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Synthesis of 1-Alloc-piperidine-4-Fmoc-amino-4-carboxylic Acid: A Novel, Protected ?,?- Disubstituted Amino Acid

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Synthesis of 1-Alloc-piperidine-4-Fmoc-amino-4-carboxylic Acid: A Novel, Protected α,α-Disubstituted Amino Acid

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Introduction

Recently, the specific induction of helix conformation in peptide secondary (2°) structure has become the target of research directed toward the design of novel anti-microbial agents. The design of such peptides was partially prompted by the discovery of natural anti-microbial peptaibols.[1] It was in studying these naturally occurring peptaibols and other classes of fungal peptides that the helix-inducing effect of α,α-disubstituted amino acids (ααAAs) was first postulated. The high helicity of these peptaibols is not predicted for peptides of such short length and large proportion of aliphatic residues. However, these peptides contain a large proportion of ααAAs. Alamethicin, a common example of these peptaibols, consists of 20 residues, 7 of which are α-amino isobutyric acid (Aib). Aib is the prototypical ααAA with its replacement of the αH of alanine with a methyl group (Figure 1).

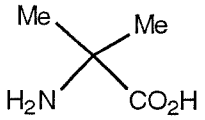


Figure 1. α-aminoisobutyric acid(Aib)

Because the proteinogenic amino acid content of alamethicin did not explain its observed helical conformation, the Aib residues were concluded to be responsible for helicity. It has been found that the 2° structure-defining torsion angles ψ and ϕ about the C^{α}-C′ and N^{α}-C^{α} bond, respectively, are restricted by the substitution of the C^{α} hydrogen of the ααAAs with at least one methyl group (Figure 2).

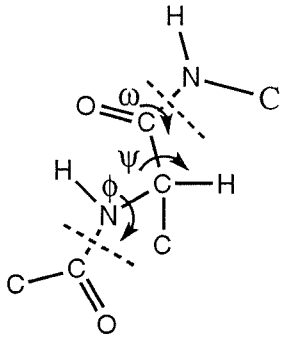


Figure 2. Critical angles of a peptide chain.

The constrained angle results in the observed tendency for α -helix or 3_{10}-helix formation. Because the helix-promoting character of ααAAs is a local effect, the greater the number of ααAAs, the greater the helix-promoting effect. A greater proportion of ααAAs favors 3_{10}-helix formation over α -helix formation. And in addition to helix-promotion, ααAA confer proteolytic stability to the peptide.[2]

Briefly, the α -helix consists of a pattern of hydrogen bonding between the α-amino group of the *i*th amino acid and the carbonyl oxygen of the $(i + 4)$th residue.[3] In one complete turn of the helix, there are 3.6 amino acid residues.[4] The 3_{10}-helix is characterized by i, $i + 3$ hydrogen bonding and has 3 residues per turn of the helix.[3,4] With the positioning of their side chains, the α and 3_{10}-helix conformations serve as ideal molecular design tools.[5] One benefit of such design is that the partial substitution of ααAAs for naturally occurring amino acids in a series of short, amphipathic peptides has been shown to increase both *in vivo* and *in vitro* bioactivity of the peptide.[1] In addition, these short, amphipathic peptides have been used as a tool in probing the α -helix to 3_{10}-helix equilibrium. This was accomplished in one case by designing the peptide such that 3_{10}-helix conformation was significantly more amphipathic than the α -helix conformation. By increasing organic content of solvent, the 3_{10}-helix conformation of the peptide was favored over the α -helix. It was also found that increasing the proportion of ααAAs favors the 3_{10}-helix conformation over the α -helix conformation.[3]

Until recently, such studies on helical peptides have been done using only hydrophobic ααAAs . While amphipathic, helical peptides could be designed using polar proteinogenic amino acids that were not αα-disubstituted, their study was limited to organic solution due to the high proportion of these hydrophobic ααAAs necessary to induce helix formation. The recent synthesis of a novel protected polar ααAA, N^{α}-Fmoc-N^{γ}-Boc-4-amino piperidine-4-carboxylic acid(Fmoc-Api(Boc)-OH) (Figure 3), and its subsequent incorporation into a short peptide allowed for the high proportion of ααAAs necessary for significant helical formation while also conferring water solubility on the peptide.[5]

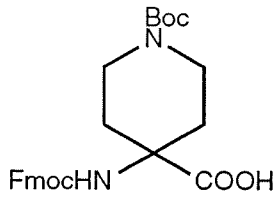


Figure 3. 1-Boc-piperidine-4-Fmoc-amino-4-carboxylic acid [Fmoc-Api(Boc)-OH]

In essence, polar αα AAs like Api play a dual role: the promotion of helix formation and the endowment of water solubility on the peptide. These two qualities now opened the door for the study of the biological activity of these water soluble, amphipathic, helical peptides. Thus far, studies have been performed on short, amphipathic peptides containing Api to learn about solvent effects on the peptides.[3] In addition, studies on the anti-microbial effects of these amphipathic peptides indicate significant biological activity.[1]

Expanding on this idea, we sought to protect the side chain of Api to allow for controlled reaction at the Api side chains after elongation of the peptide but before cleavage of the peptide from the resin. Api was previously protected with a scheme that consisted of deprotection of the α-amino group with a base-labile protecting group while the side-chain protecting groups *and* the linkage to the resin were base-labile.[5] What was needed was an orthogonal system of protecting groups; that is, " a set of completely independent classes of protecting groups, such that each class of groups can be removed in any order and in the presence of all other classes."[6] In essence, such a protection scheme would allow for cleavage of side chain protecting groups without cleavage from the resin, or vice versa. This would have the advantage of allowing first elongation of the peptide, then deprotection of the side chain groups, followed by reaction at the side chains, and finally cleavage from the resin, all done while avoiding unwanted deprotection at any step of the way. This system offers selective deprotection based on *chemistry* as opposed to kinetics, an alternative mechanism for selective cleavage, and therefore has the added advantage of requiring milder cleavage conditions. Milder conditions means a decrease in the likelihood of unwanted side reactions.[6]

A variety of these orthogonal protection schemes have been used. Acid lability, base lability, and photolysis are among the possible protecting groups.[6] Recently, the use of the veteran allyloxycarbonyl groups has become ideal with the development of π-allyl palladium chemistry. The allyloxycarbonyl (Alloc) group can be cleaved under mild conditions with Pd(0) and is highly acid and base stable.[8]

The goal of this project was therefore to first protect the α-amino group (endocyclic nitrogen of Api) with the Alloc group, then incorporate Api(Alloc) into a 10-residue peptide similar to previously studied amphipathic peptides, and cleave the Alloc group with Pd(0) in preparation for controlled modification of Api side chains on the resin.

Theory

A protecting or blocking group is used to prevent attack on certain functional groups while still allowing for reaction of unprotected functional groups. In the case of Api (Figure 4), three sites are open for reaction during peptide synthesis: the carboxy acid, the α-amino group, and the secondary γ-amine on the piperidine ring.

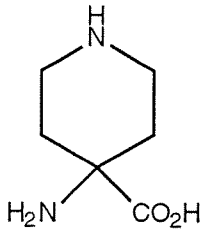


Figure 4. 4-amino-piperidine-4-carboxylic acid(Api)

Since the peptide synthesis planned will build the peptide from the carboxy terminus to the amino terminus, protection of the two nitrogen groups is necessary such that cleavage of the α-amino blocking group does not cause cleavage of the peptide from the resin and/or cleavage of the γN blocking group. The connection of the peptide to the resin peptide amide linker (PAL) is acid labile (Figure 5).

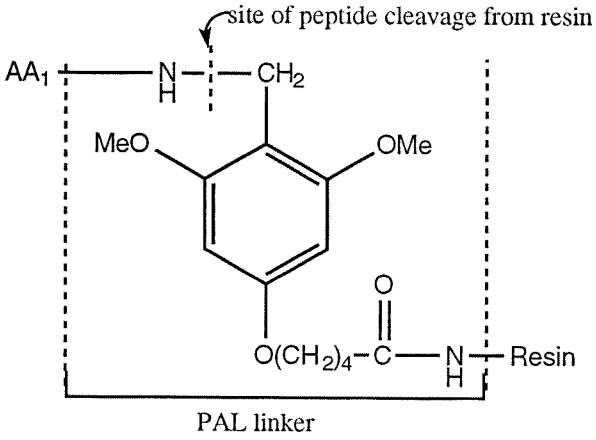
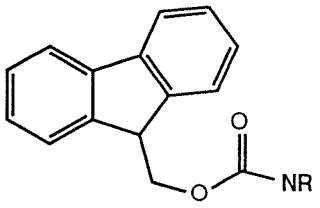
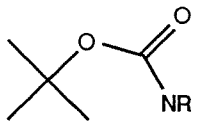


Figure 5.

