Design and Synthesis of Belactosin A Analogs

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Abstract

Belactosin A is a naturally occurring proteasome inhibitor with potent anti-tumor activity. Proteasome inhibition is a fairly new process for the development of anti-tumor drugs. Proteasome inhibition is conferred by enzymatic acylation of a serine trap, while the variability of belactosin A's peptide-like structure may improve its activity. Several syntheses have been reported, however only a few analogs have been prepared. An approach to the synthesis of analogs is reported here, with the key step being the nitrocyclopropanation of an amino-acid derived enone. This efficient approach leads to nitrocyclopropyl peptidomimetics in three steps. The use of alanine, phenylalanine, and leucine as starting amino acids leads to methyl, benzyl, and isobutyl analogs of the natural product. Reduction of the nitro group, followed by coupling to a serine trap affords belactosin A analogs. This research has led to the successful synthesis of Fmoc-protected alanine and phenylalanine nitrocyclopropyl alcohol analogs. Future work includes synthesis of a serine trap, reduction of the nitro group, and coupling of the two moieties.

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1 Introduction

1.1 Proteasomes

Radiation and chemotherapy are established ways to manage and treat cancers. However, the accompanying side effects, possible resurgence, or total ineffectiveness remain as vital obstacles for cancer patients. To overcome these obstacles in cancers, the traditional ways of destroying all mitotically active cells are being replaced by targeting specific molecules. One such molecule that plays an essential role in cancer therapy is a proteasome. Proteasomes are large, multi-catalytic, multi-subunit proteases located in the nucleus and cytosol of nearly all cells.[1] They are responsible for maintaining the intracellular concentration of a variety of proteins by degrading over 80% of cellular proteins in the nucleus, endoplasmic reticulum, and cytosol of eukaryotic cells via the ubiquitin–proteasome pathway (UPP).[2] Disruption of physiological proteasome activity is catastrophic to cellular homeostasis.

1.2 The Ubiquitin Proteasome Pathway (UPP)

Proteolysis via the UPP is performed in two specific stages: ubiquitin tagging and protein degradation. The first stage of the pathway involves three enzymes, namely Ub-activating (E1), Ub-conjugating (E2), and Ub-ligating (E3), which covalently link ubiquitin, a 76-amino acid protein, to a protein substrate destined for proteolysis in a sequential multi-step process.[3] The second stage involves the identification of the polyubiquitin chain by the 26S proteasome, the catalysis of the protein into small peptides, and release and recycling of ubiquitin molecules. (Figure 1).[4]

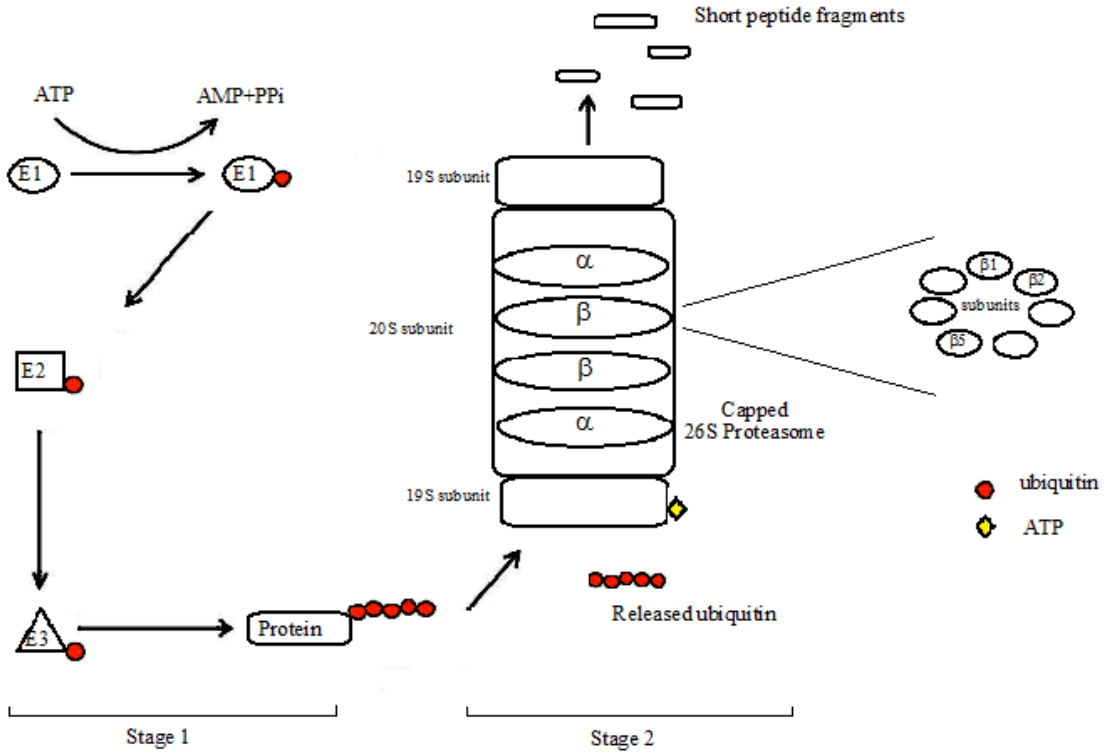


Figure 1. The Ubiquitin Proteasome pathway. Stage 1 – covalent tagging of ubiquitin to the protein (substrate) destined for proteolysis by enzymes, namely E1, E2, and E3. Stage 2 – identification of polyubiquitin chain by 26S proteasome and catalysis of the protein.

According to X-ray crystallography, the 26S proteasome consists of three complexes: 20S core structures capped by 19S regulatory structures at each extremity. The function of the 19S complex is to recognize the proteins that have been chosen for proteolysis via ubiquitin tagging, remove ubiquitin molecules and unfold the protein, and feed the protein into the catalytic 20S proteasome.[5,6] The 20S core particle is made up of 28 subunits arranged into four heptameric, stacked rings (α7,β7,β7,α7) which possess multiple peptidase activities.[7] These outer α-subunits serve as an anchor for the multi-subunit, ATPase-containing 19S regulator which binds to form the 26S proteasome

complex. This linking serves two functions: it opens the channel of the catalytic 20S proteasome and it unfolds ubiquitinated proteins to allow entry into the central β-subunits.[8] The two inner β-rings have active subunits: β1, β2, and β5. The β1 subunit is responsible for caspase-like, or peptidylglutamyl-peptide hydrolyzing-like, activity and cleaves after amino acids with acidic side chains. The β2 subunit possess trypsin-like activity and cleaves after amino acids with basic side chains. The β5 subunit has chymotrypsin-like activity and cleaves after amino acids with hydrophobic side chains.[8] The by-products formed from the degraded polypeptide are short polypeptides ranging from 4 to 25 amino acids in length.[9]

1.3 Proteasome Inhibition

The ubiquitin-proteasome pathway is responsible for degradation of proteins related to cell cycle,[10] apoptosis,[11] and abnormal proteins accumulated in response to stress.[12] Regulation of proteasome activity can affect cellular homeostasis by either accumulating inappropriate proteins or degrading regulatory proteins. Proteasome inhibition is an established means for the treatment of multiple myeloma, mantle cell lymphoma, and non-Hodgkin's lymphoma.[13] Highly proliferating cells are more sensitive to proteasome inhibition than non-proliferating cells.[14] This pathway closely regulates cyclins which in turn regulate cyclin-dependent kinases involved in the progression of the cell through its cell cycle. For example, degradation of cyclin B via this pathway allows the cell in G2 phase in the cell cycle to undergo mitosis.[10,15] The survival of the cell is largely dependent on the ratio of pro-apoptotic to anti-apoptotic proteins. One such

pro-apoptotic protein, Bax, is responsible for mitochondrial cytochrome c release which induces apoptosis of the cell.[16] In pathological conditions degradation of Bax may be increased to promote cell's survival and proliferation.[11,17] The tumor suppressor protein p53 halts the cell cycle progression at G1/S point which ultimately leads to apoptosis. The accumulation of this protein has been seen in the presence of proteasome inhibitors.[18] Lastly, NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a protein complex that controls transcription of DNA. While the ubiquitin-proteasome pathway does not directly regulate NF-kB, it does so indirectly by degrading IkBa (an inhibitor of NF-kB). Proteasome inhibition leads to a buildup of NF-kB in the cytoplasm and in doing so causes apoptosis.[19]

1.4 Novel Proteasome Inhibitors

Proteasome inhibition has become a fairly new target for the development of drugs to fight cancer. Proteasome inhibitors can be categorized as reversible and irreversible and the inhibition profile predominantly revolves around β -subunits, specifically by inhibiting the N-terminal threonine of the β5-subunit or proteasome's chymotrypsin-like activity. The inhibition is mainly achieved through the serine traps, such as boronic acids and epoxyketones. To date, only two compounds, bortezomib and carfilzomib (Figure 1), have been approved by Food and Drug Administration. This scarce amount of drugs on the market can be attributed to the low activity and toxicity which limit the use of others.

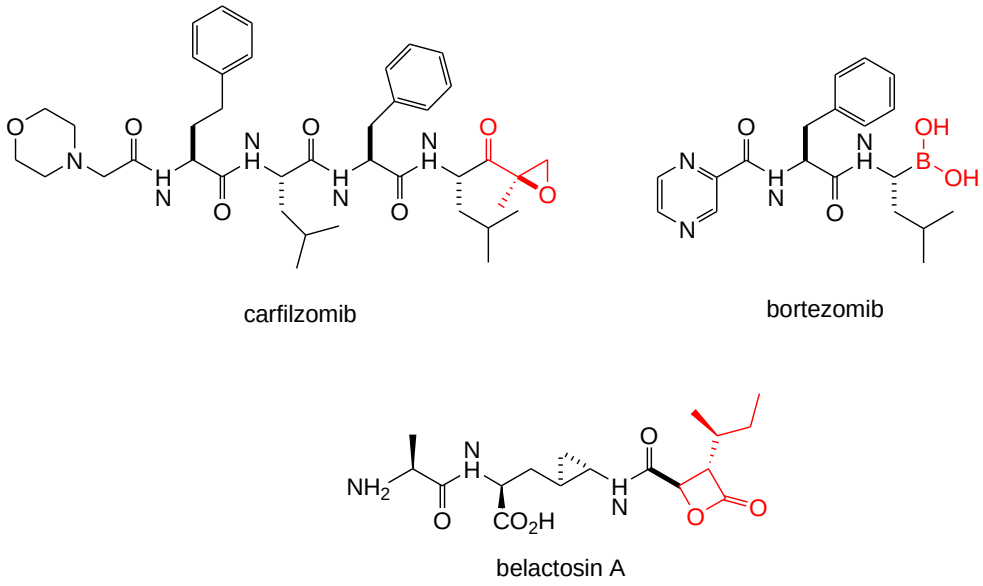


Figure 1. Examples of proteasome inhibitors (serine trap in red).

Bortezomib is a proteasome inhibitor that is a peptide with a boronic acid serine trap. It reversibly inhibits chymotrypsin-like, caspase-like, and immune-proteasome activities of the proteasome.[4] Bortezomib is used to treat multiple myeloma, recurrent multiple myeloma, and mantle cell lymphoma.[20,21] However, studies have shown mutated and over-expressed β5-subunits, indicating potential mechanisms for resistance to the drug.[22] To combat this resistance, carfilzomib, an irreversible proteasome inhibitor has been developed, since the cell needs to synthesize new proteasomes instead of modifying the existing ones. It is a peptide with epoxyketone serine trap, which inhibits chymotrypsin-like and immune-proteasome activities of the proteasome.[4] Carfilzomib is used to treat recurrent multiple myeloma, non-Hodgkin's lymphoma, and solid tumors.[23,24,25]

In 2000, belactosin A was isolated from *Streptomyces* and showed promising activity against colon and pancreatic cancers (Figure 1).[5,26] Belactosin A is a cyclopropyl-

containing antitumor, peptide-like natural compound which bears a β-lactone serine trap at one end of the molecule that acylates the N-terminal threonine of the β5-subunit in the proteasome.

1.5 Approach to Cyclopropyl Peptidomimetics

Cyclopropyl peptidomimetics can serve as core structures for bioactive compounds. The concept of using peptidomimetics as enzyme or similar inhibitors is central to numerous drugs both on the market and in development for a diverse number of conditions. In our laboratory, efficient syntheses of both ester-cyclopropyl and nitro-cyclopropyl peptidomimetics have been reported.[27] Using amino acids as starting materials, chiral cyclopropyl peptidomimetics are generated in just four steps. As outlined in Figure 2, a carboxybenzyl, Cbz, -protected amino acid is converted to the corresponding Weinreb amide, followed by addition of vinylmagnesium bromide to afford the enone. Cyclopropanation using EDSA afforded the protected cyclopropyl ketone in good yield as a 1:1 mixture of syn- and anti-isomers. Reduction of the ketone provided the protected cyclopropyl peptidomimetic in just four steps from commercially available protected amino acids.

