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Synthesis of a Novel ??-Amino Acid Capable of Stabilizing a 310-Helix via Side-Chain Salt-Bridging

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Synthesis of a Novel αα-Amino Acid Capable of Stabilizing a 3_{10}-Helix via Side-Chain Salt-Bridging

A Thesis

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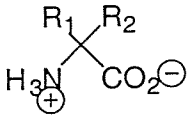
Introduction

The specific induction of helix conformation in peptide secondary (2°) structure has been a major target of research directed toward the design of novel anti-microbial agents.[1] Recently, efforts to induce peptides to form helical conformations have focused on the study of αα-disubstituted amino acids (ααAAs). This was initiated in part by the discovery of the highly helical 2° structure of several classes of fungal peptides that contain a large percentage of ααAAs.[1]



General structure of an αα-disubstituted amino acid

The highly helical nature of these fungal peptides is not predicted for peptides of their short length, nor is it a prediction based on the helix-promoting effects of the proteinogenic amino acid residues. However, what is observed to be unique in these peptides is the large proportion of ααAAs they contain. This feature has naturally led to the postulation that the ααAAs are responsible for the unusually high helicity.[1] This hypothesis has prompted intensive studies of both novel ααAAs and naturally occurring ones such as α-aminoisobutyric acid (Aib) in an effort to understand how these compounds may influence a helical 2° structure in peptides.

The helix-promoting effect of ααAAs has been found to be associated with the restrictions it imposes on the 2° structure-defining torsion angles ψ and ϕ about the C^{α}—C' and the N^{α}—C^{α} bond of the peptide backbone, respectively (see figure 1).[1] When the α-hydrogen of mono-substituted amino acids is replaced with an alkyl group, the ψ

and ϕ angles are restricted to only certain values and, in general, these values correspond to those found in helices.

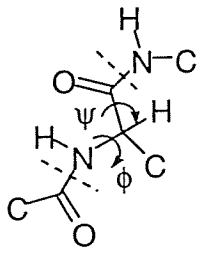
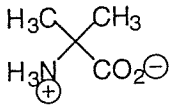


Figure 1. *Critical angles of the peptide backbone.*

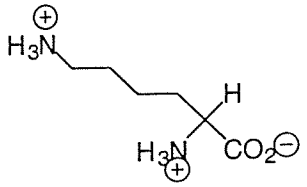
Furthermore, the restrictions on the torsion angles of the peptide backbone is a local effect and, as such, the greater the number of ααAAs, the greater the helix-promoting effect. It is a combination of this effect and the fact that peptides containing large percentages of ααAAs exhibit unusually high resistance to proteolysis,[1] that makes peptides rich in ααAAs prime candidates for novel anti-microbial drugs.

The helix 2° structure motif comes in two forms: the α-helix and the 3_{10}-helix. Of the two, the more studied structure is by far the α-helix. The 3_{10}-helix has not been widely studied and, thus, it is the structure of particular interest to us. A 3_{10}-helix consists of 3 amino acid residues per turn and hydrogen bonding occurs between the *i*th amino acid residue and the *i*th+3 residue, creating a 10-membered ring (hence the name "3_{10}").[1] 3_{10}-Helices show great potential for bioactivity; they are thought to participate in receptor binding and may also be a protein folding intermediate to the α-helix.[2] Hydrophilic 3_{10}-helices are more likely to be biologically active than hydrophobic ones since they are more likely to be soluble in biological media, yet studies of the 3_{10}-helix have been limited to those containing hydrophobic amino acids.[3] Consequently, little is known about factors contributing to the stability of this conformation in aqueous media. My

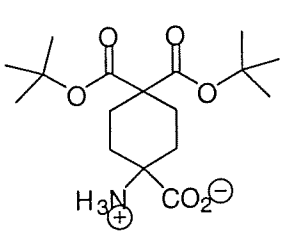
research has focused on the synthesis of a new hydrophilic ααAA, 1-amino-4,4-di-*tert*-butoxycarbonyl cyclohexane carboxylic acid (Cda), to incorporate into the septapeptide, H-Lys-Aib-Aib-Cda-Aib-Aib-Lys-NH_2 (Sb7), for the purpose of investigating its effect on the stability of the 3_{10}-helix in aqueous environments.



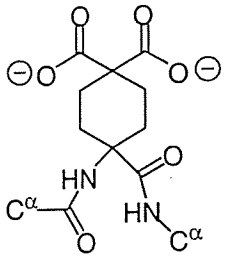
α-Aminoisobutyric acid (Aib)



Lysine (Lys)



Cda



Hydrolyzed Cda residue

Figure 2. *Amino acid constituents of Sb7*

Cda and Sb7 have been specially designed so that Sb7 features intra-peptide salt-bridging, a feature which will allow us to test the viability of salt-bridging as a means to stabilize the 3_{10}-helix in water. The theory behind salt-bridge stabilization is that if, in addition to hydrogen bonding, the helix is held together via ionic bonding of the same pattern (*i*, *i*+3), the helical conformation will be significantly stabilized.[2] Cda will be incorporated into Sb7 (placed in the *i*th position) and the esters then hydrolyzed to give two carboxylate anions which can potentially form bridges with the cationic side chains of the two Lys residues (ideally placed in the *i*th±3 positions). Subsequent analysis of the peptide in aqueous solution will demonstrate whether or not salt-bridging will stabilize a 3_{10}-helix.

In addition to controlling helicity in peptides, we are interested in designing peptides which are amphipathic. Amphipathic peptides are those which have both a polar and non-polar face when in the "correct" helical conformation.[1] The potential benefits of this design are two-fold: it may confer water solubility to the peptide and may also augment its potential for anti-microbial activity by allowing it to penetrate the amphipathic phospholipid bilayers of microbial cell membranes. Thus, Sb7 has been amphipathically designed; when looking down the helical axis of the intended structure (figure 2), Lys and Cda compose the hydrophilic face while Aib composes the hydrophobic face.

