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PROPERTIES OF MODIFIED TRYPTOPHANS IN A MEMBRANE-SPANNING CHANNEL

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Abstract:

An emerging concept in biology assigns the amino acid tryptophan specific roles at the membrane/water interface that help to determine the conformation and biological function of membrane-spanning proteins. Previous studies involving the antibiotic model system gramicidin A (gA) have illustrated the importance of the indole ring of tryptophan (Trp) in anchoring proteins to a bilayer membrane and promoting ionic currents. To further investigate these phenomena, derivatives of Trp that have lost hydrogen-bonding ability (1-methyl-Trp), have an altered dipole moment (7-aza-Trp), or both (1-methyl-7-aza-Trp) were chosen for incorporation into gA. Gramicidin analogues that incorporate these modified Trps were then analyzed by single channel experiments. In addition, methods were developed for the selective exchange of indole hydrogen with deuterium (a heavy isotope of hydrogen, ^2H) using a Raney nickel catalyst. The ^2H labels enable determinations of the orientation of each Trp indole ring with respect to the membrane surface using solid-state deuterium NMR spectroscopy. The last method I pursued involves the application of ab initio molecular modeling programs to calculate the side-chain dipole moments of Trp, 1-methyl-Trp, 7-aza-Trp, and 7-aza-1-methyl-Trp. Therefore, this project combines both experimental and theoretical aspects of scientific research.

Results from the single-channel experiments of [7-aza-Trp] and [7-aza-1-methyl-Trp] gA analogues indicate that there is a positive correlation between channel conductance and the magnitude of the side chain dipole moments. A new methodology involving a Raney nickel catalyst was also successfully developed that allows for ~75% of 7-aza-Trp's sixth hydrogen to exchange with deuterium. In addition to these experimental results, the ab initio program PQS was used to generate theoretical predictions of Trp side chain dipoles that were comparable to experimentally determine dipoles, and that allowed for the calculation of 1-methyl-Trp's side chain dipole. Beyond the immediate results, the more general implication of this project is the fundamental knowledge gained concerning the interactions of Trp with other amino acids, water, and lipids. These studies will contribute to a better understanding of folded proteins — especially those that span biological membranes.

Introduction:

With approximately 90 percent of the human genome nucleotide sequence elucidated[1,2], a continuing challenge for biochemists and molecular biologists will be to determine the mechanisms responsible for the inherent folding and tertiary structure that render encoded proteins biologically active. At present, the tertiary structures of thousands of proteins have been determined. However, the vast majority of these proteins are globular, water-soluble proteins; only a minority (less than 40) are transmembrane and/or channel proteins.

Several of these characterized transmembrane proteins contain the amino acid tryptophan (Trp). Within each protein, Trp displays a strong preference for the membrane/water interface that may be attributed to the dipole moment and hydrogen bonding ability of the indole ring of Trp. Previous studies involving the channel protein gramicidin A (gA) suggest that three of the four Trps in its amino acid sequence must form hydrogen bonds with the corresponding membrane interface in order for gA to maintain its native conformation and biological activity[3]. In fact, if all but one of the four Trps are substituted with phenylalanine (a more hydrophobic amino acid that does not have the ability to form hydrogen bonds), another gA conformation appears that exhibits no significant ion transport activity[4]. The passage of ions through gA has been largely attributed to the ability of Trp to attract ions into the channel by means of its indole ring's dipole moment and to hydrogen bonding that involves the amine group in the Trp indole ring.

The structure and biological function of gramicidin A is well established, making it a useful model system for further investigations of Trp. The gA peptide has the following sequence: HCO*Val*—Gly—*Ala*—Leu—*Ala*—Val—*Val*—Val—*Trp*—Leu—*Trp*—Leu—*Trp*—Leu—*Trp*—$NHCH_2CH_2OH$ (L-amino acids are in italics), and folds into a single-stranded helical

subunit. This subunit must then couple with another identical subunit in a head-to-head manner to display positive ion (e.g. sodium, potassium, cesium) conductance indicative of channel activity as it occurs in nature.

