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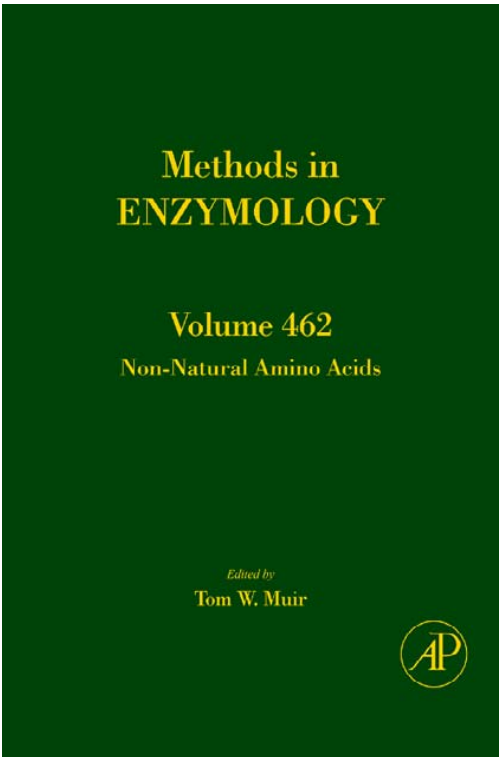

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CHAPTER TWO

Protein Engineering with the Traceless Staudinger Ligation

Annie Tam *and* Ronald T. Raines

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Abstract

The engineering of proteins can illuminate their biological function and improve their performance in a variety of applications. Within the past decade, methods have been developed that facilitate the ability of chemists to manipulate proteins in a controlled manner. Here, we present the traceless Staudinger ligation as a strategy for the convergent chemical synthesis of proteins. This reaction unites a phosphinothioester and an azide to form an amide bond with no residual atoms. An important feature of this reaction is its ability to ligate peptides at noncysteine residues, thereby overcoming a limitation of alternative strategies. Attributes of the traceless Staudinger ligation are discussed, and an overall comparison of known reagents for effecting the reaction is presented.

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General methods are elaborated for the synthesis of the most efficacious phosphinothiol for mediating the traceless Staudinger ligation, as well as for the preparation of phosphinothioester and azide fragments and the ligation of peptides immobilized on a solid support. Together, this information facilitates the use of this emerging method to engineer proteins.

1. Introduction

The advent of recombinant DNA technology and site-directed mutagenesis has made facile the substitution of one amino acid for another at any site within a protein (Smith, 1994). For protein chemists, however, there remains a major barrier—the genetic code, which only tolerates the introduction of 20 amino acids. Methods that overcome this limitation but still rely on the ribosome are limited to the substitution of a subset of α-amino acids and α-hydroxyacids.

Driven by the desire to achieve complete flexibility in the manipulation of primary structure, protein chemists are developing methods that enable nonnatural amino acids and artificial modules to be incorporated into proteins. The most popular such method is "native chemical ligation," which was developed by Kent and coworkers as a means to join large peptide fragments (Kent, 2003). In native chemical ligation, the thiolate of an N-terminal cysteine residue of one peptide reacts with a C-terminal thioester of a second peptide, forming an amide bond after rapid $S \rightarrow N$ acyl group transfer. The ligation also works with selenocysteine—the rare "21st" amino acid—in the place of cysteine (Hondal and Raines, 2002; Hondal *et al.*, 2001). An extension of native chemical ligation, "expressed protein ligation," employs an engineered intein to access a polypeptide containing the C-terminal thioester (Muir, 2003). Although these methods have produced landmark results, both require a cysteine residue at the ligation juncture. Cysteine is uncommon, comprising <2% of all protein residues. The introduction of a new cysteine residue can be detrimental, as its high nucleophilicity and propensity to oxidize leads to undesirable side reactions. Accordingly, many natural proteins can be neither synthesized nor modified by a ligation method that relies on cysteine residues.

2. Traceless Staudinger Ligation

Emerging strategies for the unconstrained engineering of proteins avoid the requisite cysteine residues (Nilsson *et al.*, 2005). Here, we describe one such strategy—the Staudinger ligation—which is based on the Staudinger reaction (Staudinger and Meyer, 1919). In the Staudinger reaction, a

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phosphine is used to reduce an azide to an amine: $PR_3 + N_3R' + H_2O \rightarrow O = PR_3 + H_2NR' + N_2(g)$. This reaction occurs via a stable intermediate, an iminophosphorane ($R_3P^+-^-NR'$, also known less precisely as an "aza-ylide"), which has a nucleophilic nitrogen. Vilarrasa and others showed that this nitrogen can be acylated, both in intermolecular (i.e., three-component) and intramolecular (i.e., two-component) ligations (Bosch *et al.*, 1995; Velasco *et al.*, 2000). Hydrolysis of the resulting amidophosphonium salt gives an amide and a phosphine oxide. Bertozzi and coworkers showed that the phosphine itself can serve as the acyl group donor in a two-component ligation (Saxon and Bertozzi, 2000).

To apply the Staudinger reaction to peptide synthesis, we developed the use of a phosphinothiol to unite a thioester and azide, as shown in Fig. 2.1 (Nilsson *et al.*, 2000, 2001). This phosphinothiol is bifunctional, having a thiol group that can be tethered to the C-terminus of a peptide fragment, and a phosphino group that can react with a peptide fragment that has an azido group at its N-terminus to form an iminophosphorane intermediate. Attack of the iminophosphorane nitrogen on the conjoined thioester carbon leads first to a tetrahedral intermediate, and then to an amidophosphonium salt (Soellner *et al.*, 2006a). Hydrolysis of the amidophosphonium salt releases a phosphine oxide and produces a native amide bond between the two peptides. Significantly, no extraneous atoms remain in the amide product—the reaction is "traceless" (Nilsson *et al.*, 2000). This attribute is a strict requirement for the use of the Staudinger ligation in the chemical synthesis of proteins or other molecules. It is noteworthy that the traceless Staudinger ligation mediated by a phosphinothiol couples the energetics of native chemical ligation with that of the Staudinger reaction (which is highly exergonic), resulting in an enormous thermodynamic driving force for the overall transformation (Nilsson *et al.*, 2005).

