Unveiling the Importance of Amide Protons in CSP:ComD Interactions in *Streptococcus pneumoniae*

Bimal Koirala,[‡] Naiya R. Phillips,[‡] and Yftah Tal-Gan*

Department of Chemistry, University of Nevada, Reno, 1664 North Virginia Street, Reno, Nevada, 89557, United States

KEYWORDS: Streptococcus pneumoniae; quorum sensing; competence stimulating peptide (CSP); N-methylation

ABSTRACT: *Streptococcus pneumoniae* is an opportunistic pathogen that can cause diseases ranging from mild respiratory infections to life-threating conditions such as pneumonia, meningitis and bacteremia. *S. pneumoniae* pathogenicity is dependent on the action of a 17-amino acid peptide pheromone, termed competence stimulating peptide (CSP) that controls the competence regulon, a quorum sensing (QS) circuit. Therefore, intercepting QS could have therapeutic implications in treating pneumococcal infections while avoiding emerging antimicrobial resistance. In this study, we set out to evaluate the impact of amide protons on CSP activity and metabolic stability through systematic *N*-methylation. Our results indicate that the majority of amide protons are critical for CSP activity, either through direct interactions with the cognate receptor or by stabilizing the bioactive conformation. Importantly, we identified several *N*-methyl CSP analogs, namely CSP1(15)-*N*-Me-K6 and CSP1(15)-*N*-Me-F7, that retain their biological activity while exhibiting enhanced metabolic stability. These analogs are privileged scaffolds for the design of CSP-based QS modulators with drug-like properties.

Quorum sensing (QS) is a cell-cell communication mechanism through which bacterial cells detect neighboring bacteria, using diffusible signaling molecules, to assess their population density.[1-2] Upon reaching a threshold density QS is activated, leading to a population-wide alteration

in gene expression and resulting in the initiation of different group phenotypes, such as swarming, motility, virulence factor production and biofilm formation.[3] Since this communication pathway is nonessential for bacterial survival, QS interference has been utilized by us and others to combat bacterial pathogenicity, while limiting the potential for resistance development.[4-10]

Streptococcus pneumoniae is a commensal bacterium that primarily resides in the nasopharynx. As an opportunistic pathogen, *S. pneumoniae* can further disseminate into the ears, sinuses and lungs. Additionally, *S. pneumoniae* is able to penetrate the mucosal barrier to reach into the blood stream and eventually pass through the blood-brain barrier. *S. pneumoniae* is thus responsible for a variety of illnesses, from mild respiratory infections to deadly bacteremia, meningitis, and pneumonia.[11-12] The recombinogenic nature of *S. pneumoniae* allows it to rapidly acquire antibiotic resistances to a wide range of antibiotics, including beta lactam derivatives, quinolones, and macrolides, as well as to evade vaccine treatments by switching its extra-cellular capsule.[13-16] Overall, *S. pneumoniae* is responsible to over 445,000 hospitalizations, leading to medical costs exceeding $3.5 billion a year in the U.S.[17-19]

The main QS circuitry in *S. pneumoniae* is called the competence regulon and is controlled by the ComDE two-component signal transduction system (TCSTS).[20] In this circuitry, a mature 17-amino acid peptide, termed competence stimulating peptide (CSP), is exported outside the cell via an ABC transporter, ComAB.[21] Once extracellular CSP reaches a threshold concentration, it binds the membrane bound histidine kinase receptor, ComD, resulting in phosphorylation of the response regulator, ComE. Phosphorylated ComE then upregulates the expression of the *comABCDE* genes as well as genes involved in competence, biofilm formation, and virulence factor production.[22-24] Interception of the competence regulon is thus an attractive target to attenuate *S. pneumoniae* infections.[9, 25-28] There are two main variants of *S. pneumoniae* strains that differ in CSP sequence

(CSP1 and CSP2), and each possesses its own specific ComD receptor (ComD1 and ComD2).[29] These two CSPs share 50% homology, with the majority of differences occurring in the central region of the sequence.[30] We therefore aim to develop CSP-based analogs capable of disrupting CSP:ComD interactions in both *S. pneumoniae* variants. To this end, we previously conducted a systematic structure-activity relationship (SAR) study of both CSP1 and CSP2 to identify critical residues involved in ComD1 and ComD2 receptor binding and activation.[27] Through this analysis, we were able to develop a potent ComD2 inhibitor, CSP2-E1Ad10, capable of attenuating *S. pneumoniae* infections in a mouse model of acute pneumonia.[9]

In this study, we set out to expand the SAR knowledge of both CSP1 and CSP2 to include systematic backbone modifications (*N*-methylation), to assess the role of amide protons in receptor binding and specificity, as well as improve the drug-like properties of CSP-based therapeutics. Our analysis revealed that, with a few exceptions, amide protons are important for activity, either by direct interactions with the receptor or through stabilization of the bioactive conformation. Furthermore, our results indicate that *N*-methylation of key positions significantly enhance the proteolytic stability of the CSP analogs. These results are significant since they provide an avenue for the development of CSP-based drug leads with enhanced pharmacological properties.

