

Synthesis of Methylene Blue Analogues as Multifunctional Radical Quenchers, Synthesis
of Unnatural Amino Acids and Their Ribosomal Incorporation into Proteins

by

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ABSTRACT

The energy required in a eukaryotic cell is provided by mitochondria. Mitochondrial electron transport chain (ETC) coupled with oxidative phosphorylation generates ATP. During electron transport, electron leakage from the ETC produces reactive oxygen species (ROS). In healthy cells, there are preventive and defense mechanisms in place to manage ROS. Maintaining a steady balance of ROS is very important because overproduction of ROS can lead to several pathological conditions. There are several strategies to prevent ROS production. Addition of external antioxidants is widely used among them. Discussed in the first part of Chapter 1 is the mitochondrial ETC, ROS production and antioxidant strategies.

The second part of Chapter 1 is concerned with ribosomal protein synthesis in bacteria. Ribosome, the organelle that synthesizes proteins with exceptional fidelity, has a strong bias for α -L-amino acids. It has been demonstrated that reengineering of the peptidyltransferase center (PTC) of the ribosome could enable the incorporation of both α -D-amino acids and β -amino acids into full length protein.

Oxidative stress is a common cause of various neurological disorders such as Alzheimer's disease and Parkinson's disease. Antioxidative strategies are used widely for the treatment of these disorders. Although several antioxidants demonstrated positive results *in vitro* as well as in *in vivo* models, none of them have been effective in clinical settings. Hence, there is an ongoing search for effective neuroprotective drugs. Described in Chapter 2 is the synthesis and biological evaluation of several methylene blue analogues as potentially effective antioxidants for the treatment of pathologies related to oxidative stress.

In Chapter 3, the synthesis and ribosomal incorporation of several rationally designed dipeptidomimetic analogues are discussed. The dipeptidomimetic analogues are structurally similar to the GFP chromophore and, therefore, highly fluorescent. In addition, the backbone of the dipeptidomimetic analogues resemble the peptide backbone of a dipeptide, due to which they can be incorporated into protein by modified ribosomes selected for the incorporation of dipeptides.

Discussed in Chapter 4 is the synthesis of the pdCpA derivatives of several β -amino acids. The pdCpA derivatives were ligated to tRNA-C_{OH} and were used as probes for studying the regio- and stereoselectivity of modified ribosomes.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
APCI	atmospheric pressure chemical ionization
aq	aqueous
ATP	adenosine-5'-triphosphate
br s	broad singlet
BSA	bovine serum albumin
^{13}C NMR	carbon-13 nuclear magnetic resonance
$^{\circ}\text{C}$	degree Celsius
cat	catalytic
CDCl_3	deuterated chloroform
CH_2Cl_2	methylene chloride
cm	centimeter
δ	chemical shift (ppm)
d	doublet
DCF	2',7'-dichlorofluorescein
DCFH	2',7'-dichlorodihydrofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DEAE	diethylaminoethyl
DHFR	dihydrofolate reductase
DMF	dimethylformamide
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
DTT	dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenedinitrilotetraacetic acid
ESI	electrospray ionization
FACS	fluorescence-activated cell sorting
FCCP	carbonyl cyanide- <i>p</i> -trifluoromethoxyphenylhydrazone
FRDA	Friedreich's ataxia
g	gram (s)
GDP	guanosine-5'-diphosphate
GTP	guanosine-5'-triphosphate
GFP	green fluorescent protein
GSH	glutathione
¹ H NMR	proton nuclear magnetic resonance
h	hour (s)
H ₂	hydrogen gas
H ₂ O	water
HCl	hydrochloric acid
HPLC	high pressure liquid chromatography
Hz	Hertz
IPTG	isopropyl-β-D-thiogalactopyranoside
<i>J</i>	coupling constant
L	liter

M	molar
m	multiplet
MHz	mega Hertz
mL	milliliter
mM	millimolar
mmol	millimole(s)
mRNA	messenger ribonucleic acid
μM	micromolar
μmol	micromole(s)
N	normal
Na_2CO_3	sodium carbonate
NADH	nicotinamide adenine dinucleotide
NaHCO_3	sodium bicarbonate
nm	nanometer
NMR	nuclear magnetic resonance
pdCpA	5'-O-phosphoryl-2'-deoxycytidyl(3'→5')adenosine
PTC	peptidyltransferase center
R_f	ratio of fronts
RNA	ribonucleic acid
s	singlet
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
t	triplet
TBA	tetrabutylammonium

THF	tetrahydrofuran
TLC	thin layer chromatography
tRNA	transfer RNA
UV	ultraviolet
Val	valine

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CHAPTER 1

1.1 MITOCHONDRIAL ELECTRON TRANSPORT CHAIN: GENERAL

INTRODUCTION

Mitochondria are the powerhouses of all eukaryotic cells. They produce energy through oxidative phosphorylation (OX-PHOS) in the form of adenosine triphosphate (ATP).¹⁻⁴ ATP provides the energy for many cellular processes. Apart from energy production mitochondria are also involved in several cellular processes such as pyrimidine⁵ and lipid biosynthesis,⁶ apoptosis,⁷ calcium homeostasis⁸ and flux. Therefore, damage to mitochondria must have widespread consequences.

The mitochondrial respiratory chain consists of a series of protein complexes, which are embedded in the mitochondrial inner membrane. Electrons provided from donor molecules are transferred through these complexes. This process is accompanied by the pumping of several protons into the intermembrane space. The potential gradient generated by the pumping of protons is used by ATP synthase to synthesize ATP from ADP and inorganic phosphate.^{9,10} The complexes in the mitochondrial inner membrane are NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), cytochrome bc₁ (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V). All of these complexes contain Fe-S prosthetic groups. Additionally, complexes I and II have flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), respectively, whereas complexes III and IV have heme groups. Two mobile carriers, namely ubiquinone and cytochrome c, are also part of the ETC. Electron transport begins with the transfer of two electrons from NADH to complex I; coupled with this is the transfer of four protons to the intermembrane space. These two electrons

are then transferred to ubiquinone, which transfers them to complex III. Similarly, succinate donates two electrons to complex II. These are also carried to complex III by ubiquinone. Electron transport through complex III results in the transfer of two protons to the intermembrane space. Cytochrome *c* acts as the mobile carrier between complexes III and IV. Complex IV accepts electrons, one at a time and one proton is pumped into the intermembrane space for each electron. In complex IV, four electrons, O_2 and four protons interact to form two water molecules; the other four protons are pumped across the membrane into the intermembrane space. The ten protons transported to the intermembrane space generate a proton electrochemical gradient; this gradient is utilized by ATP synthase to synthesize ATP from ADP and inorganic phosphate.^{11,12} The electron transport chain is illustrated in the following figure¹³:

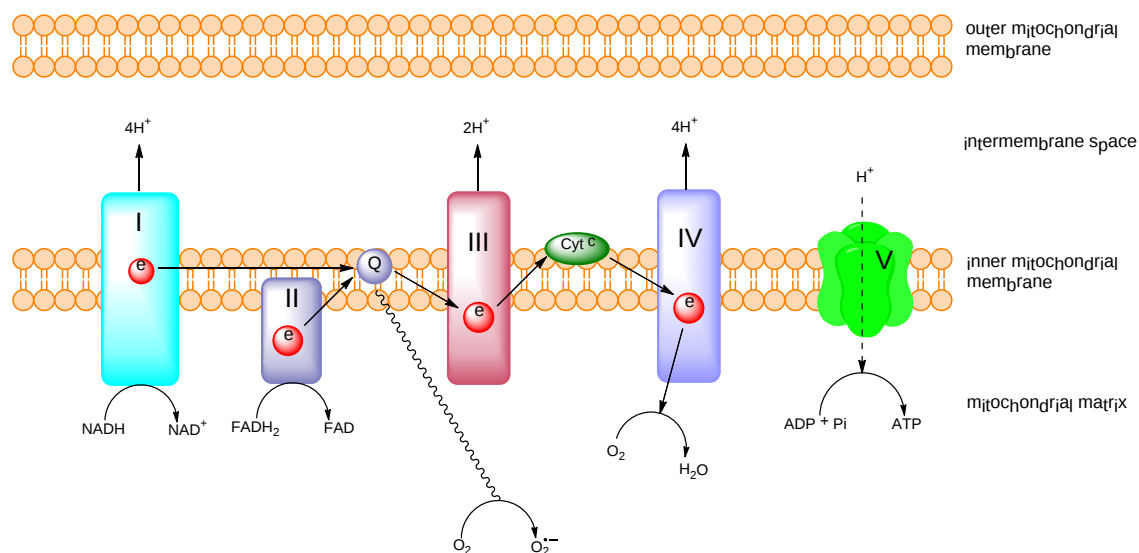


Figure 1.1. Electron transport chain in mitochondria.

Mitochondria consume the most oxygen of all the organelles in a cell in order to maintain the production of a large amount of ATP. ATP synthase utilizes the gradient generated by the passage of about three protons to generate one ATP. Thus, one NADH

and one succinate produce $\sim 3\text{ATPs}$ and $\sim 2\text{ATPs}$, respectively. Eighty percent of oxygen consumption related to ATP production occurs in mitochondria.¹⁴ During electron transport leakage of a small portion of electrons, mostly at complexes I and III, leads to the formation of superoxide anion ($\text{O}_2^{\cdot-}$) which is then converted to other reactive oxygen species (ROS). These are very reactive and can damage cellular components including proteins, lipids and nucleic acids. The overproduction of ROS is believed to be the key cause of aging and many neuropathological diseases.¹⁵

ROS is a term used to describe molecular oxygen derived chemical species with unpaired electrons. ROS include hydroxyl radical ($\cdot\text{OH}$), superoxide anion ($\text{O}_2^{\cdot-}$), singlet oxygen and peroxynitrite ($\text{ONOO}^{\cdot-}$).¹⁶ The reduction potential of the $\text{O}_2/\text{O}_2^{\cdot-}$ pair is about -0.15 V which means that the oxidation of cellular compounds by molecular oxygen is a thermodynamically favorable process. Normally, it doesn't occur because the process is kinetically unfavorable due to high activation energy.¹⁶ However, for highly reactive respiratory chain electron carriers such as reduced flavins, non-heme iron proteins, quinols and especially semiquinones, the activation energy for electron transfer is low. Therefore, instead of the desired four electron reduction of oxygen by complex IV, $\text{O}_2^{\cdot-}$ is formed by a one-electron reduction. Superoxide radical ($\text{O}_2^{\cdot-}$) disproportionates (either spontaneously or by the action of superoxide dismutases) to H_2O_2 . Hydrogen peroxide can generate hydroxyl radicals ($\cdot\text{OH}$) in presence of lower valent transition metals like copper or iron by the Fenton reaction (Figure 1.2).¹⁷ Hydroxyl radical is an extremely strong oxidant, which can oxidize practically everything.

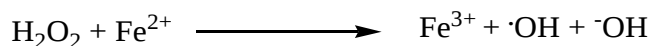


Figure 1.2. Formation of hydroxyl radicals by the Fenton reaction.

To prevent the deleterious effects resulting from the formation of ROS, there are several natural defense mechanisms in cells. This is illustrated in Table 1.1.¹⁸ In general,

Table 1.1. Different types of defense mechanisms against ROS.

	Actions	Example
Prevention	Protein binding of metal ions like copper and iron	Transferrin, ferritin, albumin
Enzymatic neutralization	Conversion of ROS to nontoxic products	Superoxide dismutase, catalase, glutathione peroxidase
Scavenging	Sacrificial interaction with ROS by expendable (replaceable or recyclable) substrates	Ascorbic acid, α -tocopherol
Quenching	Absorption of electrons	α -Tocopherol, β -carotene

any species that can prevent oxidation is an antioxidant. Antioxidants can be divided into two classes, enzymatic and non-enzymatic antioxidants.¹⁹ Glutathione peroxidase, catalase and superoxide dismutase are among the enzymatic antioxidants. Superoxide dismutase (SOD) destroys the free radical superoxide by converting it into peroxide that can be destroyed by catalase or glutathione peroxidase (GPX) (Figure 1.3).¹⁹

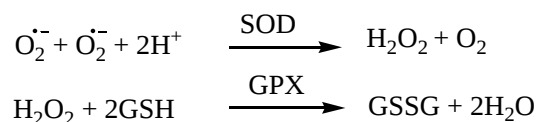


Figure 1.3. Quenching of ROS by superoxide dismutase and glutathione peroxidase.

Non-enzymatic antioxidants include α -tocopherol or vitamin E, L-ascorbic acid, glutathione (GSH), β -carotene, resveratrol, curcumin (non-flavonoids) and quercetin (flavonoid) (Figure 1.4 and 1.5).^{18,20-23}

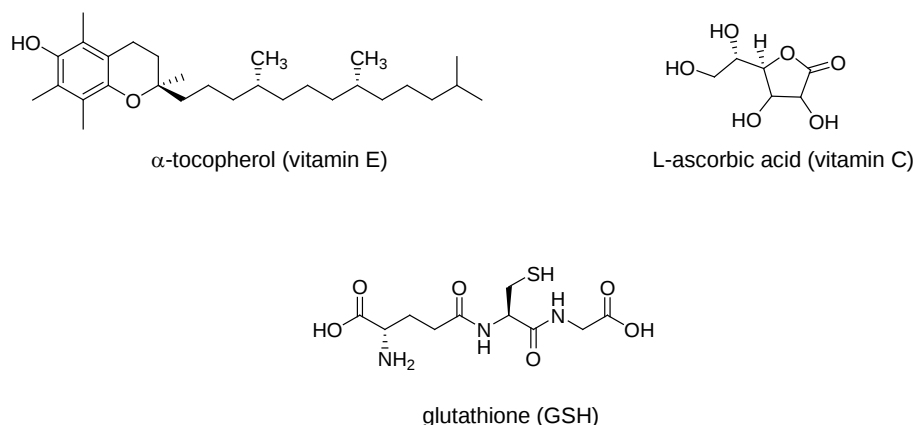


Figure 1.4. Chemical structures of vitamin E, vitamin C and glutathione.

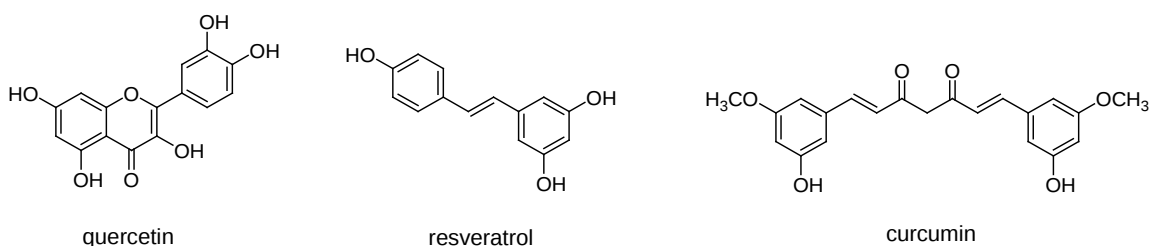


Figure 1.5. Chemical structures of quercetin, resveratrol and curcumin.

Vitamin E is a chain breaking antioxidant because it interferes with one or more of the propagation steps of lipid peroxidation. Vitamin E is the collective term for a family of structurally related chemical substances. It naturally occurs in eight different forms: α , β , γ , δ -tocopherols and α , β , γ , δ -tocotrienols (Figure 1.6).^{24,25}

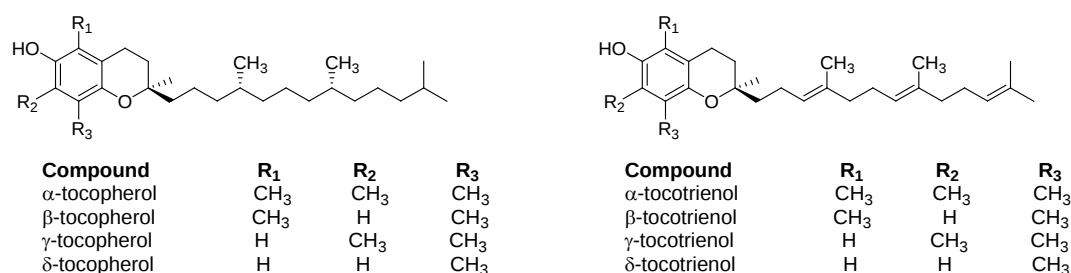


Figure 1.6. Chemical structures of members of the vitamin E family.

‘Oxidative stress’ is an expression used to describe various deleterious processes resulting from the formation of excessive ROS. The steady state concentrations of $O_2^{\cdot -}$ and H_2O_2 in normal mitochondria are $\sim 10^{-10}$ M and 5×10^{-9} M, respectively.¹⁶ During intracellular signaling small fluctuations in the steady-state concentrations of ROS are observed but a several-fold increase in the steady-state concentrations of these oxidants can lead to indiscriminate chain reactions with proteins, lipids, DNA and polysaccharides. The peroxidation of lipid membranes by hydroxyl radicals is well documented.^{26,27} A carbon centered radical ($R^{\cdot}$) is formed due to the abstraction of hydrogen from the lipids. This is the chain initiation reaction. The radical $R^{\cdot}$ reacts extremely rapidly with oxygen to form the peroxy radical ($ROO^{\cdot}$). The peroxy radical can abstract a hydrogen atom from another lipid molecule, forming another carbon centered radical ($R^{\cdot}$). Thus a single hydroxyl radical is capable of forming many lesions, which ultimately breach the integrity of lipid membranes (Figure 1.7).

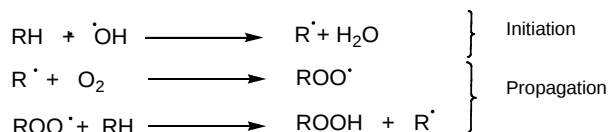


Figure 1.7. Free radical chain reaction and lipid peroxidation.

As discussed before, CoQ₁₀ is the carrier molecule, which transfers electrons from complexes I and II to complex III. In the reduced form, it is one of the most potent

antioxidants present in mitochondria, capable of quenching lipid peroxidation.²⁷ α -Tocopherol, one of the members of the vitamin E family, is a phenolic compound and is also very effective in quenching lipid peroxidation.²⁶ Phenolic antioxidants can suppress lipid peroxidation in two ways, by quenching the carbon centered radical during initiation step (preventive antioxidants) or by quenching the peroxy radical during chain propagation step (chain-breaking antioxidants).²⁶ It has been demonstrated that phenolic antioxidants are chain-breaking antioxidants because they quench peroxy radicals much faster than carbon centered lipid radicals.

When α -TOH quenches a peroxy radical, α -TOH itself is converted to a radical (α -TO $\cdot$). Due to effective resonance stabilization (Figure 1.8) α -TO $\cdot$ is much less reactive and does not react with carbon centered lipid radicals.²⁸⁻³¹ α -TO $\cdot$ is recycled back to α -TOH by cellular ascorbic acid and NADH.

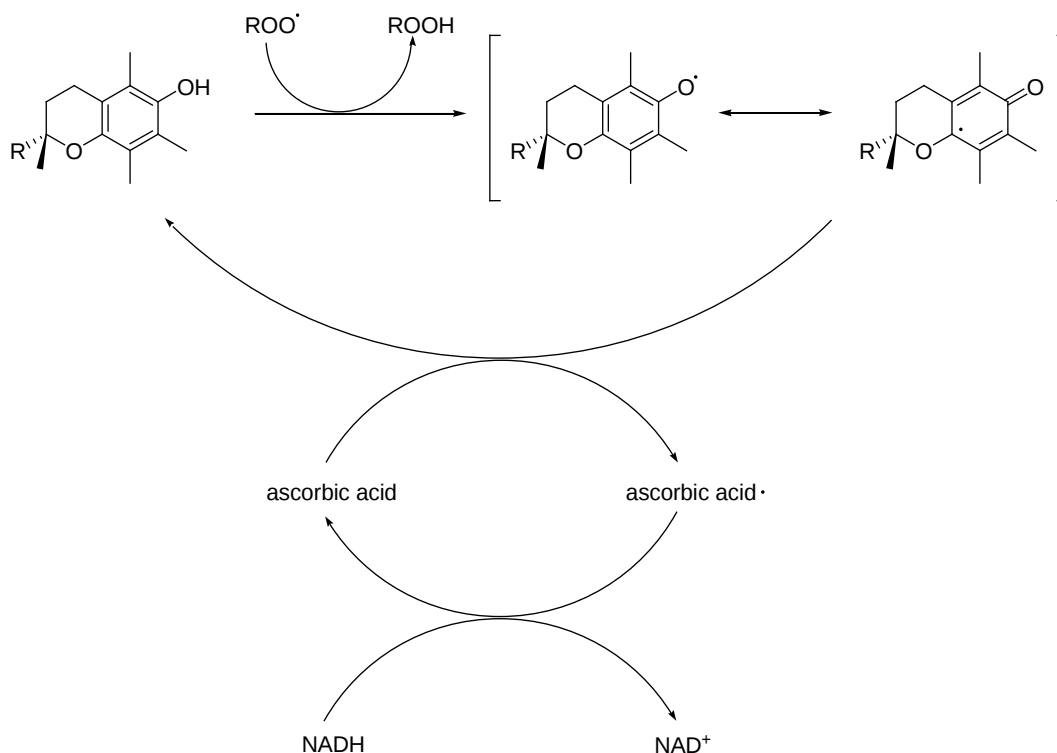


Figure 1.8. Quenching of lipid peroxidation by α -tocopherol and recycling of α -tocopherol by ascorbic acid and NADH.

Mitochondria are rapidly emerging as major contributors to human health and disease. Hence, the appropriate operation of mitochondria is extremely important. Scientists are always in search of new lead compounds for the treatment of mitochondrial diseases. Many compounds, which are effective *in vitro*, do not show the anticipated efficacy *in vivo*. In this thesis, the synthesis and biological evaluation of several methylene blue analogues as antioxidants has been described. Preliminary studies indicate that some of the analogues are better antioxidants compared to methylene blue itself.

1.2 RIBOSOMAL PROTEIN SYNTHESIS: GENERAL INTRODUCTION

The ribosome is a large ribonucleoprotein particle that directs the synthesis of proteins. The bacterial ribosome consists of a large (50S) subunit and a small (30S) subunit; together these two subunits form the 2.5 megadalton 70S ribosome. The 50S subunit consists of 23S rRNA, 5S rRNA and about 30 proteins whereas the 30S subunit is made of 16S rRNA and about 20 proteins.^{32,33} The ribosome synthesizes proteins using messenger RNA (mRNA) and transfer RNA (tRNA) with the help of several protein factors. The large subunit contains the peptidyltransferase center, which is responsible for forming peptide bonds; whereas the small subunit contains the codon recognition center in which charged tRNA decodes the codon sequence of mRNA. Each ribosomal subunit has three sites for tRNA binding, namely the A-site or acceptor site for aminoacyl-tRNA, the P-site or peptidyl site that binds peptidyl-tRNA containing the nascent polypeptide chain and the E-site or exit site from which deacylated tRNA exits the ribosome (Figure 1.9).³³ Bacterial protein synthesis can be divided into three steps- initiation, elongation and termination. Bacterial protein synthesis always starts by positioning formylmethionyl-tRNA^{fMet} over the start codon (AUG) on the P-site. Therefore, ribosomally translated bacterial proteins always contain formyl-methionine at the beginning, which are processed by posttranslational modifications later. In bacteria, the 30S ribosomal subunit is positioned in the vicinity of the mRNA start codon, and the 3'-end of the 16S ribosomal RNA base pairs with its complementary Shine–Dalgarno sequence in the bound mRNA.³² Three initiation factors (IF1, IF2 and IF3) are also involved in the formation of this 30S initiation complex (30S-IC). The 30S-IC then recruits the 50S subunit to form an intact 70S ribosome, all set to start protein synthesis.

Protein elongation occurs due to the nucleophilic attack by the α -amino moiety of the aminoacyl-tRNA in the A-site, on the carbonyl carbon of the amino acid bound to the tRNA in the P-site (Scheme 1.1a). As a result, the nascent peptide is transferred onto the tRNA occupying the A-site, adding a new amino acid to the growing chain while the tRNA occupying the P-site is deacylated (Scheme 1.1b). This event is driven by the hydrolysis of GTP followed by translocation of the mRNA and ribosome relative to one

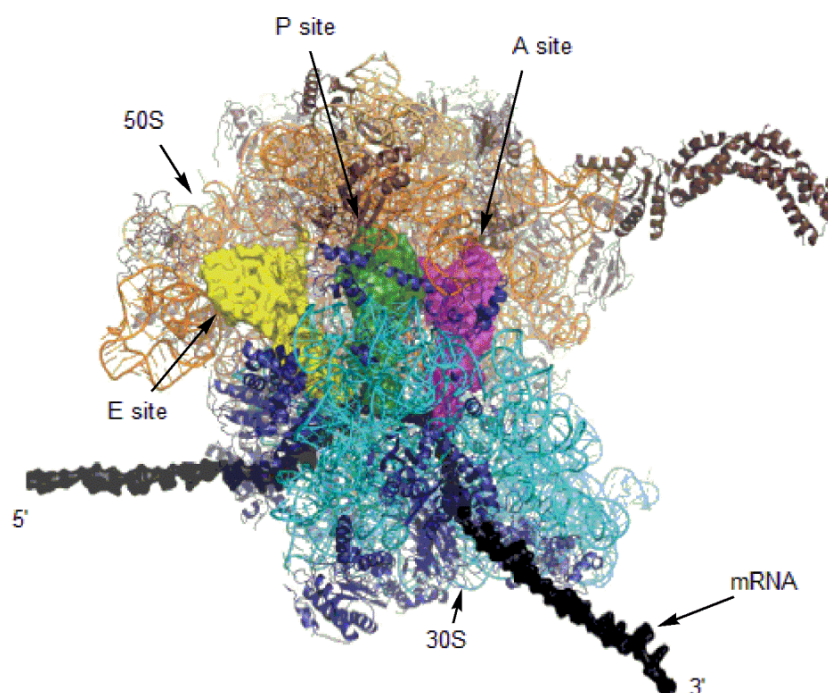
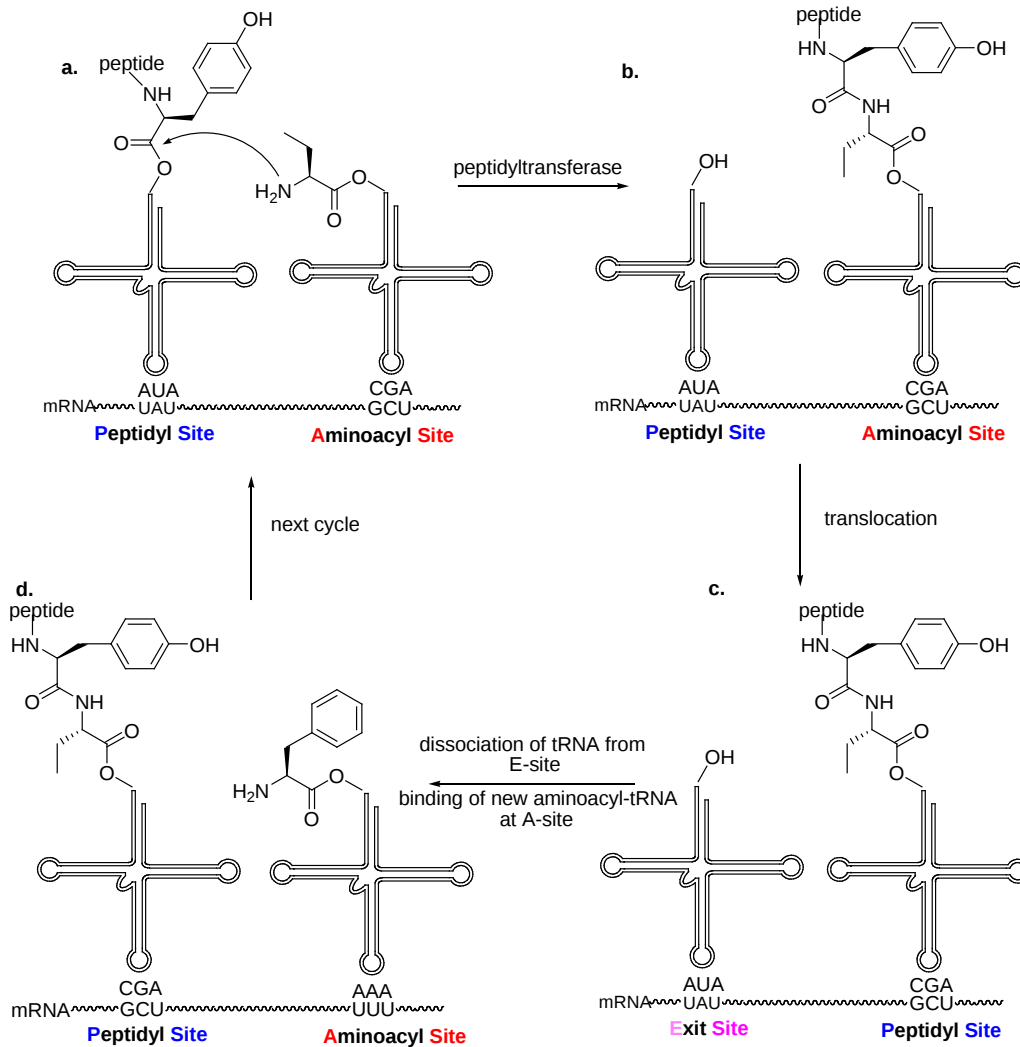


Figure 1.9. Schematic diagram of the ribosome.

another. In essence, the ribosome shifts three nucleotides (one codon) along the mRNA in the 5' to 3' direction. This is accompanied by the movement of the peptidyl-tRNA in the A-site to the P-site and the deacylated tRNA in the P-site to the E-site of the ribosome (Scheme 1.1c). Following this event a new cycle begins in which another aminoacyl-tRNA binds to the A-site and the deacylated tRNA dissociates from the E-site (Scheme

1.1d).³² Each amino acid is added to the nascent peptide chain accompanied by the hydrolysis of at least two GTP molecules. One GTP molecule is hydrolyzed during aminoacyl-tRNA selection as a ternary complex and another is hydrolyzed during translocation. Each elongation cycle takes about one-twentieth of a second.³⁴

Scheme 1.1. Peptide bond formation in PTC.



The ribosome increases the rate of peptide bond formation by at least 10^5 -fold. It has been shown that the catalytic site is in domain 5 of 23S rRNA. Since RNA is responsible for the catalytic activity, the ribosome is also a ribozyme.³⁵

Among the 64 trinucleotide codons, there are three codons, which do not encode any amino acid; their function is to terminate translation. These three codons are designated stop codons, namely UAA, UAG and UGA. There are generally no tRNAs whose anticodons are complementary to the stop codons. Hence, when the ribosome detects one of these stop codons, elongation is stopped and the polypeptide is released; this is known as translation termination. Termination requires the presence of one of the three release factors, which recognize the stop codons and cleave the polypeptide chain from the P-site tRNA. There are three release factors in bacteria: release factor 1 (RF1), which recognizes UAA and UAG stop codons; release factor 2 (RF2), which recognizes UAA and UGA stop codons; release factor 3 (RF3), which is not codon specific but increases the activity of other factors.^{32,34} Studies show the similarity between the structures of the release factors and tRNA, due to which the release factors can enter the A-site of the ribosome.

During protein synthesis, the overall translation error rate ranges from 6×10^{-4} to 5×10^{-3} which is the result of net error accumulation from several steps, including transcription (10^{-4}), aminoacyl-tRNA synthesis (10^{-4}), and ribosomal decoding (10^{-4}).³⁶⁻³⁹ There are two steps involved in the aminoacylation by aminoacyl-tRNA synthetases; first the formation of an aminoacyladenylate or aminoacyl-AMP and then the transfer of the amino acid to the cognate tRNA. Aminoacyl-tRNA synthetases often employ unique proofreading mechanisms to maintain translational fidelity (also known as editing).³⁶

Although Linus Pauling and Francis Crick foresaw the need for an editing mechanism for structurally similar amino acids during protein synthesis, the actual experimental observation was first reported by Baldwin and Berg.⁴⁰ They demonstrated that the addition of tRNA^{Ile} accelerated the breakdown of misactivated Valyl-AMP bound to Ile-RS.

The idea that the ribosome plays a crucial role in maintaining translational fidelity was first demonstrated in 1964⁴¹ when the production of an essential enzyme in mutant cells in the presence of streptomycin was reported. Without streptomycin the enzyme was not produced because of the presence of a stop codon in the gene encoding for the protein. Streptomycin resulted in misreading of the mRNA template in *in vitro* translation reactions. Thus, ribosome structural modulations by streptomycin appeared to influence the accuracy of protein translation.

Ribosome accepts aminoacyl-tRNA in the A-site as a ternary complex of aminoacyl-tRNA with elongation factor Tu (EF-Tu) and GTP to form the initiation complex.^{32,42} The rate constant (k_1 , Figures 1.10, 1.11 and 1.12) for the formation of this complex is same for cognate, non-cognate or near-cognate ternary complexes.⁴² When there is codon recognition the complex is stabilized (k_2). Codon recognition results in conformational change of EF-Tu towards the active conformation for GTP hydrolysis (GTPase activation, k_3). After GTP hydrolysis (k_{GTP}), EF-Tu changes its conformation (k_4), loses the affinity for aminoacyl-tRNA and dissociates (k_6), leaving the aminoacyl end of aminoacyl-tRNA to move into the peptidyltransferase center (Accommodation, k_5) where it can take part in peptide bond formation (k_{pep}). This is depicted in Figure 1.10.⁴² The initiation complex dissociates rapidly (slow k_3 , fast k_2 , Figure 1.11), before GTP

hydrolysis, for non-cognate ternary complexes. This is known as initial selection. Thus, the bulk of the non-cognate ternary complexes are discarded without GTP hydrolysis.

This is depicted in Figure 1.11.⁴²

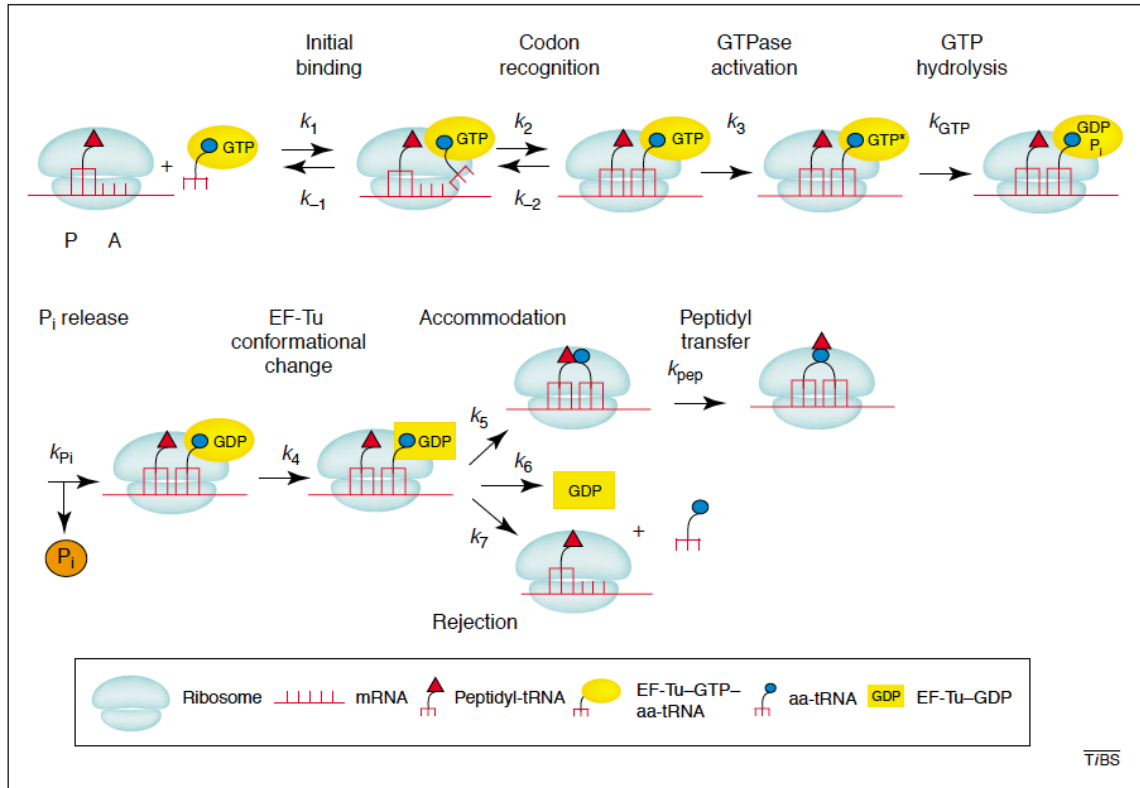


Figure 1.10. Kinetic mechanism displaying the binding of aminoacyl-tRNA to the ribosomal A-site. Numbered rate constants indicate kinetically resolved steps. k_{GTP} , k_{Pi} , k_{pep} indicate steps which are rate-limited by the preceding steps. P and A designate the P-site and A-site of ribosome. Elongation factor Tu (EF-Tu) is shown differently in the GTP and GDP bound state. GTP* indicates GTP-ase activity (reference 41).

The screening of near-cognate ternary complexes is more complex. Upon initial selection near-cognate ternary complex is stabilized (fast k_2 , slow k_{-2} , Figure 1.12⁴²), stimulating the hydrolysis of GTP in EF-Tu (GTPase activation, k_3). Although both the values of k_2 and k_3 are several fold lower for near-cognate case compared to cognate case, rapid and irreversible GTP hydrolysis drives the equilibrium forward for near-cognate case. For the majority of near-cognate aminoacyl-tRNA, discrimination takes

place in the subsequent proofreading step because of the less stable codon-anticodon interaction than cognate one (fast k_7).

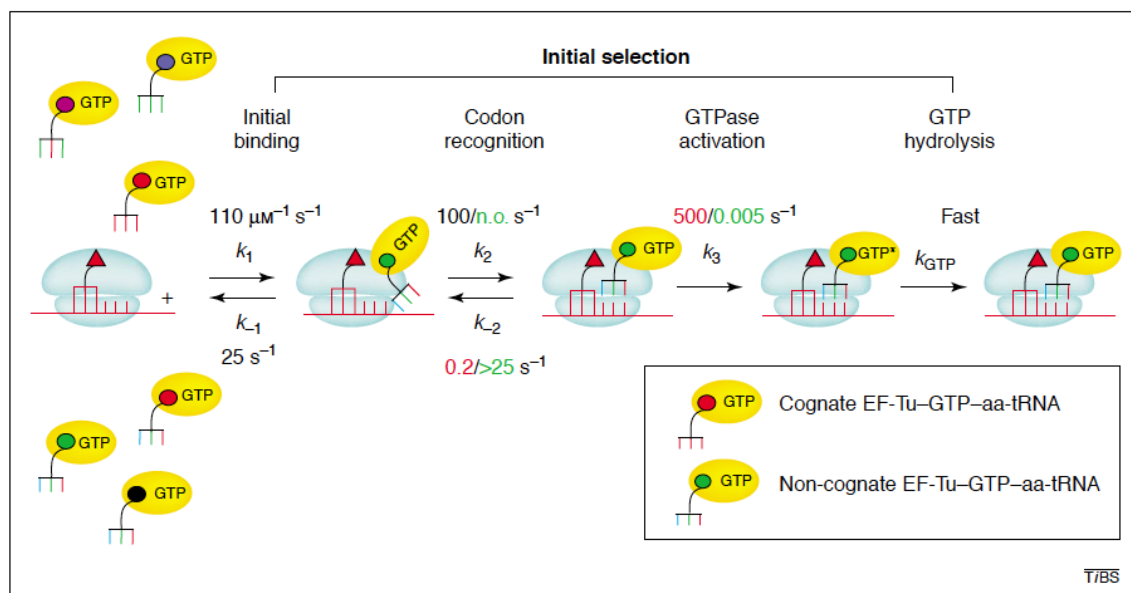


Figure 1.11. Kinetic mechanism showing discrimination against non-cognate ternary complexes. Black-rate constants do not contribute to the discrimination because they are the same for cognate and non-cognate ternary complexes. Rate constants in red are specific for cognate ternary complexes and green are for non-cognate ternary complexes (reference 41).

