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Reaction Mechanism and Kinetics of the Traceless Staudinger Ligation

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Abstract: The traceless Staudinger ligation enables the formation of an amide bond between a phosphinothioester (or phosphinoester) and an azide without the incorporation of residual atoms. Here, the coupling of peptides by this reaction was characterized in detail. Experiments with $[^{18}O]H_2O$ indicated that the reaction mediated by (diphenylphosphino)methanethiol proceeded by *S*→*N* acyl transfer of the iminophosphorane intermediate to form an amidophosphonium salt, rather than by an aza-Wittig reaction and subsequent hydrolysis of the resulting thioimidate. A continuous ^{13}C NMR-based assay revealed that the rate-determining step in the Staudinger ligation of glycyl residues mediated by (diphenylphosphino)-methanethiol was the formation of the initial phosphazide intermediate. Less efficacious coupling reagents and reaction conditions led to the accumulation of an amine byproduct (which resulted from a Staudinger reduction) or phosphonamide byproduct (which resulted from an aza-Wittig reaction). The Staudinger ligation mediated by (diphenylphosphino)methanethiol proceeded with a second-order rate constant (7.7×10^{-3} M^{-1} s^{-1}) and yield (95%) that was unchanged by the addition of exogenous nucleophiles. Ligations mediated by phosphinoalcohols had lower rate constants or less chemoselectivity. Accordingly, (diphenylphosphino)-methanethiol was judged to be the most efficacious known reagent for effecting the traceless Staudinger ligation.

Introduction

The advent of methodology for the chemoselective ligation of peptide fragments has made proteins accessible targets for total chemical synthesis.[1] Already, many proteins have been assembled from synthetic peptides using prior capture strategies. "Native chemical ligation", the coupling of a peptide containing a C-terminal thioester with another peptide containing an N-terminal cysteine residue, has been the most widely applied of such strategies.[2−4] "Expressed protein ligation" is an enabling extension of native chemical ligation in which the C-terminal thioester is accessed by using recombinant DNA technology.[5] Although powerful, these methods are limited by their reliance on cysteine, which is the second least common residue.[6]

Emerging strategies for protein assembly avoid the need for a cysteine residue at the ligation junction.[1] The Staudinger ligation is one such strategy.[7,8] In one version of the Staudinger ligation, a peptide with a C-terminal phosphinothioester is coupled with a second peptide having an N-terminal azido acid to form a new peptide bond in a traceless manner, without residual atoms.[9,10] The reaction occurs without detectable racemization.[11] The Staudinger ligation has been used in the orthogonal assembly of a fully functional enzyme[12] and for the site-specific immobilization of peptides and small molecules on a surface.[13,14] A variety of phosphines have been used to apply the Staudinger ligation to other problems in chemical biology,[7] including glycopeptide synthesis,[15] biomolecular labeling in vitro[16] and in vivo,[17,18] and drug delivery.[19] Most of this work has employed reagents that are engrammic, leaving a phosphine oxide in the ligation product. This attribute has negligible consequences for typical labeling experiments, in which the creation of a stable linkage is of paramount

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importance. In synthetic applications, however, a residual phosphine oxide is unacceptable.

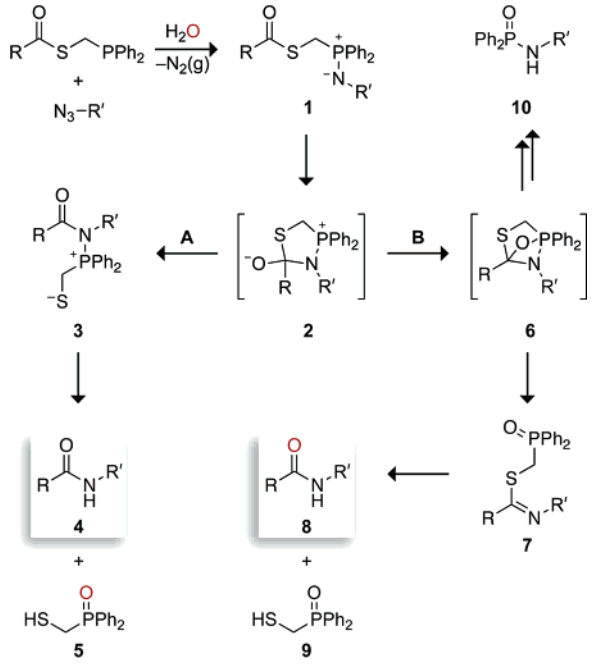
Two distinct phosphinothiols have been used specifically for the Staudinger ligation of peptides. The first, a phosphinothiophenol, was able to provide dipeptides from phosphinothioesters and azido acids, albeit in modest yield (≤35%).[9] The second was a phosphinomethanethiol that effected the Staudinger ligation in high yield (>90%).[10,11] These couplings were performed in mixed THF/water or DMF/water solvents with a stoichiometric ratio of reagents. To form an Xaa-Yaa junction, however, either Xaa or Yaa (or both) must be a glycine residue for the ligation to be efficient (B. L. Nilsson, M. B. Soellner, and R. T. Raines, unpublished results).[20]

Herein, we report on a detailed analysis of the traceless Staudinger ligation between a variety of phosphinothioesters (or phosphinoesters) and azides. Our analyses rely on isotopic labeling experiments and a continuous NMR-based assay for the reaction. These analyses reveal much new and useful information on the mechanism and kinetics of this versatile synthetic reaction.

Results and Discussion

Mechanism of the Staudinger Ligation. Our putative mechanism for the Staudinger ligation is shown in Scheme 1.[10] The nitrogen atom of an iminophosphorane such as **1** has intrinsic nucleophilicity and can be acylated by a thioester (Scheme 1, path A).[21−28] This acylation likely proceeds via

Scheme 1. Putative Mechanisms for the Staudinger Ligation Mediated by (Diphenylphosphino)methanethiol



tetrahedral intermediate **2** to form amidophosphonium salt **3**. Finally, the P−N bond of the amidophosphonium salt is hydrolyzed to form amide **4** and phosphine oxide **5**.

There is considerable literature precedent to indicate that the iminophosphorane formed from a phosphinoester (as opposed to a phosphinothioester) tends to undergo an aza-Wittig reaction[29] to produce a stable imidate (Scheme 1, path B).[16f,p] In Scheme 1, the intramolecular aza-Wittig reaction of iminophosphorane **1** could also form a thioimidate, here **7**. In contrast to the stable imidate formed during Staudinger ligations mediated by phosphinoalcohols,[16f,p] a thioimidate has not been observed as a product of the reaction mediated by phosphinothiols.[30,31] Nonetheless, the failure to observe thioimidate **7** does not preclude its existence on the reaction pathway. Accordingly, we did an experiment to search for evidence of the aza-Wittig reaction of iminophosphorane **1**.

In our proposed mechanism for the Staudinger ligation mediated by a phosphinothiol (Scheme 1, path A),[9,10] the oxygen of phosphine oxide **5** is derived from water during the hydrolysis of amidophosphonium salt **3**. In the alternative aza-Wittig mechanism (Scheme 1, path B), this oxygen originates from the thioester. To distinguish between these two mechanisms, we reacted phosphinothioester **11** (R = $AcNHCH_2$ in Scheme 1) and azide **12** (R′ = $CH_2C(O)NHBn$) in $[^{18}O]H_2O$ and examined the products with mass spectrometry. We found that amide **4** contained exclusively ^{16}O, and phosphine oxide **5** contained only ^{18}O.[32] These data demonstrate that an aza-Wittig reaction does not occur to an appreciable extent in the traceless Staudinger ligation to form a Gly-Gly junction using (diphenylphosphino)methanethiol as the coupling reagent.

