

Native Chemical Ligation of Highly Hydrophobic Peptides in Ionic Liquid-Containing Media

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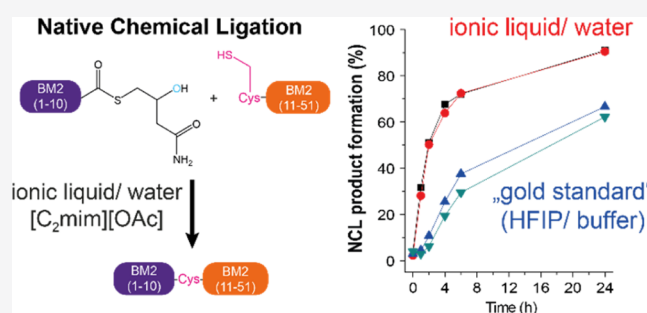


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ABSTRACT: The chemical synthesis of a highly hydrophobic membrane-associated peptide by native chemical ligation (NCL) in an ionic liquid (IL) $[C_2mim][OAc]$ /buffer mixture was achieved by employing peptide concentrations up to 11 mM. NCL was studied at different pH and water content and compared to several “gold-standard” ligation protocols. The optimized reaction protocol for the NCL in IL required the addition of 40% water and pH adjustment to 7.0–7.5, resulting in ligation yields of up to 80–95% within 1 to 4 h. This new ligation protocol is generally applicable and outperforms current “gold-standard” NCL methods.



INTRODUCTION

Ionic liquids (ILs) have been shown to be a good alternative to organic solvents for biotransformation reactions.^{1–4} Several groups describe the use of ILs as advantageous solvents for protein refolding because of the ability of ILs with nucleophilic anions to either break or lower the formation of hydrogen bonds in solution, which leads to the suppression of aggregation in proteins.^{5–8} The use of 1-methoxyethyl-3-methylimidazolium hexafluorophosphate with a water content of 3% was described to be suitable for enzymatic peptide synthesis of tripeptide (ZTyrGlyGlyOEt), resulting in a higher enzyme activity and reaction yield in comparison to conventional systems.⁹ Wehofsky et al. reported the use of 60% of 3-methylimidazolium dimethylphosphate/buffer solution for protease catalyzed ligation.¹⁰ The authors describe a good solubility of all reactants, the full suppression of proteolytic side reactions, higher turnover rates, and a higher stability of chemically unstable reactants.¹⁰ Furthermore, 1-ethyl-3-methylimidazolium acetate ($[C_2mim][OAc]$) was successfully used for native chemical ligation (NCL) and oxidative folding of various hydrophobic peptides.^{11–14} In 2012, Böhm et al. described the NCL in neat $[C_2mim][OAc]$ ligating a 30- and 36-mer to form the 66-meric polypeptide tridegin, a potent inhibitor of human blood coagulation factor XIIIa.¹⁵ In neat $[C_2mim][OAc]$, the solubility of the peptide fragments was increased to >2 mM and the yield was significantly higher compared with that of the NCL in conventional ligation buffer.¹⁵ In another study, the effect of the thioester-neighboring amino acid on the ligation yields in a neat $[C_2mim][OAc]$, which contained about 3% water, was investigated.¹⁶ Again, a faster and efficient ligation was

achieved in comparison to conventional buffers. However, the yield of the ligation product decreased with time due to side reactions, such as oxidative folding or succinimide formation.¹⁶ A somewhat different NCL strategy was reported by Duan et al. using hexamethyldisiloxane in $[BMIM]PF_6$ or $[BMIM]BF_4$ as a promoter to ligate a cysteine-free peptide fragment to a thioester peptide.¹⁷ The ligation yields for di-, tri-, and tetrapeptides achieved by this method varied between 60 and 93%. Although ILs were used as reactants and solvents for biomolecules, their behavior and reaction mechanism differ in ILs and are not fully understood to date. Recently, we have made an attempt to understand the reactions of thiol- and disulfide-containing compounds in $[C_2mim][OAc]$.¹⁸ We have found conditions under which the IL can be used as a solvent or as a reactant through the presence of *N*-heterocyclic carbenes in the neat IL.¹⁸ Based on these recent achievements, we sought to apply IL as reaction media for reactions which are problematic in conventional solvents, that is, conjugation of hydrophobic peptides or orthogonal modifications of peptides. Thus, the herein presented work will focus on the NCL of highly hydrophobic peptides in order to improve the synthetic accessibility to membrane proteins or fragments derived thereof, which in turn would allow for a more detailed structural and functional analysis of, for example, ligand-

