Serine-Selective Bioconjugation

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ABSTRACT: The first general method for the rapid, chemoselective, and modular functionalization of serine residues in native polypeptides is reported. Using a reagent platform based on P(V) oxidation state, this redox-economic approach can be used to append nearly any kind of cargo onto serine generating a stable, benign, and hydrophilic phosphorothioate linkage. The method tolerates all other known nucleophilic functional groups of naturally occurring proteinogenic amino acids. A variety of applications can be envisaged enabled by this expansion of the toolbox of site-selective bioconjugation methods.

It is no surprise that of the twenty naturally-occurring amino acid residues bearing reactive groups, serine is rarely chosen as a handle for selective derivatization (Figure 1A).[1-4] This lack of serine functionalization clearly stems not from a lack of desire, but rather the unmet challenge of differentiating the nucleophilicity of serine residues' primary alcohol from other nucleophilic sidechains and water.[1-5] A process that could achieve this differentiation would be inherently valuable, as it would add a new dimension to the toolbox of options available for site-selective labeling of proteins and the chemoselective modification of peptides. Whereas nature has pointed to a possible solution to this problem using enzymatic machinery and phosphorus(V) reactivity,[6-11] there is currently no general method (chemoenzymatic or otherwise) to site-selectively attach a precise cargo to a serine residue in a native biomolecule.[1-3,12] Kinases are typically responsible for the phosphorylation of various amino acid residues such as serine (Figure 1B).[6-11] This process suggests that P(V)-based electrophiles could have an innate preference for alcohol-based nucleophiles.[13] In parallel with this biological precedent, chemical inspiration for P(V)-based bioconjugation stems from the new class of phosphorous reagents (Ψ and Π) which react with both oxygen- and carbon-based nucleophiles in a rapid and predictable way.[14,15] In this Communication, Ψ-loaded reagents (Ψ-modules) are shown to

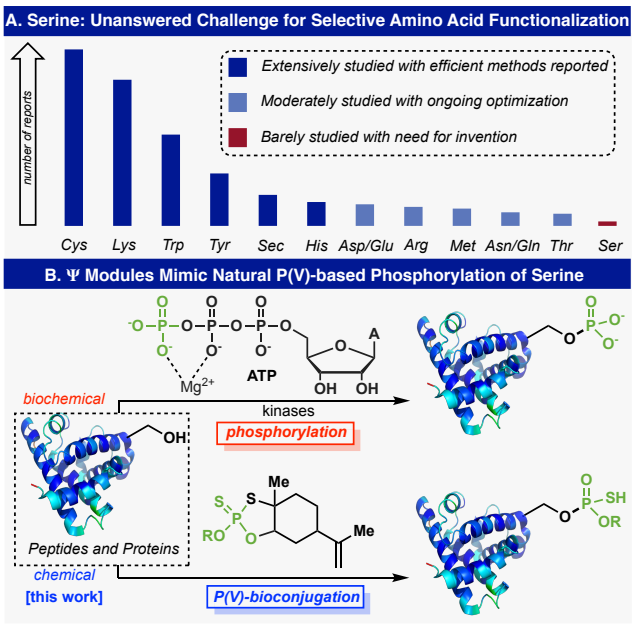
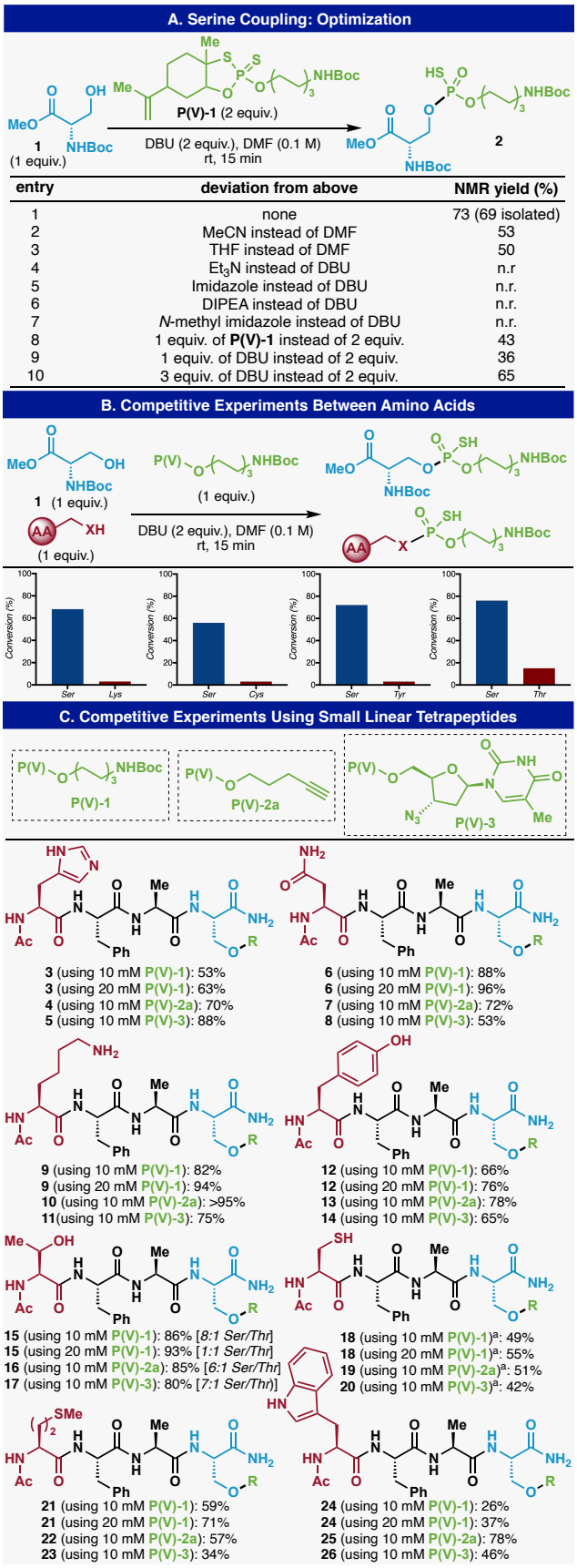


