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Peptide Ligation without Cysteine

Native Chemical Ligation at Valine

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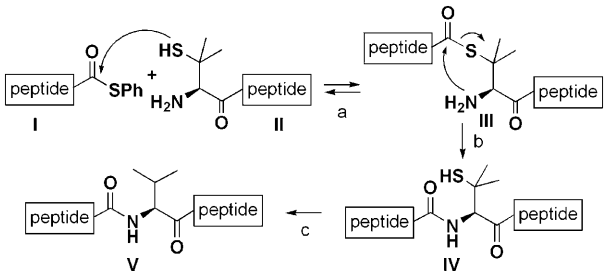
Among the techniques employed for coupling peptide segments,[1] native chemical ligation is among the most useful.[2] Since its discovery it has become a powerful tool for the chemical synthesis of proteins including labeled or posttranslationally modified proteins and proteins that contain non-proteinogenic amino acids.[3] In the native chemical ligation an unprotected C-terminal peptide thioester reacts with an unprotected N-terminal cysteine residue. The requirement for the rare amino acid cysteine limits the applicability in the synthesis of naturally occurring proteins. Several approaches have been developed to allow access to other, more common ligation sites.[4]

In the extended native chemical ligation the cysteine structure is mimicked by means of a removable auxiliary group that is attached to the N terminus of the peptide fragment.[5] Typically, electron-rich aromatic ring systems are included in the auxiliary structure to facilitate acidolytic removal subsequent to the ligation.[5e–k] The formation of glycine–glycine peptide bonds usually proceeds without problems. However, the reactivity of the secondary amine rapidly decreases with increasing steric demand at the ligation site. Thus, ligation at bulky amino acids such as valine or isoleucine has not been achieved. Recently, sugar-assisted ligation has been reported, which is of particular interest for glycopeptide synthesis.[6]

A conceptually different approach avoids the usage of less reactive secondary amines and draws upon the coupling of amino acids that contain sulfanyl groups in the side chain which are removed after ligation. For example, cysteine was used in a conventional native chemical ligation followed by conversion to the abundant alanine by using an excess of metal reagents.[7] The undesired desulfurization of other cysteine residues can be avoided by using protecting groups.[7b] Recently, the repertoire of this ligation–desulfurization approach was extended to phenylalanine.[8] The required β-sulfanylphenylalanine was prepared in a multistep synthesis.

In this communication we describe the use of penicillamine (Pen) as a precursor of valine in the ligation–desulfurization strategy (Scheme 1). β,β-Dimethylcysteine is commercially available with various protecting-group patterns suitable for routine solid-phase synthesis of peptides. We found that the ligation at penicillamine proceeded surprisingly fast despite the steric shielding of the methyl groups in the vicinity of the sulfanyl function. Even Leu–Val ligation sites, which appear in hydrophobic peptide segments, are accessible. We also present an improved method for achieving metal-free desulfurization and show applications in the synthesis of valine-containing peptides.



Scheme 1. Native chemical ligation at valine. The β,β-dimethylcysteine at the N terminus of the penicillyl peptide **II** induces the thiol exchange with peptide **I** (step a) to form thioester intermediate **III**. Subsequent S→N acyl transfer furnishes the peptide bond in **IV** (step b). Desulfurization (step c) provides the valyl peptide **V**.

Initially, we anticipated that the steric demand of the methyl groups in penicillamine would present a challenge in ligation-like reactions. Hence we first scrutinized the kinetics of reactions between model peptides that have been studied previously.[9] The penicillaminyl model peptide Pen-Arg-Ala-Glu-Tyr-Ser-NH_2 (**1**, Scheme 2) was prepared by Fmoc-based solid-phase synthesis, which included the coupling of a Boc/Trt-protected penicillamine building block (see the Supporting Information). The first experiments explored ligations at glycine as a sterically less demanding C-terminal amino acid in the peptide thioester Leu-Tyr-Lys-Ala-Gly-SR (**2**). The ligations were performed at a 5 mM concentration of the peptides in aqueous sodium phosphate buffer which contained 6 M guanidinium hydrochloride (GnHCl) and 50 mM triscarboxyethylphosphine (TCEP) as the denaturing and reducing agent, respectively. Sodium sulfanylethanesulfonate,