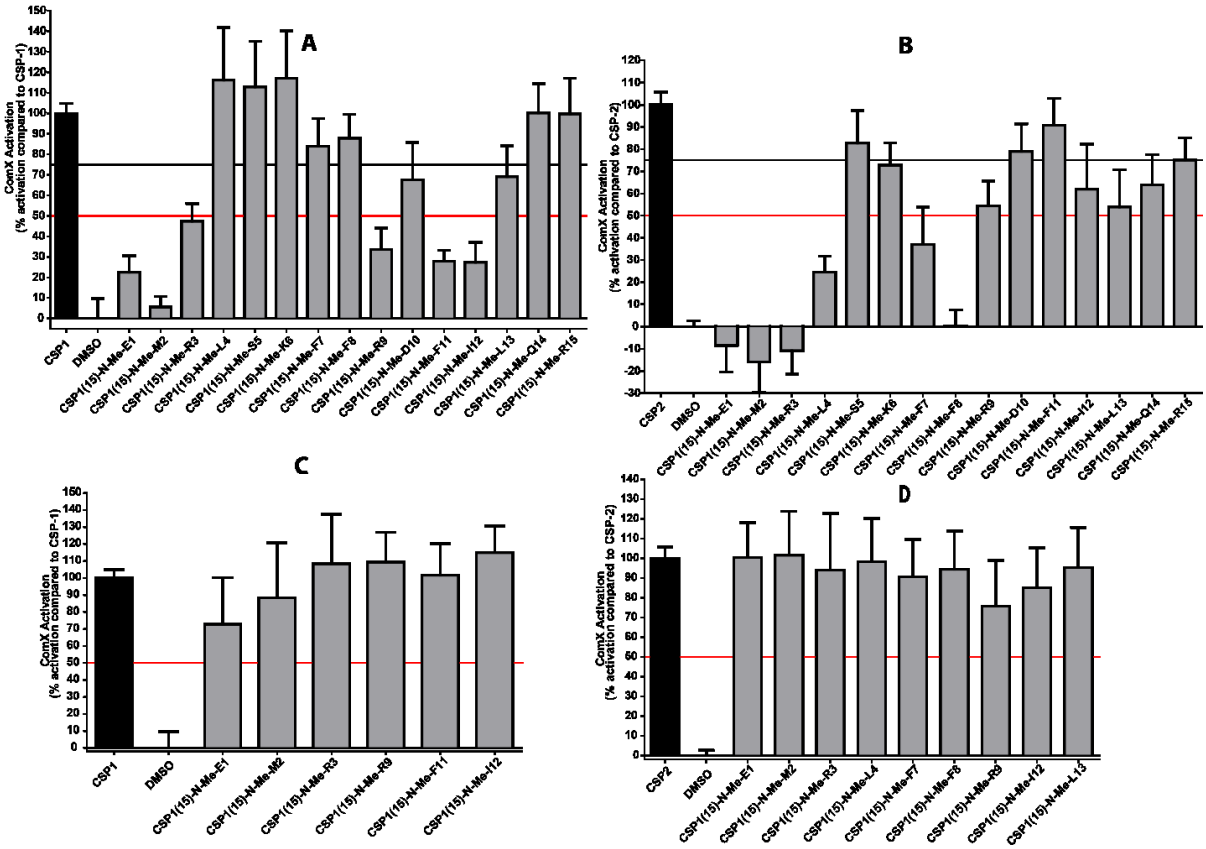

Figure 1. Agonism and antagonism screening of the *N*-methyl CSP1 analogs against the ComD1 and ComD2 receptors. (A) Agonism screening of the *N*-Me CSP1 library against the ComD1 receptor. (B) Agonism screening of the *N*-Me CSP1 library against the ComD2 receptor. (C) Antagonism screening of the *N*-Me CSP1 library against the ComD1 receptor. (D) Antagonism screening of the *N*-Me CSP1 library against the ComD2 receptor. Agonism assays were performed at 10 μM concentration of synthetic CSP. Native CSP (CSP1 or CSP2) was used as the positive control (100%) while DMSO as the negative control (0%). Antagonism assays were performed at 10 μM concentration of peptides against 50 nM concentration of CSP1 or 250 nM concentration of CSP2. CSP1 (50 nM) or CSP2 (250 nM) was used as the positive control (100%) while DMSO as the negative control (0%). Percent (%) *comX* activation was measured by normalizing the Miller units obtained for each peptide to that of the native CSP. All peptides were screened in triplicates over three separate trials. Error bars indicate standard error of the mean of nine values.

Table 1. EC_{50} values of the *N*-methyl CSP1 analogs against the ComD1 and ComD2 receptors[a]

Peptide Name	Sequence	ComD1		ComD2	
		EC_{50} (nM)[b]	95% CI[c]	EC_{50} (nM)[b]	95% CI[c]
CSP1	EMRLSKFFRDFILQRKK	10	6.3 - 17	530	500 - 560
CSP1(15)	EMRLSKFFRDFILQR	8.1	5.3 - 12	>1000	--
CSP1(15)-*N*-Me-E1	***N*Me**EMRLSKFFRDFILQR	--[d]	--	--[d]	--
CSP1(15)-*N*-Me-M2	E***N*Me**MRLSKFFRDFILQR	--[d]	--	--[d]	--
CSP1(15)-*N*-Me-R3	EM***N*Me**RLSKFFRDFILQR	--[d]	--	--[d]	--
CSP1(15)-*N*-Me-L4	EMR***N*Me**LSKFFRDFILQR	280	140 - 530	--[d]	--
CSP1(15)-*N*-Me-S5	EMRL***N*Me**SKFFRDFILQR	280	143 - 530	>1000	--
CSP1(15)-*N*-Me-K6	EMRLS***N*Me**KFFRDFILQR	65	61 - 70	>1000	--
CSP1(15)-*N*-Me-F7	EMRLSK***N*Me**FFRDFILQR	280	200 - 400	--[d]	--
CSP1(15)-*N*-Me-F8	EMRLSKF***N*Me**FRDFILQR	>1000	--	--[d]	--
CSP1(15)-*N*-Me-R9	EMRLSKFF***N*Me**RDFILQR	--[d]	--	--[d]	--
CSP1(15)-*N*-Me-D10	EMRLSKFFR***N*Me**DFILQR	>1000	--	>1000	--
CSP1(15)-*N*-Me-F11	EMRLSKFFRD***N*Me**FILQR	--[d]	--	>1000	--
CSP1(15)-*N*-Me-I12	EMRLSKFFRDF***N*Me**ILQR	--[d]	--	--[d]	--
CSP1(15)-*N*-Me-L13	EMRLSKFFRDFI***N*Me**LQR	>1000	--	--[d]	--
CSP1(15)-*N*-Me-Q14	EMRLSKFFRDFIL***N*Me**QR	68	50 - 91	--[d]	--
CSP1(15)-*N*-Me-R15	EMRLSKFFRDFILQ***N*Me**R	130	120 - 150	>1000	--

