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Solid-Phase Synthesis and Biochemical Studies of *O*-Boranophosphopeptides and *O*-Dithiophosphopeptides[1]

Kenneth E. Jenkins,[†] Adrian P. Higson,[‡] Peter H. Seeberger,[§] and Marvin H. Caruthers*

Contribution from the Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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Abstract: Signal transduction cascades maintain control over important cellular processes such as cell growth and differentiation by orchestrating protein phosphorylation and dephosphorylation. Specific control of these processes in vivo and in vitro can be achieved with peptide analogues that mimic the binding properties of phosphoproteins. We present here the solid-phase synthesis of two novel classes of phosphopeptide mimetics, *O*-boranophosphopeptides and *O*-dithiophosphopeptides, derivatized on tyrosine, serine, and threonine. The use of *H*-phosphonate and *H*-phosphonothioate monoesters containing the base labile 9-fluorenemethyl protecting group was key to the synthesis of both phosphopeptide mimetics. *O*-Boranophosphopeptides were synthesized by condensing *O*-(9-fluorenemethyl)-*H*-phosphonate to the peptide hydroxylic component (tyr, ser, or thr) followed by oxidation with borane complexes. Similarly, the synthesis of *O*-dithiophosphopeptides used the *O*-(9-fluorenemethyl)-*H*-phosphonothioate synthon and oxidation with elemental sulfur. Base elimination of the Fmol protecting group and cleavage from the solid support with concentrated ammonium hydroxide afforded the boranophosphopeptide and dithiophosphopeptide target compounds. Ac-YIIPLPG-NH_2, having either dithiophosphoryl tyrosine or boranophosphoryltyrosine but no sequence specificity for *Yersinia* protein tyrosine phosphatase (PTP), was found to competitively inhibit this enzyme with K_I values of 430 $\pm$ 50 and 670 $\pm$ 50 μM, respectively. In addition, both phosphopeptide analogues were resistant toward *Yersinia* PTP enzymatic hydrolysis. Under conditions (pH 8.0) where the phosphopeptide was rapidly dephosphorylated, the boranophosphopeptide hydrolyzed slowly ($t_{1/2}$ = 15 h) and the dithiophosphopeptide was completely stable over 24 h.

Introduction

Phosphorylation and dephosphorylation of specific tyrosine, serine, and threonine residues is the basis for a variety of signaling events which control important cellular processes such as growth and differentiation. Phosphorylation is catalyzed by kinases while the reverse reaction, hydrolysis, is performed by phosphatases. These enzymes act in highly coordinated signal transduction pathways producing a cascade of phosphorylation/dephosphorylation events.[2] The complexity of these processes has created a need for phosphopeptides that can be used to study specific steps along signal transduction pathways. Although these peptides have proven to be useful in characterizing signal protein interactions in vitro,[3] they are of limited use as research tools in cell free systems and in vivo since they are quickly hydrolyzed by cellular phosphatases.[4]

To overcome this problem, phosphopeptide analogues have been prepared and found to indeed be resistant toward hydrolysis by protein phosphatases and to competitively inhibit these enzymes. Additionally, signaling proteins that interact with unique phosphoprotein seqeuences have been studied by using phosphopeptide mimetics. These include the 14-3-3 signaling proteins that recognize phosphoserine sites,[5] and proteins having SH2 domains which bind to phosphorylated tyrosine residues.[6] Among the more useful phosphopeptide mimetics are those containing difluorophosphonomethylphenylalanine and phosphonomethylphenylalanine which competitively inhibit phosphatases[7,8] and specifically bind SH2 domains in vitro[9] and in vivo.[10] Another very useful mimetic is the *O*-thiophosphotyrosyl

* Corresponding author. E-mail: Marvin.Caruthers@Colorado.EDU.
† Current address: 1294 Vallejo Street #2, San Francisco, CA 90501.
‡ Current address: Department of Chemistry, University of Dundee, Dundee DD1 4HN, United Kingdom.
§ Current address: Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA 02139.

(1) This research was supported by the National Institutes of Health (Grant GM25680). Abbreviations: Fmol, 9-fluorenemethyl; BSTFA, bis(trimethylsilyl)trifluoroacetamide; Fmoc, fluorenemethoxycarbonyl; Boc, *tert*-butyloxycarbonyl; PS-PEG, polystyrene-poly(ethylene glycol); HMB, hydroxymethylbenzoate; DPCP, diphenylchlorophosphate; Piv-Cl, pivaloyl chloride; TEA, triethylamine; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; ESI-MS, electrospray ionization mass spectrometry; Trt, trityl; Mtt, monomethoxytrityl; DCC, dicyclohexylcarbodiimide; HOBt, hydroxybenzotriazole; pNPP, *p*-nitrophenyl phosphate; HMDST, hexamethyldisilathiane; DIEA, diisopropylethylamine; DMAP, *N*,*N*-(dimethylamino)pyridine.

(2) Hunter, T. *Cell* **1995**, *80*, 225−236.
(3) Pinna, L. A.; Donella-Deana, A. *Biochim. Biophys. Acta* **1994**, *1222*, 415−431.
(4) Domchek, S. M.; Auger, K. R.; Chatterjee, S.; Burke, T. R., Jr.; Shoelson, S. E. *Biochemistry* **1992**, *31*, 9865−9870.
(5) Muslin, A. J.; Tanner, J. W.; Allen, P. M.; Shaw, A. S. *Cell* **1996**, *84*, 889−897.
(6) Songyang, Z.; Shoelson, S. E.; Chauhuri, M.; Gish, G.; Pawson, T.; Haser, W. G.; King, F.; Roberts T.; Ratnofsky, S.; Lechleider, R. J.; Neel, B.; Birge, R.; Fajardo, J.; Chou, M.; Hanufusa, H.; Schauffhausen, B.; Carnley, L. *Cell* **1993**, *72*, 767−778.

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derivative that competitively inhibits protein phosphatases with IC_{50} values (the inhibitor concentration required to reduce catalysis by 50%) in the 2−15 μM range.[11−13]

To increase the diversity of useful phosphopeptide analogues and perhaps generate derivatives with unique biochemical properties, methods were developed for synthesizing peptides having dithiophosphate and boranophosphate moieties linked to serine, threonine, and tyrosine. The potential utility of this work was based upon earlier investigations of these analogues in the nucleic acid field. For example, phosphorodithioate diester linked oligonucleotides are completely stable to degradation by nucleases[14,15] and exhibit several useful biochemical properties such as activation of RNase H,[15] inhibition of the erb-B2 tyrosine kinase receptor through an antisense mechanism,[16] and inhibition of HIV-1 Reverse Transcriptase.[17] More recently, phosphorodithioate monoesters of nucleotides and oligonucleotides have been prepared and shown to be completely resistant to degradation by phosphatases.[18,19] In a similar manner, boranophosphate linked oligonucleotides have been shown to be resistant to nucleases[20,21] and also activate RNase H,[20,22] which suggests that these compounds are excellent mimetics for natural oligonucleotides. This paper outlines the synthesis of dithiophosphoryl and boranophosphoryl peptides and reports initial studies on their biochemical activity. A preliminary communication of our earliest work in this area has been published.[23]

Results and Discussion

Synthetic Strategy. Briefly, dithiophosphoryl and boranophosphoryl peptides were synthesized by condensing appropriately protected *H*-phosphonothioate or *H*-phosphonate synthons, respectively, to a presynthesized, support-linked peptide. A key element of this strategy was to use a novel, mildly base labile 9-fluorenemethyl (Fmol) protecting group in combination with *H*-phosphonate and *H*-phosphonothioate chemistries for preparing these analogues. After oxidation with sulfur or a borane complex followed by cleavage from the support, the phosphopeptide was isolated via HPLC and used for various biochemical studies.

