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HYDROPHOBIC MATCHING OF SHORT GRAMICIDINS WITH PHOSPHOLIPIDS

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Abstract:

Given the highly stochastic nature of larval supply, coral reef fish may often settle in sub-optimal habitats with limited prey. This study examines the foraging and territorial habits of a coral feeding butterflyfish, Chaetodon baronessa, *living in two contrasting habitats with markedly different coral prey. In exposed front reef habitats, where coral prey was highly abundant,* C. baronessa *was highly selective in its choice of prey and aggressively maintained small territories. In contrast, in back-reef habitats where coral prey was scarcer,* C. baronessa *was more generalist in its choice of prey, and had larger territories that were only weakly defended. The contrasting habits of C. baronessa in different reef habitats are consistent with predictions of optimal foraging theory, in that dietary specialisation and territoriality are reduced to maximise food intake where prey is less abundant.*

Introduction

In all living cells, transmembrane proteins "speckle" biological membranes. Some of these structures allow passage of charged particles across the hydrophobic membrane, making possible the concentration and electrical gradients necessary for essential functions such as cell-to-cell communication and life itself. Understanding the membrane channel is therefore essential to understanding the function of the cell. However, membrane-spanning proteins, naturally difficult to isolate and purify because they consist of both hydrophobic and hydrophilic segments, function only in the lipid environment of the membrane and are soluble only in the membrane or organic solvent. In addition, the large size of these proteins complicates their study.

The simpler "model" peptide gramicidin A offers an alternative approach toward understanding transmembrane channels (1). A naturally-produced antibiotic, gramicidin is a peptide consisting of 15 amino acids. Two molecules of gramicidin associate to form a b-helical channel in the membrane, with a pore wide enough to allow passage of cations such as sodium or potassium (2). This "ion-conducting" channel forms only in membranes; in other environments, gramicidin folds differently, into a non-conducting double stranded form (3) (Figure 1).

Four tryptophans at the end of the peptide are thought to be responsible for channel formation (3). Tryptophan is an amino acid that is most stable when located at the interface between the membrane and the surrounding solution (4). Tryptophans seek the membrane-water interface, burying the remaining, hydrophobic amino acids in the interior of the membrane. Presumably, if this activity generalizes to other channels, then the proportions and placements of tryptophans in the protein sequence vitally influence channel conformation and activity.

The tryptophan-driven folding model predicts that proper gramicidin channel folding is governed both by the relative lengths of the lipids and peptides, and by the tryptophan content of the peptides. Greathouse et al. (5) have varied the length of lipid chains in the membrane to regulate the hydrophobic matching lengths between lipids and peptides. Because the lowest energy conformation requires that the tryptophans remain at the interface, disrupting the matched hydrophobic lengths induces strain upon the conformation. Gramicidin was inserted into membranes containing 6 to 12 carbons per acyl chain. It was found that channels will form only in lipids containing acyl chains with 8 or more carbons. In lipids that are too short for channel formation, a double stranded conformation is observed (5).

This project expands on the previous research by varying the number of tryptophans and the length of the gramicidin peptide itself, in addition to the lipid length, in order to further investigate the effect of hydrophobic matching upon channel conformation. We have synthesized shortened gramicidins of 9, 11, and 13 amino acids, each with 1 or 2 tryptophans (Table 1). We examined the conformations of the molecules in lipids of varying lengths using circular dichroism (CD)[1] spectroscopy, a method of analysis that reveals whether the peptide forms a channel or some other conformation. Based upon our results, conformation does seem to correlate with lipid length.

Materials and Methods

Materials. Amino acids were purchased from Bachem and Advanced Chem Tech. Diacylphosphocholine lipids (Table 2; Figure 2) were from Avanti Polar Lipids, Inc. (Alabaster, AL). Methanol, chloroform, DCM and MtBE (HPLC grade) were from Burdick and Jackson (Muskegon, MI). Water was deionized Millipore Corp. Milli-Q water (Bedford, MA). F-L-Val was from Bachem, Bioscience.

