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Ionic liquid 1-ethyl-3-methylimidazolium acetate: an attractive solvent for native chemical ligation of peptides

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ABSTRACT

The ionic liquid 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) was successfully used as alternative solvent for native chemical ligation of peptide fragments to produce model peptide LYRAXCRANK (X = G, A, L, N, Q, K, and F). The commonly used buffer system including thiol additives such as thiophenol and benzyl mercaptan can be replaced by the nontoxic ionic liquid [C_2mim][OAc]. In addition to improving the solubility of the peptides in [C_2mim][OAc], yields and rates of the ligation reactions were found to be efficiently enhanced.

Many natural cysteine-containing peptides and miniproteins are therapeutically interesting and may represent potential lead compounds for drug design. However, some of these peptides are usually difficult to be extracted and purified from the respective biological sources.[1,2] Thus, alternative approaches for their preparation were intensely studied over the last decades. Besides solid-phase peptide synthesis (SPPS) and recombinant expression techniques, ligation methods for the condensation of peptide fragments were also examined in this respect.[3–5] The native chemical ligation (NCL) method, which was introduced by Kent and Dawson in 1994,[3] turned out to be very useful, in particular for peptides containing a cysteine residue at the ligation site (Scheme 1B). The NCL reaction is generally carried out in buffered aqueous solutions with unprotected peptide segments. The addition of distinct additives such as benzyl mercaptan, (4-carboxymethyl)-thiophenol, or thiophenol, enhance rate and yield of the ligation reaction by generating the more reactive aryl thioester from the original alkyl thioester (Scheme 1A).[3,6] Moreover, in order to avoid oxidation of the cysteine residue, it may be required to add dithiothreitol or dithioerythriol, too.[3–7]

However, the application of aqueous media in connection with peptides always holds the risk that either the starting compounds and/or the product molecule/s are not well soluble and thus, do not allow for a homogeneous reaction.[5] Furthermore, the contradiction between high toxicity and efficiency of some catalysts used in NCL also needs to be overcome. Therefore, it is worth considering alternative solvents as media and/or catalysts for peptide chemical reactions. Several laboratories have successfully used ionic liquids (ILs) in this respect,[8–13] and the reactions reported thus far range from solution peptide synthesis[11] to protease-catalyzed ligation[12] and peptide oxidation.[13] It is well-known that ionic liquids are unique, useful, and tunable compounds of an interesting architecture only consisting of ions. The fact that ILs are composed of polar ions (cations and anions), but also comprise an apolar region due to the alkyl moieties of their organic ions renders them highly suitable material for polar, apolar as well as amphiphilic solutes. Thus, distinct ILs, in particular imidazolium-based ILs, found their way into applications in connection with biopolymers, such as proteins, carbohydrates, and nucleic acids.[8–13]

Recently, we tested a series of imidazolium-based ILs as reaction media for the generation of 66mer peptide Tridegin, an inhibitor of blood coagulation factor XIIIa, by native chemical ligation.[5] Some of these room temperature ILs (RTILs) showed a remarkably high capacity for dissolving the peptide fragments in higher concentrations (>2 mM) as compared to aqueous systems (⩽0.4 mM).[5] Moreover, distinct ILs such as 1-ethyl-3-methylimidazolium acetate ([C_2mim][OAc]) and 1-butyl-3-methylimidazolium acetate ([C_4mim][OAc]) revealed a promoting effect on the ligation rate and yield. The majority of the ILs tested, however, had no impact on NCL reaction, probably because of their non-favorable