Ribosomal protein synthesis is limited to α -L-amino acids.⁴³ The ribosomal peptidyltransferase center can differentiate between individual amino acids and is sensitive to the structure as well as chirality of the amino acids. For example, initial efforts to incorporate α -D-amino acids were largely unsuccessful.⁴⁴⁻⁴⁶ There have been numerous efforts to incorporate nonnatural amino acids into preselected positions of proteins. The major strategy for the site-specific incorporation of nonnatural amino acids into proteins was by the suppression of stop codons (UAG) using chemically misacylated

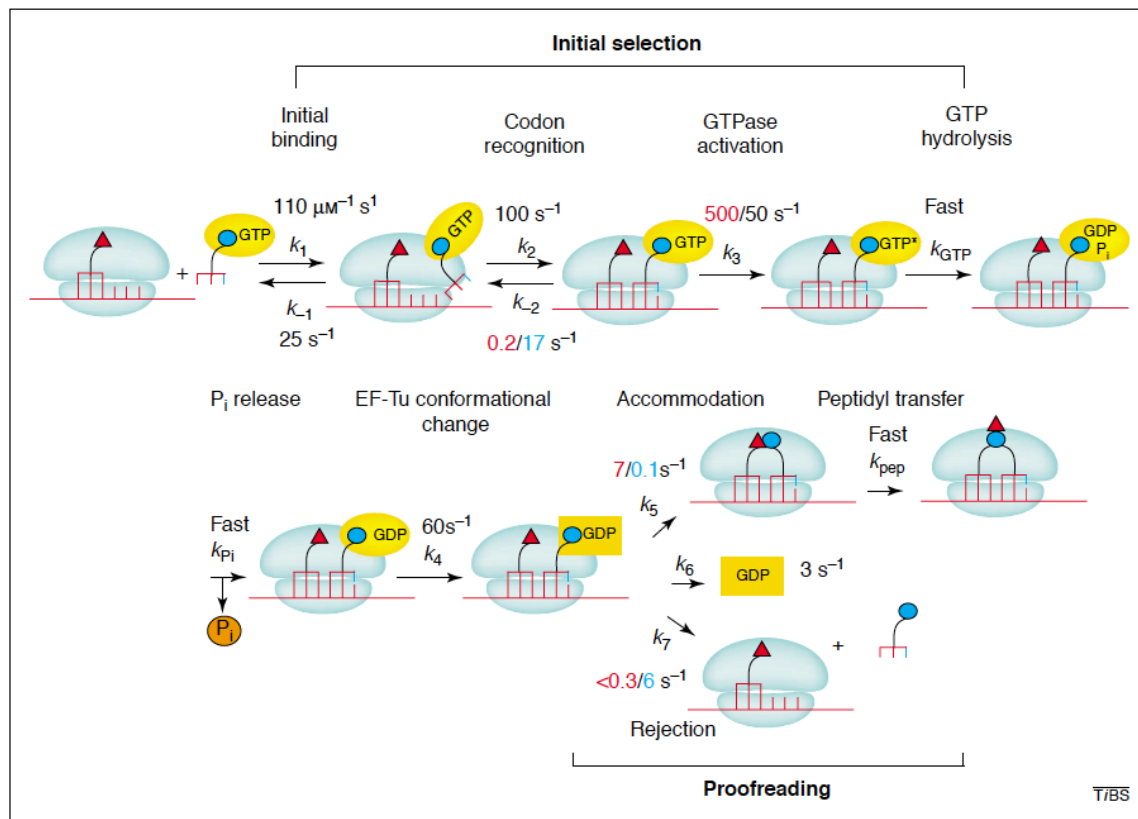


Figure 1.12. Kinetic mechanism for the discrimination against near-cognate ternary complexes. Black rate constants are same for cognate and near-cognate ternary complexes. Rate constants in red are specific for cognate ternary complexes and those in blue are specific for near-cognate ternary complexes and hence contribute to discrimination (reference 41).

suppressor tRNAs having CUA anticodons. Hecht and coworkers developed a chemical method to covalently attach a non-natural amino acid to the 3'-end of tRNA^{Phe}.^{47,48}

Chemically misacylated pCpA was attached to an abbreviated tRNA using T4 RNA ligase. Schultz and coworkers used pdCpA instead pCpA to overcome the synthetic difficulties of preparing pCpA. The same groups then used these misacylated tRNAs for the site-specific incorporation of unnatural amino acids in cell-free protein synthesizing systems.⁴⁹⁻⁵¹ Schultz and coworkers also developed a strategy to incorporate unnatural amino acids into proteins *in vivo*.⁵²

A lot of work has been done to change the translation property of ribosomes by changing the critical nucleotides in the PTC, yet until recently there had been no report of the incorporation of non-natural amino acids into protein by modified ribosomes.

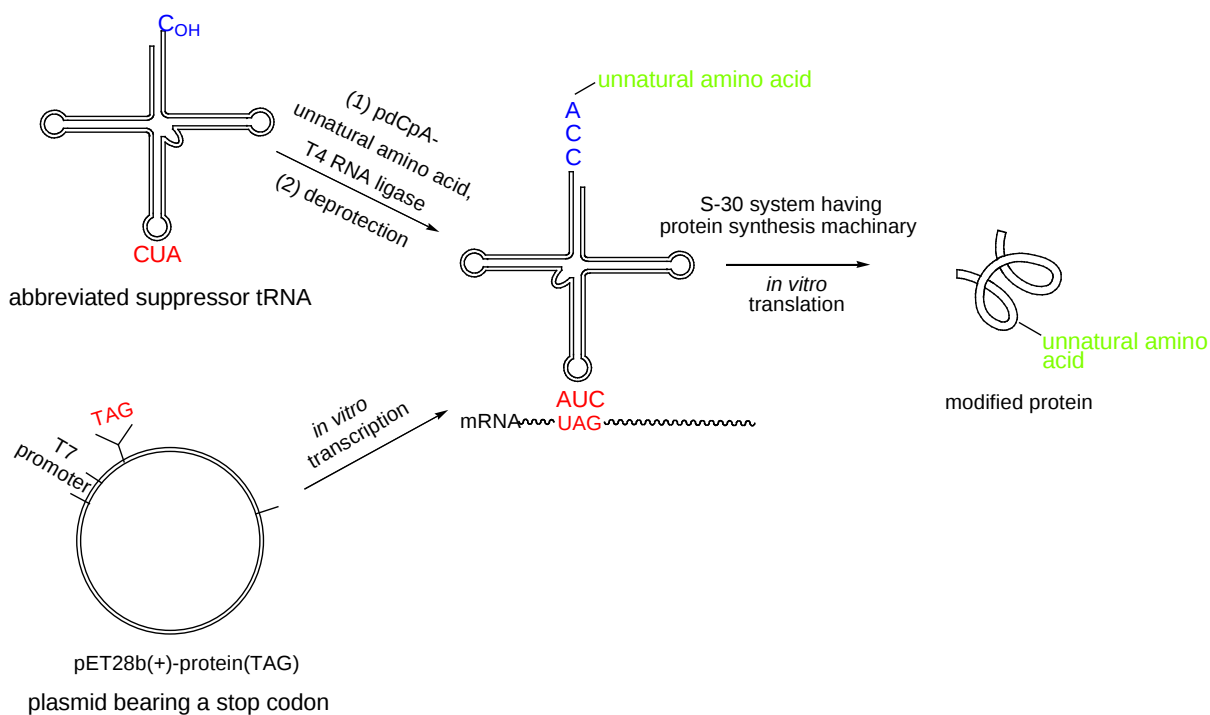


Figure 1.13. Strategy for the incorporation of unnatural amino acids into proteins *in vitro*.⁵³

Puromycin (Figure 1.14) is an aminonucleoside antibiotic, which acts as an inhibitor of protein synthesis in intact cells as well as cell free extracts of different organisms. The structure of puromycin resembles the 3'-end of an aminoacyl-tRNA (Figure 1.14) which makes it a putative aminoacyl-tRNA mimic.⁵⁴ Thus ribosomal A-site recognizes puromycin as aminoacyl-tRNA. The free amino group of puromycin then takes part in peptide bond formation with the nascent polypeptide attached to the peptidyl-tRNA in the P-site, resulting in premature chain release.⁵⁴ Puromycin, and its derivatives having

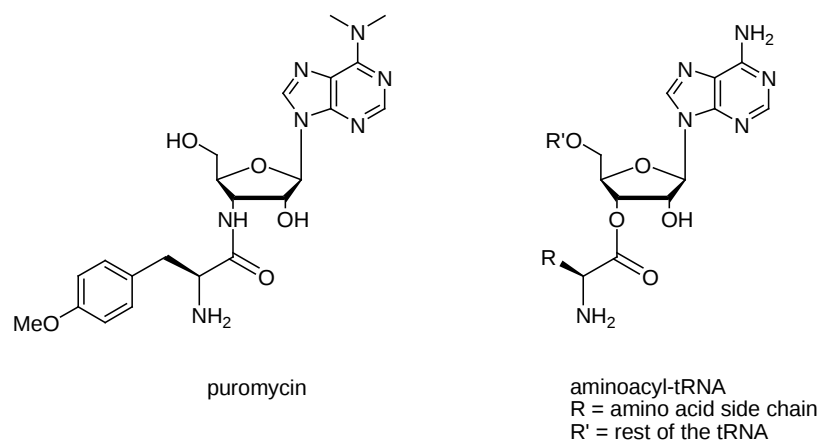


Figure 1.14. Structural similarity between puromycin and 3'-end of aminoacyl-tRNA.⁵⁴

different amino acid side chains show different ribosomal inhibition efficiencies.^{55,56}

Thus, it seemed possible that in order to mediate the incorporation of non-natural amino acids by ribosomes with altered peptidyltransferase centers, the modified ribosomes could be selected using puromycin analogues containing the amino acid structure of interest.

Modification of the peptidyltransferase center (PTC) can be a novel strategy for incorporation of nonnatural amino acids. Hecht and coworkers exploited this idea by successfully preparing modified ribosomes having modifications in two regions (2447-2450 and 2457-2462), which correspond to the PTC and helix 89 of *Escherichia coli* 23S rRNA.⁵⁷ The ribosomes were able to incorporate α -D-methionine and α -D-phenylalanine into *E. coli* dihydrofolate reductase (DHFR) in enhanced yields.⁵⁷ Following this success, the same group reported the selection of ribosomes having modifications in the PTC of the 23S rRNA using β -puromycin (**1.1**, Figure 1.15).^{58,59} The modified ribosomes were able to incorporate five β -amino acids including β -alanine into DHFR.^{53,59} In all these cases the modified ribosomes were able to incorporate a single unnatural amino acid by the suppression of a single stop codon. In 2015, the modified ribosome technology was

extended importantly when Hecht and coworkers reported the incorporation of dipeptides and dipeptide mimic analogues into position 10 of DHFR by the suppression of a single stop codon using modified ribosomes selected against a puromycin analogue containing a dipeptide (**1.2**, Figure 1.15).⁶⁰

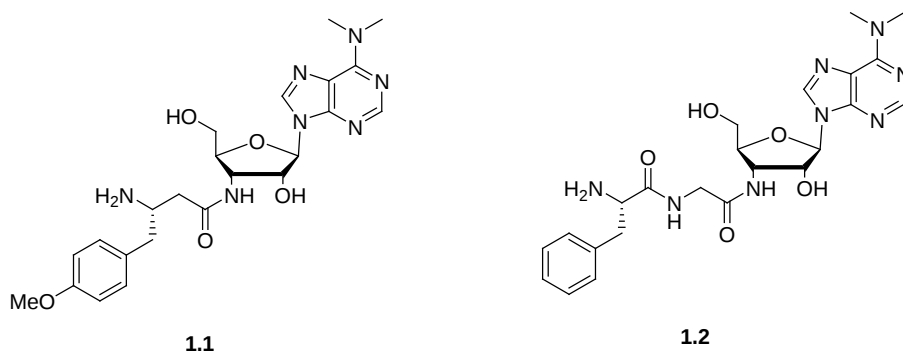


Figure 1.15. Structures of puromycin derivatives.

In this thesis, the syntheses of several dipeptidomimetic analogues are described. The dipeptidomimetic analogues included analogues of the chromophore of green fluorescent protein (GFP) and were, therefore, highly fluorescent. Additionally, some of the dipeptidomimetic analogues were incorporated into position 66 of GFP and the photophysical properties of the proteins were determined. Also described are the syntheses of the pdCpA derivatives of several β -amino acids, which were used as probes for the study of the regio- and stereoselectivity of the selected modified ribosomes.

CHAPTER 2

2. METHYLENE BLUE AND METHYLENE VIOLET ANALOGUES AS MULTIFUNCTIONAL RADICAL QUENCHERS

2.1 Introduction

Phenothiazine and methylene blue: a historical background

Phenothiazine (Figure 2.1) is arguably the most promiscuous lead structure of 20th century medicinal chemistry.⁶¹ Methylene blue (MB) was the first phenothiazine to be used as an antimalarial agent by Paul Ehrlich in the late 1880s. Phenothiazines were administered to humans as anthelmintic agents in the 1930s and 1940s.⁶² The use of phenothiazines expanded in the 1950s when researchers at Rhône-Poulenc Laboratories in France developed derivatives of phenothiazine having antihistaminergic and antipsychotic properties.⁶³ Although the redox properties of phenothiazine and some congeners have been known for decades, their application as such was limited to the engineering industry. With increasing evidence of the role of oxidative stress in many degenerative human pathologies, there has been a steady increase in the investigation of phenothiazine, MB and other derivatives for the treatment of neurodegenerative diseases like Parkinson's disease and Alzheimer's disease.⁶¹

Methylene blue (MB) (Figure 2.1) has been used as a medicine for many years in the treatment of various pathological conditions.⁶⁴ The therapeutic potential of MB has been demonstrated against many diseases including methemoglobinemia and malaria as well as cyanide and carbon monoxide poisoning. MB is a derivative of phenothiazine; it was first synthesized by Heinrich Caro at BASF in 1876 and by Bernthsen in 1883. The structure was later determined by Bernthsen in 1885.⁶¹

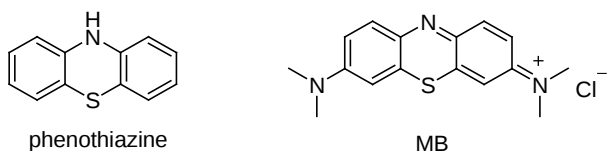


Figure 2.1. Chemical structures of phenothiazine and MB.

Ehlich first described the redox properties of MB. While staining different tissues *in vivo* he saw variable, reversible reduction of MB to its leuco form in certain organs such as liver and lung. After exposure to ambient oxygen the blue color returned.⁶¹ By the end

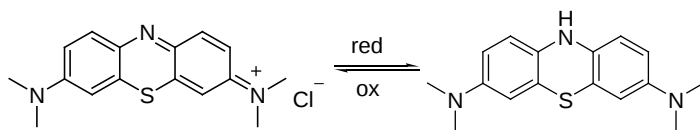


Figure 2.2. Redox cycling of methylene blue.

of the 1940s Charles Murphy et al. had demonstrated that phenothiazine could prevent the oxidation of turbo-jet engine lubricants even at temperatures above 100 °C.⁶²

Numerous derivatives of phenothiazine were synthesized and patented in 1950s and 1960s with ongoing use to date.⁶⁵ Decades later, in 1991, Yu et al. used phenothiazines as chemicals that potentially protected cells, especially neurons, from oxidative stress.⁶⁶

Antioxidant property of phenothiazines: a comparison

Following the report of Yu et al. in 1991, there have been myriad studies involving the antioxidant properties of phenothiazine and its derivatives. But until recently, little information was available regarding the structure-activity relationships with respect to antioxidant properties between different phenothiazine derivatives. In 2012 the Yang group from University of North Texas Health Science Center investigated the structure-activity relationships between several phenothiazine analogues (Table 2.1).⁶⁷ The derivatives comprise three major modifications of phenothiazine (Figure 2.3), namely,

Table 2.1. EC₅₀ values for viability, ROS of the phenothiazine analogues in the HT-22 glutamate toxicity assay.

Drug	Viability (nM)	ROS (nM)
Phenothiazine	19	57
Promethazine	1070	9700
Methylene blue	18	20
Toluidine blue	1.8	0.1
Neutral red	5800	15760

(i) substitution of the hydrogen at position 10 (promethazine), (ii) introduction of amine groups at positions 3 and 7 (methylene blue and toluidine blue) and (iii) substitution of sulfur with nitrogen at position 5 (neutral red). The parent compound phenothiazine was highly effective in a glutamate neurotoxicity assay, as well as in mitigating ROS (Table 2.1). In the glutamate neurotoxicity assay, glutamate blocks the glutamate/cysteine antiporter due to saturating concentrations of extracellular glutamate, causing depletion of cellular glutathione and increasing ROS. The results were also similar for iodoacetic acid (IAA) and rotenone neurotoxicity assays. The second compound, promethazine, having a side chain at the 10-position was less potent in all of the assays (Table 2.1). The third group of compounds having amine side chains at the 3 and 7 positions (MB and TB) were the most potent in all of the assays. The fourth group consisted of the compound neutral red (NR). NR is the phenazine analogue of TB. It showed minimum potency in the neuroprotective assays. In conclusion, the structure-activity relationship study indicated the importance of having hydrogen at the 10-position as well as having sulfur at position 5. Although phenothiazine had similar potency compared to MB and TB in the neurotoxicity assays, MB and TB are better drug candidates because they have the unique capability of acting as electron shunts between complexes I and III.⁶⁷

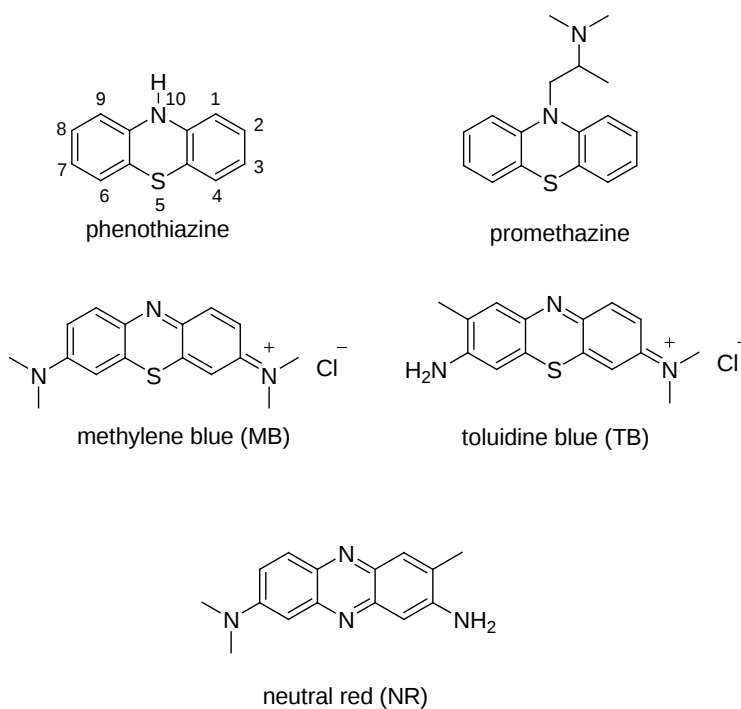


Figure 2.3. Chemical structures of the phenothiazine analogues compared for antioxidant properties.

Distinct neuroprotective action of MB

Mitochondrial dysfunction is a pathological condition that is a common feature of many neuropathological disorders including Alzheimer's disease, Parkinson's disease and stroke. Several antioxidants have been studied extensively for the treatment of neurological disorders. For example, CoQ₁₀ has been used as an endogenous electron transport chain (ETC) supplement to treat Parkinson's disease, singly or in combination with other antioxidants such as creatine. Estrogen has been found to increase gene expression for many key components of the ETC. However, none of these treatments proved successful in clinical studies, casting doubt on such neuroprotective strategies. Inhibition of mitochondrial complex IV with KCN and complex V with oligomycin demonstrated that inhibition reduced cell growth but did not result in cytotoxicity or cell death. On the contrary, inhibition of complex I with rotenone or complex III with

antimycin caused direct cytotoxicity and cell death. This is presumably because inhibition of complex I/III caused leakage of electron, which in turn increased ROS and induced cytotoxicity. Since complexes IV and V are not involved in electron leakage and ROS production, cells survive by anaerobic glycolysis and lactate production. Therefore, bypassing complex I/III can be an alternative neuroprotective strategy. Studies show that MB mediated electron transfer is insensitive to complex I/III inhibition, indicating an alternate route of electron transfer.⁶⁸ Due to the low reduction potential (11 mV), MB can easily cycle between oxidized and reduced forms.⁶⁹ Upon completion of a cycle, electrons from NADH are delivered to cytochrome *c* bypassing the inhibition of complexes I and III.

MB in photodynamic therapy (PDT)

Phenothiazinium dyes acting as photosensitizers in PDT treatment of various cancers, and in antimicrobial research have been studied extensively.^{70,71} In PDT, a combination of photosensitizing agent and light causes the formation of singlet oxygen (ROS), which is responsible for tumor death, either by necrosis or apoptosis. Photodynamic antimicrobial chemotherapy (PACT) is also based on the same principle. For decades it has been known that phenothiazinium dyes like MB can stain microbes. It is therefore possible to kill the microbes by illumination. PACT can be used for the disinfection of blood samples before blood transfusion.⁷²⁻⁷⁵

MB is a primary candidate in the PDT therapy of several cancers. In aqueous solution it produces an intense blue color. Due to its extended conjugation system, the energy difference between the electronic states of MB is reduced such that it strongly absorbs visible light in the region 600-700 nm, thus allowing the rest of the visible spectrum to be

transmitted (350-600 nm). MB acts as an effective photosensitizer because it can pass on the absorbed light energy to nearby molecules.

There have been extensive structure-activity studies related to the photodynamic action of phenothiazinium dyes.⁷⁶⁻⁷⁹ The photodynamic action of a photosensitizer is largely dependent on the lipophilicity (relative solubility in lipids and water, log P) of the molecules. MB is an effective phototoxic agent against a variety of tumor cell lines *in vitro*.^{79,80} MB demonstrated effective photodynamic activity *in vivo*, when administered directly into the tumor. When solid Ehrlich carcinomas were treated with intratumoral MB followed by illumination with red light, significant tumor reduction and destruction was observed.⁸¹ Nonetheless, there has been only limited success in clinical study of MB,^{82,83} especially when injected intravenously or intravesically, which might be a result of poor localization of MB in tumors due to its high hydrophilicity. MB has a log P value of -1.0,⁷⁶ which means when partitioned between water and octanol, MB will be mostly in the aqueous phase.

In order to draw a correlation between photodynamic activity and lipophilicity, Mellish et al. synthesized several MB analogues with varying alkyl chains on the exocyclic amines (Figure 2.4).⁷⁶ The log P values followed the order

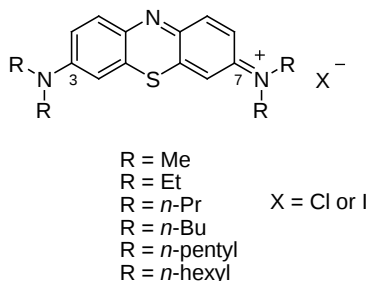


Figure 2.4. Chemical structures of lipophilic MB analogues.

n-pentyl > *n*-hexyl > *n*-butyl > *n*-propyl > ethyl > methyl. *In vitro* phototoxicity toward RIF 1 fibrosarcoma cells followed the order *n*-propyl > *n*-pentyl > *n*-butyl > *n*-hexyl > ethyl > methyl. As expected, *in vitro* phototoxicity on the lipophilic MB analogues were better compared to MB itself due to improved cellular uptake, although the order was somewhat ambiguous.⁷⁶

Designing the target compounds

Due to the negative charge in the membrane of the mitochondrial matrix and high potential gradient through the inner membrane, lipophilic compounds with delocalized positive charge concentrate in much higher amounts in mitochondria than other organelles. Therefore, in analogy with the study of Mellish et al., the use of lipophilic MB analogues having long alkyl chains might be a good strategy to target mitochondria. However the position of the alkyl chain is crucial. N-alkylated MB analogues are lipophilic and relatively easy to synthesize. But MB is metabolized *in vivo*, yielding the demethylated analogues, azure A and azure B (Figure 2.6).⁸⁴ These demethylated analogues are found in the urine of the patients treated with MB. Therefore, in principle, the alkyl side chains may be removed during metabolism, changing the polarity of the compounds. To eliminate this possibility our analogues were designed such that the long alkyl chains are positioned at one of the six available aromatic positions. In addition, to

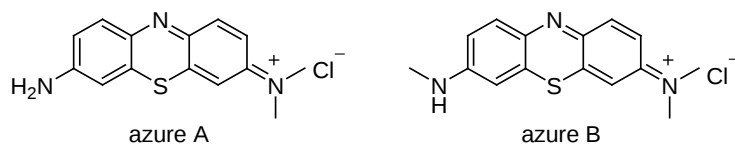


Figure 2.5. Chemical structures of azure A and azure B.

alter the electronic properties of the analogues, which might influence the antioxidant properties, the dimethylamine moieties at 3 and 7 positions were substituted with

different secondary amines including piperidine and morpholine. Figure 2.6 shows the chemical structures of the synthesized MB analogues.

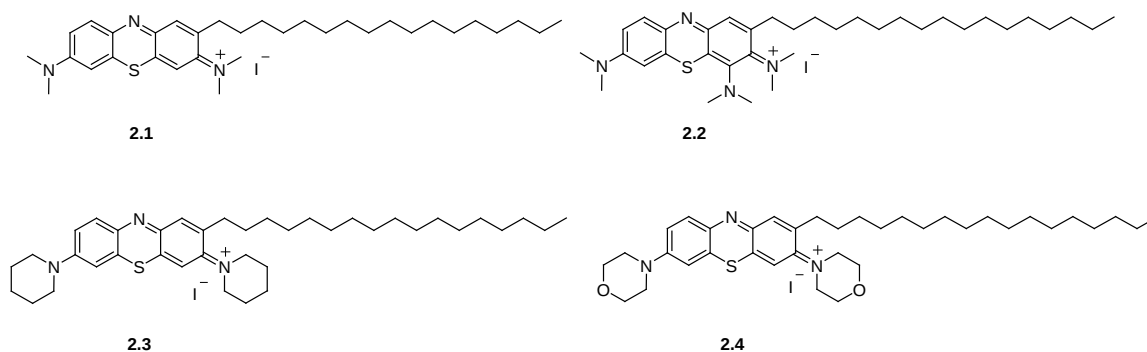


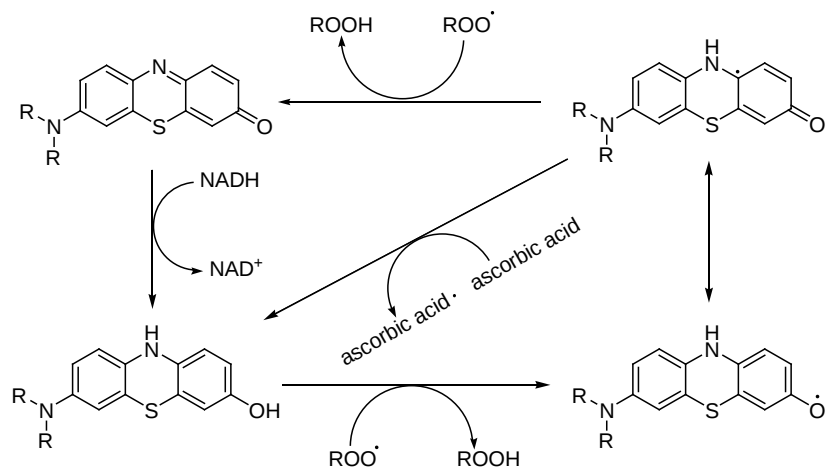
Figure 2.6. Chemical structures of the synthesized MB analogues.

Designing methylene violet analogues

The substantial hydrophilicity of MB may restrict its transport across the plasma membranes of some mammalian cells, which would also limit its cellular uptake. For this reason, we have been interested in MB analogues with increased hydrophobicity.

Methylene violet (MV) is a neutral phenothiazine dye.⁸⁵ Hydrolysis of MB under strongly basic condition yields MV. In addition to improved hydrophobicity, a structure-activity relationship can be drawn between MB and MV. MV can be looked upon as a phenolic analogue of MB where a hydroxyl group substitutes one of the two dimethylamine moieties. Although MV is naturally obtained in the oxidized (quinone) form, it can be reduced by the mitochondrial redox centers generating the phenolic (quinol) form. The reduced form of MV can act as phenolic antioxidants similar to CoQ₁₀. The proposed mechanism of antioxidant activity of MV is shown in Scheme 2.1.

Scheme 2.1. Proposed mechanism of antioxidant activity of MV analogues.



In order to modulate the polarity of the molecules, alkyl side chains having varying numbers of carbon atoms were attached either to the exocyclic amine at position 3 or on the aromatic ring at position 6. Figure 2.7 shows the MV analogues synthesized having aliphatic substituents on the exocyclic nitrogen atom.

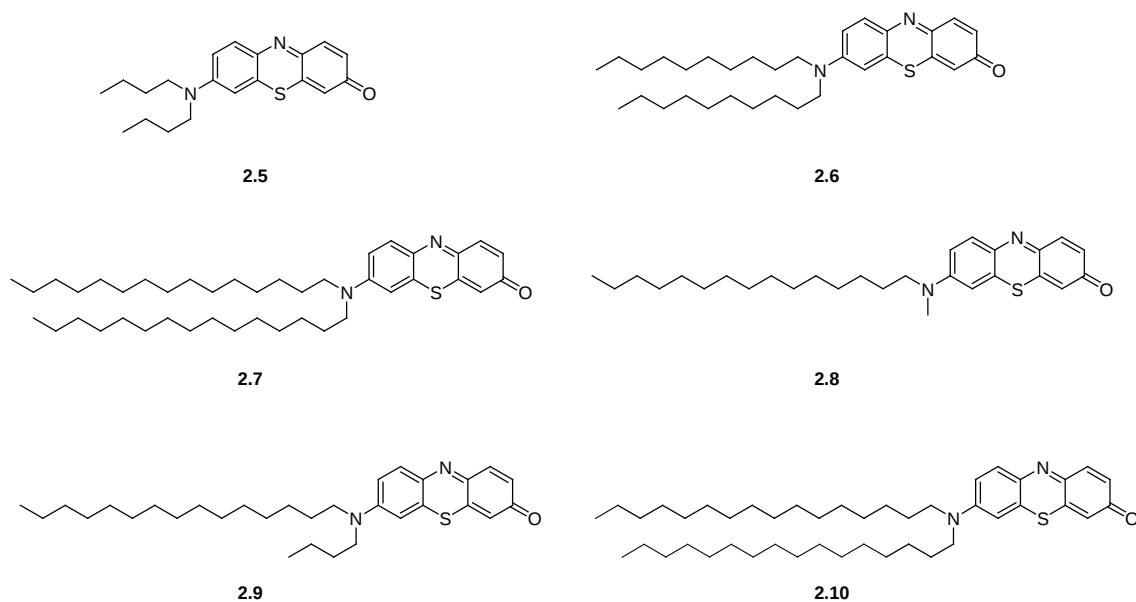


Figure 2.7. MV analogues having long chain alkyl groups on the exocyclic amine.

Figure 2.8 illustrates the MV analogues carrying long chain alkyl groups on the aromatic ring. In addition, the dimethylamine moiety was also substituted with piperidine

and morpholine (**2.12** and **2.13**) to explore the effect on the antioxidant properties of the molecules.

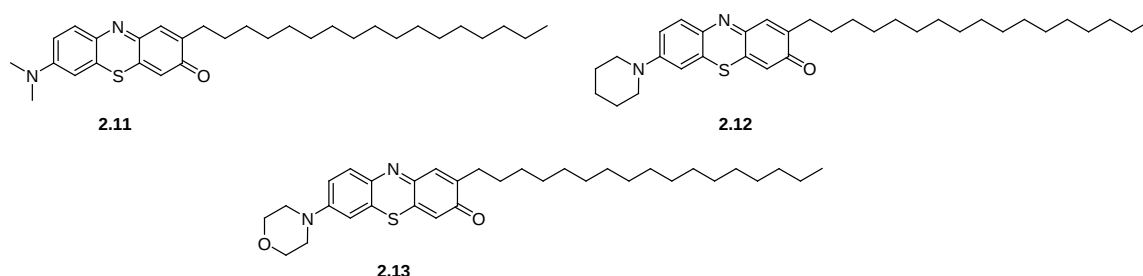


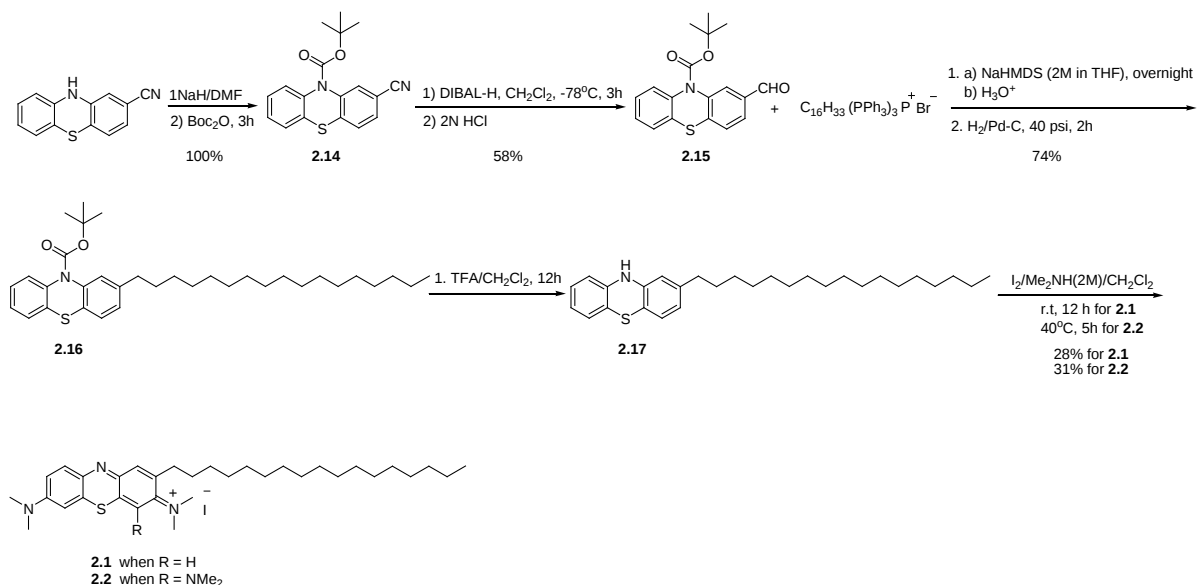
Figure 2.8. MV analogues having long chain alkyl substituents on the aromatic ring.

2.2 Results

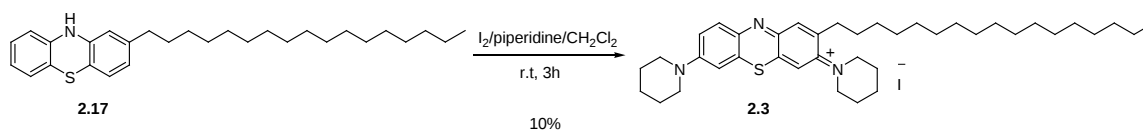
Synthesis of the MB and MV analogues

The synthesis of the lipophilic MB analogues started with the protection of the hydrogen at position 10 of commercially available 2-cyanophenothiazine. Treatment of 2-cyanophenothiazine with 60% NaH at 0 °C yielded the corresponding anion which was subsequently treated with di-*tert*-butyl dicarbonate to obtain **2.14** in quantitative yield. Reductive hydrolysis of **2.14** by DIBAL-H and ~2 N HCl afforded **2.15** in 58% yield.⁸⁶ Compound **2.15** was subjected to the Wittig reaction with hexadecyltriphenylphosphonium bromide in the presence of 1 M NaHMDS to afford an intermediate alkene as a *cis-trans* mixture; this was subsequently reduced by hydrogenolysis using palladium-on-carbon as catalyst to obtain the corresponding alkane **2.16** in 74% yield over two steps. In the next step, the Boc protecting group was removed using 10 equivalents of trifluoroacetic acid to obtain **2.17**. Oxidation of **2.17** with iodine in CH₂Cl₂ followed by the subsequent addition of different secondary amines afforded **2.1-2.4** in varying yields (Scheme 2.2-2.4).⁸⁷

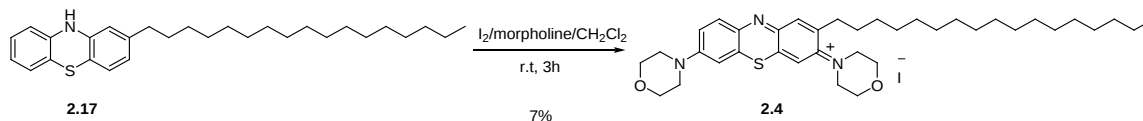
Scheme 2.2. Synthesis of 2.1 and 2.2.



Scheme 2.3. Synthesis of 2.3.

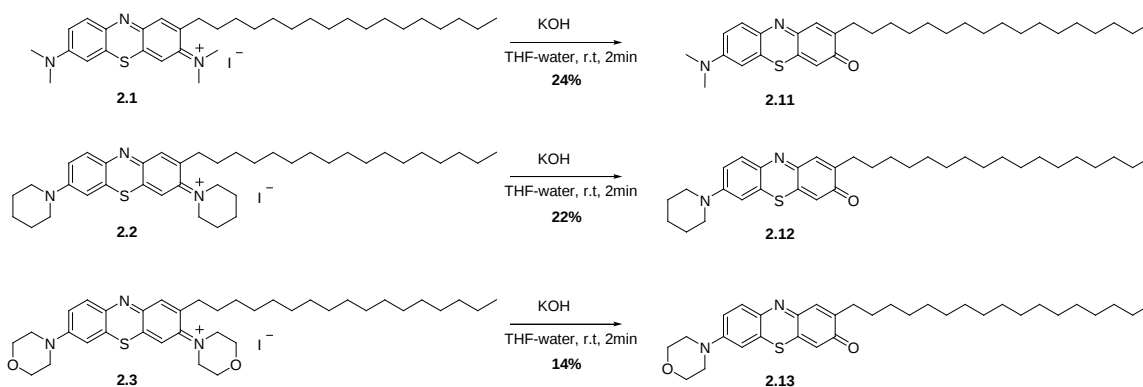


Scheme 2.4. Synthesis of 2.4.



MV analogues **2.11-2.13** were obtained by the basic hydrolysis of **2.1**, **2.3** and **2.4**, respectively (Scheme 2.5).

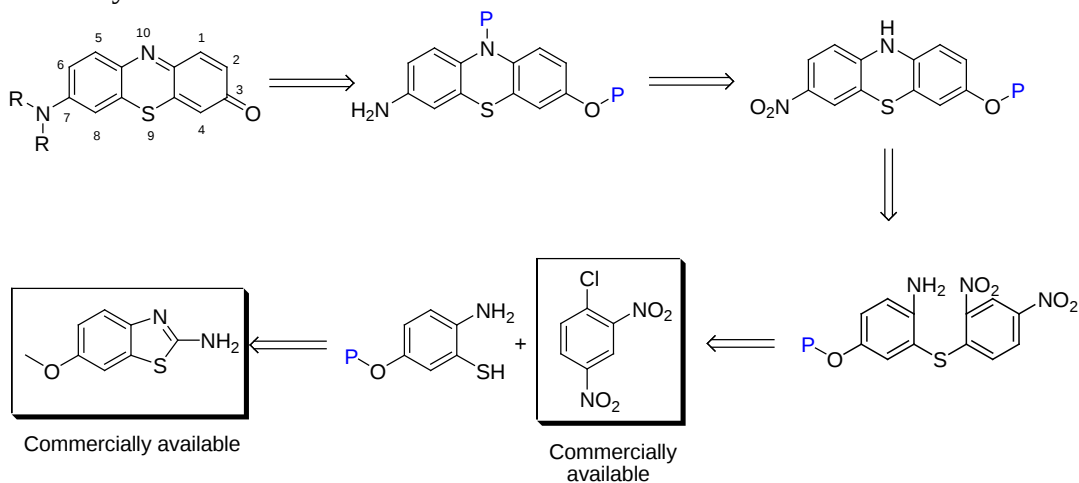
Scheme 2.5. Synthesis of **2.11-2.13**.



The synthesis of the MV analogues having long alkyl substituents on exocyclic nitrogen atoms was carried out using the Smiles rearrangement⁸⁸ as one of the key steps.

The retrosynthetic analysis is shown in Scheme 2.6.

Scheme 2.6. Retrosynthetic analysis for MV analogues having long alkyl substituents on the exocyclic amine.

