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Total Synthesis of Cyclocitropside A and Its Conversion to Cyclocitropsides B and C via Asparagine Deamidation

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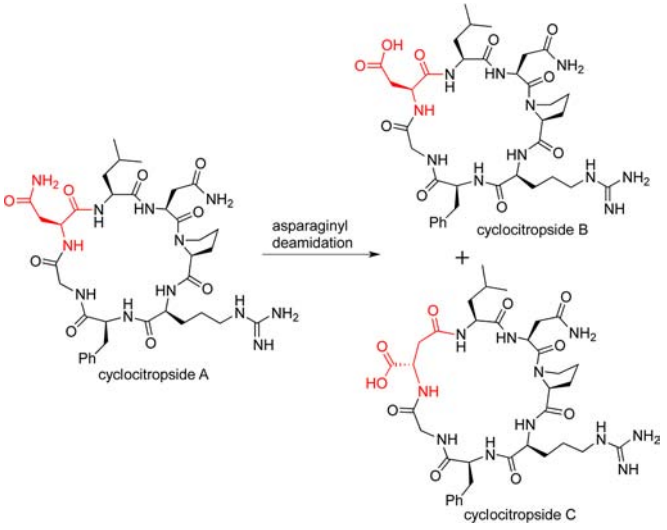
ABSTRACT



The total syntheses of three closely related cyclic peptide natural products, cyclocitropsides A−C, are described. Cyclocitropside A could be readily converted into cyclocitropsides B and C through an asparagine deamidation pathway, indicating that this is a plausible biosynthetic route to these compounds.

The structrually related cyclic heptapeptides, cyclocitropsides A−C (**1**−**3**, Figure 1), were recently isolated from the root bark of the African cherry orange *Citropsis articulata*.[1] The three peptides are almost identical in sequence, varying only in the nature of the amino acid at position five. Cyclocitropside A contains an asparagine (Asn) residue at this position, while cyclocitropsides B and C are configurational isomers of each other bearing aspartic acid (Asp) or isoaspartic acid (isoAsp) residues, respectively. The isolation of these three closely related structures led to the hypothesis that cyclocitropsides B and C might be derived from deamidation of ^{5}Asn in cyclocitropside A and that this might be catalyzed by the basic guanidino moiety of the side chain of an arginine residue also present in the cyclic peptide sequence.[1]

The deamidation of asparagine residues in peptides and proteins is one of the most common forms of nonenzymatic degradation and appears to be associated with the aging of peptides and proteins in vivo.[2,3] However, to the best of our knowledge, cyclocitropside C is the first cyclic peptide natural product reported to contain an isoAsp residue.[4] The deamidation pathway has been well characterized in proteins and model peptides. At neutral or basic pH, deamidation proceeds via a succinimide intermediate, the rate of formation of which is dependent on the

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(3) Robinson, A. B.; Rudd, C. J. *Curr. Top Cell Regulat.* **1974**, *8*, 247.

(4) Several cyclic peptides containing *erythro*-β-methyl-D-isoaspartic acid have been isolated from cyanobacteria. See: Krishnamurthy, T.; Szafraniec, L.; Hunt, D. F.; Shabanowitz, J.; Yates, J. R., III; Hauer, C. R.; Carmichael, W. W.; Skulberg, O.; Codd, G. A.; Missler, S. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 770.

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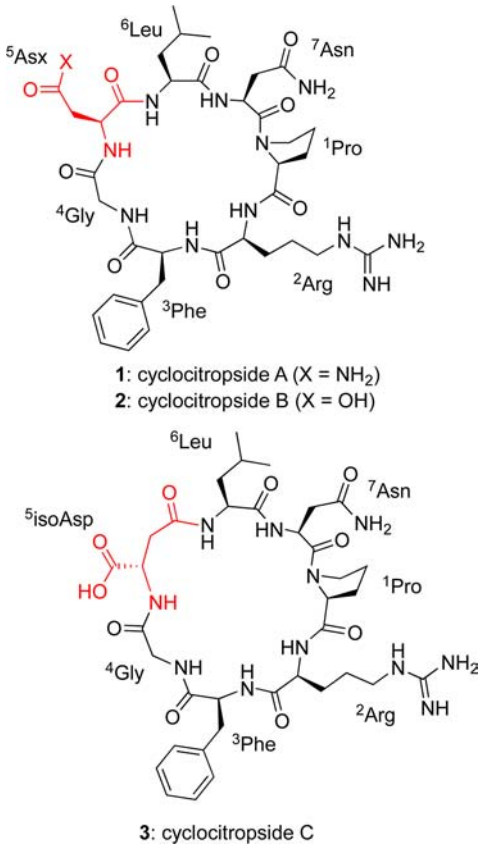

