Novel Phosphate Derivatives as Scaffolds for the Preparation of Synthetic Phosphoserine-Based Peptides Using the Fmoc/*t*-Bu Solid-Phase Strategy

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Abstract: Synthetic peptides incorporating analogues of phosphoserine are valuable tools for the study of protein kinases and phosphatases. In addition, derivatives of naturally occurring peptides incorporating phosphate groups may have interesting biological properties. Herein we describe a new Fmoc/*t*-Bu solid-phase peptide synthesis (SPPS) strategy for the convenient generation of phosphoserine-based peptides. A proof-of-concept synthesis that demonstrates the feasibility of this approach is presented.

Key words: phosphopeptide, serine, phosphorus, solid-phase synthesis, amino acids

Protein phosphorylation and dephosphorylation reactions are reversible processes, catalysed by kinases and phosphatases respectively, which play a key role in the regulation of cell function, growth and homeostasis.[1–3] Many diseases, including cancer, result from perturbations in the activity, or level of expression, of these enzymes.[4] Consequently the study of how nature orchestrates these opposing processes is a subject of considerable interest.[1,3] Synthetic peptides incorporating phosphorylated amino acid residues and their analogues (so-called phosphopeptides) have proven to be valuable tools for such investigations.[1,5–7] Furthermore derivatives of naturally occurring peptides incorporating phosphorylated amino acid residues (including serines) may have other interesting, potentially useful, biological properties.[8]

In most cells, a large proportion of total protein phosphorylation occurs on serine residues; as such, there has been significant effort directed towards the development of methods for the incorporation of phosphoserine-based residues into artificial peptide chains.[3,9] In this context, the 9-fluorenylmethoxycarbonyl/*tert*-butyl (Fmoc/*t*-Bu) solid-phase peptide synthesis (SPPS) strategy has proven to be especially valuable.[3,5,6,9] However, there are challenges associated with phosphopeptide synthesis using Fmoc/*t*-Bu SPPS and improvements in speed and efficiency as well as the complexity and diversity of the resulting phosphopeptides are desirable.[3] Towards this end we report here the development of a novel Fmoc/*t*-Bu SPPS strategy suitable for the efficient generation of structurally diverse phosphoserine-based peptides. As a proof-of-concept this approach was applied to the synthesis of **1**, a phosphorylated derivative of auto-inducing peptide III (AIP-III), a naturally occurring molecule involved in the regulation of quorum sensing in the Gram positive bacterium *Staphylococcus aureus* (Figure 1).[10] Non-natural analogues of AIPs hold significant value as chemical probes for studies on this intercellular signaling process and the delineation of structure-activity relationships may allow the rational development of antagonists which could potentially be used in a therapeutic context.[10a]

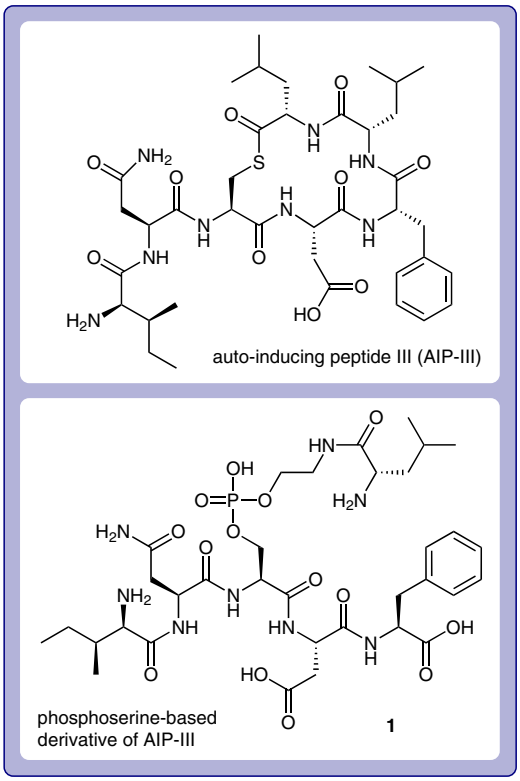

Figure 1 Structure of AIP-III and the phosphoserine-based derivative **1**

The SPPS of serine phosphopeptides via the on-resin phosphorylation of a protected peptide prepared using the standard Fmoc/*t*-Bu-strategy is known to suffer from a number of drawbacks.[11] As such it has been argued that Fmoc/*t*-Bu SPPS methods which directly incorporate a serine phosphate equivalent are more desirable.[9] Towards this end we decided to adopt a synthetic strategy involving novel tetra-protected phosphoserine analogues of the general form **2**, which present three levels of orthogonality (Scheme 1).[12] We envisaged compounds of this sort could

