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GAP Peptide Synthesis *via* Design of New GAP Protecting Group: An Fmoc/tBu Synthesis of Thymopentin Free from Polymers, Chromatography and Recrystallization

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Abstract

A novel method for Fmoc/tBu solution-phase peptide synthesis and the development of a new benzyl-type GAP protecting group is reported. This new GAP protecting group is utilized in place of a polymer support, facilitating C→N Fmoc peptide synthesis without chromatography, recrystallization, or polymer supports. The GAP group can be added and removed in high yield, and was used to synthesize over 1 gram of the immunostimulant, thymopentin, in high overall yield (83%) and purity (99%).

Keywords

Peptides; Amino Acids; Protecting Groups; GAP Chemistry; Thymopentin

Introduction

In recent years, our research group and others have made significant advancements in the area of purification chemistry, focusing specifically on avoiding column chromatography and recrystallization.^[1] This research and concept have been defined as Group-Assisted Purification (GAP) chemistry/technology as follows:^[1a] “*a chemistry for organic synthesis that avoids traditional purification methods such as chromatography and/or recrystallization by purposefully introducing a well-functionalized group in the starting material or in the newly generated product.*” As one can see from the definition, this research has the potential to encompass the entire field of synthetic organic chemistry. In our initial stage of this research, we focused on the development of chiral, N-phosphonyl and N-phosphinyl imine chemistry for the synthesis of chiral amines, and have found much success in this area.^[1b–i]

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By controlling solubility, the chiral amine products can be selectively precipitated from the crude mixture, thereby avoiding chromatography and recrystallization. In the second stage of this research, we are developing ways to extend this technology to other substrates and functional groups. In order to do this, we have taken the GAP properties of our chiral auxiliaries and, with modification, developed the concept of GAP protecting groups.

Protecting groups are found in almost every complex synthesis where multiple functional groups are present.^[2] Good protecting groups need to be robust to a wide variety of conditions, and must be added and removed with high yield.^[3] An ideal example for GAP chemistry would be one in which a semi-permanent protecting group introduced the necessary solubility characteristics required for GAP. The problem is, most traditional protecting groups are nonpolar, and therefore do not generate the required GAP solubility for most substrates. If a protecting group could be developed that generated adequate solubility control, then GAP chemistry could potentially be extended to all syntheses, which require the use of that protecting group.

One area where protecting groups are used extensively is in peptide synthesis, both for solid and solution phase approaches.^[4] Developed by Merrifield in the 1960's, Solid-Phase Peptide Synthesis (SPPS) has become a standard protocol used by multiple scientific disciplines for research and manufacturing.^[5] The advantages of the polymer support lie in its ability to allow facile purification of the growing peptide after each coupling/deprotection step, which avoids the use of column chromatography. The key disadvantage of SPPS lies in the difficulty of scale-up: many polymer supports are expensive, and occupy the vast majority of the mass of the material to be worked with.^[6] An ideal peptide synthesis would be one where the reaction occurred in solution phase, without the mass waste of polymer supports, but retained all of the purification benefits of SPPS. Recently, our group published a series of papers, which utilized our GAP technology as an alternative to both traditional solution-phase peptide synthesis (SolPPS) as well as SPPS, affording advantages of both methods.^[7] In this article, we report advancements in GAP peptide synthesis (GAP-PS) via the development of a new GAP benzyl-type protecting group for C-terminus protection (Scheme 1). In connection with C-terminus protection, we now report the ability to conduct GAP-PS using an Fmoc/tBu strategy, which is the most used method in SPPS due to its mild deprotection protocols.^[8] This strategy is currently almost entirely restricted to SPPS due to the formation of N-fluorenylmethylpiperidine (NFMP) as a side product during deprotection, which is difficult to remove without polymer supports. We are pleased to report that our GAP technology can easily provide over 1 gram of the target peptide, thymopentin, in 83% overall yield and 99% purity via utilization of a solution-phase Fmoc/tBu strategy. Protection of various amino acids with this new protecting group has also been achieved in consistent quantitative yield.

