

Enzyme-cleavable linkers for peptide and glycopeptide synthesis

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Hydroxymethylphenoxy linkers that are commonly used in solid phase peptide synthesis are surprisingly susceptible to efficient cleavage by the protease chymotrypsin with a broad range of amino acid residues being tolerated at the scissile bond; this enzyme-cleavable linker system has been applied to peptide and glycopeptide synthesis.

The current trend for miniaturisation and automation of synthesis and analysis at the interface of chemistry and biology has resulted in an increasing demand for synthetic tools that can be used on solid supports, and which are also biocompatible. Biocatalysts are particularly attractive tools because they can operate under mild, physiological aqueous conditions with very high chemo-, regio- and stereo-selectivity. Thus, several groups have described enzyme-cleavable linkers which allow for the release of a variety of different compounds from solid supports through enzyme-mediated hydrolysis.^{1–3} In general, such linkers have been specifically designed and synthesized to incorporate enzyme recognition motifs, some of which remain as tags in the compound library. The need for such motifs has limited the application of enzyme-cleavable linkers. In this paper, we describe the unexpected discovery that the protease chymotrypsin can cleave ester linkers that are commonly employed in solid phase peptide synthesis, such as the Wang linker (hydroxymethylphenoxyacetic acid, HMPA). Cleavage is not limited to peptides containing C-terminal aromatic amino acids normally associated with chymotrypsin recognition motifs but can be used for a wide range of peptide sequences. The application of such enzyme-cleavable linkers to solid phase peptide and glycopeptide synthesis is also described.

In the present studies, the chymotrypsin-catalyzed cleavage of peptides such as **1** (Scheme 1), which were linked through a standard Wang linker to PEGA₁₉₀₀ [a copolymer of poly(ethylene glycol) and polyacrylamide], was investigated. PEGA was chosen as the support because it has been shown to provide fully accessible functionalized sites for enzyme catalysis.⁴ Rather unexpectedly, the predominant hydrolysis product of **1** was the dipeptide FmocPheAsp **2** as opposed to the expected FmocPhe **3**, with **2** and **3** formed in a ratio of 2 : 1 respectively (Scheme 1).

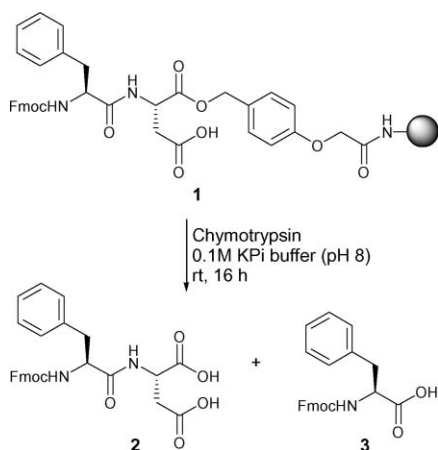
Chymotrypsin is well known to be specific for phenylalanine and tyrosine residues in the P1 site⁵ and thus the PheAsp bond should present an excellent substrate site for this protease. The ester bond of the Wang linker, on the other hand, was flanked by a negatively charged amino acid (Asp) in the pseudo P1 position (*i.e.* the position that would be occupied by the P1 residue in the corresponding amide) and such a substrate would not be expected to be cleaved by chymotrypsin. Also surprising was the stability of dipeptide **2** as the major product in the reaction mixture: even if **2** was initially formed by ester hydrolysis, one would expect it to be hydrolysed rapidly in solution by chymotrypsin. This was further investigated by incubation of a pure sample of **2** with chymotrypsin in solution; however, no hydrolysis product **3** was observed, possibly due to the insolubility of **2** in aqueous buffer.

The interesting lability of the commonly used Wang linker to chymotrypsin-catalyzed hydrolysis had not been reported before and was further investigated. First of all, the C-terminal amino acid residue attached to the Wang linker was replaced by amino acids with diverse functionalities to investigate the tolerance of chymotrypsin at the 'pseudo P1' site of the ester. A series of resin-bound Fmoc-L-amino acids incorporating the acid-labile Wang linker was synthesized and subjected to treatment with chymotrypsin (2 mg ml⁻¹ in 0.1 M potassium phosphate buffer pH 8) for 16 h (Table 1). Release of the Fmoc-protected amino acid was monitored by high performance liquid chromatography after the specified reaction time. The results show that complete hydrolysis was achieved for Fmoc-L-amino acids containing hydrophobic (entries 1, 2 and 3), polar (entry 4) and charged (entries 5 and 6) side-chains. FmocGly **5g** appeared to be the only substrate obtained in poor conversion (entry 7).⁶ Hydrolysis was also possible, but much more sluggish, with substrates containing larger hydrophobic protecting groups (*e.g.* entry 8).

