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Design and synthesis of a reagent for solid-phase incorporation of the phosphothreonine mimetic (2*S*,3*R*)-2-amino-3-methyl-4-phosphonobutyric Acid (Pmab) into peptides in a bio-reversible phosphonyl-*bis*-pivaloyloxymethyl (POM) prodrug form

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Abstract

Reported herein are the synthesis and solid-phase peptide incorporation of *N*-Fmoc-(2*S*,3*R*)-2-amino-3-methyl-4-phosphonobutyric acid *bis*-pivaloyloxymethyl phopshoryl ester [Fmoc-Pmab(POM)$_2$-OH, **2**] as a phosphatase-stable phosphothreonine (pThr) mimetic bearing orthogonal protection suitable for the synthesis of Pmab-containing peptides having bio-reversible protection of the phosphonic acid moiety. This represents the first report of a bio-reversibly protected pThr mimetic in a form suitable for facile solid-phase peptide synthesis.

Keywords

Phosphothreonine mimetic; Prodrug; Pmab; Solid-phase peptide synthesis; Signal transduction

Introduction

Protein phosphorylation is a fundamental form of post-translational modification that affects approximately one third of all proteins. The presence of phosphothreonine (pThr), phosphoserine (pSer) and phosphotyrosine (pTyr)-can introduce unique recognition features, which often facilitate specific protein-protein interactions (PPIs) (Yaffe 2002; Ladbury 2005; Elia and Yaffe 2005). Synthetic phosphopeptides modeled on recognition sequences can serve as useful pharmacological tools for studying these PPIs (Eisele et al. 1999; Lu et al. 2012). However, in cellular systems the bioavailabilty of phosphopeptides may be limited by the enzymatic lability of the phosphoryl ester bond toward phosphatases and poor membrane transport of the phosphoryl di-anionic species. While replacement of the phosphoryl ester oxygen by methylene or fluoromethylenes has addressed issues related to phosphatase hydrolysis for pTyr (Burke and Lee 2003), pSer (Shapiro et al. 1993; Perich 1994; Nair et al. 1995; Panigrahi et al. 2009) and pThr (Berkowitz et al. 1966; Otaka et al. 2000; Liu et al. 2009), cell membrane transit can still be limited by the di-anionic charge of the resulting phosphonic acids.

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Conflict of interest
The authors have declared no conflict of interest.

Electronic supplementary material
The online version of the article (doi:) contains the ^{1}H and ^{13}C NMR spectra of compounds **2** – **5** and the analytical HPLC of peptide **6**, which is available to authorized users.

A general strategy for increasing the cell membrane permeability of phosphates and phosphonic acids is to mask their acidic functionality with neutral "prodrug" groups that can be removed enzymatically once the agent is within the cell. Although a variety of general prodrug strategies have been for phosphonic acids (Schultz 2003; Hecker and Erion 2008), there are few reports detailing incorporation of these functionalities into protected amino acid derivatives that are amenable to solid-phase peptide synthesis. Recent examples of the latter include the application of 5'-nitrofuryl-2'-methyl *N*-(4"-chlorobutyl)phosphonamido ester prodrug strategy to the pTyr mimetic difluorophosphonomethylphenylalanine (F_2Pmp) (Boutselis et al. 2007) and to the pSer mimetic, difluorophosphonoaminobutyric acid (Arrendale et al. 2012). In these two cases, the prodrug-protected reagents were used to prepare dipeptides for examination in whole cell studies. The use of these reagents for the solid-phase synthesis of longer peptides is potentially limited by chemical instability of the phosphonamido group to repetitive cycles of piperidine treatment needed for Fmoc-based solid-phase protocols (Boutselis et al. 2007). In the broader sense, there is a paucity of reagents available for the solid-phase synthesis of polypeptides containing prodrug-protected pThr mimetics.

The pivaloyloxymethyl (POM) group is an esterase-labile moiety that has been widely applied to phosphoryl prodrug protection of nucleotides (Hecker and Erion 2008) and phosphate and phosphonic acid functionality in small molecules and peptide mimetics (Stankovic et al. 1997; Mandal et al. 2009; Mandal et al. 2011; Zhao and Etzkorn 2007). However, in spite of its usefulness in these latter contexts, there are no prior reports of polypeptides containing pThr, pSer or their phosphonic acid-based mimetics, bearing POM protection. Given the importance of pThr in a large number of biological processes (Elia and Yaffe 2005), a reagent that would permit the solid-phase synthesis of polypeptides containing POM-protected pThr mimetics would be highly desirable.