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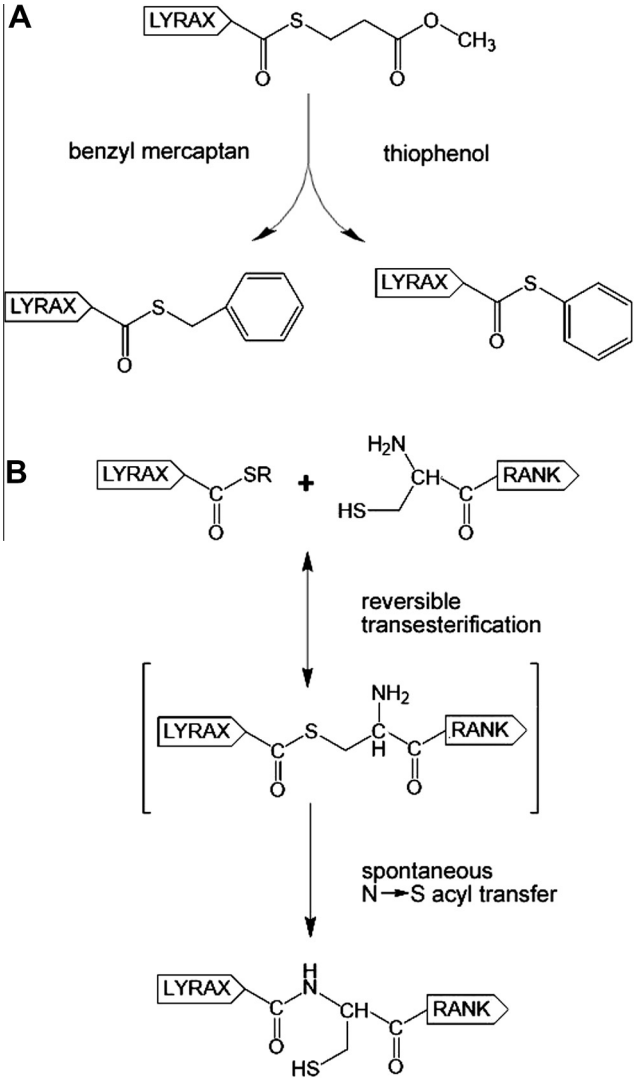


Scheme 1. (A) Thioester exchange reactions with benzyl mercaptan and thiophenol. (B) General mechanism of native chemical ligation exemplified for model peptide LYRAXCRANK (X = any amino acid) (modified from Ref. 14).

anion, that is, *para*-toluenesulfonate ($[OTs]^-$), diethylphosphate ($[DEP]^-$), and dicyanamide ($[N(CN)_2]^-$).[5,10] This work led us to hypothesize that [C_2mim][OAc] might be a suitable reaction medium for the NCL reaction of peptide fragments in general. Thus, to expand our earlier studies to a broader range of peptides, and subsequently, to develop it into a more generally applicable method, we applied [C_2mim][OAc] for NCL of cysteine-containing peptide fragments and compared these results to those obtained from the common approach in which a buffer system is used.[3,6,14]

In 1999, Hackeng et al.[14] reported that the C-terminal amino acid of the model N-terminal peptide fragment **LYRAX-thioester** (X = any amino acid) providing the thioester group at its C-terminus had a great influence on the ligation rate.[14,15] This fragment was linked to a cysteine residue of the C-terminal segment, that is, model peptide **CRANK**. Reaction rates and yields were found to be highly different for the individual **LYRAX-thioester** peptides as demonstrated by MALDI analysis of the product formation over a time period of up to 72 h.[14] However, Hackeng et al. already stated that the MALDI analysis cannot be regarded as quantitative approach.[14] We thus repeated NCL reactions of a selection of these peptide fragments (**1**–**7**: **LYRAX-thioester**, **8**: **CRANK**; X = G (**1**), A (**2**), L (**3**), F (**4**), N (**5**), Q (**6**), K (**7**), Table S1) in the buffer system

Table 1
Comparison of the conversions and the ligation yields obtained in different reaction media

Ligation	Conversion	Yields (reaction time)	
	Buffer, 37 °C[a,14]	Buffer,[b] 20 °C	IL,[c] 20 °C
1[d] to **8**[e]	~100%	57% (1 h)	85% (10 min)
	(after ⩽4 h)	81% (8 h)	98% (1 h)
			83% (2 h)[g]
2[d] to **8**[e]	~100%		49% (10 min)
	(after ⩽9 h)	8% (1 h)	84% (1 h)
		43% (24 h)	78% (4 h)[g]
3[d] to **8**[e]	~100%		27% (10 min)
	(after ⩽48 h)		36% (1 h)
		25% (8 h)	20% (8 h)[g]
		19% (24 h)[f]	
4[d] to **8**[e]	~100%		29% (10 min)
	(after ⩽9 h)	0% (1 h)	83% (1 h)
		3% (48 h)[f]	78% (8 h)[g]
5[d] to **8**[e]	~100%	5% (1 h)	—[h,i]
	(after ⩽24 h)	16% (8 h)[f]	
6[d] to **8**[e]	~100%	3% (1 h)	22%/11% (1 h)[h]
	(after ⩽24 h)	11% (24 h)[f,e]	4%[f]/45% (24 h)
7[d] to **8**[e]	~100%	4% (1 h)	29% (1 h)[i]
	(after ⩽24 h)	41% (24 h)[f]	10% (24 h)[g,i]

