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Construction of artificial pigment-protein antennae

by

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GENERAL INTRODUCTION

Photosynthesis is a complex process which results in the conversion of solar radiation into chemical energy. This chemical energy is then used as the free energy source for all living organisms.¹ In its basic form, photosynthesis can be described as the light-activated synthesis of carbohydrates from the simple molecules of water and carbon dioxide:

This basic mechanism actually requires numerous reaction steps. The two primary steps being: the capture of light by pigment molecules in light-harvesting antenna complexes and the transfer of this captured energy to the so-called photochemical reaction center.² While the preferred pathway for energy absorbed by the chromophores in the antenna complexes is transfer to the reaction center, energy can be lost to competing processes such as internal conversion or radiative decay.³ Therefore, the energy transfer must be rapid, typically on the order of picoseconds, to successfully compete.

Energy transfer to the reaction center involves singlet-singlet state interactions, is radiationless, and in many cases can be described by the Förster mechanism.^{4,5} Förster theory states that the rate of energy transport is dependent on the separation distance between the donor (D) and the acceptor (A) pigments, denoted by the directional vector R , and on the relative orientation of the pigments, particularly the dipole-dipole coupling between their transition dipole moments. The probability of energy transfer is proportional to the fluorescence quantum yield of the donor molecule, the extinction coefficient at the

wavelength of interest ϵ_A , and the dipole-dipole orientation factor, κ . It follows that the rate of D $\rightarrow$ A energy transfer, k_{DA} , is proportional to

$$k_{DA} \approx \frac{\kappa^2}{|\bar{R}|^6} \int f_D(\nu) \epsilon_A(\nu) \frac{d\nu}{\nu^4}$$

where $f_D(\nu)$ is the normalized fluorescence emission spectrum of the donor and ν is the frequency of the light. In designing new donor-acceptor systems, the $1/R^6$ dependence of k_{DA} will thus require closely-packed pigments in the antenna complex for efficient energy transfer.^{4,5} It is anticipated that biomimetic antennae require pigment spacing of less than 20-40 Å for area-filling cross sections and a large integrated cross sections for highly efficient energy transfer to the reaction centers.

Antennae systems are generally classified as naturally occurring or biomimetic. Examples of antennae that occur in nature are: the Fenna-Matthews-Olson (FMO) Bchl_a-protein trimers⁶ and light-harvesting chlorosomes from green photosynthetic bacteria.⁷ Biomimetic antennae are artificially synthesized pigment-phospholipids or modular pigment-protein monolayers that mimic their biological counterparts. The focus of the present work is on the construction of artificial light-harvesting antenna complexes incorporating modular pigment-proteins. Biomimetic antennae are preferred for their ease of preparation and for the ability to control the orientation of pigments and the anticipated spectroscopy.

Selection of a suitable pigment is the primary step in the construction of an artificial antenna. Three general classes of molecules were considered as potential pigment

candidates: chlorins, porphyrins and phthalocyanines (Figure 1). As discussed above the chromophore must have a strongly allowed $S_0 \rightarrow S_1$ electronic transition as suggested by Förster's theory, and must be thermally and photochemically durable. Chlorins and bacteriochlorins are immediately ruled out as candidates because they are unstable outside of the natural protein host. Porphyrins and metalloporphyrins, on the other hand, are known to be stable and rather robust compounds. The absorption spectrum of tetraphenyl porphyrin⁸ (Figure 2) has a relatively low intensity Q_x (0,0) band at 650 nm, which implies a weak $S_0 \rightarrow S_1$ transition. Thus, the porphyrins are spectroscopically unfavorable for energy transfer. The absorption spectrum for (chloro)-aluminum phthalocyanine (Figure 3) depicts a degenerate Q (0,0) band at 670 nm, which is nearly ten times more intense than that for the porphyrin. The robust phthalocyanines are considered to have an intense $S_0 \rightarrow S_1$ transition and are, therefore, spectroscopically very favorable. An additional benefit is that their absorption coefficient and transition wavelengths are comparable to those of chlorophyll *a*, the well-known pigment of green plants, making the phthalocyanines a good pigment for the biomimetic model of antennae systems.

In constructing an artificial antenna, the next step is to choose a suitable matrix or structural support system. For this purpose modular peptides were chosen. This matrix system must provide adequate support for the pigments while allowing control of their relative orientation and geometry.

The coupling of a phthalocyanine pigment and a matrix support yields a single antenna complex, several of which make up the desired light-harvesting antennae.

However, formation of these antennae is not a trivial process. One approach for connecting

Chlorin

Porphyrin

Phthalocyanine

Figure 1 Molecular Structures of potential pigment candidates for the artificial antennae.

Figure 2. The absorption spectrum of tetraphenyl porphyrin in 1-chloronaphthalene.

Figure 3. The absorption spectrum of (chloro)-aluminum phthalocyanine in ethyl acetate.

multiple pigment-protein antennae is the formation of helical bundles. Several scientists have developed *de novo* design motifs involving individual helices which form helical bundles. Research design has been classified by the number of peptide residues. For over thirty residues, a complete database of four helix bundles has been compiled by Hecht and associates.⁹ For twenty to thirty residues, Dutton and De Grado have assembled bundles of four identical α -helices.¹⁰ The *de novo* design has two distinct components: the design of activity and the design of structure.¹¹

An excellent example of *de novo* design is given by the work of Urry and coworkers.^{12,13} Urry's work is based on modular polypenta-peptides of the form $(VPGXG)_n$ which resemble naturally occurring elastin polymers, Figure 4. Elastin has

Figure 4. The backbone arrangement and sequence of the elastin helix.

nearly exact copies of the repeating unit (VPGVG), where the orientation of the elastin helix controls the degree of allowed peptide rotation.¹⁴ Urry has shown that peptides of the type (VPGXG)_n, where *X* can be a variety of amino acids, form helical structures.¹⁵ Also, these helices demonstrated a unique temperature behavior. By raising the temperature, the helix "locked" into a rigid conformation which was maintained regardless of any further stimulus, adding to the stability of the entity. The flexibility of the amino acid sequence and the structural rigidity of the polypentapeptide make Urry's peptide systems an excellent choice for the pigment support matrix.

Following this General Introduction, the thesis is organized into sections. Section I. includes the spectroscopic characterization (absorption, fluorescence, and single-photon counting) of a series of phthalocyanine pigments. Section II. describes the preparation of several peptide sequences of the general form (VPGXG)_n employing the method of solid-phase peptide synthesis. The General Conclusion summarizes the work of both Sections I. and II. and provides potential directions for future work. Appendix A is included as a practical guide for sequencing using the Model 433A solid-phase peptide synthesizer. Finally, Appendix B includes relevant peptide sequencing results not discussed in previous sections.

SECTION I. SPECTROSCOPIC CHARACTERIZATION OF
PHTHALOCYANINE PIGMENTS

INTRODUCTION

Metallophthalocyanines are industrially important as paints, lubricants and stabilizers for semiconducting materials. Most of these applications take advantage of the relatively low solubility and strong tendency toward aggregation of the phthalocyanine molecules. They have also been utilized as the active media for dye lasers and as the passive Q-switch in certain pulsed lasers.¹⁶ Phthalocyanines have also been proposed for use in artificial light-harvesting antennae. A suitable phthalocyanine (Pc) for antennae must adhere to the criteria established in the General Introduction: thermally and photochemically durable along with a strongly allowed $S_0 \rightarrow S_1$ electronic transition, as required by Förster's theory.^{4,5} Also, the central metal atom must be carefully selected and should be inexpensive, nontoxic and have a low atomic mass. The lightness of this atom encourages S_1 state quenching and provides the correct symmetry for energy transfer.

