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Functionalization of Peptides and Proteins by Mukaiyama Aldol Reaction

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Abstract: The scope of the catalyst-free water-based Mukaiyama aldol reaction was explored through its application to the site-selective functionalization of N-terminal aldehydes of peptides and proteins. Various functional groups were introduced under mild and environmentally friendly conditions, with the first demonstration of aldol C−C bond formation in protein labeling studies. The efficiency and speed achieved in protein labeling can be of special interest in chemical biology studies.

The chemical modification of proteins has become an important tool for the study of complex biological systems in cells. The availability of such techniques is crucial for the discovery of new therapeutic agents, as it is very difficult to design modulators of protein function if an understanding of the basic biological mechanism is lacking. Furthermore, this may provide understanding of protein function in chemical genetic studies.

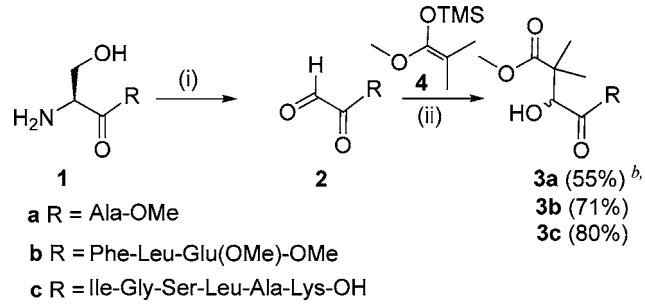
Ideally, protein functionalization should proceed rapidly with high yield, without perturbation of the tertiary structure, and with selectivity for one site in the amino acid chain. These requirements pose extreme challenges for protein chemists, and a number of different strategies have been investigated. In principle two hurdles have to be overcome: (1) a unique site has to be introduced into a protein, and (2) methodology for derivatizing the unique site under mild aqueous conditions needs to be available. A number of groups have developed methods for introducing unnatural amino acids into proteins,[1] and Cu(I)- or strain-promoted Huisgen azide−alkyne [3 + 2] cycloadditions, Staudinger ligations, and anaerobic cross-coupling reactions catalyzed by transition metals have been explored for the targeted functionalization of these modified proteins

Francis and co-workers[2] have developed a versatile oxidative deamination reaction that can be used to introduce an N-terminal aldehyde group into proteins. The most straightforward options for selective modification of an aldehyde are the formation of imines and oximes. However, only oximes are stable under physiological conditions,[3] while available alternatives such as the carbon−carbon bond-forming Pictet−Spengler reaction[4] are slow and suffer from low conversion.

In this paper, we describe a novel method for site-directed functionalization of peptides and proteins using the Mukaiyama aldol condensation in aqueous media. This reaction furnishes a carbon−carbon bond and therefore leads to a stable product. Furthermore, the mild conditions and the high reactivity of the reagent provide rapid and clean transformations.

In recent years, our group has demonstrated that silyl ketene acetals can react with various reactive aldehydes in water to afford the corresponding aldol products at ambient temperature without any Lewis acid activation.[5] Because of the mild reaction conditions, this method seemed to be perfectly suited for the funtionalization of proteins.

Scheme 1. Mukaiyama Aldol Condensation of Peptide Aldehydes[a]



[a] Conditions: (i) peptide **1a**, **1b**, or **1c**, 2 equiv of $NaIO_4$, 10 mM sodium phosphate buffer (pH 7.0), dark, 10 min−2 h; (ii) aldehyde **2a**, **2b**, or **2c**, 2 equiv of ketene acetal **4**, 10 mM sodium phosphate buffer (pH 7.0), rt, 24 h. [b] Column-purified yield of Mukiayama aldol product after two steps. [c] Isomeric ratios determined by ^{13}C NMR spectroscopy: **3a** (50:50), **3b** (60:40), **3c** (53:47).

