A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

CHEMBIOCHEM

SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

Title: Native Chemical Ligation Directed by Photocleavable Peptide Nucleic Acid (PNA) Templates

Authors: Stephen Middel, Cornelia Panse, Swantje Nawratil, and Ulf Diederichsen

To be cited as: *ChemBioChem* 10.1002/cbic.201700487

Link to VoR: http://dx.doi.org/10.1002/cbic.201700487

A Journal of

ChemPubSoc Europe

WILEY-VCH

www.chembiochem.org

10.1002/cbic.201700487

WILEY-VCH

COMMUNICATION

Native Chemical Ligation Directed by Photocleavable Peptide Nucleic Acid (PNA) Templates**

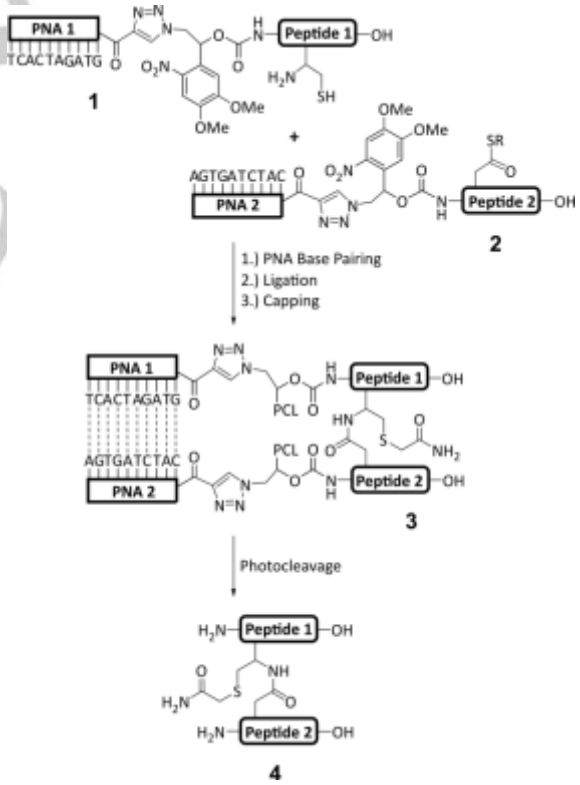
Stephen Middel, Cornelia H. Panse, Swantje Nawratil, and Ulf Diederichsen*

Abstract: A novel peptide-peptide ligation strategy is introduced that has the potential to provide peptide libraries of linearly or branched coupled fragments and will be suited to introduce simultaneous protein modifications at different ligation sites. Ligation is assisted by templating peptide nucleic acid (PNA) strands, and therefore, ligation specificity is solely encoded by the PNA sequence. PNA templating in general allows for various kind of covalent ligation reactions. As a prove of principle, a native chemical ligation strategy was elaborated. This PNA templated ligation includes easy on-resin procedures to couple linker and PNA to the respective peptides as well as a traceless photocleavage of the linker/PNA oligomer after the ligation step. A 4,5-dimethoxy-2-nitrobenzaldehyde based linker that allows the photocleavable linkage of two biooligomers was developed.

Nature uses biomacromolecules as templates to facilitate efficient and selective product formation out of countless different reactants in low concentrations. These templates recognize reactants, bind them non-covalently, bring their reactive centers in close proximity, and therefore, increase the effective concentration of the reaction partners.[1] Chemists adopted this concept, and first template-directed ligation approaches were already developed in 1966 by Naylor and Gilham.[2] Most ligation approaches developed since focus on ligation of two complementary lagging strand fragments or ligation of the leading and the lagging strand.[3] This concept was expanded to template-mediated transfer reactions[4] also allowing selective protein modifications.[5] Mainly nucleic acid derived templates have been used, but also peptidic templates are established like the self-replication on amphiphilic β-sheet peptides,[6] protein templated peptide ligations[1], and coiled-coil motifs as guiding units.[7] The potential of template-directed reactions was demonstrated when applied to *in vivo* modifications of proteins.[8]

The advantage of nucleic acid derived templates is the sequence dependent recognition of reaction partners allowing for multiple orthogonal ligation events. Peptide nucleic acids (PNA) are well established as DNA surrogates with higher contribution of base pair recognition to duplex stabilities and lower mismatch tolerance compared to DNA and RNA.[9] The reduced solubility of PNA in water can be compensated with terminal lysine or arginine residues. We envisioned a PNA based template-directed peptide-peptide ligation strategy that includes photocleavage of the PNA helper strands following the ligation reaction. In addition, this concept gives access to difficult to synthesize branched peptides, which have therapeutical potential as multivalent binders.[10]

