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Caged Phospho-Amino Acid Building Blocks for Solid-Phase Peptide Synthesis

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Abstract: Three 1-(2-nitrophenyl)ethyl-caged phospho-amino acids have been synthesized for use in standard N^{α}-fluorenylmethoxycarbonyl-based solid-phase peptide synthesis (SPPS). The most common naturally occurring phospho-amino acids, serine, threonine, and tyrosine, were prepared as protected caged building blocks by modification with a unique phosphitylating reagent. In previous work, caged phospho-peptides were made using an interassembly approach (Rothman, D. M.; Vazquez, M. E.; Vogel, E. M.; Imperiali, B. *Org. Lett.* **2002**, *4*, 2865−2868). However, this technique is limited to creating peptides without oxidation sensitive residues C-terminal to the amino acid to be modified and the methodology involves synthetic manipulations on the solid phase that may limit the utilization of the methodology. Herein we report the facile synthesis of *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine **1**, *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-threonine **2**, and *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosine **3**. These building blocks allow the synthesis of any caged phospho-peptide sequence using standard Fmoc-based SPPS procedures.

Kinase-mediated phosphorylation of serine, threonine, and tyrosine in peptides and proteins represents a central mechanism of cell regulation and is an area of intense study for which there is a need for new chemical tools.[2] Phosphopeptides and phosphoproteins commonly mediate key events in complex biochemical pathways, and the ability to study phosphorylation events affords biologists the opportunity to assign the specific roles of a particular kinase in a cellular process. Chemical inhibition, gene knockout experiments, and point mutation studies can reveal the significance or essentiality of a protein phosphorylation event,[2,3] but may be limited when analysis of the process in "real time" is desired. In this case, the use of chemically caged analogues is highly valuable. A caged compound has a photocleavable-protecting group that masks an essential functionality; upon removal by photolysis, the functionality is revealed, generating a biologically active molecule.[4] Thus, caged compounds allow for spatial and temporal control over the release of a predetermined concentration of an effector molecule.[5] When desired, a living system can be charged with the compound, and upon controlled photolysis the effect of the phosphorylated species on the system can be observed in real time.

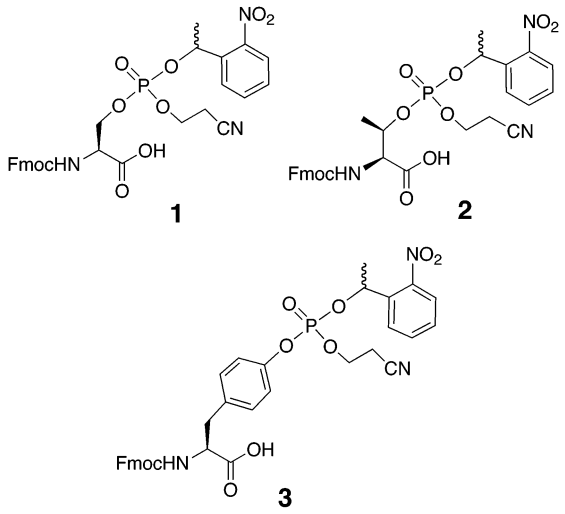

FIGURE 1. Structures of caged phospho-amino acid building blocks for Fmoc-based SPPS: **1**, *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine; **2**, *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-threonine; **3**, *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosine.

As previously described, the 1-(2-nitrophenyl)ethyl (NPE) caging group satisfies key requirements of biologically useful caging groups and those of Fmoc-based SPPS.[1,4] The NPE group may be removed with far UV light (>350 nm), which will not harm a cell. Furthermore, the photo-byproduct is nitrosoacetophenone, which is less reactive than the corresponding aldehyde released by photolysis of commonly implemented *o*-nitrobenzyl caging groups.[4] Also, the NPE group can withstand the repetitive basic treatments in Fmoc-based SPPS and the strongly acidic resin cleavage and global peptide deprotection conditions. Herein, we describe the syntheses of protected caged phospho-amino acid building blocks that enable the rapid assembly of any desired caged phospho-peptides using SPPS (Figure 1).

