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Ascorbate as an Alternative to Thiol Additives in Native Chemical Ligation

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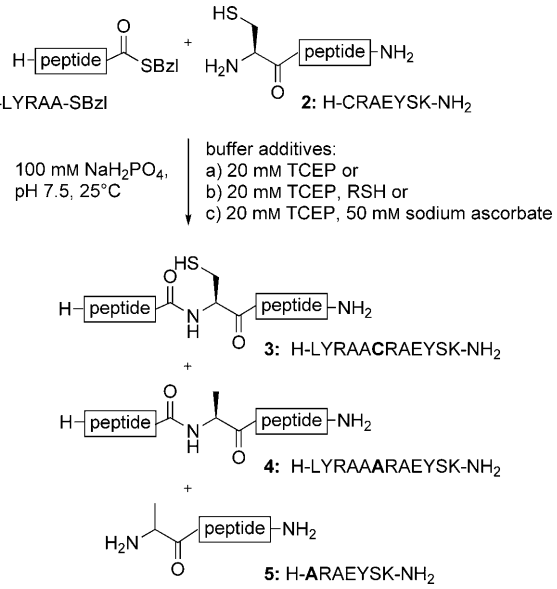
Among the segment coupling methods used for the chemical synthesis of proteins,[1] native chemical ligation is one of the most powerful and most widely applied reactions.[2] This method provided a major boost to chemical protein sciences, as can be inferred from the increasing amount of publications in which native chemical ligation is employed to construct biologically active proteins. The approach involves a peptide thioester such as **1** (Scheme 1) and an N-terminal cysteinyl peptide



Scheme 1. Synthesis of the peptide H-LYRAACRAEYSK-NH_2 (**3**) by native chemical ligation. The ligation reactions were performed in different buffer systems (conditions a–c) with **1** (2 mM) and **2** (4 mM). Desulfurization of the cysteinyl-peptide **2** and the ligation product **3** to afford the alanyl-peptides **4** and **5**, respectively, was observed under conditions a and conditions b (RSH = BnSH or $NaO_3SCH_2CH_2SH$), but not with PhSH.

such as **2**, which react in aqueous solutions at neutral pH to furnish a native peptide bond as in **3**.[3] Native chemical ligation proceeds through a reversible thiol exchange reaction, which eventually involves the side chain of the N-terminal cysteine in **2**. The formed thioester-linked ligation intermediate undergoes a spontaneous *S*→*N* acyl shift and provides the thermodynamically favoured amide bond at the ligation site in **3**.

The aqueous buffer systems used in native chemical ligation reactions include a number of additives. Typically, triscarboxyethylenephosphine (TCEP) is added to prevent the formation of disulfides. Thiol additives such as 2-mercaptoethanesulfonate sodium salt (MESNa), benzyl mercaptan, thiophenol or (4-mercaptophenyl)acetic acid are commonly included to help maintain reducing environments and to increase the reactivities of peptide thioesters.[4] Alternatively, native chemical ligations have been performed by using combinations of benzyl mercaptan/thiophenols, in which case there is no need for TCEP as a reducing agent.[5] Despite their widespread use, however, thiols are not among the most appreciated reagents. Some thiols are characterized by unpleasant odours and toxicities. Moreover, the thiol additives can co-elute with products in HPLC analysis and form mixed disulfides, which can complicate the analysis of native chemical ligation at long reaction times. In addition, thiol-induced side reactions such as addition to multiple carbon-carbon bonds have been reported.[6] The unwanted effects can be avoided when the native chemical ligation is performed with TCEP as sole additive.[7]

In this communication we show that the use of TCEP as sole additive can, however, lead to desulfurization both of starting cysteinyl peptides and of formed ligation products. Our findings suggest that the commonly used thiols such as thiophenols act not only as reducing and thiol exchange agents but also as radical scavengers to prevent the TCEP-induced desulfurization. We describe a native chemical ligation format that involves the use of TCEP and yet proceeds remarkably well without the involvement of thiol additives. This reaction is based on the use of the nontoxic, highly water-soluble radical scavenger ascorbate, which is available at very low cost.