FIGURE 1. (A) Serine: Unanswered Challenge for Selective Amino Acid Functionalization (B) PSI Reagents Mimic Natural P(V)-based Phosphorylation of Serine.

exhibit strikingly selective reactivity towards serine. This method enables both selective post-translational labeling and bioconjugation of serine residues.



entry	deviation from above	NMR yield (%)
1	none	73 (69 isolated)
2	MeCN instead of DMF	53
3	THF instead of DMF	50
4	Et_3N instead of DBU	n.r
5	Imidazole instead of DBU	n.r.
6	DIPEA instead of DBU	n.r.
7	*N*-methyl imidazole instead of DBU	n.r.
8	1 equiv. of **P(V)-1** instead of 2 equiv.	43
9	1 equiv. of DBU instead of 2 equiv.	36
10	3 equiv. of DBU instead of 2 equiv.	65

FIGURE 2. (A) Serine Coupling: Optimization. (B) Competitive Experiments Between Amino Acids. (C) Competitive Experiments Using Small Linear Tetrapeptdes. [a] Using EtSH (5 equiv.).

In 2018, a set of reagents based upon a limonene-scaffold fused to an oxathiophospholane heterocycle were introduced for the purpose of simplifying stereocontrolled access to phosphorothioate linkages (Phosphorous-Sulfur Incorporation, abbreviated PSI or Ψ).[14] This redox-economic approach avoided the extraneous steps associated with a P(III)-based manifold, yet retained the high reactivity associated with phosphoramidates and related systems.[14] Shortly thereafter, related limonene-based reagents were introduced to access both phosphines and methylphosphonates through stereocontrolled P–C bond formation (Phosphorous-Sulfur Incorporation, abbreviated PI or Π).[15]

During those studies, it was sporadically observed that oxygen-based nucleophiles would react in preference to other heteroatoms such as sulfur and nitrogen.[14,15] After optimization of the coupling step (Figure 2A), the generality of these observations was explored systematically in the context of amino-acid functionalization as depicted in Figure 2B. Thus, competitive coupling experiments between amino acids revealed a striking selectivity for serine functionalization using Ψ-module **P(V)-1** in the presence of cysteine, lysine and tyrosine, even at high concentration (0.1 M). These preliminary studies established the viability of such an approach, and represent selectivities on par with traditional protein labeling methods (e.g. NHS esters for lysine, iodoacetamide for cysteine, and MTAD for tyrosine/tryptophan).[1-4] Interestingly, reagent **P(V)-1** under similar conditions displayed a respectable selectivity of 7:1 favoring serine over threonine. Transitioning to more realistic models for chemoselective labeling, competitive coupling experiments were then pursued using small peptides with Ψ-modules **P(V)-1**, **P(V)-2a**, and **P(V)-3** (Figure 2C). Good-to-excellent conversions were observed within 15 minutes, and unless otherwise noted, only one product was formed with no epimerization. Exquisite selectivity was obtained for serine at both 10 mM and 20 mM reagent concentration (2 mM peptide concentration) when competing with cysteine, lysine, histidine, glutamine, methionine, tryptophan, and tyrosine. When employing cysteine containing peptides, excess EtSH was added to avoid the formation of adducts between cysteine and the thiirane by-product of the Ψ-module. In addition, a reductive quench (DTT) is used to reduce any S–S bond formed during the reaction.[16,17] When competing with threonine, a useful level of selectivity (determined by H-coupled ^{31}P NMR) was observed at 10 mM of reagents **P(V)-1** (8:1), **P(V)-2a** (6:1), or reagent **P(V)-3** (7:1) delivering compounds **15**, **16** and **17** in 86%, 85% and 80%, respectively.[1-4]

