University of Nevada, Reno

Investigating the Effects of N-Methyl Modifications on Activity of a Truncated Group I Competence Stimulating Peptide (CSP1) on Quorum Sensing in *Streptococcus pneumoniae*

A thesis submitted in partial fulfillment
of the requirements for the degree of

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by

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Abstract

Bacterial infections are becoming increasingly difficult to treat as more bacteria develop antibiotic resistance. Our research aims to produce a therapeutic to block bacterial communication, rendering bacteria non-pathogenic without killing them, thus avoiding driving the evolution of resistant strains. Bacteria communicate through a phenomenon called quorum sensing, in which bacteria release signal molecules to indicate their population size and density. Once a population is large enough, it engages in behaviors that are effective only when the whole group, rather than individual bacterium, exhibit them. This phenomenon can induce previously non-pathogenic bacteria populations to attack their hosts. *S. pneumoniae* uses a 17-amino acid long peptide called competence stimulating peptide (CSP) to communicate. At a threshold concentration, CSP binds and activates a receptor called comD, starting a signaling cascade ending with bacteria exhibiting group behaviors such as virulence. CSP analogs that outcompete the native peptide for binding to comD could impede bacterial communication, and therefore, pathogenicity. However, finding an effective therapeutic is complicated by the fact that different strains of *S. pneumoniae* have different signaling molecules called CSP-1 and CSP-2 that will only bind respectively to comD-1 and comD-2, respectively. Our previous research has shown CSP-1 interacts slightly more effectively with comD-2 than CSP-2 will with comD-1, and that the final two residues on both peptides are unnecessary for binding. Therefore, the purpose of this project was to complete an *N*-methyl scan of a 15-amino acid long CSP-1 analog to determine the importance of different backbone hydrogen bonds on the activity of the peptide. Solid-phase peptide synthesis was utilized to construct a library of 15 *N*-methyl analogs, and cell-based reporter assays were conducted to evaluate the ability of the different analogs to modulate quorum sensing in both *S. pneumoniae* specificity groups.

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Introduction

Quorum sensing (QS) is a method used by bacteria to communicate population density in order to control behaviors that are only effective when performed by the entire population. Bacteria secrete a signaling molecule into their environment that will gradually accumulate and induce such behaviors at a threshold concentration (Neaslon and Hastings, 1979). This phenomenon was initially discovered by Alexander Tomasz, although he didn't coin the term QS at the time, when he found a "hormone-like" substance could initiate competence behavior in *Streptococcus pneumoniae* (Tomasz, 1965).

QS has been further studied and the basic mechanisms shared between different species are now known. The basic principle of secreting of a signal molecule and using its concentration to determine population density is universal, but there are different modes of secretion and signal reception. The mechanism used by gram-negative bacteria has been named LuxIR-type. Using a LuxI enzyme, bacteria produce small molecules such as acyl-homoserine lactones (AHLs) that diffuse freely in and out of the bacteria until the threshold concentration is reached, at which point an AHL will bind to its cognate LuxR protein intracellularly, which will then activate the transcription of different genes responsible for QS phenotypes (Manefield & Turner, 2002). Meanwhile, gram-positive bacteria secrete oligopeptides as signaling molecules that bind to a membrane-bound receptor kinase that transduces signal through phosphorylation of a transcription factor (Shepherd, et al., 2012). Because *S. pneumoniae* is gram-positive, this paper will focus on the latter system.

The specific mechanism of QS in *S. pneumoniae* is illustrated in Figure 1. A prepeptide is produced and then exported by an ABC-transporter named ComAB, which cleaves after a Gly-Gly bacteriocin consensus site to produce a mature signaling peptide

called Competence-Stimulating Peptide (CSP). Once exported, this peptide accumulates in the extracellular space and eventually binds to a membrane-bound histidine-kinase receptor called ComD, which then phosphorylates an aspartate residue on transcription factor, ComE. This transcription factor goes on to upregulate expression of the *ComCDE* and *ComAB* operons, thus increasing production of CSP and QS activity, as well as other operons containing "late genes" responsible for QS phenotypes such as ComX (Ween, 1999).

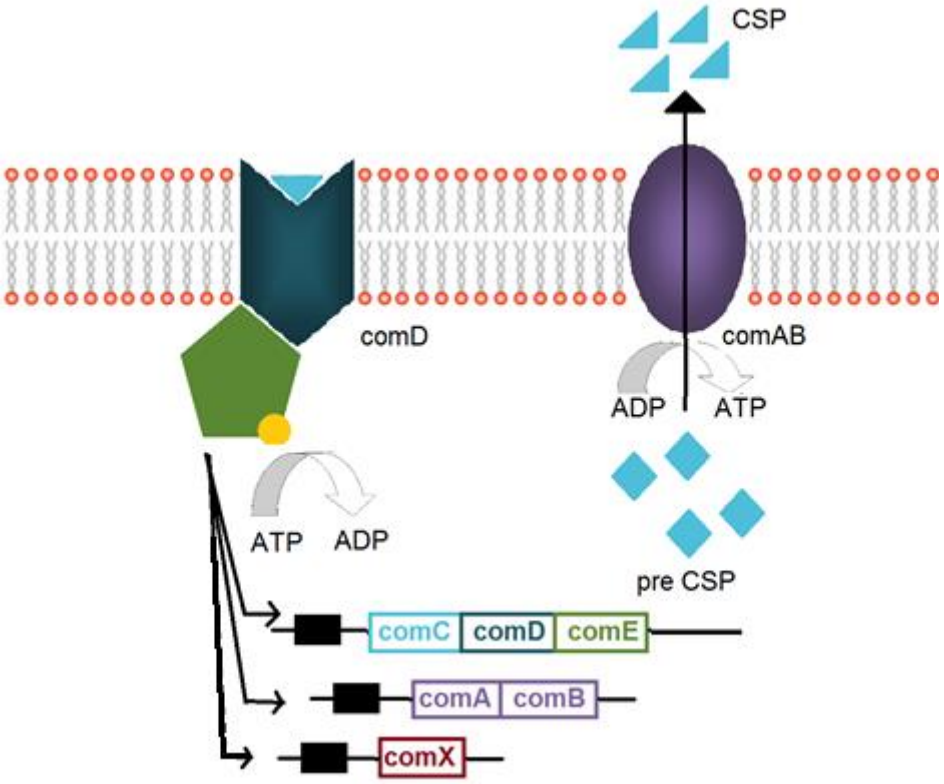


Figure 1. Schematic of Quorum Sensing in *Streptococcus pneumoniae.* *ComC* is transcribed into pre-CSP, which is then processed and exported by the comAB. At high oncentrations, CSP will bind to the comD histidine kinase receptor, which will autophosphorylate and phosphorylate comE. ComE is a transcription factor that binds directly to the promoters upstream of the *ComCDE* and *ComAB* operons. It also activates other "late genes" such as comX.

The phenotypes stimulated by QS vary across species, depending on the needs of the species. Different species will use QS to control bioluminescence and antibiotic production, as well as phenotypes associated with virulence such as biofilm formation, sporulation, virulence factor production, or any combination of these (reviewed in

Rutherford and Bassler, 2012). This makes QS a target for relatively novel "anti-virulence drugs." Drugs that disable virulence have become an increasingly attractive option as many traditional antibiotics become obsolete in the face of antibiotic resistance. Traditional antibiotics provide strong selective pressure for bacterial populations to develop resistance by killing only susceptible bacteria while leaving resistant mutants to survive and reproduce. Anti-virulence drugs would avoid killing the bacteria directly, and thus provide significantly less, if any, evolutionary pressure to favor resistance. There are numerous targets for anti-virulence drugs such as toxin secretion pathways, adhesins, and the QS pathway, to name a few (reviewed in Rasko and Sperandio, 2010). Some research suggests that even if there were some mutants with resistance to QS inhibitors (QSI), they would actually be subject to negative selection since they might attempt QS behaviors without the support of the entire population, placing them at an energy deficit and lowering their fitness compared to their QSI-susceptible neighbors (Gerdt, 2014).

A QSI would be an ideal drug to treat *S. pneumoniae* infections for several reasons. First and foremost, it would be a novel antimicrobial treatment for a disease the WHO has already listed as a priority on its list of pathogens in need of new antibiotic treatments (WHO, 2017). It would also prevent the bacteria from entering a competent state in which they have the ability to uptake antibiotic resistance genes. This would be most important in a clinical setting where there are likely to be other species and strains of bacteria present carrying such genes. The third reason is that it would attenuate virulence. The infections would be less aggressive and produce significantly less tissue damage in diseases such as pneumonia, meningitis, or other infections.

There are numerous strategies for designing an anti-virulence drug to affect QS. One method is to inhibit the secretion of CSP by targeting ComAB. For example, one lab found a small molecule that inhibits the peptidase activity of ComAB, thus preventing secretion of mature CSP and successfully attenuating biofilm formation. While this strategy has yielded promising results, it should be kept in mind that a drug targeting ComAB will be fairly broad-range and affect most *Streptococcus* species due to conservation of structure in the peptidase domain among ABC-transporters (Ishii, et al., 2016). Another proposed method is using various macromolecules to quench QS signals. There are already several known lactonases and acylases that inhibit QS in gram-negative bacteria relying AHLs, and it would be possible to make antibodies that would sequester signaling molecules. This is an active area of research, but it hasn't yet yielded any clinically or agriculturally practical molecules (Amara, 2011).