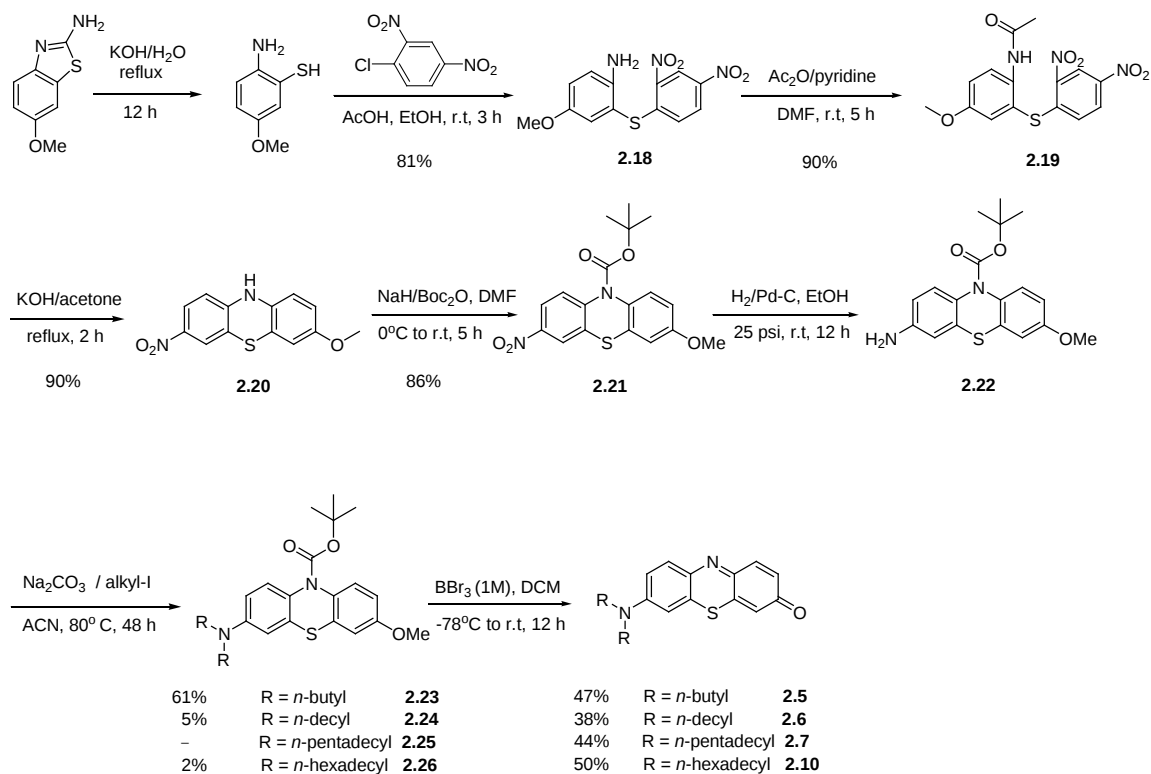


P = protecting group

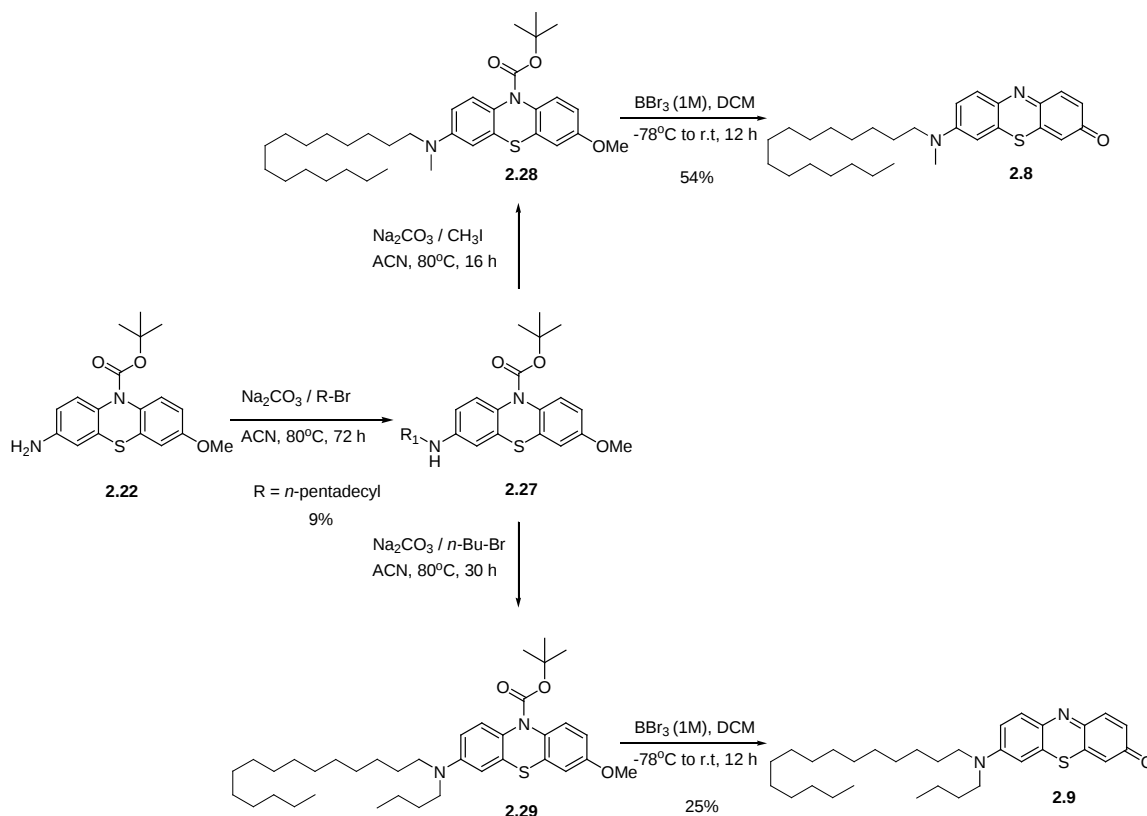
The syntheses are shown in Schemes 2.7 and 2.8. This process was especially helpful for the synthesis of MV analogues carrying secondary amines having different alkyl chains (**2.8** and **2.9**). Hydrolysis of 2-amino-6-methoxybenzothiazole under strongly basic conditions afforded 2-amino-5-methoxybenzothiophenol which was coupled with 2, 4-dinitrochlorobenzene to obtain substituted diphenylsulfide intermediate **2.18** in 81%

yield.⁸⁹ Compound **2.18** was treated with acetic anhydride in order to activate the amine as acetamide intermediate **2.19**. Smiles rearrangement of **2.19** under mildly basic conditions afforded the substituted phenothiazine intermediate **2.20** in 90% yield.⁸⁹ Compound **2.20** was subsequently protected with a *tert*-butoxycarbonyl (Boc) group to obtain **2.21** in 86% yield. Hydrogenolysis of **2.21** afforded **2.22**, which was alkylated with different alkyl iodides/bromides in the presence of Na₂CO₃ as base to obtain **2.23-2.29** in varying yields. In order to remove the O-methyl group to afford the free hydroxyl group at position 7, compounds **2.23-2.29** were treated with 1 M BBr₃ in CH₂Cl₂. The HBr, formed *in situ*, effected removal of the Boc group at the same time generating the desired MV analogues **2.5-2.10** in yields varying from 25% to 54%.

Scheme 2.7. Synthesis of compounds **2.5-2.7** and **2.10**.



Scheme 2.8. Synthesis of compounds 2.8 and 2.9.



Biochemical and biological evaluation of some chosen analogues

Cytotoxicity

Cytotoxicity was assessed by the dye exclusion method. Live cells have intact membranes, which exclude a variety of dyes that easily penetrate the damaged, permeable membranes of non-viable cells. Propidium iodide, a membrane impermeant dye, is generally excluded from viable cells, although it can enter non-viable cells and bind to double-stranded DNA by intercalating between base pairs. Methylene blue analogues were tested for their cytotoxicity in FRDA lymphocytes using FACS analysis with propidium iodide. The results are depicted in Figure 2.9. From the results it is evident that cytotoxicity decreases with increasing lipophilicity in lower as well as higher concentrations for both MB and MV analogues.

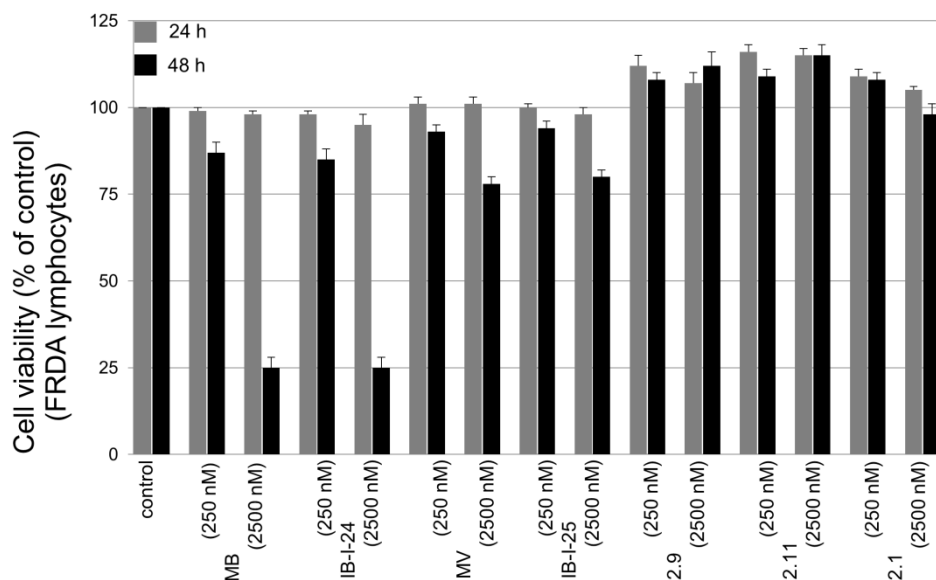


Figure 2.9. Cytotoxicity measurement for the test compounds in cultured FRDA lymphocytes. Experiment performed by Dr. Omar Khmour. Compounds **IB-I-24** and **IB-I-25** (Figure 2.14) were synthesized by Indrajit Bandyopadhyay.

Suppression of reactive oxygen species

The ability of MB analogue **2.1** and MV analogue **2.11** to suppress ROS induced by the depletion of cellular glutathione was evaluated in FRDA lymphocytes in a quantitative FACS experiment using dichlorofluorescein diacetate (DCFH-DA)⁹⁰ as an indicator of intracellular ROS levels. In cells DCFH-DA is hydrolyzed by esterases to afford 2, 7-dichlorodihydrofluorescein (DCFH). The non-fluorescent DCFH can be oxidized by cellular oxidants generating fluorescent dichlorofluorescein (DCF). The results are shown in Figure 2.10, and demonstrate that compounds **2.1** and **2.11** were more potent and effective in protecting FRDA lymphocytes than the parent compounds MB and MV, respectively, and did so in a concentration dependent manner.

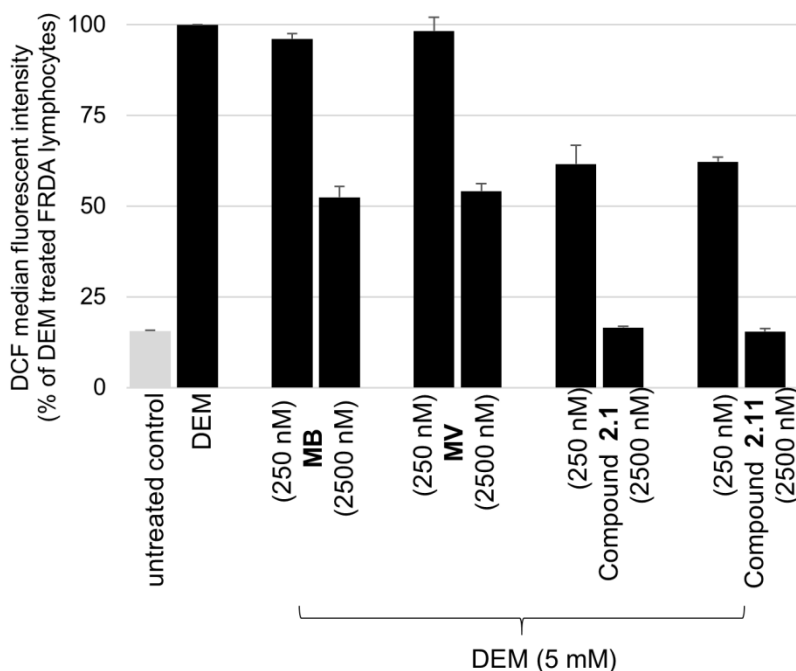


Figure 2.10. Suppression of ROS production by the test compounds in cultured FRDA lymphocytes pretreated with DEM. Experiment performed by Dr. Omar Khmour.

Preserving mitochondrial inner membrane potential ($\Delta\Psi_m$)

The ability of **2.1** and **2.11** to maintain mitochondrial membrane potential ($\Delta\Psi_m$) compared to the parent compounds MB and MV was studied.⁹¹ $\Delta\Psi_m$ was determined using the cationic fluorescent dye 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1), which is a lipophilic cation that selectively accumulates in mitochondria due to the negative potential across the inner mitochondrial membrane. The dye exists as a monomer at low concentrations giving a green fluorescence. At higher concentrations the dye forms J-aggregates, which exhibit red fluorescence. Therefore, mitochondrial depolarization is indicated by a decrease in red/green fluorescence ratio. Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a commonly used uncoupler of oxidative phosphorylation in mitochondria, was

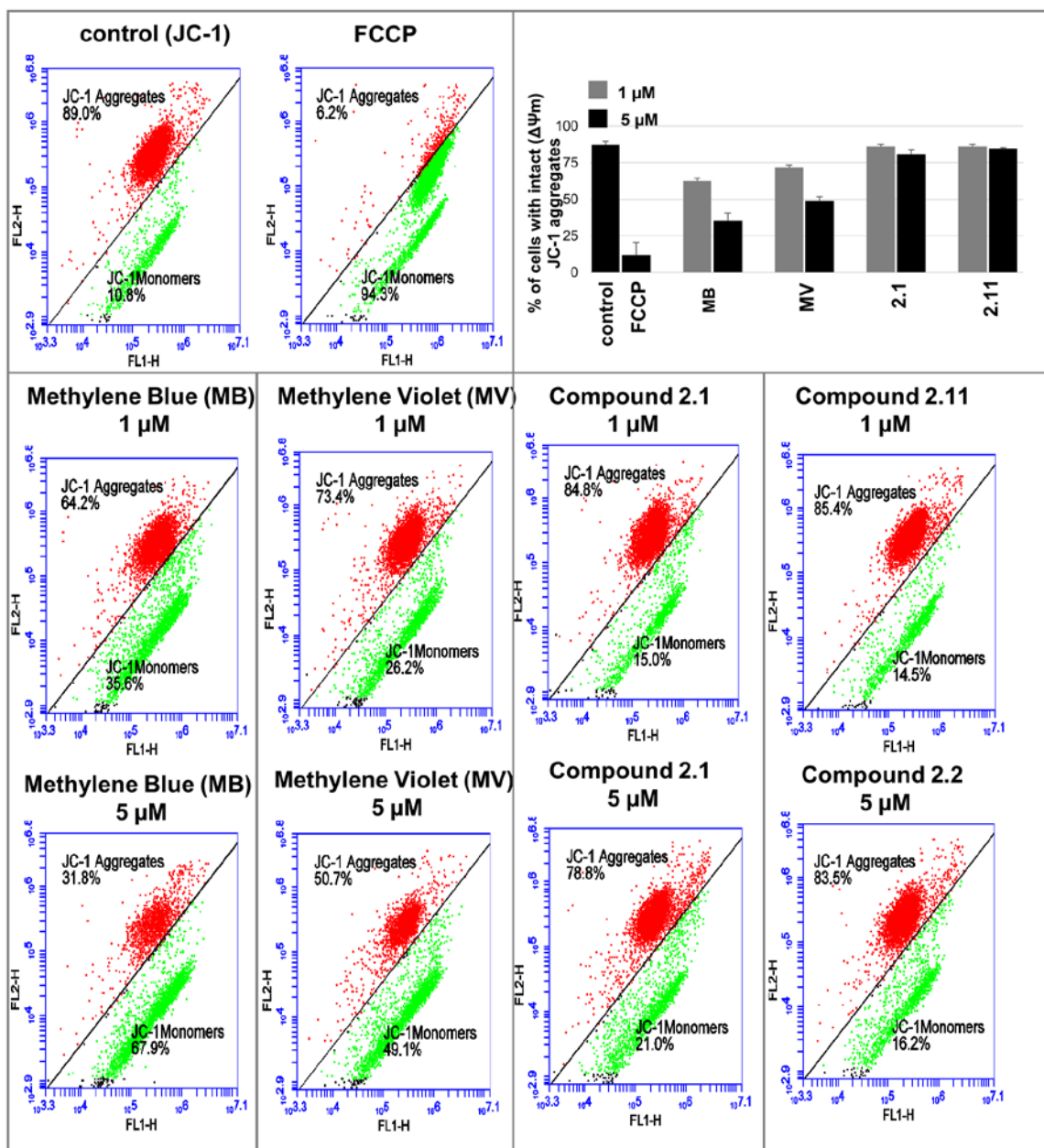


Figure 2.11. Flow cytometric analysis showing the effect of the test compounds on $\Delta\psi_m$ in FRDA lymphocyte cells using the ratiometric fluorescent probe JC-1. Red regions represent intact mitochondrial membranes with JC-1 aggregates (red fluorescence), whereas the gated regions in green depict cells with loss of $\Delta\psi_m$. The bar graph shows the percentage of cells with intact $\Delta\psi_m$ calculated using *Accuri*TM C6 software. Experiment performed by Dr. Omar Khmour.

used as a negative control to dissipate the chemiosmotic proton gradient, which results in depolarization of mitochondrial membrane potential. Figure 2.11 summarizes the results

of this study. Clearly compounds **2.1** and **2.11** were more effective in preserving mitochondrial membrane potential than the parent compounds MB and MV.

Cytoprotection

The ability of **2.1** and **2.11** to demonstrate cytoprotection was measured in FRDA lymphocytes.^{92,93} The cells were treated with rotenone to induce cytotoxicity by inhibiting complex I. The results are shown in Figure 2.12. Compounds **2.1** and **2.11** protected the cells against rotenone cytotoxicity more efficiently than MB and MV, in agreement with the earlier studies.

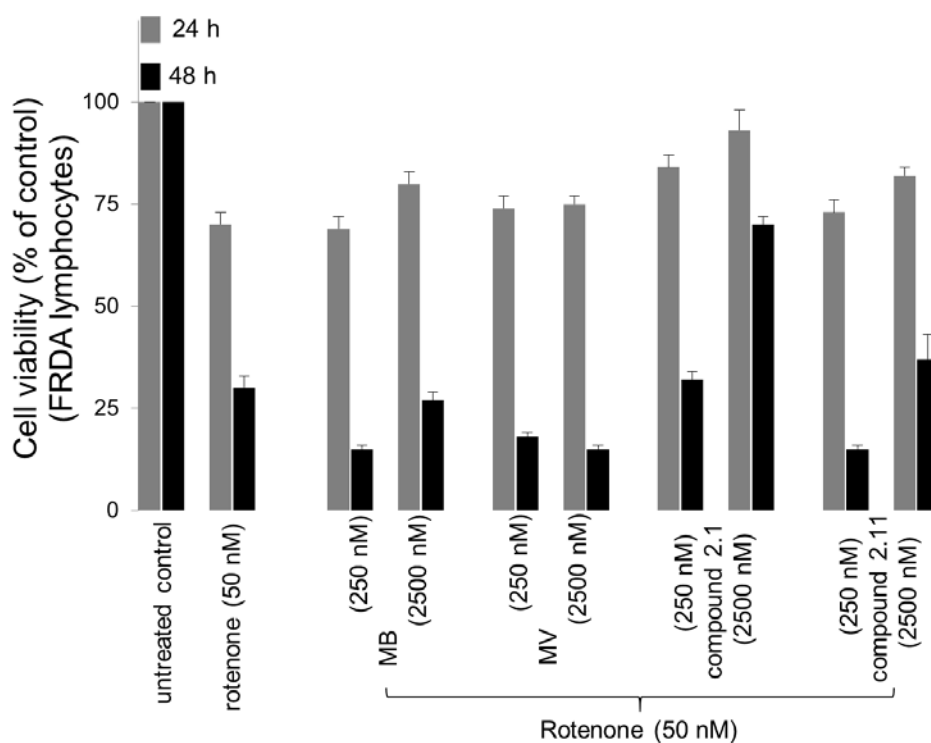


Figure 2.12. Cytoprotective effects of the test compounds on the viability of cultured FRDA lymphocytes treated with rotenone. Experiment performed by Dr. Omar Khmour.

Determination of ATP levels

ATP levels in FRDA lymphocytes were measured using firefly luciferase.⁹³ The cells were grown on glucose-free media supplemented with galactose for 24 h prior to the

addition of the test compounds. Since cells grown in galactose rely mostly on oxidative phosphorylation (OX-PHOS) to produce their ATP, they become more sensitive to mitochondrial respiratory chain inhibitors than cells grown in glucose medium. The results are shown in Figure 2.14. MB, MV, **IB-I-24** and **IB-I-25** strongly reduce cellular ATP levels in a concentration dependent manner. Compound **2.11** maintains cellular ATP levels in all concentrations. Compounds **2.1** and **2.9** maintain ATP levels upto 2.5 μM concentration, beyond that they reduce ATP levels significantly.

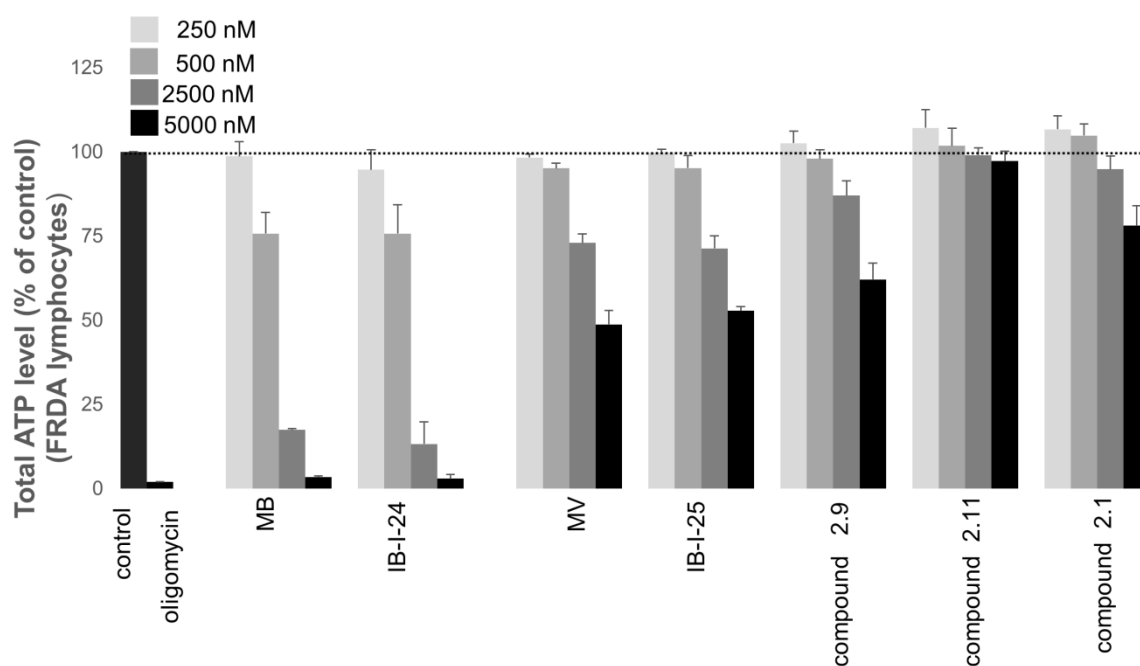


Figure 2.13. Total ATP concentration in FRDA lymphocytes, upon incubation with the test compounds for 24 and 48 h. Experiment performed by Dr. Omar Khmour. Compounds **IB-I-24** and **IB-I-25** (Figure 2.14) were synthesized by Indrajit Bandyopadhyay.

2.3 Discussion

Although there have been numerous reports of synthesis of phenothiazinium analogues, most of them lack evidence of purity such as NMR analysis, elemental analysis and HPLC analysis.^{76,78} Strekwoski et al. demonstrated a facile synthesis of 3, 7-

di(alkylamino)phenothiazinium salts by the oxidation of commercially available phenothiazine by iodine followed by the sequential addition of dialkylamines.⁸⁷ Although the final yields of the phenothiazinium salts produced by this method were lower, the method was used because of relatively smaller number of steps involved. The synthesis of hydrophobic MB analogues was divided into two parts; the addition of long alkyl substituent to the phenothiazine core through functional group interconversion followed by oxidative addition of the amines. In order to attach a long alkyl substituent on the aromatic ring, commercially available 2-cyanophenothiazine was selected as the starting material (Scheme 2.2). The secondary amine at position 10 was protected using a *tert*-butyloxycarbonyl (Boc) group. This step resulted in a quantitative yield of **2.14**. In the next step the cyano group at position 2 was converted to an aldehyde by reductive hydrolysis. This step gave a 58% yield of the aldehyde **2.15**. The aldehyde was then converted to an alkene by the Wittig reaction with commercially available hexadecyltriphenylphosphonium bromide under strongly basic conditions. The alkene was obtained as a *cis-trans* mixture, which was subsequently reduced to alkane **2.16** by hydrogenolysis using palladium-on-carbon (Pd-C) as catalyst. The use of hydrogen at atmospheric pressure was not sufficient for the reduction to proceed. Therefore, an elevated hydrogen pressure (40 psi) was used along with 20% Pd-C. Compound **2.16** was obtained in 74% yield over two steps. In the subsequent step, the protecting group at position 10 was removed using trifluoroacetic acid (TFA) to obtain the 2-alkylphenothiazine **2.17**. This step gave the compound in almost quantitative yield. The oxidation and subsequent addition of an amine to the alkylphenothiazine **2.17** could be done in one or two steps. A one-step procedure was followed, since in the two-step

procedure isolation of the oxidized phenothiazine proved difficult. The MB analogues were obtained in low yields ranging from 7% (for **2.4**) to 31% (for **2.2**) (Schemes 2.2-2.4).

The MV analogues were obtained following two different procedures. Compounds **2.11**, **2.12** and **2.13** were obtained by the basic hydrolysis of MB analogues **2.1**, **2.3** and **2.4** independently, albeit in poor yields. Compounds **2.5-2.10** were synthesized using the Smiles rearrangement where functionalized phenothiazines are prepared by the intramolecular rearrangement of functionalized diphenyl sulfides under basic conditions (Scheme 2.7). The diphenyl sulfide intermediate **2.18** was prepared by the coupling of 2-amino-5-methoxybenzothiol and 2, 4-dinitrochlorobenzene under mildly acidic condition. The highly unstable 2-amino-5-methoxybenzothiol was prepared by the basic hydrolysis of 2-amino-5-methoxybenzothiazole at reflux. The thiol was used for the coupling step without isolation or further purification from the reaction mixture. The high yield of **2.18** (81%) indicates the efficient formation of the thiol intermediate. Compound **2.18** was then activated as its acetamide derivative (**2.19**) by treatment with acetic anhydride. Smiles rearrangement of **2.19** under mildly basic condition yielded the functionalized phenothiazine **2.20** in 90% yield. Subsequently the nitro group at position 7 was reduced to afford an aromatic amine, which was alkylated using different alkyl iodides/bromides under basic conditions to obtain compounds **2.23-2.29**. Removal of the Boc and methyl groups by BBr_3 afforded the final compounds **2.5-2.10** in varying yields.

A number of MB analogues have been designed and synthesized with variations in their redox core and their side chain that could enable the compounds to protect against

oxidative stress, augment ATP levels and enhance mitochondrial function. The preliminary biochemical results for some of the compounds synthesized are presented in



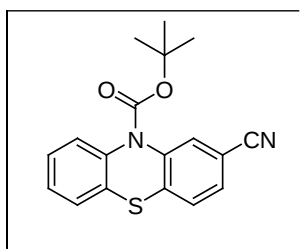
Figure 2.14. Chemical structures of IB-I-24 and IB-I-25. The compounds were synthesized by Indrajit Bandyopadhyay.

this thesis. The strong cytotoxicity of MB itself in FRDA lymphocytes at 0.5-2.5 μM concentrations was surprising, given its long clinical use. A nutrient-sensitized screening strategy was used by culturing the FRDA cells on galactose as the sole sugar source, forcing them to rely on mitochondrial OX-PHOS to produce their ATP; consequently they became more sensitive to respiratory chain inhibitors than cells grown on glucose. Figure 2.9 shows that MB was cytotoxic when used at 2.5 μM concentration for 48 h. The modified MB/MV analogues with long alkyl side chains (**2.1**, **2.9** and **2.11**) were not significantly cytotoxic under any tested condition, although two analogues with shorter side chains (**IB-I-24** and **IB-I-25**, Figure 2.14) were somewhat cytotoxic. Shown in Figure 2.10 are the abilities of the new MBs to suppress ROS production in FRDA lymphocytes depleted of glutathione by using 5 mM diethyl maleate. The MB analogues preserved the mitochondrial membrane potential of FRDA lymphocytes. FACS analysis of $\Delta\psi_m$ showed that MB and MV significantly depolarized $\Delta\psi_m$, while our analogues did not (Figure 2.11). The three long alkyl chain MB analogues also restored ATP levels in FRDA lymphocytes (Figure 2.13). In contrast, MB itself inhibited ATP production, especially at higher concentrations. The side chain modifications also had a dramatic effect on their ability to afford cytoprotection to rotenone-treated FRDA lymphocytes

(Figure 2.12). Two other analogues (**IB-I-24** and **IB-I-25**) did not exhibit favorable activity in increasing ATP levels (Figure 2.13).

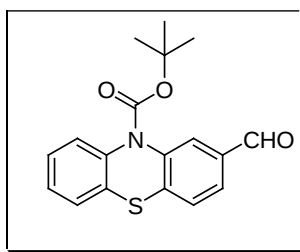
2.4 Experimental

Chemicals and solvents were purchased as reagent grade from Sigma-Aldrich Chemicals and were used without further purification. All the reactions were performed under an argon atmosphere, unless otherwise specified. Thin-layer chromatography (TLC) plates (precoated glass plates with silica gel 60 F254, 0.25 mm thickness) were used for analytical TLC and were visualized by UV irradiation (254 nm). Flash chromatography was carried out using Silicycle 200–400 mesh silica gel. ^1H and ^{13}C NMR spectra were obtained using a Varian 400 MHz NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) and are referenced to residual CHCl_3 (δ 7.26 ppm for ^1H NMR and δ 77.16 for ^{13}C NMR) as the internal standard. Splitting patterns are designated as s, singlet; d, doublet; m, multiplet. High-resolution mass spectra were obtained at the Michigan State Mass Spectrometry Facility or the Arizona State University CLAS High Resolution Mass Spectrometry Facility.



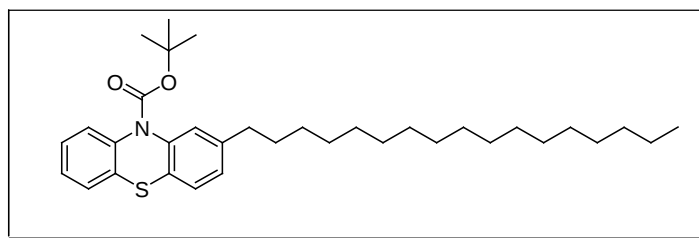
***tert*-Butyl 2-Cyano-10*H*-phenothiazine-10-carboxylate (2.14).** 0.74 g (3.30 mmol) of 2-cyanophenothiazine was dissolved in 10 mL of anhydrous DMF. The reaction mixture was cooled to 0 °C and 0.40 g (9.90 mmol) of 60% NaH was added. The dark reaction mixture was stirred at 0 °C for an additional 15 min and 0.90 g (3.96 mmol) of di-*tert*-

butyl dicarbonate was added. The reaction mixture was stirred at room temperature for 3 h and was diluted with 50 mL of brine. The aqueous layer was extracted with three 25-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO_4 and concentrated under diminished pressure. The crude product was purified on a silica gel column (10 $\times$ 2 cm). Elution with ethyl acetate-hexanes (1:9) gave **2.14** as a pale yellow solid: yield 1.07 g (100%); silica gel TLC R_f 0.26 (1:9 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 1.49 (s, 9H), 7.16-7.20 (m, 1H), 7.28-7.33 (m, 2H), 7.40 (s, 2H), 7.49 (d, 1H, J = 8.4 Hz) and 7.80 (s, 1H); ^{13}C NMR δ 28.1, 83.1, 110.2, 118.2, 126.6, 127.2, 127.3, 127.5, 128.1, 129.1, 130.3, 130.6, 137.7, 139.0, 139.1 and 151.8; mass spectrum (APCI), m/z 325.1017 ($\text{M}+\text{H}^+$) ($\text{C}_{18}\text{H}_{17}\text{N}_2\text{O}_2\text{S}$ requires m/z 325.1011).



tert-Butyl 2-Formyl-10H-phenothiazine-10-carboxylate (2.15). To a solution of 1.07 g (3.30 mmol) of **2.14** in 10 mL of anhydrous CH_2Cl_2 was added dropwise at -78°C 4.0 mL (4.0 mmol) of 1 M DIBAL-H in toluene. The reaction mixture was stirred at -78°C for 3 h and was diluted with ~ 2 N HCl. The aqueous layer was extracted with three 30-mL portions of CH_2Cl_2 . The combined organic extract was dried over anhydrous MgSO_4 and was concentrated under diminished pressure. The residue was purified on a silica gel column (7 $\times$ 4 cm). Elution with 1:9 ethyl acetate-hexanes afforded **2.15** as a yellow solid: yield 0.63 g (58%); silica gel TLC R_f 0.17 (1:9 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 1.48 (s, 9H), 7.14-7.18 (m, 1H), 7.26-7.32 (m, 2H), 7.44 (d, 1H, J = 8.0 Hz),

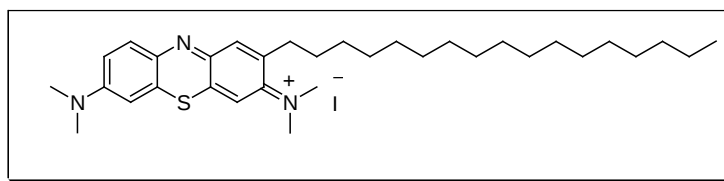
7.52 (d, 1H, $J = 7.4$ Hz), 7.64 (d, 1H, $J = 8.0$ Hz), 7.99 (s, 1H) and 9.96 (s, 1H); ^{13}C NMR δ 28.1, 82.8, 126.5, 126.6, 127.1, 127.3, 127.4, 127.8, 128.3, 130.5, 135.1, 137.9, 139.1, 140.2 and 152.1; mass spectrum (ESI), m/z 328.1003 ($\text{M}+\text{H}^+$) ($\text{C}_{18}\text{H}_{18}\text{NO}_3\text{S}$ requires m/z 328.1007).



tert-Butyl 2-Heptadecyl-10H-phenothiazine-10-carboxylate (2.16). To a solution of 0.64 g (1.13 mmol) of hexadecyltriphenylphosphonium bromide in 5 mL of anhydrous THF at -78 °C was added dropwise 1.20 mL (1.20 mmol) of 1 M sodium bis-(trimethylsilyl)amide in THF. The yellow reaction mixture was stirred at 0 °C for 3 h and was cooled to -78 °C. To this bright orange suspension was added dropwise 0.37 g (1.13 mmol) of **2.15** dissolved in 5 mL of anhydrous THF. The light yellow reaction mixture was stirred at 0 °C under an argon atmosphere overnight and was then diluted with 50 mL of brine. The aqueous layer was extracted with three 25-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO_4 and was concentrated under diminished pressure. The residue was purified on a silica gel column. Elution with 1:9 CH_2Cl_2 -hexanes yielded a *cis-trans* mixture as a colorless solid.

To a solution of 0.41 g (0.77 mmol) of the *cis-trans* mixture in 3:7 CH_2Cl_2 -ethanol was added 26 mg of palladium on carbon. The suspension was stirred at room temperature under a H_2 atmosphere (40 psi) for 2 h. The reaction mixture was filtered through a Celite pad. The filtrate was concentrated under diminished pressure to afford **2.16** as a colorless

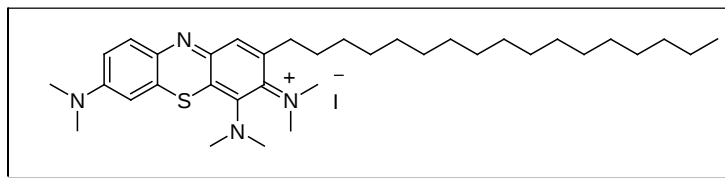
solid: yield 0.41 g (74% over two steps); silica gel TLC R_f 0.1 (1:9 CH_2Cl_2 -hexanes); ^1H NMR (CDCl_3) δ 0.88 (t, 3H, $J = 7.0$ Hz), 1.26-1.30 (m, 28H), 1.49 (s, 9H), 1.60 (t, 2H, $J = 7.2$ Hz), 2.59 (t, 2H, $J = 7.6$ Hz), 6.96 (d, 1H, $J = 7.6$ Hz), 7.12 (t, 1H, $J = 7.6$ Hz), 7.21-7.26 (m, 2H), 7.32-7.35 (m, 2H) and 7.52 (d, 1H, $J = 7.6$ Hz); ^{13}C NMR (CDCl_3) δ 14.3, 22.9, 28.4, 29.5, 29.53, 29.70, 29.74, 29.84, 29.87, 31.7, 32.1, 35.8, 82.0, 126.1, 126.5, 126.6, 127.2, 127.33, 127.37, 127.6, 128.9, 132.7, 138.8, 139.0, 142.0 and 152.7; mass spectrum (APCI), m/z 538.3723 ($\text{M}+\text{H}^+$) ($\text{C}_{34}\text{H}_{52}\text{NO}_2\text{S}$ requires m/z 538.3719).



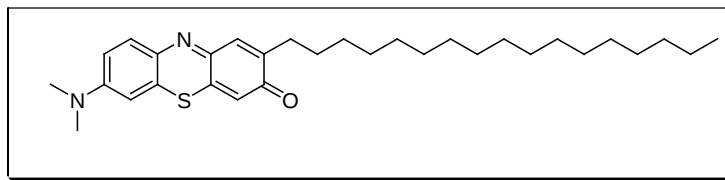
***N*-(7-(Dimethylamino)-2-heptadecyl-3*H*-phenothiazin-3-ylidene)-*N*-methylmethanaminium Iodide (2.1).** To a solution of 0.23 g (0.43 mmol) of **2.16** in 8 mL of anhydrous CH_2Cl_2 was added dropwise 0.26 mL (0.39 g, 3.44 mmol) of trifluoroacetic acid. The reaction mixture was stirred at room temperature for 12 h under an argon atmosphere and was neutralized with 50 mL of saturated NaHCO_3 solution. The aqueous layer was extracted with three 30-mL portions of CH_2Cl_2 . The combined organic layer was dried over anhydrous MgSO_4 and was concentrated under diminished pressure. The crude (**2.17**) was utilized in the next step without further purification.

To a solution of 45 mg of the crude **2.17** in 5 mL of CH_2Cl_2 was added 81.0 mg (0.32 mmol) of iodine followed by 0.25 mL (0.50 mmol) of 2 M dimethylamine in THF. The reaction mixture was stirred at room temperature under an argon atmosphere for 12 h. The crude product was purified on a silica gel column (10 $\times$ 2 cm). Elution with ethyl acetate afforded **2.1** as a green solid: yield 18 mg (28%); silica gel TLC R_f 0.40 (ethyl

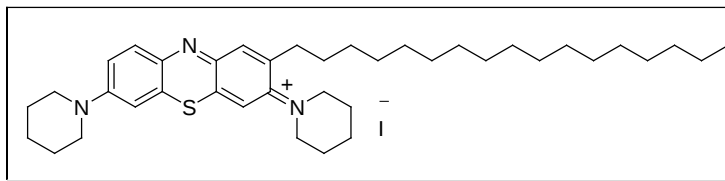
acetate); ^1H NMR (CDCl_3) δ 0.86 (t, 3H, $J = 6.6$ Hz), 1.15-1.37 (m, 28H), 1.69-1.72 (m, 2H), 2.81-2.85 (m, 2H), 3.33 (s, 6H), 3.46 (s, 6H), 7.36-7.38 (m, 1H), 7.41 (s, 1H), 7.76 (m, 1H), 7.89 (s, 1H) and 7.99 (d, 1H, $J = 7.6$ Hz); ^{13}C NMR (CDCl_3) δ 14.2, 22.8, 29.4, 29.7, 29.72, 29.74, 29.76, 29.79, 30.3, 32.0, 34.1, 34.9, 42.6, 44.7, 107.5, 111.6, 119.9, 132.2, 135.9, 137.1, 137.4, 138.5, 138.9, 154.2 and 158.1; mass spectrum (APCI), m/z 522.3882 (M^+) ($\text{C}_{33}\text{H}_{52}\text{N}_3\text{S}$ requires m/z 522.3882).



