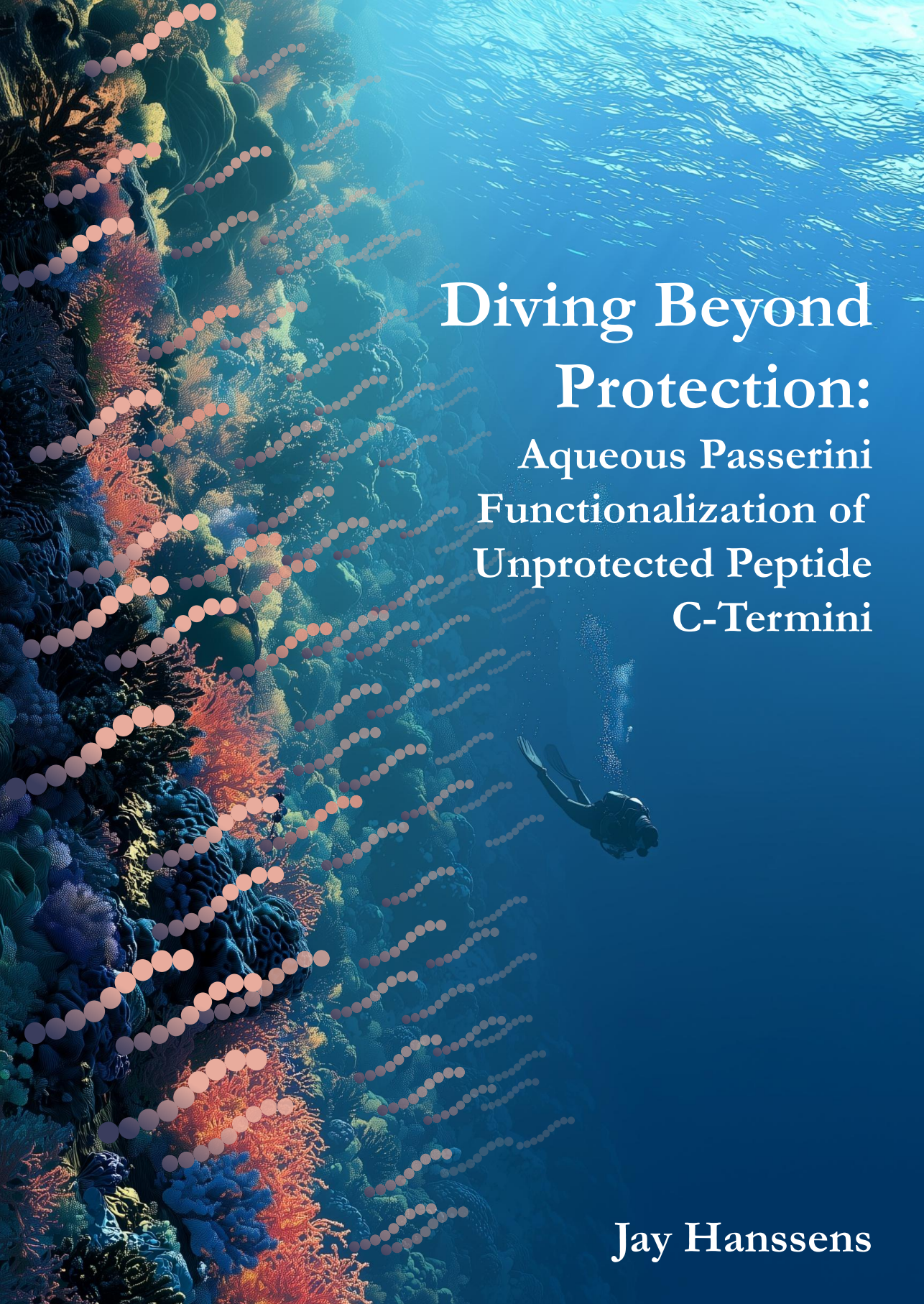


Diving Beyond Protection:

Aqueous Passerini Functionalization of Unprotected Peptide C-Termini

Jay Hanssens

Propositions for the Ph.D. thesis

Diving Beyond Protection:

Aqueous Passerini Functionalization of Unprotected Peptide C-Termini

By Jay Hanssens

1. The best strategy for C-terminal functionalization depends on the goal.
2. There is no universal method for protein C-terminal functionalization.
3. Combining chemical functionalization with enzymatic ligation can make peptide and protein synthesis more sustainable and versatile.
4. Small aldehydes bearing asymmetric disulfides are terrible to synthesize.
5. In proteins, regioselectivity is basically chemoselectivity.
6. The right isocyanide can turn a solubility problem into an opportunity for protein engineering.
7. Charged isocyanides are difficult to work with, but at least they do not smell.
8. New solutions also afford new problems.
9. Innovative ideas merely emerge at the frontier of the known.

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2026

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DISSERTATION

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on the authority of the Rector Magnificus, Prof. Dr. Jan Smits,

in accordance with the decision of the Board of Deans,

to be defended in public on Thursday 29th of Oktober 2026, at 16:00 hours

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Prof. Dr. Jan H. van Maarseveen, University of Amsterdam

For those who are part of my soul.

List of Abbreviations

Å	Ångström
2-MIM	2-methylimidazole
4IMP	4-iodine *N*-methylpyridinium
AA	Amino acid
Ac	Acetyl
ADC	Antibody drug conjugate
Ala	Alanine
aq	Aqueous
Ar	Aryl
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Bn	Benzyl
Boc	*tert*-butyloxycarbonyl
BOP	Benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate
BSA	Bovine serum albumin
Cam	Carboxyamidomethyl
Cbz	Benzyloxycarbonyl
CDI	1,1'-carbonyldiimidazole
CIC	*N*-cyclohexyl-*N*'-isopropylcarbodiimide
cLog*P*	Calculated Log*P*
COMU	(1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate
CPE	Cysteinyl prolyl ester
CTC	2-chlorotrityl chloride
Cy	Cyclohexyl
Cys	Cysteine
DATB	1,3-dioxa-5-aza-2,4,6-triborinane
DBU	1,8-diazabicycloundec-7-ene
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DCU	Dicyclohexylurea
DEAD	Diethyl azodicarboxylate
DIC	Diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethyl formamide
DMP	Dess-Martin periodinane
DMSO	Dimethyl sulfoxide
DNPH	2,4-dinitrophenylhydrazine
dr	Diastereomeric ratio
DTT	Dithiothreitol
e.g.	*exempli gratia* (for example)
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
ee	Enantiomeric excess
EPL	Expressed protein ligation
equiv	Equivalent
ESI	Electrospray ionization
Et	Ethyl
et al.	*et alii* (and others)
EWG	Electron withdrawing group
FG	Functional group
Fmoc	Fluorenylmethyloxycarbonyl
Gdn·HCl	Guanidinium hydrochloride
Gln	Glutamine
Glu	Glutamic acid
Gly	Glycine
h	Hour
His	Histidine
HMPA	Hexamethylphosphoramide
HOAt	1-hydroxy-7-azabenzotriazole
HOBT	1-hydroxybenzotriazole

HOPfp	Hydroxypentafluorophenyl
HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
HRP	Horseradish peroxidase
Hz	Hertz
i.e.	*id est* (that is)
Ile	Isoleucine
IMCR	Isocyanide based multicomponent reaction
*i*Pr	Isopropyl
IR	Infrared (spectroscopy)
J	Coupling constant
Leu	Leucine
Log*P*	Octanol/water partition coefficient
LPPS	Liquid phase peptide synthesis
Lys	Lysine
M	Molar (concentration)
m.p.	Melting point
MCR	Multicomponent reaction
Me	Methyl
MesNa	Sodium 2-mercaptoethanesulfonate
Met	Methionine
min	Minute
MOF	Metal organic framework
MPAA	4-Mercaptophenylacetic acid
Ms	Methanesulfonyl
MTBSTFA	*N*-(tert-butyldimethylsilyl)-*N*-methyltrifluoroacetamide
NBS	*N*-bromosuccinimide
NCL	Native chemical ligation
NDTP	5-nitro-4,6-dithiocyanatopyrimidine
NHPI	*N*-hydroxyphthalimide
NHS	*N*-hydroxysuccinimide
NMM	*N*-methylmorpholine
NMR	Nuclear magnetic resonance
Nu	Nucleophile
Oxyma	Ethyl cyanohydroxyiminoacetate
P-3CR	Passerini three-component reaction
PB	Phosphate buffer
PBS	Phosphate buffered saline
PC	Photocatalyst
PFP	Pentafluorophenyl
PG	Protecting group
Ph	Phenyl
Phe	Phenylalanine
pin	Pinacol
pK_{BHX}	Hydrogen-bond basicity parameter
POM	Polyoxometalates
ppm	Parts per million
Pro	Proline
*p*TsOH	*para*-toluenesulfonic acid
quant	Quantitative
RAE	Redox-active ester
R_f	Retention factor
rt	Room temperature
SAL	Sugar-assisted glycopeptide ligations
Ser	Serine
SET	Single-electron transfer
S_N2	Bimolecular nucleophilic substitution
SPPS	Solid phase peptide synthesis
TBEC	1-tert-Butyl-3-ethylcarbodiimide
*t*Bu	*tert*-butyl
Tf	Trifluoromethylsulfonyl
TFA	2,2,2-Trifluoroacetic acid
TFFH	Tetramethylfluoroformamidinium hexafluorophosphate
TFPN	Tetrafluorophthalonitrile

THF	Tetrahydrofuran
Thr	Threonine
TIPS	Triisopropylsilane
TLC	Thin layer chromatography
TM	Transition metal
TMS	Trimethylsilyl
TMSIM	1-(trimethylsilyl)imidazole
TPSA	Topological polar surface area
Trp	Tryptophan
Ts	Toluenesulfonyl
UV	Ultraviolet
U-4CR	Ugi four-component reaction
U-4C-3CR	Ugi four-centre three-component reaction
Val	Valine
vs.	Versus

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Chapter 1

Strategies for the Functionalization of C-terminal Carboxylic Acids on Amino Acids, Peptides and Proteins



Challenges:
- Epimerization
- Hydrolysis
- Regio/chemo selectivity

Abstract*: The functionalization of the C-terminus of amino acids, peptides, and proteins has been extensively studied for more than 150 years and remains central to modern peptide synthesis. In this chapter we discuss strategies for C-terminal activation and functionalization, including classical coupling reagents, enzymatic ligations, photo- and electrochemical decarboxylation, and emerging catalytic approaches. Particular attention is given to advances that minimize epimerization, improve selectivity, and enable the modification of complex peptides and proteins without the need for protecting groups. Current limitations and remaining challenges in achieving general, selective, and broadly applicable C-terminal functionalization methods are also highlighted.*

J. Hanssens, M. Lambers, J. M. Saya and R. V. A. Orru, *manuscript submitted.*

1.1. Introduction

1.1.1 General introduction – value of proteins and their synthesis

Proteins, the complex biomolecules that govern the majority of biological processes (*e.g.* as catalysts, molecule and ion transporters, signaling molecules, and receptors), are fundamental to the advancement of medicine, chemistry, and biology. Together with peptides—chains of <50-100 amino acids (AAs)—they are indispensable across a wide range of fields with significant societal and scientific impact, including catalysis, therapeutics, and diagnostics.[1,2] A notable example is the elucidation of the SARS-CoV-2 spike protein structure, which was pivotal for the rational design of therapeutics and vaccines.[3] Beyond their natural roles, proteins are the molecular workhorses of life, driving processes from cellular communication to advanced therapeutic functions. Since the introduction of insulin, the first protein-based drug, over a century ago, there are now over 350 FDA-approved protein-based therapeutics on the market with a large proportion of these (*i.e.* at least 212) being antibodies.[4,5] Regardless of application, protein studies demand access to significant quantities (occasionally exceeding 40 mg of sample consumption for X-ray crystallography) of highly pure material.[6] Despite extensive research efforts, the efficient and reliable synthesis of proteins remains a formidable task. Although composed of only twenty canonical amino acids, the primary challenge lies in exerting precise control over both sequence and three-dimensional folding. Currently, the synthesis of proteins is based on two principal strategies: ribosomal (synthetic biology) and chemical synthesis.

1.1.2 Ribosomal synthesis

In synthetic biology, living organisms, such as bacteria, are used for the synthesis of proteins. The engine behind these synthetic machineries, the ribosomes, read copies of DNA molecules and stitch amino acids together to form linear chains, also referred to as (poly)peptides. In this process, peptide bonds form so efficiently (in the N-to-C direction) that proteins can be synthesized within minutes (*i.e.* approximately two amino-acids added per second).[7,8] In addition to peptide bond formations, the linear chain is consistently folded to give biologically functional three-dimensional structures.[9] Although recombinant protein synthesis has provided useful insights and applications in both academia and industry,[10] there are several limitations in this biological approach. Aside from laborious purification efforts, this process is primarily restricted to naturally occurring amino acids, which makes it difficult to rationally tune the function of proteins.[11] As a result, chemists are left with the daunting task to overcome these problems and enable selective synthesis of modified proteins.[12]

1.1.3 Chemical synthesis

The chemical synthesis of proteins allows facile introduction of non-proteinogenic or even non-natural amino acids in the peptide chain, thus introducing structural and functional diversity compared to using only natural amino acids. Commonly, chemists use Solid Phase Peptide Synthesis (SPPS), in which a peptide chain is grown on a solid support by stepwise addition of amino acids from the carboxylic acid end to the amine end (C-to-N, **Figure 1A**). To prevent undesired side reactions, all reactive functionalities in the used amino residues and their side chains require protection. This pioneering concept was introduced in 1963 by Merrifield whose work was rewarded with the Nobel Prize in Chemistry 1984.[13] Since then, the performance of SPPS has markedly progressed, allowing the synthesis of peptide chains within hours.[14] For peptide chains shorter than ~60 amino acids, SPPS is superior to synthetic biology technologies. However, the main limitation of SPPS, besides low atom efficiency, is its limited suitability for longer peptide/protein chains.[15,16] Although significant breakthroughs have been made using flow chemistry, where proteins of 164 AAs could be quickly synthesized (*i.e.* one AA every ~2.5 mins), these methods generally require very large excesses of reagents.[17] This size constraint highlights the need for ligation strategies, which enable the synthesis of longer peptide chains by coupling unprotected peptide fragments generated through SPPS or Liquid Phase Peptide Synthesis (LPPS).[18]

The properties of peptides, *e.g.* structure, solubility, and pharmacokinetics, are sensitive to minor alterations of such structures. Over time, scientists have capitalized on this by functionalizing peptides/proteins to enhance or impair such properties to obtain optimized products. An important example is the lipidation of semaglutide (**7**), a GLP-1 analog used for the treatment of diabetes and weight loss and was the third highest selling drug in 2023 (**Figure 1B**).[19] The lipidation of the peptide allowed for a half-life increase of over 100-fold (165 h half-life).[20] Despite significant advancements, the precise modification of proteins and peptides remains a significant bottleneck, often limiting progress in drug development, biomaterials, and biotechnology. Most of the functionalization strategies have targeted the N-terminus or reactive side-chains (*e.g.* cysteine, methionine, lysine and tyrosine).[12,21,22] On the other hand, the selective modification of peptide C-termini is critical for the development of therapeutic conjugates, enzyme conjugates, and functional biomaterials, but poses a major synthetic challenge.

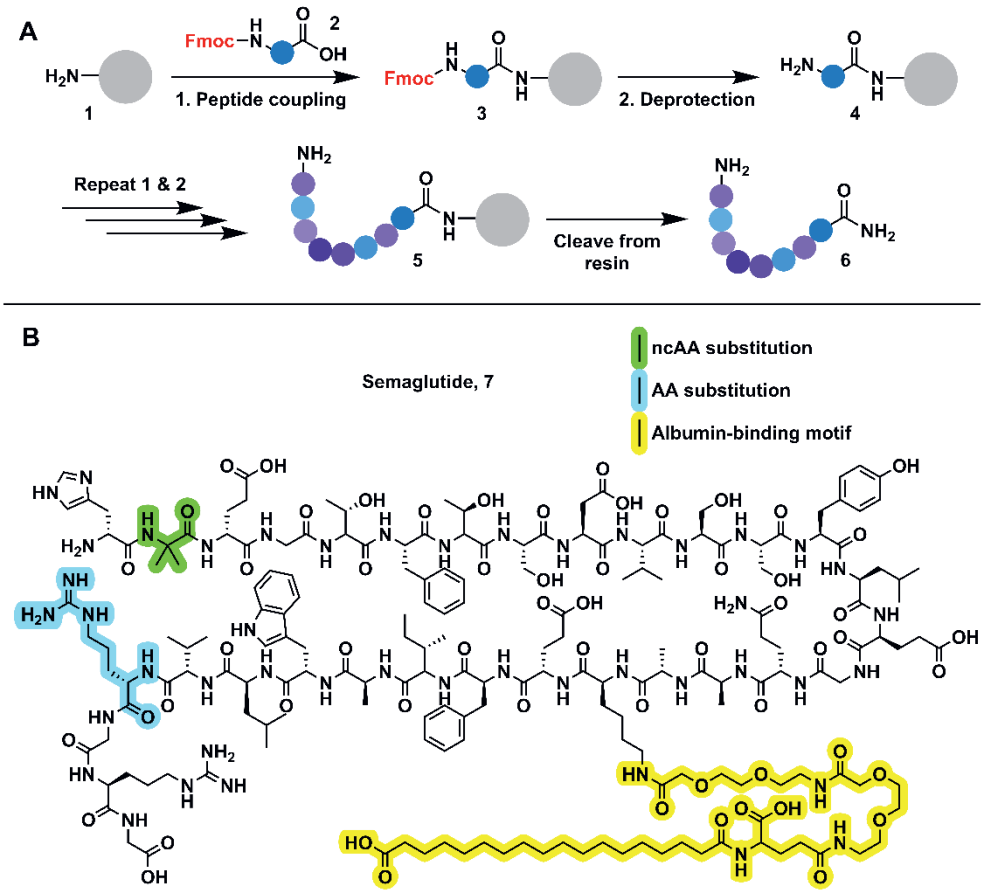


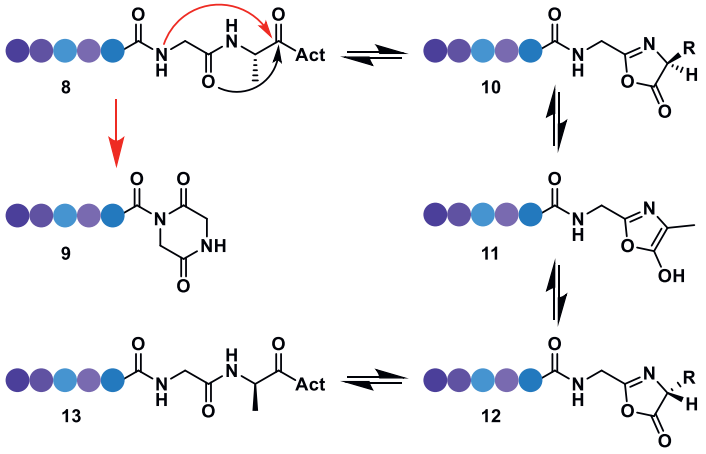
Figure 1. A: Solid Phase peptide synthesis (SPPS). B. Chemical structure of Semaglutide. Green: non-canonical AA, blue: AA substitution, yellow: albumin binding motif.

1.2. Synthetic challenges of C-terminal functionalization

1.2.1 Epimerization

Typical methods of functionalizing carboxylic acids involve the use of activating reagents, thereby introducing a suitable leaving group which can be displaced by an incoming nucleophile.[23] However, in peptide chemistry, such activation of carboxylic acids easily leads to epimerization of the chiral α-carbon via two pathways: oxazolone formation or direct deprotonation (**Scheme 1**).[24,25] Oxazolone formation is caused by the intramolecular attack of the adjacent backbone amide oxygen onto the activated carbonyl, affording an oxazolone (**10**). This heterocycle can subsequently be deprotonated to provide a planar aromatic 5-hydroxyoxazole (**11**). The non-selective protonation then results in a mixture of

diastereoisomers and therefore, in an epimerized peptide product (**13**). Once the desired nucleophile attacks this intermediate, full loss of stereoinformation is the result. Although there have been multiple studies on the performance of SPPS from N-to-C, epimerization via oxazolone formation remains a critical hurdle of such synthesis approaches and additionally of C-terminal functionalization strategies.[24–30] Furthermore, the chain growth can also be stopped by the formation of a diketopiperazine (**9**) by attack of the amide nitrogen from the β-residue.

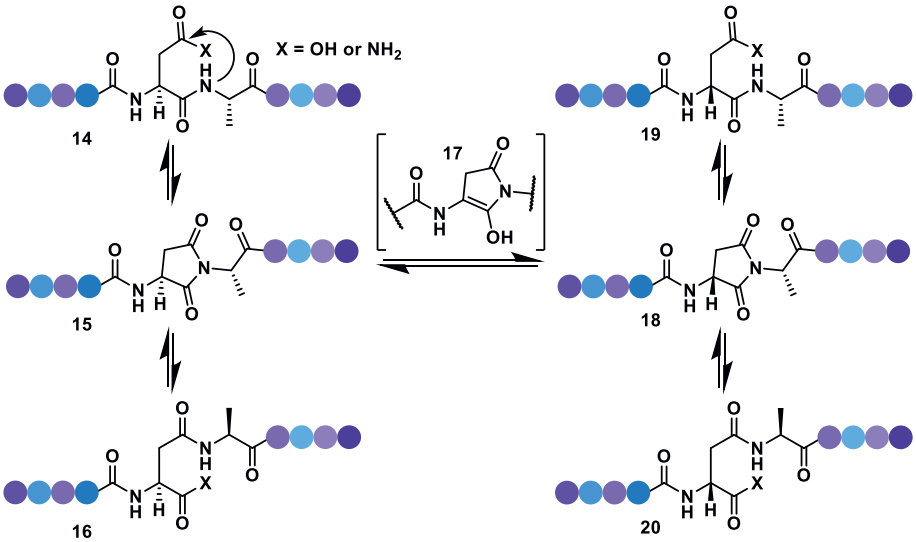


Scheme 1. Epimerization of an activated peptide through oxazolone formation (black arrows) and capping of the growing chain via diketopiperazine formation (red arrows).

In addition to epimerization by means of the mechanisms described above, certain amino acids with functionalized reactive sidechains are naturally more prone to epimerization (*e.g.* Asp, Asn, Cys and His).[31–34] Particularly cysteine is susceptible to both direct α-carbon deprotonation and oxazolone formation. It therefore constitutes a significant challenge since it is prone to epimerization in multiple synthetic steps and multiple research groups have devoted their efforts to solving this issue.[35–38] In the case of Asp and Asn, epimerization is facilitated by succinimide formation (**Scheme 2**).[33,34] This happens spontaneously and non-enzymatically both *in vivo* and *in vitro* and commonly occurs via an attack of the backbone amide nitrogen of the adjacent amino acid on the side-chain carbonyl group of the Asp or Asn residues (**14**) to displace either water or ammonia. The resulting succinimide (**15**) easily deprotonates via an enolate intermediate, resulting in epimerization (**18**). Furthermore, hydrolysis of the succinimide results in a mixture of two isomers (**19**-**20**) since ring opening can occur at both carbonyls. This typically provides a mixture of Asp and *iso*-Asp at a ratio of 1:3.[33,34]

1.2.2 Hydrolysis

In addition to epimerization, C-terminal activation often increases susceptibility to hydrolysis. Both can seriously hamper desired product formation, lowering the overall yield, and complicate product purification. Moreover, certain modified C-terminal moieties are more vulnerable to hydrolysis. For example, C-terminal thioesters are highly sought after owing to their application in native chemical ligation (NCL, discussed in more detail below).[30,39,40] These structures are quite reactive and must be handled under acidic conditions to prevent hydrolysis. Furthermore, they decompose over time upon storing.



Scheme 2. Chemical pathways for Succinimide-mediated Asp/Asn isomerization.

1.2.3 Chemo- and regioselectivity

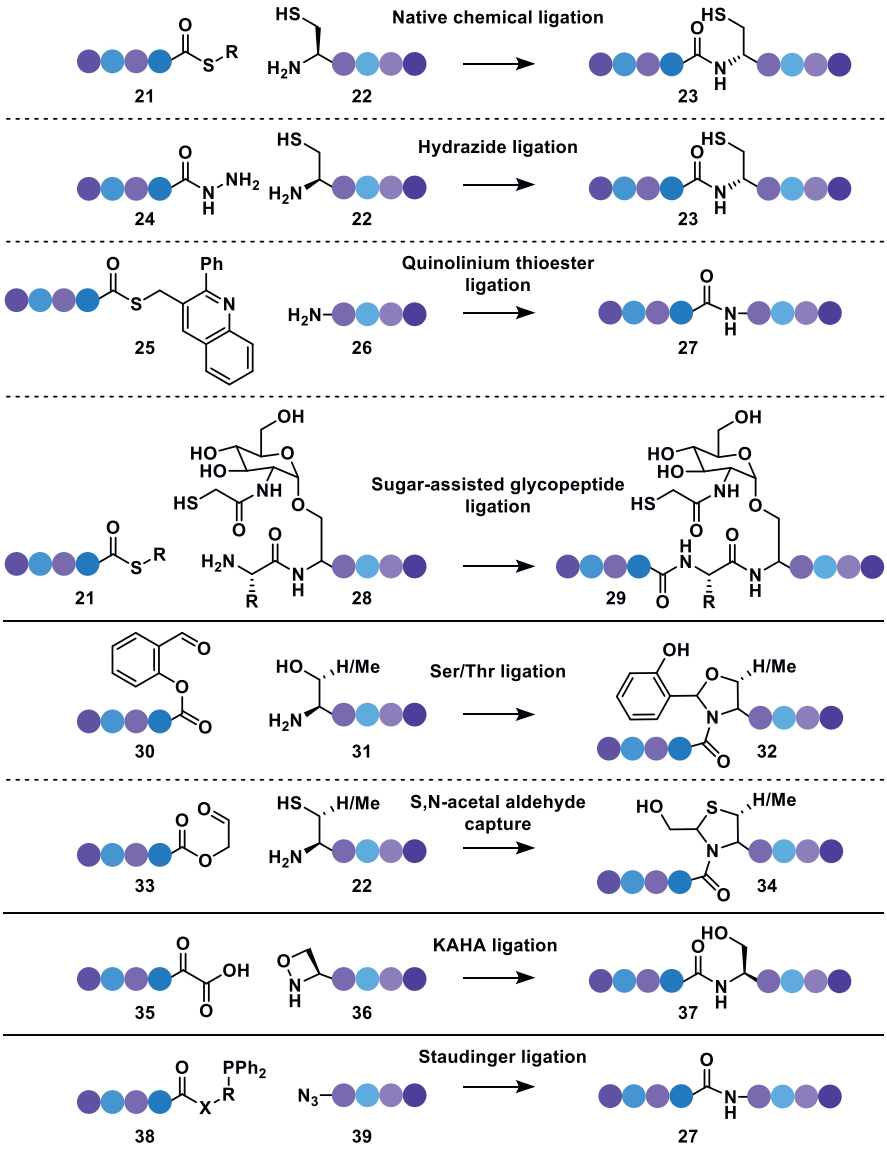
One of the major challenges in selective C-terminal functionalization is achieving both chemo- and regioselectivity. Canonical amino acids present a wide array of reactive functionalities, including nucleophilic groups (*e.g.* alcohols, amines, and thiols) and polar, ionizable moieties (*e.g.* imidazoles, guanidines, carboxylic acids, and amines). Consequently, significant efforts have been devoted to the development of site-selective modification strategies.[12] In addition to chemoselectivity, regioselectivity poses an additional challenge when targeting the C-terminus. The sidechains of Asp and Glu contain carboxylates which closely resemble the C-terminus in pKa and chemical environment, rendering selective C-terminal functionalization especially demanding.

1.3. Peptide ligation strategies

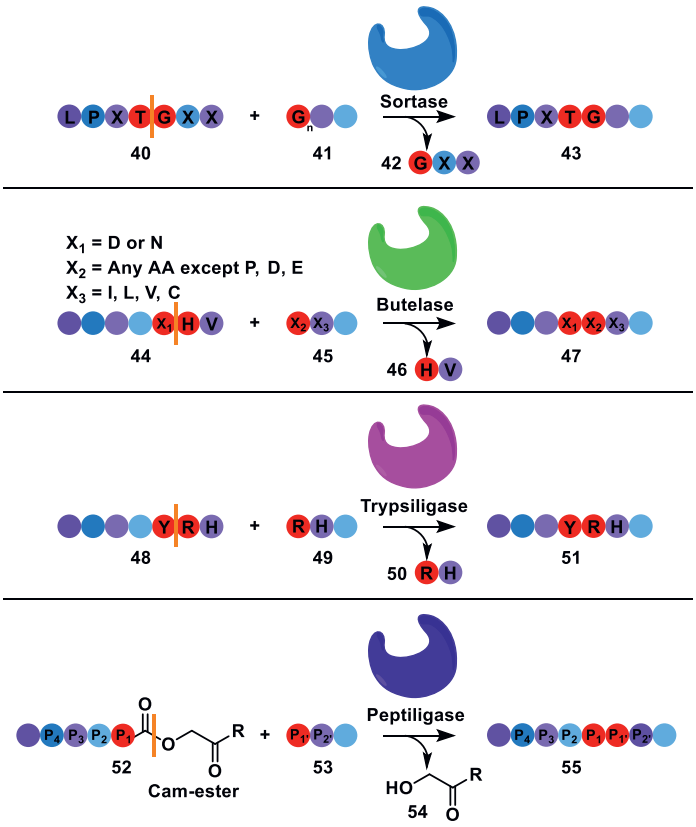
To facilitate the synthesis of longer peptide chains, more advanced techniques such as ligation strategies have been introduced. These strategies involve the selective coupling of unprotected peptide fragments, which are synthesized through SPPS or LPPS, to form extended peptide sequences.[18] This research field has made significant progress in chemical ligation of unprotected peptides using various strategies: native chemical ligation (NCL) type (NCL[39,41–45], hydrazide ligation[46,47], quinolinium thioester ligation[48,49], sugar-assisted glycopeptide ligations (SAL)[50–52]), aldehyde capture ligations (Ser/Thr ligation[53–55], *S*,*O*-acetal aldehyde capture ligation[56]), KAHA ligation[57,58], or the Staudinger ligation[59–64] (**Scheme 3**).

Enzymatic/biochemical ligation approaches rapidly advanced over the past decades and are especially acknowledged as a complementary methodology to traditional chemical ligations.[65,66] They have proven to be a valuable tool since they show excellent chemoselectivity with unprotected peptides, no racemization at the ligation site and are far more atom-efficient and thus more environmentally benign.[67–70] Multiple enzymes with the ability to catalyze peptide-coupling reactions have been identified, including sortases[71,72], butelases[73–75], OaAEP1 (*i.e.* a butelase-1 homolog)[76,77], trypsin related enzymes and subtilisin-derived variants.[69,78] These enzymatic ligations, while highly selective, necessitate a specific recognition sequence, often leading to the requirement of a unique sequence in the end product (**Scheme 4**).

Chemo-enzymatic ligations utilizing the peptiligase enzyme family (derived from subtilisin), advantageously enable ligation of nearly all primary amino acids without requiring a specific recognition sequence.[69,70,79–81] Ligations with peptiligase are kinetically driven, requiring merely an activated ester for the formation of a peptide bond. Carboxyamidomethyl (Cam) esters are often used as they optimally balance reactivity and stability. However, other esters like thioesters and HMBA-esters are also exploited depending on the application.[80] Typically, the Cam ester is synthesized through standard SPPS. A drawback of this method is the susceptibility of Cam esters to hydrolysis during SPPS, which negatively impacts the yield of the desired peptides.[80] Importantly, extending this strategy to the C-termini of native peptides would further broaden its utility, enabling direct C-terminal functionalizations and expanding the scope of peptide and protein ligations.



Scheme 3. Strategies for chemical peptide ligation.



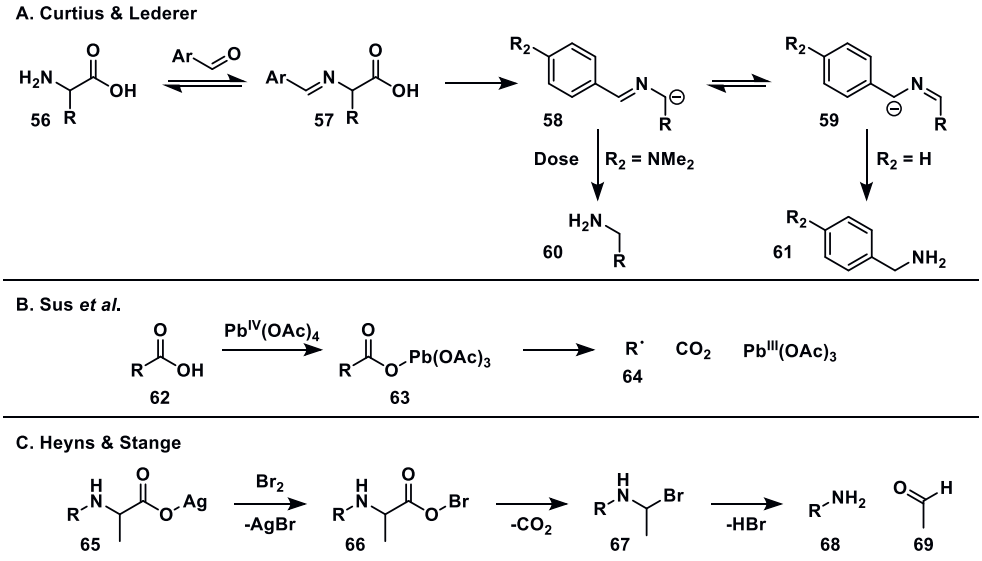
Scheme 4. Strategies for enzymatic peptide ligation.

1.4. Decarboxylation strategies

1.4.1 Classical strategies

Over the past centuries, chemists have developed methods for the decarboxylation of carboxylic acid groups. The Kolbe, Hunsdiecker, and Barton decarboxylations have been widely applied in various fields of research.[82–87] Notably, the first decarboxylation reaction of amino acids was observed by Strecker in 1862.[88] He reported the formation of acetaldehyde and carbon dioxide by reacting alloxan and alanine. This decarboxylation of α-amino acids by means of an imine intermediate is now commonly known as the Strecker degradation. Nearly a century later, Schönberg *et al*. published an extensive review on this reaction and the various means of achieving it.[89,90] A variation of the Strecker degradation was later published by Curtius and Lederer. They decarboxylated glycine at elevated temperatures (up to 130 °C) with benzaldehyde.[91] Interestingly, they observed benzylamine

(**61**) as the main product as opposed to methylamine owing to transamination of the Schiff base. Dose later found that by tuning the electronic properties of the aromatic ring, one can prevent this and they could isolate the decarboxylated amino acid (**60**, **Scheme 5A**).[92] Then, Barakat *et al*. found another route for decarboxylative decompositions using *N*-bromosuccinimide or *N*-bromophthalimide.[93] This method was expanded by Chapelle and Luck, who could perform the decarboxylation of not only amino acids, but on larger peptides and proteins as well.[94]



Scheme 5. Various decarboxylation strategies.

In 1949, Süs reported the use of $Pb(OAc)_4$ for the oxidative decarboxylation to provide free alkyl radicals (**64**, **Scheme 5B**).[95] Sheldon and Kochi published an extensive review of such oxidative decarboxylations mediated by $Pb(OAc)_4$.[96] These reactions involve a free-radical chain mechanism resulting in various products (*e.g.* esters, alkyls, alkenes). Heyns and Stange were the first to use Hunsdiecker conditions for the decarboxylation of amino acids and even short peptides (**Scheme 5C**).[97–99] The mechanism also involves a radical chain mechanism, however, here it is initiated by the formation of a silver salt at the C-terminal carboxyl group (**65**). Upon treatment with bromine or *N*-bromosuccinimide (NBS), the silver salt undergoes oxidative decarboxylation, releasing carbon dioxide (CO_2) and forming a bromoalkyl intermediate (**67**). This intermediate is unstable and rapidly hydrolyzes to yield an aldehyde (**69**) and the N-terminal fragment amide (**68**). They condensed the resulting carbonyl compound with 2,4-dinitrophenylhydrazine (DNPH) and subsequently confirmed the selective cleavage of the C-terminal amino acid using paper chromatography.[97,98]

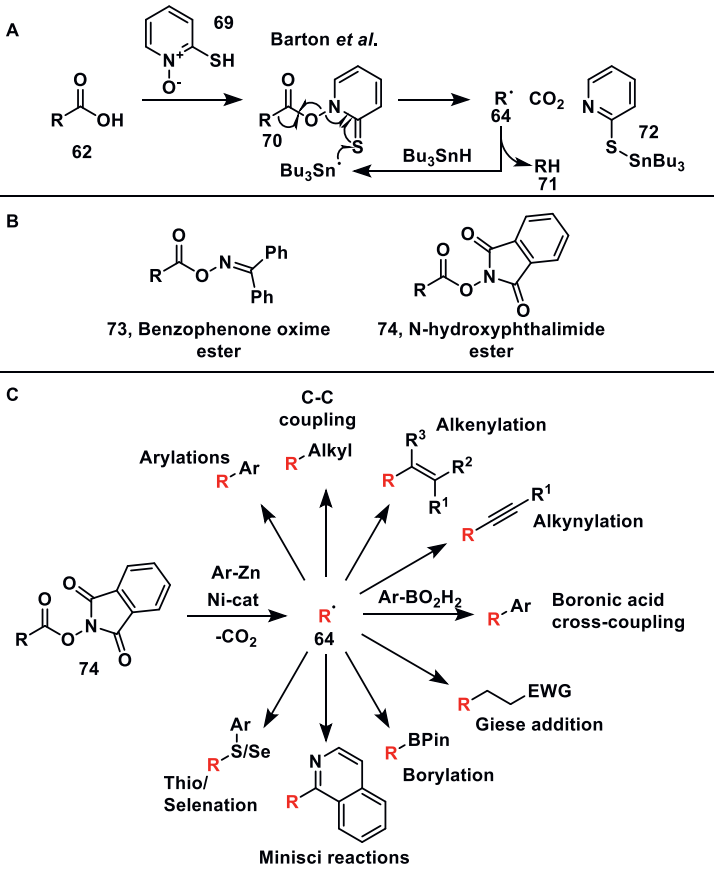
1.4.2 Photochemical decarboxylations

Over time, the use of decarboxylative couplings has become a dominating strategy in C-terminal modifications on peptides and proteins.[100,101] Some key examples in literature are highlighted below.

1.4.2.1 Redox-active esters

In 1983, Barton *et al*. published, for the first time, the use of a redox-active ester (RAE) to decarboxylate amino acids.[102,103] These thioxamic anhydrides, later termed Barton esters (**70**), could conveniently be obtained by coupling pyrithione (**69**) with a carboxylic acid (**62**, **Scheme 6A**). They readily decompose under visible light to provide carbon dioxide and an alkyl radical (**64**). The latter has been utilized for a multitude of functionalizations (*e.g.* halogen atom transfer, reductive decarboxylation, Giese addition).[104–108] After the discovery of the Barton ester, alternative redox-active esters were developed for photochemical decarboxylative functionalizations: benzophenone oxime esters (**73**)[109], and *N*-hydroxyphthalimide (**74**, NHPI) esters (**Scheme 6B**).[110–112] The latter was first reported by Okada *et al*. in 1988, marking the start of a range of transformations to various products using this RAE-approach (*e.g.* alkanes, chloroalkanes, Giese addition products).[110–112]

In an attempt to mitigate some of the issues that accompany the use of the Barton ester (*i.e.* the need for a photochemical setup, use of toxic/odorous reductants, and their low stability), the group of Baran used a RAE based on the NHPI group in combination with aryl-zinc reagents and a nickel-catalyst in the absence of light.[113] Although similar to the work of Okada, their mechanism involved a single-electron transfer (SET) from the transition-metal (TM) complex to initiate the radical decarboxylation. Baran *et al.* pioneered the use of this group for a variety of nickel- or iron-catalyzed transformations when applied to amino acids and even peptides: alkyl-alkyl cross-couplings[114,115], alkenylation[116], alkynylation[117], cross-couplings with sp^2-boronic acids[118], Giese addition[119], and decarboxylative borylations.[120] Other groups have also reported arylations with aryl isocyanides[121,122], Minisci reactions[123], homologations[124], Giese additions[125], thiolations[126], and selenation[127] reactions based on this principle (**Scheme 6C**).



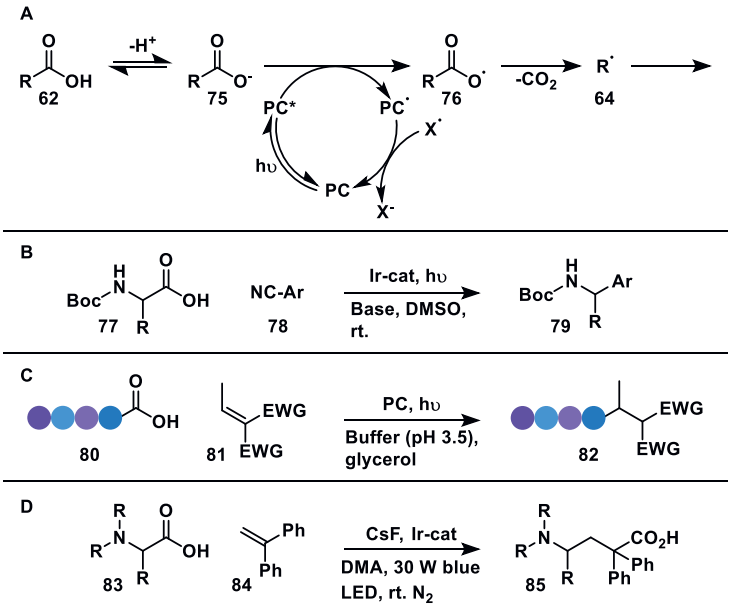
Scheme 6. A. Barton ester mediated decarboxylation. B. Examples of Redox-active esters. C. Examples of reported functionalizations using an NHPI-ester decarboxylation strategy.

1.4.2.2 Free carboxylic acids

Over the last ten years, significant advancements have been made in using visible light for chemical transformations. In this context, synthetic organic chemists have developed photochemical electron transfer reactions of free carboxylic acids (**62**, **Scheme 7A**). Such processes generally require a suitable photocatalyst (PC), which upon irradiation with light becomes an excited state complex (**PC***) that is highly oxidizing. After deprotonating the target carboxylic acid, a single-electron oxidation takes place, transferring an electron to the excited photocatalyst to afford the carboxyl radical (**76**). This species undergoes spontaneous decarboxylation to provide carbon dioxide and an alkyl radical (**64**) which can be further functionalized. Wallentin *et al*. described the first hydrodecarboxylation of stabilized carboxylic acids (*e.g.* amino acids and phenylacetic acid derivatives) using this

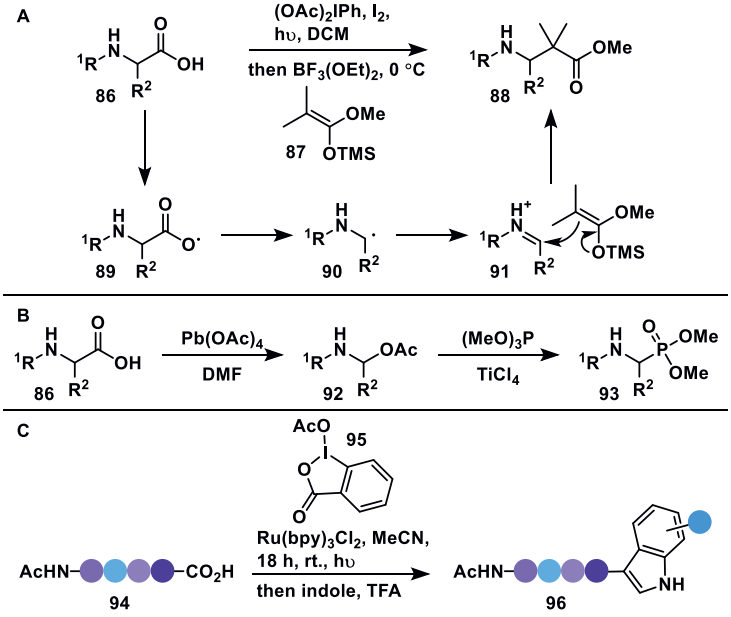
method.[128] The group of Nicewicz later reported a method which also allowed for the functionalization of unstabilized aliphatic carboxylic acids.[129]

In 2014, the Macmillan group published the first photochemical decarboxylative arylation of amino acids (**77**).[130] Therein, a photoredox-mediated decarboxylation afforded an α-carbamoyl radical, which could in turn partake in a diradical coupling with a formed radical anion from cyanoarenes (**78**) to form a C-C bond (**79**, **Scheme 7B**). Later that year, the same group employed this method using the alkyl radical for Giese additions on several dipeptide substrates.[131] They also expanded their method to mediate intramolecular macrocyclization reactions of peptides bearing an N-terminal acrylamide functionality and an unprotected C-terminus.[132] This Iridium-catalyzed reaction was chemoselective for C-terminal acids over those found in the sidechain of Asp and Glu owing to the higher stability of the resulting α-amino radical. In addition, this concept was employed for the selective decarboxylation of larger, more complex peptides and even proteins (**Scheme 7C**).[133] Therein, they used a water-soluble flavin-based photocatalyst under strongly diluted conditions (1 mM with respect to the peptide/protein) in aqueous buffer solution to modify important peptides such as the ZHER affibody and even human insulin. This method was later employed by Bloom, Marcotte and Anslyn *et al*. as a means for bulk- and single-molecule proteomics.[134] Additional conversions performed using a photochemical decarboxylation approach include: arylation[135,136], allylations[128], cyanation[137], alkynylation[137], azidation[138], macrocyclizations with N-terminal NHPI[139–141], unnatural α-amino acid synthesis[142], and decarboxylative oxygenations.[143] In 2021, Yu *et al*. reported a novel variation of the decarboxylative alkylation where they managed to simultaneously utilize both the alkyls and the carboxyl parts of the carboxylic acids in carbocarboxylation reactions that do not release carbon dioxide (**Scheme 7D**).[144] Rather, the initially released carbon dioxide is trapped by the formed alkyl anion to form the final product (**85**). Next to the low catalyst loading and mild reaction conditions, their method involves a high step and atom economy, tolerates diverse functional groups, and is readily scalable.



Scheme 7. A. Direct photochemical decarboxylation. B. Decarboxylative arylation as reported by Macmillan. C. Decarboxylative arylation of peptides and proteins as reported by Macmillan. D. Decarboxylative carbocarboxylation as reported by Yu et al.

Thus, the alkyl radical resulting from decarboxylation can be used for additions or diradical couplings (as described above), however, it can also be oxidized to form the *N*-acyl iminium ion (**91**). This reactive species can subsequently be targeted by nucleophilic reagents. Hernandez and Boto *et al*. reported an oxidative Mannich reaction using $PhI(OAc)_2$, iodine and a silyl ketene acetal under visible-light irradiation generating β-amino acids (**88**, **Scheme 8A**).[145,146] Furthermore, the *N*-acyliminium ion may also be trapped with protic nucleophilic solvents to afford *N,O*-acetals (**92**). In that respect, Corcoran *et al*. reported a decarboxylation with $Pb(OAc)_4$ like those described previously and managed to obtain the desired *N,O*-acetals, which could subsequently be further modified to the corresponding phosphonate (**93**, **Scheme 8B**).[147] Later, the group of Waser developed a photoredox-catalyzed decarboxylative coupling to *N,O*-acetals which could sequentially be modified to aromatic functionalities *i.e.* phenols and indoles (**96**, **Scheme 8C**).[148] A few excellent reviews have been published with further information on photochemical transformations of amino acids, peptides and proteins.[149–152]



Scheme 8. A. Photochemical decarboxylative Mannich reaction (Hernandez & Boto *et al*.) B. Decarboxylative N,O-acetal formation and subsequent phosphonation (Corcoran *et al*.) C. Photochemical decarboxylation and subsequent arylation (Waser *et al*.)

1.4.3 Electrochemical decarboxylations

Next to these (photo)chemical approaches, oxidative decarboxylation reactions of carboxylic acids in peptides can be performed electrochemically. Historically, the first to report the electrochemical decarboxylative transformation of carboxylic acids into alcohols were Hofer and Moest in 1902.[153,154] Their approach was later utilized as a means for stepwise degradation of polypeptides.[155] Seebach *et al.* used this method for the synthesis of *N,O*-acetals (**97**) from amino acids (**86**) and peptides in the presence of methanol or acetic acid. The products could in turn be activated to electrophilic *N*-acyliminium intermediates for further functionalization to various moieties *e.g.* allyl, alkynyl, phosphonyl derivatives (**Scheme 9A**).[156–158]

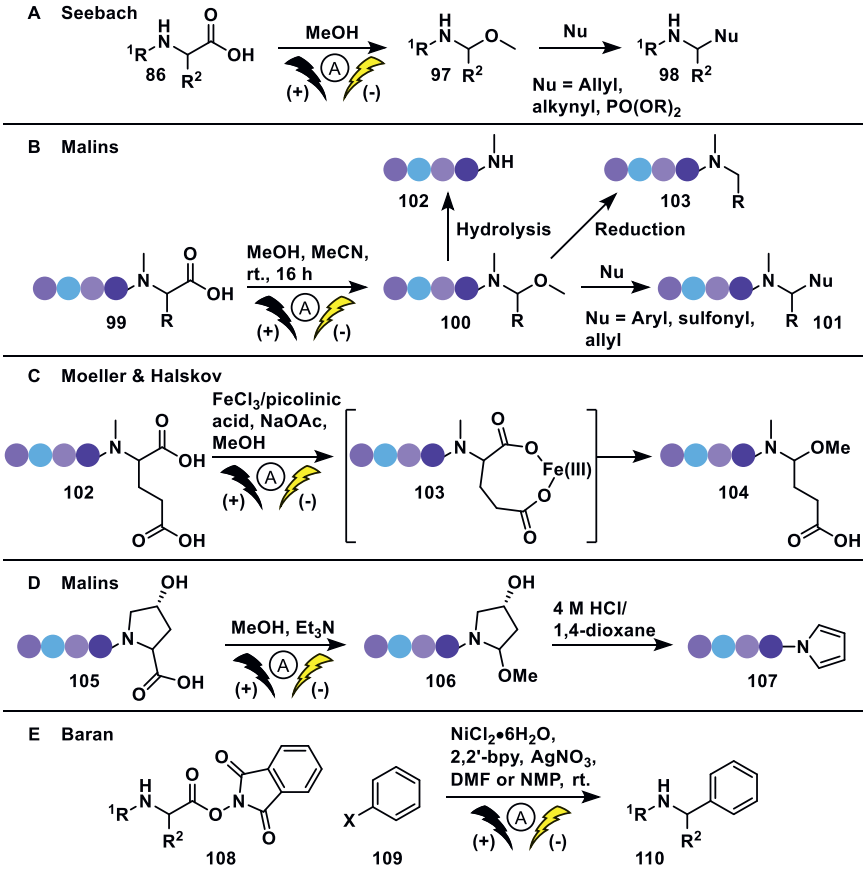
More recently, Malins *et al*. applied the work of Seebach *et al*. to more complex peptides and utilized it for the synthesis of cytotoxic cyanobacterial peptide natural products biseokeaniamides A–C.[159] Therein, they reacted the formed *N,O*-acetals (**100**) with electron-rich aromatics for Friedel-Crafts-type reactions, direct organometallic addition, and sulfonylation chemistry providing C-terminally modified peptide analogues (**101**).[159,160] They continued their work and developed a method to obtain designer peptide C-terminal

amides (**102**-**103**) by performing a tandem hydrolysis/reduction pathway after *N,O*-acetal formation. The methodology tolerates most of the proteinogenic functional groups and excludes epimerization (**Scheme 9B**).[161] The authors further demonstrated the utility of their work by synthesizing natural and bioactive peptides *i.e.* acidiphilamide A, cyclophilin A, and leuprolide (analogues). Recently, Moeller and Halskov *et al.* reported a refined approach for the synthesis of C-terminal amides.[162] They opted to use a chelation event using an iron-based redox mediator to facilitate the selective decarboxylation of the C-terminal acid (**Scheme 9C**). This also allowed for a chemoselective oxidation over other amino acid sidechains and merely required a C-terminal glutamic acid residue (**102**) of which the dicarboxylate residue was required for selective mediator binding (**103**).[162] Finally, the Malins group reported the synthesis of C-terminal *N*-acylpyrroles (**107**) by aromatization of C-terminal hydroxyproline-containing peptides (**105**). The *N*-acylpyrrole products could be further modified to the respective C-terminal aldehyde using $NaBH_4$ (**Scheme 9D**).[163] For more information on chemoselective electrochemical transformations on peptides and proteins, I refer the reader to a review by Malins and Payne *et al*.[164]

Using their experience in NHPI-ester mediated decarboxylative arylations, Baran *et al*. developed a general Ag–Ni electrocatalytic procedure for decarboxylative arylation (**110**) that overcomes common limitations in substrate scope, functional group tolerance, and operational complexity (**Scheme 9E**).[165] By employing *in situ*-formed silver nanoparticle electrodes, the method stabilizes reactive intermediates, reduces overpotential, and enables coupling of redox-active esters with diverse (hetero)aryl halides. This electrochemical strategy proceeds under mild, scalable, and open-air conditions, offering a robust alternative where conventional and photochemical methods fail. However, they merely functionalized a few amino acids and dipeptides leaving room for application on more complex systems.[165]

There are a few important aspects to consider when comparing the use of RAEs, like the NHPI-ester, with free carboxylic acids for decarboxylative coupling reactions. RAEs enable decarboxylation through reductive activation and are generally considered the most versatile and robust platform for decarboxylative $C(sp^3)$–$C(sp^2)$ cross-coupling.[166] RAEs engage in single-electron transfer under mild conditions to generate carbon-centered radicals, tolerating a broad range of coupling partners and functional groups. However, their use is limited by incompatibility with unprotected primary and secondary amines, which can react with the activated esters, and in some cases by the need for specific coupling partners.[166] In contrast, free carboxylic acids undergo decarboxylation via oxidative activation, typically under photoinduced electron transfer conditions but also available using electrochemistry. This approach avoids the intermediacy of RAEs and therefore additional synthesis steps and offers an orthogonal strategy for bond construction.

Nevertheless, the presence of redox-sensitive functional groups can quench the photocatalyst, potentially diminishing reaction efficiency.[166]



Scheme 9. Electrochemical decarboxylation reactions. A. Acetal formation and subsequent nucleophilic attack (Seebach et al.) B. Acetal formation and subsequent hydrolysis, reduction, or substitution (Malins et al.) C. Selective C-terminal decarboxylation (Moeller & Halskov), D. C-terminal pyrazole formation (Malins et al.) E. Decarboxylative arylation (Baran et al.)

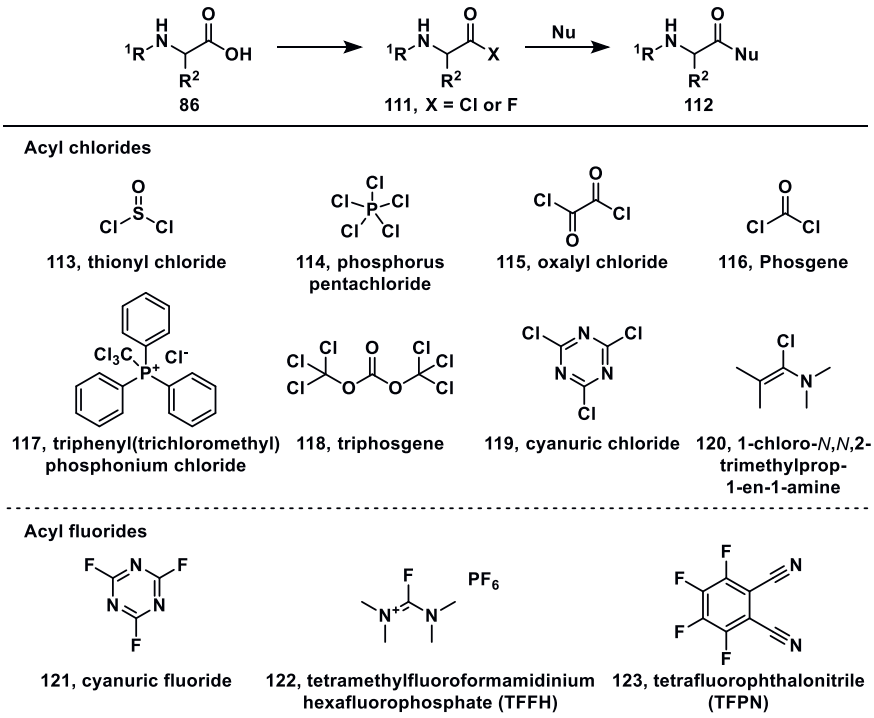
1.5. Condensation strategies

1.5.1 Acyl Halides

Direct nucleophilic substitution at a carboxylic acid is intrinsically disfavored, and in peptides the need for activation is further challenged by the constant risk of C-terminal epimerization and competitive side-chain reactivity. The earliest methods for peptide bond formation relied on acyl halide intermediates (**111**), particularly acid chlorides, which

convert the carboxylic acid into a highly reactive electrophile (**Scheme 10**). As early as 1901, Fischer and Fourneau reported the synthesis of Gly–Gly using thionyl chloride (**113**) and phosphorus pentachloride (**114**) to generate the acid chloride.[167] While highly effective, these reagents are corrosive and moisture-sensitive, posing significant challenges for substrates with sensitive side chains or protective groups. Later innovations included the use of oxalyl chloride (**115**)[167] and phosgene (**116**).[167] The reactants lead to the formation of volatile by-products (CO_2 or SO_2) or to strong P-O bonds, traits that provide significant driving force for these processes. However, these reagents still suffer from the generation of HCl, which can catalyze undesired side reactions and lead to racemization at the α-carbon.[167] Efforts to mitigate this included the use of pyridine as both solvent and base, forming acylpyridinium intermediates that offer more controlled reactivity.[168] Additional reagents that have been developed for the synthesis of acyl chlorides include triphenylphosphine-CCl_4 (**117**)[169], triphosgene (**118**)[170], cyanuric chloride (**119**)[171], and tetramethyl-α-chloroenamine (**120**).[172] Despite improvements, acid chlorides remain largely incompatible with modern peptide synthesis owing to the harsh activation conditions and the low functional group tolerance. Particularly in the context of stereointegrity, epimerization/racemization due to oxazolone or enolate formation continues to be a limitation, especially during the activation of C-terminal residues.[173]

Acyl fluorides have attracted interest as coupling agents due to their enhanced stability to hydrolysis compared to acid chlorides and their reduced susceptibility to racemization, particularly under mild conditions.[174,175] They are effective in the coupling of sterically hindered α,α-disubstituted amino acids and are compatible with acid-sensitive protecting groups, including those used in Fmoc-based SPPS.[176,177] Their in-situ generation using reagents such as cyanuric fluoride (**121**) or fluoroformamidinium salts (*e.g.* TFFH **122**, BTFFH) enables efficient activation without isolating unstable intermediates (**Scheme 10**).[178,179] A recent example is tetrafluorophthalonitrile (**123**, TFPN), a benchstable, cheap, and readily available coupling reagent which has been found to be effective in both simple and challenging couplings and can be used in SPPS.[180] However, limitations include the need for specialized fluorinating agents, occasional side reactions with sensitive residues, and incomplete activation in some cases, particularly when dealing with hindered substrates or acids with low solubility.[181]



Scheme 10. Examples of acyl chloride and acyl fluoride-forming reagents.

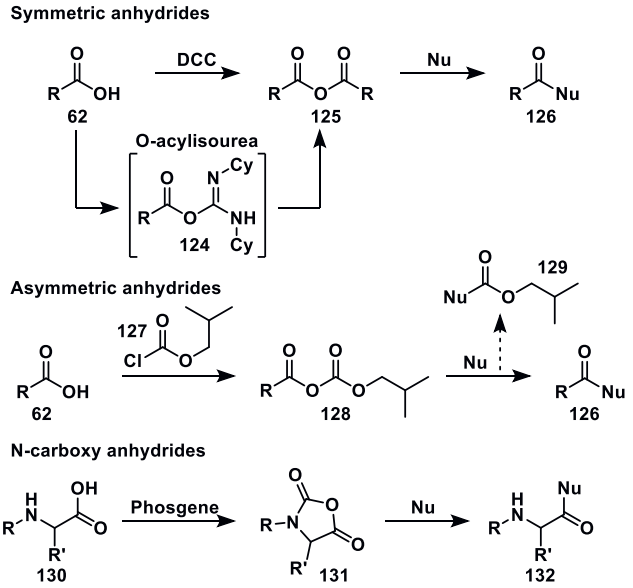
1.5.2 Anhydrides

Anhydrides were among the first reagents used for peptide bond formation and played a significant role in the early development of peptide chemistry. Two main types are typically employed: symmetric anhydrides and mixed anhydrides.[181]

1.5.2.1 Symmetric Anhydrides

Symmetric anhydrides are generally formed *in situ* by reacting a carboxylic acid with a carbodiimide (discussed later), such as dicyclohexyl carbodiimide (DCC), under anhydrous conditions (**Scheme 11**).[181] This process initially forms an *O*-acylisourea intermediate (**124**), which, in the absence of amine nucleophiles, can undergo displacement by a second equivalent of the acid to generate the anhydride (**125**).[182,183] Unlike *O*-acylisoureas, symmetric anhydrides preferentially acylate N-nucleophiles (amines) over O-nucleophiles owing to their lower reactivity, making them more chemoselective and reducing side-reactions. Although these reagents are sufficiently stable to be isolated and purified, they cannot be stored over long periods of time. Furthermore, while 0.5 equivalents of the

original carboxylic acid is regenerated after coupling, its recovery is not cost- or time-effective.[182,183]



Scheme 11. Variations of different anhydride strategies activation strategies.

1.5.2.2 Mixed Anhydrides

Mixed anhydrides were first introduced as a peptide coupling strategy by Vaughan in 1951.[184] They are typically generated by treating a carboxylic acid with an alkyl chloroformate (*e.g.* isobutyl chloroformate **127**) in the presence of a tertiary amine, usually *N*-methylmorpholine (NMM)(**Scheme 11**).[185,186] The resulting mixed carbonic anhydride (**128**) reacts efficiently with amine nucleophiles to form amide bonds under relatively mild conditions. The method is valued for its operational simplicity, short reaction times, and compatibility with various amino acid side chains.[186] Owing to the reactive and unstable species, optimal results are often achieved by pre-activating the acid for a brief period (1–2 minutes) at low temperatures (−5 to −15 °C) in solvents such as ethyl acetate or tetrahydrofuran.[187] This procedure reduces the likelihood of side reactions and promotes high coupling efficiency. However, a notable disadvantage is that the method suffers from lack of regioselectivity, as aminolysis can occur at either the acyl or the carbonate carbonyl. It also often leads to side reactions such as urethane formation, especially when excess chloroformate is used.[185,186]

Leuchs further developed the anhydride strategy into the cyclic *N*-carboxy anhydrides (**131**), which can be readily prepared from free amino acids and phosgene (**Scheme 11**).[188] Upon reacting these compounds with catalytic amounts nucleophile, a ring opening and subsequent decarboxylation reveals a new nucleophile, causing a polymerization chain reaction. The strategy is commonly employed for the synthesis of polypeptides but has also been employed for mono addition to the N-terminus of an amino acid/peptide in SPPS. Tuning the stability of the intermediate carbamic acid is crucial to ensure monocoupling.[188,189]

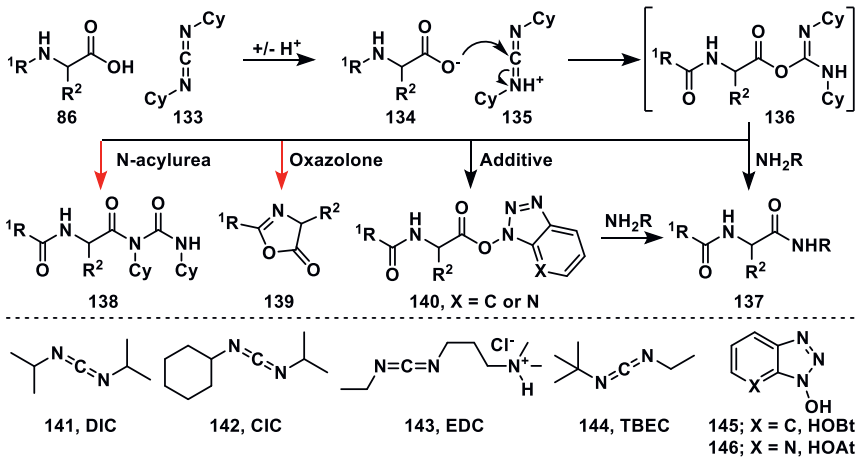
Though mixed anhydrides were once widely used in peptide synthesis, their instability and moisture sensitivity allowed them to be largely replaced by more efficient and versatile coupling reagents such as carbodiimides with additives, phosphonium, and uronium salts (discussed later). Nevertheless, they remain historically significant and are occasionally used in solution-phase protocols, particularly for small peptides or special cases requiring mild, non-aqueous conditions.

1.5.3 Carbodiimides

Carbodiimides are among the most historically significant reagents for peptide bond formation and continue to play a central role in modern synthesis. They first appeared in the early 1950s, when Sheehan and Hess first introduced DCC (**133**) as a coupling reagent capable of activating carboxylic acids toward amide formation.[190] The general mechanism involves the initial protonation of the carbodiimide with the carboxylic acid (**186**) and subsequent attack of the carboxylate (**134**) to form an *O*-acylisourea intermediate (**136**). This species is then subject to nucleophilic attack by a free amine to provide the desired peptide bond (**137**) and a urea by-product.[191] However, this mechanism competes with three additional pathways: anhydride formation by attack of an additional carboxylate (see previous section), and acyl migration to form *N*-acylurea (**138**), and oxazolone formation (**139**, **Scheme 12**).[192,193] While the first two lead to a reduction in yield, the third pathway causes epimerization through oxazole formation as previously mentioned.

A key issue of carbodiimides is that the *O*-acylisourea intermediate is highly reactive but non-selective, capable of acylating both amines and hydroxyl groups, thereby leading to side products such as esters.[192,193] Furthermore, the rearrangement to the unreactive *N*-acylurea (**138**) mentioned above results in reduced coupling efficiency and potential loss of starting material. To address these issues, nucleophilic additives such as 1-hydroxybenzotriazole (HOBt, **145**) and 1-hydroxy-7-azabenzotriazole (HOAt, **146**) are commonly included in the reaction mixture (**Scheme 12**). These additives trap the *O*-acylisourea to form more stable active esters (**140**) that preferentially react with amines,

thereby improving both chemoselectivity and yield. Importantly, they also suppress racemization by minimizing the formation of oxazolone intermediates.[193,194]



Scheme 12. Carbodiimide activation of carboxylic acids including byproducts, sidereactions and examples of reagents.