Initial model studies were undertaken with protected dipeptide **1a**, tetrapeptide **1b**, and unprotected heptapeptide **1c**, each containing serine at the N-terminus. $NaIO_4$ oxidation of the N-terminal serine would provide an aldehyde at that site.[6] After some optimization of the reaction conditions, we found that when the dipeptide aldehyde **2a** generated by periodate oxidation was vigorously stirred with 2 equiv of (1-methoxy-2-methylpropenyloxy)trimethylsilane (**4**) in 10 mM sodium phosphate buffer (pH 7.0) for 24 h at room temperature, the Mukaiyama aldol product **3a** was obtained in 55% isolated yield (for two steps) (Scheme 1). Further model reactions of this particular silyl ketene acetal with protected peptide aldehyde **2b** and unprotected peptide aldehyde **2c** in buffer showed that the reaction proceeded efficiently, with the yield of product improving to 80% for the longer, unprotected peptide (Scheme 1).

Further examination of the reaction of peptide aldehydes with a variety of ketene acetals provided Mukaiyama aldol products with yields ranging from 11 to 55%. In general, alkyl-substituted ketene acetals proved to be more suitable for this application, yielding results comparable to those for compound **4** (Table 1, entry 1). The results in Table 1 also suggest that this method is well-suited for the introduction of substituents containing alkenes and alkynes (Table 1, entries 4−6), which are functional groups that can be further modified using olefin metathesis[7] or Cu(I)-promoted Huisgen [3 + 2] cycloadditions.[8] Furthermore, entry 8 in Table 1 indicates that polyethylene glycol-conjugated proteins[9] could be synthesized using this method. Since the reaction seemed to perform better on more complex peptides, these results were very encouraging for the functionalization of proteins (Scheme 1).

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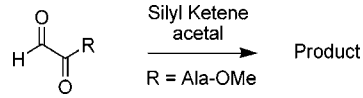
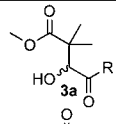
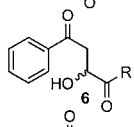
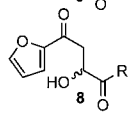
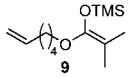
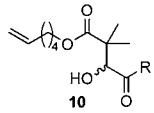
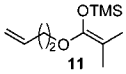
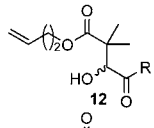
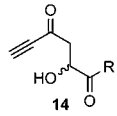
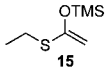
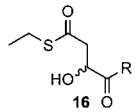
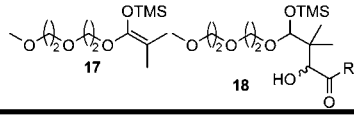
Table 1. Mukaiyama Aldol Condensation with Silyl Ketene Acetals[a]

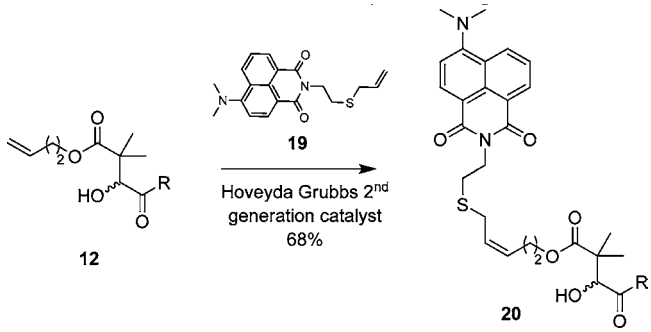


Entry	Ketene acetal	Product	Yield[b]	Ratio[c]
1			55	57:43
2			11	52:48
3			11	55:45
4			45	60:40
5			41	60:40
6			12	60:40
7			52	52:48
8			42	53:47

[a] Conditions: aldehyde **2a**, 2 equiv of ketene acetal, 10 mM sodium phosphate buffer (pH 7.0), rt, 24 h. [b] Column-purified yield of Mukiayama aldol product. [c] Isomeric ratios determined by ^{13}C NMR spectroscopy.

