Phosphate-assisted peptide ligation†

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A novel ligation method for the synthesis of phosphopeptides and peptides is described, which utilises the inherent reactivity of a peptide bearing an N-terminal phosphoserine or phosphothreonine residue to facilitate amide bond formation with a range of C-terminal peptide thioesters.

Phosphorylation is a ubiquitous post-translational modification reported to occur on 30–50% of human proteins.[1] This reversible process is known to be intimately involved in signal transduction and the control of a host of biological processes that are critical for normal cellular function.[2] Unlike protein synthesis, phosphorylation events are not under template control, but rather are dictated by the combined activities of a range of kinase (phosphorylation) and phosphatase (dephosphorylation) enzymes. To date, the study of phosphoproteins has been significantly hampered by the fact that they exist as heterogeneous mixtures of differentially phosphorylated species. It is currently accepted that chemical synthesis may provide a viable avenue for the construction of phosphopeptides[3,4] and other post-translationally modified peptides and proteins in a homogeneous fashion and thus has been the focus of intense research efforts.[5,6] To date, the most efficient strategy for the generation of these biomolecules has relied on chemoselective ligation methods.[7,8] Native chemical ligation, initially introduced by Wieland *et al.*[9] and later exploited by Kent and co-workers for the convergent synthesis of peptides and proteins,[10,11] represents the most useful approach. The method involves the condensation of a C-terminal peptide thioester and a peptide bearing an N-terminal cysteine residue to afford a native peptide bond in a rapid and chemoselective manner.[10] A number of alternative approaches have been developed to overcome the requirement for an N-terminal cysteine residue, with a view to expanding the number of ligation sites that can be accessed by peptide ligation chemistry. Recent examples include the traceless Staudinger ligation,[12,13] native chemical ligation at phenylalanine[14] and valine residues,[15,16] native chemical ligation followed by conversion of cysteine to serine,[17] sugar-assisted ligation[18–20] and thiol free "direct aminolysis" methods.[21–23] However, there is still significant demand for the development of new ligation strategies that can be implemented in the synthesis of underivatised and post-translationally modified peptides and proteins for biological study.

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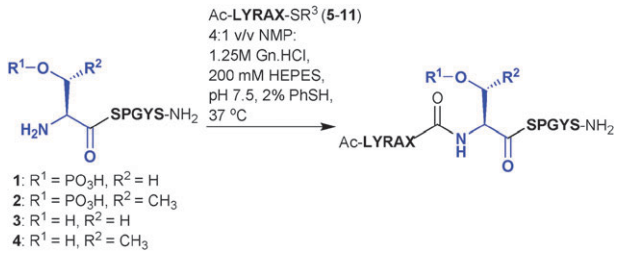
† Electronic supplementary information (ESI) available: Detailed procedures for the synthesis of peptides, phosphopeptides, peptide thioesters, ligation reactions and dephosphorylation reactions. Crude traces and analytical data for peptides, phosphopeptides, peptide thioesters, ligation products and dephosphorylated peptides. See DOI: 10.1039/b906492c

We report herein the development of an efficient new ligation method for the synthesis of peptides and phosphopeptides, namely phosphate-assisted ligation. Our design strategy aimed to harness the nucleophilicity of a phosphate group on the N-terminus of a peptide, which we envisaged would play an analogous role to the thiolate utilised in native chemical ligation. Specifically, we speculated a reaction process whereby a phosphate moiety on the N-terminus of a peptide could react with a peptide thioester to generate an acyl phosphate intermediate (*cf.* the thioester intermediate in native chemical ligation). A subsequent O → N acyl shift involving a 7-membered ring transition state could then generate the desired peptide bond (*cf.* an S → N acyl shift *via* a 5-membered ring in native chemical ligation). We were encouraged that such a reaction process would prove to be viable by the common occurrence of acyl phosphate intermediates in acylation reactions in nature. For example, aminoacylation of tRNA is promoted by aminoacyl tRNA synthetases which are known to utilise an acyl phosphate intermediate.[24,25] In this process ATP activates the carboxylate of amino acids generating an aminoacyl adenylate (an acyl phosphate of AMP). The aminoacyl moiety is subsequently transferred to the 3′-OH of the cognate tRNA generating the "charged" tRNA with the amino acid linked *via* an ester. In addition, there have been a number of recent reports from Kluger and co-workers, who have utilised acyl phosphates as biomimetic donors for the formation of esters[25,26] and amides[27] in an intermolecular fashion. The same group has also utilised acyl phosphates to crosslink proteins such as haemoglobin by amide bond formation with lysine side chains.[28]

