ORGANIC LETTERS

2012
Vol. 14, No. 17
4694–4697

A Proline-Based Phosphine Template for Staudinger Ligation

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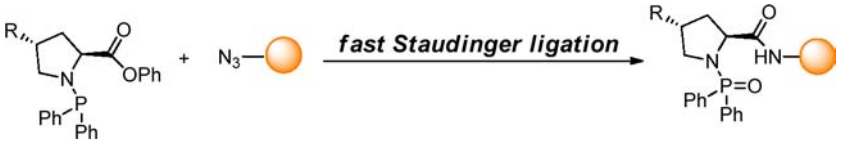
Received August 14, 2012

ABSTRACT



A proline-based phosphine template enabling a rapid Staudinger ligation of azide-containing substrates under mild conditions is reported. This reaction has a second-order rate constant of 1.12 M^{-1} s^{-1}. It is expected that the proline-based Staudinger ligation strategy will be a useful method for bioconjugation and proline based peptide coupling.

Bioorthogonal reactions, in which the coupling partners react selectively without interference with other biological functionalities, are important tools for chemical biology research.[1] The development of Staudinger ligation by Bertozzi and co-workers is a seminal contribution to bioorthogonal chemistry.[1,2] Utilizing azides and phosphines as orthogonal species, Staudinger ligation promotes a chemoselective amide bond formation under mild and physiological conditions. It has been employed in labeling a wide range of azide-containing biomolecules such as glycans,[2a] DNA,[3] lipids, and proteins.[4] Typically, the Staudinger ligation satisfies the criteria for bioorthogonal reactions such as good reactivity, selectivity, biocompatibility, etc. Currently the structural templates for Staudinger ligation are limited. Almost all of the substrates used in regular Staudinger ligation are triphenyl substituted phosphine derivatives. In the 'traceless' version of Staudinger ligation developed by Raines et al., some alkyl-diphenyl-substituted phosphine derivatives are also used.[5] With these substrates, the kinetics of the reaction are somewhat slow (typical second-order rate constant of 0.002 M^{-1} s^{-1}), which mandates the use of high concentrations of phosphine reagents.[6] Here we report a proline-based template for the design of Staudinger ligation substrates. It allows fast kinetics with azide substrates and provides an alternative way to construct peptide adducts.

The studies by Raines et al. revealed that a favorable $n \rightarrow \pi^*$ electrostatic interaction exists between the amide oxygen and ester carbonyl in *N*-formylproline phenylesters

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10.1021/ol3022484
Published on Web 08/27/2012

(Figure 1).[7] Such type of effects contribute to the endogenous preference for the *trans*-peptide bonds. These results led us to consider the structures of proline-based iminophosphoranes such as **2** (Figure 1), which could be generated through the Staudinger reaction of azides. We anticipated that a similar n $\rightarrow \pi^*$ interaction might occur and fix the iminophosphorane conformation as shown in Figure 1. This could lead to a favorable ligation process. In addition, the electron-rich phosphorus atom adjacent to the proline nitrogen could facilitate the formation of the azaylide intermediate **2**. Finally the reactive species **2** having a pyrrolidine enamine-like skeleton[8] could also enhance the desired intramolecular acyl transfer to complete the ligation process. Given these factors, it may provide a new Staudinger ligation template with improved kinetics.

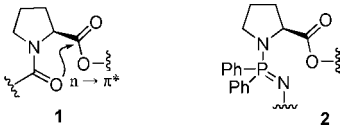
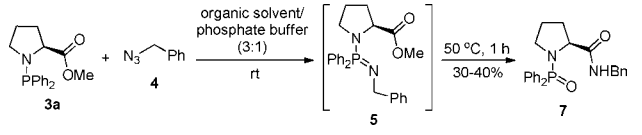


Figure 1. Design of proline-based template.

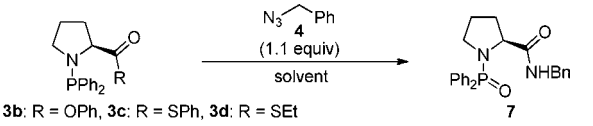
To test this idea, we prepared a proline-based diphenylphosphine derivative **3a** (Scheme 1; see Supporting Information for preparation). When **3a** was treated with a model azide **4** in a mixture solvent system (organic solvent/pH 7.4 phosphate buffer = 3/1) at rt, the aza-ylide intermediate **5** should be formed. Interestingly this intermediate did not proceed to form the desired ligation product **7** at rt. Instead, we isolated the protonated azaylide intermediate in 55% yield (Supporting Information). We were able to promote the ligation process by increasing reaction temperature to 50 °C. Under such conditions, the ligation product **7** was obtained in moderate yields. Several different solvents (THF, DMF, a mixture of MeCN/THF) were tested, and the yields were consistent in the 30−40% range.