My research addresses both the hydrogen bonding and dipolar properties of the indole ring. To investigate these characteristics, several methods were developed. First, analogues of tryptophan with chemically modified indole rings were chosen for incorporation into gA, (Figure 1).

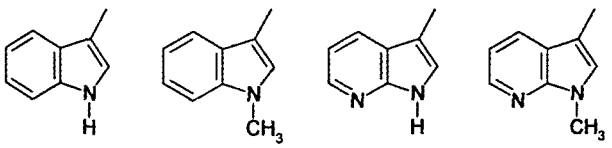

Figure 1: Indole side-chains of Trp, 1-methyl-Trp, 7-aza-Trp, and 7-aza-1-methyl-Trp.

The side chain of 1-methyl-Trp has lost hydrogen-bonding ability at N1; 7-aza-Trp as an altered dipole moment from Trp and introduces additional hydrogen bonding ability at N7; and 7-aza-1-methyl-Trp has a combination of the properties described for 1-methyl-Trp and 7-aza-Trp. As the dipole moments of some of these Trp derivatives were not well established experimentally, I decided to pursue a theoretical method that utilizes computer molecular modeling techniques to accurately determine each side-chain dipole. Finally, to eventually understand how each Trp derivative orients in the membrane after incorporation at position 9, 11, 13, or 15 in the gA sequence, a protocol for the selective exchange of indole hydrogen with deuterium (a heavy isotope of hydrogen, ^2H) was needed in anticipation for future ^2H-NMR experiments.

Amino Acid Synthesis and Purification:

Of the three Trp derivatives, only 1-methyl-Trp and 7-aza-Trp are available commercially. Therefore, 7-aza-1-methyl-Trp must be synthesized from 7-azaTrp, (Figure 2). Prior to synthesis, the amino acid (α-) amine of (D,L)-7-aza-Trp was†"protected" with a BOC-ON reagent, which prevents it from forming bonds out of sequence during peptide synthesis. The indole nitrogen (N1) of Boc-(D,L)-7-aza-Trp was then methylated with triethylamine under anhydrous (water free) conditions at -78°C. The BOC-ON and methylation protocols were adopted from Rich et al. (1995)[5], although several minor adjustments had to be made to the methylation procedure before I was able to consistently obtain a 50% yield of 1-methyl-7-aza-Trp with 95% purity.

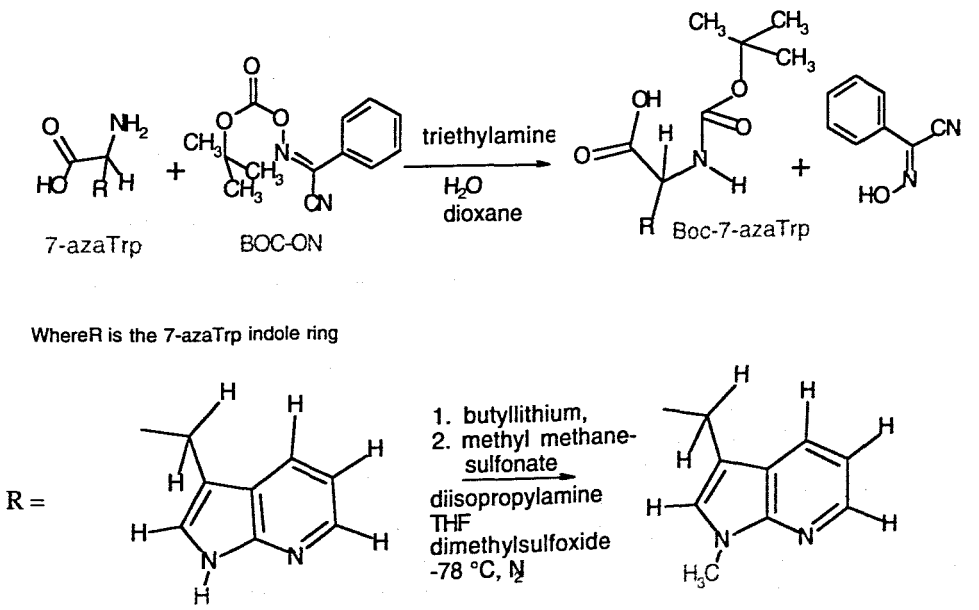

Figure 2: Schematic representation of Boc-D,L-7-azaTrp and Boc-D,L-7-aza-1-methylTrp synthesis reactions. A) D,L-7-azaTrp reacts with BOC-ON in the presence of a base (triethylamine). This results in the formation of Boc-D,L-7-azaTrp. B) The N at position 1 in Boc-D,L-7-azaTrp is deprotonated by anhydrous butyllitium, and methylated with methyl methanesulfonate, to yield Boc-D,L-7-aza-1-methylTrp.

