

# Reinventing Amide Bond Formation

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**Abstract** The chemical synthesis of peptides and proteins has long relied on innovative inventions of amide-forming reactions. Our group has discovered and developed an amide-forming ligation reaction by the coupling of  $\alpha$ -ketoacids and hydroxylamine (KAHA ligation). This reaction does not require reagents or catalysts, proceeds in the presence of unprotected functional groups, and generates no nonvolatile by-products. This chapter recounts our discovery of this reaction, our development of novel methods for the preparation of  $\alpha$ -ketoacids and hydroxylamines, and the application of these new methods to the synthesis of peptides and proteins.

**Keywords** Amide bonds · Mechanism · Peptides · Protein synthesis · Reaction development

## Contents

1	Introduction .....	14
2	Defining the Research Target .....	15
3	The $\alpha$ -Ketoacid–Hydroxylamine (KAHA) Amide-Forming Ligation .....	16
3.1	Invention of the KAHA Ligation .....	16
3.2	The KAHA Amide Formation as a General Peptide Ligation .....	19
3.3	Chemoselective Synthesis of C-Terminal Peptide $\alpha$ -Ketoacids .....	20
3.4	Synthesis of N-Terminal Peptide Hydroxylamines .....	23
3.5	KAHA Ligation for the Synthesis of Therapeutic Peptides: GLP-1 .....	25
3.6	Cyclization of Unprotected Linear Peptides .....	26
3.7	2010: A Critical Assessment of the KAHA Ligation .....	27
3.8	The Mechanism of the KAHA Ligation .....	28
3.9	The Next Steps ... Protein Synthesis by KAHA Ligation .....	30
4	Outlook and Expectations .....	31
	References .....	31

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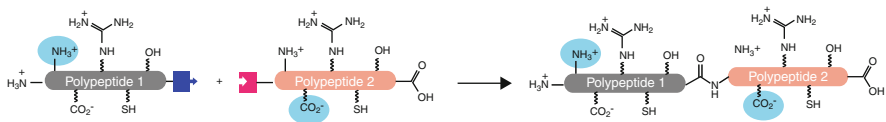
## Abbreviations

Ac	Acetyl
Acac	Acetylacetonate
Alk	Alkyl
Btw	By the way
Etc	Et cetera

## 1 Introduction

The majority of my research group currently works on some aspect of amide bond formation. This work now encompasses a broad range of applications, reaction types, and mechanistic paradigms. This focus—my graduate students might say obsession—began as a conversation over breakfast in Tokyo on 26 January 2003 with Prof. Naoki Umezawa, now at the School of Pharmaceutical Science at Nagoya City University. Naoki and I were discussing why it is so difficult to go directly from genetic information to a protein. Naoki, a bioorganic chemist, was asking me, a synthetic organic chemist, why organic chemists cannot readily synthesize the protein he was interested in studying? To make his proteins, he pointed out, he had to rely on bacteria.

After this conversation, I began to think and read more deeply about the problem. For the organic chemists of my generation, it was widely assumed that peptide and protein synthesis was largely a solved problem. I initially shared this naïve belief—and suffered later from reviewers who held similar views. I knew, of course, about the power of Merrifield's solid-phase peptide synthesis but soon learned from the literature that routinely making peptides longer than approximately 40 residues could be very difficult and was not routine [1, 2]. I was also not aware of the economical and environmental costs of solid-phase peptide synthesis and was surprised to learn that the cost of peptide synthesis can be roughly calculated as \$300/residue/gram for natural peptides and as much as 10 times that for unnatural amino acids [3]. I also knew of the beautiful and impressive native chemical ligation of peptide thioesters and peptides and proteins containing N-terminal cysteines [4]. I was surprised to learn, however, that cysteine is one of the rarest amino acids and that they are not present in all proteins. The 864 amino acid *taq* DNA polymerase routinely used for PCR, for example, does not contain a single cysteine! I also learned that the biggest challenge to NCL, at least at that time, was the preparation of the peptide thioesters [5]. These had to be prepared by Boc chemistry, rather than the more user-friendly Fmoc chemistry, restricting their synthesis to hard-core peptide chemists and precluding many desirable peptides including glycopeptides.



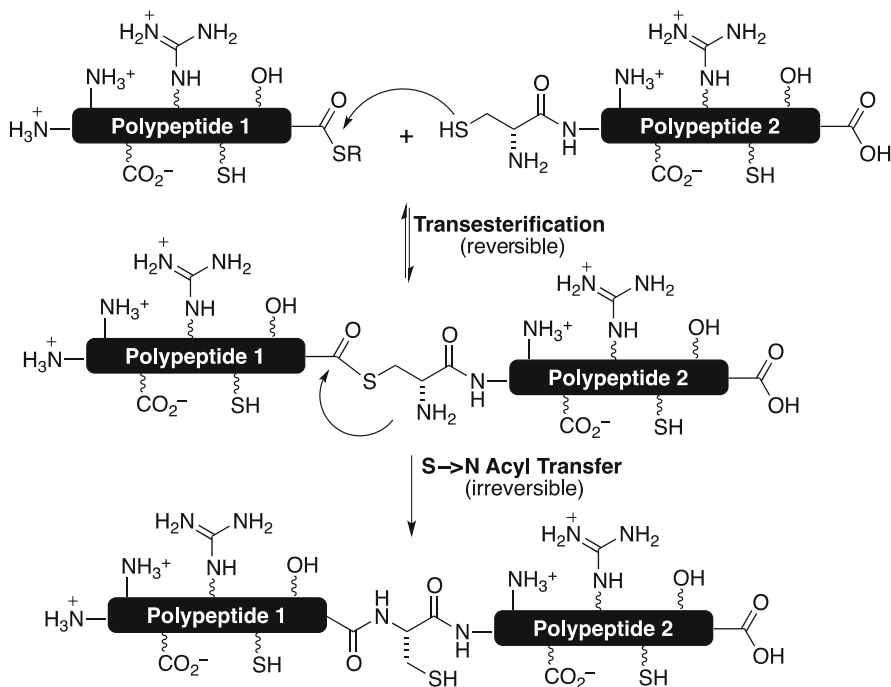
**Scheme 1** Cartoon of a peptide ligation. The substrates combine chemoselectively to form a backbone amide bond in the presence of unprotected side chains, under water-compatible conditions, and without reagents or catalysts

## 2 Defining the Research Target

The almost magical chemistry of the native chemical ligation was an inspiration and set a very high bar for innovation. It made clear that any advance in synthetic protein chemistry would have to come in the form of a *peptide ligation reaction* that allowed two completely unprotected fragments to be selectively joined under water-compatible conditions. Furthermore, the reaction must work at low reactant concentrations and should not produce any toxic or reactive by-products. It would have to be a “general” reaction—not limited to sulfur-containing residues and ideally applicable to amide types other than  $\alpha$ -peptides. Put another way, our task was to figure out what chemical functionalities the cartoon “puzzle pieces” in Scheme 1 could be.