Like with most reagents, carbodiimides have experienced multiple generations of development and improvement. DCC (**133**), a solid reagent soluble in nonpolar solvents (*e.g.* DCM), was highly favored for solution-phase synthesis, and the urea by-product, N,N'-dicyclohexylurea (DCU), can be easily removed by filtration. However, in SPPS the insolubility of this byproduct causes lower coupling efficiency and more tedious purification.[195] Improved versions like diisopropylcarbodiimide (DIC, **141**), *N*-cyclohexyl-*N'*-isopropylcarbodiimide (CIC, **142**), and *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide (EDC or EDCI, **143**) are more commonly used due to their better solubility and easier removal of the corresponding urea by-product (**Scheme 12**). The latter is even water-soluble and can be easily removed after a reaction by acidic workup.[181,195] In 2019, McFarland *et al*. reported that a coupling reaction with DIC with Oxyma produces the very toxic hydrogen cyanide as a byproduct.[196] Since then, 1-tert-Butyl-3-ethylcarbodiimide (TBEC, **144**) has been explored as a viable alternative and safer alternative (**Scheme 12**).[197]

1.5.4 Phosphorus-based strategies

1.5.4.1 Phosphonium Reagents

Gawne, Kenner, and Sheppard were the first to report the use of acylphosphonium salts as coupling reagents for peptide synthesis in 1969.[198] However, it was not until Castro and coworkers had thoroughly investigated such reagents that they became widely adopted. In

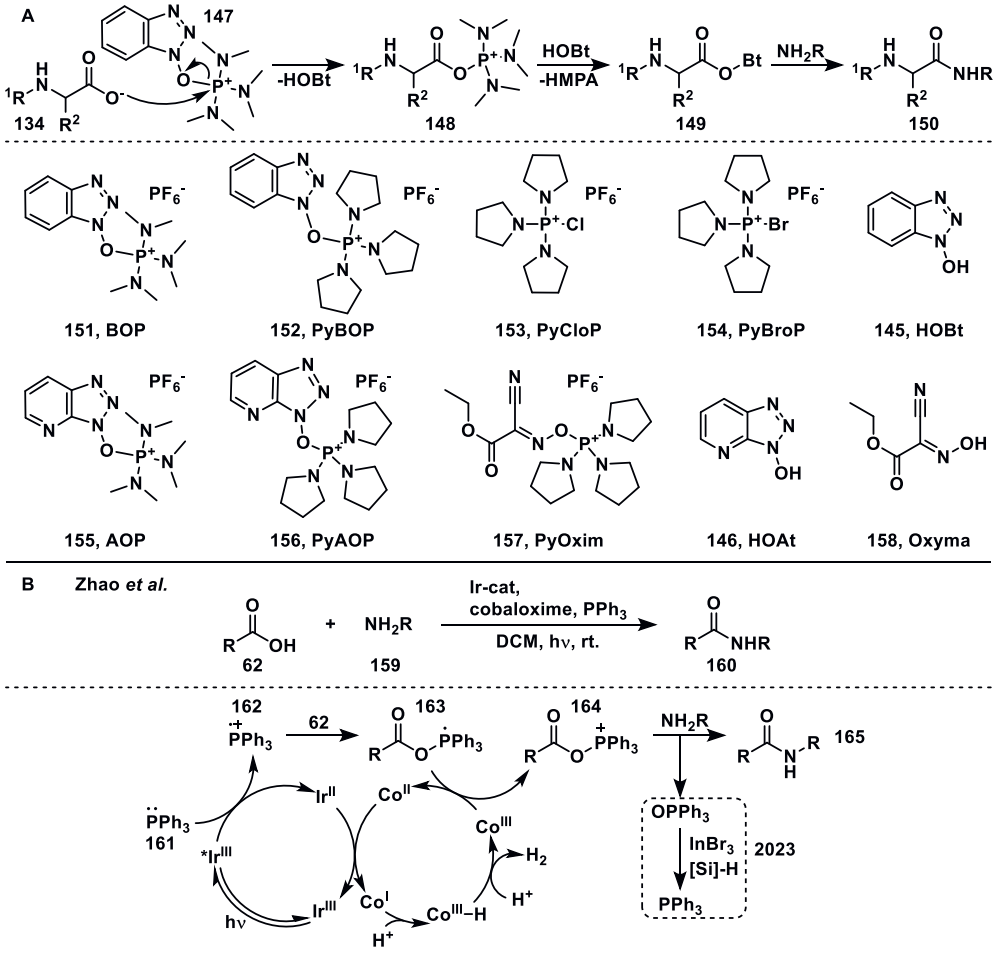
1975, they first published the use of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP, **151**) as coupling reagent (**Scheme 13A**).[199,200] Although this is an excellent coupling reagent, the toxic byproduct, hexamethylphosphoramide (HMPA), sparked researchers to find safer and more efficient alternatives *i.e.* PyCloP (**153**), PyBroP (**154**), and PyBOP (**152**, **Scheme 13A**).[201] Phosphonium reagents such as BOP (**151**) promote amide bond formation by reacting with a carboxylate to form an active acyloxyphosphonium salt, typically in the presence of HOBt (**145**). Upon forming the HOBt ester (**149**) by expulsion of a phosphine oxide, a final attack of an amine results in the desired coupling product. It has been observed that in the absence of nucleophilic derivatives, like HOBt, the symmetrical anhydride or the oxazolone intermediates form the active species of these reactions, lowering the efficiency and allowing more racemization.[201,202]

Aside from the HOBt-based BOP (**151**) and PyBOP (**152**), phosphonium salts based on halides (*e.g.* PyCloP **153**, PyBroP **154**), HOAt (*e.g.* AOP **155**, PyAOP **156**), and on ethyl cyanohydroxyiminoacetate (Oxyma, **158**)(*e.g.* PyOxm, **157**) have been reported (**Scheme 13A**).[181] Most of these reagents show improved reaction efficiency with reduced racemization and toxicities compared to BOP. PyAOP, for example, is particularly well-suited for difficult or hindered couplings, including those involving sterically encumbered amino acids, and is frequently used in advanced SPPS protocols.[203]

Although phosphonium reagents have been largely supplanted by newer uronium and aminium salts (discussed later) due to their improved solubility, lower toxicity, and broader applicability, phosphonium-based systems remain valuable in certain peptide coupling scenarios, particularly when high coupling efficiency and low racemization are critical.

Phosphines have also found use in catalytic carboxyl functionalization. Recently, Zhao, J. *et al*. reported a method which employs photoredox and cobaloxime dual catalysis for the radical catalytic formation of acyloxyphosphonium ions (**164**) to be used for subsequent amidation reactions (**Scheme 13B**).[204] Initially, the oxidation of PPh_3 (**161**) by the iridium photocatalyst results in a phosphine radical cation (**162**), which is then attacked by the carboxylate affording a phosphoranyl radical (**163**). This species is then oxidized to the acyloxyphosphonium ion (**164**) by a Co(III)-species whereafter a final substitution by the nucleophilic amine provides the amide product (**165**). The same group later (2023) reported an extension of this method where they were able to regenerate the initial phosphine by means of reduction with silanes, making a synergistic photoredox, cobaloxime, and organophosphorus triple catalysis process (**Scheme 13B**).[205] The group of Chiba showed that both the oxidation of the initial phosphine to the radical cation and the oxidation to the

acyloxyphosphonium ion can be performed with electrochemistry.[206] They could effectively use their electrochemical method for peptide coupling reactions.

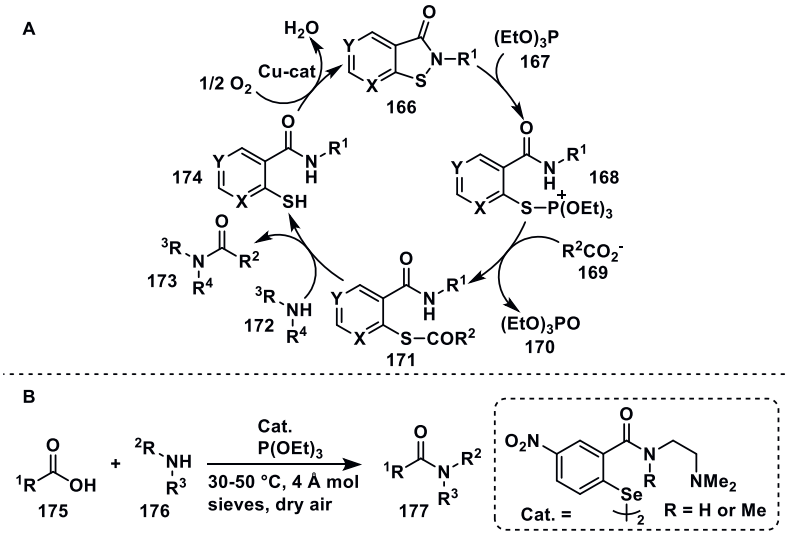


Scheme 13. A. Carboxylate functionalization using typical phosphonium reagents. B. Photochemical dual/triple-catalyzed amide bond formation.

1.5.4.2 Phosphite reagents

Phosphite reagents have been employed for peptide bond formation for quite some time.[207,208] In 1980, for example, Wissmann reported the use of phosphonic anhydride with a base to afford peptides.[209,210] However, this chemistry has recently experienced a renewed interest from the community.

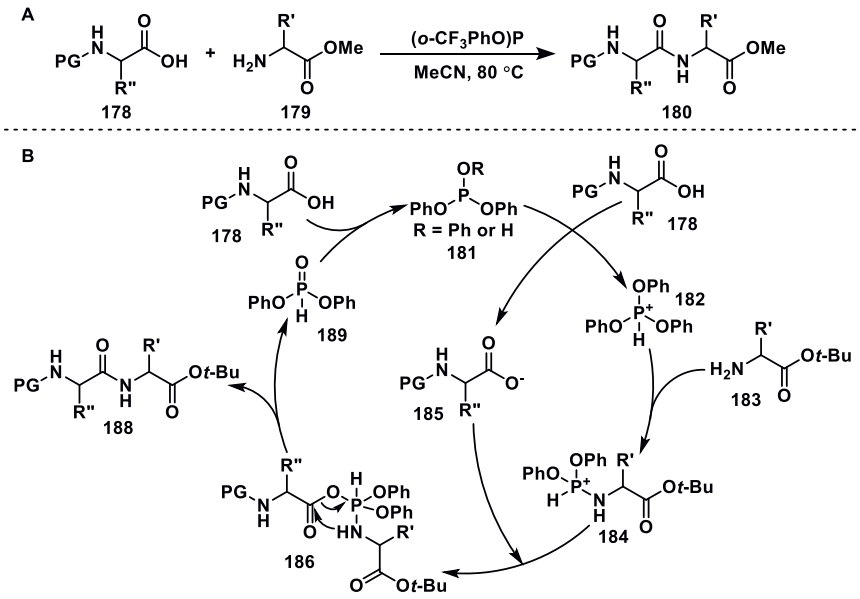
Liebeskind *et al*. developed benzoisothiazolones (**166**) as a new class of coupling reagents, which in combination with $P(OEt)_3$ and a Cu(I)-complex facilitate amide bond formation of amino acids and peptides.[211] These organocatalysts work by forming a thioester (**171**) which is susceptible to attack by an incoming amine nucleophile (**172**). The benzoisothiazolone (**166**) is regenerated in the final step through oxidation by the Cu(I)-catalyst and molecular oxygen (**Scheme 14A**). The authors later published a mechanistic study of the reaction and found the involvement of multiple reaction pathways.[212] They used this information to optimize the system for thioester formation. Finally, they published a diselenide version of their benzoisothiozolones which no longer required a Cu(I)-complex for reoxidation and now merely relied on air as the final oxidant (**Scheme 14B**).[213] The groups of Gangireddy and Mainkar presented a protocol for using the benzoisothiazolones in SPPS methodology for catalytic couplings under mild conditions with minimal waste.[214]



Scheme 14. A. Reaction mechanism of a benzothiazolone, copper, and phosphite-mediated amidation reaction. B. Similar amidation reaction but now using a di-selenide catalist and no copper.

In 2024, Zhang and Li *et al.* reported the use of tris trifluoromethylphenylphosphite [(*o*-$F_3CPhO)_3P$] as coupling reagent for amidation reactions by activation of the nucleophilic amines (**Scheme 15A**).[215] They also included the synthesis of dipeptides in their scope, but did not report formation of larger peptides. Yamamoto and Hattori *et al*. recently expanded that method, managing to use triphenyl phosphite (**181**) for peptide couplings to obtain oligopeptides (**188**) in high yield and low epimerization rates in one pot (**Scheme 15B**).[216] Their proposed mechanism involves the initial activation of the phosphite (**181**) to the alkoxyphosphonium salt (**182**) by attack of a neutral amine (**183**). After subsequent attack

of the carboxylate (**185**) on the phosphorus atom, the intermediate (**186**) rearranges and expels diphenylphosphite (**189**) to provide the amide product (**188**). The diphenylphosphite (**189**) is converted back to triphenyl phosphite (**181**) in the presence of carboxylic acid.



Scheme 15. A. Amide coupling conditions reported by Zhang and Li et al. B. Suggested mechanism of triphenylphosphite-mediated coupling reported by Yamamoto & Hattori et al.

1.5.5 Uronium/Aminium Salts

Uronium and aminium (guanidinium) salts are coupling reagents that function in a similar fashion compared to phosphonium reagents. They activate carboxylic acids in the presence of a base and a benzotriazole-type additive (*e.g.* HOBt or HOAt) by initial formation of a carboxyluronium salt and subsequent substitution to form a benzotriazolyl ester. This finally undergoes aminolysis to form the peptide bond.[181]

These reagents are typically supplied as hexafluorophosphate or tetrafluoroborate salts, which are soluble in polar solvents such as DMF and NMP, making them especially compatible with solid-phase peptide synthesis (SPPS). Whereas these coupling reagents were initially considered as uronium compounds, X-ray analysis later uncovered that certain examples like HBTU (**190**), HATU (**192**), and HAPyU (**193**) are present as aminium structures (**Figure 2**).[217,218]

Following the first version HBTU (**190**)[219], further refinements led to a large variety of reagents such as HATU (**192**), and COMU (**194**, **Figure 2**). The former is widely favored for

challenging couplings, including those involving sterically hindered residues or difficult sequences, due to the enhanced reactivity and leaving group ability of HOAt as opposed to HOBt.[194,218] COMU incorporates dimethylmorpholinourea and Oxyma in its structure, affording a more efficient, stable, and safe reagent.[220] Owing to the explosive traits of benzotriazoles, Oxyma was developed as a less-hazardous efficient alternative and is now commonly used as additive in most coupling reactions.[221]

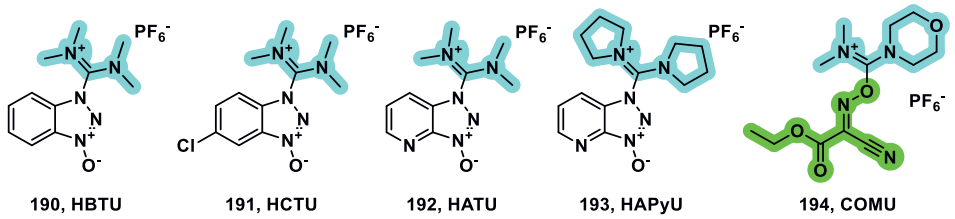


Figure 2. Examples of uronium & aminium reagents. Blue: the aminium/uronium moieties. Green: Oxyma in COMU.

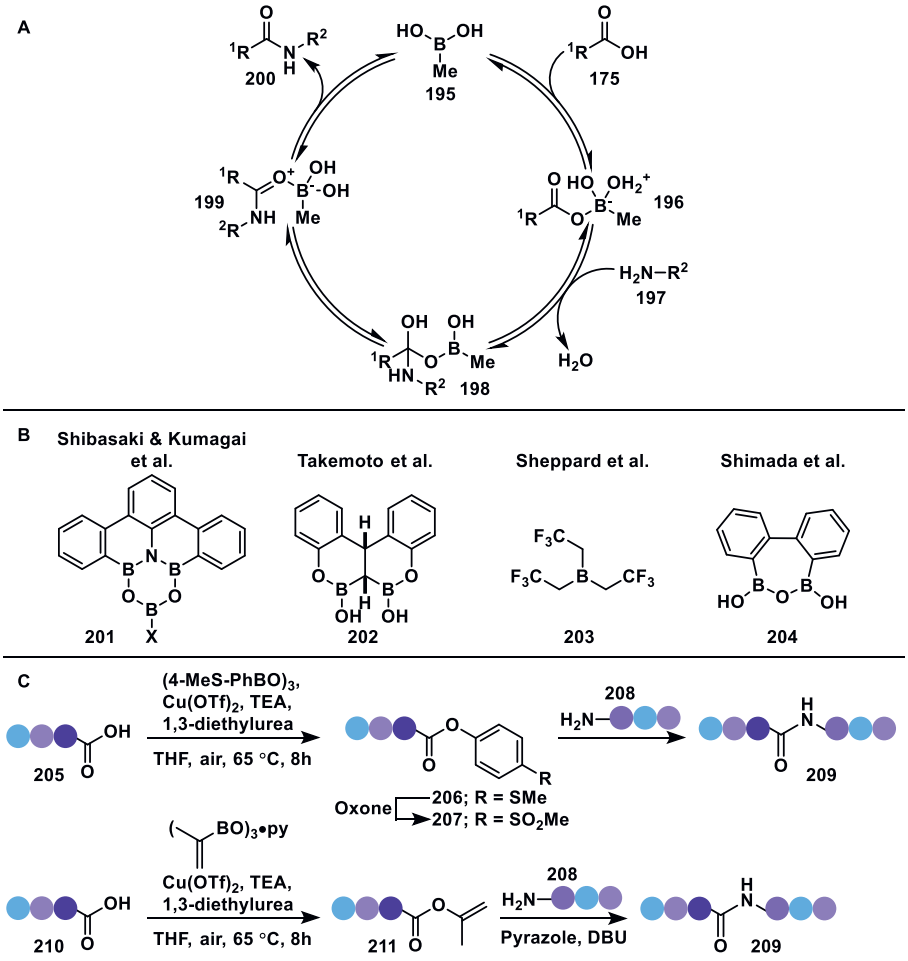
Uronium and aminium reagents now represent the state of the art in peptide bond formation. Their superior solubility, efficiency, and safety have led them to replace earlier phosphonium-based systems in most academic and industrial settings. Their compatibility with automated synthesis, high-throughput protocols, and a wide range of side-chain functionalities further supports their widespread adoption.

1.5.6 Boronic Acids

Perhaps surprisingly, boronic acids have received limited attention for amide bond formation despite the potential of these mild reagents. After pioneering work by Yamamoto in 1996, several groups have reported different strategies with this approach focusing on catalytic, mild conditions often using molecular sieves to capture water as opposed to Dean-Stark setups.[222–228] Unlike conventional electrophilic activation methods, boron-mediated couplings exploit the Lewis acidity of boronic acids (**195**) to activate carboxylic acids or esters toward amine nucleophiles. Marcelli unveiled the mechanism using DFT calculations and showed that mixed anhydride type species are involved and the elimination of water from the tetrahedral intermediate (**196**) is rate-limiting (**Scheme 16A**).[229]

Multiple studies have since reported novel boron-catalysts for amide bond formation and peptide synthesis.[230–234] A noteworthy example is that of Shibasaki and Kumagai *et al*., who developed 1,3-dioxa-5-aza-2,4,6-triborinane (DATB, **201**) and derivatives thereof containing a six-membered B_3NO_2 ring system (**Scheme 16B**).[235] These catalysts showed high stability and particular reactivity which allowed the efficient production of amides. Similarly, Takemoto *et al*. showed that a phenol-substituted *gem*-diboronic acid catalyst (**202**) was superior to the early arylboronic acids.[236] The group of Sheppard was capable of performing

peptide couplings on unprotected amino acids using their borate ester (**203**).[237] Finally, Shimada *et al*. reported hydroxy-directed dehydrative condensation of serine, threonine, and β-hydroxyvaline-derived amino acids in high yields and minimal epimerization using diboronic acid anhydrides (**204**, **Scheme 16B**).[238]



Scheme 16. A. Catalytic amide bond formation catalytized by a boronic acid. B. Various boronic acid catalysts reported. C. Chan-Lam-Evans for peptide functionalization and elongation.

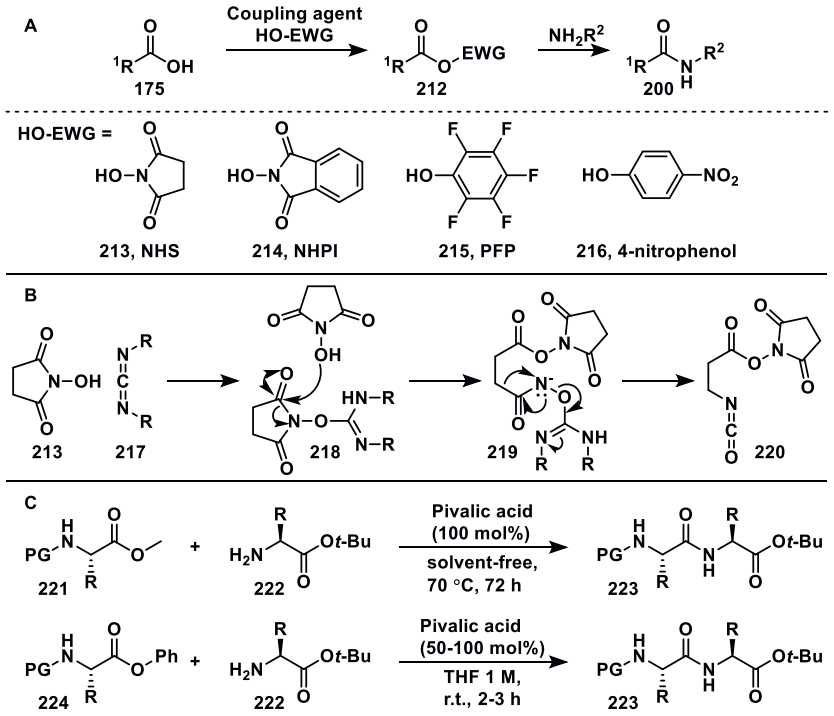
In 2010, Cheng *et al.* reported the Chan–Lam reaction to access esters from carboxylic acids.[239] Van Maarseveen *et al.* later applied this method to the functionalization of amino acids and small peptides (**Scheme 16C**). Their approach was particularly interesting as they completely omitted epimerization. Furthermore, their active ester products could be further functionalized by direct amidation and NCL.[240] Since the approach would otherwise

lead to racemization, it required slow addition of the amine (**208**) to prevent highly basic conditions. In a subsequent publication, the same group reported a Chan-Lam-Evans enol esterification with isoprenyl boroxine to provide C-terminal isopropenyl esters (**211**). The compounds were shelf stable and allowed stereoselective coupling of amino acid and peptide nucleophiles using DBU and pyrazole as catalyst (**Scheme 16C**).[241]

1.5.7 Preformed Activated Esters

Another historically significant class of activation strategies involves preformed active esters, species which are stable enough to be isolated, yet reactive enough to undergo acyl transfer once combined with a nucleophilic amine. They are commonly synthesized by reacting the C-terminus with substituted phenols or hydroxylamines using a carbodiimide activator. A few well-known examples include *N*-hydroxysuccinimide (NHS, **213**) esters, *N*-hydroxyphtalimide esters (NHPI, **214**), pentafluorophenyl (PFP, **215**) esters, and 4-nitrophenyl esters (**216**, **Scheme 17A**).[242–246] These esters can be isolated and stored, offering operational simplicity in multistep synthesis or fragment couplings. Their reactivity is modulated by the leaving group's electron-withdrawing nature, which influences both the rate and selectivity of amide bond formation. While generally less reactive than *in situ* methods, their stability and shelf-life make them useful in automated synthesis and in the construction of peptide conjugates. Limitations of these active esters include susceptibility to slow hydrolysis or side reactions with other nucleophilic residues, and some can promote epimerization if poorly controlled. Furthermore, with NHS and NHPI esters, a nucleophilic hydroxylamine (**213**) can attack the imide carbonyl (**218**) and a sequential Lossen rearrangement (**219**) results in an undesired byproduct (**220**, **Scheme 17B**).[184,247] Still, they remain a valuable complement to *in situ* activation methods.

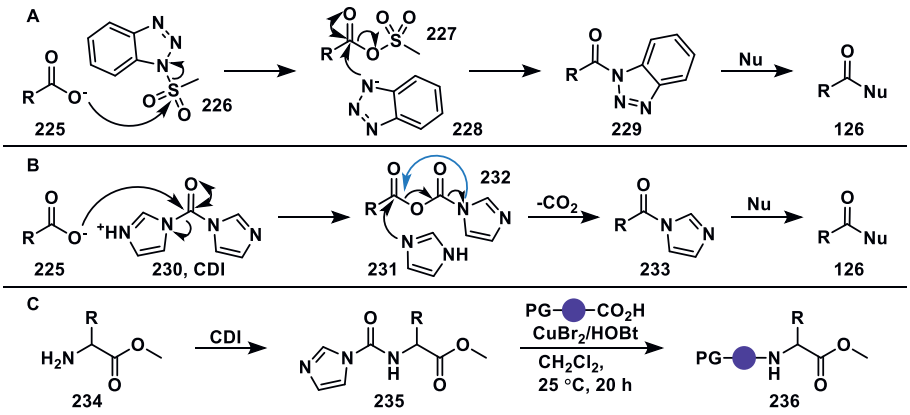
In 2022, the group of Yamamoto reported a biomimetic peptide bond formation catalyzed by various Brønsted acids (**Scheme 17C**).[248] Starting from either a methyl (**221**) or a phenyl ester (**224**), they reported two individual methods for the synthesis of di-, tri-, and tetrapeptides, either in solution or solvent-free. Although the authors employed 50-100% of acid, they showed that the method works catalytically, achieving yields of >85% using 20 mol%. Furthermore, while various organic acids provided the desired condensation products in high yields, the authors elected pivalic acid as optimal owing to its high steric hindrance and therefore lower tendency for racemization. Finally, they applied this method to a flow system to showcase the scale-up capabilities of the method.



Scheme 17. A. Common alcohols used for active esters. B. Lossen-rearrangement sidereaction from NHS and a carbodiimide. C. Bronsted-acid catalyzed amide bond formation by Yamamoto.

1.5.8 Acylazoles

Acylimidazoles and acylbenzotriazoles, are attractive as mild and stereochemically stable C-terminal activation intermediates at low temperatures that allow one-pot amide formation. Their synthesis is cheap, facile and they can be formed from the corresponding carboxylic acids in various ways. Acylimidazoles are most commonly synthesized using carbonyldiimidazole (CDI), whereas acylbenzotriazoles (**229**) can be obtained by a reaction with thionyl chloride and benzotriazole or using sulfonylbenzotriazoles (**226**) as a "counter attack" reagent (**Scheme 18A**).[249–252] The latter method involves the *in situ* formation of a carboxylic sulfonic anhydride (**227**) which is then substituted by the benzotriazole. These methods have been employed for the kilogram scale synthesis of complex structures, peptides and conjugates thereof.[253,254] Acylbenzotriazoles demonstrate a higher degree of stability compared to acid chlorides, allowing them to form products in aqueous conditions and be isolated and stored over longer periods. Furthermore, they are compatible with N-protected amino acids bearing unprotected reactive sidechains and show minimal racemization.[255–260]



Scheme 18. A. Acid activation by sulfonylbenzotriazoles. B. Acid activation by CDI. C. Amine activation by CDI and subsequent amide bond formation.

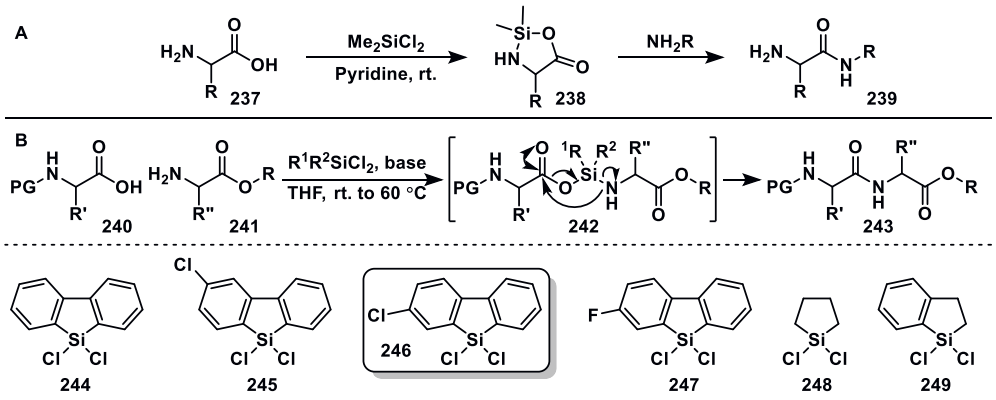
A reaction of an acid with CDI involves the initial protonation of one of the two imidazole rings, followed by attack of the carboxylate (**225**) on the carbonyl carbon (**230**) to displace the protonated imidazolium and form an anhydride intermediate (**232**). Subsequently, one of two pathways takes place to form the final product. Either the released imidazole attacks the anhydride to form the active acyl imidazole (black arrows), or an intramolecular attack by the still present imidazole (blue arrow) forms a cyclic four-membered intermediate which opens to provide the acyl imidazole (**233**, **Scheme 18B**). In both cases, the release of CO_2 drives the reaction. In 2022, Campagne and Figueiredo *et al*. reported an adaptation of the CDI methodology for the "inverse" peptide synthesis of dipeptides.[261–263] Therein, the amine (**234**) was initially activated using CDI and subsequent attack of the carboxylate on the formed intermediate (**235**) displaced the second imidazole. A final rearrangement resulted in the amide product (**236**, **Scheme 18C**).[261–263]

1.5.9 Organosilicon reagents

Since the late 1960's, organosilicon reagents have been reported for direct amidation reactions. Chan *et al.* were the first to report the use of silicon tetrachloride in pyridine for this purpose.[264] They later expanded the method to the synthesis of dipeptides by allowing the initial formation of silyl esters prior to the addition of the nucleophilic amino esters.[265] Since then, a variety of such reagents have been reported for amide bond synthesis including applications in peptide chemistry.[266,267] A few important examples are discussed here.

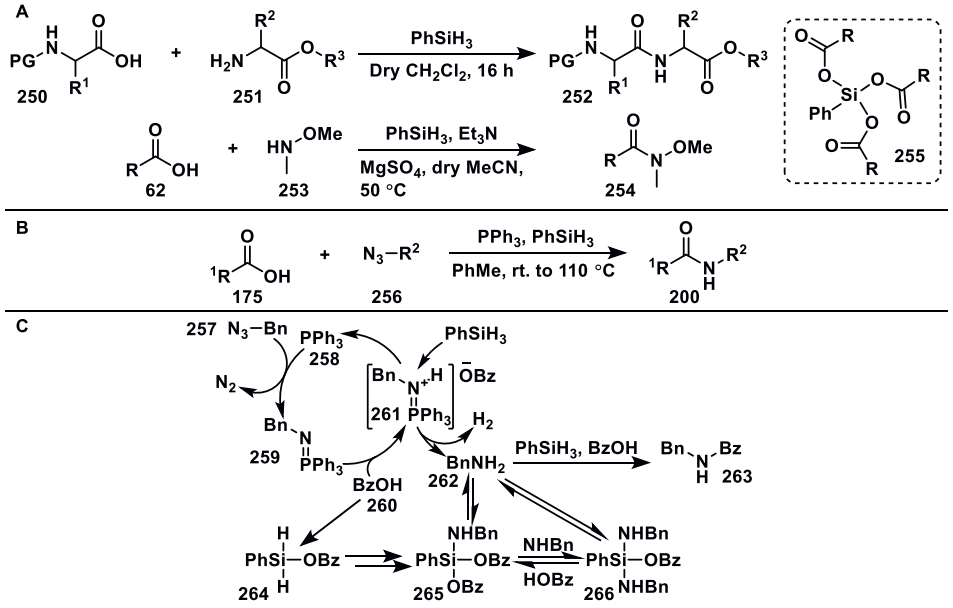
The group of Liskamp applied dimethyldichlorosilane to develop coupling reactions of unprotected peptides.[268] This simultaneous protection-activation protocol likely operates

via a cyclic silane species (**238**) and was applied to β-amino acids and *N*-alkylamino acids (**Scheme 19A**). Moreover, the method regioselectively amidates aspartic acid on both the C-terminus and sidechain. Fedorov *et al*. later expanded the scope of this method to include secondary amine nucleophiles as well.[269] Nearly a decade ago, Charette *et al*. developed more rigid and planar 9-silafluorenyl dichlorides (**244**-**249**) as reagents for direct peptide bond formation (**Scheme 19B**).[270] They synthesized di- and tripeptides without epimerization using this methodology. The reactions proceeds via a chemical ligating mechanism (**242**), which was later supported by the computational studies of Jiang *et al*.[271]



Scheme 19. A. Amide formation with unprotected AAs dimethyldichlorosilane. B. 9-silafluorenyl dichloride mediated amide formation.

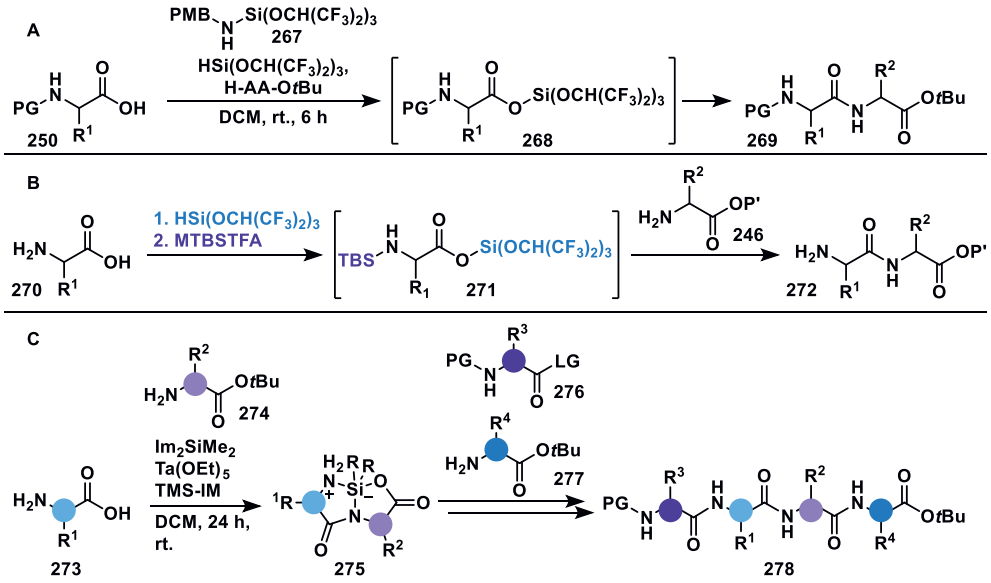
Blanchet *et al*. used phenyl silane as coupling reagent for amide synthesis. They employed their method for the synthesis of CBz-, Boc-, and Fmoc-protected dipeptides (**252**) without epimerization (**Scheme 20A**).[272] They also managed to obtain Weinreb amides (**254**) mediated by a hydrosilane for the first time. Contrary to the suggested mechanism by Charette, they provided evidence supporting that the process proceeds through a phenyl-triacyloxysilane (**255**) active intermediate. Additionally, Ashfeld and coworkers reported the use of phenyl silane in a catalytic Staudinger ligation reaction where this reagent was speculated to function as a stoichiometric reductant of the triphenylphosphine oxide byproduct (**Scheme 20B**).[273] They synthesized peptides, amides and lactams with their method. Denton *et al*. later showed that the reagent could not merely function as terminal reductant, a mechanism which was later elaborated on by White and Mecinovic (**Scheme 20C**).[274,275]



Scheme 20. A. (Weinreb) Amide synthesis using phenyl silane. B. Catalytic Staudinger ligation using phenyl silane and (C) the mechanism thereof.

Recently, the group of Yamamoto developed a direct amidation reaction using $HSi(OCH(CF_3)_2)_3$ as stoichiometric reagent for epimerization-free dipeptide bond formation of protected amino acids (**Scheme 21A**).[276] Employing an aminosilane catalyst (**267**, 3 mol%) in their system allowed for a far more efficient process that produces less waste. Importantly, a stoichiometric coupling reagent [$HSi(OCH(CF_3)_2)_3$] was still required for the desired product formation and the exact role of the aminosilane catalyst in the mechanism remains unclear. However, the authors underlined the importance of the latter for facilitating active silylester intermediate (**268**) formation and suggested a role as Lewis acid to increase acidity of the carboxylic acid. Recently, they used the same method for the condensation of peptide fragments[277] and made the method catalytic with respect to $HSi(OCH(CF_3)_2)_3$.[278] Moreover, the group later expanded the method to include *N*-(tert-butyldimethylsilyl)-N-methyltrifluoroacetamide (MTBSTFA) as an additional silylation reagent for the transient masking and coupling of unprotected amino acids (**Scheme 21B**).[279] Furthermore, they developed a strategy using imidazolylsilane and a Ta-catalyst for the synthesis of tetra- and oligopeptides (**278**) by functionalizing preformed unprotected dipeptides (**275**) at the N- and the C-terminus in one-pot (**Scheme 21C**).[280] The Ta-catalyst was previously reported as Lewis-acid for the "remote" activation of carboxyl groups without racemization, a strategy which has often been applied to the transesterification of methyl esters when not combined with a silyl reagent.[228,281] The use of such metals in

coupling reactions will be discussed in more detail in the following section. Hattori and Yamamoto also showed similar reactivity when using trimethylaluminum.[282] Finally, they used their accumulated knowledge to report the first cross-condensation between amino acids without protection groups on the main-chain using these conditions.[283]



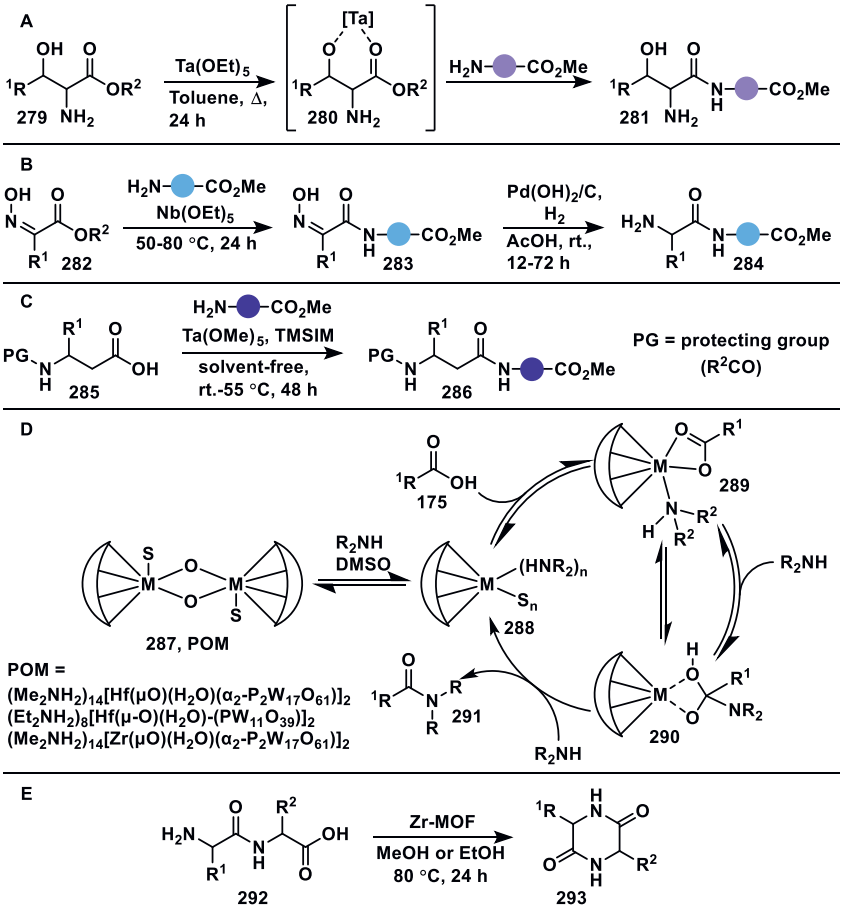
Scheme 21. Various reports by the group of Yamamoto. A. Epimerization-free dipeptide bond formation. B. Uprotected amino acid coupling using "transient masking". C. Oligopeptide synthesis by functionalization of preformed unprotected dipeptides.

1.5.10 Metal(IV/V)-catalyzed activation

An additional method for C-terminal activation which has gained increasing attention in the past decade involves the use of metal (IV/V) catalysts. Such catalysts allow the activation of both esters and free carboxylic acids towards the formation of amide bonds. Much like the activation mechanism for boron reagents mentioned above, these reagents normally coordinate the carboxylic acid or ester, which in turn increases the electrophilicity of the carbonyl carbon to allow subsequent nucleophilic attack by a corresponding amine. Numerous reports have demonstrated amide bond formation using metals such as zirconium, tantalum, hafnium.[284–291] Yet, the use of these metals to access peptide bonds remains limited with only a handful of examples reported thus far.

In 2016, Tsuji and Yamamoto reported a tantalum alkoxide catalyst which they could use for the hydroxy-directed (**280**) chemoselective synthesis of amide bonds. Using their strategy they managed to synthesize a series of dipeptides (**281**) without epimerization, but

remained limited to couplings with serine and threonine since these contain β-hydroxy-moieties (**Scheme 22A**).[292] Two years later, the same group expanded the scope of their method by using *N*-hydroxyimino esters (**282**), where those moieties acted as directing group in a coupling reaction that employed a niobium catalyst under solvent-free conditions. After subsequent facile diastereoselective hydrogenation, this afforded di- and tripeptides in high yields without racemization (**Scheme 22B**).[293] As discussed in the previous section, Muramatsu and Yamamoto later also developed methods which employed both a Ta-catalyst and a silyl reagent. Accordingly, they reported a reaction which uses 1-(trimethylsilyl)imidazole (TMSIM) and a Ta-catalyst for the amidation of amino acid homologues in high yields (**Scheme 22C**).[294]

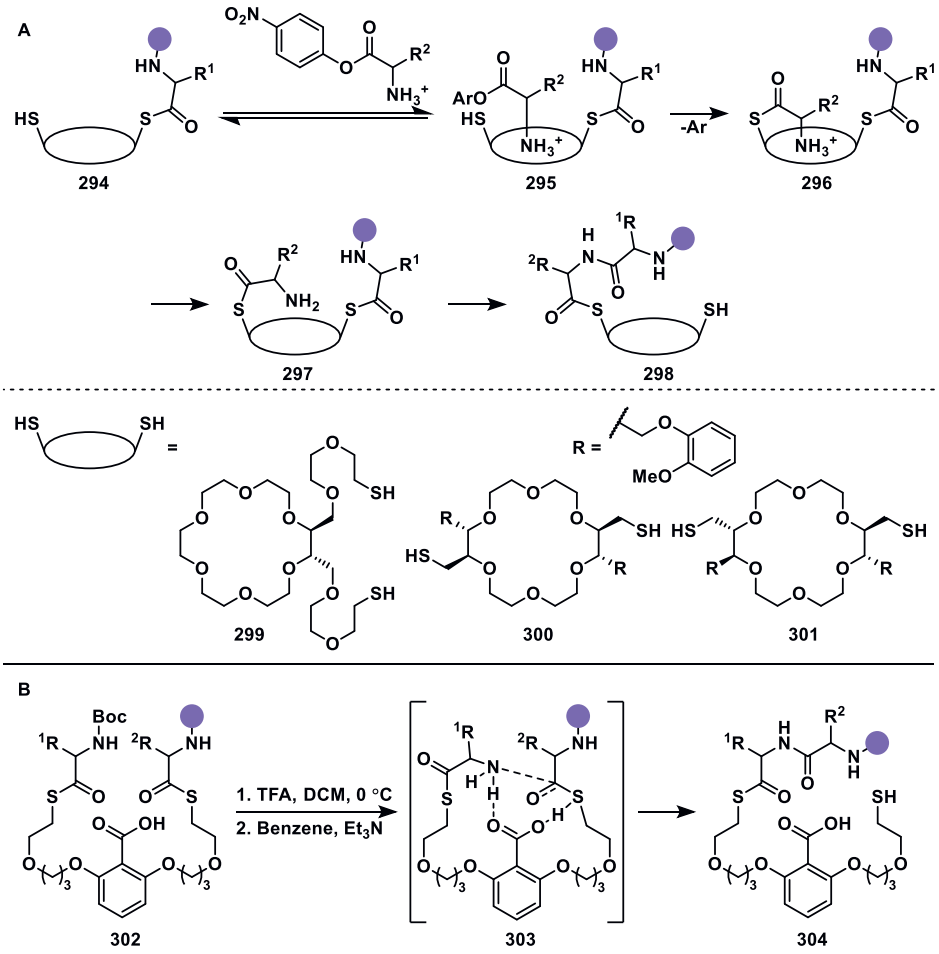


Scheme 22. A. Ta-catalyzed OH-directed amide bond formation. B. Nb-catalyzed hydroxyimino-directed peptide bond formation. C. Amidation of amino acid homologues using a Ta-catalyst. D. Zr- and Hf-POMs for amide bond formation. E. peptide bond formation mediated by a Zr-MOF.

The group of Parac-Vogt studied alternative metal-based catalysts for amide bond formation. They showed that Zr(IV)- and Hf(IV)-substituted polyoxometalates (POM, **287**) can function as excellent catalysts for amide bond formation under mild conditions and high water tolerance (**Scheme 22D**).[291,295] The POM scaffolds were found to function as ligand and preserve the reactivity of the metals by protecting them from hydrolysis. Moreover, this also allowed catalyst reuse without activity loss. The group later investigated the use of a Zr-based metal organic framework (MOF) for peptide bond formation (**Scheme 22E**).[296] This catalyst had multiple benefits; it did not require additives for carboxylic acid activation, the catalyst was recyclable, tolerant to water, and had a broad substrate scope. Furthermore, the group applied the method to the synthesis of cyclic dipeptides, *i.e.* diketopiperazines (**293**), but hope to optimize the conditions and later generalize the method for intermolecular peptide bond formations.[296]

1.5.11 Organocatalysis

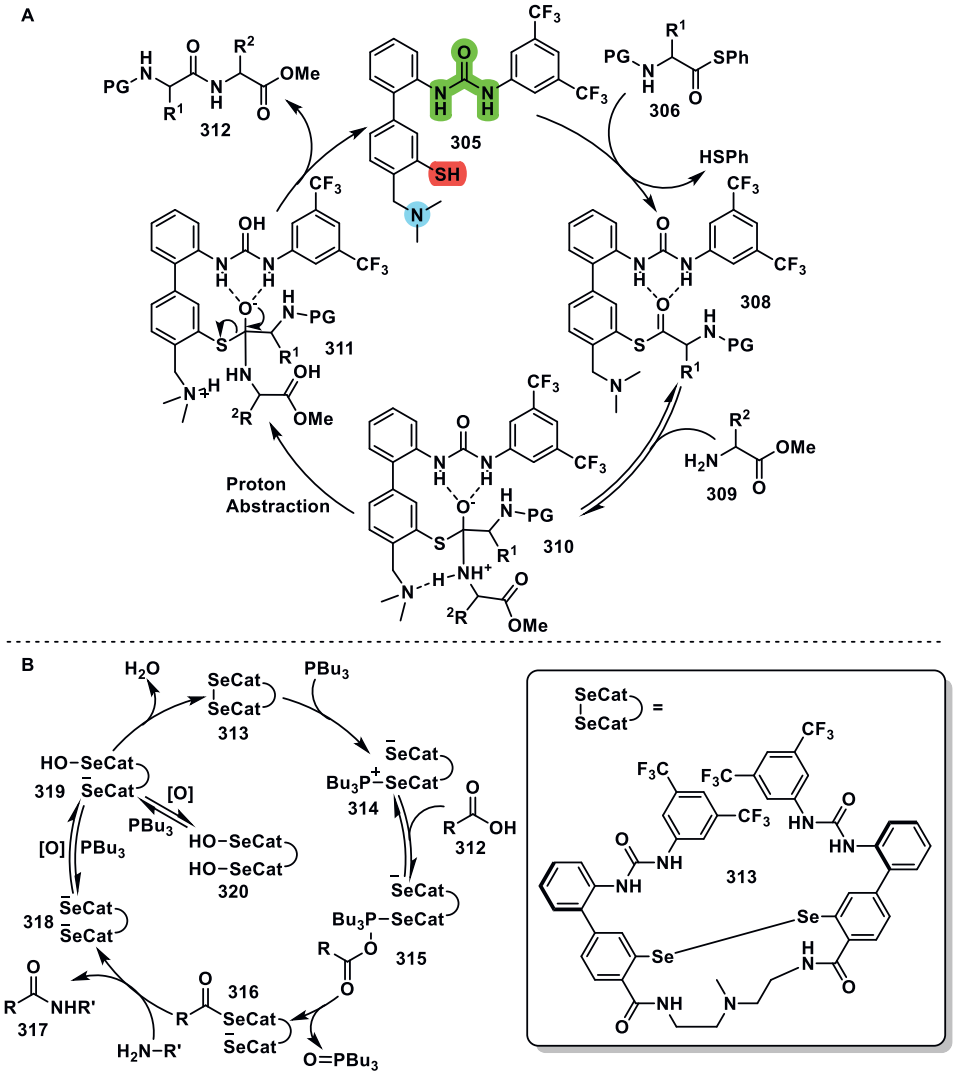
A relatively underexplored field for carboxyl functionalization utilizes organocatalysis. The first reports of a biomimetic catalyst for amidation reactions of activated AAs were published in the 1980s by the group of Koga. They pioneered mimicking non-ribosomal peptide synthesis using crown-ether compounds (**299**-**301**) bearing two thiol moieties which facilitated thioester formation from activated carboxyls, setting the stage for intramolecular S-to-N acyl transfer to afford peptide bonds (**Scheme 23A**).[297–301] The group later improved the efficiency of the catalysts by introducing additional catalytic groups (*i.e.* methyl ester) to the crown ether[302] and by making it a bridged system.[303] Similarly, Gennari *et al*. reported the development of different C_2 symmetrical catalysts (**302**) bearing two thiol groups and a bifunctional acid-base catalyst to facilitate the necessary proton transfer on the transition state, thus accelerating the rate (**Scheme 23B**).[304,305]



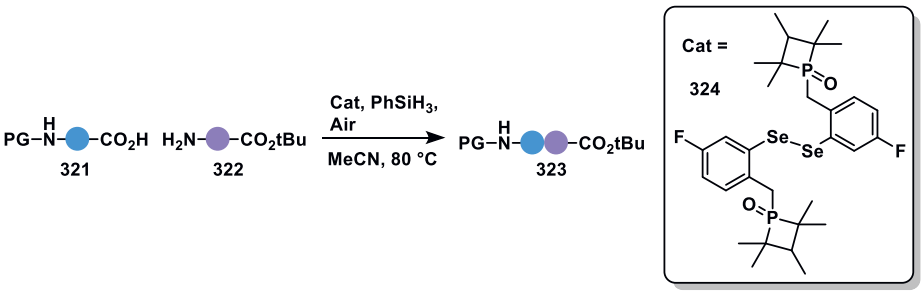
Scheme 23. A. Peptide bond formation mediated by crown ether biomimetic organocatalysts. B. Peptide bond formation mediated by benzoic acid bisthiol biomimetic organocatalysts.

In 2019, Arora *et al*. rationally designed a biomimetic catalyst (**305**, **Scheme 24A**)[306] capable of coupling protected activated amino acids (**306**) to enhance peptide bond formation. Subsequently, the group developed a diselenide version of this catalyst (**313**) which also facilitated the coupling of free carboxylic acids (**312**), thus allowing the efficient synthesis of oligopeptides on solid phase with low catalyst loading (**Scheme 24B**).[307] The latter catalyst works on three biomimetic principles; oxyanion holes in enzymes stabilize the tetrahedral intermediate in amide bond formation and is mimicked by a urea moiety, carboxyl activation as thioesters like in non-ribosomal peptide synthesis, and direct thioester/selenoester formation from disulfides/diselenides and phosphorus(III)-reagents. Their method was very effective yet suffered from two main drawbacks; it required

stoichiometric amounts of phosphorus(III) reagent and needed molecular sieves as dehydrating agents. In a subsequent paper, the authors addressed the limitations of their diselenide catalyst by employing silanes to both reduce the phosphineoxide byproduct and regenerate the phosphine(III) species, and scavenge the released water resulting in silanols or siloxanes.[308] More recently, the group developed a small molecule catalyst (**324**) for catalytic peptide synthesis by simplifying the previous designs making the new catalyst more easily accessible (**Scheme 25**).[309]

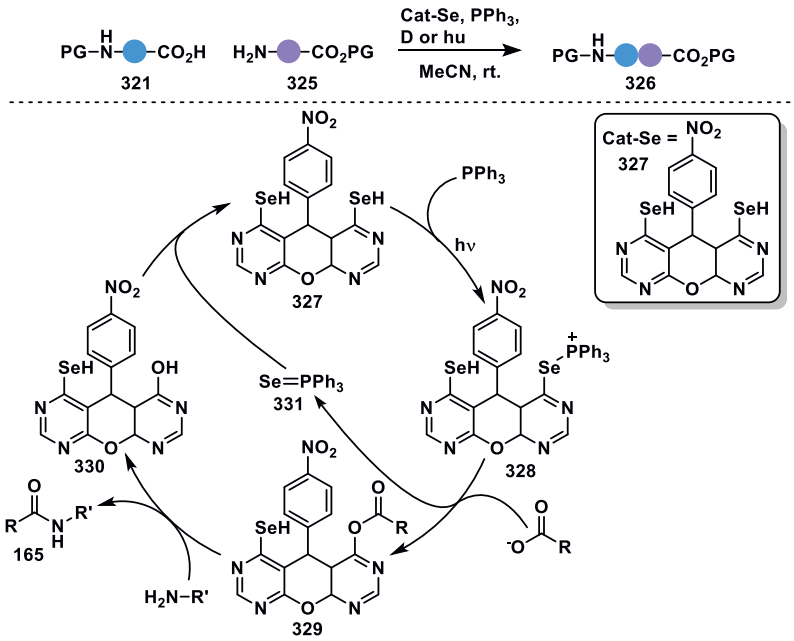


Scheme 24. Various organocatalysts developed by Arora et al. A. Mechanism of the original thiol-catalyst. B. Diseleno-catalyst including its catalytic cycle.



Scheme 25. Simpler small-molecule catalyst using air and phenyl silane as oxidants and reductant, respectively.

Very recently, the group of Sun developed an organocatalyst (**327**) like that of Arora, but with a different mechanism that uses light as the driving force of the process (**Scheme 26**).[310] Importantly, their reaction does not require heating or inert conditions. The phosphine species here reacts with the initial catalyst under light to form a selenophosphonium intermediate (**328**), which is subsequently attacked by the carboxylate expelling the phosphine selenide (**331**). The formed intermediate readily reacts with amines to the desired amide (**165**) products and a final reaction of the phosphine selenide reforms the initial catalyst. The authors showed that their new method can also engage in peptide fragment condensation and is applicable to SPPS.[310]

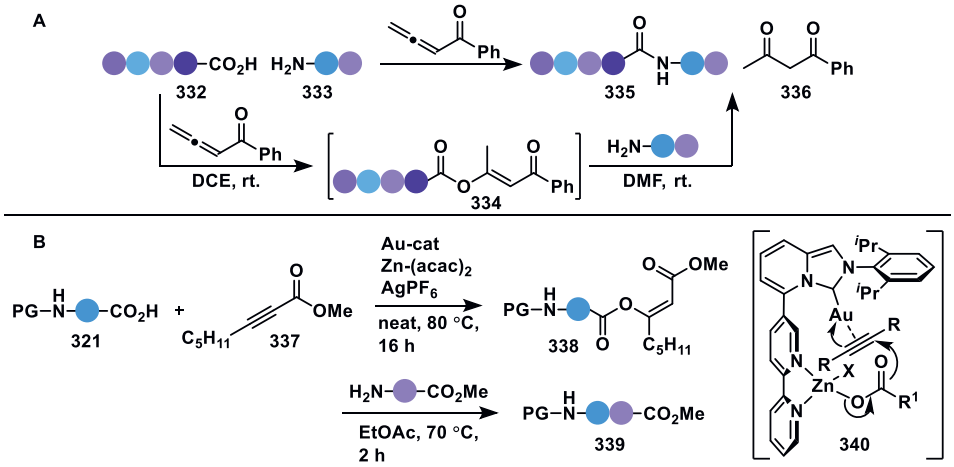


Scheme 26. Organocatalytic photochemical amide bond formation.

1.5.12 Vinyl esters – allenones and ynamides

Vinyl esters are known activated intermediates for the synthesis of amide bonds and can be obtained by reacting carboxylic acids with acetylenes.[311] The earliest reports of this strategy for peptide synthesis by Arens date back to the 1950s.[312–314] More recently, the group of Zhao reported multiple strategies toward amide bond formation using vinyl esters aiming to overcome some of the inherent challenges regarding the reactivity of the alkyne and acyl enol ether intermediate. In 2021, the group reported the first use of an allenone as an effective racemization- and epimerization-free coupling reagent through the formation of active α-carbonyl vinyl ester intermediates (**334**) when reacted with carboxylic acids (**Scheme 27A**).[315] This approach was effective in a range of amide coupling reactions, ranging from simple dipeptides to peptide fragment condensation, and is compatible with SPPS. Furthermore, it overcomes the disadvantages of conventional coupling reagents and active esters while maintaining their benefits (*i.e.* reactivity and stability).

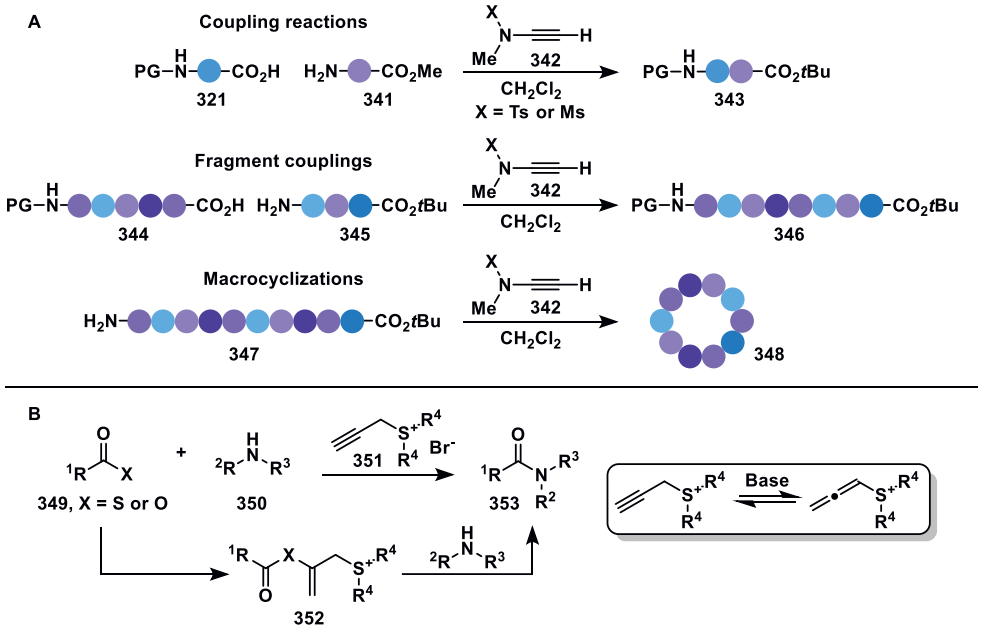
Related to allenones, Sawamura and Higashida *et al*. developed a gold-zinc cooperative catalyst (**340**) for the activation of carboxyl groups to α-methoxycarbonyl enolesters (**338**, **Scheme 27B**).[316] These products are isolable, stable enough for storage and are capable of reacting with amines to form dipeptides (**339**) without epimerization.



Scheme 27. A. Allenone-mediated carboxylate activation and subsequent amide bond formation. B. Gold-zinc cooperative catalysis for carboxyl activation to α-methoxycarbonyl enolesters and subsequent amidation.

In addition to allenones, the use of ynamides has become an increasingly prevalent activation/coupling strategy. These compounds are subject to nucleophilic attack by a carboxylic acid to form an α-acyloxyenamide intermediate, which includes an active ester that can be attacked by nitrogen or oxygen nucleophiles to provide amides or esters,

respectively. Initially, ynamines as coupling reagents were alkaline in nature, leading to higher degrees of epimerization.[317] Zhao et al. have played a major role in the development of the more electron deficient ynamides for their use in peptide couplings and overcome this limitation.[318,319] They explored N-(methyl)ynetoluenesulfonamide (**342**) and N-(methyl)ynemethylsulfonamide since these contained an electron withdrawing group on the amine rendering the intermediates less prone to epimerization (**Scheme 28A**). Prior to these reports, peptide enol esters obtained from Ru-catalyzed alkyne addition to peptide C-termini were used for racemization-free peptide synthesis in a chemoenzymatic approach.[320] Zhao *et al*. later also reported peptide macrocyclizations using this chemistry and separately did a thorough mechanistic investigation which facilitated optimization of the protocol to very short reaction times (*i.e.* 20-30 mins) without loss in efficacy (**Scheme 28A**).[321,322] Furthermore, the group also reported the synthesis of thioamides with this method.[323] In 2024, the group of Zhao published a paper on "inverse peptide synthesis" using ynamides as coupling reagents.[324] Therein, they managed to synthesize a range of peptides including a 21-mer in the N-to-C direction.



Scheme 28. A. Ynamide-mediated coupling of AAs, peptide fragments, and macrocyclizations. B. Propargyl sulfonium-mediated amide bond formation.

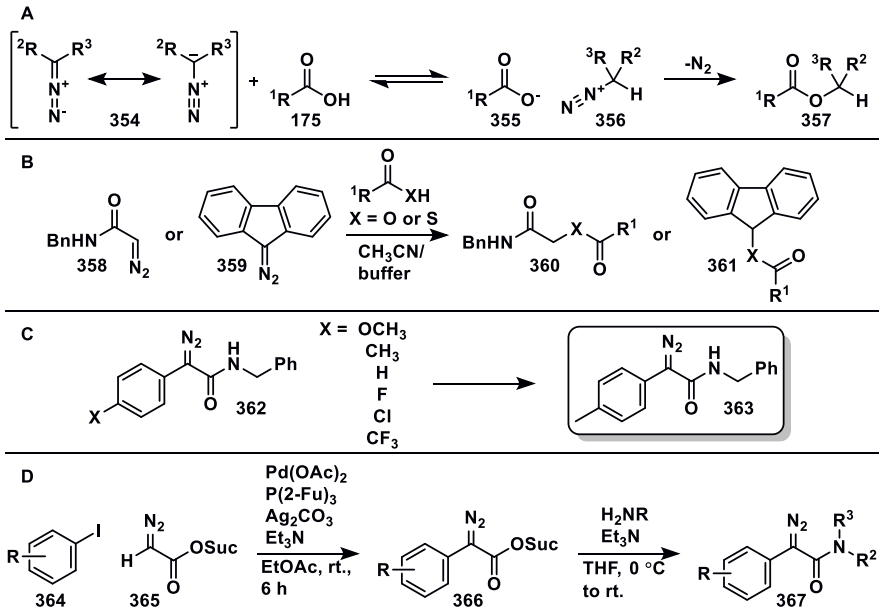
Inspired by the above reagents, Li *et al*. developed propargyl sulfoniums (**351**) as coupling reagents with comparable reactivity to allenones under alkaline conditions (**Scheme**

28B).[325] They showed that these could also be employed for SPPS and protein amidation and labeling.

1.5.13 Diazo compounds

Diazo compounds react with carboxylic acids by a direct substitution mechanism which, as will be discussed below, allows for chemoselectivity when applied to protein modification. Diazoalkanes have long been known to decompose in the presence of carboxylic acids. Staudinger discovered the relationship between the basicity and the reactivity of diazo compounds, realizing that more basic means more reactive. This is motivated by the rate of protonation of the diazo carbon preceding substitution (**Scheme 29A**).[326] However, the more reactive entities suffer from lack of selectivity; they have been shown to also react with water and the sidechains of tyrosine and lysine residues, limiting their utility for biofunctionalization.[327] In the 60s, a few reports emerged where diazo compounds were employed for the identification of reactive carboxyls in proteins.[328–331] Although employing vast molar excesses of reagent, they merely obtained modest labelling results, likely caused by lack of specificity.

