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SCIENCE DIRECT
Tetrahedron Letters
Tetrahedron Letters 47 (2006) 3009–3012

A novel solid-phase linker strategy for the side-chain anchoring of arginine: an expeditious route to arginine 7-amido-4-methylcoumarins

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Received 31 January 2006; revised 21 February 2006; accepted 2 March 2006
Available online 20 March 2006

Abstract—A novel linker strategy for the efficient side-chain anchoring of arginine is described. The utility of this approach was demonstrated by the facile synthesis of arginine-specific fluorogenic peptide substrates by standard Fmoc solid phase peptide synthesis methods.

In solid-phase peptide synthesis (SPPS), the standard practice of linking the first amino acid residue to the solid support through its α-carboxyl group does not readily facilitate synthesis of C-terminally modified peptides. Strategies have therefore been developed where anchoring is either through the α-amino group[1] or more commonly through a side-chain functionality, thereby leaving the α-carboxyl group available for modification. Most trifunctional amino acids can be simply linked via their side chains to standard synthesis supports, such as Wang[2] or 2-chlorotrityl resin.[3] However, these methods are not satisfactory for arginine since the initial attachment and final cleavage yields are poor and the protection afforded by the resin handle is insufficient to prevent side reactions during SPPS.[3] To overcome these problems, a number of handles based on standard arenesulfonyl arginine-protecting groups have been developed.[4–7] However, none has found general application, as the synthesis of such linkers is either complex, in one case requiring an on-resin chlorosulfonation step,[7] or attachment and detachment of the arginine to the linker is difficult and proceeds in poor yields.

Our strategy aimed to avoid these difficulties by forming the guanidine moiety on the solid phase, employing an approach similar to that described by Martinez et al.[8] Here, an ornithine-derived isothiocyanate was attached to Rink amide resin, and the resulting thiourea converted into a guanidino group by *S*-methylation and ammonolysis immediately prior to cleavage. To avoid the need for post-assembly guanidinylation, we developed a two-step strategy in which the guanidine is prepared maximally protected on the solid-phase prior to commencement of Fmoc SPPS. This approach also eliminates the risk of ornithine formation[9] and premature chain loss from the resin (Fig. 1) which can potentially occur in Martinez's approach where the guanidine is not protected. In the first step, an appropriate Fmoc-protected ornithine bearing the desired C-terminal modification is simply immobilised on a solid support by reductive amination to a backbone-amide-type linker.[1] For our purposes, we selected the 3-formyl-indol-1-ylacetyl linker[10] supported on aminomethyl polystyrene as we believed this would confer the desired acid-lability to the final construct. The second step involved carbodiimide-mediated guanidinylation of the resulting immobilized secondary amine with bis-Boc-thiourea.[11–14] (Treatment of this resin with electrophiles such as isocyanates, thiocyanates, or chloroformates may also be used to generate ureas, thioureas or carbamates, respectively.[15])

To demonstrate the utility of the approach, we used it to prepare a resin for the synthesis of peptides containing C-terminal arginyl-7-amino-4-methylcoumarin (Fig. 2). Such peptides are widely used as fluorogenic peptide substrates for the detection of trypsin-like serine proteases,

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doi:10.1016/j.tetlet.2006.03.019

