Ester Ligation Hot Paper

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Formation and Rearrangement of Homoserine Depsipeptides and Depsiproteins in the α-Ketoacid–Hydroxylamine Ligation with 5-Oxaproline**

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Abstract: *The primary products of the chemical ligation of α-ketoacids and 5-oxaproline peptides are esters, rather than the previously reported amides. The depsipeptide product rapidly rearranges to the amide in basic buffers. The formation of esters sheds light on possible mechanisms for the type II KAHA ligations and opens an avenue for the chemical synthesis of depsiproteins.*

Chemoselective ligation with large unprotected peptide segments allows chemical synthesis of proteins to probe biological systems.[1] The α-ketoacid–hydroxylamine (KAHA) ligation with 5-oxaproline (Opr)[2] offers an alternative to the more established native chemical ligation of thioesters and N-terminal cysteine residues.[3] The 5-oxaproline monomer offers a very stable—yet reactive—alkoxylamine that is easy to prepare and incorporate into synthetic peptides. Since our first report of ligations with 5-oxaproline in 2012, we have extensively investigated its use for peptide and protein synthesis and have prepared several protein sequences with more than 100 residues. During these investigations, we have recently discovered that the primary products of most KAHA ligations with 5-oxaproline are esters rather than the expected amides (Figure 1). Herein, we document our observations of the formation of the depsipeptide products, an assay for their identification, conditions for clean rearrangement to the amide products, and preliminary studies on the mechanism of the ester formation.

The coupling of two peptide segments shown in Scheme 1 is representative of KAHA ligations with 5-oxaproline. The

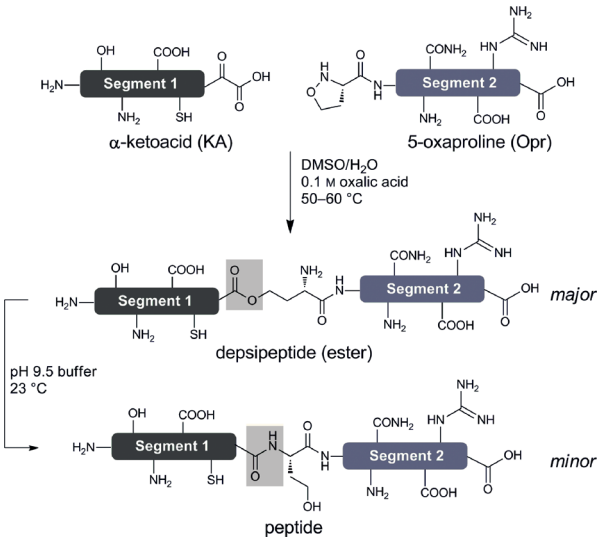

Figure 1. Formation of depsipeptides by KAHA ligation with 5-oxaproline and O→N acyl shift to form the amide at the ligation site. DMSO = dimethylsulfoxide.

reactions were conducted with peptide segment concentrations of 10–20 mM in NMP/H_2O in the presence of oxalic acid (pH 1–2). The formation of a new product was observed within a few minutes of starting the ligation and reached maximum conversion within six to eight hours. Close inspection of the HPLC traces revealed the presence of a second, small peak with the same mass as the desired product.[4] Both peaks were isolated by preparative HPLC and exposed to basic conditions (pH 10 buffer with 6 M guanidine hydrochloride) typically used to induce O→N acyl shifts.[5] To our surprise, the minor product did not change under these conditions, but the major product cleanly converted into the minor product (Scheme 1).

A plausible explanation for these observations is that an ester rather than an amide was formed by the KAHA ligation with 5-oxaproline. As the conditions for the KAHA ligation with 5-oxaproline are acidic, we first excluded the possibility that the expected amide product underwent an N→O acyl shift to the depsipeptide.[6] Exposing authentic homoserine-containing peptides to the ligation conditions (NMP/H_2O, 60 °C, 0.1 M oxalic acid) did not lead to the formation of the depsipeptide in any examples studied so far (see the Supporting Information).