More realistic applications of this method were then explored in the context of longer peptides containing a variety of nucleophilic amino acids (Figure 3A). Excellent selectivity for serine and satisfactory conversions were always observed using a variety of Ψ-modules (Figure 3A). As an example, peptides **SI-14** was selectively labelled at the serine position to afford peptide **31**, **32**, and **33** in 23%, 36% and 17% conversions using reagents **P(V)-1**, **P(V)-2b**, and **P(V)-3**, respectively. In addition to these linear examples, the functionalization of cyclic peptides was explored due

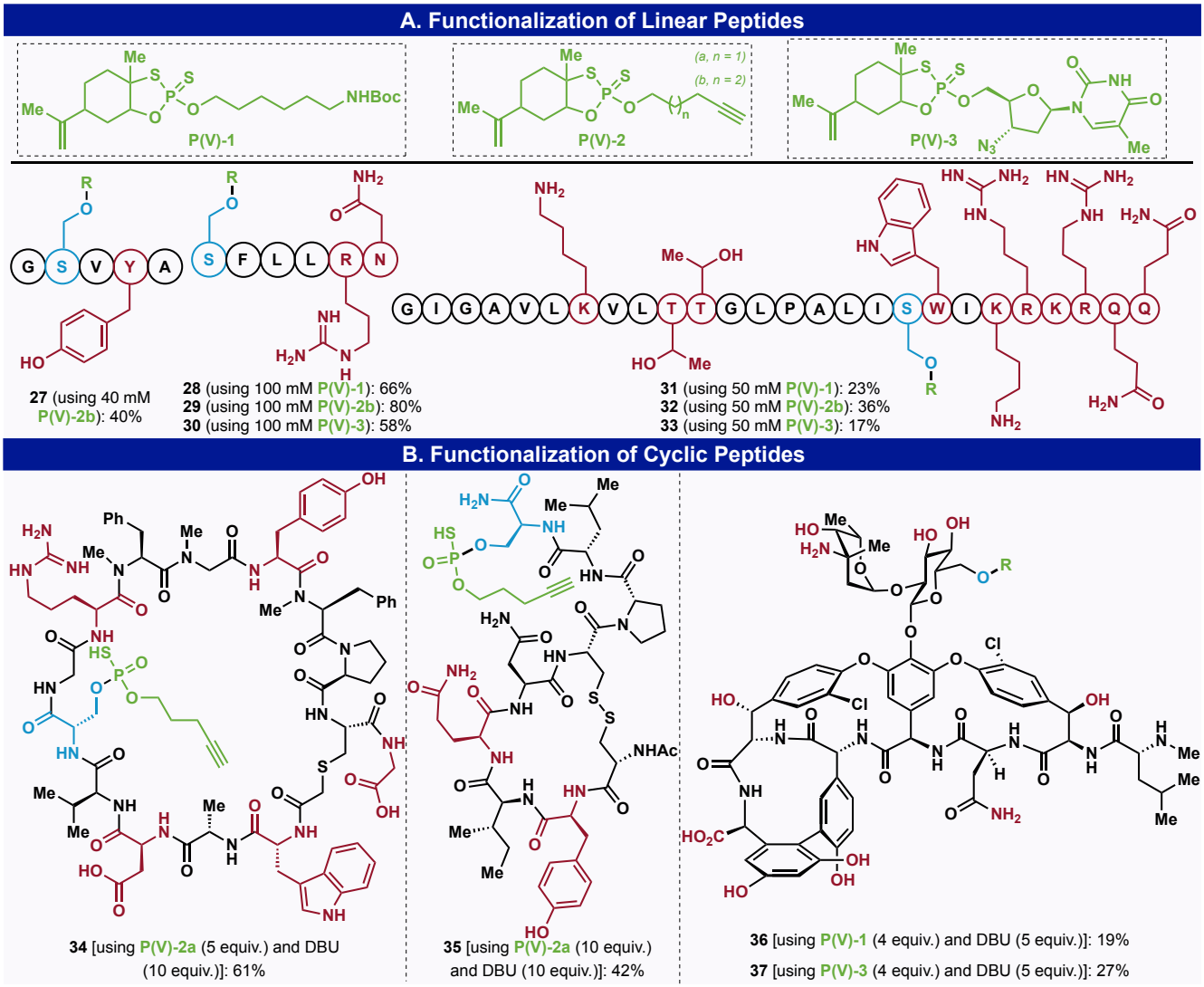


FIGURE 3. (A) Peptide Scope. (B) Functionalization of Cyclic Peptide.

to their importance and relevance in the context of medicinal chemistry (Figure 3B).[18-20] The developed conditions were first applied to the diversification of a cyclic peptide containing several nucleophilic functionalities, affording 61% yield of the desired product **34** within 10 minutes. An oxytocin-derivative, bearing a sensitive disulfide bridge and a tyrosine, successfully underwent coupling in 42% yield, with complete selectivity for the serine residue.