Ionic liquids as novel reaction media for the chemical synthesis of peptides

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Abstract

To seek the best solvent in which the peptide can dissolve is usually a serious challenge in peptide chemical synthesis. Ionic liquids, which are liquid below 100 °C, or even at room temperature, showed an outstanding solubility for polar compounds, unpolar compounds and even biological macromolecules. Furthermore, ionic liquids are also recognized as a green solvent because of their special properties, such as non-flammability, thermal/chemical stability, no measurable vapor pressure and recyclability. Thus, ionic liquids have attracted increasing interest in the field of peptide synthesis. In this thesis, the compatibility of ionic liquids for the Native Chemical Ligation strategy at a X–Cys ligation site (X = any amino acid) was analyzed and compared to the conventional ligation strategy. Besides that, the initial attempts of lactam bridge formation of cyclic peptides in ionic liquid have been made with the aim to complete the insufficient knowledge regarding peptide cyclization in ionic liquids. In conclusion, this thesis obtains an impression on how the application of ionic liquids for both reaction types provides essential contributions to the field of peptide chemistry employing alternative reaction media.

Table of Contents

1 Introduction

A suitable dissolution method is an important factor for the success of peptide synthesis, especially the solution-phase peptide synthesis. An inadequate solvent can lead to precipitation and denaturation of the peptide of interest during the synthesis process, purification and also analysis. Peptides possess variable solubility properties, which are dictated by their amino acid composition, length of sequences and secondary structure. Although some peptides can be dissolved in aqueous solutions, organic solvents or mixtures of these, no conventional solvent has been found to be ideal for all kinds of synthetic peptides. Therefore, it is quite a challenge to find out the best solvent for distinct peptides.

A series of novel solvents, so-called “room temperature ionic liquids (RT-ILs)” (generally also “ionic liquids”, ILs) referred to as salts with a melting point under room temperature, has been introduced as potential alternatives to conventional organic solvents recently. ILs exhibit varying physical and chemical properties, such as low viscosity, negligible vapor pressure, non-flammability, high thermal and chemical stability. Significantly, ILs have also good dissolubility for a wide range of polar as well as non-polar compounds and biological macromolecules, too. Due to the outstanding features of ILs, several attempts have been made to use them in chemical peptide synthesis, however, these were primarily focused on solution synthesis for the generation of small peptides possessing less than four amino acids. The studies of peptide synthesis in ILs are thus still not comprehensive. Therefore, in the first part of this thesis the compatibility of the Native Chemical Ligation strategy for combining peptide fragments at a X–Cys ligation site (X = any amino acid) should be analyzed and compared to the conventional ligation strategy. In the second part, the initial attempts of lactam bridge formation of cyclic peptides in ILs should be investigated with the aim to complete the insufficient knowledge regarding peptide cyclization in ILs. Taken together, this thesis should give an impression on how the application of ILs for both reaction types provides essential contributions to the field of peptide chemistry employing alternative reaction media.

2 State of Knowledge

Today, therapeutic compounds on the market are divided into two categories: low molecular weight compounds (small molecules) and large (bio)molecules (also biologics or biopharmaceuticals).[1] Classical small molecules have been extensively studied as drugs over the past decades. They can generally be manufactured easily and still dominate the pharmaceutical market today. Biologics are commonly limited to peptides and proteins, such as hormones, vaccines and antibodies. Mass production of peptides and proteins, however, is more challenging compared to small molecules (Table 2.1).[1,2] Most biologics are unstable and require special storage and handling. Peptides and proteins can be degraded by ~600 different proteases *in vivo* before they reach their target.[3] Hence, there were only a handful of commercial biologics on the market until 20-30 years ago, including e.g. insulin, human growth hormone, hepatitis B vaccine, α-interferon and tissue plasminogen activator (tPA).[4]

Table 2.1 Characteristics of biological drugs compared to small molecule-based drugs.[1,2]

	Small molecule-based drugs	**Biomacromolecule-based drugs**
Molar mass	low (up to 500 g/mol)	high (>> 500 g/mol)
Structure	Simple	complex
Modification	well defined	many options
Analysis	complete	difficult, partially incomplete
Manufacturing	chemical synthesis, relatively simple and cheap	isolation from natural sources, biochemical, biotechnological or chemical production, expensive and complicated
Stability	mostly stable	often unstable, sensitive
Immunogenicity	mostly non-immunogenic	possibly immunogenic

Historical data from the American Food and Drug Administration (FDA) revealed that high failure rates (>90%) of novel drug candidates in their clinical trials and the approval process result in exorbitant costs (Figure 2.1-B,C) and long cycle time of current drug development (Figure 2.1-A).[5-9] However, according to the market reports,[6,9] biological drug candidates perform with higher success rates than small molecules at all phases of development (Figure 2.1-A). More than 13% of large biomolecules have been successfully approved by the FDA. This final pass rate is about twice that of small molecule drugs (approx. 7%). Thus, the large biomolecules have attracted increasing attention from global pharmaceutical companies.

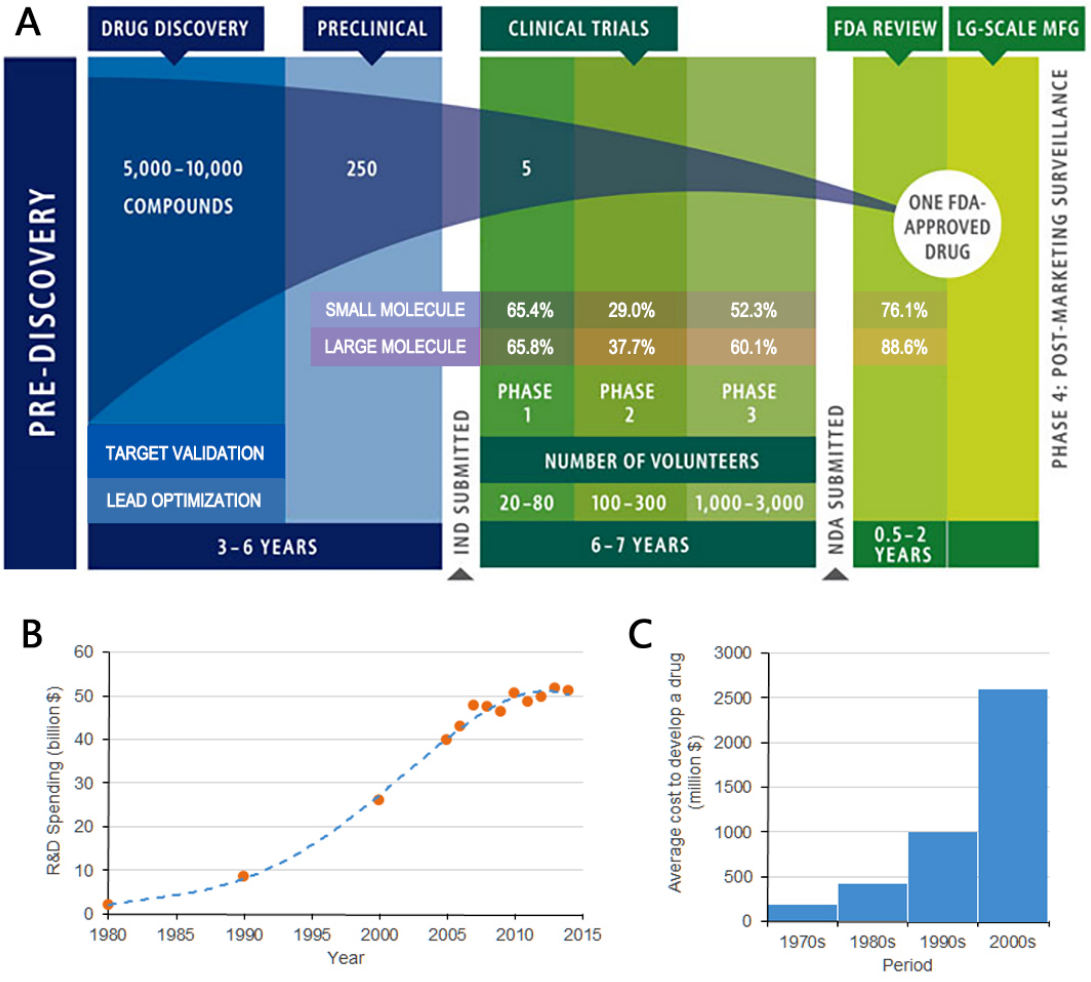


Figure 2.1 (A) Overview of the drug development process (adaptation based on resource[5]) and phase success rates by molecule size;[6] (B) Historical trend of drug research and development spent (USA) from 1980 - 2014;[7] (C) Historical trend of the average cost to develop one drug (USA) from 1970s - early 2010s.[8,9]

The market report “2014 FDA Drug Approvals”[10] revealed that the proportion of approved biologics has obviously increased from 8% in 1993 to 37% in 2014 (Figure 2.2-A). It needs to be highlighted that among biologics, the peptide drugs have become the second largest category of approved drugs besides the small molecule drugs on the market in 2012 (Figure 2.2-B).[11]

Peptides are defined as biomacromolecules composed of amino acid residues which are linked together via peptide bonds between their respective carboxyl and amino functional groups. The structural organization of peptides and proteins, however, differs in that peptides generally possess a greater flexibility and, in turn, different structural and functional roles compared to proteins,[12] though exceptions of highly structured and conformationally constrained peptides such as toxins stabilized by several disulfide bonds exist. Peptides, which fill the molecular weight gap between conventional small molecule drugs (<500 Da) and biologics (>5000 Da),[13] have been approved in the treatment of various diseases, such as cancer, cardiovascular, and metabolic disease (Figure 2.2-C).[14] Since the successful commercialization of the peptide hormone insulin in 1982, the number of patent applications for peptide drugs has raised significantly (Figure 2.2-D).[15] More than 60 peptide drugs have reached the market and several hundreds of novel peptide candidates are now in preclinical and clinical trials.[16] This can be exemplified with diagnostic reagent γ-GT II (dipeptide glycylglycine used for diagnosis and/or prediction of osteoporosis),[17] Prialt® (SNX-111 or ziconotide, 25mer peptide N-type calcium channel blocker)[18] and Fuzeon® (enfuvirtide, 36mer antiretroviral peptide).[19] A market research from Japan[20] forecasts that, compared to the statistics of 2012, the market of patented peptide drugs will grow up with a rate of 44% in 2020 and benefiting from a huge number of drug patent expirations in 2020, the market size of generic peptide drugs will then be tripled (Figure 2.2-E).[20]

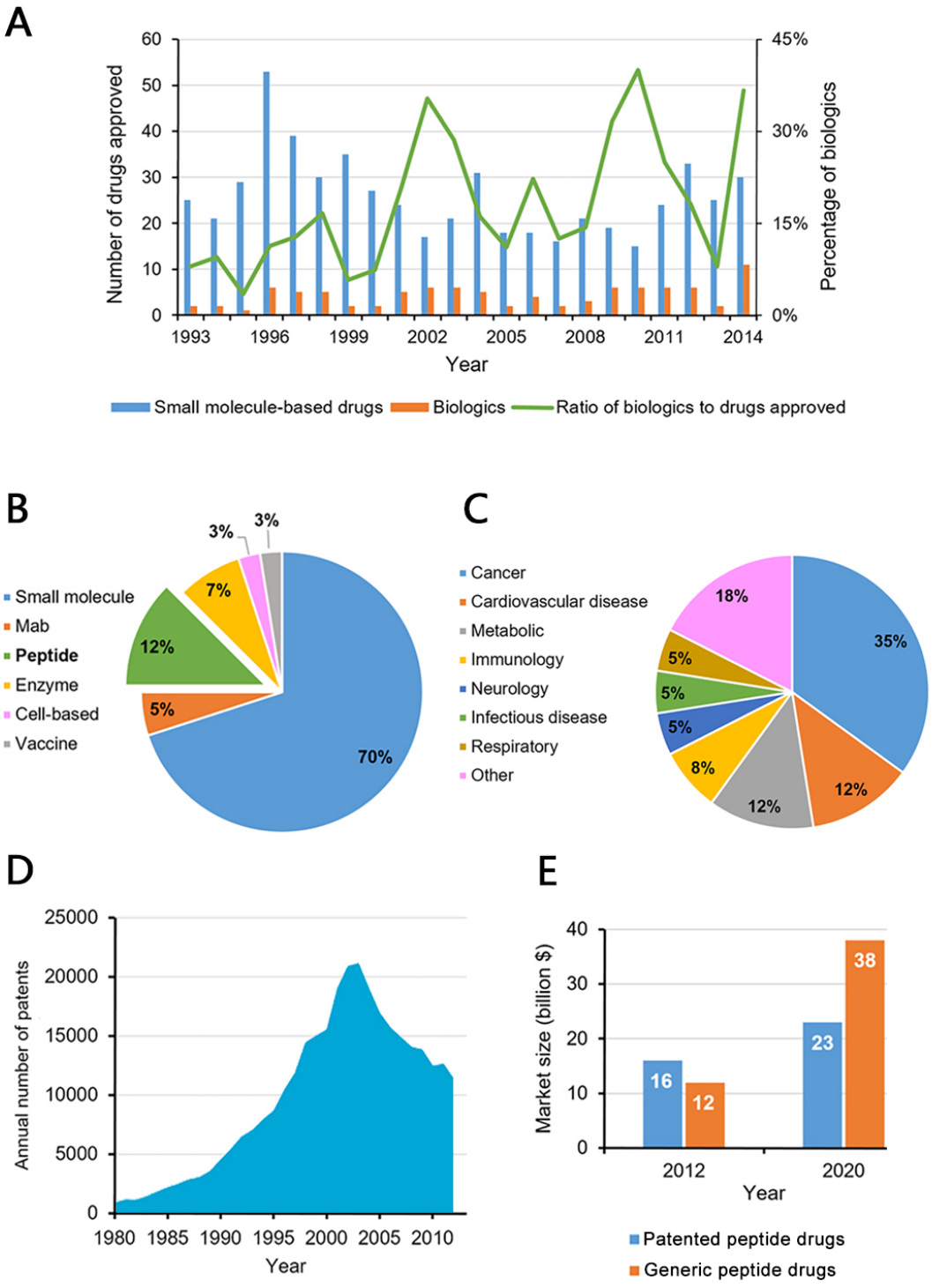


Figure 2.2 (A) Small molecule-based drugs and biologics license applications approved by the Center for Drug Evaluation and Research (CDER) since 1993 and the corresponding historical trend of approved biologics percentages;[10] (B) Modality breakdown of novel drug approvals in 2012;[11] (C) Global peptide drugs market revenue by therapeutic indications in 2014;[14] (D) Historical trend in patent applications for therapeutic peptides from 1980 until 2012 (adaptation based on resource[15]); (E) Forecasting of market shares for patented and generic peptide drugs.[20]

2.1 Peptides as drugs

Compared to common small molecule drugs, peptide drugs have both, potential advantages and disadvantages.[21] A primary reason for the increasing interest in peptides and small proteins is that they bind with exquisite specificity to their *in vivo* targets, resulting in exceptionally high potencies of action and relatively few off-target side effects.[22] Peptides have been found to possess different beneficial properties such as antimicrobial, anticancer, antioxidant, antithrombotic, anti-hypertensive, mineral-binding, and immunomodulation.[23,24] However, applications of synthesized peptides have been severely limited by their low systemic stability, solubility problems, high clearance, poor membrane permeability, low oral bioavailability, and high production costs. Thus, manufacturing of peptides is the largest bottleneck for the commercialization of many potential peptide drugs (Table 2.2).[21]

Table 2.2 Advantages and disadvantages of peptides as therapeutics (adaptation based on resource[21]).

Advantages	**Disadvantages**
High activity	Low oral bioavailability
High specificity	Injection required
Broad spectrum activity with few side effects	Low systemic stability
Minimization of drug-drug interactions	Difficult delivery, poor membrane permeability
Less accumulation in tissues and organs	
Lower toxicity than small molecules	Solubility challenges
Often potent	High clearance
Biological and chemical diversity	Immunogenic effect

2.2 Chemical synthesis of peptides

Currently there are various primary technologies for peptide production, such as extraction from natural sources[25], combinatorial biosynthesis[26] and chemical synthesis, available.[27,15] Since it is relatively difficult to isolate and characterize natural peptides from the respective biological sources, many large peptides, such as insulin[28], somatostatin[29] and other hormones,[30] have been produced through combinatorial biosynthesis. Compared to the biosynthesis of synthetic polypeptides, chemical synthesis is a feasible technology for the production of small and medium-sized peptides ranging from about 5 to 80 residues,[31] especially for the synthesis of natural peptides which are difficult to express in bacteria, peptides containing unnatural amino acids or D-amino acids and peptide backbone modification (Table 2.3).

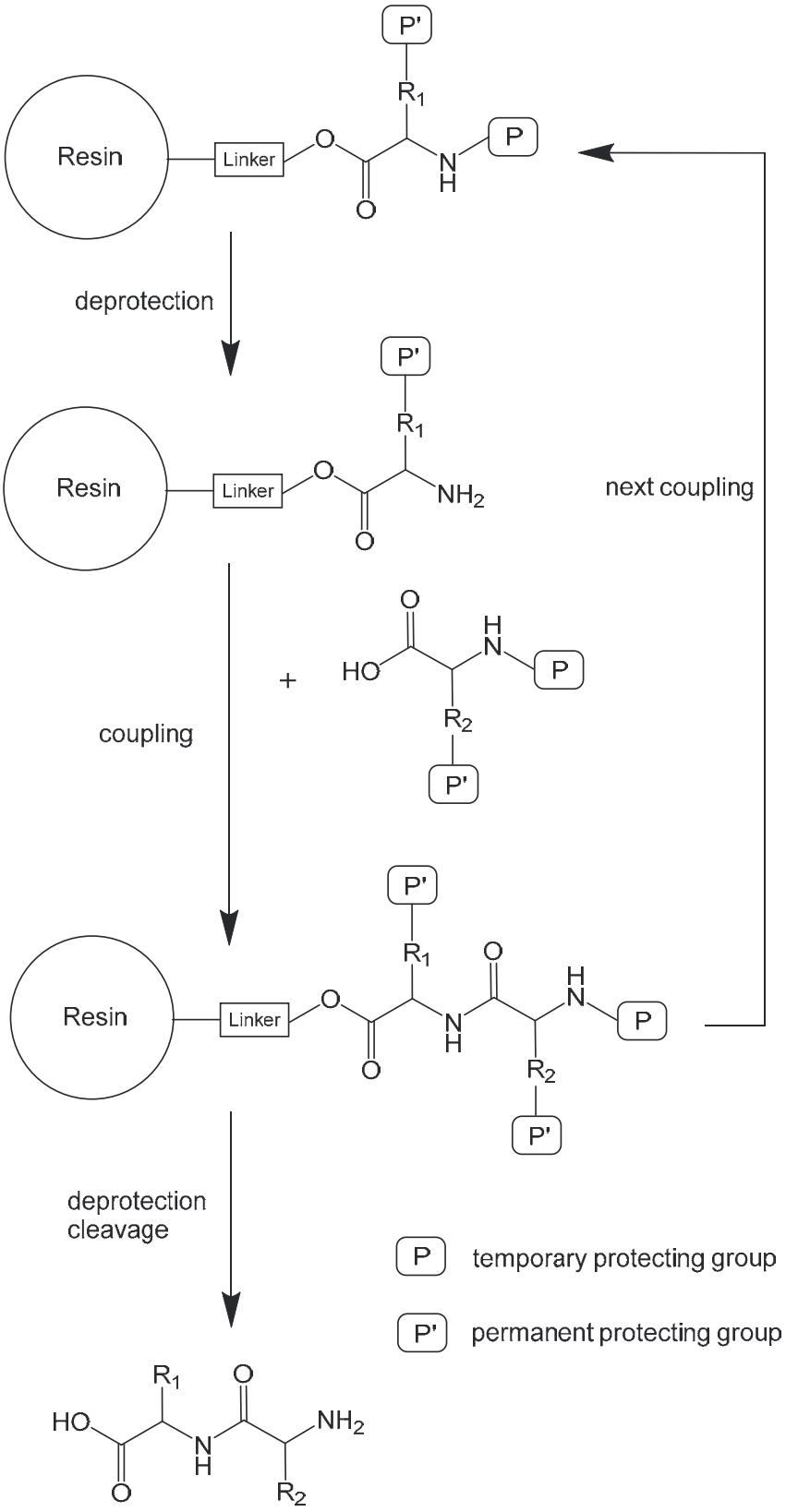
Table 2.3 Advantages and disadvantages of primary synthetic approaches to peptide production.[15]

Extraction from nature	Combinatorial biosynthesis	Chemical synthesis
Choice for selected natural peptides	Choice for production of long polypeptides and proteins	Choice for production of short and long peptides
Limited sources of raw materials	Low cost of raw materials	Relatively high cost of raw materials
Difficult purification	Difficult purification	Easier purification, higher purity
Unstable yields	Low yields	Good yields, low yields for production of long peptides
Large batch production	Large batch production	Small batch production, time-consuming
Safe	Safe and clean	Possibly toxic reagents

2.2.1 Overview of available methods

Chemical peptide synthesis is commonly considered as the formation of peptide bonds which link amino acid residues together between their respective carboxyl (N-terminal amino acid) and amino (C-terminal amino acid) groups. The history of technologies developed for chemical peptide synthesis dates back to more than 100 years ago,[32] and they are still widely used in peptide production so far. Chemical peptide synthesis can be divided into solid phase synthesis and solution phase synthesis.

In 1963, Robert Bruce Merrifield pioneered solid-phase peptide synthesis (SPPS),[27] which consists of repeated cyclic processes resulting in the elongation of a peptide chain anchored to an insoluble resinous polymer by successive couplings of amino acids until the desired peptide sequence is assembled completely (Scheme 2.1).[33] Protecting groups are required to temporarily shield the α-amino group and side-chain functionalities of amino acids in SPPS method, so as to avoid polymerization of amino acid excesses used to ensure complete coupling. Two most common types of protecting groups are the N-*tert*-butyloxycarbonyl (Boc)[34] and fluorenyl-9-methyloxy-carbonyl (Fmoc),[35] which can be easily cleaved. It is generally considered that the length limitation of peptides prepared by SPPS is approximately 50 amino acid residues (limit may fluctuate depending on individual peptide sequence) in order to obtain a satisfying yield of final product. Even though 66mer,[36] 95mer[37] or even longer polypeptides[38] have been reported to be also synthesized by SPPS method, the overall yields of these peptides are still relatively low or unstable.



Scheme 2.1 A flow diagram of solid phase peptide synthesis (SPPS) (adaptation based on resource[27]).

There are two types of solution phase synthetic approach: stepwise assembly and fragment assembly.[39.40] The stepwise assembly of peptides in solutions, which is usually regarded as an “old” or “classical” peptide synthesis method, has been replaced by mature SPPS technology in most cases. In general, the side chains of peptide fragments should also be protected during each synthesis step. In the method of stepwise assembly, coupling reactions between amino acids are carried out in solution instead of the polymer support used in SPPS. The classical stepwise assembly method is still valuable for the synthesis of small peptides like dipeptides, tripeptides, for some large-scale manufacturing and for specialized laboratory applications today.[41]

The second approach is the fragment assembly. The concept is to connect the synthesized peptide fragments via amide (peptide) or other chemical bonds,[42] e.g. peptide thioester[43] or thiazolidine ester.[44] Long peptide chains can be formed by the coupling of small protected or even unprotected peptide fragments. Among them, several fragment ligation methods, which have been developed to make the ligation of unprotected peptide fragments possible, will be introduced in the next section.

2.2.2 Ligation of peptide fragments*

In the ligation methods, unprotected peptide segments can be coupled orthogonally to form long peptide chains or large proteins, usually in good yield (42-95% total yield) and of high purity.[36,45,46] Because of their superior advantages, these methods have been widely used in the synthesis of peptides and small proteins, such as cytochrome b562 (106 AAs),[47] His-tagged interleukin-2 (133 AAs)[48] and insulin lispro (51 AAs).[49]

The ligation methods were commonly divided into the ligation methods using imine and thioester capture strategies.[42] The most frequently used ligation methods for peptide synthesis have been summarized in Table 2.4,[51] and individual approaches of these methods are further illustrated in details as for example thioester ligation (Scheme 2.2), thioether ligation (Scheme 2.3-A) and imine ligation (Scheme 2.3-B,C,D,E,F), respectively.[51] Since no natural amino acid residue can be obtained at the ligation site in case of the imine and thioether ligation methods, thioester-based ligation is by far the more commonly used method in peptide synthesis.

The first thioester ligation, in which an amide bond was formed upon the reaction of valine-S-phenylalanine with cysteine, was observed by Theodor Wieland and coworkers already in 1953.[50] The reaction mechanism of a thioester-based ligation is a procedure involving two elementary steps. In the initial step, an intermediate, such as a covalent thioester or perthioester, is formed by two segments with the functionalities of a thioester or its analogues and a thiol nucleophile. This step is usually reversible. After a rapid S-N acyl rearrangement step, the thiol bond of the intermediate is transformed into an amide bond spontaneously and irreversibly. After about 40 years, various ligation technologies, such as cysteine, selenocysteine, methionine, glycine and histidine ligations have been developed based on the thioester-based

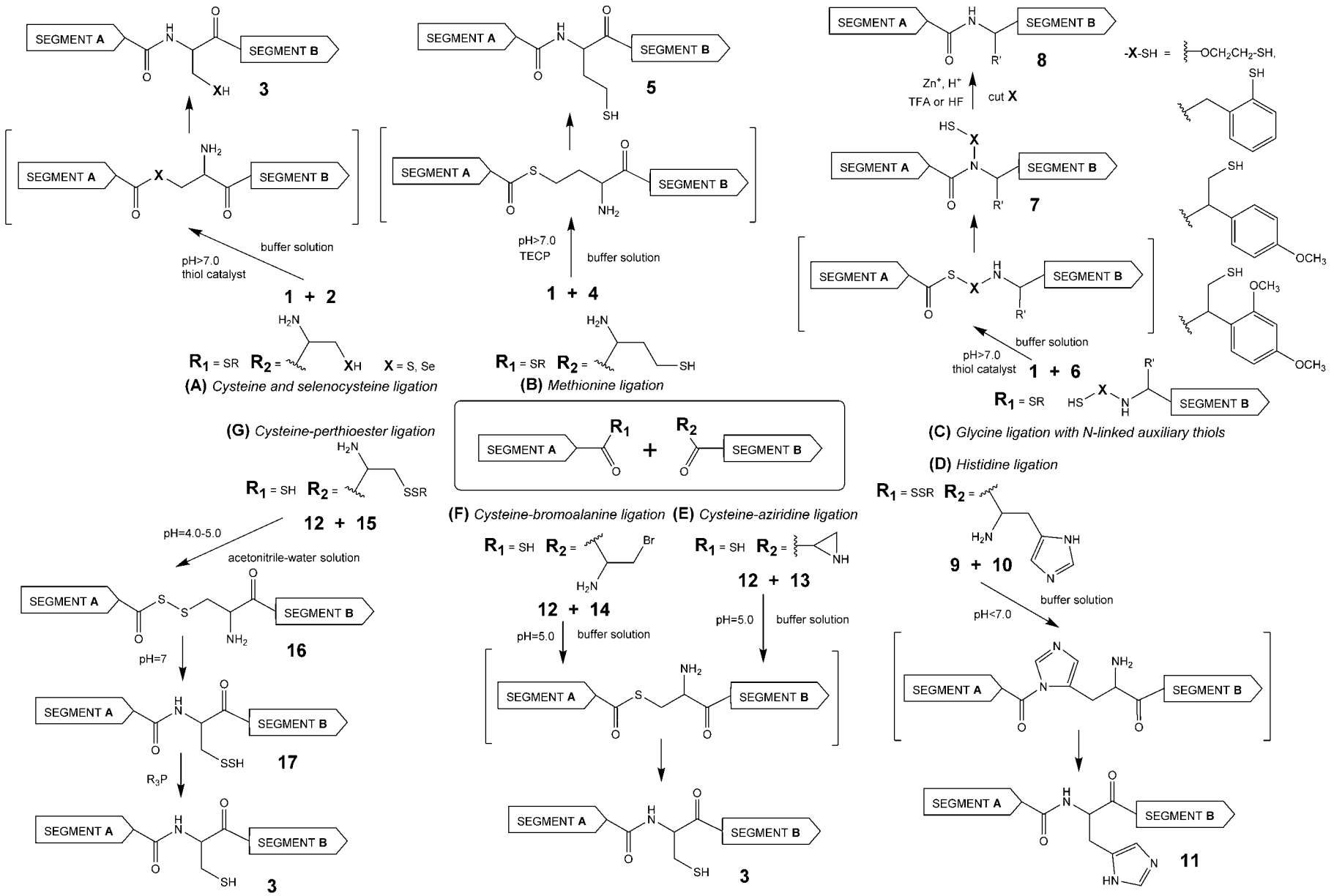
* The full text of this capture was modified from the author's publication[51]

method for producing unprotected peptides. Among them, the cysteine ligation has become the most popular and convenient method for chemoselective synthesis of polypeptides and proteins.

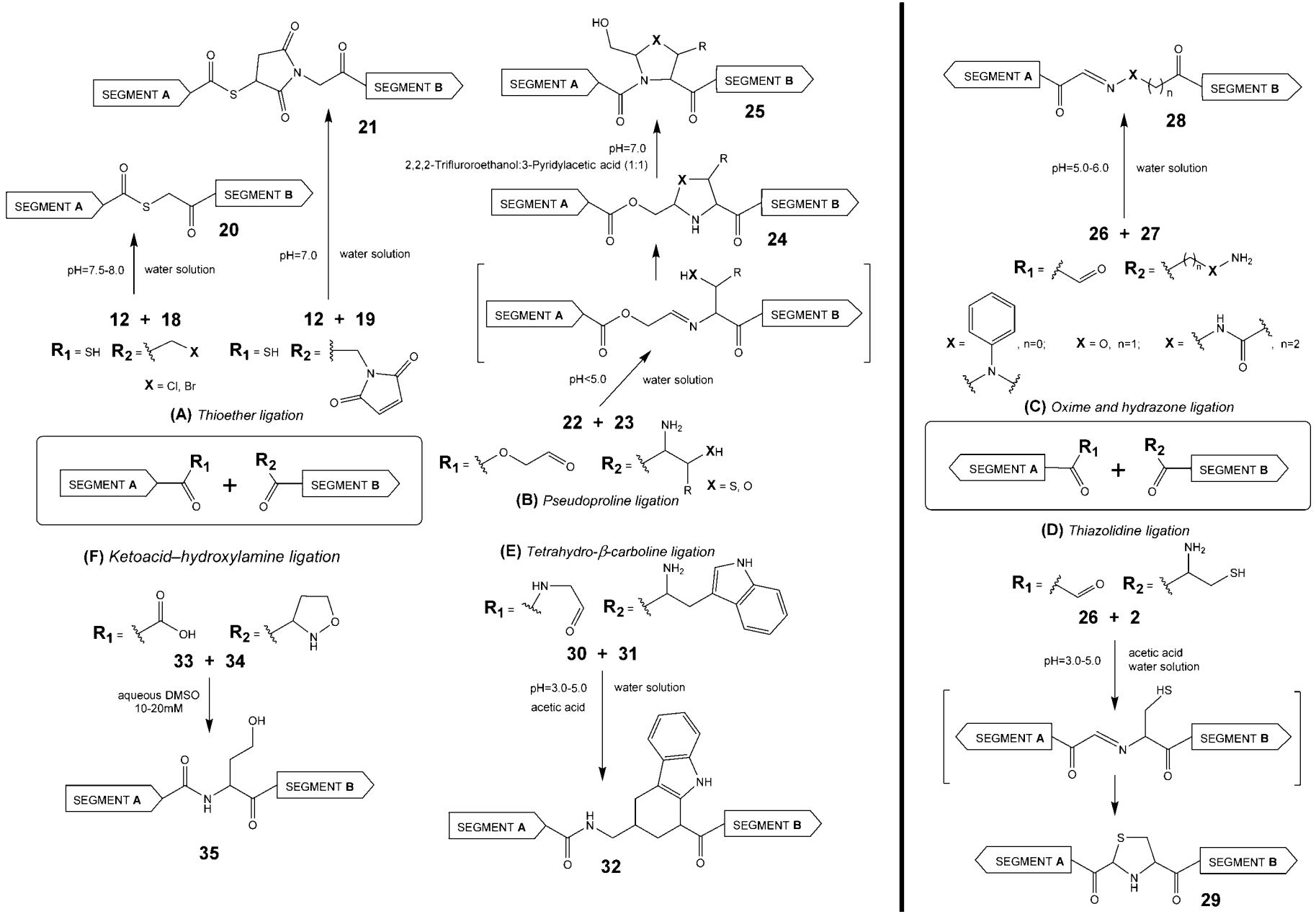
Table 2.4 Imine and thioester capture strategies for ligation methods (adapted with permission from the author's publication[51]).

Method	Segment	Product	Example[a]	Ref.[a]
Cysteine and selenocysteine ligation	**1 + 2**	**3**	Human interleukin 8 (IL-8)	1994
Methionine ligation	**1 + 4**	**5**	Parathyroid hormones	1998
Glycine ligation with N-linked auxiliary thiols	**1 + 6**	**7**	Gly12-brain natriuretic peptide$_{5–26}$, porcine	2007
Histidine ligation	**9 + 10**	**11**	-	-
Cysteine-aziridine ligation	**12 + 13**	**3**	-	-
Cysteine-bromoalanine ligation	**12 + 14**	**3**	-	-
Cysteine-perthioester ligation	**12 + 15**	**16**	K48-linked diubiquitin	2010
Thioether ligation	**12 + 18, 19**	**20, 21**	HIV-1 protease	1992
Pseudoproline ligation	**22 + 23**	**24**	Model 50-residue peptide	1994
Oxime and hydrazine ligation	**26 + 27**	**28**	-	-
Thiazolidine ligation	**26 + 2**	**29**	-	-
Tetrahydro-β-carboline ligation	**30 + 31**	**32**	-	-
Ketoacid–hydroxylamine ligation	**33 + 34, 35**	**36, 37**	-	-

[a] Selection is based on a peptide length larger than 20 amino acids.



Scheme 2.2 (A) Cysteine and selenocysteine ligation; (B) Methionine ligation; (C) Glycine ligation with N-linked auxiliary thiols; (D) Histidine ligation; (E) Cysteine-aziridine; (F) Cysteine-bromoalanine ligation; (G) Cysteine-perthioester ligation (reprinted with permission from the author's publication[51]).

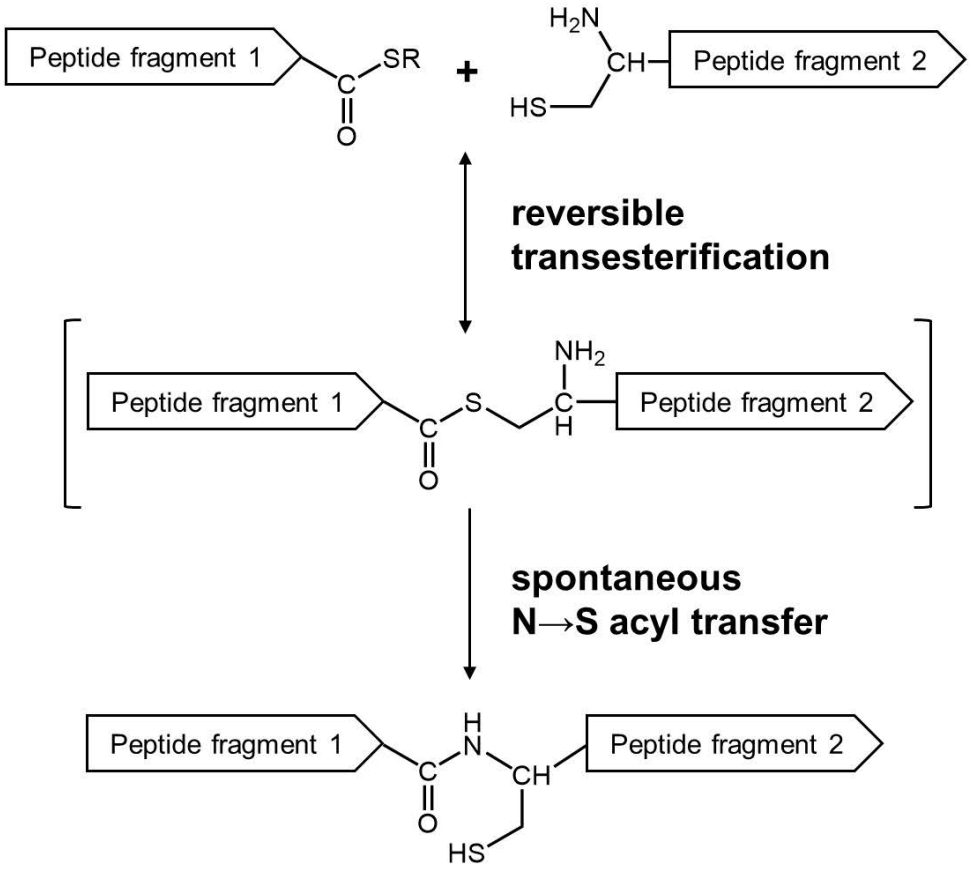


Scheme 2.3 (A) Thioether ligation; (B) Pseudoproline ligation; (C) Oxime and hydrazine ligation; (D) Thiazolidine ligation; (E) Tetrahydro-β-carboline ligation; (F) Ketoacid-hydroxylamine (reprinted with permission from the author's publication[51]).