Results and Discussion

In designing the new protecting group, it was apparent that the GAP-functionalized segment of the protecting group would need to be stable to a wide variety of conditions. Considerations were taken that it must provide the necessary solubility characteristics for GAP chemistry. Also, it must work efficiently and orthogonally with the reactivity of current

protection strategies. With these goals in mind, we decided to modify the already successful benzyl protecting group,^[3] in order to keep the desirable reactivity while introducing the GAP group. The GAP group chosen is diphenylphosphine oxide, due to our previous success with phosphine oxide groups using GAP chemistry.^[1] Also, attachment of this group onto the para position of the benzyl group creates a triphenylphosphine oxide moiety, which is widely known in the literature to be stable to an extensive variety of conditions. This stability is necessary to avoid interference with the multiple deprotection conditions that the substrate may be exposed to, thereby establishing true orthogonality.

Our synthesis of this new protecting group begins with commercially available, diphenyl(p-tolyl)phosphine **1** (Scheme 2). Oxidation of **1** with potassium permanganate provides benzoic acid **2**, as well as the GAP group through phosphine oxidation. We have abbreviated this GAP group Dpp, short for **diphenylphosphine oxide**. Esterification followed by borohydride reduction affords the GAP-equipped benzyl alcohol **4**, or “HOBndpp,” in high yield. Next, we decided to test the orthogonality and the GAP capabilities of this new protecting group. Protection of Boc-Phe-OH was both facile and quantitative using EDCI as the carbodiimide coupling reagent (Scheme 3). The product **5a** can be selectively precipitated from an ethyl acetate/petroleum ether solvent mixture as a white solid, thereby satisfying the requirements of GAP chemistry. Deprotection of the Boc group was also quantitative, and did not result in any loss of the Bndpp group. The Bndpp group can be easily removed using catalytic hydrogenation, and also can be recovered and recycled as “HBndpp” **7a** for reuse after washing away the deprotected amino acid. Subjection of **7a** to permanganate oxidation affords **2**, which can be transformed into HOBndpp **4** as previously mentioned (Scheme 2).

A full substrate scope for amino acid protection is shown below in Table 1, with consistent quantitative yields for the protection of a variety of Boc and Fmoc amino acids with varying side-chain protecting groups. Of note is the quantitative protection of tryptophan, arginine, valine, and cysteine.

For a first application of this new protecting group, we decided to test its capabilities in handling an Fmoc/tBu SolPPS strategy. Our target peptide of interest is thymopentin, a pharmacologically interesting, biologically active pentapeptide subunit of the immunomodulatory polypeptide, thymopoietin.^[9] For a short peptide, thymopentin contains amino acids with a variety of functional groups (1 aromatic, two basic (one with guanidine), two acidic, and one β -branched). This makes thymopentin an ideal candidate for a first test of our GAP protecting group and its ability to tolerate the removal of several side-chain protecting groups. Our synthesis of thymopentin is illustrated in Scheme 4. Compound **5k** is first treated with 30% piperidine in DCM for 10 minutes to remove the Fmoc group, followed by ammonium chloride wash to remove the excess piperidine. The DCM layer (after drying) is directly loaded with the next Fmoc amino acid (side chain protection as noted), along with TBTU coupling reagent and DIPEA. After coupling for 20 minutes, the reaction mixture is washed with ammonium chloride and 0.5 M sodium hydroxide (respectively), dried and evacuated. The crude product after coupling contains several impurities, most notably NFMP and tetramethyl urea (from coupling). The GAP purification procedure can easily remove these impurities simply by dissolving the mixture in a minimal

amount of ethyl acetate, followed by selective precipitation of the GAP-peptide with petroleum ether. Product **6k** was obtained in 99% yield (based on starting mass of **5k**). This same procedure was utilized for all of the peptide couplings. For the tetra- and pentapeptide fragments, a small amount of DCM is added to the ethyl acetate prior to GAP precipitation, to help with the solubility. Following the last coupling step and the synthesis of **9k**, the last Fmoc group is removed as before but after workup, the DCM layer is concentrated and the peptide is dissolved in TFA/DCM/H₂O (6/3/1) solution for side-chain deprotection. The pentapeptide **10k** (now with Bndpp as the only protecting group) is precipitated using diethyl ether. This peptide is then subjected to hydrogenation and the GAP group removed. The product is isolated via extraction from chloroform with 10% acetic acid (aq). To our delight, HPLC analysis of the product peptide reveals that the compound is nearly 99% pure without any column chromatography, recrystallization, or polymer supports. The GAP group can be recovered simply by evacuating the chloroform layer after extraction. Subjecting this raw material to the synthesis methods in Scheme 2 can regenerate BndppOH.

