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PAPER

Triazole phosphohistidine analogues compatible with the Fmoc-strategy†

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Phosphorylation of histidine is essential for bacterial two-component signalling; its importance to modulation of eukaryotic protein function remains undefined. Until recently, no immunochemical probes of this post-translational modification existed, however triazole phosphonate analogues of this modified amino acid have now been applied to the generation of site-specific antibodies. The protecting group strategy used in the original report is incompatible with standard protocols for Fmoc-solid phase peptide synthesis. In this paper, we report the application of P(III) chemistry to generate the complementary dibenzyl and di-*tert*-butyl phosphonate esters. These forms of the triazole analogue are fully compatible with standard Fmoc-SPPS and are therefore ideal for wider application by the chemical and biochemical community.

Introduction

Protein phosphorylation is central to the roles of proteins in cell signalling and regulation.[1] To date, the majority of studies have been limited to the phosphate esters derived from serine, threonine and tyrosine however phosphoanhydrides derived from aspartate and glutamate[2] are observed in addition to phosphoramidates derived from lysine,[3] arginine[4] and histidine.[5] These latter classes of modification are not stable to acidic (and sometimes neutral) conditions and the development of methods to detect them by mass spectrometric or immunochemical means has lagged behind those for the more stable phosphate esters.[3,6] In the case of histidine, phosphorylation can occur on both nitrogens in the side chain forming two distinct regioisomers (Fig. 1); both regioisomers are observed, however the τ-(*tele*-)form **2** is thought to be the predominant form at present.[7]

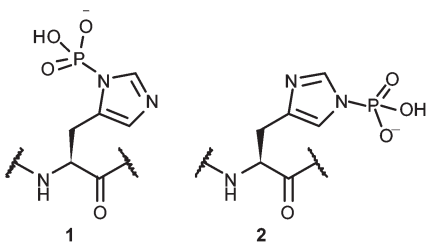

Fig. 1 The two isomers of phosphohistidine; π- **1** and τ-**2**.

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Histidine phosphorylation is observed in a wide range of systems.[7,8] It is essential for signal transduction in bacterial two-component signalling systems:[9] binding of a ligand by a sensor kinase leads to autophosphorylation to generate a phosphohistidine residue, the phosphate is then transferred to an aspartate residue in the cognate response regulator leading to regulation of gene expression. This transfer is dependent upon the relative lability of the P–N bond in phosphohistidine and this lability also allows the residue to act as a reactive intermediate in phosphotransfer enzymes such as pyruvate, orthophosphate dikinase[10] and glycogen mutase.[11] In human systems, the role of phosphohistidine is less clear. Histidine phosphorylation has been detected as a modification on only a small number of proteins including on histones H1 and H4,[12] on G-protein coupled receptors such as the cystic fibrosis transmembrane receptor,[13] on annexin I[14] and on P-selectin.[15] For some of these systems, candidate histidine kinases and histidine phosphatases have been identified,[16] indeed recent work by Attwood *et al.* using chemical phosphorylation of peptides based on histone H4 has highlighted some promiscuity in protein histidine phosphatase (PHP).[17]

A wide-range of phosphorylation-specific antibodies for the phosphate esters derived from serine, threonine and tyrosine are available. The phosphate esters are sufficiently stable to hydrolysis to allow a specific immune response to phosphorylated proteins or peptides. In the case of phosphotyrosine, monoclonal antibodies selected using a simple peptide conjugate can be used to identify tyrosine phosphorylation in numerous proteins; in general antibodies to serine and threonine are sequence-specific. For histidine phosporylation, the lability of the residue means that the generation of antibodies has not been reported (Fig. 2).

An alternative approach to the use of the native modified amino acid residue is to use stable analogues of this residue as

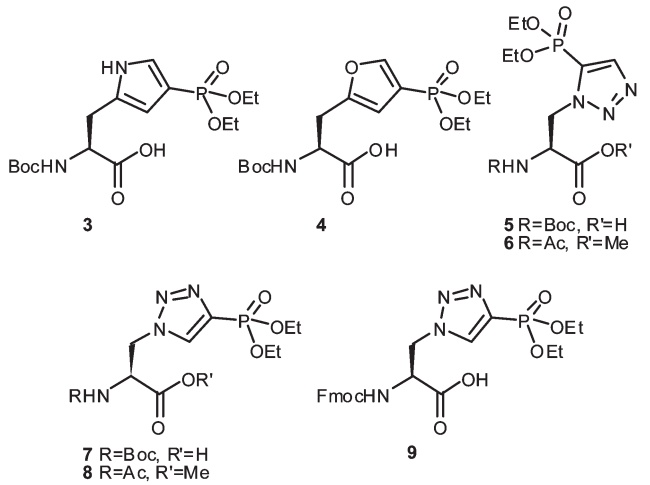
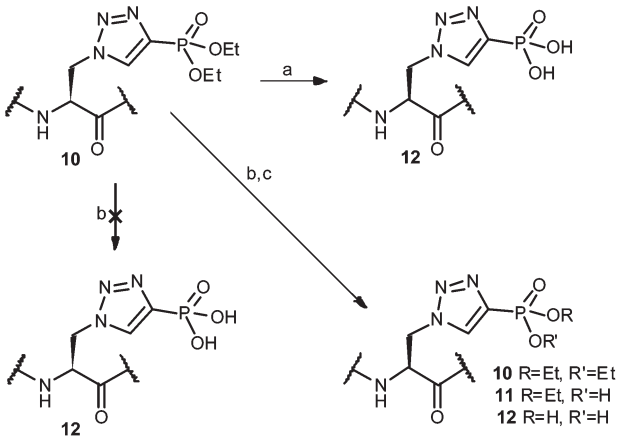


