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A threonine-forming oxazetidine amino acid for the chemical synthesis of proteins with the KAHA ligation

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Abstract: The α -ketoacid-hydroxylamine (KAHA) ligation allows the coupling of unprotected peptide segments by the chemoselective formation of an amide bond. Currently the most widely used variant employs a 5-membered cyclic hydroxylamine that forms a homoserine ester as the primary ligation product. In order to directly form amide-linked threonine residues at the ligation site, we prepared a new 4-membered cyclic hydroxylamine building block. This monomer was applied to the synthesis of wild type ubiquitin conjugating enzyme UbcH5a (146 residues) and a Titin protein domain, TI I27 (89 residues). Both the resulting UbcH5a and the variant with two homoserine residues showed identical activity to a recombinant variant in a ubiquitination assay.

The chemical synthesis of proteins has advanced greatly over the last decades by the development of ligation reactions for the coupling of unprotected peptide chains. In particular, the native chemical ligation (NCL) of peptide thioesters has been applied to the synthesis of hundreds of protein targets.^[1,2] In 2006, we reported the α -ketoacid-hydroxylamine (KAHA) amide-forming ligation,^[3] which complements the well-established NCL for the chemical synthesis of proteins by operating under acidic conditions and facilitating convergent assembly strategies.^[4] Currently the most used hydroxylamine for KAHA ligations is 5-oxaproline (Opr) due to its high stability and chemoselective reactivity with α -ketoacids.^[5] The primary outcome of this ligation is a homoserine ester, which can be rearranged under basic conditions to the amide.^[6] In 2015, we developed a novel 4-membered ring hydroxylamine, which gives serine at the ligation site, exclusively as the amide product.^[7] It undergoes KAHA ligation at lower temperatures and concentrations than the 5-membered analogue (Scheme 1); unfortunately, this serine-forming monomer exhibited sequence-dependent stability after removal of the Fmoc protection group, precluding isolation and purification of the deprotected peptide segments.

In this report, we document the synthesis of a threonine-forming oxazetidine acid that directly yields a canonical threonine residue at the ligation site. In contrast to the serine-forming structure, peptides bearing this more substituted oxazetidine are amenable to isolation and purification. We

demonstrate its utility by the chemical synthesis of ubiquitin conjugating enzyme UbcH5a with two sequential ligations and the preparation of the TI I27 domain of Titin.

a) Previous work (ref. 3,6):

b) Previous work (ref. 7):

c) This work:

Scheme 1. Previously reported hydroxylamines for KAHA ligation and the threonine variant.

Our targeted building block required the stereoselective synthesis of a substituted oxazetidine, a class of compounds for which very few routes have been reported.^[8] We began our synthesis from commercially available 1-chloro-2-butene, which was converted by Sharpless dihydroxylation into the corresponding diol.^[9,10] Treatment with powdered NaOH and subsequent conversion of the crude material into the corresponding tosylate **1** gave the enantioenriched precursor for our synthesis. Unfortunately, standard conditions gave only an 80:20 ratio of the desired enantiomer, but we elected to employ this material for establishing the synthesis with the knowledge that – if successful – we would be able to improve access to this building block at a later point.

Alkylation of compound **2** with tosylate **1** in the presence of K₂CO₃ proceeded cleanly. The Alloc protecting group was removed under standard conditions to give hydroxylamine **3** with a free NH. The oxazetidine ring was formed by a BF₃-mediated ring closure to give free alcohol **4**, which showed poor stability over time and was treated promptly with TBDMSCl and imidazole to give the significantly more stable silyl ether.^[7] The DMB protecting group required for successful cyclization was removed under mild conditions using DDQ and the resulting hydroxylamine was protected with Fmoc-Cl to give **5**.^[7] At this stage, the enantiomers could be separated by preparative chiral

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HPLC to give the enantiopure product, although much of our studies were conducted with enantioenriched material.

a) Synthesis of threonine oxazetidine building block:

b) Crystal structure of intermediate 4 after derivatization:

Scheme 2. a) Synthesis of threonine oxazetidine building block **6**, b) Crystal structure of intermediate **4** after derivatization to intermediate **7**.

