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Synthesis of Phytosulfokine Analogs as Probes for Studying Plant Signaling and Molecular Trafficking

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Master of Science in Chemistry, concentration in Organic Chemistry

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by

Thomas Ntim

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ABSTRACT

Synthesis of Phytosulfokine Analogs as Probes for Studying Plant Signaling and Molecular Trafficking

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Plants are exposed to a wide range of biotic and abiotic stresses that hinder their growth and reduce crop productivity. In their adaptive response, plants use signaling molecules that are trafficked throughout the plant. This research focuses on the chemical synthesis and assessment of analogs of the plant signal phytosulfokine (PSK, a sulfated pentapeptide), its delivery to plants and its observation using a fiber-optic fluorescence microscope. PSK regulates growth, cell expansion, heat tolerance, and tissue longevity. Analogs of PSK were synthesized using solid-phase peptide synthesis. Pure PSK and TAMRA-labeled PSK were delivered into the wild-type *Arabidopsis thaliana* Col-0 and a transgenic line expressing PSKR-GFP (PSK receptor – green fluorescent protein). PSKR-GFP could be detected in imaging experiments, but no internalization was observed upon treatment with PSK. Successful implementation of a microscopic approach suited for live plants opens a path to understanding how plants signal and adapt under different stress conditions.

DEDICATION

To my lovely family,

For their sacrifice and encouragement,

Thank you.

ACKNOWLEDGEMENTS

The journey of my M.S. in Chemistry program was not easy to complete, but the lessons gained from this journey have prepared me to be a better chemist. Having completed this journey, I would like to show my honest gratitude to the Almighty God for granting me wisdom, understanding, and strength throughout my two years program.

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LIST OF ABBREVIATIONS

ABA	Abscisic acid
AtPSK1-6	*Arabidopsis thaliana* phytosulfokine genes 1-6
Boc	*tert*-Butoxycarbonyl
C_{18}	Octadecyl
C_8	Octyl
CaM	Calmodulin
cDNA	Complementary deoxyribonucleic Acid
cGMP	Cyclic guanosine monophosphate
DCC	Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DIPEA	*N*,*N*-Diisopropylethylamine
DMF	*N*,*N*-Dimethylformamide
DNA	Deoxyribonucleic Acid
EtOH	Ethanol
5-FAM	5-Carboxyfluorescein
Fmoc	Fluoren-9-ylmethoxycarbonyl
G protein	Guanine nucleotide-binding protein
GC	Guanylate cyclase
GFP	Green fluorescent protein
Gln	Glutamine
HATU	1-[Bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate
HOAt	1- Hydroxy-7-azabenzotrialzole
HOBt	1-Hydroxybenzotriazole
Ile	Isoleucine
IR	Infrared

LPPS	Liquid-phase peptide synthesis
LRR	Leucine-rich repeat
MCA1	Meta caspase 1
MCA2	Meta caspase 2
MeOH	Methanol
Mtt	4-Methyltrityl
NMP	*N*-Methyl-2-pyrrolidone
OsPSK1-7	*Oryza sativa* phytosulfokine genes 1-7
PERK4	Proline-rich extensin-like receptor kinase 4
PSK	Phytosulfokine
PyBOP	Benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate
RLK	Receptor-like kinase
RP-HPLC	Reversed-phase high-performance liquid chromatography
SAR	Systemic acquired resistance
SPPS	Solid-phase peptide synthesis
Su	Succinimidyl
5(6)-TAMRA	5(6)-Carboxytetramethylrhodamine
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
Thr	Threonine
TPST	Tyrosyl protein sulfotransferase
Tr	Trityl (triphenylmethyl)
Tyr	Tyrosine
UV	Ultraviolet
VACNFs	Vertically aligned carbon nanofibers

CHAPTER 1. INTRODUCTION

Background

One of the most abundant living organisms on the planet Earth is plants. The sessile nature of plants exposes them to a wide range of stress conditions. These stress conditions refer to external factors that negatively affect the growth, development, and productivity of plants.[1] It has been found that stress conditions activate a diverse range of plant responses like modified plant cellular metabolism, altered gene expression, reduced crop yield, and changes in growth and development.[2] Stress conditions can be either biotically or abiotically induced. Biotic stress conditions are the biological entities that hinder the normal performance of plants and include plant attacks by insects, viruses, bacteria, fungi, oomycetes, nematodes, and herbivores. The physical and chemical environmental factors that affect the activities of the plant are considered abiotic stress conditions. Low or excess water availability (drought or flooding), extreme temperatures, mineral deficiency, changes in salinity, and toxicity are classified as abiotic stress conditions.[1,2] The exposure of plants to these two stress conditions is illustrated in Figure 1.

Biotic and abiotic stress conditions can be mild or severe depending on the effects they impose on plants. It has been reported that the environmental location of plants determines the kind of stress conditions that the plant will be exposed to as well as the effects of the stress conditions on the plants.[2] When plants are exposed to biotic or abiotic stress conditions for a very long time, they become unhealthy. Plants that are not able to withstand mild stress conditions are classified as stress-susceptible plants whereas those that can withstand both mild and severe stress conditions are referred to as stress-tolerant plants.[3] According to Suzuki et al.,[4] the effects of biotic and abiotic stresses, or a combination of these two stress conditions harm plants' growth, physiology, productivity, and yield.

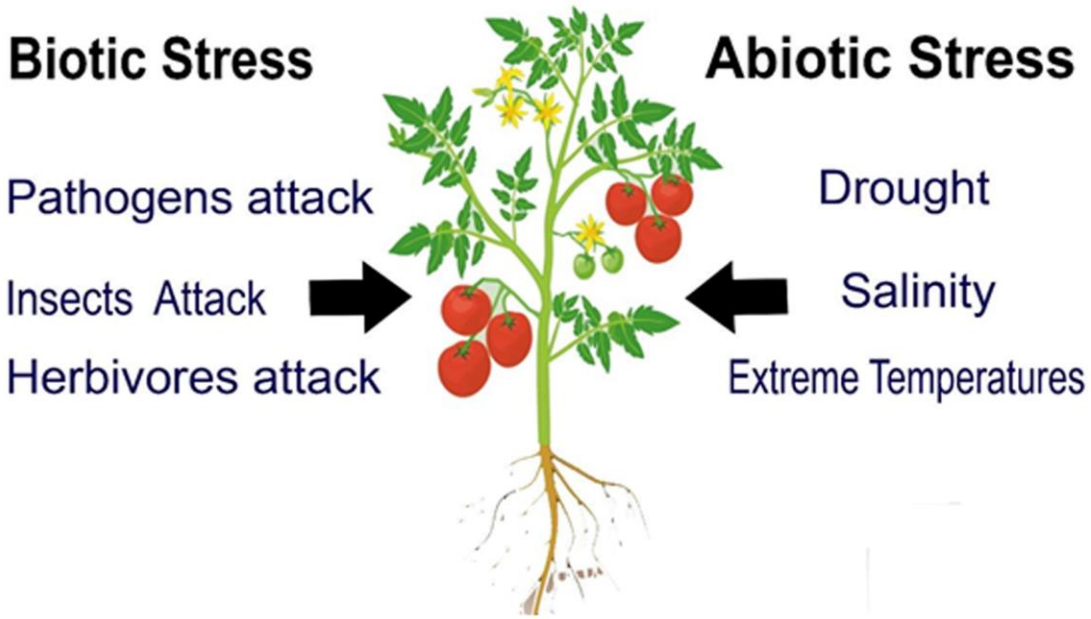

Figure 1. Plant exposed to biotic and abiotic stress conditions

Effects of Biotic and Abiotic Stress Conditions on Plants

Plants struggle with numerous biotic and abiotic stress conditions. These stress conditions negatively affect plant growth and development as well as inducing morphological, biochemical, and molecular changes in the plant. Biotic stress agents like fungi, viruses, bacteria, and nematodes have been recognized as determinants that cause various kinds of plant diseases which reduce crop productivity.[2] These agents can also deprive plants of necessary nutrients and result in plant death. For this reason, biotic stress is noted to be the major cause of pre- and post-harvest losses.[2]

Much like biotic stress, abiotic stress also induces a series of morphological, physiological, biochemical, and molecular changes that adversely hinder plant growth and productivity.[5] Abiotic stresses can cause accumulation of reactive oxygen species (ROS) and negatively influence plant survival, biomass production, and grain yield of many crops.[6] According to Singhal et al.,[7] abiotic stress decreases the average yield of crops by 50%. Drought and salinity are considered as the major abiotic stresses which cause serious deterioration of

arable land. Drought, salinity, and extreme temperatures are also known to cause osmotic stress, which results in the disruption of homeostasis and ion distribution in the cell. It is estimated that drought and salinity could cause devastating global effects and lead to a 30% loss of fertile land within 25 years and 50% by the year 2050.[5]

Reducing the Effects of Stress Conditions on Plants

In an effort to reduce the effects of stress conditions on plants, scientists have investigated the use of farming practices, plant breeding and genetic engineering.

Farming practices aim to modify the physical and chemical environment of crops. For example, irrigation is used to reduce the effect of drought on plants; mulching is employed to create conducive temperature conditions intended to facilitate plant growth, and fertilizer applications are implemented to provide the chemical environment necessary for plant development.[8] The use of novel farming practices to combat the effects of stress conditions is becoming limited because of the large amount of time consumed in decreasing the effects of multiple stress conditions on plants, as well as the labor-intensive aspect of diverse farming practices.

Plant breeding is another practice used to reduce the effects of stress conditions on plants. Plant breeders seek to improve plants by developing stress-tolerant plants able to grow well under biotic and abiotic stress conditions. Classical plant breeding methods consist of interspecific or intergeneric hybridization of crops. Despite the success of plant breeding to reduce the effects of stress conditions on plants, some drawbacks have been identified. The drawbacks are quantitative complexity of plant traits, low genetic variance yield obtained under stress conditions, and lack of selection criteria that makes identifying specific plant traits

difficult.[9,10] The limitations of farming practices and plant breeding have forced plant scientists to find alternative means of developing stress-tolerant plants.

One method of curbing the effects of stress conditions on plants is through genetic engineering. Genetic engineering refers to the mobilization of genes from virtually any source and the ability to manipulate these genes to improve the agronomic performance of a plant.[7] Gene insertion and gene knock-out in the genome of a plant are the two components of genetic engineering utilized to alter plant activities. In the development of stress-tolerant plants, either genes are inserted into the genome of the plant, or genes are substituted in the genetic locus of the plant. James et al.[11] reported that genetic engineering was employed in cultivating transgenic crops spanning 1.7 million hectares of land in 1996. By 2009, 134 million hectares of land were used to cultivate transgenic crops. This 80-fold increase of transgenic crop land-use between 1996 and 2009 demonstrates the usefulness of genetic engineering in crop yield. To better understand the use of genetic engineering, plant breeding, and farming practices in preventing or reducing the effects of stress conditions on plants, it is necessary to consider plant signaling and molecular trafficking.

Plant Signaling and Molecular Trafficking

Plant signaling and molecular trafficking are involved in normal plant development as well as externally induced pathways such as systemic acquired resistance (SAR) used by plants against stress conditions.[12] It has been reported that in stress conditions, plants release biomolecules like signaling peptides. The movement of these molecules from the external environment of the cell, and through the cell membrane, along with the cascade of reactions they prompt in the cell is referred to as molecular trafficking. Plant signaling and molecular

trafficking play vital roles in food and energy crops. The results of these two processes control the growth and development of plants and aid plants in adapting to stress conditions.[13]

In plant signaling and molecular trafficking processes, three distinct biochemical steps enable plants to respond to various environmental conditions: reception, signal transduction, and cellular response. These processes are illustrated in Figure 2.

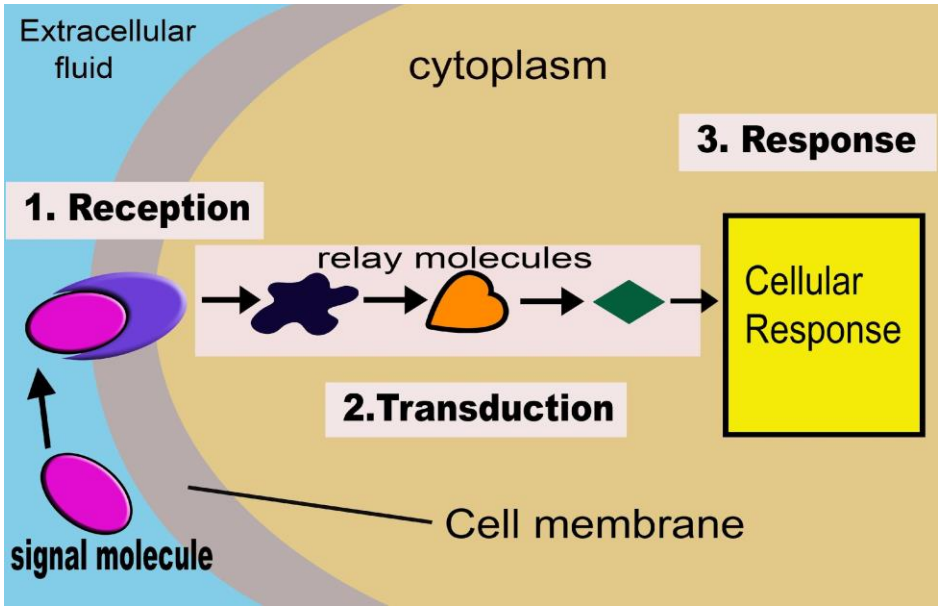

Figure 2. The three stages of plant signaling and molecular trafficking

In signal reception, primary signal molecules released during stress bind to protein receptors. This binding produces secondary messenger molecules that cause a cellular response. The G Protein-coupled receptors and tyrosine kinases receptors are two examples of receptors integral to plant signaling and molecular trafficking. The second biochemical process of plant signaling, and molecular trafficking, is transduction. Transduction begins with the conformational change of a receptor after it binds with a signal molecule to communicate perception across the cell membrane. Subsequently, transduction involves the transmission and amplification of signals from small primary messengers to relay molecules, or secondary messengers. Following the conformational change of a receptor, transduction involves the

regulation of cellular activities like turning specific genes on or off, opening or closing of ion channels in the cell membrane, and altering cell metabolism. Transduction reactions can include the addition or removal of phosphate groups using enzymes (protein kinases and protein phosphatases) or may make use of ions or non-protein and water-soluble molecules. The addition and removal of phosphate groups can either activate or inactivate enzymes that regulate transduction. The last biochemical process in plant signaling and molecular trafficking is response. Response refers to the change in the cell functions which results in the regulation of cellular activities. For example, regulation of gene expression and protein (enzyme) activity are outcomes of plant signaling, which provide immunity to the plant, enhance growth and development, and increase crop yield.