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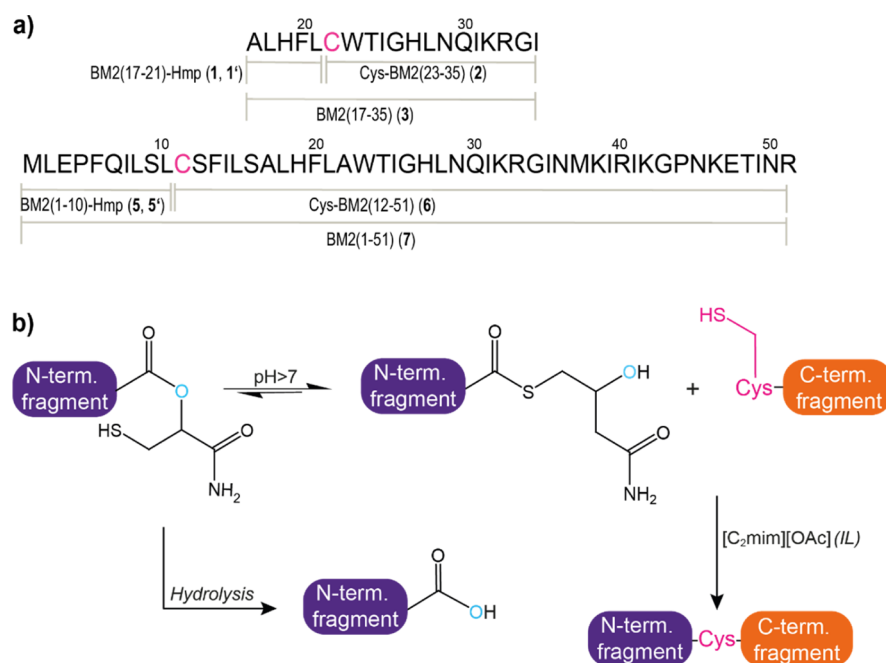


Figure 1. (a) Overview of the BM2 peptide fragments synthesized in this study. (b) General schematic representation of NCL of N-terminal oxo-ester fragment and C-terminal fragment possessing the terminal cysteine to form ligation product in $[\text{C}_2\text{mim}][\text{OAc}]$ and by-product formation due to the hydrolysis of oxo-ester fragment at $\text{pH} > 7$.

Table 1. Abbreviations, Molecular Weights, and R_f of Model Peptides Subjected for Study

abb.	peptide	chemical formula	M_w m. i. [g/mol]	R_f [min]
1	BM2(17–21)-Hmp diast. 1	$\text{C}_{33}\text{H}_{50}\text{N}_8\text{O}_7\text{S}$	702.36	$7.6^a/6.2^b$
1'	BM2(17–21)-Hmp diast. 2	$\text{C}_{33}\text{H}_{50}\text{N}_8\text{O}_7\text{S}$	702.36	$8.3^a/6.6^b$
2	BM2(22–35)	$\text{C}_{73}\text{H}_{120}\text{N}_{24}\text{O}_{17}\text{S}$	1636.96	$3.3^a/3.5^b$
3	BM2(17–35)	$\text{C}_{103}\text{H}_{163}\text{N}_{31}\text{O}_{22}\text{S}$	2218.24	$20.9^a/9.7^b$
4	BM2(17–21)-OH	$\text{C}_{30}\text{H}_{45}\text{N}_7\text{O}_6$	599.35	$4.8^a/4.7^b$
5	BM2(1–10)-Hmp diast. 1	$\text{C}_{59}\text{H}_{96}\text{N}_{12}\text{O}_{16}\text{S}_2$	1292.65	15.6^c
5'	BM2(1–10)-Hmp diast. 2	$\text{C}_{59}\text{H}_{96}\text{N}_{12}\text{O}_{16}\text{S}_2$	1292.65	15.9^c
6	BM2(11–51)	$\text{C}_{214}\text{H}_{353}\text{N}_{65}\text{O}_{52}\text{S}_2$	4729.64	17.5^c
7	BM2(1–51)	$\text{C}_{270}\text{H}_{442}\text{N}_{76}\text{O}_{66}\text{S}_3$	5901.27	21.5^c
8	BM2(1–10)-MPAA	$\text{C}_{64}\text{H}_{97}\text{N}_{11}\text{O}_{16}\text{S}_2$	1339.65	16.5^c
9	BM2(1–10)-OH	$\text{C}_{56}\text{H}_{91}\text{N}_{11}\text{O}_{15}\text{S}$	1189.64	14.8^c

^aHPLC conditions: 25 – 35% acetonitrile in 30 min, flow rate 1 mL/min. ^bHPLC conditions: 25 – 70% acetonitrile in 35 min, flow rate 1 mL/min. ^cHPLC conditions: 10% acetonitrile for 3 min followed by a gradient from 10 – 99% acetonitrile in 30 min, flow rate 1 mL/min.

membrane protein interactions for development of efficient inhibitors of the viral influenza proton channel.^{19–25}

NCL is widely used as a standard method for the synthesis of soluble polypeptides, reaction conditions have been optimized, and the use of various thioesters has been introduced during the last decade.^{15,26} However, NCL is challenging for highly hydrophobic peptides under standard conditions.^{27–29} Several strategies were introduced to overcome the low solubility of hydrophobic peptides in buffer, for example, incorporation of removable or constantly attached solubilizing units^{21,30–34} to a desired peptide sequence and the use of organic solvents [e.g. *N,N*-dimethylformamide (DMF), acetonitrile, isopropanol, and dimethyl sulfoxide] or other additives such as lipids (i.e. dodecylphosphocholine),^{35,36} denaturation agents [i.e. sodium dodecyl sulfate (SDS)],²⁹ or helix-inducers [i.e. 2,2,2-trifluoroethanol (TFE)].²⁴ However, most of these strategies still suffer from low yields resulting from a poor efficiency of the ligation reaction and low solubility of highly hydrophobic fragments. Nevertheless, by

increasing the NCL yield while eliminating solubility problems, we will compensate the product yield loss through aggregation during the sequence prolongation while synthesizing long fragments of hydrophobic peptides.^{30,33} Herein, we describe an alternative and effective method for the NCL of membrane-associated peptides using $[\text{C}_2\text{mim}][\text{OAc}]$. This method can be used as an alternative to removable solubilizing tag strategy³³ and does not require incorporation of additional solubilizing sequences into the core sequence of a hydrophobic peptide. For this purpose, we used fragments of different lengths of the influenza B proton channel (BM2) as a model system, which is a prototypical member of the proton channels of the influenza types A, B, and C and represents an important drug target.^{37–39}