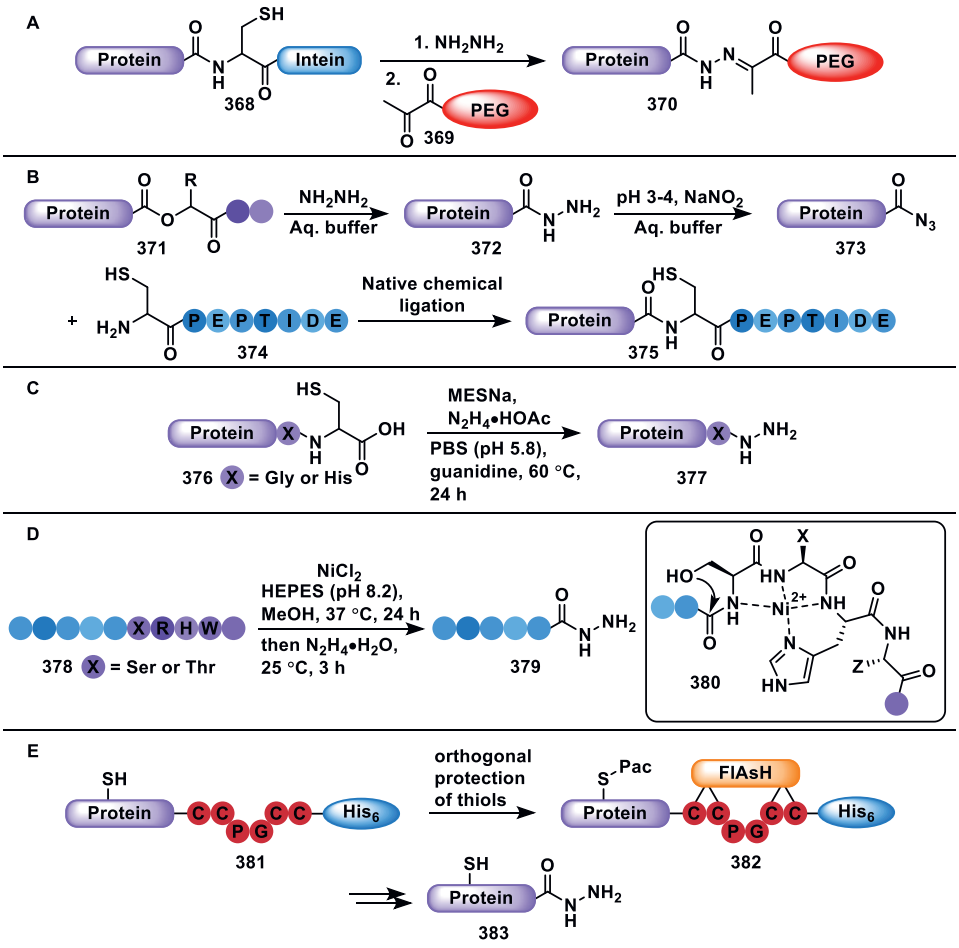
The group of Raines picked up the development of diazo compounds for biofunctionalization.[332] In 2014, they published a bioreversible esterification strategy for proteins in an aqueous environment using diazo compounds (**358**-**359**) as reagents (**Scheme 29B**).[333] Soon after, they sought to optimize the reactivity of these compounds in terms of chemoselectivity towards carboxyl groups and make them more easily modifiable.[334] The group managed to tune the basicity of the diazo carbon to establish selectivity over amines, alcohols and thiols in aqueous buffer conditions at slightly acidic pH, discovering that (*p*-methylphenyl)-glycinamide scaffold (**362**) has a particularly high reactivity with a good ester/hydrolysis product ratio (**Scheme 29C**). These two publications layed the foundations for a series of research projects involving the functionalization of proteins to: improve cell-penetration[335,336], increase solubility of esterified proteins[337,338], facilitate hydrolysis[339], chemical decaging of proteins[340], develop bioassays.[341,342] Over the years, the group has also optimized the synthesis of their scaffold to a two-step process using a Pd-catalyzed C-H activation reaction (**Scheme 29D**).[343]



Scheme 29. A. Mechanism of diazo-mediated esterification. B. Bioreversible esterification of proteins with diazo-compounds. C. Optimization of the diazo scaffold by Raines et al. D. Optimized synthesis of the diazo scaffold by Raines et al.

1.5.14 C-terminal hydrazides

Hydrazides are an important functional group for the functionalization of peptide C-termini. This moiety can be used directly or converted into more reactive functional groups for downstream derivatizations. Conventional approaches for obtaining these C-terminal hydrazide peptides involved synthesis by SPPS.[344,345] Alternatively, they can also be obtained by hydrazinolysis of intein fusion proteins (**368**). The group of Cotton used this principle to create proteins with C-terminal hydrazides to in turn be used for hydrazone-forming ligation reactions for protein PEGylation (**370**, **Scheme 30A**).[346] The group of Liu have also developed an approach based on a combination genetic code expansion and expressed protein ligation (**Scheme 30B**).[347] By incorporating α-hydroxy acids into the protein sequence, the oxo-esters in the protein (**371**) could be substituted to provide the hydrazide (**372**), which was in turn converted to an azide (**373**) for a subsequent ligation reaction.[348,349]



Scheme 30. A. Intein-mediated C-terminal hydrazide formation. B. Ester-mediated C-terminal hydrazide formation and subsequent NCL. C. Sequence-dependent N-to-S acyl shift for hydrazide formation. D. Ni(II)-mediated alcoholysis and sequential quadruple acyl transfer. E. C-terminal cysteine tag mediated selective hydrazide formation at protein C-terminus.

Many of the above methods required denaturing conditions, but there have also been strategies that obtain protein hydrazides from expressed proteins which work regardless of protein folding. The group of Macmillan found that native peptides and proteins can undergo thioester formation across Gly/His-Cys sequences by direct N-to-S acyl shift. This allowed them to directly substitute the thioester with hydrazine to form the desired hydrazide (**Scheme 30C**).[350] Otaka *et al*. developed a Ni(II)-mediated alcoholysis and sequential quadruple acyl transfer for the preparation of peptide thioesters (**Scheme 30D**).[351] They found a four-residue sequence (S/T-X-H-Z, **378**) which mimics the function of

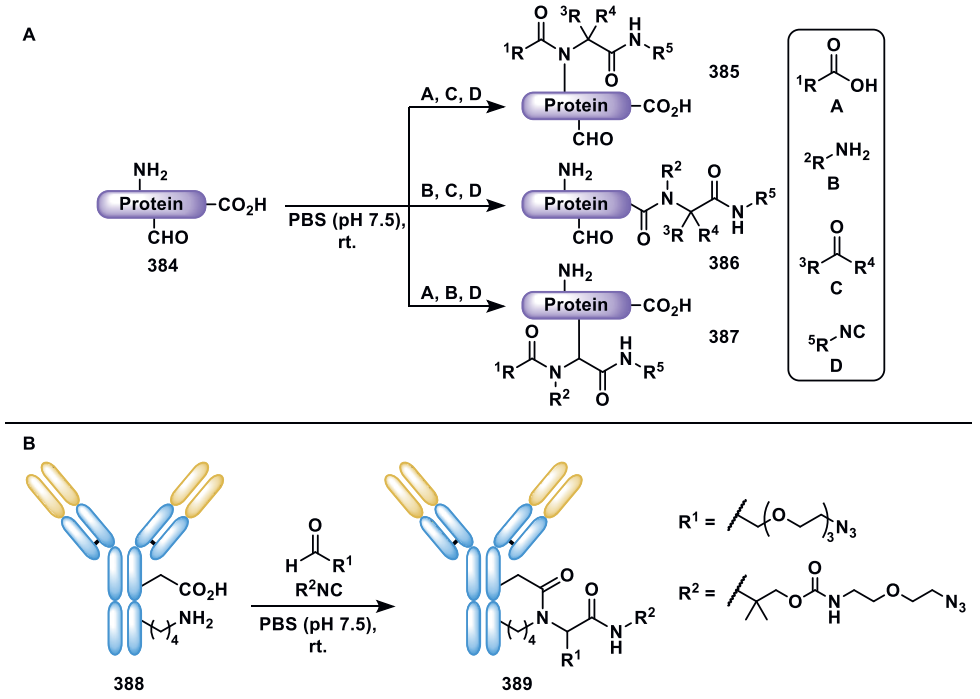
intein proteins (**380**) and utilized it for the functionalization of peptide hydrazides (**379**) and subsequent thioesters. Finally, the formation of C-terminal hydrazides can also be done via S-cyanylation of cysteine residues. The group of He developed the first regioselective S-cyanylation and hydrazinolysis strategy that was applied on proteins (**Scheme 30E**).[352] They employed orthogonal protection strategy which allows for the differentiation of internal versus terminal thiols by employing a short C-terminal tetracysteine tag (**381**). The tag is selectively targeted for temporary protection with a biarsenical compound (FlAsH) to protect the other present cysteines (**382**). After subsequent deprotection, the tag is selectively cyanylated and cleaved with hydrazine for following ligations.[352]

1.5.15 Isocyanide-based approaches

Isocyanide-based multicomponent reactions (MCRs) have been used for stapling, immobilization and bioconjugation strategies with proteins. The most applied MCR for this purpose is the Ugi four-component reaction (U-4CR), where an amine, carbonyl species, isocyanide and carboxylic acid react to form one product in a single step. Many studies have reported the use of this versatile method for the C-terminal functionalization of amino acids[353–358] and it also extends to C-terminal peptide functionalization for ligation[359–365] and macrocyclization strategies.[366–370] In this context, Axen *et al*. reported the first protein immobilization using the U-4CR in 1971.[371] Depending on the functional groups present on the solid support, they targeted the amines or the carboxylic acid groups on chymotrypsin as substrates for the reaction. In the following years, the group used this method for the immobilization of various enzymes.[372,373] Since then, a number of Ugi functionalizations on proteins have been reported.[374,375] However, many of these did not target a carboxyl group in the involved protein. In the remainder of this chapter we focus the discussion on selected examples that target carboxyl groups on proteins.[376–379]

In 2000, Ziegler *et al*. published a paper on the preparation of bioconjugates (**385**-**387**) using the U-4CR (**Scheme 31A**).[380] They reacted two proteins, bovine serum albumin (BSA) and horseradish peroxidase (HRP) with various substrates for the Ugi reaction, targeting both amines and carboxylic acids on the proteins. More recently, the group of Chaubet developed a site-selective variation of the Ugi bioconjugation by targeting the amine and carboxylate groups of vicinal lysine and aspartate/glutamate residues (**388**) for an Ugi four-center three-component reaction (U-4C-3CR)(**Scheme 31B**).[381] Therein, they functionalized the model antibody trastuzumab, where their approach limited the conjugation sites to six. This allowed the synthesis of antibody-drug conjugates (ADCs) which retained their selectivity towards the respective antigen. Although a significant breakthrough, the site-selectivity of the reaction was far from perfect as they encountered competing single-residue Passerini reactions and intra-residue U-4C-3CR at *N*-terminal E1 and D1 residues.

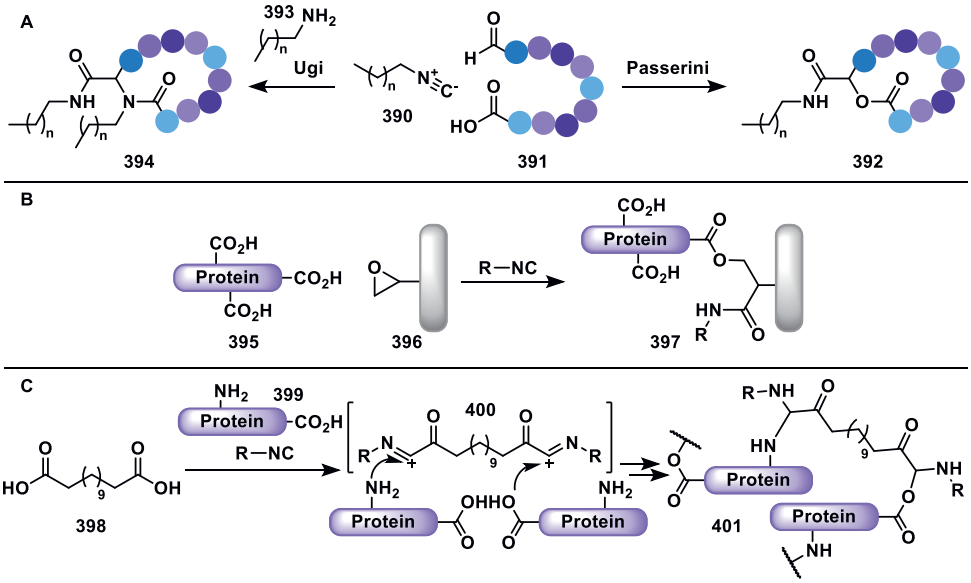
The group of Chaubet investigated this Ugi approach in more detail, varying all conjugation parameters to assess the impact on both the site- and reaction-selectivity.[382] They found conditions for the *N*-terminal selective conjugation of aspartate and glutamate residues, a strategy applicable to multiple protein formats.



Scheme 31. A. Ugi reaction for the bioconjugation of proteins at various reactive groups. B. Ugi four-center three-component reaction on antibodies.

Another important MCR for peptide and protein modification is the Passerini reaction. In 2017, the group of Rivera and Wessjohann reported both Ugi and Passerini macrocyclization reactions to afford lypopeptides (**Scheme 32A**).[368] By employing sidechain protected starting materials modified to aldehydes at the N-terminus, they were able to synthesize a macrolactone with an exocyclic lipid tail utilizing the Passerini reaction. The created lipopeptide library was in turn evaluated for anticancer activity. Although the Passerini reaction itself has not been employed for protein immobilization, analogs of this reaction have. The group of Mohammadi and Habibi employed a variation of the reaction where epoxides were incorporated instead of aldehydes to immobilize various enzymes, *e.g.* lipases and laccase (**Scheme 32B**).[383–387] Furthermore, the same group created cross-linked lipase enzymes to improve their activity utilizing a dicarboxylic acid in combination with an isocyanide and an amine or carboxylate from the involved lipase (**Scheme 32C**).[388] This

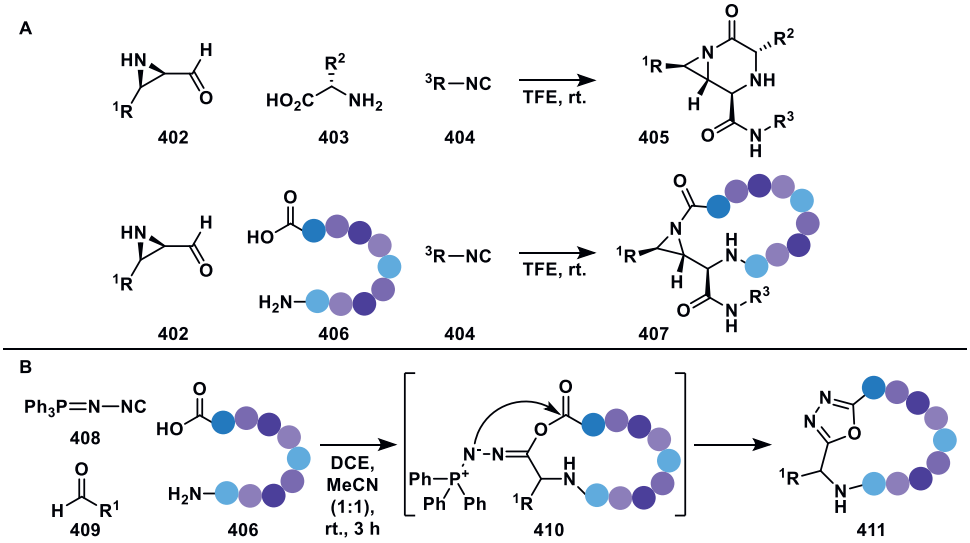
chemistry was based on previous work reported by Basso *et al*.[389] While all these Passerini-type reactions have provided significant advancements for protein carboxylate functionalization, they lack regiospecificity for the C-terminus or require protection groups for the functionalization.



Scheme 32. A. Synthesis of lipopeptides through Passerini and Ugi macrocyclizations. B. Protein immobilization by Passerini-type reaction. C. Protein crosslinking by using a dicarboxylate and isocyanide moiety.

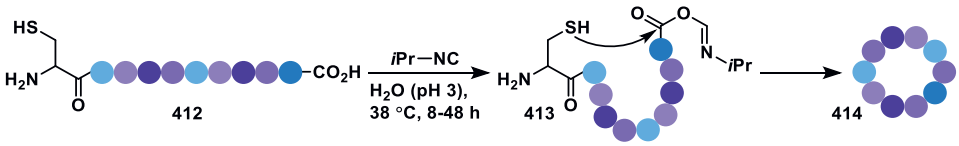
Yudin *et al*. developed their own multicomponent reaction for the cyclization of amino acids and peptides (**Scheme 33A**). The reaction involves using an aziridine-aldehyde reagent (**402**), isocyanide (**404**), amine and carboxylic acid (**403**) where mechanistically it is very similar to the Ugi reaction up until the final Mumm rearrangement.[390] At that stage, instead of the amine, the nucleophilic aziridine nitrogen attacks the carbonyl to initiate the rearrangement. When performing this reaction with simple amino acids (**403**) or oligopeptides (**406**), a hybrid piperazin-2-one/aziridine motif (**405**) or aziridine-containing cyclotide (**407**) is formed, respectively. Interestingly, owing to the chiral nature of the aziridine-aldehyde, the reaction affords products as single diastereoisomers (>20:1 dr). The group published numerous papers where they used their method for the synthesis of rigid[391] and membrane permeable cyclotides[392], developed a solid phase synthesis protocol[393], and developed multiple postfunctionalization strategies.[390,394–396] Furthermore, the concept of using substrates bearing an additional nucleophilic amine for further functionalization inspired the group of Yudin to employ a special isocyanide, (*N*-isocyanimino)-triphenylphosphorane (**408**, Pinc)[397], along with an aldehyde (**409**) and linear

peptide (**406**) for the synthesis oxadiazole-grafted cyclotides (**411**, **Scheme 33B**).[398,399] The cyclization reaction proceeds through an Ugi-type/aza-Wittig mechanism and proved to be efficient in obtaining such cyclotides.[400,401]



Scheme 33. A. Aminoacid and peptide cyclization with the Yudin reaction. B. Synthesis of oxadiazole-grafted cyclotides using Pinc (**408**).

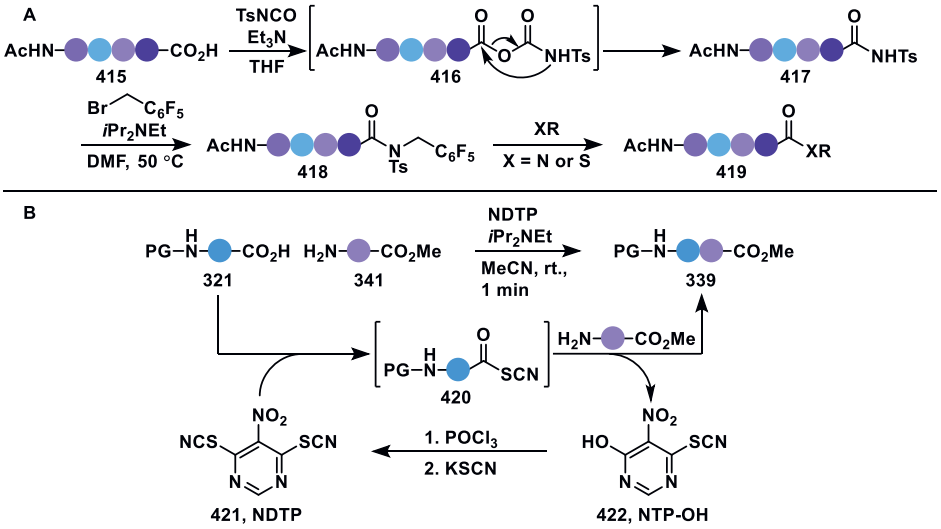
In 2023, Zhang and Chen reported the use of isocyanides for the activation of peptide carboxylic acids (**Scheme 34**).[402] At low pH (~3), nucleophilic attack of the carboxylate onto the isocyanide provides an electrophilic mixed anhydride (**413**). This reactivity was in turn exploited for cyclization reactions with peptides bearing an N-terminal cysteine. Much like in NCL, the thiol moiety substitutes the activated acid to form a thioester, which subsequently undergoes an S-to-N acyl shift to provide the cyclic product (**414**). By carefully considering the pH, their approach showed high selectivity for the C-terminus with shorter peptides yet yielded mixed products (1.7:1 C-terminal vs. Asp cyclization) with larger 12-mer peptides (*i.e.* CKWGLSDTQAEA).



Scheme 34. Native peptide cyclization facilitated by an isocyanide.

1.5.16 Miscellaneous

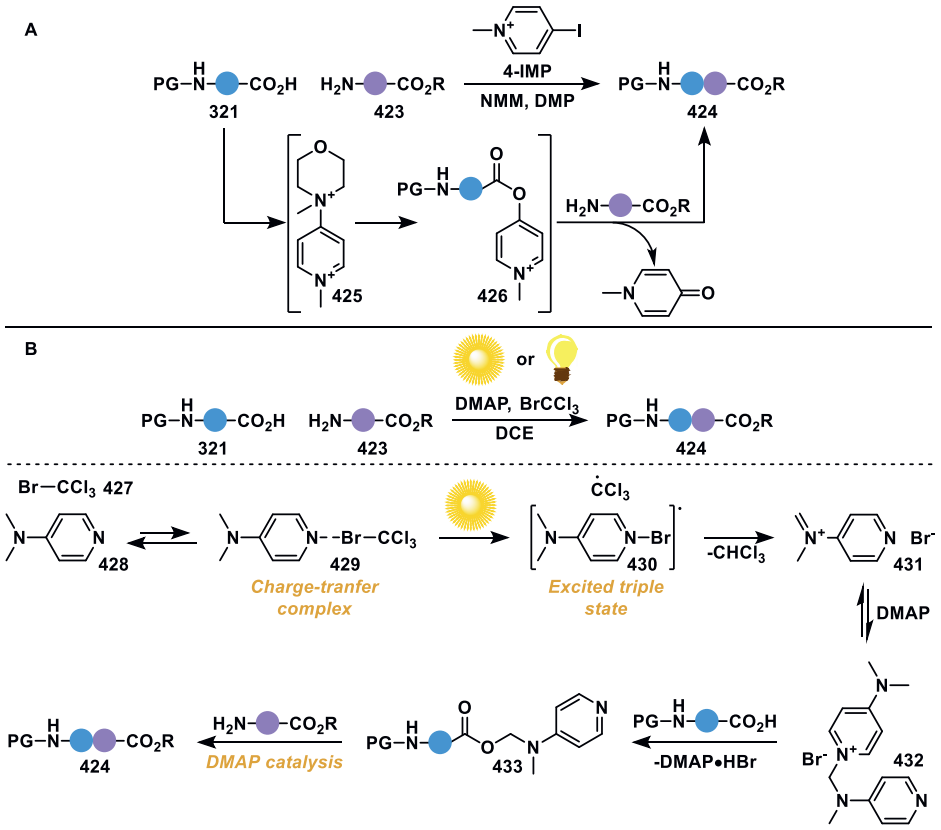
A mechanistically similar method to CDI activation called twisted-amide mediated peptide synthesis was reported recently by Kuranaga *et al.* (**Scheme 35A**).[403] Therein, they reacted the C-terminus with tosyl isocyanate. The resulting compound (**416**) rearranges and decarboxylates to form an acyl sulfonamide (**417**) which suppresses isomerization. It can then be displaced by the initial activation reaction with pentafluorobenzyl bromide and subsequent substitution with an amine.[403]



Scheme 35. A. Twisted-amide mediated peptide synthesis as reported by Kuranaga et al. B. Peptide coupling reaction mediated by NDTP.

Recently, the groups of Sun and Wang reported a new coupling reagent, 5-nitro-4,6-dithiocyanatopyrimidine (NDTP, **421**), which activates carboxylic acids via an acyl thiocyanide (**420**) and allows fast peptide synthesis under mild conditions (**Scheme 35B**).[404] This non-irritable and conveniently available reagent can be recycled and forms peptide bonds in minutes without epimerization. Another novel reagent class for peptide coupling was recently reported by Zhong *et al.* (**Scheme 36A**).[405] They showed that 4-iodine N-methylpyridinium (4IMP), an easily prepared and bench stable reagent, can be utilized in SPPS with high-chemoselectivity and efficiency and virtually no epimerization. In combination with *N*-methylmorpholine, this reagent forms a reactive biscation species (**425**) which activated carboxyl groups for follow-up amidation. Yet another novel strategy for activating peptide carboxylic acids was reported by Spilzman and Eichen *et al*.[406] They developed a peptide coupling reaction which relies on sunlight for the activation of a charge-transfer complex between DMAP (**428**) and an alkyl halide (**427**). This in turn results

in an iminium salt (**430**), which acts as a novel coupling reagent. This reaction proceeds under mild, epimerization-free conditions without the need for dry solvents or inert conditions and with yields comparable to conventional reagents (**Scheme 36B**).[406]



Scheme 36. A. Peptide coupling reaction mediated by 4-IMP. B. Photochemical peptide coupling using DMAP and bromotrichloromethane.

1.6. Conclusion

The functionalization of the C-terminus of amino acids, peptides, and proteins has been under development for over 150 years, and innovations are still constantly being reported. The selective formation of amide bonds through C-terminal activation remains a cornerstone of modern peptide synthesis. While amide bond formation appears deceptively simple, the development of effective coupling strategies has required continual innovation to improve yields, minimize epimerization, and expand functional group tolerance. A plethora of strategies for the functionalization of such carboxylates has been successfully employed, including enzymatic couplings, photo-/electrochemical decarboxylation, a range

of classical and novel coupling reagents, and metal catalysts. Although conventional coupling reagents provided a strong foundation, they often lacked selectivity and stability and resulted in increased epimerization (*e.g.*, acyl halides and anhydrides). The emergence of additives (*i.e.* benzotriazoles and oxyma) greatly improved the efficiency and reliability of such reactions. Additionally, activated acyl species, such as NHS/NHPI esters and allenones, can now be stored for longer periods of time and used upon demand. Over time, scientists have developed innovative approaches to overcome important challenges associated with C-terminal functionalization. Most of these novel strategies minimize or omit epimerization. Furthermore, since the invention of SPPS, many coupling strategies have required large excesses of reagents. However, we are now observing a clear shift toward more catalytic systems, in which both catalyst efficiency and reagent economy are improving. Good examples are the discussed organocatalysts, which initially employed phosphorus(III) species in stoichiometric amounts but are now catalytic with respect to these reagents. Another important advancement is the development of protection group-free strategies. Organosilicon reagents have made a particularly significant impact in this area, although thus far they have primarily been applied to the synthesis of small oligopeptides. Regarding the functionalization of more complex peptides and proteins, the most successful strategies reported to date include the diazocompounds employed by Raines, C-terminal hydrazides, isocyanide-based functionalizations, and the decarboxylative strategies developed by Macmillan. However, all these approaches still have disadvantages. Diazocompounds are not specific for the C-terminus, hydrazides require specific C-terminal sequences in the peptide or protein chain for synthesis, and isocyanide-based approaches are not fully selective. Currently, the most effective approach for the C-terminal selective functionalization of unprotected proteins is photochemical decarboxylation. However, this method is not universal for all C-termini and is not compatible with all amino acids. Thus, although novel methods are gradually becoming more broadly applicable, more environmentally friendly, suitable for complex systems (*i.e.* proteins), and capable of obviating the need for protection groups, much remains to be done in the field of C-terminal functionalization.

1.7. Thesis outline

In this chapter many novel strategies for the functionalization of complex peptides and proteins have been discussed. While witnessing the power of current ligation strategies, in addition to isocyanide-based functionalizations on large and complex systems, we recognized the lack of technologies to synthesize ligation substrates directly on unprotected native peptides and proteins. Therefore, in **Chapter 2**, we initially set out to develop a method that employs the multicomponent Passerini reaction for the selective functionalization of peptide carboxylic acids to afford α-acyloxyamide products. These

products could, in turn, serve as substrates for enzymatic ligations using peptiligase. The strength of this method lies in the combination of these two powerful reactions: the developed Passerini reaction enables the functionalization of carboxyl groups, while the enzymatic ligation selectively targets the C-terminus. Next, we combined our novel Passerini reaction with a chemical ligation strategy that is currently among the most widely applied in the field of peptide and protein functionalization: native chemical ligation (NCL). In **Chapter 3** we focused our attention to the synthesis of a novel disulfide aldehyde substrate for the Passerini reaction on peptides to obtain esters bearing a vicinal protected thiol. Upon subjecting these products to NCL under reductive conditions, we found that these substrates can efficiently form thioesters in situ and subsequently afford the desired NCL products through reaction with N-terminal cysteine-bearing peptides. During the development of the first two projects, we encountered solubility issues with our Passerini-functionalized peptides, since the polar carboxylic acids were converted into less soluble esters. Therefore, in **Chapter 4** we describe novel isocyanides bearing polar functionalities or even ionic groups to enhance their water-solubility properties. This completely new class of reagents represents the first of its kind and can be readily synthesized through diversification in the final step. These reagents were subsequently examined for the functionalization of peptides by means of the Passerini reaction and subsequent enzymatic ligation, where improved solubility of these substrates was observed. Finally, **Chapter 5** provides several future directions that are currently under development or have not yet been attempted.

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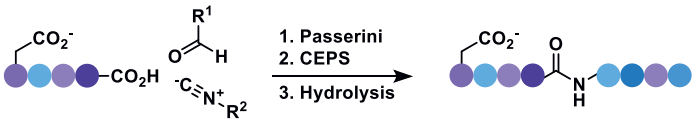
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Chapter 2

Merging the Passerini Reaction with Chemo-enzymatic Synthesis for C-terminal Peptide Modification



Abstract: *Peptide and protein synthesis, as well as bioconjugation, are increasingly important for developing site-selective modifications in biomedical applications. Herein, we report a strategy that combines the Passerini multicomponent reaction and peptiligase-mediated ligations for the selective bio-conjugation of peptide C-termini. The Passerini reaction, performed under aqueous acidic buffer conditions, ensures chemoselectivity for the carboxylic acids, while the subsequent enzymatic ligation selectively targets the functionalized C-terminal substrates. We demonstrated this approach on a diverse set of peptides while incorporating various isocyanides and aldehydes/ketones and successfully achieved ligations. By combining the Passerini reaction with enzymatic selectivity, this method provides a versatile and efficient platform for site-selective C-terminal modification, expanding the toolkit for peptide and protein modifications and synthesis.*

J. Hanssens, S. van Dun, T. H. G. Lokate, V. R. A. M. Reinartz, L. J. van den Bos, R. V. A. Orru and J. M. Saya*. *Cell Reports Physical Science*, **2025**, *6*, 10, 102860.

2.1 Introduction

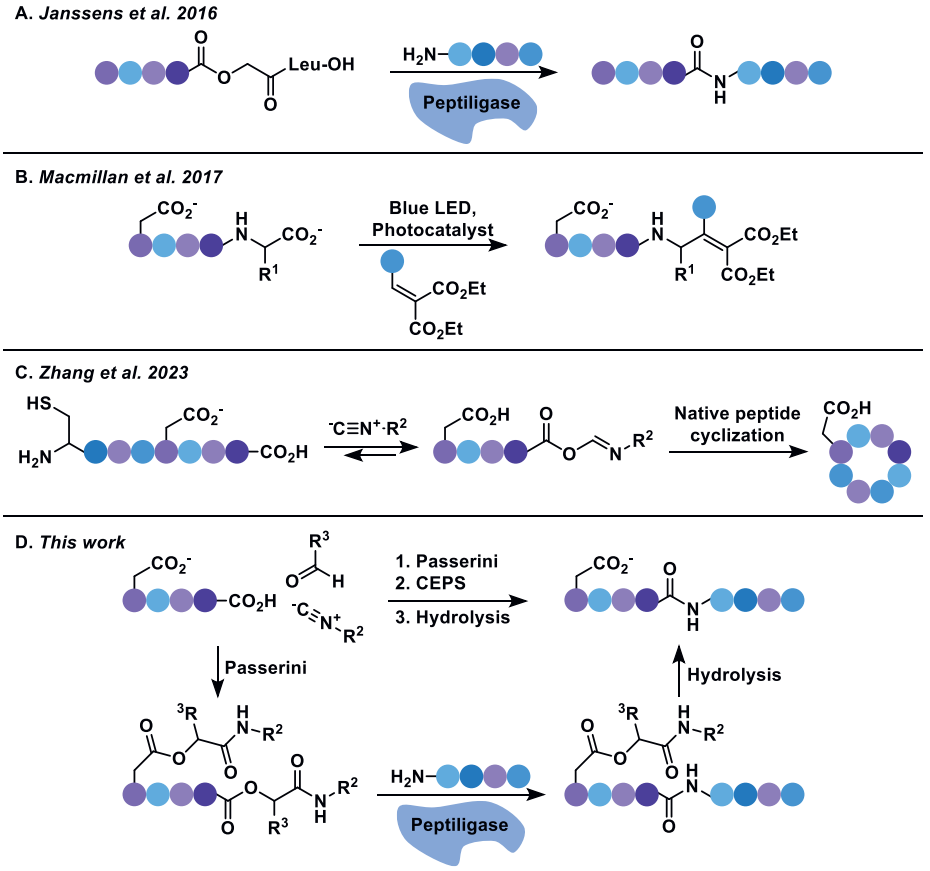
As the pharmaceutical industry seeks more sustainable manufacturing practices, biocatalysis has emerged as a key technology, aligning with nearly all 12 principles of green chemistry.[1] Pioneering work by Frances Arnold and Willem Stemmer on directed evolution has further cemented its industrial relevance.[2] With peptide-based therapeutics now accounting for 5% of the global pharmaceutical market, exceeding $50 billion in annual sales, enzymatic peptide synthesis has gained significant attention.[3] However, Merrifield's solid-phase peptide synthesis (SPPS) remains the dominant method for peptide production.

Since its introduction in 1963, SPPS has advanced significantly, enabling the rapid synthesis of peptide chains within hours.[4,5] However, SPPS is typically limited to peptides of around 50 amino acids, requiring additional techniques such as chemical fragment condensation and ligation strategies to assemble longer sequences.[6] However, these methods can lead to side reactions and pose scalability challenges. Enzymes offer a promising alternative due to their high selectivity and successful adoption in large-scale peptide manufacturing.

To overcome the limitations of chemical ligation strategies, enzymatic ligation has emerged as a powerful alternative for assembling longer peptides.[7] Over the past decades, several enzymes have been developed for peptide ligation, including sortase and butelase. However, these ligases require specific recognition sequences, restricting their applicability since the ligated product must retain these sequences. In contrast, the peptiligase enzyme family, derived from subtilisin, enables traceless ligation of nearly all primary amino acids without the need for a recognition motif.[7–11] Peptiligase-mediated ligation requires only an activated ester, with carboxyamidomethyl (Cam) esters commonly used due to their optimal balance of reactivity and stability (**Scheme 1A**).[8,10] However, Cam esters are typically synthesized via SPPS, where they are prone to hydrolysis, reducing overall peptide yields.[10] More importantly, this approach is not compatible with native or recombinant peptides and proteins, highlighting the need for alternative methods to introduce Cam esters under mild conditions.

To address these limitations, we envisioned a late-stage, siteselective C-terminal functionalization strategy. Macmillan et al. introduced a pioneering approach for selective C-terminal functionalization via photodecarboxylation, leveraging the differing oxidation potentials of carboxylic acid groups (**Scheme 1B**).[12,13] However, this method produces peptides that are incompatible with peptiligases. More recently, Zhang et al. reported a strategy for selective C-terminal activation using isocyanides under controlled aqueous pH conditions, facilitating peptide cyclizations (**Scheme 1C**).[14] While this approach demonstrated high selectivity for shorter peptides, it yielded mixed products (1.7:1 C-terminal vs. Asp cyclization) when applied to more complex 12-mer peptides (*e.g.*,

CKWGLSDTQAEA). Additionally, the acyloxy imidate intermediates were too labile for isolation, making them unsuitable for peptiligase-mediated ligation.



Scheme 37. Existing approaches of C-terminal functionalization and this work: Passerini-peptiligase-hydrolysis strategy. A. Janssen et al. reported the enzymatic peptide ligation using peptiligase and a presynthesized C-terminal OCam ester. B. MacMillan et al. reported the C-terminally selective photodecarboxylative alkylation of peptides. C. Zhang et al. reported the isocyanide-mediated native peptide cyclization. D. This work: Passerini reaction on peptide carboxylic acids followed by peptiligase-mediated ligation of the acyloxyamide products and final hydrolysis of the remaining unreacted esters

As part of our ongoing interest in multicomponent reactions and biocatalysis[15,16], we recognized an analogy between Cam esters and the typical α-acyloxyamide products of the Passerini three-component reaction (P-3CR). Herein, we report a strategy that combines the P-3CR and peptiligase-mediated ligations to selectively functionalize the C-terminal carboxylic acid of peptides (**Scheme 1D**). We hypothesized that acidic buffer conditions would enhance chemoselectivity by suppressing side-chain reactivity, except for the

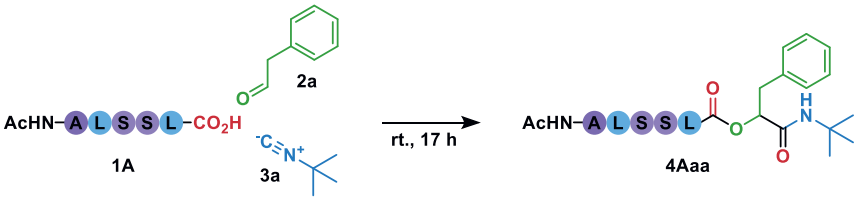
carboxylic acid side chains of Asp and Glu residues. Furthermore, we anticipated that peptiligases would selectively target C-terminal Cam esters while remaining inert to side-chain Cam esters, enabling post-ligation hydrolysis of any remaining side-chain esters to yield the final ligation product. This Passerini-ligation-hydrolysis approach establishes a unique traceless method for selective C-terminal modification of peptides.

2.2 Results and Discussion

2.2.1 Passerini reaction optimization.

For the optimization of the Passerini reaction, we selected pentapeptide Ac-ALSSL-OH (**1A**) as a model substrate. This selection was based on previous studies showing that the C-terminal LSSL-OR fragment serves as a good recognition site for omniligase-1, a peptiligase variant developed for broad substrate specificity.[9,17,18] The enzyme binding pocket consists of six recognition domains, four of which recognize the C-terminal amino acids of the acyl-donor peptide and the other two bind the acyl-acceptor nucleophile.[11]

Table 1. Reaction optimization of the Passerini reaction on unprotected peptides [a].



Entry	Solvent	Co-solvent	Yield (%)[b]
1	Water	MeCN (10%)	55
2	HEPES buffer (pH 7.5)	MeCN (10%)	21
3	TRIS buffer (pH 7.5)	MeCN (10%)	51
4	PB (pH 1.5, 100 mM)	MeCN (10%)	22
5	PB (pH 2.5, 100 mM)	MeCN (10%)	59
6	PB (pH 3.5, 100 mM)	MeCN (10%)	58
7	PB (pH 6.5, 100 mM)	MeCN (10%)	15
8	PB (pH 7.5, 100 mM)	MeCN (10%)	2
9	PB (pH 2.5, 100 mM)	None	49
10	PB (pH 2.5, 600 mM)	MeCN (10%)	35
11	PB (pH 2.5, 100 mM)	MeCN (20%)	56
12	PB (pH 2.5, 100 mM)	MeCN (30%)	58

Phosphate buffer (PB). [a] Standard conditions: **1A** (10 mM), **2a** (10 equiv), and **3a** (10 equiv) at rt. for 17 h. [b] The yields are reported based on HPLC analysis of the reaction utilizing *p*-hydroxy benzoic acid as IS.

Based on our extensive experience in isocyanide-based multicomponent reactions, we selected phenylacetic aldehyde (**2a**) and tert-butyl isocyanide (**3a**), respectively, as the

other inputs for the Passerini reaction. Our explorations began with various solvent systems for the Passerini reaction (Table S1). To our delight, the highest yield of Passerini product **4** was observed when utilizing MeCN in water.[15,16,19–26] Subsequently, we investigated a range of pH levels in various buffer solutions (**Table 1**, also see Table S2). We avoided the use of carboxylic acid-based buffers, since these could also participate in the reaction. At pH 7.5, the Tris buffer gave the highest yield of Passerini product **4Aaa**. However, we hypothesized that employing pH values below 4.5 (*i.e.*, below the pKa of carboxylic acids) would limit unwanted side reactions. Based on the pKa of *e.g.*, Lys-NH_3 or Cys-SH, these reactive side chains become protonated under acidic conditions. This significantly lowers their nucleophilicity compared to the more acidic carboxylic acids. Furthermore, the mechanism of the Passerini reaction requires protonation of the aldehyde in the first step, which can now be provided by the buffer or the carboxylic acid/carboxylate to facilitate the reaction.

Since the buffer ranges of Tris and HEPES solutions are limited to pH ~7, we continued with phosphate buffer (PB). Interestingly, the conversion was significantly lower in PB at pH 7.5. However, as shown, high yields were obtained when the pH was adjusted to 2.5–5.5. When performing the reaction at pH 1.5, the lower yield most likely results from the instability of the isocyanide. To ensure high selectivity when having other amino acid residues, pH 2.5 was adopted as the standard and most optimal buffer pH value for our process. Next, we examined the influence of the buffer concentration and the concentration of the MeCN organic cosolvent on the reaction (Table S3). With an increased buffer concentration, the yield shows a steady decrease, which is likely a consequence of the lower solubility of the organic components. Furthermore, increasing the acetonitrile content did not exert a direct effect on the yield. Finally, studies on the stoichiometry showed that using 10–25 equiv. of aldehyde and isocyanide was optimal for the reaction (Table S4). Since our products seem unstable and show hydrolysis under the reaction conditions, the reaction was terminated after 17 h to prevent further decomposition of the product. Moreover, multicomponent reactions generally benefit from higher reaction concentrations, but to make our system applicable to complex peptides and even proteins, the yields at 10 mM were considered satisfactory. Finally, no additional byproducts were observed.

2.2.2 Reaction Scope

With the optimal conditions in hand, we investigated the scope of the reaction by varying the amino acid residues at the N-terminus (**Table 2**). We were delighted to see that the reaction was compatible with all amino acids and furnished the desired α-acyloxyamide esters. We stopped the reactions before reaching full conversion to prevent product decomposition over extended periods. Passerini reactions with pentapeptides that contained Asn and Gln resulted in **4Naa** and **4Qaa** in good yields (61% and 57%,

respectively). However, certain pentamers resulted in particularly low yields, *e.g.*, Cys (**4Caa**; 26%), and Met (**4Maa**; 24%). In both cases, the low yield was due to undesired oxidation reactions. Given the presence of free amine groups, *i.e.* in the side chain of Lys (entry 6, **4Kaa**) or the free N-terminus (entry 14, **5Aaa**), the involved substrates could also partake in an (acid-less) Ugi reaction.[21,27] However, with the low pH buffer conditions, these products were only observed in trace amounts (<2%). Substrates with other nucleophilic side chains like those of Thr (entry 2, **4Taa**), Tyr (entry 3, **4Yaa**), and His (entry 7, **4Haa**) solely provided conversion to the desired products. Asp- and Glu-containing pentapeptides (entries 12–13, **4Daa**-**4Eaa**) also formed Passerini adducts on the carboxyl side chains.

Table 2. Reaction optimization of the Passerini reaction on unprotected peptides [a].

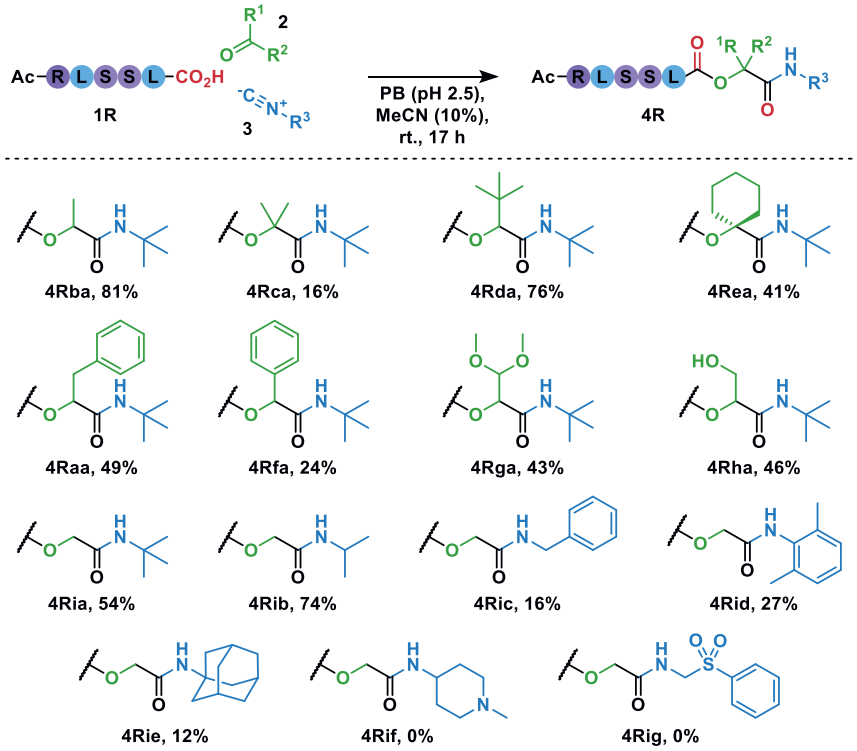


Entry	X	Product	Yield (%)[b]
1	Alanine	**4Aaa**	47
2	Threonine	**4Taa**	61
3	Tyrosine	**4Yaa**	41
4	Cysteïne	**4Caa**	26
5	Methionine	**4Maa**	24
6	Lysine	**4Kaa**	25
7	Histidine	**4Haa**	69
8	Tryptophan	**4Waa**	34
9	Arginine	**4Raa**	49
10	Asparagine	**4Naa**	61
11	Glutamine	**4Qaa**	57
12	Aspartate[c]	**4Daa**	46
13	Glutamate[c]	**4Eaa**	37
14	Alanine	**5Aaa**	24

[a] Reaction conditions: pentapeptide (10 mM), phenylacetaldehyde (10 equiv), t-butyl isocyanide (10 equiv), phosphate buffer (pH 2.5, 100 mM), and MeCN (10%). [b] The yields are of the isolated products. [c] The product of the reactions with peptides bearing carboxylate side chains were functionalized with two Passerini adducts using 20 equiv of aldehyde and isocyanide.

Having investigated the tolerance of the various functionalized amino acids, we explored the scope of the Passerini reaction with various aldehydes/ketones and isocyanides (**Scheme 2**). We used Ac-RLSSL-OH as the reference peptide component, as arginine provides high water solubility, which is beneficial for the subsequent enzymatic ligation. Firstly, we investigated the scope of aldehydes and ketones in combination with tert-butyl

isocyanide (**4Rba**-**4Ria**). The more reactive aliphatic aldehydes and formaldehyde seem to provide the best yields (**4Rba**, **4Rda**, **4Raa**, and **4Ria**; 49–81%), whereas the less reactive oxo-substrates like benzaldehyde result in lower conversion and yield (**4Rfa**; 24%). Similarly, the inherently lower reactivity of ketones compared to the aldehydes results in reduced conversion and yield (**4Rca**: 16%, **4Rea**: 41%). Furthermore, acetal moieties and alcohols were nicely tolerated under the reaction conditions, furnishing the desired products **4Rga** and **4Rha** in 43% and 46% yield, respectively. Partial decomposition of the acetal to the aldehyde under the acidic conditions accounts for the slightly lower yield for **4Rga**.

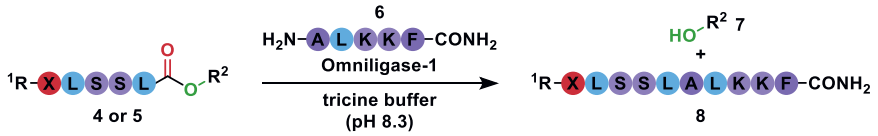


Scheme 38. Scope of the Passerini reaction with Ac-RLSSL-OH and various carbonyls and isocyanides.

Subsequently, we investigated the scope of isocyanides in combination with formaldehyde (**4Ria**-**4Rig**), since this is an unsubstituted reactive aldehyde which forms a product similar to the Cam ester previously utilized as peptiligase substrate (**Scheme 2**).[7–11,17] The reaction provides better yields with the more nucleophilic aliphatic isocyanides (**4Ria**: 54%, **4Rib**: 74%). Benzylic (**3c**) and aromatic isocyanides (**3d**) as well as sterically congested adamantyl isocyanide (**3e**) afford the product in lower yields (**4Ric**, **4Rid**, **4Rie**, 12–27%). Solubility of the starting isocyanides may account for the lower yields. Due to the basic amine in the side

chain of isocyanide **3f**, we observed low conversion and low stability of the resulting product **4Rif** under acidic conditions. Presumably, a facilitated intramolecular proton transfer from the amine to the ester leads to subsequent decomposition and cumbersome isolation of the Passerini product **4Rif**. Finally, the reaction with TosMIC (**3g**) also gave no conversion to the desired product (**4Rig**, 0%).

Table 3. Results of the enzymatic ligations of the synthesized peptides with omniligase-1[a]



Entry	Compound	Conversion (%)	Diastereoselective	Product?
1	4Aaa	57	>95%	✓
2	4Taa	50	>95%	✓
3	4Yaa	18	>95%	✓
4	4Caa	54	>95%	✓
5	4Maa	61	>95%	✓
6	4Kaa	54	>95%	✓
7	4Haa	56	>95%	✓
8	4Waa	53	>95%	✓
9	4Raa	52	>95%	✓
10	4Naa	53	>95%	✓
11	4Qaa	52	>95%	✓
12	4Daa	37	>95%	✓
13	4Eaa	51	>95%	✓
14	5Aaa	63	>95%	✓
15	4Rba	60	>95%	✓
16	4Rca	15	N/A	✗
17	4Rda	11	N/A	✗
18	4Rea	4	N/A	✗
19	4Rfa	47	85%	✓
20	4Rga	59	78%	✓
21	4Rha	47	N/D	✓
22	4Ria	100	N/A	✓
23	4Rib	89	N/A	✓
24	4Ric	100	N/A	✓
25	4Rid	100	N/A	✓
26	4Rie	91	N/A	✓

[a] Reaction conditions: 1.5 µM omnligase-1, 2.0 mM ester, and 2.4 mM amine 6; pH 8.3; and 2 mg/mL TCEP. The reported conversions are based on the remaining ester starting material after 2 h of ligation.

2.2.3 Ligation Experiments

With a set of structurally diverse Cam esters in hand, we set out to perform the ligation experiments utilizing omniligase-1 and H2N-ALKKF-OH (**6**) as a nucleophile to form hydrolysis product **7** and decapeptide **8** (**Table 3**).[17] The results of the ligations are expressed as the conversion of starting material over 2 h. To our delight, all peptide products with phenylacetaldehyde and t-butyl isocyanide (**4Aaa**-**4Yaa**, **5Aaa**) resulted in the desired peptide product. The Passerini reaction generates a 1:1 mixture of diastereoisomers, but omniligase-1 shows full selectivity for one of the two diastereoisomers (**Figure 1**). As expected, it merely recognizes the natural configuration of the amino acids.[17] The slightly higher conversion (above 50%) can be attributed to hydrolysis of the substrates under the reaction conditions. Furthermore, the side-chain esters of **4Daa** and **4Eaa** could be hydrolyzed to the free peptide products after the ligation. Finally, the lower conversion of compounds **4Yaa** and **4Daa** can be attributed to the low solubility of these peptides.

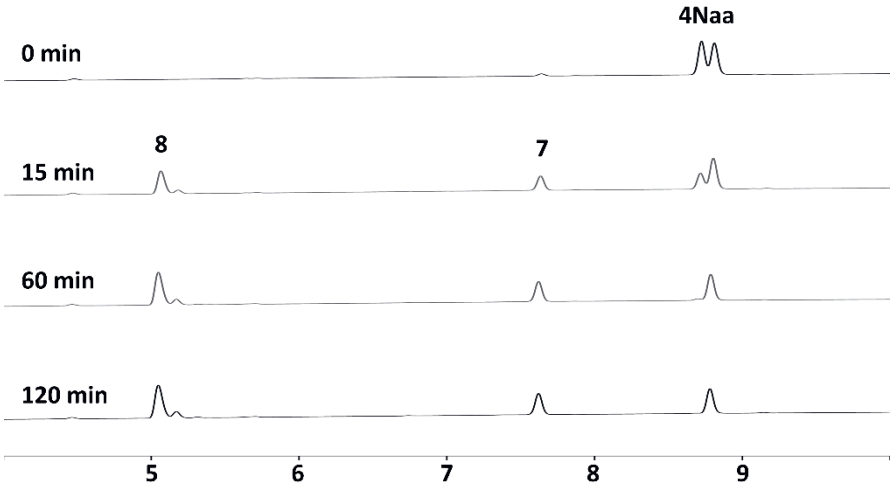
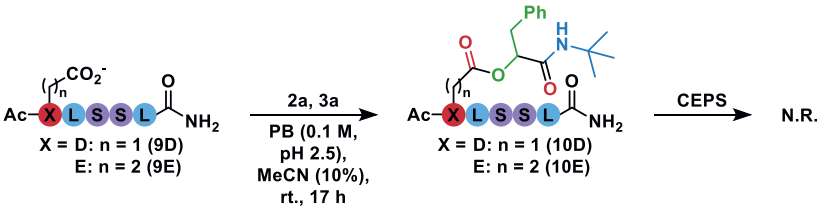


Figure 3. Example ligation of compound **4Naa**, where merely one diastereoisomer is recognized by the enzyme and fully converted to the product.

We subsequently investigated the effect of different esters on the omniligase-1 selectivity. When introducing a smaller methyl group (**4Rba**), the enzyme remained fully diastereoselective and provided the desired product. The same was observed for substrates with slightly larger groups (**4Rfa**-**4Rha**). This net kinetic resolution may offer opportunities for further development in the future. Additionally, no ligation was observed for the compounds with a quaternary α-carbon (**4Rca**, **4Rea**). Interestingly, when using a very bulky substituent (**4Rda**), the ligation rate was greatly diminished. The peptide bearing no α-

substituents (the Cam-ester, **4Ria**) showed high conversion toward the desired product. When comparing the terminal position of the substrates, determined by the different isocyanides, omniligase-1 shows a much higher tolerance to the various functionalities. All variations (**4Ria**-**4Rie**) showed high conversion of substrate to the desired product within 2 h, demonstrating the tolerance of both aromatic and sterically congested termini. Given these results, the optimal candidate for the ligation in combination with the Passerini reaction is the substrate formed from formaldehyde and isopropyl isocyanide (**4Rib**). The peptide with an unprotected N-terminus (**5Aaa**) shows signs of homodimer formation, indicating that the N-terminus of **5Aaa** competes with the N-terminus of **6**.



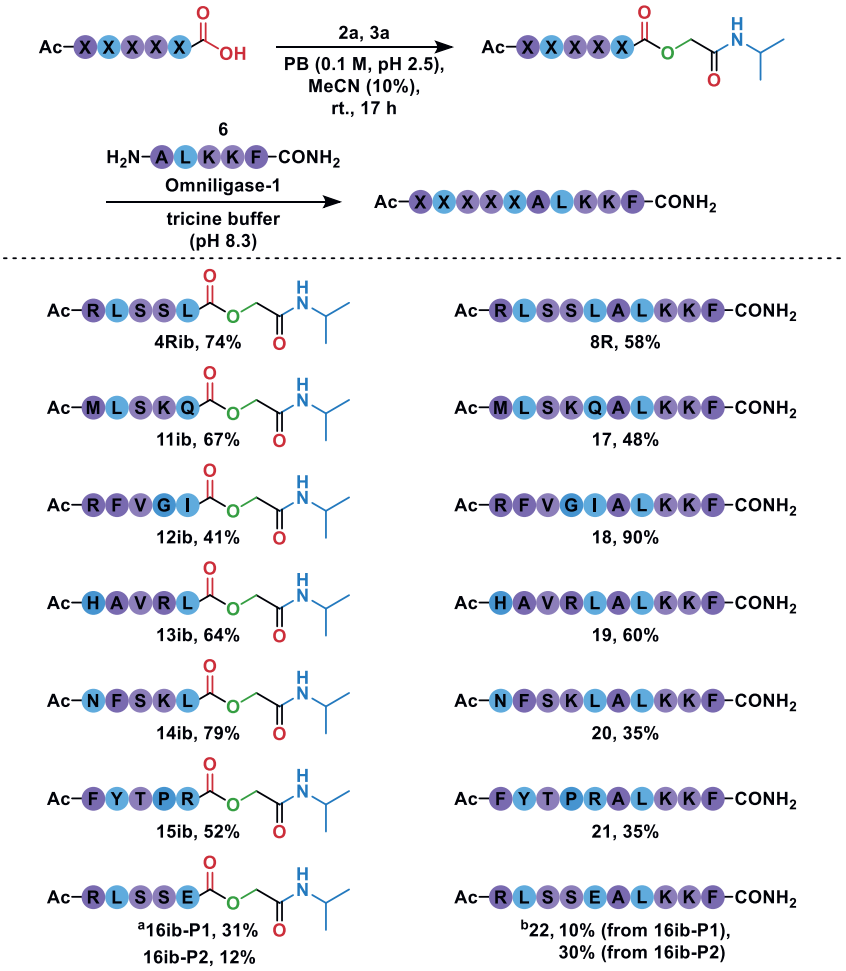
Scheme 39. Investigation of the selectivity for the C-terminus.

Next, we examined the enzyme's selectivity in favor of the C-terminal ester as opposed to the esters in the side chain. Ligations with Passerini products **4Daa** and **4Eaa** merely yielded the monoligated product. To demonstrate the enzyme's preference for the C-terminus, we modified the backbone of two peptides with C-terminal amides (**9D** and **9E**) under standard conditions and subsequently conducted ligations (**Scheme 3**). As expected, these peptides, **10Daa** and **10Eaa**, showed neither signs of conversion nor desired product formation, illustrating the selectivity of the enzyme for C-terminal esters. Any remaining side chains can be hydrolyzed back to the free peptide by increasing the pH of the ligation solution after full conversion of the C-terminal ester. Finally, during the ligation with both **4Daa** and **10Daa**, we observed succinimide formation through backbone cyclization onto the ester side chain and subsequent hydrolysis, generating two regioisomers.[28]

2.2.4 Methodology at synthesis scale

Finally, to showcase the scalability (89–167 µmol) of our Passerini-peptiligase approach we selected the optimal candidates, **2i** and **3b**, to demonstrate its effectiveness on a diverse set of unprotected pentapeptide targets (**Scheme 4**). To our delight, all peptides were compatible with the Passerini reaction, achieving yields between 41% and 79%. Furthermore, all peptides were efficiently tolerated by omniligase-1, with isolated yields ranging from 35 to 90%. Finally, we included an entry with a C-terminal Glu residue where we isolated two fractions, one containing the Passerini products with one ester (**16ib-P1**,

31%) at either the C-terminus or the side chain, and the difunctionalized (**16ib-P2**, 12%) product. Therein, we observed low product stability of these Passerini esters. This may explain the low desired product formation, which is likely caused by facilitated hydrolysis by the neighboring carboxylic acid. Nevertheless, we managed to purify the products and perform the subsequent ligations to isolate the final peptide **22** from both fractions **16ib-P1** and **16ib-P2** after hydrolysis in acceptable yields.



Scheme 40. Overall efficiency of the Passerini-peptiligase methodology at the synthesis scale. The reported yields are of the isolated products based on recovered starting material [a] Yields shown are for the compounds involving a mono- (**16ib-P1**) and di-functionalized (**16ib-P2**) Passerini products. [b] Yields shown are of the isolated peptide after ligation and subsequent hydrolysis (at pH 12) of the sidechain esters.

2.3 Conclusion

In conclusion, we developed a protocol that integrates the Passerini multicomponent reaction with peptiligase technology for selective bio-conjugation at the C-terminus of the peptides. Employing a low pH buffer as the solvent in the Passerini reaction enabled high selectivity for carboxylic acids within unprotected peptides (**Table 2**). This approach demonstrated compatibility with a wide range of aldehydes and isocyanides, in yields up to 81% (**Scheme 2**). Furthermore, omniligase-1 facilitated stereo- and site-selective conversion of C-terminal esters for efficient ligation. We applied this Passerini-peptiligase methodology on the two-step synthesis of 10-mer peptides, presenting a unique approach for selective C-terminal functionalization of unprotected peptides. In future work, we aim to extend this methodology to site-selective C-terminal modification of proteins, with promising applications in biomedical fields.

3.4 Experimental Section

General Remarks

All commercial reagents were used as received without further purification unless stated otherwise. Peptides were purified on an Autopurification Waters Prep system equipped with a QDA detector, 2998 photodiode array detector, 2545 binary gradient module, 2767 sample manager, 515 HPLC pump for makeup flow, and an Xbridge BEH C18 OBD column (C18, 5 µM, 130 A˚, 30 mm) with an optimized gradient for each peptide (MeCN/mQ with 0.1% TFA, 45 mL/min). The collection was triggered by the mass spectrometry (MS) signal. Peptide samples were lyophilized with a Christ Alpha 2–4 LCSplus lyophilizer. High-pressure liquid chromatography-mass spectrometry (HPLC-MS) analyses were performed on a Shimadzu Nexera 2 UHPLC system equipped with a Shimadzu LC-30AD pump, an SPD-M30A photodiode array detector, and LCMS-2020 single quadrupole detector. The eluent consisted of MilliQ water, LC-MS grade acetonitrile, and 0.1% formic acid. A Waters XSelect CSH C_{18} column (3.0 mm × 75 mm with a particle size of 3.5 mm) was employed for the separation at 30◦C. The method comprised a gradient of acetonitrile in water as follows: 5% (2 min) to 95% acetonitrile (6 min), 1 min at 95% acetonitrile, and back to 5% acetonitrile (2 min). NMR spectra (1D ^{1}H and ^{13}C, COSY, DIPSI, and ^{13}C-^{1}H HSQC) were recorded on a Bruker Avance III HD 700 MHz spectrometer equipped with a TCI cryoprobe, or on a Bruker Avance 600 MHz using the residual solvent, or TMS standard (0 ppm) as reference. The data are reported as follows: chemical shift (δ ppm), coupling constant (*J*, Hz), multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets; and dt, doublet of triplets). High-resolution mass spectroscopy was conducted on a Bruker micrOTOF-Q II mass spectrometer (electrospray ionization).

General synthetic procedures

General procedure for phosphate buffer solutions (PBS).
Separate stock solutions of phosphoric acid, potassium phosphate monobasic, and potassium phosphate dibasic were made with the desired concentration (0.1, 0.3, or 0.6 M). While monitoring the pH, the less acidic solutions were slowly added to the more acidic ones (with the same concentrations) until the desired pH was obtained.

General procedure A (GPA): the Passerini reaction optimization.
To a stirred solution of Ac-ALSSL-OH (0.03 mmol) in the appropriate solvent (mixture) was added phenylacetaldehyde and t-butyl isocyanide and the resulting mixture was stirred at rt. for 17 h. The reaction mixture was brought to pH 7 (monitored by pH paper) using NaOH (2 M). To the solution was added *p*-hydroxybenzoic acid (0.03 mmol, 1 equiv) in MeCN (1 mL) as an internal standard (IS). If necessary, MeCN was added until all solids had dissolved. An aliquot was taken for HPLC analysis. The yields were determined by HPLC against p-hydroxybenzoic acid as an internal standard at 220 nm.

General procedure B (GPB): the Passerini reaction with pentapeptides
To a stirred solution of peptide (0.06 mmol, 1 equiv) in PBS (100 mM, pH 2.5, 5.4 mL) and MeCN (0.6 mL, 10%) was added carbonyl (0.6 mmol, 10 equiv) and isocyanide (0.6 mmol, 10 equiv) and the resulting mixture was stirred at rt. for 17 h. The reaction mixture was brought to pH 7 (monitored by pH paper) using NaOH (2 M) and lyophilized. The crude product was purified by reversed-phase preparative HPLC.

General procedure C (GPC): ligation reactions with pentapeptides.
The standard amine was dissolved in tricine buffer (pH 8.5, 200 mM) to a final concentration of 2.4 mM, depending on the solubility of the peptide-ester. Subsequently, TCEP solution (100 mg/ml, 200 mM tricine, pH 8.5) was added to a final concentration of 2 mg/ml. The mixture was added to the dry ester to a final concentration of 2-15 mM ester. The pH was adjusted if necessary using 3 M NaOH or 3 M HCl to ~8.3. Sequentially, Omniligase (6.78 mg/ml,

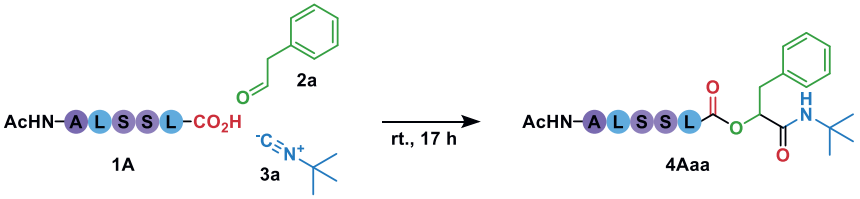
final concentration 0,041 mg/ml, 1.5 µM, 0.8 µL) was added to the ligation solution to start the ligation. A sample was taken before the start of the ligation by quenching 20 µL of the ligation solution in 80 µL quenching solution (mQ/MeCN/TFA; 48:48:4 Vol%). Samples were taken by quenching after 15, 60, and 120 minutes and analyzed on HPLC-MS.

General procedure D: SPPS of starting material peptides.

The reactions were performed in either a syringe reactor on an orbital shaker at room temperature or using a glass SPPS vessel to be stirred by nitrogen bubbling. The procedures were performed with 1 mL solvent/50 mg resin unless stated otherwise. **Rink amide AM resin** (GL Biochem, Cat. No. 49001) was swollen in DMF (dimethylformamide) for 30 min. The amino acid (AA) coupling solution of 4 equiv Fmoc-AA, 4 equiv (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) and 4 equiv ethyl cyano (hydroxyimino) acetate (oxyma) in DMF was prepared (0.3 mL/50 mg resin). 8 equiv *N,N*-diisopropylethylamine (DIPEA) were added to the coupling solution when ready for use. Subsequently, the coupling solution was added to the resin and allowed to react for 30 min. Subsequently, the reaction solution was discarded, and the AA coupling repeated. The resin was washed with DMF (5×) before Fmoc removal was performed by adding a deprotection solution of piperidine in DMF (2:8, v/v) and reacting for 5 min. The solution was discarded and the Fmoc removal repeated. Next, the resin was washed with DMF (3×), dichloromethane (DCM) (3×) and DMF (3×). Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above. **2-chlorotrityl chloride (CTC)-resin (100-200 mesh), 1% DVB** (Novabiochem, Cat. No. 8.55017) was swollen in DCM for 30 min. A solution of 2.5 equiv Fmoc-AA and 8 equiv DIPEA in DCM (0.3 ml/50 mg resin) was added to the resin and reacted for 1 h. The solution was discarded, and non-reacted linker groups were capped by adding a solution of methanol (MeOH), DIPEA and DCM (2:1:17, v/v/v) and reacting for 5 min. The solution was discarded and the capping step repeated. Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above for the rink amide AM resin. Peptides were N-terminally acetylated by adding a solution of Ac_2O, DIPEA and DMF (1:1:8, v/v/v) to the reaction and reacted for 10 min. The acetylation was repeated. The resin was washed with DMF (3×) and DCM (3×). Subsequently, the peptide was cleaved and the side chain protecting groups were removed in one step by adding a cleavage solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water (95:2.5:2.5, v/v/v) and reacting for 1 h. The cleavage solution was collected and the cleavage was repeated twice. The combined cleavage solutions were concentrated in vacuo. The material was precipitated in diethyl ether and centrifuged (15 min, 4000 rcf). The supernatant was discarded and the crude peptide was dissolved in MeCN and water and lyophilized. The peptides were purified by preparative HPLC.

Passerini Optimization

Table S1. Solvent screening, related to Table 1[a].