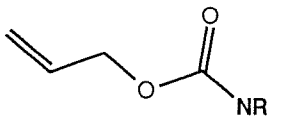
For this reason, a base-labile protecting group, 9-flourenylmethoxycarbonyl (Fmoc) is used to protect the α-amino functionality (Figure 6). In the past, an acid-labile protecting group, *tert*-butyloxycarbonyl (Boc), has been used to protect the γN of Api (Figure 6).[5] While this has been successful in protecting the side-chain group from attack during synthesis, it does not allow for orthogonal synthesis on the resin because cleavage of the side-chain protecting group also cleaves the peptide from the resin. Allyloxycarbonyl groups have been used in the protection of side-chains and have been shown to be stable to both acid and base cleavage (Figure 6).[7]



9-fluorenylmethoxycarbonyl (Fmoc) protected amine



tert-butyloxycarbonyl (Boc) protected amine



allyloxycarbonyl (Alloc) protected amine

Figure 6. Some amino protecting groups.

π-Palladium chemistry has provided a nice mechanism for deprotection of the Alloc group using Pd(0). This mechanism involves the formation of a π-allyl palladium complex that reacts further with a nucleophile to free the protected group and regenerate the catalyst (Figure 7).

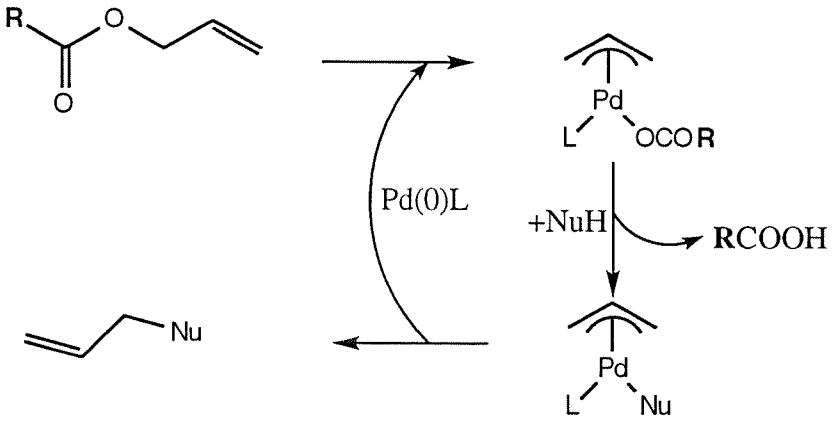
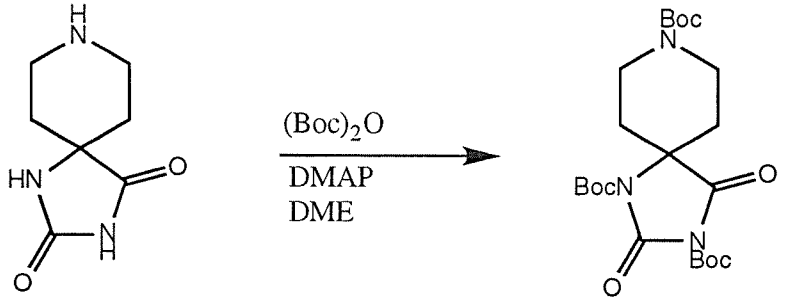


Figure 7. Mechanism of Alloc cleavage via Pd(0) catalysis.[9]

Synthesis of Api begins with the protection of the three nitrogen groups of the piperidine-4-spiro-5′-hydantoin (piphydantoin) with Boc groups yielding 1-Boc-piperidine-4-spiro-5'-(1',3'-bis-Boc)-hydantoin (Tribocpiphydantoin) (Figure 8).



piperidine-4-spiro-5′-hydantoin (piphydantoin)

1-Boc-piperidine-4-spiro-5′-(1′,3′-bis-Boc)-hydantoin (Tribocpiphydantoin)

Figure 8.

DMAP (dimethylaminopyridine) functions as an acylation catalyst here, reacting with the $(Boc)_2O$ to yield the activated pyridinium salt (Figure 9).

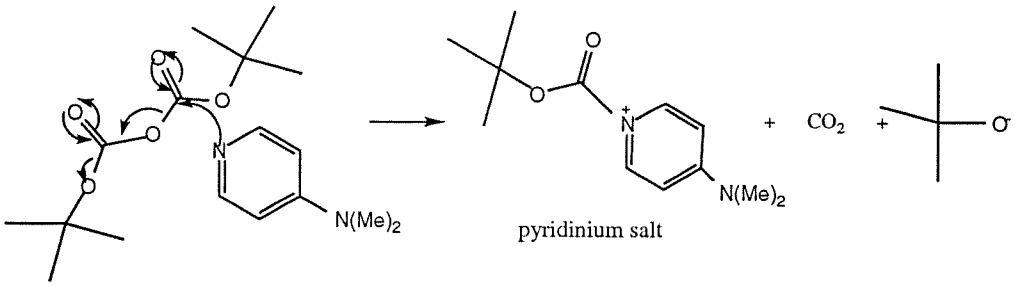
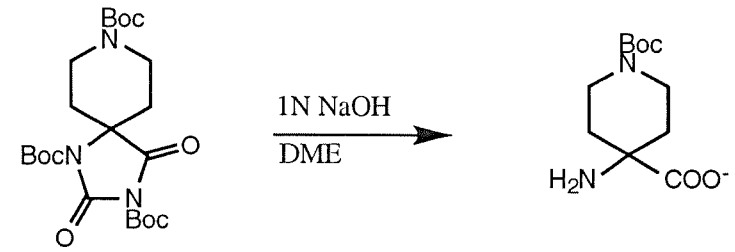


Figure 9.

The carbonyl group, now with a prime leaving group, is more susceptible to attack by the nitrogen groups of the hydantoin. Most likely, the piperidine amine nitrogen, N1, is the most nucleophilic and therefore attacks first, followed by N1′ and finally N3′. This is to be expected as N3′ sits between two carbonyl groups that serve to disperse negative charge, making this nitrogen more acidic than the other nitrogens. N1′ has its negative charge stabilized by one carbonyl group, making it more basic than N3′. 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 a driving force in this reaction and also provides a means of monitoring the reaction's progress.

The reaction's progress is also monitored via normal phase thin-layer chromatography. In a solvent system of 20:1 DCM to AcOH, the product should be expected to travel farther than the starting material due to the three large, non-polar Boc groups it bears. Development of the TLC plates in a concentrated HCl chamber serves to cleave the Boc groups from the product and subsequent application of ninhydrin stain in ethanol allows for detection of both the position of the product and the starting material. Ninhydrin complexes with free amino groups and therefore the deblocking of the Boc groups after running the TLC plates allows the ninhydrin to complex with the product as well as the free amines of the starting material. Comparison of R_f values and proton NMR spectra with that previously obtained by provides verification of product formation.[5]

The next step in the procedure is to make Api(Boc)-OH. Cleavage of the tribochydantoin is prerequisite to attaching an Fmoc group to the amino nitrogen (Figure 10).

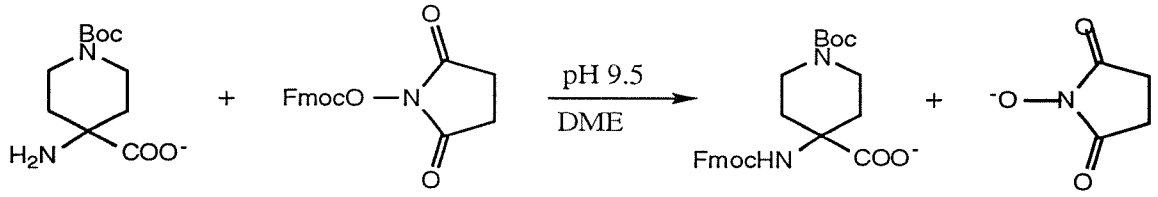


Tribocpiphydatoin Api(Boc)-OH

Figure 10.

The mechanism of cleavage for this reaction is unclear. However, cleavage of the hydantoin provides a convenient method of producing the amino acid and removing the 1′ and 3′ Boc groups while leaving the N1 Boc group in place.[5] The N1 Boc group protects the amino nitrogen from blocking by Fmoc. What can also be expected from this reaction is the quantitative formation of the imide $(Boc)_2NH$ as well as small amounts of the amide, $(Boc)NH_2$. TLC comparison of starting material, standard $(Boc)_2NH$ and $(Boc)NH_2$, and the reaction mixture with ninhydrin staining should reveal the progress of the reaction and also point out the presence of the imide and amide impurities. Again, the HCl treatment followed by ninhydrin staining will allow for a comparison of starting material and reaction mixture. The Triboc starting material is expected to travel farther than the product, again due to the bulky non-polar *tert*-butyl groups in contrast to the negatively charged mono-Boc amino acid product which will tend to adhere to the plate.