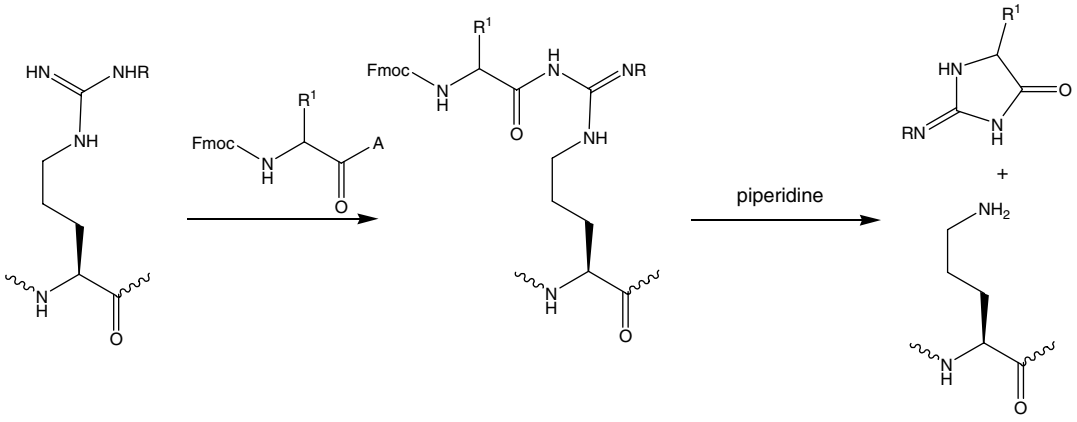

Figure 1. Formation of orthinine residues via deguanidinylation of arginine residues.

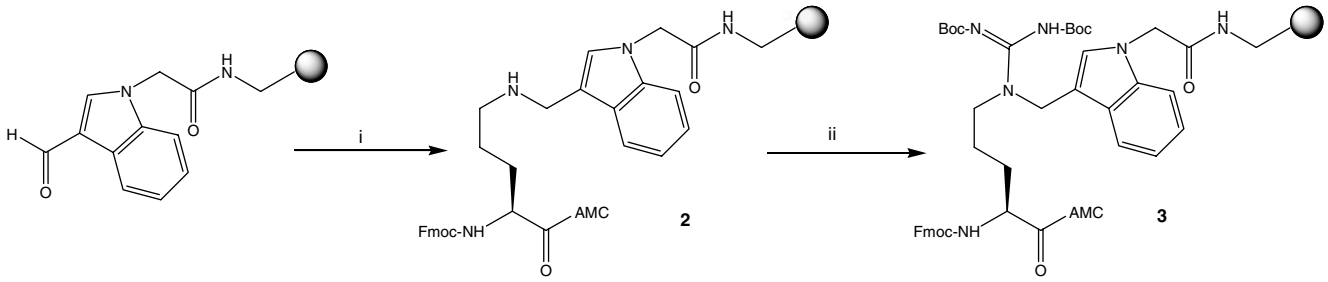

Figure 2. Synthesis of resin **3**. (i) Fmoc-Orn-AMC **1**, TMOF, DCM, DIEA, $NaBH(OAc)_3$; (ii) *N*,*N*′-bis-Boc-thiourea, DIC, DIEA, DCM.

a class of enzymes which includes important therapeutic targets such as thrombin, factor IXa and factor Xa. Cleavage by the enzyme of the amide bond between the arginine and 7-amino-4-methylcoumarin (AMC) releases free fluorophore, which can be detected at 441 nm upon excitation with light at 342 nm. The synthesis of arginine-AMC derivatives is particularly problematic owing to the poor nucleophilicity of AMC and the propensity for arginine to undergo γ-lactam formation. It is for this reason that the synthesis of such compounds is usually carried out in solution. Solid-phase strategies such as those described above employing side-chain anchoring of arginine have also been used; however, these methods are subject to the limitations previously mentioned. Another approach utilizes an AMC derivative (ACC, 7-amino-4-carbamoylmethylcoumarin) which contains a carboxylic acid group to provide the means of attachment to amino-functionalised support.[16] However, the synthesis of ACC is complex and loading of arginine onto the polymer-bound aromatic amino group is extremely difficult (50% after two exposures).[17] Therefore, a simple and practical method for making such peptides would be of enormous value to researchers studying trypsin-like proteases.

Fmoc-Orn-AMC **1** was prepared from Fmoc-Orn(Boc)-OH by published methods.[8] Reductive amination of 3-formylindol-1-ylacetylaminomethyl polystyrene with **1** was effected with sodium triacetoxyborohydride in TMOF/DCM/DIEA.[18] The resin-bound secondary amine **2** was guanidinylated with bis-Boc-thiourea in conjunction with DIC,[12] to afford the supported bis-Boc-protected arginine AMC derivative **3**. (The use of Mukaiyama's reagent[19] instead of DIC gave poorer results.) Treatment of a small sample of **3** with TFA/DCM (1:1) and TLC analysis of the cleaved product indicated complete guanidinylation. The loading of **3** was determined by the quantitative Fmoc test[20] to be 0.26 mmol/g.