The strategy employed in this research is to make a molecule that mimics the native signaling molecule of *S. pneumoniae* enough to bind to the ComD receptor, but that produces a different level of activity than the native signal. An analog that could outcompete the native signal and bind to the receptor without activating the kinase activity would be a promising competitive inhibitor to prevent the expression of genes leading to virulent phenotypes. On the other hand, an analog that could outcompete the native signal and produce a higher level of activation would result in premature attempts at the group behaviors. Because the population density wouldn't be high enough to make those group behaviors productive, the bacteria would essentially be wasting energy and reducing their fitness to the point that they could be displaced by bacteria in the normal microbiota, or be made vulnerable to the host's immune system (Rasko and Sperandio, 2010).

Making analogs and testing their activity not only provides clinically applicable treatments, but will provide valuable information on the nature of the CSP:ComD interaction. Although a fair amount of research has gone into the QS system of *S. pneumoniae,* there is still a lot to learn about the system. It is known that mature CSP is a heptadecapeptide (Havarstein, 1995) and that there are two different CSP molecules used by different strains of *S. pneumoniae* (Figure 2).

CSP1:E-M-R-L-S-K-F-F-R-D-F-I-L-Q-R-K-K
CSP2:E-M-R-I-S-R-I-I-L-D-F-L-F-L-R-K-K

Figure 2. Sequences of Native Competence Stimulating Peptides (CSPs). Different *S. pneumoniae* strains will produce either or both peptide sequences, depending on whether the strain is Group 1 or Group2. CSP1 interacts with Group 1 receptors and CSP2 interacts with Group 2

The sequences of both these peptides are known, and although they are similar, they are fairly exclusive to their cognate receptors—these are called CSP1 and CSP2 and bind to ComD1 and ComD2 respectively (Pozzi et al., 1996). Its been shown that the central hydrophobic residues in CSP1 form a non-polar face on the amphiphilic α-helix formed by CSP1 in membrane-mimicking conditions, and that this face is critical for receptor binding and recognition (Johnsborg et al., 2005). Zhu and Lau also demonstrated that the first three residues (Glu-Met-Arg for both peptides) are important for receptor activation (2011). The ComD receptor itself has not been extensively studied, although it has been shown that the ComD receptor of *Streptococcus mutans* is a six pass transmembrane histidine kinase with three extracellular loops, two of which—loop C and loop B—appear to be required for CSP recognition (Dong et al., 2016). There is likely homology between *S. mutans* and *S. pneumoniae,* but that has yet to be confirmed.

Research in the Tal-Gan lab aims to expand upon this knowledge by systematically synthesizing analogs of CSP1 and CSP2 and determining the relative activity of each

analog in order to assess which chemical moieties are important for binding to and activation of ComD. After a systematic investigation, the results can be synthesized in order to rationally design peptides with multiple modifications that will modulate QS of both group I and group II bacteria to attenuate virulence. For both CSP1 and CSP2, alanine and D-amino acid scans have been performed by sequentially replacing each residue with either an alanine or its D-amino isomer. These analogs were incubated with reporter strains in which the *lacZ* operon was conjugated to the *comX* promoter. If the peptides were capable of activating the QS circuitry, there was an upregulation of β-galactosidase. The degree of upregulation was measured indirectly by using a colorimetric assay measuring digestion of 2-nitrophenyl beta-D-galactopyranoside into galactose and *ortho*-nitrophenol.

The point of the alanine scan was to determine which side chains were critical for maintaining either the peptide structure, or for interacting with the receptor. The D-amino acid scans were meant to reveal which chiral centers were important for the same functions. These scans confirmed the findings of Johnsborg and Zhu in that they demonstrated that the central hydrophobic residues and N-terminal residues are critical for binding and replacing any of them with alanine or switching their orientation reduces binding affinity. Circular dichroism analysis of all the analogs also supported Shepherd's claims that the peptides containing an α-helix are the most effective mimetics, as the most active analogs all adopted α-helical configurations in membrane-mimicking conditions.

These studies also revealed new information. The C-terminal lysines were determined to be unnecessary for binding or activation since substituting them with alanine, changing the chirality, and even removing them altogether did not significantly change the observed binding affinity. Switching the chirality of the tenth residue in CSP2 resulted in

an analog with a higher binding affinity than the native signal (EC_{50} value of 2.86 nM compared to EC_{50} value of 50.7 nM for the native peptide). It was also shown that substituting alanine for the lysine in the sixth position on CSP1 makes the peptide capable of interacting moderately effectively with both ComD1 and ComD2. All this information can be combined to design an analog that would successfully outcompete native CSP signals in an infection (Yang, et al. 2017). However, there is still more information to be gained.

The goal of this project was to complete an *N*-methyl scan of the minimal CSP1 scaffold required for effective ComD1 binding and activation, CSP1-*des*-K16K17. Doing so would allow determination which backbone hydrogen-bond donors play an important role in signaling. Sequentially replacing the hydrogens bound to the backbone nitrogens may elucidate which hydrogen bonds are critical either for forming the α-helix necessary for an active peptide, or for interacting with the receptor. Any of these modifications also have the potential to introduce new interactions to the system that could enhance binding affinity. Thus, performing this scan will provide more basic knowledge of the QS circuitry of *S. pneumoniae* and potentially new modifications to be considered in rational design of an anti-virulence drug to treat infections.

Materials and Methods

Chemical Reagents and Instrumentation

All chemical reagents and solvents were purchased from Sigma-Aldrich and used without further purification. 18 MΩ water was purified using a Millipore Analyzer Feed

System. Solid-phase resins were purchased from Advanced ChemTech and Chem-Impex International.

Reverse-phase high performance liquid chromatography (RP-HPLC) was carried out on a Shimadzu system with a CBM-20A communications bus module, two LC-20AT pumps, an SIL-20A auto sampler, an SPD-20A UV/vis detector, a CTO-20A column oven, and an FRC-10A fraction collector.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed using a Bruker Microflex spectrometer equipped with a 60 Hz nitrogen laser and a reflectron. In positive ion mode, Ion Source 1 was set to an acceleration voltage of 19.01 kV.

Exact Mass (EM) data was obtained on an Agilent Technologies 6230 TOF LC/MS spectrometer. The samples were sprayed with capillary voltage of 3500 V, and the electrospray ionization (ESI) source parameters were set at gas temperature of 325 °C at a drying gas flow rate of 8 L/min at a pressure of 35 psi.

Solid Phase Peptide Synthesis

Standard Fmoc-based solid phase peptide synthesis (SPPS) was used to make every CSP analog, with one exception to the standard protocol. When coupling an *N*-methylated amino acid and the subsequent amino acid, HATU was used in place of HBTU. A chloranil test was used after deprotecting the *N*-methyl amino acid to confirm the presence of a secondary amine. Peptides were constructed on a 4-benzyloxybenzyl alcohol (Wang) resin. Preloaded Fmoc-Arg(Pbf) Wang resin (0.237 mmol/g) was used for all peptides with an arginine as the *C*-terminal residue (White and Chan, 2000).

To synthesize the analog with *N*-methyl-arginine as the *C*-terminal residue, *N*-methyl-Fmoc-Arg(Pbf) was loaded onto a Wang resin linker using the standard symmetrical anhydride procedure. Then, a dipeptide, Fmoc-Leu-Gln-OH, was coupled to avoid the formation of diketopiperazine (DKP) side product when coupling the next amino acid to the *N*-methyl *C*-terminal Arg. From that point, standard Fmoc-based SPPS was used.

Peptide Purification

Crude peptides were purified using RP-HPLC with a semipreparative Phenomenex Kinetex C18 column at a flow rate of 5 mL/min. Analytical RP-HPLC was run using an analytical Phenomenex Kinetex C18 column at a flow rate of 1 mL/min. Purity was analyzed using integration of peaks from UV detection at 220 nm from the chromatogram of analytical RP-HPLC. For both types of runs, the mobile phase A=18 MΩ water + 0.1% TFA; mobile phase B=ACN+0.1% TFA.

Preparative HPLC methods were used to separate the crude peptide mixture into a relatively pure peptide and waste (5% B→45% B over 40 min). Then, an analytical HPLC method was used to quantify the purity of the peptide fraction using a linear gradient (5% B→ 95% B over 27 min). Only peptide fractions that were purified to greater than 95% purity were used for the biological assays. TOF-MS was used to confirm the identity of synthesized peptides.

Biological Reagents and Strain Information

All standard biological reagents were purchased from Sigma-Aldrich and used according to their enclosed instructions.

The strains used to perform the β-Galactosidase assays were D39pcomX::lacZ (group I) and TIGR4pcomX::lacZ (group II) reporter strains.

Biological Growth Conditions

Freezer stocks were 1.5 mL aliquots of bacteria (0.2 OD 600_{nm}) grown in Todd-Hewitt broth with 0.5% yeast extract (THY) and 0.5 mL glycerol. The stocks were stored at -80° C. Bacteria from the stocks were streaked onto THY agar plates containing 5% defibrinated donor horse serum and chloramphenicol at a final concentration of 4 μg/mL and allowed to incubate for 8-9 hours in a CO_2 incubator set at 37° C and 5% CO_2. Colonies were picked from the plates (one colony for D39pcomX::lacZ; multiple colonies for TIGR4pcomX::lacZ) and transferred to 5 mL of THY broth containing 4 μg/mL of chloramphenicol to grow overnight (approximately 15 hours) in a CO_2 incubator. These cultures were then diluted with THY (1:50 dilution for D39pcomX::lacZ; 1:10 dilution for TIGR4pcomX::lacZ) and incubated in the CO_2 incubator until they reached early exponential stage (0.30−0.35 OD 600_{nm} for D39pcomX::lacZ; 0.20−0.25 OD 600_{nm} for TIGR4pcomX::lacZ).

