Convenient Synthesis of Collagen-Related Tripeptides for Segment Condensation

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ABSTRACT:

Chromatography is a common step in the solution-phase synthesis of typical peptides, as well as peptide fragments for subsequent coupling on a solid support. Combining known reagents that form readily separable byproducts is shown to eliminate this step, which wastes time and other resources. Specifically, activating carboxyl groups with isobutyl chloroformate or as pentafluorophenyl esters and using N-methyl morpholine as a base enable chromatography-free synthetic routes in which peptide products are isolated from byproducts by facile evaporation, extraction, and trituration. This methodology was used to access tripeptides related to collagen, such as Fmoc-Pro-Pro-Gly-OH and Fmoc-Pro-Hyp(tBu)-Gly-OH, in a purity suitable for solid-phase segment condensation to form collagen mimetic peptides. © 2015 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 104: 674–681, 2015.

Keywords: *collagen; segment condensation; synthesis; isobutyl chloroformate; pentafluorophenyl ester*

Additional Supporting Information may be found in the online version of this article

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INTRODUCTION

Synthetic peptides have been the basis for transformative advances in medicine, as well as the creation of new catalysts and materials.[1–8] Peptides are synthesized either in solution, as pioneered by Emil Fischer at the beginning of the 20th century,[9] or on a solid support, as developed by Bruce Merrifield in the 1960s.[10] Drawbacks occur with both methods. The synthesis of peptides in solution is plagued by challenging purification steps and other issues.[11] Solid-phase peptide synthesis (SPPS) facilitates purification[12,13] and continues to advance,[14–18] though overall yields necessarily diminish with each coupling step in the linear route and are intrinsically low for some sequences.[19,20]

A convergent strategy in which protected peptide fragments made in solution are coupled on a solid support offers an attractive alternative.[14,21,22] Such "segment condensation" requires fewer couplings, increasing efficiency and reducing waste. In part, however, the use of segment condensation on a solid support merely displaces inefficiency and waste to the solution phase, and its requisite chromatographic purification.

Our research group and many others are interested in collagen mimetic peptides (CMPs).[23–25] CMPs have been used to reveal the structure of the collagen triple helix[26,27] along with the forces that underlie that structure,[28] and have been of particular relevance in biomedicine and materials science.[29–35] Like collagen itself, CMPs are composed of Xaa-Yaa-Gly triplets. This invariant repetition makes CMPs ideal for synthesis

by segment condensation. These factors motivated us to seek synthetic routes to CMPs that avoid cumbersome purification steps.

Typical CMPs comprise 21–30 residues. The component Xaa-Yaa-Gly triplets for segment condensation are made by using solution-phase methods.[36–39] In our experience,[40–43] the synthesis of tripeptides with common reagents requires chromatography on a column of silica gel (or crystallization, which can be idiosyncratic) to obtain products of adequate purity from each coupling step and the final C-terminal deprotection step. Purification by chromatography is not only laborious, but also requires the use of chromatographic media and solvents that are typically discarded as waste. Finally, chromatography of longer peptides or on a large scale suffers from diminished resolution of products. Here, we report on methodology for the chromatography-free synthesis of collagen-related tripeptides that are poised for SPPS. Our methods are efficient and scalable, and could be applicable to other peptide fragments.

MATERIALS AND METHODS

General

Pentafluorophenyl (Pfp) esters[44] and other amino acid derivatives were from Chem-Impex International (Wood Dale, IL). Isobutyl chloroformate[45] and all other reagents were from Sigma–Aldrich (St. Louis, MO) and were used without further purification. Tetrahydrofuran (THF) and *N*,*N*-dimethylformamide (DMF) were dried with a Glass Contour system from Pure Process Technology (Nashua, NH). In addition, DMF was passed through an associated isocyanate "scrubbing" column to remove any amines.

Volatile Removal

The phrase "concentrated under reduced pressure" refers to the removal of solvents and other volatile materials with a rotary evaporator at water aspirator pressure (<20 torr) while maintaining a water bath below 40°C. The phrase "volatiles were removed under reduced pressure" refers to the removal of solvents and other volatile materials with a rotary evaporator at vacuum-pump pressure (~2 torr) while maintaining a water bath below 40°C.

Trituration

Trituration was done by the "solvent swap" method. Briefly, a compound was dissolved in a mixture of two solvents, one being a more volatile solvent in which the compound has good solubility (e.g., DCM), the other (the "anti-solvent") being a less volatile solvent in which the compound has poor solubility (e.g., hexanes). The resulting solution was concentrated under reduced pressure to remove the more volatile solvent. The solids were isolated from the slurry by filtration through a Büchner funnel, and the filter cake was washed with the anti-solvent.

Analysis of Peptides From Solution-Phase Synthesis

Molecular mass was determined at high resolution by electrospray ionization (ESI) mass spectrometry with an LCT instrument from Waters (Milford, MA).

^{1}H and ^{13}C NMR spectra were acquired with an Avance III 400 spectrometer or Avance III 500I spectrometer equipped with a cryogenic probe from Bruker (Billerica, MA). Chemical shifts are reported in units of δ (ppm) relative to tetramethylsilane as the internal standard. In the spectrum of each compound herein, multiple conformers are apparent due to cis−trans isomerism around amide and/or carbamide bonds.

Peptide purity was assessed by reverse-phase chromatography with a Discovery BIO Wide Pore C5-5 column from Supelco (Bellefonte, PA). The column was eluted with 5–95% v/v B over 10 min, 95% v/v B for 2 min, 95–5% over 2 min, and 5% v/v B for 5 min (A: H_2O containing 0.1% v/v formic acid; B: acetonitrile containing 0.1% v/v formic acid). Absorbance at 254 nm and molecular mass were monitored with an LCMS-2020 instrument from Shimadzu (Kyoto, Japan).