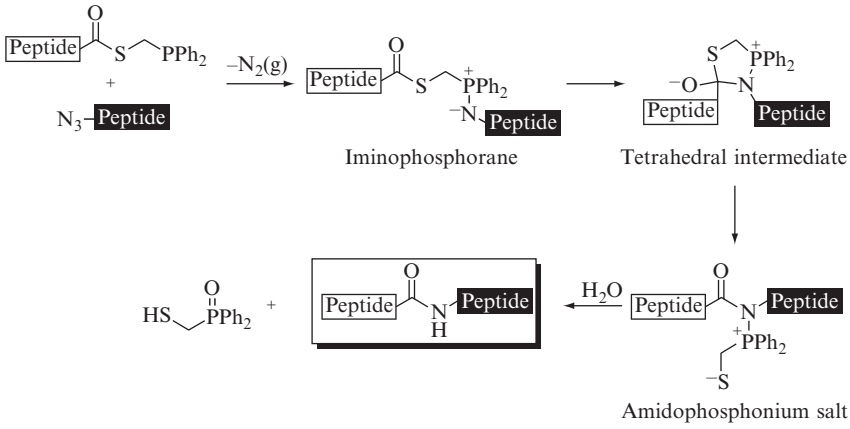

Figure 2.1 Putative mechanism for the traceless Staudinger ligation of two peptides.

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The kinetics of the traceless Staudinger ligation have been characterized by using a sensitive and continuous assay based on ^{13}C NMR spectroscopy (Soellner *et al.*, 2006a). In this assay, a phosphinothioester is allowed to react with a $^{13}C^{\alpha}$-labeled azide in a deuterated solvent, and the course of the reaction is monitored over time. Significantly, intermediates do not accumulate, indicating that the rate-limiting step is the association of the phosphinothioester and the azide. For the reaction of $AcGlySCH_2PPh_2$ and $N_3{}^{13}CH_2C(O)NHBn$ at room temperature, $t_{1/2} = 7$ min. The traceless Staudinger ligation proceeds without detectable (<0.5%) epimerization of the α-carbon of the azido acid (Soellner *et al.*, 2002). This attribute is crucial for its application in protein chemistry, as all 20 proteinogenic amino acids except glycine have a stereogenic center at their α-carbon. The reaction of phosphinothioesters (but not phosphinoesters) with azides is also chemoselective in the presence of the functional groups in native proteins, and unprotected peptide fragments can be ligated with no undesirable side reactions (Soellner *et al.*, 2006a). These attributes endow the Staudinger ligation with broad utility.

The traceless Staudinger ligation has been applied to the assembly of a protein from constituent peptides (Nilsson *et al.*, 2003a), as well as the site-specific immobilization of peptides and proteins to a surface (Gauchet *et al.*, 2006; Soellner *et al.*, 2003). Variations of the Staudinger ligation have also been used in the synthesis of glycopeptides (Bianchi and Bernardi, 2006; Bianchi *et al.*, 2005; He *et al.*, 2004; Liu *et al.*, 2006) and biomolecular labeling experiments *in vitro* (Grandjean *et al.*, 2005; Tsao *et al.*, 2005) and *in vivo* (Dube *et al.*, 2006), and for drug delivery (Azoulay *et al.*, 2006). As with auxiliary-mediated ligations (Nilsson *et al.*, 2005), steric hindrance at the ligation junction (as in nonglycyl couplings) diminishes the ligation yield. Phosphinothiols that mediate the efficient coupling of nonglycyl amino acids are, however, now known (Soellner *et al.*, 2006b; Tam *et al.*, 2008).

3. Choice of Coupling Reagent

Several coupling reagents have been used in the traceless Staudinger ligation, with varied success. These compounds include phosphinomethanethiol **I** (Nilsson *et al.*, 2001), phosphinothiophenol **II** (Nilsson *et al.*, 2000), phosphinomethanol **III** (Saxon *et al.*, 2000), phosphinoethanethiol **IV** (Han and Viola, 2004), and phosphinophenol **V** (Saxon *et al.*, 2000). The efficacy of these coupling reagents in a model reaction between its AcGly(thio)ester and $^{13}C^{\alpha}$-labeled $N_3GlyNHBn$ in a wet organic solvent has been compared directly (Soellner *et al.*, 2006b), and the key results are listed in Table 2.1. Traceless Staudinger ligations mediated by reagents

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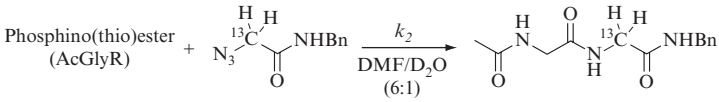
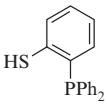
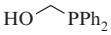
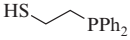
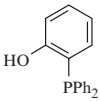
Table 2.1 Effect of coupling reagent on the rate and product distribution of the Staudinger ligation



Coupling reagent (HR)	k_2 ($\times 10^{-3}$ M^{-1} s^{-1})	Yield (%)
I	7.7 ± 0.3	95
 **II**	1.04 ± 0.05	38
 **III**	0.12 ± 0.01	11
 **IV**	0.65 ± 0.01	39
 **V**	7.43 ± 0.03	99

II, III, and **IV** are sluggish compared to those by reagents **I** and **V**. Furthermore, coupling reagents **II, III**, and **IV** also display low-ligation yields. The low rate and yield with **II** and **IV** could be due to the increased size of the ring that is formed during the nucleophilic attack of the imino-phosphorane nitrogen on the thioester (e.g., to produce the tetrahedral intermediate in Fig. 2.1). Reagent **III** enabled a direct comparison of an ester and thioester reagent, and highlights the advantage of a good leaving group (thiolate vs alkoxide) in mediating the traceless Staudinger ligation. Finally, phosphinophenol **V** gave amide yields and reaction rates nearly indistinguishable from phosphinomethanethiol **I**. Although Staudinger ligation with **V** requires the formation of a six- rather than a five-membered ring during $S \rightarrow N$ acyl group transfer (Fig. 2.1), the conjugate base of **V** is a somewhat better leaving group than is that of **I**. Upon further investigation,

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ligations mediated by **V** were found to suffer a decrease in amide yield in the presence of the functional groups found in proteinogenic amino acids. This result is presumably due to the aryl ester of **V** being more electrophilic than the thioester of **I**, increasing its susceptibility to nonspecific acyl transfer reactions (e.g., with the ε-amino group of a lysine residue). On the contrary, Staudinger ligations performed with **I** can be performed on unprotected peptide fragments (Gauchet *et al.*, 2006; Liu *et al.*, 2006; Soellner *et al.*, 2003).