Evaporation *in vacuo* of solvent followed by extraction with ethyl ether to remove impurities prepare the product for the reaction in Figure 11:



Api(Boc)-OH Fmoc-OSu Fmoc-Api(Boc)-OH

Figure 11.

This reaction is performed at pH 9.5 to ensure an unprotonated α-amino group that is therefore able to attack the carbonyl group of the Fmoc-OSu, releasing the N-hydroxysuccinimide anion.

Although the carboxylate functionality bears a negative charge, the dispersion of the charge through the carbonyl makes this group less basic than the α-amino group of the amino acid and therefore favors attack of Fmoc-OSu by the α-amino group. If the pH is too low, the α-amine is protonated before the carboxylate, allowing the carboxylate to attack the Fmoc-OSu and form a reactive mixed anhydride that could promote coupling. This could result in the unwanted dipeptide and tri-peptide formation. Maintaining a pH of 9.5 is an attempt to minimize this possible competing reaction (Figure 12).

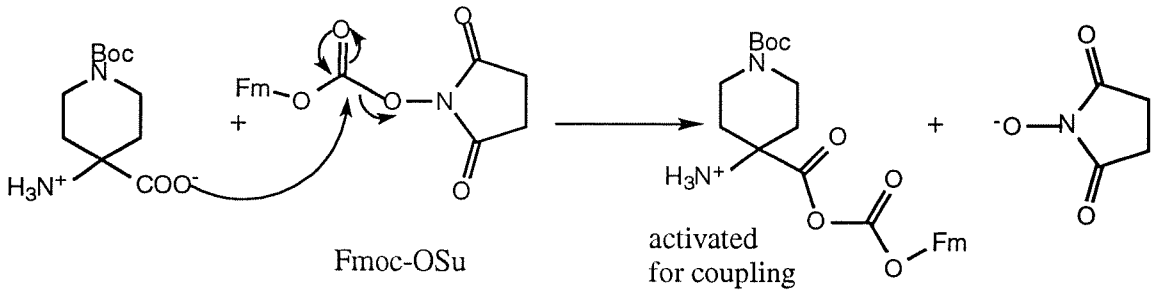


Figure 12.

An added measure taken to prevent this competing reaction is the dropwise addition of Fmoc-OSu to the reaction flask. If the pH is eleven or greater, cleavage of the Fmoc group from the amino acid may occur and the starting material is not protected.

TLC can be used to follow this reaction and uv light used to detect any Fmoc present on the plate. Because Fmoc is a highly aromatic system, it absorbs light at about 300nm. Since possible species present on the TLC plate are starting material, target product, Fmoc-OSu, and $^{-}$OSu, ninhydrin is also useful to detect any starting material still present in the reaction. Comparison with known standards of the product as well as Fmoc-OSu standard will also help to identify spots on TLC.

The solvent in this reaction is easily removed *in vacuo*. Extraction with ethyl ether should remove any unreacted Fmoc-OSu, leaving the negatively charged product and any $^{-}$OSu in the aqueous layer. Adjusting the pH of the aqueous layer to between 2 and 3 protonates these negatively charged species, drawing the product into EtOAc while leaving the HOSu in the aqueous layer.

The next step is replacement of the Boc protecting group on the γN with the Alloc group. First, the Boc group is cleaved (Figure 13).

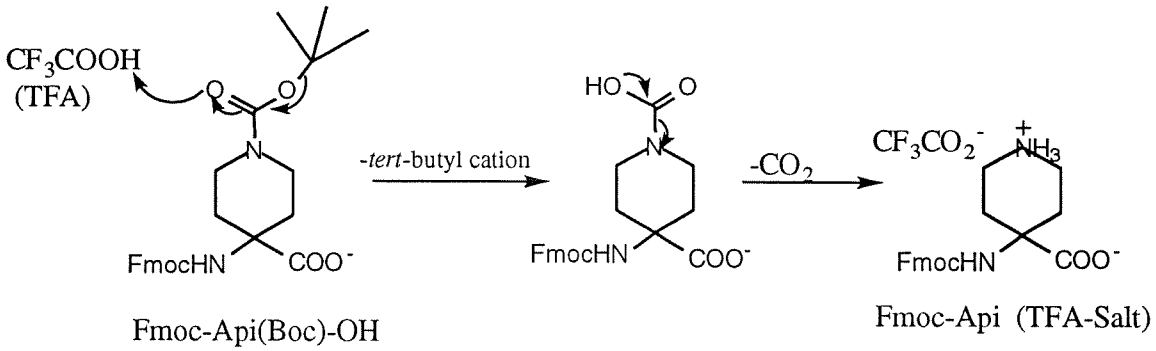


Figure 13.

TFA protonates the carbonyl oxygen, leading to release of the *tert*-butyl cation and CO_2 leaving the amino acid-TFA salt. The release of CO_2 is again a driving force in this reaction. H_2O could theoretically be used as a scavenger for the *tert*-butyl cation. E1 elimination of the cation gives 2-methylbutene which prevents back reaction (Figure 14).

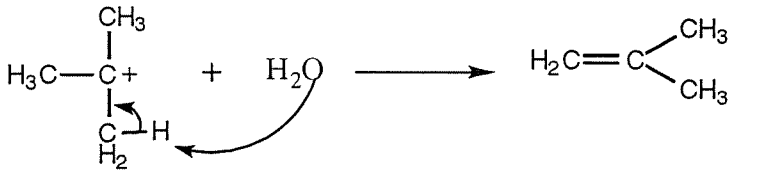
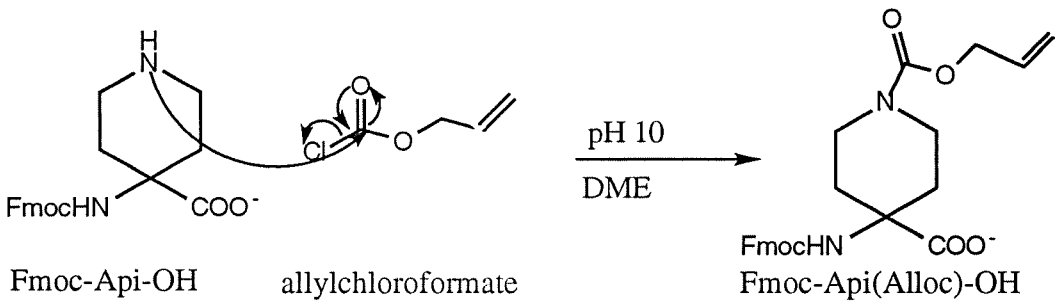


Figure 14.

TFA specifically is chosen as the acid as opposed to HCl, for instance, because it is relatively volatile. When neutralization is necessary in the next step, the TFA in solution will be far more easily removed than water.

The reaction can be monitored by TLC and spots detected by uv and ninhydrin. Because the nitrogen is protonated, the product should stay nearer to the baseline than the starting material.

The bridge to the next step is removal of solvent *in vacuo* followed by the reaction in Figure 15.



Fmoc-Api-OH allylchloroformate Fmoc-Api(Alloc)-OH

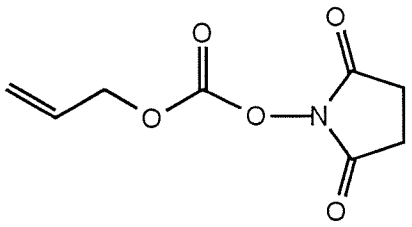
Figure 15.

A pH of 10 is required in order to deprotonate N1, allowing this nitrogen to attack the carbonyl of the allyl chloroformate in a displacement reaction. This reaction is done on ice and the allyl chloroformate is added dropwise to minimize the high reactivity of the allyl chloroformate. Allyl chloroflormate, like FmocOSu, has the potential to form a mixed anhydride at the carboxylic acid end of the amino acid and in turn promote coupling. In the case of the chloroformate, the possibility of this undesired reaction is even greater due to the nature of the leaving group: the chloride ion is a better leaving group than ⁻OSu.

Ethyl ether is used first to extract any hydrophobic organic impurities in the reaction mixture such as remaining allyl chloroformate. Lowering the pH of the reaction mixture allows the product to be protonated and no longer negatively charged. It can then be pulled from the aqueous layer into EtOAc. The successive rinses with HCl and brine serve to remove any water and salts remaining in the organic layer. Before analysis via ^{1}HNMR, the mixture must be dried extremely well to remove any water or solvent. Api protected with both Fmoc and Alloc has not been made before so no spectral data exists with which to directly compare. However, comparison can be made with similar spectral data such as Api, keeping in mind the replacement of the Boc group with the Alloc group. The Alloc group is expected to give a characteristic multiplet which should be relatively downfield due to the electron withdrawing nature of the oxycarbonyl group and the double bond.