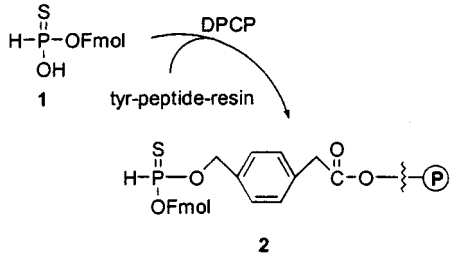
The heptapeptide Ac-Tyr(OH)-Ile-Ile-Pro-Leu-Pro-Gly-NH_2 (Ac-YIIPLPG-NH_2), which resembles the SH2-phospholipase Cγ1 binding region of the platelet derived growth factor receptor,[24] was chosen as the initial target for synthesis of the *O*-boranophosphate and *O*-dithiophosphate analogues. In addition the serine (SIIPLPG) and threonine (TIIPLPG) *O*-boranophosphate and *O*-dithiophosphate containing derivatives were also synthesized as it was important to develop a chemistry that could be used with all three hydroxylic amino acids. This model sequence does not contain any reactive side chains other than the hydroxylic amino acid. Inclusion of other reactive side chains would necessitate orthogonal protection strategies, which was an issue addressed only after the chemistry had been established. Another consideration was the overall strategy of phosphopeptide assembly, which can be carried out by either incorporating a phosphorylated amino acid into the growing peptide chain (the building block approach) or phosphorylating the peptide hydroxyl component after completion of peptide synthesis (global phosphorylation). Because the Fmol group is unstable to the acidic and basic conditions commonly used for either the *tert*-butyloxycarbonyl (Boc) or the fluorenemethoxy carbonyl (Fmoc) peptide synthesis strategies, the building block approach did not appear as a reasonable alternative. As a consequence of these considerations, the peptide containing the appropriate hydroxyl amino acid was first synthesized on the polymer support and then the *H*-phosphonate synthon introduced as the final step. This strategy proved to be quite versatile as it was used for introducing both the dithiophosphate and boranophosphate moieties to peptides containing threonine, serine, and tyrosine. This approach also appears to be compatible with several, if not all, side-chain protected amino acids.

During the development of this synthesis strategy, the choice of a polymer support had to be addressed as standard acid labile resin linkages could not be used with dithiophosphopeptides. This was because previous work[18,19] had shown that dithiophosphates were unstable to the acidic conditions required for cleavage of peptides from these supports. Instead, peptide assembly was carried out by using Fmoc chemistry on resins containing polystyrene-poly(ethylene glycol) (PS-PEG) copolymers having a base-labile hydroxymethyl benzoate (HMB) linker. The final phosphorylated peptide could then be cleaved from the support with ammonium hydroxide—conditions that also remove the Fmol phosphorus protecting group but do not cause peptide racemization as does strong alkali. With ammonium hydroxide, the predominant product is the peptide amide. This derivative is attractive since it is stable toward carboxypeptidases[25] and is a more logical mimic of biochemically active peptides as it does not have terminal carboxylic acid functionality.

Synthesis of *O*-Dithiophosphopeptides. Our initial target was the dithiophosphoryl derivative of Ac-Tyr(OH)-Ile-Ile-Pro-Leu-Pro-Gly attached to a PS-PEG resin via the HMB linker. This peptide was synthesized by using Fmoc chemistry and *tert*-butyl side chain protected tyrosine. To monitor the phosphityl-

(7) Burke, T. R.; Kole H. K., Jr.; Roller, P. P. *Biochem. Biophys. Res. Commun*. **1994**, *1*, 129−134.
(8) Chatterjee, S.; Goldstein, B. J.; Csermely, P.; Shoelson, S. E. In *Peptides: Chemistry and Biology*; Rivier, J. E., Smith, J. A., Eds.; Leiden: The Netherlands, 1992; p 553.
(9) Burke, T. R.; Smyth M. S., Jr.; Otaka, A.; Nomizo M.; Roller P. P.; Wolf, G.; Case, R.; Shoelson, S. E. *Biochemistry* **1994**, *33*, 6490−6494.
(10) Chuang, L.-M.; Myers, M. G., Jr.; Backer, J. M.; Shoelson, S. E.; White, M. F.; Birnbaum, M. J.; Kahn, C. R. *Mol. Cell. Biol*. **1993**, *13*, 6653−6660.
(11) de Bont, D. B.; Moree, W. J.; van Boom, J. H.; Liskamp, R. M. *J. Org. Chem.* **1993**, *58*, 1309−1317.
(12) Cho, H.; Krishnaraj, R.; Itoh, M.; Kitas, E.; Bannwarth, W.; Saito, H.; Walsh, C. T. *Protein Sci.* **1993**, *2*, 977−984.
(13) Hiriyana, K. T.; Baedke, D.; Baek, K.-H.; Forney, B. A.; Kordiyak, G.; Ingebritsen, T. S. *Anal. Biochem.* **1994**, *223*, 51−55.
(14) Wiesler, W. T.; Caruthers, M. H. *J. Org. Chem.* **1996**, *61*, 4272−4281.
(15) Cummins, L.; Graff, D.; Beaton, G.; Marshall, W. S.; Caruthers, M. H. *Biochemistry* **1996**, *30*, 8734−8741.
(16) Vaughn, J. P.; Stekler, J.; Demirdji, S.; Mills, J. K.; Caruthers, M. H.; Iglehart, J. D.; Marks, J. R. *Nucleic Acids Res.* **1996**, *24*, 4558−4564.
(17) Marshall, W. S.; Caruthers, M. H. *Science* **1993**, *259*, 1564−1570.
(18) Seeberger, P. H.; Jorgensen, P. N.; Bankaitis-Davis, D. M.; Beaton, G.; Caruthers, M. H. *J. Am. Chem. Soc.* **1996**, *118*, 9562−9566.
(19) Seeberger, P. H.; Yau, E.; Caruthers, M. H. *J. Am. Chem. Soc.* **1995**, *117*, 1472−1478.
(20) Higson, A. P.; Sierzchala, A.; Brummel, H.; Zhao, Z.; Caruthers, M. H. *Tetrahedron Lett.* **1998**, *39*, 3899−3903.
(21) Sood, A.; Spielvogel, B. F.; Shaw, B. R. *J. Am. Chem. Soc.* **1989**, *111*, 9234−9235.
(22) Sergueev, D. S.; Shaw, B. R. *J. Am. Chem. Soc*. **1998**, *120*, 9417−9427.
(23) Seeberger, P. H.; Jordan, J. L.; Caruthers, M. H.; Bankaitis-Davis, D. M.; Heil, J. R.; Munson, M. C.; Beaton, J. In *Innovation and Perspectives in Solid-Phase Synthesis and Combinatorial Libraries*; Epton, R., Ed.; Mayflower Worldwide Limited: Birmingham, UK, 1996; pp 141−146.
(24) Larose, L.; Gish, G.; Pawson, T. *J. Biol. Chem.* **1995**, *270*, 3858−3862.
(25) O'Cuinn, G.; O'Conner, B.; Gilmartin, L.; Smyth, M. In *Metabolism of Brain Peptides*; O'Cuinn, G., Ed.; CTC Press: New York, 1995; p 100.

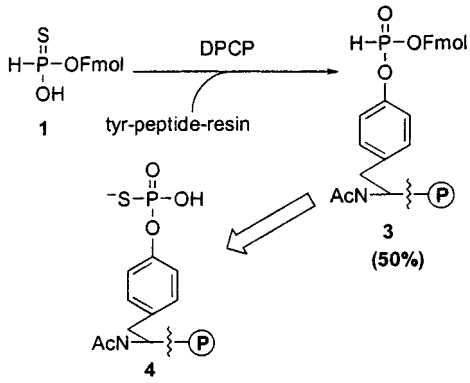
Scheme 1



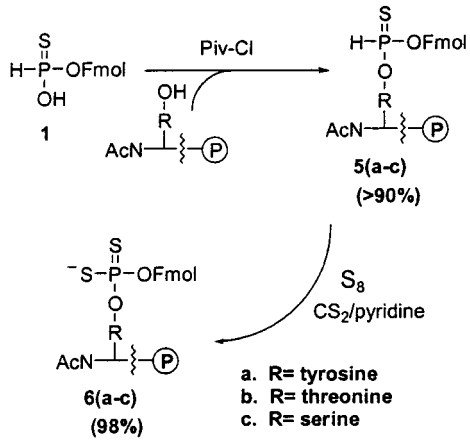
ation reaction, a ^{31}P NMR procedure was adapted for detection of phosphorus nuclei on a solid support. This procedure had been used previously during the solid-phase synthesis of phosphorodithioate RNA on a polystyrene resin and was called ^{31}P gel phase NMR.[26] The method was based upon the concept that the phosphorus nucleus relaxed faster when attached to the polystyrene resin than when in solution, which allowed acquisition times to be decreased (see procedures). Thus all phosphorus reaction steps performed on a solid support were monitored by ^{31}P NMR. Initial attempts to condense the Fmol-H-phosphonothioate **1** to the tyrosyl hydroxyl (Scheme 1) resulted in phosphitylation of resin-HMB to yield **2** as judged by gel phase ^{31}P NMR ($\delta = 70.8$). To avoid condensation of **1** with the HMB component of the resin, a capping procedure was manually performed with acetic anhydride after completion of peptide synthesis and prior to removal of the *tert*-butyl protecting group from the tyrosine, serine, or threonine side chains. Subsequent condensation with **1** resulted in no resin phosphorylation as measured by ^{31}P gel phase NMR. This acetylation procedure also furnished *N*-acetylated peptides, which show increased stability toward aminopeptidases.[27,28]

