi[CH_2SO_2]$-N_3 building block while preserving the δ-amide moiety. The peptide was built up by conventional Fmoc SPPS methods using orthogonally protected Fmoc-Glu(OAll)-OH which allowed for selective deprotection of the glutamic acid side chain similar to the procedure as was described in Scheme 8.3. The sulfonyl azide building block was successfully introduced, as was confirmed by the Malachite green test.[9] However, when a small portion of the peptide was deprotected and cleaved from the resin, mass analysis of the crude product revealed a molecular mass of 26 amu lower than was expected on basis of its theoretical molecular weight. This mass difference was indicative for loss of a N_2-molecule via reduction of the sulfonyl azide functionality. Presumably, the reduction was caused by the free thiol side chain of a cysteine residue, present in the peptide sequence at position 10. To verify this hypothesis, the peptide sequence was resynthesized, however, this time an Acm-protected cysteine building block was used instead of standard Trt-protected cysteine which allowed for the selective protection of cysteine after cleavage from the resin. Indeed, in this case, the sulfonyl azide functionalized peptide was obtained as expected.



Scheme 8.4 Solid phase synthesis of sulfonyl azide functionalized hLF (1-11) **17**.

As a model for the site-selective introduction of a biophysical label or chemoselective ligation to an additional peptide sequence, sulfonyl azide functionalized peptide **17** was reacted with peptide thio acid Boc-Val-Gly-SH. Therefore, a small excess of the peptide thio acid (2 equiv) was added to a stirred solution of **17** in HEPES buffer at physiological pH (7.4) at room temperature. Upon the addition of the dipeptide thio acid, the immediate evolution of gas bubbles (N_2) was observed as well as the formation of a precipitate (S_8) which were both indicative for the thio acid/sulfonylazide amidation reaction. Interestingly, the reaction appeared to proceed instantaneously, even without the addition of any extra organic base. The reaction progress was monitored by analytical HPLC and the conversion of sulfonyl azide functionalized peptide **17** was found to be complete within 1 h, as was judged from the chromatograms in Scheme 8.5.



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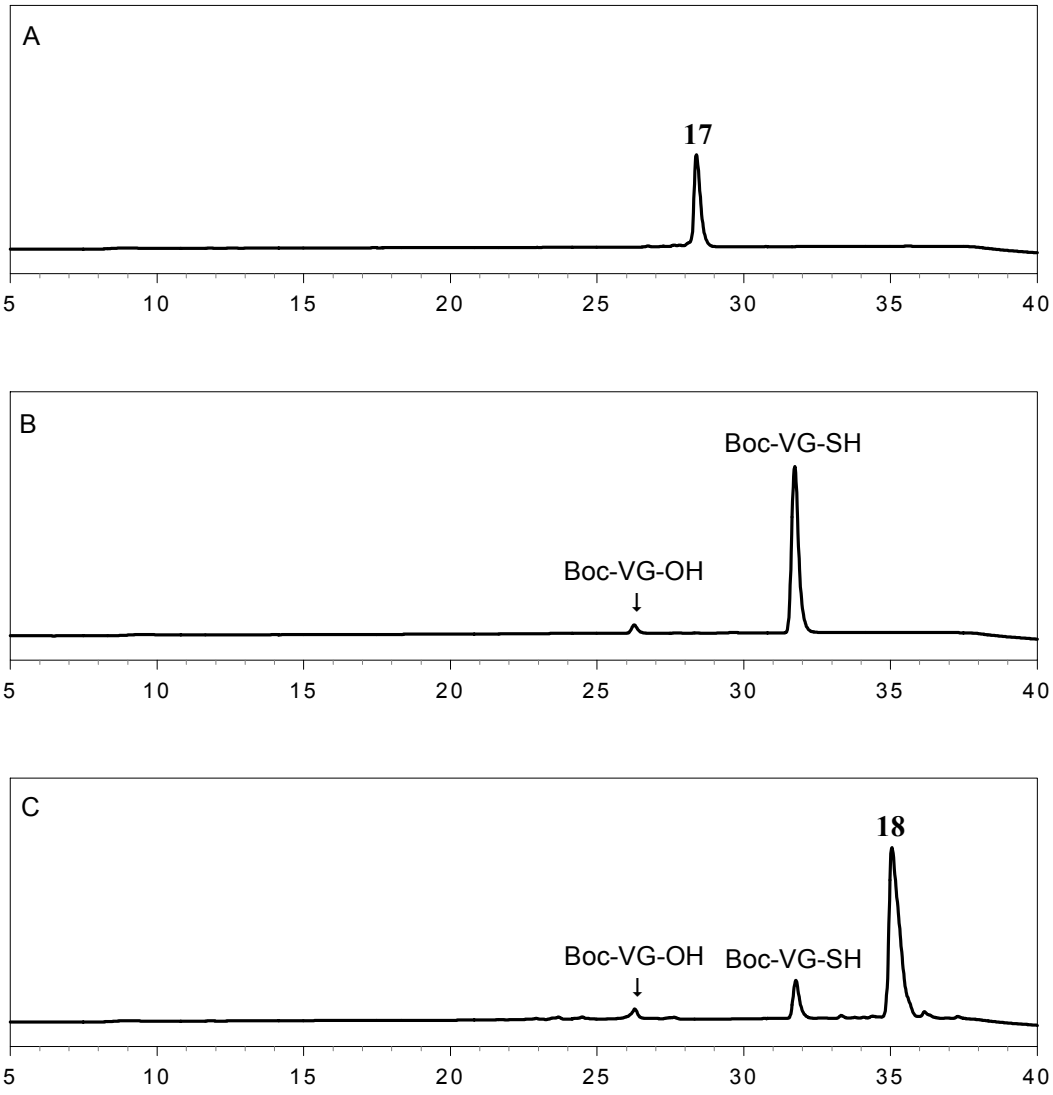
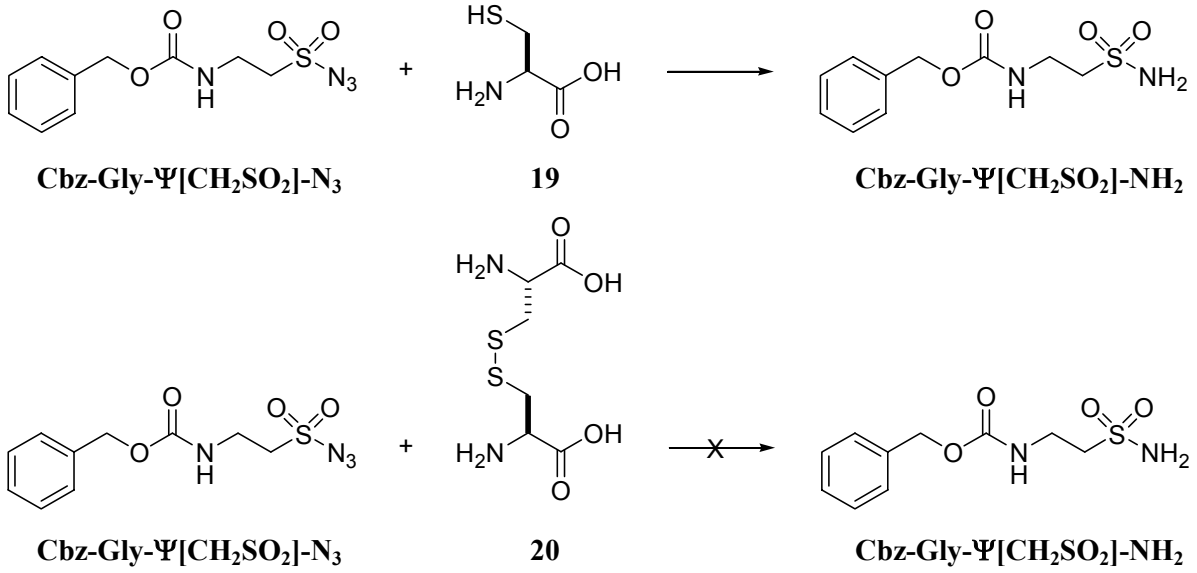


Scheme 8.5 The reaction of sulfonylazide functionalized peptide **17** with Boc-Val-Gly-SH to give **18**. HPLC analysis of: (A) sulfonyl azide functionalized peptide **17**; (B) peptide thio acid Boc-Val-Gly-SH; (C) the reaction mixture after 1 h.

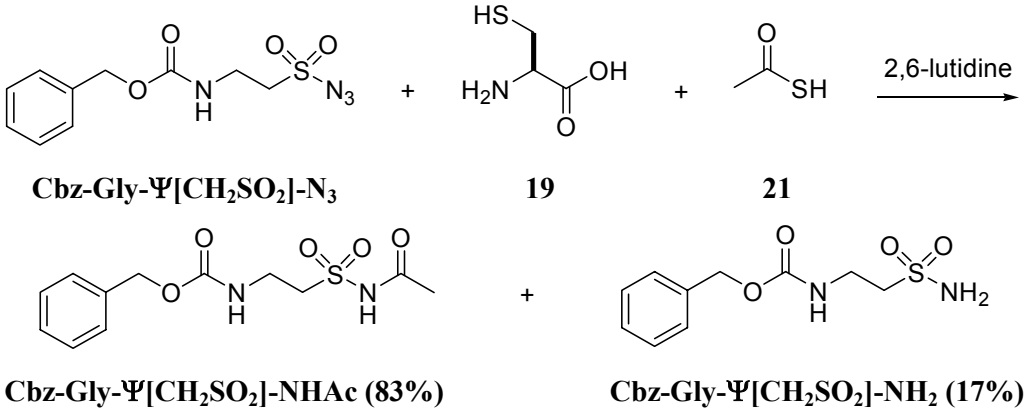
The aforementioned thiol-induced reduction of the sulfonyl azide moiety decreases the bioorthogonality of the thio acid/sulfonylazide amidation reaction which, in principle, could limit its application as chemoselective ligation methodology. Although the analogous reduction of aromatic azides by thiols has been described in the literature,[11] the influence of thiols on sulfonyl azides remains unresolved. Therefore, it was decided to investigate the stability of the sulfonyl azide moiety toward thiols. When Cbz-Gly-Ψ[CH_2SO_2]-N_3 was added to a five-fold excess of cysteine (**19**) in DMF, again the immediate evolution of N_2 (g) indicated the reduction of the sulfonyl azide moiety, as was confirmed by TLC-analysis (Scheme 8.6). Not surprisingly, when Cbz-Gly-Ψ[CH_2SO_2]-N_3 was added to a solution of cystine (**20**), no reduction of the azide took place. These results demonstrated that the thiol side chain of cysteine is indeed capable of reducing the sulfonyl azide moiety.



Scheme 8.6 Investigation of the thiol-induced reduction of sulfonyl azides.

The reduction of Cbz-Gly-Ψ[CH_2SO_2]-N_3 by a five-fold excess of cysteine was still incomplete after 2.5 hours, whereas the reaction of sulfonyl azides and thio acids proceeds almost instantaneously (chapter 5). Another test reaction was performed to investigate the selectivity of the amidation reaction over the thiol-induced reduction. In this experiment, an equimolar amount of cysteine (**19**) and thioacetic acid (**21**) were simultaneously added to a mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 and 2,6-lutidine in DMF (Scheme 8.7). Again, the evolution of N_2 (g) as well as the formation of a precipitate (S_8) were observed directly after addition of the thio species. After the evolution of N_2 had ceased, the reaction mixture was stirred for an additional 30 min before the solvent was removed and the crude product was analyzed by HPLC. It was found that *N*-acyl sulfonamide Cbz-Gly-Ψ[CH_2SO_2]-NHAc

and sulfonamide Cbz-Gly-Ψ[CH_2SO_2]-NH_2 were formed in a ratio of 4:1, which indicated that coupling of the thio acid was faster compared to the thiol-induced reduction of the sulfonyl azide. Probably, the selectivity of the reaction can be further increased by adjustment of the pH since under slightly acidic conditions, the thiocarboxylate group is the better nucleophile compared to the sulfhydryl group.[12,13] Moreover, the reduction of sulfonyl azides by thiols does not lead to the formation of a coupling product as is the case in the reaction with thio acids. Taken together, these results anticipate the application of the thio acid/sulfonyl azide amidation reaction as novel chemoselective selective ligation or bioconjugation method, also in the presence of free thiol (cysteine) moieties.



Scheme 8.7 Selectivity of the thio acid/sulfonyl azide amidation reaction over the thiol-induced reduction of sulfonyl azides.

8.2.3 Outlook

Synthesis of parallel peptide dimers using the thio acid/ sulfonyl azide amidation reaction

The biological function of proteins is closely related to their three-dimensional structure. In fact, alterations in protein folding may lead to prion-related illnesses such as bovine spongiform encephalopathy (mad cow disease) and Creutzfeldt-Jakob disease[14-16] or amyloid-related illnesses such as Alzheimer's Disease,[17,18] Parkinson's Disease[19,20] and type II diabetes.[21-23] All these diseases are associated with the uncontrolled aggregation of misfolded peptides/proteins. Although the proteins that form these amyloid deposits differ in their primairy sequence, they share a common structural motif: the (anti)parallel β-sheet. To gain insight into the sequence-stability relationship of β-sheet motifs, these motifs should be synthetically accessible. In contrast to an *antiparallel* β-sheet, a *parallel* β-sheet can not be exclusively created from α-amino acids. Therefore, many research-groups have explored non-peptide units that promote parallel sheet interactions by linking two peptide

segments via their *C*-termini using for example a short turn-inducing diamine.[24] Evidently, the formation of secondary structures like sheets, can seriously hinder the (stepwise) chemical synthesis of peptides. However, only few examples exist for the convergent synthesis of *β*-turn motifs.[24] Moreover, the chemical synthesis of parallel-linked peptide strand-segments via turn-inducing motifs by chemical ligation methods should provide fast access to structurally diverse compounds. Preferably, the ligation should take place in aqueous solvents since water is regarded as the most important solvent for peptide model studies.[25] All criteria mentioned can potentially be met by application of the thio acid/sulfonylazide amidation reaction as a chemical ligation method for the coupling of peptide segments to study the sequence-stability relationship of parallel *β*-sheets.

Figure 8.2 Proposed application of the thio acid/sulfonyl azide amidation reaction for the synthesis of *N*-acyl sulfonamide based parallel *β*-sheets. (A) Chain inversion in the *N*-acyl sulfonamide ligation product; (B) molecular structure of benzensulfonamide according to Petrov *et al.*[24]; (C) schematic representation of an *N*-acyl sulfonamide based parallel *β*-sheet.

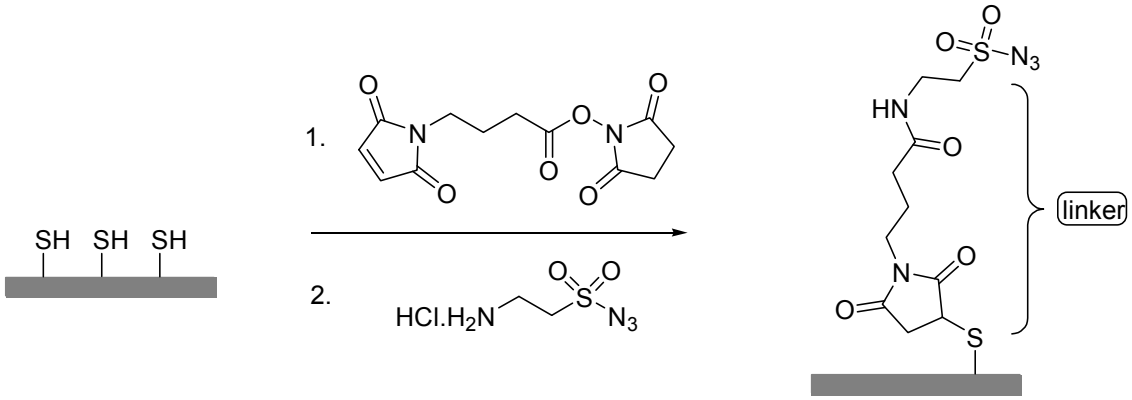
As mentioned earlier, thio acids and sulfonyl azides can be coupled to form an *N*-acyl sulfonamide linkage. Interestingly, the amidation reaction of peptide thio acids and peptide sulfonyl azides that contain the aminoethane sulfonyl azide building block features a chain reversal in the *N*-acyl sulfonamide ligation product (Figure 8.2A). In addition, the molecular structure and conformation of benzenesulfonamide was recently reinvestigated by Petrov *et al.*[24] Using theoretical methods, they found that the *S*-*N* bond is oriented perpendicular to the ring plane (Figure 8.2B). These results imply that the *N*-acyl sulfonamide moiety may act as a turn inducer. These distinctive characteristics are promising for the synthesis of parallel peptide dimers[26] and parallel sheets via chemoselective bioconjugation methods.

Site-selective peptide immobilization by the thio acid/sulfonyl azide amidation reaction

Besides the introduction of biophysical labels and coupling of additional peptide sequences, chemoselective ligation methods can also be used for the site-selective immobilization of functional peptides or proteins. In this regard, peptide microarrays are of great interest since they allow the rapid screening of thousands of molecular events in a single experiment.[27,28]

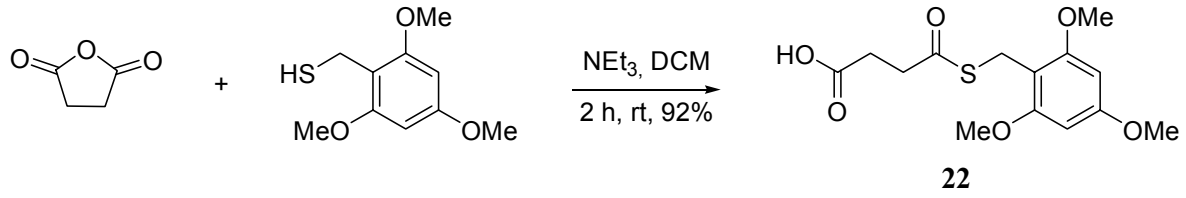
Today, peptide/protein microarrays form a key tool in proteomics, drug discovery and diagnostics.