The previously reported interassembly approach for incorporating caged phospho-amino acids into peptides is limited to sequences without oxidation sensitive residues C-terminal to the modified amino acid and the methodology involves synthetic manipulations on the solid phase. In the interassembly approach, the peptide is built by standard Fmoc-based SPPS up to the serine, threonine, or tyrosine of interest, which is then incorporated without side-chain protection. The hydroxyamino acid residue is then modified on the resin by phosphitylation, followed by oxidation to the desired phosphate species.[1] As illustrated in Figure 2, sequences with tryptophan or methionine residues already installed in the peptide are particularly susceptible to the oxidation step; side reactions involving these two residues have the potential to disrupt experiments in biological systems. Therefore, the availability of building blocks that are

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(3) McMurray, J. S.; David R.; Coleman, D. R. IV.; Wang, W.; Campbell, M. L. *Biopolymers (Peptide Sci.)* **2001**, *60*, 3−31.
(4) Kaplan, J. H.; Forbush, B., III; Hoffman, J. F. *Biochemistry* **1978**, *17*, 1929−1935.
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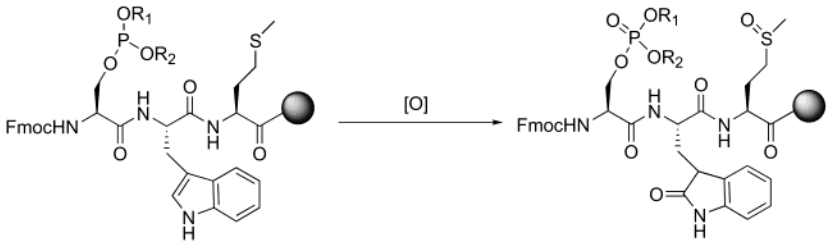
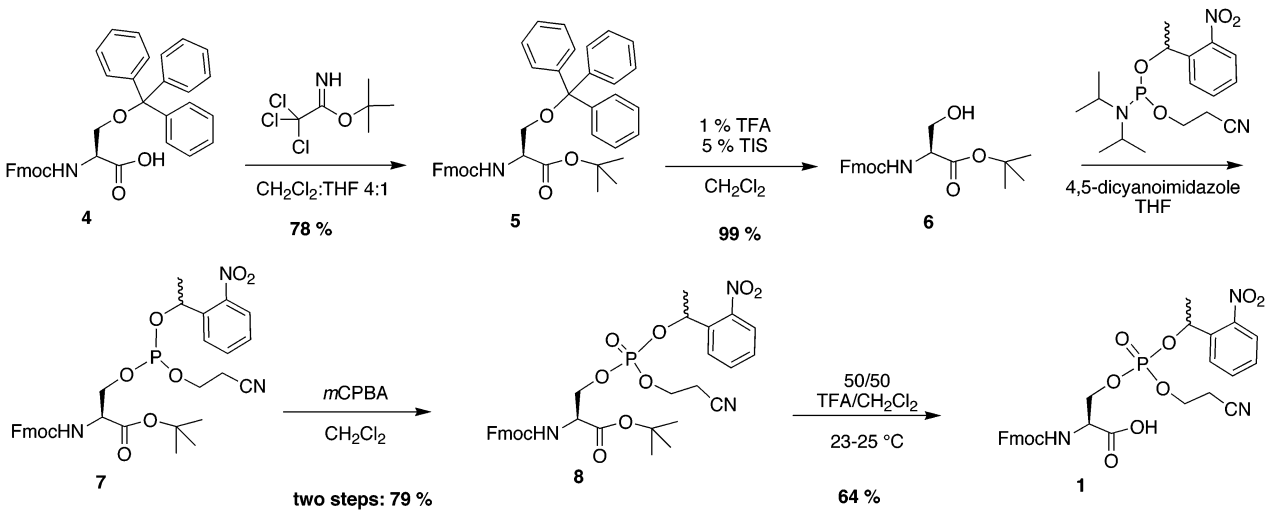


FIGURE 2. Potential complications due to the oxidation step in interassembly approach.

SCHEME 1. Synthesis of *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine 1



amenable to Fmoc-based SPPS is of great interest because any restrictions on peptide sequence are eliminated and the constructs may be generally implemented by a broad range of researchers.

