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Synthesis, Biochemical and Pharmacological Evaluation of Rationally Designed Multifunctional Radical Quenchers

by

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ABSTRACT

Mitochondria are crucial intracellular organelles which play a pivotal role in providing energy to living organisms in the form of adenosine triphosphate (ATP). The mitochondrial electron transport chain (ETC) coupled with oxidative phosphorylation (OX-PHOS) transforms the chemical energy of amino acids, fatty acids and sugars to ATP. The mitochondrial electron transport system consumes nearly 90% of the oxygen used by the cell. Reactive oxygen species (ROS) in the form of superoxide anions ($O_2^{\bullet -}$) are generated as byproduct of cellular metabolism due to leakage of electrons from complex I and complex III to oxygen. Under normal conditions, the effects of ROS are offset by a variety of antioxidants (enzymatic and non-enzymatic).

Mitochondrial dysfunction has been proposed in the etiology of various pathologies, including cardiovascular and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, ischemia-reperfusion (IR) injury, diabetes and aging. To treat these disorders, it is imperative to target mitochondria, especially the electron transport chain. One of the methodologies currently used for the treatment of mitochondrial and neurodegenerative diseases where endogenous antioxidant defenses are inadequate for protecting against ROS involves the administration of exogenous antioxidants.

As part of our pursuit of effective neuroprotective drugs, a series of pyridinol and pyrimidinol analogues have been rationally designed and synthesized. All the analogues were evaluated for their ability to quench lipid peroxidation and reactive oxygen species

(ROS), and preserve mitochondrial membrane potential ($\Delta\psi_m$) and support ATP synthesis. These studies are summarized in Chapter 2.

Drug discovery and lead identification can be reinforced by assessing the metabolic fate of orally administered drugs using simple microsomal incubation experiments. Accordingly, *in vitro* microsomal studies were designed and carried out using bovine liver microsomes to screen available pyridinol and pyrimidinol analogues for their metabolic lability. The data obtained was utilized for an initial assessment of potential bioavailability of the compounds screened and is summarized fully in Chapter 3.

Dedicated to my family……

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I would like to express my first and foremost gratitude to my advisor, Professor Sidney M. Hecht. I have been incredibly privileged to be his Ph.D. student; he gave me the freedom to explore on my own, and at the same time challenged me to the greatest extent possible. I am highly indebted for his excellent guidance to recovery when my steps faltered, patience, strict attitude and in providing me with an exceptional environment for doing research. All of these assisted me in developing my own independent and pioneering problem-solving aptitudes that will greatly benefit me during my career.

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TABLE OF CONTENTS

Page

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
APCI	atmospheric pressure chemical ionization
aq	aqueous
atm	atmosphere
ATP	adenosine triphosphate
BDE	bond dissociation energy
Bn	benzyl
br	broad
br s	broad singlet
br m	broad multiplet
BSA	bovine serum albumin
^{o}C	degrees Celsius
^{13}C	carbon nuclear magnetic resonance
C^{11} BODIPY$^{581/591}$	4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid
$CDCl_3$	deuterated chloroform
CH_2Cl_2	methylene chloride
CH_3CN	acetonitrile
CoQ_{10}	coenzyme Q_{10}
d	doublet
dq	doublet of quartet
DCF	2',7'-dichlorofluorescein

DCFH	2',7'-dichlorodihydrofluorescein
DCFH-DA	2',7'-dichlorodihydrofluorescein diacetate
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
EI	electronic ionization
EtOH	ethanol
EtOAc	ethyl acetate
Et_2O	diethyl ether
Et_3N	triethylamine
FAB	fast atomic bombardment
FACS	fluorescence-activated cell sorting
FADH	flavin adenine dinucleotide
FBS	fetal bovine serum
FCCP	carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone
FRDA	Friedreich's ataxia
g	gram(s)
GSH	glutathione
^1H NMR	proton nuclear magnetic resonance
h	hour(s)
H_2	hydrogen gas
H_2O	water
HCl	hydrogen chloride gas
Hz	Hertz

IP	ionization potential
$ImPrPh_2$•HCl	1,3-bis(2,6-diisopropylphenyl)-imidazolium chloride
J	coupling constant
K_2CO_3	potassium carbonate
KO*t*Bu	potassium *t*-butoxide
m	multiplet
M	molar
M^+	molecular ion
MeOH	methanol
Me_4Phen	3,4,7,8-tetramethyl-1,10-phenanthroline
mg	milligram(s)
$MgSO_4$	magnesium sulfate (anhydrous)
min	minute(s)
mL	milliliter(s)
mM	millimolar
mmol	millimole(s)
mp	melting point
N	normal
N_2	nitrogen gas
$NaBH_4$	sodium borohydride
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate

$NaOCH_3$	sodium methoxide
NBS	*N*-bromosuccinimide
NH_3	ammonia gas
NMR	nuclear magnetic resonance
PBS	phosphate buffered saline
$POCl_3$	phosphorus oxychloride
PCl_5	phosphorus pentachloride
Pd	palladium
$Pd_2(dba)_3$	tris(dibenzylideneacetone)dipalladium (0)
ppm	parts per million
R_f	ratio of fronts
ROS	reactive oxygen species
RPMI	Roswell Park Memorial Institute
rt	room temperature
s	singlet
satd	saturated
S.E.M.	standard error of the mean
SMPs	submitochondrial particles
$SOCl_2$	thionyl chloride
t	triplet
THF	tetrahydrofuran
TFA	trifluoroacetic acid
TLC	thin layer chromatography

TMEDA	*N*,*N*,*N'*,*N'*-tetramethylethylenediamine
TMRM	tetramethylrhodamine methyl ester
Ts-Cl	4-toluenesulfonyl chloride

LISTS OF FIGURES

Figure Page

Figure Page

Figure Page

Figure Page

LIST OF TABLES

Table Page

Table Page

LIST OF SCHEMES

Scheme Page

CHAPTER 1

INTRODUCTION

Mitochondria are crucial intracellular organelles and play a pivotal role in providing energy to living organisms for their survival in the form of adenosine triphosphate (ATP). The mitochondrial electron transport chain (ETC) coupled with oxidative phosphorylation (OX-PHOS) transforms the chemical energy of amino acids, fatty acids and sugars to ATP (Figure 1.1).[1-4]

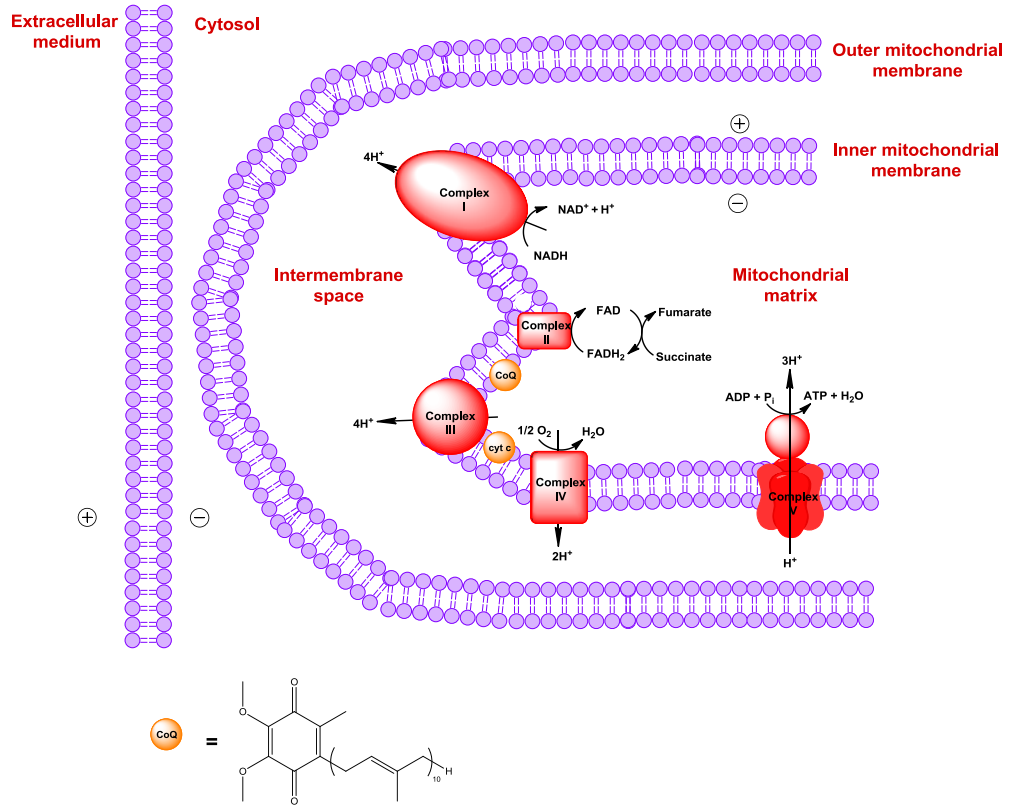


Figure 1.1. Mitochondrial Membranes and Different Compartments Involved in Electron Transport Chain (ETC) and Oxidative Phosphorylation (OX-PHOS).

The mitochondrion has five lipid enzyme complexes, I-V, that are embedded in the inner mitochondrial membrane (IMM) and complexes I-IV are part of the electron

transport chain (ETC). Complex I and complex II catalyze the transfer of electrons from NADH and succinate (through $FADH_2$), respectively, to coenzyme Q_{10} (CoQ_{10}) and simultaneously translocate four protons to the intermembrane space from the mitochondrial matrix. CoQ_{10} carries the electrons to complex III, from where they are transferred to cytochrome c. Complex IV receives the electrons from cytochrome c and utilizes them to reduce molecular oxygen to water, simultaneously translocating two protons to the intermembrane space. In summary, electrons are transported from the TCA (tricarboxylic acid) cycle to oxygen, ultimately producing water along with the translocation of ten protons at complex I ($4H^+$), complex III ($4H^+$) and complex IV ($2H^+$). The proton motive force (PMF) generated by the translocation of ten protons from the matrix to intermembrane space is the driving force for ATP synthase to synthesize ATP from ADP and inorganic phosphate.[5-10]

The mitochondrial electron transport system consumes nearly 90% of the oxygen used by the cell. Reactive oxygen species (ROS) in the form of superoxide anions ($O_2^{\bullet-}$) are generated as a byproduct of cellular metabolism due to the leakage of electrons from complex I and complex III to oxygen.[11-12] ROS generation by mitochondria was first demonstrated by Jensen along with other investigators in 1961.[13] It is used by cells as natural defense system against pathogens and for signal transduction.[14-16] ROS production is increased when electron carriers possess excess electrons, e.g. due to inhibition of OX-PHOS (e.g., dysfunctional mitochondria or several other bioenergetic related pathologies) or excessive calorie consumption. Nature has designed a multilayer network of mitochondrial antioxidants to detoxify $O_2^{\bullet-}$ and its disproportion product H_2O_2 in order to balance the appropriate concentration of ROS and avert the generation

of even more reactive species such as hydroxyl radical ($HO^{\bullet}$) and peroxynitrite ($ONOO^{-}$) (Figure 1.2).[17-21]

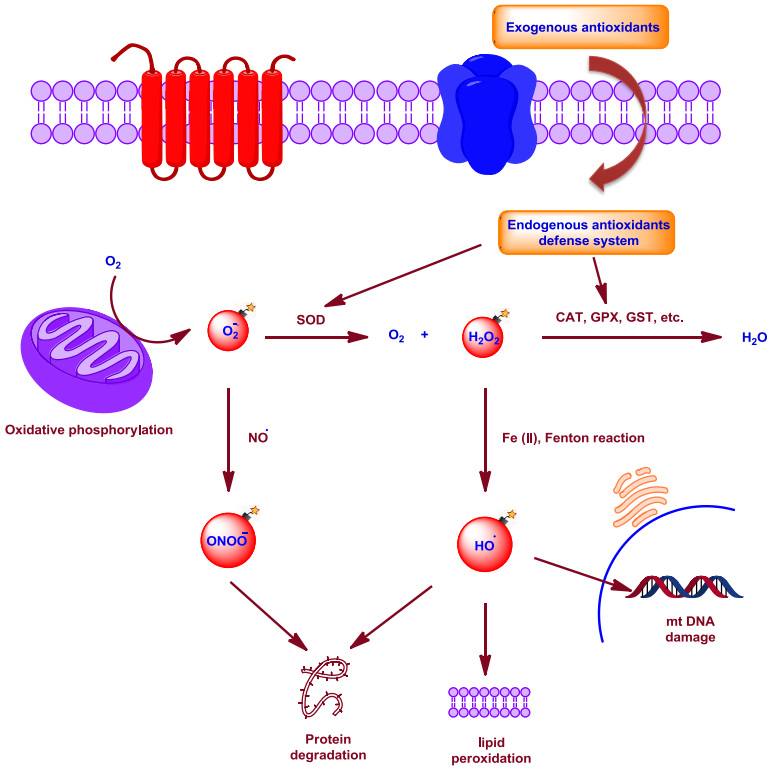


Figure 1.2. Schematic for Cooperative Network Between Exogenous and Endogenous Antioxidants to Mitigate Oxidative Stress. Adapted From Ref. 19, 20 and 21.

Antioxidants are classified into two classes, namely enzymatic antioxidants[22,23] (glutathione peroxidase, catalase and superoxide dismutase) and non-enzymatic antioxidants, including α-tocopherol (vitamin E, phenolic type), L-ascorbic acid (vitamin C, enolic type), β-carotene, resveratrol and curcumin (non-flavonoids), and quercetin (flavonoid) (Figures 1.3, 1.4 and 1.5).[24-34]

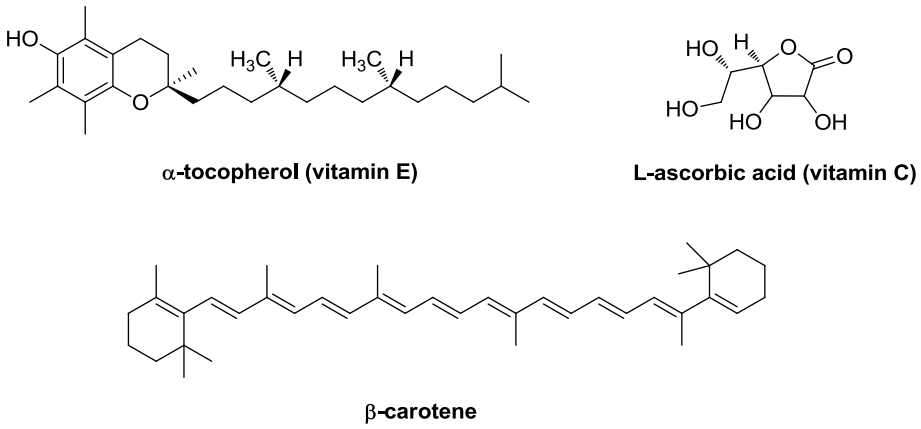
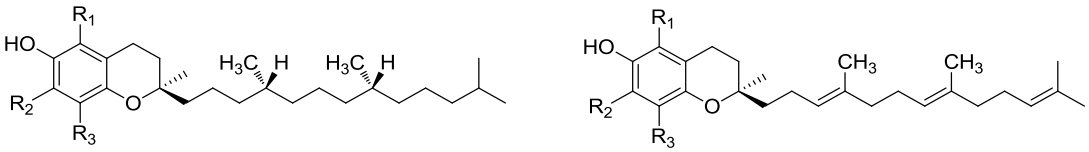


Figure 1.3. Chemical Structures of Vitamins and a Provitamin (β-carotene) Serving as Antioxidants.

Glutathione peroxidase converts H_2O_2 to H_2O and simultaneously oxidizes glutathione (GSH, reduced form) to glutathione (GSSG, oxidized form).[35] Vitamin E, a lipophilic vitamin, is the best known natural antioxidant and has been studied extensively since its discovery decades ago. There are eight members of the vitamin E family including α, β, γ, δ -tocopherols and α, β, γ, δ -tocotrienols that have methylated hydroquinone moieties and an isoprenoid chain (Figure 1.4).[36,37]



Form	R_1	R_2	R_3
α-tocopherol	CH_3	CH_3	CH_3
β-tocopherol	CH_3	H	CH_3
γ-tocopherol	H	CH_3	CH_3
δ-tocopherol	H	H	CH_3

Form	R_1	R_2	R_3
α-tocotrienol	CH_3	CH_3	CH_3
β-tocotrienol	CH_3	H	CH_3
γ-tocotrienol	H	CH_3	CH_3
δ-tocotrienol	H	H	CH_3

Figure 1.4. Chemical Structures of Member of the Vitamin E Family, Which Serve as Antioxidants.

Resveratrol, a biologically active non-flavonoid found predominantly in red fruit, grapes and red wine, is a potent antioxidant which mediates its effects by sequestering free radicals and inducing the synthesis of endogenous antioxidants.[31] Quercetin is among the most common dietary flavonoids and is found ubiquitously, including in berries, onions, apples and broccoli (Figure 1.5). It imparts its antioxidant property by means of chelation with a transition metal ion (e.g. Fe^{2+}) and by free radical scavenging of highly reactive species, such as hydroxyl radical ($HO^{\bullet}$) and the peroxynitrite ($ONOO^{-}$).[34,38-40]

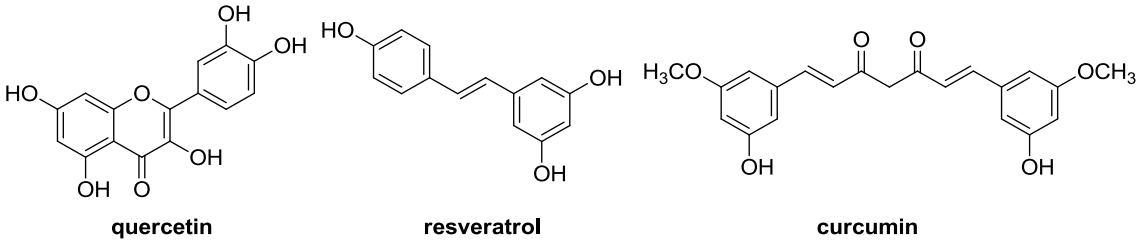


Figure 1.5. Chemical Structures of Flavonoid (Quercetin) and Non-flavonoid (Resveratrol and Curcumin) Phenolic Antioxidants Found in the Human Diet.

Diffusion of $O_2^{\bullet-}$ across the membrane is not feasible because of its polarity; hence the location of $O_2^{\bullet-}$ and the presence of enzymes regulate its release. Studies suggest plausible release of $O_2^{\bullet-}$ in the matrix by complex I, whereas complex III can release them into the IMM space as well as in the matrix (Figure 1.6).[18] The mitochondrial matrix enzyme manganese superoxide dismutase (MnSOD) can convert superoxide at the stage of complexes I and III into the more stable H_2O_2; the latter can readily diffuse into the cytosol.[41] Similarly, copper zinc superoxide dismutase (CuZnSOD) transforms $O_2^{\bullet-}$ to H_2O_2 in the intermembrane space.[42,43] Glycerol-3-

phopshate dehydrogenase (GPDH) can also generate superoxide in the intermembrane space during the transformation of glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP). ROS can also be generated during the β-oxidation of fatty acids, where electron transferring flavoprotein ubiquinone oxidoreductase (ETF–QOR) oxidizes electron transferring flavoprotein (ETF) (Figure 1.6).[44-46]

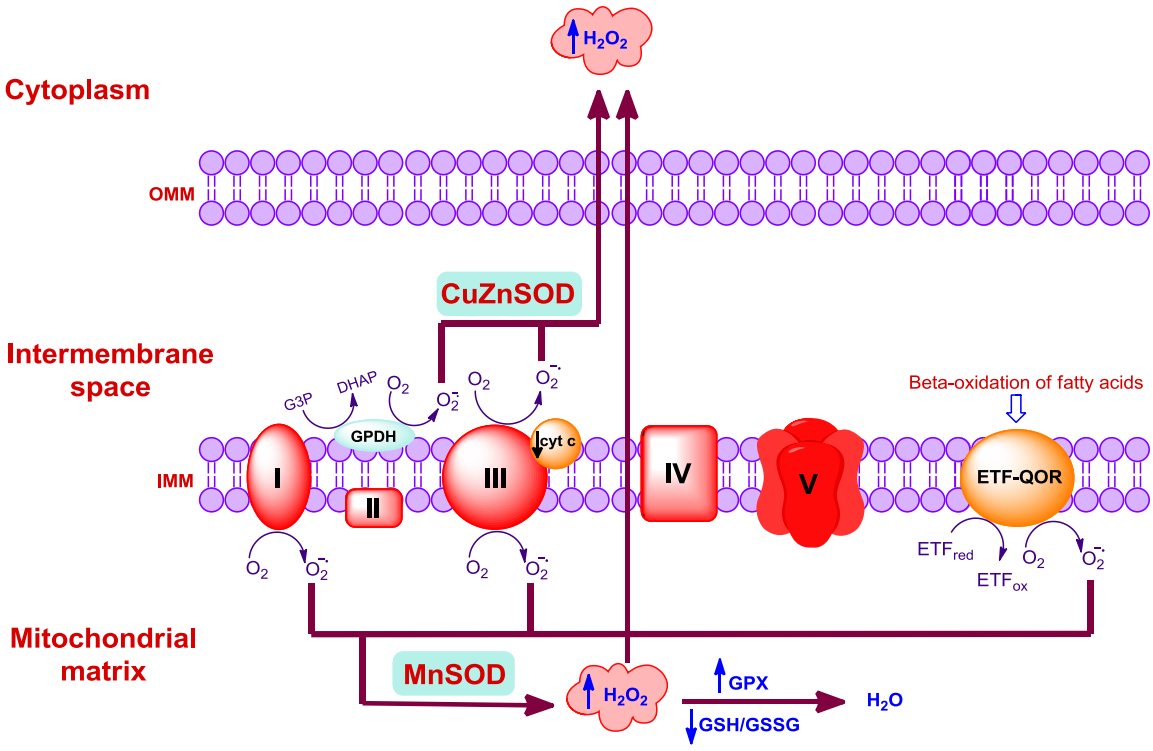


Figure 1.6. Scheme for Mitochondrial $O_2^{\bullet-}$ Production in FRDA Cells. Adapted From Ref. 44-46.

Oxidative stress resulting from the generation of ROS, primarily H_2O_2, has been proposed in the etiology of various pathologies including cardiovascular and neurodegenerative diseases, diabetes and aging. ROS generation is enhanced by a decrease in the cellular antioxidants network and accumulation of transition metals like

iron and copper with age or in these pathological environments.[47-53] Dysfunctional mitochondria generate ROS and initiate a feed-forward loop, in which the cycle of ROS generation is sustained by ROS-mediated oxidative damage.[20,48,49]

Treatment of mitochondrial and neurodegenerative diseases in which the endogenous antioxidant defenses are inadequate for protecting against ROS involves the administration of exogenous antioxidants. This is suggested not only to counterbalance the incompetence of the endogenous defense systems but also to augment the overall antioxidant response (Figure 1.7).[54-57]

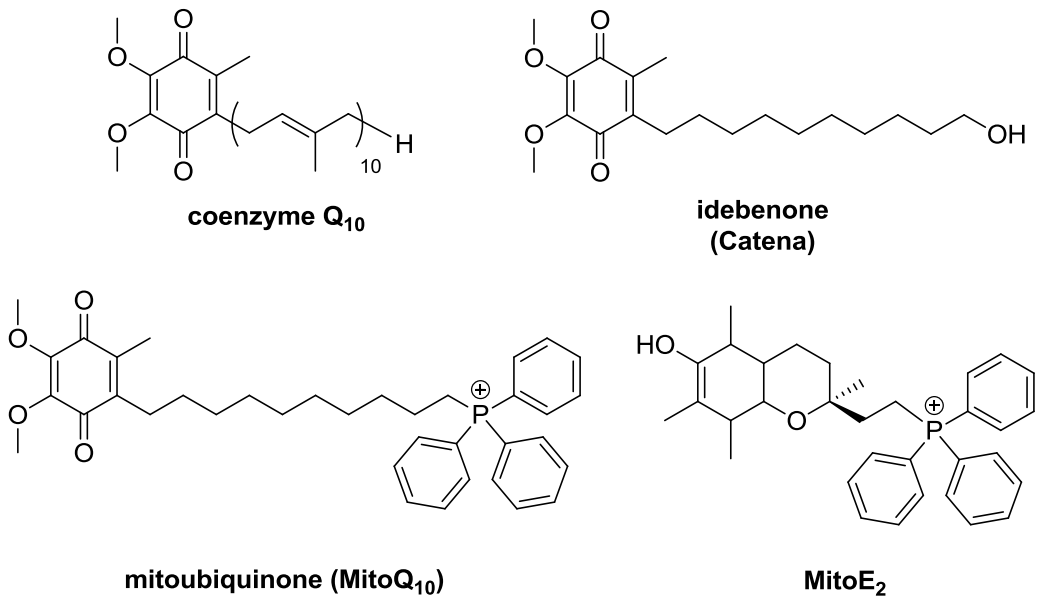


Figure 1.7. Chemical Structures of the Synthetic Antioxidants Used as Exogenous Antioxidants to Supplement the Self-defense Mechanisms of Cells.[58,59]

The protein complexes of the ETC reside on the IMM, which is composed predominantly of phosphatidylcholine, phosphatidylethanolamine and cardiolipin.[60] Cardiolipin is an important phospholipid which helps in providing curvature to the IMM,

and the organization of cristae structures and respiratory complexes into a supercomplex with a larger surface area on the IMM. It anchors complex III and complex IV with the cationic cyt c by means of electrostatic interaction to facilitate efficient electron transfer among the proximal redox partners.[61-69] Proximity to ROS generation sites along with a high unsaturated fatty acid content makes cardiolipin highly vulnerable to oxidative damage.[70] Lipid peroxidation of cardiolipin disrupts the supercomplex organization and results in detachment of cyt c from the IMM. This event leads to ETC inhibition and ultimately facilitates apoptosis (Figure 1.8).[71,72]

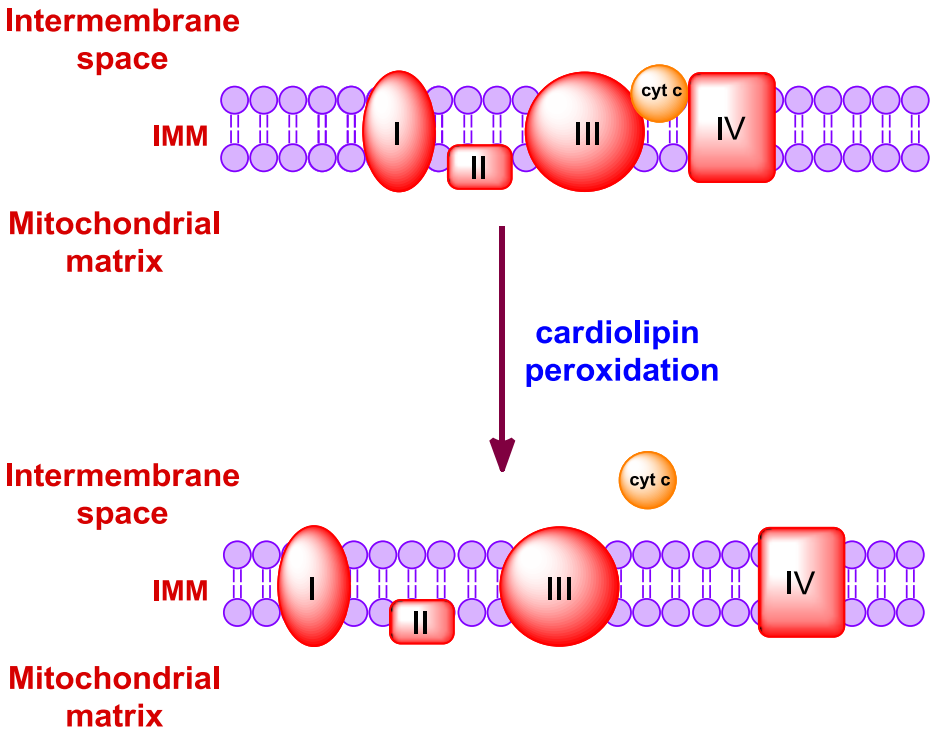


Figure 1.8. Scheme Illustrating the Significance of Cardiolipin in the Organization of Respiratory Complexes into a Supercomplex and the Deleterious Effects of Cardiolipin Peroxidation During Oxidative Stress. Adapted From Ref. 73.