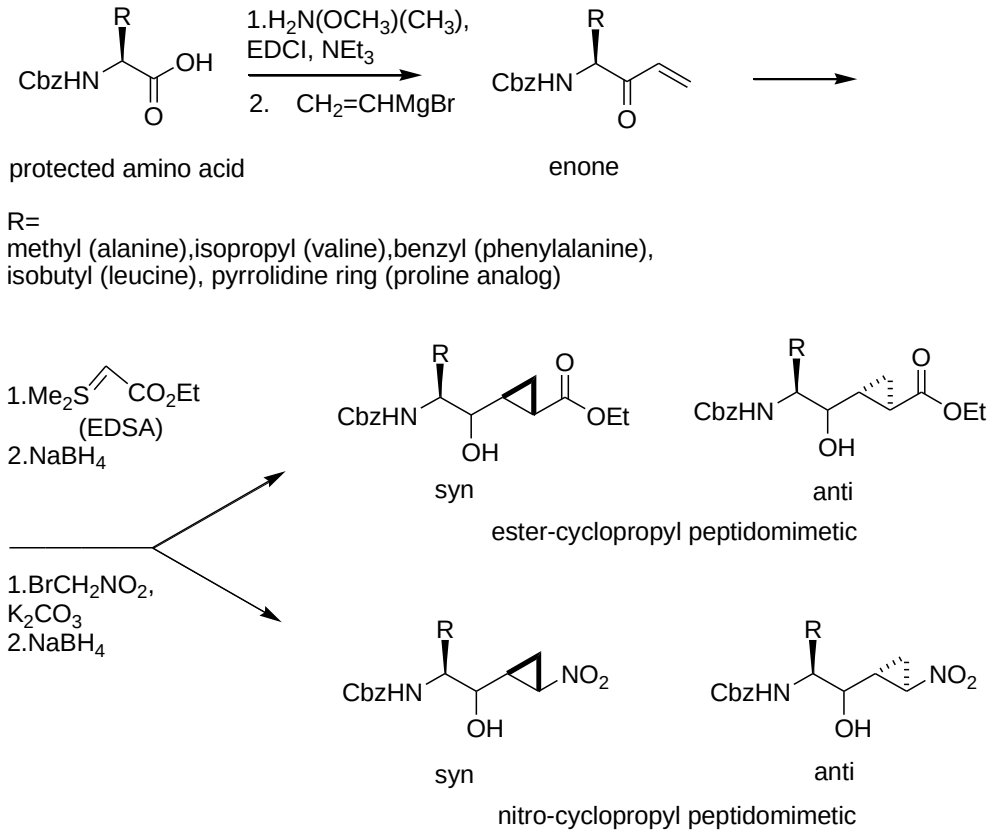


Figure 2. General synthesis of cyclopropyl peptidomimetics.

As expected for addition to the terminal methylene, none of the *cis*-cyclopropyl analogs were observed. Reduction of the ketones with sodium borohydride was only mildly stereoselective, typically affording a 2:1 mixture of the *4R* and *4S* isomers of alcohols. In a similar fashion, cyclopropanation using bromonitromethane afforded the nitro-cyclopropyl ketones, and ketone reduction with $NaBH_4$ gave the core nitro-cyclopropyl peptidomimetics. In both the ester and nitro series, we reported the synthesis of five amino acid analogs: phenylalanine, leucine, valine, alanine and proline. The synthesis has also been extended to a protected serine analog. The natural product

belactosin A, while not a peptidomimetic, has a cyclopropyl amino acid as its core. Thus, this method can be applied to the synthesis of belactosin A analogs.

1.6 Approaches to belactosin A Analogs Syntheses

Although several total syntheses of the natural product have been completed, few analogs of belactosin A have been reported, with examples shown in Figure 3.

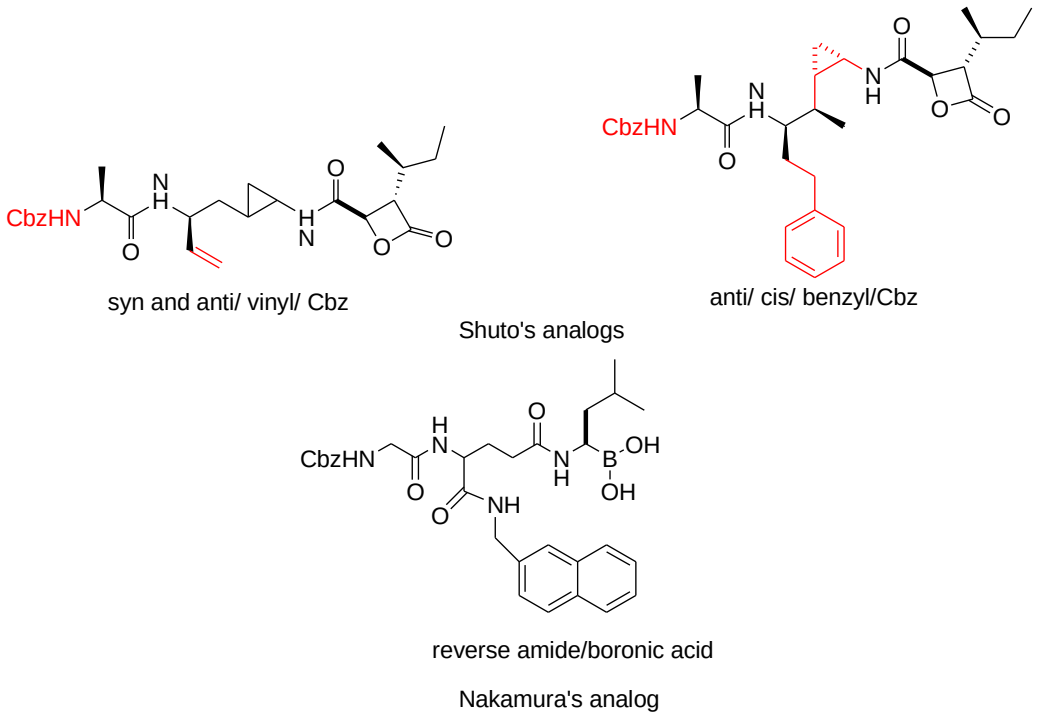


Figure 3. Previously reported balactosin analogs.

Shuto has reported a number of analogs of belactosin A,[28] and Nakamura has investigated boronic acid analogs of the related compound, belactosin C.[29] From the limited number of analogs, it is clear that the peptide-like core is fairly variable, and

some analogs have improved activity. In particular, the terminal amine is not necessary, and the carboxylic acid can be replaced with a lipophilic group, such as alkene or benzyl, or a large amide substituent, such as naphthyl. Substitutions for the β-lactone serine trap are also possible. It has been shown that incorporation of the rigid cyclopropane ring increases stability and reduces conformational freedom. Theoretically, constrained analogs should show reduced entropy effects, leading to improved binding in target proteins. These limited reports indicate that preparation of analogs is viable and may improve activity and reduce toxicity.

1.7 Targeted Design

The four-step synthesis that has been developed generates a core structure (**1**). One innovation is the design of analogs that is variable at different positions (R^1, R^2 and R^3). This will allow the structure to be tuned to target inhibition. The R^1 variable is a protecting group, such as Cbz or fluorenylmethyloxycarbonyl, Fmoc. Based on knowledge that R^2 may be a lipophilic group, two amino acids have been chosen as starting materials, alanine and phenylalanine. While the β-lactone is required for activity due to acylation of a threonine in the proteasome, its synthesis is not simple, and the unsubstituted version suffers from instability. The β-lactam is a classic serine trap, found in the antibacterial penicillins, cephalosporins, and monobactams, which are all acylated by a serine of their target enzyme, peptidoglycan transpeptidase. The investigation of the use of β-lactams is novel in the application to antitumor proteasome inhibition.

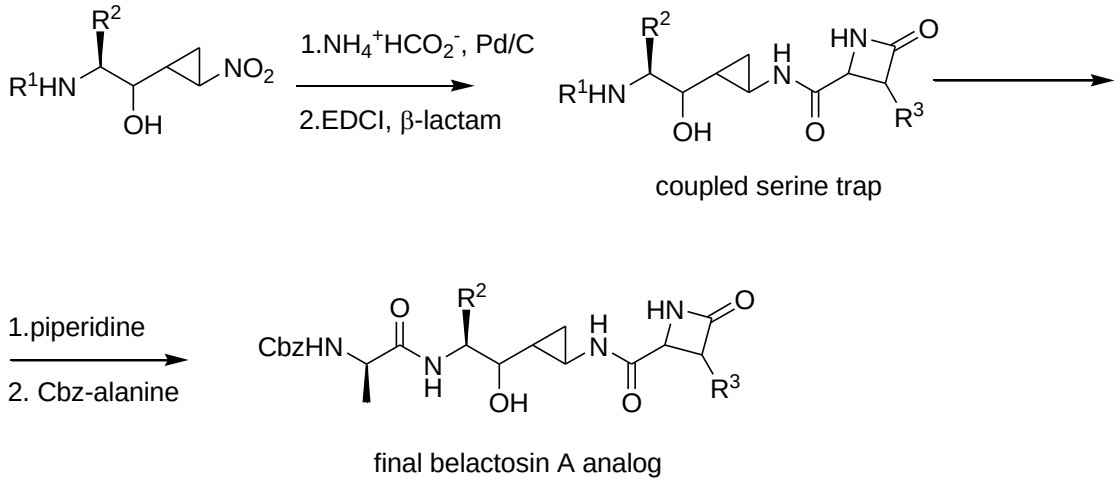


Figure 4. Proposed novel proteasome inhibitors.

Transfer hydrogenation with ammonium formate and Pd/C rapidly reduces the nitro to the primary amine without affecting the cyclopropane. In contrast, hydrogenation with Pd/C at 40 psi results in over-reduction of the cyclopropane. Although nitrocyclopropyls have been reduced using zinc and acid, the presence of the alcohol precludes that option for reduction in our case. Coupling of a variable β-lactam following the reduction affords the coupled serine trap (Figure 4). Removal of the Fmoc-protecting group, followed by coupling of Cbz-alanine provides the final product with variable R groups (Figure 4).

2 Materials & Methods

2.1 Materials, Reagents, and Instruments

Amino acids were purchased reagent grade from Aldrich, Milwaukee, WI. All other reagents, except triethylamine (NEt_3) and potassium carbonate, were purchased reagent grade from Aldrich, Milwaukee, WI. NEt_3, potassium carbonate, and hydrochloric acid were purchased reagent grade from Fischer Scientific, Pittsburgh, PA. The solvents dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), and dimethylformamide (DMF) were purchased anhydrous reagent grade from Aldrich. Solvent-solvent extractions were performed after each reaction. The organic layer was washed with one molar hydrochloric acid and brine. The organic layer was extracted with either methylene chloride or ethyl acetate, where appropriate. Hexanes, acetone, and methylene chloride were purchased reagent grade from Fischer Scientific, Pittsburgh, PA. Ethyl acetate and methanol were purchased reagent grade from Pharmco-AAPER, Shelbyville, KY. Chloroform was purchased reagent grade from Acros Organics, NJ.

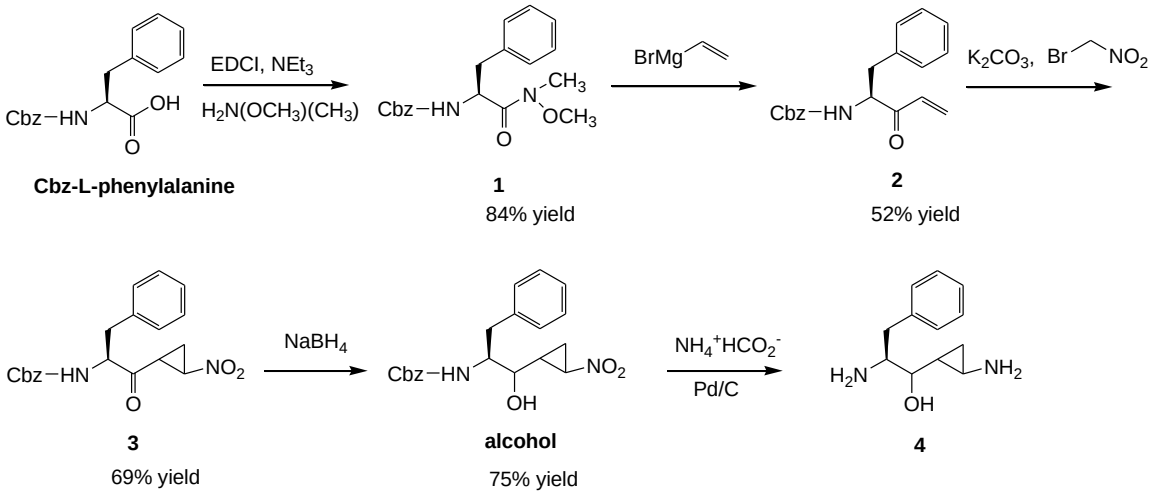
Analytical thin layer chromatography (TLC) was performed using glass plates coated with silica gel and UV active backing purchased from Dynamic Adsorbents, Norcross, GA. The plates were analyzed under UV light (254 nm) and stained with phosphomolybdic acid (reagent grade, Aldrich) or ninhydrin in a 10 % solution of ethanol. Flash column chromatography was performed with silica gel 32-60 microns 230-400 mesh (reagent grade, Dynamic Adsorbents, Norcross, GA).