We had previously reported the preparation of *N*-Fmoc-(2*S*,3*R*)-2-amino-3-methyl-4-phosphonobutyric acid bis-*tert*-butyl phopshoryl ester [Fmoc-Pmab(Bu^t_2)-OH, **1**, Fig. 1] as a hydrolytically-stable pThr mimetic bearing orthogonal protection suitable for the standard Fmoc-based synthesis of peptides containing Pmab. However this reagent yields peptides in which the Pmab residue has its phosphonic acid moiety in the free di-anionic form (Liu et al. 2009). To date, there have been no reports of Pmab suitably derivatized for the solid-phase synthesis of polypeptides that maintained the phosphonic acid in a prodrug-protected form. In the current paper we describe the first preparation of Fmoc-Pmab having the phosphonic acid group masked as its POM *bis*-esters [Fmoc-Pmab$(POM)_2$-OH, **2**, Fig. 1] and demonstrate its use in the solid-phase synthesis of a peptide bearing full POM protection of the Pmab residue.

Materials and methods

General methods

All experiments involving moisture-sensitive compounds were conducted under anhydrous conditions. Fmoc-Ser(Trt)-OH, and Fmoc-His(Mtt)-OH were purchased from NovaChioChem. All solvents were purchased in anhydrous form (Aldrich) and used directly. Analytical TLCs were performed using Analtech precoated plates (Uniplate, silica gel GHLF, 250 nm) containing a fluorescence indicator. NMR spectra were recorded using a Varian Inova 400 MHz spectrometer. Coupling constants are reported in hertz, and peak shifts are reported in δ (ppm) relative to TMS. Low-resolution mass spectra (ESI) were measured with an Agilent 260 1200 LC/MSD-SL system. High resolution mass spectra (HRMS) were obtained by positive ion, ESI analysis on a Thermo Scientific LTQ-XL Orbitrap mass spectrometer with HPLC sample introduction using a short narrow-bore C_{18}

reversed-phase column with CH_3CN - H_2O gradients. Reported *m/z* values are the average of eight or more scans over the chromatographic peak of interest.

Synthesis of Fmoc-Pmab(POM)$_2$-OH (2)

(2S,3R)-Benzyl 2-(((benzyloxy)carbonyl)amino)-4-(di-tert-butoxyphosphoryl)-3-methylbutanoate (4)—To a solution of **3** (Liu et al. 2009) (0.26 g, 0.57 mmol) in THF–H_2O (4 : 1; 5 mL) at 0 °C was added a solution of LiOH•H_2O (48 mg, 1.14 mmol) and the mixture was stirred at room temperature until all starting material was consumed as indicated by TLC. The pH was adjusted to 2–3 by the addition of 1N aqueous HCl and THF was removed by evaporation. The residue was extracted (EtOAc) and the combined organic phase was washed with brine, dried ($MgSO_4$), filtered and concentrated. To a solution of the crude residue in DMF (5.0 mL) were added sequentially, $NaHCO_3$ (95 mg, 1.14 mmol) and BnBr (0.10 mL, 0.85 mmol) at room temperature under argon and the mixture was stirred until the starting material was consumed as indicated by TLC. The mixture was diluted with EtOAc and washed with H_2O and brine, dried ($MgSO_4$) and filtered and concentrated. Purification by silica gel column chromatography (CH_2Cl_2 : MeOH from 100:1 to 30:1) afforded **4** as a colorless oil (0.25 g, 80% for two steps, Scheme 1). $[\alpha]_D^{21.6}$ -0.59 (c 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$ δ 7.40 – 7.28 (m, 10H), 5.91 (d, J = 8.0 Hz, 1H), 5.20 – 5.06 (m, 4H), 4.38 -4.31 (m, 1H), 2.42 (brs, 1H), 1.84 – 1.69 (m, 1H), 1.57 – 1.48 (m, 1H), 1.47 – 1.45 (m, 18H), 1.10 (d, J = 8.0 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$ δ 171.7, 156.4, 136.5, 135.4, 128.8, 128.7, 128.6, 128.3, 82.4 (d, $^2J_{CP}$ = 10 Hz), 82.3 (d, $^2J_{CP}$ = 10 Hz), 67.4, 67.2, 59.8 (d, $^3J_{CP}$ = 10 Hz), 34.0, 32.6, 30.59, 30.56, 17.6 ppm; ESI-HRMS *m/z* calcd for $C_{28}H_{41}NO_7P$ $(M+H)^+$: 534.2621, found: 534.2594.