Several pathological conditions, including cardiac failure, diabetes, ischemia-reperfusion (IR) injury and neurodegenerative diseases have reported intimate involvement of cardiolipin depletion and its peroxidation as one of the major causes leading to these pathologies.[73] Compounds that can preserve cardiolipin against lipid peroxidation may be beneficial for patients suffering from these diseases. The electron scavenger XJB-5-131 peptide (4-amino-TEMPO conjugated to hemigramicidin S), has been designed to target mitochondria and it has been shown to inhibit cardiolipin peroxidation by scavenging ROS in rat traumatic brain injury model.[74] Szeto-Schiller (SS) peptides, including SS-31and SS-02 (< 10 amino acids) have been reported to target cardiolipin on the IMM and help to restore mitochondrial bioenergetics by modifying the activity of ETC protein complexes.[75-77] The dimethyltyrosine (Dmt) residue present in these tetrapeptides scavenges ROS and inhibits lipid peroxidation *in vitro* (Figure 1.9).[77,78]

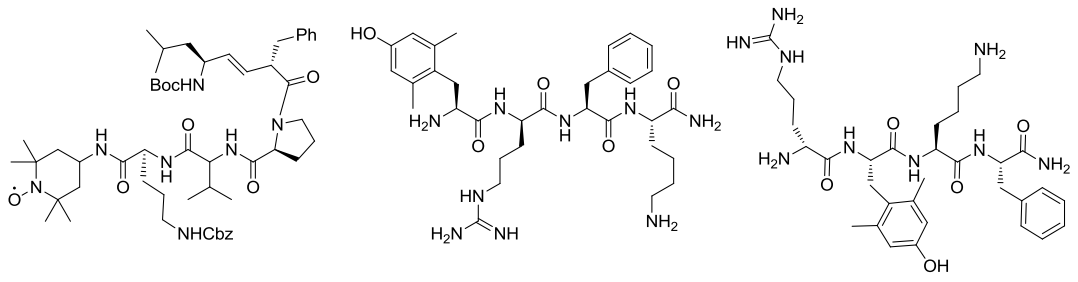
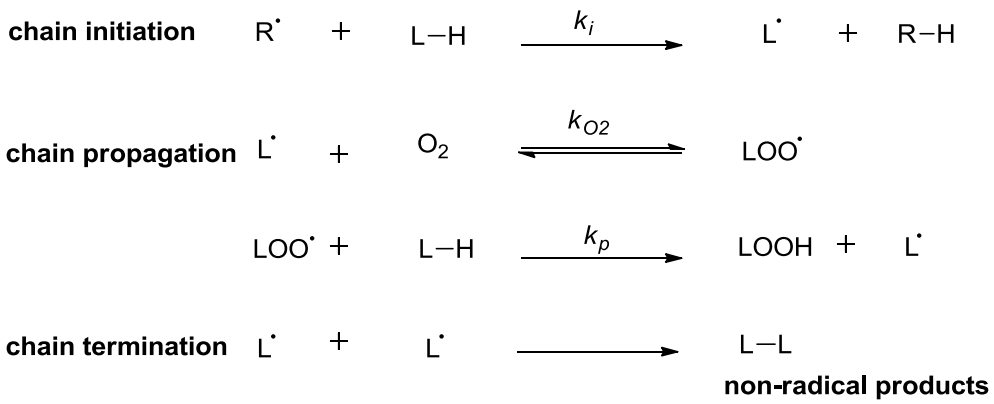


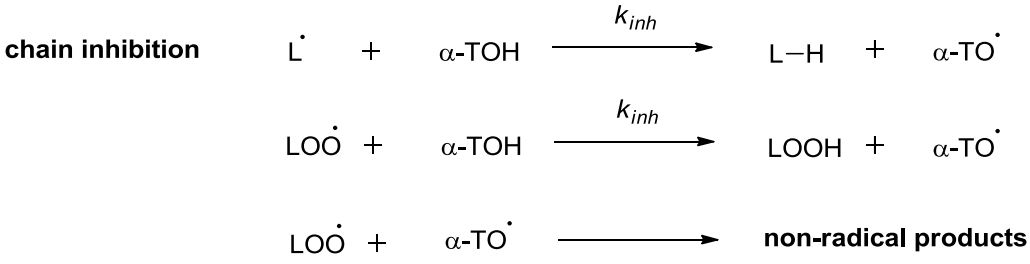
Figure 1.9. Structures of Mitochondria-targeted Small Peptide Antioxidants.

The radical chain reactions involved in lipid peroxidation have been studied extensively since the 1960s and are summarized in Scheme 1.1. Several possibilities,

other than the more common L• dimerization leading to L–L at the chain termination step, are feasible to generate stable species, such as L–O–L and L–O–O–L. In principle, inhibition of lipid peroxidation can be achieved in a number of ways (Scheme 1.2), which includes suppression of initiator L• responsible for the propagation of the radical chain reaction. In a second approach, antioxidants serving as a hydrogen atom donor can interrupt the chain reactions by trapping the peroxyl radical (LOO•).[79-81]



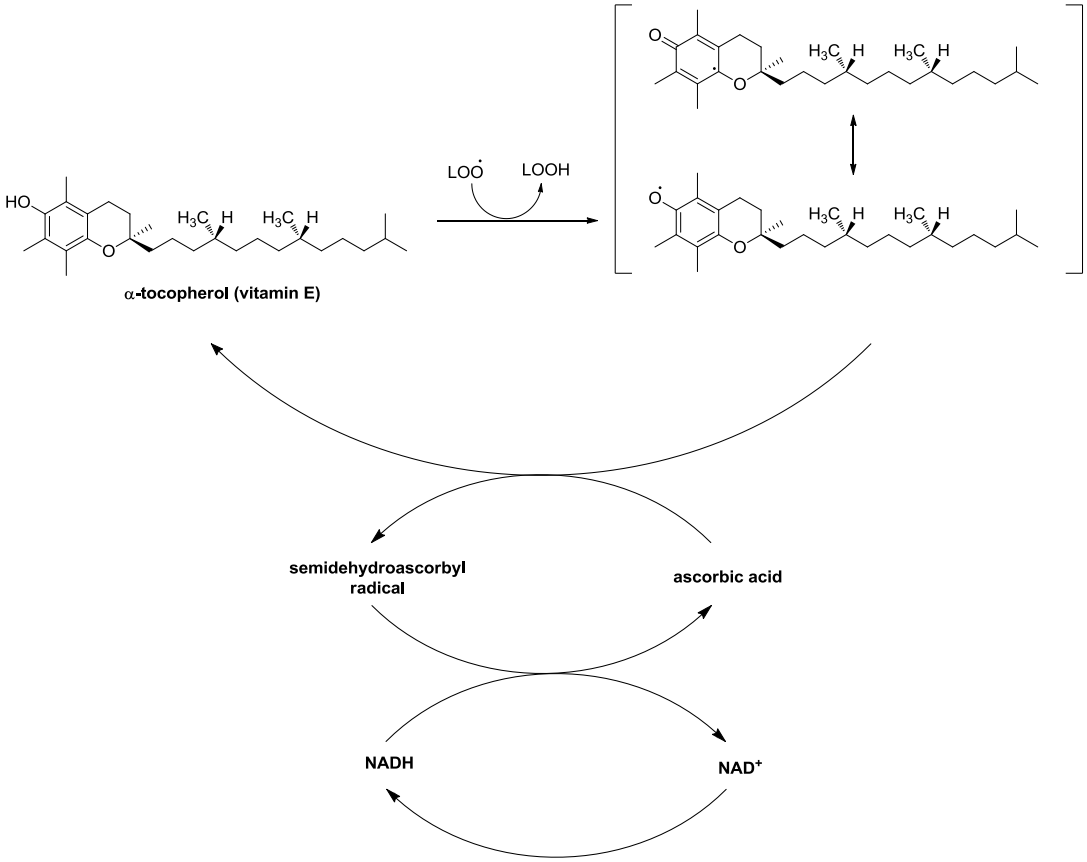
Scheme 1.1. Lipid Peroxidation Chain Reactions in Dysfunctional Mitochondria.



Scheme 1.2. Inhibition of Lipid Peroxidation Using α-TOH as an Antioxidant.

It has been well established that during lipid peroxidation, α-TOH reacts spontaneously with peroxyl radicals by transferring its phenolic hydrogen atom to the peroxyl radical in the chain propagation step. Resonance stabilization of α-TOH radicals

(α-TO•) generated after the phenolic hydrogen transfer facilitates the above transformation. Delocalization of unpaired electrons of oxygen into the aromatic ring structure makes α-TOH derived radicals less reactive for abstracting another H• from the unsaturated fatty acid moiety of the phospholipid membrane. α-TOH can be regenerated from the stabilized α-TOH radicals by cooperating with the other redox molecules, such as vitamin C and NADH (Scheme 1.3).[82-84]



Scheme 1.3. α-TOH Mediated Lipid Peroxidation Quenching, and Ascorbic Acid and NADH Mediated Recycling of α-TOH.

Appropriate functioning of mitochondria is really critical for sustaining life, and especially requires the ETC to continuously supply cells with ATP. Restoration of

mitochondrial function is essential for blunting the progression of mitochondrial diseases. Many compounds having desirable efficacy *in vitro* do not impart the anticipated efficacy *in vivo* due to their rapid metabolic clearance, which results in compromised pharmacological potency. Xenobiotics (foreign compounds) in the liver after oral absorption are metabolized by the oxidative enzymes which eventually affect their half-life and oral bioavailability.[85,86] Drug discovery and lead identification/optimization can be reinforced by assessing the metabolic fate of orally administered drugs early during lead optimization using simple microsomal incubation experiments. Thus the identification of small molecule antioxidants having good biological activity and reduced lability to oxidative metabolism has become a critical goal.

CHAPTER 2

SYNTHESIS AND CHARACTERIZATION OF PYRIDINOL AND PYRIMIDINOL AS MULTIFUNCTIONAL RADICAL QUENCHERS

2.1. Introduction

Mitochondrial dysfunction has been proposed in the etiology of various pathologies, including cardiovascular and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease, ischemia-reperfusion (IR) injury, diabetes and aging.[47-50] To treat these disorders, it is imperative to target mitochondria, especially the electron transport chain. One of the methodologies currently used for the treatment of mitochondrial and neurodegenerative diseases where endogenous antioxidant defenses are inadequate for protecting against ROS involves the administration of exogenous antioxidants.[54-57,87] Coenzyme Q_{10} (CoQ_{10}), present endogenously within the inner mitochondrial membrane (IMM), plays a central role in serving as an electron shuttle, connecting complex I and complex II to complex III. The reduced form of endogenous CoQ_{10}, $CoQ_{10}H_2$, found predominantly in the IMM is the most potent natural lipophilic antioxidant. It can be converted back to CoQ_{10} proficiently by the respiratory chain via redox cycling (Figure 2.1).[88-90]

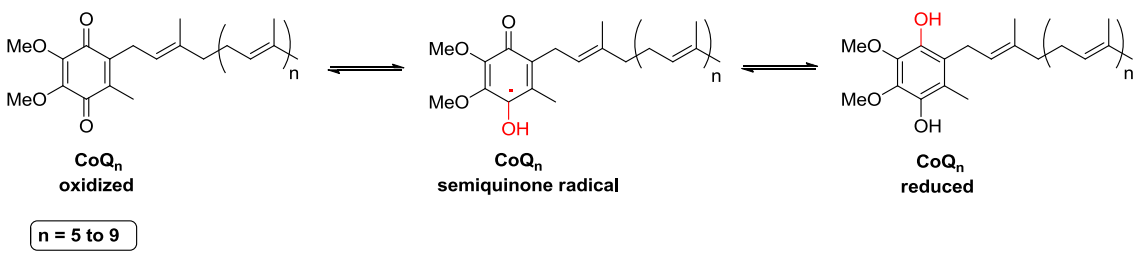


Figure 2.1. Scheme Displaying the Redox Cycling of CoQ_n in the IMM.

It is widely accepted and well supported by various *in vitro* studies that $CoQ_{10}H_2$ is able to inhibit lipid peroxidation by sequestering the radicals generated in the chain initiation process, and by quenching superoxide which generates ubisemiquinone and hydrogen peroxide. Hence, CoQ_{10} has been used therapeutically in CoQ_{10} deficient cells to restore the proper electron flow between the complexes and avert accumulation of electrons at complex I. This ultimately helps in reducing oxidative stress due to ROS generation. But its utility in restoring mitochondrial function by augmenting the level of antioxidants is limited due to its extreme hydrophobicity and consequent poor bioavailability. CoQ_{10} is accumulated in the outer mitochondrial membrane (OMM) and cannot impart any efficacy without access to the inner mitochondrial membrane (IMM).[88,91-96]

Idebenone is a synthetic analogue of CoQ_{10} initially developed by Takeda Pharmaceuticals for treating cognitive disorders and Alzheimer's disease.[97] Idebenone has conditional approval in Canada where it is sold under the trade name Catena® for the treatment of Friedreich's Ataxia.[98-100] A Phase IIIb clinical trial on Friedreich's ataxia patients has recently been completed in North America but the results are still pending. Accordingly, idebenone is not yet approved as a prescribed drug in North America or Europe.[101-104] Conversely, studies have established the inhibitory effects of idebenone on complex I in the ETC and documented its poor bioavailability after passage through the liver (Figure 2.2).[105-107]

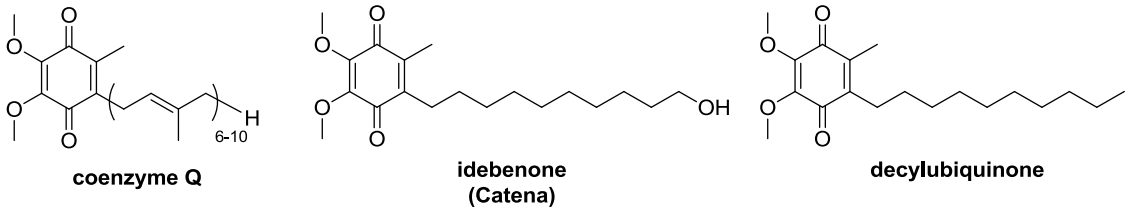


Figure 2.2. Natural and Synthetic Quinone Antioxidants.

There is considerable interest in identifying the structural elements in CoQ_{10} responsible for imparting therapeutic efficacy of utility in treating neurodegenerative and mitochondrial disorders. Porter and coworkers first reported the nitrogen heterocyclic α-TOH type analogues having one nitrogen atom (3-position) and two nitrogen atoms (3- and 5- positions) in the phenolic ring,[108-110] as well as bicyclic pyridinol type analogues formed by fusion of C5 in the ring with the alkyl group of the amine (Figure 2.3).[111] It has been demonstrated both by *in silico* and *in vitro* experiments that nitrogen incorporation in the phenolic ring contributes to improved air stability due to increased ionization potential (IP) and lowering of O–H bond dissociation energy (BDE).[108-110]

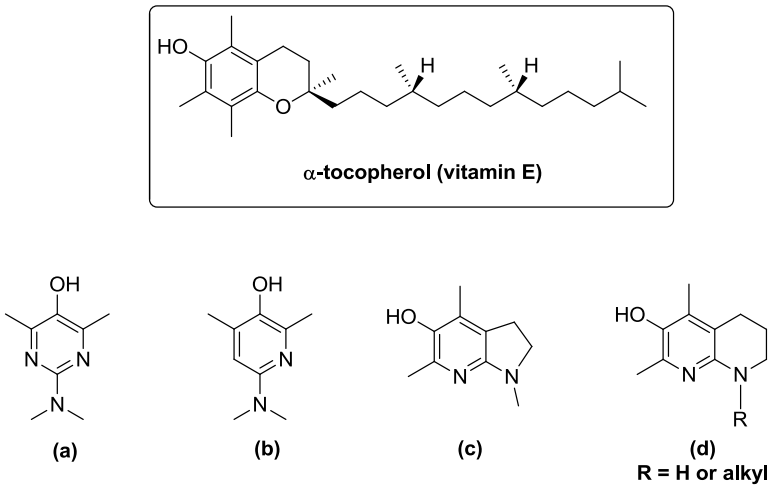


Figure 2.3. Chemical Structures of α-TOH Type Analogues Having Pyrimidine and Pyridine core (a and b) and Bicyclic Pyridinol Analogues (c and d).

These series of compounds behave similarly to α-TOH and could be regenerated in the cell by cooperating with the other redox molecules, such as vitamin C and NADH.[82-84] The pyridinol and pyrimidinol analogues could be recycled by superoxide also, as they can be reduced at the oxidizing potentials (Figure 2.4).[112]

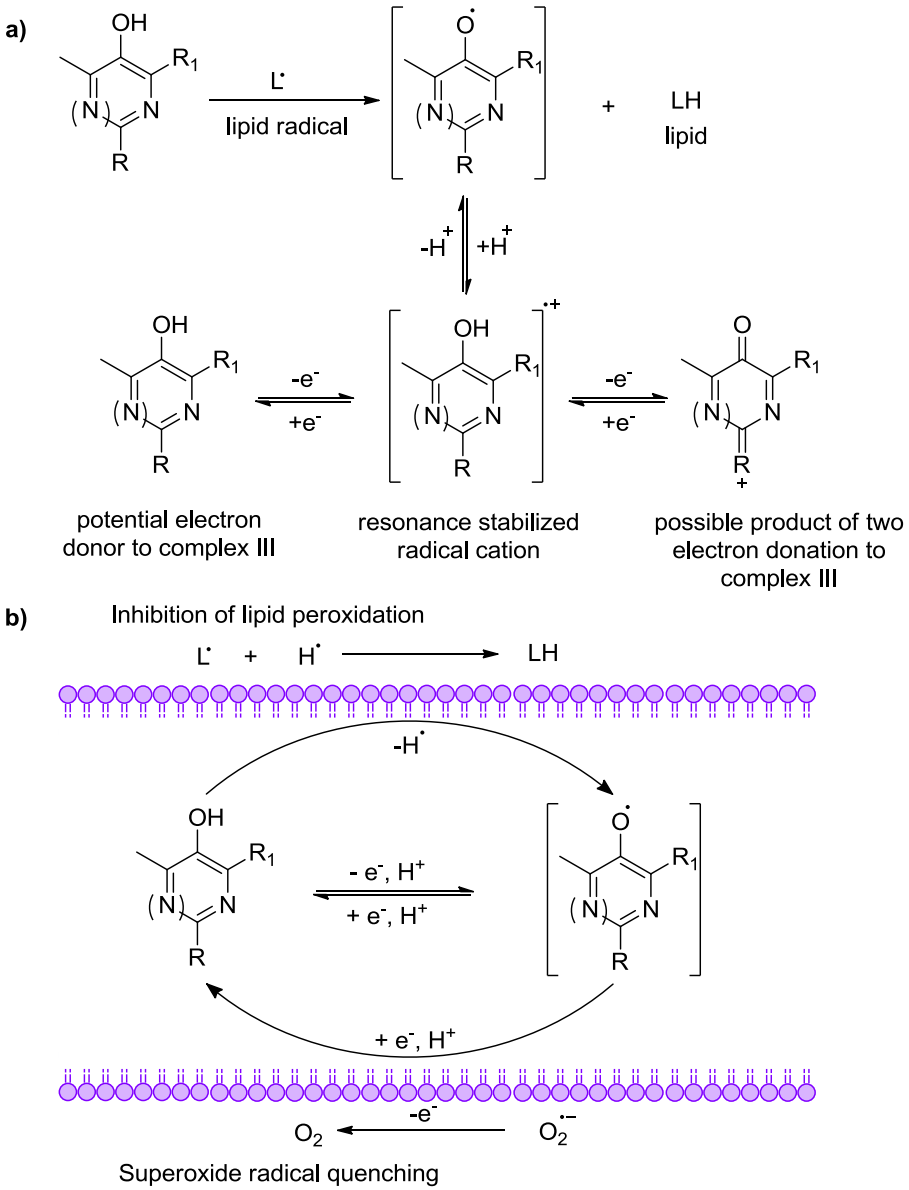


Figure 2.4. (a) Resonance Stabilized 5-Pyridinoxyl/Pyrimidinoxyl Radical Generated by the Reaction of Analogues with Lipid Radicals and its Relationship to the Reduced and Oxidized Forms of the Corresponding Analogues. (b) Proposed Catalytic Cycle for the Compounds Acting as Lipid Radicals and Superoxide Quencher. Adapted From Ref. 112.

Encouraging results have been obtained *in vitro* using the pyrimidinol and pyridinol analogues, as assessed by their ability to scavenge free radicals, and preserve mitochondrial function from oxidative stress in a number of cell lines originated from patients having mitochondrial and neurodegenerative diseases.[112-118] Some of the pyrimidinol analogues have supported ATP production in cultured CoQ_{10} deficient lymphocytes and FRDA fibroblasts and lymphocytes. These compounds are referred to as multifunctional radical quenchers (MRQs) as they suppress one-electron trafficking in dysfunctional mitochondria, with multiple beneficial effects.[117,118]

These encouraging results prompted further structural optimization studies that might lead to more potent and efficacious compounds. The optimized compounds will ultimately be studied for *in vivo* efficacy in animal models of mitochondrial and neurodegenerative diseases. The structures of the compound **2.1** and the lead compounds **2.2** (pyrimidinol) and **2.9** (pyridinol) are shown in Figures 2.5 and 2.6. The structures of the rationally designed and synthesized compounds are shown in Figures 2.6 to 2.10.

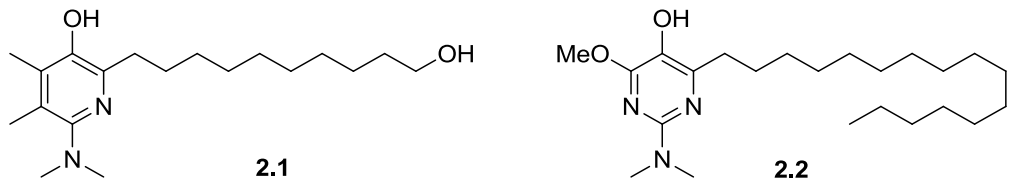


Figure 2.5. Compounds Chosen for Further Structural Optimization.

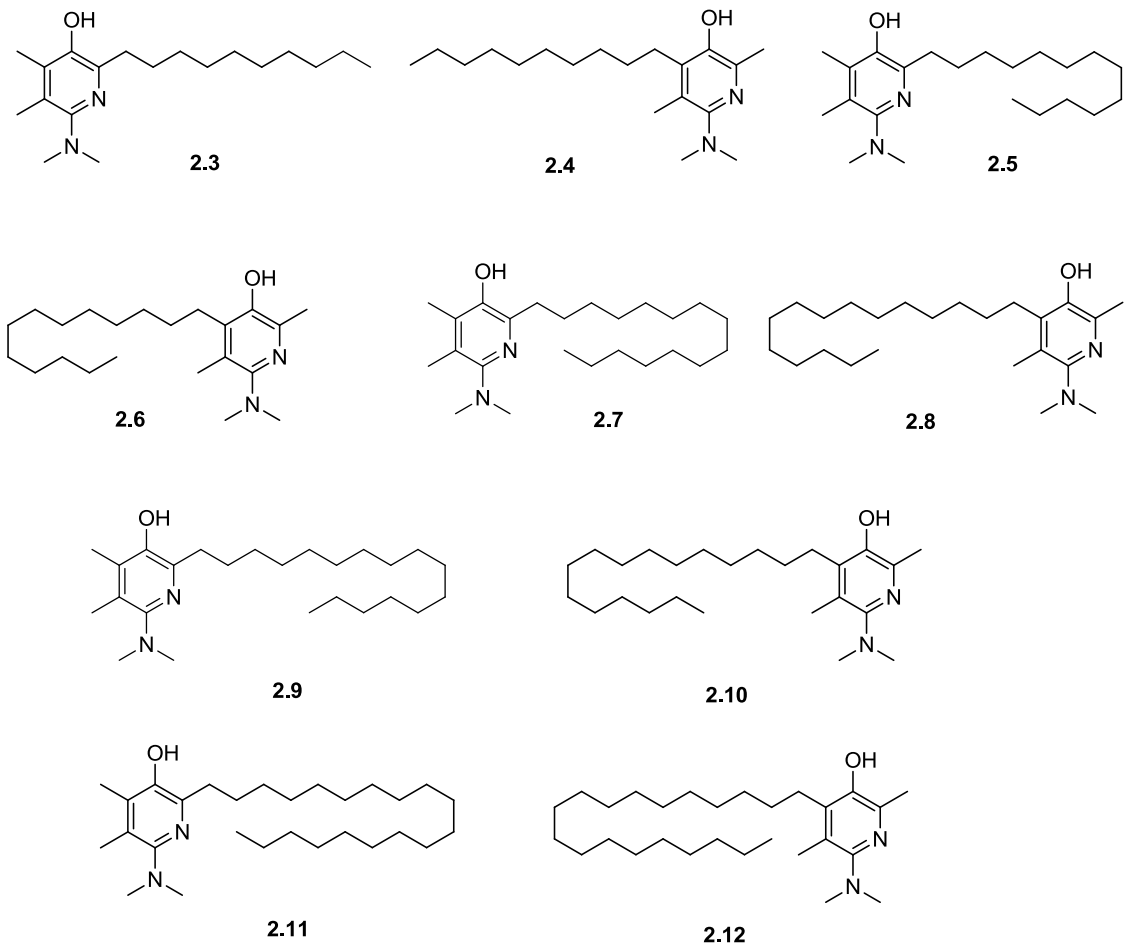


Figure 2.6. Series of Pyridinol Analogues With Linear Alkyl Side Chains Synthesized and Evaluated.

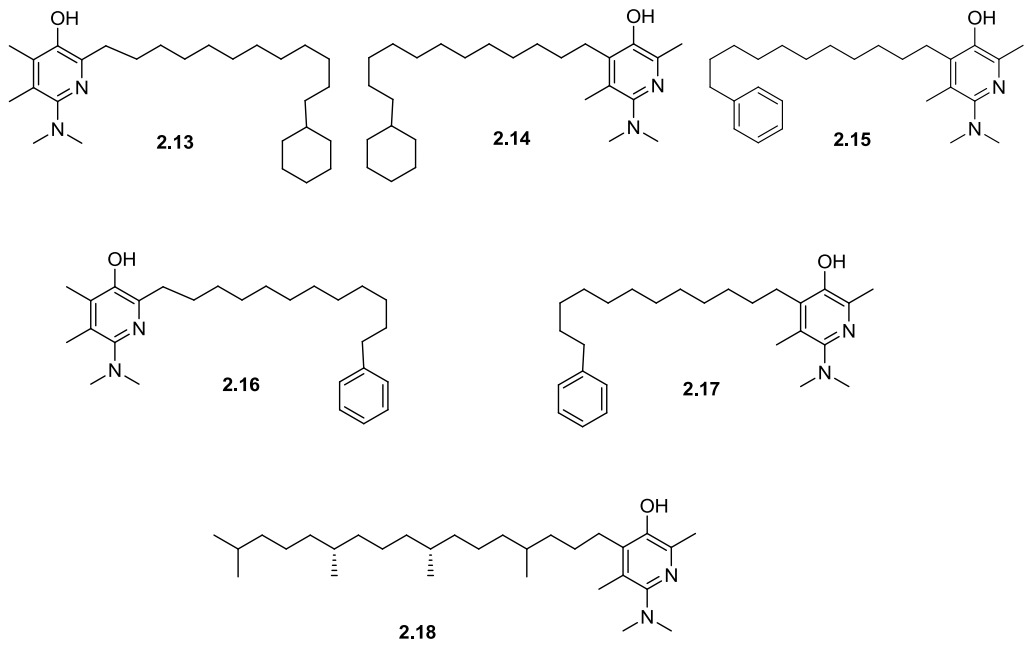


Figure 2.7. Series of Pyridinol Analogues With Modified Alkyl Side Chains Synthesized and Evaluated.

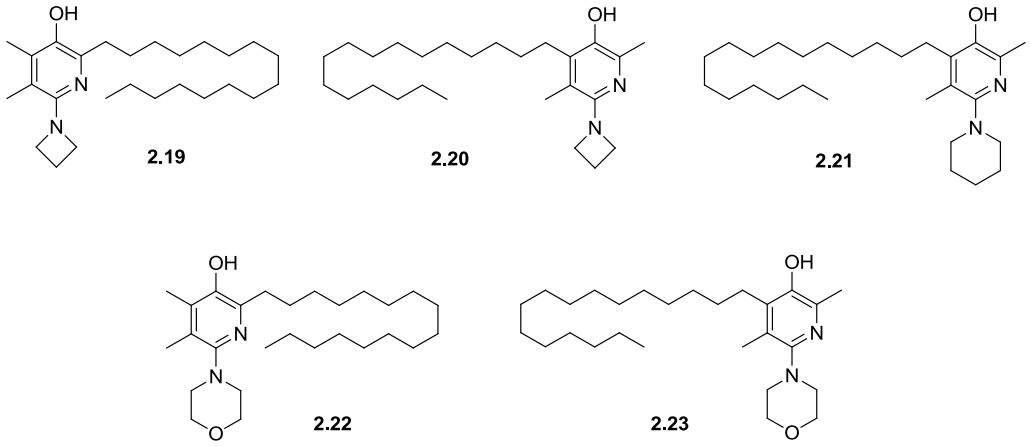


Figure 2.8. Series of Pyridinol Analogues With Cyclic Amino Groups Synthesized and Evaluated.

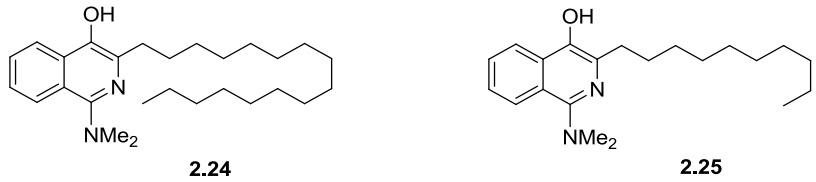


Figure 2.9. Bicyclic Pyridinol Analogues Synthesized and Evaluated.

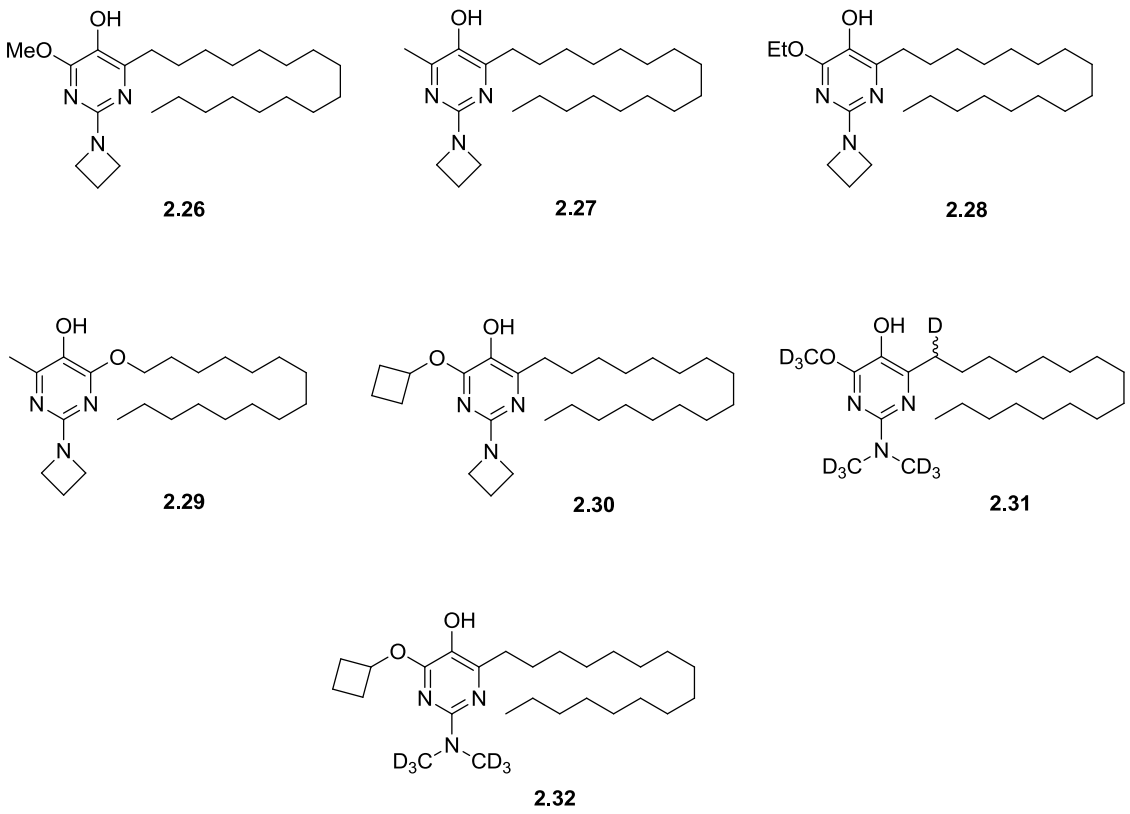


Figure 2.10. Series of Pyrimidinol Analogues Synthesized and Evaluated.