β-Galactosidase Assays

Activation Assays

Activation assays were used to measure activation of the QS pathway caused by the *N*-methyl analogs. An initial activation screening was performed at a high concentration (10 μM) for all analogs. A total of 2 μL of 1 mM solution of CSP analogs in dimethyl sulfoxide (DMSO) was added in triplicate to a clear 96-well microtiter plate. As a positive control, 2 μL of each native peptide was added in triplicate (concentration = 20 μM CSP1 for D39pcomX::lacZ; concentration = 100 μM CSP2 for TIGR4pcomX::lacZ). These

concentrations were used to ensure full activation of the QS circuit, based on dose-dependent curves created for the native CSPs. A total of 2 μL of DMSO was added in triplicate as the negative control for both groups. 198 μL of diluted overnight bacterial culture was added to each well and incubated at 37°C for 30 min, after which, the OD 600_{nm} was measured. The cells were then lysed by adding 20 μL of 0.1% Triton X-100 and incubated for 30 min at 37 °C to release the β-galactosidase from the cells. In a new plate, 100 μL of Z-buffer solution (60.2 mM Na_2HPO_4, 45.8 mM NaH_2PO_4, 10 mM KCl, and 1.0 mM $MgSO_4$ in 18 MΩ H_2O; pH was adjusted to 7.0, and the buffer was sterilized before use) containing 2-nitrophenyl beta-D-galactopyranoside (ONPG) at a final concentration of 0.4 mg/mL was added, followed by 100 μL of lysate, and the plate was incubated for 3 h at 37 °C. The free β-galactosidase present in the lysate acted on the ONPG to produce *ortho*-nitrophenol, resulting in the solution turning yellow. The reaction was stopped by adding 50 μL of 1 M sodium carbonate solution. The OD 420_{nm} and OD 550_{nm} were measured using a plate reader. The final results were reported as percent activation, which is the ratio between the Miller units of the analog and of the positive control.

$$\text{Miller unit} = 1000\times[Abs_{420}-(1.75\times Abs_{550})]/(t\times v\times Abs_{600})$$

Where t = incubation time with ONPG (min), v = volume of lysate (mL), Abs_{420} shows the absorbance of ONP, Abs_{500} corrects for scatter from cell debris, and Abs_{600} accounts for cell density (Yang et al, 2017).

Inhibition Assays

Analogs that activated QS less than 50% compared to the native peptide were assessed for their ability to function as competitive inhibitors. This was done by putting both the native CSP and an analog in the same well and measuring activation of the circuit.

In a clear 96-well microtiter plate, 2 μL of 1 mM solution of a CSP analog were added in triplicate and combined with 2 μL of native CSP (concentration = 5 μM CSP1 for D39pcomX::lacZ; concentration = 25 μM CSP2 for TIGR4pcomX::lacZ). For positive controls, 2 μL of native CSP (at the same concentration) and 2 μL of DMSO were added in the same well as triplicate. As a negative control, 4 μL of DMSO was added in triplicate. A total of 196 μL of bacterial culture was added to each well. The procedures for incubation with the peptide, lysis, incubation with ONPG, and all the measurements were the same as those used in the activation assay (Yang et al, 2017).

Circular Dichroism

CD spectra were taken with an Aviv Biomedical CD spectrometer (model 202−01). All the samples had a peptide concentration of 200 μM in a solution of PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH was adjusted to 7.4) and 20% trifluoroethanol (TFE) as membrane-mimicking conditions. Measurements were performed at 25° C with a quartz cuvette (science outlet) with a path length of 0.1 cm. Samples were scanned once at 3 nm min^{-1} with a bandwidth of 1 nm and a response time of 20 s over a wavelength range of 195 to 260 nm. Percent helicity (f_H) was calculated for peptides that exhibited a significant helical pattern using the following equation:

$$f_H=[\theta]_{222}/[\theta_\infty]_{222}(1 - {}^{x}/_{n})$$

$[\theta]_{222}$ is the mean residue ellipticity of the peptide at 222 nm, $[\theta_\infty]_{222}$ is the mean residue ellipticity of an ideal peptide with 100% helicity (-44,000 deg cm^2 $dmol^{-1}$), *n* is the number of residues in the potential helical region, and *x* is an empirical correction for end effects (2.5) (Luo & Baldwin, 1997).

Results

Peptide Purification

Table 1. Assessment of Purity and Confirmation of Identity of Peptide Analogs. After synthesis, each peptide was purified using RP-HPLC, and each fraction was run through an analytical column to determine its purity. Pure fractions were analyzed using TOF-MS, and the difference between the expected m/z ratio and the observed m/z ratio was calculated in parts per million.

Name	Formula	Mass (Da)	Purity	TOF-MS Difference (ppm)
CSP1(15)-E1A	$C_{89}H_{142}N_{26}O_{20}S$	1927.06	98.6%	3.6845129
CSP1(15)-K6A	$C_{88}H_{137}N_{25}O_{22}S$	1928.01	96.7%	4.3697801
CSP1(15)-*N*-Me-E1	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	96.9%	0.4493008
CSP1(15)-*N*-Me-M2	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	98.9%	1.4976694
CSP1(15)-*N*-Me-R3	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	98.8%	2.5460379
CSP1(15)-*N*-Me-L4	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	98.8%	4.3953848
CSP1(15)-*N*-Me-S5	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	97.8%	2.0967371
CSP1(15)-*N*-Me-K6	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	98.8%	1.1981355
CSP1(15)-*N*-Me-F7	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	97.3%	0.8990559
CSP1(15)-*N*-Me-F8	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	97.8%	1.5347244
CSP1(15)-*N*-Me-R9	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	97.6%	3.9758463
CSP1(15)-*N*-Me-D10	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	99.4%	0.9895726
CSP1(15)-*N*-Me-F11	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	97.4%	4.3816812
CSP1(15)-*N*-Me-I12	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	99.4%	3.7384224
CSP1(15)-*N*-Me-L13	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	98.6%	1.1631954
CSP1(15)-*N*-Me-Q14	$C_{92}H_{146}N_{26}O_{22}S$	2000.09	97.2%	2.9586312

β-Galactosidase Assays

An initial screen was performed to assess each analog's ability to activate QS in both specificity groups relative to the native signal (Figure 3). Unsurprisingly, more analogs were capable of activating QS in Group I than Group II, which was to be expected given that CSP1-*des*-K16K17 (referred to as CSP1(15)) was used as the basic scaffold. However, CSP1(15)-K6A was as effective at activating Group I as Group II. Modification of the first three residues, either *N*-methylation or alanine mutation, rendered the analogs incapable of activating either Group I or Group II bacteria. Other modifications did not have such clear effects. For example, *N*-methylation of L4, F7, F8, and Q14 seems to have little effect on an analog's ability to activated ComD1, but seems to greatly diminish its ability to activate ComD2.

Analogs that failed to activate the circuit at least 50% as well as the native CSP were tested for their ability to competitively inhibit QS (Figure 4). In the inhibition screening, bacteria are incubated both with their native CSP and the synthetic analogs. If an analog is inactive and simply fails to bind to ComD, the native CSP present will still activate the circuit; however, a competitive inhibitor will still bind to ComD, but fail to activate QS, resulting in a lower signal. Only CSP1(15)-E1A showed competitive inhibition, and only against Group I bacteria.