((((2R,3S)-4-(Benzyloxy)-3-(((benzyloxy)carbonyl)amino)-2-methyl-4-oxobutyl)phosphoryl)bis(oxy))bis(methylene) Bis(2,2-dimethylpropanoate)(5)—A solution of **4** (0.27 g, 0.5 mmol) in 10% TFA (CH_2Cl_2) was stirred at room temperature (1 h), then volatiles were removed under vacuum and the residue was taken up in DMF with pivaloyloxymethyl iodide (POMI) (Bandgar et al. 2011) (0.32 mL, 2.0 mmol) and DIPEA (0.35 mL, 2.0 mmol) and stirred at room temperature under argon (overnight). The mixture was diluted with H_2O, extracted with EtOAc and the combined organic extracts were washed with H_2O, and brine, dried ($MgSO_4$), filtered and concentrated. Purification by silica gel chromatography (EtOAc : hexanes from 1:4 to 1:1) afforded **5** as a white semi-solid (0.24 g, 74% yield for two steps, Scheme 1). $[\alpha]_D^{21.9}$ 2.88 (c 0.7, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$ δ 7.42 – 7.18 (m, 10H), 5.70 – 5.60 (m, 4H), 5.56 (d, J = 12.0 Hz, 1H), 5.19 (s, 2H), 5.11 (s, 2H), 4.42 – 4.34 (m, 1H), 2.45 (brs, 1H), 2.04 – 1.91 (m, 1H), 1.78 – 1.67 (m, 1H), 1.22 (s, 9H), 1.21 (s, 9H), 1.08 (d, J = 4.0 Hz, 3H) ppm; ; ^{13}C NMR (100 MHz, $CDCl_3$ δ 177.1, 171.1, 156.3, 136.3, 135.2, 128.9, 128.8, 128.7, 128.44, 128.35, 81.7 (d, $^2J_{CP}$ = 20 Hz), 67.6, 67.4, 59.2 (d, $^3J_{CP}$ = 10 Hz), 38.9, 31.9, 30.3, 29.9, 29.6 (d, $^1J_{CP}$ = 140 Hz), 27.0, 17.5 ppm; ESI-HRMS *m/z* calcd for 650.2725, found: 650.2704.

(2S,3R)-2-((((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-4-(bis((pivaloyloxy)methoxy)phosphoryl)-3-methylbutanoic Acid [N-Fmoc-Pmab(POM)$_2$-OH] (2)—A solution of **5** (0.18 g, 0.28 mmol) in MeOH was hydrogenated over 10% Pd•C (30 mg) until the reaction was complete as indicated by TLC. The mixture was filtered, evaporated to dryness and reacted with Fmoc-OSu (0.19 g, 0.55 mmol) and $NaHCO_3$ (70 mg, 0.83 mmol) in dioxane : H_2O (1:1, 5.5 mL) at room temperature (overnight). The mixture was acidified with 1N HCl, extracted with EtOAc and the combined organic phases were washed with brine, dried ($MgSO_4$), filtered and the filtrate concentrated. Purification by silica gel column chromatography (CH_2Cl_2 : MeOH from 20:1 to 4:1) provided **2** as a colorless oil (0.14g, 75% yield for two steps, Scheme 1). $[\alpha]_D^{21.0}$ 12.8 (c 0.66, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$ δ 7.77 (d, J = 8.0 Hz, 2H), 7.60 (d, J = 8.0