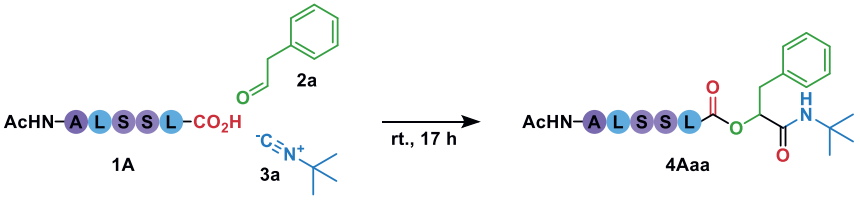
Entry	Solvent	Relative Yield (%)[b]
1	Water + MeCN (10%)	55
2	EtOAc	14
3	CH_2Cl_2	37
4	$CHCl_3$	33
5	DMF	11
6	MeOH	42

7	NMP	13
8	HFIP	6
9	BMIM (BF_4)	18
10	PEG 400	0

[a] Reaction condition: 0.03 mmol Ac-ALSSL-OH, 0.3 mmol **2a**, 0.3 mmol **3a**, solvent (10 mM), rt., 17 h. [b] Yields were determined by HPLC at 220 nm using *p*-hydroxybenzoic acid as internal standard.

Key finding Table S1: Aqueous conditions provided the best results, however, organic solvent like CH_2Cl_2, $CHCl_3$, MeOH were also acceptable.

Table S2. Buffer solvents & pH influence, related to Table 1[a].

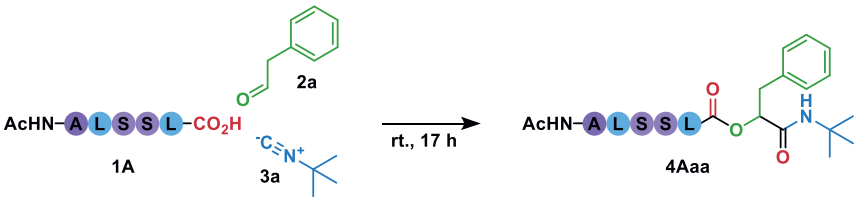


Entry	Solvent	Co-solvent (10%)	Relative Yield (%)[b]
1	HEPES buffer (pH 7.5)	MeCN	21
2	TRIS buffer (pH 7.5)	MeCN	51
3	Phosphate buffer (pH 7.5)	MeCN	2
4	Phosphate buffer (pH 6.5)	MeCN	15
5	Phosphate buffer (pH 5.5)	MeCN	64
6	Phosphate buffer (pH 4.5)	MeCN	61
7	Phosphate buffer (pH 3.5)	MeCN	58
8	Phosphate buffer (pH 2.5)	MeCN	59
9	Phosphate buffer (pH 1.5)	MeCN	22

[a] Reaction condition: 0.03 mmol Ac-ALSSL-OH, 0.3 mmol **2a**, 0.3 mmol **3a**, solvent (10 mM), co-solvent (10%), rt., 17 h. [b] Yields were determined by HPLC at 220 nm using *p*-hydroxybenzoic acid as internal standard.

Key finding Table S2: Phosphate buffer at pH 5.5 - 2.5 is optimal for the reaction.

Table S3. Buffer concentration & Co-solvent content, related to Table 1[a].

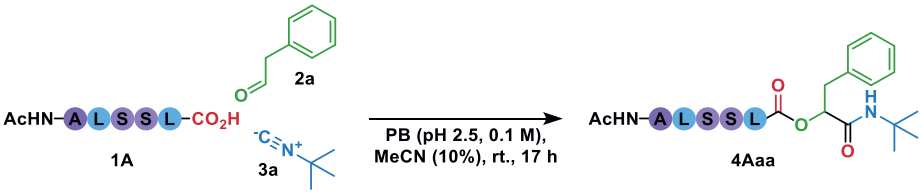


Entry	Solvent	Co-solvent (10%)	[mM]	Relative Yield (%)[b]
1	Phosphate buffer (pH 2.5, 0.3 M)	MeCN (10%)	10	46
2	Phosphate buffer (pH 2.5, 0.6 M)	MeCN (10%)	10	35
3	Phosphate buffer (pH 2.5, 0.3 M)	-	10	49
4	Phosphate buffer (pH 2.5, 0.3 M)	MeCN (20%)	10	58
5	Phosphate buffer (pH 2.5, 0.3 M)	MeCN (30%)	10	56
6	Phosphate buffer (pH 2.5, 0.3 M)	MeCN (40%)	10	62
7	Phosphate buffer (pH 2.5, 0.3 M)	MeCN (50%)	10	58
8	Phosphate buffer (pH 2.5, 0.3 M)	MeCN (10%)	50	52

[a] Reaction condition: 0.03 mmol Ac-ALSSL-OH, 0.3 mmol **2a**, 0.3 mmol **3a**, solvent, co-solvent, rt., 17 h. [b] Yields were determined by HPLC at 220 nm using *p*-hydroxybenzoic acid as internal standard.

Key finding Table S3: The buffer concentration at 100 mM provides the highest relative yield, likely due to the lower miscibility of the co-solvent. The co-solvent concentration does not affect the reaction significantly, but it does facilitate the reaction in low amounts.

Table S4. Stoichiometry, related to Table 1[a].

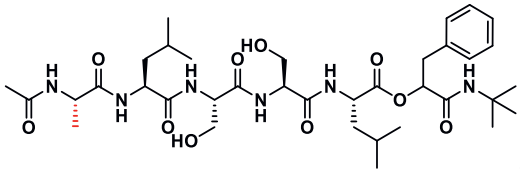


	Phenyl acetaldehyde	*t*-BuNC	Relative Yield (%)[b]
1	1	1	8
2	5	5	41
3	10	10	59
4	25	25	52
5	50	50	40

[a] Reaction condition: 0.03 mmol Ac-ALSSL-OH, X mmol **2a**, Y mmol **3a**, phosphate buffer (pH 2.5, 100 mM), MeCN (10%), rt., 17 h. [b] Yields were determined by HPLC at 220 nm using *p*-hydroxybenzoic acid as internal standard.

Key finding Table S4: Using 10 and 25 equivalents of isocyanide and aldehyde provides the optimal yield.

Characterization of compounds



4Aaa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-alanyl-L-leucyl-L-seryl-L-seryl-Lleucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 47% yield.

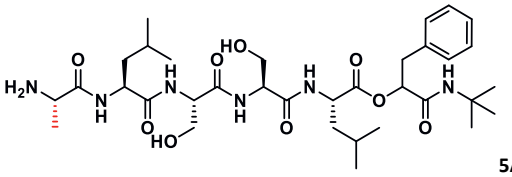
HPLC: Product is a set of two peaks with retention times 8.452 min, and 8.554 min.

^{1}H NMR: (701 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 7.29 – 7.24 (m, 4H), 7.23 – 7.19 (m, 1H), 5.11 (dd, J = 9.0, 4.6 Hz, 0.5H), 5.02 (dd, J = 9.2, 4.4 Hz, 0.5H), 4.47 – 4.42 (m, 1H), 4.42 – 4.24 (m, 4H), 3.96 – 3.74 (m, 4H), 3.16 – 3.08 (m, 1H), 3.06 – 2.98 (m, 1H), 1.99 (s, 1.5H), 1.98 (s, 1.5H), 1.78 – 1.53 (m, 4H), 1.53 – 1.44 (m, 1H), 1.43 – 1.38 (m, 1H), 1.37 (d, J = 7.2 Hz, 1.5H), 1.35 (d, J = 7.2 Hz, 1.5H), 1.31 (s, 4.5H), 1.30 (s, 4.5H), 0.97 (dd, J = 17.5, 6.6 Hz, 3H), 0.94 – 0.88 (m, 5H), 0.85 – 0.80 (m, 4H).

^{13}C-NMR: (176 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 176.25, 176.02, 175.36, 175.15, 173.90, 173.76, 173.17, 173.06, 172.55, 172.45, 172.41, 172.37, 171.02, and 170.89 (CO of ester and amides), 137.85, and 137.73 (quaternary C^1 of Ph), 130.85, 130.72, 129.51, 127.96, and 127.90 ($C^{2\text{-}6}$H of Ph), 76.94, and 76.64 (α-CH of ester), 62.88, 62.82, 62.78, and 62.71 (CH_2 of Serine), 57.61, 57.53, 57.03, 57.00, 54.06, 53.79, 53.18, and 52.87 (α-CH of main-chain amides), 52.65, and 52.51 (quat. C of t-Bu), 51.15, and 51.02 (α-CH of main-chain amides), 41.27,

41.18, 40.99, and 40.70 (CH_2 of Leucine), 39.16, and 39.07 (CH_2 of Phenylalanine), 28.84, and 28.81 (CH_3 of t-Bu), 26.00, 25.96, 25.74, and 25.63 (CH of Leucine), 23.55, 23.52, 23.27, and 23.26 (CH_3 of Leucine), 22.44, and 22.42 (CH_3 of acetyl), 22.10, 21.92, 21.85, and 21.78 (CH_3 of Leucine), 17.74, 17.70 (CH_3 of Alanine).

HRMS (ESI-TOF): m/z calculated for $C_{36}H_{59}N_6O_{10}$ $[M+H]^+$: 735.42872. Found 735.42872.



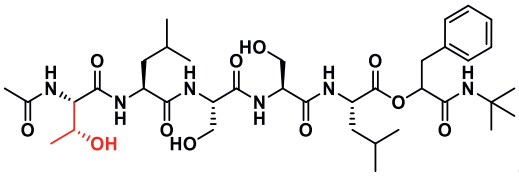
5Aaa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl L-alanyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 24% yield.

HPLC: Product is a set of two peaks with retention times 6.608 min, and 6.700 min.

1H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 7.30 – 7.24 (m, 4H), 7.23 – 7.19 (m, 1H), 5.10 (dd, J = 9.0, 4.8 Hz, 0.5H), 5.04 (dd, J = 8.9, 4.7 Hz, 0.5H), 4.48 – 4.41 (m, 3H), 4.35 (dd, J = 8.9, 5.6 Hz, 0.5H), 4.30 (dd, J = 9.8, 5.5 Hz, 0.5H), 3.97 (p, J = 7.2 Hz, 1H), 3.93 – 3.82 (m, 2.5H), 3.81 – 3.72 (m, 1.5H), 3.15 – 3.08 (m, 1H), 3.06 – 2.99 (m, 1H), 1.76 – 1.69 (m, 1H), 1.69 – 1.57 (m, 2.5H), 1.56 – 1.49 S8 (m, 3.5H), 1.49 – 1.41 (m, 1.5H), 1.40 – 1.34 (m, 0.5H), 1.29 (s, 4.5H), 1.29 (s, 4.5H), 1.00 – 0.88 (m, 8H), 0.85 – 0.81 (m, 4H).

13C-NMR: (176 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 174.65, 174.48, 173.20, 172.82, 172.56, 172.37, 172.36, 172.28, 171.56, 171.43, 170.96, and 170.90 (CO of ester and amides), 137.71, and 137.66 (quat. C1 of phenyl), 130.78, 130.70, 129.52, 129.50, 127.98, and 127.94 (C2-6H of phenyl), 76.84, and 76.67 (α-CH of ester), 63.07, 62.92, and 62.76 (CH2 of Serine), 57.10, 56.87, 56.76, 56.38, 53.85, 53.71, 52.98, and 52.68 (α-CH of main-chain amides), 52.60, and 52.59 (quat. C of t-Bu), 50.15 (αCH of Alanine), 41.61, 41.01, and 40.76 (CH2 of Leucine), 39.12, and 39.09 (CH2 of Phenylalanine), 28.84, and 28.80 (CH3 of t-Bu), 25.94, 25.79, and 25.67 (CH of Leucine), 23.47, 23.46, 23.19, 23.16, 22.10, 21.92, and 21.86 (CH3 of Leucine), 17.74 (CH3 of Alanine).

HRMS (ESI-TOF): m/z calculated for $C_{34}H_{57}N_6O_9$ $[M+H]^+$: 693.41815. Found 693.41952.



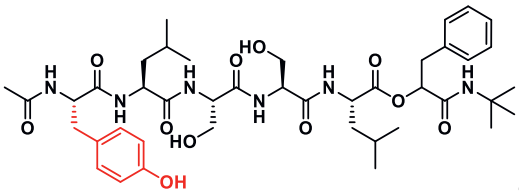
4Taa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-threonyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 61% yield.

HPLC: Product is a set of two peaks with retention times 8.457 min, and 8.559 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 7.29 – 7.24 (m, 4H), 7.24 – 7.19 (m, 1H), 5.11 (dd, J = 9.0, 4.7 Hz, 0.5H), 5.02 (dd, J = 9.2, 4.3 Hz, 0.5H), 4.47 – 4.42 (m, 1H), 4.42 – 4.35 (m, 3H), 4.35 – 4.26 (m, 1H), 4.24 – 4.16 (m, 1H), 3.96 – 3.75 (m, 4H), 3.16 – 2.99 (m, 2H), 2.05 (s, 1.5H), 2.04 (s, 1.5H), 1.82 – 1.71 (m, 1H), 1.71 – 1.66 (m, 1H), 1.66 – 1.54 (m, 2H), 1.53 – 1.46 (m, 1H), 1.42 – 1.37 (m, 1H), 1.31 (s, 4.5H), 1.30 (s, 4.5H), 1.21 (dd, J = 12.6, 6.4 Hz, 3H), 1.01 – 0.91 (m, 6H), 0.91 – 0.83 (m, 3H), 0.83 – 0.80 (m, 3H).

^{13}C-NMR: (176 MHz, Methanol-d_4) δ 175.45, 175.22, 174.06, 173.96, 173.72, 173.47, 173.18, 173.02, 172.56, 172.41, 172.35, 171.03, 170.96, and 170.89 (CO of ester and amides), 137.86, and 137.73 (quat. C1 of phenyl), 130.86, 130.72, 129.51, 127.96, and 127.90 (C2-6H of phenyl), 76.92, and 76.63 (α-CH of ester), 68.69, and 68.64 (β-CH of Threonine), 62.85, 62.79, 62.75, and 62.68 (CH2 of Serine), 60.24, 60.22, 57.79, 57.59, 57.15, 57.05, 54.40, 54.05, 53.20, and 52.88 (α-CH of main-chain amides), 52.64, and 52.53 (quat. C of t-Bu), 41.26, 41.20, 40.98, and 40.70 (CH2 of Leucine), 39.18, and 39.07 (CH2 of Phenylalanine), 28.84, and 28.81 (CH3 of t-Bu), 25.92, 25.89, 25.73, and 25.63 (CH of Leucine), 23.54, 23.50, 23.27, and 23.26 (CH3 of Leucine), 22.59, and 22.58 (CH3 of acetyl), 22.11, 21.99, 21.87, and 21.78 (CH3 of Leucine), 20.13, and 20.10 (CH3 of Threonine).

HRMS (ESI-TOF): m/z calculated for $C_{37}H_{61}N_6O_{11}$ $[M+H]^+$: 765.43928. Found 765.43928.



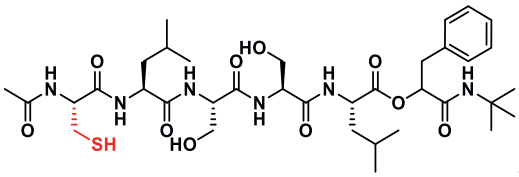
4Yaa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-tyrosyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 41% yield.

HPLC: Product is a set of two peaks with retention times 8.685 min, and 8.782 min.

^{1}H-NMR: (600 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 7.30 – 7.18 (m, 5H), 7.10 – 7.03 (m, 2H), 6.75 – 6.65 (m, 2H), 5.11 (dd, J = 9.0, 4.7 Hz, 0.5H), 5.02 (dd, J = 9.1, 4.5 Hz, 0.5H), 4.56 – 4.50 (m, 1H), 4.50 – 4.43 (m, 1H), 4.43 – 4.36 (m, 1H), 4.36 – 4.25 (m, 2H), 3.97 – 3.74 (m, 4H), 3.17 – 3.08 (m, 1H), 3.08 – 2.99 (m, 2H), 2.87 – 2.78 (m, 1H), 1.94 (s, 0.3H), 1.93 (s, 1.35H), 1.92 (s, 1.35H), 1.70 – 1.45 (m, 5H), 1.45 – 1.34 (m, 1H), 1.32 (s, 0.9H), 1.31 (s, 4.05H), 1.30 (s, 4.05H), 0.99 – 0.93 (m, 3H), 0.93 – 0.88 (m, 5H), 0.85 – 0.80 (m, 4H).

^{13}C-NMR: (151 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.13, 174.92, 174.73, 174.53, 173.81, 173.69, 173.14, 173.01, 172.51, 172.43, 172.33, 170.97, and 170.95 (CO of ester and amides), 157.36, and 157.33 (COH of phenol), 137.78, and 137.67 (quat. C1 of phenyl), 131.25 (C2,6H of phenol), 130.79, 130.67, and 129.47 (C2-3,5-6H of phenyl), 128.86, and 128.82 (quat. C4 of phenol), 127.92, and 127.87 (C4H of phenyl), 116.23, and 116.21 (C3,5H of phenol), 76.87, and 76.62 (α-CH of ester), 62.88, 62.85, 62.76, 62.72, and 62.69 (CH2 of Serine), 57.56, 57.52, 57.48, 57.46, 57.43, 57.39, 57.36, 57.04, 57.02, 56.98, 56.96, 56.93, 56.88, 56.81, 56.77, 56.73, 56.69, 54.11, 54.07, 54.01, 53.97, 53.86, 53.82, 53.76, 53.72, 53.11, 53.01, 52.79, and 52.69 (α-CH of main-chain amides), 52.59, and 52.57 (quat. C of t-Bu), 41.31, 41.26, 41.24, 41.19, 40.93, 40.90, 40.64, and 40.59 (CH2 of Leucine), 39.11, and 39.03 (CH2 of Phenylalanine), 37.75, and 37.71 (CH2 of Tyrosine), 28.80, 28.77, and 28.75 (CH3 of t-Bu), 25.75, 25.73, 25.68, and 25.57 (CH of Leucine), 23.54, 23.52, and 23.22 (CH3 of Leucine), 22.38, and 22.37 (CH3 of acetyl), 22.05, 21.90, 21.83, and 21.74 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{42}H_{63}N_6O_{11}$ $[M+H]^+$: 827.45493. Found 827.45554.



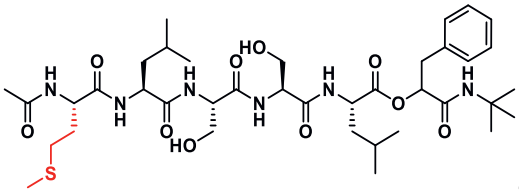
4Caa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-cysteinyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 26% yield.

HPLC: Product is a set of two peaks with retention times 9.233 min, and 9.335 min.

^{1}H-NMR: (600 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 7.30 – 7.18 (m, 5H), 5.10 (dd, J = 9.0, 4.6 Hz, 0.5H), 5.02 (dd, J = 9.1, 4.4 Hz, 0.5H), 4.53 – 4.25 (m, 5H), 3.95 – 3.73 (m, 4H), 3.16 – 3.08 (m, 1H), 3.07 – 2.98 (m, 1H), 2.97 – 2.86 (m, 1H), 2.86 – 2.77 (m, 1H), 2.01 (s, 1.5H), 2.00 (s, 1.5H), 1.80 – 1.70 (m, 1H), 1.70 – 1.45 (m, 4H), 1.45 – 1.35 (m, 1H), 1.34 – 1.26 (m, 9H), 1.00 – 0.89 (m, 8H), 0.86 – 0.80 (m, 4H).

^{13}C-NMR: (151 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.23, 175.02, 173.77, 173.70, 173.37, 173.16, 173.14, 172.97, 172.53, 172.43, 172.41, 172.28, 171.02, and 170.98 (CO of ester and amides), 137.83, and 137.71 (quat. C1 of phenyl), 130.84, 130.81, 130.72, 129.51, 127.96, and 127.91 (C2-6H of phenyl), 76.92, and 76.65 (α-CH of ester), 62.86, 62.77, 62.73, and 62.68 (CH2 of Serine), 57.55, 57.45, 57.36, 57.33, 56.96, 56.94, 56.68, 54.27, 53.98, 53.95, 53.80, 53.06, and 52.73 (α-CH of main-chain amides), 52.64, and 52.62 (quat. C of t-Bu), 41.29, 41.23, 40.94, and 40.62 (CH2 of Leucine), 39.16, and 39.08 (CH2 of Phenylalanine), 28.84, and 28.81 (CH3 of t-Bu), 26.65, and 26.61 (CH2 of Cysteine), 25.91, 25.88, 25.75, 25.73, 25.63, and 25.61 (CH of Leucine), 23.55, 23.52, and 23.26 (CH3 of Leucine), 22.56, 22.45, and 22.45 (CH3 of acetyl), 22.13, 22.11, 22.08, 22.03, 21.89, 21.84, 21.80, 21.77 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{36}H_{59}N_6O_{10}S$ $[M+H]^+$: 767.40079. Found 767.40148.



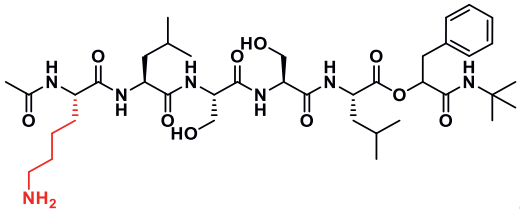
4Maa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-methionyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 24% yield.

HPLC: Product is a set of two peaks with retention times 8.995 min, and 9.102 min.

^{1}H-NMR: (701 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 7.29 – 7.24 (m, 4H), 7.23 – 7.19 (m, 1H), 5.11 (dd, J = 9.0, 4.7 Hz, 0.5H), 5.03 (dd, J = 9.1, 4.4 Hz, 0.5H), 4.46 (q, J = 4.7 Hz, 1H), 4.43 – 4.31 (m, 3.5H), 4.29 (dd, J = 9.9, 5.3 Hz, 0.5H), 3.95 – 3.90 (m, 1H), 3.90 – 3.82 (m, 1.5H), 3.82 – 3.78 (m, 1H), 3.76 (dd, J = 10.9, 6.2 Hz, 0.5H), 3.16 – 3.08 (m, 1H), 3.06 – 2.99 (m, 1H), 2.60 – 2.48 (m, 2H), 2.09 (s, 1.5H), 2.09 (s, 1.5H), 2.08 – 2.03 (m, 1H), 2.00 (s, 1.5H), 1.99 (s, 1.5H), 1.97 – 1.88 (m, 1H), 1.76 – 1.69 (m, 1H), 1.69 – 1.52 (m, 3H), 1.52 – 1.44 (m, 1H), 1.44 – 1.35 (m, 1H), 1.30 (s, 4.5H), 1.30 (s, 4.5H), 0.97 (dd, J = 15.4, 6.6 Hz, 3H), 0.94 – 0.89 (m, 4.5H), 0.85 – 0.80 (m, 4.5H).

^{13}C-NMR: (176 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 175.19, 175.00, 174.86, 174.65, 174.00, 173.88, 173.16, 172.97, 172.55, 172.44, 172.42, 172.32, 171.00, and 170.97 (CO of ester and amides), 137.84, and 137.73 (quat. C1 of phenyl), 130.84, 130.73, 129.52, 127.97, and 127.92 (C2-6H of phenyl), 76.93, and 76.67 (α-CH of ester), 62.95, 62.83, 62.80, and 62.77 (β-CH2 of Serine), 57.41, 57.38, 56.96, 56.86, 54.49, 54.41, 53.99, 53.76, 53.07, and 52.76 (α-CH of main-chain amides), 52.64, and 52.63 (quat. C of t-Bu), 41.34, 41.27, 40.97, and 40.69 (β-CH2 of Leucine), 39.16, and 39.09 (β-CH2 of Phenylalanine), 32.51, and 32.45 (β-CH2 of Methionine), 31.13, and 31.12 (γ-CH2 of Methionine), 28.86, and 28.83 (CH3 of t-Bu), 25.97, 25.95, 25.76, and 25.64 (γ-CH of Leucine), 23.55, 23.52, and 23.25 (CH3 of Leucine), 22.46, and 22.44 (CH3 of acetyl), 22.10, 21.93, 21.86, and 21.80 (CH3 of Leucine), 15.31 (εCH3 of Methionine).

HRMS (ESI-TOF): m/z calculated for $C_{38}H_{63}N_6O_{10}S$ $[M+H]^+$: 795.43209. Found 795.43307.



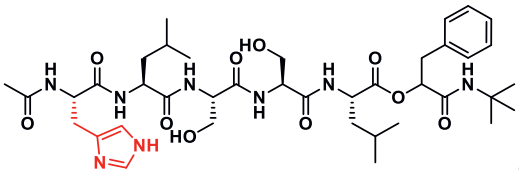
4Kaa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-lysyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 25% yield.

HPLC: Product is a set of two peaks with retention times 6.754 min, and 6.841 min.

^{1}H-NMR: (701 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 7.30 – 7.24 (m, 4H), 7.24 – 7.20 (m, 1H), 5.11 (dd, J = 9.0, 4.8 Hz, 0.5H), 5.04 (dd, J = 8.8, 4.8 Hz, 0.5H), 4.48 – 4.29 (m, 5H), 3.95 – 3.73 (m, 4H), 3.16 – 3.07 (m, 1H), 3.07 – 2.98 (m, 1H), 2.93 (t, J = 7.6 Hz, 2H), 1.99 (s, 1.5H), 1.98 (s, 1.5H), S11 1.87 – 1.80 (m, 1H), 1.76 – 1.52 (m, 7H), 1.50 – 1.42 (m, 3H), 1.40 – 1.34 (m, 1H), 1.30 (s, 4.5H), 1.29 (s, 4.5H), 0.97 (dd, J = 11.8, 6.6 Hz, 3H), 0.94 – 0.88 (m, 5H), 0.85 – 0.80 (m, 4H).

^{13}C-NMR: (176 MHz, Methanol-d_4; approx. 1:1 ratio of diastereomers) δ 175.23, 175.05, 174.77, 174.61, 173.74, 173.62, 173.25, 172.76, 172.62, 172.38, 172.37, 172.23, 170.88, and 170.82 (CO of ester and amides), 137.70, and 137.67 (quat. C1 of phenyl), 130.81, 130.71, 129.53, 129.51, 128.00, and 127.95 (C2- 6H of phenyl), 76.85, and 76.66 (α-CH of ester), 63.01, 62.86, and 62.82 (β-CH2 of Serine), 57.18, 56.93, 56.69, 54.74, 54.63, 53.82, 53.67, 52.94, and 52.66 (α-CH of main-chain amides), 52.50, and 52.48 (quat. C of t-Bu), 41.53, 41.45, 41.03, and 40.80 (β-CH2 of Leucine), 40.57 (ε-CH2 of Lysine), 39.12, and 39.11 (β-CH2 of Phenylalanine), 32.35, and 32.32 (β-CH2 of Lysine), 28.83, and 28.80 (CH3 of t-Bu), 28.17 (δCH2 of Lysine), 25.97, and 25.94 (γ-CH2 of Lysine), 25.79, 25.68, 23.72, and 23.68 (γ-CH of Leucine), 23.54, 23.52, and 23.23 (CH3 of Leucine), 22.47, and 22.45 (CH3 of acetyl), 22.08, 21.87, 21.84, and 21.79 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{39}H_{66}N_7O_{10}$ $[M+H]^+$: 792.48657. Found 792.48642.



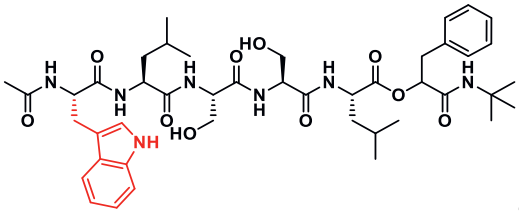
4Haa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-histidyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 69% yield.

HPLC: Product is a set of two peaks with retention times 6.860 min, and 6.953 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 8.72 (s, 0.5H), 8.71 (s, 0.5H), 7.32 (s, 1H), 7.30 – 7.23 (m, 4H), 7.23 – 7.19 (m, 1H), 5.10 (dd, J = 9.0, 4.8 Hz, 0.5H), 5.05 (dd, J = 8.8, 4.8 Hz, 0.5H), 4.69 (td, J = 6.7, 1.3 Hz, 1H), 4.50 – 4.43 (m, 2H), 4.41 – 4.35 (m, 1.5H), 4.32 (dd, J = 9.8, 5.5 Hz, 0.5H), 3.93 – 3.73 (m, 4H), 3.23 (dd, J = 15.3, 6.7 Hz, 1H), 3.16 – 3.06 (m, 2H), 3.06 – 2.98 (m, 1H), 1.98 (s, 0.3H), 1.97 (s, 1.2H), 1.97 (s, 1.5H), 1.74 – 1.66 (m, 1H), 1.66 – 1.57 (m, 2.5H), 1.56 – 1.50 (m, 0.5H), 1.50 – 1.43 (m, 1.5H), 1.40 – 1.35 (m, 0.5H), 1.29 (s, 0.9H), 1.29 (s, 3.6H), 1.28 (s, 4.5H), 0.96 (dd, J = 9.8, 6.6 Hz, 3H), 0.94 – 0.89 (m, 4.5H), 0.85 – 0.81 (m, 4.5H).

^{13}C-NMR: (176 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.36, 175.25, 173.45, 173.38, 173.25, 172.75, 172.69, 172.63, 172.53, 172.37, 172.25, 170.95, 170.87, and 170.80 (CO of ester and amides), 137.68, and 137.66 (quat. C of phenyl), 135.18 (ε-CH of Histidine), 131.04, and 130.94 (γ-C of Histidine), 130.79, 130.71, 129.52, 129.50, 127.99, and 127.95 (C2-6H of phenyl), 118.70, and 118.67 (δ-CH of Histidine), 76.83, 76.80, 76.70, and 76.67 (αCH of ester), 63.02, 62.88, 62.87, and 62.81 (CH2 of Serine), 57.15, 57.05, 56.85, 56.76, 53.96, 53.82, 53.67, 53.57, 52.95, and 52.66 (α-CH of main-chain amides), 52.60, and 52.57 (quat. C of t-Bu), 41.52, 41.49, 41.04, and 40.81 (CH2 of Leucine), 39.12, and 39.09 (CH2 of Phenylalanine), 28.84, 28.82, 28.80, and 28.78 (CH3 of t-Bu), 28.29 (CH2 of Histidine), 25.95, 25.80, and 25.68 (CH of Leucine), 23.52, 23.50, 23.20, and 23.19 (CH3 of Leucine), 22.47, and 22.46 (CH3 of acetyl), 22.09, 21.86, 21.83, and 21.76 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{39}H_{61}N_8O_{10}$ $[M+H]^+$: 801.45052. Found 801.45116.



4Waa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-tryptophyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 34% yield.

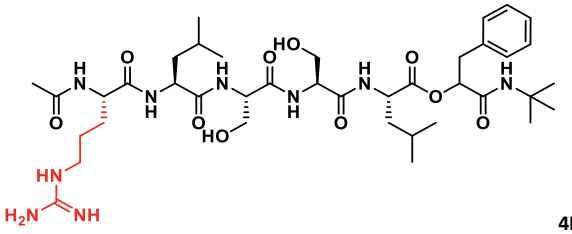
HPLC: Product is a set of two peaks with retention times 9.505 min, and 9.597 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 7.60 (d, J = 7.9 Hz, 1H), 7.33 (d, J = 8.1 Hz, 1H), 7.29 – 7.25 (m, 4H), 7.21 – 7.17 (m, 1H), 7.14 (s, 1H), 7.12 (s, 1H), 7.09 (m, 1H), 7.01 (m, 1H), 5.02 (dd, J = 9.1, 4.4 Hz, 1H), 4.65 (dd, J = 8.0, 5.8 Hz, 1H), 4.57 (s, 1H), 4.45 (dd, J = 5.4, 3.9 Hz, 1H), 4.35 (t, J = 5.4 Hz, 1H), 4.31 – 4.25 (m, 2H), 3.94 (dd, J = 11.4, 5.4 Hz, 1H), 3.90 (dd, J = 11.2, 5.4 Hz, 1H), 3.85 (dd, J = 11.4, 4.0 Hz, 1H), 3.79 (dd, J = 11.2, 5.4 Hz, 1H), 3.29 – 3.26 (m, 1H), 3.15 (dd, J = 14.8, 8.0 Hz, 1H), 3.10 (dd, J = 14.0, 4.4 Hz, 1H), 3.02 (dd, J =

14.0, 9.2 Hz, 1H), 1.94 (s, 3H), 1.63 – 1.50 (m, 5H), 1.43 – 1.38 (m, 1H), 1.30 (s, 9H), 0.91 (d, J = 6.5 Hz, 3H), 0.89 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.4 Hz, 3H), 0.81 (d, J = 6.5 Hz, 3H).

^{13}C-NMR: (176 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 175.21, 175.10, 173.96, 172.94, 172.52, 172.43, and 170.96 (CO of ester and amides), 138.07, and 137.78 (quat. C of phenyl and indole), 130.80, and 129.46 (CH of phenyl), 128.80 (quat. C of indole), 127.86 (CH of phenyl), 124.52, 122.51, 119.90, 119.31, and 112.33 (CH of indole), 110.72 (quat. C of indole), 76.90 (α-CH of ester), 62.75, and 62.64 (CH2 of Serine), 57.59, 57.43, 56.17, 54.15, and 53.03 (α-CH of main-chain amides), 52.60 (quat. C of t-Bu), 41.13, and 40.62 (CH2 of Leucine), 39.12 (CH2 of Phenylalanine), 28.81 (CH3 of t-Bu), 28.45 (CH2 of Tryptophan), 25.72, and 25.70 (CH of Leucine), 23.43, and 23.22 (CH3 of Leucine), 22.51 (CH3 of acetyl), 21.95, and 21.74 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{44}H_{64}N_7O_{10}$ $[M+H]^+$: 850.47092. Found 850.47092.



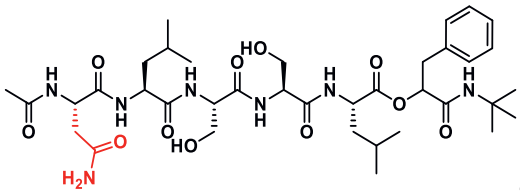
4Raa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 49% yield.

HPLC: Product is a set of two peaks with retention times 6.700 min, and 6.792 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 7.30 – 7.24 (m, 4H), 7.23 – 7.20 (m, 1H), 5.11 (dd, J = 9.1, 4.7 Hz, 0.5H), 5.04 (dd, J = 8.8, 4.7 Hz, 0.5H), 4.47 – 4.29 (m, 5H), 3.93 – 3.74 (m, 4H), 3.20 (td, J = 7.0, 2.3 Hz, 2H), 3.16 – 3.08 (m, 1H), 3.06 – 2.99 (m, 1H), 2.00 (s, 1.5H), 1.99 (s, 1.5H), 1.88 – 1.82 (m, 1H), 1.71 (m, 2H), 1.68 – 1.59 (m, 4.5H), 1.57 – 1.52 (m, 0.5H), 1.49 – 1.43 (m, 1.5H), 1.39 – 1.34 (m, 0.5H), 1.30 (s, 4.5H), 1.29 (s, 4.5H), 0.97 (dd, J = 11.8, 6.6 Hz, 3H), 0.94 – 0.90 (m, 4.5H), 0.84 – 0.81 (m, 4.5H).

^{13}C-NMR: (176 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 175.20, 175.03, 174.60, 174.45, 173.80, 173.66, 173.27, 172.78, 172.61, 172.44, 172.22, 170.95, and 170.89 (CO of ester and amides), 158.65, and 158.63 (CN of guanidine), 137.70, and 137.67 (quat. C1 of phenyl), 130.81, 130.70, 129.53, 129.51, 127.99, and 127.95 (C2-6H of phenyl), 76.87, and 76.70 (CH of ester), 63.05, 62.89, 62.85, and 62.81 (CH2 of Serine), 57.21, 57.17, 56.96, 56.66, 54.57, 54.44, 53.85, 53.70, 52.97, and 52.70 (α-CH of main-chain amides), 52.61, and 52.59 (quat. C of t-Bu), 42.04 (δ-CH2 of Arginine), 41.51, 41.42, 40.99, and 40.77 (CH2 of Leucine), 39.13, and 39.10 (CH2 of Phenylalanine), 30.14, and 30.08 (β-CH2 of Arginine), 28.85, and 28.81 (CH3 of t-Bu), 26.26, and 26.24 (γ-CH2 of Arginine), 25.96, 25.94, 25.78, and 25.67 (CH of Leucine), 23.54, 23.53, and 23.21 (CH3 of Leucine), 22.48, and 22.45 (CH3 of acetyl), 22.09, 21.84, and 21.77 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{39}H_{66}N_9O_{10}$ $[M+H]^+$: 820.49272. Found 820.49272.



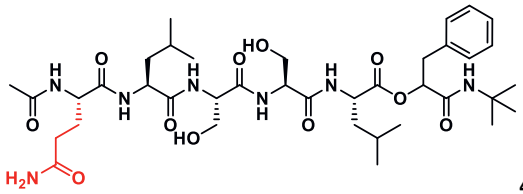
4Naa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-asparaginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 61% yield.

HPLC: Product is a set of two peaks with retention times 8.127 min, and 8.214 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 7.34 – 7.24 (m, 4H), 7.24 – 7.18 (m, 1H), 5.11 (dd, J = 9.0, 4.5 Hz, 0.5H), 5.00 (dd, J = 9.3, 4.2 Hz, 0.5H), 4.75 – 4.67 (m, 1H), 4.45 – 4.39 (m, 1H), 4.39 – 4.34 (m, 1H), 4.33 – 4.23 (m, 2H), 4.01 – 3.78 (m, 4H), 3.17 – 3.08 (m, 1H), 3.08 – 2.99 (m, 1H), 2.91 – 2.81 (m, 1H), 2.77 – 2.68 (m, 1H), 1.98 (s, 1.8H), 1.96 (s, 1.2H), 1.85 – 1.70 (m, 2H), 1.70 – 1.58 (m, 2H), 1.58 – 1.45 (m, 1H), 1.41 – 1.34 (m, 1H), 1.33 (s, 0.9H), 1.32 (s, 3.6H), 1.31 (s, 0.9H), 1.30 (s, 3.6H), 1.00 (d, J = 6.6 Hz, 1.5H), 0.96 (d, J = 6.5 Hz, 1.5H), 0.93 (d, J = 6.5 Hz, 1.5H), 0.90 (d, J = 6.5 Hz, 3H), 0.84 (d, J = 6.4 Hz, 1.5H), 0.83 – 0.77 (m, 3H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.87, 175.64, 175.18, 175.13, 174.53, 174.34, 173.22, 173.14, 173.08, 172.59, 172.53, 172.51, 172.31, 171.05, and 170.90 (CO of ester and amides), 137.92, and 137.75 (quaternary C1 of Ph), 130.93, 130.79, 130.71, 129.51, 129.44, 129.11, 127.94, 127.86, and 127.46 (C2-6H of Ph), 77.00, and 76.63 (α-CH of ester), 62.77, 62.69, 62.63, and 62.44 (CH2 of serine), 59.02, 58.20, 57.94, 57.92, 57.14, 55.00, 54.64, 53.21, 53.11, and 52.80 (α-CH of main-chain amides), 52.54, 52.51 (quat. C of t-Bu), 51.52, and 51.47 (α-CH of Asparagine), 40.97, 40.93, 40.83, 40.55, and 40.51 (CH2 of Leucine), 39.20, and 39.06 (CH2 of Phenylalanine), 37.72 (CH2 of Asparagine), 28.82 (CH3 of t-Bu), 25.91, 25.88, 25.70, and 25.58 (CH of Leucine), 23.59, 23.55, 23.39, and 23.33 (CH3 of Leucine), 22.45 (CH3 of acetyl), 22.07, 21.83, 21.68, and 21.67 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{37}H_{60}N_7O_{11}$ $[M+H]^+$: 778.43453. Found 778.43453.



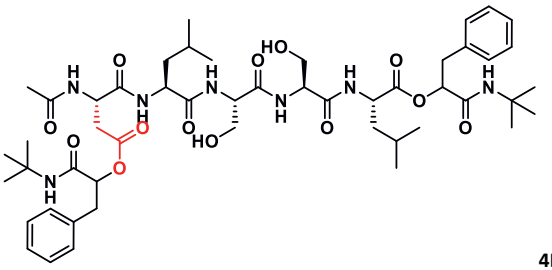
4Qaa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl acetyl-L-glutaminyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 57% yield.

HPLC: Product is a set of two peaks with retention times 7.991 min, and 8.078 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 7.31 – 7.19 (m, 5H), 5.11 (dd, J = 9.0, 4.7 Hz, 0.5H), 5.02 (dd, J = 9.0, 4.5 Hz, 0.5zH), 4.50 – 4.26 (m, 5H), 3.99 – 3.74 (m, 4H), 3.17 – 3.08 (m, 1H), 3.08 – 2.98 (m, 1H), 2.38 – 2.27 (m, 2H), 2.13 – 2.03 (m, 1H), 1.99 (d, J = 1.5 Hz, 1.8H), 1.98 (s, 1.2H), 1.97 – 1.91 (m, 1H), 1.80 – 1.69 (m, 1H), 1.69 – 1.65 (m, 1H), 1.65 – 1.35 (m, 4H), 1.30 (s, 5.4H), 1.30 (s, 3.6H), 1.02 – 0.95 (m, 3H), 0.95 – 0.77 (m, 9H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 177.75, 175.35, 175.14, 174.74, 174.52, 173.77, 173.65, 173.20, 173.18, 173.07, 172.95, 172.75, 172.57, 172.56, 172.50, 172.48, 172.29, 171.01, 170.98, 170.93, and 170.90 (CO of ester and amides), 137.81, and 137.71 (quat. C1 of phenyl), 130.84, 130.72, 129.52, 129.51, 127.97, and 127.92 (C2-6 of phenyl), 76.91, 76.88, 76.65, and 76.62 (α-CH of ester), 62.93, 62.78, 62.77, and 62.73 (CH2 of Serine), 57.52, 57.44, 57.41, 56.98, 56.96, 56.90, 54.65, 54.54, 54.10, 53.84, 53.13, 53.03, 52.83, and 52.73 (α-CH of main-chain amides), 52.62, and 52.50 (quat. C of t-Bu), 41.40, 41.32, 40.97, 40.93, 40.69, and 40.64 (CH2 of Leucine), 39.15, and 39.07 (CH2 of Phenylalanine), 32.50 (γ-CH2 of Glutamine), 28.84, 28.82, 28.80, and 28.79 (CH3 of t-Bu), 28.63, and 28.57 (β-CH2 of glutamine), 25.92, 25.89, 25.74, and 25.62 (CH of Leucine), 23.56, 23.53, 23.26, and 23.25 (CH3 of Leucine), 22.46, and 22.44 (CH3 of acetyl), 22.08, 21.90, 21.80, and 21.77 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{38}H_{62}N_7O_{11}$ $[M+H]^+$: 792.45018. Found 792.45018.



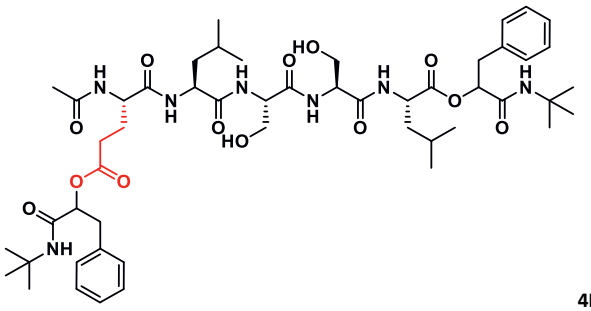
4Daa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl ((2S)-2-acetamido-4-((1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl)oxy)-4-oxobutanoyl)-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB, but now using 20 equiv. of **2a** and **3a**. The title compound was provided as a white solid in 46% yield.

HPLC: Product is a set of two peaks with retention times 10.839 min, and 10.902 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1:1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 7.32 – 7.17 (m, 10H), 5.14 – 5.09 (m, 1H), 5.07 (dd, J = 6.9, 6.7 Hz, 0.2H), 5.02 (dd, J = 9.2, 4.4 Hz, 0.8H), 4.75 (t, J = 7.0 Hz, 0.2H), 4.70 – 4.64 (m, 0.8H), 4.47 – 4.35 (m, 2H), 4.35 – 4.23 (m, 2H), 3.97 – 3.72 (m, 4H), 3.17 – 2.88 (m, 5H), 2.85 – 2.70 (m, 1H), 1.97 (s, 0.45H), 1.96 (s, 1.65H), 1.95 (s, 0.95H), 1.80 – 1.43 (m, 5H), 1.43 – 1.34 (m, 1H), 1.33 – 1.28 (m, 9H), 1.26 – 1.22 (m, 9H), 1.00 – 0.87 (m, 8H), 0.86 – 0.76 (m, 4H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1:1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.35, 175.31, 175.06, 173.79, 173.76, 173.70, 173.60, 173.43, 173.13, 172.99, 172.96, 172.51, 172.39, 172.28, 171.57, 171.46, 171.37, 170.98, 170.96, 170.89, and 170.75 (CO of esters and amides), 137.85, 137.84, 137.72, and 137.37 (quat. C1 of phenyl rings), 130.86, 130.82, 130.71, 129.51, 129.48, 128.01, 127.99, 127.95, and 127.90 (C2-6H of phenyl rings), 76.91, 76.61, 76.59, 76.50, S15 and 76.49 (α-CH of esters), 62.81, 62.78, 62.73, 62.61, and 62.60 (CH2 of Serine), 57.90, 57.88, 57.57, 57.54, 57.15, and 56.94 (α-CH of Serine), 54.49, 54.45, 54.06, 53.16, 53.06, and 52.76 (α-CH of Leucine), 52.53, 52.50, 52.39, 52.38, and 52.37 (quat. C of t-Bu), 51.49, and 51.22 (α-CH of Aspartate), 41.30, 41.24, 41.22, 40.89, 40.63, and 40.59 (CH2 of Leucine), 39.17, 39.11, 39.08, and 39.04 (CH2 of Phenylalanine), 36.52, 36.48, and 36.47 (β-CH2 of Glutamic acid), 28.83, 28.80, 28.78, 28.77, and 28.76 (CH3 of t-Bu), 25.91, 25.88, 25.85, 25.72, and 25.60 (CH of Leucine), 23.57, 23.52, 23.30, and 23.28 (CH3 of Leucine), 22.53, and 22.50 (CH3 of acetyl), 22.09, 21.97, 21.93, 21.85, 21.74, and 21.73 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{50}H_{76}N_7O_{13}$ $[M+H]^+$: 982.54956. Found 982.55076.



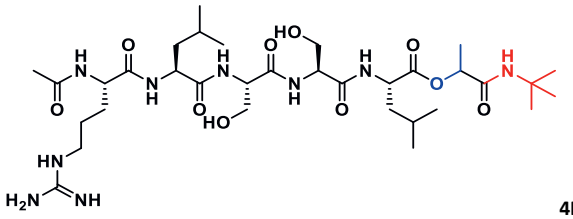
4Eaa: bis(1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl) (2S,5S,8S,11S,14S)-14-acetamido-5,8-bis(hydroxymethyl)-2,11-diisobutyl-4,7,10,13-tetraoxo-3,6,9,12-tetraazaheptadecanedioate: The product was synthesized following GPB, but now using 20 equiv of **2a** and **3a**. The title compound was provided as a white solid in 37% yield.

HPLC: Product is a set of two peaks with retention times 10.315 min, and 10.363 min.

^{1}H-NMR: (701 MHz, Methanol-d4, approx. 1:1:1:1 ratio of diastereomers) δ 7.29 – 7.24 (m, 8H), 7.23 – 7.18 (m, 2H), 5.10 – 5.06 (m, 1H), 5.02 (ddd, J = 9.2, 4.4, 1.6 Hz, 1H), 4.45 (ddd, J = 7.9, 5.3, 4.0 Hz, 1H), 4.41 – 4.33 (m, 2.5H), 4.30 – 4.25 (m, 1.5H), 3.96 – 3.89 (m, 2H), 3.87 – 3.78 (m, 2H), 3.15 – 3.08 (m, 1H), 3.08 – 3.00 (m, 3H), 2.55 – 2.46 (m, 1H), 2.46 – 2.38 (m, 1H), 2.12 – 2.03 (m, 1H), 1.99 (s, 1.5H), 1.97 (s, 1.5H), 1.96 – 1.89 (m, 1H), 1.81 – 1.59 (m, 4H), 1.59 – 1.52 (m, 1H), 1.43 – 1.38 (m, 1H), 1.30 (s, 9H), 1.25 (s, 9H), 0.98 (dd, J = 6.6, 1.9 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 0.90 (dd, J = 6.6, 2.3 Hz, 3H), 0.81 (dd, J = 6.5, 1.9 Hz, 3H).

^{13}C-NMR: (176 MHz, Methanol-d4, approx. 1:1:1:1 ratio of diastereomers) δ 175.24, 175.22, 174.76, 174.70, 173.50, 173.47, 172.99, 172.54, 172.48, 171.23, 171.18, 171.01, and 171.01 (CO of esters and amides), 137.84, and 137.56 (quat. C1 of phenyl rings), 130.85, 130.79, 130.78, 129.51, 129.47, 129.46, 127.96, and 127.91 (C2-6H of phenyl rings), 76.94, 76.26, and 76.24 (α-CH of esters), 62.80, 62.79, 62.74, and 62.71 (CH3 of Serine), 57.57, 57.52, 57.43, 57.41, 54.48, 54.31, 54.14, 54.11, and 53.08 (α-CH of main-chain amides), 52.65, and 52.43 (quat. C of t-Bu), 41.22, 41.21, and 40.67 (CH2 of Leucine), 39.20, and 39.17 (CH2 of phenyl alanine), 31.27 (γ-CH2 of Glutamic acid), 28.87, 28.83, and 28.81 (CH3 of t-Bu), 28.00, and 27.81 (β-CH2 of Glutamic acid), 25.95, 25.90, and 25.75 (CH of Leucine), 23.56, 23.52, and 23.26 (CH3 of Leucine), 22.50, and 22.43 (CH3 of acetyl), 21.99, 21.94, and 21.80 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{51}H_{78}N_7O_{13}$ $[M+H]^+$: 996.56521. Found 996.56521.



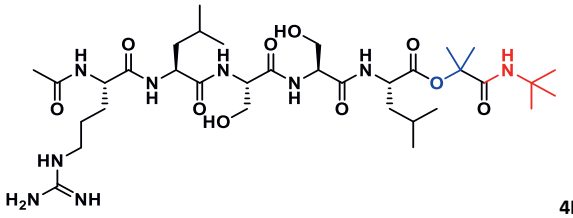
4Rba: 1-(tert-butylamino)-1-oxopropan-2-yl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using acetaldehyde and **3a** to provide the title compound as a white solid in 81% yield.

HPLC: Product is a set of two peaks with retention times 5.798 min, and 5.914 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 4.96 – 4.91 (m, 1H), 4.49 – 4.38 (m, 4H), 4.38 – 4.32 (m, 1H), 3.92 – 3.80 (m, 3H), 3.79 – 3.72 (m, 1H), 3.26 – 3.15 (m, 2H), 2.00 (s, 0.6H), 1.99 (s, 1.2H), 1.99 (s, 1.2H), 1.89 – 1.80 (m, 1H), 1.78 – 1.60 (m, 9H), 1.40 (dd, J = 7.9, 6.9 Hz, 3H), 1.35 (s, 0.9H), 1.34 (s, 4.5H), 1.33 (s, 3.6H), 1.00 – 0.94 (m, 6H), 0.94 – 0.89 (m, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.11, 175.06, 174.44, 174.43, 173.67, 173.65, 173.03, 172.95, 172.63, 172.45, 172.40, and 172.29 (CO of ester and amides), 158.61 (CN of guanidine), 72.65, and 72.39 (α-CH of ester), 63.03, 62.96, and 62.82 (CH2 of Serine), 57.20, 57.05, 56.82, 56.66, 54.42, 54.40, 53.64, 52.71, and 52.50 (α-CH of main-chain amides), 52.33, and 52.27 (quat. C of t-Bu), 42.04 (δ-CH2 of Arginine), 41.52, 41.50, 41.03, and 40.82 (CH2 of Leucine), 30.16, and 30.14 (β-CH2 of Arginine), 28.84, and 28.82 (CH3 of t-Bu), 26.23 (γ-CH2 of Arginine), 25.93, 25.88, and 25.86 (CH of Leucine), 23.56, 23.47, and 23.24 (CH3 of Leucine), 22.44 (CH3 of acetyl), 22.02, 21.77, 21.74, and 21.68 (CH3 of Leucine), 18.16, and 18.13 (β-CH3 of Alanine).

HRMS (ESI-TOF): m/z calculated for $C_{33}H_{62}N_9O_{10}$ $[M+H]^+$: 744.46142. Found 744.46144.



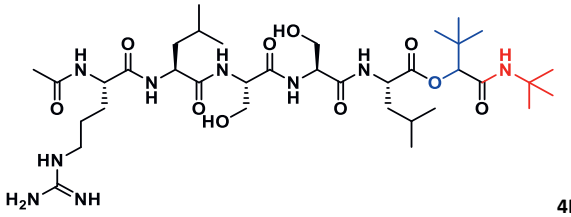
4Rca: 1-(tert-butylamino)-2-methyl-1-oxopropan-2-yl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using acetone and **3a** to provide the title compound as a white solid in 16% yield.

HPLC: Product is a single peak with retention time 6.021 min.

^{1}H-NMR: (600 MHz, Methanol-d_4) δ 4.45 (dd, J = 5.5, 4.1 Hz, 1H), 4.43 – 4.37 (m, 2H), 4.35 (dd, J = 7.5, 6.3 Hz, 1H), 4.32 (dd, J = 9.7, 5.5 Hz, 1H), 3.93 – 3.82 (m, 3H), 3.77 (dd, J = 11.0, 6.0 Hz, 1H), 3.21 (t, J = 7.0 Hz, 2H), 2.00 (s, 3H), 1.89 – 1.82 (m, 1H), 1.76 – 1.58 (m, 9H), 1.52 (s, 3H), 1.51 (s, 3H), 1.33 (s, 9H), 0.97 (d, J = 6.5 Hz, 6H), 0.92 (dd, J = 6.5, 3.5 Hz, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4) δ 175.12, 174.49, 174.31, 173.71, 172.53, 172.36, and 172.35 (CO of ester and amides), 158.60 (CN of guanidine), 82.63 (quat. α-C of ester), 62.89, and 62.77 (CH2 of Serine), 57.12, 56.90, 54.47, 53.71, 53.22 (α-CH of main-chain amides), 52.32 (quat. C of t-Bu), 42.00 (δ-CH2 of Arginine), 41.41, and 40.56 (CH2 of Leucine), 30.06 (β-CH2 of Arginine), 28.79 (CH3 of t-Bu), 26.22 (γ-CH2 of Arginine), 25.91, and 25.84 (CH of Leucine), 25.15, and 24.77 (β-CH3 of ester), 23.50, and 23.23 (CH3 of Leucine), 22.42 (CH3 of acetyl), 21.89, and 21.75 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{34}H_{64}N_9O_{10}$ $[M+H]^+$: 758.47707. Found 758.47755.



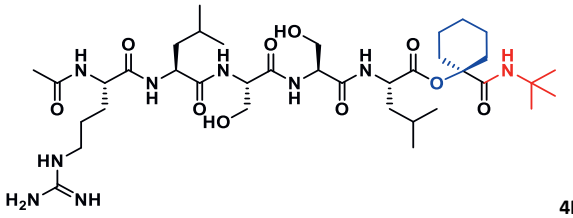
4Rda: 1-(tert-butylamino)-3,3-dimethyl-1-oxobutan-2-yl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using pivaldehyde and **3a** to provide the title compound as a white solid in 76% yield.

HPLC: Product is a set of two peaks with retention times 6.715 min, and 6.788 min. *Compound hydrolyzes in NMR solvent. Ratio product : hydrolysis = 1 : 1.7.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 4.57 (s, 0.1H), 4.56 (s, 0.4H), 4.54 (s, 0.1H), 4.54 (s, 0.4H), 4.52 – 4.32 (m, 5H), 3.94 – 3.86 (m, 2H), 3.86 – 3.80 (m, 1H), 3.79 – 3.73 (m, 1H), 3.20 (t, J = 7.0 Hz, 2H), 1.99 (s, 1.8H), 1.99 (s, 1.2H), 1.89 – 1.81 (m, 1H), 1.81 – 1.58 (m, 9H), 1.34 (s, 9H), 1.02 (s, 4.5H), 1.01 (s, 4.5H), 0.99 – 0.96 (m, 6H), 0.94 – 0.89 (m, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.14, 175.08, 174.47, 174.44, 173.69, 173.64, 173.20, 172.96, 172.48, 172.39, 172.31, 172.10, 170.02, and 169.93 (CO of ester and amides), 158.61 (CN of guanidine), 83.02, and 82.92 (α-CH of ester), 80.26 (α-CH of byproduct), 63.01, 62.94, and 62.88 (CH2 of Serine), 57.05, 56.92, 56.68, 54.42, 54.38, 53.71, 53.69, 52.82, and 52.68 (α-CH of main-chain amides), 52.62, and 51.73 (quat. C of main-chain t-Bu), 42.04 (δ-CH2 of Arginine), 41.49, 41.47, 41.23, and 41.01 (CH2 of Leucine), 35.80, 34.82, and 34.75 (quat. C of side-chain t-Bu), 30.15, and 30.12 (β-CH2 of Arginine), 28.97 (CH3 of main-chain *t*-Bu of byproduct), 28.91, and 28.87 (CH3 of main-chain t-Bu), 26.86, and 26.85 (CH3 of side-chain t-Bu), 26.72 (CH3 of side-chain t-Bu of byproduct), 26.23 (γ-CH2 of Arginine), 25.94, 25.89, and 25.80 (CH of Leucine), 23.57, 23.55, 23.34, and 23.33 (CH3 of Leucine), 22.45, and 22.44 (CH3 of acetyl), 22.00, 21.81, 21.79, and 21.74 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{36}H_{68}N_9O_{10}$ $[M+H]^+$: 786.50837. Found 786.50837.



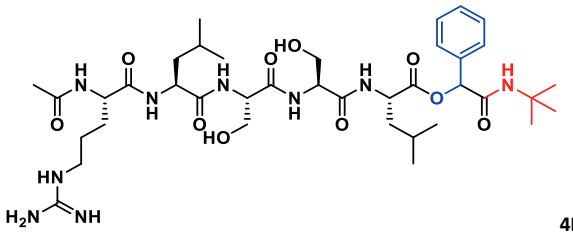
4Rea: 1-(tert-butylcarbamoyl)cyclohexyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using cyclohexanone and **3a** to provide the title compound as a white solid in 41% yield.

HPLC: Product is a single peak with retention time 6.628 min.

^{1}H-NMR: (600 MHz, Methanol-d_4) δ 4.45 (dd, J = 5.5, 3.9 Hz, 1H), 4.42 – 4.31 (m, 4H), 3.95 – 3.84 (m, 3H), 3.77 (dd, J = 11.0, 5.8 Hz, 1H), 3.21 (t, J = 7.0 Hz, 2H), 2.21 (d, J = 14.3 Hz, 1H), 2.14 (d, J = 13.7 Hz, 1H), 2.00 (d, J = 4.2 Hz, 3H), 1.89 – 1.81 (m, 1H), 1.80 – 1.56 (m, 15H), 1.50 – 1.40 (m, 1H), 1.31 (s, 9H), 1.30 – 1.23 (m, 1H), 1.01 – 0.96 (m, 6H), 0.96 – 0.90 (m, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4) δ 175.20, 174.61, 174.59, 173.77, 172.61, 172.39, and 172.24 (CO of ester and amides), 158.61 (CN of guanidine), 84.07 (quat. α-C of ester), 62.89, and 62.83 (CH2 of Serine), 57.19, 57.05, 54.52, 53.80, and 53.60 (α-CH of main-chain amides), 52.42 (quat. C of t-Bu), 42.03 (δ-CH2 of Arginine), 41.39, and 40.90 (CH2 of Leucine), 34.03, and 32.19 (CH2 of cyclohexyl), 30.09 (β-CH2 of Arginine), 28.86 (CH3 of t-Bu), 26.28, and 26.25 (γ-CH2 of Arginine, CH2 of cyclohexyl), 25.95, and 25.92 (CH of Leucine), 23.54, and 23.32 (CH3 of Leucine), 22.60 (CH2 of cyclohexyl), 22.45 (CH3 of acetyl), 22.44 (CH2 of cyclohexyl), 21.80, and 21.79 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{37}H_{68}N_9O_{10}$ $[M+H]^+$: 798.50837. Found 798.50837.



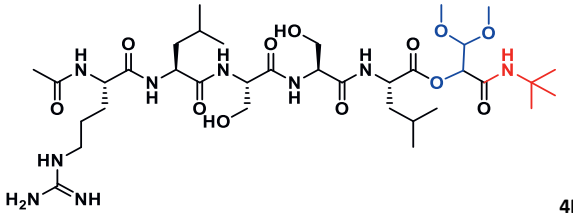
4Rfa: 2-(tert-butylamino)-2-oxo-1-phenylethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using benzaldehyde and **3a** to provide the title compound as a white solid in 24% yield.

HPLC: Product is a single peak with retention time 6.705 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 7.51 – 7.43 (m, 2H), 7.41 – 7.33 (m, 3H), 5.88 (s, 0.1H), 5.87 (s, 0.4H), 5.86 (s, 0.1H), 5.86 (s, 0.4H), 4.56 – 4.50 (m, 1H), 4.50 – 4.38 (m, 3H), 4.38 – 4.31 (m, 1H), 3.92 – 3.78 (m, 3H), 3.78 – 3.70 (m, 1H), 3.22 – 3.17 (m, 2H), 1.99 (s, 1.8H), 1.99 (s, 1.2H), 1.88 – 1.80 (m, 1H), 1.77 – 1.61 (m, 9H), 1.33 (s, 0.9H), 1.32 (s, 4.5H), 1.31 (s, 3.6H), 0.98 – 0.95 (m, 4H), 0.95 – 0.87 (m, 8H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.12, 175.04, 174.41, 174.38, 173.66, 173.62, 172.74, 172.67, 172.58, 172.43, 172.37, 172.28, 170.19, 170.11, and 170.04 (CO of ester and amides), 158.60 (CN of guanidine), 136.96, and 136.90 (quat. C1 of phenyl ring), 129.98, 129.96, 129.70, 129.68, 128.64, and 128.54 (C2-6H of phenyl ring), 77.55, 77.52, 77.42, and 77.38 (quat. α-C of ester), 63.03, 62.93, 62.86, and 62.85 (CH2 of Serine), 57.16, 56.98, 56.83, 56.64, 54.39, 54.37, 53.66, and 53.63 (α-CH of main-chain amides), 52.73 (quat. C of t-Bu), 52.66, and 52.60 (α-CH of main-chain amides), 42.04 (δ-CH2 of Arginine), 41.52, 41.51, 41.04, and 40.85 (CH2 of Leucine), 30.17, and 30.15 (β-CH2 of Arginine), 28.83, 28.81, 28.80, and 28.78 (CH3 of t-Bu), 26.22 (γ-CH2 of Arginine, 25.94, 25.86, and 25.80 (CH of Leucine), 23.57, 23.55, 23.39, and 23.22 (CH3 of Leucine), 22.45, and 22.44 (CH3 of acetyl), 22.00, 21.78, 21.75, and 21.74 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{38}H_{64}N_9O_{10}$ $[M+H]^+$: 806.47707. Found 806.47707.



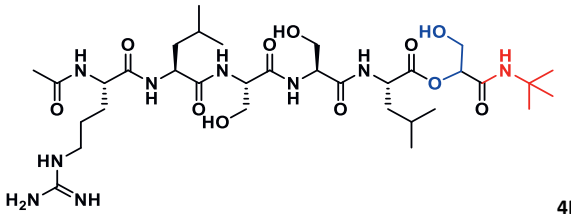
4Rga: 1-(tert-butylamino)-3,3-dimethoxy-1-oxopropan-2-yl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using 2,2-dimethoxyacetaldehyde and **3a** to provide the title compound as a white solid in 43% yield.

HPLC: Product is a set of two peaks with retention times 5.633 min, and 5.856 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 4.98 (d, J = 5.8 Hz, 1H), 4.59 (d, J = 5.8 Hz, 1H), 4.50 – 4.38 (m, 4H), 4.36 (t, J = 6.9 Hz, 1H), 3.92 – 3.81 (m, 3H), 3.77 (dd, J = 11.0, 5.9 Hz, 1H), 3.42 (s, 3H), 3.41 (s, 3H), 3.20 (td, J = 7.0, 2.8 Hz, 2H), 1.99 (s, 3H), 1.88 – 1.81 (m, 1H), 1.77 – 1.61 (m, 9H), 1.33 (s, 9H), 0.97 (dd, J = 6.5, 1.4 Hz, 6H), 0.92 (dd, J = 6.4, 3.8 Hz, 6H).

^{13}C-NMR: (176 MHz, Methanol-d_4) δ 175.04, 174.32, 173.60, 172.51, 172.46, 172.34, and 168.17 (CO of ester and amides), 158.64 (CN of guanidine), 104.38 (CH of acetal), 75.00 (α-CH of ester), 63.01, and 62.95 (CH2 of Serine), 56.99, and 56.76 (α-CH of main-chain amides), 56.49, and 55.12 (CH3 of acetal), 54.33, 53.60, 52.56 (α-CH of main-chain amides), 52.54 (quat. C of t-Bu), 42.07 (α-CH2 of guanidine), 41.58, and 40.97 (CH2 of Leucine), 30.20 (β-CH2 of Arginine), 28.83 (CH3 of t-Bu), 26.21 (γ-CH2 of Arginine), 25.96, and 25.87 (CH of Leucine), 23.56, and 23.34 (CH3 of Leucine), 22.45 (CH3 of acetyl), 21.83, and 21.80 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{35}H_{66}N_9O_{12}$ $[M+H]^+$: 804.48255. Found 804.48255.



4Rha: 1-(tert-butylamino)-3-hydroxy-1-oxopropan-2-yl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using glycolaldehyde dimer (5 equiv) and **3a** to provide the title compound as a white solid in 46% yield.