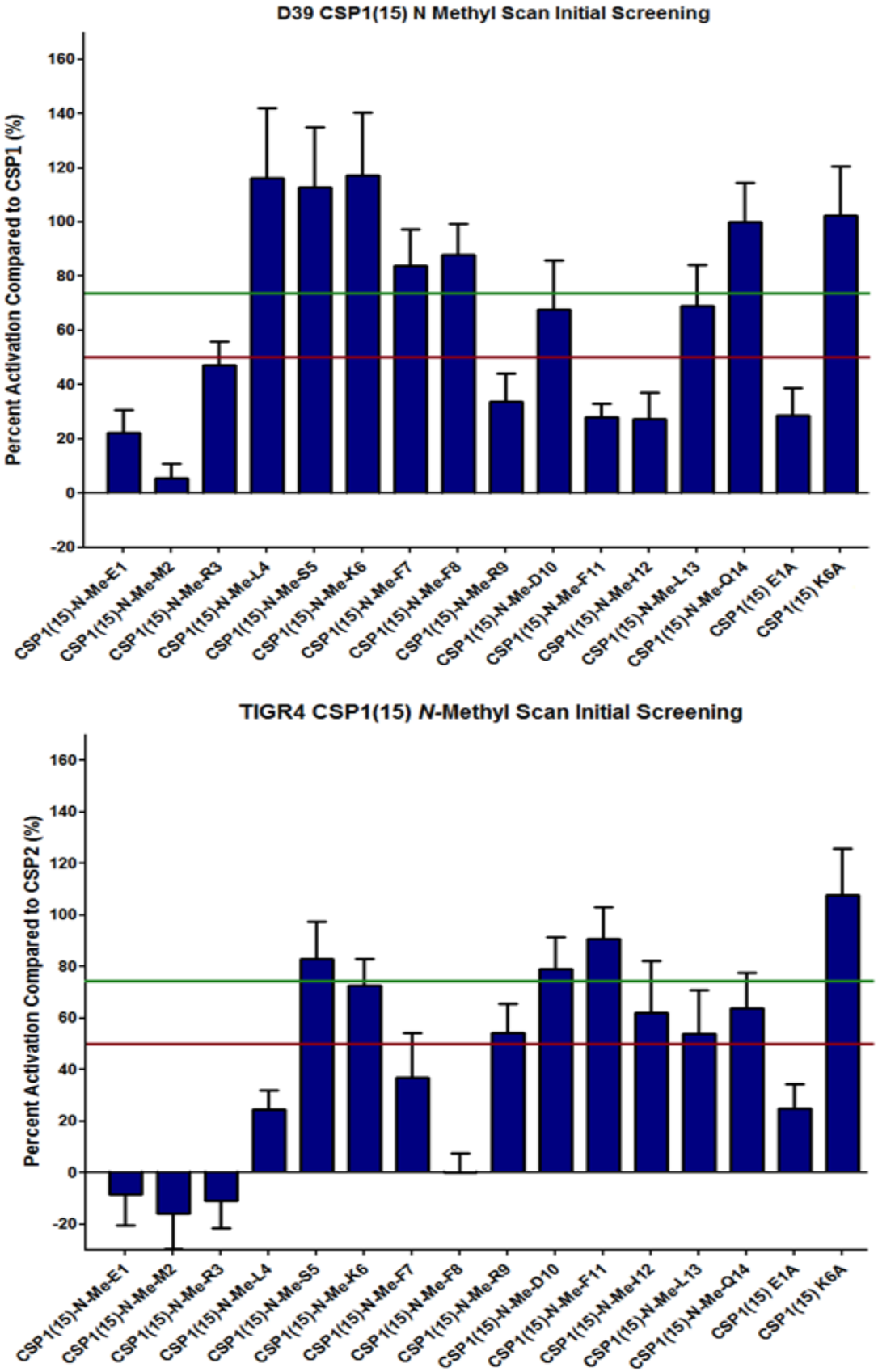


Figure 3. Activation Screening of Truncated CSP analogs against Group I and Group II *S. pneumoniae.* Bacteria were incubated with each analog at a high concentration (100 nM). The amount of β-galoctosidase produced was indirectly measured as absorbance of 420 nm light. The absorbance produced by each analog was compared to that produced by the native CSP for each group, and reported as a percent activation. Analogs that activated less than 50% as well as the native signals were tested for their ability to competitively inhibit QS.

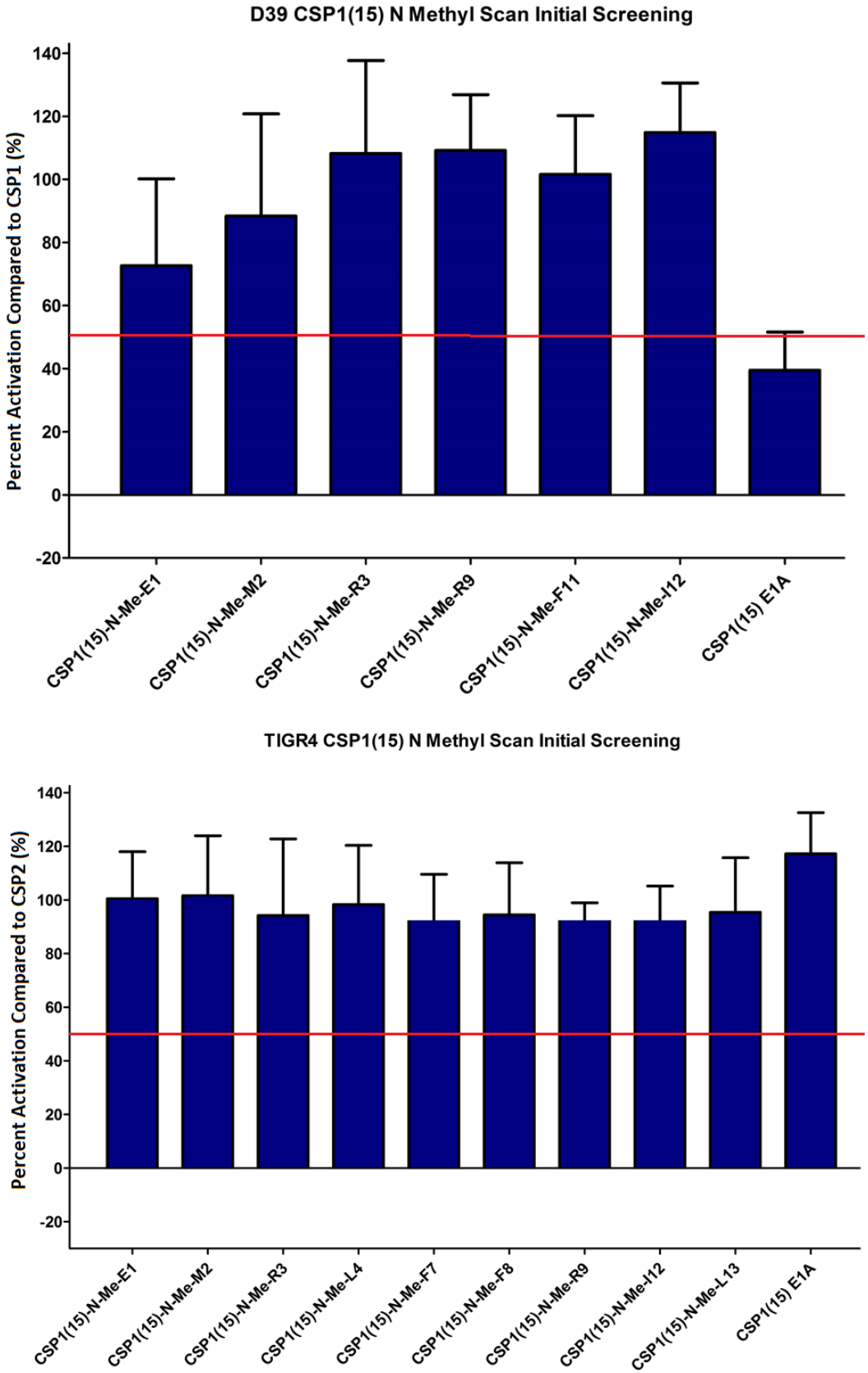


Figure 4. Inhibition Screening of Truncated CSP analogs against Group I and Group II *S. pneumoniae*. Analogs with less than 50% activation in the initial screening were tested for their ability to competitively inhibit QS. Bacteria were incubated with both an analog and the native CSP in the same well. Analogs capable of binding to ComD without activating the circuit would lower the percent activation. Analogs incapable of binding would allow the native signal to activate QS and maintain a high percent activation. Only CSP1(15)E1A shows any competitive inhibition.

Circular Dichroism

Previous research had demonstrated that more biologically active analogs had a tendency to form an α-helix in membrane-mimicking conditions. Given that *N*-methylation of critical residues could interfere with the peptide's ability to form the necessary intramolecular hydrogen bonds to form an α-helix, circular dichroism was performed on each analog to determine its secondary structure (Figure 5). Most analogs still show at least some degree of α-helicity (Table 1). However, the analogs that were *N*-methylated on a central residue (S5 through F11) show a significant decrease in both CD signal and helicity.

Table 2. Helicity of Each Peptide Calculated from CD spectra. The data from the CD spectra was run through the single spectrum analysis tool in Beta Structure Selection (BeStSel) to obtain the percent helicity of each analog (Micsoni, et al).

Peptide name	% Helicity	Peptide name	% Helicity
CSP1(15)	25.6%	CSP1(15)-*N*-Me-F7	9.3%
CSP1(15)E1A	22.7%	CSP1(15)-*N*-Me-F8	8.1%
CSP1(15)K6A	16.8%	CSP1(15)-*N*-Me-R9	4.4%
CSP1(15)-*N*-Me-E1	31.3%	CSP1(15)-*N*-Me-D10	7.5%
CSP1(15)-*N*-Me-M2	24.8%	CSP1(15)-*N*-Me-F11	4.4%
CSP1(15)-*N*-Me-R3	21.1%	CSP1(15)-*N*-Me-I12	10.9%
CSP1(15)-*N*-Me-L4	24.8%	CSP1(15)-*N*-Me-L13	9.6%
CSP1(15)-*N*-Me-S5	18.4%	CSP1(15)-*N*-Me-Q14	16.5%
CSP1(15)-*N*-Me-K6	10.8%		