Diphenylchlorophosphate (DPCP) was our initial choice as the condensing agent since chlorophosphates have low reactivity toward sulfur nucleophiles[29] and have been used successfully to activate *H*-phosphonothioate monoesters.[18,30,31] On the basis of results from the solid-phase synthesis of 5′-dithiophosphoryl deoxyoligonucleotides,[18] the DPCP-mediated condensation of the Fmol-*H*-phosphonothioate **1** with the peptide-resin was expected to produce small amounts of a desulfurized *H*-phosphonate diester ($\delta = 4.5$) side product **3** due to sulfur activation by DPCP (Scheme 2) in addition to the *H*-phosphonothioate product ($\delta = 68.3$). Unfortunately, condensation of **1** to the tyrosylpeptide-resin with equimolar amounts of DPCP and **1** produced the side product in 50% yield as judged by gel phase ^{31}P NMR spectroscopy. This Fmol-H-phosphonate diester side product would ultimately result in the formation of the thiophosphotyrosylpeptide **4** if the chemistry were carried through to its conclusion. During the synthesis of dithiophosphate RNA, the amount of sulfur activation was reduced by using a slight excess of the *H*-phosphonothioate monoester over DPCP.[31] The same strategy was attempted for the synthesis of dithiophosphopeptides. The ratio of **1** to DPCP was increased from 1:1 to 2:1, resulting in a decrease of undesired **3** from 50% to 18%. To further decrease the formation of **3**, other condensing agents were tested. For this survey, we used a single tyrosine amino acid linked to the PS-PEG resin instead of the usual peptide sequence. Pivaloyl chloride reacted less with the sulfur of **1** than DPCP to yield only 6% of the *H*-phosphonate diester as judged by ^{31}P gel phase NMR. *p*-Toluenesulfonyl chloride activation produced 100% desulfurized side product. The explanation for the variation in product/side product ratio is not readily apparent. However, it is known that the arenesulfonyl chlorides and diphenylchlorophosphate react with *H*-phosphonate monoesters to form trimetaphosphate intermediates.[32] Perhaps the formation of an extremely reactive trimetaphosphate, even in small amounts, leads to preferential consumption of tyrosine to yield the *H*-phosphonate diester before the less reactive, activated **1** can form product. On the basis of these studies, pivaloyl chloride was determined to be the condensing agent of choice. In addition, the molar ratio of **1** to pivaloyl chloride was determined to be optimal at 3:2. These reaction conditions translated well to the tyrosine (YIIPLPG), serine (SIIPLPG), and threonine (TIIPLPG) containing peptides (Scheme 3) and generated the *H*-phosphonothioate diesters **5a**−**c** with yields consistently above 90% (relative to the *H*-phosphonate diesters) as determined by ^{31}P gel phase NMR. The diester

Scheme 2



Scheme 3



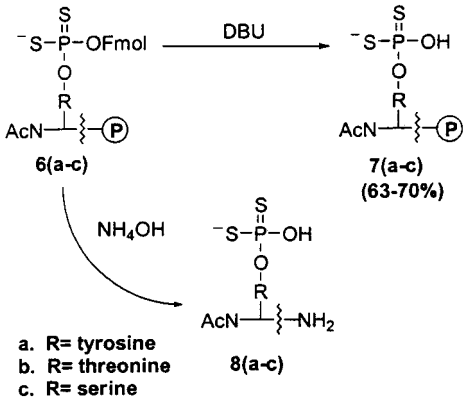
(26) Greef, C. H.; Seeberger, P. H.; Caruthers, M. H.; Beaton, G.; Bankaitis-Davis, D. *Tetrahedron Lett.* **1996**, *37*, 4451−4454.
(27) Wallace, R. *Br. J. Nutr.* **1992**, *68*, 365−372.
(28) Theoretically blocking of excess hydroxymethyl groups was not critical to phosphopeptide synthesis as the conditions used to remove the final product from the support did not hydrolyze phosphorylated resin. However, blocking these groups was advantageous for several reasons. These include the following: (1) ^{31}P NMR gel phase analysis would then reflect only phosphopeptide products. (2) Lower excesses of phosphitylating reagents could be used. (3) Blocking excess hydroxymethyl groups early during peptide synthesis decreased the amount of failure sequences contaminating the final product. Acylation can be carried out at any peptide synthesis step prior to removal of the hydroxyl amino acid protecting group.
(29) Dostrovsky, I.; Halmann, M. *J. Chem. Soc.* **1953**, 502−507.
(30) Zain, R.; Stawinski, J. *J. Org. Chem.* **1996**, *61*, 6617−6622.
(31) Greef, C. Synthesis and Biochemical Chracterization of Dithiophosphate RNA. Ph.D. Thesis, University of Colorado, Boulder, 1997.
(32) Stawinski, J. In *Handbook of Organophosphorus Chemistry*; Engel, R., Ed.; Marcel Dekker: New York, 1992; pp 387−388.

Table 1. *O*-Dithiophosphopeptide and *O*-Boranophosphopeptide Characterization Data

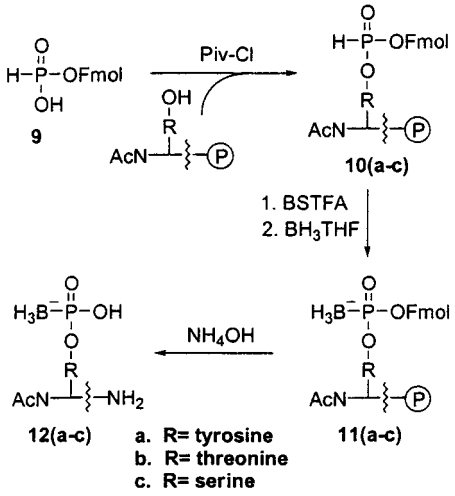
phosphopeptide	yield,[a] %	amino acid anal.	^{31}P NMR,[b] ppm	^{11}B NMR,[c] ppm	ESI-MS neg. ion
Ac-Y(POS_2)IIPLPG-NH_2	56.1	Y (1) 0.9; I (2) 2.2; P (2) 2.0; L (1) 1.1; G (1) 1.1	87.3		923.3
Ac-T(POS_2)IIPLPG-NH_2	49.2	T (1) 1.0; I (2) 1.7; P (2) 2.2; L (1) 0.9; G (1) 1.1	89.0		861
Ac-S(POS_2)IIPLPG-NH_2	42.4	S (1) 1.1; I (2) 1.5; P (2) 2.0; L (1) 0.9; G (1) 1.3	89.3		847
Ac-Y(PO_2BH_3)IIPLPG-NH_2	41.2	Y (1) 0.9; I (2) 1.2; P (2) 2.1; L (1) 1.5; G (1) 1.3	77.3, 76.3, 75.3, 74.3	−36.2, −37.8	889.2
Ac-T(PO_2BH_3)IIPLPG-NH_2	44.2	T (1) 0.9; I (2) 1.8; P (2) 2.2; L (1) 0.9; G (1) 1.2	80.3, 79.3, 78.3, 77.3	−36.3, −38.1	827.3
Ac-S(PO_2BH_3)IIPLPG-NH_2	42.0	S (1) 0.9; I (2) 1.6; P (2) 2.3; L (1) 1.2; G (1) 1.5	80.6, 79.5, 78.5, 77.5	−36.6, −38.4	813.2
Ac-Y(PO_2S)IIPLPG-NH_2			40.5		907
Ac-Y(PO_3)IIPLPG-NH_2			−0.2		891
Ac-GNDY(POS_2)IIPLPG-NH_2			87.0		1054

[a] Yields were determined from two 0.11 g samples of peptidyl-resin. One sample was converted to the phosphorylated analogue and isolated by column chromatography. The second sample was not phosphorylated but the peptide was isolated by the identical purification procedure. The absolute yields of both samples were determined by amino acid analysis. The yield was the amount of phosphorylated peptide divided by unphosphorylated peptide. [b] Reference standard, H_3PO_4. [c] Reference standard, $Et_2O{\cdot}BF_3$.

Scheme 4



Scheme 5



H-phosphonothioate peptides were sulfurized in a solution of carbon disulfide and pyridine to form the dithiophosphate diester products with conversions of **5a**−**c** to **6a**−**c** being in excess of 98% as judged by gel phase ^{31}P NMR spectroscopy.

Initial experiments on the removal of the Fmol group from **6a**−**c** (Scheme 4) focused on using the nonnucleophilic base 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in various solvents (dichloromethane, acetonitrile, *N*,*N*-dimethylformamide). Monitoring this reaction over time by ^{31}P gel phase NMR revealed similar results with all three peptides. As the protected dithiophosphate diester converts to the dithiophosphate monoester, a side product forms ($\delta = 65$) at the expense of product. The maximum yield never exceeds 65−70% and drops rapidly upon further treatment with DBU. After 25 min the side-product predominates (80%) with only 20% product remaining. Piperidine in the same solvents instead of DBU generated similar results.

