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Demonstration of a Scalable One-Pot Synthesis of Fmoc-*O*-Benzylphospho-L-serine

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Supporting Information

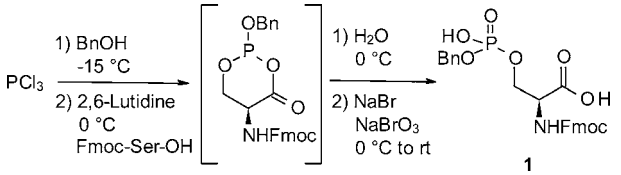
ABSTRACT: A cost-effective one-pot synthesis of Fmoc-*O*-benzylphospho-L-serine, an amino acid commonly used in the synthesis of phosphorylated peptides, has been developed. Two methods for executing this synthesis are described, and both have been scaled to provide kilogram quantities of the title compound in ~50% isolated yield. Development of both processes has led to the identification of crystallization conditions which provide the product as a solvate with high purity. An efficient process for generating the solvent-free product from the solvate has also been developed.

INTRODUCTION

The phosphorylation of proteins is utilized by cells to regulate many fundamental biochemical processes. Many of these processes are reversible, with kinases and phosphatases providing the means to install and remove the phosphate group. Accordingly, synthetic peptides containing phosphorylated amino acids are of great interest in studying these processes.[1] Researchers commonly incorporate phosphorylated amino acids such as phosphoserine, phosphothreonine, and phosphotyrosine into peptides for use in biological studies.[2] As a natural extension of this work, phosphorylated amino acids have also been incorporated into active pharmaceutical ingredients (API's), causing interest in them to span across both industry and academia.[3] One of the most commonly used phosphorylated amino acids is Fmoc-*O*-benzylphospho-L-serine (**1**). Typically, Fmoc-*O*-benzylphospho-L-serine is synthesized in 58−68% yield from Fmoc-L-serine via a 3−4 step sequence which requires synthesis of phosphorylating agents as well as protection of the carboxylic acid functionality.[4] This strategy is not efficient and furthermore requires the use of hazardous reagents such as 1*H*-tetrazole which are not amenable to scale-up. Fmoc-*O*-benzylphospho-L-serine can be purchased from a limited number of contract manufactures in kilogram quantities, but at a cost of more than $21,000/kg.

Recently we reported the efficient and cost-effective synthesis of phosphoamino acids including **1** using a one-pot procedure (Scheme 1).[5] This procedure includes *in situ* generation of the phosphorylating reagent, no carboxylic acid protection, and use of inexpensive reagents, with Fmoc-L-serine being the largest cost contributor at $450/kg. Since this discovery, we have further developed the protocol for **1** and scaled it to provide kilogram quantities in approximately 50% yield with excellent purity. Two comparable procedures have been successfully demonstrated, generating 1.0−3.5 kg quantities with similar yields and purities.

Scheme 1. One-Pot Synthesis of Fmoc-*O*-benzylphospho-L-serine



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RESULTS AND DISCUSSION

We began development of the scalable process with the gram scale reaction conditions in place. These conditions were developed after screening various reagents, solvents, and stoichiometries on small scale.[5] Treatment of PCl_3 with BnOH in THF at −15 °C produced the proposed benzyl dichlorophosphite intermediate. Consumption of PCl_3 was observed by ^{31}P NMR as a loss of the signal at 219 ppm. This intermediate was then treated with 2,6-lutidine before adding a mixture of Fmoc-L-serine and 2,6-lutidine at 0 °C. This led to the presumed phosphite intermediate, which was hydrolyzed and oxidized with NaBr/$NaBrO_3$ at 0 to 22 °C. After oxidation, the title compound (**1**) was the major product by HPLC.

Using these conditions, we began development of the scalable process with an investigation of the impact of the quality of the key starting material, Fmoc-L-serine, on the process performance. Fmoc-L-serine is commercially available with varying amounts of water. We found that a greater amount of water in the starting material had a negative effect on the yield. With 7.8 wt % water the yield was 40%, while with <0.7 wt % water, the yield was typically 50−55%. The water content varied for each of the vendors; therefore, a suitable method to dry Fmoc-L-serine which contained >0.7% water was necessary. By subjecting the solution of Fmoc-L-serine in four volumes of THF to three distillation/dilution cycles, as much as 8 wt % water could be reduced by azeotropic drying to the desired levels (<0.7 wt %).