***N*-(4,7-bis(Dimethylamino)-2-heptadecyl-3*H*-phenothiazin-3-ylidene)-*N*-methylmethanaminium Iodide (2.2).** To a solution 0.10 g of the crude **2.17** in 5 mL of CH_2Cl_2 was added 0.19 g (0.74 mmol) of iodine followed by 1.15 mL (2.30 mmol) of 2 M dimethylamine in THF. The reaction mixture was heated at 45°C under an argon atmosphere for 7 h. The blue reaction mixture was purified on a silica gel column (10×2 cm). Elution with 20% methanol in ethyl acetate afforded **2.2** as a blue solid: yield 40 mg (31%); silica gel TLC R_f 0.35 (4:1 ethyl acetate-methanol); ^1H NMR (CDCl_3) δ 0.86 (t, 3H, $J = 6.8$ Hz), 1.24-1.44 (m, 28H), 1.61-1.63 (m, 2H), 2.71 (m, 2H), 3.28 (s, 6H), 3.30 (s, 6H), 3.49 (s, 6H), 6.17 (s, 1H), 6.89 (d, 1H, $J = 2.8$ Hz), 7.05 (dd, 1H, $J = 9.2$ Hz, $J = 2.4$ Hz) and 7.78 (d, 1H, $J = 9.6$ Hz); ^{13}C NMR δ 14.3, 22.8, 26.4, 29.5, 29.8, 29.82, 29.85, 30.1, 30.7, 32.1, 41.4, 44.7, 45.9, 51.0, 101.3, 105.6, 115.8, 121.5, 130.6, 131.1, 132.2, 133.8, 137.0, 153.5 and 161.1; mass spectrum (MALDI-TOF), m/z 565.4135 (M^+) ($\text{C}_{35}\text{H}_{57}\text{N}_4\text{S}$ requires m/z 565.4298).

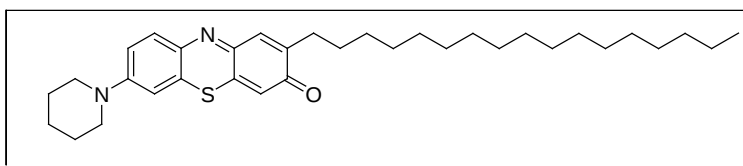


7-(Dimethylamino)-2-heptadecyl-3H-phenothiazin-3-one (2.11). To a dark green solution of 70.0 mg (0.09 mmol) of **2.1** in 3.5 mL THF-H₂O (6:1) was added 30.0 mg (0.53 mmol) of KOH. The reaction mixture was stirred for 2 min at room temperature until it changes from green to red. The aqueous layer was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO₄ and was concentrated under diminished pressure. The crude product was purified on a silica gel column (7 × 2 cm). Elution with 2:3 ethyl acetate-hexanes yielded **2.11** as a violet solid: yield 11 mg (24%); silica gel TLC *R_f* 0.59 (2:3 ethyl acetate-hexanes); ¹H NMR (CDCl₃) δ 0.86-0.89 (m, 3H), 1.27-1.41 (m, 28H), 1.72 (t, 2H, *J* = 6.0 Hz), 2.72 (t, 2H, *J* = 6.4 Hz), 2.89 (s, 6H), 6.73 (d, 1H, *J* = 1.6 Hz), 6.89 (dd, 1H, *J* = 7.6 Hz, *J* = 2.0 Hz), 6.93 (s, 1H), 7.60 (d, 1H, *J* = 8.0 Hz) and 7.71 (s, 1H); ¹³C NMR (CDCl₃) δ 14.3, 22.8, 29.5, 29.6, 29.7, 29.81, 29.85, 30.1, 31.6, 32.1, 44.0, 112.8, 119.3, 123.3, 134.0, 134.6, 135.5, 136.4, 139.7, 143.3, 155.8 and 182.5; mass spectrum (ESI), *m/z* 495.3414 (M+H)⁺ (C₃₁H₄₇N₂OS requires *m/z* 495.3409).



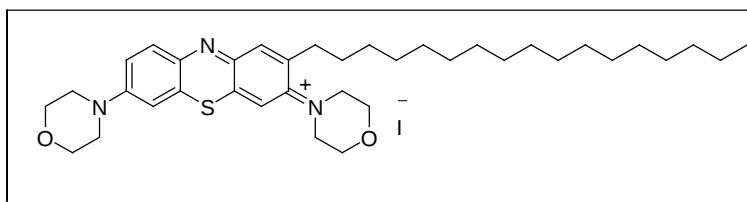
1-(2-Heptadecyl-7-(piperidin-1-yl)-3H-phenothiazin-3-ylidene)piperidin-1-ium Iodide (2.3). 30.0 mg (0.07 mmol) of crude **2.17** was dissolved in 5 mL of CH₂Cl₂ and 56.0 mg (0.22 mmol) of I₂ was added followed by piperidine 34.0 μL (29.0 mg, 0.35

mmol). The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was purified on a silica gel column (8 × 2 cm). Elution with ethyl acetate gave **2.3** as a green solid: yield 5 mg (10%); silica gel TLC R_f 0.43 (ethyl acetate); ^1H NMR (CDCl_3) δ 0.87 (t, 3H, J = 6.8 Hz), 1.25 (br s, 30H), 1.73-1.90 (m, 14H), 2.72 (t, 2H, J = 7.8 Hz), 3.38-3.40 (m, 3H), 3.94-3.96 (m, 3H), 7.47 (s, 1H), 7.56 (dd, 1H, J = 9.8 Hz, J = 2.6 Hz), 7.65 (d, 1H, J = 2.4 Hz), 7.92 (s, 1H) and 7.98 (d, 1H, J = 10 Hz); ^{13}C NMR (CDCl_3) δ 14.3, 22.8, 24.0, 26.2, 27.0, 29.5, 29.54, 29.7, 29.78, 29.8, 29.83, 30.5, 32.1, 32.1, 50.9, 53.4, 107.8, 110.1, 113.7, 121.2, 130.5, 137.4, 139.1, 139.6, 139.9, 153.4 and 158.8; mass spectrum (ESI), m/z 602.4496 (M^+) ($\text{C}_{39}\text{H}_{60}\text{N}_3\text{S}$ requires m/z 602.4508).



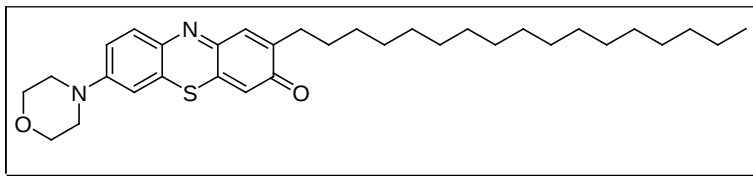
2-Heptadecyl-7-(piperidine-1-yl)-3H-phenothiazin-3-one (2.12). To a dark green solution of 60.0 mg (0.10 mmol) of **2.3** in 3.5 mL THF- H_2O (6:1) was added 16.0 mg (0.30 mmol) of KOH. The reaction mixture was stirred for 2 min at room temperature until it changes from green to red. The aqueous layer was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO_4 and was concentrated under diminished pressure. The crude product was purified on a silica gel column (7 × 2 cm). Elution with 1:4 ethyl acetate-hexanes yielded **2.12** as a brownish solid: yield 12.3 mg (22%); silica gel TLC R_f 0.75 (3:2 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 0.88 (t, 3H, J = 5.6 Hz), 1.26 (br s, 30H), 1.62-1.78 (m, 7H), 2.35 (t, 1H, J = 6.0 Hz), 2.66 (t, 2H, J = 6.4 Hz), 2.97-2.99 (m, 2H), 6.73 (d, 1H, J = 1.6 Hz), 6.87 (dd, 1H, J = 12 Hz, J = 2.0 Hz), 6.96 (s, 1H), 7.60 (d, 1H, J = 9.6 Hz) and 7.72 (s,

1H); ^{13}C NMR (CDCl_3) δ 14.1, 22.6, 22.7, 24.1, 26.1, 29.3, 29.4, 29.5, 29.54, 29.6, 29.63, 29.67, 30.2, 30.5, 31.6, 31.9, 53.5, 114.1 119.1, 122.9, 134.0, 134.9, 135.0, 137.0, 139.6, 143.6, 156.1 and 182.3; mass spectrum (ESI), m/z 535.3731 ($\text{M}+\text{H}^+$) ($\text{C}_{34}\text{H}_{51}\text{N}_2\text{OS}$ requires m/z 535.3722).

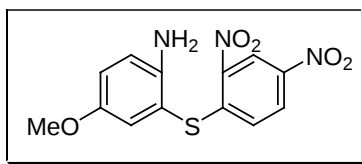


4-(2-Hexadecyl-7-morpholino-3H-phenothiazin-3-ylidene)morpholin-4-ium Iodide

(2.4). 44.0 mg (0.10 mmol) of crude **2.17** was dissolved in 5 mL of CH_2Cl_2 and 81.0 mg (0.32 mmol) of I_2 was added followed by morpholine 47.0 μL (47.0 mg, 0.50 mmol). The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was purified on a silica gel column (8 $\times$ 2 cm). Elution with ethyl acetate gave **2.4** as a green solid: yield 5 mg (7%); silica gel TLC R_f 0.43 (ethyl acetate); ^1H NMR (CDCl_3) δ 0.88 (t, 3H, J = 6.8 Hz), 1.24-1.40 (m, 30H), 1.76-1.80 (m, 7H), 2.76 (t, 2H, J = 7.8 Hz), 3.43-3.45 (m, 3H), 3.92-3.98 (m, 6H), 7.56 (s, 1H), 7.61-7.64 (m, 1H), 7.69 (d, 1H, J = 2.0 Hz), 8.06 (s, 1H) and 8.13 (d, 1H, J = 9.6 Hz); ^{13}C NMR (CDCl_3) δ 14.1, 22.7, 29.3, 29.4, 29.5, 29.6, 29.7, 30.1, 30.9, 31.9, 32.1, 48.9, 52.3, 66.6, 107.7, 113.7, 121.2, 130.9, 137.6, 137.9, 139.7, 139.8, 140.1, 153.9 and 157.9; mass spectrum (ESI), m/z 606.4113 (M^+) ($\text{C}_{37}\text{H}_{56}\text{N}_3\text{O}_2\text{S}$ requires m/z 606.4093).

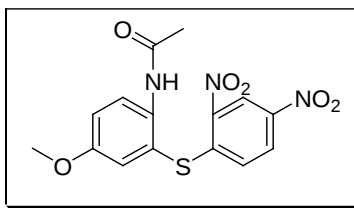


2-Heptadecyl-7-(morpholine-1-yl)-3H-phenothiazin-3-one (2.13). To a dark green solution of 58.0 mg (0.10 mmol) of **2.4** in 3.5 mL THF-H₂O (6:1) was added 11.0 mg (0.19 mmol) of KOH. The reaction mixture was stirred for 2 min at room temperature until it changes from green to red. The aqueous layer was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO₄ and was concentrated under diminished pressure. The crude product was purified on a silica gel column (7 × 2 cm). Elution with 1:4 ethyl acetate-hexanes yielded **2.13** as a brownish solid: yield 7 mg (14%); silica gel TLC *R_f* 0.75 (3:2 ethyl acetate-hexanes); ¹H NMR (CDCl₃) δ 0.86 (m, 3H), 1.21-1.38 (m, 28H), 1.69-1.70 (m, 2H), 2.67 (t, 2H, *J* = 8.0 Hz), 2.99-3.02 (m, 4H), 3.86-3.88 (m, 4H), 6.72 (d, 1H, *J* = 2.0 Hz), 6.89 (dd, 1H, *J* = 10.2 Hz, *J* = 2.2 Hz), 6.97 (s, 1H), 7.58 (d, 1H, *J* = 10 Hz) and 7.74 (s, 1H); ¹³C NMR (CDCl₃) δ 14.3, 22.8, 29.5, 29.6, 29.7, 29.8, 29.85, 30.4, 30.6, 32.1, 52.6, 67.1, 114.6, 119.6, 122.9, 134.5, 135.2, 135.4, 135.7, 138.0, 144.6, 154.5 and 182.4; mass spectrum (ESI), *m/z* 537.3518 (M+H)⁺ (C₃₃H₄₉N₂O₂S requires *m/z* 537.3515).



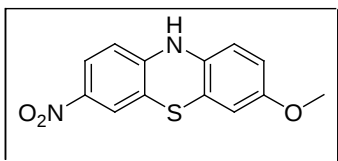
2-(2,4-Dinitrophenylthio)-5-methoxyaniline (2.18). 2.00 g (11.1 mmol) of 2-amino-6-methoxybenzothiazole was suspended in 40 ml of water and 9.30 g (167 mmol) of solid KOH was added. The suspension was heated to reflux for 12 h. The reaction mixture was cooled to room temperature and added dropwise to a solution of 2.25 g (11.1 mmol) of 2,4-dinitrochlorobenzene in a mixture of ethanol (30 mL)-AcOH (20 mL) in ice-water bath. The reaction mixture was stirred at room temperature for an additional 3 h. The

precipitate was filtered, washed with water-ethanol (1:1, v/v) and dried to afford **2.18** as an orange solid: yield 2.90 g (81%); silica gel TLC R_f 0.7 (3:7 ethyl acetate-hexanes); ^1H NMR (CD_3COCD_3) δ 3.76 (s, 3H), 6.85 (d, 1H, J = 8.8 Hz), 6.97 (d, 1H, J = 2.8 Hz), 7.00-7.06 (m, 2H), 8.18 (dd, 1H, J = 9.2 Hz, J = 2.6 Hz) and 9.12 (d, 1H, J = 2.4 Hz); ^{13}C NMR ($\text{DMSO}-d_6$) δ 55.9, 111.0, 117.5, 120.4, 120.6, 121.6, 127.1, 128.6, 143.3, 144.4, 144.6, 145.8 and 153.0; mass spectrum (APCI), m/z 322.0499 ($M + H$) $^+$ ($\text{C}_{13}\text{H}_{12}\text{N}_3\text{O}_5\text{S}$ requires m/z 322.0498).

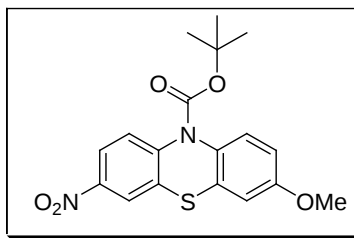


***N*-(2-((2,4-Dinitrophenyl)thio)-5-methoxyphenyl)acetamide (2.19).** To a solution of 2.90 g (9.03 mmol) of **2.18** in 10 mL of anhydrous DMF was added 3.66 mL (2.74 g, 27.1 mmol) of anhydrous triethylamine followed by 4.30 mL (4.64 g, 45.5 mmol) of acetic anhydride. The reaction mixture was stirred for 12 h at room temperature under an argon atmosphere and was quenched by pouring into ice-cold water. The aqueous layer was extracted with four 25-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO_4 , filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (8×4 cm). Elution with 3:7 ethyl acetate-hexanes afforded **2.19** as a bright yellow solid: yield 2.95 g (90%); silica gel TLC R_f 0.57 (3:7 ethyl acetate-hexanes); ^1H NMR ($\text{DMSO}-d_6$) δ 1.86 (s, 3H), 3.77 (s, 3H), 6.99 (d, 1H, J = 8.8 Hz), 7.18 (dd, 1H, J = 8.8 Hz, J = 2.4 Hz), 7.23 (d, 1H, J = 2.8 Hz), 7.66 (d, 1H, J = 8.8 Hz), 8.32 (dd, 1H, J = 9.0 Hz, J = 2.6 Hz), 8.88 (d, 1H, J = 2.4 Hz) and 9.43 (br s, 1H); ^{13}C NMR ($\text{DMSO}-d_6$) δ 22.8, 55.6, 117.7, 120.5, 121.0, 123.8, 127.5,

128.1, 129.2, 133.7, 144.05, 144.07, 145.6, 157.2 and 168.7; mass spectrum (APCI), m/z 364.0609 ($M + H$)⁺ ($C_{15}H_{14}N_3O_6S$ requires m/z 364.0603).

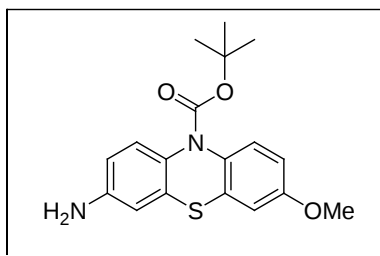


3-Methoxy-7-nitro-10H-phenothiazine (2.20). To a stirred solution of 2.95 g (8.13 mmol) of **2.19** in 20 mL of acetone, at reflux, was added in portions 0.91 g (16.2 mmol) of KOH in 10 mL of ethanol. The reaction mixture was kept at reflux for an additional 3 h and poured into ice-cold water. The aqueous layer was extracted with four 25-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous $MgSO_4$, filtered and concentrated under diminished pressure. The residue was purified on a silica gel column (8 × 2 cm). Elution with 1:1 ethyl acetate-hexanes gave **2.20** as a violet solid: yield 2.00 g (90%); silica gel TLC R_f 0.43 (3:7 ethyl acetate-hexanes); 1H NMR (CD_3COCD_3) δ 3.70 (s, 3H), 6.57-6.70 (m, 4H), 7.73 (d, 1H, $J = 2.4$ Hz), 7.81-7.84 (m, 1H) and 8.47 (br s, 1H); ^{13}C NMR (CD_3COCD_3) δ 55.0, 111.7, 113.0, 113.3, 116.1, 117.0, 117.2, 121.6, 124.2, 132.4, 141.5, 148.3 and 156.6; mass spectrum (APCI), m/z 275.0488 ($M + H$)⁺ ($C_{13}H_{11}N_2O_3S$ requires m/z 275.0490).

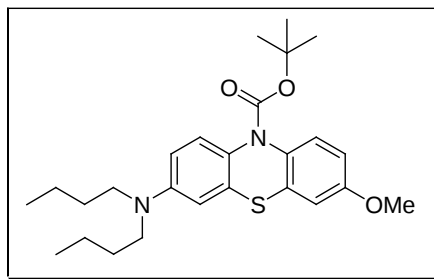


tert-Butyl 3-Methoxy-7-nitro-10H-phenothiazin-10-carboxylate (2.21). To a solution of 1.42 g (5.18 mmol) of **2.20** in 20 mL of anhydrous DMF at 0 °C was added 0.55 g (13.7 mmol) of 60% NaH. The reaction mixture was stirred at 0 °C for another 15 min

and 2.40 g (11.0 mmol) of di-*tert*-butyl dicarbonate was added. The reaction mixture was stirred at room temperature for 4 h under an argon atmosphere, and was quenched with 30 mL of water. The aqueous layer was extracted with three 20-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous MgSO₄ and concentrated under diminished pressure. The residue was purified on a silica gel column (8 × 4 cm). Elution with 3:7 ethyl acetate-hexanes afforded **2.21** as a bright yellow solid: yield 1.66 g (86%); silica gel TLC *R*_f 0.54 (3:7 ethyl acetate-hexanes); ¹H NMR (CD₃COCD₃) δ 1.54 (s, 9H), 3.86 (s, 3H), 6.97 (dd, 1H, *J* = 9.2 Hz, *J* = 2.8 Hz), 7.01 (d, 1H, *J* = 2.8 Hz), 7.47 (d, 1H, *J* = 9.2 Hz), 7.84 (d, 1H, *J* = 8.8 Hz), 8.20 (dd, 1H, *J* = 9.0 Hz, *J* = 2.6 Hz) and 8.24 (d, 1H, *J* = 2.4 Hz); ¹³C NMR (CD₃COCD₃) δ 29.2, 57.1, 84.3, 113.3, 115.6, 123.7, 124.1, 129.8, 130.0, 132.3, 133.1, 135.3, 146.9, 147.1, 153.2 and 159.9; mass spectrum (APCI), *m/z* 374.0932 (M + H)⁺ (C₁₈H₁₉N₂O₅S requires *m/z* 374.0936).

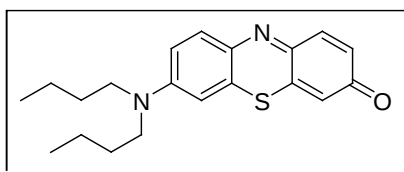


***tert*-Butyl 3-Amino-7-methoxy-10*H*-phenothiazin-10-carboxylate (2.22).** To a suspension of 0.42 g (1.12 mmol) of **2.21** in 10 mL of ethanol was added 10 mg of 10% Pd on carbon. The reaction mixture was stirred at room temperature under a hydrogen atmosphere (25 psi) overnight. The reaction mixture was filtered through a Celite pad and was concentrated under diminished pressure. The crude product (**2.22**) was used for the next reaction without further purification.



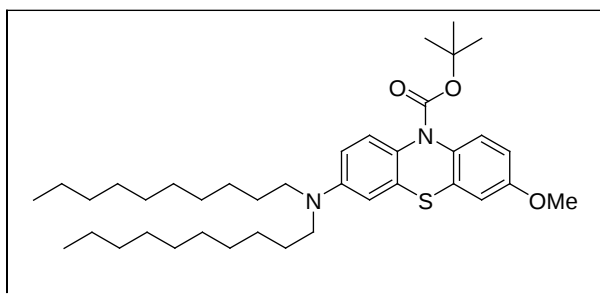
tert-Butyl 3-(N,N-Dibutylamino)-7-methoxy-10H-phenothiazin-10-carboxylate

(2.23). To a solution of crude **2.22** in 3 mL of acetonitrile was added 1.40 g (12.8 mmol) of Na_2CO_3 followed by 0.94 g (5.12 mmol) of 1-iodobutane. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C overnight. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel column (10 x 2 cm). Elution with 1:9 ethyl acetate-hexanes gave compound **2.23** as a pale yellow solid: yield 0.31 g (61%); silica gel TLC R_f 0.43 (1:9 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 0.95 (t, 6H, J = 7.4 Hz), 1.32-1.37 (m, 4H), 1.49 (s, 9H), 1.51-1.57 (m, 4H), 3.23 (t, 4H, J = 7.6 Hz), 3.78 (s, 3H), 6.52-6.55 (m, 2H), 6.78 (dd, 1H, J = 8.8 Hz, J = 2.8 Hz), 6.86 (d, 1H, J = 2.8 Hz), 7.31 (d, 1H, J = 8.4 Hz) and 7.39 (d, 1H, J = 8.8 Hz); ^{13}C NMR (CDCl_3) δ 13.9, 20.2, 28.1, 29.2, 50.8, 55.5, 81.2, 109.1, 110.2, 111.5, 112.6, 126.8, 127.2, 127.5, 132.3, 132.5, 133.2, 146.2, 153.2 and 157.0; mass spectrum (APCI), m/z 457.2530 ($\text{M} + \text{H}^+$) ($\text{C}_{26}\text{H}_{37}\text{N}_2\text{O}_3\text{S}$ requires m/z 457.2525).



7-(N,N-Dibutylamino)-3H-phenothiazin-3-one (2.5). To a solution of 86.0 mg (0.19 mmol) of **2.23** in 2 mL of anhydrous CH_2Cl_2 was added dropwise 0.76 mL (0.76 mmol)

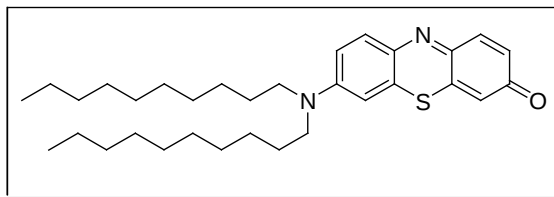
of 1 M BBr₃ in CH₂Cl₂ at -78 °C. The reaction mixture was stirred overnight at room temperature under an argon atmosphere and was quenched with 10 mL of water. The aqueous layer was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 10 mL of brine, dried over anhydrous MgSO₄ and concentrated under diminished pressure. The residue was purified on a silica gel column (7 x 2 cm). Elution with 1:1 ethyl acetate-hexanes afforded **2.5** as a violet solid: yield 30 mg (47%); silica gel TLC *R_f* 0.42 (1:1 ethyl acetate-hexanes); ¹H NMR (CDCl₃) δ 0.95 (t, 6H, *J* = 7.4 Hz), 1.26-1.37 (m, 4H), 1.51-1.57 (m, 4H), 3.23 (m, 4H), 6.52-6.55 (m, 2H), 6.76-6.87 (m, 2H), 7.31 (d, 1H, *J* = 8.8 Hz) and 7.38 (d, 1H, *J* = 8.8 Hz); mass spectrum (APCI), *m/z* 341.1690 (*M* + H)⁺ (C₂₀H₂₅N₂OS requires *m/z* 341.1688).



***tert*-Butyl 3-(*N,N*-Didecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate**

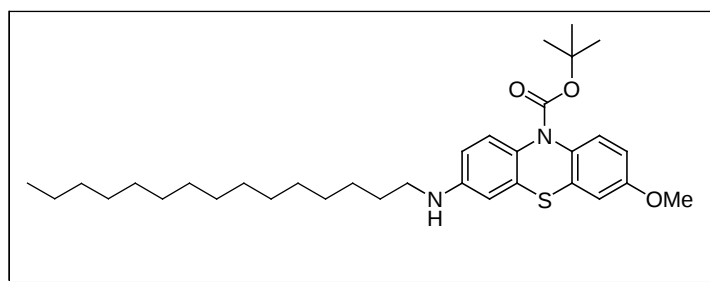
(2.24). 0.74 g (2.90 mmol) of 1-iododecane in 2 mL of acetonitrile was added into a mixture of 0.40 g (~ 1.16 mmol) of crude **2.22** in 2 mL of acetonitrile and 1.23 g (11.6 mmol) of Na₂CO₃. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 48 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel column (8 x 2 cm). Elution with 1:19 ethyl acetate-hexanes gave compound **2.24** as a pale yellow solid: yield 30 mg (5%); silica gel TLC *R_f* 0.41 (1:19 ethyl acetate-hexanes) ¹H NMR (CDCl₃) δ 0.81 (t, 6H, *J* = 6.8 Hz), 1.19 (m, 28H), 1.22 (s, 9H), 1.40-1.46 (m,

4H), 3.13 (t, 4H, $J = 7.6$ Hz), 3.69 (s, 3H), 6.42-6.44 (m, 2H), 6.90 (dd, 1H, $J = 8.6$ Hz, $J = 2.6$ Hz), 6.70 (d, 1H, $J = 2.8$ Hz), 7.21 (d, 1H, $J = 8.4$ Hz) and 7.30 (d, 1H, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3) δ 14.1, 22.7, 27.1, 28.2, 29.3, 29.5, 29.6, 31.9, 51.2, 55.5, 81.2, 109.1, 110.2, 111.6, 112.6, 126.8, 127.3, 127.6, 132.4, 132.6, 133.2, 146.3, 153.3 and 157.1; mass spectrum (APCI), m/z 625.4390 ($\text{M} + \text{H}^+$) ($\text{C}_{38}\text{H}_{61}\text{N}_2\text{O}_3\text{S}$ requires m/z 625.4403).



7-(*N,N*-Didecylamino)-3*H*-phenothiazin-3-one (2.6). 0.20 mL (0.20 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise into a solution of 30.0 mg (0.05 mmol) of compound **2.24** in 4 mL of CH_2Cl_2 at -78°C . The reaction mixture was stirred overnight at ambient temperature and was quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The violet organic layer was washed with 20 mL of brine, dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (7 x 2 cm). Elution with 1:2 ethyl acetate-hexanes afforded **2.6** as a violet solid: yield 9 mg (38%); silica gel TLC R_f 0.50 (1:2 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 0.87 (t, 6H, $J = 6.8$ Hz), 1.24-1.34 (m, 28H), 1.63 (br s, 4H), 3.36 (t, 4H, $J = 7.8$ Hz), 6.51 (d, 1H, $J = 2.8$ Hz), 6.66 (d, 1H, $J = 2.0$ Hz), 6.75-6.82 (m, 2H), 7.53 (d, 1H, $J = 9.6$ Hz) and 7.46 (d, 1H, $J = 9.2$ Hz); ^{13}C NMR (CDCl_3) δ 14.1, 22.6, 27.0, 27.3, 29.3, 29.4, 29.50, 29.54, 29.7, 31.8, 51.4, 104.7, 113.2, 118.4, 128.6, 130.9, 132.1, 134.8, 135.9, 139.4, 139.6, 149.8 and 182.1; mass spectrum (APCI), m/z 509.3553 ($\text{M} + \text{H}^+$) ($\text{C}_{32}\text{H}_{49}\text{N}_2\text{OS}$ requires m/z 509.3566).

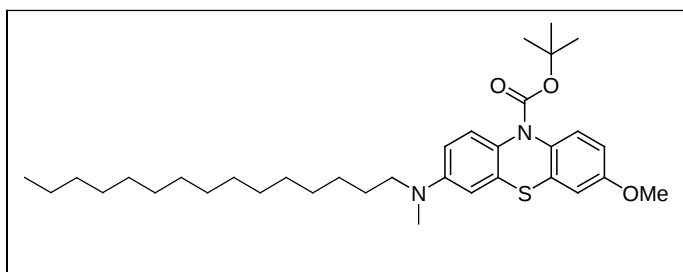
(1:4 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 0.88 (t, 6H, J = 6.8 Hz), 1.26-1.35 (m, 48H), 1.62-1.64 (m, 4H), 3.37 (t, 4H, J = 7.6 Hz), 6.53 (d, 1H, J = 2.8 Hz), 6.67 (d, 1H, J = 2.0 Hz), 6.76-6.84 (m, 2H), 7.55 (d, 1H, J = 9.6 Hz) and 7.66 (d, 1H, J = 9.2 Hz); ^{13}C NMR (CDCl_3) δ 14.3, 22.8, 27.1, 27.5, 29.50, 29.53, 29.6, 29.7, 29.77, 29.79, 29.81, 29.83, 32.1, 51.5, 104.8, 113.3, 118.7, 128.7, 131.0, 132.4, 134.9, 136.0, 139.6, 140.0, 150.0 and 182.4; mass spectrum (APCI), m/z 648.5144 ($\text{M} + \text{H}^+$) ($\text{C}_{42}\text{H}_{69}\text{N}_2\text{OS}$ requires m/z 649.5131).



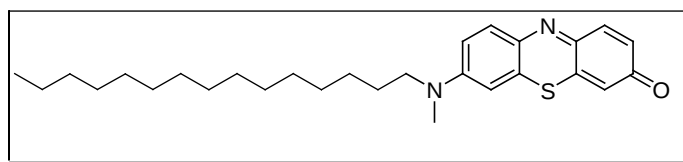
***tert*-Butyl 3-(*N*-Pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate**

(2.27). 2.11 g (7.25 mmol) of 1-bromopentadecane in 3 mL of acetonitrile was added into a mixture of 0.50 g (~ 1.45 mmol) of crude compound **2.22** in 2 mL of acetonitrile and 1.55 g (14.6 mmol) of Na_2CO_3 . The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 3 days. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and purified on a silica gel column. Elution with 1:9 ethyl acetate-hexanes gave compound **2.27** as a pale yellow solid: yield 0.14 g (9%); silica gel TLC R_f 0.31 (1:9 ethyl acetate-hexanes); ^1H NMR (CDCl_3) δ 0.87 (t, 3H, J = 6.6 Hz), 1.25 (br s, 24H), 1.45 (s, 9H), 1.52-1.57 (m, 2H), 3.04 (t, 2H, J = 7.2 Hz), 3.76 (s, 3H), 6.45 (dd, 1H, J = 8.6 Hz, J = 2.6 Hz), 6.50 (d, 1H, J = 2.8 Hz), 6.76 (dd, 1H, J = 8.8 Hz, J = 2.8 Hz), 6.83 (d, 1H, J = 2.8 Hz), 7.24 (d, 1H, J = 8.4 Hz) and 7.36 (d, 1H, J = 8.8 Hz); ^{13}C NMR (CDCl_3) δ

14.2, 22.8, 27.2, 28.20, 28.27, 28.31, 28.4, 29.46, 29.5, 29.69, 29.70, 29.75, 29.8, 32.0, 44.2, 55.7, 81.4, 109.8, 111.65, 111.7, 112.8, 127.6, 127.8, 128.7, 132.5, 132.7, 133.3, 146.7, 153.3 and 157.3; mass spectrum (APCI), m/z 555.3629 ($M + H$)⁺ ($C_{33}H_{51}N_2O_3S$ requires m/z 555.3620).

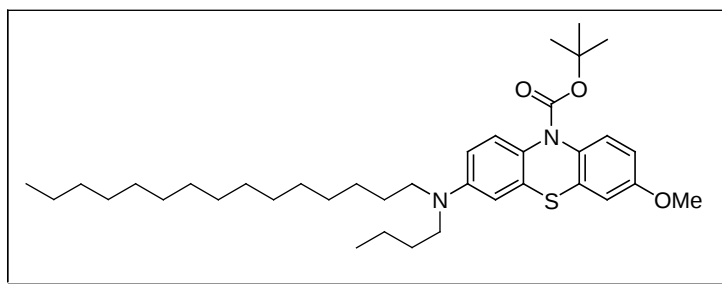


***tert*-Butyl 3-(*N*-Methyl-*N*-pentadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate (2.28).** 7.00 μ L (16.0 mg, 0.11 mmol) of iodomethane was added into a mixture of 53.0 mg (0.10 mmol) of compound **2.27** in 2 mL of acetonitrile and 16.0 mg (0.15 mmol) of Na_2CO_3 . The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 $^{\circ}C$ for $\sim$ 16 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure. The crude was used to the next step without further purification.

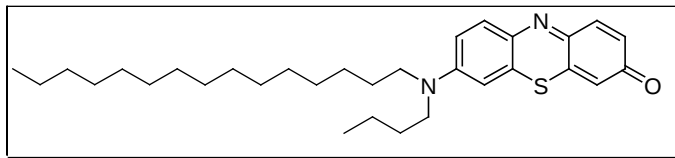


7-(*N*-Methyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (2.8). 0.27 mL (0.27 mmol) of 1 M BBr_3 in CH_2Cl_2 was added dropwise into the solution of 50.0 mg of crude **2.28** in 2 mL of CH_2Cl_2 at $-78^{\circ}C$. The reaction mixture was stirred overnight at room temperature and was quenched with 10 mL of water. The product was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of

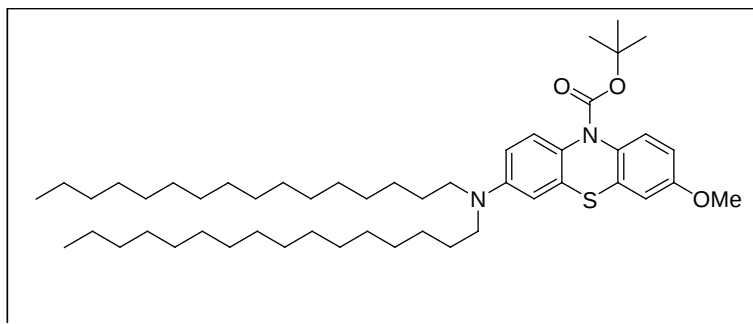
brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (7 x 2 cm). Elution with 3:7 ethyl acetate-hexanes afforded **2.8** as a violet solid: yield 22 mg (54%); silica gel TLC *R_f* 0.50 (1:4 ethyl acetate-hexanes); ¹H NMR (CDCl₃) δ 0.86 (t, 3H, *J* = 6.4 Hz), 1.24-1.32 (m, 24H), 1.60-1.65 (m, 2H), 3.09 (s, 3H), 3.37 (t, 2H, *J* = 7.6 Hz), 6.55 (d, 1H, *J* = 2.8 Hz), 6.68 (d, 1H, *J* = 2.4 Hz), 6.80-6.84 (m, 2H), 7.54 (d, 1H, *J* = 9.6 Hz) and 7.67 (d, 1H, *J* = 9.2 Hz); ¹³C NMR (CDCl₃) δ 14.2, 22.6, 27.1, 27.4, 29.50, 29.55, 29.6, 29.7, 29.77, 29.78, 29.81, 29.83, 51.5, 104.8, 113.3, 118.7, 128.7, 131.0, 132.4, 134.8, 136.1, 139.6, 140.2, 150.0 and 182.4; mass spectrum (APCI), *m/z* 453.2947 (*M* + H)⁺ (C₂₈H₄₁N₂OS requires *m/z* 453.2940).



tert-Butyl 3-(N-Butyl-N-pentadecylamino)-7-methoxy-10H-phenothiazin-10-carboxylate (2.29). 0.11 mL (0.14 g, 1.00 mmol) of 1-bromobutane was added into a mixture of 90.0 mg (0.16 mmol) of compound **2.27** in 2 mL of acetonitrile and 0.17 g (1.60 mmol) of Na₂CO₃. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 30 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure. The crude product was used to the next step without further purification.

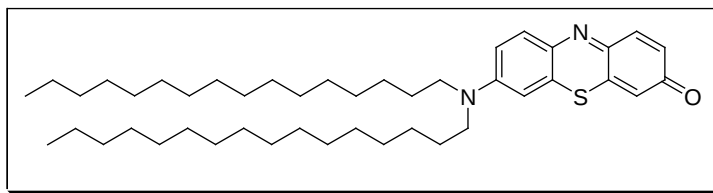


7-(*N*-Butyl-*N*-pentadecylamino)-3*H*-phenothiazin-3-one (2.9). 0.48 mL (0.48 mmol) of 1 M BBr₃ in CH₂Cl₂ was added dropwise into the solution of 100 mg (~ 0.16 mmol) of crude **2.29** in 2 mL of CH₂Cl₂ at -78 °C. The reaction mixture was stirred overnight at room temperature and was quenched with 10 mL of water. The crude product was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (7 x 2 cm). Elution with 3:7 ethyl acetate-hexanes afforded **2.9** as a violet solid: yield 20 mg (25%); silica gel TLC *R*_f 0.50 (1:4 ethyl acetate-hexanes); ¹H NMR (CDCl₃) δ 0.88 (t, 3H, *J* = 6.6 Hz), 1.00 (t, 3H, *J* = 7.2 Hz), 1.26-1.43 (m, 26H), 1.62-1.64 (m, 4H), 3.36-3.41 (m, 4H), 6.55 (d, 1H, *J* = 2.4 Hz), 6.71 (s, 1H), 6.79-6.85 (m, 2H), 7.57 (d, 1H, *J* = 9.6 Hz) and 7.68 (d, 1H, *J* = 9.2 Hz); ¹³C NMR (CDCl₃) δ 14.3, 22.8, 27.1, 27.5, 29.50, 29.53, 29.6, 29.7, 29.77, 29.79, 29.81, 29.83, 32.1, 51.5, 104.8, 113.3, 118.7, 128.7, 131.0, 132.4, 134.9, 136.0, 139.6, 140.0, 150.0 and 182.4; mass spectrum (APCI), *m/z* 495.3330 (M + H)⁺ (C₃₁H₄₇N₂OS requires *m/z* 495.3331).



***tert*-Butyl 3-(*N,N*-Dihexadecylamino)-7-methoxy-10*H*-phenothiazin-10-carboxylate**

(2.26). 1.40 mL (4.35 mmol) of 1-iodohexadecane in 2 mL of acetonitrile was added into a mixture of 0.50 g (~ 1.16 mmol) of crude **2.22** in 2 mL of acetonitrile and 1.50 g (14.5 mmol) of Na₂CO₃. The reaction mixture was sealed under nitrogen atmosphere and stirred at 80 °C for ~ 48 h. The reaction mixture was cooled to room temperature and filtered. The filtrate was concentrated under diminished pressure and the crude was used in the next reaction without further purification.



7-(*N,N*-Dihexadecylamino)-3*H*-phenothiazin-3-one (2.10). 50.0 µL (0.05 mmol) of 1 M BBr₃ in CH₂Cl₂ was added dropwise into the solution of 20.0 mg (0.03 mmol) of compound **2.26** in 2 mL of CH₂Cl₂ at -78 °C. The reaction mixture was stirred overnight at room temperature and was quenched with 10 mL of water. The crude product was extracted with two 10-mL portions of ethyl acetate. The combined organic layer was washed with 20 mL of brine, dried over anhydrous Na₂SO₄ and concentrated under reduced pressure. The resulting violet solid was purified on a silica gel column (10 x 2 cm). Elution with 1:4 ethyl acetate-hexanes afforded **2.10** as a violet solid: yield 8 mg (50%); silica gel TLC *R*_f 0.50 (1:4 ethyl acetate-hexanes); ¹H NMR (CDCl₃) δ 0.87 (t, 6H, *J* = 6.2 Hz), 1.26-1.35 (m, 52H), 1.64 (br s, 4H), 3.38 (t, 4H, *J* = 7.4 Hz), 6.54 (br s, 1H), 6.71 (br s, 1H), 6.78-6.86 (m, 2H), 7.60 (d, 1H, *J* = 9.0 Hz) and 7.67 (d, 1H, *J* = 8.8 Hz); ¹³C NMR (CDCl₃) δ 14.1, 14.2, 21.0, 22.7, 27.0, 27.4, 29.3, 29.4, 29.5, 29.60, 29.63, 29.67, 31.9, 51.5, 60.4, 104.8, 113.60, 113.62, 118.1, 131.7, 134.7, 136.1, 139.4, 139.5,

150.1, 169.2 and 171.1; mass spectrum (APCI), m/z 677.5367 ($M + H$)⁺ ($C_{44}H_{72}N_2OS$ requires m/z 677.5365).

