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Investigation of Unanticipated Alkylation at the N(π) Position of a Histidyl Residue Under Mitsunobu Conditions and Synthesis of Orthogonally Protected Histidine Analogues

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Abstract

We had previously reported that Mitsunobu-based introduction of alkyl substituents onto the imidazole N(π)-position of a key histidine residue in phosphothreonine-containing peptides can impart high binding affinity against the polo box domain of polo like kinase 1. Our current paper investigates the mechanism leading to this N(π)-alkylation and provides synthetic methodologies that permit the facile synthesis of histidine N(π)-modified peptides. These agents represent new and potentially important tools for biological studies.

Introduction

The serine/threonine specific polo-like kinase 1 (Plk1) is one of four Plks involved in cell cycle regulation. Plk1 is of particular interest as a therapeutic target, due to its ability to promote oncogenic transformation.^{1–3} Pharmacological inhibition of Plk1 is being approached through kinase catalytic site-directed agents.^{4–11} Inhibitors of Plk1 function are also being investigated that interfere with the actions of its C-terminal polo-box domains (PBDs). These latter homologous protein modules facilitate sub-cellular localization of Plk1 by recognizing and binding to phosphoserine (pSer)/phosphothreonine (pThr)-containing sequences.^{12,13}

The polo-box interacting protein 1 (PBIP1) is a Plk1 substrate, which undergoes phosphorylation at its T78 residue to form a Plk1 PBD-binding site that is critical for recruiting Plk1 to the interphase and mitotic kinetochores.¹⁴ We had previously shown that the wild-type PBIP1-derived five-mer sequence, 74-PLHSpT-78 represents a minimal peptide (**1**) that specifically interacts with the Plk1 PBD with high affinity ($K_d = 0.45 \mu\text{M}$).¹⁵ More recently, in an effort to investigate whether reduction in the phosphoryl anionic charge could also be accomplished with retention of binding affinity, we performed synthetic experiments to esterify one of the two pThr phosphoryl hydroxyls of the parent peptide **1**.¹⁶ Our approach was to conduct solid-phase Mitsunobu coupling¹⁷ of a series of alcohols with the protected resin-bound peptide, Ac-Pro-Leu-His[N(τ)Trt]-Ser(OBu^t)-Thr[OPO(OBn)(OH)]-amide,¹⁸ which contained a single free phosphoryl hydroxy group (Figure 1). Applications of Mitsunobu chemistries to resin-supported substrates and to the synthesis of phosphoryl esters are known.^{19,20}

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Supporting information: ¹H and ¹³C NMR spectra of **7**, **8**, **9**, and **11**, including 1D NOESY assignment of N(π)-alkylation in **11** and HPLC charts of peptide **2**, **3**, **4**, **5**, **13**. This material is available free of charge via the Internet at <http://pubs.acs.org/>.

Following coupling reactions, peptides were cleaved from the resin under mild acid conditions. In addition to the expected phosphoryl esters, unanticipated synthetic peptide byproducts were isolated having the same molecular weights as the intended products. These byproducts exhibited exceptionally high affinities in Plk1 PBD binding assays and in some these affinities were approximately three orders of magnitude greater than the parent peptide (**1**). A Plk1 PBD co-crystal structure of the highest affinity peptide byproduct, which was obtained in small amount along with the expected major product (**2**) following Mitsunobu reaction with $C_6H_5(CH_2)_8-OH$, showed it to be the modified peptide **3**, in which alkylation had occurred on the histidine imidazole $N(\pi)$ group (Figure 2).¹⁶ The high affinity and unexpected nature of this histidine adduct prompted us to investigate potential mechanisms leading to its formation and to develop synthetic methodologies to prepare related peptide analogues.

Results and Discussion

We rationalized that alkylation at the His $N(\pi)$ of peptide **3** could potentially have occurred either indirectly by intramolecular alkyl transfer of the phosphoryl ester group (Mechanism 1, Figure 3), or directly through alkylation by the alcohol reactants under Mitsunobu conditions (Mechanism 2, Figure 3). There are literature precedents for both mechanisms. For example, in 1-substituted imidazoles, alkylation of the 3-nitrogen (corresponding to the $N(\pi)$ position of histidine) can be effected by phosphoryl esters. However these reactions require elevated temperatures.^{21,22} Similarly, alkylation of imidazole by alcohols can be achieved using Mitsunobu chemistries.^{23–25}

In order to examine whether phosphoryl ester-mediated transfer was involved in the formation of **3** (Mechanism 1), Mitsunobu esterification of the free phosphoryl hydroxyl of resin-bound $N(\alpha)$ -Fmoc-O-(benzyl phosphoryl)-threonine using $C_6H_5(CH_2)_8-OH$ was conducted prior to coupling of the $N(\alpha)$ -Fmoc-[$N(\tau)$ -Trt]-His residue. Following Mitsunobu phosphoryl ester formation, the resin was washed and peptide synthesis was completed in standard fashion using Fmoc protocols. A portion of the resulting finished resin was cleaved and examined by HPLC. The chromatogram showed the presence of the peptide phosphoryl ester **2** with no evidence of product **3**. This indicated that $N(\pi)$ alkylation did not occur by intramolecular transfer from the phosphoryl ester.