First, model peptides were used to determine the best NCL conditions and finally applying an optimized NCL protocol for the NCL of the BM2(1–10) with the highly hydrophobic peptide fragment BM2(11–51). Therefore, we determined the optimal reaction conditions while changing the ionic strength

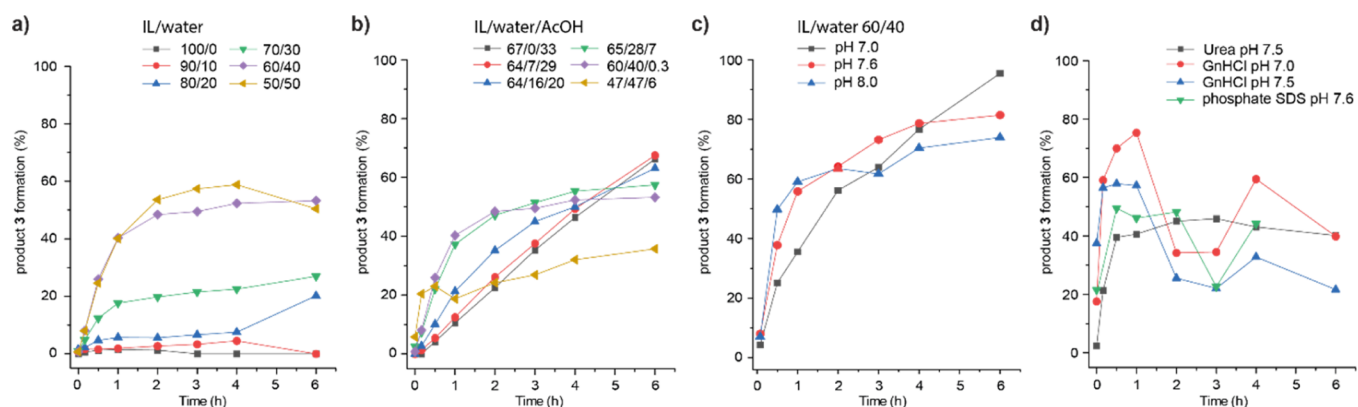


Figure 2. Summary of the reaction progress of the NCL of **1**, **1'** (BM2(17–21)-Hmp) and **2** (BM2(22–35)) in different solvent systems following product formation of **3** by RP-HPLC. (a) NCL with increasing amounts of water in [C2mim][OAc] (ligation buffers LB1a–LB1f). (b) NCL with increasing amounts of water and acetic acid at pH of 7.5 (ligation buffers LB2a–LB2f). (c) NCL in [C2mim][OAc] and water (60/40) at different pH (ligation buffers LB7–LB9). (d) NCL in standard ligation buffers LB3 (black symbols), LB4 (red symbols), LB5 (blue symbols), and LB10 (green symbols).

of the IL-based ligation solution and compared the results with the NCL under standard conditions, employing chaotropic agents such as urea (8 M) or guanidinium hydrochloride (6 M) (experimental details are given in the [Supporting Information](#)). Rather than synthesizing thioester peptide fragments employing a sulfamylbutyryl resin, which usually requires a special workup procedure and gives rather low yields, we decided to use oxo-ester peptide fragments. This allows for the use of the more convenient rink amide resins, which only require a standard peptide workup and TFA cleavage. Moreover, the oxo-ester strategy allows for the incorporation of an in situ cleavable solubility tag, as recently shown by us.³³

RESULTS AND DISCUSSION

Thus, we first studied the NCL of two model peptides (**1**, **1'** and **2**, [Figure 1a](#)) aiming to elucidate the impact of the ionic strength of the reaction solution and the yield (please note, the 2-hydroxy-3-mercaptopropionic acid (Hmp) oxo-ester was used as a racemic mixture resulting in two diastereomeric peptides named **1**, **1'**, [Table 1](#)). The model peptides represent short fragments of the influenza B virus proton channel (strain B/Maryland/1/2001), whereas the oxo-ester fragment represent the BM2 sequence starting from the amino acid position 17 to 21 (BM2(17–21)-Hmp) and the cysteine-containing fragment comprises amino acids 22 to 35 (BM2(22–35)) ([Figure 1a](#), [Table 1](#)). The sequence of BM2 was modified in order to have a C-terminal leucine (I21L for BM2(17–35), I10L BM2(1–51)) at the ligation site of both fragments ([Figure 1a](#)). The oxo-ester fragment forms a thioester at pH > 7 which is substituted with thiophenol (PhSH) to form a better leaving group through the reaction. The thiophenol intermediate then reacts with the cysteine-fragment forming the final product **3**, BM2(17–35) ([Figure 1b](#), [Table 1](#)). In aqueous solutions, the only by-product that can be formed during this ligation reaction is the hydrolysis product of the oxo-ester fragment ([Figure 1b](#)). As shown by Zheng et al., this type of oxo-ester undergoes a rearrangement through a 1,5-acyl migration with nearly 100% conversion, resulting in over 80% ligation yields.⁴⁰