2.2. Results

In order to synthesize pyridinol analogues with different alkyl side chain lengths, the fully protected heterocyclic core was first prepared starting from pyridoxine hydrochloride. Aliphatic side chains were attached to the core followed by deprotection to obtain the proposed analogues (Figure 2.11). The synthetic routes employed in this thesis were considerably more efficient than in previously reported studies.[116]

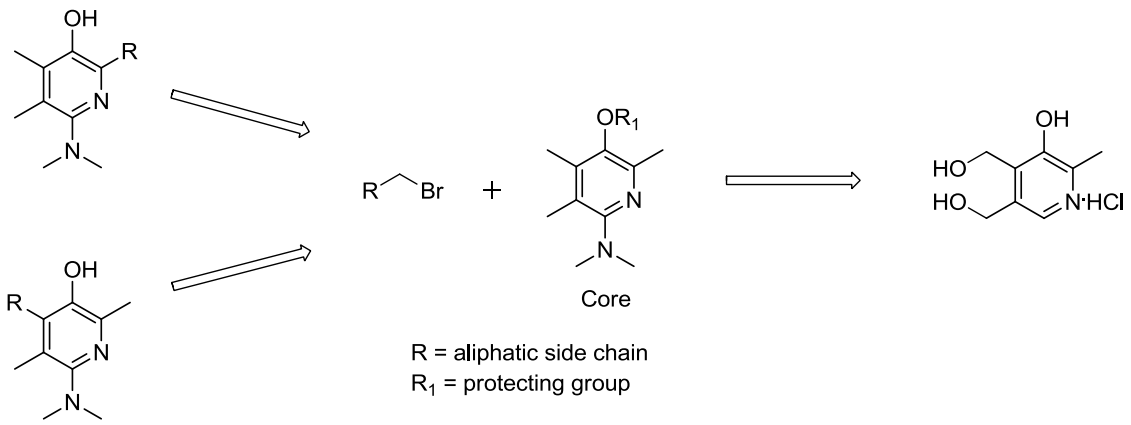


Figure 2.11. Retrosynthetic Analysis for the Pyridinol Analogues With a Dimethylamino Substituent at the Position Ortho to the Ring Nitrogen.

The synthetic approach for pyridinol analogues having a cyclic amino group involved the synthesis of 6-iodo substituted heterocyclic core from pyridoxine hydrochloride (Figure 2.12). The fully substituted core was obtained by C-N coupling of a cyclic amine with benzyl protected 6-iodo pyridinol. Aliphatic side chains were attached to the core followed by deprotection to obtain the proposed analogues.

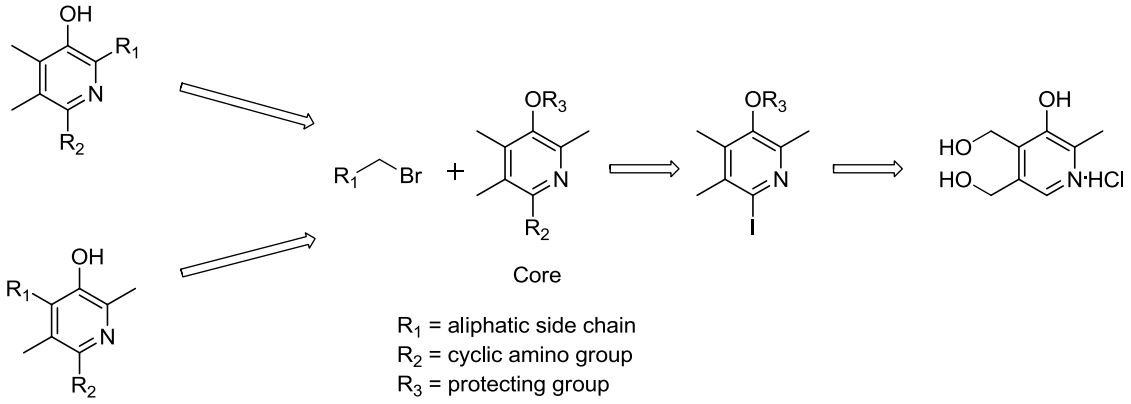


Figure 2.12. Retrosynthetic Analysis for the Pyridinol Analogues With a Cyclic Amino Substituent at the Position Ortho to the Ring Nitrogen.

In order to synthesize bicyclic pyridinol analogues with different length alkyl side chains, the fully protected heterocyclic core was first prepared starting from potassium

phthalamide and 2-bromopropionate (Figure 2.13). Aliphatic side chains were attached to the core followed by deprotection to obtain the proposed analogues.

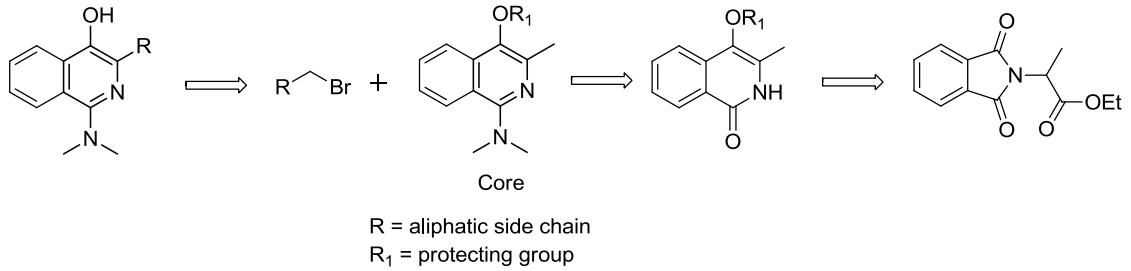


Figure 2.13. Retrosynthetic Analysis for the Bicyclic Pyridinol Analogues.

The synthetic approach for pyrimidinol analogues having a cyclic amino group involved the synthesis of the substituted heterocyclic core by C-N coupling of a cyclic amine with a 2-chloro- or 2-iodo-pyrimidinol (Figure 2.14). Aliphatic side chains were incorporated onto the core followed by introduction of a hydroxyl group to obtain the desired analogues.

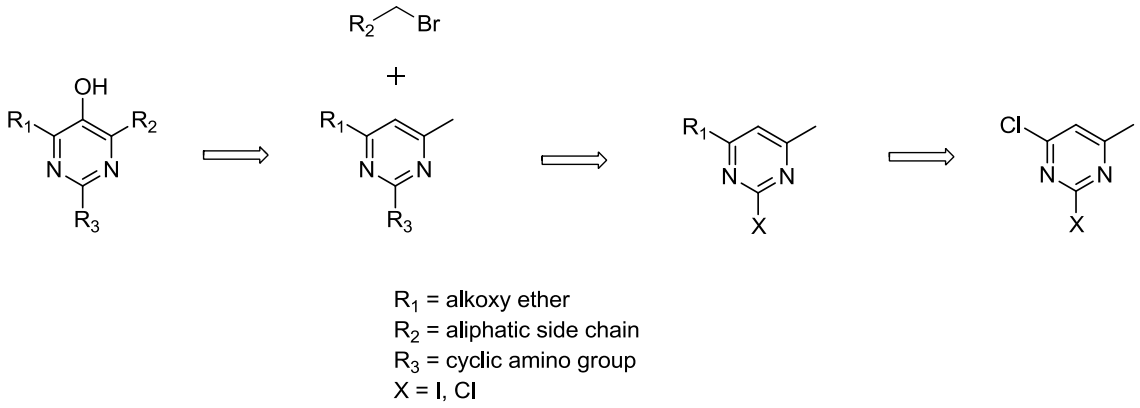


Figure 2.14. Retrosynthetic Analysis for the Pyrimidinol Analogues.

In order to synthesize pyrimidinol analogues with a hexadeuterated dimethylamino group, a concise strategy was followed (Figure 2.15). 2-Amino-4-chloro-

6-methyl pyrimidine was converted to the fully deuterated dimethylamino substituted pyrimidine, which was followed by the replacement of the chloro substituent with an alkoxy group. An aliphatic side chain was introduced onto the core followed by introduction of a hydroxyl group to obtain the desired analogues.

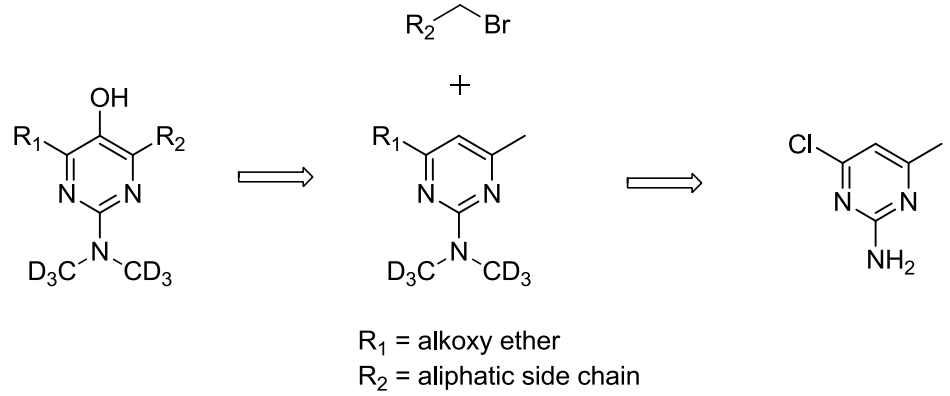


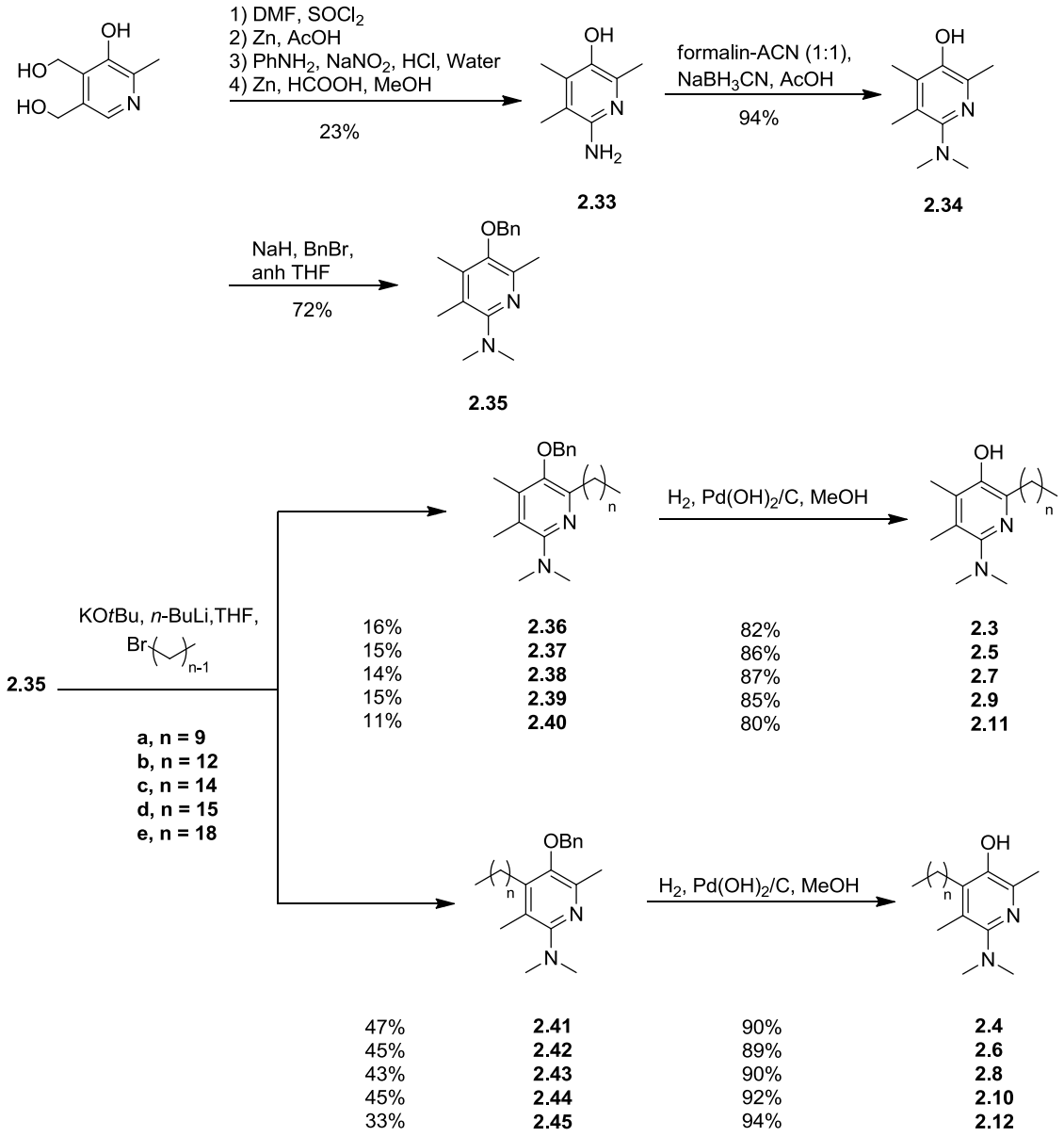
Figure 2.15. Retrosynthetic Analysis for the Pyrimidinol Analogues With a Fully Deuterated Dimethylamine.

2.2.1. Synthesis of Pyridinol and Pyrimidinol Analogues

2.2.1.1. Synthesis of Pyridinol Analogues With Linear Alkyl Side Chains

The route employed for synthesis of the desired pyridinol antioxidants with linear alkyl side chains is illustrated in Scheme 2.1. Accordingly, 6-amino-2,4,5-trimethylpyridin-3-ol (**2.33**) was synthesized in 23% yield by a method reported previously.[117,119] Reductive alkylation of **2.33** using formalin and sodium cyanoborohydride afforded **2.34** in 94% yield. The intermediate 3-pyridinol (**2.34**) was *O*-benzylated using benzyl bromide and NaH to afford the *O*-benzylated core **2.35** in 72% yield. Aliphatic side chains were introduced by alkylation of **2.35** using Schlosser's super base (a mixture of KOtBu and *n*-BuLi) and the appropriate alkyl bromide.[120]

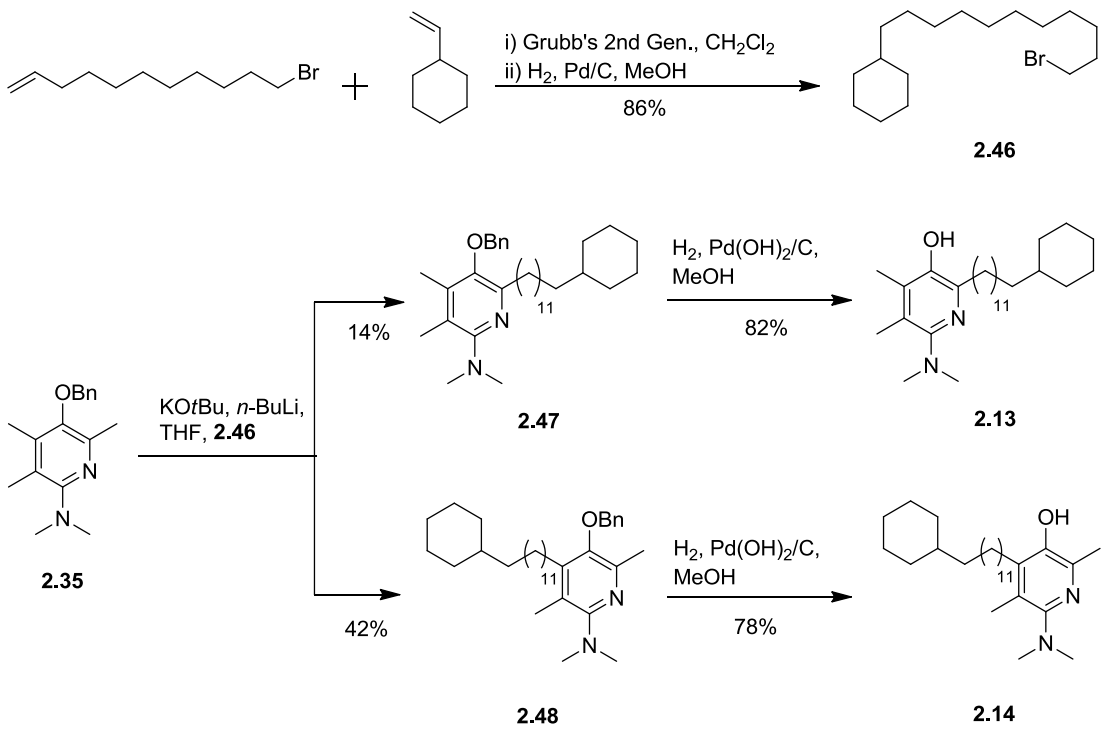
Alkylation resulted in a pair of regioisomers which were readily separated by silica gel chromatography to afford **2.36**–**2.45**. The alkylated products were treated with Pearlman's catalyst and H_2 in MeOH to afford the desired pyridinol analogues **2.3**–**2.12**.



Scheme 2.1. Route Employed for the Synthesis of Pyridinol Analogues With Linear Alkyl Side Chains (**2.3**–**2.12**).

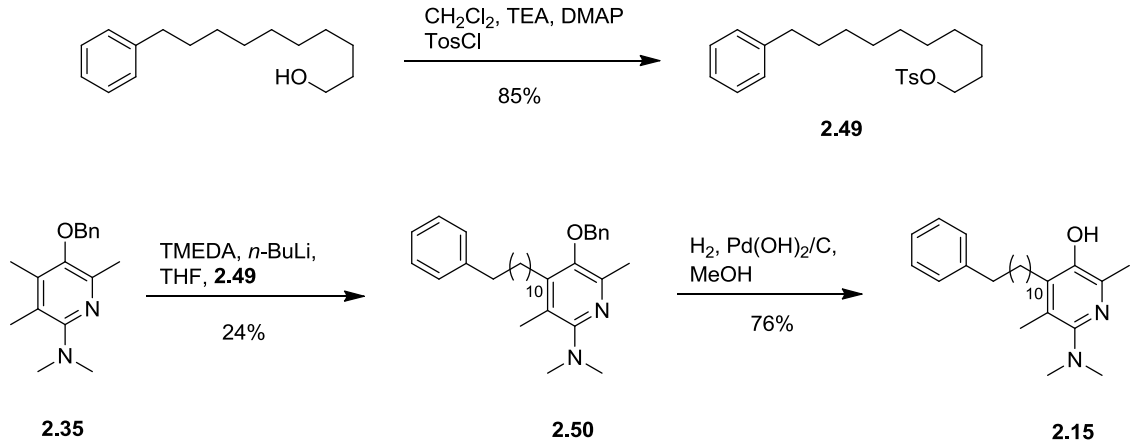
2.2.1.2. Synthesis of Pyridinol Analogues With Modified Linear Alkyl Side Chains

Compound **2.46** was synthesized by a cross metathesis reaction between vinyl cyclohexane and 11-bromo-1-undecene using 2nd generation Grubb's catalyst[121] followed by hydrogenation in presence of palladium-on-carbon (Scheme 2.2). The modified side chain was introduced by alkylation of **2.35** using Schlosser's super base (a mixture of KOtBu and *n*-BuLi).[120] Alkylation resulted in a pair of regioisomers which were readily separated by silica gel chromatography to afford **2.47** and **2.48** in 14% and 42% yields, respectively. The alkylated products were treated with Pearlman's catalyst and H_2 in MeOH to afford the desired pyridinol analogues **2.13** and **2.14** in 82% and 78% yields, respectively.



Scheme 2.2. Route Employed for the Synthesis of Pyridinol Analogues Having Terminal Cyclohexyl Groups (**2.13** and **2.14**).

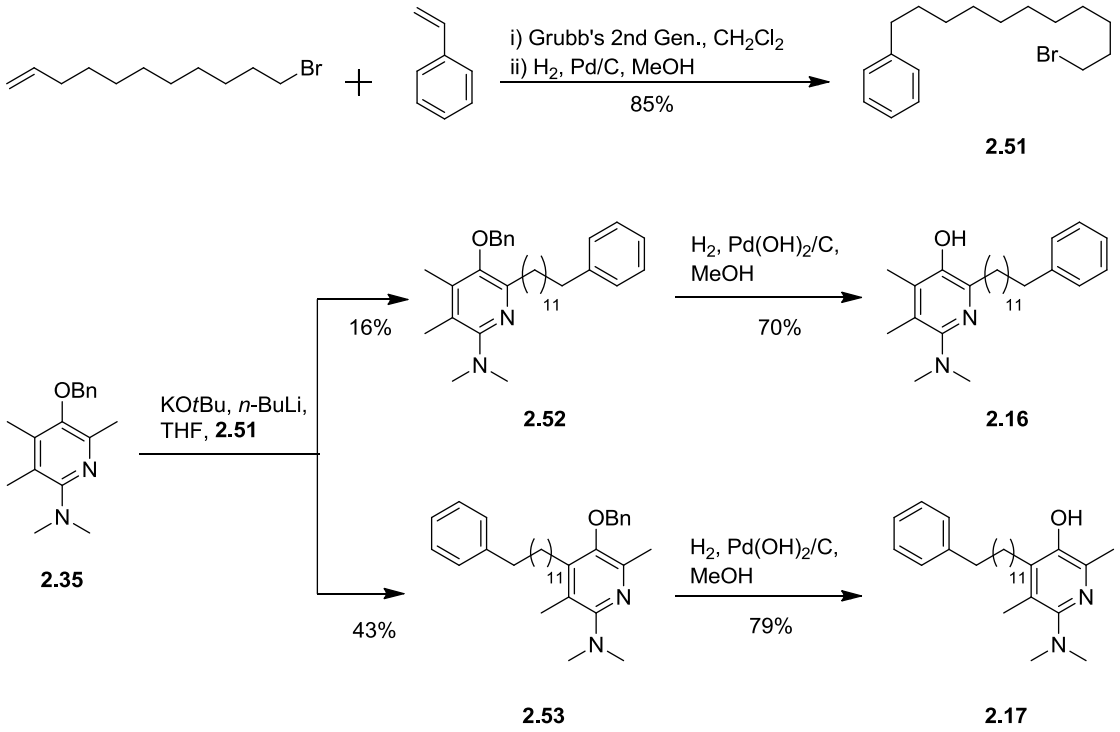
10-Phenyldecyl-4-methylbenzenesulfonate (**2.49**) was synthesized in 85% yield by tosylation of 10-phenyldecan-1-ol using *p*-toluenesulfonyl chloride, TEA and DMAP (Scheme 2.3). The modified side chain was introduced by alkylation of **2.35** using TMEDA and *n*-BuLi to afford **2.50** in 24% yield. The alkylated product was treated with Pearlman's catalyst and H_2 in MeOH to obtain the desired pyridinol analogue **2.15** in 76% yield.



Scheme 2.3. Route Employed for the Synthesis of a Pyridinol Analogue Having a Terminal Phenyl Group (**2.15**).

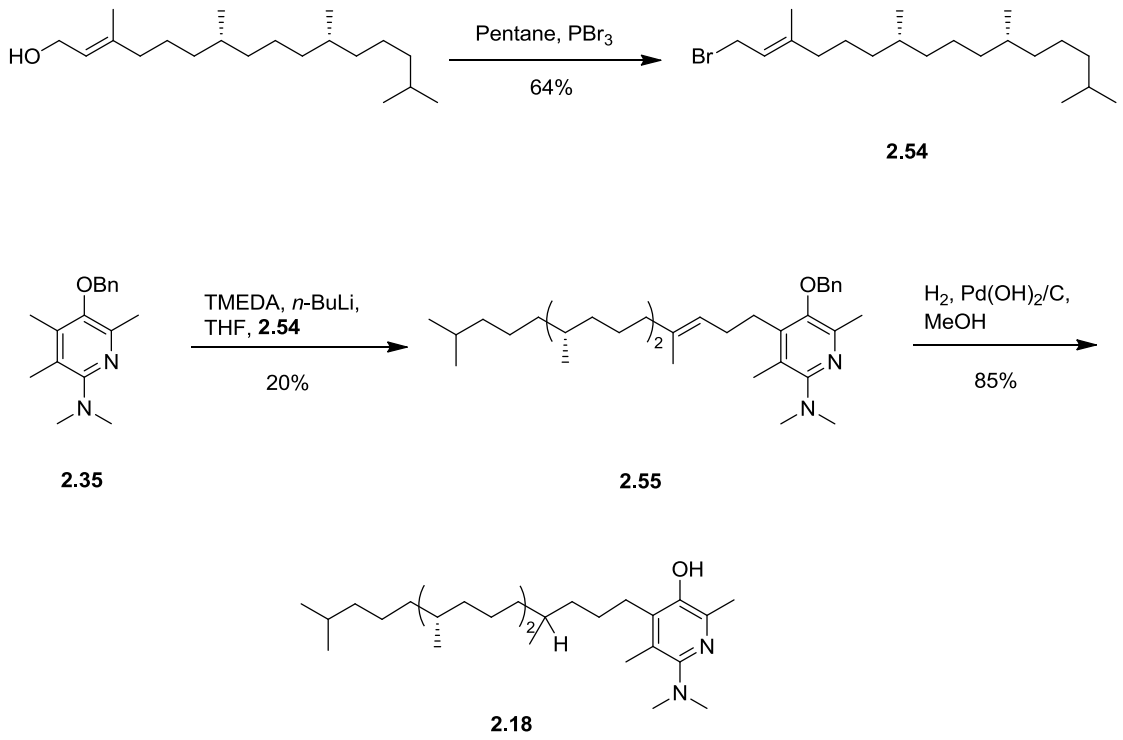
1-Bromo-11-phenylundecane (**2.51**) was synthesized in 85% yield by a cross metathesis reaction between styrene and 11-bromo-1-undecene using 2nd generation Grubb's catalyst[121] followed by hydrogenation in presence of palladium-on-carbon (Scheme 2.4). The modified side chain was introduced by alkylation of **2.35** using Schlosser's super base (a mixture of KO*t*Bu and *n*-BuLi).[120] Alkylation resulted in a pair of regioisomers which were readily separated by silica gel chromatography to afford **2.52** and **2.53** in 16% and 43% yields, respectively. The alkylated products were treated with

Pearlman's catalyst and H_2 in MeOH to afford the desired pyridinol analogues **2.16** and **2.17** in 70% and 79% yields, respectively.



Scheme 2.4. Route Employed for the Synthesis of Pyridinol Analogues Having Terminal Phenyl Groups (**2.16** and **2.17**).

(7*R*,11*R*,*E*)-1-Bromo-3,7,11,15-tetramethylhexadec-2-ene (**2.54**) was synthesized in 64% yield by bromination of phytol using PBr_3 in pentane (Scheme 2.5).[122] The modified side chain was introduced by alkylation of **2.35** using TMEDA and *n*-BuLi to afford **2.55** in 20% yield.[116] The alkylated product was treated with Pearlman's catalyst and H_2 in MeOH to obtain the desired pyridinol analogue **2.18** in 85% yield.

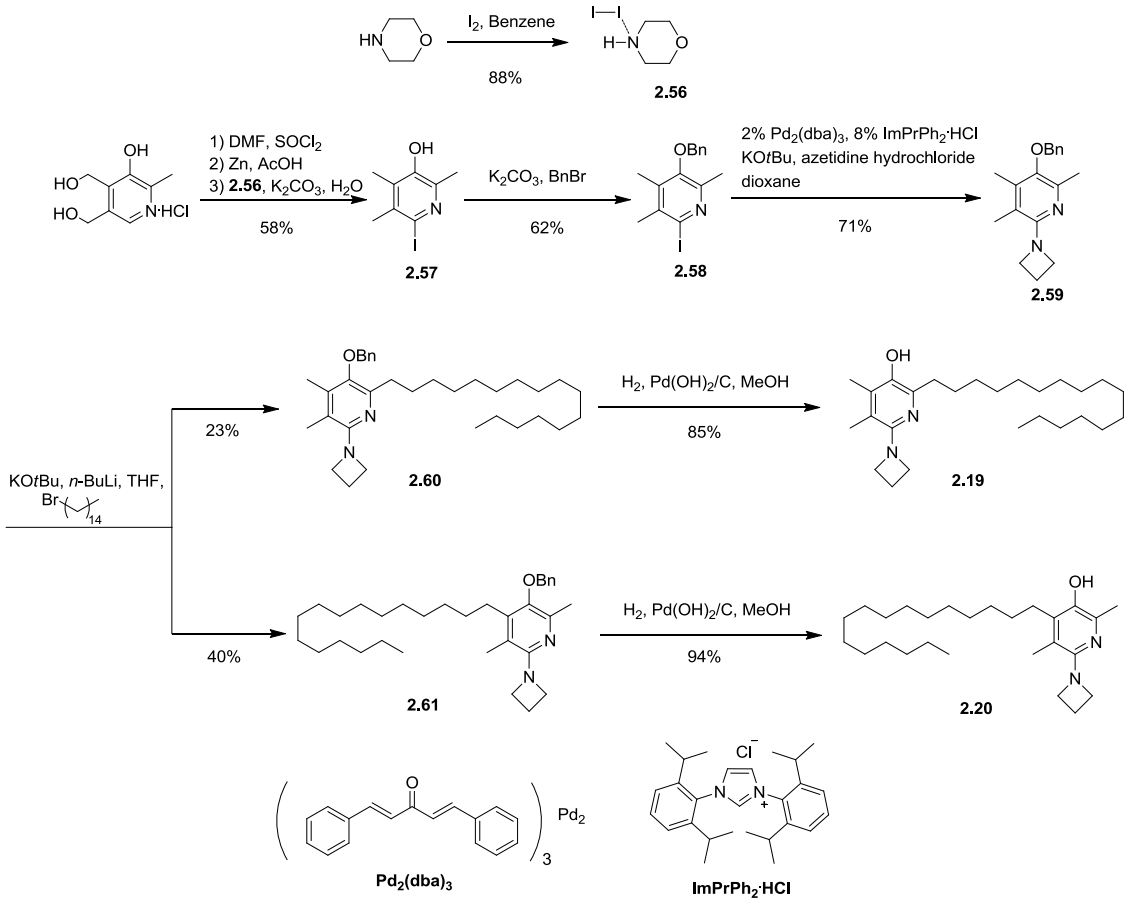


Scheme 2.5. Route Employed for the Synthesis of Pyridinol Analogue Having Phytyl Side Chain (**2.18**).