Because of its high reaction rate, high ligation yields, and chemoselectivity, (diphenylphosphino)methanethiol (**I**) is the most efficacious of known reagents for mediating the traceless Staudinger ligation (Soellner *et al.*, 2006b). Thiol-based reagents (e.g., **I**) have another intrinsic advantage over hydroxyl-based reagents (e.g., **V**). The thiol-based reagents react readily with thioester fragments generated by expressed protein ligation or other methods to form phosphinothioesters poised for a traceless Staudinger ligation.

Phosphinothiol **I** can be prepared from diphenylphosphine–borane complex and other commercial materials by two routes, designated as **a** (Soellner *et al.*, 2002) and **b** (He *et al.*, 2004) in Fig. 2.2, both with overall yields of 55%. A precursor that is common to both routes, phosphine–borane complex **X**, is stable to air and moisture and can be stored on the

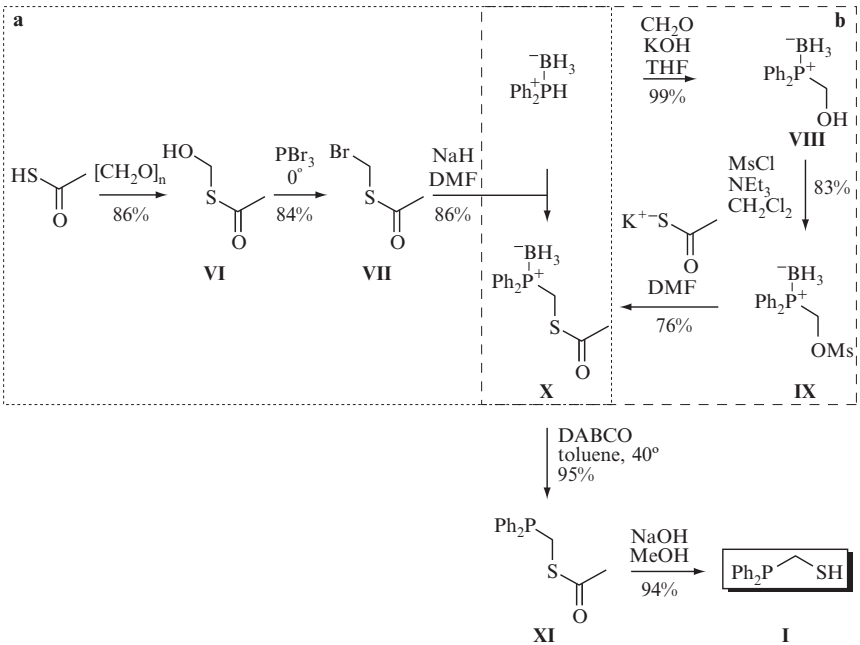

Figure 2.2 Routes for the synthesis of (diphenylphosphino)methanethiol (**I**).

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shelf at room temperature for months without any sign of oxidation or decomposition. Phosphine–borane complex **X** is also available from a commercial vendor (Sigma–Aldrich product #670359). Although fully deprotected phosphinothiol **I** is stable under Ar(g) for several days, it is best when prepared freshly from phosphine–borane complex **X**.

3.1. Experimental procedure: Synthesis of phosphinothiol I

*Route **a***. In route **a** of Fig. 2.2 (Soellner *et al.*, 2002), a P–C bond is made by alkylation of a diphenylphosphine–borane complex with agent **VII**, which was known previously (Farrington *et al.*, 1989). Thioacetic acid (50 g, 0.65 mol) and paraformaldehyde (20 g) are mixed and heated at 100 °C for 2 h under Ar(g). The reaction mixture becomes clear and light yellow, which indicates that the reaction is complete. Distillation under a high vacuum (bp 36 °C at 0.1 mm Hg) gives the $AcSCH_2OH$ (**VI**) as a colorless oil (typical yield: 59 g, 0.65 mmol, 86%). $AcSCH_2OH$ (**VI**, 59 g, 0.56 mol) is cooled under Ar(g) in an ice bath, and PBr_3 (50.5 g, 0.19 mol) is added dropwise slowly such that the reaction temperature does not exceed 8 °C. After the complete addition of PBr_3, the reaction mixture is stirred for an additional 30 min in an ice bath, and then allowed to warm to room temperature. The reaction mixture is poured over an ice/water mixture (100 ml), and extracted with ether (3 × 100 ml). The organic extracts are dried over anhydrous $MgSO_4$(s), filtered, and concentrated under reduced pressure. The residue is distilled under a high vacuum (bp 53 °C at 0.1 mm Hg) to give alkylating agent **VII** as a colorless oil (typical yield: 0.80 g, 0.47 mmol, 84%). Spectral data should be as reported previously (Farrington *et al.*, 1989).

Diphenylphosphine–borane complex (10.33 g, 51.6 mmol) is dissolved in dry DMF under Ar(g) and cooled to 0 °C. NaH (1.24 g, 51.6 mmol) is added slowly, and the mixture is stirred at 0 °C until bubbling ceases. Alkylating agent **VII** (8.73 g, 51.6 mmol) is then added, and the mixture is allowed to warm to room temperature and stirred for 12 h. The product is concentrated under reduced pressure, and the residue is purified by flash chromatography (silica gel, 10% (v/v) EtOAc in hexanes). Phosphine–borane complex **X** is isolated as a colorless oil (typical yield: 12.8 g, 44.4 mmol, 86%), and can be stored under air in a flask or bottle for extended periods in this form. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.74–7.67 (m, 4 H), 7.54–7.41 (m, 6 H), 3.72 (d, $J = 6$ Hz, 2 H), 2.23 (s, 3 H), 1.51–0.53 (broad m, 3 H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$) δ 192.94, 132.26 (d, $J = 9.2$ Hz), 131.61 (d, $J = 2.3$ Hz), 128.71 (d, $J = 10.2$ Hz), 127.43 (d, $J = 55.4$ Hz), 29.87, 23.59 (d, $J = 35.5$ Hz) ppm; ^{31}P NMR (121 MHz, $CDCl_3$) δ 19.40 (d, $J = 59.3$ Hz) ppm; typical MS (ESI) m/z 311.0806 ($MNa^+ = 311.0807$).