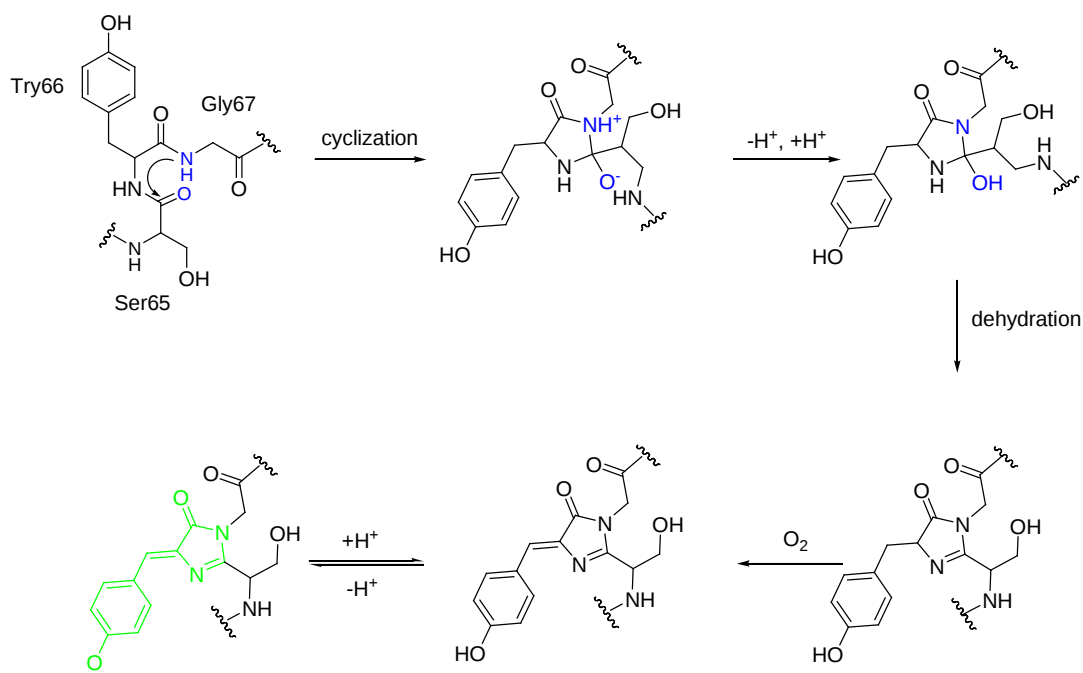
CHAPTER 3

3. SYNTHESIS OF FLUORESCENT DIPEPTIDOMIMETICS AND THEIR RIBOSOMAL INCORPORATION INTO GREEN FLUORESCENT PROTEIN

3.1 Introduction

Green fluorescent protein (GFP) exemplifies a family of chromoproteins consisting of about 220-240 amino acid residues (25 kDa).⁹⁴ GFP was first isolated by the Shimomura group from the jellyfish *Aequorea victoria* in 1962.⁹⁵ Over the next 30 years, GFP was mainly limited to studies of luminescent marine creatures.⁹⁴ The crucial breakthrough came after its cloning in 1992 by Prasher et al.⁹⁶ and its utilization as fluorescent tag for gene expression in 1994 by Chalfie et al.⁹⁷ *Aequorea victoria* GFP consists of 238 amino acid residues, which fold into a β -barrel, formed by 11 β -sheets that accommodate an internal distorted helix. The chromophore of GFP, also reported by Shimomura, is a 4-(*p*-hydroxybenzylidene)imidazolidin-5-one moiety, which forms by a posttranslational cyclization and oxidation of the polypeptide backbone, involving the Ser65-Tyr66-Gly67 residues.⁹⁸ This results in an extended conjugated system capable of absorbing and emitting visible light (Scheme 3.1).

Scheme 3.1. Proposed mechanism of formation of GFP chromophore.⁹⁹⁻¹⁰¹ The reactive nitrogen and carbonyl groups are highlighted in blue. The final structure of the chromophore is shown in green.



The amino acid at position 65 can vary but Tyr66 and Gly67 are conserved for all proteins within GFP family.¹⁰² The GFP chromophore is located in the center of the β -barrel and therefore is well shielded from solvent molecules. Additionally, the β -barrel is stabilized by multiple non-covalent interactions, which make it highly stable with respect to its thermal and chemical properties, as well as susceptibility to proteolysis.⁹⁴

The GFP chromophore has absorption peaks at 395 and 475 nm, usually assigned to the neutral and anionic forms of the chromophore, respectively (Scheme 3.1), and an emission peak at 509 nm with a high fluorescence quantum yield (0.79).¹⁰³ There have been extensive efforts to improve or alter the spectral properties of GFP. For example, Schultz and coworkers have replaced Tyr66 by phenylalanine analogues using site-directed mutagenesis (Figure 3.1).¹⁰⁴ Numerous reports have described the study and

spectral properties of novel fluorescent proteins and their chromophores.^{105,106} In comparison, there has been no report of the incorporation of a preformed fluorophore into a protein backbone by *in vitro* protein translation.

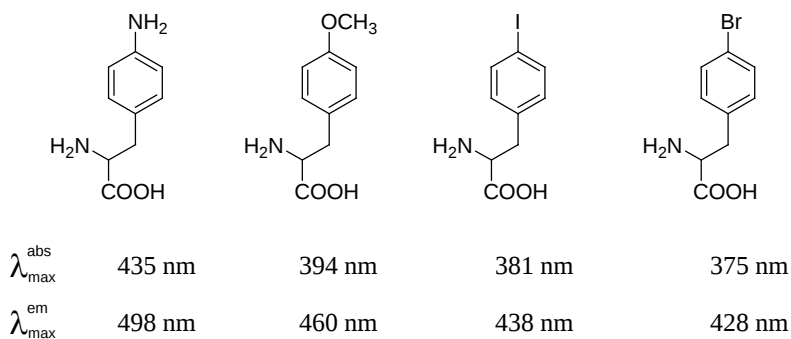


Figure 3.1. Structures of unnatural Phe analogues substituted for Tyr66 of GFP and the photophysical properties of their corresponding GFP.¹⁰⁴

Peptidomimetics are small molecules, designed to mimic a natural peptide or protein. They often exhibit very similar biological effects as the parent protein or peptide, or support some new effect. Oxazole and thiazole based peptidomimetics are widespread among bioactive molecules. For example, microcin B17, a bacterial DNA gyrase inhibitor,¹⁰⁷ and goadsporin, a promoter of secondary metabolism and morphogenesis in *Streptomyces*, contain both oxazole and thiazole moieties. Thiostrepton, an inhibitor of bacterial ribosome function,¹⁰⁸ contains multiple thiazole moieties. Given the extensive occurrence of oxazole and thiazole moieties in natural products, there has been considerable interest in the use of such precursors in preparing bioactive molecules and peptidomimetic structures synthetically.¹⁰⁹⁻¹¹² At present, we report the synthesis and incorporation of novel fluorescent oxazole and thiazole-based dipeptide analogues, which are also reminiscent of the fluorophores formed by naturally occurring fluorescent proteins, into GFP. The structures of the dipeptidomimetic analogues are shown in Figure 3.2.¹¹³

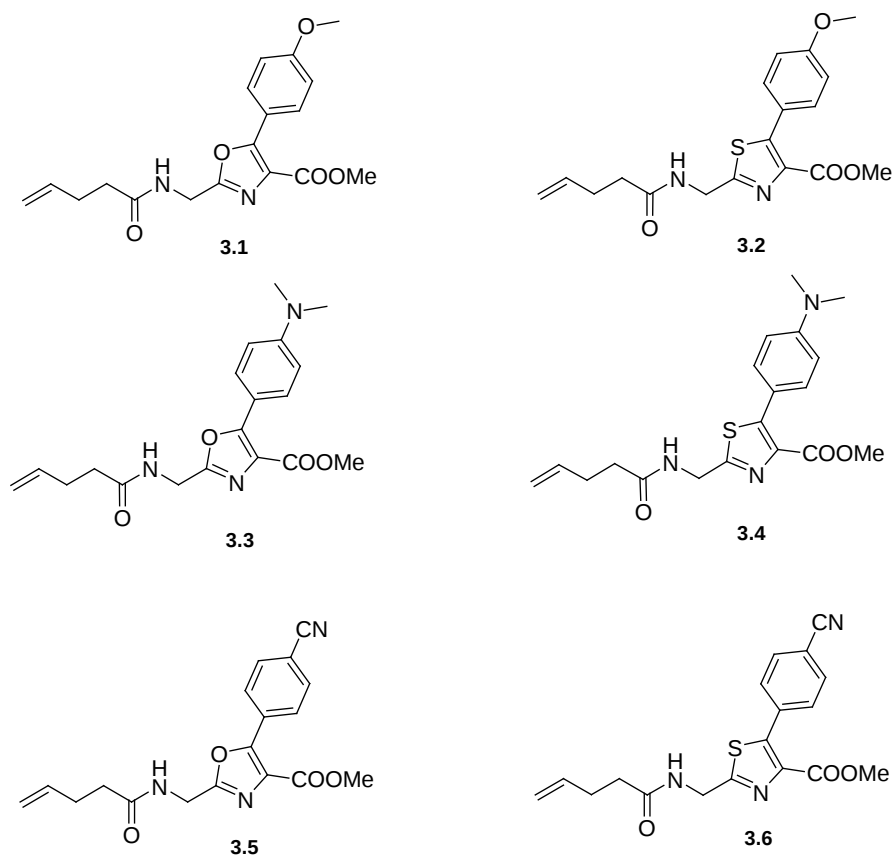
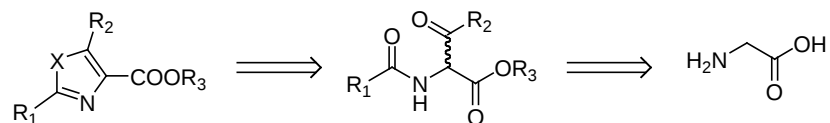


Figure 3.2. Chemical structures of the dipeptidomimetic analogues synthesized.

3.2 Results

The retrosynthetic analysis of the dipeptidomimetic analogues is shown in Scheme 3.2.¹¹⁴ Double C, N acylation of glycine affords α -amido- β -ketoesters, which were then

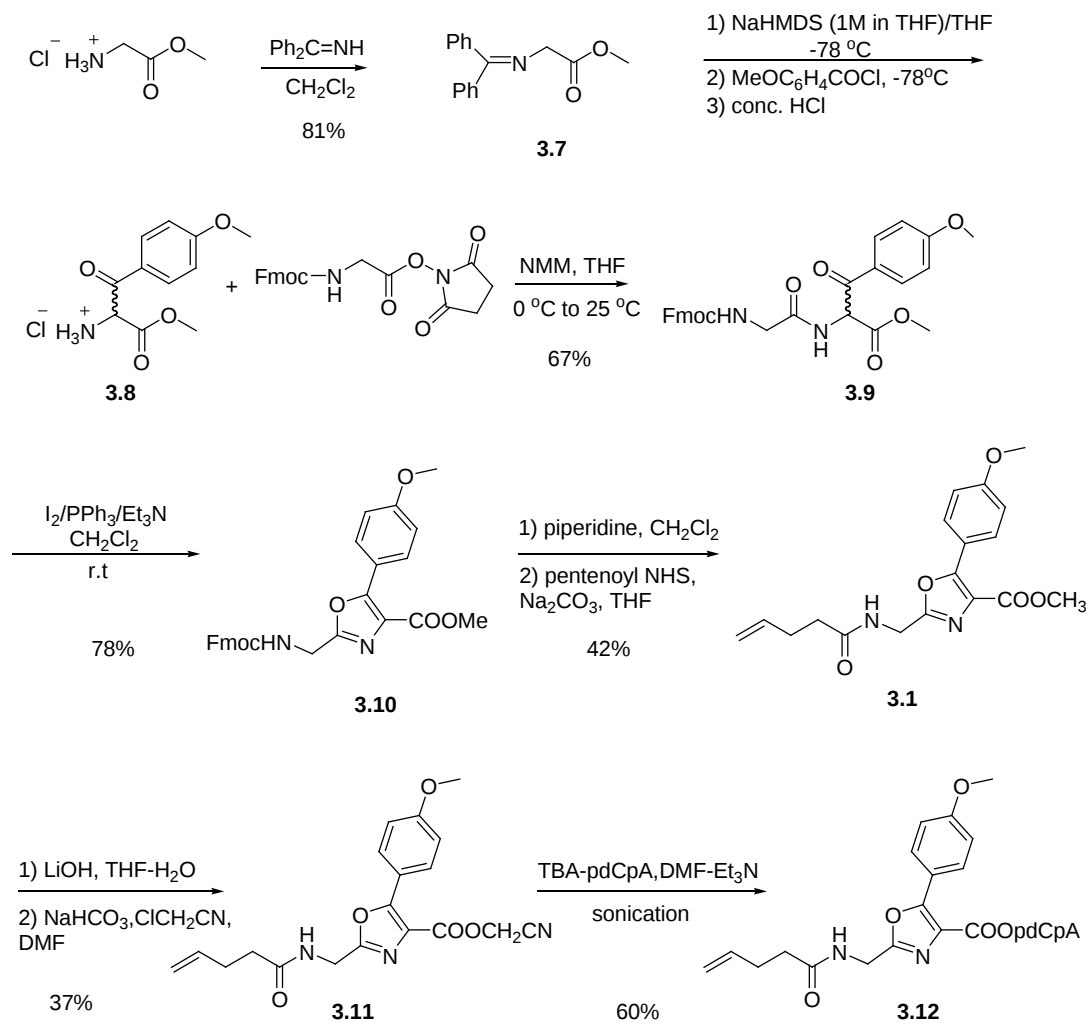
Scheme 3.2. Retrosynthetic analysis for the syntheses of the dipeptidomimetic analogues.



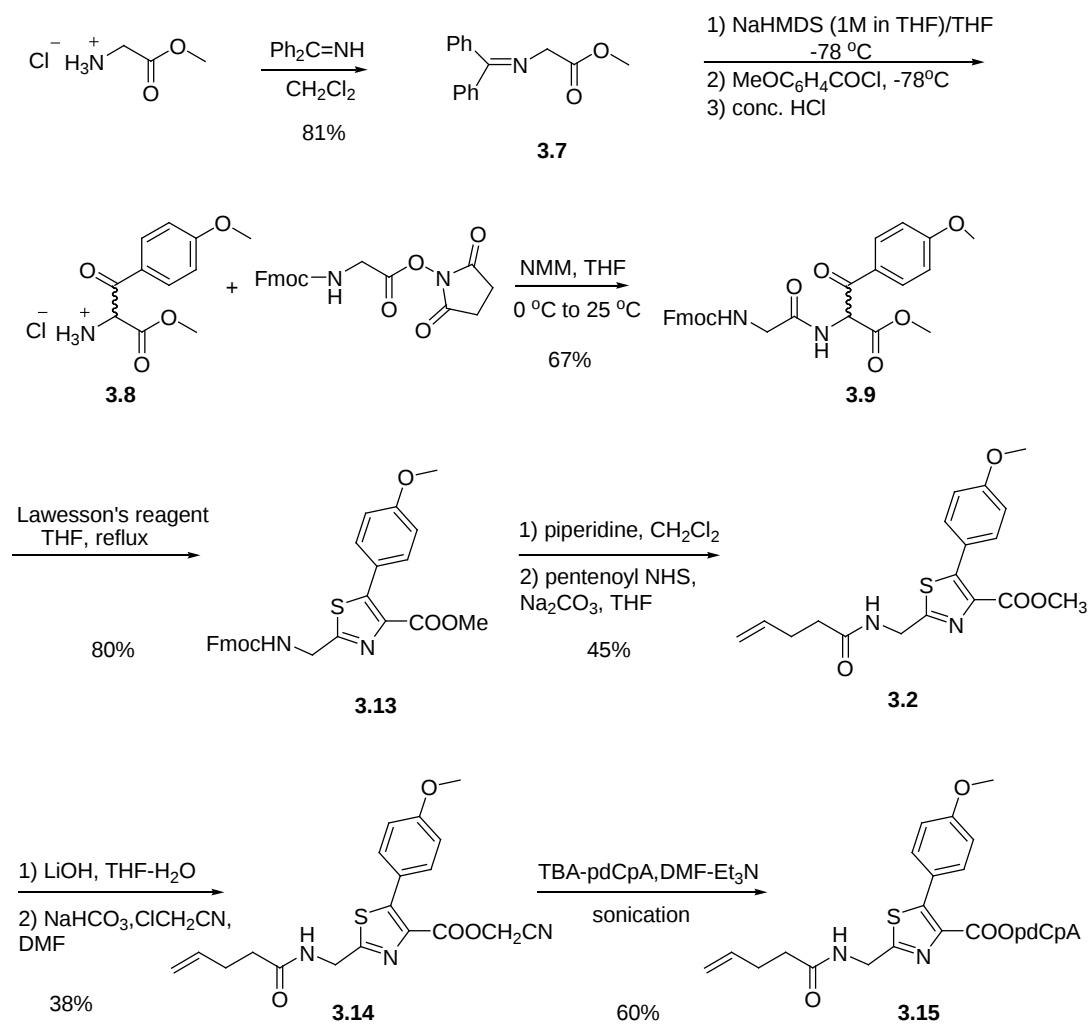
transformed to oxazoles and thiazoles. The syntheses of the pdCpA derivatives of the dipeptidomimetic analogues are shown in Schemes 3.3-3.8. Commercially available glycine methyl ester hydrochloride was treated with benzophenone imine to obtain fully protected glycine **3.7**¹¹⁵ which was condensed with various *p*-substituted benzoyl

chlorides in the presence of 1 M NaHMDS in THF. Subsequent hydrolysis of the imine with conc. HCl afforded corresponding α -amino- β -ketoesters as crude products, which were condensed directly with Fmoc-gly-*N*-hydroxysuccinimide to obtain α -amido- β -ketoester intermediates in varying yields.¹¹⁴ Cyclodehydration of the α -amido- β -ketoesters using triphenylphosphine in the presence of iodine and triethylamine in anhydrous dichloromethane at room temperature afforded the oxazoles.¹¹⁶ The corresponding thiazoles were obtained by treating the α -amido- β -ketoesters with Lawesson's reagent. Subsequent removal of the Fmoc group with piperidine followed by treatment with pentenoyloxy succinimide¹¹⁷ yielded the pentenoyl protected compounds, which were then saponified and activated as cyanomethyl esters.¹¹⁸ The cyanomethyl esters were then used for the acylation of the dinucleotide pdCpA.¹¹⁹ A mixture of the cyanomethyl esters and tris(tetrabutylammonium) salt of pdCpA in 9:1 DMF-Et₃N was sonicated for 4 h and purified by reversed phase HPLC using a semi-preparative C₁₈ column, which provided the corresponding pdCpA derivatives.

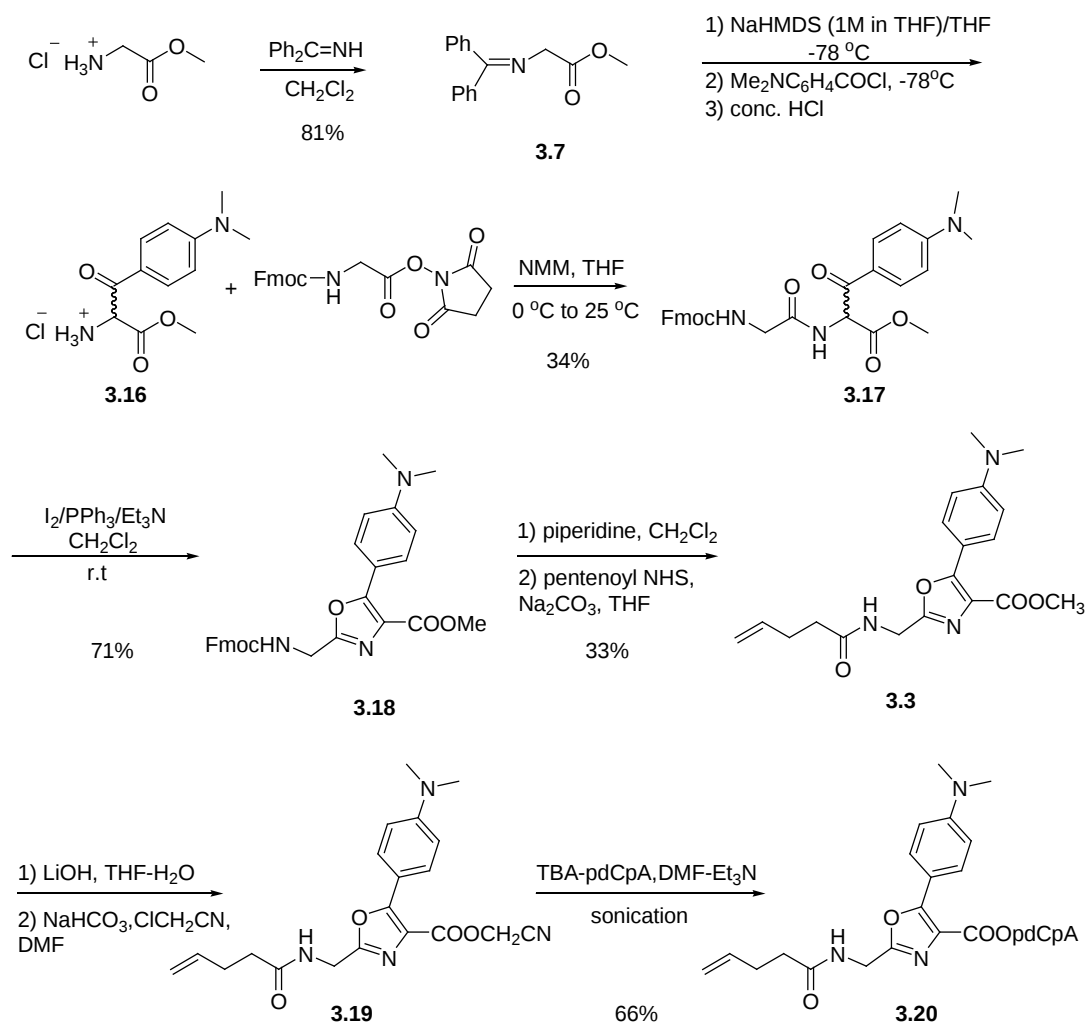
Scheme 3.3. Synthetic route for the preparation of the pdCpA derivative of dipeptidomimetic analogue **3.1**.



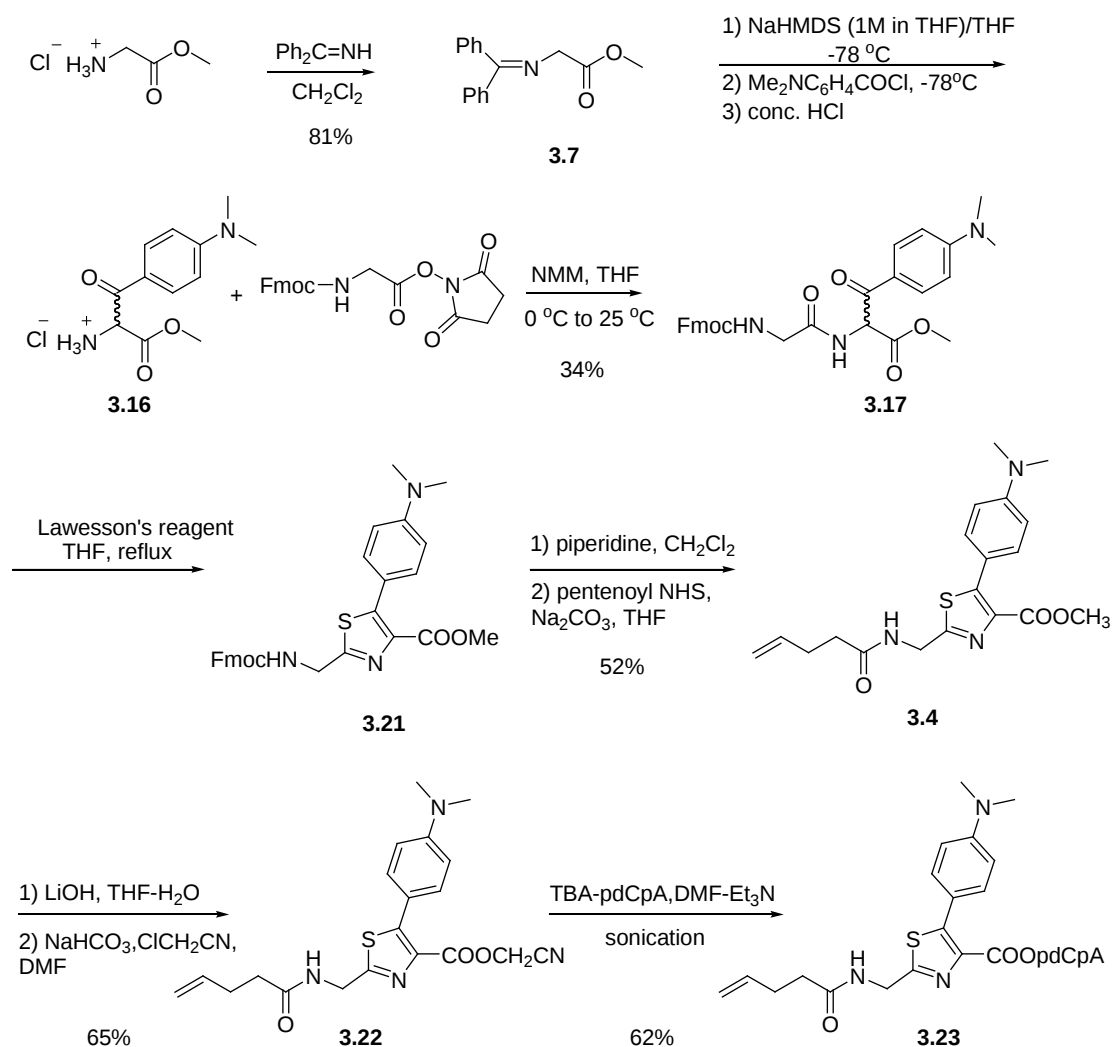
Scheme 3.4. Synthetic route for the preparation of the pdCpA derivative of dipeptidomimetic analogue **3.2**.



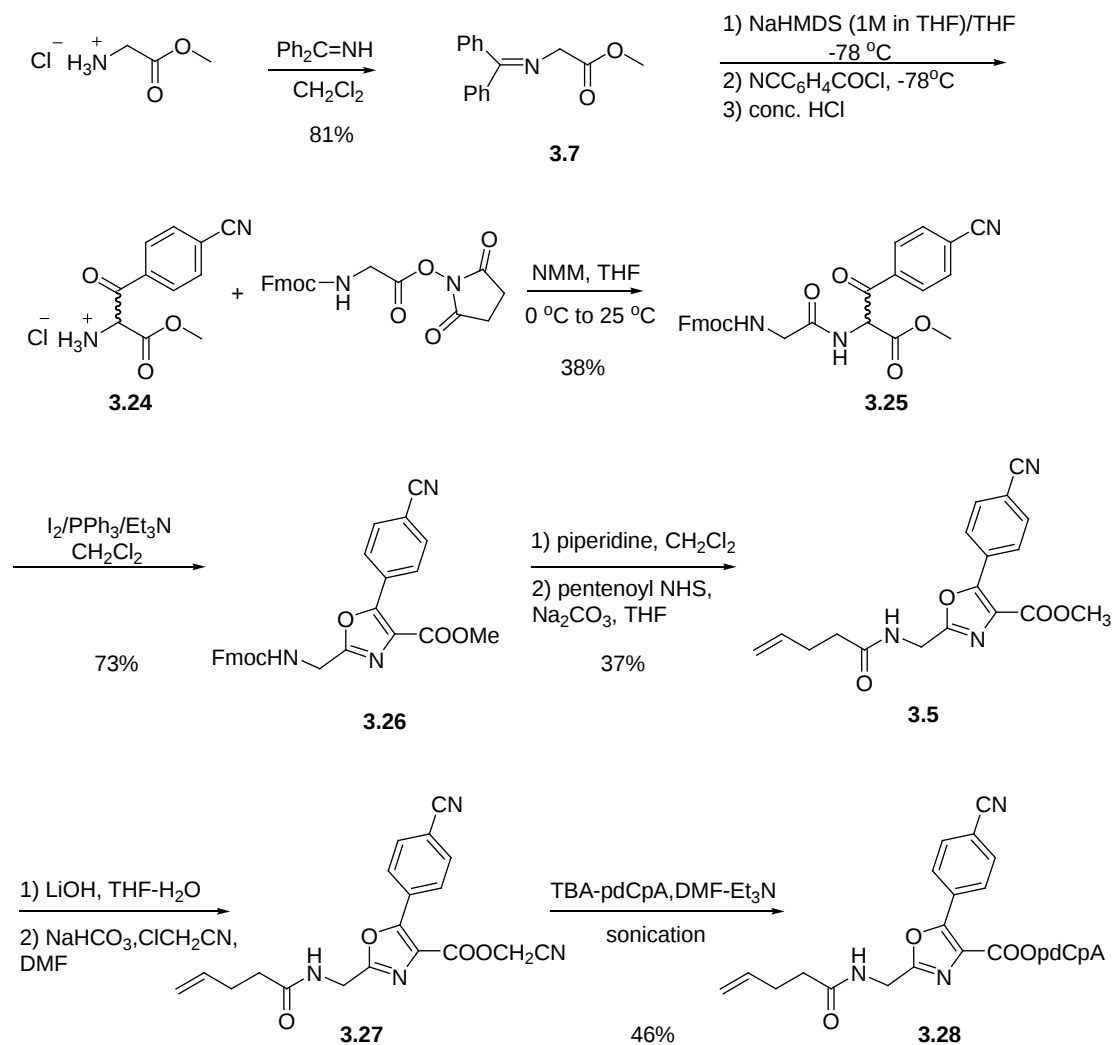
Scheme 3.5. Synthetic route for the preparation of the pdCpA derivative of dipeptidomimetic analogue **3.3**.



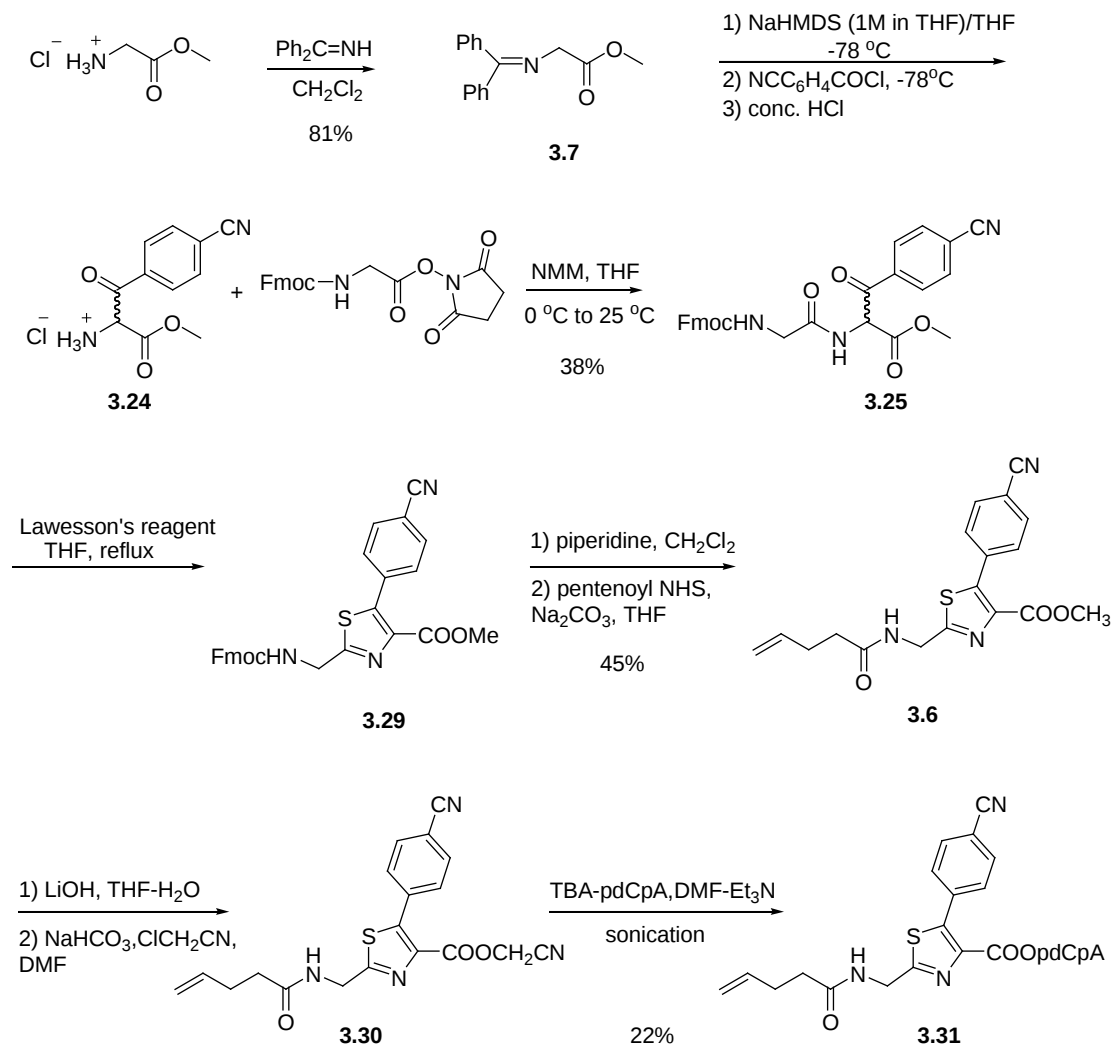
Scheme 3.6. Synthetic route for the preparation of the pdCpA derivative of dipeptidomimetic analogue **3.4**.



Scheme 3.7. Synthetic route for the preparation of the pdCpA derivative of dipeptidomimetic analogue **3.5**.



Scheme 3.8. Synthetic route for the preparation of the pdCpA derivative of dipeptidomimetic analogue **3.6**.



The photophysical properties of the dipeptidomimetic analogues were characterized by measuring the molar absorptivities, quantum yields, emission maxima and absorption maxima of the corresponding *N*-pentenoyl methyl ester derivatives in methanol (Table 3.1). The absorbance and emission maxima of the analogues span the range from 285 to 352 nm and 365 to 490 nm, respectively. All the compounds displayed maximum fluorescence intensity when they were excited at their respective absorption peak wavelengths. Compounds **3.1** and **3.2** were strongly fluorescent with absorption peaks at

~300 nm and emission peaks at ~391 and ~420 nm, respectively. The UV absorption and fluorescence emissions of **3.3** and **3.4** were the most red-shifted compared to all other analogues. The emission peak wavelength of **3.4** (~490 nm) was closest to the emission peak wavelength of wild type GFP (~509 nm) with a large Stokes shift (~138 nm). Nonetheless, **3.4** was a poor fluorescent probe since it had very low quantum yield (0.04). Compound **3.3** was the best fluorescent probe among all the analogues. Because it had high quantum yield (0.62), high molar absorptivity (20000 M⁻¹ cm⁻¹), large Stokes shift (108 nm) and it emitted in the blue region (460 nm) of the spectrum. Compounds **3.5** and **3.6** exhibited very poor fluorescence properties either in terms of shorter wavelengths of emission or lower quantum yields or both.

Table 3.1. Photophysical properties of the dipeptidomimetic analogues in MeOH.

Compounds	$\lambda_{\text{ex, max}}$ (nm)	$\lambda_{\text{em, max}}$ (nm)	ϵ (M ⁻¹ cm ⁻¹)	Φ_{F}^{120}
3.1	296	391	17600	0.90
3.2	300	420	11000	0.23
3.3	352	460	20000	0.62
3.4	352	490	7500	0.04
3.5	285	365	13800	0.26
3.6	290	390	9530	0.01

The activated pdCpA derivatives were ligated to an abbreviated tRNA_{CUA}-C_{OH} transcript using T4 RNA ligase (Scheme 3.9), affording the respective misacylated suppressor tRNA transcripts. The *N*-pentenoyl protecting group was removed by treatment with aqueous iodine.¹¹⁷ Removal of the protecting group was done immediately prior to the use of misacylated tRNAs in protein synthesis.

Scheme 3.9. Ligation of the pdCpA derivatives with tRNA_{CUA}-C_{OH}.

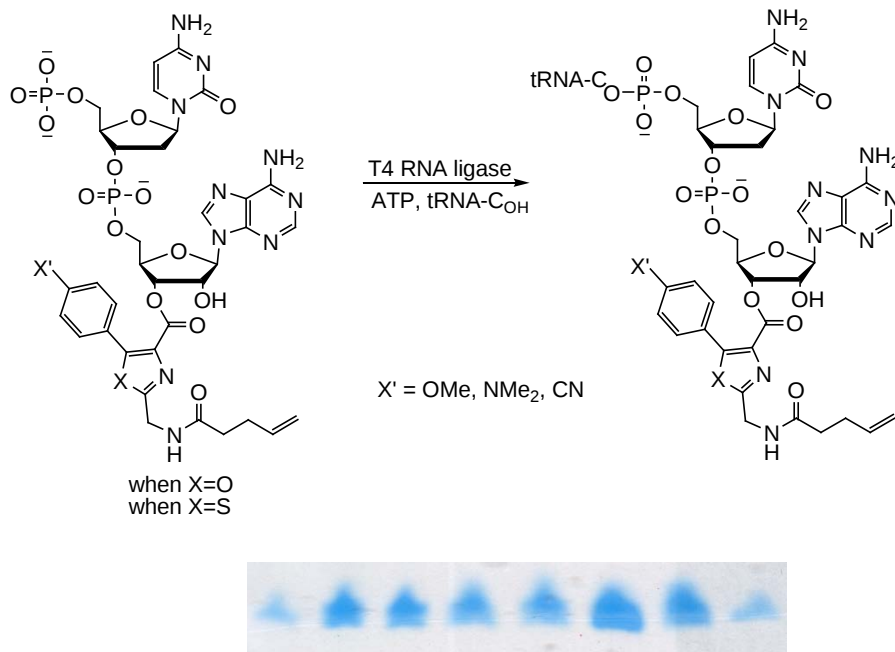


Figure 3.3. Preparation of dipeptidomimetic-tRNA_{CUA}s. Lane 1, nonacylated abbreviated tRNA_{CUA}; lane 2, tRNA_{CUA} ligated with **3.1**; lane 3, tRNA_{CUA} ligated with **3.2**; lane 4, tRNA_{CUA} ligated with **3.3**; lane 5, tRNA_{CUA} ligated with **3.4**; lane 6, tRNA_{CUA} ligated with **3.5**; lane 7, tRNA_{CUA} ligated with **3.6**; lane 7, nonacylated abbreviated tRNA_{CUA}.

The deprotected tRNAs were used in a cell-free coupled transcription-translation system containing an S-30 fraction prepared from *Escherichia coli*, programmed with a GFP analogue construct having a TAG codon at position 66 (pETGFP66 plasmid).¹¹³ The suppression efficiencies were expressed relative to the wild-type GFP synthesis from the wild-type mRNA. As a negative control, wild-type GFP synthesis from the modified mRNA in the presence of nonacylated tRNA_{CUA} was measured for each experiment. The amounts of GFP produced were quantified with a phosphoimager, which monitored the incorporation of ³⁵S-methionine into proteins. S-30 preparations having the modified ribosomes from clone 010326R6⁶⁰ produced full length GFP in ~6.5% yield relative to wild-type GFP synthesis in case of **3.1**, while in case of **3.2** the suppression yield was

~3.5% relative to wild-type GFP synthesis (Figure 3.4).¹¹³ The suppression yields for compounds **3.3-3.6** were ~12%, 11%, 5% and 5%, respectively (Table 3.2).

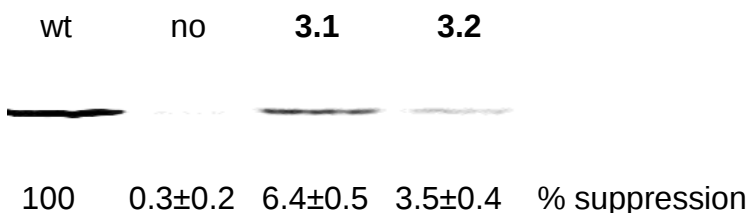


Figure 3.4. Autoradiogram of a 15% SDS-polyacrylamide gel depicting the incorporation of dipeptidomimetic analogues **3.1** and **3.2** into position 66 of GFP. Translation of protein from a wild-type (lane 1) and modified GFP mRNA having a UAG codon in position 66 (lanes 2-4) by the use of an S-30 system prepared from ribosomal clone 010326R6 in the presence of different suppressor tRNAs. Lane 1, wild-type GFP expression; lane 2, modified GFP mRNA in the presence of an abbreviated suppressor tRNA_{CUA}-COH; lane 3, tRNA_{CUA} activated with **3.1**; lane 4, tRNA_{CUA} activated with **3.2**. Phosphorimager analysis was performed using an Amersham Biosciences Storm 820 equipped with ImageQuant version 5.2 software from Molecular Dynamics.

