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Product Selectivity in KAHA Ligations: Ester vs. Amide Formation with Cyclic Hydroxylamines

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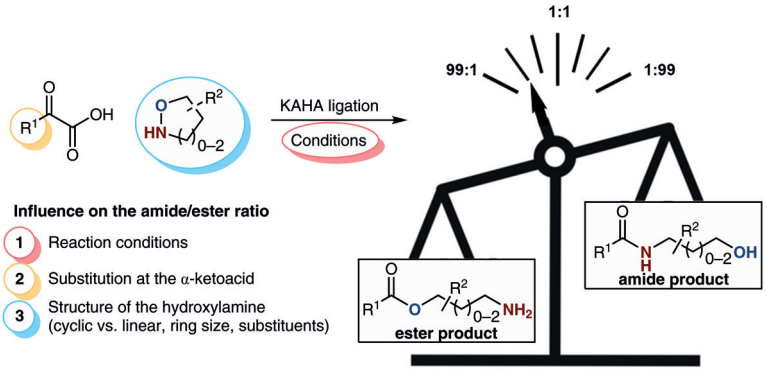

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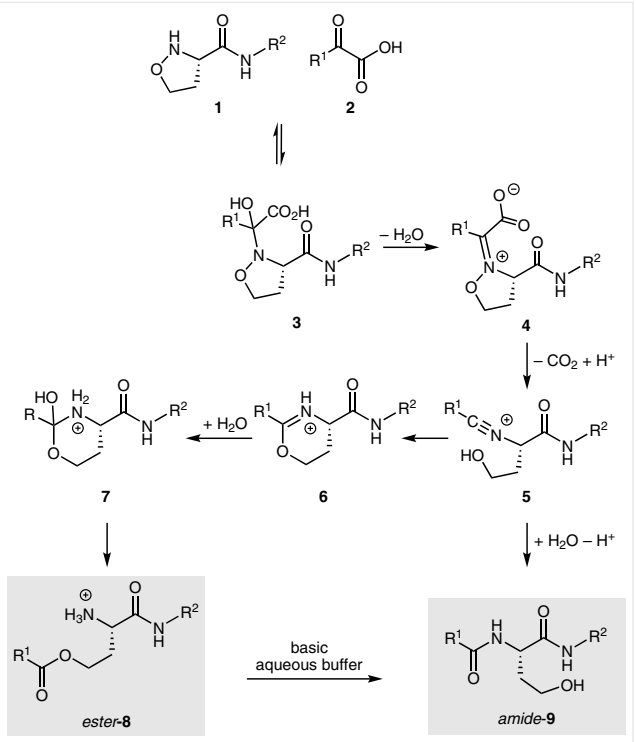
Abstract Cyclic hydroxylamines form esters instead of the expected amides as major product upon reaction with α-ketoacids. In this report, we document a systematic investigation into the effect of the hydroxylamine structure and the solvent mixture on the product ratio of amides vs. ester in the KAHA ligation. We show that the ratio is almost exclusively determined by the structure of the hydroxylamine, with only minor contributions from the reaction solvent or the structure of the α-ketoacid.

Key words ligation, mechanism, hydroxylamines, α-ketoacids, esters, peptides

In 2006, we reported the α-ketoacid-hydroxylamine (KAHA) amide-forming ligation,[1] which complements the well-established native chemical ligation[2] (NCL) for the chemical synthesis of proteins by chemoselective couplings of unprotected peptide segments.[3]

The most widely used implementation of the KAHA ligations uses a cyclic hydroxylamine – 5-oxaproline – as stable and chemoselective ligation partner that forms a homoserine residue upon ligation with α-ketoacids.[4] The primary product of this reaction is ester **8** (Scheme 1), which is rearranged to amide **9** under basic conditions.[5] In contrast, the oxazetidine analogue of 5-oxaproline (four- vs. five-membered ring) exclusively yields the amide product.[6]

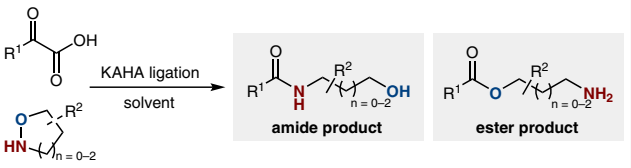
We have previously demonstrated that the structure of the α-ketoacid only has a minor influence on the ester to amide product ratio (Table 1).[5] We have not, however, reported a systematic investigation into the effect of the hydroxylamine structure on the formation of amides vs. esters in the KAHA ligation. In this report, we document comprehensive studies on the effect of hydroxylamine structure on the product distribution and consider the implications of these results for the understanding of the mechanism of the KAHA ligation.



