High-Yielding Staudinger Ligation of a Phosphinothioester and Azide To Form a Peptide
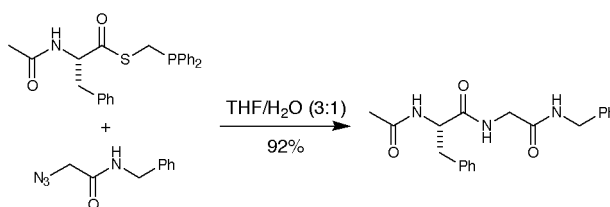
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ABSTRACT

The Staudinger ligation can be used to couple a peptide with a *C*-terminal phosphinothioester to another with an *N*-terminal α-azido group to form a single peptide that contains no residual atoms. Here, diphenylphosphinomethanethiol thioesters are shown to give high isolated yields for this transformation. This finding provides precedent for a powerful and versatile new method for the total synthesis of proteins.

The ligation of synthetic peptides provides a convergent route for the total chemical synthesis of proteins. Currently, the most common ligation method is "native chemical ligation". This ligation method was discovered in 1953, when the reaction of ValSPh and CysOH in aqueous buffer was shown to yield the dipeptide: ValCysOH.[1] In the 1990s, this seminal discovery was developed into a practical method to ligate large peptide fragments.[2,3] In native chemical ligation, the thiolate of an *N*-terminal cysteine residue of one peptide attacks the *C*-terminal thioester of a second peptide. An amide linkage forms after rapid S → N acyl transfer (Scheme 1). "Expressed protein ligation" is an extension of native

Scheme 1
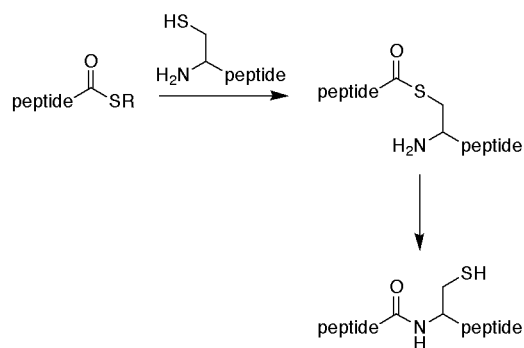
chemical ligation in which the *C*-terminal thioester is produced by recombinant DNA (rDNA) technology rather than chemical synthesis.[4]

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(1) Wieland, T.; Bokelmann, E.; Bauer, L.; Lang, H. U.; Lau, H. *Liebigs Ann. Chem.* **1953**, *583*, 129−149.

(2) Dawson, P. E.; Muir, T. W.; Clark-Lewis, I.; Kent, S. B. H. *Science* **1994**, *266*, 776−779.

(3) For recent reviews of peptide ligation methods, see: (a) Tam, J. P.; Yu, Q.; Miao, Z. *Biopolymers* **1999**, *51*, 311−332. (b) Dawson, P. E.; Kent, S. B. H. *Annu. Rev. Biochem.* **2000**, *69*, 923−960. (c) Borgia, J. A.; Fields, G. B. *Trends Biotechnol.* **2000**, *18*, 243−251.