In 1994, a cysteine ligation method for peptide synthesis, the so-called “Native Chemical Ligation” (NCL), was introduced by Stephen Kent’s laboratory.[52] In NCL method, a long peptide containing cysteine can be formed directly by an unprotected peptide-α-carboxy thioester and an unprotected peptide segment with an N-terminal cysteine residue. Just like the introduced mechanism of other thiol-based ligations, these two peptide segments first combine with each other via reversible transthioesterification and a subsequent irreversible S-N acyl shift giving a “native” peptide bond as well as a cysteine residue at the ligation site (Scheme 2.4).[52]

In order to carry out an NCL reaction, the conditions of neutral pH (pH 6.5 to 7.5) and aqueous phosphate buffer containing a chaotrope, e.g. guanidine hydrochloride or urea are generally necessary.[52,53] Moreover, the addition of thiol additive(s), such as benzyl mercaptan, thiophenol and 4-mercaptophenyl acetic acid (MPAA), are also required to keep the cysteine side chains in a reduced state and to accelerate the initial transthioesterification step (Scheme 2.4).[53,54]

The NCL method, which can overcome the length limitation of peptide synthesis occurring for the SPPS method, has become an efficacious technology for the synthesis of polypeptides and proteins of a size ranging from 50 to approximately 150 amino acids, such as the recently reported pore-forming antimicrobial protein caenopore-5 (82 AAs),[55] the sialic-acid-binding lectin siglec-7 (127 AAs),[56] human interleukin-6 glycoprotein (183 AAs)[57] and polydiscamides B, C, D from the sponge *Ircinia sp.* (13 AAs).[58]



Scheme 2.4 General mechanism of Native Chemical Ligation.[53]

The recent reports[59-61] showed that the type of the residue on the C-terminus of the thioester segment could strongly influence the yield of product in NCL. Hackeng, T. M. *et al.* found that the ligation at a -Pro-Cys- site is relatively difficult to be performed if applying NCL.[60] The formation of a high level of β- and γ-linked byproducts have been also found in native ligation at -Glu-Cys- and -Asp-Cys- sites by using classical additives like thiophenol,[59] whereas the β- and γ-isomer formation occurring at the "-Glu-Cys-" site could be prevented by utilizing the additive 4-mercaptophenylacetic acid (MPAA).[61] However, MPAA has no advantage regarding the avoidance of the formation of β-linked byproduct in the ligation reaction at the "-Asp-Cys-" site.[61] Recent improvements from Nakamura *et al.* revealed that increasing the concentration of MPAA and the reaction temperature could facilitate the reaction rates of peptide ligation.[62]

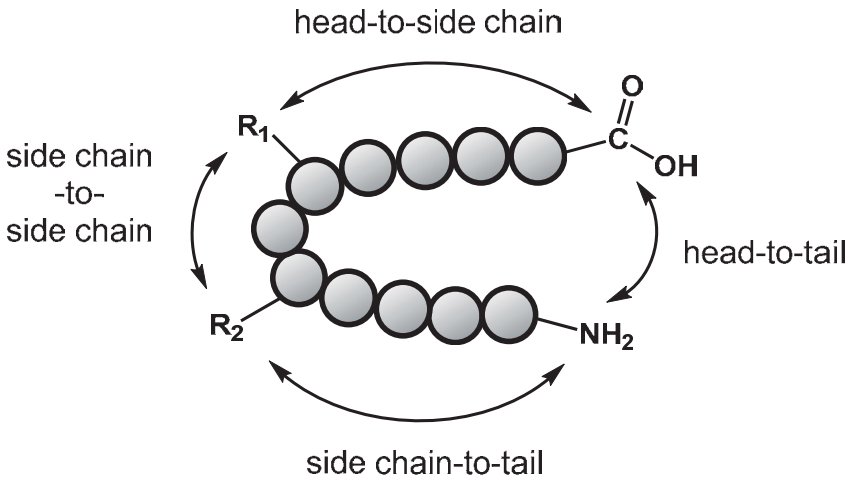
2.2.3 Preparation of cyclic peptides

The applications of linear peptides as pharmaceuticals or therapeutic agents are limited because of their poor cell permeability and *in vivo* instability.[63] In contrast to linear peptides, cyclic peptides or cyclic peptide analogues have higher resistance to the degradation by exo- and endo-proteases.[64] Since the first discovery of the cyclic peptide gramicidin S in the 1940s,[65] cyclic peptides found exhibited a wide spectrum of biological activities and behaved as potential lead compounds of hormones,[66] antibiotics,[67] ionophores,[68] antifungals,[69] and anticancer drugs.[70] Their application scope has been also expanded to the fields of therapeutic agents,[71] nanomaterials,[72,73] and supramolecular self-assembly.[74] It is worth noting that disparate biochemical functions could be realized by fine-tuning of the cyclic peptide conformations using the techniques of chemical synthesis.[64] However, until now, it is still a difficult challenge to prepare cyclic peptides by using common synthetic methods.[31]

In theory, peptide macrocycles can be closed also by the similar strategies used for linear peptide assembly, while in most cases the peptide cyclization has to face the following actual problems. For small-to-medium-sized rings, the ground-state E geometry of the peptide bond causes supernumerary high angle strain of ring-shape conformation, which prevents the formation of the cyclized peptide. For larger rings, reaction conditions need to be carefully controlled to avoid the unwanted intermolecular combination between peptide molecules.[64]

Just like the ligation methods for linear peptides, there is a great variety of cyclization methods available. Among them, the most popular macrocyclization methods are lactamization, lactonization and disulfide bond formation. Depending on the site of cyclization, the four commonly used methods are the most frequently used: head-to-tail, head-to-side-chain, side-chain-to-tail and side-chain-to-side-chain (Scheme 2.5).[64] Currently, the most common

cyclization methods of peptides are head-to-tail via a lactam bridge and side-chain-to-side-chain via a disulfide bond.[64,75]



Scheme 2.5 General synthetic considerations for peptide cyclization (adaptation based on resource [64]).

Peptide cyclization with formation of one or more disulfide bridges is usually observed in folding of the peptides bearing cysteine residues. Cysteine can be easily oxidized on air in solution to form a dimer called cystine. In contrast to the formation of disulfide bridges, head-to-tail cyclization via a lactam bridge usually requires relatively complex and special conditions. Cyclization is suggested to be performed under high dilution (10^{-3}-10^{-4} M) conditions so as to protect against dimerization and oligomerization.[75] Furthermore, it is critical to choose a suitable coupling reagent for an effective head-to-tail cyclization reactions (Table 2.5).[75] There are two sorts of coupling reagents, which have been frequently used in cyclization (Table 2.5).[75,76] The first are the phosphonium derivatives of reagents such as PyBOP and PyAOP which have been used as efficient coupling reagents in solution-phase cyclization of e.g. pentapeptides with good yields (52-56%).[77] The other kind are so-called uronium derivatives, such as HBTU and HATU, which are well-known as coupling reagents for solid-phase cyclization. In general, no excess of uronium salts is required in cyclization (1 equiv. of coupling reagent in the presence of 2 equiv. of tertiary base).[78] In addition, the solubility of the

cyclic peptides in conventional solvents is another challenge during synthesis. It has been reported recently that the three-dimensional conformational change in cyclic peptides can be induced by different reaction media.[79]

Table 2.5 Recent popular coupling reagents for peptide cyclization via lactam bridge formation (adaptation based on resource[75]).

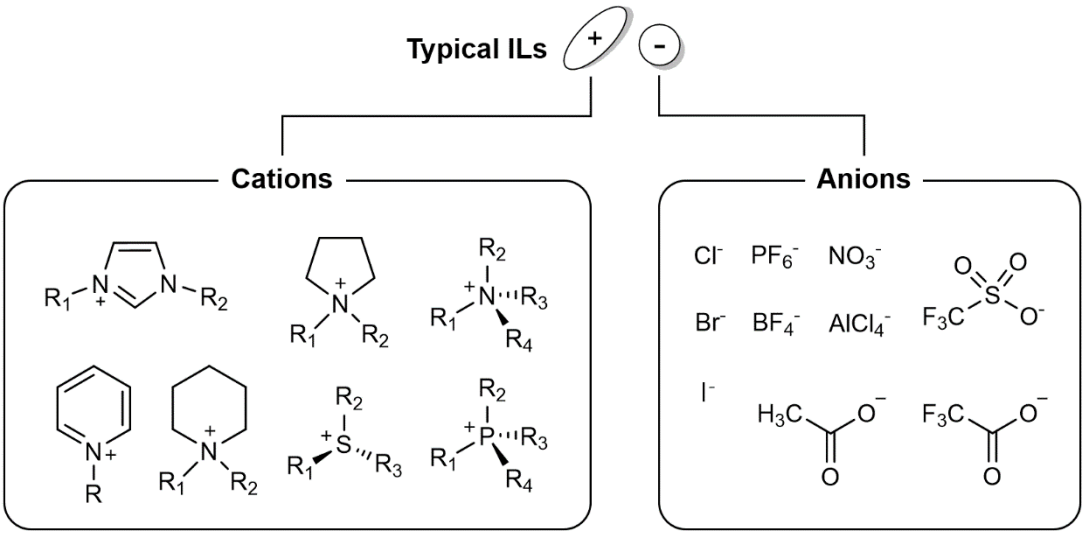
Phosphonium derivatives	**Uronium derivatives**
1-benzotriazole-tris-dimethylaminophosphonium hexafluorophosphate (**BOP**)	O-(benzotriazol-1-yl)-1,1,3,3 tetramethyl uronium hexafluorophosphate (**HBTU**)
1-benzotriazolyloxy-tris-pyrrolidino phosphonium hexafluorophosphate (**PyBOP**)	O-(benzotriazol-1-yl)-1,1,3,3 tetramethyl uronium tetrafluoroborate (**TBTU**)
7-azabenzotriazol-1-yloxy trispyrrolidino phosphonium hexafluorophosphate (**PyAOP**)	O-(7-azabenzotriazol-1-yl)-1,1,3,3 tetramethyl uronium hexafluorophosphate (**HATU**)
7-azabenzotriazol-1-yloxy-tris-dimethyl aminophosphonium hexafluorophosphate (**AOP**)	O-(7-azabenzotriazol-1-yl)-1,1,3,3 tetramethylene uronium hexafluorophosphate (**HAPyU**)
	O-(7-azabenzotriazol-1-yl)-1,1,3,3 pentamethylene uranium hexafluorophosphate (**HAPipU**)

2.3 Ionic liquids (ILs) as alternative reaction media

A series of novel solvents, so-called “room temperature ionic liquids, RT-ILs” (referred to as only “ionic liquids, ILs” in this thesis), which are salts with a melting point at room temperature, has been introduced as potential alternatives to conventional organic solvents quite recently. ILs have been evaluated as “green solvents”,[80] “solvents for the future”[81] or “designer solvents”[82] attracting increasing interest in the fields of electrochemistry, engineering, catalysis, physical chemistry, and organic chemistry.[83]

2.3.1 Characteristic properties of ILs

ILs are usually comprised of organic cations associated with inorganic anions or organic anions. Common cations used in construction of ILs are unsymmetrically substituted nitrogen-containing cations or onium ions (e.g. imidazolium, pyridinium, pyrrolidinium, quaternary ammonium, quaternary phosphonium or quaternary sulfonium). Anions being constituted in ILs can be halogen ions, hexafluorophosphate, tetrafluoroborate or carboxylate (Scheme 2.6).[84] These ions are poorly coordinated, hence resulting in the solvents being liquid at room temperature. At least one ion has a delocalized charge and one component is organic, which prevents the formation of a stable crystal lattice (Figure 2.3).[85]



Scheme 2.6 Examples of cations and anions commonly found in ILs.[84]

Depending on their diverse combinations of cations and anions, ILs exhibit distinguishing physico-chemical properties, such as relatively low viscosity, negligible vapor pressure, non-flammability, high thermal and chemical stability.[86] Significantly, ILs have good dissolubility for a wide range of polar,[87] non-polar compounds[88] and biological macromolecules (e.g. peptides,[89,90,91] proteins,[92] polysaccharides[93,94] and nucleic acids[95]), as well. The miscibility of IL with water and organic solvents can be regulated with the lengths of side chains on the cation and with the type of anion.[96,97] Thus, some ILs can be recovered from the waste and reused after extraction of the product with another solvent, which is not miscible with the respective IL.[98] It is reported that Lewis base ILs can even be recycled by distillation in some cases.[99]

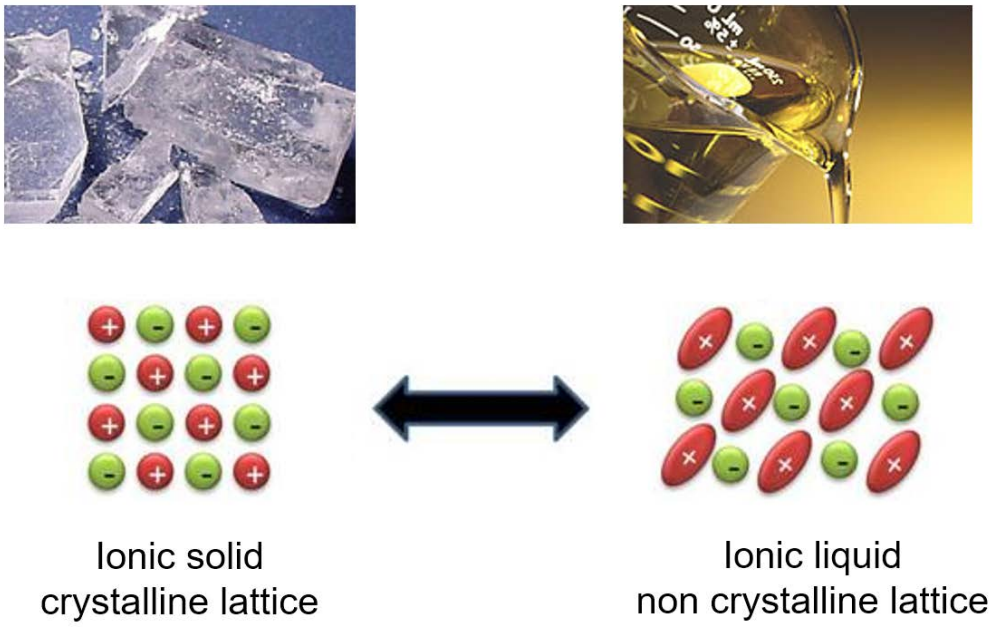


Figure 2.3 Comparison of the characters of ionic solid (crystallized salt) and ionic liquid (adaptation based on resource[85]).

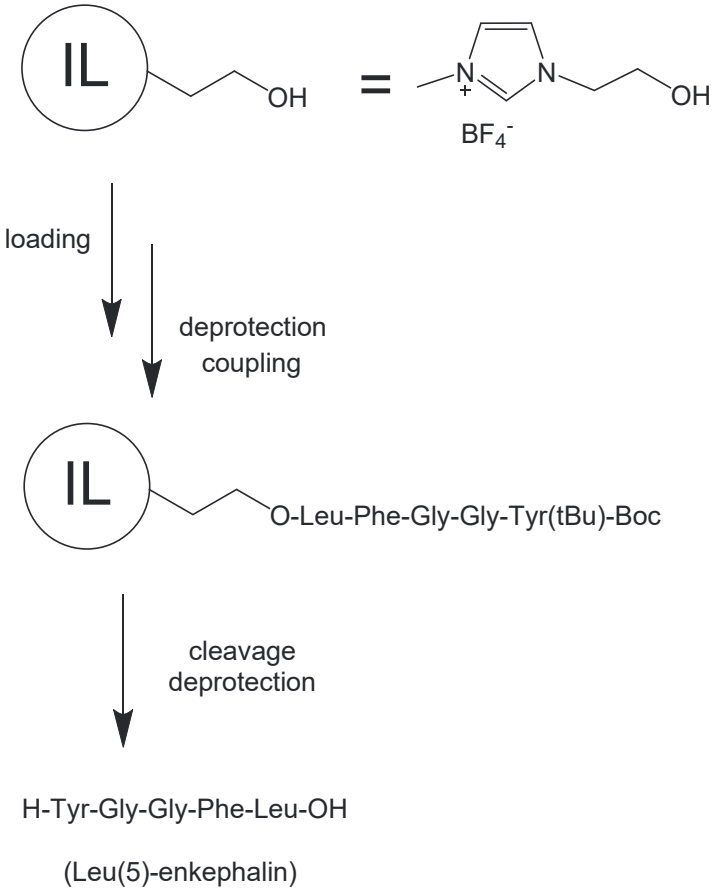
Due to these impressive features, the applications of ILs as reaction media in numerous organic reactions have been explored, including hydrogenation, [100] hydroformylation, [101] oligomerization, [102] oxidation, [103] reduction, [104] ring opening, [105] Aldol condensation, [106] Beckmann rearrangement, [107] Diels-Alder, [108] Friedel-Crafts, [109] Stille, [110] Wittig, [111] Heck[112] and Suzuki reaction,[113] and so forth. The ILs are not only used as solvents, but sometimes also act as precursor of an N-heterocyclic carbene,[114] Lewis acid[115] or Lewis base[99] in organic reactions.

2.3.2 Application of ILs in peptide chemistry

As mentioned above, ILs have an outstanding ability to dissolve biological macromolecules. A recent study showed that ILs could be used as an effective solvent for increasing enzyme activity in enzyme-catalyzed reactions.[116,117,118] ILs have been found to reduce aggregation of proteins and to improve *in vitro* refolding.[119,120] The application of ILs in separation and purification of biomolecules has been also established.[121122,123] These facts demonstrated that ILs became an inimitable solvent for biomolecules.

The successful applications of ILs in biochemistry provide a potential solution for the solvent problem in the chemical synthesis of peptides. In 2004, the first report of dipeptide formation in neat ILs was published by Jean-Christophe Plaquevent and coworkers.[124] After that, the coupling leading to various small peptides was carried out with the addition of distinct additives in an imidazolium-based IL.[125] Weishi Miao *et al.* developed a new method called “IL-supported peptide synthesis” (ILSPS) for producing Leu(5)-enkephalin (a 5-mer endogenous opioid peptide), in which an imidazolium-based IL was used as loading support to replace the solid resin support of the conventional SPPS method (Scheme 2.7).[126] In the ILSPS method, the loading capacity of ILSPS is much higher than in SPPS. As a previous work of our laboratory, tridegin (a 66mer anticoagulant peptide) was successfully synthesized by NCL using an imidazolium-based IL as reaction medium.[36] It needs to be highlighted that the addition of additives has not been required during the NCL reaction here. Furthermore, the influences of different ILs on the oxidative folding yields of μ-SIIIA (a 20mer conotoxin peptide) have been also investigated previously.[127]

Besides the wide use in peptide synthesis, there are numerous applications of ILs in analytical chemistry, which have been recently summarized in a review written by Alesia A. Tietze *et al*..[128]

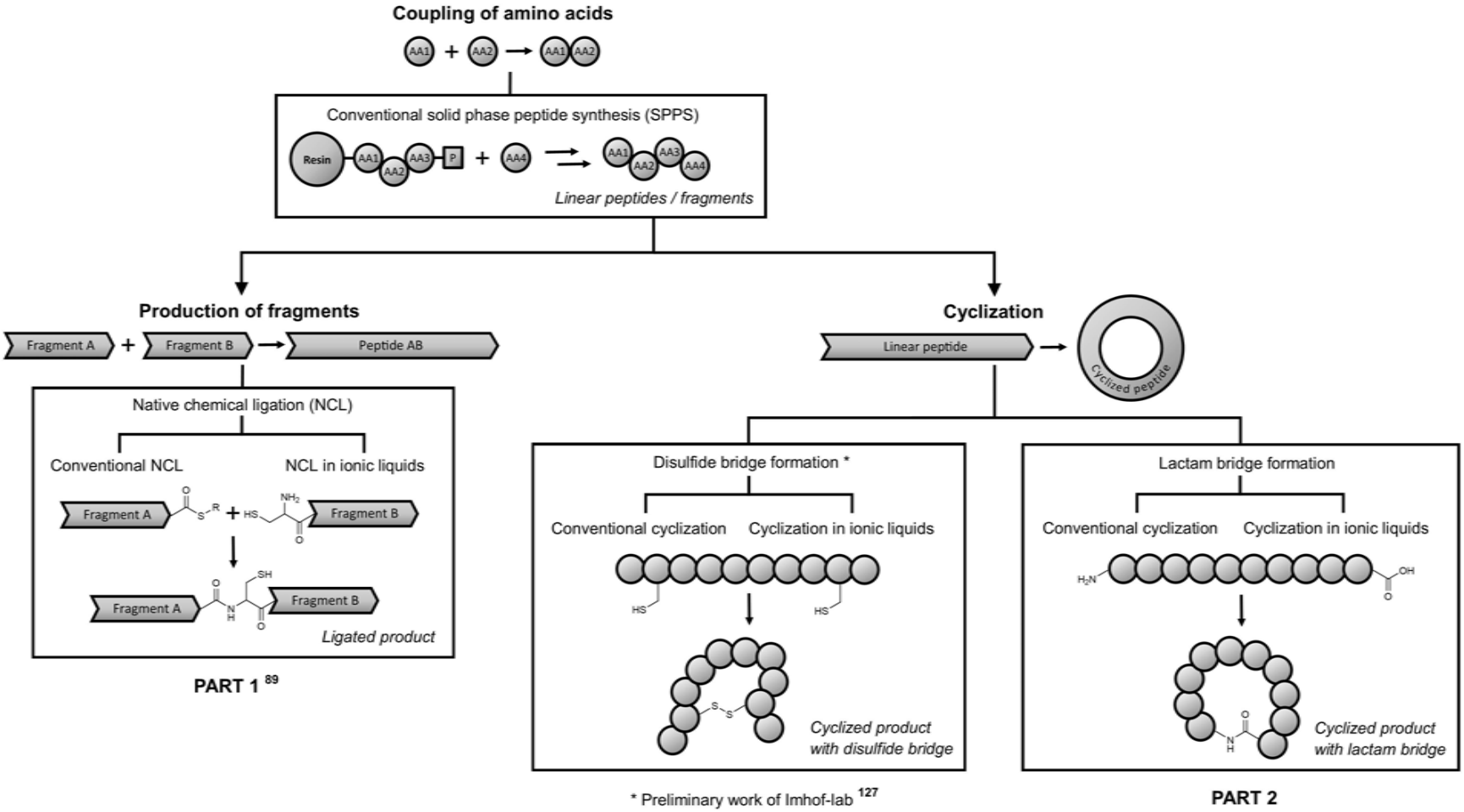


Scheme 2.7 Strategy for the synthesis of Leu(5)-enkephalin using an ionic liquid as a soluble support (adaptation based on resource[126]).

3 Aim of this work

The study on applications of ILs in solution-phase peptide synthesis has previously been expanded in a limited way to the synthetic scopes of Native Chemical Ligation and cyclization of peptides by disulfide bridge formation (Scheme 3.1). However, the work on these topics in IL-supported peptide synthesis is still insufficient in both breadth and depth. It is hard to summarize an employable ligation strategy using an Ionic Liquid with the help of only few examples. Furthermore, no attempt on head-to-tail cyclization of peptides in ILs has been made so far.

The goal of this thesis thus is: 1) to verify the applicability and compatibility of ligation strategy in ILs for X–Cys ligation sites using NCL (Scheme 3.1, PART 1), and 2) to fill the gap of IL applications in peptide cyclization for lactam bridge formation of head-to-tail cyclic peptides (Scheme 3.1, PART 2).



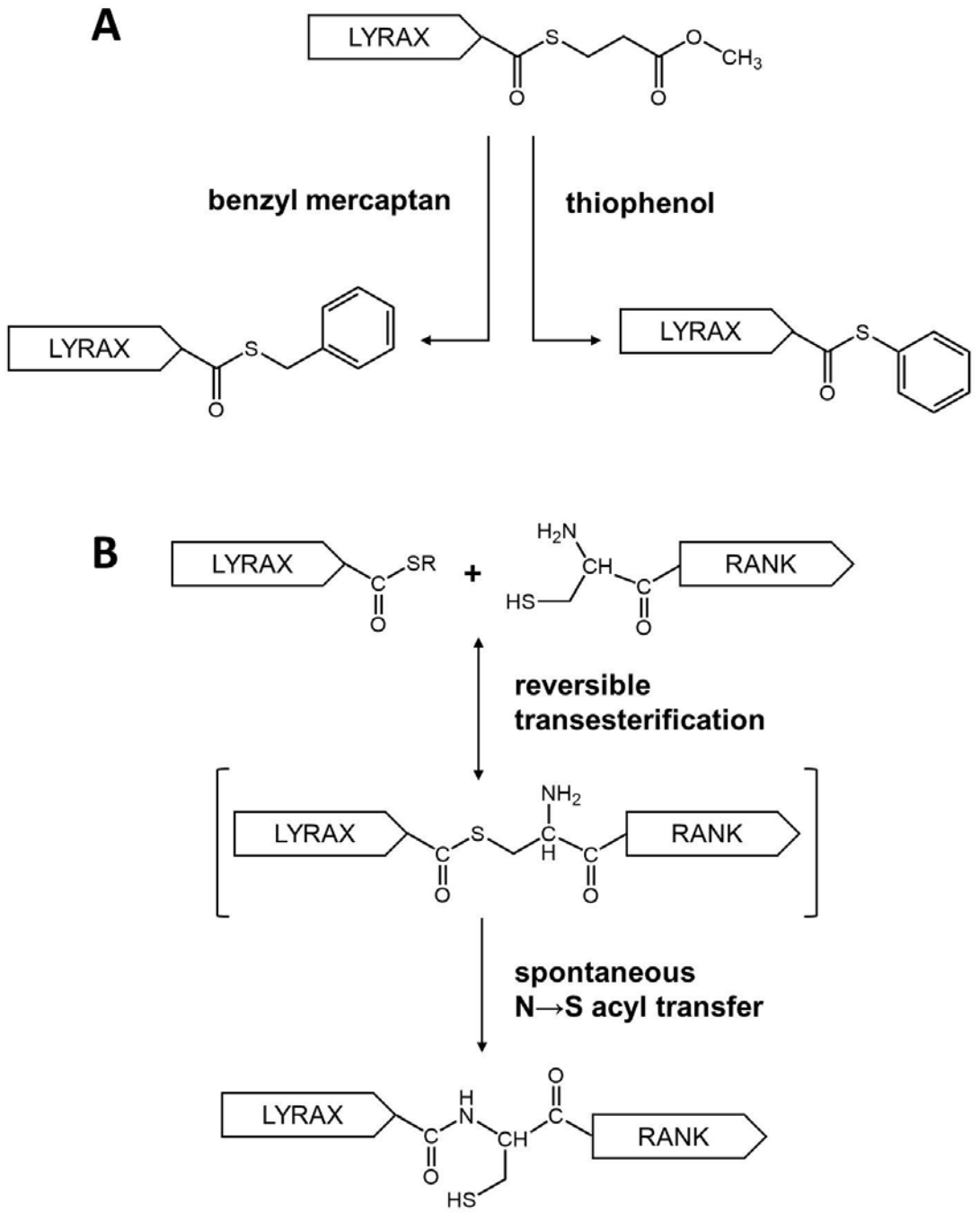
Scheme 3.1 Overview of chemical synthesis of peptides with conventional methods and in ILs forming the background of this thesis.

4 Results and Discussion

4.1 Native Chemical Ligation (NCL) of peptides

In 1999, Hackeng T.M. and his co-workers [60] reported that the amino acid at the C-terminus of the peptide thioester fragment had a great impact on the yield of a conventional NCL reaction. They performed a series of NCL reactions between two unprotected model peptide fragments, namely the N-terminal thioester, which provided a thioester group at its C-terminus, and the C-terminal peptide bearing a cysteine residue at its N-terminus. In these reactions, the thiol group of the cysteine residue of fragment Cys-Arg-Ala-Asn-Lys-NH_2 (referred to as CRANK) attacks the C-terminal thioester of fragment Leu-Tyr-Arg-Ala-Xaa-SR (LYRAX-thioester, Xaa = any amino acid) to form a native amide bond at the ligation site (Scheme 4.1). The desired product of the NCL reactions should be the decapeptide LYRAXCRANK (X = any amino acid). In the experiments of Hackeng *et al.*, the NCL reactions of five LYRAX-peptides (X = pool 1, W, E, D, T, S; pool 2, R, Q, N, P, G; pool 3, F, M, I, V, A; or pool 4, Y, K, L, C, H) have been carried out simultaneously in one tube under 37 °C.[60]

Reaction rates and yields of the individual ligations have been demonstrated by MALDI analysis of the product formation over a time period of up to 72 hours.[60] However, as Hackeng *et al.* annotated in their report,[60] the MALDI analysis is not strictly quantitative for yield determination.



Scheme 4.1 (A) Thioester exchange reactions with benzyl mercaptan and thiophenol. (B) General mechanism of native chemical ligation exemplified for model peptide LYRAXCRANK (X = any amino acid) (adaptation based on resource[60]).

4.1.1 NCL in buffer system

The NCL reactions performed by Hackeng *et al.*[60] were repeated herein in order to provide evidence of accomplished reaction to be compared with the same ligations performed in IL (see chapter 4.1.2). The peptide fragments LYRAG, LYRAA, LYRAL, LYRAF, LYRAN, LYRAQ, LYRAK and CRANK were selected as reactants[89] according to the groups of peptides determined by Hackeng *et al.*:[60] a) ligation of LYRA**F**CRANK completed within 4 hours, b) ligation of LYRA**A**CRANK and LYRA**G**CRANK completed within 9 hours, c) ligation of LYRA**N**CRANK, LYRA**Q**CRANK and LYRA**K**CRANK completed within 24 hours, and finally d) ligation of LYRA**L**CRANK completed within 48 hours (Table 4.1).[89]

Table 4.1 Peptide reactants and ligation products in NCL reactions.

Peptide reactant		**Ligation product**	
Abbrev.	**Sequence synthesized**	**Abbrev.**	**Sequence synthesized**
LYRAG	Ac-**LYRAG**-$SCH_2CH_2COOCH_3$	LYRAGCRANK	Ac-**LYRAGCRANK**-NH_2
LYRAA	Ac-**LYRAA**-$SCH_2CH_2COOCH_3$	LYRAACRANK	Ac-**LYRAACRANK**-NH_2
LYRAL	Ac-**LYRAL**-$SCH_2CH_2COOCH_3$	LYRALCRANK	Ac-**LYRALCRANK**-NH_2
LYRAF	Ac-**LYRAF**-$SCH_2CH_2COOCH_3$	LYRAFCRANK	Ac-**LYRAFCRANK**-NH_2
LYRAN	Ac-**LYRAN**-$SCH_2CH_2COOCH_3$	LYRANCRANK	Ac-**LYRANCRANK**-NH_2
LYRAQ	Ac-**LYRAQ**-$SCH_2CH_2COOCH_3$	LYRAQCRANK	Ac-**LYRAQCRANK**-NH_2
LYRAK	Ac-**LYRAK**-$SCH_2CH_2COOCH_3$	LYRAKCRANK	Ac-**LYRAKCRANK**-NH_2
CRANK	H-**CRANK**-NH_2		

In addition, a buffer system containing thioadditives was prepared as reaction medium. Each reaction was performed at room temperature (20 °C) instead of 37 °C used by Hackeng *et al.*[60] and in an argon atmosphere. The reaction process was monitored by using high-performance liquid

chromatography (HPLC) analysis of the individual reactions in order to optimize the quantification.[89]

It was observed that the NCL reaction of product LYRAGCRANK was finished within 8 hours (Fig4.1A,C), and a ligation yield of 81% was obtained (Table 4.2). However, most of the ligation reactions were still not completed after 3 days. An example among them is the peptide ligation of fragment LYRAL to CRANK which proceeded with an extremely low conversion rate of fragment LYRAL (Fig4.1B,C). According to the experimental results, the C-terminal amino acid of the N-terminal fragment can be considered as an important influencing factor of the ligation rate. A similar finding was also reported by Hackeng *et al.* earlier.[60]

In contrast, the NCL reaction was enhanced for glycine at the ligation site regardless of the reaction temperature (room temperature vs. 37°C) (Table 4.2). In this work, leucine was found to be one of the most hindered amino acids for the NCL reaction at both temperatures (Table 4.2, Fig. 4.1B). Simultaneously, some differences were observed when comparing NCL at room temperature with NCL at 37 °C. The yields of all peptide ligations at room temperature, especially of peptides LYRAXCRANK with X = Ala, Phe, and Gln were obviously lower than the yields obtained at 37 °C. This phenomenon revealed that low temperature was generally unfavorable to the efficiency of the NCL reactions performed in buffer (Table 4.2). In addition, the continuous accumulation of the “activated” intermediates, e.g. peptide thioester with R = benzyl mercaptane-derived (LYRAL, LYRAK) or R = thiophenol-derived (LYRAQ) (Scheme 4.1-A), was determined by HPLC. The results demonstrated that the traditional thioadditives were not effective enough for the reverse transesterification for NCL with leucine, phenylalanine or glutamine at the ligation site. Thus, the amino acids at the ligation site can be ranked in order of the ligation yields in buffer system as follows: (high yield) G > K, A > L, N > Q > F (low yield) (Table 4.2).

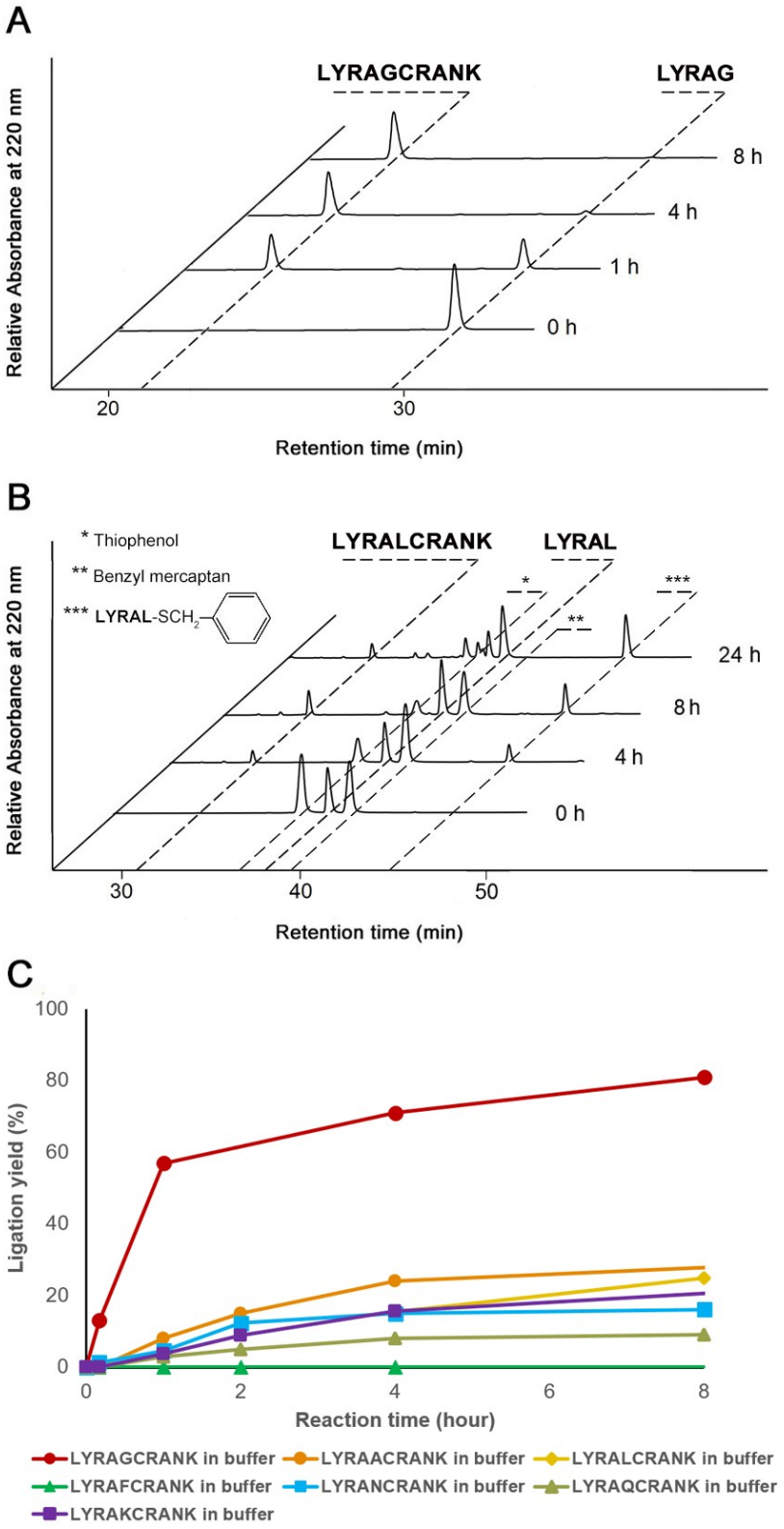


Figure 4.1 HPLC profiles of the ligation reactions obtained in buffer system[60] for linking of (A) LYRAG to CRANK resulting in peptide LYRAGCRANK, (B) LYRAL to CRANK resulting in peptide LYRALCRANK, (C) Plot of yields vs. reaction time for the formation of peptides LYRAXCRANK (X = G, A, L, F, N, Q, and K) (adapted with permission from the author's publication[89]).

4.1.2 NCL in [C_2mim][OAc]

As mentioned in chapter 1, a series of imidazolium-based ILs has been screened already as potential reaction media for the preparation of 66mer peptide tridegin by NCL.[36] Some of these imidazolium-based ILs showed an amazingly high capacity for dissolving the peptide reactants in higher concentrations (> 2 mM) as compared to aqueous buffer systems (≤ 0.4 mM).[36] This can be exemplified by the imidazolium-based ILs, which contained the following anions: *para*-toluenesulfonate ($[OTs]^-$), diethylphosphate ($[DEP]^-$), and dicyanamide ($[N(CN)_2]^-$). Interestingly, besides the outstanding dissolving capacity, the ILs 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) and 1-butyl-3-methylimidazolium acetate ([C_4mim][OAc]) have been found to possess an enhancing effect on the ligation rate and yield. However, so far, the applicability of these ILs has not been verified for NCL reactions employing different C-terminal amino acids at the N-terminal fragment. Hence, the ligation reactions leading to model peptide LYRAXCRANK (X = any amino acid) were repeated in the Ionic Liquid [C_2mim][OAc] and compared to the reactions in a buffer system (chapter 4.1.1).

