“Multicomponent Strategies to Side Chain and Backbone Modified Cyclic Peptides”

Kumulative Dissertation

Zur Erlangung des
Doktorgrades der Naturwissenschaften
(Dr. rer. nat.)

der
Naturwissenschaftlichen Fakultät II
Chemie, Physik und Mathematik

der Martin-Luther-Universität Halle-Wittenberg

vorgelegt von
Herren M.Sc. Manuel García Ricardo

geb. am 07.03.1989 in Holguín, Kuba

The work presented in this dissertation has been developed at the Leibniz-Institute of Plant Biochemistry (IPB) in cooperation with the University of Havana, Cuba and it have been published as three peer-reviewed original research articles and one draft in press.

Supervisor and thesis editor: Prof. Dr. Ludger A. Wessjohann

International co-supervisor: Prof. Dr. Daniel García Rivera

Date of the defense: 03.03.2020

“Don't bend; don't water it down; don't try to make it logical; don't edit your own soul according to the fashion. Rather, follow your most intense obsessions mercilessly.”

“By believing passionately in something that still does not exist, we create it. The nonexistent is whatever we have not sufficiently desired.”

Franz Kafka

Acknowledgments

First of all, I want to thank my Cuban supervisor Daniel García Rivera for the guidance, vision, knowledge and the confidence he has on this survey. Daniel, thanks a lot for your inspiring words, your patience, your comprehension, … for your friendship. My career, my expertise and my concern in science have been sculpted with your hands. In this context I would like to thank my supervisor Ludger Wessjohann for his continuous support, it has been great placer to share his wisdom and scientific knowledge. Thanks for contribute to fortify and prosecute my formation with your ideas, discussions and comments. I am also especially grateful with Bernhard Westermann for his advises. Thanks Berni for always keeping your door open for me.

This race could be for sure more difficult without the presence of some very important people. Thanks to my friends, “mis queridos”, Yanira, Aldrin, Javiel, Leo, Dailyn, Elena, Frank, Dube, Nalin, Bruno, Ramona, Anja K., Anja E., Tuvshin, Annegrete, Micjel, Ricardo, Akbar Ali, Yen, Ho Ai, Svitlana, Janoi, Hidajat, Ekaterina, thanks for all advises, discussions, dinners, trips, parties, drunkenness, thanks to all serious and crazy thinks we remember we did, and those we do not. To my friends in the university of Havana, my old friends Gerardo, Fidel la vaca, Fidel el guataca, Antuch, Vivian, Patricia, Odette. Thanks so much to Haider, for all, papa, for being always there. I know I can count with you. Thanks to Mr. Alfred, papa, I hope I have caught some from you, I wish you all. To Antonio, not me, the world thanks for your existence. I have more than a leopard because I have you, papa.

I would like to thank also the support of some members of the Bioorganic Chemistry department at the Leibniz Institute of Plant Biochemistry (IPB-Halle). Thanks to Frau Porzell, for your help and understanding, your way of being is an inspiration for honest people. Thanks to Frolov, Mandy, Martina Lerbs, Martina Brode and Frau Hann for all your technical support. Thanks to Frau Stein, for all your assistance.

Thanks to my deep, ancient and eternal friends: Yilian, Isis, Jose, Bernardo and Dago, wherever you are, like a huge amount of Cubans spread for the whole world, I will have you always in my heart. Dago, my little brother, my should mate, my best and forever friend, I acknowledge you my life.

To my family, for all their love and encouragement. To my mom, for rise me and make the person I am. Mam, for our constant love, thanks. Thanks also to my brother, my Tata, for been always there for me, for been more than just a brother for been my dad. And most of all, thanks to my love, my support, my joy, my treasure, my wife Dayma. Thanks for everything you did and everything you will do, for bring sense to my life. I love you Luli.

Manuel García Ricardo

Leibniz Institute of Plant Biochemistry (IPB)

Halle (Saale), June 2019

Table of contents

List of abbreviations

AA	amino acid
Ac	acetyl
AcB	acetate buffer
All	allyl
Alloc	allyloxycarbonyl
Boc	*tert*-butoxycarbonyl
calcd	calculated
CD	circular dichroism
2CT	2-chlorotrityl
DCE	dichloroethane
DCM	dichloromethane
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DIAD	diisopropylazodicarboxylate
DIC	diisopropylcarbodiimide
DIPEA	diisopropylethylamine
DMF	dimethylformamide
EDT	ethanodithiol
EDCI	*N,N'*-1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride
equiv	equivalent
ESI-MS	electrospray ionization mass spectrometry
FA	formic acid
Fmoc	fluorenylmethyloxycarbonyl
GS	Gramicidin S
HOAt	hydroxy-7-azabenzotriazol
HOBt	hydroxybenzotriazol
HBS	hydrogen bond surrogate
HR-MS	high resolution mass spectrometry
Hz	Hertz
IR	infrared
MCR	multicomponent reaction
MBHA	methylbenzhydrylamine
MD	molecular dynamic
Me	methyl
MeOH	methanol
MW	microwave
NBS	2-nitrobenzenesulfonyl
NMM	*N*-methylmorpholine
NOE	nuclear Overhouser effect
PEG	polyethylene glycol
PB	phosphate buffer
Ph	phenyl
ppm	parts per million
Petasis-3CR	Petasis three components reaction
PyAOP	7-azabenzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
PyBOP	benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate
RCM	ring closing metathesis
R*f*	retention factor
RP-HPLC	revered-phase high performanceliquid chromatography
RT	room temperature
SPPS	solid phase peptide synthesis
***t*Bu**	*tert*-butyl
TCEP	2-(carboxyethyl)phosphine hydrochloride
TFA	trifluoroacetic acid
TFE	2,2,2-trifluoroethanol
TG-S-RAM	tentagel resin bounded Rink amide
THF	tetrahydrofuran
TIPS	triisopropylsilane
TLC	thin layer chromatography
TMS	tetramethylsilane
Trt	triphenylmethyl
Ugi-4CR	Ugi four components reaction

Summary

Labeling and macrocyclization are among the most challenging and important methods in the field of peptide chemistry. Multicomponent macrocyclizations are synthetic protocols that enable the simultaneous ring-closure and the introduction of additional functionality in one single step. The main goal of this research project was to develop new multicomponent reactions-based strategies towards labeling and macrocyclization of either the peptide backbone or the side chain.

In this sense, we report an Ugi reaction-based approach for the synthesis of analogues of the antibacterial cyclopeptide Gramicidin S. The method encompasses the cyclization together with an additional functionalization by the addition of dissimilar *exo*-cyclic fragments of cationic, anionic and lipidic nature. This multicomponent approach also focuses on mimicking the β-hairpin structure of the natural product by installing *N*-alkylated dipeptide fragments capable of mimicking the conformation of the natural product.

Peptide stapling have become a powerful strategy for introducing conformational constrains in peptides. In an attempt to further exploit multicomponent reactions (MCRs) in this field, we describe a multicomponent approach for the synthesis of macrocyclic peptides through the execution of two Ugi reactions between peptide diacids and diisocyanide-linkers. This method enables the introduction of high levels of diversity varying the amino component and the cross-linker moiety, offering promising alternatives for the modulation of peptide topology seeking for biological applications.

In addition, we describe for the first time, a multicomponent method that allows the late-stage derivatization and stapling of peptides by the Petasis reaction. This report includes the incorporation of boronic acids bearing fluorescent and affinity labels, PEGs, steroids, and lipids at both the peptide *N*-terminus and Lys side-chains. Simultaneously, a variety of *oxo*-components are also introduced, such as dihydroxyacetone, glyceraldehyde, glyoxylic acid, and aldoses, thus encompassing a powerful complexity-generating approach without changing the charge of the peptides. Stapled peptides with rigid aromatic linkages in combination with small

sugars are also produced in a bidirectional Petasis-based macrocyclization, proving the versatility of this multicomponent reaction in most areas of peptide chemistry.

In correspondence with the emergence of new methods for the peptide backbone cyclization, a new multicomponent backbone *N*-modification of peptides is also described. Here is demonstrated that by performing an Ugi reaction during solid-phase peptide synthesis, the incorporation of a variety of functionalized *N*-substituents suitable for backbone stapling can be achieved. Accordingly, using different ω-functionalized isocyanides, several types of macrocyclizations can be implemented on the peptide backbone, including ring-closing metathesis, lactamization and bis-thiol alkylation. This backbone *N*-modification approach was also applied to the synthesis of α-helical peptides by linking *N*-substituents to the peptide *N*-terminus, thus featuring hydrogen-bond surrogate structures.

Zusammenfassung

Das Labeling und die Makrozyklisierung von Peptiden gehören zu den anspruchvollsten und vielversprechendsten kovalenten Modifizierungen in der Peptidchemie. Auf Multikomponentenreaktionen basierende Makrozyklisierungen sind außerordentlich attraktiv, da sie sowohl den Ringschluss als auch die Einführung zusätzlicher Funktionalitäten in einem einzigen Schritt ermöglichen. Das Ziel dieser Dissertation war die Entwicklung neuer Synthesestrategien unter Verwendung von Multikomponentenreaktionen für die Markierung („*labeling*“) sowie Makrozyklisierung von Peptiden sowohl am Peptidrückgrat als auch mit Seitenketten.

Im Rahmen dieser Arbeit wurde eine auf der Ugi-Reaktion basierende Synthesestrategie für die Herstellung von Gramicidin-S-Analoga angewendet, die die Zyklisierung sowie die zusätzliche Funktionalisierung durch verschiedene exo-zyklische Einheiten (kationischer, anionischer und lipidischer Natur) ermöglicht. Dieses Multikomponentenkonzept zielt auch auf die Nachahmung der β-Haarnadelstruktur des Naturstoffs durch den Einbau *N*-alkylierter Dipeptide ab, die die Konformation des Zielproduktes erfolgreich nachbildet.

Das *„peptide stapling“* hat sich zu einer effektiven Methode für die Einführung von Konformationseinschränkungen in Peptidstrukturen entwickelt. Um Multikomponentenreaktionen auf diesem Gebiet weiter zu etablieren, wurde ein bidirektionaler Ansatz für die Synthese von makrozyklischen Peptiden über die Durchführung von zwei Ugi-Reaktionen zwischen Peptid-Disäuren und Diisocyanat-Linkern realisiert. Diese Methode ermöglicht die Einführung eines hohen Grades an Diversität durch die Variation der Aminokomponente und der Crosslinker-Einheit, wobei vielversprechende Alternativen in Bezug auf die Anpassung der Peptidtopologie für weitere biologische Anwendungen ermöglicht werden.

Des Weiteren wurde erstmalig eine Multikomponentenmethode für die „*late-stage*“ Derivatisierung und das *„peptide stapling“* mittels Petasis-Reaktion beschrieben. Dieser Ansatz beinhaltet den Einbau verschiedener Boronsäuren, die Fluoreszenz- oder Affinitätsmarker tragen, von Polyethylenglykolen (PEGs), Steroiden und Lipiden an beiden *N*-Termini sowie an Lysin-Seitenketten. Gleichzeitig wurden verschiedene Oxo-

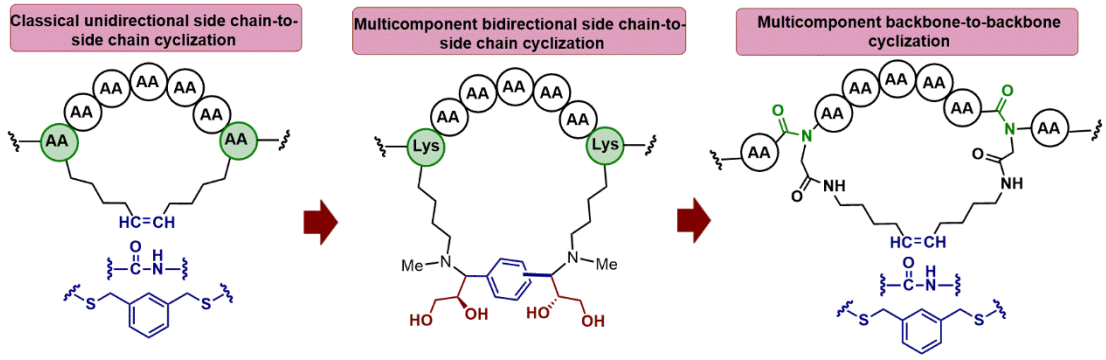
Komponenten wie Dihydroxyaceton, Glycerinaldehyd, Glyoxalsäure und verschiedene Aldosen genutzt, um eine effektive und komplexität-generierende Methode bereitzustellen, ohne dabei eine Ladungsänderung des Peptids zu verursachen. „*Stapled*“ Peptide mit starren aromatischen Bindungen in Kombination mit kleinen Zuckern wurden ebenfalls mittels einer bidirektionalen Petasis-basierten Makrozyklisierung synthetisiert, wodurch die vielseitige Verwendbarkeit dieser Multikomponentenreaktion in vielen Bereiche der Peptidchemie nachgewiesen werden konnte.

Im Rahmen dem Aufkommen neuer Methoden für die Peptidrückgrat-Zyklisierung wird eine neue Multikomponenten-Rückgrat-*N*-Modifizierung von Peptiden innerhalb dieser Arbeit vorgestellt. Dabei konnte gezeigt werden, dass bei der Durchführung der Ugi-Reaktion während der Festphasenpeptidsynthese eine Vielzahl an funktionalisierten *N*-Substituenten für die Peptidrückgratfixierung („*backbone stapling*“) geeignet sind. Dementsprechend können unter Verwendung verschiedener ω-funktionalisierter Isocyanate mehrere Sets von chemischen Makrozyklisierungstechniken am Peptidrückgrat implementiert werden, einschließlich Ringschlussmetathese, Lactamisierung und Biothiol-Alkylierung. Diese Rückgrat-*N*-Modifizierungsstrategie wurde auch auf die Synthese von α-helikalen Peptiden angewandt, indem *N*-Substituenten am Peptid *N*-Terminus eingeführt wurden. Dieses führte zur Ausbildung von Wasserstoffbindungs-Surrogat-Strukturen.

Chapter 1

Witnessing the Evolution of Peptide Cyclization by Multicomponent Reactions

-Introductory chapter-



Abstract

Multicomponent reactions have become a prevailing strategy for peptide macrocyclization thereby enabling the creation of high diversity accompanied by low synthetic cost. This chapter provides an overview of the contribution of multicomponent reactions on the development of peptide macrocyclization in terms of chemical efficiency and synthetic scope. The utilization of multicomponent reactions in the classic head-to-tail cyclization of peptides overcomes most of the disadvantages of the established methods. Moreover, in peptide stapling, a multicomponent approach adds the possibility to explore the peptide topology, introducing additional *endo* and *exo*-functionalizations on the ring-forming moiety. Recently, MCRs have appeared as an alternative for the incorporation of peptide *N*-substituents bearing functional groups suitable for backbone cyclization without the need to alter any important side chains in the sequence.

Introduction

The development of protein-protein interaction inhibitors by the introduction of conformational constrains in small peptides have become one of the main guidelines in medicinal chemistry.[1,2] Protein interfaces are commonly complex with large surface areas that can make the selective interaction of small molecules very difficult.[3] Natural peptides are frequently used as target structures to design new candidates with high affinity.[2,4] However, the bioavailability of this molecules is pour, due to low solubility, rapid enzymatic degradation by proteolytic enzymes and poor intestinal permeability. Together with a high intrinsic flexibility showed by short sequences, this points are the main drawbacks of peptides as potential pharmaceuticals.[5,6] Therefore, there is a substantial need to identify chemical approaches that may enhance bioavailability of peptides while maintaining their pharmacological activity. Covalent modifications of peptides by means of configurational and structural modification of amino acids, local backbone modifications and macrocyclization are the most common methods to overcome these barriers.[7] Among these, macrocyclizations of either peptide side chain or backbone of short sequences are the most recurrent alternatives.[2,8] By reduction of the intrinsic flexibility, cyclization can increase target selectivity by stabilizing the bioactive conformation of the linear parent peptide.[9–11] Moreover, it has been reported that cyclic peptides show increased resistance against proteases,[12] as well as an improved cell membrane permeability.[13] Nowadays, introducing constrains in short sequences has been successfully applied in the design of protein ligands or mimetics of protein secondary structures (i.e., β-hairpins,[14] β-strands[15] and α-helices[16,17]).

Besides the great advances made in this field during the last decades, peptide cyclization is still recognized as a challenging synthetic process. Traditionally, the macrocyclization step is characterized by low yields due to the entropic demand of the conformational restriction.[18] However, different protocols have been successfully applied to the cyclization of either the peptide backbone or the side chains.[19,20] In this regard, multicomponent reactions have emerged as highly promising methods also for peptide cyclization. The execution of a multicomponent approach is not prone to steric hindrance and permits the generation of additional molecular diversity during the ring-closure of the peptide skeleton. The latter feature has proved advantageous in

comparison with the traditional strategies.[21–23] This chapter focuses on how multicomponent reactions have been closely involved in the synthetic evolution of the most powerful strategies for peptide cyclization.

1. Classical modes of cyclization found in naturally occurring and synthetic peptides

Since the discovery in 1944 of Gramicidin S,[24] the first cyclic peptide antibiotic isolated from a natural source, cyclic peptides have shown a wide range of biological applications.[9,11] Accordingly, natural peptides with cyclic structures have served as inspiration and guide for the development of new synthetic methods.[2,19] The mode of cyclization found in naturally occurring cyclic peptides include: (a) head-to-tail cyclization, (b) head-to-side chain cyclization, (c) tail-to-side chain cyclization and (d) side chain-to-side chain cyclization. As shown in the figure 1A, in Gramicidin S all peptide bonds are taking part in the cyclic structure, in an assembly formally known as head-to-tail cycle. Alternatively, examples of combining side chain with either *N*- or *C*-terminus cyclizations are also common in nature. For example, head-to-side chain linkage are found in Nodularins or Microcystins (Figure 1B), families of cyanobacterial peptides produced in toxic blue-green algal blooms. Similarly, Polymyxin B – an antibiotic used against Gram-negative infections – is an example of a cyclic peptide having the *C*-terminus or tail compromised in the cycle through an amide bond with the side chain of the non-proteinogenic amino acid, L-2,4-diaminobutyric acid, Dab (Figure 1C). On the other hand, oxytocin – a peptide hormone involved in sexual reproduction and childbirth – is characterized by formation of the cyclic structure from the interaction of two Cys side chains in a disulfide bridge (Figure 1D).

These methods mostly rely on ring-closure by either natural amide or disulfide bond, both with limited biological/pharmacological presentation as potential drugs.[25,26] Currently, there have been substantial efforts to reproduce the conformation provided by these linking approaches but instead using efficient and more physiological stable isosters. Replacement of the amide bond by a triazol[27,28] and the disulfide bond by a hydrocarbon-based bridge[26,29] are two of the most recurrent examples with proven applications in the synthesis of peptide drugs.

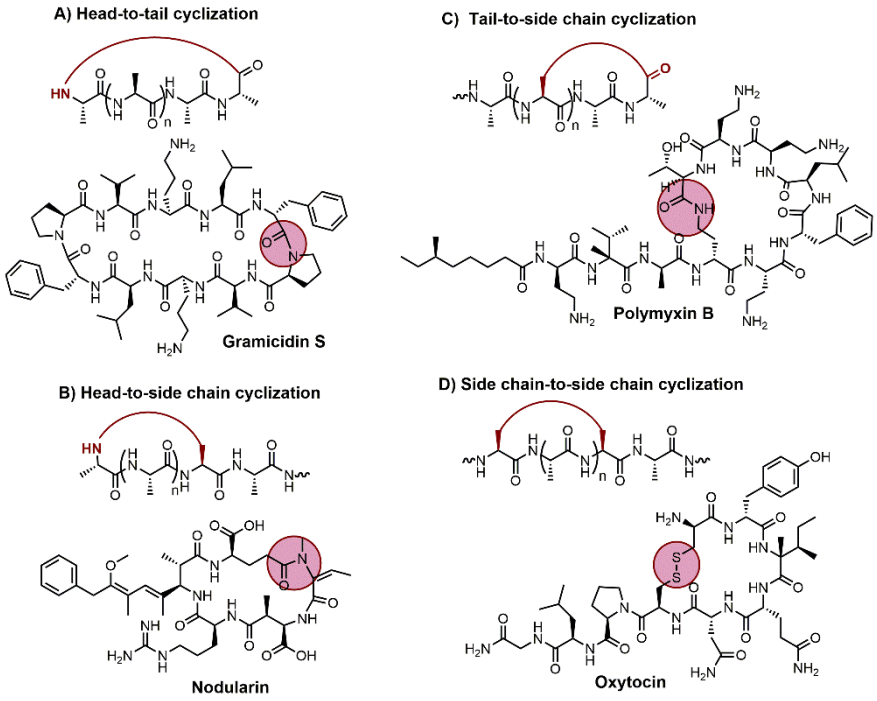


Figure 1. Ways of cyclization found in naturally occurring cyclic peptides.

Interestingly, in the past the head-to-tail linkage inspired by natural products prevailed as the common method for peptide cyclization, although it offers only the formation of a single macrocycle. A different scenario is illustrated by the side chain-to-side chain cyclization, where higher levels of molecular diversity can be achieved depending on the amino acid positions, ring size and chemistry involved (Figure 2).[30] A special class of side chain-to-side chain peptide cyclization – termed "peptide stapling" – has provided a variety of helical peptides with important biological and medicinal applications.[31–33] This strategy was initially reported by Verdini and co-workers – based on an original report of Grubbs and co-workers[34] – for the synthesis of all-hydrocarbon bridged α-helical peptides by means of ring-closing metathesis (RCM) using two alkene-amino acid residues separated at *i*, *i* + 4 and *i*, *i* + 7 (Figure 2A).[35] Recently, stapling chemistries have been extended to include macrolactamization (Figure 2B),[17,36] "click" cycloaddition (Figure 2C),[27,37] thio-ether formation,[37,38] Pd-catalyzed C-H activation,[39,40] among others.

Stapling techniques are commonly derived from the utilization of two complementary functional groups that participate in a single and unidirectional cyclization reaction. A different approach consists on the utilization of a bidirectional macrocyclization strategy, in which a pair of amino acid side chains bearing the same functional groups reacts doubly with a bifunctionalized cross-linker.[41] As depicted in the Figure 2, some of these examples include double thiol-ene coupling (Figure 2D),[42] double bis-alkylation (Figure 2E and 2G),[38] bis-arylation of two Cys[43] or Lys[44] residues (Figure 2F) and a double-"click" approach[41] (Figure H).

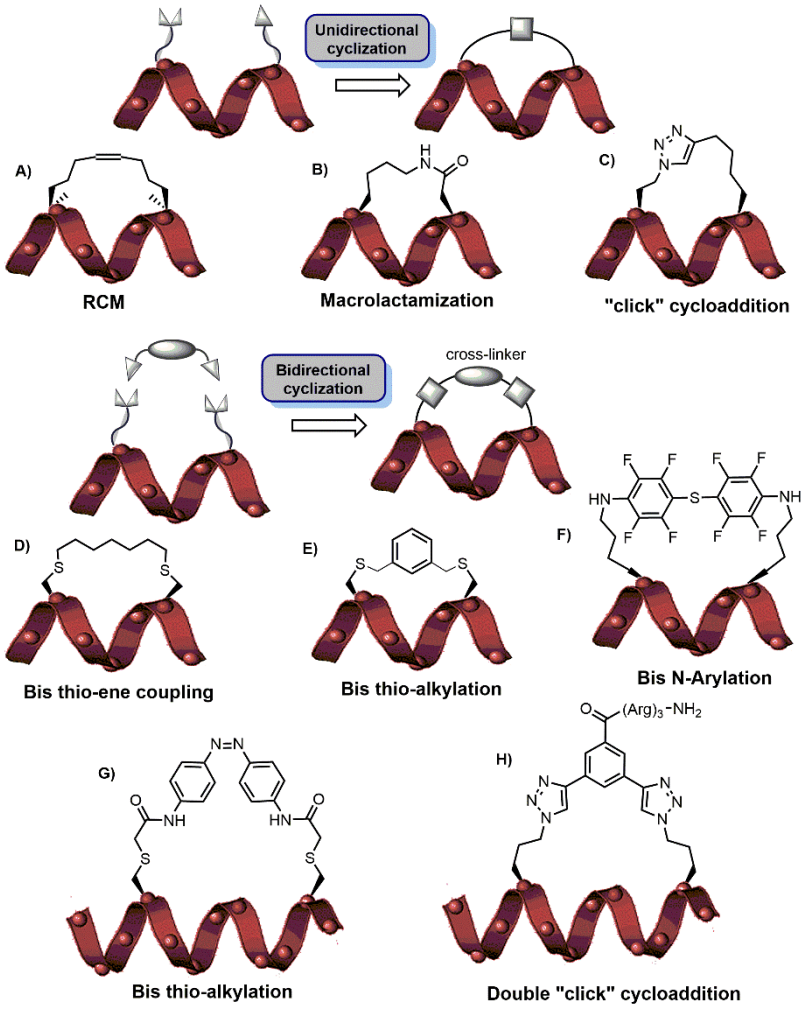


Figure 2. Most common synthetic methods for peptide stapling. From A) to C) unidirectional and from D) to H) bidirectional.

Besides the desired helicalendoi stabilization achieved by these strategies, they allow the introduction of an additional building block and therefore the modulation of the peptide properties. For example, Woolley and co-workers[45] installed via a double bis-

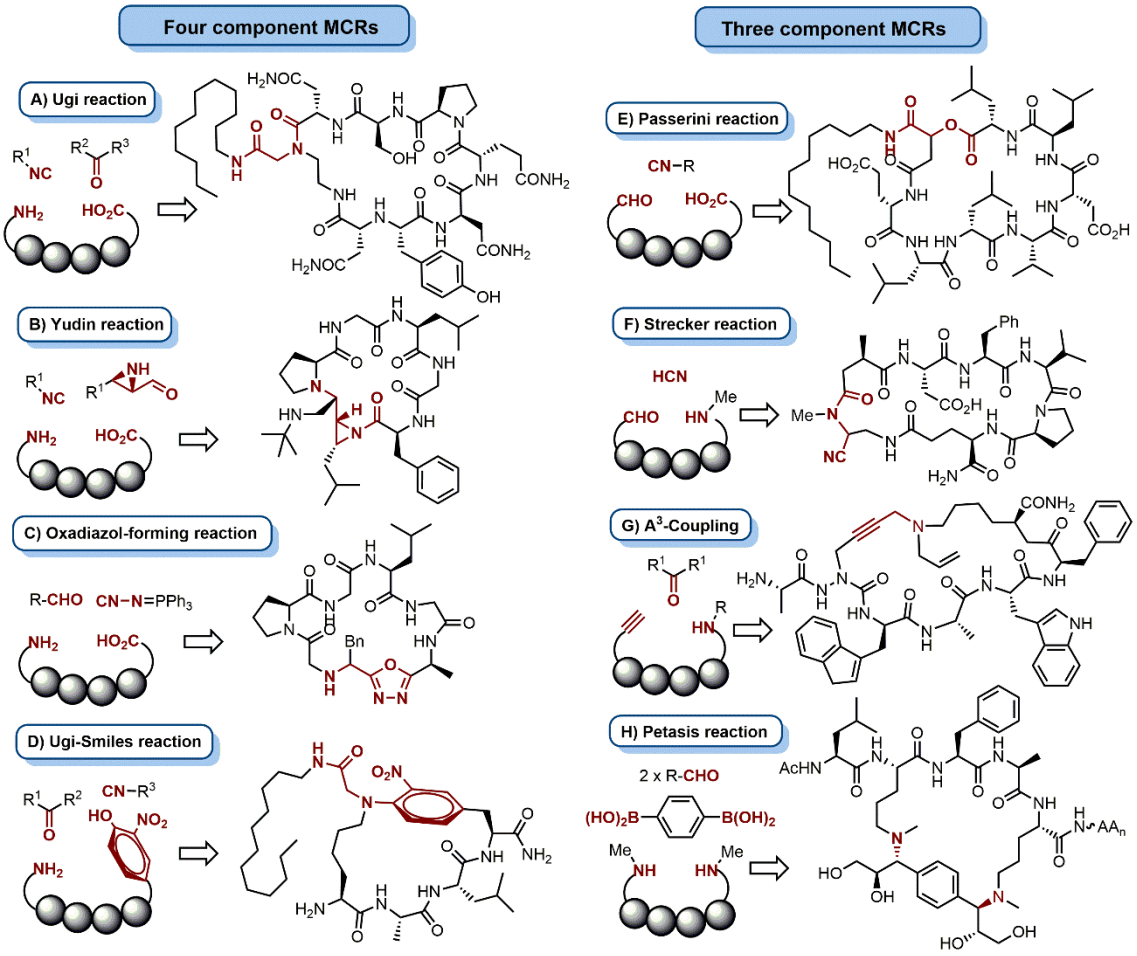
thiol alkylation of two Cys residues separated at *i*, *i* + 7 or *i*, *i* + 11, an azo cross-linker that photo-modulates the peptide helicity (Figure 2G). Moreover, Spring *et al.*, have developed a double-click cyclization, in which two azido amino acids react with a specially functionalized dialkyne linker with notable influence in the cellular activity of a peptide inhibitor of p53/MDM2 interaction (Figure 2H).[41]

2. Multicomponent approaches for peptide macrocyclization

Despite the advances in peptide cyclization methodologies in the last years, the possibility of introducing high levels of structural diversity remains as an ongoing quest. In this sense, the employment of multicomponent reactions in peptide cyclization has offered a range of opportunities, as they comprise the rapid generation of molecular diversity in just one pot. These convergent approaches involve the incorporation of three or more components into a final product, leading to high levels of complexity with high atom economy.[46,47]

Multicomponent reactions applied in peptide cyclizations normally involve carboxylic acids or amino groups as they are present in peptide termini and side chains. The Ugi four component reaction (Ugi-4CR),[48] is the first MCR used in peptide cyclization[49] and, in general, in macrocyclization chemistry.[50] It comprises the condensation of an amine, a carbonyl component (i.e., aldehyde or ketone), a carboxylic acid and an isocyanide to produce a *N*-substituted dipeptide (Scheme 1A). Beyond the application of Ugi-4CR in peptide cyclization, its potential has been exploited for the one-pot synthesis of macrocycles, incorporating natural scaffolds like steroids,[51] lipids,[52] carbohydrates[53] and heterocycles[51]. In the last decades, there have been intensive efforts to extend the scope of this reaction to peptide cyclization. In this sense, the development of an aziridine-aldehyde version by Yudin and co-workers, has been broadly employed for the synthesis of cyclic peptides and their post-MCR derivatization (Scheme 1B).[21] Some years later, Yudin also described the multicomponent synthesis of oxadiazole-macrocyclic peptides, using an special isocyanide named (*N*-isocyanimino)triphenylphosphorane (Pinc), an aldehyde and a linear peptide, affording the insertion of the heterocycle within the cyclopeptide backbone (Scheme 1C).[54] The Passerini reaction[55] – an isocyanide-based MCR like the Ugi-4CR – has also shown

some applications in the cyclization of *oxo*-peptides leading to depsipeptide skeletons (Scheme 1E).[23] As shown in Scheme 1D, another interesting variation of Ugi reaction – denominated Ugi-Smiles[56] – uses 2- or 4-nitrophenol instead of the carboxylic acid. It has made a notable contribution to the easy incorporation of interesting *N*-aryl moieties in peptides.[57]



Scheme 1. Multicomponent reactions applied in peptide cyclization strategies.