To gauge the efficiency of the proposed ligation reaction we first subjected model peptide **1**, bearing an N-terminal phosphoserine residue, and peptide thioester **5**, possessing a C-terminal glycine, to a mixed solvent buffer comprising 4 : 1 v/v *N*-methylpyrrolidinone : 1.25 M Gn·HCl, 0.2 M HEPES, pH 7.5 in the presence of thiophenol at 37 °C (see ESI†). After 48 h, we were delighted to observe that the reaction was complete, with the desired phosphopeptide product isolated in 91% yield (entry 1, Table 1). Gratifyingly, peptide **2**, bearing an N-terminal phosphothreonine residue, also reacted with thioester **5** in a facile manner to afford the desired phosphopeptide in 95% yield (entry 2, Table 1). In both cases, amide bond formation was confirmed by stability of the resulting product to extended hydrazine treatment (see ESI†). The importance of the phosphate moiety for the efficiency of this ligation reaction was verified by conducting control ligations with peptides **3** and **4**, bearing N-terminal serine and threonine residues, respectively. These were reacted with **5** under the same mixed solvent system to afford ligated peptide products in only 45 and 36% yields, respectively, after a substantially increased reaction time of 72 h (entries 3 and 4, Table 1). Given the increased steric bulk of the

Table 1 Scope of the phosphate-assisted ligation reaction. Reaction times: [a]48 h; [b]72 h; [c]14 d. Reaction yield error ± 5%. R^3 = $(CH_2)_2CO_2Et$; NMP = *N*-methylpyrrolidinone; Gn·HCl = guanidine hydrochloride; HEPES = 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid. **5**: **X** = AsnGly; **6**: **X** = AsnAla; **7**: **X** = AsnMet; **8**: **X** = AsnPhe; **9**: **X** = Tyr; **10**: **X** = Ser; **11**: **X** = Val



Entry	X	R^1	R^2	Ligation junction	Isolated ligation yield (%)
1	AsnGly	PO_3H	H	Gly-pSer	91[a]
2	AsnGly	PO_3H	CH_3	Gly-pThr	95[a]
3	AsnGly	H	H	Gly-Ser	45[b]
4	AsnGly	H	CH_3	Gly-Thr	36[b]
5	AsnAla	PO_3H	H	Ala-pSer	90[a]
6	AsnMet	PO_3H	H	Met-pSer	71[a]
7	AsnPhe	PO_3H	H	Phe-pSer	82[b]
8	Tyr	PO_3H	H	Tyr-pSer	82[b]
9	Ser	PO_3H	H	Ser-pSer	62[b]
10	AsnVal	PO_3H	H	Val-pSer	47[c]
11	AsnAla	PO_3H	CH_3	Ala-pThr	61[b]
12	AsnMet	PO_3H	CH_3	Met-pThr	50[b]
13	AsnPhe	PO_3H	CH_3	Phe-pThr	52[b]
14	Tyr	PO_3H	CH_3	Tyr-pThr	90[b]
15	Ser	PO_3H	CH_3	Ser-pThr	62[b]
16	AsnVal	PO_3H	CH_3	Val-pThr	47[c]

phosphate group, this study provided initial evidence that the phosphate moiety was crucial to the efficiency of the ligation reactions, consistent with our experimental design. Kinetic studies were subsequently conducted to quantify this increase in reaction rate (Fig. 1). After 10 h, reactions with **1** and **2** provided a 50% yield of the desired ligation product and reactions were complete within 48 h. In contrast, unphosphorylated peptides **3** and **4** underwent sluggish ligation reactions, corroborating that the phosphate moiety in **1** and **2** was assisting the formation of the peptide bond and that these reactions were not proceeding through a direct aminolysis reaction alone. Indeed, sampling the reactions at 12 h revealed that the