Neat [C_2mim][OAc] was applied as reaction medium for the individual ligation reactions to form LYRAXCRANK with the same amino acids for X as used in chapter 4.1.1: G, A, L, F, N, Q, K). In addition, no additives (e.g. benzylmercaptan, thiophenol or MPAA) were added during the reaction process. All other conditions were the same as for the reactions performed in buffer system.

The reactions were monitored by HPLC. The results showed that almost all the reactions performed in [C_2mim][OAc] reached their maximum yield much faster than comparable NCL reactions in buffer (Table 4.2, Figure 4.2). All the reactions were finished (100% conversion of LYRAX) in 2 hours. In contrast to the ligation yields obtained in buffer system, the yields of most NCL

reactions were significantly increased in IL even though the reactions were carried out without any additives (Table 4.2, Figure 4.2).

However, in [C_2mim][OAc], the C-terminal amino acid at ligation site (X) of product LYRAXCRANK was also observed to have an impact on the reaction yields. Compared to the yield (57%) in an aqueous medium, the reaction with X = glycine performed in the IL was still the fastest and gained the highest yield (98%) within the first hour (Table 4.2, Figure 4.1-A, Figure 4.2-A). The reaction with X = leucine, formerly determined as “a sluggish residue” for NCL[60], has reached a relatively high yield of 36% in 2 hours, compared to the yield (19%) at room temperature after 24 h (Table 4.2, Figure 4.1-B, Figure 4.2-B). Interestingly, phenylalanine, which was the second most hindered amino acid in buffer system, has reacted at almost the same reaction rate and yield as glycine in [C_2mim][OAc].

However, the yields of the products in [C_2mim][OAc] were found to be gradually decreased after 1-2 hours (Fig. 4.2-C). Obviously, higher concentrations of product led to the formation of different byproducts preferably by reacting with the IL ions. The formation of several byproducts was detected in HPLC elution profiles and confirmed by mass spectrometry. For some peptides, a byproduct was observed which linked product LYRAXCRANK to the excess reactant CRANK (X = G, F, K) or methyl-3-mercaptopropionate (X = G, A, N, Q) by forming a disulfide bridge (Scheme 4.2). The molecular mass, which represented a LYRAXCRANK molecule connected via a thioether bridge to methyl-3-mercaptopropionate, had also been detected in the ligation of LYRALRANK and LYRAQRANK (Scheme 4.2). In particular, it was also found that the peptide reactants LYRAN and LYRAQ were converted to a special byproduct, i.e. a cyclic imide, in [C_2mim][OAc]. A possible reason for this finding was suggested to be a side reaction called deamidation, in which the α-amino group of asparagine or glutamine attacks its own backbone carbonyl to form a cyclic imide (Scheme

4.2). This side reaction occurring during the preparation of peptides and proteins was described several times in earlier reports.[129,130,131]

Table 4.2 Comparison of the conversion rates and the ligation yields obtained in different reaction media (adaptation based on resource[89]).

Ligation product	Conversion[a]	Reaction yields (reaction time)	
	Buffer, 37 °C[60]	Buffer[b], RT	IL[c], RT
LYRA**G**CRANK	~100% (after ≤ 4 h)	 57% (1 h) 81% (8 h)	85% (10 min) 98% (1 h) 83% (2 h)[d]
LYRA**A**CRANK	~100% (after ≤ 9 h)	 8% (1 h) 43% (24 h)	49% (10 min) 84% (1 h) 78% (4 h)[d]
LYRA**L**CRANK	~100% (after ≤ 48 h)	 25% (8 h) 19% (24 h)	27% (10 min) 36% (1 h) 20% (8 h)[d]
LYRA**F**CRANK	~100% (after ≤ 9 h)	 0% (1 h) 3% (48 h)	29% (10 min) 83% (1 h) 78% (8 h)[d]
LYRA**N**CRANK	~100% (after ≤ 24 h)	5% (1 h) 16% (8 h)	-[e,f]
LYRA**Q**CRANK	~100% (after ≤ 24 h)	3% (1 h) 11% (24 h)	11% (1 h)[e] 45% (24 h)
LYRA**K**CRANK	~100% (after ≤ 24 h)	4% (1 h) 41% (24 h)	29% (1 h)[f] 10% (24 h)[d,f]

[a] Values are estimated from MALDI analysis reported in reference[60].

[b] Buffer conditions: 0.1 M phosphate buffer (pH 8.5) containing 6 M guanidinium chloride, 4% (vol/vol) benzyl mercaptan and 4% (vol/vol) thiophenol.

[c] IL: $[C_2mim][OAc]$. For evaluation, the amount of the LYRAXCRANK-product as well as the IL-oxidized product were combined.

[d] Side product formation in IL led to reduced product formation.

[e] LYRAX-thioester in this approach immediately degraded.

[f] Product formation could not or only partially be determined due to a peak-overlay of side product and product in the chromatogram

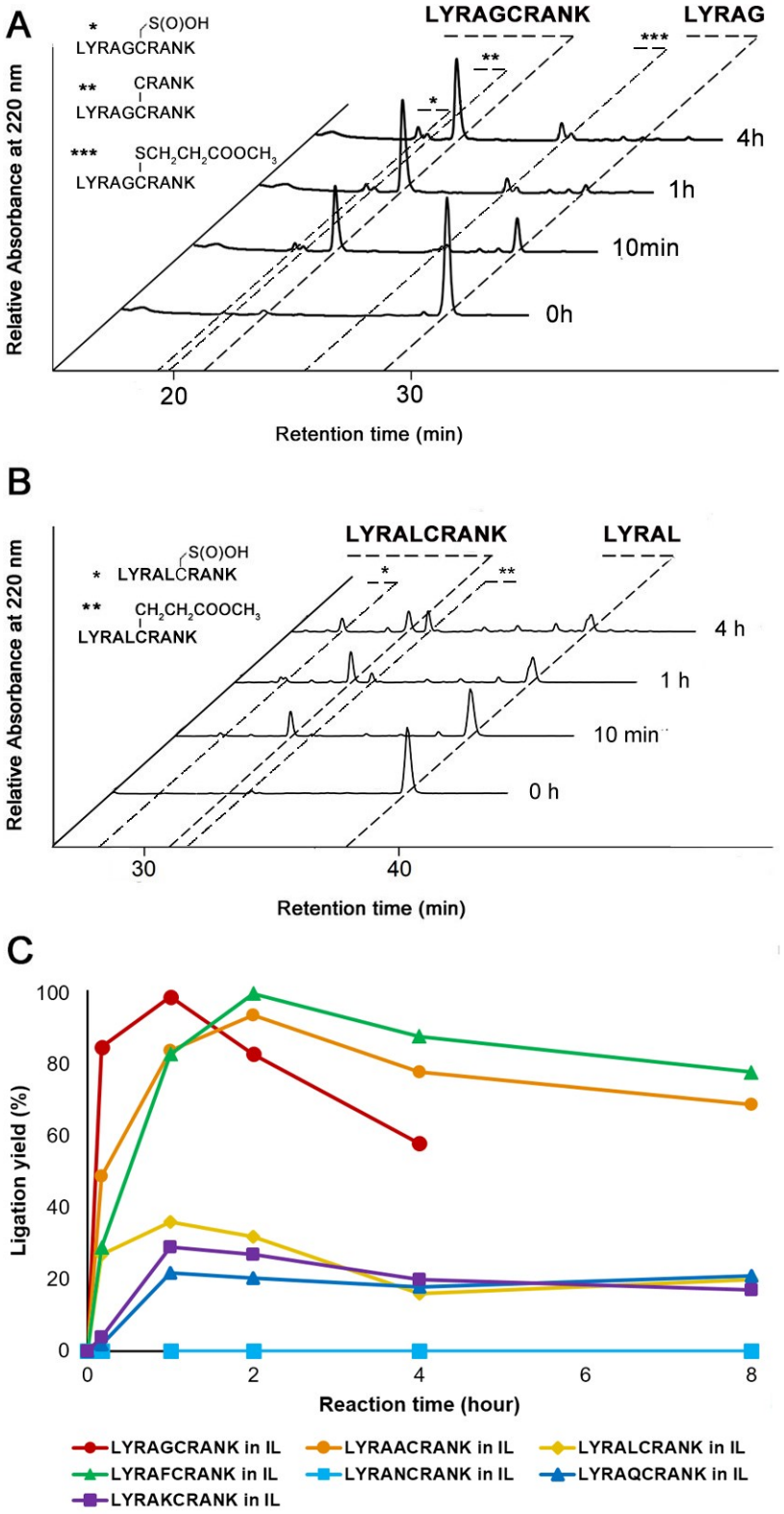
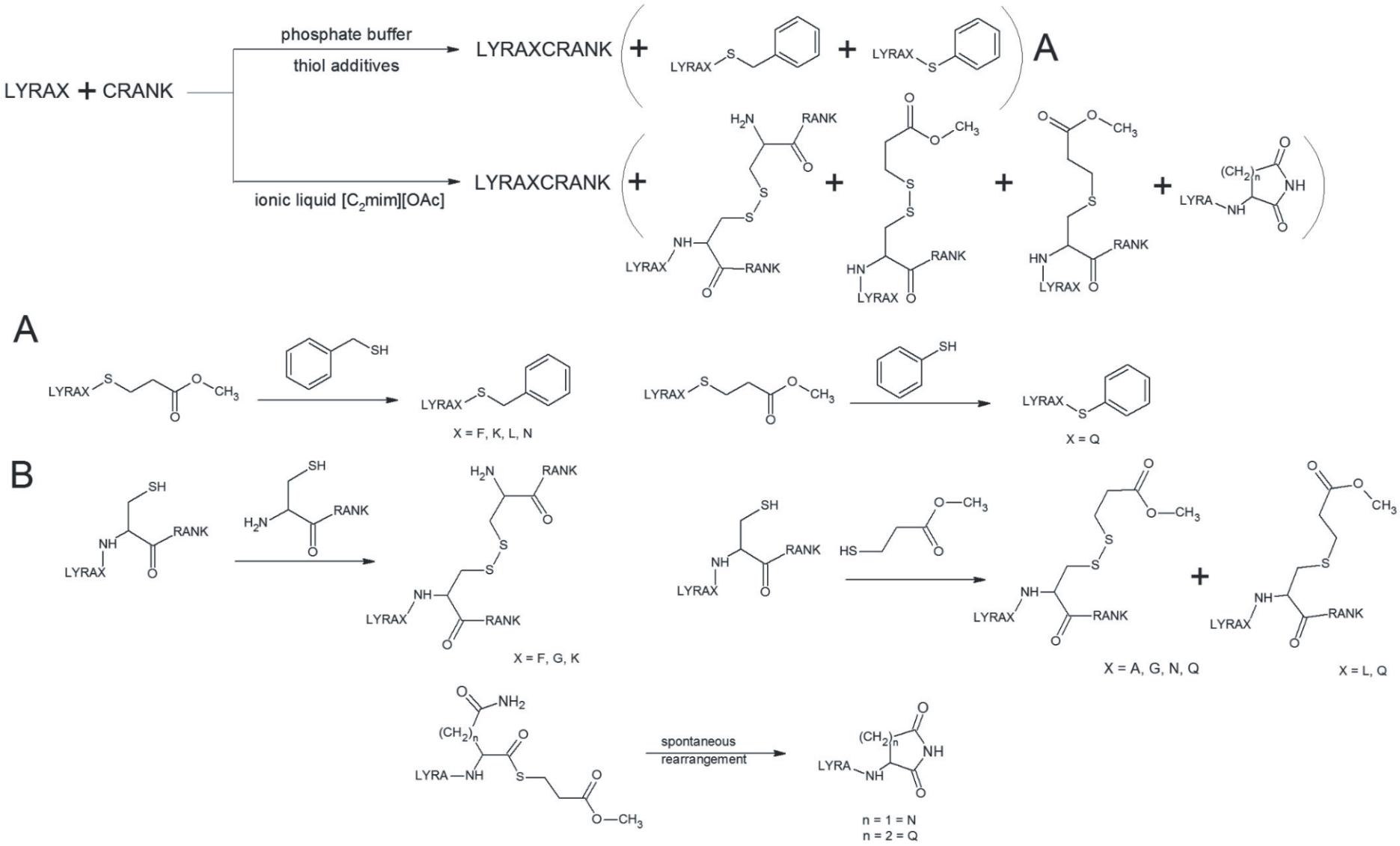


Figure 4.2 HPLC profiles of the ligation reactions obtained in IL [C_2mim][OAc] for linking of (A) LYRAG to CRANK resulting in peptide LYRAGCRANK, (B) LYRAL to CRANK resulting in peptide LYRALCRANK, (C) Plot of yields vs. reaction time for the formation of peptides LYRAXCRANK (X=G, A, L, F, N, Q, K) (adapted with permission from the author's publication[89]).



Scheme 4.2 Different side reactions occur during NCL depending on the reaction medium and the use of additives (reprinted with permission from the author's publication[89]).

4.1.3 Pros and cons of IL application for NCL of peptides

Thanks to the excellent features of the Ionic Liquid [C_2mim][OAc], NCL reactions could be performed in clean and non-toxic conditions. In addition, since the temperature has been proved to be favorable for increasing the conversion rate of NCL reactions, low volatility, high thermal stability and non-flammability make IL attractive for the ligation reactions requiring heating or assisted by microwave.[51,128] The efficient dissolving capacity of [C_2mim][OAc] for various peptides, in particular hydrophobic peptides, were determined, since this is a prerequisite for generating a homogeneous solution of the peptides at higher concentrations (> 2 mM). On the other hand, separation of the products from the IL is more complicated because the IL has almost no volatility and cannot be removed by simple evaporation.

Compared to the reactions in buffer solutions, both the reaction rates and yields of the NCL reactions have been strongly increased by using [C_2mim][OAc]. In addition to its outstanding dissolving capacity, [C_2mim][OAc] has also showed an interesting catalytic effect on the NCL reaction, which made the addition of additives unnecessary. This fact makes the simplification for current protocols of NCL possible. On the other hand, the formation of side products in [C_2mim][OAc] limited the reaction time of NCL. Few side reactions, e.g. the formation of a disulfide or a thioether bond, could be partially overcome by adjusting the ratio between the concentrations of fragment reactants (Figure 5.15, Figure 5.16), while the deamidation of asparagine or glutamine is still a challenge for NCL reactions performed in IL. Consequently, the ligation reaction needs to be carefully monitored and discontinued in time when the yield starts decreasing.

Reactions in peptide synthesis which cannot proceed in aqueous systems due to the lack of solubility of the reactants can now be envisioned in ILs. The effect of ILs for NCL reaction further realized the omission of toxic and expensive additives. These facts can be considered as a new horizon of the IL

applications in peptide chemistry, however, more example peptides need to be prepared by this strategy in order to completely assess the success of employing ILs.

4.2 Head-to-tail cyclization via lactam bridge formation in ILs

To my best knowledge, no attempt has been made for performing a head-to-tail cyclization of a peptide via lactam bridge formation in an Ionic Liquid so far. Herein, a series of model peptides, which were developed as potential Gαq inhibitors, have been prepared in neat IL and compared to the same reactions carried out in DMF, a conventional solvent for peptide cyclization. The efficiency of both solvents was tested in order to verify the applicability of ILs in other types of chemical reactions in peptide synthesis.

4.2.1 Synthetic strategies for depsipeptide YM 254890 and analogues

The roots of the *Ardisia crenata* plant have been used for a long time in Chinese traditional medicine for the treatment of respiratory tract infections, tonsillitis and menstrual disorders. FR900359, which is a naturally derived agent extracted from *A. crenata*, has been first introduced in 1980s.[132] It is a cyclic depsipeptide which inhibits platelet aggregation (Scheme 4.3-A).[132] Afterwards, the cyclic depsipeptide YM-254890 (Scheme 4.3-B) of similar structure as FR900359, also being a natural platelet aggregation inhibitor, has been found in the culture broth of *Chromobacterium sp. QS3666*.[133] This compound was shown to influence the signal transduction of ligand-activated G protein coupled receptors (GPCRs) by specific inhibition of the GDP-GTP exchange of the heterotrimeric G protein Gαq.[134]

Moreover, YM-254890 is currently the most efficient and selective inhibitor of Gαq/11,[135] and has thus attracted great interest since its discovery by

Yamanouchi Pharmaceuticals in 2003.[133] This specific Gαq/11 inhibitor may help us to understand the role of Gαq/11 in GPCR transactivation signaling. Various studies have shown that YM-254890 could be a potential anti-hypertensive agent, besides its antithrombotic and thrombolytic effects.[136]

However, this compound cannot be obtained commercially from Yamanouchi Pharmaceuticals (Fujisawa Pharmaceuticals) anymore.[137] In addition, no successful synthesis of YM-254890 has been reported so far, even though a $100,000 USD award has been offered for this challenge on the global innovation market since 2012.[138]

Due to the difficulty and complexity of depsipeptide syntheses, several attempts have been undertaken recently to produce analogues of YM-254890.[138] The structure-activity relationship of YM-254890 (Scheme 4.3-B) and its analogues YM-254891 and YM-254892 (Scheme 4.3-C) indicated that the acyl-β-hydroxyleucine residue is a crucial group to the inhibitory activity while the α,β-unsaturated carbonyl group of the N-MeDha (Dha = dehydro-alanine) residue is not important.[139] Furthermore, Rensing *et al.* have designed an analogue of YM-254890 as a potential Gαq inhibitor, namely WU-07047 (Scheme 4.3-E).[140] In order to enable a convergent approach to the synthesis, the two peptide-based linkers in the cyclic YM-254890 have been replaced with hydrocarbon chains in the analogue. YM-280193 (Scheme 4.3-D),[139] another analogue of YM-254890, was synthesized by Brimble *et al.* recently.[138] Unfortunately, neither of these peptides, YM-280193 (Scheme 4.3-D) and WU-07047 (Scheme 4.3-E) have been determined as potent Gαq/11 inhibitor compared to YM-254890.

Since YM-254890 is not available currently, effort has been made to simplify the natural structure of the compound by marginal modifications, so that multitudinous analogues could be obtained easily by SPPS method. Such modifications should reduce the complexity of synthetic strategies conspicuously compared to the solution-phase approaches described so far.

This molecular design work was based on a combination of our chemical experience and structural investigations in collaboration with Dr. Daniel Tietze (TU Darmstadt), who performed additional molecular modelling and docking studies (Table 4.3). In the first peptide modification, the dehydroalanine residue, the β-hydroxyleucine residue and the ester bond have been replaced by an alanine, a leucine and a peptide bond, respectively (Scheme 4.3-F, abbreviated as YM-1). However, the unsuccessful synthesis of this analogue still bearing an ester bond impelled us to continue with simplification of backbone and side chain modifications. Unfortunately, a complete coupling failure was still encountered at the O-methyl-L-threonine in the second analogue intended (Scheme 4.3-F, abbreviated as YM-2). This problem has been solved by substituting the methyl group for the use of acetyl-L-threonine. Thus, a series of cyclic analogues as shown in Table 4.4 and Scheme 4.3-G (abbreviated as YM3–YM10) has been designed and prepared.

Before the cyclization, the linear precursor peptides needed to be synthesized by SPPS (Scheme 4.4). The linear peptides of YM3–YM10 (Table 4.4) were synthesized on 2-chlorotrityl chloride resin (loading: 2.1 mmol/g) or 2-alanine-chlorotrityl chloride resin (loading: 0.67 mmol/g). All subsequent amino acids were coupled by using stepwise Fmoc-based SPPS. After deprotection and cleavage from the solid support using a mixture of reagent K and trifluoroacetic acid (TFA), the crude linear peptides were precipitated in cold diethyl ether. After purification and freeze-drying, the pure linear peptides were stored at -20 °C before the cyclization step.

Table 4.3 Binding parameters obtained from docking studies performed on YM-analogues YM-3 and YM-4 (kindly provided by Dr. Daniel Tietze, TU Darmstadt)

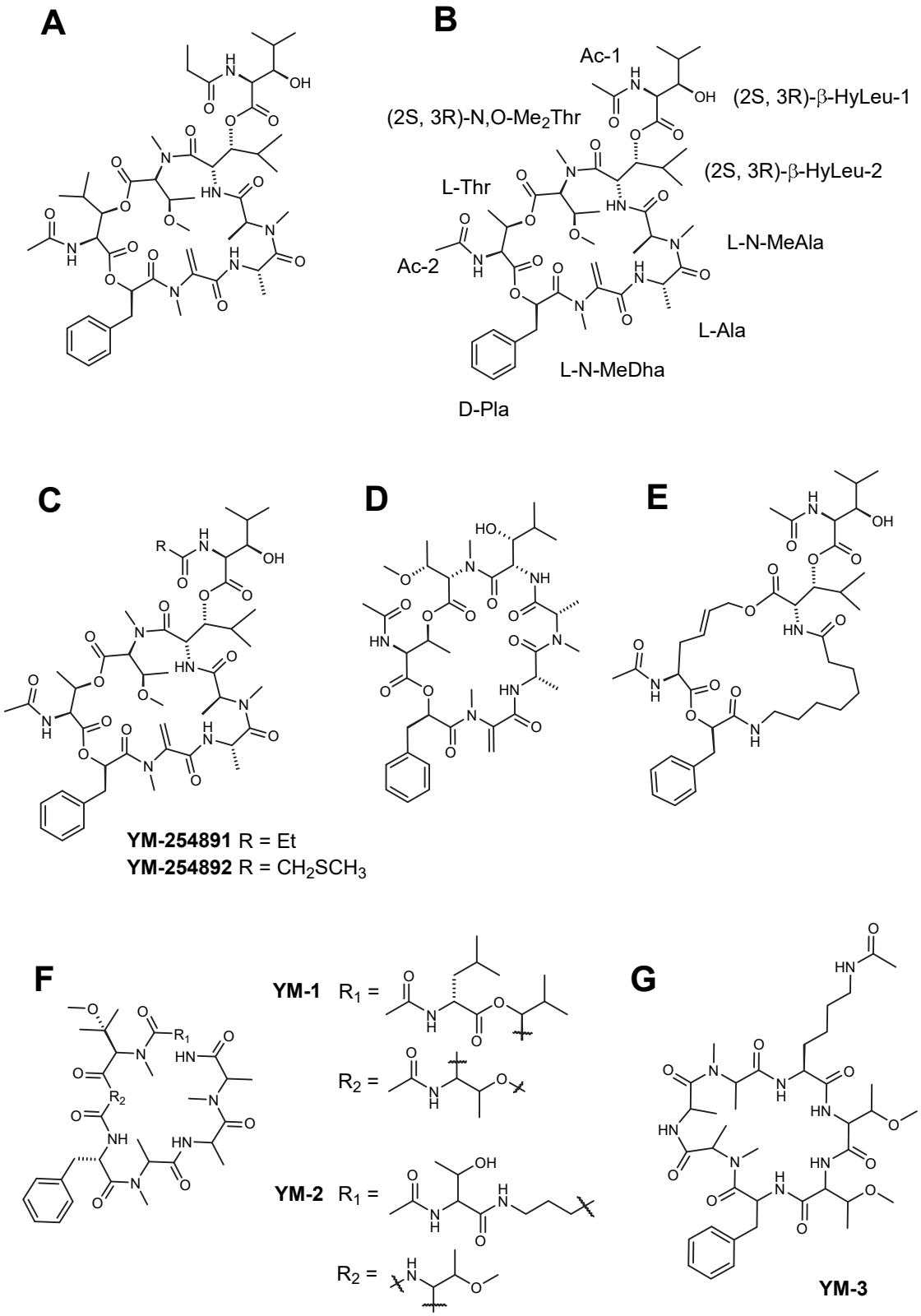
Peptide	Binding Energy (kcal/mol)	Inhibitory Constant *Ki* (µM)
YM-254890	9.85	0.06
YM-3	9.20	0.18
YM-4	8.94	0.28

Table 4.4 Peptide sequences of cyclic analogues of YM-254890.

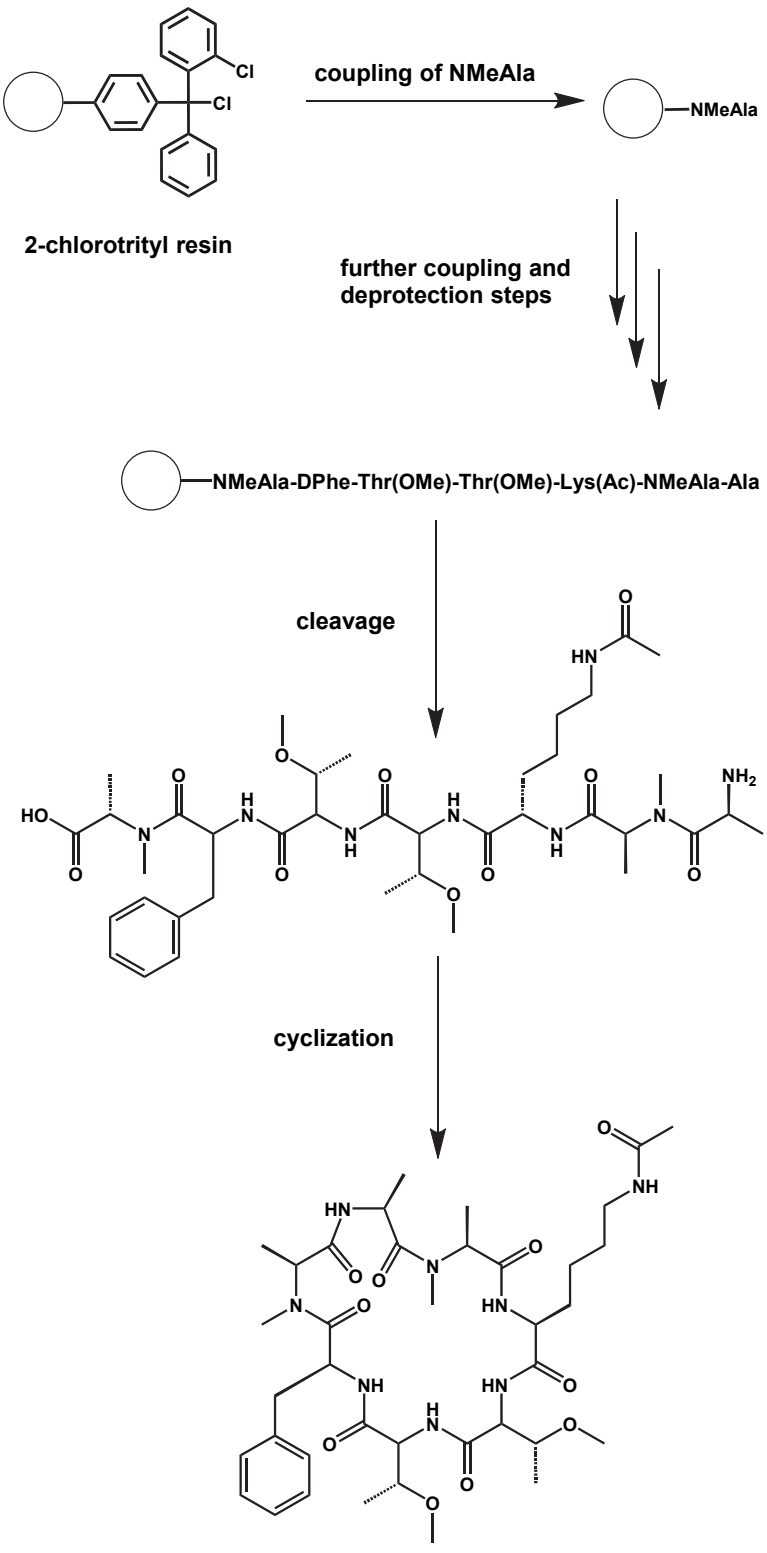
Peptide Abbr.	Sequence[a]	Expected mass $[M+H]^+$	Measured mass[b] $[M+H]^+$
YM-3	cyclo[Ala-NMeAla-Lys(Ac)-Thr(OMe)-Thr(OMe)-D-Phe-NMeAla]	788.5	789.44
YM-4	cyclo[Ala-NMeAla-Lys(Ac)-Thr(OMe)-Thr(OMe)-Phe-NMeAla]	788.5	789.44
YM-5	cyclo[Ala-Ala-Lys(Ac)-Thr(OMe)-Thr(OMe)-D-Phe-Ala]	760.4	761.44
YM-6	cyclo[Ala-Ala-Lys(Ac)-Thr(OMe)-Thr(OMe)-Phe-Ala]	760.4	761.44
YM-7	cyclo[Ala-NMeAla-Leu-Thr(OMe)-Thr(OMe)-D-Phe-NMeAla]	731.4	732.42
YM-8	cyclo[Ala-NMeAla-Leu-Thr(OMe)-Thr(OMe)-Phe-NMeAla]	731.4	732.42
YM-9	cyclo[Ala-Ala-Leu-Thr(OMe)-Thr(OMe)-D-Phe-Ala]	703.4	704.42
YM-10	cyclo[Ala-Ala-Leu-Thr(OMe)-Thr(OMe)-Phe-Ala]	703.4	704.39

[a] All peptides were cyclized using via lactam bridge between the N- and the C-terminal.

[b] If the peptide was detected in a higher charged state, $[M+H]^+$ was calculated from this peak.



Scheme 4.3 Chemical structures of compounds existing or being developed as Gαq inhibitors: (A) FR-900359;[132] (B) YM-254890;[133] (C) YM-254891 and YM-254892;[139] (D) YM-280193;[139] (E) WU-07047;[140] (F) peptide YM-1 and YM-2; and (G) peptide YM-3.



Scheme 4.4 Synthetic strategy for cyclic peptides exemplified for YM-3.

As described in earlier publications, the linear precursors can be successfully cyclized by using PyBOP as coupling reagent and *N*,*N*-diisopropylethylamine (DIEA) as the base (Scheme 4.4).[141,142,143,144]

4.2.2 Peptide cyclization in an organic solvent

Hereafter, the linear peptides of YM-3–YM-10 were cyclized manually using the coupling reagent PyBOP and the organic base DIEA in the organic solvent *N,N*-dimethylformamide (DMF) for 6 hours at room temperature to form the cyclic products (Scheme 4.4). Argon gas atmosphere was not necessary here because no sensitive residue existed in the sequences. All the linear and cyclic peptides in this experiment were purified by reversed-phase HPLC, and the mass was confirmed by electrospray ionization (ESI) mass spectrometry.

For the reactions performed in DMF, the yields of all cyclic peptides YM-3–YM-10 have been determined to reach their maximum values in the first 15-30 minutes and remained substantially stable in the next hours (Figure 4.3-C). The final yields were obtained after 6 hours reaction time for YM-5, YM-7, YM-8, and YM-9, which reached up to 70 - 80%, while the yields for YM-3, YM-4, YM-6, and YM-10 (approx. 50 - 60%) were lower, yet seemed to be fair as well (Figure 4.3A, B). This study revealed that the reaction time suggested from other protocols for cyclization[141,142,143,144] can be shortened at least for the peptides described herein and similar analogues.

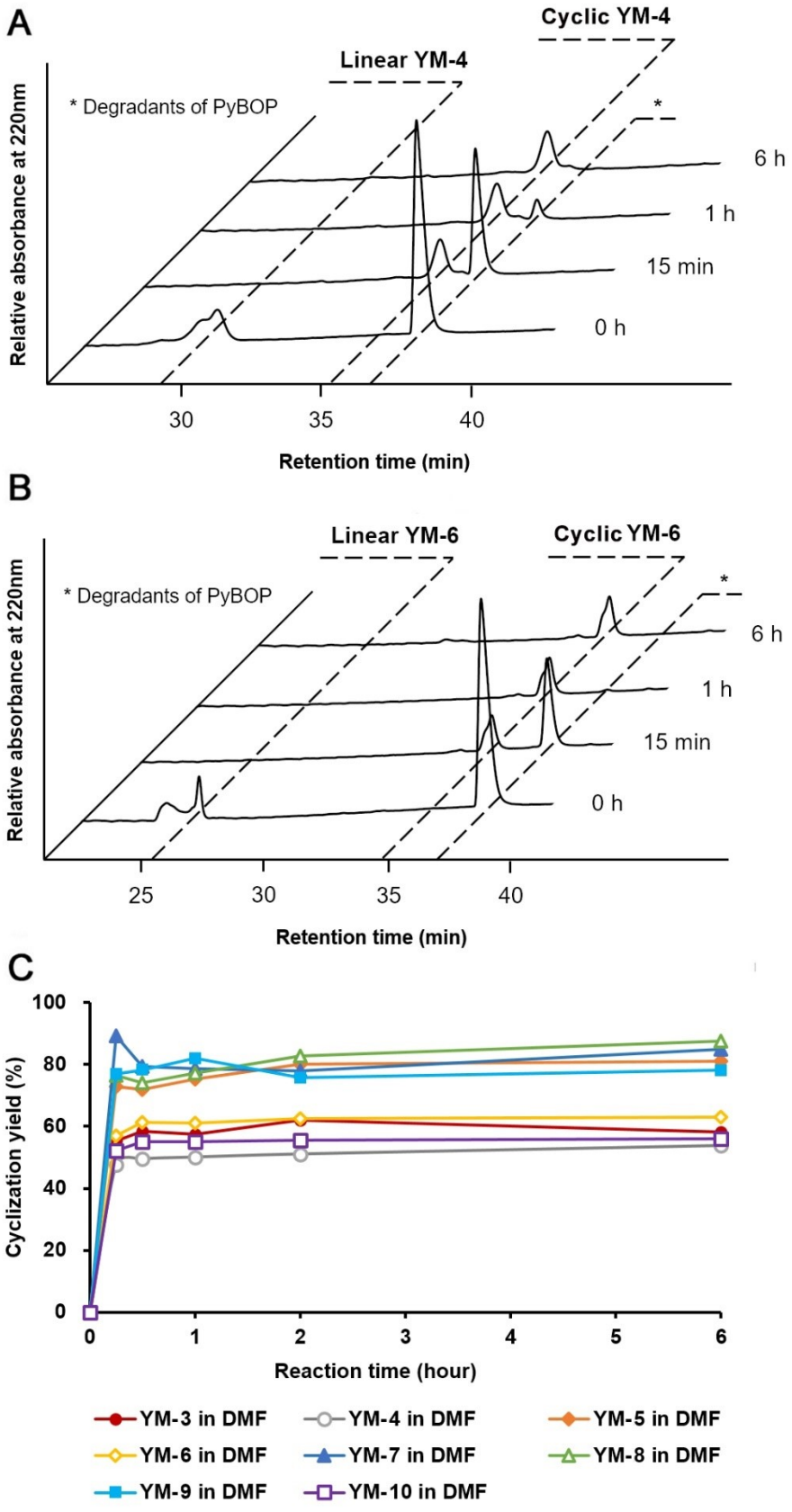


Figure 4.3 HPLC profiles of the cyclization reactions obtained in DMF of (A) YM-4, and (B) YM-6; (C) Plot of yields vs. reaction time for the cyclization of peptides YM-3–YM-10.

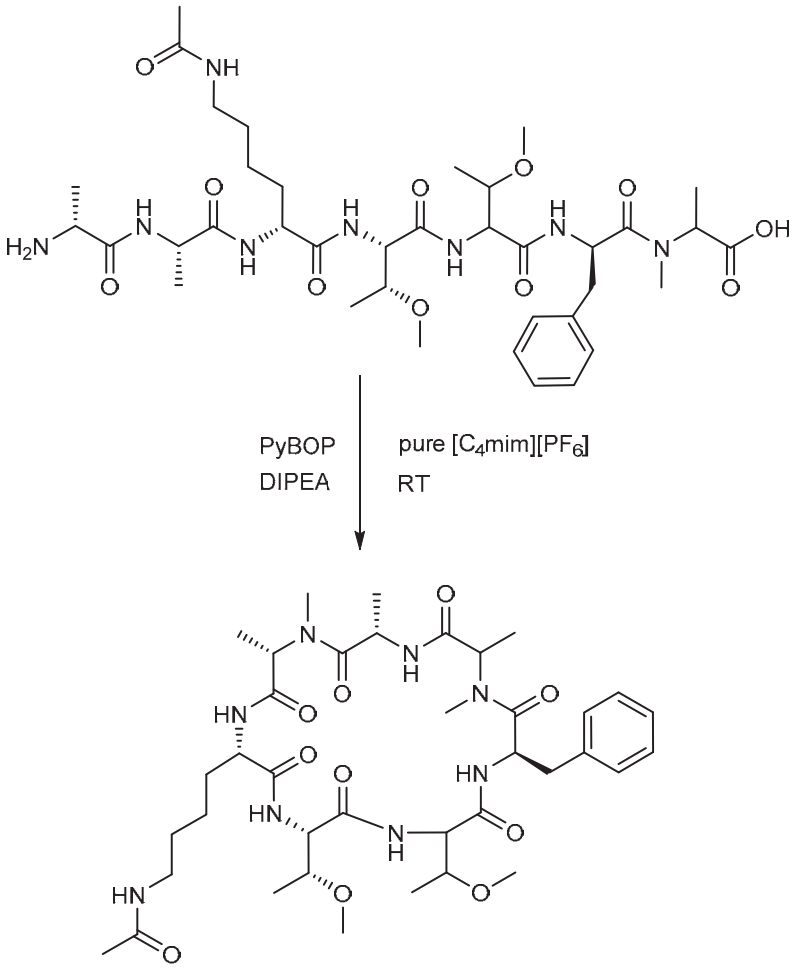
4.2.3 Peptide cyclization in [C_4mim][PF_6]

Considering the high content of hydrophobic residues in the peptide sequences described herein, such as Phe, Leu and NMeAla, a lot of efforts have been made to optimize the reaction media. So far, the primarily used organic solvent for peptide cyclizations, both in solution and on the solid phase, is DMF.[141,142,143,144] In addition, others such as acetonitrile,[145] dimethyl sulfoxide (DMSO)[146] and isopropanol[147] were applied as well. In our previous work, ionic liquids have been applied for the synthesis of peptides, such as the 66mer peptide tridegin.[36] In addition, disulfide bond formation occurred very efficiently in the imidazolium-based IL 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc])[148], and Heimer *et al.* also performed the oxidative folding of the μ-conotoxin SIIIA in different ILs under up-scaled conditions.[127] However, no report about the head-to-tail cyclization of peptides in ILs was described so far. Thus, our interest concerning peptide chemistry performed in ILs led us to investigate the applicability of these new media in the preparation process of the cyclic peptides YM3–YM10 (Table 4.4).