The Wang linker is popular in Fmoc peptide synthesis because of its acid lability. However, acid lability did not seem to be important for chymotrypsin hydrolysis, since the base-labile HMBA (hydroxymethylbenzoic acid) linker was also readily cleaved by the enzyme (Table 1, entries 9–11). Thus, chymotrypsin can be employed to cleave both acid- and base-labile ester linkers and might be useful for orthogonal linker strategies.

Work reported by Bordusa and colleagues⁷ has shown that chymotrypsin can accept activated esters of aromatic alcohols, in which the leaving group (which would be the Wang linker in structure **4**) binds to the aromatic pocket (S1 site). To investigate if the present linker presents such a substrate mimetic, the non-aromatic hydroxyoctanoic acid (HOA) was investigated as a third ester linker system. Again, enzymatic hydrolysis appeared to be unaffected by the change in linker structure (entries 12–14), and suggests that the selective ester cleavage by chymotrypsin in **4** might be a result of the much more labile ester bond rather than due to any additional recognition of the aromatic group of the HMPA or HMBA linker.⁸

A great advantage of biocatalysis over chemical synthesis is that reactions can often be highly stereoselective. Thus, chymotrypsin is known to be highly specific for L-amino acids. Such stereospecificity was also observed for the present linker cleavage: whereas the L-amino acid in **4a** was efficiently cleaved



Scheme 1 Enzymatic hydrolysis of solid-supported FmocPheAsp.

Table 1 Enzymatic cleavage of PEGA-supported Fmoc-amino acids

Entry	Substrate	R	Linker ^a	Product	Conversion (%) ^b
1	4a	CH ₂ Ph	HMPA	5a	100
2	4b	CH ₃	HMPA	5b	100
3	4c	CH ₂ CH(CH ₃) ₂	HMPA	5c	100
4	4d	CH ₂ OH	HMPA	5d	60
5	4e	CH ₂ CO ₂ H	HMPA	5e	100
6	4f	(CH ₂) ₄ NH ₂	HMPA	5f	100
7	4g	H	HMPA	5g	24
8	4h	CH ₂ CO ₂ (2- ⁱ Pr-Ph)	HMPA	5h	25
9	4i	CH ₂ Ph	HMBA	5a	100
10	4j	CH ₃	HMBA	5b	100
11	4k	CH ₂ CO ₂ H	HMBA	5e	100
12	4l	CH ₂ CO ₂ H	HOA	5e	100
13	4m	H	HOA	5g	35
14	4n	CH ₂ Ph	HOA	5a	100
15	4o	D-CH ₂ Ph	HMPA	5o	0

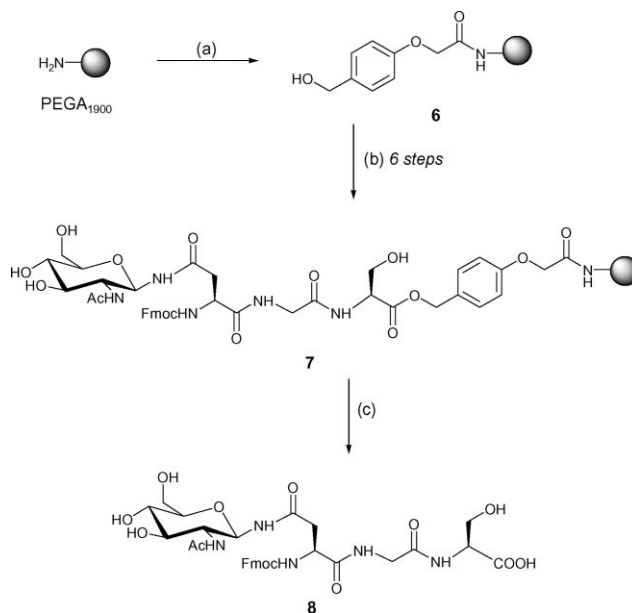
^a HMPA (hydroxymethylphenoxyacetic acid), HMBA (hydroxymethylbenzoic acid), HOA (hydroxyoctanoic acid). ^b Conversion rates are calculated using HPLC analysis after a 16 h reaction time and compared to the standard TFA cleavage for HMPA linkers.

from the resin, its D-enantiomer **4o** did not yield any **5o** under the same conditions. This result confirms that the observed cleavage of the linker is indeed catalyzed by the enzyme. Given that the C-terminal amino acid is often prone to racemisation during peptide synthesis or cleavage, chymotrypsin-catalyzed cleavage is an attractive option for the simultaneous cleavage and kinetic resolution/purification of C-terminal epimers.

