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Communication

Serine-Selective Bioconjugation

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Cite This: *J. Am. Chem. Soc.* 2020, 142, 17236–17242
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ABSTRACT: This Communication reports the first general method for rapid, chemoselective, and modular functionalization of serine residues in native polypeptides, which uses a reagent platform based on the P(V) oxidation state. This redox-economical 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 by this expansion of the toolbox of site-selective bioconjugation methods.

It is no surprise that of the 20 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 from those of other nucleophilic side chains and water (chemoselectivity) and a single residue among its multiple copies (site selectivity).[1−7] 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 chemoselective modification of peptides and site-selective labeling of proteins. Whereas nature has pointed to a possible solution to this problem using enzymatic machinery and phosphorus(V) reactivity,[8−16] there is currently no general method (chemoenzymatic or otherwise) to site-selectively attach a precise cargo to a serine residue in a native biomolecule.[1−3,17] Kinases are typically responsible for phosphorylation of various amino acid residues such as serine (Figure 1B).[8−16] This process suggests that P(V)-based electrophiles could have an innate preference for alcohol-based nucleophiles.[18] In parallel with this biological precedent, chemical inspiration for P(V)-based bioconjugation stems from the new class of phosphorus reagents (Ψ and Π) that react with both oxygen- and carbon-based nucleophiles in a rapid and predictable way.[19,20] In this Communication, Ψ-loaded reagents (Ψ-modules) are shown to exhibit strikingly selective reactivity toward serine. This method enables both selective post-translational labeling and bioconjugation of serine residues.

In 2018, a set of reagents based on a limonene scaffold fused to an oxathiophospholane heterocycle were introduced for the purpose of simplifying stereocontrolled access to phosphorothioate linkages (phosphorus−sulfur incorporation, abbreviated as PSI or Ψ).[19] This redox-economical approach avoided the extraneous steps associated with a P(III)-based manifold yet retained the high reactivity associated with phosphoramidites and related systems.[19] Shortly thereafter, related limonene-based reagents were introduced to access

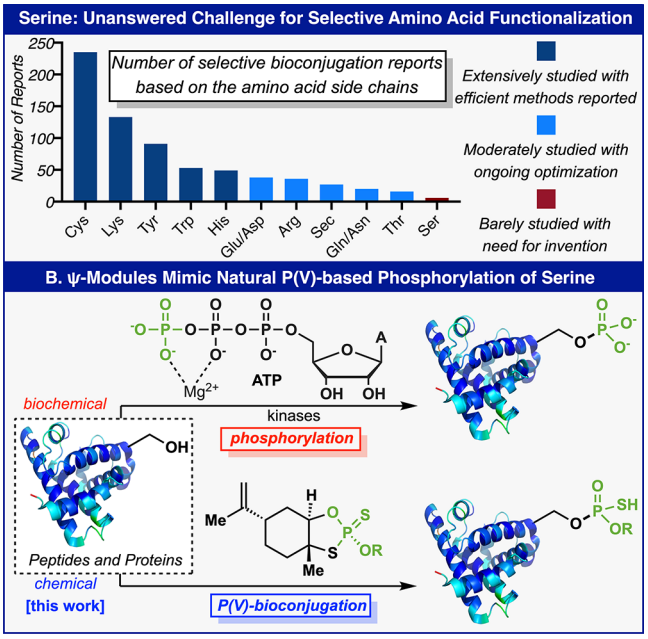
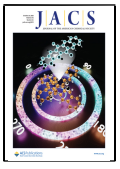


Figure 1. (A) Serine poses an unanswered challenge for selective amino acid functionalization. (B) PSI reagents mimic natural P(V)-based phosphorylation of serine.