In light of these results, DBU deprotection proved to be unsatisfactory but further work revealed that concentrated ammonium hydroxide could be used to quantitatively remove the Fmol protecting group and cleave the peptides from the resin (Scheme 4). With use of this procedure, the crude, unpurified products were obtained in yields of 85% (**8a**), 92% (**8b**), and 88% (**8c**) as measured by ^{31}P NMR (i.e. relative yields compared to all phosphopeptide products detected). However, analytical reverse-phase HPLC and electrospray-ionization mass spectrometry (ESI-MS) revealed that the crude product mixtures also contained both the C-terminal amide and carboxylic acid peptides. The major products, the C-terminal amides, were easily separated from the C-terminal carboxylic acid peptides by preparative reverse-phase HPLC. Further purification by preparative ion exchange HPLC removed contaminating monothiophosphorylpeptide side products and generated dithiophosphoryl tyrosine, threonine, and serine peptides **8a**−**c**. Characterization data for these dithiophosphoryl peptides are reported in Table 1. Attempts to obtain only the peptide C-terminal amide with liquid ammonia or ammonia in methanol reduced but did not eliminate C-terminal carboxylic acid peptides and decreased the overall yields of **8a**−**c**.

Synthesis of *O*-Boranophosphopeptides. In a manner similar to the synthesis of **8a**−**c**, the preparation of the corresponding *O*-boranophosphopeptides linked to PS-PEG begins with Fmoc-protected amino acid synthons and *tert*-butyl side chain protected serine, threonine, and tyrosine. Following acidic removal of the *tert*-butyl blocking group, condensation of Fmol-*H*-phosphonate **9** with each peptide was completed with use of pivaloyl chloride as an activating agent (Scheme 5) to yield **10a**−**c**. Boronation was then carried out by first generating the silyl phosphite with BSTFA[20] and the product reacted with $BH_3{\cdot}$THF to produce the boranophosphate diester **11a**−**c**. Peptides **12a**−**c** were then generated by treatment with concentrated ammonium hydroxide. However, reduction of peptide amide linkages via the boronation procedure was detected by electrospray ionization mass spectrometry where peaks at multiples of −14 AMU were observed for both the boranophosphopeptide and the unphosphorylated peptide. During synthesis of boranophosphate DNA, it was found that the reducing activity of borane could be minimized by using a borane diisopropylethylamine (DIPEA) complex.[20] Unfortunately, the inclusion of DIPEA failed to produce **12a**

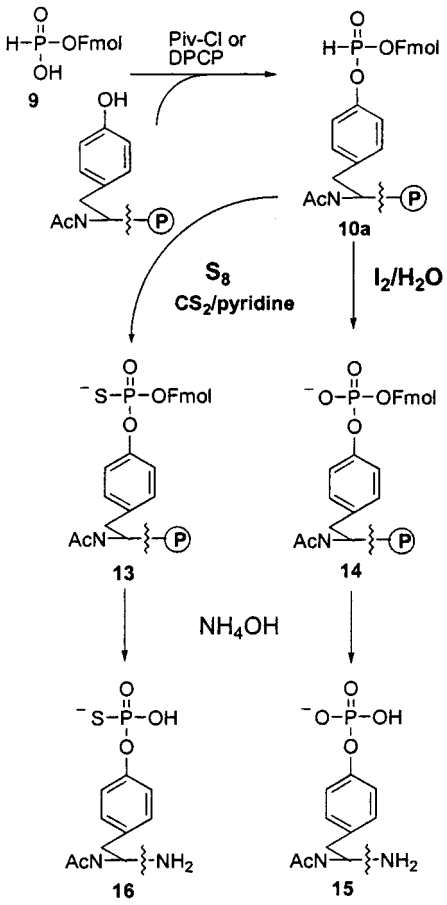
in a significant yield, probably due to side reactions with the base-sensitive Fmol ester. After further investigation, it was found that decreasing the excess BH_3·THF (45- to 15-fold over peptide) minimized carbonyl reduction. By using these optimized boronation conditions, essentially no peptide backbone reduction was observed (ESI-MS, reverse-phase HPLC) under conditions where 72% boranophosphopeptide, 7% *H*-phosphonyl peptide, and 20% phosphorylpeptide were present (^{31}P NMR) in the unpurified reaction mixture. Similar results were obtained for **12b** and **12c**. Fractionation of these reaction mixtures by reverse-phase HPLC led to pure C-terminal amide peptides **12a**−**c** as the major products free of various backbone amide reduced side products, the C-terminal carboxylic acid peptides, and unphosphorylated peptides. These boranophosphopeptides were stable for several days at room temperature in 20% acetonitrile and 80% 25 mM Tris·HCl, pH 8.0 (HPLC buffer), or for several weeks in the same buffer at 0 °C. Characterization data for **12a**−**c** are reported in Table 1.

Analysis of **12a**−**c** reaction mixtures by ^{11}B NMR showed a side product at 19 ppm, a chemical shift in the range usually attributed to boric acid or boric acid esters. Treatment of acetylated PS-PEG resin with BH_3·THF in the absence of any synthetic peptide followed by ammonium hydroxide also produced the same side product. This result indicated that excess borane perhaps formed a complex with the resin and further reacted to yield boric acid or a boric acid ester.[33] This result is supported by the observation that extensive washes with various solvents, including several that complex with borane, and mild acid failed to remove the boric acid type product. However, this boric acid derivative did not co-purify with **12a**−**c** by HPLC and therefore failed to impact synthesis procedures in any significant manner.

Phosphopeptide and Monothiophosphopeptide Synthesis. Compound **10a** was also used as an intermediate for synthesizing either the phosphotyrosylpeptide **15** or monothiophosphotyrosylpeptide **16** (Scheme 6). Using iodine in a tetrahydrofuran/pyridine/water mixture (78:20:2), an *H*-phosphonate diester tyrosylpeptide **10a** was oxidized to the tyrosyl phosphopeptide diester **14** followed by deprotection of the Fmol group and cleavage from the resin with concentrated NH_4OH to form **15**. The Fmol-protected thiophosphotyrosylpeptide **13** was obtained from **10a** by oxidation with sulfur. Simultaneously removing the Fmol group and cleaving the peptide with NH_4OH generated the thiophosphotyrosylpeptide **16** as the major product. Purification of both **15** and **16** was completed by reverse-phase HPLC, which successfully fractionated the products from various impurities such as the C-terminal carboxylic acid phosphopeptides and unphosphorylated peptides. Characterization data are summarized in Table 1.

Amino Acid Sequence Variability. Our initial phosphopeptide analogue investigations, which utilized the sequence Ac-X-Ile-Ile-Pro-Leu-Pro-Gly-NH_2 where X is serine, threonine, or tyrosine, did not contain any reactive amino acid side chains other than X as the focus was to develop the chemistry for synthesizing phosphopeptides. To study the sequence versatility of this chemistry, a new target peptide, Ac-Gly-Asn-Asp-Tyr-Ile-Ile-Pro-Leu-NH_2, was selected for exploring whether mild

Scheme 6



acid labile side chain protecting groups were compatible with these phosphorylation procedures.[34,35] Many of the blocking groups in this category are labile to 95% TFA over 1−2 h, but from previous studies, these conditions appeared too harsh for the dithiophosphoryl moiety.[18] Indeed, this proved to be the case as ^{31}P NMR studies revealed 100% degradation of dithiophosphoryl peptides within minutes with 95% TFA. There are, however, many acid labile groups that can be removed with 1−2% TFA. These include side chains protected with trityl (Trt: tyrosine, serine, and threonine), monomethoxy trityl (Mtt: lysine, cysteine, asparagine, glutamine), and 2-phenylisopropyl ester (aspartic acid and glutamic acid). Initial work with asn(Mtt) and asp(2-phenylisopropyl ester) led to the following conclusions. (1) ESI-MS revealed that treatment of the peptidyl-resin with 1% TFA failed to remove the asn(Mtt) group but was sufficient to completely hydrolyze the 2-phenylisopropyl ester. (2) As measured by ^{31}P gel phase NMR, unprotected asparagine was compatible with the procedures used to generate dithiophosphoryl and boranophosphoryl peptides. In contrast, when peptides containing unprotected aspartic and glutamic acid were tested with the *H*-phosphonothioate and *H*-phosphonate synthons, ^{31}P NMR analysis showed very low yields of final product contaminated with several uncharacterized side products.

(33) Perhaps the presence of these reactive sites on PS-PEG explains why a 15-fold excess of THF·BH_3 was required to carry out the boronation with minimum backbone reduction of the peptide.