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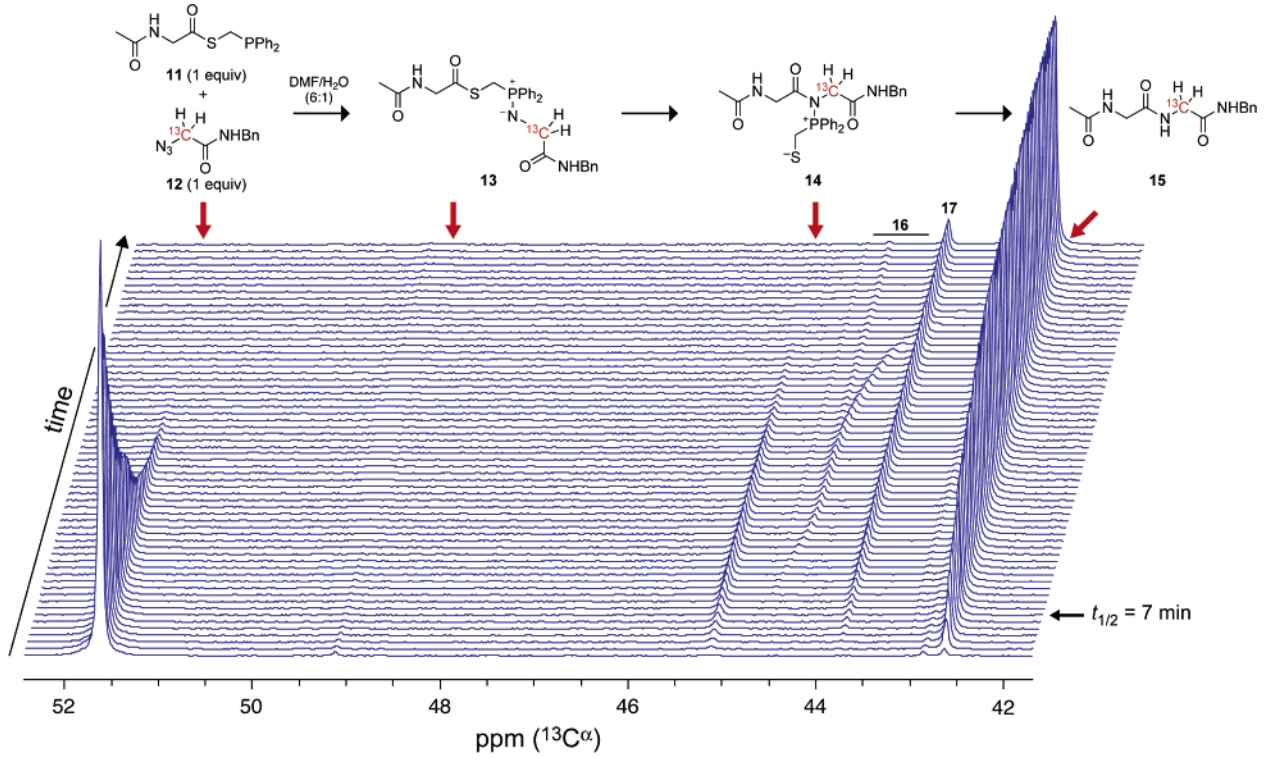


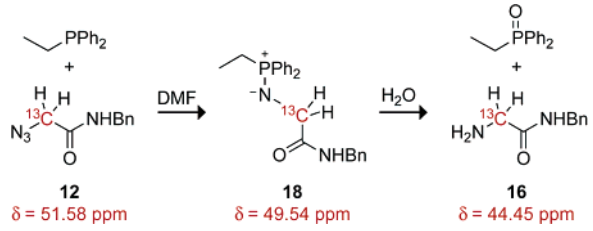
Figure 1. ^{13}C NMR-based assay of a traceless Staudinger ligation. The reaction was performed at room temperature with reagent concentrations of 0.175 M. 90 spectra were acquired over a 12-h time course, scaled to enable the calculation of the second-order rate constant and reaction yield. Peak integrations were calibrated with a standard curve generated with purified ^{13}C-labeled species.

NMR-Based Assay To Monitor the Staudinger Ligation. We next sought to obtain detailed mechanistic and kinetic information about the Staudinger ligation. To do so, we needed an appropriate assay. NMR spectroscopy offers the ability to observe a chemical reaction continuously and without its perturbation. The resulting data can be used to identify reaction intermediates, as well as obtain quantitative data on the rate of their appearance and disappearance. Perhaps most importantly, NMR spectroscopy can reveal a product-distribution profile that is unperturbed by the vagaries of chemical purification. Finally, an assay based on NMR spectroscopy is facile, allowing many coupling reagents and reaction conditions to be surveyed rapidly.

We developed the means to monitor the Staudinger ligation by ^{13}C NMR spectroscopy using an organic azide enriched with ^{13}C at its α-carbon (Figure 1). Detection by ^{13}C NMR spectroscopy was chosen after attempts to follow the reaction by ^{15}N NMR spectroscopy with a uniformly ^{15}N-labeled azide were unsuccessful due to low sensitivity. ^{31}P NMR spectroscopy, which was also considered, reports not on the desired product (amide **4**) but on a side product (phosphine oxide **5**), which can form disulfides that further complicate analyses. DMF was chosen as the solvent, as this solvent had been shown previously to be conducive to the Staudinger ligation.[12,14] In addition, DMF has a high boiling point (153 °C), which allows for monitoring overnight reactions with no significant change in solute concentrations due to solvent evaporation.

To correlate the observed chemical shifts with discrete intermediates along the reaction pathway, mimics of the intermediates were synthesized and characterized further by ^{13}C NMR spectroscopy. To obtain a mimic for the iminophosphorane intermediate, the reaction of diphenylethylphosphine and ^{13}C-labeled azide **12** was monitored by ^{13}C NMR spectroscopy,

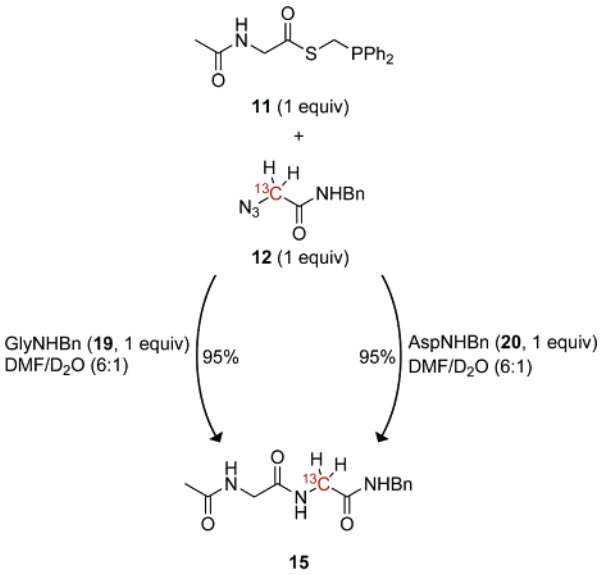
Scheme 2. ^{13}C Chemical Shift of Mimics of Reaction Intermediates



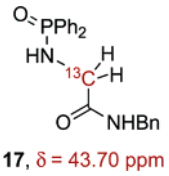
in both the presence and absence of water (Scheme 2). Iminophosphorane **18** was found to have a chemical shift of 49.54 ppm, which indicated that the signal at 49.20 ppm in Figure 1 was due to iminophosphorane **13** in the Staudinger ligation. This model reaction also provided information for the chemical shift of the amine **16** byproduct, observed at 44.45 ppm. During the Staudinger ligation, the chemical shift of amine **16** changed from 44.58 to 44.05 ppm (Figure 1), which is likely due to a decrease in solution pH and consequent amine protonation during the course of the reaction.