An interesting application for enzyme-cleavable linkers is in glycopeptide synthesis because of the additional complexity that the carbohydrate side-chains bring to solid phase peptide synthesis, in particular problems of acid lability. Here, a protocol using enzymes as highly selective and orthogonal catalysts under physiological conditions is very attractive. To test out the applicability of the enzyme-labile linker we chose as a target the highly conserved *N*-glycan motif (Asn-X-Ser/Thr tripeptide codon where X = any amino acid except Pro)⁹ in particular, the tripeptide Asn-Gly-Ser, in which the Asn side-chain was *N*-glycosylated.

In the first step the HMPA linker was attached to PEGA₁₉₀₀ [0.15 mmol g⁻¹ (dry), 10%wt in MeOH] to yield **6**. The first amino acid, FmocSer(Trt)-OH was coupled to **6** via the symmetrical anhydride approach and the trityl side-chain protecting group was selected in order to enable its deprotection in the presence of the TFA-labile HMPA linker. The glycopeptide **7** was then assembled using standard Fmoc solid phase glycopeptide synthesis using the glycoamino acid, FmocAsn(GlcNAc)-OH, prepared by a newly improved method in our laboratory.¹⁰ The resin-bound glycopeptide **7** was then subjected to the enzymatic cleavage conditions resulting in quantitative hydrolysis to give the desired glycopeptide **8** in 100% conversion (Scheme 2). Thus, enzyme cleavage is as efficient as TFA cleavage and is compatible with glycopeptide functionalities.

In summary, we have shown that the commonly used HMPA and HMBA ester linkers, as well as alkyl ester linkers can be cleaved quantitatively in PEGA₁₉₀₀ by chymotrypsin-catalyzed hydrolysis under neutral conditions. Enzyme catalysis is therefore a useful complementary method to the cleavage of such esters under acidic or basic conditions. The linkers are commercially available and can be incorporated using well established solid phase peptide synthesis methodologies.



Scheme 2 Chemo-enzymatic synthesis of glycopeptide **8**. *Reagents and conditions:* (a) HMPA, HOBt, DIC, DMF; (b) (1) FmocSer(Trt)-OH, DIC, DMAP (cat.), DMF, (2) 20% piperidine/DMF, (3) FmocGly-OH, TBTU, HOBt, DIPEA, DMF, (4) 20% piperidine/DMF, (5) FmocAsn(GlcNAc)-OH, TBTU, HOBt, DIPEA, DMF, (6) TFA-TIS-CH₂Cl₂ (1 : 5 : 94); (c) α-chymotrypsin, potassium phosphate buffer (0.1 M, pH 8, 2 mg ml⁻¹) or TFA-H₂O (95 : 5). HOBt = 1-hydroxybenzotriazole; DIC = 1,3-diisopropylcarbodiimide; TBTU = *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium tetrafluoroborate; DIPEA = *N,N'*-diisopropylethylamine; TIS = triisopropylsilane.

Furthermore, in combination with PEGA₁₉₀₀ they do not require any additional spacers to ensure sufficient accessibility of enzyme. The application to longer peptides has recently been demonstrated.¹¹ The only limitation to this methodology is that it may not be suitable for the hydrolysis of peptides containing aromatic amino acids anywhere in the sequence other than the

ester terminus, due to the competing hydrolysis at the specified amide bond.

Despite its known preference for aromatic/hydrophobic residues at the P1 site of amides, chymotrypsin appears to cleave esters of a range of hydrophilic and charged amino acids efficiently in high yields. Thus, the present enzyme-labile ester linkers can be used for the synthesis of a much broader range of peptide sequences than previously suggested,² and is not restricted to those with C-terminal aromatic amino acids.

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