[a] See the supporting information for full experimental details. [b] EC_{50} values were determined by testing peptides over a range of concentrations. [c] 95% confidence interval. [d] EC_{50} not determined due to the analog's low induction in primary agonism screening assay.

Our first aim in this study was to expand the known SAR knowledge of CSP1. In a previous work, Yang et al. evaluated the role of side chain residues and chiral centers in CSP1-ComD1 binding. Furthermore, the systematic analysis of CSP1 allowed Yang et al. to identify a minimal CSP1 sequence that is required for effective receptor binding and activation, CSP1-desK16K17 (termed here CSP1(15)).[27] Thus, in this work, we assessed additional structural features that are involved in CSP1:ComD1 binding, namely the role of amide protons. To this end, we conducted a full *N*-methyl scan of the minimal CSP1 sequence (CSP1(15)) and evaluated the backbone modified analogs for their ability to modulate QS in pneumococcal strains bearing either the ComD1 or ComD2 receptors. The *N*-methyl peptides were synthesized using established solid-phase peptide synthesis (SPPS) protocols on Wang resin by incorporating commercially available Fmoc-protected *N*-methyl amino acids.[31-32] The peptides were then cleaved and purified to homogeneity

using RP-HPLC (>95% purity), and their identity confirmed using mass spectrometry (see supporting information for full characterization details). To assess QS modulation, we utilized the previously constructed β-gal reporter strains D39pcomX::lacZ and TIGR4pcomX::lacZ,[25] both of which are wild-type strains capable of producing their native CSP, that also carry the *lacZ* gene under the control of the *comX* promoter. Activation of the competence regulon can therefore be quantified by measuring β-gal activity.

The peptides were first screened for their ability to activate/inhibit the ComD1 and ComD2 receptors at high concentration (10 μM, **Figure 1**). Only analogs that exhibited more than 75% activation compared to the native peptides (CSP1 or CSP2) in the initial screening or more than 50% inhibition of the maximal signal induced by the addition of exogenous native peptide in the competition screening were further evaluated, and their EC_{50}/IC_{50} values were determined through dose response curves (**Table 1**). Not surprisingly, the *N*-methyl analogs exhibited low to no activity against the ComD2 receptor, similar to their parent scaffold, CSP1(15), although the hydrophilic residues (S5, K6, D10, R15) were more tolerant to *N*-methylation (**Figure 1B** and **Table 1**). Moving to the ComD1 receptor, *N*-methylation of the first three residues E1, M2 and R3 resulted in analogs that completely lost their activity. These results further support previous observations indicating that the *N*-terminus of CSP1 plays an important role in receptor binding and activation. Contrary to the *N*-terminus, *N*-methylation of the *C*-terminal residues, Q14 and R15, resulted in active analogs, further emphasizing the minimal role the *C*-terminus has in receptor binding. Moving to the central region of CSP1(15), *N*-methylation of positions L4 to F7 was tolerated, resulting in analogs that exhibited QS activity, whereas *N*-methylations of positions F8 to L13 resulted in analogs with weak to no activity (**Figure 1A** and **Table 1**).

Our second aim in this study was to assess the role of amide protons in CSP2 activity. In their work, Yang et al. identified the minimal CSP2 sequence required for effective receptor binding and activation, CSP2-desL14-K17 (termed here CSP2(13)).[27] Thus, we used this scaffold to conduct a systematic *N*-methyl scan. Unsurprisingly, the initial evaluation revealed that the CSP2 analogs are inactive against ComD1 (**Figure 2A and C**). This observation is in agreement with previous SAR analysis of the CSP2 scaffold. With regard to the ComD2 receptor, with the exception of the *C*-terminal residue, generally *N*-methylation resulted in significant reduction in activity (**Figure 2B and D**, and **Table 2**). Interestingly, *N*-methylation of the *N*-terminal residues, E1 or M2, was sufficient to convert these peptides into antagonists, highlighting the stringent structural requirements of the *N*-terminus in driving ComD2 activation. In contrast, *N*-methylation of the *C*-terminal residue, F13, resulted in an analog with activity comparable to that of CSP2(13). Although removal of the F13 residue resulted in significant reduction in peptide potency (see SI for dose curve), the activity of CSP2(13)-*N*-Me-F13 further emphasizes the minor role the *C*-terminus plays in receptor binding.[27] Within the central region of the peptide, *N*-methylation was most tolerated in positions S5, R6 and D10 (**Table 2**).

In a recent NMR study we conducted to assess the binding interactions between the CSPs and the ComD receptors, we observed that the CSPs adopt an amphiphilic alpha helix conformation, where all the critical residues for receptor binding occupy one phase of the helix, while dispensable residues occupy the opposite phase of the helix.[33] Importantly, residues S5, R6 and D10 in CSP2 were all found to occupy the opposite phase relative to the CSP2 binding patch. Thus, these results are in agreement and further support our hypothesized CSP2 mechanism of action.