2.2.1.3. Synthesis of Pyridinol Analogues With Cyclic Amino Groups

A different synthetic route was employed to synthesize the pyridinol analogues containing an exocyclic amine (Scheme 2.6). First, commercially available pyridoxine hydrochloride (vitamin B_6) was treated with $SOCl_2$ then with zinc dust in AcOH, followed by treatment with morpholine–iodine charge transfer complex (**2.56**) to afford iodopyridinol (**2.57**) in 58% yield.[119,123] Morpholine–iodine complex was synthesized by a reported method.[124,125] Briefly, morpholine and iodine were stirred in benzene in the dark to afford the charge transfer complex (**2.56**) in 88% yield. *O*-Benzylation of **2.57** was achieved in 62% yield using K_2CO_3 and BnBr. Catalytic cross-coupling of halide **2.58** with azetidine was achieved using tris(dibenzylideneacetone)dipalladium (0)

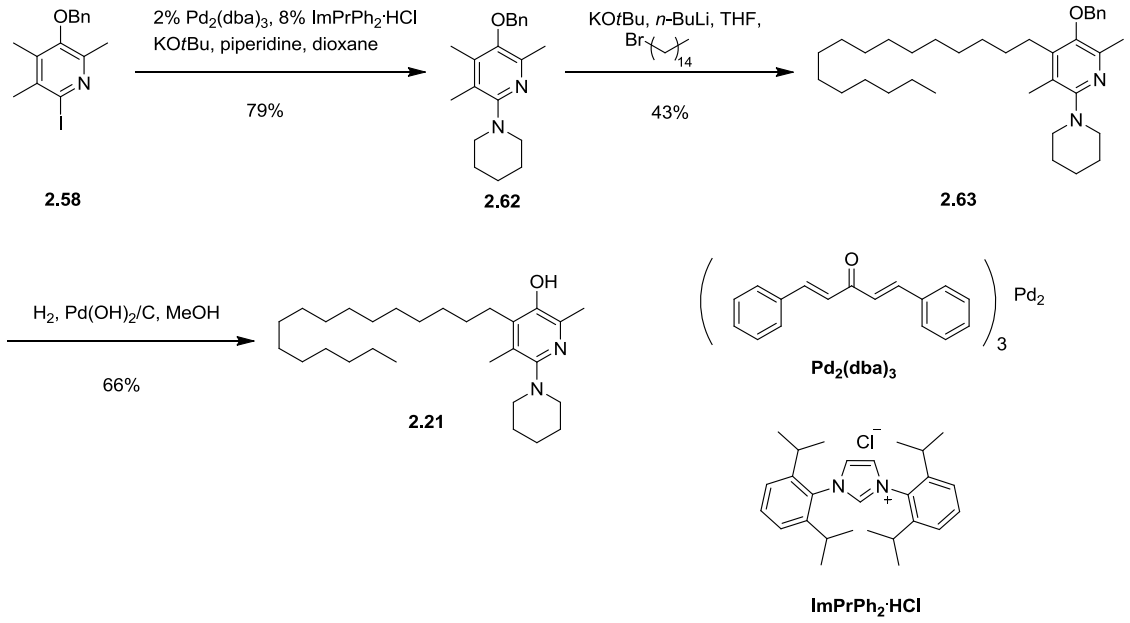
($Pd_2(dba)_3$), 1,3-bis(2,6-diisopropylphenyl)-imidazolium chloride ($ImPrPh_2$•HCl) and KO*t*Bu to afford **2.59** in 71% yield.[126] The hexadecyl side chain was introduced using Schlosser's super base and 1-bromopentadecane to afford **2.60** and **2.61** in 23% and 40% yields, respectively.[120] Finally, treatment of **2.60** and **2.61** with Pearlman's catalyst and H_2 in MeOH afforded analogues **2.19** and **2.20** in 85% and 94% yields, respectively.



Scheme 2.6. Route Employed for the Synthesis of Pyridinol Analogues With an Azetidine Substituent (**2.19** and **2.20**).

In order to synthesize a pyrimidinol analogue with a 6-member exocyclic amine (piperidine) core **2.58** was used (Scheme 2.7). Catalytic cross-coupling of halide **2.58**

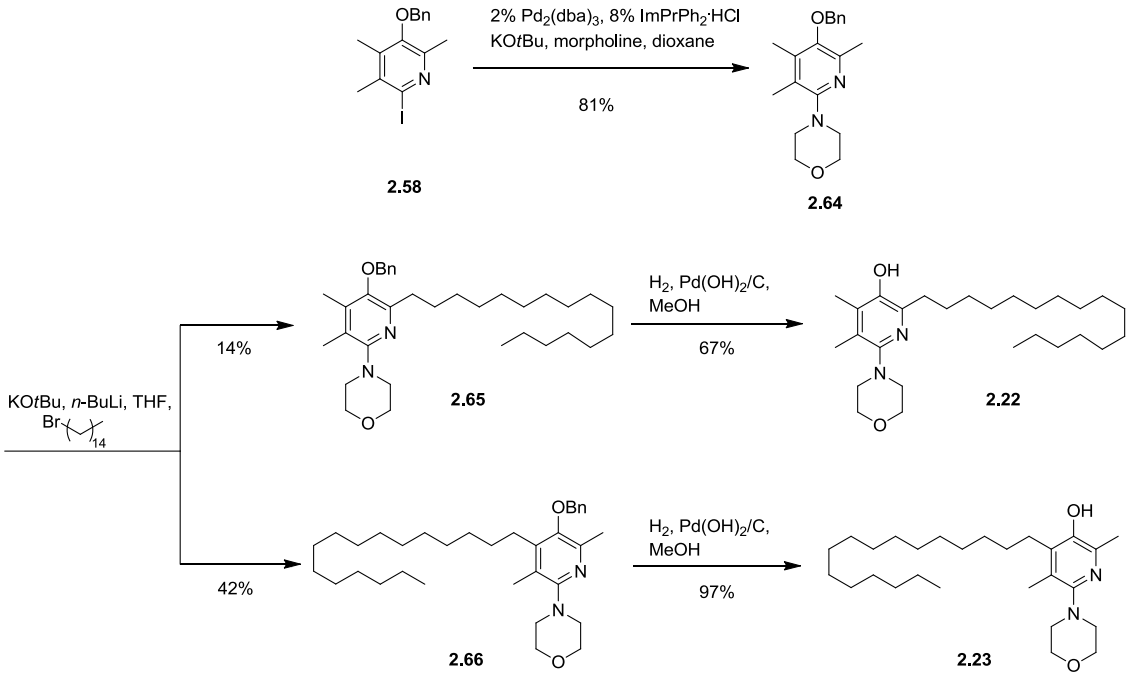
with piperidine was achieved using tris(dibenzylideneacetone)dipalladium (0) ($Pd_2(dba)_3$), 1,3-bis(2,6-diisopropylphenyl)-imidazolium chloride ($ImPrPh_2$•HCl) and KO*t*Bu to afford **2.62** in 79% yield.[126] The hexadecyl side chain was introduced using Schlosser's super base and 1-bromopentadecane to afford **2.63** in 43% yield.[120] Finally, treatment of **2.63** with Pearlman's catalyst and H_2 in MeOH afforded **2.21** in 66% yield.



Scheme 2.7. Route Employed for the Synthesis of Pyridinol Analogue With a Piperidine Substituent (**2.21**).

The synthesis of morpholine substituted pyridinol analogues involved the catalytic cross-coupling of halide **2.58** with morpholine using tris(dibenzylideneacetone)dipalladium (0) ($Pd_2(dba)_3$), 1,3-bis(2,6-diisopropylphenyl)-imidazolium chloride ($ImPrPh_2$•HCl) and KO*t*Bu to afford **2.64** in 81% yield (Scheme 2.8).[126] The hexadecyl side chain was introduced using Schlosser's super base and 1-bromopentadecane to afford **2.65** and **2.66** in 14% and 42% yields, respectively.[120]

Finally, treatment of **2.65** and **2.66** with Pearlman's catalyst and H_2 in MeOH afforded analogues **2.22** and **2.23** in 67% and 97% yields, respectively.

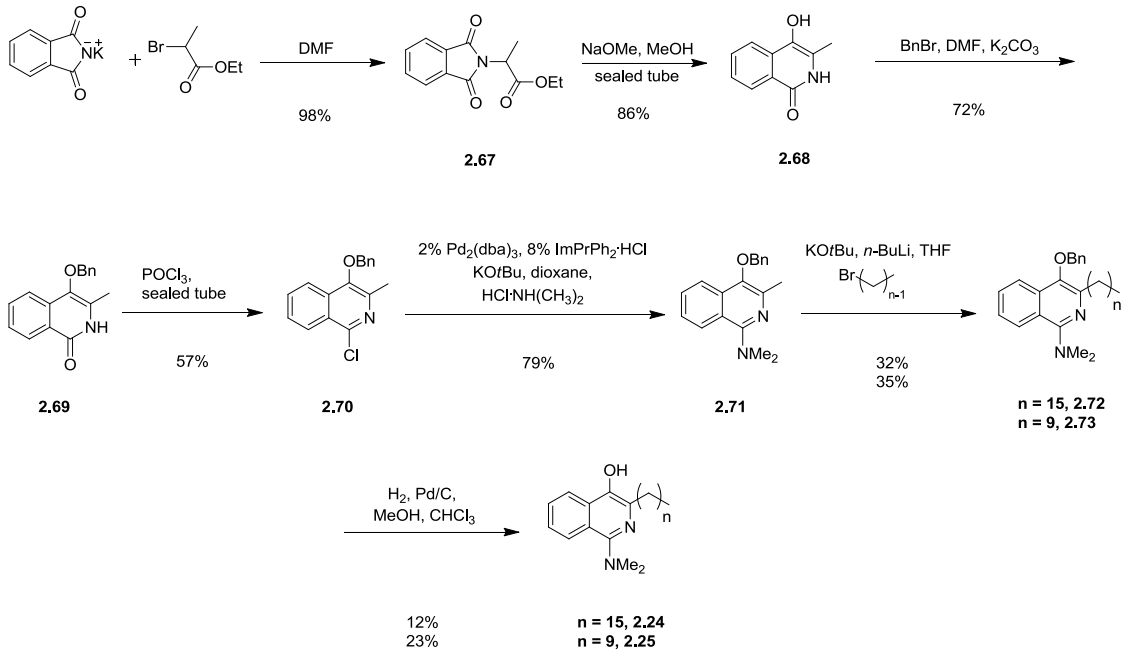


Scheme 2.8. Route Employed for the Synthesis of Pyridinol Analogues With a Morpholine Substituent (**2.22** and **2.23**).

2.2.1.4. Synthesis of Bicyclic Pyridinol Analogues

In order to synthesize the bicyclic pyridinol analogues, commercially available potassium phthalimide was treated with 2-bromopropionate in dry DMF to afford **2.67** in 98% yield. Rearrangement of compound **2.67** by treatment with freshly prepared sodium methoxide in MeOH afforded **2.68** in 86% yield.[127,128] Compound **2.68** was *O*-benzylated using benzyl bromide to afford **2.69** in 72% yield. Treatment of **2.69** with neat $POCl_3$ afforded **2.70** in 57% yield.[129] The catalytic cross-coupling of halide **2.70** with dimethylamine hydrochloride using tris(dibenzylideneacetone)dipalladium (0) ($Pd_2(dba)_3$), 1,3-bis(2,6-diisopropylphenyl)-imidazolium chloride ($ImPrPh_2$•HCl) and

KO*t*Bu afforded **2.71** in 79% yield (Scheme 2.9).[126] The protected bicyclic pyridinol core was alkylated by generating carbanion using Schlosser's super base and adding 1-bromoalkane to afford **2.72** and **2.73** in 32% and 35% yields, respectively.[120] Finally, treatment of **2.72** and **2.73** with Pearlman's catalyst and H_2 in MeOH–$CHCl_3$ afforded analogues **2.24** and **2.25** in 12% and 23% yields, respectively.

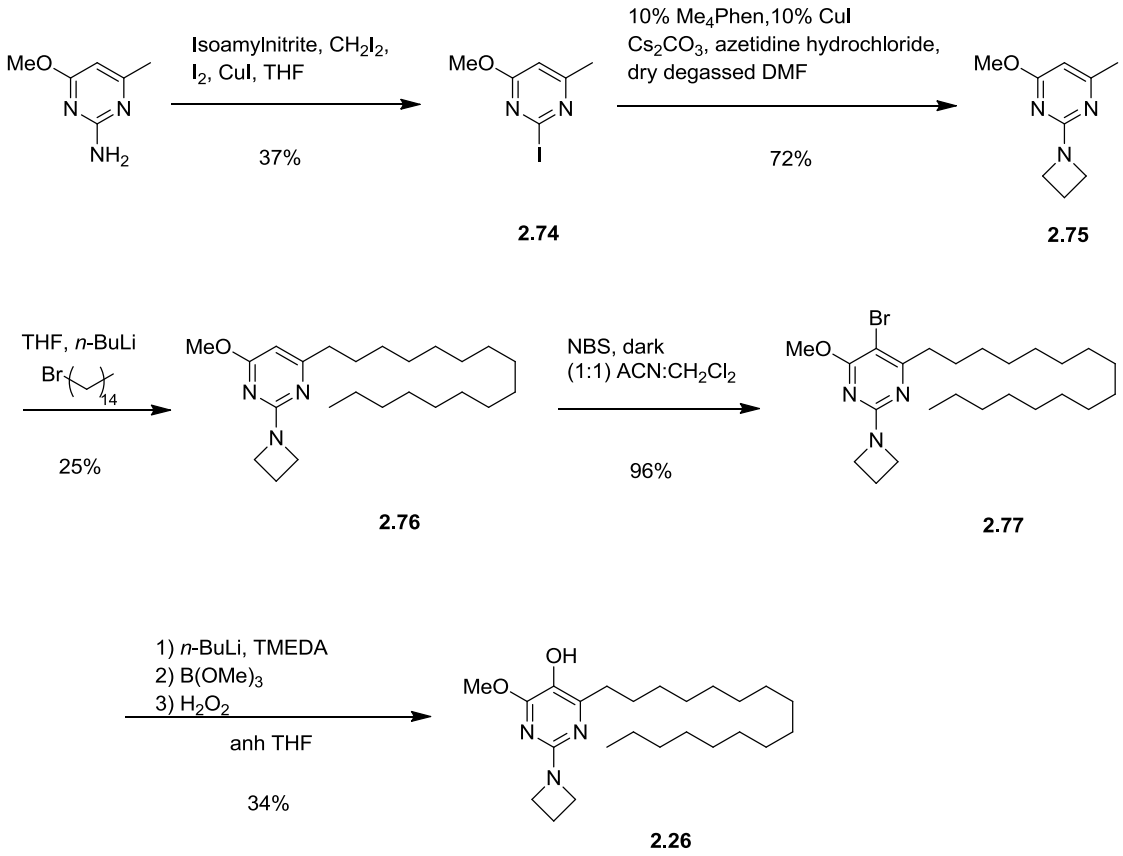


Scheme 2.9. Route Employed for the Synthesis of Bicyclic Pyridinol Analogues (**2.24** and **2.25**).

2.2.1.5. Synthesis of Pyrimidinol Analogues With Cyclic Amino Groups

The route employed for the synthesis of pyrimidinol analogue **2.26** is illustrated in Scheme 2.10. Accordingly, 2-amino-4-methoxy-6-methylpyrimidine was treated with isoamylnitrite, diiodomethane, iodine and CuI to afford 2-iodo-4-methoxy-6-methyl pyrimidine (**2.74**) in 37% yield.[130] The catalytic cross coupling of halide **2.74** using azetidine hydrochloride, CuI, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen) and

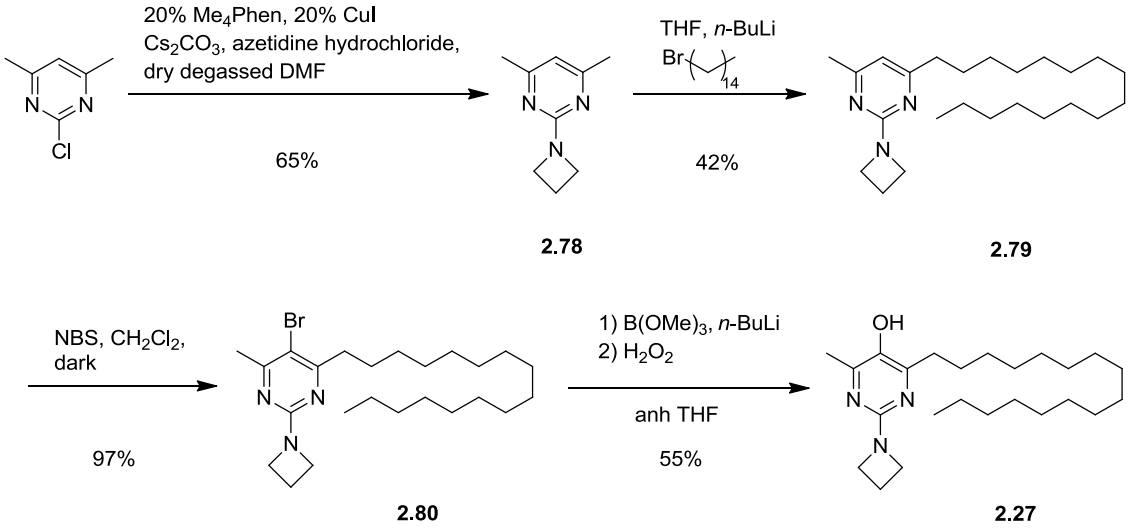
Cs_2CO_3 afforded **2.75** in 72% yield.[131] The hexadecyl side chain was introduced using *n*-BuLi and 1-bromopentadecane to afford **2.76** in 25% yield. Treatment of **2.76** with *N*-bromosuccinimide in the dark afforded **2.77** in 96% yield. The final product, **2.26**, was obtained in 34% yield by treatment of **2.77** with *n*-BuLi in presence of TMEDA, then with trimethyl borate, and lastly with hydrogen peroxide in the subsequent steps.[118]



Scheme 2.10. Route Employed for the Synthesis of Methoxy Pyrimidinol Analogue With a Cyclic Amino Group (**2.26**).

The synthetic scheme employed for pyrimidinol analogue **2.27** is illustrated in Scheme 2.11. The catalytic cross coupling of 2-chloro-4,6-dimethylpyrimidine using azetidine hydrochloride, CuI, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen) and

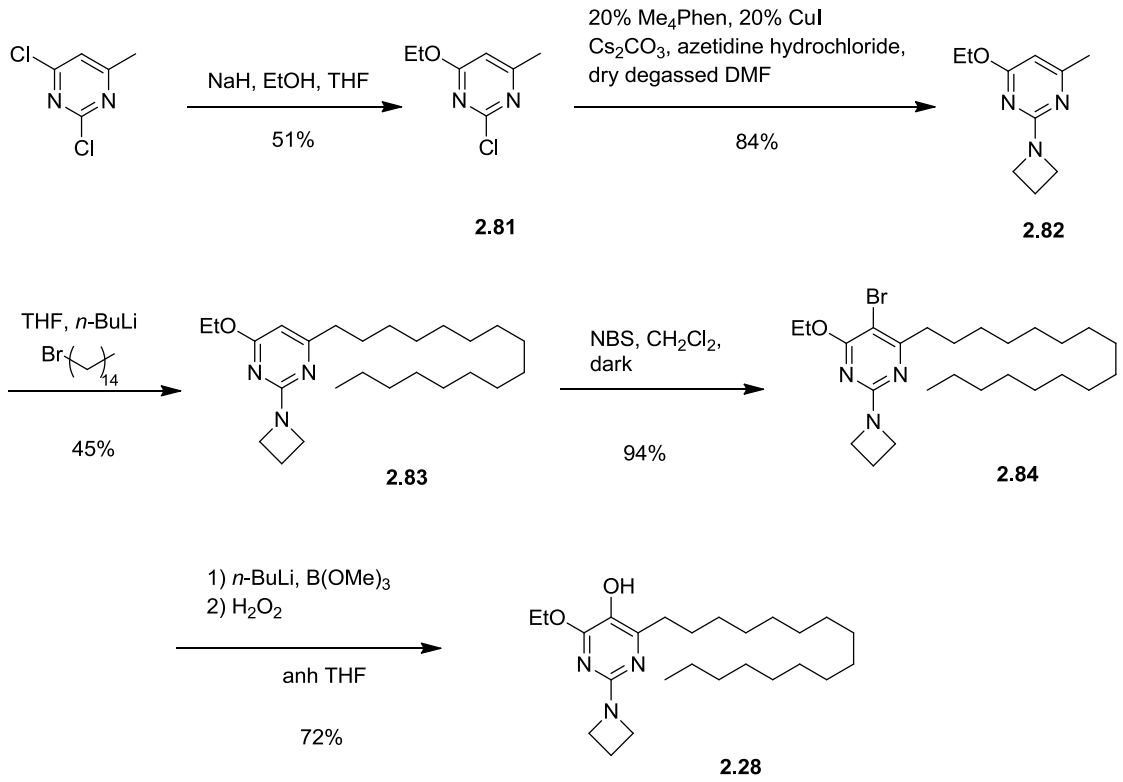
Cs_2CO_3 afforded **2.78** in 65% yield.[131] The hexadecyl side chain was introduced using *n*-BuLi and 1-bromopentadecane to afford **2.79** in 42% yield. Treatment of **2.79** with *N*-bromosuccinimide in the dark afforded **2.80** in 96% yield. The final product, **2.27**, was obtained in 55% yield by the treatment of **2.80** with trimethyl borate followed by *n*-BuLi, and lastly with hydrogen peroxide.[118]



Scheme 2.11. Route Employed for the Synthesis of Pyrimidinol Analogue With a Cyclic Amino Group (**2.27**).

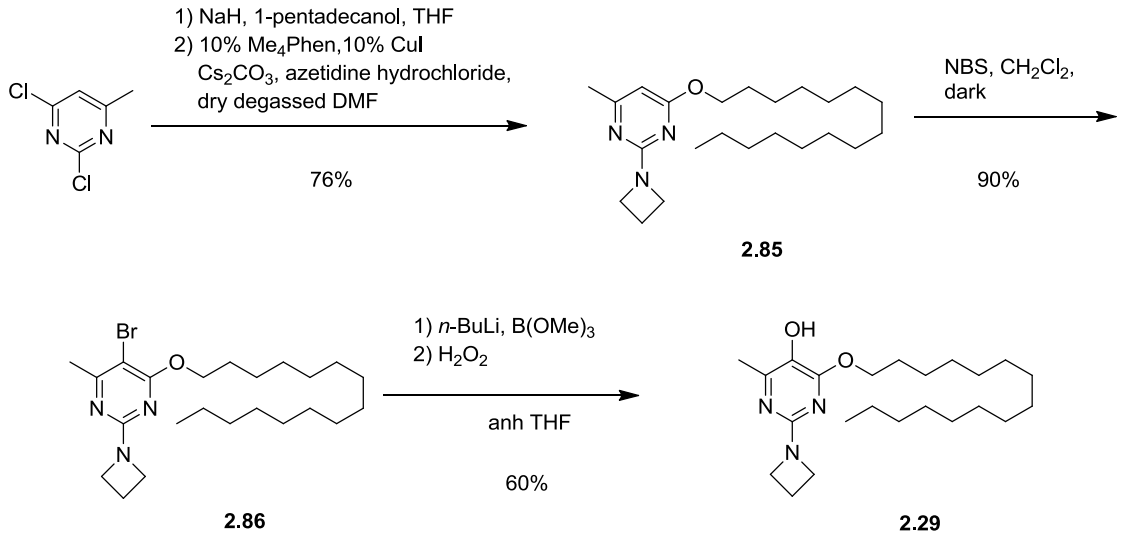
The synthesis of **2.28** started with the treatment of 2,4-dichloro-6-methylpyrimidine with EtOH and NaH in anh THF to afford **2.81** in 51% yield (Scheme 2.12). The catalytic cross coupling of 2-chloro-4-ethoxy-6-methylpyrimidine using azetidine hydrochloride, CuI, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen) and Cs_2CO_3 afforded **2.82** in 84% yield.[131] The hexadecyl side chain was introduced using *n*-BuLi and 1-bromopentadecane to afford **2.83** in 45% yield. Treatment of **2.83** with *N*-bromosuccinimide in the dark afforded **2.84** in 94% yield. The final product **2.28** was

obtained in 72% yield by the treatment of **2.84** with *n*-BuLi followed by trimethyl borate, and lastly with hydrogen peroxide.[118]



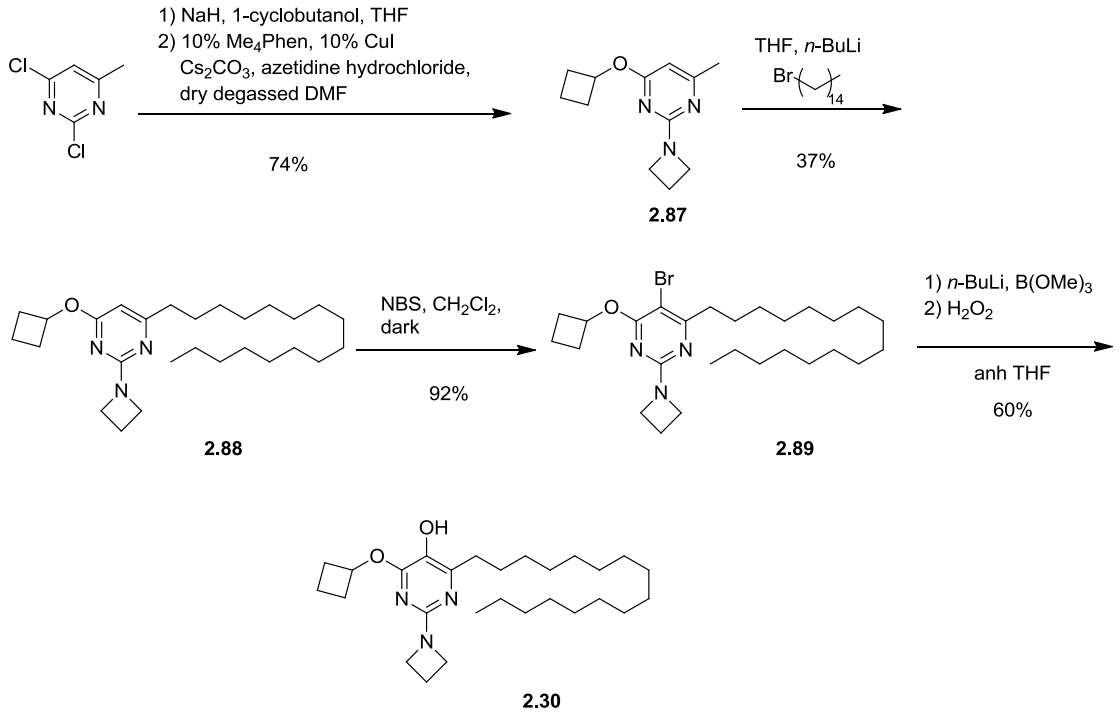
Scheme 2.12. Route Employed for the Synthesis of Ethoxy Pyrimidinol Analogue **2.28**.

The synthetic scheme employed for the preparation of **2.29** is illustrated in Scheme 2.13. 2,4-Dichloro-6-methylpyrimidine was first treated with 1-pentadecanol and NaH in anh THF to afford crude 2-chloro-4-pentadecoxy-6-methylpyrimidine, which was coupled with azetidine hydrochloride using CuI, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen) and Cs_2CO_3 to afford **2.85** in 76% yield over two steps.[131] Treatment of **2.85** with *N*-bromosuccinimide in the dark afforded **2.86** in 90% yield. The final product **2.29** was obtained in 60% yield by the treatment of **2.86** with *n*-BuLi followed by trimethyl borate, and lastly with hydrogen peroxide.[118]



Scheme 2.13. Route Employed for the Synthesis of Pyrimidinol Analogue **2.29**.