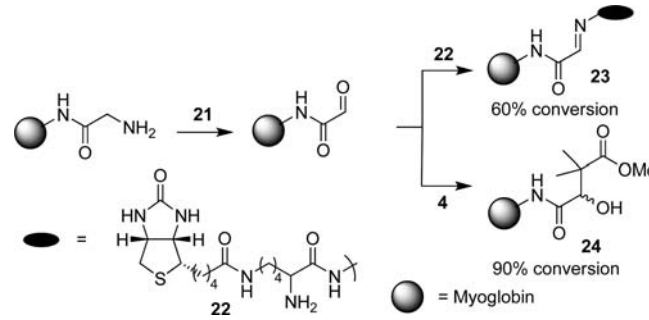
In order to demonstrate the utility of the Mukaiyama adducts, compound **12** was coupled to thioallyl fluorescent probe **19** under the cross-metathesis conditions using the Hoveyda−Grubbs second-generation catalyst as reported by Davis,[6] providing compound **20** in 68% yield (Scheme 2).

Scheme 2. Functionalization of Mukaiyama Aldol Product **12**[a]



[a] Refer to the Supporting Information for details.

Scheme 3. Mukaiyama Aldol Condensation of Myoglobin



As a next step, we sought to apply the Mukaiyama aldol functionalization to proteins. Horse heart myoglobin served as a convenient model system.[2]

While the periodate method of aldehyde generation was convenient for the model studies, we switched to the pyridoxal-5′-phosphate (PLP, **21**) oxidation method[2] for our protein modification reactions, since myoglobin has an N-terminal Gly that cannot be oxidized with periodate. In the event, a 50 μM solution of the protein was incubated with 10 mM **21** for 20 h at 37 °C in 25 mM sodium phosphate buffer (pH 6.5). After removal of excess **21** with NanoSep 10 filtration devices, the product solution was divided into two portions. One portion was incubated with 25 mM biotin-X-hydrazide[10] (**22**) overnight to confirm the formation of the aldehyde,[10] while the other portion was incubated with 5 M silyl ketene acetal **4** in 25 mM phosphate buffer (pH 7.0) (Scheme 3).

After overnight reaction, 60% conversion to the biotinylated product **23** (Scheme 3 and Figure 1b) was identified by liquid chromatography−electrospray ionization mass spectrometry (LC−ESI-MS). So-

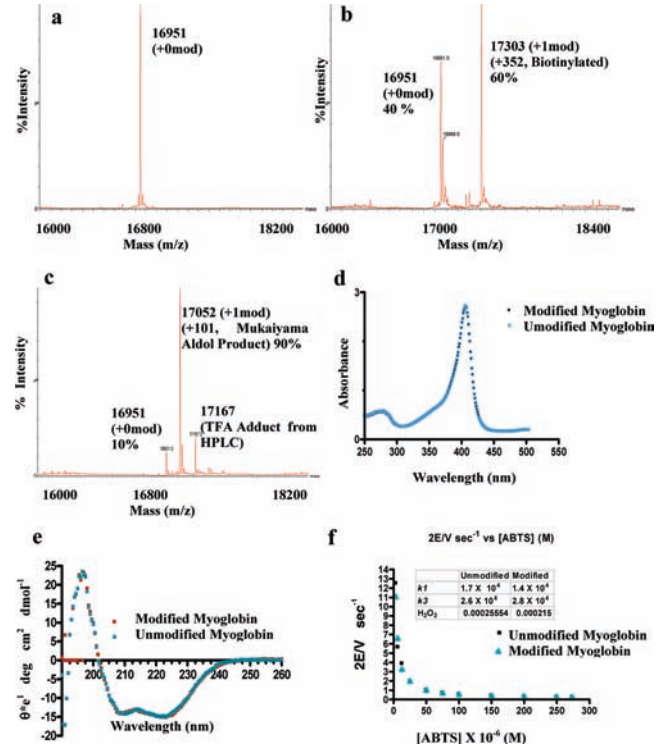


Figure 1. Mukaiyama aldol modification of myoglobin. (a−c) LC−ESI-MS data for (a) unmodified myoglobin and the (b) biotinylated (60%) and (c) Mukaiyama aldol-modified (90%) protein products. (d) UV and (e) CD traces of modified and unmodified myoglobin. (f) Steady-state kinetic parameters for the peroxidase activities of unmodified and modified myoglobin.