A second method of forming the Fmoc-Api(Alloc)-OH uses Alloc-OSu (Figure 16) instead of allyl chloroformate. This method was explored because of the less reactive nature of the former reagent. As previously stated, ⁻OSu is not as ready of a leaving group and the chloride

ion, decreasing the possibility of competing reactions. However, to our knowledge, Alloc-OSu is not available commercially so its synthesis was required.

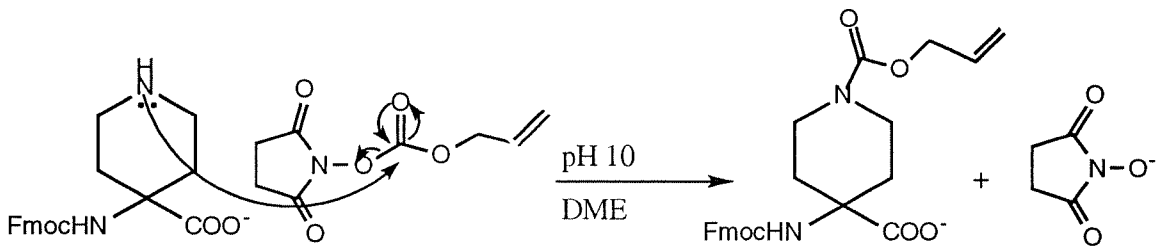


Allocsuccinimide(Alloc-OSu)

Figure 16.

The Et_3N serves as a base to remove the proton from the -OH group on the N-hydroxysuccinimide. The N-hydroxysuccinimide anion can now attack the allyl chloroformate, displacing the chloride and forming the N-allocsuccinimide. The triethylammonium chloride should precipitate out and can be removed easily via suction filtration. Once the solvent is evaporated off, the remaining product is dissolved in EtoAc and washed with dH_2O followed by saturated $KHSO_4$ solution to remove remaining triethylammonium chloride. After evaporation of solvent and high vacuum drying, the product can be analyzed via NMR and its identity verified by comparison of NMR with that previously published[8].

The formation of the Fmoc-Api(Alloc)-OH using Alloc-OSu follows the same procedure as that of the allyl chloroformate (Figure 17).



Fmoc-Api-OH Allocsuccinimide(Alloc-OSu) Fmoc-Api(Alloc)-OH

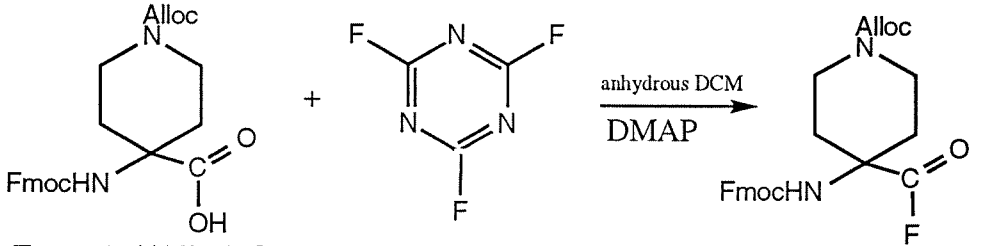
Figure 17.

The -OSu moeity serves as a good leaving group and is displaced by N1 of the amino acid.

Finally, the diprotected amino acid must be prepared for peptide synthesis. In the past, automated peptide synthesis with Api has been difficult and is more effective when the first three

peptides in the sequence are manually coupled.[5] The reason for this has not been determined but it is suggested that the steric bulk associated with ααAA decreases accesibility of the N-terminus for deblocking by base. Manually coupling the first three residues allows for heating of the reaction at reflux for as long as necessary to achieve adequate coupling.

There are a variety of methods of activation for coupling, one of which is through acid fluoride activation of the carboxyl end of the amino acid. An advantage of this method of activation is that the acid fluoride can be formed without cleavage of protecting groups and efficiently promotes coupling. Acid chlorides are also effective methods of activation but their formation results in the cleavage of any acid-labile protecting groups.[2] Other traditional methods of coupling such as DCC are inefficient.[2] The acid fluoride method of activation, which involves a smaller, more activating nucleophile, is used here as a uniformly applicable and efficient mechanism safeguarding against cleavage of any acid-labile protecting groups in the residues we choose for the peptide. The acid fluoride of Fmoc-Api(Alloc)-OH was prepared using cyanuric fluoride and DMAP in the reaction seen in Figure 18.



Fmoc-Api(Alloc)-OH cyanuric fluoride Fmoc-Api(Alloc)-F

Figure 18.

The fluoride serves as a much better leaving group than the hydroxide group and therefore activates the coupling of the amino acid to the resin.

The resin used in our coupling procedure was the PAL-PEG-PS resin (Figure 19), with PAL serving as an amide linker to the growing peptide. An amide linker is one such that when the peptide is cleaved from the resin, an amide is produced at the C-terminus instead of a carboxy acid.

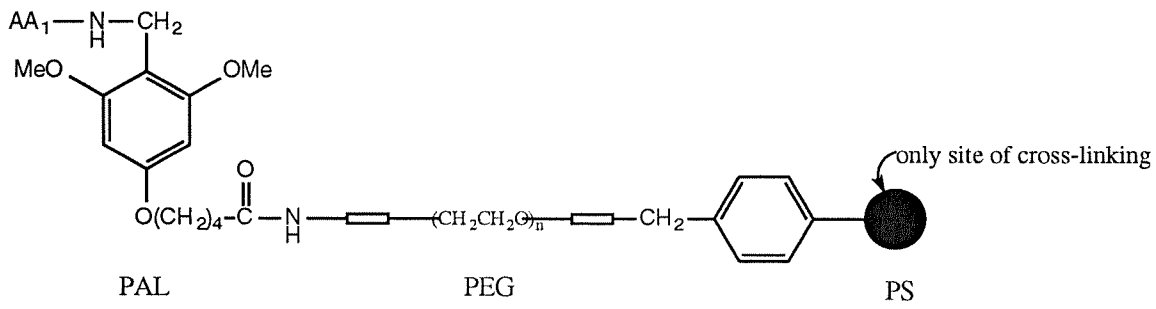


Figure 19.

The PS (polystyrene) is the only cross-linked part of the resin. The PEG serves to "solubilize" the resin: that is, PEG (polyethylene glycol) makes it easier for solvent to interact as completely as possible with the resin, minimizing diffusion limitations. The amino group of the linker is initially protected with Fmoc groups. The process of coupling then would involve the deblocking of the resin with base, followed by addition of the first amino acid under reaction conditions. In order to be able to assess coupling success, an Fmoc analysis procedure is performed. Before coupling, a small resin sample of weight between 4-8 mg is taken and deblocked using piperidine dissolved in DMF. The DMF serves to swell the resin, making its blocking groups susceptible to attack by the piperidine (Figure 20).

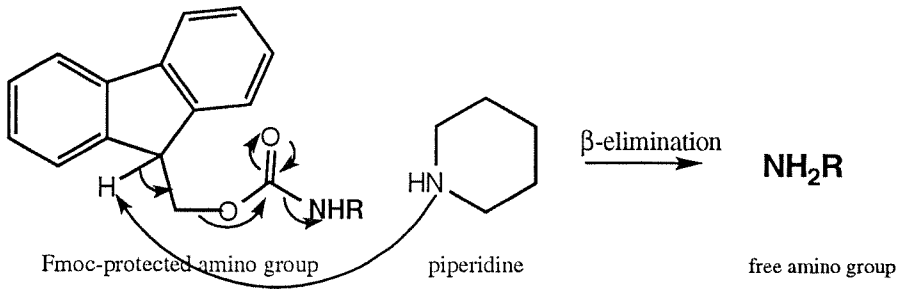


Figure 20.

After deblocking, the resin-deblock solution can be diluted in methanol to a known volume and the absorbance of the deblock solution at 300nm measured. This serves to detect any free Fmoc groups removed from the resin. A blank consisting of piperidine in DMF is also measured and subtracted from the former value. Using the Beer-Lambert Law, the concentration of Fmoc in mmol Fmoc/g of resin could be determined.

$$\text{mmol Fmoc/g resin} = \frac{(A_{300})(\text{total vol. diluted solution, L})(10^6\ \mu\text{g/ml})}{(\varepsilon_{300}\text{cm}^{-1}\text{m}^{-1})(\text{path length, cm})(\text{weight of sample, mg})}$$

A sample of between 4-8 mg of resin in a total volume of .007 L is used to ensure a concentration of analyte within the linear range of the Beer-Lambert plot. If the concentration of Fmoc removed from the resin is within the range specified by the manufacturer, the calculated concentration is then taken as the loading capacity and used to analyze degree of coupling to the resin. The principle is that those sites on the resin bearing the newly coupled amino acid will also bear the Fmoc groups of the α-amino nitrogen. Those sites that have not coupled will simply be free amino groups. A small sample of resin is removed from the coupling reaction, dried, rinsed well first with DMF to swell the resin to remove any coupling solution caught between its particles, then MeOH to shrink the resin, then DCM to swell the resin, and finally deblocked with a solution of DBU in piperidine and DMF and dilution in MeOH for spectrophotometric analysis. The percent coupling of amino acid to the resin can be determined by comparing the mmol Fmoc/g resin measuring in this analysis to the loading capacity of the resin.

$$\%\ \text{coupling} = \frac{(\text{mmol Fmoc/g resin cleaved from coupling reaction})}{\text{loading capacity (total mmol Fmoc/g resin possible)}}$$

DBU is used to deblock the α-amino group of the coupled residue for further coupling because, as mentioned, ααAA are sterically hindered due to a replacement of the αH with a bulkier group. It is hoped that by using a stronger base than piperidine, DBU, the cleavage of the Fmoc group will proceed more efficiently.