The chemistry used for the building block approach is similar to that of the interassembly approach. The key P−O bond construction is carried out using a reactive trivalent phosphoramidite species, and the phosphite is then oxidized to the corresponding phosphate. In this process, the phosphate retains the *β*-cyanoethyl protecting group until after incorporation of the caged amino acid into the peptide. At this stage, the base-induced Fmoc deprotection step necessary for peptide elongation concurrently removes the *β*-cyanoethyl protecting group, leaving the monophosphate protected. This is ideal for Fmoc-based SPPS because *β*-elimination side reactions that may otherwise complicate the incorporation of caged phospho-serine and threonine residues are suppressed.[6]

The general scheme for the synthesis of the caged building blocks is illustrated for serine in Scheme 1. The synthetic scheme is also applicable to threonine and tyrosine with minor changes, as noted below and in the Experimental Section. Commercially available *N*-α-Fmoc-hydroxytrityl-L-serine, **4**, is first converted to the *tert*-butyl ester **5** with *tert*-butyl 2,2,2-trichloroacetimidate. Following this, the hydroxyl group is deprotected under mild acidic conditions to leave the *N*-α-Fmoc *tert*-butyl ester **6**. In the case of tyrosine, esterification was performed using the unprotected *N*-α-Fmoc-L-tyrosine amino acid due to the reduced reactivity of the phenolic hydroxyl group in tyrosine. Using the previously described phosphoramidite (*O*-1-(2-nitrophenyl)ethyl-*O*′-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite),[1] the amino acid is converted to the phosphite **7**. This trivalent phosphorus species may be isolated by flash chromatography on silica gel; however, the subsequent oxidation using *m*-chloroperoxybenzoic acid or *tert*-butyl hydroperoxide affords the more stable pentavalent species **8**. Therefore, the phosphite **7** was immediately oxidized to **8** and then purified for characterization. Finally, the free acid **1** is generated by treating the fully protected amino acid **8** with 50% trifluoroacetic acid in dichloromethane at room temperature. The final building blocks are made in approximately 40% yield over five steps.

It should be noted that as soon as the phosphorus center is introduced to the amino acid building blocks, diastereomers arise. The NPE group is installed into the phosphitylating reagent as a racemic mixture at the benzylic methine, and once the trivalent phosphorus is oxidized, an additional chiral center is introduced. With the absolute configuration of the α-carbon fixed for each amino acid (and the *β*-carbon for threonine), there are four diastereomers for each amino acid (**1**, **2**, and **3**) and the corresponding tBu esters. These diastereomeric mixtures are inseparable by flash chromatography or RP-HPLC. The proton and carbon NMR data (both decoupled to proton only) that are reported appear complex due to the strong propensity of ^{31}P to split peaks, in addition to the presence of diasteromeric mixtures. The existence of diastereomers is particularly clear in the case of the ^{31}P NMR spectra (also decoupled to proton), in which several peaks arise solely due to the existence of multiple species.

(6) Wakamiaya, T.; Saruta, K.; Yasuoka, J.; Kusumoto, S. *Chem. Lett.* **1994**, 1099−1102.

However, once the cyanoethyl group is removed from the phosphate during base treatment for Fmoc deprotection, the phosphorus center is no longer chiral. Finally, the inseparability of the diasteromeric mixtures due to the chirality at the benzylic methine of the caging group does not appear to have any effect on the ultimate viability of the research tool, as distal phosphate uncaging is not affected by this stereocenter in the NPE group.

In conclusion, we have presented a facile and efficient synthesis of caged phospho-amino acids amenable to Fmoc-based SPPS. The availability of such building blocks allows the synthesis of any peptide sequence using standard SPPS techniques. The potential biological applications of caged phospho-peptides are plentiful, as phosphorylation-dependent cascades play a central role in cell regulation mechanisms, and cell-based studies in real time are desired to assign phosphopeptide and phosphoprotein function. Such peptides have been successfully synthesized and are currently under investigation for biological activity and will be reported in due course (Rothman, D. M.; Nguyen, A.; Stehn, J.; Yaffe, M.; Imperiali, B. Unpublished data).