Additional concerns are introduced because 7-aza-Trp is commercially available only as a "racemic" mixture (equal parts D- and L-isomers). As illustrated previously, only the L-isomer of Trp, or modified Trp, is present in the functional peptide. If the stereochemistry is not precise, folding of the helical gA structure will be distorted so as to prohibit the passage of positive ions through the channel interior[6]. Thus, to obtain functional peptides, the D- and L-isomers of 1-methyl-7-aza-Trp and 7-aza-Trp were separated using a Chirobiotic T chiral column on the basis of their "handedness," (Figure 3).[7,8] Chirobiotic T is a chiral packing material that consists of Teicoplanin, a glycopeptide, which has been covalently bound to silica gel. Teicoplanin effectively forms a series of cavities to "capture" both L- and D-amino acids, but with variable affinity. An alternative resolution was to separate the two peptide diastereoisomers resulting from the single substitution of one D,L-Trp derivative. This separation is possible because functional and non-functional gA analogues induce unique conformers that elute at different rates from a reversed-phase column[vi].

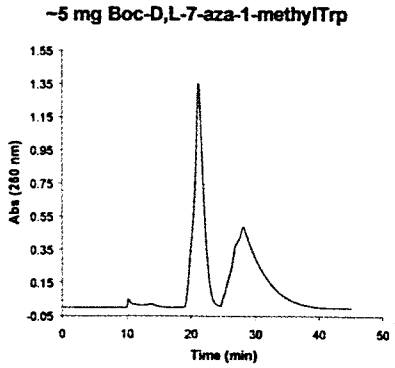

Figure 3: Separation of ~5 mg Boc-D,L-7-aza-1-methyl-Trp using a semi-preparative Chirobiotic T chiral column. The first peak to elute is Boc-L-7-aza-1-methyl-Trp; the second is the D-isomer[vii,viii].

Peptide Synthesis and Purification:

Haiyan Sun and I synthesized a total of 12 singly-substituted gA peptides using standard solid-phase peptide chemistry: [1-methyl-Trp][9,11,13, or 15] gA, [7-aza-Trp][9,11,13, or 15] gA, and [7-aza-1-methyl-Trp][9,11,13, or 15] gA. Fmoc is the protecting group for all the amino acids except 7-aza-Trp and 7-aza-1-methyl-Trp, which are Boc-derivatized amino acids. Each consecutive amino acid is joined to the growing peptide by an amide bond until the sequence is complete. The peptide is subsequently "cleaved" from the resin using ethanolamine and "formylated" at the amino-terminus using *para*-nitrophenylformate. As the Boc-group differs from the protecting group of the other amino acids, the peptide must be taken off the synthesizer and deprotected manually using trifluoroacetic acid at these steps.

Once synthesized, each gA analogue was purified on a reversed-phase high performance liquid chromatography column. The final peptides were analyzed at Weill Medical College of Cornell University by single-channel experiments for conformational and functional changes.

Results from single-channel experiments indicate that singly-substituted [1-methyl-Trp] gA analogues form two distinct channels. One channel (A) is remarkably similar to native gA and has a shorter lifetime and higher conductance than the other channel (B), (Figure 4).

Figure 4: Current trace for the single-channel experiment of [1-methyl-Trp][15] gA. The B channel is identified to distinguish it from the A channel which has a higher conductance and shorter lifetime.

Hybrid channel experiments, in which a reference subunit of known helix sense is paired with the peptide of interest, confirmed the A channel to be right-handed. The B channel, which is present only as a minor population, represents a second channel type that is the subject of continuing investigation as to its structural conformation.