To obtain chemical shift information on the amidophosphonium salt intermediate, the reaction of **11** and **12** was monitored in anhydrous DMF. Although the reaction in the absence of water did provide amide **15** (thereby suggesting that amidophosphonium salt **3** in Scheme 1 can fragment to form amide **4** and a thiaphosphiranium salt), it also led to a marked accumulation of a species with a chemical shift of 45.08 ppm, which was likely amidophosphonium salt **14**. Likewise, Staudinger ligation mediated by (diphenylphosphino)ethanethiol, which has an additional methylene group between its phosphorus and sulfur atoms, implicated the chemical shift at 45.08 in Figure 1 as arising from amidophosphonium salt **14** (vida infra). The compound conferring the signal at 43.69 ppm was isolated from

Scheme 3. Staudinger Ligation in the Presence of Amino Acids



the completed reaction and found to be a previously unknown byproduct, phosphonamide **17**. Finally, the chemical shift of isolated amide **15** was observed to be 42.52 ppm.



^{13}C NMR spectroscopy enabled us to observe each putative intermediate in the traceless Staudinger ligation of phosphinothioester **11** and azide **12** (Figure 1). Conveniently, the ^{13}C chemical shifts of the relevant species (azide → iminophosphorane → amidophosphonium salt → amide) decrease monotonically as the reaction progresses. Significantly, the Staudinger ligation of phosphinothioester **11** and azide **12** proceeds without the significant accumulation of any intermediate.

Mechanism for Phosphonamide Byproduct Formation. To reveal the source of the phosphonamide **17** byproduct, phosphinothioester **11** and azide **12** were reacted in the presence of [^{18}O]H_2O. The phosphonamide **17** byproduct produced during this reaction was isolated and found to contain exclusively ^{16}O.[33] This result indicates that phosphonamide byproduct forms via an aza-Wittig reaction of the iminophosphorane (Scheme 1), in which oxazaphosphetane **6** is transformed into phosphonamide **10** by a mechanism that is (as yet) unknown. Discerning the fate of the sulfur atom during the **6**→**10** conversion would likely illuminate this mechanism.

Chemoselectivity of the Staudinger Ligation. To examine its chemoselectivity, the Staudinger ligation of equimolar amounts of phosphinothioester **11** and azide **12** in DMF/D_2O (6:1) was examined by ^{13}C NMR spectroscopy in the presence of compounds containing functional groups present in proteinogenic amino acids (Scheme 3). No significant changes in the spectra were observed upon addition of stoichiometric amounts of GlyNHBn (**19**, which is unlabeled **16**) or AspNHBn (**20**). These data assert further to the chemoselectivity of the reaction with (diphenylphosphino)methanethiol as a coupling reagent.[14]

(33) MS (ESI) *m*/*z* 388.1251 (MNa^+ [$C_{20}{}^{13}CH_{21}N_2O_2PNa^+$] = 388.1266).

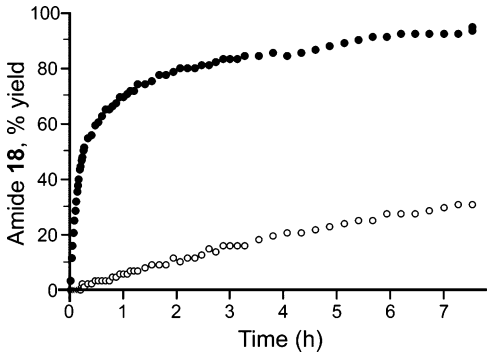
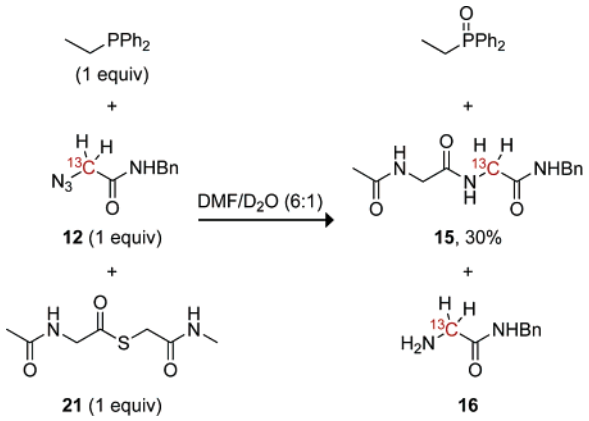


Figure 2. Rate of amide **15** formation during an intramolecular (●) and intermolecular (○) Staudinger ligation. The intramolecular reaction was between phosphinothioester **11** and azide **12**. The intermolecular reaction was between (diphenylethyl)phosphine, azide **12**, and thioester **21** (Scheme 4). Reactions were performed at room temperature with reagent concentrations of 0.175 M and were monitored by ^{13}C NMR spectroscopy as in Figure 1. The initial rate constants for amide formation were $(7.7 \pm 0.3) \times 10^{-3}$ M^{-1} s^{-1} (intramolecular) and $(6.5 \pm 0.1) \times 10^{-4}$ M^{-2} s^{-1} (intermolecular).

Scheme 4. Intermolecular Staudinger Ligation



Rate of Glycyl Couplings Using (Diphenylphosphino)-methanethiol. The reaction of phosphinothioester **11** and azide **12** has a half-life of $t_{1/2} = 7$ min with reactant concentrations of 0.175 M (Figure 1). These data were used to calculate a second-order rate constant for the appearance of the amide **15** product of $k_2 = (7.7 \pm 0.3) \times 10^{-3}$ M^{-1} s^{-1}. This value is similar to that reported previously for the Staudinger reduction of ethyl(2-azido)acetate by triphenylphosphine in benzene ($k_2 = 0.01$ M^{-1} s^{-1}).[34] A recent analysis by Bertozzi and co-workers of a nontraceless Staudinger ligation with an oxygen ester revealed a similar rate constant.[35] As with the Staudinger reduction,[34] the lack of intermediate accumulation (Figure 1) indicates that the encounter of the phosphine and azide is the rate-determining step in the traceless Staudinger ligation.