Scheme 1. Staudinger Ligation of **3a** with Benzyl Azide **4**



To improve the ligation process with the proline-based template, we decided to introduce better leaving groups in the ester moiety. Three different substrates **3b**, **3c**, and **3d** were prepared and tested (Table 1). As expected, the desired ligation product **7** was obtained in excellent yields with these substrates. These ligations proved to be fast, as all reactions completed within 30 min. The change of organic solvents (THF, DMF, and acetonitrile) had little effect on the reaction yields. It should also be noted that these proline-based substrates (**3a**−**3d**) are quite stable toward air and moisture and can be easily handled in the laboratory.

Table 1. Staudinger Ligation of **3b**−**3d** with Benzyl Azide **4**[a]



entry	substrate	solvent	yield (%)[b]
1	**3b**	DMF/buffer (3:1)	96
2	**3c**	DMF/buffer (3:1)	95
3	**3d**	DMF/buffer (3:1)	84
4	**3b**	THF/buffer (3:1)	90
5	**3c**	THF/buffer (3:1)	92
6	**3d**	THF/buffer (3:1)	84
7	**3b**	MeCN/THF/buffer (1.5:1.5:1)	96
8	**3c**	MeCN/THF/buffer (1.5:1.5:1)	94
9	**3d**	MeCN/THF/buffer (1.5:1.5:1)	82

[a] Reaction conditions: organic solvent with pH 7.4 phosphate buffer (20 mM with 0.15 M NaCl) at rt for 30 min. [b] Isolated yield.

It is known that pH values could affect the efficiency of the Staudinger ligation.[6b] We then tested the reactivity of **3b**−**3d** under various pH's. As shown in Figure S1 (Supporting Information), the yields of the ligation product **7** did not change much in pH 5.5−8.5. These results suggested that the azaylide intermediates were very reactive toward the ester function groups in **3b**−**3d**. We noticed that under basic conditions (pH 8.5) the yield from **3d** dropped slightly. This was possibly due to partial hydrolysis of the thioester group in **3d** under such conditions.

Among these phosphine substrates, we expected **3b** to be the most promising for Staudinger ligation, given the better stability of the ester functional group compared to thioester groups. We then measured the kinetics of the reaction between **3b** and azide **4** using a ^{31}P NMR method as described before.[6a,9] As such, **3b** (0.031 M) and **4** (0.31 M) were combined in CD_3CN with 5% H_2O (v/v) with triphenylphophine oxide as the internal standard. Under these conditions, we only observed two species by ^{31}P NMR, i.e. the starting material **3b** (δ = 54.5 ppm) and the ligation product **7** (δ = 35.2 ppm). As shown in Figure 2A, the reaction completed within 30 min at rt. The pseudo-first-order rate constants k_{obs} for the consumption of **3b** using different concentrations of excess azide **4** (0.31,

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0.62, and 1.24 M) were measured and used to determine the second-order rate constant. By plotting ln k_{obs} versus ln $[\mathbf{4}]_0$, the overall second-order rate constant was determined to be $1.12 \pm 0.03\ M^{-1}\ s^{-1}$ (Figure 2B). This was approximately 500 times faster than the triphenylphosphine-based Staudinger ligation (including the corresponding phenyl ester substrate[6a]).

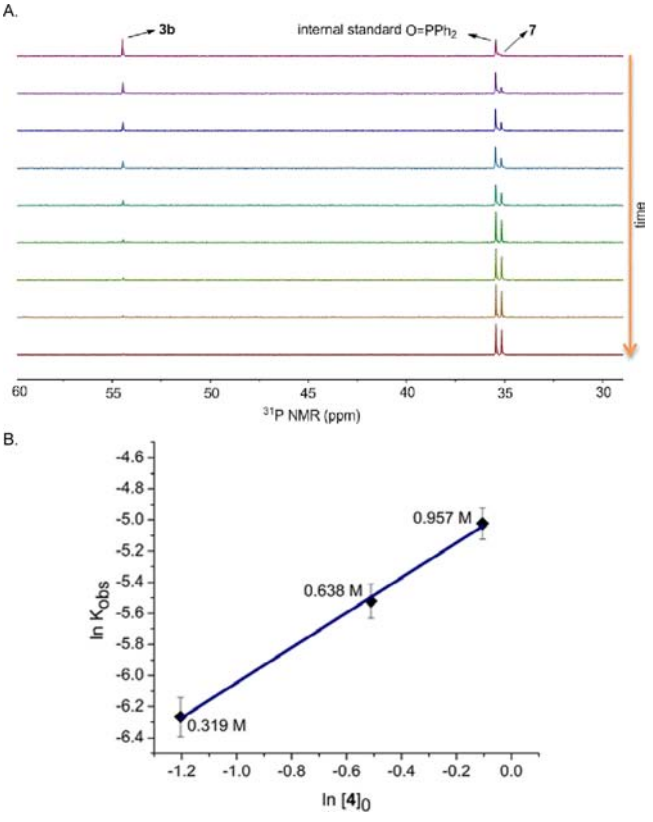
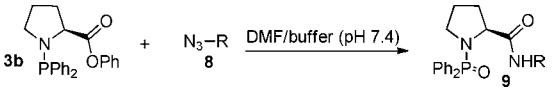