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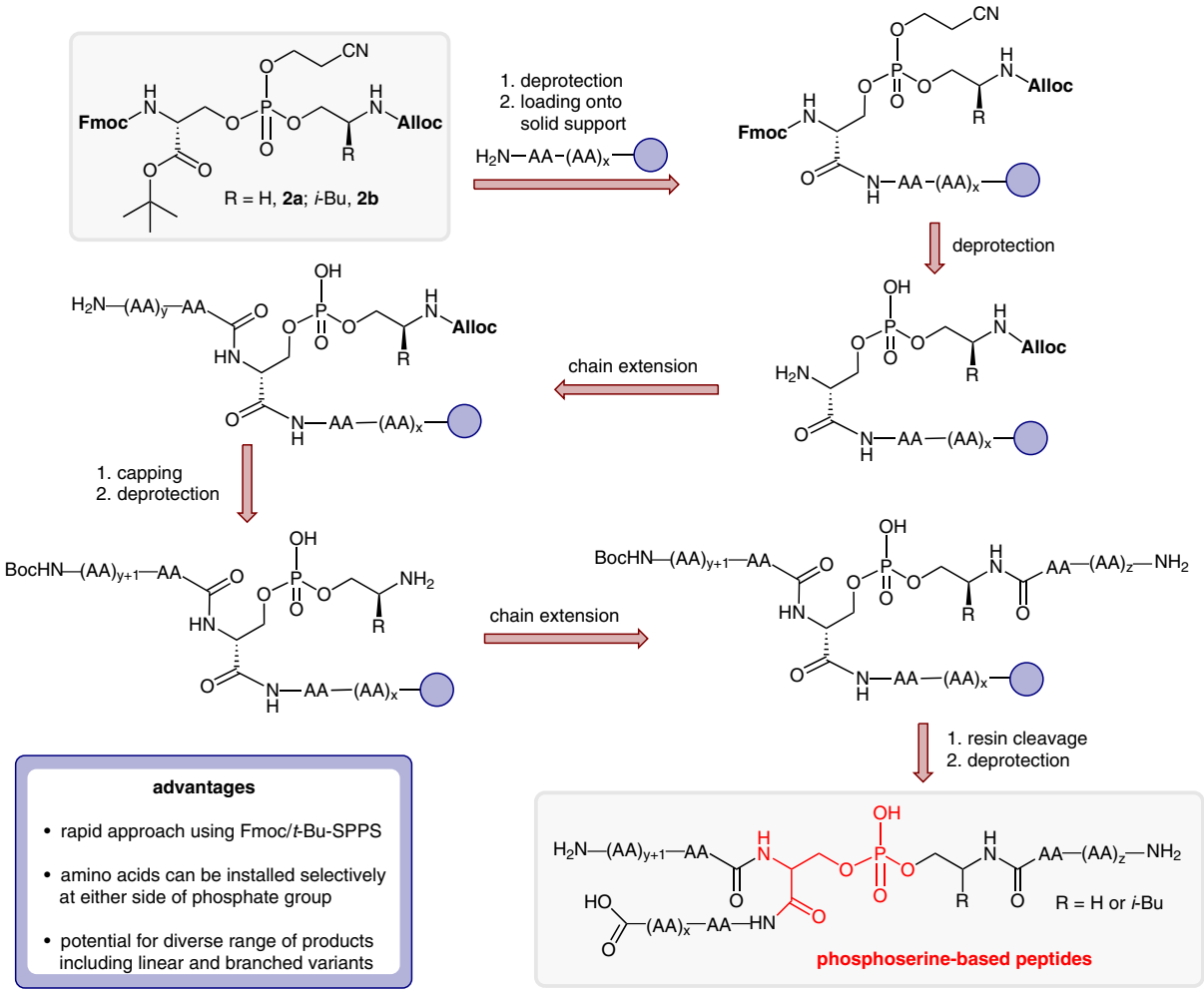
be prepared efficiently using solution-phase methods and then loaded onto the solid phase, whereupon they could serve as scaffolds around which amino acid residues could be installed using standard Fmoc/*t*-Bu SPPS techniques, thus providing a convenient method for the generation of phosphoserine-based peptides.