Experimental Section

Serine Building Block (1). *N*-α-Fmoc-hydroxytrityl-L-serine *tert*-Butyl Ester (5). *N*-α-Fmoc-hydroxytrityl-L-serine (2.17 g, 3.80 mmol) was dissolved in 12.6 mL of anhydrous dichloromethane (CH_2Cl_2)/tetrahydrofuran (THF) 4:1 by volume. To the stirring solution was added *tert*-butyl 2,2,2-tricholoracetimidate (2.72 mL, 15.20 mmol). The reaction was allowed to stir at room temperature overnight under argon. The reaction was then concentrated under reduced pressure and redissolved in ethyl acetate (EtOAc) (50 mL). The solution was washed with 10% sodium bicarbonate ($NaHCO_3$) (2 × 100 mL) and then brine (1 × 100 mL). The organic layer was dried over magnesium sulfate ($MgSO_4$), filtered, and concentrated. The crude product was purified by chromatography on a short plug of basic alumina (1:1 hexanes/EtOAc, $R_f = 0.68$) to give **5** (1.85 g) in 78% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.75 (d, $J_{HH} = 7.5$ Hz, 2H), 7.62 (q, $J_{HH} = 4.8$ Hz, 2H), 7.41−7.19 (m, 19H), 5.83 ($J_{HH} = 8.1$ Hz, 1H), 4.39 (m, 3H), 4.25 (t, $J_{HH} = 6.9$ Hz, 1H), 3.47 (s, 2H), 1.44 (s, 9H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 170.3, 156.6, 144.7, 144.6, 144.3, 142.1, 142.0, 129.4, 128.7, 128.5, 128.0, 127.9, 126.0, 127.0, 120.8, 87.1, 83.1, 68.0, 64.8, 55.7, 47.9, 28.8. ESI-MS: $[MNa]^+$ 648.2708 (obsd), 648.2720 (calcd).

***N*-α-Fmoc-L-serine *tert*-Butyl Ester (6).** *N*-α-Fmoc-hydroxytrityl-L-serine *tert*-butyl ester (1.85 g, 2.96 mmol) was dissolved in 70 mL of CH_2Cl_2 with 1% trifluoroacetic acid (TFA) and 5% triisopropylsilane. The solution was allowed to stir at room temperature for 1 h. The mixture was diluted with dichloromethane (100 mL) and washed with 10% $NaHCO_3$ (2 × 100 mL) and then brine (1 × 100 mL). The crude mixture was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product was purified by silica gel flash chromatography (1:1 hexanes/EtOAc, $R_f = 0.28$) to give **6** (1.12 g) in 99% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.74 (d, $J_{HH} = 7.2$ Hz, 2H), 7.59 (d, $J_{HH} = 7.2$ Hz, 2H), 7.40 (t, $J_{HH} = 7.5$ Hz, 2H), 7.31 (t, $J_{HH} = 8.4$ Hz, 2H), 5.75 (d, $J_{HH} = 6.3$ Hz, 1 H), 4.40 (d, $J_{HH} = 7.2$ Hz, 2H), 4.33 (t, $J_{HH} = 3.6$ Hz, 1H), 4.22 (t, $J_{HH} = 6.9$ Hz, 1H), 3.92 (d, $J_{HH} = 2.1$ Hz, 2H), 2.22 (bs, 1H), 1.48 (s, 9H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 170.5, 157.2, 144.6, 144.5, 142.01, 141.99, 128.4, 127.8, 125.9, 120.72, 120.71, 83.5, 67.9, 64.2, 57.4, 47.8, 28.7. ESI-MS: $[MH]^+$ 384.1816 (obsd), 384.1805 (calcd).