The thioacid/sulfonyl azide amidation reaction may represent a novel chemoselective conjugation method for the site selective covalent immobilisation of peptides (or proteins). One envisaged example of such an approach is depicted in Scheme 8.8. Here, a commonly used chip, featuring a thiol functionalized surface, is successively treated with commercially available 4-maleimidobutyric acid succinimidyl ester and HCl.H-Gly-$\Psi[CH_2SO_2]$-N_3 to yield a sulfonyl azide functionalized microarray.



Scheme 8.8 Proposed synthesis of a sulfonyl azide functionalized microarray.

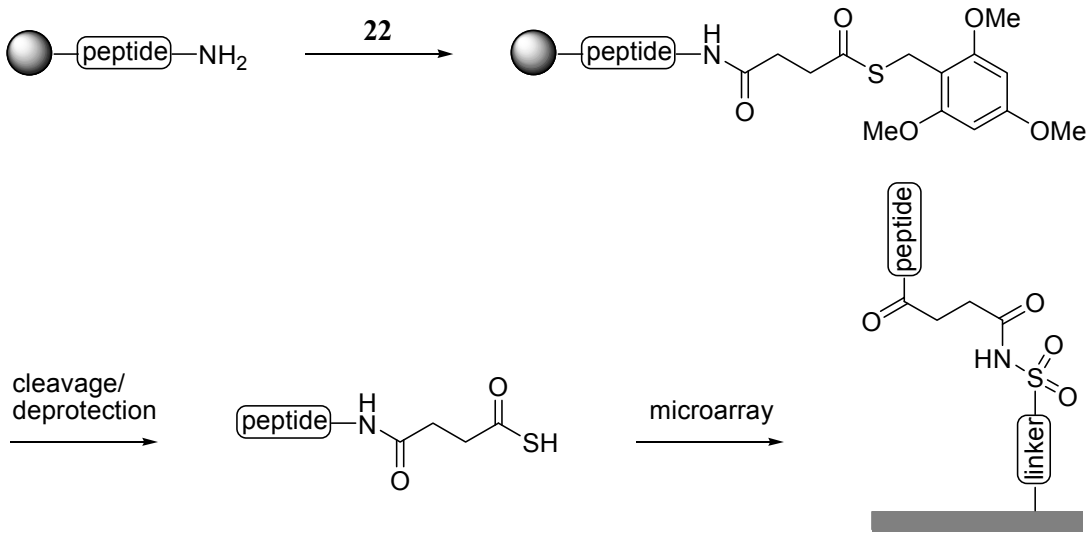
Next, peptides functionalized with a thio acids moiety should be accessible by SPPS methods using TMOB-thio ester building block **22** (Scheme 8.9). The synthesis of this TMOB-thio ester building block was investigated and it was found that **22** could readily be obtained from the reaction of succinic anhydride with TMOB-thiol in the prescence of TEA as base (Scheme 8.9).



Scheme 8.9 Synthesis of the mono TMOB-thio ester of succinic acid (**22**).

In the described strategy for the synthesis of peptide thio acids, the TMOB-ester moiety is introduced as the final step of the SPPS procedure. This means, in principle, the crude peptide thio acid may be used without further purification for reaction with the sulfonyl azide functionalized microarrays. Since potential (deletion) side products that lack the thio

acid moiety, will not couple to the surface, they can simply be washed away. Alternatively, the site selective introduction of the thio acid moiety to synthetic peptides can also achieved via a carboxylic acid moiety in the peptide side chain followed by esterification with the TMOB-thiol as was previously described in this chapter.



Scheme 8.10 Proposed strategy for SPPS synthesis and subsequent attachement to a sulfonyl azide functionalized microarray of peptide thio acids.

All described steps for the synthesis and subsequent coupling of peptide thio acids to sulfonyl azide functionalized microarrays, may be performed in aqueous buffers. These mild conditions are of great advantage for application in automated systems.

8.3 The thio acid/sulfonyl azide amidation reaction as a new synthetic approach to obtain multivalent dendrimeric peptides

8.3.1 Introduction

The combination of multiple relatively weak interactions to enhance affinity, is often referred to as multivalency. On a molecular scale, multivalency can be achieved by use of highly branched structures such as dendrimers. The molecular structure of dendrimers resembles tree-like architectures since they contain symmetrically arranged and radially connected branches arising from a central core. Repeated reaction sequences add a precise number of terminal groups at each step or generation.[29] With respect to the high local concentrations of drugs or molecular labels that can be created by suitably functionalized

dendrimers, these structures form attractive candidates for diverse biological and medical applications.[30-34]

For instance, in our group "amino (benzoic) acid" based dendrimers have been developed[35-37] and successfully used for, among others, the synthesis of multivalent carbohydrate dendrimers to increase the interaction of weakly interacting individual ligands.[38-42]

Methods for the conjugation of peptides, traditionally make use of activated esters which are non-selective and require suitably protected peptides. Instead, the use of mutually reactive groups enables the chemoselective (bio)conjugation of unprotected peptides which allows for a better solubility, isolation and characterization of the peptide preceding the conjugation reaction.

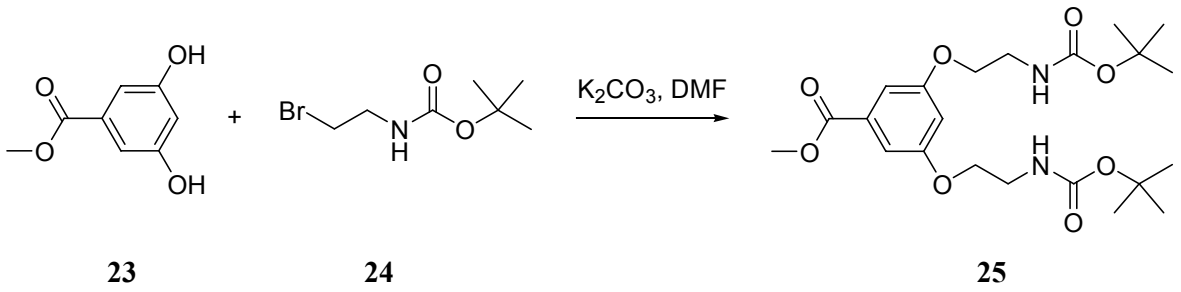
The procedures described in the literature for the chemoselective attachment of peptides to dendrimers mostly make use of the reaction of sulfhydryl groups of cysteine residues with maleimide or iodoacetamide functionalities,[43] a thiol-disulfide exchange, native chemical ligation[44-46] or a chemoselective oxime[47-49] respectively hydrazone ligation.[50] In some recent examples from our group, the copper-catalyzed 1,3-dipolar cycloaddition (click chemistry) was used for the coupling of cyclic-RGD azido peptides to an alkyne functionalized dendrimer scaffold to obtain multivalent peptide dendrimers for tumor targeting and tumor imaging purposes.[51,52]

Although the use of click chemistry has proven to be very efficient for the chemoselective attachment of synthetically easily accessible peptide derivatives in both aqueous and organic solvents, the need for a copper catalyst may involve some drawbacks. For instance, the metal ion might be coordinated by the peptide which could lead to a decreased efficiency of the click reaction and, moreover, result in a difficult removal of the catalyst during workup which is of special interest in case of biological relevant molecules since copper is known to be cytotoxic. Therefore, there is an interest in novel chemoselective ligation reactions for the efficient coupling of unprotected peptides. The thio acid/sulfonyl azide amidation reaction combines all previously mentioned advantages of the copper-catalyzed 1,3-dipolar cycloaddition whilst it is independent of any catalyst. Here, the applicability of this reaction on the coupling of peptides to a multivalent dendrimer scaffold is investigated.

8.3.2 Results and discussion

The prototype dendrimers that have been developed in our group are assembled from a two arm-containing building block (**25**, Scheme 8.11), referred to as a first generation dendrimer. This building block can be assembled by combining the corresponding

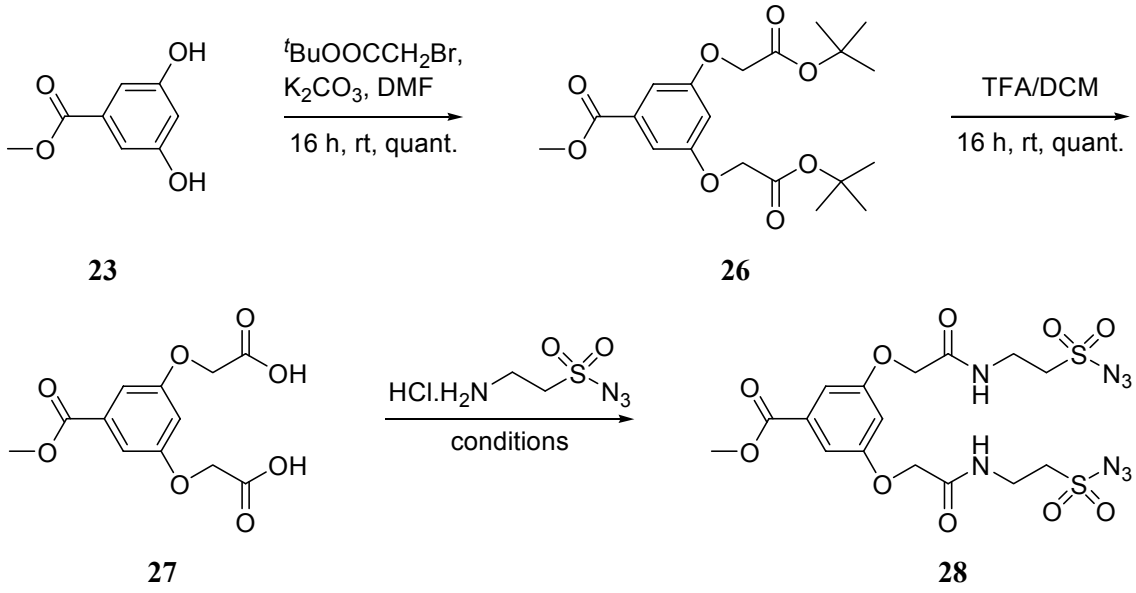
orthogonally protected dihydroxybenzoic acid methyl ester (**23**) and *N*-Boc-protected bromoethylamine (**24**) derivatives.[37]



23 **24** **25**

Scheme 8.11 Synthesis of dendrimer building block **25**.

Similarly, the synthesis of a sulfonyl azide functionalized building block was started from the dihydroxymethylbenzoate branching unit which was alkylated using *t*-butyl bromoacetate to give **26** in quantitative yield (Scheme 8.12). Subsequently, acidolysis with TFA afforded **27** which features two carboxylic groups available for coupling two equivalents of the sulfonylazide building block HCl.H-Gly-Ψ[CH_2SO_2]-N_3.



23 **26** **27** **28**

Scheme 8.12 Synthesis of divalent sulfonylazide **28**.

Initially, the coupling of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 was attempted with BOP in the presence of D*i*PEA, similar to the conditions used for the synthesis of Boc-Phe-Gly-Ψ[CH_2SO_2]-N_3 as described in chapter 5. Unexpectedly, **28** was obtained in only 3% yield, whereas Boc-Phe-Gly-Ψ[CH_2SO_2]-N_3 could be isolated in 85% yield (chapter 5). The reason for this discrepancy in yields was unclear. In order to improve the yield, other

coupling methods were investigated (Table 8.1). The best result was obtained with EDCI/HOBt as coupling reagents (entry 4) which afforded **28** in an improved, satisfactory, yield of 62%. In the end, the described method allowed for the straightforward preparation of divalent sulfonyl azide **28** in high purity.

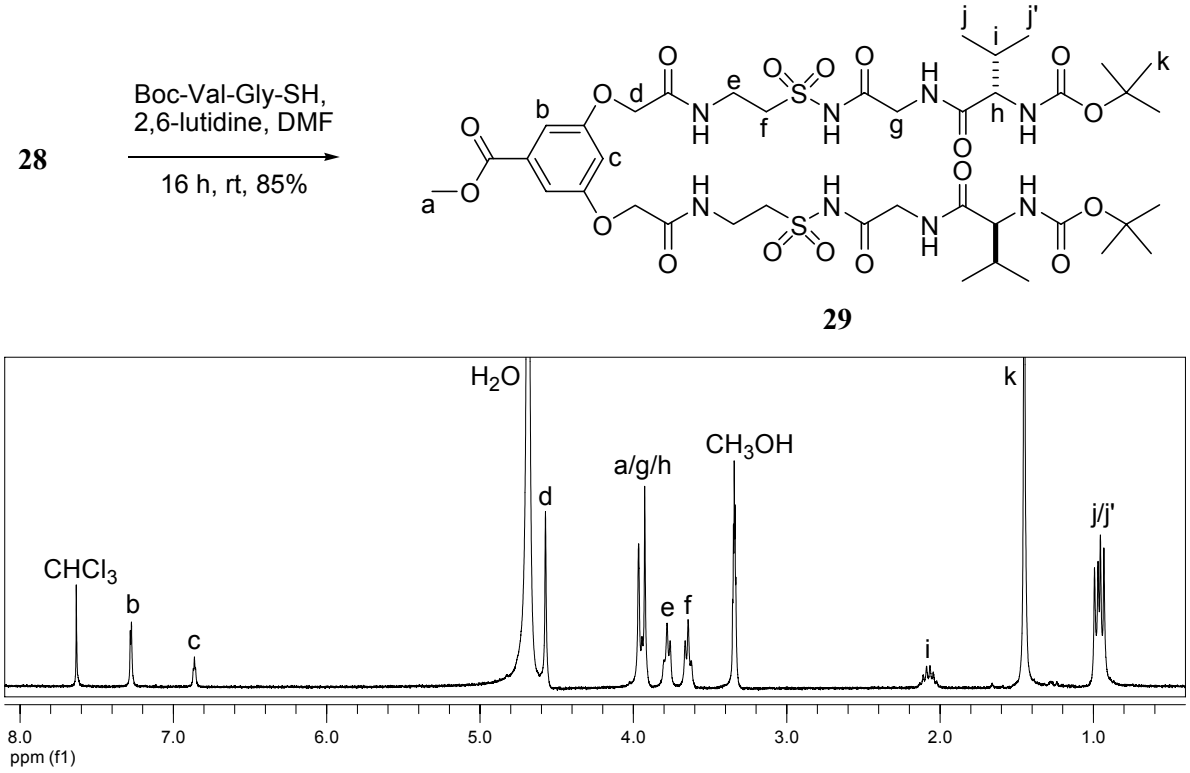
Table 8.1 Optimization for the reaction of **27** with HCl.H-Gly-Ψ[CH_2SO_2]-N_3 to form **28**.

Entry	Coupling method	Yield [%]
1	BOP[a]	3
2	Acid chloride[b]	5[c]
3	EDCI[d]	42
4	EDCI/HOBt[e]	62

[a]Reaction conditions: BOP (2.1 equiv), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (2 equiv), D*i*PEA (4.4 equiv), DMF, 16 h, rt. [b]Reaction conditions: $SOCl_2$/DCM (1:1), 16 h, rt – reflux, 64%; next: HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (2 equiv), D*i*PEA (2.2 equiv), DMF, 16 h, rt, 8%. [c]Yield calculated over two steps. [d]Reaction conditions: EDCI (3 equiv), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (2 equiv), D*i*PEA (3 equiv), DMF, 16 h, rt. [e]Reaction conditions: EDCI (2 equiv), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (2.5 equiv), HOBt (2.1 equiv), D*i*PEA (3 equiv), DMF, 16 h, rt.

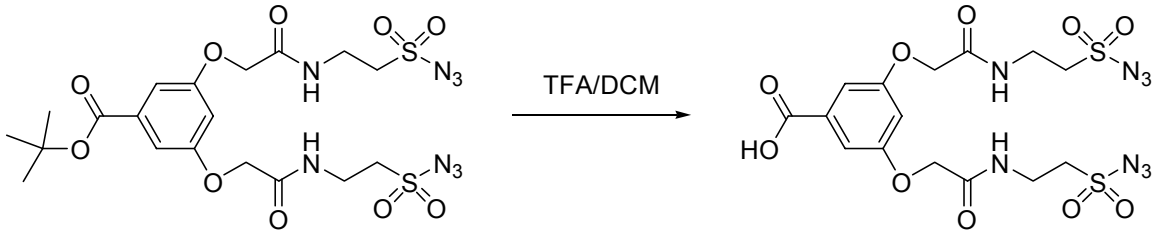
Next, the suitability of divalent sulfonyl azide **28** for the synthesis of peptide dendrimers was evaluated by the reaction with Boc-Val-Gly-SH. The reaction conditions used consisted of a small excess (2.6 equiv) of the thio acid and 2,6-lutidine in relation to the sulfonyl azide and DMF was used as solvent. After 16 h of stirring at room temperature, the *N*-acyl sulfonamide product (**29**) was obtained in a satisfactory yield of 85%. Its chemical identity was confirmed by ^{1}H NMR-spectroscopy (Scheme 8.13).

These results clearly demonstrated the value of the thio acid/sulfonyl azide amidation reaction for the chemoselective coupling of biologically relevant peptides to higher generation dendrimer scaffolds. However, sulfonyl azide **28** is not a suitable building block for the convergent synthesis of higher generation dendrimers. The intrinsic sensitivity of the sulfonyl azide moiety toward nucleophiles makes it not compatible with conditions needed for hydrolysis of the methyl ester moiety for coupling of additional molecules.



Scheme 8.13 Synthesis and ^{1}H NMR spectrum of divalent peptide dendrimer **29** (300 MHz, $CDCl_3/CD_3OD$, δ in ppm).