Received: May 22, 2020
Published: September 23, 2020



https://dx.doi.org/10.1021/jacs.0c05595
J. Am. Chem. Soc. 2020, 142, 17236−17242

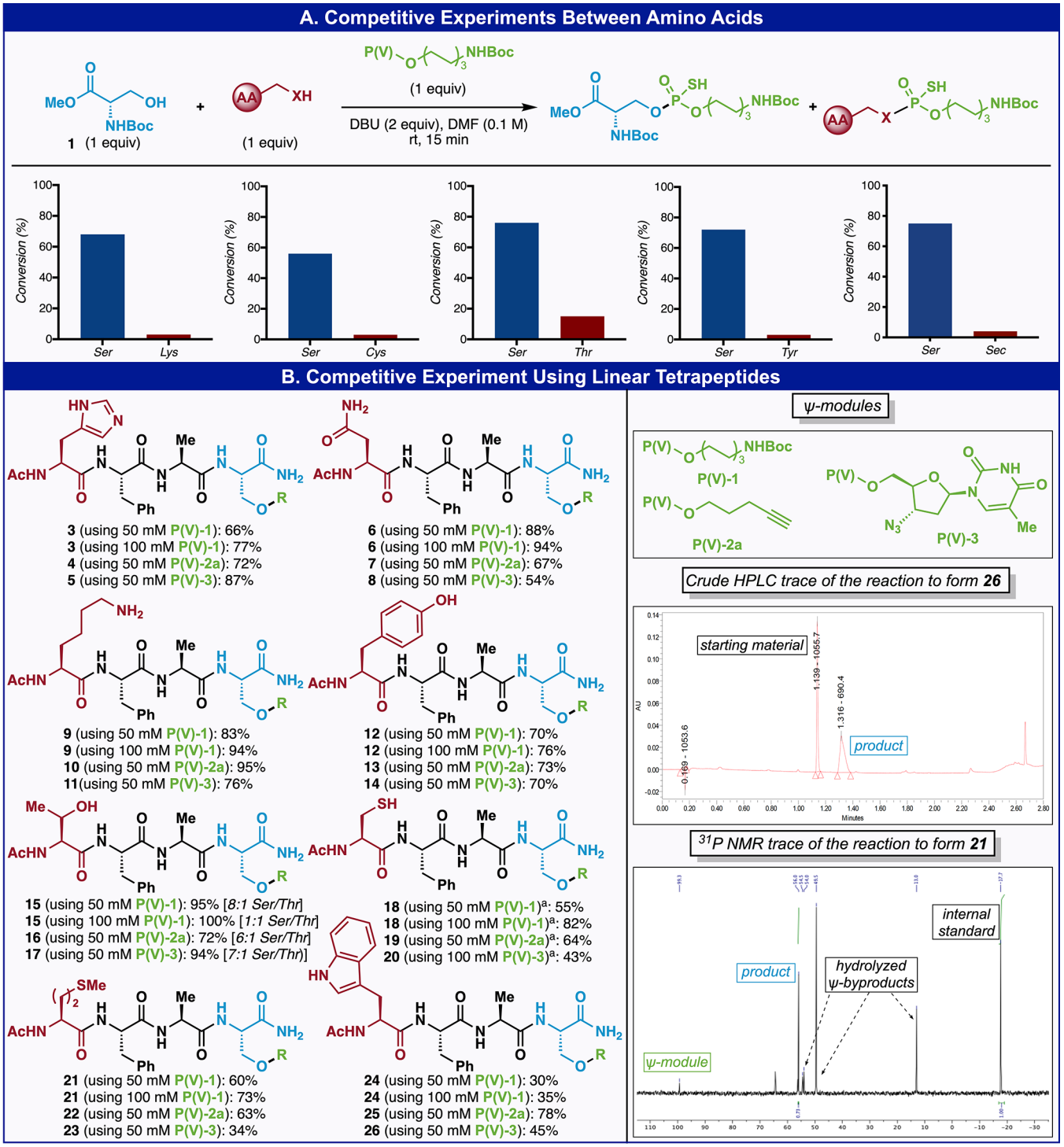


Figure 2. (A) Competitive experiments between amino acids. (B) Competitive experiments using small tetrapeptides (10 mM). [a]Using EtSH (5 equiv).

both phosphines and methylphosphonates through stereocontrolled P−C bond formation (phosphorus incorporation, abbreviated as PI or Π).[20]