Fig. 2 Previously reported analogues of pHis.

haptens for antibody generation. Schenkels *et al.*[18] reported the phosphonofurylalanine **4** analogue over a decade ago, but there have been no subsequent reports of using **4** as a hapten. Similarly, antibodies generated from the phosphonopyrrolylalanine **3** by Attwood *et al.* were not selective for pHis.[19] Kee *et al.*[20] have now reported the synthesis of the two Boc-protected phosphonotriazolylalanine analogues **5** and **7**. These analogues were incorporated into peptides using Boc-solid phase peptide synthesis (SPPS) as part of a sequence based upon histone H4. The peptides were subsequently used to generate an antibody, which was able to distinguish the analogue from the phosphate esters of serine, threonine and tyrosine in the context of the peptide. The antibody was also able to distinguish chemically phosphorylated histone H4 from non-phosphorylated histone H4 in a site-specific manner.

We have subsequently reported the synthesis of the corresponding Fmoc-protected triazolylalanine derivative **9**.[21] While this molecule is readily incorporated into peptides using standard Fmoc-SPPS methodology, the diethyl phosphonate diester **10** is unreactive under standard cleavage/deprotection conditions (95% TFA) and only the diethyl phosphonate-containing peptide could be obtained in this fashion (Scheme 1). This contrasts with the Boc-SPPS protocol described by Kee *et al.* where the diethyl groups were removed by the anhydrous HF used for cleavage, yielding the phosphonic acid **12**. We subsequently demonstrated that prolonged treatment with trimethylsilyl bromide could be used to obtain the phosphonic acid **12** (as a separable mixture with the mono- and di-ethyl phosphonate esters **11** and **10**). A third method was recently demonstrated by Mukai *et al.*,[22] who used 33% HBr/AcOH to deprotect their triazolyl phosphonate diethyl ester compounds bearing carboxylate ester and acetamide functionalities **6** and **8**. However, the utility of this procedure has only been shown for small molecules, not in the context of a peptide.

A more convenient strategy for generating phosphonic acid-containing peptides is clearly required. This route should be fully compatible with standard conditions for Fmoc-SPPS and not require any additional post-synthesis modifications. We propose that the dibenzyl- and di-tert-butyl protected analogues **13** and **14** fulfill these requirements (Fig. 3) and report their



Scheme 1 Existing procedures for producing phosphonic acid-containing peptides of general structure **12** from the corresponding diethyl ester **10**. Standard cleavage conditions (a) from Boc-compatible resin yield **12**, while standard Fmoc-compatible resin cleavage conditions (b) cleaves and deprotects the rest of the peptide but not the phosphonate **10**. This can be subsequently hydrolysed using specific conditions (c) to give a mixture of **10**, **11** and **12**. Conditions: (a^{20}) Anhydrous HF, *p*-cresol, 0 °C, 1h; (b^{21}) 94%TFA, 2.5% EDT, 2.5% H_2O, 1% TIS, rt, 2h; (c^{21}) TMS-Br, DCM, rt, 3 days.

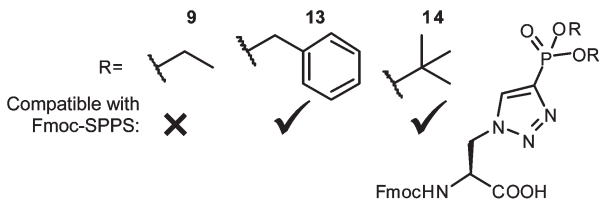


Fig. 3 The protecting group used for the phosphonate moiety previously **9** and the target compounds **13** and **14** for this work.

synthesis *via* a novel P(III) intermediate in this manuscript. Application of these derivatives will benefit the wider chemical and biochemical community without access to the specialist facilities required for Boc-SPPS and lead to the wider application of this class of analogues in generation of site-specific antibodies for histidine phosphorylation.

Results and discussion

The existing synthetic strategies for both regioisomers of triazole-based phosphohistidine analogues are dependent upon the Ru- or Cu-catalysed [3 + 2] cycloaddition of azidoalanine derivatives with diethyl ethynylphosphonate **17** to yield the π- or τ-isomer respectively.[20,22] This cycloaddition is compatible with multiple derivatives of azidoalanine including *N*-acetyl, *N*-Boc and *N*-Fmoc protected amino acids. Synthesis of the dibenzyl and di-*tert*-butyl triazole analogues is therefore contingent on identification of a suitable synthetic route to the corresponding dibenzyl- or di-*tert*-butyl ethynylphosphonates **15** and **16** respectively (Fig. 4).

We initially investigated the transesterification of the diethyl ethynylphosphonate **17** under acidic and basic conditions. **17** was dissolved in BnOH (15 eq) and heated at 120 °C with either

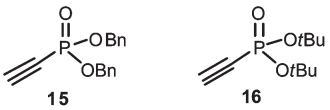
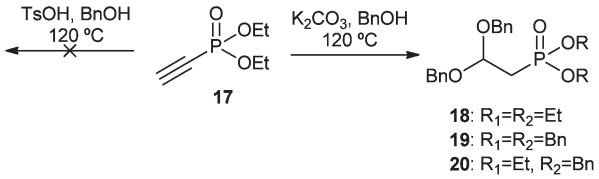
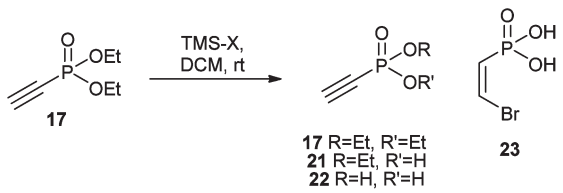


Fig. 4 Target alkynes that will give triazoles furnished with phosphonate esters cleavable by TFA.