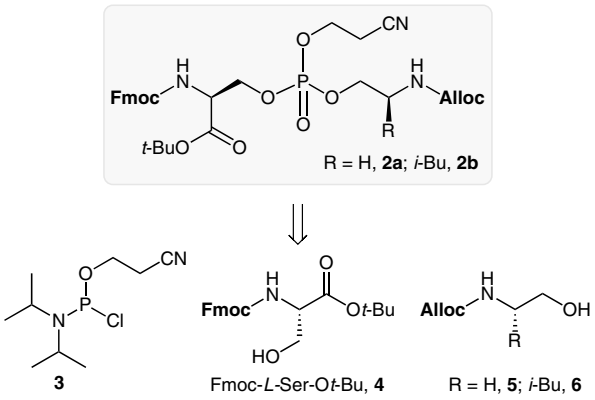
It was anticipated that **2** could be loaded onto a solid-phase resin after removal of the *t*-Bu group and reaction of the carboxylic acid group so liberated. Standard removal of the Fmoc protecting group under basic conditions should reveal the amino group thus allowing the peptide chain to be extended at one end of the phosphate group.[12] The presence of the Alloc-protected amino alcohol would provide a synthetic handle for further derivatisation of the peptide (after the peptide chain had been 'capped' with an orthogonally protected Boc-amino acid) potentially allowing access to a diverse range of products. For example, amino acids could be selectively introduced at the other side of the phosphate group to generate novel 'branched' phosphoserine-based peptides. In order to facilitate the solution-phase synthesis of the phosphoserine analogues **2** (*vide infra*) it was expected that the presence of an additional protecting group on the phosphorous would be required. However, Fmoc/*t*-Bu SPPS methods employing fully protected serine phosphotriester residues are known to be troublesome due to the propensity of the phosphate functionality to undergo β-elimination under the basic conditions required for Fmoc group removal.[8,13] Thus we decided to include a third base-labile cyanoethyl protecting group on the phosphoserine analogues **2** which would only be lost after based-induced Fmoc removal, allowing the solid-phase synthesis of the remaining phosphoserine peptide using Fmoc-based chemistry.[13]

We envisaged that **2a** and **2b** could each be accessed from three building blocks; commercially available 2-cyanoethyl-*N*,*N*-diisopropyl-chlorophosphoramidite (**3**), Fmoc-L-Ser-O-*t*-Bu (**4**) and either Alloc-ethanolamine (**5**) or Alloc-L-leucinol (**6**; Scheme 2).

Alcohols **5** and **6** were readily obtained in one and two steps, respectively, from commercial sources (see Supporting Information). Subsequent coupling with **3** furnished intermediates **7a** and **7b** in high yields (Scheme 3). Reaction with Fmoc-L-Ser-O*t*-Bu ester **4** (readily obtained in quantitative yield from Fmoc-L-Ser-OH, see Supporting Information) was performed in the presence of



Scheme 1 Overview of the strategy for the generation of phosphoserine-based peptides using phosphoserine analogues **2a** and **2b** as scaffolds around which amino acid residues can be installed using Fmoc/*t*-Bu SPPS. AA = amino acid (unspecified); x and y refer to an unspecified number of amino acid residues. Alloc = allyloxycarbonyl. The phosphoserine portion of the final product is highlighted in red.

