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PAPER

Development of Oxetane Modified Building Blocks for Peptide Synthesis

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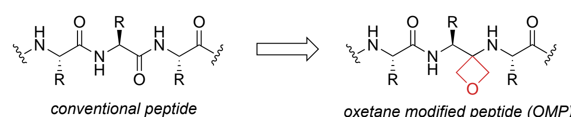
The synthesis and use of oxetane modified dipeptide building blocks in solution and solid-phase peptide synthesis (SPPS) is reported. The preparation of building blocks containing non-glycine residues at the N-terminus in a stereochemically controlled manner is challenging. Here, a practical 4-step route to such building blocks is demonstrated, through the synthesis of dipeptides containing contiguous alanine residues. The incorporation of these new derivatives at specific sites along the backbone of an alanine-rich peptide sequence containing eighteen amino acids is demonstrated *via* solid-phase peptide synthesis. Additionally, new methods to enable the incorporation of all 20 of the proteinogenic amino acids into such dipeptide building blocks are reported through modifications of the synthetic route (for Cys and Met) and by changes to the protecting group strategy (for His, Ser and Thr).

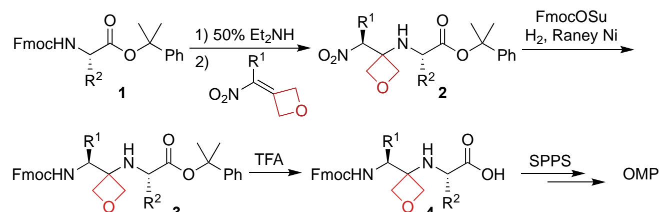
Introduction

Peptides and peptidomimetics attract considerable attention as therapeutic agents due to their synthetic accessibility, high degree of specific binding, and their ability to target protein surfaces, one of the most challenging biological targets.[1,2] Much of this work has focused on the development of peptidomimetics, to overcome issues with proteolytic stability and pharmacokinetic properties of conventional peptides.[3] An increasing number of approved therapeutics and clinical candidates are based on peptidomimetics, and this area continues to offer enormous potential for drug development.[4] Recently, the four-membered oxetane ring has found application in peptide science,[5,6,7] and more generally in medicinal chemistry,[8] as a bioisosteric replacement for the carbonyl group. This work has led to the development of a new type of peptidomimetic, in which one or more of the backbone amide C=O bonds is substituted with an oxetane ring (Figure 1a).[6,7] As proteolysis revolves around peptide bond cleavage, replacing an amide bond with a non-cleavable oxetane residue should increase the metabolic stability of peptidomimetics, while minimally disturbing the overall structure. Indeed, the increased proteolytic stability of an oxetane modified dipeptide able to form hydrogels has recently been demonstrated.[9] Additionally, Carreira has shown that an oxetane modified Leu-enkephalin analogue is less vulnerable towards proteolytic degradation increasing its serum half-life while retaining *in vivo* analgesic properties.[10]

(a) Generalised strategy for replacement of backbone C=O with oxetane ring:



(b) Established synthetic route to oxetane modified building blocks (R^1 = H only):



(c) Examples of natural product OMP analogues made:

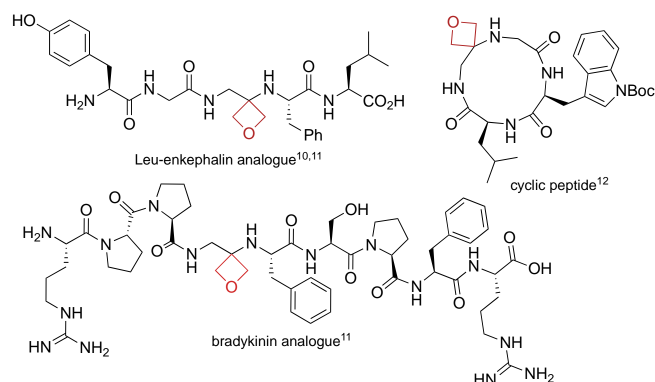


Figure 1. Oxetane Modified Peptidomimetics.