Scheme 2 Transesterification did not yield the target phosphonate **15**.

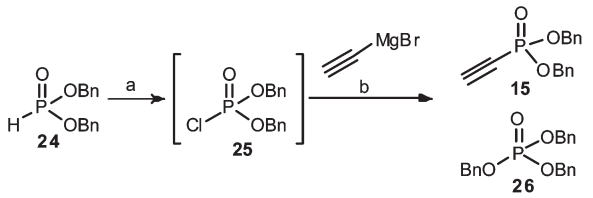


Scheme 3 TMS-X deprotection of diethyl ethynyl phosphonate **17**.

0.1 eq *p*-tolylsulfonic acid or potassium carbonate for 3 h. No reaction was observed in acidic conditions, in basic conditions three new products (Scheme 2) were observed. Isolation of these compounds by column chromatography indicated that these were the three dibenzyl acetals **18**, **19** and **20** formed *via* Michael addition to the alkyne followed by transesterification to form the dibenzyl ester and the mixed ester. No benzyl-protected alkyne could be detected and the distribution of products suggested that the Michael addition occurs more rapidly than transesterification under these conditions.

We next investigated deprotection of the phosphonate diester **17** using TMSBr to generate the phosphonic acid **22** (Scheme 3), which we postulated could then be converted to the desired dibenzyl ester by treatment with benzyl bromide. Overnight reaction with a large excess of TMSBr led to complete consumption of product, however characterisation of the product indicated the presence of the bromoalkene **23**[23] in an approximately 3 : 5 ratio to the desired phosphonic acid **22**. It was not possible to readily separate these compounds by chromatography. Reduction in the number of equivalents of TMSBr and reaction time yielded the same product mixture. Reduction to approximately stoichiometric quantities of TMSBr (2.5 eq) and a reduced reaction time led to a mixture of starting material **17**, the mono-deprotected alkyne **21**, the fully deprotected alkyne **22** and the undesired bromoalkene **23**. Reaction with the less nucleophilic TMSOTf led to a mixture of partially protected alkynes suggesting that protecting group manipulation at this point in the synthetic route is not an effective strategy.

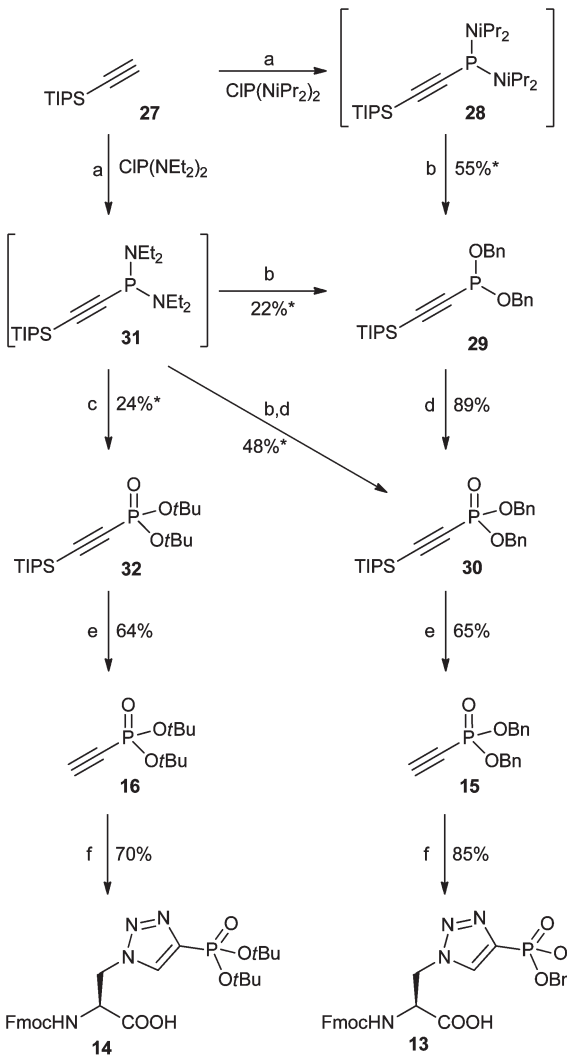
Following these disappointing results, we sought a route to generate the correctly protected alkynes directly. We (and others) previously prepared diethyl ethynylphosphonate **17** through reaction of diethyl chlorophosphate with an ethynyl Grignard



Scheme 4 (a) Trichloroisocyanuric acid, MeCN, rt; (b) THF, 0 °C.

reagent. At the time of study, the equivalent dibenzyl chlorophosphate **25** was not commercially available so we sought to generate both dibenzyl and di-*tert*-butyl chlorophosphates *via* the method of Acharya *et al.*[24] These authors reported the conversion of dialkyl phosphites into chlorophosphates *via* treatment with trichloroisocyanuric acid. We initially investigated the formation of dibenzylchlorophosphate **25** using this procedure (Scheme 4). When preparing this compound the authors did not carry out the distillation procedure as means of purification as they had done with the majority of their other examples. Further searching in the literature revealed that **25** decomposes before distillation occurs, so with this in mind we elected to use the unpurified mixture in a reaction with ethynyl Grignard reagent. This yielded a mixture of the target alkynyl phosphonate **15** and tribenzylphosphate **26** (identified by NMR), which were inseparable by chromatography.