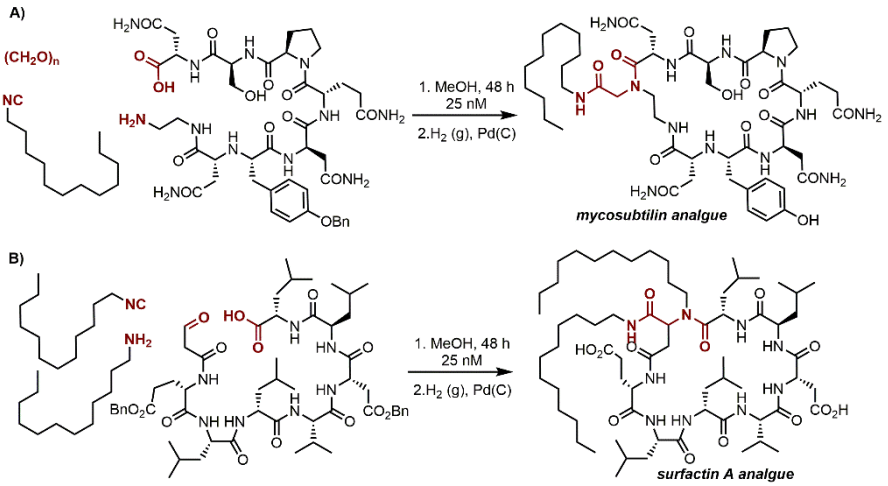
Three component reactions (3CR) involving imines have also been employed in multicomponent macrocyclizations of peptides. Interestingly, the first MCR known, the Strecker-3CR[58], has only recently been applied for head-to-tail cyclization of peptides (Scheme 2F).[59] Additionally, two MCRs also based on imine formation have been recently incorporated to the repertoire of stapling processes. This represent the metal-catalyzed coupling of an aldehyde, an alkyne and an amine (A^3-coupling, Scheme 2G)[60] and the Petasis-3CR (Scheme 2H).[61]

2.1 Head-to-tail cyclization by multicomponent reactions

As commonly observed for most of the established macrocyclization procedures in peptide science, the initial attempt has been the head-to-tail cyclization.[62] Direct amide coupling between both termini constitutes the first method applied to peptide cyclization.[7] However, it is well known that macrolactamization, in general, has some synthetic limitations such as cyclodimerization, *C*-terminal epimerization and frequently low yield.[7] Traditionally, the development of highly specialized coupling reagents has become a solution to most of these limitations.[63] Moreover, the use of MCRs has demonstrated clear evidence to overcome these drawbacks. In this sense, a key advantage of the head-to-tail cyclization of peptides by MCRs like the Ugi and Yudin variant reactions, is the lack of epimerization of the terminal carboxylic group.[19] Furthermore, the occurrence of any non-desired oligomerization in MCR-based macrocyclization is substantially reduced in comparison with the macrolactamization.[19] In MCRs, the entropic loss associated by bringing both reacting ends together is more favored due to the iminium-carboxylate ion pairing. However, in a classical macrolactamization, there is not such a thermodynamic contribution because the ion pairing between the two ends disappears upon activation of the *C*-terminal carboxylate.

The first report of a macrocyclization using a MCR on head-to-tail macrocyclization of peptides by Ugi-4CR was described in 1979 by Failli et al.[49] They found that tripeptides in the presence of isobutyraldehyde and cyclohexyl isocyanide were very difficult to cyclize, thus affording only dimerization products. However, it was shown that under the same conditions, hexapeptides were cyclized without any appreciable dimerization. In addition, a study was implemented in our group in order to analyze the influence of the sequence on the cyclization of tetrapeptides and pentapeptides by Ugi-4CR.[64] It was concluded that while all-L-pentapeptides cyclize without appreciable dimerization, all-L-tetrapeptides lead to a mixture of the desired cyclic tetrapeptide, and that the cyclomonomer vs. ratio strongly depends on the residue's flexibility and on the presence of D-amino acids.

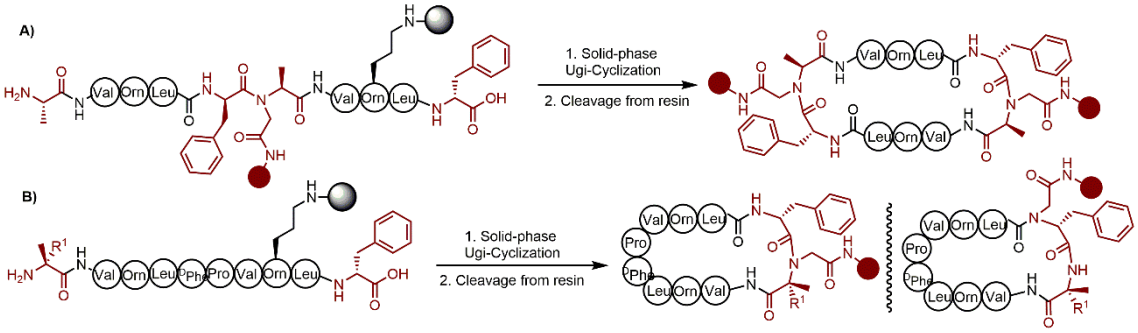
Application of the Ugi reaction in the synthesis of analogues of natural cyclic peptides has been one of the main objectives in our group. Consequently, modulation of the biological properties by the incorporation of *exo*-cyclic functionalities during the ring-closing stage was studied in our laboratory for synthesis of analogues of the antimicrobial cyclic lipopeptides, surfactin A and mycosubtilin.[23] As shown in Scheme 2A, by performing a macrocyclization based on Ugi reactions, it was possible to simultaneously incorporate an *exo*-cyclic lipid tail during the ring-closure of the linear octapeptide and to afford a novel MCR-based analogue of mycosubtilin in a good yield. Extension of this macrocyclization/lipidation strategy, allowed to doubly lipidate a linear analogue of surfactin A using both n-dodecyl amine and n-dodecyl isocyanide, proving the remarkable scope of the multicomponent process (Scheme 2B).



Scheme 2. Synthesis of analogues of A) mycosubtilin and B) surfactin A by Ugi cyclization.

Another example of head-to-tail cyclization was recently developed by our group for the multicomponent synthesis of Gramicidin S analogues, as detailed in the first chapter of the presented document. This work was designed to assess the influence on the inclusion of an Ugi reaction-derived *N*-alkylated dipeptide in the β-hairpin structure of the natural product (Scheme 3). Our first attempt was to replace both Pro residues by the *N*-alkylated Ala residue generated by the multicomponent process since it was already reported that the replacement by *N*-methylated Ala does not have major consequences on the structure or biological profile of the natural product. As depicted in Scheme 3A, several doubly *N*-functionalized analogues of Gramicidin S containing lipids, benzyl, carboxyl and amino groups were obtained. Moreover,

diverse combinations of amino acids (Aib, Ala and Gly) with dissimilar *exo*-cyclic fragments in different positions were carried out during the construction of a parallel library of analogues (Scheme 3B). It is worth mentioning that this strategy allows to modulate the polarity (introducing hydrophobic or charged groups) of a natural cyclopeptide skeleton without affecting the canonical amino acid side chains.



Scheme 3. MCR-based synthesis of analogues of Gramicidin S with the introduction of two *N*-alkylated dipeptide units as β-turn inducers.

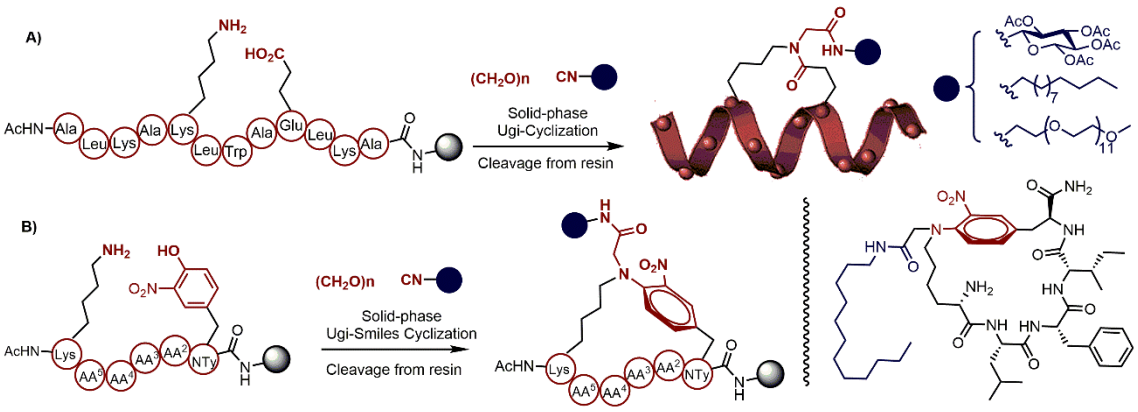
2.2 Multicomponent reactions in peptide stapling

The growing need for developing new methodologies for peptide cyclization has also been extended to multicomponent reactions. Recently, MCRs have emerged as very suitable stapling methods for helix stabilization and the simultaneous structure-oriented diversification of peptides. Hence, several multicomponent reactions have been applied for staging of peptide stapling strategies such as: the Ugi-4CR, the Ugi-Smiles reaction and recently the Petasis-3CR.[65]

The first application of a multicomponent process for the side chain-to-side chain cyclization was reported by our group and consisted as an unidirectional Ugi-4CR using Lys and Asp/Glu residues.[66] Recently, this Ugi stapling approach was extended to the stabilization of helical secondary structures and the simultaneous installation of very diverse exocyclic *N*-functionalities (Scheme 4A).[22] This work encompassed the first example of a multicomponent peptide stapling that combines the stabilization of α-helical secondary structure with the simultaneous incorporation of additional tags.

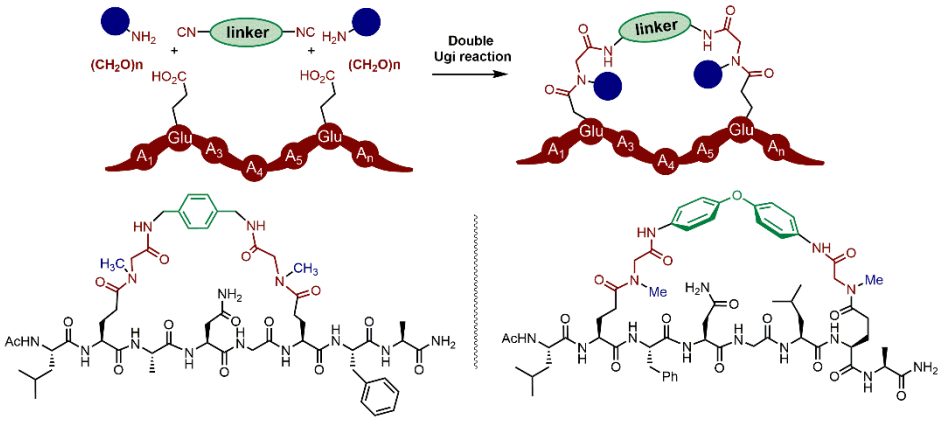
As depicted in the Scheme 4B, placing a 3-nitrotyrosine instead of an Asp/Glu residue in the peptide sequence, allow a new stapling method *via* an Ugi-Smiles reaction

(Scheme 4B).[57] Here, a lipid isocyanide was employed aimed at installing a biologically important moiety. Promisingly, this technique allows the integration of a cyclopeptide skeleton with a novel lipidated *N*-aryl-bridge, not accessible in one step by any other macrocyclization process.



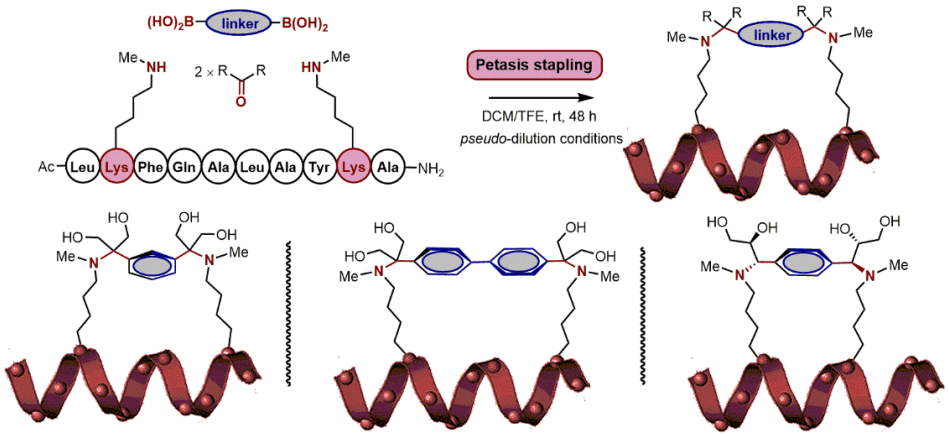
Scheme 4. Unidirectional isocyanide-based multicomponent reactions applied to peptide cyclization.

As mentioned earlier, the implementation of a stapling cyclization in a two-component manner, generates new opportunities regarding ring sizes and structure-oriented diversifications of peptides. In our group, by using a multicomponent reaction in this bidirectional fashion, we are able to generate structural diversity at a higher level.[67] The second chapter of this manuscript describes the bidirectional macrocyclization of peptide side chains by Ugi reactions. As illustrated in the Scheme 5, a variety of stapled peptides featuring different bifunctional diacid peptides with diisocyanide cross-linkers were obtained. The linker length and flexibility introduced depends on the separation between the two reacting amino acid residues, whose position could differ from *i*, *i* + 3; *i*, *i* + 4 to *i*, *i* + 5. This method allows the generation of diversity at the cross-linker fragment, not only through variation of the diisocyanide building block (e.g., aromatic, aliphatic and heterocyclic linkers) but also of the amine component, including proteinogenic amino acids.



Scheme 5. Bidirectional macrocyclization of peptides by double Ugi-4CR.

A very recent addition to the collection of multicomponent macrocyclization methods is the peptide stapling by Petasis reaction, presented in third chapter of this thesis.[61] Primarily we envisioned the utilization of this extraordinary three-component process for late-stage diversification and labeling of peptides at the side chains and the *N*-terminus. Since the discovery of this borono-Mannich reaction by Petasis et al., its application in peptide chemistry has been limited to the modification of amino acids.[68] Here we report the simultaneous incorporation of dissimilar aldoses and ketoses (as carbonyl component) and boronic acids endowed with fluorescent labels, steroid skeletons, PEG chains, etc. into peptides. Moreover, similarly to the double macrocyclization by the Ugi-reaction , it was possible to develop an efficient multicomponent stapling protocol based on the utilization of N$^{\varepsilon}$-MeLys-containing peptides in combination with aldoses and ketoses of biological relevance.



Scheme 6. Bidirectional macrocyclization of peptides by double Petasis-3CR.

3. Backbone cyclization as an alternative to peptide side chain cyclization

As previously stated, the reduction of conformational space in peptides by cyclization has caught a continuous attention in the past years. Interestingly, most of the reported stapling-like methods rely on tethering the functional groups present on two side chains. Therefore, either the native functional groups are blocked or replaced by two residues for the ones that will be further involved in the cycle. These covalent modifications, when are applied to small peptides or on the active region of the sequence can be dramatically dangerous.[69] Consequently, the potential risk of either blocking important functionalities on the side chain or obstructing the solvent-exposed recognition surfaces of the target peptide, represent important drawbacks of these strategies.[69] An example of this difficulty was shown by Huang and co-workers[70] on their attempt to target Bcl-xL protein by a helical Bak BH3 peptide stabilized by macrolactamization. Interestingly, they found that this lactam-based side chain cyclization was unable the efficiently favor the binding to Bcl-xL, due to steric clashes in the transition interaction with the peptide receptor. Bearing this in mind, the design of new cyclization methods that allow the reduction of the conformational space of peptides without using side chains is an envisioned challenge.

In 1991, Gilon and coworkers suggested a new general concept of cyclization in addition to the known modes described above.[71] According to this method, called "backbone cyclization", the process involves tethering *N*-atoms of the amide backbone (Figure 3). Thus, to introduce a *N*-backbone cyclization, hydrogens of the peptide bond are replaced by ω-functionalized branches that can then be: connected to each other (Figure 3A) or to ends (Figure 3B) or to the side chains (Figure 3C) to form the desired cyclic peptide. Using this suggesting method, side chain functionalities are not perturbed and a highly variable set of cyclization chemistries can be applied for any linear sequence. Thus, many ring-closing procedures have been applied to backbone cyclizations including RCM, disulfide bond formation and macrolactamization.[20] Interestingly, as depicted in the Scheme 1G, the use of an A^3-coupling-based multicomponent protocol to cyclize *N*-aza modified peptide backbones can also be considered as a backbone cyclization, representing an example of MCRs applied to this cyclization concept.[60]

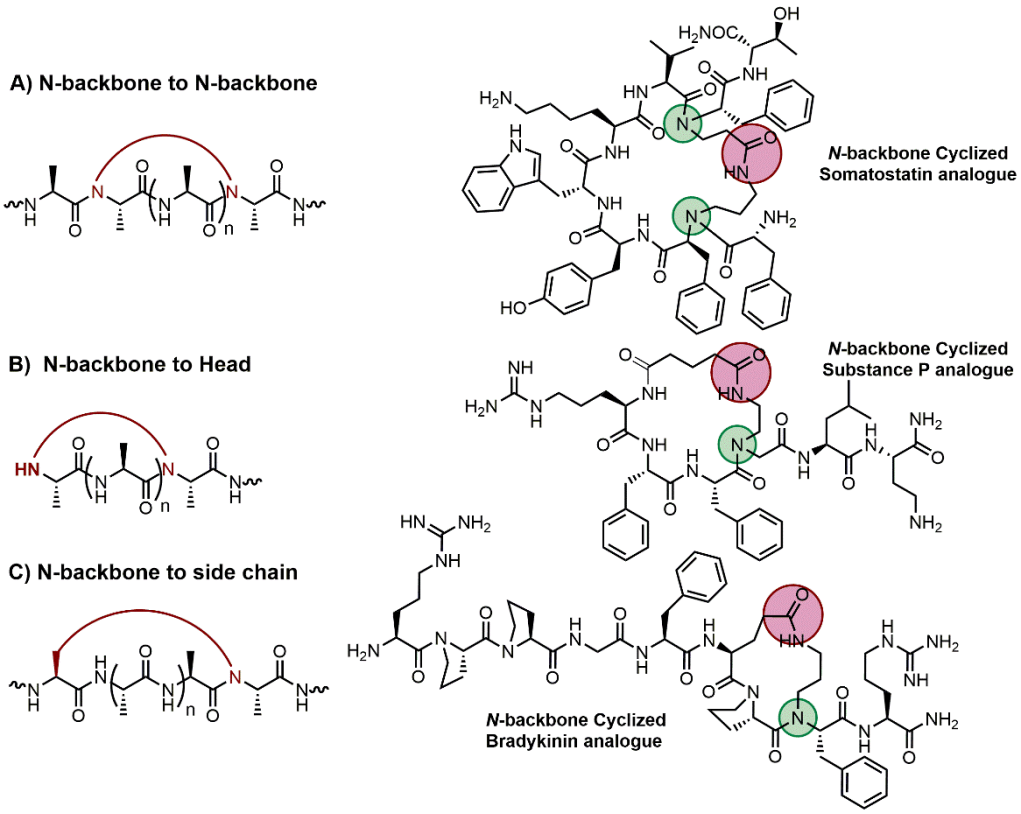


Figure 3. Methods of *N*-backbone cyclization: A) *N*-backbone-to-*N*-backbone, B) *N*-backbone-to-head C) *N*-backbone-to-side chain and.

In general, the capability to separate the effects of chemical changes from structural changes makes this sequence-independent strategy highly promising. Thus, by applying backbone cyclization on a biologically relevant peptides, it is possible to generate conformational libraries of the same sequence in dissimilar conformations, protocol that was named by Gilon and co-workers as "Cycloscan"[72]. This alternative depends on the possibility of introducing conformational alterations by discreet variations in the modes for backbone cyclization, position of backbone bridge and the type of chemistry used in the ring formation.

This notable approach combines two of the more successful alternatives to modulate the pharmacological properties of peptides: *N*-alkylation and cyclization, and since it was developed, it has successfully been applied to many natural peptides and peptides derived from target proteins. Some examples include the derivatization of substance P,[71] somatostatin,[73] Human immunodeficiency virus (HIV)-1 integrase,[74] bradykinin[75] and Bovine pancreatic trypsin inhibitor (BPTI)[76]. In several other examples, backbone-

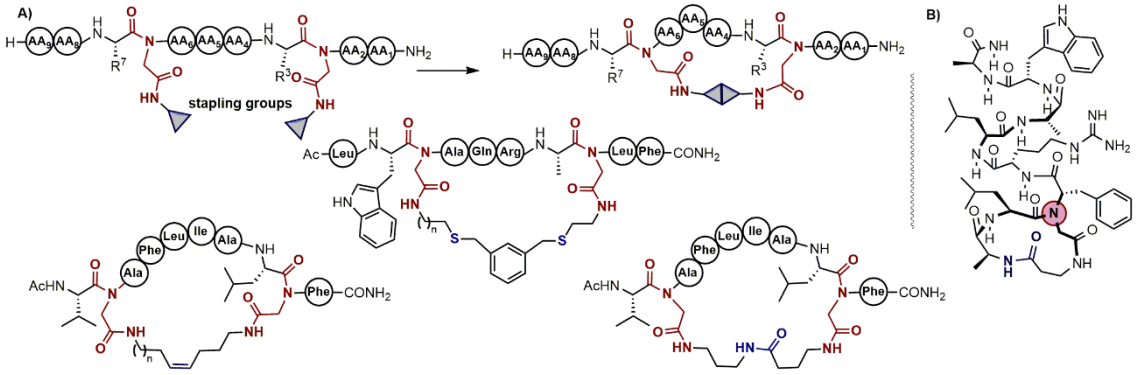
cyclized peptides have shown an increased pharmacological selectivity, higher stability and improved cellular permeability compared to their linear analogues.[20]

Independent of the concept stablished by Gilon, another interesting strategy developed in 2003 by Arora and co-workers[77] emerged as a suitable cyclization-based approach to stabilize helices. However, unlike peptide stapling, it was not based on side chains linkages. This strategy, so-called "hydrogen bond surrogate" (HBS), consisted of the stabilization of helical conformations by replacing the hydrogen bond between the C=O and NH, located at *i* + 4 position from the *N*-terminus, by an intramolecular covalent linkage derived from RCM cyclization. The HBS concept can be considered as a backbone cyclization where the cycle comprises an *N*-amide atom with the peptide head. Here the macrocycle formed works as a nucleator and, consequently, stabilizes the helix structure without compromising any side chain of the structure. This strategy has shown impressive results in the creation of stable Bak BH3 helices that target the Bcl-xL receptor with high selectivity and affinity. [69]

Besides the great opportunities that *N*-backbone cyclization offers in general, there have been some important synthetic limitations.[78] For example, incorporation of *N*-substituted amino acids in standard solid-phase peptide synthesis (SPPS) is still a challenging aim.[78] The first approach adopted, comprises the incorporation of *N*-alkylated and *N*-protected amino acids building units to overcome this limitation, followed by deprotection and coupling of the next residue. In this sense, many efforts have been dedicated to the synthesis of these building units. The second approach encompasses the alkylation of the *N*-terminal residue followed by the subsequent amino acid coupling. Accordingly, both approaches require the difficult acylation of the *N*-substituted terminal residue in order to complete the process.[78] To overcome such a challenging step, reported approaches either introduce *N*-substituted Gly[79] – with the subsequent replacement of the native side chain – or use very strong activation methods in repeated couplings.[80]

3.1 Multicomponent-assisted backbone cyclization

As described in the fourth chapter of this document, we developed a novel backbone cyclization approach relying on a general methodology for the incorporation of peptide *N*-substituents bearing functional groups suitable for cyclization processes. As depicted in the scheme 7, the proposed solid phase protocol relies on the utilization of the Ugi-4CR for the simultaneous acylation and *N*-substitution of the peptide. Thus, this strategy allows in only one step the incorporation of both the Fmoc-amino acid and the functionalized N-substituent, avoiding the difficult *N*-alkylation/*N*-acylation sequence. Noticeably, the straightforward nature of this alternative, enabled the use of diverse macrocyclization chemistry on the backbone *N*-substituents, including ring-closing metathesis, thiol bis-alkylation and macrolactamization.



Scheme 7. Peptide backbone stapling strategy enabled by the incorporation of amide *N*-substituents derived from on-resin Ugi reactions. A) Backbone-to-backbone and B) backbone-to-head featuring HBS peptides.

Another feature of this multicomponent strategy is proposed as an alternative for the HBS concept. Thus, incorporation of Ugi-derived backbone *N*-substituents in two amino acids ahead of the *N*-terminus enables the versatile and easy introduction of carboxyl and olefin functionalities. Accordingly, after the RCM or macrolactamization, the formed 13-membered macrocycle, replaces the intramolecular hydrogen bond and produces the entire HBS peptide on resin. These results proved that the on-resin multicomponent *N*-modification method provides a powerful strategy for peptide cyclization at their backbone substituents instead of their side chains.

References

(1) Pelay-Gimeno, M.; Glas, A.; Koch, O.; Grossmann, T. N. *Angew. Chem. Int. Ed.* **2015**, *54*, 8896–8927.

(2) Hill, T. A.; Shepherd, N. E.; Diness, F.; Fairlie, D. P. *Angew. Chem. Int. Ed.* **2014**, *53*, 13020–13041.

(3) Moreira, I. S.; Fernandes, P. A.; Ramos, M. J. *Proteins* **2007**, *68*, 803–812.

(4) Rizo, J.; Gierasch, L. M. *Annu. Rev. Biochem.* **1992**, *61*, 387–418.

(5) Chan, O. H.; Stewart, B. H. *Drug Discov. Today* **1996**, *1*, 461–473.

(6) Witt, K. A.; Gillespie, T. J.; Huber, J. D.; Egleton, R. D.; Davis, T. P. *Peptides* **2001**, *22*, 2329–2343.

(7) Sewald, N.; Jakubke, H. *Peptides: Chemistry and Biology*; WILEY-VCH Verlag GmbH: Weinheim, 2002.

(8) Cardote, T. A. F.; Ciulli, A. *ChemMedChem.* **2016**, *11*, 787–794.

(9) Yudin, A. K. *Chem. Sci.* **2015**, *6*, 30–49.

(10) Villar, E. A.; Beglov, D.; Chennamadhavuni, S.; Jr, J. A. P.; Kozakov, D.; Vajda, S.; Whitty, A. *Nat. Chem. Biol.* **2014**, *10*, 723–732.

(11) Giordanetto, F.; Kihlberg, J. *J. Med. Chem.* **2014**, *57*, 278–295.

(12) Tyndall, J. D. A.; Nall, T.; Fairlie, D. P. *Chem. Rev.* **2005**, *105*, 973–999.

(13) Rezai, T.; Yu, B.; Millhauser, G. L.; Jacobson, M. P.; Lokey, R. S. *J. Am. Chem. Soc.* **2006**, *128*, 2510–2511.

(14) Robinson, J. A. *Acc. Chem. Res.* **2008**, *41*, 1278–1288.

(15) Loughlin, W. A.; Tyndall, J. D. A.; Glenn, M. P.; Hill, T. A.; Fairlie, D. P. *Chem Rev.* **2010**, *110*, PR32–PR69.

(16) Henchey, L. K.; Jochim, A. L.; Arora, P. S. *Curr. Opin. Chem. Biol.* **2008**, *12*, 692–697.

(17) Garner, J.; Harding, M. M. *Org. Biomol. Chem.* **2007**, *5*, 3577–3585.

(18) Udugamasooriya, D. G.; Spaller, M. R. *Biopolymers* **2008**, *89*, 653–667.

(19) White, C. J.; Yudin, A. K. *Nat. Chem.* **2011**, *3*, 509–524.

(20) Rubin, S. J. S.; Qvit, N. *Curr. Top. Med. Chem.* **2018**, *18*, 526–555.

(21) Hili, R.; Rai, V.; Yudin, A. K. *J. Am. Chem. Soc.* **2010**, *132*, 2889–2891.

(22) Vasco, A. V.; Mendez, Y.; Porzel, A.; Balbach, J.; Wessjohann, L. A.; Rivera, D. G. *Bioconjug. Chem.* **2018**, *30*, 253–259.

(23) Morejón, M. C.; Laub, A.; Kaluderović, G. N.; Puentes, A. R.; Hmedat, A. N.; Otero-González, A. J.; Rivera, D. G.; Wessjohann, L. A. *Org. Biomol. Chem.* **2017**, *15*, 3628–3637.

(24) Gause, G. F.; Brazhnikova, M. G. *Nature* **1944**, 703.

(25) Hruby, V. J. *Nat. Rev.* **2002**, *1*, 847–858.

(26) Góngora-Benítez, M.; Tulla-Puche, J.; Albericio, F. *Chem. Rev.* **2014**, *114*, 901–926.