Peptide synthesis. Peptides were synthesized on an Applied Biosystems Model 431 as described in Greathouse et al. (6). Peptides were cleaved from the resin in 1500 µl of 10% ethanolamine (EA) in DMF for 48 hours at room temperature. Peptides were then filtered and precipitated with MtBE. Peptides were dried under vacuum overnight. Dried peptides were dissolved prior to quantification. Masses of peptides were confirmed by mass spectral analysis (Mass Consortium, San Diego, CA) and purity by high-performance liquid chromatography.

Sample preparation. Peptide/lipid dispersions (1:30 molar ratio) were prepared by suspension of dried peptide in filtered methanol followed by addition of lipid from stock chloroform solutions. The volumes of methanol and chloroform were adjusted to create a 50/50 (v:v) methanol/chloroform solution. The suspensions were dried on a speed vacuum for 1.5 hr and then dried under vacuum 24-72 hr.

Dried peptide/lipid mixtures were resuspended in deionized water, sonicated for approximately 70 min. at 50°C in a Branson W-185 cell disruptor (power level 5) fitted with a Model 431-A cup horn accessory, and incubated for 30 minutes at 50°C. Samples were centrifuged at 14000 rpm for 5-15 minutes at room temperature. The concentration of peptide in the supernatants was determined at 280 nm on a Hewlett Packard 8452A Diode Array spectrophotometer using an extinction coefficient of 5600 $M^{-1}cm^{-1}$ per tryptophan. CD measurements were obtained at room temperature using a Jasco 710A spectrometer. Each spectrum is an average of 8-12 scans from 200-300 nm, with a path length of 0.1 cm.

Results

The conformational dependence of shortened gramicidin analogues on lipid acyl chain length of short-chain phospholipids was investigated. It is known that gA exhibits a dependence on acyl chain length. In lipids longer than C8, gA exhibits a CD spectrum typical of single-stranded channels, with maxima at 220 and 235 nm, and a minimum at 230 nm. In shorter lipids, gA exhibits a CD spectrum typical of non-conducting DS helices such as are observed in organic solvent, with negative peaks at 212 nm and 230 nm (5) (Figure 3).

Both channel and DS conformations were obtained for shortened gramicidin peptides with two tryptophans (Figures 4-6). Trends are presented in figure 7. As can be seen, the 2W 9mer exhibits the most dependence upon hydrophobic matching; it only forms the channel conformation in one lipid, of intermediate length (C12). The 2W 11mer and 2W 13mer form channels in a wider range of lipids, C8 to C16. The DS conformation is observed in 2W 9mer and 2W 13mer peptides, but not in a predictable pattern. The 2W 9mer forms DS dimers only when lipid is too short for channel formation. The 2W 13mer takes a DS form when lipid is both too short and too long for channel formation. The 2W 11mer does not form a DS structure in any of the lipids tested. No channel-like or DS-like spectra were observed for one-tryptophan peptides. Only varied, atypical spectra were seen (figures not shown).

Discussion

Tryptophan importance. No standard channel or DS spectra were observed for any of the 1W gA analogues in the lipids tested. These CD results indicate that at least two tryptophans are necessary for formation of either standard channel or DS structures. This supports the importance of tryptophan in transmembrane channel folding; one tryptophan is not enough to adequately anchor the peptide in the membrane.

Hydrophobic matching importance. Hydrophobic matching between lipid chain length and the hydrophobic length of the folded peptide was particularly crucial to the folding of the shortest peptide, the 2W 9mer. It was expected that this short peptide would form channels in a range of short lipids; however, this was not the case. The short peptide simply may not form enough hydrogen bonds to stabilize the channel. The slightly longer 2W 11mer and 13mer peptides formed channels in a range of peptides, C8-C16. This is the same range of lipids in which gA forms channels. C8 was a threshold for gA (5); C8 seems to be the critical transition for these shortened gA analogues as well.

The DS conformation, however, does not follow the same pattern as for gA. The 2W 11mer produced no DS dimer spectra. DS formation in the other two 2W peptides varied; DS spectra appeared in lipids either longer or shorter than channel-inducing lipids, with no predictable pattern. The mechanism that determines DS dimer formation remains uncertain.