To quantify the effect of tethering the phosphine functionality to the thioester functionality, we used our NMR-based assay to examine an intermolecular version of the traceless Staudinger ligation (Scheme 4). This reaction proceeded in low yield relative to their intramolecular counterparts.[25] Based on the derived rate constants (Figure 2), the effective concentration of

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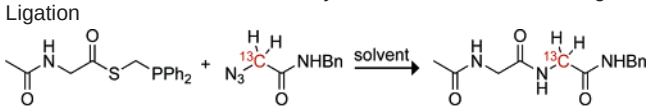
Table 1. Effect of Solvent Polarity on the Rate of the Staudinger Ligation



11 (1 equiv) **12** (1 equiv) **15**

solvent	SPP	k_2 (10^{-3} M^{-1} s^{-1})
DMF	0.954	7.7
CH_3CN	0.895	4.7
THF	0.838	1.3

the nitrogen nucleophile in iminophosphorane **1** is estimated to be $EC = (7.7 \times 10^{-3}\ M^{-1}\ s^{-1})/(6.5 \times 10^{-4}\ M^{-2}\ s^{-1}) = 12$ M.

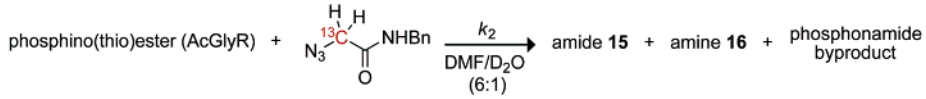
Effect of Solvent Polarity on Reaction Rate. The Staudinger ligation of phosphinothioester **11** and azide **12** was assayed in three solvents of different polarities. The observed rate constants are listed in Table 1. In general, the reaction rate was higher in more polar solvents, as denoted by the solvent polarity−polarizability scale (SPP).[36] These data indicate that the rate-determining step involves a polar transition state that is stabilized by polar solvents. This transition state is likely that for the formation of the initial $R_3{-}P^+{-}N{=}N{-}N{-}R'$ phosphazide intermediate.[37,38]

Comparison of Coupling Reagents in the Staudinger Ligation. Several reagents other than phosphinomethanethiol **22** have been used in the traceless Staudinger ligation, with varied success.[39−41] These compounds include phosphinothiophenol **23**,[9] phosphinomethanol **24**,[39] phosphinoethanethiol **25**,[41] and phosphinophenol **26**[39] (Figures S1−S4 in the Supporting Information). We used our NMR-based assay to compare the efficacy of reagents **22**−**26** in mediating the coupling of acetylglycine and azide **12** in DMF/D_2O (6:1). The results are listed in Table 2.

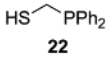
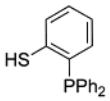
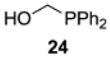
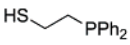
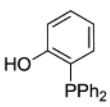
The first traceless Staudinger ligation used phosphinothiophenol **23** as the coupling reagent.[9] The yield of this reaction, as determined herein by our NMR-based assay, was 38%. This yield is similar to the 35% yield determined previously by product isolation.[9] No buildup of intermediates was observed during the course of the reaction. After 12 h, the reaction consisted solely of the amide **15** product and amine **16** byproduct. The rate constant for the reaction of $AcGlySC_6H_4$-*o*-PPh_2 and azide **12** was found to be $k_2 = (1.04 \pm 0.05) \times 10^{-3}\ M^{-1}\ s^{-1}$, which is 14% of that for the same reaction mediated by phosphinothiol **22**. Using phosphinothiophenol **23** as the coupling reagent has another consequence, an increase in the production of the amine **16** byproduct, presumably because hydrolysis of the iminophosphorane intermediate is able to compete more successfully with *S*→*N* acyl transfer.

The next coupling reagent examined was (diphenylphosphino)methanol (**24**). This reagent was described by Bertozzi and co-workers and differs from (diphenylphosphino)methanethiol (**22**) only in its bridging oxygen,[39] enabling a direct comparison of an ester and thioester reagent in mediating a traceless Staudinger ligation. The rate constant for the formation of amide **15** was $k_2 = (1.2 \pm 0.1) \times 10^{-4}\ M^{-1}\ s^{-1}$, which is merely 2% that with (diphenylphosphino)methanethiol **22**. Moreover, the reaction of phosphinoester **27** and azide **12** resulted in a low (11%) yield of the amide **15** product (Table 2). Bertozzi and co-workers reported that the Staudinger ligation mediated by (diphenylphosphino)methanol yielded only the amine byproduct.[39] In contrast, we observed nearly exclusive formation of aza-Wittig byproducts with our NMR-based assay. Mass spectrometric analysis indicates that the aza-Wittig adduct is likely to be oxazaphosphetane **29** or imidate **30** (Scheme 5), which have identical mass.[42] The presence of a cross-peak ($^{13}C-^{31}P$ $^3J = 20.2$ Hz) in the $^{13}C-^{31}P$ two-dimensional COSY NMR spectrum provides additional support for the presence of oxazaphosphetane **29** (data not shown). Attempted purification of this adduct led to its decomposition.

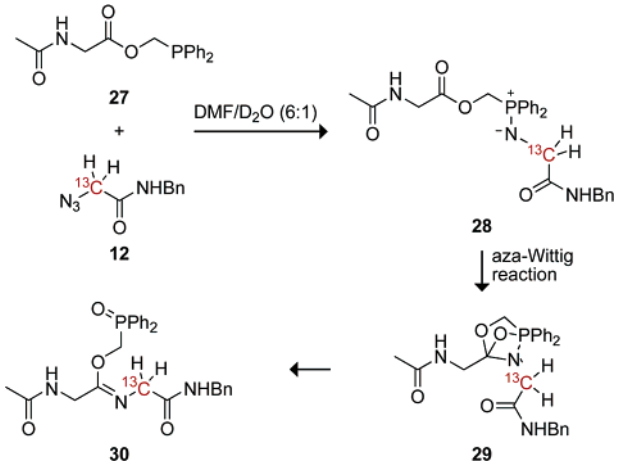
Table 2. Effect of Coupling Reagent on the Rate and Product Distribution of the Staudinger Ligation



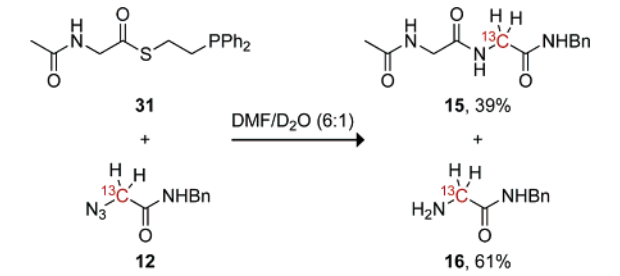
HR = **22–26** (1 equiv) **12** (1 equiv)

coupling reagent (HR)	k_2 (10^{-3} $M^{-1}s^{-1}$)	% amide **15**	% amine **16**	% phosphonamide byproduct
 **22**	7.7 ± 0.3	95	3	2
 **23**	1.04 ± 0.05	38	62	0
 **24**	0.12 ± 0.01	11	0	89
 **25**	0.65 ± 0.01	39	61	0
 **26**	7.43 ± 0.03	99	0	0

Scheme 5. Staudinger Ligation with (Diphenylphosphino)methanol (**24**)



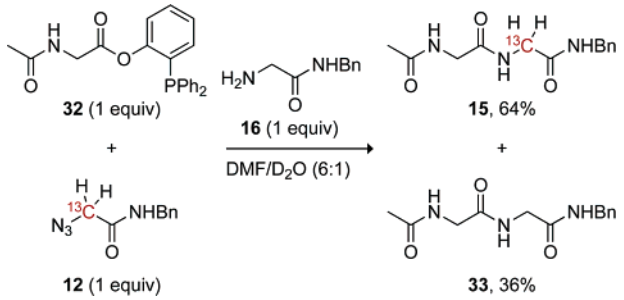
Scheme 6. Staudinger Ligation with (Diphenylphosphino)ethanethiol (**25**)



The inability of phosphinoalcohol **24** to mediate the Staudinger ligation indicates that the nature of the leaving group plays an important role in the partitioning of tetrahedral intermediate **2** toward a Staudinger ligation (Scheme 1, path A) or aza-Wittig reaction (Scheme 1, path B). With a poor leaving group, such as an alkoxide, the oxyanion of the tetrahedral intermediate is long-lived enough to react with the oxophilic phosphorus to form an oxazaphosphetane (e.g., **29** in Scheme 5). In contrast, a good leaving group, such as a thiolate, is displaced quickly from the tetrahedral intermediate, leading to the formation of an amidophosphonium salt. Thus, these results highlight a key advantage of using a phosphinothiol (**22**) rather than a phosphinoalcohol (**24**) to mediate the traceless Staudinger ligation.