All peptide fragments were successfully synthesized on a solid support using an AmphiSpheres RAM resin with 0.37 mmol/g loading capacity following a standard Fmoc-SPPS

protocol, including coupling of the HMP group. The following amino acid was coupled through a Mitsunobu reaction.^{41–43} Fmoc-deprotection after HMP coupling was performed with the mild base 2-methyl piperidine, to ensure stability of the HMP unit. Also, cleavage from the resin was performed following the standard cleavage protocol with 95% TFA as recently described by us. Thus, the synthesis of the thioether-forming fragment is straight forward to perform and does not require any special protocol, as is the case for sulfamylbutyryl resin-based thioester synthesis. Moreover, oxo-ester peptides are stable and can be stored for long time in comparison to thioester-peptides, which is a noticeable advantage of the selected strategy. Besides, we recently demonstrated that no epimerization of the amino acid at the condensation point was observed within a very similar strategy for the NCL of BM2(1–51), when oxoester peptides were employed.³³

In the present work, we studied the NCL of BM2(17–21)-Hmp (**1**, **1'**) and the cysteine-containing fragment BM2(22–35) (**2**) in [C₂mim][OAc] with respect to different IL/water ratios ([Figure 2a](#)), IL/water/acetic acid ratios ([Figure 2b](#)), and pH ([Figure 2c](#)) and compared the results to those of gold-standard NCL protocols ([Figure 2d](#)). For comparability with the NCL conditions used by Zheng et al., thiophenol was used as the ligation catalyst.⁴⁰

The NCL reactions were followed by analytical RP-HPLC. The product formation was calculated with respect to the changes of the peak area of the product and the Cys-fragment. To avoid any bias in the data analysis resulting from significantly different extinction coefficients for the product and the Cys-fragment, we estimated the absorptivity of a reference solution for both compounds for the analytical HPLC setup. Thus, the peptide content of a standard solution of BM2(22–35), BM2(17–35), BM2(11–51), and BM2(1–51) was determined, and the peak area for each compound was recorded ([Tables S2 and S4](#), more details are given in the [Supporting Information](#)). So far, within the error margin of the method, the absorptivity of the Cys-fragments BM2(22–35) (**2**) and BM2(11–51) (**6**) and the respective ligation products BM2(17–35) (**3**) and BM2(1–51) (**7**) are similar ([Table S4](#)), allowing to directly compare their peak areas in the HPLC chromatograms.

First, we studied the influence of water on the ligation yields and the hydrolysis rates of the Hmp-group during the ligation

of the model peptides **1**, **1'** and **2** (Figures 2a and S5). In order to find the ideal ratio between the IL [C₂mim][OAc] and water, we prepared different ligation buffers LB1a–LB1f (Table S1) with a different content of water. Although we previously determined that the concentration of water should be at least 30% in order to use [C₂mim][OAc] as a solvent, experiments with a lower water content were performed (0–20%) for comparison. In each ligation experiment the water content was increased by 10% starting with the ligation in the neat IL LB1a (Figures 2a and S5). All newly formed peaks in the RP-HPLC were separated and identified by electrospray ionization mass spectrometry (ESI-MS) (Figures S3 and S4). The formation of the hydrolyzed product **4** (Figure S3) and ligation product **3** (Figure S4) was monitored for 6 h by RP-HPLC and their yields were calculated from relative peak areas (Figures 2a and S5). Because of the high amount of [C₂mim][OAc] in ligation buffers LB1a–LB1f, it was possible to dissolve large amounts (10 mM–peptide **1** and 11 mM–peptide **2**) of the hydrophobic peptides in a very small volume. In order to ensure a quantitative excess of the catalyst and the reducing agent, high concentrations (150 mM) for thiophenol and TCEP were used. If only low amounts of water (0–20%) were present in the IL, no ligation product could be observed (Figure S5a–c). Instead, significantly more hydrolyzed oxoester (**4**) was formed compared to the experiments performed when more water (30–50%) was present in the reaction mixture (Figure S5d–f). However, at higher water contents (30–50%), an intense product peak of **3** at *t_R* 21 min was observed in the HPLC chromatograms (Figure S5d–f). The detailed analysis of the formation of ligation product **3** in the presence of different amounts of water in the IL revealed (Figure 2a) that at 50% water, the maximum amount of 60% of product **3** was formed within 4 h. However, after 4 h, the product began to precipitate under these conditions as indicated by the reduction of the peak area in the chromatograms. In contrast, when 40% water was present in the IL reaction mixture, only slightly less product **3** (53%) was formed within 6 h of reaction time and the product remained in the solution during the following hours (Figures 2a and S5). When the amount of water was reduced to 30%, the yield of product **3** was much smaller only reaching 25%. Thus, it seems that at an IL/water ratio of 60:40, the product formation appears optimal. Moreover, and contrary to our expectations, the relative peak area of the hydrolysis product **4** gradually increased with decreasing water content. In fact, this observation led us to analyze the pH of the IL/water mixture used for the ligation experiments. The pH values for the respective IL/water mixtures LB1a–f are outlined in Table S1. Please note that the read-out values for the pH were not corrected as suggested by Garcia-Mira et al. for water/GnHCl mixtures⁴⁴ since the specific correction factor for IL-based water mixtures has not yet been determined. Although measuring the pH of a neat IL is questionable, the pH of the IL/water mixtures decreased from 10.5 at a water content of 10% to 6.8 at 50% water. Apart from this pH dependency, the high viscosity of the IL/water mixtures when only low amounts of water were present might additionally reduce the reactivity of the ligation fragments. So far, these results are in agreement with previous results, which indicated an optimal pH of 7.5 for the ligation strategy used in this study.³³

In a second set of experiments, we aimed to keep the pH of the solution constant. Since acetate is the anion of the IL, concentrated acetic acid was used to adjust the pH, while

increasing the amount of water and the ionic strength of the reaction solution. For this set of reaction mixtures (LB2a–f, Table S1), the amount of IL was in the range of 65–47%, while the amount of water was increased from 0 to 47%. With respect to this increasing water amount, glacial acetic acid was added to adjust the pH of the reaction mixture to 7.5. In contrast to the IL/water mixture of LB1, product **3** was already formed when no water was added to the initial mixture of IL and acetic acid (LB2a, Figures 2b and S6).