In a study involving investigation of the reactivities of various peptide thioesters, we observed a side reaction. We performed the native chemical ligation of the peptide thioester **1** with the cysteinyl peptide **2** in the presence of TCEP without use of thiol additives (Scheme 1 a). Two unexpected peaks appeared in the HPLC trace together with the ligation product **3** (Figure 1 A). MS analysis suggested the formation of the alanine-containing peptides **4** and **5**. HPLC-MS analysis of authentic reference compounds (Figures S3 and S4 in the Supporting Information) confirmed this finding.

We assumed that the desulfurization of the cysteine residues would have been induced by TCEP. This reaction has precedence: it has been shown that phosphites and phosphines trigger the radical-based desulfurization of alkyl mercaptans, a reaction that proceeds slowly at ambient temperature but can be driven to completion at elevated temperatures.[8–11] This side reaction has also been observed in the TCEP-induced cleavage of *S*–*St*Bu-protected cysteines.[12] Very recently the occurrence of TCEP-induced desulfurization during sample preparation for protein characterization by mass spectrometry was proposed.[13]

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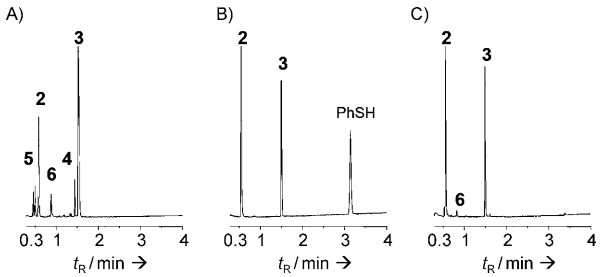

Figure 1. UPLC traces for the ligation of the peptide thioester **1** with the peptide **2** performed in A) TCEP, B) TCEP/PhSH, and C) TCEP/ascorbate buffers. Conditions: **1** (2 mM), **2** (4 mM), NaH_2PO_4 (100 mM), TCEP (20 mM), pH 7.5, 25 °C, PhSH (3 vol% when added) or sodium ascorbate (50 mM when added). Compound **6**: disulfide (-CRAEYSK)$_2$. TCEP = triscarboxyethylenephosphine.

We examined the reaction between **1** and **2** in commonly used ligation buffers containing TCEP and thiol additives. No desulfurization products were detectable when thiophenol was added (Figure 1 B). Benzyl mercaptan and MESNa were found to be less effective than thiophenol in preventing desulfurization (Table 1). Careful GC-MS and NMR analysis revealed that thiophenol did not serve as surrogate of cysteine desulfurization, because no formation of benzene could be detected. Rather, the mercaptan probably acted as a radical scavenger. Thiophenol readily forms a stabilized benzenethiyl radical (S–H bond dissociation energies: thiophenol 322 kJ mol^{-1}, cysteine 367 kJ mol^{-1}).[14] It has been shown that thiophenol inhibits the phosphite-induced radical desulfurization of mercaptoalkanes.[9]

The TCEP-induced desulfurization was found to be pH-dependent and occurred with lower rates when performed at slightly acidic pH 6.5 (Table 1). However, the native chemical ligation is slower under these conditions (pH 7.5: 97%; pH 6.5: 81% after 2 h), which can limit the achievable yields in the native chemical ligation of more challenging peptides.

We considered the possibility of using other, odourless radical scavengers and examined sodium ascorbate as a water-soluble, nontoxic and inexpensive alternative to thiols. We were pleased to find that the reaction between the model peptide thioester **1** and the model cysteinyl peptide H-CRAEYSK-NH_2 **2** occurred without any desulfurization when ascorbate (50 mM) was included (Figure 1 C). The yield of ligation product obtained after HPLC purification and the purity of the crude product were comparable to those obtained in the "conventional approach" based on addition of thiophenol (51 or 55%, respectively).