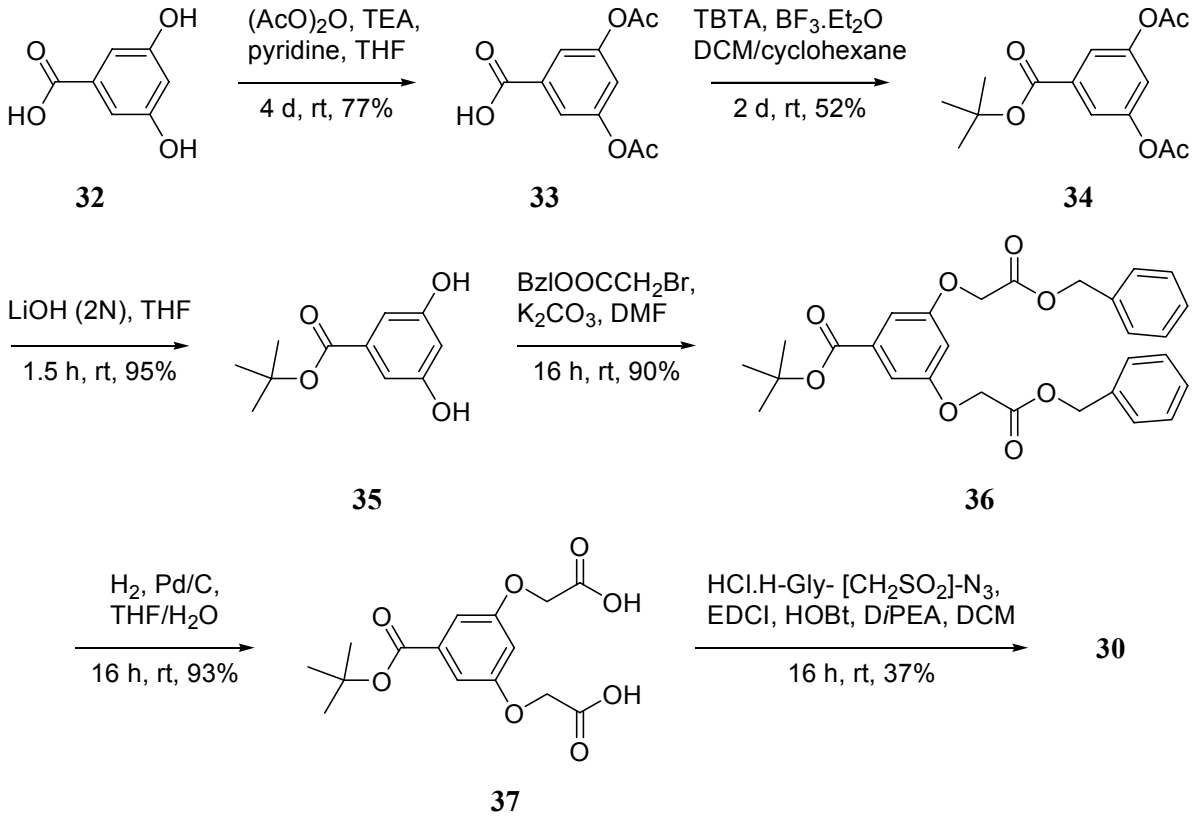
For that reason a new building block was envisaged that features a *t*-butyl ester rather than a methyl ester moiety (compound **30**, Scheme 8.14). In this case, cleavage of the ester can be achieved by acidolysis instead of saponification, which is well tolerated by the sulfonyl azide functionality.



30 **31**

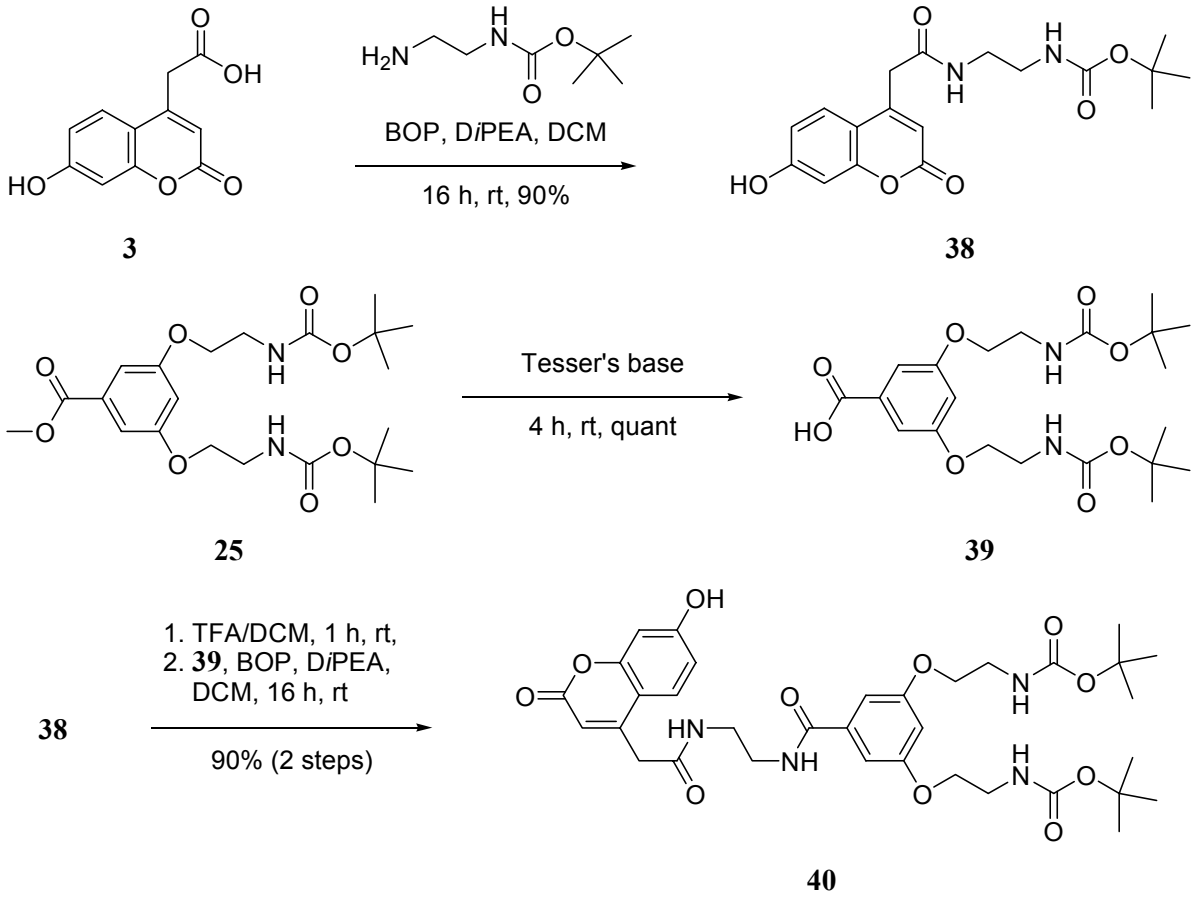
Scheme 8.14 Chemical structure of compounds **30** and **31**.

The synthesis of compound **30** starting from 3,5-dihydroxybenzoic acid (**32**) is depicted in Scheme 8.15. Firstly, the hydroxyl groups of **32** were temporarely protected by acylation. After the carboxylic acid moiety was converted into the corresponding *t*-butyl ester by reaction with *t*-butyl-2,2,2-trichloroacetimidate (TBTA),[53] the acetyl groups were removed by saponification. Subsequent reaction with benzyl bromoacetate afforded protected *tri*-ester **36**. Next, Pd/C-mediated hydrogenolysis gave *di*-acid **37**. Lastly, the sulfonyl azide moieties were introduced by coupling of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 using EDCI/HOBt to yield divalent sulfonyl azide building block **30**.



Scheme 8.15 Synthesis of divalent sulfonyl azide **30**.

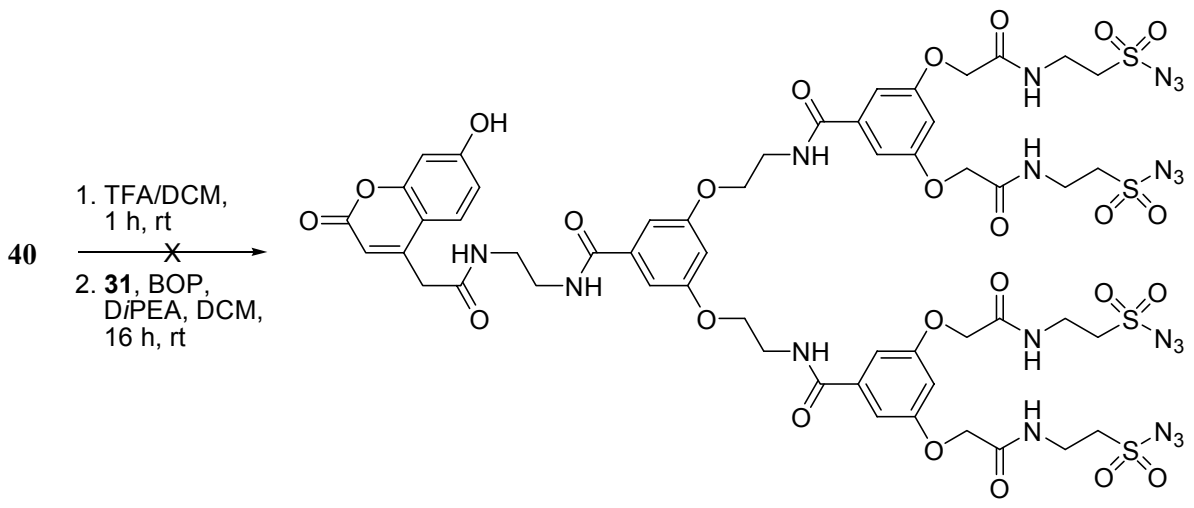
Compound **30** is a versatile building block since acidolysis of the *t*-butyl ester functionality gave access to a free carboxylic acid moiety, which was used for the synthesis of a second generation (tetravalent) dendrimer scaffold, equipped with a fluorescent label. The synthetic route that was followed is outlined in Scheme 8.16. Hydroxycoumarin was chosen as a fluorescent label, to be connected to a template core derived from **25**[37] via a diaminoethane spacer. Therefore, 7-hydroxycoumarin-4-acetic acid was coupled with *mono*-Boc-protected diaminoethane by BOP. After removal of the Boc-group coupling to **39** gave the fluorescently labeled dendrimer core **40**.



Scheme 8.16 Synthesis of fluorescently labeled divalent dendrimer core **40**.

Next, **30** and **40** were both treated with TFA to remove the protecting groups as was confirmed by TLC. Subsequently, it was tried to connect both deprotected building blocks via standard BOP-coupling. Trituration of the crude product with ice-cold methanol afforded a white solid which proved sparingly soluble in commonly used organic solvents such as $CHCl_3$, MeOH and acetone. However, the chemical identity of **41** could not be confirmed by NMR spectroscopy and/or mass spectrometry. The ^{1}H NMR spectrum showed broad signals and was inconclusive. Similarly, the HPLC chromatogram recorded with an UV detector (λ = 220 and 254 nm), consisted of a broad signal which was made up of many peaks whilst in case of an ELSD-detector, no signal could be detected at all. In addition, neither the ESMS nor the MALDI-TOF spectrum was indicative of the expected product (**41**, Scheme 8.17).

In conclusion, this section describes the successful synthesis of the first generation dendrimer **28** and its convenient conversion into a ligation product as represented by **29**. Unfortunately, however, higher generations of this type of dendrimers (such as **41**) were not obtained.

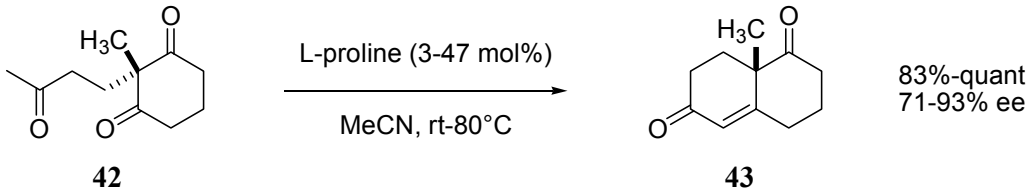


Scheme 8.17 Attempted synthesis of fluorescently labeled tetravalent sulfonyl azide **41**.

8.4 Synthesis and evaluation of potentional new proline-based N-acyl sulfonamide organocatalysts

8.4.1 Introduction

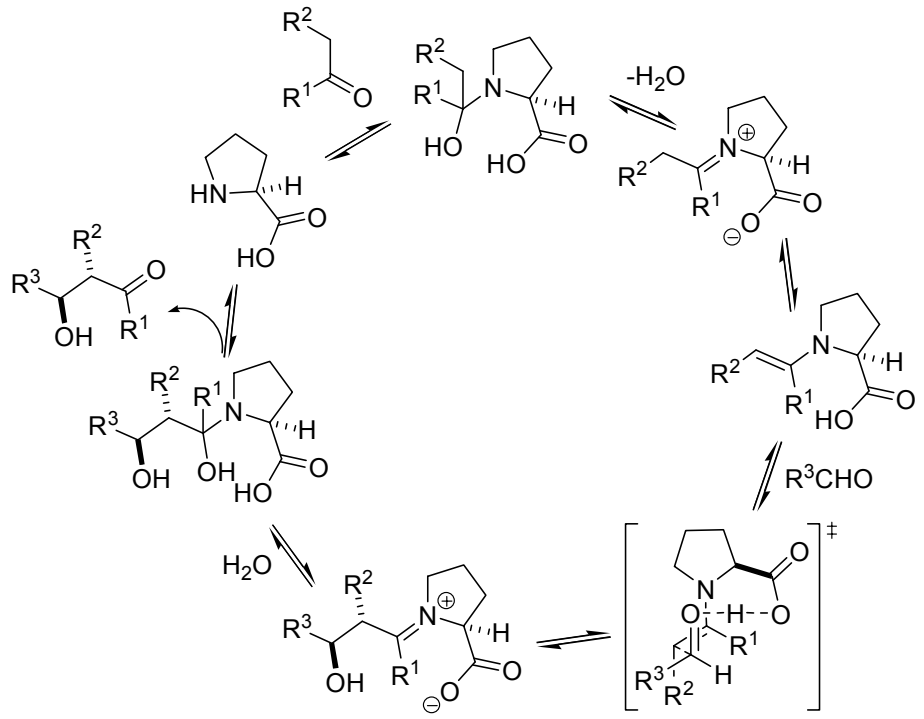
Compounds of biological interest, usually posses a certain amount of complexity such as chirality. Therefore, it has been a constant challenge to develop new methods for their asymmetric and/or enantioselective synthesis. Often these methods rely on catalysis to enable efficient and selective syntheses. Where Nature uses enzymes for asymmetric catalysis, organic chemists mostly make use of metal-based catalysts to obtain chiral compounds. Although transition metal catalysts are, for instance, very useful for asymmetric hydrogenation, they are often expensive, highly sensitive to oxygen and may possibly leave traces of toxic heavy metals in the product. In contrast, organocatalysts are purely organic and metal-free small molecules. Moreover, they are often non-toxic, stable, and in most cases relatively easy to synthesize. For these reasons, organonocatalysis is now recognized as an important topic that has received increased attention over the past few years.[54-58]



42 **43**

Scheme 8.18 The Hajos-Parsh-Eder-Sauer-Wiechert reaction.

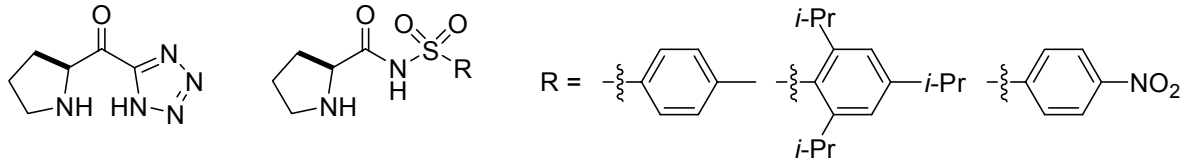
In one of the earliest examples of organocatalysis, reported more than three decades ago, the Hajos–Parrish–Eder–Sauer–Wiechert aldol reaction, proline was found to be effective in catalyzing the *intra*molecular asymmetric aldol cyclodehydration of the achiral ketone (**42**) to the unsaturated Wieland-Miescher ketone (**43**).[59,60] Thirty years later, a revival of this chemistry was initiated with the discovery of the proline-catalyzed direct asymmetric *inter*molecular aldol reaction.[61-63] the proposed mechanism is depicted in Scheme 8.19.[64]



Scheme 8.19 Proposed mechanism of the proline-catalyzed aldol reaction.