One of the major pharmacological drawbacks of peptides is their rapid metabolic degradation.[34] We have previously reported that CSP1 has a relatively short half-life of 1.5 hours.[9] We reasoned

that *N*-methylation in specific positions within the sequence could confer resistance to enzymatic degradation. We therefore selected the two most potent CSP1(15) *N*-methylated analogs, CSP1(15)-*N*-Me-K6 and CSP1(15)-*N*-Me-Q14, along with CSP1(15)-*N*-Me-F7, which exhibited relatively high activity in comparison to the entire library and was suspected to possess *N*-methylation in a position critical for stability (chymotrypsin cleaves after aromatic residues), and evaluated their stability using a trypsin/chymotrypsin stability assay.[35-36] Due to their low solubility in the assay conditions, we were unable to evaluate the stability of any of the CSP2(13) *N*-methyl analogs. Evaluation of CSP1(15) revealed that this peptide degraded rapidly, having a half-life of 4-hours (**Figure 3**). *N*-methylation at the *C*-terminus (CSP1(15)-*N*-Me-Q14) did not significantly alter the susceptibility to enzymatic degradation, leading to a half-life of 6 hours. In contrast, *N*-methylation in the central region of the peptide (CSP1(15)-*N*-Me-K6 and CSP1(15)-*N*-Me-F7) conferred enough resistance to enzymatic degradation that both analogs exhibited half-lives exceeding the 48-hour assay duration (**Figure 3**). Since trypsin and chymotrypsin cleave peptide bonds following a positive charge (Arg or Lys) or aromatic residue (Phe, Tyr or Trp), respectively, it is not surprising that *N*-methylation of such residues (K6 and F7) resulted in poor protease binding and peptide cleavage. Moving to human plasma, degradation of all the peptides was significantly faster. Interestingly, CSP1(15)-*N*-Me-Q14 exhibited the most resistance to degradation in plasma, having a 2-hour half-life, whereas the other analogs having a half-life lower than 30 min. Overall, the stability results are significant since they provide an avenue to stabilize CSP-based QS modulators without significantly compromising the activity of the peptides.

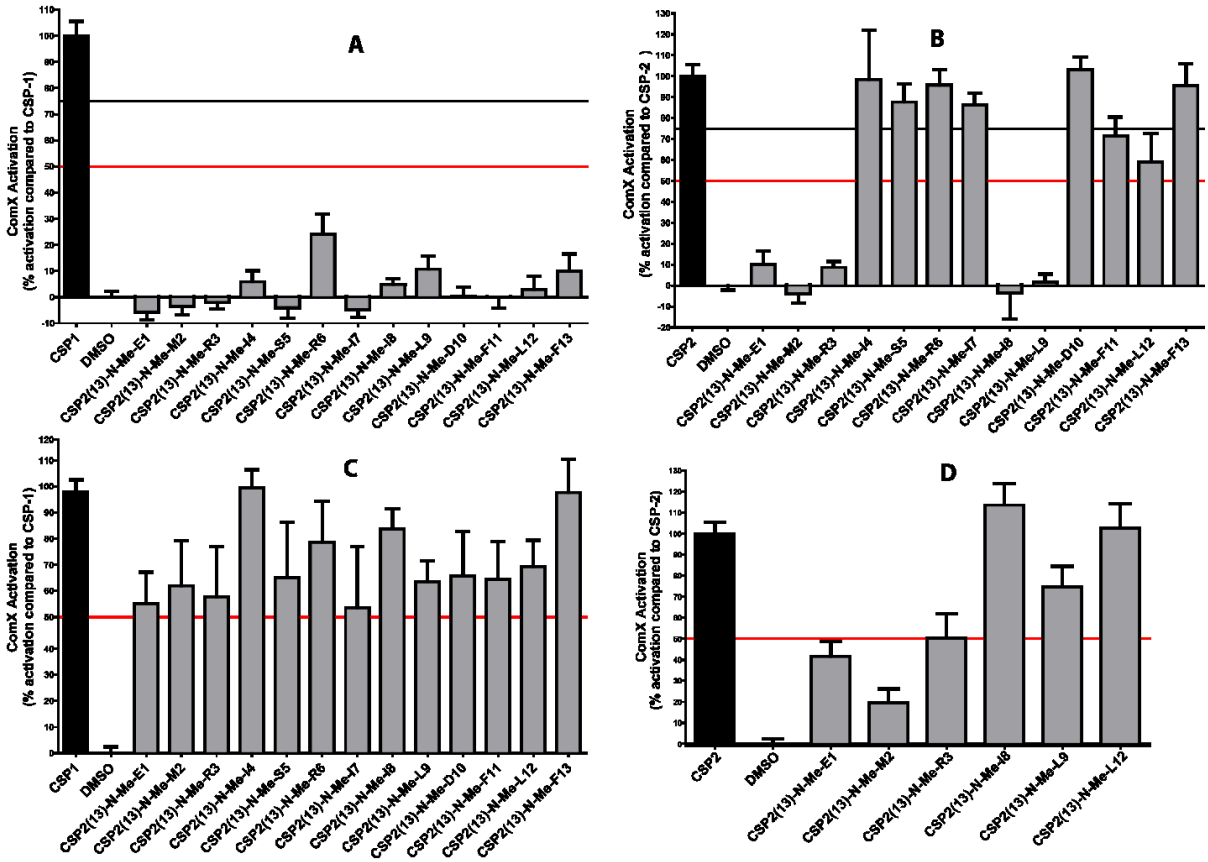

Figure 2. Agonism and antagonism screening of the *N*-methyl CSP2 analogs against the ComD1 and ComD2 receptors. (A) Agonism screening of the *N*-Me CSP2 library against the ComD1 receptor. (B) Agonism screening of the *N*-Me CSP2 library against the ComD2 receptor. (C) Antagonism screening of the *N*-Me CSP2 library against the ComD1 receptor. (D) Antagonism screening of the *N*-Me CSP2 library against the ComD2 receptor. See **Figure 1** for experimental details.