To confirm the identity of the ester-linked product we prepared dipeptide model substrates that would afford ligation products that could be readily characterized by

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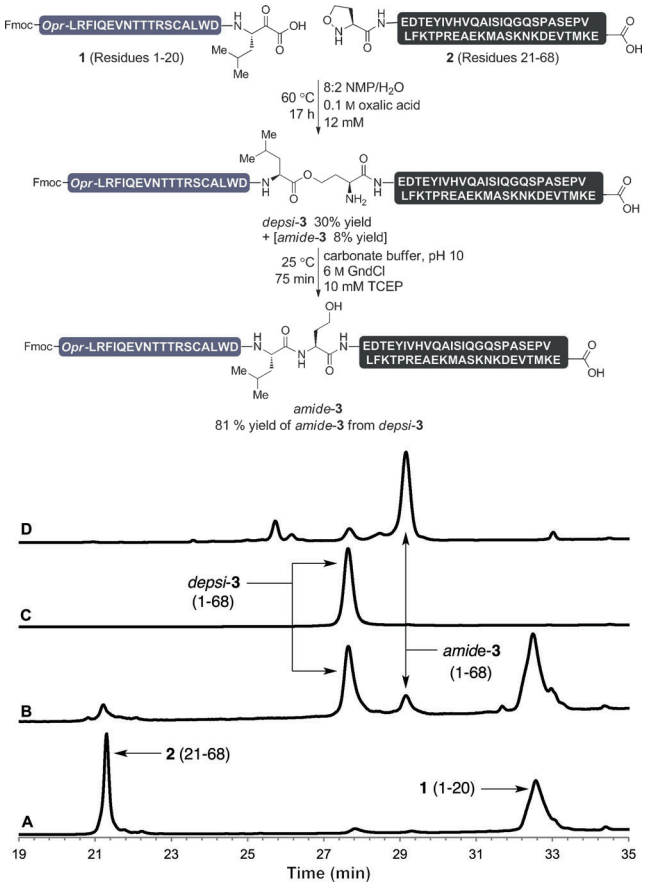
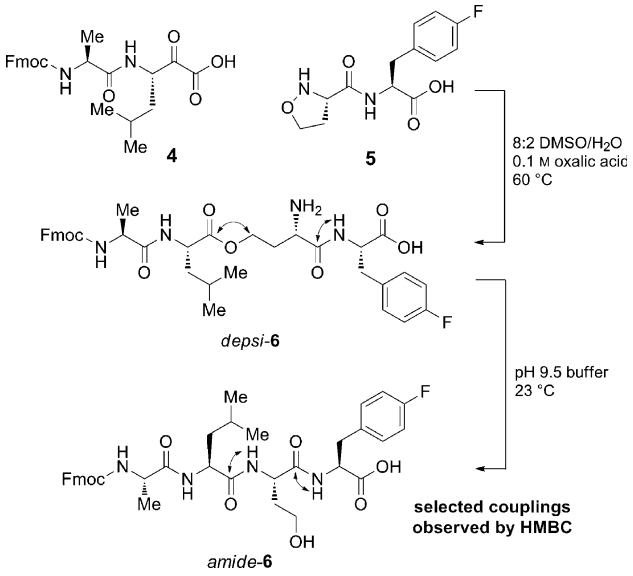


Scheme 1. KAHA ligation of peptide segments **1** and **2** with 5-oxaproline and conversion of *depsi*-**3** into *amide*-**3**. A) Ligation after 0 h. Protein segment concentration 12 mM in 8:2 NMP/H_2O containing 0.1 M oxalic acid. B) Ligation after 17 h at 60 °C. C) Purified ligation product *depsi*-**3**. D) Conversion of *depsi*-**3** into *amide*-**3** after 75 min in pH 10 buffer. Yields are of isolated material following preparative HPLC. NMP = 1-methyl-2-pyrrolidone, GndCl = guanidine hydrochloride, TCEP = tris(2-carboxyethyl)phosphine.



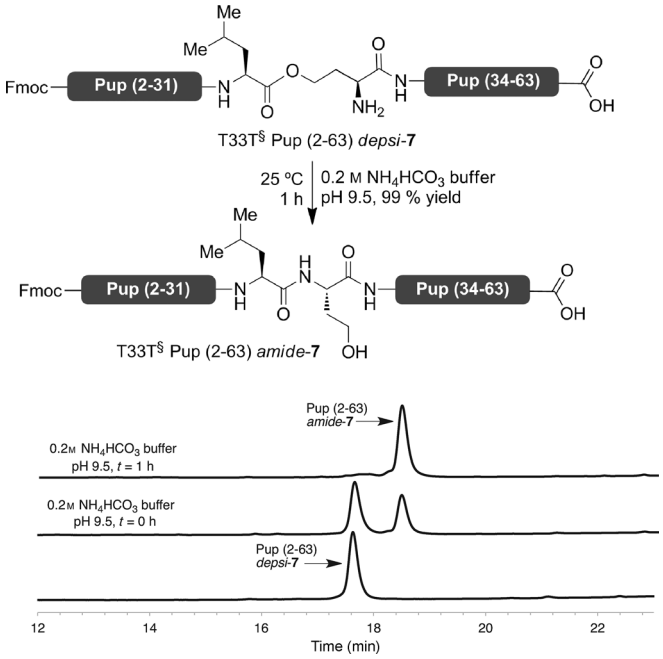
Scheme 2. Formation of *depsi*-**6** by KAHA ligation with 5-oxaproline and conversion into *amide*-**6**. Both the ester and amide products were fully characterized by NMR spectroscopy.