***N*-α-Fmoc-phosphi(1-nitrophenylethyl-2-cyanoethyl)-L-serine *tert*-Butyl Ester (7).** In a round-bottom flask under argon was dissolved *N*-α-Fmoc-L-serine *tert*-butyl ester (930 mg, 2.43 mmol) in 12 mL of anhydrous THF, and stirring was continued. In a pear flask under argon were dissolved *O*-1-(2-nitrophenyl)-ethyl-*O*′-*β*-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (synthesized as previously described[1]) (1.51 g, 4.12 mmol) and 4,5-dicyanoimidazole (490 mg, 4.12 mmol) in 12.3 mL of anhydrous THF, and the solution was allowed to mix for several minutes in the dark. The phosphoramidite solution was added into the stirring serine solution via cannula under argon positive pressure and allowed to stir at room temperature in the dark overnight, under inert conditions. The reaction was judged complete by disappearance of the starting material and appearance of the product (1:1 hexanes/EtOAc, $R_f = 0.43$). The crude mixture was concentrated under reduced pressure and redissolved in EtOAc (100 mL). The solution was washed with 10% $NaHCO_3$ (2 × 100 mL) and then brine (1 × 100 mL). The crude material was dried over Na_2SO_4, filtered, concentrated under reduced pressure, and used immediately in the following reaction.

***N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine *tert*-Butyl Ester (8).** *N*-α-Fmoc-phosphi(1-nitrophenylethyl-2-cyanoethyl)-L-serine *tert*-butyl ester (1.58 g, 2.43 mmol) was dissolved in 97.2 mL of CH_2Cl_2. *m*-Chloroperoxybenzoic acid (837 mg, 4.86 mmol) was added to the solution, and the solution was allowed to stir in the dark at room temperature for 1 h. The solution was washed with 10% $NaHCO_3$ (2 × 100 mL) and then brine (1 × 100 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The product was purified by silica gel flash chromatography (1:2 hexanes/EtOAc, $R_f = 0.23$) to give **8** (1.27 g) in 79% yield over two steps. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.94 (m, 1H), 7.73 (d, $J_{HH} = 7.5$ Hz, 3H), 7.60 (d, $J_{HH} = 5.7$ Hz, 3H), 7.38 (m, 5H), 6.14 (m, 1H), 5.79 (m, 1H), 4.42 (m, 4H), 4.23 (m, 4H), 2.65 (m, 2H), 1.72 (m, 3H), 1.47 (m, 9H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 168.3, 156.6, 147.5, 144.5, 141.9, 137.7, 134.7, 129.8, 128.5, 128.3, 128.2, 127.8, 125.9, 125.4, 120.7, 117.1, 84.1, 74.2, 68.7, 68.0, 63.1, 55.4, 47.7, 28.6, 24.9, 20.3. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: −2.20, −2.31, −2.41. ESI-MS: $[MNa]^+$ 688.2011 (obsd), 688.2031 (calcd).

***N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine (1).** *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine *tert*-butyl ester (500 mg, 751 μmol) was dissolved in 35.6 mL of CH_2Cl_2 at 25 °C in the dark. Triisopropylsilane (3.75 mL) was added to the solution, followed by 35.6 mL of TFA. The solution was allowed to stir in the dark at 25 °C for 2 h. CH_2Cl_2/TFA was removed by evaporation. The mixture was then redissolved in EtOAc (80 mL), washed with 10% $NaHCO_3$ to pH 5, and then washed with brine (1 × 50 mL). The product was purified by silica gel flash chromatography (65:25:4 $CHCl_3$/MeOH/H_2O, $R_f = 0.44$) to give **1** (293 mg) in 64% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.92 (d, $J_{HH} = 8.1$ Hz, 1H), 7.73 (d, $J_{HH} = 7.5$ Hz, 3H), 7.64 (m, 3H), 7.38 (m, 5H), 6.15 (m, 1H), 6.00 (m, 1H), 4.58 (m, 8H), 2.67 (m, 2H), 1.73 (m, 3H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 171.1, 156.7, 147.5, 144.5, 142.0, 137.4, 134.8 129.9, 128.5, 128.3, 127.9, 126.0, 125.4, 120.7, 117.3, 74.7, 68.6, 68.2, 63.5, 54.8, 47.7, 20.2, 14.9. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: −3.09. ESI-MS: $[MNa]^+$ 632.1412 (obsd), 632.1405 (calcd).