Solid-Phase Peptide Synthesis

Solid-phase peptide synthesis was performed at the University of Wisconsin–Madison Biotechnology Center with a Prelude peptide synthesizer from Protein Technologies (Tucson, AZ). Synthetic peptide was purified by HPLC with an LC-20 instrument from Shimadzu. Molecular mass was determined by matrix-assisted laser desorption/ionization–time-of-flight (MALDI–TOF) mass spectrometry on a α-cyano-4-hydroxycinnamic acid matrix with a Voyager DE-Pro instrument at the Biophysics Instrumentation Facility at the University of Wisconsin–Madison.

Synthesis of Cbz-Pro-Gly-O*t*Bu

Cbz-Pro-OH (3.01 g, 12 mmol) and NMM (4.0 mL, 36 mmol) were dissolved in 200 mL THF, and the resulting solution was cooled to –78°C in a dry ice/acetone bath. Isobutyl chloroformate (1.5 mL, 12 mmol) was added dropwise, and a white precipitate (NMM·HCl) formed. Solid HCl·Gly-O*t*Bu (2.02 g, 12 mmol) was added in one portion, and the reaction mixture was allowed to warm to room temperature and then stirred for 12 h. The precipitate was removed by filtration, and the solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10 M $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and volatiles were removed under reduced pressure to give Cbz-Pro-Gly-O*t*Bu (4.01 g, 92%) as a pale yellow solid, which was judged to be 99% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 363.1915; found, 363.1909. Integration of signals for the N–H proton revealed that the cis:trans ratio of the prolyl peptide bond was 1.0:1.5. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.42–7.28 (m, 5H), 5.32–4.99 (m, 2H), 4.56–4.22 (m, 1H), 3.93 (dt, J = 16.0, 8.8 Hz, 2H), 3.66–3.39 (m, 2H), 2.43–2.11 (m, 2H), 2.03–1.80 (m, 3H), 1.46 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 171.75, 168.75, 136.38, 128.51, 128.09, 127.94, 82.08, 67.33, 60.57, 47.08, 42.09, 28.71, 28.05, 24.52.

Synthesis of Fmoc-Pro-Pro-Gly-O*t*Bu

A suspension of Cbz-Pro-Gly-O*t*Bu (4.01 g, 11 mmol) and Pd/C (1.17 g, 10% w/w) in 25 mL of MeOH was stirred under an

atmosphere of H_2(g) for 16 h at room temperature. Any insoluble material was removed by filtration, and volatiles were removed under reduced pressure. (The weight of the crude intermediate was 2.48 g.) The residue was dissolved in 35 mL of THF, and NMM (3.47 mL, 32 mmol) and Fmoc-Pro-OPfp (4.00 g, 7.9 mmol) were added to the resulting solution. After stirring for 16 h, volatiles were removed under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10 M $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. To enhance its solidity, material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-Pro-Pro-Gly-O*t*Bu (4.44 g, 74%) as an off-white solid, which was judged to be 94% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 548.2756; found, 548.2742. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.81–7.72 (m, 2H), 7.66–7.52 (m, 2H), 7.40 (t, *J* = 7.5 Hz, 2H), 7.31 (tt, *J* = 7.4, 1.3 Hz, 2H), 4.76–4.14 (m, 5H), 4.04–3.34 (m, 6H), 2.42–1.78 (m, 8H), 1.54–1.29 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 171.62, 155.21, 144.28, 143.97, 141.43, 127.81, 127.20, 127.15, 125.38, 125.24, 125.18, 120.12, 120.09, 82.10, 81.68, 77.16, 67.95, 67.69, 61.17, 59.97, 59.65, 58.48, 57.76, 47.33, 47.25, 47.17, 46.99, 42.33, 42.10, 29.57, 28.16, 28.14, 28.07, 27.24, 25.39, 24.88, 24.50, 23.18.

Synthesis of Fmoc-Pro-Pro-Gly-OH

Fmoc-Pro-Pro-Gly-O*t*Bu (4.44 g, 8.1 mmol) was dissolved in 12 mL 4*M* HCl in dioxane, and the resulting solution was stirred for 14 h at room temperature. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. To enhance its solidity, material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-Pro-Pro-Gly-OH (3.57 g, 90%) as an off-white solid, which was judged to be 85% pure by LCMS. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.75 (ddd, *J* = 10.3, 7.2, 4.1 Hz, 2H), 7.65–7.49 (m, 2H), 7.43–7.36 (m, 2H), 7.30 (ttd, *J* = 7.4, 3.1, 1.4 Hz, 2H), 4.73–4.49 (m, 1H), 4.49–4.18 (m, 3H), 4.18–3.79 (m, 2H), 3.79–3.64 (m, 2H), 3.64–3.50 (m, 2H), 2.60–1.65 (m, 8H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 172.49, 172.24, 172.10, 171.91, 171.85, 171.80, 171.69, 155.21, 154.49, 144.28, 144.06, 143.98, 143.72, 143.57, 141.29, 141.25, 141.23, 127.77, 127.72, 127.69, 127.59, 127.08, 127.04, 125.21, 125.07, 124.78, 124.61, 119.96, 119.77, 67.93, 67.70, 66.28, 60.99, 60.06, 59.86, 59.70, 58.75, 58.44, 57.67, 52.27, 47.58, 47.25, 47.16, 47.01, 46.90, 41.33, 41.15, 31.84, 30.15, 29.34, 29.29, 27.68, 27.49, 27.16, 25.17, 25.02, 24.67, 24.39, 23.08, 22.02.