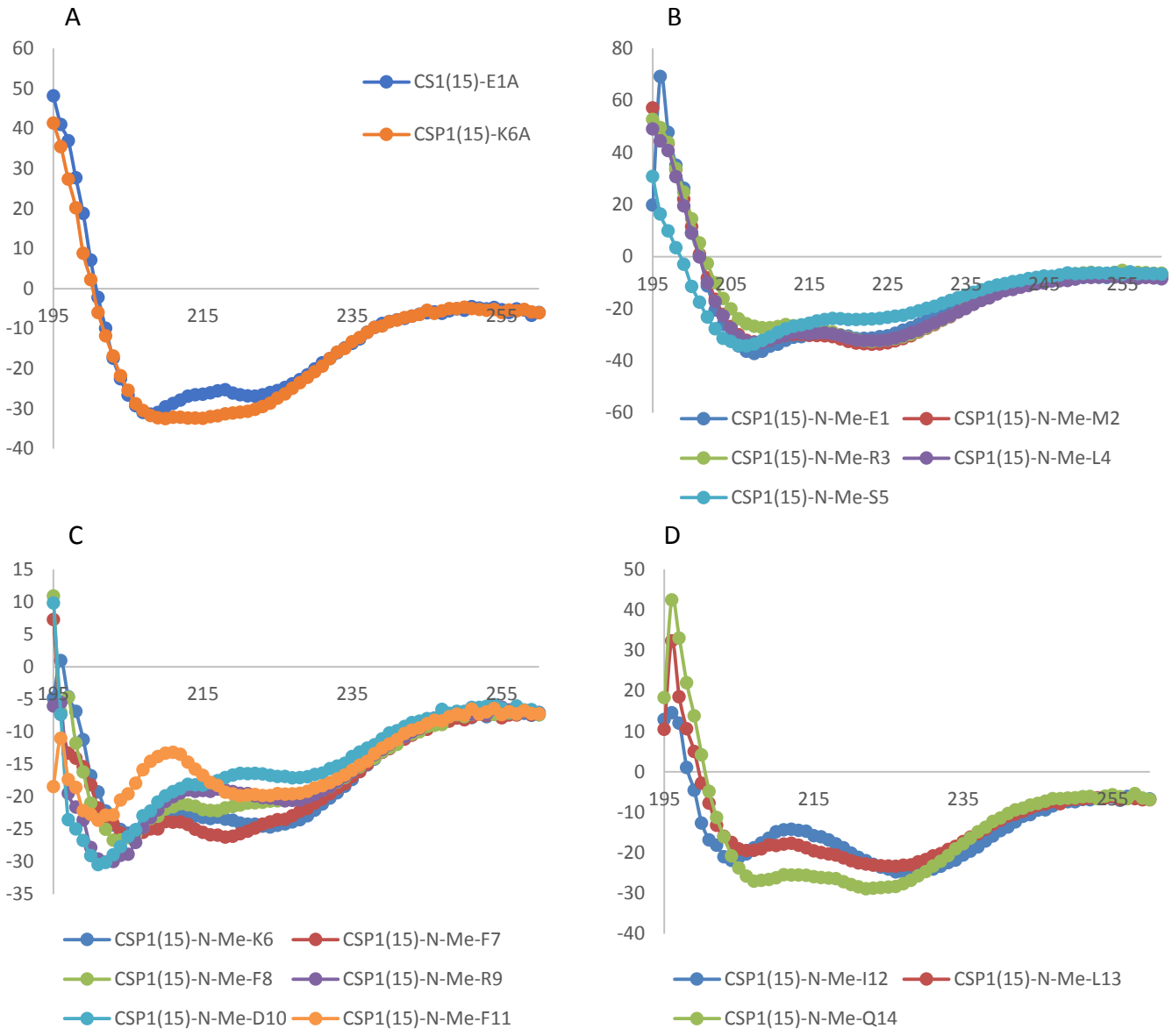


Figure 5. Circular Dichroism Spectra of Truncated CSP1 in Membrane-mimicking Conditions. Every spectra was taken with samples at a concentration of 200 μM in 20% TFE in PBS buffer (pH = 7.4) **(**A) CD spectra of truncated CSP1 alanine mutations. Both show expected α-helicity. (B) CD spectra of analogs with the first four residues *N*-methylated. These also show a fairly regular α-helical pattern. (C) CD spectra of analogs with the sixth through eleventh residue *N*-methylated. These spectra show a departure from a typical α-helix pattern, and have a much smaller signal range than the other peptides. (D) CD spectra of analogs with final three residues *N*-methylated. These spectra again begin to approach a normal range and α-helical pattern

Discussion

The purpose of this research was to determine which of the hydrogens in the peptide backbone of CSP1-*des*-K16K17 were critical for the signal's ability to activate QS in *S. pneumoniae.* Over the course of this research, we have confirmed our older findings and discovered some new information. Our previous research had shown that mutating the sixth residue (lysine) to an alanine in CSP1 allowed that analog to act as an activator for both Group I and Group II QS. This pan-activation ability was still present even the last two lysine residues were removed, as shown by the ability of CSP1(15)-K6A to activate both groups. Likewise, our own research group and others had found that mutating the first residue (glutamate) to an alanine produced a Group I inhibitor, which remains to be the case in the truncated peptide.

Our new findings show that while some *N*-methylation mutations are tolerable, others disrupt the structure enough that the peptides can no longer effectively bind to either ComD receptor. This is demonstrated by the inhibition screens, in which none of the *N*-methylated peptides being screened could bind effectively enough to outcompete the native CSP present and prevent activation of QS (Figure 4). It is unclear whether this inability to bind is due to a lack of signal:receptor hydrogen bonds, or to disruption of intramolecular hydrogen bonds that would stabilize the α-helical conformation seen in more active analogs.

It is possible both are true for different peptides. For example, the three N-terminal residues are unlikely to be part of the α-helix, and the CD spectra of the *N*-methylated analogs show a fairly normal α-helix (Figure 5), so it seems more likely their backbone hydrogens are forming critical intermolecular hydrogen bonds with ComD, and removing

their hydrogens prevents signal binding. On the other hand, perhaps the *N*-methylation of the central residues prevents effective α-helix formation, and that is the reason for poor binding. It is interesting to note that many of the *N*-methylations in this central region seem to be more tolerable to one receptor at the expense of its ability to bind to the other, which means they can't be destabilizing the helix too much or the analogs wouldn't be capable of activating either receptor. For example, the analogs containing *N*-methylations in L4, F7, and F8 completely failed to bind to the ComD2 receptor of the TIGR4 strain but were capable of fully activating ComD1 in the D39 strain. Meanwhile, *N*-methylating F11 has the opposite effect: CSP1(15)-*N*-Me-F11 can activate ComD2 but not ComD1, despite the fact that both CSP1 and CSP2 have a phenylalanine in the eleventh position (Figures 3 and 4). While these are interesting results, they can't yet be explained. Further tests to determine the EC_{50} of each analog are necessary before making any solid conclusions. The screenings are performed at high concentration, so it is possible that the analogs that seem to activate only one specificity group but are only marginally capable of activating one over the other, and aren't actually very good at activating either.

One unexpected finding was the result the *N*-methylations had on the CD spectra. We had expected disruption of an α-helix, resulting in either more random coil or β-sheet formation. Instead, what seemed to happen was the signal itself was reduced especially in the central residues. This is shown in Figure 5. Part C shows that the range for CD signal in the *N*-methylated central residues is only about 40 units, whereas for all the others, the range is closer to 90-100. These central spectra also show a more irregular shape compared to the relatively recognizable α-helix pattern seen in the other spectra. The reduction in signal would suggest these peptides either absorb of left- and right-handed circularly

polarized light more evenly than other peptides, or just absorb less light in general. However, its difficult to determine why they would have more even light absorption, and why the shape of their spectra is so disordered. Given that there haven't been any spectroscopic studies on the effects *N*-methylation can have on CD spectra, more research will have to be conducted on these effects to determine what is happening.

References

Dong, G. F., Tian, X. L., Cyr, K., Liu, T. L., Lin, W., Tziolas, G., & Li, Y. H. (2016). Membrane Topology and Structural Insights into the Peptide Pheromone Receptor ComD, A Quorum-Sensing Histidine Protein Kinase of Streptococcus mutans. *Scientific Reports, 6*. doi:10.1038/srep26502

Gerdt, J. P., & Blackwell, H. E. (2014). Competition Studies Confirm Two Major Barriers That Can Preclude the Spread of Resistance to Quorum-Sensing Inhibitors in Bacteria. - *ACS Chemical Biology*(10), 2291-2299. doi:10.1021/cb5004288

Havarstein, L. S., Coomaraswamy, G., & Morrison, D. A. (1995). An Unmodified Heptadecapeptide Pheromone Induces Competence for Genetic-Transformation in *Streptococcus Pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America, 92*(24), 11140-11144. doi:10.1073/pnas.92.24.11140

Johnsborg, O., Kristiansen, P. E., Blomqvist, T., & Havarstein, L. S. (2006). A hydrophobic patch in the competence-stimulating peptide, a pneumococcal competence pheromone, is essential for specificity and biological activity. *Journal of Bacteriology, 188*(5), 1744-1749. doi:10.1128/jb.188.5.1744-1749.2006

Luo, P., and Baldwin, R. L. (1997). Mechanism of helix induction by trifluoroethanol: a framework for extrapolating the helix-forming properties of peptides from trifluoroethanol/water mixtures back to water. *Biochemistry*, 36, 8413−8421. doi: 10.1021/bi9707133

Ng, W. L., Wei, Y. Z., Perez, L. J., Cong, J. P., Long, T., Koch, M., . . . Bassler, B. L. (2010). Probing bacterial transmembrane histidine kinase receptor-ligand interactions with natural and synthetic molecules. *Proceedings of the National Academy of Sciences of the United States of America, 107*(12), 5575-5580. doi:10.1073/pnas.1001392107