(4) (a) Muir, T. W.; Sondhi, D.; Cole, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *9*, 6705−6710. For reviews, see: (b) Holford, M.; Muir, T. W. *Structure* **1998**, *15*, 951−956. (c) Cotton, G. J.; Muir, T. W. *Chem. Biol.* **1999**, *6*, R247−R256. (d) Evans, T. C., Jr.; Xu, M.-Q. *Biopolymers* **2000**, *51*, 333−342.
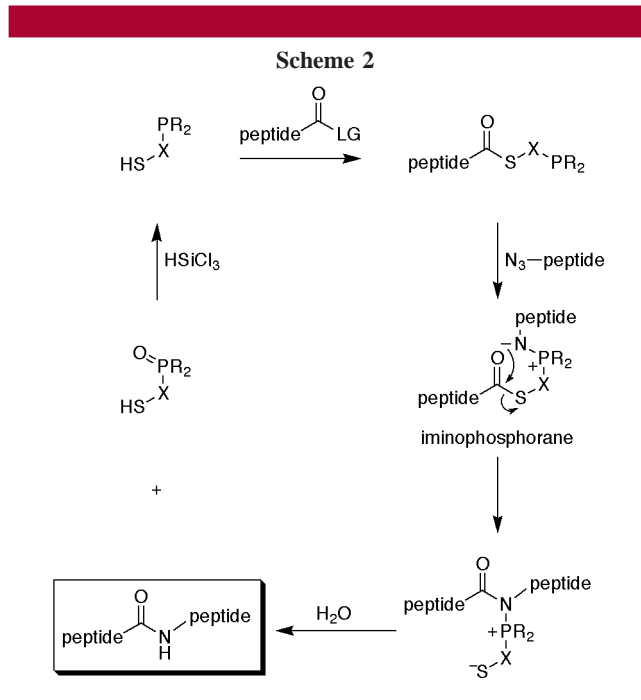
A limitation of native chemical ligation is its intrinsic reliance on having a cysteine residue at the ligation juncture. Cysteine is uncommon, comprising only 1.7% of all residues in proteins.[5] Modern peptide synthesis is typically limited to peptides of ≤50 residues.[3] Hence, most proteins cannot be prepared by any method that allows for peptides to be coupled only at cysteine residues.

The removal of the cysteine limitation by applying a more general ligation technology would greatly expand the utility of total protein synthesis. The Staudinger reaction provides such an alternative. In the Staudinger reaction, a phosphine is used to reduce an azide to an amine: $PR_3 + N_3R' + H_2O \rightarrow O{=}PR_3 + H_2NR' + N_2(g)$.[6] This reaction occurs via a stable intermediate, an iminophosphorane ($R_3P^{+}{-}{^-}NR'$), which has a nucleophilic nitrogen. Vilarrassa and others have shown that this nitrogen can be acylated, both in intermolecular and intramolecular reactions.[7,8] Hydrolysis of the resulting amidophosphonium salt gives an amide and phosphine oxide. Saxon and Bertozzi have shown that the phosphine can itself serve as the acyl donor.[8a]

Recently, we reported the use of the "Staudinger ligation" to form a peptide.[8b] In our initial work, an amide bond was formed from a thioester of phosphinothiol **1** and an azide.
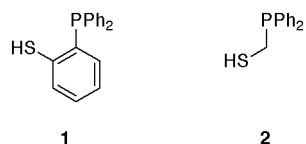
The reaction likely proceeds by the intramolecular rearrangement of an iminophosphorane intermediate to give an amidophosphonium salt, as shown in Scheme 2. This salt hydrolyzes to leave an amide and *o*-(diphenylphosphinyl)-benzenethiol. It is noteworthy that the Staudinger ligation

(5) Only tryptophan (1.3%) is less common. McCaldon, P.; Argos, P. *Proteins* **1988**, *4*, 99−122.

(6) (a) Staudinger, H.; Meyer, J. *Helv. Chim. Acta* **1919**, *2*, 635−646. For comprehensive reviews, see: (b) Gololobov, Yu. G.; Zhmurova, I. N.; Kasukhin, L. F. *Tetrahedron* **1981**, *37*, 437−472. (c) Gololobov, Yu. G.; Kasukhin, L. F. *Tetrahedron* **1992**, *48*, 1353−1406.