In contrast to the first set of experiments, the reaction progress was much slower for the first three sets of reaction (67/0/33, 64/7/29, and 64/16/20, Figure 2b), which contained either no or only a small amount of water (0, 7, and 16%, Figure 2b). Surprisingly, over the course of 6 h, a somewhat higher amount of product was formed (about 60–67%) when compared with the experiments (65/28/7 and 60/40/0.3, Figure 2b) with larger amounts of water (28, 40%, Figure 2b), which resulted in about 50–55% ligation product. Only 30% product was formed when the amount of water was further increased (47% at a reaction of 47/47/6, Figure 2b). Moreover, for this reaction product, precipitation was observed immediately after the reaction was started (Figure 2b). Interestingly, the progress and product formation of the ligation experiment in LB1e and LB2e (Figure 2a,b, 60/40 and 60/40/0.3) were almost identical, which can be rationalized by the almost identical pH and IL/water ratio (Table S1). Also, the overall amount of product formed during the ligation reaction was among the highest in these two IL-reaction mixtures. In an additional set of experiments, the water content was kept at 40%, which was suggested to be an optimal amount with respect to our data, while the reactions were performed at pH 7.0, 7.6, and 8.0 (Figure 2c, Table S1 and Figure S8). Whereas the reaction progress and product formation at pH 7.6 and 8.0 were similar, a somewhat slower reaction was observed at pH 7.0, but resulting in an almost quantitative product formation. Additionally, product **3** (BM2(17–35)) was isolated and purified by preparative HPLC from the reaction at pH 7.6 in this set of experiments, yielding about 35% purified product (95% purity). So far, the large amount of IL in the reaction mixture had no negative impact on the preparative scale HPLC purification. Usually, the IL fraction has no significant retention on standard C18 columns (250 × 8 mm and 250 × 20 mm were tested) and elutes shortly after the injection peak at low acetonitrile concentrations.

Lastly, a set of ligation experiments in conventional ligation buffer was performed using guanidine hydrochloride, urea, and a phosphate/SDS buffer system (LB3–LB5, and LB10, Figures 2d and S7). Because of the low solubility of the peptides **1**, **1'** and **2** in ligation buffers LB3, LB4, and LB5, higher buffer volumes were necessary to entirely dissolve the peptides. Ligation experiments in these buffers were carried out at peptide concentrations of 1.3 mM (peptide **1**, **1'**) and 1.5 mM (peptide **2**), which are 10 times more diluted compared to the IL-based buffers. The SDS-based phosphate buffer allowed for somewhat higher peptide concentrations of about 4 mM. The initial reaction rates of the NCL were higher in these standard buffers (Figure 2d) compared to the IL-based ligation solutions. The reaction was completed within 30–60 min and no further product was formed after that time, which agrees well with earlier reported reaction times.⁴⁰ In this set of ligation experiments, product precipitation occurred after some time in all reactions although much smaller peptide concentrations were used. Yields were about 20–25%

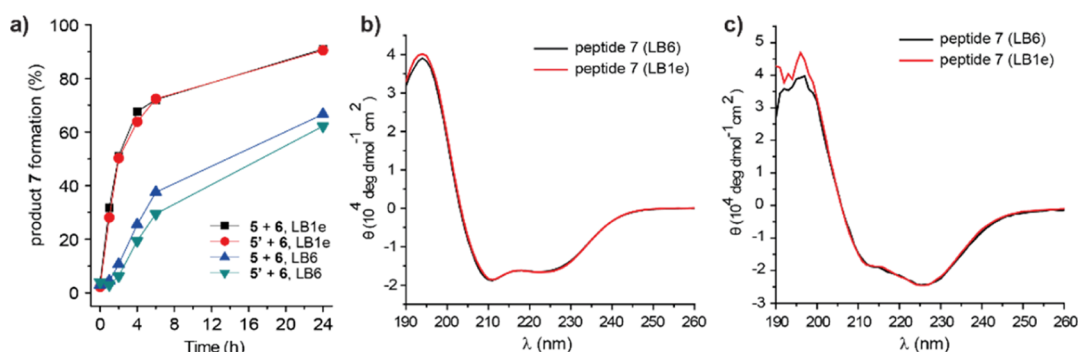


Figure 3. (a) Reaction progress of the NCL of BM2(1–10)-Hmp (5, 5) with BM2(11–51) (6) in ligation buffer LBe1 (black and red symbols) and LB6 (blue and green symbols) to form 7. CD spectra of ligation product 7 obtained from the HFIP-based solvent mixture LB6 (black trace) and the IL/water mixture LBe1 (red trace) in (b) TFE and (c) 0.65 M POPC/Na₂HPO₄ buffer (pH of 7.5, 20 °C).

lower in the conventional buffer systems, except for the NCL in GnHCl at pH 7.0 (Figure 2d), which has recently been shown to work exceptionally well for the NCL of BM2(1–51).⁴⁵ Regarding the very fast reaction times in the standard buffer systems, choosing thiophenol instead of the better soluble MPAA as the ligation catalyst does not seem to have a significant impact on the reaction progress (Figure 3a). Moreover and as one would expect, more by-product 4 was formed, since aqueous buffer supports much faster hydrolysis of Hmp-peptide.