Hz, 2H), 7.40 (t, *J* = 8.0 Hz, 2H), 7.32 (td, *J1* = 8.0 Hz, *J2* = 4.0 Hz, 2H), 5.78 – 5.64 (m, 5H), 4.51 – 4.44 (m, 1H), 4.40 (d, *J* = 8.0 Hz, 2H), 4.22 (t, *J* = 8.0 Hz, 1H), 2.54 (brs 1H), 2.18 – 2.03 (m, 1H), 1.97 -1.83 (m, 1H), 1.24 (s, 9H), 1.23 (s, 9H), 1.13 (d, *J* = 8.0 Hz, 3H) ppm; ^{13}C NMR (100 MHz, $CDCl_3$ δ 177.2, 172.8, 156.4, 144.0, 143.8, 141.5, 128.0, 127.3, 125.3, 120.2, 82.0, 67.5, 58.3 (d, $^3J_{CP}$ = 10 Hz), 47.3, 39.0, 31.4, 29.4 (d, $^1J_{CP}$ = 140 Hz), 27.0, 17.6 (d, $^3J_{CP}$ = 7 Hz) ppm; ESI-HRMS *m/z* calcd for $C_{32}H_{42}NO_{11}PNa$ $(M+Na)^+$: 670.2393, found: 670.2376.

Use of reagent 2 for the solid-phase synthesis of peptide 6

Standard Fmoc-protected amino acids were purchased from Novabiochem. Peptides were synthesized on NovaSyn® TG Sieber resin (Novabiochem, cat. no. 01-64-0092) using Fmoc-based solid-phase 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. Reagent **2** was employed for introduction of the first residue. Following completion of peptide elongation and Fmoc-deprotection, amino terminal acetylation was achieved using 1-acetylimidazole. The finished resin (**7**, Scheme 2) was washed with DMF, MeOH, CH_2Cl_2 and diethyl ether and then dried under vacuum (overnight). Peptide **6** was cleaved from the resin using 1% TFA in CH_2Cl_2. The resin was removed by filtration and the filtrate was concentrated under vacuum, then precipitated with cold ether and the precipitate washed with cold ether. The resulting solid was dissolved in 50% aqueous acetonitrile (5 mL) and purified by reverse phase preparative HPLC using a Phenomenex C_{18} column (21 mm dia x 250 mm, cat. no: 00G-4436-P0) with a linear gradient from 0% aqueous acetonitrile (0.1% TFA) to 100% acetonitrile (0.1% trifluoroacetic acid) over 30 minutes at a flow rate of 10.0 mL/minute. Lyophilization gave peptide **6** as a white powder (>99% pure by HPLC, Scheme 2). ESI-MS *m/z* calcd for $C_{39}H_{66}N_8O_{14}P$ $(M+H)^+$: 901.4, found: 901.4.

Pig liver esterase hydrolysis studies on peptide 6

Following literature procedures (Srivastva and Farquhar 1984), 0.05M potassium phosphate buffer (pH 7.4) was placed in a centrifuge tube and a solution of peptide **6** in MeOH was added to achieve a concentration of 200 μM of **6** with MeOH being less than 1%. To a 1 mL aliquot of the above solution was added pig liver esterase (57.6 units) and the reaction mixture was incubated at 37 °C with gentle agitation. At various time points, aliquots of the reaction mixture (50 μL) were transferred to an Eppendorf tube containing MeCN (50 μL). Following filtration, the hydrolysis product was monitored by LC-MS. Data are shown in Fig. 2.

Results and discussion

Preparation of reagent **2** began with Cbz-Pmab(Bu^t_2)-OMe (**3**), which is an intermediate in our previously reported synthesis of **1** (Liu et al. 2009). Benzyl *trans*-esterification of **3** to **4** was accomplished by initial LiOH-mediated hydrolysis of the methyl ester followed by reaction of the free acid with benzyl bromide ($NaHCO_3$ in DMF, 80% yield for two steps, Scheme 1). Subsequent treatment of **4** with pivaloyloxymethyl iodide (POMI) (Bandgar et al. 2011) and diisopropylethyl amine (DIPEA) in DMF provided the corresponding bis-POM-protected intermediate (**5**). Hydrogenolytic removal of amino and carboxyl protecting groups and introduction of *N*-Fmoc-protection by treatment with 9-fluorenylmethyl succinimidyl carbonate (Fmoc-OSu) in aqueous THF with $NaHCO_3$, gave the desired reagent **2** (55% yield from **4**) (Scheme 1).