Nuclear magnetic resonance, NMR, data was collected using a 500 MHz FT-NMR model ECA-500 JEOL (Peabody, MA). Compounds were dissolved in deuterated

chloroform (reagent grade, Cambridge) containing tetramethylsilane (TMS). Chemical shifts are reported in parts per million with TMS as an internal reference. Splitting patterns are reported as the following: s (singlet), d (doublet), t (triplet), m (multiplet), and dd (doublet of doublets). High-performance liquid chromatography data was collected using Waters Binary HPLC Pump 1525 and Waters Refractive Index Detector 2414. High resolution ESI-MS, electrospray ionization-mass spectrometry, was conducted at Notre Dame University, Notre Dame, Indiana.

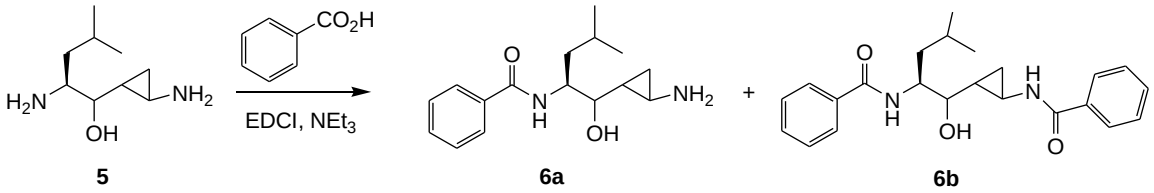
2.2 Reaction Schemes

Beginning with Cbz-protected L-phenylalanine, the amine was converted to the Weinreb amide (**1**). Treatment of Weinreb amide with vinylmagnesium bromide afforded the enone (**2**). In the key step, enone underwent cyclopropanation with bromonitromethane and afforded the cyclopropyl ketone (**3**). Treatment of the cyclopropyl ketone with sodium borohydride afforded the cyclopropyl alcohol. Hydrogenation with ammonium formate and palladium-carbon catalyst afforded the amine (**4**).



Scheme 1. Synthesis of belactosin A analogs with Cbz-L- phenylalanine as a starting amino acid.

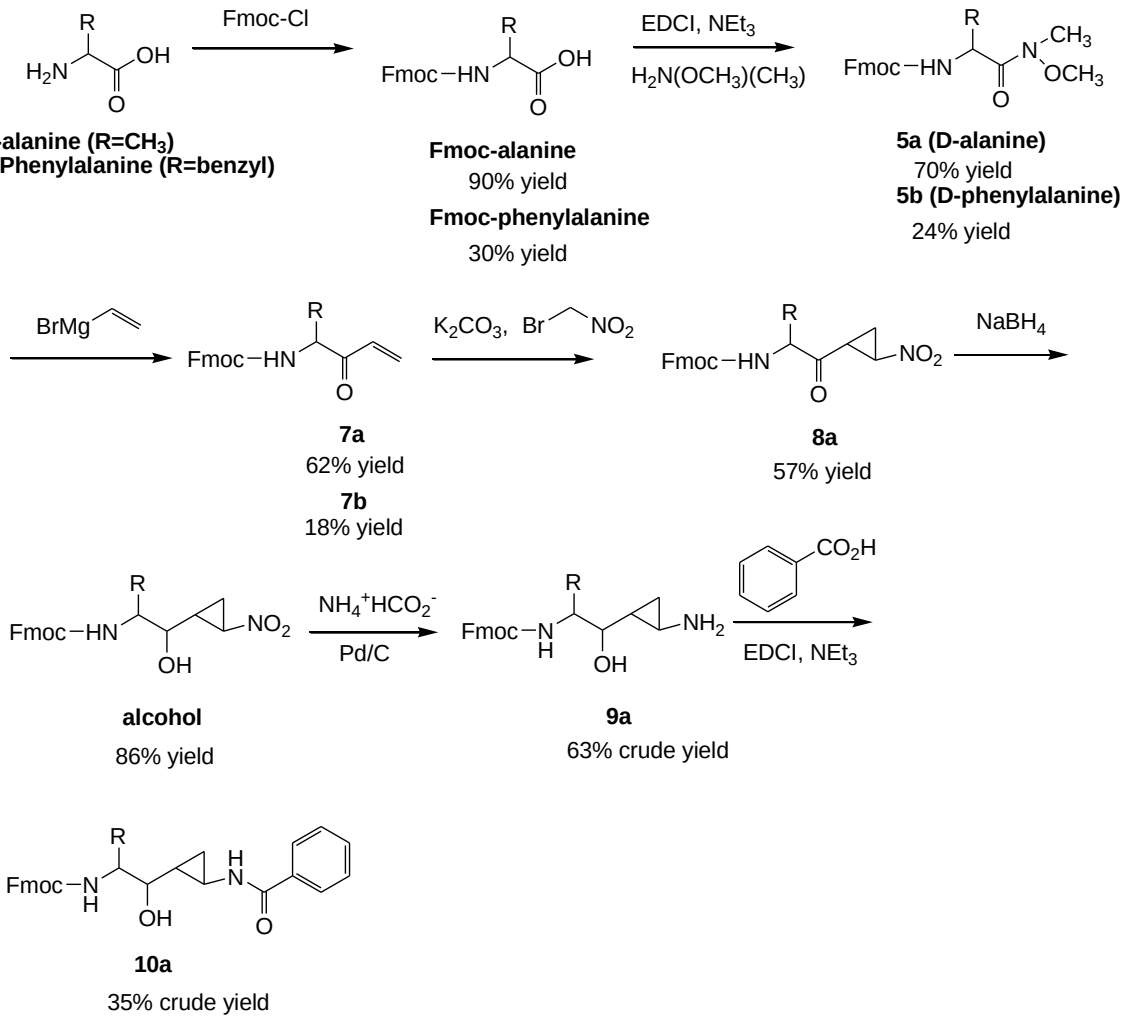
The analogous diamine **5** was coupled to benzoic acid to afford compounds (**6**). Benzoic acid is used as a model system for coupling until consistent reduction of the nitro group has been achieved.



S*cheme 2. Synthesis of belactosin analogs with D-leucine as a starting amino acid.*

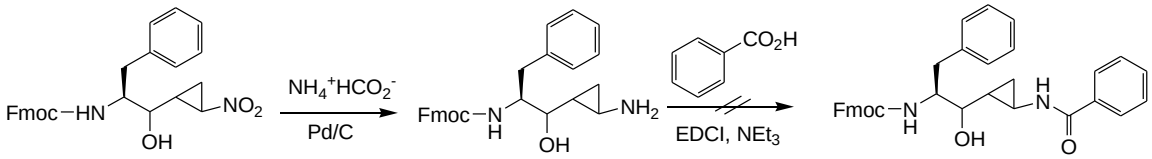
Beginning with D-alanine, the amine was protected using fluorenylmethyloxycarbonyl chloride, Fmoc-Cl. Reactions from Scheme 1 were performed to afford Fmoc-protected nitro cyclopropyl alcohol. Hydrogenation with

ammonium formate and palladium-carbon catalyst was performed to reduce the nitro group to an amine (**9**). The amine was then coupled to benzoic acid and afforded compoumd (**10**).



Scheme 3. Synthesis of belactosin A analogs with Fmoc-D- alanine as a starting amino acid.

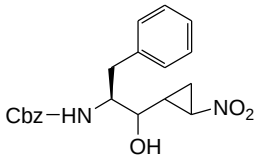
Previously synthesized Fmoc-L-phenylalanine nitro cyclopropyl alcohol (**11**) was hydrogenated with ammonium formate and palladium-carbon catalyst to afford a cyclopropyl amine (**12**). The amine was then coupled with benzoic acid (**13**).



11 **12** 58% crude yield **13** 52% crude yield

Scheme 4. Synthesis of belactosin A analogs with Fmoc-D-phenylalanine as a starting amino acid.

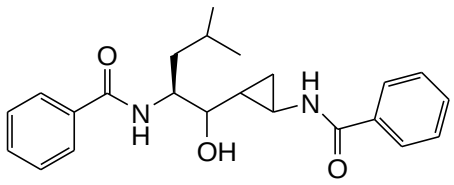
2.3 Procedures and Supporting Data



2.3.1 Benzyl (2S)-1-hydroxy-1-(2-nitrocyclopropyl)-3-phenylpropan-2-ylcarbamate (Cbz alcohol)

To a solution of ketone (0.250 g, 0.66 mmol) in 10 mL of methanol was added sodium borohydride (0.100 g, 2.64 mmol). The reaction was stirred for 1 hour. The reaction mixture was poured into brine and extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated. The crude product

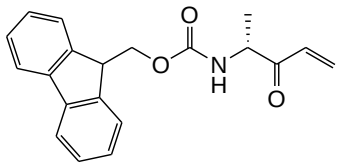
was purified by flash column chromatography eluting with 1:2, 1:1, and 2:1 ethyl acetate/hexane affording 217.5 mg (86%) of the alcohol. The products were separated by HPLC with an initial solvent ratio of 70/30 hexane-ethyl (3.0 min), to 60/40 over 10.0 min. (hold for 5min), to 55/45 over 6.0 min., holding for 2.0 min. for a total run time of 30.0 min. with a flow rate of 2.0 mL/min.^{1}H-NMR (500 MHz, $CDCl_3$) (mixture of stereoisomers): δ 7.35-7.18 (m, 10H, aryl Cbz and phenyl), 5.37-5.10(d, J=6.2, 1H, NH), 5.05-502 (m, 2H, Cbz CH_2), 4.30-4.27 (m, 1H, $CHNO_2$), 3.92 (m, 1H, C<u>H</u>NH), 3.80-3.34 (s, 1H, OH), 2.95-2.90 (m, 2H, CH_2Ph), 1.75-1.35 (m, 2H, CH_2 cpropyl), 1.32-1.27 (m, 1H, cpropyl C<u>H</u>COH).



2.3.2 N-(2-((2S)-2-benzamido-1-hydroxy-4-methylpentyl)cyclopropyl)benzamide (10b)

To a solution of leucine diamine (0.028 g, 0.14 mmol) in 0.8 mL of dichloromethane was added benzoic acid (0.026 g, 0.21 mmol), 0.2 mL trimethylamine (0.141 g, 1.4 mmol), and EDCI (0.040 g, 0.21 mmol). The reaction was stirred overnight. The reaction mixture was poured into 1 M HCl and extracted with ethyl acetate. The organic layer was washed with water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated**.** The crude product was purified by flash column chromatography eluting with 10:1 ethyl acetate/hexane, ethyl acetate, and methanol affording 9 mg (22%) of dibenzoyl leucine . ^{1}H-NMR (500 MHz, $CDCl_3$) (mixture of stereoisomers): δ 7.70-7.40

(m, 5H, aryl), 6.38-6.34 (s, 1H, NH), 4.85-4.75 (s, 1H, cpropyl C<u>H</u>NH_2), 4.4.98-4.91 (m, 1H, OH), 3.18-3.10 (m, 1H, C<u>H</u>OH), 2.70-2.65 (m, 1H, C<u>H</u>NH), 2.05-1.90 (m, 1H, cpropyl CH), 1.82-1.62 (m, 2H, cpropyl CH_2), 1.25-1.20 (m, 2H, C<u>H_2</u>CH), ($C_{16}H_{25}N_2O_2$), 0.94-0.74 (m, 7H, isopropyl). (ESI-MS) *m/z* calculated for (M+H) 277.1911. Found 277.1913.

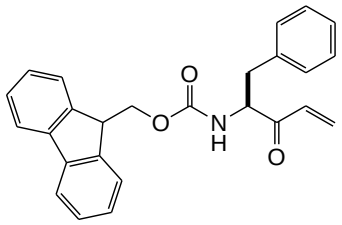


2.3.3 (R)-(9H-fluoren-9-yl)methyl 3-oxopent-4-en-2-ylcarbamate (6a)

To a solution of Fmoc-alanine Weinreb amide (0.280 g, 0.8 mmol) in 3 mL of anhydrous THF at 0° C under argon was added 3 mL (2.9 mmol) of 1 M vinylmagnesium bromide solution. The reaction was stirred for 3 hours at room temperature. The reaction mixture was poured into 1 M HCl and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and the solvent evaporated. The crude product was purified by flash column chromatography eluting with 1:6, 1:5, and 1:3 ethyl acetate/hexane affording 93 mg (36%) of Fmoc-alanine enone. ^{1}H-NMR (500 MHz, $CDCl_3$) (mixture of conformers): δ 6.44-6.42 (m, 2H, enone), 5.91-5.89 (m, 1H, enone), 5.80-5.70 (m, 1H, NH), 4.72-4.70 (m, 1H, C<u>H</u>NH), 3.45-3.35 (m, 2H, CH_2O), 4.30-4.20 (m, 1H, C<u>H</u>CH_2O), 1.40-1.30 (m, 3H, CH_3).