Direct Mitsunobu-induced $N(\pi)$ alkylation (Mechanism 2) was examined next by again subjecting a portion of the above, fully-formed resin-bound peptide to a second Mitsunobu coupling with $C_6H_5(CH_2)_8-OH$ prior to resin cleavage. HPLC analysis of the resulting peptide products showed that in addition to **2**, a new product was obtained having mass consistent with bis-alkylated product (**4**, Figure 2). This indicated that histidine alkylation had occurred during the second Mitsunobu reaction. In order to unambiguously discriminate whether the additional alkyl adduct originated directly from the alcohol used in the Mitsunobu reaction or from pre-formed phosphoryl ester under Mitsunobu catalysis, the above reaction was repeated using a homologous alcohol having a shorter chain length [$C_6H_5(CH_2)_7-OH$] as compared with the $C_6H_5(CH_2)_8-OH$ used in the second Mitsunobu reaction. This allowed differentiation between sources of the alkylating species. In this case, it was found that the mass of the bis-adduct was consistent with addition of $C_6H_5(CH_2)_7-$ (peptide **5**, Figure 2), indicating that histidine alkylation occurred directly from the alcohol used in the second Mitsunobu reaction (Mechanism 2). We concluded from these studies that the high affinity byproduct **3** was formed by direct alkylation with $C_6H_5(CH_2)_8-OH$ during the Mitsunobu phosphoryl esterification reaction.

As exemplified by **3**, $N(\pi)$ -alkyl-histidine residues can impart exceptional Plk1 PBD-binding affinity.¹⁶ However, the adducts in the referenced study were obtained as minor reaction

byproducts. In order to facilitate a broader application of N(π)-alkyl-histidine analogues, we undertook the preparation of N(α)-Fmoc-[N(π)-R]-His [**8** for R = C₆H₅(CH₂)₈ -], which is suitably protected for incorporation of histidine derivatives into peptides using standard Fmoc protocols. We employed Hodges' regiospecific synthesis of N(π)-substituted histidines, which relies on the use of N(τ)-Boc-protected histidines and alkyl triflates as the alkylating species.²⁶ Our approach started with N(α), N(τ)-bis(*tert*-butoxycarbonyl)-L-histidine methyl ester (**6**).²⁶ A solution of 8-phenyloctan-1-ol in CH₂Cl₂ at -75 °C was treated with triflic anhydride and diisopropylethylamine (DIEPA, one equivalent) to form the triflate ester *in situ*. This was then reacted with the N(τ)-Boc histidine derivative **6** to form the corresponding N(π)-adduct **7** in 60% yield (Scheme 1). Without purification, global deprotection was performed and the crude product was reacted with 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) and aqueous NaHCO₃ in tetrahydrofuran to yield the final, protected amino acid analogue, **8** (83% from **7**, Scheme 1).

N(π)-derivatization in the presence of N(τ)-Trt protection has also previously been reported using alkyl halides as the alkylating species, however these conditions either involve elevated temperatures, prolonged reaction times or large excesses of reagent.²⁷⁻²⁹ In model reactions on N(α)-Fmoc-[N(τ)-Trt]-His methyl ester (**9**)³⁰ (prepared from commercially available N(τ)-trityl-L-histidine methyl ester) we found that N(π)-alkylated product (**11**) could be obtained in high yield at room temperature using *in situ* generated alkyl triflate. The crude product from this reaction contained a mixture of the N(τ)-trityl, N(π)-alkyl bis-adduct (**10**) as well as the desired product (**11**) (Scheme 2). Treatment of this mixture with TFA gave **11** in 90% yield from **9** and demethylation under nucleophilic conditions gave **8** in good yield. This approach represents a more direct synthesis of **8** than that described above using starting material **6** (Scheme 1).

Traditionally, during peptide synthesis protection of the histidine imidazole ring is important to avoid side product formation arising from ring acylation and subsequent migration of acyl species to other regions of the peptide. Protection also minimizes intramolecular base-catalyzed racemization of activated histidine by the N(π)-group. Acid-labile N(τ)-Trt derivatization is commonly employed for this purpose in combination with N(α)-Fmoc protection.³¹ Analogue **8** is somewhat unique in that its synthesis, and providing potentially important biological functionality. To verify the synthetic utility of **8**, we employed the reagent for the solid-phase synthesis of peptide **3**, which had previously been obtained only as a minor byproduct from the synthesis of intended product **2**. The successful completion of this synthesis confirmed the utility of **8** as providing a much improved route to peptides containing N(π)-modified histidine residues.

The general solid-phase methodology outline above using modified histidine analogue **8** is inherently limited because it requires that a new histidine reagent be prepared for each unique N(π)-substituent installed. A more useful approach that would allow the generation of multiple peptides containing unique N(π)-substituents, could be realized through on-resin modification of the histidyl residue in post-peptide synthesis fashion. Scheme 3 shows the application of such a protocol to the synthesis of peptide Peptide **13** is a variant of **3** having the labile pThr residue replaced by (2S,3R)-2-amino-3-methyl-4-phosphonobutyric acid (Pmab), which is a phosphatase-stable pThr mimetic³² that retains the PBD-binding affinity of parent pThr-containing peptides.^{15,16} A key feature of the route outlined in Scheme 3 is its use of commercially-available N(α)-Fmoc-[N(τ)-Trt]-His for the incorporation of an N(τ)-Trt-protected histidine residue. Although N(α)-Fmoc-[N(τ)-Boc]-His is also commercially available, the N(τ)-Boc group suffers from a lack of stability against prolonged or repeated exposure to piperidine used in Fmoc-based protocols.³¹

In conclusion, our current paper provides evidence that formation of high affinity Plk1 PBD-binding peptide byproducts previously observed as minor components following on-resin Mitsunobu esterification, result from direct alkylation at the N(π)-position. We detail the high yield preparation of N(α)-Fmoc-N(π)-modified histidine reagents from N(α)-Boc and N(α)-Fmoc protected precursors, which permit facile Fmoc-based solid-phase synthesis of N(π)-modified peptides. We extend the utility of this chemistry by demonstrating on-resin triflate-mediated derivatization of fully formed peptides that should permit the ready preparation of libraries of N(π)-alkyl peptides. Although only a very narrow range of alcohols is specifically examined in our current report, it is anticipated that the approach should provide access to a broad range of N(π)-modified histidine analogues that may be useful in a variety of biological contexts.