[a] Values are estimated from MALDI analysis reported in Ref. 14 (for a comment on this approach see text).
[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. (100% yield was not achieved in any of the ligation reactions, instead degradation products, for example, LYRAX-COOH, can be formed.)
[c] IL: [C_2mim][OAc]. For evaluation, the amount of the LYRAXCRANK-product as well as the IL-oxidized product were combined.
[d] Peptide terminates as thioester ($-SCH_2CH_2COOCH_3$).
[e] Peptide terminus is amidated.
[f] Intermediates (LYRAX-thioesters + additives) could be detected in high amounts in these approaches pointing to slow conversion.
[g] Side product formation in IL led to reduced product formation.
[h] LYRAX-thioester in this approach immediately degrades.
[i] Product formation could not or only partially be determined due to a peak-overlay of side product and product in the chromatogram.

described earlier[14] but using HPLC to monitor the formation of the resulting peptides (**LYRAXCRANK**: X = G (**9**), A (**10**), L (**11**), F (**12**), N (**13**), Q (**14**), K (**15**)) (Table 1, Table S1, Figures 1 and 2). Also, we monitored each reaction individually to optimize the quantification, while Hackeng et al. performed the reaction of 5 **LYRAX-thioesters** simultaneously in one tube.[14] Subsequently, these reactions were compared with those carried out in [C_2mim][OAc] based on our earlier findings.[5] Peptides were selected according to the groups of amino acids representing the X-residue determined by Hackeng et al.[14] (i) G (ligation completed within 4 h), (ii) A, F (ligation completed within 9 h), (iii) N, Q, K (ligation completed within 24 h), and (iv) L (ligation completed within 48 h).

In addition to improve reaction rate and yield, simple and clean reaction conditions were also intended to be implemented in order to circumvent disadvantages of the conventional method. As a consequence, the application of toxic additives and increased reaction temperature were avoided in case of the IL.

To compare the results obtained for both media, the experiments of Hackeng et al.[14] were reproduced for peptide segments **1**–**7** with the same reaction conditions, yet room temperature (25 °C) instead of 37 °C was applied. We observed that the reaction with glycine at the ligation site (peptide **9**) was completed within 8 h reaction time (Fig. 1A, C), while most of the other NCL reactions were not finished even after 72 h as exemplified for peptide **11** (Fig. 1B and C, Table 1). The reaction rates and yields were found to be highly different for all peptides investigated (Table 1), yet revealed a similar classification concerning the C-terminal amino acid of the N-terminal fragment as earlier reported (i.e., G > F, A > N, Q, K > L[14]). In more detail, glycine at the ligation site