Recently, Viola and co-workers examined the Staudinger ligation mediated by (diphenylphosphino)ethanethiol (**25**).[41] This reaction was reported to proceed in quantitative yield, as monitored by thin-layer chromatography. With our NMR-based assay, however, we found that the reaction of phosphinothioester **31** and azide **12** in DMF/D_2O (6:1) yields mostly the amine **16** byproduct (Table 2; Scheme 6). The rate constant for the formation of amide **15** was $k_2 = (6.5 \pm 0.1) \times 10^{-4}$ M^{-1} s^{-1}, which is only 8% that with (diphenylphosphino)methanethiol (**22**). The low rate and high level of amine are likely due to the increased size of the ring that is formed during the nucleophilic attack of the iminophosphorane nitrogen on the thioester (e.g., to produce tetrahedral intermediate **2** in Scheme 1). Indeed, a comparison of the reaction rates and product distributions of the ligations mediated by **22**, **23**, and **25** indicates that ring size [5 atoms (**22**) being better than 6 atoms (**23** and **25**)] is a more significant determinant of the efficacy of a phosphinothiol in mediating the traceless Staudinger ligation than is the type of thiol [alkyl (**22** and **25**) versus aryl (**23**)]. The larger ring size of the tetrahedral intermediate derived from **23** and **25** also appears to change the rate-determining step for the formation of amide **15**. The lower reaction rate and accumulation of the amine **16** byproduct are consistent with the formation of tetrahedral intermediate **2** (rather than the initial phosphazide intermediate) limiting the rate of the Staudinger ligation mediated by **23** and **25**.

Scheme 7. Staudinger Ligation with (*o*-Diphenylphosphino)phenol (**26**)



Finally, we examined (*o*-diphenylphosphino)phenol (**26**). This coupling reagent was first described by Bertozzi and co-workers and later used by Liskamp and co-workers in the synthesis of tetrapeptides with non-glycyl residues.[39,40] Bertozzi and co-workers obtained a yield of >95% using this reagent for acyl transfers to an azido nucleotide.[39] Likewise, our yield for the reaction of phosphinoester **32** and azide **12** was nearly quantitative (99%) with no observable formation of the amine **16** byproduct or accumulation of reaction intermediates (Table 2). The rate constant for the formation of amide **15** was $k_2 = (7.43 \pm 0.03) \times 10^{-3}$ M^{-1} s^{-1}, which is indistinguishable from that with (diphenylphosphino)methanethiol (**22**)[$k_2 = (7.7 \pm 0.3) \times 10^{-3}$ M^{-1} s^{-1}].

As phosphinophenol **26** was as efficacious as (diphenylphosphino)methanethiol (**22**) in mediating a traceless Staudinger ligation, its reactivity was investigated further. The presence of a stoichiometric amount of GlyNHBn (**19**) during the reaction of azide **12** and phosphinoester **32** decreased the yield of amide **15** from 97% to 64% (Scheme 7). This decrease in yield with phosphinoester **32** is in contrast to the reaction with phosphinothioester **11**, which produced no less amide **15** in the presence of a stoichiometric amount of GlyNHBn (Scheme 3). Presumably, the diminished yield with phosphinoester **32** was due to the greater electrophilicity of the aryl ester of **32** than the alkyl thioester of **11**. This result is consistent with the findings of Liskamp and co-workers, who reported that both N-terminal and lysyl ϵ-amino groups can undergo nonspecific reaction with esters derived from phosphinophenol **26**.[40] Thus, phosphinophenol **26** can be an exceptional reagent for mediating the traceless Staudinger ligation, but its intrinsic lack of chemoselectivity constrains its use.

(36) Catalan, J.; Lopez, V.; Perez, P.; Martinvillamil, R.; Rodriguez, J. G. *Liebigs Ann.* **1995**, 241−252.
(37) Gololobov, Y. G.; Zhmurova, I. N.; Kasukhin, L. F. *Tetrahedron* **1981**, *37*, 437−472.
(38) Gololobov, Y. G.; Kasukhin, L. F. *Tetrahedron* **1992**, *48*, 1353−1406.
(39) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141−2143.
(40) Merkx, R.; Rijkers, D. T. S.; Kemmink, J.; Liskamp, R. M. J. *Tetrahedron Lett.* **2003**, *44*, 4515−4518.
(41) Han, S.; Viola, R. E. *Protein Pept. Lett.* **2004**, *11*, 107−114.
(42) MS (ESI) *m/z* 388.1251 (MNa^+ [$C_{20}{}^{13}CH_{21}N_2O_2PNa^+$] = 388.1266).

Scheme 8. Staudinger Ligation of Non-Glycyl Residues

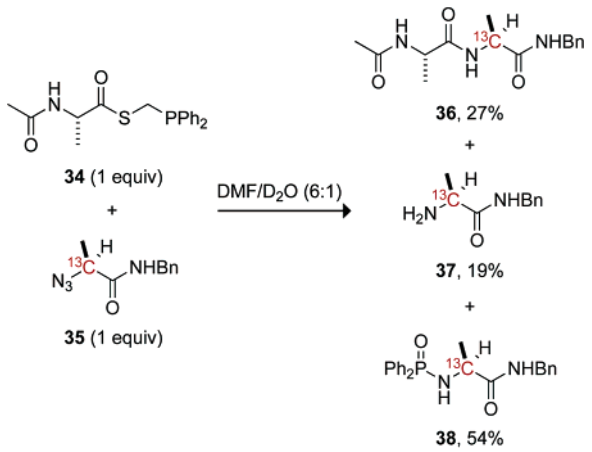
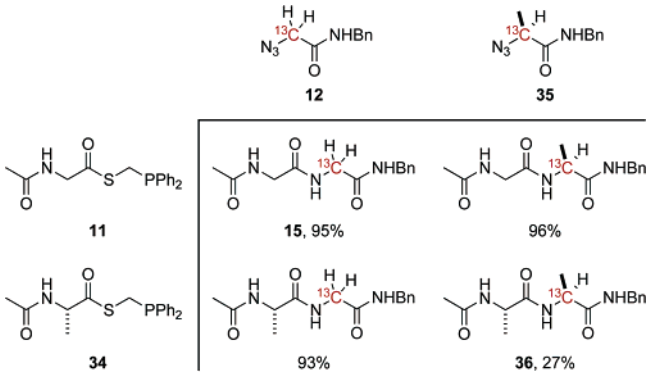


Table 3. Yield of Amide from the Staudinger Ligation of Glycyl and Non-Glycyl Residues



Non-Glycyl Couplings. High (>90%) yields in the Staudinger ligation mediated by (diphenylphosphino)methanethiol (**22**) are obtained routinely if a glycine residue is at the *N*- or *C*-terminus of the ligation junction.[10,11] Couplings involving two non-glycyl residues provide low (20−50%) yields.[20] The origin of these low yields is not readily apparent upon consideration of the putative reaction mechanism (Scheme 1). Accordingly, we used our NMR-based assay to examine reactions involving a non-glycyl phosphinothioester and non-glycyl azide.