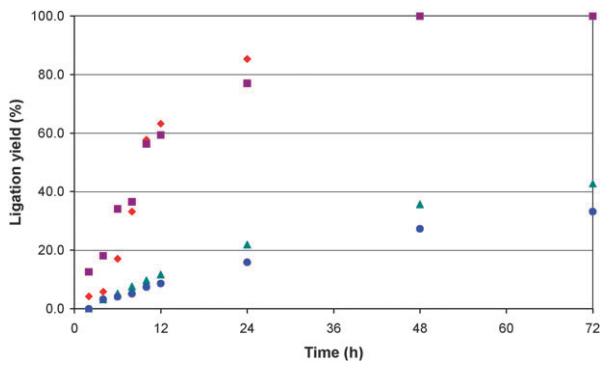


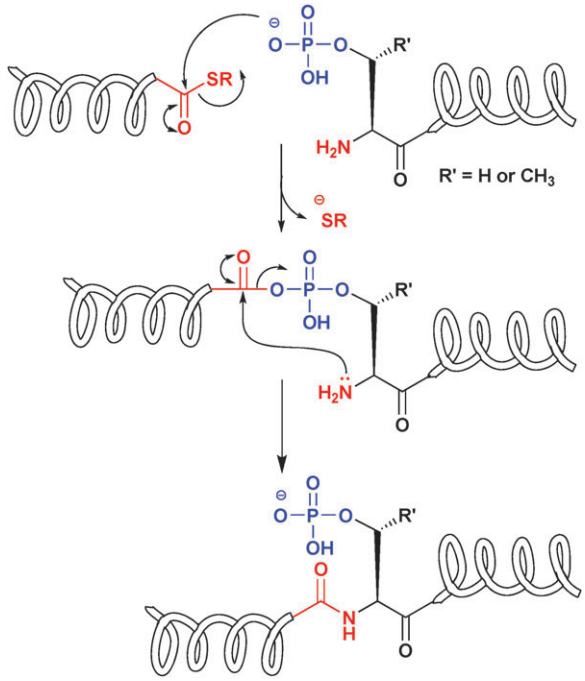
Fig. 1 Kinetics of the phosphate-assisted peptide ligation between peptide thioester **5** and peptides **1–4**; ■ = **1**, ◆ = **2**, ▲ = **3**, ● = **4**.

phosphate moiety in **1** and **2** confers a six-fold rate acceleration when compared to unphosphorylated peptides **3** and **4**.

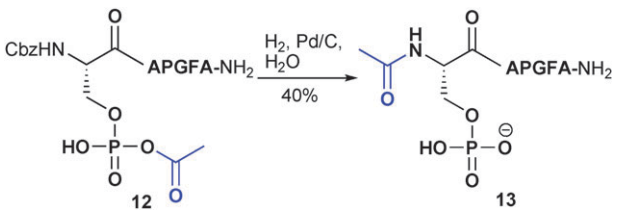
Having demonstrated that N-terminal phosphorylamino acids enhance the rates of ligation reactions, the next goal was to investigate the scope of the phosphate-assisted ligation reactions. To this end, we synthesised peptide thioesters bearing a range of amino acids at the C-terminus (see ESI†).[29] Gly, Ala, Met, Phe, Tyr, Ser and Val were selected as a representative range of the 20 proteinogenic amino acids to incorporate into the C-terminus of peptide thioesters (**5–11**). These were reacted with phosphopeptides **1** and **2** under the previously described conditions (Table 1). We were delighted to find that the phosphate-assisted ligation reactions with **1** were high yielding in almost all cases (entries 5–10, Table 1). Indeed, ligations with thioesters **5–10** bearing C-terminal Gly, Ala, Met, Phe, Tyr and Ser residues gave the desired phosphopeptides in yields ranging from 62 to 91%. In line with reactivity patterns observed in native chemical ligation, the ligation with C-terminal valine thioester **11** resulted in a much slower reaction, requiring 14 days to reach completion. Fortunately, the stability of valine thioester **11** under the ligation conditions led to slow hydrolysis and allowed for a satisfactory ligation yield (47%, entry 10, Table 1). Ligations with phosphopeptide **2** containing an N-terminal phosphothreonine residue provided lower yields, presumably due to the increased steric bulk of the threonine residue. Nonetheless, isolated reaction yields were moderate to high in all cases and represent synthetically useful reactions (entries 11–16, Table 1).