In the present work, (chloro)-aluminum phthalocyanine and a series of its derivatives were characterized by absorption, fluorescence, and time-correlated single photon counting in order to study the spectral diversity of the phthalocyanine chromophore. The concentration dependence of the absorption spectra was investigated in order to identify any aggregation phenomena. Preliminary results are also presented for the silicon substituted phthalocyanine.

EXPERIMENTAL METHODS

The (chloro)-aluminum phthalocyanine and the (chlorophenyl)-silicon phthalocyanine were prepared by Steve Vanderlouw, a member of the Kraus research group at Iowa State University. In addition, a series of aluminum phthalocyanine derivatives were synthesized; the nomenclature was adapted from a research article by Owen and Kenney:¹⁷

phenyl-di(phthalocyanino)-aluminum

hydroxyphenyl-(phthalocyanino)-aluminum

hydroxy-diphenyl-(phthalocyanino)-aluminum

naphthyl-di(phthalocyanino)-aluminum

Absorption spectra were recorded on a Perkin-Elmer Lambda 3B UV-Vis spectrophotometer. The sample holder was a 1.0 mm quartz cell. The scan speed was 120 nm/min with a 1.0 nm slit, providing 2.0 nm spectral resolution.

Steady-state fluorescence spectra were obtained on a Spex Fluorolog 2 spectrofluorimeter with 1.0 nm spectral resolution. The sample holder was a 1.0 cm quartz fluorescence cell. The excitation wavelength was 640 nm.

Fluorescent lifetimes were determined by time-correlated single photon counting (TCSPC). The apparatus was previously discussed in detail.¹⁸ Briefly, the second harmonic (532 nm) from a mode-locked Nd:YAG laser (Coherent Antares 76s, 38 MHz, 100 ps FWHM) synchronously pumped a cavity-dumped, DCM dye laser (Coherent CR599) to produce tunable picosecond pulses (Figure 5). Laser performance was monitored and optimized using a rotating-mirror, real-time autocorrelator. The autocorrelation of the dye laser pulses was about 8 ps FWHM. Detection was monitored with a microchannel plate phototube (MCP). Magic-angle (MA) fluorescence was observed at 54.7° in a quartz cell formed by compressing a drop of sample between two one-quarter inch quartz discs; the resulting pathlength was approximately 10 μm . The MCP photocurrent pulses were amplified and inverted by using a fast preamplifier and an inverting transformer. These amplified pulses were passed through a constant-fraction discriminator (CFD) and served as START pulses in a time-to-amplitude converter (TAC). The TAC data was stored and processed on the multichannel analyzer (MCA) computer. Instrument functions were measured by scattering laser pulses from a suspension of aqueous Coffeemate in a quartz cell. Laser excitation was 640 nm.

The transient decay profiles were fitted by single or multiexponential decay laws by utilizing SPECTRA processor, a MS-DOS based fitting program developed by Sergei Savikhin.¹⁹ The statistical reduced chi-squared, χ_v^2 (where v is the number of degrees of

Figure 5. Experimental setup for time-correlated single photon counting (TCSPC): AOM, acousto-optic modulator; AO, autocorrelator; BS, beam splitter; CFD, constant-fraction discriminator; MCA, multichannel analyzer; MCP, multichannel plate phototube; OC, oscilloscope; TAC, time-to-amplitude converter.

freedom), determined the quality of the fit. The reduced χ_v^2 has an expectation value of 1.

The accepted definition for the reduced chi-squared is $\chi_v^2 = \chi^2 / \nu$, where

$$\chi^2 = \frac{\sum_{i=n_1}^{n_2} \frac{[I_0(t_i) - Y(t_i)]^2}{Y(t_i)}}{n_2 - n_1 + 1}$$

n_1 and n_2 represent the first and last channels of the region under analysis, $I_0(t_i)$ represents the measured curve, and $Y(t_i)$ indicates the calculated fitting function.²⁰

RESULTS AND DISCUSSION

There is very little literature available on the photophysics of phthalocyanines, making the determination of their spectral properties quite beneficial. Absorption spectra, fluorescence spectra, and fluorescent lifetimes were measured for (chloro)-aluminum phthalocyanine and its derivatives, which are referred to throughout this section as Compounds I-IV. In general, the spectroscopic properties of the derivative compounds were similar to those properties of the parent chromophore, aluminum phthalocyanine (AlPc). The absorption spectrum of Compound I, taken in the ethyl acetate solvent, is characteristic of a metal phthalocyanine (Figure 6). Important features are an intense Soret band in the 350 nm region and an intense, degenerate Q (0,0) at 671 nm with less intense vibrational peaks at 640 nm and 610 nm.

The fluorescence spectrum of Compound I in ethyl acetate (Figure 7) shows a Q(0,0) band Stokes-shifted to 674 nm whose intensity indicates that AlPc is a relatively efficient fluorescent molecule. The correct way to ascertain the efficiency, or conversion of absorbed energy to fluorescence, is to overlap the normalized absorption and fluorescence spectra as in Figure 8. In the ideal case, the normalized absorption and fluorescence spectra perfectly overlap. This represents 100% fluorescent efficiency and a fluorescent quantum yield equal to unity. Brannon and Magde reported a quantum yield of 0.58 ± 0.04 for (chloro)-aluminum phthalocyanine in 1-chloro-naphthalene.¹⁶

To assess the aggregational properties of AlPc, an absorbance versus concentration study in ethyl acetate was performed. Aggregation is characterized by changes in the Q

Figure 6. Absorption spectrum of Compound I. (4.7×10^{-5} M) in ethyl acetate solvent.

Figure 7. Fluorescence spectrum of Compound I. (1.0×10^{-8} M) in ethyl acetate solvent.

Figure 8. Spectral overlap of normalized absorption and fluorescence spectra for Compound I. in ethyl acetate solvent.

band region, specifically at the 640 nm and 610 nm peaks. A plot of the normalized absorption spectra (Figure 9) demonstrates the trend toward aggregation. At higher concentrations (10^{-4} M), the 640 nm peak was more intense than the 610 nm peak indicating aggregation in the solution; while at lower concentrations (10^{-7} M), the 640 nm peak was less intense than the 610 nm peak suggesting no aggregation occurred. A transitional period occurred between concentrations 5.0×10^{-6} M and 1.0×10^{-6} M. To minimize the effects of aggregates on the spectral features, sample solutions were prepared having concentrations around 10^{-6} M.

Due to the low solubility of the phthalocyanine, the molar extinction coefficient could not be calculated precisely. However, an approximate value for AlPc in ethyl acetate was calculated to be $2.0 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$. This value is consistent with the reported value of $2.5 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$ for (chloro)-AlPc in 1-chloro-naphthalene.¹⁶ Ethyl acetate was employed as a solvent in the synthesis of the derivative compounds and was the recommended solvent for the current studies because the robustness of the aluminum to oxygen bond has not yet been determined in other solvents. In fact, several solvents, including methanol, dimethylformamide and dimethyl sulfoxide, caused this bond to break, drastically changing the spectroscopic properties of the molecule.

The observed spectral properties for Compounds II.-IV. and (chloro)-aluminum phthalocyanine were very similar to those obtained for Compound I. and are summarized in Table 1. All compounds exhibited an absorbance Q (0,0) band at $(671 \pm 2) \text{ nm}$ and a fluorescence Q (0,0) band at $(672 \pm 2) \text{ nm}$. Magic-angle fluorescence profiles were acquired using the single photon counting apparatus. These profiles were deconvoluted to

Figure 9. Normalized absorption spectra for a concentration series (10^{-4} to 10^{-7}) of Compound I. in ethyl acetate solvent.