Cox *et al.*[25] and Mukai *et al.*[22] described the synthesis of diethyl phosphonoethylene **17** using diethyl chlorophosphite. The corresponding dibenzyl and *tert*-butyl compounds are not commercially available but we chose to use the inherent reactivity of a P(III) intermediate to generate a more general synthetic route capable of producing various dialkylalkynylphosphonates. The key compounds for this new synthetic strategy are the ethynylphosphoramidite intermediates **28**/**31** (Scheme 5). We formed these intermediates *via* reaction of the corresponding bis(dialkylamino)chlorophosphine with a Grignard reagent generated from triisopropylsilyl-protected acetylene **27** and isopropylmagnesium chloride. (The fluoride-labile TIPS protecting group was selected in preference to TMS to minimise desilylation during chromatography on silica gel.) The moisture-sensitive intermediates were not isolated but following a solvent switch from THF to MeCN, were activated with tetrazole and reacted with either benzyl or *tert*-butyl alcohol to form the corresponding phosphonites. The dibenzylphosphonite **29** was isolated by column chromatography, then oxidised to form ethynyl dibenzylphosphonate **30** using hydrogen peroxide. The corresponding di-*tert*-butyl phosphonite was not isolated as the di-*tert*-butylphosphonate **32** was formed spontaneously upon standing. We initially employed bis(diisopropylamino) chlorophosphine, however substitution with bis(diethylamino)chlorophosphine (a liquid rather than a solid) did not lead to significant changes in the observed yields and simplified the experimental procedure. Direct oxidation of the dibenzyl phosphonite product **30** during work-up with aqueous H_2O_2 was later used to yield **31** directly. Removal of the TIPS group with TBAF then generated the target dialkyl protected alkynylphosphonates **15** and **16** in a 30% overall yield (for the dibenzyl phosphonate), which is comparable to the yields from the single step addition of ethynyl Grignard to diethyl chlorophosphate.



Scheme 5 (a) i – *i*PrMgCl, THF, 0 °C; ii – phosphoramidite, THF, 0 °C; (b) BnOH, tetrazole, MeCN, 0 °C; (c) *t*BuOH, tetrazole, MeCN, 0 °C; (d) H_2O_2 (30% in H_2O), rt; (e) TBAF, THF, −78 °C; (f) Fmoc-azidoalanine-OH, $CuSO_4{\cdot}5H_2O$, sodium ascorbate, THF/H_2O, rt. * yield from **27**.

We next generated the two Fmoc-protected triazolylalanines **13** and **14** by Cu(I)-catalysed cycloaddition in the presence of ascorbic acid using Fmoc-protected azidoalanine as employed previously for the synthesis of **9**. Reaction of both alkynes generated both target molecules **13** and **14** in good yield. While both target protected triazole analogues were available, we chose to employ the dibenzyl-protected analogue in peptide synthesis since the di-*tert*-butyl analogue **14** was unstable to storage and decomposed over time.[26]

The next step was to incorporate the analogue **13** into a peptide and demonstrate its deprotection under standard TFA-based conditions. Initial attempts showed poor yields for the coupling reaction of **13**, and we observed mixtures of the target peptide with a peptide of truncated sequence, missing **13** (identified by LC-MS – data not shown). It was not possible to separate these by size, however using ion-exchange it was possible to separate these peptides to give analytically pure samples of phosphonate containing peptide (data not shown). Replacement of the

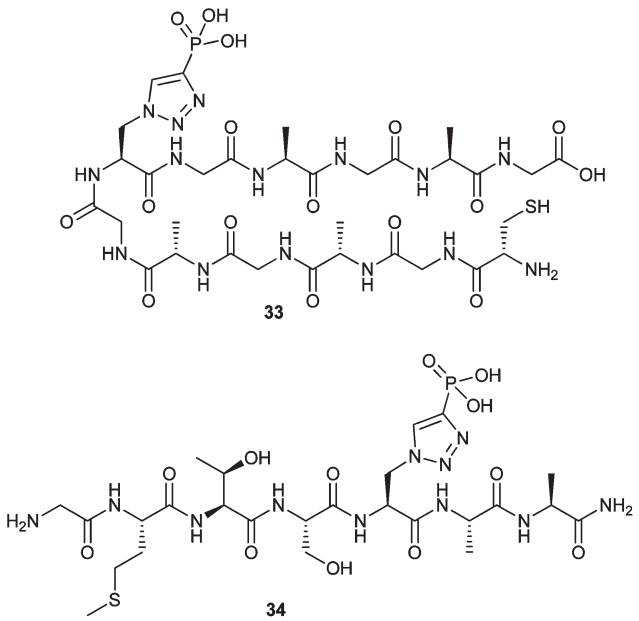


Fig. 5 Peptides **33** and **34** were successfully produced following exposure of a peptide containing the benzyl protected phosphonate building block **13** to a standard TFA-based cleavage cocktail.

HCTU coupling reagent with the more reactive HATU led to near quantitative incorporation, as did the use of two sequential coupling steps using HATU without Fmoc-deprotection in between.