Initial attempts to remove the TBDMS protecting group of compound **5** using TBAF led mainly to decomposition, and treatment with aqueous HF was poor yielding. Instead, treating intermediate **5** with concentrated HCl gave clean conversion to the corresponding free alcohol. Although we originally used a two-step oxidation to the carboxylic acid, this route required extensive HPLC purification. Instead, a slightly modified TPAP oxidation procedure from Stark *et al.* gave directly the desired acid in one step,^[11] which allowed building block **6** to be isolated by standard column chromatography on SiO₂. An X-ray structure of a crystalline derivative of **4** confirmed the relative stereochemistry (Scheme 2).

Before proceeding to the synthesis of proteins, we first established that Fmoc-oxazetidine **6** could be coupled to a short peptide segment and was stable towards resin cleavage and Fmoc-deprotection. A model peptide was synthesized by standard SPPS conditions on a Rink amide resin and oxazetidine **6** was coupled with COMU/2,2,6,6-Tetramethylpiperidine (TMP). Resin cleavage with TFA/DODT/H₂O (95:2.5:2.5 v/v/v) for 2 hours followed by preparative HPLC afforded the desired product in good yield (Scheme 3). The Fmoc protecting group was removed with *N,N*-diethylamine, followed by quenching after 10 minutes by the addition of ice-cooled TFA/CH₃CN/H₂O solution to give a 7 mM

solution of unprotected oxazetidine monomer on the model peptide. KAHA ligation was performed by the addition of Fmoc-Tyr- α -ketoacid; after 5 h at room temperature the reaction mixture was purified to give ligated product **9** in 40% yield over two steps. Comparison of **9** by both NMR and HPLC to peptides prepared by linear solid phase peptide synthesis confirmed the relative stereochemistry and the direct formation of amide, rather than ester, bonds during KAHA ligation. No epimerization of the α -ketoacid employed for the ligation was observed.

Scheme 3. Application of threonine oxazetidine building block **6** on a model peptide.

As a first protein target for synthesis with building block **6** we selected the preparation of human Ubch5a, a 147-residue ubiquitin-conjugating (E2) enzyme.^[12–17] Analysis of the sequence identified two ideal ligation sites – both at Leu–Thr junctions – allowing us to divide the protein into three segments of almost equal length. The first chosen ligation site was between Leu52 and Thr53 and the second between Leu97 and Thr98. To simplify handling the assembled protein by avoiding oxidation, all Met residues in the sequence were mutated to Ile residues.

We have recently introduced solid supported linkers for Fmoc-SPPS that afford peptide α -ketoacids directly upon resin cleavage.^[3] We employed these linkers to prepare the necessary peptide segments, two of which included the threonine oxazetidine monomer introduced by coupling with COMU/TMP. We were pleased to see that all desired peptide segments containing the α -ketoacids as well as the Fmoc-protected threonine oxazetidine building blocks were readily accessible. Treatment of Fmoc-Ozt-(Ubch5a-99-147) with *N,N*-diethylamine afforded the free oxazetidine segment **10** in good yield. This free hydroxylamine was perfectly stable to preparative HPLC and the unprotected oxazetidine was stable on the peptide segment for weeks at 4 °C, a marked contrast to the corresponding serine-forming monomer, which decomposes immediately. KAHA ligation with **10** and **11** in NMP:H₂O (9:1) at room temperature gave peptide **12** after purification in 38% yield. The Fmoc protecting group was removed with *N,N*-diethylamine and the purified, lyophilized product was treated with α -ketoacid **14**. The second ligation with the threonine-forming oxazetidine also performed well in NMP:H₂O (9:1) to give protected compound **15** after purification in 31% yield. The Fmoc protecting group on the N-terminus was removed with *N,N*-diethylamine. The solution containing the crude product was acidified and the Acn protecting groups were removed by treatment with AgOAc for one hour at 37 °C and the product was purified by preparative HPLC (Scheme 4).

