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Detection and control of aspartimide formation in the synthesis of cyclic peptides

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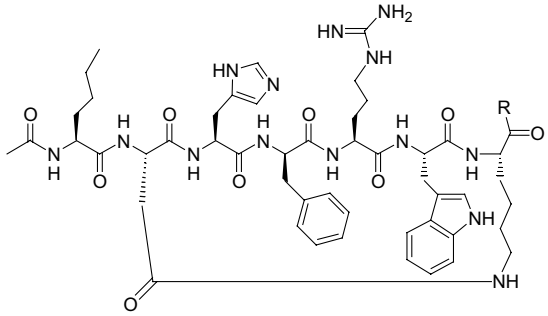
Abstract—Extensive two-dimensional NMR analysis was employed to characterize the structural identity of the macrocyclic peptide lactam and the imide analog, a major side reaction product when allyl ester was used to protect the side chain of aspartic acid. A straightforward protocol modification was developed to minimize aspartimide formation during the synthesis of cyclic peptides.

Cyclic peptides, in contrast to linear peptides, have historically been considered to have greater potential as therapeutic agents due to their increased chemical and enzymatic stability, more defined structure, and improved pharmacodynamic properties.[1,2] A prominent example, MT-II (**1**, Fig. 1), a potent melanocortin receptor agonist, has been tested for a number of therapeutic indications, such as a tanning agent, a therapy for sexual dysfunction and obesity.[3–6]

The initial synthesis of MT-II (**1**) employed Boc chemistry followed by solution phase cyclization (lactamization).[7,8] However, more recent approaches to this class of peptides utilize Fmoc chemistry in conjunction with polymer-supported cyclization.[9,10] This method takes advantage of orthogonal allyl-based protection for both amino and carboxylic moieties together with the convenience of TFA final cleavage and deprotection. Removal of allyl protection can be achieved with a single step Pd(0) treatment. However, aspartimide formation, a well-documented side reaction when allyl ester is used as a protection for the aspartic acid side chain, can be severe in the context of an Asp-Gly sequence motif.[11–14] For other sequence motifs, aspartimide formation does not seem to be a problem.[9,10]

Our Fmoc-based synthesis of peptide **1** involved automated assembly of the primary sequence using 4 equiv of Fmoc-protected amino acid activated with DCC/HOBt. The following side chain protection scheme was used: Asp(OAllyl), His(Trt), Arg(Pbf), Trp(Boc), and Lys(Alloc). After the chain assembly was completed, the peptide resin was treated with $Pd(PPh_3)_4/PhSiH_3$ to remove the allyl protecting groups by following a literature procedure.[15] Prior to the side chain cyclization, a



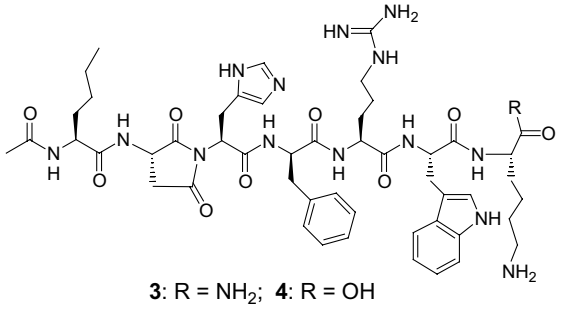


Figure 1. Compounds reported in this study.

Keywords: Allyl ester; Aspartimide; Cyclic peptides; NMR.
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test cleavage followed by LC–MS analysis revealed two major components in a roughly 1:4 ratio.[16] The minor peak at 13.0 min, with the expected linear molecular weight of 1042.2 Da, was followed by the major component at 16.6 min with a molecular weight 1025.1 Da (consistent with that of the cyclized material **1**). These data initially led us to believe that the side chain to side chain cyclization may have proceeded concomitantly with the Pd(0) deprotection treatment.[17,18] Final cleavage and deprotection were then carried out. The isolated major component (14.5 mg, 18.9% overall yield based on the resin loading), however, was found to be aspartimide **3** from rigorous two-dimensional NMR analysis.[19] The results are summarized in Figure 2. Briefly, no His3 amide proton was observed, which strongly suggests the formation of an imide. As a result, the two β protons of His3 showed downfield shifted chemical shifts (3.42 and 3.19 ppm) and the β carbon of Asp2 showed an upfield shift. Consistent with the side chain flexibility of Lys7, the two protons on the ε carbon had the same chemical shifts at 2.77 ppm and the two protons on the side chain nitrogen exchanged quickly with the trace amount of water in DMSO which has a chemical shift of 3.37 ppm. Thus, in two-dimensional ^{13}C HSQC–TOCSY, a cross-peak could be observed with $C_{\varepsilon}H_2$ carbon and water proton chemical shift. In addition, imide **3** showed a significantly reduced retention time on a reverse phase HPLC compared with that of commercial material **1** (Fig. 3).