Threonine Building Block (2). *N*-α-Fmoc-hydroxytrityl-L-threonine *tert*-Butyl Ester. *N*-α-Fmoc-hydroxytrityl-L-threonine *tert*-butyl ester was synthesized as described for *N*-α-Fmoc-hydroxytrityl-L-serine *tert*-butyl ester (**5**). The crude product was purified by flash chromatography on a short plug of basic alumina (1:1 hexanes/EtOAc, $R_f = 0.66$) to give the product in 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.88 (d, $J_{HH} = 7.5$ Hz, 2H), 7.81 (d, $J_{HH} = 7.3$ Hz, 1H), 7.78 (d, $J_{HH} = 7.5$ Hz, 1H), 7.61−7.34 (m, 19H), 5.98 ($J_{HH} = 9.7$ Hz, 1H), 4.56 (m, 2H), 4.43 (m, 1H), 4.34 (m, 1H), 4.02 (m, 1H), 1.47 (s, 9H), 1.09 (s, 3H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 169.9, 156.6, 144.6, 144.0, 143.8, 141.3, 129.0, 127.7, 127.6, 127.2, 127.1, 125.1, 125.2, 119.98, 119.96, 86.3, 81.9, 71.4, 67.1, 60.1, 47.2, 27.9, 18.6. ESI-MS: $[MNa]^+$ 646.1554 (obsd), 646.1561 (calcd).

***N*-α-Fmoc-L-threonine *tert*-Butyl Ester.** *N*-α-Fmoc-L-threonine *tert*-butyl ester was synthesized as described for *N*-α-Fmoc-L-serine *tert*-butyl ester (**6**). The crude product was purified by silica gel flash chromatography (hexanes, then 1:1 hexanes/EtOAc, $R_f = 0.48$) to give the product in 94% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.75 (d, $J_{HH} = 7.4$ Hz, 2H), 7.63 (d,

J_{HH} = 7.4 Hz, 2H), 7.39 (t, J_{HH} = 7.2 Hz, 2H), 7.30 (t, J_{HH} = 7.4 Hz, 2H), 5.99 (d, J_{HH} = 9.1 Hz, 1 H), 4.42 (m, 2H), 4.35 (dd, J_{HH} = 2.2 and 6.3 Hz, 1H), 4.30 (m, 1H), 4.24 (t, J_{HH} = 7.2 Hz, 1H), 3.07 (bs, 1H), 1.52 (s, 9H), 1.29 (d, J_{HH} = 6.6 Hz, 3 H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 170.4, 157.0, 143.9, 143.7, 141.3, 127.7, 127.1, 125.2, 120.0, 82.5, 68.2, 67.2, 59.8, 47.1, 28.0, 20.0. ESI-MS: $[MNa]^+$ 420.1770 (obsd), 420.1781 (calcd).

***N*-α-Fmoc-phosphi(1-nitrophenylethyl-2-cyanoethyl)-L-threonine *tert*-Butyl Ester.** In a round-bottom flask under argon was dissolved *N*-α-Fmoc-L-threonine *tert*-butyl ester (1.35 g, 3.39 mmol) in 16 mL of anhydrous THF, and the solution was allowed to stir. In a pear-shaped flask under argon were dissolved *O*-1-(2-nitrophenyl)-ethyl-*O*′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (3.74 g, 10.2 mmol) and 4,5-dicyanoimidazole (1.20 g, 10.20 mmol) in 16 mL of anhydrous THF, and the solution was allowed to mix for several minutes in the dark. The phosphoramidite solution was added into the stirring amino acid solution via cannula under argon positive pressure and allowed to stir at room temperature in the dark overnight, under inert conditions. The reaction was judged complete by disappearance of the starting material and appearance of the product. The crude mixture was concentrated under reduced pressure and redissolved in EtOAc (100 mL). The solution was washed with 10% $NaHCO_3$ (2 × 100 mL) and then brine (1 × 100 mL). The crude material was dried over Na_2SO_4, filtered, concentrated under reduced pressure, and used immediately in the following reaction.