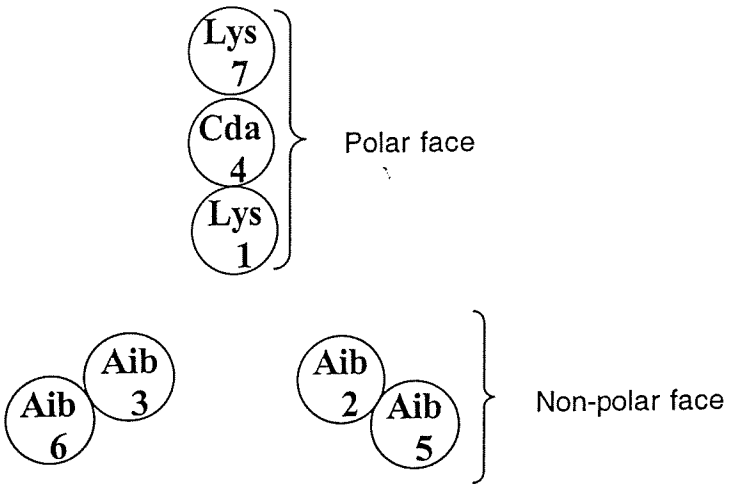


Figure 2. The amphipathic, 3_{10}-helical design of H-Lys-Aib-Aib-Cda-Aib-Aib-Lys-NH_2.

In summary, we have sought to synthesize Cda because it has the ability to stabilize the 3_{10}-helix in three ways: 1) It is αα-disubstituted and is thus likely to promote a helical conformation and confer proteolytic stability to the peptide. 2) When incorporated into the peptide we have designed, it will make it amphipathic. 3) It shows promise to maintain a 3_{10}-helical structure of the designed peptide in aqueous media by

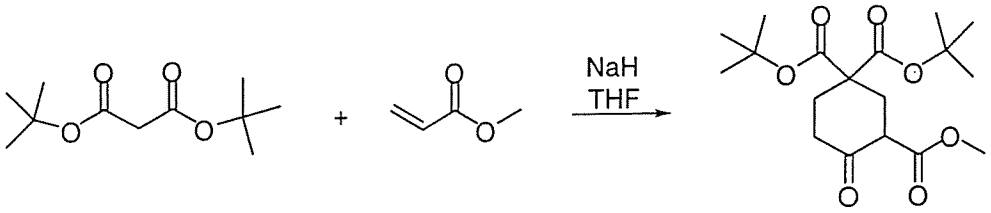
ion-pairing of the ammonium cation of the Lys side chain and the two anionic carboxylate groups of the hydrolyzed side-chain of Cda.

Synthetic Methods

Step 1

Theory:

The first precursor to Cda is 2-methoxycarbonyl-4,4-di-*tert*-butoxycarbonyl cyclohexanone, **1**, and is prepared via a one-pot Michael addition of di-*tert*-butylmalonate to methyl acrylate and intramolecular Claisen condensation:[4]



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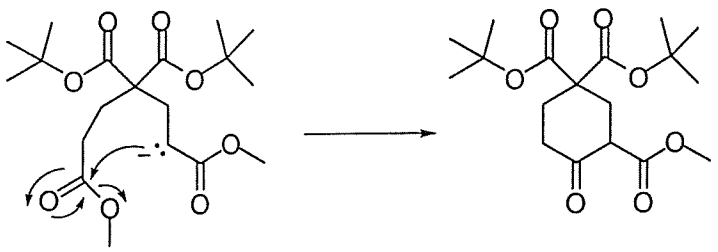
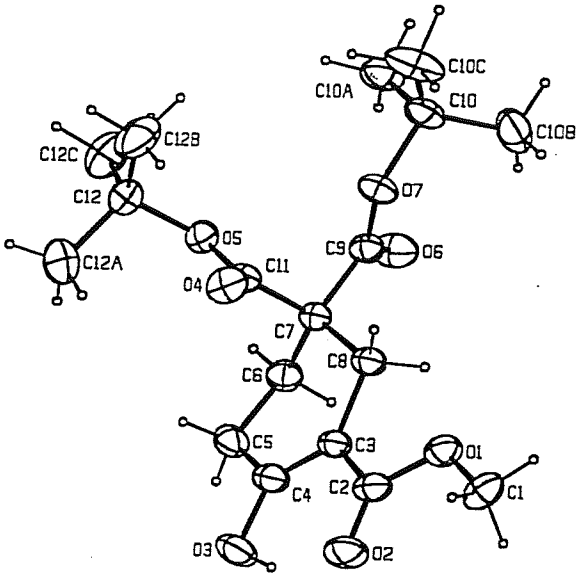
The reaction requires a two-fold excess of base in order to remove both α-hydrogens of di-*tert*-butylmalonate. In addition, a two-fold excess of methyl acrylate is used to ensure conjugate addition of the α-carbon of di-*tert*-butylmalonate to two methyl acrylate molecules. The resulting dialkylated complex undergoes the following Claisen condensation to yield the desired compound:



Methods and Results:

NaH reacts violently with water and, as such, this reaction must be performed under inert atmosphere. Freshly distilled di-*tert*-butylmalonate in dry tetrahydrofuran (THF) was added dropwise over a 30 minute period to a stirred suspension of sodium hydride (2.5 equiv.) in dry THF at 40-45°C. This mixture was stirred for an additional 15 minutes to allow generation of the enolate anion. To this mixture, a solution of methyl acrylate (2.0 equiv.) in dry THF was added slowly over 30 minutes and the resulting mixture allowed to stir for an additional hour. The solution was cooled and poured into ice-water, taken to pH 3 by means of 1N hydrochloric acid (HCl), and then extracted with ethyl acetate. The product obtained after drying and rotary-evaporation of the ethyl acetate layer was a dark orange oil which gave 3 major spots on a normal phase thin layer chromatography plate using a 90% hexanes/ethyl acetate solvent system. No staining is required for visualization of peaks since all three spots were found to be UV active. The spot with the largest retention factor (R_f) was purified over silica gel stationary phase using the same mobile phase as for TLC and was confirmed to be the triester by ^{1}H and ^{13}C-nuclear magnetic resonance spectroscopy (NMR), and by x-ray crystallographic analysis. Crystals for the x-ray structure were grown in a NMR tube by slow evaporation from a 90% hexanes to ethyl acetate mixture. This reaction was performed several times and consistently gave 30-35% yield of the white powder.