(34) Gross, E.; Meienhofer, J. *The Peptides: Analysis, Synthesis, Biology*; Vol. 3, Protection of Functional Groups in Peptide Synthesis; Academic Press: New York, 1987.

(35) Base labile side chain protecting groups were unacceptable as the 20% piperidine solution used in conjunction with the Fmoc peptide synthesis strategy would remove these blocking groups. Fmoc chemistry was the method of choice for peptide synthesis as Boc chemistry requires strong acid conditions for removal of peptides from standard resins. These conditions would desulfurize dithiophosphoryl peptides.

These results encouraged us to use asparagine without protection but to incorporate the 2-phenylisopropyl ester as a protecting group for aspartic acid. (3) Treatment of tyrosine dithiophosphate diester **6a** with 1% TFA for 30 min led to complete degradation (^{31}P NMR) but the tyrosine *H*-phosphonothioate (**5a**), *H*-phosphonate (**10a**), and boranophosphate (**12a**) peptide derivatives were stable to 1% TFA under these conditions. Because of this observation, acid labile side chain protecting groups were removed prior to oxidation of the *H*-phosphonothioate or *H*-phosphonate peptides.

To complete this strategy, two additional problems had to be addressed. For the synthesis of **8a**−**c** and **12a**−**c**, hydroxymethyl benzoate functional groups on the resin were blocked by acetylation following peptide synthesis but prior to acidic removal of the *tert*-butyl protecting group from the side chains of tyrosine, threonine, or serine. This was necessary to prevent unwanted phosphorylation of the support during conversion to the phosphopeptide. When we choose to incorporate mild acid labile protecting groups on side chains, then the serine, tyrosine, or threonine amino acids that are to be phosphorylated must be added unprotected as acidic reagents cannot be used to remove their *tert*-butyl blocking groups. Therefore the support was blocked following synthesis of Fmoc-Ile-Ile-Pro-Leu-resin with benzoic anhydride and (dimethylamino)pyridine.[36] The Fmoc group was then removed and synthesis continued. As a final step in the synthesis cycle, *N*-acetylglycine was added to the *N*-terminus of the peptide prior to phosphorylation. This was important as peptides with an Fmoc amino terminus were labile to the basic conditions used for phosphorylation. This lability leads to phosphorylation of not only the appropriate hydroxylic amino acid but also the amino terminus, which generates uncharacterized side products (presumably phosphoramidate or thiophosphoramidate derivatives). This problem was solved by adding *N*-acetylglycine at the amino terminus of the peptide as this acyl group is stable to the mildly basic phosphorylation conditions.[37]

As a result of these considerations, the following strategy was adapted for the synthesis of the dithiophosphoryl derivative of this peptide. (1) Add Fmoc amino acids stepwise to the resin, using standard DCC/HOBt coupling chemistry. These include side chain unprotected tyrosine and asparagine as well as the side chain protected 2-phenylisopropyl ester of aspartic acid. (2) Cap the resin with benzoic anhydride and (dimethylamino)-pyridine prior to addition of unprotected tyrosine. (3) Add *N*-acetylglycine at the amino terminus. (4) Phosphorylate the tyrosyl residue with the *H*-phosphonothioate synthon with use of pivaloyl chloride as activator. (5) Remove acid-labile amino acid side chain protecting groups with 1% TFA. (6) Oxidize the *H*-phosphonothioate peptide with sulfur to generate the dithiophosphopeptide. (7) Cleave the peptide from the support with concentrated ammonium hydroxide, which generates primarily carboxamide peptides. Partial purification of the reaction mixture from this synthesis by reverse-phase HPLC

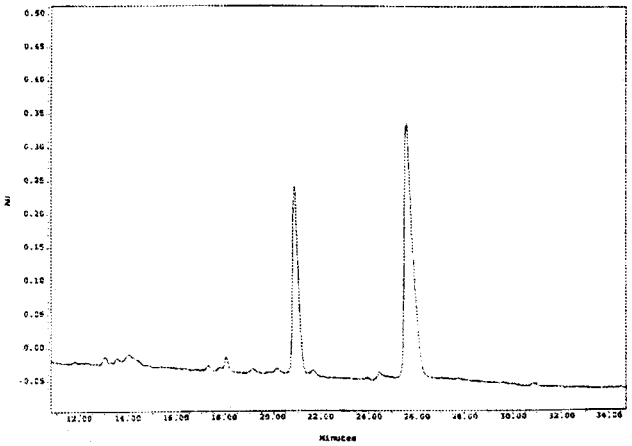


Figure 1. Reverse phase HPLC of the reaction mixture obtained from preparing the dithiophosphoryl derivative of Ac-GNDYIIPL-NH_2: Ac-GNDY(POS_2)IIPL-NH_2 (21 min); Ac-GNDYIIPL-NH_2 (26 min).

(Figure 1) indicated that the overall yield of dithiophosphoryl peptide was low (40%). However, the synthesis strategy was sound as very few detectable side products were present other than unphosphorylated peptide. As the dithiophosphorylpeptide was not purified further by ion exchange chromatography to remove the thiophosphoryl derivative, this side product was present as expected (23%, ^{31}P NMR) in the isolated product. Characterization data are summarized in Table 1.

Inhibition and Stability Studies. *Yersinia enterocolitica* Protein Tyrosine Phosphatase (*Yersinia* PTP) was selected as a model to study both the stability of these phosphopeptide substrates toward hydrolysis and their ability to inhibit the phosphatase activity of this type of enzyme. This was because *Yersinia* PTP has been kinetically characterized with a variety of phosphopeptide sequences and its substrate specificity has been determined.[38] On the basis of these studies, it is known that this enzyme requires acidic amino acid residues *N*-terminal to the phosphorylated tyrosine to maximize binding affinity. This work also established that an *N*-terminal acyl group and a *C*-terminal amide did not alter the binding affinity of a peptide with *Yersinia* PTP. As a result of these observations and because the focus of this work was to examine the effect of the phosphate analogue moieties on enzyme−substrate interactions without complications from peptide side chain binding energies, we chose to use phosphopeptide analogues of Ac-YIIPLPG-NH_2 as inhibitors where side chain effects could be minimized. As a substrate for the inhibition studies, *p*-nitrophenyl phosphate (pNPP) was used primarily because its hydrolysis rate with *Yersinia* PTP has been kinetically determined and one of the reaction products, *p*-nitrophenolate, can readily be measured at 405 nm.[38]

Inhibition experiments were performed with *Yersinia* PTP, using pNPP as substrate and phosphopeptide analogues of (Ac-YIIPLPG-NH_2) as inhibitors (boranophosphopeptide, dithiophosphopeptide, and monothiophosphopeptide). Substrate concentrations varied from 0.2 to 0.8 mM. Each substrate concentration was tested at three inhibitor concentrations. The monothiophosphopeptide was the least effective inhibitor (K_I = 2.31 mM) but still 15 times more so than Ac-YIIPLPG-NH_2, the unphosphorylated peptide (K_I = 35 mM). The boranophos-

(36) Capping the support can be completed at any step prior to condensation of the amino acid that is to be phosphorylated (ser, tyr, thr). This includes blocking after addition of the first Fmoc amino acid. Additionally, both benzoic anhydride and acetic anhydride were used successfully for this purpose.

(37) Any *N*-acyl amino acid could be used in place of *N*-acetylglycine. Recall that the acetyl groups on Ac-YIIPLPG-NH_2, Ac-TIIPLPG-NH_2, and Ac-SIIPLPG-NH_2 were added following peptide synthesis but prior to removal of the acid-labile *tert*-butyl protecting group on tyrosine, threonine, and serine.

(38) Zhang, Z.-Y.; Maclean, D.; McNamara, D. J.; Sawyer, T. K.; Dixon, J. E. *Biochemistry* **1994**, *33*, 2285−2290.