During the studies using PSI reagents, it was sporadically observed that oxygen-based nucleophiles would react in preference to other heteroatoms such as sulfur and nitrogen.[19,20] After optimization of the coupling step (Figure S23), the generality of these observations was explored systematically in the context of amino acid functionalization, as depicted in Figure 2A. 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, tyrosine, and selenocysteine, even at high concentration (0.1 M). These preliminary studies established the viability of such an approach and demonstrated selectivities on par with those of 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

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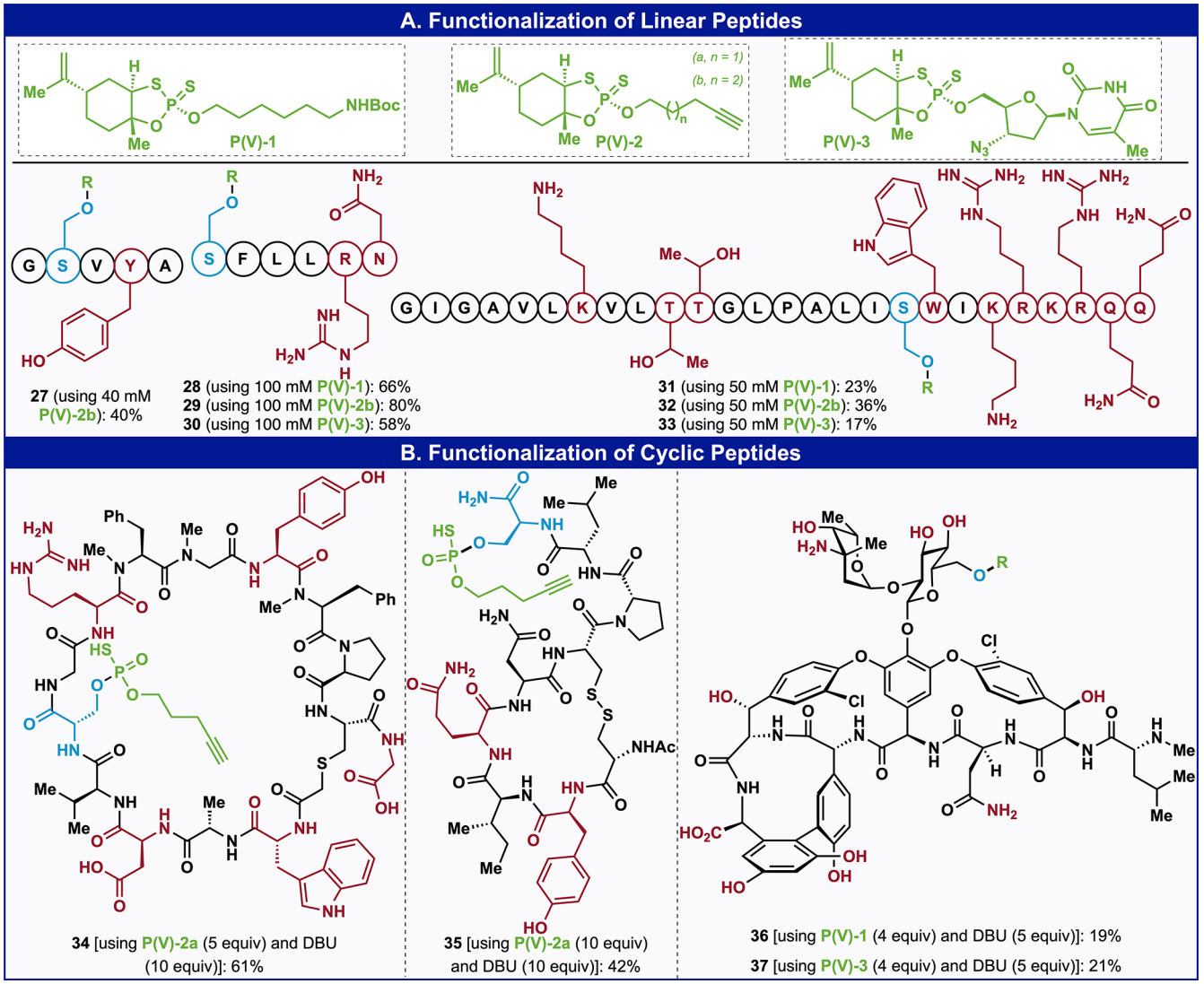

Figure 3. (A) Functionalization of linear peptides. (B) Functionalization of cyclic peptides.