With an understanding of the required starting material quality in hand, we turned our attention to identifying an appropriate isolation. Upon completion, the reaction mixture consists of a biphasic solution of THF and water. Addition of 2-

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MeTHF allowed partitioning of the product into the organic layer with <5% loss to the aqueous layer. Upon washing the organic layer with brine and drying with sodium sulfate, the resultant solution could be concentrated to an oil containing the desired product (83 A%), starting material (4 A%), and the dimeric phosphate ester **2** (13 A%, Figure 1).

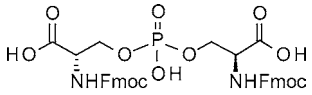

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Figure 1. Dimeric phosphate.

Attempts to isolate pure Fmoc-*O*-benzylphospho-L-serine free from the starting material and dimer byproduct began with crystallization studies. Though the initial solubility study (Table 1) did not identify any solvents that were ideal for

Table 1. Solubility of Fmoc-*O*-benzylphospho-L-serine

entry	solvent	solubility (mg/mL)
1	ACN	1.8 mg/mL
2	CH_2Cl_2	2.0 mg/mL
3	EtOAc	6.0 mg/mL
4	*i*-Pr_2O	0 mg/mL
5	MTBE	1.2 mg/mL
6	acetone	76 mg/mL
7	2-MeTHF	58 mg/mL
8	MIBK	3.2 mg/mL
9	THF	>100 mg/mL

crystallizations, we found that solids did precipitate from 2-MeTHF. Further study showed that the precipitate was the 2-MeTHF solvate, which had lower solubility (20−25 mg/mL) in 2-MeTHF than the commercial Fmoc-*O*-benzylphospho-L-serine that was initially studied (Table 1). If the THF and water levels were low enough in the crude product stream, this 2-MeTHF solvate could be isolated with minimal product loss. Initial studies showed that concentrating the 2-MeTHF/THF solution and redissolving the resulting oil in 2-MeTHF generated a thick slurry. This finding permitted the development of a scalable vacuum distillation solvent switch from the mixed solvent to 2-MeTHF followed by seeding. We found that consecutive batch vacuum distillation at 15−25 °C reduced the THF and water to acceptable levels. To achieve good purity with minimal product loss, the final 2-MeTHF solution should contain <2% THF and ≤0.7% water.

Once the product had precipitated from the 2-MeTHF solution at 22 °C, the product that remained in solution could be further reduced by cooling to −10 °C. Below −10 °C, the isolated product purity decreased. We found that, at −10 °C, the product could be isolated in 91−98% purity as the 2-MeTHF solvate with 10% product loss. The variability in purity at −10 °C was tracked to the efficiency of the cake wash. If the cake was allowed to dry and crack, the resultant uneven wash led to purities as low as 91%.

Though the wash can be controlled to provide high purity, it was desirable to develop an upgrade to ensure the desired purity at scale. This upgrade was also attractive, as it presented an opportunity to convert from the 2-MeTHF solvate[7] to the solvent-free Fmoc-*O*-benzylphospho-L-serine. A screen identified *i*PAc as an ideal solvent for the desolvation upgrade (Upgrade 1) with product loss of ≤5% (Table 2). *i*PAc could upgrade material from 91 to 99% purity while breaking the

Table 2. Solvents Screened for Desolvation Upgrade

entry	solvent	loss	purity
1	DCM	6.0%	99.48
2	EtOAc	6.0%	99.52
3	*i*PAc	3.6%	99.50

solvate and reducing the volatiles present to ≤1.0 wt % of any single process solvent used. We used Method A combined with Upgrade 1 to successfully convert Fmoc-L-serine to Fmoc-*O*-benzylphospho-L-serine at 1.5 kg scale (Table 3, Run 1).

Table 3. Kilogram Scale Reaction Results

run	scale	solvate yield	overall yield[a]	overall purity[b]	method
1	1.5 kg	54%	50%	97.3%	A
2	4.9 kg	50%	44%	97.0%	B
3	4.9 kg	55%	49%	96.0%	B

[a]Yield after isolation from the *i*PAc reslurry. [b]Area % purity after isolation from *i*PAc.