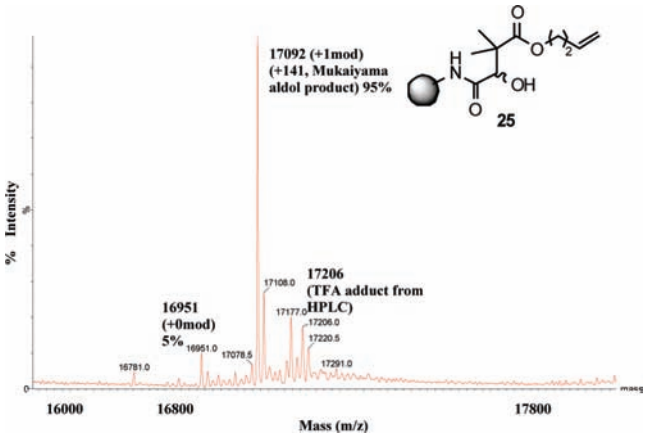


Figure 2. Alkene-functionalized myoglobin **25**.

dium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis and Western blotting of the SDS-PAGE gel were used to further confirm biotinylation.[10]

Only a trace amount of the aldol product **24** was formed under the conditions used for the peptides (Table 1). A likely explanation for the unsatisfactory result is the poor solubility of the silyl ketene acetal in water, together with a lack of stirring. Since it is known that proteins lose their stability upon stirring,[11] the use of a cosolvent was explored.

Ethylene glycol is known to be a suitable solvent for protein functionalizations.[12] However, in the case of the Mukaiyama aldol reaction, water/ethylene glycol mixtures were not suitable. After a number of experiments, we identified *tert*-butyl alcohol/water (17:83) as the best solvent, with 90% conversion[13] to the desired product **24** (Scheme 3 and Figure 1c) within 1 h.

The modified protein was characterized using ESI-MS (Figure 1c). The UV and circular dichroism (CD) spectroscopy traces of the modified and unmodified proteins were identical, suggesting that the reaction conditions did not modify the tertiary structure of the myoglobin (Figure 1d,e). Rate constants k_1 and k_3 for the ping-pong mechanism of peroxidase defined by Dunford[14] were determined for both unmodified and modified myoglobin under steady-state conditions. The results showed that under saturating concentrations of peroxide, the modification did not have any effect on the reaction of myoglobin with the substrate ABTS (Figure 1f).[10] Finally, to confirm the site specificity of the modification, products **23** and **24** were subjected to tryptic digestion. Analysis of the resulting peptide fragments by LC−ESI-MS showed that the reactions had successfully modified the protein N-terminus.[10]

After the successful demonstration that the Mukaiyama aldol reaction can be applied to proteins, the amount of silyl ketene acetal needed for the reaction was optimized. Near-quantitative conversion could be achieved with as little as 190 equiv (0.3 μL) of the substrate.[10]

Similar results were obtained with silyl ketene acetal **11**. A 95% conversion to the desired product **25** was seen (Figure 2), suggesting that this newly developed reaction is well-suited for the functionalization of proteins.

In conclusion, we have described here the application of an aldol carbon−carbon bond formation for the functionalization of N-terminal aldehydes of peptides and proteins. The method can be used for the targeted introduction of a wide variety of functional groups into specific N-terminal proteins under very mild conditions. It has been demonstrated that in the case of myoglobin, the functionalization of the protein can be carried out without disturbing either the tertiary structure or, more importantly, the enzymatic activity of the protein. The Mukaiyama aldol reaction of proteins is very fast and highly efficient and as a consequence may be of particular interest when the stability of the protein is a concern.

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Supporting Information Available: Additional experimental procedures and spectral data for reaction products. This material is available free of charge via the Internet at http://pubs.acs.org.

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(13) The optimized conditions for the Mukaiyama aldol reaction with myoglobin use 190 equivalents of silyl ketene acetal. The greater efficiency of the reaction relative to that of the peptides (Table 1) is likely due to the greater excess of ketene acetal. We have shown that the yield of the Mukaiyama aldol reaction with peptides can be improved if a larger excess of ketene acetal is used (see the experimental conditions and comments in the Supporting Information).
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