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 2B). Good to excellent conversions were observed within 15 min, and unless otherwise noted, only one product was formed with no epimerization. Exquisite selectivity was obtained for serine at both 50 and 100 mM reagent concentration (10 mM peptide concentration) in competition with cysteine, lysine, histidine, glutamine, methionine, tryptophan, and tyrosine. When cysteine-containing peptides were employed, excess EtSH was added to avoid the formation of adducts between cysteine and the thiirane byproduct of the Ψ-module. In addition, a reductive quench with dithiothreitol was used to reduce any S−S bond formed during the reaction.[21,22] In competition experiments with threonine, a useful level of selectivity (as determined by H-coupled ^{31}P NMR spectroscopy) was observed with **P(V)-1** (8:1), **P(V)-2a** (6:1), or **P(V)-3** (7:1) at 50 mM, delivering compounds **15**, **16**, and **17** in 95%, 72%, and 94% yield, 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 selectivities for serine and satisfactory conversions were always observed using a variety of Ψ-modules (Figure 3A). As an example, peptide **SI-16** was selectively labeled at the serine position to afford peptides **31**, **32**, and **33** in 23%, 36%, and 17% conversion 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 because of their importance and relevance in the context of medicinal chemistry (Figure 3B).[23−25] The developed conditions were first applied to the diversification of a cyclic peptide containing several nucleophilic functionalities, affording the desired product **34** in 61% yield within 10 min. An oxytocin derivative bearing a sensitive disulfide bridge and a tyrosine successfully underwent coupling in 42% yield with complete selectivity for the serine residue.[26−28] 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.[29−33] 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 21% yield, respectively. The labeled position was confirmed by H-coupled ^{31}P NMR spectroscopy; the assignment was simple because the molecule bears only one primary alcohol.

https://dx.doi.org/10.1021/jacs.0c05595
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Next, we challenged this chemical method for the installation of various Ψ-loaded reagents on proteins. The protein ubiquitin (**38**) was primarily chosen for its relevance in proteasome targeting.[34−36] When **38** was vortexed with **P(V)-2b** and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), monolabeled ubiquitin **39** was obtained in 37% conversion (Figure 4A). Labeled ubiquitin **39** was digested with trypsin and

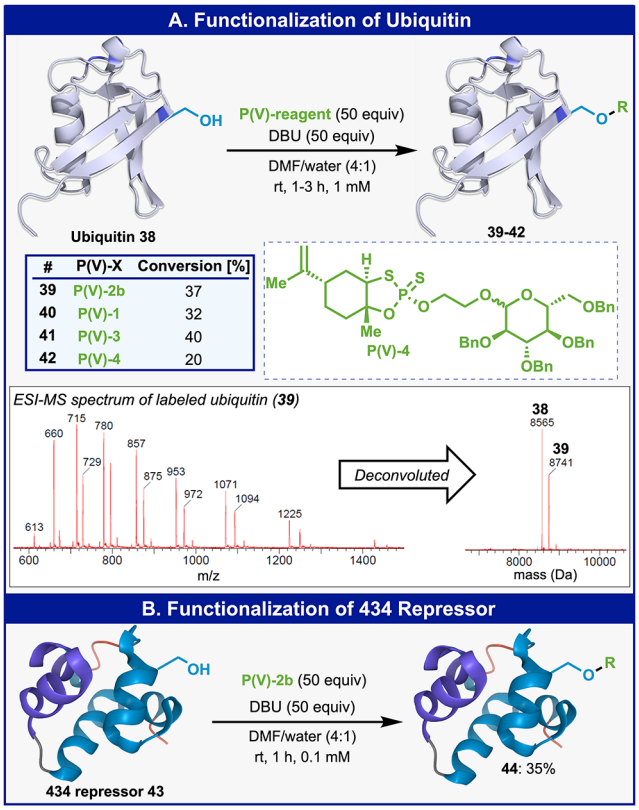

Figure 4. Functionalization of (A) ubiquitin and (B) the 434 repressor protein.