Types of Plant Signaling

There are three different forms of plant signaling, and they are: electrical signaling, hydraulic signaling, and chemical signaling.[12]

In electrical signaling, voltage transients are generated from an ion imbalance across a plant's plasma membrane.[14] Transmitting information from one part of the plant relies on ion channels and transporters in the plant cell. Rapid leaf movements (such as those found in *Mimosa pudica*), respiration, and water and nutrient uptake are all physical outcomes of electrical signaling.[12,15]

Hydraulic signaling refers to self-propagating changes in water pressure caused by environmental stress conditions. The availability of water throughout the entire plant makes ubiquitous hydraulic signaling throughout the continuous water phase in the apoplast between neighboring cells, across the cell membrane, and within all hydrated cells.[16] Hydraulic signals are generated by a change in water potential, which is caused by varying turgor or osmotic

pressure, and they are quickly transmitted throughout the plant. It has been suggested that hydraulic signals are perceived by several sensors including MCA1, MCA2, and PERK4. The perception of hydraulic signals by these sensors converts the hydraulic signal to a chemical signaling molecule, e.g., through the production of compounds such as abscisic acid (ABA), a molecule responsible for mediating various adaptive responses of plants.[17]

Chemical signaling uses chemical molecules to transmit information within and between plant cells and their external environment. The downregulation and upregulation of genes resulting from chemical signaling enable plants to adapt to stress conditions. The signal molecules used in chemical signaling act as ligands and bind to receptors in plant cell membranes, initiating a series of chemical reactions leading to a physical response by the plant. Various signal molecules have been identified and their role confirmed in plant signaling and molecular trafficking. Depending on the size, shape, and function of a signaling molecule, it can travel either a short or long distance to its target receptor. Some of the chemical signaling molecules have been studied, and they include calcium, ABA, cyclic nucleotides, nitric oxide, polyphosphoinositides, sugars, plant hormones, and polypeptides.[18] The role of plant hormones and polypeptides in plant development have been studied extensively. The five major plant hormones that have been identified are auxin, gibberellins, cytokinin, abscisic acid, and ethylene. These hormones have unique and specific functions in plants. Auxin induces cell division, cell differentiation, and cell elongation; gibberellin is responsible for stem elongation; cytokinin aids in cell division; abscisic acid initiates the onset of plant dormancy during unfavorable conditions, and ethylene affects the ripening of fruit. Much like plant hormones, polypeptide signal molecules vary in size, origin, and function (a table is provided in Appendix A). Polypeptide signal molecules are produced naturally in plants during plant development or during stress

conditions. Signal molecules have been chemically synthesized ,and their signal transduction has been studied. This research focuses on the chemical synthesis and signaling studies of the peptide hormone phytosulfokine (PSK) and its analogs.

Phytosulfokine (PSK)

The native PSK is a pentapeptide composed of five amino acid residues in the sequence Tyr(SO_3H)-Ile-Tyr(SO_3H)-Thr-Gln. Of these five amino acid residues, the two tyrosine residues are sulfated post-translationally.[19] The structures of native PSK and the proposed PSK analog molecules are illustrated in Figure 3. The native PSK and the synthetic PSK analog differ at positions 1 and 3. In the native molecule, there exist sulfated tyrosines in positions 1 and 3, which are colored blue in Figure 3, whereas in the analog, these residues are replaced with sulfomethylphenylalanine, which is colored pink. A further distinction between the native and analog molecules is the substation of Gln with Lys at position 5 on the analog to provide a handle for fluorescent labeling. The native PSK molecule is produced by endogenous processing of an 80 amino acid-long precursor encoded by the *PSK* family genes.[20] The processing of this precursor involves tyrosine sulfation by tyrosyl protein sulfotransferase (TPST) in Golgi bodies and proteolytic cleavage of the preprotein in the apoplast. Sakagami et al. indicated that the incorporation of the sulfate groups into the tyrosine residues makes PSK biologically active.[21] According to Matsubayashi, stringent phenotype analysis and biochemical purification procedures were used to identify PSK from a culture suspension containing a small density of plant cells.[22] It is reported that PSK binds to a transmembrane receptor (PSKR), of which there may be two or more homologs in a given plant species. The PSKR has two domains, the C-terminal intracellular kinase domain, and the extracellular domain. PSKRs are in the receptor-like kinase (RLK) family, which has 600 members in rice and 1100 members in *Arabidopsis*.[23,24]

A single transmembrane helix connects the PSK receptor proteins to the cell membrane, and there is an N-terminal signal peptide that targets the PSK receptor to the secretory pathway.[25] Two techniques, namely, photoaffinity labeling and radiolabeling, have been used to determine the binding of PSK molecules to their receptors on the plasma membrane in maize, tomato, tobacco, carrot, and asparagus plants.[26]

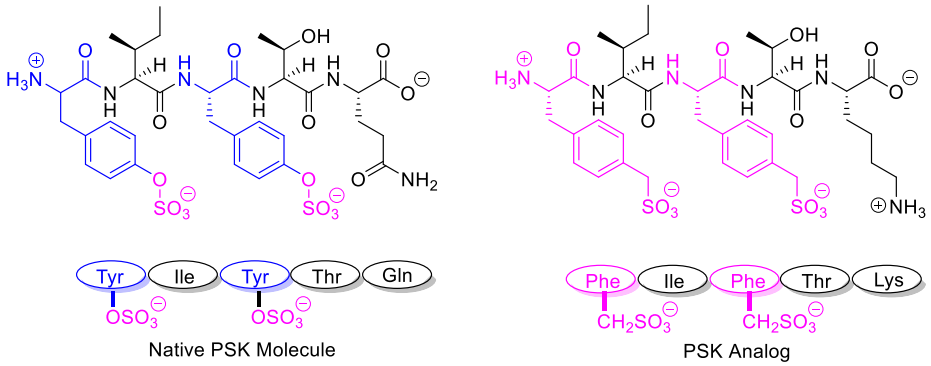

Figure 3. Structures of native PSK and PSK analog molecules

Signaling Network of PSK

The biological activity of PSK is dictated by ligand-receptor binding that leads to a cascade of signals with effects such as providing immunity and enhancing growth of plant cells.[23] The extracellular glycosylated domain of the PSK receptor is made up of 21 leucine-rich repeats (LRRs) with an island domain found in LRR18. Binding of PSK to its receptor occurs at this island domain,[22] and activates the intracellular serine-threonine kinase part of the receptor. The serine-threonine kinase has guanylate cyclase (GC) activity, which produces cyclic guanosine monophosphate (cGMP). cGMP targets many sites in the cell to elicit biochemical and molecular changes. In the nucleus, cGMP causes gene regulation, with effects that include cell proliferation. The receptor's ability to detect PSK is regulated by a calcium-binding protein called Calmodulin (CAM). The PSK signaling network is illustrated in Figure 4.

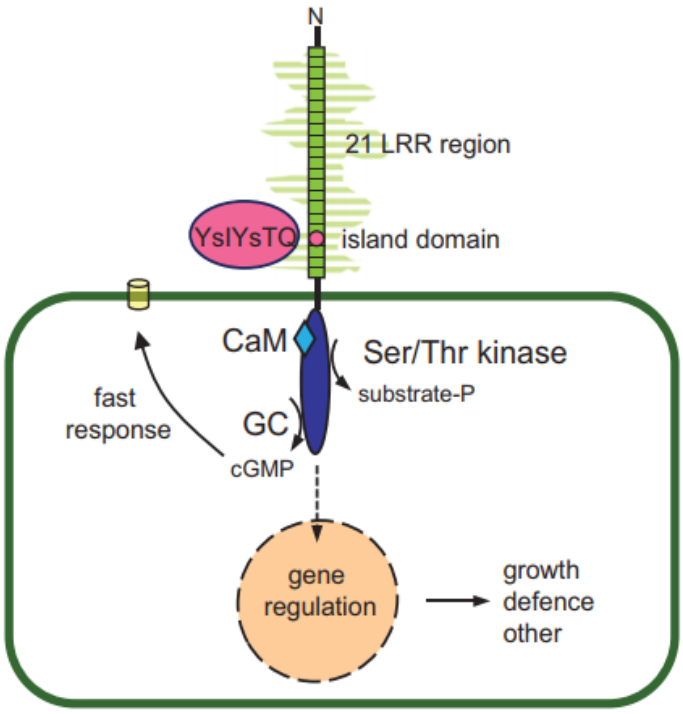

Figure 4. Signaling network of phytosulfokine.[22] Copyright Oxford University Press, used with permission.

PSK has several other biological functions aside from promoting plant cell proliferation. Yamakawa et al. reported enhancement of chlorophyll synthesis in etiolated cotyledons of cucumber as well as the promotion of growth and chlorophyll content in *Arabidopsis* seedlings under nighttime temperature conditions.[27] In the reproduction of flowering plants, PSK can direct pollen tubes and contribute to the immunity of the plant. Furthermore, PSK can also stimulate tracheary element differentiation and somatic embryogenesis, aid in heat tolerance, and promote tissue longevity.[22]

Chemical Synthesis of PSK

PSK is known to be synthesized in plants from 80–120 residue pre-propeptides encoded by a small gene family. In rice there are seven genes in the family, *OsPSK1-7*, and in *Arabidopsis* there are six, *AtPSK1-6*. Expression of these genes produces PSK-precursors which

can undergo post-translational modification — specifically, tyrosine sulfation — by tyrosyl protein sulfotransferases (TPSTs) to yield active PSK.[28]

Over the years, peptides have played a vital role in chemical, biological, medicinal, and pharmaceutical research. The diverse functions of peptides have encouraged organic chemists to study their artificial synthesis. Fundamentally, the formation of peptides involves a repetitive amidation reaction between an amino group of the initial amino acid and the carboxyl group of the following amino acid. There are 20 natural L-amino acids that can be assembled in any order and length to produce novel peptides and proteins.[29,30]

Emil Fischer is the father of peptide chemistry. Fischer produced the first peptide, glycylglycine, and introduced the name "peptide" to the scientific community.[31] One of the major developments since Fischer's discovery is the ability to produce semisynthetic proteins: natural or non-natural proteins that can undergo post-translational modification like phosphorylation, ubiquitination, and glycosylation.[32] Synthetic peptides have many applications in the field of biochemistry, pharmaceutical sciences, and medicine; however, artificial synthesis of large polypeptides (proteins) is highly complex and in many cases infeasible. In the last few decades, the recombinant technique — a method of producing peptides and proteins in a microorganism such as transgenic animals or plants — has been utilized most to synthesize large peptides. An alternative to the recombinant method is chemical synthesis. Chemical synthesis allows for the production of innumerable different peptide sequences, including those that contain unnatural D-amino acid residues or synthetic amino acid residues. The two most common methods of chemical peptide synthesis are liquid-phase peptide synthesis (LPPS) and solid-phase peptide synthesis SPPS.

Liquid-Phase Peptide Synthesis

LPPS was the first chemical method used to produce peptides synthetically. LPPS makes use of two strategies in the production of a desired peptide sequence: linear synthesis and convergent synthesis. Linear synthesis involves the stepwise addition of each amino acid in the sequence until the entire peptide is assembled. Convergent synthesis begins with a small segment of peptide, subsequently assembling further small fragments to give the final peptide sequence. In LPPS, the size of the desired peptide sequence determines the preferred strategy. Peptides with less than five amino acid residues are amenable to linear synthesis, whereas longer peptides use convergent synthesis.[33] LPPS makes use of protecting groups to shield either the carboxylate group or the amino group before deprotection and coupling to produce the desired peptide. The advantages of using LPPS are that the process is cost-effective, the product is purified at each step, and side reactions are mitigated by a large number of protecting groups. However, there are drawbacks to using LPPS. LPPS is not favorable for the synthesis of hydrophobic, hydrophilic, or long-chain peptides due to the insoluble nature of the peptide fragments and the necessary establishment of workup procedures for each intermediate peptide.[34] The disadvantages encountered in LPPS led to the invention of solid-phase peptide synthesis (SPPS). The synthesis of the PSK analog in this study was carried out by SPPS.

Solid Phase Peptide Synthesis

SPPS was invented by R. Bruce Merrifield in 1963,[35] for which he was awarded a Nobel Prize in 1984. Merrifield pioneered the idea of synthesizing peptides on an insoluble polymer-based material. The polymers are pre-functionalized for easy covalent attachment of the first-chain amino acid to obtain the desired peptide after a single post-synthesis cleavage step. What makes SPPS so exceptional and widely used by researchers are the principal advantages it offers:

high efficiency during isolation, self-purification of reaction intermediates, and high percentage yield of the final products. These advantages are achieved through the use of excess reactants and a one flask process. The solid support used to anchor the peptide ensures simple isolation of the finished peptide after cleavage.[29]

The general process used in SPPS is indicated in Figure 5. SPPS begins by attaching the C-terminus of the first protected amino acid to the solid support. This step is achieved through various organic reactions. The type of reaction chosen for SPPS depends on the functional group present in the solid support and the carboxyl group of the first amino acid. The next step in SPPS is the addition of the second protected amino acid to the unprotected N-terminus of the first amino acid. Temporary protecting groups to prevent side reactions also protect the side-chains of the various amino acids. Coupling of the amino acid to the peptide chain occurs at the α-amino group of one amino acid and the carboxyl group of the next amino acid. To achieve fast and efficient coupling, the carboxylic acid group is activated by converting the -OH of the acid to a good leaving group using a coupling reagent. Once the amino acid is added, the solid support is filtered and washed to get rid of by-products and excess reagents. The next step is the removal of the N_{α}-protecting group of the newly coupled amino acid using deprotection reagents. After this process, the solid support is again filtered and washed to eliminate by-products and excess reagents. Washing is followed by coupling the next new amino acid to the growing peptide chain. The cycle is repeated until the desired peptide sequence is obtained. To complete the SPPS process, the desired peptide product is cleaved from the solid support using a cleavage reagent. The cleavage reagent also removes all of the side-chain protecting groups.[32]

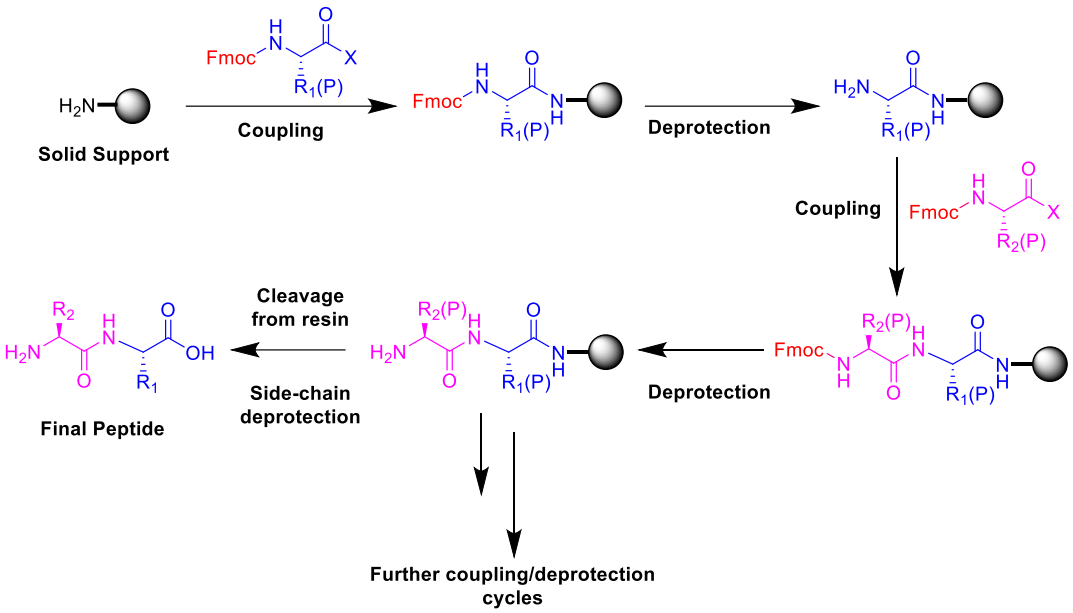

Figure 5. General scheme of solid phase peptide synthesis. To add an amino acid residue to the solid support or the growing peptide chain, four steps are performed to ensure successful reactions: the selection and swelling of the solid support, deprotection reactions, coupling reactions, and cleavage of the peptide from the solid support with concomitant removal of side-chain protecting groups (P).