Crystal Structure of ***1****, 2-methoxycarbonyl-4,4-di-*tert*-butoxycarbonyl cyclohexanone*

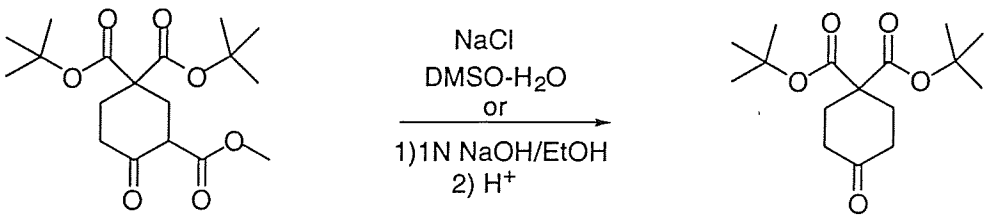
Discussion:

The very low yield of this reaction is a severe hinderance to the ultimate synthesis of Cda and must be improved upon. There appears to be two other major products in this reaction besides the triester, the most likely identities of which are the uncyclized product (formed by two Michael additions and no Claisen condensation) and the mono-substituted di-*tert*-butylmalonate (one Michael addition). If this is the case, then a solution to the yield problem would be to simply extend the time of the reaction. In addition, the reaction could be closely monitored by TLC; it would support our hypothesis if the two contaminating compounds were shown to appear before the desired product. If lengthening the reaction fails to improve the yield and if the contaminants still remain, then the contaminants should be isolated, their identities ascertained, and a new plan to improve the yield should be formulated based on this information.

Step 2

Theory:

The next precursor to Cda is 4,4-di-*tert*-butoxycarbonyl cyclohexanone, **2**, and has been attempted by two different routes:



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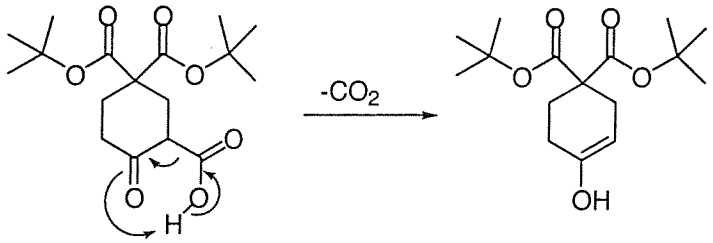
The first is a route taken by Sanchez, *et. al.* to a similar molecule.[4] The reaction is a simple S_N2 (bimolecular nucleophilic substitution) reaction in which the chloride ion acts as the nucleophile in the attack of the methyl carbon of the methyl ester to form chloromethane and CO_2. The entropy gain resulting from the release of CO_2 is the driving force for this reaction.

The second route is a well known path to ketones via β-ketoesters. The first step is to hydrolyze the methyl ester to a carboxylate anion. Saponification using only 1 equivalent of dilute base coupled with the fact that the *t*-butyl esters are less base-sensitive than methyl esters is sufficient to cause selective hydrolysis of the methyl ester over the *t*-butyl esters. The second step is acidification using dilute HCl to yield the carboxylic acid which will then readily lose CO_2. The viability of this reaction is based on the fact that such carboxylic acids having a carbonyl group at the β-position are very unstable. Instability is a result of the fact that reaction can occur by means of a 6-

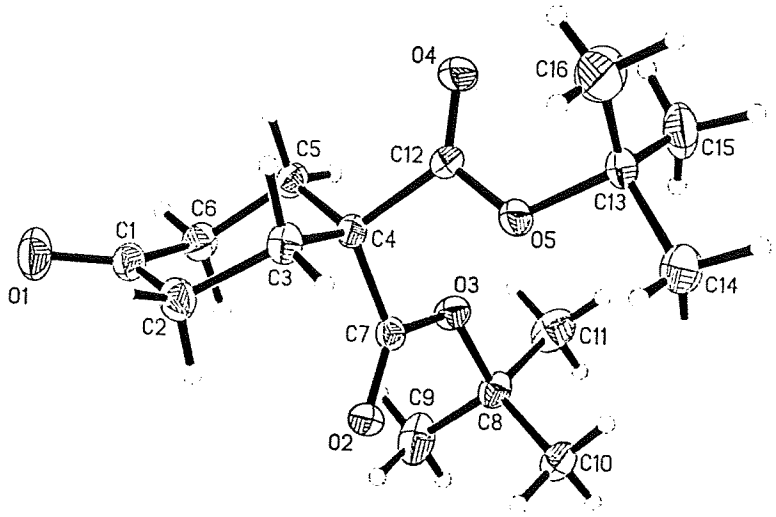
membered, cyclic transition state in which the transfer of a proton, loss of carbon dioxide, and the formation of an enol all occur simultaneously:



Acid catalyzed tautomerization then generates the desired ketone.