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In order to study the impact of oxetane modification on the structure and properties of a range of biologically important peptides, we have previously developed a solid-phase peptide synthesis (SPPS) strategy to oxetane modified peptides (OMPs) using orthogonally protected dipeptide building blocks **3** (Figure 1b).[11] These compounds are readily accessible from C-terminal protected amino acid and nitroalkenes *via* conjugate addition followed by reduction of the nitro group and *in situ* Fmoc protection. After hydrolysis of the C-terminus cumyl ester, the utility of these building blocks was demonstrated through the preparation of OMP analogues of several naturally occurring linear and cyclic peptides *via* SPPS (Figure 1c).[11,12]
Clearly, there is considerable scope to use this chemistry to make much larger and more complex OMP libraries for medicinal chemistry.[13] However, the feasibility of this is hampered by the rather incomplete coverage of R^1 and R^2 within the dipeptide building blocks. Within **3**, R^1 has been essentially restricted to hydrogen,[14] allowing access to oxetane modified glycine residues only (Figure 1b). Furthermore, with respect to R^2, only nine of the twenty proteinogenic amino acids (A, D, F, G, K, P, R, S, and V) have been incorporated.[11,12] Of the remaining R^2 side chains, incompatibility with one or more steps in the synthesis were foreseen (Figure 1b).[11] Here, we report improved synthetic routes to the building blocks that allow all twenty proteinogenic amino acids to be installed at R^2. Additionally, we extend the method such that it offers a strategy to building blocks where $R^1 \neq H$. This is illustrated by the synthesis of Fmoc-AOx-Ala-OCumyl[15] and its use in the site-specific replacement of various C=O bonds along an eighteen-residue helical peptide by SPPS.

Results and Discussion

Preparation of alanine-derived oxetane modified building blocks

We began by exploring the synthesis of protected building block **3** in which $R^1 \neq H$ (Figure 1b). Specifically, we chose to focus on the synthesis of alanine based system, Fmoc-AOx-Ala-OCumyl where R^1, R^2 = Me. Previous work suggested that conjugate addition of a chiral amine to nitroalkene **5** would proceed without stereocontrol leading to a mixture of diastereoisomers.[6] Indeed, when H-Ala-OCumyl (**6**) was added to nitroalkene **5**, a 50:50 mixture of diastereomers **7** was formed (Scheme 1a). Unfortunately, it was neither possible to separate these diastereomers after conjugate addition nor after reduction of the nitro group and Fmoc protection. As an alternative, we investigated the addition of α-methylbenzylamine, (*R*)-**8** to nitroalkene **5** to generate a 60:40 mixture of diastereoisomers (Scheme 1b). In this case, the isolation of the major diastereomer could be readily achieved by column chromatography to provide (*S*,*R*)-**9** with >95:5 dr in good yield. Reduction of the nitro group and cleavage of α-methylbenzylamine using catalytic hydrogenation gave the corresponding 1,2-diamine. Finally, the sterically less hindered amine was selectively protected with FmocOSu to provide (*S*)-**10**. Correspondingly (*R*)-**10** was synthesised starting from nitroalkene **5** and (*S*)-**8**.[16] The absolute configuration of compound (*R*)-**10** was unambiguously confirmed by X-ray crystal structure analysis after acylation with 4-bromobenzoyl chloride (Scheme 1c).[16]

Scheme 1. (a) Addition of H-Ala-OCumyl (**6**) to nitroalkene **5**; (b) synthesis of enantiopure amino oxetane (*S*)-**10** *via* conjugate addition of α-methylbenzamine, (*R*)-**8** to **5**; (c) proof of absolute stereoconfiguration *via* XRD. [a] Determined by ^{1}H NMR analysis of the crude reaction mixture. [b] Major diastereoisomer isolated in >95:5 dr as determined by ^{1}H NMR.

Applying a strategy previously developed by Carreira,[10] triflate **13** was prepared *in situ* from hydroxyester (*R*)-**12**, derived from L-(+)-lactic acid after cumyl ester formation (2-phenyl-2-methylethyl ester), Mitsunobu inversion and hydrolysis.[16] The cumyl ester was chosen as C-terminal protecting group as it can be quantitatively hydrolysed under weakly acidic conditions with only 2% TFA leaving other acid sensitive amino acid side chain protecting groups untouched.[17] Nucleophilic substitution of triflate **13** with (*S*)-**10** provided the desired oxetane modified dipeptide building block Fmoc-AOx-Ala-OCumyl, (*S*,*S*)-**14** (Scheme 2). Correspondingly oxetane modified (*R*,*S*)-**14** was prepared from triflate **13** and stereoisomer (*R*)-**10**. Chiral HPLC analysis confirmed that both building blocks were single diastereomers, indicating that the substitution proceeds exclusively in an S_N2 fashion without epimerisation.[18] Using the same reaction sequence outlined in Scheme 2 starting from (*S*)-**12**, the cumyl ester of L-(+)-lactic acid, the preparation of stereoisomeric building blocks (*S*,*R*)-**14** and (*R*,*R*)-**14** would be possible in an analogous manner.
Next, we sought to confirm that these new alanine based building blocks can be integrated into conventional SPPS. Alanine-rich peptide **18** forms a stable and well-characterised α-helix that has previously been used to probe the impact of site-specific structural changes on helix stability and secondary structure.[19] Hence, the synthesis of derivatives of **18** in which specific C=O amide bonds are replaced by oxetane rings would allow an exploration of the impact of this carbonyl analogue on