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*Route **b***. In route **b** of Fig. 2.2 (He *et al.*, 2004), a P–C bond is made by addition of a diphenylphosphine–borane complex to formaldehyde. Diphenylphosphine–borane complex (2.45 g, 12.2 mmol) is dissolved in THF (7 ml). Formaldehyde (37% (v/v) in H_2O, 7.16 ml) is added to the solution, followed by potassium hydroxide (825 mg, 14.7 mmol). The resulting bilayered solution is stirred overnight, and then concentrated under reduced pressure. The residue is dissolved in ethyl acetate (10 ml), and the layers are separated. The organic extracts are washed with brine, dried over anhydrous $MgSO_4$(s), filtered, and concentrated under reduced pressure. The crude oil is purified by flash chromatography (silica gel, 50% (v/v) CH_2Cl_2 in hexanes) to give phosphine–borane complex **VIII** as a colorless oil (typical yield: 2.81 g, 12.2 mmol, 99%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.73–7.68 (m, 4 H), 7.52–7.41 (m, 6 H), 4.40 (broad s, 2 H), 2.38 (broad s, 1 H), 1.50–0.50 (broad m, 3 H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 132.89 (d, $J = 8.9$ Hz), 131.79, 129.10 (d, $J = 10.8$ Hz), 126.88 (d, $J = 54.5$ Hz), 60.47 (d, $J = 41.4$ Hz) ppm; ^{31}P NMR ($CDCl_3$, 121 MHz) δ 17.61 (d, $J = 58.9$ Hz) ppm; typical MS (ESI) m/z 253.0927 ($MNa^+ = 253.0930$).

Triethylamine (2.56 ml, 18.35 mmol) is added to a solution of phosphine–borane complex **VIII** (2.81 g, 12.2 mmol) in CH_2Cl_2 (36 ml), and the reaction mixture is cooled to 0 °C with an ice bath. Methanesulfonyl chloride (1.33 ml, 17.1 mmol) is added dropwise, and the resulting solution is allowed to warm to room temperature slowly (e.g., overnight). The solution is washed with 0.1 N HCl and brine, and the combined organic extracts are dried over anhydrous $MgSO_4$(s), filtered, and concentrated under reduced pressure. The residue is purified by flash chromatography (silica gel, 30% (v/v) ethyl acetate in hexanes) to give phosphine–borane complex **IX** as a pale yellow oil (typical yield: 3.14 g, 10.16 mmol, 83% yield). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.76–7.71 (m, 4 H), 7.59–7.48 (m, 6 H), 4.90 (d, $J = 1.90$ Hz, 2 H), 2.87 (s, 3 H), 1.50–0.50 (broad m, 3 H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 133.09 (d, $J = 9.50$ Hz), 132.48, 129.343 (d, $J = 10.0$ Hz), 125.32 (d, $J = 58.3$ Hz), 64.68 (d, $J = 37.8$ Hz), 37.65 ppm; ^{31}P NMR ($CDCl_3$, 121 MHz) δ 18.87 (d, $J = 57.8$ Hz) ppm; typical MS (ESI) m/z 331.0719 ($MNa^+ = 331.0705$).

Potassium thioacetate (1.4 g, 12.2 mmol) is added to a solution of phosphine–borane complex **IX** (3.14 g, 10.16 mmol) in anhydrous DMF (50 ml) under Ar(g). The resulting solution is stirred overnight, and then concentrated under reduced pressure. The residue is dissolved in ethyl acetate (25 ml), and the resulting solution is washed with water and brine. The combined organic extracts are dried over anhydrous $MgSO_4$(s), filtered, and concentrated under reduced pressure. The residue is purified by flash chromatography (silica gel, 30% (v/v) CH_2Cl_2 in hexanes) to give phosphine–borane complex **X** as a colorless oil (typical yield: 2.22 g, 7.7 mmol, 76%). Spectral data should be as reported for route **a**.

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Phosphine–borane complex **X** (4.00 g, 13.9 mmol) is dissolved in toluene (140 ml) under Ar(g). 1,4-Diazabicylo[2.2.2]octane (DABCO) (1.56 g, 13.9) is added, and the mixture is heated at 40 °C for 4 h. The product is concentrated under reduced pressure, dissolved in CH_2Cl_2 (50 ml), and washed with both 1 N HCl (20 ml) and saturated brine (20 ml). The organic layer is dried over $MgSO_4$(s), and concentrated under reduced pressure. Phosphine **XI** is isolated as a colorless oil (typical yield: 3.62 g, 13.2 mmol, 95%) and is used without further purification. ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.43–7.40 (m, 4 H), 7.33–7.30 (m, 6 H), 3.50 (d, J = 4 Hz, 2 H), 2.23 (s, 3 H) ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ 194.01, 136.42 (d, J = 13.6 Hz), 132.28 (d, J = 19.4 Hz), 128.69, 128.11 (d, J = 6.8 Hz), 29.83, 25.41 (d, J = 23.4 Hz) ppm; ^{31}P NMR ($CDCl_3$, 202 MHz) δ −15.11 ppm; typical MS (ESI) m/z 274.06 (MH^+ = 275.0, fragments at 233.0, 199.2, 121.2).

Phosphine **XI** (17.27 g, 63.0 mmol) is dissolved in anhydrous methanol (0.40 l), and Ar(g) is bubbled through the solution for 1 h. Sodium hydroxide (5.04 g, 126 mmol) is then added, and the mixture is stirred under Ar(g) for 2 h. The product is concentrated under reduced pressure, and then dissolved in methylene chloride (0.30 l). The resulting solution is washed with 2 N HCl (2 × 0.10 l) and brine (0.10 l). The organic layer is dried over $MgSO_4$(s), filtered, and concentrated under reduced pressure. The residue is purified by flash chromatography (alumina, 25% ethyl acetate in hexanes). (Diphenylphosphino)methanethiol (**I**) is isolated as a colorless oil (typical yield: 10.8 g, 46.6 mmol, 74%). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.41–7.38 (m, 4 H), 7.33–7.26 (m, 6 H), 3.02 (d, J = 7.8 Hz, 2 H), 1.38 (t, J = 7.5 Hz, 1 H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 132.54 (d, J = 17.1 Hz), 128.86, 128.36, 128.14, 20.60 (d, J = 21.7 Hz) ppm; ^{31}P NMR ($CDCl_3$, 121 MHz) δ −7.94 ppm; typical MS (ESI) m/z 232.05 (MH^+ = 233.0, fragments at 183.0, 155.0, 139.0, 91.2).