a) Sequence and marked fragments of UbchH5a:

b) Assembly of UbchH5a:

c) Ligation of 10 and 11:

d) Ligation of 13 and 14:

e) Purified UbchH5a and mass spectrum:

Scheme 4. a) Primary structure of UbchH5a, b) Assembly of UbchH5a, c) Ligation traces for ligation of peptide segments **10** and **11**, d) Ligation traces for ligation of peptide segments **13** and **14**, e) HPLC trace of purified UbchH5a and mass spectrum of the synthetic UbchH5a.

UbchH5a was also synthesized by using an analogous synthetic route using 5-oxaproline instead of the oxazetidine building block. This gave us the possibility to compare the newly developed threonine oxazetidine monomer with the well-studied 5-oxaproline monomer. In direct comparison, the ligations showed similar yields after purification. An advantage of the oxazetidine monomer was that no rearrangement step was needed, due to direct amide formation, and milder conditions were employed at lower concentration for the KAH ligations. The final products UbchH5a (Ozt) and UbchH5a (Opr, Thr53 to Hse, Thr98 to Hse) were denatured in 6 M Gdn/HCl in 20 mM Tris (pH 7.3), 150 mM NaCl, 1 mM TCEP and 10% glycerol and then dialyzed against 20 mM Tris (pH 7.3), 150 mM NaCl, 1 mM TCEP and 10% glycerol to give the folded products (Scheme 5). We tested the activity of UbchH5a (E2) synthesized with the threonine oxazetidine and oxaproline building blocks using a commercially available ubiquitination kit from Enzo (BML-UW9920). The kit provides the means of generating a thioester-linked ubiquitin conjugated UbchH5a. Ubiquitination is achieved via multiple enzymatic steps. First, the ubiquitin-activating enzyme (E1) catalyzes the formation of a reactive thioester bond with ubiquitin. From there, the ubiquitin is transferred to the active site cysteine of a ubiquitin conjugating enzyme (E2).^[18-22] In the presence of ATP we were able to form the desired thioester-linked ubiquitin conjugated UbchH5a for the commercial, synthetic (Ozt) and synthetic (Opr) variants. Negative control was also carried out in the absence of ATP; no reaction between ubiquitin and UbchH5a was observed (Scheme 5).

a) Folding of synthetic UbchH5a:

b) Ubiquitination of synthetic UbchH5a:

c) Observed UbchH5a complex by anti-biotin Western blotting:

Scheme 5. a) Folding of synthetic UbchH5a (PDB 5ftr), b) Ubiquitination assay of synthetic UbchH5a (PDB 5fer), c) Thioester-linked ubiquitin conjugation observed by anti-biotin Western blotting of the UbchH5a-biotinylated ubiquitin complex with an expected mass of around 25 kDa.

We also applied the threonine-forming monomer for the synthesis of a domain of the muscle protein Titin. Human Titin consists of about 320 individually folded protein domains, all of which have slightly different amino acid sequences. We selected one of these domains, TI I27, a β -sandwich protein consisting of 89 amino acid residues with no disulfide bridges present.^[23–27] To accommodate future work we mutated Cys47 and Cys63 to Ser, and to avoid aspartimide formation we also mutated Asp51 to a Glu residue. Both the α -ketoacid segment **16** and the hydroxylamine segment were accessible by standard SPPS conditions. The attachment of the threonine monomer, resin cleavage and Fmoc removal with *N,N*-diethylamine proceeded well to give **17**. These two segments were cleanly ligated in NMP/H₂O at room temperature for 6 hours to give the desired product, which was isolated by preparative HPLC in 42% isolated yield. The ligated product **18** was denatured in 6 M Gdn/HCl (pH 4.4) and then dialyzed against PBS-buffer at 4 °C to give the folded product (Scheme 6).

