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Head-to-Tail Cyclization and Use of C^{α}–Allyl Ester Protection Improves the Yield of Cyclic Peptides Synthesized by the Oxime Resin Method

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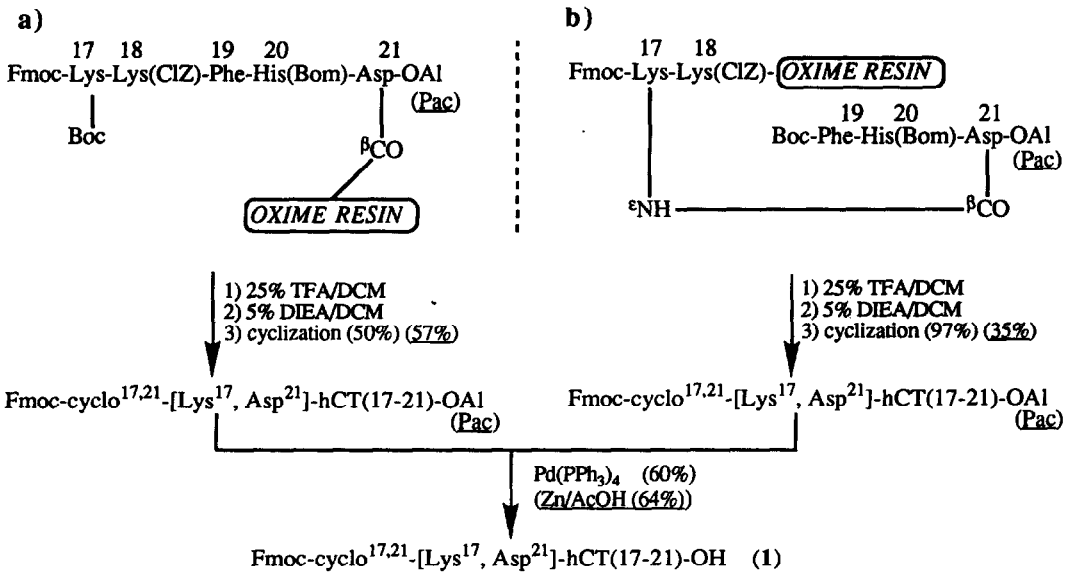
Abstract: Fully protected and C-terminal free cyclic (lactam-bridged) peptides are assembled by the oxime resin method in high yields and purity applying a four dimensional orthogonal (Boc/Bzl/Fmoc/Al) protection scheme and a head-to-tail cyclization strategy.

The introduction of conformational constraints into biologically active peptides *via* cyclization has been shown to provide important information about their functional conformations.[1] Hence, the development of methods for the solid-phase synthesis of cyclic, lactam-bridged peptides has been the target of extensive research in recent years.[2] For the incorporation of lactam bridges between specific residues in a linear peptide, two strategies are commonly used: (1) side chain-to-side chain cyclization on the solid support[3] and (2) separate assembly of the protected side chain-to-side chain bridged peptide, followed by segment condensation on the solid support containing the C-terminal peptide segment and subsequent peptide chain elongation.[4] Kaiser's oxime resin[5] has been successfully used by our group[4,6,7] and others[8,9] for the rapid synthesis of fully protected lactam[4,6,7,9] or Asu[8a] -bridged peptides. To date, our synthetic strategy towards the assembly of a fully protected and C-terminal free X-cyclo[1,5]-(Lys^1,AA^2,AA^3,AA^4,Asp^5)-OH (where X is the N-terminal protecting group) has been based on the following features:[4,10] (i) side-chain attachment of Boc-Asp^5-OPac (or Boc-AA^4-Asp^5-OPac) on the oxime resin; (ii) subsequent peptide chain elongation applying the Boc/Bzl strategy; (iii) use of an amine-protecting group of third orthogonality for the N^{α} or N^{ε} function of Lys^1 (Fmoc/Boc or Boc/Trt); (iv) selective cleavage of the N^{ε} protecting group with concomitant side chain-to-side chain cyclization and release of the fully protected peptide from the polymeric support; (v) selective cleavage of the C-terminal protecting group (phenacyl ester[11]) to provide the C-terminal free, cyclic peptide.