The general concept introduced herein is based on biooligomer hybrids of the respective peptide to be ligated with corresponding PNA recognition sequence. The PNA and peptide oligomers are joined by a photocleavable linker providing hybrid oligomers like **1** and **2** (Scheme 1). Hybrids with complementary PNA sequences assemble due to PNA base pairing bringing the ligation sites of peptides **1** and **2** in proximity, thereby, facilitating the ligation reaction. For covalent linkage of the peptide fragments a native chemical ligation (NCL) approach is used creating a native amide bond.[11] Finally, the PNA recognition units can be cleaved by irradiation of ligation product **3** yielding peptide **4**.



Scheme 1. Concept of the template-directed ligation strategy applying photocleavable PNA strands. Two PNA/peptide hybrids with complementary PNA strands recognize each other by PNA base pairing. The reactive centers of the peptides are brought in close proximity and the ligation takes place. After the ligation, the PNA strands can be cleaved by irradiation. PCL = photocleavable linker.

[*] Dr. S. Middel, Dr. C. H. Panse, S. Nawratil, Prof. Dr. U. Diederichsen
Georg-August-Universität Göttingen
Institut für Organische und Biomolekulare Chemie
Tammannstraße 2, 37077 Göttingen (Germany)
E-mail: udieder@gwdg.de

[**] This work was supported by a priority program (SPP1623) of the German Research Foundation (DFG).

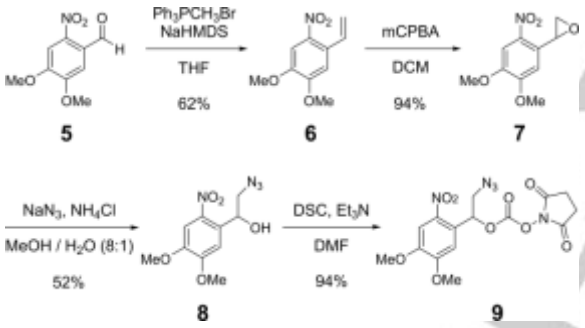
Supporting information for this article is given via a link at the end of the document.

10.1002/cbic.201700487

COMMUNICATION

The design of the photocleavable linker was derived from 1-(2-nitrophenyl)propargyl alcohol used as photolytically cleavable linker of two peptide fragments.[12] Nitrobenzene derivatives are appropriate linkers based on their photochemical properties[13] and use as peptide caging groups.[12, 14] In order to avoid interference of deprotection with the nucleobase absorption the wavelength for uncaging was shifted to 347 nm applying the 3,4-methoxy nitrobenzene derivative.[15] The respective peptide was bound to the *N,N'*-disuccinimidyl carbonate (DSC) activated linker followed by attachment of the PNA oligomer by copper-catalyzed [2+3] cycloaddition between an azide in the linker and an alkyne modified PNA strand. This linker unit allows efficient *N*-terminal on-resin functionalization of the peptide and on-resin click-chemistry to attach the peptide with linker to the resin-bound PNA oligomer.

The photocleavable linker was synthesized starting from 4,5-dimethoxy-2-nitrobenzaldehyde (**5**, Scheme 2). Wittig reaction provided the corresponding alkene **6** which was converted into epoxide **7** by treatment with *meta*-chloroperoxybenzoic acid (mCPBA). Nucleophilic ring-opening provided azide **8** in a 2:1 mixture with the non-desired regioisomer. Finally, the alcohol was activated with DSC to give the photocleavable linker **9**.