a) Sequence and marked fragments of Titin I27:

LIEVEKPLYG₁₀ VEVFVGETAH₂₀ FEIELSEPDV₃₀ HGQWKLKGQP₄₀ LTASPDSEII₅₀

EGGKKHILIL₆₀ HNSQLGNLeTGE₇₀ VSFQAANAKS₈₀ AANLKVKEI₈₉

b) Assembly of TI I27:

Scheme 6. a) Primary structure of Titin I27, b) Assembly of TI I27 by KAHA ligation, c) Ligation traces for ligation of peptide segments **16** and **17**, d) HPLC trace and mass spectrum of synthetic TI I27. e) CD-spectrum of folded synthetic TI I27 (**18**), f) CD-spectrum of wild type TI I27.

In conclusion, we have developed a synthetic route to a new hydroxylamine monomer that yields threonine upon KAHA ligation. It affords a canonical amino acid residue at the ligation site and, in contrast to 5-oxaproline, directly forms the amide product. We applied it to the chemical synthesis of ubiquitin-conjugating enzyme Ubch5a and Titin domain TI I27. We expect

that this variant of the KAHA ligation will be particularly useful in situations where the introduction of a homoserine residue is not acceptable or ligations under milder conditions are required.

Experimental Section

Experimental, characterization details for all compounds and experiments are given in the supporting information.

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- [1] P. E. Dawson, T. W. Muir, I. Clark-Lewis, S. B. Kent, *Science* **1994**, *266*, 776–779.
- [2] Other amide-forming ligations include the traceless Staudinger and the serine/threonine ligation. (a) R. Kleineweischede, C. P. Hackenberger, *Angew. Chem. Int.* **2008**, *47*, 5984–5988. (b) Y. Zhang, C. Xu, H. Y. Lam, C. L. Lee, X. Li, *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 6657–6662. (c) Saxon, C. R. Armstrong, C. R. Bertozzi, *Org. Lett.* **2000**, *2*, 2141–2143. (d) B. L. Nilsson, L. L. Kiessling, R. T. Raines, *Org. Lett.* **2000**, *2*, 1939–1941. (e) M. B. Soellner, B. L. Nilsson, R. T. Raines, *J. Am. Chem. Soc.* **2006**, *128*, 8820–8828. Recent reviews on chemical protein synthesis. (f) S. Bondalapati, M. Jabara, A. Brik, *Nature Chemistry* **2016**, *8*, 407–418. (g) V. Agouridas, O. E. Mahdi, M. Cargoet, O. Melnyk, *Bioorganic & Medicinal Chemistry* **2017**, *25*, 4938–4945. (h) S. S. Kulkarni, J. Sayers, B. Premdjee, R. J. Payne, *Nature Reviews Chemistry* **2018**, *2*, 0122.
- [3] J. W. Bode, R. M. Fox, K. D. Baucom, *Angew. Chem. Int.* **2006**, *45*, 1248–1252.
- [4] J. W. Bode, *Acc. Chem. Res.* **2017**, *50*, 2104–2115.
- [5] V. R. Pattabiraman, A. O. Ogunkoya, J. W. Bode, *Angew. Chem. Int.* **2012**, *51*, 5114–5118.
- [6] T. G. Wucherpfennig, F. Rohrbacher, V. R. Pattabiraman, J. W. Bode, *Angew. Chem. Int.* **2014**, *53*, 12244–12247.
- [7] I. Pusterla, J. W. Bode, *Nat. Chem.* **2015**, *7*, 668–672.
- [8] (a) V. Capriati, L. Degennaro, R. Favia, S. Florio, R. Luisi, *Org. Lett.* **2002**, *4*, 1551–1554. (b) V. Capriati, L. Degennaro, S. Florio, R. Luisi, *Eur. J. Org. Chem.* **2002**, 2961–2969. (c) B. B. Snider, J. R. Duvall, *Tetrahedron Lett.* **2003**, *44*, 3067–3070. (d) H. Staudinger, S. Jelagin, *Ber. Dtsch. Chem. Ges.* **1911**, *44*, 365–374.
- [9] K. P. M. Vanhessche, Z. Wang, K. B. Sharpless, *Tetrahedron Lett.* **1994**, *35*, 3469–3472.
- [10] T. I. Richardson, S. D. Rychnovsky, *J. Org. Chem.* **1996**, *61*, 4219–4231.
- [11] A. K. C. Schmidt, C. B. W. Stark, *Org. Lett.* **2011**, *13*, 4164–4167.
- [12] Y. Sheng, J. H. Hong, R. Doherty, T. Srikumar, J. Shloush, G. V. Avvakumov, J. R. Walker, S. Xue, D. Neculai, J. W. Wan, S. K. Kim, C. H. Arrowsmith, B. Raught, S. Dhe-Paganon, *Mol. Cell. Proteomics* **2009**, *11*, 329–341.
- [13] Y. David, T. Ziv, A. Admon, A. Navon, *J. Biol. Chem.* **2010**, *285*, 8595–8604.