In the course of our studies on lactam-bridged human calcitonin (hCT) analogs, we synthesized N^{α}–Fmoc-cyclo[17,21]-[Lys^{17},Asp^{21}]-hCT(17-21)-OH (**1**) by applying the strategy described above. However, this method gave us low substitution levels and low or irreproducible yields, possibly due to the sequence dependent nature of cyclization reactions[9] and/or a phenacyl ester associated side reaction.[12] Therefore, we have extended our earlier strategy to explore alternative head-to-tail (peptide backbone) cyclizations[2d] and have also replaced the phenacyl ester protection by allyl ester[13] protection.

Peptide **1**, N^{α}–Fmoc-cyclo[1,5]-(Lys-Lys(ClZ)-Phe-His(Bom)-Asp)-OH, was used as a model peptide to investigate the side chain-to-side chain and head-to-tail cyclization strategies and to compare allyl ester protection with phenacyl ester protection. Peptide **2**, N^{α}–Fmoc-cyclo[1,5]-(Lys-Thr(Bzl)-Tyr(BrZ)-Thr(Bzl)-Asp)-OH, (N^{α}–Fmoc-cyclo[10,14]-[Lys^{10},Asp^{14}]-hCT(10-14)-OH) was then assembled using head-to-tail-cyclization and allyl ester protection, since this approach provided a simpler synthetic procedure[14,15] and better yields. For the

side chain-to-side chain cyclization method, the synthesis of peptide **1** (scheme 1a) started with the anchoring of Boc-Asp^{21}-OAl[14] or Boc-Asp^{21}-OPac[15] on the oxime resin (Asp substitution level: 0.45 mmol/g and 0.17 mmol/g[16], respectively), followed by the stepwise assembly of the protected amino acid residues, according to the "oxime resin" synthetic protocol.[16] After N^{ε} deprotection of the N-terminal residue, the N^{ε} to C^{β} cyclization reaction[17] with concomitant release of the cyclic peptide into solution proceeded in dioxane and in the presence of 10 equivalents of AcOH (cyclization-catalyst). By this route, the phenacyl ester of **1** was obtained in 57% yield[18] and good purity after 4h cyclization time,[19] whilst the crude allyl ester of **1** was obtained in 50% yield after 24h cyclization time and was contaminated in a ratio of 3:4 (byproduct:product) by a Phe^{19}-deletion cyclic peptide (as shown by amino acid analysis and FAB-MS).



Scheme 1. Synthesis of the model cyclopentapeptide **1** by (**a**) the side chain-to-side chain cyclization strategy and (**b**) the backbone cyclization strategy with allyl or (underlined parentheses) phenacyl ester protection (yields of crude products are shown in parentheses).

For the head-to-tail cyclization method (scheme 1b) the synthesis of peptide **1** started with the attachment of Boc-Lys^{18}(ClZ)-OH to the oxime resin (Lys substitution level: 0.47 mmol/g for the synthesis with allyl ester protection and 0.36 mmol/g for the one with phenacyl ester protection), followed by Boc-cleavage and coupling with Fmoc-Lys^{17}(Boc)-OH. Then, the side chain-to-side chain lactam bridge was formed after deprotection of the N^{ε} function of Lys^{17} and subsequent coupling with Boc-Asp^{21}-OAl[14] (or Boc-Asp^{21}-OPac)[15]. Attachment of the last two residues and cleavage of the N-terminal Boc-group was followed by the N^{α} to C^{α} cyclization reaction, performed as described above.[17] Crude allyl ester of **1** was obtained after 24h in 97% yield[18a] and high purity (Figure 1a). However, the phenacyl ester of **1** was formed in only 35% yield and was contaminated up to 30% by the deletion byproduct cyclo-Phe-His(Bom) (as shown by amino acid analysis and FD-MS).

Allyl ester cleavage proceeded quantitatively under N_2 using $Pd(PPh_3)_4$ in DMSO/THF/0.5M (or 1M) aqueous HCl in a ratio of 2/2/1 (23 eq. of HCl) with 50 equivalents of N-methylaniline[20] (reaction times: 1/2-2h) and gave, after only the washing steps, precipitation and extensive ether washes of the product, a highly pure cyclopeptide (**1**) in 60% yield. The phenacyl ester was cleaved by Zn/90% AcOH[11b] (3-6h; 64% yield).