This was obviously not a simple endeavor, and we were by no means the first to contemplate this problem. The goal of chemoselective peptide ligation had been outlined many years before us [6, 7]. The native chemical ligation had demonstrated that such peptide-forming ligations were indeed possible and had already begun to revolutionize the field of synthetic protein chemistry. A particularly clear and honest review by Kent published in 2000 outlined both the power and promise of native chemical ligation and highlighted the limitations [8]: the preparation of the fragments by solid-phase peptide synthesis and the requirement for a relatively rare and sometimes problematic cysteine residue at the ligation site.

When I began my first independent position in August 2003 at the University of California, Santa Barbara, we had two amide-forming projects in mind: (1) to devise an approach for catalytic, enantioselective peptide synthesis, which could potentially lower the cost and waste associated with classical solid-phase peptide synthesis, and (2) to develop a new peptide ligation that met or exceeded the specifications of the native chemical ligation. Both of these goals shared the same problem: the known mechanistic paradigms for amide formation were inadequate. Therefore, our first goal became the invention of mechanistically novel approaches to amide bond formation. This line of thinking led to two of the major lines of research in my group: (1) N-heterocyclic carbene-catalyzed generation of reactive intermediates (for an account of the new amide, ester, and C–C bond-forming reactions that this research program has led to, see [9]) and (2) the development of new amide-forming ligation reactions. This chapter will describe our discovery and development of the latter.



**Scheme 2** The native chemical ligation (NCL) of C-terminal thioesters and peptides containing an N-terminal cysteine

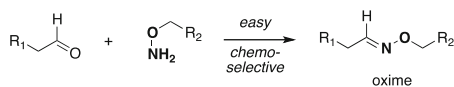
### 3 The $\alpha$ -Ketoacid–Hydroxylamine (KAHA) Amide-Forming Ligation

The native chemical ligation, which is the chemical reaction that most closely meets the challenge set forth in Scheme 1, works because of the remarkably selective and reversible exchange of peptide thioesters under the reaction conditions (Scheme 2). When the thioester exchange occurs with the sulfur atom of an N-terminal cysteine residue, an irreversible migration can occur to give the amide bond. All of this occurs rapidly and in the presence of unprotected functional groups. The number of intermolecular organic reactions that proceed selectively, in water, and without reagents can be counted on one hand.

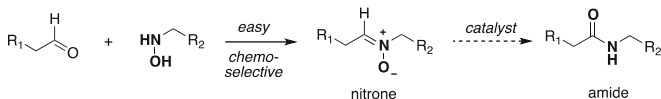
#### 3.1 *Invention of the KAHA Ligation*

We began our efforts with one of these privileged reactions—oxime formation. It had long been recognized that the coupling of *O*-alkylhydroxylamines and aldehydes was rapid and selective, making it suitable for bioconjugation reactions (Scheme 3) [10]. Less well known is that *N*-alkylhydroxylamines also couple

• **Oxime Formation:** (a chemoselective reactions)



• **Our initial research goal:** catalytic rearrangement of nitrones to amides



**Scheme 3** The thinking behind our eventual development of the KAHA ligation. We recognized that nitrones, like oximes, are easily formed under aqueous, chemoselective conditions. We sought to selectively convert the unstable nitron to the stable amide

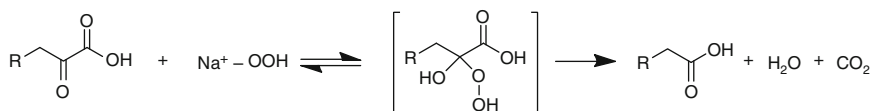
rapidly with aldehydes to give nitrones. The reaction is reversible, but nitron formation is often fast and selective [11]. This analysis led to our first idea: develop a catalyst for the rearrangement of nitrones to amides. This is a reaction that is known under photochemical conditions [12], but its low efficiency makes it unlikely to be useful for peptide ligation. We sought, instead, to find a catalyst or reagent to affect the conversion of the nitron to the amide. The overall process is thermodynamically favored, and a number of reasonable mechanisms can be drawn. Despite our efforts, however, a suitable catalyst was never found—we still believe this would be a great transformation!

These investigations proved essential to our further thinking. In preparing various nitrones as substrates for catalytic rearrangement to amides, we learned a few important things about the chemistry of nitrones. First, we found that the formation of nitrones from *aldehydes* and *N*-alkylhydroxylamines is fast and selective. These two functional groups have a very high affinity for one another, and although the nitron formation is reversible, the equilibrium lies completely on the side of nitron (something that would come back to haunt us later). In contrast, the formation of nitrones from *ketones* had been reported to be slow, including one report that the equilibrium mixture favored the hemiaminal [13]. Second, we had used several oxidative nitron formations including the work of Murahashi, who had reported the synthesis of nitrones by oxidative decarboxylation of amino acids [14] (Scheme 4).

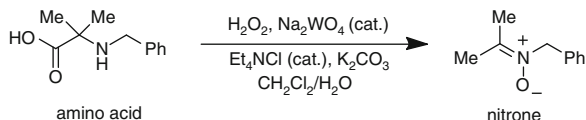
These key precedents were combined, mentally at least, into an amide-forming reaction on a flight from Santa Barbara to Albuquerque, New Mexico, in November 2003. I hurried back a few days later to try out the reaction in the lab. Both  $\alpha$ -ketoacids and hydroxylamines are commercially available, and it was simply a matter of mixing the two components together and looking for amide formation. We tried several solvents and on our first attempt found the desired amide product when CH<sub>3</sub>CN was used (Scheme 5). The yield was not great, but it was undeniably a new reaction for amide formation. Most importantly, it did not require any reagents or catalysts, produced only water and CO<sub>2</sub> and by-products, and did not involve an activated carboxylic acid [15].