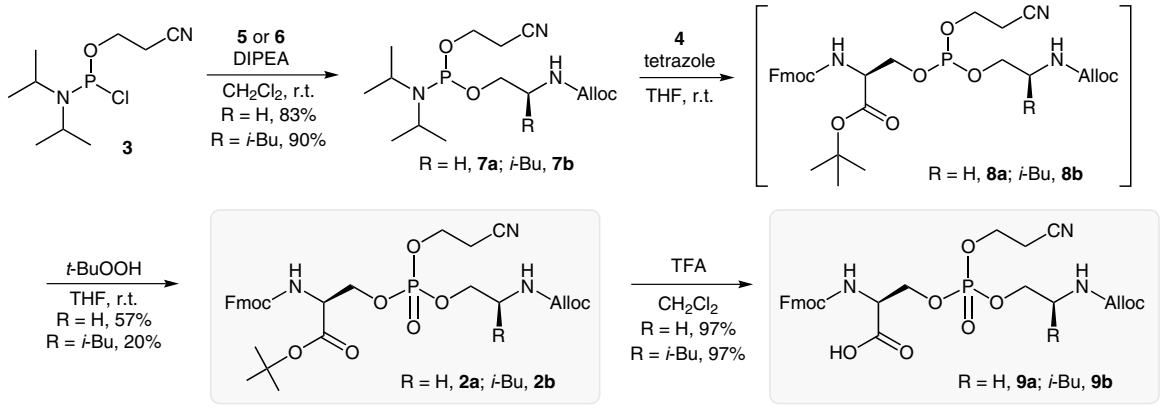


Scheme 2 Three building blocks used in the synthesis of **2a** and **2b**

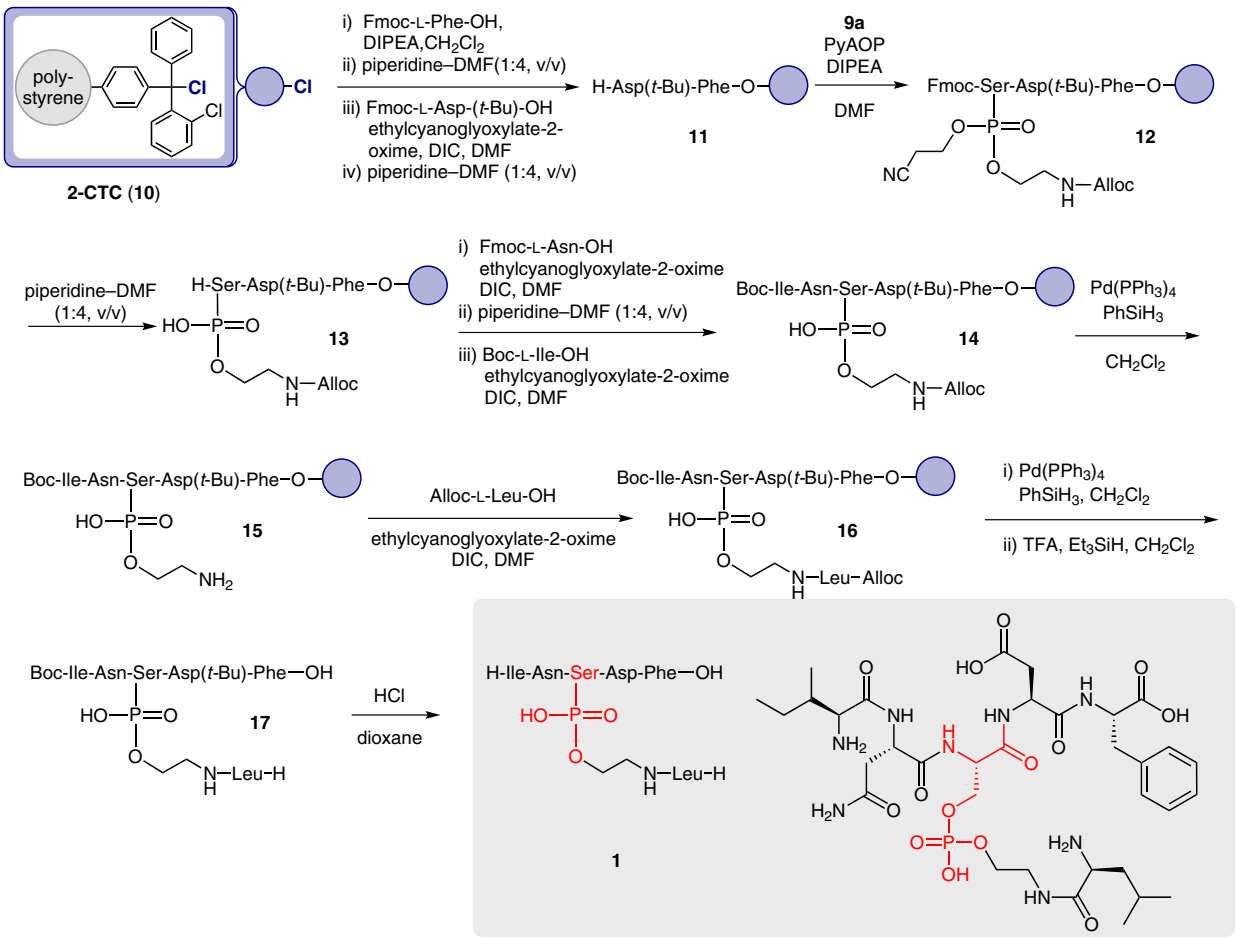
tetrazole. The obtained phosphite intermediates **8a** and **8b** were oxidised in situ with *tert*-butyl hydroperoxide (*t*-BuOOH) to furnish the desired fully protected phosphoserine analogues **2a** and **2b**. Subsequent removal of the *t*-Bu group under acidic conditions proceeded smoothly to generate the free acid derivatives **9a** and **9b**. We were next interested in exploring the suitability of compounds of this sort for the preparation of synthetic phosphopeptides using Fmoc/*t*-Bu SPPS. In line with our groups continued interest in quorum sensing[14] we targeted the synthesis of **1**, a phosphorylated derivative of naturally occurring AIP-III (Scheme 4). This molecule is known to be involved in the regulation of intercellular communication in the Gram positive bacterium *S. aureus* and non-natural analogues are of value for chemical biology studies on this system.