Figure 1. Structures of cyclocitropsides A–C.

amino acid sequence,[5] local peptide structure,[2] and a number of external factors including pH, temperature, and the dielectric strength of the solvent.[6] Hydrolysis of this intermediate is relatively rapid and can occur at either the α- or *β*-carbonyl group to give either isoAsp or Asp, generally in an approximately 3:1 ratio.[7]

The unique structure of cyclocitropside C (**3**) together with the intriguing possibility that this compound and cyclocitropside B (**2**) might be derived from cyclocitropside A (**1**) prompted us to undertake the total synthesis of all three cyclocitropsides and to investigate whether **1** could be converted into **2** and **3** via biomimetic deamidation of the ^{5}Asn residue. In addition we prepared two analogues of cyclocitropside A in which the ^{2}Arg residue was replaced with either Lys or 2-aminoheptanoic acid in order to probe the contribution of this amino acid residue to the deamidation process. We report here the results of these studies.

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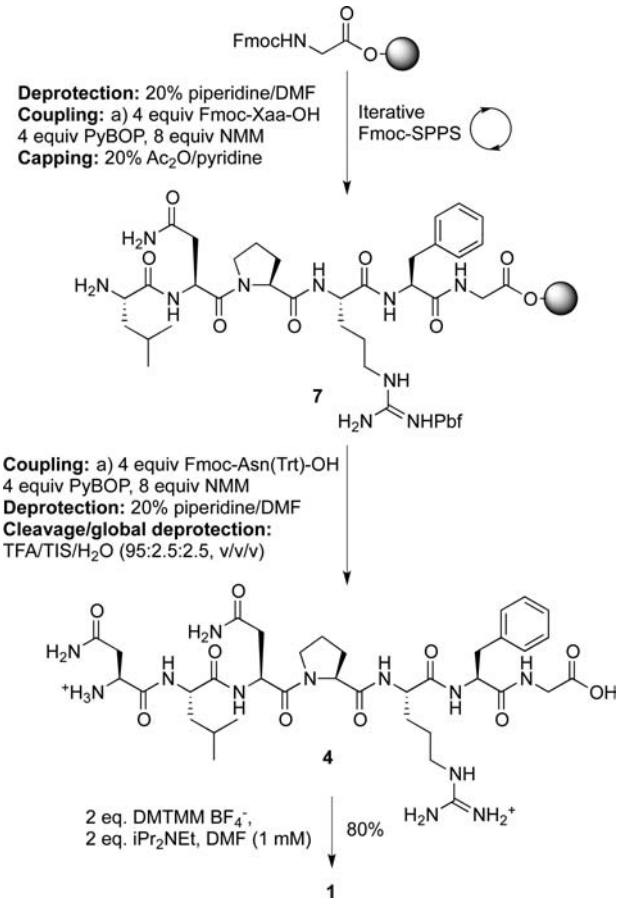
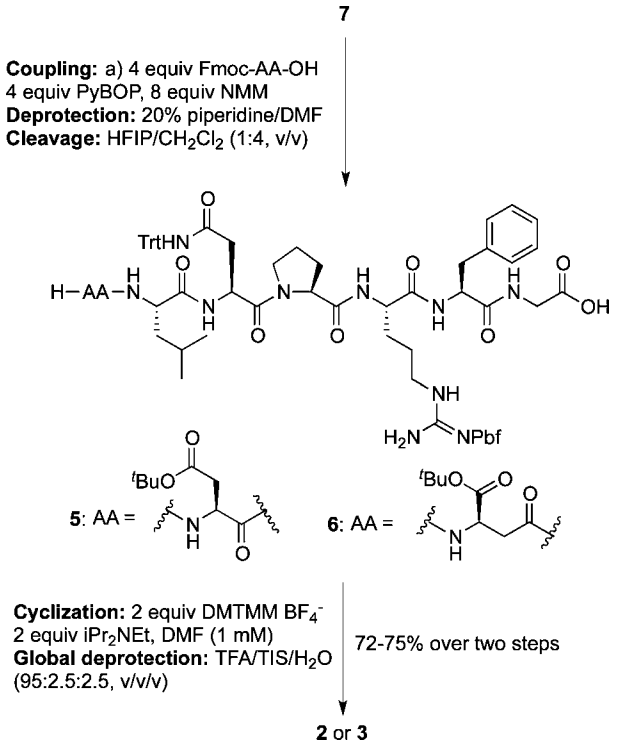
(7) Capasso, S.; Di Cerbo, P. *J. Pept. Res.* **2000**, *56*, 382.