Methods and Results:

Method 1: A suspension of the triester **1**, NaCl (3.0 equiv.), and water in dimethyl sulfoxide (DMSO) was maintained at 150-160°C for 2 hrs under argon. The resulting mixture was allowed to cool down to room temperature, poured into ice-water, and extracted with ethyl acetate. Rotary-evaporation of the ethyl acetate layer yielded a dark orange oily residue which separated into 3 TLC spots (mobile phase 90% hexanes to ethyl acetate). The center spot was determined by ^{1}H-NMR to be starting material and the spot with the lowest R_f was confirmed by both ^{1}H-NMR and x-ray crystallography to be the ketone, **2**.



*Crystal Structure of 2, 4,4-di-*tert*-butoxycarbonyl cyclohexanone*

Crystals were grown by slow evaporation from DMSO. This reaction was performed several times and consistently gave 20-25% yield.

Method 2: Due to the very low yield obtained by the first method, the following alternative was attempted. To prevent yield loss due to the reaction of NaOH and CO_2 from the air, this reaction was performed under inert atmosphere. A mixture of **1** dissolved in ethanol and 1M sodium hydroxide (NaOH, 1 equiv.) was allowed to reflux at 79°C for about 2 days. The reaction's progress was monitored by TLC (mobile phase 90% hexanes/ethyl acetate) via the disappearance of starting material in the reaction. The final solution was cooled, brought to pH 3 using 1N HCl and then extracted with ethyl acetate. The crude product was a dark yellow oil which gave 2 TLC spots (mobile phase 90% hexanes/ethyl acetate). Visualization of spots required staining with anisaldehyde, I_2, or phosphomolybdic acid, as the diester is not UV active. The R_f value of the less retained spot compared well with that of the starting material reference and the lower spot was determined by ^{1}H-NMR and electron impact-mass spectrometry to be **2**. This reaction consistently yields 60-70% of the white solid product.

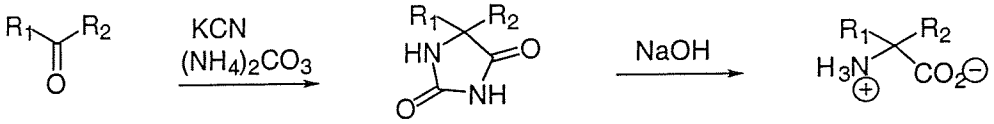
Discussion:

The second route is clearly the more fruitful path with its yield of 60-70% vs 20-25%. The yield of this reaction may perhaps still be improved upon. In all runs, the reaction never proceeded to completion and appeared to reach its end after about 2 days, i.e. starting material ceased to disappear on the TLC. Therefore, adding base in slight excess may improve the yield by driving the reaction to completion. If yield loss is due to the reaction of NaOH and CO_2 in the air, a slight excess of base would also improve the yield.

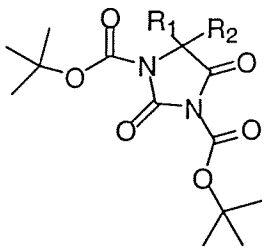
Step 3

Theory:

This step and the subsequent step to Cda are all part of the modified Bucherer-Bergs method, now one of the most widely used routes for α-amino acid and αα-amino acid synthesis. The Bucherer-Bergs method has proven to be advantageous over the alternative Strecker synthesis in that it generally gives higher yields of the desired product.[5] The original Bucherer-Bergs method involves the formation of a hydantoin intermediate by reaction of the appropriate aldehyde or ketone with ammonium carbonate and potassium or sodium cyanide. Subsequent hydrolysis of the hydantoin with strong, concentrated base yields the desired amino acid: [6,7]



The conditions required to hydrolyze the hydantoin in this method are very harsh and can often lead to problems if the alkyl subsituents at the α-position are especially susceptible to degradation. In order to prevent the undesired degradations, the Bucherer-Bergs synthesis was modified to place the *tert*-butoxycarbonyl (Boc) protecting group on both the nitrogens of the hydantoin ring before hydrolysis:[8]

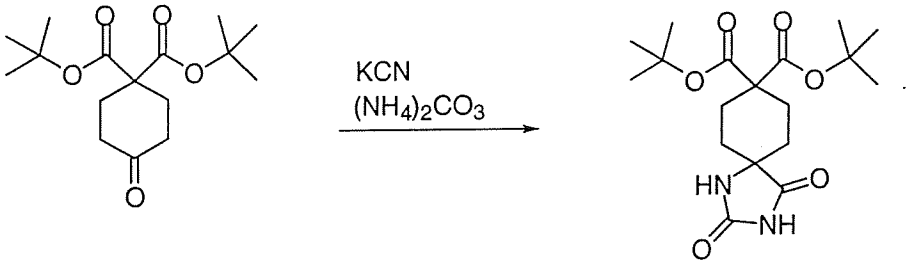


Boc-protected hydantoin

With the hydantoin protected, the two carbonyls composing the hydantoin ring are more susceptible to attack and, therefore, allow the use of milder hydrolysis conditions. It has

been suggested that the greater ease of hydantoin cleavage is due to the release of steric strain which the Boc-groups create. Also, due to the electron-withdrawing ability of the Boc-group, the nitrogens are converted into better leaving groups and, thus, the new step effectively activates the carbonyls of the hydantoin toward nucleophilic attack. Because the carbonyls are activated, dilute base can be used and undesired side reactions are eliminated.