4. Preparation of the Azido Fragment

Three methods have been described for preparing a peptide with an N-terminal azido group. These methods are listed in Table 2.2. Two of these methods involve a protected peptide on a solid support; the third involves an unprotected peptide in solution.

N1. A synthetic azido acid (or peptide) is coupled to the N-terminus of a synthetic peptide on a solid support (Nilsson *et al.*, 2003a; Soellner *et al.*, 2003). Side-chain functional groups are protected from side reactions (as indicated by the triangles).

N2. The N-terminal amino group of a peptide on solid support is converted into an azide by diazo transfer from, for example, triflyl azide in

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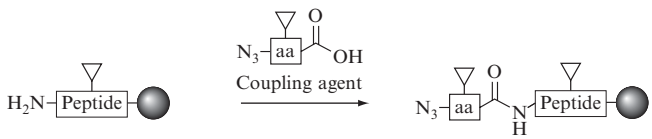
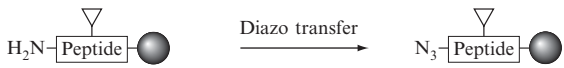
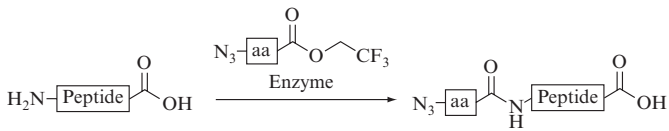
Table 2.2 Strategies for preparation of the azide fragment

Strategy	Route
N1	
N2	
N3	

the presence of divalent copper ions (Rijkers *et al.*, 2002). Side-chain functional groups are protected from reaction.

N3. A protease-catalyzed peptide condensation reaction is used to introduce azido dipeptides to the N-terminus of an unprotected peptide fragment (Liu *et al.*, 2006). A large excess (10 equiv.) of synthetic azido dipeptides is needed, along with the protease subtilisin.

4.1. Experimental procedure: Strategy N1

The azido derivatives of amino acids can be prepared by a method described previously (Lundquist and Pelletier, 2001). In our example, azido glycine is prepared by partially dissolving sodium azide (20.56 g, 317 mmol) by stirring in DMSO (880 ml) for 1.5 h. Bromoacetic acid (20.96 g, 151 mmol) is added to this slurry, and the remaining NaN_3 dissolves within minutes. The reaction mixture is stirred overnight at room temperature, before diluting with H_2O (1.0 l) and adjusting the pH to 2.5 with concentrated HCl. The desired azido glycine is extracted with EtOAc (2 × 1 l). The organic extracts are dried over anhydrous $MgSO_4$(s), and then concentrated under reduced pressure to yield azido glycine as a pale oil (typical yield: 11.1 g, 110 mmol, 73%). Spectral data should be as reported previously (Lundquist and Pelletier, 2001).

A desired ($n - 1$) peptide fragment (in our example, RNase A fragment 113–124: NPYVPVHFDASV (Nilsson *et al.*, 2003a)) is synthesized by

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solid-phase peptide synthesis on a Novasyn® TGA resin loaded with FmocValOH (110 mg, 22 μmol) by using standard methods on an automated synthesizer. The resin containing the ($n - 1$) peptide fragment is swollen in DMF (1 ml) for 1 h. Azido glycine (10.1 mg, 100 μmol), PyBOP (52 mg, 100 μmol), HOBT (14 mg, 100 μmol), and diisopropylethylamine (DIEA, 35 μl, 200 μmol) are dissolved in DMF (4 ml), and this mixture is added to the resin. The resin is agitated for 2 h by bubbling Ar(g) through the slurry. The resin is filtered, and this coupling protocol is repeated to ensure maximal coupling. After the second coupling, the resin containing the azido peptide is rinsed and dried under Ar(g).

5. Preparation of the Phosphinothioester Fragment

Many methods are known for installing a phosphinothioester at the C-terminus of a synthetic peptide (or module). Five of these methods are listed in Table 2.3. All peptide fragments synthesized via solid-phase peptide synthesis have the potential of incorporating nonnatural amino acids and synthetic modules anywhere within the peptide fragment.

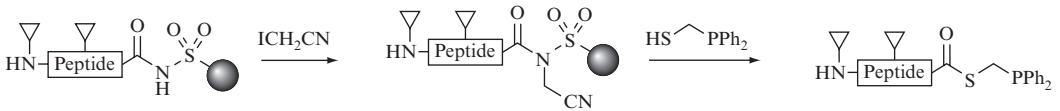
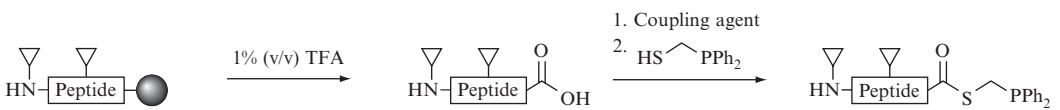
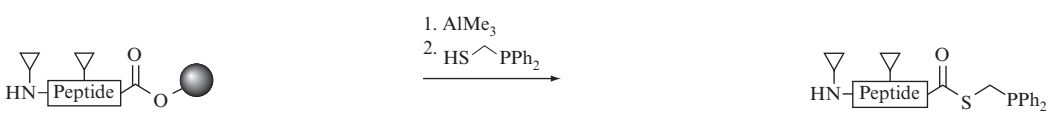
C1. A peptide fragment is synthesized on a sulfonamide-linker ("safety-catch") resin (Backes and Ellman, 1999). After activation of the fully loaded resin with iodoacetonitrile, treatment with an excess of phosphinothiol **I** liberates the thioester fragment.