***N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-threonine *tert*-Butyl Ester.** *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-threonine *tert*-butyl ester was synthesized as described for *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine *tert*-butyl ester (**8**). The product was purified by silica gel flash chromatography (1:3 hexanes/EtOAc, R_f = 0.38) to give the product in 74% yield over two steps. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.96 (m, 1H), 7.80−7.60 (m, 6H), 7.53−7.33 (m, 5H), 6.12 (m, 1H), 5.45 (m, 1H), 5.03 (m, 2H), 4.44 (m, 2H), 4.24 (m, 3 H), 2.69 (m, 2H), 1.75 (m, 3H), 1.47 (m, 9H), 1.34 (m, 3H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 168.5, 168.2, 156.8, 147.1, 143.8, 141.5, 136.9, 134.1, 129.4, 128.0, 127.9, 127.8, 127.3, 125.3, 124.8, 120.2, 116.4, 83.6, 73.9, 67.5, 67.4, 62.5, 58.9, 47.3, 28.1, 24.4, 19.8, 18.4. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: −3.30, −3.20. ESI-MS: $[MNa]^+$ 702.2194 (obsd), 702.2187 (calcd).

***N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-threonine (2).** *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-threonine was synthesized as described for *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-serine (**1**). The product was purified by silica gel flash chromatography (EtOAc, then 9:1 EtOAc/MeOH with 1% AcOH, R_f = 0.18) to give **2** in 64% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm: 7.97−7.26 (m, 12H), 6.21 (bs, 1H), 6.13 (m, 1H), 5.62 (m, 1H), 5.01 (m, 2H), 4.51−4.11 (m, 5H), 2.70 (m, 2H), 1.75 (m, 3H), 1.30 (m, 3H). ^{13}C NMR (125.8 MHz, $CDCl_3$) δ ppm: 170.1, 156.9, 147.1, 143.8, 141.5, 137.2, 134.2, 129.4, 128.0, 127.9, 127.3, 125.3, 124.8, 120.2, 116.6, 73.8, 67.6, 67.1, 63.0, 58.1, 47.3, 19.8, 18.5. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: −3.91. ESI-MS: $[MNa]^+$ 662.2857 (obsd), 662.2877 (calcd).

Tyrosine Building Block (3). *N*-α-Fmoc-L-tyrosine *tert*-Butyl Ester. *N*-α-Fmoc-L-tyrosine (440 mg, 1.10 mmol) was placed in a dry 25 mL round-bottom flask in CH_2Cl_2/THF (4 mL, 4:1) and cooled to 0 °C under argon, and *tert*-butyl trichloroacetimidate (720 mg, 3.28 mmol) was added over the resulting solution. The reaction mixture was allowed to stir overnight at room temperature. CH_2Cl_2 (100 mL) was added, and the solution was washed with 2.5% $NaHCO_3$ (2 × 50 mL). The organic layer was dried with Na_2SO_4 and concentrated. The residue was purified by flash column chromatography (1:1 EtOAc/hexanes, R_f = 0.72) to give the desired product as a sticky solid (342 mg) in 68% yield. ^{1}H NMR (400 MHz, MeOD) δ ppm: 7.70 (d, J_{HH} = 7.5 Hz, 2H), 7.55 (d, J_{HH} = 7.4 Hz, 2H), 7.33 (m, 2H), 7.25 (m, 2H), 7.06 (d, J_{HH} = 8.3 Hz, 2H), 6.78 (d, J_{HH} = 8.3 Hz, 2H), 4.36−4.25 (m, 3H), 4.09 (m, 1H), 3.05 (m, 2H), 1.47 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ ppm: 171.5, 156.4, 155.7, 144.3, 141.7, 131.0, 128.2, 127.8, 127.5, 125.6, 120.4, 115.9, 83.1, 62.5, 55.8, 47.5, 38.0, 28.4. ESI-MS: $[MH]^+$ 460.2115 (obsd), 460.2118 (calcd).