Having established that the phosphate moiety enhanced the rate of peptide ligations, we next investigated its specific role in the reaction. This began by attempting to detect the acyl phosphate intermediate that would be formed if the reaction were operating *via* the mechanism depicted in Scheme 1. Unfortunately, all attempts at identifying the proposed acyl phosphate intermediate by HPLC, LC-MS, ^{31}P NMR and FTIR proved unsuccessful. We therefore prepared a model of the proposed intermediate, peptide **12** containing an N-terminal serine residue, bearing an acyl phosphate moiety (see Scheme 2 and ESI†). Acyl phosphate **12** proved to be an extremely reactive intermediate which, upon removal of the N-terminal Cbz-protecting group by hydrogenolysis, rearranged spontaneously *via* an intramolecular O → N acyl shift to afford the *N*-acetylated phosphopeptide **13**. Phosphopeptide **13** was produced in relatively low yield due to the rapid hydrolysis of **12** under the reaction conditions. Although not conclusive evidence for the intramolecular mechanism proposed in Scheme 1, this study does provide initial evidence that such an intermediate can undergo an intramolecular O → N acyl shift to afford an amide bond in a rapid manner.

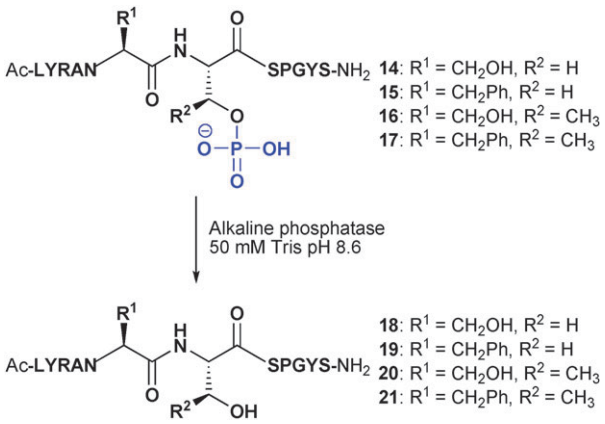
Finally, as an additional application of the phosphate-assisted ligation, we set upon dephosphorylation reactions of these ligation products to afford unmodified peptides. To this end, a selection of phosphopeptide ligation products **14–17** were treated with alkaline phosphatase in 50 mM tris buffer at pH 8.6 to provide dephosphorylated peptide products **18–21** in high yields in all cases (74–98% isolated yields, Scheme 3). Notably, the successful implementation of dephosphorylation reactions significantly expands the utility of the phosphate-assisted ligation, whereby the phosphate moiety can be introduced as a traceless ligation auxiliary with a view to incorporating native serine and threonine residues into target peptides or proteins.



Scheme 1 Proposed mechanism of phosphate-assisted ligation.



Scheme 2 Intramolecular O → N acyl shift of acyl phosphate.



Scheme 3 Enzymatic dephosphorylation of ligation products.

The generation of unmodified peptides by enzymatic dephosphorylation reactions post-ligation therefore provides a direct disconnection at serine and threonine residues, a novel addition to the ligation chemistry toolbox.

In summary, we have developed an efficient new method for the construction of phosphopeptides and peptides. The reaction utilises the reactivity of an N-terminal phosphoserine or phosphothreonine residue to facilitate the formation of a native peptide bond. The method displayed impressive scope for a range of amino acids at the ligation junction, and, as such, should serve as a useful tool for the construction of biologically relevant peptides, phosphopeptides, proteins and phosphoproteins in future studies. We have conducted preliminary experiments which suggest that acyl phosphates can undergo rapid acyl migration to the N-terminal amine of a peptide however, it is not yet clear that such an intermediate is formed during the ligation reactions. Further delineation of the mechanism of the phosphate-assisted ligation will be the focus of future research in our laboratory. In addition, the phosphate-assisted ligation is currently being employed in the total synthesis of biologically relevant phospho- and glycoproteins in our research group.

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