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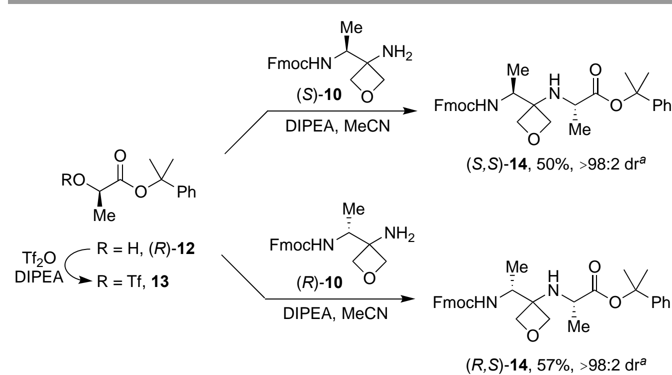
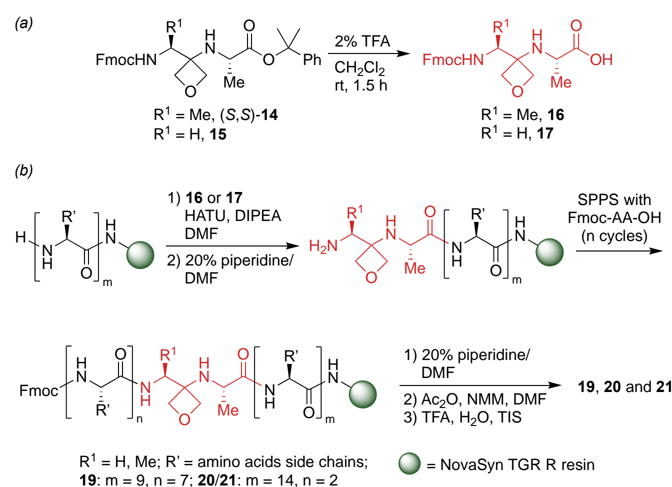
helix stability.[20] Using Fmoc-AOx-Ala-OCumyl, (*S*,*S*)-**14**, or previously reported Fmoc-GOx-Ala-OCumyl (**15**),[12,15] we have successfully synthesised three such derivatives **19-21** using SPPS (Table 1). After initial acid catalysed deprotection of the C-terminal cumyl ester to give **16** and **17** (Scheme 3a),[17] the building blocks were successfully incorporated into the growing peptide chain by double, manual couplings. The final peptides **19-21** were isolated in good purity after preparative, reverse-phase HPLC (Scheme 3b).[16]



Scheme 2. Preparation of oxetane modified dipeptide building blocks (*S*,*S*)-**14** and (*R*,*S*)-**14** Fmoc-AOx-Ala-OCumyl. [a] Determined by chiral HPLC.



Scheme 3. (a) C-terminal deprotection of dipeptide building blocks; (b) solid-phase synthesis of OMPs with eighteen amino acid residues. Key: *N*-methylmorpholine (NMM), triisopropylsilane (TIS).

Table 1. Synthesis of alanine-rich OMPs by SPPS.

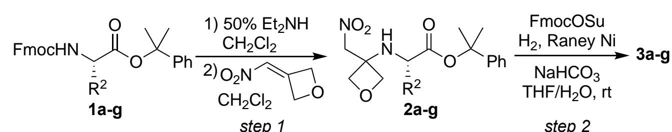
Entry	Peptide Sequence	HRMS Calculated	HRMS Observed	Purity (%)[a]
1	Ac-KAAAA-KAAAA-KAAAA-KGY-NH_2, **18**	822.9808 $[M+2H]^{2+}$	822.9808 $[M+2H]^{2+}$	92
2	Ac-KAAAA-KA**AOx**AA-KAAAA-KGY-NH_2, **19**	858.9784 $[M+2Na]^{2+}$	858.9766 $[M+2Na]^{2+}$	90
3	Ac-KA**AOx**AA-KAAAA-KAAAA-KGY-NH_2, **20**	558.3334 $[M+3H]^{3+}$	558.3331 $[M+3H]^{3+}$	76
4	Ac-KA**GOx**AA-KAAAA-KAAAA-KGY-NH_2, **21**	851.9706 $[M+2Na]^{2+}$	851.9701 $[M+2Na]^{2+}$	90

[a] Measured at 212 nm, lowest purity of two gradient runs.