Only the right-handed channel type was present in samples of [7-aza-Trp] and [7-aza-1-methyl-Trp] gA. This suggests that there are properties of the 7-aza-group that influence gA to form characteristic right-handed channels. Results also indicate that there is a positive correlation between the dipole moment and observed channel conductance, for as the magnitude of the dipole decreases between [7-aza-Trp] and [7-aza-1-methyl-Trp] gA analogues (which have similar dipole directions), so does the channel conductance.

Dipole Moment Calculation:

Using the *ab initio* molecular modeling program PQS, I was able to accurately calculate the dipole moments for the side chains of all three derivatized amino acids, as well as Trp, for comparison with experimental data. The results illustrated below are based on the converged geometries of each side-chain (Table 1; Figure 5).

Table 1

	Trp	1-methyl-Trp	7-aza-Trp	7-aza-1-methyl-Trp
Experimental Dipole	2.1 D[9]	-	1.47 D[10]	1.34 D[x]
Calculated Dipole	2.05 D	2.22 D	1.80 D	1.67 D

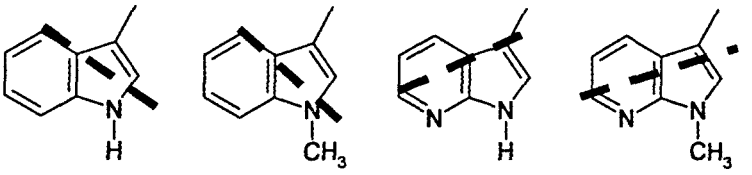

Figure 5: Orientation of the calculated dipole moment in the side chains of Trp, 1-methyl-Trp, 7-aza-Trp, and 7-aza-1-methyl-Trp.

Electron orbitals were approximated by Gaussian functions of basis set 6-31G★, and all initial geometries were generated by the semi-empirical program PM3. Density functional theory was applied to approximate the electron correlation, and self-consistent field theory was applied to calculate the orbital coefficient.

Selective Deuteration:

It was necessary to develop a protocol for the selective exchange of indole hydrogen with deuterium (^2H) to eventually determine the orientations of each Trp derivative within a phospholipid bilayer after incorporation into the gA sequence. The selective deuteration of the indole ring of 1-methyl-Trp was achieved by employing a deuterated trifluoroacetic acid catalyst[11], (Table 2). This method proved unsuccessful for 7-aza-Trp, for reasons we believe may be related to the protonation of the 7-aza-group (N7). An alternative protocol from Yau and Gawrisch (1999)[12] was then considered, which uses Raney nickel as a catalyst for the selective deuteration of indole and 1-methyl-indole. After making several alterations to the procedure, I found that 7-aza-Trp could be dissolved in a 1% NaO^2H solution before combining with deuterated Raney nickel. Upon sufficient reaction time, the Raney nickel was filtered and amino acid precipitated out of solution using citric acid. Analysis by ^1H nuclear magnetic resonance spectroscopy (NMR) confirmed an ~75% selective exchange (loss of signal) of the 6H with ^2H, after three weeks and at room temperature.

Table 2.

Proton position	Trp % ^2H	1-methylTrp % ^2H	7-azaTrp % ^2H	7-azaTrp % ^2H*
2	~70	>90	~0	<10
4	0	<10	<5	~0
5	~20	~70	~0	<10
6	<10	<10	<5	~75
7	~0	<10	-	-

* Raney nickel

Discussion:

The results presented in this paper have provided new methods for the methylation of Boc-D,L-7-aza-Trp, the chiral separation of D,L-7-aza-Trp and D,L-7-aza-1-methyl-Trp, and the selective deuteration of 7-aza-Trp. The developments of the first two methods are of significant interest, for the future synthesis of doubly and multiply substituted and labeled peptides. In addition, it is anticipated that the last described method will transfer to other 7-aza-Trp derivatives, thus providing a method for the selective exchange of indole H with ^2H for these compounds.