In order to demonstrate the usefulness of **2** for the incorporation of Pmab(POM)$_2$ into peptides by solid-phase techniques, we chose the sequence, Ac-Pro-Leu-His-Ser-Pmab-amide, which has previously shown to bind with good affinity to the polo-like kinase 1 (Plk1) polo box domain (PBD) (Yun et al. 2009; Liu et al. 2011; Liu et al. 2012b, a). PBD-binding ligands may potentially serve as anticancer agents by blocking the spatial organization required for Plk1 to function in oncogenic processes (van de Weerdt et al. 2008). The syntheses of Ac-Pro-Leu-His-Ser-Pmab(POM)$_2$-amide (**6**) was accomplished on NovaSyn TG Sieber resin using standard Fmoc protocols. Histidine and serine were employed in their 4-methoxytrityl (Mtt) and trityl (Trt) side chain-protected forms, respectively, to allow their cleavage under mildly acidic conditions (Scheme 2). Following synthesis completion, the resin-bound Ac-Pro-Leu-His(Mtt)-Ser(Trt)-Pmab(POM)$_2$-amide (**7**) was subjected to treatment with 1% TFA, which resulted in removal of histidine and serine protecting groups and cleavage of the peptide from the resin with retention of Pmab-*bis*-POM functionality. Purification by HPLC provided Ac-Pro-Leu-His-Ser-Pmab(POM)$_2$-amide (**6**) as a white solid (Scheme 2).

In order to examine the bio-reversibility of the Pmab POM protection of **6**, we performed *in vitro* assays using porcine liver estase (PLE) in phosphate buffer at pH 7.4 (Srivastva and Farquhar 1984). We observed that the parent Pmab *bis*-POM-containing **6** was rapidly converted to the mono-POM-containing product ($t_{1/2} \approx 5$ minutes) with further deprotection to the free Pmab-containing peptide occurring at a slower rate (20% conversion over 8 h) (Fig. 2). Reduction in the rate of enzymatic cleavage of a second POM group is known (Srivastva and Farquhar 1984).

Conclusions

Our current paper presents the synthesis of a reagent [Fmoc-Pmab(POM)$_2$-OH (**2**)] that allows the facile synthesis of peptides containing the phosphatase-stable pThr mimetic, Pmab, bearing bio-reversible POM protection. This represents a rare example of a reagent that allows the solid-phase synthesis of polypeptides having a pThr mimetic in bio-reversible prodrug form. Reagent **2** should find utility in a variety of pharmacological applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ac	Acetyl
$[\alpha]_D$	Specific rotation
BnBr	Benzyl bromide
Bu^t	*t*ert-Butyl
Cbz	Benzyloxycarbonyl
CH_2Cl_2	Dimethyl chloride

CH_3CN	Acetonitrile
DIPEA	Diisopropylethylamine
DMF	Dimethylformamide
ESI	Electrospray ionization
EtOAc	Ethyl acetate
F_2Pmp	Difluorophosphonomethylphenylalanine
Fmoc	9-Fluorenylmethoxycarbonyl
Fmoc-OSu	9-Fluorenylmethyl succinimidyl carbonate
HBTU	1-*O*-Benzotriazole-*N,N,N',N'*-tetramethyl-uromium-hexafluoro-phosphate
HCl	Hydrochloric acid
His	Histidine
HOBt	1-Hydroxybenzotriazole
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectra
Leu	Leucine
LiOH	Lithium hydroxide
MeOH	Methanol
$MgSO_4$	Magnesium sulfate
Mtt	Methyltrityl
m/z	Mass-to-charge ratio
$NaHCO_3$	Sodium bicarbonate
NMP	*N*-Methyl-2-pyrrolidone
NMR	Nuclear magnetic resonance
PBD	polo box domain
Pd•C	Palladium on carbon
PLE	Porcine liver esterase
Plk1	Polo-like kinase 1
Pmab	(2*S*,3*R*)-2-Amino-3-methyl-4-phosphonobutyric Acid
POM	Pivaloyloxymethyl
POMI	Pivaloyloxymethyl iodide
PPIs	Protein-protein interactions
pSer	Phosphoserine
pThr	Phosphothreonine
pTyr	Phosphotyrosine
Ser	Serine
SPPS	Solid-phase peptide synthesis

$t_{½}$	Half life
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilane
Trt	Trityl

