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COMMUNICATION

Biomimetic peptide bond formation in water with aminoacyl phosphate esters†

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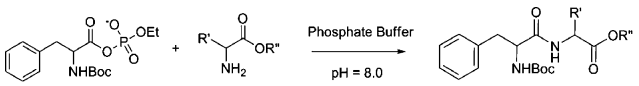
Aminoacyl phosphates, biomimetic analogues of aminoacyl adenylates, react efficiently with amino acid esters to form dipeptides with retention of stereochemical integrity. The reactions are selective and occur readily in the presence of nucleophiles other than amino groups on their side chains. Aminoacyl phosphate esters that lack an amino-protecting group are also suitable for peptide bond formation, leading to a simplified overall process.

We report the use of aminoacyl phosphates as the key to a biomimetic protocol for producing peptides in water. Our method is based on an analogue of the common intermediate in biochemical pathways that efficiently produces peptides from amino acids. In these pathways the initial activation of an amino acid by reaction with ATP generates an enzyme-bound aminoacyl adenylate.[1] In this intermediate, the carboxylic acid is activated as an acyl phosphate monoester and this acylates a second substrate that is bound to the enzyme.[2] The transient nature of the acyl phosphate monoester in enzymes is not due to any inherent instability: acyl phosphate monoesters are long-lived in water.[3] Due to this stability, several reports in the literature have described the application of synthetic acyl phosphate monoesters in aqueous acylation reactions.[4,5] The known characteristics make them logical candidates for a central role in a biomimetic approach to peptide synthesis; they provide an opportunity for selective reactions and a reduced dependence on protecting groups. Thus, we investigated the efficiency of the reactions of aminoacyl phosphate monoesters with amino acid esters. We find that dipeptides are formed readily in buffered aqueous solutions, establishing a basis for a general protocol that can be extended for multiple steps in aqueous peptide coupling.

Scheme 1 presents the overall reaction scheme. As an initial test of the general approach, reactions were performed with *N*-*t*-Boc-protected phenylalanyl ethyl phosphate (BocPheEP) and glycine ethyl ester (GlyOEt).

Analysis of the residual aminoacyl phosphate by ^{31}P-NMR reveals that it is fully converted within one hour (pH 8 HEPES buffer). The reaction produces BocPheGlyOEt as a precipitate that is readily isolated by filtration (55% isolated yield). Additional product was recovered using solid phase extraction cartridges with activated carbon as the stationary phase. ESI-MS data confirmed that the dipeptide is formed exclusively without further reactions that would result in oligomerization. In extending the study, we find that aminoacyl phosphate esters are also efficiently coupled to other amino acid esters (Table 1).



Scheme 1 Peptide bond formation *via* BocPheEP and an amino acid ester.

In all cases the coupling reaction occurs rapidly and the conversion of the amino acid ester to the dipeptide by acylation is nearly quantitative. The precipitation of the product greatly simplifies the process as the material is isolated by filtration. This result is consistent with the development of ideal reactions in water where two reactants that are completely soluble combine to form a product which is insoluble; this approach serves to reduce waste generated during work-up procedures.[6]

We find that the reactions are selective for peptide bond formation in the presence of other reactive nucleophiles. The reaction of the aminoacyl phosphate with serine ethyl ester produces the dipeptide with no acylation of the hydroxyl of the side chain (based on results of the hydroxamic acid test[7]). However, where cysteine ethyl ester is the substrate, there is greater potential for side chain acylation due to the presence of a significant proportion of the thiolate relative to the thiol at pH 8.0. Nonetheless, the coupling reaction results exclusively in formation of the peptide. This conclusion is based on qualitative analysis (TLC analysis and Ellman's test) as well as comparative analysis of the products of the reaction in which *N*-acetyl cysteine serves as the substrate. This selectivity may result from a direct reaction of the more reactive amine or an initial aminoacylation of the thiolate followed by S to N acyl migration, a common pattern in cysteine-centred ligation strategies.[8]

A recent report from our laboratory noted that aminoacyl phosphates without a protecting group on nitrogen provide selective lanthanide-promoted esterification of nucleosides and nucleotides without oligomerization of the reagent.[9] This prompted us to attempt peptide coupling with deprotected aminoacyl phosphate monoesters (Scheme 2).