***N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosine *tert*-Butyl Ester.** *N*-α-Fmoc-L-tyrosine *tert*-butyl ester (440 mg, 0.96 mmol) was dissolved in dry THF (4 mL) in a round-bottom flask provided with 4 Å molecular sieves (400 mg). In a separate flask was dissolved 1*H*-tetrazole (115 mg, 1.63 mmol) in dry THF (4 mL) followed by *O*-1-(2-nitrophenyl)-ethyl-*O*′-β-cyanoethyl-*N*,*N*-diisopropylphosphoramidite (600 mg, 1.63 mmol). After 5 min activation the mixture was added over the Fmoc-L-tyrosine *tert*-butyl ester solution, and the resulting mixture was allowed to stir in the dark overnight under argon. The reaction mixture was filtered over Celite and concentrated under reduced pressure, and the residue was redissolved in CH_2Cl_2 (25 mL) and washed with 1% $NaHCO_3$ (2 × 25 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The oily residue was redissolved in dry CH_2Cl_2 (25 mL), and *tert*-butyl hydroperoxide was added dropwise over the solution (300 μL of 5−6 M solution in decane). The reaction was stirred for 1 h at room temperature and then washed with 2% $NaHCO_3$ (2 × 50 mL). The organic layer was dried with Na_2SO_4 and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (1:1 hexanes/EtOAc, R_f = 0.19) to give the product (442 mg) in 63% yield. ^{1}H NMR (400 MHz, MeOD) δ ppm: 7.75−7.41 (m, 7H), 7.34 (t, 2H, J_{HH} = 7.4 Hz), 7.25 (t, 2H, J_{HH} = 6.2 Hz), 7.19 (d, 1H, J_{HH} = 8.5 Hz), 7.12 (d, 2H, J_{HH} = 8.5 Hz), 7.05 (d, 1H, 8.3 Hz), 6.96 (dd, 1H, J_{HH} = 2.7, 8.6 Hz), 6.11−6.15 (m, 1H), 4.28−4.08 (m, 6H), 3.08−2.82 (m, 2H), 2.78 (t, 2H, J_{HH} = 5.7 Hz), 1.70−1.66 (m, 3H), 1.39 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ ppm: 172.5, 158.3, 148.4, 145.3, 142.6, 137.7, 136.2, 135.2, 132.1, 130.5, 129.0, 128.9, 128.3, 126.4, 125.7, 121.1, 121.0, 120.9, 118.3, 83.1, 75.3, 68.0, 64.9, 57.5, 48.4, 37.9, 28.4, 24.6, 20.1. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: 7.763, 7.762, −7.40, −7.46, −7.50. ESI-MS: $[MH]^+$ 742.2541 (obsd), 742.2524 (calcd).

***N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosine (3).** *N*-α-Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-L-tyrosine *tert*-butyl ester (440 mg, 590 μmol) was dissolved in dry CH_2Cl_2 (8 mL) in a 25 mL round-bottom flask, and the resulting solution was cooled to 0 °C. TFA (8 mL) was slowly added over the solution, and the resulting mixture was allowed to stir in the dark for 1 h at room temperature under argon. The solvents were removed under reduced pressure, and the residue was redissolved in chloroform and concentrated again to eliminate the residues of TFA. The residue was purified by flash column chromatography (1%AcOH/5% MeOH/CH_2Cl_2, R_f = 0.22) to give the product **3** (329 mg) in 81% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm: 9.70 (broad s, 1H), 7.95−7.93 (d, 1H, J_{HH} = 7.1 Hz), 7.77−7.70 (m, 3H), 7.65−7.58 (m, 3H), 7.45−7.37 (m, 3H), 7.27−7.32 (m, 2H), 7.24−6.99 (m, 4H), 6.25 (m, 1H), 5.54 (m, 1H), 4.65 (broad s, 1H), 4.47 (broad s, 2H), 4.35 (broad s, 1H), 4.21 (broad s, 2H), 3.1 (m, 2H), 2.50 (m, 2H), 1.71 (dd, 3H J_{HH} = 2.6, 3.6 Hz). ^{13}C NMR (101 MHz, $CDCl_3$) δ ppm: 173.5, 156.0, 149.1, 146.9, 143.9, 141.5, 136.8, 134.2, 134.1, 131.1, 129.2, 127.8, 127.3, 125.2, 124.7, 120.2, 120.1, 120.1, 116.2, 74.4, 67.2, 63.0, 54.7, 47.3, 37.1, 24.3, 19.6. ^{31}P NMR (121.5 MHz, $CDCl_3$) δ ppm: −7.74. ESI-MS: $[MH]^+$ 686.1888 (obsd), 686.1898 (calcd).

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Supporting Information Available: ^{1}H NMR and ^{13}C NMR spectra for all compounds; general experimental information. This material is available free of charge via the Internet at http://pubs.acs.org.

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