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Preparation of active esters on solid support for aqueous-phase peptide couplings

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Abstract—TentaGel supported active esters analogous to those prepared from solution-phase reagents such as 1-hydroxybenzotriazole, *N*-hydroxysuccinimide, and pentafluorophenol were prepared and examined for their ability to effect aqueous-phase peptide couplings. HOBt esters showed high hydrolysis rates, relative to peptide coupling, while active esters derived from 4-hydroxy-2,3,5,6-tetrafluorobenzoic acid produced mainly dipeptide coupling products when treated with amino acids in water.

Active esters have become a staple of peptide synthesis. The formation of an active ester in a peptide coupling protocol may be achieved either in situ or as a separate step. In either case, their use generally improves peptide couplings, particularly those mediated by carbodiimides, by reducing both racemization and side reactions such as *N*-acyl urea formation.[1] Numerous alcohols have been used for the formation of active esters, including *N*-hydroxysuccinimide (HOSu),[2] 1-hydroxybenzotriazole (HOBt),[3] 1-hydroxy-7-azabenzotriazole (HOAt),[4] and pentafluorophenol (PFP).[5] In addition to solution-phase reactions, several of these alcohols have been linked to solid supports which allows the active ester to be easily isolated and stored.[6] The stability, ease-of-use and recyclable nature of these solid-supported active esters has led to their use in applications outside of classical peptide synthesis.[7]

Although the conventional peptide synthesis is typically carried out in organic solvents, peptide couplings have also been performed in aqueous media, most commonly using soluble PEG supports.[8] It has been shown that, as in organic solution, the formation of active esters significantly reduces racemization in aqueous media.[9] To aid water solubility, active esters containing charged functional groups have been developed. The most widely used of these is *N*-hydroxysulfosuccinimide, which has been used for labeling of proteins in water.[10] However, even this popular method suffers from low yields due to hydrolysis of the active ester by water, high cost and difficulty in recycling of the reagent.

As a part of a project to develop dynamic peptide libraries, we desired a solid-supported active ester which would function effectively in aqueous solution such that amide bond synthesis could be carried out in the presence of an enzyme target. In this paper, we report the first example of solid-supported active esters for use in water. The efficiency of amide bond synthesis for three active esters (**1**–**3**, Fig. 1) in water was studied and for the optimum resin **3**, coupling was found to be efficient even with dilute concentrations of the amine.

The choice of TentaGel as the solid support for the active esters was based on its excellent swelling properties in both aqueous and organic media.[11] The first resin prepared was *N*-linked HOBt residue **1**. Prior to preparation on solid support, the synthesis of this unit was optimized in solution.[12] Thus, 4-chloro-3-nitrobenzoylchloride was allowed to react with *tert*-butylamine in dichloromethane and pyridine to afford amide **5a** in 89% yield (Scheme 1). Strong acylation catalysts such

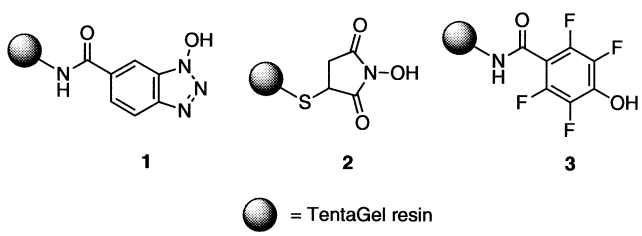

Figure 1. Solid-supported alcohols for active ester formation.