As anticipated from the chemistry of oxime and nitron formation, we quickly found that the amide formation was remarkably chemoselective. Carboxylic acids,

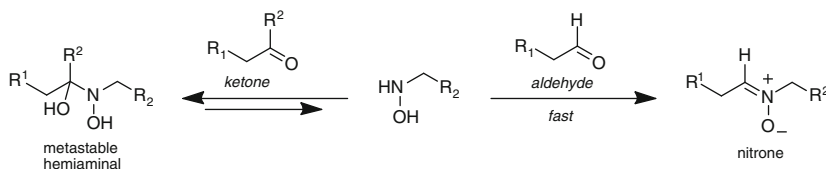
• **Oxidative decarboxylation of  $\alpha$ -ketoacids**



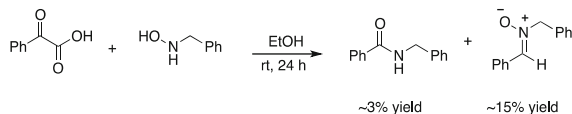
• **Oxidation of amino acids to nitrones via N-hydroxy intermediates**



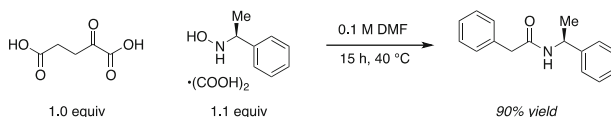
• **Formation of hemiaminals from hydroxylamines and ketones**



**Scheme 4** Three key precedents for the design and invention of the KAHA ligation



**Scheme 5** Our first attempt at the KAHA ligation (December 2003)



**Scheme 6** Conditions for the formation of simple amides by KAHA ligation

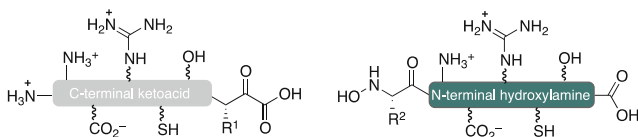
amines, alcohols, and other functional groups did not disturb the amide formation. The amide formation proceeded best in polar, aprotic solvents such as DMF and DMSO, but nonpolar solvents suppressed the amide formation. Protic solvents such as MeOH and aqueous acids also worked well. The  $\alpha$ -ketoacid and hydroxylamines could be used as their more easily handled salts, provided that the overall reaction was neutral or acidic. In all but a few cases, the amide ligations were remarkably clean. Years later, the “optimized” conditions for the formation of simple amides by KAHA ligation were published by Lei Ju in *Organic Synthesis* [16]. The experimental procedure is simply to combine an  $\alpha$ -ketoacid and the salt of a hydroxylamine in DMF and warm to 40 °C (Scheme 6). After a few hours or overnight, removal of the solvent or extraction gives the amide products.

We were surprised—and perhaps a bit lucky—to discover that the KAHA amide formation was not a known reaction. Months of literature searching turned up only a single example of a something similar: the conversion of pyruvic acid to a hydroxyamic acid [17, 18]. Perhaps the unconventional reactivity of the reaction partners explains this. Formally, the highly electrophilic  $\alpha$ -ketoacids acts as the “nucleophile” in the overall transformation. The nucleophilic hydroxylamine is the “electrophile” with the hydroxyl moiety as the leaving group. As we have learned over the years of studying this reaction and its starting materials, it is as if there is a parallel world of organic synthesis. For example, in many cases, we have found our chemistries work better in water, give higher yields in the presence of unprotected functional groups, and work most effectively on large molecules!

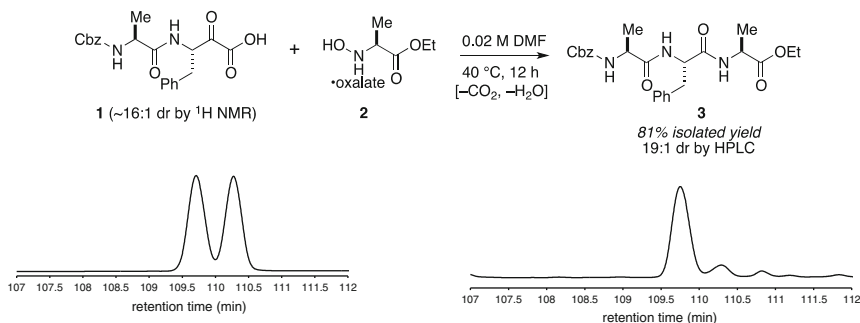
### 3.2 *The KAHA Amide Formation as a General Peptide Ligation*

At this point, we were faced with a strategic dilemma. The amide-forming reaction was interesting and novel, surely this merited publication in its own right. By the middle of 2004, we had done enough simple substrates and preliminary mechanistic investigations to prepare a communication. But we had not lost sight of our primary goal: a general peptide ligation that could be used to assemble proteins from constituent fragments. In our mind, establishing that the KAHA amide formation could be used for peptide ligation, at least in principle, was the necessary and appropriate step in its development. This was not only because of the importance of peptide ligation in general. We recognized that while the KAHA amide formation itself was simple, clean, and powerful, the starting materials were somewhat obscure; chemists were not going to synthesize  $\alpha$ -ketoacids and hydroxylamines just to convert them into amides. In the context of peptide ligation, however, the syntheses of these functional groups might be justified or even transparently incorporated into the reagents or linkers used for solid-phase peptide synthesis. At this juncture we decided to put all of our efforts into applying the KAHA ligation to the chemoselective ligation of unprotected peptide fragments, and graduate student Ryan Fox joined the group to make this happen.

The strategy was now clear: we would demonstrate that the KAHA ligation could be used for the union of unprotected peptide fragments simply by mixing a peptide containing a C-terminal  $\alpha$ -ketoacid with a peptide containing an N-terminal hydroxylamine amine. There was only one problem with this strategy: no one had ever made such compounds before! Furthermore, to be relevant to the field of peptide chemistry, we needed synthetic routes to these compounds that (1) could operate in the presence of all of the functional groups found in unprotected peptide side chains, (2) preserved the stereochemistry of the  $\alpha$ -amino acid and did not epimerize, and (3) were compatible with Fmoc-based solid-phase peptide synthesis (Scheme 7). As we set out on this task, we did not fully appreciate what we were committing to.



**Scheme 7** Our new research goals: the synthesis of enantiopure, side-chain unprotected peptide  $\alpha$ -ketoacids and hydroxylamines



(a) HPLC chromatograph of *epi*-3 prepared from Z-Ala-D/L-Phe-CO-COOH.

(b) HPLC chromatograph of 3 prepared by KAHA ligation with ketoacid 13 (Z-Ala-Phe-CO-COOH, 16:1 dr).

