Chapter 7: Investigations into the Role of the Unusual Disulfide in the nAChR Agonist Binding Site

7.1 Introduction

Disulfide bonds are generally viewed as stabilizing elements in protein function. However, available high-resolution structural data over the last two decades have revealed that many important proteins contain so-called “forbidden disulfides,” or highly constrained S-S linkages that may be a net destabilizing presence in the protein. Various potential roles have been ascribed to these special case disulfide bonds, including as redox, mechanochemical, or structural switches.[1, 2] One of the most interesting “forbidden disulfides” is the vicinal disulfide, in which two adjacent cysteines form a disulfide bond. Examples of this type of disulfide have been found near the active site of a small number of enzymes,[3, 4] as well as in the agonist binding site of the nicotinic acetylcholine receptor (nAChR).[3-6]

In the case of the nAChR, conformational changes in the ring formed by this vicinal disulfide have been proposed to play a role in modulating receptor function.[5-7] Specifically, changes in the *cis-trans* preferences of the peptide backbone between these residues could be altered by the binding of acetylcholine, contributing to the opening of the central ion conducting pore. In support of this hypothesis, it has been known for some time that proper function of the nicotinic receptor is highly sensitive to the oxidation state of these cysteines.[8] In addition, Cys-to-Ser mutations at either residue lead to a non-functional receptor.[9] Alternatively, this unusual disulfide bond could be important in localizing a diffuse negative potential near the agonist binding site or conferring additional rigidity on the C-loop.

Computational and NMR studies of small model peptides have yielded conflicting results about the putative native conformation of this unusual eight-membered, disulfide-containing ring.[5, 6] Part of this is likely due to the unique nature of these eight-membered rings. Although otherwise unconstrained peptide bonds have a strong *trans* preference, small amide-containing rings may adopt a *cis* conformation to minimize ring strain. In a systematic study of the conformational preferences of amide-containing rings,[10] it was found that rings with fewer than eight atoms prefer the *cis* geometry, while those with greater than eight atoms adopt the *trans* conformation. In rings containing exactly eight atoms, such as the one formed by the vicinal disulfide, both *cis* and *trans* isomers can be accommodated, making it difficult to predict which geometry will be preferred.

Avizonis et al.[5] performed natural abundance ^{13}C and proton NMR spectroscopy in aqueous solution on a pentapeptide (TCCPD) corresponding to amino acids 191-195 of the nAChR. They additionally performed molecular mechanics calculations to predict the lowest energy conformers and to estimate the barriers to interconversion. Good agreement was found between theory and experiment, with both *cis* and *trans* conformations detected and having $\Delta G^{\neq}$ values at 20 ^{o}C of approximately 15 and 21 kcal/mol, respectively. Molecular mechanics calculations found the lowest energy conformation to be a *cis*-boat-chair conformation. Similarly, high level (B3LYP/6-31++ G(d,g)) ab initio calculations on the eight-membered ring 4,5-ditiaheptano-7-lactam and a small model dipeptide HCO-*ox*-[Cys-Cys]-NH_2 identified the lowest energy conformers for both molecules as containing *cis* amide bonds.[4]

NMR and Monte Carlo simulations by Creighton et al.[6] on the small peptide fragment Ac-*ox*-[Cys-Cys]-NH_2 also revealed interconverting *cis* and *trans* conformations. However, in this study the *trans* amide was found to be preferred over the *cis* amide by a ratio of 60:40, a ratio subsequently confirmed by Hondal and co-workers.[11] Similar to the study by Avizonis et al., this ratio implies a relatively low barrier to interconversion between the two conformers.

Both the Acetycholine Binding Protein (AChBP) crystal structures[12-15] and the α_1 nAChR crystal structure[16] reveal a *trans* amide bond, regardless of the presence of agonist or antagonist (Figure 7.1). However, these structures are static snapshots, and, in the case of the AChBPs, they are images of a binding protein - a protein lacking an ion conducting pore and thus presumably lacking some of the elements that enable signal transduction. As such, one must always interpret these structures with care.