Selection and Swelling of Solid Support in SPPS

The solid supports used in SPPS are composed of small, spherical resin beads, commonly polystyrene. These resins are insoluble in all solvents used in synthesis and have a stable physical form that allows for easy washing and filtration. Solid support beads typically come in 100–200 mesh (75–100 µm) or 200–400 mesh (35–75 µm) and have a loading capacity of 0.1–1.0 mmol/g. The loading capacity chosen according to the scale and the length of the peptide being synthesized.[32,35] A special feature of solid support beads is linkers: functional groups to which the first amino acid can be easily and firmly attached via covalent bond formation. The functional group on the solid support is used to determine chemical reaction type and the reaction conditions necessary in the cleavage step. Appendix B provides a table of some common solid supports, their structures, and cleavage conditions.[32] Research conducted by

Merrifield et al. investigated different functional groups found on solid-support resins and their mode of attachment. It was determined that the chloromethylated copolymer of styrene and divinylbenzene is ideally suited for standard SPSS.[35] Many other common resins are made by modification of this base resin.

Before synthesis, the solid support resin is "swelled" in an organic solvent for a particular duration of time. This process allows the diffusion of the reagents used in SPPS into the core of the resin and leads to high synthetic efficiency. Some of the organic solvents used to swell resins include DMF, THF, NMP, DCM, dioxane, acetonitrile, methanol, and ethanol.

Protection Strategy and Deprotection Reaction in SPPS

Protecting groups are used to prevent side reactions during synthesis, such as self-condensation of activated amino acids and branching at reactive side chains.[36] One of the characteristic features of SPPS chemistry is the presence of a temporary protecting group located at the N_{α}-terminus of the amino acid and the amino acid side chain protecting groups. The most common and widely used protecting groups for securing the N_{α}-terminus are *tert*-butoxycarbonyl (Boc) and fluoren-9-ylmethyloxycarbonyl (Fmoc) groups. Boc groups are reactive to acids like trifluoroacetic acid (TFA), meaning they are acid labile. Fmoc groups are sensitive to bases like piperidine, meaning they are base-labile.[36] Figure 6 shows how Boc and Fmoc protect the N_{α}-termini of the amino acids. The side chains of many amino acids are very reactive and are also chemically shielded by protecting groups. Appendix C provides a table of common side chain protecting groups and the conditions used to remove them. Figure 7 illustrates the deprotection mechanism of Fmoc using piperidine, and Figure 8 indicates the deprotection mechanism of Boc using TFA. The stability of the protecting group in acidic or basic conditions determines the

deprotection mechanism used. The different mechanisms used to remove protecting groups on the N_{α}-termini of amino acids and amino acid side chains are termed orthogonal strategies.

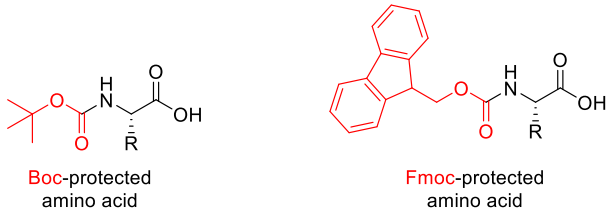

Figure 6. Structures of Boc- and Fmoc-protected amino acids

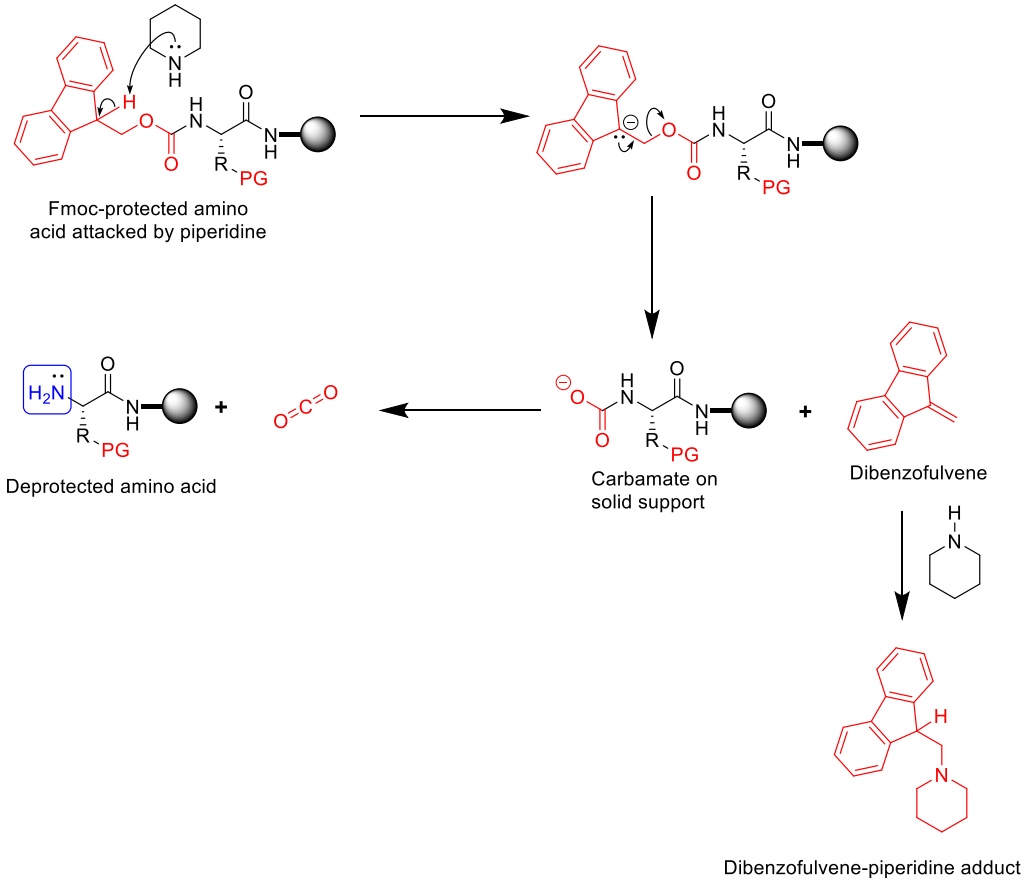

Figure 7. Deprotection mechanism of Fmoc using piperidine

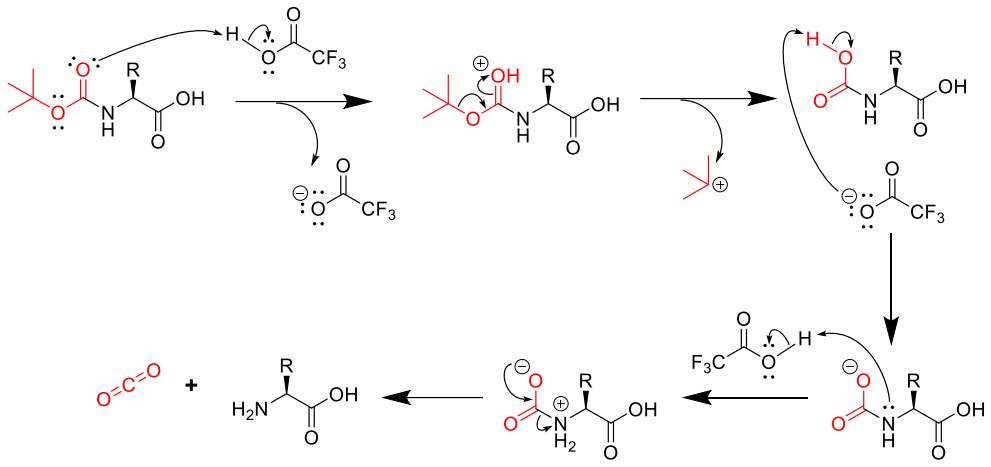

Figure 8. Deprotection mechanism of Boc using TFA

The Coupling Reaction in SPPS

Activation of the α-carboxyl group is essential for easy and rapid formation of peptide bonds in the SPPS technique.[32] The method employed to connect two amino acid residues through amide bond formation is termed the coupling method. A coupling reaction aims to produce an "activated" amino acid, or an amino acid that possesses a good leaving group in place of the terminal hydroxyl group. Examples of activated amino acids are active esters, acyl halides, acyl azides, and mixed or symmetrical anhydrides. This transformation is commonly achieved through the use of coupling reagents and bases. Some of the coupling reagents used are dicyclohexylcarbodiimide (DCC), diisopropylcarbodiimide (DIC), benzotriazol-1-yl-*N*-oxy-tris (pyrrolidinone) phosphonium hexafluorophosphate (PyBOP), and *N*-{(dimethylamino)-1*H*-1,2,3-triazolo[*4,5-b*]-pyridin-1-yl methylene}-*N*-methylmethan-aminium hexafluorophosphate *N*-oxide (HATU). The most common base used in coupling is *N,N*-diisopropylethylamine (DIPEA). Activated intermediates can undergo side reactions such as direct enolization and the formation of oxazolones (Figure 9), which are highly prone to racemization. These reactions lead to reduced yield, defects, and a loss of chiral integrity of the final peptide, reducing its biological

and chemical activities. Urethane protecting groups (including Boc and Fmoc) are known to decrease the formation of oxazolones, hence racemization. In addition, the leaving group is chosen to provide good balance between reactivity with amine nucleophiles and resistance to side reactions. Active esters (esters of alcohols having a pK_a roughly in the range of 3-8) are widely used. In particular, esters of 1-hydroxy-7-azabenzotriazole (HOAt, pK_a 3.38) and 1-hydroxybenzotriazole (HOBt, pK_a 4.60) have gained wide popularity for having struck this balance. Structures of HOAt and HOBt esters are illustrated in Figure 9. The nitrogen at position 7 in HOAt has an electron-withdrawing influence, which enhances the quality of the leaving group and imparts greater reactivity to the activated amino acids. Figure 10 indicates the general reaction mechanism of how the activated amino acid undergoes direct enolization and oxazolone formation leading to loss of configuration. Figure 11 shows the coupling reaction using a particular coupling agent, PyBOP with HOBt, to generate activated amino acids from shelf-stable precursors.[27, 37]

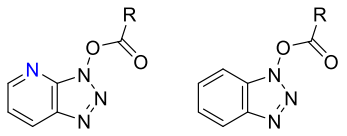

Figure 9. Structures of HOBt and HOAt esters

The Kaiser test is used to confirm deprotection and coupling reactions. This test verifies the presence of primary amines produced in Figure 7. The test reagent contains ninhydrin, which reacts with the free amino group of the peptide or terminal amino acid to produce an intense blue/purple color. A positive test confirms the success of deprotection. A lack of intense blue/purple color marks a negative test, meaning the amino group of the peptide or terminal amino acid is still protected. A subsequent negative test thus confirms that the coupling reaction was successful.[38] The reaction products of the Kaiser test are illustrated in Figure 13.

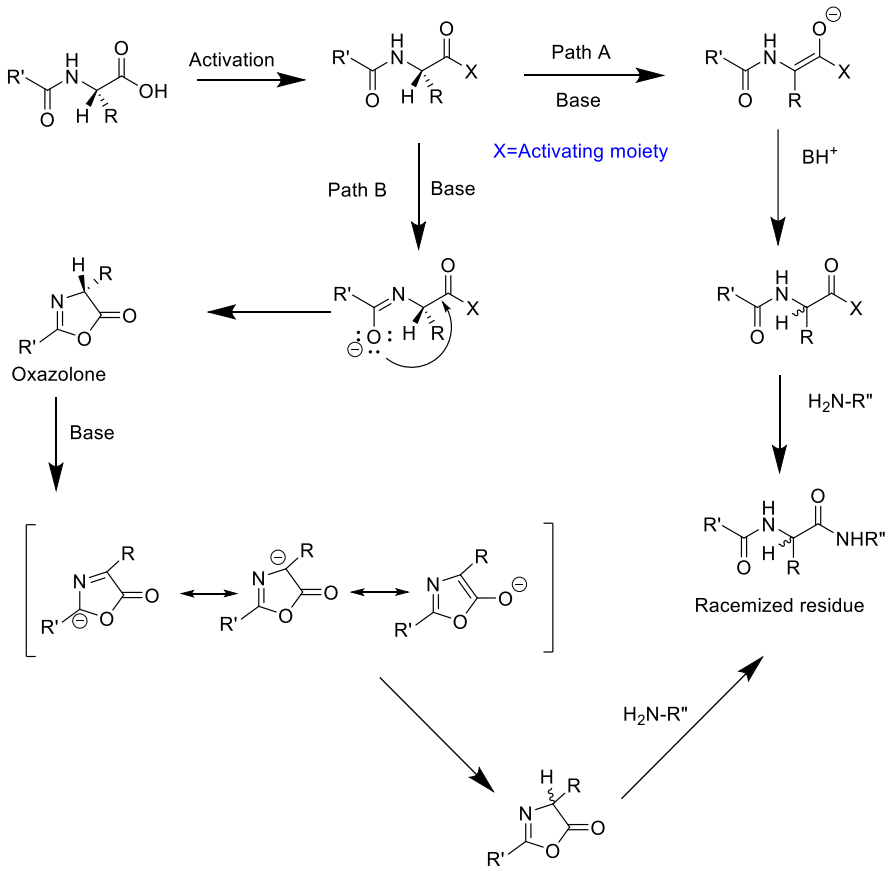

Figure 10. General mechanism of racemization mechanism. Path A proceeds via direct enolization, and path B proceeds via an oxazolone formation[37]