PyBOP was again selected as coupling reagent for reasons of comparison and since it was already successfully used earlier in our laboratory. However, it has been observed that the PyBOP was not long-term stable in both ILs [C_2mim][$N(CN)_2]^-$ and [C_2mim][OAc], even though [C_2mim][OAc] has been successfully used in peptide ligation reactions. Thus, the IL 1-butyl-3-methylimidazolium hexafluorophosphate ([C_4mim][PF_6]), which has been used by J.-C. Plaquevent *et al.* for dipeptide formation, was applied as reaction medium.[124] J.-C. Plaquevent and coworkers reported that [C_4mim][PF_6] enhanced the peptide coupling reactions for the generation of dipeptide Boc-2-methyl-2-(p-tolyl)-glycine-glycine-methyl ester, and the purities of crude products were higher than the couplings carried out in a classical solvent, e.g. dichloromethane (DCM).[124]

One equiv. of linear peptide (YM3–YM10) and 6 equiv. of PyBOP were first

dissolved in 2000 equiv. of [C_4mim[]PF_6]. Then, 12 equiv. of the organic base DIEA was added. All the experiments were performed under room temperature and continuous stirring (Scheme 4.5). The formation of cyclic products (YM3–YM10) was monitored by HPLC analysis over a time period of up to 6 hours. Simultaneously, in order to verify the efficiency of ILs, these cyclization reactions were also carried out in DMF instead of [C_4mim][PF_6] under the same conditions.



Scheme 4.5 Synthetic strategy for cyclization reaction of YM-254890 analogue YM-3 of in [C_4mim][PF_6].

In contrast to the results obtained in DMF, the reaction rates and yields of the products formed in [C_4mim][PF_6] were found to be significantly different (Figure 4.4). The cyclization reactions of the peptides containing an acetylated lysine residue, including YM-3, YM-4, YM-5, and YM-6 were rather slow within

the first 2 hours and achieved a relative high yield (>60%) after 6 hours reaction time. The cyclic peptides YM-9 and YM-10 were formed with yields of 61% and 51%, respectively, which were somewhat lower than the yields of YM-9 (78%) and YM-10 (56%) in DMF, but still acceptable.

However, it has been determined that the yields of the cyclic products YM-7 (42%) and YM-8 (33%) in IL were only half of those in DMF (85% and 88%, respectively). It was also observed that the yield of YM-7 in IL was gradually decreased from 62% (30 min) to 42% (6 hours) after 30 minutes, even though it was increasing dramatically in the first 30 min. Compared to the results in DMF, all the cyclic peptides containing leucine were formed with relatively low yields.

Table 4.5 Cyclization yields of the peptide analogues formed in the Ionic Liquid.

Peptide Abbr.	**Yield[a]**					
	DMF / reaction time			**[C_4mim][PF_6] / reaction time**		
	15 min	**2 hrs**	**6 hrs**	**15 min**	**2 hrs**	**6 hrs**
YM-3	55%	62%	58%	36%	64%	65%
YM-4	48%	51%	54%	16%	60%	65%
YM-5	72%	80%	81%	11%	66%	67%
YM-6	57%	63%	63%	47%	74%	67%
YM-7	89%	78%	85%	58%	52%	42%
YM-8	77%	83%	88%	29%	31%	33%
YM-9	77%	76%	78%	34%	57%	61%
YM-10	52%	56%	56%	5%	49%	51%

[a] Yields were determined by HPLC analysis.

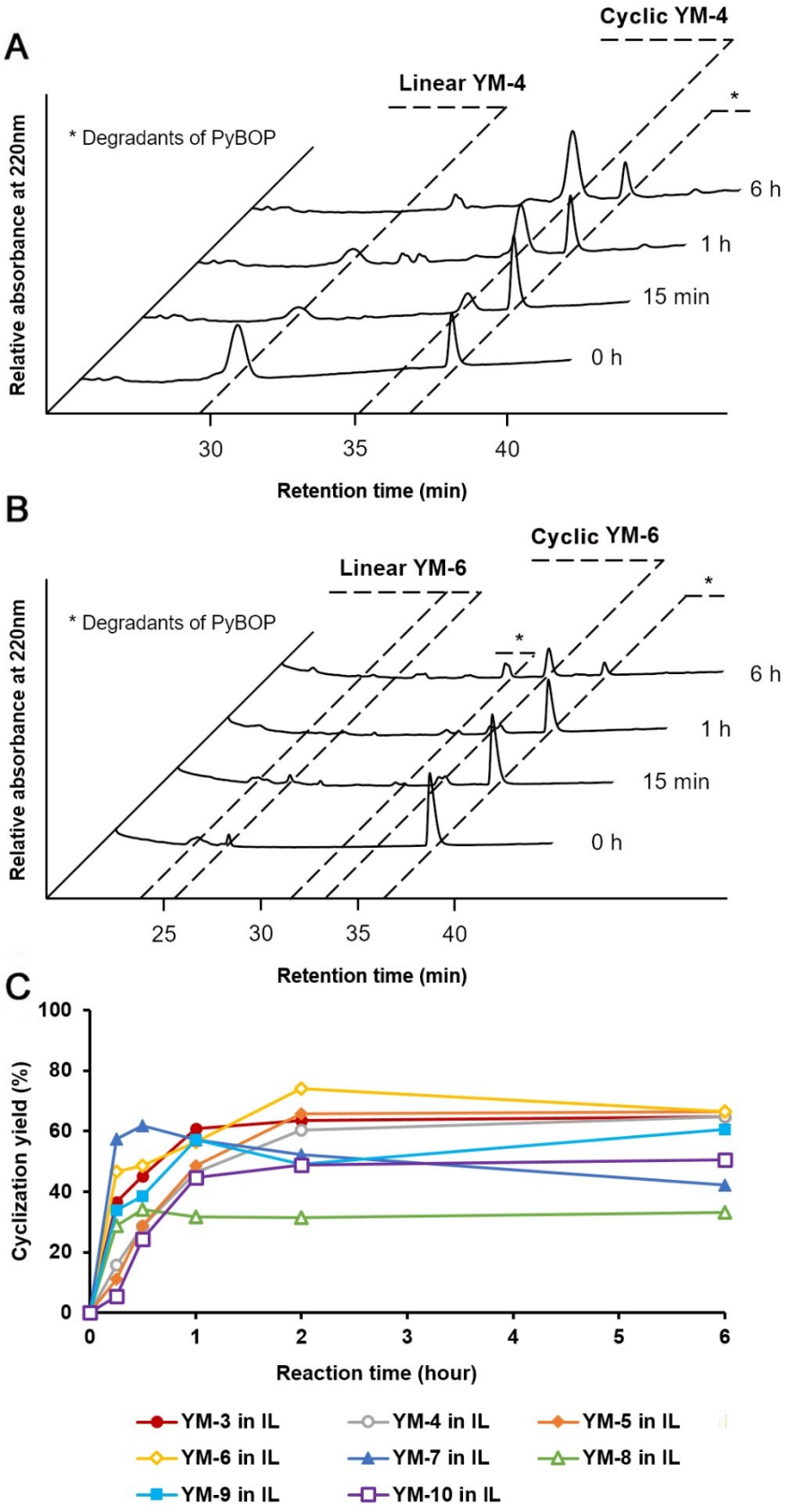


Figure 4.4 HPLC profiles of the cyclization reactions obtained in $[C_4mim][PF_6]$ of (A) YM-4, and (B) YM-6; (C) Plot of yields vs. reaction time for the cyclization of peptides YM-3–YM-10.

4.2.4 Pros and Cons of IL application for peptide cyclization in solution

According to the experimental results, [C_4mim][PF_6] might be used as an alternative reaction medium for the cyclization reaction examined in this work. It was found that the residue leucine could be considered as a hindered residue for the cyclization yields of the peptides studied. To a certain extent, [C_4mim][PF_6] as a hydrophobic solvent could be a suitable solvent to synthesize the polar cyclic peptides (e.g. YM-3, YM-4) rather than the most non-polar ones (e.g. YM-7, YM-8). Furthermore, it was also demonstrated that the coupling reagent PyBOP could work efficiently even in a Brønsted acidic ionic liquid, i.e. [C_4mim][PF_6].

On the other hand, the interactions between ILs, additives or peptides during the cyclization were still unclear. It has been observed that the reaction rates have been slightly reduced over time if using [C_4mim][PF_6] in contrast to DMF.

A possible reason for these facts may be the strong interactions between the peptide's backbone/amino acid side chains and ionic liquid cations and anions, since P. Heimer *et al.*[127] observed that the anion from IL contacted preferentially the lysine residue in both folded and unfolded toxin peptide μ-SIIIA.

5 Experimental Section

5.1 Materials

All commercial materials, except tetrahydrofuran and dichloromethane were used without further purification.

Peptide synthesis reagents, 4-sulfamylbutyryl NovaSyn® TG amino resins (0.18 – 0.23 mmol/g), Rink Amid MBHA Resin (0.56 mmol/g) and Fmoc–amino acids used for the synthesis of peptide LYRAX (X = G, A, F, L, N, Q, K) and peptide CRANK were purchased from Orpegen Peptide Chemicals (Heidelberg, Germany), Novabiochem (Hohenbrunn, Germany) and Iris Biotech (Marktredwitz, Germany), respectively. Anhydrous tetrahydrofuran and dichloromethane were dried over molecular sieve 0.3 nm. Monosodium phosphate, disodium phosphate and guanidine hydrochloride were obtained from Merck (Darmstadt, Germany), Sigma-Aldrich Chemie GmbH (Munich, Germany) and Carl Roth (Karlsruhe, Germany), respectively. Benzyl mercaptan and thiophenol were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). [C_2mim][OAc] was purchased from IoLiTec GmbH (Heilbronn, Germany). Solvents for chromatography (methanol, acetonitrile, water) were of analytical grade and obtained from VWR International (Dresden, Germany). All other solvents were of reagent grade or HPLC grade depending on the experiment to be performed.

Standard coupling reagents (TFFH, PyBOP), 2-chlorotrityl chloride resin, alanine-2-chlorotrityl chloride and Fmoc-amino acids used for the synthesis of all YM 254890 analogues were purchased from Orpegen Peptide Chemicals GmbH (Heidelberg, Germany), Novabiochem and IRIS Biotech (Marktredwitz, Germany), respectively. Peptide synthesis reagents (piperidine, TFA) and solvents (DMF, dichloromethane) were of reagent grade, and solvents for chromatography (methanol, acetonitrile, water) were of analytical grade

obtained from VWR International (Dresden, Germany). [C_4mim][PF_6] was purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

5.2 Methods

The crude peptides were purified by semipreparative reversed-phase HPLC. The purifications involved a mobile phase of 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water (90:10) (eluent B) (see next chapter for further details). Peptides were characterized by thin layer chromatography, analytical HPLC, amino acid analysis, and mass spectrometry (see next chapter for further details).

5.3 Solid phase synthesis of peptides

5.3.1 Preparation of peptide fragments for NCL

All protected peptide fragments were synthesized by solid-phase peptide synthesis (SPPS). SPPS was performed on an automated peptide synthesizer EPS 221 (Intavis, Cologne, Germany). H-Gly-sulfamylbutyryl NovaSyn® TG amino resin (0.18 mmol/g), H-Ala-sulfamylbutyryl NovaSyn® TG amino resin (0.20 mmol/g), H-Phe-sulfamylbutyryl NovaSyn® TG amino resin (0.23 mmol/g), H-Asn-sulfamylbutyryl NovaSyn® TG amino resin (0.20 mmol/g), H-Leu-sulfamylbutyryl NovaSyn® TG amino resin (0.20 mmol/g), H-Glu -sulfamylbutyryl NovaSyn® TG amino resin (0.21 mmol/g) and H-Lys-sulfamylbutyryl NovaSyn® TG amino resin (0.23 mmol/g) were used for the synthesis of peptide LYRAG, LYRAA, LYRAF, LYRAN, LYRAL, LYRAQ and LYRAK, respectively. Rink Amid MBHA Resin (0.56 mmol/g) was used for the synthesis of CRANK. Amino acids were coupled subsequently using a standard Fmoc protocol with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium hexafluorophosphate (HBTU) as coupling reagent and N-methylmorpholine as the base. The Fmoc group was deprotected with 20% piperidine/DMF twice (5 min and 15 min). Finally, the LYRAX-peptides were acetylated using N-methylimidazole:acetic anhydride:DMF (1:2:3, 1 ml mixture/100 mg resin).

Side-chain deprotection and cleavage of the peptides from the resin were achieved with reagent K consisting of phenol (0.75 g), ethandithiol (0.25 ml), thioanisol (0.5 ml) in 95% TFA/water (v/v) (1 ml/100 mg resin). The deprotection was carried out at room temperature for 3 hours. The crude peptides were precipitated in diethyl ether, centrifuged and washed several times with diethyl ether.

Cleavage of LYRAX-peptides was performed under addition of 5 ml of 1 M trimethylsilyldiazomethane in dry hexane/THF (1:1) to the resin (0.1 mmol) for

2 hours.[149] After washing with dry THF methylated resin was incubated with methyl-3-mercaptopropionate (280 equiv.) and sodium thiophenoxide (2.8 equiv.) for 24 hours under gentle agitation.[150] The resulting peptide solution was filtrated, washed 3-times with DMF and evaporated to dryness on a rotary evaporator. The product was triturated with diethyl ether, and the residue was treated with TFA:water:triisopropylsilane:phenol (88:5:2:5) for 2 hours at room temperature. The cleavage solution was precipitated in diethyl ether, centrifuged and washed several times with diethyl ether. The yields of the crude peptides ranged between 10–40% for LYRAX-thioesters and 70–80% for CRANK and LYRAXCRANK peptides (X = G, A, L, F, N, Q, K). After lyophilisation, the remaining solid was purified using HPLC.

The purification of the crude peptides was performed by semipreparative reversed-phase HPLC using a Shimadzu LC-8A system equipped with a C18 column (Knauer Eurospher 100, 32 x 250 mm, 5 µm particle size, 100 Å pore size, Berlin, Germany). The gradient elution system was 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water (9:1) (eluent B). The peaks were detected at 220 nm. The flow rate used was 10 ml/min. Purity of the peptides was confirmed by thin layer chromatography (Table 5.1) and analytical reversed-phase HPLC on a Shimadzu LC-10AT chromatograph with a Vydac 218TP column (4.6 x 250 mm, 5 µm particle size, 300 Å pore size) and a mobile phase system consisting of A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile. The detection was at 220 nm. Detailed information on chemical characterization of the selected peptides has been summarized in Table 5.1. The pure peptides were lyophilized and stored at −20 °C until use.

The determination of molar mass was performed with MALDI-TOF mass spectrometry on an Autoflex TOF/TOF mass spectrometer (Bruker Daltonics) with α-cyano-4-hydroxycinnamic acid as matrix.

5.3.2 Preparation of YM 254890 peptide analogues

The linear peptides were abbreviated as YM-3–YM-10 for ease of identification (Table 4.4). The linear peptides YM-4, YM-6, YM-9, and YM-10 were synthesized on an automated peptide synthesizer EPS 221 (Intavis Bioanalytical Instruments AG, Cologne, Germany) according to a standard Fmoc protocol. The remaining linear peptides YM-1, YM-3, YM-5, YM-6, and YM-8 were synthesized manually. The polymer support was 2-chlorotrityl chloride (loading: 2.1 mmol/g) (Advanced ChemTech, Louisville, USA) or alanine-2-chlorotrityl chloride resin (loading: 0.67 mmol/g) (Iris Biotech GmbH, Marktredwitz, Germany). Specially modified amino acids that were used included the following: Fmoc-N-Me-Thr(tBu)-OH, Ac-Thr-OH, Fmoc-N-Me-Ala-OH, Fmoc-Lys(NHAc)-OH, and Fmoc-Thr(Ac)-OH. In general, coupling reactions were performed using Fmoc-amino acids (2-4 equiv.) activated using TFFH (2-4 equiv.) in the presence of DMF and DIEA (4-8 equiv., correspondingly) for 5–15 minutes (double couplings). Fmoc removal was carried out by treating the resin twice with 20% piperidine in DMF. All deprotecting and coupling steps were followed by intensive washings using DMF and DCM, alternately. Peptide cleavage and deprotection was accomplished with a reagent K/TFA mixture (82.5% TFA, 5% water, 5% phenol, 5% thioanisole, 2.5% ethanedithiol) for 4 hours at room temperature. The crude peptides were precipitated in cold diethyl ether, centrifuged and washed with diethyl ether several times.

After lyophilisation, the crude peptides were purified by the same HPLC system as described in chapter 5.3.1. The gradient elution system was also 0.1% TFA in water (eluent A) and 0.1% TFA in acetonitrile/water (9:1) (eluent B). The peptides were eluted with a flow rate of 10 ml/min and a gradient of 0%-60% eluent B in 120 min. The peaks were detected at 220 nm. Collected fractions were combined, freeze-dried and stored at -20 °C.

Thin layer chromatography (TLC), both preparatory and analytical, was

performed using silica gel 60 F254 glass-plates (10 x 20 cm) and visualized by UV fluorescence quenching (254nm), KI-containing acetic o-Tilidin solution staining. The retention factor values for both linear and cyclic peptides were determined in System A: n-Butanol/acetic acid/water (48:18:24) and Sytem B: n- propanol/ 25% NH_3 (7:3) (Table 5.1).

Table 5.1 Retention Factor values for linear and cyclic YM 254890 peptide analogues.

System	Peptides	YM-3	YM-4	YM-5	YM-6	YM-7	YM-8	YM-9	YM-10
A	linear	0.5	0.45	0.45	0.45	0,55	0.55	0.56	0.55
	cyclic	0.58	0.59	0.55	0.55	0.68	0.54	0.55	0.55
B	linear	0.54	0.47	0.50	0.46	0.51	0.53	0.57	0.51
	cyclic	0.77	0.79	0.75	0.72	0.76	0.75	0.76	0.76

The amino acid composition of the peptides was verified by amino acid analysis using a LC 3000 system from Eppendorf-Biotronik (Hamburg, Germany). Hydrolysis was performed using 6N HCl solution in sealed tubes at 110 °C for 24 h. The analyses obtained supported the expected quantitative results. Amino acid analysis in combination with HPLC (calibration curve) was used for the determination of peptide concentrations in solution prior to electrophysiological experiments.

5.4 Native Chemical Ligation (NCL)

5.4.1 Peptide ligation in buffer system

All NCL reactions were performed under argon atmosphere. The individual peptide fragment 1 (3 mM, 1 equiv., LYRAX with X = G, A, F, L, N, Q, K) and fragment 2 (6 mM, 2 equiv., CRANK) were dissolved in ligation buffer (0.1 M phosphate buffer, pH 8.5 containing 6 M guanidine HCl, 4% (v/v) benzyl

mercaptan, and 4% (v/v) thiophenol). The reaction mixture was stirred at room temperature for 48 hours.

Progress of the reaction was monitored by analytical reversed-phase HPLC on a Jasco LC-Net II/ADC chromatograph equipped with a Vydac 218TP column (4.6 x 25 mm, 5 µm particle size, 300 Å pore size) and a mobile phase system consisting of eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile). The detection was at 220 nm. For each analysis, a continuously increasing gradient was applied starting at 0% eluent B and finishing at 50 % eluent B in a time range of 50 min. The flow rate used was 1 ml/min. Single peaks were collected, freeze-dried and forwarded to MALDI-TOF MS analysis.

5.4.2 Peptide ligation in [C_2mim][OAc]

Similar to the peptide ligation in the buffer system, the individual fragments 1 (3 mM, 1 equiv., LYRAX with X = G, A, F, L, N, Q, K) and fragment 2 (6 mM, 2 equiv., CRANK) were dissolved in pure [C_2mim][OAc] (3% water content). The reactants were mixed by continuous stirring under argon atmosphere. All other reaction conditions and measurements were identical to the NCL reactions in buffer system mentioned in chapter 5.4.1.

For some peptides a shift of the retention time was observed. This shift was also found for the standard peptides LYRAXCRANK (X = F, N, G, L) if stored in the IL (Fig. 5.8). MALDI-TOF MS analysis of HPLC peaks of the same retention time in IL (Table 5.1) revealed the same mass as found for the respective compound in buffer. Furthermore, a second peak in HPLC appeared showing a mass increase of approx. 32 g/mol, which was supposed to be an oxidized cysteine residue. In order to consider this fact for quantification of the ligation products, both peaks were used when occurring in the NCL experiment.

Table 5.2 Analytical characterization of the peptides prepared and the products obtained from NCL reactions.[89]

Peptide abbrev.	M_w (theor.)[a]	HPLC [b] t_R (min) in buffer	HPLC [b] t_R (min) in IL
LYRAG	723.30 (722.87)	29.1[c]	29.0[c]
LYRAA	737.22 (736.89)	30.9[d]	31.0[d]
LYRAL	779.39 (778.98)	38.8[d]	38.8[d]
LYRAF	813.32 (812.99)	39.6[d]	39.5[d]
LYRAN	780.28 (779.92)	27.3[c]	27.1[c]
LYRAQ	794.35 (793.95)	27.9[c]	27.7[c]
LYRAK	794.39 (793.97)	27.5[c]	27.1[c]
CRANK[X]	589.58 (588.71)	6.9[c]	n.d.[e]
LYRAGCRANK	1192.62 (1191.41)	21.7[c]	21.4[c]/19.8[c,f]
LYRAACRANK	1206.82 (1205.43)	24.3[d]	24.3[d]/21.8[d,f]
LYRALCRANK	1248.60 (1247.52)	31.2[d]	31.1[d]/28.1[d,f]
LYRAFCRANK	1282.59 (1281.54)	32.4[d]	32.1[d]/29.6[d,f]
LYRANCRANK	1249.64 (1248.46)	21.6[c]	22.1[c]/20,6[c,f]
LYRAQCRANK	1263.75 (1262.49)	21.8[c]	21.8[c]/20,5[c,f]
LYRAKCRANK	1263.84 (1262.53)	21.6[c]	21.3[c]/20,6[c,f]

[a] Mass peaks detected as $[M+H]^+$.
[b] eluent A: water with 0.1% TFA; eluent B: acetonitrile with 0.1% TFA.
[c] 0%–40% eluent B in 40 min.
[d] 0%–60% eluent B in 60 min.
[e] Peptide peak overlapped with IL peak.
[f] IL-oxidized peptide peak.
[g] The C-terminal is amidated.

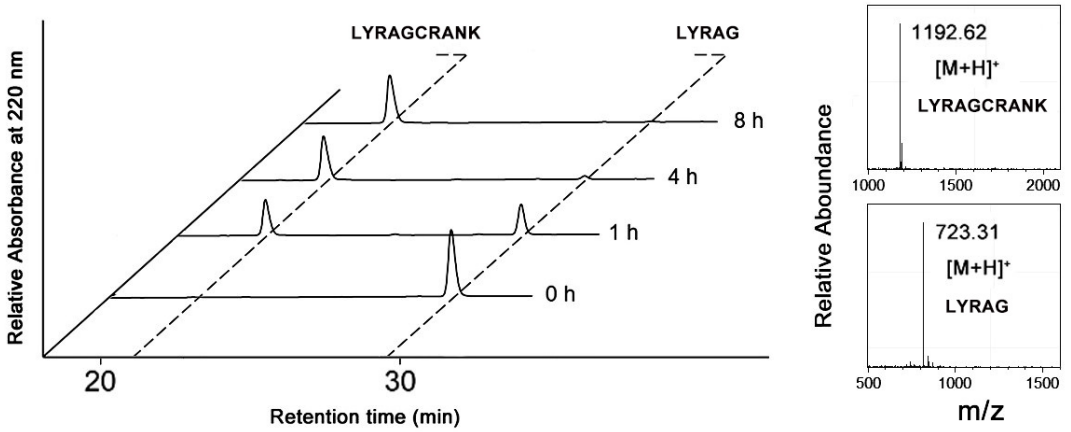


Figure 5.1 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products obtained in buffer system of reacting LYRAG with CRANK resulting in LYRAGCRANK (reprinted with permission from the author's publication[89]).

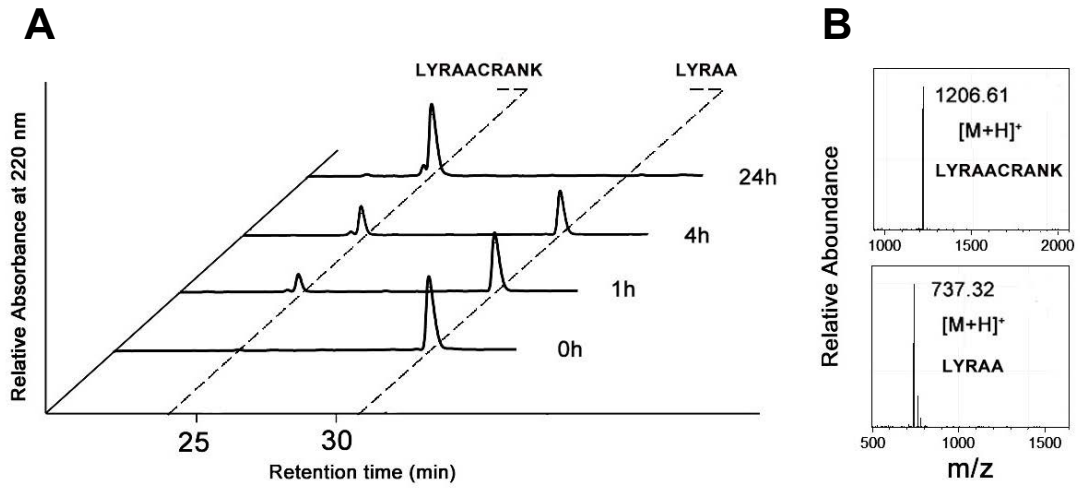


Figure 5.2 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products obtained in buffer system of linking LYRAA with CRANK resulting in LYRAACRANK (reprinted with permission from the author's publication[89]).

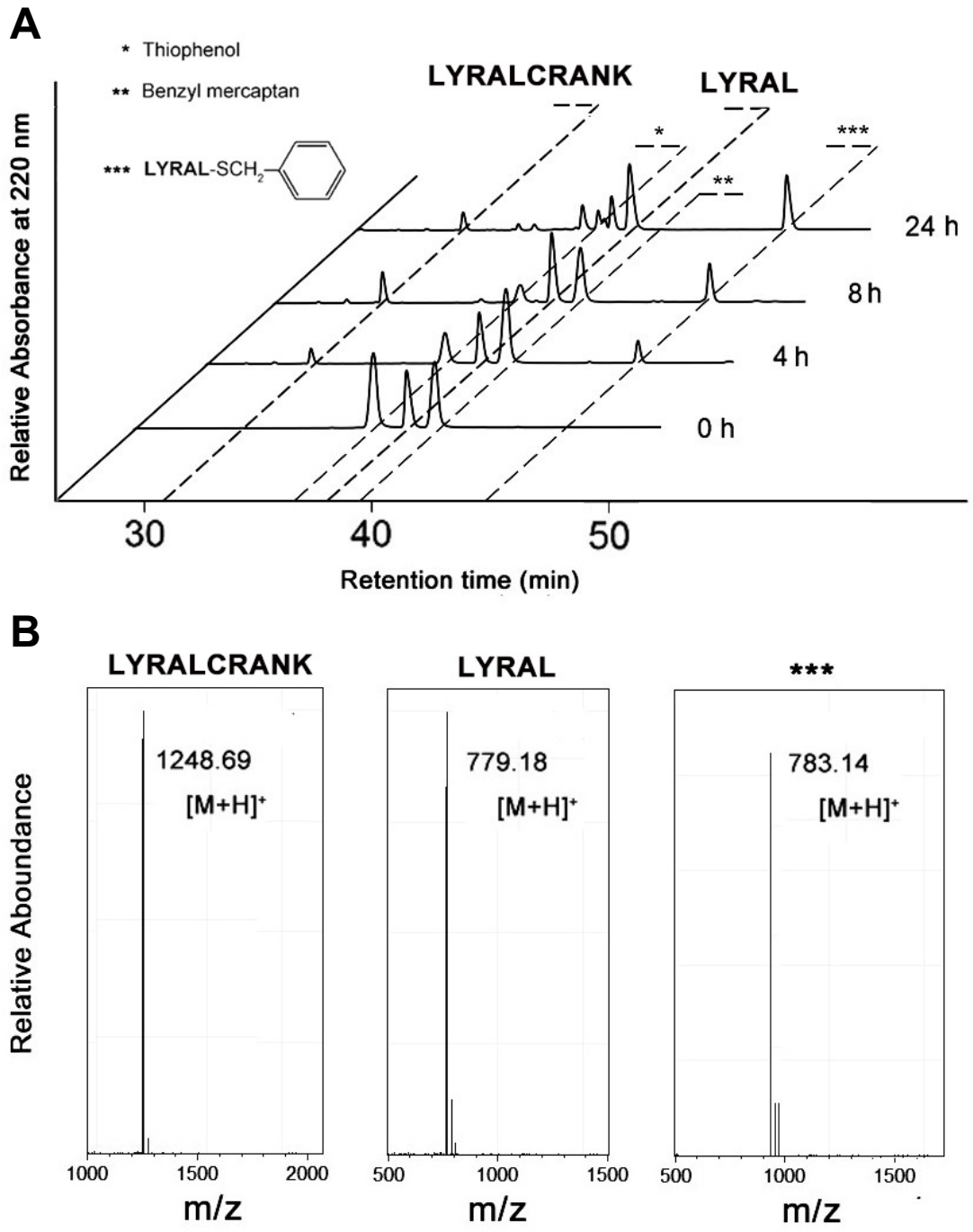


Figure 5.3 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in buffer system of reacting LYRAL with CRANK resulting in LYRALCRANK (reprinted with permission from the author's publication[89]).

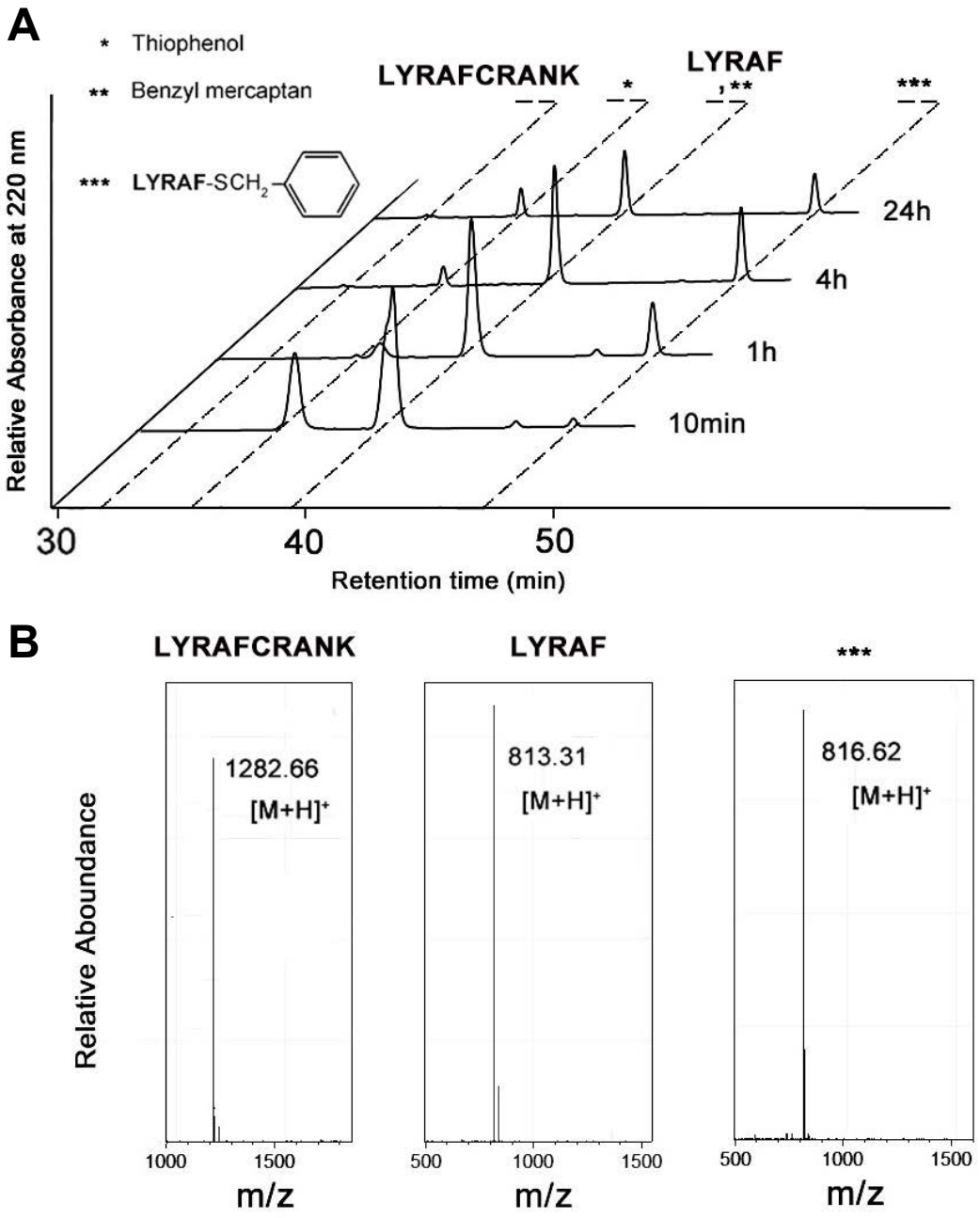


Figure 5.4 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in buffer system of reacting LYRAF with CRANK resulting in LYRAFCRANK (reprinted with permission from the author's publication[89]).

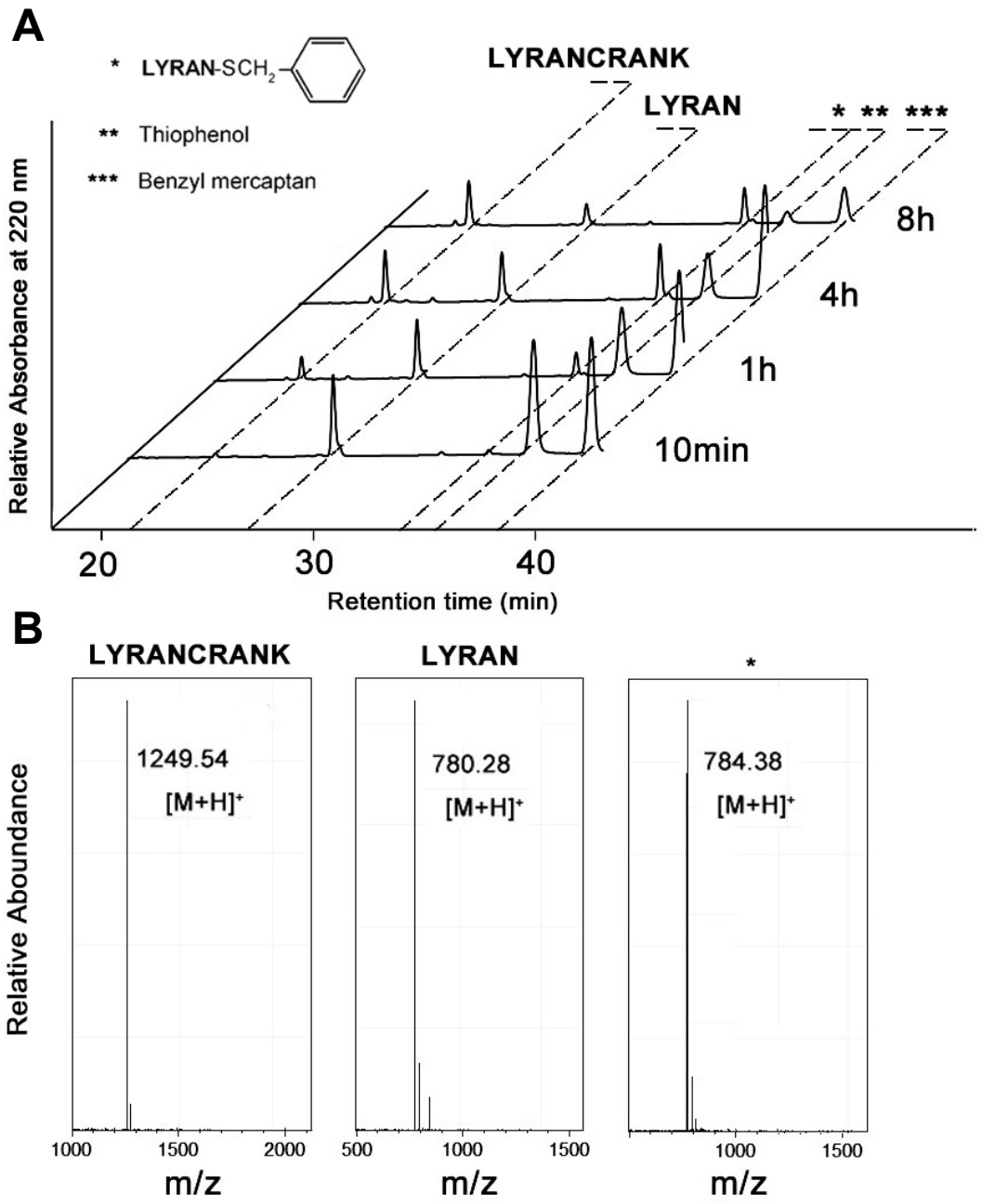


Figure 5.5 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in buffer system of reacting LYRAN with CRANK resulting in LYRANCRANK (reprinted with permission from the author's publication[89]).