Thus far, the results of these four sets of experiments clearly indicate that the water content and the pH of the IL-based reaction mixture have a strong impact on the reaction outcome with an optimal pH of about 7.0 – 7.5. The pH dependency, which was observed in all our experiments, can mainly be explained by the decreasing hydrolysis rate of the oxoester fragment when the pH of the reaction solution gets lower. At the same time, a certain amount of nucleophilic thiolate needs to be present, which usually requires a pH above 6.5 – 7.0. This behavior is in agreement with the standard reaction conditions necessary for an efficient NCL in standard solvents.⁴⁶ Furthermore, our data indicate no direct influence of the acetic acid on the reaction progress, except for the pH adjustment. As the addition of water had a stronger impact on the progress of the ligation reaction than acetic acid (Figure 2a,b) and the viscosity of water and glacial acetic acid is similar, the viscosity of the reaction mixture alone is not the rate limiting factor.

Apart from this interpretation, it might be possible that the addition of water enables the formation of hydrated ion pairs of the IL components, which then are capable of better solubilizing the peptides or stabilizing reaction intermediates more efficiently than in the IL/acetic acid mixture without water (Figure 2b), thus resulting in an increased reaction rate. This would also be in line with recent results of Fujita et al., who found that specifically hydrated ILs are effective media for protein refolding from aggregates.⁴⁷ Taken together, our results show that IL-based buffers were clearly more suitable for ligation of hydrophobic peptides in comparison to standard ligation buffers under addition of chaotropic reagents. Although thiophenol appears to be not fully soluble at larger water contents (>40%) in the IL and in some of the buffer systems, which resulted in an increased suspension of thiophenol in the reaction mixture, the reaction progress does not indicate significant influence on the reaction performance (Figure 2) which is most likely due to the large excess that was used. Also, with respect to the NCL reactions

performed with the soluble thiophenol counterpart MPAA, which are outlined and discussed in the next section, no significant impact of the reaction performance could be observed (Figure 3).

Last but not least, we compared the ligation protocol using an IL/water (60:40, LB1e) mixture with our recently developed and highly efficient 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)-based ligation protocol (LB6) synthesizing a 50 aa long fragment of the BM2 proton channel 7 (BM2(1–51)), ligating peptides 5 BM2(1–10)-Hmp, and 6 BM2(11–51) (Figures 3a and S9–S12) employing MPAA as a ligation catalyst (please note: the Hmp-oxoester was used as a racemic mixture resulting in two diastereomeric peptides, namely, 5, 5, Table 1). The overall yield of the ligation in the HFIP-based reaction mixture (LB6) was slightly higher (~10%) than that in the IL/water mixture (LB1e). Whereas the reaction in the IL/water mixture was almost completed after 4 h, the NCL in the HFIP-based solvent mixture was not finished even after 24 h (Figure 3a). Again, the IL/water mixture allowed us to use a 2-fold higher peptide concentration (2-fold) compared to the HFIP-based solvent mixture, although HFIP-based buffer systems were considered as more efficient than other standard buffer-based ligation solvent systems.⁴⁸ Thus, the higher concentration might partly explain the much faster reaction in the IL/water mixture (Figure 3a). With respect to the choice of the ligation catalyst, MPAA does not seem to perform significantly better, since similar reaction times were observed for the NCL of the short BM2 fragments, where it was used as the catalyst (Figures 2c and 3a).

Additionally, product 7 BM2(1–51) from the IL/water mixture at pH 7.5 was isolated and purified by preparative HPLC, resulting in an isolation yield of about 20%, which is half of the yield recently reported for BM2(1–51) employing a trityl-based solubility tag strategy in GnHCl/phosphate buffer.⁴⁵

The structural integrity of the BM2(1–51) peptide 7 synthesized in ligation buffer LB6 and LBe1 was confirmed by far-ultraviolet circular dichroism (CD) analysis (Figure 3b,c). So far, CD spectroscopic analysis yielded almost identical CD spectra for product 7 obtained from both ligation approaches and confirmed the α -helical nature of the BM2(1–51) fragment in TFE as well as after the fragment had been incorporated into a 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) lipid. After deconvolution of the CD spectra, it was found that both BM2(1–51) peptides contain around 60% α -helical structure in TFE and in POPC.

CONCLUSIONS

In conclusion, the NCL of membrane-associated peptides by the reaction of a thioester-forming and a cysteine-containing peptide was successfully performed in IL [C_2mim][OAc]. We were able to establish an efficient IL-based ligation buffer system which shows equal or even slightly better ligation yields compared to that of standard ligation buffers commonly used in modern peptide chemistry. The most efficient ligation buffer contained 60% IL and 40% water with an optimal pH between 7.0 and 7.5 and allows for much higher peptide concentrations in the ligation solution. Moreover, the IL-based ligation buffer was shown to be even more efficient for NCL of the highly hydrophobic membrane peptide BM2(1–51) in comparison to our previously developed NCL protocol in HFIP-based ligation buffer resulting in a faster product formation. CD measurement of BM2(1–51) confirmed the structural integrity of the BM2(1–51) fragment obtained from both ligation approaches.