As a result we elected to prepare peptide **1** through a conventional route by cyclizing the linear unprotected precursor in solution. The same protocol as in the earlier synthesis was used with the exception of Asp(O*t*Bu) and Lys(Boc) protection. The fully deprotected linear peptide was cyclized in solution using 3 equiv of PyBOP/HOBt/DIEA in DMF. The purified product **1** (observed mass 1024.5 Da, calcd mass 1024.2 Da) showed different chromatographic properties compared with **3** (Fig. 3, HPLC retention time was 15.9 min for **3**, and 18.0 min for **1**), but the same as that of the commercial material (18.0 min, Fig. 3).

In order to further confirm the structural identity of the macrocyclic lactam, two-dimensional NMR analysis was also carried out. The results are summarized in

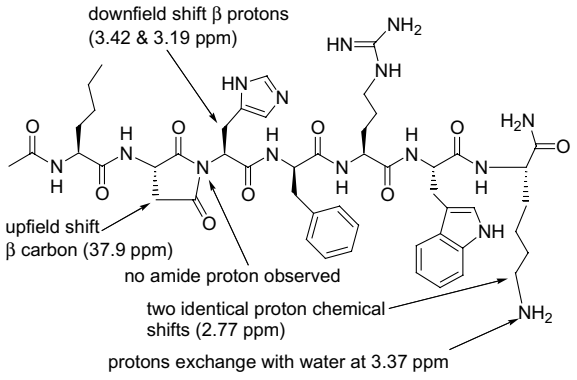


Figure 2. NMR structural analysis on compound **3**.

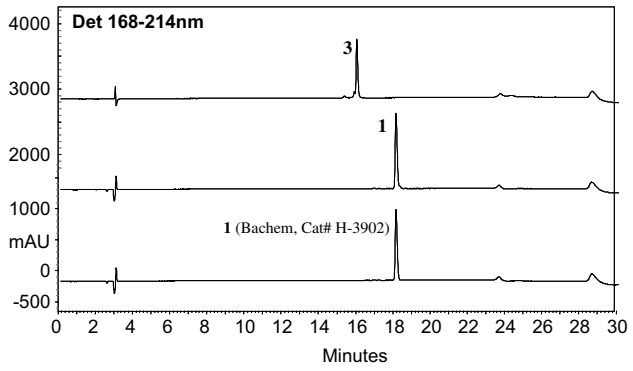


Figure 3. HPLC traces of MT-II (**1**) and the imide analog (**3**).[20]