Non-channel-like and non-DS-like spectra were varied; the conformational behavior of these peptides is complex. The atypical spectra observed for 1W peptides and occasionally for 2W peptides could represent alternate dimeric or monomeric structures (as opposed to the RH $b^{6.3}$ helix of gA). These spectra cannot be compared to typical a-helical and b-sheet spectra of other peptides because of the alternating L,D chirality of gramicidin residues.

This project is aimed toward better understanding of channel protein/membrane interactions and the role of tryptophan and channel length in the folding of these proteins. As the lipid interactions of model systems such as the gramicidin channel are

better understood, insight may be gained into the workings of other, less well-characterized membrane proteins. These insights into the molecular interactions in biological membranes may eventually aid in membrane-active drug design and membrane-assisted drug delivery.

Reference List

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4. Wimley, W.C. and S.H. White. 1996. Experimentally determined hydrophobicity scale for proteins at membrane interfaces. *Nature Structural Biology.* 3:842-848.

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6. Greathouse, D.V., R.E. Koeppe, II, L.L. Providence, S.Shobana, and O.S. Andersen. 1999. Design and characterization of gramicidin channels. *Methods in Enzymology.* 294:525-550.

Note

Abbreviations: Standard amino acid abbreviations are used. Additional abbreviations are: CD, Circular dichroism; DCM, dichloromethane; MtBE, Methyl *t*-butyl ether; HPLC, high-performance liquid chromatography; DMF, N,N-dimethylformamide; gA, gramicidin A; DS, double stranded; W, tryptophan; RH, right handed.

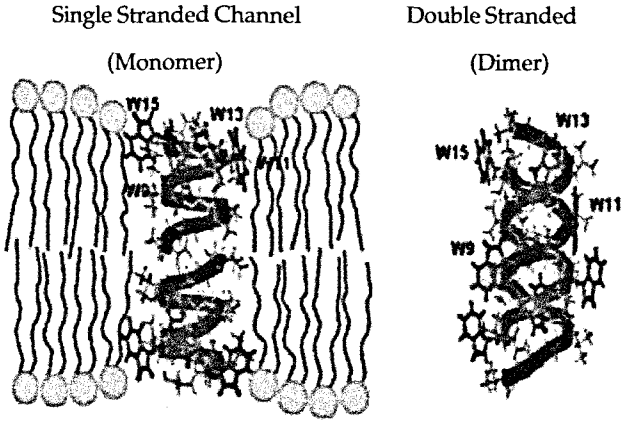

Figure 1. Models of gA in membrane (single stranded channel and in organic solveent (double stranded dimer).

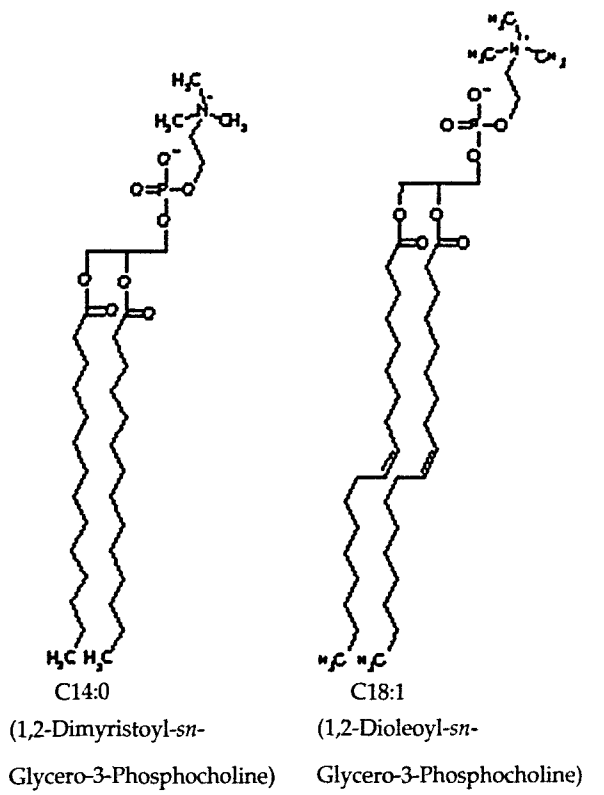

Figure 2. Examples of structures of lipids used for CD and SEC measurements.