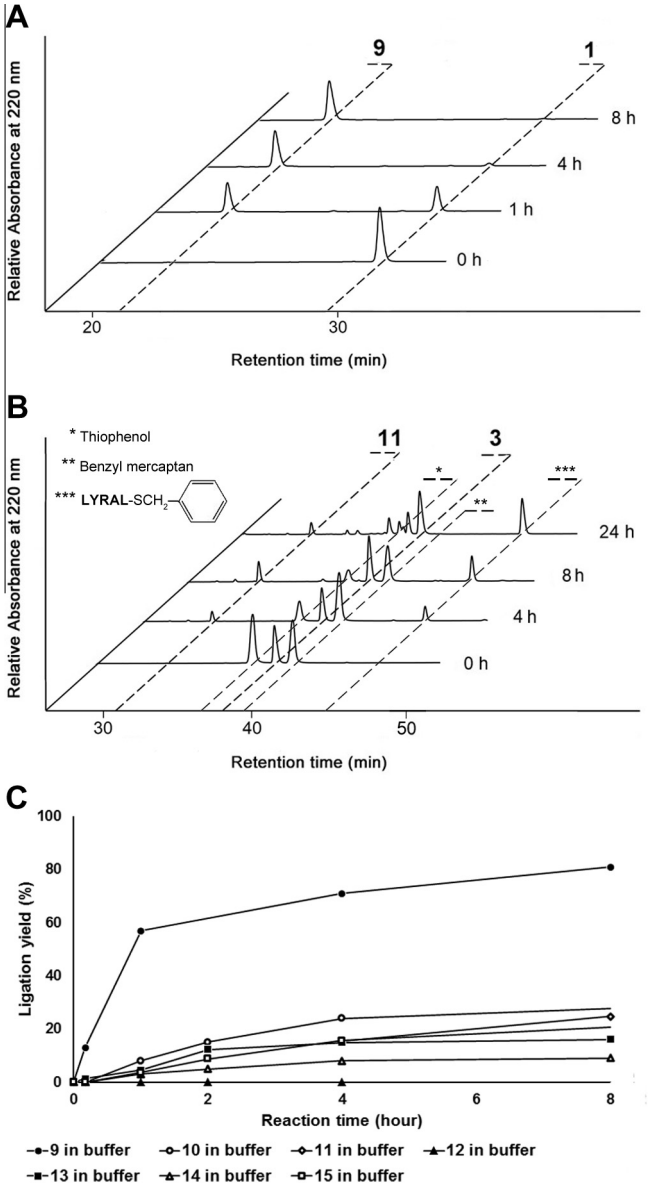


Figure 1. HPLC profiles of the ligation reactions obtained in buffer system[14] of (A) linking fragment **1** to **8** resulting in peptide **9** (LYRAGCRANK), and (B) fragment **3** to **8** resulting in peptide **11** (LYRALCRANK). (C) Plot of yields versus reaction time for the formation of peptides **9**–**15**.

facilitates NCL reaction at both temperatures (Table 1). Leucine was still one of the most hindered amino acids for the NCL reaction at room temperature which was comparable to reactions at 37 °C as described earlier (Table 1, Fig. 1B). However, in contrast to the earlier report,[14] the reaction yields of all peptides, especially peptides **10** (**LYRAACRANK**), **12** (**LYRAFCRANK**), and **16** (**LYRAQCRANK**) were significantly decreased when the reaction temperature was reduced to 25 °C. In general, a lower temperature obviously dramatically decreased the efficiencies of all ligation reactions performed in buffer (Table 1). Besides temperature, different yields compared to Hackeng et al. were detected due to analysis of individual reactions using HPLC monitoring. Also, we observed the accumulation of the rather stable benzyl mercaptane-peptide (peptides **3**–**7**) or thiophenol-peptide (peptide **6**) thiol-thioester-exchange-intermediate which only slowly or never formed the desired ligated product (Fig. S1, Table S2, Supplementary data; Scheme 2A). The following order of ligation yields was thus determined herein: G > K, A > L, N > Q > F (Table 1).