Using our optimised procedure to couple **13**, we synthesized two model peptides; one functionalised peptide with the same sequence as the phosphohistidine carrier domain of PPDK (H_2N-GMTSpTzAA-$CONH_2$) in addition to the relatively unfunctionalised sequence (H_2N-CGAGAGpTzGAGAG-CO_2H). As predicted, standard TFA-based resin cleavage/deprotection of the phosphonate esters furnished fully deprotected peptides **33** and **34** in good yield. Both peptides are readily purified by ion-exchange chromatography (Fig. 5).

Conclusions

We have demonstrated the synthesis of two protected phosphohistidine analogues fully compatible for incorporation into peptides *via* Fmoc SPPS. We have subsequently demonstrated their application to the Fmoc synthesis of peptides: the benzyl ester protecting groups on the phosphonate **13** are readily removed by using standard TFA-based deprotection/cleavage conditions. This represents a significant advance over the ethyl-protected phosphonate **9** which required extremely hazardous reagents (HF) or long reaction times under other harsh conditons (TMS-Br or HBr/AcOH). This compatibility with standard procedures for peptide synthesis will allow the application of this class of phosphohistidine analogues by the wider biochemical and chemical community. Compounds **13** and **14** should be valuable tools in the further elucidation of the biochemistry of phosphohistidine-containing systems enabling the generation of antibodies specific for τ-phosphohistidine in a sequence-specific manner.

Experimental†

Dibenzyl (triisopropylsilyl)ethynylphosphonite (29)

isoPropylmagnesium chloride (2.0 M in diethyl ether, 2.5 mL, 5 mmol) was added dropwise to an ice-cold solution of triisopropylsilylacetylene (1.1 mL, 4.8 mmol) in anhydrous THF (40 mL). The mixture was stirred over ice for 5 min and allowed to warm to room temperature for 30 min before cooling once more over ice. bis(Diethylamino)chlorophosphine (1 mL, 4.685 mmol) was added dropwise, the mixture stirred for 10 min over ice then allowed to warm to rt for 2 h. The volatiles were removed *in vacuo* to leave an amorphous grey solid, the apparatus back-filled with N_2, the flask quickly removed and purged with N_2. Anhydrous acetonitrile (30 mL) was added to the flask, followed by benzyl alcohol (anhydrous, 1.1 mL, 10.63 mmol). The stirred mixture was then cooled over ice for 30 min and tetrazole (0.45 M in MeCN, 23 mL, 10.354 mmol) added dropwise. Upon complete addition the mixture was stirred for a further 15 min over ice, then at rt for 45 min. Saturated. aq. NH_4Cl (10 mL) was added to the stirred solution, producing a biphasic mixture; the solution was decanted off and the remaining solid washed with EtOAc (3 × 20 mL). The combined washes were transferred to a separating funnel, and washed with brine (2 × 30 mL), dried ($MgSO_4$) and concentrated *in vacuo* to a colourless oil. Column chromatography with a gradient of hexane–EtOAc (19 : 1–4 : 1), yielded the title compound **29** as a colourless oil (433 mg, 22%). δ_H (300 MHz, $CDCl_3$): 7.37–7.24 (10H, m, 2 × Ph), 5.01 (2H, dd, J^2_{H-H} 12.1, J^3_{H-P} 8.3, 2 × OC*H*HPh), 4.93 (2H, dd, J^2_{H-H} 12.1, J^3_{H-P} 8.3, 2 × OCH*H*Ph), 1.17–1.05 (21H, m, Si(CH(CH_3)$_2$)$_3$ + Si(C*H*(CH$_3$)$_2$)$_3$); δ_C (75 MHz, $CDCl_3$): 138.2 (d, J^3_{C-P} 4.1, Ph–C_1), 128.5 (s, Ph–C_3), 127.9 (s, Ph–C_4), 127.7 (s, Ph–C_3), 110.3 (d, J^2_{C-P} 2.3, PC*C*Si), 108.1 (d, J^1_{C-P} 47.8, P*C*CSi), 69.9 (d, J^2_{C-P} 5.1, O*C*H$_2$Ph), 18.65 (s, Si(CH(*C*H$_3$)$_2$)$_3$), 11.1 (s, Si(*C*H(CH$_3$)$_2$)$_3$); δ_P (121 MHz, $CDCl_3$): 130.2 (p, J^3_{P-H} 8.3) HRMS Found *MH*$^+$: Found 427.2232, $C_{25}H_{36}O_2PSi$ requires 427.2217

Di-*tert*-butyl (triisopropylsilyl)ethynylphosphonate (32)

32 was synthesised as **29**, but using *tert*-butanol (anhydrous, 1 mL, 10.8 mmol) in place of benzyl alcohol. The crude product was a clear pale yellow oil, which was purified by column chromatography with a gradient of hexane–EtOAc (9 : 1–2 : 1) to yield the title compound **32** as a colourless oil (407 mg, 24%). R_f (2 : 1 hexane–EtOAc) = 0.37; ν_{max} (film)/cm^{-1} 2945, 2131 (C≡C), 1464, 1395 (C(CH$_3$)$_3$), 1370 (C(CH$_3$)$_3$), 1270 (P═O), 992 (P–O); δ_H (300 MHz, $CDCl_3$): 1.61–1.50 (18H, s, 2 × OC(CH_3)$_3$), 1.13–1.07 (21H, m, Si(CH(CH_3)$_2$)$_3$ + Si(C*H*(CH$_3$)$_2$)$_3$); δ_C (75 MHz, $CDCl_3$): 102.7 (d, J^2_{C-P} 38.8, PC*C*Si), 102.3 (d, J^1_{C-P} 272.4, P*C*CSi), 84.0 (d, J^2_{C-P} 7.6, O*C*(CH$_3$)), 30.2 (d, J^3_{C-P} 4.3, OC(*C*H$_3$)$_3$), 18.4 (s, Si(CH(*C*H$_3$)$_2$)$_3$), 11.0 (s, Si(*C*H(CH$_3$)$_2$)$_3$); δ_P (121 MHz, $CDCl_3$): −20.4 (s); HRMS Found *MH*$^+$: 397.2302, $C_{19}H_{39}NaO_3PSi$ requires 397.2298.