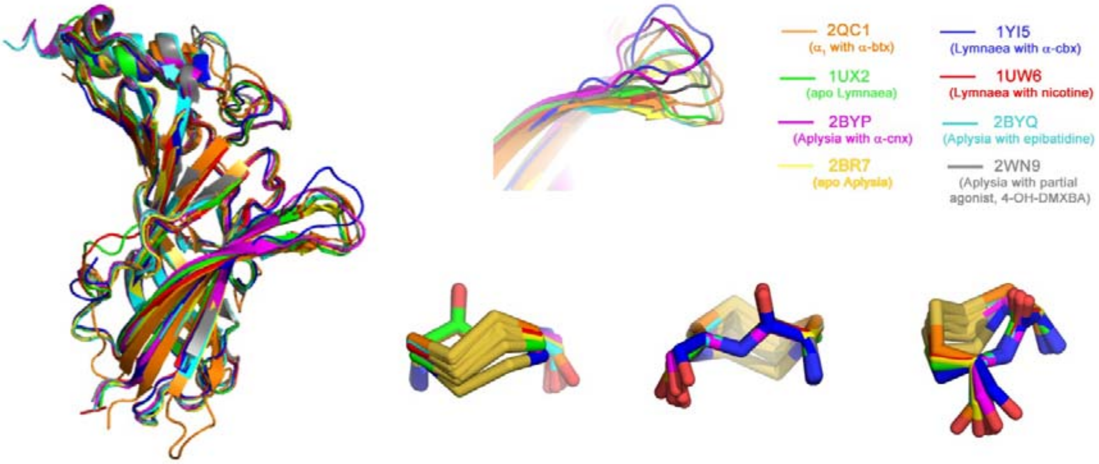

Figure 7.1: Crystal structures of α_1 (orange) and AChBP in the absence (green and yellow) and presence of various antagonists (magenta and blue) and agonists (red, cyan, and grey).

In addition, the ability to correctly identify the amide-bond conformation relies on the structure refinement program used in structure determination. Many of these programs allow for the *cis* conformation only in the instance of a proline residue or where explicitly defined by the crystallographer. Furthermore, at medium resolution the electron density of a *cis* peptide bond appears remarkably similar to that of a *trans* peptide bond and the wrong conformation can easily be accommodated with only minor distortions in the surrounding protein environment.[17, 18] The C-loop vicinal disulfide in the original medium resolution structure of AChBP could not be definitively identified as in the *trans* conformation,[19] although this bond preference is shown in the crystal structure. This structure was used to aid in structure determination for subsequent, higher-resolution structures[12-14, 16], drawing into question the *trans* bond assignment given in these AChBP structures.

Functional studies probing the role of *cis-trans* isomerization of this vicinal disulfide are largely absent in the current literature. One hint as to the conformational proclivities of this region comes from a solution NMR study of AChBP, where ^{15}N-cysteine was incorporated at the native cysteines involved in both the C-loop and Cys-loop disulfide bonds.[20] In the absence of agonist, five distinct cross-peaks associated with the C-loop vicinal disulfide are observed, where only two are anticipated. Additional cross-peaks indicate multiple distinct conformations and could potentially be ascribed to *cis-trans* isomerization of the peptide backbone at this position. In the presence of the agonist acetylcholine, the number of cross-peaks associated with this disulfide is reduced to the expected two cross-peaks, indicating a single predominant conformation in the agonist-bound state.

Structure-function studies at the C-loop vicinal disulfide have largely failed due to the intolerance for substitutions at these positions. While conventional mutagenesis of these residues has been unable to provide insight as to the specific functional role of this unusual disulfide, unnatural amino acid mutagenesis may afford us the opportunity to probe the role of conformational flexibility in this region. Specifically, N-methyl amino acids (Figure 2A) have a lower energy barrier between *cis* and *trans* isomers.[21] In addition, N-methylation of the amide bond has been shown to increase the population of the *cis* conformer in peptides.[22, 23] Most recently, the presence of the N-methyl group in a model vicinal disulfide-containing dipeptide led to a dominant *cis*-conformation (by NMR), whereas both *cis* and *trans* conformations were observed in the amide-containing dipeptide.[11] Thus, incorporation of N-methyl cysteine affords one possible opportunity to better understand the role of backbone isomerization at this position.