NMR spectroscopy. Ligation of **4** and **5** under standard conditions led to the formation of two products in a 9:1 ratio (Scheme 2). Comparison with NMR spectra of independently synthesized diastereomers ruled out epimerization at the ligation site during ligation and subsequent rearrangement (see the Supporting Information). The two products were fully characterized by NMR spectroscopy (^{1}H, ^{13}C, TOCSY, ^{1}H-^{13}C and ^{1}H-^{15}N HSQC, as well as ^{1}H-^{13}C HMBC) and clearly showed that the major product of this ligation is the ester. The ester cleanly rearranged to the amide after isolation and treatment with pH 9.5 buffer.

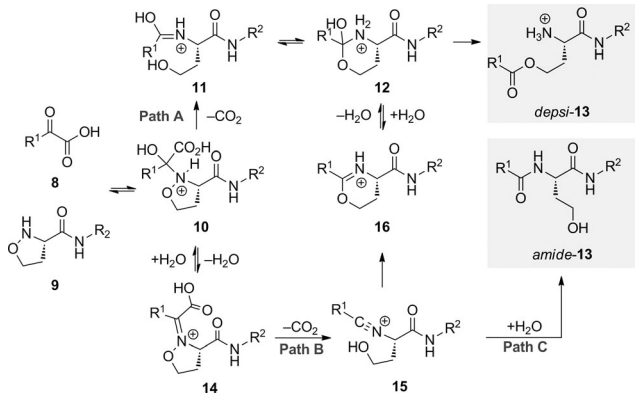
The formation of the depsipeptides by KAHA ligations with 5-oxaproline is not restricted to this sequence and does not appear to be affected by the neighboring residues. The immediate products of ligations to form the proteins Pup (Leu α-ketoacid), CspA (Tyr α-ketoacid), UFM1 (Phe and Ala α-ketoacid), and IFITM3 (Arg α-ketoacid) are in fact esters, rather than the previously reported amides.[2] We have reinvestigated the ligation products of the published protein syntheses (Pup, cspA, and UFM1) and re-characterized all of the ester and amide products (see the Supporting Information).

We also investigated the KAHA ligation of 5-oxaproline with several smaller α-ketoacids (Fmoc-Leu-ketoacid, ketoglutaric acid, α-ketobutyric acid, and benzoylformic acid) and found that all of these gave approximately 9:1 mixtures of ester and amide products in good yields. In every case studied, the ester products cleanly rearrange to the amides upon exposure to basic conditions. In some large peptides, such as the 68 residue depsipeptide shown in Scheme 1, the rearrangements are somewhat slower, perhaps because of the presence of secondary structure within the peptide. In these cases, the use of 6 M aqueous guanidine hydrochloride adjusted to pH 10 can accelerate the rearrangement. In most cases, such as the 62 residue Pup protein depsipeptide or the 82 residue UFM1, this is not necessary and clean rearrangement occurs at pH 9.5 within 1–2 h (Scheme 3).

We have previously noted that there are, depending on the hydroxylamine used in the reaction, two distinct mechanisms for KAHA ligations, which we have termed type I and type II.[7] We reported detailed studies on the mechanism of the type I ligation of O-unsubstituted hydroxylamines, which have water as a leaving group. However, we have not fully elucidated the mechanism of the type II ligation of O-substituted variants, which includes the 5-oxaproline substrate. The formation of the esters in the KAHA ligation with 5-oxaproline provides some insights into the possible mechanisms of the type II ligations. The amide product could arise from a concerted decarboxylation/elimination of **10** (Scheme 4, Path A) or the prior formation of iminium **14** that undergoes elimination to give nitrilium intermediate **15** (Path B). A similar elimination/decarboxylation with simultaneous cleavage of an oxime N–O bond to yield an alcohol and a nitrile has been extensively studied by Kemp et al.[8] Although it is possible that the ester arises from Path A



Scheme 3. Rearrangement of Pup (2–63) *depsi*-**7** to *amide*-**7** in a volatile buffer at pH 9.5.