Micsoni, A., Wien, F., Kernya, L., Lee, Y-H., Goto, Y., Réfrégiers, M., and Kardos, J. (2015). *PNAS* 112:E3095-103

Pozzi, G., Masala, L., Iannelli, F., Manganelli, R., Havarstein, L. S., Piccoli, L., . . . Morrison, D. A. (1996). Competence for genetic transformation in encapsulated strains of Streptococcus pneumoniae: Two allelic variants of the peptide pheromone. *Journal of Bacteriology, 178*(20), 6087-6090. doi:10.1128/jb.178.20.6087-6090.1996

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harbor Perspectives in Medicine, 2*(11). doi:10.1101/cshperspect.a012427

Shepherd, N. E., Harrison, R. S., & Fairlie, D. P. (2012). Targeting quorum sensing and competence stimulation for antimicrobial chemotherapy. *Curr Drug Targets, 13*(11), 1348-1359. doi

Tomasz, A. (1965). Control of competent state in Pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria. *Nature, 208*(5006), 155-&. doi:10.1038/208155a0

Ween, O., Gaustad, P., & Havarstein, L. S. (1999). Identification of DNA binding sites for ComE, a key regulator of natural competence in Streptococcus pneumoniae. *Molecular Microbiology, 33*(4), 817-827. doi:10.1046/j.1365-2958.1999.01528.x

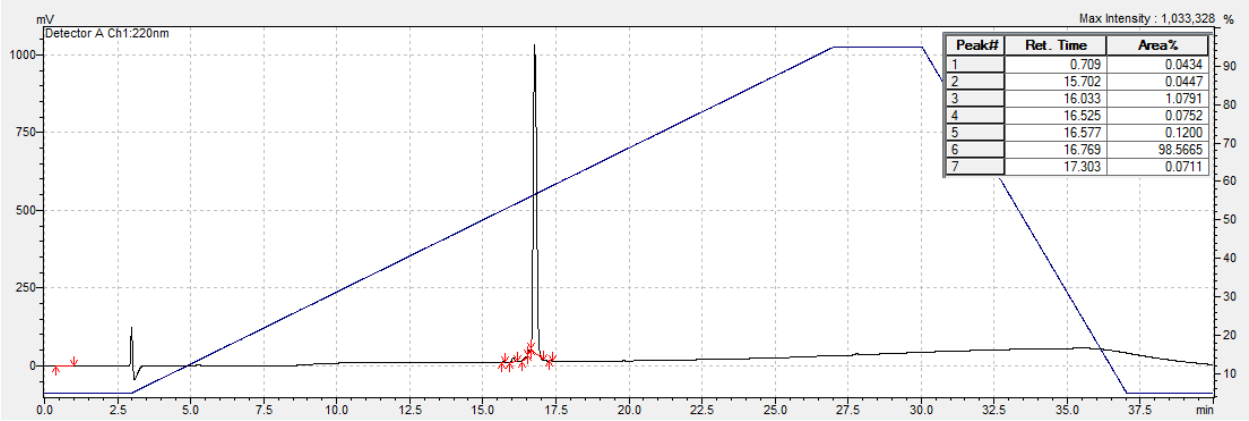
White, P. D., & Chan, W. C. (2000). *Fmoc Solid Phase Peptide Synthesis* (Vol. 222). New York Oxford University Press.

Yang, Y., Koirala, B., Sanchez, L. A., Phillips, N. R., Hamry, S. R., & Tal-Gan, Y. (2017). Structure–Activity Relationships of the Competence Stimulating Peptides (CSPs) in Streptococcus pneumoniae Reveal Motifs Critical for Intra-group and Cross-group ComD Receptor Activation. *ACS Chemical Biology*, a012427. doi:10.1021/acschembio.7b00007

Zhu, L. C., & Lau, G. W. (2011). Inhibition of Competence Development, Horizontal Gene Transfer and Virulence in Streptococcus pneumoniae by a Modified Competence Stimulating Peptide. *Plos Pathogens, 7*(9). doi:10.1371/journal.ppat.1002241

Appendix: Analytical HPLC Chromatograms of analogs

CSP1(15)-E1A



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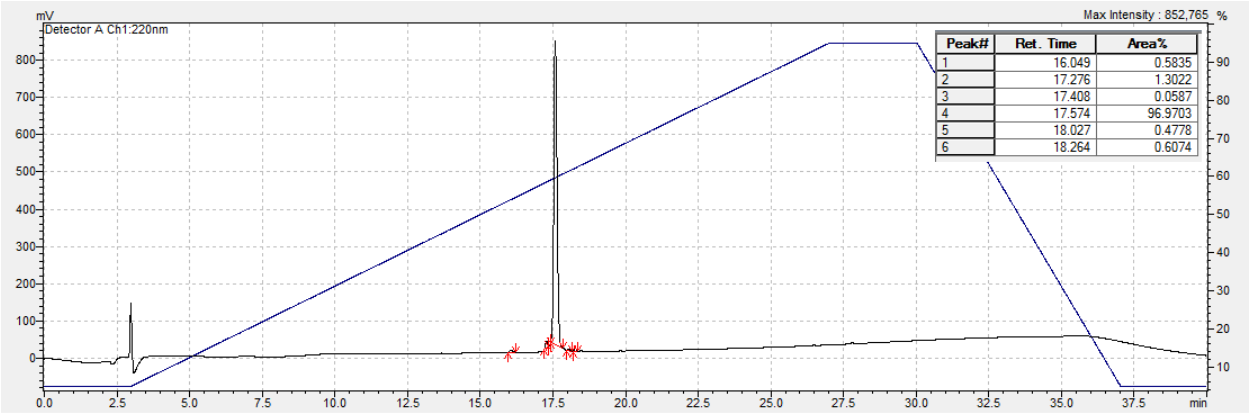

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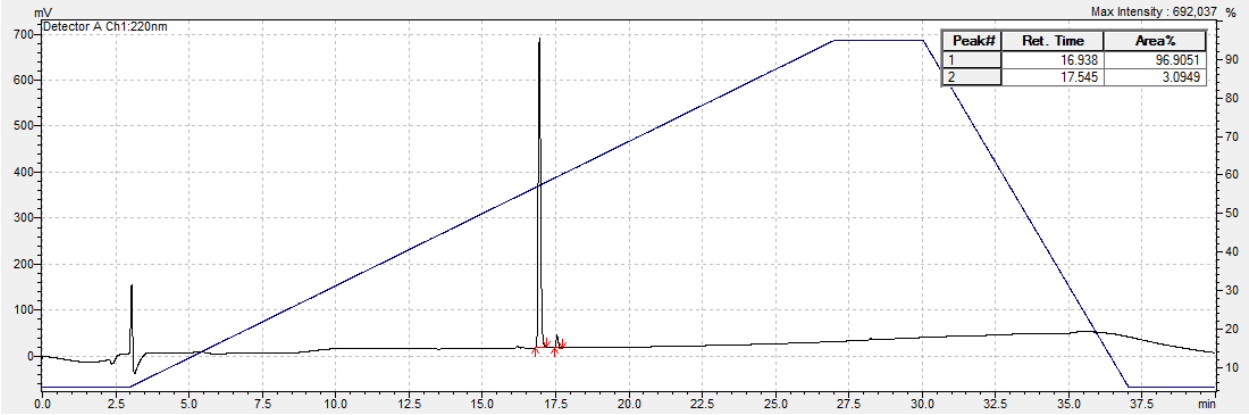

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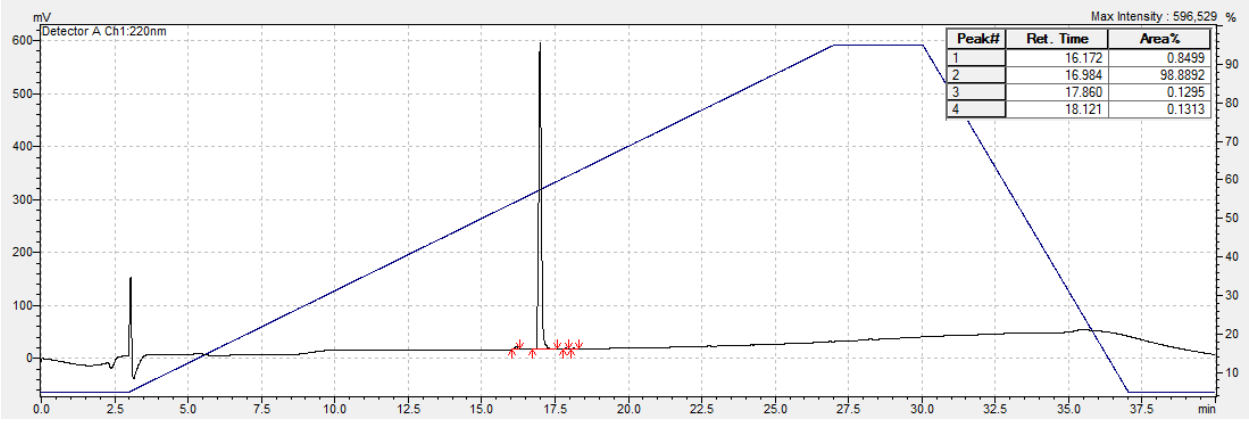