Table 1. Spectroscopic Data for Aluminum Phthalocyanine Compounds.

<u>Compound</u>	<u>Concentration</u> ($\times 10^{-6}$ M)	<u>Absorbance Q(0,0)</u> (nm)	<u>Fluorescence Q(0,0)</u> (nm)	<u>Lifetime</u> (ns)
I.	1.76	670	672	6.52
II.	2.06	669	671	6.68
III.	2.34	671	673	6.76
IV.	2.12	672	674	6.78
PcAl-Cl	1.98	670	672	6.79

single exponential decays based on the reasonable assumption that only one fluorescent species was present. The fitting process is displayed for Compounds I-IV, and for the (chloro)-aluminum phthalocyanine in Figures 10-14, respectively. Underneath each profile is the autocorrelation of residuals and the calculated reduced χ^2 , which indicated a reasonable statistical fit. From the exponential decays, the fluorescent lifetimes were calculated and were in reasonable agreement with the reported lifetime of (6.80 ± 0.6) ns for (chloro)-aluminum phthalocyanine.¹⁶

The aluminum phthalocyanine compounds were developed as prototypes to simulate the behavior of phthalocyanines bound to a polypeptide. It was intended that the rate of energy transfer as a function of distance between two chromophores would be measured. However, initial depolarization experiments on the compounds indicated that the energy transfer rate was faster than the time-resolution of our instrument. Because the

Figure 10. Magic-angle fluorescence profiles for Compound I (1.98×10^{-6} M); ethyl acetate solvent, fluorescent lifetime is 6.52 ns and χ^2 is 1.17398.

Figure 11. Magic-angle fluorescence profiles for Compound II (2.06×10^{-6} M); ethyl acetate solvent, fluorescent lifetime is 6.68 ns and χ^2 is 1.18206.

Figure 12. Magic-angle fluorescence profiles for Compound III. (2.34×10^{-6} M); ethyl acetate solvent, fluorescent lifetime is 6.76 ns and χ^2 is 1.1580.

Figure 13. Magic-angle fluorescence profiles for Compound IV. (2.12×10^{-6} M); ethyl acetate solvent, fluorescent lifetime is 6.78 ns and χ^2 is 1.02398.

Figure 14. Magic-angle fluorescence profiles for (chloro)-aluminum phthalocyanine. (1.76×10^{-6} M); ethyl acetate solvent, fluorescent lifetime is 6.52 ns and χ^2 is 1.1262.

latter is in the picosecond time domain, the energy transfer rate was believed to be on the order of several hundred femtoseconds.

Another phthalocyanine of interest was (chlorophenyl)-silicon phthalocyanine (PcSi). The absorption spectrum of PcSi in 1-chloronaphthalene solvent is shown in Figure 15. This should be compared to absorption spectrum for (chloro)-aluminum phthalocyanine (PcAl) (Figure 16). The most important observation was that the PcSi spectral features were characteristic of a *nonmetal* phthalocyanine. The Q(0,0) band centered at 690 nm was significantly broadened, indicative of the symmetry-lowering of the phthalocyanine macrocycle. Only one other well-resolved visible absorption band occurred in this

region and was at 620 nm. On the other hand, the metal substituted phthalocyanine, PcAl, in 1-chloronaphthalene had three bands in the visible region: an intense, narrow, degenerate Q (0,0) at 694 nm and vibronic peaks at 640 nm and 610 nm. For PcAl, the narrow linewidth of the Q (0,0) band reflects the square- pyramidal, in-plane bonding between the aluminum and the phthalocyanine macrocycle. This geometry results in a high degree of molecular symmetry.

Figure 15. Absorption spectrum of (chlorophenyl)-silicon phthalocyanine (9.02×10^{-5} M) in 1-chloronaphthalene solvent.

Figure 16. Absorption spectrum of (chloro)-aluminum phthalocyanine (1.32×10^{-4} M) in 1-chloronaphthalene solvent.

SECTION II. SOLID-PHASE PEPTIDE SYNTHESIS

INTRODUCTION

The expansive interest in the synthesis of peptides and small proteins has been driven by biological advancements, including the utilization of peptides as synthetic vaccines and the folding patterns of complex proteins.²¹ Due to the pioneering efforts of Merrifield and his extensive research and refinement over the past twenty five years²², solid-phase peptide synthesis (SPPS) has become the leading technique for sequencing peptides and small proteins. SPPS is characterized by the N- α blocking group of the amino acid which is either t-butyloxycarbonyl (Boc) or 9-fluorenylmethoxycarbonyl (Fmoc) (Figure 17). Both the Boc chemistry^{23,24} and Fmoc chemistry^{25,26} have been recently reviewed. Three basic steps are required for SPPS:²⁷ 1) chain assembly of amino acids on a resin; 2) simultaneous or sequential cleavage and deprotection of the resin-bound, fully protected peptide chain; and 3.) purification and characterization of the target peptide.

The general procedure for SPPS chain assembly is outlined in Figure 18.²⁸ In the first step, an N- α -derivatized amino acid is attached to a solid resin support by means of a linker. This linker functions as a chemical "handle" and determines the mechanistic nature of the synthesis. Second, the N- α blocking group (Boc or Fmoc) is removed in a deprotection step. This deprotection step, however, does not remove any side-chain protecting groups on the amino acid, these remain attached during the entire process. To complete the deprotection step inert precipitates and excess reagents are washed away. Third, the C-terminus end of the next amino acid is "activated" to initiate coupling to the exposed N- α group of the amino acid-linker-support product. The deprotection, activation

Figure 17. Molecular Structures of the t-butyloxycarbonyl (Boc) group, left, and the 9-fluorenylmethoxycarbonyl (Fmoc) group, right.

Figure 18. General scheme for chain-assembly in solid-phase peptide synthesis.

and coupling steps are repeated simultaneously until all of the desired amino acids are attached. Finally, the linker-support and the side-chain protecting groups on the amino acids may be cleaved to yield the target peptide.

In the present work, all solid-phase peptide synthesis followed *FastMoc* chemistry, an adapted form of the Fmoc protocol. *FastMoc* is a term coined by Applied Biosystems to describe the feedback monitoring capabilities of their instruments. The Fmoc mechanisms of deprotection and activation were slightly modified to produce ionic species, which were monitored by a conductivity cell. The comparison of conductivities read from subsequent trials enables the instrument to determine the efficiency of coupling. For example, a low conductivity reading represents more efficient coupling. The instrument is programmed to repeat the deprotection, activation and coupling cycles until a high coupling efficiency, typically greater than 99%, is obtained. The *FastMoc* deprotection and activation mechanisms are described in more detail below.

In the deprotection step, piperidine attacks and then cleaves the Fmoc protecting group on the N-terminus end of the amino acid (Figure 19). Excess piperidine further reacts with the free Fmoc group forming an inert precipitate. In addition, the piperidine attacks the resulting N-terminus end of the amino acid leaving a deprotected peptide-resin and a piperidine carbamate salt. This carbamate salt is the ionic species, whose conductance is detected by the feedback monitoring control of a *FastMoc* instrument.

After deprotection is completed, a new amino acid can be coupled to the growing chain. This is accomplished by first "activating" the next amino acid's C-terminus end to encourage coupling. *FastMoc* activation is also called HBTU activation, where HBTU is

Figure 19. Carbamate salt formed by the Fmoc deprotection mechanism.