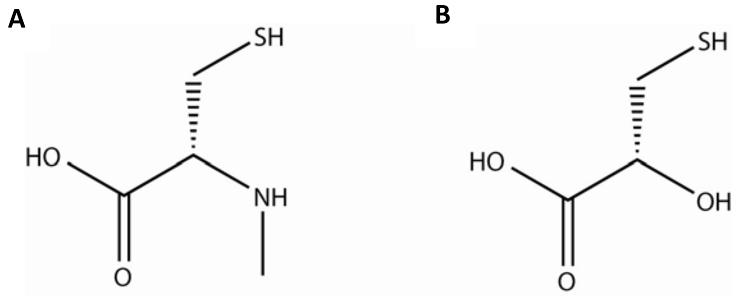

Figure 7.2: Cysteine analogues used in this study. A) N-methyl cysteine B) α-hydroxy cysteine

Incorporation of an α-hydroxy cysteine analogue (Figure 2B) could provide another avenue for addressing this question. Generally speaking, the resulting ester backbone has a lower barrier for *cis-trans* isomerization, but a stronger overall *trans* preference.[24]

Before incorporating these analogues into full-length receptors, *ab initio* calculations using the Gaussian software package[25] were undertaken, comparing the native eight-member, disulfide-containing ring with model peptides containing the two unnatural analogues. By finding the lowest energy conformations and the relative energy differences between the *cis* and *trans* backbone conformations in each of these three cases (amide, ester, and N-methyl), we seek to gain a better understanding of the influence of the unnatural substitutions on the backbone conformations of the vicinal disulfide.

7.2 Results and Discussion

The *cis* and *trans* isomers of a model peptide of the form CH_3CO-ox-[Cys-Cys]-NH_2 were constructed using GausView[25] molecule building tools with the S-S disulfide torsional angles of +/-90. The dihedral angles for these starting structures were derived from the lowest energy conformers from high-level ab initio calculations on a similar model peptide, HCO-ox-[Cys-Cys]-

NH_2.[4] These structures were then subject to B3LYP/6-311++G(d,p) level of theory. The optimized structures then served as the starting point for the ester and N-methyl model systems, where GausView was used to replace the backbone NH between the two cysteines with an oxygen or N-methyl moiety, respectively. In total, the energies for twelve geometry-optimized structures were calculated, with four conformers for each model system (Table 7.1, Figure 7.3).