Scheme 4. Possible mechanisms for the formation of depsipeptides during the type II KAHA ligation with 5-oxaproline.

through interception of the imidate **11**, the trapping of a nitrilium generated through Path B seems more likely. Examples are known of both the 6-*endo* attack of an alcohol at an in situ generated nitrilium[9] to give the cyclic imino ether and the hydrolysis of cyclic iminoethers under acidic conditions[10] to give the ester. Supporting this pathway are experiments performed in DMSO/$^{18}OH_2$, which always give nearly complete ^{18}O incorporation into both the ester and amide products. Amide formations with *O*-Bz-hydroxylamines—which cannot form esters since the leaving group is a carboxylic acid rather than an alcohol—give labeled products, presumably through Path C, since Path A would lead mainly to unlabeled amide products. Exchange of the ketone oxygen atom was much slower than the ligation, and starting material was isolated in unlabeled form. We cannot completely rule out Path A,[11] but we consider it less likely that **11**—the primary product of Path A—would give almost exclusively the ester product in preference to tautomerization to the amide. Neither temperature nor solvents (DMSO, NMP, CH_3CN, DMF) have an influence on the ratio of the depsi and amide products. The KAHA ligation with 5-oxaproline proceeds readily at pH 1–3 and slows with higher pH values.

The formation of depsipeptides in KAHA ligations is unique to reactions with 5-oxaproline. In both published and unpublished work on KAHA ligations with other hydroxylamines, we have not observed the formation of products other than the expected amides.[12] The depsipeptide ligation products are remarkably stable and do not decompose under the ligation conditions (pH 1–2, 60 °C), during Fmoc cleavage under anhydrous conditions (5 % $HNEt_2$, DMSO), or during preparative HPLC (0.1 % TFA in CH_3CN/H_2O). We have yet to observe products arising from simple hydrolysis of the ester bond; rearrangement appears to be far faster than hydrolysis. This is clearly advantageous, but also delayed our detection of the ester products, as the behavior and high-resolution mass spectra of the peptides and depsipeptides are identical. Synthetic proteins prepared by KAHA ligation with 5-oxaproline and folded under standard conditions at slightly basic pH values are likely to rearrange to the amides within a few hours.

Although surprising and unexpected, the formation of depsipeptides by ester-forming chemoselective ligations has potential applications. Changing an amide bond to an ester bond is known to disrupt secondary structures in peptides and proteins, and has been exploited in so-called "switch peptides" and "switch proteins", which have applications in self-assembly and drug delivery.[13] In general, depsipeptides are more polar and more soluble than their amide counterparts.[14] For this reason, Kiso's *O*-acyl dipeptides are often used for the preparation of large, hydrophobic peptides.[15] The KAHA ligation with 5-oxaproline may make possible similar advantages in the total synthesis[16] of proteins that intentionally contain esters or provide access to depsiproteins inaccessible by recombinant expression or by other ligation methods. It may also inspire the design of reaction partners for a new class of chemoselective ester-forming ligations.

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[1] a) S. B. H. Kent, *Angew. Chem. Int. Ed.* **2013**, *52*, 11988 – 11996; *Angew. Chem.* **2013**, *125*, 12208 – 12217; b) S. B. H. Kent, *Chem. Soc. Rev.* **2009**, *38*, 338 – 351; c) R. Kleineweischede, C. P. R. Hackenberger, *Angew. Chem. Int. Ed.* **2008**, *47*, 5984 – 5988; *Angew. Chem.* **2008**, *120*, 6073 – 6077; d) L. Raibaut, N. Ollivier, O. Melnyk, *Chem. Soc. Rev.* **2012**, *41*, 7001 – 7015.

[2] a) V. R. Pattabiraman, A. O. Ogunkoya, J. W. Bode, *Angew. Chem. Int. Ed.* **2012**, *51*, 5114 – 5118; *Angew. Chem.* **2012**, *124*, 5204 – 5208; b) A. O. Ogunkoya, V. R. Pattabiraman, J. W. Bode, *Angew. Chem. Int. Ed.* **2012**, *51*, 9693 – 9697; *Angew. Chem.* **2012**, *124*, 9831 – 9835; c) J. W. Bode, R. M. Fox, K. D. Baucom,

Angew. Chem. Int. Ed. **2006**, *45*, 1248 – 1252; *Angew. Chem.* **2006**, *118*, 1270 – 1274.