With sufficient quantities of deuterated 7-aza-Trp and 7-aza-1-methyl-Trp through the selective exchange protocol, it will be possible to synthesize additional singly-substituted gA analogues with ^2H-7-aza-Trp and ^2H-7-aza-1-methyl-Trp at positions 9,11,13, and 15. These peptides will then be analyzed by solid-state deuterium NMR, so that we may ultimately understand the orientations of 7-aza-Trp and 7-aza-1-methyl-Trp within the membrane[13].

New discoveries were made with regard to both the side-chain dipole moments of Trp, 1-methyl-Trp, 7-aza-Trp, and 7-aza-1-methyl-Trp, and the effect of the latter two amino acids on channel function. The *ab initio* calculations for the Trp derivative side-chain dipoles are comparable with the magnitudes of the experimental dipoles and have allowed for the accurate prediction

of the side-chain dipole moment of 1-methyl-Trp. Key results to date also indicate that the channel conductance is positively correlated with the magnitude of the dipole moment; however, future experiments regarding the orientation of these singly substituted amino acids will be necessary to confirm the dipole orientations with respect to the ion translocation pathway.

These findings allow us to better understand those properties of Trp that are essential to the tertiary structure and biological function of gA. Overall, the implication of this project is the fundamental knowledge gained concerning the interactions of Trp with other amino acids, water, and lipids. These detailed studies will ultimately contribute to better understanding of folded proteins — especially those that span biological membranes.

Endnotes:

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Faculty Comments:

Ms. Scherer's mentor, Roger Koeppe, has extraordinarily positive comments about her and her work. He says ...

> I write to express enthusiasm for the superb job that **Erin Scherer** has done in her undergraduate research concerning the development and discovery of new molecular tools for basic research in membrane biochemistry. Ms. Scherer has pursued her research with a remarkable and unique combination of creativity, independence, perseverance and versatility. I will comment on each of these qualities in turn.
>
> Creativity – Beginning with a mere suggestion from me that she investigate the electrostatic and hydrogen-bonding properties of modified indole rings in membrane-spanning peptides, Ms. Scherer defined a broad scope for the project and infused her creative abilities into each aspect of the research. On the experimental side, she pursued new strategies for the labeling of modified rings that were intractable by the methods in current use in my laboratory (see "perseverance," below). Not content with experiments alone, Ms. Scherer on her own sought to fill a gap in the existing literature by doing *ab initio* calculations (for which I have no expertise) to determine the dipolar properties of her modified indole rings; she obtained the help of Dr. Peter Pulay to perform these calculations. Furthermore, she, on her own, wrote to a leading pharmaceutical company to request a gift — for research purposes — of a particular chemical isomer of one of her molecules that has been reported but is not commercially available. She has gumption and drive, combined with innovative curiosity, that are rare among graduate students or indeed established scientists at any level.
>
> Independence – As noted above, Ms. Scherer expanded the scope of her project, in terms of theoretical as well as experimental aspects, far beyond my original suggestions. She designed and performed her own experiments and calculations in a highly original fashion.
>
> Perseverance – Some of Ms. Scherer's early experiments involving organic synthesis and catalyzed hydrogen/deuterium isotope exchange reactions did not go well. In particular, the standard trifluoroacetic acid-catalyzed isotope exchange on indole rings, that others in my laboratory were using, did not work for the "7-aza" modified rings that Ms. Scherer needed to use. Undaunted, she pursued a new method, based on catalysis by Raney nickel. Although precedents for this method had been reported for indole itself, the approach to 7-aza-indole was unknown and Ms. Scherercontinued to endure failure after failure with difficult experimental procedures. Fortunately, her persistence was rewarded. By suitably modifying the existing procedures, she developed a new method for introducing deuterium into 7-aza-indole rings. The method will have general applicability in our laboratory and others for the preparation of labeled samples as probes for analysis for magnetic resonance spectroscopy in biological membranes.
>
> Versatility – I have already mentioned the two major aspects of Ms. Scherer's versatility, namely her willingness and flexibility to modify her experimental approaches to achieve the necessary chemical products and her interest in combining theory with experiment. I have not seen other undergraduate students assume such a broad combined perspective toward understanding an overall research question.
>
> In summary, Erin Scherer bubbles with ideas and possesses the practical ability to carry them to fruition. Her inquiring mind makes it a pleasure to work with her.