The 10-residue target peptide sequence, shown below, was selected based on a similar amphipathic peptide synthesized previously, Ipi-10, and that exhibitied significant anti-microbial activity.[5]

Target peptide: H-Api-Aib-Api(Alloc)-Lys-Aib-Aib-Lys-Api(Alloc)-Aib-Api-NH_2

Ipi-10[3]: H-Api-Aib-Aib-Lys-Aib-Aib-Lys-Aib-Aib-Api-NH_2

Results and Discussion

All reactions through the synthesis of Fmoc-Api(Boc)-OH had been performed and reproduced previously in this lab and their data published making their execution and analysis straightforward. Synthesis of the Tribocpiphydantoin gave quantitative yield with an Rf of approximately .48 and a positive test with HCl/ninhydrin. While the positive result from the ninhydrin test verifies the presence of a compound with free amino groups(after treatment with HCl), comparing the Rf value and ^{1}HNMR spectrum with the previously published Rf value and spectral data for Tribocpipaa verified that the product was Tribocpipaa.

In the subsequent cleavage step, TLC after extraction with ether indicated the presence of one spot at an Rf of approximately .4 that gave a positive ninhydrin test after HCl treatment. Comparison with published data confirmed the identity of the cleaved tribocpiphydantoin. A TLC comparing product after extraction with starting material stained with ninhydrin and no HCl treatment indicated showed that only the product gave a positive test. This also verified that the spot was indeed the cleaved tribocpiphydantoin. Yield was assumed quantitative.

Protection of the α-amino group with Fmoc gave a heavy uv positive spot at an Rf of approximately .96, matching that of a previously made standard. However, a second uv positive spot appeared from the reaction mixture at an Rf of approximately .5. All spots gave positive ninhydrin tests after treatment with HCl. It is possible that the extra spot from the reaction mixture is starting material. After extraction, however, only the product spot remained. The yield for this reaction was approximately 82%.

Two hours after starting the cleavage reaction of the Boc protecting group from the endocyclic nitrogen an appreciable amount of product was evident by TLC. Comparison of the reaction mixture with the starting material revealed two spots in the reaction mixture: one at an Rf of approximately .4 and one with the same Rf as the starting material, .85, for this starting material. As expected, the only strongly ninhydrin positive spot was at the lower Rf and in the reaction mixture while all three spots gave positive uv tests. The starting material and the higher spot in the reaction mixture lane both gave very faint ninhydrin positive tests. It is possible that a

small amount of Boc-protected amino acid without Fmoc protection at the α-amino group is left behind from the previous step. This might account for the slight ninhydrin positive test these spots give. In addition, the product spot was a more streaked spot while the spot that travelled further was smaller and more compact. This is perhaps due to the charged endocyclic nitrogen of the piperidine ring of the product as opposed to the uncharged starting material. A quantitative yield was assumed for this reaction as well.

The first of the new syntheses, the synthesis of Fmoc-Api(Alloc)-OH , gave a yield of 69% from Fmoc-Api-OH. The ^{1}HNMR spectral data and MALDI(Matrix-Assisted Laser Desorption Ionization) analysis corroborated that the principle spot from the reaction mixture viewed by TLC was Fmoc-Api(Alloc)-OH. Unfortunately, none of the starting material was saved for comparison via TLC so analysis of product relied on ^{1}HNMR (Figure 23) and MALDI(Figure 24) analysis. TLC comparisons were, however, made between previously made Fmoc-Api(Boc)-OH and the reaction mixture which allowed us to compare the Rf of the product to Fmoc-Api(Boc)-OH. Because Fmoc-Api(Alloc)-OH differs from Fmoc-Api(Boc)-OH by an ethylene group instead of a *tert* -butyl group, the Rf of Api(Alloc) appears to be very close to that of Fmoc-Api(Boc)-OH. In the product lane, before comparison with Api, two principle uv visible spots were seen, one at Rf .45 and the other at Rf .33. However, in the plate run against Fmoc-Api(Boc)-OH, only one of these spots remained, with an Rf of .47 while Api had an Rf of .5. As expected, the TLC of the Fmoc-Api(Alloc)-OH reaction mixture both before and after work-up was cluttered with faint uv positive spots preceding and following the principle spot. One possiblity is that these spots could be dipeptides or tripeptides that formed according to the side reaction discussed earlier. This is suspected to be due to the high reactivity of allyl chloroformate.

^{1}HNMR revealed clearly the presence of Alloc. The most downfield peak was a small peak from the carboxy acid hydrogen. The Fmoc protons were the next furthest downfield probably due to a high level of deshielding from its two aromatic groups. The Alloc group also consists of a carboxylic acid moiety which puts it relatively downfield as well but it is not as far

downfield as the Fmoc protons because it not influenced by a group comparable to the aromatics of the Fmoc. A telltale multiplet from the 2´hydrogen of Alloc is located between 5.8-6.0 ppm (Figure 21).

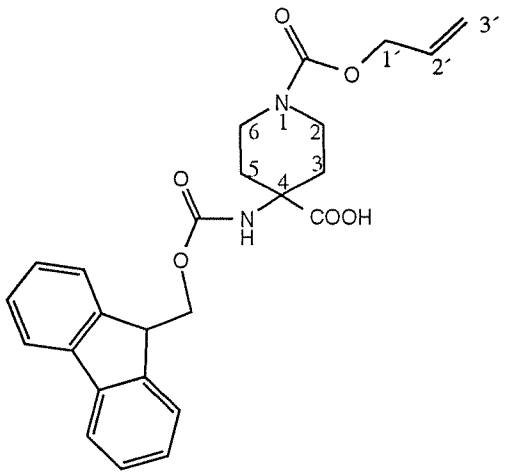


Figure 21. Fmoc-Api(Alloc)-OH

No carbon-13 analysis was performed to help verify peak assignments so no definite assignment is offered here. However, it is suggested that the signal for this hydrogen is split by the 1´ hydrogens and the 3´ hydrogens. The next group of signals are located roughly between 5.1-5.4 ppm. Three single peaks occupy this range, the more downfield of the three resulting from the 1´ hydrogens with the middle peak resulting from the *trans* 3´ hydrogen and the remaining peak resulting from the *cis* 3´ hydrogen.

Mass spectrometric analysis using MALDI (positive ion mode) did indicate the presence of a species of molecular weight 451.33 compared to the calculated molecular weight for Fmoc-Api(Alloc)-OH of which is approximately 449.5. The signal at 473.473 could be due to the MW of Api plus Na^+.

The synthesis of Fmoc-Api(Alloc)-OH using N-Allocsuccinimide was cleaner than when allylchloroformate was used, according to TLC. Synthesis of N-Allocsuccinimide gave a 75% yield and ^{1}HNMR spectral data were compared with that pulished[8] to verify attainment of product. Subsequent allyloxycarboxylation of the deblocked endocyclic nitrogen of Fmoc-Api gave a much cleaner reaction by TLC, shown below.

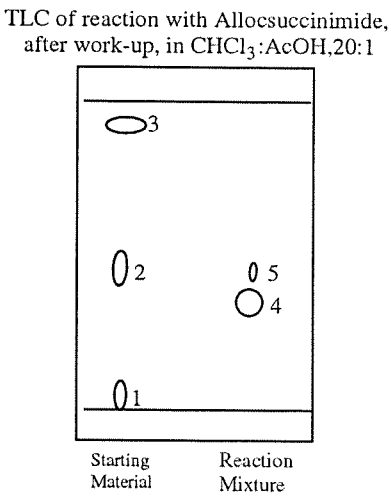


Figure 22.