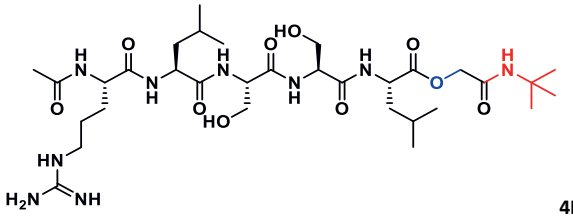
HPLC: Product is a set of two peaks with retention times 5.449 min, and 5.560 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 4.54 – 4.13 (m, 8H), 3.94 – 3.86 (m, 2H), 3.86 – 3.81 (m, 1H), 3.80 – 3.74 (m, 1H), 3.25 – 3.17 (m, 2H), 1.99 (s, 3H), 1.89 – 1.81 (m, 1H), 1.76 – 1.61 (m, 9H), 1.39 – 1.34 (m, 9H), 0.99 – 0.89 (m, 12H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers; approx. 0.2 : 0.8 ratio of rotamers for each diastereomer) δ 175.09, 175.07, 174.43, 173.71, 173.64, 172.55, 172.52, 172.34, and 172.33 (CO of ester and amides), 158.60 (CN of guanidine), 71.59, and 71.46 (α-CH of ester), 68.12, and 68.06 (β-CH2 of Serine-ester), 63.03, 63.01, and 62.73 (CH2 of Serine), 57.25, 56.70, 56.67, 54.39, 54.38, 53.68, 53.65, 52.44, and 52.30 (α-CH of main-chain amides), 52.10 (quat. C of t-Bu), 42.05 (δ-CH2 of Arginine), 41.53, 41.34, and 41.25 (CH2 of Leucine), 30.19, and 30.18 (β-CH2 of Arginine), 28.95 (CH3 of t-Bu), 26.24 (γ-CH2 of Arginine), 25.94, 25.87, and 25.85 (CH

of Leucine), 23.56, 23.52, and 23.47 (CH3 of Leucine), 22.43 (CH3 of acetyl), 21.85, 21.76, and 21.75 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{33}H_{62}N_9O_{11}$ $[M+H]^+$: 760.45633. Found 760.45633.



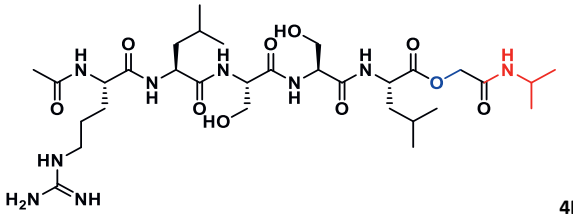
4Ria: 2-(tert-butylamino)-2-oxoethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using formaldehyde and **3** to provide the title compound as a white solid in 54% yield.

HPLC: Product is a single peak with retention time 5.599 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 0.2 : 0.8 ratio of rotamers) δ 4.53 – 4.41 (m, 5H), 4.43 – 4.37 (m, 1H), 4.38 – 4.32 (m, 1H), 3.93 – 3.80 (m, 3H), 3.77 (dd, J = 10.9, 6.1 Hz, 1H), 3.25 (t, J = 7.6 Hz, 0.4H), 3.21 (td, J = 7.0, 1.8 Hz, 1.6H), 2.00 (s, 3H), 1.90 – 1.81 (m, 1H), 1.78 – 1.60 (m, 9H), 1.37 (s, 1.8H), 1.35 (s, 7.2H), 1.00 – 0.94 (m, 6H), 0.94 – 0.89 (m, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 0.2 : 0.8 ratio of rotamers) δ 175.05, 174.44, 173.66, 173.14, 172.48, 172.43, and 168.71 (CO of ester and amides), 158.60 (CN of guanidine), 64.14 (α-CH2 of ester), 62.95, 62.73 (CH2 of Serine), 57.13, 56.73, 54.44, 53.66, and 52.53 (α-CH of main-chain amides), 52.43 (quat. C of t-Bu), 42.00 (δ-CH2 of Arginine), 41.44, 40.95 (CH2 of Leucine), 30.08 (β-CH2 of Arginine), 28.92, and 28.88 (CH3 of t-Bu), 26.20 (γ-CH2 of Arginine), 25.90, 25.82 (CH of Leucine), 23.49, 23.33, (CH3 of Leucine), 22.41 (CH3 of acetyl), 21.80, and 21.73 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{32}H_{60}N_9O_{10}$ $[M+H]^+$: 730.44577. Found 730.44577.



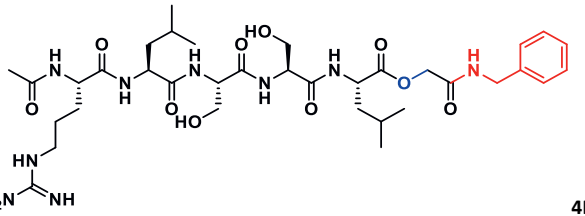
4Rib: 2-(isopropylamino)-2-oxoethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using formaldehyde and isopropyl isocyanide to provide the title compound as a white solid in 74% yield.

HPLC: Product is a single peak with retention time 5.220 min.

^{1}H-NMR: (600 MHz, Methanol-d_4) δ 4.53 (s, 2H), 4.48 (dd, J = 9.2, 5.7 Hz, 1H), 4.46 – 4.41 (m, 2H), 4.41 – 4.37 (m, 1H), 4.37 – 4.31 (m, 1H), 4.01 (hept, J = 6.6 Hz, 1H), 3.93 – 3.86 (m, 2H), 3.84 (dd, J = 11.3, 4.2 Hz, 1H), 3.76 (dd, J = 10.9, 6.1 Hz, 1H), 3.21 (td, J = 7.0, 2.2 Hz, 2H), 1.99 (s, 3H), 1.89 – 1.81 (m, 1H), 1.77 – 1.59 (m, 9H), 1.17 (dd, J = 6.6, 3.7 Hz, 6H), 0.97 (dd, J = 6.4, 1.7 Hz, 6H), 0.92 (d, J = 6.3 Hz, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4) δ 175.12, 174.50, 173.70, 173.21, 172.63, 172.50, and 168.64 (CO of ester and amides), 158.61 (CN of guanidine), 63.95 (α-CH2 of ester), 62.98, and 62.71 (CH2 of Serine), 57.20, 56.79, 54.46, 53.67, and 52.65 (α-CH of main-chain amides), 42.69 (CH of isopropyl), 42.03 (δ-CH2 of Arginine), 41.48, and 40.90 (CH2 of Leucine), 30.13 (β-CH2 of Arginine), 26.25 (γ-CH2 of Arginine), 25.93, and 25.85 (CH of Leucine), 23.55, and 23.35 (CH3 of Leucine), 22.51, and 22.49 (CH3 of isopropyl), 22.44 (CH3 of acetyl), 21.85, and 21.75 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{31}H_{58}N_9O_{10}$ $[M+H]^+$: 716.43012. Found 716.43013.



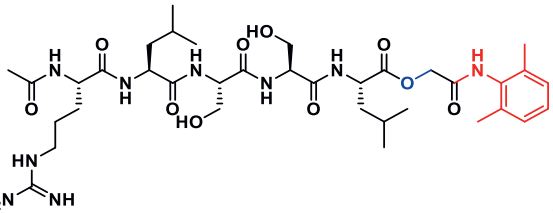
4Ric: 2-(benzylamino)-2-oxoethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate: The product was synthesized following GPB using formaldehyde and benzyl isocyanide to provide the title compound as a white solid in 16% yield.

HPLC: Product is a single peak with retention time 5.943 min.

^{1}H-NMR: (600 MHz, Methanol-d_4) δ 7.33 – 7.26 (m, 4H), 7.26 – 7.20 (m, 1H), 4.65 – 4.60 (m, 2H), 4.55 – 4.48 (m, 1H), 4.48 – 4.41 (m, 2H), 4.41 – 4.31 (m, 4H), 3.91 – 3.83 (m, 2H), 3.79 (dd, J = 11.3, 3.9 Hz, 1H), 3.73 (dd, J = 11.0, 5.9 Hz, 1H), 3.19 (d, J = 7.8 Hz, 2H), 1.99 (s, 3H), 1.89 – 1.80 (m, 1H), 1.78 – 1.56 (m, 9H), 0.95 (d, J = 6.1 Hz, 6H), 0.91 (d, J = 6.2 Hz, 6H).

^{13}C-NMR: (151 MHz, Methanol-d_4) δ 175.27, 174.56, 173.77, 173.05, 172.58, and 169.65 (CO of ester and amides), 158.60 (CN of guanidine), 139.68 (quaternary C1 of phenyl), 129.59, 128.57, and 128.30 (C2-6 of phenyl), 64.11 (α-CH2 of ester), 62.87, and 62.64 (CH2 of Serine), 57.23, 56.97, 54.52, 53.77, and 52.57 (α-CH of main-chain amides), 43.82 (α-CH2 of phenyl), 42.02 (δ-CH2 of Arginine), 41.40, and 40.96 (CH2 of Leucine), 30.07 (β-CH2 of Arginine), 26.26 (γ-CH2 of Arginine), 25.94, and 25.82 (CH of Leucine), 23.53, and 23.40 (CH3 of Leucine), 22.45 (CH3 of acetyl), 21.79, and 21.75 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{35}H_{58}N_9O_{10}$ $[M+H]^+$: 764.43012. Found 764.43043.



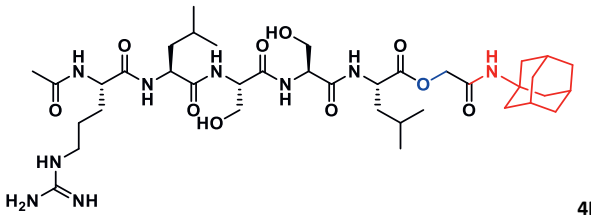
4Rid: 2-((2,6-dimethylphenyl)amino)-2-oxoethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-Lleucinate: The product was synthesized following GPB using formaldehyde and 2,6- dimethylphenylisocyanide to provide the title compound as a white solid in 27% yield.

HPLC: Product is a single peak with retention time 5.977 min.

^{1}H-NMR: (600 MHz, Methanol-d_4) δ 7.14 – 7.03 (m, 3H), 4.82 (d, J = 14.9 Hz, 1H), 4.78 (d, J = 15.3 Hz, 1H), 4.61 – 4.54 (m, 1H), 4.46 – 4.41 (m, 1H), 4.38 – 4.30 (m, 3H), 3.92 – 3.78 (m, 3H), 3.76 – 3.69 (m, 1H), 3.21 – 3.15 (m, 2H), 2.20 (s, 6H), 1.99 (s, 3H), 1.89 – 1.80 (m, 1H), 1.80 – 1.53 (m, 9H), 0.99 – 0.88 (m, 12H).

^{13}C-NMR: (151 MHz, Methanol-d_4) δ 175.32, 174.63, 173.76, 173.21, 172.57, 172.48, and 168.71 CO of ester and amides), 158.55 (CN of guanidine), 137.12, and 134.56 (quat. C1-2,6 of phenyl), 129.11, and 128.68 (C3-5H of phenyl), 64.10 (α-CH2 of ester), 62.74, and 62.67 (CH2 of Serine), 57.28, 57.04, 54.53, 53.87, and 52.49 (α-CH of main-chain amides), 41.97 (δ-CH2 of Arginine), 41.24, and 41.05 (CH2 of Leucine), 30.00 (β-CH2 of Arginine), 26.22 (γ-CH2 of Arginine), 25.87, and 25.77 (CH of Leucine), 23.48, and 23.38 (CH3 of Leucine), 22.40 (CH3 of acetyl), 21.75, and 21.69 (CH3 of Leucine), 18.41 (CH3 on phenyl ring).

HRMS (ESI-TOF): m/z calculated for $C_{36}H_{60}N_9O_{10}$ $[M+H]^+$: 778.44577. Found 778.44662.



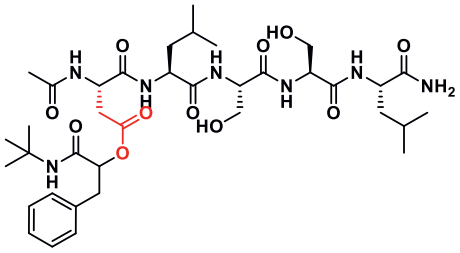
4Rie: 2-(((3R,5R,7R)-adamantan-1-yl)amino)-2-oxoethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-Lleucinate: The product was synthesized following GPB using formaldehyde and 1-adamantyl isocyanide to provide the title compound as a white solid in 12% yield.

HPLC: Product is a single peak with retention time 6.982 min.

^{1}H-NMR: (600 MHz, Methanol-d_4, approx. 0.2 : 0.8 ratio of rotamers) δ 4.49 – 4.42 (m, 3H), 4.40 (t, J = 7.6 Hz, 1H), 4.38 – 4.33 (m, 1H), 3.93 – 3.79 (m, 5H), 3.76 (dd, J = 11.0, 6.0 Hz, 1H), 3.27 – 3.17 (m, 2H), 2.07 (s, 3H), 2.04 (s, 6H), 1.99 (s, 3H), 1.89 – 1.82 (m, 1H), 1.77 – 1.57 (m, 15H), 0.97 (d, J = 6.6 Hz, 3H), 0.95 (d, J = 6.5 Hz, 3H), 0.93 (d, J = 6.5 Hz, 3H), 0.91 (d, J = 6.5 Hz, 3H).

^{13}C-NMR: (151 MHz, Methanol-d_4, approx. 0.2 : 0.8 ratio of rotamers) δ 175.00, 174.55, 174.50, 174.39, 173.60, 172.38, and 172.27 (CO of ester and amides), 158.59 (CN of guanidine), 62.98, and 62.78 (CH2 of Serine), 57.03, 56.66, 54.36, 53.66, 52.35 (α-CH of main-chain amides), 42.44 (CH2's of adamantyl), 42.02 (α-CH2 of guanidine), 41.48, 41.34; 37.39 (CH2's of adamantyl), 30.91 (CH's of adamantyl), 30.13, 26.18, 25.90, 25.86; 23.49, and 23.31 (CH3 of Leucine), 22.40 (CH3 of acetyl), 21.83, and 21.72 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{38}H_{66}N_9O_{10}$ $[M+H]^+$: 808.49272. Found 808.49252.



10Daa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl (3S,6S,9S,12S,15S)-3-acetamido-15-carbamoyl-9,12-bis(hydroxymethyl)-6-isobutyl-17-methyl-4,7,10,13-

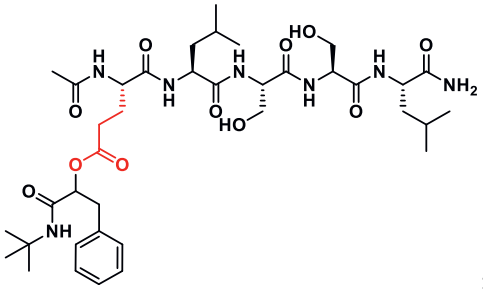
tetraoxo-5,8,11,14- tetraazaoctadecanoate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 13% yield.

HPLC: Product is a double peak with retention time 8.330 and 8.389 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 7.29 – 7.21 (m, 5H), 5.12 (dd, J = 6.7, 6.7 Hz, 0.5H), 5.09 (dd, J = 7.3, 6.2 Hz, 0.5H), 4.74 (dd, J = 6.9, 6.9 Hz, 0.5H), 4.67 (dd, J = 7.3, 6.0 Hz, 0.5H), 4.39 – 4.28 (m, 4H), 3.94 – 3.84 (m, 2H), 3.84 – 3.78 (m, 1.5H), 3.76 (dd, J = 11.2, 4.4 Hz, 0.5H), 3.08 – 3.04 (m, 2H), 2.97 (dd, J = 16.7, 6.0 Hz, 0.5H), 2.91 (dd, J = 16.8, 7.1 Hz, 0.5H), 2.81 (dd, J = 16.8, 6.9 Hz, 0.5H), 2.76 (dd, J = 16.7, 7.3 Hz, 0.5H), 1.96 (s, 3H), 1.75 – 1.68 (m, 2H), 1.68 – 1.60 (m, 4H), 1.25 (s, 4.5H), 1.25 (s, 4.5H), 0.95 (dd, J = 6.6, 1.4 Hz, 3H), 0.94 (dd, J = 6.6, 3.6 Hz, 3H), 0.91 – 0.87 (m, 6H).

^{13}C-NMR: (176 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 177.88, 177.83, 175.31, 175.27, 173.73, 173.62, 173.57, 173.53, 173.22, 173.19, 172.54, 172.50, 171.55, 171.43, 170.99, and 170.83 (CO of ester and amides), 137.39 (quaternary C1 of Ph), 130.83, 130.81, 129.49, 129.48, 128.00, and 127.99 (C2-6H of Phenyl), 76.65, and 76.55 (α-CH of ester), 62.69, 62.66, 62.64, and 62.47 (CH2 of Serine), 57.94, 57.91, 57.61, 57.58, 54.26, 54.22, 53.52, and 53.49 (α-CH of main-chain amides), 52.51, and 52.50 (quat. C of t-Bu), 51.51, and 51.23 (α-CH of Aspartate), 41.51, 41.47, 41.25, and 41.23 (CH2 of Leucine), 39.12, and 39.07 (CH2 of benzyl), 36.55, and 36.53 (CH2 of Aspartate), 28.81, and 28.80 (CH3 of t-Bu), 25.90, and 25.87 (CH of Leucine), 23.77, 23.75, and 23.50 (CH3 of Leucine), 22.55, and 22.51 (CH3 of acetyl), 21.90, 21.85, 21.53, and 21.51 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{37}H_{60}N_7O_{11}$ $[M+H]^+$: 778.43453. Found 778.43488.



10Eaa: 1-(tert-butylamino)-1-oxo-3-phenylpropan-2-yl (4S,7S,10S,13S,16S)-16-acetamido-4- carbamoyl-7,10-bis(hydroxymethyl)-13-isobutyl-2-methyl-6,9,12,15-tetraoxo-5,8,11,14- tetraazanonadecan-19-oate: The product was synthesized following GPB using **2a** and **3a** to provide the title compound as a white solid in 21% yield.

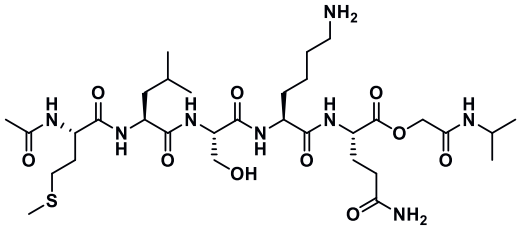
HPLC: Product is a single peak with retention time 10.897 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 7.29 – 7.23 (m, 4H), 7.23 – 7.19 (m, 1H), 5.10 – 5.06 (m, 1H), 4.41 – 4.31 (m, 4.5H), 4.27 (dd, J = 8.3, 6.1 Hz, 0.5H), 3.95 – 3.85 (m, 2H), 3.83 – 3.75 (m, 2H), 3.08 – 3.02 (m, 2H), 2.55 – 2.45 (m, 1H), 2.46 – 2.38 (m, 1H), 2.11 – 2.03 (m, 1H), 1.99 (s, 1.5H), 1.97 (s, 1.5H), 1.95 – 1.87 (m, 1H), 1.78 – 1.68 (m, 2H), 1.68 – 1.59 (m, 4H), 1.25 (s, 9H), 0.97 (d, J = 1.3 Hz, 3H), 0.94 (dd, J = 6.6, 1.3 Hz, 3H), 0.92 (d, J = 6.5 Hz, 3H), 0.89 (d, J = 1.1 Hz, 3H).

^{13}C-NMR: (176 MHz, Methanol-d_4, approx. 1:1 ratio of diastereomers) δ 177.87, 175.22, 175.20, 174.62, 174.56, 173.88, 173.76, 173.53, 173.49, 173.27, 172.56, 172.48, 171.22, 171.17, 171.14, and 171.09 (CO of ester and amides), 137.56 (quaternary C1 of Phenyl), 130.78, 129.46, 129.45, and 127.95 (C2-6H of Phenyl), 76.23, and 76.21 (α-CH of ester), 62.76, 62.75, 62.51, and 62.49 (CH2 of Serine), 57.89, 57.88, 57.32, 54.44, 54.28, 53.94, 53.91,

53.49, and 53.39 (α-CH of main-chain amides), 52.41, and 52.30 (quat. C of t-Bu), 41.49, 41.44, 41.21, and 41.20 (CH2 of Leucine), 39.18 (CH2 of benzyl), 31.26 (γ-CH2 of Aspartate), 28.80, and 28.78 (CH3 of t-Bu), 28.01, and 27.85 (β-CH2 of Aspartate), 25.91, and 25.87 (CH of Leucine), 23.76, 23.54, and 23.50 (CH3 of Leucine), 22.49, and 22.43 (CH3 of acetyl), 21.92, 21.88, and 21.53 (CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{38}H_{62}N_7O_{11}$ $[M+H]^+$: 792.45018. Found 792.45128.



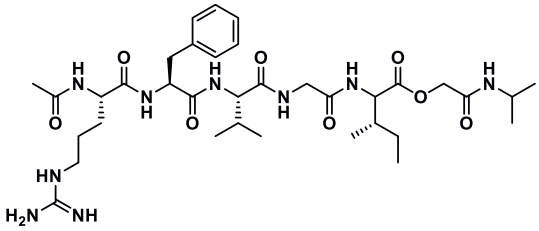
11ib: 2-(isopropylamino)-2-oxoethyl acetyl-L-methionyl-L-leucyl-L-seryl-L-lysyl-L-glutaminate: The product was synthesized following GPB using **2i** and **3b** to provide the title compound as a white solid in 67% yield.

HPLC: Product is a single peak with retention time 4.760 min.

^{1}H-NMR: (701 MHz, Methanol-d_4) δ 4.55 (s, 2H), 4.44 (dd, J = 8.7, 5.5 Hz, 1H), 4.41 – 4.33 (m, 4H), 4.01 (ddd, J = 12.6, 7.4, 6.3 Hz, 1H), 3.89 (dd, J = 11.0, 5.4 Hz, 1H), 3.80 (dd, J = 11.0, 5.7 Hz, 1H), 2.97 – 2.89 (m, 2H), 2.61 – 2.51 (m, 2H), 2.36 (t, J = 7.7 Hz, 2H), 2.25 – 2.19 (m, 1H), 2.09 (s, 3H), 2.08 – 2.02 (m, 2H), 2.01 (s, 3H), 1.98 – 1.91 (m, 2H), 1.79 – 1.73 (m, 1H), 1.73 – 1.61 (m, 5H), 1.54 – 1.45 (m, 2H), 1.17 (dd, J = 6.6, 4.3 Hz, 6H), 0.97 (d, J = 6.5 Hz, 3H), 0.92 (d, J = 6.5 Hz, 3H).

^{13}C-NMR: (176 MHz, Methanol-d_4) δ 177.56, 175.17, 174.76, 174.27, 174.25, 172.62, 172.34, 168.63, and 168.55 (CO of ester and amides), 64.03 (α-CH2 of ester), 62.81 (CH2 of Serine), 57.19, 54.82, 54.29, 53.78, and 53.67 (α-CH of main-chain amides), 42.69 (CH of isopropyl), 41.12 (β-CH2 of Leucine), 40.66 (ε-CH2 of Lysine), 32.39 (γ-CH2 of Glutamine), 32.28 (β-CH2 of Methionine), 31.75 (β-CH2 of Lysine), 31.12 (γ-CH2 of Methionine), 27.97 (β-CH2 of Glutamine and δ-CH2 of Lysine), 25.99 (γ-CH of Leucine), 23.65 (γ-CH2 of Lysine), 23.56 (ε-CH3 of Leucine), 22.58, 22.55, and 22.52 (CH3 of acetyl and CH3 of isopropyl), 21.80 (CH3 of Leucine), 15.30 (ε-CH3 of Methionine).

HRMS (ESI-TOF): m/z calculated for $C_{32}H_{59}N_8O_{10}S$ $[M+H]^+$: 747.40694. Found 747.40716.



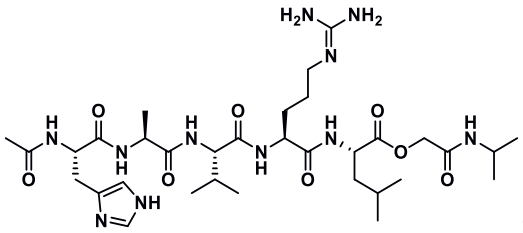
12ib: 2-(isopropylamino)-2-oxoethyl (6S,9S,12S)-6-acetamido-1,1-diamino-9-benzyl-18-((S)-secbutyl)-12-isopropyl-7,10,13,16-tetraoxo-2,8,11,14,17-pentaazanonadec-1-en-19-oate: The product was synthesized following GPB using **2i** and **3b** to provide the title compound as a white solid in 41% yield.

HPLC: Product is a single peak with retention time 5.427 min.

^{1}H-NMR: (701 MHz, Methanol-d_4) δ 7.28 – 7.18 (m, 5H), 4.68 (dd, J = 9.0, 5.7 Hz, 1H), 4.58 (d, J = 14.7 Hz, 1H), 4.50 (d, J = 14.8 Hz, 1H), 4.43 (d, J = 6.2 Hz, 1H), 4.30 (dd, J = 7.8, 6.4 Hz, 1H), 4.16 (d, J = 6.9 Hz, 1H), 4.00 (hept, J = 6.6 Hz, 1H), 3.90 (s, 2H), 3.18 – 3.11 (m, 3H), 2.94 (dd, J = 14.0, 9.0 Hz, 1H), 2.11 (hept, J = 6.9 Hz, 1H), 1.99 – 1.94 (m, 1H), 1.94 (s, 3H), 1.75 – 1.68 (m, 1H), 1.64 – 1.49 (m, 4H), 1.31 – 1.24 (m, 1H), 1.15 (dd, J = 11.1, 6.6 Hz, 6H), 0.98 (d, J = 6.8 Hz, 3H), 0.97 – 0.92 (m, 9H).

^{13}C-NMR: (176 MHz, Methanol-d_4) δ 173.79, 173.71, 173.63, 173.48, 172.37, 171.56, and 168.52 (CO of ester and amides), 158.63 (CN of Arginine), 138.27 (quaternary C1 of Ph), 130.43, 129.57, and 127.89 (C2- 6H of Phenyl), 63.84 (α-CH2 of ester), 60.48, 58.58, 55.95, and 54.23 (α-CH of main-chain amides R, F, V, I), 43.26 (α-CH2 of Glycine), 42.67 (CH of isopropyl), 41.98 (δ-CH2 of Arginine), 38.57 (β-CH2 of Phenylalanine), 38.13 (β-CH of Isoleucine), 31.88 (β-CH of Valine), 29.99, 26.33, and 26.12 (β,γ-CH2 of Arginine and γ-CH2 of Isoleucine), 22.53, and 22.51 (CH3 of acetyl and isopropyl), 19.81, 18.78, 15.91, and 11.71 (CH3 of Valine and Isoleucine).

HRMS (ESI-TOF): m/z calculated for $C_{35}H_{58}N_9O_8$ $[M+H]^+$: 732.44029. Found 732.44124.



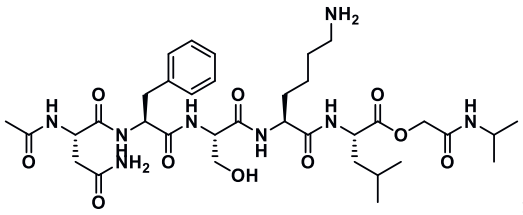
13ib: 2-(isopropylamino)-2-oxoethyl ((S)-2-((S)-2-((S)-2-((S)-2-acetamido-3-(1H-imidazol-4-yl)propanamido)propanamido)-3-methylbutanamido)-5-((diaminomethylene)amino)pentanoyl)-Lleucinate: The product was synthesized following GPB using **2i** and **3b** to provide the title compound as a white solid in 64% yield.

HPLC: Product is a single peak with retention time 4.677 min.

^{1}H-NMR: (701 MHz, Methanol-d_4, approx. 0.2 : 0.8 ratio of rotamers) δ 8.77 – 8.71 (m, 1H), 7.33 (m, 1H), 4.73 – 4.70 (m, 0.2H), 4.69 (dd, J = 7.0, 6.1 Hz, 0.8H), 4.59 – 4.55 (m, 1H), 4.54 – 4.49 (m, 2H), 4.44 – 4.40 (m, 1H), 4.38 (q, J = 7.2 Hz, 1H), 4.20 (d, J = 7.2 Hz, 0.8H), 4.18 (d, J = 6.7 Hz, 0.2H), 4.00 (hept, J = 6.6 Hz, 1H), 3.25 – 3.17 (m, 3H), 3.11 – 3.04 (m, 1H), 2.13 – 2.04 (m, 1H), 1.97 (s, 0.6H), 1.96 (s, 2.4H), 1.89 – 1.83 (m, 1H), 1.77 – 1.62 (m, 6H), 1.38 (d, J = 7.2 Hz, 2.76H), 1.33 (d, J = 7.2 Hz, 0.24H), 1.16 (dd, J = 8.6, 6.6 Hz, 6H), 0.99 – 0.94 (m, 9H), 0.92 (d, J = 6.1 Hz, 3H).

^{13}C-NMR: (176 MHz, Methanol-d_4, approx. 0.2 : 0.8 ratio of rotamers) δ 175.54, 173.76, 173.56, 173.39, 172.01, and 168.51 (CO of ester and amides), 158.67 (CN of Arginine), 135.11(CH of imidazole), 131.06 (quaternary C1 of Ph), 118.76 (CH of imidazole), 63.98 (α-CH2 of ester), 60.37, 60.33 , 53.78, 53.40, and 52.35, 50.77 (α-CH of main-chain amides, 42.67 (CH of isopropyl), 42.11, and 42.07 (δ-CH2 of Arginine), 41.06 (β-CH2 of Leucine), 32.15, and 32.08 (β-CH of Valine), 30.43 (β-CH2 of Arginine), 28.42 (β-CH2 of Histidine), 26.19, 26.14, and 25.92 (γ-CH2 of Arginine and γ-CH of Ieucine), 23.36 (δ-CH3 of Leucine), 22.56, and 22.51 (CH3 of acetyl and isopropyl), 21.76, 19.82, 19.72, 18.82, and 18.61 (CH3 of Valine and Leucine), 17.90 (β-CH3 of Alanine).

HRMS (ESI-TOF): m/z calculated for $C_{33}H_{58}N_{11}O_8$ $[M+H]^+$: 736.44643. Found 736.44948.



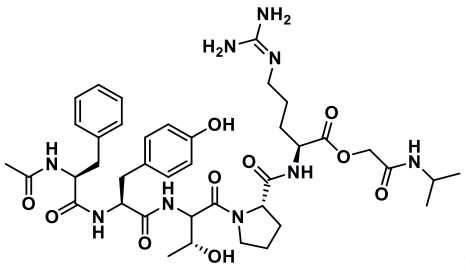
14ib: 2-(isopropylamino)-2-oxoethyl acetyl-L-asparaginyl-L-phenylalanyl-L-seryl-L-lysyl-Lleucinate: The product was synthesized following GPB using **2i** and **3b** to provide the title compound as a white solid in 79% yield.

HPLC: Product is a single peak with retention time 5.232 min.

^{1}H-NMR: (701 MHz, Methanol-d_4) δ 7.30 – 7.25 (m, 2H), 7.25 – 7.19 (m, 3H), 4.64 (dd, J = 7.9, 6.0 Hz, 1H), 4.57 – 4.53 (m, 2H), 4.51 (d, J = 14.8 Hz, 1H), 4.46 (dd, J = 9.5, 5.4 Hz, 1H), 4.40 (dd, J = 10.0, 4.6 Hz, 1H), 4.30 (t, J = 5.4 Hz, 1H), 4.01 (hept, J = 6.6 Hz, 1H), 3.90 (dd, J = 11.4, 5.8 Hz, 1H), 3.83 (dd, J = 11.4, 5.0 Hz, 1H), 3.28 (dd, J = 14.3, 4.5 Hz, 1H), 2.96 (dd, J = 14.2, 9.6 Hz, 1H), 2.93 – 2.87 (m, 2H), 2.74 (dd, J = 15.7, 7.9 Hz, 1H), 2.55 (dd, J = 15.7, 6.0 Hz, 1H), 2.01 – 1.94 (m, 1H), 1.79 – 1.60 (m, 6H), 1.53 – 1.42 (m, 2H), 1.17 (dd, J = 7.8, 6.6 Hz, 6H), 0.97 (d, J = 6.2 Hz, 3H), 0.92 (d, J = 6.2 Hz, 3H).

^{13}C-NMR: (176 MHz, Methanol-d_4) δ 175.02, 174.35, 174.12, 173.82, 173.41, 173.32, 172.59, and 168.61 (CO of ester and amides), 138.46 (quaternary C1 of Ph), 130.35, 129.60, and 127.91 (C2-6H of Phenyl), 63.96 (α-CH2 of ester), 62.69 (β-CH2 of Serine), 58.13, 56.62, 54.09, 52.63, and 51.62 (α-CH of main-chain amides), 42.68 (CH of isopropyl), 40.90 (β-CH2 of Leucine), 40.69 (ε-CH2 of Lysine), 37.71 (β-CH2 of Phenylalanine), 37.29 (β-CH2 of Asparagine), 31.63, and 27.81 (β,δ-CH2 of Lysine), 25.89 (γ-CH2 of Leucine), 23.64 (γ-CH2 of Lysine), 23.38 (δ-CH3 of Leucine), 22.56, and 22.52 (CH3 of acetyl and isopropyl), 21.81 (δ-CH3 of Leucine).

HRMS (ESI-TOF): m/z calculated for $C_{35}H_{57}N_8O_{10}$ $[M+H]^+$: 749.41922. Found 749.41857.



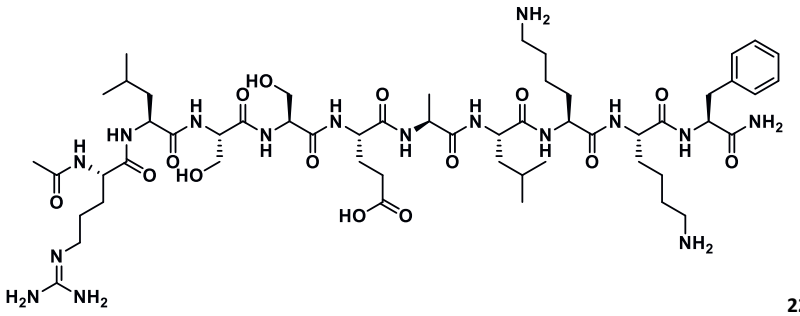
15ib: 2-(isopropylamino)-2-oxoethyl (2S)-2-((2S)-1-((3R)-2-((S)-2-((S)-2-acetamido-3-phenylpropanamido)-3-(4-hydroxyphenyl)propanamido)-3-hydroxybutanoyl)pyrrolidine-2- carboxamido)-5-((diaminomethylene)amino)pentanoate: The product was synthesized following GPB using **2i** and **3b** to provide the title compound as a white solid in 52% yield.

HPLC: Product is a single peak with retention time 5.147 min.

^{1}H-NMR: (701 MHz, Methanol-d_4) δ 7.26 – 7.22 (m, 2H), 7.20 – 7.16 (m, 1H), 7.16 – 7.12 (m, 2H), 7.03 – 6.99 (m, 2H), 6.72 – 6.66 (m, 2H), 4.60 – 4.56 (m, 2H), 4.56 – 4.49 (m, 4H), 4.43 (dd, J = 8.6, 5.3 Hz, 1H), 4.06 (p, J = 6.3 Hz, 1H), 4.03 – 3.95 (m, 1H), 3.81 – 3.75 (m, 1H), 3.71 – 3.64 (m, 1H), 3.20 (t, J = 7.1 Hz, 2H), 3.06 (dd, J = 13.9, 6.1 Hz, 1H), 3.01 (dd, J = 14.1, 5.5 Hz, 1H), 2.87 – 2.81 (m, 1H), 2.81 – 2.74 (m, 1H), 2.29 – 2.22 (m, 1H), 2.08 – 1.93 (m, 4H), 1.87 (s, 3H), 1.85 – 1.78 (m, 1H), 1.77 – 1.70 (m, 2H), 1.23 (d, J = 6.4 Hz, 3H), 1.15 (dd, J = 6.6, 1.9 Hz, 6H).

^{13}C-NMR: (176 MHz, Methanol-d_4) δ 174.65, 173.75, 173.67, 173.33, 172.51, 170.94, and 168.50 (CO of ester and amides), 158.63 (CN of Arginine), 157.41 (quaternary ζ-C of Tyrosine), 138.44 (quaternary C1 of Ph), 131.47 (ε-CH of Tyrosine), 130.19, and 129.49 (CH of Ph), 128.90 (quaternary γ-C of Tyrosine), 127.79 (CH of Ph), 116.30 (δ-CH of Tyrosine), 68.58 (β-CH of Threonine), 63.91 (α-CH2 of ester), 61.62, 58.23, 56.52, 56.11, and 53.23 (α-CH of main-chain amides), 42.69 (CH of isopropyl), 41.89 (δ-CH2 of Arginine), 38.48, and 37.61 (β-CH2 of Phenylalanine and Tyrosine), 30.59, 29.36, 26.06, and 25.93 (β,γ-CH2 of Proline and Arginine), 22.54, 22.51, and 22.42 (CH3 of acetyl and CH3 of isopropyl), 19.92 (γ-CH3 of Threonine). Note: Carbon of δ-CH2 of Proline overlaps with solvent signal (around 49.2 ppm, see HSQC spectrum).

HRMS (ESI-TOF): m/z calculated for $C_{40}H_{58}N_9O_{10}$ $[M+H]^+$: 824.43012. Found 824.42822.



22:

(6S,9S,12S,15S,18S)-6-acetamido-1-amino-12,15-bis(hydroxymethyl)-1-imino-9-isobutyl-18-((2-(isopropylamino)-2-oxoethoxy)carbonyl)-7,10,13,16-tetraoxo-2,8,11,14,17-pentaazahenicosan-21- oic acid: The mono- and di-Passerini products were synthesized following GPB using **2i** (20 equiv) and **3b** (20 equiv) to provide white solids in 31% and 12% yield, respectively. The following ligations then provided the title compound as a white solid in 10% and 30% yield, respectively.

HPLC: Product is a single peak with retention time 4.235 min.

Supporting information

The supporting information is available free of charge.

References and Notes

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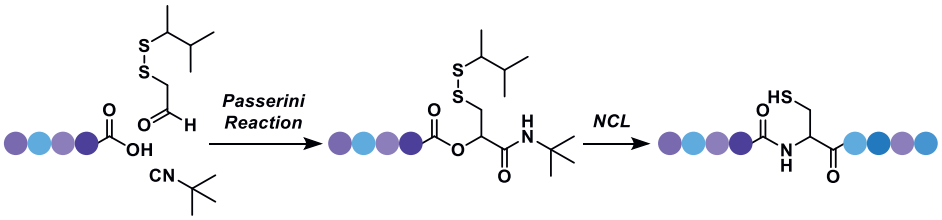
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Chapter 3

Late-Stage Passerini Functionalization of Unprotected Peptides for Native Chemical Ligation



Abstract: *Native chemical ligation (NCL) is one of the most widely adopted methods for large peptide and protein synthesis. Herein, we report a strategy that combines the Passerini multicomponent reaction and NCL for the functionalization of the C-termini of unprotected peptides. Using the Passerini reaction under aqueous acidic buffer conditions, we generate C-terminal α-acyloxyamides bearing a disulfide-protected thiol. Under standard NCL conditions, these substrates undergo efficient ligation for peptides featuring all canonical C-terminal amino acids typically compatible with NCL. Overall, this strategy enables C-terminal functionalization of unprotected peptides and broadens the applicability of NCL to native peptides and proteins for modification and synthesis.*

J. Hanssens, M. Lambers, R. V. A. Orru and J. M. Saya*. *Manuscript in preparation.*

3.1 Introduction

Native chemical ligation (NCL) is a cornerstone transformation in modern chemical protein synthesis, enabling the chemoselective coupling of unprotected peptide fragments in aqueous media under mild conditions.[1–3] Strategies involving NCL typically operate at neutral pH and ambient temperature. In combination with solid-phase peptide synthesis (SPPS), it has made the synthesis of large, functionalized peptides and proteins accessible with full precision. Moreover, it allows the incorporation of noncanonical amino acids, site-specific post-translational modifications, and complex molecular architectures that remain challenging for recombinant expression systems.[1–3]

Mechanistically, NCL proceeds through a well-established two-step pathway involving a peptide bearing a C-terminal α-thioester and a second fragment equipped with an N-terminal cysteine (**Figure 1A**).[4–6] In the initial step, the cysteine thiolate engages in a reversible intermolecular transthioesterification with the C-terminal thioester, generating a transient thioester-linked intermediate. This is followed by a rapid intramolecular S-to-N acyl transfer via a five-membered ring transition state, furnishing a native amide bond and irreversibly driving the reaction toward product formation. The rearrangement step is thermodynamically favored and underpins the high chemoselectivity of the ligation.[4–6]

The efficiency of NCL is strongly influenced by the structural features of the C-terminal thioester. Both the steric demand of the terminal amino acid and the properties of the thiol leaving group play key roles in determining reaction rates. Bulky residues such as Val and Ile significantly hamper ligation kinetics, while aryl thioesters generally outperform their alkyl counterparts due to superior leaving group ability.[2,7] In this context, the pKa of the thiol is key: the lower the pKa value the higher the reactivity. To mitigate slow kinetics and suppress unproductive side reactions arising from intramolecular or intermolecular thiol exchange, exogenous thiol additives (*e.g.* aryl thiols) are frequently required.[3,7] Additionally, denaturants such as guanidinium hydrochloride (Gdn·HCl) or urea are often included to improve peptide solubility and to further accelerate ligation.[7,8] Collectively, these considerations underscore the importance of developing improved methods for the synthesis of optimized NCL substrates.

Many strategies have been reported to obtain the C-terminal thioester substrate required for NCL. The most common strategies involve the functionalization of peptide C-termini via SPPS.[7] This has been done in three main ways: backbone anchoring, sidechain anchoring, or in solution on protected peptides. Sidechain anchoring exploits the reactive functionalities on the sidechain of the C-terminal amino acids for attachment (*e.g.*, Asp, Glu, Thr, Ser, Tyr, Cys). Backbone anchoring involves covalent attachment of the C-terminus of the peptide or of the adjacent amide of the following amino acid residue. Therein, the thioester can be

installed prior to or after peptide elongation, the latter of which is more commonly employed. When desired, the C-terminus is activated to allow for the formation of the thioester function by thiolysis. Subsequently, the peptide can be removed from the resin leaving the integrity of the thioester intact. Finally, peptides can also be selectively cleaved from the resin at the C-terminus after their synthesis, thus allowing for selective activation and thiolysis of that position prior to removal of the other protection groups. However, this approach requires the production and handling of protected peptides, which are inherently troublesome to purify or keep in solution.[7]

In addition to the above, methods which allow thioester formation on unprotected peptides at a later stage have been developed.[7] Such strategies require specific functional groups (*i.e.* latent thioesters) on the protected peptides, which need to be installed. These should then later be selectively activated towards thiolysis to form the required thioesters. The C-terminal hydrazine method developed by Liu *et al*. is, in that respect, currently most widely applied for thioester synthesis (see **Chapter 1**).[9,10] With this approach, the completely deprotected peptide hydrazide is converted to an acyl azide using sodium nitrite under acidic conditions (**Figure 1B**). Subsequently, the azide is displaced by a thiol to form the desired thioester. The main drawback of the latent thioester strategy is that the reactive group is usually installed during solid phase synthesis under protected conditions.[7,11,12]

Aside from the chemical pathways, recombinant strategies have also emerged to access thioesters. Shortly after the introduction of NCL, the groups of Muir[13,14] and Xu[15] independently reported the use of inteins for the synthesis of peptide or protein thioesters, a method later referred to as expressed protein ligation (EPL, **Figure 1C**).[16] Although very useful and widely applied, EPL is quite laborious and not very atom efficient since the inteins themselves are discarded.

So far, a method for the synthesis of thioester (surrogates) on free unprotected peptide fragments has not yet been reported and would be of great value to the toolkit for chemical protein synthesis. In **Chapter 2**, we developed a selective strategy to functionalize peptide C-termini by combining the Passerini reaction with peptiligases. We identified conditions that enable chemoselective modification of carboxylic acids in the presence of other unprotected amino acid sidechains. With the research described in this chapter, our aim was to extend this approach to native chemical ligation, using it as a route to install thioester surrogates on unprotected peptides. We exploit the concept of N/O-to-S acyl shift systems,[7] which requires a C-terminal ester or amide with a vicinal (protected) thiol moiety that can undergo an acyl shift upon activation forming the desired thioester for NCL.[17–19] To achieve this, we have developed novel disulfide-bearing aldehyde compounds which, when used as substrates in the Passerini on unprotected peptides, affords α-acyloxyamide products

bearing a disulfide in the sidechain (**Figure 1D**). This moiety functions as a thioester surrogate, since it, upon reduction to the free thiolate, can engage in an O-to-S acyl transfer to provide a thioester.[20–22] The product can in turn be exploited for NCL reactions.

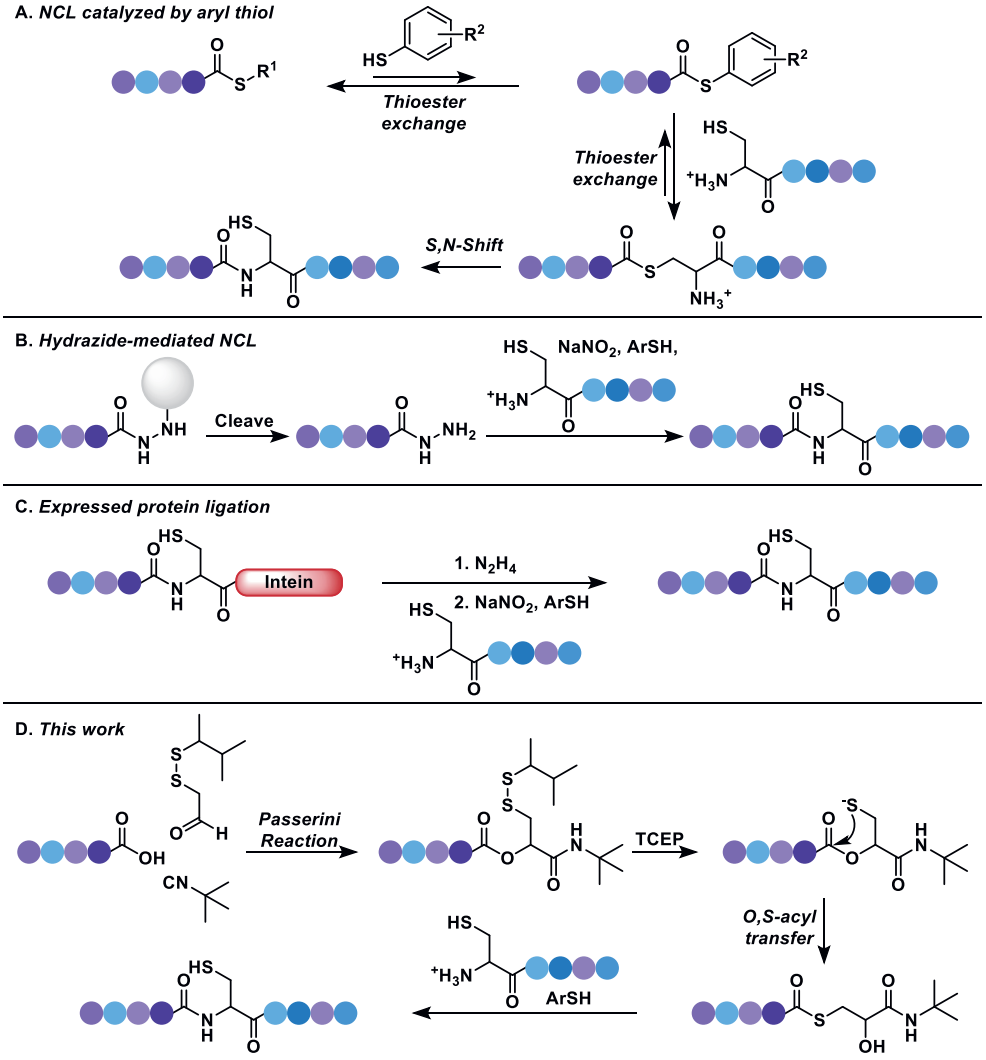
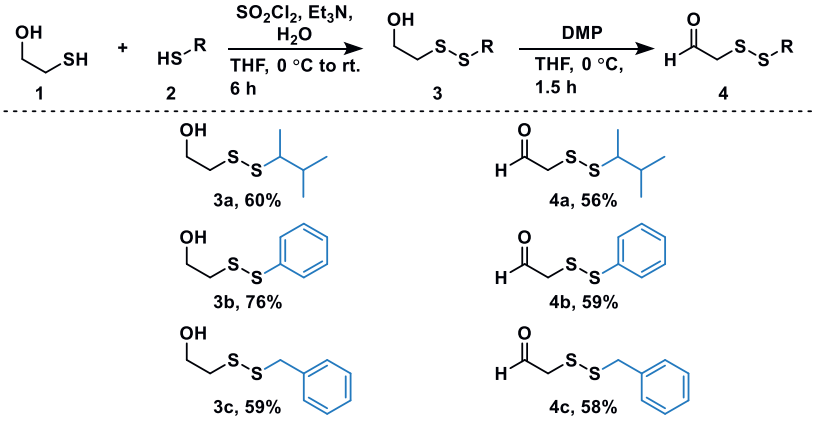


Figure 4. A. Mechanism of native chemical ligation catalyzed by an aryl thiol. B. SPPS-mediated synthesis of C-terminal thioesters. C. Expressed protein ligation. D. This work: Thioester surrogate synthesis on unprotected peptides and subsequent NCL.

3.2 Results and Discussion

3.2.1 Disulfide-aldehyde substrate synthesis

We started our investigation with the synthesis of asymmetric disulfide aldehydes **4a**-**4c** required for the Passerini reaction (**Scheme 1**). Using previously reported conditions[23], the two thiols **1** and **2** were allowed to react with sulfuryl chloride under basic conditions and furnished the desired disulfide alcohols (**3a**-**3c**) in moderate to high yields. These were subsequently oxidized using Dess-Martin periodinane to provide disulfide-protected mercaptoacetaldehydes **4a**-**4c**.



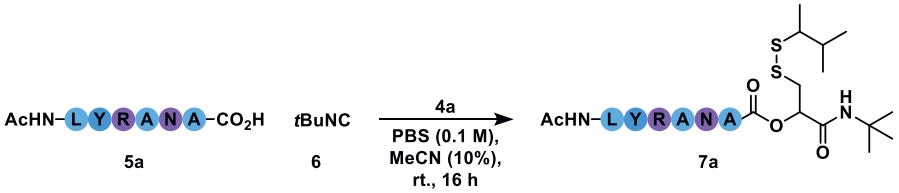
Scheme 41. Sidulfide formation and subsequent alcohol to aldehyde oxidation.

3.2.2 Optimization of the Passerini reaction

With our novel disulfide aldehyde substrates in hand, we set out to optimize the Passerini reaction. The hexapeptide Ac-LYRANA-OH (**5a**) was selected as model substrate, as it has successfully been used in previous NCL studies.[24] Given our experience with the Passerini reaction for the functionalization of peptide C-termini (**Chapter 2**), we selected *tert*-butyl isocyanide (**6**) and aldehyde **4a** as the other components (**Table 1**).[25] Furthermore, we used phosphate buffer as the reaction medium and investigated the effect of the pH on the performance of the P-3CR. Consistent with our previous investigations, we obtained high yields with a pH in the range of 2.5-5.5 (**Table 1**, entries 2-5). Lowering to a pH of 1.5 results in a diminished yield, likely owing to decomposition of the isocyanide. Furthermore, higher pH values likely result in lower yield owing to the maintained deprotonated state of the carboxylate and lower protonation of the aldehyde, resulting in lower reactivity (**Table 1**, entries 6-7). To ensure chemoselective modification of the C-terminus over other amino acid residues in the peptide, we decided to work at pH 2.5 as preferred reaction conditions. Next, we found that optimal performance of the reaction (yield) is directly proportional to

the concentration, a general trend for multicomponent reactions (**Table 1**, entries 8-11). However, with the application of complex peptide and protein functionalization in mind, a concentration of 20 mM with respect to the peptide was considered satisfactory. Finally, varying the stoichiometry with respect to the aldehyde and isocyanide showed no significant increase at 10 equivalents or higher of both these inputs (**Table 1**, entries 12-15). Finally, we observed no additional byproducts or signs of epimerization.

Table 4. Optimization of the Passerini reaction[a].



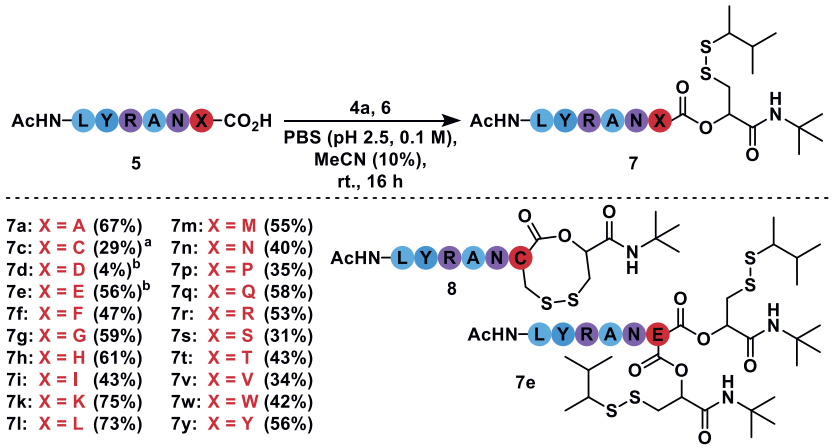
Entry	pH	[5] (mM)	Equiv 4a & 6	Yield (%)[b]
1	1.5	20	10	43
2	**2.5**	**20**	**10**	**98**
3	3.5	20	10	96
4	4.5	20	10	90
5	5.5	20	10	90
6	6.5	20	10	27
7	7.5	20	10	6
8	2.5	1	10	23
9	2.5	10	10	72
10	2.5	50	10	96
11	2.5	100	10	85
12	2.5	20	1	17
13	2.5	20	5	75
14	2.5	20	25	92
15	2.5	20	50	97

[a] Standard conditions: peptide **5** (0.01 mmol), *t*-butyl isocyanide **6** and **4a** in phosphate buffer (0.1 M) with MeCN (10%), stirred at indicated temperature and time. [b] Relative yield based on HPLC-MS analysis and reported as percentage of the total present starting material+product.

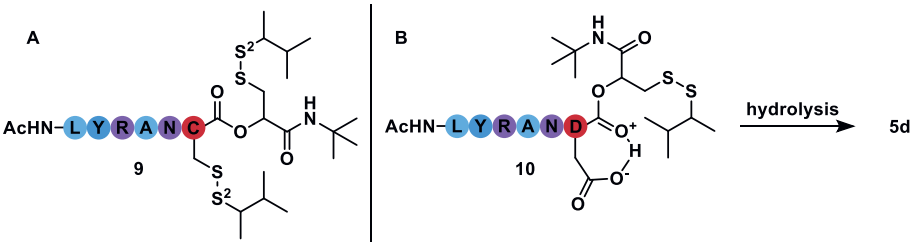
3.2.3 Scope of the Passerini reaction

With the optimal conditions in hand, we explored the scope of the reaction by varying the C-terminal amino acid residue (**Scheme 2**). We were happy to find that the reaction was compatible with most of the residues at that position and afforded the desired α-acyloxyamide esters in acceptable to high yields. However, a few residues provided lower yields. Interestingly, a Cys residue at the C-terminus (**5c**) resulted in partial disulfide exchange and a cyclic 8-membered disulfide (**8**) along with the desired product was identified. This cyclic byproduct was formed as a 1:2 diastereoisomeric mixture based on

HPLC-MS. This ratio is likely caused by the *L*-Cys configuration leading to a favored attack by the Cys-SH on one of the two diastereoisomer disulfides of **7**. Furthermore, analysis of the isolated desired product (**7c**) shows full conversion towards **8** after its isolation. Moreover, a mixture of disulfides was observed, as the Cys-SH can attack both sulfur atoms (S^{1-2}) of the original disulfide (compound **11** was observed on HPLC, **Scheme 3A**). Next, we examined the placement of an Asp residue (**5d**) at the C-terminal position (LYRAND). A minor amount of both mono- and di-Passerini products was observed after the reaction. Furthermore, the mono-Passerini product showed clear signs of instability and hydrolyzed back to the starting material upon purification. A hydrolysis mechanism facilitated by the Asp sidechain likely accounts for this observation as its proximity to the ester allows for hydrogen bonding leading to increased electrophilicity (**Scheme 3B**). In contrast, Glu analogue **5e** gave the di-Passerini adduct (**7e**; 56%) in good yield, likely because its extra methylene (vs Asp) reduces intramolecular hydrolysis. Moreover, peptide **5p** bearing a C-terminal Pro gave lower conversion and the product was obtained in 35% yield. However, it showed signs of diketopiperazine formation, a common side product for C-terminal proline residues. All other amino acids provided good conversion towards the respective products. However, the purification through preparative HPLC of certain compounds proved more troublesome owing to bad separation on the column *e.g.*, Ser (**7s**; 31%), and Val (**7v**; 34%). Consistent with previous results described in **Chapter 2**, the presence of free amine groups, *i.e.* in the sidechain of Lys (**7k**; 75%), did not result in an (acid-less) Ugi reaction under the low pH buffer conditions.[25] Similarly, other residues bearing nucleophilic sidechains solely provided conversion to the desired products *i.e.*, Thr (**7t**; 43%), Tyr (**7y**; 56%), and His (**7h**; 61%).



Scheme 42. Scope of the Passerini reaction. Standard conditions: peptide **5** (0.05 mmol, 20 mM), *t*-butyl isocyanide **6** (0.5 mmol) and aldehyde **4a** (0.5 mmol) in phosphate buffer (pH 2.5, 0.1 M) with MeCN (10%). Isolated yields are shown. [a] The product **7c** gradually converted and was isolated as cyclic compound **8**. [b] The product of the reactions with peptides bearing carboxylate sidechains were functionalized with two Passerini adducts (**7e**).

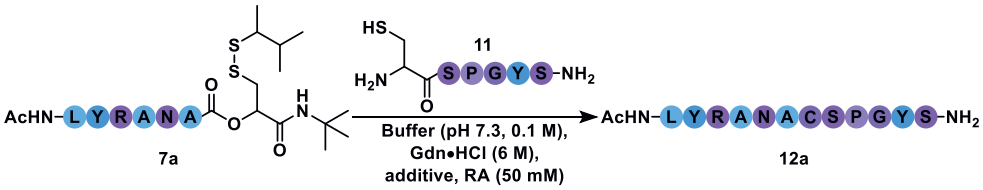


Scheme 43. A. Observed side product during formation of compound **7c**. B. Mechanism of facilitated hydrolysis with C-terminal Asp residues.

3.2.4 Optimization of the NCL

With disulfide-esters **7** in hand, we set out to test our hypothesis that these Passerini products indeed serve as NCL substrates. For that purpose, we studied the reaction of **7a** with NH_2-CSPGYS-NH_2 (**11**) to provide the 12-mer peptide **12a**. We were happy to find that under our initial conditions using phosphate buffer (pH ~7.3, 100 mM), guanidine (6 M) and TCEP (50 mM), with 4-mercaptophenylacetic acid (MPAA, 500 mM) as the additive, at a 5 mM peptide ester concentration (**Table 2**, entry 1), full conversion to the desired product **12a** was observed. The reaction was then optimized based on rate of conversion and product/hydrolysis ratio (P/H: ratio of desired product vs. hydrolyzed ester). Initially, we explored various solvent systems and found that the reaction worked well in different buffer systems (*i.e.* HEPES, TRIS, and PBS), providing the desired product with no significant difference in kinetics or P/H ratio (**Table 2**, entries 1-3). We then explored numerous additives known to facilitate NCL reactions (**Table 2**, entries 4-6).[26] Notably, the kinetics of the reaction was optimal employing MPAA, resulting in full conversion within 15 mins and providing a comparable P/H ratio to Sodium 2-mercaptoethanesulfonate (MesNa, entry 4). MesNa gave near full conversion after 19 h, whereas 2-methylimidazole (2-MIM) and benzylmercaptan gave near full conversion after 2 and 4 h, respectively. The latter two also resulted in significantly more hydrolysis. Next, when using concentrations lower than 250 mM MPAA, we observed both slower kinetics and again increased hydrolysis (**Table 2**, entries 7-10). Moreover, changing the stoichiometry revealed that full conversion with respect to the C-terminal fragment **11** could be obtained when using an excess of ester reagent (**Table 2**, entries 11-13). Since the ester is generally employed in excess in NCL, the hydrolysis product is usually not reported but always present. However, we deemed the ester fragment of higher value in this study and regarded an excess of C-terminal fragment optimal. Finally, omitting TCEP resulted in significant hydrolysis, while replacing it with DTT still afforded the desired product but lowered both the rate and P/H ratio (**Table 2**, entries 14-15).

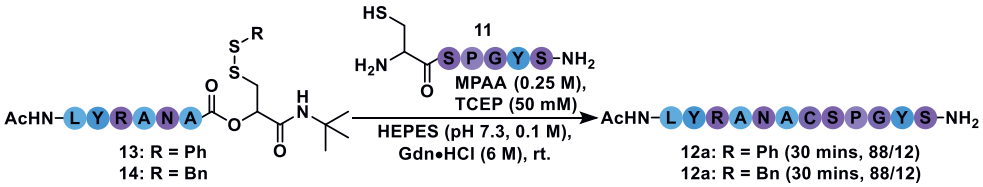
Table 5. Optimization of the native chemical ligation[a].



Entry	Buffer	Additive	RA	7a : 11	Time (mins)	P/H[b]
1	HEPES	MPAA (0.5 M)	TCEP	1 : 2	15	89/11
2	TRIS	MPAA (0.5 M)	TCEP	1 : 2	30	90/10
3	PBS	MPAA (0.5 M)	TCEP	1 : 2	30	89/11
4	HEPES	MesNa (0.5 M)	TCEP	1 : 2	1140	89/9
5	HEPES	2-MIM (0.5 M)	TCEP	1 : 2	180	64/36
6	HEPES	BnSH (0.5 M)	TCEP	1 : 2	240	77/22
7	**HEPES**	**MPAA (0.25 M)**	**TCEP**	**1 : 2**	**15**	**89/11**
8	HEPES	MPAA (0.1 M)	TCEP	1 : 2	60	84/16
9	HEPES	MPAA (0.05 M)	TCEP	1 : 2	120	81/19
10	HEPES	-	TCEP	1 : 2	>120	58/42
11	HEPES	MPAA (0.25 M)	TCEP	1.2 : 1	60[c]	-
12	HEPES	MPAA (0.25 M)	TCEP	1.5 : 1	60[c]	-
13	HEPES	MPAA (0.25 M)	TCEP	2 : 1	30[c]	-
14	HEPES	MPAA (0.5 M)	-	1 : 2	60	32/68
15	HEPES	MPAA (0.5 M)	DTT	1 : 2	>120	86/14

[a] Standard conditions: ester **7a**, peptide **11** and in buffer (pH 7.3, 0.1 M) and Gdn·HCl (6 M) with additive and reducing agent (RA, 50 mM). [b] Based on HPLC-MS analysis. [c] Time for full conversion of **11**.

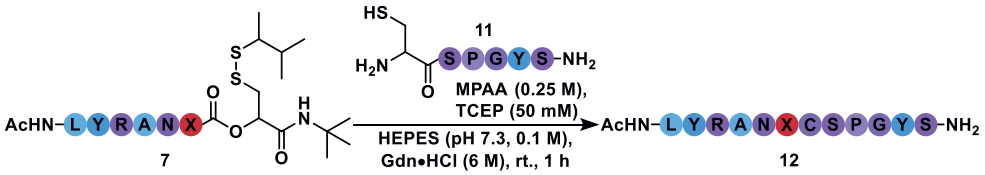
In an attempt to further improve the kinetics of the NCL, we synthesized Passerini products bearing phenyl (**13**) and benzyl (**14**) substituted disulfides (**Scheme 4**). Upon reduction, the lower pKa of the resulting thiol leaving group should accelerate thioester formation and thereby the overall ligation. However, the ligations using **13** and **14** gave comparable P/H ratios and showed slightly diminished conversion rates, so we continued with **7** as our optimal ester.



Scheme 44. Native chemical ligation reactions with different disulfide-ester depsipeptides (**13**-**14**). In brackets after the products are reported the reaction time for full conversion and the P/H ratio.

3.2.5 Scope of the NCL

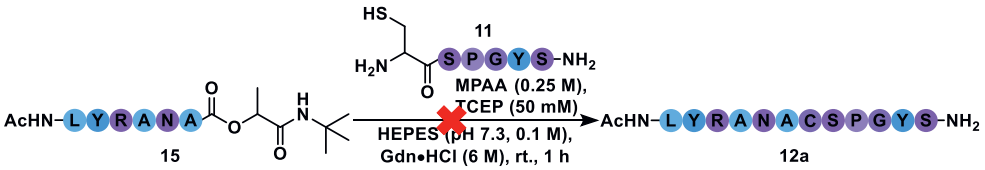
Then, we set out to perform the NCL with the set of Passerini products (**7**-**8**) along with 2 equivalents of C-terminal fragment **11** (**Scheme 5**). The ligations were all run for 1 h before confirming reaction completion by HPLC-MS and purification by preparative HPLC. We were delighted to find product formation for most of the tested substrates in good isolated yields (42-69%). However, a sterically hindered ester at C-terminal Ile (**7i**, 13%) and Val (**7v**, 31%) resulted in low yields. When evaluating Pro C-terminal peptide ester (**7p**), a notoriously troublesome NCL junction (200-fold slower rate),[24,27,28] we expectedly observed minimal product formation and extensive hydrolysis. However, we were still able to isolate **12p**, albeit in a relatively low yield of 15%. Furthermore, Cys-bearing cyclic peptide **8** provided the desired ligation product **12c** in moderate 30% yield. From the difunctionalized Passerini product **12e**, the NCL afforded a mixture of three products, two mono-ligation products at the side- or mainchain and a double-ligation product in a 1:2.2:8.8 ratio respectively, indicating partial reaction selectivity. Finally, additional peptides bearing C-terminal nucleophilic sidechains also afforded the desired products in good yields (53-69%).



12a: X = A (36%)	12h: X = H (67%)	12n: X = N (64%)	12t: X = T (60%)
12c: X = C (30%)[a]	12i: X = I (13%)	12p: X = P (15%)	12v: X = V (31%)
12e: X = E (3/9/16%)[b]	12k: X = K (53%)	12q: X = Q (69%)	12w: X = W (63%)
12f: X = F (54%)	12l: X = L (42%)	12r: X = R (67%)	12y: X = Y (63%)
12g: X = G (69%)	12m: X = M (67%)	12s: X = S (69%)	

Scheme 45. Native chemical ligation between esters **7**-**8** and peptide **11**. [a] The reaction was performed with **8** as starting material ester. [b] The isolated yields of the two mono- and the di-ligation products are shown, respectively.