Scheme 1 Current understanding of the mechanism

In a preliminary study we could isolate iminoether **6** as an intermediate of the reaction leading to the ester product and hypothesized that nitrilium **5** could be a reactive intermediate leading to the formation of iminoether **6**.[7]

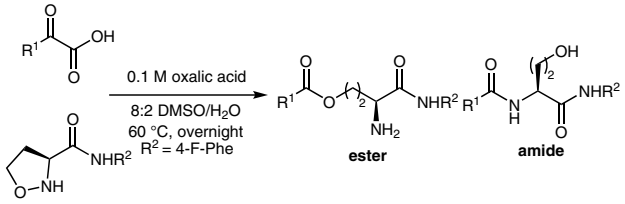
As show in Scheme 2, we expected the main influences on the product selectivity to be: 1) solvents and 2) the structure of the hydroxylamine (variation of n and R^2). We therefore carried out ligations with small model peptides.

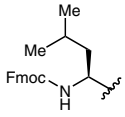
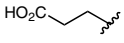


Scheme 2 Evaluation of various hydroxylamines to assess product selectivity of ester vs. amide product

We assessed the influence of solvents by varying the ratio of water/organic solvent and changing the organic solvent itself. Restricting the organic solvents to those capable of solubilizing proteins we chose dimethylsulfoxide (DMSO), *N*-methylpyrrolidone (NMP), and MeCN. The product ratio of the reaction between tripeptide α-ketoacid **10** and 5-oxaproline-containing dipeptide **11** was determined by analytical HPLC. As shown in Table 2 most amide product *amide*-**12** was formed in pure water (Table 2, entry 6). Under our standard conditions (7:3 organic solvent/H_2O; Table 2, entries 1 and 2) a ratio of approximately 9:1 is observed independent on the organic solvent. Using almost pure organic solvents (99:1; Table 2, entries 3, 4, and 5) the ratio of the ester increased up to 20:1 in the case of DMSO and MeCN and remained at 9:1 in the case of NMP.

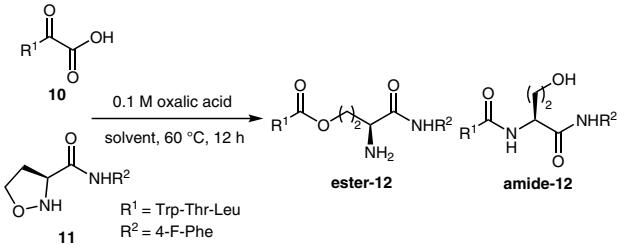
Table 1 Influence of the α-Ketoacid on Product Selectivity



Entry	R^1	Ratio ester/amide[a]	Isolated yield of ester (%)
1		93:7	61
2		93:7	59
3		86:14	59
4		87:13	55

[a] Determined by analytical HPLC of the reaction mixture at 220 nm.

Table 2 Influence of the Solvent on Product Selectivity



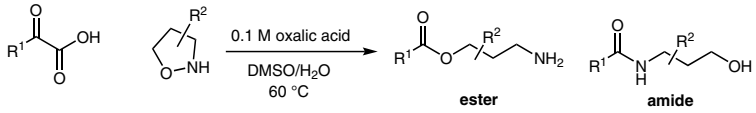
Entry	Solvent	Ester (%)[a]	Amide (%)[a]
1	7:3 DMSO/H_2O	89	11
2	7:3 NMP/H_2O	87	13
3	99:1 DMSO/H_2O	97	3
4	99:1 NMP/H_2O	90	10
5	99:1 CH_3CN/H_2O	95	5
6	H_2O	73	27

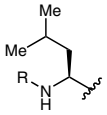
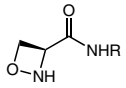
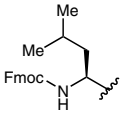
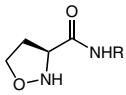
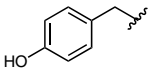
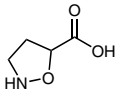
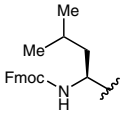
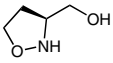
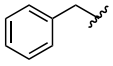
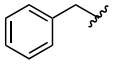
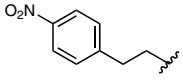
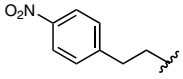
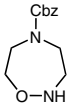
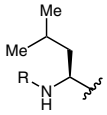
[a] Determined by analytical HPLC of the reaction mixture at 220 nm.