Instead of seeing several small spots in the TLC of the reaction mixture as with the allyl chloroformate reaction, only two spots remained after work-up and both were uv visible. These spots were the same two principle spots seen in the TLC of the allylchloroformate reaction. Again, when compared with the starting material, the spot that advanced furthest and was half the size of the second spot has an Rf of .44, close to that of spot 2 from the starting material of .44. The second principle spot is slightly behind the other at an Rf of .34. Because the solvent system used to run this TLC is different from that used in the preceding Boc cleavage reaction, the starting material looks different by TLC. Instead of having two spots when the solvent was BuOH:AcOH:H_20, three spots now appeared with $CHCl_3$:H_20 as the solvent. Since spot 1 was the only ninhydrin positive spot, it is possible that it is the starting material. Perhaps spot 2 is due to Api left over from the preceding Boc cleavage reaction or some Fmoc contaminant from that reaction. Spot 3 could also be due to either Fmoc-Api(Boc)-OH or Fmoc contaminant. Neither spot 2 nor spot 3 were ninhydrin positive suggesting that no starting material was present in either spot. Since ^{1}HNMR and FAB analysis suggested the appreciable presence of Fmoc-Api(Alloc)-OH, it is suspected that spot 4, at Rf .34, is the Fmoc-Api(Alloc)-OH because it is the largest, it is uv-positive and not ninhydrin-positive, and its Rf is near that of Fmoc-Api(Boc)-OH in this solvent system, approximately .5. Spot 5, slightly ahead of this at Rf .44, is greatly reduced in size after work-up, indicating that it might have contained some of the same Fmoc

contaminants as spot 2 but perhaps also consisted of some Fmoc-Api(Boc)-OH that may not have been removed during work-up.

In retrospect, it would have been more helpful if the TLC plates were treated with HCl before staining with ninhydrin in order to distinguish between Fmoc-Api(Boc)-OH and Fmoc-Api(Alloc)-OH. Also, consistency with TLC solvents used would have facilitated comparisons. The deblocking of Api before protection with Alloc was monitored by TLC in one solvent and a different solvent was used to compare the product of the cleavage with the product of the protection reaction. The TLC of the deblocked Api looked different under different solvent conditions and because Rf values were calculated in the first solvent system, it would have been helpful to at least see the reaction mixture of the Alloc protection reaction run against the deblocked Api in that same solvent system.

The acid fluoride reaction proceededwith 50.5% yield from Api(Alloc)-OH. ^{1}HNMR (Figure 25) was not so helpful in verifying the success of this reaction because the only difference between the Fmoc-Api(Alloc)-OH and the Fmoc-Api(Alloc)-F spectra is that the carboxylic acid proton is missing. This was indeed true in the ^{1}HNMR taken but ^{19}FNMR (Figure 26) was also done to verify the presence of the product. Two peaks are reported at 98.461ppm and 124.661 ppm. FAB (Fast Atom Bombardment) analysis was performed as well and indicated the presence of Fmoc-Api(Alloc)-F at a MW of 451.1 (Figure 27).

The coupling portion of this project ran into problems. In coupling the first amino acid residue, Api-F, coupling was as high as 60% by the second day coupling but 2 days later had dropped considerably to less than 10%. This coupling was discarded and a second coupling started which rose to a coupling of 50% and then dropped to 35% after 60 hours of coupling. One possible explanation for this is that in the coupling process, the Fmoc protecting groups are somehow being cleaved prematurely. This would mean that Fmoc analysis would reveal a lower degree of coupling than has actually occurred. Hoping that this was the case, at 35% coupling, the acid fluoride of the second residue, Aib-F was coupled. This coupling is currently in process in the lab.

Conclusion

Api, a polar, heterocyclic ααAA, was successfully protected at the γN with Alloc. Although both allyl chloroformate and alloc succinimide gave equal yields of the desired product, alloc succinimide provided a cleaner reaction by TLC and repeating the reaction with Alloc-ONSu may provide better yields in the future. No determination has yet been made regarding the effectiveness of Fmoc-Api(Alloc)-OH in allowing orthogonal synthesis on the peptide.

It is hoped that with the protected ααAA Fmoc-Api(Alloc)-OH, synthesis can be done at chosen positions of a peptide while on the resin. This would allow for the attachment of intercalators or cylization of peptides. Ultimately, DNA-binding studies and studies of the anti-microbial effects of these peptides may offer alternative treatment for diseases that are becoming more and more threatening with the rise of antibiotic resistant bacteria. While the studies performed in this project may seem far removed from this goal, they have taken one more step in the on-going battle against disease.

Experimental

1-Boc-piperidine-4-spiro-5´-(1´,3´-bis-Boc)-hydantoin. $(Boc)_2O$ (293 g, 1342 mmol) was first added to a stirring suspension of piperidine-4-spiro-5-hydantoin (65.88 g) in ethylene glycol dimethylether (DME) (1L) followed by DMAP (dimethylaminopyridine) (4.81 g, 39.4 mmol). The reaction flask was fitted with an oil bubbler and allowed to react for 5 days. CO_2 evolution was initially vigorous (1 bubble/10 sec.). TLC used to track reaction (20 $CHCl_3$: 1 AcOH, treatment with conc. HCl, followed by ninhydrin staining and heat to develop). The reaction mixture was filtered *in vacuo* to yield a creamy white solid (183.06 g, quantitative). ^{1}HNMR verified by comparison with published results.[5]

1-Boc-piperidine-4-Fmoc-amino-4-carboxylic acid. 1N NaOH (1.5L, 1.5 mol) was added to tri-boc-hydantoin (1.17 g, 173 mmol) suspended in DME (500 ml) and the reaction mixture was allowed to stir under N2, tracking the reaction by TLC (8 BuOH:1 AcOH:1 H_20, conc. HCl treatment, then ninhydrin in EtOH then heat to develop). After 8 days, DME was removed from the reaction mixture *in vacuo* and the remaining reaction mixture extracted with ethyl ether (3X). After adjusting pH of aqueous layer to 9.5 with HCl, a 250 ml flask of Fmoc-OSu (53.5 g, 160 mmol) filled to volume with DME was added to the reaction mixture and allowed to stir overnight. A white precipitate formed after storage in the refrigerator overnight. DMF was removed *in vacuo* and the aqueous layer remaining was extracted with ethyl ether (4X) to remove unreacted Fmoc-OSu. The aqueous fraction was adjusted to pH 2-3 using conc. HCl, extracted with ethyl acetate (5X), and the ethyl acetate removed *in vacuo* from organic layer to yield dark off-white powder (40.0 g, 82%). ^{1}HNMR verified by comparison with published results.[5]

(3)N-allyloxysuccinimide. Hydroxysuccinimide (Aldrich, 20 g, 174 mmol), Et3N (24.2 ml, 174 mmol), and acetonitrile (175 ml) were combined and maintained at 0°C. To this

mixture, allyl chloroformate (Aldrich, 18.4 ml, 174 mmol) suspended in acetonitrile (175 ml) was added dropwise over 15 minutes forming a white precipitate almost immediately. The reaction mixture was allowed to stir for 15 additional minutes, mantaining 0°C. White precipitate was removed by suction filtration and filtrate concentrated to yield an oil. Dissolved product in EtOAc and estracted with dH20 followed by $KHSO_4$ solution. Any solvent was removed *in vacuo* leaving a clear viscous liquid that was refrigerated for further use. ^{1}HNMR verified by comparison with published results.[8]

(4)1-Alloc-piperidine-4-Fmoc-amino-4-carboxylic acid. Fmoc-Api(Boc)-OH (3.92 g, 9.54 mmol) was suspended in $CHCl_3$ (15 ml) and allowed to stir overnight, monitoring by TLC (8 BuOH:1 AcOH:1 H2O, ninhydrin in EtOH and uv). Removed solvent *in vacuo*, dissolved dried product in distilled H_20 (20 ml) and DME (20 ml), and adjusted pH to 10 with conc. NH_4OH. Allyl chloroformate (1.55 g, 12.88 mmol) dissolved in DME (20 ml) was added dropwise over fifteen minutes. Monitored reaction by TLC (8BuOH:1AcOH:1H20, uv, ninhydrin/EtOH) and maintained reaction at pH 10 with 3N NaOH. After about 20 minutes, added a pipette squirt of allylchloroformate to reaction, continuing to monitor pH. Reaction was complete after 30 minutes, when pH no longer dropped. Extracted with ethyl ether (4X), lowered pH to 1.5 with conc. HCl, extracted with ethyl acetate (4X), rinsed with 1N Hcl (1X), rinsed with brine (2X), dried with $NaSO_4$, filtered and dried *in vacuo* for 3 days (2.94 g, 69%). Product was cream colored powder. ^{1}HNMR: see Figure 23.
MALDI-MS(M + H^+: m/z 451.33, Figure 24.

via Allocsuccinimide: Deblocked Fmoc-Api(Boc)-OH (2.6 g, 5.6 mmol) as above to yield Fmoc-Api (2.0 g, 4.97 mmol). Instead of suspending allyl chloroformate in DCM as above, Alloc-ONSu (1.19 g, 5.96 mmol) was suspended in DME (15 ml) and added dropwise over 15 minutes to a solution of Fmoc-Api in DME (35 ml) and distilled H_20 (11 ml), maintaining at 0°C. Maintained pH at 10 with 3N NaOH and monitored reaction with TLC

(8BuOH:1AcOH:1H20, uv, ninhydrin/EtOH). Reaction was complete after 30 minutes and work-up was as in the allyl chloroformate procedure. Product was a cream-colored powder (1.54g, 69%). ^{1}HNMR: see Figure 23.