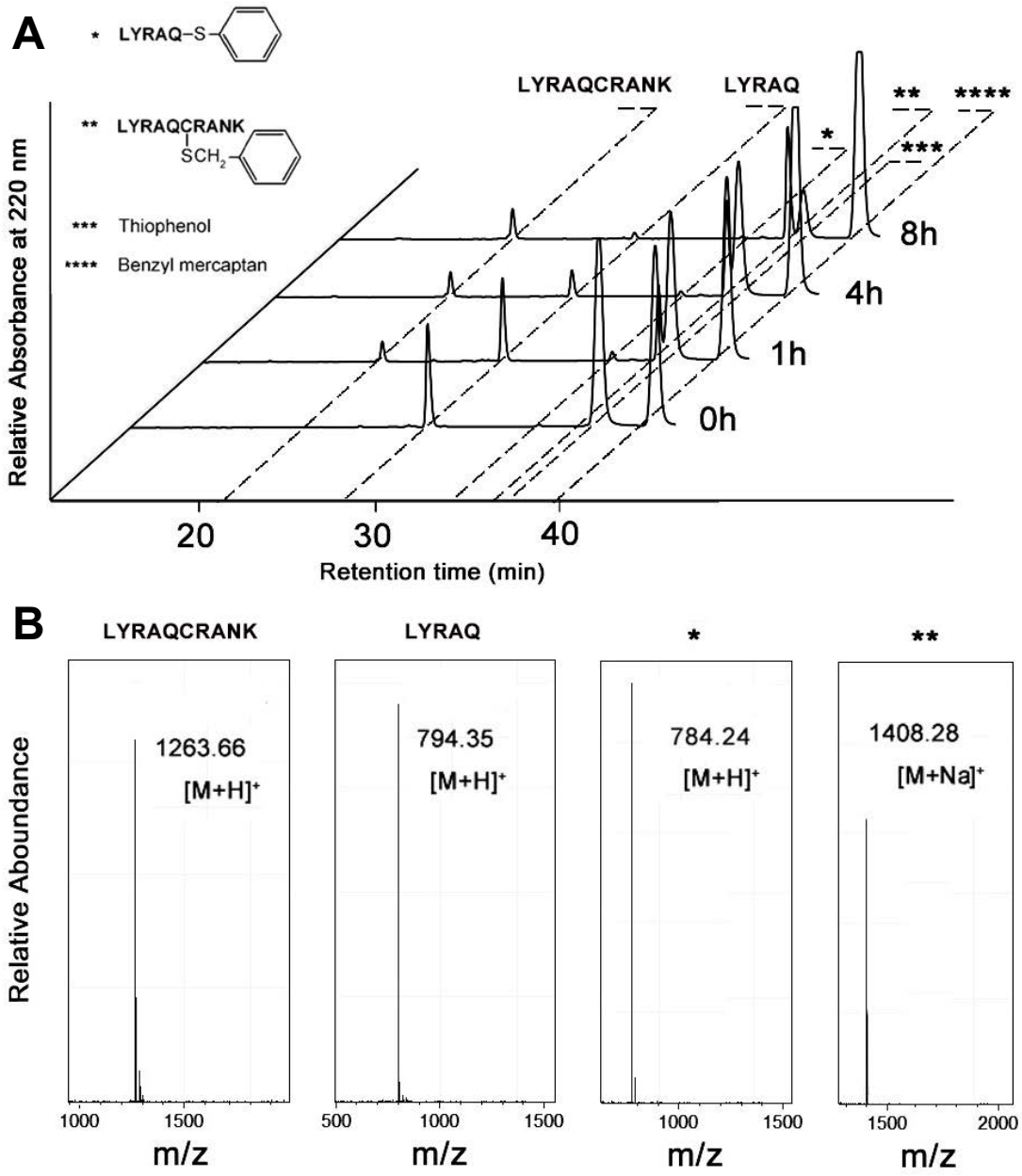


Figure 5.6 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in buffer system of reacting LYRAQ with CRANK resulting in LYRAQCRANK (reprinted with permission from the author's publication[89]).

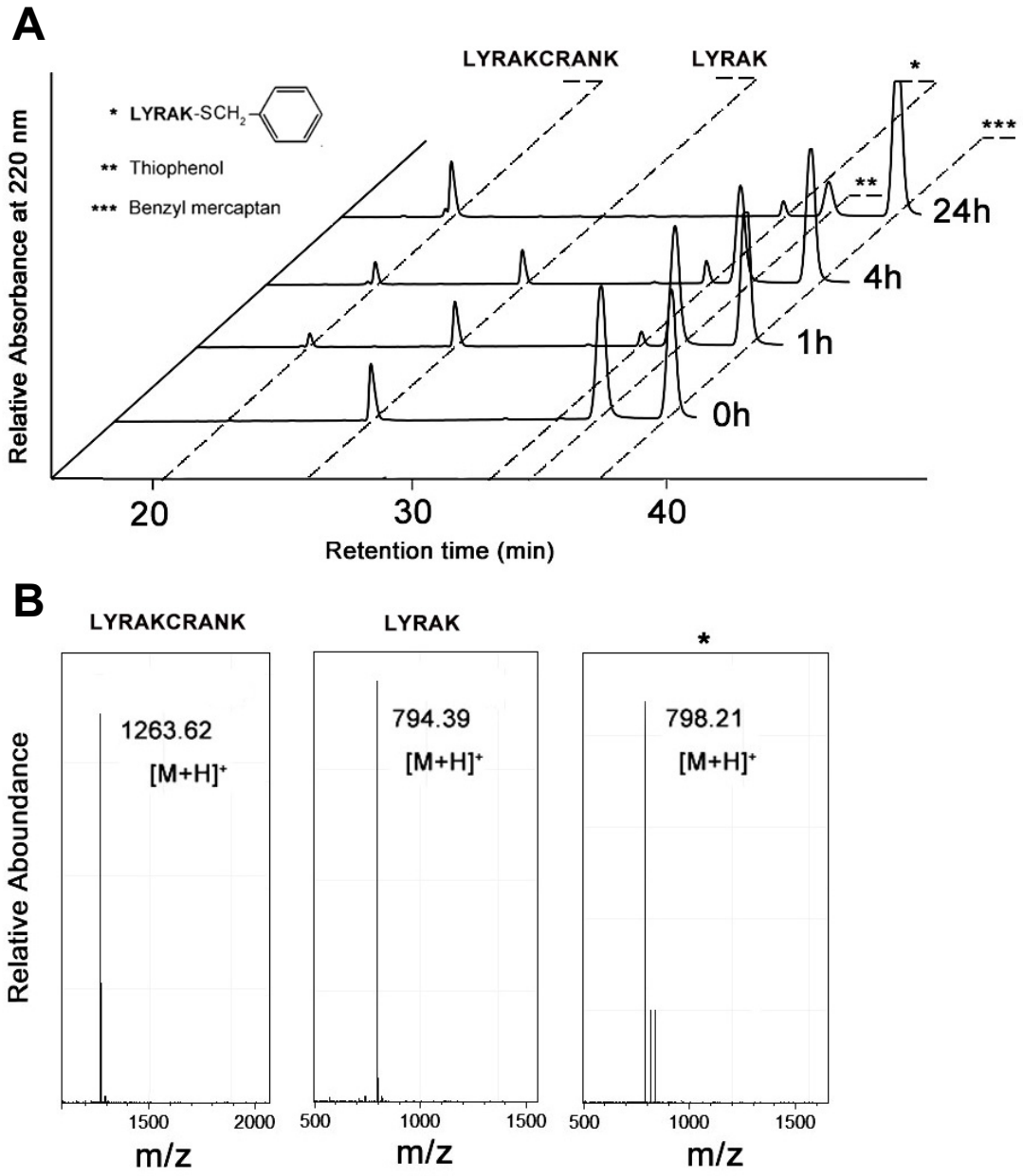


Figure 5.7 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in buffer system of reacting LYRAK with CRANK resulting in LYRAKCRANK (reprinted with permission from the author's publication[89]).

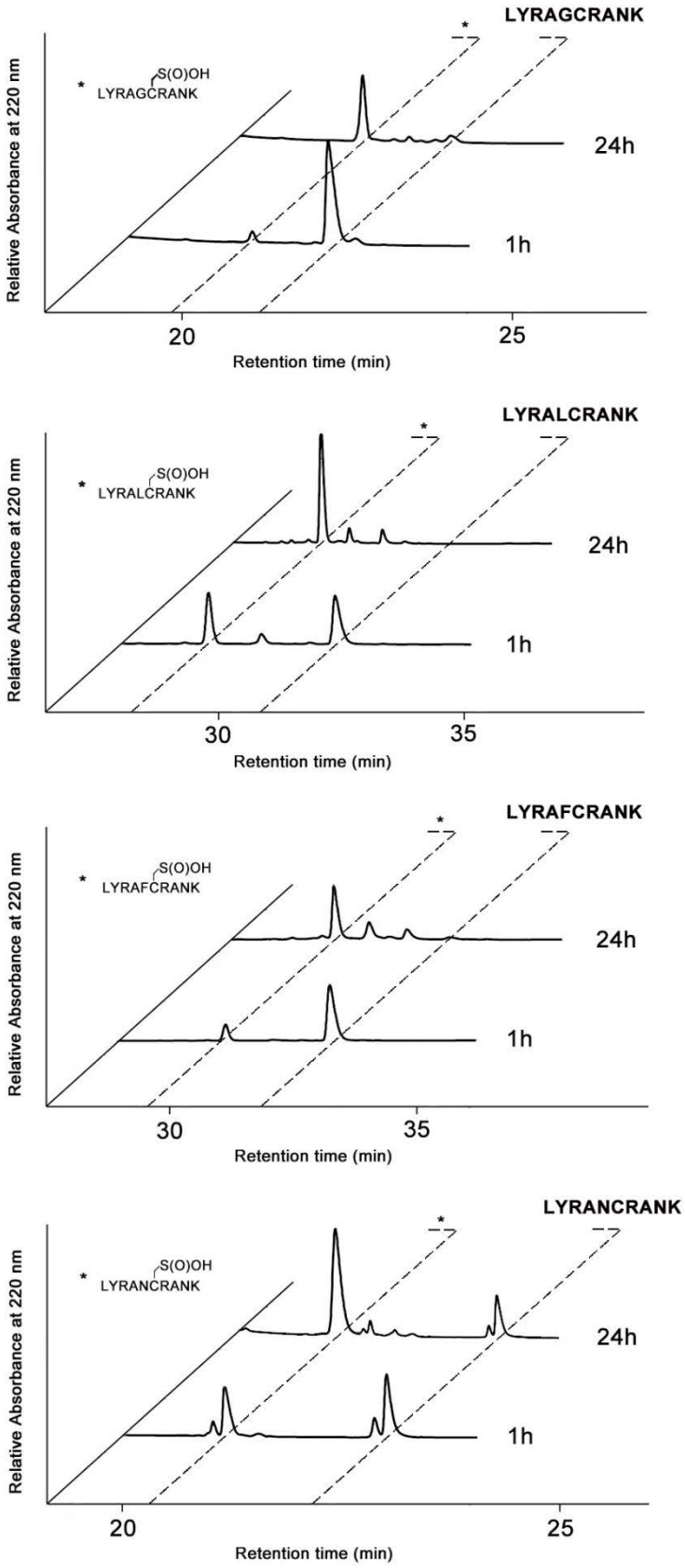


Figure 5.8 HPLC profiles of the incubation of control peptides LYRAXCRANK (X=G, L, F, N) in [C_2mim][OAc] displaying a shift of the retention time during incubation due to oxidation processes (reprinted with permission from the author's publication[89]).

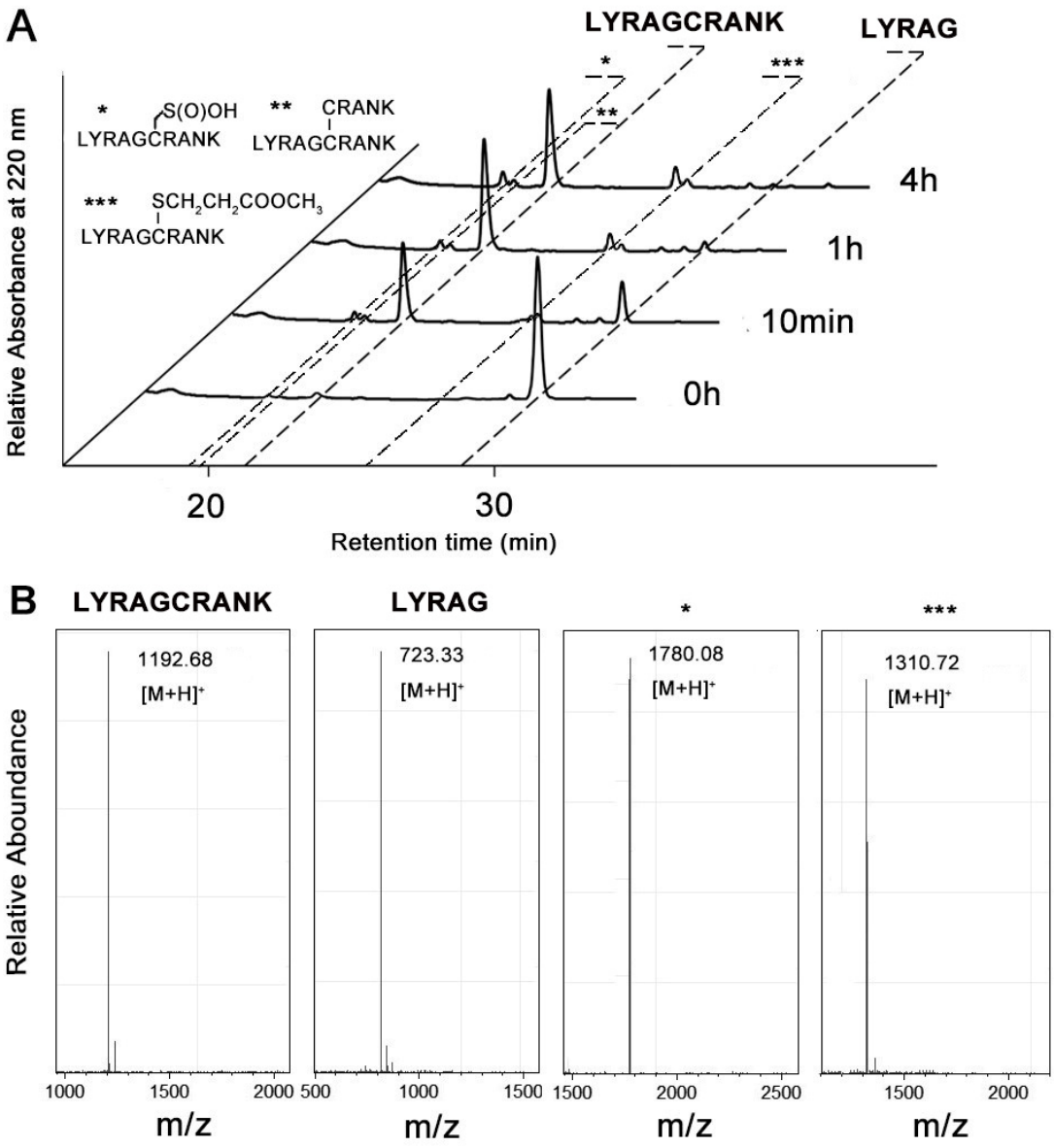


Figure 5.9 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] for reacting LYRAG with CRANK resulting in LYRAGCRANK (reprinted with permission from the author's publication[89]).

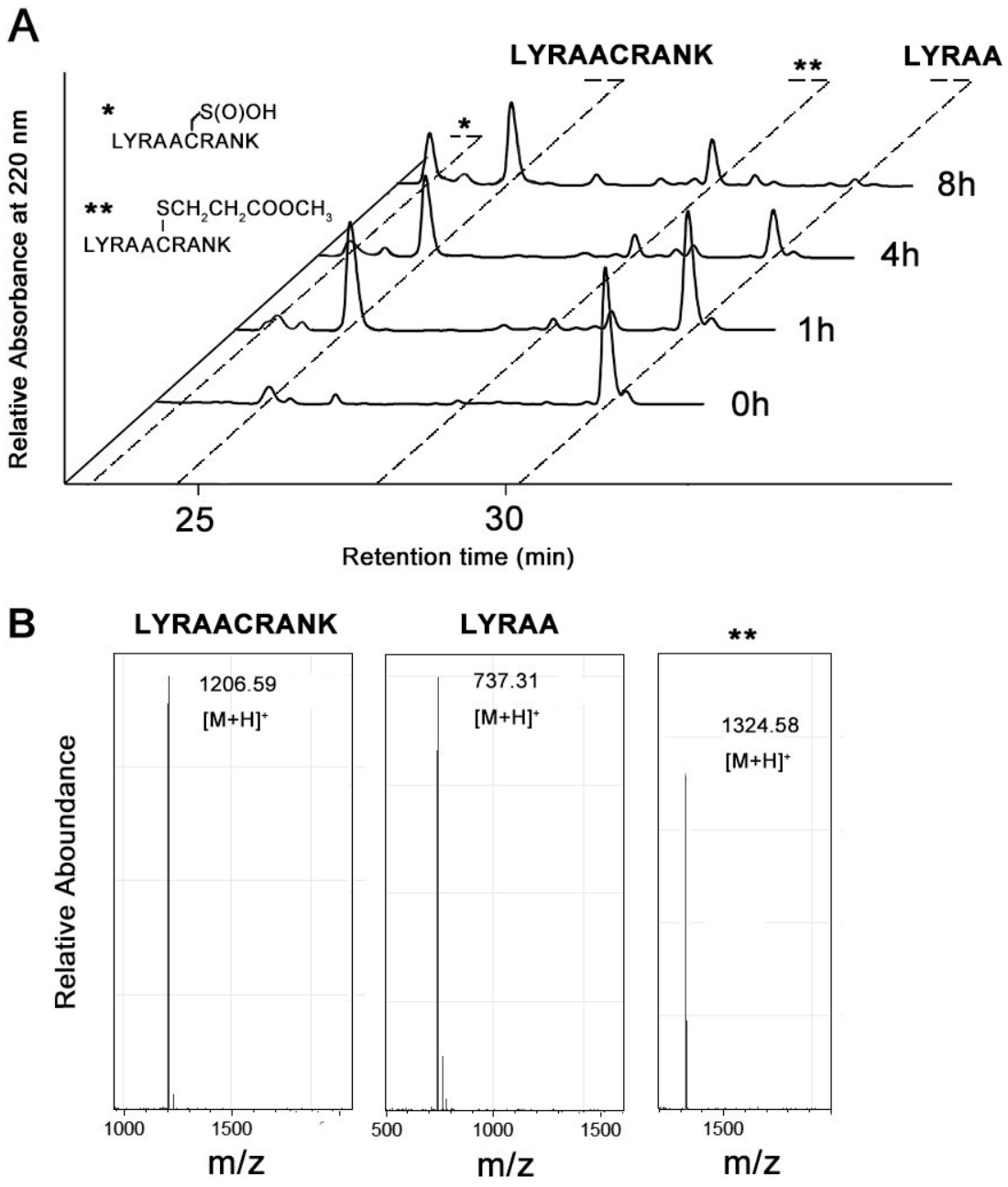


Figure 5.10 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] of reacting LYRAA with CRANK resulting in LYRAACRANK (reprinted with permission from the author's publication[89]).

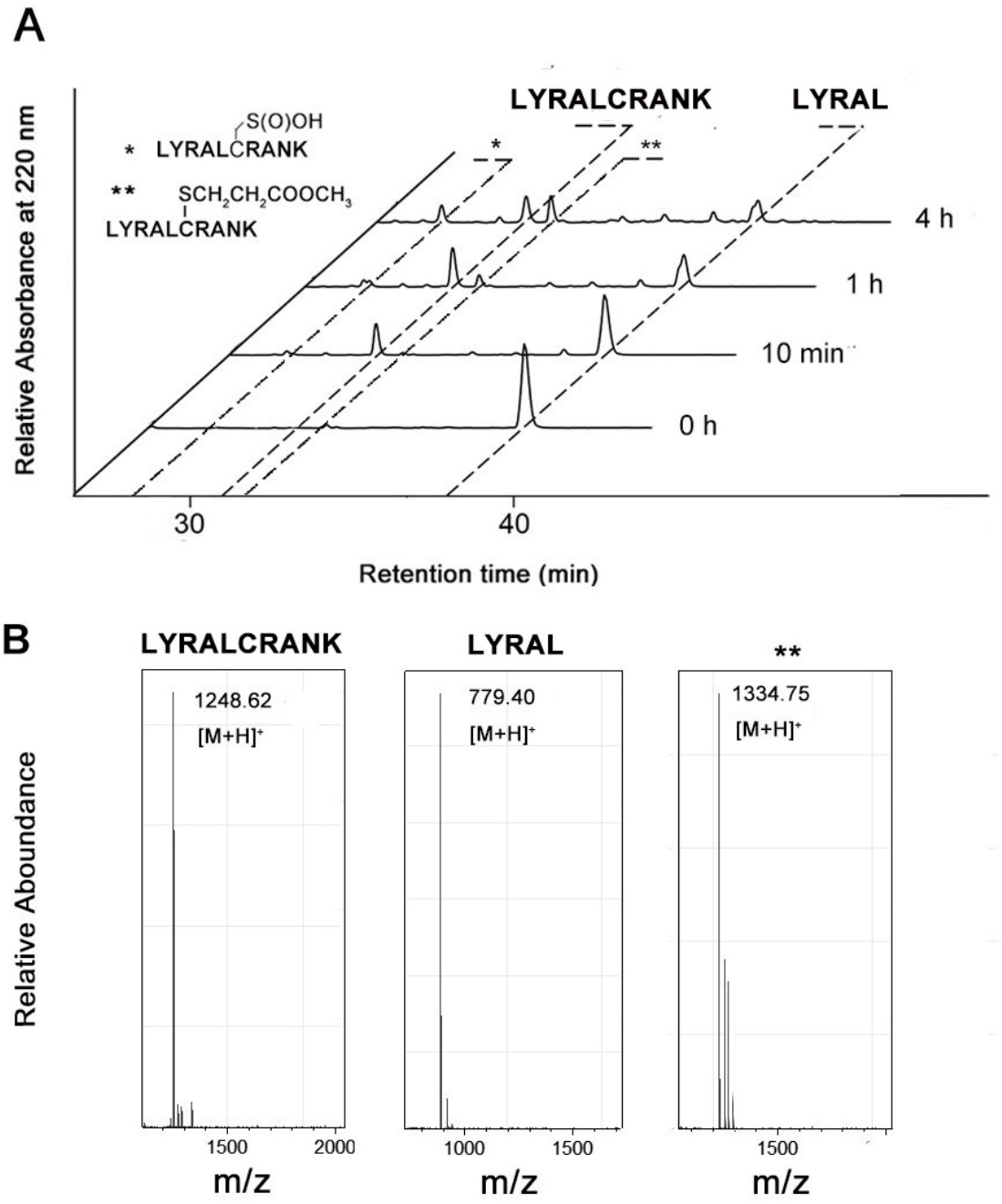


Figure 5.11 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] of reacting LYRAL with CRANK resulting in LYRALCRANK (reprinted with permission from the author's publication[89]).

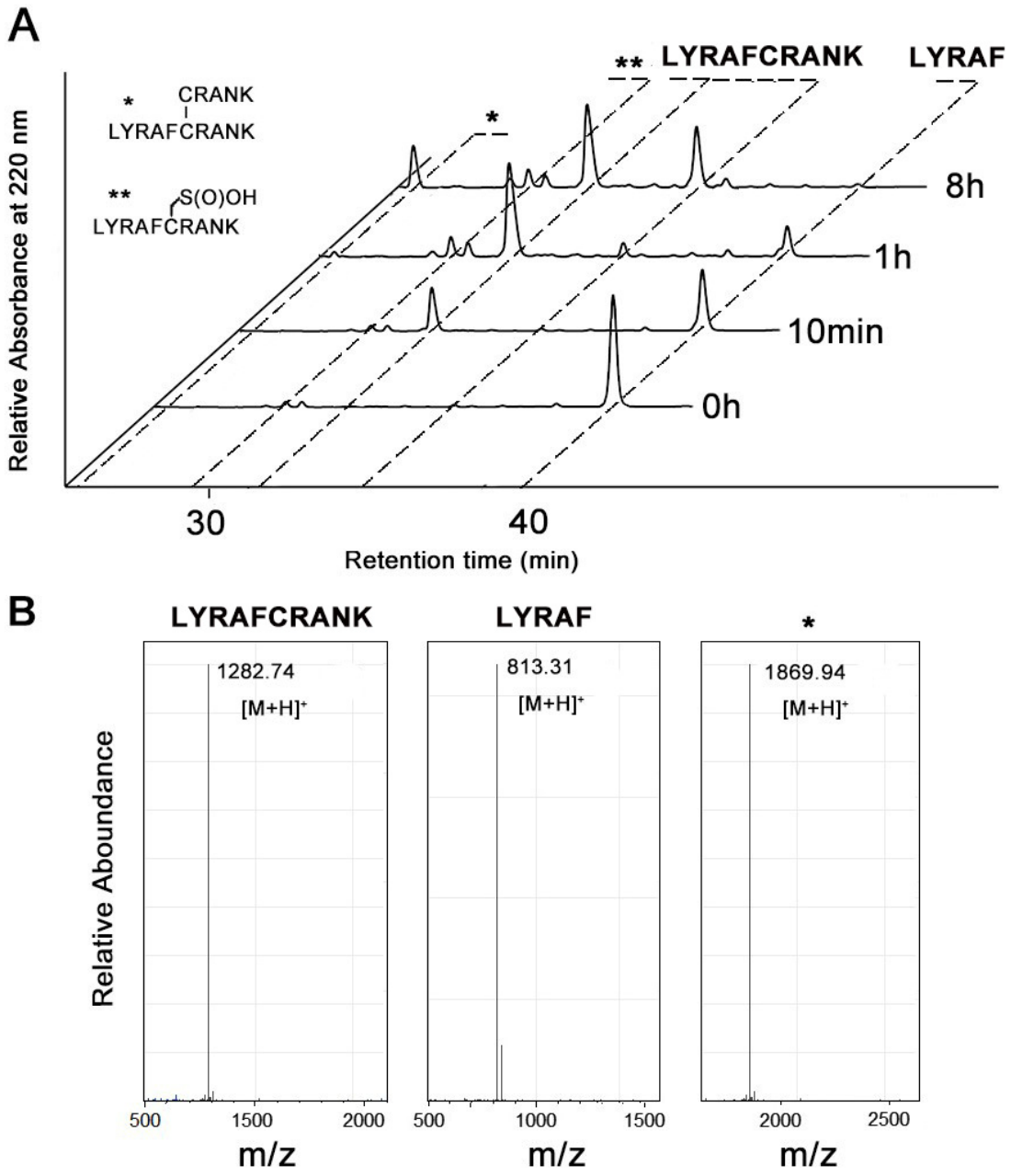


Figure 5.12 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] of reacting LYRAF with CRANK resulting in LYRAFCRANK (reprinted with permission from the author's publication[89]).

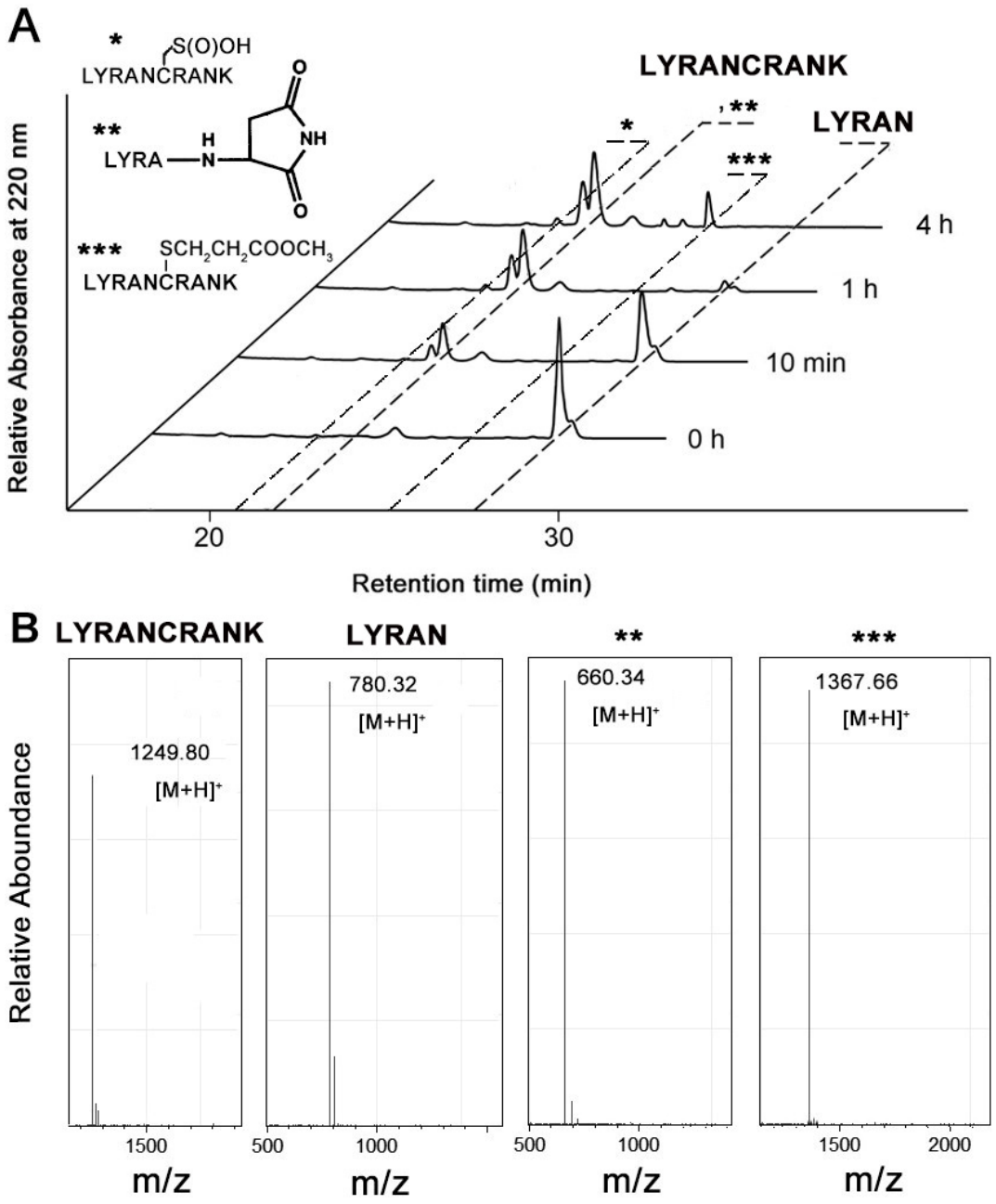


Figure 5.13 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] of reacting LYRAN with CRANK resulting in LYRANCRANK (reprinted with permission from the author's publication[89]).

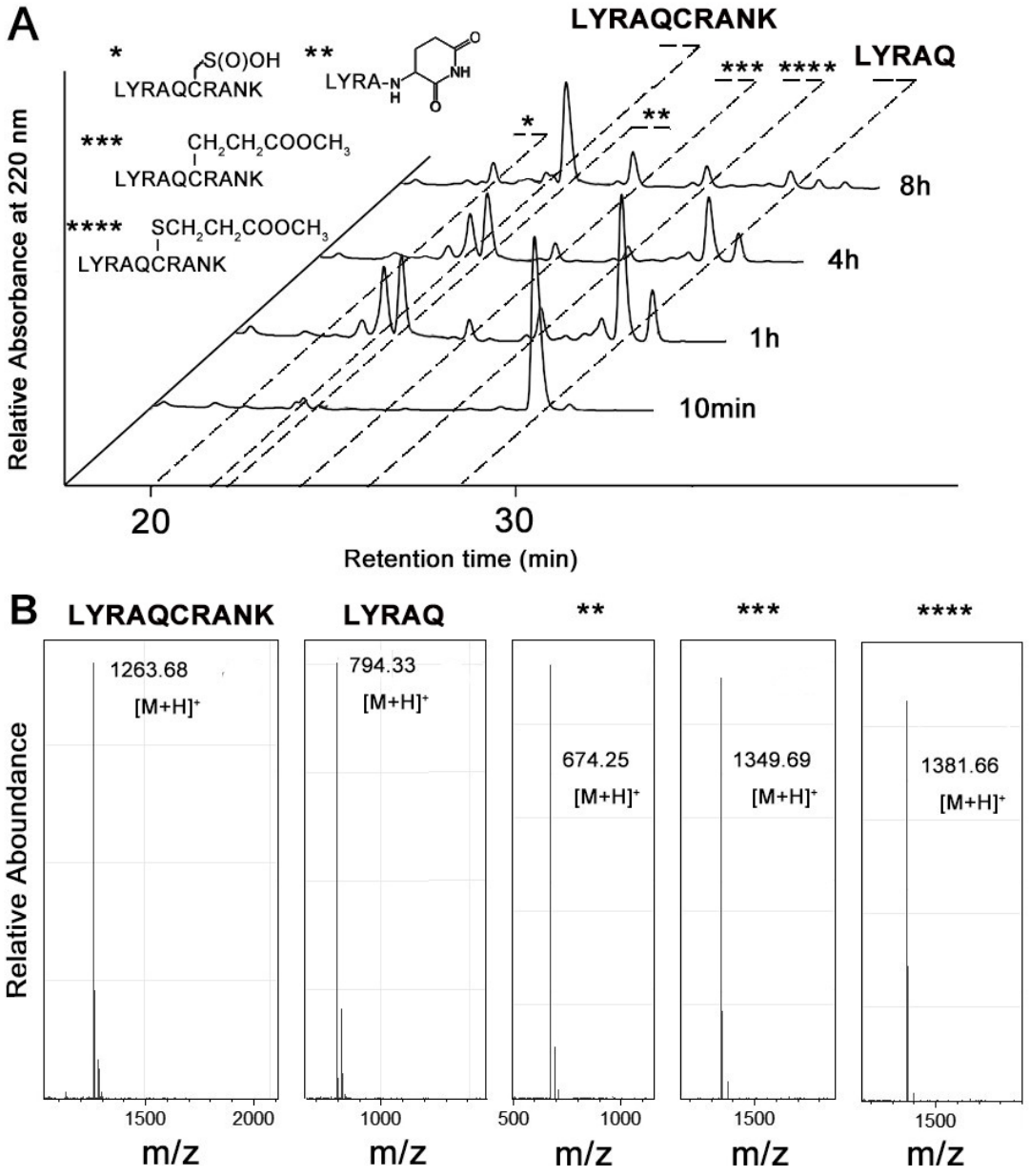


Figure 5.14 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] of reacting LYRAQ with CRANK resulting in LYRAQCRANK (reprinted with permission from the author's publication[89]).

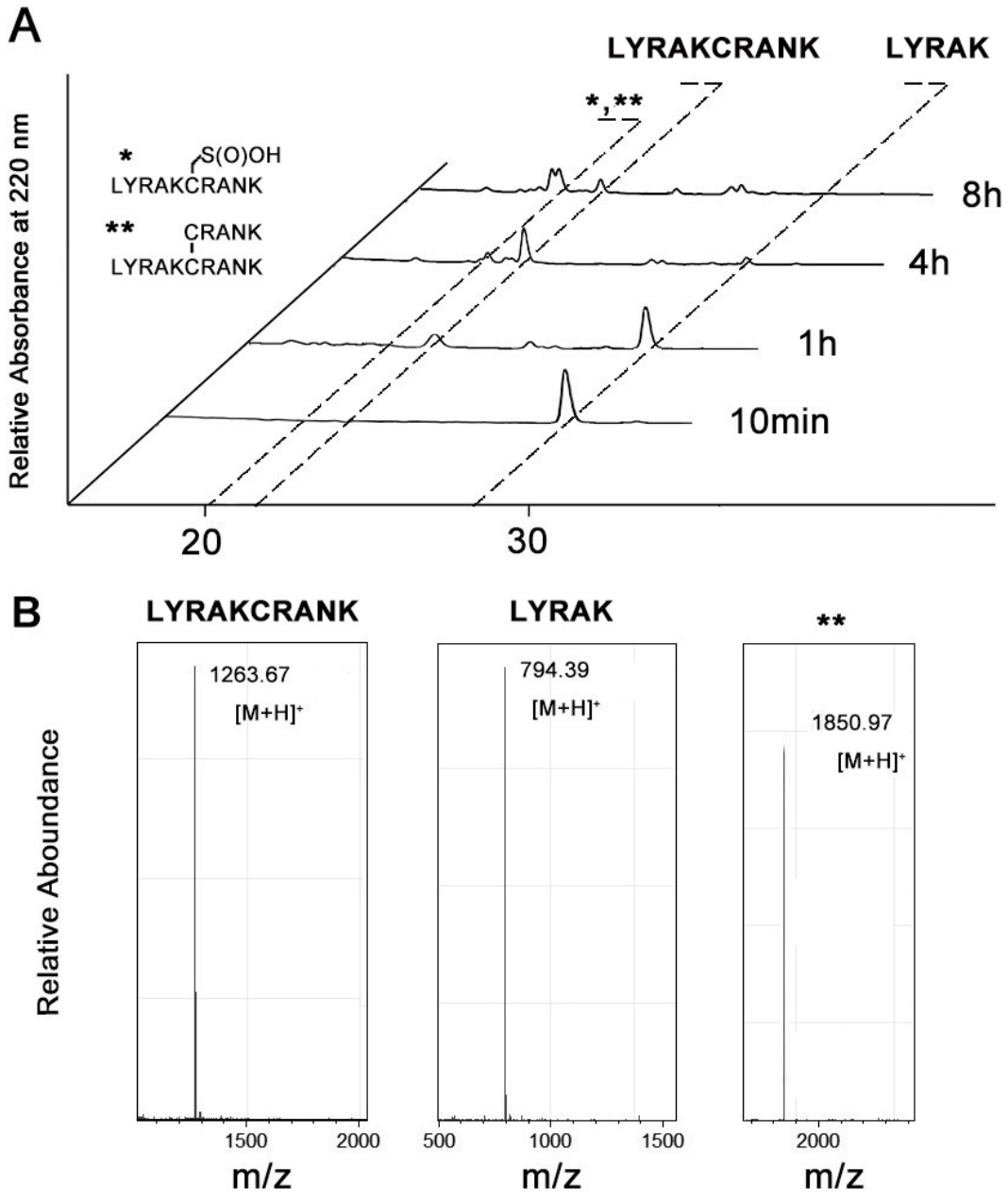


Figure 5.15 HPLC profiles (A) of the ligation reactions and mass spectra (B) of the observed products and intermediates/byproducts obtained in [C_2mim][OAc] of reacting LYRAK with CRANK resulting in LYRAKCRANK (reprinted with permission from the author's publication[89]).