With Method A successfully demonstrated on kilogram scale, we made one additional optimization. We wanted to eliminate the cumbersome sodium sulfate drying step which was used prior to the solvent switch. This proved more difficult than first expected as both the brine wash and sodium sulfate treatment were critical to successfully executing the new isolation. If the sodium sulfate drying step was removed, the residual brine in the organic solution precipitated sodium chloride within the product stream after water was removed. If the brine wash was removed, 2,6-lutidine remained in the organic solution and contaminated the product upon precipitation.

To eliminate these two issues, the brine wash was left in place, and after the initial concentration and dilution with 2-MeTHF, a water wash was incorporated. This second water wash removed the residual sodium chloride. Because the majority of the organic layer was 2-MeTHF, the product loss and residual water were minimized. Though this method was successful in eliminating the sodium sulfate drying, it resulted in higher levels of residual water than the original work up. To address the need to remove additional water from the solution, stability data up to 55 °C was collected. Although we knew that the isolated Fmoc-*O*-benzylphospho-L-serine was not stable above 40 °C, we found that the solution was stable up to at least 55 °C. This allowed the solvent switch to be performed at elevated temperature, which allowed more efficient azeotropic drying, and distillation times similar to those used initially. This alternate Method A (Method A1) was demonstrated on 35 g scale to give 56% isolated yield of 98.2% pure material after applying Upgrade 1.[6]

While developing this THF based method, a second method was also under development with the goal of eliminating the need for use of both THF and 2-MeTHF. The reaction could be run in 2-MeTHF with slight increases in the equivalence of PCl_3 (1.5 vs 1.3) and BnOH (1.8 vs 1.5) to maintain similar yields. Not only was there an advantage of using only one solvent, but due to the slightly more hydrophobic nature of 2-MeTHF, this method eliminated the need for the sodium sulfate drying or the alternative second water wash during the isolation. However, the new method still required distillation

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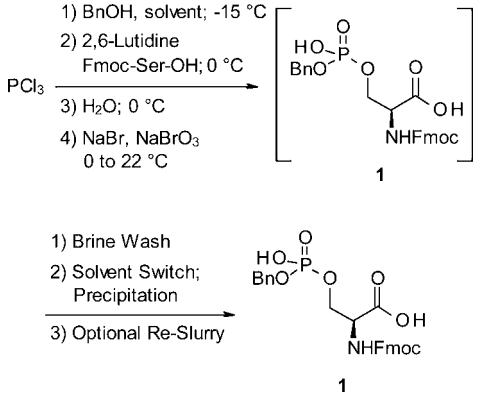
prior to the crystallization to dry the crude product solution, and we found that an antisolvent (MTBE) was required to achieve a similar product recovery during isolation of the 2-MeTHF solvate. This second method (Method B) was also executed successfully at kilogram scale to provide Fmoc-*O*-benzylphospho-L-serine in ~50% isolated yield and very high purity after use of Upgrade 1 (Table 3, Runs 2 and 3).

In summary, we have developed two scalable processes to produce Fmoc-*O*-benzylphospho-L-serine in an overall isolated yield of ~50% in ≥96% purity (Scheme 2). The processes do

Scheme 2. Scalable Process To Generate Fmoc-*O*-benzylphospho-L-serine



not require protection of the carboxylic acid, and they can be performed in one pot at −15 to 0 °C[8] using inexpensive, readily available reagents. The isolation entails a distillation to achieve a solvent composition which facilitates precipitation of the 2-MeTHF solvate. This solvate is typically isolated in ≥94% purity and in ~55% yield. An *i*PAc upgrade has also been developed to improve the purity if necessary and to provide the solvent-free Fmoc-*O*-benzylphospho-L-serine. This upgade typically provides the pure product in ≥96% purity and in ~50% yield from Fmoc-L-serine.

EXPERIMENTAL SECTION

Fmoc-L-serine was purchased with varying water contents up to ~8 wt %, and dried as indicated in the procedure below before use. Reactions were monitored on Agilent 1200 series HPLC equipment. The details of the HPLC methods are in the Supporting Information.