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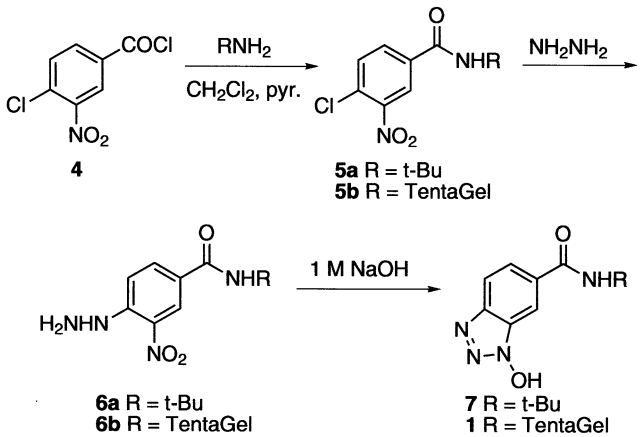


Scheme 1. Preparation of TentaGel linked HOBt.

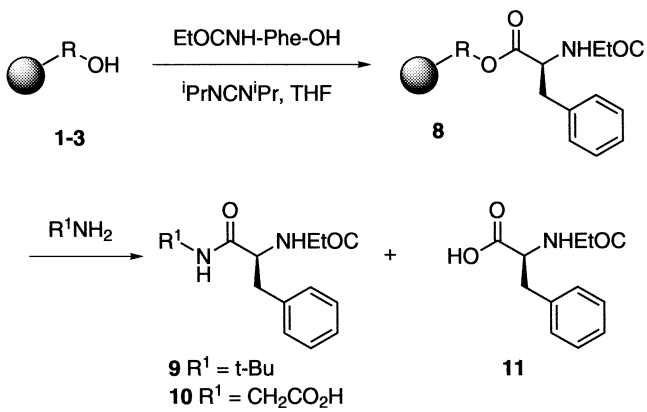
as DMAP must be avoided in this reaction, otherwise by-products arising from nucleophilic aromatic substitution are observed. The amide **5a** was treated with neat hydrazine to form **6a**. Although the reaction appeared to be quantitative, purification of the product was extremely difficult. Thus it was treated directly with aqueous sodium hydroxide to form the 1-hydroxybenzotriazole **7** in 94% yield over two steps. Direct application of this protocol to TentaGel-NH_2 (0.44 mmol/g) afforded a low loading (<5%) of the final HOBt unit **1** (see below for quantitation procedures). The low yield was traced to the nucleophilic aromatic substitution step, as it was observed that TentaGel resins do not swell significantly in pure hydrazine. The addition of DMF as co-solvent (3:1 DMF/NH_2NH_2) in this reaction was a sufficient remedy and using this procedure, the HOBt resin was obtained in high yield (vide infra). The remaining resins, HOSu analog (**2**) and PFP analog (**3**), were prepared following minor modifications of literature procedures. Thus, TentaGel-SH was prepared by treatment of TentaGel-Br (0.27 mmol/g) with thiourea followed by hydrolysis.[13] The resulting thiol was allowed to react with *N*-hydroxymalimide in the presence of pyridine to form resin **2** in modest yield.[6b,14] The PFP analog **3** was prepared in good yield by acylating TentaGel-NH_2 with 4-hydroxy-2,3,5,6-tetrafluorobenzoic acid using diisopropylcarbodiimide (DIC) and pyridine in THF, followed by treatment with *t*-$BuNH_2$ to cleave any oligomeric acylation products.[6c]

In all three cases, the yield for the synthesis of the alcohol was quantified by preparing the active ester of EtOC-Phe-OH using DIC in THF, followed by reaction with *tert*-butylamine (10 equiv) in THF to form EtOC-Phe-NH^{t}Bu (Scheme 2). The yield of the amide coupling step was taken as a lower limit for the yield of the resin preparation, and indicated a 77% yield of **1** (0.34 mmol/g), a 24% yield for **2** (0.065 mmol/g), and a 73% yield for **3** (0.32 mmol/g).