Preparation of glycine-derived oxetane modified building blocks

We sought to expand the synthesis of oxetane modified dipeptide building blocks of the general type Fmoc-GOx-AA-OCumyl to include all twenty of the proteinogenic amino acids. First, following our previously reported synthetic strategy, new cumyl esters **1a-g** were prepared.[11] After Fmoc deprotection, conjugate addition to 3-(nitromethylene)oxetane afforded nitroalkenes **2a-g** in moderate to good yield (Table 2, step 1). Then, reduction of the nitro group using hydrogen and Raney nickel in the presence of FmocOSu gave the required oxetane modified dipeptide building blocks Fmoc-GOx-AA-OCumyl **3a-g** for a range of amino acids (Table 2, step 2). While some of the transformations proceeded in modest yields, the strength of this methodology lies in the structural variety and scalability of the procedure providing enatiomerically pure and bench-stable derivatives **3a-g**.

Table 2. Synthesis of oxetane containing cumyl ester dipeptide building blocks.



Entry	Amino acid	Yield (%) Step 1	Structure of **3**	Yield (%) Step 2
1	E, Glu(*t*Bu)	49%	**3a**	43%
2	I, Ile	73%	**3b**	31%
3	L, Leu	64%	**3c**	51%
4	N, Asn(Trt)	51%	**3d**	21%
5	Q, Gln(Trt)	49%	**3e**	62%
6	W, Trp(Boc)	50%	**3f**	44%
7	Y, Tyr(*t*Bu)	62%	**3g**	58%

Unfortunately, for amino acids containing sulfur, Cys and Met, Raney nickel reduction of nitro alkenes **2h** and **2i** led to partial desulfurisation[16] and an alternative procedure for the

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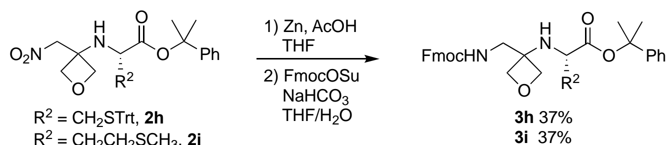
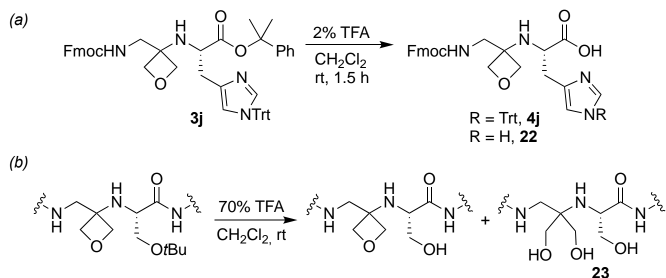
reduction of the nitro group was required. This problem was solved by using Zn dust and acetic acid for the reduction step providing access to Cys- and Met-containing dipeptide building blocks **3h** and **3i** (Scheme 4).[21] Notably, the cumyl ester was not hydrolysed under the acidic reductive conditions. We note however that these conditions are generally less efficient than the Raney Ni reduction, and so are recommended only for the synthesis of sulfur containing building blocks.



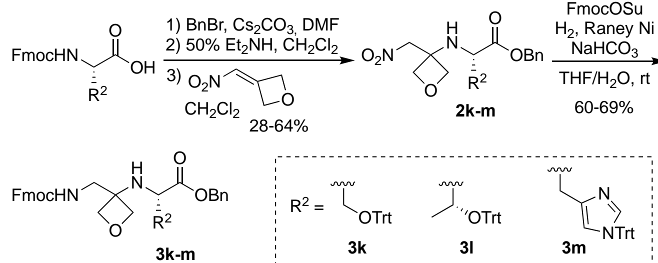
Scheme 4. Synthesis of Cys- and Met-containing dipeptide building blocks **3h** and **3i**.

Unfortunately, the C-terminal cumyl ester protecting group proved unsuitable for three amino acids. When oxetane modified dipeptide building block Fmoc-GOx-His(Trt)-OCumyl (**3j**) was treated with 2% TFA in dichloromethane, concomitant deprotection of the acid labile trityl group was observed giving a mixture of dipeptide building block **4j** and Trt-deprotected **22** (Scheme 5a). An additional problem arose during the deprotection of *tert*-butyl protected aliphatic alcohols after incorporation into peptide sequences. Removal of the *tert*-butyl groups of either Ser(*t*Bu) or Thr(*t*Bu) required high concentrations of TFA leading partially to diol **23** caused by hydrolysis of the four-membered oxetane ring upon extended acid treatment (Scheme 5b). Alternatively, replacing the *tert*-butyl group on Ser and Thr with a more labile trityl group led to partial deprotection during hydrolysis of the cumyl ester as previously observed for **3j**.