Phosphorylation Procedure (Method A). Volumes indicated in this procedure are in reference to the weight of the dry amino acid used. When a hydrated (>0.7 wt % water) form of Fmoc-L-serine was purchased, the following azeotropic drying was employed to remove the water and increase the yield. A mixture of Fmoc-L-serine (1.59 kg crude mass, 5.4 wt % H_2O, 4.58 mol corrected for wt %, 1.0 equiv) and dry THF (6.0 L, 4 vol) was heated (T_j = 70 °C), and THF (3.0 L) was removed via distillation at 700 mbar. The solution was diluted to the original volume with THF (3.0 L). This distillation and dilution cycle was repeated two additional times. The final water content of the Fmoc-serine solution was 0.10 wt % H_2O (target < 0.7 wt %). This solution was held overnight.

THF (12.0 L, 8.0 vol) was cooled to −18 °C, and PCl_3 (819 g, 5.96 mol, 1.30 equiv) was charged over 4 min. After 15 min, BnOH (752 g, 6.96 mol, 1.53 equiv) was added over 46 min. The maximum temperature observed was −14 °C.

The solution was then warmed to 0 °C over 38 min. The consumption of PCl_3 was confirmed by ^{31}P NMR. 2,6-Lutidine (1.47 kg, 13.7 mol, 3.00 equiv) was added to the reactor over 39 min at <5 °C, resulting in the formation of solids.

The dried Fmoc-L-serine in THF was mixed with 2,6-lutidine (488 g, 4.56 mol, 1.00 equiv) at 22 °C, and the resulting solution was added to the PCl_3/BnOH/2,6-lutidine vessel over 1.5 h at 0 °C. The maximum temperature observed was −0.2 °C. Additional THF (1.5 L) was used to rinse the flask containing the Fmoc-L-serine.

HPLC assays (Method 1) were taken at 10 min intervals to confirm reaction completion (completion defined as no change in the ratio of starting material to intermediate phosphite at 225 nm) while maintaining the reaction at 0 °C. After stirring for 45 min, H_2O (5.46 L, 3.6 vol) was added to the reactor over 30 min with cooling. An exotherm was observed, and the maximum temperature reached 7 °C. During the addition, all of the solids went into solution, forming a two-phase mixture.

Solid NaBr (1.09 kg, 10.6 mol, 2.31 equiv) was then added at 0 °C. Complete dissolution of the solids was observed. An aqueous solution of $NaBrO_3$ (332 g, 2.20 mol, 0.48 equiv, in 1.66 L of H_2O) was then added over 18 min with cooling. After the addition, the reaction was allowed to warm to 22 °C. A slow exotherm was then observed, and cooling was required to maintain the temperature below 27 °C. HPLC assays (Method 1) were taken 1 h after $NaBrO_3$ solution addition was complete to evaluate reaction progress (completion defined as phosphite intermediate <1.5 A% at 225 nm). After 2.5 h at 20 to 27 °C, the reaction was complete and an aqueous solution of $Na_2S_2O_5$ (150 g, 0.789 mol, in 1.35 L H_2O) was added to the reactor over 5 min to quench any remaining oxidant.

2-MeTHF (15 L, 10 vol) was then added to the reactor, and the mixture was stirred for 10 min. After the layers were allowed to settle, the aqueous layer was drained from the reactor and the organic layer was washed with aqueous NaCl (6.25 kg, diluted to 15 L total volume with H_2O). After the reaction mixture was stirred for 5 min, the brine layer was drained from the reactor. Solid Na_2SO_4 (1.50 kg) was added to the reactor, and the mixture was stirred for 20 min.

With stirring, the mixture was drained from the reactor and filtered to remove the Na_2SO_4, rinsing with 2-MeTHF (1.5 L). The filtrate was assayed by HPLC (Method 1) against a weight-based standard. 1.50 kg of product was present (66% yield). This solution was held overnight.

The filtrate was charged to a reactor equipped for distillation. Solvent was distilled off at 116 mbar with a jacket temperature of 50−55 °C. When the total volume in the reactor was reduced to 11.25 L, the jacket temperature was reduced and the reaction was sampled. The sample was assayed for H_2O content by KF titration (5.5 wt %) and analyzed by GC for the ratio of 2-MeTHF to THF (62−38 by mass).