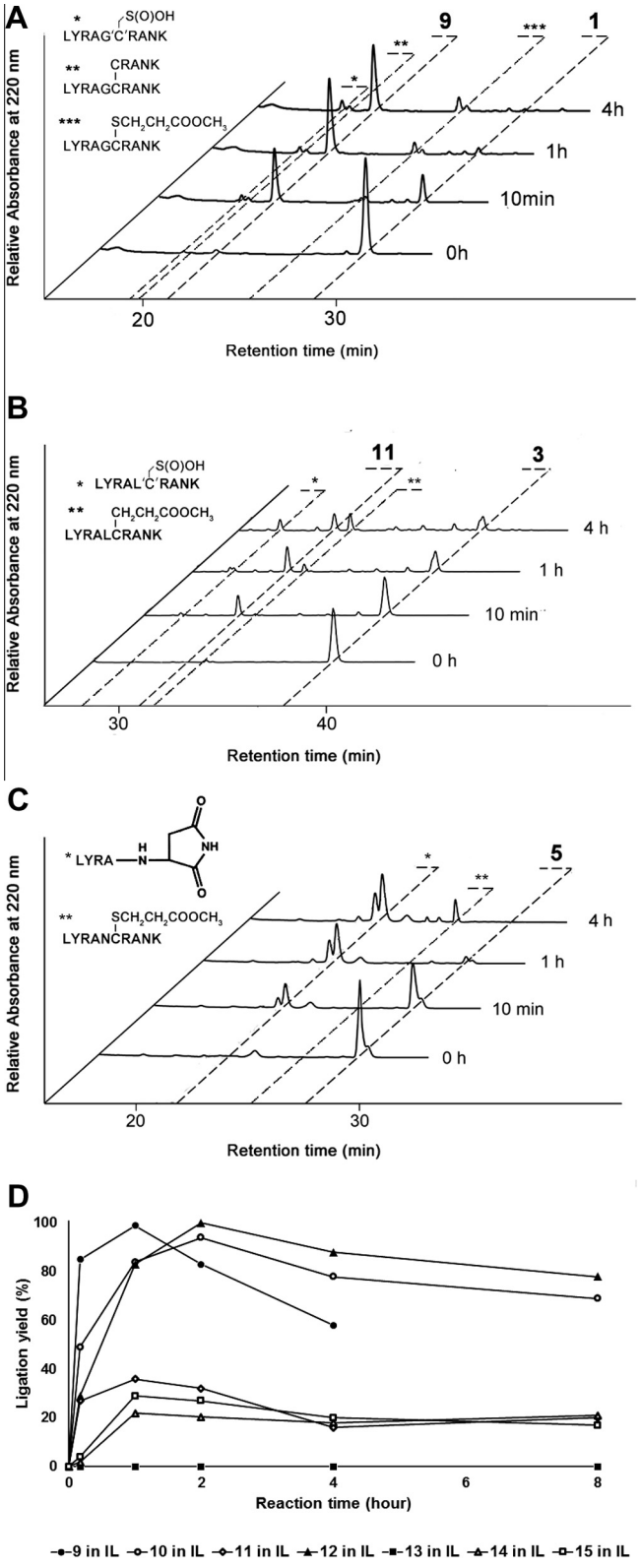
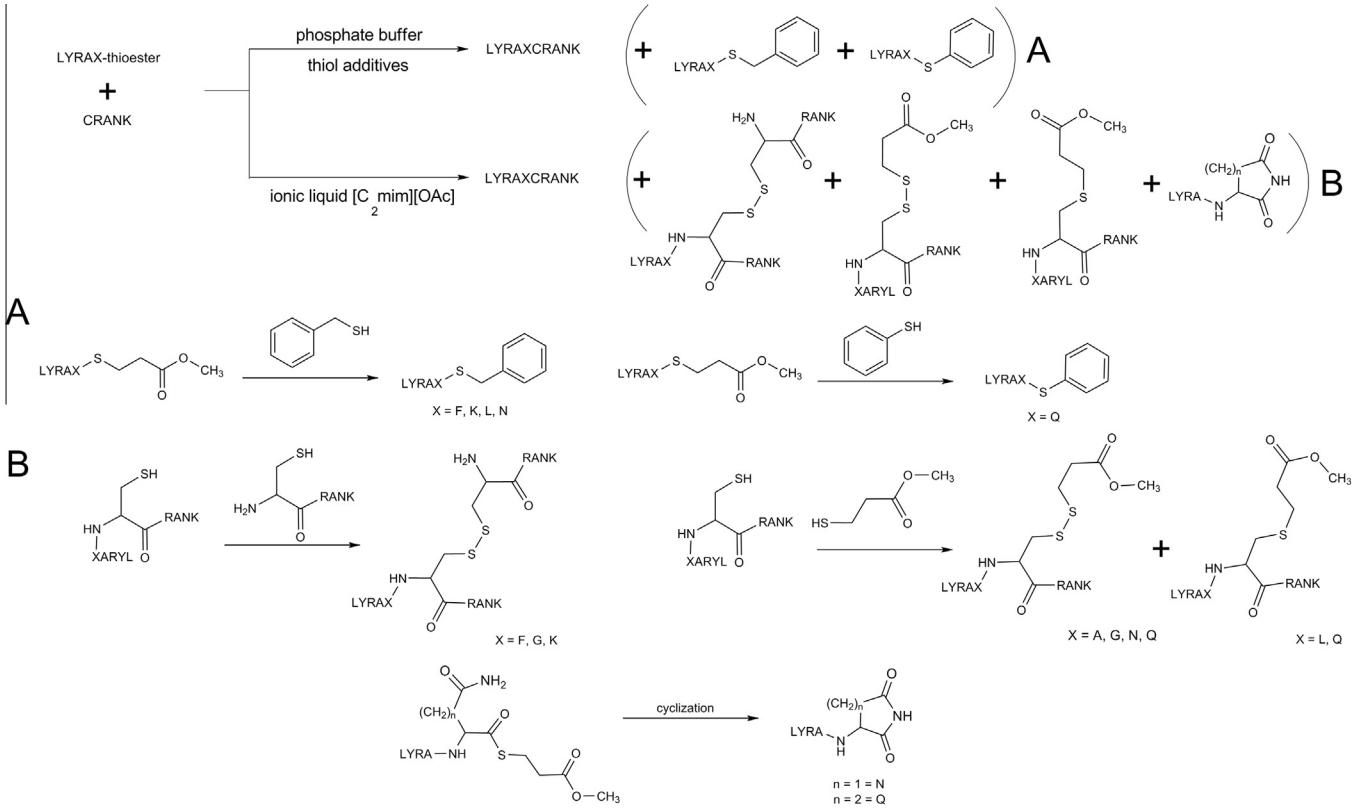