The aldol reaction is one of the most important *C–C* bond forming reactions in organic synthesis and following this discovery, in recent years, many successful examples of organocatalysis have been described in the literature.[64-69]

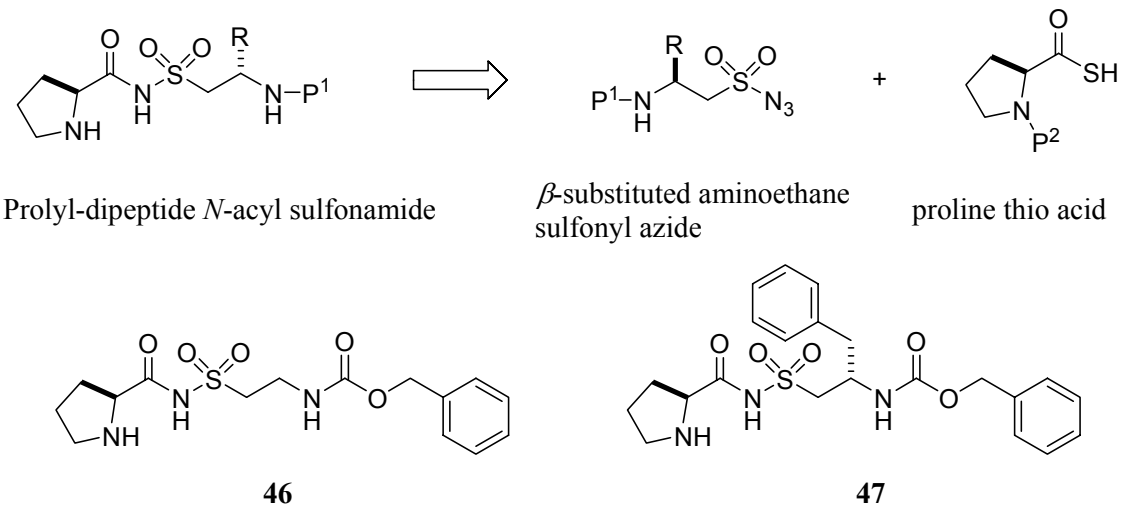
Although proline is an ideal catalyst in terms of price and availability, often-encountered drawbacks relate to low reactivity and low solubility of the catalyst in non-polar solvents. In addition, fine-tuning of the catalytic properties of proline by derivatization is difficult since the five-membered pyrrolidine ring and both the free carboxyl and the secondary amine function proved essential for effective catalysis. This led to the development of second generation catalysts, in which the carboxylic acid function of proline has been replaced by known bio-isosteres such as a tetrazole- (**44**) or an *N*-acyl sulfonamide moiety (**45**).[70-74]



44 **45**

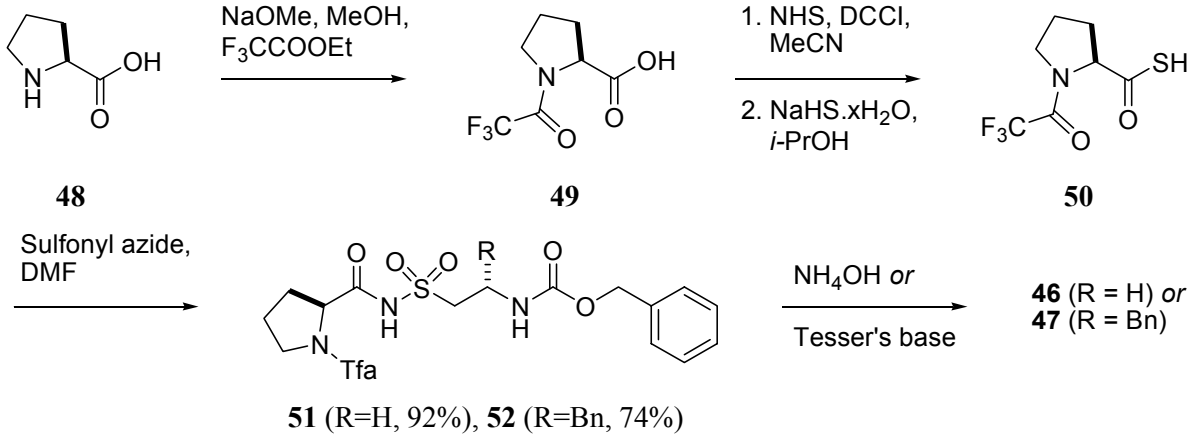
Figure 8.3 Chemical structures of known proline-derived tetrazole (**44**) and *N*-acyl sulfonamide (**45**) organocatalysts.

Compared to tetrazoles, *N*-acyl sulfonamides allow for more structural variation as was demonstrated recently by Berkessel and coworkers (Figure 8.3).[70] Although structural variation was achieved by using diverse sulfonyl amides, thus far no chiral sulfonyl amides have been applied. Combined with the fact that the coupling of sulfonamides with carboxylic acids using traditional methods often proceeds rather sluggish, this inspired us to use the thio acid/sulfonyl azide amidation reaction for the synthesis of *N*-acyl sulfonamide based prolyl-dipeptide derivatives as potential new organocatalysts for the asymmetric aldol reaction. The introduction of extra chirality via the coupling of an amino acid residue to the catalyst molecule may lead to a greater enantioselectivity compared to proline. For the proof-of-concept, two compounds (**46** and **47**, Scheme 8.20) were synthesized for evaluation of their catalytic properties in the model aldol reaction between acetone and *p*-nitrobenzaldehyde (Table 8.2).



Scheme 8.20 Retro-synthesis and chemical structure of compounds **46** and **47**.

First, the synthesis of **46** was attempted from Boc-Pro-SH[75] and Cbz-Gly-Ψ[CH_2SO_2]-N_3. However, the yield of the *N*-acyl sulfonamide product was only 31% after 16 h. In an effort to increase the yield, proline was protected at the N^{α}-moiety with a trifluoroacetyl- (Tfa) group rather than the more bulky Boc-group. In addition, the reaction time was extended to 72 h. When Tfa-Pro-SH (**50**)[76] was employed in this chemoselective amidation reaction, the product **51** (Scheme 8.21) could be obtained in an improved yield of 92%. Comparably, **52** was obtained by reacting **50** and Cbz-Phe-Ψ[CH_2SO_2]-N_3 in 74% yield. Finally, the trifluoroacetyl group was removed under basic conditions using NH_4OH or Tesser's base.[77]

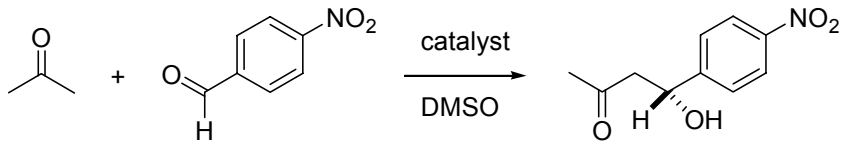


Scheme 8.21 Synthesis of *N*-acyl sulfonamides **46** and **47** starting from proline.

Next, the catalytic properties of **46** and **47** were tested in the aldol reaction between acetone (**53**) and *p*-nitrobenzaldehyde (**54**). The conditions used, consisted of an excess of acetone

and 20 mol% of the catalyst relative to the aldehyde and the results are summarized in Table 8.2.

Table 8.2 Results for the *N*-acyl sulfonamide catalyzed direct aldol reaction.[a,b]



53 **54** **55**

Entry	Catalyst	Time (h)	Yield (%)	ee (%)[c]
1[d]	L-proline	24	72	98
2	**46**	1	12	29
3	**46**	3	24	14
4	**46**	19	62	9
5	**46**	24	75	12
6	**46**	43	97	12
7	**47**	1	21	17
8	**47**	3	37	14
9	**47**	19	94	8
10	**47**	24	99	1

[a]The experimental data as presented in Table 8.2 were provided by A. Berkessel (University of Köln, Germany).
[b]Conditions: *p*-nitrobenzaldehyde (0.5 mmol), catalyst (20 mol%), 5 mL DMSO/acetone (4:1, v/v), rt.
[c] The enantiomeric excess (ee) was determined by HPLC analysis.
[d] Conditions: *p*-nitrobenzaldehyde (0.5 mmol), catalyst (30 mol%), 5 mL DMSO/acetone (4:1, v/v), rt.[70]

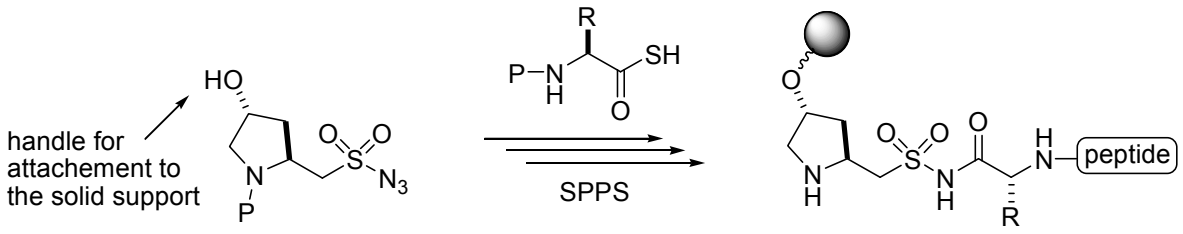
The data in Table 8.2 show that the newly synthesized *N*-acyl sulfonamide-based prolyl-dipeptide derived catalysts **46** and **47**, gave at least comparable (**46**, entry 5) or even better (**47,** entry 10) yields at lower catalyst loading compared to L-proline (entry 1). Initially, the reaction did exhibit some enantioselectivity, unfortunately, this was almost completely

diminished after prolonged reaction time resulting in a much lower enantioselectivity relative to the proline-catalyzed reaction.

8.4.2 Outlook

Apparently **46** and **47** possesed a suboptimal structure to obtain high enantioselectivity in the aldol reaction. However, the described method offers possibilities for great structural variation, meaning the catalytic properties can be tuned and eventually optimized. For instance, the sulfonyl azide building block can be derived from various (proteinogenic and non-proteinogenic) amino acids, also α-substituted aminoethane sulfonyl azides are accessible via substituted taurines.[78-80] In addition, the *N*-acyl sulfonamide organocatalysts that have been described in literature[70,71,73] should be available by employing simple aromatic and aliphatic sulfonyl azides.

Moreover, the strategy may also be reversed. Starting from a proline derived β-aminoethane sulfonyl azide, various (amino) thio acids can be coupled to obtain *N*-acyl sulfonamide-based β-homoproline derivatives.[81,82] In either case, further derivatization following the thio acid/sulfonyl azide amidation reaction may involve, among others, the coupling of additional amino acid residues and the attachment to a dendrimer scaffold or solid support. In case when more amino acid residues are introduced, the increased structural complexity of these peptidic catalysts could lead to an improved activity and selectivity.[83]



Scheme 8.22 Proposed methodology for the combinatorial synthesis of *N*-acyl sulfonamide based prolyl-peptide catalysts.

Since the purely rational design of peptidic catalysts is far from trivial, the best-suited candidates are best selected by combinatorial screening.[84-89] One example of such an approach is depicted in Scheme 8.22. Here, a hydroxyproline-derived sulfonyl azide is coupled to the solid support via its hydroxyl moiety. Next, the resin-bound prolyl sulfonyl azide can be reacted with amino thio acids followed by chain elongation using SPPS methods as was described in the previous chapter.[90]

8.5 Conclusions

Selective and bio-orthogonal conjugation methods that allow for the site selective modification of biomolecules under physiological conditions are still relatively rare. Therefore, there remains a constant interest in the development of new chemoselective ligation or (bio)conjugation methods.

In this respect, the chemoselective reaction of thiocarboxylic acids with sulfonyl azides is highly interesting since it is efficient in both organic and aqueous solvents. Moreover, a very stable and non-immunogenic *N*-acyl sulfonamide product is formed with non-toxic nitrogen and sulfur as the only side products. Here, the thio acid/sulfonyl azide amidation reaction was employed for the site selective modification of peptides with a fluorescent label or an additional peptide segment. The method was efficient for obtaining peptide conjugates under mild conditions using an aqueous buffer solution at physiological pH at ambient temperature. Hence, these examples illustrate the thio acid/sulfonyl azide amidation reaction as a new promising chemoselective ligation method, also in a biologically relevant environment. The bio-orthogonality of both reaction partners under these conditions however, should be investigated further.

The special characteristics of the thio acid/sulfonyl azide amidation reaction also resemble those of the ideal click reaction.[91] However, in contrast to the copper-catalyzed Huisgen 1,3-dipolar cycloaddition, the thio acid/sulfonyl azide amidation reaction is independent of any catalyst since the method is based on the intrinsic reactivity of both reaction partners. This is of particular interest for the synthesis of biologically relevant conjugates such as multivalent peptide dendrimers, since no (cyto)toxic reagents are needed that may be difficult to remove during workup. As a proof-of-concept, it was shown that the first generation dendrimer, functionalized with two sulfonyl azide moieties, efficiently reacted with a dipeptide thio acid to yield the divalent *N*-acyl sulfonamide peptide dendrimer.

The thio acid/sulfonyl azide amidation reaction yields an *N*-acyl sulfonamide moiety in the coupling product which is highly interesting since it acts as a carboxylic acid isostere. Here, two *N*-acyl sulfonamide-based prolyl-dipeptide organocatalysts were synthesized by reaction of the corresponding prolyl thio acid and β-substituted aminoethane sulfonyl azide. Both compounds were evaluated in the direct aldol reaction of *p*-nitrobenzaldehyde with acetone and showed high catalytic activity. Unfortunately, the catalysts did not lead to an enantioselective reaction. Nontheless, the described method may lead to novel *N*-acyl sulfonamide-based prolyl-peptide-derived catalysts with improved properties.

8.6 Experimental section

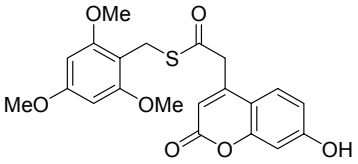
General information

Unless stated otherwise, all analytical HPLC runs were performed on a Shimadzu HPLC workstation equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 5 μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min, connected to a UV-VIS- (Shimadzu SPD-10Avp) or ELSD-detector (Polymer Laboratories PL-ELS 1000) unless stated otherwise. Electrospray ionization mass spectrometry was performed on a Shimadzu LCMS-QP8000 single quadrupole bench-top mass spectrometer operating in a positive ionization mode. Preparative HPLC was performed on a Gilson preparative HPLC system equipped with a reverse phase C8 column (Alltech Adsorbosphere XL C8, 90Å, 10 μm, 250 × 22 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 40 min at a flow rate of 5 mL/min. Microwave-assisted syntheses were conducted in closed reaction vessels using a Biotage Initiator microwave reactor equipped with a temperature and pressure sensor for monitoring the reaction conditions. ^{1}H NMR spectra were recorded on a Varian G-300 (300 MHz) or a Varian INOVA-500 (500 MHz) spectrometer and chemical shifts are given in ppm (δ) relative to TMS (0.00 ppm). ^{13}C NMR spectra were recorded on a Varian G-300 (75.5 MHz) or a Varian INOVA-500 (125 MHz) spectrometer and chemical shifts are given in ppm relative to $CDCl_3$ (77.0 ppm). The ^{13}C NMR spectra were recorded using the attached proton test (APT) sequence. R_f values were determined by thin layer chromatography (TLC) on Merck precoated silicagel 60F^{254} plates. Spots were visualized by UV-quenching, ninhydrin or Cl_2/TDM.[92]

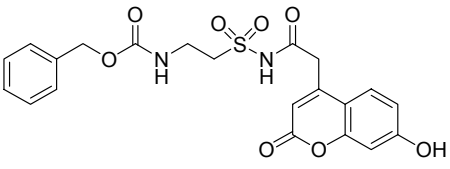
Chemicals and reagents

The coupling reagent 1H-benzotriazol-1-yl-oxy-tris-(dimethylamino)phosphonium hexafluorophosphate (BOP) was obtained from Richelieu Biotechnologies Inc. *t*-Butyl oxycarbonyl (Boc) and carbobenzoxy (Cbz) amino acids were obtained from MultiSynTech. Peptide grade dichloromethane (DCM), *N*,*N*-dimethylformamide (DMF), *N*-methylpyrrolidone (NMP), trifluoroacetic acid (TFA), acetonitrile (MeCN), ethyl acetate (EtOAc) and hexane were purchased from Biosolve. *N*,*N*-Diisopropylethylamine (D*i*PEA), $Pd(Ph_3P)_4$, triisopropylsilane (T*i*S), $PhSiH_3$, ethyl trifluoroacetate and diethyldithiocarbamate were obtained from Acros Organics, acetic acid (HOAc) and isopropanol (*i*-PrOH) purchased from Merck KGaA and Pd/C and *p*-nitrobenzaldehyde

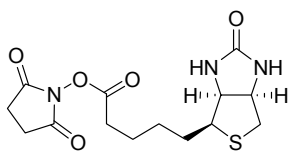
were obtained from Fluka. 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) was obtained from Aldrich.



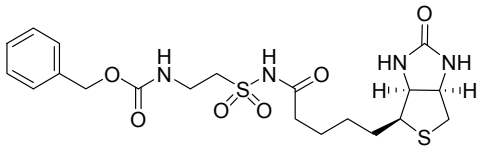
7-Hydroxycoumarin-4-acetic acid TMOB-thio ester 4: EDCI (121 mg, 0.63 mmol) and trimethoxybenzylmercaptane (141 mg, 0.66 mmol) were consecutively added to an ice cold solution of 7-hydroxycoumarin-4-acetic acid (132 mg, 0.6 mmol) in DMF (5 mL). The reaction mixture was stirred overnight at room temperature. After concentration, the residue was taken up in EtOAc (25 mL) and the solution was washed with 1N $KHSO_4$ (3 × 10 mL), brine (1 × 10 mL), 5% $NaHCO_3$ (3 × 10 mL) and brine (1 × 10 mL) and dried over Na_2SO_4. The product (139 mg, 56%) was isolated as a white solid by crystallization from EtOAc /$CHCl_3$/hexane. R_f: 0.25 (EtOAc/hexane 1:1 v/v); R_t: 16.48 min; ^{1}H NMR (DMSO-d_6): δ 10.62 (s, 1H), 7.53 (d, J = 8.7 Hz, 1H), 6.82 (dd, J = 6.7 Hz, J = 2.3 Hz, 1H), 6.73 (d, J = 2.3 Hz, 1H), 6,24 (s, 1H), 6.22 (s, 2H), 4.18 (s, 2H), 4.08 (s, 2H), 3.76 (s, 3H), 3.74 (s, 6H), ^{13}C NMR (DMSO-d_6): δ 194.7, 161.3, 160.7, 159.9, 158.5 (double line), 155.0, 149.3, 126.8, 112.9, 112.3, 111.0, 102.9, 102.3, 90.7 (double line), 55.8 (double line), 55.2, 45.5, 21.8; ESMS calcd for $C_{21}H_{20}O_7S$: 416,09, found: 439.05 $[M+Na]^+$.