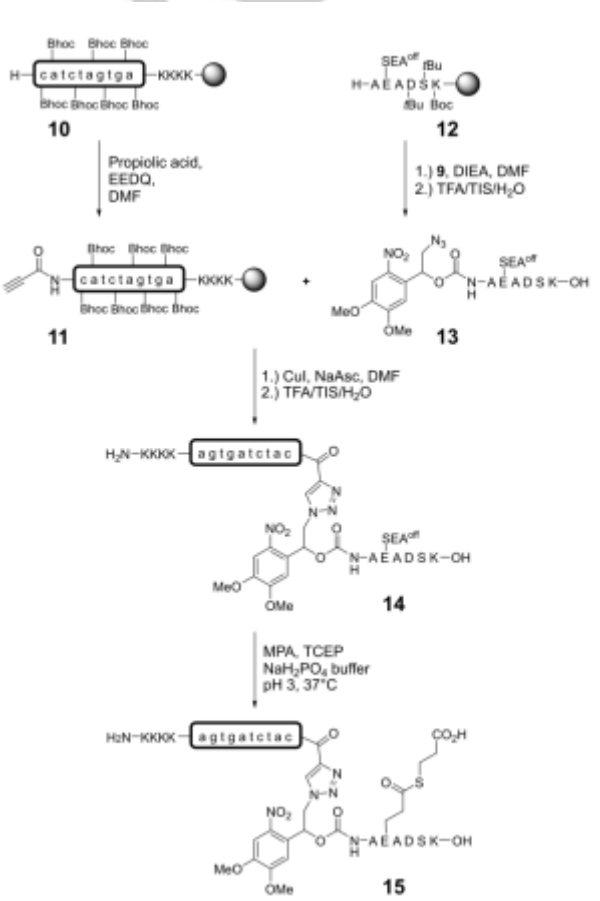


Scheme 2. Four-step synthesis of the photocleavable linker **9**. NaHMDS = sodium bis(trimethylsilyl)amide.

The respective peptide was prepared by solid phase peptide synthesis (SPPS) on a Wang resin and as final coupling step the activated linker **9** was attached to the *N*-terminus of peptide **12** followed by cleavage from solid support (Scheme 3). The linker was efficiently attached and not affected by the conditions applied for cleavage from the resin. Also, PNA **10** was obtained by SPPS on a rink amide MBHA resin and was *N*-terminally functionalized with propiolic acid applying only a small excess of activator *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ) in DMF. Full conversion to PNA **11** was obtained using base-free coupling conditions in order to avoid side-reactions upon deprotonation of the alkyne. The peptide-linker construct was attached to the resin bound alkyne functionalized PNA strand **11** by copper-catalyzed azide-alkyne cycloaddition. The resulting triazole **14** was obtained in high purity as indicated by HPLC and MS analysis.

For covalent NCL a thioester and a cysteine are required. As thioesters are labile under basic conditions applied for Fmoc deprotection during SPPS, a thioester precursor was incorporated into the peptide using Melnyk's bis(2-sulfanylethyl)amido (SEA) group introduced to the glutamic acid side chain.[16] After synthesis of hybrid **14**, the SEA group was transformed into its corresponding 3-mercaptopropionic acid (MPA) thioester **15** in order to obtain reasonable reaction rates during ligation.

The preparation of the second hybrid **16** with the cysteine containing peptide and complementary PNA sequence followed the same strategy as for hybrid **14**. The cysteine was introduced coupled to the side chain of 2,3-diamino propionic acid (Dap) being the second last *N*-terminal amino acid of the peptide sequence. The availability of thiol and amino functions for the ligation allows the irreversible *S,N*-shift reaction and provides a stable peptide bond. Dap was used instead of lysine because less background reaction was expected when the reactive center is closer to the backbone.

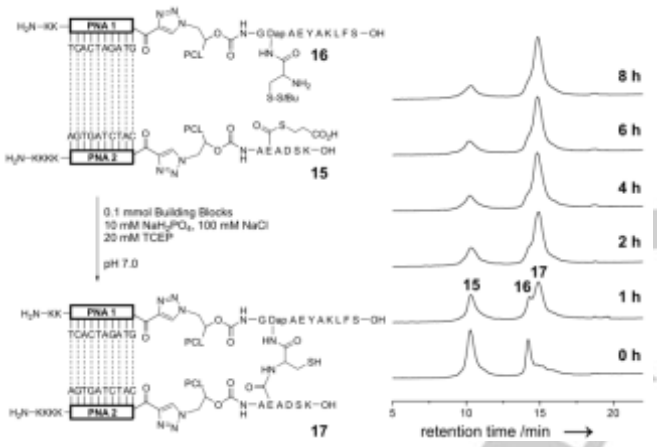


Scheme 3. Convergent strategy for the peptide/PNA hybrid synthesis. Modification of the PNA strand (**11**), attachment of the caging group (**13**) and subsequent PNA/peptide synthesis by CuAAC (**14**) are equal for all hybrids. For thioester hybrids an additional transthioesterification step is necessary.