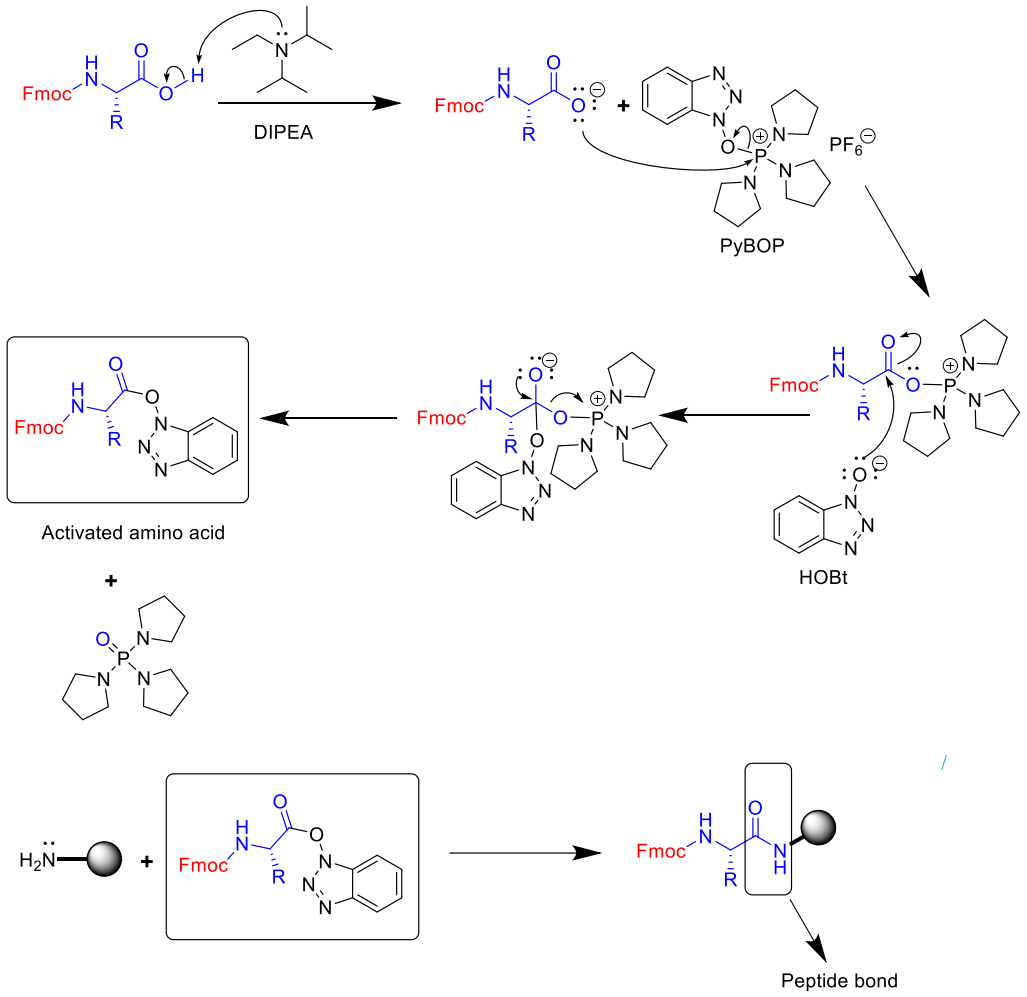

Figure 11. Coupling reaction using PyBOP, HOBt and DIPEA

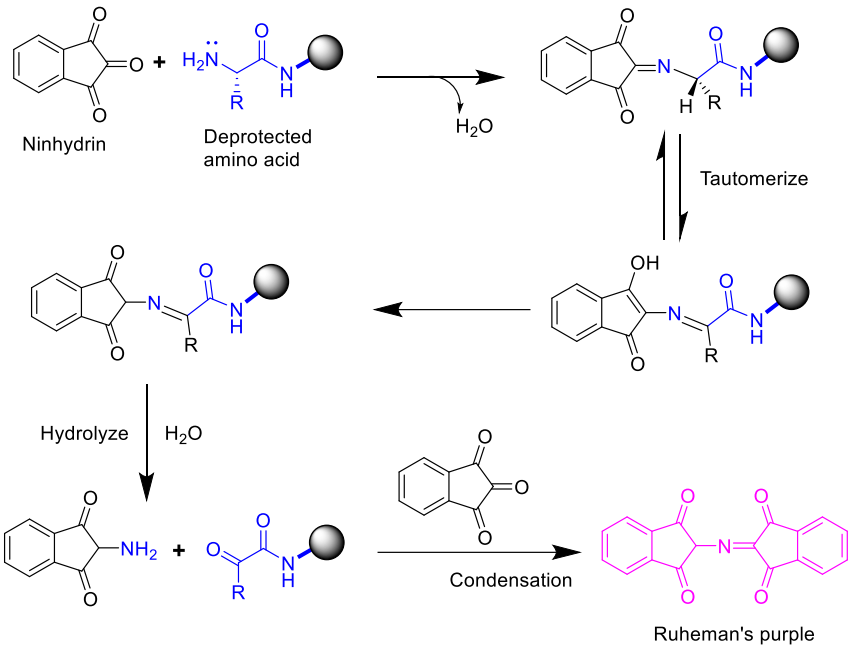

Figure 12. Chemistry of the Kaiser test

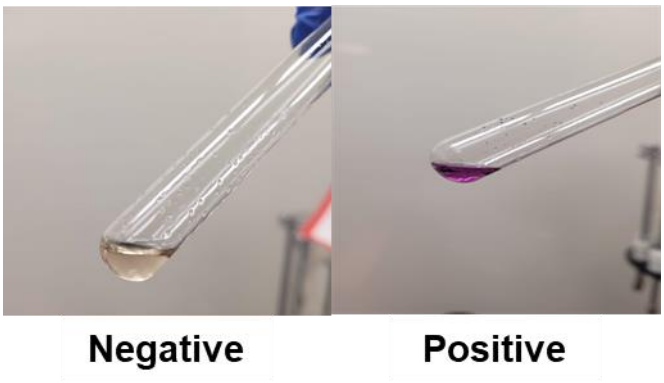

Figure 13. Kaiser test results

Cleavage of the Peptide from its Solid Support

The simultaneous removal of side chain protecting groups from the amino acid residues and removal of the desired peptide product from the solid support marks the completion of the SPPS synthesis procedure. Cleavage conditions depend on the functional groups present on the solid support and linker as well as the protecting groups on the side chain of the amino acids' residues (which are stable to the N_{α}-deprotection reagent). The cleavage step of SPPS can be

carried out either as a batch process, where the resin is filtered between each step, or as a continuous flow process, whereas the resin is solvated during reagent exchange.[39] The most widely used cleaving reagent for Fmoc chemistry is trifluoroacetic acid (TFA). Cleavage conditions for several resins are included in the table in Appendix B. TFA causes the resin and the protecting groups of the amino acid side chains to be released as carbocations. These carbocations can react with the electron-rich side chain of amino acids like cysteine, methionine, threonine, serine, and tryptophan, resulting in the formation of unwanted byproducts that affect the purity of the desired peptide. To reduce the formation of these unwanted byproducts, "scavengers" are added to the cleavage cocktail to remove the carbocations. Silane scavengers like triisopropylsilane are widely used in the SPPS process. Triisopropylsilane acts as a mild reducing agent when used with TFA, donating hydride ions which react with the carbocations of the side chain protecting groups and thereby preventing the protecting groups from reacting again with the peptide. Figure 14 demonstrates simultaneous removal of the peptide from the solid support and removal of the side-chain protecting group from an amino acid.[40,41] The table in Appendix C also provides conditions used to remove side chain deprotecting groups[32].

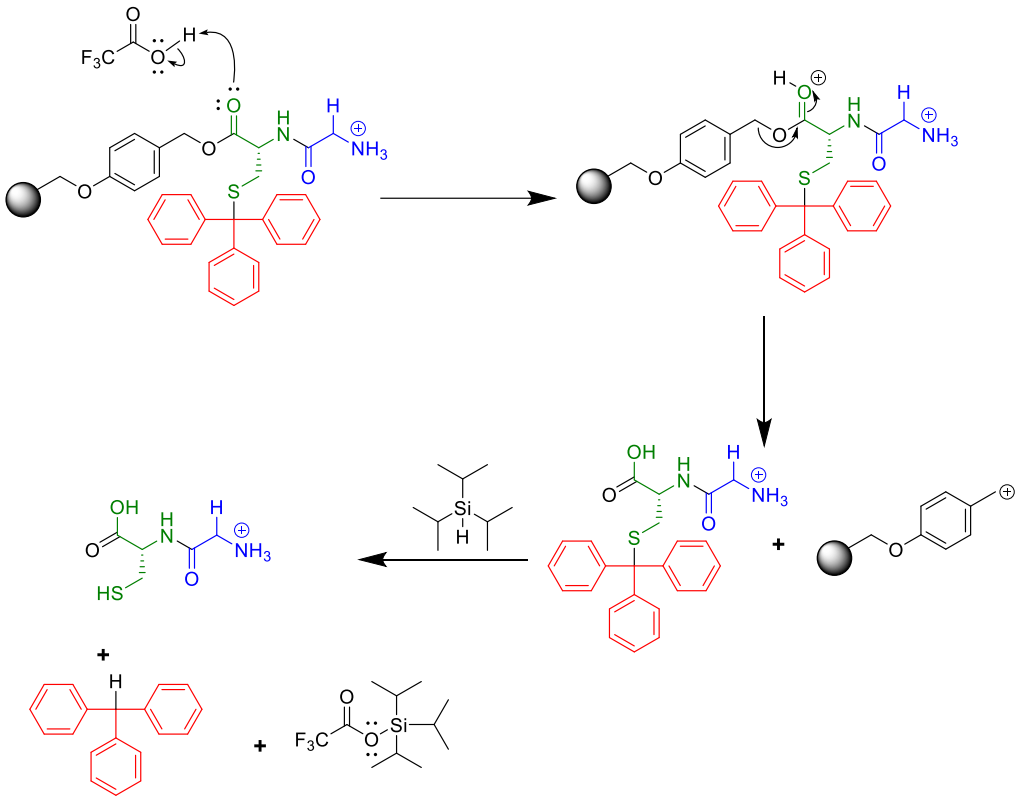

Figure 14. Cleavage of the solid support and side-chain protecting group[40,41]

Purification and Isolation of Synthesized Peptide

The diverse functions of peptides and proteins in agricultural products, drug manufacturing, cosmetics, and food require that peptides and proteins produced by different techniques be highly pure. The aforementioned techniques of section 1.7 utilize a variety of different purification and isolation processes. It has been reported that the purity of peptides depends on the method used in the purification and isolation process. Over the years, the main purification techniques that have been employed in peptide synthesis include reversed-phase high-performance liquid chromatography (RP-HPLC), size exclusion chromatography, hydrophilic interaction chromatography, gel filtration chromatography, crystallization, flash chromatography, and hydrophobic interaction chromatography.[42] Most of these purification techniques have several limitations. For example, some of these methods use large quantities of

solvents, some produce low yields of peptides, and others require long purification times. Peptides are difficult to crystallize and therefore crystallization is not utilized in peptide synthesis. The challenges associated with peptide purification have forced peptide chemists to rely heavily on powerful purification techniques like reversed phase HPLC.[33,42] RP-HPLC separates peptides and proteins based on differences in hydrophobicity. Thus, they allow the separation of peptides and proteins from a wide variety of complex mixtures.[43] The mobile phase of RP-HPLC can be described as an aqueous solution containing organic modifier and, optionally, an ion-pair reagent or buffer, responsible for desorbing and eluting the peptides from the stationary phase on the column. Some of the widely used organic modifiers are acetonitrile, isopropanol, ethanol, and methanol. A gradient of organic modifier concentration creates an increasingly hydrophobic medium that sequentially desorbs the peptides from the hydrophobic surface of the column for easy separation and purification. The stationary phase of RP-HPLC contains a silica base support whose surface has been reacted with chlorosilane reagents to create a hydrophobic medium. In this reaction, chlorine acts as the leaving group to bond silyl groups with attached hydrocarbon chains to oxygen atoms of the silica. The length of the hydrocarbon controls the hydrophobicity of the support. The most widely used hydrocarbon in this reaction is C_{18} (octadecyl), but less hydrophobic chains like C_8 and C_4 are used, particularly with strongly hydrophobic compounds. To ensure effective purification and isolation of peptides using RP-HPLC, factors like flow rate, temperature, pH, and elution gradient are optimized. The chromatogram produced with the RP-HPLC (using a UV detector) provides useful information about the peptide's structure.[44]

Peptide Modification Through Fluorescent Labeling and its Applications

Peptide modifications have become necessary in protein chemistry due to the different functions that peptides and proteins play in living organisms. In signaling, peptides are often encountered in the extracellular environment. Peptides need to enter the cell (signal transmission) or bind to its receptor (signal transduction) on the plasma membrane to elicit their functions. The functions of peptides within the cell can be well understood if they can be detected in the cell. For this reason, peptides are modified through fluorescent labeling to aid in their detection.[45] Fluorescent labeling of peptides involves the incorporation of fluorescent dyes into the peptide sequence during or after the chemical synthesis of the peptide. Fluorescent labeling of peptides can be done using semiconductor nanocrystals, fluorescent proteins, or organic molecules. Fluorescent organic molecules can form covalent bonds with peptides, leading to the formation of the bioconjugates which can fluoresce at varying wavelengths. The absorption and emission wavelengths of fluorescent tags are of critical importance to protein chemists and microscopists.[46] In choosing a particular fluorescent molecule for peptide labeling, it is essential to understand the excitation and emission cycle of the molecule. This cycle occurs when the fluorescent molecule absorbs a photon of light, exciting an electron from the ground state to a higher-energy singlet excited state, including many vibrationally excited states. After a fluorescent molecule has absorbed a photon, it decays to the vibrational ground state of the electronic excited state and re-emits the light as the molecule falls back to various vibrational levels of its electronic ground state. This mechanism leads to a red shift of the emitted light with respect to the excitation light, facilitating detection. Examples of fluorescent molecules used in conjunction with peptide synthesis are fluorescein dyes (e.g., 5-FAM) and rhodamine dyes (e.g., 5(6)-TAMRA). The absorption (excitation) maxima for FAM and TAMRA are ~494 nm and ~552 nm, respectively. Their emission maxima are ~521 nm and ~574 nm, respectively.[47]

However, both absorption and emission spectra are broad, and special filters are used to select the appropriate bands, with lasers sometimes used for excitation. Peptides can be labeled during synthesis or afterward using a variety of suitable reagents. The overall application of labeling peptides is to provide powerful tools for studying biological interactions like receptor-ligand binding, protein structures and enzyme activity.[48]

Delivering of Fluorescently Labeled Peptides into Arabidopsis Plants

Synthesis and functional evaluation of biomolecules in plants is an area of research that many scientists have focused on. Knowing the function of synthesized biomolecules is important to better understand their roles in plants. One way of deducing the function of a synthesized plant biomolecule is by introducing the molecule into the plants and studying what happens inside of the plant.[49] *Arabidopsis thaliana* is a model plant that is ideal for such experiments. *Arabidopsis thaliana* is a plant belonging to the mustard family and is naturally distributed throughout North America, Asia, and Europe. Different ecotypes have been sampled from the natural population for experimental analysis. Columbia 0 and Landsberg are the standard ecotypes used in genetic and molecular studies. In these experiments, Columbia 0 and Landsberg served as the "wild-types" of the species. The features that make *Arabidopsis thaliana* a model plant are its short life cycle of approximately six weeks (thus, it grows and reproduces quickly), compactness and genetic tractability. Starting from seed germination, the life cycle of *Arabidopsis* is characterized by the formation of a rosette plant, the bolting of the main stem, flowering, and the maturation of the first seeds. *Arabidopsis* has five chromosomes, and its 120-megabase genome has been sequenced, with the identification of approximately 20,000 genes. The genetic features mentioned above facilitates easy manipulation of the genome through approaches such as chemical and insertional mutagenesis, efficient methods for performing crosses, and techniques

for introducing DNA through plant transformation. Extensive collections of mutants with diverse phenotypes have been constructed. It was noted by the *Arabidopsis* Research Team in 1987 that the advances of *Arabidopsis* research could be utilized in other plants to solve problems in agriculture, industry, and human health. These qualities of *Arabidopsis* promote the interest of plant biologists to study functional evaluation of biomolecules using different delivery methods.[50]