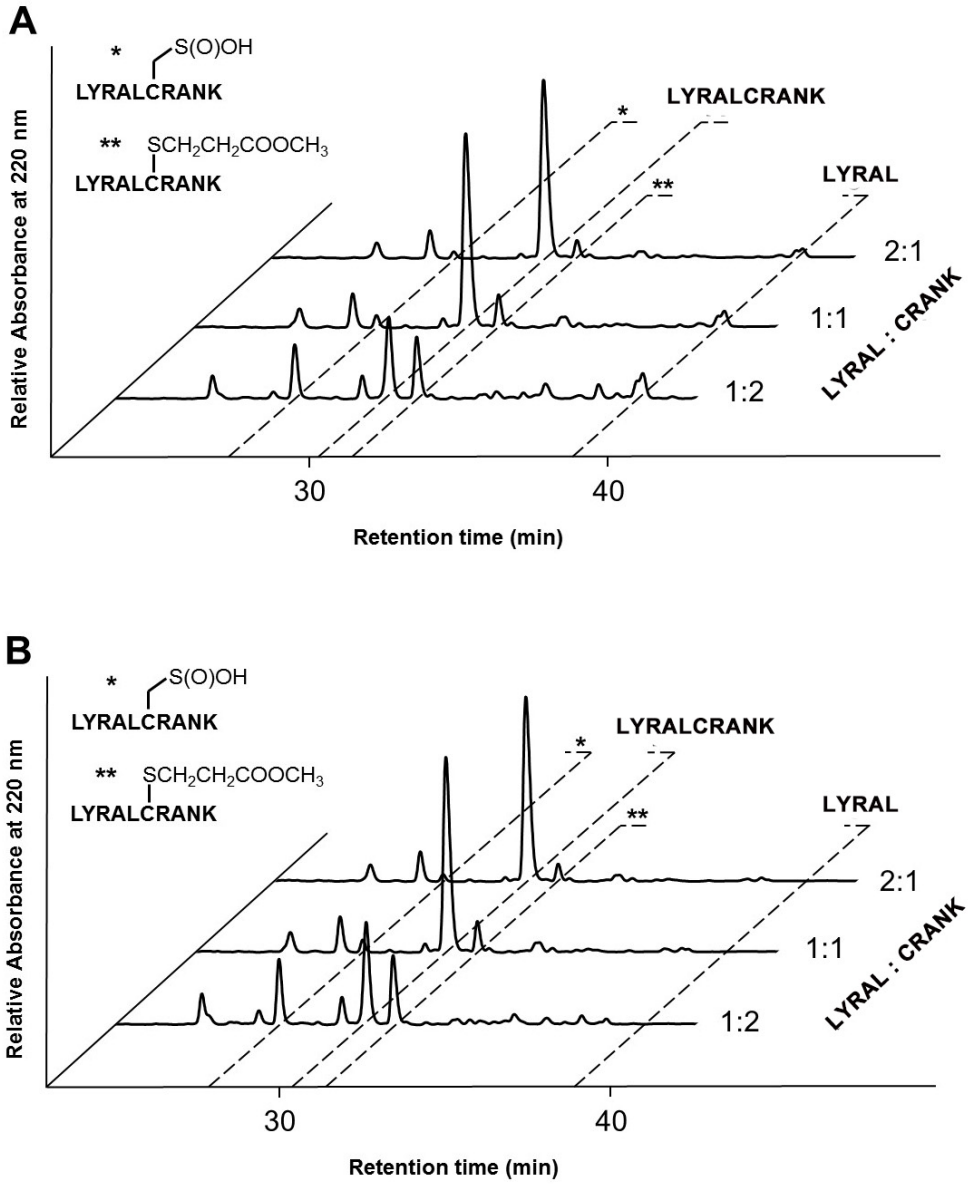


Figure 5.16 HPLC profiles of the ligation reactions obtained in [C_2mim][OAc] of reacting LYRAL with CRANK at different ratios (1:2, 1:1, 2:1) resulting in peptide LYRALCRANK after 4 hours (A) and 8 hours (B) (reprinted with permission from the author's publication[89]).

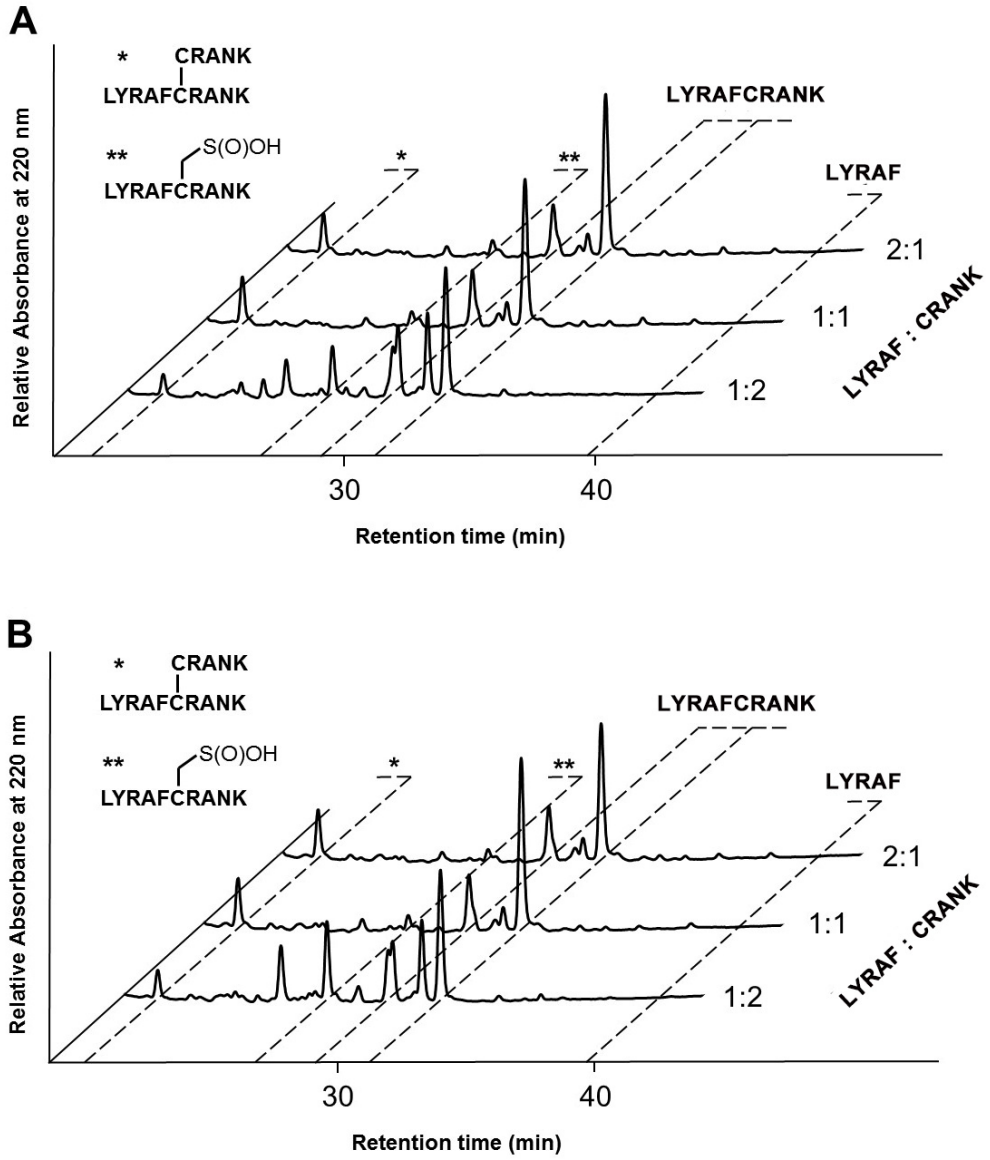


Figure 5.17 HPLC profiles of the ligation reactions obtained in [C_2mim][OAc] of reacting LYRAK with CRANK at different ratios (1:2, 1:1, 2:1) resulting in peptide LYRAKCRANK after 4 hours (A) and 8 hours (B) (reprinted with permission from the author's publication[89]).

5.5 Peptide cyclization via lactam bridge formation

5.5.1 Peptide cyclization in DMF

Peptide cyclization was performed in DMF with a final peptide concentration of 0.65 mM and 6 equiv. PyBOP. The reactions were started by adding DIEA (12 equiv.). The mixture was stirred continuously at room temperature for 6 hours. Progress of the reaction was monitored by analytical reversed-phase HPLC on a Jasco LC-Net II/ADC chromatograph equipped with a Vydac 218TP column (4.6 x 25 mm, 5 µm particle size, 300 Å pore size) and a mobile phase system consisting of A: 0.1% TFA in water and B: 0.1% TFA in acetonitrile. The detection was at 220 nm. Samples for monitoring of cyclization reactions were diluted with a quenching solution (acetonitrile:water, 45:55)[125] in a ratio of 1:16. For each analysis, a continuously increasing gradient was applied starting at 0% eluent B and finishing at 50 % eluent B in 50 min. The flow rate used was 1 ml/min. Single peaks were collected, freeze-dried and forwarded to mass spectrometry analysis.

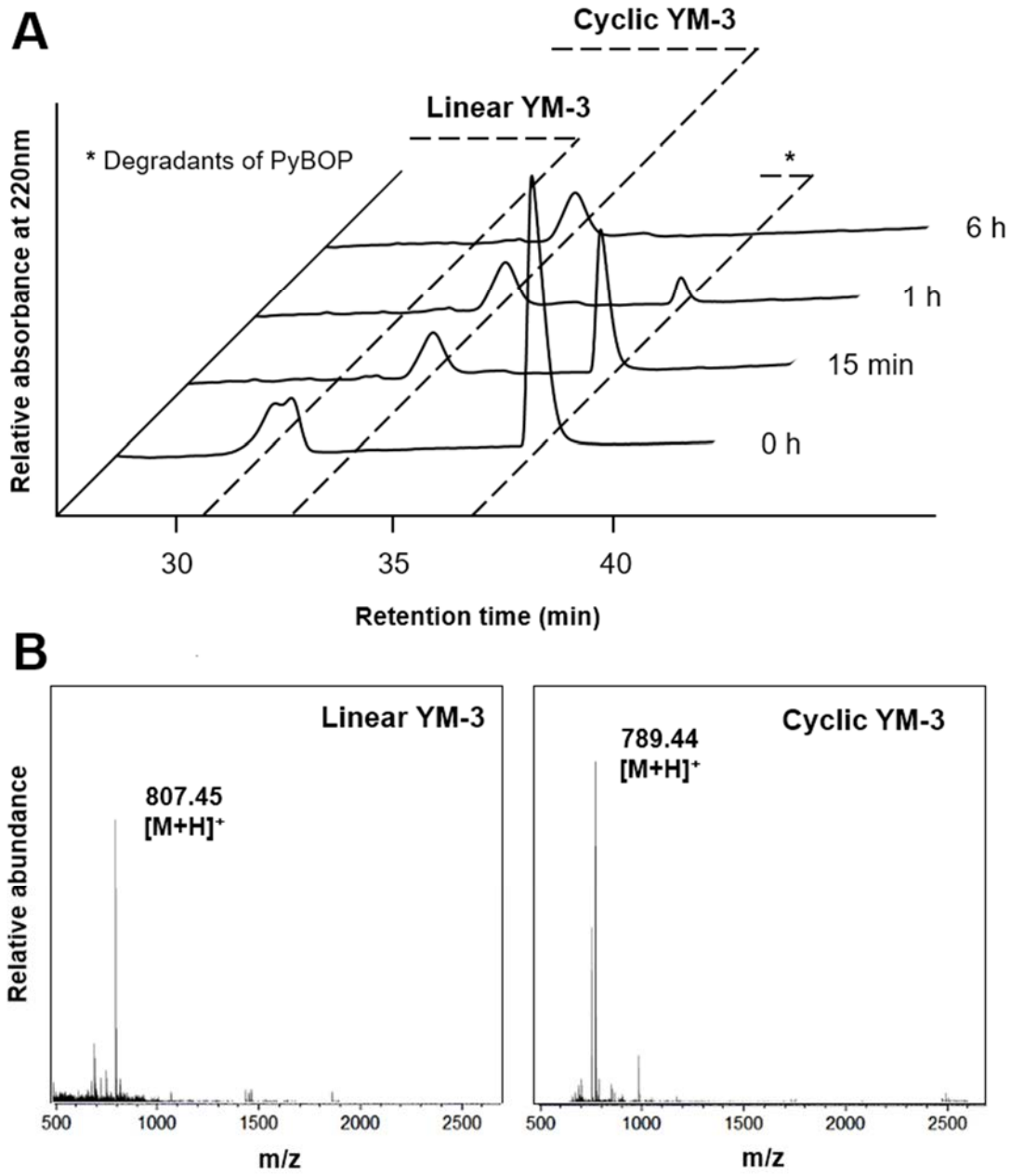


Figure 5.18 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-3 in DMF.

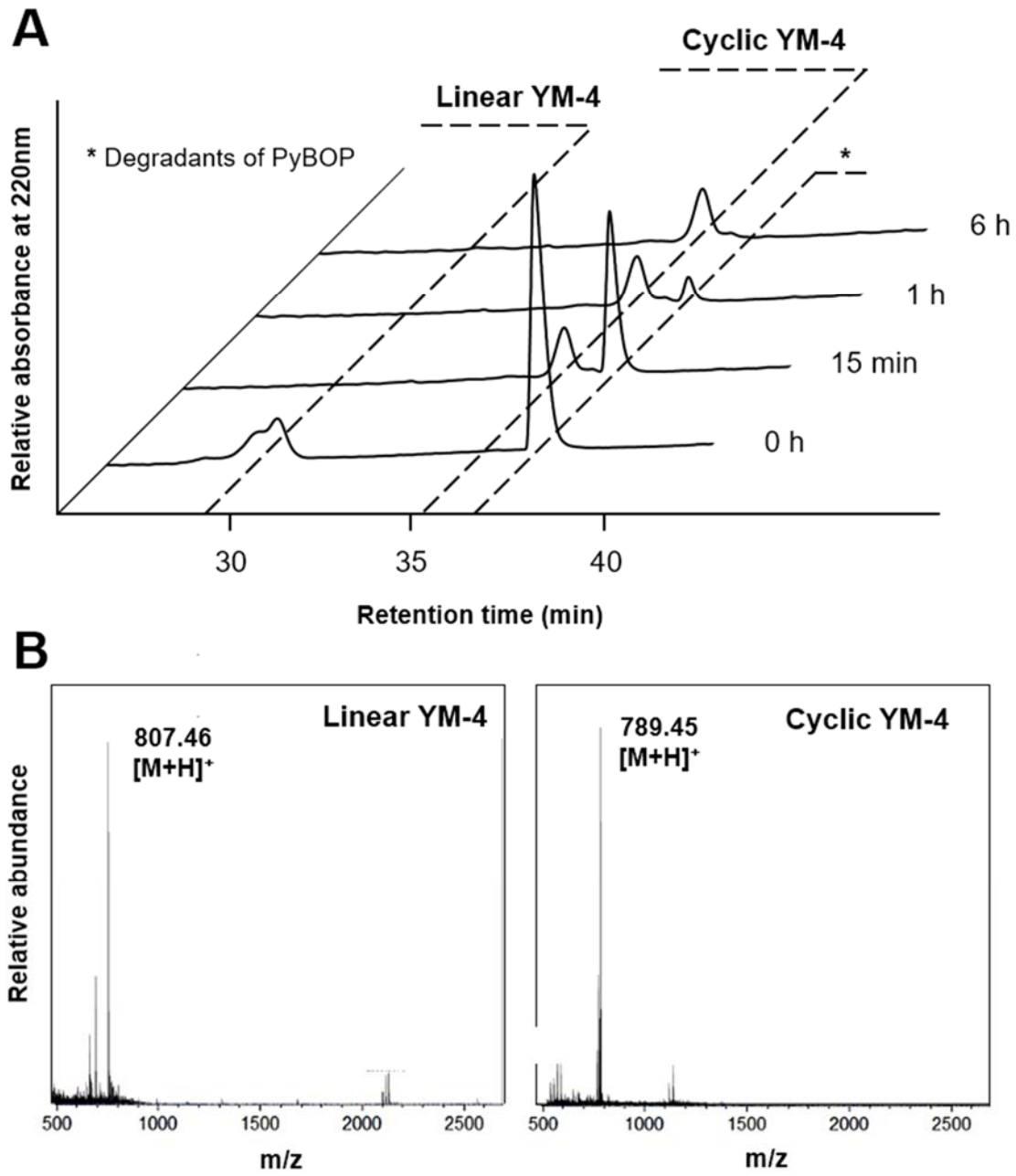


Figure 5.19 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-4 in DMF.

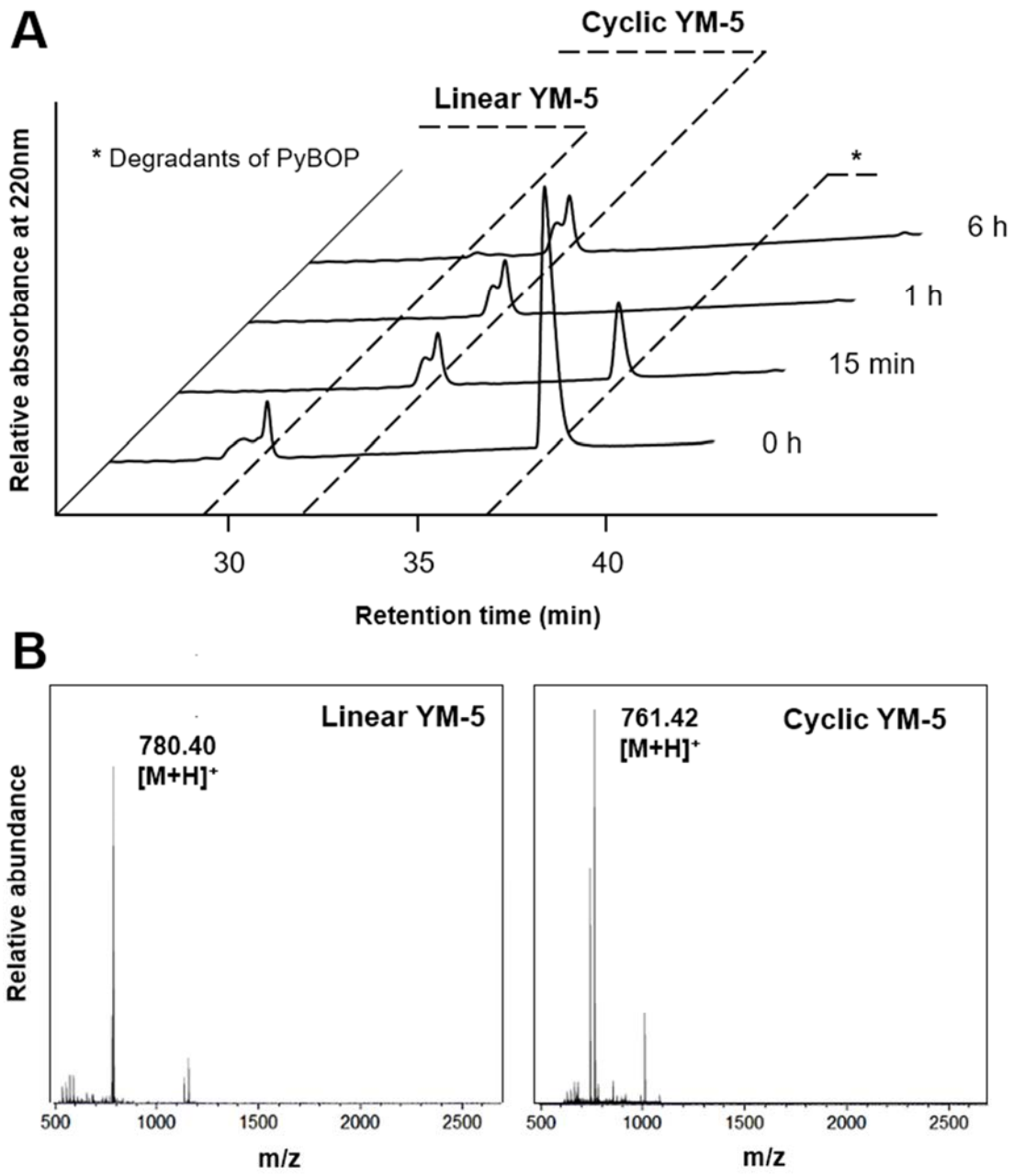


Figure 5.20 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-5 in DMF.

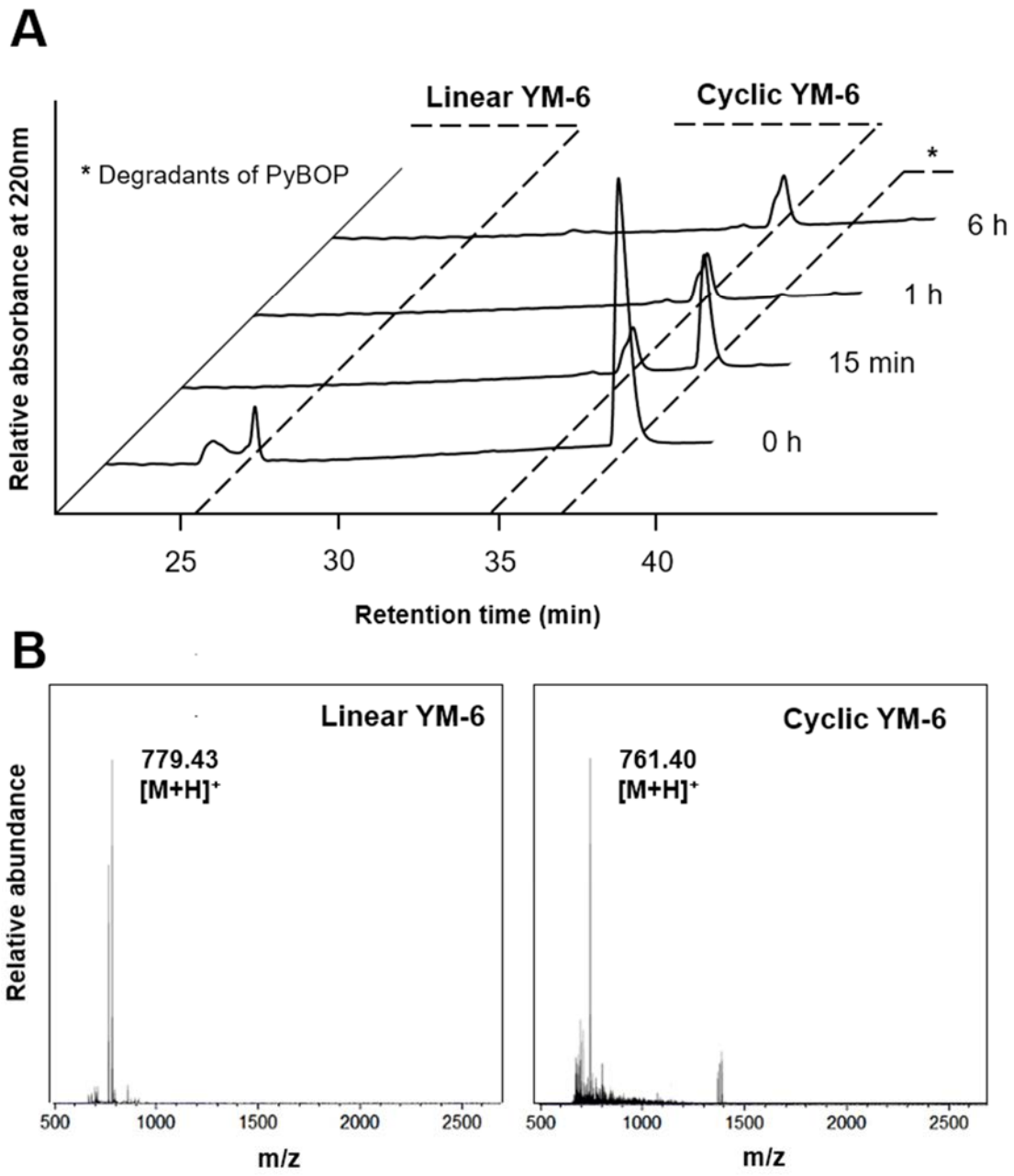


Figure 5.21 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-6 in DMF.

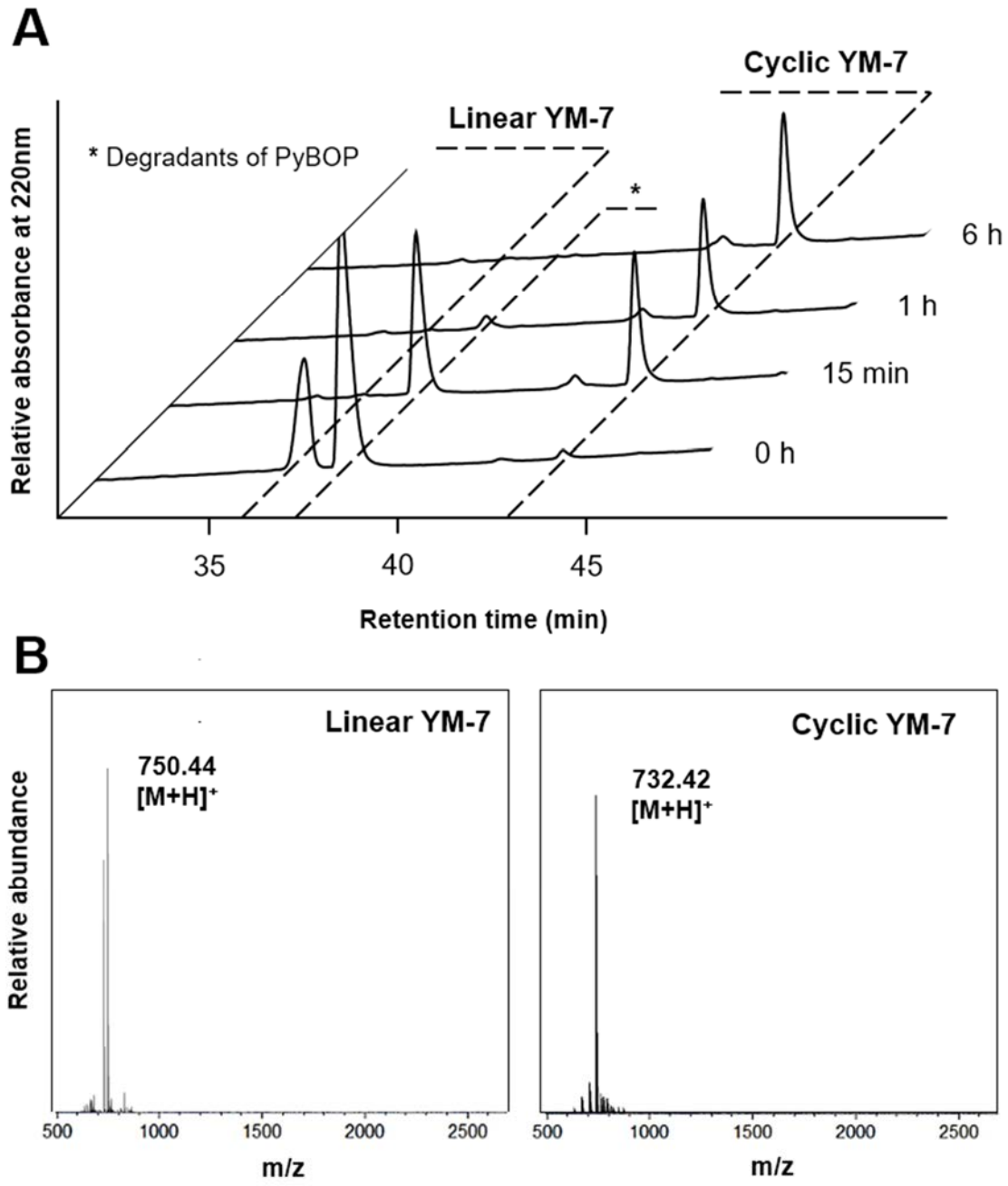


Figure 5.22 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-7 in DMF.

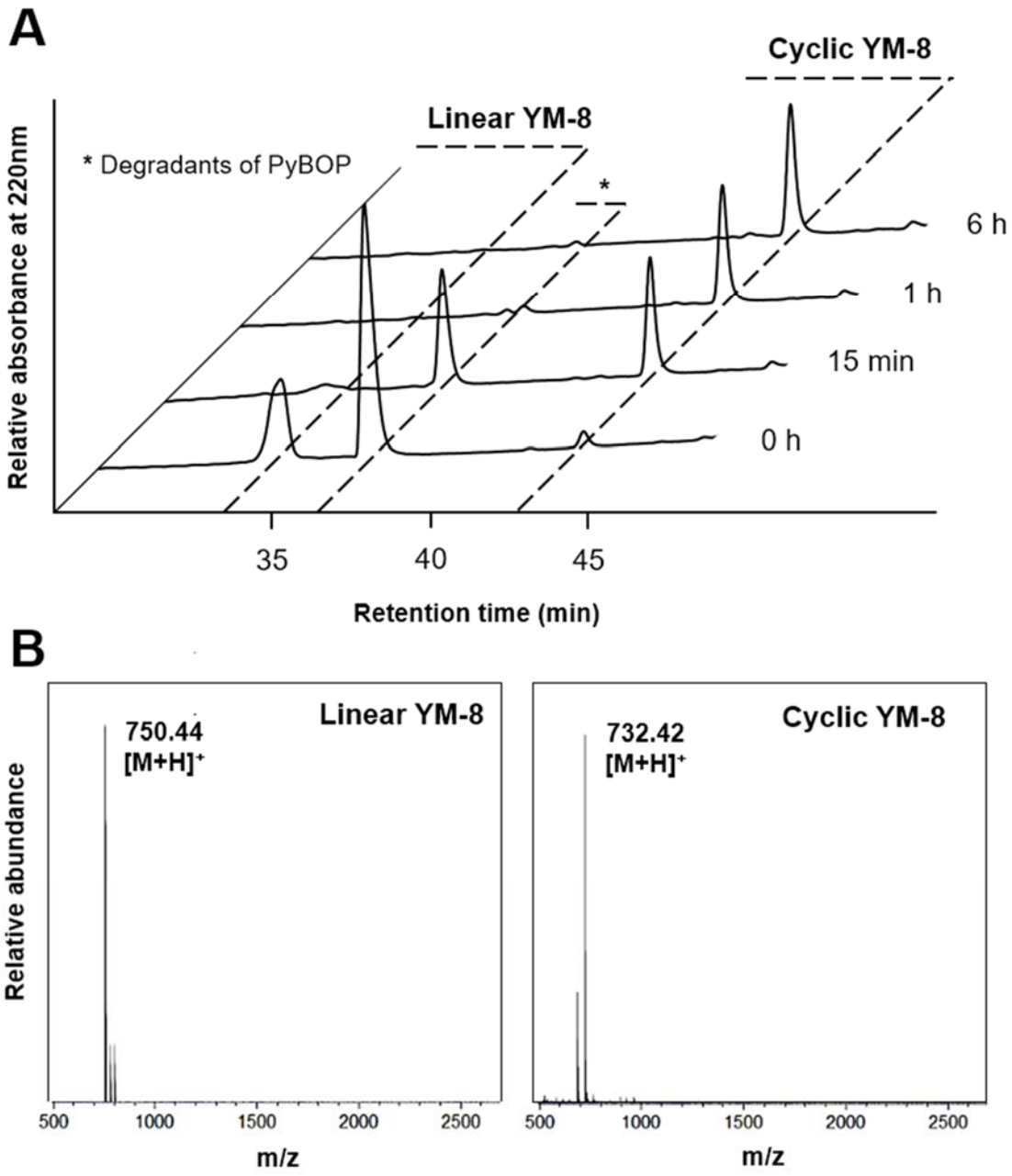


Figure 5.23 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-8 in DMF.

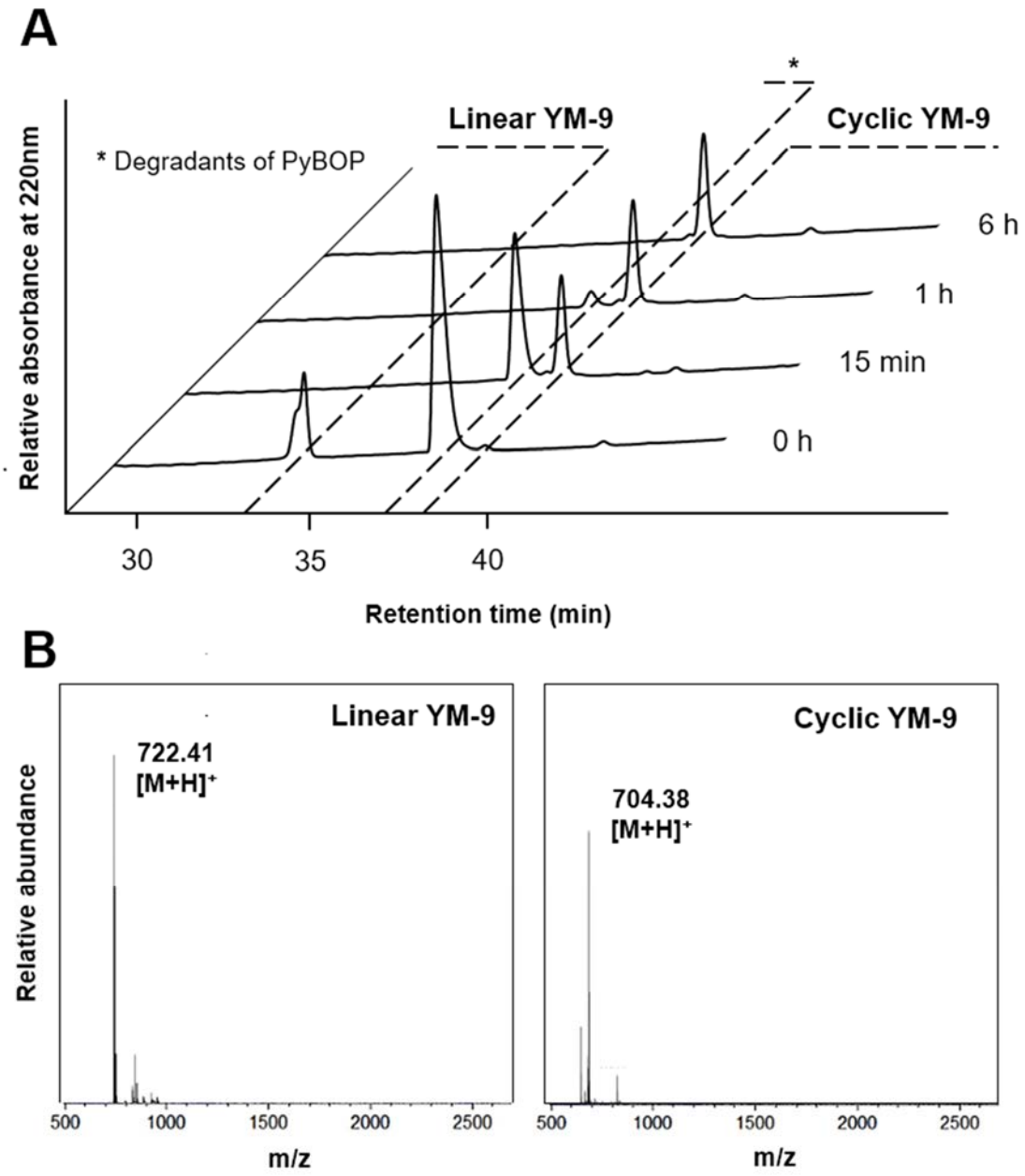


Figure 5.24 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-9 in DMF.