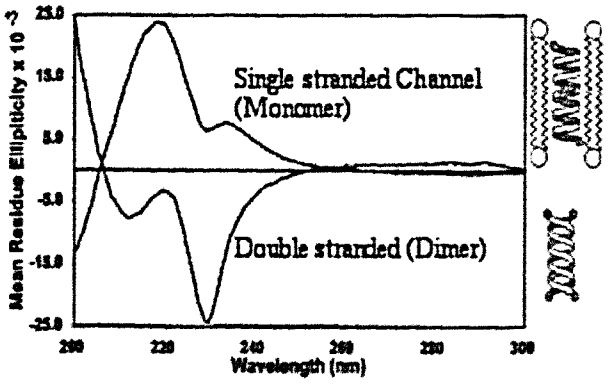

Figure 3. Reference CD spectra of monomeric (channel) and dimeric (double stranded) native gA.

Faculty comments

In his letter of recommendation, Ms. Dunn's mentor, Roger Koeppe writes:

> Elizabeth has been productive in the laboratory since the beginning of her sophomore year, and she has done a very nice job of providing a systematic definition of the minimal requirements for the folding of short peptides in the presence of a series of membrane-forming lipids. Her research has served to provide fundamental important new information about the molecular interactions of proteins and lipids and her results make a significant contribution to the future design of molecular "force transducers" that are being developed in my laboratory for the purpose of measuring the energetic requirements for deforming the membranes of living cells of different types. Elizabeth presented her results to a national audience at the Biophysical Society annual meeting in Boston in February 2001.
>
> On the Fayetteville campus, Elizabeth has excelled in "everything." She is one of those rare students of the very highest quality who come along only once or twice in a decade. I would rank her in the top 2% of all students in our department over the. Past twenty years. Elizabeth has been honored with a national Goldwater Fellowship and a National Science Foundation Fellowship for graduate study at Stanford. Her accomplishments in the classroom as well as the laboratory give her unusual status for an undergraduate student at this early stage in her career, and she is highly sought by distinguished graduate programs in cell biology, such as those at Stanford, Duke and Berkeley.
>
> Academically, Elizabeth has a 4.0 GPA and is one of the most outstanding students in any discipline on the Fayetteville campus. In her research on peptide/lipid interactions, Elizabeth has been instrumental at all stages of the experimental design, including the choices of peptides to make and study, the approach to issues of chiral fidelity during the synthesis, and the methods (circular dichroism spectroscopy and size-exclusion chromatography) for analyzing the peptide conformations and interactions in a variety of lipid bilayer systems. Her approach to research is thoughtful, creative and careful. Her package of skills is complete from experimental design to laboratory technique, record keeping, analysis and importantly the writing of reports, including the first draft of a manuscript.
>
> Elizabeth is well aware of the growing interdisciplinary nature of science. She will combine her knowledge of chemistry, biology and biochemistry as she pursues a program of graduate study in cell and molecular biology. In recommending her for a Howard Hughes Fellowship, I have written that I believe her to be the best candidate for a Hughes Fellowship whom I have encountered during twenty-one years of university teaching. This conclusion is based on her scientific understanding, laboratory productivity, and overall accomplishments.