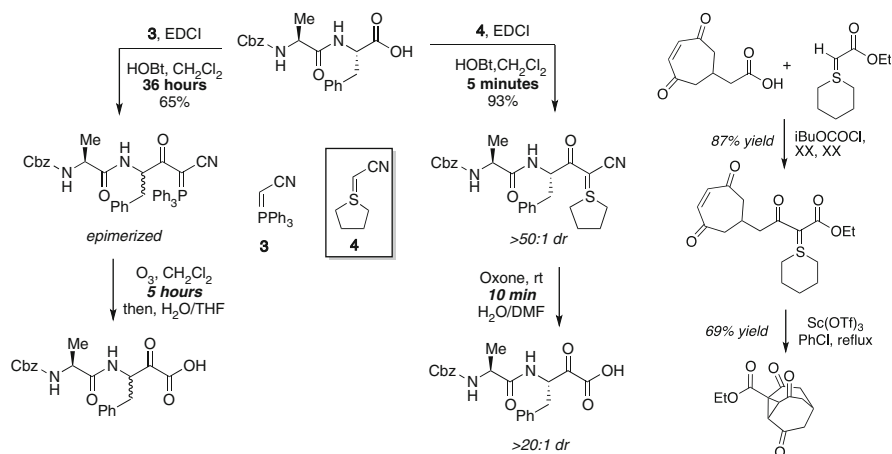
**Scheme 8** An early demonstration that enantiomerically enriched  $\alpha$ -ketoacids do not epimerize under the conditions for the KAHA ligation. Actually synthesizing enantiopure peptide  $\alpha$ -ketoacids is the real challenge

### 3.3 Chemoselective Synthesis of C-Terminal Peptide $\alpha$ -Ketoacids

The most important thing to recognize about  $\alpha$ -ketoacids is that they are neither ketones nor carboxylic acids. Yes, they maintain some characteristics of these functional groups, but they have unique and often unpredictable chemistry of their own. For example, it is usually difficult to directly couple an  $\alpha$ -ketoacid to an amine with standard coupling reagents.

An  $\alpha$ -ketoacid does not always act like a ketone, but it still undeniably has ketone character. When complemented with an  $\alpha$ -stereocenter, there is always the possibility of epimerization. Much of our early work on peptide ligation was spent establishing that the peptide  $\alpha$ -ketoacids do not epimerize under the ligation conditions. Through laborious methods, we prepared a few model peptides to demonstrate that under the acidic conditions we preferred for the KAHA ligation,  $\alpha$ -ketoacids were largely configurationally stable (Scheme 8). With a few exceptions, our years of experience with  $\alpha$ -ketoacid confirm that they are configurationally and chemically stable when they are pure. Much to my surprise, my students found that the best way to do this is to first purify them by preparative HPLC. This is now common practice in our lab.

The actual synthesis of enantiopure  $\alpha$ -ketoacids was the real challenge, and initially the best we could do was about 85% ee for protected dipeptides. There

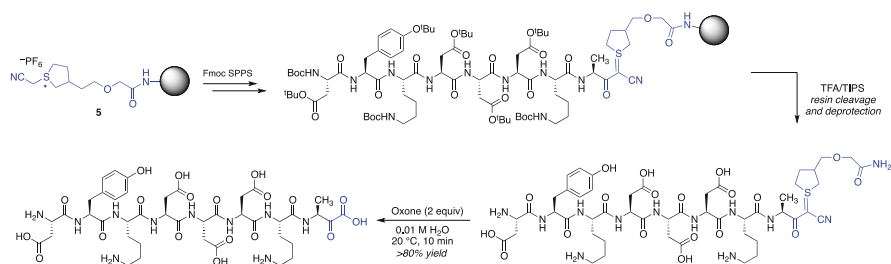


**Scheme 9** Formation of  $\alpha$ -ketoacids by oxidation of phosphorous and sulfur ylides. This work was inspired by sulfur ylide chemistry used in our synthesis of bullvalene

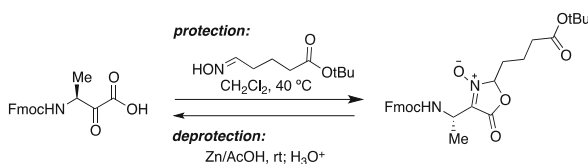
were almost no examples of peptide  $\alpha$ -ketoacids and very few examples of enantioenriched  $\alpha$ -ketoacids in general. The closest examples to our targets were the peptide  $\alpha$ -ketoesters and  $\alpha$ -ketoamides prepared by Wasserman using his elegant phosphonium ylide chemistry [19]. This chemistry initially looked very attractive for our purposes, especially as Rademann had reported a solid-supported variant that could be employed for solid-phase peptide synthesis [20]. This phosphorus chemistry was used for our first foray into ligations of  $\alpha$ -peptides. But we quickly found serious problems.

First, with peptide monomers, the formation of the phosphonium ylides proceeded acceptably. But with larger peptides, the rate of coupling became intolerably slow, low yielding, and gave complete epimerization (Scheme 9a). We believed that this could be fixed by working on a solid support with routes that did not require transformation of the peptide C-terminus. Second, the larger problem was the conversion of the phosphorous ylide to the  $\alpha$ -ketoacid. This required exhaustive ozonolysis, often requiring several hours of treatment with ozone at low temperatures followed by treatment with water. This difficult procedure could not be extended to larger peptides due to poor solubility and serious problems with epimerization.

A solution came from a completely different project in our group: the synthesis of shape-shifting, adaptive organic molecules [21, 22]. Alex Lippert, a graduate student, had devised a synthesis of oligosubstituted bullvalenes that featured and intramolecular cyclopropanation using a stabilized sulfur ylide (Scheme 9b). Unlike the phosphorous ylides, we had found the formation of the sulfur ylides to be fast and high yielding. We were pleased to find the same when we used peptide substrates and cyanosulfonylide **2**. Furthermore, Alex and another graduate student, Lei Ju, devised a procedure that used the easily handled sulfonium salt and standard peptide coupling conditions [23]. Most importantly, we found that the sulfur ylides



**Scheme 10** Synthesis of C-terminal peptide  $\alpha$ -ketoacids with solid-supported sulfur ylide linker **5**. This methodology has been routinely applied for the synthesis of unprotected  $\alpha$ -ketoacids up to 35 residues long

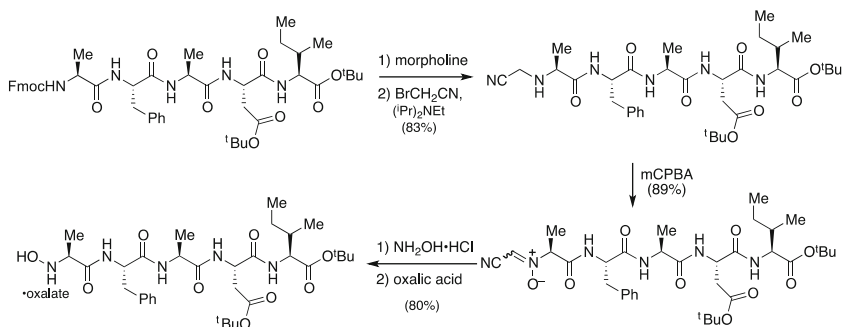


**Scheme 11** Protection of  $\alpha$ -ketoacids by direct annulations with oximes

were much more easily oxidized than their phosphorous counterparts. Exposure of fully unprotected C-terminal peptide sulfur ylides to aqueous Oxone in DMF gives the  $\alpha$ -ketoacid within minutes. If there is a problem with this reaction, it is that it can be too fast and we often have to adjust the temperature and concentration to regulate the reaction times.