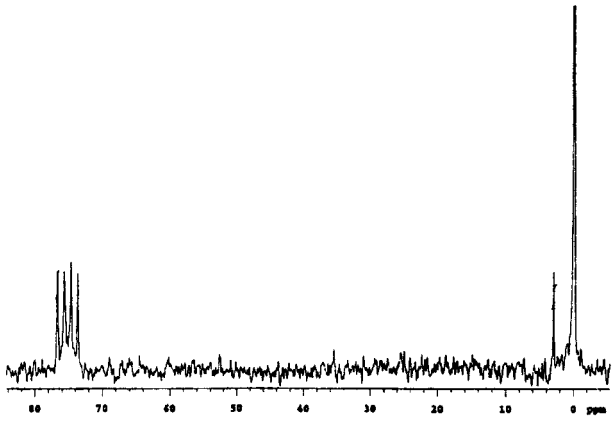


Figure 2. A study of *Yersinia* PTP phosphatase activity with the boranophosphoryl derivative of Ac-YIIPLPG-NH_2. The ^{31}P NMR of the boranophosphoryl peptide was recorded 3 h after addition of enzyme: boranophosphoryl peptide (δ = 76.8, 75.8, 74.8, 73.8; 89%), hydrolyzed boranophosphate (δ = 2.9, 11%), and reference phosphoric acid (δ = 0.0).

phopeptide was slightly less effective (K_I = 670 μM) than the dithiophosphopeptide (K_I = 430 μM) although both were better inhibitors than the monothiophosphopeptide (K_I = 2.31 mM). The best inhibitor under these conditions was the dithiophosphopeptide, which was over five times more effective than the corresponding monothiophosphopeptide. Although the sequence Ac-Y(PO_3)IIPLPG-NH_2 was not tested in this study, a similar sequence, Y(PO_3)LIPQQG, had a K_M of 2200 $\pm$ 240 μM.[38] The same study showed that the K_M of phosphotyrosine was 7080 $\pm$ 1405 μM with *Yersinia* PTP.

Phosphopeptide stability studies with *Yersinia* PTP were followed by ^{31}P NMR. In a 50 mM Tris buffer (pH 8.0) the control phosphopeptide having the sequence Ac-Y(PO_3)IIPLPG-NH_2 was completely hydrolyzed within 15 min. Using these assay conditions, the tyrosyl monothiophosphopeptide and dithiophosphopeptide having the same sequence were stable to *Yersinia* PTP after 24 h. The enzyme remained active throughout these experiments. This was shown by adding *p*-nitrophenyl phosphate (pNPP) after 24 h to each reaction mixture and observing its immediate hydrolysis by *Yersinia* PTP to *p*-nitrophenolate and phosphate. In contrast, the boranophosphopeptide was less stable with a $t_{1/2}$ of 15 h (Figure 2).

Conclusions

In conclusion, boranophosphopeptides **12a**−**c**, phosphopeptide **15**, and thiophosphopeptide **16** target compounds were successfully synthesized on a solid-support by using *H*-phosphonate chemistry. These results demonstrate the exceptional versatility of the Fmol-protected *H*-phosphonate synthon for the preparation of phosphate, boranophosphate, and monothiophosphate peptides having serine, threonine, and tyrosine amino acids. By using a similarly protected *H*-phosphonothioate synthon, dithiophosphate peptides **8a**−**c** were synthesized. Further extension of this work to a peptide having amide and carboxylic acid side chains demonstrated two important features of this approach. One was that these phosphorylation synthons could be used without protection of the amino acid side chain of aspargine. Presumably this conclusion can be extended to glutamine as well. Additionally the successful deprotection of the 2-phenylisopropyl ester of aspartic acid with 1% TFA suggests that this approach can be extended to the synthesis of various phosphopeptide analogues having lysine, cysteine, and glutamate as well as unphosphorylated tyrosine, threonine, and serine amino acids. This is because these side chains can be protected as the 2-phenylisopropyl ester (glu), monomethoxytrityl (lys, cys), and trityl (ser, thr, tyr) derivatives during phosphorylation of serine, threonine, or tyrosine (introduced during peptide synthesis as the side chain unprotected amino acid). These protecting groups can then be removed with 1% TFA without damaging the dithiophospho-, boranophospho-, or thiophosphopeptides. To be completely versatile, however, further research with appropriately protected histidine and arginine must be completed.

These studies also demonstrate that the dithiophosphoryl and boranophosphoryl peptides are superior to the monothiophosphoryl derivative as an inhibitor of *Yersinia* PTP. As these analogues, especially the dithiophosphoryl peptide, are additionally stable toward phosphatase activity, they should prove useful for a wide range of studies involving phosphorylation/dephosphorylation signal transduction cascades in various biochemical pathways. By adding specificity to these phosphopeptides, binding affinity and therefore potency as inhibitors should improve substantially. For example, with addition of an acidic amino acid to the amino terminal side of a phosphorylated tyrosine, it is possible to lower the K_I by a full order of magnitude with *Yersinia* PTP.[38]

An additional intriguing avenue of research is also possible with the dithiophosphoryl peptides due to their stability toward phosphatases. This is because the incorporation of dithiophosphate into DNA has been shown to be immunogenic and capable of producing sequence- and metal-specific monoclonal antibodies.[39] Other more recent work has also demonstrated that organic esters of dithiophosphate can be used to generate metalloantibodies.[40] Perhaps a dithiophosphate derivative, as part of a sequence-specific dithiophosphoryl peptide, can be used to generate monoclonal antibodies for use in characterizing unique phosphoproteins in appropriate biochemical signaling cascades. Similarly, because these peptides are stable toward phosphatases, sequence-specific dithiophosphoryl peptides may prove useful as affinity column conjugates for purifying targeted protein kinases and phosphatases.

Finally, the synthetic strategy provides an additional avenue for the use of these analogues in various biochemical assays. As outlined in Schemes 3 and 5, these analogues are synthesized by oxidation of the *H*-phosphonothioate or *H*-phosphonate intermediates with appropriate reagents. Previous research has shown that similar *H*-phosphonodithioate and *H*-phosphonothioate derivatives of nucleosides and oligonucleotides can be oxidized with alkylamines and alcohols to form various thiophosphoramidate, thiophosphate, and dithiophosphate esters.[41,42] Perhaps the *H*-phosphonothioate and *H*-phosphonate analogues shown in Schemes 3 and 5 can be similarly oxidized to generate peptides labeled with reporter groups (fluorescent chromophores, spin labels, or fluorine NMR probes) useful in various biochemical studies. An alternative method for introducing reporter groups would be to replace *N*-acetylglycine at the amino terminus with a probe activated as the succinimide, isothio-

(39) Graff, D. A.; Jacobs, J. W.; Caruthers, M. H. U.S. Patent 5,962,291, 1999.
(40) Gao, C.; Brümmer, O.; Mao, S.; Janda, K. D. *J. Am. Chem. Soc.* **1999**, *121*, 6517−6518.
(41) Brill, W. K.-D.; Yau, E. K.; Caruthers, M. H. *Tetrahedron Lett.* **1989**, *30*, 6621−6624.
(42) Nielsen, J.; Brill, W. K.-D.; Caruthers, M. H. *Tetrahedron Lett.* **1989**, *29*, 2911−2914.

cyanate, or sulfonyl chloride derivative. There are numerous fluors and other reporter groups that fit these criteria.[43]

Methods developed in this work should prove useful for the synthesis of a large number of biologically important molecules. In this work, monitoring synthesis steps via solid-phase ^{31}P NMR proved especially valuable as we could extensively characterize various synthetic steps without workup, which usually introduces additional variables. Generally this technique should prove especially useful for those who wish to introduce various NMR measurable nuclides into solid-phase synthesis strategies for peptides, oligonucleotides, and, more recently, oligosaccharides.[44]

Experimental Section

Materials and Methods. Solid-phase peptide synthesis was completed on an Applied Biosystems 433A peptide synthesizer. Phosphitylation of peptides linked to a polymer support used an Applied Biosystems 380A or 394 automated DNA synthesizer. Amino acid analysis was performed by Amgen, Inc., Boulder, CO, using phenylisothiocyanate (Edman's reagent). ^{31}P NMR spectra were recorded on a Bruker AM-400 spectrometer operating at 162.0 MHz referenced to 70% H_3PO_4 in D_2O as an external standard. ^{11}B NMR spectra were carried out on a Varian VXR-300S operating at 96.23 MHz, using 5 mm Quartz NMR tubes with a quartz coaxial probe containing D_2O. The reference standard was $Et_2O{\cdot}BF_3$. Mass spectrometry was performed on a Hewlett-Packard 29987A ESI-MS.

Reverse-phase and ion exchange HPLC were performed with a Waters 625 LC system equipped with a 600A gradient controller. A Waters 991 photodiode array UV detector operating at 205 nm was used for peptide detection. Analytical reverse-phase or ion exchange HPLC were performed on a Vydac Protein and Peptide C18 reverse-phase column or a Dionex Nucleopac PA-100 column, respectively. Similarly, preparative reverse-phase or ion exchange HPLC were carried out on a Whatman Partisil column or a Zorbax Oligo column, respectively.

9-Fluorenemethanol, DBU, DPCP, HMDST, elemental sulfur, carbon disulfide, acetic anhydride, benzoic anhydride, DIEA, triethylsilane, phosphorus trichloride, pivaloyl chloride, BSTFA, $BH_3{\cdot}$THF, triethylamine, and ethyl acetate were purchased from Aldrich. Dry pyridine, dichloromethane, DMF, and acetonitrile were purchased from Fisher Chemicals and used as supplied.