***N*-Acyl sulfonamide 5:** 47 mg (0.11 mmol) of **4** was dissolved in TFA/T*i*S (4 mL, 19:1 v/v) and stirred for 3 h at room temperature. When removal of the TMOB-group was complete, as confirmed by TLC (eluent: EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v, thio acid R_f: 0.40), the solvent was removed under high vacuum. The crude thio acid was taken up in DMF (2 mL) and added dropwise to a mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (22 mg, 0.07 mmol) and an excess of 2,6-lutidine (100 μL) in DMF (0.5 mL). The reaction mixture was stirred for 16 h at room temperature. After concentration, the product was isolated by column chromatography (gradient: EtOAc/hexane/AcOH 49.5:49.5:1 v/v/v → EtOAc/hexane/AcOH 89.5:9.5:1 v/v/v) to afford **5** (29 mg, 91%) as a white solid. R_f: 0.25 (EtOAc/hexane/AcOH 89.5:9.5:1 v/v/v); R_t: 14.38 min; ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.47 (d, J = 8.7 Hz, 2H), 7.17 (m, 5H), 6.67 (d, J = 2.4 Hz, 1H), 6.61 (d, J = 2.4 Hz, 1H), 6.29 (s, 1H), 6.02 (s, 1H), 4.93 (s, 2H), 3.57 (s, 2H), 3.43 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 168.0, 161.9, 161.4, 156.6, 155.2, 148.4, 135.9, 128.3, 127.9, 127.7, 125.8, 113.4, 112.5, 111.3, 102.9, 66.7, 52.3, 39.1, 35.2, 29.4; ESMS calcd for $C_{21}H_{20}N_2O_8S$ 460.09, found: 483.20 $[M+Na]^+$.



Biotin HONSu-ester 8:[5] Biotin (500 mg, 2.05 mmol) and NHS (236 mg, 2.05 mmol) were dissolved in hot DMF (6 mL) and DCCI (422 mg, 2.05 mmol) was added. The resulting mixture was stirred for 2 h at room temperature, the precipitate was removed by filtration, and the filtrate was evaporated *in vacuo*. The residue was triturated with ether and crystallized from *i*-PrOH to yield the product (554 mg, 79%) as a white solid. R_f: 0.63 ($CHCl_3$/MeOH/HOAc 81:17:3 v/v/v); R_t: 12.18 min; ^{1}H NMR (DMSO-d_6): δ 6.47 (s, 1H), 6.40, (s, 1H), 4.29 (m, 1H), 4.15 (m, 1H), 3.12 (m, 1H), 2.81 (br s, 4H), 2.70 – 2.56 (m, 4H), 1.65 (m), 1.44 (m, total 6H); ^{13}C NMR (DMSO-d_6): δ 170.2 (double line), 168.9, 162.7, 60.9, 59.1, 55.2, 29.9, 27.8, 27.5, 25.4 (double line), 24.3 (double line); ESMS calcd for $C_{14}H_{19}N_3O_5S$ 341.10, found 342.15 $[M+H]^+$.

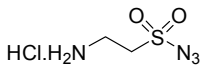


***N*-Acyl sulfonamide 9:** 58 mg (0.17 mmol) of **8** was dissolved in hot *i*-PrOH (10 mL) and added to a solution of $NaHS.xH_2O$ (29 mg, 0.34 mmol) in *i*-PrOH (15 mL). After 3 h of stirring the conversion was complete, as confirmed by TLC (eluent: EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v, **2** R_f: 0.48). The reaction mixture was quenched with 1N HCl until pH = 1 and the reaction mixture was concentrated to dryness. The residue was triturated twice with cold H_2O and once with EtOAc to give **2** (39 mg, 89%) as a white solid. The crude thio acid was taken up in DMF (1 mL) and added dropwise to a mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (28 mg, 0.10 mmol) and an excess of 2,6-lutidine (100 μL) in DMF (0.5 mL). The reaction mixture was stirred for 2 h at rt before the solvent was removed. The product was isolated by column chromatography (gradient: MeOH/$CHCl_3$/HOAc 4.5:94.5:1 v/v/v → MeOH/$CHCl_3$/HOAc 9.5:89.5:1 v/v/v) to afford **9** (53 mg, 73%) as a white solid. R_f: 0.30 (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v); ^{1}H NMR (CD_3OD): δ 7.34 (m, 5H), 5.09 (s, 2H), 4.47 (m, 1H), 4.30 (m, 1H), 3.56 (s, 4H), 3.20 (m, 1H), 2.92 (dd, J = 5.0 Hz, J = 12.8 Hz, 1H), 2.70 (d, J = 12.8 Hz, 1H), 2.31 (m, 2H), 1.66 (m), 1.44 (m, total 6H); ^{13}C NMR (CD_3OD): δ 175.6, 166.1, 158.5, 138.2, 129.5, 129.1, 128.9, 67.7, 63.4 (triple line), 61.7, 56.9, 53.0, 41.1, 37.2, 36.8, 29.6, 29.5, 25.7; ESMS calcd for $C_{20}H_{28}N_4O_6S_2$ 484.15, found 507.00 $[M+Na]^+$.

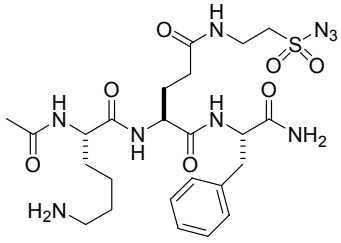
General procedure for the selective removal of the allyl protective group and subsequent coupling of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 to resin-bound peptides.

A portion of the peptide resin (~0.12 mmol) was allowed to swell in NMP (5 mL) for 5 min by gentle agitation under Ar (g). To this suspension $PhSiH_3$ (492 μL, 2.40 mmol) and $Pd(PPh_3)_4$ (69 mg, 0.06 mmol) were added and the resin was agitated for an additional 45

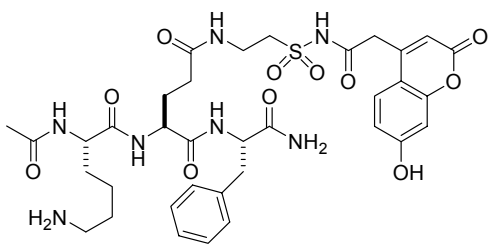
min. The resin was subsequently washed with NMP (5 mL, 3 × 2 min), 1% diethyldithiocarbamate (5 mL, 1 × 2 min), 20% D*i*PEA in NMP (5 mL, 1 × 2 min), NMP (5 mL, 3 × 2 min) and DCM (5 mL, 3 × 2 min). All coupling and washing steps were repeated once and removal of the allyl-group was confirmed by a Malachite green test.[9] Then, a solution of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (2 equiv), BOP (2 equiv) and D*i*PEA (6 equiv) in NMP (5 mL) was added to the resin, and the obtained reaction mixture was shaken for 16 h. The resin was washed with NMP (5 mL, 3 × 2 min), *i*-PrOH (5 mL, 1 × 2 min) and DCM (5 mL, 3 × 2 min).



HCl.H-Gly-Ψ[CH_2SO_2]-N_3: The synthetic procedure and analytical data for this compound have been described in chapter 5.

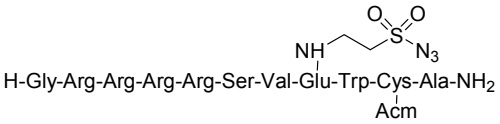


Ac-Lys-Glu(Gly-Ψ[CH_2SO_2]-N_3)-Leu-NH_2 (12): The peptide sequence Fmoc-Lys(Boc)-Glu(OAll)-Leu-NH-Rink Amide Tentagel™ was synthesized manually using standard Fmoc/^{t}Bu SPPS protocols with BOP and D*i*PEA as coupling reagents (~0.12 mmol scale). Selective removal of the allyl protecting group and subsequent coupling of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 was carried out following the general procedure. The product was simultaneously detached from the resin and deprotected by treatment with TFA/H_2O/T*i*S (5 mL, 95:2.5:2.5 v/v/v) for 3 h followed by precipitation with MTBE/hexane 1:1 v/v at −20°C. The crude product was purified by preparative HPLC and finally lyophilized from MeCN/H_2O 1:1 v/v to yield **12** (42 mg, 58%) as a white solid. R_t: 11.28 min; ESMS calcd for $C_{24}H_{37}N_9O_7S$: 595.25, found: 596.80 $[M+H]^+$.

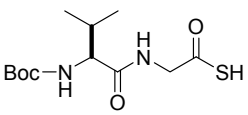


***N*-Acyl sulfonamide 13:** 21 mg (0.05 mmol) of **4** was dissolved in TFA/T*i*S (4 mL, 19:1 v/v) and stirred for 3 h at room temperature. When removal of the TMOB-group was complete, as confirmed by TLC (eluent: EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v, thio acid R_f: 0.40), the solvent was removed under high vacuum. The crude thio acid was taken up in DMF (600 µL) and added dropwise to a mixture of **12** (15 mg, 0.25 mmol) in an excess of 2,6-lutidine (20 µL) with DMF (0.5 mL) as solvent. The reaction mixture was stirred for 16 h at room temperature. After concentration, the product was isolated by preparative HPLC and finally lyophilized

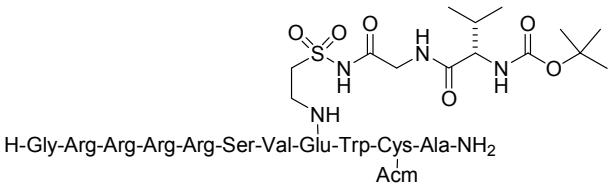
from MeCN/H_2O (1:1 v/v) to give a white solid (15 mg, 80%). R_t: 12.74 min; ESMS calcd for $C_{35}H_{47}N_7O_{11}S$: 773.31, found: 772.10 $[M+H]^+$.



H-Gly-Arg-Arg-Arg-Arg-Ser-Val-Glu(Gly-Ψ[CH_2SO_2]-N_3)-Trp-Cys(Acm)-Ala-NH_2 (17): The peptide sequence H-Gly-Arg-Arg-Arg-Arg-Ser-Val-Glu(Gly-Ψ[CH_2SO_2]-N_3)-Trp-Cys(Acm)-Ala-NH-Rink Amide Tentagel™ was synthesized manually using conventional Fmoc/^{t}Bu SPPS protocols with HBTU, HOBt and D*i*PEA as coupling reagents (~0.24 mmol scale) including the use of Fmoc-Glu(OAll)-OH. Selective removal of the allyl protecting group and subsequent coupling of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 was carried out following the general procedure. The product was simultaneously detached from the resin and deprotected by treatment with TFA/H_2O/T*i*S (5 mL, 95:2.5:2.5 v/v/v) for 3 h followed by precipitation with MTBE/hexane 1:1 v/v at –20°C. The crude product was purified by preparative HPLC and finally lyophilized from MeCN/H_2O 1:1 v/v to yield **17** as a white solid. R_t: 26.35 (Alltech Prosphere C18, 300Å, 5 μm, 250 × 4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/MeCN 95:5 v/v) to 30% buffer B (0.1% TFA in MeCN/H_2O 95:5 v/v) in 34 min at a flow rate of 1 mL/min); ESMS calcd for $C_{61}H_{104}N_{30}O_{16}S_2$: 1578.80 $[M_{avg}+H]^+$, found: 791.38 $[M+2H]^{2+}$.



Boc-Val-Gly-SH: The synthetic procedure and analytical data for this compound have been described in chapter 4.

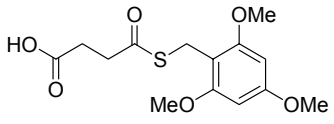


H-Gly-Arg-Arg-Arg-Arg-Ser-Val-Glu(Gly-Ψ[CH_2SO_2]-NH-Gly-Val-Boc)-Trp-Cys(Acm)-Ala-NH_2 (18): 4 mg of Boc-Val-Gly-SH (14 μmol) was added to a solution of **17** (10 mg, 6 μmol) in HEPES buffer (2 mL, pH = 7.4). The reaction mixture was stirred for 1 h at rt and a sample of 80 μL was withdrawn, diluted with 0.5 mL MeCN/H_2O (1:1 v/v), and analysed immediately by HPLC-MS. Conversion was determined by comparison of the retention times of the starting materials: Boc-Val-Gly-SH (R_t: 32 min); **17** (R_t: 35 min); ESMS calcd for $C_{73}H_{126}N_{30}O_{20}S_2$: 1809.10 $[M_{avg}+H]^+$, found: 905.47 $[M+2H]^{2+}$.

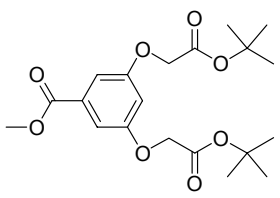
Reaction of Cbz-Gly-Ψ[CH_2SO_2]-N_3 with cysteine: Cysteine (12 mg, 88 μmol) was added to a solution of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (5 mg,18 μmol) in DMF (0.5 mL). The

reaction mixture was stirred for 2.5 h at rt and analyzed by TLC (eluent EtOAc/hexane 3:2 v/v). Reduction of Cbz-Gly-Ψ[CH_2SO_2]-N_3 was determined by comparison of the retention factors of sulfonyl azide (R_f: 0.65) and sulfonamide Cbz-Gly-Ψ[CH_2SO_2]-NH_2 (R_f: 0.16).

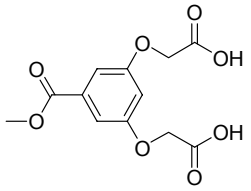
Reaction of Cbz-Gly-Ψ[CH_2SO_2]-N_3 with thio acetic acid in the presence of cysteine: A solution of cysteine (54 mg, 0.39 mmol) in DMF (0.5 mL) and a solution of thioacetic acid (28 μL, 0.39 mmol) were added simultaneously to a mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (85 mg, 0.30 mmol) in DMF (2 mL). The reaction mixture was allowed to stir for 30 min at rt before the solvent was removed. The crude product was analysed by HPLC (Alltech Altima C8, 90Å, 5μm, 250×4.6 mm) using a linear gradient of 100% buffer A (0.1% TFA in H_2O/CH_3CN 95:5 v/v) to 100% buffer B (0.1% TFA in CH_3CN/H_2O 95:5 v/v) in 20 min at a flow rate of 1 mL/min. Conversion was determined by comparison to the retention times of Cbz-Gly-Ψ[CH_2SO_2]-NAc (R_t: 14.33 min) and Cbz-Gly-Ψ[CH_2SO_2]-NH_2 (R_t: 13.52 min).



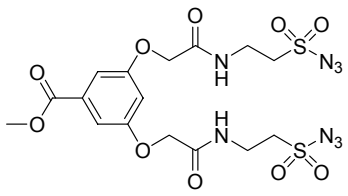
Succinic acid TMOB-thio ester 22: Under a nitrogen atmosphere 459 mg (2 mmol) of TMOB-thiol was added to a mixture of succinic anhydride (403 mg, 4 mmol) and TEA (832 μL, 6 mmol) in DCM (30 mL). The reaction mixture was stirred for 2 h at rt under a nitrogen atmosphere. Next, the reaction mixture was extracted with H_2O (60 mL, once). The aqueous layer was acidified with 1N $KHSO_4$ (pH = 1) and extracted with DCM (3 × 20 mL). The combined organic layers were combined and dried over Na_2SO_4. The product was isolated by crystallization with $CHCl_3$/hexane to afford **22** (576 mg, 92%) as a white solid. R_f: 0.56 (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v); ^{1}H NMR (DMSO-d_6): δ 6.22 (s, 2H), 4.05 (s, 2H), 3.76 (br s, 9H), 2.77 (m, 2H), 2.50 (m, 2H).



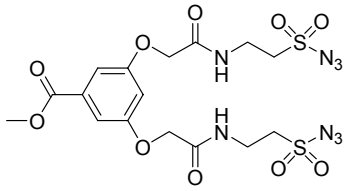
Compound 26: K_2CO_3 (6.90 g, 50 mmol) and *t*-butyl bromoacetate (7.38 mL, 50 mmol) were successively added to a solution of **23** (3.36 g, 20 mmol) in DMF (220 mL) and the reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was suspended in EtOAc (500 mL) and washed with 1N $KHSO_4$ (2×250 mL), brine (1×250 mL), 5% $NaHCO_3$ (2×250 mL) and brine (1×250 mL) and dried over Na_2SO_4. The product (7.89 g, 100%) was isolated as a clear yellow oil that solidified upon standing. R_f: 0.78 (EtOAc/hexane 3:2 v/v); R_t: 19.1 min; ^{1}H NMR ($CDCl_3$): δ 7.18 (d, J = 2.5 Hz, 2H), 6.72 (m, 1H), 4.53 (s, 4H), 3.89 (s, 3H), 1.49 (s, 18H); ^{13}C NMR ($CDCl_3$): δ 167.4, 166.2, 158.8, 132.0, 118.3, 108.4, 107.2, 82.5, 65.7, 52.1, 27.9.



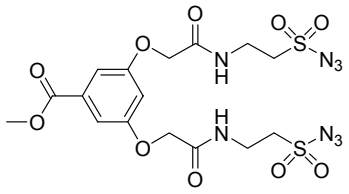
Compound 27: 7.66 g (19.3 mmol) of **26** was dissolved in TFA/DCM (400 mL, 1:1 v/v) and stirred for 16 h at room temperature. The solvent was removed under reduced pressure and the residue was coevaporated with $CHCl_3$ to obtain the product (5.66 g) as a white solid in a quantitative yield. *Rf*: 0.45 (EtOAc/hexane 3:2 v/v); ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.23 (d, *J* = 2.5 Hz, 2H), 6.78 (m, 1H), 4.64 (s, 4H), 3.91 (s, 3H).