Conclusions

In summary, we have provided an expansion to GAP-PS, which allows for the mild, Fmoc/tBu synthesis of thymopentin. A new benzyl-type protecting group, Bndpp, has been developed which facilitates GAP-PS and avoids chromatography, recrystallization, and polymer supports, providing the target peptide in both high yield (83%) and purity (99%). Future work in this area includes the development of Boc-, tBu-, Trt, and Cbz-type GAP protecting groups as well as the extension of these new GAP protecting groups to small molecule synthesis. Also worthy of note is the ability of GAP-PS to adapt to convergent synthesis, and research on using this method for the synthesis of larger peptides with few sequential steps is ongoing in our laboratories.

Experimental Section

General methods

All solvents were ACS grade and used without additional purification. HRMS analysis was performed using an Orbitrap mass analyzer. HPLC analysis was conducted using a Perkin Elmer Flexar isocratic pump equipped with a UV deuterium lamp detector. Fmoc and Boc protected amino acids were purchased from BachemBio and used directly for coupling.

Synthesis of benzoic acid **2**

10.0 g **1** was placed in a 500 mL round-bottomed flask, followed by 130 mL 0.43 M NaOH(aq) solution and then 22.2 g KMnO₄. The reaction was stirred at reflux for 12 hours, after which the reaction mixture was filtered through celite while hot. The resulting solution was washed X2 with diethyl ether, followed by the addition of 50% H₂SO₄ to precipitate the product. After filtration, benzoic acid **2** was collected as a white solid; yield, 10.8 g, 93%; this product was directly subjected to the next reaction.

Synthesis of ester **3**

10.8 g **2** was placed in a 500 mL round-bottomed flask along with 300 mL ethanol and 3 mL thionyl chloride. The reaction was brought to reflux and stirred for 12 hours. After completion, the reaction was cooled to room temperature and the volatiles evacuated, affording ester **3** as a white solid; yield, 11.8 g, 99%; this product was directly subjected to the next reaction.

Synthesis of BndppOH **4**

11.8 g ester **3** was placed in a 500 mL round-bottomed flask along with 300 mL ethanol. The reaction was cooled to 0 °C, after which 3.82 g NaBH₄ was added portionwise. The reaction was brought to room temperature and stirred for 12 hours. The solvent was evacuated, followed by solvation of the crude in DCM and washing X3 with 2 M HCl(aq). The organic layer was then dried over MgSO₄, filtered, and evacuated to afford BndppOH **4** as a white solid; yield, 9.96 g, 96%; this compound has been previously synthesized via a different method, and NMR data matches that found in the literature:^[10] ¹H NMR (400 MHz, CDCl₃) δ = 7.62 – 7.57 (m, 4H), 7.54 – 7.47 (m, 4H), 7.45 – 7.40 (m, 4H), 7.38 – 7.36 (m, 2H), 4.70 (s, 2H).

General procedure for Bndpp protection

100 mg BndppOH **4**, 2.0eq PG-AA-OH, and 10 mL DCM were stirred at 0 °C in a 20 mL screw-cap vial. 124 mg (2.0 eq) EDCI(HCl) was added, and the reaction was stirred for 10 min, at which point 4 mg (10 mol%) DMAP was added and the reaction was brought to room temp and stirred for 2 hours. The reaction mixture was washed X2 with sat. NH₄Cl(aq), followed by sat. Na₂CO₃(aq) X2. The combined organic layers were dried with MgSO₄, filtered, and evacuated to afford the crude protected amino acid. GAP purification was performed by dissolving the crude mixture in a minimal amount of ethyl acetate, followed by precipitation with petroleum ether and filtration of the resulting white precipitate. This same procedure was used for every substrate except **5k**, where the reaction was conducted on a larger scale using 600 mg BndppOH **4** and the same equivalents of the other reagents as before.

Compound **5a**

White solid; yield 180 mg, 99%; mp 62 – 63 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.69 – 7.63 (m, 6H), 7.58 – 7.52 (m, 2H), 7.49 – 7.45 (m, 4H), 7.35 – 7.32 (m, 2H), 7.24 – 7.18 (m, 3H), 7.07 – 7.05 (d, J = 6.4 Hz, 2H), 5.20 – 5.12 (m, 2H), 4.96 – 4.95 (d, J = 7.8 Hz, 1H), 4.68 – 4.58 (m, 1H), 3.09 – 3.07 (d, J = 5.9 Hz, 2H), 1.40 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ = 171.9, 155.2, 139.3, 135.9, 133.0, 132.6, 132.5, 132.2, 132.1, 132.0, 129.4, 128.8, 128.6, 128.2, 128.1, 127.2, 80.2, 66.3, 54.6, 38.5, 28.4; ³¹P NMR (162 MHz, CDCl₃) δ = 29.28; HRMS (ESI): m/z calcd for [C₃₃H₃₄NO₅P + H]⁺: 556.2253, found: 556.2235.