Lei went on to develop solid-supported sulfur ylide linker **5** that has become the standard practice in our group for preparing C-terminal peptide  $\alpha$ -ketoacids (Scheme 10) [24]. Once the linker is loaded onto the resin, standard Fmoc-based solid-phase peptide synthesis can be used to grow peptide chains without difficulty. Deprotection and cleavage from the resin gives the fully unprotected peptide sulfur ylides that are easily handled and purified by standard methods. Exposure of these peptides to Oxone or DMDO gives the peptide  $\alpha$ -ketoacids, which are usually isolated by preparative HPLC and lyophilized. Just one example out of many is shown in Scheme 10.

The oxidation of the sulfur ylides to the  $\alpha$ -ketoacids is remarkable for its generality, functional group tolerance, and reliability. It is, however, an oxidative process and is not currently compatible with the sulfur-containing side chains cysteine and methionine. We have therefore devised other protecting groups for  $\alpha$ -ketoacids such as cyclic nitrones (Scheme 11) [25]. Graduate student Melissa Flores found that  $\alpha$ -ketoacids can be protected as cyclic nitrones by an unexpectedly facile cyclization with oximes. This and other strategies under development in our group can be used to prepare peptide  $\alpha$ -ketoacids with methionine or cysteine residues. All of the enantiopure  $\alpha$ -ketoacid starting materials, however, are prepared with sulfur ylide chemistry.



**Scheme 12** Our original, inconvenient route to N-terminal peptide hydroxylamines by solution phase conversion of an N-terminal amine to the hydroxylamine

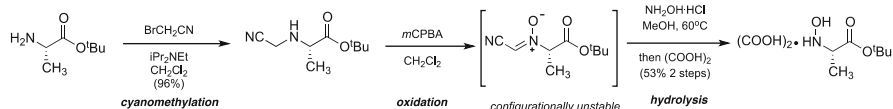
### 3.4 Synthesis of N-Terminal Peptide Hydroxylamines

When we first disclosed the KAHA ligation, the general sentiment was that making the  $\alpha$ -ketoacids would be the major problem. In contrast, most chemists did not feel that the peptide hydroxylamines would present a serious obstacle. This intuition was supported by the literature. There were only a few scattered reports of peptide  $\alpha$ -ketoacids but a respectable body of literature, including a *Chemical Reviews* article, on *N*-hydroxyamino acids and peptides [26].

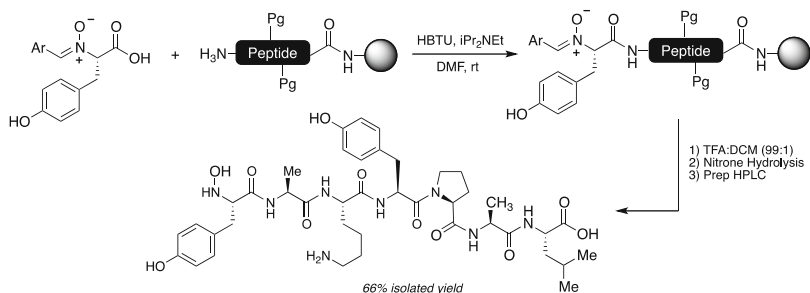
We quickly found that the major problem was the identification of a suitable protecting group for the introduction of *N*-hydroxyamino acid monomers onto the end of a growing peptide chain. Like  $\alpha$ -ketoacids, hydroxylamines are not simply the sum of their functional groups; they are not alcohols and are not amines. If anything they are the worst of both groups! They are susceptible both to oxidation and reduction, as well as elimination and epimerization. Standard amine protecting groups do not stay for long on the nitrogen atom, and if the nitrogen is suitably protected, the oxygen atom becomes difficult to mask.

In our early studies we avoided the issue by preparing short peptide fragments and converting the N-terminus to the hydroxylamine by a variation of Fukuyama's outstanding method (Scheme 12) [27, 28]. This was effective but not convenient. Following our first publication, we returned to the problem of peptide hydroxylamine synthesis.

The Fukuyama method was the most general and reliable approach to making peptide hydroxylamines. It suffered from two problems: (1) we had occasionally noted some epimerization during the three step process, and (2) it was rather long, synthetically involved, and not suitable for longer peptides. Diligent studies by Lei Ju and graduate student Irene Medina traced the epimerization to the "cyan-nitrone" intermediate. If this was hydrolyzed immediately, epimerization could be avoided. With some improvements, the Fukuyama method proved highly suited for the preparation of *N*-hydroxyamino acid monomers in enantiomerically pure form (Scheme 13). Introducing these monomers onto the end of a growing peptide,



**Scheme 13** Synthesis of enantiopure *N*-hydroxy  $\alpha$ -amino acids with Fukuyama's method

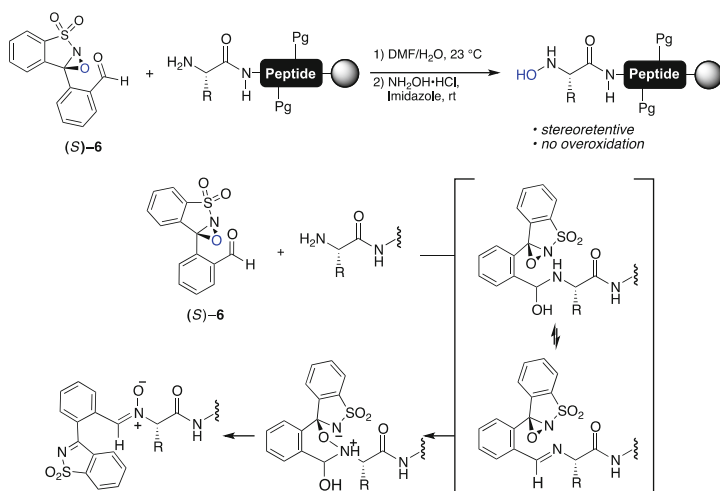


**Scheme 14** Coupling of nitronitrone protected hydroxylamines to the N-terminus of a solid-supported peptide followed by resin cleavage and deprotection

however, initially proved to be problematic due to the failure of common protecting groups to mask the hydroxylamine.