To verify our hypothesis on the mechanism of the reaction, we also performed the NCL with depsipeptide **15** (**Scheme 6**). This compound did not bear a disulfide in the sidechain and would therefore be incapable of the initial O-to-S acyl transfer. Indeed, no product formation was observed after 2 h, confirming the requirement for a tethered thiol to allow for productive ligation.



Scheme 46. Control experiment of NCL with **15**, a peptide ester bearing no disulfide substituent. No product formation observed.

3.3 Conclusion

We have developed a novel Passerini strategy for functionalizing the C-termini of unprotected peptides towards α-acyloxyamide products bearing a disulfide moiety in the sidechain. These products could in turn be utilized as NCL substrates. We found that the Passerini reaction proceeds efficiently with high selectivity for carboxylates. Moreover, ligations with the majority of the substrates proceeded efficiently, obtaining desired products in 42-69% isolated yields. However, Passerini reactions with peptides bearing functionalized sidechains on certain C-terminal residues (*i.e.* Cys **7c**, Asp **7d**) led to undesired side reactions or product hydrolysis. Furthermore, the subsequent NCL reactions with substrates with higher steric hindrance (*i.e.* Ile **12i**, Val **12v**) or with particular substrates (*i.e.* Cys **8**, Glu **7e**, Pro **7p**) led to lower yields or a mixture of products. Nevertheless, this developed method represents a useful novel tool for the functionalization of unprotected peptides at the C-terminus.

3.5 Experimental Section

General Remarks

Unless stated otherwise, all solvents and commercially available reagents were used as received without further purification. Fmoc-protected amino-acids were purchased from Combi-blocks, Doug discovery and GLbiochem. Coupling reagents, solvents, buffer salts, carbonyl and isocyanide compounds were purchased from Sigma-Aldrich, Doug discovery, Combi-blocks, Iris Biotech, Biosolve, and TCI chemicals. Preparative-scale purification was performed using a Shimadzu Nexera preparative HPLC system, consisting of: a CBM-40 systems controller, LC-20AP preparative pump, FCV-200AL quaternary vale, SPD-M40 photodiode array detector (PDA), LCMS-2050 mass detector, LC-40D HPLC pump, LH-40 fraction collector, and an FCV-20AH2 valve unit. Separation was carried out using Shim-pack Scepter C_{18}-120 column (5 µm, 20x150 mm) with an optimised gradient for each compound (MeCN/MilliQ water with 0.1% TFA) at a flow rate of 20 mL/min. Fraction collection was triggered by PDA and/or MS signal detection. Peptide samples were lyophilized with a Büchi Lyovapor^{tm} L-250 Pro lyophilizer. High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) analyses were performed on a Shimadzu Nexera 2 UHPLC system equipped with a Shimadzu LC-30AD pump, a SPD-M30A photodiode array detector and LCMS-2020 single quadrupole detector. The eluent consisted of MilliQ water and LCMS grade acetonitrile, and 0.1% formic acid. A Waters XSelect CSH C18 column (3.0 mm × 75 mm with a particle size of 3.5 mm) was employed for the separation at 30 °C. The method comprised a gradient of acetonitrile in water: 5% (2 min) to 95% acetonitrile (6 min), 1 min at 95% acetonitrile and back to 5% acetonitrile (2 min). The substrates and products were quantified by their UV absorption at 220 nm. High resolution mass spectroscopy was conducted on a Bruker micrOTOF-Q II mass spectrometer (electrospray ionization). NMR spectra (1D ^{1}H and ^{13}C, COSY, DIPSI, $^{13}C^{1}H$ HSQC) were recorded on a Bruker Avance III HD 700 MHz spectrometer equipped with a TCI cryoprobe, or on a Jeol JNM-ECZL400S 400 MHz using the residual solvent or TMS standard (0 ppm) as reference. The data is reported as follows: chemical shift (δ ppm), coupling constant (J, Hz), multiplicity (s = singlet, d = doublet, t = triplet, q = 5 quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets).

General synthetic procedures

General procedure A: SPPS of starting material peptides.
The reactions were performed in either a syringe reactor on an orbital shaker at room temperature or using a glass SPPS vessel to be stirred by nitrogen bubbling. The procedures were performed with 10 mL solvent/1 g resin unless stated otherwise. Rink amide AM resin (GL Biochem, Cat. No. 49001) was swollen in DMF (dimethylformamide) for 30 min. The amino acid (AA) coupling solution of 4 equiv Fmoc-AA, 4 equiv (1-cyano-2-ethoxy-2-oxoethylidenaminooxy)dimethylamino-morpholino-carbenium hexafluorophosphate (COMU) and 4 equiv ethyl cyano (hydroxyimino) acetate (oxyma) in DMF was prepared (0.3 mL/50 mg resin). 8 equiv N,Ndiisopropylethylamine (DIPEA) were added to the coupling solution when ready for use. Subsequently, the coupling solution was added to the resin and allowed to react for 30 min. Subsequently, the reaction solution was discarded, and the AA coupling repeated. The resin was washed with DMF (5×) before Fmoc removal was performed by adding a deprotection solution of piperidine in DMF (2:8, v/v) and reacting for 5 min. The solution was discarded and the Fmoc removal repeated. Next, the resin was washed with DMF (3×), dichloromethane (DCM) (3×) and DMF (3×). Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above. 2-chlorotrityl chloride (CTC)-resin (100-200 mesh), 1% DVB (Novabiochem, Cat. No. 8.55017) was swollen in DCM for 30 min. A solution of 2.5 equiv Fmoc-AA and 8 equiv DIPEA in DCM (0.3 ml/50 mg resin) was added to the resin and reacted for 1 h. The solution was discarded, and non-reacted linker groups were capped by adding a solution of methanol (MeOH), DIPEA and DCM (2:1:17, v/v/v) and reacting for 5 min. The solution was discarded and the capping step repeated. Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above for the rink amide AM resin. Peptides were N-terminally acetylated by

adding a solution of Ac_2O, DIPEA and DMF (1:1:8, v/v/v) to the reaction and reacted for 10 min. The acetylation was repeated. The resin was washed with DMF (3×) and DCM (3×). Subsequently, the peptide was cleaved and the sidechain protecting groups were removed in one step by adding a cleavage solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water (95:2.5:2.5, v/v/v) and reacting for 1 h. The cleavage solution was collected and the cleavage was repeated twice. The combined cleavage solutions were concentrated in vacuo. The material was precipitated in diethyl ether and centrifuged (15 min, 4000 rcf). The supernatant was discarded and the crude peptide was dissolved in MeCN and water and lyophilized. The peptides were purified by preparative HPLC

General procedure B: Asymmetric disulfide synthesis.
Prepared according to a modified procedure by Hahn and coworkers.[23] To a solution of aliphatic thiol (1 equiv) in THF (0.2 M) at 0 °C under ice-bath was added slowly sulfuryl chloride (1.65 equiv). The reaction mixture was stirred for 1 h at the same temperature. This solution [A] was used immediately in the next step. To a solution of 2-mercaptoethan-1-ol (1 equiv), triethylamine (2.2 equiv) and water (10 equiv) in THF (0.25 M) at room temperature was added the pre-prepared solution of sulfenyl chloride [A] while stirring. The reaction mixture was stirred for 3-6 h at room temperature. The reaction progress was monitored by TLC. The reaction mixture was diluted with CH_2Cl_2 (2.5x volume of THF), washed sequentially with aqueous saturated $NaHCO_3$ solution and water and then dried over anhydrous Na_2SO_4. The solution was filtered and concentrated in vacuo, and the residue was purified by flash column chromatography on silica gel (10-25% EtOAc in heptane) to obtain the corresponding asymmetric disufide.

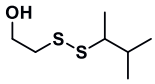
General procedure C: Alcohol oxidation to aldehyde.
Prepared according to a modified procedure by Bierer and coworkers.[29] To a solution of alcohol (1 equiv) in THF (0.1 M) at 0 °C was added quickly the Dess-Martin periodinane (1.2 equiv) and the mixture was stirred at 0 °C for 30 min and then at room temperature for another 40 min. Upon completion of the reaction, sat. $NaHCO_3$ (0.5x volume of THF) was added and the mixture was extracted with diethyl ether (3x). If a suspension formed during extractions, sat. sodium thiosulfate solution was added and stirred until a clear solution was obtained. The combined organic layers were washed brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The crude material was purified by flash column chromatography on silica gel (3-8% EtOAc in heptane) to obtain the corresponding aldehyde.

General procedure D: Passerini reaction towards ester disulfides.
Prepared according to a modified procedure by Hanssens *et al.*[25] To a stirred solution of peptide (1 equiv) in the appropriate solvent was added aldehyde (10 equiv) in MeCN (10%) and *t*-butyl isocyanide (10 equiv) and the resulting mixture was stirred at rt. for 16 h. The reaction mixture was diluted with MeCN and H_2O until all solids had dissolved, then brought to pH ~7 (monitored by pH paper) using NaOH (1 M) while stirring vigorously. An aliquot was taken for HPLC analysis. The yields were determined by HPLC-MS at 220 nm.

General procedure E: Native chemical ligation with peptide esters.
Prepared according to a modified procedure by Payne and coworkers.[26] Degassed (N_2-bubbling for 20 mins) ligation buffer (HEPES buffer 0.125 M, Gdn 7.5 M, pH 7.5) was used to dissolve the MPAA and TCEP and the pH was adjusted to ~7.3 to obtain the following ligation solution: HEPES buffer (pH 7.3, 0.1 M), Gdn·HCl (6 M), TCEP (50 mM), MPAA (0.25 M). NH_2-CSPGYS-NH_2 **11** (2 equiv) was dissolved in the ligation solution to a concentration of 10 mM with respect to NH_2-CSPGYS-NH_2. This solution was added to the peptide-ester to give a concentration of 5 mM and a final pH of the reaction of 7.0-7.5. The reaction was monitored over 2h. Ligation time-courses were plotted by taking 10 µL aliquots at various timepoints and quenching with 10 µL of 7 vol.% $N_2H_4{\cdot}H_2O$ in Milli-Q water. This solution was diluted with 80 µL of 0.1 % TFA in Milli-Q water then analyzed by HPLC.

Characterization of compounds

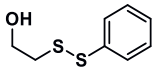
2-((3-methylbutan-2-yl)disulfaneyl)ethan-1-ol (3a)



Prepared from 3-methylbutane-2-thiol (624 µL, 5 mmol, 1 equiv) and 2-mercaptoethan-1-ol (351 µL, 5 mmol, 1 equiv) according to procedure B. Purification was done by column chromatography on silicagel (10-25% EtOAc in heptane). Isolated as yellow oil (541.4 mg, 3.002 mmol, 60%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 3.89 (t, *J* = 5.8 Hz, 2H), 2.86 – 2.75 (m, 3H), 2.02 – 1.92 (m, 1H), 1.26 (d, *J* = 7.0 Hz, 3H), 0.97 (dd, *J* = 16.7, 6.8 Hz, 6H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 60.49, 53.64, 41.68, 32.10, 20.48, 18.18, 16.51; **HRMS (ESI)**: *m/z* calculated for $C_7H_{16}NaOS_2^+$ $[M+Na]^+$ 203.0535, found: 203.0531.

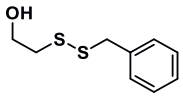
2-(phenyldisulfaneyl)ethan-1-ol (3b)



Prepared from benzenethiol (513 µL, 5 mmol, 1 equiv) and 2-mercaptoethan-1-ol (351 µL, 5 mmol, 1 equiv) according to procedure B. Purification was done by column chromatography on silicagel (10-25% EtOAc in heptane). Isolated as yellow oil (704.5 mg, 3.782 mmol, 76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 – 7.52 (m, 2H), 7.36 – 7.29 (m, 2H), 7.27 – 7.21 (m, 1H), 3.85 (td, *J* = 5.7, 1.1 Hz, 2H), 2.89 (td, *J* = 6.3, 5.2 Hz, 1H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 137.09, 129.27, 129.17, 128.04, 127.58, 127.34, 127.25, 60.44, 60.00, 41.34, 41.29; **HRMS (ESI)**: *m/z* calculated for $C_8H_{10}NaOS_2^+$ $[M+Na]^+$ 209.0065, found: 209.0059.

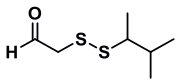
2-(benzyldisulfaneyl)ethan-1-ol (3c)



Prepared from 3-methylbutane-2-thiol (587 µL, 5 mmol, 1 equiv) and 2-mercaptoethan-1-ol (351 µL, 5 mmol, 1 equiv) according to procedure B. Purification was done by column chromatography on silicagel (10-25% EtOAc in heptane). Isolated as yellow oil (588.1 mg, 2.94 mmol, 59%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.31 – 7.17 (m, 5H), 3.84 (s, 2H), 3.65 (t, *J* = 5.7 Hz, 2H), 2.45 (t, *J* = 5.7 Hz, 2H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 137.30, 129.47, 128.75, 127.75, 60.21, 43.54, 40.98; **HRMS (ESI)**: *m/z* calculated for $C_9H_{12}NaOS_2^+$ $[M+Na]^+$ 223.0222, found: 233.0217.

2-((3-methylbutan-2-yl)disulfaneyl)acetaldehyde (4a)



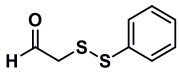
Prepared from **3a** (466.8 mg, 2.589 mmol, 1 equiv) and Dess-Martin periodinane (1.318 g, 3.106 mmol, 1.2 equiv) according to procedure C. Purification was done by column chromatography on silicagel (3-8% EtOAc in heptane). Isolated as colourless oil (258.9 mg, 1.452 mmol, 56%). When performed on a larger scale (>30 mmol), additional purification was required by vacuum distillation (80 °C, 0.2 mbar).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.57 (t, *J* = 3.3 Hz, 1H), 3.40 (d, *J* = 3.3 Hz, 2H), 2.79 (qd, *J* = 7.0, 4.8 Hz, 1H), 1.95 (dtd, *J* = 13.6, 6.8, 4.8 Hz, 1H), 1.25 (d, *J* = 7.0 Hz, 3H), 0.96 (dd, *J* = 16.3, 6.8 Hz, 6H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 195.15,

53.67, 48.32, 32.09, 20.37, 18.13, 16.46; **HRMS (ESI)**: *m/z* calculated for $C_8H_{18}NaO_2S_2^+$ $[M+MeOH+Na]^+$ 233.06404, found: 233.0636. Note: the mass is for the hemiacetal formed by dissolving the aldehyde in methanol.

Note: Compound shows signs of instability over time at rt. and more so upon heating. Storage at -20 is essential.

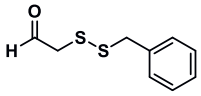
2-(phenyldisulfaneyl)acetaldehyde (4b)



Prepared from **3b** (704.5 mg, 3.782 mmol, 1 equiv) and Dess-Martin periodinane (1.925 g, 4.538 mmol, 1.2 equiv) according to procedure C. Purification was done by column chromatography on silicagel (3-8% EtOAc in heptane). Isolated as colourless oil (408.2 mg, 2.215 mmol, 59%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.52 (t, *J* = 3.2 Hz, 1H), 7.58 – 7.51 (m, 2H), 7.39 – 7.27 (m, 3H), 3.45 (d, *J* = 3.2 Hz, 2H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 194.69, 135.73, 129.59, 129.40, 128.18, 47.49; **HRMS (ESI)**: *m/z* calculated for $C_9H_{12}NaO_2S_2^+$ $[M+MeOH+Na]^+$ 239.01709, found: 239.0169. Note: the mass is for the hemiacetal formed by dissolving the aldehyde in methanol.

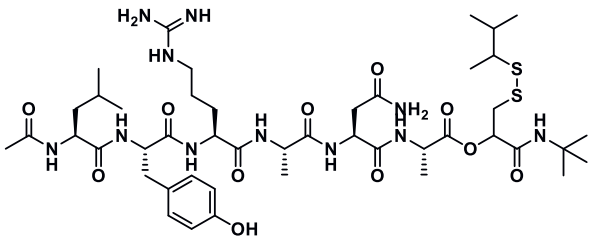
2-(benzyldisulfaneyl)acetaldehyde (4c)



Prepared from **3c** (588.1 mg, 2.936 mmol, 1 equiv) and Dess-Martin periodinane (1.494 g, 3.523 mmol, 1.2 equiv) according to procedure C. Purification was done by column chromatography on silicagel (3-8% EtOAc in heptane). Isolated as colourless oil (339.9 mg, 1.714 mmol, 58%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 9.41 (t, *J* = 3.1 Hz, 1H), 7.38 – 7.27 (m, 5H), 3.92 (s, 2H), 3.09 (d, *J* = 3.1 Hz, 2H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 195.02, 136.58, 129.60, 128.83, 127.93, 47.57, 43.30; **HRMS (ESI)**: *m/z* calculated for $C_{10}H_{14}NaO_2S_2^+$ $[M+MeOH+Na]^+$ 253.0327, found: 253.0322. Note: the mass is for the hemiacetal formed by dissolving the aldehyde in methanol.

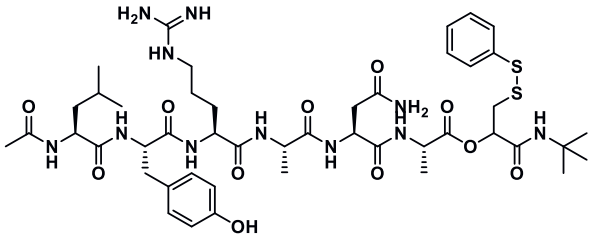
Ac-LYRANA-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ^3-propanamide (7a)



Prepared from Ac-LYRANA-OH (**5a**, 37 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (33.9 mg, 33.6 µmol, 67%).

Analytical HPLC trace of prep-HPLC purified title compound; Rt 5.77 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1010.5 (100%), $[M+2H]^{2+}$: 505.8 (100%); Mass Found (ESI^+); 506.1 $[M+2H]^{2+}$.

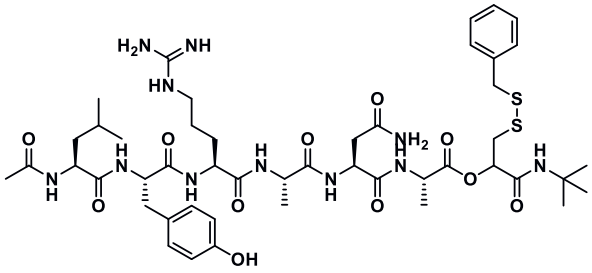
Ac-LYRANA-N-(tert-butyl)-3-(phenyldisulfaneyl)-2λ³-propanamide (13)



Prepared from Ac-LYRANA-OH (**5a**, 37 mg, 50 µmol, 1 equiv), **4b** (92 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (23 mg, 23 µmol, 45%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.60 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1016.5 (100%), $[M+2H]^{2+}$: 508.7 (100%); Mass Found (ESI^+); 509.0 $[M+2H]^{2+}$.

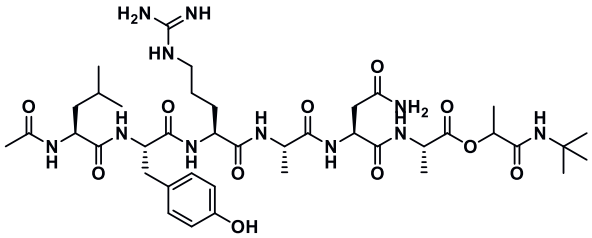
Ac-LYRANA-3-(benzyldisulfaneyl)-N-(tert-butyl)-2λ³-propanamide (14)



Prepared from Ac-LYRANA-OH (**5a**, 37 mg, 50 µmol, 1 equiv), **4c** (99 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (41.3 mg, 40.1 µmol, 80%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.61 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1030.5 (100%), $[M+2H]^{2+}$: 515.7 (100%); Mass Found (ESI^+); 516.0 $[M+2H]^{2+}$.

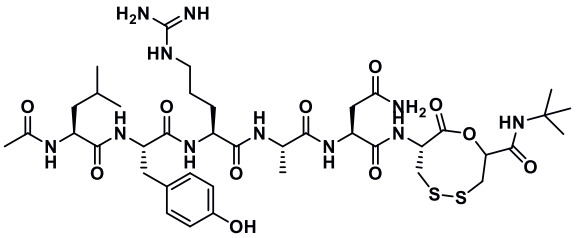
Ac-LYRANA-N-(tert-butyl)-2λ³-propanamide (15)



Prepared from Ac-LYRANA-OH (**5a**, 37 mg, 50 µmol, 1 equiv), acetaldehyde (28 µL, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (29.7 mg, 33.9 µmol, 68%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.75 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 876.5 (100%), $[M+2H]^{2+}$: 438.8 (100%); Mass Found (ESI^+); 876.6 $[M+H]^+$, 438.9 $[M+2H]^{2+}$.

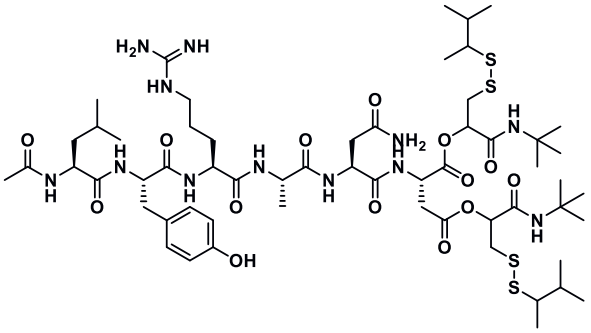
Ac-LYRAN-NH[-*N*-(*tert*-butyl)-8-oxo-7l3-1,4,5-oxadithiocane-2-carboxamide] (8)



Prepared from Ac-LYRANC-OH (**5c**, 13 mg, 17 µmol, 1 equiv), **4a** (30 mg, 0.17 mmol, 10 equiv) and *t*-butyl isocyanide (19 µL, 0.17 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.7 mg, 4.6 µmol, 26%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.95 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 938.4 (100%), $[M+2H]^{2+}$: 469.7 (100%); Mass Found (ESI^+); 938.5 $[M+H]^+$, 469.9 $[M+2H]^{2+}$.

Ac-LYRAND-[N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ^3-propanamide]$_2$ (7d)

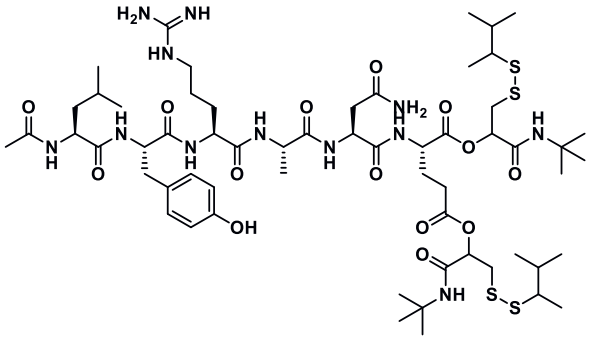


Prepared from Ac-LYRAND-OH (**5d**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (1.2 mg, 0.91 µmol, 1.8%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 6.79 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1315.6 (100%), $[M+2H]^{2+}$: 658.3 (100%); Mass Found (ESI^+); 1315.8 $[M+H]^+$, 658.8 $[M+2H]^{2+}$.

Note: compound was not pure after prep-HPLC.

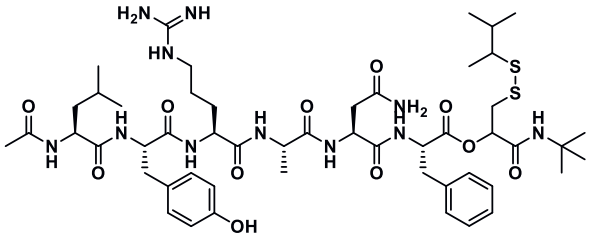
Ac-LYRANE-[N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ^3-propanamide]$_2$ (7e)



Prepared from Ac-LYRANE-OH (**5e**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (37.5 mg, 28.2 µmol, 56%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 6.81 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1329.6 (100%), $[M+2H]^{2+}$: 665.3 (100%); Mass Found (ESI^+); 1329.8 $[M+H]^+$, 665.6 $[M+2H]^{2+}$.

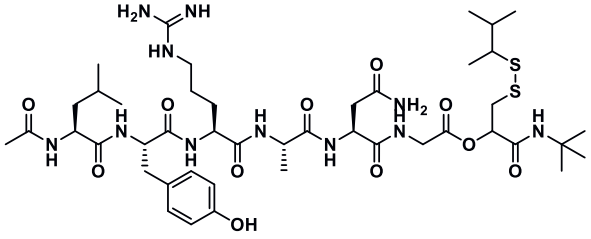
Ac-LYRANF-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ^3-propanamide (7f)



Prepared from Ac-LYRANF-OH (**5f**, 41 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (25.8 mg, 23.7 µmol, 47%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 6.11 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1086.5 (100%), $[M+2H]^{2+}$: 543.8 (100%); Mass Found (ESI^+); 1086.6 $[M+H]^+$, 544.1 $[M+2H]^{2+}$.

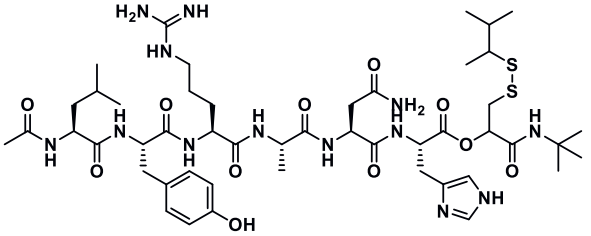
Ac-LYRANG-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ^3-propanamide (7g)



Prepared from Ac-LYRANG-OH (**5g**, 37 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (29.3 mg, 29.4 µmol, 59%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.67 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 996.5 (100%), $[M+2H]^{2+}$: 498.6 (100%); Mass Found (ESI^+); 996.5 $[M+H]^+$, 498.9 $[M+2H]^{2+}$.

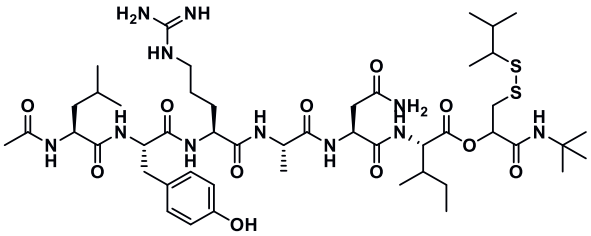
Ac-LYRANH-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7h)



Prepared from Ac-LYRANH-OH (**5h**, 41 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (32.7 mg, 30.4 µmol, 61%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.10 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1076.5 (100%), $[M+2H]^{2+}$: 538.8 (100%); Mass Found (ESI^+); 1076.7 $[M+H]^+$, 539.2 $[M+2H]^{2+}$.

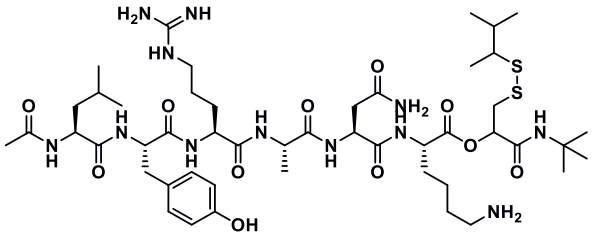
Ac-LYRANI-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7i)



Prepared from Ac-LYRANI-OH (**5i**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (22.7 mg, 21.6 µmol, 43%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 6.08 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1052.6 (100%), $[M+2H]^{2+}$: 526.8 (100%); Mass Found (ESI^+); 1052.6 $[M+H]^+$, 527.1 $[M+2H]^{2+}$.

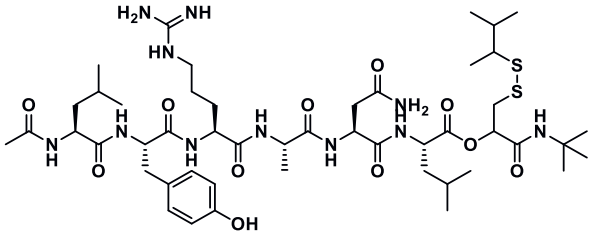
Ac-LYRANK-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7k)



Prepared from Ac-LYRANK-OH (**5k**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (39.9 mg, 37.4 µmol, 75%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.10 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1067.6 (100%), $[M+2H]^{2+}$: 534.3 (100%); Mass Found (ESI^+); 1067.6 $[M+H]^+$, 534.5 $[M+2H]^{2+}$.

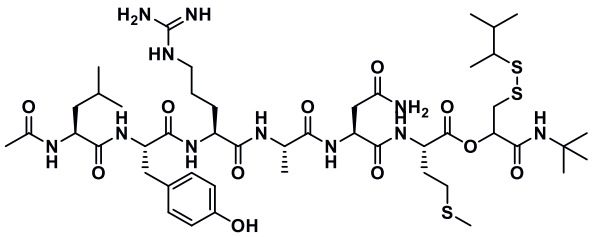
Ac-LYRANL-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7l)



Prepared from Ac-LYRANL-OH (**5l**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (38.6 mg, 36.7 µmol, 73%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 6.08 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1052.6 (100%), $[M+2H]^{2+}$: 526.8 (100%); Mass Found (ESI^+); 1052.6 $[M+H]^+$, 527.1 $[M+2H]^{2+}$.

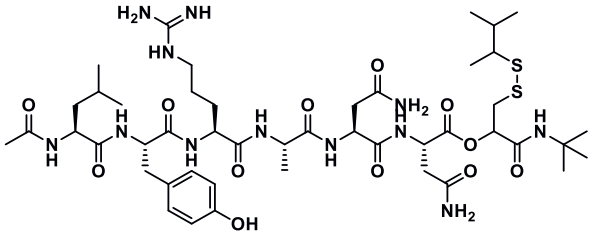
Ac-LYRANM-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7m)



Prepared from Ac-LYRANM-OH (**5m**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (29.5 mg, 27.6 µmol, 55%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.94 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1070.5 (100%), $[M+2H]^{2+}$: 535.8 (100%); Mass Found (ESI^+); 1070.5 $[M+H]^+$, 536.1 $[M+2H]^{2+}$.

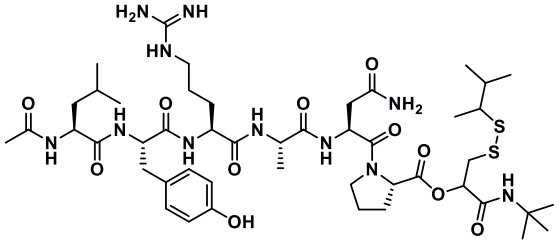
Ac-LYRANN-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7n)



Prepared from Ac-LYRANN-OH (**5n**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (21 mg, 20 µmol, 40%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.55 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1053.5 (100%), $[M+2H]^{2+}$: 527.3 (100%); Mass Found (ESI^+); 1053.6 $[M+H]^+$, 527.5 $[M+2H]^{2+}$.

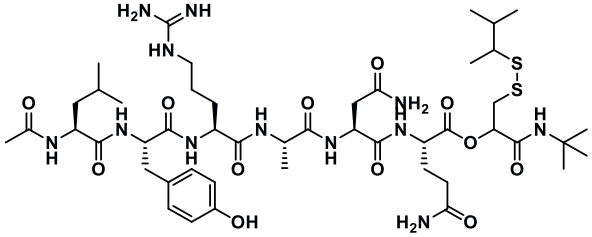
Ac-LYRANP-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7p)



Prepared from Ac-LYRANP-OH (**5p**, 39 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (17.9 mg, 17.3 µmol, 35%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.72 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1036.5 (100%), $[M+2H]^{2+}$: 518.8 (100%); Mass Found (ESI^+); 1036.6 $[M+H]^+$, 519.0 $[M+2H]^{2+}$.

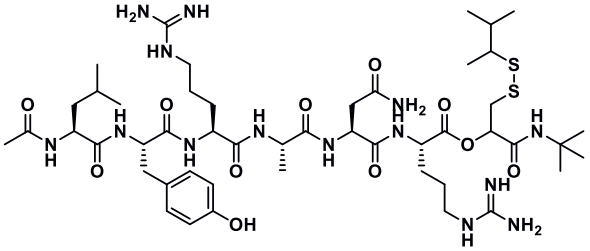
Ac-LYRANQ-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7q)



Prepared from Ac-LYRANQ-OH (**5q**, 40 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (31.2 mg, 29.2 µmol, 58%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.54 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1067.5 (100%), $[M+2H]^{2+}$: 534.3 (100%); Mass Found (ESI^+); 1067.6 $[M+H]^+$, 534.5 $[M+2H]^{2+}$.

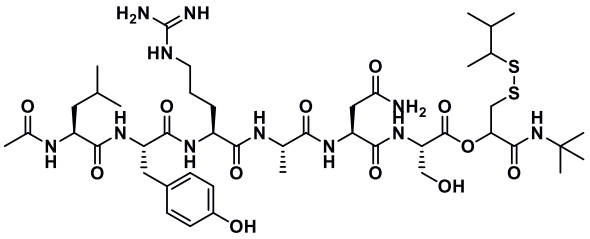
Ac-LYRANR-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7r)



Prepared from Ac-LYRANR-OH (**5r**, 42 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (28.8 mg, 26.3 µmol, 53%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.09 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1095.6 (100%), $[M+2H]^{2+}$: 548.3 (100%); Mass Found (ESI^+); 1095.6 $[M+H]^+$, 548.7 $[M+2H]^{2+}$.

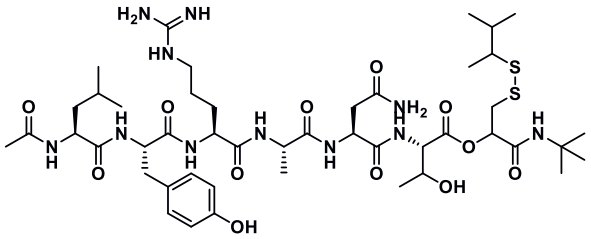
Ac-LYRANS-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7s)



Prepared from Ac-LYRANS-OH (**5s**, 38 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (15.9 mg, 15.5 µmol, 31%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.65 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1026.5 (100%), $[M+2H]^{2+}$: 513.8 (100%); Mass Found (ESI^+); 1026.6 $[M+H]^+$, 514.0 $[M+2H]^{2+}$.

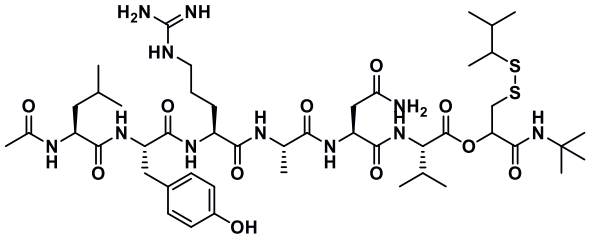
Ac-LYRANT-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7t)



Prepared from Ac-LYRANT-OH (**5t**, 39 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (22.5 mg, 21.6 µmol, 43%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.74 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1040.5 (100%), $[M+2H]^{2+}$: 520.8 (100%); Mass Found (ESI^+); 1040.6 $[M+H]^+$, 521.0 $[M+2H]^{2+}$.

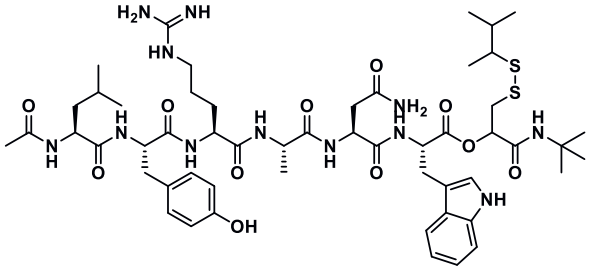
Ac-LYRANV-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7v)



Prepared from Ac-LYRANV-OH (**5v**, 39 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (17.7 mg, 17.0 µmol, 34%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.89 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1038.5 (100%), $[M+2H]^{2+}$: 519.8 (100%); Mass Found (ESI^+); 1038.6 $[M+H]^+$, 520.0 $[M+2H]^{2+}$.

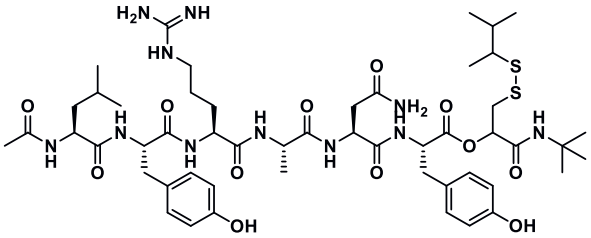
Ac-LYRANW-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7w)



Prepared from Ac-LYRANW-OH (**5w**, 43 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (23.8 mg, 21.1 µmol, 42%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 6.13 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1125.6 (100%), $[M+2H]^{2+}$: 563.3 (100%); Mass Found (ESI^+); 1125.6 $[M+H]^+$, 563.6 $[M+2H]^{2+}$.

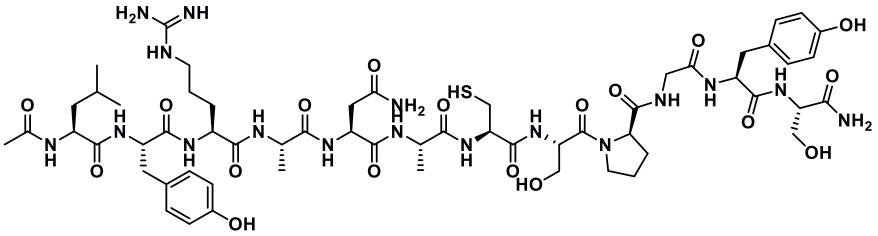
Ac-LYRANY-N-(tert-butyl)-3-((3-methylbutan-2-yl)disulfaneyl)-2λ³-propanamide (7y)



Prepared from Ac-LYRANY-OH (**5y**, 42 mg, 50 µmol, 1 equiv), **4a** (89 mg, 0.5 mmol, 10 equiv) and *t*-butyl isocyanide (57 µL, 0.5 mmol, 10 equiv) according to procedure D. Purification was done by preparative HPLC. Isolated as white fluffy powder (31.1 mg, 28.2 µmol, 56%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 5.86 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1102.5 (100%), $[M+2H]^{2+}$: 551.8 (100%); Mass Found (ESI^+); 1102.6 $[M+H]^+$, 552.1 $[M+2H]^{2+}$.

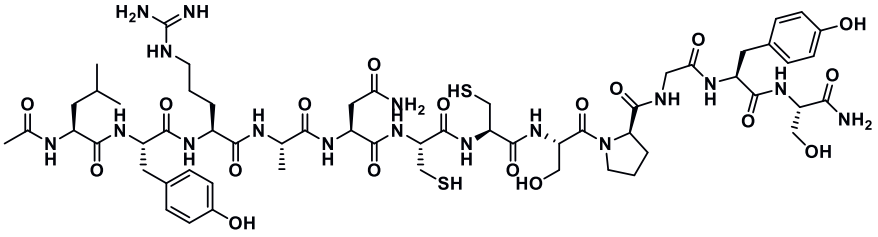
Ac-LYRANACSPGYS-NH_2 (12a)



Prepared from **7a** (5.05 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (2.4 mg, 1.8 µmol, 36%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.45 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1342.6 (100%), $[M+2H]^{2+}$: 671.8 (100%); Mass Found (ESI^+); 1342.7 $[M+H]^+$, 672.1 $[M+2H]^{2+}$.

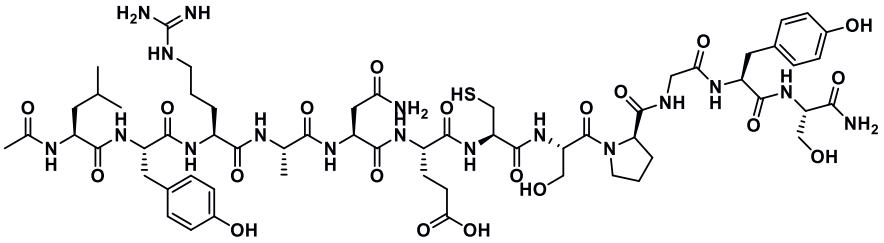
Ac-LYRANCCSPGYS-NH_2 (12c)



Prepared from **8** (1.93 mg, 1.85 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 2.27 mg, 3.7 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (0.8 mg, 0.6 µmol, 30%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.49 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1374.6 (100%), $[M+2H]^{2+}$: 687.8 (100%); Mass Found (ESI^+); 1375.7 $[M+H]^+$, 688.2 $[M+2H]^{2+}$.

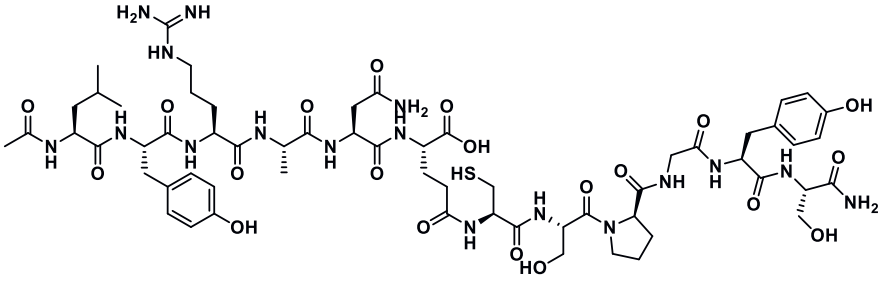
Ac-LYRANECSPGYS-NH_2 (12e)



Prepared from **7e** (6.65 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (0.6 mg, 0.6 µmol, 9%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.47 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1400.6 (100%), $[M+2H]^{2+}$: 700.8 (100%); Mass Found (ESI^+); 1401.8 $[M+H]^+$, 701.2 $[M+2H]^{2+}$.

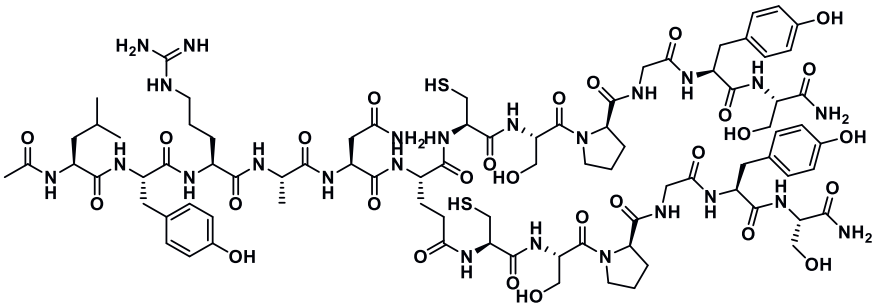
Ac-LYRANE(CSPGYS-NH_2)-OH (12e)



Prepared from **7e** (6.65 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (0.2 mg, 0.14 µmol, 3%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.44 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1400.6 (100%), $[M+2H]^{2+}$: 700.8 (100%); Mass Found (ESI^+); 1401.8 $[M+H]^+$, 701.2 $[M+2H]^{2+}$.

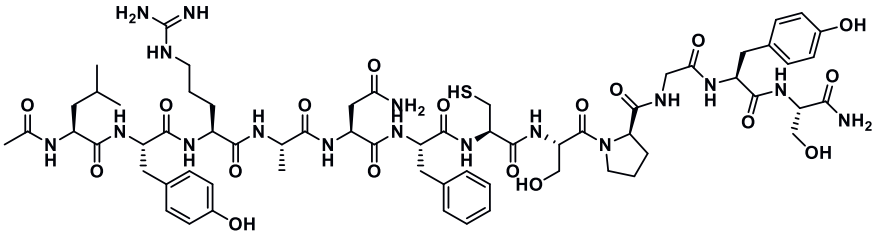
Ac-LYRANE(CSPGYS-NH_2)CSPGYS-NH_2 (12e)



Prepared from **7e** (6.65 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (1.6 mg, 0.8 µmol, 16%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.53 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1994.9 (100%), $[M+2H]^{2+}$: 997.4 (100%); Mass Found (ESI^+); 998.0 $[M+2H]^{2+}$.

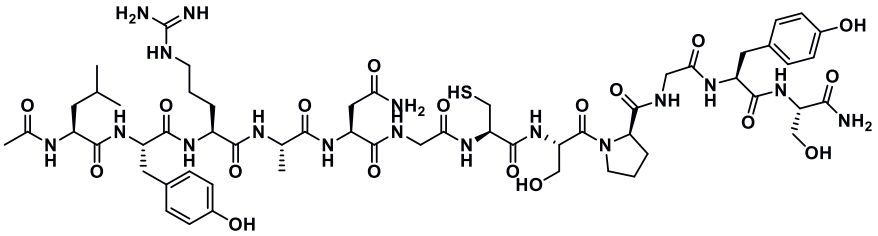
Ac-LYRANFCSPGYS-NH_2 (12f)



Prepared from **7f** (5.43 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (3.8 mg, 2.7 µmol, 54%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.76 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1418.7 (100%), $[M+2H]^{2+}$: 709.8 (100%); Mass Found (ESI^+); 1418.8 $[M+H]^+$, 710.2 $[M+2H]^{2+}$.

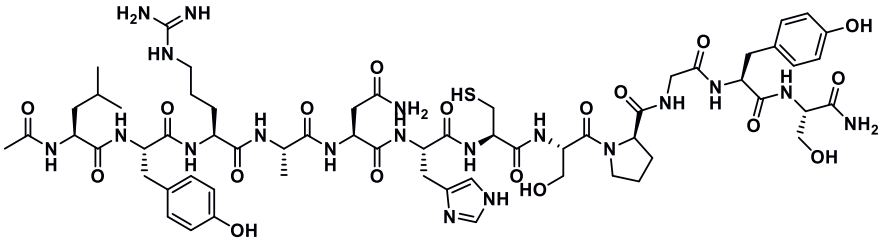
Ac-LYRANGCSPGYS-NH_2 (12g)



Prepared from **7g** (4.98 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.6 mg, 3.5 µmol, 69%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.41 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1328.6 (100%), $[M+2H]^{2+}$: 664.8 (100%); Mass Found (ESI^+); 1328.7 $[M+H]^+$, 665.1 $[M+2H]^{2+}$.

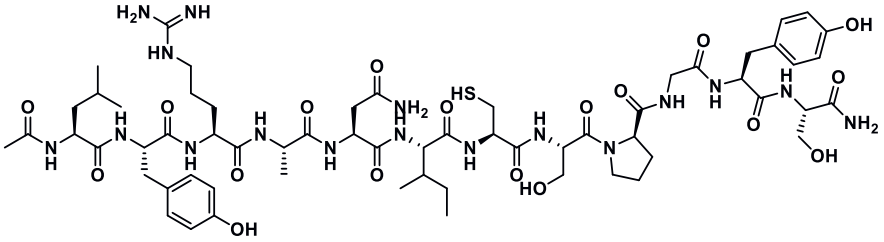
Ac-LYRANHCSPGYS-NH$_2$ (12h)



Prepared from **7h** (5.38 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.7 mg, 3.3 µmol, 67%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.13 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1408.6 (100%), $[M+2H]^{2+}$: 704.8 (100%); Mass Found (ESI^+); 1408.9 $[M+H]^+$, 705.2 $[M+2H]^{2+}$.

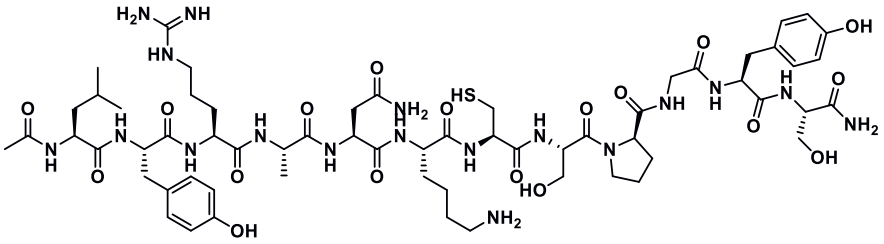
Ac-LYRANICSPGYS-NH$_2$ (12i)



Prepared from **7i** (5.26 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (0.9 mg, 0.65 µmol, 13%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.66 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1384.7 (100%), $[M+2H]^{2+}$: 692.8 (100%); Mass Found (ESI^+); 1384.7 $[M+H]^+$, 693.2 $[M+2H]^{2+}$.

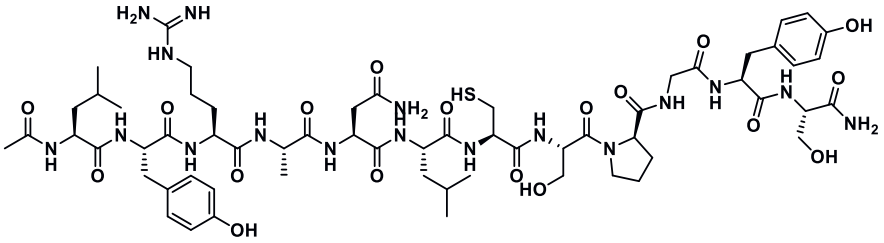
Ac-LYRANKCSPGYS-NH$_2$ (12k)



Prepared from **7k** (5.34 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (3.7 mg, 2.6 µmol, 53%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.14 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1399.7 (100%), $[M+2H]^{2+}$: 700.3 (100%); Mass Found (ESI^+); 1399.8 $[M+H]^+$, 700.7 $[M+2H]^{2+}$.

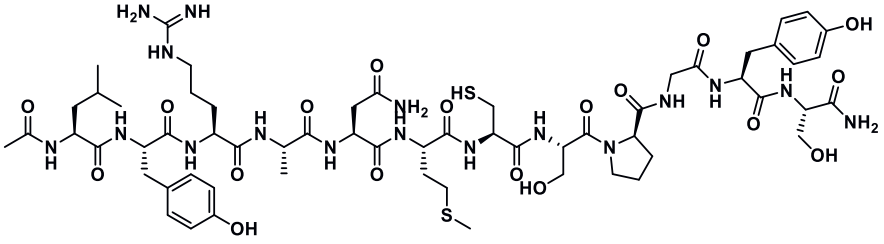
Ac-LYRANLCSPGYS-NH_2 (12l)



Prepared from **7l** (5.26 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (2.9 mg, 2.1 µmol, 42%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.69 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1384.7 (100%), $[M+2H]^{2+}$: 692.8 (100%); Mass Found (ESI^+); 1384.7 $[M+H]^+$, 693.2 $[M+2H]^{2+}$.

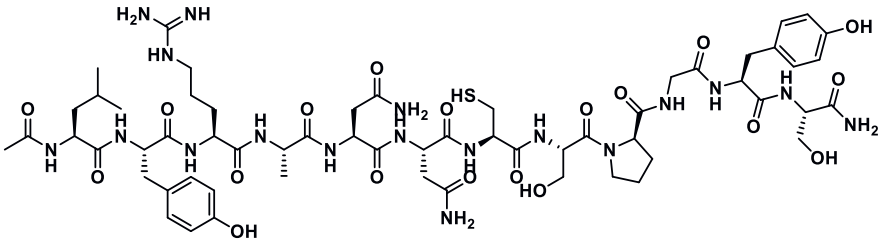
Ac-LYRANMCSPGYS-NH_2 (12m)



Prepared from **7m** (5.35 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.7 mg, 3.4 µmol, 67%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.59 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1402.6 (100%), $[M+2H]^{2+}$: 701.8 (100%); Mass Found (ESI^+); 1402.9 $[M+H]^+$, 702.2 $[M+2H]^{2+}$.

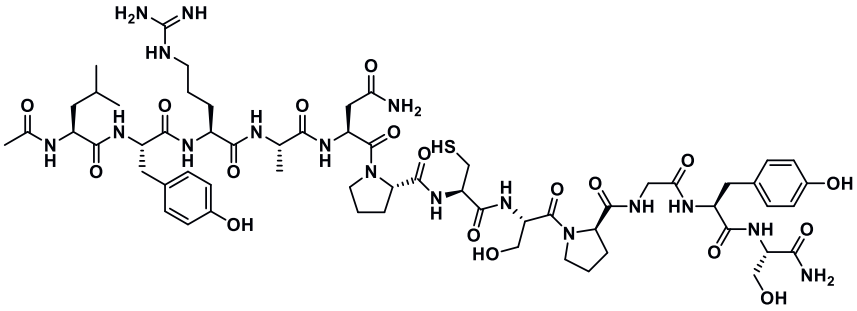
Ac-LYRANNCSPGYS-NH_2 (12n)



Prepared from **7n** (5.27 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.4 mg, 3.2 µmol, 64%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.37 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1385.6 (100%), $[M+2H]^{2+}$: 693.3 (100%); Mass Found (ESI^+); 1385.9 $[M+H]^+$, 693.7 $[M+2H]^{2+}$.

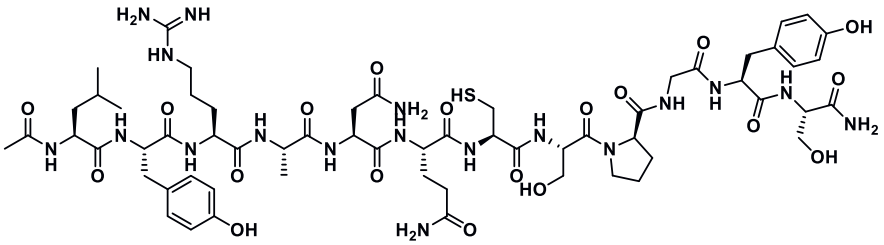
Ac-LYRANPCSPGYS-NH_2 (12p)



Prepared from **7p** (5.18 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (1.0 mg, 0.73 µmol, 15%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.50 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1368.6 (100%), $[M+2H]^{2+}$: 684.8 (100%); Mass Found (ESI^+); 1368.9 $[M+H]^+$, 685.2 $[M+2H]^{2+}$.

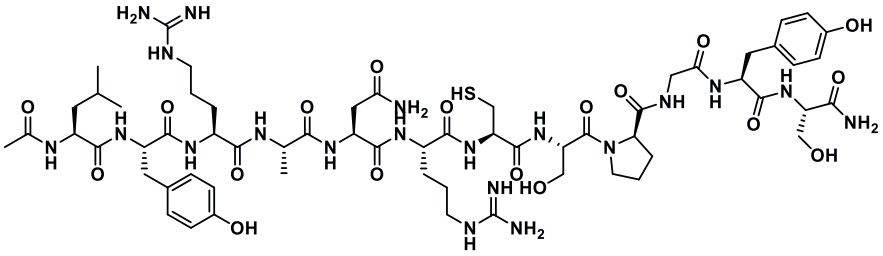
Ac-LYRANQCSPGYS-NH_2 (12q)



Prepared from **7q** (5.34 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.8 mg, 3.4 µmol, 69%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.39 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1399.6 (100%), $[M+2H]^{2+}$: 700.3 (100%); Mass Found (ESI^+); 1399.9 $[M+H]^+$, 700.7 $[M+2H]^{2+}$.

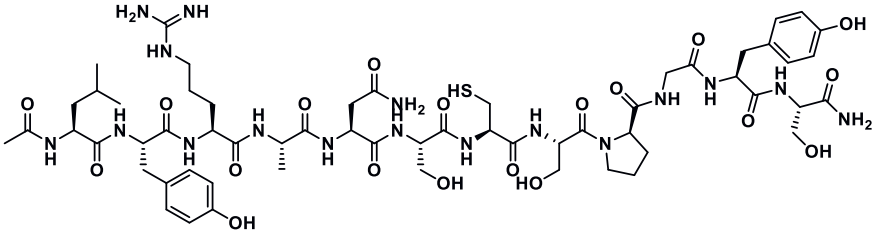
Ac-LYRANRCSPGYS-NH$_2$ (12r)



Prepared from **7r** (5.48 mg, 5.0 µmol, 1 equiv) and NH$_2$-CSPGYS-NH$_2$ (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.8 mg, 3.4 µmol, 67%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.16 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1427.7 (100%), $[M+2H]^{2+}$: 714.3 (100%); Mass Found (ESI^+); 1427.9 $[M+H]^+$, 714.8 $[M+2H]^{2+}$.

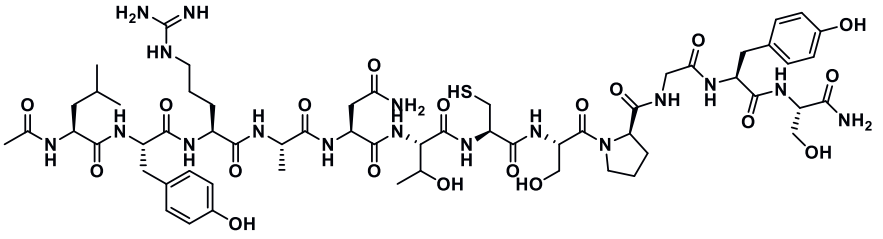
Ac-LYRANSCSPGYS-NH$_2$ (12s)



Prepared from **7s** (5.13 mg, 5.0 µmol, 1 equiv) and NH$_2$-CSPGYS-NH$_2$ (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.7 mg, 3.5 µmol, 69%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.40 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1358.6 (100%), $[M+2H]^{2+}$: 679.8 (100%); Mass Found (ESI^+); 1358.9 $[M+H]^+$, 680.2 $[M+2H]^{2+}$.

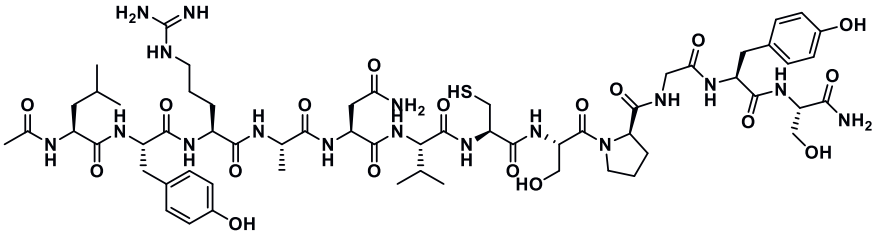
Ac-LYRANTCSPGYS-NH$_2$ (12t)



Prepared from **7t** (5.20 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.1 mg, 3.0 µmol, 60%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.43 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1372.6 (100%), $[M+2H]^{2+}$: 686.8 (100%); Mass Found (ESI^+); 1372.8 $[M+H]^+$, 687.2 $[M+2H]^{2+}$.

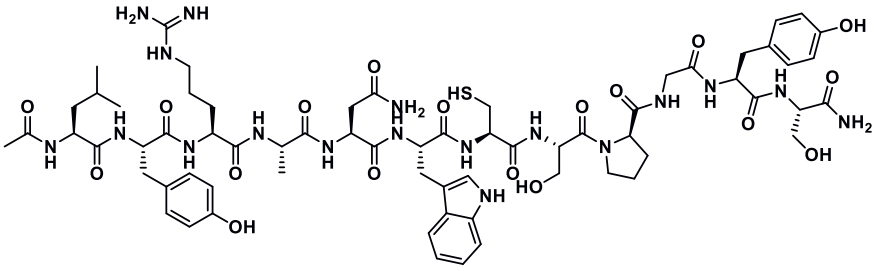
Ac-LYRANVCSPGYS-NH_2 (12v)



Prepared from **7v** (5.19 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (2.1 mg, 1.5 µmol, 31%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.55 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1370.7 (100%), $[M+2H]^{2+}$: 685.8 (100%); Mass Found (ESI^+); 1370.9 $[M+H]^+$, 686.2 $[M+2H]^{2+}$.

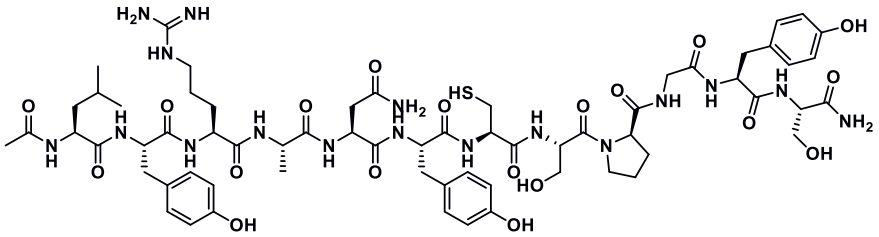
Ac-LYRANWCSPGYS-NH_2 (12w)



Prepared from **7w** (5.63 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.6 mg, 3.2 µmol, 63%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.81 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1457.7 (100%), $[M+2H]^{2+}$: 729.3 (100%); Mass Found (ESI^+); 1458.9 $[M+H]^+$, 729.7 $[M+2H]^{2+}$.

Ac-LYRANYCSPGYS-NH_2 (12y)



Prepared from **7y** (5.51 mg, 5.0 µmol, 1 equiv) and NH_2-CSPGYS-NH_2 (**11**, 6.12 mg, 10.0 µmol, 2 equiv) according to procedure E. Purification was done by preparative HPLC. Isolated as white fluffy powder (4.5 mg, 3.1 µmol, 63%).

Analytical HPLC trace of HPLC-prep purified title compound; Rt 4.55 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 1434.6 (100%), $[M+2H]^{2+}$: 717.8 (100%); Mass Found (ESI^+); 1435.8 $[M+H]^+$, 718.2 $[M+2H]^{2+}$.

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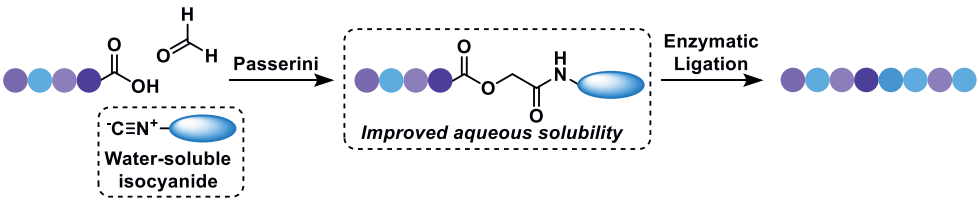
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Chapter 4

Synthesis of Water-Soluble Isocyanides for C-terminal Peptide Modification.



Abstract: *Protein solubility critically influences peptide and protein modification and application, yet chemical functionalization is often compromised under aqueous/buffer conditions. To address this limitation, we developed an unprecedented class of ionic, water-soluble isocyanide reagents for the functionalization of peptide carboxyl groups under aqueous conditions. These isocyanides uniquely combine strong hydrogen-bonding motifs with ionic functionalities, enabling enhanced solubility and modulation of peptide isoelectric points. The compounds were synthesized via a concise route and applied successfully in Passerini multicomponent reactions without observable precipitation. The resulting esterified peptides were competent substrates for omniligase-1-mediated ligation, revealing a preference for polar cationic products. This work demonstrates that rational isocyanide design can mitigate solubility losses associated with peptide esterification and expands the scope of isocyanide-based ligation chemistry in aqueous environments.*

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4.1 Introduction

Tuning the surface charge of a protein is a proven way to improve its biopharmaceutical suitability, because electrostatics strongly influence solubility, viscosity, and aggregation in formulation. Importantly, these same properties are not only central to biopharmaceutical development but are also frequent bottlenecks in basic protein research, where workable concentrations are often required.[1] Across applications, wether in fundamental research or applied contexts, sufficient protein solubility is often essential for success. Structural and biochemical investigations typically require protein concentrations of several mg/mL, while therapeutic proteins administered subcutaneously may demand concentrations as high as 50–100 mg/mL.[2,3] High-throughput structural genomics efforts have highlighted solubility and aggregation as the limiting obstacle, effectively excluding a significant number of proteins from detailed study.[4,5] Consequently, a substantial fraction of proteins exhibit poor solubility, severely limiting their accessibility for such applications. Indeed, many recombinant proteins (around 80%) cannot be obtained in soluble form, and even proteins that are initially soluble frequently aggregate or precipitate upon concentration.[6] Improving protein solubility is therefore widely recognized as essential for expanding the range of proteins that can be characterized and advanced for downstream applications (*e.g.*, rational drug design).[7]

Numerous strategies have been explored to overcome protein solubility limitations. These include optimization of expression, purification, and refolding protocols, as well as the use of buffer additives such as salts, ionic compounds, detergents, and other excipients.[7–12] While systematic screening of buffer conditions can improve solubility in many cases, it is labor-intensive and often require multiple iterations, particularly for intrinsically insoluble proteins.[7] Alternatively, the protein itself can be engineered or modified, for example by mutating hydrophobic residues[13], attaching polyethylene glycol chains[14], or exploiting naturally occurring post-translational modifications.[15] Among these, glycosylation is frequently associated with enhanced solubility, although mechanistic interpretation is complicated by sample heterogeneity.[16]

Chemical modification strategies that modulate protein/peptide solubility and/or charge have also been developed to enable intracellular delivery. These include, for example, covalent attachment of cationic cell-penetrating peptides[17–21] and chemically masking carboxylates through esterification.[22–27] However, esterification of carboxylate groups reduces the anionic character of proteins, while typically increasing hydrophobicity and shifting the isoelectric point toward physiological pH.[28–30] Although such modifications can enable valuable functions (*i.e.*, ligation and intracellular delivery), they often promote aggregation or precipitation, particularly under near-physiological conditions.[31] This creates a fundamental challenge: the development of chemical transformations that are

compatible with highly polar, aqueous environments and that preserve protein solubility. In this context, the availability of suitably designed, water-soluble reagents becomes a critical requirement for their application in peptide and protein functionalization.

The Raines group recently reported a methodology to generate protein esters with improved aqueous solubility by incorporating substituents with higher hydrogen-bond basicity (**Figure 1A**).[31] In **Chapter 2**, we disclosed a methodology which employed the isocyanide-based Passerini three component reaction for the esterification and subsequent ligation of peptide carboxyl groups (**Figure 1B**).[32] Therein, esterification indeed lowered solubility, and application to larger fragments remained challenging. Here, we describe the design and synthesis of polar, water-soluble isocyanides and evaluate their suitability for peptide functionalization and ligation (**Figure 1C**). Notably, charged water-soluble isocyanides that enable isocyanide-based multicomponent reactions directly on peptides and proteins in aqueous media are essentially unknown. Reported water-soluble variants (*e.g.*, sugar-derived) are largely neutral and do not tune biomolecular charge, while cationic tertiary-amine isocyanides are prone to decomposition.[33–36]

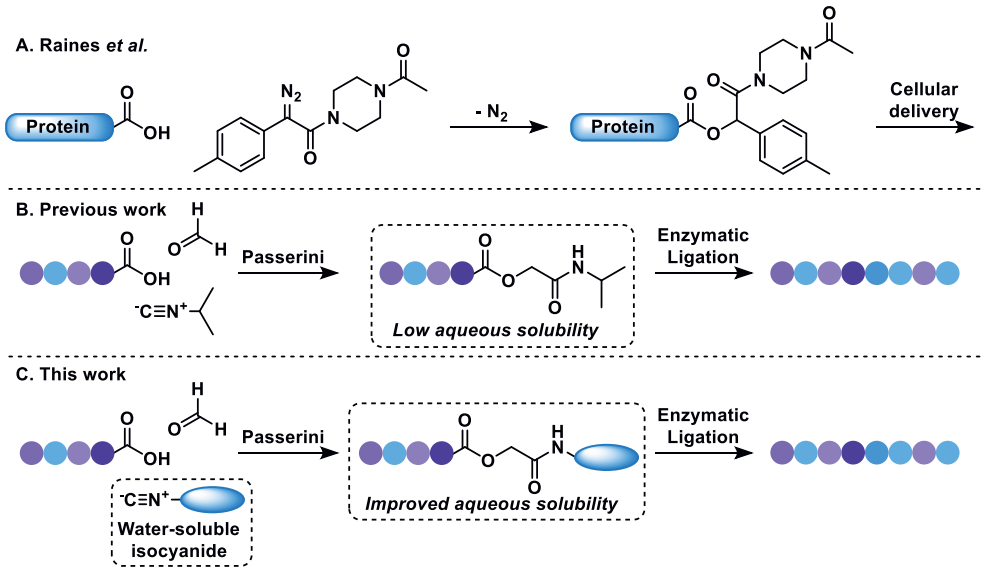


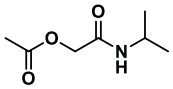
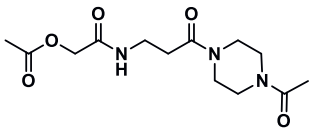
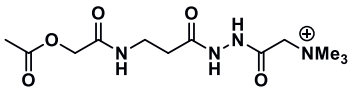
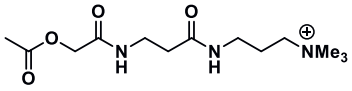
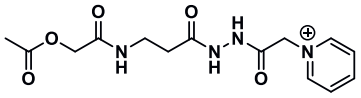
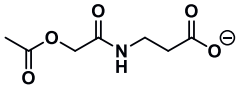
Figure 5. A. Esterification of protein with diazocompounds for intracellular delivery. B. **Chapter 2 -** Passerini-based esterification of peptides leading to low-solubility products. C. This work - water-soluble isocyanides for the Passerini functionalization of peptides leading to better soluble products.