Various types of hydroxylamines undergo KAHA ligation with α-ketoacids. O-Unsubstituted hydroxylamines – which we have previously classified as type I hydroxylamines – appear to react by a different mechanism and are not part of this study.[8] Substituted hydroxylamines – which we have previously termed type II hydroxylamines – proceed via a different mechanism. We have reported two major types of substituted hydroxylamines: 1) *O*-acyl and *O*-carbamoyl hydroxylamines and 2) *O*-alkyl hydroxylamines. Linear *O*-alkyl hydroxylamines react with α-ketoacids only very slowly and under forcing conditions. In contrast, strained, cyclic hydroxylamines are excellent reaction partners in the KAHA ligation, although in the case of 5-oxaproline the ester is observed as the major product.

We synthesized cyclic hydroxylamines (Table 3) to probe their influence on the product selectivity. As previously reported, four-membered 1,2-oxazetidines exclusively yield the amide product at a much faster reaction rate (Table 3, entry 1).[6] 5-Oxaproline (Table 3, entry 2) affords a mixture of ester and amide product in a ratio of 9:1, while the regioisomer of 5-oxaproline (Table 3, entry 3) exclusively yields the ester product. 5-Oxaprolinol (Table 3, entry 4) showed essentially the same product distribution of 9:1 as 5-oxaproline. Unsubstituted isoxazolidine (Table 3, entry 5) and 1,2-oxazinane (Table 3, entry 6) showed a slight increase in amide product (8:2 and 6:4) with increasing ring size, along with a decrease in the reaction rate. The seven-membered 1,2,5-oxadiazepanes (Table 3, entries 7 and 8) afforded a 7:3 mixture of the ester and amide products, regardless whether the transannular amine is protected or not. The linear analogue of isoxazolidine (Table 3, entry 9) exclusively yields the amide product.

Table 3 Influence of the Hydroxylamine on Product Selectivity



Entry	R^1	Hydroxylamine	Ratio ester/amide[a]	Isolated yield of ester (%)
1[b]	 R = Fmoc-Ala-	 R = Gly-Phe-Gly	<1:99[c]	ester: 0 amide: 69
2[d]		 R = 4-F-Phe	93:7	61
3[e]			>99:1[f]	40
4[g]			92:8	43
5[h]			79:21[i]	27
6[h]			58:42[i]	14
7[j]			71:29	25
8[j]			73:27	27
9[k]	 R = Trp-Thr-		<1:99[c]	79

[a] Determined by analytical HPLC of the reaction mixture at 220 nm.
[b] Previously reported (see Supporting Information of ref. 6), in 10:1 DMSO/H_2O at r.t. for 30 min.
[c] Only the amide product was observed.
[d] Previously reported (see Supporting Information of ref. 5), in 8:2 DMSO/H_2O overnight.
[e] In 8:2 DMSO/H_2O for 5 h, racemic hydroxylamine was used.
[f] Only the ester product was observed.
[g] In 8:2 DMSO/H_2O for 2 h.
[h] In 8:2 DMSO/H_2O for 14 h.
[i] Based on isolated yields after purification by preparative HPLC.
[j] In 9:1 DMSO/H_2O for 12 h.
[k] In 7:3 DMSO/H_2O for 16 h.

The remarkable difference in product distribution of ligations with isoxazolidine and its linear analogue is illustrated in Scheme 3 (A and B). Even though **18** contains a hydroxyl group that would allow a similar 6-*endo*-dig attack as observed for KAHA ligations with isoxazolidine (**14**), it exclusively yields the amide product. When **18** is used in KAHA ligations under anhydrous conditions (Scheme 3, C) we observed both amide and ester products in a ratio of 8:2.