The ability of all three resins to effect peptide coupling in aqueous solution via their active esters was studied. The active esters of EtOC-Phe-OH were prepared from all three resins using standard conditions (DIC/THF). The resins were carefully rinsed to remove all traces of residual DIC, diisopropylurea and unreacted EtOC-Phe-OH. The peptide couplings were carried out using 0.1 M glycine in pH 10 aqueous solution at 21°C for 16 h.[15] The products were isolated by repeated rinsing of the resin with water, followed by acidification of the aqueous filtrate and extraction into ethyl acetate. Under these conditions, the HOBt active ester afforded a small amount of the dipeptide product along with a greater amount of the direct hydrolysis product, EtOC-Phe-OH (Table 1). Although HOBt has been used as an additive in aqueous solution-phase coupling reactions, the slower reaction rates associated with solid supports presumably are to blame for the increased levels of hydrolysis observed with resin **1**.

In contrast to the HOBt resin, active esters derived from both the HOSu resin **2** and the tetrafluorophenol resin **3** produced the dipeptide as the major product, with the latter affording a higher ratio of coupling to hydrolysis. The mass balance in all of these reaction is around 60–70%. This reflects the effect of the solvent change on the swelling of the resin. In water, TentaGel resin only swells to 2/3 the volume observed in THF. When the recovered resins were treated with *t*-$BuNH_2$ in THF, the remaining active ester was released as the *t*-Bu amide.

Given the higher ratio of coupling to hydrolysis for resin **3**, along with its facile and less costly preparation relative to **2**, we chose to study its properties further. A pH survey using 0.1 M glycine solution showed that the efficiency of coupling is highest above pH 9 (Table 2). Even at pH 8.5, where glycine is 95% *N*-protonated, the coupling still proceeds in good yield. Separate experiments established that even under the alkaline condi-



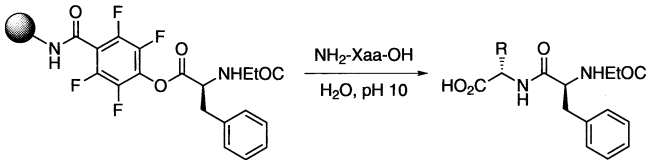
Scheme 2. Peptide coupling using active esters.

Table 1. Formation of dipeptides in water using of active esters derived from resins **1**–**3**[a]

Resin	EtOC-Phe-Gly (**10**)	EtOC-Phe (**11**)
1	30%	41%
2	51%	14%
3	63%	3%

[a] All couplings were carried out with 0.1 M glycine at pH 10 in water at 21°C for 16 h. Yields are relative to the loading of the alcohol as determined above.

Table 2. Peptide coupling with active esters of tetrafluorophenol resin **3** as a function of pH[a]



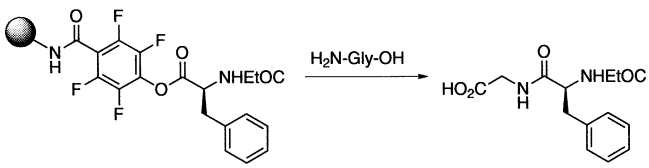
Entry	[Glycine]	pH	EtOC-Phe-Gly (**10**)	EtOC-Phe (**11**)
1	0.1	8.5	48%	8%
2	0.1	9.0	52%	4%
3	0.1	9.5	61%	4%
4	0.1	10.0	63%	3%

[a] Entries 1 and 2 were carried out using 0.05 M bicine as buffer. For entries 3 and 4, the excess of glycine is sufficient to act as buffer. All reactions were carried out at 0°C for 16 h.

tions, less than 2% racemization of the active ester was observed. When the concentration of glycine was varied, it was observed that the coupling occurred at reasonable rates even with concentrations as low as 0.01 M (Table 3).