Compound **5b**

White solid; yield 189 mg, 99%; mp 76 – 77 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.69 – 7.64 (m, 6H), 7.58 – 7.54 (m, 2H), 7.49 – 7.44 (m, 6H), 6.65 (bs, 1H), 5.52 – 5.50 (d, J = 5.9 Hz, 1H), 5.27 – 5.19 (m, 2H), 4.54 (bs, 1H), 4.38 – 4.32 (m, 2H), 3.09 – 2.91 (m, 2H), 2.06

– 2.00 (m, 1H), 1.98 (s, 3H), 1.43 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ = 170.9, 170.4, 139.3, 133.5, 132.8, 132.6, 132.5, 132.4, 132.2, 132.1, 131.8, 128.7, 128.2, 80.7, 66.7, 54.2, 42.2, 34.5, 28.4, 23.3, 22.5, 14.2; ³¹P NMR (162 MHz, CDCl₃) δ = 29.46; HRMS (ESI): m/z calcd for [C₃₀H₃₅N₂O₆PS + H]⁺: 583.2032, found: 583.2012.

Compound 5c

White solid; yield 246 mg, 99%; mp 86 – 87 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.76 – 7.74 (d, J = 7.5 Hz, 2H), 7.69 – 7.63 (m, 6H), 7.60 – 7.52 (m, 4H), 7.47 – 7.42 (m, 6H), 7.40 – 7.36 (t, J = 7.4 Hz, 2H), 7.31 – 7.27 (t, J = 7.4 Hz, 2H), 5.48 – 5.46 (d, J = 7.3 Hz, 1H), 5.22 (s, 2H), 4.65 – 4.57 (bs, 1H), 4.43 – 4.34 (m, 3H), 4.22 – 4.19 (t, J = 6.9 Hz, 1H), 3.10 – 3.02 (m, 2H), 1.88 – 1.84 (m, 1H), 1.72 – 1.68 (m, 1H), 1.42 (s, 9H), 1.38 – 1.24 (m, 4H); ¹³C NMR (100 MHz, CDCl₃) δ = 172.4, 156.2, 143.9, 141.4, 139.5, 132.9, 132.6, 132.2, 131.8, 128.7, 128.0, 127.8, 127.2, 125.2, 120.1, 79.3, 67.2, 66.4, 54.0, 47.3, 40.0, 32.1, 29.8, 28.5, 22.5; ³¹P NMR (162 MHz, CDCl₃) δ = 29.37; HRMS (ESI): m/z calcd for [C₄₅H₄₇N₂O₇P + H]⁺: 759.3199, found: 759.3183.

Compound 5d

White solid; yield 227 mg, 99%; mp 85 – 86 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.76 – 7.74 (d, J = 7.5 Hz, 2H), 7.66 – 7.52 (m, 10H), 7.46 – 7.36 (m, 8H), 7.29 – 7.26 (t, J = 7.2 Hz, 2H), 5.86 – 5.84 (d, J = 8.6 Hz, 1H), 5.29 – 5.20 (dd, J = 12.8 Hz, 12.4 Hz, 2H), 4.69 – 4.66 (m, 1H), 4.44 – 4.31 (m, 2H), 4.24 – 4.21 (t, J = 7.0 Hz, 1H), 3.01 – 2.95 (dd, J = 4.3 Hz, 17.0 Hz, 1H), 2.81 – 2.76 (dd, J = 4.2 Hz, 17.0 Hz, 1H), 1.39 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ = 170.9, 170.2, 156.1, 143.9, 143.8, 141.4, 139.5, 132.7, 132.6, 132.5, 132.2, 132.1, 131.7, 128.7, 128.6, 128.0, 127.9, 127.2, 125.2, 120.1, 82.1, 67.4, 66.7, 50.7, 47.2, 37.8, 28.1; ³¹P NMR (162 MHz, CDCl₃) δ = 29.75; HRMS (ESI): m/z calcd for [C₄₂H₄₀N₂O₇P + H]⁺: 702.2621, found: 702.2602.