We next turned our attention to a difficult ligation reaction. The Pro-Cys bond is the most challenging ligation site. Here, the problem of desulfurization is particular pressing because ligation can proceed at a slower rate than desulfurization. The reaction between the peptide proline thioester **7** and the cysteinyl peptide **2** was sluggish (Figure S12 in the Supporting Information). In the absence of radical scavengers, only a 7% yield of the ligation product **12** was obtained after 30 h reaction time (Table 1). More than 60% of the cysteinyl peptide **2** was consumed in the desulfurization. The addition of benzyl mercaptan reduced the amount of desulfurization to 21%, with the reaction product **12** being formed in 11% yield. A similar ligation yield was obtained when ascorbate was used as additive. This radical scavenger completely inhibited desulfurization, reducing the spectrum of products detected by HPLC analysis (Figure S12 in the Supporting Information).

To evaluate the usefulness of ascorbate as a thiol replacement in native chemical ligation further, we studied the ligation reactions between the longer peptides **8** and **9** and be-

Table 1. Yields of native chemical ligation and amount of desulfurization of ligation products and unconsumed cysteinyl peptides.

Ligation conditions[a]	Yields[b,c] [reaction time]
LYRAA**C**RAEYS (**3**)	
LB A, pH 7.5	**3** (97%), **4** (3%), **5** (7%) [2 h]
LB A, pH 7.5, PhSH (3 *v*/%)	**3** (quant., 55% isol.),[d] no desulfurization [2 h]
LB A, pH 7.5, BnSH (3 *v*/%)	**3** (96%), **4** (4%), **5** (9%) [2 h]
LB A, pH 7.5, MESNa (50 mM)	**3** (98%), **4** (2%), **5** (13%) [2 h]
LB A, pH 6.5	**3** (81%), **4** (1%), **5** (2%) [2 h]
LB A, pH 7.5, ascorbate (50 mM)	**3** (quant., 51% isol.),[d] no desulfurization [2 h]
LYRAP**C**RAEYS (**12**)	
LB B, pH 7.5	**12** (7%), **5** (61%), desulfurized **12** (2%) [30 h]
LB B, pH 7.5, BnSH (3 *v*/%)	**12** (11%), **5** (21%), desulfurized **12** (1%) [30 h]
LB B, pH 7.5, PhSH (3 *v*/%)	**12** (16%), no desulfurization [30 h]
LB B, pH 7.5, ascorbate (50 mM)	**12** (11%), no desulfurization [30 h]
LNELDADEQADL**C**ESLHDHADELYRSCLARFGDDGENL (**13**)	
LB B, pH 7.5	**13** (17%), desulfurization[e] [30 h]
LB B, pH 7.5, BnSH (3 vol%)	**13** (35%), desulfurization[e] [30 h]
LB B, pH 7.5, PhSH (3 vol%)	**13** (74%) [30 h]
LB B, pH 7.5, MESNa (50 mM)	**13** (58%), desulfurization[e] [30 h]
LB B, pH 7.5, ascorbate (50 mM)	**13** (51%) [30 h]
LB B, pH 7.5, ascorbate (50 mM) + PhSH (3 vol%)	84% **13** [30 h]
DVPLPAGWEMAKTS**C**GQRYFLNHIDQTTTWQDPRKAML (**14**)	
LB B, pH 7.5	**14** (37%, 26% isol.),[d] desulfurization[e] [24 h]
LB B, pH 7.5, ascorbate (50 mM)	**14** (86%, 30% isol.)[d] [24 h]
LB B, pH 7.5, PhSH (3 vol%)	**14** (91%, 40% isol.)[d] [24 h]
LB B, pH 7.5, MESNa (50 mM)	**14** (65%, 35% isol.),[d] desulfurized **11**[e] [24 h]

[a] Ligation buffer A: NaH_2PO_4 (100 mM), TCEP (20 mM). Ligation buffer B: NaH_2PO_4 (100 mM), Gn·HCl (6 M), TCEP (20 mM). [b] The peptide **12** was synthesized from the thioester **7** and the cysteinyl peptide **2**, the peptide **13** was synthesized from the thioester **8** and the cysteinyl peptide **9** and the peptide **14** was synthesized from the thioester **10** and the cysteinyl peptide **11**. [c] The ligation yield was determined by integration of the peak areas. [d] Yield obtained after HPLC purification. [e] Observed by ESI mass spectrometry.