Since peptide-peptide ligation should be facilitated by recognition of complementary PNA sequences, the PNA duplex needed to be formed at room temperature and the ligation being applicable in aqueous buffer at neutral pH. The PNA sequences were taken from literature[17] and it was ensured that the melting temperature of 46 °C remains also for the PNA/peptide hybrids

10.1002/cbic.201700487

(Supporting Information). The native chemical ligation approaches were performed in degassed NaH_2PO_4 buffer (pH 7.0) with tris(2-carboxyethyl)phosphine (TCEP) as a reducing agent at room temperature. The cysteine hybrid **16** was allowed to equilibrate for 30 min to remove the S*t*Bu protecting group before the thioester hybrid **15** was added. No additional additives, such as thiols were required since the template effect ensured a fast reaction even with the moderately reactive MPA thioester. The reaction was monitored by LC-MS. After 2 h 53% of the thioester was consumed and after 8 h a conversion of 67% was observed (Scheme 4). This result was reproduced in a second ligation reaction differing in peptide sequences (Supporting Information). In case the thioester containing amino acid is sequentially flanked by glycines, aspartimide formation-like cyclization was observed. Nevertheless, cyclization was significantly slower than the ligation reaction and in case of alanines or other sterically more demanding amino acids no cyclization was observed (Supporting Information).

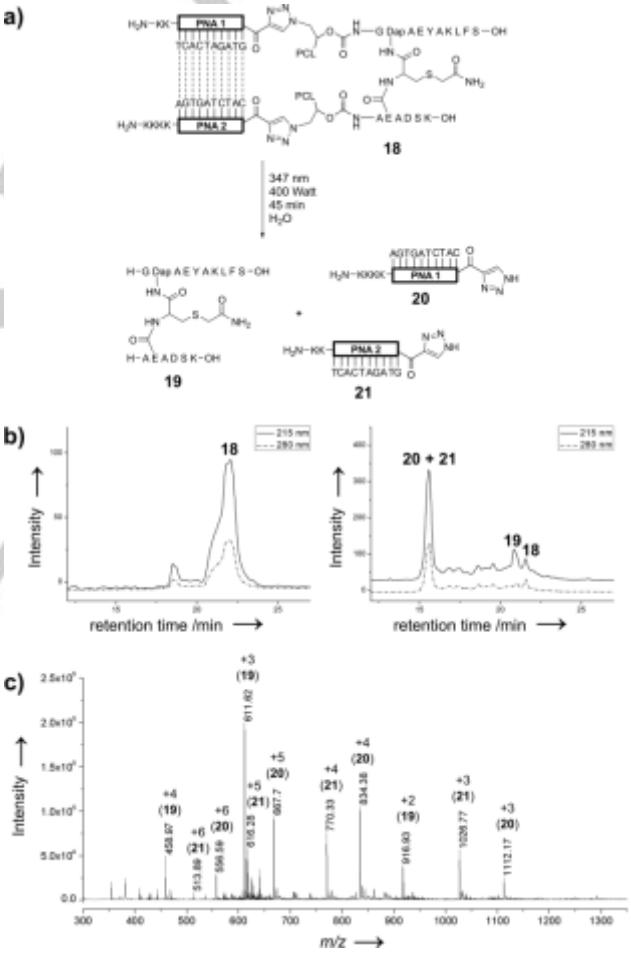


Scheme 4. Template-directed native chemical ligation between thioester hybrid **15** and cysteine hybrid **16**. HPLC chromatograms after 0, 1, 2, 4, 6, and 8 h indicate consumption of hybrids **15** and **16** and formation of ligation product **17**. Conversion of thioester hybrid **15**: 38% (1 h), 53% (2 h), 62% (4 h), 64% (6 h), 67% (8 h).

In order to ensure the impact of templating PNAs a comparable ligation was conducted with hybrids containing non-complementary PNA oligomers (identical sequences). We found that non-templated ligation reactions proceed more than three times slower than templated ligation reactions. (Supporting Information).

Finally, photochemical cleavage of the linker/PNA was attempted to deliberate the ligated peptide. First attempts provided massive by-product formation most likely caused by the free thiol group of the ligation product since this group is prone to formation of radicals leading to side reactions such as hydrogen abstraction, decarboxylation or fragmentation reactions.[18] Therefore, prior to photocleavage the thiol was capped with iodoacetamide in the ligation solution immediately following NCL. In case the peptide sequence contains additional cysteins, NCL based on selenocysteines will be considered which allows selective deselenization afterwards.[19] Quantitative conversion was obtained forming the capped oligomer **18**; only monoalkylated product was observed.