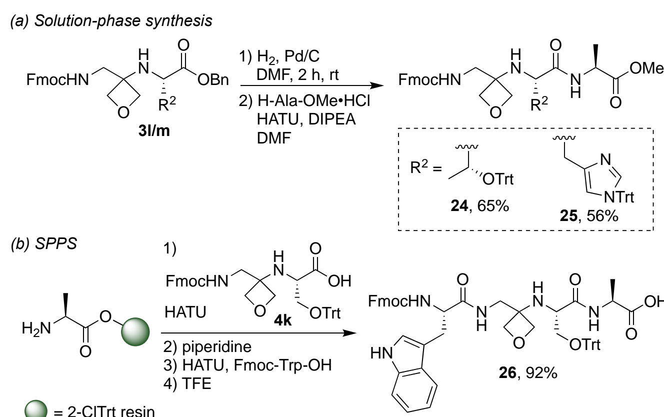
Scheme 5. (a) Hydrolysis of Fmoc-GOx-His(Trt)-OCumyl (**3j**); (b) ring-opening of the oxetane ring with 70% TFA.

On investigation, replacing the C-terminal cumyl group with a simple benzyl ester was the best approach for building blocks containing His, Ser or Thr. Following the same strategy starting from the Fmoc-protected amino acids, C-terminal benzyl protection, Fmoc-deprotection followed by conjugate addition to 3-(nitromethylene)oxetane, and Raney nickel-mediated reduction in the presence of FmocOSu gave oxetane modified dipeptide building blocks **3k-m** (Scheme 6).



Scheme 6. Synthesis of oxetane containing benzyl ester dipeptide building blocks **3k-m**.

These orthogonally protected building blocks enabled peptide coupling after cleavage of the C-terminal benzyl group *via* Pd-catalysed hydrogenolysis. Previously we reported undesired deprotection of the N-terminal Fmoc group during reduction of C-terminal benzyl esters.[11] This side reaction can be largely suppressed by carefully monitoring the reaction progress.[16] The reductions were best carried out in DMF in order to avoid solubility problems of the carboxylic acids, which were used after filtration without further purification. The application of **3k-m** in peptide couplings was demonstrated in solution-phase (Scheme 7a) and in SPPS (Scheme 7b). Importantly, peptides **24**-**26** fully retain their labile trityl protecting groups during these sequences (*cf.* Scheme 5a). Moreover, analysis by ^{1}H NMR confirmed that no detectable epimerisation arose during these couplings. These experiments demonstrate that benzyl protected dipeptide building blocks provide a solution for amino acids that are not compatible with C-terminal cumyl ester protection. Taken together with previous studies,[11,12] the synthesis of glycine-derived oxetane modified building blocks Fmoc-GOx-AA-OR, **3** has now been extended to all twenty proteinogenic amino acids.



Scheme 7. Use of benzyl ester deprotection strategy in preparation of oxetane modified peptides in (a) solution and (b) on solid phase.

Conclusions

We have generalised our strategy for the preparation and use of oxetane containing dipeptide building blocks in solution and solid-phase peptide synthesis. The methodology has been expanded to residues beyond glycine at the N-terminus as exemplified by the synthesis of building blocks containing an

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oxetane modified alanine **14**. Either enantiomer of Fmoc-protected diamine **10** can be made in three simple steps and 22% overall yield. This chemistry reported is much more amenable than earlier work that required twelve steps to provide the corresponding Boc or Cbz-protected variant of this diamine.[10] The approach has potential to be expanded to residues bearing other side chains. Reaction of enantiopure **10** with trifluorosulfonates of hydroxy esters provides oxetane modified dipeptide building block **14**. The strategy allows access to all four stereoisomers of **14** using one unified procedure. The incorporation of these new derivatives at specific sites along the backbone of an alanine-rich peptide sequence containing eighteen amino acids is demonstrated *via* SPPS. At the C-terminus, we have improved the chemistry such that all twenty of the proteinogenic amino acids can be introduced in three simple synthetic steps. Specifically, for sulfur-containing amino acids, the procedure for the nitro reduction had to be adjusted to avoid partial desulfurisation of the side chain. For amino acids containing acid-sensitive trityl-protected side chains, His, Ser and Thr, the C-terminal cumyl ester was replaced by a simple benzyl group without detriment. With this expanded set of building blocks at our disposal, we are now well placed to explore their application in the synthesis of structurally interesting and biologically active peptidomimetics.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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