Compound 5e

White solid; yield 257 mg, 97%; mp 98 – 99 °C; ¹H NMR (400 MHz, CDCl₃) δ = 8.10 – 8.08 (d, J = 7.7 Hz, 1H), 7.76 – 7.74 (d, J = 7.5 Hz, 2H), 7.68 – 7.61 (m, 6H), 7.56 – 7.36 (m, 14H), 7.31 – 7.25 (m, 3H), 7.21 – 7.18 (t, J = 7.5 Hz, 1H), 5.48 – 5.46 (d, J = 8.2 Hz, 1H), 5.21 – 5.06 (dd, J = 12.9, 47.8 Hz, 2H), 4.84 – 4.79 (m, 1H), 4.41 – 4.34 (m, 2H), 4.22 – 4.18 (t, J = 7.0 Hz, 1H), 3.28 – 3.27 (d, J = 5.7 Hz, 2H), 1.63 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ = 171.6, 155.8, 149.6, 143.9, 143.8, 141.4, 139.1, 135.5, 132.9, 132.6, 132.5, 132.2, 132.1, 131.8, 130.4, 128.7, 128.6, 127.8, 127.2, 125.2, 124.8, 124.3, 122.8, 120.1, 118.9, 115.5, 114.8, 84.0, 67.4, 66.6, 54.3, 47.2, 28.2; ³¹P NMR (162 MHz, CDCl₃) δ = 29.32; HRMS (ESI): m/z calcd for [C₅₀H₄₅N₂O₇P + H]⁺: 817.3043, found: 817.3031.

Compound 5f

White solid; yield 304 mg, 99%; mp 117 – 118 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.75 – 7.73 (d, J = 7.5 Hz, 2H), 7.69 – 7.63 (m, 4H), 7.60 – 7.55 (m, 4H), 7.53 – 7.46 (m, 8H), 7.39 – 7.35 (t, J = 7.4 Hz, 2H), 7.29 – 7.27 (d, J = 7.4 Hz, 2H), 6.61 (bs, 2H), 5.88 (bs, 1H), 5.50 – 5.35 (dd, J₁ = 9.7 Hz, J₂ = 52.8 Hz, 2H), 5.03 – 5.00 (d, J = 11.8 Hz, 1H), 4.36 – 4.34 (m, 3H), 4.20 – 4.16 (t, J = 7.0 Hz, 1H), 3.25 – 3.15 (m, 2H), 2.90 (s, 2H), 2.78 – 2.67 (m, 2H),

2.58 (s, 3H), 2.51 (s, 3H), 2.06 (s, 3H), 1.68 – 1.57 (m, 2H), 1.42 (s, 6H); ¹³C NMR (100 MHz, CDCl₃) δ = 172.0, 158.6, 156.6, 156.2, 143.8, 141.3, 140.0, 138.3, 133.3, 132.5, 132.4, 132.2, 132.0, 131.9, 131.8, 130.8, 128.9, 128.8, 127.8, 127.2, 125.2, 124.6, 121.1, 120.0, 119.8, 117.4, 86.4, 68.0, 67.2, 66.2, 53.5, 47.1, 43.3, 40.5, 29.6, 28.6, 25.2, 19.4, 18.1, 12.6; ³¹P NMR (162 MHz, CDCl₃) δ = 31.03; HRMS (ESI): m/z calcd for [C₅₃H₅₅N₄O₈PS + H]⁺: 939.3556, found: 939.3538.

Compound 5g

White solid; yield 204 mg, 99%; mp 81 – 82 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.77 – 7.75 (d, J = 7.2 Hz, 2H), 7.70 – 7.53 (m, 10H), 7.48 – 7.44 (m, 6H), 7.41 – 7.37 (t, J = 7.2 Hz, 2H), 7.32 – 7.28 (t, J = 7.2 Hz, 2H), 5.36 – 5.34 (d, J = 8.8 Hz, 1H), 5.22 (s, 2H), 4.44 – 4.32 (m, 3H), 4.24 – 4.21 (t, J = 6.8 Hz, 1H), 2.26 – 2.17 (m, 1H), 0.97 – 0.95 (d, J = 6.8 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ = 172.0, 156.3, 143.9, 143.8, 141.4, 139.4, 132.6, 132.5, 132.2, 132.1, 128.7, 128.6, 128.1, 128.0, 127.8, 127.1, 125.1, 120.1, 67.1, 66.2, 59.1, 47.2, 31.3, 19.1, 17.6; ³¹P NMR (162 MHz, CDCl₃) δ = 29.45; HRMS (ESI): m/z calcd for [C₃₉H₃₆N₅O₅P + H]⁺: 630.2409, found: 630.2392.