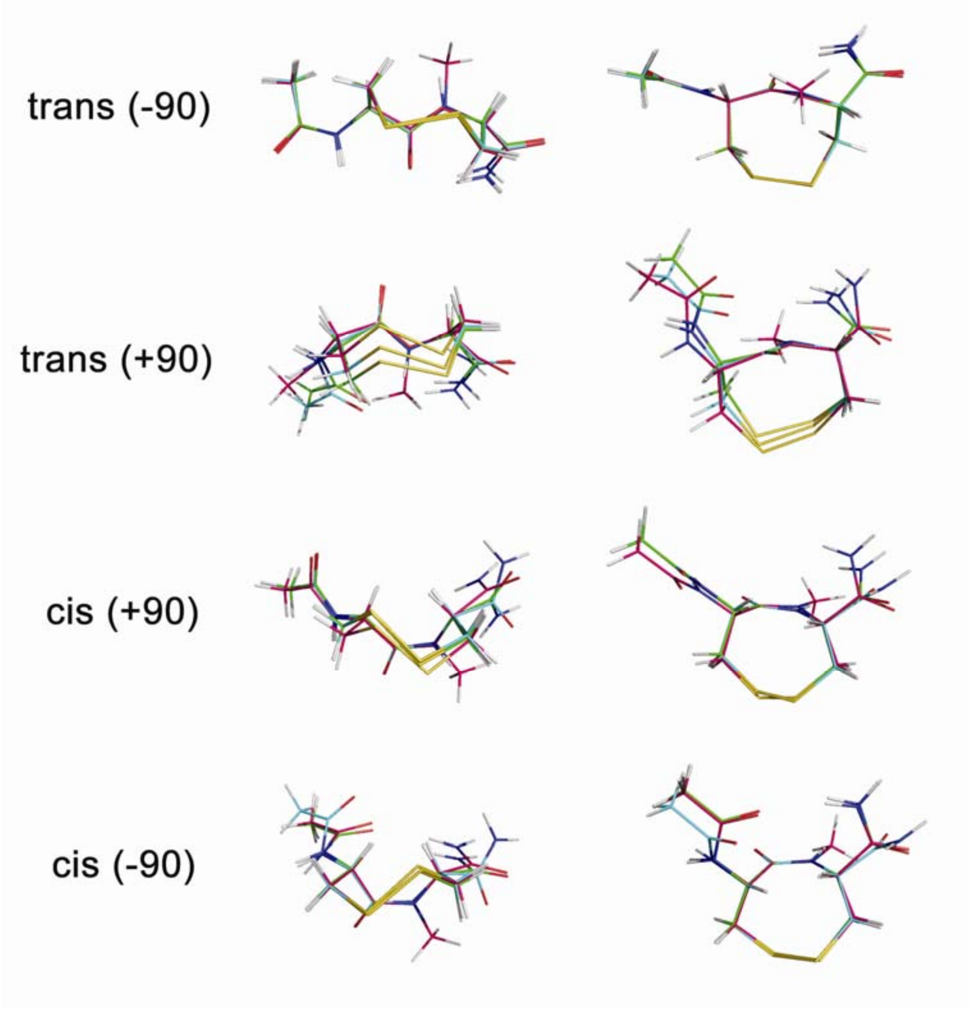

Figure 7.3: Geometry optimized structures. The amide is shown in cyan, the ester in green, and the N-methyl in pink.

	Energy (Hartrees)	ϕ_1	ϕ_2	ψ_1	ψ_2	ω_1	χ_{11}	χ_{21}	χ_{13}
Cis WT (+90)	-1499.30857804	-153.77	-139.05	151.87	143.30	6.80	-159.45	-54.61	95.53
Cis WT (-90)	-1499.30727674	-104.83	-152.82	130.89	148.79	-1.83	177.16	-94.14	-80.94
Trans WT (+90)	-1499.30315553	68.60	-166.68	-77.03	23.25	-146.12	175.91	43.10	90.12
Trans WT (-90)	-1499.29771128	-147.27	81.89	173.00	-43.19	144.01	77.08	-55.71	-86.81
Cis Ester (+90)	-1519.16938950	-155.17	-135.07	150.70	-3.48	7.48	-161.49	-58.20	95.37
Cis Ester (-90)	-1519.16561903	-63.07	-142.84	129.31	61.65	-2.90	175.34	-86.42	-92.62
Trans Ester (+90)	-1519.17167390	67.48	-161.54	-86.89	8.42	-154.74	179.98	44.28	85.90
Trans Ester (-90)	-1519.16449190	-146.02	80.28	176.17	-36.01	147.11	74.05	-56.52	-83.20
Cis N-methyl (+90)	-1538.62309456	-155.54	-133.60	146.86	34.85	13.82	-162.22	-55.58	95.68
Cis N-methyl (-90)	-1538.62226175	-70.28	-134.67	131.89	69.80	-6.52	176.43	-84.12	-92.57
Trans N-methyl (+90)	-1538.59575667	-32.31	-158.51	-67.98	63.27	-148.05	173.35	30.46	91.96
Trans N-methyl (-90)	-1538.60825774	-147.99	85.38	172.74	-49.22	144.11	73.56	-55.06	-86.15

Table 7.1: Relevant parameters for the geometry-optimized structures in this study. See Figure 7.4 for definitions of dihedral angles.

Given that the amide structures served as a starting point for the ester and N-methyl structure geometry optimizations, it is not surprising that geometric preferences of the resulting amide, ester, and N-methyl structures are, on the whole, similar (Figure 7.4). The largest differences between these molecules is seen in the external dihedral angles, ϕ_1 and ψ_2. In general, a larger range of values is observed for the *cis*-conformers, versus the *trans* conformers. The internal angles, by contrast, remain tightly clustered among the three molecules in both the *cis* and *trans* conformations.

Energetically, however, there are some striking differences among the three molecules. In agreement with previous studies, a moderate energy difference of 3.4 kcal/mol is seen between the two lowest energy conformers of the amide, with the preference for the *cis*-form (Figure 7.5). As would be predicted from the literature, the N-methylation of the internal amide leads to a stronger *cis* preference, resulting in a 9.3 kcal/mol energy difference between the *cis* and *trans* conformations. The ester modification has exactly the opposite effect. In fact, the lowest energy ester conformer contained a *trans* bond (ω = -154.74˚), which was 1.4 kcal/mol lower in energy than the lowest energy *cis* conformer.