Synthesis of Cbz-D-Pro-Gly-O*t*Bu

Cbz-D-Pro-OH (3.02 g, 12 mmol) and NMM (4.0 mL, 36 mmol) were dissolved in 200 mL THF, and the resulting solution was cooled to –78°C in a dry ice/acetone ice bath. Isobutyl chloroformate (1.5 mL, 12 mmol) was added dropwise and a white precipitate (NMM·HCl) formed. Solid HCl·Gly-O*t*Bu (2.04 g, 12 mmol) was added in one portion, and the reaction mixture was allowed to warm to room temperature and then stirred for 12 h. The precipitate was removed by filtration, and the solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10*M* $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and volatiles were removed under reduced pressure to give Cbz-D-Pro-Gly-O*t*Bu (4.27 g, 97%) as a pale yellow solid, which was judged to be 91% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 363.1915; found, 363.1907. Integration of signals for the N–H proton revealed that the cis:trans ratio of the prolyl peptide bond was 1.0:1.7. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.45–7.30 (m, 5H), 5.29–5.03 (m, 2H), 4.37 (d, *J* = 22.7 Hz, 1H), 4.06–3.81 (m, 2H), 3.66–3.36 (m, 2H), 2.43–2.08 (m, 1H), 2.02–1.84 (m, 3H), 1.46 (s, 9H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 172.30, 171.71, 168.75, 156.15, 136.36, 128.51, 128.10, 127.96, 82.12, 77.28, 77.03, 67.35, 60.90, 60.58, 47.53, 47.09, 46.93, 42.10, 41.68, 31.06, 29.72, 28.64, 28.05, 24.53, 23.63, 19.03.

Synthesis of Fmoc-D-Pro-D-Pro-Gly-O*t*Bu

A suspension of Cbz-D-Pro-Gly-O*t*Bu (4.267 g, 12 mmol) and Pd/C (1.25 g, 10% w/w) in 25 mL of MeOH was stirred under an atmosphere of H_2(g) for 18 h at room temperature. Any insoluble material was removed by filtration, and volatiles were removed under reduced pressure. The residue was dissolved in 80 mL of 5 : 3 THF/DMF. NMM (3.87 mL, 35 mmol), HBTU (4.45 g, 12 mmol), and Fmoc-D-Pro-OH (3.97 g 12 mmol) were added to the resulting solution. After stirring for 16 h, volatiles were removed under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10*M* $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. To enhance its solidity, material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-D-Pro-D-Pro-Gly-O*t*Bu (5.26 g, 82%) as an off-white solid, which was judged to be 95% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 548.2756; found, 548.2755. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.85–7.27 (m, 8H), 4.81–4.13 (m, 2H), 4.05–3.36 (m, 6H), 3.34–2.66 (m, 2H), 2.36–1.62 (m, 9H), 1.56–1.37 (m, 9H). ^{13}C NMR (101 MHz, $CDCl_3$, δ): 171.32, 168.87, 140.13, 138.01, 134.70, 129.09, 128.73, 127.05, 124.34, 121.00, 120.32, 119.74, 107.79, 82.10, 77.36, 77.24, 77.04, 76.72, 59.96, 59.24, 47.68, 46.79, 42.04, 41.98, 30.49, 28.04, 27.38, 26.28, 25.01.

Synthesis of Fmoc-D-Pro-D-Pro-Gly-OH

Fmoc-D-Pro-D-Pro-Gly-O*t*Bu (5.26 g, 9.6 mmol) was dissolved in 12 mL of 4 M HCl in dioxane, and the resulting solution was stirred for 14 h at room temperature. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. Material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-D-Pro-D-Pro-Gly-OH (3.37 g, 71% yield) as an off-white solid, which was judged to be 90% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 492.2130; found, 492.2133. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.75 (t, *J* = 7.6 Hz, 2H), 7.57 (ddd, *J* = 25.7, 15.6, 7.3 Hz, 2H), 7.39 (td, *J* = 7.7, 3.1 Hz, 2H), 7.30 (tt, *J* = 7.5, 1.3 Hz, 2H), 4.73–4.48 (m, 1H), 4.47–4.18 (m, 3H), 4.18–3.80 (m, 2H), 3.80–3.63 (m, 2H), 3.63–3.48 (m, 2H), 2.61–1.65 (m, 8H). ^{13}C NMR (101 MHz, $CDCl_3$, δ): 172.34, 172.19, 172.15, 172.12, 171.91, 171.85, 171.82, 171.68, 155.43, 155.12, 154.45, 144.31, 144.11, 144.01, 143.78, 143.64, 141.28, 141.24, 141.22, 127.77, 127.72,

127.69, 127.59, 127.14, 127.10, 127.07, 127.01, 125.26, 125.12, 124.81, 124.65, 119.96, 119.77, 67.85, 67.63, 66.28, 60.94, 60.00, 59.67, 58.73, 58.40, 57.67, 47.58, 47.24, 47.16, 47.05, 46.88, 46.64, 41.42, 41.34, 41.27, 31.84, 30.14, 29.33, 29.28, 27.87, 27.66, 25.12, 24.99, 24.64, 24.40, 23.09, 22.03, 20.79.