EXPERIMENTAL SECTION

If not otherwise stated, all amino acid derivatives and coupling reagents were purchased from carbolutions and used without further purification. Standard solvents for solid-phase-peptide synthesis and other standard chemicals were purchased from Sigma Aldrich and used without further purification. ILs were purchased from Iolitec ILs Technologies GmbH.

pH of solutions were measured with either 716 DMS Titrimo (Metrohm) or Five Easy F20 (Mettler Toledo). The pH values were used as a direct read-out from the pH meter and no correction was applied.

High-Performance Liquid Chromatography (RP-HPLC). All crude and purified peptides were analyzed by analytical RP-HPLC on a Waters 2695 Alliance system (Waters, Milford, MA, USA) employing a Waters 2998 photo diode array (PDA) detector equipped with a prontosil C8-SH (120 × 5 mm, 5.0 μ m) column. HPLC eluent A was water [0.1% trifluoroacetic acid (TFA)] and eluent B was acetonitrile (0.1% TFA). If not stated otherwise, HPLC conditions for model peptides (1, 1', and 2), 25–35% eluent B over 30 min at flow rate of 1 mL/min was applied (detection at 214 nm). For peptides (5, 5', and 6), 10% eluent B for 3 min followed by 10–99% eluent B over 30 min was used. Chromatograms were extracted at 220 nm and analyzed at 214 nm. Peptide absorptivity for the 2998 PDA detector at 214 nm was determined from the peak area of a standard reference solution of the respective peptide. Concentration of the peptide solution was determined through a HPLC-based amino acid analysis protocol.

Preparative scale purification of the peptides was achieved by employing a Waters 1525 binary pump and a Waters 2998 PDA detector or a customized Waters 600 module equipped with a Waters 996 PDA detector (Waters, Milford, MA, USA). HPLC eluent A was water (0.1% TFA) and eluent B was acetonitrile (0.1% TFA).

Mass Spectrometry. The molecular weight of the purified peptides was confirmed by ESI mass spectrometry on a Bruker TOF-Q impact II spectrometer (Bruker Daltonik GmbH, Bremen, Germany) and calibrated using a Bruker's ESI-Tune-Mix or Waters SYNAPT G2-Si HD-MS spectrometer equipped with a Waters Acquity UPLC system (Waters, Milford, MA, USA).

CD Spectroscopy. CD spectra were recorded at 190–260 nm (0.1 cm path-length cuvette, 20 °C) in trifluoroethanol (TFE) or buffer [10 mM disodium phosphate (Na_2HPO_4), pH 7.5] containing the phospholipid (POPC, 0.5 mg/mL) employing a JASCO J-810 spectropolarimeter. Lipid samples were prepared as described previously.³³ Spectra deconvolution was performed using CDNN (Circular Dichroism analysis using Neural Networks) software.

Peptide Synthesis. The Cys-containing peptide fragment BM2(11–51) 6 peptide (Figure S1) was synthesized on Amphiphores RAM resin (0.37 mmol/g) by microwave-assisted automated

Fmoc-SPPS (50 °C, 35 W, CEM Liberty 908505 peptide synthesizer) in a 35 mL sealed reaction vessel. The coupling of amino acids was performed by activation of Fmoc-protected amino acids (4 equiv) with 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 3.95 equiv) and *N*-ethyl-*N*-(propan-2-yl)propan-2-amine (DIEA, 8 equiv) in DMF. The deprotection of the Fmoc-group was carried out by 20% piperidine in DMF under microwave radiation (50 °C, 40 W) for 120 s. The coupling and Fmoc-deprotection steps were all followed by intensive washing of the resin with DMF and dichloromethane. The Cys-containing peptide fragment BM2(22–35) 2, Hmp (BM2(17–21)-Hmp) 1,1', and BM2(1–10)-Hmp fragments 5,5' (Figure S2) were synthesized as described previously.³³ All peptides were cleaved from the resin as described previously.³³

After purification of the crude peptides via RP-HPLC, the peptides were freeze-dried, redissolved in water, and aliquoted, yielding aliquots of 0.2 μ mol for 1 and 1', 0.22 μ mol for 2, 0.1 μ mol for 5 and 5', and 0.12 μ mol for 6. The aliquots were freeze-dried again and stored at –28 °C.

Native Chemical Ligation of Model Peptides 1 and 2 in Ligation Buffers LB1 and LB2. To prepare LB1 buffers, an equimolar solution of tris-(2-carboxyethyl)-phosphine (TCEP) (150 mM, 0.03 mmol) and thiophenol (150 mM, 0.03 mmol) was dissolved in 0.2 mL of [C_2mim][OAc] containing different amounts of water according to Table S1.

The NCL was started by dissolving 1.1 equiv of Cys-BM2(23–35) (0.36 mg, 0.22 μ mol) and 1.0 equiv of ALHFL-Hmp (0.14 mg, 0.2 μ mol) in 20 μ L of LB1 or LB2. For LB2, different amounts of glacial acetic acid (AcOH) were added to the reaction mixture in order to adjust the pH to 7.5. The final solution was stirred under nitrogen at room temperature (rt). To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted in 30 μ L acetonitrile/water (60/40) (v/v) containing 0.8% TFA.

Native Chemical Ligation of Peptides 5, 5', and 6 in Buffer LB1e. For LB1e, an equimolar solution of TCEP (0.03 mmol, 150 mM) and 2-(4-mercaptophenyl)acetic acid (MPAA, 0.03 mmol, 150 mM) was prepared while 0.2 mL of [C_2mim][OAc]/water (60:40, v/v) was added. The NCL was started by dissolving 1.0 equiv of BM2(1–10)-Hmp (0.13 mg, 0.10 μ mol) and 1.1 equiv of Cys-BM2(12–51) (0.56 mg, 0.12 μ mol) in 50 μ L of LB1e. The final solution was stirred under nitrogen (rt). To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted in 40 μ L acetonitrile/water (60/40) (v/v) containing 0.8% TFA.