Thus, in neutral buffer, PheEP forms oligopeptides and diketopiperazines that result from self-condensation. These reactions must be suppressed in order for this class of reagent to be

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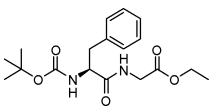
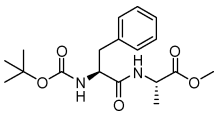
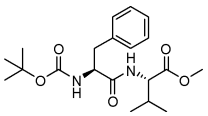
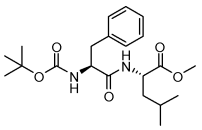
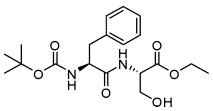
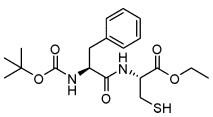
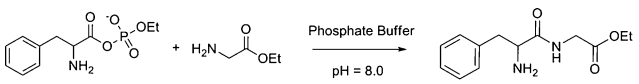
† Electronic supplementary information (ESI) available: Detailed experimental procedures and NMR data for coupling reactions of BocPheEP. See DOI: 10.1039/c1ob05660c

Table 1 Summary of peptide coupling reactions of BocPheMP and amino acid esters

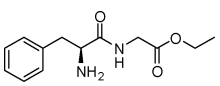
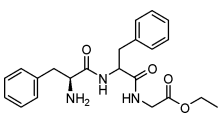
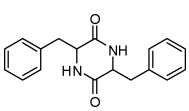
Amino acid ester	Product	Isolated yield	ESI-MS data
GlyOEt		55%	351.1 (M + H$^+$)
AlaOMe		27%	351.1 (M + H$^+$)
ValOMe		43%	379.1 (M + H$^+$)
LeuOMe		80%	393.2 (M + H$^+$)
SerOEt		33%	381.1 (M + H$^+$)
CysOEt		50%	397.1 (M + H$^+$)



Scheme 2 Peptide coupling between phenylalanine ethyl phosphate (PheEP) and GlyOEt.

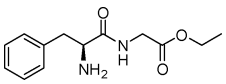
utilized for the desired peptide coupling process. We find that in the reaction of PheEP with GlyOEt in the presence of 50% molar excess of the amino acid ester leads to efficient coupling with suppression of side reactions (ESI-MS peak identification in Tables 2 and 3). A control experiment with PheEP in 50% molar excess gave products due to the side reactions. The results indicate that an excess of the non-activated substrate is needed to suppress side reactions. Our results are consistent with the general reactivity patterns of acyl phosphates monoesters with amines ($\beta_{nuc} = 0.9$).[10] The amino groups of the aminoacyl phosphate and the amino acid ester have similar pK_a values and without any other structural distinction they would be expected to react at comparable rates. However, the anionic character of the phosphate adds a barrier to self-condensation. In combination with higher concentrations of the attacking amine, this distinction results in selective reaction of the amino acid ester in an otherwise competitive situation. We then extended this approach to reactions with alanine and again found that an excess of the amino acid is required before peptide bond formation is observed.[11] Reactions with the use of additional amino acid substrates are expected to follow the same pattern and the utility of deprotected materials is currently the subject of further studies.

Table 2 ESI-MS analysis of peptide coupling reaction between PheEP and GlyOEt (1 : 1 ratio)

Compound	ESI-MS data
	251.1 (M + H$^+$)
	398.2 (M + H$^+$), 545.1 (M + 147), 692.1 (M + 2 × 147), 839.1 (M + 3 × 147)
	295.1 (M + H$^+$)

Activated amino acids are potentially subject to racemization due to the increased acidity of their α-protons compared to those of the free acid. This can be prevented by mediating the reactivity of the activated amino acid by changing the activating group or by the use of additives.[12] It has already been established that activation of an amino acid as an acyl phosphate ester does not lead to racemization of its aminoacyl component.[13]

Table 3 ESI-MS analysis of peptide coupling reaction between PheEP and GlyOEt (1 : 1.5 ratio)

Compound	ESI-MS data
	251.1 (M + H^+)

This was demonstrated by base hydrolysis of the aminoacyl phosphate, followed by derivatization with Mosher's acid chloride. The resultant materials, when tested against authentic samples, did not undergo any racemization. By extension, as the peptide coupling reaction is performed under mildly basic conditions, there should be no racemization in the dipeptide products. Further, we examined the ^{1}H-NMR spectrum of an aminoacyl phosphate ester in D_2O and found that there is no exchange of the α-proton, which is a necessary consequence of racemization and is thus a reliable test. The anionic charge of the phosphate may also contribute to configurational stability by hydrogen bonding to the α-proton. Similar effects account for the configurational stability of acyl azides.[14] The anionic charge also repels hydroxide, which is the necessary catalyst for proton removal.

In conclusion, we have demonstrated that aminoacyl phosphate monoesters function as effective acyl donors in water in their reactions with amino acid esters. The pattern is clearly suitable as a basis for peptide coupling in water in general. Furthermore, the process permits formation of amides from amines in the presence of other reactive nucleophiles. Based on these results, we are broadening the scope of our peptide coupling protocol as well as expanding the method to related applications.

Acknowledgements

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