Synthesis of Boc-Pro-Pro-OMe

Boc-Pro-OH (3.97 g, 18 mmol) and NMM (6.0 mL, 55 mmol) were dissolved in 200 mL of THF, and the resulting solution was cooled to –78°C in a dry ice/acetone bath. Isobutyl chloroformate (2.4 mL, 18 mmol) was added dropwise, and a white precipitate (NMM·HCl) formed. Solid HCl·Pro-OMe (3.05 g, 18 mmol) was added in one portion, and the reaction mixture was allowed to warm to room temperature and then stirred for 12 h. The precipitate was removed by filtration, and the solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10*M* $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and volatiles were removed under reduced pressure to give Boc-Pro-Pro-OMe (3.25 g, 54%) as a pale yellow solid. Also present is the byproduct of the carbamate formed by reaction of HCl·Pro-OMe and isobutyl chloroformate. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 327.1915; found, 327.1912. ^1H NMR (400 MHz, $CDCl_3$, δ): 4.57 (ddd, *J* = 13.3, 8.5, 4.2 Hz, 0H), 4.50 (dd, *J* = 8.4, 3.1 Hz, 0H), 4.38 (td, *J* = 8.6, 3.9 Hz, 0H), 4.32 (dd, *J* = 8.7, 3.8 Hz, 0H), 3.95–3.85 (m, 1H), 3.82–3.75 (m, 0H), 3.75–3.69 (m, 3H), 3.66–3.32 (m, 3H), 2.32–1.74 (m, 7H), 1.45 (s, 3H), 1.39 (s, 2H), 0.94 (d, *J* = 6.7 Hz, 1H), 0.89 (d, *J* = 6.7 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$, δ): 173.42, 173.26, 172.92, 172.63, 171.60, 171.12, 154.62, 154.57, 153.71, 79.44, 71.45, 59.01, 58.73, 58.67, 57.73, 57.68, 52.18, 52.08, 46.84, 46.77, 46.64, 46.46, 46.25, 30.94, 29.98, 29.90, 29.05, 28.81, 28.70, 28.49, 28.34, 28.02, 27.96, 25.03, 24.97, 24.31, 24.05, 23.55, 19.04, 18.92, 18.89.

Synthesis of Fmoc-Gly-Pro-Pro-OMe

Boc-Pro-Pro-OMe (3.25 g, 9.9 mmol) was dissolved in 9.0 mL of 4*M* HCl in dioxane. The resulting solution was stirred for 14 h at room temperature. The reaction mixture was concentrated under reduced pressure. The residue was dissolved in 30 mL of THF, and NMM (3.28 mL, 29 mmol) and Fmoc-Gly-OPfp (4.56 g, 9.9 mmol) were added to the resulting solution. After stirring for 16 h, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10 M $KHSO_4$ (2×), saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. To enhance its solidity, material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-Gly-Pro-Pro-OMe (4.38 g, 87%) as an off-white solid, which was judged to be 90% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 506.2292; found, 506.2286. ^1H NMR (400 MHz, $CDCl_3$, δ): 7.76 (dd, *J* = 7.6, 4.9 Hz, 2H), 7.59 (dd, *J* = 7.6, 3.6 Hz, 2H), 7.43–7.35 (m, 2H), 7.30 (tdd, J = 7.6, 6.0, 1.5 Hz, 2H), 5.73 (t, *J* = 4.4 Hz, 1H), 5.42 (d, *J* = 23.5 Hz, 1H), 4.70 (dd, *J* = 8.0, 3.7 Hz, 1H), 4.57 (dd, *J* = 8.6, 4.2 Hz, 1H), 4.41 (d, *J* = 7.2 Hz, 1H), 4.36 (dd, *J* = 7.3, 3.3 Hz, 1H), 4.22 (q, *J* = 7.7 Hz, 1H), 4.13 (dd, *J* = 17.2, 5.7 Hz, 1H), 4.05 (s, 1H), 4.03–3.91 (m, 1H), 3.83 (dt, *J* = 9.5, 7.1 Hz, 1H), 3.77 (s, 1H), 3.72 (s, 2H), 3.63 (dt, *J* = 9.7, 6.2 Hz, 2H), 3.57–3.43 (m, 1H), 2.22 (qd, *J* = 11.5, 10.3, 7.1 Hz, 2H), 2.13–1.93 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$, δ): 172.57, 170.51, 167.21, 156.38, 143.89, 143.84, 141.29, 141.25, 127.74, 127.67, 127.09, 127.07, 127.05, 125.19, 125.08, 120.00, 119.97, 119.93, 67.24, 63.81, 58.90, 58.22, 53.58, 52.43, 52.28, 47.09, 46.79, 46.36, 43.34, 28.79, 28.20, 24.93, 24.61.

Synthesis of Fmoc-Gly-Pro-Pro-OH

Fmoc-Gly-Pro-Pro-OMe (4.38 g, 8.6 mmol) was dissolved in 50 mL of 80:20 isopropanol/THF. Powdered $CaCl_2$ (14.22 g, 128 mmol), $LiOH{\cdot}H_2O$ (1.42 g, 33 mmol), and H_2O (5 mL) were added to the resulting solution. The mixture was vigorously stirred for 48 h. The pH was adjusted to 2 with aqueous HCl (1% v/v), and the resulting solution was concentrated under reduced pressure. The aqueous layer was extracted with EtOAc, and the organic layer was washed with water and brine, dried over Na_2SO_4(s), and concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. To enhance its solidity, material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-Gly-Pro-Pro-OH (3.63 g, 86%) as an off-white solid, which was judged to be 87% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 492.2130; found, 492.2128. ^1H NMR (400 MHz, $CDCl_3$, δ): 7.75 (d, *J* = 7.5 Hz, 2H), 7.64–7.54 (m, 2H), 7.39 (t, *J* = 7.5 Hz, 2H), 7.33–7.27 (m, 2H), 5.77 (s, 1H), 5.39 (s, 1H), 4.82–4.51 (m, 2H), 4.49–4.29 (m, 2H), 4.21 (t, *J* = 7.3 Hz, 1H), 4.14 (dd, *J* = 17.3, 5.8 Hz, 1H), 4.06–3.88 (m, 1H), 3.83 (dd, *J* = 9.4, 7.2 Hz, 1H), 3.74–3.45 (m, 3H), 2.43–1.79 (m, 6H). ^{13}C NMR (101 MHz, $CDCl_3$, δ): 174.38, 172.80, 171.53, 167.89, 156.72, 156.68, 143.88, 143.79, 143.74, 141.25, 127.74, 127.70, 127.09, 127.06, 125.20, 125.17, 125.10, 119.98, 119.95, 67.31, 59.31, 58.99, 58.26, 52.35, 47.13, 47.07, 47.03, 46.47, 43.27, 42.58, 28.77, 28.39, 28.19, 24.92, 24.88, 24.70.