Table 2. EC_{50}/IC_{50} values of the *N*-methyl CSP2 analogs against the ComD2 receptor[a]

Peptide Name	Sequence	ComD2	
		EC_{50} /IC_{50}[c] (nM)[b]	95% CI[c]
CSP2	EMRISRIILDFLFLRKK	51	41 - 63
CSP2(13)	EMRISRIILDFLF	78	71 - 86
CSP2(13)-*N*-Me-E1	***N*Me**EMRISRIILDFLF	>1000[e]	--
CSP2(13)-*N*-Me-M2	E***N*Me**MRISRIILDFLF	>1000[e]	--
CSP2(13)-*N*-Me-R3	EM***N*Me**RISRIILDFLF	--[d]	--
CSP2(13)-*N*-Me-I4	EMR***N*Me**ISRIILDFLF	>1000	--
CSP2(13)-*N*-Me-S5	EMRI***N*Me**SRIILDFLF	550	440 - 700
CSP2(13)-*N*-Me-R6	EMRIS***N*Me**RIILDFLF	560	440 - 710
CSP2(13)-*N*-Me-I7	EMRISR***N*Me**IILDFLF	>1000	--
CSP2(13)-*N*-Me-I8	EMRISRI***N*Me**ILDFLF	--[d]	--
CSP2(13)-*N*-Me-L9	EMRISRII***N*Me**LDFLF	--[d]	--
CSP2(13)-*N*-Me-D10	EMRISRIIL***N*Me**DFLF	340	240 - 500
CSP2(13)-*N*-Me-F11	EMRISRIILD***N*Me**FLF	--[d]	--
CSP2(13)-*N*-Me-L12	EMRISRIILDF***N*Me**LF	--[d]	--
CSP2(13)-*N*-Me-F13	EMRISRIILDFL***N*Me**F	27	14 - 53

[a] See the supporting information for full experimental details. [b] EC_{50} or IC_{50} values were determined by testing peptides over a range of concentrations. [c] 95% confidence interval. [d] EC_{50} not determined due to the analog's low induction in primary agonism screening assay. [e] IC_{50} value.

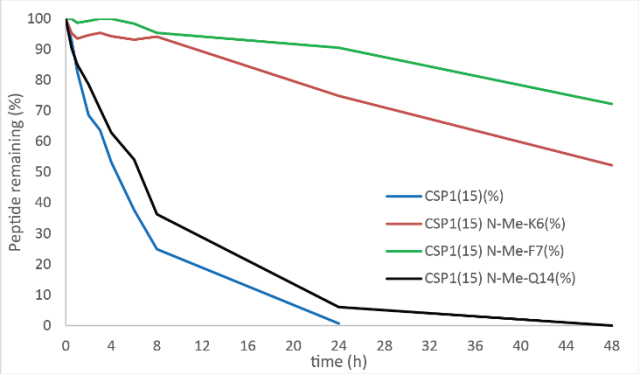

Figure 3. Metabolic stability of *N*-Methyl CSPs. *N*-Me CSP1(15) analogs were treated with trypsin/chymotrypsin and their enzymatic degradation monitored by HPLC. Both CSP1(15) (blue) and CSP1(15)-*N*-Me-Q14 (black) had relatively similar half-lives of 4 h and 6 h, respectively. On the contrary, CSP1(15)-*N*-Me-F7 (green) and CSP1(15)-*N*-Me-K6 (red) had significantly longer half-lives (>48 h).

Encouraged by the stability results, we set out to evaluate whether the incorporation of *N*-methylation in specific positions would be tolerated in the lead CSP-based QS inhibitor scaffolds, namely CSP1-E1A and CSP2-E1Ad10, resulting in inhibitory analogs with enhanced metabolic stability. We therefore synthesized three CSP1-E1A-based analogs: CSP1-E1A-*N*-Me-K6, CSP1-E1A-*N*-Me-Q14 and CSP1-E1A-*N*-Me-K6-*N*-Me-Q14, and one CSP2-E1Ad10-based analog: CSP2-E1Ad10-*N*-Me-F13. To our satisfaction, all the analogs exhibited inhibitory activities, with CSP1-E1A-*N*-Me-K6 and CSP2-E1Ad10-*N*-Me-F13 exhibiting activities comparable to their parent analogs, CSP1-E1A and CSP2-E1Ad10, respectively (**Table 3**).[27]

Table 3. IC_{50} values of the CSP1-E1A and CSP2-E1Ad10 *N*-methyl analogs against the ComD1 and ComD2 receptors[a]

Peptide Name	ComD1 IC_{50} (nM)[b] [95% CI][c]	ComD2 IC_{50} (nM)[b] [95% CI][c]
CSP1-E1A-*N*-Me-K6	48 [25-100]	--[d]
CSP1-E1A-*N*-Me-Q14	370 [180-770]	--[d]
CSP1-E1A-*N*-Me-K6-*N*-Me-Q14	360 [230-580]	--[d]
CSP2-E1Ad10-*N*-Me-F13	>1000	64 [40-110]

[a] See the supporting information for full experimental details. [b] IC_{50} values were determined by testing peptides over a range of concentrations. [c] 95% confidence interval. [d] IC_{50} not determined due to the analog's low activity.