Scheme 1. Synthesis of Cyclocitropside A



We envisaged that a cyclization point at the ^{4}Gly-^{5}Asx/isoAsp junction would enable rapid synthesis of the three required linear peptide precursors **4**–**6** (Schemes 1 and 2), which vary only in their *N*-terminal amino acids, from a common hexapeptide presursor. This places Gly as the *C*-terminal residue which was expected to both facilitate the cyclization and avoid any *C*-terminal epimerization during this reaction. Thus, the resin-bound linear hexapeptide **7** was prepared using standard Fmoc solid-phase peptide synthesis (SPPS) techniques with PyBOP/*N*-methylmorpholine (NMM) as the coupling reagent and 2-chlorotrityl chloride resin as the solid support (Scheme 1). This resin-bound peptide was split into three equal portions to which either Fmoc-Asn(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, or Fmoc-Asp(OH)-O*t*Bu was coupled in order to provide all three of the required resin-bound linear heptapeptides **4**–**6**. Cleavage of the Asn-containing precursor from the resin and concomitant global deprotection was achieved using an acidic cocktail of trifluoroacetic acid, water, and triisopropylsilane to afford **4** in crude form following precipitation from cold ether. Cyclization of **4** was performed using 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium tetrafluoroborate (DMTMM)[8] as the coupling reagent at high dilution (1 mM) in DMF. Following purification of the reaction mixture by RP-HPLC, **1** was obtained in a pleasing 80% overall yield based on the initial resin loading (Scheme 1).

Scheme 2. Synthesis of Cyclocitropsides B and C



The synthesis of structural isomers **2** and **3** was achieved in a similar manner from the resin-bound precursor **7**. Cleavage of these peptides from the resin to liberate the free *C*-terminal carboxylic acid moiety, with protecting groups intact, was achieved using a mildly acidic solution of 20% hexafluoro-2-propanol in dichloromethane to give linear peptides **5** and **6** (Scheme 2), each of which was cyclized upon treatment with DMTMM as described above. In situ acidolytic cleavage of the side-chain protecting groups was conducted using TFA/TIS/H_2O to afford **2** and **3** in 72% and 75% yield (based on the initial resin loading), respectively, after purification by RP-HPLC. All three synthesized cyclic peptides had physical data identical to those reported for the isolated natural products,[1] thus confirming the structures and identities of these three natural products.

Having established the structures of **1**–**3** through total synthesis, we turned our attention to the biomimetic synthesis of **2** and **3** via deamidation of **1**. However, our initial attempts to elicit this transformation in 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethane sulfonate (HEPES) buffer were unsuccessful, leading only to starting material after 12 h incubation at 100 °C. It is well established that phosphate buffer and high temperatures promote the deamidation reaction.[3,9] Therefore, in order to effect this transformation in a reasonable time period, we incubated **1**

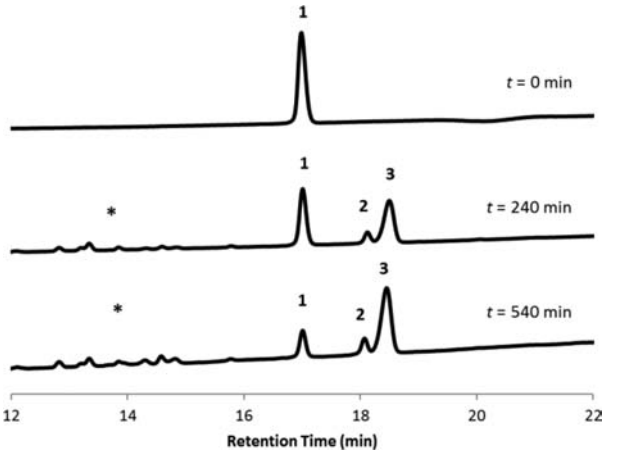


Figure 2. Analytical HPLC trace of crude reaction mixture of **1** in 200 mM phosphate buffer at pH 7.5 at 100 °C after 0, 240, and 540 min. Peaks corresponding to compounds **1**–**3** are shown, as well as linear peptide byproducts (*). Absorbance measured at 230 nm.

at 100 °C in 200 mM phosphate buffer at pH 7.5. The reaction was monitored by LC–MS, by which the starting material **1** and the deamidated products **2** and **3** were observed (Figure 2). After 18 h, the starting material had been completely consumed, and subsequent purification by RP-HPLC gave **2** and **3** in 15% and 60% yields, respectively (Scheme 3). Notably, the 1:4 ratio observed for formation of **2** and **3** is similar to those commonly observed for Asn deamidation reactions.[2,7,10]