(7) For examples, see: (a) Garcia, J.; Urpí, F.; Vilarrasa, J. *Tetrahedron Lett.* **1984**, *25*, 4841−4844. (b) Garcia, J.; Vilarrasa, J. *Tetrahedron Lett.* **1986**, *27*, 639−640. (c) Urpí, F.; Vilarrasa, J. *Tetrahedron Lett.* **1986**, *27*, 4623−4624. (d) Bosch, I.; Romea, P.; Urpí, F.; Vilarrasa, J. *Tetrahedron Lett.* **1993**, *34*, 4671−4674. (e) Inazu, T.; Kobayashi, K. *Synlett.* **1993**, 869−870. (f) Molina, P.; Vilaplana, M. J. *Synthesis-Stuttgart* **1994**, 1197−1218. (g) Bosch, I.; Urpí, F.; Vilarrasa, J. *J. Chem. Soc., Chem. Commun.* **1995**, 91−92. (h) Shalev, D. E.; Chiacchiera, S. M.; Radkowsky, A. E.; Kosower, E. M. *J. Org. Chem.* **1996**, *61*, 1689−1701. (i) Bosch, I.; Gonzalez, A.; Urpí, F.; Vilarrasa, J. *J. Org. Chem.* **1996**, *61*, 5638−5643. (j) Maunier, V.; Boullanger, P.; Lafont, D. J. *Carbohydr. Res.* **1997**, *16*, 231−235. (k) Afonso, C. A. M. *Synth. Commun.* **1998**, *28*, 261−276. (l) Tang, Z.; Pelletier, J. C. *Tetrahedron Lett.* **1998**, *39*, 4773−4776. (m) Ariza X.; Urpí, F.; Viladomat, C.; Vilarrasa J. *Tetrahedron Lett.* **1998**, *39*, 9101−9102. (n) Mizuno, M.; Muramoto, I.; Kobayashi, K.; Yaginuma, H.; Inazu, T. *Synthesis-Stuttgart* **1999**, 162−165. (o) Mizuno, M.; Haneda, K.; Iguchi, R.; Muramoto, I.; Kawakami, T.; Aimoto, S.; Yamamoto, K.; Inazu, T. *J. Am. Chem. Soc.* **1999**, *121*, 284−290. (p) Boullanger P.; Maunier, V.; Lafont, D. *Carbohydr. Res.* **2000**, *324*, 97−106. (q) Velasco, M. D.; Molina, P.; Fresneda, P. M.; Sanz, M. A. *Tetrahedron* **2000**, *56*, 4079−4084. (r) Malkinson, J. P.; Falconer, R. A.; Toth, I. *J. Org. Chem.* **2000**, *65*, 5249−5252.

(8) (a) Saxon, E.; Bertozzi, C. R. *Science* **2000**, *287*, 2007−2010. (b) Nilsson, B. L.; Kiessling, L. L.; Raines, R. T. *Org. Lett.* **2000**, *2*, 1939−1941. (c) Saxon, E.; Armstrong, J. I.; Bertozzi, C. R. *Org. Lett.* **2000**, *2*, 2141−2143.

Scheme 2

has no reliance upon a cysteine or any other specific residue at the *N*-terminus of the peptide fragment. In addition, the method is traceless—no residual atoms from the phosphinothiol remain in the peptide product. The model amides synthesized with this original phosphinothiol are listed in Table 1.[8b] Although phosphinothiol **1** does enable amide bond formation, the isolated yields for these Staudinger ligations are too low for some applications.

Staudinger ligation with phosphinothiol **1** occurs through a transition state with a six-membered ring. We reasoned

Table 1. Yields for Staudinger Ligation with Phosphinothiols **1** and **2**[a]

phosphinothioester	azide	peptide	yield (%)
AcS-(benzene)-PPh₂	N₃CH₂C(O)NH-Ph	AcGlyNHBn	<10
AcS-CH₂-PPh₂	N₃CH₂C(O)NH-Ph	AcGlyNHBn	91
AcGlyS-(benzene)-PPh₂	N₃CH₂C(O)NH-Ph	AcGlyGlyNHBn	15
AcGlyS-CH₂-PPh₂	N₃CH₂C(O)NH-Ph	AcGlyGlyNHBn	80
AcPheS-(benzene)-PPh₂	N₃CH₂C(O)NH-Ph	AcPheGlyNHBn	35
AcPheS-CH₂-PPh₂	N₃CH₂C(O)NH-Ph	AcPheGlyNHBn	92