The synthesis of **2** started with the attachment of Boc-Tyr(BrZ)[12]-OH on the oxime resin (Tyr substitution level: 0.38 mmol/g) and was continued by stepwise synthesis on oxime resin, the lactam bridge being formed by side chain-to-side chain coupling of Boc-Asp[14]-OAl to the N^{ε}-function of Lys[10]. The cyclization reaction gave the crude allyl ester of **2** in 49% overall yield (24h cyclization time) (Figure 1c) and allyl ester cleavage (as above) provided the C-terminal free, fully protected cyclic pentapeptide in 86% yield and high purity (Figure 1d).

We are currently studying a possible explanation for the low synthesis yields of **1** prepared by either cyclization method in combination with phenacyl ester protection. Intra- and/or interchain nucleophilic attack of the amino-function at the carbonyl of the phenacyl group (formation of a cyclic carbinol-amine derivative with subsequent dehydration to a Schiff base), a side reaction which has already been proposed to cause termination of H-Gly-oxoacyl-resins,[12] is likely to occur under the cyclization conditions applied, resulting in the maintenance of the peptide on the polymeric support. The degree of this side reaction, however, should be dependent on the particular peptide structure and conformation, the substitution level of the resin and the cyclization conditions as well.

In summary, we have shown that head-to-tail cyclization synthetic strategies in combination with C^{α}-allyl ester protection may significantly improve the yields and purity of i,i+4 lactam-bridged peptide assembly on the Kaiser oxime resin.[21,22] The application of this strategy for the synthesis of i,i+7 lactam-bridged peptides is now under investigation.

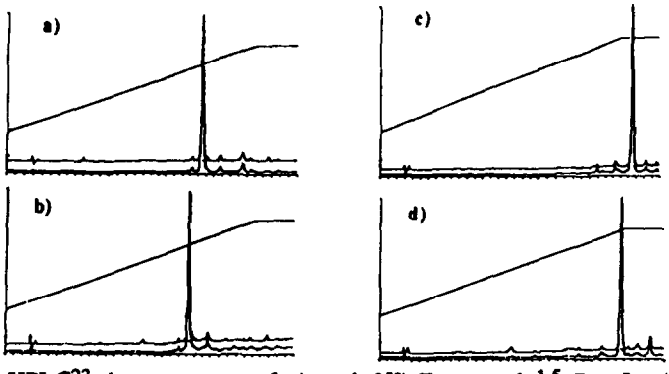


Figure 1. Analytical HPLC[23] chromatograms of: a) crude N^{α}–Fmoc-cyclo[1,5]-(Lys-Lys(ClZ)-Phe-His(Bom)-Asp)-OAl synthesized by the head-to-tail strategy; b) crude N^{α}–Fmoc-cyclo[1,5]-(Lys-Lys(ClZ)-Phe-His(Bom)-Asp)-OH after allyl ester cleavage(**1**); c) crude N^{α}–Fmoc-cyclo[1,5]-(Lys-Thr(Bzl)-Tyr(BrZ)-Thr(Bzl)-Asp)-OAl synthesized by the head-to-tail method and d) crude N^{α}–Fmoc-cyclo[1,5]-(Lys-Thr(Bzl)-Tyr(BrZ)-Thr(Bzl)-Asp)-OH (**2**) after allyl ester cleavage.