Robinson and co-workers have shown that the asparagine-leucine junction is one of the least prone dipeptide junctions to undergo deamidation in peptides or proteins (the succinimide intermediate can not be formed at the Asn-Pro junction, so deamidation is not observed for this dipeptide under the conditions used).[5c] Local secondary structure, such as is likely to be found in a small cyclic peptide, has also been shown to reduce the rate of the deamidation reaction.[11] The surprisingly facile deamidation we observed for **1** under phosphate-buffered conditions led us to investigate the hypothesis presented by Bodo and co-workers[1] that this process may be catalyzed by the basic side chain of the arginine residue in the cyclocitropside sequence. In a manner similar to that described above, we synthesized two unnatural analogues of **1**, replacing the arginine residue with either lysine (**9**), providing an alternate basic residue, or the unnatural amino acid L-2-aminoheptanoic acid (**10**) to remove the basic functionality but provide a similar steric environment to that of the native sequence (Scheme 4, see the Supporting Information for

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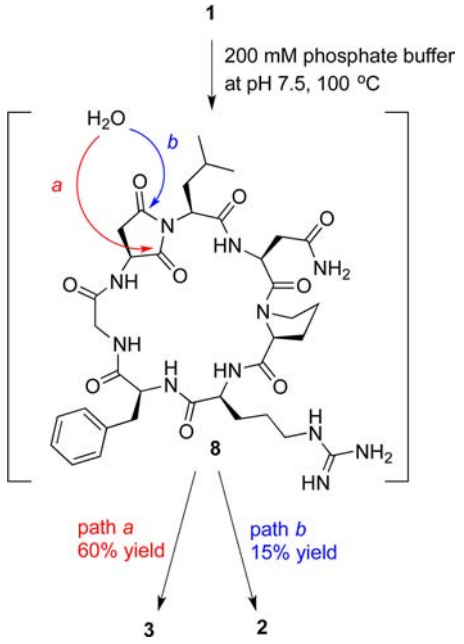
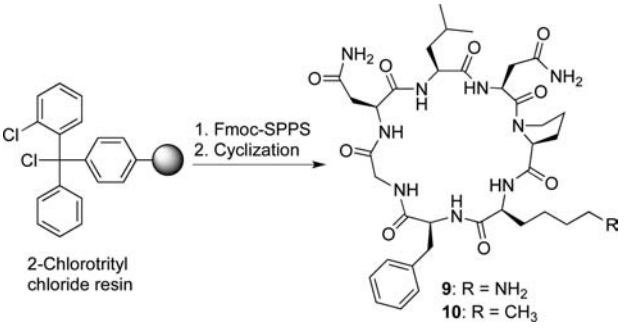
Scheme 3. Deamidation Pathways Leading to **2** and **3** from **1**



synthetic details). However, incubation of cyclic peptides **1**, **9**, and **10** at 100 °C in 50 mM phosphate buffer at pH 7.5 over a 30 h period revealed little to no difference in the rates of degradation into their respective deamidated products as monitored by LC−MS (see the Supporting Information for details). Furthermore, no difference was observed in the rates of deamidation of linear peptide **4** and cyclic peptide **1** under the same conditions. It appears that the influence of the Arg residue on deamidation is negligible under these phosphate-buffered conditions and that the cyclic peptide structure does not impose a significant constraint on deamidation. Similar deamidation reactions have recently been observed in other cyclic peptides,[12] indicating that a cyclic peptide structure does not hinder

(12) Curnis, F.; Cattaneo, A.; Longhi, R.; Sacchi, A.; Gasparri, A. M.; Pastorino, F.; Di Matteo, P.; Traversari, C.; Bachi, A.; Ponzoni, M.; Rizzardi, G.-P.; Corti, A. *J. Biol. Chem.* **2010**, *285*, 9114.

Scheme 4. Synthesis of Structural Analogues **9** and **10**



the deamidation reaction significantly. While the conditions employed in this study were relatively harsh in order to facilitate the reaction in a short time frame, it is likely that the deamidation process could either be an active enzyme-catalyzed process or a passive process under ambient conditions in the plant root over extended time periods.

In summary, the total syntheses of three cyclic peptide natural products **1**−**3** have been completed, providing confirmation of their structures. In addition, the conversion of **1** to **2** and **3** has been observed under conditions favoring asparagine deamidation, confirming this as a plausible biosynthetic pathway for these compounds and suggesting the existence of other isoAsp-containing cyclic peptide natural products.

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Supporting Information Available. Experimental procedures and characterization data. Details of deamidation studies including analytical HPLC data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.