Experimental Section

Synthesis N(α)-Fmoc-[N(π)-(8-phenyloctyl)]-histidine (**6**) from (**6**)

N(α), N(τ)-bis[(1,1-dimethylethoxy)carbonyl]-L-histidine Methyl Ester (6**).²⁶—**Triethylamine (11.51 mL, 83 mmol) was added to a stirred suspension of L-histidine methyl ester hydrochloride (10.0 g, 41.3 mmol) in MeOH (98 mL) and stirring was continued at room temperature until dissolution was complete (30 min). A solution of di-*tert*-butyl dicarbonate (18.03 g, 83 mmol) in MeOH (49 mL) was then added dropwise over 30 min and stirring was continued at room temperature (48 h). Solvent was removed in vacuo and the residue was partitioned between CH₂Cl₂ and H₂O. The organic layer is washed with 10% citric acid, dried (MgSO₄), concentrated and purified by gel column chromatography (EtOAc : hexanes; from = 4:1 to 1.5:1) to afford known **6** as a white amorphous solid (11.06 g, 72% yield).

N(α)-[(1,1-Dimethylethoxy)carbonyl]-N(π)-(8-phenyloctyl)-L-histidine Methyl Ester (7**)—**To a stirred solution of triflic anhydride (1.51 mL, 8.93 mmol) in CH₂Cl₂ (18 mL) under nitrogen at -75 °C, was added a solution of 8-phenyloctan-1-ol (1.88 mL, 8.93 mmol) and diisopropylethylamine (DIEPA) (1.56 mL, 8.93 mmol) in CH₂Cl₂ (12.0 mL) dropwise over 10 min. Stirring was continued at -75 °C (20 min), then a solution of **6** (3.0 g, 8.12 mmol) in CH₂Cl₂ (21 mL) was added dropwise and the mixture was allowed to gradually warm to room temperature over a period of 16 – 18 h. The mixture was poured into a solution of aqueous NaHCO₃ and stirred vigorously (30 min). The organic layer was diluted with CH₂Cl₂ and washed with aqueous NaHCO₃ and brine, then dried (Na₂SO₄) and concentrated to a gum. Purification by silica gel flash chromatography (CHCl₃ : MeOH; from 100:1 to 100:2) provided **7** as colorless gum (2.23 g, 60% yield). [α]_D²¹ 23.99 (c 1.045, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 7.43 (s, 1H), 7.30-7.24 (m, 2H), 7.20-7.14 (m, 3H), 6.78 (s, 1H), 5.18 (d, *J* = 8 Hz, 1H), 4.54 (q, *J* = 4 Hz, 1H), 3.82 (t, *J* = 8 Hz, 2H), 3.74 (s, 3H), 3.16-2.96 (m, 2H), 2.60 (t, *J* = 8 Hz, 2H), 1.83 (brs, 1H), 1.77-1.65 (m, 2H), 1.65-1.55 (m, 2H), 1.43 (s, 9H), 1.31 (brs, 8H); ¹³C NMR (100 MHz, CDCl₃) δ 171.7, 155.0, 142.7, 137.4, 128.3, 128.2, 128.0, 125.8, 125.5, 80.2, 53.0, 52.5, 44.7, 35.9, 31.3, 30.9, 29.3, 29.1, 29.0, 28.2, 26.9, 26.6; IR ν_{max} 2929, 2361, 1705, 1164 cm⁻¹; HR-ESI MS calcd for C₂₆H₄₀N₃O₄ (M+H)⁺: 458.3013, found: 458.3023.

N(α)-[(9H-Fluoren-9-ylmethoxy)carbonyl]-N(π)-(8-phenyloctyl)-L-histidine (8**)—**To a solution of **7** (0.7 g, 1.53 mmol) in dissolved in THF (10 mL) and H₂O (2.5 mL) at 0 °C was added LiOH•H₂O (128 mg, 3.06 mmol) and the mixture was stirred at 0 °C (1.5 h). The solution was acidified to pH 1 – 2 (aqueous HCl), then THF was removed in vacuo and the residue was extracted (EtOAc) and the organic layer was dried and concentrated in vacuo. The residue was dissolved in a solution of 4M HCl in dioxane (4 mL) and EtOAc (4 mL) and stirred at room temperature (1 h), then concentrated in vacuo. The residue was