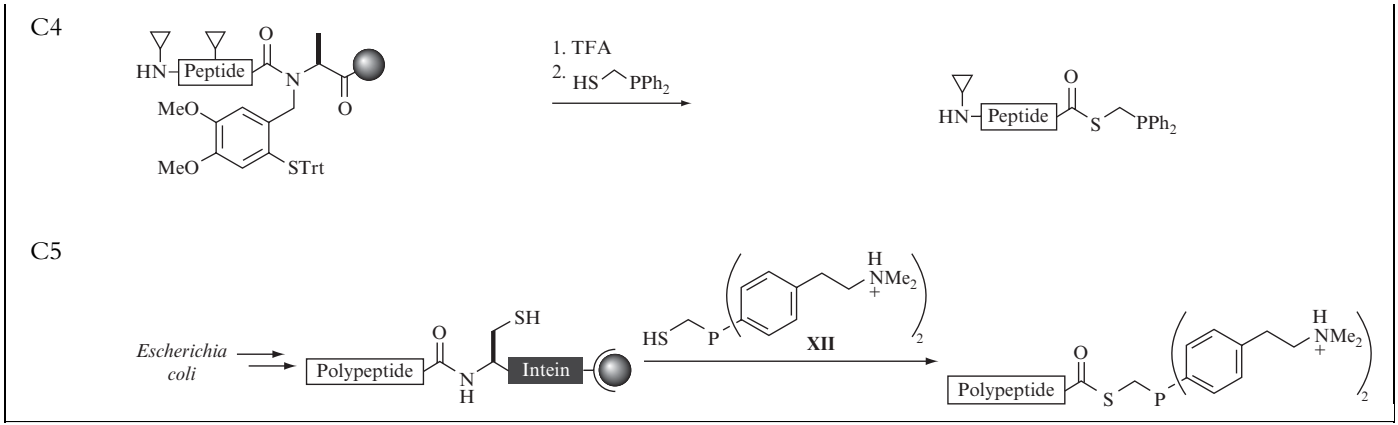
C2. A peptide fragment is synthesized on an acid-sensitive resin (e.g., NovaSyn TGA resin or 2-chlorotrityl resin) and liberated with 1% (v/v) TFA, which leaves intact the amino acid protecting groups. The C-terminus is then activated (e.g., with DCC, PyBOP, or NHS) and coupled with phosphinothiol **I**.

C3. A peptide fragment is assembled by standard Fmoc chemistry on a 4-hydroxymethyl-phenylacetamidomethyl (PAM) or 4-hydroxymethylbenzoic acid (HMBA) resin (Sewing and Hilvert, 2001). The ester linkage is activated for cleavage by $AlMe_3$ in the presence of an excess of phosphinothiol **I**. Epimerization at the C-terminal residue can occur, limiting this strategy to peptide fragments with a C-terminal glycine.

C4. A peptide fragment is assembled by standard Fmoc chemistry on an ester-linked (acid-stable) resin, which is loaded with *N*-4,5-dimethoxy-2-mercaptobenzyl (Dmmb)–Ala. The Dmmb group undergoes an $N \rightarrow S$ acyl group shift under acidic conditions, and the resulting thioester can undergo transthioesterification (Kawakami *et al.*, 2005).

Table 2.3 Strategies for preparation of the phosphinothioester fragment

Strategy	Route
C1	
C2	
C3	


C4
HN
Peptide
O
N
O
MeO
MeO
STrt
1. TFA
2. HS PPh2
HN
Peptide
O
S
PPh2
C5
Escherichia coli
Polypeptide
O
N
H
SH
Intein
HS
P
H
NMe2
+
XII
2
Polypeptide
O
S
P
H
NMe2
+
2

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C5. A polypeptide with a C-terminal intein and resin-binding domain is produced by recombinant DNA technology. Transthioesterification with water-soluble phosphinothiol **XII** liberates the peptide from the resin, simultaneously forming the C-terminal phosphinothioester (Tam and Raines, 2009; Tam *et al.*, 2007).

5.1. Experimental procedure: Strategy C1

First, a peptide is synthesized on resin. In our example (Nilsson *et al.*, 2003a), FmocGlu(O*t*Bu)OH is loaded onto 4-sulfamylbutyryl resin as described previously (Backes and Ellman, 1999). 4-Sulfamylbutyryl resin (1 g, 1.12 mmol) is swollen in $CHCl_3$ (25 ml) for 1 h. DIEA (1.56 ml, 8.96 mmol) and FmocGlu(O*t*Bu)OH (1.91 g, 4.48 mmol) are added to the resin. The reaction mixture is cooled to −20 °C under a flow of Ar(g). After 20 min, PyBOP (2.33 g, 4.48 mmol) is added to the solution and the resulting mixture is stirred, allowing the temperature to warm slowly to room temperature over a period of 8 h. The resin is filtered immediately and rinsed with $CHCl_3$. It is important to terminate the reaction after 8 h so as to minimize epimerization (Backes and Ellman, 1999). The coupling protocol is repeated to ensure maximal loading. After the second coupling is complete, the resin is filtered, rinsed with $CHCl_3$, and dried under Ar(g).

Fmoc-deprotection is achieved by swelling the resin in DMF. A solution of piperidine in DMF (30% (v/v), 10 ml) is then added to the resin, and agitated for 2 h. The resin is filtered, and rinsed with DMF (10 × 5 ml) and CH_2Cl_2 (10 × 5 ml).

To couple the subsequent amino acid, FmocCys(Trt)OH (2.62 g, 4.48 mmol), PyBOP (2.33 g, 4.48 mmol), and HOBT (0.605 g, 4.48 mmol) are dissolved in DMF (10 ml). DIEA (1.56 ml, 8.96 mmol) is added to the mixture, and the resulting solution is added to the resin described above. After agitating for 3 h, the resin is filtered, and rinsed with DMF (5 × 10 ml) and CH_2Cl_2 (5 × 10 ml).

The linker between a resin and its pendant synthetic peptide (in our example, RNase A fragment 110–111) is then activated with iodoacetonitrile as follows (Nilsson *et al.*, 2003a). The resin is swollen in CH_2Cl_2. A solution of iodoacetonitrile (3.4 ml, 46.8 mmol), DIEA (3.2 ml, 18.7 mmol), and NMP (75 ml) is filtered through a plug of basic alumina, and added to the resin. The resin is agitated for 18 h, filtered, and washed with NMP (5 × 10 ml) and CH_2Cl_2 (5 × 10 ml).