Several techniques for introducing probes into plants have been characterized including genetic introduction, physical introduction, and invasive procedures such as abrasion with carborundum. The genetic method has been the most frequently used method to deliver probes like heterologous proteins into plants to induce or suppress the overexpression of genes. It is reported that the introduction of biomolecules into certain plants is simpler than others. In *Arabidopsis* plants, the genetic method can be used to deliver genetic materials with less effort than in *Populus* plants. With the use of biolistic bombardment or the *Agrobacterium tumefaciens*-mediated gene delivery method, genetic material can nonetheless be introduced into *Populus*.[51] The physical methods of introducing biomolecules into plants are foliar application, root irrigation, and pressure infiltration. These methods provide flexibility in the site and rate of delivery for biomolecules. With the use of the genetic method, expressible tags like green fluorescent protein (GFP) can be introduced into the plant genome. Despite the advantages of these methods, some limitations exist. Physical methods are limited to natural permeability barriers around and within the plants. These limitations do not allow for the successful introduction of all biomolecules into all parts plants. Therefore, there is the need to research different methods of transferring labeled and unlabeled biomolecules like TAMRA-PSK and PSK molecules into plants.[52]

According to research conducted by Standaert and colleagues, an array of vertically aligned carbon nanofibers (VACNFs) can be utilized to permeabilize the surface of a leaf and introduce sub-microliter quantities of radiolabeled and fluorescent molecules into leaf cells. The delivery of molecules using the VACNF method ensures that plant samples remain intact without causing a detectable wound response like the production of reactive oxygen species, which can cause oxidative stress to the plant. This research focuses on using VACNFs and foliar application by immersion to introduce PSK analogs into the plant and to study their bioactivity.[52]

Imaging Fluorescently Labeled Peptides in Plants for Their Biological Activity Using a Fiber-Optic Fluorescence Microscope

The ability to detect changes in intact cells or tissues of plants or animals is a gateway to understanding internal processes in living organisms. Over the years, scientists from clinical and botanical fields have investigated ways to study *in-vivo* processes and to use their findings to improve the performance of both plants and animals. Researchers have used several experimental models for the exploration of organs in living organisms. In animals, one of the experimental models used to better understand the internal system is a surgical procedure that permits the placement of studied organs under the objective lens of a microscope. This surgical model has been widely used in the clinical field and it now represents an important qualitative step for intravital microscopy.[53] Other intravital techniques that have been used for imaging *in situ* include confocal microscopy, multiphoton microscopy, and second harmonic microscopy. These intravital techniques require complex and expensive components like scanning laser systems and high-powered light sources which make them difficult to justify in particular research laboratory settings.[54] The complex nature of these techniques has pushed scientists to design protocols to enable the easier development of suitable microscopy techniques. One protocol that has been published by Pierce et al. is the assembly of a high-resolution micro-

endoscope. A simple version of this device can be completed by an experienced practitioner in a day and can offer flexibility in terms of field-of-view, image resolution, and operating wavelength. Modification of the protocol for designing a high-resolution micro-endoscope has been used to develop a fiber-optic fluorescence microscope.[54,55]

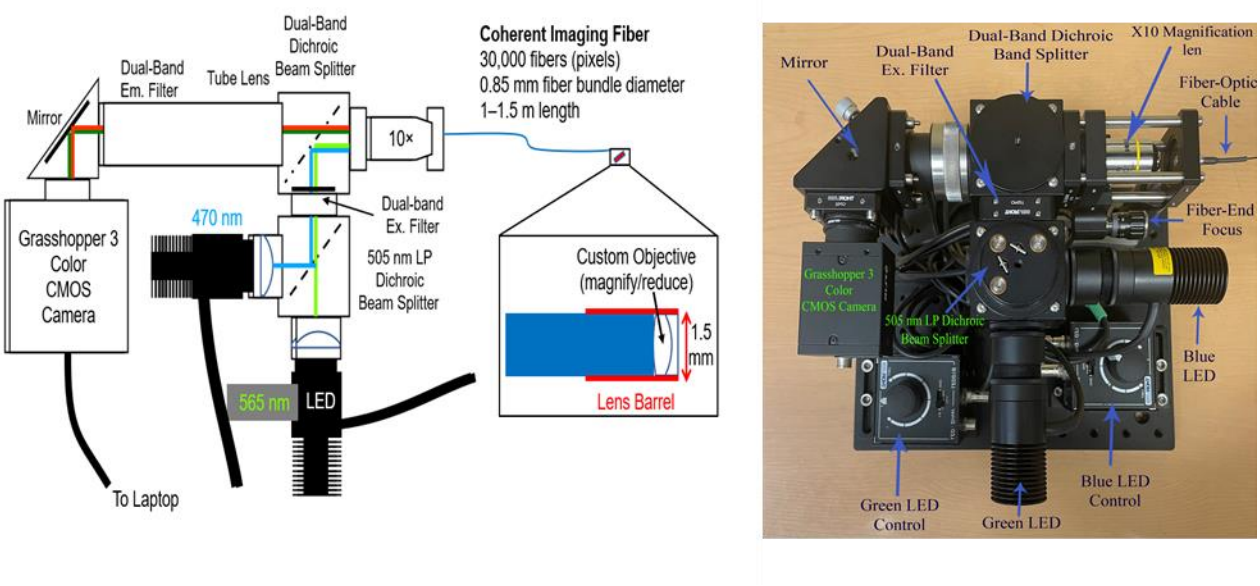

Figure 15. Optical components of the fiber-optic fluorescence microscope. A schematic diagram (left) and photo (right) are shown. Images copyright Robert F. Standaert, used with permission.

The basic fiber-optic fluorescence microscope depicted in Figure 15 was constructed by Dr. Robert F. Standaert using an adaptation of the literature designs.[54,56] Images were obtained using this microscope at 2.3× native magnification using a GRIN endoscopic objective lens (Grintech GT-MO-080-032-ACR-VISNIR-08-00, 0.75 NA, 80-µm working distance in water) cemented to a 1-m coherent imaging fiber (Fujikura FIGH-30-850N, 30,000 fibers); use of the magnifying objective provides a field of view (FOV) 340 µm in diameter. Fluorescence excitation was provided by independent LED light sources centered at ~470 nm (blue) and ~565 nm (green) for excitation of fluorescein/GFP and tetramethyl rhodamine chromophores, respectively. A dual-band filter/beam splitter set (Chroma Technologies, set 59004) and a color camera (FLIR Technologies Grasshopper 3.0 equipped with a Sony IMX252 sensor) were used

to allow independent or simultaneous observation of both channels without changing filters. Except as indicated above, optical components were obtained from Thorlabs. Modifications to the sample stage were performed in collaboration with Mr. Edward Offei and Mr. Christopher Erb. The complete set up, which consists of the sample stage, the microscope and the laptop is illustrated in Figure 16.

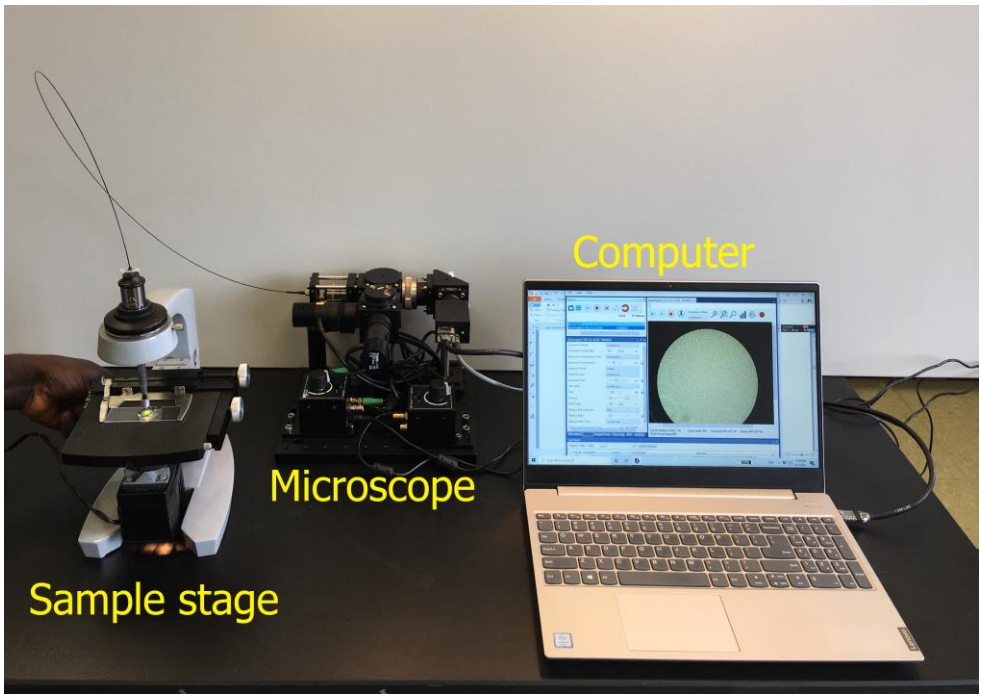

Figure 16. Complete fiber optic fluorescence microscope Addition of a sample stage and laptop computer for image acquisition produces a functional microscope for plant imaging

Aims of the Research

The objectives of the present study were to:

1. Synthesize and label a phytosulfokine analog using solid-phase peptide synthesis with purification by reversed-phase high-performance chromatography.
2. Introduce the synthesized TAMRA-labeled PSK analog into the wild-type *Arabidopsis thaliana* Columbia-0 and in the transgenic *Arabidopsis* expressing PSKR-GFP using the immersion method and the vertically aligned carbon nanofiber (VACNFs).
3. Study the localization and molecular trafficking of the PSK analogs using fiber optic-fluorescence microscopy.

CHAPTER 2. EXPERIMENTAL METHODS

Chemicals and Equipment

Standard peptide synthesis resins, PyBOP, and protected L-amino acids were purchased from CREOSALUS Advanced Chemtech: Fmoc-Lys-Wang resin, Fmoc-Gln(Trt)-wang resin, Fmoc-Ile-OH, and Fmoc-Thr(But)-OH; Fmoc-Phe(4-CH_2SO_3TCE)-OH was purchased from BACHEM, and Fmoc-L-Phe(4-CH_2CO_2t-Bu)-OH from eNovation Chemicals LLC. Other auxiliary coupling chemicals were sourced as follows: HOBt hydrate from Oakwood Chemical; DIPEA (99.5+% pure), DMF (99.8% pure) and 2-methylpiperidine from Acros Organics; TFA (98%) from Fisher Chemical; triisopropylsilane from TCI America; and TAMRA from Molecular Probes (Thermo Scientific). Other reagents and solvents were of reagent grade and were used as received.

Phytosulfokine Analog Synthesis and TAMRA Labeling Procedure

The PSK analog was synthesized using Fmoc-Gln(Trt)-Wang resin as solid support and procedures based on those reported by Taylor and coworkers.[57] A 38 mg (27 μmol) portion of resin at 0.70 mmol/g was preswollen in DMF for 1 h before the various amino acid residues were coupled to the solid support. The Fmoc protecting groups on the support were removed using 20% 2-methylpiperidine in DMF on a mini-rotator (3 × 10 minutes). The success of Fmoc deprotection was confirmed by placing a small sample of resin into a test tube and performing the Kaiser test as follows. Three drops each of Reagent A (0.40 mL of 0.66 mg/mL potassium cyanide in water plus 19.6 mL of pyridine), Reagent B (5% ninhydrin in 1-butanol) and Reagent C (20 g of phenol in 10 mL of 1-butanol) were added into the tube, it was heated with a heat gun for approximately 1 minute (as long as it took a positive control to stain deep blue). The coupling

of the next amino acid residue, which was Fmoc-Thr(But)-OH, was accomplished by taking 20 mg (50 μmol, 2 equiv) of Fmoc-Thr (But)-OH into a vial and then dissolving it in 200 μL of DMF, followed by addition of 100 μL (100 μmol, 2 equiv.) of 1 M HOBt in DMF, 100 μL (100 μmol) of 1 M PyBOP in DMF and 34 μL (200 μmol) of DIPEA. The resulting solution was added to the swollen and deprotected solid support (H-Gln(Trt)-Wang resin) and was then agitated on a mini tube rotator for 180 min. The completion of the coupling reaction was confirmed by a negative Kaiser test. Subsequent amino acid residues of PSK, namely Fmoc-Phe(4-CH_2SO_3TCE)-OH, Fmoc-Ile-OH and Fmoc-Phe(4-CH_2SO_3TCE)-OH were coupled using the same procedure. The masses used for Fmoc-Phe(4-SO_3TCE)-OH and Fmoc-Ile-OH were 31 mg and 18 mg, respectively, corresponding to 50 μmol in each case. The same procedure was used to synthesize the protected PSK analog with Fmoc-Lys(Mtt)-Wang resin.

The synthesized PSK analogs were cleaved from the resins with 2 mL of TFA:triisopropylsilane:water (95:2.5:2.5, with their respective volume being 1900 μL, 50 μL and 50 μL) for 2.5 hours, after which the mixture was filtered, and the Wang resin was washed with the cleavage cocktail. The filtrate was placed in a 13 × 100 mm test tube and concentrated on a nitrogen evaporator to half its original volume. Cold ethyl ether was added to the remaining concentrate to precipitate the PSK analog. The mixture was centrifuged, and the supernatant was discarded, whereas the pellet was washed by resuspension in ethyl ether and centrifugation. The synthesized PSK analogs were then ready for HPLC analysis.

To begin biological studies in plants while the analogs were in preparation, PSK and TAMRA-PSK solutions whose biological activity had been confirmed were provided by research collaborators Dr. Jean Greenberg and Ms. Jessica Morgan at the University of Chicago.

Procedure for Growing Arabidopsis Plants

Arabidopsis thaliana Columbia-0 (Col-0, wt) and PSKR-GFP were grown by transferring about 10−12 seeds of each strain into a 50 ml conical flask using a wax paper. Agar solution (0.1%, 5 mL) was added, and the tube was wrapped with aluminum foil and then placed in a dark area at 4 °C for 4 days. On the 4th day, 8 seeds from each conical flask were transferred using a Pasteur pipette into a pot containing moderately watered soil with 1 cm distance between seeds. Pots were then placed in a growth chamber with growth conditions as follows: temperature of 23–24 °C, light cycle of 16 hours light and 8 hours dark, illumination at 100–150 μmol $m^{-2}s^{-1}$, and relative humidity of 60–70%. The plants were consistently watered 1–2 times a day. The leaves from each plant type were then used after about 4 weeks of planting for peptide delivery and microscopy analysis.