1: **Pen**-Arg-Ala-Glu-Tyr-Ser-NH_2
2: Leu-Tyr-Lys-Ala-Gly-$SCH_2CH_2CONH_2$
3: Leu-Tyr-Lys-Ala-His-$SCH_2CH_2CONHCHCONH_2$
4: Leu-Tyr-Lys-Ala-Met-$SCH_2CH_2CONHCHCONH_2$
5: Leu-Tyr-Lys-Ala-Leu-$SCH_2CH_2CONHCHCONH_2$
6: Ac-Thr-Leu-Gln-Asn-Arg-Glu-His-Glu-Thr-Asn-Gly-SBn
7: **Pen**-Ala-Lys-Ser-Asp-Gln-Lys-Gln-Glu-Gln-Leu-NH_2
8: Ac-Leu-Lys-Lys-Pro-Phe-Asn-Arg-Pro-Gln-Gly-SBn
9: **Pen**-Gln-Pro-Lys-Thr-Gly-Pro-Phe-Glu-Asp-Leu-Lys-NH_2
10: Val-Arg-Ala-Glu-Tyr-Ser-NH_2

Scheme 2. Peptides **1**–**10** used in the study of the penicillamine ligation.

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benzylthiol, and/or thiophenol were added to induce the formation of reactive thioesters in situ. The course of the ligation reaction was monitored by HPLC-MS analysis, which provided an accurate means to detect all the reaction products (Figure 1). The fastest initial ligation rates were achieved

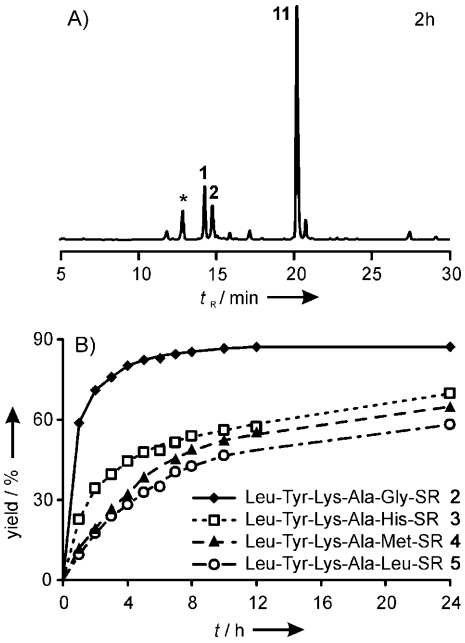

Figure 1. Scope of the penicillamine-mediated ligation. A) HPLC trace analysis of a representative ligation of **1** with **2** after a reaction time of 2 h. (*: hydrolyzed thioester). B) Time course of product formation in ligations of **1** with peptide thioesters **2**, **3**, **4**, or **5**. Ligation conditions: 5 mM **1**, 5 mM thioester **2**, **3**, **4** or **5**, 6 M GnHCl, 100 mM NaH_2PO_4, 50 mM TCEP, 5% PhSH, pH 8.5, 37°C. After 3, 6, 9, and 12 h further aliquots of buffer solution (8 vol% of the initial reaction volume) containing 0.5 M TCEP, 6 M GnHCl, 200 mM NaH_2PO_4, and PhSH were added.

when the aqueous phosphate buffer contained 5% thiophenol as sole thiol additive. HPLC analysis indicated the formation of the symmetric disulfide and the disappearance of thiophenol during the course of the reaction. Hence, further aliquots of TCEP and thiophenol were added after 3, 6, 9, and 12 h. Under these conditions, the ligation was remarkably fast, furnishing 60% ligation product already after 1 h and 87% ligation yield after 12 h. To exclude the possibility of a direct reaction between the thioester moiety and the α-amino function of the penicillaminyl peptide **1**, the corresponding valyl peptide **10** was synthesized and incubated with the peptide thioester **2** under the optimized ligation conditions (see Figure S2 in the Supporting Information). The formation of a ligation product was not observed, highlighting the importance of the thiol group in penicillamine.