analyzed by LC−MS/MS (see the Supporting Information (SI) for details). The resulting MS/MS data were searched with 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 site-selective labeling of the Ser residue (S65; kinetic labeling). Subsequently, other Ψ-modules were screened for the labeling of ubiquitin. **P(V)-1**, **P(V)-3**, and **P(V)-4** afforded monolabeling 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**)[37−39] using **P(V)-2b**; reaction of these components delivered **44** in 35% conversion (Figure 4B). Further advances in the design of Ψ-reagents that are more reactive yet more stable under aqueous conditions and cysteine-compatible will facilitate further use of this new approach toward the chemo- and regioselective modification of serine residues on proteins.

Density functional theory (DFT) studies were undertaken to rationalize the selectivity observed for serine in the coupling step (Figure 5).[40] 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) intermediate (**INT1**) (Figure 5B). The remaining steps along 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).[41] Transition structures **TS3** represent the highest barrier within the sequence of conformational distorsions[42] required to transform chair **INT2** into chair **INT3** (see the SI for details).[43] The transition structures leading to the TBP intermediates, **TS1**, display the backside attack of the nucleophile to the P−O ring bond in a late intermediate-like arrangement. The **TS1** structures show a large extent of RX−P (X = O, 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 TBP intermediate with barriers that increase in the order Ser < Thr ≪ Tyr ≪ Lys (**TS1**, Figure 5B). The $\Delta\Delta G^{\ddagger}$ of 0.6 kcal/mol between **TS1**-Ser and **TS1**-Thr is in qualitative agreement with the lower but competitive reactivity of threonine, whereas the $\Delta\Delta G^{\ddagger}$ 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[44] and reduction of the O−P−O angle as the tetrahedral phosphorylated P(V) product forms (Figure 5A; see the 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 the SI for details).[45] 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** for formation of 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 in 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.[46] 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 the **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 the SI). The higher pseudorotation barrier for cysteine relative to serine is consistent with the preference of good leaving groups (e.g., cysteine) to occupy an axial position.[47] It worth noting that the selectivity for serine versus cysteine and tyrosine may simply be associated with a higher degree of reversibility. Taken all together, these results rationalize the serine selectivity observed for the coupling step.

Expanding on our previous work on the chemistry of the Ψ-reagent system, a chemoselective, rapid, and robust method for the direct functionalization of serine residues in the context of both peptides and proteins has been developed. The presented

