Pergamon

Tetrahedron Letters 40 (1999) 809–812

TETRAHEDRON LETTERS

Cyclisation of Histidine Containing Peptides in the Solid-Phase by Anchoring the Imidazole Ring to Trityl Resins

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Received 31 July 1998; accepted 17 November 1998

Abstract: Head-to-tail histidine containing cyclopeptides can be efficiently synthesised by a three-dimensional orthogonal solid-phase strategy (Fmoc/*t*Bu/allyl) *via* anchoring the imidazole ring to trityl-resins. Furthermore, Fmoc-His(Trt-®)-OAl can be a useful starting support for the preparation of diketopiperazine combinatorial libraries.

Keywords: Solid-phase synthesis; Peptides; Cyclisation; Piperazinones.

Cyclisation is a key step in small cyclic peptide synthesis. Only a few examples of cyclisation in the solid-phase of constrained tetrapeptides are described.[1] A major problem is represented by cyclodimerization. The extent of oligomerization is strongly correlated to the choice of the *C*-terminal residue.[2] An efficient approach to the solid-phase preparation of head-to-tail cyclic peptides, which recently found wide application, is based on the principle of anchoring the peptide to the resin through a side chain.[3] The combination of Fmoc/*t*Bu/allyl[4] provides a really flexible three-dimensional orthogonal protection scheme that permits the construction of more complex peptides including peptides which are cyclic, which have branching, which contain post-translational modifications or which are conjugated to sugars and oligonucleotides.[5] An extension of the side-chain anchoring approach to the synthesis of head-to-tail cyclic peptides, based on the exploitation of allyl chemistry, is the possibility of anchoring the imidazole ring of a peptide containing His residues to a trityl-resin (Trt-®),[6] following the methodology we describe in this communication.

Our method, which can be generalized, is based on the anchoring of the imidazole ring of a His residue to the handle of a Trt-®. This approach can be used in batch or in continuous-flow peptide synthesisers depending on the starting resin, following the efficient three-dimensional orthogonal solid-phase Fmoc/*t*Bu/allyl strategy.[7] The anchoring was successful with tritylchloride-resin as well as with NovaSyn tritylalcohol-resin. In order to anchor

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0040-4039/99/$ - see front matter
PII: S0040-4039(98)02459-9

the resin to the imidazole ring, we synthesised the new building block Fmoc-His-OAl **1**.[8] As the Trt group is known to be a protective group for the NH of the imidazole ring of His, we anchored it to the handle of a Trt-®. The tritylchloride-resin **2a** was treated with **1** under mechanical stirring.[9] The quantity of the amino acid was specifically chosen in order to obtain a final level of substitution around 0.4-0.6 mmol/g. This was to take advantage, in the subsequent cyclisation step, of the pseudo-dilution phenomenon which favors intramolecular resin-bound reactions, minimizing inter-chain interactions.

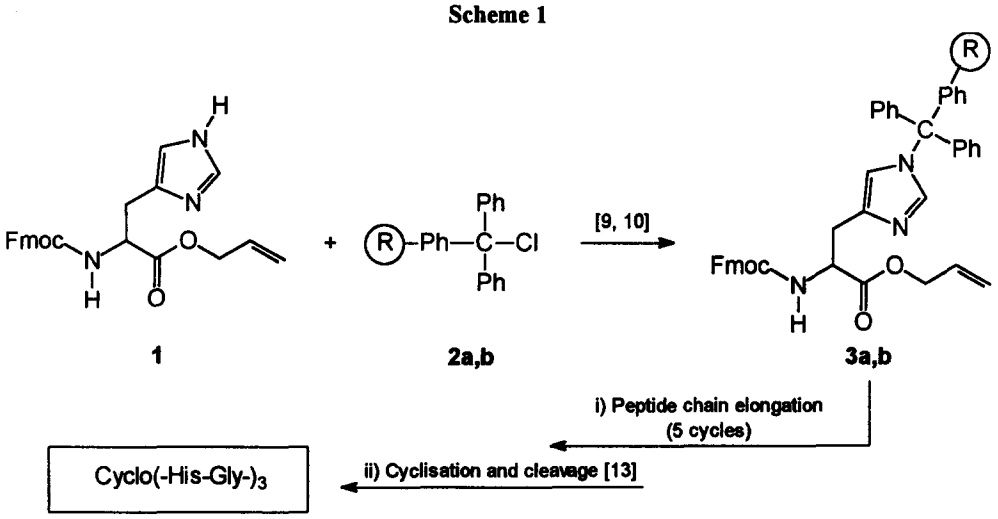
As we wanted to be able to use this amino acid anchored to Trt-® also on a continuous-flow peptide synthesiser, we followed the same procedure with a NovaSyn tritylalcohol-resin. In this case, we had to activate the triphenylmethanol-handle, transforming it into the corresponding chloride **2b** by treatment, in anhydrous toluene at 60 °C, with $SOCl_2$.[10] After obtaining the required level of substitution, we end-capped. In this way, we inactivated the reactive sites which remained after coupling of the first amino acid on the resin.