(27) Cantel, S.; Le, A.; Isaad, C.; Scrima, M.; Levy, J. J.; Dimarchi, R. D.; Rovero, P.; Halperin, J. A.; Papini, A. M.; Chorev, M. *J. Org. Chem.* **2008**, *73*, 5663–5674.

(28) Bonandi, E.; Christodoulou, M. S.; Fumagalli, G.; Perdicchia, D.; Rastelli, G.; Passarella, D. *Drug Discov. Today* **2017**, *22*, 1572–1581.

(29) Cui, H.; Guo, Y.; He, Y.; Wang, F.; Chang, H.; Wang, Y. *Angew. Chem. Int. Ed.* **2013**, *52*, 9558–9562.

(30) Lau, Y. H.; de Andrade, P.; Wu, Y.; Spring, D. R. *Chem. Soc. Rev.* **2015**, *44*, 91–102.

(31) Walensky, L. D.; Kung, A. L.; Escher, I.; Malia, T. J.; Barbuto, S.; Wright, R. D.; Wagner, G.; Verdine, G. L.; Korsmeyer, S. J. *Science* **2004**, *305*, 1466–1471.

(32) Walensky, L. D.; Bird, G. H. *J. Med. Chem.* **2014**, *57*, 6275-6288.

(33) Cromm, P. M.; Spiegel, J.; Grossmann, T. N. *ACS Chem. Biol.* **2015**, *10*, 1362–1375.

(34) Shepherd, N. E.; Abbenante, G.; Fairlie, D. P. *Angew. Chemie. Int. Ed.* **2004**, *43*, 2687–2690.

(35) Scrima, M.; Le Chevalier-Isaad, A.; Rovero, P.; Papini, A. M.; Chorev, M.; D'Ursi, A. M. *European J. Org. Chem.* **2010**, 446–457.

(36) Kawamoto, S. A.; Coleska, A.; Ran, X.; Yi, H.; Yang, C. Y.; Wang, S. *J. Med. Chem.* **2012**, *55*, 1137–1146.

(37) Assem, N.; Ferreira, D. J.; Wolan, D. W.; Dawson, P. E. *Angew. Chem. Int. Ed.* **2015**, *54*, 8665–8668.

(38) Jo, H.; Meinhardt, N.; Wu, Y.; Kulkarni, S.; Hu, X.; Low, K. E.; Davies, P. L.; Degrado, W. F.; Greenbaum, D. C. *J. Am. Chem. Soc*. **2012**, *134*, 17704-17713.

(39) Noisier, A. F. M.; García, J.; Ionut, I. A.; Albericio, F. *Angew. Chem. Int. Ed.* **2017**, *56*, 314–318.

(40) Mendive-Tapia, L.; Preciado, S.; García, J.; Ramón, R.; Nicola, K.; Albericio, F.; Lavilla, R. *Nat. Commun.* **2015**, *6*, 1–9.

(41) Lau, Y. H.; de Andrade, P.; Sköld, N.; McKenzie, G. J.; Venkitaraman, A. R.; Verma, C.; Lane, D. P.; Spring, D. R. *Org. Biomol. Chem.* **2014**, *12*, 4074–4077.

(42) Wang, Y.; Chou, D. H. *Angew. Chem. Int. Ed.* **2015**, *54*, 10931–10934.

(43) Brown, S. P.; Smith, A. B. *J. Am. Chem. Soc.* **2015**, *137*, 4034–4037.

(44) Lautrette, G.; Touti, F.; Lee, H. G.; Dai, P.; Pentelute, B. L. *J. Am. Chem. Soc.* **2016**, *138*, 8340–8343.

(45) Woolley, G. A. *Acc. Chem. Res.* **2005**, *38*, 486–493.

(46) Ruijter, E.; Scheffelaar, R.; Orru, R. V. A. *Angew. Chem. Int. Ed.* **2011**, *50*, 6234–6246.

(47) Zhu, J.; Wang, Q.; Wang, M.-X. *Multicmponent Reactions in Organic Chemistry*; Wiley-VCH: Weinheim, 2015.

(48) Ugi, I. *Angew. Chem. Int. Ed.* **1959**, *71*, 386–386.

(49) Failli, A.; Immer, H.; Götz, M. *Can. J. Chem.* **1979**, *57*, 3257–3261.

(50) Wessjohann, L. A.; Rivera, D. G.; Vercillo, O. E. *Chem. Rev.* **2009**, *109*, 796–814.

(51) Wessjohann, L. A.; Voigt, B.; Rivera, D. G. *Angew. Chem. Int. Ed.* **2005**, *44*, 4785–4790.

(52) Michalik, D.; Schaks, A.; Wessjohann, L. A. *Eur. J. Org. Chem.* **2007**, 149–157.

(53) Rivera, D. G.; Vercillo, E.; Wessjohann, L. A. *Org. Biomol. Chem.* **2008**, *6*, 1787–1795.

(54) Frost, J. R.; Scully, C. C. G.; Yudin, A. K. *Nat. Chem.* **2016**, *8*, 1105–1111.

(55) Banfi, L.; Riva, R. *Org. React.* **2005**, *65*, 1–140.

(56) Arylation, M.; El Kaïm, L.; Grimaud, L.; Oble, J. *Angew. Chem. Int. Ed.* **2005**, *44*, 7961–7964.

(57) Morejón, M. C.; Laub, A.; Westermann, B.; Rivera, D. G.; Wessjohann, L. A. *Org. Lett.* **2016**, *18* , 4096–4099.

(58) Strecker, A. *Ann. Chem.* **1850**, *75*, 27–45.

(59) Malins, L. R.; Justine, N.; Robbins, K. J.; Scola, P. M.; Eastgate, M. D.; Ghadiri, M. R.; Baran, P. S. *J. Am. Chem. Soc*. **2017**, *139*, 5233−5241.

(60) Zhang, J.; Mulumba, M.; Ong, H.; Lubell, W. D. *Angew. Chem. Int. Ed.* **2017**, *56*, 6284–6288.

(61) Ricardo, M. G.; Llanes, D.; Wessjohann, L. A; Rivera, D. G. *Angew. Chemie. Int. Ed.* **2019**, *58*, 2700-2704.

(62) Ye, Y.; Gao, X.; Liu, M.; Tang, Y.; Tian, G. *Lett. Pept. Sci.* **2004**, *10*, 571–572.

(63) Lambert, J. N.; Mitchell, P.; Roberts, K. D. *J. Chem. Soc., Perkin Trans. 1.* **2001**, *10*, 471–484.

(64) Rivera, D. G.; Wessjohann, L. A. *Org. Lett.* **2017**, *19*, 4022−4025.

(65) Reguera, L.; Rivera, D. G. *Chem. Rev.* **2018**, https://doi.org/10.1021/acs.chemrev.8b00744

(66) Morales, F. E.; Garay, H. E.; Vasilev, D.; Gav, J. A.; Wessjohann, L. A.; Rivera, D. G. *J. Org. Chem.* **2015**, *80*, 6697−6707.

(67) Ricardo, M. G.; Morales, F. E.; Garay, H.; Reyes, O.; Vasilev, D.; Wessjohann, L. A.; Rivera, D. G. *Org. Biomol. Chem.* **2015**, *13*, 438–446.

(68) Candeias, N. R.; Montalbano, F.; Cal, P. M. S. D.; Gois, P. M. P. *Chem. Rev.* **2010**, *110*, 6169–6193.

(69) Patgiri, A.; Jochim, A. L.; Arora, P. S. *Acc. Chem. Res.* **2008**, *41*, 1289–1300.

(70) Yang, B.; Liu, D.; Huang, Z. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 1403–1406.

(71) Gilon, C.; Halle, D.; Chorev, M.; Selinger, Z.; Byk, G. *Biopolymers* **1991**, *31*, 745–750.

(72) Gilon, C.; Muller, D.; Bitan, G.; Salitra, Y.; Goldwasser, I.; Hornik, V. Cycloscan: Backbone Cyclic Conformational Libraries of Peptides. In *24th Conference of the European Peptide Society*; Edinbrough, Scotland, 1999.

(73) Gazal, S.; Gelerman, G.; Ziv, O.; Karpov, O.; Litman, P.; Bracha, M.; Afargan, M.; Gilon, C. *J. Med. Chem.* **2002**, *45*, 1665–1671.

(74) Hayouka, Z.; Levin, A.; Hurevich, M.; Shalev, D. E.; Loyter, A.; Gilon, C.; Friedler, A. *Bioorg. Med. Chem.* **2012**, *20*, 3317–3322.

(75) Schumann, C.; Seyfarth, L.; Greiner, G.; Paegelow, I.; Reissmann, S. *J. Pept. Res.* **2002**, *60*, 128–140.

(76) Kasher, R.; Bitan, G.; Halloun, C.; Gilon, C. *Lett. Pept. Sci.* **1998**, *5*, 101–103.

(77) Chapman, R. N.; Dimartino, G.; Arora, P. S. *J. Am. Chem. Soc.* **2004**, *126*, 12252–12253.

(78) Fernández-Llamazares, A. I.; Spengler, J.; Albericio, F. *Biopolymers* **2015**, *104*, 435–452.

(79) Hurevich, M.; Tal-gan, Y.; Klein, S.; Barda, Y.; Levitzki, A.; Gilon, C. *J. Pept. Sci.* **2010**, *16*, 178–185.

(80) Falb, E.; Yechezkel, T.; Salitra, Y.; Gilon, C. *J. Pep. Res.* **1999**, *53*, 507–517.

Outlook and Aims of this thesis*

MCRs have played a crucial role in the evolution of the synthetic methods for peptide cyclizations. Together with the classical methods, they were initially developed for the head-to-tail cyclization of peptides achieving some important contributions in terms of chemical diversity, synthetic cost and avoiding epimerization and dimerization problems. Among all the multicomponent processes, the Ugi-4CR has been the pioneer and the lead approach, participating in peptide cyclizations either by termini or side chains. In this sense, **development of an on-resin protocol for the head-to-tail cyclization of analogues of Gramicidin S by the Ugi reaction in order to analyse the conformational influence of the *N*-substituted dipeptide generated**, constitutes the first aim of this work.

Recently, with the significant importance of the side chain-to-side chain cyclization methods – coined as peptide stapling –, was the advent of many multicomponent alternatives have been published. Taking into account the synthetic scope of the two-component stapling approaches, we aim in the second chapter to **develop new multicomponent strategies for the double macrocyclization of peptides by Ugi reaction**. Interestingly, without considering the isocyanide-based multicomponent reactions like Passerini, Yudin or Ugi-Smiles, all other multicomponent processes have shown up in the peptide cyclization field only during the last two years. This very recent knowledge includes the classical and first multicomponent reaction known, the Strecker-3CR, the metal catalysed A3-coupling and the Petasis reaction. The Petasis reaction was used previously only for the synthesis of non proteinogenic amino acids and sugar-derived natural products. Accordingly, **the introduction of this multicomponent process to the repertoire of not only peptide stapling, but also for peptide labelling and general diversification** constitutes the third aim of the present research.

In comparison with traditional protocols, all multicomponent approaches have been shown as important features associated with the rapid generation of complexity by

* To approach the overall research objective, four aims are detailed in the frame of the following text

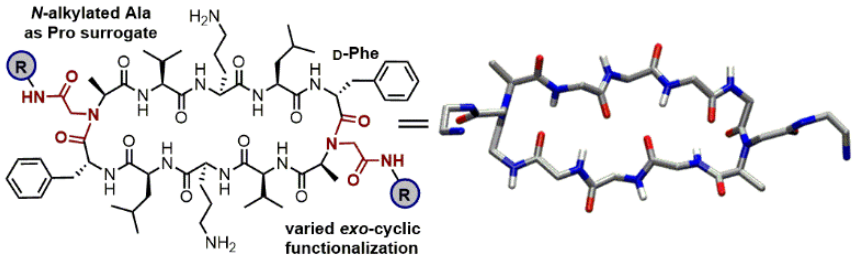
allowing the introduction of important diversity during the ring-closure. Since it was demonstrated that the participation of side-chains on the cyclization can negatively affect the biological activity because of blocking the binding-peptides surface, the development of methods for peptide backbone cyclization has acquired a growing relevance. Because of the noticeably limitations of the standard methods for backbone *N*-alkylation, **the Ugi-4CR appears as a suitable alternative for the introduction of *N*-modifications bearing functional groups that enable the desired cyclizations without using side chains**. Developing the according methods is the fourth aim of the present thesis.

Chapter 2

On the Stabilization of Cyclic β-Hairpins by Ugi Reaction-Derived *N*-Alkylated Peptides: The Quest for Functionalized β-Turns

This Chapter has been accepted to be published as:

Ricardo, Manuel G.; Vasco, Aldrin V.; Rivera, Daniel G. and Wessjohann, Ludger A. *Org. Lett.* **2019**, *21*, 7307-7310



Abstract

A solid-phase approach including on-resin Ugi reactions was developed for the construction of β-hairpins. Various *N*-alkylated dipeptide fragments proved capable of aligning antiparallel β-sheets in a macrocyclic scaffold, thus serving as β-hairpin templates. Gramicidin S was used as model β-hairpin to compare the Ugi-derived β-turns with the type II' β-turn. The results show that the multicomponent incorporation of such *N*-alkylated residues allows for the simultaneous stabilization and *exo*-cyclic functionalization of cyclic β-hairpins.

Introduction

Macrocyclization approaches based on multicomponent reactions (MCRs) have recently emerged as excellent tools for the incorporation of conformational constrains in peptide sequences.[1,2] Especially Ugi-type macrocyclizations[3,4,5,6] have proven great success in accessing constrained cyclic peptides. At the same time, they allow for tunable *exo* and *endo*-functionalization of the macrocyclic scaffold. Thus, the Ugi macrocyclization has been employed for stapling peptides in α-helical[3] and turn[4] conformations, but so far, the Ugi moieties have not been responsible for imposing specific conformation to the peptide backbone. A different scenario shows up in various MCR macrocyclizations developed by Yudin and co-workers,[5,6] in which either *endo*-cyclic heterocycles[7] or *exo*-cyclic amides,[8] formed during the MCR ring closure, do have a determinant role in the peptide conformation by participating in intramolecular hydrogen bonds.

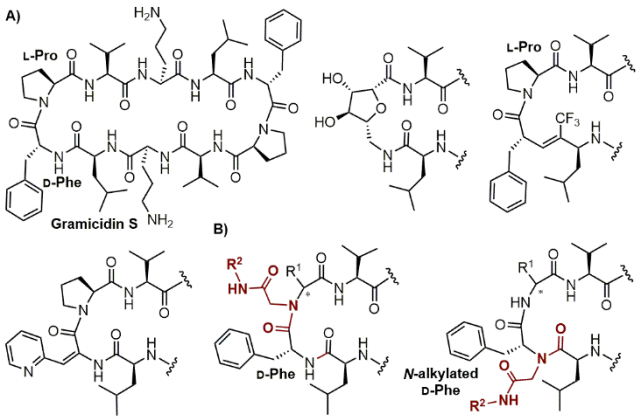


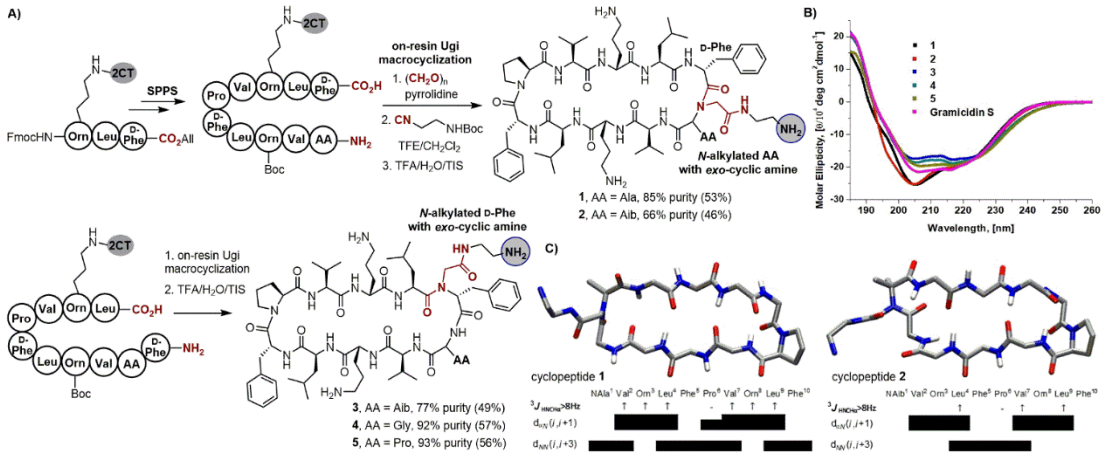
Figure 1. A) Gramicidin S and some β-turn mimics used in its analogs. B) Ugi reaction-derived *N*-alkylated peptide fragments as novel β-hairpin-stabilizing templates.

In an endeavor to demonstrate that the *N*-alkylated peptide fragment resulting from the Ugi macrocyclization is capable to enforce a conformational bias leading to a stable secondary structure, herein we describe a strategy for the construction of Ugi-derived cyclic β-hairpins. A β-hairpin is a class of secondary structure in which a reverse turn or loop is flanked by two anti-parallel β-sheets.[9] This class of peptide motif – indeed comprising diverse conformations – is recognized as one of the most important protein regular structures and functional epitopes.[9] There are several hairpin-stabilizing templates known in the literature, such as the dipeptide D-Pro-L-Pro and combinations

of Pro with other amino acids (AA), e.g., D-AA-L-Pro and Gly-L-Pro.[9] However, we are not aware of approaches based on Ugi or any other type of MCR macrocyclization targeting peptide mimics of β-hairpins.

We chose the antibacterial cyclic decapeptide Gramicidin S (GS, cyclo(Pro-Val-Orn-Leu-D-Phe)$_2$) as model compound for β-sheet conformational mimicry.[10] Whereas our goal is not to develop novel antibacterials, GS is considered as a prototypical β-hairpin compound and its structure has been previously used to design novel peptidomimetics[10,11,12] and sugar-amino acid[13] turn inducers (Fig. 1A). GS is a C2-symmetric β-hairpin in which two antiparallel β-strands are connected by two type II' β-turns induced by the dipeptide D-Phe-Pro. As depicted in Fig. 1B, we envisioned that an *N*-alkylated peptide fragment created by the Ugi reaction could serve as β-hairpin stabilizing template due to the conformational bias imposed by the turn inducing effect of the tertiary amide, in a similar way as Pro and *N*-Me-AAs do in reverse turns.[9]

Scheme 1. A) Synthesis of cyclic peptide β-hairpins by on-resin Ugi macrocyclization.[a] B) Circular dichroism spectra of Ugi-derived β-hairpins compared with Gramicidin S. C) Average NMR-derived structures of cyclic peptides 1 and 2.[b]



[a]In parenthesis the yields of crude products; an analytical sample was purified to >95% purity. [b]Most side chains are omitted for clarity.

Our group has gathered previous evidence on the ability of Ugi-derived *N*-alkylated residues to fold oligopeptide chains[14] and facilitate macrocyclization[15] by engaging the two termini. In addition, a recent study proved that the replacement of Pro by *N*-Me-Ala does not affect the activity of the GS analogs.[16] As a result, we initially focused on

substituting one of the Pro residues in GS by an *N*-alkylated AA generated by the Ugi reaction, which simultaneously allows installing an *exo*-cyclic functional group, something that cannot be achieved with the use of Pro or *N*-Me-AAs.

An orthogonal solid-phase peptide synthesis (SPPS) strategy was envisioned seeking to conduct most steps on resin, including the key Ugi multicomponent macrocyclization. For this, it was important to achieve the previous attachment of the Orn-side chain to a resin, thus leaving the two peptide-termini free for macrocyclization. Albericio and co-workers were the first to develop a SPPS approach toward cyclic peptides comprising the attachment of an amine-containing side chain to a resin.[17] In addition, the same group reported the synthesis of GS analogs by cyclization of the peptide at the Orn residue, whose side chain was anchored to a resin through a carbamate functionality.[18] However, nowadays it is known that the intrinsic folding of acyclic GS favors cyclization by residues opposed to one of the β-turns.[19] As a result, we designed the SPPS approach considering two dissimilar sites for incorporating the *N*-alkylation at one β-turn motif.

As depicted in scheme 1, tripeptide Fmoc-Orn-Leu-D-Phe-OAll was produced in solution and next anchored to the 2-clorotrityl (2CT) resin by the Orn side chain. Subsequent growth of the peptide sequence included the incorporation of either Ala or 2-aminoisobutyric acid (Aib) the *N*-terminus, followed by deprotection of the two termini. Thus, the on-resin Ugi macrocyclization of the main-chain amino and carboxylic acid groups, was conducted following a procedure recently developed for cyclizing peptide side chains.[3] The protocol comprises an initial transimination step using 4 equiv of paraformaldehyde and pyrrolidine, followed by washing the resin-linked iminopeptide and subsequent reaction with 4 equiv. of the isocyanide in trifluoroethanol (TFE)/CH_2Cl_2 1:1 (*v*/*v*). Mini-cleavages and HPLC analysis were carried out after 12 h of reaction, usually showing complete consumption of the linear peptide. In cases of incomplete macrocyclization after 12 h, a second cycle of imine formation and reaction with isocyanide is required (see the Supporting Information, SI). It is worth-mentioning that cleavage of the peptide from the 2CT resin was not detected during the course of the entire SPPS protocol, not even during the macrocyclization conducted in TFE/CH_2Cl_2.

Cyclic peptides **1** and **2** were produced by conducting the Ugi reaction ring closure with Ala and Aib, respectively, as amino component and D-Phe as carboxylic acid, thus forming an *N*-alkylated β-turn inducer opposed to the natural D-Phe-Pro sequence. The fact that even a peptide bearing a bulky Aib residue at the *N*-terminus undergoes, with two cycles, the Ugi macrocyclization in excellent yield corroborates the strength of the method. In parallel, cyclic peptides **3**, **4** and **5** were prepared by choosing the other site recommended for cyclization, i.e., between Leu and D-Phe. Once more, high crude purities were obtained for the three peptides, in which not only Pro (**5**) but also Gly (**4**) and Aib (**3**) were installed as preceding residue. GS was produced in a similar way in 67% crude purity using a final macrolactamization instead of the Ugi reaction ring closure.

An isocyanide bearing a protected amine was employed to introduce an additional *exo*-cyclic amino (cationic) group at the GS scaffold. In principle, any functionalization can be installed here in such a cyclo-ligation process. To address the effect of such structural variations on the stability of the β-hairpin, circular dichroism (CD) spectra were recorded and compared with that of GS. As shown in scheme 1B, cyclic peptides **3**, **4** and **5** exhibit CD spectra almost identical to GS, suggesting that the *N*-functionalization of D-Phe does not modify the type II' β-turn motif and keeps the stability of the β-hairpin. This result is reasonable for compounds **4** and **5**, in which the *N*-alkylated D-Phe-Gly and D-Phe-L-Pro fragments can be seen as mimetic of another type II' β-turns such as D-Pro-L-Pro and D-Pro-Gly. However, the fact that the *N*-alkylated D-Phe-Aib motif in **3** also induces this type of β-turn reproducing the β-hairpin conformation of GS is noteworthy.

However, in the case of cyclic peptides **1** and **2**, there is a small increase in the minimum at 205 nm as compared with the CD spectrum of GS, proving a slight deviation from the model GS β-hairpin structure. To better understand this conformational change, the solution-phase structures of **1** and **2** were determined by means of NMR and Molecular Dynamics (MD) simulation (see the SI). The NMR spectra of peptide **1** demonstrate an extended conformation for Val, Orn and Leu residues (i.e., coupling constants higher than 8 Hz and strong $\alpha_i N_{i+1}$ NOEs) and two β-turns comprising the Leu-D-Phe-Pro-Val and Leu-D-Phe-*N*-(Alk)Ala-Val fragments, as

confirmed by the N_iN_{i+3} NOEs between the amide protons of residues Val and Leu. In addition, cross-peaks indicative of spatial proximity between the Hα of both Orn residues were observed.

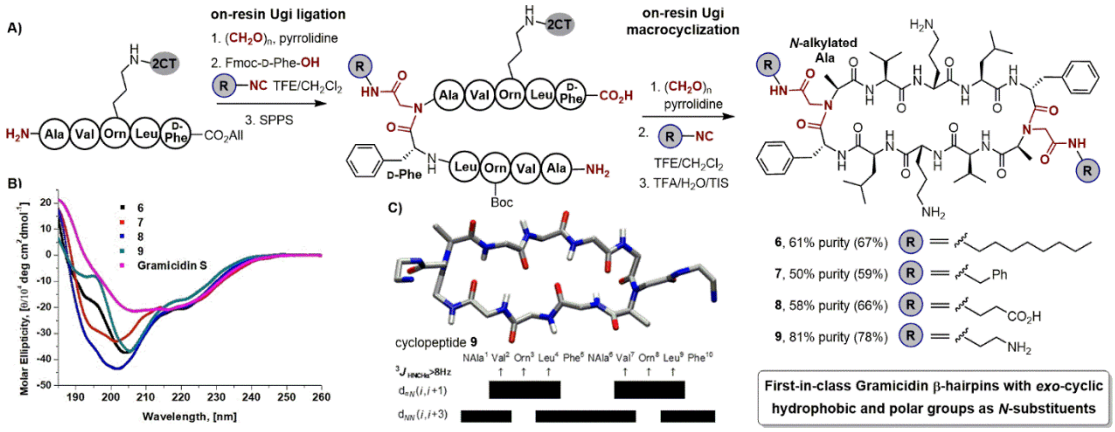
As depicted in the NMR structure of **1** (Scheme 1c), this cyclic backbone definitely occurs in a β-hairpin conformation, in which the *exo*-cyclic aminopeptidic moiety inserted as amide *N*-substitution points to the opposite face of the Orn side chains. In this regard, Wishart and co-workers have shown that for type II' β-turns,[20] the residues *i*+1 and *i*+2 within the turn have equatorial and axial orientations, respectively; i.e., D-Phe (*i*+1) and Pro (*i*+2) in GS. However, in cyclic peptide **1**, both the D-Phe (*i*+1) and *N*-(Alkyl)Ala (*i*+2) side chains have equatorial disposition with respect to peptide backbone (see side view in the SI), while it is actually the *exo*-cyclic *N*-substituent which is axially oriented. This analysis suggests that the Ugi derived β-turn centered at D-Phe-*N*-(Alkyl)Ala is not of type II'. Nonetheless, this fact does not have a detrimental influence in the stability of the β-hairpin conformation, and therefore, the combination of D-Phe with Ugi-derived *N*-alkylated L-AA could be considered as a new type of β-hairpin stabilizing template.

On the other hand, NMR evidence proves that the installation of an *N*-alkylated Aib does have a negative influence of the β-hairpin stability, since the typical β-sheet characteristic are missing in cyclic peptide **2**. In this case, only the two Leu and one Val residues show coupling constants higher than 8 Hz. Furthermore, the NOEs between Leu and Val amide protons are only observed for the residues enclosing the β-turn centered at D-Phe-Pro, while the dipeptide fragment D-Phe-*N*-(Alkyl)Aib does not induce a typical β-turn motif. As shown in scheme 1C, the NMR structure of cyclic peptide **2** shows a distorted reverse turn at the D-Phe-*N*-(Alkyl)Aib corner, which certainly destabilizes the β-hairpin conformation.

We next wondered if the double substitution of the D-Phe-Pro fragments by the Ugi-derived D-Phe-*N*-(Alkyl)Ala would result in a stable β-hairpin conformation. As depicted in scheme 2, an SPPS approach was employed in which an initial on-resin Ugi reaction enabled the introduction of the first *N*-alkylated Ala residue using Fmoc-D-Phe-OH as the carboxylic acid component and a variety of isocyanides. After consecutive incorporation of the following AAs, a final Ugi macrocyclization was employed as

described before, again coupling the terminal D-Phe and Ala with the introduction of a second amide *N*-substitution. The overall yields and purities of the crude peptides were acceptable considering the complexity of this SPPS sequence, which involved several coupling steps and two on-resin Ugi reactions, one of them for the ring closure. Thus, cyclic peptides bearing long *exo*-cyclic aliphatic chains, phenyl rings, as well as anionic or cationic groups were readily produced.

Scheme 2. A) Synthesis of cyclic β-hairpins by a SPPS approach comprising two Ugi reactions.[a] B) Circular dichroism spectra of double Ugi-derived β-hairpins compared with Gramicidin S. C) Average NMR-derived structure of cyclic peptide 9.[b]



[a]In parenthesis the yields of crude products; an analytical sample was purified to >95% purity. [b]Most side chains are omitted for clarity.

The CD spectra of cyclic peptides **6**-**9** show a much more intense minimum around 205 nm for all compounds as compared with GS. This suggests a deviation from the native GS conformation, likely due to the presence of β-turn conformations different from that of GS. To get a deeper insight into this finding, we chose cyclic peptide **9** to determine the solution-phase structure based on NMR and MD simulation. Hence, all NMR evidence confirms that this type of cyclic peptide occurs in a β-hairpin conformation, albeit featuring β-turns different from the native type II'. The ^{1}H-NMR spectrum of **9** proves the conformational symmetry expected for a cyclic C2-symmetric β-hairpin, in which only five amide protons (see the SI). Moreover, as expected for extended conformations, coupling constants higher than 8 Hz and strong $\alpha_i N_{i+1}$ NOEs were detected for Val, Orn and Leu residues (i.e., the β-sheet region), and $N_i N_{i+3}$ NOEs between the amide protons of Val and Leu again confirmed the β-turn conformation at

the two corners of the β-hairpin. The temperature coefficients of the amide protons were also determined (see the SI), showing low values for Leu and Val residues that further confirm the occurrence of closed β-turns.[20] Scheme 2C depicts the NMR structure of peptide **9** featuring a β-hairpin conformation with the two amide *N*-substituents pointing toward the opposite face of Orn side chains.