dissolved in solution of THF (7 mL) and H₂O (7 mL) and 9-fluorenylmethyl-succinimidyl carbonate (Fmoc-OSu) (774 mg, 2.30 mmol) and NaHCO₃ (514 mg, 6.12 mmol) were added and the mixture was stirred at room temperature (overnight). The mixture was neutralized by the addition of saturated NH₄Cl, extracted (EtOAc) and the organic layer was dried and concentrated in vacuo. The resulting residue was purified by silica gel column chromatography first by elution with (CHCl₃: MeOH; from 100:1 to 100:3) to remove byproduct and then (CHCl₃: MeOH; from 10:1 to 3:1) to provide the desired product **8** as a white wax (716 mg, 83% yield from **7**). [α]_D²¹ 38.78 (c 0.90, CHCl₃); ¹H NMR (400 MHz, DMSO-*d*₆) δ 8.39 (s, 1H), 7.89 (d, *J* = 8 Hz, 2H), 7.85 (d, *J* = 8 Hz, 1H), 7.67 (d, *J* = 8 Hz, 2H), 7.41 (t, *J* = 8 Hz, 2H), 7.32 (t, *J* = 8 Hz, 2H), 7.25 (t, *J* = 8 Hz, 2H), 7.20 – 7.10 (m, 3H), 7.8 (s, 1H), 4.35 – 4.15 (m, 4H), 4.10 – 3.90 (m, 2H), 3.20 – 2.90 (m, 2H), 2.60 (s, 1H), 2.55 – 2.47 (m, 2H), 1.75 – 1.60 (m, 2H), 1.60 – 1.45 (m, 2H), 1.23 (brs, 9H); ¹³C NMR (100 MHz, DMSO-*d*₆) δ 172.4, 155.9, 143.7, 142.2, 140.7, 135.9, 129.1, 128.20, 128.16, 127.6, 127.1, 125.5, 125.2, 121.8, 120.1, 65.7, 53.0, 46.6, 45.0, 35.1, 30.9, 29.8, 28.7, 28.5, 28.4, 25.8, 25.2; IR ν_{max} 2930, 2361, 1713, 1452, 1255 cm⁻¹; HR-ESI MS calcd for C₃₅H₄₀N₃O₄ (M+H)⁺: 566.3013, found: 566.3022.

Synthesis N(α)-[(9H-Fluoren-9-ylmethoxy)carbonyl]-[N(π)-(8-phenyloctyl)]-histidine (**8**) from (**9**)

N(α)-[(9H-Fluoren-9-ylmethoxy)carbonyl]-N(τ)-(triphenylmethyl)-L-histidine Methyl Ester (9**)**—To a solution of N(τ)-(triphenylmethyl)-L-histidine methyl ester hydrochloride (0.8 g, 1.78 mmol) and NaHCO₃ (0.45 g, 5.3 mmol) in THF : H₂O (v/v 1:1) at 0 °C, was added Fmoc-OSu (0.9 g, 2.67 mmol) and the mixture was allowed to come to room temperature and stirred (overnight). The organic layer was collected, the aqueous layer was extracted (EtOAc) and the combined organic layers were washed with H₂O, brine and dried (Na₂SO₄). Concentration in vacuo provided a residue, which was purified by silica gel column chromatography (EtOAc : petroleum ether; from 1 : 10 to 1 : 4) to yield known **9**³⁰ as white foam (84% yield). [α]_D²³ 9.55 (c 1.0, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 7.77 (d, *J* = 8.0 Hz, 2H), 7.64 (t, *J* = 8.0 Hz, 2H), 7.44 (s, 1H), 7.40 (t, *J* = 8.0 Hz, 2H), 7.37 – 7.32 (m, 9H), 7.30 (t, *J* = 8.0 Hz, 2H), 7.16 – 7.10 (m, 6H), 6.60 (s, 1H), 6.55 (d, *J* = 8.0 Hz, 1H), 4.68 – 4.63 (m, 1H), 4.40 – 4.24 (m, 3H), 3.65 (s, 3H), 3.14 – 3.17 (m, 2H); ¹³C NMR (125 MHz, CDCl₃) δ 172.3, 156.4, 144.3, 144.1, 142.5, 141.4, 139.0, 136.6, 129.9, 128.26, 128.25, 127.8, 127.2, 125.6, 125.5, 120.1, 119.8, 75.5, 67.4, 54.5, 52.4, 47.4, 30.3; IR ν_{max} 2361, 1719, 1492, 1445, 1248 cm⁻¹; HR-ESI MS calcd for C₄₁H₃₅N₃O₄Na (M+Na)⁺: 656.2525, found: 656.2538.

N(α)-[(9H-Fluoren-9-ylmethoxy)carbonyl]-N(τ)-(8-phenyloctyl)-L-histidine Methyl Ester (11**)**—To a stirred solution of triflic anhydride (0.101 mL, 0.60 mmol) in CH₂Cl₂ (3.8 mL) under nitrogen at –75 °C was added a solution of 8-phenyloctan-1-ol (124 mg, 0.60 mmol) and DIEA (0.104 mL, 0.60 mmol) in CH₂Cl₂ (2.6 mL) dropwise over 10 min at –75 °C. Stirring was continued at –75 °C (20 min), then a solution of **9** (0.34 g, 0.54 mmol) in CH₂Cl₂ (4.5 mL) was added dropwise and mixture was allowed to gradually warm to room temperature and stirred (16 – 18 h). The mixture was poured into aqueous NaHCO₃ and stirred vigorously (30 min), then extracted with CH₂Cl₂ and the organic extract washed with aqueous NaHCO₃ and brine, dried (Na₂SO₄) and concentrated in vacuo. To a solution of the resulting gum in CH₂Cl₂ (4.2 mL) was added trifluoroacetic acid (0.42 mL, 5.4 mmol) and triisopropylsilane (TIS) (0.12 mL, 0.60 mmol) and the mixture was stirred at room temperature until reaction was complete as shown by TLC (2 h). Solvent was removed in vacuo and the residue was purified by silica gel column chromatography (CH₂Cl₂ : MeOH; from 100:1 to 20:1) to provide **11** a white foam (90% yield from **9**).