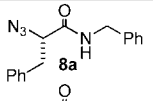
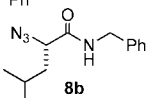
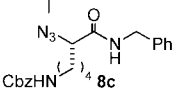
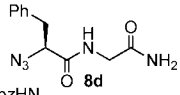
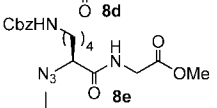
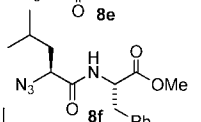
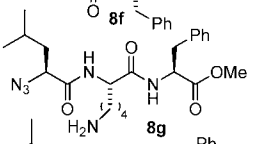
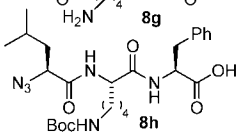
Figure 2. Kinetic analysis of the Staudinger ligation of **3b** and azide **4**. (A) ^{31}P NMR spectra recorded at 20 °C with **3b** (0.031 M) and **4** (0.31 M) in CD_3CN containing 5% H_2O. The spectra were recorded every 3 min for 30 min. (B) Plot of ln k_{obs} versus ln $[\mathbf{4}]_0$ to determine the second-order rate constant, where **4** was used in excess. The second-order rate constant is calculated to be $1.12 \pm 0.03\ M^{-1}\ s^{-1}$.

To further demonstrate the generality of using phosphine **3b** in Staudinger ligation, a series of azide-containing substrates (**8a**–**8i**) were prepared and tested. All reactions were carried out with a 1:1 ratio of the phosphine and azides. As shown in Table 2, the reaction worked smoothly for all the cases and the ligation products were obtained in high yields. Steric hindrance did not seem to affect the reaction much, as the structural changes adjacent to the azide group had little effect on the reaction yields. It should be noted that substrates with unprotected functional groups such as $-CO_2H$ and $-NH_2$ (entries 7 and 8) also showed good reactivity.

We envisioned that the proline moiety on phosphine substrates could allow us to easily introduce chemical handles or report molecules for bioconjugation. This change should not affect the ligation efficacy of this

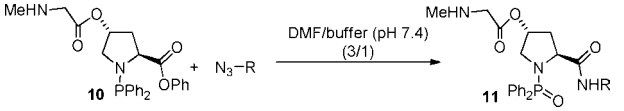
Table 2. Staudinger Ligation of **3b** with Azide-Containing Substrates



entry	α-azido compound, N_3-R	product	% yield
1	 **8a**	Ph_2OP-Pro-Phe-NHBn **9a**	91
2	 **8b**	Ph_2OP-Pro-Leu-NHBn **9b**	92
3	 **8c**	Ph_2OP-Pro-Lys(Cbz)-NHBn **9c**	91
4	 **8d**	Ph_2OP-Pro-Phe-Gly-NH_2 **9d**	94
5	 **8e**	Ph_2OP-Pro-Lys(Cbz)-Phe-OMe **9e**	90
6	 **8f**	Ph_2OP-Pro-Leu-Phe-OMe **9f**	89
7	 **8g**	Ph_2OP-Pro-Leu-Lys-Phe-OMe **9g**	78
8	 **8h**	Ph_2OP-Pro-Leu-Lys(Boc)-Phe-OH **9h**	90
9	 **8i**	Ph_2OP-Pro-NHPh **9i**	95

substrate system. To prove this hypothesis, a *trans*-hydroxyproline-phosphine derivative **10** was prepared. The compound has excellent stability under a normal environment. Control experiments demonstrated that **10** did not react with free thiols (like cysteine), disulfides, or amines

Table 3. Staudinger Ligation of Phosphine **10** with Azide-Containing Substrates