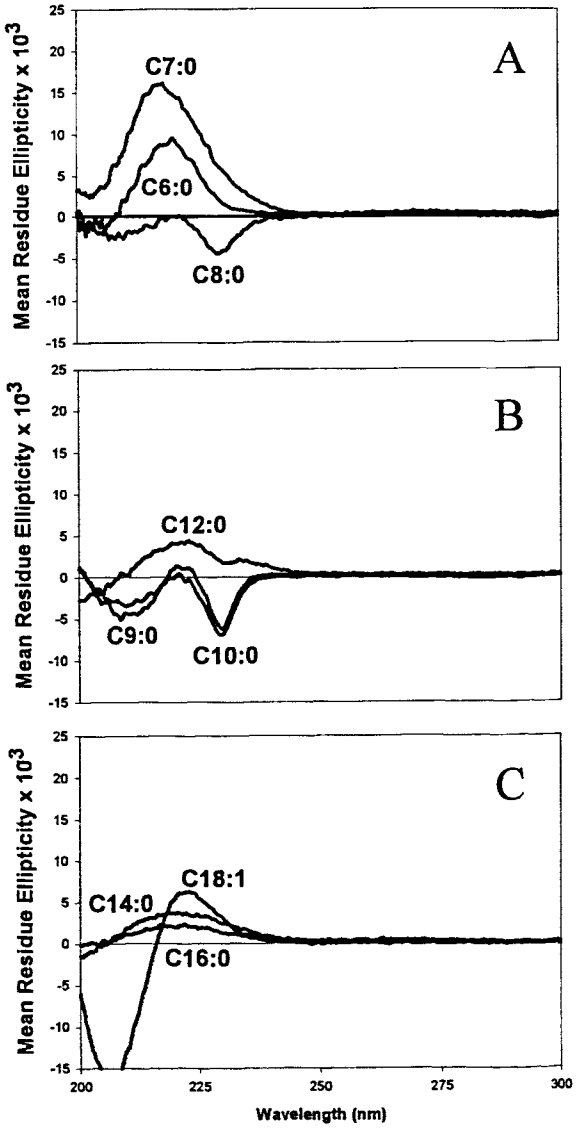

Figure 4. CD spectra of 2W 9mer in A) short lipids (C6:0-C8:0), B) intermediate lipids (C9:0-C12:0), C) long lipids (C14:0-C18:1). (Conditions: 1:30 gA:lipid ratio, 0.01-cm path length, room temperature.)

Neil Allison, Ms. Dunn's undergraduate advisor and organic chemishtry instructor, had the following things to say about her:

> Elizabeth is one of those few students who have an unquenchable thirst for knowledge. In fact, I believe she takes on challenges just for fun. An example of how Elizabeth has used her Sturgis Fellowship from

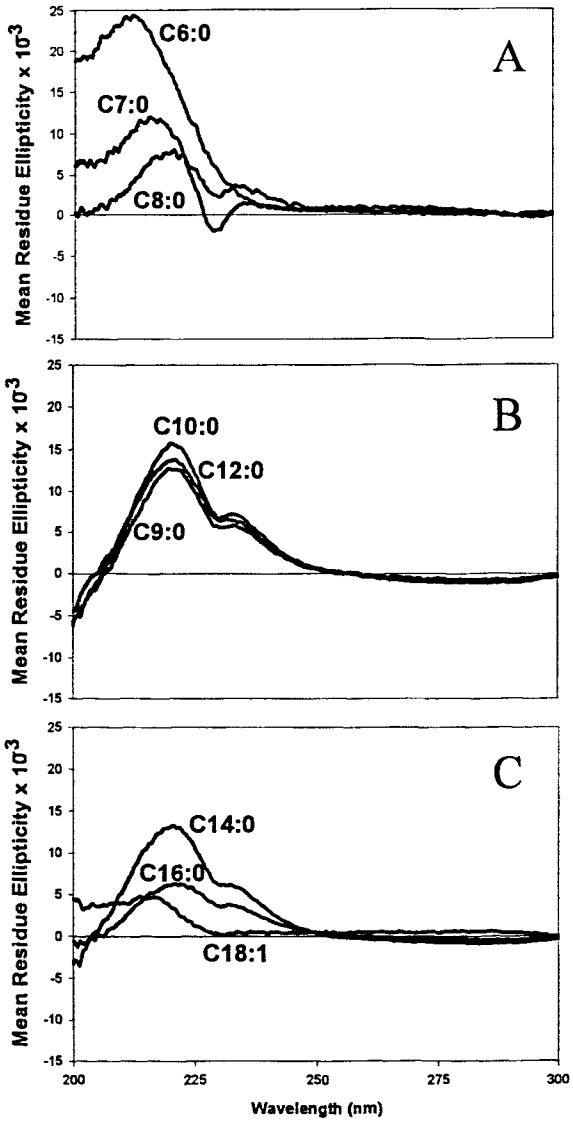

Figure 5. CD spectra of 2W 11mer in A) short lipids (C6:0-C8:0), B) intermediate lipids (C9:0-C12:0), C) long lipids (C14:0-C18:1). Conditions as in Fig. 4.