^{13}C-NMR (125 MHz, $CDCl_3$): δ 198.1 (C=O), 155.5(CONH), 143.7 (<u>C</u>HCHCH_2O), 141.2 (<u>C</u>HCHCHCH_2O), 132.4 (enone CHCO), 130.4 (enone CH_2), 66.8 (CH_2O), 53.5

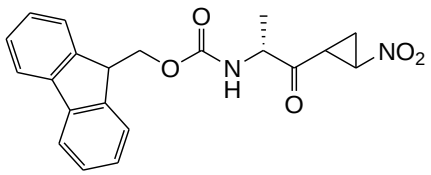
(CHNH), 47.0 (CHCH$_2$O), 18.3 (CH$_3$). (ESI-MS) *m/z* ($C_{20}H_{20}NO_3$) calculated for (M+H) 322.1438. Found 322.1443.



2.3.4 (S)-(9H-fluoren-9-yl)methyl 3-oxo-1-phenylpent-4-en-2-ylcarbamate (6b)

To a solution of Fmoc-phenylalanine Weinreb amide (0.092 g, 0.22 mmol) in 1 mL of anhydrous THF at 0° C under argon was added 1 mL (0.8 mmol) of 1 M vinylmagnesium bromide solution. The reaction was stirred for 3 hours at room temperature. The reaction mixture was poured into 1 M HCl and extracted with ethyl acetate. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and the solvent evaporated. The crude product was purified by flash column chromatography eluting with 1:3 ethyl acetate/hexane affording 16 mg (18%) of Fmoc-alanine enone. ^{1}H-NMR (500 MHz, $CDCl_3$) (mixture of conformers): δ 6.45-6.35 (m, 2H, enone), 5.95-5.85 (m, 1H, enone), 5.60-5.52 (m, 1H, NH), 4.98-4.92 (m, 1H, CHNH), 4.45-4.30 (m, CH$_2$O), 4.25-4.15 (s, 1H, CHCH$_2$O), 3.20-3.06 (m, 2H, CH$_2$Ph).

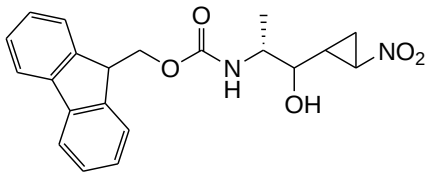
^{13}C-NMR (125 MHz, $CDCl_3$): δ 198.3 (C=O), 155.1 (CONH), 143.8 (CHCHCH$_2$O), 141.3 (CHCHCHCH$_2$O), 133.1 (enone CHCO), 130.6 (enone CH$_2$), 66.9 (CH$_2$O), 58.6 (CHNH), 47.2 (CHCH$_2$O), 37.9 (CH$_2$Ph). (ESI-MS) *m/z* ($C_{26}H_{23}NO_3Na$) calculated for (M+Na) 420.1570. Found 420.1572.



2.3.5 (9H-fluoren-9-yl)methyl (R)-1-(2-nitrocyclopropyl)-1-oxopropan-2-ylcarbamate (7a)

To a solution of Fmoc-alanine enone (0.068 g, 0.22 mmol) in 10 mL acetonitrile at 0° C was added crushed K_2CO_3 (.031 g, 0.22 mmol) and 0.016 mL bromonitromethane (0.031 g, 0.22 mmol). The reaction was stirred for two hours, after which another 0.016 mL of bromonitromethane was added. After two more hours, another 0.016 mL of bromonitromethane was added. The reaction was stirred for another 90 minutes, for a total time of five and a half hours. The reaction mixture was poured into brine and extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated**.** The crude product was purified by flash column chromatography eluting with 1:5, 1:3, and 1:2 ethyl acetate/hexane affording 48 mg (57%) of Fmoc-alanine nitrocyclopropyl ketone. ^{1}H-NMR (500 MHz, $CDCl_3$) (mixture of conformers): δ 5.44-5.38 (m, 1H, NH), 4.65-4.5 (m, 1H, cpropyl $CHNO_2$), 4.45-4.35 (m, 2H, CH_2O), 4.25-4.15 (m, 1H, C<u>H</u>CH_2O), 3.15-3.05 (m, 1H, C<u>H</u>NH), 2.15-2.05 (m, 2H, cpropyl CH_2), 1.65-1.55 (m, 1H, cpropyl CH), 1.30-1.20 (m, 3H, CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$): δ 202.4 (C=O), 155.8 (CONH), 143.3 (<u>C</u>HCHCH_2O), 140.8 (<u>C</u>HCHCHCH_2O), 65.9 (CH_2O), 55.6 (CHNH), 51.3 (CHNH), 46.2 (<u>C</u>HCH_2O),

26.3 (cpropyl CH), 18.4 (CH_3). (ESI-MS) *m/z* ($C_{21}H_{20}N_2O_5Na$) calculated for (M+Na) 403.1264. Found 403.1247.

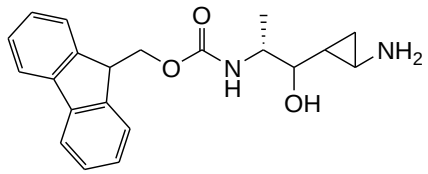


2.3.6 (9H-fluoren-9-yl)methyl (2R)-1-hydroxy-1-(2-nitrocyclopropyl)propan-2-ylcarbamate (Fmoc-alcohol)

To a solution of Fmoc-alanine nitrocyclopropyl ketone (0.250 g, 0.66 mmol) in 10 mL methanol was added sodium borohydride (0.100 g, 2.64 mmol). The reaction was stirred for one hour. The reaction mixture was poured into brine and extracted with ethyl acetate. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated**.** The crude product was purified by flash column chromatography eluting with 1:2, 1:1, and 2:1 ethyl acetate/hexane affording 218 mg (86%) of Fmoc-alanine nitrocyclopropyl alcohol. ^{1}H-NMR (500 MHz, $CDCl_3$) (mixture of conformers): δ 5.13-5.10 (m, 1H, NH), 5.05-4.90 (OH), 4.50-4.40 (m, 1H, cpropyl $CHNO_2$), 4.30-4.20 (m, 2H, CH_2O), 4.25-4.15 (m, 1H, C<u>H</u>CH_2O), 3.85-3.75 (m, 1H, C<u>H</u>NH), 3.70-3.30 (dd C<u>H</u>OH), 2.05-1.95-2.05 (m, 1H, cpropyl CH), 1.40-1.25 (m, 1H, cpropyl CH_2), 1.20-1.10 (m, 3H, CH_3).

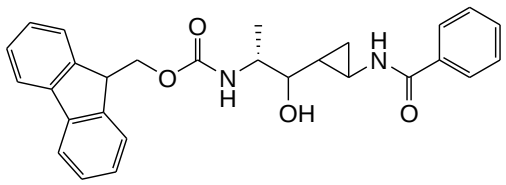
^{13}C-NMR (125 MHz, $CDCl_3$): δ 143.6 (CONH), 141.3 (<u>C</u>HCHCH_2O), 140.8 (<u>C</u>HCHCHCH_2O), 72.5 (COH), 66.7 (CH_2O), 56.6 ($CHNO_2$), 51.4 (CHNH), 47.1

(CHCH$_2$O), 26.8 (cpropyl CH), 14.2 (CH$_3$), 13.6 (cpropyl CH$_2$). (ESI-MS) *m*/*z* ($C_{21}H_{22}N_2O_5Na$) calculated for (M+Na) 405.1421. Found 405.1448.



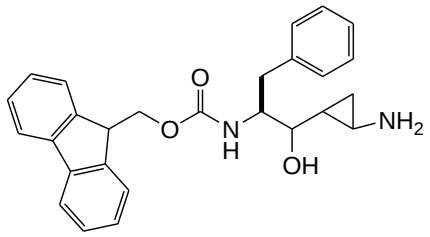
2.3.7 (9H-fluoren-9-yl)methyl (2R)-1-(2-aminocyclopropyl)-1-hydroxypropan-2-ylcarbamate (8a)

To a solution of Fmoc-alanine nitrocyclopropyl alcohol (0.130 g, 0.34 mmol) in 10 mL methanol was added ammonium formate (0.580 g, 9.2 mmol) followed by a 10% palladium-carbon (0.100 g). The reaction was stirred for forty minutes. The reaction mixture was gravity filtered to remove the palladium-carbon catalyst. The reaction mixture was poured into water and extracted with ethyl acetate. The aqueous layer was lyophilized. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated**.** The crude product was not purified affording 76 mg (63%) of Fmoc-alanine aminocyclopropyl alcohol.



2.3.8 (9H-fluoren-9-yl)methyl (2R)-1-(2-benzamidocyclopropyl)-1-hydroxypropan-2-ylcarbamate (9a)

To a solution of crude Fmoc-alanine nitrocyclopropyl alcohol (0.076 g, 0.22 mmol) in 2 mL dichloromethane was added benzoic acid (0.041 g, 0.33 mmol), 0.3 mL trimethylamine (0.222 g, 2.2 mmol), and EDCI (0.064 g, 0.33 mmol). The reaction was stirred overnight. The reaction mixture was poured into 1 M HCl and extracted with ethyl acetate. The organic layer was washed with water. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated**.** The crude product was purified by flash column chromatography eluting with 1:5 ethyl acetate/hexane affording 35 mg (35%) of Fmoc-alanine benzoyl aminocyclopropyl alcohol.



2.3.9 (9H-fluoren-9-yl)methyl (2S)-1-hydroxy-1-(2-nitrocyclopropyl)-3-phenylpropan-2-ylcarbamate (12)

To a solution of Fmoc-phenyalanine nitrocyclopropyl alcohol (0.050 g, 0.11 mmol) in 5 mL methanol was added ammonium formate (0.190 g, 3 mmol) followed by a 10% palladium-carbon (0.050 g). The reaction was stirred for forty minutes. The reaction mixture was gravity filtered to remove the palladium-carbon catalyst. The reaction

mixture was poured into water and extracted with ethyl acetate. The aqueous layer was lyophilized. The organic layer was dried over anhydrous $MgSO_4$, filtered, and the solvent was evaporated**.** The crude product was not purified affording 23 mg (58%) of Fmoc-phenylalanine aminocyclopropyl alcohol.

3 Results and Discussion

3.1 Cbz Series

In order to make belactosin A analogs, it is necessary to reduce the nitro group (right-side) without deprotecting the amine (left-side). Compared to the Fmoc series, work with the Cbz series did not yield the desired results. The four-step synthetic sequence yielded the nitro cyclopropyl alcohol from L-phenylalanine. In order to characterize all four possible stereoisomers, the four stereoisomeric products were analyzed via HPLC and optical rotations were obtained on a polarimeter (Table 1). First, the pure syn ketone isomer (**3**) was reduced to give the 4S and 4R alcohols. HPLC showed a 1:1 ratio of isomers (retention time 17.3 mins and 20.7 mins, respectively) as seen in Figure 5. Second, reduction of the mixed syn and anti ketone isomers was done. HPLC analysis showed the two syn alcohols, and one new signal which is the inseparable anti alcohols. Based on a ketone reduction of syn nitro cyclopropyl phenylalanine performed earlier it was deduced that the anti mixture of the four stereoisomers does not separate. Based on the x-ray structure of ester cyclopropyl series, most probably the first stereoisomer to elute is the syn, 4S alcohol. After performing hydrogenation, the desired amine product **4** could not be recovered from either aqueous or organic layers.

Using the same conditions as for L-phenylalanine, the amine product from the L-leucine series was coupled to benzoic acid. This attempt had resulted in over-reduction and produced a diamine, which was characterized as the di-amine. Therefore, Fmoc-Cl, was chosen as an alternative to the previously used Cbz group, as a more stable protecting group under reduction conditions.

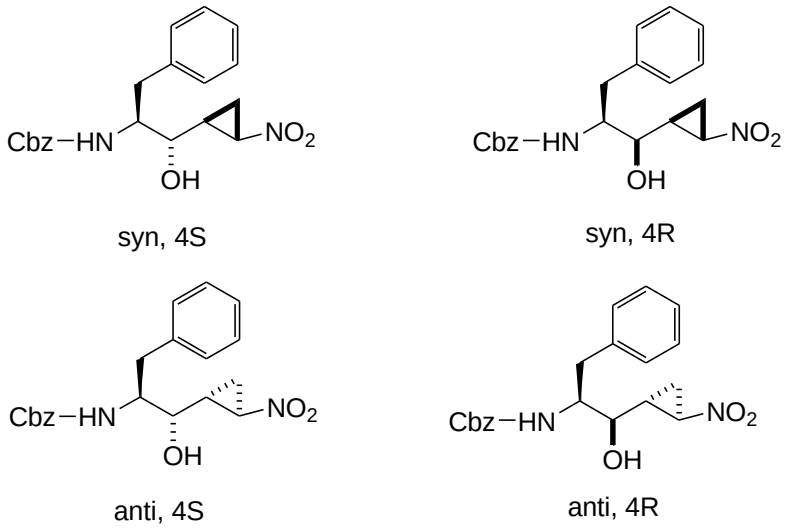


*Figure 5. Structures of the four stereoisomers produced following the reduction of compound **3**.*

Table 1. Cbz-L-phenylalanine nitro cyclopropyl alcohol stereoisomers data.

	syn, 4S (or R)	anti, 4S+4R mixture	syn, 4R (or S)
HPLC retention time (minutes)	17.3	18.7	20.7
Specific rotation (degrees)	-11.6	-39.1	-3.8

3.2 Fmoc Series

The Fmoc series were prepared using D-alanine and L-phenylalanine. The phenylalanine series was plagued with low yields throughout the reaction sequence. The alanine series had promising results. Coupling of benzoic acid following the hydrogenation of the cyclopropyl nitro group appeared to produce a coupled product. However, the coupling reaction could not be differentiated further to a very small amount of product that was left. However, the Fmoc-L-phenylalanine nitro cyclopropyl alcohol was obtained, and was followed by hydrogenation and coupling to benzoic acid. Mass spectroscopy revealed that the coupling did not produce the desired product, so this requires further investigation.