In conclusion, we have developed a new type of cyclic β-hairpin peptide by proving that the incorporation of *N*-alkylated AA residues by Ugi reactions may induce β-turn conformations and, thereby, stabilize the β-hairpin architecture. Our results suggest that the combination of *N*-alkylated D-Phe at *i*+1 with Pro, Gly and even Aib at *i*+2 enables to mimic type II' β-turn motifs as found in GS. Thus, the incorporation of these fragments opposite to the native D-Phe-Pro keeps the overall β-hairpin conformation of GS. The substitution of Pro (*i*+2) by *N*-alkylated Ala (but not Aib) also leads to a β-hairpin conformation, in which the D-Phe-*N*-(Alkyl)Ala sequence induces a β-turn, but not of type II'. Nonetheless, the Ugi-derived β-turn equally imposes the anti-parallel alignment of the two β-strands in a β-sheet structure, thus forming a cyclic β-hairpin. In this sense, the Ugi reaction-based approach allows for the stabilization of the β-hairpin and the simultaneous functionalization of the β-turn motifs. This *exo*-cyclic modification can be used to modulate the bioactivity by adding additional cationic or hydrophobic tails, but also to install bioconjugation handles, fluorescent and affinity tags, etc.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures, RP-HPLC chromatograms, NMR and HR-ESI-MS spectra of cyclic peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

* E-mail: dgr@fq.uh.cu, wessjohann@ipb-halle.de

Author Contributions

‡These authors contributed equally

DEDICATION

Dedicated to Prof. Dr. Armin de Meijere on the occasion of his 80th birthday

ACKNOWLEDGMENT

A. V. V. and D.G.R are grateful to DAAD, Germany, for PhD and University Academics fellowships, respectively.

REFERENCES

(1) Reguera, L.; Rivera, D. G. Multicomponent Reaction Toolbox for Peptide Macrocyclization and Stapling. *Chem. Rev.* **2019**, doi: 10.1021/acs.chemrev.8b00744.

(2) White, C. J.; Yudin, A. K. Contemporary strategies for peptide macrocyclization. *Nat. Chem.* **2011**, *3*, 509-524.

(3) Vasco, A. V.; Méndez, Y.; Porzel, A.; Balbach, J.; Wessjohann, L. A.; Rivera, D. G. A Multicomponent stapling approach to exocyclic functionalized helical peptides: adding lipids, sugars, PEGs, labels, and handles to the lactam bridge. *Bioconjugate Chem.* **2019**, *30*, 253−259.

(4) Vasco, A. V.; Pérez, C. S.; Morales, F. E.; Garay, H. E.; Vasilev, D.; Gavín, J. A.; Wessjohann, L. A.; Rivera, D. G. Macrocyclization of peptide side chains by the Ugi reaction: achieving peptide folding and exocyclic *N*-functionalization in one shot. *J. Org. Chem.* **2015**, *80*, 6697-6707.

(5) Frost, J. R.; Scully, C. C. G.; Yudin, A. K. Oxadiazole grafts in peptide macrocycles. *Nat. Chem.* **2016**, *8*, 1105-1111.

(6) Hili, R.; Rai, V.; Yudin, A. K. Macrocyclization of linear peptides enabled by amphoteric molecules. *J. Am. Chem. Soc.* **2010**, *132*, 2889-2891.

(7) Appavoo, S. D.; Kaji, T.; Frost, J. R.; Scully, C. C. G.; Yudin, A. K. Development of endocyclic control elements for peptide macrocycles. *J. Am. Chem. Soc*. **2018**, *140*, 8763–8770.

(8) Zaretsky, S.; Scully, C. C. G.; Lough, A. J.; Yudin, A. K. Exocyclic control of turn induction in macrocyclic peptide scaffolds. *Chem. Eur. J.* **2013**, *19*, 17668-17672.

(9) Robinson, J. A. β-Hairpin peptidomimetics: design, structures and biological activities. *Acc. Chem. Res.* **2008**, *41*, 1278-1288.

(10) Pal, S.; Ghosh, U.; Ampapathi, R. S.; Chakraborty, T. K. Recent Studies on Gramicidin S Analog Structure and Antimicrobial Activity. In *Peptidomimetics II. Topics in Heterocyclic Chemistry*; Lubell, W., Eds.; Springer: Cham, 2015; Vol. 49, pp 159-202.

(11) Xiao, J.; Weisblum, B.; Wipf, P. Electrostatic versus steric effect in Peptidomimicry: Secondary structure analysis of Gramicidin S Analogues with (*E*)-Alkene Dipeptide Isosteres. *J. Am. Chem. Soc*. **2005**, *127*, 5742-5743.

(12) Yamada, K.; Kodaira, M.; Shinoda, S.; Komagoe, K.; Oku, H.; Katakai, R.; Katsu, T.; Matsuo, I. Structure–activity relationships of gramicidin S analogs containing (β-3-pyridyl)-α,β-dehydroalanine residues on membrane permeability. *Med. Chem. Commun.*, **2011**, *2*, 644-649.

(13) Grotenbreg, G. M.; Timmer, M. S., Llamas-Saiz, A. L.; Verdoes, M.; van der Marel, G. A.; van Raaij, M. J.; Overkleeft, H. S.; Overhand, M. An Unusual Reverse Turn Structure Adopted by a Furanoid Sugar Amino Acid Incorporated in Gramicidin S. *J. Am. Chem. Soc.* **2004**, *126*, 3444-3446.

(14) Rivera, D. G.; Vasco, A. V.; Echemendía, R.; Concepción, O.; Pérez, C. S.; Gavín, J. A.; Wessjohann, L. A. A Multicomponent Conjugation Strategy to Unique *N*-Steroidal Peptides: First Evidence of the Steroidal Nucleus as a β-Turn Inducer in Acyclic Peptides. *Chem. - A Eur. J.* **2014**, *20*, 13150–13161.

(15) Puentes, A. R.; Morejón, M. C.; Rivera, D. G.; Wessjohann, L. A. Peptide macrocyclization assisted by traceless turn inducers derived from Ugi peptide ligation with cleavable and resin-linked amines. *Org. Lett.* **2017**, *19*, 4022-4025.

(16) Li, Y.; Bionda, N.; Yongye, A.; Geer, P.; Stawikowski, M.; Cudic, P.; Martinez, K.; Houghten, R. A. Dissociation of Antimicrobial and Hemolytic Activities of Gramicidin S through N - Methylation Modification. *ChemMedChem* **2013**, *8*, 1865–1872.

(17) Alsina, J.; Rabanal, F.; Giralt, E.; Albericio, F. Solid-Phase Synthesis of "Head-to-Tail" Cyclic Peptides via Lysine Side-Chain Anchoring. *Tetrahedron Lett*. **1994**, *35*, 9633–9636.

(18) Andreu, D.; Ruiz, S.; Carren, C.; Alsina, J.; Albericio, F.; Jiménez, M. Á.; de la Figuera, N.; Herranz, R.; García-López, M. T.; González-Muñiz, R. IBTM-Containing Gramicidin S Analogues : Evidence for IBTM as a Suitable Type II' β-Turn Mimetic. *J. Am. Chem. Soc.* **1997**, *119*, 10579–10586.

(19) Wadhwani, P.; Afonin, S.; Ieronimo, M.; Buerck, J.; Ulrich, A. S.; Optimized Protocol for Synthesis of Cyclic Gramicidin S: Starting Amino Acid Is Key to High Yield. *J. Org. Chem.* **2006**, *71*, 55-61.

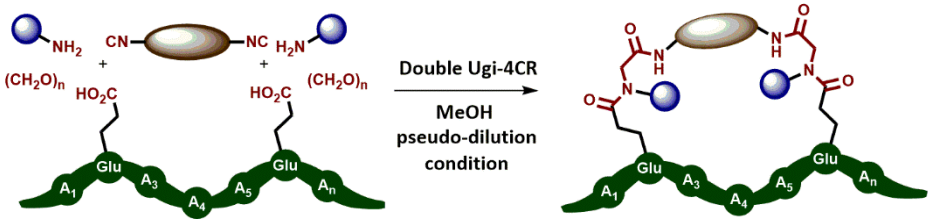
(20) Gibbs, A. C.; Bjorndahl, T. C.; Hodges, R. S.; Wishart, D. S. Probing the Structural Determinants of Type II' β-Turn Formation in Peptides and Proteins. *J. Am. Chem. Soc.* **2002**, *124*, 1203–1213.

Chapter 3

Bidirectional Macrocyclization of Peptides by Double Multicomponent Reaction

This Chapter has been published as:

Ricardo, Manuel G.; Morales, Fidel E.; Garay, Hilda; Reyes, Osvaldo; Vasilev, Dimitar; Wessjohann, Ludger A. and Rivera, Daniel G. *Org. Biomol. Chem.*, **2015**, *13*, 438-446*



Abstract

Increasing the diversity of peptide cyclization methods is an effective way of accessing new types of macrocyclic chemotypes featuring a wide variety of ring sizes and topologies. Multicomponent reactions (MCRs) are processes capable of generating great levels of molecular diversity and complexity at low synthetic cost. In an attempt to further exploit MCRs in the field of cyclopeptides, we describe a bidirectional multicomponent approach for the synthesis of *N*-alkylated macrocyclic peptides of varied sequences and cross-linking positions. The process relies on the execution of two Ugi reactions between peptide diacids and diisocyanides. Varying the amino component enabled the installation of exocyclic elements of diversity, while skeletal diversity was created through different side chain and backbone cyclizations. This procedure shows prospects for the rapid scanning of the chemical space of macrocyclic peptides for applications in chemical biology and drug discovery.

Introduction

The introduction of conformational constraints in peptides by means of cyclization is a common strategy in the design of peptide ligands[1] and the mimetics of protein epitopes.[2] Upon creating conformationally constrained peptide mimics, cyclization is among the most synthetically feasible ways of reducing the intrinsic flexibility of both the peptide backbone and the side chains.[3] This usually leads to an increased ligand binding affinity to the biological target while improving the pharmacological properties compared to the acyclic parent.[4] The backbone preorganization derived from cyclization has also been extensively used to access peptidic templates for the appropriate arrangement of recognition elements[5] and to induce peptide secondary structures (α-helices, β-hairpins etc.) in sequences not likely to have them.[6,7] As a result, cyclized peptides have been designed for a variety of applications ranging from the investigation of protein folding to the development of peptide-based therapeutic agents and materials.

Different synthetic procedures based on peptide coupling protocols[3,8] and chemoenzymatic[9] approaches are available for the head-to-tail cyclization of peptides. Nevertheless, these methods mostly focus on the formation of the natural amide bond, which has limited the biological/pharmacological evaluation of other types of chemical linkages. A different scenario shows up in the side chain-to-side chain cyclization, for which a variety of strategies leading to the incorporation of structurally varied linkages have been reported.[3,7,10] Thus, the formation of amide bonds between the side chains of Lys and Asp/Glu residues,[7,11] the CuI-catalyzed alkyne–azide 1,3-dipolar cycloaddition,[12] the olefin metathesis with Grubbs catalysts[13] and the nucleophilic (aromatic) substitution[10,14] are among the most powerful approaches utilized by chemists to reduce the conformational space of peptides through side chain-to-side chain cyclization. In some cases, the covalent bridges derived from those processes have been introduced as surrogates of the natural disulfide linkage in the pursuit of more stable, but conformationally identical, analogues of bioactive peptide sequences.[7,12,15] These non-natural linkages are also frequently introduced with the aim of achieving by covalent means what nature does through non-covalent

interactions, as it is the case for the stabilization of helical structures by linking the side chains of residues located at the

i, i + 4 and i, i + 7 positions of the peptide sequence.[7,11,16]

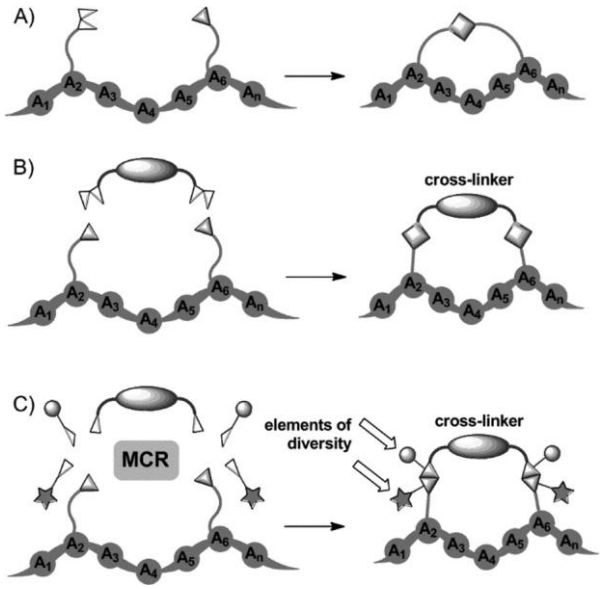


Figure 1. Schematic representation of (A) unidirectional, (B) bidirectional and (C) bidirectional multicomponent macrocyclizations of peptide side chains (C is exemplified with a four-component reaction).

As shown in Fig. 1A, a common characteristic of these conventional methods is their unidirectionality, which derives from the utilization of two complementary functional groups that participate in a single reaction. A different approach is the utilization of a bidirectional macrocyclization strategy, in which a pair of amino acid side chains bearing the same functional groups (i.e., Cys, Lys and Asp/Glu) reacts doubly with a cross-linker bifunctionalized with reactive groups matching in reactivity with those of the peptide. This second strategy, exemplified in Fig. 1B, has been much less exploited than the unidirectional one, albeit it has rendered outstanding contributions to the field of folded peptides.[7,17] In terms of molecular design, the bidirectional strategy is perhaps more versatile, as it allows for the tunable variation of the structure and properties of the cross-linking moiety. Thus, excellent reports have described the utilization of flexible,[18] linear rigid,[19] photoisomerizable[17,20] and near-infrared fluorescent[21] cross-linkers either to stabilize or derivatize folded peptides.

Despite the advances in peptide cyclization methods, a key factor limiting the broader exploration of macrocyclic peptide scaffolds is their low structural diversity. During the construction of cyclic peptide libraries, the diversity elements are usually incorporated either prior to or after the cyclization step. In this sense, the utilization of multicomponent reactions (MCRs) in peptide cyclization may offer a range of opportunities, as they allow for the rapid generation of molecular diversity during the ring closing step.[22] Herein we describe a bidirectional multicomponent macrocyclization approach for the synthesis of structurally diverse N-alkylated macrocyclic peptides. Fig. 1C illustrates the concept of a bidirectional macrocyclization based on two MCRs, which enable tethering a pair of side chains with a bifunctional cross-linker. We implement this idea not only for side chain-to-side chain but also for side chain-to-backbone and backbone-to-backbone macrocyclizations. In this work, the bidirectional macrocyclization relies on two Ugi four-component reactions (Ugi-4CRs) between peptide diacids and aromatic cross-linkers, eventually including exocyclic amino acid appendages as N-substituents, altogether in one pot.

The Ugi-4CR is the condensation of a primary amine, an oxo compound (i.e., ketone or aldehyde), a carboxylic acid and an isocyanide. Despite the fact that two of the reactive components are functional groups present in peptide termini and side chains, this reaction has been rarely utilized for peptide cyclization.[23] The reason for this may be the inherent slow kinetics of the Ugi-4CR at the high dilution typically used for macrocyclization and the frequent mismatch between the intrinsic folding of the peptide and the one required for the Mumm rearrangement to take place. Attempts to expand the substrate scope of the Ugi-4CR to the cyclization of peptoids (i.e., N-alkylated peptides) have demonstrated that even short peptoids can be effectively cyclized with this MCR,[24] possibly owing to the fact that the easier access to s-cis amide bonds facilitates the two termini to come closer. Alternatively, a modification of the original Ugi-4CR using amphoteric aziridine aldehydes has enabled the highly efficient and stereoselective head-to-tail cyclization of peptides of variable sizes.[25]

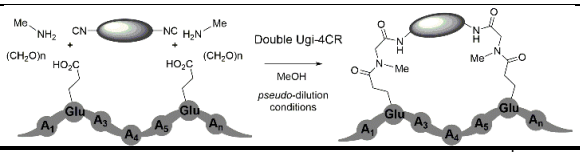
The potential of MCRs for the rapid generation of molecular complexity and diversity has been previously exploited in bidirectional macrocyclizations[22] based on double Ugi-4CRs[26] as well as Passerini,[27] Staudinger[27] and Zhu[28] three component reactions. This

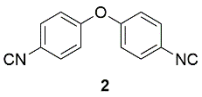
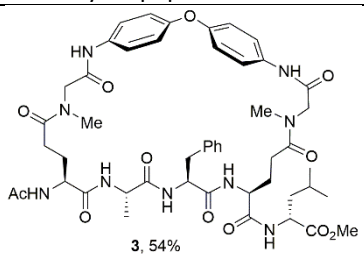
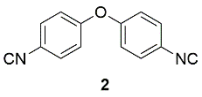
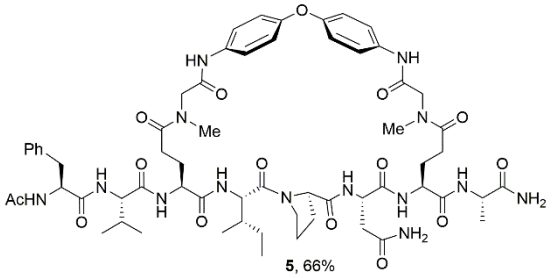
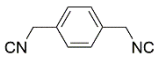
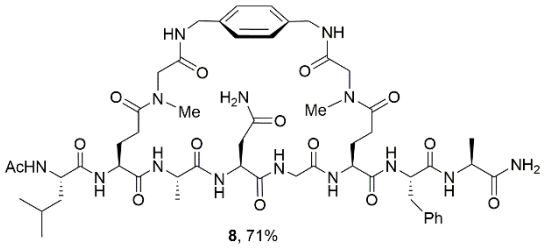
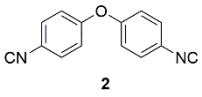
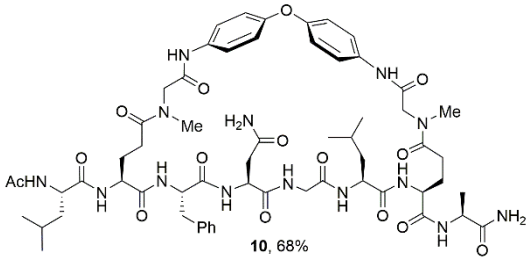
strategy has been utilized for the one-pot synthesis of macrocycles incorporating natural scaffolds like steroids,[26] lipids,[29] carbohydrates[30] and nitrogen heterocycles.[26a,30] However, to our knowledge neither the Ugi-4CR nor related MCRs have been utilized for the macrocyclization of peptide side chains. Based on our experience with a variety of substrates,[26–30] we anticipated that using flexible side chains like those of Glu and Lys would allow for the Ugi-4CR-based macrocyclization to proceed more efficiently than with the terminal amino and carboxylic groups.

Results and discussion

Table 1 illustrates the results of the bidirectional macrocyclization of peptide side chains by means of a double Ugi-4CR procedure, which leads to the formation of two parallel *N*-substituted dipeptide backbones as tethers of the crosslinker. The partial *N*-methylation,[31] and N-alkylation in general,[24] is known to be a successful way of improving the pharmacological properties of peptides. Important characteristics such as metabolic stability, membrane permeability and pharmacokinetics are increased in N-alkylated peptides when compared to conventional ones.[31] Despite the possible incorporation of two different elements of exocyclic diversity using a four component reaction (see Fig. 1C), we initially focused on the combination of methylamine and paraformaldehyde to seek the best experimental conditions. Peptides including two glutamic acids were produced either by standard solution or solid-phase protocols[32] (see ESI†), and then subjected to macrocyclization based on the diacid/diisocyanide combination. Aromatic cross-linkers were chosen as diisocyanide components because of their recognized capability to rigidify peptides upon macrocyclization.[10,19] Among them, we focused on the utilization of the biaryl ether cross-linker, as this moiety frequently occurs in natural macrocyclic peptides like vancomycin, K-13, bouvardin, OF4949-III, and biphenomycin-A.[33]

Table 1. Side Chain-to-side chain bidirectional macrocyclization of peptides by double Ugi-4CR[a]



Peptide diacid	Diisocyanide	Macrocyclic peptide[b]
Ac-Glu-Ala-Phe-Glu-Leu-OMe **1**	 **2**	 **3**, 54%
Ac-Phe-Val-Glu-Ile-Pro-Asn-Glu-Ala-NH_2 **4**	 **2**	 **5**, 66%
Ac-Leu-Glu-Ala-Asn-Gly-Glu-Phe-Ala-NH_2 **6**	 **7**	 **8**, 71%
Ac-Leu-Glu-Phe-Asn-Gly-Leu-Glu-Ala-NH_2 **9**	 **2**	 **10**, 68%

[a] Macrocyclizations carried out for 96 h under pseudo-dilution conditions comprising the slow addition of the two bifunctional components. [b] Yield of the isolated pure product.

Peptides are commonly cyclized under high dilution conditions (0.01–0.1 mM), unless their intrinsic folding favors the ring closure as it is the case for peptides including reverse turns. Thus, a large volume of solvents and long reaction times are required if a sufficient amount of the cyclic peptide is wanted. A solution to this is the implementation of pseudo-dilution conditions in which a solution of the linear peptide is slowly added (usually by means of a syringe pump) to the reaction mixture.

Nevertheless, in bidirectional macrocyclizations the synthetic design is even more elaborate, as the two different building blocks need to be under dilution to avoid formation of complex mixtures of (a)cyclic oligomers. In this sense, a proper understanding of the intrinsic reaction kinetics and the substrate characteristics (e.g., rigidity, folding, etc.) are crucial for the selection of the addition flow rate. For example, whereas cyclizations based on peptide coupling are usually complete within 24 h, those based on the Ugi-4CR require from 72 h to 96 h for ensuring a yield of isolated pure product higher than 50%.[26,30] Nevertheless, it must be noticed that eight new bonds are formed in a bidirectional Ugi-4CR-based macrocyclization, whilst either bidirectional peptide couplings[18] or nucleophilic substitutions[19] render only two new bonds.

Based on these considerations, different macrocyclization conditions – all based on the pseudo-dilution protocol – were tested aiming to reach a compromise between chemical efficiency and reaction time. The best results were obtained by the slow addition with syringe pumps of both the peptide diacid and the diisocyanide to a reaction mixture containing the preformed imine. Typically, two solutions, one of the peptide diacid and another of the diisocyanide (0.15 mmol in 10 mL of MeOH each), are simultaneously added (flow rate 0.2 mL h−1) to a methanolic solution of the imine (0.5 mmol in 50 mL). Under these conditions, the addition was complete within 50 h, albeit the stirring was continued for an additional two days to ensure a conversion of at least 70%, as indicated by analytical HPLC analysis. As an example, Fig. 2 shows the HPLC traces of the multicomponent macrocyclizations of peptides **6** and **9** with diisocyanides **7** and **2**, respectively, after 96 hours of reaction. This HPLC monitoring proved high conversion onto macrocycles **8** and **10**, while only a minor amount of the acyclic peptides **6** and **9** remained unreacted after this time. Thus, the general reaction time was fixed at 96 h to enable comparison of the macrocyclization outcomes varying the different components.

As depicted in Table 1, a variety of macrocyclic peptides featuring different sequences and cross-linking positions were obtained in good yields of isolated pure products. Although the goal of this article is not to study the peptide folding characteristics derived from macrocyclization, the synthetic design focused on locating the Glu

residues at *i*, *i* + 3, *i*, *i* + 4 and *i*, *i* + 5 positions and thus addressing the influence of this variation on the macrocyclization efficiency. Interestingly, macrocycle **3** was obtained in the lowest yield among the four side chain-to-side chain macrocyclizations, while there were no great differences in the macrocyclization outcomes of peptides having the Glu at *i*, *i* + 4 and *i*, *i* + 5 positions (i.e., **4**, **6** and **9**). A reason for this may be that the intrinsic folding of peptide **1** does not favor the proximity of the Ugi-reactive groups during the multicomponent ring closure. This is supported by the fact that the acyclic intermediate of compound **3** (i.e., the one derived from only one Ugi-4CR) was isolated in 13% yield, which suggests a slow final ring closure. In contrast, the intermediates of macrocycles **5**, **8** and **10** were detected only in minor amounts by HPLC and ESI-MS analyses.

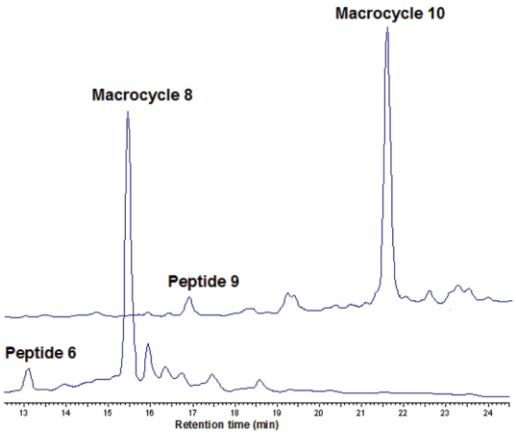


Figure 2. HPLC monitoring of the crude bidirectional macrocyclizations giving rise to macrocycles 8 and 10. Gradient: 5% → 60% of acetonitrile in 0.1% (v/v) TFA in water over 35 min at a flow rate of 0.8 mL min^{-1}.

Besides the variation of the amino acid sequence and the position for the side chain cross-linking, we were interested in exploiting the potential of MCRs for the generation of exocyclic diversity during the bidirectional macrocyclization. We also turned to address the scope of the side chain-to-backbone and backbone-to-backbone cyclizations, once again relying on the efficient diacid/diisocyanide combination of building blocks. As shown in Table 2, a variety of peptide diacids bearing the side chain and terminal carboxylic groups were subjected to bidirectional macrocyclization with biaryl ether diisocyanide **2** and either methylamine or C-protected amino acids as amino components. Peptides were produced through typical solution phase protocols,[32] relying on two different strategies: (i) the incorporation of an Asp residue

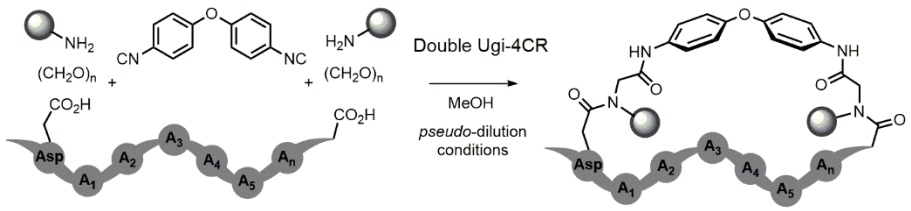
at the *N*-terminus (i.e., **11** and **13**) to enable the macrocyclization using the Asp side chain and the *C*-terminal carboxylic acids and (ii) a bidirectional growing approach based on a double coupling of either an amino acid to *N*-protected Glu (i.e., **15**) or a peptide to *N*-protected Asp (i.e., 17). The latter strategy enabled the assembly of bifunctional peptides bearing two C-terminal carboxylic acids to be used for the bidirectional multicomponent macrocyclization. In all cases, peptides were produced according to the Boc/Bzl solution-phase protocol, then deprotected at both termini and capped by acetylation at the N-terminus (see ESI†).

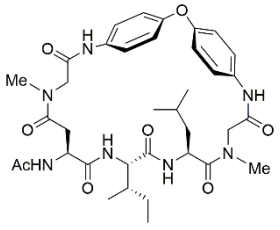
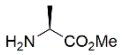
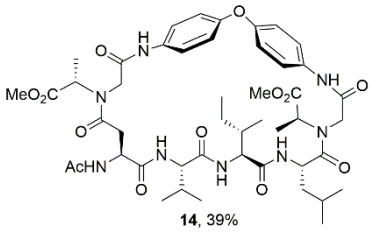
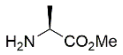
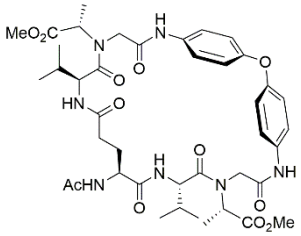
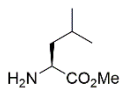
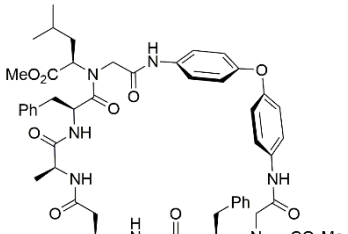
These side chain-to-backbone and backbone-to-backbone macrocyclizations were carried out under the same reaction conditions as those of the side chain-to-side chain ones, and nonetheless their chemical efficiency was lower. The rationale of this might be found in the mechanism of the Ugi-4CRbased ring closure, which proceeds via a macrocyclic intermediate (i.e., the α-adduct) that evolves through an intramolecular acylation (the Mumm rearrangement) to the final macrocycle. Thus, several factors may disfavor the final ring closing step, including: (i) a high conformational constraint, (ii) a poor proximity of the Ugi-reactive groups provoked either by a mismatch in size between the two building blocks or unfavorable peptide folding, and (iii) a high steric hindrance at the migrating group in the macrocyclic α-adduct.

However, all macrocycles in Table 2 were produced in about 40% yield, and in all cases the acyclic intermediates derived from one Ugi-4CR were isolated in 15–20% yield, regardless of the different lengths of the peptide diacids. Accordingly, we believe that it is the low conformational flexibility of the peptide backbones – perhaps combined with an unfavorable folding – that makes the final ring closing step difficult. This may also explain why the side chain-to-side chain multicomponent macrocyclization (Table 1) is considerably more efficient, as the higher conformational freedom of the Glu side chains may enable the ring closure without a costly conformational change of the peptide backbone. To assess whether this is solely a kinetic problem or a deviation of the reaction course to different by-products, we carried out a parallel experiment with the synthesis of macrocycle **14** for 144 h. To our delight, the yield of isolated pure product **14** increased up to 67%, which proves that the macrocyclization efficiency can be improved with longer reaction times. On the other hand, the use of a higher

concentration – derived from a faster flow rate addition of building blocks – is not recommended, as this leads to formation of complex mixtures of larger (a)cyclic oligomers.

Table 2. Side Chain-to-backbone and backbone-to-backbone bidirectional macrocyclization of peptides by double Ugi-4CR[a]



Peptide diacid	Amine	Macrocyclic peptide[b]
Ac-Asp-Ile-Leu-OH **11**	Me−NH_2	 **12**, 40%
Ac-Asp-Val-Ile-Leu-OH **13**		 **14**, 39%
Val-OH Ac-Glu-Val-OH **15**		 **16**, 44%
Ala-Phe-OH Ac-Asp-Ala-Phe-OH **17**		 **18**, 41%

[a] Macrocyclizations carried out for 96 h under pseudo-dilution conditions comprising the slow addition of the two bifunctional components. [b] Yield of the isolated pure product.