SPPS started with the limited incorporation of Fmoc-L-Phe-OH on 2-chlorotrityl chloride polystyrene resin (2-CTC; **10**, Scheme 4) in order to have a loading of 0.7 mmol/g.[15] The remaining resin chloride functions were capped with methanol to prevent the formation of tertiary amines after the piperidine treatment used to remove the Fmoc group. Fmoc-L-Asp(*t*-Bu)-OH was then coupled onto the solid phase using *N*,*N*-diisopropylcarbodiimide (DIC) and ethylcyanoglyoxylate-2-oxime to minimise epimerisation[16] generating derivative **11**.[17,18] Attempts to append phosphoserine analogue **9a** using these coupling conditions failed (Scheme 2); instead the use of (7-aza-benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyAOP) and DIPEA furnished the desired adduct **12**. Base-induced Fmoc removal yielded the free amine derivative **13** with concomitant cleavage of the cyanoethyl protecting group observed as desired.[19] Subsequent chain extension using Fmoc-L-Asn-OH and Boc-L-IIe-OH was achieved by a standard Fmoc/*t*-Bu SPPS approach to furnish the phosphopeptide analogue **14**.[17] With this derivative in hand we next investigated the introduction of the additional amino acids at the other side of the phosphate group. The Alloc protecting group was readily removed using Pd(0) and the resulting free amine **15** was coupled with Alloc-L-Leu-OH to give **16**. Alloc group removal followed by chemoselective acidolysis of the 2-chlorotrityl ester resin linkage using a TFA–Et_3SiH mix then afforded the partially protected hexapeptide **17**. Partial purification was accomplished by filtration of the acidolytic resin suspension into pyridine–MeOH and evaporation of the filtrate to dryness under reduced pressure. The desired phosphopeptide-based derivative **1** was obtained by treatment of **17** with HCl–dioxane to remove the remaining *t*-Bu and BOC protecting groups.[20]