These results could be most easily be explained by the formation of the ion pair **15** upon decarboxylation in the case of cyclic hydroxylamines. This ion pair could readily undergo 6-*endo*-dig cyclization to form the iminoether, leading ultimately to the ester product. DFT calculations implicate the formation of similar ion pairs for Ugi reactions.[9] Since this ion pair is unlikely to be formed with the (protonated) hydroxyl group of linear hydroxylamine **18**, 6-*endo*-dig cyclization is slower and is only observed under strictly anhydrous conditions.

In summary, we have shown that cyclic hydroxylamines – with the notable exception of four-membered 1,2-oxazetidines – afford the ester product in significant amounts.[10] The ester-to-amide ratio is almost exclusively determined by the structure of the hydroxylamine, with only minor contributions from the reaction solvent or the structure of the α-ketoacid. These experiments provide further data supporting a nitrilium intermediate in type II KAHA ligations and suggest that ester formation is preferred in cyclic hydroxylamines due to the formation of an ion pair with favorable geometry for intramolecular cyclization. We believe that these insights will support the further improvement of the KAHA ligation and its application in chemical protein synthesis.

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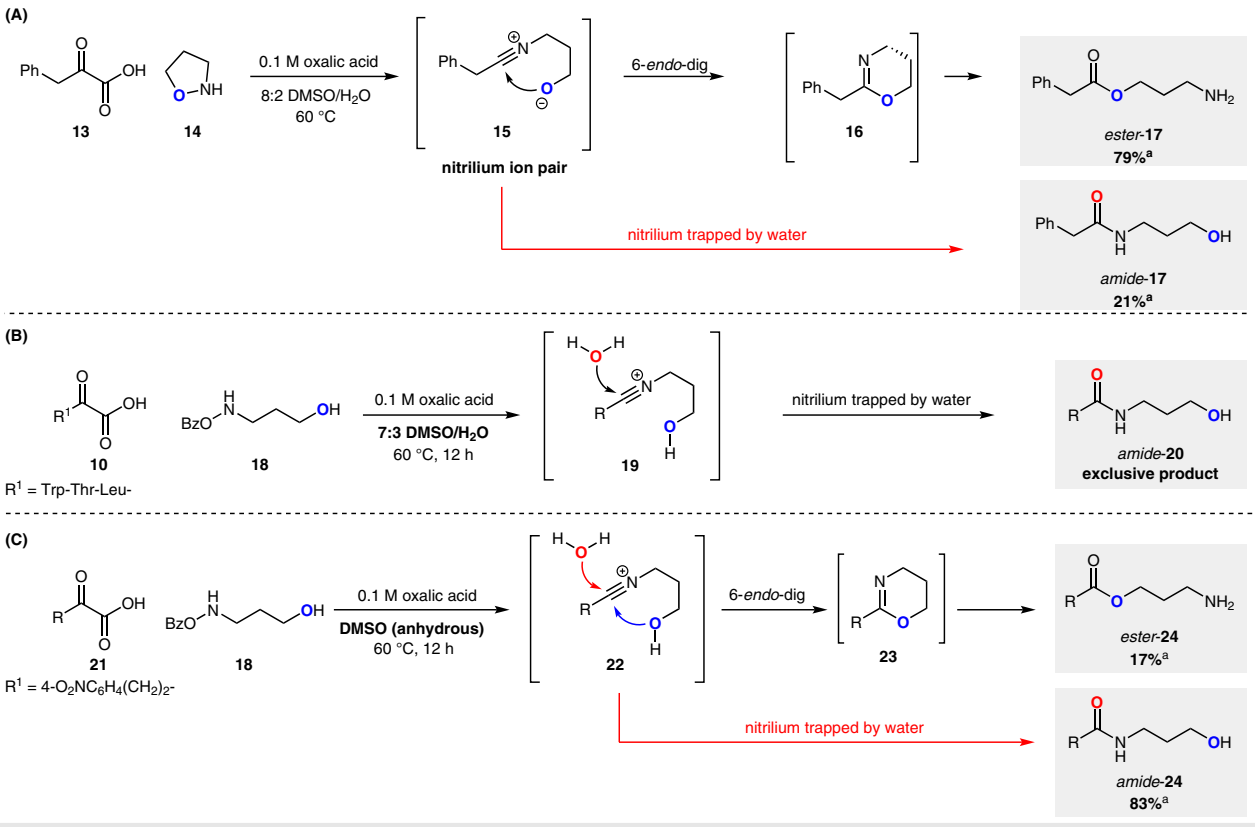
Supporting Information

Supporting information for this article is available online at https://doi.org/10.1055/s-0036-1588480.