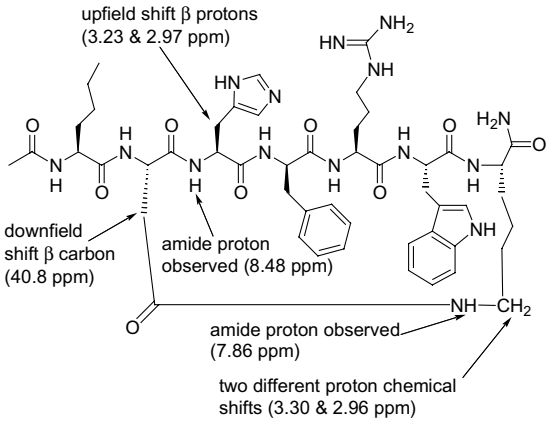
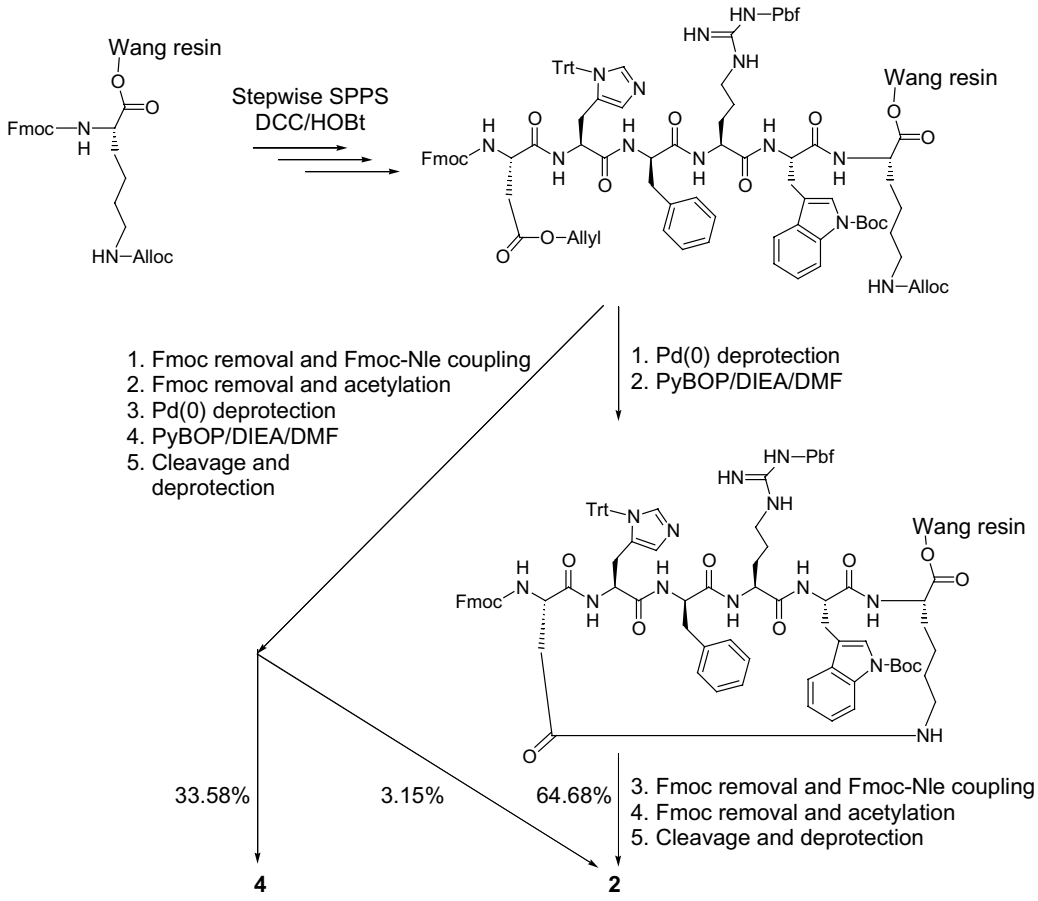


Figure 4. NMR structural analysis of compound **1**.

Figure 4.[19] Compounds **1** and **3** differ significantly around the linkage region. For peptide **1**, His3 amide proton was observed at 8.48 ppm. As a result of the macrocyclic structure, chemical shift of the two β-protons of His3 appeared upfield (3.23 and 2.97 ppm) and β carbon of Asp2 showed a downfield shift at 40.80 ppm. The proton on the ε-nitrogen of Lys7 is an amide proton shown a signal at 7.86 ppm. In addition, the two protons on ε carbon of Lys7 had different chemical shifts, one at 3.30 ppm and one at 2.96 ppm due to their different chemical environments.

To establish a practical allyl-based method for polymer-supported cyclization and to minimize aspartimide formation, we chose model peptide **2** to carry out further investigation. In contrast to peptide **1**, analog **2** with a C-terminal carboxylic acid necessitates a polymer-supported route of cyclization in order to achieve the regio-selectivity. A step-wise synthesis of peptide **2** is shown in Scheme 1.

Fmoc-Lys(Alloc) was loaded onto Wang resin following a published method.[21] The substitution level was determined to be 0.35 mmol/g by the method of Meienhofer et al.[22] The linear sequence Fmoc-Asp(OAllyl)-His(Trt)-d-Phe-Arg(Pbf)-Trp(Boc)-Lys(Alloc)-Wang resin was then assembled on an ABI 433A peptide synthesizer. At this stage, the resin was split into two parts.



Scheme 1. Synthesis of MT-II acid (**2**) and the imide (**4**).