2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluoro-phosphate. The structure of the HBTU molecule is shown below.

This reacts with the deprotected amino acid to produce an HOBt ester, depicted below, following the mechanism proposed by Henklein.²⁹

Activation functions to maximize peptide-resin solvation and to minimize peptide-peptide hydrogen bonding that could lead to peptide aggregation. If aggregation occurs, it can interfere with the interaction between the activated amino acid and the deprotected amino acid, thereby reducing the coupling efficiency. N-methylpyrrolidone has proven to be the most efficient reagent at preventing peptide aggregation and is thus widely used in solid-phase peptide synthesis during deprotection and activation.³⁰

The current study was undertaken to prepare the following peptide sequences:

VPKGK, (VPKGK)₃, VPGSG and VPGYG where the amino acid residues (Figure 20) are

Figure 20. Molecular structures of the amino acids utilized for peptide synthesis.

valine, proline, glycine, lysine, serine and tyrosine. The peptides were synthesized by automated solid-phase peptide synthesis, dried at ambient temperature, and then stored for future studies.

EXPERIMENTAL METHODS

A variety of Fmoc amino acids were purchased from Applied Biosystems, a Division of Perkin-Elmer Corporation: Fmoc-glycine, Fmoc-proline, Fmoc-valine, Fmoc-lysine with a t-butyl oxycarbonyl (Boc), Fmoc-serine with a t-butyl (tBu), and Fmoc-tyrosine with a t-butyl (tBu) protecting group. Fmoc-lysine with a benzyloxy-carbonyl (Z) protecting group and Fmoc-tyrosine with a benzyl (Bzl) protecting group were purchased from Cal-Biochem Nova-Biochem Corporation.

All syntheses utilized 4-hydroxy methyl-phenoxy-methyl-copolystyrene 1% divinyl benzene resin (HMP):

The HMP resin, purchased from Applied Biosystems, was preloaded with glycine because all peptides required this amino acid on the C-terminus end.

The reagents used to complete the peptide syntheses were purchased from Applied Biosystems and included: 2-(1-H-benzo triazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate, dichloromethane, dicyclohexylurea, diisopropylethylamine, dimethylformamide, 1-hydroxy benzotriazole, methanol, and N-methylpyrrolidone. For reference throughout the thesis, a list of reagent abbreviations is compiled in Table 2.

All peptide sequencing was performed on a Model 433A Automated Solid-Phase Peptide Synthesizer (Applied Biosystems). The 0.25 mmol scale, the *FastMoc* protocol

Table 2. Abbreviation List for Reagents utilized in Peptide Synthesis.

DCM	Dichloromethane
DCC	Dicyclohexylurea
DMF	dimethylformamide
DIEA	diisopropylethylamine
HBTU	2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate
HOBt	1-hydroxybenzotriazole
NMP	N-methylpyrrolidone

and the Monitor Previous Peak monitoring option (*FastMoc* 0.25 mmol/MonPrevPeak) were utilized for all sequencing.

RESULTS AND DISCUSSION

All peptide sequences of the general form (VPGXG)_n prepared using the Model 433A Synthesizer are summarized in Table 3. The variable amino acid was either lysine, serine or tyrosine. A variety of side-chain protecting groups were selected to investigate the changes in chemical behavior with environment. In early experiments, the Fmoc blocking group on the final amino acid was deprotected. Whereas, in later experiments, no final deprotection was performed in order to provide a "cap" on the potentially reactive N-terminus end during further chemical modifications. None of the resulting peptides were cleaved from the HMP resin due to modification requirements.

The peptide sequencing was accomplished using the *Synthassist* software program. This Macintosh-based software allows selection of the specific sequences, chemistries, scales and run parameters. The run parameters involve run cycles such as single coupling and reagent washings. These run cycles are comprised of modules which are further

Table 3. Peptide Sequences for (VPGXG)_n.

Variable Amino Acid = X	Repeat No. <i>n</i>	Protecting Group	Final Fmoc Deprotection ?
K	1	Boc = t-butyloxycarbonyl	yes
K	1	Z = benzylcarbonyl	no
K	3	Z = benzylcarbonyl	no
S	1	tBu = t-butyl	yes
S	1	tBu = t-butyl	no
Y	1	tBu = t-butyl	no
Y	1	Bzl = benzyl	no

broken down into functions. Each type of chemistry contains specific modules to perform deprotection, activation and coupling. The module definitions, time requirements, and reagents utilized for *FastMoc* 0.25 mmol/ MonPrevPeak are listed in Table 4. Each of the functions within a module are simple executions like pressurizing and delivering reagents, vortexing the reaction vessel or ejecting cartridges.

The peptides of the general form (VPGXG)_n in which the final Fmoc group was deprotected included X = lysine and serine, where n =1. The sequencing results of VPGK(Boc)G, where Boc is the protecting group on the lysine side-chain, demonstrates the parameters and conditions that were required for final deprotection on the N-terminus end of the amino acid.

The run cycles and corresponding modules that were required are displayed in Table 5. The initial cycle was a DCM and NMP wash of the resin. It is important to note that the series of modules BADEFD apply to the amino acid on the "previous" line. From the definitions above, module B refers to the deprotection process and module A to the

Table 4. Module Definitions for *Fastmoc* Chemistry on the 0.25 mmol Scale.

<u>Module</u>	<u>Definition</u>	<u>Reagents Utilized</u>	<u>Time (minutes)</u>
A	Dissolving Amino Acid	NMP, HBTU/HOBt	7.6
B	Piperidine Deprotection	NMP, Piperidine	8.8
D	NMP Washes	NMP	4.6
E	DIEA to initiate Activation	NMP, DIEA	2.2
F	Coupling, NMP Washes	NMP	22.2
I	Wait to extend Deprotection		15.0
c	DCM Washes	DCM	7.6

Table 5 Cycles and Modules for Sequencing VPGK(Boc)G on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Lys(Boc)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6	Final Deprot/ MonPrev	BIDc

activation process. For example, during the first single couple cycle, the Fmoc group on the resin-bound glycine was removed, then the lysine was "activated", and coupling was performed. The entire process is depicted in the monitor trace (Figure 21) where the peak height corresponds to the conductivity value. The number of peaks present for each amino acid depended on the number of times the deprotection, activation, and coupling cycle were repeated. Because Monitor Previous Peak was chosen for feedback monitoring, a minimum of three peaks were observed for each amino acid. This meant that cycling was repeated until the peak difference was within the acceptable range of 5%. As a demonstration, consider the peaks for the first amino acid, glycine. The relative difference between the first two peaks is 55.4%; whereas, the relative difference between the second and third peaks is only 1.27%. The fact that the peak difference was within the acceptable range after three cycles indicated that the chain assembly process could continue to the next amino acid. The sixth cycle (Table 5) provided the final deprotection of the Fmoc group on valine, shown on the monitor trace by three peaks for valine. This is important because the synthesizer built the sequence from the C-terminus to the N-terminus amino acid.

Figure 21. Monitor Trace for sequencing VPGK(Boc)G.