The phosphinothioester is liberated by incubating the above resin (1.0 g, 1.12 mmol peptide loading) with a solution of phosphinothiol **I** (2.1 g, 9.0 mmol) in DMF (15 ml) for 12 h under Ar(g). The resin is filtered, and rinsed with DMF (5 × 10 ml) and CH_2Cl_2 (5 × 10 ml), and the filtrate is concentrated under reduced pressure. The residue is purified by flash

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chromatography (silica gel, 30% (v/v) EtOAc in hexanes) to yield FmocCys(Trt)Glu(O*t*Bu)SCH_2PPh_2 (typical yield: 0.71 g, 0.72 mmol, 64% based on a 1.12-mmol resin loading). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.75–7.70 (m, 2 H), 7.57–7.55 (m, 2 H), 7.42–7.14 (m, 29 H), 6.68 (d, $J = 6.6$ Hz, 1 H), 5.13 (d, $J = 8.1$ Hz, 1 H), 4.56–4.50 (m, 1 H), 4.36–4.34 (m, 2 H), 4.19–4.17 (m, 1 H), 3.81–3.80 (m, 1 H), 3.44–3.38 (m, 2 H), 2.78–2.68 (m, 1 H), 2.61–2.57 (m, 1 H), 2.27–2.23 (m, 2 H), 2.11–1.95 (m, 1 H), 1.83–1.70 (m, 1 H), 1.37 (s, 9 H) ppm; ^{13}C NMR ($CDCl_3$, 75 MHz) δ 198.14, 171.89, 170.19, 155.81, 144.17, 143.59, 143.46, 141.10, 136.49 (d, $J =$ 14 Hz), 132.69 (d, $J = 4.2$ Hz), 132.44 (d, $J = 4.1$ Hz), 129.41, 128.98, 128.38 (d, $J = 6.6$ Hz), 127.93, 127.59, 126.94, 126.74, 124.93, 119.80, 80.74, 67.21, 67.00, 58.50, 53.83, 46.89, 31.00, 27.85, 27.23, 25.45 (d, $J =$ 24.8 Hz) ppm; ^{31}P NMR ($CDCl_3$, 121 MHz) δ −14.51 ppm; typical MS (ESI) m/z 1007.3340 ($MNa^+ = 1007.3371$).

5.2. Experimental procedure: Strategy C5

A water-soluble phosphinothiol can effect the traceless Staudinger ligation in purely aqueous medium in moderate yields, thereby integrating the traceless Staudinger ligation with expressed protein ligation (Tam *et al.*, 2007). Incubation of the phosphinothiol and the chitin-bound peptide expressed via rDNA technology, and direct elution from the chitin resin yields the C-terminal phosphinothioester, which can then be used in Staudinger ligation with an azido peptide fragment.

Proteins and peptide fragments can be produced with rDNA methods in which the fragment is fused with the *Mxe* intein and a chitin-binding domain (CBD) (Arnold *et al.*, 2002). In our example (Tam *et al.*, 2007), this method is performed on Met(−1)RNase A–Gly–intein–CBD fusion protein to generate its C-terminal phosphinothioester. The desired plasmid is transformed into *E. coli* BL21(DE3) cells. Luria–Bertani (LB) medium (5 ml) containing ampicillin (0.10 mg/ml) is inoculated with a single colony and grown for 16 h at 37 °C. The cells are collected by centrifugation (2000×*g* for 2 min), and resuspended in LB medium (4 ml). Four 4–l flasks each containing 1 l of LB medium with ampicillin (0.10 mg/ml) are then inoculated with the resuspended cells (1 ml to each flask) from the 16 h culture. Cultures are grown with shaking at 37 °C until OD = 0.5 at 600 nm. Gene expression is then induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG; to 0.5 m*M*), and the cultures are grown for an additional 3–4 h at 25 °C. The lower temperature prevents the formation of inclusion bodies. Cells are harvested by centrifugation, and the cell pellet is stored at −20 °C.

Frozen cells are thawed and suspended in lysis and column buffer (LCB), which is 20 m*M* 3-(*N*-morpholino)propanesulfonic acid (MOPS)–NaOH buffer (pH 6.8) containing NaCl (0.5 *M*), ethylenediaminetetraacetic acid (EDTA; 0.1 m*M*), Triton X-100 (0.1%, w/w). Cells are lysed by sonication,

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and the lysate is subjected to centrifugation at 15,000×*g* for 30 min. The supernatant is applied slowly to an LCB-equilibrated column of chitin resin (New England Biolabs, Ipswich, MA). Approximately 6 ml of chitin resin is needed for 1 g of cells. The loaded resin is washed thoroughly with LCB (8 column-volumes), and LCB containing 0.5 *M* NaCl (2 column-volumes).

Intein-mediated cleavage is induced by incubating the resin with degassed cleavage buffer, which is 50 m*M* MOPS–NaOH buffer (pH 6.8) containing NaCl (0.5 *M*), EDTA (0.1 m*M*), and a water-soluble thiol such as 2-mercaptoethanesulfonic acid (MESNA) for 14 h under Ar(g). The thiol effects the transthioesterification of the fusion protein to form a C-terminal thioester of the protein, which is eluted from the resin with 0.5 *M* NaCl (2 ml). The peptide thioester is precipitated by the addition to 1% (v/v) of an aqueous solution of sodium deoxycholate (NaDOC) (1%, v/v) and by the addition to 2% (v/v) of an aqueous solution of trichloroacetic acid (TCA, 50%, w/v). After mixing, the precipitate is collected by centrifugation (5000×*g* for 5 min), decanted, and resuspended in acetone to remove small-molecule additives. MALDI mass spectrometry can be used to confirm the identity of the peptide thioester. After dissolving the peptide thioester in the appropriate solvent/buffer, transthioesterification can be performed with a phosphinothiol to generate the C-terminal phosphinothioester. The resulting peptide phosphinothioester can be isolated by the above precipitation procedure using NaDOC and TCA.