Method of Delivering PSK and TAMRA-PSK

Solutions of PSK and TAMRA-PSK at 100 nM were introduced into both Col-0 and PSKR-GFP plants. Concentrations were determined by measuring UV-Vis spectra at the lowest practical concentration before diluting to a working concentration. Extinction coefficients were taken as ε_{205} = 31 Lg^{-1} for peptide bonds, ε_{260} = 283 $M^{-1}cm^{-1}$ per sulfotyrosine and ε_{280} = 1490 $M^{-1}cm^{-1}$ per tyrosine.[58] The stock concentrations of PSK, and TAMRA-PSK solutions were 3 mM, and 2 mM, respectively. A 6.6 μL aliquot of each of the stock solutions was diluted with 1000 μL of deionized water to produce diluted stocks of ~10–15 μM, and the UV spectrum recorded over the wavelength range of 190–300 nm. After spectrophotometric determination of concentration, the diluted stocks were further diluted to working concentrations between 100 nM and 10 μL immediately prior to use.

Leaves from Col-0 and PSKR-GFP plants were detached and placed in water to prevent desiccation. The leaves were patted dry on a paper towel and placed on the stage of the microscope. An aliquot of 100 μL of PSK solution was applied on abaxial side of the leaf, and micrographs were obtained after periods of 1, 5, 10, 30 and 60 min. Water was applied to the leaves in the same way as a control.

Fiber Optic Fluorescence Microscopy Study Procedure

Fluorescence images of leaves placed in peptide solutions and water were obtained using the fiber optic fluorescence microscope developed by Standaert and coworkers and depicted in Figure 15. Before treatment with peptides, leaves were imaged to verify the presence of GFP signals in the PSKR-GFP plants using blue light (470 nm) excitation. After treatment, the leaves were removed from peptide solutions, patted dry, washed with water, and patted dry again. The leaves were placed on a glass slide and secured using the stage clip, and a small metallic washer was placed on the leaf to create a well with a relatively flat field of view. The power of the LED source was adjusted to obtain sufficient signal intensity and contrast. In each experiment, all the images were obtained with the same settings of the fiber optic fluorescence microscope to allow comparison between leaf samples subjected to various conditions.

Complete Synthesis of Phytosulfokine Analogs Using SPPS

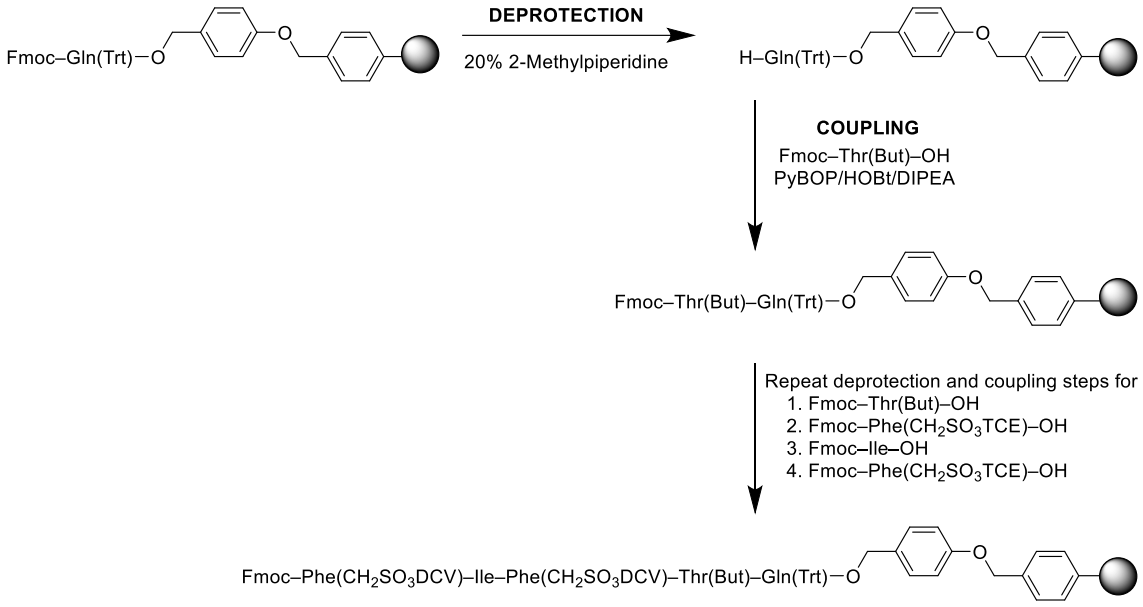

Figure 17. SPPS synthetic route for the PSK analog. Coupling was performed using PyBOP/HOBt/DIPEA and suitably protected monomers. Fmoc deprotection was effected using 20% 2-methylpiperidine.

The syntheses of PSK analogs Phe(4-CH_2SO_3H)-Ile-Phe(4-CH_2SO_3H)-Thr-Gln and Phe(CH_2SO_3H)-Ile-Phe(CH_2SO_3H)-Thr-Lys, protected on the sulfonic acid side chains, were accomplished by the solid phase peptide technique with Fmoc chemistry. Verification of product structure by MS and purification by HPLC are pending. Figure 17 shows the synthetic route of PSK analog Phe(CH_2SO_3H)-Ile-Phe(CH_2SO_3H)-Thr-Gln. A similar synthetic route was used for the production of Phe (CH_2SO_3H)-Ile-Phe(CH_2SO_3H)-Thr-Lys, except that the resin used was Fmoc-Lys(Mtt)-Wang resin. The choice of the two different resins was very important in this synthesis since the resin determined the success of PSK analog synthesis. Wang resin features an acid-labile *p*-alkoxybenzyl linkage that generates peptide acids. A related resin, Rink resin, produces peptide amides. Fmoc-Gln(Trt)-Wang resin is the standard choice for the synthesis of a peptide bearing a C-terminal Gln residue. Fmoc-Lys(Mtt)-Wang resin was selected for the

synthesis of peptides to be fluorescently tagged with TAMRA. The Mtt group is extremely acid-labile and can be cleaved with dilute (1%) TFA in DCM, which does not cleave the Wang linker or other side-chain protecting groups such as Boc and *t*-Bu.

Deprotection of the Fmoc-protected swollen resin in step 1 of Figure 17 using 20% 2-metlypiperidine was necessary to provide the free amino group required to grow the peptide chain. Research studies by Gregg et al.[39] illustrated that deprotection of the Fmoc group is commonly done using weak bases. They investigated the stability of the Fmoc group to a variety of bases, and they also demonstrated three ways of measuring Fmoc removal – UV absorbance measurement of fulvene-piperidine adduct at 273 nm, TLC analysis of Fmoc-Ala-O-*t*-Bu, and amino acid analysis of Fmoc-Val. Based on their studies, the Fmoc group is rapidly removed by primary amines (such as cyclohexylamine, and ethanolamine) and some secondary amines like piperidine and piperazine. However, tertiary amines like TEA and DIPEA remove Fmoc group at a much slower rate. Solvent polarity also contributes to Fmoc group removal. Polar solvents such as DMF were noted to result in more rapid Fmoc cleavage than occurred in the less polar DCM solvent. These reasons explain why we chose piperidine derivatives in DMF as the deprotection reagent in the PSK analog synthesis. For PSK analog syntheses employing Fmoc-Phe(CH_2SO_3TCE)-OH, 20% 2-Methylpiperidine (2-MP) was used as deprotection reagent. The reason is that the reactivity of the trichloroethyl (TCE) sulfonates to base and nucleophiles is such that they are unstable to piperidine, which leads to decomposition of the Phe(CH_2SO_3TCE) moiety. Taylor and coworkers[57] used ^{1}H NMR to study the reaction between TCE sulfonate esters and piperidine in DMF, revealing that the TCE sulfonate ester undergoes rapid elimination to a 2,2-dichlororovinyl (DCV) ester, followed by additional reactions which led to complete decomposition of the TCE sulfonate ester within 24 hours (Figure 18). They again performed ^{1}H

NMR studies on the reaction between the TCE sulfonate ester and the more hindered base, 2-methylpiperidine (2-MB). They observed the same rapid elimination to the DCV ester, but only that 8% further decomposition with 2-MP after 8 hours. Thus, 2-MP must be used in place of piperidine when using TCE sulfonates. Removal of the DCV ether requires an extra step, hydrogenolysis with a palladium catalyst and ammonium formate/H_2 (Figure 17).

As illustrated in Figure 7, the mechanism of removing Fmoc groups from the amino acid residues used in PSK analog synthesis follows an E1Cb mechanism. The fluorene ring system of Fmoc renders the hydrogen on the 9-carbon very acidic, such that it can be abstracted by a weak, organic base like piperidine or 2-MP. The abstraction of the proton results in the formation of dibenzofulvene and carbamate through a carbanion intermediate. The carbamate undergoes decarboxylation to produce free amino acid and carbon dioxide, whereas the dibenzofulvene can further react to form an adduct with piperidine that can be determined quantitatively using UV spectrophotometer at a wavelength of 273 nm.

Coupling of one amino acid residue to the other amino acid residue was achieved using PyBOP/DIPEA/HOBt in DMF. As discussed, this coupling reagent mixture facilitated the conversion of the poor leaving group (HO^-) to a good leaving group (BtO^-). DIPEA serves as a base responsible for abstracting proton from the protected amino acid to yield a nucleophilic carboxylate that reacts with PyBOP to produce a phosphonium ester intermediate. The phosphonium ester is highly reactive and prone to racemization, but reaction with HOBt results in a racemization-resistant activated amino acid.

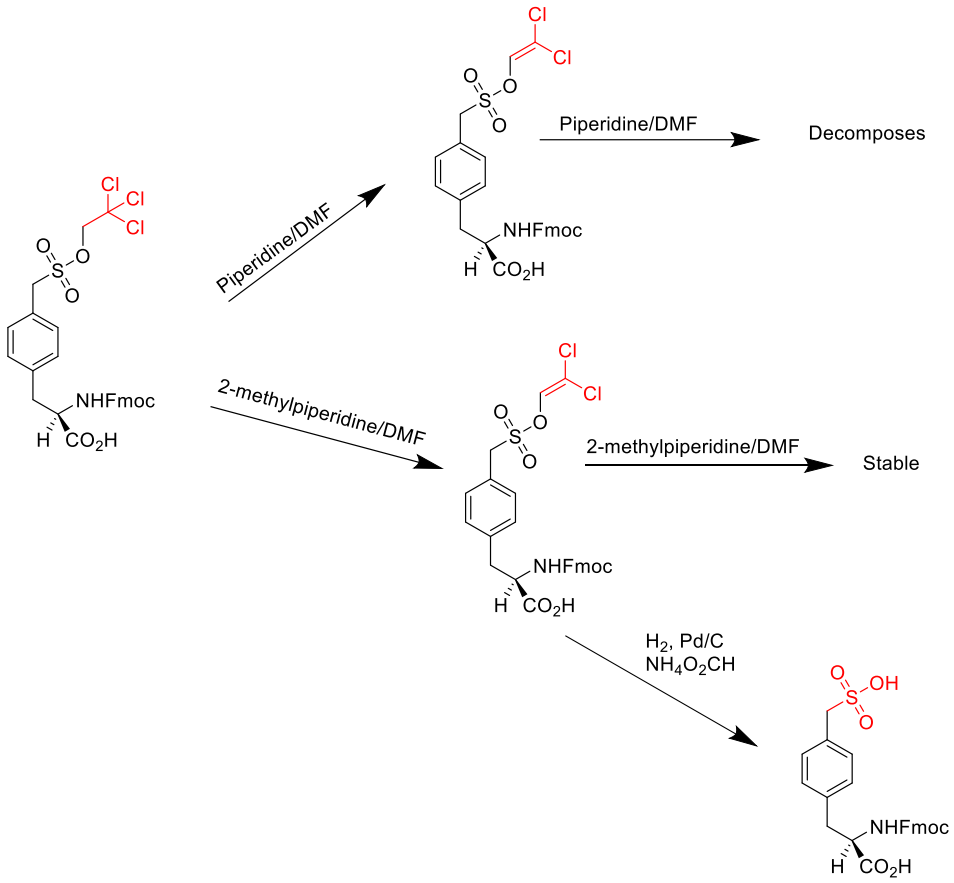

Figure 18. Reaction of trichloroethyl sulfonate esters with piperidine and 2-methylpiperidine

Final cleavage, deprotection and TAMRA fluorescent labeling of the suflomethyl PSK analog were to be conducted by performing reactions indicated in Figure 19. In the first step, the methyltrityl (Mtt) protecting group is removed by dilute TFA, allowing introduction of the TAMRA group using TAMRA-OSu. Subsequently, 2-methylpiperidine/DMF will be used to remove the N-terminal Fmoc group, and 95% TFA/2.5% TIS/2.5% H_2O followed by H_2 and Pd/C in ammonium formate buffer will be used to cleave the peptide from the resin and remove the side-chain protecting groups

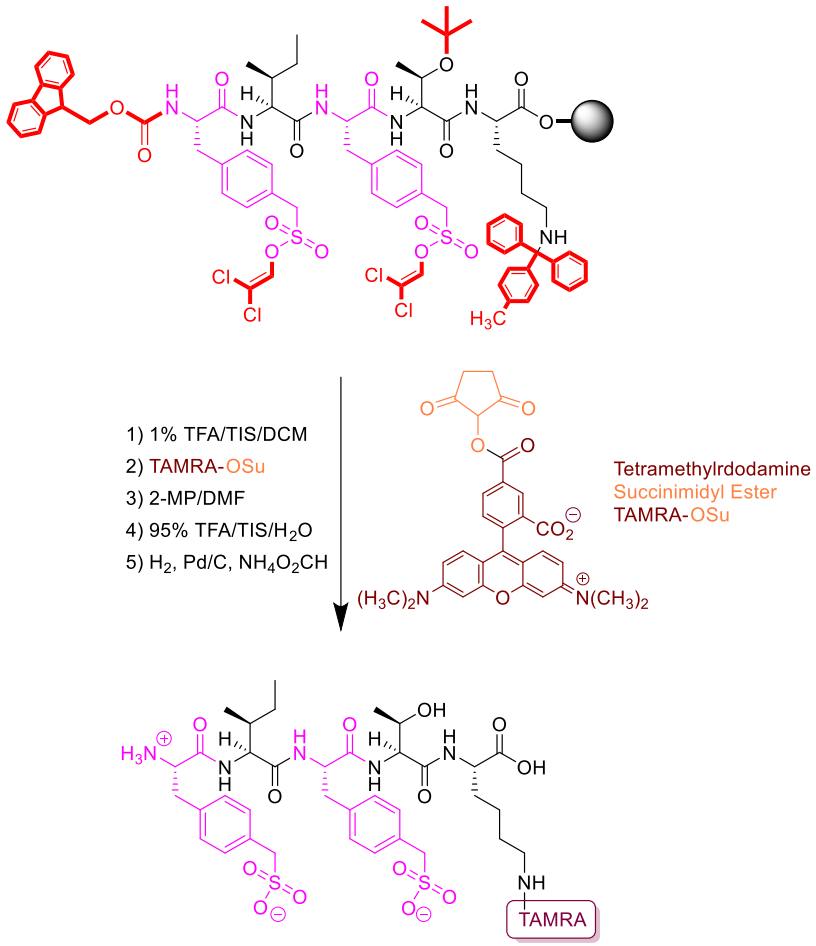

Figure 19. Deprotection, TAMRA-labeling and resin cleavage of the PSK analog

Significance of Focusing on PSK Analogs Studies

The interest in synthesizing the PSK analogs as illustrated in Figure 3 of section 1.5 was based on the findings reported by Matsubayashi et al. They reported two kinds of PSK, namely PSK-α and PSK-β. The latter is missing the C-terminal Gln residue, but both of them possess tyrosine residues with the phenolic hydroxyl esterified with sulfate groups by tyrosyl protein transferase (TPST). These two PSK molecules are the only secreted peptides in plants that contain sulfated-tyrosine residues, and assessment of their biological activity showed that the sulfate groups are needed. Since the sulfotyrosine residues are likely to be susceptible to enzymatic hydrolysis *in vivo*, deactivating the peptide, we chose 4-sulfomethyl phenylalanine as

an isostere that could substitute for the sulfated-tyrosine in the native PSK to make an active but biologically and chemically more stable analog. In the analog, a methylene group (CH_2) replaces the tyrosyl oxygen, and the sulfonic acid has a comparable acidity to that of the sulfuric monoester.