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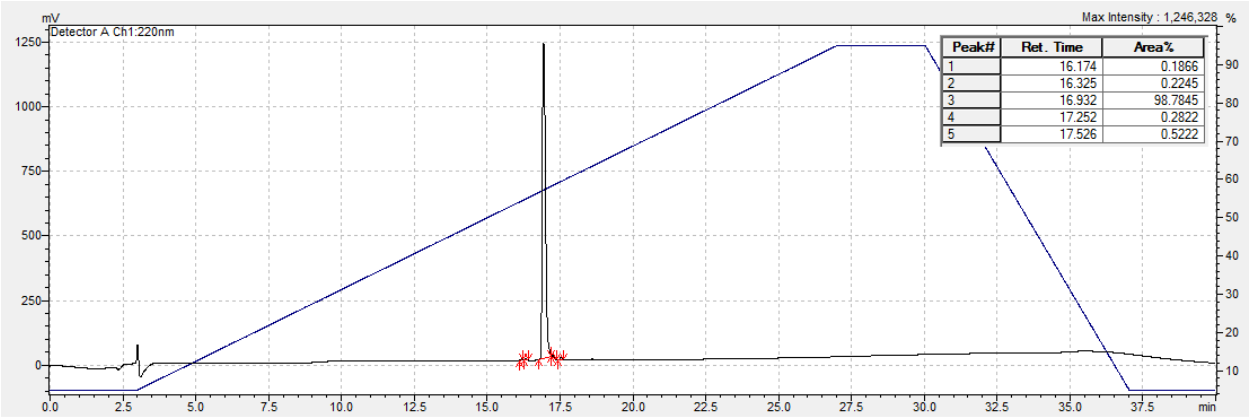

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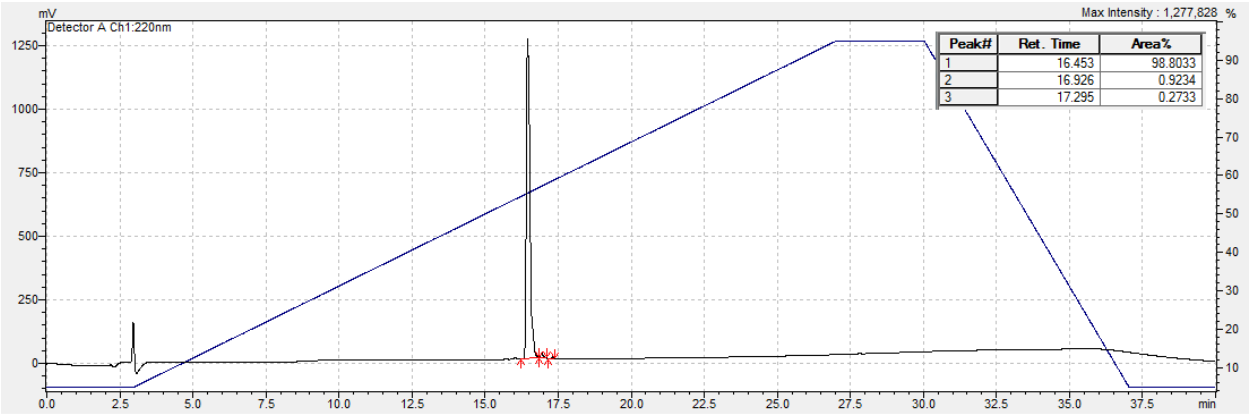

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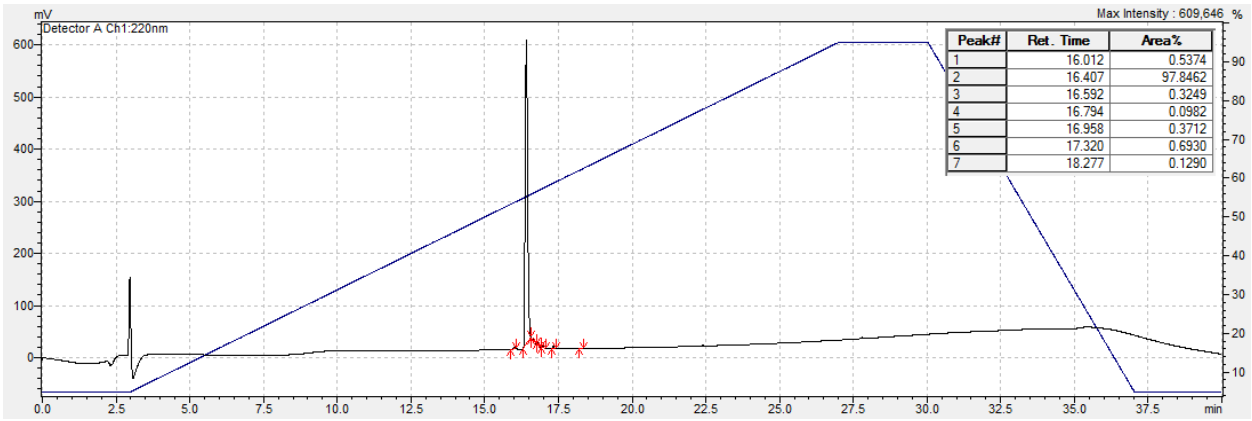

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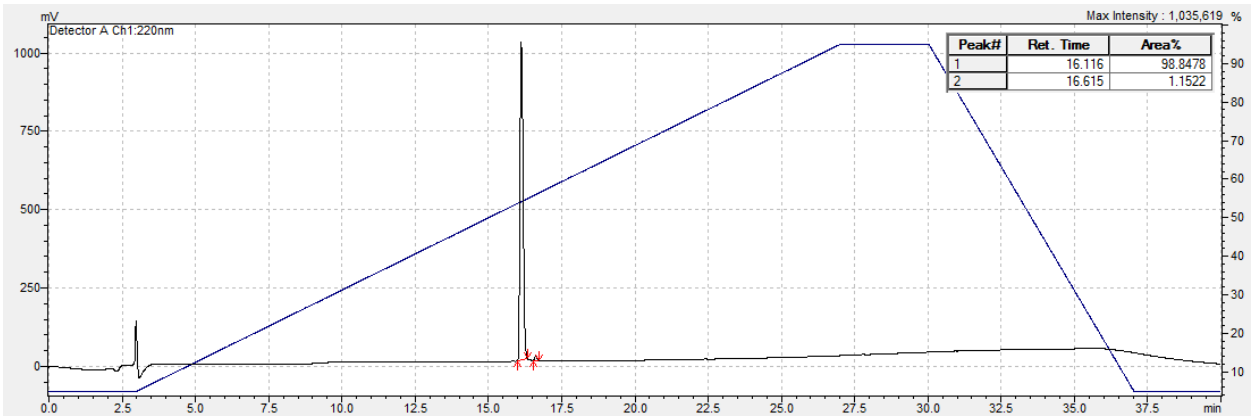

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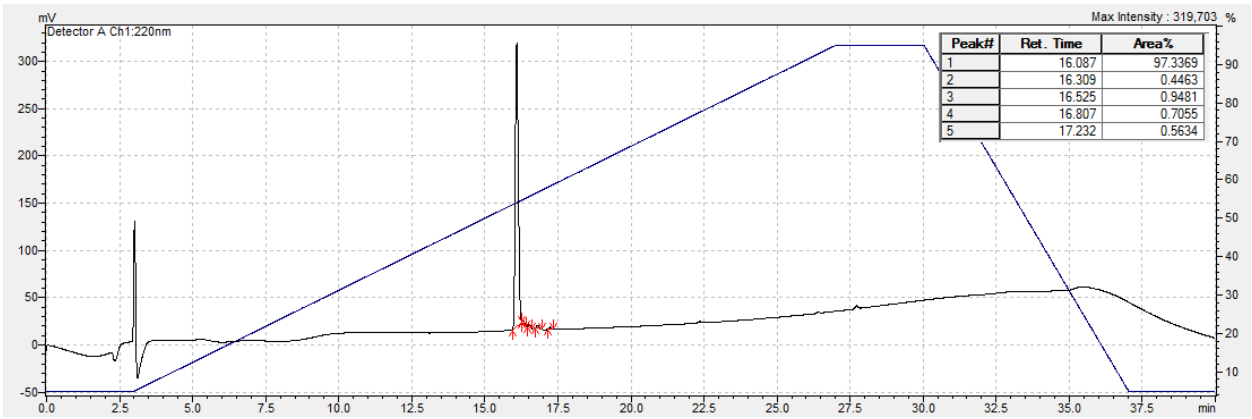

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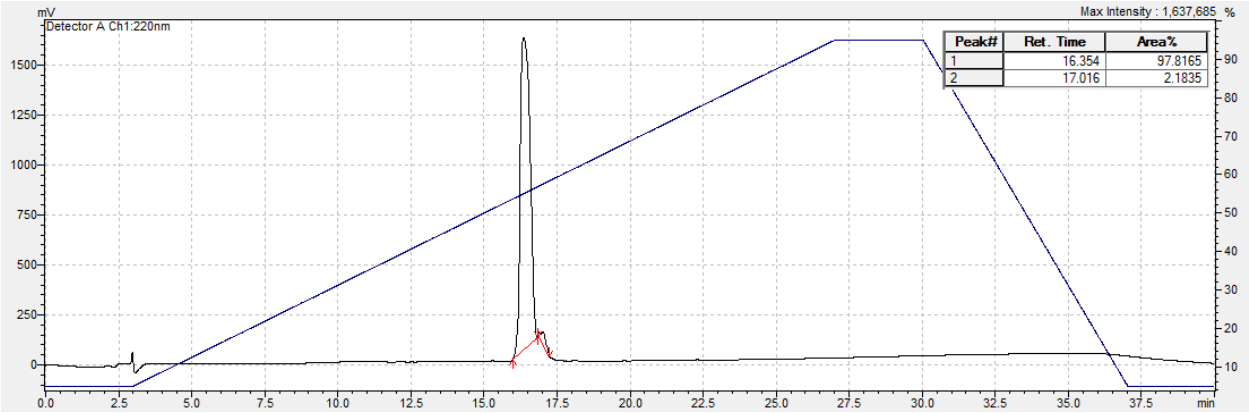