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14. Boc-Asp-OAl was synthesized by the following three steps: (a) Boc-Asp(OtBu)-OH was reacted with 3eq. of allyl bromide (1eq. DIEA, in DMF; 18h) to give Boc-Asp(OtBu)-OAl (81%; oil); (b) TFA treatment (2h) of the latter, followed by the replacement of the TFA salt by HCl gave H-Asp-OAl x HCl (100%; oil) and (c) reaction of H-Asp-OAl x HCl with 1.2eq. of $(Boc)_2O$ (DIEA 3eq., in DMF; 2h) which gave Boc-Asp-OAl as an oil (90%; ^{1}H-NMR data were the expected ones; FAB-MS: $[2M + H]^+$ 547.1 (calcd $[2M + H]^+$ 547.2)).
15. Our group used to obtain Boc-Asp-OPac by a two step synthesis starting with commercially available Boc-Asp(OBz)-OH, which after esterification with 2-bromoacetophenone (80%) was catalytically hydrogenated in EA to obtain a mixture of Boc-Asp-OPac and Boc-Asp-OH (approximately 60:40). Pure Boc-Asp-OPac was thereafter isolated after slow recristallization from iPrOH in 30-40% yield (m.p. 149-151°C). However, the above mentioned hydrogenation caused in some cases a complete reduction of the desired phenacyl ester to the 1,1-hydroxyphenyl ethyl ester of Boc-Asp-OH (m.p. 101-103°C, FAB-MS: $[2M + H]^+$ 707.1 (calcd $[2M + H]^+$ 707.3); ^{1}H-NMR data (200 MHz) show triplet for CH(OH) at 4.28 ppm)). Hence, we are now obtaining Boc-Asp-OPac through a three step synthesis starting with Boc-Asp(OtBu)-OH, which after esterification with 2-bromoacetophenone (85%), TFA mediated cleavage of the Boc-and tBu-groups (100%) with subsequent TFA replacement by HCl (4N HCl in dioxane) and introduction of the Boc-group with $(Boc)_2O$ (1.2 eq, 2eq. of DIEA, in DMF, 2h) gives Boc-Asp-OPac in 60-70% yield (m.p. 149-151°C, FAB-MS $[2M + H]^+$ 703.1 (calcd $[2M + H]^+$ 703.2).
16. The "oxime resin" synthetic protocol in this work included the following steps: (1) loading of the C-terminal protected amino acid onto the resin (3eq. AA, HOBt, DCC; 24h); repeat of (1) if low substitution level (Boc-Asp-OPac was always loaded twice); (2) capping (25 eq. $(Tmac)_2O$ and DIEA in DCM; 24h, with subsequent washing (step (4)); (3) Boc-cleavage (25% TFA/DCM; 1x1min, 1x30min); (4) wash (DCM 3x1min, iPrOH 2x1min, DCM 2x1min, DMF 1x1min) (5) coupling (2.5eq. AA, BOP; 3.5eq DIEA; DMF or DCM or both; 1-2h) and (6) repeat of steps (3), (4), (5) for the assembly of the whole sequence (completion of the couplings was checked by the Kaiser test and substitution levels by the picric acid titration).
17. A typical cyclization protocol included: (1) Boc-cleavage and wash (steps (3) and (4) of [16] (from step (4) the last DMF wash is eliminated)); (2) neutralization (5% DIEA in DCM 2x1min; (3) wash (fast; DCM 3x1min, dioxane 1x1min); (4) addition of the cyclization solvent (dioxane) and of 10 eq. of AcOH; (5) Kaiser test after 3-4h and collecting of the cyclization solution; (6) 2-3x1min wash of the resin with dioxane, washings and the solution of step (5) being collected together and evaporated to dryness;(6) addition of dioxane and lyophilization or precipitation of the peptide from a solvent mixture; (7) continuing of the cyclization reaction by repeating steps (4)-(6).
18. a) Yields are calculated based on the substitution level of the first amino acid attached to the resin; b) the yield of this strategy was 10-30% in other syntheses.
19. Although the cyclization rates and yields are strongly sequence-dependent (see ref. 9), we observed that at least 70% of the final cyclization product is formed, in the first 3-4h of the cyclization reaction (see also ref. 9).
20. See ref. 2a and: Lloyd-Williams, P.; Jou, G.; Albericio, F.; Giralt, E. *Tetrahedron Lett.* **1991**, *32*, 4207.
21. All syntheses described here were performed on the same batch of oxime resin (substitution level: 0.47mmol/g). All peptides were characterized by FAB-MS and amino acid analyses, which gave the expected results.
22. Abbreviations: The following abbreviations are used in addition to the IUBAC-IUB abbreviations (*Eur. J. Biochem.* **1984**, *38*, 9): AA, amino acid; Al, allyl; Asu, a-aminosuberic acid; Bom, benzyloxymethyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; DIEA, N,N-diisopropylethylamine; DCM, dichloromethane, DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; EA, ethyl acetate; FAB-MS, fast atom bombardment mass spectrometry; tBu, tert-butyl; hCT, human calcitonin; HPLC, high performance liquid chromatography; iPrOH, isopropanol; Pac, phenacyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; $(Tmac)_2O$, trimethylacetic anhydride.
23. HPLC conditions: column: Reversed phase C^{18} (Dynamax-300A, 5µm, ID 4.6 mm, length 25 cm); eluents: A: 0.058% TFA in 10% AcCN and B: 0.05% TFA in 90% AcCN; gradient: a linear gradient over 30min of B and A from 10-100% B, followed by an isocratic elution for 5min at 100%B (as indicated on the chromatograms); flow rate: 1.2 ml/min; detection: at 220 and 260 nm.

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