Dibenzyl (triisopropylsilyl)ethynylphosphonate (30)

Hydrogen peroxide (30% wt solution in H_2O, 4 mL, 39 mmol) was added to dibenzyl (triisopropylsilyl)ethynylphosphonite **29** (831 mg, 1.95 mmol) and the mixture stirred at rt for 3 h, diluted with DCM (20 mL) and H_2O (10 mL) and the layers separated. The aqueous layer was extracted with DCM (2 × 10 mL), the combined organic extracts washed with H_2O (1 × 10 mL) and brine (1 × 10 mL), dried ($MgSO_4$) and concentrated *in vacuo* to yield the product **30** as a colourless oil (766 mg, 89%).; ν_{max} (film)/cm^{-1} 2945, 2132 (C≡C), 1497, 1462, 1239 (P═O), 1030 (P–O); δ_H (500 MHz, $CDCl_3$): 7.41–7.30 (10H, m, 2 × Ph), 5.11 (4H, d, J^3_{H-P} 8.5, 2 × OCH_2Ph), 1.11–1.05 (21H, m, Si(CH(CH_3)$_2$)$_3$ + Si(Ci(CH$_3$)$_2$)$_3$); δ_C (75 MHz, $CDCl_3$): 135.5 (d, J^2_{C-P} 7.6, Ph-C_1), 128.6 (s), 128.5 (s), 127.9 (s), 107.8 (d, J^2_{C-P} 38.3, PC*C*Si), 95.7 (d, J^1_{C-P} 274.8, P*C*CSi), 68.5 (d, J^2_{C-P} 5.3, O*C*H$_2$Ph), 18.4 (s, Si(CH(CH$_3$)$_2$)$_3$), 10.8 (s, Si(CH(CH$_3$)$_2$)$_3$); δ_P (121 MHz, $CDCl_3$): −8.00 (p, J^3_{P-H} 8.5); HRMS Found *MH*$^+$: 443.2170, $C_{25}H_{36}O_3PSi$ requires 443.2166, Found *MNa*$^+$: 465.1991, $C_{25}H_{35}NaO_3PSi$ requires 465.1985

Di-*tert*-butyl ethynylphosphonate (16)

TBAF (1.0 M in THF, 1.2 mL, 1.2 mmol) was added to a stirred solution of di-*tert*-butyl (triisopropylsilyl)ethynylphosphonate **32** (392 mg, 1.047 mmol) in anhydrous THF (10 mL) at −78 °C. TLC after 4 h showed complete consumption of the starting material. The reaction mixture was warmed to 0 °C and saturated. aq. NH_4Cl (5 mL) added, generating a thick colourless suspension. H_2O was added to generate a homogenous mixture, which was transferred to a seperating funnel and extracted with ether (4 × 10 mL). The combined ethereal extracts were washed with brine (1 × 10 mL), dried ($MgSO_4$) and concentrated *in vacuo* to yield a clear yellow oil that yielded crystals upon standing. Column chromatography (hexane–EtOAc 9 : 1–1 : 1) yielded a colourless solid which was recrystallised from hot heptane to give colourless plates (192 mg). Concentration of the mother liquor gave a colourless solid (40 mg) identical by NMR (combined yield 232 mg, 64%). Mp: 72.2–75.4 °C; R_f (Hex–EtOAc 1 : 1) = 0.37; ν_{max} (solid)/cm^{-1} 3130 (C–H alkyne), 2159 (C≡C), 1394 (C(CH$_3$)$_3$), 1370 (C(CH$_3$)$_3$), 1247 (P═O), 996 (P–O); δ_H (300 MHz, $CDCl_3$): 2.81 (1H, d, J^3_{H-P} 13.3, PCC*H*), 1.56 (18H, s, 2 × OC(CH_3)$_3$); δ_C (75 MHz, $CDCl_3$): 84.9 (d, J^2_{C-P} 52.5, PC*C*Si), 84.7 (d, J^2_{C-P} 7.5, O*C*(CH$_3$)), 79.2 (d, J^1_{C-P} 306.3, P*C*CSi), 30.2 (d, J^3_{C-P} 4.3, OC(*C*H$_3$)$_3$); δ_P (121 MHz, $CDCl_3$): −18.8 (d, J^3_{P-H} 13.3); HRMS Found *MNa*$^+$: 241.0968, $C_{10}H_{19}NaO_3P$ requires 241.0964; Found *MH*$^+$: 219.1136, $C_{10}H_{20}O_3P$ requires 219.1145

Dibenzyl ethynylphosphonate (15)