Work with both series afforded compounds that had not previously been synthesized. For each new compound, ^{1}H-NMR, ^{13}C-NMR, DEPT-135, gradient COSY, and gradient HMQC data was collected. In addition to NMR data, where applicable, exact mass spectroscopy, infrared spectroscopy, and polarimetry data was collected. Future work involves further improvement of the hydrogenation reaction to afford an amine which could then be coupled to a serine trap to afford belactosin A analogs.

Conclusions

Two protected series, Cbz and Fmoc, were investigated. Individual stereoisomers in the Cbz series were purified and characterized, but the key reduction step led to over-reduction and removal of Cbz. Fmoc nitrocyclopropyl alcohols were synthesized in two amino acid series. Reduction in alanine series looks promising and needs to be repeated.

Reduction in phenylalanine series followed by coupling to benzoic acid as a model did not produce the desired product. More work needs to be done in the Fmoc series to allow consistent reduction of nitro group in order to couple to “serine trap” for belactosin A analogs.

4 References

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Appendix A: Spectral Data

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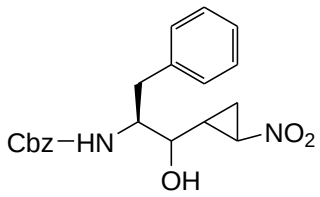

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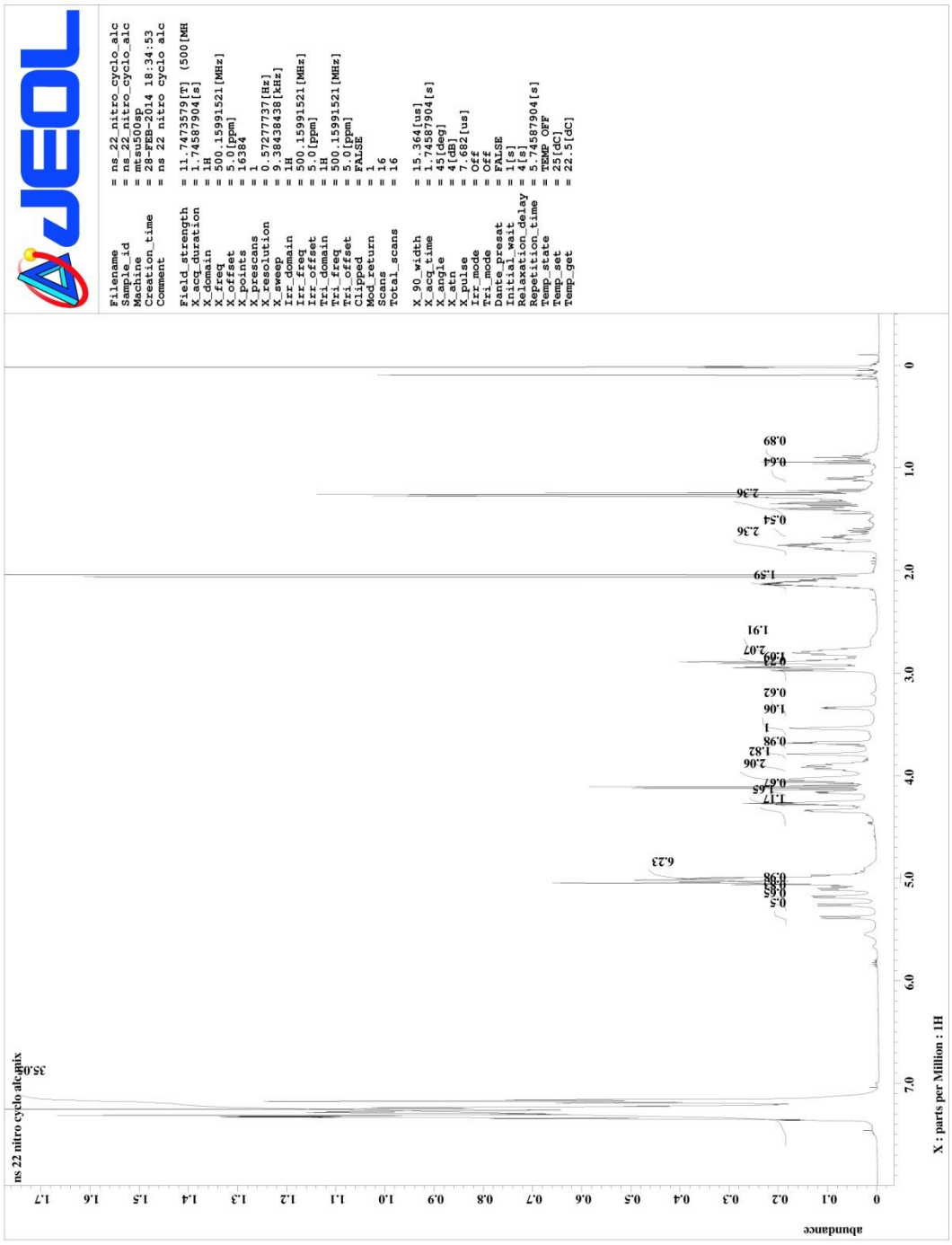
HPLC chromatogram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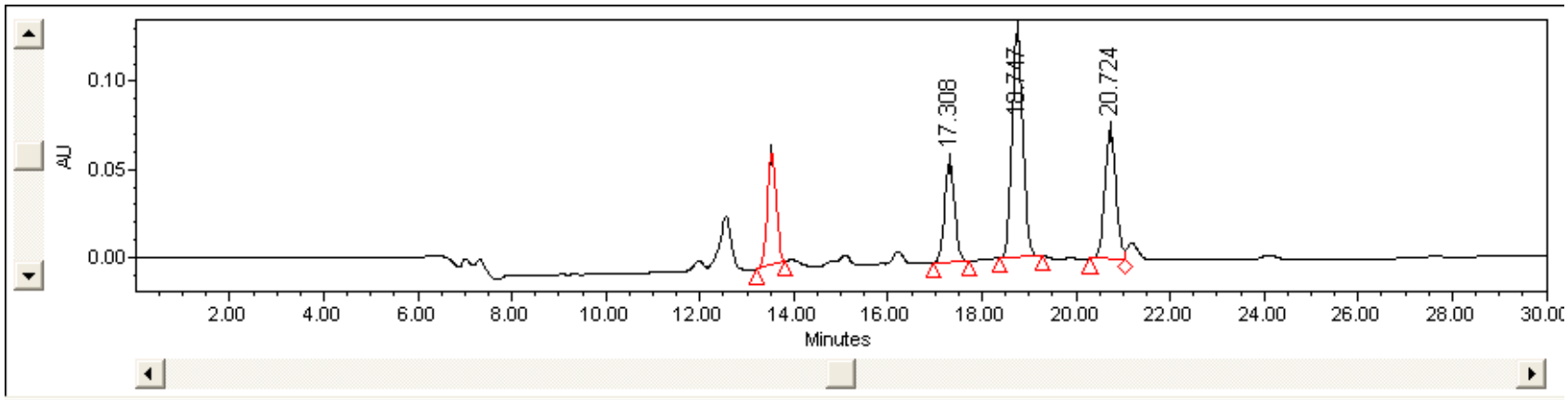

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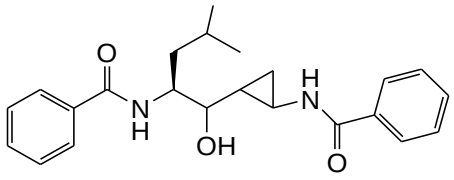

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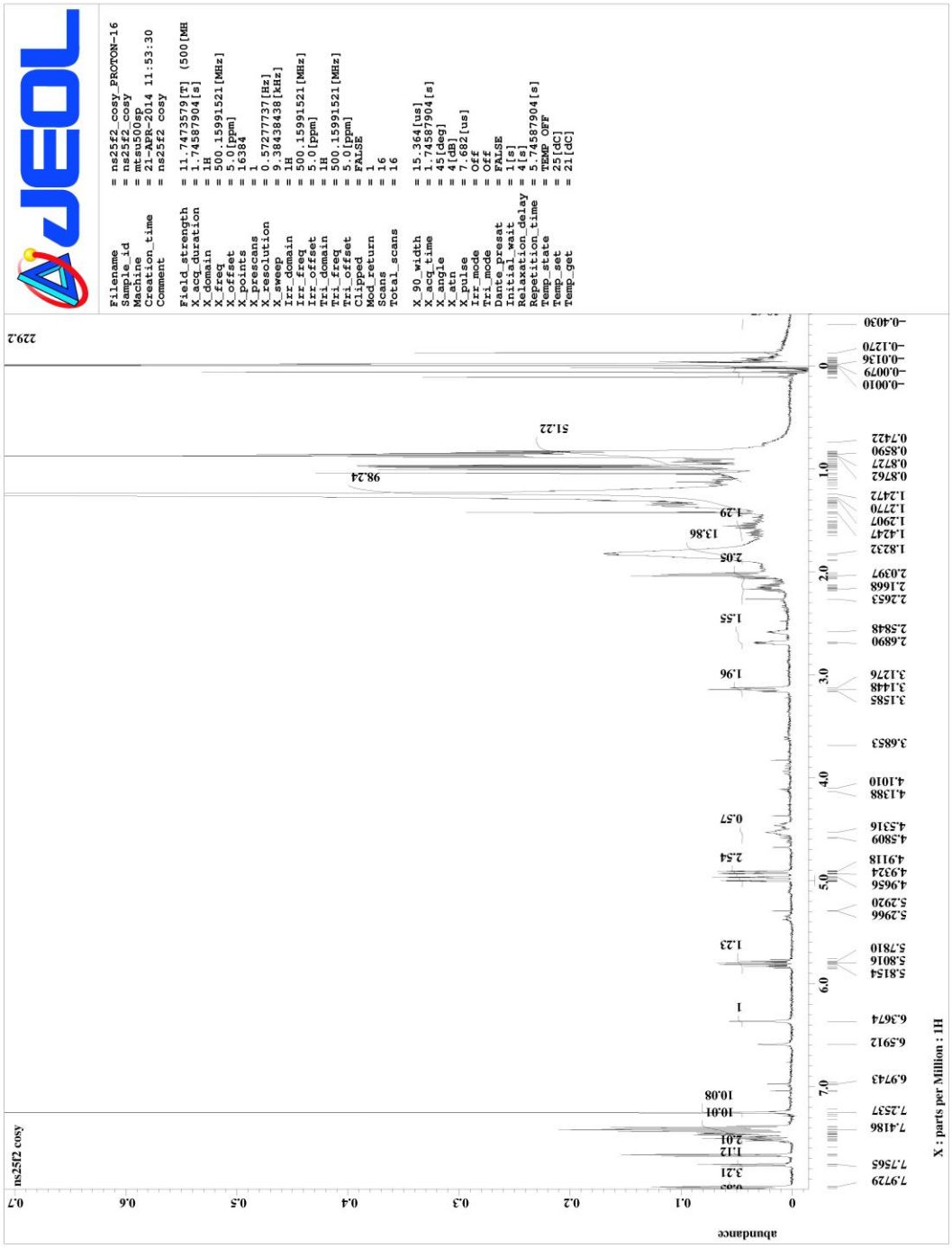
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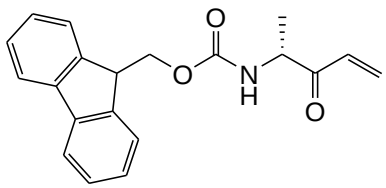