Product 7 was purified by preparative scale RP-HPLC from the experiment in ligation buffer LB1e (416 μ L, IL/H₂O 60/40 v/v, pH 7.5) after 4 h of stirring using 0.65 μ mol (3.07 mg, 1.1 equiv) of Cys-BM2(11–51) and 0.58 μ mol (0.75 mg, 1.0 equiv) of BM2(1–10)-Hmp. About 20% (0.65 mg, 0.11 μ mol) purified product 7 (95%) was yielded. HPLC conditions were as follows. A solvent gradient of 30–70% eluent B over 100 min at a flow rate of 8 mL/min was applied using a C18 column (ISAspher 300–5 C18, 250 × 20 mm).

Native Chemical Ligation in Urea-Ligation Buffer (LB3). To an 8 M urea solution (0.15 mL) in 0.2 M Na_2HPO_4 (0.03 mmol 4.26 mg), 50 mM TCEP (0.0075 mmol, 2.12 mg) and 150 mM thiophenol (0.023 mmol, 2.3 μ L) were added. The ligation was started by dissolving 1 equiv of ALHFL-Hmp (0.14 mg, 0.2 μ mol) and 1.1 equiv of BM2(22–35) (0.36 mg, 0.22 μ mol) in ligation buffer LB3. The mixture was stirred under argon for 6 h (rt). To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted in 55 μ L water (0.1% TFA).

Native Chemical Ligation in Guanidinium Chloride (GnHCl)-Ligation Buffer (LB4, LB5). 50 mM TCEP and 150 mM thiophenol were added to a 6 M GnHCl solution in 0.2 M Na_2HPO_4 buffer at pH of either 7.0 (LB4) or 7.5 (LB5). The ligation was started by dissolving 1 equiv of ALHFL-Hmp (0.14 mg, 0.2 μ mol) and 1.1 equiv of BM2(22–35) (0.36 mg, 0.22 μ mol) in ligation buffer LB4 or LB5. The mixture was stirred under argon for 6 h (rt). To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted with 55 μ L water (0.1% TFA).

Native Chemical Ligation in HFIP-Containing Urea Buffer (LB6). For LB6, to a mixture of 6 M urea/HFIP (2:1 (v/v)) 150 mM Na_2HPO_4 , 75 mM TCEP HCL and 112 mM 2-(4-mercaptophenyl)-acetic acid were added. The ligation was started by dissolving 1.0 equiv of BM2(1–10)-Hmp (0.13 mg, 0.10 μmol) and 1.1 equiv of BM2(11–51) (0.56 mg, 0.12 μmol) in 100 μL of ligation buffer LB6. The pH of the reaction mixture was adjusted to 7.5 using 10 M NaOH solution. The mixture was stirred under argon for 24 h (rt). To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted in 30 μL of water/TFE (0.2% TFA).

Native Chemical Ligation of Model Peptides 1 and 2 in Ligation Buffers LB7–9. To prepare LB (7–9) buffers, an equimolar solution of TCEP (150 mM, 0.03 mmol) and thiophenol (150 mM, 0.03 mmol) was dissolved in 0.2 mL $[\text{C}_2\text{mim}][\text{OAc}]$ containing 40% water. The NCL was started by dissolving 1.1 equiv of Cys-BM2(23–35) (0.36 mg, 0.22 μmol) and 1.0 equiv of ALHFL-Hmp (0.14 mg, 0.2 μmol) in 20 μL of LB1 or LB2. Different amounts of 10 M aqueous NaOH solution were added to the reaction mixture to adjust the pH to 7.0, 7.6, and 8.0. The final solution was stirred under nitrogen at rt. To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted in 30 μL acetonitrile/water (60/40) (v/v) containing 0.8% TFA.

Product 3 was purified by preparative scale RP-HPLC from the experiment in ligation buffer LB8 (270 μL , IL/H₂O 60/40 v/v, pH 7.6) after 4 h of stirring using 2.14 μmol (3.06 mg, 1.1 equiv) of Cys-BM2(23–35) and 1.9 μmol (1.33 mg, 1.0 equiv) of ALHFL-Hmp. About 35% (1.66 mg, 0.75 μmol) purified product 3 (95%) was yielded. HPLC conditions were as follows. A solvent gradient of 10 – 50% eluent B over 40 min at a flow rate of 8 mL/min was applied using a C18 column (MultoKrom 100–5 C18, 250 \times 20 mm).

Native Chemical Ligation in Phosphate Buffer (LB10). The two peptides of 1 equiv of ALHFL-Hmp (0.14 mg, 0.2 μmol) and 1 equiv of BM2(22–35) (0.36 mg, 0.22 μmol) were dissolved in 50 μL 0.1 M phosphate buffer at pH 7.6 containing SDS (173 mM) and 2% (v/v) thiophenol. To monitor the reaction progress by RP-HPLC, equal aliquots from the ligation solution were diluted in 30 μL of acetonitrile/water (60/40) (v/v) containing 0.8% TFA.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.joc.0c02498>.

Details on the reaction progress, LC–MS analysis, RP-HPLC chromatograms, and HPLC-based amino acid analysis (PDF)

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Notes

The authors declare no competing financial interest.

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