Compound 5h

White solid; yield 287 mg, 99%; mp 121 – 122 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.76 – 7.71 (t, J = 6.4 Hz, 2H), 7.65 – 7.51 (m, 12H), 7.46 – 7.40 (m, 4H), 7.38 – 7.31 (m, 4H), 7.24 – 7.20 (m, 9H), 7.15 – 7.13 (m, 6H), 6.75 (s, 1H), 6.13 – 6.11 (d, J = 8.8 Hz, 1H), 5.21 – 5.11 (q, J = 12.8 Hz, 2H), 4.69 – 4.65 (m, 1H), 4.43 – 4.38 (m, 1H), 4.30 – 4.26 (t, J = 8.9 Hz, 1H), 4.20 – 4.16 (t, J = 7.1 Hz, 1H), 3.18 – 3.13 (dd, J₁ = 4.2 Hz, J₂ = 15.8 Hz, 1H), 2.87 – 2.82 (dd, J₁ = 4.2 Hz, J₂ = 15.8 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃) δ = 171.0, 169.4, 156.4, 144.3, 144.0, 143.8, 141.4, 139.6, 132.6, 132.5, 132.2, 132.0, 128.7, 128.6, 128.2, 127.9, 127.6, 127.5, 127.4, 127.2, 125.3, 120.1, 71.1, 67.4, 66.6, 51.2, 47.2, 38.8; ³¹P NMR (162 MHz, CDCl₃) δ = 29.38; HRMS (ESI): m/z calcd for [C₅₇H₄₇N₂O₆P + H]⁺: 887.3250, found: 887.3230.

Compound 5i

White solid; yield 195 mg, 99%; mp 78 – 79 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.76 – 7.75 (d, J = 7.6 Hz, 2H), 7.70 – 7.63 (m, 6H), 7.59 – 7.53 (m, 4H), 7.48 – 7.37 (m, 8H), 7.31 – 7.28 (t, J = 7.6 Hz, 2H), 5.37 – 5.35 (d, J = 7.6 Hz, 1H), 5.23 (s, 2H), 4.49 – 4.38 (m, 3H), 4.23 – 4.19 (t, J = 7.2 Hz, 1H), 1.46 – 1.44 (d, J = 7.2 Hz, 3H); ¹³C NMR (100 MHz, CDCl₃) δ = 172.9, 155.8, 144.0, 143.8, 141.4, 139.5, 132.9, 132.6, 132.5, 132.2, 132.1, 131.9, 128.7, 128.6, 127.9, 127.8, 127.2, 125.2, 120.1, 67.2, 66.4, 53.6, 49.8, 47.3, 31.7, 22.8, 18.7, 14.3; ³¹P NMR (162 MHz, CDCl₃) δ = 29.28; HRMS (ESI): m/z calcd for [C₃₇H₃₂N₅O₅P + H]⁺: 602.2096, found: 602.2080.

Compound 5j

White solid; yield 189 mg, 99%; mp 79 – 80 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.76 – 7.75 (d, J = 7.5 Hz, 2H), 7.70 – 7.63 (m, 6H), 7.60 – 7.53 (m, 4H), 7.48 – 7.42 (m, 6H), 7.41 – 7.37 (t, J = 7.5 Hz, 2H), 7.31 – 7.27 (t, J = 7.4 Hz, 2H), 5.42 – 5.37 (m, 1H), 5.23 (s, 2H), 4.41 – 4.39 (d, J = 7.1 Hz, 2H), 4.24 – 4.21 (t, J = 7.0 Hz, 1H), 4.06 – 4.05 (d, J = 5.6 Hz,

2H); ¹³C NMR (100 MHz, CDCl₃) δ = 169.9, 156.4, 143.9, 141.4, 139.3, 132.9, 132.6, 132.2, 132.1, 131.8, 128.7, 128.6, 128.1, 128.0, 127.9, 127.2, 125.2, 120.1, 67.4, 66.4, 47.2, 42.9; ³¹P NMR (162 MHz, CDCl₃) δ = 29.33; HRMS (ESI): m/z calcd for [C₃₆H₃₀NO₅P + H]⁺: 588.1940, found: 588.1925.