Biology Professor Claudia Bailey taught Ms. Scherer and has followed her progress closely; she says ...

> Many student research projects are offshoots of faculty endeavors. Ms. Scherer has displayed significant understanding of the research project and was awarded a SILO/SURF Undergraduate Research Fellowship. Her research has encompassed several semesters of work, and she has mastered numerous techniques. She will submit her Honors Thesis in April. Ms. Scherer's project is considerably more sophisticated, complex and technical than most of the honors projects on campus. Ms. Scherer is engaged in a research project in Roger Koeppe's lab where she is investigating the role of the indole ring of tryptophan, an amino acid present in the antibiotic gamacidin, in augmenting the interaction between ions, water and the plasma membrane of cells. She will employ deuterization of the amino acid solid phase peptide synthesis to modify the structure of the antibiotic. The newly synthesized molecule, once inserted into a membrane, should modify the flow of ions through the membrane. Ms. Scherer then utilizes solid state deuterium NMR to determine the localization of Trp relative to the protein and its orientation in the membrane.
>
> Ms. Scherer's work is generating data that contributesto the fundamental knowledge for predicting the effect of the modification of protein tertiary structure on the functional state of the protein. This work impacts protein chemistry, membrane structure and function, and drug design. Her body of work should have significance in the biochemistry and medical chemistry communities, and I would expect it to be publishable in a peer-reviewed professional journal. This is a highly sophisticated project for an undergraduate and attests to the confidence Dr. Koeppe has in Ms. Scherer's abilities. I have seen few student research projects that compete with Ms. Scherer's research in categories of significance, rigor, complexity, and quality.
>
> In 2001, 1 recognized Ms. Scherer's abilities when she was among the top five students in cell biology, all of whom achieved a 100% level of performance. Ms. Scherer wrote the most articulate essay answers I have read in many years. Not only was she factual, but her logical development of the topics indicated a thorough understanding of the principles involved. In class, her questions indicated good critical thinking and often helped other students to "break the silence" barrier, encouraging others to participate. Her enthusiasm for learning was always evident. In her discussions with me about her current research these same qualities continue to be apparent.
>
> I also am impressed with Ms. Scherer's involvement in summer research programs. She has sought opportunities to expand her experience in the research laboratory while remaining focused on her goal to apply her research skills to the medical sciences. The work in Dr. Dave Wessinger's lab at UAMS to determine a preliminary pharmaco-kinetic profile for a psychoactive anti-pertussive agent available in over-the-counter cough medications was fascinating. Both the UAMS and UAF research programs have significant clinical potential that she can explain and evaluate.
>
> Ms. Scherer remains focused upon a professional research career and undoubtedly will continue to make contributions in her field. She is a highly worthy candidate for the Outstanding Undergraduate Research Award.

Chemistry Professor Lothar Schafer taught Ms. Scherer in his honors colloquium. He writes ...

> I know Erin Scherer as her teacher in CHEM 3923H, the Chemistry Department's honors colloquium, in which she is currently enrolled.
>
> Ms. Scherer's research is involved with Gramicidin A as a model system for studying the properties of tryptophane. Tryptophane is an important amino acid because in membrane proteins it aligns between the phospholipid (water-repellant) and the aqueous phases. Tryptophane also has an important function in controlling the flow of ions through membrane channels. When it is removed from gramicidin, conductance is decreased. In the recent past Dr. Koeppe's research in this area has found a great deal of national and international recognition. It is a first class program, and Ms. Scherer's project is really at the cutting edge in this field, not a typical undergraduate-level project. Her research is of general significance because it can potentially lead to the development of antiviral and antibiotic drugs. Most importantly, I expect it to generate important information on how amino acid residues contribute to tertiary protein folding, one of the last true frontiers of protein chemistry.
>
> In our discussions in CHEM 3923H, I have found Erin Scherer to be a brilliant young lady, very articulate, highly intelligent and able to think critically and independently. It is a pleasure to witness how vigorously she can discuss concepts that have caught her interest.