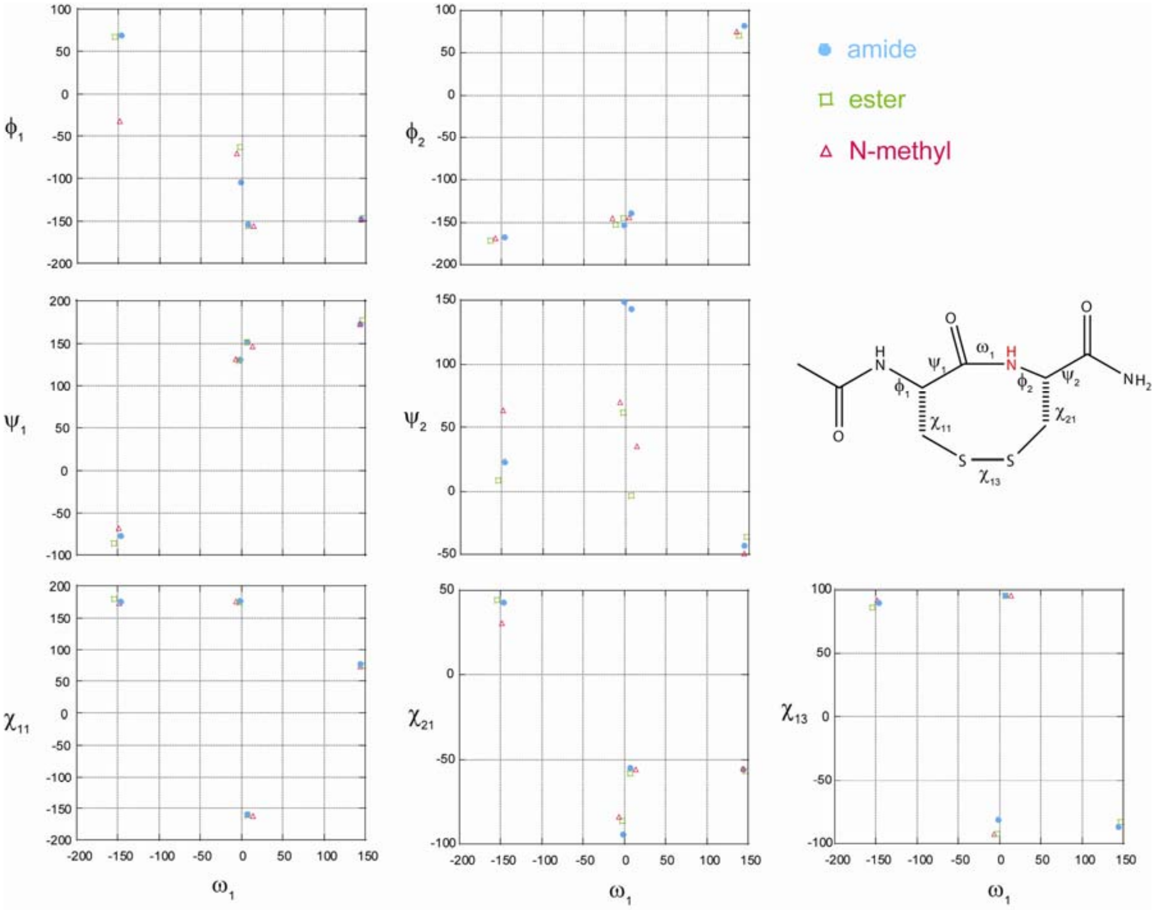

Figure 7.4: Selected conformational parameters of the structures evaluated in this study. Torsion angles are defined in the structure on the right.