6. Protein Assembly by Orthogonal Chemical Ligations

Perhaps the most well-characterized protein, bovine pancreatic ribonuclease (RNase A; Raines, 1998), has been used to evaluate the efficacy of some of the strategies above. The 124 amino acids of RNase A were assembled by using a variety of sequential and convergent amide-bond forming reactions, including the traceless Staudinger ligation, as depicted in Fig. 2.3 (Nilsson *et al.*, 2003a). The enzyme thus created is remarkable in that its peptide bonds were synthesized by four distinct processes, two of which are sequential (mRNA translation by a ribosome and solid-phase peptide synthesis) and two of which are convergent (native chemical ligation and traceless Staudinger ligation).

6.1. Experimental procedure: Traceless Staudinger ligation on a solid phase

The resin-bound azido peptide (RNase A fragment 112–124: GNPYVPVHFDASV, 180 mg, 25 μmol) as synthesized with Strategy N1 is swollen in DMF for 1 h. The C-terminal phosphinothioester of RNase A

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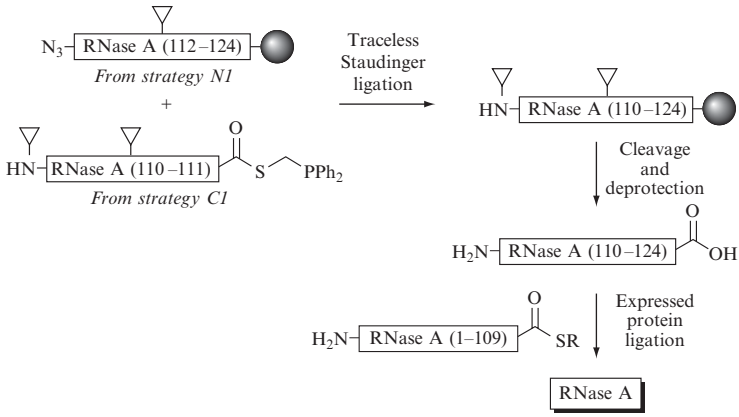

Figure 2.3 Route for the assembly of RNase A with solid-phase peptide synthesis, Staudinger ligation, and expressed protein ligation.

fragment 110–111 is synthesized with Strategy C1 as FmocCys(Trt)Glu (O*t*Bu)SCH_2PPh_2 (99 mg, 100 μmol), dissolved in 10:1 DMF/H_2O (1.5 ml), and added to the swollen resin. The slurry is agitated gently for 12 h, after which the solvent is removed by filtration, and the resin is rinsed with DMF (5 × 10 ml) and CH_2Cl_2 (5 × 10 ml). The resin is dried under high vacuum and then treated with a cleavage cocktail (38:1:1 TFA/H_2O/ethanedithiol, 2 ml) for 2 h. The resin is filtered, and added to ice-cold diethyl ether (20 ml) to precipitate the deprotected peptide, RNase A fragment 110–124. The peptide is purified by reverse-phase HPLC and can be analyzed by MALDI mass spectrometry. The ligated peptide can be elaborated further with orthogonal-ligation methods. In our example, expressed protein ligation with the C-terminal thioester of RNase A fragment 1–109 gives full-length RNase A, as shown.

7. Prospectus

The traceless Staudinger ligation has joined the repertoire of ligation methods for the convergent synthesis of proteins. This method has been used along with others to assemble an entire protein. A putative strategy for the assembly of proteins is depicted in Fig. 2.4 (Nilsson *et al.*, 2003b). Here, a target protein is divided into shorter fragments, and the ultimate C-terminal fragment is attached to a solid support. This immobilized fragment is capped with an α-azido acid and then reacted with a protected C-terminal phosphinothioester peptide fragment. The cycle is repeated until all fragments have been added. Deprotection and folding of the nascent

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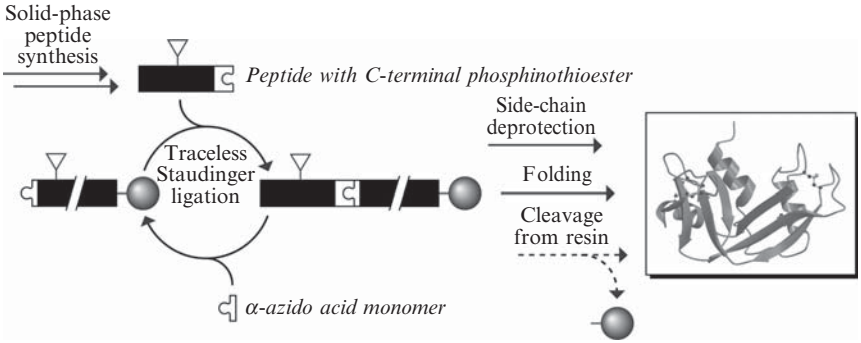

Figure 2.4 **Strategy for the chemical synthesis of proteins by iterative cycles of solid-phase peptide synthesis and solid-phase Staudinger ligation.**

polypeptide while still attached to the solid support (to avoid aggregation) yields a functional protein. The protein can be left attached to the resin for high-throughput assays or liberated for structure–function analyses in solution. The entire process is amenable to automation. Most notably, nonnatural amino acids or synthetic modules can be substituted for native ones, affording otherwise inaccessible proteins for otherwise unattainable goals.

7.1. Experimental procedure: General

All chemicals and reagents are available from Aldrich Chemical (Milwaukee, WI), with the exception of Fmoc-protected amino acids and alkanesulfonamide safety-catch resins, which are available from Novabiochem (San Diego, CA). Solution-phase reactions are monitored by thin-layer chromatography and visualized by UV light or staining with I_2. Flash chromatography is performed with columns of silica gel 60, 230–400 mesh (Silicycle, Québec City, Québec, Canada). HPLC purification is performed on a C18 reverse-phase column.

The term "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials using a rotary evaporator at water-aspirator pressure (<20 mm Hg) while maintaining the water-bath temperature below 40 °C. The term "high vacuum" refers to a vacuum (≤0.1 mm Hg) achieved by a mechanical belt-drive oil pump.

Peptide synthesis is performed by standard Fmoc-protection strategies using an automated synthesizer with HATU activation. Phosphorus-31 NMR spectra are proton-decoupled and referenced against an external standard of deuterated phosphoric acid. Mass spectra are obtained with electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) techniques.

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