Thus, the method for our synthesis of Cda is the modified Bucherer-Bergs reaction of the ketone, **2**. Step 3 is the synthesis of the free hydantoin, **3**:



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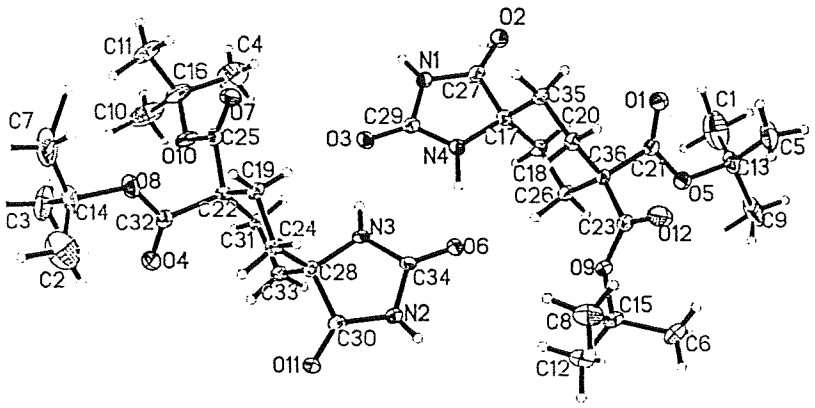
Methods and Results:

Ketone **2**, potassium cyanide (1 equiv.), ammonium carbonate (5.0 equiv.), and a 50:50 ethanol:water mixture were heated under reflux (50-55°C) for 5 hours. The resulting mixture was diluted with water and allowed to cool in an ice bath. The off-white precipitate which formed was filtered and recrystallized from ethanol to yield 65-70% pure hydantoin. The identity of the hydantoin was ascertained by ^{1}H-NMR in DMSO in which the peaks at 8.4 and 10.6 ppm clearly indicate the presence of the amide and imide hydrogens, respectively, of the hydantoin ring. In addition, x-ray crystallographic methods unambiguously determined the structure to be the hydantoin. Crystals for the analysis were grown in a NMR tube by slow evaporation from ethanol.



*Crystal structure of 3, 1,1-di-*tert*-butoxycarbonyl cyclohexane-4-spiro-5'-hydantoin*

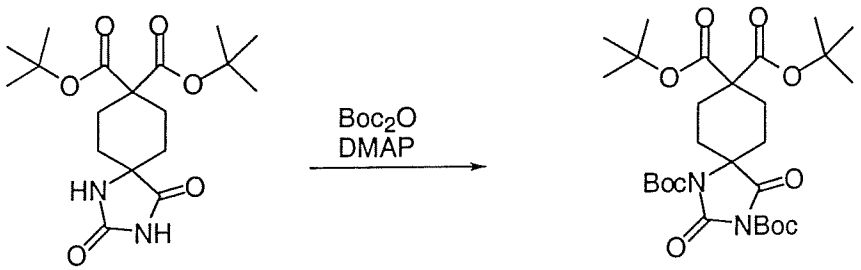
Discussion:

The most likely account for the less than optimal yield of this reaction is the fact that NH_4CO_3 sublimes at the temperature of the reaction. Significant amounts of this compound have been observed on the inside of the reflux condenser during the course of the reaction and, in order to improve the yield of future runs, the insides of the condenser should be periodically scraped to return the sublimed NH_4CO_3 to the reaction mixture.

Step 4

Theory:

Next, we protect the hydantoin nitrogens with the Boc group in order to ease the hydantoin hydrolysis in the subsequent step:[8]



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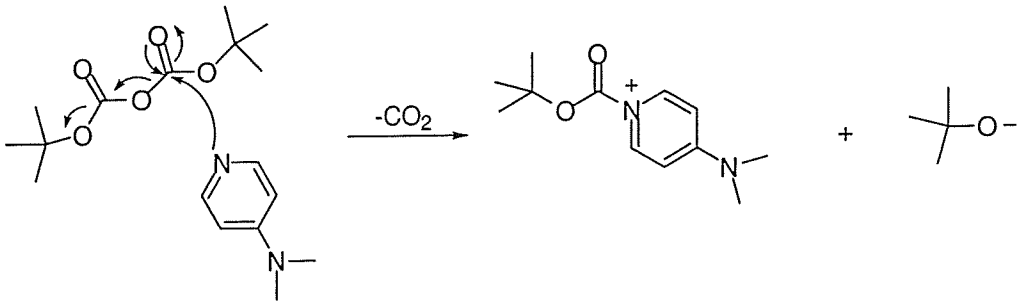
In this reaction, dimethylaminopyridine (DMAP) functions as an acylation catalyst, reacting with *tert*-butoxycarbonyl anhydride ($(Boc)_2O$) to yield the activated pyridinium