1: H-Leu-Tyr-Arg-Ala-Ala-SBzl

2: H-Cys-Arg-Ala-Glu-Tyr-Ser-Lys-NH_2
7: H-Leu-Tyr-Arg-Ala-Pro-SBzl

8: H-Leu-Asn-Glu-Leu-Asp-Ala-Asp-Glu-Gln-Ala-Asp-Leu-SBzl
9: H-Cys-Glu-Ser-Leu-His-Asp-His-Ala-Asp-Glu-Leu-Tyr-Arg-Ser-Cys-Leu-Ala-Arg-Phe-Gly-Asp-Asp-Gly-Glu-Asn-Leu-NH_2

10: H-Asp-Val-Pro-Leu-Pro-Ala-Gly-Trp-Glu-Met-Ala-Lys-Thr-Ser-SBzl
11: H-Cys-Gly-Gln-Arg-Tyr-Phe-Leu-Asn-His-Ile-Asp-Gln-Thr-Thr-Thr-Trp-Gln-Asp-Pro-Arg-Lys-Ala-Met-Leu-NH_2

tween **10** and **11** in the syntheses of the C-terminal segment (26–63) of the ColE1-Rop protein (**13**) and the 38-amino-acid-long WW domain (**14**) of human Yes-associated protein (hYAP), respectively. The ligation buffer included guanidinium hydrochloride (Gn·HCl, 6 M) for denaturation and TCEP as reducing agent. The reaction between the leucine thioester in **8**[15,16] and the 26-residue cysteinyl peptide **9** proceeded smoothly when ascorbate was included in the buffer (Figure 2A). After 30 h reaction time the ligation product **13** (Table 1) was obtained in 51% yield based on HPLC analysis. Similar yields were obtained when MESNa was included as thiol additive.