Figure 2. HPLC profiles of NCL reactions obtained in [C_2mim][OAc] for linking of (A) fragment **1** to **8** resulting in peptide **9**, (B) fragment **3** to **8** resulting in peptide **11**, and (C) fragment **5** to **8** resulting in peptide **13**. (D) Plot of yields versus reaction time for the formation of peptides **9**–**15**.

A further prerequisite for the present study was to observe the behavior of the ligation products in the ionic liquid during HPLC analysis, since shifts in retention times were observed in earlier



Scheme 2. Different side reactions occur during NCL depending on the reaction medium and the use of additives. While side-reactions for the LYRAX thioester were common for buffer- and thiol-additive-based NCL (A), only for 2 amino acids in IL-based NCL, a side product could be detected (B, lower reaction). In contrast, in [C_2mim][OAc] increasing concentrations of LYRAXCRANK led to formation of side products due to side reactions with excess CRANK (peptide **8**) and released methyl-3-mercaptopropionate at the cysteine side chain (B, upper reactions).

studies,[5,13] and were thus taken into account. One full-length standard peptide per group (see above[14]) was selected for this experiment (**LYRAXCRANK**, X = G (**9**), L (**11**), F (**12**), N (**13**)) (Text S1, Table S1, Fig. S2, Supplementary data).

Finally, to evaluate the effect of the ionic liquid on NCL outcome, [C_2mim][OAc] (3% (w/w) water) was applied as solvent for the individual ligation reactions at room temperature. As shown in Figure 2 and Table 1, almost all the reactions performed in IL reached their maximum yield faster than comparable NCL reactions in buffer. The reactions peaked within a maximum of 2 h even though no catalytic additives were used. The yields of the reactions within a reaction time of 1 h in IL at room temperature were comparable to Hackeng's NCL results at 37 °C applying thiol additives.[14] However, in [C_2mim][OAc] the C-terminal amino acid (X) of the product **LYRAXCRANK** was also found to exhibit a predominant influence on the reaction yields. In particular, in IL, some amino acids undergo ligation at a similar rate as glycine, which is the least hindered amino acid. The product yield of **9** already reached completion (98%) within 1 h instead of only 57% after 1 h in the aqueous medium (Fig. 2A). Compared to the results of Hackeng et al.[14] even the reaction with the sluggishly reacting residue leucine (peptide **11**) could be accomplished at a relative high yield (36%) in less than 2 h at 25 °C (Fig. 2B and D, Table 1), in contrast to 2 days at 37 °C or low yield (19%) at room temperature after 24 h (Table 1). Phenylalanine, which was the second most hindered amino acid at room temperature in our hands, also reacted at the same rate and yield as glycine in [C_2mim][OAc]. However, the yields of the products **LYRAXCRANK** in [C_2mim][OAc] were found to be gradually decreased after 1–2 h for peptides **9**–**13** and **15** and after 8 h for peptide **14** (Fig. 2D). A reason for this reduction of product yield was side reactions of compound **LYRAXCRANK** (in dependence of X) that started after a distinct time period following product formation (Fig. 2B, C, Scheme 2B). Thereby, higher concentrations of product led to the formation of different side products as depicted in Scheme 2B. Unfortunately, increasing amounts of these undesired side products were with time detected in HPLC elution profiles and confirmed by mass spectrometry (Figs. S3–S9, Table S2, Supplementary data). For several peptides, a side product was observed which linked **LYRAXCRANK** to the other excess reactant **CRANK** (peptides **9**, **12**, **15**) or methyl-3-mercaptopropionate (peptides **9**, **10**, **13**, **14**) by forming a disulfide bridge (Table S2, Supplementary data). In a few cases (peptide **11**, **14**), formation of a compound was detected whose mass represented a **LYRAXCRANK** molecule connected via a thioether bridge to methyl-3-mercaptopropionate. These problems may be overcome by applying reduced **LYRAX-thioester**: **CRANK** ratios. Therefore, we have performed two further experiments to prove this hypothesis. Two peptides were selected: (a) ligation of **3** to **8** (resulting in **11**) and (b) ligation of **4** to **8** (resulting in **12**). Peptide **12** was one of the candidates which formed the **LYRAXC**(**CRANK**)**RANK** side product (Scheme 2B, Table S2). In contrast, this side product did not occur for **11**, but we intended to observe the overall effect on quality of the product with a modified **LYRAX-thioester**: **CRANK** ratio (original 1:2) here as well. Incubation using ratios 1:1 and 2:1 revealed a higher purity and less side product formation for both peptides **11** and **12** (Fig. S10). This can obviously be assigned to the reduced amount of **CRANK** in the reaction.