Table 3.2. Suppression yields for compounds **3.3-3.6**. (Experiment was done by Dr. Larisa dedkova)

	3.3	3.4	3.5	3.6
Suppression yield	12%	11%	5%	5%

An increased concentration (0.6 – 1.0 µg/µL) of the activated suppressor tRNA_{CUA} was crucial for effective translation. This concentration was quite excessive compared to the suppressor tRNA_{CUA} concentration used for the expression of α-amino acids (0.1 – 0.2 µg/µL). At lower concentrations of the aminoacyl-tRNA_{CUA} minimal suppression was observed.¹¹³

The GFP analogues having **3.1** and **3.2** at position 66 were prepared at larger scale and purified to study the fluorescence intensity of **3.1** and **3.2** in the protein. The purified proteins containing **3.1** and **3.2** were excited at 305 and 302 nm, respectively. The GFP analogue containing **3.1** exhibited a fluorescence emission maximum at ~375 nm whereas

the protein carrying **3.2** had an emission maximum at ~403 nm (Figure 3.5).¹¹³ In addition, the fluorescence intensities of both the GFP analogues were compared with wild-type GFP. The fluorescence intensities of the modified GFP analogues were significantly greater than wild-type GFP at the same protein concentration.

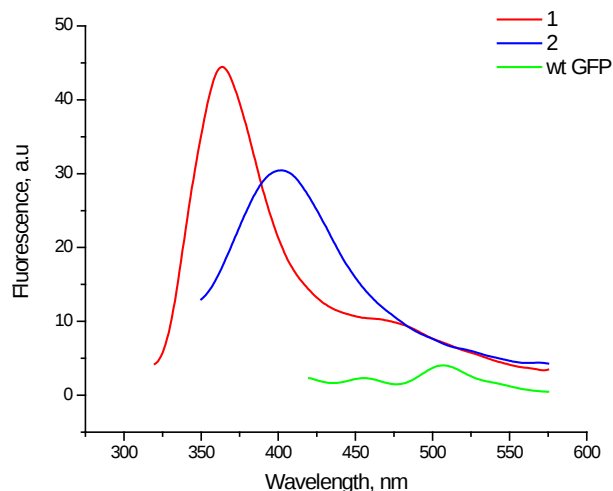


Figure 3.5. Fluorescence emission spectra of three different GFP samples. The red trace is of GFP having dipeptidomimetic analogue **3.1** at position 66; excitation at 305 nm. The blue trace is of GFP having dipeptidomimetic analogue **3.2** at position 66; excitation was at 302 nm. The green trace is of wild-type GFP sample; excitation was at 395 nm. The fluorescence emission spectra were recorded using ~20 nM GFP concentration in 25 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl.

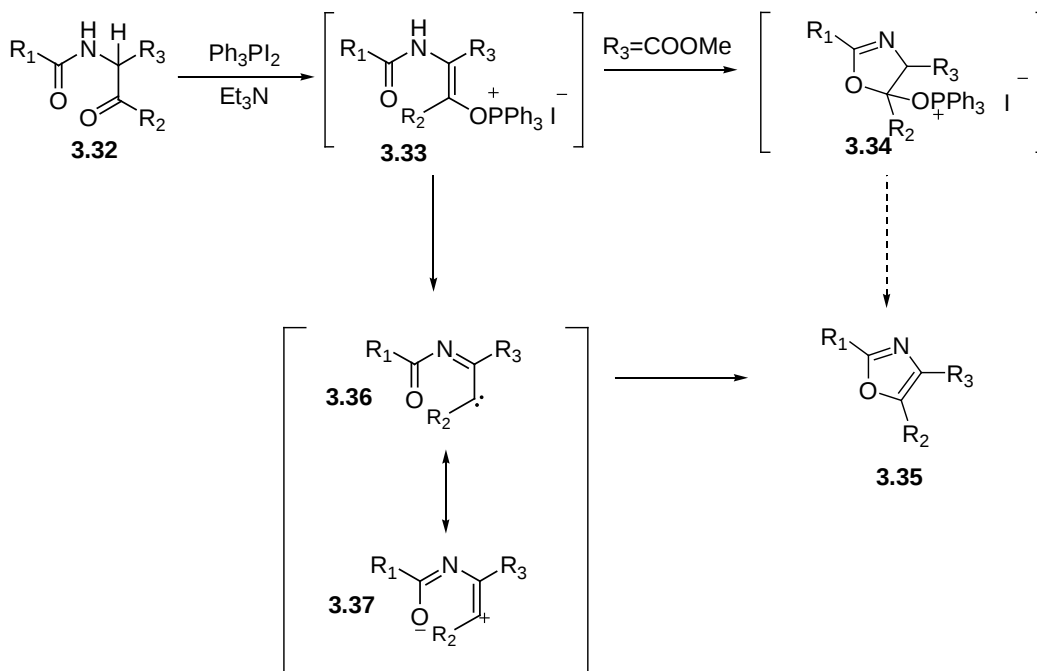
3.3 Discussion

The synthesis of 1, 3-oxazoles and 1, 3-thiazoles has been the focus of many research groups due to their presence in numerous natural products.¹¹⁰⁻¹¹² Nature synthesizes oxazoles through cyclodehydration-oxidation of acylserine derivatives. Most of the synthetic routes for oxazoles also follow this scheme. A versatile synthesis of 1, 3-oxazoles was reported by Dr. Robinson in 1909¹²¹ and Dr. Gabriel in 1910¹²², respectively; it is known as the Robinson-Gabriel oxazole synthesis. According to this

method oxazoles are obtained by the cyclodehydration of α -acylaminoketones, esters or amides. The oxazole synthesis described in this thesis is a versatile extension of the Robinson-Gabriel method. The α -amido- β -ketoesters were directly cyclized using a dehydrating mixture of two equivalents of triphenylphosphine, iodine and triethylamine.¹⁰⁹ The reaction was complete in 15 minutes at room temperature. Several plausible mechanisms can be envisioned for the conversion. Wipf et al. proposed two mechanisms which are shown in Scheme 3.10.¹⁰⁹ In the absence of any base (triethylamine here) no reaction occurs at room temperature; accordingly an initial enolization of **3.32** was proposed. The enol is trapped by highly electrophilic Ph_3PI_2 to form **3.33**. In the next step, an intramolecular addition of the amide onto the vinylphosphonium species can lead to product formation. The nucleophilic attack will be especially favored if the R_3 is electron withdrawing. However, this mechanism can be questioned for a number of reasons. First, the ring closure is disfavored because it is a 5-*endo-trig* type^{123,124}, second, when $\text{R}_3 = \text{CH}_3$, an increase rather than a decrease in yield of **3.35** is observed. So, an alternative mechanism via acylimino carbene **3.36** was proposed by Wipf et al. This mechanism might be more plausible because ring closure of **3.36** to oxazoles was already observed.¹²⁵

Similar to 1, 3-oxazoles, there are number of methods for the synthesis of 1, 3-thiazoles, the most common of them being the Hantzsch synthesis where suitably substituted α -haloketones and thioamides are condensed.¹²⁶ In this report, the thiazoles were obtained by the cyclization of α -amido- β -ketoesters with Lawessons's reagent. This method was followed since α -amido- β -ketoester was a common intermediate for both oxazole and thiazole synthesis, which simplified the entire process.

Scheme 3.10. Proposed mechanism for the cyclization of α -amido- β -ketoesters.



Aequorea victoria GFP chromophore contains a Tyr moiety (Scheme 3.1). The phenolic OH group of the Tyr moiety is particularly important for the photophysical properties of GFP. There has been an extensive structure-activity study on the fluorophore of GFP.¹⁰⁵ In addition, several mutant GFPs containing Phe analogues with different para-substituents, in place of Tyr66, were prepared by Schultz and coworkers.¹⁰⁴ All these studies indicate that the wavelengths for both absorbance and emission peaks of the mutant GFPs increased with an increase in the electron-donating ability of the aryl substituents. In order to see the effect of the substituents on the photophysical properties of the peptidomimetic analogues that I prepared, several analogues containing either electron donating (NMe_2 , OMe) or electron withdrawing (CN) substituents were synthesized. An analogue having phenolic OH group was omitted in order to avoid complicated synthetic steps as well as the possible multiple charge state of the

chromophore under the assay conditions. The wavelengths of absorbance and emission peaks for both oxazoles and thiazoles follow the order $\text{NMe}_2 > \text{OMe} > \text{CN}$, which is in accordance with earlier studies.¹⁰⁴ The NMe_2 is the most electron donating substituent, and exhibited the maximum red shift of the absorption and emission peaks. Compounds **3.3** and **3.4** had absorption peaks at ~352 nm and the emission peaks at ~460 and 490 nm in methanol, respectively (Table 3.1). In comparison compounds **3.5** and **3.6** exhibited the shortest absorption and emission peaks. In all the cases, the emission peaks of the thiazoles were ~20-30 nm red shifted compared to the corresponding oxazoles.

Hecht and coworkers have been trying to incorporate unnatural amino acids into proteins by modified ribosomes.⁵⁷⁻⁵⁹ So far, a single unnatural amino acid was incorporated into protein by the suppression of a single amber stop codon. In 2015 there was a significant improvement in this method when our group incorporated dipeptides and a dipeptidomimetic analogue, which can be seen as two amino acids tied together, into proteins by the suppression of a single stop codon.⁶⁰ The S-30 system contained the modified ribosomes, which had altered peptidyltransferase centers. The modified ribosomes were able to recognize dipeptides and dipeptide analogues in addition to α -L-amino acids.⁶⁰ A dipeptidyl puromycin derivative (**1.2**, Figure 1.15) was used to select the modified ribosomes. Recognition of the dipeptidomimetic analogues by these modified ribosomes is presumably due to the fact that the distance between the amine and carboxylate groups in the dipeptidomimetics is similar to the distance between the free amine and carboxylate groups of a dipeptide. When these S-30 systems were employed for the *in vitro* translation experiments, they produced full length GFP containing **3.1-3.6** in yields ranging from ~3.5% to ~12%. Additionally, the mutant GFPs containing **3.1** and

3.2 were scaled up to study the fluorescence of the dipeptidomimetic analogues in protein. It was observed that the mutant GFP analogue containing **3.1** had ~10-fold higher fluorescence intensity compared to wild-type GFP at the same protein concentration, while the GFP analogue containing **3.2** had ~7-fold higher fluorescence intensity.¹¹³ Thus these fluorophores are sensitive to their environment, a property, which should be applicable to many applications.

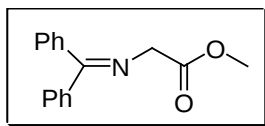
3.4. Experimental

All experiments requiring anhydrous conditions were conducted in flame-dried glassware fitted with rubber septa under a positive pressure of dry argon. Analytical thin layer chromatography was performed using glass plates precoated with silica gel (0.25 mm, 60 pore size, 230-400 mesh, Silicycle) impregnated with a fluorescent indicator (254 nm). TLC plates were visualized by exposure to ultraviolet light (UV). Flash column chromatography was performed employing silica gel (60 Å pore size, 40-63 µm, standard grade Silicycle silica gel). An acetone cooling bath was adjusted to the appropriate temperature by the addition of small portions of dry ice.

¹H NMR and ¹³C NMR spectra were recorded on Varian INOVA 400 (400 MHz) spectrometer at 25 °C. Proton chemical shifts are expressed in parts per million (ppm, δ scale). Splitting patterns are indicated as follows; s, singlet; br s, broad singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; m, multiplet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Facility or at the Michigan State University Mass Spectrometry Facility. HPLC purification was conducted with a Waters 600 pump coupled with a Varian-ProStar 340 detector and a Grace Econosil C₁₈ column (250 × 10 mm, 5 µm). The tetra(*n*-

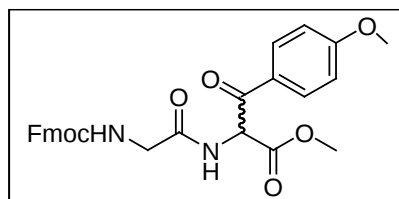
butyl)ammonium (TBA) salt of pdCpA was prepared using Dowex 50W×8, 200-400 mesh activated in its TBA form. Reagents were purchased from Aldrich Chemical Co., Sigma Chemical Co., Combi Blocks or Chem Impex International and were used without further purification. THF was distilled under argon from benzophenone ketyl and CH₂Cl₂ was distilled under argon from calcium hydride.

Tris, acrylamide, bis-acrylamide, urea, ammonium persulfate, TEMED, dihydrofolic acid, glycerol, ampicillin, pyruvate kinase, lysozyme, erythromycin, isopropyl-β-D-thiogalactopyranoside (IPTG), dithiothreitol (DTT) and 2-mercaptoethanol were purchased from Sigma Chemicals (St. Louis, MO). ³⁵S-Methionine (10 μCi/μL) was purchased from Amersham (Piscataway, NJ). BL-21-(DE-3) competent cells and T4 RNA ligase were from Promega (Madison, WI). Plasmid MaxiKit (Life Science Products, Inc., Frederick, CO) and GenElute™HP plasmid miniprep kit (Sigma) were used for plasmid purification.



Methyl 2-(Diphenylmethylenamino)acetate (3.7). To a stirred suspension containing 5.00 g (39.9 mmol) of glycine methyl ester hydrochloride in 20 mL of anhydrous CH₂Cl₂ was added dropwise 6.70 mL (7.20 g, 39.9 mmol) of benzophenone imine. The reaction mixture was stirred at 25 °C for 24 h under an argon atmosphere. The reaction mixture was filtered and concentrated under diminished pressure. The crude product was crystallized from ether–hexanes to afford **3.7** as colorless needles: yield 8.20 g (81%); silica gel TLC *R_f* 0.22 (1:9 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 3.73 (s, 3H), 4.21 (s, 2H), 7.15-7.18 (m, 2H), 7.30-7.45 (m, 6H) and 7.63-7.66 (m, 2H); ¹³C NMR

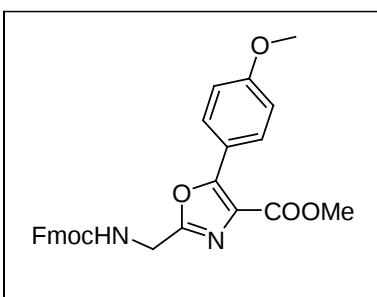
(CDCl₃) δ 52.0, 55.6, 127.6, 128.1, 128.69, 128.75, 128.83, 130.5, 135.9, 139.2, 171.1 and 171.9; mass spectrum (APCI), m/z 254.1182 (M + H)⁺ (C₁₆H₁₆NO₂ requires m/z 254.1181).



Methyl 2-(2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)acetamido)-3-(4-methoxyphenyl)-3-oxopropanoate (3.9). A solution of 0.50 g (2.00 mmol) of **3.7** in 5 mL of anhydrous THF was cooled to -78 °C under an argon atmosphere and 2.00 mL (2.00 mmol) of 1 M sodium bis(trimethylsilyl)amide in THF was added dropwise while maintaining the temperature at -78 °C. After 30 min, the resulting yellow solution was added via cannula to a stirred solution of 0.34 g (2.00 mmol) of 4-methoxybenzoyl chloride in 3 mL of anhydrous THF at -78 °C. The reaction mixture was stirred at -78 °C for 2 h. The yellow reaction mixture was acidified with concentrated HCl to pH ~2 and was concentrated under diminished pressure. The crude product (**3.8**) was utilized for the next reaction without further purification.

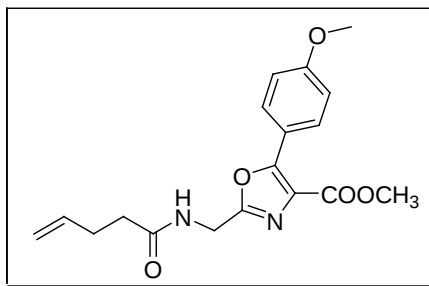
To a solution of the crude product in 10 mL of anhydrous THF at 0 °C was added 0.79 g (2.00 mmol) of Fmoc-gly-*N*-hydroxysuccinimide ester followed by the dropwise addition of 0.22 mL (0.20 g, 2.00 mmol) *N*-methylmorpholine. The yellow reaction mixture was stirred at 25 °C for 2 h and then concentrated under diminished pressure. The crude product was purified on a silica gel column (15 × 2 cm). Elution with 1:1 ethyl acetate–hexanes afforded **3.9** as a colorless oil: yield 0.67 g (67%); silica gel TLC R_f 0.29 (3:2 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 3.69 (s, 3H), 3.86 (s, 3H), 4.06 (br s,

2H), 4.23 (d, 1H, $J = 6.8$ Hz), 4.40 (d, 2H, $J = 7.2$ Hz), 5.68 (br s, 1H), 6.18 (d, 1H, $J = 7.6$ Hz), 6.94 (d, 2H, $J = 8.8$ Hz), 7.29 (t, 2H, $J = 7.4$ Hz), 7.38 (t, 2H, $J = 7.4$ Hz), 7.54 (d, 1H, $J = 6.8$ Hz), 7.60 (d, 2H, $J = 7.2$ Hz), 7.75 (d, 2H, $J = 7.6$ Hz) and 8.09 (d, 2H, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3) δ 44.3, 47.2, 53.4, 55.7, 57.7, 67.4, 114.2, 120.0, 125.2, 126.8, 127.2, 127.8, 132.2, 141.3, 143.9, 156.6, 164.9, 167.2, 169.0 and 189.1; mass spectrum (APCI), m/z 503.1816 ($\text{M} + \text{H}^+$) ($\text{C}_{28}\text{H}_{27}\text{N}_2\text{O}_7$ requires m/z 503.1818).



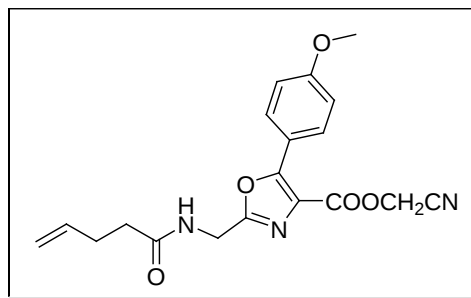
Methyl 2-((((9H-Fluoren-9-yl)methoxy)carbonylamino)methyl)-4-(4-methoxyphenyl)oxazole-5-carboxylate (3.10). To a stirred solution of 0.17 g (0.80 mmol) of triphenylphosphine and 0.20 g (0.80 mmol) of iodine in 10 mL of anhydrous CH_2Cl_2 was added 0.11 mL (83.0 mg, 0.80 mmol) of triethylamine. The dark yellow solution was stirred for 5 min and 0.20 g (0.40 mmol) of **3.9** dissolved in 5 mL anhydrous CH_2Cl_2 was added dropwise. The reaction mixture was stirred for 30 min at 25 °C under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (15 $\times$ 2 cm). Elution with 3:2 ethyl acetate–hexanes afforded **3.10** as a colorless solid: yield 0.15 g (78%); silica gel TLC R_f 0.45 (3:2 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 3.85 (s, 3H), 3.92 (s, 3H), 4.23 (t, 1H, $J = 7.0$ Hz), 4.45 (d, 2H, $J = 6.8$ Hz), 4.59 (d, 2H, $J = 5.6$ Hz), 5.63 (br s, 1H), 6.96 (d, 2H, $J = 8.8$ Hz), 7.28 (t, 2H, $J = 7.6$ Hz), 7.38 (t, 2H, $J = 7.4$ Hz), 7.59 (d, 2H, $J = 7.6$ Hz), 7.75 (d, 2H, $J = 7.2$ Hz) and 8.02 (d, 2H, $J = 9.2$ Hz); ^{13}C NMR (CDCl_3) δ 38.4, 47.2, 52.4, 55.5,

67.4, 114.0, 119.1, 120.1, 125.1, 125.4, 127.2, 127.8, 130.2, 141.4, 143.8, 156.3, 156.4, 158.5, 161.4 and 162.6; mass spectrum (APCI), m/z 485.1722 ($M + H$)⁺ ($C_{28}H_{25}N_2O_6$ requires m/z 485.1713).

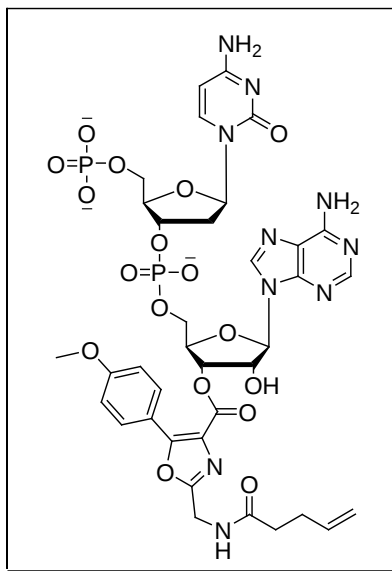


Methyl 4-(4-Methoxyphenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxylate (3.1).

To a stirred solution of 0.15 g (0.31 mmol) of **3.10** in 4 mL of anhydrous CH_2Cl_2 was added dropwise 31.0 μ L (27.0 mg, 0.31 mmol) of piperidine. The reaction mixture was stirred at 25 °C under an argon atmosphere for 2 h and was then concentrated under diminished pressure. The residue was dissolved in 5 mL of anhydrous THF and 0.61 g (3.10 mmol) of 4-pentenoyloxysuccinimide was added followed by 66.0 mg (0.62 mmol) of Na_2CO_3 . The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (7 $\times$ 2 cm). Elution with 7:3 ethyl acetate–hexanes afforded **3.1** as a pale yellow solid: yield 45.0 mg (42% over two steps); silica gel TLC R_f 0.19 (7:3 ethyl acetate–hexanes); 1H NMR ($CDCl_3$) δ 2.33–2.42 (m, 4H), 3.84 (s, 3H), 3.90 (s, 3H), 4.62 (d, 2H, J = 5.6 Hz), 4.97–5.08 (m, 2H), 5.76–5.85 (m, 1H), 6.43 (br s, 1H), 6.95 (d, 2H, J = 9.2 Hz) and 8.00 (d, 2H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ 29.4, 35.6, 36.6, 52.3, 55.5, 114.0, 115.8, 119.1, 125.3, 130.2, 136.9, 156.4, 158.6, 161.4, 162.5 and 172.5; mass spectrum (APCI), m/z 345.1452 ($M + H$)⁺ ($C_{18}H_{21}N_2O_5$ requires m/z 345.1450).

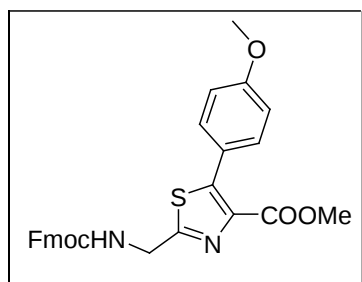


Cyanomethyl 4-(4-Methoxyphenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxylate (3.11). To a stirred solution of 15.0 mg (0.04 mmol) of **3.1** in 0.40 mL of 3:1 THF-water was added 0.13 mL of 1 N LiOH. The reaction mixture was stirred at 25 °C for 2 h. The yellow reaction mixture was diluted with MeOH, dried over anhydrous Na₂SO₄ and was concentrated under diminished pressure. The crude product was dissolved in 2 mL of anhydrous DMF and 27.0 mg (0.32 mmol) of NaHCO₃ was added, followed by 13.0 µL (15.0 mg, 0.21 mmol) of ClCH₂CN. The reaction mixture was stirred at 25 °C for 3 h under an argon atmosphere. The reaction mixture was concentrated under diminished pressure and the residue was purified on a silica gel column (7 × 1 cm). Elution with 2.5% methanol in dichloromethane afforded **3.11** as a pale yellow solid: yield 5.5 mg (37%); silica gel TLC *R*_f 0.74 (ethyl acetate); ¹H NMR (CDCl₃) δ 2.39-2.44 (m, 4H), 3.87 (s, 3H), 4.65 (d, 2H, *J* = 5.6 Hz), 4.94 (s, 2H), 5.00-5.10 (m, 2H), 5.80-5.87 (m, 1H), 6.25 (br s, 1H), 6.99 (d, 2H, *J* = 8.8 Hz) and 8.01 (d, 2H, *J* = 8.8 Hz); ¹³C NMR (CDCl₃) δ 29.4, 31.1, 35.6, 36.7, 48.8, 55.6, 114.2, 116.0, 118.5, 123.5, 130.4, 136.8, 158.3, 158.9, 160.6, 162.0 and 172.5; mass spectrum (APCI), *m/z* 370.1402 (*M* + H)⁺ (C₁₉H₂₀N₃O₅ requires *m/z* 370.1403).

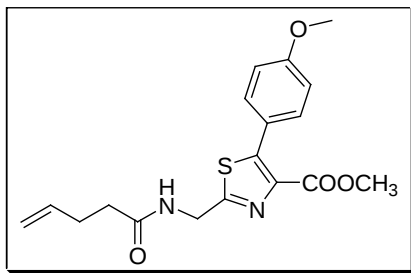


4-(4-Methoxyphenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxylate pdCpA

(3.12). A solution containing 6.0 mg (16 μmol) of cyanomethyl ester **3.11** and 5.3 mg (4.0 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 100 μL of 9:1 DMF–Et₃N was subjected to sonication at room temperature for 4 h. The reaction mixture was diluted with 0.5 mL of 50 mM ammonium acetate, pH 4.5 and centrifuged. The supernatant was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1% $\rightarrow$ 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 19 min was collected and lyophilized to afford **3.12** as a colorless solid: yield 2.3 mg (60%); mass spectrum (ESI), m/z 947.2159 ($M - H$)[–] (C₃₆H₄₁N₁₀O₁₇P₂ requires m/z 947.2126).

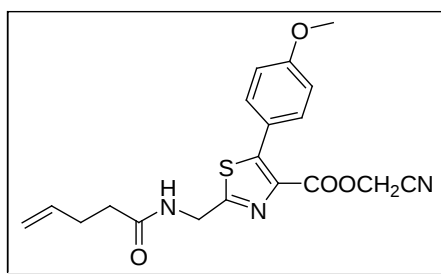


Methyl 2-((((9H-Fluoren-9-yl)methoxy)carbonylamino)methyl)-4-(4-methoxyphenyl)thiazole-5-carboxylate (3.13). To a stirred solution of 0.22 g (0.44 mmol) of **3.9** in 5 mL of anhydrous THF was added 0.36 g (0.88 mmol) of the Lawesson's reagent. The reaction mixture was heated to reflux under an argon atmosphere for 1 h. The yellow reaction mixture was diluted with 20 mL of saturated NaHCO₃ solution. The aqueous layer was extracted with two 25-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO₄ and was concentrated under diminished pressure. The residue was purified on a silica gel column (15 × 2 cm). Elution with 3:2 ethyl acetate–hexanes afforded **3.13** as a colorless solid: yield 0.18 g (80%); silica gel TLC *R_f* 0.48 (7:3 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 3.84 (s, 3H), 3.85 (s, 3H), 4.23 (t, 1H, *J* = 6.6 Hz), 4.49 (d, 2H, *J* = 6.8 Hz), 4.67 (d, 2H, *J* = 6.0 Hz), 5.62 (br s, 1H), 6.94 (d, 2H, *J* = 7.6 Hz), 7.29 (t, 2H, *J* = 7.2 Hz), 7.37 (t, 2H, *J* = 7.2 Hz), 7.51 (d, 2H, *J* = 8.4 Hz), 7.58 (d, 2H, *J* = 7.2 Hz) and 7.75 (d, 2H, *J* = 7.2 Hz); ¹³C NMR (CDCl₃) δ 42.8, 47.3, 52.4, 55.5, 67.2, 113.8, 120.1, 122.2, 125.1, 127.2, 127.9, 131.4, 138.9, 141.4, 143.8, 148.2, 156.4, 160.6, 162.5 and 165.9; mass spectrum (APCI), *m/z* 501.1468 (*M* + *H*)⁺ (C₂₈H₂₅N₂O₅S requires *m/z* 501.1484).



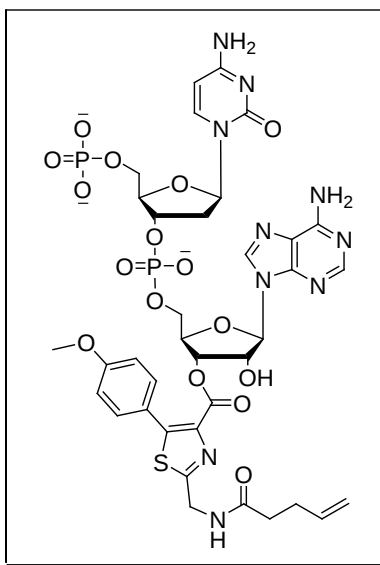
Methyl 4-(4-Methoxyphenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxylate (3.2). To a stirred solution of 0.17 g (0.34 mmol) of **3.13** in 4 mL of anhydrous CH₂Cl₂

was added dropwise 67.0 μL (58.0 mg, 0.68 mmol) of piperidine. The reaction mixture was stirred at 25 $^{\circ}\text{C}$ under an argon atmosphere for 3 h and was concentrated under diminished pressure. The residue was dissolved in 5 mL of anhydrous THF and 0.13 g (0.66 mmol) of 4-pentenoyloxysuccinimide was added, followed by 47.0 mg (0.44 mmol) of Na_2CO_3 . The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (7×2 cm). Elution with 7:3 ethyl acetate–hexanes afforded **3.2** as a pale yellow solid: yield 55.0 mg (45% over two steps); silica gel TLC R_f 0.19 (7:3 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 2.32–2.41 (m, 4H), 3.81 (s, 3H), 3.83 (s, 3H), 4.70 (d, 2H, $J = 6.0$ Hz), 4.97–5.07 (m, 2H), 5.75–5.85 (m, 1H), 6.59 (br s, 1H), 6.91 (d, 2H, $J = 8.8$ Hz) and 7.40 (d, 2H, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3) δ 29.4, 35.5, 41.1, 52.3, 55.4, 113.8, 115.9, 122.1, 131.4, 136.8, 138.6, 148.3, 160.6, 162.5, 165.4 and 172.7; mass spectrum (APCI), m/z 361.1110 ($\text{M} + \text{H}^+$) ($\text{C}_{18}\text{H}_{21}\text{N}_2\text{O}_4\text{S}$ requires m/z 361.1144).



Cyanomethyl 4-(4-Methoxyphenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxylate (3.14). To a stirred solution of 15.0 mg (0.04 mmol) of **3.2** in 0.40 mL of 3:1 THF–water was added 0.13 mL of 1 N LiOH. The reaction mixture was stirred at 25 $^{\circ}\text{C}$ for 2 h. The yellow reaction mixture was diluted with MeOH, dried over anhydrous Na_2SO_4 and was concentrated under diminished pressure. The crude product was

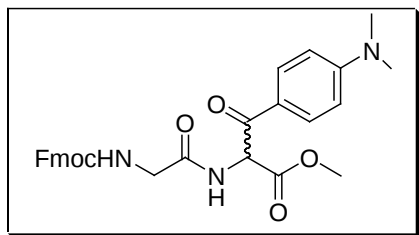
dissolved in 2 mL of anhydrous DMF and 7.00 mg (0.08 mmol) of NaHCO₃ was added, followed by 13.0 μ L (16.0 mg, 0.21 mmol) of ClCH₂CN. The reaction mixture was stirred at 25 °C for 3 h under an argon atmosphere. The reaction mixture was concentrated under diminished pressure and was purified on a silica gel column (7 $\times$ 1 cm). Elution with ethyl acetate afforded **3.14** as a pale yellow solid: yield 5.8 mg (38%); silica gel TLC *R_f* 0.74 (ethyl acetate); ¹H NMR (CDCl₃) δ 2.37-2.44 (m, 4H), 3.86 (s, 3H), 4.73 (d, 2H, *J* = 6.0 Hz), 4.86 (s, 2H), 5.01-5.10 (m, 2H), 5.79-5.86 (m, 1H), 6.33 (br s, 1H), 6.95 (d, 2H, *J* = 8.8 Hz) and 7.42 (d, 2H, *J* = 8.4 Hz); ¹³C NMR (CDCl₃) δ 29.5, 31.1, 35.6, 41.2, 48.7, 55.5, 114.1, 114.2, 116.1, 121.5, 131.4, 136.8, 151.2, 160.2, 161.0, 165.8 and 172.7; mass spectrum (APCI), *m/z* 386.1177 (*M* + *H*)⁺ (C₁₉H₂₀N₃O₄S requires *m/z* 386.1175).



4-(4-Methoxyphenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxylate pdCpA

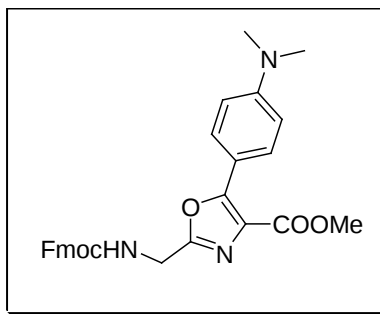
(3.15). A solution containing 6.0 mg (16 μ mol) of cyanomethyl ester **3.14** and 8.0 mg (6.0 μ mol) of the tris(tetrabutylammonium) salt of pdCpA in 100 μ L of 9:1 DMF–Et₃N was subjected to sonication at room temperature for 4 h. The reaction mixture was

diluted with 0.5 mL of 50 mM ammonium acetate, pH 4.5 and centrifuged. The supernatant was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1% → 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 19 min was collected and lyophilized to afford **3.15** as a colorless solid: yield 3.5 mg (60%); mass spectrum (ESI), m/z 963.1874 ($M - H$)⁻ (C₃₆H₄₁N₁₀O₁₆P₂S requires m/z 963.1898).



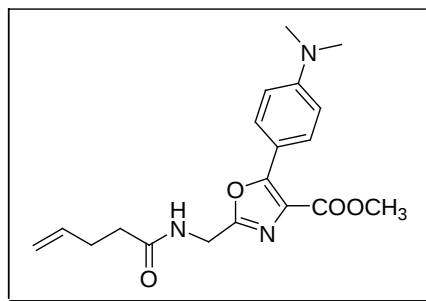
Methyl 2-(2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)acetamido)-3-(4-(dimethylamino)phenyl)-3-oxopropanoate (3.17). A solution of 0.50 g (2.00 mmol) of **3.7** in 5 mL of anhydrous THF was cooled to -78 °C under an argon atmosphere and 2.00 mL (2.00 mmol) of 1 M sodium bis(trimethylsilyl)amide in THF was added dropwise while maintaining the temperature at -78 °C. After 30 min, the resulting yellow solution was added via cannula to a stirred solution of 0.43 g (2.00 mmol) of 4-dimethylaminobenzoyl chloride in 3 mL of anhydrous THF at -78 °C. The reaction mixture was stirred at -78 °C for 2 h. The yellow reaction mixture was acidified with concentrated HCl until pH ~2 and was then concentrated under diminished pressure. The crude product (**3.16**) was utilized for the next reaction without further purification. To a solution of the crude product in 10 mL of anhydrous THF at 0 °C was added 0.79 g (2.00 mmol) of Fmoc-gly-*N*-hydroxysuccinimide followed by the dropwise addition of 0.22 mL (0.20 g, 2.00 mmol) of *N*-methylemorpholine. The yellow reaction mixture was

stirred at 25 °C for 2 h and then concentrated under diminished pressure. The crude product was purified on a silica gel column (15 × 2 cm). Elution with 1:1 ethyl acetate–hexanes afforded **3.17** as a yellowish solid: yield 0.35 g (34%); silica gel TLC R_f 0.27 (1:1 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 3.07 (s, 6H), 3.68 (s, 3H), 4.02 (br s, 2H), 4.21 (s, 1H), 4.39 (d, 2H, J = 6.8 Hz), 5.50 (br s, 1H), 6.08 (d, 1H, J = 7.2 Hz), 6.64 (d, 2H, J = 8.8 Hz), 7.28 (t, 2H, J = 7.4 Hz), 7.37 (t, 2H, J = 7.4 Hz), 7.48 (d, 1H, J = 7.2 Hz), 7.59 (d, 2H, J = 7.6 Hz), 7.73 (d, 2H, J = 7.6 Hz) and 8.00 (d, 2H, J = 8.8 Hz); ^{13}C NMR (CDCl_3) δ 44.3, 47.2, 53.4, 55.7, 57.7, 67.4, 114.2, 120.0, 125.2, 126.8, 127.2, 127.8, 132.2, 141.4, 143.9, 156.6, 164.9, 167.3, 169.0 and 189.1; mass spectrum (APCI), m/z 516.2060 ($\text{M} + \text{H}^+$) ($\text{C}_{29}\text{H}_{30}\text{N}_3\text{O}_6$ requires m/z 516.2069).



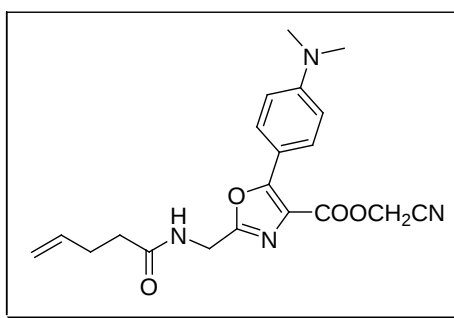
Methyl 2-((((9H-Fluoren-9-yl)methoxy)carbonylamino)methyl)-4-(4-(dimethylamino)phenyl)oxazole-5-carboxylate (3.18). To a stirred solution of 0.29 g (1.12 mmol) of triphenylphosphine and 0.28 g (1.12 mmol) of iodine in 10 mL of anhydrous CH_2Cl_2 was added 0.15 mL (0.11 g, 1.12 mmol) of triethylamine. The dark yellow solution was stirred for 5 min and 0.29 g (0.56 mmol) of **3.17** dissolved in 5 mL of anhydrous CH_2Cl_2 was added dropwise. The reaction mixture was stirred for 30 min at 25 °C under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (15 × 2 cm). Elution with 2:3

ethyl acetate–hexanes afforded **3.18** as a yellow solid: yield 0.20 g (71%); silica gel TLC R_f 0.43 (1: 1 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 3.02 (s, 6H), 3.91 (s, 3H), 4.23 (t, 1H, J = 5.8 Hz), 4.44 (d, 2H, J = 6.8 Hz), 4.57 (d, 2H, J = 5.6 Hz), 5.67 (br s, 1H), 6.71 (d, 2H, J = 8.8 Hz), 7.29 (t, 2H, J = 7.4 Hz), 7.38 (t, 2H, J = 7.2 Hz), 7.60 (d, 2H, J = 7.2 Hz), 7.74 (d, 2H, J = 7.6 Hz) and 7.98 (d, 2H, J = 8.8 Hz); ^{13}C NMR (CDCl_3) δ 38.2, 40.0, 47.0, 52.0, 67.2, 111.2, 113.7, 119.9, 123.7, 125.0, 127.0, 127.6, 128.5, 129.6, 141.2, 143.7, 151.5, 156.1, 157.4 and 162.7; mass spectrum (APCI), m/z 498.1930 ($\text{M} + \text{H}^+$) ($\text{C}_{29}\text{H}_{28}\text{N}_3\text{O}_5$ requires m/z 498.1951).

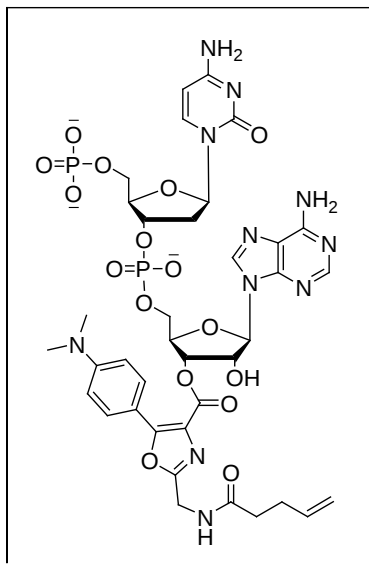


Methyl 4-(4-(Dimethylamino)phenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxylate (3.3). To a stirred solution of 0.20 g (0.40 mmol) of **3.18** in 4 mL of anhydrous CH_2Cl_2 was added dropwise 77.0 μL (66.0 mg, 0.78 mmol) of piperidine. The reaction mixture was stirred at 25 $^\circ\text{C}$ under an argon atmosphere for 2 h and was then concentrated under diminished pressure. The residue was dissolved in 5 mL of anhydrous THF and 0.15 g (0.78 mmol) of 4-pentenoyloxysuccinimide was added followed by 83.0 mg (0.78 mmol) of Na_2CO_3 . The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (7 $\times$ 2 cm). Elution with 7:3 ethyl acetate–hexanes afforded **3.3** as a pale yellow solid: yield 48.0 mg (33%

over two steps); silica gel TLC R_f 0.19 (7:3 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 2.35-2.42 (m, 4H), 3.02 (s, 6H), 3.90 (s, 3H), 4.61 (d, 2H, $J = 5.6$ Hz), 4.97-5.08 (m, 2H), 5.79-5.85 (m, 1H), 6.39 (br s, 1H), 6.71 (d, 2H, $J = 8.8$ Hz) and 7.96 (d, 2H, $J = 8.8$ Hz); ^{13}C NMR (CDCl_3) δ 29.3, 35.5, 36.6, 40.0, 52.1, 111.2, 113.7, 115.7, 123.7, 129.6, 136.7, 151.6, 157.45, 157.50, 162.7 and 172.2; mass spectrum (APCI), m/z 358.1680 ($\text{M} + \text{H}$) $^+$ ($\text{C}_{19}\text{H}_{24}\text{N}_3\text{O}_4$ requires m/z 358.1689).