Resin **3** was evaluated in the synthesis of peptides **4** and **5** (Fig. 3).[21] In both examples, coupling of the C-terminal residue was performed twice using Fmoc-protected amino acids activated with PyBOP® in the presence of DIEA. This double coupling procedure was used to ensure complete condensation, as it was anticipated that the arginine amino group would be hindered by the two Boc groups protecting the side-chain. Additions of subsequent Fmoc-protected amino acids were carried out using a single coupling with PyBOP®/DIEA. A solution of 3% DBU in DMF was used for Fmoc removal as in our experience its use in the synthesis of fluorescently labelled peptides frequently gives better results than piperidine.

In all cases, cleavage of products from the solid support was achieved by treating the peptidyl resins with TFA/DCM (1:1) for 2 h. Peptides **4** and **5** were obtained in excellent purities (Fig. 4) and in good yields (**4**, 73%; **5**, 62%). Detailed LC-MS of the products indicated that there were no orthinine-containing by-products in the crude peptides.

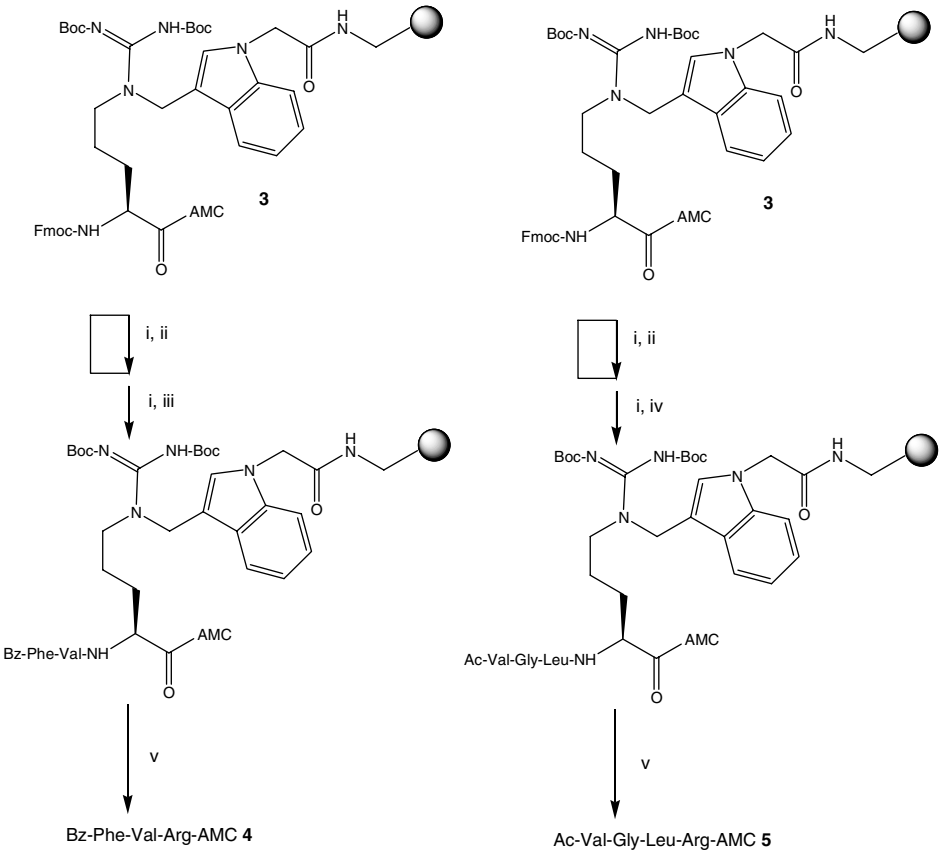

Figure 3. (i) 3% DBU in DMF; (ii) Fmoc-Aaa-OH, PyBOP®, HOBt, DIEA, DMF; (iii) Bz_2O, DIEA, THF; (iv) Ac_2O, DIEA, THF; (v) TFA/DCM.