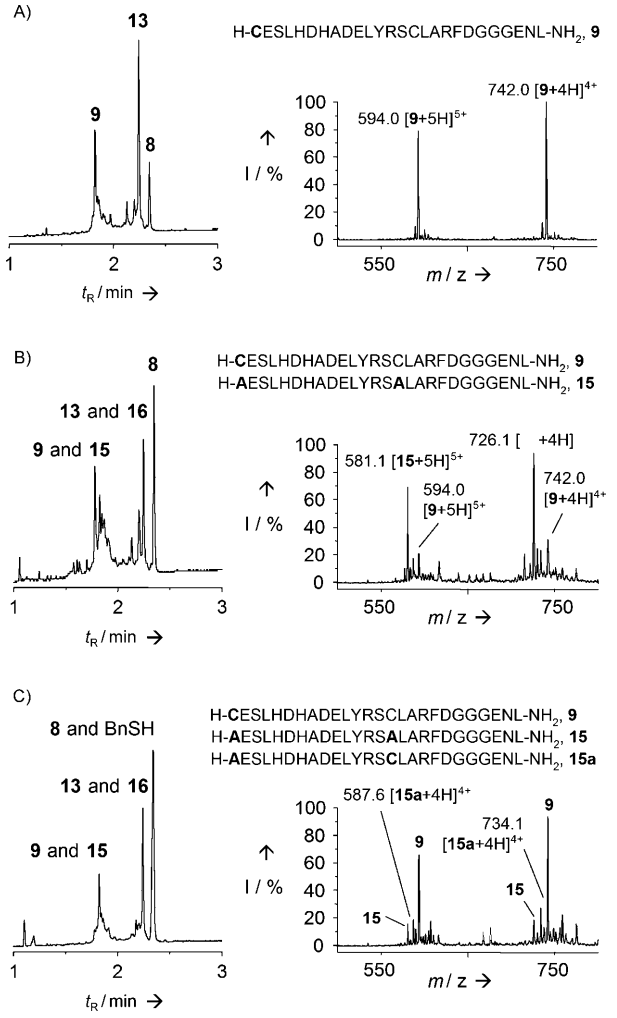

Figure 2. UPLC traces for the ligation between the peptide thioester **8** and the peptide **9** performed in A) TCEP/ascorbate, B) TCEP, or C) TCEP/BnSH buffers. Left: UPLC traces after 30 h. Right: ESI-MS of the cysteinyl peptide after 30 h. Conditions: peptides (2 mM concentrations), TCEP (20 mM), Gn·HCl (6 M), NaH_2PO_4 (100 mM), pH 7.5, 25 °C, 30 h and A) sodium ascorbate (50 mM), B) only TCEP (20 mM), and C) BnSH (3 vol%). Peptide **9**: **C**ESLHDHADELYRSCLARFGDDGENL. Peptide **15**: **A**ESLHDHADELYRS**A**LARFGDDGENL. Peptide **15a**: **A**ESLHDHADELYRS**C**LARFGDDGENL. Peptide **16**: LNELDADEQADL**A**ESLHDHADELYRS**A**LARFGDDGENL.

In the absence of ascorbate ligation was slow (17% yield, Table 1). HPLC-MS analysis revealed that the desulfurization products **15** and **16** were formed (Figures 2B and S13 in the Supporting Information). It is worth noting that the desulfurization was difficult to detect by HPLC analysis alone because the desulfurization products had elution properties similar to those of the cysteine-containing peptides before desulfurization. The use of benzyl mercaptan led to improved ligation yields, but not to the extents observed with ascorbate or MESNa. Again, MS analysis revealed that benzyl mercaptan failed in preventing desulfurization (Figure 2C). As was observed previously, the use of thiophenol as additive (74% ligation yield) proved superior to the use of benzyl mercaptan and also completely prevented desulfurization (Figure S13 in the Supporting Information).[17] Interestingly, the combination of thiophenol with ascorbate conferred a further improvement to 84% yield. This might point to a potential activating effect of ascorbate or simply indicate that the addition of ascorbate can increase the concentration of thiophenol available for the native chemical reaction.

We then investigated native chemical ligation reactions in the synthesis of the 38-amino-acid-long WW domain **14** (Figure 3A). The reaction between the peptide thioester **10**[16] and the cysteinyl-peptide **11** was performed in the absence of thiol additives and ascorbate. HPLC analysis showed that under these conditions only a 37% yield of the ligation product **14** was formed after 24 h. The mass spectrometric characterization showed that substantial amounts of the ligation product **14** and the unconsumed cysteinyl peptide **11** were desulfurized to form the alanine-containing peptides **17** and **18**, respectively. In the presence of sodium ascorbate (50 mM) neither desulfurization of the cysteinyl peptide **11** to **17** nor desulfurization of the ligation product **14** occurred and the ligation succeeded in 86% yield based on HPLC analysis (Figure 3B, Table 1). The WW domain **14** was isolated in 30% yield after HPLC purification. A similar yield (91%) was obtained when thiophenol was included in a "conventional" ligation buffer (Table 1, Figure S15 in the Supporting Information). Again, desulfurization was inhibited. The MESNa additive also provided useful product yields (65%), although MS analysis revealed a minor degree of desulfurization of the cysteinyl peptide.