As a model coupling for non-glycyl residues, alanyl phosphinothioester **34** and alanyl azide **35** were allowed to react in DMF/D_2O (6:1). At room temperature, the reaction resulted in 27% amide **36** product, 19% amine **37** byproduct, and 54% phosphonamide **38** byproduct (Scheme 8). Moreover, the rate constant for amide formation was found to be $k_2 = (3.7 \pm 0.3) \times 10^{-3}\ M^{-1}\ s^{-1}$, which is half that for the analogous all-glycyl coupling. When the reaction was performed in the presence of [^{18}O]H_2O, the phosphonamide **38** byproduct contained exclusively ^{16}O,[42] indicating once again that this byproduct results from an aza-Wittig reaction. A comparison of the quantity of amide produced during the four possible glycyl and alanyl couplings (Table 3) indicates that steric encumbrance during the reaction of alanyl phosphinothioester **34** and alanyl azide **35** (27% yield) is significantly more severe than that during a glycyl coupling (93−96% yield). This strain could arise in tetrahedral intermediate **2**, in which the bulky R and R′ groups of a non-glycyl coupling are brought into close proximity.

To acertain whether the stereochemistry at the α-carbon plays a role in the decreased amide yield of a non-glycyl coupling, the reaction of D-alanyl phosphinothioester **39** (which is the enantiomer of **34**) and azide **35** was examined with our NMR-based assay. The yield of amide from this reaction was indistinguishable from that for L-alanyl phosphinothioester **34**, indicating that amino acid stereochemistry does not play a role in the diminished yields of non-glycyl couplings.

The ratio of amide **36** product to phosphonamide **38** byproduct did not rely on the presence of water. In anhydrous DMF, the reaction of alanyl phosphinothioester **34** and azide **35** results in 36% amide **36** product and 64% phosphonamide **38** byproduct. This nearly 1:2 ratio is similar to that observed in DMF/D_2O (6:1) (Scheme 8). This finding is consistent with the putative mechanism in Scheme 1, as the hydrolysis of the iminophosphorane intermediate occurs prior to the partitioning toward path A (amide product) or path B (phosphonamide byproduct).

Finally, increasing the temperature of the reaction of alanyl phosphinothioester **34** and alanyl azide **35** increased the ratio of amide **36** product to phosphonamide **38** byproduct. Still, at the highest temperature tested (50 °C), the yield of amide **36** remained moderate (48%). This yield for a non-glycyl coupling with (diphenylphosphino)methanethiol (**22**) is comparable to that reported previously with phosphinophenol **26**.[40]

Conclusions

We have elucidated the mechanism for the traceless Staudinger ligation mediated by phosphinothiols and phosphinoalcohols. By developing and using a sensitive and continuous assay based on ^{13}C NMR spectroscopy, we were able to probe for reaction intermediates and determine reaction rate constants. In addition, we were able to compare reagents for their efficacy in mediating the Staudinger ligation. Based on its high rate constant and chemoselectivity, (diphenylphosphino)methanethiol (**22**)[10] was judged to be the most efficacious of known coupling reagents. Efforts are underway in our laboratory to use the extant understanding of the mechanism and kinetics of the Staudinger ligation to develop new reagents and reaction conditions for the high-yielding, traceless coupling of non-glycyl residues and other chemical transformations.

Experimental Procedures

General. Reactions were monitored by thin-layer chromatography with visualization by UV light or staining with ninhydrin or I_2. Compound purification was carried out with an Argonaut Flashmaster Solo automated chromatography system. Silica gel used in flash chromatography had a 230−400 mesh and 60 Å pore size. Reagent chemicals were obtained from commercial suppliers, and reagent grade solvents were used without further purification. NMR spectra were obtained with a 500 or 400 MHz spectrometer at the National Magnetic Resonance Facility at Madison (NMRFAM) or the University of Wisconsin Nuclear Magnetic Resonance Facility, respectively. Carbon-13 and phosphorus-31 spectra were both proton-decoupled, and phosphorus-31 spectra were referenced against an external standard of deuterated phosphoric acid (0 ppm). Mass spectra were obtained with electrospray ionization (ESI) techniques.

General Procedures for Kinetics Experiments Using ^{13}C NMR Spectroscopy. A phosphinothioester or phosphinoester (0.105 mmol in 400 μL of DMF) was mixed with a ^{13}C-labeled azide (0.105 mmol in 300 μL of DMF/D_2O (2:1)) in a vial. After mixing, the solution was transferred to an NMR tube. (Due to the evolution of N_2(g), NMR tubes were not capped during experiments.) The NMR tube containing the reaction mixture was then inserted into an NMR spectrometer that had

been prelocked on a sample containing ^{13}C-labeled azide in 700 μL of DMF/D_2O (6:1). After an initial delay of 45 s, the acquisition of NMR spectra was initiated. 90 spectra were acquired over a scaled time course of 16 h (15 spectra during the first 15 min, 15 spectra during the next 30 min, 15 spectra during the next 60 min, 15 spectra during the next 120 min, 15 spectra during the next 240 min, and 15 spectra during the final 480 min). Each time point was designed to consume 44 s, with the remaining time being a preacquisition delay before the next scan. An appropriate flip angle (30° pulse) and relaxation delay (10 s) were chosen to obtain fully quantitative spectra at each time point and for each intermediate. In addition, the decoupler was turned on solely during the acquisition to prevent any NOE buildups. To confirm that the NMR-based assay provided quantitative results, a standard curve was made for each starting material and available intermediate and shown to correlate well with the spectral integration during a reaction.

General Procedures for Staudinger Ligations. Unless noted otherwise, Staudinger ligations were performed at room temperature with equimolar amounts of phosphinothioester (or phosphinoester) and azide (0.105 mmol) in DMF/D_2O (6:1; 600 μL).

AcGlySCH$_2$PPh$_2$ (11). *N*-Acetylglycine (1.90 g, 16.2 mmol) was dissolved in anhydrous DMF (75 mL). HOBt (2.48 g, 16.2 mmol) was added to the resulting solution, followed by DCC (3.34 g, 16.2 mmol). Once precipitate (DCU) was observed, phosphinothiol **26** was added (3.77 g, 16.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a white solid. The solid was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate). Phosphinothioester **11** was isolated in 96% yield. **Spectral Data.** Spectral data were as reported previously.[10]

[2-^{13}C]-2-Azido-*N*-benzyl-acetamide (12). Azide **12** was synthesized from 2-^{13}C bromoacetic bromide via methods reported previously[10] and isolated in 98% yield. **Spectral Data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.39−7.27 (m, 5H), 6.71 (bs, 1H), 4.47 (d, J = 5.7 Hz, 2H), 4.00 (s, 2H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ 166.66, 137.39, 128.43, 127.45, 127.35, 52.06, 43.08 ppm; MS (ESI) *m*/*z* 214.0789 (MNa$^+$ [$C_8{}^{13}CH_{10}N_4ONa^+$] = 214.0780).