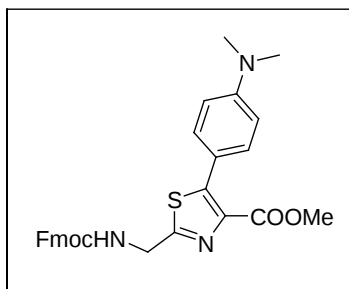


Cyanomethyl 4-(4-(Dimethylamino)phenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxylate (3.19). To a stirred solution of 15.0 mg (0.04 mmol) of **3.3** in 0.40 mL 3:1 THF–water was added 0.08 mL of 1 N LiOH. The reaction mixture was stirred at 25 °C for 3 h. The yellow reaction mixture was diluted with MeOH, dried over anhydrous Na_2SO_4 and was concentrated under diminished pressure. The crude product was dissolved in 2 mL of anhydrous DMF and 10.0 mg (0.09 mmol) of NaHCO_3 was added followed by 13.0 μL (16.0 mg, 0.21 mmol) of ClCH_2CN . The reaction mixture was stirred at 25 °C for 3 h under an argon atmosphere. The reaction mixture was concentrated under diminished pressure. The crude product used in the next step without further purification.

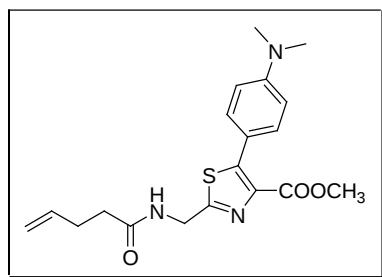


4-(4-(Dimethylamino)phenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxyl pdCpA

(3.20). A solution containing 6.0 mg (16 μmol) of cyanomethyl ester **3.19** and 8.0 mg (6.0 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 100 μL of 9:1 DMF–Et₃N was subjected to sonication at room temperature for 2.5 h. The reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1% to 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 19.5 min was collected and lyophilized to afford **3.20** as a yellow solid: yield- 3.7 mg (66%); mass spectrum (ESI), m/z 960.2440 ($M - H$)[−] (C₃₇H₄₄N₁₁O₁₆P₂ requires m/z 960.2443).

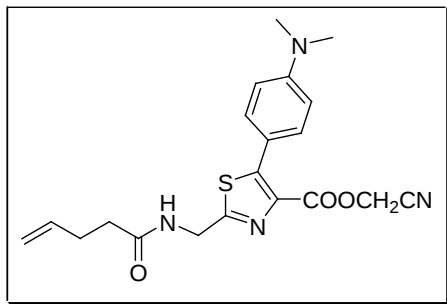


Methyl 2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)methyl)-4-(4-(dimethylamino)phenyl)thiazole-5-carboxylate (3.21). To a stirred solution of 0.16 g (0.31 mmol) of **3.17** in 5 mL of anhydrous THF was added 0.25 g (0.62 mmol) of the Lawesson's reagent. The reaction mixture was heated to reflux under an argon atmosphere for 1 h. The yellow reaction mixture was diluted with 20 mL of saturated NaHCO₃ solution. The aqueous layer was extracted with two 25 mL-portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO₄ and was concentrated under diminished pressure. The crude product was utilized in the next reaction without further purification.



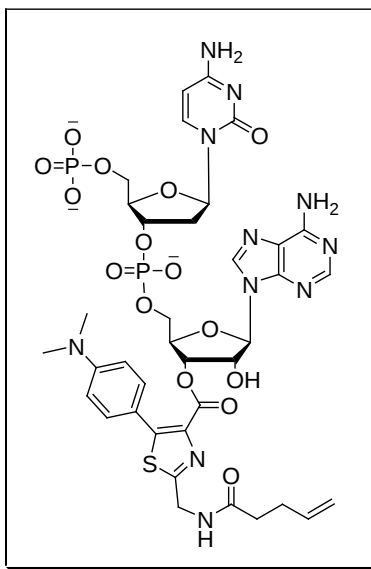
Methyl 4-(4-(Dimethylamino)phenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxylate (3.4). To a stirred solution of the crude **3.21** in 4 mL of anhydrous CH₂Cl₂ was added dropwise 64.0 μ L (55.0 mg, 0.65 mmol) of piperidine. The reaction mixture was stirred at 25 °C under an argon atmosphere for 2 h and was concentrated under diminished pressure. The residue was dissolved in 5 mL of anhydrous THF and 0.26 g (1.32 mmol) of 4-pentenoyloxysuccinimide was added followed by 83.0 mg (0.78 mmol) of Na₂CO₃. The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and concentrated under diminished pressure. The residue was purified on a silica gel column (7 $\times$ 2 cm). Elution with 7:3 ethyl acetate–hexanes

afforded **3.4** as a pale yellow solid: yield 60.0 mg (52% over three steps); silica gel TLC R_f 0.17 (7:3 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 2.33-2.42 (m, 4H), 3.00 (s, 6H), 3.84 (s, 3H), 4.70 (d, 2H, J = 6.0 Hz), 4.99-5.09 (m, 2H), 5.78-5.85 (m, 1H), 6.40 (br s, 1H), 6.69 (d, 2H, J = 8.8 Hz) and 7.38 (d, 2H, J = 8.8 Hz); ^{13}C NMR (CDCl_3) δ 29.3, 35.5, 40.2, 40.9, 52.1, 111.3, 115.8, 116.9, 130.9, 136.7, 137.4, 149.7, 150.9, 162.6, 163.9 and 172.4; mass spectrum (APCI), m/z 396.1348 ($\text{M} + \text{H}$) $^+$ ($\text{C}_{19}\text{H}_{23}\text{N}_3\text{O}_3\text{NaS}$ requires m/z 396.1358).

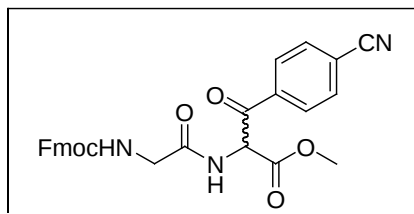


Cyanomethyl 4-(4-(Dimethylamino)phenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxylate (3.22). To a stirred solution of 17.0 mg (0.05 mmol) of **3.4** in 0.4 mL of 3:1 THF–water was added 0.06 mL of 1 N LiOH. The reaction mixture was stirred at 25 °C for 4.5 h. The yellow reaction mixture was diluted with MeOH, dried over anhydrous Na₂SO₄ and was concentrated under diminished pressure. The crude product was dissolved in 2 mL of anhydrous DMF and 9.00 mg (0.11 mmol) of NaHCO₃ was added followed by 25.0 μL (30.0 mg, 0.40 mmol) of ClCH₂CN. The reaction mixture was stirred at 25 °C for 3 h under an argon atmosphere. The reaction mixture was concentrated under diminished pressure and was purified on a silica gel column (7 $\times$ 1 cm). Elution with 3:2 ethyl acetate–hexanes afforded **3.22** as a bright yellow solid: yield 12.0 mg (65%); silica gel TLC R_f 0.7 (ethyl acetate); ^1H NMR (CDCl_3) δ 2.36-2.44 (m,

4H), 3.02 (s, 6H), 4.71 (d, 2H, $J = 2.8$ Hz), 4.87 (s, 2H), 5.00-5.10 (m, 2H), 5.79-5.84 (m, 1H), 6.29 (br s, 1H), 6.71 (d, 2H, $J = 8.4$ Hz) and 7.39 (d, 2H, $J = 8.4$ Hz); ^{13}C NMR (CDCl_3) δ 29.3, 35.5, 40.1, 41.0, 48.5, 111.3, 114.2, 115.9, 116.1, 131.0, 135.0, 136.6, 151.2, 152.8, 160.3, 164.3 and 172.4; mass spectrum (ESI), m/z 421.1316 ($\text{M} + \text{H}^+$) ($\text{C}_{20}\text{H}_{22}\text{N}_4\text{O}_3\text{NaS}$ requires m/z 421.1310).

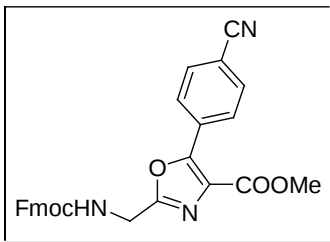


4-(4-(Dimethylamino)phenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxyl pdCpA (3.23). A solution containing 6.0 mg (15 μmol) of cyanomethyl ester **3.22** and 5.7 mg (4.2 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 100 μL of 9:1 DMF- Et_3N was subjected to sonication at room temperature for 4 h. The reaction mixture was purified by C_{18} reversed phase HPLC (250 x 10 mm) using a gradient of 1% to 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 19.5 min was collected and lyophilized to afford **3.23** as a yellow solid: yield- 2.5 mg (62%); mass spectrum (ESI), m/z 976.2213 ($\text{M} - \text{H}^-$) ($\text{C}_{37}\text{H}_{44}\text{N}_{11}\text{O}_{15}\text{P}_2\text{S}$ requires m/z 976.2214).

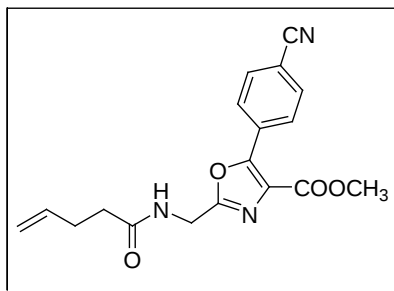


Methyl 2-(2-(((9H-Fluoren-9-yl)methoxy)carbonylamino)acetamido)-3-(4-cyanophenyl)-3-oxopropanoate (3.25). A solution of 1.00 g (4.00 mmol) of **3.7** in 10 mL of anhydrous THF was cooled to $-78\text{ }^{\circ}\text{C}$ under an argon atmosphere and 4.00 mL (4.00 mmol) of 1 M sodium bis(trimethylsilyl)amide in THF was added dropwise while maintaining the temperature at $-78\text{ }^{\circ}\text{C}$. After 30 min, the resulting yellow solution was added via cannula to a stirred solution of 0.66 g (4.00 mmol) of 4-cyanobenzoyl chloride in 3 mL of anhydrous THF at $-78\text{ }^{\circ}\text{C}$. The reaction mixture was stirred at $-78\text{ }^{\circ}\text{C}$ for 2 h. The yellow reaction mixture was acidified with concentrated HCl until pH ~ 2 and was then concentrated under diminished pressure. The crude product (**3.24**) was utilized for the next reaction without further purification. To a solution of the crude product in 10 mL of anhydrous THF at $0\text{ }^{\circ}\text{C}$ was added 1.58 g (4.00 mmol) of Fmoc-gly-*N*-hydroxysuccinimide followed by a dropwise addition of 0.44 mL (0.40 g, 4.00 mmol) of *N*-methylmorpholine. The yellow reaction mixture was stirred at $25\text{ }^{\circ}\text{C}$ for 2 h and then concentrated under diminished pressure. The crude product was purified on a silica gel column ($15 \times 2\text{ cm}$). Elution with 1:1 ethyl acetate–hexanes afforded **3.25** as a colorless oil: yield 0.60 g (38%); silica gel TLC R_f 0.3 (1:1 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 3.69 (s, 3H), 4.00 (d, 2H, $J = 5.6\text{ Hz}$), 4.20 (t, 1H, $J = 7.0\text{ Hz}$), 4.39 (d, 2H, $J = 6.8\text{ Hz}$), 5.55 (t, 1H, $J = 5.4\text{ Hz}$), 6.15 (d, 1H, $J = 7.2\text{ Hz}$), 7.27 (t, 2H, $J = 7.4\text{ Hz}$), 7.37 (t, 2H, $J = 7.4\text{ Hz}$), 7.47 (brs, 1H), 7.56 (d, 2H, $J = 7.2\text{ Hz}$), 7.73–7.77 (m, 4H) and

8.14 (d, 2H, $J = 7.2$ Hz); mass spectrum (APCI), m/z 498.1662 ($M + H$)⁺ ($C_{28}H_{24}N_3O_6$ requires m/z 498.1665).

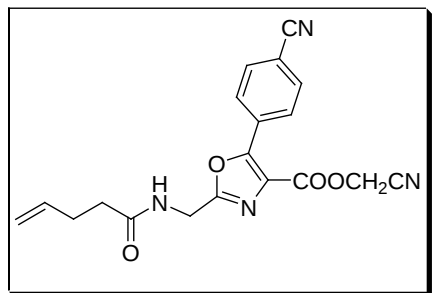


Methyl 2-((((9H-Fluoren-9-yl)methoxy)carbonylamino)methyl)-4-(4-cyanophenyl)oxazole-5-carboxylate (3.26). To a stirred solution of 0.21 g (0.80 mmol) of triphenylphosphine and 0.2 g (0.8 mmol) of iodine in 10 mL of anhydrous CH_2Cl_2 was added 0.11 mL (83.0 mg, 0.80 mmol) of triethylamine. The dark yellow solution was stirred for 5 min and 0.20 g (0.40 mmol) of **3.25** dissolved in 5 mL of anhydrous CH_2Cl_2 was added dropwise. The reaction mixture was stirred for 30 min at 25 °C under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (15 × 2 cm). Elution with 1:1 ethyl acetate–hexanes afforded **3.26** as a pale yellow solid: yield 0.14 g (73%); silica gel TLC R_f 0.5 (1:1 ethyl acetate–hexanes); 1H NMR ($CDCl_3$) δ 3.95 (s, 3H), 4.22 (t, 1H, $J = 6.0$ Hz), 4.45 (d, 2H, $J = 6.8$ Hz), 4.61 (d, 2H, $J = 5.6$ Hz), 5.52 (br s, 1H), 7.28 (t, 2H, $J = 7.4$ Hz), 7.38 (t, 2H, $J = 7.4$ Hz), 7.58 (d, 2H, $J = 7.2$ Hz), 7.71–7.76 (m, 4H) and 8.20 (d, 2H, $J = 8.4$ Hz); ^{13}C NMR ($CDCl_3$) δ 29.7, 38.2, 47.0, 52.7, 67.4, 113.7, 118.2, 120.0, 124.9, 127.0, 127.8, 128.7, 130.4, 132.2, 141.3, 143.6, 153.5, 156.1, 160.2 and 161.9; mass spectrum (APCI), m/z 479.1479 ($M + H$)⁺ ($C_{28}H_{21}N_3O_5$ requires m/z 392.1481).



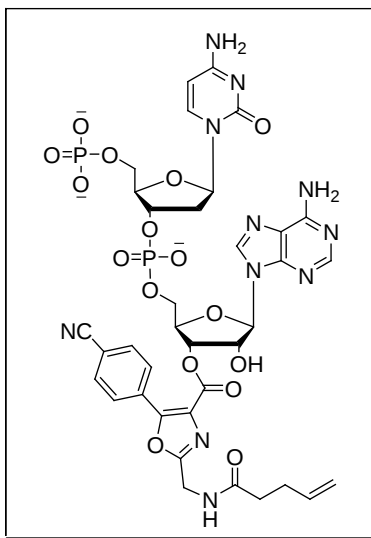
Methyl 4-(4-Cyanophenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxylate (3.5).

To a stirred solution of 0.14 g (0.29 mmol) of **3.26** in 4 mL of anhydrous CH_2Cl_2 was added dropwise 59.0 μL (51.0 mg, 0.60 mmol) of piperidine. The reaction mixture was stirred at 25 $^\circ\text{C}$ under an argon atmosphere for 2 h and was then concentrated under diminished pressure. The residue was dissolved in 5 mL of anhydrous THF and 0.11 g (0.58 mmol) of 4-pentenoyloxysuccinimide was added followed by 37.0 mg (0.44 mmol) of Na_2CO_3 . The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (7 $\times$ 2 cm). Elution with 7:3 ethyl acetate–hexanes yielded **3.5** as a pale yellow solid: yield 47.0 mg (37% over two steps); silica gel TLC R_f 0.29 (7:3 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 2.34–2.40 (m, 4H), 3.92 (s, 3H), 4.64 (d, 2H, J = 6.0 Hz), 4.96–5.12 (m, 2H), 5.77–5.81 (m, 1H), 6.45 (br s, 1H), 7.72 (d, 2H, J = 8.8 Hz) and 8.18 (d, 2H, J = 8.8 Hz); mass spectrum (APCI), m/z 340.1210 ($\text{M} + \text{H}$) $^+$ ($\text{C}_{18}\text{H}_{18}\text{N}_3\text{O}_4$ requires m/z 340.1219).

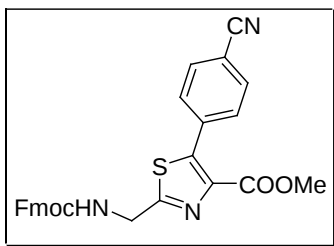


Cyanomethyl 4-(4-Cyanophenyl)-2-(pent-4-enamidoethyl)oxazole-5-carboxylate

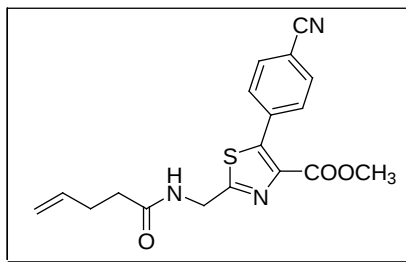
(3.27). To a stirred solution of 16.0 mg (0.05 mmol) of **3.5** in 0.4 mL of 3:1 THF-water was added 0.05 mL of 1 N LiOH. The reaction mixture was stirred at 25 °C for 2 h. The yellow reaction mixture was diluted with MeOH, dried over anhydrous Na₂SO₄ and was concentrated under diminished pressure. The crude product was dissolved in 2 mL of anhydrous DMF and 12.0 mg (0.14 mmol) of NaHCO₃ was added followed by 15.0 μL (18.0 mg, 0.24 mmol) of ClCH₂CN. The reaction mixture was stirred at 25 °C for 3 h under an argon atmosphere. The reaction mixture was concentrated under diminished pressure. The crude product was utilized in the next step without further purification.



4-(4-Cyanophenyl)-2-(pent-4-enamidomethyl)oxazole-5-carboxyl pdCpA (3.28). A solution containing 7.0 mg (20 μ mol) of the crude cyanomethyl ester **3.27** and 6.0 mg (4.4 μ mol) of the tris(tetrabutylammonium) salt of pdCpA in 100 μ L of 9:1 DMF–Et₃N was subjected to sonication at room temperature for 4 h. The reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1% to 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 17 min was collected and lyophilized to afford **3.28** as a white solid: yield 1.9 mg (46%). mass spectrum (ESI), m/z 942.1973 ($M - H$)[–] (C₃₆H₃₈N₁₁O₁₆P₂ requires m/z 942.1968).

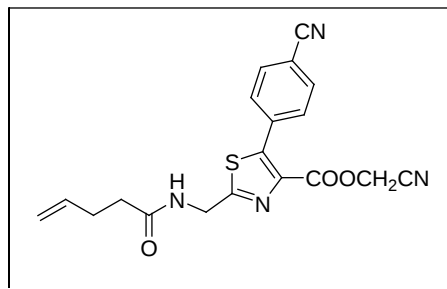


Methyl 2-((((9H-Fluoren-9-yl)methoxy)carbonylamino)methyl)-4-(4-cyanophenyl)thiazole-5-carboxylate (3.29). To a stirred solution of 0.30 g (0.60 mmol) of **3.25** in 5 mL of anhydrous THF was added 0.49 g (1.20 mmol) of the Lawesson's reagent. The reaction mixture was heated to reflux under an argon atmosphere for 1 h. The yellow reaction mixture was diluted with 20 mL of saturated NaHCO₃ solution. The aqueous layer was extracted with two 25-mL portions of ethyl acetate. The combined organic layer was dried over anhydrous MgSO₄ and was then concentrated under diminished pressure. The crude product was utilized in the next reaction without further purification.

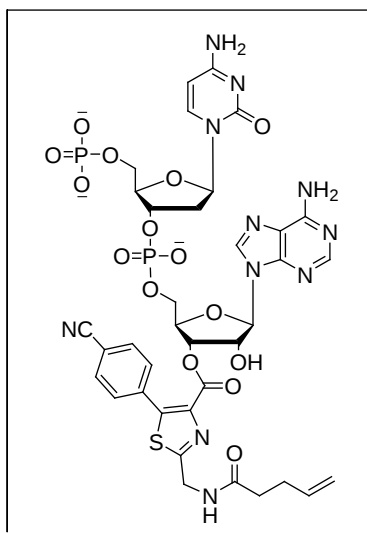


Methyl 4-(4-Cyanophenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxylate (3.6).

To a stirred solution of the crude **3.29** in 4 mL of anhydrous CH_2Cl_2 was added dropwise 0.12 mL (0.10 g, 1.20 mmol) of piperidine. The reaction mixture was stirred at 25 °C under an argon atmosphere for 2 h and was then concentrated under diminished pressure. The residue was dissolved in 5 mL of anhydrous THF and 0.26 g (1.32 mmol) of 4-pentenoyloxysuccinimide was added followed by 83.0 mg (0.78 mmol) of Na_2CO_3 . The reaction mixture was stirred at room temperature for 3 h under an argon atmosphere and was then concentrated under diminished pressure. The residue was purified on a silica gel column (7 × 2 cm). Elution with 1:1 ethyl acetate–hexanes yielded **3.6** as a pale yellow solid: yield 47.0 mg (22% over two steps); silica gel TLC R_f 0.29 (7:3 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 2.34–2.40 (m, 4H), 3.92 (s, 3H), 4.64 (d, 2H, J = 6.0 Hz), 4.96–5.12 (m, 2H), 5.77–5.81 (m, 1H), 6.45 (br s, 1H), 7.72 (d, 2H, J = 8.8 Hz) and 8.18 (d, 2H, J = 8.8 Hz); ^{13}C NMR (CDCl_3) δ 29.3, 35.4, 41.1, 52.5, 113.0, 116.0, 118.2, 130.7, 131.9, 134.9, 136.6, 140.1, 145.3, 161.9, 167.2 and 172.6; mass spectrum (APCI), m/z 356.0980 ($\text{M} + \text{H}$) $^+$ ($\text{C}_{18}\text{H}_{18}\text{N}_3\text{O}_3\text{S}$ requires m/z 356.0991).



Cyanomethyl 4-(4-Cyanophenyl)-2-(pent-4-enamidoethyl)thiazole-5-carboxylate (3.30). To a stirred solution of 16.0 mg (0.05 mmol) of **3.6** in 0.4 mL of 3:1 THF-water was added 0.05 mL of 1 N LiOH. The reaction mixture was stirred at 25 °C for 2 h. The yellow reaction mixture was diluted with MeOH, dried over anhydrous Na₂SO₄ and was concentrated under diminished pressure. The crude product was dissolved in 2 mL of anhydrous DMF and 12.0 mg (0.14 mmol) of NaHCO₃ was added followed by 15.0 μL (18.0 mg, 0.24 mmol) of ClCH₂CN. The reaction mixture was stirred at 25 °C for 3 h under an argon atmosphere. The crude product was utilized in the next reaction without further purification.



4-(4-Cyanophenyl)-2-(pent-4-enamidomethyl)thiazole-5-carboxyl pdCpA (3.31). A solution containing 6.0 mg (15.8 μmol) of cyanomethyl ester **3.30** and 6.0 mg (4.4 μmol) of the tris(tetrabutylammonium) salt of pdCpA in 100 μL of 9:1 DMF–Et₃N was subjected to sonication at room temperature for 4 h. The reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1% to 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 17 min was collected and lyophilized to afford **3.31** as a white solid: yield 1.9 mg (45%). mass spectrum (ESI), m/z 958.1741 ($M - H$)⁻ (C₃₆H₃₈N₁₁O₁₅P₂S requires m/z 958.1745).

Biochemical experiments

Preparation of Aminoacyl-tRNA_{CUA}s. The activation of suppressor tRNA_{CUA}s was carried out as described previously.^{117,127} Briefly, a 100- μL reaction mixture (total volume) in 100 mM Na Hepes, pH 7.5, contained 1.0 mM ATP, 15 mM MgCl₂, 100 μg of suppressor tRNA_{CUA}-CO₂H, 0.5 A₂₆₀ unit of *N*-pentenoyl-protected aminoacyl-pdCpA, 15% DMSO, and 100 units of T4 RNA ligase. The reaction mixture was incubated at 37 °C for 1.5 h and quenched by the addition of 0.1 vol of 3 M NaOAc, pH 5.2. The *N*-protected aminoacylated tRNA was precipitated with 3 vol of cold ethanol. The efficiency of ligation was estimated by 8% polyacrylamide–7 M urea gel electrophoresis, pH 5.0.¹²⁸ The *N*-pentenoyl-protected aminoacyl-tRNA_{CUA}s were deprotected by treatment with aqueous I₂, typically 2 mM I₂ at 25 °C for 30 min. The solution was centrifuged, and the supernatant was adjusted to 0.3 M NaOAc and then treated with 3 vol of cold ethanol to precipitate the aminoacylated tRNA. The tRNA pellet was collected by centrifugation, washed with 70% aq EtOH, air dried and dissolved in 10 μL of RNase free water.

Preparation of S-30 Extracts from Cells Having Modified Ribosomes. Aliquots (5-10 μ L) from liquid stocks of *E. coli* BL-21(DE-3) cells, harboring plasmids with a wild-type or modified *rnB* gene, were placed on LB agar supplemented with 100 μ g/mL of ampicillin and grown at 37 °C for 16-18 h. One colony was picked from each agar plate and transferred into 3 mL of LB medium supplemented with 100 μ g/mL of ampicillin and 0.5 mM IPTG.

The cultures were grown at 37 °C for 3-6 h in a thermostated shaker until OD₆₀₀ ~ 0.15-0.3 was reached, diluted with LB medium supplemented with 100 μ g/mL ampicillin, 1 mM IPTG and 3 μ g/mL of erythromycin (for selectively enhancing the modified ribosome fraction) until OD₆₀₀ 0.01 was reached, and then grown at 37 °C for 12-18 h. The optimal concentration of the final cultures was OD₆₀₀ 0.5-1.0. Cells were harvested by centrifugation (5000 $\times$ g, 4 °C, 10 min), washed three times with S-30 buffer (1 mM Tris-OAc, pH 8.2, containing 1.4 mM Mg(OAc)₂, 6 mM KOAc and 0.1mM DTT) supplemented with β -mercaptoethanol (0.5 mL/L) and once with S-30 buffer having 0.05 mL/L β -mercaptoethanol.

The weight of the wet pellet was estimated and 1.27 mL of S-30 buffer was added to suspend each 1 g of cells. The volume of the suspension was measured and used for estimating the amount of other components. Pre-incubation mixture (0.3 mL) (0.29 M Tris, pH 8.2, containing 9 mM Mg(OAc)₂, 13 mM ATP, 84 mM phosphoenol pyruvate, 4.4 mM DTT and 5 μ M amino acids mixture), 15 units of pyruvate kinase and 10 μ g of lysozyme were added per 1 mL of cell suspension and the resulting mixture was incubated at 37 °C for 30 min. The incubation mixture was then frozen at – 80 °C (~30 min), melted (37 °C, 30 min), and again frozen and melted at room temperature (~30

min). Ethylene glycol tetraacetic acid (EGTA) was then added to 2.5 mM final concentration and the cells were incubated at 37 °C for 30 min. The same molar concentration of CaCl₂ was added, mixed well and frozen (– 80 °C, 30 min). The frozen mixture was centrifuged (15,000 × g, 4 °C, 1 h) and the supernatant was stored in aliquots at – 80 °C.

***In vitro* Protein Translation.** Protein translation reactions were carried out in 15-1700 µL of incubation mixture containing 0.3 µL/µL of S-30 system, 170 ng/µL of plasmid, 35 mM Tris acetate, pH 7.4, 190 mM potassium glutamate, 30 mM ammonium acetate, 2 mM DTT, 0.2 mg/mL total *E. coli* tRNA, 3.5% PEG 6000, 20 µg/mL folinic acid, 20 mM ATP and GTP, 5 mM CTP and UTP, 100 µM amino acids mixture, 0.5 µCi/µL of ³⁵S-methionine and 1 µg/mL rifampicin. In the case of plasmids having a gene with a TAG codon, an activated suppressor tRNA was added to a concentration of 1.5 µg/µL. Reactions were carried out at 37 °C for 1 h (for 15-µL reaction mixtures) to 1.5 h (for 1700-µL reaction mixtures) and terminated by chilling on ice. Aliquots from *in vitro* translation mixtures were analyzed by SDS-PAGE followed by quantification of the radioactive bands by phosphorimager analysis (for 15-µL reaction mixtures).

Purification of GFP. Samples of GFP were diluted with 50 mM Tris-HCl, pH 8.2, and applied to a 100 µL Ni-NTA agarose column that had been equilibrated with the same buffer. The column was washed with 1 mL of the same buffer and GFP was eluted with 500 µL of the same buffer containing 250 mM imidazole. A 50-µL column of DEAE-Sepharose was equilibrated with two 500-µL portions of 25 mM Tris-HCl, pH 7.4. Samples of GFP purified by Ni-NTA chromatography were diluted 3-fold in the same buffer and applied to the resin. The column was washed with four 500-µL portions of

the same buffer. GFP was eluted from the resin with 0.5 mM NaCl in 25 mM Tris-HCl, pH 7.4. The fractions were analyzed on a 15% polyacrylamide gel, stained using Coomassie R-250, and then destained in water. The appropriate fractions were combined and fluorescence was measured.

CHAPTER 4

4. β -AMINO ACID ANALOGUES AS PROBES FOR THE STUDY OF THE REGIO- AND STEREOSELECTIVITY OF MODIFIED RIBOSOMES

4.1. Introduction

The biological and physical properties of peptides and proteins are critically dependent on their folded conformations.¹²⁹ For decades scientists have been captivated by the question of how the amino acid sequence directs the folding of a polypeptide into its bioactive conformation, and how these structures are stabilized.¹²⁹ It has slowly emerged that like proteins, there are several other types of biopolymers, such as β -peptides,¹³⁰ γ -peptides,¹³¹ peptoids¹³² etc., which can assume well-defined three-dimensional structures. These non-natural biopolymers have drawn much attention because they can provide systems to study protein folding and stabilization.

β -Peptides are non-natural biopolymers having β -amino acids as building blocks. Although β -amino acids are found in cells, they are not found in ribosomally synthesized proteins or enzymes.¹³³ β -peptides are promising antimicrobial candidates, they can adopt secondary structures. Additionally, the β -peptide backbone is resistant to proteolysis unlike their natural counterparts.¹³⁴ Nonetheless, the availability of β -peptides is limited due to the constraints of synthetic and semi-synthetic methods. The largest β -mimetic reported is only 17 amino acid long.¹³⁵ Therefore, *in vitro* protein synthesis using modified ribosomes can be an efficient tool for the synthesis of proteins or long peptides with β -amino acids.

The ribosomal PTC is biased against β -amino acids.⁵⁹ From the experiments of

Roberts and coworkers, it is evident that β -amino acids can be accommodated in the A-site of the PTC but for some reason, they do not take part in peptide bond formation.⁵⁶ The modified ribosomes used for the incorporation of D-amino acids were also unable to incorporate β -amino acids to a significant extent. This implies that the architecture of ribosomal PTC may have a stronger bias for β -amino acids than D-amino acids.⁵⁹ Therefore, a new library of modified ribosomes was prepared, having modifications in two regions of the PTC. A dual selection process was used to identify the modified ribosomes, which would be able to incorporate α -amino acids as well as β -amino acids. It is well known that erythromycin prevents bacterial translation by binding to a site close to the PTC.^{136,137} Therefore, altered erythromycin resistance would indicate the alteration in the architecture at a site close to the PTC. In addition, erythromycin resistance also permitted the identification of mutations that caused extensive alterations of PTC architecture, characterization of the modified ribosomes in the presence of wild-type ribosomes and maximization of the levels of modified ribosomes. In order to have a moderate erythromycin resistance a library of *E. coli* having plasmids with modifications in 23S rRNA gene in nucleotides 2057-2063 was prepared.⁵⁹ Eight different 23S rRNA variants having 4-8 times higher erythromycin resistance than wild-type variants were selected, tested for an enhanced sensitivity to β^3 -puromycin (Figure 4.1) and were subjected to a second round of modification. In this round of mutagenesis the eight variants were used as templates and were modified in regions 2502-2507 or 2496-2501. Thus a new library of modified ribosomes was prepared having modifications in two regions of 23S rRNA (2057-2063 and 2502-2507 or 2496-2501).^{53,59} The modified ribosomes were again subjected to erythromycin and β^3 -puromycin selection. Five clones

giving desired β^3 -puromycin sensitivity were used for the preparation of S-30 systems and were shown to incorporate five different β -alanine analogues into full length DHFR by nonsense codon suppression.⁵³

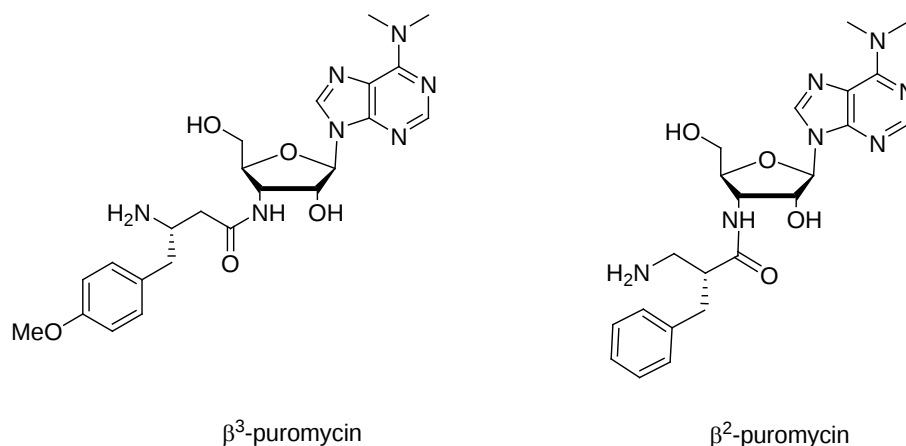


Figure 4.1. Chemical structures of the puromycin derivatives used for selection.

In the study mentioned above, the modified ribosomes were selected using a single puromycin analogue (β^3 -puromycin) where the side chain was at the 3-position with an *S*-configuration. Since PTC is sensitive to the spatial orientation of the side chains of amino acids, it was anticipated that the modified ribosomes might prefer the aminoacyl-tRNAs with regio- and stereoselectivity similar to the configuration of the β -amino acid moiety in the β^3 -puromycin used for ribosome selection.¹³⁸ Thus, several aminoacyl-tRNA_{CUAS} containing four different methyl- β -alanine isomers (Figure 4.2) and four constrained cyclic- β -amino acid isomers (Figure 4.3) as probes were prepared in order to study the regio- and stereochemical predisposition of the modified ribosomes.¹³⁸

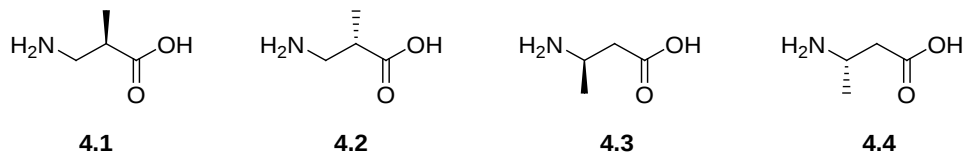


Figure 4.2. Methyl β -alanine analogues used as probes.

Additionally, β^2 -puromycin, having opposite regiochemistry compared to β^3 -puromycin, was further employed in order to test the idea, that modified ribosomes facilitate the incorporation of amino acids having similar regio- and stereochemistry as the puromycin derivative used for selection.¹³⁸

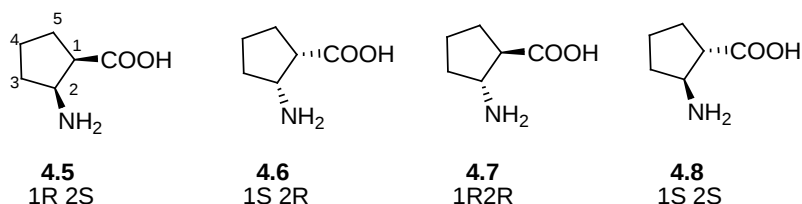
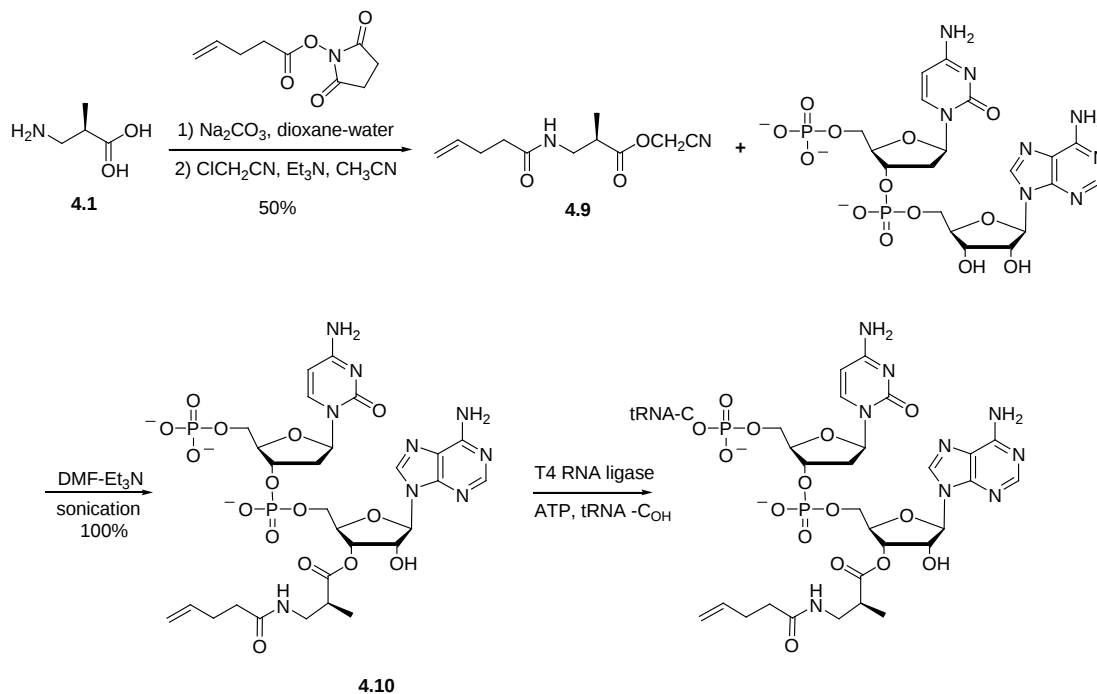


Figure 4.3. Cyclic β -amino acids used as probes.