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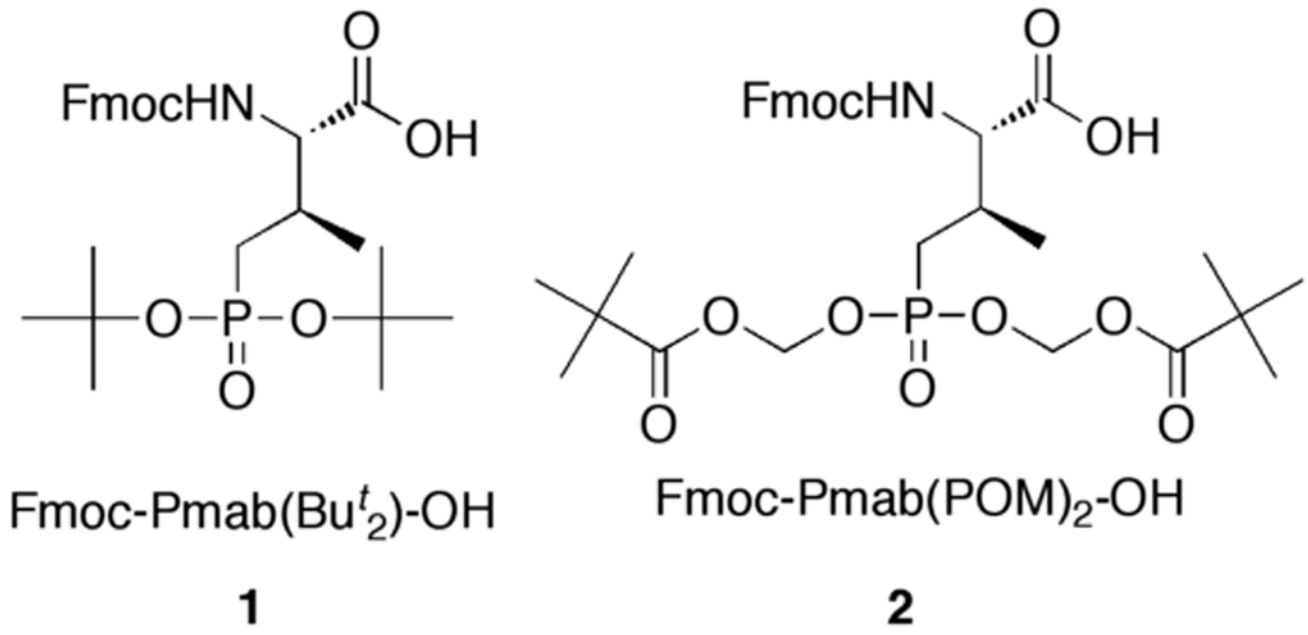