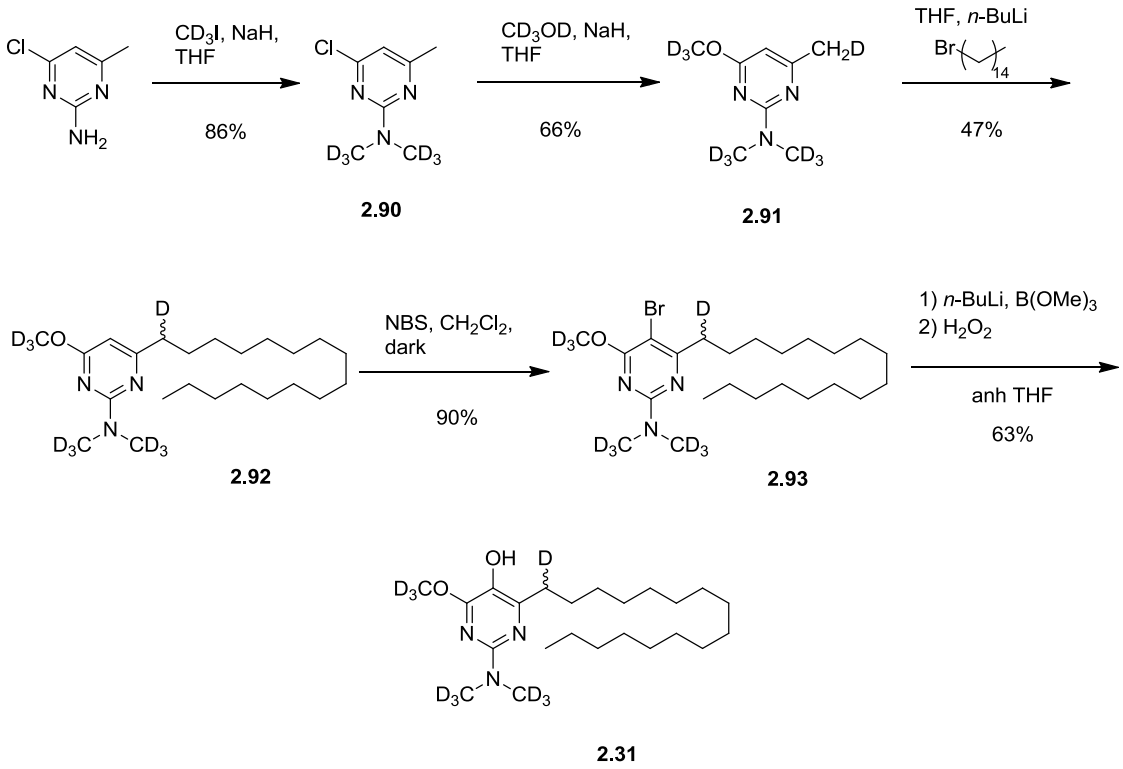
The route employed for the synthesis of **2.30** is illustrated in Scheme 2.14. 2,4-Dichloro-6-methylpyrimidine was first treated with 1-cyclobutanol and NaH in anh THF to afford crude 2-chloro-4-cyclobutoxy-6-methylpyrimidine, which was coupled with azetidine hydrochloride using CuI, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen) and Cs_2CO_3 to afford **2.87** in 74% yield over two steps.[131] The hexadecyl side chain was introduced using *n*-BuLi and 1-bromopentadecane to afford **2.88** in 37% yield. Treatment of **2.88** with *N*-bromosuccinimide in the dark afforded **2.89** in 92% yield. The final product **2.30** was obtained in 60% yield by the treatment of **2.89** with *n*-BuLi followed by trimethyl borate, and lastly with hydrogen peroxide.[118]



Scheme 2.14. Route Employed for the Synthesis of Pyrimidinol Analogue **2.30**.

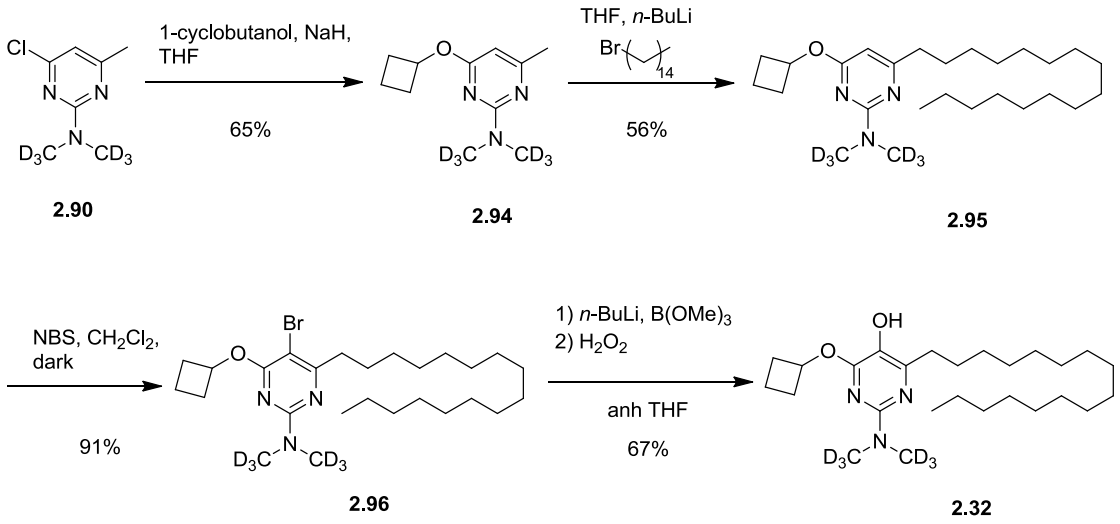
2.2.1.6. Synthesis of Deuterated Pyrimidinol Analogues

Synthesis of **2.31** started with the treatment of 2-amino-4-chloro-6-methylpyrimidine with CD_3I and NaH in anh THF to afford **2.90** in 86% yield (Scheme 2.15). Treatment of compound **2.90** with CD_3OD and NaH in anh THF afforded **2.91** in 66% yield in the next step. The hexadecyl side chain was introduced using *n*-BuLi and 1-bromopentadecane to afford **2.92** in 47% yield. Treatment of **2.92** with *N*-bromosuccinimide in the dark afforded **2.93** in 90% yield. The final product **2.31** was obtained in 63% yield by the treatment of **2.93** with *n*-BuLi followed by trimethyl borate, and lastly with hydrogen peroxide.[118]



Scheme 2.15. Route Employed for the Synthesis of Pyrimidinol Analogue **2.31**.

The synthetic route employed for the preparation of **2.32** is illustrated in Scheme 2.16. Treatment of compound **2.90** with 1-cyclobutanol and NaH in anh THF afforded **2.94** in 65% yield. The hexadecyl side chain was introduced using *n*-BuLi and 1-bromopentadecane to afford **2.95** in 56% yield. Treatment of **2.95** with *N*-bromosuccinimide in the dark afforded **2.96** in 91% yield. The final product **2.32** was obtained in 67% yield by the treatment of **2.96** with *n*-BuLi followed by trimethyl borate, and lastly with hydrogen peroxide.[118]



Scheme 2.16. Route Employed for the Synthesis of Pyrimidinol Analogue **2.32**.

2.2.2. Biochemical and Biological Evaluation of Pyridinol and Pyrimidinol Analogues

2.2.2.1. Mitochondrial Electron Transport Chain Function

Inhibition of any of the mitochondrial respiratory chain complexes can limit the potential therapeutic utility of CoQ_{10} analogues. Accordingly, it is desirable to prepare analogues that are minimally inhibitory to the respiratory chain. As an initial screen, the effects of the compounds on NADH oxidase activity have been studied, which encompasses the functions of mitochondrial complexes I, III and IV. The importance of side chain length on the interaction of coenzyme Q_{10} analogues with the mitochondrial respiratory chain to achieve improved bioenergetic and antioxidant activity have been well documented.[115-118,132] In the light of those findings, analogues having different side chain lengths attached to the modified redox core have been designed and synthesized (Figures 2.6–2.10). The inhibitory effects of the test compounds on NADH oxidase

(complexes I, III and IV) function were evaluated using submitochondrial particles (SMP). The results are presented in Table 2.1, and show that the inhibitory behavior of these compounds was dose dependent. Compound **2.1**, having a 10 carbon atom side chain and a polar terminal hydroxyl group, strongly inhibited NADH oxidase, as had been shown before.[116,117] Removing the polar hydroxyl group (compounds **2.3** and **2.4)** resulted in a slightly less inhibitory effect than was observed for compound **2.1**. Increasing the side chain length to 13, 15, 16 and 19 carbon atoms largely abolished the inhibitory effect of these derivatives (**2.5**–**2.12**). Modifying the linear alkyl side chain as in compounds **2.13**–**2.18** afforded no significant improvement over compounds **2.5**–**2.12**. Replacement of the dimethylamino moiety at position 6 of the pyridinol redox core with a cyclic amino group (**2.19–2.23**) produced no significant incremental inhibitory effect on the respiratory chain. The bicyclic pyridinols (**2.24** and **2.25**) were also found not to show much improvement over other pyridinols.

Table 2.1. The Inhibitory Effect of Compounds on Bovine Heart Mitochondrial NADH Oxidase Activity (Complexes I, III and IV). The Experiment was Performed by Sriloy Dey.

	NADH oxidase activity (%)[a]		
Compound	1 μM	5 μM	10 μM
Untreated control	100	100	100
2.1	27 ± 1	9 ± 1	6 ± 1
2.3	35 ± 1	28 ± 1	13 ± 1
2.4	49 ± 1	35 ± 1	8 ± 1
2.5	81 ± 1	73 ± 1	43 ± 1
2.6	99 ± 1	96 ± 2	75 ± 2
2.7	81 ± 1	73 ± 1	43 ± 1
2.8	88 ± 1	85 ± 1	80 ± 1
2.9	98 ± 2	95 ± 2	92 ± 1
2.10	95 ± 1	93 ± 1	86 ± 1
2.11	93 ± 1	88 ± 1	85 ± 1
2.12	98 ± 1	83 ± 1	81 ± 1
2.13	62 ± 2	57 ± 1	40 ± 1
2.14	91 ± 1	90 ± 3	91 ± 1
2.15	85 ± 2	72 ± 3	62 ± 2
2.16	57 ± 3	37 ± 1	20 ± 1
2.17	85 ± 2	72 ± 3	62 ± 2
2.18	85 ± 1	85 ± 2	79 ± 1
2.19	93 ± 4	94 ± 2	95 ± 3
2.20	95 ± 2	89 ± 2	79 ± 5
2.21	81 ± 3	78 ± 4	67 ± 5
2.22	96 ± 2	74 ± 9	69 ± 7
2.23	79 ± 4	73 ± 8	52 ± 6
2.24	98 ± 1	89 ± 2	83 ± 2
2.25	79 ± 2	77 ± 2	74 ± 2

[a] Relative to untreated control.

2.2.2.2. Inhibition of Lipid Peroxidation

The ability of the pyridinol analogues to quench lipid peroxidation was studied in FRDA lymphocytes that had been depleted of glutathione by treatment with diethyl maleate (DEM). C_{11}-BODIPY$^{581/591}$, a hydrophobic fatty acid fluorophore which inserts preferentially in membranes, has been shown previously to enable quantification of fatty

acid oxidation and antioxidant activity in live cells.[133] The oxidation of the polyunsaturated butadienyl portion of the dye results in a shift of the fluorescence emission peak from red to green. The degree of probe oxidation was followed using flow cytometry as reported before.[115-117,133] Cells were analyzed for a shift of the fluorescence emission peak from red to green with excitation/emission wavelengths of 490/510 nm. The median mean fluorescence values were used for further analysis. Increasing green fluorescence intensity indicated lipid peroxidation. The results, presented in Table 2.2, show the most potent lipid peroxidation quenching activity for compounds **2.6**–**2.10**, **2.19**, **2.20** and **2.23**. Compound **2.1** had lower activity for suppressing lipid peroxidation in comparison with analogues with the same side chain length lacking a hydroxyl group (**2.3** and **2.4**). These results were in agreement with the previous reports for pyrimidinol derivatives.[116,118] No difference in the lipid peroxidation scavenging activity was noted for the regioisomeric pairs of pyridinol analogues. Compounds **2.19** and **2.20**, having an azetidine group, and **2.22** and **2.23**, having a morpholine group at the 6-position of the pyridinol core, were slightly less effective than their counterparts with the dimethylamino moiety (**2.9** and **2.10**). The analogues having nonadecyl side chains (**2.11** and **2.12**) had significantly reduced ability to quench lipid peroxidation in comparison to the analogues having 16 carbon atom side chain (**2.9**, **2.10**, **2.19** and **2.20**). Compounds (**2.13**–**2.17**) having a modified side chain exhibited a moderate ability to quench lipid peroxidation in comparison to **2.9** and **2.10**. Compound **2.18**, having a phytyl-type side chain and bicyclic pyridinols **2.24** and **2.25** did not exhibit good suppression of lipid peroxidation.

Table 2.2. Suppression of Lipid Peroxidation by Pyridinol Antioxidants in Cultured FRDA Lymphocytes Treated with DEM. The Experiment was Performed by Dr. Omar M. Khdour.

	Lipid peroxidation scavenging activity (%)	
	100 nM	250 nM
Untreated control	100	100
2.1	25 ± 4	45 ± 3
2.3	70 ± 3	80 ± 3
2.4	67 ± 4	79 ± 3
2.5	76 ± 5	86 ± 4
2.6	81 ± 3	88 ± 2
2.7	78 ± 5	89 ± 2
2.8	76 ± 6	86 ± 3
2.9	85 ± 1	94 ± 2
2.10	81 ± 3	92 ± 2
2.11	35 ± 3	58 ± 5
2.12	33 ± 6	60 ± 3
2.13	57 ± 6	77 ± 3
2.14	60 ± 2	80 ± 3
2.15	68 ± 4	82 ± 3
2.16	75 ± 2	83 ± 3
2.17	71 ± 2	84 ± 2
2.18	9 ± 1	26 ± 3
2.19	68 ± 3	85 ± 4
2.20	70 ± 3	87 ± 2
2.21	44 ± 5	68 ± 5
2.22	62 ± 4	78 ± 3
2.23	70 ± 3	84 ± 4
2.24	8 ± 3	14 ± 4
2.25	10 ± 3	12 ± 4

2.2.2.3. Suppression of Reactive Oxygen Species

The ability of the pyridinol analogues **2.1**–**2.25** to suppress ROS induced by depletion of glutathione was evaluated in FRDA lymphocyte cells. The intracellular ROS level was measured based on the ROS-induced formation of the highly fluorescent product 2′,7′-dichlorofluorescein (DCF) from the non-fluorescent dye 2′,7′-

dichlorodihydrofluorescein diacetate (DCFH-DA).[134] The results are presented in Table 2.3, and fairly closely paralleled those found for lipid peroxidation. The regioisomeric compounds **2.9** and **2.10** were the most potent in suppressing ROS, and did so in a concentration dependent manner. Compounds **2.5**–**2.8** were also good in suppressing ROS but were slightly less effective at 250 nM concentration as compared to **2.9**.The compounds having a decyl side chain (**2.3** and **2.4**) afforded slightly less protection against ROS. Compound **2.1** was the least effective analogue in suppressing ROS. Compounds with side chains longer than 16 carbon atoms (**2.11** and **2.12**) were poor ROS quenchers, in agreement with the present lipid peroxidation results. Compounds with a modified chain (**2.13**–**2.17**) showed moderate ROS quenching similar to compounds having a decyl side chain (**2.3** and **2.4**). Once again the regioisomeric analogues having an azetidine group (**2.19** and **2.20**) or morpholine group (**2.22** and **2.23**) were slightly less effective than the regioisomers pair (**2.9** and **2.10**) in suppressing ROS levels. Compound **2.21** with piperidine group was even less effective than the other analogue having cyclic amino group. The bicyclic pyridinols (**2.24** and **2.25**) and compound **2.18** did not effectively suppress ROS.

Table 2.3. Suppression of ROS Production by Pyridinol Antioxidants in Cultured FRDA Lymphocytes Pretreated with DEM. The Experiment was Performed by Dr. Omar M. Khdour.

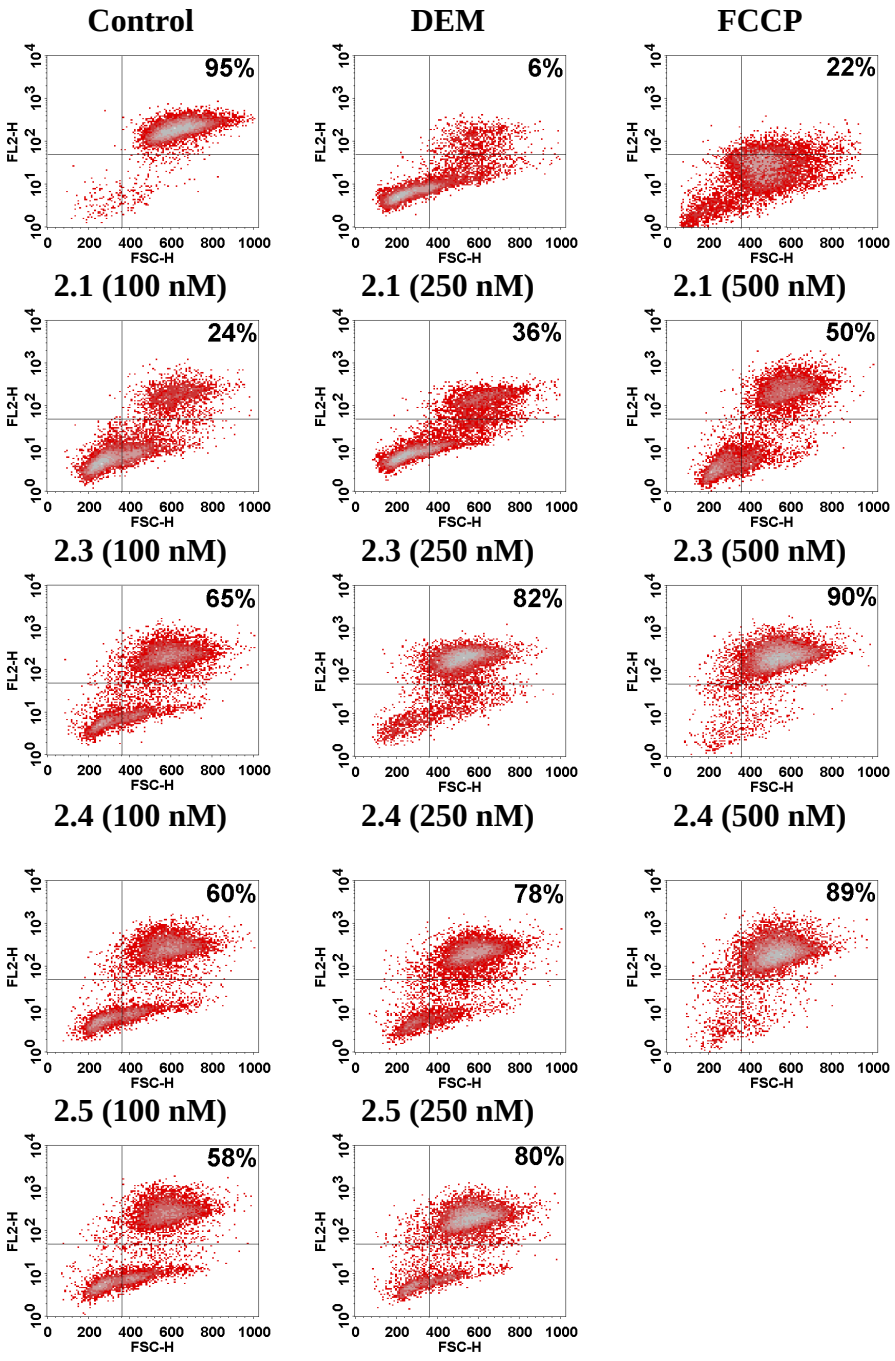
	ROS Scavenging Activity (%)		
Compound	100 nM	250 nM	500 nM
Untreated control	100	100	100
2.1	3 ± 1	17 ± 4	25 ± 5
2.3	47 ± 7	70 ± 6	80 ± 3
2.4	49 ± 6	72 ± 6	89 ± 2
2.5	68 ± 1	87 ± 2	95 ± 1
2.6	70 ± 4	85 ± 2	97 ± 3
2.7	79 ± 2	92 ± 2	94 ± 2
2.8	77 ± 3	90 ± 3	94 ± 1
2.9	80 ± 4	97 ± 2	100 ± 1
2.10	82 ± 5	95 ± 5	97 ± 2
2.11	4 ± 1	24 ± 4	37 ± 4
2.12	5 ± 2	31 ± 4	43 ± 3
2.13	46 ± 3	72 ± 2	83 ± 2
2.14	43 ± 5	66 ± 5	80 ± 4
2.15	58 ± 5	83 ± 3	88 ± 4
2.16	64 ± 2	85 ± 4	96 ± 3
2.17	61 ± 5	85 ± 2	90 ± 3
2.18	7 ± 2	17 ± 3	30 ± 2
2.19	57 ± 4	78 ± 3	88 ± 7
2.20	62 ± 3	80 ± 3	89 ± 4
2.21	35 ± 3	59 ± 4	74 ± 4
2.22	56 ± 4	70 ± 3	80 ± 4
2.23	64 ± 2	83 ± 4	90 ± 3
2.24	12 ± 2	26 ± 5	33 ± 2
2.25	10 ± 3	25 ± 3	34 ± 4

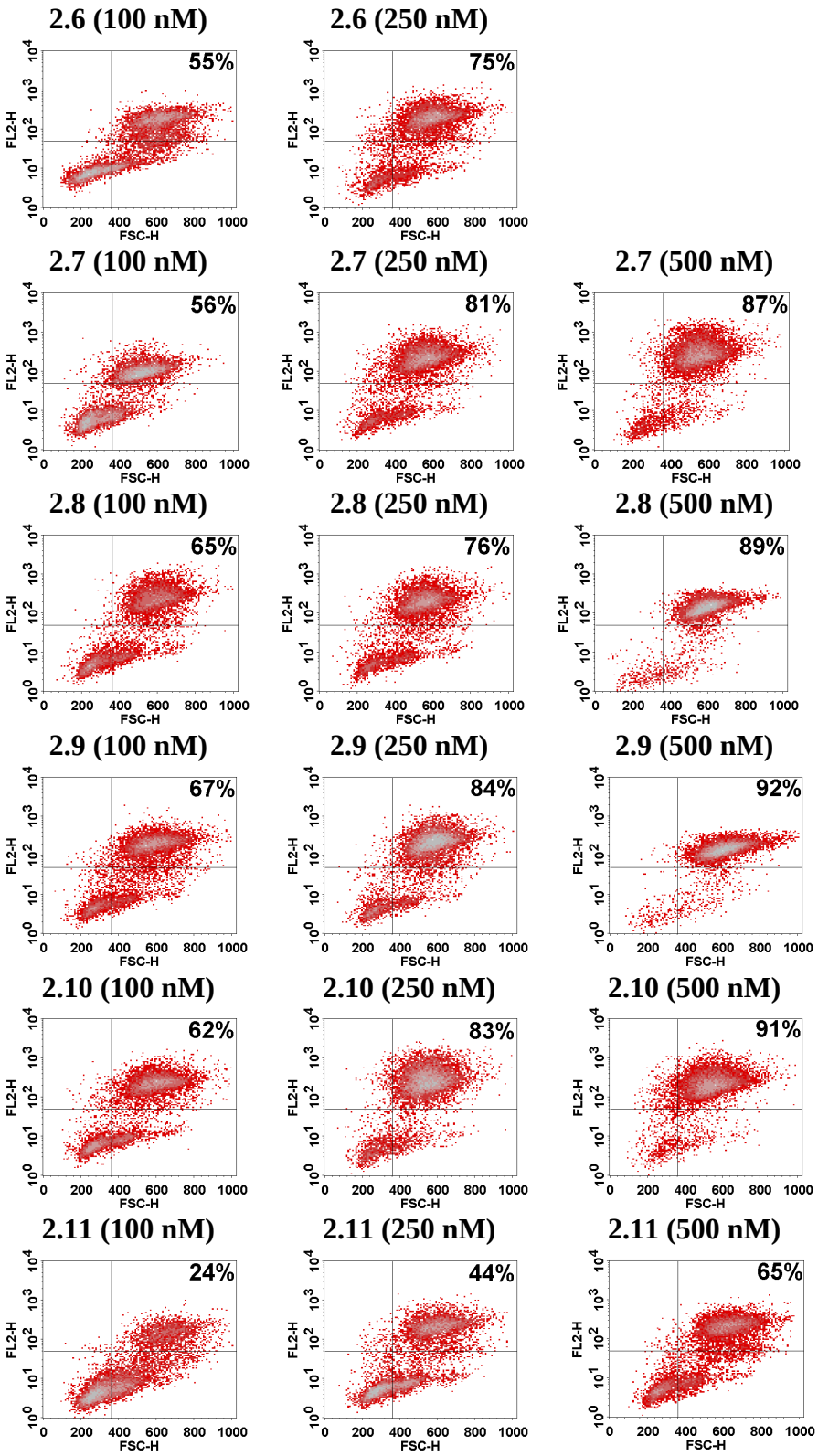
2.2.2.4. Preserving Mitochondrial Inner Membrane Potential ($\Delta\psi_m$)

The ability of the test compounds to maintain mitochondrial inner membrane potential ($\Delta\psi_m$) under conditions of oxidative stress was studied as described previously.[115-118] $\Delta\psi_m$ was measured using tetramethylrhodamine methyl ester (TMRM), a lipophilic cation that accumulates selectively within polarized mitochondria. The extent

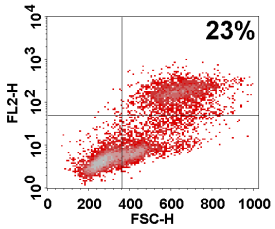
of its uptake, as measured by intensity of cellular TMRM red fluorescence, is proportional to mitochondrial function.[135] Figure 2.16 illustrates representative two-dimensional density dot plots of TMRM-stained lymphocyte cells showing the percentage of cells with intact $\Delta\psi_m$ (TMRM fluorescence in top right quadrant) vs the percentage of cells with depolarized $\Delta\psi_m$ (TMRM fluorescence in bottom left and right quadrants). Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), a commonly used uncoupler of oxidative phosphorylation in mitochondria, was employed as a positive control to dissipate the chemiosmotic proton gradient, which results in lowering of TMRM fluorescence as a result of the depolarization of mitochondrial inner membrane potential. Table 2.4 summarizes the percentage of FRDA lymphocytes with intact $\Delta\psi_m$ in the flow cytometric dot plot profiles. Treatment with 5 mM DEM resulted in a decrease in the percentage of cells with TMRM fluorescence in the top right quadrant, indicating depolarization of $\Delta\psi_m$ upon DEM treatment. Compounds that suppress ROS and lipid peroxidation may be expected to preserve mitochondrial membrane potential under conditions of induced oxidative stress. Compound **2.1**, having the idebenone side chain, was the least active of the pyridinols in this assay, consistent with the ROS and lipid peroxidation results. Again, the analogues with longer side chains (**2.11** and **2.12**), compound with phytyl type side chain (**2.18**) and bicyclic pyridinols (**2.24** and **2.25**) were also less effective in preventing mitochondrial depolarization under oxidative stress. The regioisomeric pair with 10, 13, 15 and 16 carbon atoms chain length (**2.3**–**2.10**) again conferred the good protection, and acted in a dose-dependent manner (Figure 2.16). The azetidine analogues (**2.19** and **2.20**) and morpholine analogues (**2.22** and **2.23**) were slightly less effective in preserving $\Delta\psi_m$ at lower concentration than the

other hexadecyl side chain analogues (**2.9** and **2.10**), giving results similar to the ROS and lipid peroxidation assays.