The solution was diluted with additional 2-MeTHF (11.25 L, 7.5 vol) and then distilled under vacuum. This distillation cycle was repeated 3 times, resulting in a slurry that contained a water content of 0.7 wt % (≤0.7 wt % desired), and the ratio of 2-MeTHF to THF was >99 to 1 by mass (>98 to 2 desired). The slurry was then stirred overnight at 22 °C to allow the seed bed to mature.

The slurry was then cooled to −10 °C. A supernatant HPLC assay (Method 1) taken at 1 h indicated a concentration of 19.5 mg/g product (<20 mg/g is required for good recovery). After 105 min at −10 °C, the slurry was filtered. Room temperature 2-MeTHF (4.5 L) was used to wash the cake. The solids were

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dried on the filter under vacuum to provide 1.57 kg of the 2-MeTHF solvate of Fmoc-*O*-benzylphospho-L-serine. HPLC assay (Method 1) indicated 91.2 A% and 78.1 wt % purity (53.8% isolated yield, corrected for wt %). ^{1}H NMR analysis indicated 11.9 wt % 2-MeTHF. The product loss to the mother liquor was 7.0% (of the theoretical yield).

Phosphorylation Procedure (Method B). Volumes indicated in this procedure are in reference to the weight of the dry amino acid used. When a hydrated form of Fmoc-L-serine was purchased (hydrated specified as >0.7 wt % water), the following azeotropic drying was utilized to remove the water and increase the yield. A mixture of Fmoc-L-serine (5.04 kg crude mass, 2.8 wt % H_2O, 14.97 mol corrected for wt %, 1.0 equiv) and 2-MeTHF (20.0 L, 4 vol) was heated (T_j = 65 °C), and 2-MeTHF (10.0 L) was removed via distillation at 450 mbar. The solution was diluted with 2-MeTHF (10.0 L), and this distillation and dilution cycle was repeated two more times. The final water content of the Fmoc-serine solution was <0.10 wt % H_2O (target < 0.7 wt %).

2-MeTHF (35.0 L, 7.0 vol) was cooled to −15 °C, and PCl_3 (3.15 kg, 22.9 mol, 1.53 equiv) was charged to the reactor. After 15 min, BnOH (2.90 kg, 26.8 mol, 1.79 equiv) was added over 45 min, and 2-MeTHF (5.0 L, 1.0 vol) was used to rinse the lines. A maximum temperature of −14 °C was observed.

The solution was then warmed to 0 °C over 30 min. 2,6-Lutidine (4.82 kg, 45.0 mol, 3.0 equiv) was added to the reactor over 50 min at <5 °C. A slurry formed during this addition.

2,6-Lutidine (1.61 kg, 15.0 mol, 1.0 equiv) was premixed with the dried solution of Fmoc-L-serine, and the resulting mixture was added to the reactor over 1.5 h at <3 °C.

After a 16 h age at 0 °C, an HPLC assay (Method 1) was taken to confirm reaction completion (<10% starting material at 225 nm). After confirming reaction completion, H_2O (18.0 L, 3.6 vol) was added to the reactor over 30 min with cooling. An exotherm was observed and the maximum temperature reached 5 °C. During the addition, all of the solids went into solution, forming a two-phase mixture.

Solid NaBr (3.54 kg, 34.4 mol, 2.30 equiv) was then added to the two-phase mixture in the reactor at 0 °C over 20 min. This was followed by addition of solid $NaBrO_3$ (1.08 kg, 7.16 mol, 0.48 equiv) over 20 min. After the addition, an exotherm was observed, and the jacket was used to maintain an internal temperature of 0 ± 5 °C. After a minimum of 2.5 h, HPLC assays (Method 1) were taken to confirm reaction completion (completion defined as phosphite intermediate <4.0 A% at 225 nm). When reaction completion was confirmed, solid $Na_2S_2O_5$ (510 g, 2.68 mol, 0.18 equiv) was added to the reactor at 0 °C to quench any remaining oxidant.