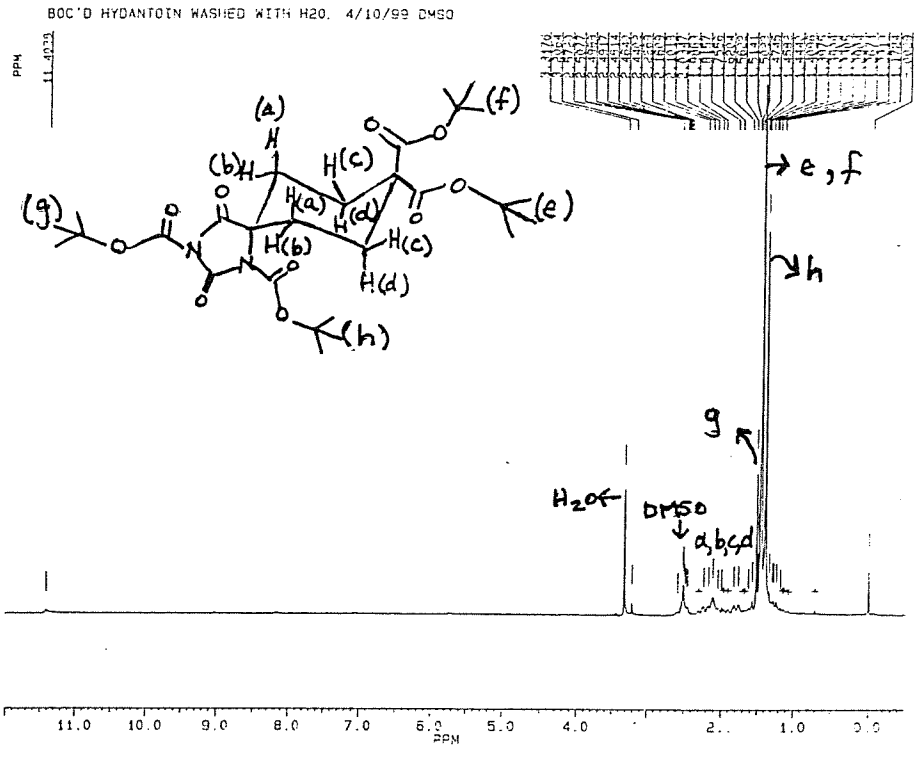


salt. The carbonyl, now with an excellent leaving group, is more susceptible to nucleophilic attack by the nitrogens of the hydantoin. In addition, formation of the pyridinium salt displaces a *tert*-butoxycarboxylate group from $(Boc)_2O$ which in turn fragments to release CO_2 and a *tert*-butoxy group. CO_2 release is the driving force for this reaction and also provides a means of monitoring the reaction's progress.

Methods and Results:

To a solution of hydantoin, **3**, in dichloromethane (DCM) was added a solution of $(Boc)_2O$ (2.0 equiv.) in DCM. Next, a spatula tip full of DMAP was added to this mixture and a mercury bubbler attached to the system to detect the release of CO_2. When bubbling ceases, more DMAP was added, bubbling observed, and the procedure repeated until no CO_2 release was observed on addition of DMAP. The progress of the reaction was also monitored by normal phase TLC comparison of starting material with the reaction mixture (solvent system 90% hexanes/ethyl acetate, phosphomolybdic acid stain). Once the reaction was determined to be complete, the mixture was washed with 1N HCl to remove the DMAP catalyst, and then with a small amount of 1M sodium bicarbonate to remove any acid which may have remained in the acid wash. Drying and rotary-evaporation of the DCM layer yielded about 75% of a white solid. ^{1}H-NMR

analysis confirmed the structure to be **4**. While TLC showed the presence of a very small amount of free hydantoin, this contaminant went undetected by NMR analysis. Purification of the protected hydantoin was deemed unnecessary since clearly the starting material was present in a very small amount and its presence will not disturb the next reaction.



^{1}H-NMR of the protected hydantoin, 4

Discussion:

This general reaction for protection of the hydantoin, as the previous reaction to synthesize the hydantoin, is a well known, published reaction in which yields are typically 80-90%.[3] Thus, our yield of 75% is fairly good and it is likely this is the optimal yield for this reaction.

Step 5

Theory:

Next is the hydrolysis of **4** to form Cda. The mechanism of this reaction has, until recently, been unclear. Confusion arose from the observation that hydrolysis of αα-disubstituted hydantoins yields the free amino acid, whereas in the hydrolysis of mono-substituted hydantoins the α-nitrogen of the amino acid remains Boc-protected. It had been previously postulated that the mechanism occurs differently for mono- and di-substituted hydantoins.[3] Two mechanisms in support of this have just recently been proposed (Mark McLaughlin, personal reference) and are shown in figure 3. The two mechanisms basically differ in the position of initial nucleophilic attack by a hydroxyl group. Path A, which involves initial attack at C2 of the hydantoin ring, is thought to occur when the area about C4 is crowded, i.e. when the hydantoin is di-substituted. Initial cleavage at this carbon leaves N3 dangling in ideal proximity to attack the carbonyl of the N^{α}-Boc group and form the by-product of this mechanism, di-*tert*-butyl imino-dicarboxylate ($NHBoc_2$). Path B involves initial attack by the hydroxyl group at C4 instead of C2 and is thought to occur only with mono-substituted hydantoins since attack at this position will then be less sterically hindered. Initial cleavage at C4 precludes N3 attack at the N^{α}-Boc group and so the by-product via this mechanism is NH_2Boc instead of $NHBoc_2$. The driving force for both mechanisms is the release of CO_2.