Different from any other class of peptide macrocyclization methods, this approach enables the double installation of exocyclic appendages as further elements of diversity during each of the Ugi-4CRs. As noticed in the structures of macrocycles 14, 16 and 18, two new amino acid residues were incorporated into the peptide sequence during the bidirectional macrocyclization. Thus, the approach allows not only for the introduction of conformational constraints but also for the enlargement of the peptide sequence in a one-pot procedure. In cases where the bidirectional macrocyclization is rather challenging, longer reaction times can be utilized to produce crude products of higher purity, thus enabling the rapid construction of macrocyclic peptide combinatorial libraries.

Conclusions

We have described a bidirectional multicomponent approach for the macrocyclization of peptides using the carboxylic groups either of the Glu and Asp side chains or of the C-terminus. The process comprises the execution of two Ugi-4CRs under pseudo-dilution conditions, which enabled the assembly of a variety of N-alkylated macrocyclic peptides featuring dissimilar sequences and cross-linking positions. The implementation of different side chain and backbone cyclizations enabled a rapid scanning of the topological space of hybrid biaryl ether–peptide macrocycles, while providing useful information regarding the substrate scope and the optimized reaction conditions. In general, the side chain-to-side chain macrocyclizations took place with higher efficiency than the side chain-to-backbone and backbone-to-backbone macrocyclizations, whereas it was proven that yields can be improved with longer reaction times. An important difference between this method and other bidirectional approaches is the capability of installing elements of exocyclic diversity during the ring closing step. We believe that this is a promising approach for the combinatorial production and screening of new chemotypes of peptidic macrocycles, as both the generation of structural diversity and macrocyclization are accomplished simultaneously, altogether in a single synthetic operation.

Experimental section

General

Fmoc-protected amino acids, MBHA resin, TBTU, EDC, and HOBt were obtained from Bachem (Switzerland). DIC was obtained from Merck (Germany). Peptide synthesis grade DMF, dichloromethane, DIEA, TFA, and HPLC-grade acetonitrile were from Caledon (Canada). Synthesis of peptides 4, 6 and 9 was carried out manually on MBHA resin by a stepwise solid-phase Fmoc strategy, while peptides **1**, **11**, **13**, **15**, and **17** were synthesized by a stepwise solution-phase Boc/Bzl strategy, as described in the ESI. Diisocyanides **2**[29] and **7**[30] were prepared as described previously. HPLC analysis was performed with an ÄKTA 100 system (GE Healthcare, USA) in a reverse-phase (RP) C18 column (Vydac, 4.6 × 150 mm, 5 µm). A linear gradient from 5% to 60% of solvent B in solvent A over 35 min at a flow rate of 0.8 mL min^{-1} was used. The preparative purification was performed on the LaChrom HPLC system (Merck Hitachi, Germany). Separation was achieved using an RP C18 column (Vydac, 25 × 250 mm, 25 µm). A linear gradient from 15% to 45% of solvent B in solvent A over 50 min at a flow rate of 5 mL min^{-1} was used. Detection was accomplished at 226 nm. Solvent A: 0.1% (v/v) TFA in water. Solvent B: 0.05% (v/v) TFA in acetonitrile. Flash column chromatography was performed on silica gel 60 (Merck, >230 mesh). Peptidic macrocycles were purified to >95% either by column chromatography or by RP-HPLC and characterized by electrospray ionization mass spectrometry (ESI-MS) in a hybrid quadrupole-time-of-flight instrument (QTOF1, Waters, USA) fitted with a nanospray ion source. The high resolution ESI mass spectra were obtained from a Bruker Apex 70e Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer equipped with an Infinity™ cell, a 7.0 Tesla superconducting magnet. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Varian Mercury 400 spectrometer at 399.94 MHz and 100.57 MHz, respectively. Chemical shifts (δ) are reported in ppm relative to the TMS (^{1}H NMR) and to the solvent signal (^{13}C NMR).

General bidirectional macrocyclization by double Ugi-4CRs. A solution of paraformaldehyde (0.5 mmol) and the amine (0.5 mmol) in MeOH (20 mL) was stirred for 2 h at room temperature (when an amino acid methyl ester hydrochloride was used, 0.5 mmol of Et_3N was added to enable the formation of the corresponding

imine). The reaction mixture was diluted up to 50 mL by addition of methanol (30 mL). Two solutions, one of the peptide diacid (0.15 mmol) and another of the diisocyanide (0.15 mmol) in 10 mL of MeOH each, were simultaneously slowly added to the reaction mixture using Syringe pumps (flow rate 0.2 mL h^{-1}). After the addition was complete, the reaction mixture was stirred for an additional 48 h and then concentrated under reduced pressure. A mixture of water–acetonitrile (1 : 1, 10 mL) was added and the suspension was sonicated and centrifuged. After removal of the supernatant, the crude product was washed with diethyl ether (10 mL) and centrifuged twice, then suspended in water–acetonitrile (1 : 1, 5 mL) and lyophilized. The resulting product was further purified either by flash column chromatography (CH_2Cl_2/MeOH) or by preparative RP-HPLC to furnish the pure macrocyclic peptide.

Peptidic macrocycle 3. Peptide **1** (98 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2$·HCl (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2–MeOH 10 : 1) afforded the pure macrocycle **3** (78 mg, 54%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.14 (s, 1H), 9.00 (s, 1H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.41 (d, *J* = 8.7 Hz, 2H), 7.33–7.18 (m, 5H), 6.89 (m, 4H), 6.75–6.69 (m, 2H), 6.52 (d, *J* = 6.2 Hz, 1H), 6.41 (m, 1H), 4.56–4.50 (m, 2H), 4.37 (m, 1H), 4.19 (m, 1H), 4.14 (m, 1H), 3.93 (d, *J* = 13.5 Hz, 1H), 3.91 (d, *J* = 13.6 Hz, 1H), 3.83 (d, *J* = 13.5 Hz, 1H), 3.79 (d, *J* = 13.6 Hz, 1H), 3.67 (s, 3H), 3.25 (dd, *J* = 13.1, 4.5 Hz, 1H), 3.03 (dd, *J* = 13.1, 5.9 Hz, 1H), 2.89 (s, 3H), 2.86 (s, 3H), 2.31–2.24 (m, 4H), 2.00 (m, 2H), 1.97 (s, 3H), 1.92 (m, 2H), 1.63 (m, 1H), 1.60 (m, 1H), 1.53 (m, 1H), 1.29 (d, J = 6.7 Hz, 3H), 0.91–0.58 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.0, 173.4, 172.3, 171.8, 171.7, 170.9, 170.6, 169.7, 167.9 (CO), 156.9, 137.6, 133.5 (C), 129.4, 129.1, 127.3, 121.3, 120.9, 119.5, 119.0, 57.7, 55.8, 55.1, 54.0 (CH), 52.6 (CH_3), 50.1 (CH), 45.8, 44.8, 41.9, 39.6 (CH_2), 38.2, 37.8 (CH_3), 34.1, 33.2, 30.3, 30.1 (CH_2), 26.0 (CH), 23.9, 23.7, 18.6 (CH_3). R_f = 0.45 (CH_2Cl_2–MeOH 15:1). HRMS (ESIFT-ICR) m/z: 992.4497 $[M+Na]^+$; calcd for $C_{49}H_{63}O_{12}N_9Na$: 992.4494.

Peptidic macrocycle 5. Peptide **4** (144 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2$·HCl (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Purification by

preparative RP-HPLC afforded the pure macrocycle **5** (125 mg, 66%) as a white amorphous solid. R*t* = 20.5 min. ^{1}H NMR (600 MHz, DMSO-d6): δ = 10.02 (s, 1H), 9.67 (s, 1H), 8.12 (d, *J* = 7.7 Hz, 1H), 8.00 (d, *J* = 7.0 Hz, 1H), 7.93 (m, 1H), 7.83 (m, 2H), 7.53–7.44 (m, 4H), 7.40 (d, *J* = 6.7 Hz, 1H), 7.19–7.14 (m, 5H), 7.09 (d, *J* = 6.7 Hz, 1H), 6.95 (d, *J* = 7.0 Hz, 1H), 6.87 (m, 4H), 6.80 (d, *J* = 8.7 Hz, 1H), 4.54–4.44 (m, 2H), 4.38 (m, 1H), 4.27–4.19 (m, 2H), 4.05 (m, 1H), 3.91 (m, 1H), 3.58 (m, 1H), 3.06–2.95 (m, 2H), 2.86 (m, 3H), 2.76 (m, 3H), 2.70–2.56 (m, 2H), 2.32 (m, 1H), 2.20 (m, 1H), 1.88 (m, 1H), 1.79 (m, 1H), 1.74 (s, 3H), 1.39 (m, 1H), 1.19 (d, *J* = 7.1 Hz, 3H), 1.10 (t, *J* = 7.3 Hz, 2H), 0.84–0.59 (m, 12H). ^{13}C NMR (150 MHz, DMSO-d6): δ = 174.3, 174.2, 173.9, 172.8, 172.6, 172.4, 171.7, 171.3, 170.8, 170.7, 169.2, 169.1 (CO), 158.3, 158.2, 153.1, 152.9, 138.0, 134.3, 134.2 (C), 129.1, 127.9, 126.1, 121.4, 121.0, 120.9, 119.2, 118.7, 118.5, 118.4, 54.5, 53.8, 52.9, 52.6, 52.3, 51.8, 51.7, 50.4, 49.5, 49.5, 48.4 (CH), 47.1, 45.7 (CH_2), 40.9, 40.6, 40.1 (CH_2), 37.2, 37.1 (CH_3), 36.8 (CH), 34.5, 31.3 (CH_2), 30.6 (CH), 29.7, 29.1, 28.9, 28.7, 27.5, 24.4, 24.1 (CH_2), 22.4 (CH3), 22.1 (CH_2), 19.1, 18.1, 17.4, 14.9, 13.9, 10.7, 10.6, 8.6 (CH_3). ESI-MS m/z: 1287.40 $[M+Na]^+$; calcd for $C_{62}H_{84}O_{15}N_{14}Na$: 1287.38.

Peptidic macrocycle 8. Peptide **6** (134 mg, 0.15 mmol), diisocyanide **7** (24 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2$·HCl (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Purification by reparative RP-HPLC afforded the pure macrocycle **8** (121 mg, 71%) as a white amorphous solid. R_t = 15.5 min. ^{1}H NMR (600 MHz, DMSO-d6): δ = 8.41 (m, 1H), 8.26 (m, 1H), 8.09 (d, *J* = 6.8 Hz, 1H), 8.00 (m, 1H), 7.97–7.91 (m, 2H), 7.90 (d, *J* = 6.8 Hz, 1H), 7.88 (d, *J* = 6.1 Hz, 1H), 7.78 (d, *J* = 7.8 Hz, 1H), 7.34 (d, *J* = 6.4 Hz, 1H), 7.22–7.09 (m, 9H), 7.03 (d, *J* = 5.5 Hz, 1H), 6.92 (s, 1H), 6.85 (m, 1H), 4.49–4.40 (m, 2H), 4.27–4.24 (m, 2H), 4.22–4.17 (m, 3H), 4.16 (m, 1H), 4.11 (m, 1H), 3.93 (dd, *J* = 13.2, 5.3 Hz, 1H), 3.90–3.83 (m, 2H), 3.67 (m, 1H), 3.52 (m, 1H), 3.00 (m, 1H), 2.90 (m, 3H), 2.76 (m, 3H), 2.50 (m, 1H), 2.42 (dd, *J* = 15.5, 6.4 Hz, 1H), 2.32 (m, 1H), 2.23 (m, 2H), 2.14 (m, 1H), 1.84 (m, 1H), 1.79 (s, 3H), 1.76 (m, 1H), 1.69 (m, 1H), 1.55 (m, 1H), 1.37 (m, 2H), 1.17 (m, 6H), 0.82 (m, 3H), 0.78 (m, 3H). ^{13}C NMR (150 MHz, DMSO-d6): δ = 174.0, 172.5, 172.4, 172.4, 172.2, 172.1, 171.8, 171.8, 171.7, 171.4, 171.3, 171.2, 171.1, 170.5, 169.4, 168.8, 168.7, 168.4, 168.2, 168.1 (CO), 158.1, 157.8, 138.0, 137.9, 137.7, 137.4 (C), 129.2, 128.1, 127.3, 127.1, 126.9, 126.3, 53.9, 52.3, 51.8, 51.2, 51.1 (CH), 50.7, 50.6

(CH_2), 49.8, 48.3, 48.2 (CH), 42.5, 42.1, 41.7, 40.7, 37.1 (CH_2), 36.8, 36.4 (CH_3), 34.3, 28.7, 28.4 (CH_2), 27.6 (CH), 24.2, 22.9, 22.5, 21.6, 18.2, 18.1, 17.5 (CH_3). ESI-MS m/z: 1133.53 $[M+H]^+$, 567.25 $[M+2H]^{2+}$; calcd for $C_{53}H_{77}O_{14}N_{14}$: 1133.57.

Peptidic macrocycle 10. Peptide **9** (140 mg, 0.15 mmol), diisocyanide 2 (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2$·HCl (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Purification by preparative RP-HPLC afforded the pure macrocycle **10** (126 mg, 68%) as a white amorphous solid. Rt = 21.6 min. ^{1}H NMR (600 MHz, DMSO-d6): δ = 10.00 (s, 1H), 9.89 (s, 1H), 8.26 (d, J = 6.5 Hz, 1H), 7.99 (m, 1H), 7.91 (dd, J = 10.4, 6.5 Hz, 1H), 7.77 (m, 1H), 7.55 (m, 4H), 7.38 (d, J = 6.8 Hz, 1H), 7.26–7.13 (m, 5H), 6.90 (m, 4H), 4.53 (m, 1H), 4.25 (m, 1H), 4.19–4.14 (m, 2H), 4.09 (m, 1H), 4.01 (m, 1H), 3.78 (m, 1H), 3.03 (m, 3H), 2.84 (m, 3H), 2.41 (m, 1H), 2.32 (m, 1H), 1.88 (m, 1H), 1.81 (s, 1H), 1.57 (m, 2H), 1.46 (m, 1H), 1.37 (m, 2H), 1.19 (m, 3H), 0.90–0.76 (m, 12H). ^{13}C NMR (150 MHz, DMSO-d6): δ = 174.1, 173.2, 172.7, 172.3, 172.0, 171.9, 171.1, 170.7, 169.3, 168.8, 167.3, 166.8 (CO), 157.8, 157.6, 153.2, 152.9, 137.5, 134.3, 134.1 (C), 129.2, 127.9, 126.2, 121.3, 121.0, 119.2, 119.0, 118.8, 118.7, 52.5, 51.9, 51.7, 51.1, 50.8, 50.2, 49.7, 48.0 (CH), 42.1, 40.8, 37.5, 37.3 (CH_2), 36.9, 36.8 (CH_3), 34.45, 28.8, 28.1, 27.5 (CH_2), 24.2 (CH), 23.0, 22.4, 21.5, 18.1 (CH_3). ESI-MS m/z: 1239.61 $[M + H]^+$, 620.28 $[M+2H]^{2+}$; calcd for $C_{60}H_{83}O_{15}N_{14}$: 1239.60.

Peptidic macrocycle 12. Peptide **11** (60 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol) and $MeNH_2$·HCl (34 mg, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2–MeOH 12 : 1) afforded the pure macrocycle **12** (43 mg, 40%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.12 (s, 1H), 8.99 (s, 1H), 7.65 (d, J = 7.4 Hz, 1H), 7.56 (d, J = 6.1 Hz, 1H), 7.48 (d, J = 8.5 Hz, 2H), 7.42 (d, J = 8.5 Hz, 2H), 6.88 (m, 4H), 6.73 (d, J = 6.1 Hz, 1H), 4.57 (m, 1H), 4.54 (m, 1H), 4.27 (m, 1H), 3.90 (d, *J* = 13.5 Hz, 2H), 3.81 (d, J = 13.5 Hz, 1H), 3.76 (d, J = 13.5 Hz, 1H), 2.93 (s, 3H), 2.88 (s, 3H), 2.73 (dd, J = 14.2, 6.3 Hz, 1H), 2.66 (dd, J = 14.2, 3.3 Hz, 1H), 1.99 (s, 3H), 1.86 (m, 1H), 1.59 (m, 1H), 1.53–1.41 (m, 2H), 1.32 (m, 1H), 1.20 (m, 1H), 0.94–0.76 (m, 12H). ^{13}C NMR (40 MHz, $CDCl_3$): δ = 174.4, 173.1, 172.8, 172.1, 170.8, 169.5, 168.9 (CO), 157.5, 133.2 (C), 121.9, 121.6, 120.0, 119.2, 61.2, 56.1, 53.8

(CH), 46.8, 46.5, 41.2, 40.8 (CH_2), 38.2, 37.5 (CH_3), 36.7 (CH_3), 26.4 (CH_2), 25.7 (CH), 24.2, 22.7, 16.1, 12.6 (CH_3). R_f = 0.48 (CH_2Cl_2–MeOH 15 : 1). HRMS (ESI-FT-ICR) m/z: 730.3543 $[M+Na]^+$; calcd for $C_{36}H_{49}O_8N_7Na$: 730.3540.

Peptidic macrocycle 14. Peptide **13** (75 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol), HCl·Ala-OMe (70 mg, 0.5 mmol) and Et_3N (70 µL, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2–MeOH 12 : 1) afforded the pure macrocycle **14** (56 mg, 39%) as a white amorphous solid. In a parallel experiment comprising 144 h of reaction, macrocycle **14** was obtained in 67% yield (96 mg). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.19 (s, 1H), 9.11 (s, 1H), 7.72 (d, *J* = 6.1 Hz, 1H), 7.53 (d, *J* = 8.6 Hz, 2H), 7.48 (d, *J* = 8.5 Hz, 2H), 7.45–7.31 (m, 2H), 6.91 (m, 4H), 6.50 (d, *J* = 5.3 Hz, 1H), 4.63 (m, 1H), 4.54 (m, 1H), 4.44 (q, *J* = 5.3 Hz, 1H), 4.37–4.29 (m, 2H), 4.18 (m, 1H), 3.94 (d, *J* = 14.0 Hz, 1H), 3.89 (d, *J* = 13.6 Hz, 1H), 3.82 (d, *J* = 14.0 Hz, 1H), 3.78 (d, J = 13.6 Hz, 1H), 3.69 (s, 3H), 3.67 (s, 3H), 2.72 (dd, *J* = 13.4, 6.8 Hz, 1H), 2.64 (dd, *J* = 13.4, 6.1 Hz, 1H), 2.03 (m, 1H), 1.98 (s, 3H), 1.82 (m, 1H), 1.59 (m, 1H), 1.48–1.40 (m, 3H), 1.39 (d, *J* = 7.0 Hz, 3H), 1.37 (d, *J* = 7.0 Hz, 3H), 1.28 (m, 1H), 1.15 (m, 1H), 0.98–0.89 (m, 9H), 0.82–0.73 (m, 9H). 13C NMR (40 MHz, $CDCl_3$): δ = 174.0, 172.7, 172.5, 172.1, 171.6, 170.7, 170.5, 170.1, 169.4, 168.8 (CO), 156.6, 133.4 (C), 121.3, 120.9, 119.4, 119.2, 59.8, 57.1, 54.7, 54.4 (CH), 52.6, 52.4 (CH_3), 51.3, 49.8 (CH), 44.8, 44.4, 41.6, 40.9 (CH_2), 37.7, 31.5 (CH), 27.1 (CH_2), 25.8 (CH), 24.1, 22.6, 19.9, 17.1, 16.8, 15.7, 11.7 (CH_3). R_f = 0.50 (CH_2Cl_2–MeOH 15 : 1). HRMS (ESI-FT-ICR) m/z: 973.4645 $[M+Na]^+$; calcd for $C_{47}H_{66}O_{13}N_8Na$: 973.4647.

Peptidic macrocycle 16. Peptide **15** (58 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol), HCl·Ala-OMe (70 mg, 0.5 mmol) and Et_3N (70 µL, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2–MeOH 15 : 1) afforded the pure macrocycle **16** (55 mg, 44%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.35 (s, 1H), 9.13 (s, 1H), 7.55 (d, *J* = 8.6 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.43 (d, *J* = 8.6 Hz, 2H), 6.85 (m, 4H), 6.70 (d, *J* = 7.1 Hz, 1H), 6.49 (d, *J* = 6.5 Hz, 1H), 4.37 (q, *J* = 6.0 Hz, 2H), 4.30–4.24 (m, 2H), 4.13 (m, 1H), 4.01 (m, 1H), 3.98 (d, *J* = 14.0 Hz, 1H), 3.96 (d, *J* = 13.5 Hz, 1H), 3.83 (d, *J* = 13.5 Hz, 1H), 3.80 (d, *J* = 14.0 Hz, 1H),

3.69 (s, 6H), 2.33 (m, 2H), 2.12 (m, 2H), 1.97 (s, 3H), 1.94 (m, 2H), 1.48 (d, *J* = 6.9 Hz, 3H), 1.40 (d, *J* = 6.8 Hz, 3H), 0.96–0.88 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 175.1, 173.1, 172.4, 171.3, 171.1, 169.1, 168.1 (CO), 156.7, 133.8 (C), 121.3, 120.7, 119.3, 119.2, 60.1, 58.6, 54.6, 52.6 (CH), 52.5 (CH_3), 51.1 (CH), 45.8, 42.9, 34.2 (CH_2), 31.6, 31.2 (CH), 30.6 (CH_2), 23.4, 19.7, 19.2, 17.3, 16.9 (CH3). R_f = 0.51 (CH_2Cl_2–MeOH 15 : 1). HRMS (ESI-FTICR) m/z: 860.3809 [M+Na]+; calcd for $C_{41}H_{55}O_{12}N_7Na$: 860.3806.

Peptidic macrocycle 18. Peptide **17** (92 mg, 0.15 mmol), diisocyanide **2** (33 mg, 0.15 mmol), paraformaldehyde (15 mg, 0.5 mmol), HCl·Leu-OMe (91 mg, 0.5 mmol) and Et_3N (70 µL, 0.5 mmol) were reacted according to the general macrocyclization procedure. Flash column chromatography purification on silica (CH_2Cl_2–MeOH 10 : 1) afforded the pure macrocycle **18** (71 mg, 41%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 9.23 (s, 1H), 9.16 (s, 1H), 7.95 (d, *J* = 5.8 Hz, 1H), 7.69 (d, J = 6.3 Hz, 1H), 7.49 (d, *J* = 8.6 Hz, 2H), 7.41 (d, *J* = 8.4 Hz, 2H), 7.34 (d, *J* = 7.10 Hz, 1H), 7.22–7.15 (m, 10H), 6.95 (m, 4H), 6.87 (d, *J* = 7.8 Hz, 1H), 6.42 (d, *J* = 6.5 Hz, 1H), 4.58 (m, 1H), 4.50 (m, 2H), 4.43 (m, 1H), 4.39 (m, 1H), 4.29–4.23 (m, 2H), 3.97 (d, *J* = 13.2 Hz, 1H), 3.95 (d, *J* = 13.5 Hz, 1H), 3.84 (d, *J* = 13.2 Hz, 1H), 3.80 (d, *J* = 13.5 Hz, 1H), 3.70 (s, 3H), 3.68 (s, 3H), 3.11 (m, 2H), 2.82 (m, 2H), 2.63 (m, 2H), 2.00 (s, 3H), 1.65 (m, 2H), 1.60–1.51 (m, 2H), 1.33 (d, J = 7.1 Hz, 3H), 1.30 (d, *J* = 6.4 Hz, 3H), 0.95–0.89 (m, 12H). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.0, 173.3, 173.2, 172.8, 170.6, 170.4, 168.7 (CO), 157.3, 136.4, 133.9 (C), 129.5, 128.6, 127.1, 121.9, 120.9, 120.2, 119.2, 58.1, 56.0, 55.4, 54.8, 53.7 (CH), 52.5 (CH_3), 51.2, 49.6 (CH), 45.7, 45.2, 42.9, 40.5, 39.6, 39.5 (CH_2), 24.8 (CH), 22.7, 22.2, 18.0, 17.2 (CH_3). R_f = 0.47 (CH_2Cl_2–MeOH 15 : 1). HRMS (ESI-FT-ICR) m/z: 1168.5335 $[M+Na]^+$; calcd for $C_{60}H_{75}O_{14}N_9Na$: 1168.5331.

Acknowledgements

We gratefully acknowledge financial support from the Land Sachsen-Anhalt, Germany (WZW project lipopeptides).

Supporting Information

Experimental procedures and spectroscopic data of peptide diacids. ESI-MS and chromatograms of the final peptidic macrocycles, see https://doi.org/10.1039/c4ob01915f

References

1 V. J. Hruby, *Nat. Rev. Drug Discovery*, 2002, **1**, 847–858.

2 (a) T. A. Hill, N. E. Shepherd, F. Diness and D. P. Fairlie, *Angew. Chem. Int. Ed.*, 2014, **53**, DOI: 10.1002/anie.201401058 (b) J. A. Robinson, *J. Pept. Sci.*, 2013, **19**, 127–140.

3 (a) H. Kessler, *Angew. Chem., Int. Ed. Engl.*, 1982, **31**, 512–521; (b) V. J. Hruby, *Life Sciences*, 1982, **31**, 189–199; (c) S. Jiang, Z. Li, K. Ding and P. Roller, *Curr. Org. Chem.*, 2008, **12**, 1502–1542; (d) D. J. Craik, D. P. Fairlie, S. Liras and D. Price, *Chem. Biol. Drug Des.*, 2013, **81**, 136–147.

4 (a) E. M. Driggers, S. P. Hale, J. Lee and N. K. Terrett, *Nat. Rev. Drug Discovery*, 2008, **7**, 608–624; (b) F. Giordanetto and J. Kihlberg, *J. Med. Chem.*, 2014, **57**, 278–295.

5 (a) Y. Singh, G. T. Dolphin, J. Razkin and P. Dumy, *Chem-BioChem*, 2006, **7**, 1298–1314; (b) S. E. Gibson and C. Lecci, *Angew. Chem., Int. Ed.*, 2006, **45**, 1364–1377.

6 J. A. Robinson, *Acc. Chem. Res.*, 2008, **41**, 1278–1288.

7 J. Garner and M. M. Harding, *Org. Biomol. Chem.*, 2007, **5**, 3577–3585.

8 (a) J. M. Humphrey and A. C. Chamberlin, *Chem. Rev.*, 1997, **97**, 2243–2266; (b) J. N. Lambert, J. P. Mitchell and K. D. Roberts, *J. Chem. Soc.*, Perkin Trans. 1, 2001, 471–484; (c) C. J. White and A. K. Yudin, *Nat. Chem.*, 2011, **3**, 509–524.

9 (a) R. M. Kohli and C. T. Walsh, *Chem. Commun.*, 2003, 297–307; (b) S. A. Sieber and M. A. Marahiel, *Chem. Rev.*, 2005, **105**, 715–738.

10 (a) W. A. Loughlin, J. D. A. Tyndall, M. P. Glenn and D. P. Fairlie, *Chem. Rev.*, 2004, **104**, 6085–6117; (b) E. Marsault and M. L. Peterson, *J. Med. Chem.*, 2011, **54**, 1961–2004.

11 J. W. Taylor, *Biopolymers*, 2002, **66**, 49–75.

12 (a) S. Cantel, A. L. C. Isaad, M. Scrima, J. J. Levy, R. D. DiMarchi, P. Rovero, J. A. Halperin, A. M. D'Ursi, A. M. Papini and M. Chorev, *J. Org. Chem.*, 2008, **73**, 5663–5674; (b) K. Holland-Nell and M. Meldal, *Angew. Chem., Int. Ed.*, 2011, **50**, 5204–5206.

13 (a) H. E. Blackwell and R. H. Grubbs, *Angew. Chem., Int. Ed*., 1998, **37**, 3281–3283; (b) C. E. Schafmeister, J. Po and G. L. Verdine, *J. Am. Chem. Soc.*, 2000, **122**, 5891–5892; (c) H. E. Blackwell, J. D. Sadowsky, R. J. Howard, J. N. Sampson, J. A. Chao, W. E. Steinmetz, D. J. O'Leary and R. H. Grubbs, *J. Org. Chem.*, 2001, **66**, 5291–5302; (d) L. D. Walensky, A. L. Kung, I. Escher, T. J. Malia, S. Barbuto, R. D. Wright, G. Wagner, G. L. Verdine and S. J. Korsmeyer, *Science*, 2004, **305**, 1466–1470.

14 (a) R. C. Reid, M. J. Kelso, M. J. Scanlon and D. P. Fairlie, *J. Am. Chem. Soc.*, 2002, **124**, 5673–5683; (b) P. Cristau, T. Temal-Laïb, M. Bois-Choussy, M.-T. Martin, J.-P. Vors and J. Zhu, *Chem. – Eur. J.*, 2005, **11**, 2668–2679.

15 J. L. Stymiest, B. F. Mitchell, S. Wong and J. C. Vederas, *Org. Lett.*, 2003, **5**, 47–49.

16 N. E. Shepherd, H. N. Hoang, G. Abbenante and D. P. Fairlie, *J. Am. Chem. Soc.*, 2009, **131**, 15877–15886.

17 (a) G. A. Woolley, *Acc. Chem. Res.*, 2005, **38**, 486–493; (b) C. Renner and L. Moroder, *ChemBioChem*, 2006, 7, 868–878.

18 J. C. Phelan, N. J. Skelton, A. C. Braisted and R. S. McDowell, *J. Am. Chem. Soc.*, 1997, **119**, 455–460.

19 (a) K. Fujimoto, N. Oimoto, K. Katsuno and M. Inouye, *Chem. Commun.*, 2004, 1280–1281; (b) F. Zhang, O. Sadovski, S. J. Xin and G. A. Woolley, *J. Am. Chem. Soc.*, 2007, **129**, 14154–14155.

20 (a) L. Chi, O. Sadovski and G. A. Woolley, *Bioconjugate Chem.*, 2006, **17**, 670–676; (b) J. A. Ihalainen, J. Bredenbeck, R. Pfister, J. Helbing, L. Chi, I. H. M. van Stokkum, G. A. Woolley and P. Hamm, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 5383–5388.