$[\alpha]_D^{22.4}$ 1.35 (c 1.01, CHCl₃); ¹H NMR (400 MHz, CDCl₃) δ 8.62 (s, 1H), 7.75 (d, *J* = 8.0 Hz, 2H), 7.58 (d, *J* = 8.0 Hz, 2H), 7.39 (t, *J* = 8.0 Hz, 3H), 7.40–7.23 (m, 4H), 7.22–7.10 (m, 4H), 6.05 (d, *J* = 8.0 Hz, 1H), 4.56 (d, *J* = 4.0 Hz, 1H), 4.38 (d, *J* = 8.0 Hz, 2H), 4.18 (d, *J* = 8.0 Hz, 2H), 4.06–4.01 (m, 2H), 3.77 (s, 3H), 3.25–3.14 (m, 2H), 2.58 (t, *J* = 8.0 Hz, 2H), 1.81–1.73 (m, 2H), 1.63–1.53 (m, 2H), 1.28 (s, 8H); ¹³C NMR (100 MHz, CDCl₃) δ 170.6, 156.2, 143.79, 143.74, 142.9, 141.5, 134.9, 129.8, 128.6, 128.4, 128.0, 127.4, 125.8, 125.2, 120.2, 119.1, 67.5, 53.3, 53.1, 47.2, 36.1, 31.5, 30.2, 29.4, 29.3, 29.0, 26.5, 26.3; IR ν_{max} 2928, 2361, 1718, 1166, 1028 cm⁻¹; HR-ESI MS calcd for C₃₆H₄₂N₃O₄ (M+H)⁺: 580.3175, found: 580.3178.

N(α)-[(9H-Fluoren-9-ylmethoxy)carbonyl]-N(τ)-(8-phenyloctyl)-L-histidine (8)—

Lithium iodide (1.13 g, 8.45 mmol) was added to a stirred solution of **11** (0.82 g, 1.41 mmol) in dry EtOAc (28 mL) under nitrogen and the mixture was heated at reflux (20 h). The mixture was brought to room temperature and the reaction quenched by the addition of 10% aqueous HCl and extracted (EtOAc). The organic extract was dried (Na₂SO₄), concentrated in vacuo and the residue was purified by silica gel flash chromatography (CH₂Cl₂ : MeOH; from 95:5 to 80:20) to provide **8** as a colorless gum (80% yield).

General Solid-Phase Peptide Synthesis

Protected amino acids used were: Fmoc-Thr(PO(OBzl)OH)-OH; Fmoc-Ser(O^tBu); N(τ)-Trt-N(α)-Fmoc-His; Fmoc-Leu and Fmoc-Pro (purchased from Novabiochem). Peptides were synthesized on NovaSyn[®]TGR resin (Novabiochem, cat. no. 01-64-0060) using standard Fmoc solidphase protocols in N-Methyl-2-pyrrolidone (NMP). 1-O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) (5.0 eq.), hydroxybenzotriazole (HOBT) (5.0 eq.) and N,N-diisopropylethylamine (DIPEA) (10.0 eq.) were used as coupling reagents. Amino terminal acetylation was achieved using 1-acetylimidazole. Finished resins were washed with N,N-dimethylformamide (DMF), MeOH, CH₂Cl₂ and Et₂O and then dried under vacuum (overnight), then cleaved by treatment with a solution of TFA : H₂O : TIS (95:2.5:2.5) (4 h). The resin was removed by filtration, and the filtrate was concentrated under vacuum, then precipitated with ether, and the precipitate was washed with ether. The resulting solid was dissolved in 50% aqueous MeCN (5 mL) and purified by reverse phase preparative HPLC using a Phenomenex C18 column (21 mm x 250 mm, cat. no: 00G-4436-P0) with a linear gradient from 0% aqueous MeCN (0.1% TFA) to 65% MeCN (0.1% TFA) over 30 min at a flow rate of 10.0 mL/min (detection at 220 nm). Lyophilization provided products as white powders. For the synthesis of Pmab-containing peptides, (2S,3R)-4-[di-(tert-butyl)-oxyphosphinyl]-N-Fmoc-L-valine³² was used in place Fmoc-Thr(PO(OBzl)OH)-OH.

Synthesis of Peptide 3 using N(α)-[(9H-Fluoren-9-ylmethoxy)carbonyl]-N(τ)-(8-phenyloctyl)-L-histidine (8)

The synthesis of peptide **3** was accomplished according to the procedures listed under General Solid-Phase Synthesis, except that reagent **8** was used in place of N(τ)-Trt-N(α)-Fmoc-His.

On Resin N(τ)-Alkylation Using Mitsunobu Reaction Conditions—Peptides **2** and **3** were prepared as previously reported.¹⁶ For the synthesis of peptides **4** and **5**, resin-bound Fmoc-Thr[(OP(OBn)OH)-amide], prepared as indicated above, (400 mg, 0.1 mmol) was swelled in CH₂Cl₂ (15 min) and then treated with triphenylphosphine (262 mg, 1.0 mmol), diethyl azidodicarboxylate (DEAD) (0.46 mL, 40% solution in toluene, 1.0 mmol) and 8-phenyloctan-1-ol (206 mg, 1.0 mmol) in dry CH₂Cl₂ at room temperature (2 h). Coupling of the remaining peptide residues was conducted as described above in General Solid-Phase Synthesis. The resulting resin bond Ac-Pro-Leu-[(τ)-Trt-His]-Ser(O^tBu)-Thr[(OP(OBn)

(O(CH₂)₈Ph)]-amide was subjected to Mitsunobu coupling using triphenylphosphine (262 mg, 1.0 mmol), DEAD (0.46 mL, 40% solution in toluene, 1.0 mmol) and appropriate alcohol [8-phenyloctan-1-ol (206 mg, 1.0 mmol) for peptide **4** and 7-phenylheptan-1-ol (192 mg, 1.0 mmol) for peptide **5**] in dry CH₂Cl₂ at room temperature (2 h). The resins were then washed (CH₂Cl₂), dried under vacuum (2 h) and cleaved by treatment with [TFA : H₂O : TIS (95:2.5:2.5) (4 h)]. Peptides **4** and **5** were following procedures indicated in General Solid-Phase Synthesis. HPLC and mass spectral data are provided in Table 1.