1-Alloc-piperidine-4-Fmoc-amino-4-carboxylic acid fluoride.

Fmoc-Api(Alloc)-OH (2.94 g, 6.5 mmol) made using allyl chloroformate was dissolved in anhydrous DCM (20 ml). Pyridine (.579 ml, 7.15 mmol) was then added under nitrogen and finally cyanuric fluoride (.9 ml, 10.7 mmol) and the reaction allowed to stir overnight, maintained under nitrogen. Reaction mixture was then mixed with ice water and the organic layer removed. The aqueous layer was extracted with DCM followed by washing with H_2O then drying with Na_2SO_4. Any remaining solvent was removed *in vacuo* to leave an orange powder (1.48 g, 50.5%). ^{1}HNMR: see Figure 25.

^{19}FNMR: see Figure 26.

FAB: 451.1, Figure 27.

Peptide Synthesis. Solid-phase peptide synthesis was performed on Fmoc-PAL-PEG-PS (PerSeptive Biosystems, 0.15 mmol/g loading). Resin (.21 g) deblocked with 20% piperidine/DMF, first for 1 minute, then for 10 minutes, then dried and rinsed with DMF followed by DCE. To rinsed resin was added DCE (2 ml), H-Api-F (.15 g, .16 M), and DIEA (.11 ml), and flask was heated at reflux for several days. Subsequent couplings used 2%DBU in 20% piperidine/DMF. Percent coupling of Fmoc-Api-F was 35% after 5 days. Percent coupling of second residue coupled, Fmoc-Aib-F, was less than 10% after three days.

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Figure 23. ^{1}HNMR of Fmoc-Api(Alloc)-OH