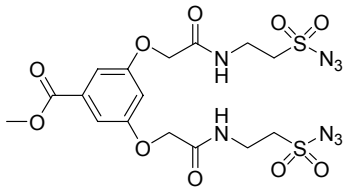
Sulfonyl azide 28, entry 1 (Table 8.1): To a solution of **27** (1.42 g, 5 mmol) in DMF (75 mL), BOP (4.64 g, 10.5 mmol), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (1.87 g, 10 mmol) and D*i*PEA (4 mL, 22 mmol) were successively added. The reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was suspended in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL), brine (1 × 50 mL), 5% $NaHCO_3$ (2 × 50 mL) and brine (1 × 50 mL) and dried over Na_2SO_4. The product (81 mg, 3%) was isolated as a white solid by flash column chromatography (eluent: EtOAc/hexane 9:1 v/v). *Rf*: 0.22 (EtOAc/hexane 9:1 v/v); ^{1}H NMR ($CDCl_3$): δ 7.28 (d, *J* = 2.5 Hz), 7.12 (br s, 2H), 6.73 (m, 1H), 4.56 (s, 4H), 3.93 (m, 7H), 3.60 (m, 4H)



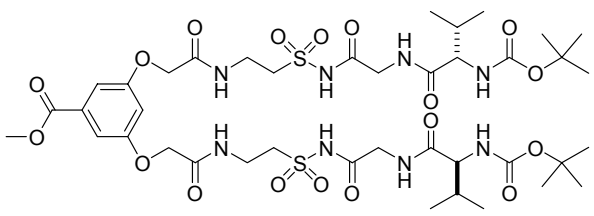
Sulfonyl azide 28, entry 2: To a solution of **27** (284 mg, 1 mmol) in DCM, thionylchloride (20 mL) was added and the resulting reaction mixture was first stirred for 16 h at room temperature followed by an additional hour under reflux. The solvent was removed under reduced pressure and the residue was coevapporated with $CHCl_3$. The acid chloride (207 mg, 64%) was isolated as a light yellow solid by crystallization from DCM/hexane. *Rf*: 0.45 (EtOAc/hexane 3:2 v/v); ^{1}H NMR ($CDCl_3$): δ 7.24 (d, *J* = 2.5 Hz, 2H), 6.71 (m, 1H), 4.94 (s, 4H), 3.92 (s, 3H). The crude acid chloride was added to a solution of HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (166 mg, 1.3 mmol) and D*i*PEA (356 µL, 1.4 mmol) in DMF (5 mL). The reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was suspended in EtOAc (50 mL) and washed with 1N $KHSO_4$ (2 × 25 mL), brine (1 × 25 mL), 5% $NaHCO_3$ (2×25 mL) and brine (1 × 25 mL) and dried over Na_2SO_4. The product (27 mg, 8%) was isolated as a white solid by flash column chromatography (eluent: EtOAc/hexane 9:1 v/v). *Rf*: 0.23 (EtOAc/hexane 9:1 v/v); ^{1}H NMR ($CDCl_3$): δ 7.27 (m, 2H), 7.20 (t, *J* = 6.0 Hz, 2H), 6.73 (m, 1H), 4.55 (s, 4H), 3.92 (m, 7H), 3.62 (m, 4H).



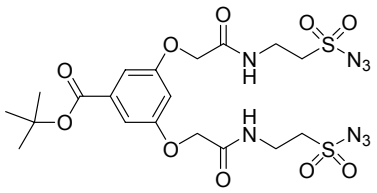
Sulfonyl azide 28, entry 3: To an ice-cold solution of **27** (284 mg, 1 mmol) in DMF (25 mL), EDCI (575 mg, 3 mmol), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (373 mg, 2.0 mmol) and D*i*PEA (500 μL, 2.5 mmol) were successively added. The reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was suspended in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL), brine (1 × 50 mL), 5% $NaHCO_3$ (2 × 50 mL) and brine (1 × 50 mL) and dried over Na_2SO_4. The product (790 mg, 42%) was isolated as a white solid by flash column chromatography (eluent: EtOAc). *Rf*: 0.34 (EtOAc); ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.28 (d, *J* = 2.5 Hz, 2H) 6.82 (m, 1H), 4.57 (s, 4H), 3.93 (s, 3H), 3.86 (m, 4H), 3.68 (m, 4H).



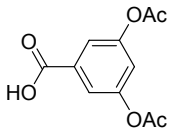
Sulfonyl azide 28, entry 4: To an ice-cold solution of **27** (57 mg, 0.2 mmol) in DCM (3 mL), EDCI (77 mg, 0.4 mmol), HOBt (64 mg, 0.42 mmol), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (93 mg, 0.5 mmol) and D*i*PEA (105 μL, 0.6 mmol) were successively added. The reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was suspended in EtOAc (50 mL) and washed with 1N $KHSO_4$ (2 × 10 mL), brine (1 × 10 mL), 5% $NaHCO_3$ (2 × 10 mL) and brine (1 × 10 mL) and dried over Na_2SO_4. The product (69 mg, 62%) was isolated as a white solid by flash column chromatography (eluent: EtOAc/hexane 4:1 v/v). *Rf*: 0.27 (EtOAc/hexane 4:1 v/v); R_t: 20.7 min; ^{1}H NMR ($CDCl_3$): δ 7.37 (t, *J* = 6.1 Hz, 2H), 7.22 (d, *J* = 2.2 Hz, 2H), 6.71 (m, 1H), 4.51 (s, 4H), 3.9 (m, 7H), 3.68 (m, 4H); ^{13}C NMR ($CDCl_3$): δ 168.2, 165.8, 158.1, 132.8, 109.0, 107.1, 67.3, 54.6, 52.5, 33.6; IR (KBr) υ = 2138 (s)



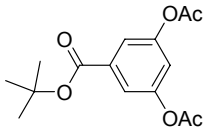
Peptide dendrimer 29: A solution of Boc-Val-Gly-SH (114 mg, 0.39 mmol) in DMF (5 mL) was added dropwise to a solution of **28** (82 mg, 0.15 mmol) and 2,6-lutidine (45 μL, 0.39 mmol) in DMF (5 mL). The reaction mixture was stirred for 16 h at room temperature before the solvent was removed by evaporation. The product (129 mg, 85%) was isolated as a white solid by crystallization from EtOAc/hexane. *Rf*: 0.23 ($CHCl_3$/MeOH/HOAc 81:17:3 v/v/v); R_t: 16.43 min; ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.28 (d, *J* = 2.2 Hz, 2H), 6.86 (m, 1H), 4.57 (s, 4H), 3.92 (m, 9H), 3.78 (m, 4H), 3.64 (m, 4H), 2.07 (m, 2H), 1.45 (s, 18H), 0.97 (dd, *J* = 6.8 Hz, *J* = 11.5 Hz, 12H).



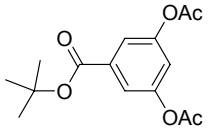
Sulfonyl azide 30: To an ice-cold solution of **37** (369 mg, 1.13 mmol) in DCM (20 mL), EDCI (438 mg, 2.28 mmol), HOBt (520 mg, 3.39 mmol), HCl.H-Gly-Ψ[CH_2SO_2]-N_3 (633 mg, 3.39 mmol) and D*i*PEA (1.19 mL, 6.79 mmol) were successively added. The reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was suspended in EtOAc (100 mL) and the solution was washed with 1N $KHSO_4$ (3 × 30 mL), brine (1 × 30 mL), 5% $NaHCO_3$ (3 × 30 mL) and brine (1 × 30 mL) and dried over Na_2SO_4. The product (247 mg, 37%) was isolated by flash column chromatography (eluent: EtOAc/hexane 4:1 v/v) as a clear oil that solidified on standing. *Rf*: 0.31 (EtOAc/hexane 4:1 v/v); ^{1}H NMR ($CDCl_3$): δ 7.33 (t, *J* = 6.0 Hz, 2H), 7.19 (d, *J* = 2.4 Hz, 2H), 6.69 (t, *J* = 2.3 Hz, 1H), 4.53 (s, 4H), 3.89 (m, 4H), 3.64 (m, 4H), 1.58 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 168.3, 164.3, 157.9, 134.8, 109.0, 106.5, 81.9, 67.3, 54.7, 33.6, 28.0.



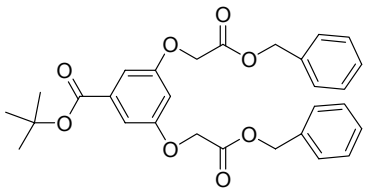
Compound 33: Acetic anhydride (9.45 mL, 100 mmol) was dissolved in THF (20 mL) and added to a solution of 3,5-dihydroxybenzoic acid (6.17 g, 40 mmol) and TEA (19.9 mL, 100 mmol) in THF/pyridine (50 mL 3:2 v/v). The reaction mixture was stirred for 4 d at room temperature before the solvent was removed by evaporation. The residue was dissolved in EtOAc (40 mL) and the solution was washed with 1N $KHSO_4$ (2 × 20 mL) and brine (1 × 20 mL) and dried over Na_2SO_4. The product (7.36 g, 77%) was isolated as a white solid by crystallization from EtOAc/hexane. *Rf*: 0.23 (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v); ^{1}H NMR ($CDCl_3$): δ 7.72 (d, *J* = 2.2 Hz, 2H), 7.21 (t, *J* = 2.2 Hz, 1H), 2.32 (s, 6H); ^{13}C NMR ($CDCl_3$): δ 168.5, 163.7, 150.7, 134.1, 120.0, 119.5, 81.7, 28.0, 20.9.



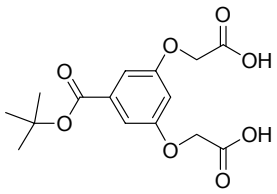
Compound 34: A solution of TBTA (2.73 g, 12.5 mmol) in cyclohexane (10 mL) was added dropwise to a solution of **33** (1.19 g, 5.0 mmol) and $BF_3.Et_2O$ (100 μL) in DCM (5 mL). Additional THF was added to obtain a clear solution. The reaction mixture was stirred for 2 d at room temperature before the solvent was removed by evaporation. The residue was dissolved in EtOAc (50 mL) and the solution was washed with 5% $NaHCO_3$ (2 × 20 mL) and brine (1 × 20 mL) and dried over Na_2SO_4. The product (768 mg, 52%) was isolated as a white solid by flash column chromatography (eluent: EtOAc/hexane 2:8 v/v). *Rf*: 0.24 (EtOAc/hexane 2:8 v/v); ^{1}H NMR ($CDCl_3$): δ 7.59 (d, *J* = 2.2 Hz, 2H), 7.10 (t, *J* = 2.2 Hz, 1H), 2.29 (s, 6H), 1.57 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 168.7, 163.7, 150.7, 134.2, 120.0, 119.9, 81.7, 28.0, 20.9.



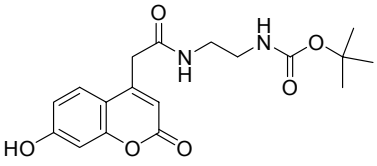
Compound 35: 2N LiOH (aq, 2 mL) was added to a solution of **34** (426 mg, 1.45 mmol) in THF (20 mL). The reaction mixture was stirred at room temperature until TLC indicated hydrolysis was complete (1 h) and the reaction mixture was quenched with 1N $KHSO_4$ (50 mL). The resulting mixture was extracted with EtOAc (3×50 mL) and the combined organic layers were washed with 5% $NaHCO_3$ (2 × 25 mL) and brine (1 × 25 mL) and dried over Na_2SO_4. The solvent was removed by evaporation to yield the product (290 mg, 95%) as a white solid. *Rf*: 0.47 (EtOAc/hexane/HOAc 45.5:45.5:1 v/v/v); ^{1}H NMR ($CDCl_3$/CD_3OD): δ 6.95 (d, *J* = 2.2 Hz, 2H), 6.51 (t, *J* = 2.2 Hz, 1H), 1.57 (s, 9H); ^{13}C NMR ($CDCl_3$/CD_3OD): δ 166.3, 157.6, 133.3, 107.8, 106.8, 81.1, 27.8.



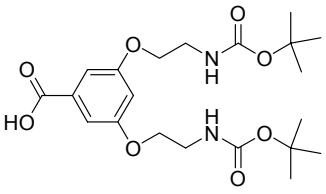
Compound 36: K_2CO_3 (477 mg, 3.45 mmol) and benzyl bromoacetate (547 μL, 3.45 mmol) were successively added to a solution of **35** (290 mg, 1.38 mmol) in dry DMF (20 mL) and the reaction mixture was stirred for 16 h at room temperature. After filtration, the filtrate was concentrated and the residue was suspended in EtOAc (100 mL) and washed with 1N $KHSO_4$ (2 × 50 mL), brine (1 × 50 mL), 5% $NaHCO_3$ (2 × 50 mL) and brine (1 × 50 mL) and dried over Na_2SO_4. The product (627 mg, 90%) was isolated by flash column chromatography (eluent: DCM/hexane 4:1 v/v) as a clear yellow oil that solidified on standing. *Rf*: 0.28 (DCM/hexane 4:1 v/v); ^{1}H NMR ($CDCl_3$): δ 7.33 (s, 10H), 7.17 (dd, *J* = 1.1 Hz, *J* = 2.4 Hz, 2H), 6.66 (m, *J* = 2.2 Hz, 1H), 5.21 (s, 4H), 4.63 (s, 4H), 1.55 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 168.1, 164.6, 158.5, 134.9, 134.2, 128.5, 128.4, 128.3, 108.6, 106.2, 81.3, 66.9, 65.2, 28.0.



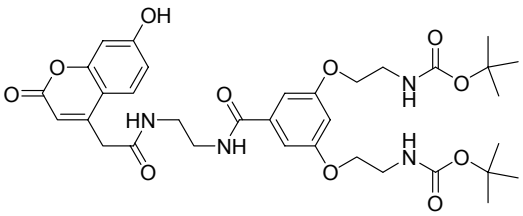
Compound 37: A mixture of **36** (627 mg, 1.24 mmol) and Pd/C (290 mg) in THF/H_2O (25 mL, 1.5:1 v/v) was allowed to stir for 16 h under a H_2 atmosphere. Then, the reaction mixture was filtered over hyflo and the filtrate was evaporated to dryness and subsequently co-evaporated with toluene (3 ×) and $CHCl_3$ (3 ×). The product (370 mg, 92%) was obtained as a white solid. *Rf*: 0.19 ($CHCl_3$/MeOH/HOAc 81:17:3 v/v/v); ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.12 (d, *J* = 1.9 Hz, 2H), 6.75 (br s, 1H), 4.67 (s, 4H), 1.57 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 166.7, 160.6, 135.3, 109.6, 107.5, 82.8, 66.2, 28.5.



Compound 38: To an ice-cold solution of **3** (220 mg, 1 mmol) in DCM/DMF (12 mL 5:1 v/v), BOP (464 mg, 1.05 mmol), *N*-Boc-ethylenediamine (168 mg, 1.05 mmol) and D*i*PEA (384 µL, 2.2 mmol) were successively added. The reaction mixture was stirred for 16 h at room temperature. After concentration, the residue was taken up in EtOAc (50 mL) and washed with 1N $KHSO_4$ (2 × 10 mL), brine (1 × 10 mL), 5% $NaHCO_3$ (2×10 mL) and brine (1×10 mL) and dried over Na_2SO_4. The product (326 mg, 90%) was isolated as a white solid by crystallization from MeOH/EtOAc/hexane. R_f: 0.43 (EtOAc/hexane 9:1 v/v); R_t: 13.1 min; ^{1}H NMR (DMSO-d_6): δ 10.54 (s, 1H), 8.21 (t, J = 4.8 Hz, 1H), 6.81 (m, 2H), 6.72 (m, 1H), 6.17 (s, 1H), 3.63 (s, 2H), 3.09 (m, 2H), 2.99 (m, 2H), 1.38 (s, 9H); ^{13}C NMR ($CDCl_3$): δ 205.3, 198.6, 197.7, 193.1, 192.5, 188.5, 164.2, 150.4, 149.3, 149.0, 139.7, 115.2, 65.7.

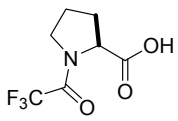


Compound 39: 4N NaOH (2 mL) was added to a solution of **25** in dioxane/MeOH (14:5 v/v). The reaction mixture was stirred at room temperature until TLC indicated hydrolysis was complete (4 h) and the reaction mixture was quenched with 1N $KHSO_4$ (25 mL). The resulting mixture was concentrated to 25 mL and extracted with EtOAc (3×50 mL) and the combined organic layers were washed with 1N $KHSO_4$ (1 × 25 mL) and brine (1 × 25 mL) and dried over Na_2SO_4. The solvent was removed by evaporation to obtain the product as a white foam in a quantitative yield. R_f: 0.50 (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v); ^{1}H NMR ($CDCl_3$): δ 7.23 (br s, 2H), 6.66 (br s, 1H), 5.01 (br s, 2H), 4.05 (m, 4H), 3.54 (m, 4H), 1.46 (s, 18H).

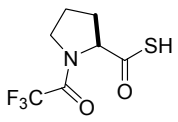


Compound 40: First was **38** (192 mg, 0.53 mmol) dissolved in TFA/DCM (40 mL 1:1 v/v) and the obtained reaction mixture was stirred at room temperature until removal of the Boc-group was complete (1 h), as indicated by TLC (Eluent: EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v). After evaporation of the solvents, the residue was coevaporated with $CHCl_3$. Next, the residue was dissolved in DCM/DMF (30 mL 5:1 v/v) and **39** (220 mg, 0.54 mmol), BOP (243 mg, 0.55 mmol) and D*i*PEA (300 µL, 1.72 mmol) were successively added. The reaction mixture was stirred for 16 h at room temperature. After concentration of the reaction mixture, the residue was taken up in EtOAc (50 mL) and the solution was washed with 1N $KHSO_4$ (2×25 mL), brine (1×25 mL), 5% $NaHCO_3$ (2 × 25 mL) and brine (1 × 25 mL) and

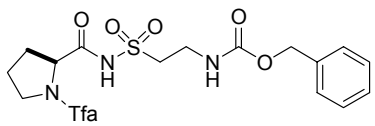
dried over Na_2SO_4. The product (233 mg, 68%) was isolated by flash column chromatography (eluent: gradient DCM → DCM/MeOH 95:5 v/v) as a clear oil that solidified upon standing. *Rf*: 0.10 (DCM/MeOH 95:5 v/v); R_t: 21.3 min; ^{1}H NMR (DMSO-d_6): δ 10.54 (br s, 1H), 8.46, (m, 1 H), 8.31 (m, 1H), 7.58, (d, J = 8.7, 1H), 6.99 (br s, 4H), 6.78 (dd, J = 2.2 Hz, J = 8.7 Hz, 1H), 6.71 (br s, 2H), 6.63 (br s, 2H), 9.17 (s, 1H), 3.98 (m, 4H), 3.65 (s, 2H), 3.31 (m, 6H), 1.38 (s, 18H) ; ^{13}C NMR (DMSO-d_6): δ 173.4, 171.2, 166.5, 165.6, 164.8, 161.1, 160.4, 156.4, 141.8, 132.1, 118.3, 117.3, 116.9, 111.4, 109.3, 107.7, 83.2, 72.1, 33.6; ESMS calcd for $C_{34}H_{44}N_4O_{11}$: 684,30, found: 684.75 $[M+H]^+$.