One portion of the peptide-attached resin was subjected to piperidine deprotection of the α-amino group and a test cleavage (Scheme 1). Two major species were detected in HPLC analysis of the crude cleavage products. The first species (39.4% peak area) corresponded to the linear sequence with a loss of a water molecule and a single allyl group, which can be explained by the formation of aspartimide to give H-(aspartimide)-His-d-Phe-Arg-Trp-Lys(Alloc)-OH (calcd mass 954.1 Da). The second species (31.9% peak area) corresponded to the expected linear product: H-Asp(OAllyl)-His-d-Phe-Arg-Trp-Lys(Alloc)-OH (calcd mass 1012.1 Da). Therefore, we concluded that a single step piperidine deprotection transformed about half of the β-aspartyl allyl ester to aspartimide. Continuing the synthesis to completion followed by Pd(0) treatment and cyclization yielded only a minimal amount of desired product **2** (3.15%, calcd mass 1025.2 Da, observed mass 1025.5 Da) with a major product **4**, the aspartimide (33.58%, calcd mass 1025.2 Da, observed mass 1025.2 Da) (Scheme 1). The existence of the aspartimide ring was confirmed by basic hydrolysis.

The second part of the N^{α}-Fmoc protected resin was subjected to Pd(0) treatment and a test cleavage (Scheme 1). LC–MS analysis showed a single major peak (53.1%) with a mass of 1110.5 Da corresponding to the desired structure Fmoc-Asp-His-d-Phe-Arg-Trp-Lys-OH (calcd mass 1110.3 Da). The exposed side chains of Asp and Lys were cyclized by using PyBOP/HOBt/DIEA in dry DMF and a test cleavage was carried out subsequent to Fmoc removal. LC–MS analysis revealed a single product (66.6%) with the desired mass of 870.3 Da corresponding to H-cyclo[Asp-His-d-Phe-Arg-Trp-Lys]-OH (calcd mass 870.0 Da).

These observations confirmed the piperidine treatment as the source of aspartimide formation, which could be prevented by avoiding the direct contact of allyl ester with piperidine. The synthesis was then continued to completion as shown in Scheme 1 and the final product was cleaved and deprotected in a cocktail of TFA/TIS/H_2O/anisole (92/4/2/2%, v/v) for 2 h at room temperature. The crude cleavage product showed a purity of 64.7% with a mass of 1025.4 Da (calcd mass 1025.2 Da) (Fig. 5). The desired product **2** showed a retention time of 18.65 min, while the imide **4** was eluted much earlier at 16.50 min on an analytical HPLC. The final product **2** was purified on a reverse phase C18 preparative HPLC column and lyophilized. Based on the initial resin loading, the overall yield of the synthesis was 23.9% with a purity of 98.0% on an analytical HPLC.

As a final validation, peptide **1** was re-synthesized using the modified protocol. Crude cleavage product showed a

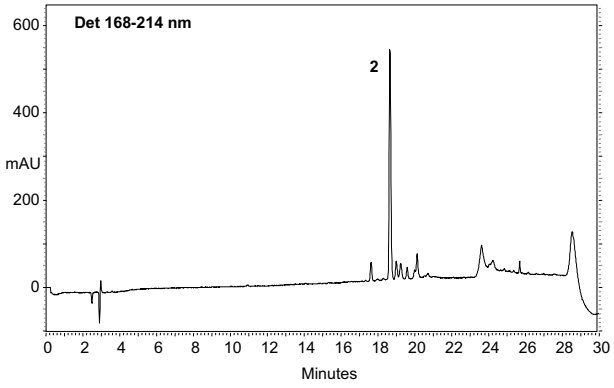


Figure 5. Crude product **2** cleaved from resin.[20]

purity of 72.3% with an expected mass of 1024.6 Da (calcd mass 1024.2 Da). The purified product **1** showed the same retention time (18.0 min) as that of the commercially obtained material as well as that of the product from solution cyclization prepared previously.

In summary, we have demonstrated that aspartimide formation is predominant in the standard Fmoc peptide synthesis using an allyl ester as the side chain protection for aspartic acid. Using multidimensional NMR, we have characterized the structural identity of MT-II and its aspartimide analog. Formation of the lactam ring prior to the removal of N^{α}-Fmoc protection of aspartic acid resulted in elimination of aspartimide side product and a corresponding increase in the target lactam yield. The strategies developed here improved the efficiency of allyl protection in the synthesis of cyclic peptides on resin and can be applied to the synthesis of various lactam constrained cyclic peptides.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2004.12.025. Detailed NMR experiment, NMR spectra, and assignment strategies are available as supplemental data.

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