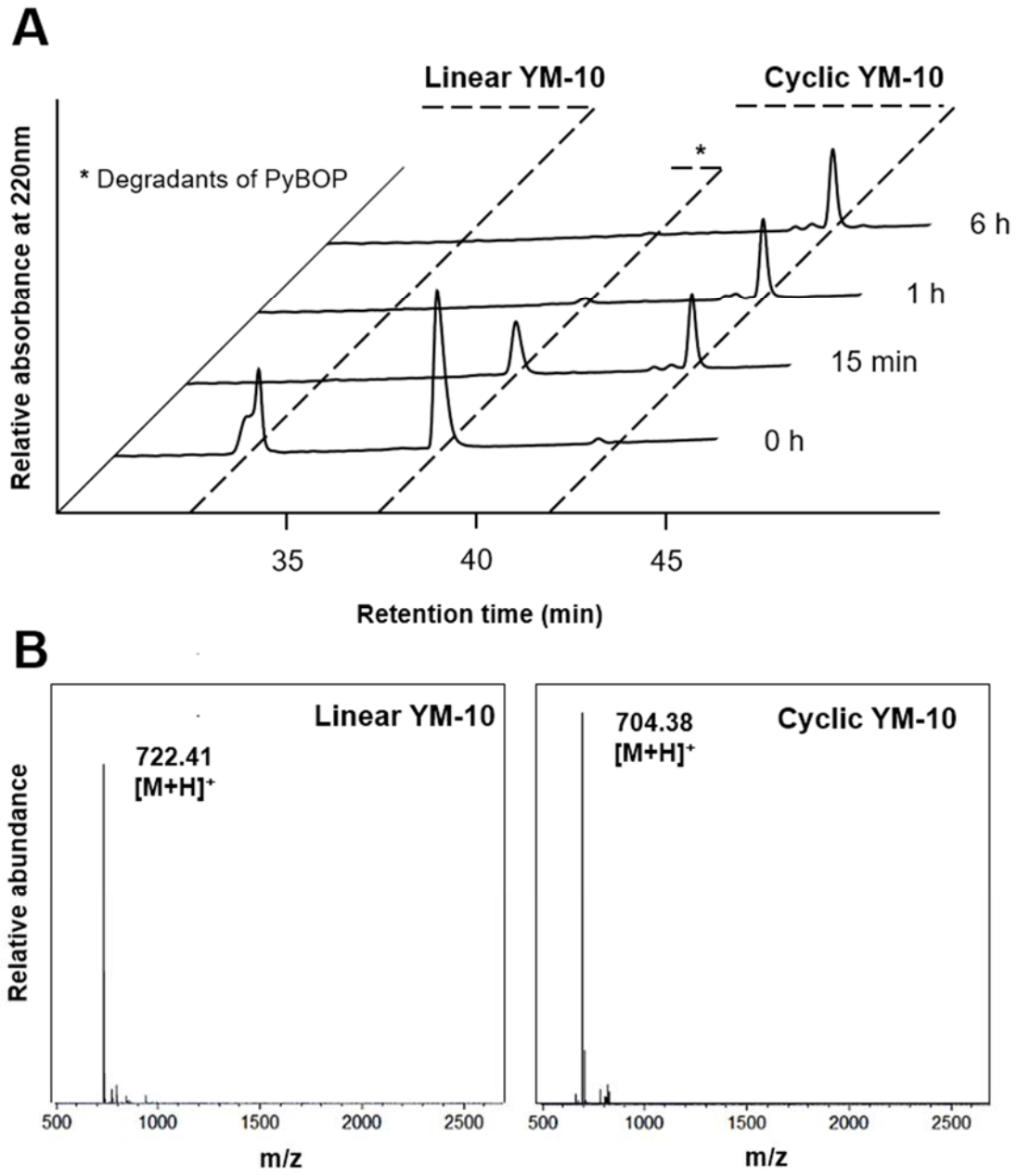


Figure 5.25 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-10 in DMF.

5.5.2 Peptide cyclization in [C_4mim][PF_6]

One equivalent of peptide and 6 equiv. of PyBOP were dissolved into 2000 equiv. of pure [C_4mim][PF_6] (⩽0.02% water content). The reactions were started by adding DIEA (12 equiv.). The mixture was stirred continuously at room temperature for 6 hours. Progress of the reaction was monitored by analytical reversed-phase HPLC on a Jasco LC-Net II/ADC chromatograph equipped with a Vydac 218TP column (4.6 x 25 mm, 5 µm particle size, 300 Å pore size) and a mobile phase system consisting of eluent A (0.1% TFA in water) and eluent B (0.1% TFA in acetonitrile). Samples for monitoring of cyclization reactions performed in IL were diluted with a quenching solution (acetonitrile:water, 45:55)[125] in a ratio of 1:16. The detection was at 220 nm. For each analysis, a continuously increasing gradient was applied starting at 0% eluent B and finishing at 50 % eluent B in 50 min. The flow rate used was 1 ml/min. Single peaks were collected, freeze-dried and forwarded to mass spectrometry analysis.

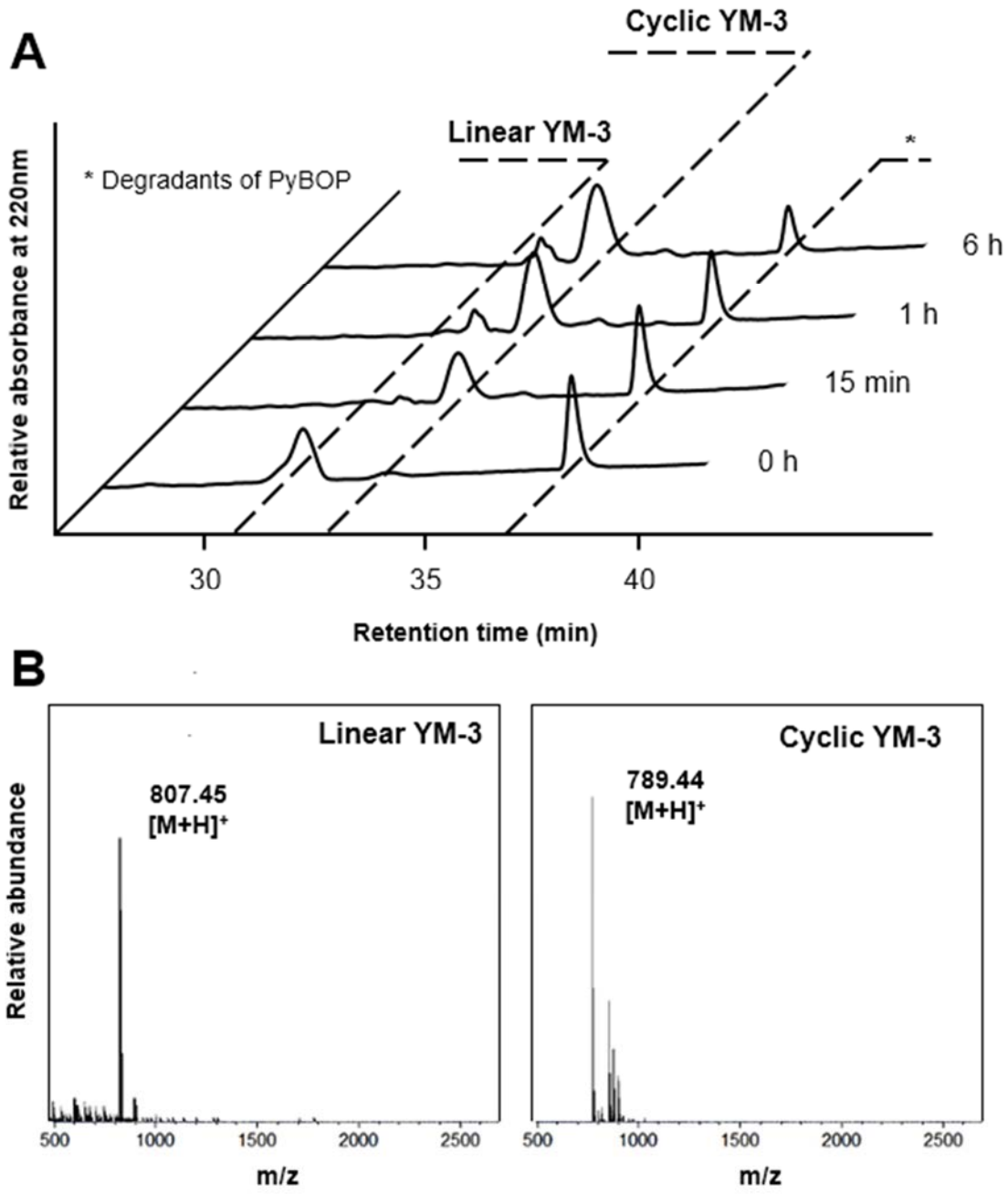


Figure 5.26 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-3 in the $[C_4mim][PF_6]$.

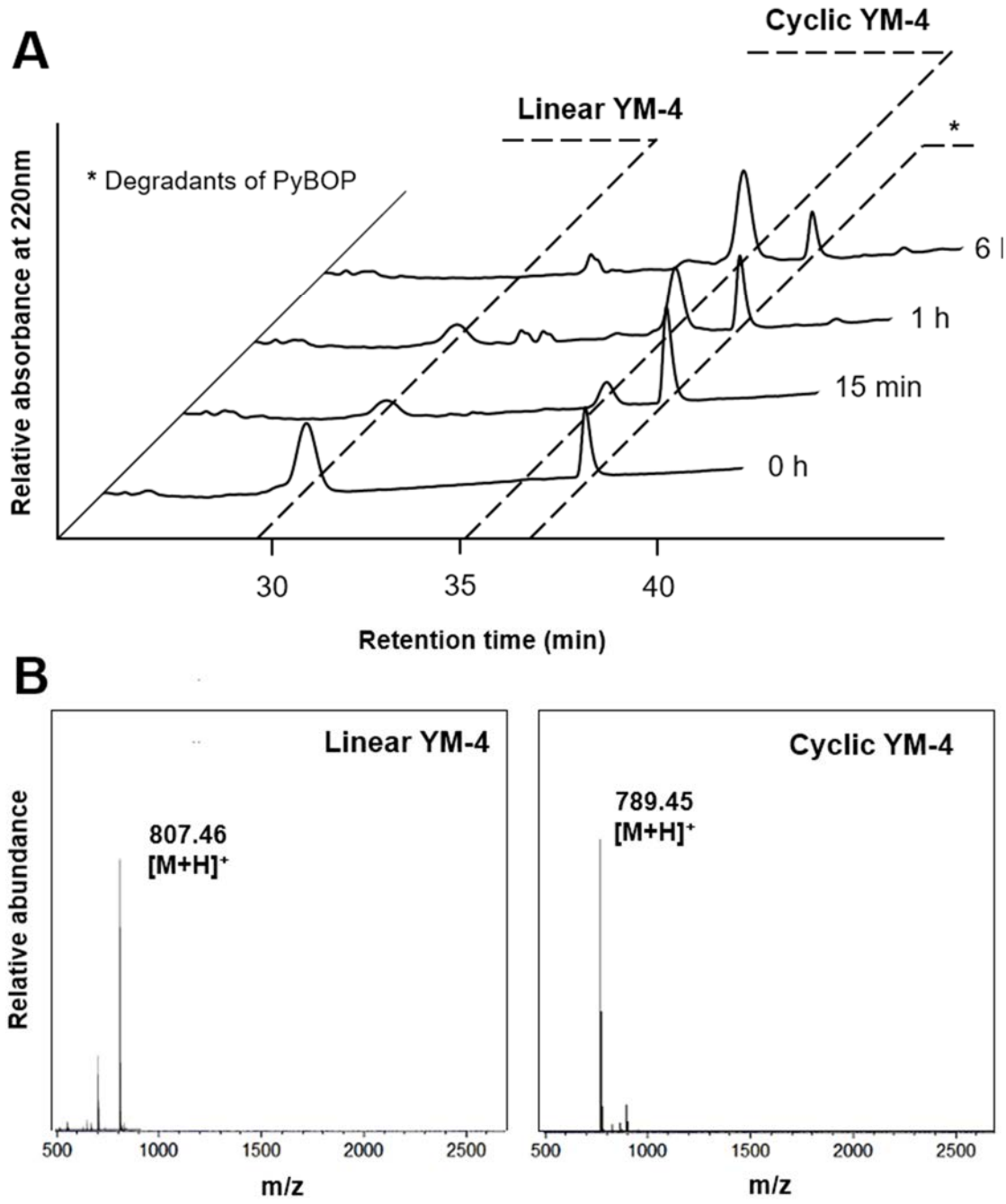


Figure 5.27 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-4 in $[C_4mim][PF_6]$.

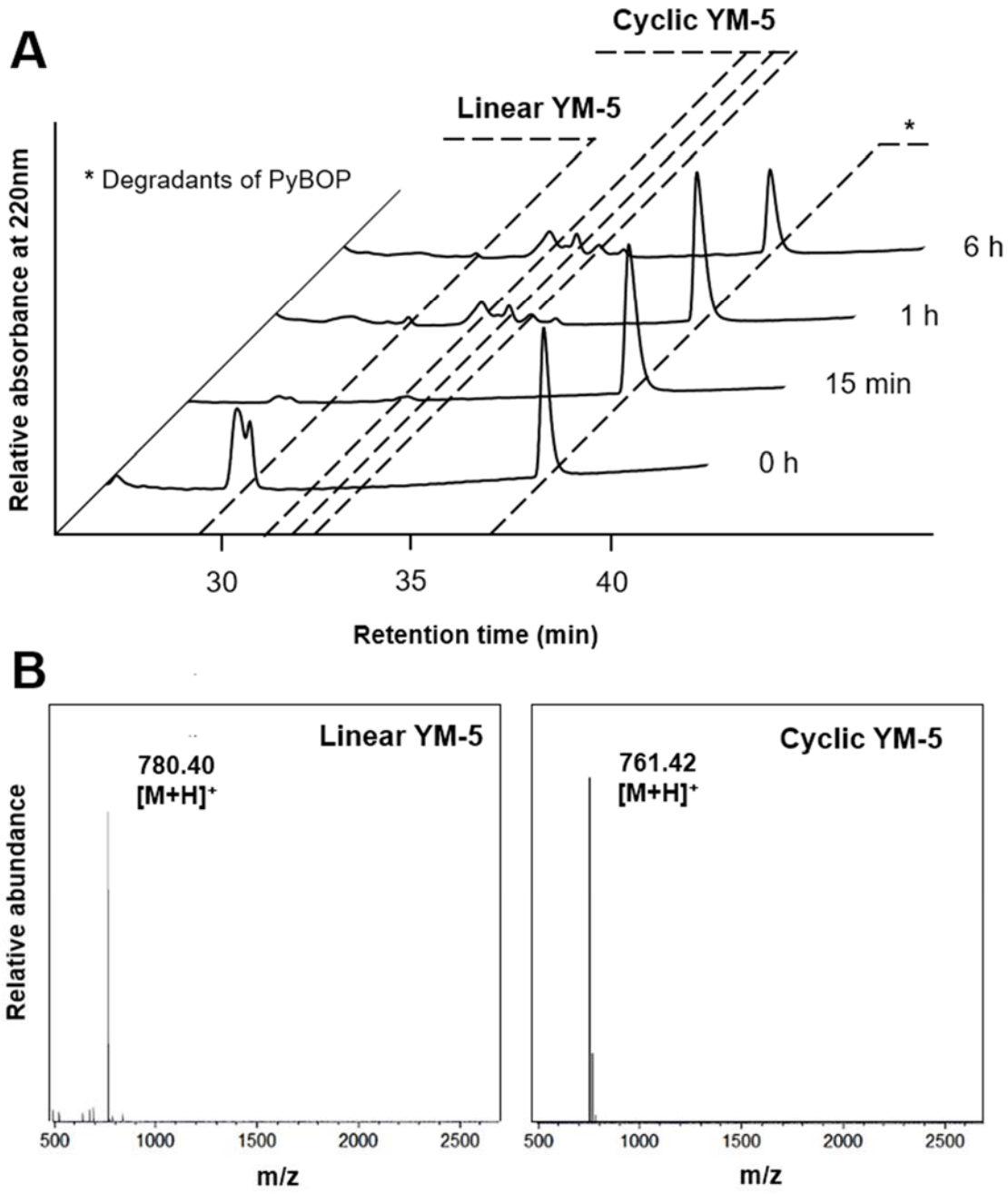


Figure 5.28 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-5 in $[C_4mim][PF_6]$.

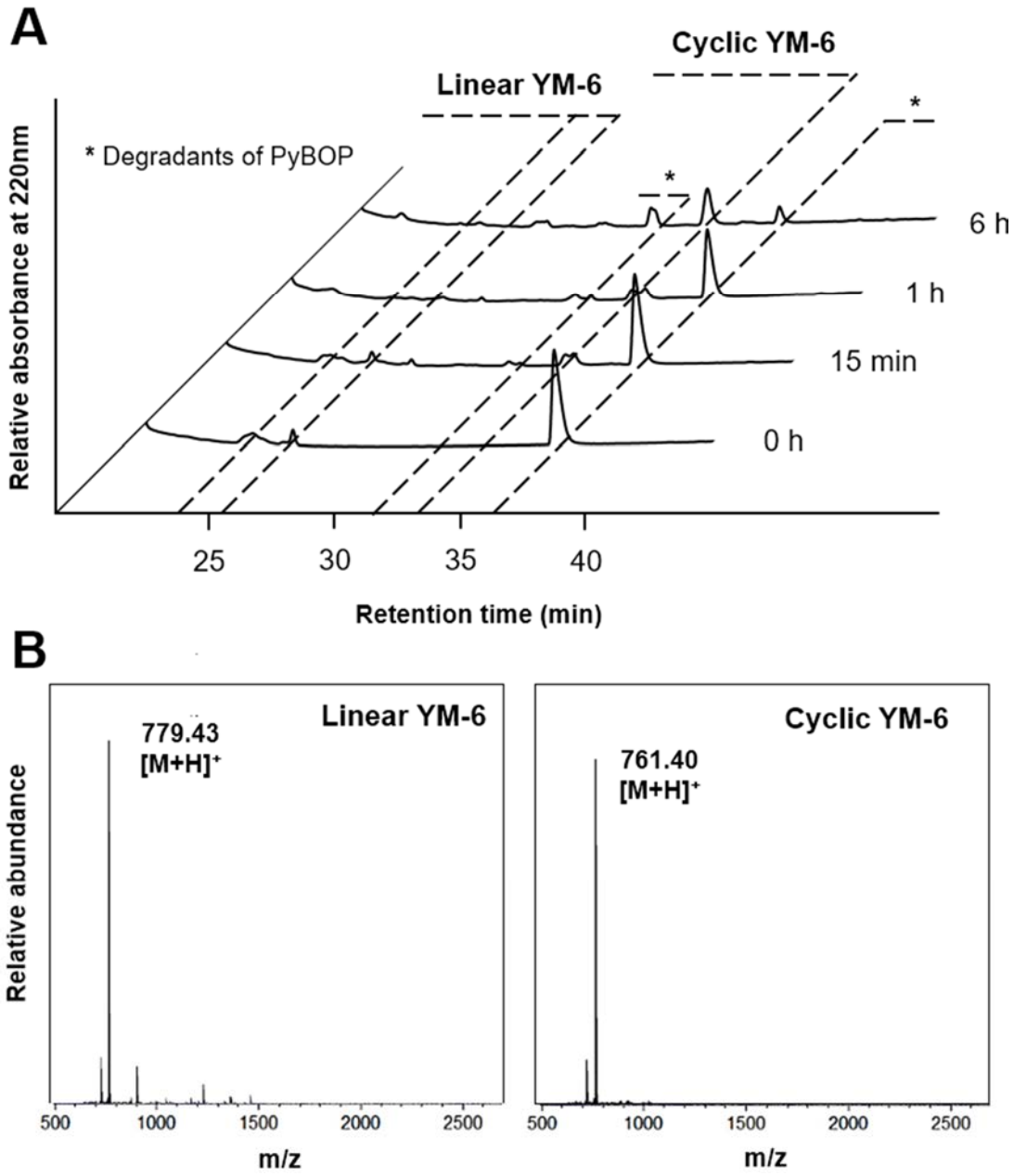


Figure 5.29 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-6 in [C_4mim][PF_6].

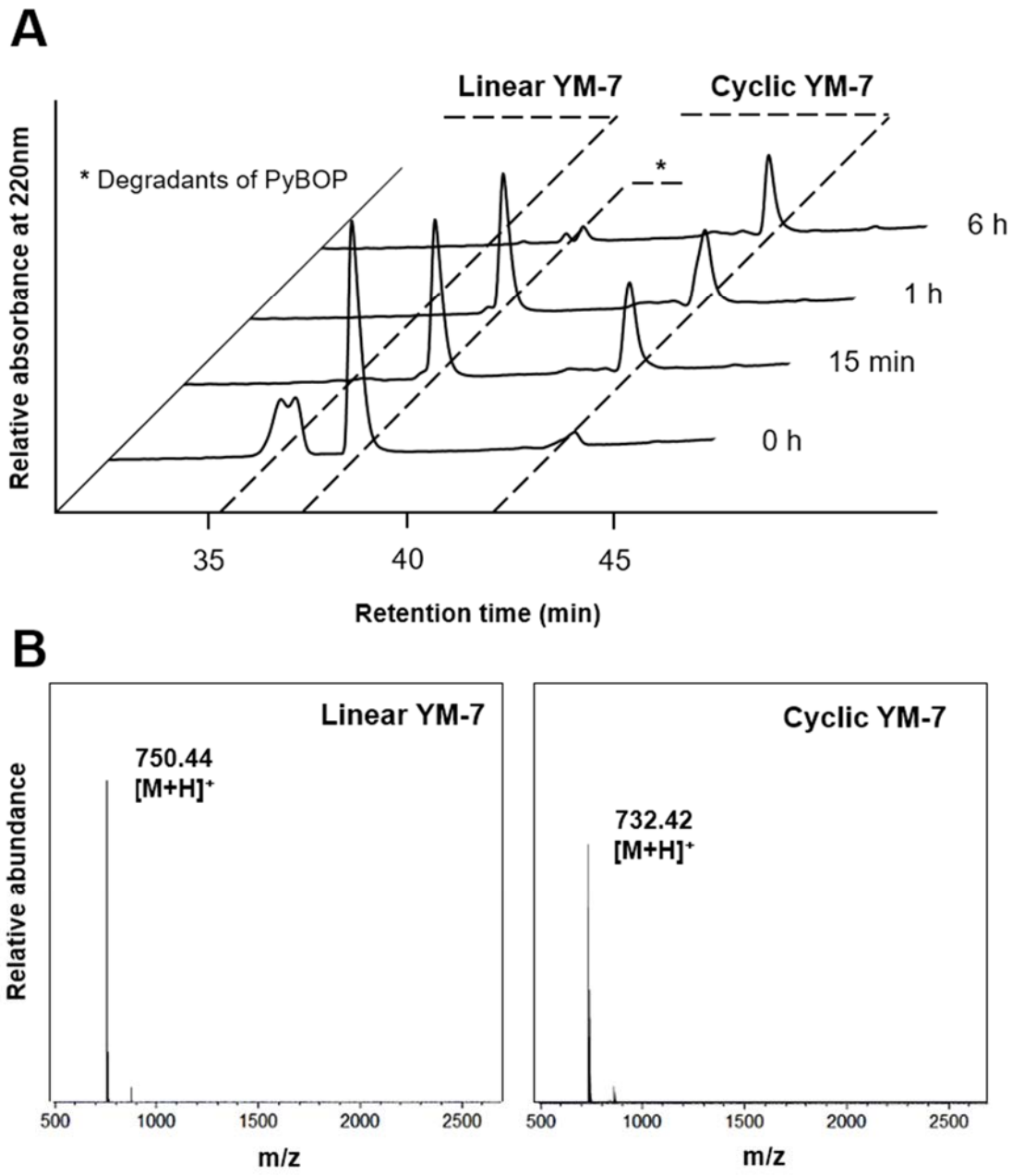


Figure 5.30 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-7 in [C_4mim][PF_6].

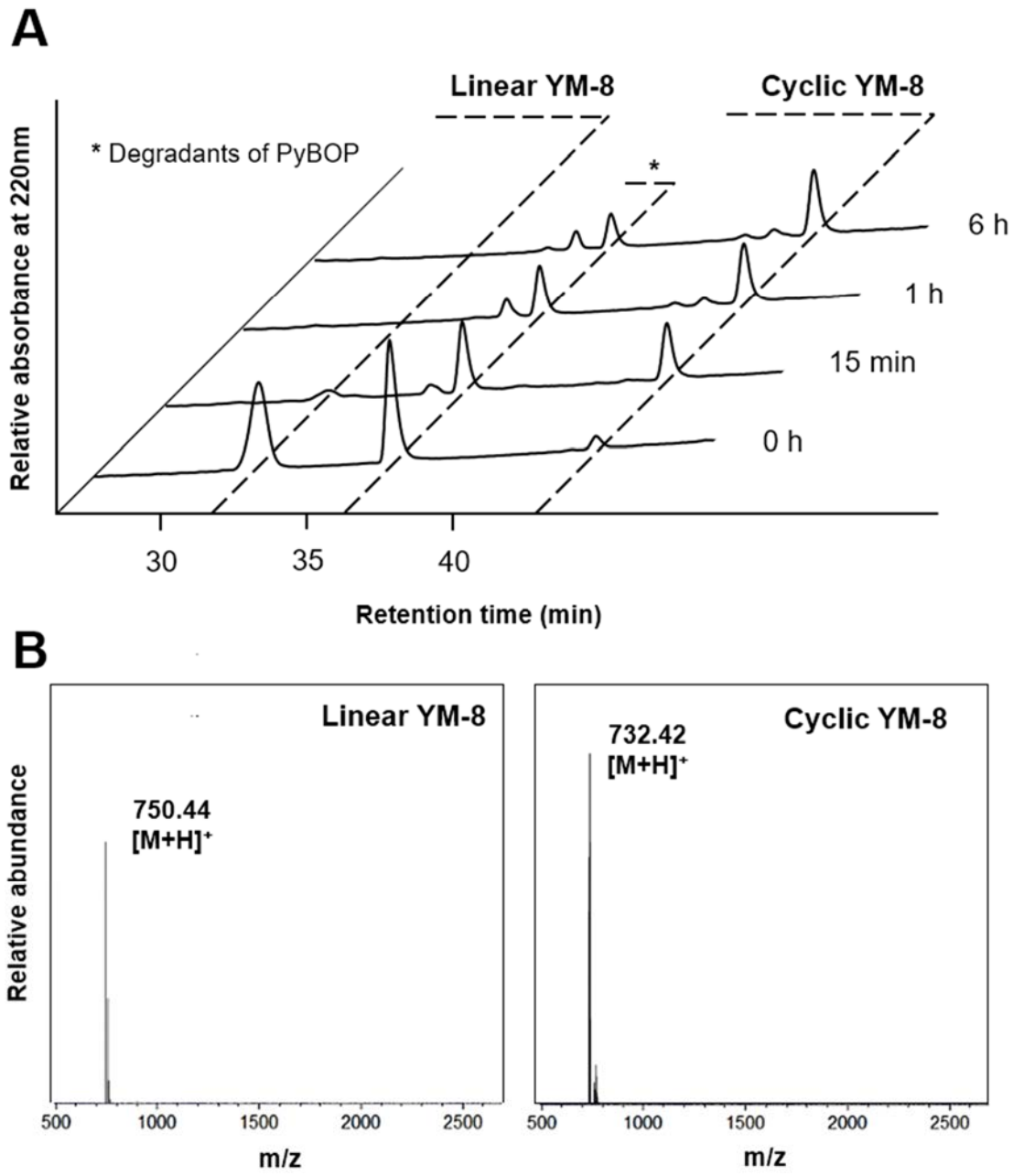


Figure 5.31 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-8 in [C_4mim][PF_6].

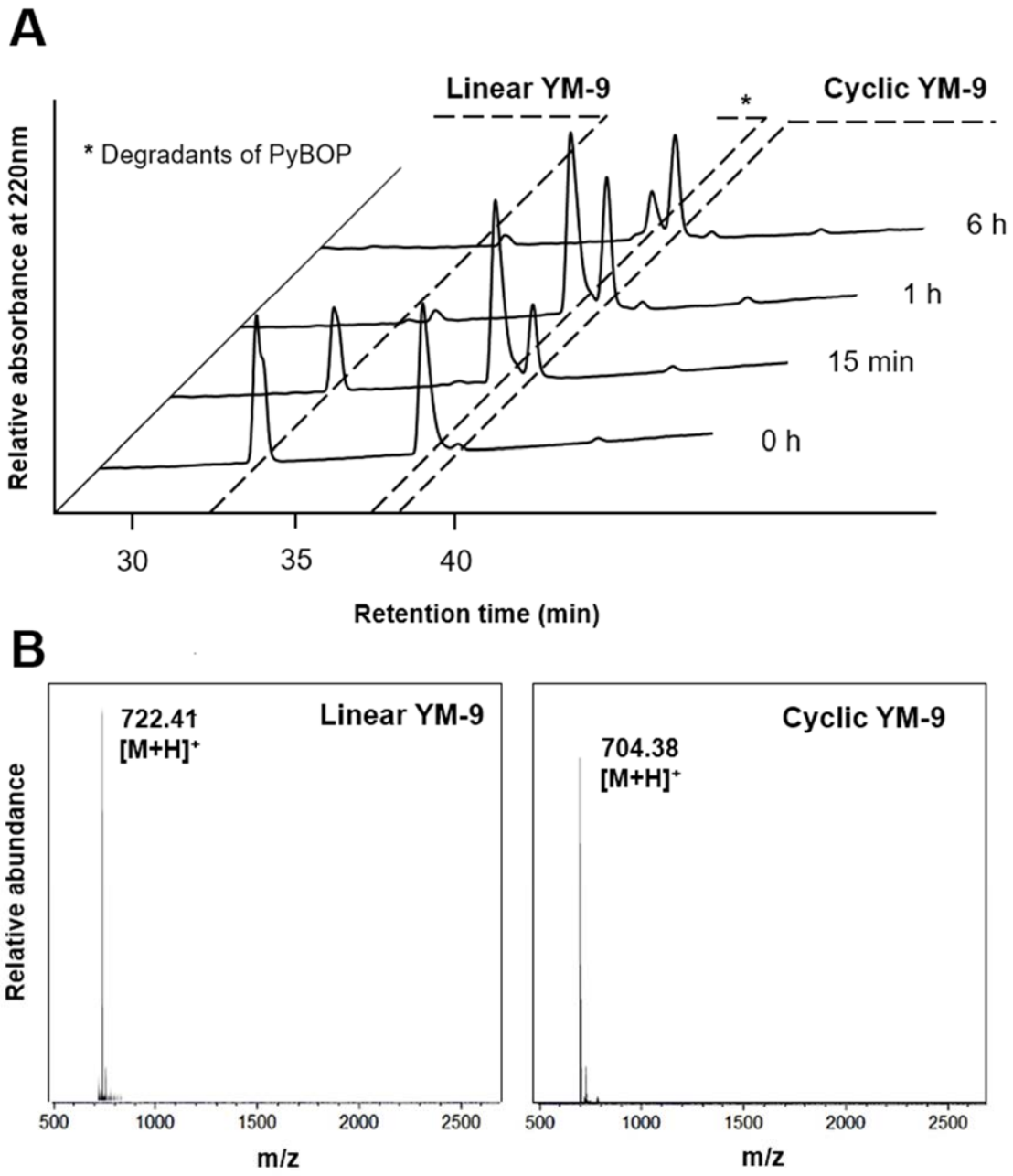


Figure 5.32 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-9 in [C_4mim][PF_6].

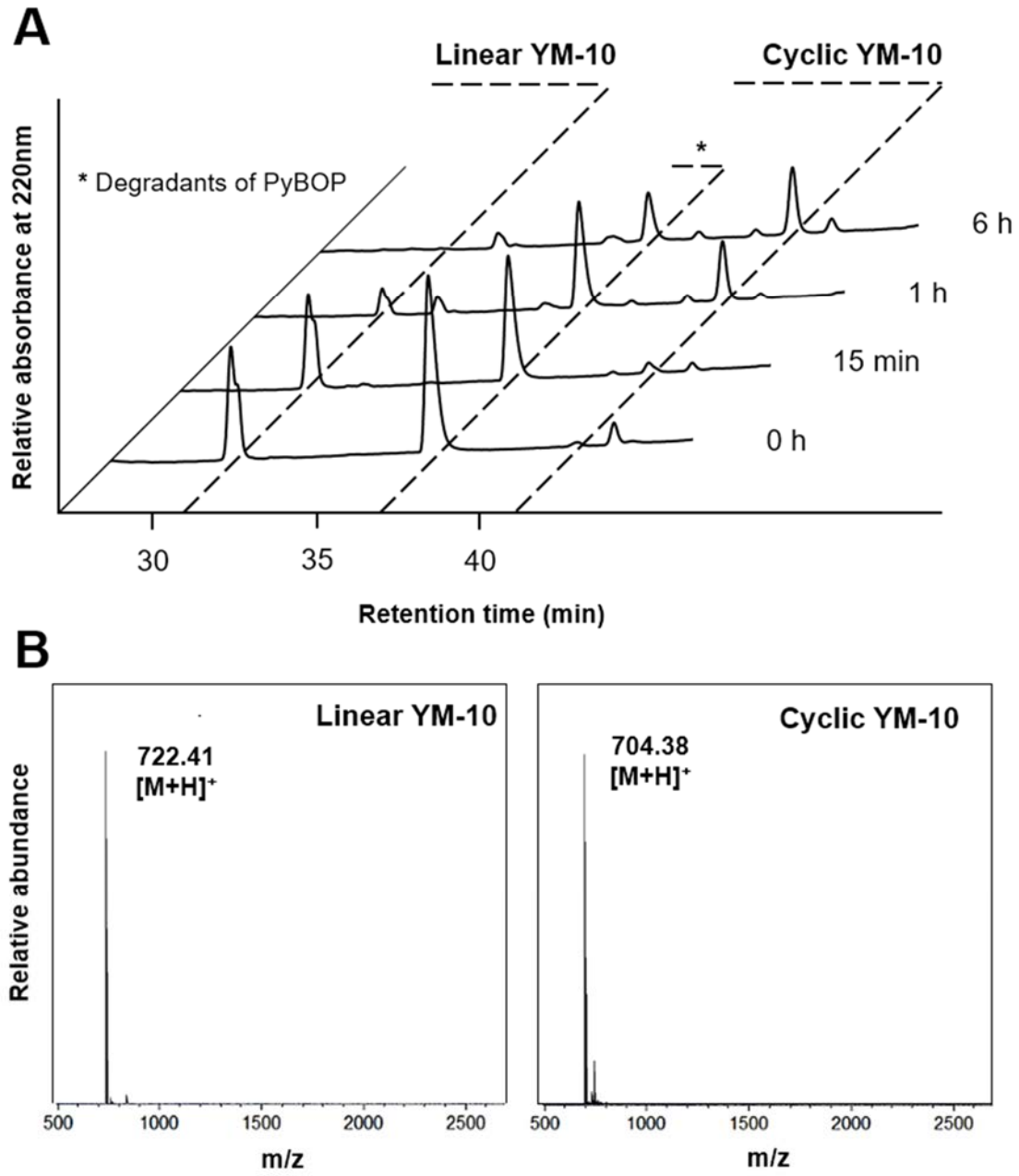


Figure 5.33 HPLC profiles (A) and mass spectra (B) obtained from the cyclization reaction of peptide YM-10 in [C_4mim][PF_6].

6 Summary

The application of ionic liquids in peptide chemistry has just started its exploration phase. So far, only few small peptides were synthesized by using ILs. In addition, only selected reactions, including amino acid condensation to simple dipeptides in solution and oxidative folding of cysteine-rich peptides, were studied. However, for confirming the applicability and compatibility of ILs for specific synthetic approaches in peptide chemistry, a systematic study is indispensable. Thus, in this thesis the focus regarding ILs in the process of peptide synthesis was laid on two distinct reaction types: first, native chemical ligation (NCL) for the generation of linear peptides by combining two fragments and second, lactam bridge formation for the production of cyclic peptides.

In case of NCL, the impact of the amino acid at the C-terminus of the N-terminal peptide thioester (fragment 1) on the efficiency of the ligation reaction was investigated in a conventional buffer system as well as in an IL. Seven NCL reactions with different C-terminal amino acid at fragment 1 were carried out in the neat IL [C_2mim][OAc], which was previously shown to be beneficial for dissolving hydrophobic peptides. The results revealed that yields and rates of most NCL reactions were increased significantly compared to the ligation in the buffer system. Among them, C-terminal glycine residue was the least hindered amino acid at ligation site in both buffer system and IL. The phenylalanine residue, which caused the slowest reaction rate in buffer system, has been found to facilitate ligation in IL at a similar reaction rate as glycine.

Apart from the fact that the reaction time can be significantly reduced compared to the conventional method, the addition of additive is not required anymore if performed in [C_2mim][OAc]. These observations shed light on a

further reaction type which can be performed successfully in an Ionic Liquid and simultaneously provide a protocol of reduced effort, costs of materials and reaction time.

In the second part of this thesis, the head-to-tail cyclization of peptides established via lactam bridge formation was examined in more detail. Thus far, the application of ILs on this reaction type was also not described before. Herein, a series of small linear peptides, designed as Gαq inhibitors related to the natural depsipeptide YM-254890, was cyclized by using [C_4mim][PF_6] as solvent. The reaction was accomplished by using the coupling reagent PyBOP and DIEA as a base commonly used for peptide cyclization in solution. In most cases comparable or even higher yields were obtained for the peptides YM-3, YM-4, YM-5, YM-6, YM-9, YM-10 studied in comparison to the results obtained in DMF. However, an exception was found for the most nonpolar peptides YM-7 and YM-8, for which the yields were evidently lower compared to the conventional approach in DMF. Nevertheless, these first results prove the general applicability of an IL for another type of reaction involving peptide sequences, i.e. the macrocyclization of peptides via head-to-tail cyclization.

Taken together, this work has deepened the understanding of the use of ILs in peptide synthesis and thus expanded the possible applications. Ionic Liquids, as an attractive reaction medium, represent an outstanding solubility opportunity for peptides and reagents used in peptide synthesis. It could be highlighted that ILs had a beneficial effect on ligation reactions of peptide fragments, resulting in a clean and simple reaction. The compatibility of ligation strategy in IL for X–Cys ligation sites has been also clarified, which has a great value for synthetic approaches for peptides. Furthermore, this work has showed the first example of the head-to-tail cyclization of peptides in an IL. The applicability of ILs for lactam-bridge formation has thus been proved. However, the association of IL ions to the peptide sequence needs to be solved, since they could interfere with biological activity.