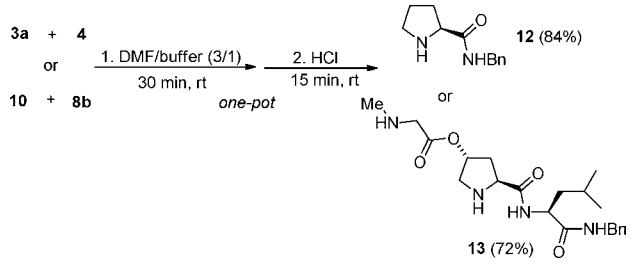


entry	N_3–R	product	yield (%)
1	N_3Bn **4**	Ph_2OP-Hyp[Gly(Ge)]-NHBn **11a**	91
2	N_3-Leu-NHBn **8b**	Ph_2OP-Hyp[Gly(Ge)]-Leu-NHBn **11b**	86
3	N_3-Leu-Phe-OMe **8d**	Ph_2OP-Hyp[Gly(Ge)]-Leu-Phe-OMe **11c**	83

(Supporting Information). Its reactions with azide substrates went smoothly, and the desired ligation products were obtained in good yields (Table 3).

The diphenylphosphinyl moiety on the ligation products could be considered the protection group of proline and should be readily removed under acidic conditions.[10] We then tested a 'one-pot' ligation–deprotection sequence (Scheme 2). Indeed the ligation followed by acid-mediated deprotection provided diphenylphosphine oxide-free products in good yields. Given the efficiency of this protocol, it could see some applications in proline-based peptide coupling.

Scheme 2. 'One-Pot' Ligation–Deprotection Sequence



Finally we validated this strategy in bioconjugation using an azide-containing protein (Figure 3). A noncanonical amino acid, 4-azido-L-phenylalanine, was genetically incorporated at position 7 of the Z-domain protein[11] in *Escherichia coli* by means of an orthogonal amber suppressor tRNA/aminoacyl-tRNA synthetase pair.[12] The azide-containing Z-domain protein was partially purified by Ni-NTA affinity chromatography and characterized by mass spectrometry (Figure 3A). The azide-containing Z-domain protein was subsequently reacted with phosphine **3b** in a mixed solvent system (DMF/pH 7.4 phosphate buffer). After shaking gently for 4 h at room temperature, the reaction mixture was directly analyzed by LC-ESI. The major peak of the observed spectrum (Figure 3B) matched the expected conjugation product. The reaction also proceeded in high yield, and only a trace amount of the azide-containing Z-domain protein was detected after the reaction.

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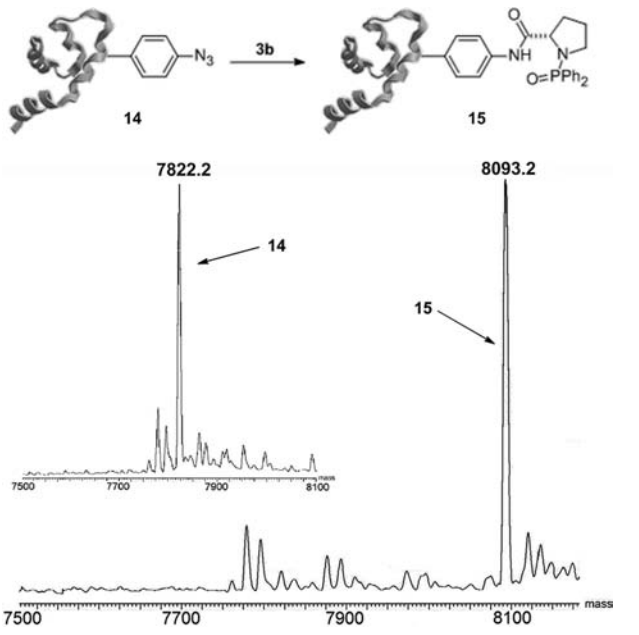

Figure 3. ESI-MS analysis of the reaction product from the reaction of azide-containing Z-domian protein with phosphine **3b**. Top spectrum: unreacted protein, calculated mass: 7822 Da (without N-terminal methionine); observed mass: 7822 Da. Bottom spectrum: reacted protein; calculated mass: 8093 Da (without N-terminal methionine); observed mass: 8093 Da.

In summary, we report in this study a proline-based phosphine template which can be used to promote a fast Staudinger ligation. The proline functional group of the substrates provides an opportunity to readily introduce the reporting molecules. We expect this strategy will be useful for bioconjugation and proline-based peptide coupling.

Acknowledgment. This work is supported by a CAREER award from the NSF (0844931) to M.X and the New Faculty Startup Fund to J.G. from the Chemistry Department of University of Nebraska-Lincoln.

Supporting Information Available. Synthetic procedures, spectroscopic data, and experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.