Compound 5k

White solid; yield, 99%; mp 99 – 100 °C; ¹H NMR (400 MHz, CDCl₃) δ = 7.77 – 7.75 (d, J = 7.6 Hz, 2H), 7.69 – 7.64 (m, 6H), 7.56 – 7.53 (t, J = 7.4 Hz, 4H), 7.48 – 7.44 (m, 4H), 7.41 – 7.36 (m, 4H), 7.31 – 7.27 (t, J = 7.4 Hz, 2H), 6.95 – 6.93 (d, J = 8.4 Hz, 2H), 6.87 – 6.85 (d, J = 8.4 Hz, 2H), 5.28 – 5.26 (d, J = 7.9 Hz, 1H), 5.22 – 5.13 (q, J = 8.5 Hz, 2H), 4.70 – 4.68 (m, 1H), 4.44 – 4.32 (m, 2H), 4.21 – 4.18 (t, J = 6.9 Hz, 1H), 3.09 – 3.06 (m, 2H), 1.30 (s, 9H); ¹³C NMR (100 MHz, CDCl₃) δ = 171.5, 155.7, 154.7, 143.9, 143.8, 141.4, 139.2, 132.9, 132.6, 132.5, 132.2, 132.1, 129.9, 128.8, 128.6, 128.2, 128.0, 127.9, 127.2, 125.2, 124.3, 120.1, 78.6, 67.1, 66.5, 55.0, 47.3, 37.8, 28.9; ³¹P NMR (162 MHz, CDCl₃) δ = 29.29; HRMS (ESI): m/z calcd for [C₄₇H₄₄NO₆P + H]⁺: 750.2984, found: 750.2966.

Synthesis of compound 6a

Boc-Phe-OBndpp **5a** (80 mg) was dissolved in 5 mL 60% TFA/DCM and stirred at room temperature. After 1 hour, the solvent mixture was evacuated, and the crude dissolved in DCM. After washing X2 with 1 M HCl(aq), the organic layer was dried with MgSO₄, filtered, and concentrated to afford crude **6a** HCl salt. GAP purification was conducted by dissolving the crude in a minimal amount of ethyl acetate, followed by precipitation with petroleum ether. The purified product was isolated via filtration as a white solid; yield 71 mg, 99%; mp 68 – 71 °C (decomposition); ¹H NMR (400 MHz, CDCl₃) δ = 7.57 – 7.40 (m, 12 H), 7.10 – 7.04 (m, 7H), 4.99 – 4.96 (d, J = 10.4 Hz, 2H), 4.41 (bs, 1H), 3.43 (bs, 1H), 3.25 (bs, 1H); ¹³C NMR (100 MHz, CDCl₃) δ = 169.1, 138.6, 134.3, 132.5, 132.3, 132.2, 132.1, 132.0, 131.5, 129.6, 128.8, 128.7, 128.6, 128.3, 128.2, 127.5, 67.0, 54.6, 36.6; ³¹P NMR (162 MHz, CDCl₃) δ = 29.79; HRMS (ESI): m/z calcd for [C₂₈H₂₆NO₃P + H]⁺: 456.1729, found: 456.1725.

Synthesis of HBndpp 7a

Boc-Phe-OBndpp **5a** (100 mg) was dissolved in a 5 mL mixture of methanol and 10% Pd/C (20 mg). The reaction mixture was placed under H₂ atmosphere (balloon) and stirred at room temperature for 12 hours. The reaction mixture was then filtered through celite and the methanol evacuated. The crude solid was dissolved in DCM and washed X2 with sat. Na₂CO₃(aq) solution. The organic layer was dried over MgSO₄, filtered, and evacuated to afford HBndpp **7a** as a white solid; yield, 51 mg, 97%; this compound has been previously synthesized via a different method, and NMR data matches that found in the literature:^[10] ¹H NMR (400 MHz, CDCl₃) δ = 7.68 – 7.63 (m, 4H), 7.57 – 7.52 (m, 4H), 7.48 – 7.44 (m, 4H), 7.29 – 7.26 (m, 2H), 2.41 (s, 3H).