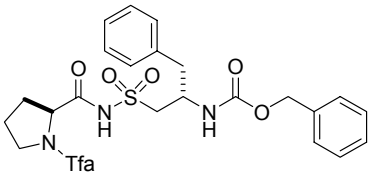
Tfa-Pro-OH (49): Ethyl trifluoroacetate (3.6 mL, 30 mmol) was added to a cooled (icebath) solution of H-Pro-OH (2.30 g, 20 mmol) in 30% NaOMe in MeOH (4.1 mL, 22 mmol). The reaction mixture was heated at 40°C for 2 h before HCl (2.5 M, 10 mL) was added. The resulting mixture was extracted with EtOAc (20 mL, twice) and the combined EtOAc-layers were washed with brine/1N HCl (9:1 v/v, 10 mL) and dried (Na_2SO_4). The product (4.16 g, 99%) was obtained as a clear oil which solidified upon standing. *Rf*: 0.67 ($CHCl_3$/MeOH/HOAc 80:18:2 v/v/v); ^{1}H NMR ($CDCl_3$): δ 10.66 (s, 1H), 4.61 (dd, J = 3.6, J = 5.0, 1H), 3.88–3.65 (m, 2H), 2.33 (m), 2.06 (m, total 4H); ^{13}C NMR ($CDCl_3$): δ 176.0, 155.9 ($J_{F\text{-}C}$ = 37.8 Hz), 114.1 ($J_{F\text{-}C}$ = 286.9 Hz), 60.0, 47.3, 28.4, 24.9.



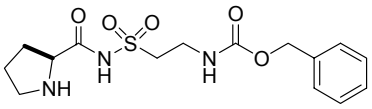
Tfa-Pro-SH (50): The thio acid of Tfa-Pro-OH was synthesised from the corresponding hydroxysuccinimide ester by treatment with NaHS according to the method of Goldstein and Gelb[6] as was described in chapter 4. The crude thio acid product was used immediately for reaction with the sulfonyl azides.



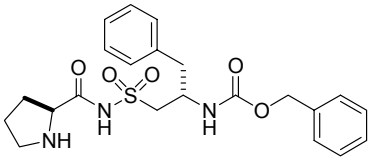
***N*-Acyl sulfonamide 51:** To a mixture of Cbz-Gly-Ψ[CH_2SO_2]-N_3 (284 mg, 0.5 mmol) and 2,6-lutidine (117 µL, 1 mmol) in $CHCl_3$(1 mL), a solution of **50** (1 mmol) in $CHCl_3$ (2 mL) was added dropwise. After completion of the addition, the reaction mixture was allowed to stir for 72 h at room temperature. Then, the reaction mixture was evaporated to dryness and the residue crystallyzed from EtOAc/hexane. The product (208 mg, 92%) was obtained as a white solid. *Rf*: 0.45 (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v); ^{1}H NMR ($CDCl_3$): δ 9.56 (br s, 1H), 7.33 (m, 5H), 5.50 (br s, 1H), 5.11 (s, 2H), 4.47 (br s, 1H), 3.80 (m), 3.71 (m), 3.61 (m, total 6H), 2.17 (m), 2.02 (m, total 4H); ^{13}C NMR ($CDCl_3$): δ 169.7, 156.7, 136.2, 128.5, 128.1, 127.9, 114.1 (q, $J_{F\text{-}C}$ = 286.6 Hz), 67.0, 61.6, 52.9, 47.7, 35.5, 25.3 (q, $J_{F\text{-}C}$ = 270.5 Hz).



***N*-Acyl sulfonamide 52:** To a mixture of Cbz-Phe-Ψ[CH_2SO_2]-N_3 (187 mg, 0.5 mmol) and 2,6-lutidine (117 µL, 1 mmol) in DMF (1 mL), a solution of **50** (1 mmol) in DMF (2 mL) was added dropwise. After completion of the addition, the reaction mixture was allowed to stir for 72 h at room temperature. Then, the reaction mixture was evaporated to dryness and the product was isolated by flash column chromatography (eluent: EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v) followed by crystallization from EtOAc/hexane. The product (191 mg, 74%) was obtained as a white solid. R_f: 0.57 (EtOAc/hexane/HOAc 89.5:9.5:1 v/v/v); ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.33 (m), 7.24 (m, total 10H), 5.99 (d, J = 8.8 Hz, 1H), 5.03 (s, 2H), 4.46 (br m, 4H), 3.85–3.36 (m, 4H), 2.91 (m, 2H), 2.20 (m, 2H), 2.05 (m, 2H); ^{13}C NMR ($CDCl_3$/CD_3OD): δ 170.3, 156.3, 155.7, 136.3, 129.4, 129.2, 128.5, 128.3, 128.3, 127.8, 127.4, 126.7, 114.0 (q, $J_{F\text{-}C}$ = 286.9 Hz), 66.5, 61.2, 55.3, 48.3, 47.5, 25.0 ($J_{F\text{-}C}$ = 245.6 Hz).



***N*-Acyl sulfonamide 46:** Compound **51** (112.8 mg, 0.25 mmol) was dissolved in dioxane/MeOH (9 mL, 14:5, v/v), subsequently 4N NaOH (2 mL) was added and the resulting mixture was stirred for 2 h at room temperature. After completion of the reaction, as indicated by TLC-analysis (eluent: $CHCl_3$/MeOH/HOAc 81:17:3 v/v/v), 1N $KHSO_4$ (10 mL) was added and the reaction mixture was concentrated to a volume of 12 mL. The resulting aqueous solution was saturated with NaCl and extracted with EtOAc and dried (Na_2SO_4). Finally the hydrosulfate-salt of the product (104 mg, 92%) was isolated as a white solid by flash column chromatography (eluent: $CHCl_3$/MeOH/HOAc 81:17:3 v/v/v). R_f: 0.11 ($CHCl_3$/MeOH/HOAc, 81:17:3, v/v/v); R_t: 11.48 min; ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.33 (br s, 5H), 5.08 (s, 2H), 4.02 (m, 1H), 3.58 (m, 2H), 3.28 (m, 4H), 2.27 (m, 1H), 2.08, (m, 1H), 1.92 (m, 2H); ^{13}C NMR ($CDCl_3$/CD_3OD): δ 156.6, 136.1, 128.2, 127.9, 127.6, 66.5, 62.2, 54.0, 51.6, 45.8, 35.8, 29.5, 24.1; ESMS calcd for $C_{15}H_{21}N_3O_5S$: 355.12, found: 356.32 $[M+H]^+$; Anal. calcd (%) for $C_{15}H_{21}N_3O_5S.HSO_4$: C, 39.82; H, 4.90; N, 9.29; found: C, 39.91; H, 4.76; N, 7.40.



***N*-Acyl sulfonamide 47:** A mixture of **52** (191 mg, 0.35) in NH_4OH (25 vol-%, 30 mL) was stirred for 3 h at 40°C before the solvent was removed. The HSO_4-salt of the product (174 mg, 92%) was isolated as a white solid by flash column chromatography (eluent: $CHCl_3$/MeOH/HOAc 81:17:3 v/v/v). R_f: 0.23 ($CHCl_3$/MeOH/HOAc, 81:17:3, v/v/v); R_t: 14.68 min; ^{1}H NMR ($CDCl_3$/CD_3OD): δ 7.31

(m, 10H), 5.00 (m, 2H), 4.29 (m, 1H), 3.98 (m, 1H), 3.39 (m, 1H), 3.26 (m), 3.17 (m, total 3H), 2.22 (m, 1H), 2.07 (m, 1H), 1.89 (m, 2H); ^{13}C NMR ($CDCl_3/CD_3OD$): δ 179.9, 156.1, 137.1, 136.2, 129.1, 128.1, 127.7, 127.3, 126.3, 66.2, 62.1, 54.7, 49.0, 45.8 40.0, 29.3, 24.1, 23.3; ESMS calcd for $C_{22}H_{27}N_3O_5S$: 445.17, found: 446.39 $[M+H]^+$; Anal. calcd (%) for $C_{22}H_{27}N_3O_5S.HSO_4$: C, 48.70; H, 5.20; N, 7.74; found: C, 47.61; H, 5.15; N, 6.46.

General Procedure for aldol reactions between *p*-nitrobenzaldehyde and acetone:[70]

In a 10 mL test tube, *p*-nitrobenzaldehyde (76 mg, 0.5 mmol) was dissolved in acetone/DMSO (5 mL, 4:1 v/v). Then 20 mol% of the catalysts **46** (36 mg), **47** (45 mg) were added, and the resulting homogeneous solutions were stirred at 20°C for 24 h. Samples of 100 μL were drawn, diluted with 1 mL dichloromethane, and conversion and enantiomeric excess was determined immediately by HPLC (Chiralcel-OJ, using an isocratic mixture of *n*-hexane/*i*-PrOH 9:1 v/v at a flowrate of 1 mL/min). Quantification was performed using integrated wavelengths from 240 – 261 nm. Conversion was determined by comparison to the peak areas of stock solutions of 4-nitrobenzaldehyde and the racemic aldol adduct in dichloromethane (8.27 mmol/L each): 4-nitrobenzaldehyde (R_t: 19.73 min); (*R*)-4-hydroxy-4-(4-nitrophenyl)butan-2-one (R_t: 31.52 min), 36.99 (*S*)-4-hydroxy-4-(4-nitrophenyl)butan-2-one (R_t: 36.99 min).

8.7 References and Notes

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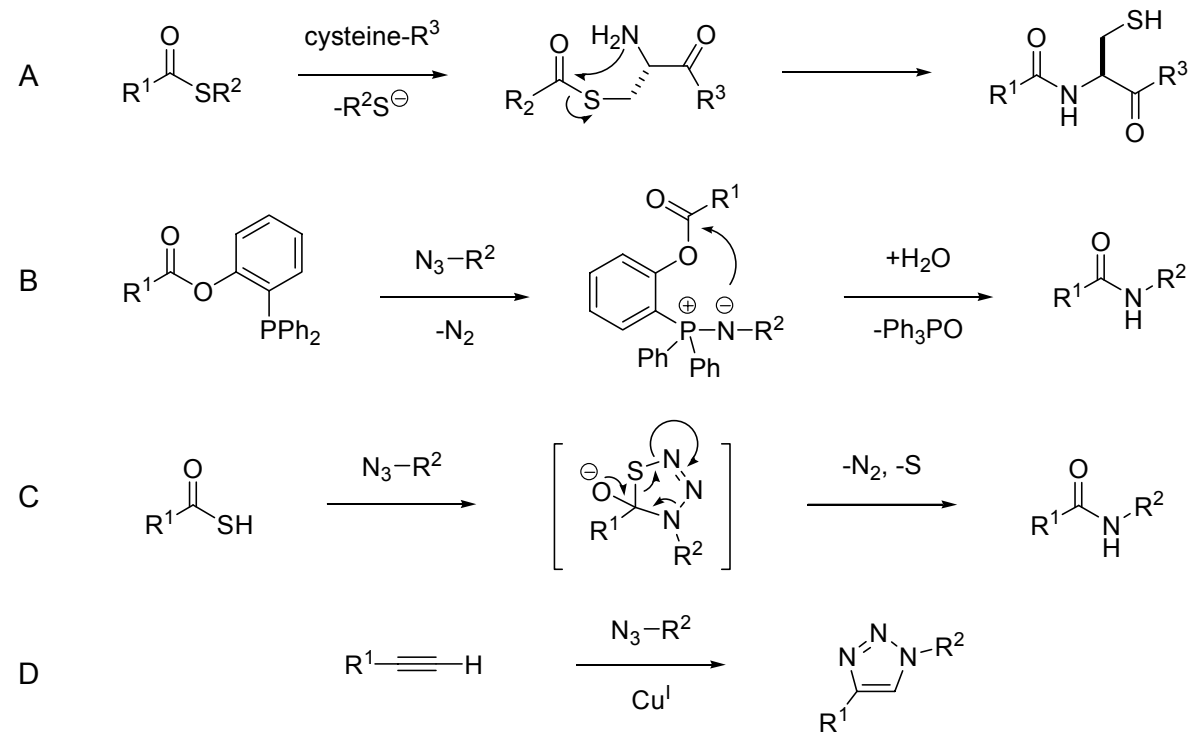
Appendices

Summary

Chemical methods that enable the synthesis and the site-selective modification of biomolecules, are of high importance for widespread applications in chemistry and biology. Most often these methods employ chemoselective ligation reactions[1] which feature mutually and uniquely reactive functional groups[2,3] to enable the covalent coupling of unprotected biomolecules without interference of any other functional groups either present in the biomolecule to be coupled or the environment in which the ligation should take place.



Scheme 1 Chemoselective ligation reactions.

Today, the repertoire of chemical ligation reactions is recognized as a valuable tool for the synthesis of complex bioconjugates and in particular larger peptides and proteins.[4-8] Over the years, a number of chemoselective ligation methods have been described in the literature. Obviously, for the synthesis of polypeptides, those ligation reactions that form an amide bond in the coupling step are the most important.[8,9] Currently, the *Native Chemical Ligation* (NCL, A, Scheme 1) is the most effective method for the chemoselective ligation of unprotected peptide segments.[10,11] In this method the highly selective reaction of a *C*-

terminal peptide thio ester and an *N*-terminal cysteine residue is used to form an amide bond. Despite widespread use of NCL as well as several approaches to broaden its applicability,[12-14] the requirement for an *N*-terminal cysteine (or a suitable mimic) at the ligation site is sometimes an obstacle.

Today, a general applicable method for the chemical synthesis of (poly)peptides featuring a chemoselective amidation reaction that is independent of the amino acid side chain at the site of ligation, is not available yet and would be highly desirable. In this respect, other chemoselective amidation reactions have been described in the literature that might be used to overcome this requirement since they are, in principle, independent of the amino acid residues at the ligation site. In this thesis, a "traceless" version of the so-called Staudinger ligation[15,16] (B, Scheme 1) and the reaction of thio acids with azides[17] (C, Scheme 1) were investigated for application as novel chemoselective amide ligation method as extension or possible alternative to NCL.

In addition to the chemical synthesis of polypeptides and proteins, chemoselective ligation reactions can also be used for the imaging of diverse biomolecules, for example, via a two-step bioorthogonal chemical reporter strategy. In this context, the azide moiety is the most versatile bioorthogonal chemical reporter group available for introduction into biomolecules and subsequent conjugation to a suitably functionalized biophysical tag.

Chemoselective coupling reactions with azides include the Staudinger ligation and the copper-catalyzed azide/alkyne Huisgen 1,3-dipolar cycloaddition (the paradigm of "click"-chemistry, D, Scheme 1).[18,19] Despite the successful applications on bioconjugation that have been described in the literature, sluggisch reaction kinetics (Staudinger ligation) and the need for a cytotoxic copper catalyst (click chemistry) can, in some cases, form a serious limitation.[20] Therefore there remains a constant interest in the development of new azide-based chemoselective ligation reactions as tool for bioconjugation as alternative to the methods available. Obviously, *in vivo* applications have the highest requirements for the chemoselective ligation method to be used. Nonetheless, chemoselective amidation reactions that do not meet the exact conditions needed for application in a certain biological environment, are still of high significance. In case these reactions feature the right properties, they could form a valuable extension to the arsenal of "click"-reactions.

Chapter 1 forms a general introduction to this thesis and gives a literature overview of the chemoselective ligation reactions available today. From this overview, azide-based chemoselective amidation reactions are recognized as highly interesting tool for the synthesis and modification of peptides.

Chapter 2 describes the first application of a traceless version of the so-called Staudinger ligation on the synthesis of peptides by the chemoselective ligation of peptide segments.

First, the synthesis of *N*-terminal peptidyl azides and *C*-terminal peptide *o*-(diphenylphosphine)phenyl esters was described. Subsequently, their reaction was monitored by mass spectrometric analysis in order to explain the observed intermediates and gain a better insight into the mechanism of the Staudinger ligation for optimization of the amidation reaction. Finally, the optimized reaction conditions were used for the Staudinger ligation of peptide segments containing amino acids other than glycine at the ligation site.

Chapter 3, describes the influence of several factors on the Staudinger ligation, including the substituents at phosphorus, the removable auxiliary, the solvent as well as the amino acid residues at the ligation site. The investigations were performed by both experimental and computational approaches. From the results, it became apparent that steric congestion at the ligation site hindered the intramolecular *O*→*N*-acyl migration in such a way that competing side reactions took place and caused a dramatic decrease in the ligation yield.

In **chapter 4** the scope and limitations of the reaction between thio acids and azides as a new amide-forming chemoselective ligation reaction for the coupling of peptides is described. The reaction of amino acid- and peptide-derived thio acids and azides was found to be slow. Optimization involved the addition of $RuCl_3$ as a catalyst, to activate the thio acid. Although this led to an increase in the efficiency, concurrently the chemoselectivity of the reaction was decreased. Alternatively, the addition of triphenylphosphine to activate the azide did not lead to a more efficient reaction.

Chapter 5 reports on the versatile synthesis of suitably protected *β*-substituted aminoethane sulfonyl azides as stable building blocks which can be conveniently introduced into peptides. Subsequently, the highly efficient coupling of sulfonyl azides with thio acids was found to be chemoselective, fast, compatible with a variety of solvents (including water) and yielded nitrogen and sulphur as only non-toxic side products. These properties resemble those of “click”-chemistry, however, without the need for a possibly cytotoxic metal catalyst.