[21-23] Finally, vancomycin was selected as a key example with an impressive functional group array: one carboxylic acid, one primary amine, one primary amide, three phenolic alcohols, five secondary alcohols, and a single primary alcohol.[24-28] Nevertheless, both reagents **P(V)-1** and **P(V)-3** demonstrated remarkable selectivity for the primary alcohol site affording compounds **36** and **37** in 19% and 27% yields, respectively. The labeled position was confirmed by H-coupled ^{31}P NMR; assignment was simple as the molecule bears only one primary alcohol.

Next, we challenged this chemical method for the installation of various ψ-loaded reagents on proteins. The protein ubiquitin was primarily chosen for its relevance in proteasome targeting.[29-31] When ubiquitin **38** was vortexed

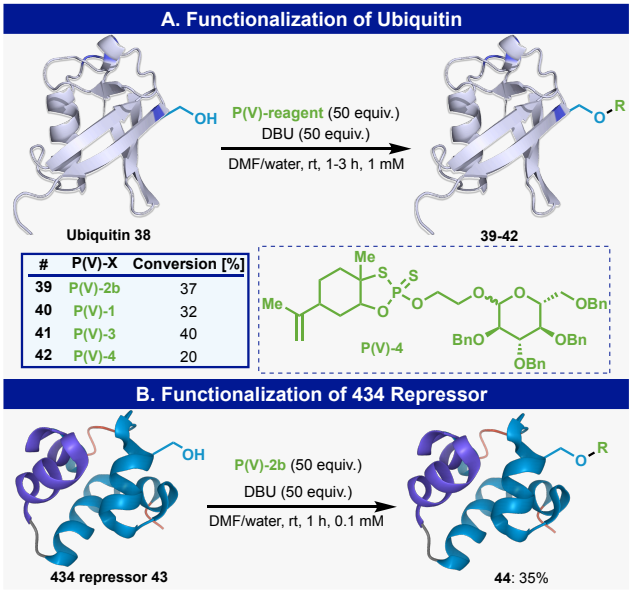


FIGURE 4. Functionalization of Proteins (A) Functionalization of Ubiquitin. (B) Functionalization of 434 Repressor.