Fmoc-asp(2-phenylisopropyl ester)-OH and Fmoc-asn(Mtt)-OH were purchased from Bachem, Inc. Fmoc-tyr(PO_3H_2)-OH, Fmoc-tyr(OH)-OH, HOBt, DCC, and DMAP were purchased from Novabiochem (Calbiochem-Novabiochem Corp.). All other amino acids and Applied Biosystems 433A machine synthesis reagents were purchased from Perkin-Elmer Applied Biosystems, Inc. Tentagel S HMB was purchased from Rapp Polymere (Tuebingen, FRG).

Yersinia Enterocolitica (YPO51) protein tyrosine phosphatase was obtained from Calbiochem. Enzyme stability studies with ^{31}P NMR were carried out on a Bruker AM-400 spectrometer operating at 162.0 MHz referenced to a 70% H_3PO_4 in D_2O as an external standard.

Gel Phase ^{31}P NMR Spectroscopy. Argon-flushed, peptide-linked Tentagel resin (ca. 100 mg) was placed in a vial, dichloromethane (1.5 mL) was added, and the suspension was transferred to an NMR tube. The resin was allowed to form a layer on the surface of the dichloromethane, excess solvent was then removed from beneath this resin plug, and 70% H_3PO_4 in D_2O was added as an external standard. NMR acquisition times were attenuated due to the more rapid resin-linked phosphorus nucleus relaxation. The following parameters were used: relaxation delay = 0.01, number of data points = 8K, acquisition time = 0.123, tip angle (pw) = 43.1°s with proton decoupling.

Diazabicyclo[5.4.0]undec-7-ene (DBU) Gel Phase ^{31}P NMR Spectroscopy Time Course Studies. Tentagel resins containing tyrosine, serine, and threonine were converted to the phosphorodithioate Fmol diesters. These derivatized resins were then flushed with argon, added to 1.5 mL of DBU:dichloromethane (1:99, v/v), and analyzed by gel phase ^{31}P NMR. Five minute scans were performed continuously until no relative changes were observed in reaction species whereupon the sample was mixed and continuously scanned again. Five sets of data points were obtained and used to assess the decomposition rate of product with DBU.

General Procedure for Synthesis of AcXIIPLPG-Tentagel Resin, Where X is Tyrosine, Threonine and Serine. Tentagel resin (1 g) containing 4-hydroxymethylbenzoate (0.24 mmol/g) was coupled to Fmoc-G-OH (0.297 g, 1 mmol), using dicyclohexylcarbodiimide (0.206 g, 1 mmol) and *N*,*N*-dimethylaminopyridine (0.024 g, 0.2 mmol) in DMF. Peptides were then synthesized with appropriate Fmoc protected amino acids, standard Fmoc chemistry, and activation of amino acids with DCC and HOBt. Fmoc-protected serine, threonine, and tyrosine contained *tert*-butyl blocked side chain hydroxyl groups. After the final deprotection of the *N*-terminal Fmoc group with piperidine, the peptide linked resin (1.1 g, 0.24 mmol/g) was washed with CH_2Cl_2 (50 mL) followed by acetonitrile (50 mL), air-dried, and reacted twice with acetic anhydride (226 μL, 2.4 mmol) and diisopropylethylamine (627 μL, 3.6 mmol) in DMF (5 mL) overnight. The resin-bound *N*-acetylated peptide was washed with DMF (3 × 10 mL) and CH_2Cl_2 (50 mL) and then dried under a stream of argon. The *tert*-butyl protecting groups of the Tyr, Ser, and Thr side chains were removed by treatment with (95:5) trifuoroacetic acid/water for 2 h. The resin was washed with dichloromethane (100 mL) to remove residual acid and dried under a stream of argon.

Synthesis of *O*-Dithiophosphopeptide Derivatives of Ac-YII-PLPG-NH_2, Ac-TIIPLPG-NH_2, and Ac-SIIPLPG-NH_2. Resin-linked peptide (0.11 g, 0.024 mmol) was loaded into the reaction vessel of an automated DNA synthesizer. Fmol-*H*-phosphonothioate **1** (5.78 mg, 0.21 mmol) in 1.5 mL of pyridine:DMF (1:1) was delivered simultaneously with a solution of pivaloyl chloride (17.2 μL, 0.14 mmol in 1.5 mL of dichloromethane:pyridine (23:1)) to the peptide-linked resin and allowed to react for 5 min. The resin was flushed with argon and washed with 4.2 mL of pyridine:dichloromethane (1:1). The coupling and washing steps were repeated two additional times to yield the *H*-phosphonodithioate diesters **5a**−**c**. The reaction products were examined by gel phase ^{31}P NMR (Tyr, δ 68.1; Ser, δ 72.2; Thr, δ = 70.1). Sulfurization was performed manually with a solution of sulfur (0.25 g, 976 mmol) in carbon disulfide:pyridine (1:1, 5 mL) for 3 h. The resin was washed with carbon disulfide (100 mL) and dichloromethane (50 mL) to yield the phosphorodithioate diesters **6a**−**c** as shown by gel phase ^{31}P NMR (Tyr, δ 110.4; Ser, δ 116.4; Thr, δ 113.3). The Fmol protecting group was removed and the dithiophosphopeptide cleaved from the resin by treatment with concentrated NH_4OH (0.5 mL) for 90 min at room temperature to produce the crude phosphorodithioate peptides **8a**−**c**. The resin was removed by filtration and washed with NH_4OH (2 × 0.5 mL). The combined solutions were concentrated in vacuo and the product purified by reverse-phase HPLC on a Hypersil C_{18} column (4.6 × 150 mm): Solvent A, 25 mM Tris·HCl, pH 8, in water; Solvent B, acetonitrile 0−40% over 40 min, flow rate 1 mL/min. Contaminating phosphorothioate peptides were removed by ion exchange HPLC on a Dionex column (4 × 250 mm).:Solvent A, 25 mM Tris·HCl, pH 7.2, in H_2O; Solvent B, 25 mM Tris·HCl, pH 7.2, in H_2O, 0.8 M NH_4Cl 0−10% over 30 min, flow rate 1 mL/min. Characterization data for peptides **8a**−**c** are included in Table 1.

Synthesis of *O*-Boranophosphopeptide Derivatives of Ac-YII-PLPG-NH_2, Ac-TIIPLPG-NH_2, and Ac-SIIPLPG-NH_2. Resin-linked peptide (0.11 g, 0.024 mmol) was loaded into the reaction vessel of an automated DNA synthesizer. Fmol-*H*-phosphonate **9** (5.12 mg, 0.4

(43) Haugland, R. P. *Handbook of Fluorescent Probes and Research Chemicals*, 6th ed.; Molecular Probes, Inc.: 4849 Pitchford Avenue, Eugene, OR 97402, 1996.
(44) Plante, O. J.; Palmacci, E. R.; Seeberger, P. H. *Science* **2001**, *291*, 1523−1527.

mmol) in 1.5 mL of pyridine:DMF (1:1) was delivered simultaneously with a solution of pivaloyl chloride (17.2 μL, 0.14 mmol in 1.5 mL dichloromethane:pyridine (23:1)) to the peptide-linked resin and allowed to react for 5 min. The resin was flushed with argon and washed with 4.2 mL of pyridine:dichloromethane (1:1). These coupling and washing steps were repeated two additional times to yield the *H*-phosphonate diesters **10a**−**c**. The reaction products were examined by gel phase ^{31}P NMR (Tyr, δ 4.5; Ser, δ 7.4; Thr, δ 6.9). Bis(trimethylsilyl)-trifluoroacetamide (0.255 mL, 0.96 mmol) dissolved in tetrahydrofuran (2.4 mL) was delivered via syringe to the column containing the Fmol-*H*-phosphonate derivative of the peptide-linked resin and conversion to the silyl phosphite was allowed to proceed for 60 min. The syringe was removed and a borane tetrahydrofuran complex [(34.4 μL, 0.36 mmol) diluted in tetrahydrofuran (2.4 mL)] was added (1.2 mL) via syringe to the column. The reaction was allowed to proceed for 30 min. The resin was washed with tetrahydrofuran (50 mL) followed by dichloromethane (100 mL) and dried under argon. The Fmol protecting group was removed and the boranophosphopeptide was cleaved with concentrated NH_4OH (0.5 mL) for 90 min at room temperature to produce the crude boranophosphopeptides **12a**−**c**. The resin was removed by filtration and washed with concentrated NH_4OH (2 × 0.5 mL). The combined solutions were concentrated in vacuo and the product purified by reverse-phase HPLC on a hypersil C_{18} column (4.6 × 150 mm): Solvent A, 25 mM Tris·HCl, pH 8.0, in water; Solvent B, acetonitrile 0−40% over 40 min, flow rate 1 mL/min. Characterization data for peptides **12a**−**c** are included in Table 1.