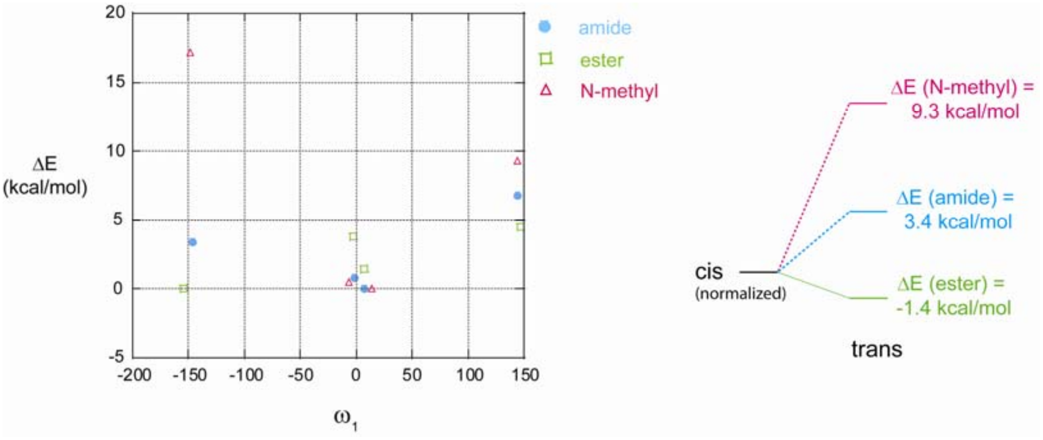

Figure 7.5: A) Correlation of the relative energies of the conformers in this study with the dihedral angle, ω. B) Relative ΔE between the lowest energy *cis* and *trans* isomers.

These results lead to some clear experimental predictions for models of nAChR function that implicate the vicinal disulfide. In the first model, the *cis*/*trans* preferences of the vicinal disulfide are altered by agonist binding. This leads to a conformational change in the C-loop that contributes to receptor activation. In this case, both the N-methyl or ester modifications should impact receptor function and these effects should be opposite. In other words, if the native conformation has a slight *cis* preference in the absence of agonist, then the N-methylation of the amide nitrogen will stabilize the apo conformation, making the receptor more difficult to open.

A second model ascribes a primarily structural role to this unusual disulfide; the conformational constraints of the eight-membered ring provide the correct geometry to the C-loop, allowing it to make the necessary intermolecular interactions with the agonist and intramolecular interactions with other residues in the receptor. If this is the case, one reasonable expectation would be that only the modification that contains the same conformational inclination as the native peptide bond will be able to produce functional receptors (e.g., α-hydroxy cysteine, if the wild-type receptor is predominantly *trans* or N-methyl cysteine, if the wild-type receptor is predominantly *cis* at the vicinal disulfide).

In our simple model system, the *cis* over *trans* preference was strengthened by 6 kcal/mol in the case of the N-methyl- containing compound. In the case of the ester, the backbone conformational preference was reversed, an energetic effect worth nearly 5 kcal/mol. A ten-fold shift in macroscopic EC_{50} is approximately equivalent to a $\Delta\Delta G$ of ± 1.4 kcal/mol. While these simple model dipeptides cannot possibly capture the complexity of a 280 kilodalton protein such as the nAChR, even moderately attenuated effects on the *cis*-*trans* preferences of the vicinal disulfide should be easily detectable in a full-length receptor, provided that the bond conformation at this position is important for receptor function.

7.3 Conclusions and Future Directions

Ab initio studies with model peptides indicate that the N-methyl and ester modifications in the eight-membered ring created by the vicinal disulfide do alter the *cis-trans* preferences of the protein backbone. The amide backbone has a slight *cis* preference, which is strengthened by N-methylation of the amide nitrogen. In contrast, the ester backbone leads to a preference for the *trans* conformation. As such, the incorporation of these cysteine analogues into the full-length nAChR should provide insight as to the importance of *cis-trans* preferences at the C-loop vicinal disulfide. However, the calculated *cis-trans* preferences tell us little about the relative barrier to *cis-trans* isomerization for the constrained eight-membered ring. To address this issue, future ab-initio characterization of the transition states for *cis-trans* isomerization in the model peptides is additionally planned.

Synthesis of N-methyl cysteine and α-hydroxy cysteine by Angela Blum is currently underway. Incorporation of these unnatural analogues at positions 192 and 193 in the muscle nAChR, followed by electrophysiological characterization is planned. In the event of no detectable ionic currents, surface expression will need to be confirmed using radiolabeled α-bungarotoxin and binding studies or fluorescently labeled α-bungarotoxin and TIRF microscopy.

7.4 Materials and Methods

Geometric parameters used in this study are defined in Figure 7.4. Structure building and subsequent ab initio calculations were carried out using the Gaussian 03 software package.[25] at the B3LYP/6-31++G(d,p) level of theory in the gas phase. Geometric parameters for the initial structures of the amide-containing model dipeptides were derived from the work of Hudaky et al.[4] *Cis* and *trans* isomers with both the +90 and -90 conformations of the disulfide dihedral (χ_{13}) were considered. These geometry-optimized structures then served as starting points for the ester and N-methyl calculations. Conformational characterization of the optimized structures was done in GausView and Pymol.[26]

7.5 References

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