Photocleavage of ligated hybrid **18** was performed at 347 nm, 400 Watt (Scheme 5). After 45 min of irradiation 91% of the oligomer was converted providing 68% of ligated peptide **19**, the templating oligomers **20** and **21,** and only minor amounts of by-product as indicated by ESI-MS of the crude reaction mixture (Scheme 5). Interestingly, the caging group was cleaved at two different positions. As expected, cleavage occurred at the carbamate function and tracelessly released the unprotected peptide. Nevertheless, photolytic cleavage also appeared at the triazole breaking the C-N bond since the triazole is located at the β-carbon with respect to the nitrophenyl moiety and triazole being a good leaving group.[20] Despite these two cleavage sites the overall deliberation of the ligated peptide was successfully obtained.



Scheme 5. a) Photocleavage of compound **18** provided the template units **20** and **21** and the released peptide **19**. b) *Left-hand side:* HPLC chromatogram of **18** before irradiation. *Right-hand side:* HPLC chromatogram after 45 min of irradiation. c) ESI-MS spectrum of the crude reaction mixture after irradiation.

In conclusion, an easily applicable and efficient protocol is described for a new template-directed ligation strategy using photocleavable guiding units. The PNA/peptide hybrids, linked by a novel photocleavable linker, were readily synthesized, the

10.1002/cbic.201700487

ligation proceeded smoothly with good conversions and a template effect was demonstrated. Finally, photolysis provided traceless cleavage of the templating strands providing ligated peptide **19**. The strategy offers the possibility to synthesize branched peptides with therapeutical potential.[10b] The template-mediated ligation of *C*- to *N*-terminal linked and *N*-terminally linked peptides has been explored,[6, 21] here a strategy was introduced for template-directed synthesis of branched peptides. Currently, we are applying this ligation strategy to combinatorial chemistry approach for peptide libraries encoded by a variation of PNA sequences. Furthermore, the PNA mediated ligation strategy will be explored for simultaneous protein modification at different ligation sites as recently described for a library with different protein-conjugates.[22]

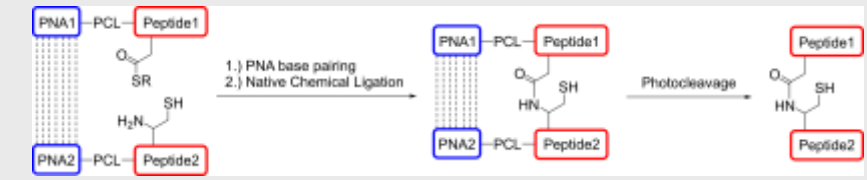
Keywords: peptide ligation • templated reactions • photocleavable linker • peptide nucleic acid • native chemical ligation