General procedure for Fmoc deprotection and coupling

Fmoc-(AA)_n-OBndpp dissolved in 30% Piperidine/DCM (100 mL per gram), and stirred at room temperature for 10 minutes. Reaction mixture washed X3 with sat. NH₄Cl(aq), dried

over MgSO_4 , and filtered. To the resulting DCM solution was added 1.2 eq TBTU, 1.2eq Fmoc-AA-OH, and 2.4 eq DIPEA; the coupling reaction was stirred for 20 min. The reaction mixture was then washed X2 with sat. $\text{NH}_4\text{Cl}(\text{aq})$, followed by 0.5 M NaOH X2. The combined organic layers were dried over MgSO_4 , filtered, and evacuated to afford the crude peptide. GAP purification was performed by dissolving the crude mixture (containing Fmoc-(AA) $n+1$ -OBndpp, NFMP, and tetramethylurea) in a minimal amount of ethyl acetate (with some DCM for longer peptides), followed by precipitation of the product with petroleum ether. The product peptide was removed via vacuum filtration as a white solid in quantitative yield.

Compound 9k

Fmoc-Arg(Pbf)-Lys(Boc)-Asp(tBu)-Val-Tyr(tBu)-OBndpp. White solid; yield 3.08 g, 97% (over 3 steps from 6k, represents yield of total material, of which 92% was found to be **9k**); mp 124 – 125 °C; Retention time on analytical NP-HPLC with 0.1% ethanolamine in IPA as the eluent: 8.85 min, 92.0% purity; HRMS (ESI): m/z calcd for $[\text{C}_{90}\text{H}_{114}\text{N}_{9}\text{O}_{17}\text{PS} + \text{H}]^+$: 1657.7903, found: 1657.7871.

Deprotection of side-chain protecting groups

Fmoc-Arg(Pbf)-Lys(Boc)-Asp(tBu)-Val-Tyr(tBu)-OBndpp **9k** was dissolved in 100 mL 30% Piperidine/DCM and stirred at room temp for 10 minutes. The reaction mixture was then washed X2 with saturated $\text{NH}_4\text{Cl}(\text{aq})$, dried over MgSO_4 , filtered and evacuated. The crude was then dissolved in TFA/DCM/ H_2O (6/3/1) and stirred at room temp for 1 hour. The reaction mixture was evacuated to saturation, and then the product peptide precipitated with diethyl ether. Peptide **10k** was obtained after filtration as a white solid and directly used for the next step.

Deprotection of Bndpp

To 100 mg dry Pd/C in a hydrogenation bottle was added H-RKDVY-OBndpp **10k** in 150 mL methanol. The bottle was placed under 70 PSI H_2 atmosphere and shaken at room temperature for 24 hours. The reaction mixture was filtered through celite, and evacuated to dryness. The crude was dissolved in a mixture of 10% acetic acid (aq) and chloroform, after which the aqueous layer was washed X2 with chloroform. Evacuation of the aqueous layer afforded thymopentin as a white solid; yield, 1.09 g, 87%; Retention time on analytical RP-HPLC with 50% MeCN in 0.06% TFA/ H_2O as the eluent: 1.24 min, 98.9% purity; HRMS (ESI): m/z calcd for $[\text{C}_{30}\text{H}_{49}\text{N}_9\text{O}_9 + \text{H}]^+$: 680.3731, found: 680.3730.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Previous Work: **This Work:**

Scheme 1.
Concept Illustration.

Scheme 2.
Synthesis of HOBndpp.

Scheme 3.
Test of Orthogonality.

Scheme 4.
Synthesis of Thymopentin.

Table 1

Substrate Scope for Protection.

Product	PG-	-AA-	Yield ^[a]
5a	Boc-	-Phe-	99%
5b	Boc-	-Cys(Acm)-	99%
5c	Fmoc-	-Lys(Boc)-	99%
5d	Fmoc-	-Asp(<i>t</i> Bu)-	99%
5e	Fmoc-	-Trp(Boc)-	97%
5f	Fmoc-	-Arg(Pbf)-	99%
5g	Fmoc-	-Val-	99%
5h	Fmoc-	-Asn(Trt)-	99%
5i	Fmoc-	-Ala-	99%
5j	Fmoc-	-Gly-	99%
5k	Fmoc-	-Tyr(<i>t</i> Bu)-	99%

^[a] Isolated yield after GAP precipitation.