An extensive survey of protection strategies suitable for protecting the hydroxylamine, coupling it to a solid-supported peptide under standard conditions, and cleavage after side-chain deprotection was undertaken. Most were unsuccessful but we eventually settled on the use of benzylidene nitronitrone protecting groups. This strategy had been reported by Bently and Brooks in 1976 but only with racemic hydroxylamines [29]. Based on our experience with the cyanonitrone intermediates in the Fukuyama protocol, we initially feared that these nitrones would not be configurationally stable. This fear proved unfounded and we were eventually able to prepare a number of benzylidene-protected peptide hydroxylamines and demonstrate their introduction into longer peptides. The nitrones had the advantage that they could be orthogonally deprotected in the presence of the side-chain protecting groups or left intact while acidic conditions removed the side-chain protecting groups without affecting the nitronitrone (Scheme 14). The nature and reactivity of the hydroxylamines made the formation and manipulation of these peptides the most challenging aspect of the ligation.

This protecting strategy led to another idea for hydroxylamine synthesis: a single reagent for the direct conversion of amines to hydroxylamines (Scheme 15). This fundamental transformation is essentially unknown, as most reagents lead to over oxidation of the initially formed hydroxylamine [30, 31]. A postdoctoral fellow, Dr. Takeo Fukuzumi, took on this challenge and designed bifunctional reagent 6 containing both an aldehyde and an oxaziridine [32]. An unprotected N-terminal amine can form an imine or hemiaminal with the aldehyde, which undergoes a subsequent intramolecular oxidation to give the hydroxylamine. This intermediate



**Scheme 15** An oxaziridine reagent for the oxidation of amines to hydroxylamines and a mechanistic proposal

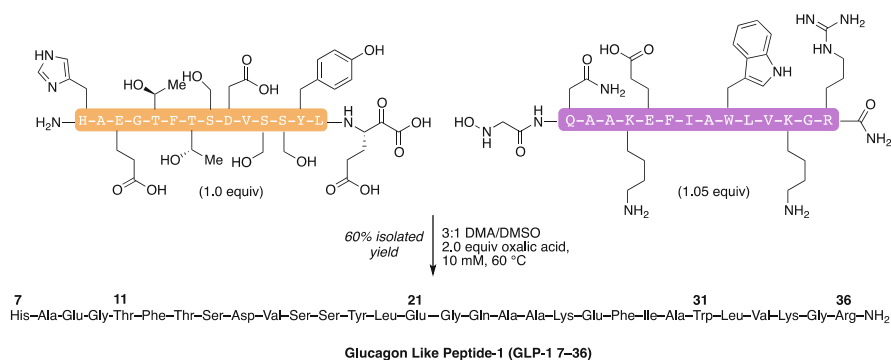
forms the nitron, protecting against further oxidation. A stereochemical mismatch between the racemic oxaziridine and the enantiopure peptides limits the synthetic utility of this reagent for peptides on larger scale, but the design principles are an excellent example of chemical problem solving and innovation.

### 3.5 KAHA Ligation for the Synthesis of Therapeutic Peptides: GLP-1

Shortly after our first publication on the KAHA ligation, we were approached by several companies interested in using this reaction for therapeutic peptide synthesis. This led to a collaboration with IPSEN/Biomeasure to test the KAHA ligation for the synthesis of human glucagon like peptide (GLP-1) and analogues, which were being developed as treatments for type II diabetes. IPSEN certainly had no trouble making the desired peptides, but as is often the case with therapeutic peptide synthesis, the purity of the final product was sometimes an issue and was not reproducible. We were asked if we could use our ligation to assemble two unprotected fragments to give the final product directly from the ligation.

At the time we took on this challenge, we had not yet developed our current methods for preparing the requisite N-terminal hydroxylamine or C-terminal  $\alpha$ -ketoacids. If brought this problem today, it would take us no more than a few days to make the fragments and try the ligation. But in 2006 we had not yet even started doing solid-phase peptide synthesis in our laboratory!

Our first postdoc, Jian Wu, took on this project. Working in parallel on the GLP-1 fragments as well as and on general methods to prepare peptide hydroxylamines, Jian



**Scheme 16** Synthesis of human GLP-1 by KAHA ligation of two unprotected peptide fragments. This work demonstrated that the KAHA ligation has the potential to serve as a general peptide ligation strategy

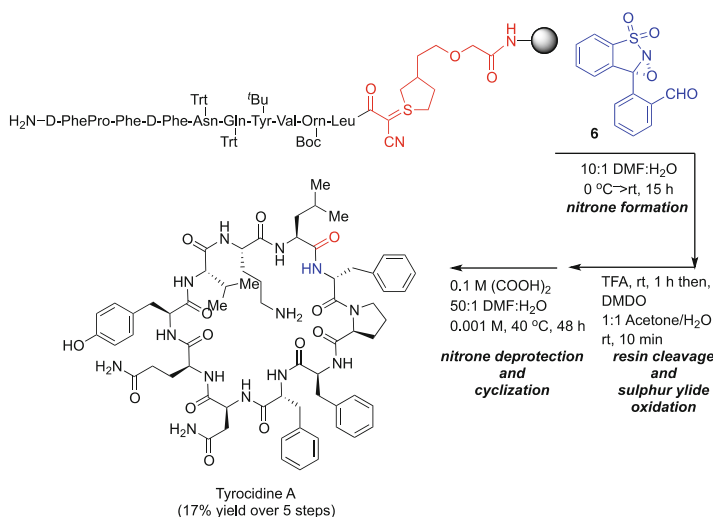
used a side-chain glutamic acid to link a C-terminal sulfur ylide residue to the solid support and prepare the first fragment. The hydroxylamine fragment was prepared by displacement of a bromoacetyl peptide followed by deprotection and resin cleavage. At this point, another common problem in peptide chemistry arose: solubility. By careful experimentation, Jian was able to find a suitable solvent for both the ligation and the peptide and prepare GLP-1 from two completely unprotected fragments (Scheme 16) [33].

Jian's demonstration that larger, side-chain unprotected peptides that could be made by the KAHA ligation was a great encouragement. Several issues remained, but this was the first ligation of a fully unprotected peptide by a method other than native chemical ligation. A planned scale-up to gram quantities was unfortunately put on hold when the GLP-1 project was transferred from IPSEN to another company, but the support and patience of IPSEN was critical to advancing our experience and methodologies.