In previous studies we had noticed that the thiol reagents commonly used in native chemical ligation chemistry can trigger undesired side reactions.[6] For a project concerned with the controlled spatial display of peptides on DNA architectures[18] we prepared various peptide-oligonucleotide conjugates by making use of native chemical ligation of peptide thioesters such as **1** with internally cysteine-modified oligonucleotides such as **20** (Figure 4A). The aqueous ligation buffer included MESNa. However, HPLC analysis showed a broad product peak and MALDI-mass spectrometry revealed the occurrence of a mercaptoethanesulfonic acid (MESA) adduct (Figure 4B), which remained stable even after repeated treatment with TCEP. The side reaction was prevented when light was excluded.[6] It was concluded that the thiol adduct was formed upon free radical addition to the triple bond of the aminopropagyl linkage in **20**. We performed the reaction between **1** and **20** in the presence of light but included ascorbate as radical scavenger. We found that the reaction proceeded smoothly (Figure 4C). Neither desulfurization nor formation of adducts

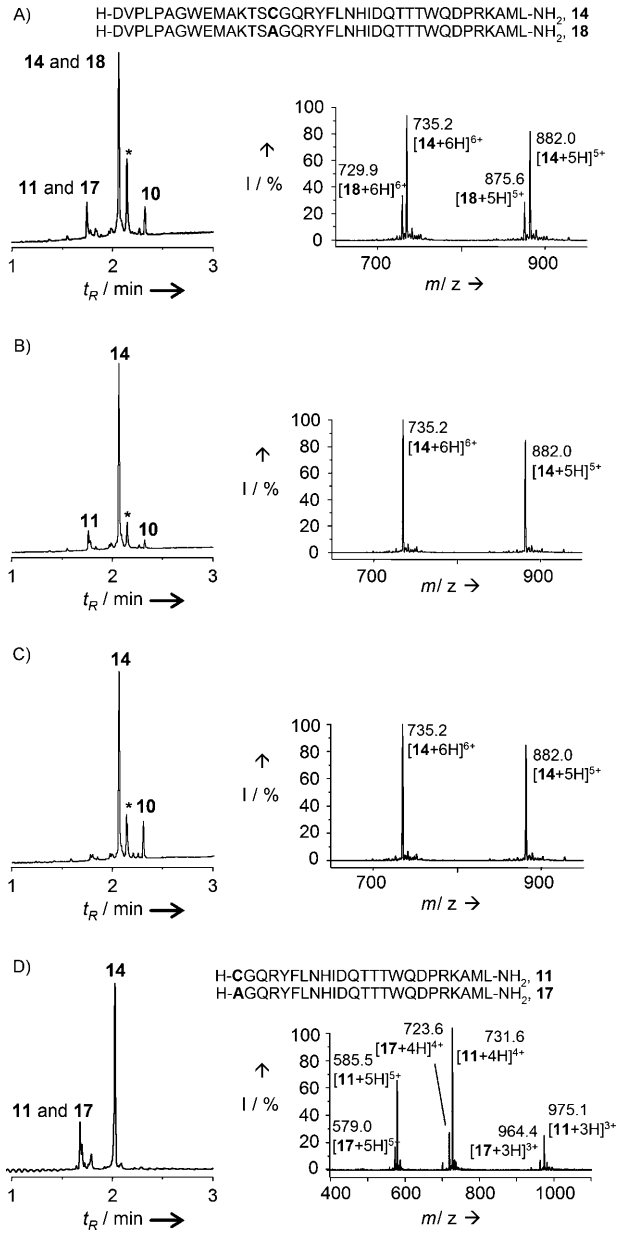

Figure 3. Synthesis of the WW domain of human Yes-associated protein (hYAP) by native chemical ligation between the peptide thioester **10** and the peptide **11** in TCEP-containing buffer A) in the absence or B) in the presence of sodium ascorbate (50 mM) and in the presence either of C) PhSH (3 vol%) or of D) MESNa (50 mM). Left: UPLC traces after 24 h. Right: ESI-MS of: A), B) and C) the ligation product, or D) the starting material. Conditions: peptides (2 mM concentrations), TCEP (20 mM), Gn·HCl (6 M), NaH_2PO_4 (100 mM), pH 7.5, 25 °C (* thioesterification of the internal cysteine).