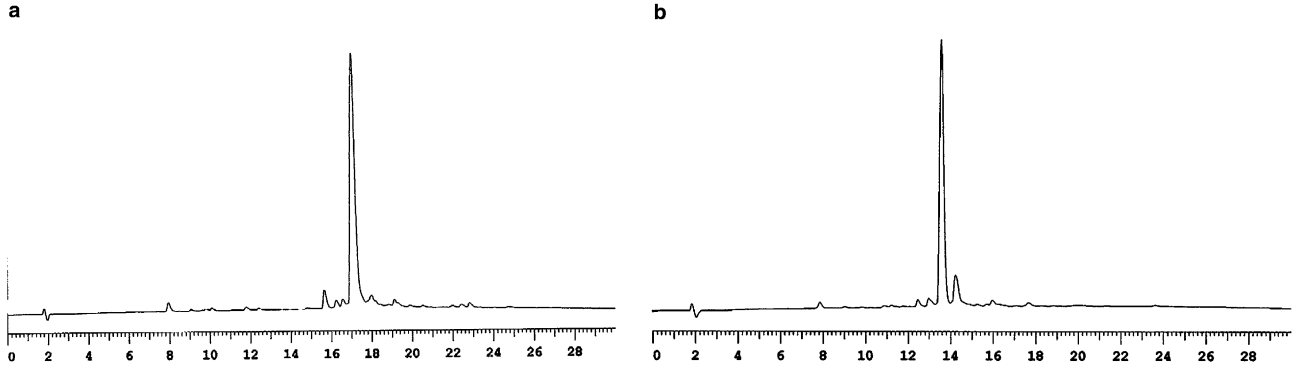

Figure 4. HPLC profile of crude peptide (a) **4** and (b) **5**. (HPLC conditions: Vydac peptide/protein C18 column; gradient: 20–100% B in A over 20 min, 1 mL/min; A: 0.1 TFA aq; B: MeCN/water/TFA (90:10:0.1).

Investigations are currently underway to extend the approach to the synthesis of *p*-nitroanilides and 7-amido-4-trifluoromethylcoumarins.

In summary, our recently developed side-chain anchoring strategy is an efficient method for preparing peptides modified on the C-terminal carboxyl group of arginine.

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21. Resin **3** (0.2 g; loading 0.26 mmol/g) pre-swollen with DMF was treated twice with 3% DBU in DMF (5 min, 15 min). After washing the resin with DMF, it was treated with Fmoc-Val-OH (70 mg, 0.2 mmol), HOBt (10 mg, 0.065 mmol), PyBOP® (100 mg, 0.21 mmol) and DIEA (100 μl, 0.6 mmol) in a minimum volume of DMF. The mixture was left to stand with gentle agitation for 1 h, after which time the resin was washed with DMF and the coupling reaction repeated with fresh reagents for 30 min. The resin was removed by filtration, washed with DMF and then treated twice with 3% DBU in DMF as described above. The resin was washed with DMF, and a mixture of Fmoc-Phe-OH (80 mg, 0.2 mmol), HOBt (10 mg, 0.065 mmol), PyBOP® (100 mg, 0.21 mmol) and DIEA (100 μl, 0.6 mmol) in DMF was added to the resin and it was gently agitated for 30 min, after which time the TNBS test was negative. Following removal of the final Fmoc group with DBU in DMF, the resin was end-capped by treatment with benzoic anhydride (100 mg, 0.44 mmol) and DIEA (100 μl) in THF. The resin is removed by filtration, washed with DMF, isopropanol, H_2O, THF, isopropanol, MeOH, ether, hexane and dried overnight. The peptide was cleaved from the resin by treatment with TFA/DCM (1:1) for 2 h. The resin was removed by filtration and the peptide precipitated from the filtrate by the addition of ether. The precipitate was washed with ether and dried under vacuum to afford the desired peptide **4** (30 mg, 73%); *m/z* (+ES) 682.53 (MH^+), calcd for $C_{37}H_{44}N_7O_6$ 682.33.