21 Y. Ye, W. P. Li, C. J. Anderson, J. Kao, G. V. Nikiforovich and S. Achilefu, *J. Am. Chem. Soc.*, 2003, **125**, 7766–7767.

22 For reviews, see: (a) L. A. Wessjohann, D. G. Rivera and O. E. Vercillo, Chem. Rev., 2009, **109**, 796–814; (b) G. Masson, L. Neuville, C. Bughin, A. Fayol and J. Zhu, Top. Heterocycl. Chem., 2010, **25**, 1–24.

23 (a) A. Failli, H. Immer and M. D. Götz, *Can. J. Chem.*, 1979, **57**, 3257–3261; (b) S. Cho, G. Keum, S. B. Kang, S.-Y. Han and Y. Kim, *Mol. Diversity*, 2003, **6**, 283–286.

24 (a) O. E. Vercillo, C. K. Z. Andrade and L. A. Wessjohann, *Org. Lett.*, 2008, **10**, 205–208; (b) A. F. S. Barreto,O. E. Vercillo, M. A. Birkett, J. C. Caulfield,L. A. Wessjohann and C. K. Z. Andrade, *Org. Biomol. Chem.*, 2011, **9**, 5024–5027.

25 (a) R. Hili, V. Rai and A. K. Yudin, J. Am. Chem. Soc., 2010, 132, 2889–2891; (b) C. C. G. Scully, V. Rai, G. Poda, S. Zaretsky, D. C. Burns, R. S. Houliston, T. Lou and A. K. Yudin, *Chem. – Eur. J.*, 2013, **19**, 17668–17672; (c) C. J. White, J. L. Hickey, C. C. G. Scully and A. K. Yudin, *J. Am. Chem. Soc.*, 2014, **136**, 3728–3731.

26 (a) L. A. Wessjohann, F. Voigt and D. G. Rivera, *Angew. Chem., Int. Ed.*, 2005, **44**, 4785–4790; (b) L. A. Wessjohann, D. G. Rivera and F. Coll, *J. Org. Chem.*, 2006, **71**, 7521–7526; (c) D. G. Rivera and L. A. Wessjohann, *J. Am. Chem. Soc*., 2009, **131**, 3721–3722.

27 F. León, D. G. Rivera and L. A. Wessjohann, *J. Org. Chem.*, 2008, **73**, 1762–1767.

28 P. Janvier, M. Bois-Choussy, H. Bienaymé and J. Zhu, *Angew. Chem., Int. Ed.*, 2003, **42**, 811–814.

29 D. Michalik, A. Schaks and L. A. Wessjohann, *Eur. J. Org. Chem.*, 2007, 149–157.

30 D. G. Rivera, O. E. Vercillo and L. A. Wessjohann, *Org. Biomol. Chem.*, 2008, **6**, 1787–1795.

31 (a) L. A. Wessjohann, C. K. Z. Andrade, O. E. Vercillo and D. G. Rivera, *Targets Heterocycl. Syst.*, 2006, **10**, 24–53; (b) J. Chatterjee, G. Chaim, A. Hoffman and H. Kessler, *Acc. Chem. Res.*, 2008, **41**, 1331–1342.

32 N. Sewald and H.-D. Jakubke, Peptides: Chemistry and Biology, Wiley-VCH, Weinheim, 2002.

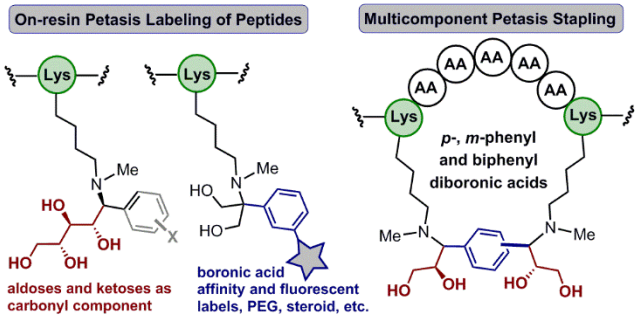
33 (a) Y. Z. Shu, *J. Nat. Prod.*, 1998, **61**, 1053–1071; (b) U. Nubbemeyer, *Top. Curr. Chem.*, 2001, **216**, 125–196; (c) L. Feliu and M. Planas, *Int. J. Pept. Res. Ther.*, 2005, **11**, 53–97; (d) L. A. Wessjohann, E. Ruijter, D. Garcia-Rivera and W. Brandt, *Mol. Diversity*, 2005, **9**, 171–186.

Chapter 4

Introducing the Petasis Reaction for the Late-Stage Multicomponent Diversification, Labeling and Stapling of Peptides

This Chapter has been published as:

Ricardo, Manuel G.; Llanes, Dayma; Wessjohann, Ludger A. and Rivera, Daniel G. *Angew. Chem Int. Ed.*, **2019**, *58*, 2700-2704*



Abstract

For the first time, the Petasis (borono-Mannich) reaction is employed for the multicomponent labeling and stapling of peptides. The report includes the solid-phase derivatization of peptides at the *N*-terminus, Lys and N^{ε}-MeLys side chains by on-resin Petasis reaction with variation of the carbonyl and boronic acid components. Peptides were simultaneously functionalized with aryl/vinyl substituents bearing fluorescent/affinity tags and oxo components such as dihydroxyacetone, glyceraldehyde, glyoxylic acid and aldoses, thus encompassing a powerful complexi-ty-generating approach without changing the peptides charge. The multicomponent stapling was conducted in solution by linking N^{ε}-MeLys or Orn side chains – positioned at i, i+7 and i, i+4 – with aryl tethers, while hydroxyl-carbonyl moieties were introduced as exocyclic fragments. The good efficiency and diversity oriented character of these methods show prospects for peptide drug discovery and chemical biology.

Introduction

Lately multicomponent reactions (MCRs) have evolved from versatile methods for the synthesis of small molecules[1] to powerful tools for the post-synthetic derivatization of peptides[2] and proteins.[3] Among the relevant multicomponent transformations recently conducted with peptides are cyclization,[4,5] lipidation[5] and labeling.[5b,6] Thus far, there are several isocyanide-MCRs available for the multicomponent cyclization of peptide,[4-6] while the Strecker reaction[7] and the metal-catalyzed A^3-coupling[8] have been recently added to this repertoire. On the other hand, the multicomponent labeling of resin-linked peptides[5c] and the side chain cyclization with linkers[9] (i.e., a type of peptide stapling) has been conducted only using the Ugi four-component reaction.

Both the on-resin derivatization of peptides (e.g., labeling, glycosylation, lipidation, etc.) and the peptide stapling are processes of remarkable biological impact. The possibility of implementing those in a multicomponent manner provides a key asset to peptide chemists. For example, multiple reactive handles, affinity or fluorescent tags, and even a combination of the latter with additional biomolecular fragments can be incorporated at once in a multicomponent late-stage transformation. Similarly, the *endo* or *exo*-cyclic diversification of the side-chain cross-linkage can be readily accomplished in a multicomponent stapling approach. Herein we introduce the Petasis reaction as a powerful method for the multicomponent labeling and stapling of peptides, without changing the overall charge of the peptide.

The Petasis reaction,[10] also known as the borono-Mannich reaction, is a three-component process comprising the condensation of an aldehyde or ketone, an amine and an aryl/vinyl boronic acid or ester. Remarkable applications of this reaction can be found in the synthesis of non-proteinogenic amino acids and natural products.[11] In solid-phase synthesis (SPS), the Petasis reaction has been only employed to modified resin-bound amino acids,[12] but to our knowledge, the method has never been translated to the on-resin derivatization of peptides or their macrocyclization.

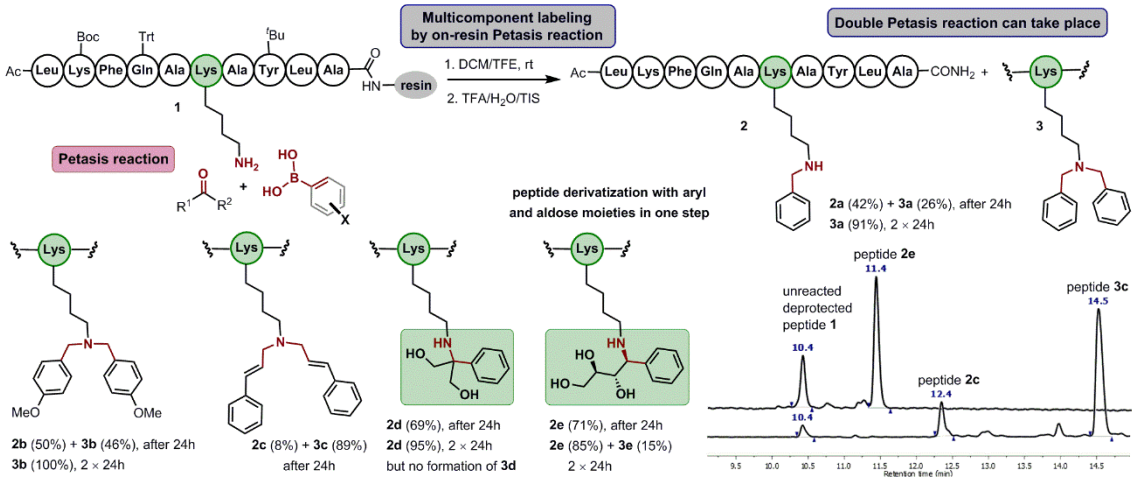
Our multicomponent peptide labeling strategy aimed at conducting the Petasis reaction on resin, as this shows promise for the combinatorial parallel production and screening of MCR-modified peptides. For this purpose, a SPS methodology based on

three degrees of orthogonality was implemented for the preparation of model peptide **1** (see the Supporting Information). Scheme 1 depicts the initial Petasis labeling study focused on assessing the scope of the on-resin method with a peptide having a free Lys ε-NH_2. It must be noticed that this reaction is traditionally conducted with carbonyl compounds possessing coordinating groups (e.g., hydroxyl aldehydes and ketones, glyoxylic acid, etc.) capable of forming the boronate complex upon the imine formation. In addition, we also sought to use formaldehyde as the simplest carbonyl component. Eventually, this may offer the possibility of double arylation of the Lys side-chain as a result of the carbonylation of the resulting secondary amine to an equally reactive iminium intermediate.[11]

Since the on-resin imine formation is rather difficult with paraformaldehyde, we relied on an aminocatalysis-mediated transimination protocol[5c] with the pyrrolidinium ion derived from a preceding reaction of paraformaldehyde and pyrrolidine. Thus, the on-resin Petasis approach with paraformaldehyde consisted of imine formation at the Lys ε-NH_2 by transimination for 30 minutes in tetrahydrofuran (THF)/MeOH 1:1 (*v*/*v*), followed by resin washing to remove the excess of pyrrolidinium ion and subsequent reaction with the boronic acid. Analytical HPLC and ESI-MS analyses were conducted after mini-cleavage at 24 hours (one reaction cycle). If desired, a second Petasis cycle can be conducted with a second iminium formation and reaction with boronic acid for another 24h. However, this second reaction can already occur in the first round, if the components are prone to overreaction. After screening of reaction conditions, the mixture dichloromethane (DCM)/trifluoroethanol (TFE) 1:1 (*v*/*v*) proved the best solvent for the on-resin Petasis reaction.

As shown in scheme 1, the outcome of the on-resin MCR of peptide **1** with paraformaldehyde and boronic acid very much depends on the reactivity of the latter one. Thus, phenyl boronic acid led to 52% of conversion of **1** into benzylated product **2a** and 26% conversion into the double Petasis product **3a** after one Petasis cycle. Alternatively, the more reactive *p*-methoxy-phenyl boronic acid rendered a about 1:1 mixture of the single Petasis-modified product **2b** and the double one **3b**. A second cycle of imine formation and reaction with these boronic acids for an additional 24 hours led the quantitative conversion of **1** into **3a** and **3b**. Moreover, the Petasis

reaction with the very reactive (*E*)-styrylboronic acid provided almost full conversion to the bis-styrene modified peptide **3c** even after the first Petasis cycle, though a second cycle may be used to finish the task.



Scheme 1. Scope of the multicomponent late-stage derivatization of peptides at the Lys side chain by on-resin Petasis reaction. Percent conversion determined by HPLC after one (24 h) and two Petasis cycles (2 × 24 h). When paraformaldehyde is used as carbonyl component, imine formation is initially carried out in THF/MeOH by transimination with the formaldehyde-derived pyrrolidinium ion. TFA = trifluoroacetic acid, TFE = trifluroethanol, TIS = triisopropylsilane.

A key goal of our work was to prove the diversity-oriented and complexity-generating characters of the on-resin Petasis modification method. For this goal, the carbonyl component, aiming at the simultaneous incorporation of aryl/vinyl and aldose/ketose (sugar-like) moieties at the Lys side chain, something that has never been achieved by any other labeling protocol. Thus, D-erythrose and dihydroxyacetone were chosen as simple, representative components of this class, and the on-resin Petasis derivatization was undertaken with peptide **1** and phenyl boronic acid. As the risk of double Petasis reaction was also latent with these oxo components, once more we relied on the preformation of the imine, followed by resin washing and addition of the boronic acid rather than using the carbonyl compounds in excess.

Accordingly, the protocol consisted in treating resin-bound **1** for 2 hours with either the hydroxyl aldehyde or ketone in THF/MeOH 1:1 (*v*/*v*) to enable the imine formation, followed by washing to remove excess of this component, addition of the boronic acid and shaking for 24 hours in DCM/TFE 1:1 (*v*/*v*). As shown in scheme 1, this procedure

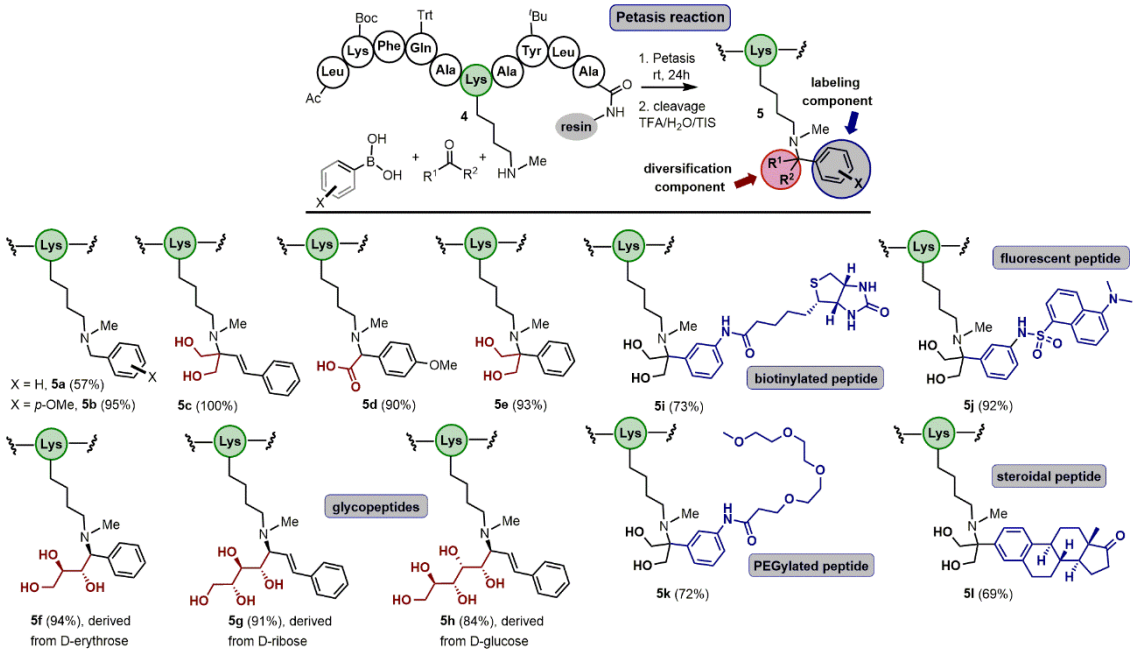
led to good conversion into the peptides **2d** and **2e** after 24h, yet about 10-20% of **1** remained unreacted (see HPLC traces in the Supporting Information). A second Petasis cycle of 24 hours improved the conversion to **2d** to 98%, remarkably, with no formation of the double Petasis product. In the case of D-erythrose, the second Petasis cycle furnished about 15% of the double Petasis product **3e**. The lack of overreaction of dihydroxyacetone with secondary amine **2d** seems to be due to the sterically crowed character of this product, which limits the formation of the subsequent iminium. An analytical sample of the main products was purified by HPLC for NMR and ESI-MS characterization.

Today, it is well-known that the utilization of an enantiomerically pure α-hydroxy-aldehyde comprises the exclusive formation of the anti-1,2-amino alcohol isomer,[10c] because of the complete diastereoselection of the Petasis reaction with these substrates. Based on the configuration of D-erythrose, we propose the *S* configuration for the newly formed stereocenter of peptide **2e**, which was obtained as a single diastereomer (see the Supporting Information).

Because of the permanent risk of double Petasis with the aldehyde/primary amine combination, we sought to use a peptide bearing a secondary amine as a side-chain, for example, N^{ε}-MeLys. Thus, methylation of the Lys ε-amino group was achieved with the Fukuyama-Mitsunobu procedure.[13] In short, **1** reacted with 2-nitrobenzenesulfonyl (*o*-NBS) chloride to form the *o*-NBS sulfonamide, followed by treatment with MeOH under Mitsunobu conditions (DIAD, PPh_3) to render the *N*-methylsulfonamide in only 2 hours. Final *o*-NBS removal led to the resin-bound peptide **4** bearing the N^{ε}-MeLys. Scheme 2 illustrates the success of the Petasis derivatization of the N^{ε}-MeLys in peptide **4**. The on-resin protocol comprised the reaction of **4** with four equivalents of the oxo-component and the boronic acid in DCM/TFE 1:1 (*v*/*v*) for 24h, without any risk of double Petasis reaction. For the use of paraformaldehyde as the carbonyl component, the previous transimination step was required as the direct imine formation again was not viable. Results shown in scheme 2 provide a clear picture of the influence of the carbonyl and boronic acid components on the efficiency of the on-resin Petasis labeling. For example, when paraformaldehyde is used, the reactivity of the boronic acid is crucial for the reaction conversion. Thus, phenyl boronic acid led to 57%

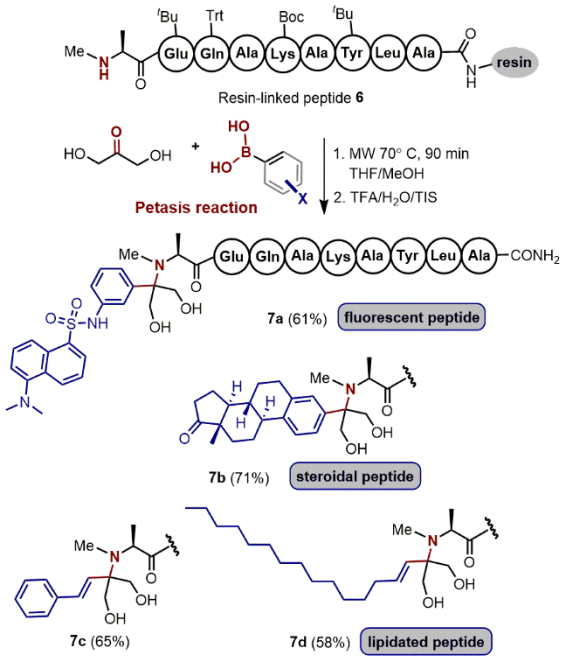
conversion of **4** into **5a** after 24 hours, while the more reactive *p*-methoxy-phenyl boronate rendered 95% conversion into **5b**. However, when D-erythrose and dihydroxyacetone were employed along with phenyl boronic acid, the conversion to **5e** and **5f** was improved to 94 and 93%, respectively. Alternatively, the combination of dihydroxyacetone with (*E*)-styrylboronic acid led to **5c** with 100% conversion, while 90% conversion into **5d** was achieved with *p*-methoxy-phenyl boronic acid and glyoxylic acid. The peptide **4** could be also labeled with aldopentoses and hexoses with similar success, as exemplified in the synthesis of glycopeptides **5g** and **5h** by Petasis with (*E*)-styrylboronic acid and D-ribose and D-glucose, respectively.

Similar to the carbonyl component, boronic acids allows high levels of diversification because of their commercial availability or easy preparation. Hence, our endeavor was to test the on-resin Petasis reaction for the possibility of installing biologically relevant molecules and tags at the peptide side chain. Thus, peptide **4** was efficiently labeled with the affinity tag biotin and with the fluorescent dansyl group attached to 3-aminophenyl boronic acid to render the peptides **5i** and **5j**, respectively (Scheme 2). Similarly, the PEGylation of **4** at the N^{ε}-MeLys was effectively conducted with a polyethylene glycol (PEG)-derived phenyl boronic acid to furnish **5k**. Also an estrone-derived boronic acid was employed for the multicomponent synthesis of the steroidal peptide **5l**.



Scheme 2. On-resin Petasis diversification and labeling of N^{ε}-MeLys-Containing Peptides. Percent conversion determined by HPLC after cleavage at 24 hours.

Aiming at providing a novel approach for the late-stage modification of peptides at the *N* terminus, we sought to translate the Petasis labeling procedure from the side-chain to the backbone. Thus, the peptide **6** was produced on solid-phase with the final incorporation of *N*-methyl Ala at the *N*-terminus. As depicted in Scheme 3, in contrast to the Petasis modification at the Lys side chain, this type of multicomponent modification proved inefficient at room temperature. As a result, we turned to carry out the multicomponent reaction under microwave (MW) irradiation, which provided good conversion of **6** after 90 minutes at 70 °C. Again, we focused on proving the versatility of this method for the incorporation of affinity or fluorescent tags, along with the use of dihydroxyacetone as carbonyl component. This MW-assisted on-resin protocol enabled tagging the *N*-terminal residue with the chemically relevant dansyl group (**7a**), with the bulky steroidal skeleton of estrone (**7b**), with the styryl moiety (**7c**) and with a lipidic fragment (**7d**). Indeed, the lower reactivity of the *N*-terminal amino group compared to that of the side-chain is inherent of peptides and is not a specific case of the Petasis multicomponent reaction. Nevertheless, the use MW to enhance the yield of some challenging *N*-terminal modification methods is widely spread nowadays, as it does not require a very complicated synthetic setup.

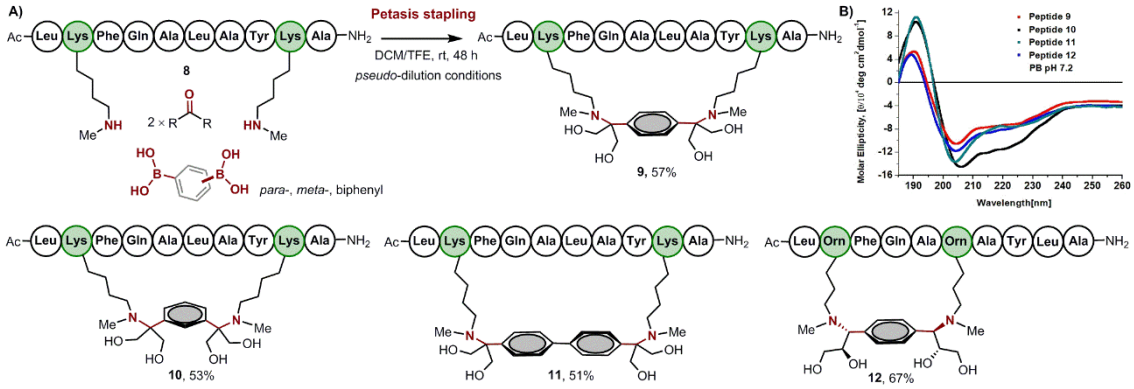


Scheme 3. On-resin Petasis modification and labeling of peptides at the N-terminus. Percent of conversion determined by HPLC. MW: microwave.

Owing to its multicomponent nature, this novel type of late-stage peptide modification protocol offers a variety of advantages over more classic approaches also targeting the amino group of the Lys side chains and the *N*-terminus. Indeed, the most obvious ones is the easy installation of either two equal or dissimilar functionalities in only one step. Such a one-pot double functionalization may be useful, for example, for the creation of multivalency in peptides, or for the simultaneous labeling and ligation to another biomolecule.

As the combination of N^{ε}-MeLys-containing peptides with hydroxyl aldehydes or ketones provided the best results, we sought to employ this variant for the development of a novel multicomponent peptide stapling protocol. The peptide stapling technique with ring-closing metathesis, originally designed to produce all-hydrocarbon stapled peptides, [14] has provided a variety of bioactive peptides acting even at intracellular tar-gets.[15] Since then, very diverse chemistries have been employed for locking active peptide conformations by stapling techniques, [16] including some MCRs.[8,9]

In this sense, we envisioned that the use of the Petasis-MCR for introducing the aryl tether would significantly increase the diversity-generating character of such a stapling concept, since further exocyclic moieties could be incorporated at the same time. Also, no special amino acids with unnatural side-chains need to be introduced into the peptide. Accordingly, our endeavor was to prove both the efficacy of the Petasis reaction as a novel peptide stapling approach and the possibility of varying the exocyclic moieties derived from the carbonyl component. As an example, model peptide **8** was produced on solid-phase with incorporation of two N^{ε}-MeLys positioned at residues *i*, *i*+7 and next released from the resin for solution-phase macrocyclization (Scheme 4). As shown, this peptide was subjected to solution-phase stapling protocol through double Petasis reaction-based macrocyclization of the methylated Lys side-chains using dihydroxyacetone as an oxo component and *p*- and *m*-phenylene and biphenylene diboronic acids.



Scheme 4. A) Multicomponent Stapling of Peptides by Solution-Phase Petasis Macrocyclization. Yields isolated pure products. B) CD spectra of the stapled peptides.

Based in our experience on double MCR-based macrocyclizations,[9,17] we chose to apply pseudo-dilution conditions to avoid large solvent volumes. This conditions were implemented by the simultaneous slow addition, with syringe pumps (flow rate 0.2 mL h^{-1}), of the diboronic acid and the peptide (0.05 mmol in 5 mL of THF/MeOH and TFE, respectively) to a solution of the oxo-component (0.2 mmol) in 40 mL of DCM/TFE 1:1 (*v*/*v*). The stapling protocol required stirring the reaction for 48 hours after completion of the slow addition, followed by HPLC purification.

Good yields of isolated stapled peptides **9**-**12** were obtained under these reaction conditions, proving that **8** is long enough to accommodate the even longer biphenyl tether (Scheme 4). We also undertook the stapling of a peptide with two methylated ornithine side-chains, positioned at residues *i*, *i*+4, by reaction with *p*-phenylene diboronic acid and glyceraldehyde, resulting in the isolation of stapled peptide **12** in very good yield for such a complex multicomponent macrocyclization. These results prove that multicomponent stapling procedures can be as efficient as the two-component variants.[16a] Although the goal of this investigation was not to obtain α-helices, we recorded the circular dichroism (CD) spectra of the Petasis stapled peptides in phosphate buffer (scheme 4B), and they show a characteristic α-helical character, with is further increased upon addition of TFE (see the Supporting Information).

In conclusion, we have developed a diversity-oriented approach for the solid-phase derivatization and solution-phase stapling of peptides using the versatile Petasis reaction. The late-stage on-resin Petasis labeling protocol could be conducted at room temperature at Lys and N^{ε}-MeLys side-chains, but double Petasis-reaction may take

place to some degree in the former with sterically unhindered aldehydes. In contrast, this class of multicomponent labeling was also implemented at the N-terminal residue, but MW irradiation was required to obtain a good percent of conversion. These multicomponent methods enabled, for the first time, the simultaneous incorporation of diverse aryl moieties and sugars at the peptide side chain without altering the overall charge of the peptide. The boronic acid component allowed the ready introduction, at both the backbone and side chain, of affinity and fluorescent labels, PEGs, lipidic and steroidal fragments. In addition, the multicomponent stapling proved highly efficient, enabling the installation of rigid aryl tethers and additional elements of diversity arising from the carbonyl component. This aspect is an interesting example of a stapling method enabling the simultaneous incorporation of exocyclic fragments with the potential to modulate the interaction with the target and simultaneously influence other parameters such as solubility. This successful application of the Petasis reaction in peptide chemistry shows promise for the future development of peptide pharmaceuticals or for protein modification

Keywords: peptide modification, multicomponent reactions, stapled peptides, labeling, macrocycles

Supporting Information

Supplementary information for this article can be found under: https://doi.org/10.1002.anie.201812620.

References

[1] a) J. Zhu, Q. Wang, M.-X. Wang, Multicomponent Reactions in Organic Synthesis, Wiley-VCH, Weinheim, 2015; b) B. H. Rotstein, S. Zaretsky, V. Rai, A. K. Yudin, *Chem. Rev.* **2014**, *114*, 8323; c) S. Brauch, S. S. van Berkel, B. Westermann, *Chem. Soc. Rev.* **2013**, *42*, 4948. (d) A. Dömling, *Chem. Rev.* **2006**, *106*, 17.

[2] a) L. Reguera, Y. Méndez, A. R. Humpierre, O. Valdés, D. G. Rivera, *Acc. Chem. Res.* **2018**, *51*, 1475; b) C. J. White, A. K. Yudin, *Nature Chem.* **2011**, *3*, 509.

[3] a) Y. Méndez, J. Chang, A. R. Humpierre, A. Zanuy, R. Garrido, A. V. Vasco, J. Pedroso, D. Santana, L. M. Rodríguez, D. García-Rivera, Y. Valdés, V. Vérez-Bencomo, D. G. Rivera,

Chem. Sci. **2018**, *9*, 2581; b) M. Chilamari, L. Purushottam, V. Rai, *Chem. Eur. J.* **2017**, 23, 3819.

[4] a) J. R. Frost, C. C. G. Scully, A. K. Yudin, *Nature Chem*. **2016**, *8*, 1105. b) A. V. Vasco, C. S. Pérez, F. E. Morales, H. E. Garay, D. Vasilev, J. A. Gavín, L. A. Wessjohann, D. G. Rivera, *J. Org. Chem.* **2015**, *80*, 6697; c) R. Hili, V. Rai, A. K. Yudin, *J. Am. Chem. Soc.* **2010**, *132*, 2889.