AcGly[^{13}C$^\alpha$]GlyNHBn (15). Amide **15** was synthesized by using the general procedures for Staudinger ligation (vide supra). **Spectral Data.** Spectral data were the same as reported previously.[10]

[^{13}C$^\alpha$]GlyNHBn (16). Azide **12** (951 mg, 5.0 mmol) was dissolved in anhydrous THF (30 mL) in a flame-dried flask under Ar(g). Ethyl diphenyl phosphine (1.29 g, 6.0 mmol) was added to the resulting solution, which was then allowed to stir under Ar(g) for 8 h. Water (3.3 mL, to 10% v/v) was added, and the reaction mixture was stirred for an additional 1 h. The solvent was then removed under reduced pressure, and the resulting oil was purified by flash chromatography (silica gel, 3% v/v MeOH in CH_2Cl_2). Amine **16** was isolated in 97% yield as a clear oil. **Spectral Data.** Spectral data were the same as reported previously.[43]

Ph$_2$P(O)[^{13}C$^\alpha$]GlyNHBn (17). Phosphonamide **17** was isolated from a Staudinger ligation of phosphinothioester **11** and azide **12**, performed as described above. The solvent of the reaction was removed under reduced pressure, and the resulting oil was washed with CH_2Cl_2. The suspension was filtered, and phosphonamide **17** was isolated in 1% yield.

Phosphonamide **17** was also synthesized directly by the reaction of diphenylphosphinic chloride and GlyNHBn (**16**). Diphenylphosphinic chloride (1.0 g, 4.2 mmol) was dissolved in anhydrous THF (20 mL). DMAP (60 mg, 0.5 mmol) was added to the resulting solution, followed by GlyNHBn (700 mg, 4.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 8 h. The solvent was then removed under reduced pressure, and the resulting oil was purified by flash chromatography (silica gel, 3% v/v MeOH in CH_2Cl_2). Phosphonamide **17** was isolated in 81% yield.

The spectral data for the isolated and synthesized compounds were indistinguishable.

Spectral Data. ^{1}H NMR (CD_3OD, 400 MHz) δ 7.91−7.86 (m, 5 H), 7.55−7.50 (m, 5H), 7.34−7.26 (m, 5H), 4.42 (s, 2H), 3.94 (s, 2H) ppm; ^{13}C NMR (CD_3OD, 125 MHz) δ 168.63, 138.19, 134.06, 132.75, 131.63, 131.60, 131.42, 131.32, 128.26, 128.18, 128.12, 127.22, 126.94, 51.71, 42.69 ppm; ^{31}P NMR (CD_3OD, 161 MHz) δ 24.55 ppm; MS (ESI) *m*/*z* 388.1251 (MNa$^+$ [$C_{20}{}^{13}CH_{21}N_2O_2PNa^+$] = 388.1266).

GlyNHBn (19) and AspNHBn (20). Amines **19** (which is unlabeled **16**)[43] and **20**[44] were prepared according to procedures reported previously. **Spectral Data.** Spectral data were as reported previously.[43,44]

AcGlySCH$_2$C(O)NHMe (21). *N*-Acetylglycine (4.0 g, 34.2 mmol) was dissolved in anhydrous DMF (100 mL). DCC (7.06 g, 34.2 mmol) was then added. Once precipitate (DCU) was observed, *N*-methyl mercaptoacetamide was added (3.6 g, 34.2 mmol). The reaction mixture was allowed to stir under Ar(g) for 18 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a white solid. The solid was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate/hexanes). Thioester **21** was isolated as an off-white solid in 91% yield. **Spectral Data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 6.09 (bs, 1H), 4.26 (bs, 1H), 3.59 (s, 2H), 2.82 (s, 2H), 2.18 (s, 3H), 2.10 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$/CD_3OD (1:1), 125 MHz) δ 196.99, 172.23, 169.07, 31.43, 31.40, 26.04, 25.90, 21.65 ppm; MS (ESI) *m*/*z* 227.0457 (MNa$^+$ [$C_7H_{12}N_2O_3SNa^+$] = 227.0461).

(Diphenylphosphino)methanethiol (22), (*o*-Diphenylphosphino)-benzenethiol (23), (diphenylphosphino)methanol (24), (Diphenylphosphino)ethanethiol (25), and (*o*-Diphenylphosphino)phenol (26). Compounds **22**,[11] **23**,[9] **24**,[39] **25**,[41] and **26**[39] were prepared according to reports published previously. **Spectral Data.** Spectral data were as reported previously.[9,11,39,41]

AcGlyOCH$_2$PPh$_2$ (27). *N*-Acetylglycine (1.12 g, 10.0 mmol) was dissolved in anhydrous CH_2Cl_2 (30 mL). DMAP (0.12 g, 1.0 mmol) was added to the resulting solution, followed by DCC (2.06 g, 10.0 mmol). Once precipitate (DCU) was observed, (diphenylphosphino)-methanol (**24**; 2.16 g, 10.0 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 18 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate/hexanes). Phosphinoester **27** was isolated as a colorless oil in 56% yield. **Spectral Data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.47−7.42 (m, 4H), 7.39−7.37 (m, 6H), 5.89 (bt, 1H), 4.92 (d, J = 6.2 Hz, 2H), 3.98 (d, J = 5.0 Hz, 2H), 2.00 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 185.35, 169.59, 132.65, 132.50, 128.85, 128.27, 128.22, 48.13, 33.27, 25.17, 24.47 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ −20.02 ppm; MS (ESI) *m*/*z* 338.0920 (MNa$^+$ [$C_{17}H_{18}NO_3PNa^+$] = 338.0916).

AcGlySCH$_2$CH$_2$PPh$_2$ (31). *N*-Acetylglycine (475 mg, 4.06 mmol) was dissolved in anhydrous DMF (20 mL). HOBt (549 mg, 4.06 mmol) was added to the resulting solution, followed by DCC (838 mg, 4.06 mmol). Once precipitate (DCU) was observed, (diphenylphosphino)-ethanethiol (**25**; 1.0 g, 4.06 mmol) was added. The reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 70% v/v ethyl acetate in hexanes). Phosphinoester **31** was isolated as a white solid in 92% yield. **Spectral Data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.47−7.43 (m, 4H), 7.37−7.36 (m, 6H), 6.06 (bs, 1H), 4.20 (d, J = 5.6 Hz, 2H), 3.00 (m, 2H), 2.46 (m, 2H), 2.07 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 196.78, 170.65, 137.23, 137.14, 132.68, 132.52, 128.81, 128.53, 128.47, 126.33, 125.53, 49.15, 28.27 (d, J = 15.4 Hz), 25.60 (d, J = 23.1 Hz), 22.81 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ −19.88 ppm; MS (ESI) *m*/*z* 368.0852 (MNa$^+$ [$C_{18}H_{20}NO_2PSNa^+$] = 368.0845).