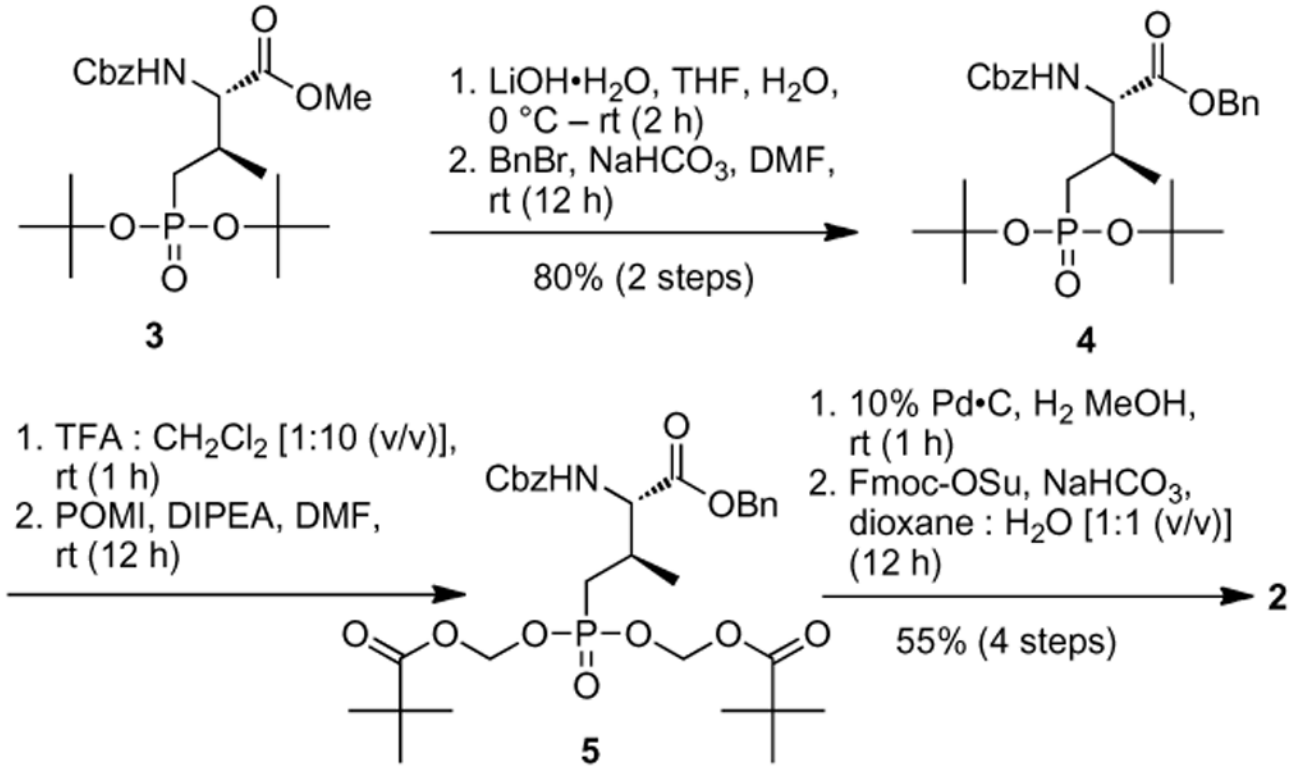
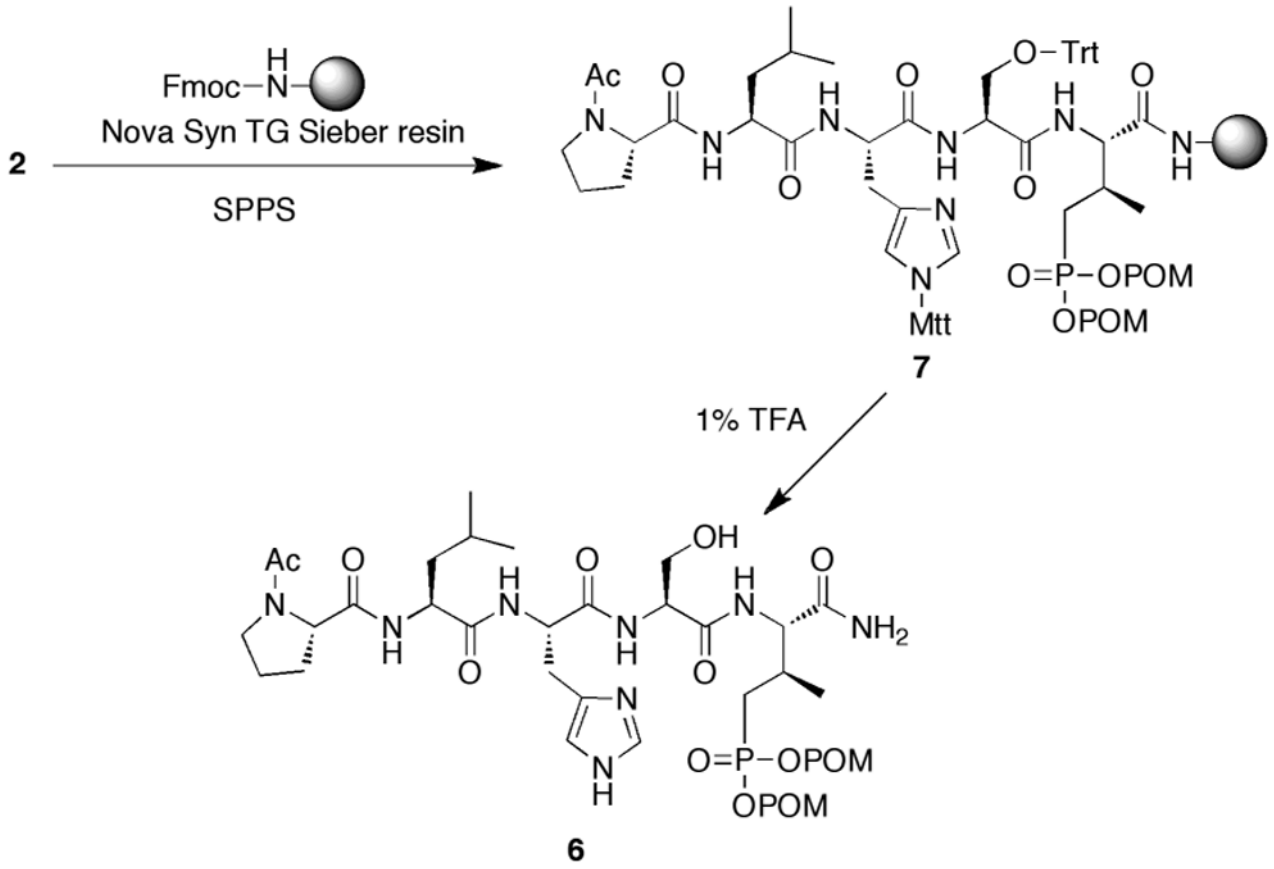