Despite the side product formation, our experiments revealed that ligation of various peptides of the general sequence **LYRAX-thioester** to the segment **CRANK** can be performed much faster and more efficiently by using [C_2mim][OAc] compared to the

results of NCL reactions in buffer (Table 1). However, it is also remarkable that the reactions of **5** (X = N) to **8** and of **6** (X = Q) to **8** revealed rather low reaction yields (Table 1, Fig. 2C, Fig. S8, Supplementary data). With respect to HPLC-monitoring of the reaction of segment **5** to **8** (Fig. 2C), a large amount of side product has been formed instead of the desired product **13**. In addition, the continuous increase of a second peak next to the desired product peak of **6** to **8** was also observed. Since both amino acids (N, Q) at the C-terminus of **5** and **6** contained an amide group in their side chain, we suspect here the formation of a side product of cyclic imide which might be due to the deamidation side reaction that appeared in the IL only (Scheme 2B). This perfectly matches the obtained molar mass in the MS analysis of the corresponding side product peak (Fig. S7, Table S2, Supplementary data). This reaction has been described in several studies including, for example, the spontaneous chemical deamidation that occurs during degradation of proteins.[16,17] The cyclic imide compound results from the attack of the α-amino group of asparagine or glutamine on its own backbone carbonyl.[16] Recently, Desfougères et al. described the stable formation of the succinimide of asparagine under water-reduced conditions.[18] Thus, it is rather likely that our IL is a good nearly 'waterless' solvent for the formation and stabilization of succinimide derivatives. We thus suppose [C_2mim][OAc] to enhance these deamidation reactions of asparagine and glutamine in a peptide chain, and can therefore not recommend NCL in IL for fragments containing these two amino acids.

In conclusion, we have demonstrated that [C_2mim][OAc] acts as an excellent nontoxic reaction medium in a specific peptide ligation method. The ligation reactions of various model peptides can be performed much faster and more efficiently by using [C_2mim][OAc] instead of buffer solutions. However, the selectivity decreases over time, and thus, reactions should be monitored to obtain maximum yields. Nevertheless, [C_2mim][OAc] exhibits an interesting catalytic effect on the NCL reaction of peptide fragments, in addition to the fact that it represents a medium in which even hydrophobic peptides are soluble in high concentrations as required for NCL of larger oligopeptides and miniproteins.[5] In addition, additives can be completely omitted, and the reaction proceeds smoothly at a lower temperature. These facts may be favorable for preparation of an individual peptide/protein, since this approach is much more convenient than the original protocol. The results of the present study are, in our opinion, beneficial to future investigations on IL applications in peptide/protein solution phase chemistry and, in particular, on the mechanism of IL-supported peptide chemical reactions, such as native chemical ligation.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2014.04.102.

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