Encouraged by these results we proceeded to explore ligations with sterically more demanding peptide thioesters. The peptidylhistidine thioester in Leu-Tyr-Lys-Ala-His-SR (**3**) underwent facile reaction (Figure 2) and provided the ligation product **12** in 70% yield (Table 1). However, small amounts (max. 8%) of an epimerized ligation product also formed (see Figure S3 in the Supporting Information). This behavior of histidine thioesters, high reactivity in both native chemical ligation and racemization reactions, was described previously.[9] The segment coupling of the methionine thioester in peptide **4** and pencillaminylpeptide **1** proceeded smoothly, yielding 65% ligation product after 24 h. Careful HPLC analysis revealed that racemization occurred to a small extent (less than 4%), which could be further reduced to 2% when the reaction was performed at pH 7.5 rather than pH 8.5 (see Table S1 in the Supporting Information). We then investigated the sterically demanding coupling of the leucine thioester in **5** with **1.** Surprisingly, the initial ligation rate was only six times less than the ligation rate of **1** with the glycine thioester in **2**. At extended reaction times (48 h) the ligation product **14** formed in 70% yield. This could be further improved to 80% yield when the leucine thioester was used in twofold excess. Racemization was not observed.

The versatility of the penicillamine-mediated ligation was demonstrated in the synthesis of the two longer peptide sequences **15** and **16** from sections of the signal transduction proteins STAT-1 and Syk kinase, respectively. The synthesis of the 176–197 segment **15** of STAT-1 was performed as described for the model peptides **11**–**14**. HPLC analysis showed that the reaction of peptide thioester **6** with penicillyl peptide **7**, added in 1.4-fold excess, resulted in near-quantitative formation of the ligation product **15** (Figure S6 in the Supporting Information). The coupling of peptide thioester **8** with penicillaminyl peptide **9** provided the 22-mer Syk kinase

Table 1: Yields of the ligations and the desulfurization.

Penicillyl peptide	Peptide thioester	Ligation	Desulfurization product	Desulfurization yield [%]	
		Product/yield [%]/reaction time [h]		metal-based	metal-free
1	**2**	LYKAG**Pen**RAEYS **11**/87/12	LYKAG**V**RAEYS **17**	61	98
1	**3**	LYKAH**Pen**RAEYS **12**/70/24	LYKAH**V**RAEYS **18**	–	93
1	**4**	LYKAM**Pen**RAEYS **13**/65/24	LYKAM**V**RAEYS **19**	–	77
1	**5**	LYKAL**Pen**RAEYS **14**/70[a] and 82[b]/48	LYKAL**V**RAEYS **20**	–	79
7	**6**	TLQNREHETNG**Pen**AKSDQKQEQL **15**/78[c]/24	LKKPFNRPQG**V**QPKTGPFEDLK **21**	0[d]	72
9	**8**	LKKPFNRPQG**Pen**QPKTGPFEDLK **16**/87[c]/24	TLQNREHETNG**V**AKSDQKQEQL **22**	54	91
10	**2**	LYKAGVRAEYS **17**/0[e]	–	–	–

[a] After 48 h with 1 equiv **5**. [b] after 48 h with 2 equiv **5**. [c] Penicillyl peptides were used in 1.3- to 1.4-fold excess. [d] After a reaction time of 8 h neither starting material nor product could be isolated. [e] Direct fragment coupling was not observed. The one letter codes for the amino acids are given in http://www.chem.qmul.ac.uk/iupac/AminoAcid/AA1n2.html

peptide **16** in nearly quantitative yield, when **9** was added in 1.3 fold excess.