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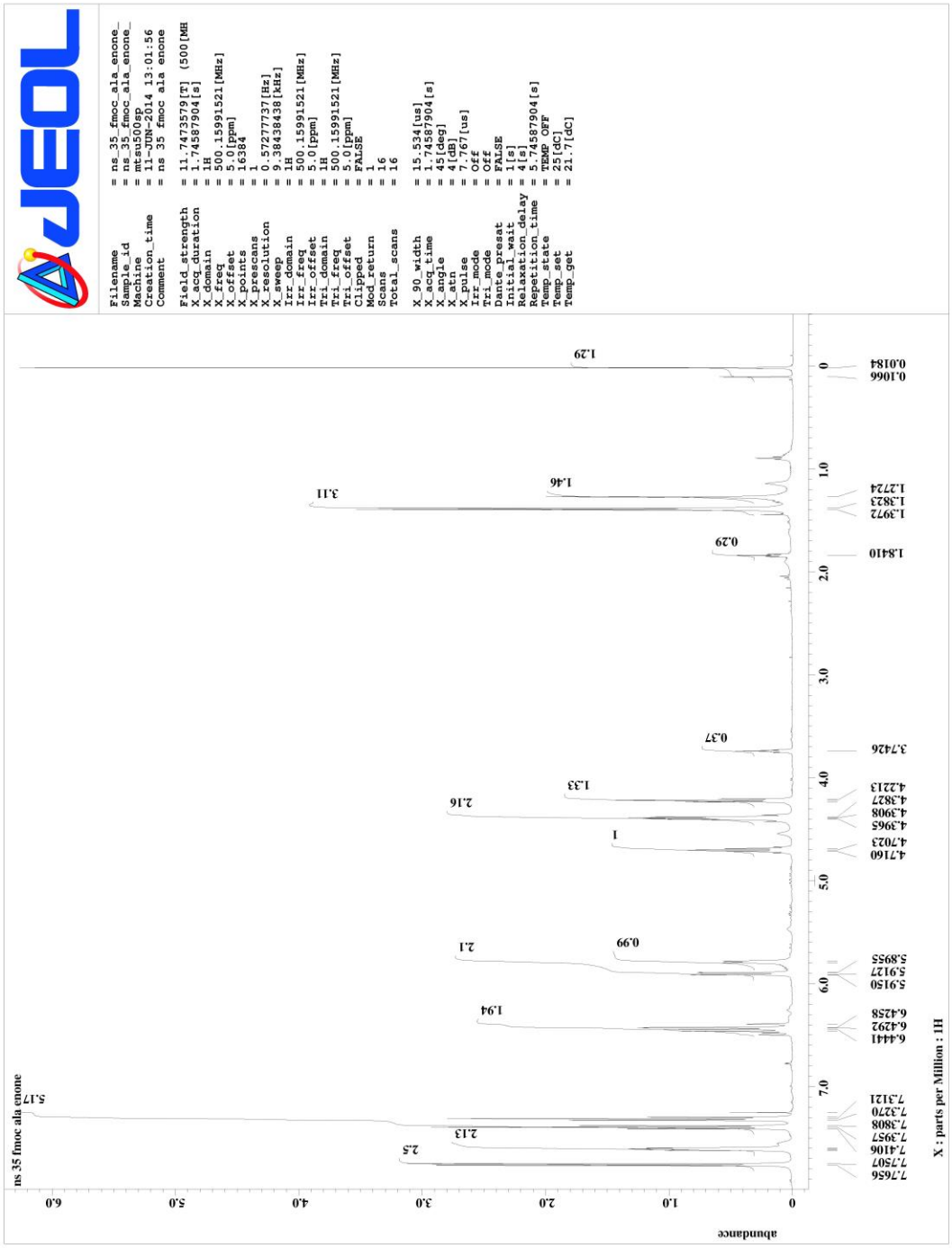
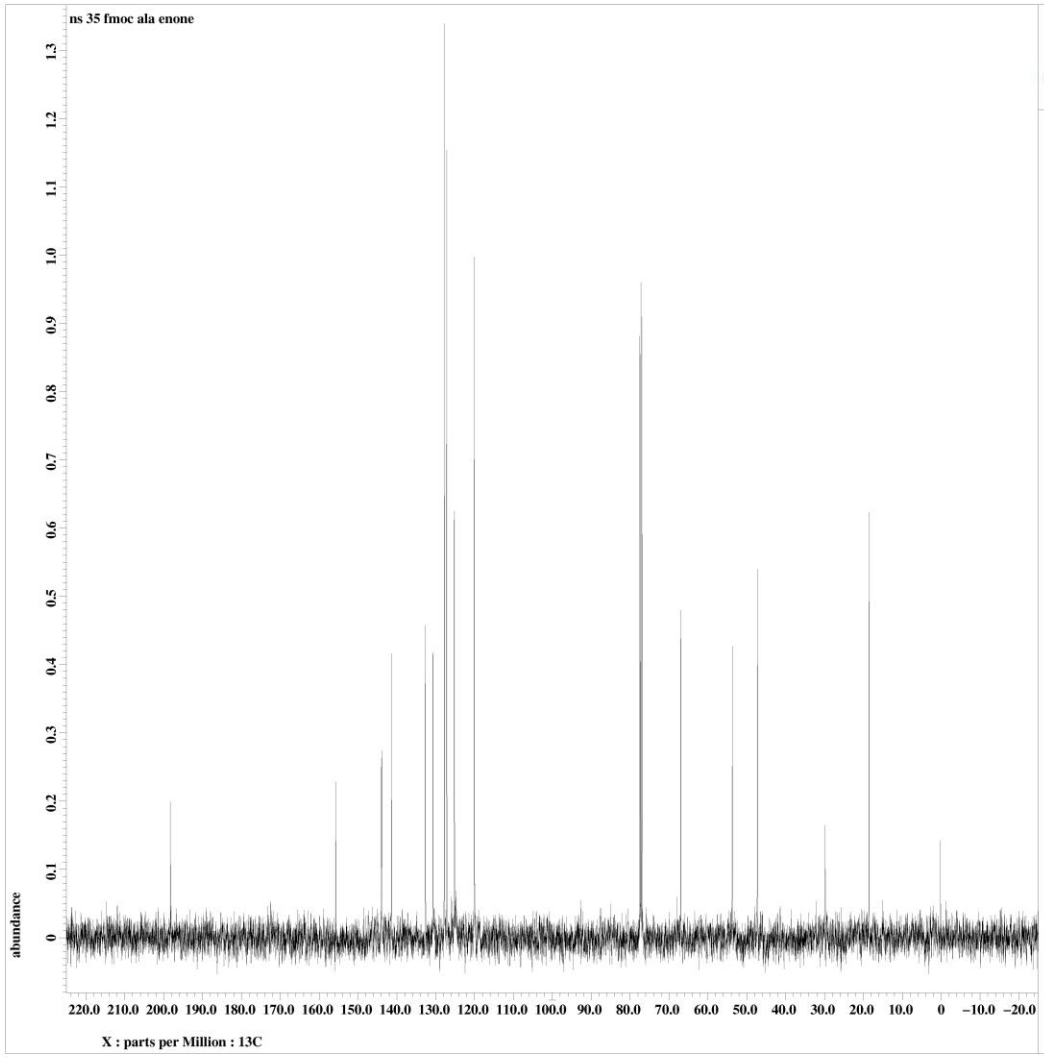
Proton

Carbon


JEOL
Filename = ns_35_fmoc_ala_enone_
Sample_id = ns_35_fmoc_ala_enone_
Machine = mtsu500sp
Creation_time = 11-JUN-2014 13:01:56
Comment = ns 35 fmoc ala enone
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 0
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 15.534[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.767[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.7[dC]
ns 35 fmoc ala enone
abundance
X : parts per Million : 1H
5.17
2.13
2.5
1.94
2.1
0.99
2.16
1.33
1
0.37
0.29
3.11
1.46
1.29
7.7656
7.7507
7.4106
7.3957
7.3808
7.3270
7.3121
6.4441
6.4292
6.4258
5.9150
5.9127
5.8955
4.7160
4.7023
4.3965
4.3908
4.3827
4.2213
3.7426
1.8410
1.3972
1.3823
1.2724
0.1066
0.0184

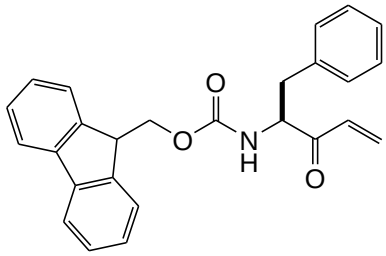



```
Filename          = ns_35_fmoc_ala_enone_
Sample_id         = ns_35_fmoc_ala_enone_
Machine           = mtsu500sp
Creation_time     = 11-JUN-2014 13:03:31
Comment           = ns 35 fmoc ala enone

Field_strength    = 11.7473579[T] (500[MH
X_acq_duration    = 0.83361792[s]
X_domain          = 13C
X_freq            = 125.76529768[MHz]
X_offset          = 100[ppm]
X_points          = 32768
X_prescans        = 4
X_resolution      = 1.19959034[Hz]
X_sweep           = 39.3081761[kHz]
Irr_domain        = 1H
Irr_freq          = 500.15991521[MHz]
Irr_offset        = 5.0[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 14
Total_scans       = 14

X_90_width        = 9.804[us]
X_acq_time        = 0.83361792[s]
X_angle           = 30[deg]
X_atn             = 9[dB]
X_pulse           = 3.268[us]
Irr_atn_dec       = 21.5[dB]
Irr_atn_noe       = 21.5[dB]
Irr_noise         = WALTZ
Decoupling        = TRUE
Initial_wait      = 1[s]
Noe               = TRUE
Noe_time          = 2[s]
Relaxation_delay  = 2[s]
Repetition_time   = 2.83361792[s]
Temp_state        = TEMP OFF
Temp_set          = 25[dC]
Temp_get          = 21.9[dC]
```

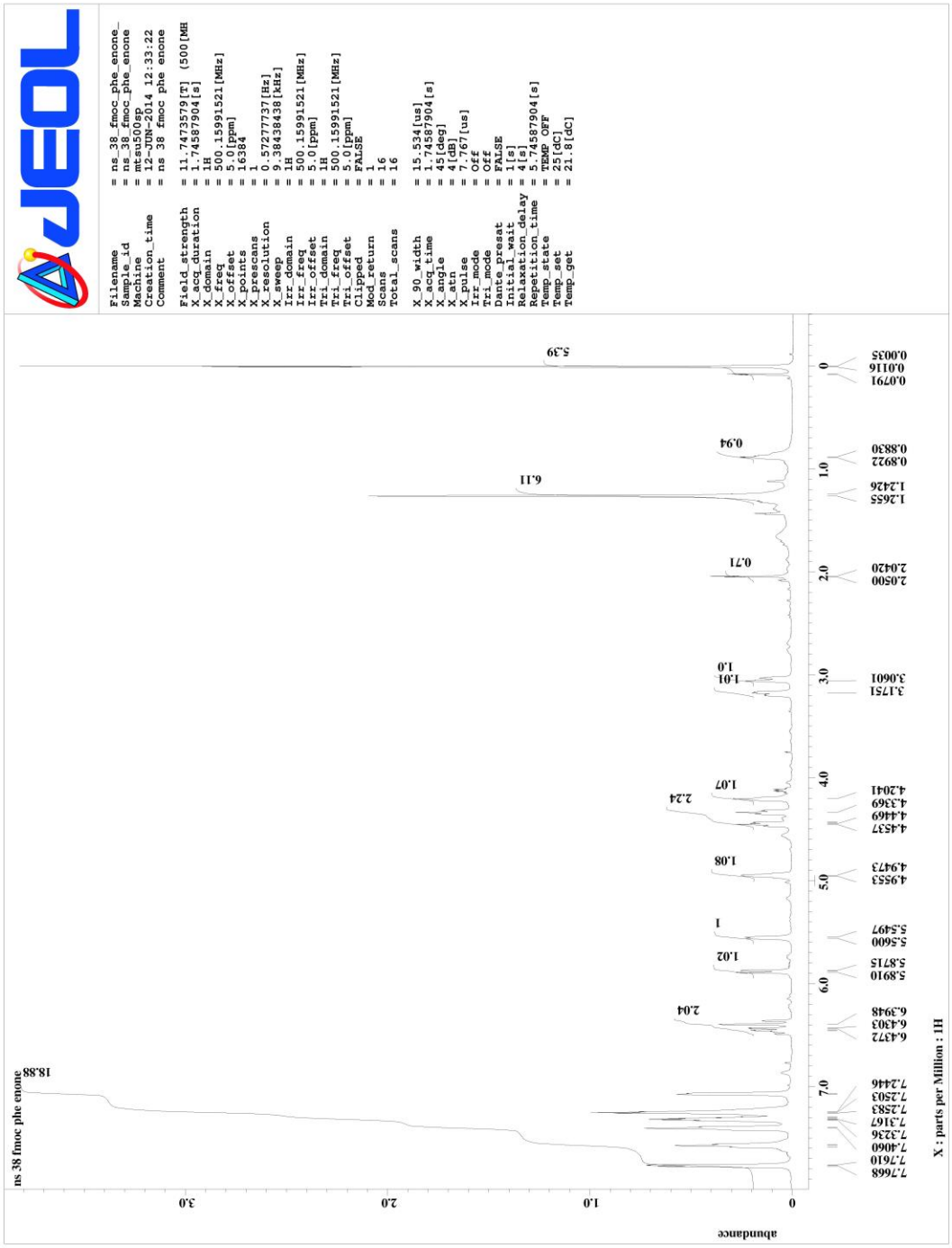
(S)-(9H-fluoren-9-yl)methyl 3-oxo-1-phenylpent-4-en-2-ylcarbamate (6b)