[a] Conditions: THF/H₂O (3:1); room temperature; 12 h.

that reducing the size of this ring[8c] would bring the nucleophilic imide nitrogen more proximal to the electrophilic thioester carbon and improve the yields for the Staudinger ligation products. To access a transition state with a smaller ring, we replaced the *o*-phenyl group of phosphinothiol **1** with a single methylene group. We retained the other two electron-withdrawing phenyl groups, which make the phosphorus less nucleophilic and thereby minimize the susceptibility of the phosphine to deleterious oxidation by $O_2(g)$.

The synthesis of the previously unknown phosphinothiol, **2**, is shown in Scheme 3. Phenylmagnesium bromide was

Scheme 3
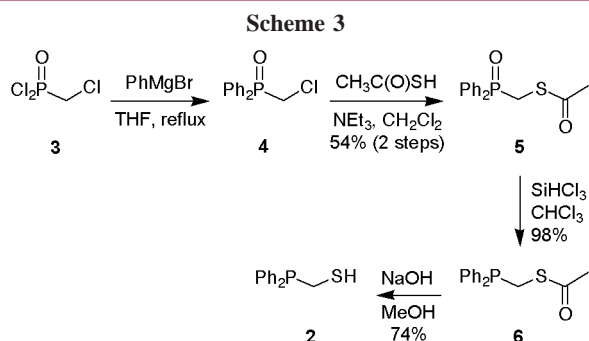
added to chloromethylphosphonic dichloride (**3**), and the resulting Grignard reaction was heated at reflux for 12 h to give phosphine oxide **4**. A mixture of **4** with thioacetic acid and triethylamine in dry THF was heated at reflux for 12 h.[9] After purification by flash chromatography and treatment with decolorizing charcoal, thiophosphine oxide **5** was isolated in a 54% combined yield for the two steps. An excess of trichlorosilane in chloroform for 72 h was used to reduce **5** to phosphinothioester **6**,[10] which was isolated by flash chromatography in nearly quantitative yield. Hydrolysis of the phosphinothioester **6** with sodium hydroxide in methanol for 2 h gave phosphinothiol **2**.[11] During this reaction, Ar(g) was bubbled through the reaction mixture to prevent oxidation of the resultant thiol. Phosphinothiol **2** was purified by chromatography over alumina and isolated in 74% yield. The overall yield for the process in Scheme 3 was 39%.

Next, we determined the efficacy of phosphinothiol **2** in effecting the Staudinger ligation (Scheme 2). Thioesters of **2** derived from AcOH, AcGlyOH, and AcPheOH were prepared by either transthioesterification or coupling with dicyclohexylcarbodiimide. After these reactions were judged to be complete by TLC analysis, Merrifield resin was used to immobilize unreacted **2**. After workup and chromatography, the purified thioesters were isolated in >90% yields. To effect the Staudinger ligation, each thioester was stirred with $N_3CH_2C(O)NHBn$[8b] (1 equiv) in THF/H_2O (3:1) at room temperature for 12 h. Solvents were removed under reduced pressure, and the product amides were purified by chroma-

(9) (a) Lamoureux, G. V.; Whitesides, G. M. *J. Org. Chem.* **1993**, *58*, 633−641. (b) Woycechowsky, K. J.; Wittrup, K. D.; Raines, R. T. *Chem. Biol.* **1999**, *6*, 871−879.

(10) Charrier, C.; Mathey, F. *Tetrahedron Lett.* **1978**, *27*, 2407−2410.