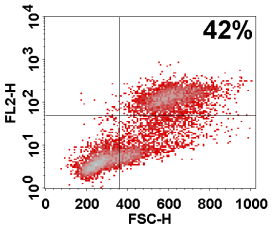



2.6 (100 nM)
55%
2.6 (250 nM)
75%
2.7 (100 nM)
56%
2.7 (250 nM)
81%
2.7 (500 nM)
87%
2.8 (100 nM)
65%
2.8 (250 nM)
76%
2.8 (500 nM)
89%
2.9 (100 nM)
67%
2.9 (250 nM)
84%
2.9 (500 nM)
92%
2.10 (100 nM)
62%
2.10 (250 nM)
83%
2.10 (500 nM)
91%
2.11 (100 nM)
24%
2.11 (250 nM)
44%
2.11 (500 nM)
65%
FL2-H
FSC-H

2.12 (100 nM)



2.12 (250 nM)



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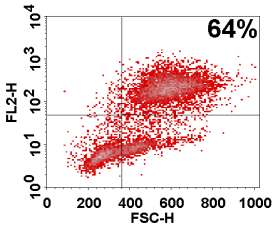
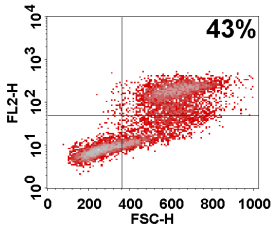
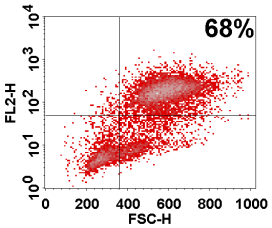


2.13 (100 nM)



2.13 (250 nM)



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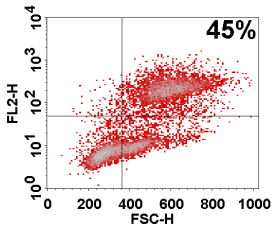
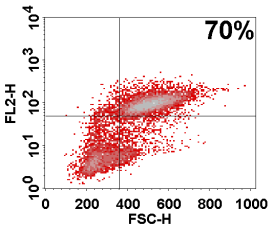
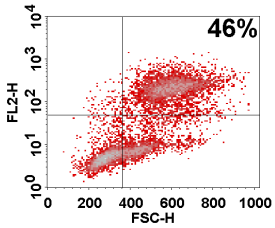


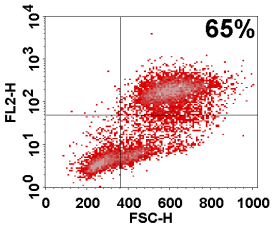
2.14 (250 nM)



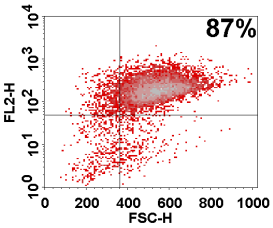
2.15 (100 nM)



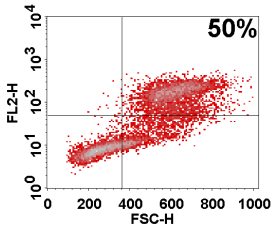
2.15 (250 nM)



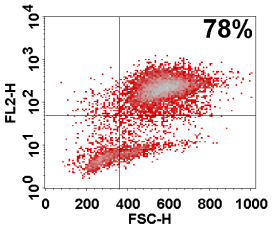
2.15 (500 nM)



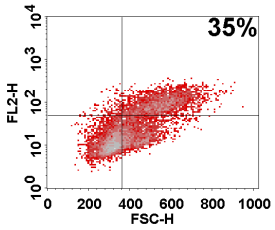
2.16 (100 nM)



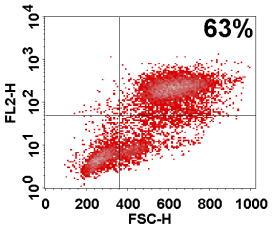
2.16 (250 nM)

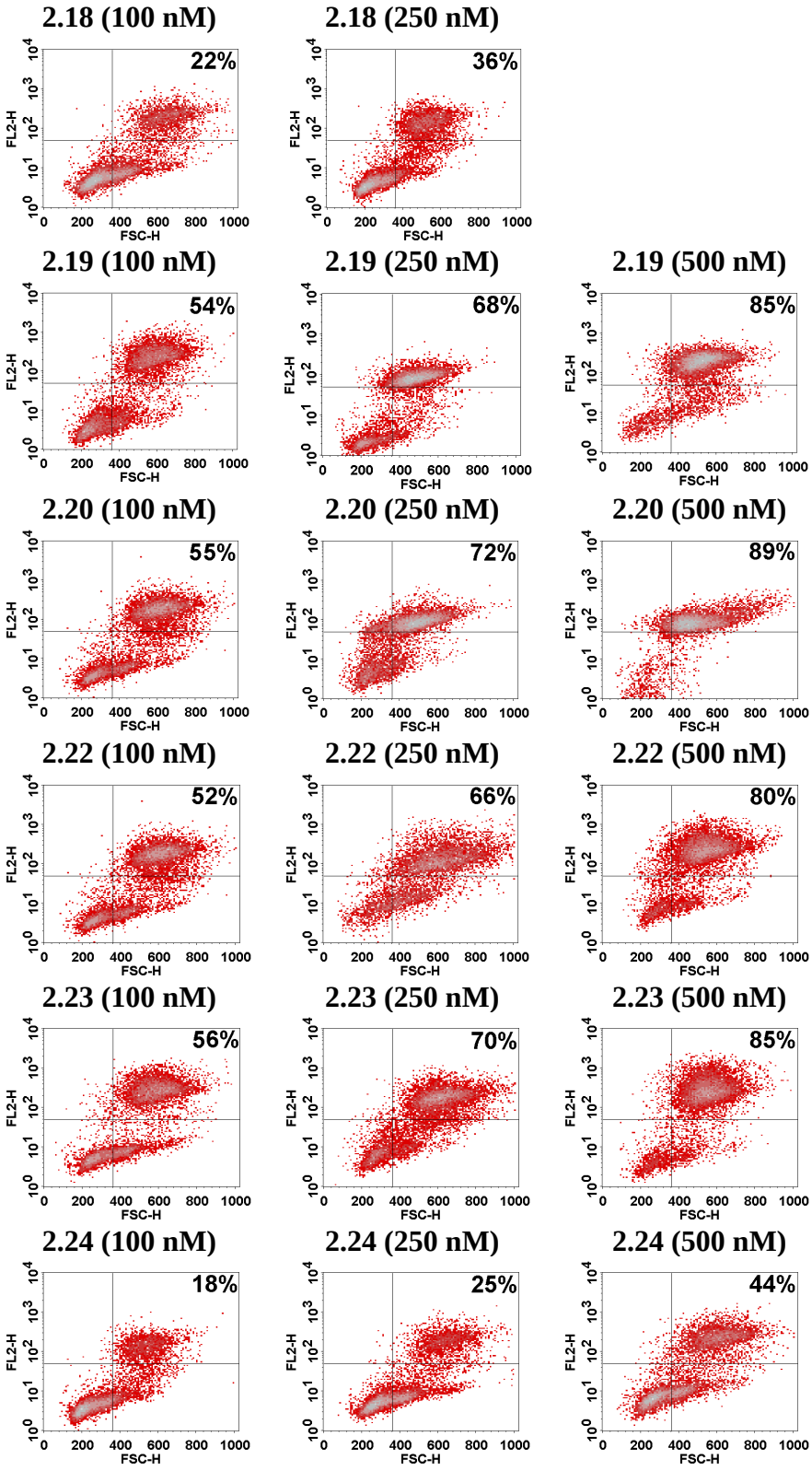


2.17 (100 nM)



2.17 (250 nM)




2.18 (100 nM)
22%
2.18 (250 nM)
36%
2.19 (100 nM)
54%
2.19 (250 nM)
68%
2.19 (500 nM)
85%
2.20 (100 nM)
55%
2.20 (250 nM)
72%
2.20 (500 nM)
89%
2.22 (100 nM)
52%
2.22 (250 nM)
66%
2.22 (500 nM)
80%
2.23 (100 nM)
56%
2.23 (250 nM)
70%
2.23 (500 nM)
85%
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18%
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25%
2.24 (500 nM)
44%
FL2-H
FSC-H

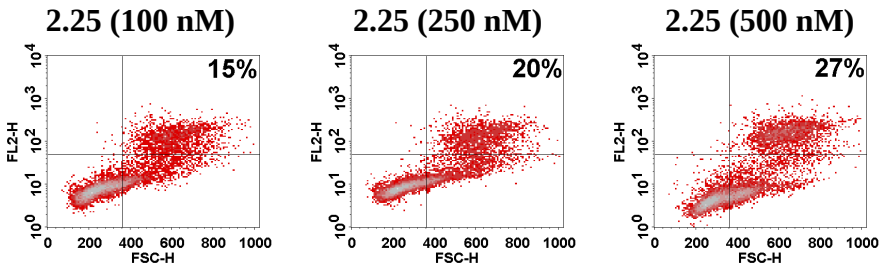


Figure 2.16. Representative Flow Cytometric Two-dimensional Color Density Dot Plot Analyses of the Ability of Compounds to Maintain Mitochondrial Membrane Potential ($\Delta\psi_m$) in DEM-treated FRDA Lymphocytes Cells Stained With 250 nM TMRM and Analyzed Using the FL2-H Channel as Described in the Experimental Section. The Percentage of Cells With Intact $\Delta\psi_m$ is Indicated in the Top Right Quadrant of Captions. Representative Examples From at Least two/three Independent Experiments are Shown. A Total of 10,000 Events Were Recorded for Each Sample and Analyzed With the CellQuest Software (BD Biosciences). The Experiment was Performed by Dr. Omar M. Khdour.

Table 2.4. Pyridinol Antioxidants Preserve Mitochondrial Membrane Potential ($\Delta\psi_m$) in Cultured FRDA Lymphocytes Pretreated with DEM. The Experiment was Performed by Dr. Omar M. Khdour.

	% of cells with intact $\Delta\psi_m$		
Compound	100 nM	250 nM	500 nM
Control	94 ± 2	94 ± 2	94 ± 2
DEM	13 ± 4	13 ± 4	13 ± 4
FCCP	20 ± 6	20 ± 6	20 ± 6
2.1	23 ± 2	38 ± 3	49 ± 2
2.3	63 ± 3	84 ± 2	89 ± 2
2.4	58 ± 3	77 ± 2	89 ± 2
2.5	61 ± 4	79 ± 2	–
2.6	54 ± 2	77 ± 3	–
2.7	59 ± 4	78 ± 2	86 ± 2
2.8	63 ± 4	75 ± 1	88 ± 1
2.9	69 ± 2	83 ± 2	91 ± 1
2.10	61 ± 2	81 ± 4	90 ± 2
2.11	24 ± 2	45 ± 4	–
2.12	22 ± 3	43 ± 2	–
2.13	46 ± 4	70 ± 2	–
2.14	48 ± 4	72 ± 3	–
2.15	44 ± 3	67 ± 3	84 ± 5
2.16	49 ± 2	77 ± 2	–
2.17	38 ± 4	66 ± 4	–
2.18	20 ± 4	33 ± 4	–
2.19	62 ± 2	74 ± 3	85 ± 3
2.20	60 ± 3	80 ± 3	87 ± 3
2.21	38 ± 6	49 ± 3	67 ± 3
2.22	54 ± 3	69 ± 4	83 ± 4
2.23	57 ± 1	73 ± 4	86 ± 1
2.24	15 ± 5	20 ± 3	42 ± 4
2.25	13 ± 4	18 ± 4	25 ± 5

2.2.2.5. Cellular ATP Levels

There is currently no curative treatment for patients with mitochondrial diseases. Current goals for mitochondrial disease therapy are focused on increasing production of ATP and reducing reactive oxygen species levels in an effort to improve, or at least

stabilize, disease signs and symptoms. A nutrient-sensitized screening strategy to identify CoQ_{10} analogues that function within the mitochondrial respiratory chain and augment ATP was used as described before.[117,118] The intracellular ATP level was measured in glucose-free media. The cells were grown on galactose-containing media to maximize ATP production via oxidative phosphorylation, and they become more sensitive to mitochondrial respiratory chain inhibitors than cells grown on glucose medium.[136,137] As shown in Table 2.5, 5 μM concentration of compounds **2.7**–**2.12**, **2.18**, **2.19** and **2.20** had minimal effects on the cellular ATP concentrations. As expected, the ATP levels diminished at higher concentrations of the test compounds (10 and 25 μM), consistent with their inhibition of NADH oxidase activity (Table 2.1). Compound **2.1**, having a hydroxydecyl side chain, strongly diminished ATP levels in a concentration-dependent fashion in the CoQ_{10} deficient lymphocytes. Compounds **2.3** and **2.4**, which differ from compound **2.1** only by the absence of the side chain OH group, reduced ATP levels by approximately 10-15% at 5 μM concentration compared to the control, and essentially completely depleted intracellular ATP level at 25 μM concentration. Compound **2.5**, with 13 carbon atom side chain showed favorable response at 10 μM concentration (~20% reduction in ATP levels) over compounds **2.3** and **2.4**, whereas its regioisomer **2.6** did not show similar efficacy. Increasing the side chain length to 15 and 16 carbon atoms (**2.7**–**2.10**) showed a favorable response in CoQ_{10}-deficient lymphocytes at 5 μM concentration (no diminution beyond the basal level) but did diminish ATP levels at higher concentrations. Similar patterns were observed with compounds having exocyclic azetidine group (**2.19** and **2.20**) and morpholine group (**2.22** and **2.23**), but they were slightly less effective than **2.9** and **2.10**. In the case of compounds with a longer (19

carbon atom) side chain (**2.11** and **2.12**) and compound **2.18**, ATP levels did not diminish significantly even at higher concentrations. This may be explained by their limited interaction with the mitochondrial respiratory chain, as noted from the NADH oxidase assay (Table 2.1).

Table 2.5. Total ATP Concentration in CoQ_{10} Deficient Lymphocytes Following Incubation With Pyridinol Antioxidants for 48 h. The Experiment was Performed by Dr. Omar M. Khdour.

	Total ATP level (% control) in CoQ_{10} deficient lymphocytes		
Compound	5 µM	10 µM	25 µM
2.1	50 ± 4	20 ± 2	3 ± 1
2.3	89 ± 2	52 ± 4	13 ± 9
2.4	87 ± 2	40 ± 3	2 ± 1
2.5	92 ± 4	81 ± 4	31 ± 5
2.6	75 ± 6	24 ± 1	3 ± 1
2.7	98 ± 2	78 ± 5	45 ± 3
2.8	95 ± 3	79 ± 5	48 ± 2
2.9	100 ± 3	79 ± 6	41 ± 6
2.10	96 ± 1	80 ± 3	37 ± 1
2.11	102 ± 3	102 ± 4	99 ± 3
2.12	106 ± 6	103 ± 6	86 ± 3
2.13	83 ± 3	74 ± 1	39 ± 1
2.14	77 ± 1	69 ± 2	17 ± 1
2.16	63 ± 6	54 ± 3	35 ± 2
2.17	23 ± 9	8 ± 4	2 ± 1
2.18	108 ± 6	102 ± 2	89 ± 2
2.19	92 ± 6	70 ± 5	38 ± 4
2.20	98 ± 3	76 ± 4	50 ± 3
2.22	93 ± 4	75 ± 4	40 ± 2
2.23	95 ± 3	77 ± 5	45 ± 3

The compounds in the pyrimidinol series (**2.30**–**2.32**) reliably increased the ATP levels in cultured FRDA lymphocytes (Table 2.6), similar to previously reported studies on other pyrimidinol analogues.[117,118] These three compounds augmented ATP levels and the increase was similar to lead compound **2.2** at 1 µM and 5 µM concentrations. Also,

they diminished ATP levels less at a high concentration (20 μM), whereas **2.2** diminished the ATP levels by ~20% at the same concentration.

Table 2.6. Total ATP Concentration in FRDA Lymphocytes Following Incubation With Pyrimidinol Antioxidants for 48 h. The Experiment was Performed by Dr. Omar M. Khdour.

	Total ATP level (% control) in FRDA lymphocytes		
Compound	1 μM	5 μM	20 μM
2.2	111 ± 3	107 ± 5	82 ± 2
2.30	101 ± 2	111 ± 3	93 ± 3
2.31	107 ± 3	108 ± 3	93 ± 5
2.32	103 ± 3	110 ± 4	99 ± 6

2.2.2.6. Cytoprotection

The ability of the test compounds (**2.1**–**2.25**) to confer cytoprotection was evaluated using six different lymphocyte cell lines, from individuals with Leigh's syndrome, FRDA, Leber's syndrome, Alzheimer's disease, Parkinson's disease and early-onset morbid obesity.[117] Lymphocytes were treated with diethyl maleate to induce oxidative stress through depletion of glutathione. The cytoprotection results obtained with various cultured cells are shown in Tables 2.7–2.12. The cytoprotective effects of all of the compounds were dose dependent in the concentration range from 0.1 μM to 2.5 μM in all six cell lines. None of the compounds provided significant cytoprotection from DEM-induced stress when employed at 0.1 μM concentration in Leigh's lymphocytes (Table 2.7). However, all but five of the compounds (**2.11**, **2.12**, **2.18**, **2.24** and **2.25**) provided significant (> 75%) cytoprotection when employed at 2.5 μM concentration in Leigh's lymphocytes. The removal of the side chain OH group from compound **2.1**

(affording compound **2.3**) resulted in a substantial improvement in the ability of the compound to confer cytoprotection when employed at 0.5 μM concentration. The same improvement in cytoprotection was obtained for isomeric compound **2.4**. Compounds **2.5** and **2.6** (with 13 carbon atoms side chain length), and compounds **2.7** and **2.8** (with 15 carbon atoms side chain length) did not show any improvement in cytoprotection over compound **2.4**. Increasing the side chain length (from 10 to 16 carbon atoms) in compounds **2.9** and **2.10** gave slightly improved cytoprotection (relative to **2.3**) only at 0.1 μM concentration. A further increase in side chain length (to 19 carbon atoms; **2.11** and **2.12**) significantly lowered cytoprotective activity, consistent with previously published results for other pyrimidinol analogues.[118] Lesser cytoprotective activity was observed for compounds **2.13**–**2.17** having modified side chains. It is interesting that compounds **2.19**, **2.20, 2.22** and **2.23**, having the same 16-carbon side chain as in compounds **2.9** and **2.10**, but with an exocyclic azetidine or morpholine substituent, exhibited comparable cytoprotection. The bicyclic compounds (**2.24** and **2.25**) failed to show any significant cytoprotection even at higher concentration.

Table 2.7. Cytoprotective Effects of CoQ_{10} Analogues on the Viability of Cultured Leigh's Syndrome Lymphocytes Treated With DEM. The Experiment was Performed by Walter G. Johnson.

	Viable cells (%)		
Compound	0.1 μM	0.5 μM	2.5 μM
2.1	20 ± 1	36 ± 6	74 ± 4
2.3	6 ± 2	77 ± 3	79 ± 3
2.4	19 ± 4	87 ± 4	86 ± 4
2.5	20 ± 5	75 ± 2	78 ± 1
2.6	17 ± 5	74 ± 2	76 ± 2
2.7	9 ± 2	79 ± 5	84 ± 1
2.8	8 ± 2	79 ± 3	85 ± 1
2.9	29 ± 1	78 ± 2	83 ± 4
2.10	29 ± 1	76 ± 1	84 ± 2
2.11	9 ± 3	10 ± 2	9 ± 1
2.12	9 ± 1	15 ± 5	61 ± 7
2.13	8 ± 1	13 ± 4	81 ± 1
2.14	15 ± 2	45 ± 19	79 ± 3
2.15	17 ± 4	88 ± 4	92 ± 2
2.16	6 ± 3	82 ± 3	78 ± 4
2.17	15 ± 1	77 ± 1	80 ± 5
2.18	5 ± 1	11 ± 1	10 ± 1
2.19	13 ± 2	82 ± 1	81 ± 2
2.20	18 ± 4	95 ± 3	92 ± 2
2.22	12 ± 2	86 ± 4	89 ± 4
2.23	14 ± 4	85 ± 9	94 ± 4
2.24	8 ± 1	9 ± 1	27 ± 4
2.25	12 ± 3	8 ± 2	10 ± 2

In FRDA lymphocyte cells, compounds **2.5–2.10**, **2.15**, **2.19**, **2.20**, **2.22** and **2.23** retained very good (>50%) potency even when tested at 0.1 μM concentration (Table 2.8). All of the test compounds were found to maintain cell viability to the extent of >85% when tested at 2.5 μM concentration in FRDA lymphocytes with the exception of compound **2.11**. All but four of the compounds (**2.1**, **2.11, 2.12** and **2.19**) afforded at least >75% cytoprotection when tested at 0.5 μM concentration in FRDA lymphocytes. Compounds **2.30**–**2.32** in the pyrimidinol series exhibited comparable cytoprotection as

the previously reported compound **2.2** at 0.5 µM and 2.5 µM concentrations. Compounds **2.30** and **2.31** conferred >90% cytoprotection at 0.5 µM and 2.5 µM concentrations. Interestingly, these three pyrimidinol analogues (**2.30**–**2.32**) were less protective when employed at 0.1 µM concentration as compared to **2.2**.[117,118]

Table 2.8. Cytoprotective Effects of CoQ_{10} Analogues on the Viability of Cultured FRDA Lymphocytes Treated With DEM. The Experiment was Performed by Basab Roy, Dr. Yana Chen and Chenhong Tang.

	Viable cells (%)		
Compound	0.1 µM	0.5 µM	2.5 µM
2.1	17 ± 1	28 ± 2	87 ± 5
2.2	68 ± 1	88 ± 3	95 ± 2
2.3	36 ± 7	74 ± 1	87 ± 3
2.4	34 ± 2	84 ± 7	86 ± 1
2.5	71 ± 5	81 ± 4	94 ± 2
2.6	67 ± 4	79 ± 4	92 ± 3
2.7	58 ± 2	75 ± 2	85 ± 2
2.8	67 ± 4	83 ± 4	89 ± 2
2.9	80 ± 10	91 ± 5	92 ± 3
2.10	52 ± 4	75 ± 2	84 ± 2
2.11	17 ± 15	38 ± 10	76 ± 13
2.12	20 ± 7	50 ± 4	87 ± 3
2.15	53 ± 6	80 ± 5	96 ± 2
2.19	54 ± 2	70 ± 2	87 ± 2
2.20	58 ± 3	79 ± 2	92 ± 3
2.22	51 ± 1	76 ± 5	91 ± 2
2.23	65 ± 5	74 ± 2	93 ± 3
2.30	21 ± 10	94 ± 5	95 ± 4
2.31	49 ± 10	90 ± 13	92 ± 6
2.32	30 ± 3	77 ± 10	86 ± 15

The cytoprotection results obtained with cultured Leber's lymphocytes are shown in Table 2.9. None of the compounds afforded >90% cytoprotection at any tested concentration, but three compounds (**2.10**, **2.19** and **2.20**) did give 88, 85 and 84%

protection, respectively, when employed at 2.5 μM concentration. Of the three, compound **2.20** gave 77% cytoprotection when used at 0.5 μM concentration, while the comparable values for compounds **2.10** and **2.19** were only 57% and 23%, respectively. Seven compounds (**2.5**–**2.8**, **2.15**, **2.22** and **2.23**) gave >70% protection from induced oxidative stress when used at 2.5 μM concentration, and all of these compounds also afforded >55% cytoprotection when tested at 0.5 μM concentration. Compound **2.21** was less effective, although its difference from **2.23** was less pronounced than in some other assays (*vide infra*). Compounds **2.24** and **2.25** were essentially inactive as cytoprotectants. None of the tested compounds afforded reasonable protection when used at 0.1 μM concentration.

Table 2.9. Cytoprotective Effects of CoQ_{10} Analogues on the Viability of Cultured Leber's Lymphocytes Treated With DEM. The Experiment was Performed by Walter G. Johnson and Dr. Darshini Patel.

	Viable cells (%)		
Compound	0.1 μM	0.5 μM	2.5 μM
2.5	22 ± 2	65 ± 6	73 ± 4
2.6	26 ± 5	67 ± 1	78 ± 4
2.7	14 ± 1	62 ± 5	76 ± 5
2.8	17 ± 3	68 ± 4	78 ± 2
2.10	24 ± 3	57 ± 5	88 ± 2
2.15	31 ± 3	67 ± 2	77 ± 3
2.19	16 ± 5	23 ± 6	85 ± 3
2.20	23 ± 2	77 ± 8	84 ± 4
2.21	45 ± 1	55 ± 4	68 ± 4
2.22	20 ± 2	69 ± 5	76 ± 4
2.23	18 ± 2	64 ± 4	74 ± 4
2.24	15 ± 2	13 ± 4	8 ± 3
2.25	7 ± 3	8 ± 2	16 ± 6

The cytoprotective effects of pyridinol analogues for Alzheimer's disease lymphocytes are summarized in Table 2.10. Five compounds afforded at least 90% cytoprotection when employed at 2.5 μM concentration. These included **2.5**, **2.6**, **2.15**, **2.20** and **2.23**. At this concentration three other compounds (**2.10**, **2.19** and **2.22**), exhibited 80-90% cytoprotection, while two compounds (**2.7** and **2.8**) gave >70% protection. All of the compounds exhibited dose dependent cytoprotection, and all retained significant activity even at 0.1 μM concentration.

Table 2.10. Cytoprotective Effects of CoQ_{10} Analogues on the Viability of Cultured Alzheimer's Disease Lymphocytes Treated With DEM. The Experiment was Performed by Basab Roy.

	Viable cells (%)		
Compound	0.1 μM	0.5 μM	2.5 μM
2.5	61 ± 4	73 ± 5	95 ± 2
2.6	55 ± 2	66 ± 5	92 ± 4
2.7	34 ± 2	56 ± 4	72 ± 4
2.8	40 ± 1	51 ± 4	76 ± 2
2.10	51 ± 2	76 ± 2	87 ± 2
2.15	50 ± 5	62 ± 3	90 ± 2
2.19	48 ± 4	71 ± 1	89 ± 3
2.20	53 ± 5	75 ± 4	93 ± 3
2.22	35 ± 4	68 ± 5	87 ± 3
2.23	31 ± 3	64 ± 6	90 ± 1

Very favorable results were obtained using Parkinson's disease lymphocytes, as summarized in Table 2.11. All but two of the compounds (**2.21** and **2.25**) gave >90% cytoprotection when tested at 2.5 μM concentration. It may be noted that this was the only cell protection assay in which **2.24** gave favorable results, and even then only at a single concentration. All but four of the compounds (**2.19**, **2.21**, **2.24** and **2.25**) also afforded >90% cytoprotection when tested at 0.5 μM concentration. Five compounds

(**2.5**, **2.8**, **2.10**, **2.20** and **2.23**) retained very good (>70%) potency even when tested at 0.1 μM concentration. Finally, it is worth noting the large different in efficacy at all three tested concentrations between **2.21** and its close structural analogue **2.23**.

Table 2.11. Cytoprotective Effects of CoQ_{10} Analogues on the Viability of Cultured Parkinson's Disease Lymphocytes Treated With DEM. The Experiment was Performed by Chenhong Tang.

	Viable cells (%)		
Compound	0.1 μM	0.5 μM	2.5 μM
2.5	75 ± 11	93 ± 1	91 ± 4
2.6	57 ± 14	93 ± 2	95 ± 2
2.7	49 ± 7	93 ± 4	98 ± 1
2.8	82 ± 9	90 ± 9	96 ± 4
2.10	77 ± 12	94 ± 5	97 ± 3
2.15	54 ± 23	95 ± 3	98 ± 5
2.19	30 ± 6	83 ± 5	98 ± 5
2.20	70 ± 10	96 ± 3	101 ± 3
2.21	18 ± 14	40 ± 27	46 ± 26
2.22	68 ± 13	97 ± 2	95 ± 2
2.23	78 ± 9	97 ± 2	98 ± 3
2.24	26 ± 8	52 ± 8	93 ± 4
2.25	21 ± 11	28 ± 10	72 ± 18

The cytoprotection results obtained with cultured early-onset morbid obesity lymphocytes are summarized in Table 2.12. Three of the compounds (**2.6**, **2.22** and **2.23**) afforded at least 90% cytoprotection from stress induced by diethyl maleate, and the remaining compounds provided at least 84% cytoprotection when used at 2.5 μM concentration. Further, with the exception of **2.19**, their cytoprotective efficacy was largely retained at 0.5 μM concentration. All of the compounds exhibited <40% cytoprotection when tested at 0.1 μM concentration.

Table 2.12. Cytoprotective Effects of CoQ_{10} Analogues on the Viability of Cultured Early-onset Morbid Obesity Lymphocytes Treated With DEM. The Experiment was Performed by Dr. Yana Chen.

	Viable cells (%)		
Compound	0.1 μM	0.5 μM	2.5 μM
2.5	34 ± 13	86 ± 4	86 ± 6
2.6	21 ± 5	82 ± 1	90 ± 2
2.7	36 ± 9	84 ± 1	89 ± 1
2.8	32 ± 7	83 ± 2	85 ± 4
2.10	22 ± 4	86 ± 1	89 ± 2
2.15	31 ± 10	83 ± 1	87 ± 3
2.19	30 ± 4	55 ± 2	84 ± 4
2.20	38 ± 5	82 ± 5	86 ± 3
2.22	28 ± 1	88 ± 3	95 ± 1
2.23	27 ± 12	87 ± 9	90 ± 3

2.3. Discussion

As part of the ongoing efforts to identify compounds having potential utility in treating neurodegenerative and mitochondrial disorders, a number of pyridinol and pyrimidinol analogues have been prepared. The synthetic route employed for the preparation of the new pyridinol analogues is different, and considerably more efficient, than that used in previously reported studies.[116] The original scheme was devised in the belief that selective alkylation of the intermediate **2.35** could be effected to obtain analogue **2.3** selectively. But in the event of alkylation using TMEDA and *n*-BuLi a pair of regioisomers was obtained in rather low yield; in some of the cases only one of the isomer was obtained, also in poor yield. Several mixtures of bases were tried for alkylation along with different reaction conditions, such as increasing the number of equivalence of *n*-BuLi and using a 10 M *n*-BuLi solution but none gave the desired results. Only Schlosser's super base[120] (a mixture of KO*t*Bu and *n*-BuLi) gave improved

yields, although a pair of regioisomers was formed; those were readily separated on a silica gel column (Schemes 2.1–2.5). For the synthesis of analogues **2.13**, **2.14**, **2.16** and **2.17** modified alkyl side chains were synthesized using Grubb's metathesis[121] of 11-bromo-1-undecene and the corresponding alkene followed by hydrogenation. Compound **2.18** was synthesized by the alkylation of **2.35** using TMEDA and *n*-BuLi. It was anticipated that the introduction of a phytyl side chain could facilitate the transport of **2.18** into the cells by α-tocopherol transfer protein (αTTP); instead, **2.18** turned out to be inhibitory to the ETC.[138]

In order to synthesize pyridinol analogues with exocyclic amino groups a different strategy was employed, involving the synthesis of fully protected heterocyclic cores for each of the analogues prior to the alkylation reaction with Schlosser's super base[120] (Schemes 2.6–2.8). The protected heterocyclic core was synthesized by the C-N coupling of a cyclic amine with the benzyl protected 6-iodopyridinol.[126] The synthesis of the 6-iodo pyridinol was troublesome. Several methods and reagents were tried (e.g. NIS in AcOH) but only a morpholine–iodine complex[124,125] gave the desired product in moderately good yield.

The synthesis of bicyclic pyridinols started with a coupling reaction between potassium phthalamide and 2-bromopropionate (Scheme 2.9). Rearrangement of **2.67** to afford **2.68** was very challenging. Several attempts were made to isolate the product via re-precipitation but failed. Finally, reaction condition and re-precipitation was optimized to afford **2.68** in better yield.[127,128] Chlorination of **2.69** was tried with PCl_5 in CCl_4 at reflux, neat $POCl_3$ at reflux, $POCl_3$ in CH_2Cl_2 at reflux, $POCl_3$ in CH_2Cl_2 and TEA at reflux but all of these resulted in degradation of the starting material. The reaction was

performed successfully in a sealed tube at 90 °C using one equivalent of $POCl_3$ to afford **2.70** in moderate yield.[129] Several attempts were made to introduce the dimethylamine functionality by using dimethylamine in aqueous solution. Treatment of **2.70** with DMF under microwave conditions at 180 °C resulted in degradation of the starting material. The use of $NiCl_2$ as a catalyst also failed to help significantly. Finally, catalytic cross-coupling of halide **2.70** with dimethylamine hydrochloride using tris(dibenzylideneacetone)dipalladium (0) ($Pd_2(dba)_3$), 1,3-bis(2,6-diisopropylphenyl)-imidazolium chloride ($ImPrPh_2$•HCl) and KO*t*Bu afforded **2.71** in reasonably good yield (Scheme 2.9).[126] Debenzylation to afford the final product proceeded readily but the product was unstable toward silica gel and hence purification was difficult. HPLC was used to purify the sample after several failed attempts using silica gel chromatography. Elution with 0.1% TFA and MeOH afforded pure compounds **2.24** and **2.25**, although in poor yields.