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- [14] M. Windheim, M. Pegg, P. Cohen, *Biochem. J.* **2008**, *409*, 723–729.
- [15] C. P. Grou, A. F. Carvalho, M. P. Pinto, S. Wiese, H. Piechura, H. E. Meyer, B. Warscheid, C. Sa-Miranda, J. E. Azevedo, *J. Biol. Chem.* **2008**, *283*, 14190–14197.
- [16] M. K. Pabarcus, N. Hoe, S. Sadeghi, C. Patterson, E. Wiertz, M. A. Correia, *Arch. Biochem. Biophys.* **2009**, *483*, 66–74.
- [17] W. Zeng, M. Xu, S. Liu, L. Sun, Z. J. Chen, *Mol. Cell.* **2009**, *36*, 315–325.
- [18] D. Komander, M. Rape, *Annu. Rev. Biochem.* **2012**, *81*, 203–229.
- [19] M. D. Stewart, T. Ritterhoff, R. E. Klevit, P. S. Brzoziv, *Cell Res.* **2016**, *26*, 423–440.
- [20] A. Hershko, A. Ciechanover, *Annu. Rev. Biochem.* **1998**, *67*, 425–479.
- [21] C. M. Pickart, *Annu. Rev. Biochem.* **2001**, *70*, 503–533.
- [22] G. J. Stous, R. J. Govers, *Cell Sci.* **1999**, *112*, 1417–1423.
- [23] T. Oguro, K. Yagawa, T. Momose, T. Sato, K. Yamano, T. Endo *J. Mol. Biol.* **2009**, *385*, 811–819.
- [24] S. B. Fowler, J. Clarke, *Structure* **2001**, *9*, 355–366.
- [25] C. D. Geierhaas, R. B. Best, E. Paci, M. Vendruscolo, J. Clarke *Biophysical Journal* **2006**, *91*, 263–275.
- [26] K. Maruyama, *Biophysical Chemistry* **1994**, *50*, 73–85.
- [27] M. K. Miller, M. L. Bang, C. C. Witt, D. Labeit, C. Trombitas, K. Wantanabe, H. Granzier, A. S. McElhinny, C. Gregorio, S. Labzeit, *Journal of Molecular Biology* **2003**, *333*, 951–964.
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COMMUNICATION

Thr(ee) from a four. The synthesis of threonine-forming oxazetidine amino acid for the KAHA ligation of unprotected peptide segments allows the synthesis of E2 conjugating enzyme UbcH5a and Titin domain T1 I27 with canonical threonine residues at the ligation sites.

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