Synthesis of Cbz-Hyp(*t*Bu)-Gly-OMe

Cbz-Hyp(*t*Bu)-OH (3.01 g, 9.4 mmol) and *N*-methyl morpholine (NMM) (3.1 mL, 28 mmol) were dissolved in 200 mL of THF, and the resulting solution was cooled to –78°C in a dry ice/acetone bath. Isobutyl chloroformate (1.2 mL, 9.3 mmol) was added dropwise, and a white precipitate (NMM·HCl) formed. Solid HCl·GlyOMe (1.17 g, 9.3 mmol) was added in one portion, and the reaction mixture was allowed to warm to room temperature and then stirred for 12 h. The precipitate was removed by filtration, and the solvent was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10 M $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and volatiles were removed under reduced pressure to give Cbz-Hyp(*t*Bu)-Gly-OMe (3.26 g, 88%) as a pale yellow solid, which was judged to be 99% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 393.2021; found, 393.2015. Integration of signals for the N–H proton revealed that the cis:trans ratio of the prolyl peptide bond was 1.0:2.1. ^1H NMR (400 MHz, $CDCl_3$, δ): 7.39–7.28 (m, 3H), 7.20 (s, 1H), 6.32 (s, 1H), 5.34–4.93 (m, 2H), 4.58–4.20 (m, 2H), 4.03 (d, *J* = 5.4 Hz, 1H), 3.74 (s, 3H), 3.64 (dd, *J* = 10.5, 7.2 Hz, 1H), 3.26 (dd, *J* = 10.6, 6.6 Hz, 1H), 2.53–2.35 (m, 1H), 2.19 (d, *J* = 22.4 Hz, 1H), 1.94 (q, *J* = 10.5, 9.7 Hz, 1H), 1.72 (s, 1H), 1.18 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, δ):

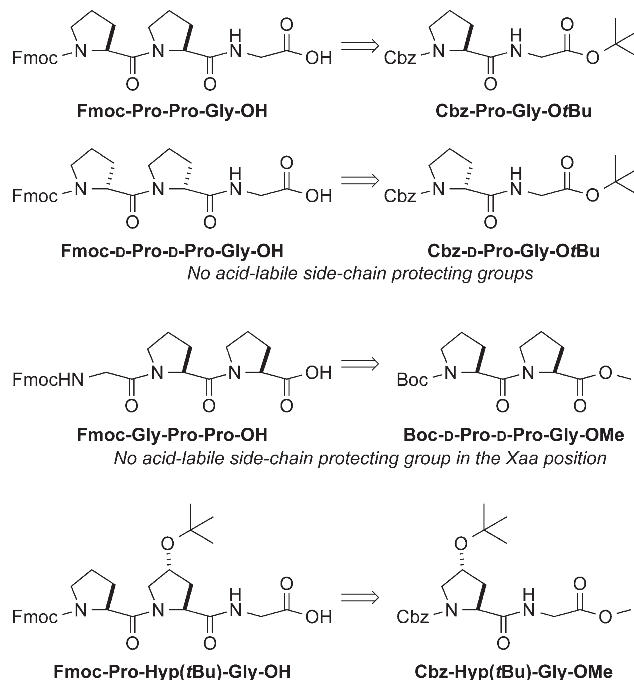
171.76, 170.06, 156.26, 136.30, 128.52, 128.12, 127.84, 69.48, 67.45, 58.95, 52.96, 52.34, 41.24, 35.99, 28.26.

Synthesis of Fmoc-Pro-Hyp(*t*Bu)-Gly-OMe

A suspension of Cbz-Hyp(*t*Bu)-Gly-OMe (3.26 g, 8.3 mmol) and Pd/C (877 mg, 10% w/w) in 25 mL of MeOH was stirred under an atmosphere of H_2(g) for 16 h at room temperature. Any insoluble material was removed by filtration, and volatiles were removed under reduced pressure. (The weight of the crude intermediate was 2.0 g.) The residue was dissolved in 35 mL of THF, and NMM (2.4 mL, 22 mmol) and Fmoc-Pro-OPfp (2.91 g, 5.8 mmol) were added to the resulting solution. After stirring for 16 h, the reaction mixture was concentrated under reduced pressure. The residue was dissolved in EtOAc, and the resulting solution was washed successively with 0.10*M* $KHSO_4$ (2×) and saturated aqueous $NaHCO_3$ (2×). The organic layer was dried over Na_2SO_4(s), filtered, and concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. To enhance its solidity, material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-Pro-Hyp(*t*Bu)-Gly-OMe (1.23 g, 25%) as an off-white solid, which was judged to be 96% pure by LCMS. HRMS-ESI (*m*/*z*): $[M + H]^+$ calcd, 578.2861; found, 578.2866. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.77 (dd, J = 7.5, 3.9 Hz, 2H), 7.68–7.52 (m, 2H), 7.40 (t, J = 7.7 Hz, 2H), 7.36–7.29 (m, 2H), 4.64–4.19 (m, 6H), 4.10 (ddd, J = 23.8, 18.0, 6.6 Hz, 1H), 4.00–3.79 (m, 1H), 3.79–3.43 (m, 6H), 2.72–2.39 (m, 1H), 2.37–1.83 (m, 6H), 1.44–1.01 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 172.93, 171.86, 170.40, 141.65, 128.04, 127.44, 125.63, 125.49, 120.40, 120.34, 77.16, 74.48, 70.57, 67.92, 58.78, 52.63, 47.61, 47.21, 41.42, 34.82, 29.66, 28.67, 28.62, 25.15, 24.75.