The *Synthassist* run program was used to calculate the initial amount of resin required for a given synthesis and the theoretical yield of the resin-peptide product. A sample calculation for the sequence VPGK(Boc)G is shown in Table 6. The key points are as follows. The number of moles of resin was determined by the scale of the synthesis. The substitution value for the resin, 0.75 mmol/g, represents the number of reactive sites per resin molecule. This value decreased after the addition of each amino acid because the overall weight of the resin-peptide increased. When the initial substitution value was not provided, it was determined by monitoring the absorbance of a 4 to 8 mg sample of resin dissolved in 0.5 mL 20% piperidine in DMF.³¹ The resin equivalent weight is a conversion factor given by the inverse of the substitution value. This resin equivalent weight multiplied by the scale is equal to the theoretical yield of the peptide-resin product. The actual yield of resin-peptide product was within 3% of the theoretical yield for all

Table 6. Calculations for Sequencing VPGK(Boc)G on the 0.25 mmol Scale.

Volume: 5.0 mL	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Lys(Boc)	0.250	0.640	1561.62	0.384
2 Gly	0.250	0.618	1618.68	0.398
3 Pro	0.250	0.583	1715.79	0.422
4 Val	0.250	0.551	1814.93	0.447
H	0.250	0.627	1593.69	0.392

syntheses performed. It should be noted that the H in the Amino Acid column represents the free hydrogen remaining on the N-terminus end of valine after Fmoc deprotection.

The peptides of the general form (VPGXG)_n where no final Fmoc deprotection was required included: X = K(Z), S(tBu), Y(tBu), Y(Bzl) for n = 1 and X = K(Z), for n = 3. The sequence VPGS(tBu)G provides a good example of this type of synthesis. Table 7 summarizes the cycles and modules required to prepare this sequence. Compared to the previous example, the sixth cycle was changed from Final Deprotection/ MonitorPrevPeak to a NMP wash. An additional DCM wash for the final resin-peptide product was also added as a seventh cycle. Both of these washes are actually required at the end of all syntheses to remove any impurities and excess reagents. The fact that no final deprotection occurred was verified by the absence of conductivity peaks for valine in the monitoring trace for this sequence (Figure 22).

The fact that there was no final deprotection necessarily changed some of the theoretical calculations for preparing the sequence VPGS(tBu)G (Table 8). Specifically, the expected value of the peptide-resin product was determined by subtracting the weight

Table 7. Cycles and Modules for Sequencing VPGS(tBu)G on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Ser(tBu)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6	NMP Wash	D
7	DCM Wash	c

Figure 22. Monitor Trace for sequencing VPGS(tBu)G.

Table 8. Calculations for Sequencing VPGS(tBu)G on the 0.25 mmol Scale.

Volume: 5.0 mL	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Ser(tBu)	0.250	0.677	1476.52	0.369
2 Gly	0.250	0.652	1533.57	0.383
3 Pro	0.250	0.613	1630.69	0.408
4 Val	0.250	0.578	1729.82	0.432

of the Fmoc protecting group (221.23 mg) from the final resin equivalent weight and then multiplying the scale to obtain the theoretical yield of product.

The fifteen residue sequence (VPGK(Z)G)₃ was prepared using similar cycles and modules as for the five residue peptides (Table 9). The single coupling cycle was repeated fourteen times. Also, there was no final deprotection of the Fmoc group, denoted in cycles 16 and 17 by an NMP wash and a DCM wash, respectively. As expected, there were nonconductivity peaks for the final amino acid (valine) in the monitoring trace (Figure 23). An interesting feature of Figure 23 is that for some amino acids, more than three conductivity peaks were observed. As the amount of material increased in the reaction vessel, coupling of the amino acids and feedback monitoring became more difficult. In fact, feedback monitoring becomes impossible for reactions on the 1.0 mmol scale.³² Three to five peaks in the monitoring trace is generally considered to be acceptable. If more than five peaks are observed, however, it is also possible that an instrumental

Table 9. Cycles and Modules for Sequencing (VPGK(Z)G)₃ on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Lys(Z)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6 Gly	Single Couple / NMP	BADEFD
7 Lys(Z)	Single Couple / NMP	BADEFD
8 Gly	Single Couple / NMP	BADEFD
9 Pro	Single Couple / NMP	BADEFD
10 Val	Single Couple / NMP	BADEFD
11 Gly	Single Couple / NMP	BADEFD
12 Lys(Z)	Single Couple / NMP	BADEFD
13 Gly	Single Couple / NMP	BADEFD
14 Pro	Single Couple / NMP	BADEFD
15 Val	Single Couple / NMP	BADEFD
16	NMP Wash	D
17	DCM Wash	c

problem exists. These include poorly aged solvents, blocked in-line filters, or even a faulty conductivity cell. To prevent instrument problems like these, flow tests and other self tests were conducted prior to beginning any synthesis.

The theoretical calculations for this fifteen residue peptide are given in Table 10. Again, the expected yield of Fmoc protected, peptide-resin product was determined by subtracting the weight of the Fmoc protecting group from the initially calculated resin equivalent weight. It is important to note that the initial amount of resin was still 0.333 grams for this peptide prepared on the 0.25 mmol scale. The actual yield was within 3% of the theoretical value.

Figure 23. Monitor trace for sequencing (VPGK(Z)G)_n, where $n = 3$.

Table 10. Calculations for Sequencing (VPGK(Z)G)₃ on the 0.25 mmol Scale

Volume: 5.0 mL	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Lys(Z)	0.250	0.627	1595.64	0.399
2 Gly	0.250	0.605	1652.69	0.413
3 Pro	0.250	0.571	1749.81	0.437
4 Val	0.250	0.541	1848.94	0.462
5 Gly	0.250	0.525	1906.00	0.476
6 Lys(Z)	0.250	0.461	2168.30	0.542
7 Gly	0.250	0.449	2225.36	0.556
8 Pro	0.250	0.431	2322.47	0.581
9 Val	0.250	0.413	2421.61	0.605
10 Gly	0.250	0.403	2478.66	0.620
11 Lys(Z)	0.250	0.365	2740.97	0.685
12 Gly	0.250	0.357	2798.02	0.700
13 Pro	0.250	0.345	2895.13	0.724
Val	0.250	0.334	2994.27	0.749

The cycles and modules, calculations and monitoring traces for the peptides not discussed in the text are given in Appendix B. These data differed only slightly from that presented in the above examples. The cycles and modules followed either the final Fmoc deprotection scheme or the no final Fmoc deprotection scheme. The calculated weight values depended on the fourth amino acid (lysine, serine or tyrosine) and its protecting group. Having a record of these results is important because each monitor trace should be evaluated independently for efficient deprotecting and coupling, which is the primary measure of the quality of the peptide synthesis.

GENERAL CONCLUSION

Peptides of the general form (VPGXG)_n with X= lysine, serine or tyrosine were successfully prepared by the method of solid-phase peptide synthesis. These particular amino acids for X were selected to ultimately control the orientations between phthalocyanine pigment and the polypeptide backbone. Solid-phase peptide synthesis was performed using an Applied Biosystems Model 433A synthesizer, which proved to be an efficient, reliable, and simple way to sequence polypentapeptides.

The phthalocyanine pigments were spectroscopically characterized by absorbance, fluorescence, and time-correlated single photon counting, and were found to have similar photophysical properties. In the absorption spectra, the Q(0,0) band was an intense transition ($\epsilon \approx 2.5 \times 10^5 \text{ L mol}^{-1} \text{ cm}^{-1}$) centered around 670 nm. Fluorescence was highly efficient (accepted quantum yield of 0.58 ± 0.04 for (chloro)-aluminum phthalocyanine)¹⁶ with the Q(0,0) band centered at 672 nm. The singlet excited state was found to be relatively long-lived based on the fluorescent lifetimes, which were in the range of 6.5 to 6.8 nanoseconds. The similarity of the spectroscopic properties confirmed the fact that derivatization of the pigment periphery does not significantly perturb the character of the chromophore.