For the synthesis of compound **2.26**, first 2-amino-4-methoxy-6-methylpyrimidine was transformed to 2-iodo-4-methoxy-6-methylpyrimidne by a Sandmeyer reaction using isoamylnitrite, diiodomethane, iodine and CuI (Scheme 2.10).[130] A cross-coupling reaction of azetidine hydrochloride with the 2-iodopyrimidine failed when $ImPrPh_2$•HCl and $Pd_2(dba)_3$ was used, although, the same combination worked well for the pyridine analogues. So a different ligand, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen), was used in the presence of CuI and Cs_2CO_3 for the cross-coupling reaction to afford the required heterocyclic core, **2.75**, in good yield.[131] The alkylation was performed using *n*-BuLi and 1-bromopentadecane. The alkylated core was brominated using NBS and then transformed to final product using *n*-BuLi,

trimethoxyborane and H_2O_2.[118] Compound **2.27** was made by following the same method as for compound **2.26** (Scheme 2.11), except that in the last step, for the conversion of 5-bromopyrimidine to 5-hydroxypyrimidine, trimethoxyborane was added before *n*-BuLi and stirred together with the 5-bromopyrimidine for 30 min. This was done to avoid the conversion of 5-bromopyrimidine back to the debrominated pyrimidine heterocyclic core (**2.79**).

The synthetic schemes employed for the syntheses of compounds **2.28**–**2.30** were identical (Schemes 2.12–2.14). They started with the treatment of 2,4-dichloro-6-methylpyrimidine with NaH and the corresponding alcohol to form the ether linkage with the pyrimidine core, followed by the catalytic cross coupling of 2-chloro-4-alkoxy-6-methylpyrimidine using azetidine hydrochloride, CuI, 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4Phen) and Cs_2CO_3 to afford the desired heterocyclic core.[131] Alkylation was performed on **2.82** and **2.87** using *n*-BuLi and 1-bromopentadecane but not on **2.85**, as it already had the long chain in the ether linkage with the heterocyclic core. The obtained intermediates were brominated using NBS and then transformed to final products using *n*-BuLi and subsequent quick addition of trimethoxyborane followed by treatment with H_2O_2.[118]

For the synthesis of **2.31**and **2.32** the hexadeuterated heterocyclic core (**2.90**) was first synthesized by the treatment of 2-amino-4-chloro-6-methylpyrimidine with CD_3I and NaH in anh THF (Schemes 2.15 and 2.16). Treatment of compound **2.90** with CD_3OD and NaH afforded **2.91**, whereas 1-cyclobutanol and NaH afforded **2.94** in good yield. Alkylation was performed using *n*-BuLi and 1-bromopentadecane. The obtained intermediates were brominated using NBS and then transformed to the final products

using *n*-BuLi and subsequent quick addition of trimethoxyborane followed by treatment with H_2O_2.[118]

As shown in Table 2.2, all of the new pyridinol derivatives have been tested for their ability to quench lipid peroxidation in FRDA lymphocytes following oxidative challenge with diethyl maleate (DEM). DEM reacts chemically (and irreversibly) with glutathione, inactivating it stoichiometrically such that it no longer provides protection against oxidative stress.[115-117,133] As a consequence, ROS levels increase, and in turn lipid peroxidation also increases. The omission of the side chain OH group from **2.1** (affording **2.3**) resulted in a substantial improvement in the ability of the compound to quench lipid peroxidation. The side chain of **2.1**, of course, is the same as that found in idebenone and OH group removal improved the stability of the compound and made them less inhibitory to the ETC. The same improvement in suppression of lipid peroxidation was obtained for isomeric compound **2.4**. Increasing the side chain length (from 10 to 13, 15 and 16 carbon atoms) afforded compounds **2.5**–**2.10**. Compounds **2.7**–**2.10** had somewhat improved activity as well in suppressing lipid peroxidation. A further increase in side chain length as in compound **2.11** and **2.12** significantly lowered activity. The attachment of a cyclohexyl ring to the end of a 12-carbon side chain (**2.13** and **2.14**), affording derivatives in which the side chain extended the same overall length from the redox core as in **2.9**, gave compounds with activities comparable to **2.3** and **2.4**. The analogues having an aromatic ring in place of the cyclohexyl ring in the modified chain (**2.16** and **2.17**) had slightly improved activity, comparable to **2.5** and **2.6**. Interestingly, the introduction of a tetramethylated 17 carbon side chain in the form of a phytyl group (compound **2.18**) dramatically lowered activity, even though no polar functionality was

present. The bicyclic pyridinol analogues **2.24** and **2.25** also failed to show any protection against lipid peroxidation. It may be noted that the concentrations at which the compounds were tested (100 and 250 nM) were rather low and most of these compounds may properly be regarded as capable of potent suppression of lipid peroxidation.

The compounds were also tested for suppression of ROS in FRDA lymphocytes,[134] affording results that fairly closely paralleled those found for lipid peroxidation (Table 2.3). The analogues were tested at low (100, 250 and 500 nM) concentrations; the results reflect quite potent ROS suppression abilities. With the exception of a few analogues (notably **2.1**, **2.11**, **2.12**, **2.18**, **2.24** and **2.25**), the analogues suppressed ROS with potency and efficacy sufficient to consider them as potential therapeutic agents (at least based on this single parameter).

Table 2.4 and Figure 2.16 summarize the ability of the new pyridinol analogues to preserve mitochondrial membrane potential in FRDA lymphocytes placed under oxidative stress. Treatment with DEM reduced the percentage of cells with intact mitochondrial membrane potential from 95% to 6%. Thus the reduction was even greater than the value of 22% achieved with FCCP, the classic uncoupler of oxidative phosphorylation, which dissipates mitochondrial membrane potential. Although most of the analogues have only been tested at two concentrations to date, very good protection was achieved with compounds **2.3**, **2.4**, **2.5**, **2.6**, **2.9** and **2.10**. Compounds with an exocyclic amino group (**2.19**, **2.20**, **2.22** and **2.23**) also conferred protection but to a lesser extent than **2.9** and **2.10**. As in the case of suppression of ROS, analogues **2.1**, **2.11**, **2.12**, **2.18**, **2.24** and **2.25** functioned less well.

Pyridinol analogues that inhibit the mitochondrial respiratory chain may function well as protective agents in the three assays described earlier, but are less likely to be able to confer cytoprotection to cells under oxidative stress, or to augment ATP production in cells with (partially) dysfunctional mitochondria (which is a hallmark of cells from individuals with mitochondrial disorders). While inhibition usually involves mitochondrial complex I, a number of compounds also inhibit complex III. Accordingly, an assay was designed which measures NADH oxidase activity, encompassing complexes I, II and III.[115-118,132] As shown in Table 2.1, some of the compounds tested were substantially inhibitory to NADH oxidase, notably compounds **2.1**, **2.3**, **2.4**, **2.13** and **2.16**. In contrast, much less inhibition was observed for other compounds in the series.

It is important to note that there are special characteristics of this assay that make interpretation somewhat more complicated. First, the assay gives a result that reflects events involving three individual mitochondrial complexes, and undoubtedly reflects both the avidity of individual compounds for individual complexes, as well as the dynamics of interaction with each of the complexes. Second, since the compounds being tested can be both substrates and inhibitors of each of the mitochondrial complexes, the aggregate "readout" can be misleading. Finally, a compound that simply fails to bind to any of the mitochondrial complexes, will show no inhibition of NADH oxidase, but has no chance of augmenting ATP production. Thus any (tentative) conclusions reached from this assay need to be confirmed by measuring the effects of the compounds on each of the complexes individually, and on the ability of the compound to support oxygen reduction in complex IV and ATP production in complex V. It may also be noted that the

concentrations of the compounds employed in Table 2.1 are purposefully higher than those used in the other assays (in order to provide mechanistic insights), but involve at least one concentration (10 μM) unlikely to be achieved physiologically.

Compounds were tested for their ability to augment ATP levels in CoQ_{10} deficient and FRDA lymphocyte cell lines (Tables 2.5 and 2.6). As is usual in this assay, the most favorable results were obtained at the lowest concentration (5 μM in this experiment). The least effective compounds were **2.6** and **2.17**, which reduced the cellular ATP level to 3% and 2% of control, respectively at 25 μM concentration. These compounds were not strongly inhibitory to NADH oxidase activity (Table 2.1), and did not affect mitochondrial membrane potential negatively (Table 2.4), but may possibly interact with mitochondrial complexes II or V. There was not a single compound in the pyridinol series, which was able to augment ATP levels in CoQ_{10} deficient lymphocytes. However, compounds **2.30**, **2.31** and **2.32** in the pyrimidinol series augmented ATP levels in FRDA lymphocytes (Table 2.6). Interestingly, they reduce ATP level by ~10% only even at the higher concentration (20 μM) whereas lead compound **2.2** reduced the ATP level by ~20%.

The six different lymphocyte cell lines, from individuals with Leigh's syndrome, FRDA, Leber's syndrome, Alzheimer's disease, Parkinson's disease and early-onset morbid obesity were used in the assay to access the cytoprotection ability of the analogues (Figures 2.7 to 2.12). Each of the cell lines was derived from lymphocytes obtained from a patient with a specific mitochondrial disorder, or a disease having a significant component of mitochondrial dysfunction. The underlying biochemistry of mitochondrial dysfunction is likely to be quite similar in all of the diseases, the

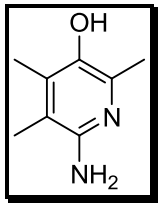
implication of which is that it should in principle be possible to identify a single compound that can be developed for therapeutic intervention in many mitochondrial diseases. The above hypothesis has been tested by investigating few compounds in each of the six cell lines. An earlier study carried out with a limited number of compounds seemed to support this idea.[117] Compounds having favorable properties as multifunctional radical quenchers were evaluated for cytoprotection in each of six cell lines. In comparison with the more limited study carried out previously, a greater spectrum of behavior was found. A number of compounds were found to be fairly effective in all six tested cell lines, while some worked well in few cell lines, but not in others. Interestingly, some cell lines (e.g. Parkinson's disease lymphocytes) were more responsive to the majority of compounds tested than were other cell lines (e.g., Leber's lymphocytes). If this proved to be true for multiple cell lines for specific diseases, it might suggest that the least responsive cells correspond to diseases less likely to be served well by therapeutic agents developed for other mitochondrial disorders.

2.4. Experimental

Anhydrous grade solvents were purchased from Sigma-Aldrich Inc. (St. Louis, MO) and from Fisher Scientific. Most of the chemical reagents were purchased from Sigma-Aldrich and used without further purification. $ImPrPh_2$•HCl, morpholine and iodine were purchased from TCI America. Azetidine hydrochloride, 3,4,7,8-tetramethyl-1,10-phenanthroline and Cs_2CO_3 were purchased from Combi-Blocks. All glassware and needles were pre-dried in an oven at 120 °C prior to use. Tetrahydrofuran was distilled from sodium/benzophenone. All reactions were performed under a stream of argon. Flash

column chromatography was carried out using silica gel (Silicycle R10030B, 60 Å particle size, 230-400 mesh), applying a low pressure stream of nitrogen. Analytical thin layer chromatographic separations were carried out on silica gel (60 Å particle size, 250 μm thickness, F-254, Silicycle) coated glass plates. Spots were visualized with UV light, or developed by using iodine vapor, or by immersing the plates in 2% anisaldehyde in ethanol/sulfuric acid/acetic acid, followed by heating with a heat gun.

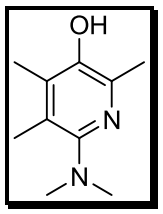
The NMR spectra were recorded using 400 MHz and 500 MHz Varian Inova instruments. Chemical shifts were reported in parts per million (ppm, δ) relative to the residual ^{1}H resonance of the solvent $CDCl_3$ or CD_3OD or DMSO-d_6 at 7.26 ppm, 3.31 ppm or 2.50 ppm, respectively. ^{13}C NMR chemical shifts were reported relative to the central line of $CDCl_3$ or CD_3OD or DMSO-d_6 at 77.16 ppm, 49.00 ppm or 39.52 ppm, respectively. Splitting patterns were designated as follows: s, singlet; br s, broad singlet; d, doublet; t, triplet; m, multiplet; q, quartet; dq, doublet of quartet and quint, quintet. High resolution mass spectra were obtained at the Arizona State University CLAS High Resolution Mass Spectrometry Laboratory.



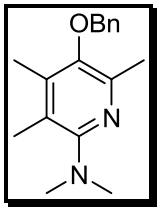
6-Amino-2,4,5-trimethylpyridin-3-ol (2.33).[117,119] To a stirred solution of 20.0 g (97.3 mmol) of pyridoxine hydrochloride in 80 mL of thionyl chloride was added 800 μL of

DMF. The reaction mixture was stirred at reflux for 2 h. The cooled reaction mixture was treated with 60 mL of Et_2O. The suspension was stirred for 1 h and then filtered and the precipitate was washed with 60 mL of Et_2O. The precipitate so obtained was dissolved in 84 mL of glacial acetic acid and 19.0 g (29.1 mmol) of zinc dust was added in three portions. The reaction mixture was stirred at reflux for 2 h. The cooled reaction mixture was filtered and washed with glacial acetic acid. The filtrate was concentrated under diminished pressure and then neutralized with 6 M NaOH. The formed precipitate was filtered and washed with small amount of brine. The orange precipitate obtained was dissolved in 10 M HCl and solid NaCl was added to salt out 12.0 g of crude 2,4,5-trimethylpyridin-3-ol. The obtained solid was dissolved in 400 mL of satd $NaHCO_3$ and freshly prepared diazonium salt, made by slowly mixing 8.00 mL (87.8 mmol) of aniline in 80 mL of 6 M HCl at 0 ^oC with a solution of 6.00 g (88.0 mmol) $NaNO_2$ in 30 mL of H_2O, was added dropwise to the cooled reaction mixture. After 1 h, the red precipitate was filtered and dissolved in 160 mL of 1:1 methanol–formic acid. Zinc dust (28.2 g, 44.0 mmol) was added to the reaction mixture in three portions. The reaction mixture was stirred at reflux for 2 h. The reaction mixture was cooled, filtered and washed with hot MeOH. The MeOH was concentrated and the white precipitate was filtered and washed with Et_2O. The precipitate so obtained was dissolved in hot water, adjusted to pH 8.0 with 6 M NaOH and allowed to cool. The white precipitate was filtered, dissolved in EtOH and again filtered through Celite. The filtrate was concentrated to afford **2.33** as a pale orange solid: yield 3.41 g (23%); mp 169-172 ^oC, lit[119] mp 170-172 ^oC; silica gel TLC R_f 0.20 (9:1 CH_2Cl_2–MeOH); ^1H NMR (DMSO-d_6, 400 MHz) δ 1.88 (s, 3H), 2.00

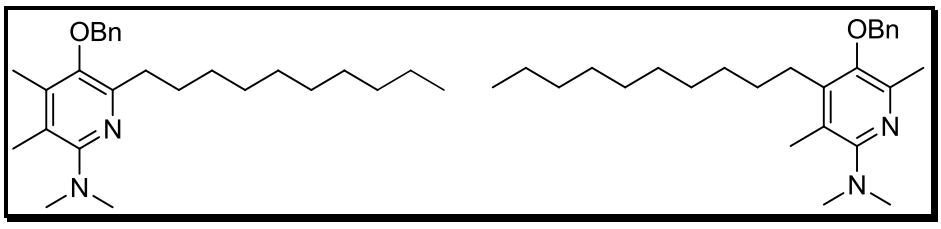
(s, 3H), 2.12 (s, 3H), 4.86 (br s, 2H) and 7.40 (br s, 1H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 12.9, 13.4, 19.4, 112.7, 135.0, 140.2, 141.4 and 151.3.



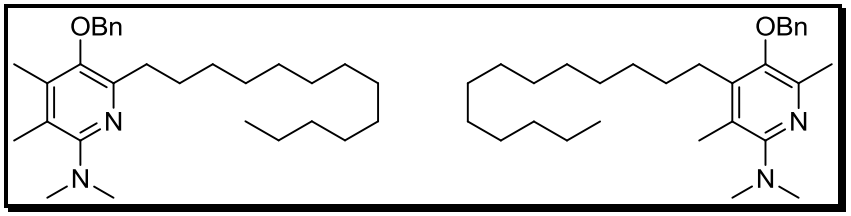
6-(*N*,*N*-Dimethylamino)-2,4,5-trimethylpyridin-3-ol (2.34).[117,119] To a stirred solution containing 1.17g (7.69 mmol) of **2.33** in 30 mL of 1:1 acetonitrile–formalin was added 2.76 g (43.9 mmol) of sodium cyanoborohydride followed by dropwise addition of 1.58 mL (27.6 mmol) of glacial acetic acid at 0 ^{o}C. The reaction mixture was allowed to warm to room temperature and was then stirred for 16 h. The reaction mixture was poured into 200 mL of satd $NaHCO_3$ and extracted with 200 mL of EtOAc. The organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with 3:1 hexane–EtOAc afforded **2.34** as a colorless solid: yield 1.31 g (94%); mp 126-128 °C (lit[119] mp 138-140 ^{o}C); silica gel TLC R_f 0.30 (2:1 hexane–EtOAc); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.15 (s, 3H), 2.17 (s, 3H), 2.33 (s, 3H), 2.65 (s, 6H) and 4.89 (br s, lH); ^{13}C NMR (CD_3OD, 100 MHz) δ 12.8, 14.6, 18.8, 43.3, 124.2, 137.0, 141.6, 146.9 and 156.4; mass spectrum (APCI), *m/z* 181.1336 $(M+H)^+$ ($C_{10}H_{17}N_2O$ requires 181.1341).



3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2,4,5-trimethylpyridine (2.35). To a stirred solution containing 1.30 g (7.21 mmol) of **2.34** in 40 mL of anh THF were added 1.29 mL (10.8 mmol) of benzyl bromide followed by 519 mg (21.6 mmol) of sodium hydride (60% suspension in mineral oil). The reaction mixture was stirred at 23 ^{o}C for 3 h and then poured into 150 mL of water. The mixture was extracted with 300 mL of EtOAc, and the organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 2:1 hexane–EtOAc afforded **2.35** as a yellowish oil: yield 1.40 g (72%); silica gel TLC R_f 0.58 (2:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.19 (s, 3H), 2.20 (s, 3H), 2.45 (s, 3H), 2.77 (s, 6H), 4.75 (s, 2H) and 7.34-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.2, 15.0, 19.5, 42.7, 74.9 121.9, 128.0, 128.2, 128.7, 137.5, 140.5, 145.9, 147.7 and 158.3; mass spectrum (APCI), *m/z* 271.1813 $(M+H)^+$ ($C_{17}H_{23}N_2O$ requires 271.1810).

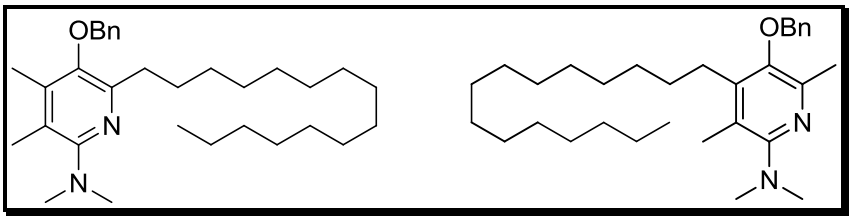


3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2-decyl-4,5-dimethylpyridine (2.36) and 3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-decyl-2,5-dimethylpyridine (2.41).[120] To a stirred solution containing 350 mg (1.29 mmol) of **2.35** and 217 mg (1.93 mmol) of KO*t*Bu in 18 mL of anh THF at –78 °C was added 1.03 mL (2.58 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 295 μL (1.55 mmol) of 1-bromononane was added. The reaction was stirred at 0 ^{o}C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by 96:4 hexane–Et_2O afforded **2.36** and **2.41** as yellowish oils: yields 82.0 mg (16%) and 240 mg (47%), respectively; silica gel TLC R_f 0.53 (4:1 hexane–Et_2O) and R_f 0.43 (4:1 hexane–Et_2O); (**2.36**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (t, 3H, J = 6.8 Hz), 1.25-1.35 (m, 14H), 1.75 (m, 2H), 2.20 (s, 3H), 2.21 (s, 3H), 2.74-2.80 (m, 8H), 4.75 (s, 2H) and 7.34-7.50 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 15.0, 22.8, 28.8, 29.5, 29.78, 29.80, 29.84, 32.06, 32.08, 42.8, 75.4, 121.6, 127.9, 128.1, 128.7, 137.7, 140.3, 147.4, 149.6 and 158.3; mass spectrum (APCI), *m/z* 397.3212 $(M+H)^+$ ($C_{26}H_{41}N_2O$ requires 397.3219); (**2.41**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (t, 3H, J = 7.2 Hz), 1.26-1.46 (m, 14H), 1.56 (m, 2H), 2.27 (s, 3H), 2.51 (s, 3H), 2.66 (m, 2H), 2.81 (s, 6H), 4.82 (s, 2H) and 7.35-7.54 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 14.4, 19.6, 22.8, 27.7, 29.4, 29.5, 29.7, 30.3, 32.0, 42.7, 75.2, 121.3, 127.6, 128.0, 128.6, 137.7, 145.1, 146.0, 147.6 and 158.7; mass spectrum (APCI), *m/z* 397.3226 $(M+H)^+$ ($C_{26}H_{41}N_2O$ requires 397.3219).



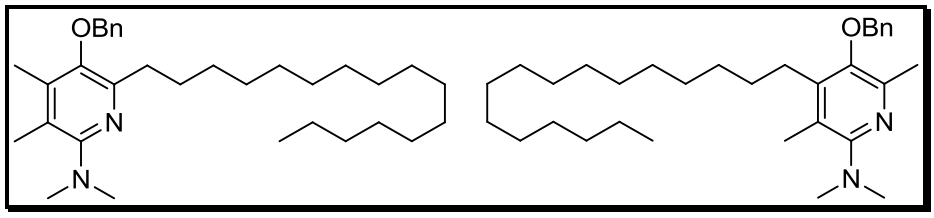
3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2-tridecyl-4,5-dimethylpyridine (2.37) and 3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-tridecyl-2,5-dimethylpyridine (2.42).[120] To a stirred solution containing 300 mg (1.11 mmol) of **2.35** and 187 mg (1.67 mmol) of KO*t*Bu in 18 mL of anh THF at –78 °C was added 888 µL (2.22 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 319 µL (1.33 mmol) of 1-bromododecane was added. The reaction was stirred at 0 ^{o}C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by 96:4 hexane–Et_2O afforded **2.37** and **2.42** as yellowish oils: yields 73.0 mg (15%) and 219 mg (45%), respectively; silica gel TLC R_f 0.59 (4:1 hexane–Et_2O) and R_f 0.50 (4:1 hexane–Et_2O); (**2.37**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, *J* = 7.2 Hz), 1.18-1.40 (m, 20H), 1.75 (m, 2H), 2.19 (s, 3H), 2.20 (s, 3H), 2.72-2.81 (m, 8H), 4.74 (s, 2H) and 7.33-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 15.0, 22.9, 28.8, 29.5, 29.8, 29.82, 29.85, 29.86, 32.06, 32.08, 42.8, 75.5, 121.6, 127.9, 128.1, 128.7, 137.7, 140.4, 147.4, 149.6 and 158.2; mass spectrum (APCI), *m/z* 439.3691 $(M+H)^+$ ($C_{29}H_{47}N_2O$ requires 439.3688); (**2.42**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 6.8 Hz), 1.23-1.34 (m, 20H), 1.49 (m, 2H), 2.21 (s, 3H), 2.45 (s, 3H), 2.59 (m, 2H), 2.76 (s, 6H), 4.76

(s, 2H) and 7.34-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 14.5, 19.6, 22.9, 27.7, 29.5, 29.6, 29.8, 29.81, 29.9, 30.3, 32.1, 42.7, 75.3, 121.4, 127.7, 128.1, 128.7, 137.8, 145.3, 146.1, 147.6 and 158.7; mass spectrum (APCI), *m/z* 439.3697 $(M+H)^+$ ($C_{29}H_{47}N_2O$ requires 439.3688).



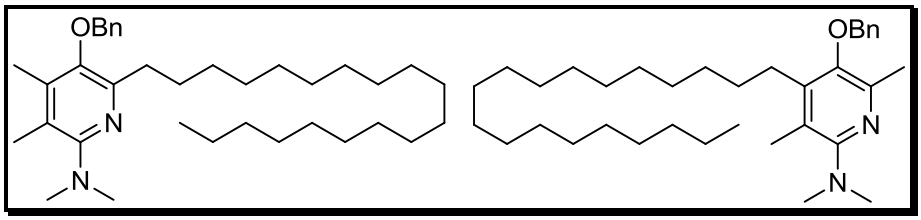
3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2-pentadecyl-4,5-dimethylpyridine (2.38) and 3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-pentadecyl-2,5-dimethylpyridine (2.43).[120] To a stirred solution containing 355 mg (1.31 mmol) of **2.35** and 221 mg (1.97 mmol) of KO*t*Bu in 20 mL of anh THF at –78 °C was added 1.05 mL (2.62 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 468 μL (1.57 mmol) of 1-bromotetradecane was added. The reaction was stirred at 0 ^{o}C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by 96:4 hexane–Et_2O afforded **2.38** and **2.43** as yellowish oils: yields 86.0 mg (14%) and 263 mg (43%), respectively; silica gel TLC R_f 0.67 (4:1 hexane–Et_2O) and R_f 0.55 (4:1 hexane–Et_2O); (**2.38**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.23-1.32 (m, 24H), 1.72 (m, 2H), 2.18 (s, 3H), 2.19 (s, 3H), 2.72-2.81 (m, 8H), 4.73 (s, 2H) and

7.31-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 15.0, 22.9, 28.8, 29.5, 29.8, 29.9, 32.06, 32.1, 42.8, 75.5, 121.6, 127.9, 128.1, 128.7, 137.7, 140.4, 147.4, 149.7 and 158.2; mass spectrum (APCI), *m/z* 467.4009 $(M+H)^+$ ($C_{31}H_{51}N_2O$ requires 467.4001); (**2.43**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.25-1.35 (m, 24H), 1.48 (m, 2H), 2.20 (s, 3H), 2.43 (s, 3H), 2.59 (m, 2H), 2.74 (s, 6H), 4.75 (s, 2H) and 7.34-7.52 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 14.4, 19.6, 22.9, 27.7, 29.53, 29.54, 29.8, 29.81, 29.83, 29.9, 30.3, 32.1, 42.7, 75.2, 121.3, 127.7, 128.1, 128.6, 137.8, 145.2, 146.1, 147.6 and 158.7; mass spectrum (APCI), *m/z* 467.3998 $(M+H)^+$ ($C_{31}H_{51}N_2O$ requires 467.4001).



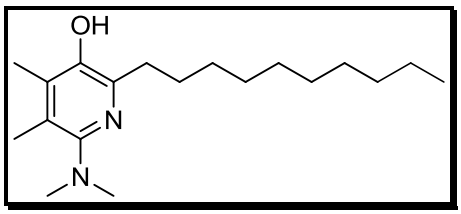
3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2-hexadecyl-4,5-dimethylpyridine (2.39) and 3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-hexadecyl-2,5-dimethylpyridine (2.44).[120] To a stirred solution containing 350 mg (1.29 mmol) of **2.35** and 217 mg (1.93 mmol) of KO*t*Bu in 18 mL of anh THF at –78 °C was added 1.03 mL (2.58 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 449 μL (1.55 mmol) of 1-bromopentadecane was added. The reaction mixture was stirred at 0 ^{o}C for another 30 min, then quenched with satd aq ammonium chloride and then extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was

purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by elution with 96:4 hexane–Et_2O afforded **2.39** and **2.44** as yellowish oils: yields 93.0 mg (15%) and 279 mg (45%), respectively; silica gel TLC R_f 0.54 (4:1 hexane–Et_2O) and R_f 0.44 (4:1 hexane–Et_2O); (**2.39**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 6.8 Hz), 1.23-1.32 (m, 26H), 1.74 (m, 2H), 2.18 (s, 3H), 2.19 (s, 3H), 2.72-2.77 (m, 8H), 4.73 (s, 2H) and 7.32-7.48 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 15.0, 22.9, 28.8, 29.6, 29.82, 29.83, 29.84, 29.9, 32.08, 32.11, 42.8, 75.5, 121.5, 127.9, 128.1, 128.7, 137.7, 140.3, 147.4, 149.6 and 158.2; mass spectrum (APCI), *m/z* 481.4159 $(M+H)^+$ ($C_{32}H_{53}N_2O$ requires 481.4158); (**2.44**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.21-1.38 (m, 26H), 1.49 (m, 2H), 2.21 (s, 3H), 2.44 (s, 3H), 2.59 (m, 2H), 2.75 (s, 6H), 4.76 (s, 2H) and 7.30-7.48 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 14.5, 19.6, 22.9, 27.7, 29.6, 29.77, 29.83, 29.85, 29.89, 30.3, 32.1, 42.7, 75.3, 121.3, 127.7, 128.1, 128.6, 137.8, 145.2, 146.1, 147.7 and 158.7; mass spectrum (APCI), *m/z* 481.4157 $(M+H)^+$ ($C_{32}H_{53}N_2O$ requires 481.4158).

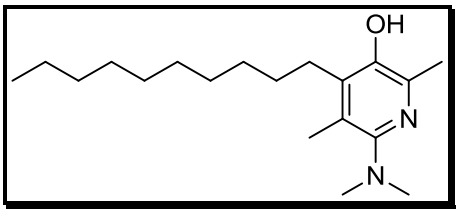


3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2-nonadecyl-4,5-dimethylpyridine (2.40) and 3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-nonadecyl-2,5-dimethylpyridine (2.45).[120] To a stirred solution containing 350 mg (1.29 mmol) of **2.35** and 217 mg (1.93 mmol) of KO*t*Bu in 18 mL of anh THF at –78 °C was added 1.03 mL (2.58 mmol) of a 2.5 M

solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 517 mg (1.55 mmol) of 1-bromooctadecane was added. The reaction mixture was further stirred at 0 ^{o}C for another 30 min, quenched with satd aq ammonium chloride and then extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by elution with 96:4 hexane–Et_2O afforded **2.40** and **2.45** as yellowish oils: yields 74.0 mg (11%) and 223 mg (33%), respectively; silica gel TLC R_f 0.55 (4:1 hexane–Et_2O) and R_f 0.46 (4:1 hexane–Et_2O); (**2.40**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, *J* = 6.8 Hz), 1.24-1.34 (m, 32H), 1.75 (m, 2H), 2.19 (s, 3H), 2.20 (s, 3H), 2.73-2.79 (m, 8H), 4.74 (s, 2H) and 7.32-7.49 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 15.0, 22.9, 28.8, 29.5, 29.80, 29.82, 29.9, 32.07, 32.09, 42.8, 75.4, 121.5, 127.9, 128.1, 128.7, 137.7, 140.3, 147.3, 149.6 and 158.2; mass spectrum (APCI), *m/z* 523.4633 $(M+H)^+$ ($C_{35}H_{59}N_2O$ requires 523.4627); (**2.45**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.94 (t, 3H, *J* = 7.2 Hz), 1.29-1.45 (m, 32H), 1.55 (m, 2H), 2.27 (s, 3H), 2.51 (s, 3H), 2.66 (m, 2H), 2.81 (s, 6H), 4.82 (s, 2H) and 7.34-7.52 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 14.4, 19.6, 22.8, 27.7, 29.5, 29.7, 29.79, 29.80, 29.85, 30.3, 32.1, 42.7, 75.2, 121.2, 127.6, 128.0, 128.6, 137.7, 145.1, 146.0, 147.6 and 158.7; mass spectrum (APCI), *m/z* 523.4623 $(M+H)^+$ ($C_{35}H_{59}N_2O$ requires 523.4627).