### 3.6 Cyclization of Unprotected Linear Peptides

The combination of our solid-phase methods for  $\alpha$ -ketoacid and N-terminal hydroxylamine syntheses invited their application to the synthesis of macrocyclic peptides by the cyclization of side-chain unprotected linear peptides. Cyclic peptide is an important and rapidly growing area of new therapeutics, and novel methods for their facile preparation are in great demand. We reasoned that our methods could be used to prepare suitably functionalized linear peptides that should cyclize simply upon warming them in an appropriate solvent.

Takeo and Lei showed that it was indeed possible to use our methods for  $\alpha$ -ketoacid and hydroxylamine synthesis to prepare several cyclic peptide natural products [34, 35]. No post-cyclization manipulations other than purification were required. The ability to perform the cyclization on unprotected side chains may



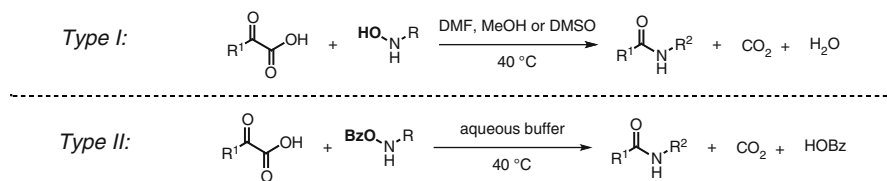
**Scheme 17** Synthesis of peptide macrocycles by cyclization of unprotected linear peptides with the KAHA ligation

have advantages for difficult substrates where the protecting groups may interfere with the cyclization. It was even possible to use Takeo's hydroxylamine reagent to introduce the hydroxylamine (Scheme 17), suggesting that it is possible to prepare cyclic peptides simply by using the sulfur ylide linker and concluding the solid-phase synthesis with reagent **6**.

### 3.7 2010: A Critical Assessment of the KAHA Ligation

The end of 2009 brought the completion of our studies on the synthesis of  $\alpha$ -ketoacids and N-terminal peptide hydroxylamines and our demonstration that the KAHA ligation could be applied to the synthesis of therapeutic peptides by the direct coupling of unprotected fragments as well as the macrocyclization of unprotected linear peptides. It also brought our second laboratory move in 3 years, this time from Philadelphia, USA, to Zürich, Switzerland. We left the USA having succeeded in our immediate goals: to develop Fmoc-compatible approaches to fully unprotected peptide fragments containing the requisite functional groups and their ligation to give medium-sized peptides.

Despite these successes, there were still problems. The preparation of the  $\alpha$ -ketoacids was largely solved, although we have continued to work on specialized strategies for fragments containing sulfur atoms. The major problems resided with the hydroxylamines. The benzylidene nitrones were workable protecting groups but could be difficult to either remove or retain, depending on the peptide. Counterintuitively, smaller amino acid residues such as glycine and alanine were particularly challenging. Furthermore, these protecting groups were not kinetically stable and



**Scheme 18** The two prototypical types of the KAHA ligation. Type I ligations proceed with *O*-substituted hydroxylamines and prefer polar aprotic solvents. Type II ligations require *O*-substituted hydroxylamines and generally prefer water as the reaction solvent

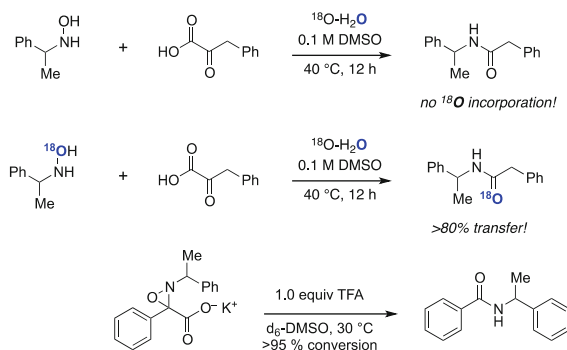
could not be used for multiple segment condensations. Even in cases where we could not deprotect them, we found that they would undergo rapid exchange with other hydroxylamines. This prevented the sequential segment condensations that would be needed for synthesizing proteins. Even more problematic was the stability of the peptide hydroxylamines themselves. They were prone to oxidation, elimination, and disproportionation under the ligation conditions, and their purification could also be challenging. When the ligations failed, it was almost always due to the properties of the peptide hydroxylamine.

For reasons that we did not understand at the time, the ligation of  $\alpha$ -ketoacids and *O*-unsubstituted hydroxylamines, which we now call type I ligations, occurred best in polar aprotic solvents such as DMSO or DMF (Scheme 18). Water was detrimental to the reaction rates and tended to accelerate the decomposition of the peptide hydroxylamine. In contrast, we had identified a number of other variants of the KAHA ligation with *O*-substituted hydroxylamines that occurred preferentially in water (type II ligations). Unfortunately, the most effective variants, *O*-Bz hydroxylamines, cannot be used for  $\alpha$ -peptides due to facile elimination [36].

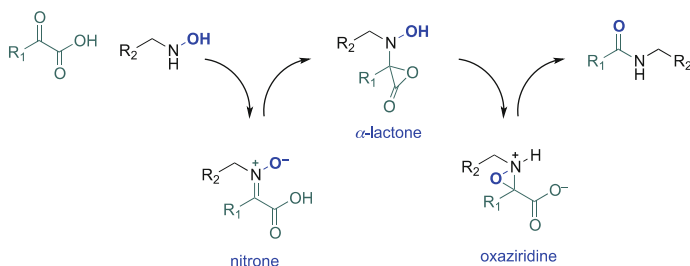
### 3.8 The Mechanism of the KAHA Ligation

The observation that different classes of hydroxylamines exhibited divergent behavior and reactivity coupled with the poor performance of the unsubstituted hydroxylamines in water prompted us to undertake a thorough investigation of the mechanism of the KAHA ligation. This work was started by one of our first new Ph.D. students at ETH-Zürich, Ivano Pusterla. Through a combination of kinetics, intermediate synthesis, and especially isotopic labeling studies, Ivano quickly realized that the KAHA ligation did not have a simple mechanism. Most surprisingly, KAHA ligations performed in  $^{18}\text{O}$ -H $_2$ O did not give any labeled amide products. After a laborious synthesis of  $^{18}\text{O}$ -labeled hydroxylamine, Ivano found almost complete label transfer from the oxygen of the hydroxylamine into the oxygen of amide (Scheme 19)!