To verify the validity of the title strategy, Fmoc-His(Trt-®)-OAl **3a** and Fmoc-His(NovaSyn Trt-®)-OAl **3b** (**Scheme 1**) were used to synthesise the peptides anchored to the resins Fmoc-Gly-His(Trt)-Gly-His(Trt-®)-OAl and Fmoc-Gly-His(Trt)-Gly-His(Trt)-Gly-His(Trt-®)-OAl[11] as precursors of the linear tetra- and hexapeptides and of the corresponding cyclopeptides. During the synthesis, after the coupling of every amino acid (HATU-DIPEA activation), we performed the Kaiser ninhydrin test[12] and a spectrophotometric estimate of substitution on samples of resin.

Moreover, in order to test the stability of the linkage of the Trt-® to the imidazole ring, after every coupling of an amino acid, the peptide anchored to the resin was treated with i) $Pd^0(PPh_3)_4$ in $CHCl_3$-AcOH-NMM (37 : 2 : 1) under Ar atmosphere (to remove the allyl group from the *C*-terminal function) and/or ii) with piperidine-DMF (1 : 4), to remove the N^{α}-Fmoc protection. All the solutions we obtained after treatment with i) and/or ii), analyzed by ESI-MS, showed no peaks which could be attributed to the corresponding peptide fragment and the resin substitution was maintained (indicating that the linkage to the resin was stable to standard cycles of the synthesis).

The linear hexapeptide anchored to the resin was firstly deprotected in the *N*-terminal position and suspended in a solution of DIPEA and TBTU in DMF. After cleavage from the resin with TFA-H_2O (95 : 5) and purification by HPLC,[13] we obtained cyclo(-His-Gly-)$_3$ (**Scheme 1**).

Scheme 1



We followed the same procedure for the cyclisation of the tetrapeptide. The crude product was examined by both ESI-MS and FAB-MS which yielded mass spectra containing three signals centered on charge ratios (m/z) of 777, 389 and 195. These signals correspond to the singly protonated molecules of the cyclo(-His-Gly-)$_4$, cyclo(-His-Gly-)$_2$ and diketopiperazine (DKP) formed. It is well known that ionization techniques can generate both singly and doubly protonated species and therefore, we have to assume that the signal at m/z 389 may contain both singly protonated cyclo(-His-Gly-)$_2$ as well as the doubly protonated cyclo(-His-Gly-)$_4$. However, HPLC separation showed only two peaks. The first one (R_t = 3.22) was attributed to the DKP formed during Fmoc removal in piperidine at the dipeptide level, according to that recently reported by Barany *et al.*[14] This process, promoted by the allyl alcohol leaving group, the Gly residue in the second position and the stability of the resulting DKP formed [3-(1*H*-imidazol-4-ylmethyl)-piperazine-2,5-dione, **Fig. 1**], could be totally suppressed by doing a fast Fmoc deprotection cycle at the level of the second coupling reaction. The second peak (R_t = 3.53) appeared to be a mixture of the target cyclo(-His-Gly-)$_2$ and the corresponding cyclodimer. Further work is in hand to find chromatographic conditions for the purification of the cyclotetrapeptide, which was the major product, as shown by FAB-MS spectra.