[5] a) M. C. Morejón, A. Laub, G. N. Kaluđerović, A. R. Puentes, A. N. Hmedat, A. J. Otero-González, D. G. Rivera, L. A. Wessjohann, *Org. Biomol. Chem.* **2017**, *15*, 3628; b) M. C. Morejón, A. Laub, B. Westermann, D. G. Rivera, L. A. Wessjohann, *Org. Lett.* **2016**, *18*, 4096; c) F. E. Morales, H. E. Garay, D. F. Muñoz, Y. E. Augusto, A. J. Otero-Gonzalez, O. Reyes Acosta, D. G. Rivera, *Org. Lett.* **2015**, *17*, 2728; d) D. G. Rivera, A. V. Vasco, R. Echemendía, O. Concepción, C. S. Pérez, J. A. Gavín, L. A. Wessjohann, *Chem. Eur. J.* **2014**, *20*, 13150.

[6] B. H. Rotstein, R. Mourtada, S. O. Kelley, A. K. Yudin, *Chem. Eur. J.* **2011**, *17*, 12257.

[7] L. R. Malins, J. N. deGruyter, K. J. Robbins, P. N. Scola, M. D. Eastgate, M. R. Ghadiri, P. S. Baran, *J. Am. Chem. Soc.* **2017**, *139*, 5233. [8] J. Zhang, M. Mulumba, H. Ong, W. D. Lubell, *Angew. Chem. Int. Ed.* **2017**, *56*, 6284; *Angew. Chem.* **2017**, *129*, 6381.

[8] J. Zhang, M. Mulumba, H. Ong, W. D. Lubell, *Angew. Chem. Int. Ed.* **2017**, *56*, 6284; *Angew. Chem.* **2017**, *129*, 6381.

[9] a) L. A. Wessjohann, O. Kreye, D. G. Rivera, *Angew. Chem. Int. Ed.* **2017**, *56*, 3501; *Angew. Chem.* **2017**, *129*, 3555; b) M. G. Ricardo, F. E. Morales, H. E. Garay, O. Reyes, L. A. Wessjohann, D. G. Rivera, *Org. Biomol. Chem.* **2015**, *13*, 438.

[10] a) N. A. Petasis, I. Akrltopoulou, *Tetrahedron Lett.* **1993**, *34*, 583; b) N. A. Petasis, I. A. Zavialov, *J. Am. Chem. Soc.* **1997**, *119*, 445; c) N. A. Petasis, I. A. Zavialov, *J. Am. Chem. Soc.* **1998**, *120*, 11798.

[11] a) S. G. Pyne, M. Tang, *Org. React.* **2014**, *83*, 211; b) N. R. Candeias, F. Montalbano, P. M. S. D. Cal, P. M. P. Gois, *Chem. Rev.* **2010**, *110*, 6169.

[12] a) N. Schlienger, M. R. Bryce, T. K. Hansen, *Tetrahedron* **2000**, *56*, 10023; b) S. R. Klopfenstein, J. J. Chen, A. Golebiowski, M. Li, S. X. Peng, X. Shao, X. *Tetrahedron Lett.* **2000**, *41*, 4835.

[13] T. Fukuyama, C.-K.; Jow, M. Cheung, *Tetrahedron Lett.* **1995**, *36*, 6373, b) J. F. Reichwein, R. M. J. Liskamp, *Tetrahedron Lett.* **1998**, *39*, 1243.

[14] a) C. E. Schafmeister, J. Po, G. L. Verdine, *J. Am. Chem. Soc.* **2000**, *122*, 5891; b) L. D. Walensky, A. L. Kung, I. Escher, T. J. Malia, S. Barbuto, R. D. Wright, G. Wagner, G. L. Verdine, S. J. Korsmeyer, *Science* **2004**, *305*, 1466.

[15] a) G. L. Verdine, G. J. Hilinski, *Drug Discovery Today Technol.* **2012**, *9*, e41. (b) L. D. Walensky, G. H. Bird, *J. Med. Chem.* **2014**, 57, 6275. (c) P. M. Cromm, J. Spiegel, T. N. Grossmann, *ACS Chem. Biol.* **2015**, *10*, 1362.

[16] a) Y. H. Lau, P. de Andrade, Y. Wu, D. R. Spring, *Chem. Soc. Rev.* **2015**, *44*, 91. b) L. Mendive-Tapia, S. Preciado, J. García, R. Ramón, N. Kielland, F. Albericio, R. Lavilla, *Nat. Commun.* **2015**, *6*, 7160; c) A. F. M. Noisier, J. García, J. A. Ionuţ, F. Albericio, *Angew. Chem. Int. Ed.* **2017**, *56*, 314; *Angew. Chem.* **2017**, *129*, 320

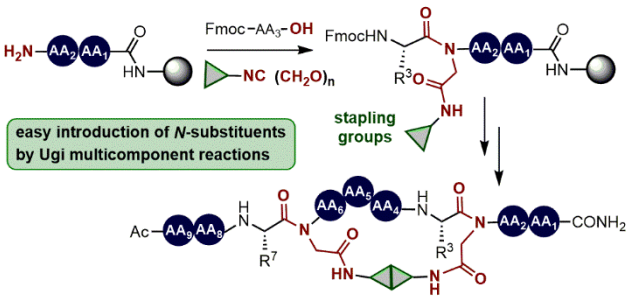
[17] a) L. A. Wessjohann, D. G. Rivera, O. E. Vercillo, *Chem. Rev.* **2009**, *109*, 796. (b) L. A. Wessjohann, G. N. Kaluđerović, R. A. W. Neves Filho, M. C. Morejon, G. Lemanski, T. Ziegler, With Isocyanide as One Component: Further Components Carboxylic Acid and Amine (Ugi Reaction). In: *Science of Synthesis* Ed. T. J. J. Müller; (Part 2013, 5). Multicomponent Reactions. Stuttgart: Thieme **2014**, 415-502.

Chapter 5

A Peptide Backbone Stapling Strategy Enabled by the Multicomponent Incorporation of Amide *N*-Substituents

This Chapter has been published as:

Ricardo, Manuel G.; Marrero, Javiel F.; Valdéz, Oscar; Rivera, Daniel G. and Wessjohann, Ludger A. *Chem. Eur. J.* **2019**, *25*, 769-774*



Abstract

The multicomponent backbone *N*-modification of peptides on solid-phase is presented as a powerful and general method to enable peptide stapling at the backbone instead of the side chains. This work show that a variety of functionalized N-substituents suitable for backbone stapling can be readily introduced by means of on-resin Ugi multicomponent reactions conducted during solid-phase peptide synthesis. Diverse macrocyclization chemistries were implemented with such backbone *N*-substituents, including the ring-closing metathesis, lactamization and thiol alkylation. The backbone *N*-modification method was also applied to the synthesis of α-helical peptides by linking *N*-substituents to the peptide *N*-terminus, thus featuring hydrogen-bond surrogate structures. Overall, the strategy proves useful for peptide backbone macrocyclization approaches that show promise in peptide drug discovery.

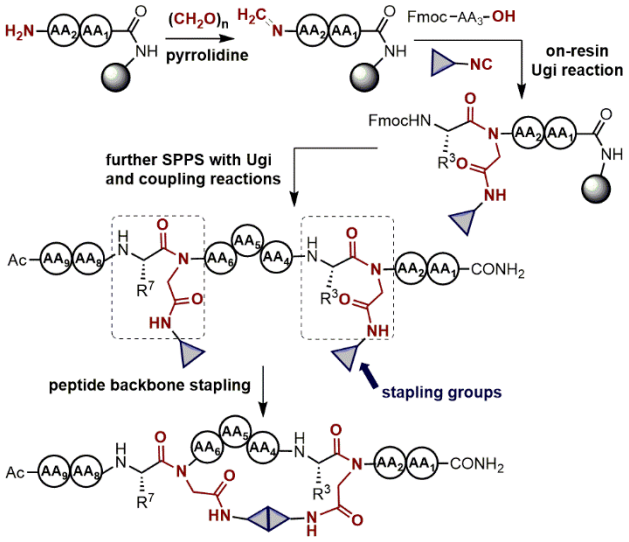
Introduction

Peptide head-to-tail cyclization and stapling – i.e., side chain-to-side chain tethering – are powerful techniques aiming at introducing conformational constraints in peptides designed for functional analysis and drug discovery.[1] Cyclization may also help to improve membrane permeability[2] and increase the metabolic resistance of peptides,[3] while it can enhance the binding affinity to biological targets, compared to their acyclic counterparts.[4] Such techniques have been especially useful for the development of protein ligands, mimetics of protein epitopes, and cell-penetrating peptides. [1,5]

A major advance in this field constitutes the methodology developed by Gilon and co-workers coined as peptide backbone cyclization.[6] This technique focuses on connecting two backbone amide nitrogens by dissimilar macrocyclization chemistries,[7] and remarkably, without altering the amino acid side chains. Backbone cyclization combines the advantages of peptide cyclization with those of amide *N*-alkylation, thus leading to improved proteolytic stability and intestinal permeability[8] of backbone cyclized peptides compared to their non-*N*-modified linear analogues. The amide *N*-substitution may also provide functionalities relevant for the interactions with biological targets[9] and gives a better *E*/*Z* conformational freedom,[10] which can be beneficial or detrimental depending on the actual application of the cyclized peptide. Conceptually, backbone cyclization shares the same vision with the peptide stapling approach.[11] In both the reactive groups can be incorporated at will at any desired amide bond much in the same way as non-proteinogenic amino acids (AAs) – with for example, alkene, alkyne and azide groups – are introduced into the peptide sequence to be stapled.[11] However, the fact of not modifying the native amino acid sequence might make the backbone cyclization approach even more advantageous for screening combinatorial conformational libraries based on a defined peptide sequence.[12] As peptide stapling, backbone cyclization has also proven effective in producing cyclic peptides mimicking α-helical secondary structures.[13]

Unfortunately, so far there is no general method for the incorporation of *N*-substituted amino acids (other than *N*-methyl ones) using standard solid-phase peptide synthesis (SPPS). Two strategies have been traditionally employed for introducing backbone *N*-

substituents in SPPS,[14] but both lack the universal character required to be routinely used for any peptide sequence. The first strategy comprises the incorporation of the *N*-alkylated/*N*-protected AAs followed by deprotection and coupling of the next AA.[14] The second one encompasses the *N*-alkylation of the *N*-terminal residue, also followed by the subsequent AA coupling.[14] Noticeably, both approaches require the difficult *N*-acylation of the *N*-substituted terminal residue in order to complete the process, which is the limiting factor for the generalization of such backbone *N*-modification techniques in the realm of peptide chemistry. To overcome such a challenging *N*-acylation step, these approaches either introduce *N*-substituted Gly or use very strong activation methods – including microwave irradiation – which are far from ideal as all require prolonged and repeating coupling steps. [14]



Scheme 1. Peptide stapling strategy based on the use of backbone *N*-substituents derived from Ugi multicomponent reactions.

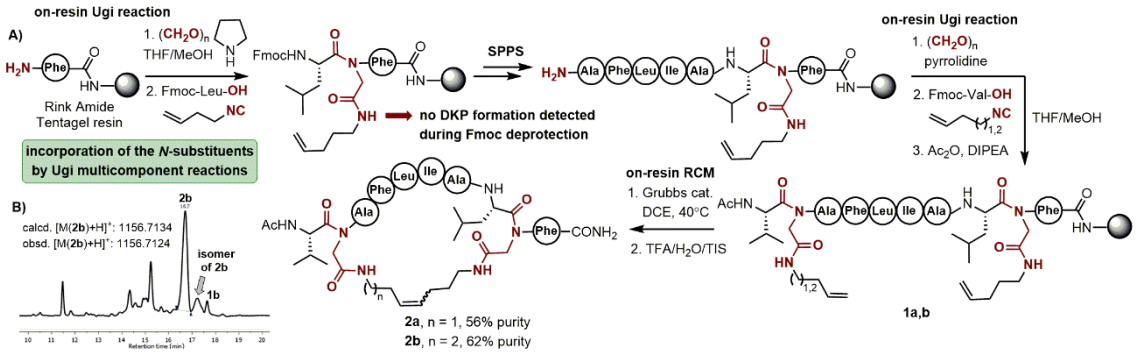
Herein we describe a novel backbone cyclization approach relying on a truly general methodology for the incorporation of peptide *N*-substituents bearing functional groups suitable for stapling processes such as: ring-closing metathesis, macrolactamization and thiol-alkylation (Scheme 1). The innovation of this backbone *N*-modification strategy lies at the utilization of the on-resin Ugi four-component reaction for the simultaneous acylation and *N*-substitution of the resin-linked peptide. Thus, with the incorporation in only one multicomponent step of both the Fmoc-amino acid and the functionalized *N*-substituent, this strategy bypasses the difficult *N*-

alkylation/*N*-acylation sequence currently employed in most backbone *N*-modification methodologies.[14]

Our initial endeavor aimed at achieving a successful translation of the stapling approach based on the ring-closing metathesis (RCM) of alkene-functionalized side chains[11] to a similar backbone stapling protocol of alkene-containing peptide *N*-substituents. As shown in scheme 2, the goal was to implement the whole route on solid-phase, including the incorporation of the olefin-containing *N*-substitutions and the final macrocyclization step. For this, the key step is the efficient implementation of the on-resin Ugi multicomponent reaction that simultaneously incorporates an Fmoc-AA and an olefin-functionalized isocyanide, along with the oxo-component.[15] Although the Ugi reaction can be undertaken with many different carbonyls,[16] its poor stereoselectivity favors the use of formaldehyde – or symmetric ketones like acetone leading to Aib – to avoid formation of complex diastereomeric mixtures. In this regard, we have recently developed a versatile aminocatalysis-mediated on-resin Ugi reaction procedure[17] that ensures full conversion in the multicompoment incorporation of the Fmoc-AA-OH (carboxylic acid) and the isocyanide component. To achieve this, the crucial step is the aminocatalytic transimination by treatment of the resin-linked peptide with paraformaldehyde and a secondary amine (i.e., piperidine or pyrrolidine) prior to addition of the carboxyclic acid and the isocyanide. This on-resin multicomponent protocol has proven success in the incorporation of all amino acids (in protected form) and very complex isocyanides,[17] so it can be considered as a powerful and general backbone *N*-modification procedure.

During optimization experiments of the on-resin Ugi reaction with dissimilar sequences, *N*-terminal residues and Fmoc-amino acids, RP-HPLC and ESI-MS analyses were conducted after mini-cleavage to determine the reaction times, which are very much dependent on the bulky character of the components and vary from 24 h to 72 h. In parallel (unpublished results), we have proven that this variant of on-resin Ugi reaction can be also conducted under microwave irradiation with reaction times ranging from 30 minutes to 1 h. However, all solid-phase protocols herein reported were carried out at room temperature and the Ugi reactions were run until full consumption of the peptide precursor, as determined by HPLC. In some cases, analysis

of the crude peptides by RP-HPLC and ESI-MS before the stapling process showed the formation of about 5-10% of the non-alkylated peptide. This is due to the minor occurrence of a coupling step at the *N*-terminus that incorporates the Fmoc-amino acid but not the *N*-substitution, which has been previously documented by our group. [17c]

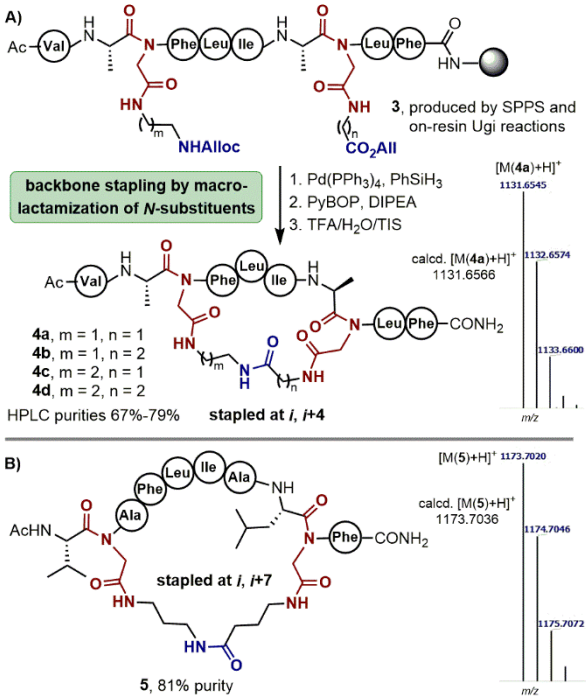


Scheme 2. Peptide backbone stapling strategy. A) Solid-phase synthesis of *i*, *i*+7 backbone stapled peptides using Ugi multicomponent reactions for introducing the olefinic backbone *N*-substituents and ring-closing metathesis for the macrocyclization. B) HPLC traces of crude peptide **2b** as released from the resin after the multi-step on-resin sequence. DCE: 1,2-dichloroethane, TFA: trifluoroacetic acid, TIS: triisopropylsilane, DIPEA: diisopropylethylamine, DKP: diketopiperazine.

Scheme 2 depicts the solid-phase construction of octapeptide skeletons bearing two olefin-functionalized *N*-substituents positioned at *i* and *i*+7 amide residues. As noticed, variation of the amide *N*-tethers – arising from the isocyanide component – enable an easy tuning of the macrocycle ring size, an important feature for a successful screening of stapled peptide conformational space. Due to their volatile and pungent characteristics, olefin-containing isocyanides – prepared by Appel's method[18] – were added to the resin-linked peptide without previous purification.

To achieve an efficient implementation of the on-resin RCM, an extensive optimization was required using several parallel experiments and combined HPLC/ESI-MS analyses. Thus, best conditions were found with the use of the first-generation Grubbs catalysts in 1,2-dichloroethane (DCE) at 40 °C using a sealed glass tube to avoid solvent evaporation. Thus, the crude stapled peptides 2a and 2b were obtained in good purity when considering the complex solid-phase sequence employed, including several coupling steps, two multicomponent reactions and especially a macrocyclization. An analytical sample (5-10 mg) of each peptide was purified by HPLC for NMR and HR-MS characterization. The HPLC traces of the crude

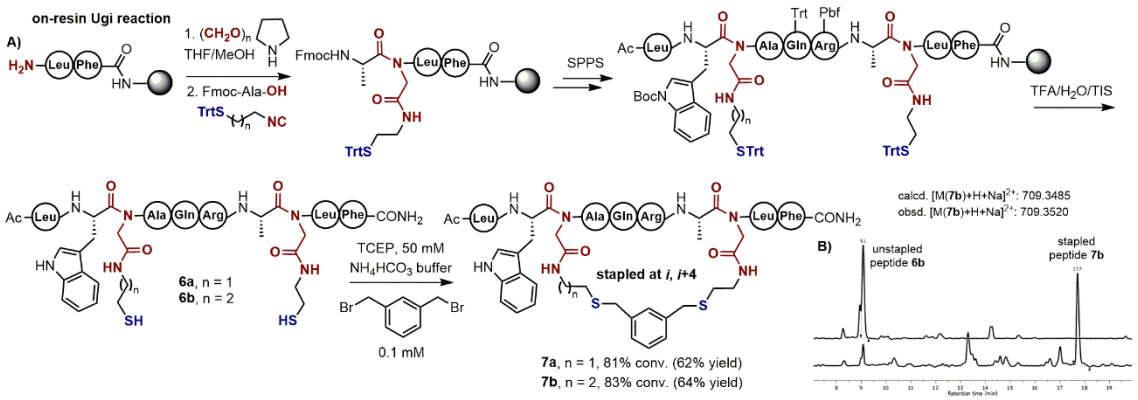
stapled peptides show that only an insignificant amount of the bis-olefin precursors 1a and 1b remain unreacted after two cycles of the on-resin RCM. Minor peaks corresponding to diastereomers of 2a and 2b were also detected, presumably corresponding to the olefin isomers with the opposite geometry (see scheme 2B).



Scheme 3. Solid-phase synthesis of *i*, *i*+4 and *i*, *i*+7 backbone stapled peptides by means of macrolactamization of Ugi reaction derived amide *N*-substituents.

A distinctive characteristic of *N*-alkylated peptides is the occurrence of both the *s-cis* and *s-trans* rotamers in solution, which results in complex NMR spectra with multiple duplicated and overlapping signals. This makes it impossible assessing whether the main products **2a** and **2b** have *E* or *Z* olefin configuration (see the SI). Based on literature reports[11] and considering the large macrocyclic rings of stapled peptides **2a** and **2b**, we may assume the *E* configuration for the main stereoisomer obtained for both compounds. The low energetic barrier between the *cis* and *trans* configurations of the two *N*-substituted amides of **2a**,**b** also leads to a high conformational heterogeneity and less rigidity than side-chain stapled peptides. In this sense, analysis of the circular dichroism (CD) spectra of **2a** and **2b** suggests the lack of a defined secondary structure (see the Supporting Information), although it should be noticed that these peptide sequences were not designed for such purpose.

Considering that very diverse chemistries have been recently applied for locking active peptide conformations by stapling techniques,[19,20] we sought to expand the scope of our backbone stapling methodology to other macrocyclization approaches. For this, we took advantage of the easy introduction of almost any reactive functionality at the *N*-substitution using this kind of multicomponent approach, as functionalized isocyanides are readily available and easy to prepare. As depicted in scheme 3, the backbone stapling strategy was implemented using lactamization for the macrocyclic ring closure, and once again, conducting the whole process on solid phase. Thus, a variety of *N*-substituted peptides were produced on resin utilizing isocyanides functionalized with amino and carboxylic groups protected as allyloxycarbonyl (Alloc) and allyl ester, respectively. The small peptide library (**3**) was thus subjected to orthogonal deprotection, macrolactamization and resin cleavage to produce a new type of *i*, *i*+4 stapled peptide having three secondary amides at the backbone *N*-tether. A similar approach was conducted for the synthesis of the *i*, *i*+7 stapled peptide **5**, in all cases with good overall yield and HPLC purity of crude products.



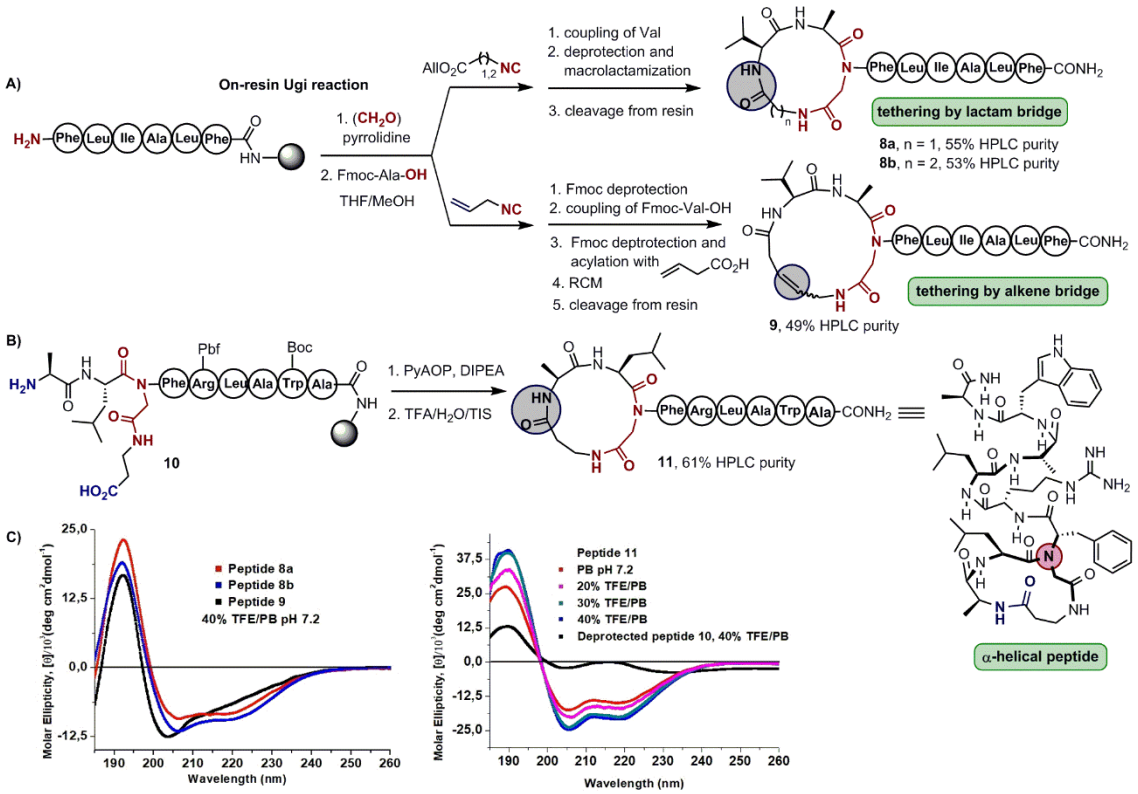
Scheme 4. A) Synthesis of *i*, *i*+4 backbone stapled peptides by bis-alkylation of thiol-containing backbone *N*-substituents derived from on-resin Ugi reactions. B) HPLC traces of linear peptide **6b** and of crude stapled peptide **7b** after macrocyclization. TCEP: Tris(2-carboxyethyl)phosphine.

As before, the ^{1}H NMR spectra of pure stapled peptides **4a-d** and **5** reveal the presence of various conformers in solution. Interestingly, there is a marked change in the CD spectrum of peptide **4a** compared to those of **4b** to **4d** (see the Supporting Information), proving the difference in the three-dimensional solution structure of the former compared to their analogues having longer backbone *N*-tethers.

Besides of RCM and lactamization, the alkylation and arylation of Cys thiol groups have proven success as stapling technique as well as for rendering bioactive stapled peptides.[20] Hence, we turned to implement a backbone stapling method in which two thiol-functionalized *N*-substituents could be tethered by a bis-alkylation macrocyclization reaction. As shown in Scheme 4, peptides **6a** and **6b** were produced by solid-phase peptide synthesis, released from the resin and next cyclized in solution with 1,3-bis(bromoethyl)benzene to furnish stapled peptides **7a** and **7b**. The solid-phase methodology comprised the utilization of two Ugi multicomponent reactions using alkyl isocyanides functionalized with trityl-protected thiol groups.[21] Once more, the on-resin *N*-modification protocol proved highly efficient, as corroborated in the HPLC traces of crude peptide **6b** shown in scheme 4B. Similarly, the stapling *S*-alkylation approaches of the fully deprotected peptides **6a** and **6b** proceeded with high conversion, thus enabling the isolation of peptides **7a** and **7b** in high yield after preparative HPLC purification. Again, despite the cyclic structures of these stapled peptides, analysis of their ^{1}H NMR spectra revealed a high conformational heterogeneity (see the Supporting Information), owing to the presence of two *N*-substituted amides in the peptide backbone.

After proving both the great scope and efficiency of the multicomponent backbone *N*-modification approach for the incorporation of diverse stapling functionalities, we turned to demonstrate the application of this method in the generation of helical peptides featuring backbone hydrogen-bond surrogate (HBS) structures. The HBS approach – developed by Arora and co-workers[13b-d] – has proven to be one of the most effective methods for producing stable α-helices, including mimetics of protein epitopes. The concept behind this notable achievement is the replacement of the hydrogen bond between the C=O and NH –located at *i* and *i*+4 positions – by an intramolecular hydrocarbon tether derived from a RCM. To achieve this, an olefin *N*-substitution is introduced three residues ahead of the *N*-terminus using standard *N*-alkylation/*N*-acylation chemistry, and next macrocyclized by RCM with an *N*-terminal olefin linker. Thus, the goal is to produce a 13-membered macrocycle that covalently replaces the intramolecular hydrogen bond of an α-helical turn, thus nucleating the α-helix formation alongside the peptide chain.

As depicted in scheme 5, the incorporation of Ugi-derived backbone *N*-substituents two amino acids ahead of the *N*-terminus allows for the versatile and easy introduction of carboxyl and olefin functionalities at the *N*-substituent. These are subsequently used for macrolactamization and RCM with the properly functionalized *N*-terminus. Again, an advantage of the multicomponent strategy is that it provides a branched peptide incorporating an AA at the main chain and another (i.e., functionalized Gly) at the *N*-substitution, all at a lower synthetic cost to afford the whole HBS peptide on resin. Accordingly, cyclic peptides **8a** and **8b** were produced on resin using PyAOP/HOAt for the macrocyclization, as their 12 and 13-membered rings are difficult to obtain classically. Alternatively, the 15-membered macrocyclic peptide **9** was prepared by the RCM approach described above, which also rendered a minor amount of the geometric isomer (not isolated).



Scheme 5. A) Solid-phase synthesis of *N*-terminus-to-backbone cyclized peptides by RCM and macrolactamization. B) Synthesis and schematic representation of the α-helical structure of peptide **11**. C) CD spectra of cyclic peptides **8**, **9** and **11** (compared with the deprotected acyclic precursor **10**). PB: phosphate buffer, RCM: ring-closing metathesis, TFE: trifluoroethanol.

Scheme 5C shows the CD spectra of the three peptides **8a**,**b** and **9** in 40% trifluoroethanol/phosphate buffer (PB), because the hydrophobic sequence of such peptides makes them insoluble in pure water or PB at 0.1 mM. Importantly, HBS peptides **8a** and **8b** show typical CD spectra of α-helices (i.e., maximum at 192 nm and double minimum at 207 and 221 nm). As one might argue that TFE strongly favors α-helix formation, we also chose to prepare an analogous peptide **11** (Scheme 5B) having a water soluble sequence, but keeping the 13-membered macrocyclic lactam ring that proved α-helix formation in peptide **8b**. As shown in scheme 5C, cyclic peptide **11** displays a classic α-helical CD spectrum even in PB alone, proving the success of this method also in producing helical peptides. Addition of TFE up to 40% does cause a further increase in the intensity of the bands at 190, 206 and 220 nm, which is characteristic of short peptides upon addition of this α-helix inducing solvent. In this regard, we also sought to calculate the relative percent helicity of peptide **11**, which can be estimated by the mean residue ellipticity at 222 nm, value commonly downshifted for short peptides to 215-220 nm.[22]

To this end, we employed the equation described by Baldwin[23] and followed the considerations of Fairlie[22c] and Baldwin[24] for carboxyamidated peptides (see the Supporting Information). Thus, it was found that the percent of helicity of peptide **11** is 63% in PB, which was next increased to 72, 84 and 88% when the percentage of TFE increases to 20, 30 and 40%, respectively. To confirm that the backbone-to-*N*-terminus cyclization is the responsible for the helical character of peptide **11**, the CD spectrum of deprotected peptide **10** – acyclic precursor of **11** – was also measured. Thus, the marked decrease in the intensity of the maximum and the two minima in the CD spectrum of deprotected **10** – even in 40% TFE/PB – confirms that this peptide is unstructured and that the cyclization certainly favors the α-helicity in this type of backbone-modified peptide skeleton.