4.2 Results and Discussion

4.2.1 Rational design of polar isocyanides

To quantify how Passerini modification alters carboxylic acid side chains, we estimated hydrophilicity using the octanol/water partition coefficient (log*P*). Log*P* is defined as the ratio of solute concentration in octanol to that in water,[37] where higher log*P* values indicate greater lipophilicity and typically lower solubility in water. Accordingly we calculated Log*P* (cLog*P*) and topological polar surface area (TPSA) of the model Passerini products derived from the designed isocyanides, formaldehyde and acetic acid utilizing both Molinspiration [38] and Chemdraw (**Table 1**). As expected, the cLog*P* of ester **2** is significantly higher than that of the corresponding free carboxylate **1**, indicating that esterification indeed decreases the water-solubility compared to the carboxylate. We opted to increase the solubility of the esters by introducing additional hydrogen-bonding moieties, replacing the isopropyl moiety in **2** with more polar structures to lower cLog*P* and raise TPSA.

Table 1. Physiochemical Properties of esters bearing polar functional groups[a].

Nr.	Ester	cLogP	TPSA
1		-2.78 ***-4.194***	40.13 ***40.13***
2		0.16 ***0.05***	55.40 ***55.40***
3		-1.64 ***-1.07***	96.02 ***96.02***
4		-5.10 ***-5.57***	113.60 **113.60**
5		-4.59 ***-4.55***	84.50 ***84.50***
6		1.10 ***-4.71***	117.48 ***116.61***
7		-3.71 ***-3.653***	95.53 ***95.53***

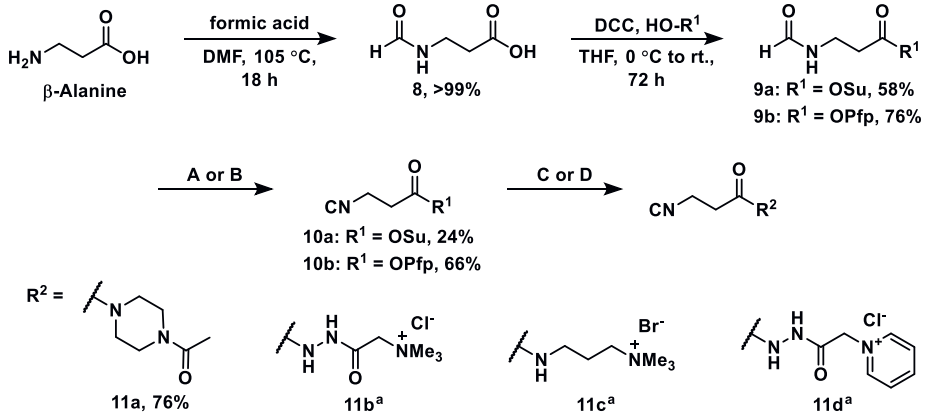
[a] cLog*P* and TPSA values were determined using Molinspiration Cheminformatics and Chemdraw (in bold) software.

Initially, we investigated an *N*-β-alanine-*N*-acetylpiperazine motif (**3**), which was recently reported by Raines *et al.* as optimal for enhancing aqueous solubility while preserving

lipophilicity.[31] Amides are known hydrogen-bond acceptors, and their acceptor strength can be quantified by the hydrogen-bond basicity parameter (pK_{BHX}), determined from complexation with 4-fluorophenol.[39] This provides the equation $pK_{BHX} = -\log_{10}K_{BHX} = \log_{10}K$, K being the equilibrium association constant of the hydrogen-bond acceptor with 4-fluorophenol. A higher K value relates to a high pK_{BHX} value and normally relates to higher water-solubility for hydrogen-bond acceptors. The hydrogen-bond capacity of amides is typically pK_{BHX} 2.00–2.55, indicating good acceptor capacity.[39] Consistent with this, the clog*P* of **3** is markedly lower than that of **2**. We next included cationic (**4**-**6**) and anionic (**7**) functional groups, since these are well-known motifs for increasing water solubility. Accordingly, their clog*P* values are substantially lower compared to the neutral esters (**2**-**3**). Tetramethylammonium salts can also engage in hydrogen bonding as weak hydrogen-bond acids (pK_{AHX} of 0.57-0.82).[40] Furthermore, we selected hydrazides as linker (**4** and **6**) instead of amides, since hydrazides can engage strongly in hydrogen bonding and reduce the cLog*P* while increasing the TPSA, consistent with the values observed for **4** and **5**.[41,42] Overall, all novel ester sidechains exhibit higher TPSA relative to ester **2**.

4.2.2 Polar isocyanide synthesis

With these new polar isocyanide designs in hand, we set out to synthesize them (**Scheme 1**). Starting from β-alanine, formylation using formic acid at elevated temperatures provided formamide **8** in quantitative yield. Subsequently, the carboxylic acid was converted to an active ester using DCC in combination with *N*-hydroxysuccinimide (HOSu) or pentafluorophenol (HOPfp) providing **9a** and **9b**, respectively. Initial attempts for the subsequent dehydration to form **10a** with $POCl_3$ resulted in decomposition and trace product formation, likely owing to side reactions with the succinimide carbonyls. Switching to the milder Burgess reagent afforded the desired isocyanide **10a** in 24% yield. In contrast, compound **9b**, which did not bear additional amide-like functionalities, could be dehydrated under the conventional conditions with $POCl_3$ to form isocyanide compound **10b** in 66% yield. Finally, substitution with various nucleophilic nitrogen species bearing polar functionalities provided polar isocyanides **11a**-**11d**. For the structures bearing ionic moieties (**11b**-**11d**), attempts at isolation by preparative HPLC proved tedious and lowered the quality of the material, likely caused by partial decomposition of the isocyanide moiety, even under pH neutral conditions. Having observed complete conversion in the formation of these compounds, we proceeded with their application without further purification.

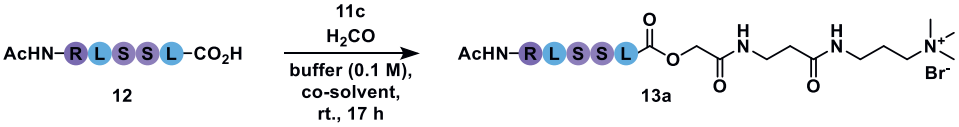


Scheme 1. Synthesis route to isocyanides bearing polar functional groups. A. Burgess reagent, DCM, 50 °C, 1.5 h. B. $POCl_3$, THF, 0 °C, 1 h. C. Amine, DCM, rt. D. Amine, MeOH, rt. [a] Compounds were unstable during isolation and were used without further isolation.

4.2.3 Optimization of the Passerini reaction

We optimized the Passerini reaction with our novel polar isocyanide (**11c**), expecting it would be the most stable ionic substrate. Given our prior work on Passerini functionalization of peptide C-termini, we selected the pentapeptide Ac-RLSSL-OH (**12**) as model substrate in combination with formaldehyde, since it is both ideal for enzymatic ligations and does not increase hydrophobicity (**Table 2**).[32] Investigating the effect of pH in phosphate buffer revealed an optimal pH range 4.5-5.5 (**Table 2**, entries 1-7). At lower pH, the isocyanide rapidly degraded to the corresponding formamide, consistent with acid promoted hydrolysis. Moreover, the higher pH maintains the free carboxylate form of the C-terminus. Next, we compared the different buffer types on the reaction (**Table 2**, entries 8-10). Using a MES buffer provided the highest yield of Passerini product **13a**, while TRIS significantly reduced the yield of **13a**. Upon exploring different co-solvents (**Table 2**, entries 11-16), we found that the reaction proceeds most effectively when combining the MES buffer with DMSO and THF. Because DMSO resulted in a biphasic mixture, we proceeded with THF. Finally, raising the co-solvent concentration increased the yield (**Table 2**, entries 17-19). However, 50% cosolvent was selected as a practical compromise to enable application to large peptides and proteins.

Table 2. Optimization of the Passerini reaction[a].



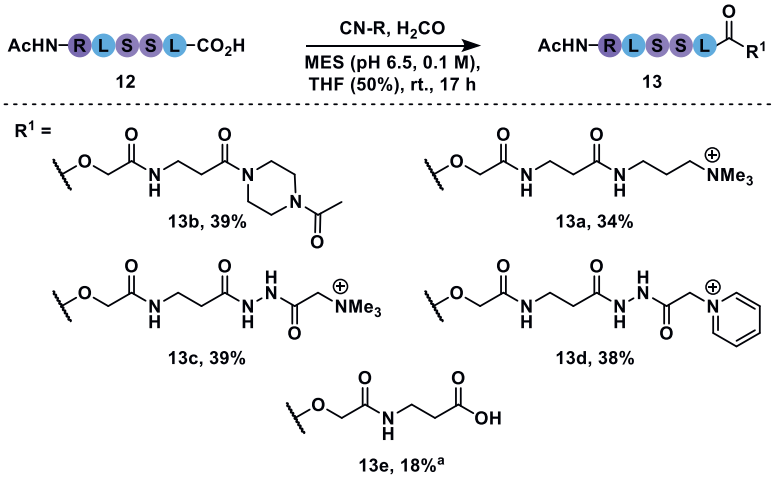
Entry	Buffer	pH	Co-solvent	Yield (%)[b]
1[c]	PB	1.5	MeCN (20%)	<1
2[c]	PB	2.5	MeCN (20%)	9
3[c]	PB	3.5	MeCN (20%)	9
4[c]	PB	4.5	MeCN (20%)	26
5[c]	PB	5.5	MeCN (20%)	31
6[c]	PB	6.5	MeCN (20%)	18
7[c]	PB	7.5	MeCN (20%)	3
8[c]	MES	6.5	MeCN (20%)	42
9[c]	HEPES	6.8	MeCN (20%)	39
10[c]	TRIS	6.5	MeCN (20%)	7
11[d]	MES	6.5	MeCN (20%)	27
12[d]	MES	6.5	DMSO (20%	36
13[d]	MES	6.5	MeOH (20%)	20
14[d]	MES	6.5	THF (20%)	36
15[d]	MES	6.5	DMF (20%)	30
16[d]	MES	6.5	NMP (20%)	29
17[e]	MES	6.5	THF (10%)	5
18[e]	**MES**	**6.5**	**THF (50%)**	**43**
19[e]	MES	6.5	THF (75%)	55

[a] Standard conditions: peptide **12** (6.84 µmol, 10 mM), formaldehyde (68.4 µmol), and **11c** in buffer (0.1 M) with co-solvent, stirred at indicated temperature and time. [b] Based on HPLC-MS analysis at 215 nm using *trans*-cinnamic acid as internal standard. [c] Isocyanide was added in MeCN (20% of total reaction volume). [d] Isocyanide was added in MES buffer (20% of total reaction volume). [e] Isocyanide was added in MES buffer (25% of total reaction volume).

4.2.4 Scope of the Passerini reaction

With our optimal conditions in hand, we performed the Passerini reaction on Ac-RLSSL-OH (**12**) with our synthesized novel polar isocyanides (**Scheme 2**). We were delighted to find that the reactions provided the desired ester products, albeit in moderate yields. Notably, in contrast to reactions with nonpolar substrates, no precipitate formed during the course of the reaction, providing support for the higher solubility of the products. To obtain an ester bearing an active ester handle for late-stage diversification, we also performed the reaction with isocyanide **10a**. However, the OSu-ester of this isocyanide hydrolyzed during the course of the reaction, leading to the corresponding free carboxylic acid **13e**. This product is also of interest since it allows for the functionalization of C-terminal acids towards an ester without the loss of the negative charge under physiological pH. The lower

yield of **13e** compared to the other products is likely due to competing Passerini oligomer formation of the additional free carboxylic acid with itself and formaldehyde.



Scheme 2. Scope of the Passerini reaction with polar isocyanides. [a] Synthesized with **10a** as isocyanide component in PB (pH 6.5, 0.1 M) and MeCN (10%).

To mitigate hydrolysis of the active ester during the Passerini reaction, we tried the OPfp ester, considering that it would be more stable under the reaction conditions (**Figure 2A**). In principle, the OPfp group could then be hydrolyzed selectively to afford the desired free carboxylic acid ester **13e**. We were happy to find that employing isocyanide **10b** in the Passerini 3-CR provided the desired Pfp-ester **14** in acceptable yield with only minor hydrolysis of the active ester. We then investigated selective hydrolysis of ester **14** at various pH values and revealed that the desired product **13e** could best be obtained by increasing the pH to 7 after the reaction was complete and allowing hydrolysis for 120 mins. Increasing the pH further results in a loss of selectivity and hydrolysis of the formed Passerini ester. Moreover, the hydrolysis also afforded two byproducts, one of which remains to be identified (**16**, m/z 826). The other could be correlated with a cyclized structure, likely with the guanidine sidechain of Arg (**15**, **Figure 2B-C**).[43] Although the side reactions lower the yield, it does support the possibility of the Pfp ester to function as a handle for late-stage modifications of Passerini esterified peptides.

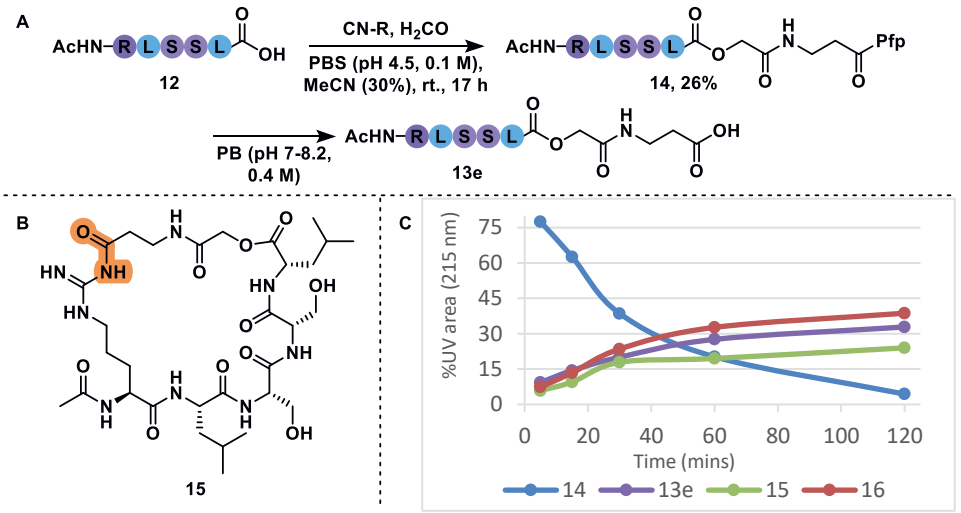
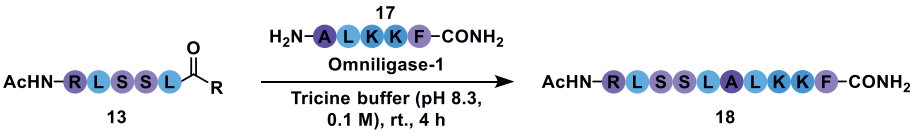


Figure 6. A Hydrolysis reaction of **14**. B. Byproduct (**15**) of the hydrolysis of **14**. C. Presence over compounds during hydrolysis reaction at pH 7. Expressed as UV area (215 nm) over time.

With the set of structurally diverse peptides bearing polar esters in hand, we evaluated their performance in enzymatic ligation using omniligase-1 and with the nucleophilic amine H_2N-ALKKF-NH_2 (**17**) to provide decamer **18** (**Table 3**). The results of these ligations are expressed as isolated yield after 4 hours of reaction time. Gratifyingly, all novel polar C-terminal esters provided the desired compound in acceptable yields (48-61%). Interestingly, the neutral polar ester and the cationic esters (**13a**-**13d**) reached full conversion within 2 hours, whereas the anionic ester bearing a carboxylate group (**13e**) required 4 hours for completion. This indicates that omniligase-1 prefers polar cationic or neutral moieties at those positions over anionic ones, thus providing insights into its substrate binding mode. Furthermore, polar esters bearing an acyl hydrazide functionality (*i.e.* **13c** and **13d**) were not cleaved at the hydrazide carbonyl-nitrogen bond, showing that these moieties are stable under the enzymatic ligations conditions and do not serve as substrates.

Table 3. Yields of **18** from various starting materials[a].



Entry	Ester	Scale (µmol)	Conversion (4 h)[b]	Yield (%)
1	**13a**	25.2	>95%	59
2	**13b**	21.9	>95%	49
3	**13c**	25.3	>95%	61
4	**13d**	10.4	>95%	50
5	**13e**	7.6	>95%	48

[a] Standard conditions: ester (**13**, 1 equiv), H_2N-ALKKF-NH_2 (**17**, 2 equiv), TCEP (2 mg/mL), and omniligase (0.14 mg/mL) in tricine buffer (pH 8.3, 0.1 M). [b] Based on consumption of the starting material ester on HPLC-MS analysis.

4.3 Conclusion

To conclude, we developed a new set of water-soluble isocyanides that enable functionalization of the carboxyl groups of unprotected peptides under aqueous conditions. These products could in turn be used as substrates for omniligase-1 mediated enzymatic ligations. The novel polar reagents included strong hydrogen bonding motifs (*i.e.*, amides, hydrazides) as well as ionic functionalities (*i.e.*, ammonium salts and a free carboxyl group) and were readily accessible from β-alanine. We found that OSu esters derived from **10a** hydrolyze under the Passerini reaction conditions whereas Pfp esters remain intact, allowing for selective hydrolysis of the latter and a potential late-stage modification strategy. Furthermore, no precipitation was observed during either the Passerini reactions or the ligations, underscoring the importance of the polar soluble groups. The ligations with the polar Passerini products revealed a preference for polar cationic substrates by the enzyme. In the future, these findings position the charged, water-soluble isocyanides described in this study as a general platform for late-stage modification of peptides, proteins, and other biomolecules under physiological conditions, thus expanding the chemical biology toolbox for modular tuning of solubility and charge.

4.4 Experimental Section

General Remarks

Unless stated otherwise, all solvents and commercially available reagents were used as received without further purification. Fmoc-protected amino-acids were purchased from Combi-blocks, Doug discovery and GLbiochem. Coupling reagents, solvents, buffer salts, carbonyl and isocyanide compounds were purchased from Sigma-Aldrich, Doug discovery, Combi-blocks, Iris Biotech, Biosolve, and TCI chemicals. Preparative-scale purification was performed using a Shimadzu Nexera preparative HPLC system, consisting of: a CBM-40 systems controller, LC-20AP preparative pump, FCV-200AL quaternary vale, SPD-M40 photodiode array detector (PDA), LCMS-2050 mass detector, LC-40D HPLC pump, LH-40 fraction collector, and an FCV-20AH2 valve unit. Separation was carried out using Shim-pack Scepter C_{18}-120 column (5 µm, 20x150 mm) with an optimised gradient for each compound (MeCN/MilliQ water with 0.1% TFA) at a flow rate of 20 mL/min. Fraction collection was triggered by PDA and/or MS signal detection. Peptide samples were lyophilized with a Büchi Lyovapor^{tm} L-250 Pro lyophilizer. High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) analyses were performed on a Shimadzu Nexera 2 UHPLC system equipped with a Shimadzu LC-30AD pump, a SPD-M30A photodiode array detector and LCMS-2020 single quadrupole detector. The eluent consisted of MilliQ water and LCMS grade acetonitrile, and 0.1% formic acid. A Waters XSelect CSH C18 column (3.0 mm × 75 mm with a particle size of 3.5 mm) was employed for the separation at 30 °C. The method comprised a gradient of acetonitrile in water: 5% (2 min) to 95% acetonitrile (6 min), 1 min at 95% acetonitrile and back to 5% acetonitrile (2 min). The substrates and products were quantified by their UV absorption at 220 nm. High resolution mass spectroscopy was conducted on a Bruker micrOTOF-Q II mass spectrometer (electrospray ionization). NMR spectra (1D ^{1}H and ^{13}C, COSY, DIPSI, $^{13}C^{1}H$ HSQC) were recorded on a Bruker Avance III HD 700 MHz spectrometer equipped with a TCI cryoprobe, or on a Jeol JNM-ECZL400S 400 MHz using the residual solvent or TMS standard (0 ppm) as reference. The data is reported as follows: chemical shift (δ ppm), coupling constant (J, Hz), multiplicity (s = singlet, d = doublet, t = triplet, q = 5 quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets).

General synthetic procedures

General procedure A: Passerini reaction with water soluble isocyanides.
Prepared according to a modified procedure by Hanssens *et al.*[32] To a stirred solution of Ac-RLSSL-OH (50 mg, 68.4 µmol, 1 equiv) and formaldehyde (37 wt% in water, 55.53 mg, 50.9 µL, 0.684 mmol, 10 equiv) in THF (3.4 mL) was added the freshly prepared isocyanide (10 eq, 0.684 mmol) in MES buffer (3.4 mL, pH 6.5, 0.1 M). The pH was monitored and adjusted to pH 6.5 if necessary, using HCl (1 M) or NaOH (1 M). The resulting light yellow mixture was stirred for 18 h at room temperature. Subsequently, the organic solvent was removed by concentration in vacuo and remaining aqueous solution was purified by preparative HPLC.

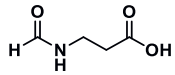
General procedure B: Pentapeptide enzymatic ligation reactions.
The standard amine (H_2N-ALKKF-NH_2, **17**) was dissolved in tricine buffer (pH 8.5, 200 mM) to a final concentration of 10 mM. Subsequently, TCEP solution (100 mg/ml, 200 mM tricine, pH 8.5) was added to a final concentration of 2 mg/ml. The mixture was added to the dry ester to a final concentration of 5 mM ester. The pH was adjusted if necessary, using 3 M NaOH or 3 M HCl to ~8.3. Sequentially, Omniligase (6.78 mg/ml, final concentration 0.14 mg/ml) was added to the ligation solution to start the ligation. A sample was taken before the start of the ligation by quenching 20 µL of the ligation solution in 80 µL quenching solution (mQ/MeCN/TFA; 48:48:4 Vol%). Samples were taken by quenching after 15, 30, 60, 120 and 240 minutes and analyzed on HPLC-MS. After 4 hours Ac-RLSSLALKKF-NH_2 was isolated by reversed-phase preparative HPLC.

General procedure C: SPPS of starting material peptides.

The reactions were performed in either a syringe reactor on an orbital shaker at room temperature or using a glass SPPS vessel to be stirred by nitrogen bubbling. The procedures were performed with 10 mL solvent/1 g resin unless stated otherwise. Rink amide AM resin (GL Biochem, Cat. No. 49001) was swollen in CH_2Cl_2 (dichloromethane, DCM) for 30 min and was subsequently washed with dimethylformamide (DMF, 3x). The amino acid (AA) coupling solution of Fmoc-AA-OH (4 equiv), and ethyl cyano (hydroxyimino) acetate (oxyma, 4 equiv) in DMF was prepared. N,N-diisopropylcarbodiimid (DIC, 6 equiv) was added to the coupling solution when ready for use. Subsequently, the coupling solution was added to the resin and allowed to react for 20 mins. Then, DIC (6 equiv) was added to the mixture and allowed to react for 20 mins. Subsequently, the reaction solution was discarded, and the resin was washed with DMF (3×) before Fmoc removal was performed by adding a deprotection solution of piperidine in DMF (2:8, v/v) and reacting for 10 min. The solution was discarded and the Fmoc removal repeated. Next, the resin was washed with DMF (6×). Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above. 2-chlorotrityl chloride (CTC)-resin (100-200 mesh), 1% DVB (Novabiochem, Cat. No. 8.55017) was swollen in DCM for 30 min. A solution of 2.5 equiv Fmoc-AA-OH and *N,N*-diethylisopropylamine (DIPEA, 5 equiv) in DCM was added to the resin and reacted for 1 h. The solution was discarded, and the resin was washed with DCM (6x). Non-reacted linker groups were capped by adding a solution of methanol (MeOH), DIPEA and DCM (3:1:16, v/v/v) and reacting for 10 min. The solution was discarded and the capping step repeated. The resin was washed with DCM (5x) and DMF (3x). Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above for the rink amide AM resin. Peptides were N-terminally acetylated by adding a solution of Ac_2O, DIPEA and DMF (1:1:8, v/v/v) to the reaction and reacted for 10 min. The acetylation was repeated. The resin was washed with DMF (3×) and DCM (3×). Subsequently, the peptide was cleaved and the side chain protecting groups were removed in one step by adding a cleavage solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water (95:2.5:2.5, v/v/v) and reacting for 2 h. The cleavage solution was collected precipitated in cold diethyl ether (-20 °C) and centrifuged (15 min, 4000 rcf). The supernatant was discarded and the precipitation and centrifugation were repeated twice. The crude peptide was dissolved in MeCN and water and lyophilized. The peptides were purified by preparative HPLC.

Characterization of compounds

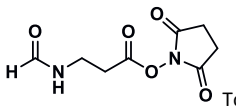
3-formamidopropanoic acid (8)



To a solution of 3-aminopropanoic acid (10.0 g, 112 mmol, 1 equiv) in DMF (34 mL) was added formic acid (7 mL) dropwise. The resulting mixture was stirred overnight at 105 °C. The crude reaction mixture was concentrated in vacuo to obtain a thick yellow oil. The crude product was dissolved in water and MeCN (1:1) and lyophilized to obtain the title compound as an off-white solid (13.1 g, 112 mmol, >99%). Used without further purification.

^{1}H NMR (400 MHz, Methanol-d_4) δ = 8.05 – 8.01 (s, 1H), 3.58 – 3.36 (t, *J*=6.6, 2H), 2.61 – 2.42 (t, *J*=6.6, 2H); **^{13}C NMR** (101 MHz, Methanol-d_4) δ 167.66, 163.94, 34.94. (Overlap between the two CH_2-carbons based on HSQC). **HRMS (ESI):** *m/z* calculated for $C_4H_7NNaO_3^+$ $[M+Na]^+$: 140.0318, found: 140.0320

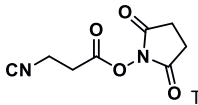
2,5-dioxopyrrolidin-1-yl 3-formamidopropanoate (9a)



To a solution of 3-formamidopropanoic acid (**8**, 13.1 g, 112 mmol, 1 equiv) in THF (100 mL) at 0 °C was added N,N-dicyclohexylcarbodiimide (DCC, 27.7 g, 134 mmol, 1.2 equiv). The reaction was stirred for 15 min before adding 1-hydroxypyrrolidine-2,5-dione (*N*-hydroxylsuccinimide, 15.5 g, 134 mmol, 1.2 equiv). The resulting mixture was allowed to gradually warm up to room temperature and stirred for 72 h. The reaction was filtered, the residu washed with EtOAc, and the filtrate was concentrated in vacuo. The dicyclohexylurea byproduct was removed by precipitation with MeCN (70 mL) and filtered. The filtrate was concentrated in vacuo and the precipitation was repeated twice. The crude product was purified using flash column chromatography (50-100% EtOAc in n-heptane) to obtain the title compound as a colorless thick oil (14.0 g, 65.3 mmol, 58%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 8.16 – 8.11 (d, *J*=1.7, 1H), 6.70 – 6.66 (s, 1H), 3.71 – 3.57 (m, 2H), 2.89 – 2.80 (m, 6H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.45, 167.49, 162.14, 33.63, 31.61, 25.67; **HRMS (ESI):** *m/z* calculated for $C_8H_{10}N_2NaO_5^+$ $[M+Na]^+$: 237.0482, found: 237.0481

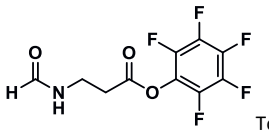
2,5-dioxopyrrolidin-1-yl 3-isocyanopropanoate (10a)



To a solution of 2,5-dioxopyrrolidin-1-yl 3-formamidopropanoate (**9a**, 580 mg, 2.71 mmol, 1 equiv) in anhydrous DCM (5 mL) in a flame dried Schlenk flask, was added a solution of Burgess Reagent (834 mg, 1.3 Eq, 3.50 mmol) in anhydrous DCM (5 mL). The resulting yellow mixture was heated to 50 °C and allowed to stir for 1.5 hours before full consumption of the starting formamide was observed as indicated by TLC (eluent: EtOAc). The mixture was diluted with DCM, washed once with water, once with saturated $NaHCO_3$ solution, and once with brine. The organic layer was dried over Na_2SO_4, filtered and concentrated in vacuo. The crude product was directly purified by flash column chromatography (50% EtOAc in n-heptane) to obtain the title compound as a white crystalline solid (126.7 mg, 0.65 mmol, 24%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.95 – 3.65 (t, *J*=7.0, 2H), 3.20 – 3.00 (t, *J*=7.0, 2H), 2.94 – 2.70 (s, 4H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 168.73, 165.18, 159.21, 36.53, 31.31, 25.68; **HRMS (ESI):** *m/z* calculated for $C_8H_8N_2NaO_4^+$ $[M+Na]^+$: 219.0376, found: 219.0373

Perfluorophenyl 3-formamidopropanoate (9b)

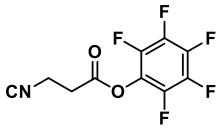


To a solution of 3-formamidopropanoic acid (**8**, 2.5 g, 21 mmol, 1 equiv) in THF (125 mL) were added N,N-dicyclohexylcarbodiimide (DCC, 5.3 g, 26 mmol, 1.2 equiv) and pentafluorophenol (4.7 g, 26 mmol, 1.2 equiv). The resulting mixture was allowed to stir for 72 h at room temperature. The reaction was filtered, the residu washed with DCM (2x), and the filtrate concentrated in vacuo. Subsequently, MeCN was added to precipitate the remaining dicyclohexylurea byproduct. The mixture was filtered and the filtrate was concentrated in vacuo. The precipitation was repeated twice. The crude product was purified by flash column chromatography (10-20% acetone in toluene), to obtain the target compound as a white solid (4.6 g, 16.2 mmol, 76%).

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.31 – 8.19 (s, 1H), 8.06 – 8.01 (s, 1H), 3.48 – 3.40 (q, *J*=6.4, 2H), 3.03 – 2.94 (t, *J*=6.5, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 167.96, 161.43, 141.91, 139.31, 138.78, 136.28, 33.08, 32.86; **^{19}F NMR**

(376 MHz, $CDCl_3$) δ = -152.5 (m, 2F), -157.1 (t, *J*=21.8, 1F), -161.8 (m, 2F); **HRMS (ESI):** *m/z* calculated for $C_{10}H_7F_5NO_3^+$ $[M+H]^+$: 284.0341, found: 284.0336

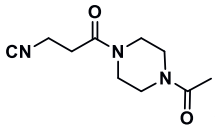
Perfluorophenyl 3-isocyanopropanoate (10b)



To a solution of perfluorophenyl 3-formamidopropanoate (**9b**, 5.63 g, 19.9 mmol, 1 equiv) in anhydrous THF (125 mL) in a flame-dried round bottom flask at 0 °C was added triethylamine (9.7 mL, 68.6 mmol, 3.5 equiv) and $POCl_3$ (2.3 mL, 24.9 mmol, 1.25 equiv) and the resulting mixture was stirred for 1 h before full consumption of the starting formamide was observed as indicated by TLC (eluent: DCM). The reaction mixture was diluted with DCM and quenched by addition of 2M $NaHCO_3$ solution. The layers were separated and the organic layer was washed with 2M $NaHCO_3$ solution (2x), brine, and then dried over Na_2SO_4. It was then filtered and concentrated in vacuo. The crude product was purified by flash column chromatography (20% EtOAc in *n*-heptane) to obtain the target compound as a white crystalline solid (3.5 g, 13.02 mmol, 66 %).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.87 – 3.79 (t, *J*=6.7, 2H), 3.17 – 3.05 (t, *J*=6.7, 2H); **^{13}C NMR** (101 MHz, DMSO-d_6) δ 166.90, 156.81, 141.74, 139.02, 136.32, 124.16, 37.00, 32.86; **^{19}F NMR** (376 MHz, $CDCl_3$) δ = -152.2 (m, 2F), -156.8 (t, *J*=21.7, 1F), -161.6 (m, 2F); **HRMS (ESI):** *m/z* calculated for $C_{10}H_5F_5NO_2^+$ $[M+H]^+$: 266.0235, found: 266.0232

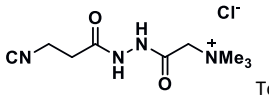
1-(4-acetylpiperazin-1-yl)-3-isocyanopropan-1-one (11a)



To a solution of 2,5-dioxopyrrolidin-1-yl 3-isocyanopropanoate (**10a**, 50 mg, 255 umol, 1 equiv) in DCM (1 mL), was added 1-(piperazin-1-yl)ethan-1-one (*N*-acetyl piperazine, 49 mg, 382 umol, 1.5 equiv). The resulting mixture was stirred for one hour before full consumption of the starting formamide was observed as indicated by TLC (eluent: 10% MeOH in DCM). The reaction mixture was directly loaded onto a silica column and purified using flash column chromatography (5% MeOH in DCM) to obtain the title compound as a brown oil (40.4 mg, 193.1 umol, 76%).

^{1}H NMR (400 MHz, $CDCl_3$) δ = 3.78 – 3.70 (t, *J*=7.1, 2H), 3.69 – 3.57 (m, 4H), 3.53 – 3.40 (m, 4H), 2.81 – 2.73 (t, *J*=7.1, 2H), 2.14 – 2.08 (s, 3H); **^{13}C NMR** (101 MHz, $CDCl_3$) δ 169.51, 169.26, 167.34, 167.13 (**C**O of amides), 157.03 (Isocyanide N**C**), 46.11, 45.88, 45.30, 45.03, 41.70, 41.51, 41.26, 41.09 (Piperazine **C**H_2), 37.47 (α-**C**H_2), 33.05, 32.95 (β-**C**H_2), 21.44, 21.37 (**C**H_3 of Acetyl). All carbons, except the NC and the adjacent α-CH_2, are observed as a set of 2 peaks, attributed to rigid ring inversion of the acetyl-piperazine ring; **HRMS (ESI):** *m/z* calculated for $C_{10}H_{16}N_3O_2^+$ $[M+H]^+$: 210.1237, found: 210.1236

2-(2-(3-isocyanopropanoyl)hydrazineyl)-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride (11b)

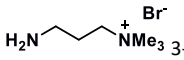


To a solution of 2-hydrazineyl-*N*,*N*,*N*-trimethyl-2-oxoethan-1-aminium chloride (115 mg, 684 µmol, 1 equiv) in MeOH (1.5 mL) was added 2,5-dioxopyrrolidin-1-yl 3-isocyanopropanoate (**10a**, 134 mg, 684 µmol, 1 equiv). The resulting mixture was stirred for 6.5 h at room temperature before full conversion to the

cationic isocyanide was observed as indicated by HPLC-MS analysis. The reaction mixture was concentrated in vacuo to obtain the title compound as a thick brown oil. The crude product was used without further purification.

******Note:* Product was characterized *in-situ* using live NMR. **^{1}H NMR** (400 MHz, Methanol-d_4) δ = 4.10 – 4.05 (s, 2H), 3.92 – 3.81 (t, *J*=6.3, 2H), 3.34 – 3.32 (s, 9H), 3.17 – 3.09 (t, *J*=6.3, 2H).**^{13}C NMR** (101 MHz, Methanol-d_4) δ 170.55, 163.86, 156.85, 64.41, 55.02, 38.18, 34.20. **HRMS (ESI):** *m/z* calculated for $C_9H_{17}N_4O^+$ $[M]^+$: 213.1346, found: 213.1344

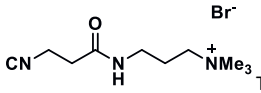
3-amino-*N*,*N*,*N*-trimethylpropan-1-aminium bromide



3-bromo-*N,N,N*-trimethylpropan-1-aminium bromide (2.730 g, 10.46 mmol, 1 equiv) was dissolved in ammonium hydroxide solution (28-30% in water, 30 mL) and refluxed at 55 °C overnight. Subsequently, 1M NaOH (5 mL) was added, and the resulting mixture was evaporated to near dryness at 100°C for 3 hours. The resulting solid was dissolved in a minimal amount of MeOH and then precipitated by addition of a large excess of MeCN. Solids were filtered off, and the precipitation was repeated with the filtrate. The resulting material was dried in vacuo to obtain the title compound as a white crystalline solid (1.61 g, 8.17 mmol, 78%).

^{1}H NMR (400 MHz, Methanol-d_4) δ = 3.58 – 3.48 (m, 2H), 3.33 – 3.13 (s, 9H), 3.11 – 2.99 (t, *J*=1.7, 2H), 2.28 – 2.13 (m, 2H); **^{13}C NMR** (101 MHz, Methanol-d_4) δ 64.37, 53.88, 37.68, 22.58; **HRMS (ESI):** *m/z* calculated for $C_6H_{17}N^+$ $[M]^+$: 117.1386, found: 117.1388

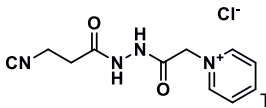
3-(3-isocyanopropanamido)-*N*,*N*,*N*-trimethylpropan-1-aminium bromide (11c)



To a mixture of 3-amino-*N*,*N*,*N*-trimethylpropan-1-aminium bromide (135 mg, 684 µmol, 1 equiv) and DIPEA (88.4 mg, 119 µL, 684 µmol, 1 equiv) in MeOH (3 mL) was added 2,5-dioxopyrrolidin-1-yl 3-isocyanopropanoate (**10a**, 134 mg, 1 Eq, 684 µmol). The resulting mixture was stirred for 0.5 hours at room temperature before full conversion to the cationic isocyanide was observed as indicated by HPLC-MS analysis. The reaction mixture was concentrated in vacuo to obtain the title compound as a thick brown oil. The crude product was used without further purification.

******Note:* Product was characterized *in-situ* using live NMR.**^{1}H NMR** (400 MHz, Methanol-d_4) δ = 3.80 – 3.75 (m, 2H) (overlap with q of DIPEA), 3.49 – 3.39 (m, 2H), 3.39 – 3.33 (t, *J*=6.4, 2H), 3.19 – 3.14 (s, 9H), 2.65 – 2.60 (m, 2H) (overlap with s of NHS Leaving Group), 2.10 – 2.00 (m, 2H). **^{13}C NMR** (101 MHz, Methanol-d_4) δ 172.01, 156.70, 65.71, 53.72, 37.13, 36.54, 26.23, 24.35. **HRMS (ESI):** *m/z* calculated for $C_{10}H_{20}N_3O^+$ $[M]^+$: 198.1601, found: 198.1598

1-(2-(2-(3-isocyanopropanoyl)hydrazineyl)-2-oxoethyl) pyridin-1-ium chloride (11d)

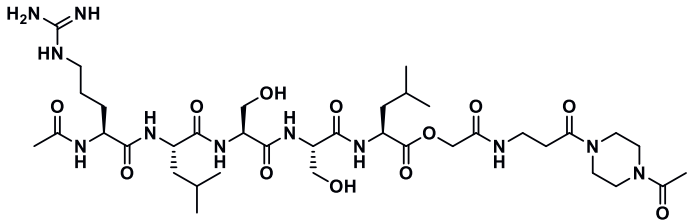


To a solution of 1-(2-hydrazineyl-2-oxoethyl)pyridin-1-ium chloride (128 mg, 684 µmol, 1 equiv) in MeOH (3 mL) was added 2,5-dioxopyrrolidin-1-yl 3-isocyanopropanoate (**10a**, 134 mg, 684 µmol, 1 equiv). The resulting mixture was stirred for 6.5 hours at room temperature before full conversion to the cationic isocyanide was observed as indicated by HPLC-MS analysis. Solvent was removed by concentration in vacuo to

obtain the title compound as a thick brown oil. The crude product was directly used for Passerini reactions without further purification.

*****_Note:_ Product was characterized *in-situ* using live NMR. Isocyanide carbon not visible on ^{13}C NMR. **^{1}H NMR** (400 MHz, Methanol-d_4) δ = 9.07 – 8.83 (m, 2H), 8.73 – 8.58 (m, 1H), 8.24 – 8.10 (m, 2H), 5.50 – 5.40 (s, 2H), 3.90 – 3.82 (t, *J*=6.1, 2H), 3.15 – 3.10 (t, *J*=6.2, 2H). **^{13}C NMR** (101 MHz, Methanol-d_4) δ 170.94, 165.56, 147.85, 147.66, 129.02, 61.60, 38.25, 34.18. **HRMS (ESI):** *m/z* calculated for $C_{11}H_{13}N_4O_2^+$ $[M]^+$: 233.1033, found: 233.1033

2-((3-(4-acetylpiperazin-1-yl)-3-oxopropyl)amino)-2-oxoethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate (13b)



Prepared from Ac-RLSSL-OH (**12**, 16.5 mg, 19.3 µmol, 1 equiv), **11a** (4.0 mg, 0.193 mmol, 10 equiv) and formaldehyde (37 wt% in water, 14.4 µL, 0.193 mmol, 10 equiv) according to procedure A. Purification was done by preparative HPLC. Isolated as white solid (7.3 mg, 7.5 µmol, 39%).

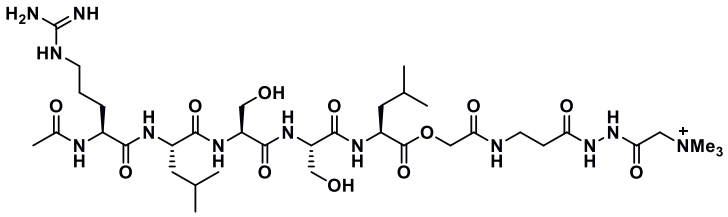
^{1}H NMR (701 MHz, Methanol-d_4) δ = 4.58 – 4.54 (d, *J*=1.8, 2H), 4.50 – 4.43 (m, 3H), 4.43 – 4.38 (dd, *J*=10.4, 4.9, 1H), 4.36 – 4.31 (ddd, *J*=8.2, 6.3, 2.1, 1H), 3.94 – 3.88 (tdd, *J*=11.2, 5.4, 2.1, 2H), 3.86 – 3.83 (dd, *J*=11.3, 4.0, 1H), 3.78 – 3.74 (dd, *J*=10.9, 6.2, 1H), 3.69 – 3.62 (m, 2H), 3.61 – 3.51 (m, 8H), 3.23 – 3.18 (td, *J*=7.0, 4.3, 2H), 2.76 – 2.63 (m, 2H), 2.14 – 2.13 (d, *J*=1.9, 3H), 2.01 – 1.99 (s, 3H), 1.87 – 1.81 (m, 1H), 1.76 – 1.59 (m, 9H), 0.99 – 0.94 (dd, *J*=6.5, 1.7, 6H), 0.93 – 0.90 (dd, *J*=6.5, 1.3, 6H).

^{13}C NMR (176 MHz, Methanol-d_4) δ 175.11, 174.42, 173.73, 173.70, 172.88, 172.86, 172.63, 172.62, 172.56, 172.54, 172.12, 172.09, 172.03, 171.98, 169.75, 169.72 (**C**O of ester, amides, 7 as set of 2 peaks), 158.60 (**C**N of guanidine), 64.02 (α-**C**H_2 of ester), 63.00, 62.67 (β-**C**H_2 of Serine), 57.19, 56.83, 54.47, 53.67, 52.68, 52.63 (α-**C**H of main-chain amides, 1 as set of 2 peaks), 47.24, 46.90, 46.55, 46.23, 42.76, 42.64, 42.43, 42.27 (Piperazine **C**H_2, all 4 as set of 2 peaks), 42.01(δ-**C**H_2 of Arginine), 41.43, 40.84, 40.81 (β-**C**H_2 of Leucine, 1 as set of 2 peaks), 36.67, 36.63, 33.57, 33.54 (**C**H_2 of β-Alanine, both as set of 2 peaks), 30.05, 30.03 (β-**C**H_2 of Arginine, set of 2 peaks), 26.22 (γ-**C**H_2 of Arg), 25.92, 25.81 (γ-**C**H of Leucine), 23.51, 23.33 (δ-**C**H_3 of Leucine), 22.44 (**C**H_3 of Acetyl), 21.77, 21.76 (**C**H_3 of Acetyl-piperazine, set of 2 peaks), 21.25, 21.19 **C**H_3 (δ-**C**H_3 of Leucine).

*Many Carbons are observed as a set of 2 peaks, attributed to rigid ring inversion of the acetyl-piperazine ring.

Analytical HPLC trace of prep-HPLC purified title compound; Rt 4.54 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 856.5 (100%), $[M+2H]^{2+}$: 428.7 (100%); Mass Found (ESI^+); 856.5 $[M+H]^+$, 428.8 $[M+2H]^{2+}$.

1-((15S,18S,21S,24S,27S)-27-acetamido-18,21-bis(hydroxymethyl)-15,24-diisobutyl-2,2-dimethyl-4,7,11,14,17,20,23,26-octaoxo-13-oxa-2,5,6,10,16,19,22,25-octaazatriacontan-2-ium-30-yl)guanidinium (13c)



Prepared from Ac-RLSSL-OH (**12**, 50 mg, 68.4 µmol, 1 equiv), **11b** (170.1 mg, 0.684 mmol, 10 equiv) and formaldehyde (37 wt% in water, 55.53 mg, 50.9 µL, 0.684 mmol, 10 equiv) according to procedure A. Purification was done by preparative HPLC. Isolated as white solid (29.1 mg, 26.8 µmol, 39%).

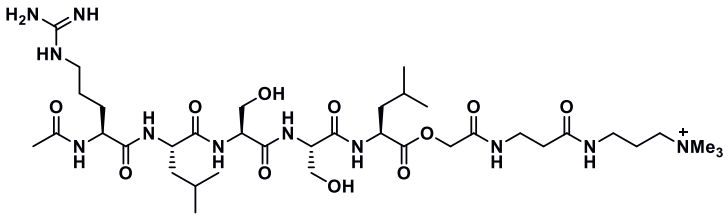
^{1}H NMR (701 MHz, Methanol-d_4) δ = 4.59 – 4.57 (s, 2H), 4.50 – 4.45 (m, 2H), 4.44 – 4.41 (t, *J*=5.5, 1H), 4.40 – 4.37 (dd, *J*=9.9, 5.2, 1H), 4.32 – 4.29 (dd, *J*=7.9, 6.1, 1H), 4.20 – 4.17 (s, 2H), 3.95 – 3.90 (ddd, *J*=11.0, 8.6, 5.3, 2H), 3.87 – 3.84 (dd, *J*=11.3, 4.1, 1H), 3.80 – 3.76 (dd, *J*=11.1, 5.9, 1H), 3.58 – 3.56 (t, *J*=6.4, 2H), 3.36 – 3.34 (s, 9H), 3.24 – 3.19 (t, *J*=7.1, 2H), 2.57 – 2.51 (qd, *J*=7.5, 6.1, 2H), 2.03 – 1.98 (s, 3H), 1.89 – 1.82 (m, 1H), 1.78 – 1.61 (m, 9H), 0.98 – 0.95 (dd, *J*=6.4, 1.8, 6H), 0.93 – 0.90 (d, *J*=6.4, 6H).

^{13}C NMR (176 MHz, Methanol-d_4) δ 175.26, 174.73, 173.99, 172.85, 172.73, 172.69, 172.54, 169.80, 163.92 (**C**O of ester, amides, hydrazides), 158.63 (CN of guanidine), 64.45 (α-**C**H_2 of hydrazide), 64.03 (α-**C**H_2 of ester), 62.88, 62.69 (β-**C**H_2 of Serine), 57.27, 57.06 (α-CH of main-chain amides), 55.03 (CH2N+(CH3)3), 54.89, 53.8, 52.71 (α-**C**H of main-chain amides), 41.97 (δ-**C**H2 of Arginine), 41.24, 40.81 (β-**C**H_2 of Leucine), 36.51, 34.30 (**C**H_2 of β-Alanine), 29.88 (β-**C**H_2 of Arginine), 26.27 (γ-**C**H_2 of Arg), 25.92, 25.78 (γ-**C**H of Leucine), 23.48, 23.31(δ-**C**H_3 of Leucine), 22.46 (**C**H_3 of Acetyl), 21.80, 21.75 (δ-**C**H_3 of Leucine).

*TFA observed on ^{13}C NMR as two sets of 4 peaks at δ163.33, 163.14, 162.94, 162.75, 120.70, 119.04, 117.38, 115.71.

Analytical HPLC trace of prep-HPLC purified title compound; Rt 4.09 min (λ = 220 nm); Calculated Mass $[M]^{+}$: 859.5 (100%), $[M+H]^{2+}$: 430.3 (100%); Mass Found (ESI^{+}); 859.5 $[M]^{+}$, 430.4 $[M+H]^{2+}$.

(6S,9S,12S,15S,18S)-6-acetamido-1-amino-12,15-bis(hydroxymethyl)-1-imino-9,18-diisobutyl-N,N,N-trimethyl-7,10,13,16,19,22,26-heptaoxo-20-oxa-2,8,11,14,17,23,27-heptaazatriacontan-30-aminium (13a)



Prepared from Ac-RLSSL-OH (**12**, 50 mg, 68.4 µmol, 1 equiv), **11c** (190.3 mg, 0.684 mmol, 10 equiv) and formaldehyde (37 wt% in water, 55.53 mg, 50.9 µL, 0.684 mmol, 10 equiv) according to procedure A. Purification was done by preparative HPLC. Isolated as white solid (24.7 mg, 23 µmol, 34%).

^{1}H NMR (701 MHz, Methanol-d_4) δ = 4.57 – 4.55 (s, 2H), 4.47 – 4.43 (m, 2H), 4.42 – 4.40 (t, *J*=5.3, 1H), 4.39 – 4.36 (dd, *J*=10.1, 5.0, 1H), 4.32 – 4.28 (dd, *J*=7.9, 6.1, 1H), 3.98 – 3.90 (ddd, *J*=14.6, 11.2, 5.3, 2H), 3.89 – 3.84 (dd, *J*=11.3, 4.0, 1H), 3.81 – 3.76 (dd, *J*=11.1, 5.7, 1H), 3.57 – 3.52 (m, 2H), 3.39 – 3.34 (m, 2H), 3.30 – 3.28 (td, *J*=6.6, 2.1, 2H),

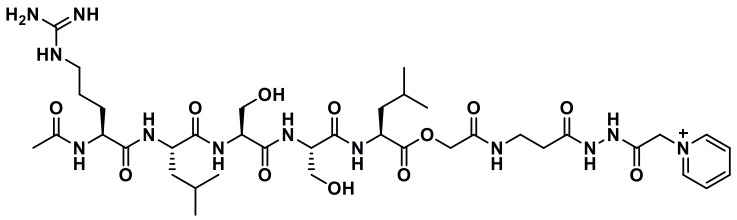
3.23 – 3.20 (t, *J*=7.1, 2H), 3.16 – 3.13 (s, 9H), 2.46 – 2.43 (td, *J*=6.1, 1.3, 2H), 2.03 – 2.00 (s, 3H), 2.01 – 1.96 (m, 2H), 1.89 – 1.83 (m, 1H), 1.78 – 1.61 (m, 9H), 0.98 – 0.96 (dd, *J*=6.5, 1.4, 6H), 0.94 – 0.90 (dd, *J*=6.4, 1.1, 6H).

^{13}C NMR (176 MHz, Methanol-d_4) δ 175.31, 174.82, 174.21, 174.03, 173.14, 172.90, 172.67, 169.65 (**C**O of ester, amides), 158.64 (**C**N of guanidine), 65.78, (**C**$H_2N^+(CH_3)_3$), 64.08 (α-**C**H_2 of ester), 62.83, 62.70 (β-**C**H_2 of Serine), 57.40, 57.20, 54.96, 53.91 (α-CH of main-chain amides), 53.66 (CH_2N^+(**C**H_3)$_3$), 52.94 (α-CH of main-chain amide), 41.96 (δ-**C**H_2 of Arginine), 41.18, 40.66 (β-**C**H_2 of Leucine), 37.32 (**C**H_2 of ammonium-amide), 37.06, 36.89 (**C**H_2 of β-Alanine), 29.85 (β-**C**H_2 of Arginine), 26.29 (γ-**C**H_2 of Arginine), 25.92, 25.79 (γ-**C**H of Leucine), 24.47(**C**H_2 of ammonium-amide), 23.47, 23.30 (δ-**C**H_3 of Leucine), 22.47 (**C**H_3 of Acetyl), 21.80, 21.76 (δ-**C**H_3 of Leucine).

*TFA observed on ^{13}C NMR as two sets of 4 peaks at δ 163.10, 162.90, 162.71, 162.51, 120.63, 118.96, 117.30, 115.62.

Analytical HPLC trace of prep-HPLC purified title compound; Rt 4.11 min (λ = 220 nm); Calculated Mass $[M]^+$: 844.5 (100%), $[M+H]^{2+}$: 422.8 (100%); Mass Found (ESI^+); 844.5 $[M]^+$, 423.0 $[M+H]^{2+}$.

1-((6S,9S,12S,15S,18S)-6-acetamido-1-amino-12,15-bis(hydroxymethyl)-1-imino-9,18-diisobutyl-7,10,13,16,19,22,26,29-octaoxo-20-oxa-2,8,11,14,17,23,27,28-octaazatriacontan-30-yl)pyridin-1-ium (13d)



Prepared from Ac-RLSSL-OH (**12**, 50 mg, 68.4 μmol, 1 equiv), **11d** (183.8 mg, 0.684 mmol, 10 equiv) and formaldehyde (37 wt% in water, 55.53 mg, 50.9 μL, 0.684 mmol, 10 equiv) according to procedure A. Purification was done by preparative HPLC. Isolated as white solid (29.1 mg, 26.3 μmol, 38%).

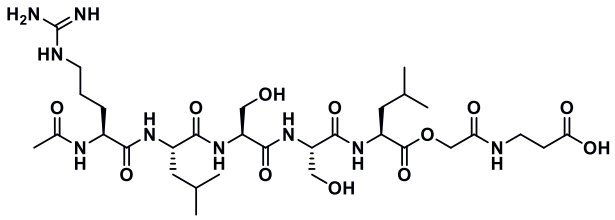
^{1}H NMR (701 MHz, Methanol-d_4) δ = 8.98 – 8.96 (d, *J*=5.3, 2H), 8.69 – 8.67 (t, *J*=7.8, 1H), 8.18 – 8.15 (t, *J*=7.6, 2H), 5.60 – 5.57 (s, 2H), 4.56 – 4.53 (s, 2H), 4.49 – 4.41 (m, 3H), 4.39 – 4.35 (dd, *J*=10.0, 5.2, 1H), 4.31 – 4.27 (dd, *J*=7.9, 6.0, 1H), 3.96 – 3.89 (m, 2H), 3.87 – 3.83 (dd, *J*=11.4, 4.0, 1H), 3.80 – 3.76 (dd, *J*=11.2, 5.8, 1H), 3.23 – 3.19 (t, *J*=7.1, 2H), 3.19 – 3.15 (t, *J*=6.5, 2H), 2.57 – 2.49 (m, 2H), 2.02 – 2.00 (s, 3H), 1.88 – 1.82 (sp, 1H), 1.76 – 1.61 (m, 9H), 0.99 – 0.93 (d, *J*=6.8, 6H), 0.93 – 0.88 (m, 6H).

^{13}C NMR (176 MHz, Methanol-d_4) δ 175.27, 174.77, 174.00, 172.89, 172.84, 172.73, 172.72, 169.80, 163.85 (**C**O of ester, amides, hydrazides), 158.64 (**C**N of guanidine), 147.81, 147.71, 129.03 (**C**H of pyridinium), 64.03 (α-**C**H_2 of ester), 62.89, 62.71 (β-**C**H_2 of Serine), 61.63 (α-**C**H_2 of hydrazide), 57.26, 57.06, 54.95, 53.81, 52.70 (α-**C**H of main-chain amides), 41.96 (δ-**C**H2 of Arginine), 41.18, 40.81(β-**C**H_2 of Leucine), 36.61, 34.33 (**C**H_2 of β-Alanine), 29.86 (β-**C**H_2 of Arginine), 26.28 (γ-**C**H_2 of Arginine), 25.91, 25.78 (γ-**C**H of Leucine), 23.47, 23.30 (δ-**C**H_3 of Leucine), 22.47(**C**H_3 of Acetyl), 21.83, 21.73 (δ-**C**H_3 of Leucine).

*TFA observed on ^{13}C NMR as two sets of 4 peaks at δ 163.37, 163.17, 162.97, 162.78, 120.72, 119.06, 117.40, 115.73.

Analytical HPLC trace of prep-HPLC purified title compound; Rt 4.07 min (λ = 220 nm); Calculated Mass $[M]^+$: 879.5 (100%), $[M+H]^{2+}$: 440.2 (100%); Mass Found (ESI^+); 879.5 $[M]^+$, 440.5 $[M+H]^{2+}$.

(6S,9S,12S,15S,18S)-6-acetamido-1-amino-12,15-bis(hydroxymethyl)-1-imino-9,18-diisobutyl-7,10,13,16,19,22-hexaoxo-20-oxa-2,8,11,14,17,23-hexaazahexacosan-26-oic acid (13e)



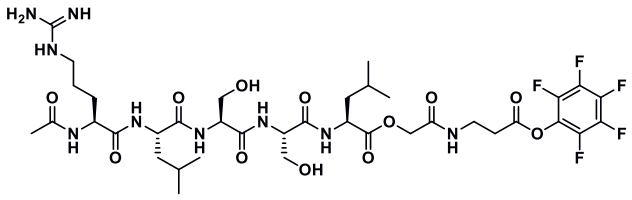
Prepared from Ac-RLSSL-OH (**12**, 50 mg, 68.4 µmol, 1 equiv), **10a** (134.2 mg, 0.684 mmol, 10 equiv) and formaldehyde (37 wt% in water, 55.53 mg, 50.9 µL, 0.684 mmol, 10 equiv) according to procedure A, but with PBS (6.16 mL, pH 6.5, 0.1 M) and MeCN (0.68 mL, 10%) as solvent system. Purification was done by preparative HPLC. Isolated as white solid (10.4 mg, 12.3 µmol, 18%).

^{1}H NMR (701 MHz, Methanol-d_4) δ = 4.57 – 4.54 (s, 2H), 4.51 – 4.48 (dd, *J*=9.6, 5.3, 1H), 4.47 – 4.45 (t, *J*=4.5, 1H), 4.45 – 4.43 (t, *J*=5.6, 1H), 4.42 – 4.38 (dd, *J*=8.9, 6.3, 1H), 4.36 – 4.33 (t, *J*=7.2, 1H), 3.94 – 3.88 (td, *J*=10.9, 5.3, 2H), 3.87 – 3.81 (dd, *J*=11.3, 4.2, 1H), 3.79 – 3.75 (dd, *J*=11.0, 6.0, 1H), 3.52 – 3.48 (t, *J*=6.9, 2H), 3.24 – 3.19 (td, *J*=7.0, 2.5, 2H), 2.58 – 2.54 (t, *J*=6.9, 2H), 2.01 – 1.99 (s, 3H), 1.90 – 1.82 (m, 1H), 1.77 – 1.62 (m, 9H), 0.99 – 0.95 (dd, *J*=6.5, 2.9, 6H), 0.94 – 0.90 (dd, *J*=6.5, 1.0, 6H).

^{13}C NMR (176 MHz, Methanol-d_4) δ 175.35, 175.15, 174.49, 173.70, 172.90, 172.60, 172.53, 169.67 (**C**O of ester, carboxylate, and amides), 158.60 (**C**N of guanidine), 63.98 (α-**C**H_2 of ester), 62.94, 62.67 (β-**C**H_2 of Serine), 57.16, 56.86, 54.47, 53.71, 52.61 (α-**C**H of main-chain amides), 42.00 (δ-**C**H_2 of Arginine), 41.40, 40.87 (**C**H_2 of Leucine), 36.30, 34.54 (**C**H_2 of β-Alanine), 30.06 (β-**C**H_2 of Arginine), 26.21(γ-**C**H_2 of Arg), 25.91, 25.81 (γ-**C**H of Leucine), 23.49, 23.32 (δ-**C**H_3 of Leucine), 22.42 (**C**H_3 of Acetyl), 21.77, 21.74 (δ-**C**H_3 of Leucine).

Analytical HPLC trace of prep-HPLC purified title compound; Rt 4.53 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 746.4 (100%), $[M+2H]^{2+}$: 373.7 (100%); Mass Found (ESI^+); 746.4 $[M+H]^+$, 373.7 $[M+2H]^{2+}$.

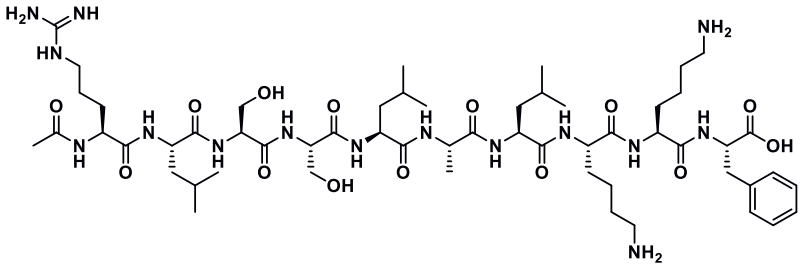
2-oxo-2-((3-oxo-3-(perfluorophenoxy)propyl)amino)ethyl acetyl-L-arginyl-L-leucyl-L-seryl-L-seryl-L-leucinate (14)



Prepared from Ac-RLSSL-OH (**12**, 50 mg, 68.4 µmol, 1 equiv), **10b** (181.4 mg, 0.684 mmol, 10 equiv) and formaldehyde (37 wt% in water, 55.53 mg, 50.9 µL, 0.684 mmol, 10 equiv) according to procedure A, but with PBS (4.79 mL, pH 4.5, 0.1 M) and MeCN (2.05 mL, 30%) as solvent system. Purification was done by preparative HPLC. Isolated as white solid (18.5 mg, 18.1 µmol, 26%).

Analytical HPLC trace of prep-HPLC purified title compound; Rt 5.53 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 912.4 (100%), $[M+2H]^{2+}$:456.7 (100%); Mass Found (ESI^+); 912.5 $[M+H]^+$, 456.8 $[M+2H]^{2+}$.

Ac-RLSSLALKKF-NH_2 (18)



Prepared from H_2N-ALKKF-NH_2 (**17**, 2 equiv) and **13a**-**13e** (1 equiv) according to procedure B. Purification was done by preparative HPLC. Isolated as white solid (48-61%).

Table 4. Yields of **18** from various starting materials[a].

Entry	Ester	Scale (µmol)	Conversion (4 h)[b]	Isolated Yield
1	**13a**	25.2	>95%	59
2	**13b**	21.9	>95%	49
3	**13c**	25.3	>95%	61
4	**13d**	10.4	>95%	50
5	**13e**	7.6	>95%	48

[a] Standard conditions: ester (1 equiv), H_2N-ALKKF-NH_2 (**17**, 2 equiv), TCEP (2 mg/mL), and omniligase (0.14 mg/mL) in tricine buffer (pH 8.3, 0.1 M). [b] Based on consumption of the starting material ester on HPLC-MS analysis.

Analytical HPLC trace of prep-HPLC purified title compound; Rt 4.34 min (λ = 220 nm); Calculated Mass $[M+2H]^+$: 602.9 (100%), $[M+3H]^{2+}$: 402.3 (100%); Mass Found (ESI^+); 602.7 $[M+2H]^+$, 402.1 $[M+3H]^{2+}$.

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Chapter 5

Future Research Directions

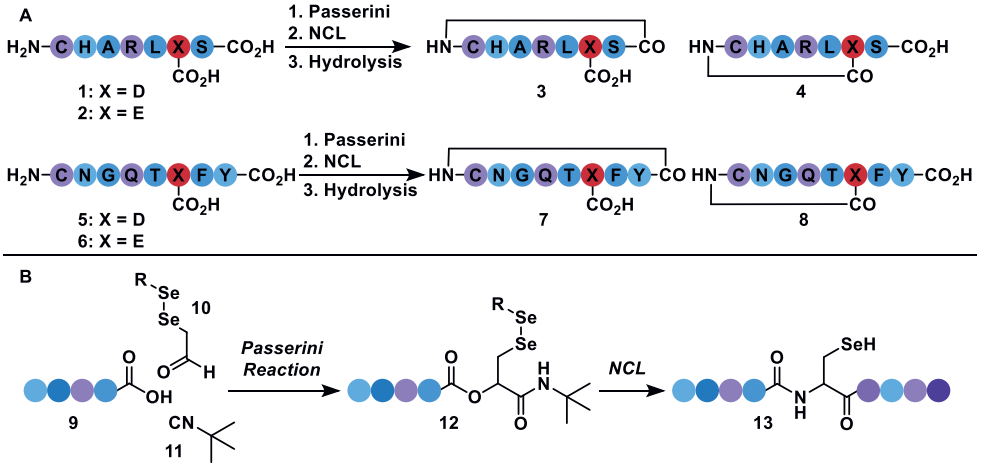
5.1 Introduction

The functionalization of peptide and protein C-termini has made significant progress over the past few decades, but there is continued interest in further development of novel strategies for these targets. Despite the many methods reported on C-terminal modifcations, much effort is still devoted to developing synthetic methodologies that are regioselective for the C-terminus, avoid epimerization, and are facile and generally applicable. Moreover, the functionalization of fully unprotected or native peptides has been particularly challenging. Starting from the general interest of our research group in isocyanide-based MCRs, we envisioned applying such chemistry to create novel methods that omit the classical issues associated with C-terminal modification. **Chapters 2**-**4** of this thesis discuss several projects which have exploited the Passerini reaction for the functionalization and subsequent ligation of C-terminal carboxylates on unprotected peptides. Nevertheless, we believe much remains to be done in expanding the scope of both isocyanide-based C-terminal functionalization and other chemical transformations in this context. This chapter discusses novel concepts and future directions that could be pursued in order to expand the known chemistry for C-terminal functionalization.