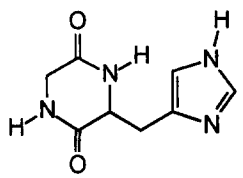


Fig. 1

In conclusion, we have shown that Fmoc-His(Trt-®)-OAl is a useful starting support for the synthesis of His containing head-to-tail cyclopeptides. Moreover, when the reaction conditions are optimized towards DKP formation at the dipeptidyl level, the above described strategy can be used as an alternative efficient way to prepare DKP combinatorial libraries,[15] leading to scaffolds which can be screened in the context of a rational drug design.

Acknowledgments. We thank Dr. Mahmoud Hamdan (GlaxoWellcome Medicines Research Centre, Verona, Italy) and Dr. Antonio Triolo (Menarini Ricerche, Firenze, Italy) for ESI-MS and FAB-MS acquisitions and interpretations. We thank also Prof. Fernando Albericio (Departamento de Química Orgánica, Universidad de Barcelona, Spain) for fruitful discussions.

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4 Abbreviations used: AcOH, acetic acid; Al, allyl; DCC, dicyclohexylcarbodiimide; DCM, dichloromethane; DIPEA, *N,N*-diisopropylethylamine; DMF, *N,N*-dimethylformamide; DVB, divinylbenzene; ESI-MS, electrospray ionization mass spectrometry; FAB-MS, fast atom bombardment mass spectrometry; Fmoc, 9-fluorenylmethoxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo(4,5-*b*)pyridinium 1-hexafluorophosphate 3-oxide; MeOH, methanol; NMM, *N*-methylmorpholine; RP-HPLC, reversed-phase high performance liquid chromatography; r.t., room temperature; TBTU, 1-[bis(dimethylamino)methylene]-1*H*-benzotriazolium 1-tetrafluoroborate 3-oxide; *t*Bu, *tert*-butyl; TFA, trifluoroacetic acid; Trt, trityl; Trt-®, trityl resin. Amino acid symbols denote the L configuration unless indicated otherwise.

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8 The commercially available Fmoc-His(Trt)-OH in DCM (0.08 mmol/mL) was treated at 0 °C with DCC (1.2 eq) in allyl alcohol (9.2 eq) and the reaction mixture left at r.t., under stirring, overnight. After cooling at 0 °C, the dicyclohexylurea formed was filtered off and the excess of DCC used was eliminated by treating the solution with AcOH-MeOH (1 : 10). The product was recrystallised from diethyl ether/hexane. Fmoc-His(Trt)-OAl (74% yield); R_f: 0.9 (DCM-MeOH, 10 : 1). ^{1}H-NMR δ (ppm) $CDCl_3$ (200 MHz): 7.76-7.26 (10 H, m, Fmoc Ar-H, His 2-H and 5-H), 7.26-7.09 (15 H, m, Trt), 6.57 (1 H, d, His NH), 5.81-5.72 (1 H, m, allyl 2-H), 5.27-5.10 (2 H, dd, allyl 3-H_2), 4.68-4.60 (1 H, m, His α-H), 4.53-4.51 (2 H, m, allyl 1-H_2), 4.40-4.19 (3 H, m, Fmoc 9-H and CH_2), 3.11-2.98 (2 H, AB system, His β-H_2).
The same product could be obtained only in a very low yield using allyl bromide. Deprotection of the imidazole ring with TFA-DCM (5 : 8) was complete in 1 h at r.t. After evaporation to dryness, the oil was lyophilized to obtain Fmoc-His-OAl **1** (93% yield, 69% overall yield); R_f: 0.33 (DCM-MeOH, 10 : 1). ^{1}H-NMR (assignments were based on H-H COSY), δ (ppm) $CDCl_3$ (200 MHz): 8.30 (1 H, s, His 2-H), 7.71-7.19 (8 H, m, Fmoc Ar-H), 6.97 (1 H, s, His 5-H), 6.34 (1 H, d, His NH), 5.79 (1 H, m, allyl 2-H), 5.29-5.17 (2 H, m, allyl 3-H), 4.58-4.50 (3 H, m, allyl 1-H_2 and His α-H), 4.26 (2 H, m, Fmoc CH_2), 4.12-4.05 (1 H, m, Fmoc 9-H), 3.49-3.09 (2 H, m, His β-H_2).