Fig. 1.
Structures of key reagents



Scheme 1.
Synthesis of title reagent **2**



Scheme 2.
Synthesis of *bis*-POM-protected peptide **6** using reagent **2**

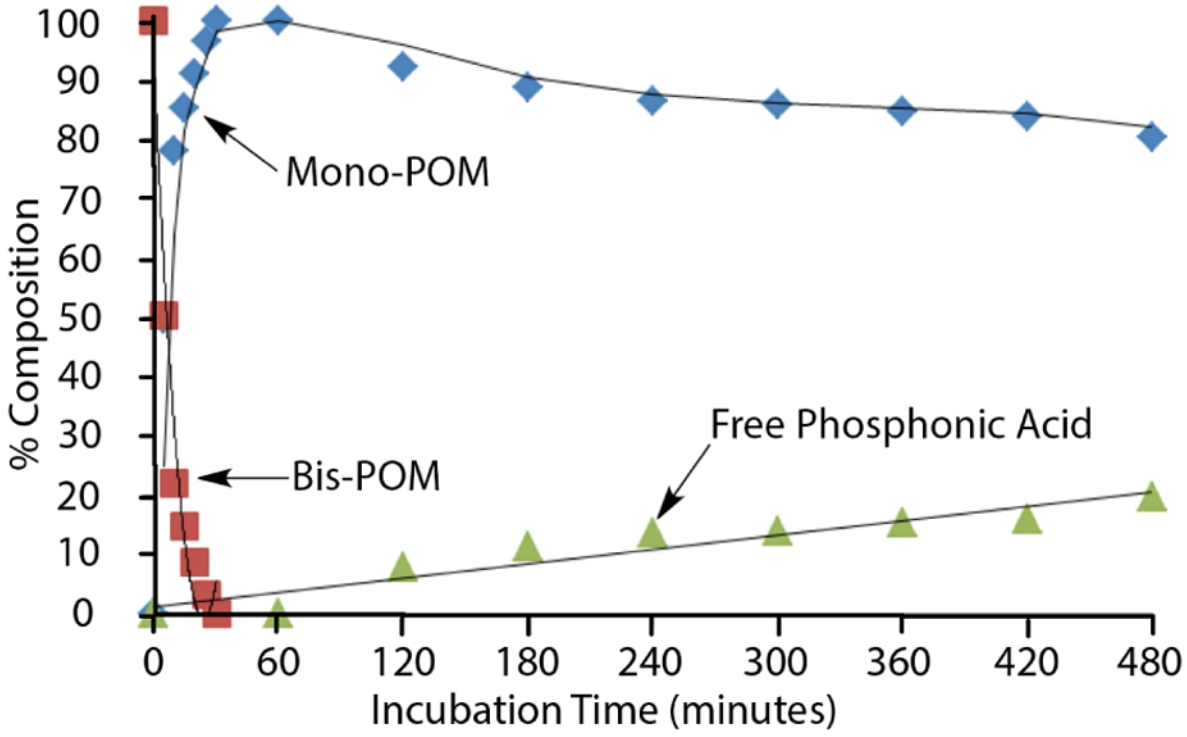


Fig. 2.
Results of pig liver esterase treatment of peptide **6**