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AcGlyOC$_6$H$_4$-*o*-PPh$_2$ (32). *N*-Acetylglycine (562 mg, 4.8 mmol) was dissolved in anhydrous CH_2Cl_2 (50 mL). DMAP (59 mg, 0.5 mmol) was added to the resulting solution, followed by DCC (990 mg, 4.8 mmol). Once precipitate (DCU) was observed, (*o*-diphenylphosphino)-phenol (**26**; 2.16 g, 10.0 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, ethyl acetate/hexanes (1:1)). Phosphinoester **32** was isolated as a white solid in 36% yield. **Spectral Data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.42−7.28 (m, 12H), 7.19−7.17 (m, 2H), 6.87 (bt, 1H), 4.00 (d, J = 5.0 Hz, 2H), 1.98 (s, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 169.98, 168.03, 135.24, 135.14, 133.99, 133.82, 130.12, 129.18, 128.73, 128.67, 126.63, 122.37, 41.27, 22.88 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ −19.07 ppm; MS (ESI) *m*/*z* 400.1071 (MNa^+ [$C_{22}H_{20}NO_3PNa^+$] = 400.1073).

AcGlyGlyNHBn (33). Amide **33** (which is unlabeled **15**) was synthesized using the general procedures for Staudinger ligations (vide supra). **Spectral Data.** Spectral data were the same as that reported previously.[10]

AcAlaSCH$_2$PPh$_2$ (34). *N*-Acetylalanine (557 mg, 4.25 mmol) was dissolved in anhydrous DMF (20 mL). HOBt (527 mg, 3.90 mmol) was added to the resulting solution, followed by DCC (805 mg, 3.90 mmol). Once precipitate (DCU) was observed, (diphenylphosphino)-methanethiol (**22**; 900 mg, 3.87 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 4 h. The precipitate was removed by filtration, and the filtrate was concentrated under reduced pressure to give a clear oil. The oil was dissolved in ethyl acetate and purified by flash chromatography (silica gel, 70% v/v ethyl acetate in hexanes). Phosphinothioester **34** was isolated as a white solid in 83% yield.

This procedure was repeated with Ac-D-AlaOH to give Ac-D-AlaSCH$_2$PPh$_2$ (**39**) as a white solid in 92% yield. The spectral data for AcAlaSCH$_2$PPh$_2$ and Ac-D-AlaSCH$_2$PPh$_2$ were indistinguishable.

Spectral Data. ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.44−7.37 (m, 10 H), 5.89 (bt, 1H), 4.71 (bt, 1H), 3.53 (s, 2H), 2.02 (s, 3H), 1.64 (m, 1H), 1.32 (d, J = 5.8 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 199.85, 169.70, 136.51, 132.74, 132.59, 132.57, 129.10, 128.51, 128.46, 54.83, 25.29 (d, J = 24.3 Hz), 22.99, 18.66 ppm; ^{31}P NMR ($CDCl_3$, 161 MHz) δ −17.97 ppm; MS (ESI) *m*/*z* 368.0853 (MNa^+ [$C_{18}H_{20}NO_2PSNa^+$] = 368.0845).

[2-^{13}C]-(2*S*)-2-Azido-*N*-benzyl-1-propionamide (35). [2-^{13}C]-(2*S*)-2-Azido-1-propionic acid was synthesized from [$^{13}C^{\alpha}$]-(2*S*)-alanine according to the procedure of Lundquist and Pelletier.[45] [2-^{13}C]-(2*S*)-2-Azido-1-propionic acid (280 mg, 2.8 mmol) was dissolved in anhydrous DMF (15 mL). Hydroxybenzotriazole (397 mg, 2.9 mmol) was then added, followed by DCC (607 mg, 2.9 mmol). Once precipitate (DCU) was observed, benzylamine (0.370 mL, 3.4 mmol) was added, and the reaction mixture was allowed to stir under Ar(g) for 3 h. The precipitate was removed by filtration, and the filtrate was removed under reduced pressure to yield a yellow oil. This oil was dissolved in 35% v/v ethyl acetate in hexanes and purified by flash chromatography (silica gel, 35% v/v ethyl acetate in hexanes). Azide **35** was isolated in 90% yield as an off-white solid. **Spectral Data.** ^{1}H NMR ($CDCl_3$, 400 MHz) δ 7.37−7.28 (m, 5 H), 6.67 (bs, 1H), 4.46 (s, 2H), 4.13 (dt, J = 144.7, 6.9 Hz, 1H), 1.60 (d, J = 2.3 Hz, 3H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 128.78, 127.74, 59.29, 43.52 ppm; MS (ESI) *m*/*z* 228.0935 (MNa^+ [$C_9{}^{13}CH_{12}N_4ONa^+$] = 228.0937).

AcAlaAlaNHBn (36). Amide **36** was prepared by using the general procedures for Staudinger ligations (vide supra). **Spectral Data.** ^{1}H NMR (300 MHz, $CDCl_3/CD_3OD$ (1:1)) δ 7.32−7.35 (m, 5 H), 4.51−4.22 (m, 4H), 1.98 (1.87) (s, 3H), 1.41 (d, J = 7.5 Hz, 3H), 1.34 (d, J = 6.9 Hz, 3H) ppm; ^{13}C NMR (75 MHz, $CDCl_3/CD_3OD$ (1:1), numbers in parentheses indicate doubling due to rotational isomerism) δ 173.27 (172.78), 172.67 (172.54), 171.44, 137.59 (137.51), 127.39 (127.49), 126.28 (126.36), 126.02 (126.17), 48.92 (48.70), 48.59 (48.34), 41.99 (42.06), 20.50 (20.76), 15.99 (16.23), 15.45 ppm; MS (ESI) *m*/*z* 315.1511 (MNa^+ [$C_{14}{}^{13}CH_{21}N_3O_3Na^+$] = 315.1509).

Ph$_2$P(O)[$^{13}C^{\alpha}$]AlaNHBn (38). Phosphonamide **38** was isolated from a Staudinger ligation of phosphinothioester **34** and azide **35**, performed as described above. The solvent of the reaction was removed under reduced pressure, and the resulting oil was washed with CH_2Cl_2. The suspension was then filtered, and phosphonamide **38** was isolated in 54% yield. **Spectral Data.** ^{1}H NMR (CD_3OD, 400 MHz) δ 7.92−7.86 (m, 5H), 7.58−7.48 (m, 5H), 7.35−7.26 (m, 5H), 4.41 (s, 2H), 4.00 (q, J = 7.1 Hz, 1H), 1.47 (d, J = 7.1 Hz, 3H) ppm; ^{13}C NMR (CD_3OD, 125 MHz) δ 171.58, 138.26, 134.07, 132.76, 131.63, 131.60, 131.42, 131.32, 128.26, 128.18, 128.13, 127.12, 126.92, 58.23, 42.64, 15.97 ppm; ^{31}P NMR (CD_3OD, 161 MHz) δ 24.63 ppm; MS (ESI) *m*/*z* 402.1432 (MNa^+ [$C_{21}{}^{13}CH_{23}N_2O_2PNa^+$] = 402.1423).

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Supporting Information Available: Four figures such as Figure 1 depicting the results of ^{13}C NMR-based assays of the reaction of azide **12** with phosphinothiophenol **23**, phosphinoalcohol **24**, phosphinothiol **25**, and phosphinophenol **26**. This material is available free of charge via the Internet at http://pubs.acs.org.

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