Synthesis of Peptide **13** by On-resin N(π)-alkylation

To a stirred solution of triflic anhydride (42 μ L, 0.25 mmol) in CH₂Cl₂ (3 mL) under nitrogen at -75 °C was added a solution of 8-phenyloctan-1-ol (52 mg, 0.25 mmol) and DIEA (45 μ L, 0.26 mmol) in CH₂Cl₂ (2.0 mL) dropwise over 10 min. Stirring was continued at -75 °C (20 min), then the mixture was warmed to room temperature and added to peptide resin **12**¹⁶ (200 mg, 0.05 mmol) that had been swelled in CH₂Cl₂ for 15 min. Coupling was conducted at room temperature (12 h), then the resin was washed (CH₂Cl₂ and Et₂O), dried under vacuum (2 h) and cleaved (TFA : H₂O : TIS; 95:2.5:2.5) (4 h). Peptide **13** was obtained following work up and HPLC purification as indicated in General Solid-Phase Synthesis. HPLC and mass spectral data are provided in Table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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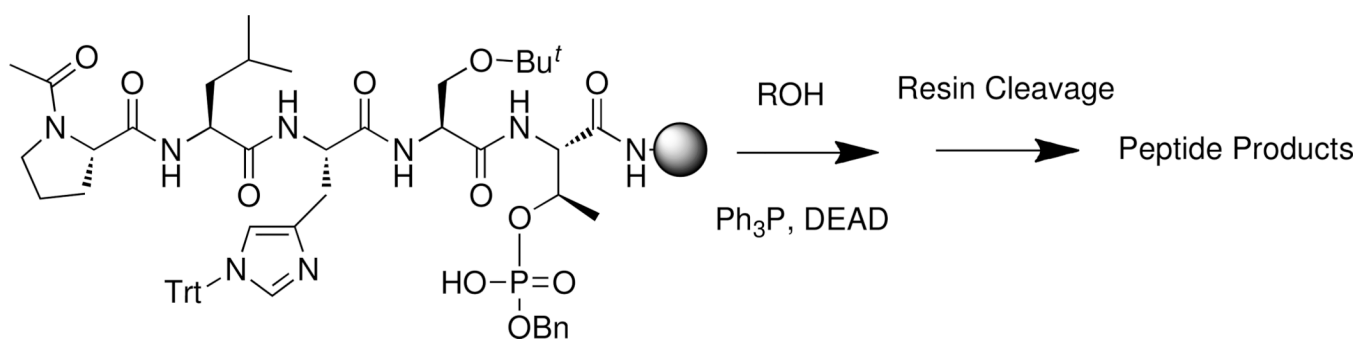
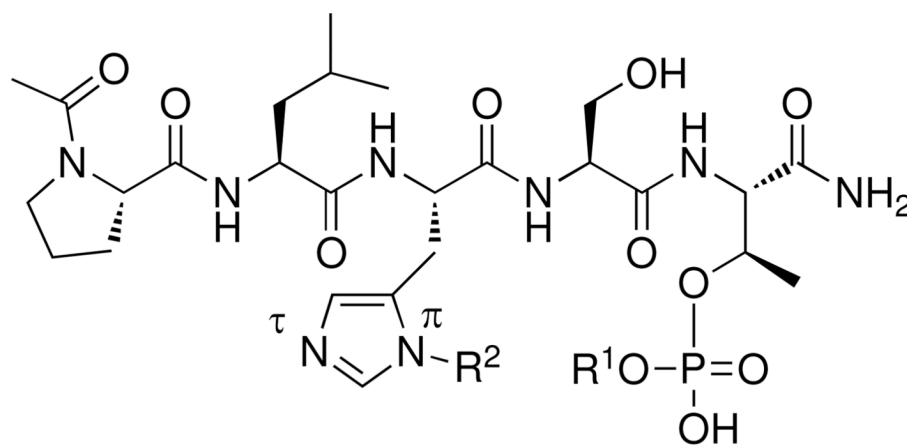