Scheme 3 KAHA ligations with (A) isoxazolidine, (B) linear hydroxylamine **18**, and (C) linear hydroxylamine **18** under anhydrous conditions. [a] Ester-to-amide ratio.

References and Notes

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(9) Chéron, N.; Ramozzi, R.; Kaïm, L. E.; Grimaud, L.; Fleurat-Lessard, P. *J. Org. Chem.* **2012**, *77*, 1361.
(10) **General Procedure for KAHA Ligations**
Ketoacid (1.00–2.00 equiv) and hydroxylamine (1.00–2.00 equiv) were dissolved in 8:2 DMSO/H_2O (20–100 mM) and heated to 60 °C for 12 h. Reaction progress was monitored by analytical HPLC (Shiseido Capcell Pak UG80 C18 column (4.6 × 250 mm), heated to 60 °C, 30–70% MeCN in 20 min). The crude reaction was directly purified by preparative HPLC (Shiseido Capcell Pak MGII C18 column, 20 × 250 mm, r.t., 40–90% MeCN with 0.1% TFA in 20 min, flow rate 10 mL/min).
Typical Analytical Data
***N*-(3-Hydroxypropyl)-3-(4-nitrophenyl)propanamide (*Amide*-24)**
^{1}H NMR (600 MHz, $CDCl_3$): δ = 8.14–8.10 (m, 2 H, CH), 7.38–7.34 (m, 2 H, CH), 6.07 (br s, 1 H, NH), 3.54 (t, *J* = 5.6 Hz, 2H, CH_2), 3.37 (q, *J* = 6.1 Hz, 2 H, CH_2), 3.13 (br s, 1 H, OH), 3.07 (t, *J* = 7.5 Hz, 2 H, CH_2), 2.53 (t, *J* = 7.5 Hz, 2 H, CH_2), 1.66–1.59 (m, 2 H, CH_2). ^{13}C NMR (150 MHz, $CDCl_3$): δ = 172.4 (CO), 148.8 (C), 146.7 (CNO_2), 129.4 (CH), 123.9 (CH), 59.6 (CH_2), 37.5 (CH_2), 36.7 (CH_2), 32.1 (CH_2), 31.4 (CH_2). ESI-HRMS: *m/z* calcd for $C_{12}H_{17}N_2O_4$ [M + H]$^+$: 253.1183; found: 253.1183.
3-{[3-(4-Nitrophenyl)propanoyl]oxy}propan-1-aminium 2,2,2-trifluoroacetate (*Ester*-24)
^{1}H NMR (600 MHz, $(D_3C)_2SO$): δ = 8.19–8.14 (m, 2 H, CH), 7.73 (br s, 3 H, NH_3^+), 7.56–7.51 (m, 2 H, CH), 4.06 (t, *J* = 6.3 Hz, 2 H, CH_2), 3.00 (t, *J* = 7.5 Hz, 2 H, CH_2), 2.82 (t, *J* = 7.4 Hz, 2 H, CH_2), 2.73 (t, *J* = 7.5 Hz, 2 H, CH_2), 1.88–1.80 (m, 2 H, CH_2). ^{13}C NMR (150 MHz, $(D_3C)_2SO$): δ = 171.9 (CO), 157.9 (q, *J* = 32.0 Hz, CF_3COO^-), 148.9 (C), 146.1 (CNO_2), 129.7 (CH), 123.5 (CH), 117.36 (q, *J* = 301 Hz, CF_3), 61.3 (CH_2), 36.2 (CH_2), 34.1 (CH_2), 29.9 (CH_2), 26.3 (CH_2). ESI-HRMS: *m/z* calcd for $C_{12}H_{17}N_2O_4$ [M + H]$^+$: 253.1183; found: 253.1186.