Synthesis of *O*-Phosphopeptide and *O*-Thiophosphopeptide Derivatives of Ac-YIIPLPG-NH_2. Peptide Ac-YIIPLPG linked to resin (0.11 g, 0.024 mmol) was loaded into the reaction vessel of an automated DNA synthesizer. Fmol-*H*-phosphonate **9** (5.12 mg, 0.21 mmol) in 1.5 mL of pyridine:DMF (1:1) was delivered simultaneously with a solution of pivaloyl chloride (17.2 μL, 0.14 mmol in 1.5 mL of dichloromethane:pyridine (23:1)) to the peptide-linked resin and allowed to react for 5 min. The resin was flushed with argon and washed with 4.2 mL of pyridine:dichloromethane (1:1). These coupling and washing steps were repeated two additional times to yield **10a**. Peptide **10a** was then treated with 0.10 M aqueous I_2:tetrahydrofuran:water (1:1:1) for 20 s to produce **14**. The resin was washed with dichloromethane (100 mL) and then treated with concentrated ammonium hydroxide to generate **15**. Characterization data for peptide **15** are included in Table 1.

Peptide **10a** was also converted to the *O*-thiophosphopeptide. The resin containing **10a** (0.11 g, 0.024 mmol) was treated with elemental sulfur (0.25 g, 976 mmol) dissolved in 5 mL of carbon disulfide:pyridine (1:1) for 120 min to produce **13**. The resin was washed with carbon disulfide and dichloromethane and then treated with concentrated NH_4OH to generate **16**. The peptide was purified by reverse-phase HPLC on a hypersil C_{18} column (4.6 × 150 mm): Solvent A, 25 mM Tris·HCl, pH 8.0, in water; Solvent B, acetonitrile 0−40% over 40 min, flow rate 1 mL/min. Characterization data for peptide **16** are included in Table 1.

Synthesis of a Dithiophosphopeptide Containing Reactive Side Chains. Synthesis of Ac-GNDY(POS_2)IIPL-NH_2 was completed by using a series of automated and manual steps. Tentagel resin (1 g) containing 4-hydroxymethylbenzoate (0.24 mmol/g) was coupled sequentially to Fmoc-leu-OH (0.353 g, 1 mmol), Fmoc-pro-OH (0.337 g, 1 mmol), and Fmoc-ileu-OH (0.353 g, 1 mmol) (twice) to generate Fmoc-IIPL-resin. Unreacted resin hydroxyl groups were then blocked before continuation of the synthesis. This was carried out by removing the resin from the peptide synthesizer and manually completing this step by adding benzoic anhydride (0.994 g, 4.4 mmol) to the Fmoc-protected peptidyl resin in DMF (5 mL) containing *N*,*N*-dimethylaminopyridine (0.012 g, 0.1 mmol). After allowing the benzoylation reaction to proceed for 1 h, the resin slurry was isolated by filtration and washed with DMF (3 × 10 mL) and dichloromethane (3 × 10 mL). The resin was returned to the peptide synthesizer and peptide synthesis continued by sequentially adding Fmoc-tyr-OH (0.403 g, 1 mmol) (unprotected side chain), Fmoc-asp(2-phenylisopropylester)-OH (0.473 g, 1 mmol), Fmoc-asn(NH_2)-OH (0.354 g, 1 mmol) (unprotected side chain), and Ac-gly-OH (0.117 g, 1 mmol). Resin-linked peptide (0.11 g, 0.024 mmol) was then loaded into the reaction vessel of an automated DNA synthesizer. Fmol-H-phosphonothioate **1** (5.78 mg, 0.21 mmol) in 1.5 mL of pyridine:DMF (1:1) was delivered simultaneously with a solution of pivaloyl chloride (17.2 μL, 0.14 mmol) in 1.5 mL of dichloromethane:pyridine (23:1) to the peptide-linked resin and allowed to react for 5 min. The resin was flushed with argon and washed with 4.2 mL of pyridine:dichloromethane (1:1). The coupling and washing steps were repeated two additional times to yield the *H*-phosphonothioate diester. The aspartate (2-phenylisopropyl) protecting group was removed with a solution of 1% TFA in dichloromethane (5 mL) for 30 min. The peptidyl resin was washed with dichloromethane (50 mL) followed by carbon disulfide (100 mL). Sulfurization was performed manually with a solution of sulfur (0.25 g, 976 mmol) in 5 mL of carbon disulfide/pyridine (1:1) for 3 h. The resin was washed with carbon disulfide (100 mL) and dichloromethane (50 mL) to yield the phosphorodithioate diester. The Fmol protecting group was removed and the dithiophosphopeptide cleaved from the resin by treatment with concentrated NH_4OH (∼0.5 mL) for 90 min at room temperature to produce the crude dithiophosphopeptide. The supernatant was removed and the resin was washed with NH_4OH (2 × 0.5 mL). The solution was concentrated in vacuo and the product partially purified by reverse-phase HPLC: Solvent A, 25 mM Tris·HCl, pH 8.0, in H_2O; Solvent B, acetonitrile 0−40% over 40 min at a flow rate of 1 mL/min. The dithiophosphopeptide was analyzed by ^{31}P NMR (δ = 87) and ESI-MS (1055).

***Yersinia* Protein Tyrosine Phosphatase Inhibition Assays.** Eight thousand units of *Yersinia* PTP (Calbiochem) was diluted in buffer (50 mM Tris·HCl, 150 mM NaCl, 5 mM DTT, 2.5 mM EDTA, 100 μg/mL BSA, pH 7.2) to 3.2 mL and kept on ice. *p*-Nitrophenyl phosphate was diluted to four different concentrations: 1.04×10^{-2}, $7.80\ 1 \times 10^{-3}$, 5.20×10^{-3}, and 2.60×10^{-3} M in the same buffer. Boranophosphoryl-, thiophosphoryl-, and phosphoryltyrosine-containing peptides having the sequence Ac-YIIPLPG-NH_2 were freshly prepared from 100 mg samples of Tantagel resin by using the chemistry outlined above and purified by preparative reverse-phase HPLC: Solvent A, 50 mM Tris·HCl, pH 7.2; Solvent B, acetonitrile 0−40% over 40 min, flow rate of 1 mL/min. Column fractions containing each product were pooled and used directly in the enzyme inhibition assays. A dithiophosphoryltyrosine-containing peptide having the same sequence was also prepared by the chemistry outlined above. However, the reaction mixture as isolated after ammonium hydroxide treatment of the resin was first purified from contaminating thiophosphopeptide by preparative ion exchange chromatography on an Zorbax oligo column (21.2 × 250 mm): Solvent A, 25 mM Tris·HCl, pH 7.2, in water; Solvent B, 25 mM Tris·HCl, pH 7.2, in water, 0.8 M NH_4Cl 0−50% for 50 min. The resulting pooled product was further purified by preparative reverse-phase HPLC by using the same gradient as described for preparing the boranophosphoryl, thiophosphoryl, and phosphoryl peptides. Unphosphorylated Ac-YIIPLPG-NH_2 was also prepared and purified by preparative reverse-phase HPLCby using the same gradient. As a consequence, all five peptides were collected from reverse-phase HPLC in the same buffer.

Peptide inhibitor (50 μL) in HPLC buffer (80%; 50 mM Tris·HCl, pH 7.2, 20%; acetonitrile) was added to each well of a 96-well microtiter plate. Each well had previously been loaded with 5 μL of aqueous *p*-nitrophenyl phosphate at one of the four concentrations being used in this study. *Yersinia* PTP (10 μL, 25 units) was then added to start the reaction. The final concentrations of the *p*-nitrophenyl phosphate were 0.8, 0.6, 0.4, and 0.2 mM. Reactions were terminated by adding 150 μL of 1 M NaOH and the absorbance read at 405 nm with a platereader.

Phosphopeptide Analogue Stability Assays. *Yersinia* PTP (500 units, 100 μL) was added to 500 μL each of purified phosphopeptide analogue (see inhibition assays) in HPLC buffer (80%; 50 mM Tris·

HCl, pH 8.0, 20% (acetonitrile)) and hydrolysis was monitored by ^{31}P NMR and reverse-phase HPLC.

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Supporting Information Available: Information on ^{31}P NMR, ^{11}B NMR, and ESI-MS data for phosphopeptides listed in Table 1, plots from the inhibitor studies with thiophosphoryl, dithiophosphoryl, and boranophosphoryl peptides, and ^{31}P NMR studies on DBU degradation of dithiophosphoryl peptides (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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