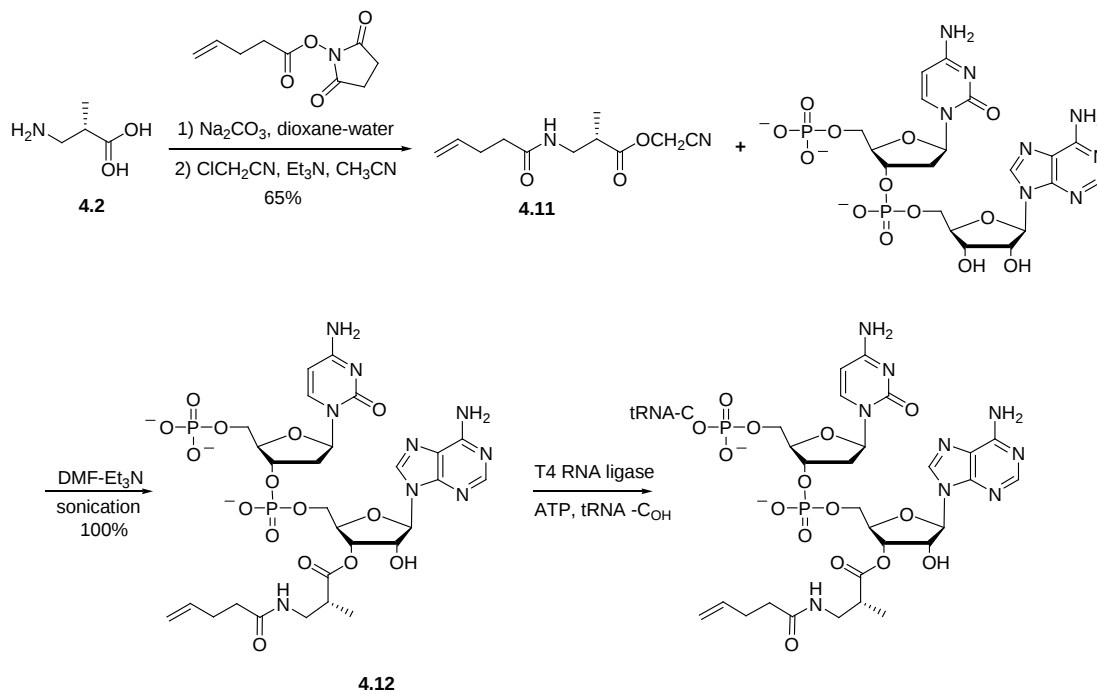
4.2. Results

Misacylated suppressor $\text{tRNA}_{\text{CUAS}}$ carrying individual β -amino acids were prepared from β -aminoacyl-pdCpAs. Schemes 4.1-4.6 describe the preparation of the suppressor $\text{tRNA}_{\text{CUAS}}$. Commercially available **4.1** and **4.2** were first protected with a pentenoyl group using pentenoyloxysuccinimide. The crude products were then activated as the corresponding cyanomethyl esters using chloroacetonitrile and triethylamine in 50% and 65% yields for **4.1** and **4.2**, respectively. The cyanomethyl esters (**4.9** and **4.11**) were then coupled with tetrabutylammonium salt of pdCpA to afford the corresponding pdCpA derivatives **4.10** and **4.12** in quantitative yields.

Scheme 4.1. Synthesis of misacylated suppressor tRNA_{CUA} carrying β -amino acid **4.1**.

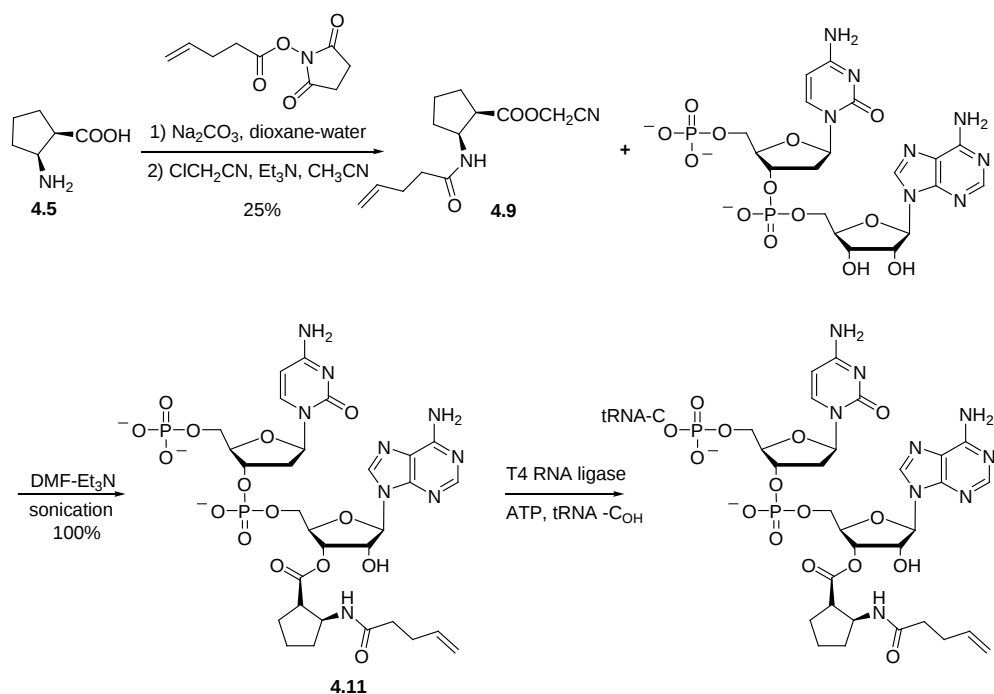


Scheme 4.2. Synthesis of misacylated suppressor tRNA_{CUA} carrying β -amino acid **4.2**.

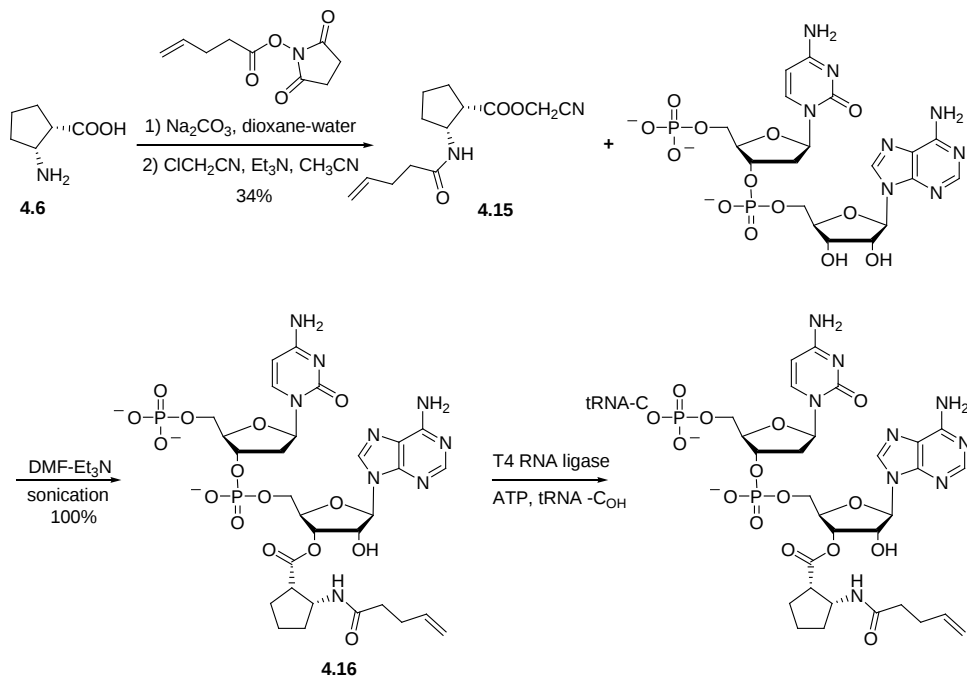


The pdCpA derivatives of the cyclic β -amino acids **4.5-4.8** were synthesized similarly. Commercially available **4.5**, **4.6**, **4.7** and **4.8** were first treated with pentenoyloxysuccinimide to protect the free amine groups. The crude acids were then activated as corresponding cyanomethyl esters **4.13**, **4.15**, **4.17** and **4.19**, respectively. Compounds **4.13**, **4.15**, **4.17** and **4.19** were afforded in 25%, 34%, 27% and 34% yields, respectively. The cyanomethyl esters were coupled with tetrabutylammonium salt of pdCpA to afford individual pdCpA derivatives in quantitative yields.

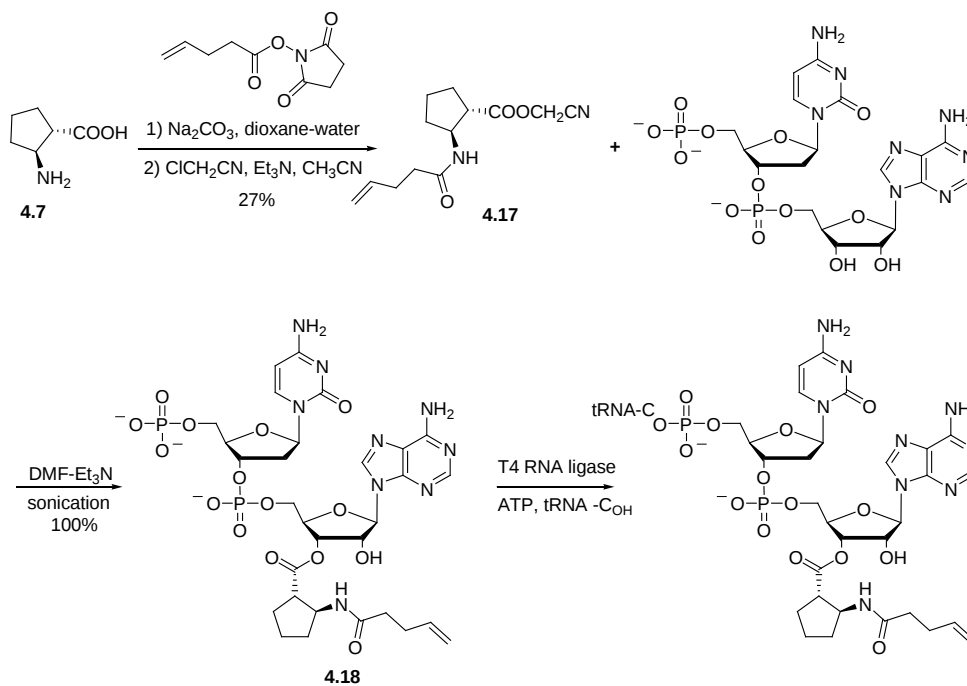
Scheme 4.3. Synthesis of misacylated suppressor tRNA_{CUA} carrying β -amino acid **4.5**.



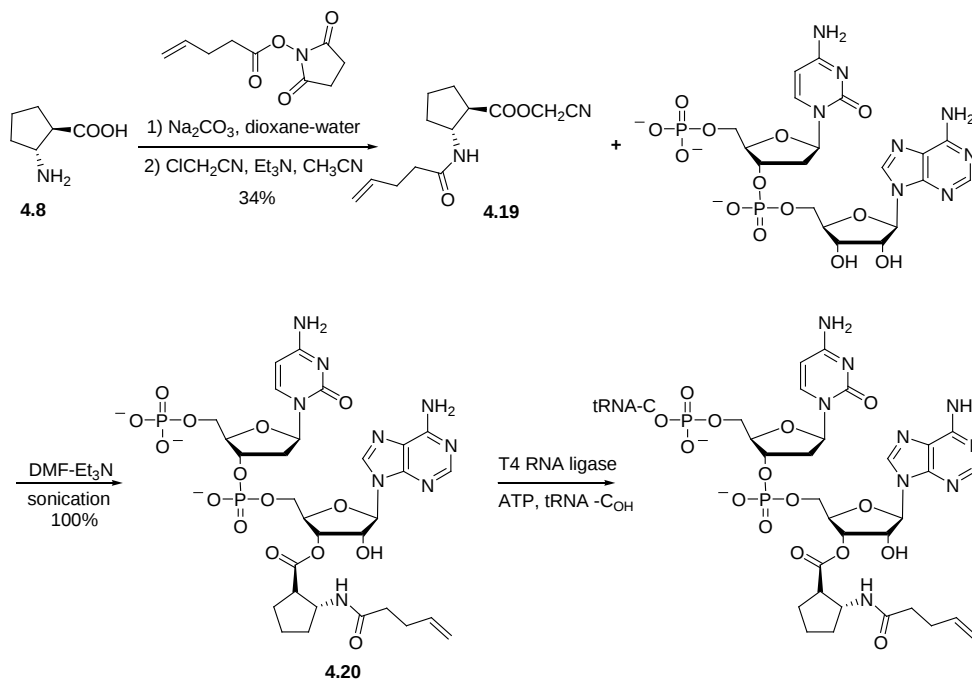
Scheme 4.4. Synthesis of misacylated suppressor tRNA_{CUA} carrying β -amino acid **4.6**.



Scheme 4.5. Synthesis of misacylated suppressor tRNA_{CUA} carrying β -amino acid **4.7**.



Scheme 4.6. Synthesis of suppressor tRNA_{CUA} carrying β -amino acid **4.8**.



The pdCpA derivatives were ligated to abbreviated suppressor tRNA_{CUA} using T4 RNA ligase. The ligated tRNAs were deprotected using 5 mM iodine and were used in cell-free protein synthesizing systems. The results are shown in Table 4.1-4.3.¹³⁸

Table 4.1. Incorporation of β -amino acids **4.1-4.4** into position 10 of *E. coli* DHFR by the use of different S-30 systems having different modified ribosomes selected using β^3 -puromycin. The experiments were conducted by Dr. Rumit Maini.¹³⁸

Clone	Suppression efficiency in different S-30 systems						
	Region 1	Region 2	-	4.1	4.2	4.3	4.4
0403x4	2057AGCGUGA206 3	2502AGCCAG2507	0.07± 0.05	2.7±1.1	3±1.3	6.7± 1.1	11.8± 1.5
040321	2057AGCGUGA206 3	2502AGAUAA2507	0.12± 0.04	2.1±1.0	3.4± 1.9	1.8± 0.8	8.3±1.5
040329	2057AGCGUGA206 3	2502UGGCAG2507	0.06± 0.03	1.8±0.4	2.8± 0.6	5.3± 1.5	9.8±1.1
040217	2057AGCGUGA206 3	2496AUAGAA2501	0.2±	3.6±0.5	10.6±	10.6±	17.8±
Wild-type	2057GAAAGAC206 3	2496CACCUC2501 2502GAUGUC2507	0.1		3.3	3.3	1.2

Table 4.2. Incorporation of β -amino acids **4.5-4.8** into position 10 of *E.coli* DHFR by the use of S-30 systems having different modified ribosomes selected using β^3 -puromycin. The experiments were conducted by Dr. Rumit Maini.¹³⁸

Clone	Suppression efficiency in different S-30 systems				
	–	4.5	4.6	4.7	4.8
0403x4	0.07±0.05	0.5±0.20	2.0±0.15	0.7±0.25	1.7±0.20
040321	0.12±0.04	1.0±0.30	2.8±0.26	0.9±0.20	2.0±0.60
040329	0.06±0.03	1.8±0.35	2.9±0.30	2.2±0.25	3.1±0.50
040217	0.2±0.1	0.8±0.15	3.9±0.30	1.5±0.10	1.9±0.20

Table 4.3. Incorporation of β -amino acids **4.1-4.4** into position 10 of *E.coli* DHFR by the use of S-30 systems having different modified ribosomes selected using β^2 -puromycin. The experiments were conducted by Dr. Rumit Maini.¹³⁸

Clone	Suppression efficiency in different S-30 systems				
	–	4.1	4.2	4.3	4.4
010374	0.05±0.02	5.5±0.20	7.1±1.0	4.6±0.5	5.2±1.1
010355	0.4±0.1	0.4±0.1	3.8±0.3	2.3±0.2	1.2±0.3

4.4. Discussion

The β -amino acids were incorporated into DHFR by the suppression of UAG stop codon at position 10 of DHFR mRNA using chemically misacylated tRNA with CUA anticodon and S-30 systems having modified ribosomes. In order to chemically misacylate tRNA_{CUA}, the pdCpA derivatives of individual β -amino acids were prepared first. The free amino group of the β -amino acids (**4.1**, **4.2** and **4.5-4.8**) was first protected by pentenoyl group using pentenoyloxysuccinimide. The crude acids were subsequently activated as cyanomethyl esters using chloroacetonitrile in varying yields (Schemes 4.1-4.6). The cyanomethyl esters were then coupled with tetrabutylammonium salt of pdCpA to afford the pdCpA derivatives. The pdCpA derivatives were obtained quantitatively in all of the cases. Subsequently the pdCpA derivatives were ligated to abbreviated suppressor tRNA_{CUAS} using T4 RNA ligase, deprotected and used in *in vitro* protein synthesis as probes. S-30 systems were prepared from *E. coli* colonies harboring different

modified ribosomes. The modified ribosomes were selected either with β^3 -puromycin or β^2 -puromycin (Figure 4.1) and tested for their ability to incorporate different β -amino acids into position 10 of DHFR.

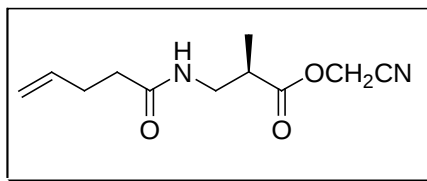
S-30 systems having modified ribosomal clones 040329, 040321, 0403x4, and 040217 were selected using β^3 -puromycin. When employed to incorporate β -amino acids **4.1-4.4** into DHFR, the modified ribosomes displayed good regioselectivity, predominantly accepting 3-substituted methyl- β -alanines **4.3** and **4.4** (up to 5-fold) than 2-substituted β -amino acids **4.1** and **4.2** (Table 4.1). β^3 -puromycin has a 4-methoxybenzyl group at position 3 (Figure 4.1), in agreement with the observed predisposition displayed by the modified ribosomes for 3-substituted methyl- β -alanine isomers. In addition, all four modified ribosomes preferred stereoisomer 3(S)-methyl- β -alanine (**4.4**) compared to 3(R)-methyl- β -alanine (**4.3**); **4.4** accurately reflects the stereochemistry of the β -tyrosine moiety in β^3 -puromycin. The ribosomal clones were also unable to discriminate between 2-substituted methyl- β -alanines **4.1** and **4.2** presumably because of the lack of a substituent at position 2 of the β -amino acid in β^3 -puromycin.¹³⁸

The amino acid moiety in β^2 -puromycin has a benzyl group at position 2 with *R*-stereochemistry (Figure 4.1). It was assumed that these clones would favor 2-methyl- β -alanine derivatives **4.1** and **4.2**, possibly with enhanced selectivity for *R*-isomer **4.1**. In accordance with the regioisomerism of the amino acid moiety in β^2 -puromycin used in the selection experiments, both ribosomal clones incorporated 2-methyl- β -alanine isomers **4.1** and **4.2** with moderately increased yields over the 3-methyl- β -alanine derivatives; this is illustrated in Table 4.3 for the incorporation of the four β -amino acids into position 10 of DHFR by clone 010335 and clone 010374.¹³⁸

It was anticipated that the modified ribosomes would show greater regio- and stereoselectivity with rigid 2, 3-disubstituted β -amino acids (Figure 4.3). The results were completely opposite to the expectations. There was no regio- or stereoselectivity displayed by the modified ribosomes for cyclic β -amino acids (Table 4.2). This is presumably because of the fact that the cyclic β -amino acids were poorly recognized by the modified ribosomes as depicted by their poor suppression yields (Table 4.2).¹³⁸

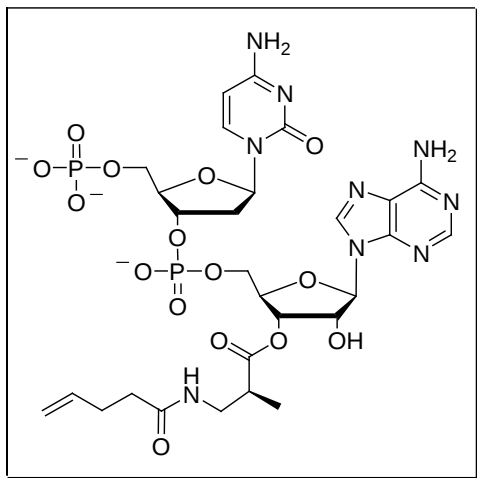
Thus, the ribosomal incorporation of eight new β -amino acids into full length DHFR was successfully demonstrated. The β -amino acids had different regio- and stereochemical properties and were used as probes to verify that the modified ribosomes, generally, displayed preferred regio- and stereoselectivity in accordance with the regio- and stereochemistry of the β -puromycin derivatives used for their selection.

4.4 Experimental



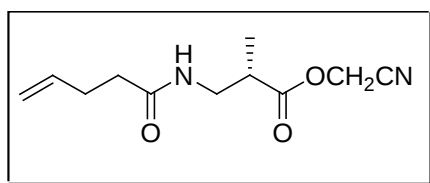
***N*-(4-Pentenoyl)-2*R*-methyl- β -alanine Cyanomethyl ester (4.9).** To a solution containing 100 mg (0.97 mmol) of **4.1** and 245 mg (2.91 mmol) of NaHCO₃ in 5 mL of 1:1 dioxane–H₂O was added 216 mg (1.10 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h under an argon atmosphere. The reaction was quenched by the addition of 15 mL of 1 N aq NaHSO₄ and the aqueous layer was extracted with two 25-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO₄ and concentrated under diminished pressure. The crude product was then dissolved in 5 mL of acetonitrile. To this solution were added

600 μL (435 mg, 4.40 mmol) of triethylamine and 260 μL (309 mg, 4.09 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq NaHSO_4 followed by 10 mL of brine, dried over anhydrous MgSO_4 and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified by flash silica gel column chromatography using 1:1 ethyl acetate–hexanes for elution to obtain **4.9** as colorless oil: yield 108 mg (50%); R_f 0.5 (1:1 ethyl acetate–hexanes); ^1H NMR (CDCl_3) δ 1.16 (d, 3H, $J = 7.2$ Hz), 2.20–2.24 (m, 2H), 2.29–2.34 (m, 2H), 2.75–2.80 (m, 1H), 3.26–3.33 (m, 1H), 3.43–3.49 (m, 1H), 4.70 (s, 2H), 4.94–5.03 (m, 2H), 5.72–5.79 (m, 1H) and 6.16 (br s, 1H); ^{13}C NMR (CDCl_3) δ 14.5, 29.5, 35.6, 39.3, 41.6, 48.6, 114.5, 115.6, 136.9, 172.8 and 173.9; mass spectrum (APCI), m/z 225.1242 ($\text{M} + \text{H}$) $^+$ ($\text{C}_{11}\text{H}_{17}\text{N}_2\text{O}_3$ requires m/z 225.1239).



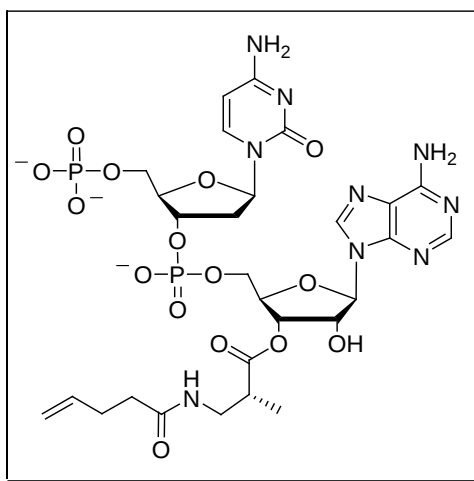
***N*-(4-Pentenoyl)-2*R*-methyl- β -alanyl-pdCpA (4.10).** To a 1.5-mL eppendorf tube containing 5.30 mg (3.70 μmol) of the tris(tetrabutylammonium) salt of pdCpA was added 10.0 mg (44.6 μmol) of **4.9** dissolved in 100 μL of 9:1 DMF– Et_3N . After 2 h of sonication, the reaction mixture was purified by C_{18} reversed phase HPLC (250 x 10 mm)

using a gradient of 1%→65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 13.9 min was collected, combined and lyophilized to afford **4.10** as a colorless solid: yield 3.0 mg (100%); mass spectrum (ESI), m/z 802.1937 ($M - H$)⁻ ($C_{28}H_{38}N_9O_{15}P_2$ requires m/z 802.1963).

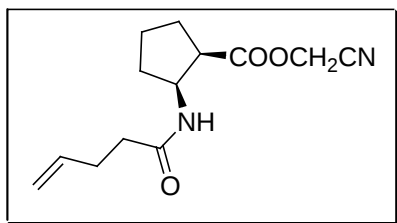


***N*-(4-Pentenoyl)-2*S*-methyl-β-alanine Cyanomethyl ester (4.11).** To a solution containing 100 mg (0.97 mmol) of **4.2** and 245 mg (2.91 mmol) of $NaHCO_3$ in 5 mL of 1:1 dioxane– H_2O was added 216 mg (1.10 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h under argon. The reaction was quenched by the addition of 15 mL of 1 N aq $NaHSO_4$ and the aqueous layer was extracted with two 25-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous $MgSO_4$ and concentrated under diminished pressure. The crude product was then dissolved in 5 mL of acetonitrile. To this solution were added 600 μ L (435 mg, 4.40 mmol) of triethylamine and 260 μ L (309 mg, 4.09 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq $NaHSO_4$ followed by 10 mL of brine, dried over anhydrous $MgSO_4$ and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified by flash silica gel column chromatography using 1:1 ethyl acetate–hexanes for elution to obtain **4.11** as colorless oil: yield 140 mg (65%); R_f 0.5 (1:1 ethyl acetate–hexanes); 1H NMR ($CDCl_3$) δ 1.15 (d, 3H, J = 7.2 Hz), 2.19–2.31 (m, 4H), 2.74–2.77 (m,

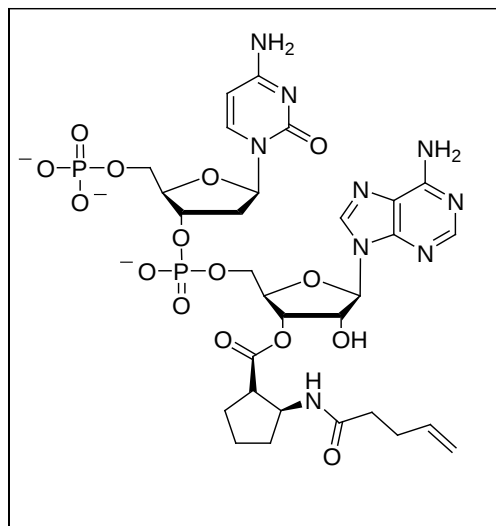
1H), 3.29-3.32 (m, 1H), 3.41-3.44 (m, 1H), 4.69 (s, 2H), 4.93-5.02 (m, 2H), 5.71-5.78 (m, 1H) and 6.23 (br s, 1H); ^{13}C NMR (CDCl_3) δ 14.5, 29.5, 35.6, 39.3, 41.6, 48.6, 114.5, 115.6, 136.9, 172.8 and 173.9; mass spectrum (APCI), m/z 225.1239 ($\text{M} + \text{H}^+$) ($\text{C}_{11}\text{H}_{17}\text{N}_2\text{O}_3$ requires m/z 225.1239).



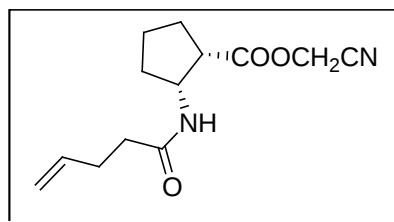
***N*-(4-Pentenyl)-2*S*-methyl- β -alanyl-pdCpA (4.12).** To a 1.5-mL eppendorf tube containing 5.30 mg (3.70 μmol) of the tris(tetrabutylammonium) salt of pdCpA was added 10.0 mg (44.6 μmol) of **4.11** dissolved in 100 μL of 9:1 DMF- Et_3N . After 2 h of sonication, the reaction mixture was purified by C_{18} reversed phase HPLC (250 x 10 mm) using a gradient of 1% $\rightarrow$ 65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 13.9 min was collected, combined and lyophilized to afford **4.12** as a colorless solid: yield 3.0 mg (100%); mass spectrum (ESI), m/z 802.1945 ($\text{M} - \text{H}^-$) ($\text{C}_{28}\text{H}_{38}\text{N}_9\text{O}_{15}\text{P}_2$ requires m/z 802.1963).



(1R, 2S)-Cyanomethyl 2-(Pent-4-enamido)cyclopentanecarboxylate (4.13). To a solution containing 17.0 mg (0.13 mmol) of **4.5** and 34.0 mg (0.40 mmol) of NaHCO₃ in 2 mL of 1:1 dioxane–H₂O was added 39.0 mg (0.20 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h under argon. The reaction was quenched by the addition of 8 mL of 1 N aq NaHSO₄ and the aqueous layer was extracted with two 15-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO₄ and concentrated under diminished pressure. The crude product was then dissolved in 2 mL of acetonitrile. To this solution were added 66.0 μ L (49.0 mg, 0.48 mmol) of triethylamine and 61.0 μ L (71.0 mg, 0.95 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq NaHSO₄ followed by 10 mL of brine, dried over anhydrous MgSO₄ and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified by flash silica gel column chromatography using 1:1 ethyl acetate–hexanes for elution to obtain **4.13** as colorless oil: yield 8.1 mg (25% over two steps); *R*_f 0.45 (1:1 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 1.62–1.70 (m, 2H), 1.82–1.86 (m, 1H), 1.95–2.05 (m, 3H), 2.22–2.26 (m, 2H), 2.32–2.37 (m, 2H), 3.10 (q, 1H, *J* = 7.4 Hz), 4.54 (quint, 1H, *J* = 7.6 Hz), 4.66 (s, 2H), 4.97–5.07 (m, 2H) and 5.76–5.84 (m, 2H); ¹³C NMR (CDCl₃) δ 22.3, 27.9, 29.5, 31.9, 35.7, 46.5, 48.4, 52.7, 114.5, 115.7, 137.1, 172.3 and 173.1; mass spectrum (EI), *m/z* 250.1315 (*M* + H)⁺ (C₁₃H₁₈N₂O₃ requires *m/z* 250.1318).

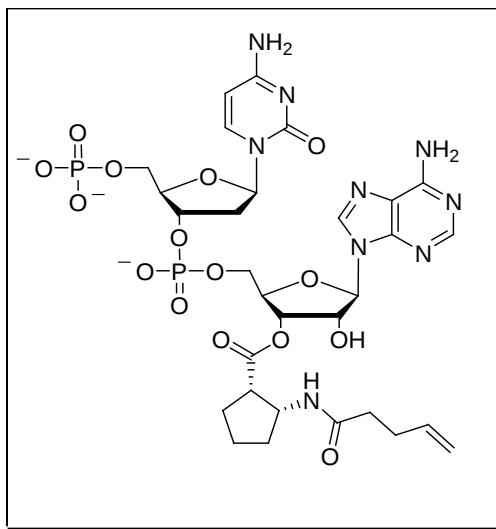


(1R, 2S)-2-(pent-4-enamido)cyclopentanecarboxyl pdCpA (4.14). To a 1.5-mL eppendorf tube containing 5.00 mg (3.66 μmol) of the tris(tetrabutylammonium) salt of pdCpA was added 4.50 mg (18.0 μmol) of **4.13** dissolved in 100 μL of 9:1 DMF–Et₃N. After 3 h of sonication, the reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1%→65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 13.5 min was collected, combined and lyophilized to afford **4.14** as a colorless solid: yield 3.0 mg (100%); mass spectrum (ESI), m/z 828.2126 ($M - H$)[−] (C₃₀H₄₀N₉O₁₅P₂ requires m/z 828.2119).

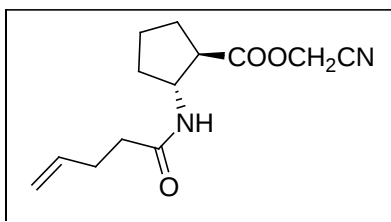


(1S, 2R)-Cyanomethyl 2-(Pent-4-enamido)cyclopentanecarboxylate (4.15). To a solution containing 20.8 mg (0.16 mmol) of **4.6** and 40.0 mg (0.48 mmol) of NaHCO₃ in 2 mL of 1:1 dioxane–H₂O was added 49.0 mg (0.25 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h

under argon. The reaction was quenched by the addition of 8 mL of 1 N aq NaHSO₄ and the aqueous layer was extracted with two 15-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO₄ and concentrated under diminished pressure. The crude product was then dissolved in 2 mL of acetonitrile. To this solution were added 66.0 μ L (49.0 mg, 0.48 mmol) of triethylamine and 61.0 μ L (71.0 mg, 0.95 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq NaHSO₄ followed by 10 mL of brine, dried over anhydrous MgSO₄ and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified by flash silica gel column chromatography using 1:1 ethyl acetate–hexanes for elution to obtain **4.15** as colorless oil: yield 11 mg (34% over two steps); *R*_f 0.45 (1:1 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 1.60-1.66 (m, 2H), 1.82-1.84 (m, 1H), 1.93-2.00 (m, 3H), 2.20-2.24 (m, 2H), 2.30-2.33 (m, 2H), 3.08 (q, 1H, *J* = 7.4 Hz), 4.52 (quint, 1H, *J* = 7.6 Hz), 4.64 (s, 2H), 4.95-5.05 (m, 2H) and 5.74-5.82 (m, 2H); ¹³C NMR (CDCl₃) δ 22.3, 27.8, 29.5, 31.9, 35.7, 46.5, 48.4, 52.6, 114.5, 115.6, 137.1, 172.2 and 173.1; mass spectrum (EI), *m/z* 250.1316 (*M* + H)⁺ (C₁₃H₁₈N₂O₃ requires *m/z* 250.1318).

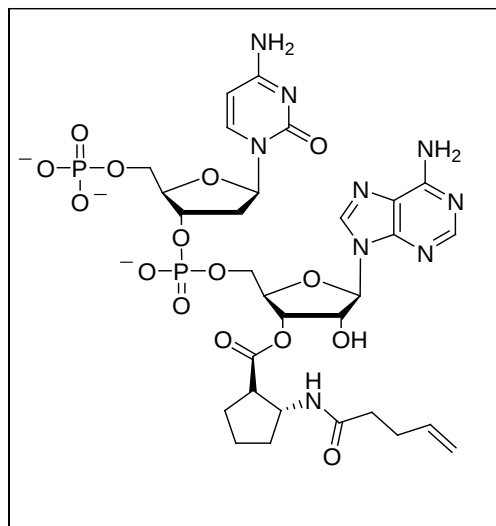


(1*S*, 2*R*)-2-(Pent-4-enamido)cyclopentanecarboxyl pdCpA (4.16). To a 1.5-mL eppendorf tube containing 5.00 mg (3.66 μ mol) of the tris(tetrabutylammonium) salt of pdCpA was added 4.50 mg (18.0 μ mol) of **4.15** dissolved in 100 μ L of 9:1 DMF–Et₃N. After 3 h of sonication, the reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1%→65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 13.5 min was collected, combined and lyophilized to afford **4.16** as a colorless solid: yield 3.0 mg (100%); mass spectrum (ESI), m/z 828.2124 ($M - H$)⁻ (C₃₀H₄₀N₉O₁₅P₂ requires m/z 828.2119).

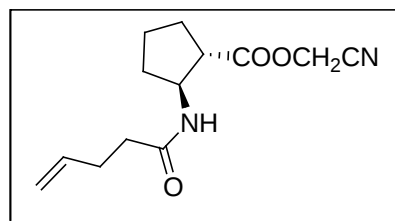


(1*R*, 2*R*)-Cyanomethyl 2-(Pent-4-enamido)cyclopentanecarboxylate (4.17). To a solution containing 19.0 mg (0.15 mmol) of **4.7** and 40.0 mg (0.48 mmol) of NaHCO₃ in 2 mL of 1:1 dioxane–H₂O was added 49.0 mg (0.25 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h

under argon. The reaction was quenched by the addition of 8 mL of 1 N aq NaHSO₄ and the aqueous layer was extracted with two 15-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO₄ and concentrated under diminished pressure. The crude product was then dissolved in 2 mL of acetonitrile. To this solution were added 66.0 μ L (49.0 mg, 0.48 mmol) of triethylamine and 61.0 μ L (71.0 mg, 0.95 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq NaHSO₄ followed by 10 mL of brine, dried over anhydrous MgSO₄ and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified by flash silica gel column chromatography using 1:1 ethyl acetate–hexanes for elution to obtain **4.17** as colorless oil: yield 10 mg (27% over two steps); *R*_f 0.45 (1:1 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 1.48-1.54 (m, 1H), 1.74-1.81 (m, 2H), 1.90-1.95 (m, 1H), 2.02-2.06 (m, 1H), 2.11-2.16 (m, 1H), 2.22-2.25 (m, 2H), 2.33-2.39 (m, 2H), 2.63 (q, 1H, *J* = 8.4 Hz), 4.33 (quint, 1H, *J* = 8.0 Hz), 4.64-4.76 (m, 2H), 4.99-5.08 (m, 2H), 5.66 (br s, 1H) and 5.77-5.83 (m, 1H); ¹³C NMR (CDCl₃) δ 23.1, 28.7, 29.6, 32.7, 35.7, 48.6, 50.4, 55.3, 114.7, 115.8, 137.0, 172.5 and 173.4; mass spectrum (EI), *m/z* 250.1318 (*M* + H)⁺ (C₁₃H₁₈N₂O₃ requires *m/z* 250.1318).

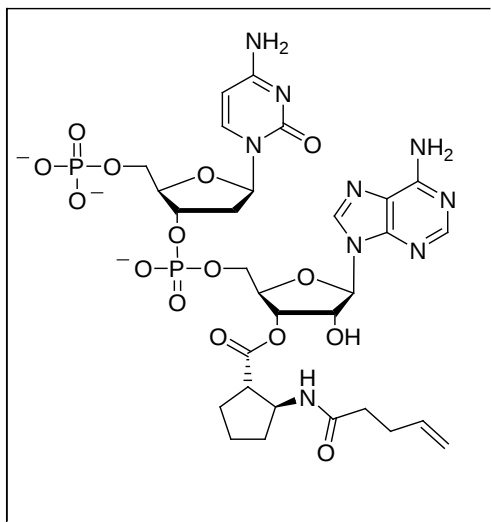


(1R, 2R)-2-(pent-4-enamido)cyclopentanecarboxyl pdCpA (4.18). To a 1.5-mL eppendorf tube containing 5.00 mg (3.66 μ mol) of the tris(tetrabutylammonium) salt of pdCpA was added 4.50 mg (18.0 μ mol) of **4.17** dissolved in 100 μ L of 9:1 DMF–Et₃N. After 3 h of sonication, the reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1%→65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 13.5 min was collected, combined and lyophilized to afford **4.18** as a colorless solid: yield 3.0 mg (100%); mass spectrum (ESI), m/z 828.2123 (M – H)[–] (C₃₀H₄₀N₉O₁₅P₂ requires m/z 828.2119).



(1S, 2S)-Cyanomethyl 2-(Pent-4-enamido)cyclopentanecarboxylate (4.19). To a solution containing 18.0 mg (0.14 mmol) of **4.8** and 40.0 mg (0.48 mmol) of NaHCO₃ in 2 mL of 1:1 dioxane–H₂O was added 49.0 mg (0.25 mmol) of 4-pentenoyloxysuccinimide. The reaction mixture was stirred at room temperature for 24 h

under argon. The reaction was quenched by the addition of 8 mL of 1 N aq NaHSO₄ and the aqueous layer was extracted with two 15-mL portions of ethyl acetate. The combined organic extract was dried over anhydrous MgSO₄ and concentrated under diminished pressure. The crude product was then dissolved in 2 mL of acetonitrile. To this solution were added 66.0 μ L (49.0 mg, 0.48 mmol) of triethylamine and 61.0 μ L (71.0 mg, 0.95 mmol) of chloroacetonitrile. The reaction mixture was stirred at room temperature for 24 h at which time 20 mL of ethyl acetate was added. The organic layer was washed with 10 mL of 1 N aq NaHSO₄ followed by 10 mL of brine, dried over anhydrous MgSO₄ and concentrated to dryness under diminished pressure, affording a crude residue. The crude product was purified by flash silica gel column chromatography using 1:1 ethyl acetate–hexanes for elution to obtain **4.19** as colorless oil: yield 10 mg (27% over two steps); R_f 0.45 (1:1 ethyl acetate–hexanes); ¹H NMR (CDCl₃) δ 1.48-1.53 (m, 1H), 1.73-1.79 (m, 2H), 1.87-1.93 (m, 1H), 2.01-2.11 (m, 2H), 2.20-2.33 (m, 2H), 2.31-2.35 (m, 2H), 2.62 (q, 1H, J = 8.0 Hz), 4.31 (quint, 1H, J = 8.0 Hz), 4.62-4.78 (m, 2H), 4.97-5.06 (m, 2H) and 5.77-5.86 (m, 2H); ¹³C NMR (CDCl₃) δ 22.9, 28.5, 29.5, 32.5, 35.5, 48.7, 50.2, 55.1, 114.2, 115.6, 136.9, 172.4 and 173.3; mass spectrum (APCI), m/z 250.1312 (M + H)⁺ (C₁₃H₁₈N₂O₃ requires m/z 250.1318).



(1S, 2S)-2-(Pent-4-enamido)cyclopentanecarboxyl pdCpA (4.20). To a 1.5-mL eppendorf tube containing 5.00 mg (3.66 μmol) of the tris(tetrabutylammonium) salt of pdCpA was added 4.50 mg (18.0 μmol) of **4.19** dissolved in 100 μL of 9:1 DMF–Et₃N. After 3 h of sonication, the reaction mixture was purified by C₁₈ reversed phase HPLC (250 x 10 mm) using a gradient of 1%→65% acetonitrile in 50 mM ammonium acetate, pH 4.5, over a period of 45 min. The fraction eluting at 13.5 min was collected, combined and lyophilized to afford **4.20** as a colorless solid: yield 3.0 mg (100%); mass spectrum (ESI), m/z 828.2124 ($M - H$)⁻ (C₃₀H₄₀N₉O₁₅P₂ requires m/z 828.2119).

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