Synthesis of Fmoc-Pro-Hyp(*t*Bu)-Gly-OH

Fmoc-Pro-Hyp(*t*Bu)-OMe (1.23 g, 2.1 mmol) was dissolved in 30 mL of 80 : 20 isopropanol/THF. Powdered $CaCl_2$ (3.53 g, 32 mmol), $LiOH{\cdot}H_2O$ (355 mg, 8.5 mmol), and water (5 mL) were added to the resulting solution. The mixture was stirred vigorously for 12 h. The pH was adjusted to 2 with 1% w/v aqueous HCl, and the resulting solution was concentrated under reduced pressure. The aqueous layer was extracted with EtOAc, and the organic layer was washed with water and brine, dried over Na_2SO_4(s), and concentrated under reduced pressure. The residue was dissolved in the minimum volume of DCM and triturated with hexanes. Material insoluble in hexanes was dissolved in methanol, and volatiles were removed under reduced pressure to give Fmoc-Pro-Hyp(*t*Bu)-Gly-OH (978 mg, 83% yield) as an off-white solid, which was judged to be 94% pure by LCMS. ^{1}H NMR (500 MHz, $CDCl_3$, δ): 7.85–7.76 (m, 2H), 7.71–7.54 (m, 2H), 7.44 (td, J = 7.4, 3.0 Hz, 2H), 7.35 (ddt, J = 8.6, 7.4, 1.2 Hz, 2H), 4.91–3.45 (m, 12H), 2.78–1.59 (m, 7H), 1.50–0.92 (m, 9H). ^{13}C NMR (126 MHz, $CDCl_3$, δ): 172.27, 172.21, 172.09, 172.04, 172.00, 171.96, 171.65, 155.50, 155.19, 154.63, 144.18, 144.10, 144.00, 143.73, 143.57, 141.29, 141.26, 141.23, 141.14, 127.78, 127.71, 127.68, 127.13, 127.09, 127.05, 125.23, 125.08, 124.76, 124.72, 120.03, 119.99, 119.94, 74.50, 74.27, 74.18, 70.15, 69.91, 67.92, 67.69, 67.37, 67.10, 59.48, 58.86, 58.64, 58.44, 58.04, 57.78, 53.38, 52.74, 47.32, 47.15, 47.02, 46.87, 41.37, 41.25, 35.62, 35.35, 30.14, 29.12, 28.22, 28.20, 28.16, 24.62, 24.33, 23.02.



SCHEME 1 Four Fmoc-protected tripeptides synthesized by chromatography-free methodology and their dipeptide precursors. Restrictions on utility are in italics typeface.

Synthesis of Ac-Lys-(Ser-Gly)$_3$-(D-Pro-D-Pro-Gly)$_7$

Using the Fmoc-D-Pro-D-Pro-Gly-OH tripeptide synthesized in solution without chromatography (*vide supra*) and an Fmoc-D-Pro-OH monomer, Ac-Lys-(Ser-Gly)$_3$-(D-Pro-D-Pro-Gly)$_7$ was synthesized by two additions of monomer followed by six segment condensations of tripeptide on preloaded Fmoc-Gly-2-chlorotrityl resin (0.19 mmol/g) from EMD Millipore (La Jolla, CA). Fmoc-deprotection was achieved by treatment with piperidine (20% v/v) in DMF. The tripeptide or amino acid monomer (4 equiv) was converted to an active ester using HATU and NMM. Each residue was double-coupled between Fmoc-deprotections.

Peptide was cleaved from the resin with 96.5 : 2.5 : 1.0 TFA/H_2O/TIPSH (5 mL), precipitated from diethyl ether at 0°C, and isolated by centrifugation. The peptide was purified by preparative HPLC using a gradient of 10–50% v/v B over 50 min (A: H_2O containing 0.1% v/v TFA; B: acetonitrile containing 0.1% v/v TFA). MALDI (*m*/*z*): $[M + H]^+$ calcd 2380.6, found 2380.0. A 50-μmol scale synthesis afforded 18.2 mg (15%) of Ac-Lys-(Ser-Gly)$_3$-(D-Pro-D-Pro-Gly)$_7$ after purification.

RESULTS AND DISCUSSION

The key to establishing a route for chromatography-free peptide synthesis is to mitigate byproduct formation. To do so, we coupled amino acids by using isobutyl chloroformate[45] to form transient anhydrides or by using stable pentafluorophenyl esters.[44] These known reagents[46] produce volatile and water-soluble byproducts, respectively. We applied variations of our method to four tripeptides that are used commonly in CMP