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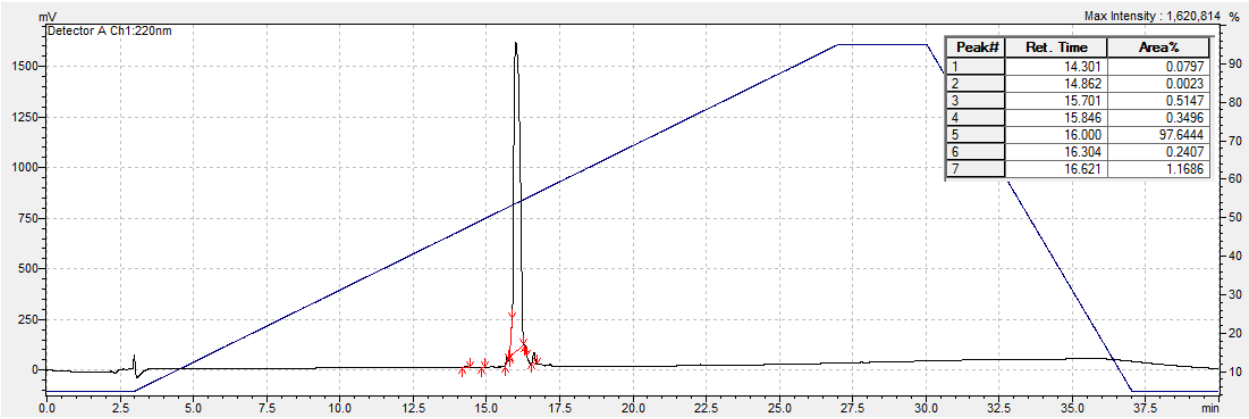

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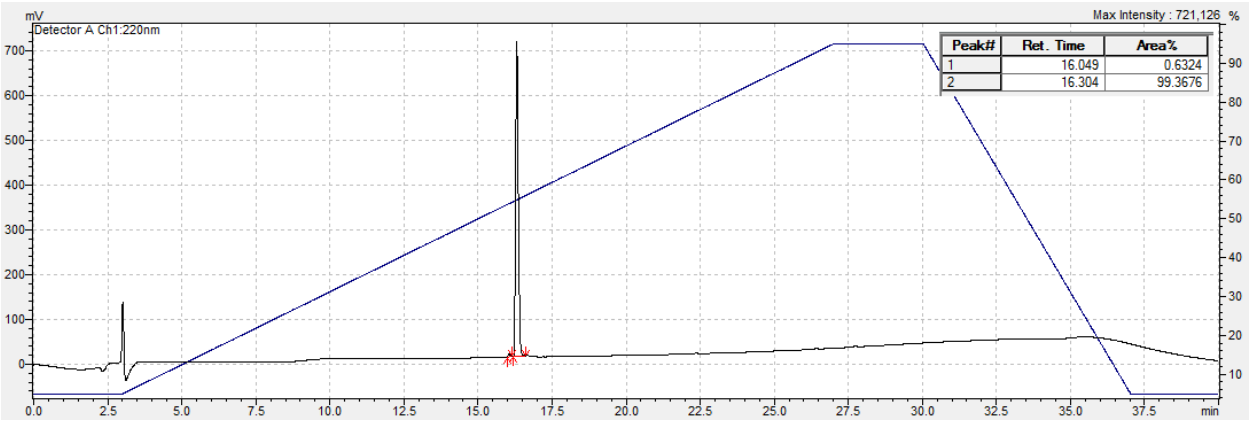

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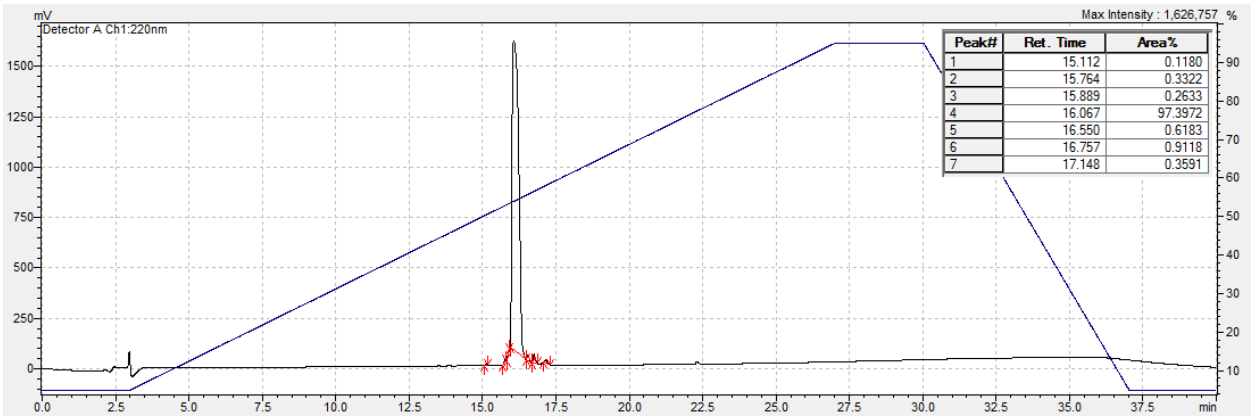

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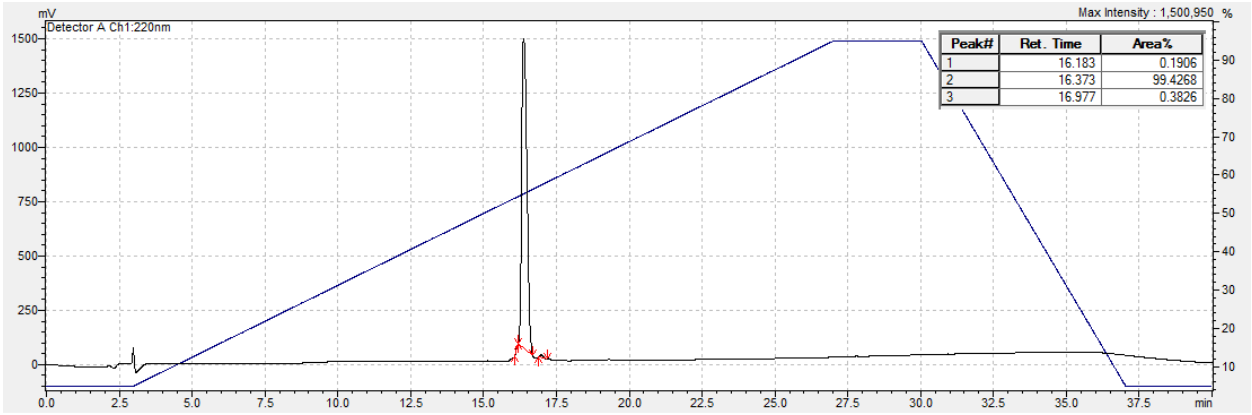

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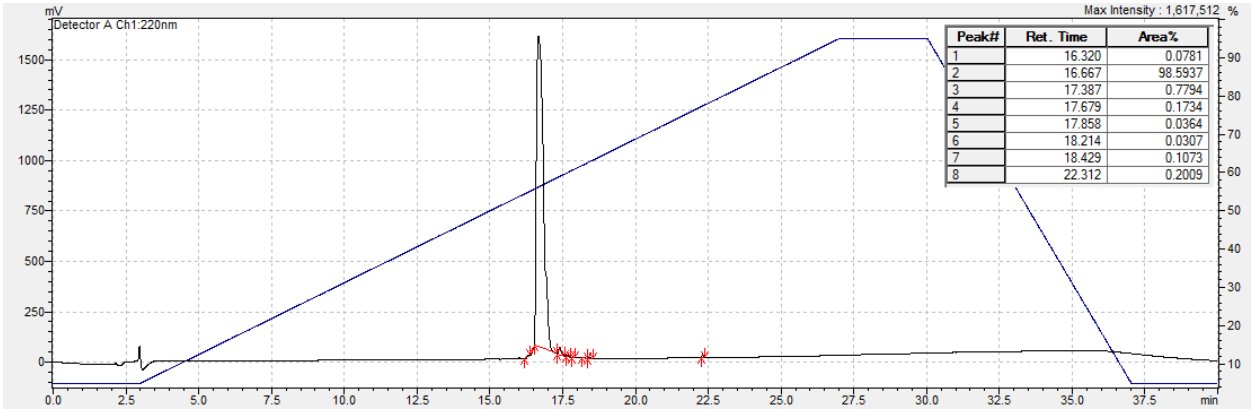

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