Future synthetic focus will be on connecting the phthalocyanines to appropriate polypeptide helices in order to study the overall behavior of the antennae. To accomplish

this attachment, the lysine's side-chain amino group will be deprotected and then acylated with silicon phthalocyanine according to the following:³³

In the case of serine, the side-chain hydroxyl group will be deprotected and then acylated with silicon phthalocyanine:

Tyrosine's side-chain phenol will be deprotected and reacted with the aluminum phthalocyanine:

Finally, the helical array of pigments will be bound into bundles to form a light-harvesting complex. This complex will be coupled to a suitable reaction center in order to mimic the entire photosynthetic process.

APPENDIX A

SOLID-PHASE PEPTIDE SYNTHESIS USING THE MODEL 433A SYNTHESIZER

This appendix serves as a supplement to the existing Applied Biosystems manuals and discusses practical aspects of peptide synthesis. The Model 433A manuals provided are the *User Manual for the Model 433A, Volumes I and II*, the *Synthassist 2.0 Software Manual*, the *Safety Supplement Manual* and the *Introduction to Cleavage Techniques Manual*. A *Log Manual for the Model 433A* containing operational log sheets and sequence run sheets has also been prepared by this author. The Model 433A can be used to perform Fmoc chemistry on the 0.25 mmol and 1.0 mmol scales and Fastmoc chemistry on the 0.25 mmol scale. Additional reaction vessels may be purchased from Applied Biosystems for scales smaller than 0.25 mmol. The instrument is programmable manually on the synthesizer and by the provided Macintosh software. The user may refer to the *Synthassist 2.0 User Manual* for examples of the windows required to define a given sequence, to select the scale and chemistry, and to construct run parameters.

The preface to any peptide synthesis is preparatory research. How the peptide will be utilized will influence the selection of resins and amino acid protecting groups. The peptides prepared on the Model 433A synthesizer are bound to a resin, and the protecting groups on the amino acids remain attached. Thus, a proper cleavage procedure must be implemented to remove resins and protecting groups. The user should refer to the *Introduction to Cleavage Techniques Manual* to select a method suitable for individual peptides. However, if post-synthesis modification of the peptide is required, then different

resins and protecting groups may be chosen to allow selective control during cleavage. For example, dilute acid susceptible (DAS) resins may be cleaved under mild conditions while leaving the amino acid protecting groups intact. Suppliers also offer a variety of protecting groups for the amino acids. These special purchase amino acids may be placed into empty cartridges suitable for the synthesizer.

The following discussion is a summary of the pertinent steps required to perform a generic synthesis. Certain conventions are implied: software commands appear capitalized, the term "windows" refers to the Macintosh-based *Synthassist* Software, and manual, function and module commands pertain to the instruments front-panel software.

INSTRUMENT PREPARATION

Determine the age and purity of existing reagents. The *Safety Supplement Manual* outlines the shelf life and storage conditions of the reagents. Never compromise these standards. The N-methyl-pyrrolidone (NMP) ages poorly, forming ionic species which interfere with the feedback monitoring of conductance. Piperidine is known to oxidize over time and will crystallize in its delivery line, blocking subsequent solvent transfer. A preventive remedy would be to periodically vent the piperidine line using manual commands. Also, check the condition of the current solvent bottles and bottle seals. Applied Biosystems supplies all reagents, bottle seals, filters, etc., and a complete listing of their products is found in the *Log Manual for the Model 433A*.

Check the nitrogen gas supply. Never begin a synthesis with less than 300 psi of gas. After replacement of a gas cylinder, always insure proper pressure throughout the instrument by performing flow test number 10. The pressure gauge for the nitrogen gas is

located on the valve assembly panel at the lower right; the top gauge has no function for this instrument. The reading of the ballast inside the instrument should be between 15-20 psi. During a synthesis, "gasps" of air should be heard from within the instrument which represent a resetting of the ballast to maintain overall pressure.

Guarantee that the waste containers can support the generated waste. As a guideline, a fifteen amino acid peptide on the 0.25 mmol scale will generate 6-7 liters of waste. The expected percentages and MSDS forms of all waste components are listed in the *Safety Supplement Manual*.

Perform self tests using manual commands on the synthesizer. These tests are found in the Self Test Menu on the synthesizer's front-panel display and include the needle, ejector, and bar-code reader. When filling empty cartridges with amino acids or other chemicals, remember to turn the bar-code reader OFF immediately prior to synthesis. Empty cartridges may be purchased with bar-codes for particular amino acids or generically labeled SP1 for other chemicals.

Conduct flow tests. All flow test specifications and copies of operational log sheets can be found in the *Log Manual for the Model 433A*. The log sheet, as shown in Figure 24, was developed to provide systematic selection of modules and efficient record keeping of tests, observations, and filter changes. Volumes are measured using the metering vessel. Weight comparisons are made using an empty, used cartridge. On the instrument, the Module Test Menu is used to select modules corresponding to particular flow tests. All modules are comprised of functions. Functions involve individual operations for vortexing, pressurizing reagents, delivering of reagents and gas, ejecting cartridges, waiting

OPERATION LOG SHEET

Date _____

FLOW TEST DATA**Lower Regulator-Flow test 10**

Date	Bot Lev	Pressure	Volume	Volume

Other Flow Tests

Number	Chemical Reagent	Module		Volume	Volume	Volume
1	Piperidine	a	Flow meter			
4	DMAP	d	Flow meter			
6	MeOH	f	Flow meter			
9	DCM	i	Flow meter			
				Mass	Mass	Mass
12	DCM to Cart	C	Cartridge			
13	HBTU to Cart	D	Cartridge			
11	NMP to Cart	B	Cartridge			

NOTE: Repeat each flowtest until two consecutive deliveries fall within specifications.

Conductivity-Flow Test 20

# 6 (MeOH)	# 1 (Piperidine)	# 10 (NMP)	# 9 (DCM)

Barcode Calibration

1	2	3	4	5	6

Figure 24. Operational Log Sheet for organizing flow test data and observations.

periods, etc. An extensive listing of all functions is found in the *User Manual for the Model 433A, Volume II*. Figure 25 depicts the Function window found in the chemistry folder of the *Synthassist* software. Any function number may be entered and the computer will demonstrate the pathway for the gas or solvent. The synthesizer's Manual Test Menu allows any function to be performed.

Because the Module Test Menu on the synthesizer contains default settings, the modules necessary to perform the desired flow tests must be transferred from the *Synthassist* Software. To do this, start by opening the Chemistry Folder and choosing Flow Tests 1-18. On the menu bar, select Synthesizer and choose Connect to establish communication with the instrument. Reopen the Synthesizer Menu and choose Send to transfer the Flow Tests to the instrument. The flow tests may now be accessed from the synthesizer's Module Test Menu. Occasionally, the modules need to be altered. An example of this would be that the weight comparison obtained is lower than the specified value. In the Module Editor Menu on the instrument, the time of solvent delivery to the cartridge can be increased for that individual function. For the conductivity flow test, select Flow Tests 19-23 from the Chemistry Folder and Send to the synthesizer. A low conductivity reading during this flow test may indicate poorly aged methanol and/or ineffective bottle seals.

Note the condition of the three in-line filters on the valve-assembly panel. The filters are located at the top of the reaction vessel, at the bottom of the reaction vessel, and close to the conductivity cell. The two in-line filters near the reaction vessel should be replaced every three to four runs for a five amino acid sequence on the 0.25 mmol scale.