The next goal was to convert the penicillyl residues in the formed ligation products to valine residues. We first attempted sulfur removal by applying known metal-based methods of desulfurization. The penicillyl ligation products were dissolved in 20% acetic acid and treated with a large excess of Raney nickel. Desulfurization yields were moderate and amounted to 61% for the model peptide **4** and 54% for the Syk kinase peptide **16** despite prolonged reaction times. Attempts to remove the thiol group in the STAT-1 peptide **15** failed. Neither was it possible to achieve the sulfur removal nor could the starting material be recovered. Apparently, the peptide material remained adsorbed onto the metal surface despite several attempted extractions with trifluoroacetic acid.

Very recently, Wan and Danishefsky described a method for the metal-free desulfurization of peptides,[10] in which a water-soluble radical starter first abstracts a hydrogen atom from the cysteine thiol group which is then reduced with TCEP to form an alanyl radical.[11] This alkyl radical receives a hydrogen atom either from unreacted cysteinyl peptide or from EtSH and *t*BuSH, which are added to accelerate product formation. When we applied the published reaction conditions (VA-044, TCEP, EtSH, and *t*BuSH) to penicillyl peptide **11** we observed that the desulfurized peptide **17** was formed; however, it was accompanied by several by-products. We assumed that hydride-transfer reactions would proceed less effectively in penicillamine than in cysteine. To accelerate thiyl radical formation, we increased the reaction temperature and the amount of radical starter VA-044. Furthermore, glutathione was used as the hydrogen source as we reasoned that this powerful hydrogen donor[12] may react faster with the formed valyl radical than malodorous EtSH and/or *t*BuSH. The reactions were performed at peptide concentrations of 1–2 mM in an aqueous 100 mM phosphate buffer adjusted to pH 6.5 which contained 250 mM TCEP, 200 mM VA-044, 40 mM glutathione, and 3 M GnHCl. The desulfurization reactions proceeded smoothly and went to completion within 2.5 h (Figure 2). The penicillamine-containing peptides **11** and **16** were converted to the corresponding valine-containing peptides **17** and **22** in 98% and 91% yield, respectively (Table 1). Interestingly, even the STAT-1 peptide segment **15**, which resisted metal-induced thiol removal, was successfully desulfurized (→**21**). The desulfurization of the penicillamine peptides **12** and **14** was also straightforward. In the case of the methionine-containing peptide **13** oxidation of the thioether moiety concomitantly occurred when the reaction was performed at 65 °C. The oxidation of the methionine side chain was insignificant at a reaction temperature of 37 °C. Under these conditions conversion into the valine peptide **19** was complete after 6 h.

The native chemical ligation is among the few chemical methods, if not the only one, that has enabled the synthesis of proteins of a complexity that can usually be obtained only by applying recombinant techniques.[13] In its original form, native chemical ligation provides access to Xaa–Cys (Xaa = any amino acid except proline) sites. Auxiliary-based methods have extended the scope, and the formation of

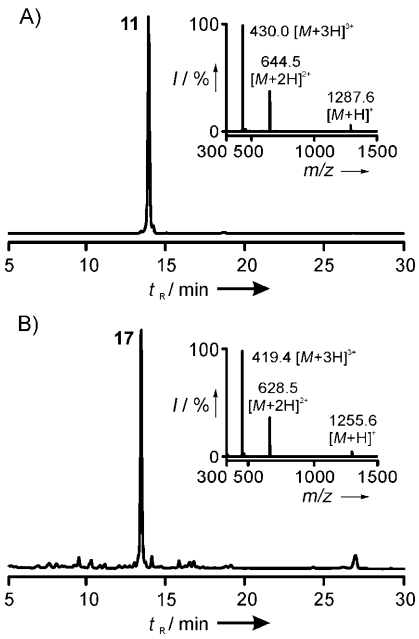

Figure 2. Metal-free desulfurization of penicillamine-containing peptide Leu-Tyr-Lys-Ala-Gly-**Pen**-Arg-Ala-Glu-Tyr-Ser-NH_2 (**11**). HPLC traces A) before and B) after 150 min reaction time. The insets show the ESI mass spectra, which provide ample evidence of desulfurization. Conditions: 5 mM **11**, 3 M GnHCl, 100 mM NaH_2PO_4, 200 mM VA-044, 250 mM TCEP, 40 mM glutathione, 65 °C, pH 6.5.