Trafficking of PSK Molecules

To study the signal transduction and trafficking of PSK molecules in *Arabidopsis*, unlabeled PSK and TAMRA-labeled Gln5Lys PSK were delivered to PSKR-GFP and Col-0 plants. TAMRA-labeled PSK and PSKR-GFP provided the basis to observe ligand and receptor, respectively, using fluorescence microscopy. Jelenska et al.[49] had performed similar studies with the signaling peptide flg22 to form TAMRA-flg22. In order to visualize the location of the TAMRA-(Gln5Lys)-PSK in leaves, the fluorophore was excited with green light (565 nm) through the fiber optic of the microscope. In Figure 20, an assessment of the PSK receptor in both Col-0 and transgenic PSKR-GFP plant is shown. The Col-0 plant showed only diffuse background fluorescence, but the transgenic PSKR-GFP plants showed strong green fluorescence at the periphery of the leaf epidermal cells and at stomata. This outcome was consistent with membrane localization of PSKR-GFP and with prior studies in root hairs by Rodiuc et al.[59] who saw peripheral fluorescence (Figure 21A). The results do not mean that the Col-0 lacks the PSK receptors, only that they are not visible under the microscope due to the absence of the GFP tag. Brightfield images were captured as well, showing the strong correlation between cell outlines and the GFP fluorescence. Subcellular compartments and other structures like stomata in the leaf tissue are also visible.

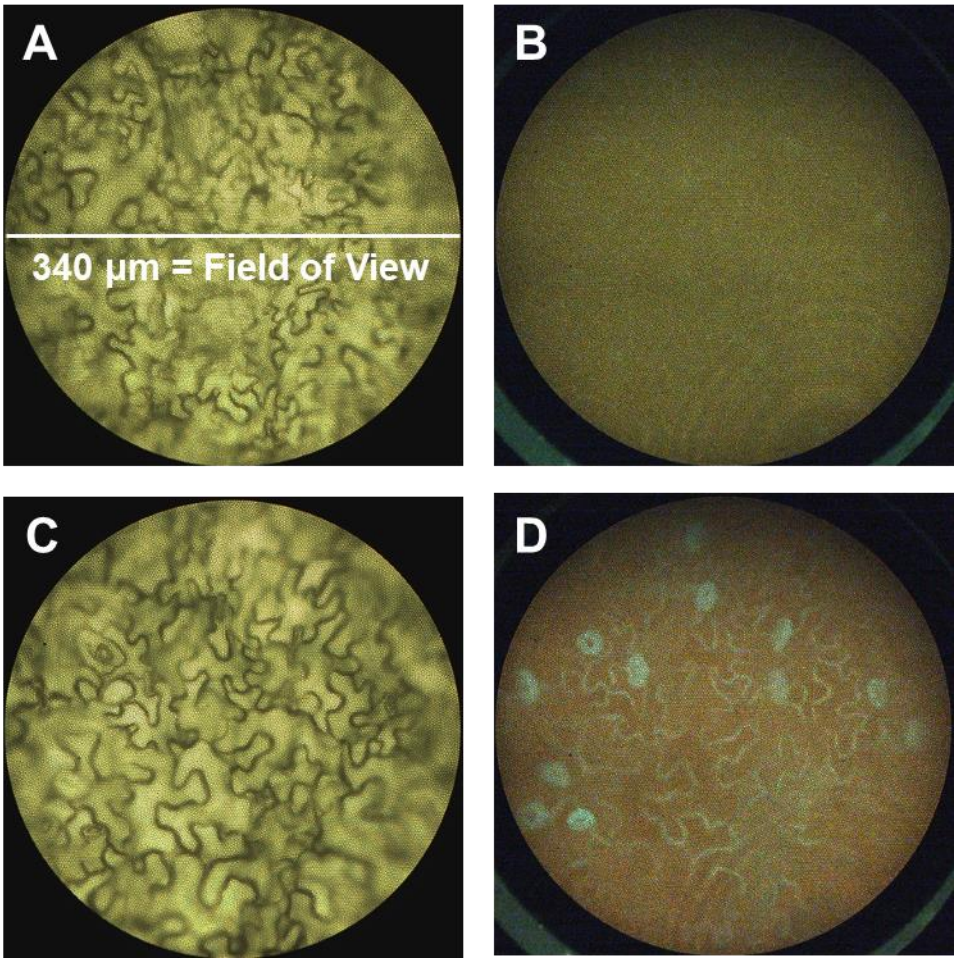

Figure 20. Micrographs showing the cellular structure and green fluorescence in Col-0 and PSKR-GFP leaves. **A** and **C** are brightfield images of Col-0 and PSKR-GFP strains, respectively, revealing similar cellular morphologies. **B** and **D** are epifluorescence images of the same strains, showing PSKR-GFP to be absent in wild-type (**B**) and present in the PSKR-GFP strain, with apparent localization of PSKR in the plasma membrane and stomata. The field of view (FOV) for all the images is 340 µm, as shown in A, and epifluorescence images were obtained with blue light excitation.

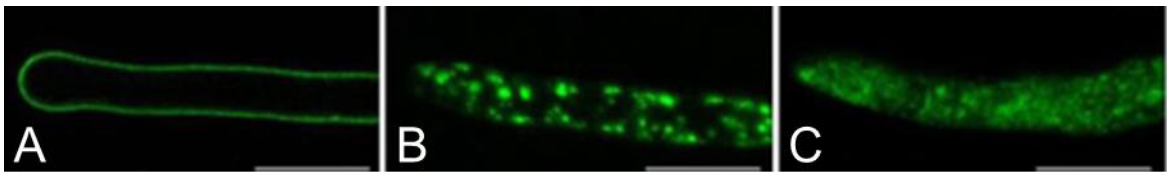

Figure 21. Confocal micrographs of a root hair from *Arabidopsis thaliana* expressing GFP-tagged PSKR1 in the plasma membrane. PSKR1 localizes to the plasma membrane **(A)**. Addition of PSK-α causes re-localization of the receptor into a punctate pattern **(B)** followed by internalization **(C)**. Image from Rodiuc et al.[59] Copyright John Wiley and Sons, used with permission.

On the basis of additional observations by Rodiuc and coworkers, we expected that the receptor should internalize upon incubating the transgenic PSKR-GFP leaf in appropriate concentration of PSK solution for an appropriate time duration (Figure 20B,C).[59] Thus, we applied 100 nM solutions of PSK and TAMRA-PSK to leaves from Col-0 and transgenic PSKR-GFP plants and observed fluorescence over the period of 60 min, recording images at 1, 5, 10, 30 and 60 min. The 60-min an endpoint was chosen based on the expectation that an internalization of the PSK molecule might occur within that period, and that further incubation may not be helpful.

Figures 22 and 23 show the images from water controls with wt Col-0 *and* transgenic PSKR-GFP plants, respectively, while Figures 24 and 25 show the results for the corresponding plants after treatment with 100 nM PSK. As expected, the Col-0 plants showed only indistinct, low-signal features, whereas the transgenic PSKR-GFP plants showed strong fluorescence at the periphery of epidermal cells, as in the untreated leaves of Figure 20. Importantly, no clear change resulted from peptide treatment. This observation suggests either the concentration of PSK solution delivered into the leaf was too small to affect the ligand-receptor binding, or the time required to ensure complete binding and internalization of the receptor was not sufficient. Given that no obvious changes were observed from PSK treatment, and based on the above interpretation, the experiment was repeated with a higher concentration (10 μM) of PSK in the PSKR-GFP plant. We again observed no significant change in receptor localization in leaves upon PSK treatment (Figure 26), in contrast to the results of Rodiuc et al. from experiments performed in root hairs. The reason may be due to the fact that the roots would be more easily take up peptides, and would have rapidly proliferating cells. This tissue difference will need further investigation.

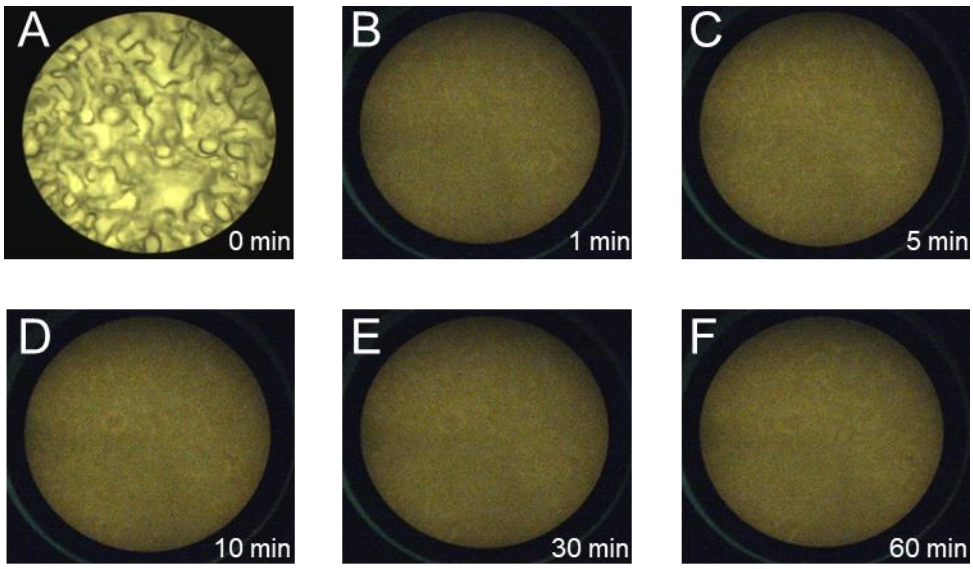

Figure 22. Micrographs of a Col-0 leaf incubated in water (control) at different times under **(A)** brightfield and **(B–F)** epifluorescence conditions. All images have the same field of view (FOV) of 340 µm as in Figure 19, and epifluorescence images were obtained with blue light excitation.

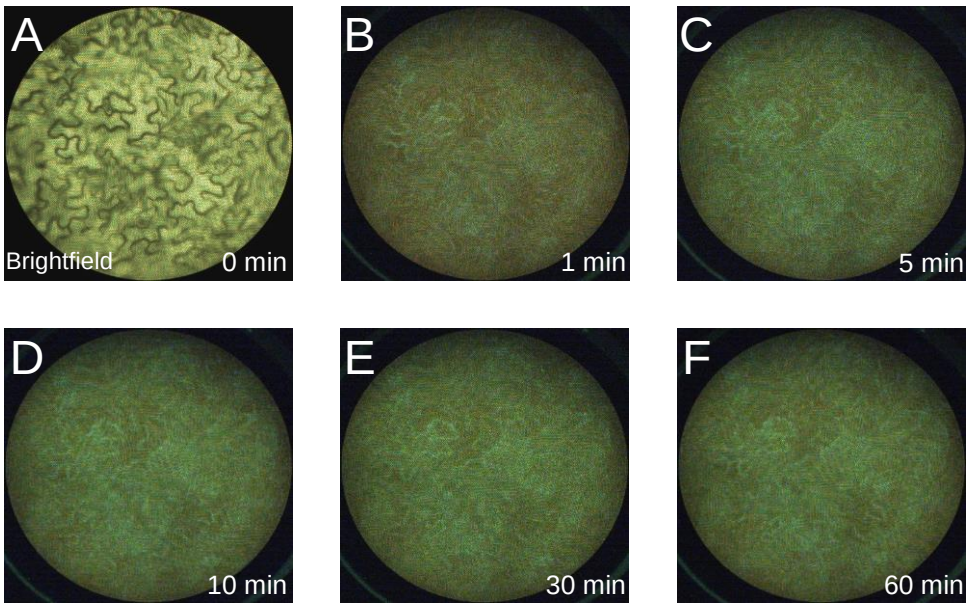

Figure 23. Micrographs of a PSKR-GFP leaf incubated in water (control) at different times under (**A**) brightfield and (**B–F**) epifluorescence conditions. All images have the same field of view (FOV) of 340 µm as in Figure 19, and epifluorescence images were obtained with blue light excitation.

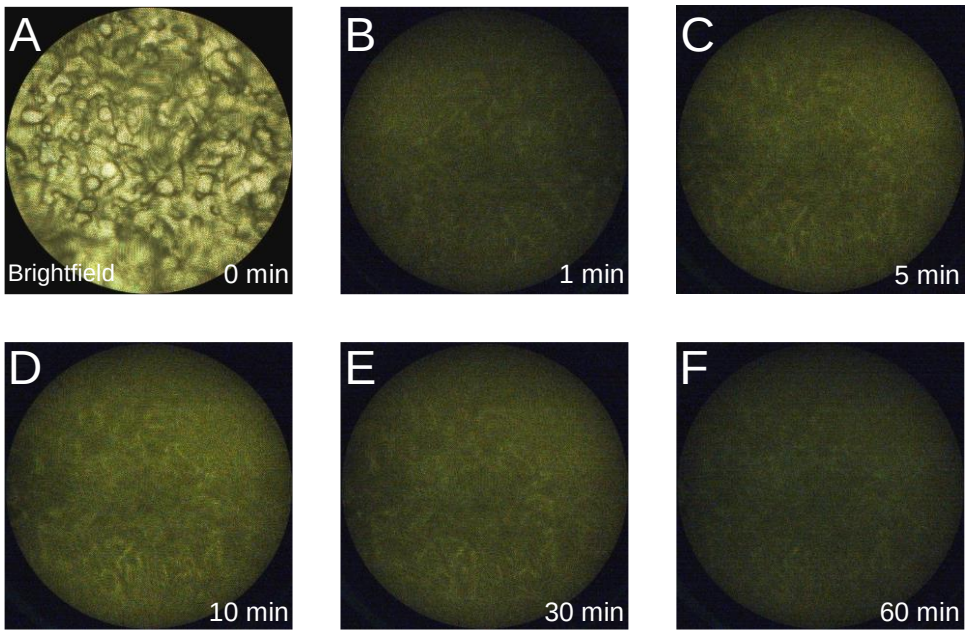

Figure 24. Micrographs of a Col-0 leaf incubated in 100 nM PSK at different times under brightfield (**A**) and epifluorescence (**B–F**) conditions.All images have the same field of view (FOV) of 340 µm as in Figure 19, and epifluorescence images were obtained with blue light excitation.