Figure 25. Function Window depicting the manual functions available on the instrument's front-panel display.

Also, low flow test levels may indicate blocked filters. The existing filters may be rebuilt using the kit supplied, provided the filter housing shows no cracks or signs of excessive wear. For additional help consult the *User's Manual for the Model 433A, Volume II* which contains schematic diagrams of the valve-assembly panel.

SYNTHASSIST 2.0 SOFTWARE PROTOCOL

First select the desired peptide sequence. To do this, open the Sequence Folder and choose NEW. When defining a sequence one must keep in mind that the synthesizer **BUILDS** from the C-terminus to the N-terminus; however, the software **WRITES** the sequence from the N-terminus to the C-terminus. Within the sequence window, a palette of amino acids is shown at the bottom of the screen. Utilize the mouse to select the desired sequence. In some instances, it may be necessary to alter the palette. For example, if the free proton on the N-terminus amino acid is highlighted using the mouse, the palette yields different options such as Ac (acetylated capped amino acid) and Fmoc (protected amino acid). On the menu bar, select Dictionary to select amino acid protecting groups, to add additional options, and to change the color representing each amino acid.

Save the sequence. Then, with the Sequence Window still open, select NEW and choose RUN. The software will then set up the proper order for the amino acids. The Run Window contains several pop-up windows including Chemistry, Sequence, Calculations, Cycle, Amino Acid, Cartridge, Resin and Monitor. In addition, any cycle or module line may be highlighted using the mouse to reveal new options. For example, if the Fmoc protection should remain on the N-terminus amino acid, double-click on the final deprotection line in the Cycle Window and choose a simple NMP Wash (module D).

Then, add an extra line for a DCM wash (module c). Use the mouse to highlight the Chemistry pop-up menu and select the appropriate chemistry, such as 0.25mmol/ Mon PrevPeak, which is generally sufficient for all sequences. The cycles and modules will be set up according to the chosen chemistry. Use the Resin pop-up window to select the resin (HMP, amide or preloaded). Enter the substitution value (mmol/g) from the resin bottle into the second column. The amount of resin required will appear in the Weight (grams) column. For example, if the substitution value is 0.75 mmol/g and the scale is 0.25 mmol, weigh out 0.3333 grams of resin. Save the Run. A Run Sheet, as shown in Figure 26, was developed to record sequencing results. Descriptive examples of all software operations are found in the *Synthassist 2.0 User Manual*.

SYNTHESIS MATERIALS AND METHODS

The desired resin should be loaded into the appropriate size reaction vessel for the selected scale. The procedure for loading a resin is as follows. Place a tabbed filter in one end of the reaction vessel using a forceps. Never touch the filter with the finger tips. Screw on one end-cap of the vessel to finger tightness. Add the required amount of resin to the reaction vessel. A second tabbed filter should be inserted into the vessel and then the second end-cap replaced. On the instrument, replace the metering valve used for the flow tests with the reaction vessel, checking for the proper fit by manually vortexing the vessel.

The cartridge track and general areas should be cleaned with methanol on a Kim-wipe. However, never allow any solvent to contact the bar-code reader.

The amino acid cartridges should now be inserted in their proper sequence order onto the instrument. The Cartridges pop-up window in the RUN Window may help to

RUN SHEET

Instrument No. _____

Synthesis Date:_____

Run #: _____

PEPTIDE: _____

Starting Resin: _____

Weight: _____

Initial Loading: _____

Lot Number: _____

[illegible]

Note: The Intensities and the general ratios of the conductivity peaks.

Figure 26. Run sheet for recording peptide sequencing parameters and observations.

determine the order of cartridges on the instrument. Begin by moving the "pusher block" to the far right and securing it using the safety latch. Carefully remove the silver tab on each amino acid cartridge cap with a spatula and press the caps on a flat surface. It should be noted that the most common interruption during synthesis is that the ejector can catch on a raised lid. After tapping powder to the bottom of the cartridges, place each cartridge on the synthesizer. Carefully release the safety latch which will then push the block against the cartridges. Lower the support bar to prevent the cartridges from tipping over. If filling empty cartridges, always add 1 mmol of material and crimp on a metal cap. New, empty cartridges can be utilized for amino acids and other chemicals, such as acetic anhydride mixtures, to cap amino acids or unloaded resins. Applied Biosystems supplies User Bulletins to describe formulations for preparing such mixtures. Because efficient cleaning of the cartridges is very difficult and the fact that cartridges tend to bulge after a few uses, reusing dirty cartridges is not recommended for any purpose other than flow tests.

In the *Synthassist* software, open the Chemistry Folder and highlight the desired chemistry. Note that this chemistry must correlate with that chosen in the run. Connect to the synthesizer and Send the data.

Open the Run Window and Send the run to the synthesizer. Open the Monitor pop-up window in order to view the process of deprotection and sequencing during the peptide synthesis. On the synthesizer, press the right arrow key on the keypad to confirm proper transfer of the correct chemistry and run sequence. On the Cycle Monitor Menu, answer the questions regarding resin sampling, Hold add time, and event printing. Press the BEGIN key to start synthesis.

IMPORTANT NOTE: Once synthesis has begun it is too late to check pressure levels, flow tests, alter chemistry, etc. Proper research and thought will save time and expense. In addition, never disconnect communication between the computer and synthesizer during synthesis for printing or any other reason. Should this happen, the synthesis will continue but it will be impossible to receive a log report or a conductivity monitoring trace of the sequence.

Once sequencing is completed, close the Monitor pop-up window and Save the Run. Open the Log Folder and Save the synthesis log report. Backing up the sequence, run, and log files on disks is recommended.

PEPTIDE STORAGE

Refer to the flow chart in the *Introduction to Cleavage Manual* to determine the best cleavage conditions and procedure for each peptide. A video produced by Applied Biosystems also discusses cleavage methods and provides helpful tips.

The resin-bound peptide should be dried prior to removing from the reaction vessel. Manual function number 48 on the synthesizer produces a stream of nitrogen which should be manually shut off after several hours. More complete drying is accomplished by placing the product in a centrifuge tube and then into a vacuum dessicator for several hours.

If cleavage is not immediately performed after synthesis, the resin-peptide product may be stored in a labelled centrifuge tube in a freezer. Otherwise, if cleavage is performed, the peptide must be lyophilized and then may be stored at room temperature.

Every amino acid may exhibit different chemical behavior in each peptide sequence. There is no guarantee of chemical behavior even if the amino acid environment,

including nearest neighbors, is the same as in a separate peptide sequence. Hence, it is best to try the following basic cleavage method with a small portion of peptide and to scale up the process accordingly. The following basic cleavage procedure was adapted from a training session with Dr. Richard Noble, a research scientist at Applied Biosystems.

BASIC CLEAVAGE PROCEDURE

Place resin-bound peptide product into a centrifuge tube. Add ice COLD tri-fluoroacetic acid (TFA). Thioanisole and ethanedithiol must also be added to some sequences to react with any scavengers. To see if this addition is required, refer to the flow chart in the *Introduction to Cleavage Manual*.

Shake the cleavage mixture on an orbital shaker for 1.5 to 2 hours.

Filter the TFA mixture through an Isolab column into another 50 mL centrifuge tube containing ice COLD methyl t-butyl ether (MTBE). The peptide should form a precipitate, otherwise another method must be used. The latter was found to be true of certain dipeptides which then required an extraction technique.