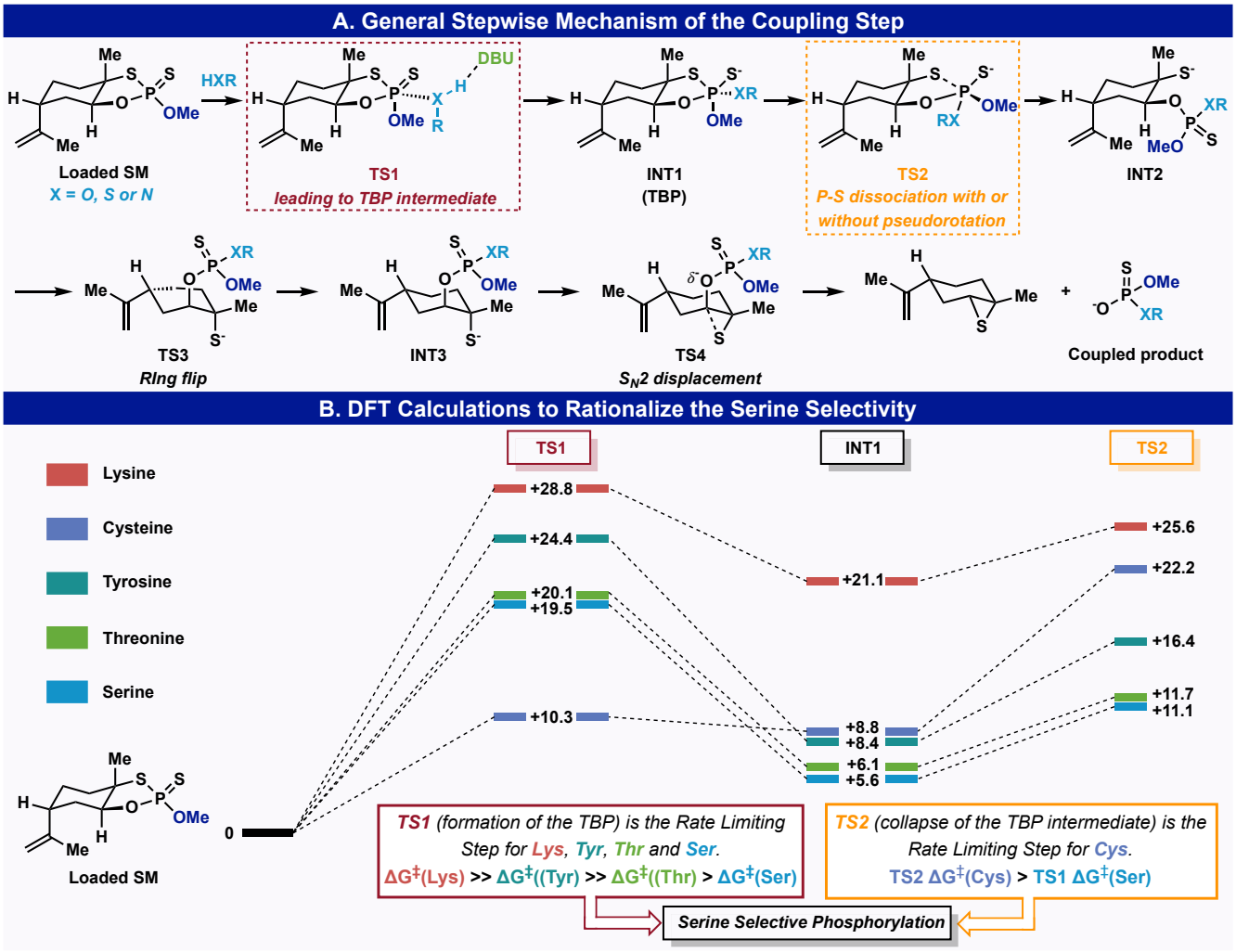


FIGURE 5. (A) General Stepwise Mechanism of the Coupling Step. (B) DFT Calculations to Rationalize the Serine Selectivity.

with **P(V)-2b** reagent and DBU, it resulted in mono-labeled ubiquitin **39** in 37% conversion (Figure 4A). Labeled ubiquitin **39** was digested with trypsin and analyzed by LC-MS/MS (see SI for details). The resulting MS/MS data were searched using the Mascot search algorithm using a variable modification of 176 Da and a database containing the ubiquitin sequence. MS/MS of P(V) tagged peptide (ESTLHLVLR, E64-R72, m/z 622.31 $[M+2H]^{2+}$) confirmed the selective labeling of ubiquitin's sole Ser residue (S65). Subsequently, other ψ-modules were screened for the labeling of ubiquitin. **P(V)-1**, **P(V)-3**, and **P(V)-4** reagents afforded the mono-labeling of ubiquitin in 32% (**40**), 40% (**41**), and 20% (**42**) conversions respectively. In addition, the method was efficiently translated to the labeling of the DNA binding protein 434 repressor **43** using **P(V)-2b**; reaction of these components delivered **44** in 35% conversion (Figure 4B). This model could help in elucidating DNA/protein interactions.[32-34]