Figure 1.
Solid-phase Mistunobu reaction



1 $R^1 = H; R^2 = H$

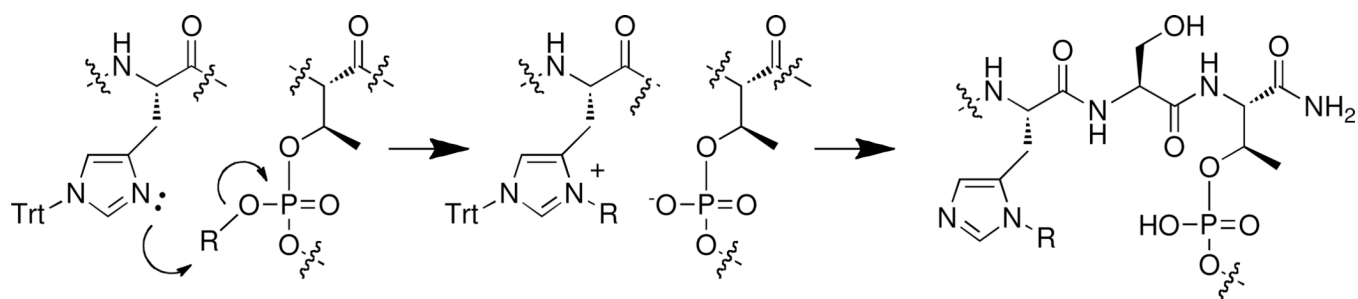
2 $R^2 = H; R^1 = C_6H_5(CH_2)_8 -$

3 $R^1 = H; R^2 = C_6H_5(CH_2)_8 -$

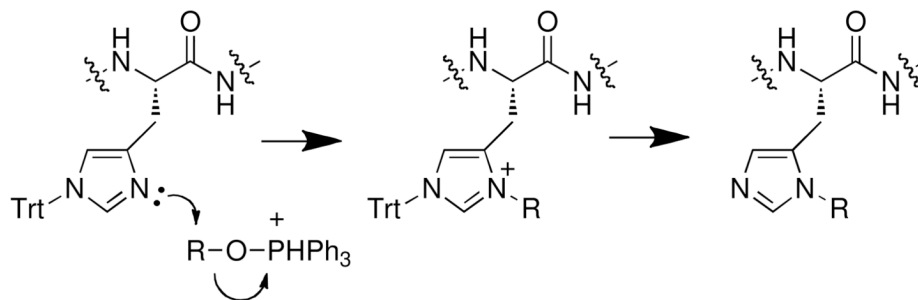
4 $R^1 = C_6H_5(CH_2)_8 -; R^2 = C_6H_5(CH_2)_8 -$

5 $R^1 = C_6H_5(CH_2)_8 -; R^2 = C_6H_5(CH_2)_7 -$

Figure 2.
Structures of peptides discussed in the text.