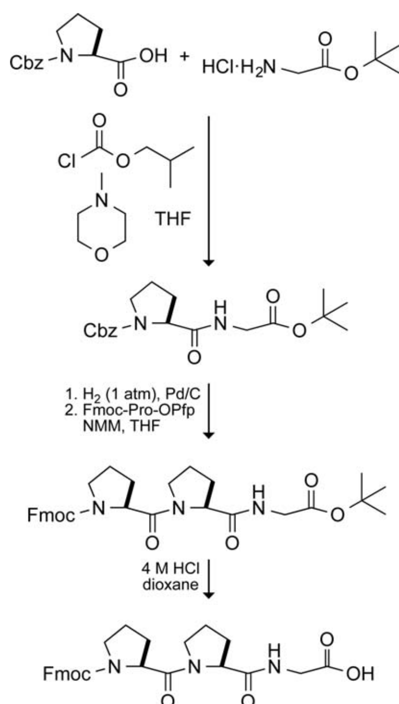
Table I Yield and Purity of Intermediates and Tripeptide Products Made by Chromatography-Free Synthesis

Protected Dipeptide Ester	Yield	Purity[a]	Fmoc Tripeptide Ester	Yield	Purity[a]	Fmoc Tripeptide	Yield	Purity[a]
Cbz-Pro-Gly-O*t*Bu	92%	99%	Fmoc-Pro-Pro-Gly-O*t*Bu	74%	94%	Fmoc-Pro-Pro-Gly-OH	90%	85%
Cbz-D-Pro-Gly-O*t*Bu	97%	91%	Fmoc-D-Pro-D-Pro-Gly-O*t*Bu	82%	95%	Fmoc-D-Pro-D-Pro-Gly-OH	71%	90%
Boc-Pro-Pro-OMe	54%	ND	Fmoc-Gly-Pro-Pro-OMe	87%	90%	Fmoc-Gly-Pro-Pro-OH	86%	87%
Cbz-Hyp(*t*Bu)-Gly-OMe	88%	99%	Fmoc-Pro-Hyp(*t*Bu)-Gly-OMe	25%	96%	Fmoc-Pro-Hyp(*t*Bu)-Gly-OH	83%	94%

[a] Purity was assessed with LCMS monitored at 254 nm. LCMS traces are shown in Figure 1 and the Supporting Information. The absorbance of Boc-Pro-Pro-OMe at 254 nm was not detectable (ND).

strands (Scheme 1). These tripeptides are rich in glycine (which lacks a stereogenic center) and proline (which has virtually no tendency to epimerize during coupling reactions).[47–49] Fmoc-Pro-Pro-Gly-OH and its permutation, Fmoc-Gly-Pro-Pro-OH, provide the simplest triplets in CMPs. Fmoc-D-Pro-D-Pro-Gly-OH is an enantiomer that can serve as a control for experiments in chiral environments. Finally, Fmoc-Pro-Hyp(*t*Bu)-Gly-OH provides the most common triplet found in natural collagen, and contains an acid-labile side-chain protecting group.[27] We were able to access each of these tripeptides without the use of chromatography (Table I).

As an example, we elaborate on the synthesis of Fmoc-Pro-Pro-Gly-OH (Scheme 2). The first coupling step was accomplished by activating the carboxylic acid with isobutyl chloroformate and NMM. Isobutyl chloroformate is a relatively inexpensive reagent compared to many common coupling reagents (e.g., HATU). Coupling with isobutyl choroformate enables ready access to a relatively pure product, as the two major byproducts—CO_2 and isobutanol—are volatile.[50] Insoluble NMM·HCl is removed by filtration, and any residual NMM (bp 115°C) is removed by evaporation or by aqueous extraction along with unreacted starting material (e.g., the free



SCHEME 2 Synthetic route to Fmoc-Pro-Pro-Gly-OH that requires no chromatography.

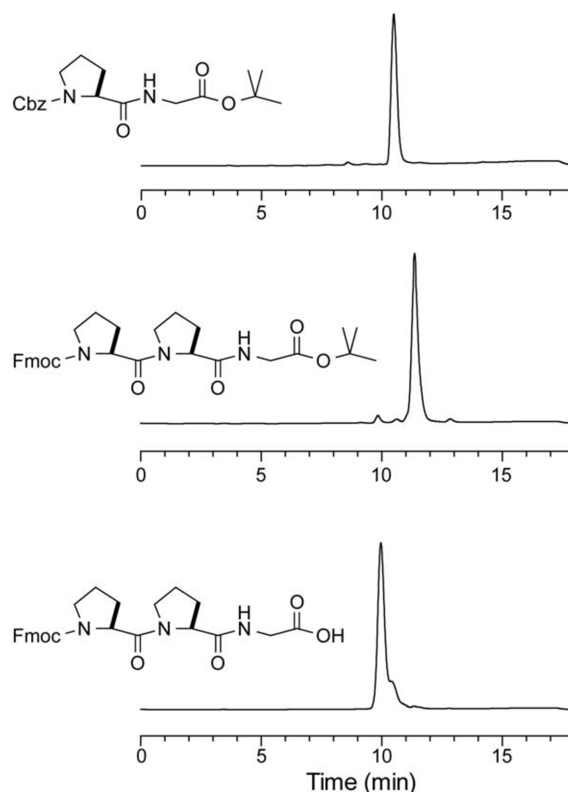


FIGURE 1 LCMS traces of products from each step in the chromatography-free synthesis of Fmoc-Pro-Pro-Gly-OH by the route in Scheme 2. Molecules were detected by asborance at 254 nm during LCMS with a reverse-phase column. Traces for the syntheses of other tripeptides are shown in Figure S1 of the Supporting Information.

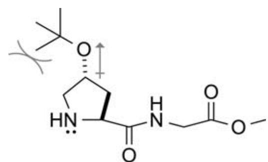


FIGURE 2 Steric and electronic effects that could diminish the reactivity of the amino group of Hyp(*t*Bu)-Gly-OMe.

amine and free acid, which are nonvolatile). This route provided Cbz-Pro-Gly-O*t*Bu in 92% yield.