DFT studies were undertaken to rationalize the selectivity observed for serine in the coupling step (Figure 5).[35] These studies support a stepwise A_N+D_N phosphorylation mechanism (Figure 5A) with limiting barriers for the formation (**TS1**) or collapse (**TS2**) of a trigonal bipyramidal pentacoordinated (TBP, **INT1**) intermediate (Figure 5B). The remaining steps of the reaction coordinate involve a ring flip of the limonene skeleton (**TS3**) followed by S_N2 displacement of the phosphorylated amino acid and simultaneous release of the cyclohexene sulfide (**TS4**) with an exothermic balance ($\Delta G \approx$ -37 kcal/mol).[36] Transition structures **TS3** represent the highest barrier within the sequence of conformational distorsions[37] required to transform chair **INT2** into chair **INT3** (See SI for details).[38] The transition structures leading to the TBP, **TS1**, display the backside attack of the nucleophile to the P-O ring bond in a late, intermediate-like arrangement. **TS1** shows a large extent of RX–P (where X = O, or N) bond formation and concomitant proton transfer to the nitrogen atom of DBU acting as a general base. Amino acids with alcohol and amine side chains display a rate-limiting formation of the trigonal bipyramidal intermediate with barriers that increase in the order Ser < Thr << Tyr << Lys (**TS1**, Figure 5B). A $\Delta\Delta G^{‡}$ of 0.6 kcal/mol between **TS1**-Ser and **TS1**-Thr is in qualitative agreement with the lower but competitive reactivity of threonine, whereas a $\Delta\Delta G^{‡}$ of 4.9 kcal/mol between **TS1**-Ser and **TS1**-Tyr is consistent with the limited reactivity of tyrosine. Similarly, **TS1**-Ser is favored relative to **TS1**-Lys by ≈ 9 kcal/mol in agreement with the lower acidity of the primary amine and greater steric congestion during the partial deprotonation by DBU. A search for the transition structures corresponding to the collapse of the TBP intermediates led to structures **TS2**, which depict the dissociation of the ring S–P bond accompanied by pseudorotation[39] and

reduction of the O–P–O angle as the tetrahedral phosphorylated P(V) product forms (Figure 5A and SI for details). The formation of a strong O–P bond and the axial arrangement of the methoxy group facilitate the dissociation of the weaker S–P bond (See SI for details).[40] For amino acids with alcohol side chains, transition structures **TS2** leading to the collapse of the TBP intermediates are ≈ 8 kcal/mol more stable than the transition structures **TS1** forming the TBP intermediates. In contrast with the reaction coordinate for the alcohol, the collapse of the **TBP**-Cys intermediate is disfavored relative to its formation (cf. **TS1**-Cys and **TS2**-Cys, Figure 5B). The existence of a low barrier for decomposition of the TBP intermediate back to reactants has been used to explain the slow thiolysis of phosphate triesters.[41] In agreement with experiment, the limiting barrier for the phosphorylation of cysteine (**TS2**-Cys, $\Delta G^{\ddagger}$ = +22.2 kcal/mol) is estimated to be 2.7 kcal/mol higher than the limiting barrier for the phosphorylation of serine (**TS1**-Ser, $\Delta G^{\ddagger}$ = +19.5 kcal/mol). A comparison of **TS2**-Cys and **TS2**-Ser geometries shows the reluctance of cysteine to pseudorotate (S–P–O*ring* 147.0° in **TS2**-Cys versus 111.2° in **TS2**-Ser), become equatorial, and stabilize ring P–S dissociation via the axial-axial arrangement of the OMe group and the ring P–S bond (See SI). The higher pseudorotation barrier of cysteine relative to serine is consistent with the preference of good leaving groups (e.g. cysteine) to occupy an axial position.[42] Taken all together, these results rationalize the serine selectivity observed for the coupling step.

Building on the chemistry of the PSI-reagent system, a chemoselective, rapid, and robust method for the direct functionalization of serine residues in the context of peptides and even proteins has been developed. The modular approach enables practitioners to append virtually any kind of cargo desired in an orthogonal way. Applications of these findings to bioconjugation, chemical biology, and as a result, the pursuit of novel materials can all be envisaged.[43]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

The Supporting Information contains all experimental procedures, analysis, the detail of DFT calculations and compound characterization data.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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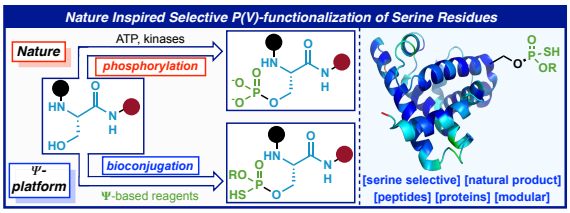

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