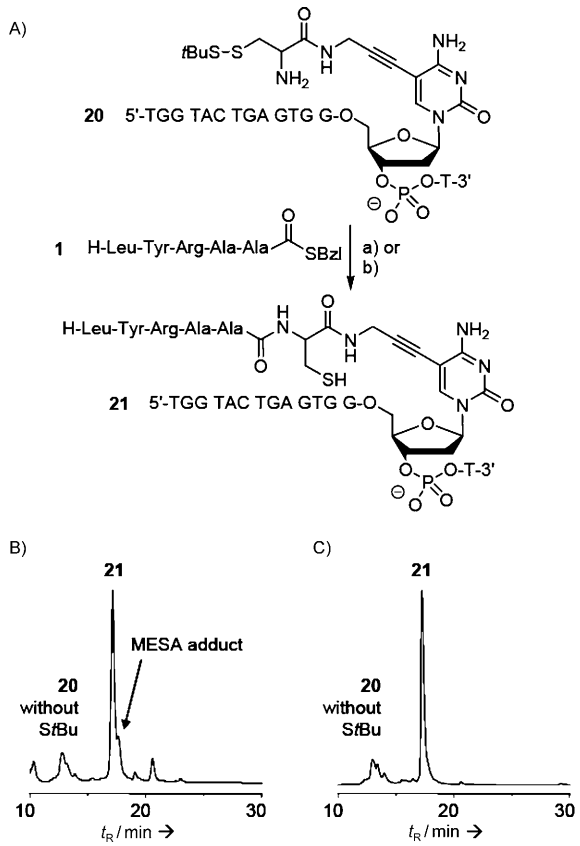

Figure 4. A) Synthesis of the peptide-DNA conjugate **21** by native chemical ligation. Conditions: DNA and peptide (1 mM concentrations), TRIS·HCl (20 mM), TCEP (20 mM), NaCl (200 mM), pH 8.0 and a) MESNa (1%), 62% or b) sodium ascorbate (50 mM), 54%. HPLC analyses of reactions performed in B) TCEP/MESNa or C) TCEP/ascorbate buffer.

were detected. The desired product **21** was isolated after HPLC purification in a yield of 54%. This result demonstrates the versatility of the TCEP/ascorbate-promoted native chemical ligation, which can extend beyond the scope of peptide-peptide conjugation.

Native chemical ligation is abundantly used in protein chemistry. Typically, the ligation reactions are performed in aqueous denaturating buffer systems containing TCEP as well as thiol additives. Our results suggest that TCEP can induce radical desulfurization of the cysteinyl peptide and the ligation product. This reaction was readily detected by HPLC analysis when reactions involved small peptide fragments. However, we noticed that careful MS analysis is required to detect desulfurization of longer peptides.

In the majority of the published reactions, arylthiols such as thiophenol or 4-mercaptophenylacetic acid (MPAA) are included. In fact, these thiols are amongst the most efficient additives used for native chemical ligation. Our findings suggest that thiophenol not only accelerates the ligation but also serves the purpose of preventing TCEP-mediated radical desulfurization both of starting materials and of ligation products. The latter role has not been reported previously. It is plausible to assume that MPAA also forms a stable thiyl radical and so is able to act as radical scavenger. In our hands, the commonly used benzyl mercaptan and MESNa also increased the ligation rates but provided only a partial remedy to the desulfurization problem.

We have demonstrated that sodium ascorbate can substitute for the thiol additives used in native chemical ligation chemistry and shown that sodium ascorbate completely inhibits TCEP-mediated desulfurization. It is worth noting that reactions that were performed in TCEP/ascorbate-containing buffers furnished similar rates as reactions in the "conventional"

TCEP/thiophenol-containing buffer. Furthermore, thiol-free native chemical ligation might open new conjugation opportunities as exemplified by the synthesis of propargyl-linked peptide-DNA conjugates.[18] Sodium ascorbate is inexpensive, odourless, nontoxic and highly water-soluble. We propose sodium ascorbate as a useful alternative to thiol additives in native chemical ligation reactions.

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Keywords: desulfurization · DNA · ligation · peptides · radical scavengers

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