Predictably, metabolic stability evaluation of the CSP1-E1A-based and CSP2-E1Ad10-based analogs revealed that these analogs degrade faster than their truncated counterparts, namely, the

CSP1(15)-based analogs. The reduced stability is likely the result of having the RKK *C*-terminal motif, which is highly susceptible to trypsin degradation. Surprisingly, a reversed trend was observed regarding the utility of *N*-methylation in specific positions: In the CSP1(15) scaffold, *N*-methylation at position Q14 was not beneficial, while *N*-methylation at position K6 enhanced the peptide stability (**Figure 3**). In contrast, for the CSP1-E1A scaffold, a scaffold based on the full CSP1 sequence, *N*-methylation in position Q14 was found to enhance peptide stability while *N*-methylation in position K6 did not confer significant resistance to enzymatic degradation (**Figure 4**). The reverse trend may also be attributed to the presence of the RKK *C*-terminal motif. Since this is likely the first site to be cleaved by trypsin, *N*-methylation in the vicinity (Q14) may reduce the ability of the protease to bind the peptide and cleave the amide bond between R15 and K16. Human plasma studies exhibited a similar trend, although overall a rapid degradation was observed, with CSP1-E1A-*N*-Me-Q14 being the most stable analog. Regarding CSP2-E1Ad10-*N*-Me-F13, this analog exhibited a stability trend similar to the parent CSP2-E1Ad10,[9] suggesting that *N*-methylation in F13 does not provide significant protection against degradation.

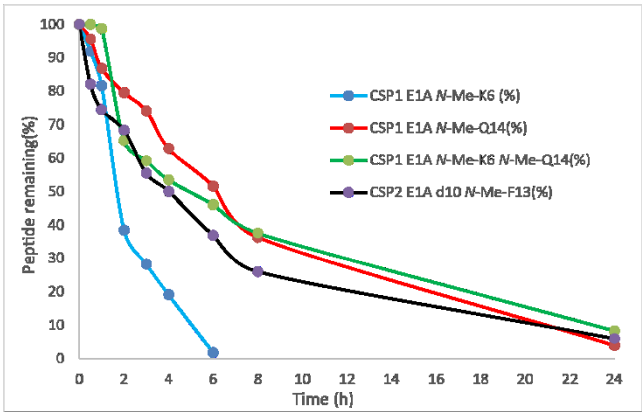

Figure 4. Metabolic stability of Inhibitory *N*-Methyl CSPs. Analogs were treated with trypsin/chymotrypsin and their enzymatic degradation monitored by HPLC. CSP1-E1A-*N*-Me-K6 (blue) had the shortest half-life (~2 h), CSP2-E1Ad10-*N*-Me-F13 (black) had a slightly improved half-life (~4 h), while CSP1-E1A-*N*-Me-Q14 (red) and CSP1-E1A-*N*-Me-K6-*N*-Me-Q14 (green) had both the longest half-life (~6 h).

In this work, we conducted a systematic analysis of amide protons of the two CSP signals in *S. pneumoniae* using *N*-methyl scans. Biological results of the CSP1(15) and CSP2(13) *N*-methyl libraries indicate that most amide backbone protons are critical for bioactivity, either due to direct interactions with the ComD receptors, or through stabilization of the bioactive conformation. Importantly, our analysis revealed a few *N*-methyl CSP analogs that retain their biological activities. Metabolic stability studies of key CSP1(15) *N*-methyl analogs highlight the potential utility of incorporating *N*-methylation into the CSP scaffolds to confer resistance to enzymatic degradation. Indeed, *N*-methylation in specific positions was found to be tolerated in the lead CSP-based QS inhibitors, resulting in analogs that exhibit improved metabolic stability while retaining the inhibitory properties.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Full details of peptide synthesis and characterization, the stability assay, the beta-galactosidase bioassay, and dose response curves for CSP analogs (PDF)

AUTHOR INFORMATION

Corresponding Author

* E-mail: ytalgan@unr.edu.

Author Contributions

‡These authors contributed equally.

Funding Sources

This work was supported by grants from the National Institutes of Health (R35GM128651), the National Science Foundation (CHE-1808370), the Cayman Biomedical Research Institute (CaBRI), and by the Nevada INBRE through a grant from the NIH (GM103440).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

S. pneumoniae D39pcomX::lacZ and TIGR4pcomX::lacZ reporter strains were generous gifts from G. W. Lau (University of Illinois at Urbana−Champaign).

ABBREVIATIONS

ACN, acetonitrile; CSP, competence stimulating peptide; EM, Exact mass; ESI, electrospray ionization; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; QS, quorum sensing; RP-HPLC, reversed-phase high-performance liquid chromatography; SAR, structure-activity relationship; SPPS, solid-phase peptide synthesis; TCSTS, two-component signal transduction system; THY, Todd-Hewitt broth with Yeast extract.

REFERENCES

1. Waters, C. M.; Bassler, B. L., QUORUM SENSING: Cell-to-Cell Communication in Bacteria. *Annu Rev Cell Dev Biol* **2005,** *21* (1), 319-346.
2. Rutherford, S. T.; Bassler, B. L., Bacterial Quorum Sensing: Its Role in Virulence and Possibilities for Its Control. *Cold Spring Harb Perspect Med* **2012,** *2*, a012427.
3. Parsek, M. R.; Greenberg, E. P., Sociomicrobiology: the Connections between Quorum Sensing and Biofilms. *Trends Microbiol* **2005,** *13* (1), 27-33.