Although the interactions and mechanisms of ILs for peptide synthesis are still not completely clear currently, ILs still seem to be an attractive medium for peptide chemistry in the future.

Abbreviations

AAs	amino acids
equiv.	equivalent
Et	ethyl
m/z	mass-to-charge ratio
nM	nmol/L
t_R	retention time
theor.	theoretical
USD	US-dollar

Abbreviations of amino acids and their derivatives are used according to the recommendation of the IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN).[151] If it is not specifically stated, amino acids and their derivatives are L-configured.

List of Schemes

List of Figures

List of Tables

Bibliography

1 Rozek, R. P. Economic Aspects of Small and Large Molecule Pharmaceutical Technologies. *Adv. Econ. Bus.* **2013**, *1* (3), 258–269.

2 Declerck, P. J. Biologicals and Biosimilars: A Review of the Science and Its Implications. *Generics Biosimilars Initiat. J.* **2012**, *1* (1), 13–16

3 López-Otín, C.; Matrisian, L. M. Emerging Roles of Proteases in Tumour Suppression. *Nat. Rev. Cancer* **2007**, *7* (10), 800–808.

4 Stoff, H.; Wahrig, B. Biologics, a History of Agents Made from Living Organisms in the Twentieth Century. **2013**.

5 Pharmaceutical Research and Manufacturers of America, Drug Discovery and Development: Understanding the R&D Process. **2003**.

6 Hay, M.; Thomas, D. W.; Craighead, J. L.; Economides, C.; Rosenthal, J. Clinical Development Success Rates for Investigational Drugs. *Nat. Biotechnol.* **2014**, *32* (1), 40–51.

7 Pharmaceutical Research and Manufacturers of America. PhRMA Annual Membership Survey. **2015**.

8 Dimasi J. A.; Cost of Developing a New Drug. R&D Cost Study Briefing. **2014**.

9 Pharmaceutical Research and Manufacturers of America. 2015 Pharmaceutical Industry Profile. **2015**.

10 Mullard, A. 2014 FDA Drug Approvals. *Nat. Rev. Drug Discov.* **2015**, *14* (2), 77–81.

11 Thomas, D. A Big Year for Novel Drugs Approvals. **2013**.

12 Mezo, G. Peptide and Protein Based Pharmaceuticals. **2013**, 203–252.

13 Craik, D. J.; Fairlie, D. P.; Liras, S.; Price, D. The Future of Peptide-Based Drugs. *Chem. Biol. Drug Des.* **2013**, *81* (1), 136–147.

14 KOL opinions, Investing Publications, Company Annual Reports. **2014**.

15 Uhlig, T.; Kyprianou, T.; Martinelli, F. G.; Oppici, C. A.; Heiligers, D.; Hills, D.; Calvo, X. R.; Verhaert, P. The Emergence of Peptides in the Pharmaceutical Business: From Exploration to Exploitation. *EuPA Open Proteomics* **2014**, *4*, 58–69.

16 Fosgerau, K.; Hoffmann, T. Peptide Therapeutics: Current Status and Future Directions. *Drug Discov. Today* **2015**, *20* (1), 122–128.

17 Tanaka, S.; Moriwaki, S.; Uenishi, K.; Koinuma, N.; Tanaka, K.; Ikeda, Y.; Niida, S. The Availability of Urinary Gamma-Glutamyltransferase as a Screening for Osteoporosis. *Bone* **2011**, *48*, S210.

18 Wermeling, D. P. Ziconotide, an Intrathecally Administered N-Type Calcium Channel Antagonist for the Treatment of Chronic Pain. *Pharmacotherapy* **2005**, *25* (8), 1084–1094.

19 Greenberg, M. L.; Cammack, N. Resistance to Enfuvirtide, the First HIV Fusion Inhibitor. *J. Antimicrob. Chemother.* **2004**, *54* (2), 333–340.

20 Seed Planning, Inc. Market Research & Consulting: Current Status and Outlook of Peptide Drug Development 2014. **2013**.

21 Marx V. Watching Peptide Drugs Grow Up. *Chem. Eng. News* **2005**, *83* (11), 17–24.

22 Lax, R. The Future of Peptide Development in the Pharmaceutical Industry. *PharManufacturing Int. Pept. Rev.* **2010**, *4*, 10–15.

23 Hartmann, R.; Meisel, H. Food-Derived Peptides with Biological Activity: From Research to Food Applications. *Curr. Opin. Biotechnol.* **2007**, *18* (2), 163–169.

24 Perez Espitia, P. J.; de Fátima Ferreira Soares, N.; dos Reis Coimbra,

J. S.; de Andrade, N. J.; Souza Cruz, R.; Alves Medeiros, E. A. Bioactive Peptides: Synthesis, Properties, and Applications in the Packaging and Preservation of Food. *Compr. Rev. Food Sci. Food Saf.* **2012**, *11* (2), 187–204.

25 Hipkiss, A. R.; Brownson, C. A Possible New Role for the Anti-Ageing Peptide Carnosine. *Cell. Mol. Life Sci.* **2000**, *57* (5), 747–753.

26 Gill, I.; Vulfson, E. Enzymic Catalysis in Heterogeneous Eutectic Mixtures of Substrates. *Trends Biotechnol.* **1994**, *12* (4), 118–122.

27 Merrifield, R. B. Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85* (14), 2149–2154.

28 Genentech Inc. First Successful Laboratory Production of Human Insulin Announced. *Genentech.* **1978**.

29 Chiba, T.; Park, J.; Yamada, T. Biosynthesis of Somatostatin in Canine Fundic D Cells. *J. Clin. Invest.* **1988**, *81* (2), 282–287.

30 von Eggelkraut-Gottanka, R.; Beck-Sickinger, A. G. Biosynthesis of Peptide Hormones Derived from Precursor Sequences. *Curr. Med. Chem.* **2004**, *11* (20), 2651–2665.

31 Guzman, F.; Barberis, S.; Illanes, A. Peptide Synthesis: Chemical Enzymatic. *Electron. J. Biotechnol.* **2007**, *10* (2), ISSN 0717-3458.

32 Curtius, T. Verkettung von Amidosäuren I. Abhandlung. *J. für Prakt. Chemie* **1904**, *70* (1), 57–72.

33 Mäde, V.; Els-Heindl, S.; Beck-Sickinger, A. G. Automated Solid-Phase Peptide Synthesis to Obtain Therapeutic Peptides. *Beilstein J. Org. Chem.* **2014**, *10*, 1197–1212.

34 MERRIFIELD, R. B. Solid-Phase Peptide Synthesis. 3. An Improved Synthesis of Bradykinin. *Biochemistry* **1964**, *3*, 1385–1390.

35 Tam, J. P.; Kent, S. B. H.; Wong, T. W.; Merrifield, R. B. Improved

Synthesis of 4-(Boc-Aminoacyloxymethyl)-Phenylacetic Acids for Use in Solid Phase Peptide Synthesis. *Synthesis (Stuttg).* **1979**, *1979* (12), 955–957.

36 Böhm, M.; Kühl, T.; Hardes, K.; Coch, R.; Arkona, C.; Schlott, B.; Steinmetzer, T.; Imhof, D. Synthesis and Functional Characterization of Tridegin and Its Analogues: Inhibitors and Substrates of Factor XIIIa. *ChemMedChem* **2012**, *7* (2), 326–333.

37 White, P.; Keyte, J. W.; Bailey, K.; Bloomberg, G. Expediting the Fmoc Solid Phase Synthesis of Long Peptides through the Application of Dimethyloxazolidine Dipeptides. *J. Pept. Sci.* **2004**, *10* (1), 18–26.

38 Fukuda, H.; Irie, K.; Nakahara, A.; Ohigashi, H.; Wender, P. A. Solid-Phase Synthesis, Mass Spectrometric Analysis of the Zinc-Folding, and Phorbol Ester-Binding Studies of the 116-Mer Peptide Containing the Tandem Cysteine-Rich C1 Domains of Protein Kinase C Gamma. *Bioorg. Med. Chem.* **1999**, *7* (6), 1213–1221.

39 Nyfeler, R. Peptide Synthesis via Fragment Condensation. In *Peptide Synthesis Protocols*; Humana Press: New Jersey; **1995**.

40 Chandrudu, S.; Simerska, P.; Toth, I. Chemical Methods for Peptide and Protein Production. *Molecules* **2013**, *18* (4), 4373–4388.

41 Walker, J. M.; Rapley, R. *Molecular Biomethods Handbook: Second Edition*; **2008**.

42 Tam, J. P.; Yu, Q.; Miao, Z. Orthogonal Ligation Strategies for Peptide and Protein. *Biopolymers* **1999**, *51* (5), 311–332.

43 Hojo, H.; Aimoto, S. Polypeptide Synthesis Using the S-Alkyl Thioester of a Partially Protected Peptide Segment. Synthesis of the DNA-Binding Domain of c-Myb protein(142-193)-NH2. *Bull. Chem. Soc. Jpn.* **1991**, *64* (1), 111–117.

44 Zhang, L.; Torgerson, T. R.; Liu, X. Y.; Timmons, S.; Colosia, A. D.; Hawiger, J.; Tam, J. P. Preparation of Functionally Active

Cell-Permeable Peptides by Single-Step Ligation of Two Peptide Modules. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95* (16), 9184–9189.

45 Raibaut, L.; Vicogne, J.; Leclercq, B.; Drobecq, H.; Desmet, R.; Melnyk, O. Total Synthesis of Biotinylated N Domain of Human Hepatocyte Growth Factor. *Bioorg. Med. Chem.* **2013**, *21* (12), 3486–3494.

46 Bodapati, K. C.; Soudy, R.; Etayash, H.; Stiles, M.; Kaur, K. Design, Synthesis and Evaluation of Antimicrobial Activity of N-Terminal Modified Leucocin A Analogues. *Bioorg. Med. Chem.* **2013**, *21* (13), 3715–3722.

47 Low, D. W.; Hill, M. G.; Carrasco, M. R.; Kent, S. B.; Botti, P. Total Synthesis of Cytochrome b562 by Native Chemical Ligation Using a Removable Auxiliary. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98* (12), 6554–6559.

48 Tolbert, T. J.; Franke, D.; Wong, C.-H. A New Strategy for Glycoprotein Synthesis: Ligation of Synthetic Glycopeptides with Truncated Proteins Expressed in E. Coli as TEV Protease Cleavable Fusion Protein. *Bioorg. Med. Chem.* **2005**, *13* (3), 909–915.

49 Sohma, Y.; Kent, S. B. H. Biomimetic Synthesis of Lispro Insulin via a Chemically Synthesized “Mini-Proinsulin” Prepared by Oxime-Forming Ligation. *J. Am. Chem. Soc.* **2009**, *131* (44), 16313–16318.

50 Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. Über Peptidsynthesen. 8. Mitteilung Bildung von S-Haltigen Peptiden Durch Intramolekulare Wanderung von Aminoacylresten. *Justus Liebigs Ann. Chem.* **1953**, *583* (1), 129–149.

51 Chen, M.; Heimer, P.; Imhof, D. Synthetic Strategies for Polypeptides and Proteins by Chemical Ligation. *Amino Acids* **2015**, *47* (7), 1283–1299.

52 Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. Synthesis of Proteins by Native Chemical Ligation. *Science* **1994**, *266* (5186), 776–779.

53 Dawson, P. E.; Churchill, M. J.; Ghadiri, M. R.; Kent, S. B. H. Modulation of Reactivity in Native Chemical Ligation through the Use of Thiol Additives. *J. Am. Chem. Soc.* **1997**, *119* (19), 4325–4329.

54 Johnson, E. C. B.; Kent, S. B. H. Insights into the Mechanism and Catalysis of the Native Chemical Ligation Reaction. *J. Am. Chem. Soc.* **2006**, *128* (20), 6640–6646.

55 Medini, K.; Harris, P. W. R.; Hards, K.; Dingley, A. J.; Cook, G. M.; Brimble, M. A. Chemical Synthesis of a Pore-Forming Antimicrobial Protein, Caenopore-5, by Using Native Chemical Ligation at a Glu-Cys Site. *Chembiochem* **2015**, *16* (2), 328–336.

56 Izumi, M.; Otsuki, A.; Nishihara, M.; Okamoto, R.; Kajihara, Y. Chemical Synthesis of a Synthetic Analogue of the Sialic Acid-Binding Lectin Siglec-7. *Chembiochem* **2014**, *15* (17), 2503–2507.

57 Reif, A.; Siebenhaar, S.; Tröster, A.; Schmälzlein, M.; Lechner, C.; Velisetty, P.; Gottwald, K.; Pöhner, C.; Boos, I.; Schubert, V.; Rose-John, S.; Unverzagt, C. Semisynthesis of Biologically Active Glycoforms of the Human Cytokine Interleukin 6. *Angew. Chem. Int. Ed. Engl.* **2014**, *53* (45), 12125–12131.

58 Santhakumar, G.; Payne, R. J. Total Synthesis of Polydiscamides B, C, and D via a Convergent Native Chemical Ligation–Oxidation Strategy. *Org. Lett.* **2014**, *16* (17), 4500–4503.

59 Villain, M.; Gaertner, H.; Botti, P. Native Chemical Ligation with Aspartic and Glutamic Acids asC-Terminal Residues: Scope and Limitations. *European J. Org. Chem.* **2003**, *2003* (17), 3267–3272.

60 Hackeng, T. M.; Griffin, J. H.; Dawson, P. E. Protein Synthesis by Native Chemical Ligation: Expanded Scope by Using Straightforward Methodology. *Proc. Natl. Acad. Sci. U. S. A.* **1999**, *96* (18), 10068–10073.

61 Dang, B.; Kubota, T.; Mandal, K.; Bezanilla, F.; Kent, S. B. H. Native Chemical Ligation at Asx-Cys, Glx-Cys: Chemical Synthesis and

High-Resolution X-Ray Structure of ShK Toxin by Racemic Protein Crystallography. *J. Am. Chem. Soc.* **2013**, *135* (32), 11911–11919.

62 Nakamura, T.; Shigenaga, A.; Sato, K.; Tsuda, Y.; Sakamoto, K.; Otaka, A. Examination of Native Chemical Ligation Using Peptidyl Prolyl Thioesters. *Chem. Commun.* **2014**, *50* (1), 58–60.

63 Camacho, C. J.; Katsumata, Y.; Ascherman, D. P. Structural and Thermodynamic Approach to Peptide Immunogenicity. *PLoS Comput. Biol.* **2008**, *4* (11), e1000231.

64 White, C. J.; Yudin, A. K. Contemporary Strategies for Peptide Macrocyclization. *Nat. Chem.* **2011**, *3* (7), 509–524.

65 Ovchinnikov, Y. A.; Ivanov, V. T. Conformational States and Biological Activity of Cyclic Peptides. *Tetrahedron* **1975**, *31* (18), 2177–2209.

66 Calne, R. Y.; White, D. J.; Thiru, S.; Evans, D. B.; McMaster, P.; Dunn, D.C.; Craddock, G. N.; Pentlow, B. D.; Rolles, K. Cyclosporin A in Patients Receiving Renal Allografts from Cadaver Donors. *Lancet (London, England)* **1978**, *2* (8104-5), 1323–1327.

67 Mootz, H. D.; Marahiel, M. A. The Tyrocidine Biosynthesis Operon of Bacillus Brevis: Complete Nucleotide Sequence and Biochemical Characterization of Functional Internal Adenylation Domains. *J. Bacteriol.* **1997**, *179* (21), 6843–6850.

68 Crusi, E.; Giralt, E.; Andreu, D. Peptide Ionophores: Synthesis and Cation-Binding Properties of a Bicyclic Peptide Containing Glycine and Lysine Residues. *Pept. Res.* **1995**,*8* (2), 62–69.

69 Frankmölle, W. P.; Knübel, G.; Moore, R. E.; Patterson, G. M. Antifungal Cyclic Peptides from the Terrestrial Blue-Green Alga Anabaena Laxa. II. Structures of Laxaphycins A, B, D and E. *J. Antibiot. (Tokyo).* **1992**, *45* (9), 1458–1466.

70 Zabriskie, T. M.; Klocke, J. A.; Ireland, C. M.; Marcus, A. H.; Molinski, T. F.; Faulkner, D. J.; Xu, C.; Clardy, J. Jaspamide, a Modified Peptide

from a Jaspis Sponge, with Insecticidal and Antifungal Activity. *J. Am. Chem. Soc.* **1986**, *108* (11), 3123–3124.

71 Nasrolahi Shirazi, A.; Tiwari, R. K.; Oh, D.; Sullivan, B.; Kumar, A.; Beni, Y. A.; Parang, K. Cyclic Peptide-Selenium Nanoparticles as Drug Transporters. *Mol. Pharm.* **2014**, *11* (10), 3631–3641.

72 Brea, R. J.; Reiriz, C.; Granja, J. R. Towards Functional Bionanomaterials Based on Self-Assembling Cyclic Peptide Nanotubes. *Chem. Soc. Rev.* **2010**, *39* (5), 1448–1456.

73 Ghadiri, M. R.; Granja, J. R.; Milligan, R. A.; McRee, D. E.; Khazanovich, N. Self-Assembling Organic Nanotubes Based on a Cyclic Peptide Architecture. *Nature* **1993**, *366* (6453), 324–327.

74 Mandal, D.; Tiwari, R. K.; Nasrolahi Shirazi, A.; Oh, D.; Ye, G.; Banerjee, A.; Yadav, A.; Parang, K. Self-Assembled Surfactant Cyclic Peptide Nanostructures as Stabilizing Agents. *Soft Matter* **2013**, *9* (39), 9465.

75 Davies, J. S. The Cyclization of Peptides and Depsipeptides. *J. Pept. Sci.* **2003**, *9* (8), 471–501.

76 Al-Warhi, T. I.; Al-Hazimi, H. M. A.; El-Faham, A. Recent Development in Peptide Coupling Reagents. *J. Saudi Chem. Soc.* **2012**, *16* (2), 97–116.

77 Jou, G.; González, I.; Albericio, F.; Lloyd-Williams, P.; Giralt, E. Total Synthesis of Dehydrodidemnin B. Use of Uronium and Phosphonium Salt Coupling Reagents in Peptide Synthesis in Solution †. *J. Org. Chem.* **1997**, *62* (2), 354–366.

78 del Fresno, M.; El-Faham, A.; Carpino, L. A.; Royo, M.; Albericio, F. Substituted Guanidines: Introducing Diversity in Combinatorial Chemistry. *Org. Lett.* **2000**, *2* (23), 3539–3542.

79 Merten, C.; Li, F.; Bravo-Rodriguez, K.; Sanchez-Garcia, E.; Xu, Y.; Sander, W. Solvent-Induced Conformational Changes in Cyclic

Peptides: A Vibrational Circular Dichroism Study. *Phys. Chem. Chem. Phys.* **2014**, *16* (12), 5627–5633.

80 Wishart, J. F. Ionic Liquids as Green Solvents. *ACS Symp. Ser.* **2003**, *856*, 381–396.

81 Earle, M. J.; Seddon, K. R. Ionic Liquids. Green Solvents for the Future. *Pure Appl. Chem.* **2000**, *72* (7), 1391–1398.

82 Plechkova, N. V.; Seddon, K. R. Ionic Liquids: “Designer” Solvents for Green Chemistry. In *Methods and Reagents for Green Chemistry: An Introduction*; 2007; pp 103–130.

83 Ghandi, K. A Review of Ionic Liquids, Their Limits and Applications. *Green Sustain. Chem.* **2014**, *04* (01), 44–53.

84 Moosavi, F. The Structure of Supported Ionic Liquids at the Interface. In *Ionic Liquids - New Aspects for the Future*; InTech, **2013**.

85 Wasserscheid, P.; Keim, W. Ionic Liquids—New “Solutions” for Transition Metal Catalysis. *Angew. Chemie* **2000**, *39* (21), 3772–3789.

86 Forsyth, S. A.; Pringle, J. M.; MacFarlane, D. R. Ionic Liquids—An Overview. *Aust. J. Chem.* **2004**, *57* (2), 113.

87 Lui, M. Y.; Crowhurst, L.; Hallett, J. P.; Hunt, P. A.; Niedermeyer, H.; Welton, T. Salts Dissolved in Salts: Ionic Liquid Mixtures. *Chem. Sci.* **2011**, *2* (8), 1491.

88 Tran, C. D.; Challa, S. Fullerene-Impregnated Ionic Liquid Stationary Phases for Gas Chromatography. *Analyst* **2008**, *133* (4), 455.

89 Kühl, T.; Chen, M.; Teichmann, K.; Stark, A.; Imhof, D. Ionic Liquid 1-Ethyl-3-Methylimidazolium Acetate: An Attractive Solvent for Native Chemical Ligation of Peptides. *Tetrahedron Lett.* **2014**, *55* (27), 3658–3662.

90 Böhm, M.; Tietze, A. A.; Heimer, P.; Chen, M.; Imhof, D. Ionic Liquids as

Reaction Media for Oxidative Folding and Native Chemical Ligation of Cysteine-Containing Peptides. *J. Mol. Liq.* **2014**, *192*, 67–70.

91 Vallette, H. Room Temperature Ionic Liquids (RTIL's) Are Convenient Solvents for Peptide Synthesis! *Arkivoc* **2006**, *2006* (4), 200.

92 Fujita, K.; MacFarlane, D. R.; Forsyth, M. Protein Solubilising and Stabilising Ionic Liquids. *Chem. Commun.* **2005**, No. 38, 4804.

93 Isik, M.; Sardon, H.; Mecerreyes, D. Ionic Liquids and Cellulose: Dissolution, Chemical Modification and Preparation of New Cellulosic Materials. *Int. J. Mol. Sci.* **2014**, *15* (7), 11922–11940.

94 Swatloski, R. P.; Spear, S. K.; Holbrey, J. D.; Rogers, R. D. Dissolution of Cellose with Ionic Liquids. *J. Am. Chem. Soc.* **2002**, *124* (18), 4974–4975.

95 Mukesh, C.; Mondal, D.; Sharma, M.; Prasad, K. Rapid Dissolution of DNA in a Novel Bio-Based Ionic Liquid with Long-Term Structural and Chemical Stability: Successful Recycling of the Ionic Liquid for Reuse in the Process. *Chem. Commun. (Camb).* **2013**, *49* (61), 6849–6851.

96 Klähn, M.; Stüber, C.; Seduraman, A.; Wu, P. What Determines the Miscibility of Ionic Liquids with Water? Identification of the Underlying Factors to Enable a Straightforward Prediction. *J. Phys. Chem. B* **2010**, *114* (8), 2856–2868.

97 Makowska, A.; Dyoniziak, E.; Siporska, A.; Szydłowski, J. Miscibility of Ionic Liquids with Polyhydric Alcohols. *J. Phys. Chem. B* **2010**, *114* (7), 2504–2508.

98 Mai, N. L.; Ahn, K.; Koo, Y.-M. Methods for Recovery of Ionic liquids—A Review. *Process Biochem.* **2014**, *49* (5), 872–881.

99 MacFarlane, D. R.; Pringle, J. M.; Johansson, K. M.; Forsyth, S. A.; Forsyth, M. Lewis Base Ionic Liquids. *Chem. Commun.* **2006**, No. 18, 1905.

100 Xu, D.-Q.; Hu, Z.-Y.; Li, W.-W.; Luo, S.-P.; Xu, Z.-Y. Hydrogenation in Ionic Liquids: An Alternative Methodology toward Highly Selective Catalysis of Halonitrobenzenes to Corresponding Haloanilines. *J. Mol. Catal. A Chem.* **2005**, *235* (1-2), 137–142.

101 Haumann, M.; Riisager, A. Hydroformylation in Room Temperature Ionic Liquids (RTILs): Catalyst and Process Developments. *Chem. Rev.* **2008**, *108* (4), 1474–1497.

102 Stenzel, O.; Brüll, R.; Wahner, U. M.; Sanderson, R. D.; Raubenheimer, H. G. Oligomerization of Olefins in a Chloroaluminate Ionic Liquid. *J. Mol. Catal. A Chem.* **2003**, *192* (1-2), 217–222.

103 Bernini, R.; Coratti, A.; Fabrizi, G.; Goggiamani, A. CH3ReO3/H2O2 in Room Temperature Ionic Liquids: An Homogeneous Recyclable Catalytic System for the Baeyer–Villiger Reaction. *Tetrahedron Lett.* **2003**, *44* (50), 8991–8994.

104 Kabalka, G. W.; Malladi, R. R. Reduction of Aldehydes Using Trialkylboranes in Ionic Liquids. *Chem. Commun.* **2000**, No. 22, 2191–2191.

105 Csihony, S.; Fischmeister, C.; Bruneau, C.; Horv??th, I. T.; Dixneuf, P. H. First Ring-Opening Metathesis Polymerization in an Ionic Liquid. Efficient Recycling of a Catalyst Generated from a Cationic Ruthenium Allenylidene Complex. *New J. Chem.* **2002**, *26* (11), 1667–1670.

106 Cota, I.; Medina, F.; Gonzalez-Olmos, R.; Iglesias, M. Alanine-Supported Protic Ionic Liquids as Efficient Catalysts for Aldol Condensation Reactions. *Comptes Rendus Chim.* **2014**, *17* (1), 18–22.

107 Gui, J.; Deng, Y.; Hu, Z.; Sun, Z. A Novel Task-Specific Ionic Liquid for Beckmann Rearrangement: A Simple and Effective Way for Product Separation. *Tetrahedron Lett.* **2004**, *45* (12), 2681–2683.

108 Earle, M. J.; McCormac, P. B.; Seddon, K. R. Diels–Alder Reactions in Ionic Liquids. *Green Chem.* **1999**, *1* (1), 23–25.

109 Earle, M. J.; Seddon, K. R.; Adams, C. J.; Roberts, G. Friedel–Crafts Reactions in Room Temperature Ionic Liquids. *Chem. Commun.* **1998**, No. 19, 2097–2098.

110 Handy, S. T.; Zhang, X. Organic Synthesis in Ionic Liquids: The Stille Coupling. *Org. Lett.* **2001**, *3* (2), 233–236.

111 Valizadeh, H.; Vaghefi, S. One-Pot Wittig and Knoevenagel Reactions in Ionic Liquid as Convenient Methods for the Synthesis of Coumarin Derivatives. *Synth. Commun.* **2009**, *39* (9), 1666–1678.

112 Handy, S. T.; Okello, M. Halide Effects on the Heck Reaction in Room Temperature Ionic Liquids. *Tetrahedron Lett.* **2003**, *44* (46), 8395–8397.

113 McLachlan, F.; Mathews, C. J.; Smith, P. J.; Welton, T. Palladium-Catalyzed Suzuki Cross-Coupling Reactions in Ambient Temperature Ionic Liquids: Evidence for the Importance of Palladium Imidazolylidene Complexes. *Organometallics* **2003**, *22* (25), 5350–5357.

114 Chiarotto, I.; Feroci, M.; Sotgiu, G.; Inesi, A. The Dual Role of Ionic Liquid BmimBF4, Precursor of N-Heterocyclic Carbene and Solvent, in the Oxidative Esterification of Aldehydes. *Tetrahedron* **2013**, *69* (37), 8088–8095.

115 Harjani, J. R.; Nara, S. J.; Salunkhe, M. M. Lewis Acidic Ionic Liquids for the Synthesis of Electrophilic Alkenes via the Knoevenagel Condensation. *Tetrahedron Lett.* **2002**, *43* (6), 1127–1130.

116 Kragl, U.; Eckstein, M.; Kaftzik, N. Enzyme Catalysis in Ionic Liquids. *Curr. Opin. Biotechnol.* **2002**, *13* (6), 565–571.

117 Gorke, J.; Srienc, F.; Kazlauskas, R. Enzyme-Catalyzed Reactions in Ionic Liquids. In *Encyclopedia of Industrial Biotechnology*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2010.

118 Moon, Y. H.; Lee, S. M.; Ha, S. H.; Koo, Y.-M. Enzyme-Catalyzed Reactions in Ionic Liquids. *Korean J. Chem. Eng.* **2006**, *23* (2), 247–

263.

119 Weingärtner, H.; Cabrele, C.; Herrmann, C. How Ionic Liquids Can Help to Stabilize Native Proteins. *Phys. Chem. Chem. Phys.* **2012**, *14* (2), 415–426.

120 Patel, R.; Kumari, M.; Khan, A. B. Recent Advances in the Applications of Ionic Liquids in Protein Stability and Activity: A Review. *Appl. Biochem. Biotechnol.* **2014**, *172* (8), 3701–3720.

121 Lin, X.; Wang, Y.; Zeng, Q.; Ding, X.; Chen, J. Extraction and Separation of Proteins by Ionic Liquid Aqueous Two-Phase System. *Analyst* **2013**, *138* (21), 6445–6453.

122 Tan, Z.; Li, F.; Xu, X.; Xing, J. Simultaneous Extraction and Purification of Aloe Polysaccharides and Proteins Using Ionic Liquid Based Aqueous Two-Phase System Coupled with Dialysis Membrane. *Desalination* **2012**, *286*, 389–393.

123 Quental, M. V.; Caban, M.; Pereira, M. M.; Stepnowski, P.; Coutinho, J. A. P.; Freire, M. G. Enhanced Extraction of Proteins Using Cholinium-Based Ionic Liquids as Phase-Forming Components of Aqueous Biphasic Systems. *Biotechnol. J.* **2015**, *10* (9), 1457–1466.

124 Galy, N.; Mazières, M.-R.; Plaquevent, J.-C. Toward Waste-Free Peptide Synthesis Using Ionic Reagents and Ionic Liquids as Solvents. *Tetrahedron Lett.* **2013**, *54*, 2703–2705.

125 Vallette, H.; Ferron, L.; Coquerel, G.; Guillen, F.; Plaquevent, J.-C. Room Temperature Ionic Liquids (RTIL's) Are Convenient Solvents for Peptide Synthesis! *Arkivoc* **2006**, *2006* (4), 200.

126 Miao, W.; Chan, T. H. Ionic-Liquid-Supported Peptide Synthesis Demonstrated by the Synthesis of Leu5-Enkephalin. *J. Org. Chem.* **2005**, *70* (8), 3251–3255.

127 Heimer, P.; Tietze, A. A.; Böhm, M.; Giernoth, R.; Kuchenbuch, A.; Stark, A.; Leipold, E.; Heinemann, S. H.; Kandt, C.; Imhof, D.

Application of Room-Temperature Aprotic and Protic Ionic Liquids for Oxidative Folding of Cysteine-Rich Peptides. *Chembiochem* **2014**, *15* (18), 2754–2765.

128 Tietze, A. A.; Heimer, P.; Stark, A.; Imhof, D. Ionic Liquid Applications in Peptide Chemistry: Synthesis, Purification and Analytical Characterization Processes. *Molecules* **2012**, *17* (12), 4158–4185.

129 Eimoto, T.; Kusano, T.; Ando, K.; Kikuchi, M.; Shirakusa, T.; Kawanami, S. Nonneoplastic and Nonhyperplastic Thymus in Myasthenia Gravis. An Immunohistochemical Study with Double Immunoenzymatic Labeling of Basement Membrane and Cellular Components. *Am. J. Clin. Pathol.* **1990**, *94* (1), 36–43.

130 Catak, S.; Monard, G.; Aviyente, V.; Ruiz-López, M. F. Reaction Mechanism of Deamidation of Asparaginyl Residues in Peptides: Effect of Solvent Molecules †. *J. Phys. Chem. A* **2006**, *110* (27), 8354–8365.

131 Clarke, S. Propensity for Spontaneous Succinimide Formation from Aspartyl and Asparaginyl Residues in Cellular Proteins. *Int. J. Pept. Protein Res.* **1987**, *30* (6), 808–821.

132 Fujioka, M.; Koda, S.; Morimoto, Y.; Biemann, K. Structure of FR900359, a Cyclic Depsipeptide from Ardisia Crenata Sims. *J. Org. Chem.* **1988**, *53* (12), 2820–2825.

133 Taniguchi, M.; Nagai, K.; Arao, N.; Kawasaki, T.; Saito, T.; Moritani, Y.; Takasaki, J.; Hayashi, K.; Fujita, S.; Suzuki, K.; Tsukamoto, S. YM-254890, a Novel Platelet Aggregation Inhibitor Produced by Chromobacterium Sp. QS3666. *J. Antibiot. (Tokyo).* **2003**, *56* (4), 358–363.

134 Nishimura, A.; Kitano, K.; Takasaki, J.; Taniguchi, M.; Mizuno, N.; Tago, K.; Hakoshima, T.; Itoh, H. Structural Basis for the Specific Inhibition of Heterotrimeric Gq Protein by a Small Molecule. *Proc. Natl. Acad. Sci.* **2010**, *107* (31), 13666–13671.

135 Flaumenhaft, R.; Dilks, J. R. Discovery-Based Strategies for Studying

Platelet Function. *Mini Rev. Med. Chem.* **2008**, *8* (4), 350–357.

136 Wirth, A.; Benyó, Z.; Lukasova, M.; Leutgeb, B.; Wettschureck, N.; Gorbey, S.; Orsy, P.; Horváth, B.; Maser-Gluth, C.; Greiner, E.; Lemmer, B.; Schütz, G.; Gutkind, J. S.; Offermanns, S. G12-G13-LARG-Mediated Signaling in Vascular Smooth Muscle Is Required for Salt-Induced Hypertension. *Nat. Med.* **2008**, *14* (1), 64–68.

137 Jacobsen, S. E.; Nørskov-Lauritsen, L.; Thomsen, A. R. B.; Smajilovic, S.; Wellendorph, P.; Larsson, N. H. P.; Lehmann, A.; Bhatia, V. K.; Bräuner-Osborne, H. Delineation of the GPRC6A Receptor Signaling Pathways Using a Mammalian Cell Line Stably Expressing the Receptor. *J. Pharmacol. Exp. Ther.* **2013**, *347* (2), 298–309.

138 Kaur, H.; Harris, P. W. R.; Little, P. J.; Brimble, M. A. Total Synthesis of the Cyclic Depsipeptide YM-280193, a Platelet Aggregation Inhibitor. *Org. Lett.* **2015**, *17* (3), 492–495.

139 Taniguchi, M.; Suzumura, K.; Nagai, K.; Kawasaki, T.; Takasaki, J.; Sekiguchi, M.; Moritani, Y.; Saito, T.; Hayashi, K.; Fujita, S.; Tsukamoto, S.; Suzuki, K. YM-254890 Analogues, Novel Cyclic Depsipeptides with Gαq/11 Inhibitory Activity from Chromobacterium Sp. QS3666. *Bioorg. Med. Chem.* **2004**, *12* (12), 3125–3133.

140 Rensing, D. T.; Uppal, S.; Blumer, K. J.; Moeller, K. D. Toward the Selective Inhibition of G Proteins: Total Synthesis of a Simplified YM-254890 Analog. *Org. Lett.* **2015**, *17* (9), 2270–2273.

141 Nguyen, H.-H.; Imhof, D.; Kronen, M.; Schlegel, B.; Härtl, A.; Gräfe, U.; Gera, L.; Reissmann, S. Synthesis and Biological Evaluation of Analogues of the Peptaibol Ampullosporin A. *J. Med. Chem.* **2002**, *45* (13), 2781–2787.

142 Besser, D.; Müller, B.; Kleinwächter, P.; Greiner, G.; Seyfarth, L.; Steinmetzer, T.; Arad, O.; Reissmann, S. Synthesis and Characterization of Octapeptide Somatostatin Analogues with Backbone Cyclization: Comparison of Different Strategies, Biological

Activities and Enzymatic Stabilities. *J. für Prakt. Chemie* **2000**, *342* (6), 537–545.

143 Besser, D.; Müller, B.; Agricola, I.; Reissmann, S. Synthesis of Differentially Protected N-Acylated Reduced Pseudodipeptides as Building Units for Backbone Cyclic Peptides. *J. Pept. Sci.* **2000**, *6* (3), 130–138.

144 Müller, B.; Besser, D.; Kleinwächter, P.; Arad, O.; Reissmann, S. Synthesis of N-Carboxyalkyl and N-Aminoalkyl Functionalized Dipeptide Building Units for the Assembly of Backbone Cyclic Peptides. *J. Pept. Res.* **1999**, *54* (5), 383–393.

145 Acosta, G. A.; del Fresno, M.; Paradis-Bas, M.; Rigau-DeLlobet, M.; Côté, S.; Royo, M.; Albericio, F. Solid-Phase Peptide Synthesis Using Acetonitrile as a Solvent in Combination with PEG-Based Resins. *J. Pept. Sci.* **2009**, *15* (10), 629–633.

146 Tam, J. P.; Wu, C. R.; Liu, W.; Zhang, J. W. Disulfide Bond Formation in Peptides by Dimethyl Sulfoxide. Scope and Applications. *J. Am. Chem. Soc.* **1991**, *113* (17), 6657–6662.

147 Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. Use of Anhydrous Hydrogen Fluoride in Peptide Synthesis. I. Behavior of Various Protective Groups in Anhydrous Hydrogen Fluoride. *Bull. Chem. Soc. Jpn.* **1967**, *40* (9), 2164–2167.

148 Miloslavina, A. A.; Leipold, E.; Kijas, M.; Stark, A.; Heinemann, S. H.; Imhof, D. A Room Temperature Ionic Liquid as Convenient Solvent for the Oxidative Folding of Conopeptides. *J. Pept. Sci.* **2009**, *15* (2), 72–77.

149 Heidler, P.; Link, A. N-Acyl-N-Alkyl-Sulfonamide Anchors Derived from Kenner's Safety-Catch Linker: Powerful Tools in Bioorganic and Medicinal Chemistry. *Bioorg. Med. Chem.* **2005**, *13* (3), 585–599.

150 Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. Solid Phase Synthesis of Peptide C-Terminal Thioesters by Fmoc/ T -Bu Chemistry. *J. Am. Chem.*

Soc. **1999**, *121* (49), 11369–11374.

151 Jones, J. H. Abbreviations and symbols in peptide science: a revised guide and commentary. *J. Pept. Sci.* **2006**, *12*, 1–12.