This unexpected result required a complete reevaluation of the mechanism. To make a long story short, we found that a key intermediate is an oxaziridine and



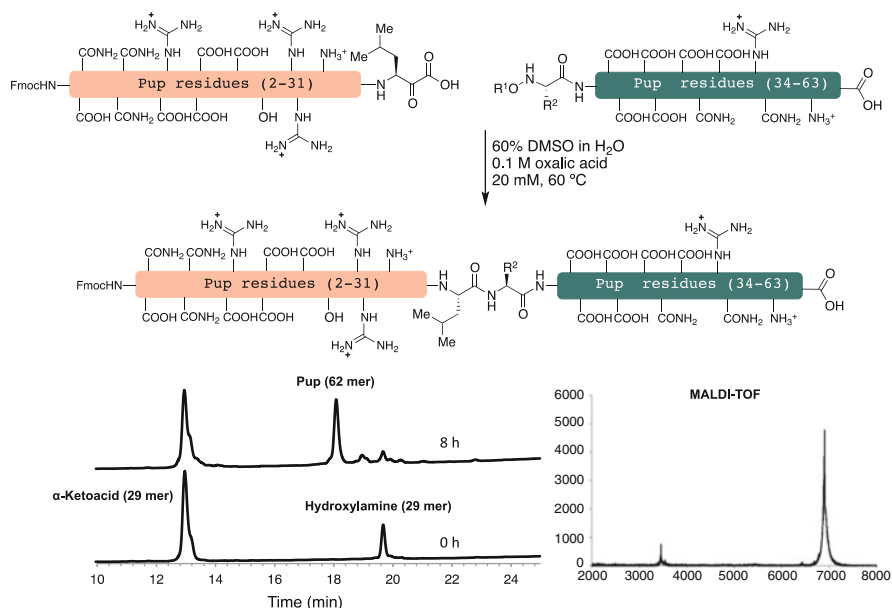
**Scheme 19** Mechanistic investigations of the KAHA ligation  $^{18}\text{O}$ -isotope labels and conversion of oxaziridine acids to amides



**Scheme 20** The “three-ring circus” mechanism for the type I KAHA ligation. We were very surprised to find that the oxaziridine is involved in the amide formation

that it must arise by rearrangement of the nitronium. The full details of the mechanism were recently published. The overview of our “three-ring circus” mechanism for the type I KAHA ligations is shown in Scheme 20. The type II ligations proceed by a different mechanism, and efforts to elucidate this are currently underway.

Our mechanistic studies on the KAHA ligation taught us several important lessons. First, it showed us very clearly why water was detrimental for the type I ligations. The formation of the nitronium, which is slowed in the presence of water, is essential. We had long believed that the nitronium was an unproductive pathway that deterred amide formation. We had even thrown out ligation reactions when we observed the formation of the nitronium! Only with hindsight did we learn that leaving such reactions alone leads to good conversion to the amide. Second, this experience reinforced the idea that mechanistic studies are essential to progress in challenging areas. We have performed extensive mechanistic investigations on our NHC-catalyzed reactions [37]. With the KAHA ligation we had put our initial studies on simple asides aside in favor of learning how to build the complex, unprotected peptide fragments more relevant to the applications. In retrospect we should have worked on the mechanism earlier. The discovery of how the type I



**Scheme 21** Protein synthesis with the KAHA ligation

ligation works and the implicit limitations has already helped us advance the KAHA ligation much further.

### 3.9 The Next Steps . . . Protein Synthesis by KAHA Ligation

The realization that the use of *O*-unsubstituted hydroxylamines would preclude the aqueous conditions needed for most protein and large peptide synthesis set off a flurry of activity in our labs. Postdoc Vijay Pattabiraman and graduate student Ayo Ogunkoya began looking for a type II ligation of  $\alpha$ -peptide substrates. The challenge here is that the obvious substrates, the *O*-acylated peptide hydroxylamines, are unstable toward elimination. The *O*-alkyl hydroxylamines generally require high reactant concentrations and long reaction times that are not suitable for peptide and protein synthesis. Just when it looked like we would never find a suitable approach, Ayo and Vijay came through with a fantastic solution. This will be published shortly, but as a preview, we can show the synthesis of Pup, a small ubiquitin-like protein, by direct ligation of two unprotected sequences (Scheme 21).

Based on this approach, Ayo and Vijay have already synthesized several other proteins. Finally, we are nearing our stated goal of protein synthesis by simply preparing modestly sized (approximately 30 residue) fragments with solid-phase peptide synthesis followed by complete deprotection and direct ligation. The next steps will be to apply this to much larger systems.

## 4 Outlook and Expectations

I sometimes tell my students that good science starts when no one knows how to do something. The project of “reinventing amide bond formation” has, by this measure, been a fantastic generator of good science. The first examples of the KAHA amide synthesis were achieved with nothing more than mixing commercially available chemicals together. Yet going forward and taking advantage of the real power of this reaction required the establishment of new synthetic and analytical techniques that have kept us busy for years. It has led to moments of great success, such as our first chemoselective peptide ligation and the generation of enantiopure  $\alpha$ -ketoacids, but also enormous frustration. The perseverance and creativity of my students have finally allowed us to develop the KAHA ligation into a viable reaction for protein synthesis. The story of this reaction is just beginning.

Chemical protein synthesis has benefited from two revolutions in the last 50 years: solid-phase peptide synthesis and the native chemical ligation. Both of these advances have brought the field forward, but both have inherent limits. It seems unlikely that solid-phase peptide synthesis will ever allow the facile, routine preparation of peptides longer than 50 residues. Native chemical ligation is a fantastic tool for protein synthesis, but its strict requirements limit its application in many contexts. The field of synthetic peptide chemistry has progressed from the synthesis of a dipeptide by Fischer in 1902 [38] to the synthesis of the 200+-mer proteins by Kent and others in the 1990s. But put another way, the field has progress at the rate of about two amino residues/year. Without new reactions and concepts for chemical protein synthesis, it will still be many years before we can prepare proteins such as *taq* polymerase! The bacterial ribosome, on the other hand, can produce an 800-mer protein in less than 10 min! Clearly, the field of chemical protein synthesis and amide bond formation still has a long way to go [39]. We hope that the continued development of the KAHA ligation will be one way to overcome the asymptotic limits inherent to the current approach to peptide synthesis.

The KAHA ligation also has applications beyond the synthesis of peptide and proteins. It is a very rare example of a reaction that exhibits “absolute chemoselectivity” and does not require any reagents or catalysts. We are exploiting these unique properties for many different applications including small molecule and macrocycle synthesis, polymerization and oligomerization reactions, and site-specific labeling. In all of these endeavors, the major challenges remain the synthesis and incorporation of the key functional groups. Improvements in this demand continued innovation and creativity in the synthesis of organic molecules and the development of new reactions.

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Gooßen, L.J. (Ed.)

2013, XIV, 342 p., Hardcover

ISBN: 978-3-642-34285-1