[1] N. Brauckhoff, G. Hahne, J. T.-H. Yeh, T. N. Grossmann, *Angew. Chem.* **2014**, *126*, 4425-4429; *Angew. Chem. Int. Ed.* **2014**, *53*, 4337–4340.
[2] R. Naylor, P. T. Gilham, *Biochemistry* **1966**, *5*, 2722–2728.
[3] a) C. Böhler, P. E. Nielsen, L. E. Orgel, *Nature* **1995**, *376*, 578–581; b) A. Luther, R. Brandsch, G. von Kiedrowski, *Nature* **1998**, *396*, 245–248; c) Z.-Y. J. Zhan, D. G. Lynn, *J. Am. Chem. Soc.* **1997**, *119*, 12420–12421; d) Z. J. Gartner, M. W. Kanan, D. R. Liu, *Angew. Chem.* **2002**, *114*, 1874-1878; *Angew. Chem. Int. Ed. Engl.* **2002**, *41*, 1796–1800; e) A. Roloff, O. Seitz, *Chem Sci* **2013**, *4*, 432–436; f) X. Li, D. R. Liu, *Angew. Chem.* **2004**, *116*, 4956-4979 *Angew. Chem. Int. Ed.* **2004**, *43*, 4848–4870.
[4] R. K. Bruick, P. E. Dawson, S. B. Kent, N. Usman, G. F. Joyce, *Chem. Biol.* **1996**, *3*, 49–56.
[5] G. Li, Y. Liu, Y. Liu, L. Chen, S. Wu, Y. Liu, X. Li, *Angew. Chem.* **2013**, *125*, 9723-9728; *Angew. Chem. Int. Ed.* **2013**, *52*, 9544–9549.
[6] B. Rubinov, N. Wagner, H. Rapaport, G. Ashkenasy, *Angew. Chem.* **2009**, *121*, 6811-6814; *Angew. Chem. Int. Ed.* **2009**, *48*, 6683–6686.
[7] a) D. H. Lee, J. R. Granja, J. A. Martinez, K. Severin, M. R. Ghadiri, *Nature* **1996**, *382*, 525–528; b) R. Issac, Y. W. Ham, J. Chmielewski, *Curr. Opin. Struct. Biol.* **2001**, *11*, 458–463.
[8] U. Reinhardt, J. Lotze, S. Zernia, K. Mörl, A. G. Beck-Sickinger, O. Seitz, *Angew. Chem.* **2014**, *116*, 10402-10406; *Angew. Chem. Int. Ed.* **2014**, *53*, 10237–10241.
[9] S. Shakeel, S. Karim, A. Ali, *J. Chem. Technol. Biotechnol.* **2006**, *81*, 892–899.
[10] a) C. Falciani, M. Fabbrini, A. Pini, L. Lozzi, B. Lelli, S. Pileri, J. Brunetti, S. Bindi, S. Scali, L. Bracci, *Mol. Cancer Ther.* **2007**, *6*, 2441–2448; b) J. Brunetti, C. Falciani, B. Lelli, A. Minervini, N. Ravenni, L. Depau, G. Siena, E. Tenori, S. Menichetti, A. Pini, *BioMed Res. Int.* **2015**, *2015*, 1–7.
[11] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776–779.
[12] J. A. Baccile, M. A. Morrell, R. M. Falotico, B. T. Milliken, D. L. Drew, F. M. Rossi, *Tetrahedron Lett.* **2012**, *53*, 1933–1935.
[13] a) H. Schupp, W. K. Wong, W. Schnabel, *J. Photochem.* **1987**, *36*, 85–97; b) P. Klán, T. Šolomek, C. G. Bochet, A. Blanc, R. Givens, M. Rubina, V. Popik, A. Kostikov, J. Wirz, *Chem. Rev.* **2013**, *113*, 119–191; c) C. G. Bochet, *J. Chem. Soc. Perkin 1* **2002**, 125–142.
[14] N. Bindman, R. Merkx, R. Koehler, N. Herrman, W. A. van der Donk, *Chem. Commun.* **2010**, *46*, 8935–8937.
[15] R. Beukers, W. Berends, *Biochim. Biophys. Acta* **1961**, *49*, 181–189.
[16] a) E. Boll, J. Dheur, H. Drobecq, O. Melnyk, *Org. Lett.* **2012**, *14*, 2222–2225; b) J. Dheur, N. Ollivier, A. Vallin, O. Melnyk, *J. Org. Chem.* **2011**, *76*, 3194–3202.
[17] P. Wittung, P. E. Nielsen, O. Buchardt, M. Egholm, B. Nordén, *Nature* **1994**, *368*, 561–563.
[18] F. Dénès, M. Pichowicz, G. Povie, P. Renaud, *Chem. Rev.* **2014**, *114*, 2587–2693.
[19] a) R. J. Hondal, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.*, **2001**, *123*, 5140–5141. b) N. J. Mitchell, S. S. Kulkarni, L. R. Malins, S. Wang, R. J. Payne, *Chem. Eur. J.*, **2017**, *23*, 946-952.
[20] a) S.-S. Tseng, E. F. Ullman, *J. Am. Chem. Soc.* **1976**, *98*, 541–544; b) K. Qvortrup, T. E. Nielsen, *Chem. Commun.* **2011**, *47*, 3278.
[21] a) K. Severin, D. H. Lee, A. J. Kennan, M. R. Ghadiri, *Nature* **1997**, *389*, 706–709; b) R. R. Araghi, B. Koksch, *Chem. Commun.* **2011**, *47*, 3544-3546.
[22] S. Dickgiesser, N. Rasche, D. Nasu, S. Middel, S. Hörner, O. Avrutina, U. Diederichsen, H. Kolmar, *ACS Chem. Biol.* **2015**, *10*, 2158–2165.

10.1002/cbic.201700487

WILEY-VCH

COMMUNICATION



A novel template-directed native chemical ligation strategy applying photocleavable PNA strands is presented. PNA/peptide hybrids, connected by a photocleavable linker, recognize by PNA base pairing and the reactive centers are brought in close proximity facilitating the ligation. Afterwards, the templating PNA oligomers can be cleaved by irradiation.

*S. Middel, C. H. Panse, S. Nawratil, U. Diederichsen**

Page No. – Page No.

Photocleavable PNA Strands for Template-Directed Native Chemical Ligation