Deprotection of the amino group of the dipeptide by hydrogenolysis was followed immediately by addition of the third amino acid, Fmoc-Pro-OPfp. Again, the byproduct, pentafluorophenol is soluble in the aqueous layer during an extraction. Pentafluorophenol (bp 143°C) also has modest volatility. Initially, we attempted this coupling reaction with *N*,*N*-diisopropylethylamine as the base in DMF. These conditions provided the desired tripeptide, but LCMS showed evidence of some proline oligomers, presumably from inadvertent Fmoc deprotection. Changing to a weaker base—NMM (which has a conjugate acid of pK_a 7.41[51]—in THF eliminated this problem and afforded Fmoc-Pro-Pro-Gly-O*t*Bu in 74% yield.

Finally, the *t*-butyl ester of Fmoc-Pro-Pro-Gly-O*t*Bu was removed with anhydrous HCl in dioxane, providing Fmoc-Pro-Pro-Gly-OH in 90% yield. In the analogous step en route to other tripeptides, selective base saponification of a methyl ester in a wet organic solvent containing LiOH (4 equiv) and $CaCl_2$ (15 equiv) afforded the desired carboxylic acid without removing the Fmoc group,[52] affording Fmoc-Gly-Pro-Pro-OH and Fmoc-Pro-Hyp(*t*Bu)-Gly-OH in 86% and 83% yield, respectively.

In lieu of chromatography, we used evaporation, extraction, and trituration to purify products. In the first coupling step, isobutyl chloroformate was the coupling agent, and evaporation and extraction were sufficient to provide a clean product. The second coupling and final deprotection steps also employed trituration with hexanes, which does not dissolve the tripeptide product. The analytical liquid chromatography traces for each step in the synthesis of Fmoc-Pro-Pro-Gly-OH exemplify the efficacy of these simple purification steps (Figure 1).

Like Fmoc-Pro-Pro-Gly-OH, three other tripeptides were obtained through this chromatography-free methodology (Table I). In general, coupling reactions proceeded in high yield and provided product of high purity. We did note with ^{1}H NMR spectroscopy that the reaction of HCl·Pro-OMe and isobutyl chloroformate generated a carbamate byproduct, which was not observed in any other coupling. The carbamate impurity was, however, not detrimental to subsequent steps and was readily removable by trituration, as shown by LCMS analysis and by NMR spectroscopy.

The reaction of Hyp(*t*Bu)-Gly-OMe with Fmoc-Pro-OPfp was atypical in providing a low (<50%) yield of product. We speculate that this deviation derives from particular steric and electronic attributes of Hyp(*t*Bu)-Gly-OMe (Figure 2). Its *t*-butyl protecting group is bulky and could interfere with amide bond formation. Moreover, the side-chain oxygen of Hyp is electron-withdrawing, thereby lowering the nucleophilicity of its amino group. Coupling to Hyp(*t*Bu) was likewise the lowest yielding step in a reported synthesis of Fmoc-Pro-Hyp-Gly-OH that was chromatography-free but used crystallization to purify most reactions products.[38] Yields might be improved by using an unprotected Hyp monomer.[38] Despite this one deviance, all other reactions with the secondary amine of proline were highly successful. Moreover, each tripeptide product was of suitable purity for SPPS.

In our work we have focused on the synthesis of amino acid trimers for CMPs. Nonetheless, we envision that the methodology herein could be applicable to other peptides as well. In this regard, the chromatography-free synthesis of Fmoc-Pro-Hyp(*t*Bu)-Gly-OH, which demonstrates tolerance for acid-labile protecting groups, is especially noteworthy. We note as well that the pentafluorophenyl ester of Fmoc-D-Pro is not available from commercial vendors. Accordingly, we used HBTU as the coupling agent in the chromatography-free synthesis of Fmoc-D-Pro-D-Pro-Gly-OH. These instances serve to showcase the generality of our procedures. Notably, our route avoids hydrogenation of a molecule containing an Fmoc protecting group, which is vulnerable to reduction.

Finally, we demonstrated the segment condensation of a tripeptide synthesized in solution without chromatography. Specifically, we coupled Fmoc-D-Pro-D-Pro-Gly-OH units to effect the solid-phase synthesis of Ac-Lys-(Ser-Gly)$_3$-(D-Pro-D-Pro-Gly)$_7$-OH on a solid support. A mass spectrum of the purified

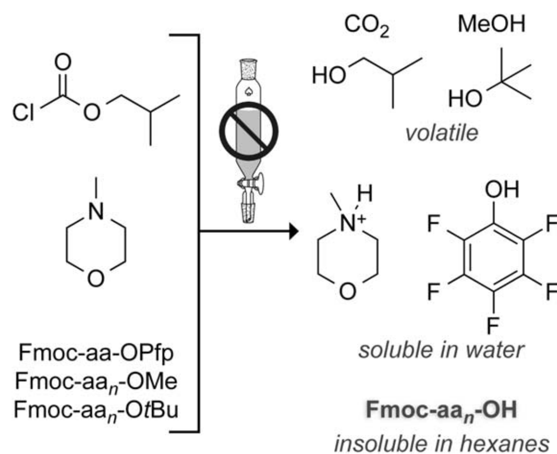


FIGURE 3 Summary of the physical properties of the products and byproducts from the reactions herein.

28-mer verified its integrity (Figure S2 of the Supporting Information).

CONCLUSIONS

We report on the synthesis of short peptides for use in SPPS by a novel combination of known reagents and reaction conditions. The methodology enables the isolation of products by using the facile techniques of evaporation, extraction, and trituration (Figure 3). Eliminating the need for purification by column chromatography reduces waste, reduces cost, and increases the ease of synthesis. This methodology is especially well suited for the synthesis of CMPs, which are in widespread use.[23–35]

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