[3] a) P. E. Dawson, T. W. Muir, I. Clarklewis, S. B. H. Kent, *Science* **1994**, *266*, 776 – 779; b) P. E. Dawson, *Isr. J. Chem.* **2011**, *51*, 862 – 867.

[4] We chose this particular ligation to illustrate the formation of the ester products as the starting materials for the ligation and both the ester and the amide products are well-resolved by HPLC analysis.

[5] C. F. Liu, J. P. Tam, *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 6584 – 6588.

[6] L. Mouls, G. Subra, C. Enjalbal, J. Martinez, J.-L. Aubagnac, *Tetrahedron Lett.* **2004**, *45*, 1173 – 1178.

[7] I. Pusterla, J. W. Bode, *Angew. Chem. Int. Ed.* **2012**, *51*, 513 – 516; *Angew. Chem.* **2012**, *124*, 528 – 531.

[8] a) D. S. Kemp, K. G. Paul, *J. Am. Chem. Soc.* **1975**, *97*, 7305 – 7312; b) D. S. Kemp, D. D. Cox, K. G. Paul, *J. Am. Chem. Soc.* **1975**, *97*, 7312 – 7318.

[9] G. T. Lee, A. Kucerovy, K. Prasad, O. Repic, T. J. Blacklock, *Synth. Commun.* **1998**, *28*, 4009 – 4018.

[10] a) W. J. Dixon, F. Hibbert, *Can. J. Chem.* **1999**, *77*, 1035 – 1041; b) W. J. Dixon, F. Hibbert, J. F. Mills, *Perkin Trans. 2* **1997**, 1503 – 1509.

[11] Attempts to trap the putative nitrilium intermediate with nucleophiles other than water in ligations with 5-oxaproline gave only ester and amide products. Attempts to trap the intermediates formed from other hydroxylamines, such as *O*-Bz-hydroxylamines, were also unsuccessful.

[12] a) T. Narumi, J. W. Bode, *Heterocycles* **2011**, *82*, 1515 – 1525; b) N. Carrillo, E. A. Davalos, J. A. Russak, J. W. Bode, *J. Am. Chem. Soc.* **2006**, *128*, 1452 – 1453.

[13] a) G. Tuchscherer, A. Chandravarkar, M.-S. Camus, J. Bétard, K. Murat, A. Schmid, R. Mimna, H. A. Lashuel, M. Mutter, *Biopolymers* **2007**, *88*, 239 – 252, and references therein; b) M. Vila-Perelló, Y. Hori, M. Ribó, T. W. Muir, *Angew. Chem. Int. Ed.* **2008**, *47*, 7764 – 7767; *Angew. Chem.* **2008**, *120*, 7878 – 7881; c) L. Baumann, A. G. Beck-Sickinger, *Angew. Chem. Int. Ed.* **2013**, *52*, 9550 – 9553; *Angew. Chem.* **2013**, *125*, 9729 – 9732.

[14] L. A. Carpino, E. Krause, C. D. Sferdean, M. Schümann, H. Fabian, M. Bienert, M. Beyermann, *Tetrahedron Lett.* **2004**, *45*, 7519 – 7523.

[15] a) Y. Sohma, M. Sasaki, Y. Hayashi, T. Kimura, Y. Kiso, *Chem. Commun.* **2004**, 124 – 125; b) F. Liu, E. Y. Luo, D. B. Flora, A. R. Mezo, *Angew. Chem. Int. Ed.* **2014**, *53*, 3983 – 3987; *Angew. Chem.* **2014**, *126*, 4064 – 4068.

[16] a) Y. Sohma, Q.-X. Hua, J. Whittaker, M. A. Weiss, S. B. H. Kent, *Angew. Chem. Int. Ed.* **2010**, *49*, 5489 – 5493; *Angew. Chem.* **2010**, *122*, 5621 – 5625; b) M. Avital-Shmilovici, K. Mandal, Z. P. Gates, N. B. Phillips, M. A. Weiss, S. B. H. Kent, *J. Am. Chem. Soc.* **2013**, *135*, 3173 – 3185.