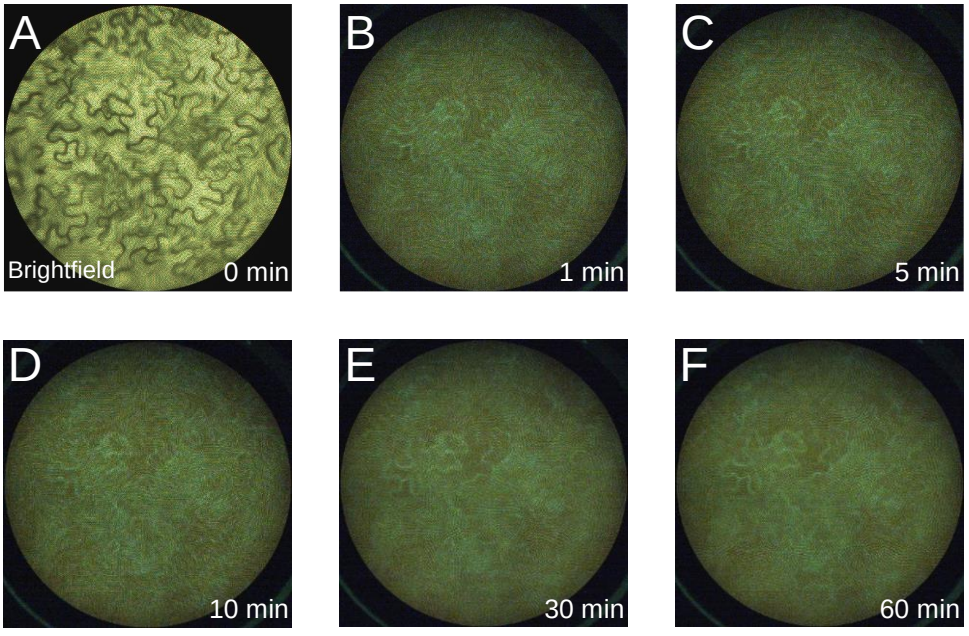

Figure 25. Micrographs of a PSKR-GFP leaf incubated in 100 nM PSK at different times under brightfield (**A**) and epifluorescence (**B–F**) conditions. All images have the same field of view (FOV) of 340 µm as in Figure 19, and epifluorescence images were obtained with blue light excitation.

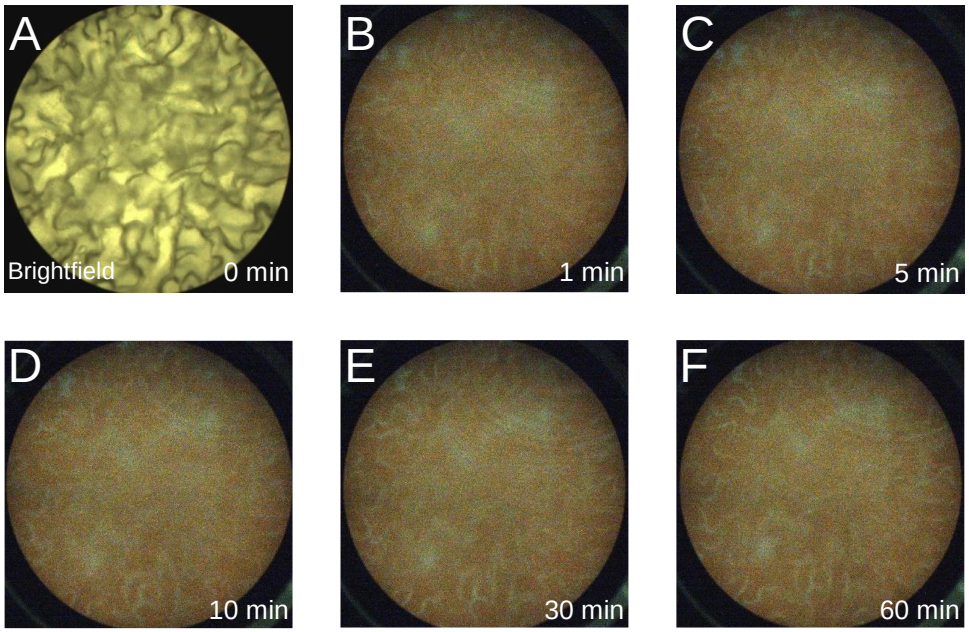

Figure 26. Micrographs of a PSKR-GFP leaf incubated in 10 µM PSK at different times under brightfield (**A**) and epifluorescence (**B–F**) conditions. All images have the same field of view (FOV) of 340 µm as in Figure 19, and epifluorescence images were obtained with blue light excitation.

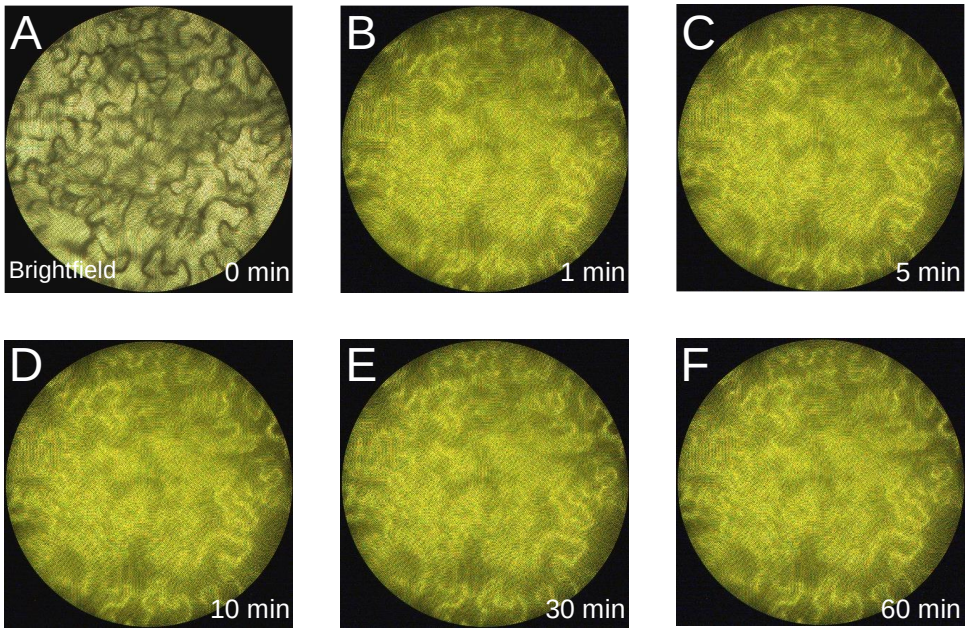

Figure 27. Micrographs of a Col-0 leaf incubated in 10 µM TAMRA-PSK at different times under brightfield (**A**) and epifluorescence (**B–F**) conditions. All images have the same field of view (FOV) of 340 µm as in Figure 19, and epifluorescence images were obtained with green light excitation.

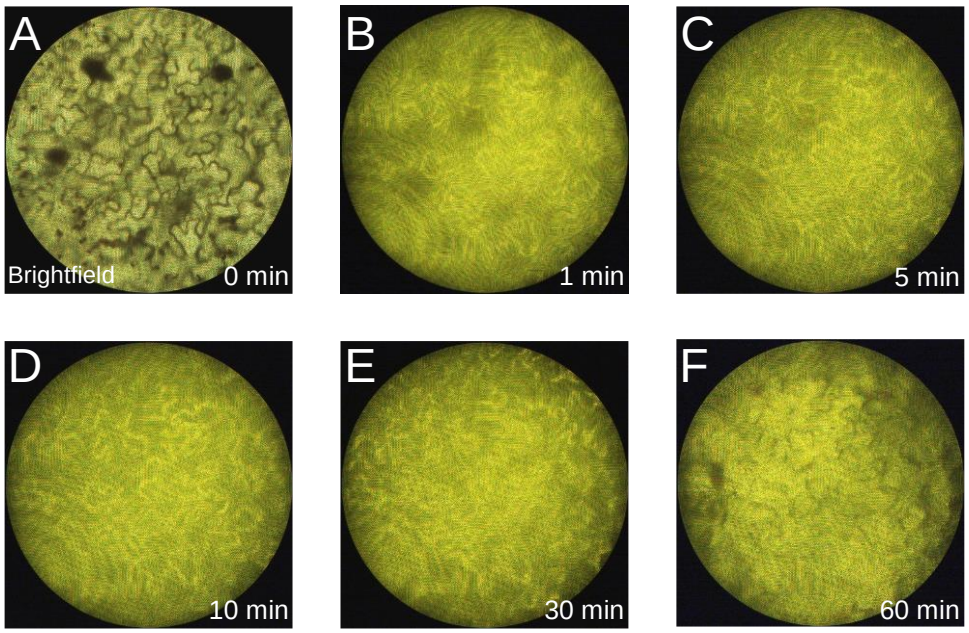

Figure 28. Micrographs of a PSKR-GFP leaf incubated in 10 μM TAMRA-PSK at different times under brightfield (**A**) and epifluorescence (**B**–**F**) conditions. All images have the same field of view (FOV) of 340 μm as in Figure 19, and epifluorescence images were obtained with green light excitation.

In a final set of experiments, 10 μM TAMRA-PSK was applied for the same time durations to newly detached leaves from wt Col-0 and transgenic PSKR-GFP plants. The imaging results are shown in Figures 27 and Figure 28, respectively (control experiments, not shown, demonstrate negligible background fluorescence with green light excitation). Figures 27 and 28 show that in both plant types, the peptide accumulates at the cell margins, similar to the pattern observed for localization of PSKR-GFP in Figures 20, 23, 25 and 26. While there are a number of qualifications that prevent a definitive conclusion that the observed localization is due to receptor binding, the outcome is at least consistent with that hypothesis, and the results reported by Rodiuc and coworkers.

CHAPTER 4. CONCLUSION AND FUTURE WORK

Plants utilize many signal peptides to withstand biotic and abiotic stress conditions. The only signaling peptide in plants with sulfated tyrosine is PSK. The sulfate groups are introduced through post-translational modification by the enzyme TPST, and they responsible for the biological activity of PSK. Chemical synthesis of PSK has been achieved previously, but the sulfate group is potentially labile to both chemical and enzymatic reactions, and no information is available on the stability of native PSK *in vivo*. To overcome this challenge and gain important information about structure–activity relationships in PSK analogs, solid phase peptide synthesis was undertaken to synthesize the PSK analogs Phe(CH_2SO_3H)-Ile-Phe(CH_2SO_3H)-Thr-Gln and Phe(CH_2SO_3H)-Ile- Phe(CH_2SO_3H)-Thr-Lys(TAMRA). These analogs are chemically and enzymatically stable, and their activity anticipated to be similar to the native PSK, but more persistent. The solid phase assembly of the backbones was achieved, with final deprotection and labeling left for future work. The localization of the PSK molecule and its receptor PSKR in plants was investigated after delivery of PSK and TAMRA-PSK by immersion and drop application methods. It was suggested that the appropriate concentration of PSK molecule is required to bind to PSK receptor and elicit signal transduction and molecular trafficking processes. Identification of PSK receptors and internalization of the PSK molecule was observed with the help of PSKR-GFP type plant and TAMRA labeled PSK solution.

In the future, we seek to use *pskr*1/*pskr2* receptor-knockout mutants as the negative control to help clearly distinguish the binding of PSK molecules to PSK receptors. The PSK analogs need to be completed synthetically, purified, and characterized by mass spectrometry. Subsequently, an investigation of the synthesized PSK analogs' receptor-binding, trafficking and bioactivity will be conducted. Even though we were trying to accomplish these goals, time

limitations, microscope challenges and COVID-19 pandemic prevented us from realizing them fully. With the plant growth techniques, strains, microscopic techniques, and nearly complete syntheses described herein, these two areas of study could be well explored in the future.

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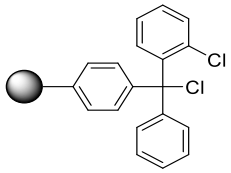
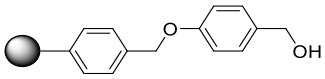
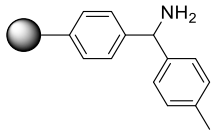
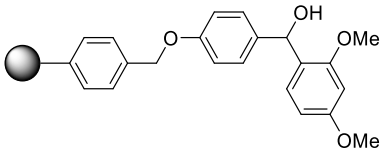
APPENDICES

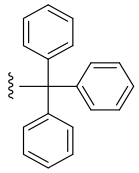
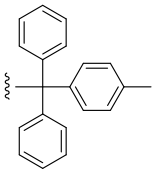
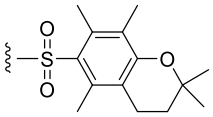
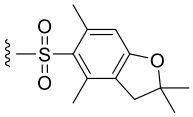
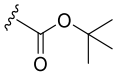
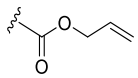
Appendix A: Plant Peptide Signals and Their Sources, Sizes and Roles[60]

Name	Source	Size (amino acids)		Role
		Preprotein	Processed	
Phytosulfokine (PSK)	Many species	89	4-5 sulfated	Helps in the proliferation of the plant cell and provides immunity to the plant cell
Systemin	Tomato, tobacco leaves, pepper, potato, and nightshade leaves	200	18 glycosylated	Defense signaling
Early Nodulin 40 (ENOD40)	Pea, soybean, tobacco, alfalfa, pea, and vetch	10–13	10–13	Cell division
Rapid Alkalinization Factor (RALF)	Many species	~120	50	Cell growth and development
CLAVATA3	*Arabidopsis* meristem	96	78	Coordinate plant growth
S-Locus Cysteine-Rich Protein (SCR)	*Brassica* pollen	74–77	50–53	Self -incompatibility

Appendix B: Common Solid Supports for SPSS With Their Structures and Cleavage Conditions[32]

Name	Structure	Cleavage Conditions
2-Chlorotrityl chloride		1–5% TFA in CH_2Cl_2 1 min
Wang		90–95% TFA 1-2 h
MBHA		HF at 0 °C 1 h
Rink Acid		1-5% TFA in CH_2Cl_2 5–15 min

Appendix C: Common Protecting Groups for Amino-Acid Side Chains, Their Structures and Deprotection Conditions[32]

Protecting Group	Structure	Amino acid(s)	Deprotection Conditions
Trt		Asn Gln His Tyr	Tyr: 2% TFA in CH_2Cl_2, 30 min Asn/Gln/His: 90–95% TFA, 30 min
Mtt		His Lys	1% TFA in CH_2Cl_2, 30 min
Pmc		Arg	90–95% TFA, 2–4 h
Pbf		Arg	90–95% TFA, 1 h
***t*-Bu**		Asp Glu Ser Thr Tyr	90–95% TFA, 30 min Tyr: 35% TFA in CH_2Cl_2
Boc		Lys Orn His	90–95% TFA, 30 min
Alloc		Lys Orn Trp	Lys/Orn: $Pd(PPh_3)_4$ (5 mol %), $PhSiH_3$, THF/CH_3OH, 12 h Trp: $Pd(PPh_3)_4$ (5 mol %), *N*-methylaniline, DMSO:THF:0.5M HCl (1:1:0.5), 8 h.

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