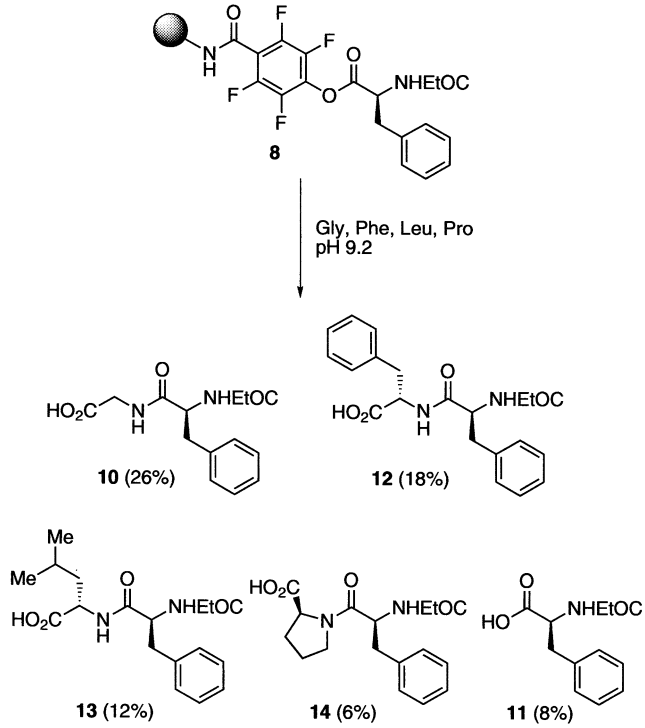
Our intention is to apply this method to the synthesis of dynamic dipeptide libraries.[16] The dynamic library will be constructed from a forward acylation reaction using the active esters developed here and a reverse hydrolysis reaction using a protease. To this end, we examined the synthesis of a simple mixture of dipeptides using a series of amino acids (Scheme 3). The activated ester described above was treated with an equimolar mixture of glycine, phenylalanine, leucine and proline in aqueous solution. All four dipeptides were formed cleanly, and the combined yield of all coupling products was 62%, which is consistent with the single amino acid coupling reactions. With the exception of glycine, the ratio of coupling products is roughly proportional to the pK_a's of the individual amino acids. The higher reactivity of glycine relative to its pK_a is presumably the result of reduced steric hindrance in the coupling step.

Table 3. Peptide coupling with active esters of tetrafluorophenol resin **3** as a function of amine concentration[a]



Entry	[Glycine]	pH	EtOC-Phe-Gly (**10**)	EtOC-Phe (**11**)
1	1.0	10.0	69%	2%
2	0.1	10.0	62%	3%
3	0.05	10.0	55%	4%
4	0.01	10.0	50%	10%

[a] All couplings were carried out in 0.05 M sodium carbonate buffer at 21°C for 16 h.



Scheme 3. Synthesis of mixtures of dipeptides using tetrafluorophenyl esters.

In conclusion, we have found that TentaGel supported tetrafluorophenol active esters are effective and reusable reagents for dipeptide couplings in aqueous solution.[17] The reagent affords a minimum of active ester hydrolysis and can be used even with low concentrations of the amine coupling partner. The reagent can be used for synthesis of libraries of molecules and will be applied to dynamic library generation.

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11. NovaSynTG was used for this study. The resin is a low-crosslinked polystyrene grafted with 3000–4000 MW PEG. The resins used in this work had functional group loadings of 0.25–0.5 mmol/g.
12. The synthesis of **7** was a modification of known procedures. See (a) Ref. 6a; (b) Munson, J. W.; Hodgkind, T. G. *J. Heterocyclic. Chem.* **1978**, *15*, 545. For a related procedure, see: (c) Pop, E. I.; Deprez, B. P.; Tartar, A. L. *J. Org. Chem.* **1997**, *62*, 2594.
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14. No attempt was made to optimize the yield of resin **2**, as the material obtained was sufficient for the analysis of its reactivity in water.
15. The pH of the reaction was chosen for its proximity to the pK_a of glycine. Glycine acts as a buffer as well as reagent for this reaction. The pH of the medium does not change over the course of the reaction.
16. For an introductory review on dynamic libraries, see: Huc, I.; Nguyen, R. *Comb. Chem. High. Throughput Screen.* **2001**, *4*, 53.
17. We have recycled and reused this resin a minimum of 50 times with no significant loss of activity.