In conclusion, we have described a novel strategy for the synthesis of synthetic phosphoserine-based peptides using phosphate derivatives **2a** and **2b** as molecular scaffolds around which amino acid residues can be installed by Fmoc/*t*-Bu SPPS. Proof-of-concept work demonstrated the feasibility of this approach; compound **1**, a novel phosphoserine-based derivative of the quorum sensing signaling molecule AIP-III was accessed in an efficient and straightforward fashion from readily accessible starting materials. The ability of **1** and related derivatives to modulate bacterial quorum sensing systems is currently being investigated and results will be presented in due course.[21] Overall the strategy described in this report potentially offers a general and convenient means of accessing a structurally diverse range of synthetic phosphoserine-based peptides, including both linear and branched derivatives. As such, it represents an attractive alternative method for the synthesis of this important class of biological tool compounds, with potentially broad applications in both target-oriented and diversity-oriented synthesis.[22]



Scheme 3 Synthesis of phosphoserine analogues **2a** and **2b** and the free acid analogues **9a** and **9b**



Scheme 4 Synthesis of branched phosphoserine-based peptide **1**. The phosphoserine potion of the final compound is highlighted in red.

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synlett.

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(13) (a) It is known that the stability of the phosphate moiety is increased when the phosphate triester is replaced with the diester. In addition, cyanoethyl group removal is generally quicker than serine β-elimination, thus preventing it. See: McMurray, J. S.; Coleman, D. R. IV; Wang, W.; Campbell, M. L. *Biopolymers (Pept. Sci.)* **2001**, *60*, 3. (b) Fmoc/*t*-Bu SPPS using derivatives of this sort as starting materials is superior to that employing the analogous compounds containing a free phosphoric acid group (see ref. 7).

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(15) The use of 2-CTC resin allows the cleavage of peptide from the solid phase with 1–2% of TFA without removing any side-chain protecting groups that may be present within the main peptide, which could be important from the perspective of any post-cleavage synthetic manipulations. See: (a) Benz, H. *Synthesis* **1994**, 337. (b) Barlos, K.; Chatzi, O.; Gatos, D.; Stavropoulos, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 513. (c) An additional advantage of the 2-CTC resin is that its hindered structure minimises the formation of diketopiperazines during removal of the temporary protecting group of the second amino acid on the solid phase (Scheme 4, **10** to **11**, step iv); see: Rovero, P.; Vigano, S.; Pegoraro, S.; Quartara, L. *Lett. Pept. Sci.* **1996**, *2*, 319.

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(17) (a) The chloranil test for detection of primary and secondary amines was performed after each solid-phase coupling reaction to confirm that it had proceeded to completion. See: Vojkovsky, T. *Peptide Res.* **1995**, *8*, 236. (b) When the test was negative the Fmoc group was removed with piperidine–DMF (1:4); otherwise the Fmoc-amino acid was re-coupled under the same conditions. See Supporting Information for full details.

(18) *t*-Bu and Boc were chosen for either carboxylic (**11**) or amino (**14**) protection as they were stable to piperidine treatment, used to remove the Fmoc group, and to low concentrations of TFA, used to cleave the peptide from the 2-CTC resin (Scheme 4).

(19) It is noteworthy that compounds **2a** and **2b**, as well as the corresponding free carboxylic acids **9a** and **9b**, were obtained as diastereomeric mixtures. However, once the cyanoethyl group undergoes β-elimination during the basic treatment to remove the Fmoc group in SPPS (Scheme 4), the phosphorus centre is no longer chiral leading to only one stereoisomer of the desired phosphopeptide.

(20) HPLC analysis of the crude material obtained after workup revealed that the peptide was approx. 70% pure.

(21) The cyclised analogue of **1** is of interest; comparison of the biological effects of the acyclic and cyclic variants would provide useful information on the importance of the macrocyclic ring structure for the biological activity of these types of compounds. Preliminary attempts at cyclising **1** via intramolecular amide formation have provided some evidence for the formation of the desired product. Studies towards the isolation and biological testing of the cyclised analogue of **1** are ongoing and will be reported in due course.

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