As **16**; TBAF (1.0M in THF, 0.8 mL, 0.8 mmol) was added to dibenzyl (triisopropylsilyl)ethynylphosphonate **30** (347 mg, 0.785 mmol) in THF (anhydrous, 15 mL). Work-up yielded a clear yellow oil purified by column chromatography eluting with a gradient of hexane–EtOAc (4 : 1) to yield the title compound **15** as a colourless oil (146 mg, 65%). R_f (Hex–EtOAc 1 : 1) = 0.40; ν_{max} (film)/cm^{-1} 3175 (C–H alkyne), 2066 (C≡C), 1497, 1456, 1270 (P═O), 999 (P–O); δ_H (300 MHz, $CDCl_3$): 7.39–7.29 (10H, m, 2 × Ph), 5.09 (4H, d, $^3J_{H-P}$ 8.6, 2 × OCH_2Ph), 2.97 (1H, d, $^3J_{H-P}$ 13.5, PCC*H*); δ_C (75 MHz, $CDCl_3$): 135.2 (d, $^3J_{C-P}$ 7.5, Ph-C_1), 128.7 (s), 128.6 (s), 128.1

(s), 88.8 (d, $^{2}J_{C-P}$ 51.7, PCCH), 73.9 (d, $^{1}J_{C-P}$ 294.7, PCCH), 68.8 (d, $^{2}J_{C-P}$ *J* 5.3, OCH_2Ph); δ_P (121 MHz, $CDCl_3$): −7.81 (dp, $^{3}J_{P-H}$ 8.6, $^{3}J_{P-H}$ 13.5); HRMS Found MH^+: 287.0834, $C_{16}H_{16}O_3P$ requires 287.0832.

(2*S*)-3-[4-(Di-*tert*-butyl-phosphoryl)-[1,2,3]triazol-1-yl]-2-(9H-fluoren-9-ylmethoxycarbonyl)aminopropionic acid (14) (Fmoc-pTz(O*t*Bu)$_2$-OH)

A freshly prepared solution of copper(II) sulphate (7 mg, 0.04 mmol) and sodium ascorbate (25 mg, 0.24 mmol) in H_2O (2 mL) was added to a stirred solution of di-*tert*-butyl ethynylphosphonate **16** (90 mg, 0.41 mmol) in THF (2 mL). After 1 min, a solution of Fmoc-azidoalanine (160 mg, 0.45 mmol) in 1 : 1 THF : H_2O (6 mL) was added in one portion and the mixture stirred at rt for 3 h, at which time TLC showed complete consumption of the azide. The reaction mixture was concentrated *in vacuo* to a yield a pale green oil, column chromatography eluting with DCM–MeOH–AcOH (94.5 : 5 : 0.5 then 94 : 5 : 1) yielded the title compound **14** as an amorphous colourless solid (204 mg, 70%). R_f (DCM–MeOH–AcOH 94 : 5 : 1) 0.06; ν_{max} (solid)/cm^{-1} 3333 (NH), 3140 (COOH), 1726 (C═O), 1673, 1538, 1451, 1219 (P═O), 1054 (P–O); δ_H (500 MHz, $CDCl_3$): 8.24 (0.73H, s, Tz-*H*$_5$), 7.73 (2H, d, *J* 7.5, Fmoc-Ar-*H*), 7.56 (2H, t, *J* 7.9, Fmoc-Ar-*H*), 7.60–7.52 (2H, m, Fmoc-Ar-*H*), 7.32–7.26 (2H, m, Fmoc-Ar-*H*), 6.16 (0.8H, s(b), N*H*), 5.11–4.90 (2H, m, CH_β + C$H_{\beta'}$), 4.85 (1H, app s(b), CH_α), 4.42–4.34 (1H, m, OC*H*H′C), 4.32–4.23 (1H, m, OCH*H*′C), 4.17 (1H, t, *J* 6.74, OCH$_2$C*H*), 1.47 (18H, s, 2 × C(CH_3)$_3$)); δ_C (75MHz, $CDCl_3$): 171.2 (*C*OOH), 155.9 (O*C*ONH), 143.7 (s), 141.2 (s), 140.7 (d, *J* 251.1, Tz-*C*$_4$), 131.2 (d, *J* 35.2, Tz-*C*$_5$), 127.7 (s), 127.1 (s), 125.2 (s), 119.9 (s), 85.2 (d, *J* 8.5, *C*(CH$_3$)$_3$), 67.4 (s, Fmoc-*C*H$_2$), 54.1 (s, *Cα*), 51.0 (s, *Cβ*), 47.0 (s, Fmoc-*C*H), 30.4 (d, *J* 3.6, C(*C*H$_3$)$_3$); δ_P (121MHz, $CDCl_3$): −2.09 (s); $[\alpha]_D^{22}$ +2.8 (*c* 0.7, MeOH); HRMS Found MH^+: 571.2291, $C_{28}H_{36}N_4O_7P$ requires 571.2316, found MNa^+ 593.2126, $C_{28}H_{35}N_4NaO_7P$ requires 593.2136.