https://dx.doi.org/10.1021/jacs.0c05595
J. Am. Chem. Soc. 2020, 142, 17236−17242

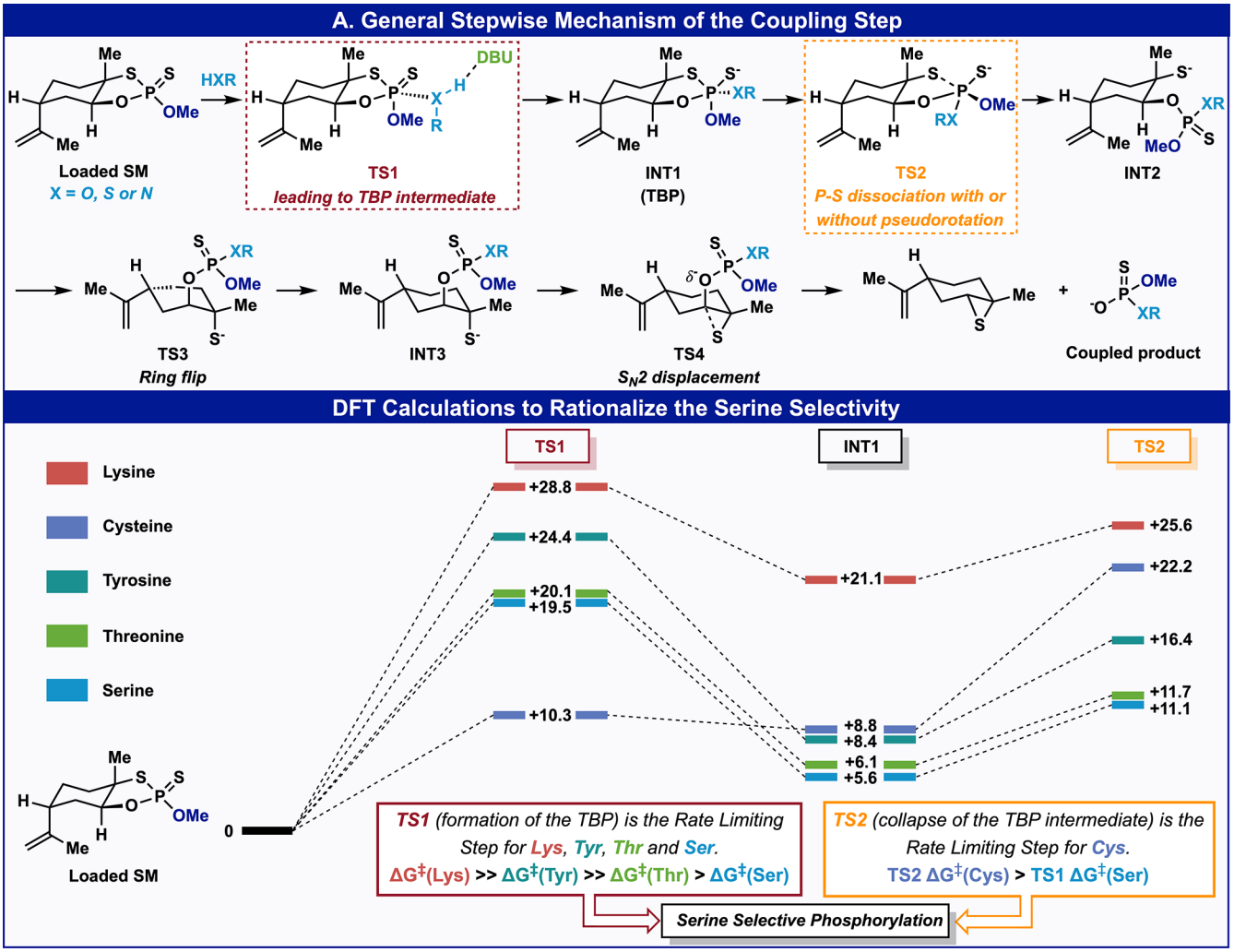

Figure 5. (A) General stepwise mechanism of the coupling step. (B) DFT calculations to rationalize the serine selectivity.

methodology displays excellent chemoselectivity for serine residues under the optimized solvent conditions and will enable practitioners in this field to append virtually any kind of desired cargo in an orthogonal fashion. Applications of these findings to bioconjugation and chemical biology as well as the pursuit of novel materials can all be envisaged.[48] The development of new PSI-reagents that are compatible with aqueous conditions and cysteine residues will also facilitate further use of this new approach for chemo- and regioselective modification of serine residues on proteins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c05595.

Experimental procedures, analysis, details of the DFT calculations, and compound characterization data (PDF)

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https://dx.doi.org/10.1021/jacs.0c05595
J. Am. Chem. Soc. 2020, 142, 17236−17242

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Financial support for this work was provided by Bristol-Myers Squibb, the NIH (GM-118176), the Marie Skłodowska-Curie Global Fellowships (749359-EnanSET to N.M.P) within the European Union's Research and Innovation Framework Programme (2014−2020), FCT Portugal (IF/00624/2015), and the Royal Society (URF\R\180019). D.F. was supported by the National Center for Advancing Translational Sciences, National Institutes of Health, through Grant UL1 TR002551 and Linked Award TL1 TR002551. The authors are grateful to Dr. Dee-Hua Huang and Dr. Laura Pasternack (Scripps Research) for assistance with NMR spectroscopy and to Dr. Jason Chen, Brittany Sanchez, and Emily Sturgell (Scripps Automated Synthesis Facility) for assistance with HPLC, HRMS, and LC−MS. We finally thank Dr. Victor L. Ayora and Lavinia Dunsmore for the 434 repressor protein sample.

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https://dx.doi.org/10.1021/jacs.0c05595
J. Am. Chem. Soc. 2020, 142, 17236−17242