5.2 Synthesis of NCL Substrates Using the Passerini Reaction

The novel approach for the Passerini-mediated synthesis of NCL substrates described in **Chapter 3** could be further elaborated in many directions. The selectivity of the NCL for the C-terminus versus the sidechain carboxyl groups of Asp and Glu residues is of particular interest. We have already shown that ligation with a peptide bearing esters at both the C-terminus and sidechain of a Glu residue results in a mixture of three products, two mono- and one di-ligated product. The fact that the ratio of the mono products is 1:2.25 shows the selectivity towards one ester. In 2023, the group of Zhang reported native peptide cyclization, a method which employs isocyanides for the N-to-C terminal cyclization of peptides (**Chapter 1**).[1] They showed that this chemistry is selective owing to the slight differences in electrophilicity of the imidate intermediates via which the process runs. Similarly, our method can be optimized to provide higher degrees of selectivity. This can be investigated by comparing the rates of cyclization of the C-terminus over the sidechain in compounds **1**-**2** and **5**-**6**, peptides previously employed by Zhang and colleagues for similar studies (**Scheme 1A**).[1] Additionally, we have shown that the NCL can also be performed in phosphate buffer, the same solvent we employed for the Passerini reaction. This provides the possibility for a one-pot procedure where both the Passerini and the ligation are performed in sequence starting from the free peptide. In time, this could even by applied to a flow setup, allowing for scaling our methodology towards more efficient product formation.[2–5]

Another research direction that could improve the efficiency of this method is through the use of selenium-based substrates. The incorporation of selenium instead of sulfur has long been known to increase both the kinetics and yield of NCL[6–9]. Developing a diselenide containing aldehyde substrate like **10** as input for the Passerini reaction and subsequent NCL, along with the use of an appropriate selenol catalyst[10], should allow the improved yield and potentially selectivity of the method (**Scheme 1B**). Finally, applying these strategies to larger more complex peptides or even protein would demonstrate its true potential and value to society.

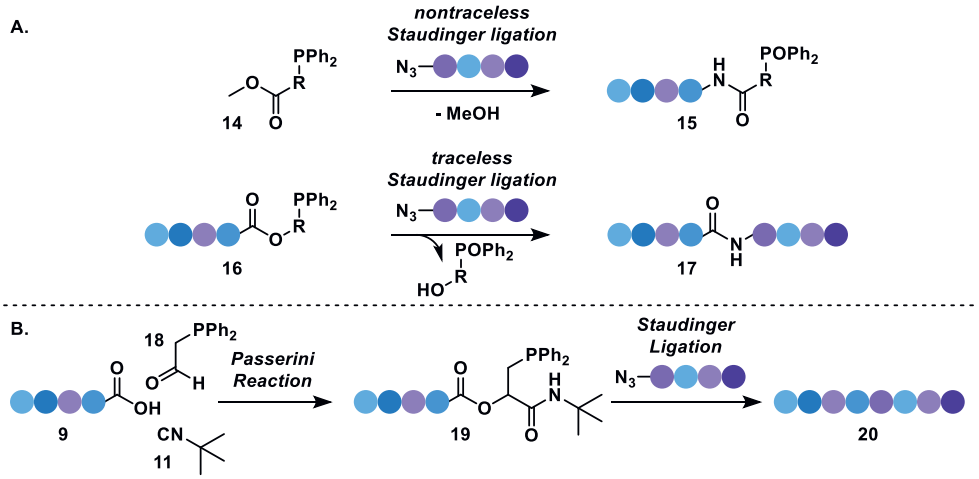


Scheme 47. A. C-terminal selectivity investigations by sequential Passerini functionalization, NCL cyclization, and hydrolysis. B. Passerini-NCL sequence using a diselenide species.

5.3 Passerini synthesis of Staudinger ligation substrates

Another important ligation method which has played a major role in peptide synthesis and development is the Staudinger ligation. This reaction ligates two peptide fragments bearing a phosphine and an azide, respectively. It has important advantages, including good selectivity, biocompatibility, and a high reaction rate and turnover across many different applications.[11] The reaction can be performed in two distinct ways; a non-traceless variant where the resulting phosphine oxide moiety remains in the product (**Scheme 2A**), and a traceless variant which is cleaved after the initial substitution (**Scheme 2A**).[11] Similarly to the work described in **Chapter 3**, the Passerini reaction can be utilized for the synthesis of esters bearing other reactive groups. We envision that employing an aldehyde bearing a reactive phosphine moiety (**18**) in the Passerini reaction affords esters (**19**) that then function as substrates for Staudinger ligation with an azide-functionalized peptide (**Scheme 2B**). The six-membered transition state allows for the attack of the iminophosphorane nitrogen onto the vicinal ester carbonyl to afford the desired peptide bond. This strategy is

promising to explore as this would further expand the chemical toolbox that peptide chemists have for large peptide or protein synthesis.



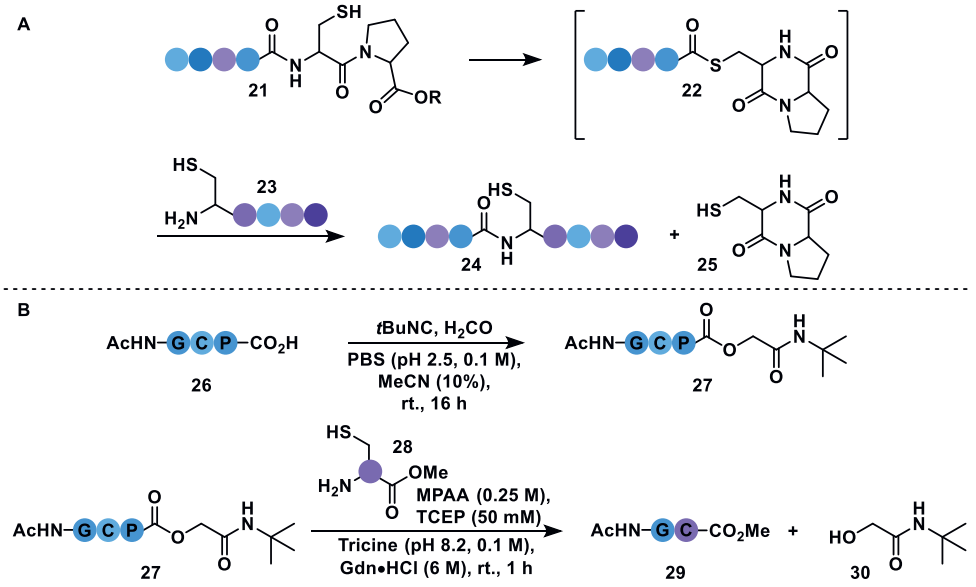
Scheme 48. A. The nontraceless (top) and traceless (bottom) Staudinger ligation. B. Proposed Passerini functionalization with a phosphine-containing aldehyde for the synthesis of Staudinger ligation substrates.

5.4 Utilizing the peptide backbone for selective chemical C-terminal functionalization

One of the major challenges in the selective functionalization of the C-termini of unprotected peptides and proteins is achieving regio- and chemoselectivity. The presence of diverse functional groups, including amines, thiols, alcohols, and additional carboxyl groups, represents a major challenge. Many chemists have tackled this selectivity issue by exploiting the difference in oxidation potential or nucleophilicity of the C-terminal carboxylate, yet no universal and broadly applicable chemical strategy that is also compatible with all canonical reactive moieties has been reported thus far (**Chapter 1**).[1,12,13] Aside from the previous reported strategies, the specific environment of the C-terminus could also be exploited for its selective functionalization. In this section, we propose a few strategies based on this concept that could potentially afford a selective methodology.

In 2007, Kawakami and Aimoto published a ligation strategy they termed the cysteinyl prolyl ester (CPE) peptide ligation.[14] This approach employs a C-terminal CPE sequence (**21**) as an auto-activating unit which could undergo an N-to-S acyl transfer followed by cyclization to form a diketopiperazine thioester group (**22**) by attack of the cysteinyl nitrogen onto the ester carbonyl (**Scheme 3A**). The resulting thioester could in turn participate in ligation with

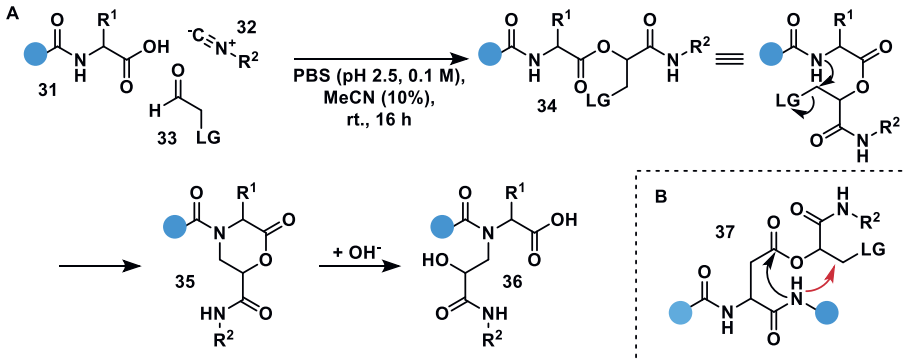
an N-terminal cysteine bearing peptide (**23**) to provide a ligated product (**24**). This strategy was later employed by the group of Suga, who translated it into a fully recombinant strategy where glyoxylic acid was ribosomally expressed at the C-terminus to provide the required ester.[15] More recently, the group of Kajihara simultaneously reported two methods that exploit the inherent tendency for C-terminal Cys residues towards N-to-S acyl transfer to afford thioesters that can in turn be utilized for ligation chemistry.[16–18] In our Passerini-based functionalization followed by enzymatic ligation (**Chapter 2**), we exploited the properties of omniligase-1 for the selective C-terminal functionalization. Similarly, we envisioned that our Passerini methodology could be applied to generate substrates for CPE ligation (**Scheme 3B**). We eagerly set out to synthesize a simple peptide substrate, *i.e.* Ac-GCP-OH (**26**), and functionalized it with a Passerini reaction to form the required ester (**27**) for CPE ligation. Upon subjecting it to ligation conditions with Cys-OMe (**28**), we were delighted to observe the desired ligation product (**29**) based on HPLC in our first attempt. With this proof of concept in hand, we have evidence that we can solve the selectivity issue for the C-terminus in **Chapter 3** through chemical means, without relying on an enzyme as in **Chapter 2**.



Scheme 49. A. Concept of the CPE ligation. B. Passerini functionalization of Ac-GCP-OH and subsequent CPE ligation with Cys-OMe.

Another strategy that we envisioned exploits the peptide backbone and is reminiscent of an aspartamide forming reaction. Aspartamide formation has caused many peptide chemists distress, particularly during SPPS, since it results in both racemization and in a mixture of α-

and β-peptides.[19–21] This is caused by the attack of the backbone amide nitrogen onto the carbonyl of a sidechain protected aspartate (**Chapter 1**). Realizing the potential, we envisioned exploiting this reaction for selective modification of the C-terminus. Combining our Passerini reaction (**Chapter 1**-**3**) with an aldehyde bearing a leaving group (or precursor thereof, **33**) provides an ester with a tethered leaving group on the β-position (**34**). This moiety could in turn be substituted by the adjacent amide nitrogen, much like aspartamide formation, resulting in a cyclic morpholinone (**35**). Subsequent hydrolysis then cleaves any undesired Passerini products and provides the desired C-terminally functionalized peptide (**36**, **Scheme 4A**). Although we do not expect this substitution to occur with Passerini products on the sidechain of Asp residues, owing to the unfavorable ring size, we know from work described in **Chapter 2** that aspartamide formation can result by substitution of the involved ester (**Scheme 4B**). Therefore, it is crucial to find the right ester that does not readily result in aspartamide formation but will afford the desired morpholinone. The isocyanide substrates of the Passerini reaction could bear a variety of functionalities, *e.g.* reactive linkers, peptides and proteins, thus providing access to a range of functionalized peptide products.[22] If this reaction would be successful, it will represent a completely unique strategy that is capable of selective functionalization of the C-terminus in one reaction step.

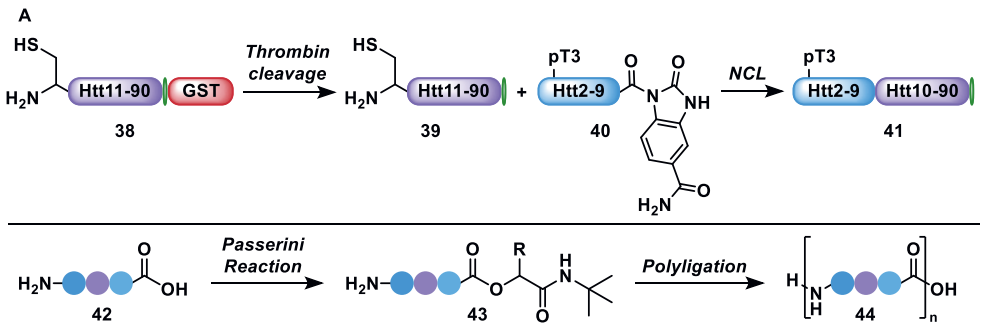


Scheme 50. A. Passerini functionalization of peptide C-termini with aldehydes bearing a leaving group for subsequent substitution and hydrolysis. B. Internal aspartamide formation (black arrow) or unfavorable cyclization by leaving group substitution (red arrow) from Passerini functionalized Asp residue.

5.5 Peptide polyligation for repetitive protein sequences

Throughout this thesis, the main challenge was achieving high selectivity in the presence of all canonical amino acids. However, many protein-based materials, such as silk, elastin, or collagen have highly repetitive sequences composed mainly of amino acid residues that do not interfere with the Passerini reaction. Furthermore, these types of structures can be

difficult to obtain through recombinant strategies owing to genetic instability and the lack of efficient post-translational modification mechanisms resulting in low yield.[23–25] Therefore, various groups have investigated the synthesis of polymeric (bio)materials utilizing ligation strategies and reported strategies to obtain hydrogels, collagen-like materials, and block copolymers.[26–30] A representative example is the N-terminal exon 1 fragment of huntingtin (Httex1), a disease-relevant protein fragment containing a polyglutamine tract that makes it highly aggregation-prone and difficult to produce by recombinant technology. Earlier studies often relied on fusion proteins or constructs with inexact termini, complicating interpretation of aggregation behavior since pathogenicity depends on repeat length and flanking sequences.[31] Lashuel *et al*. addressed these limitations using ligation-based approaches that enabled the preparation of untagged, pure Httex1 (**41**) in milligram quantities, including mutant variants, which in turn enabled higher-resolution studies of its structure and aggregation behavior (**Scheme 5A**).[32,33] Similarly, the developed ligation strategies in **chapters 2-3** can also be exploited as a means of obtaining large repetitive sequences. Moreover, we envisioned the polymerization of monomeric peptide repeating units for the synthesis of protein segments and biomaterials. By synthesizing peptide units bearing an active N-terminus and modified at the C-terminus with ligation substrates (**43**), we could subsequently employ them in "polyligation" reactions (**Scheme 5B**). This approach would be particularly interesting for the synthesis of biomaterials, adding a valuable tool to the chemical arsenal for the obtention of these structures.

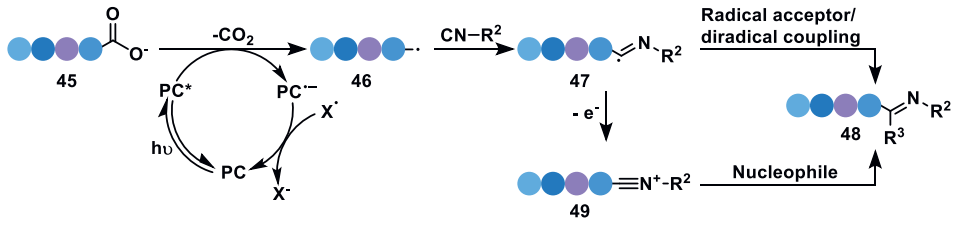


Scheme 51. A. Ligation approach for the synthesis of Httex1 by Lashuel et al. B. Passerini functionalization and subsequent polyligation strategy.

5.6 C-terminal decarboxylative coupling with isocyanides

In recent years, isocyanides have re-emerged as versatile reagents for both photochemical and electrochemical conversions. The advent of visible-light photochemistry and the resurgence of electrochemical methods have enabled their controlled utilization under

mild, sustainable conditions.[34–36] Despite significant progress, unresolved mechanistic and reactivity challenges ensure that redox chemistry with isocyanides remains a promising field for future exploration. Our group has recently gained both interest and experience in such transformations.[37,38] Therefore, in this section we propose a novel means for isocyanide-based redox reactions for the functionalization of peptide and protein C-termini. **Chapter 1** discussed various examples of selective C-terminal decarboxylative functionalization of peptides. Aside from Giese additions, initially pioneered by Macmillan, the resulting radical species after decarboxylation has been subjected to a variety of different chemical transformations (**Chapter 1**).[12] However, one important functional group that has not yet been reported for this purpose is the isocyanide. Therefore, it would be interesting to do precisely that: a selective decarboxylative coupling with isocyanides (**Scheme 6**). By employing the strategy that our predecessors have developed to obtain selectivity for the C-terminus (*i.e.* fine-tuning the employed redox potentials), resulting in selective formation of the alkyl radical (**46**), we can combine this species with an isocyanide to afford an imidoyl radical intermediate (**47**). Then, it can combine with a different species (*e.g.* radical acceptor or diradical coupling) to arrive at the final product (**48**) or be oxidized to a nitrilium ion (**49**) which can subsequently be attacked by various nucleophiles to afford other functionalized products (**48**, **Scheme 6**). This concept holds tremendous strength, as it combines the power of selective functionalization with a strongly diversifying handle. For example, the isocyanide species could be made from a peptide or protein N-terminus. Upon oxidation of the imidoyl radical resulting in the nitrilium ion (**49**), this species can react with water and after tautomerization afford a natural peptidic amide bond and therefore a traceless process. The strategy could also be utilized for a variety of additional applications, *e.g.*, dimerization, cyclization, stapling, and labeling, highlighting its true potential.

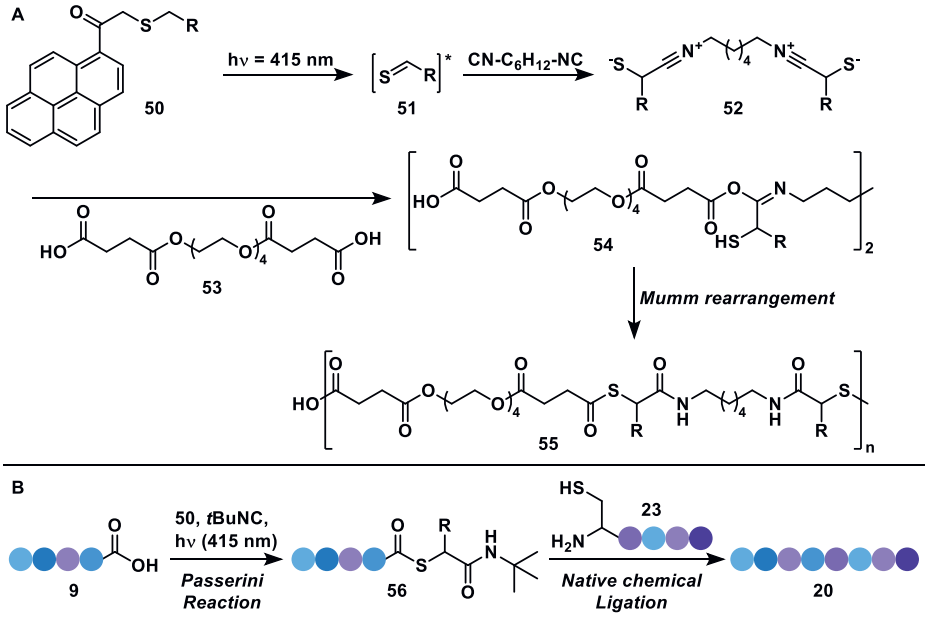


Scheme 52. Photochemical decarboxylative coupling with isocyanides.

5.7 Thioaldehydes and thioketones for direct Passerini thioester synthesis

Over the past decade, numerous papers have been published involving the use of thioaldehydes. These species are highly reactive and as a result have found limited practical use. However, they have been employed for a broad range of conversions including disulfide

formation, 1,3-dipolar cycloadditions, hetero-Diels-Alder cycloadditions, and nucleophilic trapping with amines and alkoxyamines.[39–41] The group of Barner-Kowollik have developed a means to obtain thioaldehydes by employing a photochemical cleavage of pyrene-acyl sulfides (**50**). They were able to trap the formed species (**51**) with various nucleophiles and Diels-Alder reagents (*i.e.* dienes) and developed a visible light induced self-reporting release system using their thioaldehyde precursors.[42,43] Furthermore, the group reported a light-induced Passerini polymerization where they employed the pyrene-acyl sulfide (**50**) as thioaldehyde surrogate to afford degradable thioester-amide polymers (**55**, **Scheme 7A**).[44] Finally, Tuten *et al*. recently reported similar products, but now by employing already synthesized thioketones.[45]



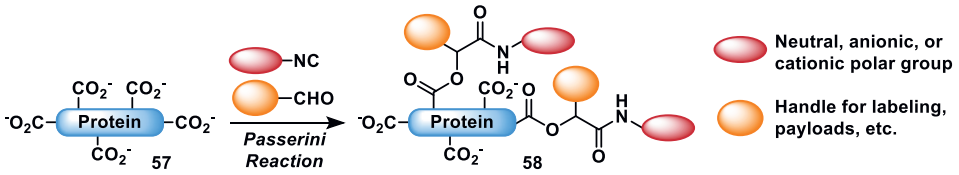
Scheme 53. A. Light-induced Passerini polymerization using pyrene-acyl sulfide as thioaldehyde component. B. Passerini functionalization towards thioesters using thioaldehydes followed by native chemical ligation.

Inspired by the work of Tuten and predecessors thereof, and related to the work described in **Chapter 3**, we envisioned employing the thioaldehyde moiety (**51**) to synthesize C-terminal thioesters (**56**) in a single step. The products should in turn serve as substrates for native chemical ligation chemistry (**Scheme 7B**). Omitting the initial O to S acyl transfer described in **Chapter 3** could facilitate the kinetics of the process. The group of Wang recently employed thioaldehydes for C-terminal peptide bioconjugation and macrocylization under aqueous buffer conditions.[46] Their achievement demonstrates the potential for finding a viable process using these moieties in the context of ligation

chemistry. Furthermore, there is much room for optimization of the thiocarbonyl species since it can be incorporated in a variety of ways including the photochemical route developed by Barner-Kowollik and colleagues and the less reactive thioketones by the group of Tuten.

5.8 Water-soluble isocyanides for protein modifications

The water-soluble isocyanides that we developed and described in **chapter 4** represent an unprecedented novel class of reagents that could be utilized for a variety of applications. We are particularly excited to explore new strategies that employ these novel compounds for complex peptides and protein functionalization. An interesting starting point would be to utilize them to generate cell-penetrating peptides and proteins. Scientists have long sought methods to change the polarities of peptides in order to allow them to cross the cell membrane, which is particularly interesting for medicinal applications.[47–49] Using the chemistry developed in **chapter 4**, we could modify protein carboxylic acids (**57**), which are normally negatively charged under physiological pH, to polar neutral, anionic, or cationic functionalities (**58**), thus providing control over the isoelectric point of the protein (**Scheme 8**). For example, changing the net surface charge of a protein to be more positive enforces higher rates of cellular uptake. This is particularly interesting when combined with intracellular biological esterases, which can cleave the polar Passerini adducts on the involved protein and revert it back to its native form.[50–55] Similar to work previously reported by Raines *et al.*, it would therefore be a bioreversible esterification capable of cytosolic delivery, a strategy that could in turn be employed for payload release or bioassay development.[50–55] Moreover, the multicomponent nature of the Passerini reaction inherently provides the products with an additional point of modification, the aldehyde component. It is interesting to incorporate an aldehyde with virtually any tethered functionality along with the polar isocyanide of choice to modify the protein, *e.g.* biotin tags, fluorescent or radioactive labels (**Scheme 8**). Additionally, methods developed to facilitate the solubility of functionalized protein carboxylic acids, such as encapsulation[56], would be rendered redundant. Finally, this method could provide a novel means to install soluble groups for on-demand protein activation.[57]



Scheme 54. Protein functionalization utilizing the Passerini reaction as charge manipulation and labeling strategy.

5.9 Conclusion

Although many strategies have now emerged for the functionalization of peptide C-termini, selectivity and universal applicability remain as the most challenging and unresolved aspects. The research described in this thesis was inspired by our group's experience with isocyanide-based multicomponent reactions, and we wanted to investigate their applicability to peptides. We have shown multiple strategies that can be employed for the functionalization of peptide C-termini, but much remains to be discovered in this field. This chapter relays several novel research directions related to our growing experience in peptide modification. Moreover, our group's recent interest and experience in photochemistry will allow the overlap of these two fields and open additional novel research directions. We believe that much remains to be learned about peptide synthesis and modification, particularly with regards to selective C-terminal functionalization, leaving us to look at the future with great enthusiasm and curiosity.

5.10 Experimental Section

General Remarks

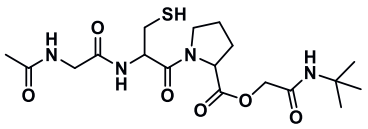
Unless stated otherwise, all solvents and commercially available reagents were used as received without further purification. Fmoc-protected amino-acids were purchased from Combi-blocks, Doug discovery and GLbiochem. Coupling reagents, solvents, buffer salts, carbonyl and isocyanide compounds were purchased from Sigma-Aldrich, Doug discovery, Combi-blocks, Iris Biotech, Biosolve, and TCI chemicals. Preparative-scale purification was performed using a Shimadzu Nexera preparative HPLC system, consisting of: a CBM-40 systems controller, LC-20AP preparative pump, FCV-200AL quaternary vale, SPD-M40 photodiode array detector (PDA), LCMS-2050 mass detector, LC-40D HPLC pump, LH-40 fraction collector, and an FCV-20AH2 valve unit. Separation was carried out using Shim-pack Scepter C_{18}-120 column (5 µm, 20x150 mm) with an optimised gradient for each compound (MeCN/MilliQ water with 0.1% TFA) at a flow rate of 20 mL/min. Fraction collection was triggered by PDA and/or MS signal detection. Peptide samples were lyophilized with a Büchi Lyovapor^{tm} L-250 Pro lyophilizer. High Pressure Liquid Chromatography-Mass Spectrometry (HPLC-MS) analyses were performed on a Shimadzu Nexera 2 UHPLC system equipped with a Shimadzu LC-30AD pump, a SPD-M30A photodiode array detector and LCMS-2020 single quadrupole detector. The eluent consisted of MilliQ water and LCMS grade acetonitrile, and 0.1% formic acid. A Waters XSelect CSH C18 column (3.0 mm × 75 mm with a particle size of 3.5 mm) was employed for the separation at 30 °C. The method comprised a gradient of acetonitrile in water: 5% (2 min) to 95% acetonitrile (6 min), 1 min at 95% acetonitrile and back to 5% acetonitrile (2 min). The substrates and products were quantified by their UV absorption at 220 nm. High resolution mass spectroscopy was conducted on a Bruker micrOTOF-Q II mass spectrometer (electrospray ionization). NMR spectra (1D ^{1}H and ^{13}C, COSY, DIPSI, $^{13}C^{1}H$ HSQC) were recorded on a Bruker Avance III HD 700 MHz spectrometer equipped with a TCI cryoprobe, or on a Jeol JNM-ECZL400S 400 MHz using the residual solvent or TMS standard (0 ppm) as reference. The data is reported as follows: chemical shift (δ ppm), coupling constant (J, Hz), multiplicity (s = singlet, d = doublet, t = triplet, q = 5 quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets).

General synthetic procedures

General procedure A: SPPS of starting material peptides.
The reactions were performed in either a syringe reactor on an orbital shaker at room temperature or using a glass SPPS vessel to be stirred by nitrogen bubbling. The procedures were performed with 10 mL solvent/1 g resin unless stated otherwise. Rink amide AM resin (GL Biochem, Cat. No. 49001) was swollen in CH_2Cl_2 (dichloromethane, DCM) for 30 min and was subsequently washed with dimethylformamide (DMF, 3x). The amino acid (AA) coupling solution of Fmoc-AA-OH (4 equiv), and ethyl cyano (hydroxyimino) acetate (oxyma, 4 equiv) in DMF was prepared. N,N-diisopropylcarbodiimid (DIC, 6 equiv) was added to the coupling solution when ready for use. Subsequently, the coupling solution was added to the resin and allowed to react for 20 mins. Then, DIC (6 equiv) was added to the mixture and allowed to react for 20 mins. Subsequently, the reaction solution was discarded, and the resin was washed with DMF (3×) before Fmoc removal was performed by adding a deprotection solution of piperidine in DMF (2:8, v/v) and reacting for 10 min. The solution was discarded and the Fmoc removal repeated. Next, the resin was washed with DMF (6×). Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above. 2-chlorotrityl chloride (CTC)-resin (100-200 mesh), 1% DVB (Novabiochem, Cat. No. 8.55017) was swollen in DCM for 30 min. A solution of 2.5 equiv Fmoc-AA-OH and *N,N*-diethylisopropylamine (DIPEA, 5 equiv) in DCM was added to the resin and reacted for 1 h. The solution was discarded, and the resin was washed with DCM (6x). Non-reacted linker groups were capped by adding a solution of methanol (MeOH), DIPEA and DCM (3:1:16, v/v/v) and reacting for 10 min. The solution was discarded and the capping step repeated. The resin was washed with DCM (5x) and DMF (3x). Subsequent AAs were added by numerous cycles of AA coupling, Fmoc removal, and washing as described above for the rink amide AM resin. Peptides were N-terminally acetylated

by adding a solution of Ac_2O, DIPEA and DMF (1:1:8, v/v/v) to the reaction and reacted for 10 min. The acetylation was repeated. The resin was washed with DMF (3×) and DCM (3×). Subsequently, the peptide was cleaved and the side chain protecting groups were removed in one step by adding a cleavage solution of trifluoroacetic acid (TFA), triisopropylsilane (TIPS) and water (95:2.5:2.5, v/v/v) and reacting for 2 h. The cleavage solution was collected precipitated in cold diethyl ether (-20 °C) and centrifuged (15 min, 4000 rcf). The supernatant was discarded and the precipitation and centrifugation were repeated twice. The crude peptide was dissolved in MeCN and water and lyophilized. The peptides were purified by preparative HPLC.

2-(tert-butylamino)-2-oxoethyl acetylglycylcysteinylprolinate (27)



To a stirred solution of Ac-GCP-OH (46 mg, 145 µmol, 1 equiv) in PBS (6.52 mL, pH 2.5, 0.1 M) and MeCN (0.725 mL, 10%) was added formaldehyde (37 wt% in water, 118 mg, 108 µL, 1.45 mmol, 10 equiv) and *t*-butylisocyanide (120 mg, 164 µL, 1.45 mmol, 10 equiv). The resulting mixture was stirred at rt. for 16 h. The reaction mixture was diluted with MeCN and H_2O until all solids had dissolved, then brought to pH ~7 (monitored by pH paper) using NaOH (1 M) while stirring vigorously. An aliquot was taken for HPLC analysis. Purification was done by preparative HPLC. Isolated as colourless sticky material (29.8 mg, 69.2 µmol, 48%).

Analytical HPLC trace of prep-HPLC purified title compound; Rt 5.02 min (λ = 220 nm); Calculated Mass $[M+H]^+$: 431.2 (100%); Mass Found (ESI^+); 431.2 $[M+H]^+$.

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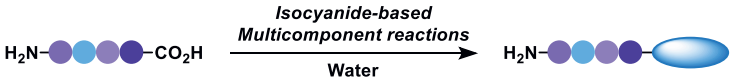
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Summary

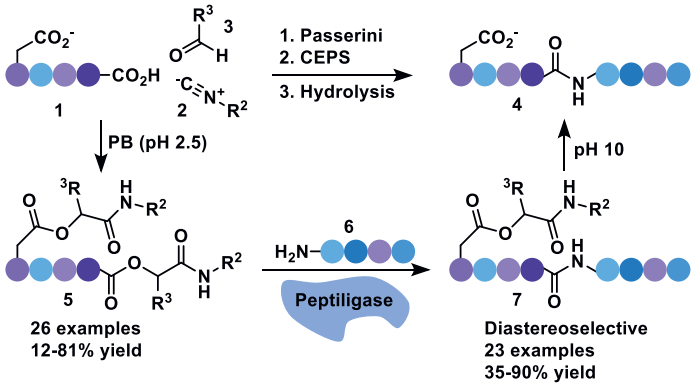
Proteins and peptides are central to nearly all biological processes and have become indispensable tools in modern science and medicine. From catalysis and cellular signaling to therapeutic and diagnostic applications, their impact is vast, exemplified by breakthroughs such as the structural elucidation of viral proteins and the development of protein-based drugs. Despite their importance, accessing sufficient quantities of pure, well-defined proteins remains a significant challenge. Moreover, while advances in synthesis and functionalization have enabled the tuning of protein properties, achieving precise and site-selective modifications continues to limit progress in drug development and biomaterials engineering. While most of the functionalization strategies have targeted the N-terminus or reactive side-chains (e.g. cysteine, methionine, lysine and tyrosine), the selective modification of peptide C-termini is critical for the development of therapeutic conjugates, enzyme conjugates, and functional biomaterials, but poses a major synthetic challenge. **Chapter 1** presents a comprehensive overview of the various strategies reported for C-terminal functionalization of peptides and proteins. The work in this thesis was based on our interest in developing novel strategies for C-terminal functionalization on unprotected peptides using isocyanide-based multicomponent reactions under aqueous conditions (**Scheme 1**).



Scheme 55. Isocyanide-based multicomponent reactions for the C-terminal modification of unprotected peptides in water.

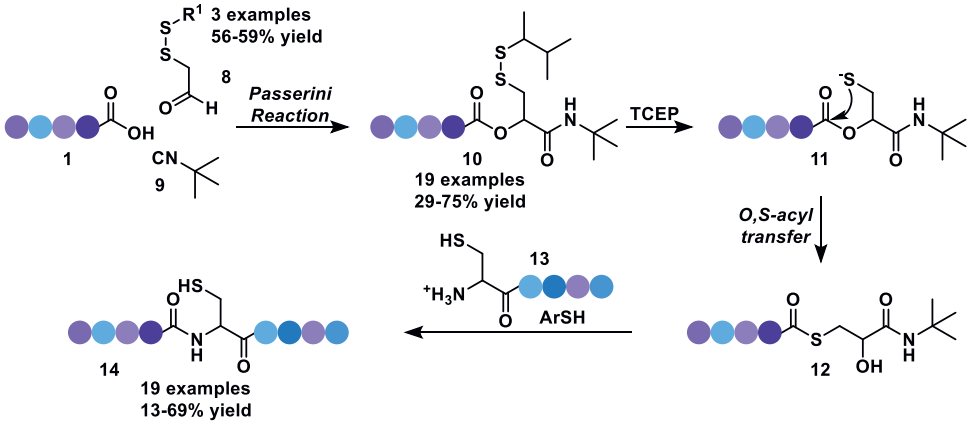
To get generally acquainted with C-terminal peptide functionalization, we set out to employ the carboxylic acid (**1**) as substrate for the Passerini reaction (**Chapter 2**). We presented a hybrid chemo-enzymatic strategy for the selective modification of peptide C-termini by integrating the Passerini multicomponent reaction with enzymatic ligation (**Scheme 2**). In this method, the Passerini reaction is performed under acidic aqueous buffer conditions to generate α-acyloxyamide products (**5**) on completely unprotected peptides. The products in turn function as ideal substrates for enzymatic ligation utilizing the omniligase-1 enzyme. The approach addresses a longstanding challenge in peptide functionalization by enabling site-selective modification at the C-terminus. The Passerini reaction facilitates selective functionalization of the peptide carboxylates, while the enzyme in turn selectively targets the C-terminus. Any remaining esters (**7**) can subsequently be hydrolyzed after the ligation to afford the final product (**4**). We demonstrated that this strategy is compatible with a range of peptides, aldehydes (**3**) and isocyanides (**2**), allowing for the incorporation of structurally diverse moieties under mild conditions. Furthermore, by synthesizing esters bearing a chiral center at the α-position and subjecting them to enzymatic ligation, we

found that the enzyme is diastereoselective in nature and merely reacts with the natural isomer to afford amide products.



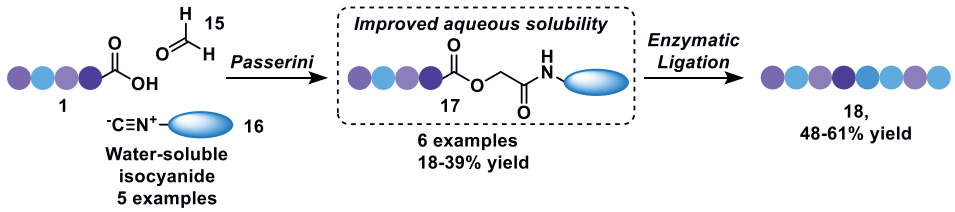
Scheme 56. Passerini C-terminal functionalization of unprotected peptides and subsequent enzymatic ligation and hydrolysis.

Having developed this novel Passerini strategy for C-terminal functionalization, we were curious to see if we could also apply this method for a chemical ligation strategy, namely native chemical ligation (NCL). Therein, we envisioned that by incorporating an aldehyde with a tethered (protected) thiol functionality in the Passerini reaction on peptides, we could subject the synthesized product (**11**) to reductive conditions to allow for an O-to-S acyl transfer, thus providing the required thioester (**12**) for NCL (**Chapter 3**). For that purpose, we synthesized asymmetric disulfide aldehyde compounds (**8**) and incorporated them as substrates in the aqueous Passerini reaction to generate C-terminal α-acyloxyamides bearing a disulfide-protected thiol (**10**, **Scheme 3**). Under standard NCL conditions, these substrates undergo efficient ligation for peptides featuring all canonical C-terminal amino acids typically compatible with NCL. Overall, this strategy enables C-terminal functionalization of unprotected peptides and broadens the applicability of NCL to native peptides and proteins for modification and synthesis. In the future, we hope to further investigate this method towards a C-terminal selective strategy that allows for a one-pot procedure and increase the kinetics and yield by incorporation of selenium-based substrates (**Chapter 5**).



Scheme 57. Passerini C-terminal peptide functionalization and subsequent native chemical ligation.

The strategies developed in **chapters 2**-**3**, while effective for C-terminal functionalization, were accompanied by a notable decrease in protein solubility, limiting their applicability to larger or more complex systems. To address this challenge, we developed an unprecedented novel class of ionic, water-soluble isocyanide reagents (**16**) designed to enable efficient modification under aqueous conditions (**Chapter 4**). These reagents incorporate charged functionalities alongside hydrogen-bonding motifs, enhancing solubility while also allowing modulation of the isoelectric properties of the modified peptides. We established a concise synthetic route to access these isocyanides and demonstrated their successful application in Passerini multicomponent reactions performed in water, without observable precipitation (**Scheme 4**). Importantly, the resulting esterified products (**17**) remained compatible with subsequent enzymatic ligation using omniligase-1, which showed a preference for polar, cationic substrates. Overall, we demonstrate that rational isocyanide design can effectively mitigate solubility losses associated with C-terminal esterification. This work expands the scope of isocyanide-based ligation chemistry and provides a more broadly applicable platform for protein modification in aqueous environments. We believe that this strategy holds great potential for the modulation of the isoelectric points of proteins, providing a novel means for intracellular delivery, solubility modulation, and bioassay development (**Chapter 5**).



Scheme 58. Water-soluble isocyanides as substrates for C-terminal peptide functionalization and subsequent enzymatic ligation.

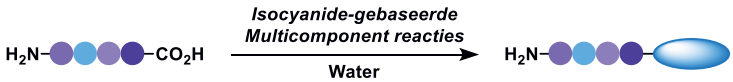
Overall, the work presented in this thesis establishes a versatile and conceptually new platform for the selective C-terminal functionalization of peptides based on isocyanide-driven multicomponent chemistry. By integrating the Passerini reaction with both enzymatic ligation and native chemical ligation strategies, we have demonstrated that efficient and site-selective modification of unprotected peptides can be achieved under mild, aqueous conditions. Importantly, the development of water-soluble, ionic isocyanides addresses a key limitation of earlier approaches by preserving solubility and enabling broader applicability to more complex systems. Together, these advances significantly expand the synthetic toolbox available for peptide and protein modification, enabling the introduction of diverse functionalities with high precision. Looking forward, we anticipate that further optimization of these methodologies, including one-pot procedures and the incorporation of more reactive substrates, will enhance their efficiency and scope. Ultimately, the strategies developed herein pave the way toward more general and practical approaches for the modification of biomolecules, with potential applications in therapeutics, chemical biology, and biomaterials science.

Samenvatting

Duiken voorbij bescherming:

Waterige Passerini-functionalisatie van onbeschermde peptide C-termini

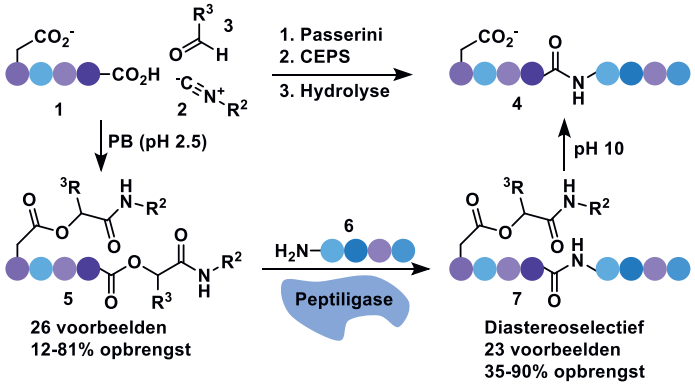
Eiwitten en peptiden staan centraal in vrijwel alle biologische processen en zijn onmisbare hulpmiddelen geworden in de moderne wetenschap en geneeskunde. Van katalyse en cellulaire signalering tot therapeutische en diagnostische toepassingen, hun impact is enorm, zoals blijkt uit doorbraken zoals de structurele opheldering van virale eiwitten en de ontwikkeling van eiwitgebaseerde geneesmiddelen. Ondanks hun belang blijft het verkrijgen van voldoende hoeveelheden zuivere, goed gedefinieerde eiwitten een aanzienlijke uitdaging. Bovendien, hoewel vooruitgang in synthese en functionalisatie het mogelijk heeft gemaakt om eiwiteigenschappen af te stemmen, blijft het bereiken van precieze en plaatsselectieve modificaties een beperkende factor voor vooruitgang in geneesmiddelontwikkeling en biomaterialenengineering. Hoewel de meeste functionaliseringsstrategieën zich hebben gericht op het N-terminus of reactieve zijketens (bijv. cysteïne, methionine, lysine en tyrosine), is de selectieve modificatie van peptide C-termini van cruciaal belang voor de ontwikkeling van therapeutische conjugaten, enzymconjugaten en functionele biomaterialen, maar vormt dit een grote synthetische uitdaging. **Hoofdstuk 1** presenteert een uitgebreid overzicht van de verschillende strategieën die zijn gerapporteerd voor C-terminale functionalisatie van peptiden en eiwitten. Het werk in dit proefschrift is gebaseerd op onze interesse in het ontwikkelen van nieuwe strategieën voor C-terminale functionalisatie van onbeschermde peptiden met behulp van isocyanide-gebaseerde multicomponentreacties onder waterige omstandigheden (**Schema 1**).



Schema 1. Op isocyanide-gebaseerde multicomponent reacties voor de C-terminale modificatie van obeschermde peptides in water.

Om algemeen vertrouwd te raken met C-terminale peptidefunctionalizatie, hebben wij ervoor gekozen om het carbonzuur (**1**) als substraat te gebruiken voor de Passerini-reactie (**Hoofdstuk 2**). Wij presenteerden een hybride chemo-enzymatische strategie voor de selectieve modificatie van peptide C-termini door de Passerini multicomponentreactie te combineren met enzymatische ligatie (**Schema 2**). In deze methode wordt de Passerini-reactie uitgevoerd onder zure, waterige buffercondities om α-acyloxyamide (**5**) producten te genereren op volledig onbeschermde peptiden. Deze producten fungeren vervolgens als ideale substraten voor enzymatische ligatie met behulp van het enzym omniligase-1. Deze benadering pakt een langdurige uitdaging in peptide functionalisatie aan door plaatsselectieve modificatie aan het C-terminus mogelijk te maken. De Passerini-reactie faciliteert selectieve functionalisatie van de peptidecarboxylaten, terwijl het enzym op zijn beurt selectief de C-terminus target. Eventuele resterende esters (**7**) kunnen na de ligatie

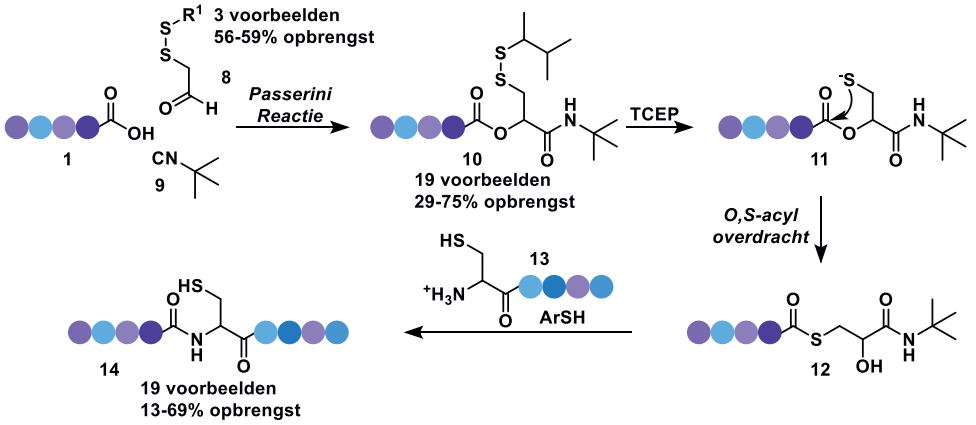
worden gehydrolyseerd om het eindproduct (**4**) te verkrijgen. Wij hebben aangetoond dat deze strategie compatibel is met een reeks peptiden, aldehyden (**3**) en isocyanides (**2**), waardoor de introductie van structureel diverse groepen onder milde omstandigheden mogelijk is. Bovendien hebben wij, door esters te synthetiseren met een chiraal centrum op de α-positie en deze te onderwerpen aan enzymatische ligatie, vastgesteld dat het enzym diastereoselectief is en uitsluitend reageert met het natuurlijke isomeer om amideproducten te vormen.



Schema 2. Passerini C-terminale functionalisatie van onbeschermde peptides en vervolgende enzymatische ligatie en hydrolyse.

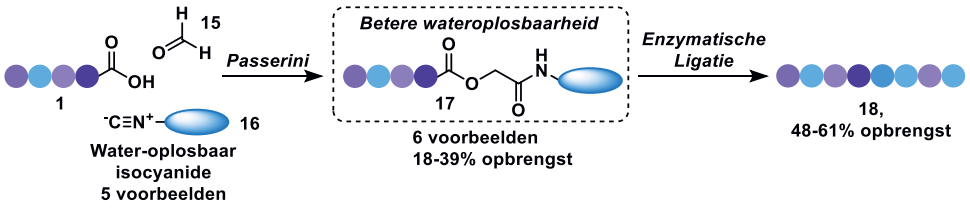
Na het ontwikkelen van deze nieuwe Passerini-strategie voor C-terminale functionalisatie, wilden wij onderzoeken of deze methode ook toepasbaar is voor een chemische ligatiestrategie, namelijk native chemical ligation (NCL). Daarbij voorzagen wij dat, door een aldehyde met een gekoppelde (beschermde) thiol functionaliteit in de Passerini-reactie op peptiden te incorporeren, wij het gesynthetiseerde product (**11**) onder reducerende omstandigheden konden omzetten via een O-naar-S acyltransfer, waardoor het benodigde thioester (**12**) voor NCL wordt verkregen (**Hoofdstuk 3**). Voor dit doel hebben wij asymmetrische disulfide-aldehyden (**8**) gesynthetiseerd en deze als substraten gebruikt in de waterige Passerini-reactie om C-terminale α-acyloxyamiden te genereren met een disulfide-beschermde thiol (**10**, **Schema 3**). Onder standaard NCL-condities ondergaan deze substraten efficiënte ligatie voor peptiden die alle canonieke C-terminale aminozuren bevatten die doorgaans compatibel zijn met NCL. Over het geheel genomen maakt deze strategie C-terminale functionalisatie van onbeschermde peptiden mogelijk en verbreedt zij de toepasbaarheid van NCL naar natuurlijke peptiden en eiwitten voor modificatie en synthese. In de toekomst hopen wij deze methode verder te ontwikkelen richting een C-terminaal selectieve strategie die een éénpotprocedure mogelijk maakt, en de kinetiek en

opbrengst te verhogen door de incorporatie van selenium-gebaseerde substraten (**Hoofdstuk 5**).



Schema 3. Passerini C-terminale peptide functionalisatie en vervolgende native chemical ligation.

De strategieën ontwikkeld in **hoofdstukken 2-3** waren, hoewel effectief voor C-terminale functionalisatie, gepaard met een merkbare afname in eiwitoplosbaarheid, wat hun toepasbaarheid op grotere of complexere systemen beperkte. Om deze uitdaging aan te pakken, hebben wij een ongekende nieuwe klasse van ionische, wateroplosbare isocyanide reagentia (**16**) ontwikkeld, ontworpen om efficiënte modificatie onder waterige omstandigheden mogelijk te maken (**Hoofdstuk 4**). Deze reagentia combineren geladen functionaliteiten met waterstofbrugvormende motieven, wat de oplosbaarheid verhoogt en tegelijkertijd modulatie van de iso-elektrische eigenschappen van de gemodificeerde peptiden mogelijk maakt. Wij hebben een beknopte synthetische route opgesteld om deze isocyanides te verkrijgen en hun succesvolle toepassing aangetoond in Passerini multicomponent reacties die in water worden uitgevoerd, zonder waarneembare precipitatie (**Schema 4**). Belangrijk is dat de resulterende veresterde producten (**17**) compatibel bleven met daaropvolgende enzymatische ligatie met omniligase-1, dat een voorkeur vertoonde voor polaire, kationische substraten. In het geheel tonen wij aan dat rationeel ontwerp van isocyaniden effectief de oplosbaarheidsverliezen geassocieerd met C-terminale verestering kan beperken. Dit werk breidt de reikwijdte van isocyanide-gebaseerde ligatiechemie uit en biedt een breder toepasbaar platform voor eiwitmodificatie in waterige omgevingen. Wij geloven dat deze strategie groot potentieel heeft voor het moduleren van de iso-elektrische punten van eiwitten en daarmee een nieuwe benadering biedt voor intracellulaire aflevering, oplosbaarheidsmodulatie en de ontwikkeling van bioassays (**Hoofdstuk 5**).



Schema 4. Water-oplosbare isocyanides als substraten voor C-terminale peptide functionalisatie en vervolgende enzymatische ligatie.

Over het geheel genomen vormt het werk dat in dit proefschrift wordt gepresenteerd een veelzijdig en conceptueel nieuw platform voor de selectieve C-terminale functionalisatie van peptiden, gebaseerd op isocyanide-gedreven multicomponent chemie. Door de Passerini-reactie te integreren met zowel enzymatische ligatie als native chemical ligation hebben wij aangetoond dat efficiënte en plaatsselectieve modificatie van onbeschermde peptiden kan worden bereikt onder milde, waterige omstandigheden. Belangrijk is dat de ontwikkeling van wateroplosbare, ionische isocyaniden een belangrijke beperking van eerdere benaderingen adresseert door de oplosbaarheid te behouden en bredere toepasbaarheid op complexere systemen mogelijk te maken. Samen vergroten deze vooruitgangen aanzienlijk de synthetische gereedschapskist voor peptide- en eiwitmodificatie, en maken zij de introductie van diverse functionaliteiten met hoge precisie mogelijk. Met het oog op de toekomst verwachten wij dat verdere optimalisatie van deze methodologieën, waaronder éénpotprocedures en de incorporatie van meer reactieve substraten, hun efficiëntie en reikwijdte verder zal vergroten. Uiteindelijk effenen de hier ontwikkelde strategieën de weg naar meer algemene en praktische benaderingen voor de modificatie van biomoleculen, met potentiële toepassingen in de therapeutica, chemische biologie en biomaterialenwetenschap.

Impact Paragraph

Diving Beyond Protection:

Aqueous Passerini Functionalization of Unprotected Peptide C-Termini

The main purpose of the research described in this thesis was to develop new and broadly applicable strategies for the selective modification of peptide and protein C-termini under mild, aqueous conditions. As outlined in **Chapter 1**, despite more than 150 years of research, C-terminal functionalization remains a significant challenge in chemistry, particularly when applied to complex and unprotected biomolecules such as peptides and proteins. Existing methods often suffer from limited selectivity, poor compatibility with sensitive functional groups, or require harsh conditions and protecting groups. In this thesis, we set out to address these limitations by exploring isocyanide-based multicomponent reactions, specifically the Passerini reaction, as a platform for site-selective C-terminal modification.

The comprehensive overview of existing strategies for C-terminal functionalization presented in **Chapter 1** provides the chemical community with a valuable resource for both peptide and protein synthesis and their subsequent modification. By bringing together and comparing the available approaches, this overview can reduce the time and resources required to identify suitable methods for specific applications. The collected knowledge may, for example, facilitate the modification of naturally occurring peptides and proteins with important medicinal properties (*i.e.* active compounds) or mechanical properties (*i.e.* materials and adhesives). Modification of their C-termini could enable the development of new analytical techniques and assays, support advances in proteomics, and potentially contribute to the identification of novel medicines for previously troublesome target diseases.

Beyond serving as a reference, the comparison of existing methods in **chapter 1** also identifies research gaps, advantages, limitations, and the requirements for a broadly applicable and genuinely selective C-terminal functionalization strategy. These insights can therefore serve as inspiration for researchers in both academia and industry to develop new synthetic approaches. For example, strategies exploiting chemoselectivity for carboxylic acids over other functional groups could be developed not only for peptide-related chemistry but also for other areas of synthesis, potentially providing access to previously unachievable target structures. Such developments could stimulate new research projects, grant applications, and potentially even new start-up companies.

In **Chapter 2**, we demonstrated that the Passerini reaction can be combined with enzymatic ligation to achieve selective modification at the C-terminus of unprotected peptides. This represents an important scientific contribution as it merges two traditionally separate fields, multicomponent chemistry and biocatalysis, into a single complementary strategy. The ability to introduce diverse chemical functionalities under mild, aqueous conditions and subsequently ligate peptides in a controlled and selective manner significantly expands the

available toolbox for peptide and protein modification. The modularity of the Passerini reaction is particularly advantageous, as a range of products can be readily prepared in a single step by varying the aldehyde and isocyanide starting materials. This flexibility also enabled the identification of the enzyme's diastereoselective properties and the optimal ester substrate for the synthesis strategy. More broadly, these observations demonstrate how the same approach could be used to investigate other enzymes (*i.e.* hydrolases) and identify enzymatic properties that can subsequently be exploited for process optimization.

The methodology developed in **chapter 2** represents one of the few existing strategies capable of selectively modifying the C-terminus on native substrates. This could reshape how large unnatural peptides and proteins are manufactured industrially, as the approach enables the ligation of recombinant peptide fragments. Using recombinant synthesis for peptide fragments could overcome several limitations associated with SPPS, including low atom economy, excessive solvent use, and size limitations, thereby offering a more sustainable general process that could be particularly beneficial at industrial scale. Indeed, this project was conducted in collaboration with Enzytag B.V., which was seeking a more atom-economical approach to obtaining the required ester substrates.

The methodology could also improve access to challenging compounds that are currently obtained in low yields or not at all, further reducing material consumption and environmental impact. In contrast to many existing approaches, it enables native peptide bond formation on linear peptides rather than relying on unnatural modifications. This could allow peptide fragments with specific medicinal activities to be coupled to proteins, providing opportunities for targeted therapeutic applications. Furthermore, because the methodology can functionalize native peptides and proteins, it may facilitate the study and identification of structures isolated from nature, for example through immobilization or the introduction of pharmacological tags. Such applications are particularly relevant to chemical biology and pharmaceutical science, where precise biomolecule modification is essential for the development of new therapeutics, diagnostics, and biomaterials.

Building on the concept developed in chapter 2, **chapter 3** extended the applicability of this chemistry to native chemical ligation (NCL), one of the most widely used methods for the synthesis of larger peptides and proteins. By designing Passerini-derived intermediates that can be converted into reactive thioesters, we demonstrated that our approach is not limited to enzymatic systems but can also be integrated into established chemical ligation methodologies. This significantly enhances the versatility and impact of the chemistry, given that NCL is routinely used in both academic and industrial settings. As in chapter 2, the major strength of this strategy is its ability to functionalize native structures and subsequently ligate additional functional groups or peptide structures. However, this

approach removes the need for enzymes or detailed knowledge of their properties, allowing chemists to perform these transformations using commercially available or in-house-synthesized reagents.

The selectivity for the C-terminus over other carboxylic acids in **chapter 3** remains to be completely established. If the proposed solutions for achieving this selectivity prove successful, the methodology could become a powerful approach and potentially a new standard for the modification of native peptides. In that case, the broader implications described for chapter 2 would also apply to this strategy. Nevertheless, even if complete selectivity cannot be achieved, the method retains significant value. It could be used for the ligation of peptide fragments lacking aspartate or glutamate residues, for protein labelling where mono-functionalization is less critical, and for the synthesis of novel biomaterials.

An additional innovation presented in **chapter 3** is the latent thioester. The increased stability of this species, resulting from the disulfide-protected thiol, can extend shelf-life and storage time, reducing material losses and improving process efficiency. The latent thioester also provides a basis for developing kinetically controlled ligation substrates from unprotected peptides. In addition, the developed disulfide aldehyde reagent provides polymer chemists with a novel building block that could enable the preparation of materials incorporating controlled degradation or covalent adaptable networks.

A key practical limitation identified in **chapters 2** and **3** was the reduced solubility of modified peptides, which can restrict their application in biological systems. **Chapter 4** addresses this limitation through the development of a novel class of ionic, water-soluble isocyanide reagents. These reagents were designed to improve solubility while maintaining reactivity, enabling Passerini reactions to be performed in aqueous environments without precipitation. Importantly, this work demonstrates that rational chemical design can be used not only to control reactivity but also to tune physicochemical properties such as solubility and charge. This has significant implications for biological and biomedical applications, where aqueous compatibility is essential.

The developed isocyanides can be used to prevent aggregation and increase the solubility of both peptides and proteins. More efficient processes with reduced material loss and higher yields would consequently require smaller quantities of starting material, improving both economic and environmental sustainability. Increased solubility could also enable the functionalization of previously troublesome insoluble targets, opening access to an otherwise inaccessible group of molecules. Such compounds could subsequently be investigated for their pharmacological activity and cellular mechanisms or employed in the synthesis of structures combining polar and apolar characteristics, such as transmembrane proteins.

The developed reagents in **chapter 4** lay the foundation for manipulating the isoelectric point of proteins, providing opportunities to address a range of challenges in chemical biology. Such control could improve understanding of the factors governing macromolecular solubility while also enabling the properties and localization of biomolecules to be deliberately modified. For example, tuning the isoelectric point could facilitate the crossing of biological barriers, including cell membranes and potentially the blood-brain barrier, with important implications for the development of new medicines as well as cellular studies. Isoelectric point manipulation could also enable new purification strategies, for example in gel electrophoresis and ion-exchange chromatography, potentially changing how these techniques are performed. Changes in surface charge may additionally influence biomolecular stability. Since protein degradation can be induced through interactions with hydrolases or digestive enzymes, modifying the surface charge could hinder these interactions and thereby increase protein stability and half-life. Finally, controlling the relationship between solution conditions and the isoelectric point could provide new opportunities for protein crystallization by enabling crystallization to be induced at specific points in a process.

The research on water-based reactions and soluble building blocks presented in **chapter 4** may also provide broader inspiration for the development of more water-soluble alternatives in synthetic chemistry. Increasing the use of water as a solvent could provide both economic and environmental benefits, supporting a broader transition towards more sustainable and efficient chemical processes. In this sense, the work in **chapter 4** extends beyond the specific chemistry developed in this thesis and may serve as a muse for how reagent and reaction design can contribute to this transition.

Taken together, the results presented in this thesis contribute to a broader shift in how chemists can approach the modification of peptides and proteins. Rather than relying solely on traditional coupling strategies, the work demonstrates the potential of multicomponent reactions as flexible and modular tools for biomolecule functionalization. The scientific impact lies in the introduction of new methodologies, the integration of chemical and enzymatic strategies, and the demonstration that rational reagent design can overcome practical limitations such as poor solubility. These findings are relevant to a broad range of research and industrial communities. For academic researchers in organic chemistry and chemical biology, the methodologies provide new tools for studying and modifying biomolecules. For the pharmaceutical and biotechnology industries, they offer potential routes towards more efficient and selective synthesis of protein-based therapeutics and conjugates. Moreover, the ability to modulate properties such as solubility and charge could contribute to improved drug delivery and formulation.

To ensure that these findings can be effectively utilized, their dissemination through scientific publications, conferences (*i.e.* poster- and oral presentations, and through networking) and collaborations will be essential. Integrating these concepts into academic education and training can further equip future scientists with the knowledge and tools required to apply and develop these strategies. Beyond conventional scientific communication, social media and science communication aimed at the general public, including television and radio, could also play an important role in communicating the broader relevance of this research. In the longer term, continued optimization and adaptation of the developed methods may enable their application to increasingly complex systems, including proteins, ultimately contributing to advances in medicine, biotechnology, and materials science.

Acknowledgments / Dankwoord

Already in primary school I had the idea that I wanted to be a doctor. I just did not realize that the kind that I would end up becoming was a bit different than what I had in mind back then. It was not until finishing high school and starting university that I realized that a medical doctor was not for me. Although, my field of study was still related to the medical field, I noticed that I developed a stronger passion for exact sciences. Throughout my studies, I slowly deviated more towards chemistry but was still not convinced of wanting to pursuit a PhD in that field. However, after an industrial internship at Mercachem, I realized that I was willing to take on this great challenge and adventure of doing a PhD. Over all these years, there have been many people in my life who have supported and helped me to get to this point. Herein, I would like to take the opportunity to thank all these people.

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Curriculum Vitae



Jay Hanssens was born in Willemstad, Curaçao, on March 3, 1996. During his secondary education, he attended KAP College (formerly Peter Stuyvesant College), where he specialized in the exact sciences and obtained his VWO diploma in 2014. In the same year, he began his B.Sc. in Pharmaceutical Sciences at Vrije Universiteit Amsterdam (VU Amsterdam). As part of his studies, he completed a five-month internship in the Synthetic Bio-Organic Chemistry (SyBOrCH) group of Romano Orru, where he contributed to the development of efficient synthetic routes towards medicinally active compounds. After also completing extracurricular courses, he graduated with honours in 2017. Jay subsequently pursued an M.Sc. in Drug Discovery and Safety (DDS), specializing in drug design and synthesis. For his master's thesis, he completed an eight-month internship in the medicinal chemistry group of Iwan de Esch at VU Amsterdam, where he focused on the synthesis of novel covalent histamine H1 receptor binders. In 2019, he began a second master's degree, an M.Sc. in Chemistry–Joint Degree, jointly offered by VU Amsterdam and the University of Amsterdam (UvA). As part of this programme, he completed an eight-month internship at Mercachem (now Symeres) in Nijmegen, where he was responsible for the synthesis of novel CDK16 binders as potential anticancer agents. He completed his M.Sc. in Chemistry in 2020 and his M.Sc. in Drug Discovery and Safety in 2022.

In February 2021, Jay began his Ph.D. research in the Biobased Organic Chemistry (BOC) group of Romano Orru at the Aachen-Maastricht Institute for Biobased Materials (AMIBM), Maastricht University. His research focused on the application of multicomponent reactions for the synthesis of unnatural amino acids, as well as the synthesis and C-terminal functionalization of peptides and proteins. During his Ph.D., Jay received two awards for his research: the Best Poster Award at the Dutch Symposium on Organic Chemistry (DSOC) in Lunteren in 2024 and second place for Best Oral Communication at the European Peptide Symposium (EPS) in Florence in 2024. Since May 2026, Jay has continued his research as a postdoctoral researcher in the group of Romano Orru. In collaboration with Mevaldi B.V., his current research focuses on the valorization of biobased building blocks for the development of useful monomers.

List of Publications

[6] **Synthesis of Water-Soluble Isocyanides for C-terminal Peptide Modification**
J. Hanssens, M. Lambers, L. J. van den Bos, R. V. A. Orru, and J. M. Saya*
Manuscript in preparation.

[5] **Late-Stage Passerini Functionalization of Unprotected Peptides for Native Chemical Ligation**
J. Hanssens, M. Lambers, R. V. A. Orru and J. M. Saya*.
Manuscript in preparation.

[4] **Strategies for the Functionalization of C-terminal Carboxylic Acids on Amino Acids, Peptides and Proteins**
J. Hanssens, M. Lambers, R. V. A. Orru and J. M. Saya*.
Manuscript submitted.

[3] **Non-innocent behaviour of aromatic isocyanides under visible light: a pathway to thioformimidates and dehydroalanine**
M. Wu, J. Hanssens, C. Russo, R. Walia, M. Giustiniano*, L. Troian-Gautier, J. M. Saya, R. V. A. Orru*, P. Ranjan*.
Chem. Sci., **2026**, *Advance Article*

[2] **Merging the Passerini Reaction with Chemo-enzymatic Synthesis for C-terminal Peptide Modification**
J. Hanssens, S. van Dun, T. H. G. Lokate, V. R. A. M. Reinartz, L. J. van den Bos, R. V. A. Orru and J. M. Saya*.
Cell Reports Physical Science, **2025**, *6*, 10, 102860.

[1] **Terpenes and Terpenoids: How can we use them?**
J. Hanssens, D. Meneses, J. M. Saya* and R. V. A. Orru*.
Eur. J. Org. Chem., **2025**, *28*, 24, e202401151.