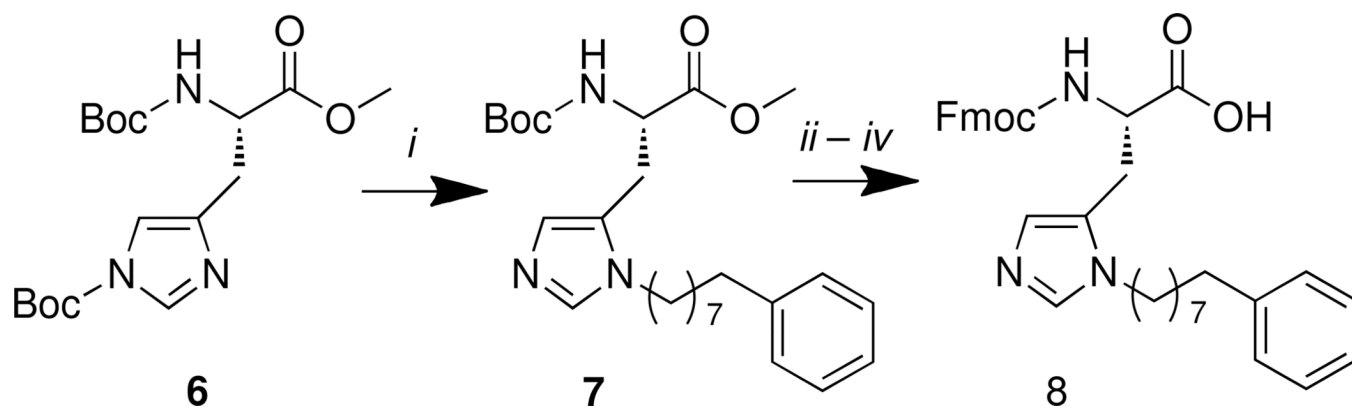


Mechanism 1: Phosphoryl Transfer

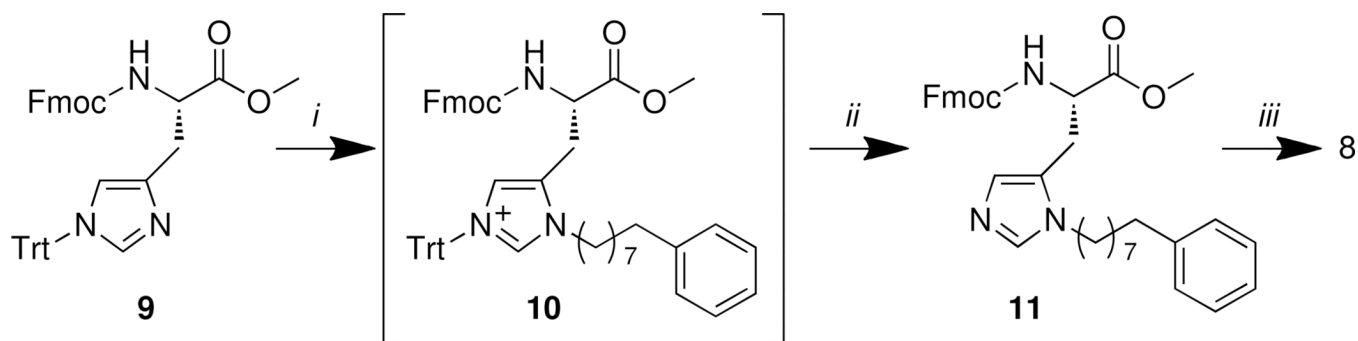


Mechanism 2: Direct Mitsunobu-catalyzed Alkylation

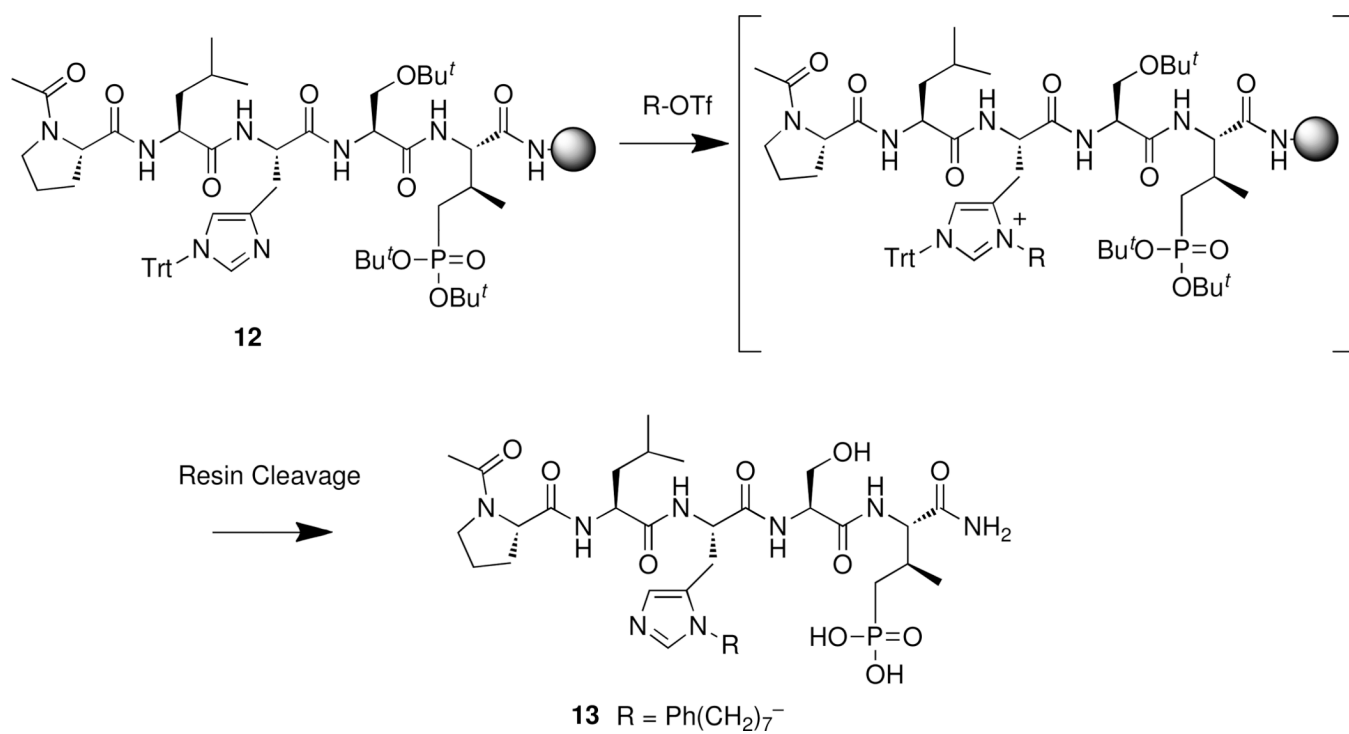
Figure 3.
Two possible mechanisms of histidine alkylation.

**Scheme 1.**

Reagents and conditions: (i) $\text{Ph}(\text{CH}_2)_7\text{CH}_2\text{OH}$ (1.1 equiv.), Ti_2O (1.1 equiv.), DIPEA (1.1 equiv.), CH_2Cl_2 , $-75\text{ }^\circ\text{C}$ – rt, 16 – 18 h, 60% yield; (ii) $\text{LiOH}\cdot\text{H}_2\text{O}$ (2.0 equiv.), THF, H_2O (v/v 4:1), $0\text{ }^\circ\text{C}$ – rt, 1 h; (iii) 4 M HCl in dioxane (10 equiv.), rt, 1 h; (iv) Fmoc-OSu (1.5 equiv.), NaHCO_3 (4.0 equiv.), THF – H_2O (v/v 1:1), rt, 12 h (82% over three steps).

**Scheme 2.**

Reagents and conditions: (i) $\text{Ph}(\text{CH}_2)_7\text{CH}_2\text{OH}$ (1.1 equiv.), Tf_2O (1.1 equiv.), DIPEA (1.1 equiv.), CH_2Cl_2 , $-75\text{ }^\circ\text{C}$ – rt, 16 – 18 h; ii) TFA (10 equiv.), triisopropylsilane (1.1 eq.), CH_2Cl_2 , rt, 2 h, (90% over two steps). (iii) LiI (6.0 equiv.), EtOAc, reflux, 20h, 80%.



Scheme 3.
Post-peptide synthesis on-resin N(π)-alkylation.

Table 1

Peptide ESI MS spectral data and HPLC retention times.

No	Expect (M+H) ⁺	Observed (M+H) ⁺	Retention (min)	HPLC ^a
2 ¹⁶	863.4	863.4	25.2	II
3 ¹⁶	863.4	863.4	24.3	II
4	1051.6	1051.5	29.8	I
5	1037.6	1037.4	29.2	I
13 ¹⁶	861.5	861.4	23.3	I

^aHPLC Method: $\lambda=220$ nm, flow rate: 10 mL/min Solvent A: H₂O, 0.1% TFA; Solvent B: MeCN, 0.1% TFA; Gradients as indicated below:**I:**

Time	0	30	30.1	35
B%	0	100	100	100

II:

Time	0	30	30.1	35
B%	0	90	100	100