Centrifuge for 5 to 10 minutes. Decant off ether. Wash the precipitate with more ice COLD MTBE, vortex, and repeat the centrifuging. Generally, three to four washings is sufficient. At this point, the resin and any protecting groups on the amino acid side-chains should have been removed. The resulting free peptide must be lyophilized and may be stored at room temperature. The free peptide or TFA cleavage mixture may be purified utilizing a high-performance liquid chromatograph (HPLC), if required.

APPENDIX B

ADDITIONAL PEPTIDE SEQUENCING RESULTS

Table 11. Cycles and Modules for Sequencing VPGS(tBu)G on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Ser(tBu)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6	Final Deprot/ MonPrev	BIDc

Table 12. Calculations for Sequencing VPGS(tBu)G on the 0.25 mmol Scale.

Volume: 5.0 mL	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Ser(tBu)	0.250	0.677	1476.52	0.369
2 Gly	0.250	0.652	1533.57	0.383
3 Pro	0.250	0.613	1630.69	0.408
4 Val	0.250	0.578	1729.82	0.432
H	0.250	0.663	1508.59	0.377

Figure 27. Monitor Trace for Sequencing VPGS(tBu)G.

Table 13. Cycles and Modules for Sequencing VPGK(Z)G on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Lys(Z)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6	NMP Wash	D
7	DCM Wash	c

Table 14. Calculations for Sequencing VPGK(Z)G on the 0.25 mmol Scale.

<u>Volume: 5.0 mL</u>	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Lys(Z)	0.250	0.627	1595.64	0.399
2 Gly	0.250	0.605	1652.69	0.413
3 Pro	0.250	0.571	1749.81	0.437
4 Val	0.250	0.541	1848.94	0.462

Figure 28. Monitor Trace for Sequencing VPGK(Z)G.

Table 15. Cycles and Modules for Sequencing VPGY(tBu)G on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Tyr(tBu)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6	NMP Wash	D
7	DCM Wash	c

Table 16. Calculations for Sequencing VPGY(tBu)G on the 0.25 mmol Scale.

Volume: 5.0 mL	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Tyr(tBu)	0.250	0.644	1552.62	0.388
2 Gly	0.250	0.621	1609.67	0.402
3 Pro	0.250	0.586	1706.79	0.427
4 Val	0.250	0.554	1805.92	0.451

Figure 29. Monitor Trace for Sequencing VPGY(tBu)G.

Table 17. Cycles and Modules for Sequencing VPGY(Bzl)G on the 0.25 mmol Scale.

<u>Amino Acid</u>	<u>Cycle</u>	<u>Modules</u>
1 Gly	Complete Wash	cD
2 Tyr(Bzl)	Single Couple / NMP	BADEFD
3 Gly	Single Couple / NMP	BADEFD
4 Pro	Single Couple / NMP	BADEFD
5 Val	Single Couple / NMP	BADEFD
6	NMP Wash	D
7	DCM Wash	c

Table 18. Calculations for Sequencing VPGY(Bzl)G on the 0.25 mmol Scale.

Volume: 5.0 mL	<u>Resin</u> (mmol)	<u>Substitution</u> (mmol/g)	<u>Resin</u> <u>Equivalent Weight</u> (g/ mol)	<u>Weight</u> (g)
Preloaded HMP Resin with Glycine	0.250	0.750	1333.33	0.333
<u>Amino Acid</u>				
1 Tyr(Bzl)	0.250	0.630	1586.63	0.397
2 Gly	0.250	0.608	1643.69	0.411
3 Pro	0.250	0.574	1740.80	0.435
4 Val	0.250	0.543	1839.94	0.460

Figure 30. Monitor Trace for Sequencing VPGY(Bzl)G.

REFERENCES

- (1) Stryer, L. *Biochemistry*, Third ed.; W.H. Freeman: New York, 1988.
- (2) Kwa, S. *Plant Solar Cells under Illumination*; Vrije University: Amsterdam, the Netherlands, 1993.
- (3) O'Connor, D. V.; Phillips, D. *Time-Correlated Single Photon Counting*; Academic Press: London, 1984; p 4.
- (4) Forster, T. In *Modern Quantum Chemistry*; Sinannoglu, O., Ed.; Academic Press: New York, 1965, p 93.
- (5) Forster, T. *Ann. Phys.* **1948**, 2, 55.
- (6) Matthews, B. W.; Fenna, R. E. *Acc. Chem. Res.* **1980**, 13, 309.
- (7) Hemelrijk, P. W.; Kwa, S. L. S.; Van Grondelle, R.; Dekker, J. P. *Biochim. Biophys. Acta* **1992**, 1098, 159.
- (8) Gouterman, M. *J. Molec. Spec.* **1961**, 6, 138.
- (9) Kamtekar, S.; Schiffer, J. M.; Xiong, H.; Babik, J.; Hecht, M. *Science* **1993**, 262, 1680.
- (10) Dutton, P. L.; DeGrado, W. F. *J. Amer. Chem. Soc.* **1994**, 116, 856.
- (11) Richardson, J. S.; Richardson, D. C. *TIBS* **1989**, 14, 304.
- (12) Urry, D. W. *Int. J. Quantum Chem., Quantum Biol. Symp.* **1988**, 15, 235.
- (13) Urry, D. W. *J. Protein Chem.* **1988**, 7, 1.
- (14) Cook, W. J.; Einspahr, H.; Trapani, T. L.; Urry, D. W.; Bugg, C. E. *J. Amer. Chem. Soc.* **1980**, 102, 5502.
- (15) Urry, D. W. *Angew. Chemie* **1993**, 32, 819.
- (16) Brannon, J. H.; Magde, D. *J. Amer. Chem. Soc.* **1980**, 102, 62.
- (17) Owen, J. E.; Kenney, M. E. *Inorg. Chem.* **1962**, 1, 331.

- (18) Anfinsen, P. A.; Hart, D. E.; Hedstrom, J. F.; Struve, W. S. *J. Phys. Chem.* **1986**, *90*, 2374.
- (19) Savikhin, S. *Software system for optical spectroscopy*, SPECTRA processor; 1993.
- (20) Bevington, P. R.; Robinson, D. K. *Data Reduction and Error Analysis for the Physical Sciences*, 2nd ed.; McGraw-Hill: New York, 1992.
- (21) Fersht, A. *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 10869.
- (22) Merrifield, R. B. *Science* **1965**, *150*, 178.
- (23) Barany, G.; Merrifield, R. B. *The Peptides, Analysis, Synthesis, Biology*; Academic Press: New York, 1980; p 3.
- (24) Kent, S. B. H. *New Aspects of Solid-Phase Peptide Synthesis*; Academic Press: New York, 1980; p 213.
- (25) Carpino, L. A.; Han, G. Y. *J. Org. Chem.* **1972**, *37*, 3404.
- (26) Chang, C. D.; Meienhofer, J. *Int. J. Peptide Protein Res.* **1978**, *11*, 246.
- (27) Merrifield, R. B. *J. Amer. Chem. Soc.* **1963**, *85*, 2149.
- (28) Fields, G. B.; Noble, R. L. *Int. J. Peptide Protein Res.* **1990**, *35*, 161.
- (29) Henklein, P.; Beyermann, M.; Sohr, R. *Peptides*; de Gruyter: Berlin, 1992.
- (30) Tam, J. *Int. J. Peptide Protein Res.* **1987**, *29*, 421.
- (31) Applied Biosystems. *User Manual for the Model 433A*; 1993, Vol. 1.
- (32) Noble, R., Personal communication, 1995.
- (33) Kraus, G., personal communication, 1996.

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