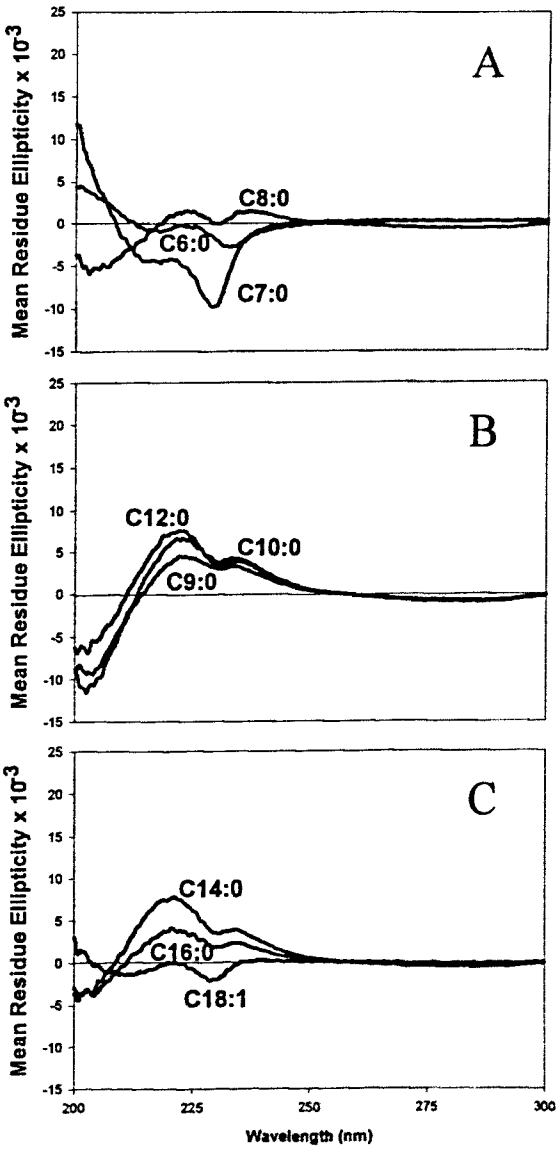

Figure 6. CD spectra of 2W 13mer in A) short lipids (C6:0-C8:0), B) intermediate lipids (C9:0-C12:0), C) long lipids (C14:0-C18:1). Conditions as in Fig. 4.

the Fulbright College to increase her experiences above and beyond most students is indicated the year she spent studying in Great Britain. I believe that her experience there was very valuable not only for the academic challenges it provided but also for allowing her to see how another part of the world operates. Moreover in addition to this year abroad, Elizabeth has been able to handle both biochemistry and a biological science double major on top of this one-year abroad study! In my memory as an advisor over the past nine years, I do not remember a single student that has taken two majors in technical fields. In fact, as seen by her grade point average she has done all of this with aplomb.

A specific example of Elizabeth's abilities is shown in my classes. She finished with the highest A in the organic chemistry major's laboratory and a very high, solid A in the Organic Chemistry 11 lecture. These were excellent grades in classes that are dominated by the most talented and grade aggressive students (in particular the premedical students) that the University of Arkansas possesses. This is particularly impressive when one considers the other difficult courses that she took at the same time in order to complete her double major in record time. Knowing Elizabeth quite

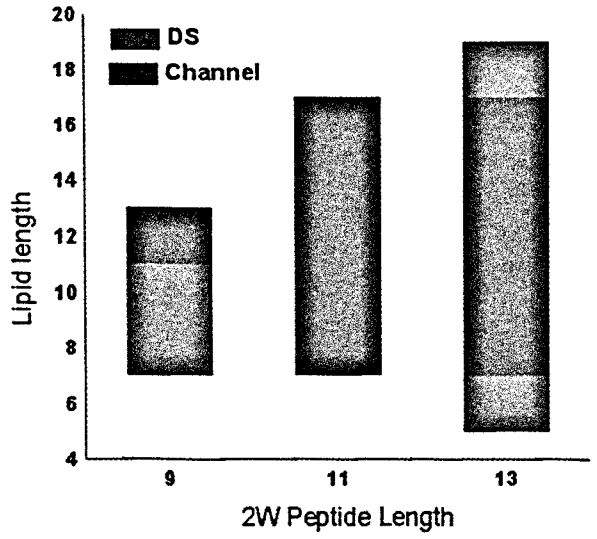

Figure 7. Correlation of length of 2W peptides with lipid length.