4. O'Loughlin, C. T.; Miller, L. C.; Siryaporn, A.; Drescher, K.; Semmelhack, M. F.; Bassler, B. L., A Quorum-Sensing Inhibitor Blocks *Pseudomonas aeruginosa* Virulence and Biofilm Formation. *Proc Natl Acad Sci U S A* **2013,** *110* (44), 17981-6.
5. Welsh, M. A.; Blackwell, H. E., Chemical Probes of Quorum Sensing: from Compound Development to Biological Discovery. *FEMS Microbiol Rev* **2016,** *40* (5), 774-94.
6. McBrayer, D. N.; Gantman, B. K.; Cameron, C. D.; Tal-Gan, Y., An Entirely Solid Phase Peptide Synthesis-Based Strategy for Synthesis of Gelatinase Biosynthesis-Activating Pheromone (GBAP) Analogue Libraries: Investigating the Structure–Activity Relationships of the *Enterococcus faecalis* Quorum Sensing Signal. *Org Lett* **2017,** *19* (12), 3295-3298.
7. Tan, L.; Li, S. R.; Jiang, B.; Hu, X. M.; Li, S., Therapeutic Targeting of the *Staphylococcus aureus* Accessory Gene Regulator (agr) System. *Front Microbiol* **2018,** *9*, 55.
8. Bikash, C. R.; Hamry, S. R.; Tal-Gan, Y., Structure–Activity Relationships of the Competence Stimulating Peptide in *Streptococcus mutans* Reveal Motifs Critical for Membrane Protease SepM Recognition and ComD Receptor Activation. *ACS Infect Dis* **2018,** *4* (9), 1385-1394.
9. Koirala, B.; Lin, J.; Lau, G. W.; Tal-Gan, Y., Development of a Dominant Negative Competence Stimulating Peptide (dnCSP) that Attenuates *Streptococcus pneumoniae* Infectivity in a Mouse Model of Acute Pneumonia. *Chembiochem* **2018,** *19* (22), 2380-2386.
10. McBrayer, D. N.; Cameron, C. D.; Gantman, B. K.; Tal-Gan, Y., Rational Design of Potent Activators and Inhibitors of the *Enterococcus faecalis* Fsr Quorum Sensing Circuit. *ACS Chem Biol* **2018,** *13* (9), 2673-2681.
11. O'Brien, K. L.; Wolfson, L. J.; Watt, J. P.; Henkle, E.; Deloria-Knoll, M.; McCall, N.; Lee, E.; Mulholland, K.; Levine, O. S.; Cherian, T.; Hib; Pneumococcal Global Burden of Disease

Study, T., Burden of Disease Caused by *Streptococcus pneumoniae* in Children Younger than 5 Years: Global Estimates. *Lancet* **2009,** *374* (9693), 893-902.

12. Mehr, S.; Wood, N., *Streptococcus pneumoniae*--A Review of Carriage, Infection, Serotype Replacement and Vaccination. *Paediatr Respir Rev* **2012,** *13* (4), 258-64.

13. Tankovic, J.; Perichon, B.; Duval, J.; Courvalin, P., Contribution of Mutations in *gyrA* and *parC* Genes to Fluoroquinolone Resistance of Mutants of *Streptococcus pneumoniae* Obtained In Vivo and In Vitro. *Antimicrob Agents Chemother* **1996,** *40* (11), 2505-2510.

14. Croucher, N. J.; Harris, S. R.; Fraser, C.; Quail, M. A.; Burton, J.; van der Linden, M.; McGee, L.; von Gottberg, A.; Song, J. H.; Ko, K. S.; Pichon, B.; Baker, S.; Parry, C. M.; Lambertsen, L. M.; Shahinas, D.; Pillai, D. R.; Mitchell, T. J.; Dougan, G.; Tomasz, A.; Klugman, K. P.; Parkhill, J.; Hanage, W. P.; Bentley, S. D., Rapid Pneumococcal Evolution in Response to Clinical Interventions. *Science* **2011,** *331* (6016), 430-4.

15. Cornick, J.; Bentley, S., *Streptococcus pneumoniae*: The Evolution of Antimicrobial Resistance to Beta-lactams, Fluoroquinolones and Macrolides. *Microbes Infect* **2012,** *14* (7-8), 573-583.

16. Feikin, D. R.; Kagucia, E. W.; Loo, J. D.; Link-Gelles, R.; Puhan, M. A.; Cherian, T.; Levine, O. S.; Whitney, C. G.; O'Brien, K. L.; Moore, M. R.; Serotype Replacement Study, G., Serotype-specific Changes in Invasive Pneumococcal Disease After Pneumococcal Conjugate Vaccine Introduction: A Pooled Analysis of Multiple Surveillance Sites. *PLoS Med* **2013,** *10* (9), e1001517.

17. Huang, S. S.; Johnson, K. M.; Ray, G. T.; Wroe, P.; Lieu, T. A.; Moore, M. R.; Zell, E. R.; Linder, J. A.; Grijalva, C. G.; Metlay, J. P.; Finkelstein, J. A., Healthcare Utilization and Cost of Pneumococcal Disease in the United States. *Vaccine* **2011,** *29* (18), 3398-412.

18. Song, J. Y.; Choi, J. Y.; Lee, J. S.; Bae, I.-G.; Kim, Y. K.; Sohn, J. W.; Jo, Y. M.; Choi, W. S.; Lee, J.; Park, K. H.; Kim, W. J.; Cheong, H. J., Clinical and Economic Burden of Invasive Pneumococcal Disease in Adults: a Multicenter Hospital-based Study. *BMC Infect Dis* **2013,** *13* (1), 202.