(2*S*)-3-[4-(Dibenzyl-phosphoryl)-[1,2,3]triazol-1-yl]-2-(9H-fluoren-9-ylmethoxycarbonyl)aminopropionic acid (13) (Fmoc-pTz(OBn)$_2$-OH)

A freshly prepared solution of copper(II) sulphate (6 mg, 0.04 mmol) and sodium ascorbate (24 mg, 0.24 mmol) in H_2O (2 mL) was added to a stirred solution of dibenzyl ethynylphosphonate **15** (112 mg, 0.391 mmol) in THF (2 mL). After 1 min, a solution of Fmoc-azidoalanine (150 mg, 0.4533 mmol) in 1 : 1 THF–H_2O (6 mL) was added in one portion and the mixture stirred at rt for 3 h, at which time TLC showed complete consumption of the azide. The reaction mixture was diluted with 10% $Na_2CO_{3\ aq}$ (5 mL) and extracted with ether (2 × 10 mL). The aqueous phase was acidified to pH 1 by dropwise addition of conc. $HCl_{(aq)}$ and extracted with EtOAc (5 × 10 mL). The EtOAc extracts were combined, dried ($MgSO_4$) and concentrated *in vacuo* to yield an off-white foam. Column chromatography (DCM–MeOH–AcOH, 94 : 5 : 1) yielded the title compound **13** as a colourless oil (143 mg, 85%). R_f (DCM–MeOH–AcOH 94 : 5 : 1) = 0.08; ν_{max} (film)/cm^{-1} 3310 (NH), 3034 (COOH), 1719 (C═O), 1508, 1451, 1247 (P═O), 998 (P–O); δ_H (500 MHz, $CDCl_3$): 8.09 (1H, s, Tz-H$_5$), 7.70 (2H, d, *J* 7.38, 2 × Fmoc-H$_5$), 7.51 (2H, m, 2 × Fmoc-H$_2$), 7.39–7.17 (14H, m, 2 × Fmoc-H$_4$ + 2 × Fmoc-H$_3$ + 10 × Ph-H), 6.30 (0.7H, d, *J* 7.0, N*H*), 5.20–5.00 (4H, m, 2 × PO(OCH_2Ph)), 4.98–4.72 (3H, m, 2 × CH_β + CH_α), 4.36 (1H, m, OC*H*H′C), 4.26 (1H, m, OCH*H*′C), 4.12 (1H, t, *J* 7.03, OCH$_2$C*H*); δ_C (75MHz, $CDCl_3$):172.0 (s, COOH), 155.3 (s, NH*C*OO), 143.0 (d, *J* 4.3, Ph-*C*$_1$), 140.4 (s, Fmoc-*C*$_1$), 135.8 (d, *J* 241.8, Tz-*C*$_4$), 131.4 (d, *J* 33.9, Tz-*C*$_5$), 127.4 (s), 127.6 (s), 127.2 (s), 127.0 (s) 126.4 (s), 124.4 (s), 119.1 (s), 67.5 (d, *J* 5.5, PO*C*H$_2$Ph), 66.1 (s, Fmoc-*C*H$_2$), 53.5 (s, *Cα*), 49.9 (s, *Cβ*), 46.4 (s, Fmoc-*C*H); δ_P (121MHz, $CDCl_3$): 8.03 (p, *J* 7.9); $[\alpha]_D^{22}$ +23.6 (*c* 1.3, $CHCl_3$); HRMS Found MH^+: 639.2012 $C_{34}H_{32}N_4O_7P$ requires 639.2003; found MNa^+ 661.1844, $C_{34}H_{31}N_4NaO_7P$ requires 661.1823.

Peptides

Peptides were synthesised according to standard solid phase synthesis protocols. Couplings of standard amino acids (Fmoc-Gly-OH, Fmoc-Ala-OH, Fmoc-Cys(Trt)-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu)-OH, and Fmoc-Met-OH) were carried out using 5 equiv. (with respect to resin loading) Fmoc-amino acid, 4.9 equiv. HCTU and 10 equiv. DIPEA. Coupling of Fmoc-pTz(OBn)$_2$-OH was carried out with two sequential coupling steps using 1 equiv. Fmoc-pTz(OBn)$_2$-OH, 1 equiv. HATU and 2 equiv. DIPEA followed by 1.7 equiv. Fmoc-pTz(OBn)$_2$-OH, 1.7 equiv. HATU and 3.4 equiv. DIPEA (peptide **33**). Full details of which can be found in the ESI† along with a detailed peptide synthesis procedure, mass spec and HPLC data.

H-CGAGAG(pTz)GAGAG-OH (33)

The peptide was prepared as described in the ESI† and lyophilised to yield a flocculent yellow solid (63 mg, 51% yield). δ_H (300 MHz, D_2O): 8.09 (1H, s, pTz-Tz*H*$_5$), 5.02–4.85 (2H, m, pTz-CH_2), 4.84–4.72 (1H, m, pTz-C*H*), 4.39–4.19 (5H, m, 4 × Ala-C*H*CH$_3$ + Cys-C*H*CH$_2$SH), 4.00–3.76 (12H, 6 × Gly-CH_2), 3.02 (2H, d, *J* 5.6, Cys-CHCH_2SH), 1.39–1.25 (12H, m, 4 × Ala-CHCH_3); δ_P (121MHz, D_2O): 0.81 (s); HRMS Found MH^-: 964.3080, $C_{32}H_{51}N_{15}O_{16}PS$ requires 964.3102

H-GMTS(pTz)AA-NH$_2$ (34)

The peptide was prepared and purified as described in the ESI†: **34** was obtained as a colourless solid (4.7 mg, 19% yield after purification), HRMS Found MH^-: 752.2539, $C_{25}H_{43}N_{11}O_{12}PS$ requires 752.2556.

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26 This is probably attributable to hydrolysis of the *tert*-butyl phosphonate esters – di-*tert*-butylphosphonoethylene **16** showed similar behaviour.