In conclusion, we have proven that the on-resin multicomponent *N*-modification method provides a powerful strategy for stapling peptides at their backbone substituents instead of their side chains. Diverse functionalities, such as olefin, amine, carboxylic acid and thiol, could be efficiently installed as *N*-substituents of peptide amides in one shot, thus enabling the implementation of very effective backbone

stapling protocols with different tethers. Other relevant functionalities, such as alkynes and azides, surely are equally easy to introduce as handles of the isocyanide component. In general, the incorporation of two amide *N*-substituents leads to the occurrence of various conformers for this type of backbone stapled peptides in solution. The backbone *N*-modification method was also applied to the synthesis of peptides with self-inducing α-helix featuring HBS structures. For this, the covalent connectivity was established between the *N*-substituent of an internal amide and the *N*-terminus, thus mimicking the intramolecular hydrogen bond occurring in α- helices. The versatility and efficacy of this backbone stapling approach makes it suitable for application in the parallel synthesis and screening of cyclic peptides based on bioactive sequences.

Keywords: peptide cyclization, multicomponent reactions, stapled peptides, α-helix, macrocycles

Supporting Information

Supplementary information for this article can be found under: https://doi.org/10.1002.anie.201805318.

References

[1] a) C. J. White, A. K. Yudin, *Nat. Chem.* **2011**, *3*, 509; b) D. J. Craik, D. P. Fairlie, S. Liras, D. Price, *Chem. Biol. Drug Des.* **2013**, *81*, 136; c) G. L. Verdine, G. J. Hilinski, *Drug Discovery Today Technol.* **2012**, *9*, e41; d) M. Góngora-Benítez, J. Tulla-Puche, F. Albericio, *Chem. Rev.* **2014**, *114*, 901; e) L. Cheng, T. A. Naumann, A. R. Horswill, S.-J. Hong, B. J. Venters, J. W. Tomsho, S. J. Benkovic, K. C. Keiler, *Protein Sci.* **2007**, *16*, 1535.

[2] a) T. Rezai, B. Yu, G. L. Millhauser, M. P. Jacobson, R. S. Lokey, *J. Am. Chem. Soc*. **2006**, *128*, 2510; b) P. S. Burton, R. A. Conradi, N. F. H. Ho, A. P. Hilgers, R. T. J. Borchardt, *Pharm. Sci.* **1996**, *85*, 1336.

[3] D. R. March, G. Abbenante, D. A. Bergman, R. I. Brinkworth, W. Wickramasinghe, J. Begun, J. L. Martin, D. P. Fairlie, *J. Am. Chem. Soc.* **1996**, *118*, 3375.

[4] V. J. Hruby, F. al-Obeidi, W. Kazmierski, *Biochem. J.* **1990**, *268*, 249.

[5] a) J. A. Robinson, *J. Pept. Sci.* **2013**, *19*, 127; b) J. Garner, M. M. Harding, *Org. Biomol. Chem.* **2007**, *5*, 3577; c) L. D. Walensky, G. H. Bird, *J. Med. Chem.* **2014**, *57*, 6275.

[6] C. Gilon, D. Halle, M. Chorev, Z. Selinger, G. Byk, *Biopolymers* **1991**, *31*, 745.

[7] a) G. Bitan, I. Sukhotinsky, Y. Mashrinki, M. Hanani, Z. Selinger, C. Gilon, *J. Pept. Res.* **1997**, *49*, 421; b) J. F. Reichwein, B. Wels, J. A. W. Kruijtzer, C. Versluis, R. M. J. Liskamp, *Angew. Chem.* **1999**, *111*, 3906; *Angew. Chem. Int. Ed.* **1999**, *38*, 3684; c) M. Hurevich, Y. Tal-Gan, S. Klein, Y. Barda, A. Levitzki, C. Gilon, *J. Pept. Sci.* **2010**, *16*, 178.

[8] a) J. G. Beck, J. Chatterjee, B. Laufer, M. U. Kiran, A. O. Frank, S. Neubauer, O. Ovadia, S. Greenberg, C. Gilon, A. Hoffman, H. Kessler, *J. Am. Chem. Soc.* **2012**, *134*, 12125; b) O. Ovadia, S. Greenberg, J. Chatterjee, B. Laufer, F. Opperer, H. Kessler, C. Gilon, A. Hoffman, *Mol. Pharmaceutics* **2011**, *8*, 479; c) G. Byk, D. Halle, I. Zeltser, G. Bitan, Z. Selinger, C. Gilon, *J. Med. Chem.* **1996**, *39*, 3174; d) A. Friedler, D. Friedler, N. W. Luedtke, Y. Tor, A. Loyter, C. Gilon, *J. Biol. Chem.* **2000**, *275*, 23783.

[9] a) T. Kodadek, P. J. McEnaney, *Chem. Commun.* **2016**, *52*, 6038; b) J. Morimoto, T. Kodadek, *Mol. BioSyst.* **2015**, *11*, 2770; c) Y. Gao, T. Kodadek, *ACS Comb. Sci.* **2015**, *17*, 190.

[10] W. Brandt, T. Herberg, L. Wessjohann, *Biopolymers (Pept. Sci.)* **2011**, *96*, 651.

[11] a) C. E. Schafmeister, J. Po, G. L. Verdine, *J. Am. Chem. Soc.* **2000**, *122*, 5891; b) L. D. Walensky, A. L. Kung, I. Escher, T. J. Malia, S. Barbuto, R. D. Wright, G. Wagner, G. L. Verdine, S. Korsmeyer, *Science* **2004**, *305*, 1466; c) Y.-W. Kim, T. N. Grossmann, G. L. Verdine, *Nature Prot*. **2011**, *6*, 761; d) S. L. Mangold, R. H. Grubbs, *Chem. Sci.* **2015**, *6*, 4561.

[12] a) M. S. Zoda, M. Zachariasb, S. Reissmann, *J. Pept. Sci.* **2010**, *16*, 403; b) Y. Tal-Gan, M. Hurevich, S. Klein, A. Ben-Shimon, D. Rosenthal, C. Hazan, D. E. Shalev, M. Y. Niv, A. Levitzki, C. Gilon, *J. Med. Chem*. **2011**, *54*, 5154; c) S. Naveh, Y. Tal-Gan, S. Ling, A. Hoffman, J. Holoshitz, C. Gilon, *Bioorg. Med. Chem. Lett.* **2012**, *22*, 493.

[13] a) M. Hurevich, M. Ratner-Hurevich, Y. Tal-Gan, D. E. Shalev, S. Z. Ben-Sasson, C. Gilon, *Bioorg. Med. Chem.* **2013**, *21*, 3958; b) A. Patgiri, M. Z. Menzenski, A. B. Mahon,

P. S. Arora, *Nature Prot.* **2010**, *5*, 1857; c) A. Patgiri, A. L. Jochim, P. S. Arora, *Acc. Chem. Res.* **2008**, *41*, 1289; d) R. N. Chapman, G. Dimartino, P. S. Arora, *J. Am. Chem. Soc*. **2004**, *126*, 12252.

[14] a) For a comprehensive review, see: A. I. Fernández-Llamazares, J. Spengler, F. J. Albericio, *Pept. Sci.* **2015**, *104*, 435; b) for a more recent method, see: P. Pels, T. Kodadek, *ACS Comb. Sci.* **2015**, *17*, 152.

[15] For a review of Ugi reaction with peptides, see: L. Reguera, Y. Méndez, A. R. Humpierre, O. Valdés, D. G. Rivera, *Acc. Chem. Res.* **2018**, *51*, 1475.

[16] a) A. Dömling, I. Ugi, *Angew. Chem.* **2000**, *112*, 3300, *Angew. Chem. Int. Ed.* **2000**, *39*, 3168; b) I. Ugi, R. Meyr, U. Fetzer, C. Steinbrücker, *Angew. Chem.* **1959**, *71*, 386.

[17] a) A. R. Puentes, M. C. Morejón, D. G. Rivera, L. A. Wessjohann, *Org. Lett.* **2017**, *19*, 4022; b) M. C. Morejón, A. Laub, B. Westermann, D. G. Rivera, L. A. Wessjohann, *Org. Lett.* **2016**, *18*, 4096; c) F. E. Morales, H. E. Garay, D. F. Muñoz, Y. E. Augusto, A. J. Otero-Gonzalez, O. R. Acosta, D. G. Rivera, *Org. Lett.* **2015**, *17*, 2728.

[18] R. Appel, R. Kleinstück, K.-D. Ziehn, *Angew. Chem.* **1971**, *83*, 143; *Angew. Chem. Int. Ed.* **1971**, *10*, 132.

[19] a) A. F. M. Noisier, J. García, J. A. Ionuţ, F. Albericio, *Angew. Chem.* **2017**, *129*, 320; *Angew. Chem. Int. Ed*. **2017**, *56*, 314; b) G. Lautrette, F. Touti, H. G. Lee, P. Dai, B. L. Pentelute, *J. Am. Chem. Soc.* **2016**, *138*, 8340; c) L. Mendive-Tapia, S. Preciado, J. García, R. Ramón, N. Kielland, F Albericio, R. Lavilla, *Nat. Commun.* **2015**, *6*, 7160; d) Y. H. Lau, P. de Andrade, Y. Wu, D. R Spring, *Chem. Soc. Rev.* **2015**, *44*, 91; e) A. D. de Araujo, H. N. Hoang, W. M. Kok, F. Diness, P. Gupta, T. A. Hill, R. W. Driver, D. A. Price, S. Liras, D. P. Fairlie, *Angew. Chem.* **2014**, *126*, 7085; *Angew. Chem. Int. Ed.* **2014**, *53*, 6965.

[20] a) S. P. Brown, A. S. Smith III, *J. Am. Chem. Soc.* **2015**, *137*, 4034; b) A. M. Spokoyny, Y. Zou, J. J. Ling, H. Yu, Y.-S. Lin, B. L. Pentelute, *J. Am. Chem. Soc.* **2013**, *135*, 5946; c) H. Jo, N. Meinhardt, Y. Wu, S. Kulkarni, X. Hu, K. E. Low, P. L. Davies, W. F. DeGrado, D. C. Greenbaum, *J. Am. Chem. Soc.* **2012**, *134*, 17704; d) P. Timmerman, J. Beld, W. C. Puijk, R. H. Meloen, *ChemBioChem* **2005**, *6*, 821.

[21] See for example: T. M. Vishwanatha, E. Bergamaschi, A. Dömling, *Org. Lett.* **2017**, *19*, 3195.

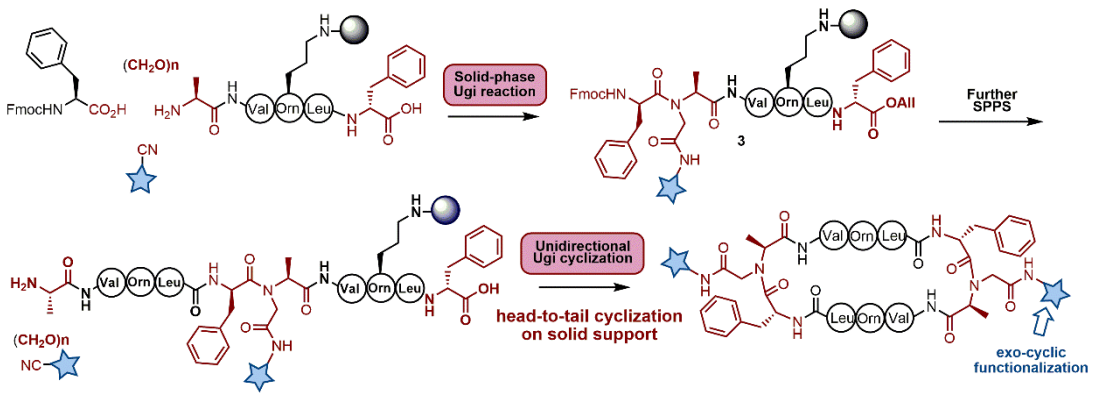
[22] a) D. H. Chin, R. W. Woody, C. A. Rohl, R. L. Baldwin, *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 15416; b) D. Wang, K. Chen, J. L. Kulp III, P. S. Arora, *J. Am. Chem. Soc*. **2006**, *128*, 9248; c) N. E. Shepherd, H. N. Hoang, G. Abbenante, D. P. Fairlie, *J. Am. Chem. Soc.* **2005**, *127*, 2974.

[23] P. Z. Luo, R. L. Baldwin, *Biochemistry* **1997**, *36*, 8413.

[24] C. A. Rohl, R. L. Baldwin, *Methods Enzymol.* **1998**, *295*, 1.

General discussions

As demonstrated before, the repertoire of macrocyclization strategies for the introduction of conformational constrains in peptides has been increased enormously. Inspired by cyclic natural products in the past and recently by the repercussion on the creation of new therapeutic candidates, many interesting methodologies are today available. Among the large variety of methods, some intrinsic features differentiate them, including: a) chemistry required for the ring-closure, b) way of cyclization regarding peptide side chain or backbone, c) number of functional groups or buildings blocks compromised (e.g., one-component, two-component, multicomponent, etc.), d) size of the ring or separation of amino acids, e) synthetic issues, e. g., if it is performed in solution or on polymer-supported. Here we developed several methods for peptide cyclizations that can be differentiated in all this aspects, having in common that all involve multicomponent reactions.

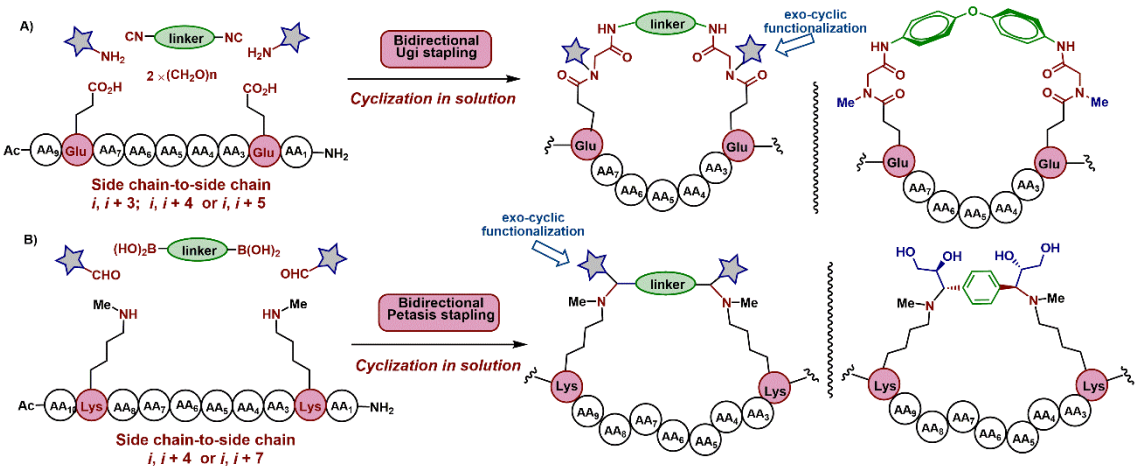


Scheme 1. Multicomponent strategy for the synthesis of analogues of Gramicidin S, based on an on-resin head-to-tail cyclization by Ugi-4CR.

In the first chapter, a new method for the synthesis of Gramicidin S analogues was developed. We found that the replacement of the turn inducer dipeptide L-Pro-D-Phe by an Ugi reaction-derived *N*-alkylated dipeptide was able to mimic the β-hairpin structure of the natural product. As depicted in the Scheme 1, we designed a solid-phase protocol, based on the head-to-tail cyclization of a linear precursor anchored to the solid support *via* one Orn-side chain. The final cyclization step, in all examples, was performed by an unidirectional Ugi reaction, in order to make use of the established advantages of the multicomponent process over the classic macrolactamization. As a

result, a library of analogues of Gramicidin S containing *exo*-cyclic cationic, anionic and lipid functionalities was synthesized. However, substitution of both turns reduced the active conformation.

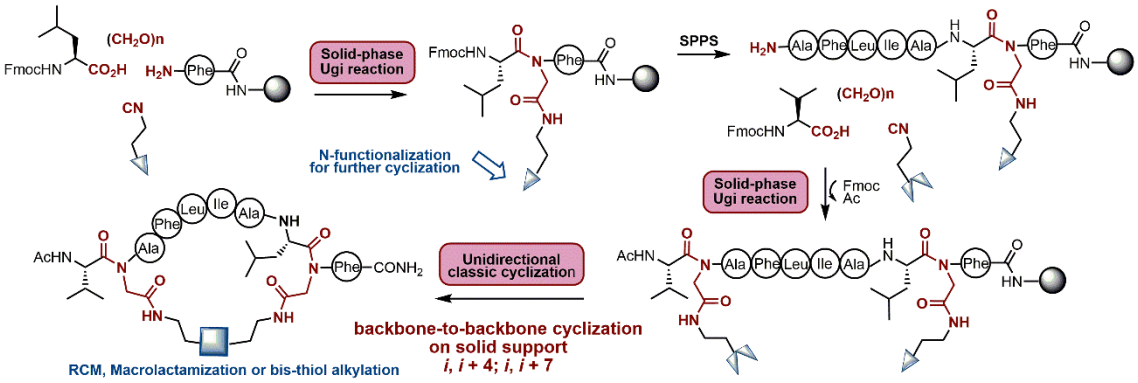
In the second chapter (scheme 2A), I studied the execution of two Ugi reactions, together comprising the ring-closure. This variant allows for more diversity introduction into the formed ring due to the inclusion of most of the components. This bidirectional approach was conducted on bifunctional diacid peptides with diisocyanide cross-linkers in combination that includes side chain-to-side chain and side chain-to-tail cyclizations. In this case, the multicomponent macrocyclization was performed in solution because according to our experience, on-resin protocols using bifunctionalized linkers can afford a complex mixture of linked and cross-linked products. The sequences studied were chosen randomly, containing two Glu/Asp residues separated by several positions like *i*, *i* + 3; *i*, *i* + 4 and *i*, *i* + 5, or a combination of one Glu/Asp with the *C*-terminus. It is worth to highlight the high level of chemical diversity that this method allows based on the variation of not only the diisocyanide cross-linker, but also of the amino component.



Scheme 2. Bidirectional macrocyclization based on double Ugi reactions (A) and on double Petasis reaction (B) of peptide side chains.

The most recent contribution of our group to the field of peptide macrocyclization or covalent modification in general, is described in the third chapter of the present document. There in, we report the utilization of the Petasis-3CR for the late-stage diversification and stapling of peptides. First we described a multicomponent labeling

protocol that enables, for the first time, the simultaneous incorporation on the solid support of diverse small sugars together with other bio-important moieties at the Lys side chain or the peptide *N*-terminus. In addition, it was possible to design a multicomponent stapling protocol based on the Petasis reaction (scheme 2B). It is based on the combination of side chain-bifunctionalized peptides containing two units of N^{ε}-MeLys or N^{ε}-MeOrn (instead of Glu/ Asp in the Ugi protocol) separated at *i*, *i* + 4 or *i*, *i* + 7 with a diboronic acid cross-linker and a dihydroxyacetone or glyceraldehyde. In comparison with the Ugi-based protocol, this one is perhaps less prominent in terms of atom economy and only four new covalent bonds are formed during the ring-closuring whereas in the former eight are formed and included in the cyclic structure. However, with using Petasis reaction in solution, the overall charge of the peptide is not affected, since the amino groups are not compromised in an amide bond like in the Ugi reaction. In addition, this multicomponent stapling also enables rigid aryl tethers in combination with additional elements of diversity arising from the carbonyl component, like small sugars, that could influence important parameters such as solubility or immunogenicity.



Scheme 3. Multicomponent *N*-modification based on the Ugi-4CR, for further backbone-to-backbone cyclization.

Like in the previous three chapters, in the fourth one (without including the multicomponent HBS alternative) also two multicomponent reactions are involved in the designed macrocyclization strategy. However, now the final cyclization step did not consist on a multicomponent reaction (scheme 3). Aiming to not compromise any side chain for the cyclization, two Ugi reactions were employed to introduce amide *N*-

substituents, at different positions of the peptide backbone bearing functional groups suitable for the final ring-closure. As a result, diverse uni- and bidirectional macrocyclization chemistries were implemented with such backbone *N*-substituents, including the ring-closu metathesis, macrolactamization and thiol bis-alkylation. Noticeably, the execution of all synthetic steps, including the two Ugi reactions and the final cyclization, can be conducted on the solid support and the *N*-substitutions were introduced in an amide separated at *i*, *i* + 4 or *i*, *i* + 7, or in combination with the *N*-terminus separated by two or three residues. In analogy with the first chapter, this cyclization method comprises attachments only at the backbone. But instead of using a head-to-tail combination, here it comprises the utilization of functionalities located at inner amide-*N*-substituents. However, in contrast to the synthesis of analogues of gramicidin S, where most of the bonds and substituents generated in the multicomponent process are *exo*-cyclic, and in similitude with both Ugi and Petasis bidirectional protocols, most of the bonds generated and building blocks used were finally incorporated into the cycle.

In general, we have demonstrated the versatility of multicomponent reactions for peptide cyclization either from the side chains or the backbone. The most important features of all developed protocols offer the chance to rapidly generate high levels of complexity and enable multiple exo- and endo-cyclic functionalizations. This render them as very suitable protocols for library synthesis as required for screening new chemotypes of biologically relevant peptides.

Declaration of the author contributions

Chapter 2

"On the Stabilization of Cyclic β-Hairpins by Ugi Reaction-Derived N-Alkylated Peptides: The Quest for Functionalized β-turns"

Ricardo, M. G., Vasco, A. V., Rivera D. G. and Wessjohann L. A. *Org. Lett.* **2019**, xx, xxx-xxx.

In this work, Manuel Garcia Ricardo designed most of the synthetic detail. Together with Aldrin Vasco he carried all synthesis, both contributing similarly. He did most of the general data analysis and interpretation, while Aldrin Vasco performed all the NMR analysis and dynamics calculations. The project was designed and supervised by Daniel Garcia Rivera and Ludger A. Wessjohann. All four authors contributed to the writing and editing of the manuscript.

Chapter 3

"Bidirectional macrocyclization of peptides by double multicomponent reactions"

Ricardo, M. G., Morales, F. E., Garay H., Reyes O., Vasilev D., Wessjohann L. A., Rivera D. G. *Org Biomol Chem* **2015**, 13, 438.

In this work, Manuel Garcia Ricardo carried out most of the synthesis including all the multicomponent macrocyclizations of peptides and experimental setup. He did all the data analysis and interpretation. Fidel Ernesto Morales did the synthesis on solid support of three of the peptide precursors under the supervision of Hilda Garay and Osvaldo Reyes and Dimitar Vasilev contributed with some analytical experiments. The project was designed and supervised by Daniel Garcia Rivera and Ludger Wessjohann. Both supervisors contributed to the writing and edition of the manuscript.

Chapter 4

"Introducing the Petasis Reaction for the Late-Stage Multicomponent Diversification, Labeling and Stapling of Peptides"

Ricardo, M. G.; Llanes, D.; Rivera, D. G.; Wessjohann, L. A. *Angew. Chem. Int. Ed.* **2019**, *58*, 2700.

In this study, Manuel Garcia Ricardo did most of the synthetic planning together with two supervisors. Mr. Ricardo, performed all the peptide syntheses and multicomponent derivatizations, labeling and stapling processes. Dayma Llanes synthesized the boronic acids funtionalized with steroids, fluorescence tags, biotin tags and small PEG chains. Manuel Garcia Ricardo did all the data processing and interpretation. Daniel Garcia Rivera and Ludger Wessjohann, acted as supervisors of the project; they designed the project, coordinated the work, and contributed to the writing and editing of the manuscript.

Chapter 5

"A Peptide Backbone Stapling Strategy Enabled by the Multicomponent Incorporation of Amide N-Substituents"

Ricardo, M. G.; Marrrero, J. F.; Valdés, O.; Rivera, D. G.; Wessjohann, L. A. *Chem. Eur. J.* **2018**, *25*, 769.

In this work, Manuel Garcia Ricardo did most of the synthetic work, designed together with the supervisors. The former carried out all the peptide syntheses, macrocyclizations, multicomponent reactions and analyzed all the analytical data. Javiel Fernández Marrero and Oscar Valdés performed the synthesis of some of the ω-functionalized isocyanides. Daniel Garcia Rivera and Ludger Wessjohann, acted as supervisors of the project; they designed the project, coordinated and checked the work, and contributed to the writing and editing of the manuscript.

Prof. Dr. Ludger A. Wessjohann

Supervisor and thesis editor

Leibniz Institute of Plant Biochemistry

Halle (Saale), June 2019

Curriculum Vitae

Personal Information

Name: Manuel García Ricardo

Date of birth: March 7th, 1989

Place of birth: Holguín, Cuba

Nationality: Cuban

E-mail: mgricardo89mail.com

Languages: Spanish, English

Educational qualifications

2013 **Diploma in Chemistry**. University of Havana, Cuba. First Honors (5.99 out of 5)

2015 **Master in Chemistry**. University of Havana, Cuba. Mention: Excellent. Topic: Bidirectional macrocyclization of peptides side chain by double multicomponent reaction. Supervisor: Daniel G. Rivera. Center for Natural Product Research.

Awards and honors

2006 **Gold Medal** at the National Chemistry Olympiad.

2007 **Gold Medal** at the National Chemistry Olympiad.

2008 **Gold Medal** at the National Chemistry Olympiad.

2006 **Silver Medal** at the 11th Iberoamerican Chemistry Olympiad. Aveiro. Portugal

2007 **Bronze Medal** at the 39th International Chemistry Olympiad. Moscow, Russia.

2009 **Special Prize** to the work of higher applicability by the Cuban Society of Chemistry presented in the Faculty of Chemistry Science Fair.

2013 **Special Prize** to the work of higher biotechnological impact presented in the Faculty of Chemistry Science Fair.

2015 **Graduated Student with the Best Academic Record** in the Faculty of Chemistry, University of Havana

2017 **Annual Prize of the Academy of Science of Cuba**, as co-author for the contribution: “Strategies for macrocyclation and N-alkylation of peptides”.

Posters and oral presentations

2012 Humboldt Kolleg: Challenges and Frontiers of Physics and Chemistry to Modern Biology. "Peptide Cyclization by Ugi Reaction: A Story of Multiplicity Becoming Complexity." (Poster Presentation)

2012 IV International Symposium of Chemistry, Santa Clara, Cuba. "Multiple Multicomponent Reactions for the Macrocyclization of Peptide Side Chains". (Poster Presentation)

2013 20th Latinoamerican Congress on Chemistry, Santiago de Cuba, Cuba. "Multiple Multicomponent Reactions for the Macrocyclization of Peptide Side Chains." (Poster Presentation)

2015 9th Congress of Chemical Sciences, Technology and Innovation Quimicuba-2012. "A New General Multicomponent Approach for Backbone Cyclization of Peptides" (Poster presentation)

2018 35th International Peptide Symposium, Dublin, Ireland. "Introducing Petasis Reaction for the Multicomponent Diversification, Labeling and Stapling of Peptides." (Poster Presentation)

2019 14th German Peptide Symposium, Cologne, Germany. "Mulicomponent Strategies to Side-chain and Backbone Modified Cyclic Peptides" (Oral Presentation)

Work experiences

2013-currently Instructor Teacher and researcher. University of Havana. Faculty of Chemistry. Center of Natural Product Research

2015-currently PhD Student at the Leibniz Institute of Plant Biochemistry – Department of Bioorganic Chemistry, IPB, Halle (Saale), Germany. Supervisors: Prof. Dr. L.A. Wessjohann, Prof. Dr. D.G. Rivera

Manuel Garcia Ricardo
Halle (Saale), 26.08.2019

List of publications

Ricardo, M. G.; Moya, C. G.; Pérez, C. S.; Porzel, A.; Wessjohann, L. A. and Rivera, D. G. "Improved Stability and Tunable Functionalization of Parallel β-Sheets via Multicomponent N-Alkylation of the Turn Moiety" *Angew. Chem Int. Ed.*, **2019**, *59*, 259-263

Ricardo, M. G.; Ali, A. M.; Surmiak, E.; Neochoritis, C. G.; Holak, T. A.; Groves, M. Alexander Dömling, A.; G. Rivera, "D. R. Ugi Multicomponent Stapling for the Development of α-Helical Peptide Inhibitors of Protein-Protein Interactions" *Angew. Chem Int. Ed.*, **2019**, *58*, XXX-XXX

Ricardo, M. G.; Vasco, A. V.; Rivera, D. G. and Wessjohann, L. A. "On the Stabilization of Cyclic β-Hairpins by Ugi Reaction-Derived N-Alkylated Peptides: The Quest for Functionalized β-Turns" *Org. Lett.* **2019**, *21*, 7307-7310

Ricardo, M. G.; Llanes, D.; Wessjohann, L. A. and Rivera, D. G. "Introducing the Petasis Reaction for the Late-Stage Multicomponent Diversification, Labeling and Stapling of Peptides" *Angew. Chem Int. Ed.*, **2019**, *58*, 2700-2704

Ricardo, M. G.; Marrero, J. F.; Valdéz, O.; Rivera, D. G. and Wessjohann, L. A. "Peptide Backbone Stapling Strategy Enabled by the Multicomponent Incorporation of Amide N-Substituents" *A. Chem. Eur. J.* **2019**, *25*, 769-774

Ricardo, M. G.; Morales, F. E.; Garay, H.; Reyes, O.; Vasilev, D.; Wessjohann, L. A.; Rivera, D. G. "Bidirectional Macrocyclization of Peptides by Double Multicomponent Reaction". *Org. Biomol. Chem.* **2015**, 13, 438-446.

Manuel Garcia Ricardo
Halle (Saale), 26.08.2019

Declaration

I declare that I have completed this dissertation without unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or based on the content of published or unpublished work of others authors.

Erklärung

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe.

Manuel Garcia Ricardo
Halle (Saale), 26.08.2019