19. Weycker, D.; Farkouh, R. A.; Strutton, D. R.; Edelsberg, J.; Shea, K. M.; Pelton, S. I., Rates and Costs of Invasive Pneumococcal Disease and Pneumonia in Persons With Underlying Medical Conditions. *BMC Health Serv Res* **2016,** *16* (1), 182.

20. Pestova, E. V.; Havarstein, L. S.; Morrison, D. A., Regulation of Competence for Genetic Transformation in Streptococcus pneumoniae by An Auto-induced Peptide Pheromone and A Two-component Regulatory System. *Mol Microbiol* **1996,** *21* (4), 853-62.

21. Håvarstein, L. S.; Coomaraswamy, G.; Morrison, D. A., An Unmodified Heptadecapeptide Pheromone Induces Competence for Genetic Transformation in *Streptococcus pneumoniae*. *Proc Natl Acad Sci U S A* **1995,** *92* (24), 11140-4.

22. Lau, G. W.; Haataja, S.; Lonetto, M.; Kensit, S. E.; Marra, A.; Bryant, A. P.; McDevitt, D.; Morrison, D. A.; Holden, D. W., A Functional Genomic Analysis of Type 3 *Streptococcus pneumoniae* Virulence. *Mol Microbiol* **2001,** *40* (3), 555-71.

23. Oggioni, M. R.; Trappetti, C.; Kadioglu, A.; Cassone, M.; Iannelli, F.; Ricci, S.; Andrew, P. W.; Pozzi, G., Switch from Planktonic to Sessile Life: A Major Event in Pneumococcal Pathogenesis. *Mol Microbiol* **2006,** *61* (5), 1196-210.

24. Zhu, L.; Lin, J.; Kuang, Z.; Vidal, J. E.; Lau, G. W., Deletion Analysis of *Streptococcus pneumoniae* Late Competence Genes Distinguishes Virulence Determinants that are Dependent or Independent of Competence Induction. *Mol Microbiol* **2015,** *97* (1), 151-65.

25. Zhu, L.; Lau, G. W., Inhibition of Competence Development, Horizontal Gene Transfer and Virulence in *Streptococcus pneumoniae* by a Modified Competence Stimulating Peptide. *PLoS Pathog* **2011,** *7* (9), e1002241.

26. Duan, C.; Zhu, L.; Xu, Y.; Lau, G. W., Saturated Alanine Scanning Mutagenesis of the Pneumococcus Competence Stimulating Peptide Identifies Analogs that Inhibit Genetic Transformation. *PloS One* **2012,** *7* (9), e44710.

27. Yang, Y.; Koirala, B.; Sanchez, L. A.; Phillips, N. R.; Hamry, S. R.; Tal-Gan, Y., 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 Chem Biol* **2017,** *12* (4), 1141-1151.

28. Koirala, B.; Hillman, R. A.; Tiwold, E. K.; Bertucci, M. A.; Tal-Gan, Y., Defining the Hydrophobic Interactions that Drive Competence Stimulating Peptide (CSP)-ComD Binding in *Streptococcus pneumoniae*. *Beilstein J Org Chem* **2018,** *14*, 1769-1777.

29. Pozzi, G.; Masala, L.; Iannelli, F.; Manganelli, R.; Havarstein, L. S.; Piccoli, L.; Simon, D.; Morrison, D. A., Competence for Genetic Transformation in Encapsulated Strains of *Streptococcus pneumoniae*: Two Allelic Variants of the Peptide Pheromone. *J Bacteriol* **1996,** *178* (20), 6087-90.

30. Johnsborg, O.; Kristiansen, P. E.; Blomqvist, T.; Håvarstein, L. S., A Hydrophobic Patch in the Competence-Stimulating Peptide, A Pneumococcal Competence Pheromone, is Essential for Specificity and Biological Activity. *J Bacteriol* **2006,** *188* (5), 1744-1749.

31. Chan, W. C.; White, P. D., *Fmoc Solid Phase Peptide Synthesis*. Oxford University Press: Oxford, 2000; Vol. 222.

32. Tal-Gan, Y.; Stacy, D. M.; Blackwell, H. E., *N*-Methyl and Peptoid Scans of an Autoinducing Peptide Reveal New Structural Features Required for Inhibition and Activation of AgrC Quorum Sensing Receptors in *Staphylococcus aureus*. *Chem Commun* **2014,** *50* (23), 3000-3003.

33. Yang, Y.; Cornilescu, G.; Tal-Gan, Y., Structural Characterization of Competence-Stimulating Peptide Analogues Reveals Key Features for ComD1 and ComD2 Receptor Binding in *Streptococcus pneumoniae*. *Biochemistry* **2018,** *57* (36), 5359-5369.

34. Chatterjee, J.; Gilon, C.; Hoffman, A.; Kessler, H., N-Methylation of Peptides: A New Perspective in Medicinal Chemistry. *Acc Chem Res* **2008,** *41* (10), 1331-1342.

35. Tal-Gan, Y.; Freeman, N. S.; Klein, S.; Levitzki, A.; Gilon, C., Metabolic Stability of Peptidomimetics: *N*-Methyl and Aza Heptapeptide Analogs of a PKB/Akt Inhibitor. *Chem Biol Drug Des* **2011,** *78* (5), 887-892.

36. Tal-Gan, Y.; Ivancic, M.; Cornilescu, G.; Yang, T.; Blackwell, H. E., Highly Stable, Amide-Bridged Autoinducing Peptide Analogues that Strongly Inhibit the AgrC Quorum Sensing Receptor in *Staphylococcus aureus*. *Angew Chem Int Ed Engl* **2016,** *55* (31), 8913-7.

Table of Contents artwork