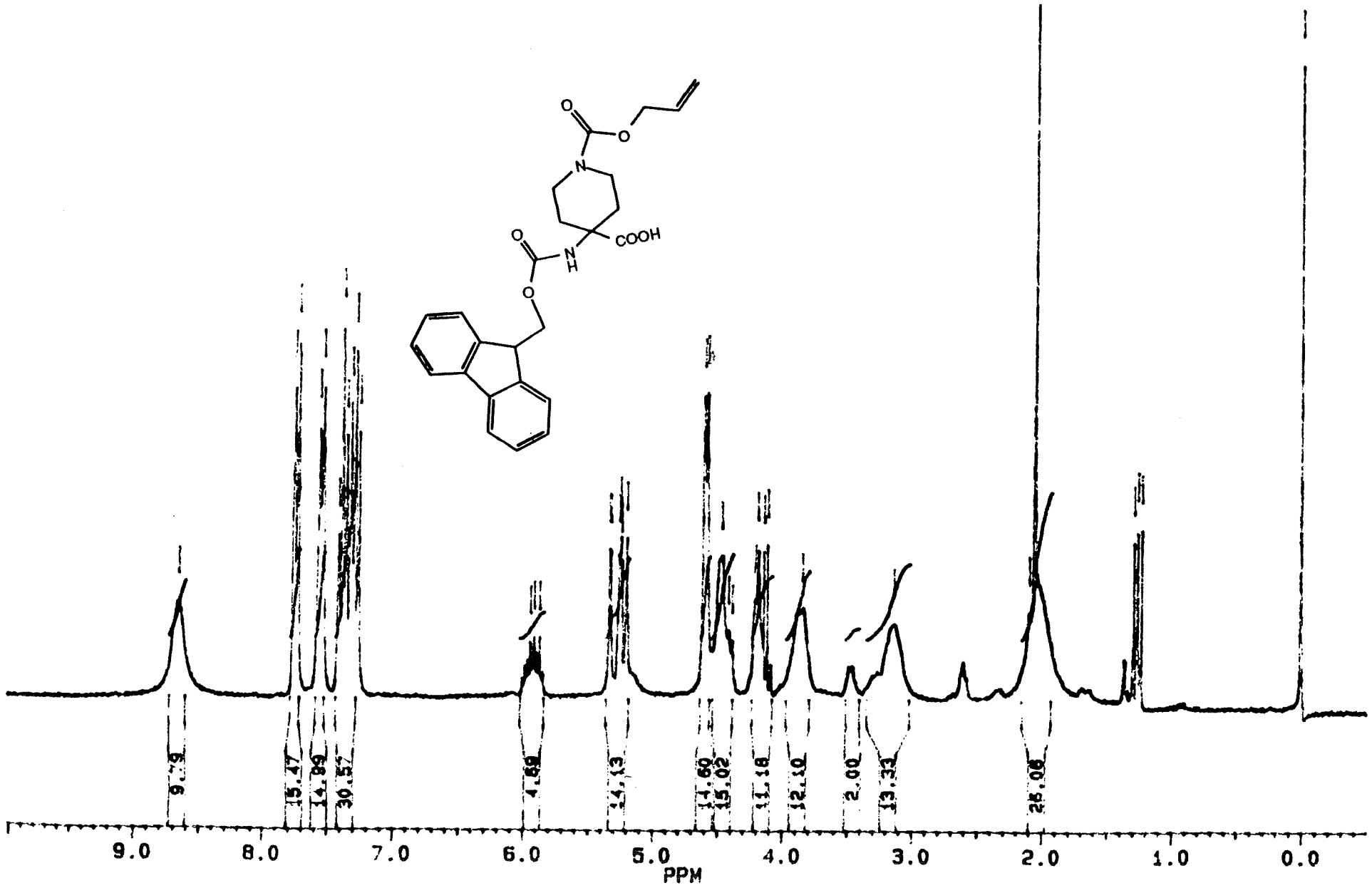

Figure 24. MALDI-MS(M + H^+) of Fmoc-Api(Alloc)-OH

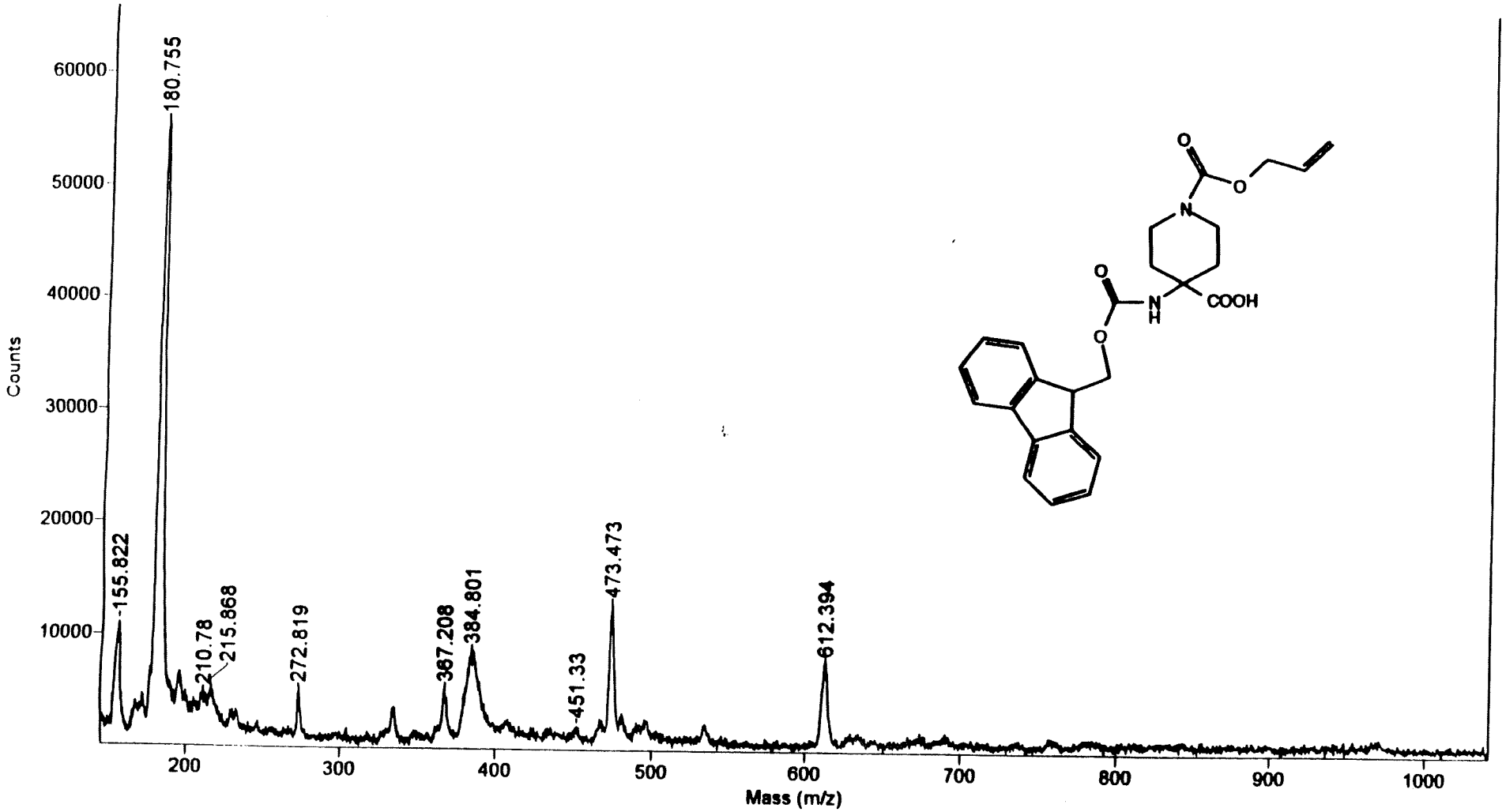

Figure 25. ^{1}HNMR of Fmoc-Api(Alloc)-F

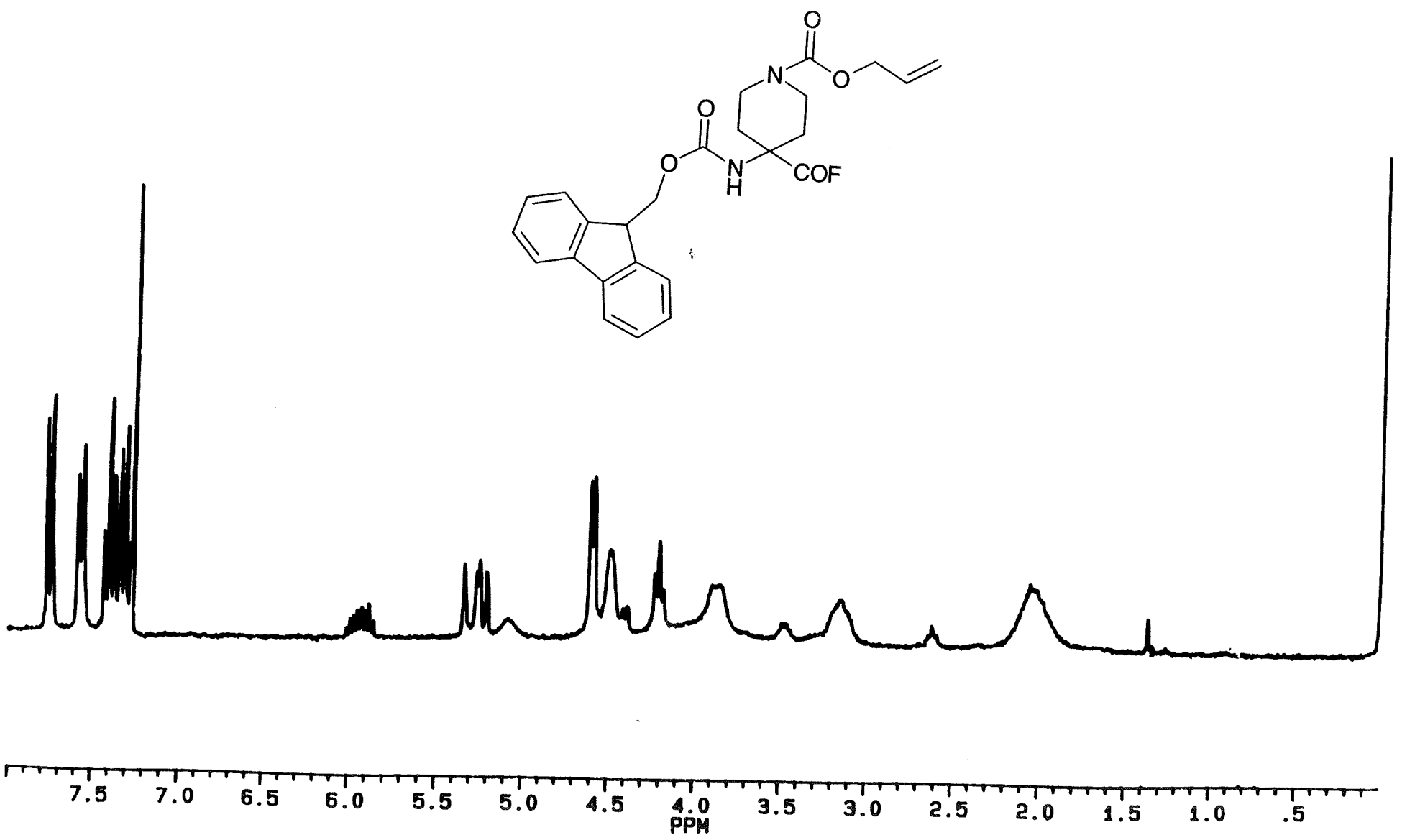

Figure 26. ^{19}FNMR of Fmoc-Api(Alloc)-F

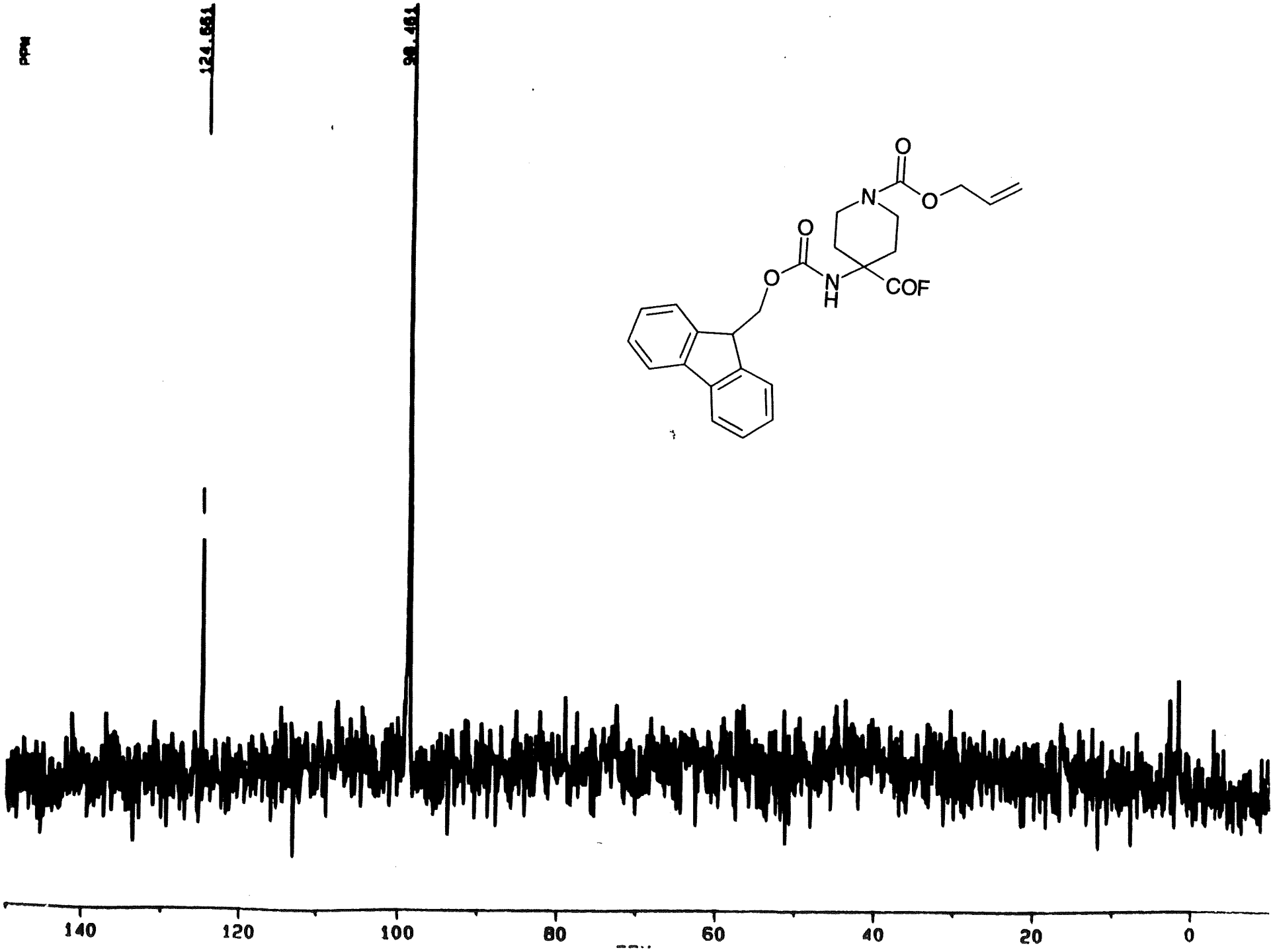

Figure 27. FAB of Fmoc-Api(Alloc)-F