Gly–Gly, Gly–Ala, Gly–Gln, Gly–Asp, Gly–His, Ala–Gly, Lys–Gly, His–Gly, Phe–Gly, Pro–Gly, His–His, His–Ala, Ala–His, and Ala–Asp peptide bonds has been demonstrated.[5] The application of these methods requires access to non-commercial building blocks. Frequently occurring Xaa–Ala sites can be formed by means of the native chemical ligation/desulfurization approach.[7] One advantage of this two-step method is that only commonly applied amino acid derivatives are used. The synthesis of β-sulfanylphenylalanine has enabled ligations at Xaa–Phe sites.[8] The work described here demonstrates the use of penicillamine (Pen) as a precursor to valine in the ligation–desulfurization approach. Valine is a frequently occurring amino acid (6.6% content). The availability of suitably protected penicillamine building blocks and in particular the feasibility to establish ligations at hydrophobic peptide segments are considered as advantageous.

The presented ligation reactions of thioesters terminated by glycine, histidine, methionine, and leucine residues suggest a broad applicability of the method, which may allow almost general access to Xaa–Val peptide bonds. Future experiments should reveal whether sterically crowded Val–Val or Ile–Val peptide bonds can be formed. The demonstrated access to Leu–Val ligation sites may provide interesting opportunities in the synthesis of transmembrane proteins. However, we wish to note that care should be taken to avoid epimerization of reactive peptide thioesters during long reaction times. This, however, applies to all reactions that involve peptide thioesters. The presented ligation–desulfurization approach also included an optimized metal-free method for removing thiol groups in peptides. Glutathione was used as the hydrogen

source in the radical desulfurization rather than the less effective and malodorous thiols EtSH and *t*BuSH. Considering the abundant availability of this inexpensive reagent in bioorganic and biological chemistry laboratories, we propose glutathione as the reagent of choice in metal-free desulfurization reactions.

In conclusion, we have expanded the scope of native chemical ligation by gaining access to hydrophobic ligation sites. Careful optimization of both ligation and reduction conditions allowed the use of the β-sulfanyl amino acid penicillamine in the ligation–desulfurization approach. Neither special building blocks nor are risky reagents are required; this may encourage the application of this new option in peptide synthesis.