9 Fmoc-His(Trt-®)-OAl **3a** was prepared as follows. Trityl chloride resin **2a** from Novabiochem with a polymer matrix copoly(styrene-1% DVB) 200-400 mesh (0.95 mmol/g) was swelled in anhydrous DCM (10 mL/g resin). Then the resin was treated for 1 h with a solution of Fmoc-His-OAl **1** (2 eq) and DIPEA (4 eq) in DCM. It was then washed with DCM-MeOH-DIPEA (17 : 2 : 1), DCM, DMF, DCM, MeOH (2 × 10 mL/g resin each). Final resin substitution, 0.58 mmol/g [spectrophotometric determination of the loading by deprotection with piperidine-DMF (1 : 4) and measuring the absorbance of the adduct dibenzofulvene-piperidine at 301 nm].

10 Fmoc-His(NovaSynTrt-®)-OAl **3b** was prepared as follows. The NovaSyn trityl alcohol resin from Novabiochem (0.19 mmol/g) was converted into the corresponding chloride **2b**. After washing it with DMF (2 × 10 mL/g resin), anhydrous DCM (3 × 10 mL/g resin) and anhydrous toluene (3 × 10 mL/g resin), the resin was maintained under N_2, in anhydrous toluene at 60 °C and treated with $SOCl_2$ (60 eq). After 3 h under stirring, the resin was filtered off and washed with anhydrous toluene and DCM. Then the resin **2b** was treated as above described.[9] Final resin substitution, 0.105 mmol/g.

11 The linear tetra- and hexapeptides were constructed in batch starting from Fmoc-His(Trt-®)-OAl **3a** (0.58 mmol/g) and in a NovaSyn Gem Semiautomatic Peptide Synthesiser starting from Fmoc-His(Trt-®)-OAl **3b** (0.105 mmol/g) using the Fmoc/*t*Bu/allyl protocol (HATU-DIPEA activation, 2.5 : 5). After the last coupling reaction, the allyl protecting group of the *C*-terminus of the peptides anchored to the resin was removed with $Pd^0(PPh_3)_4$ (3 eq) in $CHCl_3$-AcOH-NMM (37 : 2 : 1) under Ar for 3 h at r.t. Linear tetra- and hexapeptides were obtained by washing the resin with DIPEA-DMF (0.5 : 99.5), sodium diethyldithiocarbamate in DMF (0.5 : 99.5), subsequent Fmoc removal (piperidine-DMF, 4 : 1) and cleavage with TFA-H_2O (95 : 5).
H-(His-Gly)$_2$-OH $[M + H]^+$ 407 (FAB-MS). H-(His-Gly)$_3$-OH $[M + H]^+$ 601 (FAB-MS).

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13 After Fmoc removal, cyclisation of the linear peptides anchored to the resin (300 mg) was performed by treatment with DIPEA (4 eq) and TBTU (2 eq) in DMF (3 mL) for 4 h (negative Kaiser test). After washing with DMF and DCM (3 × 3 mL/g resin) the crude cyclopeptides were cleaved in TFA-H_2O (95 : 5). Analytical RP-HPLC was performed on a Vydac column ODS 214TP54 (250 × 4 mm), 1 mL/min, isocratic 0.1% TFA in H_2O. Semi-preparative RP-HPLC was performed on a Vydac column ODS 214TP1010 (250 × 10 mm), 5 mL/min, isocratic 0.1% TFA in H_2O.
Cyclo(-His-Gly-)$_3$ $[M + H]^+$ 583.5 (ESI-MS and FAB-MS).

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