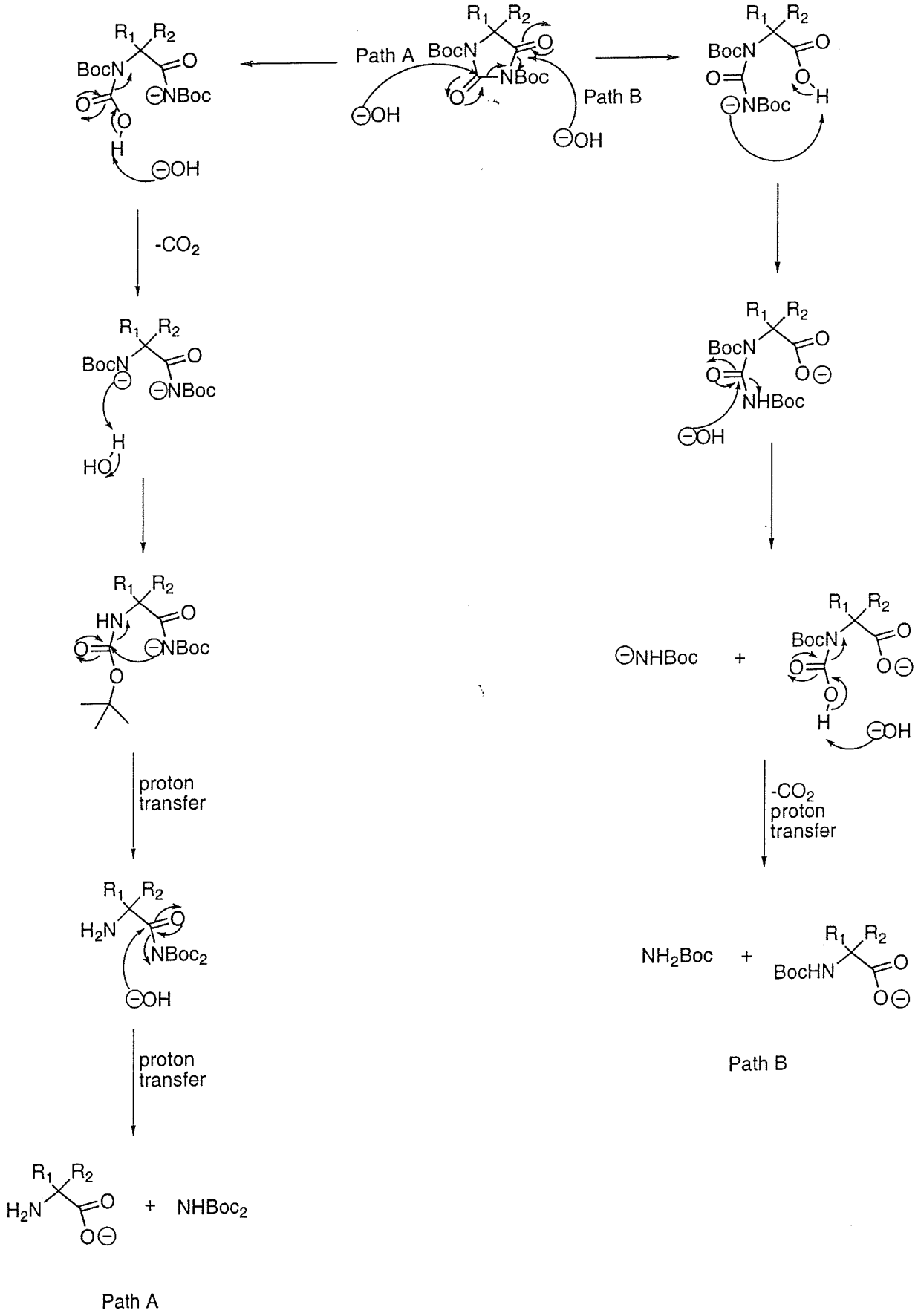
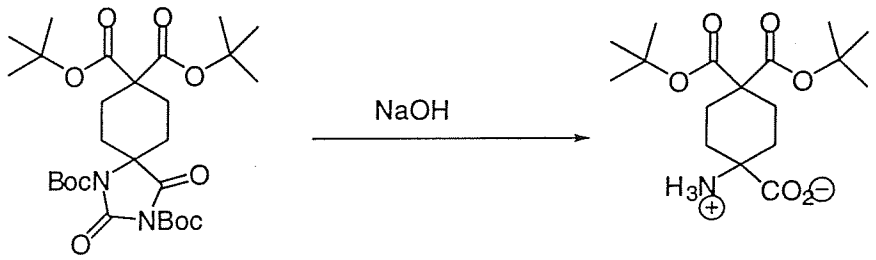


Figure 3. *Proposed mechanism of hydantoin hydrolysis*

Since **4** is a *di*substituted hydantoin, we can predict that our reaction will occur by Path A, and that hydrolysis will yield the free amino acid, **5**, and $NHBoc_2$.

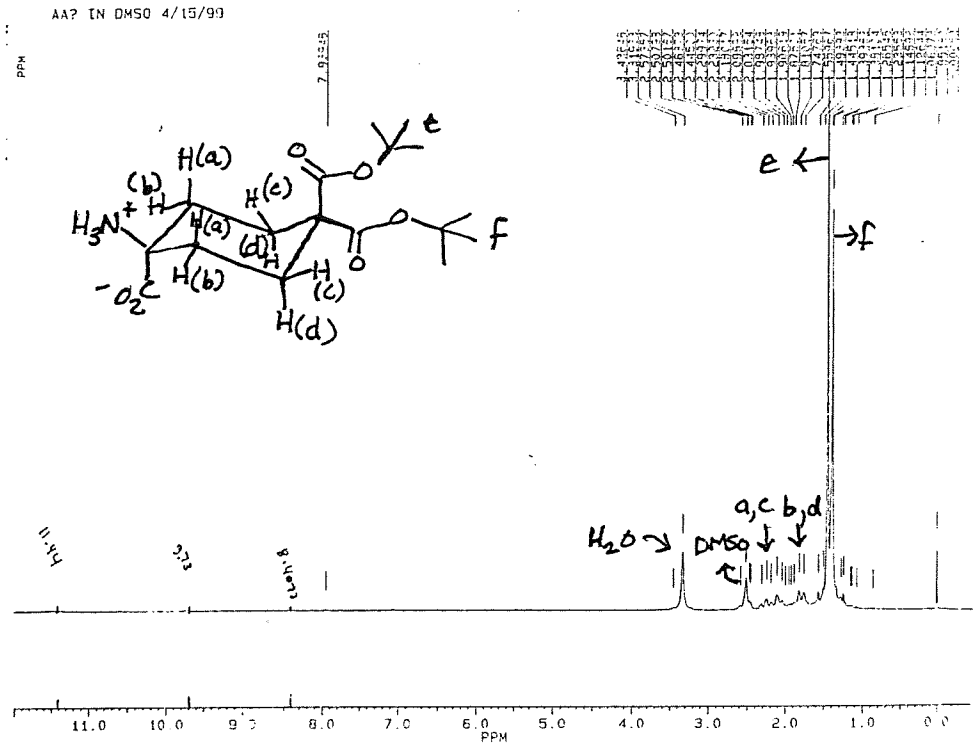


5

Methods and Results:

In an effort to avoid cleavage of the *t*-butyl esters of our hydantoin, this reaction is performed at room temperature. A 2M NaOH solution (16 equiv.) was added to a solution of **4** dissolved in THF. Equal portions of water and THF were used. The flask was stoppered, and the reaction was monitored by normal phase TLC (mobile phase 95% chloroform/methanol, phosphomolybdic acid stain) via the disappearance of **4** and the appearance of $NHBoc_2$. The reaction was stopped after 2 days at which time no further disappearance of starting material could be detected. The THF layer was separated from the water layer and the latter acidified with 1N HCl to pH 7. Because at pH 7 the amino acid (if present) should be mostly in zwitterionic form, we should expect it to precipitate out of the water. This was not observed but, nevertheless, we proceeded with our ethyl acetate extraction. ^1H NMR of the very small amount of clear oily residue obtained (barely enough for NMR analysis) was inconclusive. TLC comparison of this extract with the THF layer of the reaction revealed the abundance of this material in the THF layer. Thus, the THF was rotary-evaporated, and the residue diluted with water and

extracted with ether to remove the $NHBoc_2$. The water layer was then brought to pH 5 and a significant amount of white precipitate was observed. An attempt at extraction with ethyl acetate was unsuccessful, as the precipitate was essentially insoluble in this solvent. Instead, we filtered the precipitate (68% yield) and examined it by ^1H NMR (DMSO solvent):



Discussion:

Definitive characterization of the product of this reaction was not accomplished since it was insoluble in nearly all solvents we attempted. However, the results were promising—the precipitation of the white solid upon acidification and the presence of two distinct *t*-butyl hydrogen peaks in the NMR spectrum, are strongly indicative of Cda. On the assumption that we obtained Cda, we proceeded to run a final reaction to place the

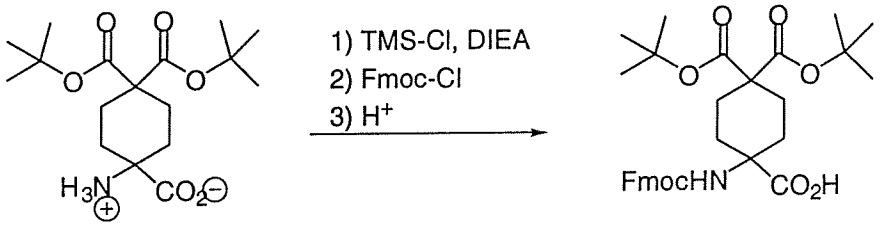
fluorenylmethoxycarbonyl (Fmoc) protecting group on its α-nitrogen in the hopes that it would facilitate characterization of the product.

Step 6

Theory:

The Fmoc protecting group is a highly aromatic system which shows a strong absorbance of 300nm light, thus allowing easy detection of amino acids. The protecting group also confers solubility to the amino acid and in this way eases its analysis. Furthermore, the Fmoc-amino acid will be suitably protected for its incorporation into peptides using Fmoc solid-phase synthesis.

Protecting the α-nitrogen of a hydrophobic amino acid such as Cda is difficult using traditional methods with aqueous/organic mixtures because of the extreme non-polar nature of the side chain.[3] The method we use solubilizes the amino acid by forming the silyl ester via the reaction of trimethylsilyl chloride (TMS-Cl) and the carboxylate ion of the AA in neat organic media.[3] The reaction of the silated complex with Fmoc-Cl is a typical acylation reaction with the α-nitrogen serving as the nucleophile in the attack of the Fmoc carbonyl. Subsequent acidification frees the molecule of the silyl group and forms the final product, **6**.



6

Methods and Results:

Because TMS-Cl undergoes an acyl transfer reaction with water to form TMS-OH, this reaction must be carried out under inert atmosphere. The precipitate from the previous step was diluted with dry DCM. TMS-Cl (2.5 equiv.) and diisopropyl-ethylamine (DIEA, 3.0 equiv.) were added to the flask. Typically, this solution must be heated at reflux to achieve complete dissolution of the starting material. Our reaction required no heat whatsoever, as the starting material dissolved almost immediately after addition of TMS-Cl. Next, Fmoc-Cl (1.1 equiv.) was added and the reaction's progress monitored by TLC (95% chloroform/methanol solvent system, UV visualization). The reaction was complete in one day, at which time the solution was acidified to pH 4, and the water layer extracted with DCM. The combined DCM extracts were dried and rotary-evaporated. The crude product (107% yield) was found by TLC to contain Fmoc-Cl starting material and one product, which is UV active. A good solvent system for separation was found by TLC to be 95% chloroform/methanol. However, the crude product was only slightly soluble in this mixture and so, this solvent system could not be employed. Due to insufficient time, separation and analysis have not yet been realized.

Discussion:

The UV-active TLC spot is strongly indicative of the presence of **6**. A new solvent system should be devised to separate this peak from the starting material and then analyses made to ascertain its identity.

Conclusions and Future Work

A major factor contributing to the inexpedience of Cda synthesis has been the very low yield of the Michael addition/Claisen condensation reaction to form **1**. Instead

of attempting to augment the yield of this step, which may prove to be only time-consuming and unfruitful, an alternative route to **2** may be taken—a Diels-Alder reaction between 2-acetoxy-1,3-butadiene and 1,1-di-*tert*-butoxycarbonyl ethylene, followed by acidification, promises the facile synthesis of **2** in high yield while eliminating, altogether, the synthesis of **1**.

Syntheses of the free and protected hydantoins were executed fairly smoothly and only the minor improvements aforementioned can be made. The synthesis of Cda has not yet been confirmed, although observations and NMR data are strongly indicative of our success. Analysis of the product from step 6 is to be completed and confirmation of the Cda structure made. Once this is accomplished, Cda will be incorporated into Sb7 using a solid-phase peptide synthesizer. It is hoped that subsequent hydrolysis of the *t*-butyl esters of the Cda residue with trifluoroacetic acid will yield a highly water-stable 3_{10}-helix that will support the idea that *i*, *i*+3 salt-bridging stabilizes the 3_{10}-helix.

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