Table 2. Lipids Used for CD and SEC Measurements

Abbreviation	Common Name
C6:0	di-Caproyl-PC
C7:0	di-Heptanoyl-PC
C8:0	di-Octanoyl-PC
C9:0	di-Nonanoyl-PC
C10:0	di-Caprl-PC
C12:0	di-Lauroyl-PC
C14:0	di-Myristoyl-PC
C16:0	di-Palmitoyl-PC
C18:0	di-Oleoyl-PC

well, I can state that it is also very impressive in that the grade appears to be secondary to understanding and learning for Elizabeth (not something that I can say for many of the very high GPA students that I have known).

Over the past 18 years, I've probably written 120 or so letters of recommendation for students with about half of these being for outstanding students. When compared to Elizabeth, however, even the best students that I have written letters for fade somewhat. She is a "First" in my book - a student with all the potential imaginable, coupled with the drive to do great things in the future.

Timothy Kral, who acted as Ms. Dunn's Sturgis Fellowship faculty mentor, made similar comments about her abilities:

I have known Elizabeth for over three years as her teacher and mentor. Elizabeth is a Sturgis Fellow, and each Sturgis Fellow is assigned a faculty mentor. It certainly has been an honor to serve as her mentor. Elizabeth is a delightful individual who is simply brilliant. In my General Microbiology course, she earned an A; however, it wasn't just an A. After four class exams, her average was 100%. I have taught at he University of Arkansas for twenty years and this is the first final average of 100% I have ever had in my classes.

Elizabeth's interests lie in both natural science (biology and chemistry) and mathematics. She is currently working on a research project in biochemistry with Dr. Roger Koeppe dealing with membrane channels and gramicidin. More specifically, she is trying to understand the principles of hydrophobic matching and tryptophan anchoring in biological membranes. Her interest in mathematics is actually slanted toward mathematical biology. She has taken a number of advanced mathematics courses and has attended seminars in our department given by an internationally-renowned scientist dealing with mathematical models and analyses of biological importance.

As a person, Elizabeth is very unassuming and a bit on the shy side. However, she regularly seeks me out with questions about lecture material as well as questions relating to her education or future career. Elizabeth is the type of person who welcomes a challenge. Whatever she undertakes, she does extremely well.

Table 1. Sequences of shortened gA analogues with reduced numbers of tryptophans.

PEPTIDES	SEQUENCES[3]
Gramididin A	f-Val-Gly-Ala-Leu-Ala-Val-Val-Val-**Trp**-Leu-**Trp**-Leu-**Trp**-Leu-**Trp**-ea
1W 9mer	f-*Val*-Ala-Ala-Val-Val-Val-Ala-Leu-**Trp**-ea
1W 11mer	f-*Val*-Ala-Ala-Val-Val-Val-Ala-Leu-Ala-Leu-**Trp**-ea
1W 13mer	f-*Val*-Ala-Ala-Ala-Ala-Val-Val-Val-Ala-Leu-Ala-Leu-**Trp**-ea
2W 9mer	f-*Val*-Ala-Ala-Val-Val-Val-**Trp**-Leu-**Trp**-ea
2W 11mer	f-*Val*-Ala-Ala-Val-Val-Val-Ala-Leu-**Trp**-Leu-**Trp**-ea
2W 13mer	f-*Val*-Ala-Ala-Ala-Ala-Val-Val-Val-Ala-Leu-**Trp**-Leu-**Trp**-ea

[a]f=formyl
ea=ethanolamine
D-amino acids underlined
D,L-amino acids *italicized*