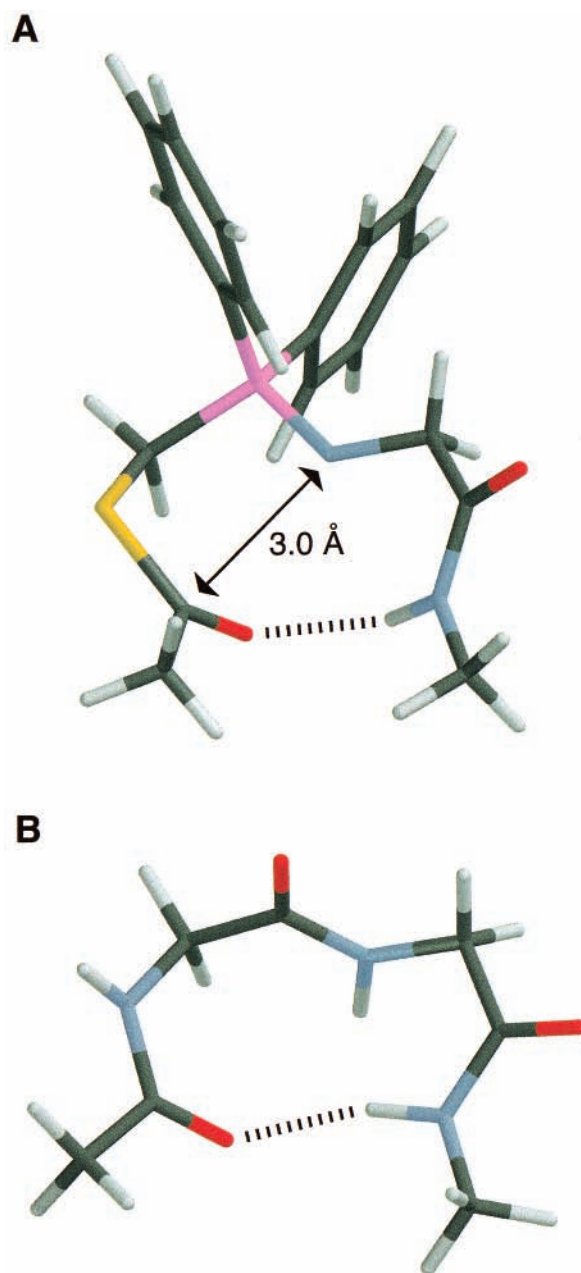(11) Hydrolysis under acidic conditions was unsuccessful.

Figure 1. (A) Structure of the iminophosphorane formed during a Staudinger ligation ($AcSCH_2PPh_2$ + $N_3CH_2C(O)NHMe$) with phosphinothiol **2** ($r_{N\cdots C}$: 3.0 Å; $\angle N\cdots C=O$: 65°). (B) Structure of an analogous β-turn (AcGlyGlyNMe). Each molecule is depicted in a conformation of minimal energy according to the MMFF94 force field in MacSpartan Pro v1.3.5 (Wavefunction, Irvine, CA).

tography. After the ligation reactions, phosphinothiol **2** was regenerated from its phosphine oxide by reduction with an excess of trichlorosilane in chloroform (Scheme 2).

The yields of amide product using phosphinothiol **2** are far greater than those using phosphinothiol **1** (Table 1). AcGlyNHBn was obtained in 91% isolated yield with **2**, compared to a trace yield with **1**. AcGlyGlyNHBn was obtained in 80% yield using **2**, compared to 15% using **1**. AcPheGlyNHBn was obtained in 92% yield with **2**, com-

pared to 35% with **1**. These dramatic improvements indicate that phosphinothiol **2** is a superior reagent for effecting the Staudinger ligation of a thioester and azide to form an amide.[12]