In **chapter 6** an efficient loading strategy for the *N*-acyl sulfonamide linker, based on a resin-bound sulfonyl azide/thio acid amidation reaction is described. The activation of the linker towards nucleophilic cleavage was optimized using a microwave-assisted *N*-alkylation of the *N*-peptidyl sulfonamide moiety. Subsequent treatment with suitably functionalized nucleophiles provided access to *C*-terminally modified peptides which may find application in chemoselective (bio)conjugation or ligation methods.

In **chapter 7** the spontaneous and irreversible coupling of thio acids and sulfonyl azides is used for a fast and efficient synthesis of a focused series of peptide-based *N*-acyl sulfonamides with interesting physicochemical properties. Starting from amino acid-

derived thio acids and sulfonyl azides that differ in their ability of forming non-covalent interaction, a peptide-based gelator could be obtained which was capable of forming a pH-responsive gel in aqueous solution at low concentration.

Finally, in **chapter 8** a three-fold successful application of the thio acid/sulfonyl azide amidation reaction on the chemoselective coupling of peptides was described. The synthetic approaches in this chapter gave an outlook toward the chemoselective ligation of peptides, peptide dendrimers and proline dipeptide-based organocatalysts. From these results as well as those described in the previous chapters, it can be concluded that the thio acid/sulfonyl azide amidation reaction is a suitable method for the synthesis of peptide conjugates.

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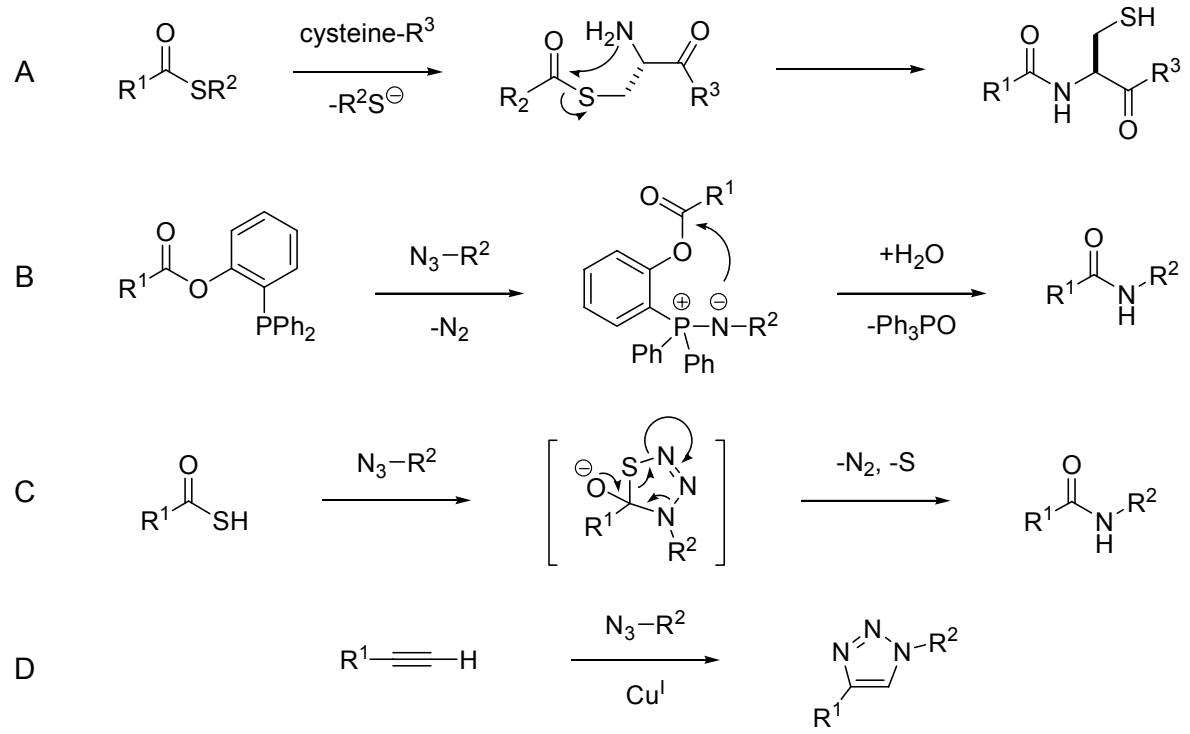
Nederlandse samenvatting

Chemische methoden voor de synthese en selectieve modificatie van biomoleculen vormen een belangrijk hulpmiddel in het onderzoek naar de structuur en biologische functie van deze verbindingen. Een belangrijke modificatie bestaat uit de introductie van biofysische labels (bijvoorbeeld radioactief of fluorescent) voor de visualisatie van biomoleculen. Daarnaast worden deze methoden gebruikt voor de immobilisatie van biomoleculen aan het oppervlak van een chip voor het snel testen van duizenden verbindingen op een bepaalde biologische activiteit. Tenslotte, kunnen door middel van de selectieve koppeling (conjugatie) van andere biomoleculen, complexe bioconjugaten en eiwitten en (poly)peptiden in het bijzonder worden verkregen.

In al deze voorbeelden is het van belang dat de modificatie selectief plaatsvindt op een specifieke, vooraf bepaalde positie. Hiervoor wordt gebruik gemaakt van zogenaamde chemoselectieve ligatie reacties. Dit zijn speciale chemische reacties tussen twee functionele groepen die een specifieke gemeenschappelijke reactiviteit ten opzichte van elkaar bezitten die resulteert in de vorming van een stabiele covalente binding. Het is belangrijk dat deze koppelingsreactie voldoende bioorthogonaal is, met andere woorden, dat deze reactie niet wordt gehinderd door andere aanwezige functionele groepen in de te koppelen biomoleculen, of het (biologische) milieu waarin de koppeling plaatsvindt. De koppeling dient daarbij efficiënt te verlopen onder omstandigheden die geschikt zijn voor biomoleculen, wat over het algemeen een neutrale pH, kamertemperatuur en een waterig milieu inhoudt. Het aantal reacties dat hieraan voldoet is echter beperkt.

Voor de synthese van eiwitten en polypeptiden via de chemoselectieve ligatie van onbeschermde peptide-segmenten, zijn vooral díe ligatie reacties interessant waarbij een hoge selectiviteit en efficiëntie onder milde omstandigheden gepaard gaan met de vorming van een “natuurlijke” amidebinding in het koppelingsproduct. In dit opzicht is de *Native Chemical Ligation* (NCL), waarin gebruik wordt gemaakt van de zeer selectieve amideringsreactie tussen een *C*-terminaal peptide thioester en een *N*-terminaal cysteïne residu (A, schema 1), tot op heden het meest succesvol gebleken. Desalniettemin vormt de noodzaak voor een *N*-terminaal cysteïne residu vaak een beperking van deze methode.

Deze beperking zou kunnen worden voorkomen door gebruik te maken van alternatieve chemoselectieve amideringsreacties die onafhankelijk zijn van aminozuurresiduen op de koppelingspositie. Twee voorbeelden uit de recente literatuur die hier mogelijk voor in aanmerking komen zijn, de reactie tussen een fosfine en een azide (*Staudinger ligatie*, B, schema 1) en de reactie tussen een thiozuur en een azide (C, schema 1).



Schema 1 Chemoselectieve koppelingsreacties.

Naast de chemische synthese van eiwitten en polypeptiden vinden chemoselectieve koppelingsmethoden ook een belangrijke toepassing in het modificeren en labelen van biologisch relevante verbindingen. Hiervoor worden de bewuste biomoleculen voorzien van een chemisch handvat waaraan vervolgens op een later tijdstip, via een chemoselectieve ligatiereactie, het betreffende label gebonden kan worden. De toepassing is daarbij bepalend voor de eisen die aan de chemoselectieve ligatie reactie worden gesteld. Zo zijn de eisen voor *in vivo* labeling over het algemeen strenger dan voor de synthese van gemodificeerde peptiden onder gecontroleerde *in vitro* condities. Vanwege de kleine omvang, de hoge stabiliteit en bioorthogonaliteit lijkt de azido-groep op dit moment de beste eigenschappen te bezitten om als een dergelijk chemisch handvat te dienen.

Vervolgens zijn er een aantal methoden beschikbaar voor de selectieve reactie met deze azido-groep. Een van de bekendste voorbeelden is de koper-gekatalyseerde Huisgen 1,3-dipolaire cycloadditie tussen azides en alkynen, beter bekend als “click”-chemie (D, schema 1). De populariteit van deze methode berust vooral op de hoge selectiviteit en efficiëntie van de reactie in een verscheidenheid van oplosmiddelen (waaronder ook water) onder milde omstandigheden. Hoewel veel van de eigenschappen van “click”-chemie overeenkomen met de eisen die aan chemoselectieve ligatie methoden worden gesteld, zijn

de begrippen niet zondermeer uitwisselbaar. In het geval van "click"-chemie wordt bijvoorbeeld geen voorwaarde gesteld aan de toepassing in een biologisch relevant milieu. Het gebruik van cytotoxisch koper als katalysator in de Huisgen 1,3-dipolaire cycloadditie kan bijvoorbeeld een serieuze beperking vormen voor bepaalde biologische toepassingen. Gezien de grote complexiteit en het diverse toepassingsgebied van de verschillende (gemodificeerde) biomoleculen, bestaat er een constante interesse voor de ontwikkeling van nieuwe op de azido-groep gebaseerde chemoselectieve koppelingsmethoden.

Hoofdstuk 1 geldt als inleiding tot het proefschrift. Hierin wordt een literatuuroverzicht gegeven van de verschillende methoden voor de chemoselectieve ligatie van biomoleculen en peptiden in het bijzonder.

Hoofdstuk 2 beschrijft de eerste toepassing van een aangepaste vorm van de Staudinger ligatie op de synthese van tetra- en pentapeptiden via de chemoselectieve koppeling van peptidesegmenten. Daartoe werd eerst de synthese van *N*-terminale azidopeptiden en *C*-terminale peptidyl *o*-(diphenylphosphine)fenol esters beschreven. Vervolgens werd de koppelingsreactie tussen beide reactanten gevolgd met behulp van massaspectrometrische analysemethoden, om een beter inzicht in het mechanisme te krijgen. Het bleek dat de eerste reactiestap, de vorming van het *iminofosforaan* intermediair, snel verliep. Echter, de hierop volgende intramoleculaire acylmigratie vormde het knelpunt van de reactie. Daarbij nam de efficiëntie van de reactie snel af met een toename in de complexiteit van de te koppelen peptidesegmenten.

Vervolgens wordt in **hoofdstuk 3** het onderzoek naar de invloed van de verschillende factoren op de Staudinger ligatie van peptidesegmenten beschreven teneinde de reactiecondities te optimaliseren en de efficiëntie van de Staudinger ligatie van peptidesegmenten te verhogen. Vanwege de enorme hoeveelheid werk die het met zich mee zou brengen om alle relevante factoren en combinaties daarvan met experimentele methoden te onderzoeken, werden deze gecombineerd met theoretische berekeningen aan computermodellen. De berekeningen werden daarbij toegespitst op de intramoleculaire acylmigratie, de veronderstelde snelheidsbepalende stap voor de ligatiereactie zoals werd beschreven in hoofdstuk 2. Uit de resultaten bleek dat sterische hindering de grootste invloed heeft op de efficiëntie van de Staudinger ligatie. Dit houdt in dat ook deze methode afhankelijk is van de aminozuurresiduen op de koppelingspositie en dus eerder een aanvulling dan een alternatief vormt op de bestaande Native Chemical Ligation methode. Daarom werd besloten de aandacht te verleggen naar een andere selectieve amideringsreactie: de reactie tussen een thiozuur en een azide.

In **hoofdstuk 4** worden de eigenschappen en beperkingen van de reactie tussen thiozuren en azides beschreven voor toepassing als nieuwe chemoselectieve ligatiemethode voor de

koppeling van onbeschermde peptidesegmenten. De reactie tussen thiozuren en azides die afgeleid zijn van aminozuren en peptiden, bleek echter erg traag en met een lage efficiëntie te verlopen. Het thiozuur kon worden geactiveerd door toevoeging van rutheniumchloride als katalysator. Hoewel hiermee de opbrengst en reactiesnelheid konden worden verhoogd, bleek dat tegelijkertijd de selectiviteit van de reactie teniet werd gedaan. Activatie van de azidogroep door toevoeging van trifenylfosfine had geen verbetering in de efficiëntie van de reactie tot gevolg.

Hoofdstuk 5 beschrijft de reactie van thiozuren met sulfonylazides. De hiervoor benodigde sulfonylazides werden verkregen uitgaande van aminozuren volgens een nieuw ontwikkelde syntheseroute. In tegenstelling tot de relatief elektronenrijke alkylazides uit hoofdstuk 4, blijkt de reactie van electronenarme sulfonylazides met thiozuren zeer snel en efficient te verlopen. Bovendien werd er een hoge selectiviteit gevonden onder milde omstandigheden in een verscheidenheid aan oplosmiddelen (inclusief water). Daarmee benaderen de eigenschappen van de thiozuur/sulfonylazide amideringsreactie die van de ideale "click"-reactie. Bovendien is, in tegenstelling tot de koper-gekatalyseerde Huisgen 1,3-dipolaire cycloadditie tussen azides en alkynen –tot op heden het belangrijkste voorbeeld van "click"-chemie– voor de thiozuur/sulfonylazide amideringsreactie geen cytotoxisch koper als katalysator nodig. Dit kan een belangrijk voordeel zijn voor biologisch relevante toepassingen.

In **hoofdstuk 6** wordt de toepassing van een vaste drager-gebonden sulfonylazide voor de ontwikkeling van een efficiënte strategie voor de belading van de *N*-acylsulfonamide linker op basis van de thiozuur/sulfonylazide amideringsreactie beschreven. De ontwikkelde strategie vormt een verbetering op de bestaande methoden voor de belading én activatie van de *N*-acylsulfonamide linker en draagt zodoende bij tot een betere beschikbaarheid van *C*-terminaal gemodificeerde peptiden. Deze peptiden kunnen op hun beurt weer gebruikt worden in allerlei chemoselectieve bioconjugatie reacties.

In **hoofdstuk 7** wordt de synthese van een serie op peptiden-gebaseerde *N*-acylsulfonamides met interessante fysische eigenschappen beschreven. Hiervoor werd gebruik gemaakt van de spontane en irreversibele koppeling van thiozuren met sulfonylazides. Uit de combinatie van aminozuur-afgeleide thiozuren en sylfonylazides, met een variatie in de mogelijkheden tot het aangaan van niet-covalente interacties, kon een gelator worden verkregen die in staat was om bij lage concentratie een pH-gevoelige hydrogel te vormen.

Tenslotte worden in **hoofdstuk 8**, drie voorbeelden beschreven van de toepassing van de sulfonylazide/thiozuur amideringsreactie op de conjugatie van peptiden. Deze voorbeelden bestaan uit de chemoselectieve ligatie van onbeschermde peptidesegmenten, de koppeling

van peptidesegmenten aan een multivalent dendrimeer skelet en de synthese van op proline-dipeptide gebaseerde organokatalysatoren. Samen met de resultaten uit hoofdstukken 5 tot en met 7 kan hieruit geconcludeerd worden dat de thiozuur/sulfonylazide amideringsreactie een zeer geschikte methode vormt voor de chemoselectieve koppeling van peptiden.

Curriculum vitae

De auteur van dit proefschrift werd geboren op 29 juli 1975 te Waalwijk. Na het behalen van het HAVO-diploma aan college "De Naulande" te Drunen werd in 1994 begonnen met de studie Chemie aan de Hogeschool West-Brabant in Etten-Leur. Daarvoor werd in 1999 een onderzoeksstage en afstudeeropdracht uitgevoerd (1 jaar) bij Akzo Nobel Chemicals Research te Arnhem. Gedurende deze periode werd onderzoek gedaan naar de synthese van nieuwe bio-afbreekbare complexvormers. In 2000 werd het ingenieursdiploma behaald. Vervolgens was hij 6 maanden werkzaam bij de Lead Discovery Unit van N.V. Organon te Oss waar werd gewerkt aan de ontwikkeling van nieuwe vruchtbaarheidsmedicijnen. In september 2000 werd begonnen met de doctoraalstudie scheikunde aan de Universiteit Utrecht die in november 2002 werd afgerond, na een onderzoeksstage (1 jaar) bij de disciplinegroep Medicinal Chemistry & Chemical Biology, begeleid door dr. ir. D.T.S. Rijkers en prof. dr. R.M.J. Liskamp. Hier werd onderzoek verricht naar de chemoselectieve ligatie van peptidesegmenten met behulp van de zogenaamde "Staudinger ligatie". In januari 2003 trad hij in dienst als assistent in opleiding (AiO) bij de disciplinegroep Medicinal Chemistry & Chemical Biology (promotor: prof. dr. R.M.J. Liskamp en co-promotor: dr. ir. D.T.S. Rijkers) aan de Universiteit Utrecht. Het hierbij verrichte onderzoek is beschreven in dit proefschrift. Tijdens deze periode werden de behaalde onderzoeksresultaten gepresenteerd op nationale en internationale congressen, zoals het 19th American Peptide Symposium te San Diego (juni 2005, Verenigde Staten). Van april 2007 tot en met oktober 2008 was de auteur werkzaam bij de afdeling DPP-ISC van DSM te Geleen. Hier werd gewerkt aan diverse projecten zoals de ontwikkeling van nieuwe methodologie voor de chemo-enzymatische synthese van peptiden en de ontwikkeling van nieuwe biomaterialen. Vanaf november 2008 is de auteur werkzaam als postdoc binnen het Howard Hughes Medical Institute aan de University of Illinois, Urbana-Champaign, Verenigde Staten (prof. dr. W.A. van der Donk) waar onderzoek wordt verricht naar de ontwikkeling van nieuwe antibiotica.

List of publications

Synthesis and Evaluation of Peptide-based *N*-Acyl Sulfonamides as a New Class of Organo/Hydrogelators
R. Merkx, D.T.S. Rijkers, R.M.J. Liskamp, *manuscript in preparation*

Resin-Bound Sulfonyl Azides: Efficient Loading and Activation Strategy for the Preparation of the *N*-Acyl Sulfonamide Linker
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