The batch was then warmed to 20 °C, and after allowing the layers to settle, the aqueous layer was drained from the reactor. The organic layer was washed with aqueous NaCl (6.3 kg, diluted with 35 L of H_2O).

Solvent was then removed from the resulting organic layer via vacuum distillation with the jacket temperature at 40 ± 5 °C. When the total volume in the reactor was reduced to 25.0 L, fresh 2-MeTHF (35.0 L, 7 vol) was added, and the distillation was continued. Upon reaching 25.0 L, the solution was again diluted with fresh 2-MeTHF (35.0 L, 7 vol) and distilled one final time to 25.0 L.

The resulting solution was held at 20 °C while MTBE (8.0 L, 1.6 vol) was added over 40 min. This mixture was allowed to stir for 16 h to allow the seed bed to mature and was then cooled to −10 °C. After 6 h at −10 °C, the slurry was filtered and the cake was washed with ambient temperature MTBE (15 L, 3.0 vol).

The solids were dried at 30 °C on the filter under house vacuum for 16 h to provide 4.67 kg of Fmoc-*O*-benzylphospho-L-serine as the 2-MeTHF solvate (containing 13.2 wt % 2-MeTHF). When adjusted for the 2-MeTHF, 4.05 kg of Fmoc-*O*-benzylphospho-L-serine was isolated (54.5% isolated yield). The product loss to the mother liquor was 11.8% (of the theoretical yield).

Desolvation Upgrade (Upgrade 1). Fmoc-*O*-benzylphospho-L-serine (1.48 kg, 91.2 A%, 78.1 wt %) was mixed with *i*PAc (10.4 L, 7.0 vol). The resulting slurry was stirred for 22 h at 20 °C to allow for digestion of the solids. The slurry was then cooled to 0−5 °C for 1 h. A supernatant HPLC assay (Method 1) taken at 1 h indicated that the level of product in the mother liquors was at about 6.7 mg/mL (≤7.0 mg/mL desired).

After 75 min, the slurry was filtered and washed with room temperature *i*PAc (3.0 L, 2.0 vol) without allowing the cake to crack. The solids were dried on the filter under house vacuum at ambient temperature to provide 1.10 kg of product (50% yield from Fmoc-L-serine, corrected for wt % and for the amount of solvate used). HPLC assay (Method 1) indicated 99.4 A% and 97.3 wt % purity (versus a recrystallized standard, >100 wt % versus commercially available Fmoc-*O*-benzylphospho-L-serine). Chiral HPLC assay (Method 2) indicated 100% ee. ^{1}H NMR analysis indicated 1.0 wt % 2-MeTHF and 0.40 wt % IPAc. KF Fischer analysis indicated 0.25 wt % H_2O. ^{1}H NMR (400 MHz, DMSO): 7.88 (m, 3H), 7.74 (d, J = 8.03 Hz, 2H), 7.25−7.45 (m, 9H), 4.94 (d, J = 7.52 Hz, 2H), 4.05−4.40 (m, 6H). ^{13}C NMR (400 MHz, DMSO): 170.7 (s, 1C), 155.9 (s, 1C), 143.7 (s, 2C), 140.6 (s, 2C), 136.7 (d, 1C), 128.3 (s, 2C), 127.9 (s, 1C), 127.6 (s, 2C), 127.5 (s, 2C), 127.0 (s, 2C), 125.2 (s, 2C), 120.0 (s, 2C), 67.4 (d, 1C), 65.8 (s, 1C), 65.1 (d, 1C), 54.3 (d, 1C), 46.5 (s, 1C). ^{31}P NMR (400 MHz, DMSO): −1.50. HRMS: [MH^+] = 498.1306 (calc = 498.1312). Specific Rotation: −9.69°, c = 0.0509 g/mL in DMF.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C, and ^{31}P NMR, HPLC methods, and additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(6) This Method A1 was not scaled up further due to project constraints. Experimental details for Method A1 can be found in the Supporting Information.
(7) The 2-MeTHF solvate was successfully used in peptide synthesis but may not be ideal for all applications.
(8) Method A was tested at 0 °C after the kiloscale run was completed. It gave similar results to the kiloscale run on up to 1.0 g scale. The only difference from the current Method A experiment was running the first step at 0 °C rather than −15 °C.