What is the basis for the high yields obtained with phosphinothiol **2**? One contributing factor could be the proximity of the nucleophile and electrophile. The key intermediate in the Staudinger ligation is the iminophosphorane (Scheme 2). The transition state leading from the iminophosphorane of **2** to the amidophosphonium salt contains a five-membered ring. Both the C−S and P−N bonds in this ring have significant double-bond character. Thus, the iminophosphorane can adopt relatively few conformations. In contrast, reaction of $N_3(CH_2)_{10}C(O)SPy$ and PBu_3 to form a lactam proceeds via a transition state with a 12-membered ring. The yield of this reaction is only 28%.[7d]

Another factor that could contribute to the high yields obtained with phosphinothiol **2** is a stable conformation that facilitates amide formation. Molecular mechanics calculations indicate that the iminophosphorane intermediate can adopt a β-turn-like conformation (Figure 1). A β-turn is stabilized by an O···HN hydrogen bond that defines a 10-membered ring. The thioester, imide, and amide groups of the iminophosphorane are situated in positions that correspond to the three amide groups of a β-turn. In this conformation, the nucleophilic imide nitrogen is within 3.0 Å of the electrophilic thioester carbon. Moreover, the O···HN hydrogen bond would polarize the thioester, making its carbon even more electrophilic. Finally, the bulk of the two phenyl groups could accelerate acyl transfer by increasing the fraction of iminophosphorane in the β-turn-like conformation.[13] The favorable conformation depicted in Figure 1 would be inaccessible during a Staudinger ligation with **1**, as well as in the ligation of a thioester with a nonpeptidyl azide.

Phosphinothiol **2** has an additional intrinsic advantage over phosphinothiol **1**. In general, aliphatic thiols (such as **2**) have higher pK_a values than do aromatic thiols (such as **1**). Because thioester hydrolysis rates correlate inversely with their thiol pK_a values,[14] aliphatic thioesters have a longer half-life in aqueous solution. This long half-life is important, as the hydrolysis of the thioester, either before or after iminophosphorane formation, is likely to be a competing side reaction for the Staudinger ligation.

Staudinger ligation with phosphinothiol **2** could enable facile protein synthesis. Peptides with a *C*-terminal thioester can be produced by Fmoc-based solid-phase synthesis[15] or rDNA technology.[4] Azido acids are readily accessible[16] and can be used in solid-phase synthesis.[7l,17] A process based on the Staudinger ligation of thioesters and azides could be a viable source of proteins for both basic research and drug discovery.

Supporting Information Available: Procedures for the preparation of compound **2** and related analytical data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) Bertozzi and co-workers have assessed the ability of the oxo analogues of phosphinothiols **1** and **2** to effect a Staudinger ligation.[8c] Surprisingly, they found that $Ph_2PC_6H_4$-*o*-OH gives a higher yield than does Ph_2PCH_2OH. The basis for the antipodal reactivity of thioesters and esters is unclear.

(13) For examples of the "reactive rotamer effect", see: (a) Bruice, T. C.; Pandit, U. K. *J. Am. Chem. Soc.* **1960**, *82*, 5858−5865. (b) Jung, M. E.; Gervay, J. *J. Am. Chem. Soc.* **1991**, *113*, 224−232.

(14) Janssen, M. J. *The Chemistry of Carboxylic Acids and Esters*; Patai, S., Ed.; Interscience Publishers: New York, 1969; pp 730−736.

(15) Ingenito, R.; Bianchi, E.; Fattori, D.; Pessi, A. *J. Am. Chem. Soc.* **1999**, *121*, 11369−11374. (b) Shin, Y.; Winans, K. A.; Backes, B. J.; Kent, S. B. H.; Ellman, J. A. Bertozzi, C. R. *J. Am. Chem. Soc.* **1999**, *121*, 11684−11689. (c) Swinnen, D.; Hilvert, D. *Org. Lett.* **2000**, *2*, 2439−2442.

(16) Zaloom, J.; Roberts, D. C. *J. Org. Chem.* **1981**, *46*, 5173−5176.

(17) Meldal, M.; Juliano, M. A.; Jansson, A. M. *Tetrahedron Lett.* **1997**, *38*, 2531−2534.