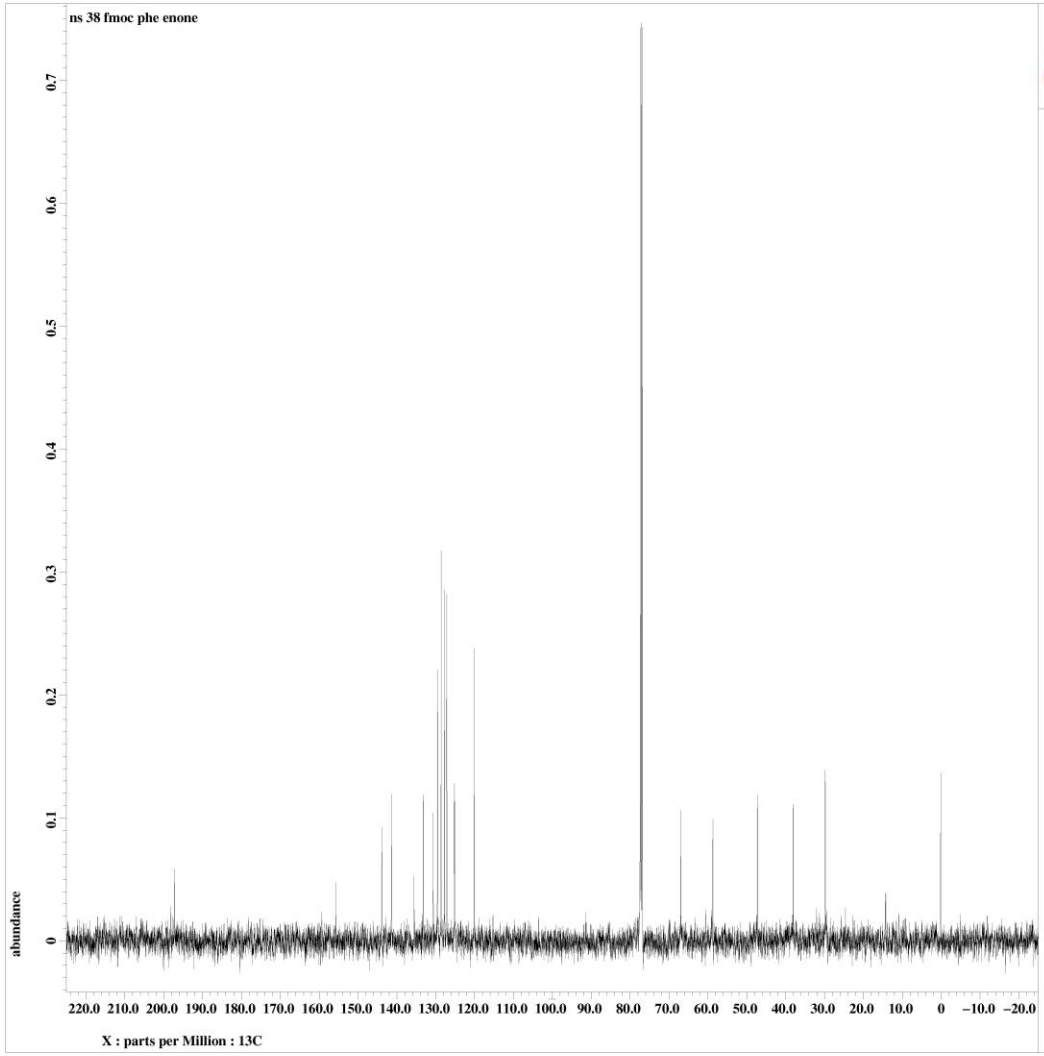


NMR data:

Proton

Carbon


JEOL
Filename = ns_38_fmoc_phe_enone_
Sample_id = ns_38_fmoc_phe_enone
Machine = mtsu500sp
Creation_time = 12-JUN-2014 12:33:22
Comment = ns 38 fmoc phe enone
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 15.534[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.767[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 21.8[dC]
ns 38 fmoc phe enone
18.88
5.39
6.11
2.24
2.04
0.94
0.71
1.08
1.07
1.02
1.01
1.0
1
abundance
X : parts per Million : 1H

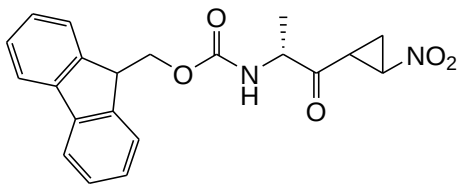



```
Filename          = ns_38_fmoc_phe_enone_
Sample_id         = ns_38_fmoc_phe_enone
Machine           = mtsu500sp
Creation_time     = 12-JUN-2014 12:37:27
Comment           = ns 38 fmoc phe enone

Field_strength    = 11.7473579[T] (500[MH
X_acq_duration    = 0.83361792[s]
X_domain          = 13C
X_freq            = 125.76529768[MHz]
X_offset          = 100[ppm]
X_points          = 32768
X_prescans        = 4
X_resolution      = 1.19959034[Hz]
X_sweep           = 39.3081761[kHz]
Irr_domain        = 1H
Irr_freq          = 500.15991521[MHz]
Irr_offset        = 5.0[ppm]
Clipped           = FALSE
Mod_return        = 1
Scans             = 72
Total_scans       = 72

X_90_width        = 9.804[us]
X_acq_time        = 0.83361792[s]
X_angle           = 30[deg]
X_atn             = 9[dB]
X_pulse           = 3.268[us]
Irr_atn_dec       = 21.5[dB]
Irr_atn_noe       = 21.5[dB]
Irr_noise         = WALTZ
Decoupling        = TRUE
Initial_wait      = 1[s]
Noe               = TRUE
Noe_time          = 2[s]
Relaxation_delay  = 2[s]
Repetition_time   = 2.83361792[s]
Temp_state        = TEMP OFF
Temp_set          = 25[dC]
Temp_get          = 22.2[dC]
```

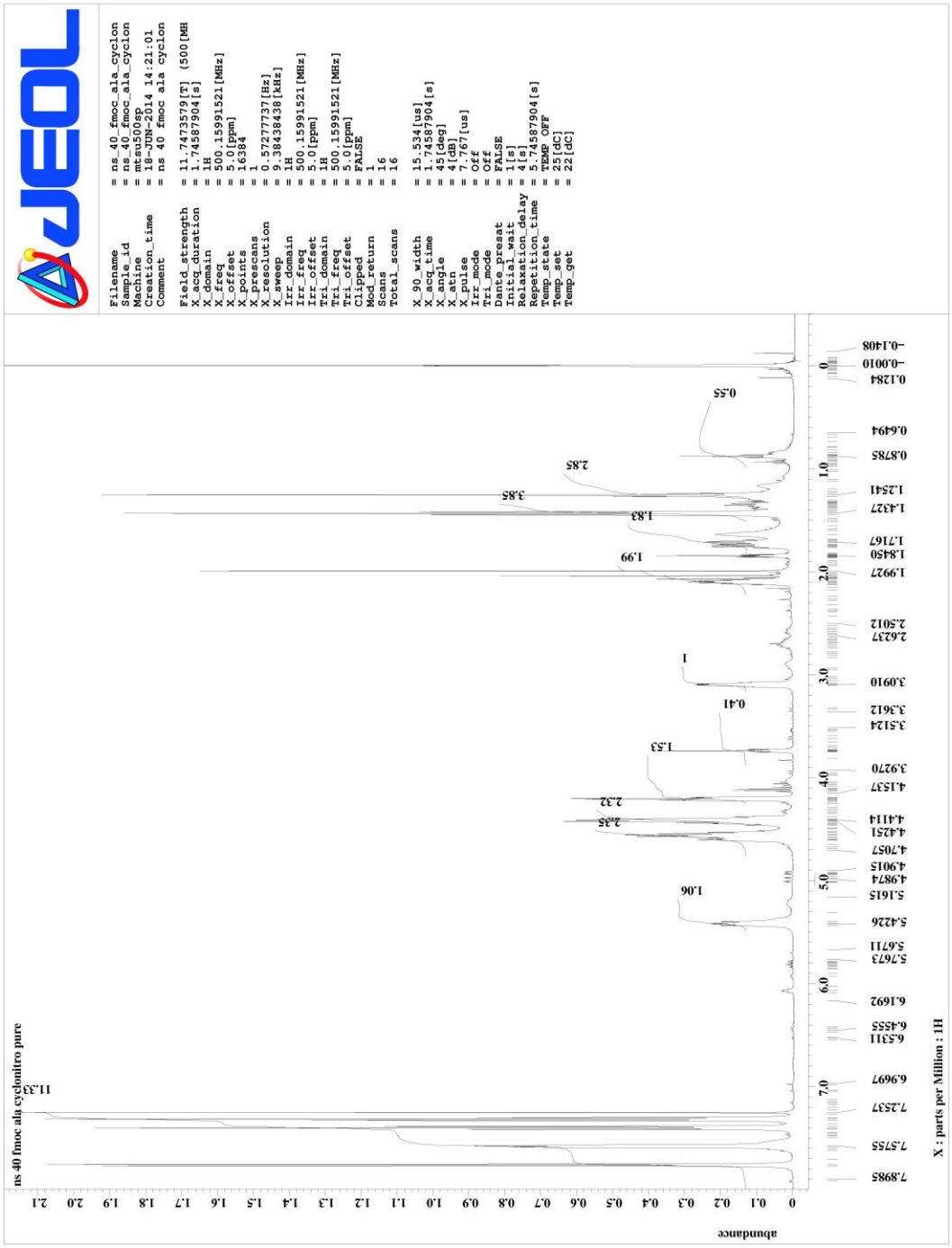
(9H-fluoren-9-yl)methyl (R)-1-(2-nitrocyclopropyl)-1-oxopropan-2-ylcarbamate (7a)

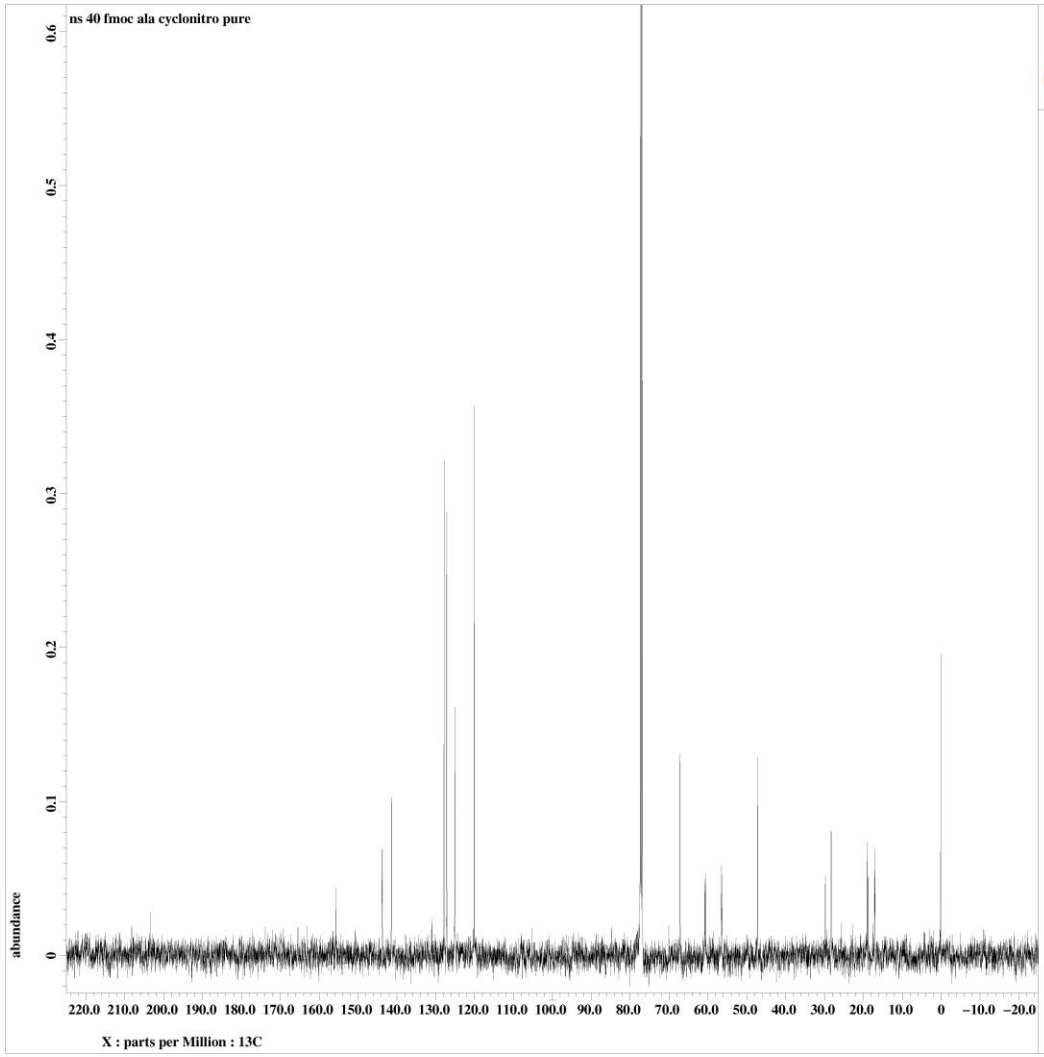


NMR data:

Proton

Carbon

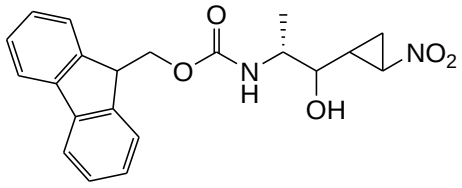

JEOL
Filename = ns_40_fmoc_ala_cyclon
Sample_id = ns_40_fmoc_ala_cyclon
Machine = mtsu500sp
Creation_time = 18-JUN-2014 14:21:01
Comment = ns 40 fmoc ala cyclon
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 15.534[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.767[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 22[dC]
ns 40 fmoc ala cyclonitro pure
11.33
1.06
2.32
2.35
1.53
0.41
1
1.99
1.83
3.85
2.85
0.55
abundance
X : parts per Million : 1H



JEOL

Filename	= ns_40_fmoc_ala_cyclon
Sample_id	= ns_40_fmoc_ala_cyclon
Machine	= mtsu500sp
Creation_time	= 18-JUN-2014 14:31:22
Comment	= ns 40 fmoc ala cyclon
Field_strength	= 11.7473579[T] (500[MH
X_acq_duration	= 0.83361792[s]
X_domain	= 13C
X_freq	= 125.76529768[MHz]
X_offset	= 100[ppm]
X_points	= 32768
X_prescans	= 4
X_resolution	= 1.19959034[Hz]
X_sweep	= 39.3081761[kHz]
Irr_domain	= 1H
Irr_freq	= 500.15991521[MHz]
Irr_offset	= 5.0[ppm]
Clipped	= FALSE
Mod_return	= 1
Scans	= 192
Total_scans	= 192
X_90_width	= 9.804[us]
X_acq_time	= 0.83361792[s]
X_angle	= 30[deg]
X_atn	= 9[dB]
X_pulse	= 3.268[us]
Irr_atn_dec	= 21.5[dB]
Irr_atn_noe	= 21.5[dB]
Irr_noise	= WALTZ
Decoupling	= TRUE
Initial_wait	= 1[s]
Noe	= TRUE
Noe_time	= 2[s]
Relaxation_delay	= 2[s]
Repetition_time	= 2.83361792[s]
Temp_state	= TEMP OFF
Temp_set	= 25[dC]
Temp_get	= 22.5[dC]

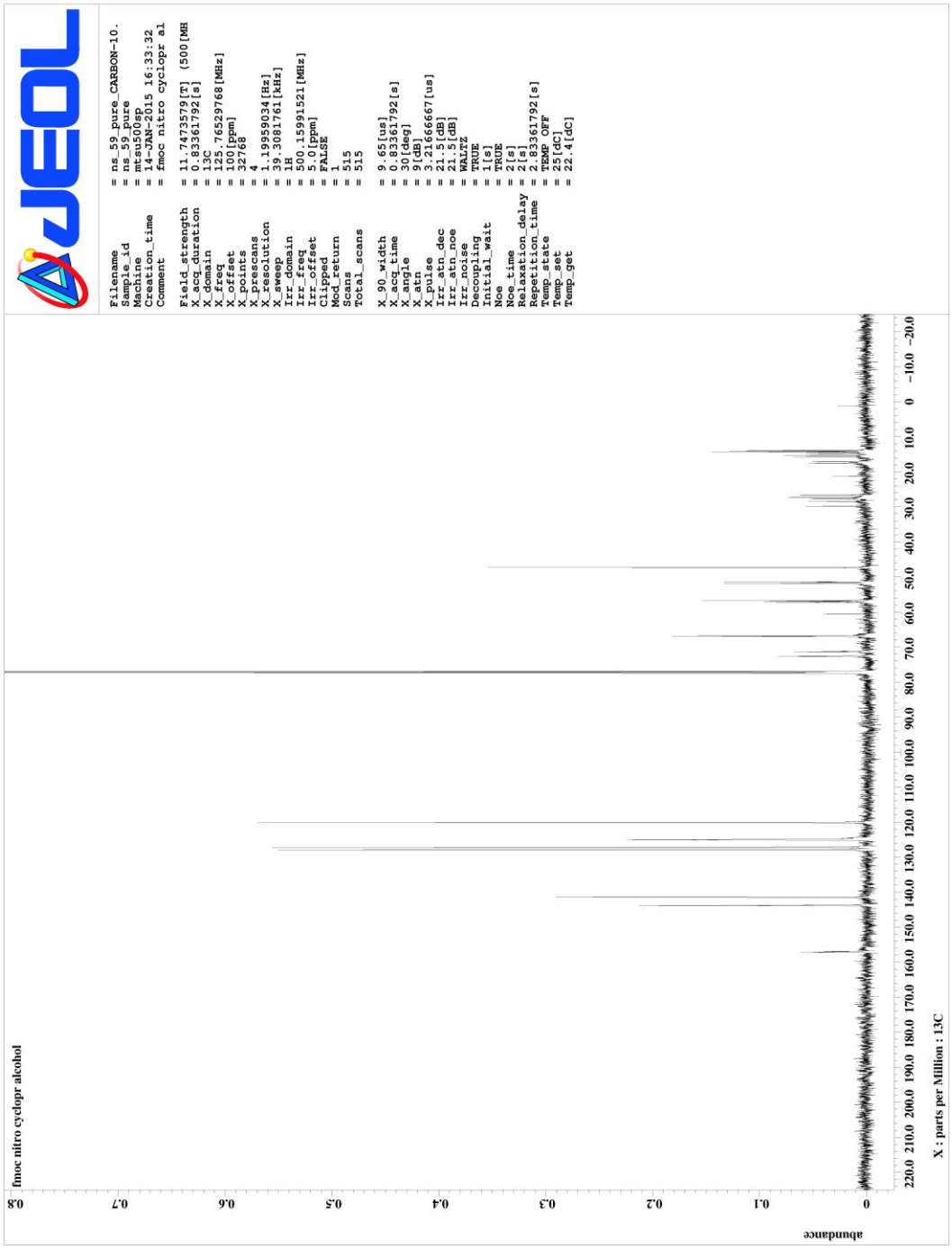
(9H-fluoren-9-yl)methyl (2R)-1-hydroxy-1-(2-nitrocyclopropyl)propan-2-ylcarbamate (Fmoc-alcohol)

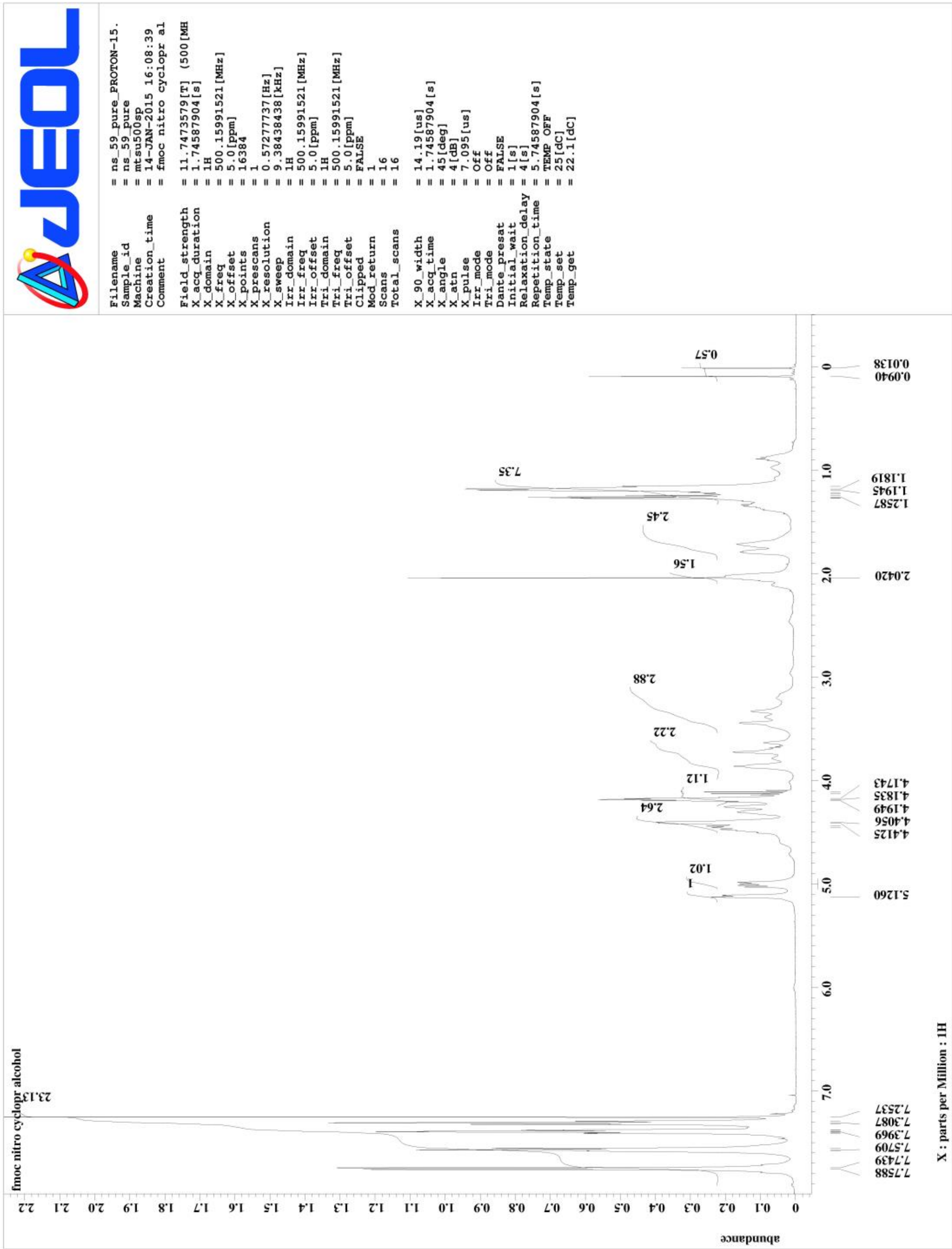


NMR data:

Proton

Carbon


JEOL
Filename = ns_59_pure_CARBON-10.
Sample_id = ns_59_pure
Machine = mtsu500sp
Creation_time = 14-JAN-2015 16:33:32
Comment = fmoc nitro cyclopr al
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 0.83361792[s]
X_domain = 13C
X_freq = 125.76529768[MHz]
X_offset = 100[ppm]
X_points = 32768
X_prescans = 4
X_resolution = 1.19959034[Hz]
X_sweep = 39.3081761[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 515
Total_scans = 515
X_90_width = 9.65[us]
X_acq_time = 0.83361792[s]
X_angle = 90[deg]
X_atn = 9[dB]
X_pulse = 3.21666667[us]
Irr_atn_dec = 21.5[dB]
Irr_atn_noe = 21.5[dB]
Irr_noise = WALTZ
Decoupling = TRUE
Initial_wait = 1[s]
Noe = TRUE
Noe_time = 2[s]
Relaxation_delay = 2[s]
Repetition_time = 2.83361792[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 22.4[dC]
fmoc nitro cyclopr alcohol
abundance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
220.0 210.0 200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0 0 -10.0 -20.0
X : parts per Million : 13C


fmoc nitro cyclopr alcohol
Filename = ns_59_pure_PROTON-15.
Sample_id = ns_59_pure
Machine = mtsu500sp
Creation_time = 14-JAN-2015 16:08:39
Comment = fmoc nitro cyclopr al
Field_strength = 11.7473579[T] (500[MH
X_acq_duration = 1.74587904[s]
X_domain = 1H
X_freq = 500.15991521[MHz]
X_offset = 5.0[ppm]
X_points = 16384
X_prescans = 1
X_resolution = 0.57277737[Hz]
X_sweep = 9.38438438[kHz]
Irr_domain = 1H
Irr_freq = 500.15991521[MHz]
Irr_offset = 5.0[ppm]
Tri_domain = 1H
Tri_freq = 500.15991521[MHz]
Tri_offset = 5.0[ppm]
Clipped = FALSE
Mod_return = 1
Scans = 16
Total_scans = 16
X_90_width = 14.19[us]
X_acq_time = 1.74587904[s]
X_angle = 45[deg]
X_atn = 4[dB]
X_pulse = 7.095[us]
Irr_mode = Off
Tri_mode = Off
Dante_presat = FALSE
Initial_wait = 1[s]
Relaxation_delay = 4[s]
Repetition_time = 5.74587904[s]
Temp_state = TEMP OFF
Temp_set = 25[dC]
Temp_get = 22.1[dC]
X : parts per Million : 1H
abundance
23.13
7.35
2.45
1.56
2.88
2.22
1.12
2.64
1.02
1
0.57
7.7588
7.7439
7.5709
7.3969
7.3087
7.2537
5.1260
4.4125
4.4056
4.1949
4.1835
4.1743
2.0420
1.2587
1.1945
1.1819
0.0940
0.0138