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[1] B. L. Nilsson, M. B. Soellner, R. T. Raines, *Annu. Rev. Biophys. Biomol. Struct.* **2005**, *34*, 91 – 118.
[2] a) T. Wieland, E. Bokelmann, L. Bauer, H. U. Lang, H. Lau, *Justus Liebigs Ann. Chem.* **1953**, *583*, 129 – 149; b) P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. H. Kent, *Science* **1994**, *266*, 776 – 779.
[3] P. E. Dawson, S. B. H. Kent, *Annu. Rev. Biochem.* **2000**, *69*, 923 – 960.
[4] C. Haase, O. Seitz, *Angew. Chem.* **2008**, *120*, 1575 – 1579; *Angew. Chem. Int. Ed.* **2008**, *47*, 1553 – 1556.
[5] a) L. E. Canne, S. J. Bark, S. B. H. Kent, *J. Am. Chem. Soc.* **1996**, *118*, 5891 – 5896; b) J. Offer, P. E. Dawson, *Org. Lett.* **2000**, *2*, 23 – 26; c) C. Marinzi, S. J. Bark, J. Offer, P. E. Dawson, *Bioorg. Med. Chem.* **2001**, *9*, 2323 – 2328; d) G. Chen, J. D. Warren, J. Chen. B. Wu, Q. Wan, S. J. Danishefsky, *J. Am. Chem. Soc.* **2006**, *128*, 7460 – 7462; e) T. Kawakami, K. Akaji, S. Aimoto, *Org. Lett.* **2001**, *3*, 1403 – 1405; f) P. Botti, M. R. Carrasco, S. B. H. Kent, *Tetrahedron Lett.* **2001**, *42*, 1831 – 1833; g) J. Offer, C. N. C. Boddy, P. E. Dawson, *J. Am. Chem. Soc.* **2002**, *124*, 4642 – 4646; h) C. Marinzi, J. Offer, R. Longhi, P. E. Dawson, *Bioorg. Med. Chem.* **2004**, *12*, 2749 – 2757; i) S. Tchertchian, O. Hartley, P. Botti, *J. Org. Chem.* **2004**, *69*, 9208 – 9214; j) D. Macmillan, D. W. Anderson, *Org. Lett.* **2004**, *6*, 4659 – 4662; k) B. Wu, J. Chen, J. D. Warren, G. Chen, Z. Hua, S. J. Danishefsky, *Angew. Chem.* **2006**, *118*, 4222 – 4231; *Angew. Chem. Int. Ed.* **2006**, *45*, 4116 – 4125; l) M. Y. Lutsky, N. Nepomniaschiy, A. Brik, *Chem. Commun.* **2008**, 1229 – 1231.
[6] a) A. Brik, Y. Y. Yang, S. Ficht, C. H. Wong, *J. Am. Chem. Soc.* **2006**, *128*, 5626 – 5627; b) S. Ficht, R. J. Payne, A. Brik, C. H. Wong, *Angew. Chem.* **2007**, *119*, 6079 – 6083; *Angew. Chem. Int. Ed.* **2007**, *46*, 5975 – 5979; c) R. J. Payne, S. Ficht, S. Tang, A. Brik, Y. Y. Yang, D. A. Case, C. H. Wong, *J. Am. Chem. Soc.* **2007**, *129*, 13527 – 13536
[7] a) L. Z. Yan, P. E. Dawson, *J. Am. Chem. Soc.* **2001**, *123*, 526 – 533; b) B. L. Pentelute, S. B. H. Kent, *Org. Lett.* **2007**, *9*, 687 – 690.
[8] a) P. Botti, S. Tchertchian, WO 2006/133962, **2006**; b) D. Crich, A. Banerjee, *J. Am. Chem. Soc.* **2007**, *129*, 10064 – 10065.
[9] T. M. Hackeng, J. H. Griffin, P. E. Dawson, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10068 – 10073.
[10] a) Q. Wan, S. J. Danishefsky, *Angew. Chem.* **2007**, *119*, 9408 – 9412; *Angew. Chem. Int. Ed.* **2007**, *46*, 9248 – 9252.
[11] a) C. Walling, R. Rabinowitz, *J. Am. Chem. Soc.* **1957**, *79*, 5326; b) A. González, G. Valencia, *Tetrahedron: Asymmetry* **1998**, *9*, 2761 – 2764; c) J. Cuesta, G. Arsequell, G. Valencia, A. González, *Tetrahedron: Asymmetry* **1999**, *10*, 2643 – 2646; d) G. Arsequell, A. González, G. Valencia, *Tetrahedron Lett.* **2001**, *42*, 2685 – 2687.
[12] a) C. Chatgilialoglu, *Helv. Chim. Acta* **2006**, *89*, 2387 – 2398; b) C. Chatgilialoglu, C. Ferreri, R. Bazzanini, M. Guerra, S. Y. Choi, C. J. Emanuel, J. H. Horner, M. Newcomb, *J. Am. Chem. Soc.* **2000**, *122*, 9525 – 9533.
[13] V. Y. Torbeev, S. B. H. Kent, *Angew. Chem.* **2007**, *119*, 1697 – 1700; *Angew. Chem. Int. Ed.* **2007**, *46*, 1667 – 1670.