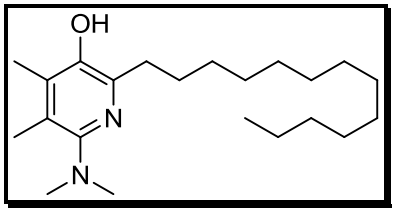


6-*N*,*N*-Dimethylamino-2-decyl-4,5-dimethylpyridin-3-ol (2.3). To a solution containing 82.0 mg (0.21 mmol) of **2.36** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.3** as a colorless oil: yield 52.0 mg (82%); silica gel TLC R_f 0.52 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, J = 6.8 Hz), 1.23-1.35 (m, 14H), 1.68 (m, 2H), 2.12 (s, 3H), 2.17 (s, 3H), 2.61-2.76 (m, 8H) and 4.85 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.5, 12.6, 14.3, 14.7, 22.9, 28.2, 29.5, 29.73, 29.77, 29.82, 32.0, 32.1, 43.1, 122.2, 133.9, 142.4, 144.3 and 155.7; mass spectrum (APCI), *m/z* 307.2743 $(M+H)^+$ ($C_{19}H_{35}N_2O$ requires 307.2749).

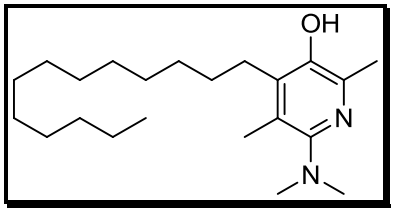


6-*N*,*N*-Dimethylamino-4-decyl-2,5-dimethylpyridin-3-ol (2.4). To a solution containing 220 mg (0.55 mmol) of **2.41** in 10 mL of MeOH was added 15.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$

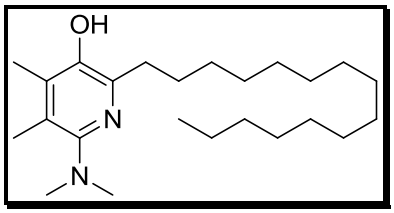
afforded **2.4** as a colorless oil: yield 153 mg (90%); silica gel TLC R_f 0.42 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, *J* = 6.8 Hz), 1.24-1.43 (m, 14H), 1.48 (m, 2H), 2.21 (s, 3H), 2.32 (s, 3H), 2.59 (m, 2H), 2.70 (s, 6H) and 5.32 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 14.2, 18.8, 22.8, 27.2, 28.6, 29.4, 29.6, 29.70, 29.72, 30.1, 32.0, 42.9, 122.1, 139.25, 139.28, 144.6 and 156.1; mass spectrum (APCI), *m/z* 307.2755 $(M+H)^+$ ($C_{19}H_{35}N_2O$ requires 307.2749).



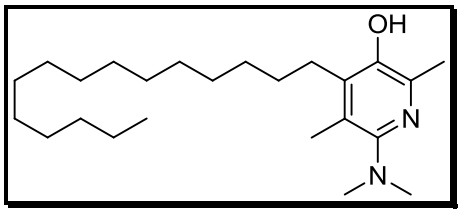
6-*N*,*N*-Dimethylamino-2-tridecyl-4,5-dimethylpyridin-3-ol (2.5). To a solution containing 73.0 mg (0.16 mmol) of **2.37** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.5** as a colorless oil: yield 47.0 mg (86%); silica gel TLC R_f 0.23 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H, *J* = 7.0 Hz), 1.18-1.40 (m, 20H), 1.70 (m, 2H), 2.15 (s, 3H), 2.19 (s, 3H), 2.61-2.79 (m, 8H) and 4.60 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.6, 14.3, 14.7, 22.9, 28.2, 29.5, 29.7, 29.77, 29.82, 29.9, 32.1, 43.1, 122.3, 134.0, 142.3, 144.4 and 155.5; mass spectrum (APCI), *m/z* 349.3225 $(M+H)^+$ ($C_{22}H_{41}N_2O$ requires 349.3219).



6-*N*,*N*-Dimethylamino-4-tridecyl-2,5-dimethylpyridin-3-ol (2.6). To a solution containing 201 mg (0.43 mmol) of **2.42** in 10 mL of MeOH was added 15.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.6** as a colorless oil: yield 134 mg (89%); silica gel TLC R_f 0.26 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.88 (t, 3H, J = 6.8 Hz), 1.23-1.34 (m, 20H), 1.47 (m, 2H), 2.22 (s, 3H), 2.39 (s, 3H), 2.60 (m, 2H), 2.73 (s, 6H) and 4.20 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1, 14.3, 18.7, 22.9, 27.2, 28.7, 29.5, 29.7, 29.71, 29.8, 29.81, 29.83, 30.2, 32.1, 43.1, 122.3, 138.5, 139.0, 144.7 and 153.6; mass spectrum (APCI), *m/z* 349.3214 $(M+H)^+$ ($C_{22}H_{41}N_2O$ requires 349.3219).

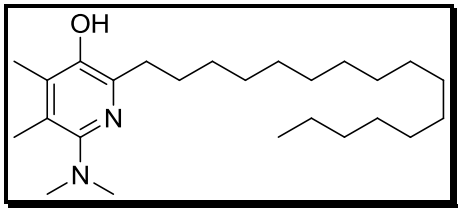


6-*N*,*N*-Dimethylamino-2-pentadecyl-4,5-dimethylpyridin-3-ol (2.7). To a solution containing 80.0 mg (0.17 mmol) of **2.38** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.7** as a colorless oil: yield 56.0 mg (87%); silica gel TLC R_f 0.13 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.89 (t, 3H, J = 7.0 Hz), 1.22-1.34 (m, 24H), 1.70 (m, 2H), 2.15 (s, 3H), 2.19 (s, 3H), 2.64-2.74 (m, 8H) and 4.32 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 12.4, 14.1, 14.5, 22.7, 28.0, 29.4, 29.57, 29.61, 29.67, 29.7, 31.9, 31.93, 42.9, 122.1, 133.6, 142.1, 144.1 and 155.5; mass spectrum (APCI), *m/z* 377.3527 $(M+H)^+$ ($C_{24}H_{45}N_2O$ requires 377.3532).

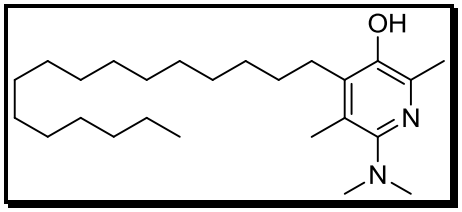


6-*N*,*N*-Dimethylamino-4-pentadecyl-2,5-dimethylpyridin-3-ol (2.8). To a solution containing 251 mg (0.54 mmol) of **2.43** in 10 mL of MeOH was added 20.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$

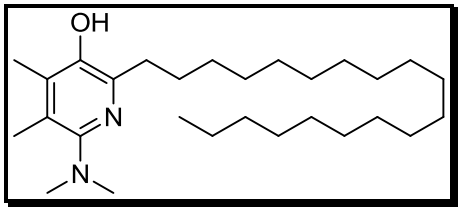
afforded **2.8** as a colorless oil: yield 182 mg (90%); silica gel TLC R_f 0.22 (9:1 hexane–Et_2O); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.23-1.33 (m, 24H), 1.48 (m, 2H), 2.22 (s, 3H), 2.37 (s, 3H), 2.59 (m, 2H), 2.70 (s, 6H) and 4.15 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.3, 18.9, 22.9, 27.2, 28.7, 29.5, 29.7, 29.8, 29.83, 29.85, 30.2, 32.1, 43.1, 122.2, 138.6, 138.7, 144.5, and 156.2; mass spectrum (APCI), *m/z* 377.3526 $(M+H)^+$ ($C_{24}H_{45}N_2O$ requires 377.3532).



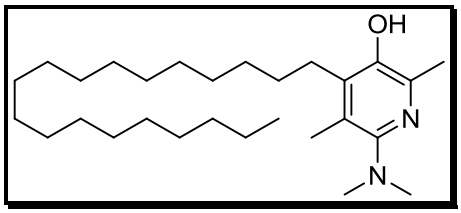
6-*N*,*N*-Dimethylamino-2-hexadecyl-4,5-dimethylpyridin-3-ol (2.9). To a solution containing 90.0 mg (0.19 mmol) of **2.39** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^oC under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.9** as a colorless solid: yield 62.0 mg (85%); mp 38-40 °C; silica gel TLC R_f 0.28 (4:1 hexane–Et_2O); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 6.8 Hz), 1.24-1.35 (m, 26H), 1.70 (m, 2H), 2.15 (s, 3H), 2.20 (s, 3H), 2.67 (m, 2H), 2.72 (s, 6H) and 4.56 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.6, 14.3, 14.7, 22.9, 28.2, 29.5, 29.7, 29.77, 29.83, 29.9, 32.0, 32.1, 43.1, 122.3, 134.0, 142.3, 144.4 and 155.5; mass spectrum (APCI), *m/z* 391.3690 $(M+H)^+$ ($C_{25}H_{47}N_2O$ requires 391.3688).



6-*N*,*N*-Dimethylamino-4-hexadecyl-2,5-dimethylpyridin-3-ol (2.10). To a solution containing 279 mg (0.58 mmol) of **2.44** in 10 mL of MeOH was added 15.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.10** as a colorless solid: yield 208 mg (92%); mp 36-38 °C; silica gel TLC R_f 0.27 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.23-1.41 (m, 26H), 1.48 (m, 2H), 2.21 (s, 3H), 2.36 (s, 3H), 2.59 (m, 2H), 2.70 (s, 6H) and 4.21 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.3, 18.9, 22.9, 27.2, 28.7, 29.5, 29.7, 29.8, 29.84, 29.86, 29.88, 30.2, 32.1, 43.1, 122.2, 138.6, 138.8, 144.5 and 156.2; mass spectrum (APCI), *m/z* 391.3681 $(M+H)^+$ ($C_{25}H_{47}N_2O$ requires 391.3688).

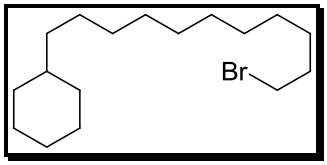


6-*N*,*N*-Dimethylamino-2-nonadecyl-4,5-dimethylpyridin-3-ol (2.11). To a solution containing 62.0 mg (0.12 mmol) of **2.40** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.11** as a colorless solid: yield 41.0 mg (80%); mp 50-51 °C; silica gel TLC R_f 0.48 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, J = 6.4 Hz), 1.24-1.34 (m, 32H), 1.70 (m, 2H), 2.15 (s, 3H), 2.19 (s, 3H), 2.63-2.74 (m, 8H) and 4.77 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.6, 14.3, 14.7, 22.9, 28.2, 29.55, 29.60, 29.64, 29.69, 29.71, 29.75, 29.79, 29.85, 29.9, 32.0, 32.1, 43.1, 122.2, 134.0, 142.5, 144.3 and 155.7; mass spectrum (APCI), *m/z* 433.4165 $(M+H)^+$ ($C_{28}H_{53}N_2O$ requires 433.4158).



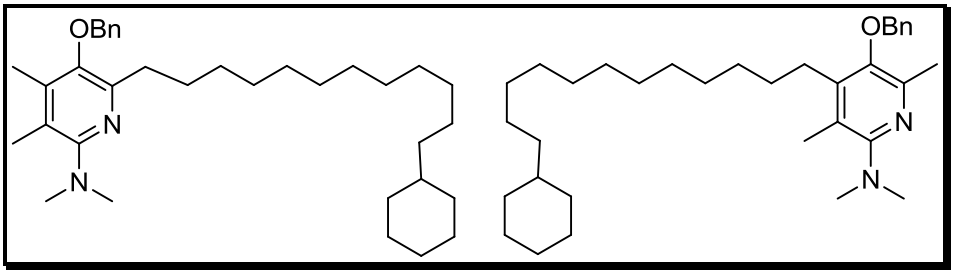
6-*N*,*N*-Dimethylamino-4-nonadecyl-2,5-dimethylpyridin-3-ol (2.12). To a solution containing 223 mg (0.43 mmol) of **2.45** in 10 mL of MeOH was added 15.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$

afforded **2.12** as a colorless solid: yield 175 mg (94%); mp 54-55 °C; silica gel TLC R_f 0.43 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, J = 6.8 Hz), 1.24-1.41 (m, 32H), 1.48 (m, 2H), 2.22 (s, 3H), 2.35 (s, 3H), 2.60 (m, 2H), 2.70 (s, 6H) and 4.83 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.2, 18.8, 22.8, 27.2, 28.7, 29.5, 29.6, 29.75, 29.81, 29.9, 30.2, 32.1, 43.0, 122.1, 138.8, 144.5 and 156.1; mass spectrum (APCI), *m/z* 433.4148 $(M+H)^+$ ($C_{28}H_{53}N_2O$ requires 433.4158).



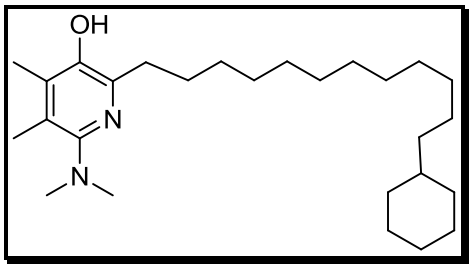
1-Bromo-11-cyclohexylundecane (2.46).[121] To a solution containing 300 mg (1.29 mmol) of 11-bromo-1-undecene and 880 μL (6.4 mmol) of vinyl cyclohexane in 6 mL of degassed CH_2Cl_2 was added 55.0 mg (0.064 mmol) of Grubbs' 2nd generation catalyst. The reaction mixture was stirred at 40 °C for 48 h, and then concentrated under diminished pressure. The residue was dissolved in 20 mL of MeOH followed by addition of catalytic amount of Pd/C. Reaction mixture was bubbled with hydrogen for 30 min, and then the reaction mixture was kept under hydrogen atmosphere (1 bar) overnight. The reaction mixture was filtered through a Celite pad and the pad was washed with MeOH. The solution was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (30 × 2 cm). Elution with 6:1 hexane–EtOAc afforeded **2.46** as a colorless oil: yield 350 mg (86%); silica gel TLC R_f 0.74 (2:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (m, 4H), 1.14-1.27 (m, 21H), 1.54-

1.69 (m, 6H) and 3.39 (t, 2H, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 22.9, 23.3, 25.4, 26.6, 26.7, 27.0, 28.4, 29.0, 29.7, 33.1, 33.7, 34.9 and 38.2; mass spectrum (EI+), *m/z* 316.1768 $(M)^+$ ($C_{17}H_{33}Br$ requires 316.1766).



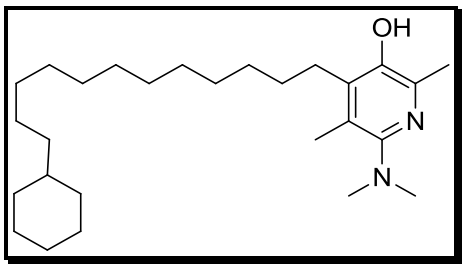
3-(Benzyloxy)-2-(12-cyclohexyldodecyl)-6-*N*,*N*-dimethylamino-4,5-dimethylpyridine (2.47) and 3-(Benzyloxy)-4-(12-cyclohexyldodecyl)-6-*N*,*N*-dimethylamino-2,5-dimethylpyridine (2.48).[120] To a stirred solution containing 340 mg (1.26 mmol) of **2.35** and 212 mg (1.89 mmol) of KO*t*Bu in 18 mL of anh THF at –78 °C was added 1.01 mL (2.52 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 480 mg (1.51 mmol) of **2.46** was added. The reaction mixture was further stirred at 0 ^{o}C for another 30 min, quenched with satd aq ammonium chloride and then extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by elution with 96:4 hexane–Et_2O afforded **2.47** and **2.48** as yellowish oils: yields 90.0 mg (14%) and 269 mg (42%), respectively; silica gel TLC R_f 0.59 (4:1 hexane–Et_2O) and R_f 0.50 (4:1 hexane–Et_2O); (**2.47**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (m, 2H), 1.14-1.35 (m, 26H), 1.69 (m, 5H), 2.19 (s, 6H), 2.76 (s, 8H), 4.73 (s, 2H) and

7.33-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 15.0, 22.9, 28.8, 29.5, 29.6, 29.7, 29.8, 29.82, 29.9, 32.07, 32.1, 42.8, 75.4, 121.5, 127.9, 128.1, 128.7, 137.7, 140.3, 147.3, 149.6 and 158.2; mass spectrum (APCI), *m/z* 507.4303 $(M+H)^+$ ($C_{34}H_{55}N_2O$ requires 507.4314); (**2.48**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85 (m, 2H), 1.12-1.31 (m, 24H), 1.49 (m, 2H), 1.67 (m, 5H), 2.21 (s, 3H), 2.45 (s, 3H), 2.61 (m, 2H), 2.76 (s, 6H), 4.76 (s, 2H) and 7.32-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.5, 19.6, 26.6, 26.9, 27.1, 27.7, 29.6, 29.8, 29.81, 29.83, 29.85, 29.9, 30.2, 30.3, 37.7, 37.9, 42.7, 75.3, 121.4, 127.7, 128.1, 128.7, 137.8, 145.2, 146.1, 147.6 and 158.7; mass spectrum (APCI), *m/z* 507.4305 $(M+H)^+$ ($C_{34}H_{55}N_2O$ requires 507.4314).

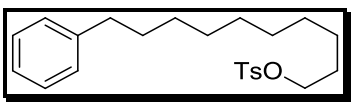


2-(12-Cyclohexyldodecyl)-6-*N*,*N*-dimethylamino-4,5-dimethylpyridin-3-ol (2.13). To a solution containing 82.0 mg (0.16 mmol) of **2.47** in 5 mL of MeOH was added 6.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.13** as a colorless oil: yield 55.0 mg (82%); silica gel TLC R_f 0.50 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.85 (m, 2H), 1.14-1.36 (m, 26H), 1.69 (m, 5H),

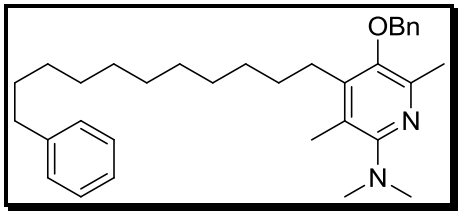
2.17 (s, 6H) and 2.74 (s, 8H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.2, 14.3, 15.0, 22.8, 28.7, 29.4, 29.5, 29.6, 29.7, 29.8, 29.9, 32.07, 32.1, 42.8, 128.1, 137.7, 140.3, 149.6 and 155.2; mass spectrum (APCI), *m/z* 417.3855 $(M+H)^+$ ($C_{27}H_{49}N_2O$ requires 417.3845).



4-(12-Cyclohexyldodecyl)-6-*N*,*N*-dimethylamino-2,5-dimethylpyridin-3-ol (2.14). To a solution containing 28.0 mg (0.05 mmol) of **2.48** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.14** as a colorless oil: yield 18.0 mg (78%); silica gel TLC R_f 0.46 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.86 (m, 2H), 1.15-1.34 (m, 24H), 1.49 (m, 2H), 1.67 (m, 5H), 2.22 (s, 3H), 2.38 (s, 3H), 2.60 (m, 2H), 2.71 (s, 6H) and 4.16 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1, 18.8, 26.6, 26.9, 27.1, 27.2, 28.7, 29.7, 29.75, 29.8, 29.84, 29.87, 29.88, 30.18, 30.19, 33.6, 37.7, 37.8, 43.1, 122.2, 138.5, 138.6, 144.5 and 156.0; mass spectrum (APCI), *m/z* 417.3841 $(M+H)^+$ ($C_{27}H_{49}N_2O$ requires 417.3845).

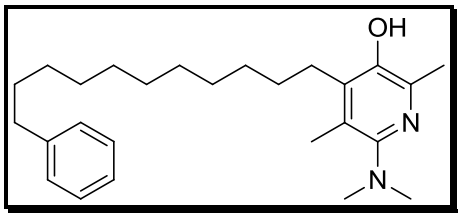


10-Phenyldecyl-4-methylbenzenesulfonate (2.49). To a solution containing 300 mg (1.28 mmol) of 10-phenyl-1-decanol in 5 mL of anh CH_2Cl_2 was added 317 mg (1.67 mmol) of *p*-toluenesulfonyl chloride, 16.0 mg (0.12 mmol) of DMAP, and 357 μL (2.56 mmol) of triethylamine. The reaction mixture was stirred at room temperature for 16 h, and then quenched with 40 mL of H_2O. The aqueous layer was washed with three 50-mL portions of CH_2Cl_2. The combined organic layer was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with 3:1 hexane–EtOAc followed by 2:1 hexane–EtOAc afforded **2.49** as a yellowish oil: yield 423 mg (85%); silica gel TLC R_f 0.33 (3:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.24-1.40 (m, 12H), 1.66 (m, 4H), 2.45 (s, 3H), 2.65 (t, 2H, *J* = 7.5 Hz), 4.06 (t, 2H, *J* = 6.5 Hz), 7.16-7.37 (m, 7H) and 7.83 (d, 2H, *J* = 8.0 Hz); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 21.4, 25.2, 28.7, 28.8, 29.1, 29.2, 29.3, 29.32, 31.4, 35.8, 70.5, 125.4, 127.7, 128.1, 128.2, 129.7, 133.2, 142.6 and 144.5; mass spectrum (APCI), *m/z* 389.2151 $(M+H)^+$ ($C_{23}H_{33}O_3S$ requires 389.2150).



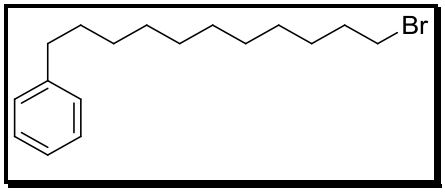
3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-(11-phenylundecyl)-2,5-dimethylpyridine (2.50).[117] To a stirred solution containing 98.0 mg (0.36 mmol) of **2.35** and 50.0 mg 3Å molecular sieves in 3 mL of anh THF was added 54 μL (0.36 mmol) of *N*,*N*,*N'*,*N'* -

tetramethylethylenediamine (TMEDA), 152 μL (0.38 mmol) of a 2.5 M solution of *n*-BuLi in hexane, followed by 155 mg (0.39 mmol) of **2.49** at 0 ^{o}C. After 15 min, 152 μL (0.38 mmol) of a 2.5 M solution of *n*-BuLi in hexane was added. The reaction was stirred at 0 ^{o}C for another 30 min, then quenched with satd aq ammonium chloride and then extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (35 × 2 cm). Elution with 9:1 hexane–Et_2O afforded **2.50** as a yellowish oil: yield 42.0 mg (24%); silica gel TLC R_f 0.52 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.23-1.40 (m, 14H), 1.51 (m, 2H), 1.63 (m, 2H), 2.24 (s, 3H), 2.47 (s, 3H), 2.62 (m, 4H), 2.78 (s, 6H), 4.78 (s, 2H) and 7.15-7.51 (m, 10H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.5, 19.6, 27.7, 29.5, 29.51, 29.66, 29.7, 29.8, 30.3, 31.7, 36.1, 42.7, 75.3, 121.4, 125.7, 127.7, 128.1, 128.3, 128.5, 128.7, 137.7, 143.1, 145.3, 146.1, 147.6 and 158.6; mass spectrum (APCI), *m/z* 487.3690 $(M+H)^+$ ($C_{33}H_{47}N_2O$ requires 487.3688).



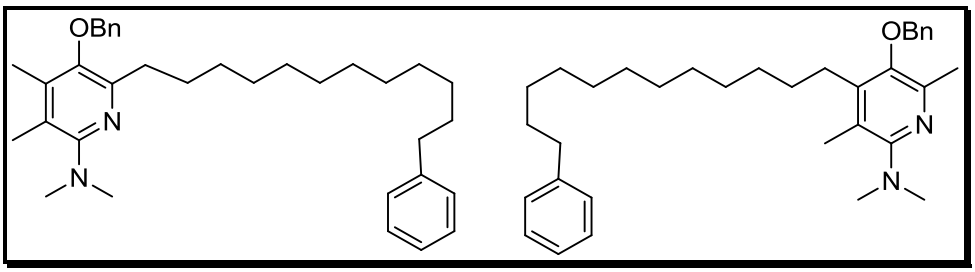
6-*N*,*N*-Dimethylamino)-2,5-dimethyl-4-(11-phenylundecyl)pyridin-3-ol (2.15). To a solution containing 42.0 mg (0.08 mmol) of **2.50** in 5 mL of MeOH was added 6.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and

the filtrate was concentrated under diminished pressure. The residue obtained was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:9 MeOH–$CHCl_3$ afforded **2.15** as yellowish oil: yield 26.0 mg (76%); silica gel TLC R_f 0.23 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.25-1.45 (m, 14H), 1.51 (m, 2H), 1.64 (m, 2H), 2.25 (s, 3H), 2.40 (s, 3H), 2.63 (m, 4H), 2.75 (s, 6H), 4.91 (br s, 1H) and 7.17-7.32 (m, 5H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1, 18.7, 27.2, 28.6, 29.4, 29.6, 29.61, 29.7, 29.74, 30.1, 31.6, 36.1, 43.0, 122.2, 125.6, 128.3, 128.5, 138.7, 139.0, 143.0, 144.6 and 155.8; mass spectrum (APCI), *m/z* 397.3224 $(M+H)^+$ ($C_{26}H_{41}N_2O$ requires 397.3219).



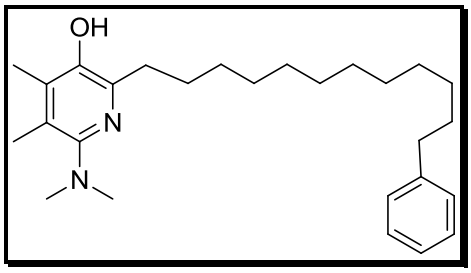
1-Bromo-11-phenylundecane (2.51).[121,139] To a solution containing 300 mg (1.29 mmol) of 11-bromo-1-undecene and 736 μL (6.41 mmol) of styrene in 6 mL of degassed CH_2Cl_2 was added 55.0 mg (0.06 mmol) of Grubbs' 2nd generation catalyst. The reaction mixture was stirred at 40 °C for 48 h, and then concentrated under diminished pressure. The residue was dissolved in 20 mL of MeOH followed by addition of catalytic amount of Pd/C. Reaction mixture was bubbled with hydrogen for 30 min, and then the reaction mixture was kept under hydrogen atmosphere (1 bar) overnight. The reaction mixture was filtered through Celite and the Celite pad was washed with MeOH. The combined organic phase was concentrated under diminished pressure and the residue was purified by flash chromatography on a silica gel column (30 × 2 cm). Elution with 6:1 hexane–

EtOAc gave **2.51** as a colorless oil: yield 340 mg (85%); silica gel TLC R_f 0.68 (2:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.37 (m, 14H), 1.71 (m, 2H), 1.89 (m, 2H), 2.69 (m, 2H), 3.41 (m, 2H) and 7.21-7.33 (m, 5H).



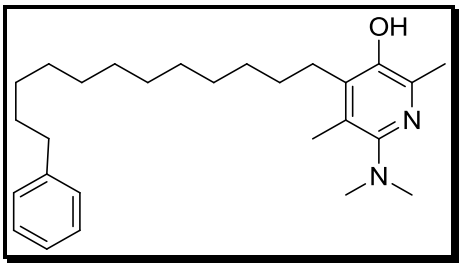
3-(Benzyloxy)-6-*N*,*N*-dimethylamino-2-(12-phenyldodecyl)-4,5-dimethylpyridine (2.52) and 3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-(12-phenyldodecyl)-2,5-dimethylpyridine (2.53).[120] To a stirred solution containing 360 mg (1.33 mmol) of **2.35** and 224 mg (1.99 mmol) of KO*t*Bu in 20 mL of anh THF at –78 °C was added 1.06 mL (2.66 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 497 mg (1.59 mmol) of **2.51** was added. The reaction mixture was further stirred at 0 ^{o}C for another 30 min, quenched with satd aq ammonium chloride and then extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 2 cm). Elution with hexane followed by elution with 96:4 hexane–Et_2O afforded **2.52** and **2.53** as yellowish oils: yields 107 mg (16%) and 287 mg (43%), respectively; silica gel TLC R_f 0.35 (4:1 hexane–Et_2O) and 0.29 (4:1 hexane–Et_2O), respectively; (**2.52**) ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.18-1.38 (m, 16H), 1.60 (m, 2H), 1.72 (m, 2H), 2.18 (s, 3H), 2.21 (s, 3H), 2.58

(m, 2H), 2.76 (s, 8H), 4.72 (s, 2H) and 7.15-7.50 (m, 10H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 13.3, 14.3, 14.5, 15.0, 22.9, 27.7, 28.8, 29.5, 29.54, 29.7, 29.76, 29.8, 29.9, 30.3, 31.7, 32.0, 36.2, 42.8, 75.5, 121.6, 125.7, 127.7, 127.9, 128.2, 128.3, 128.5, 128.7, 137.6, 143.1 and 149.7; mass spectrum (APCI), *m/z* 501.3848 $(M+H)^+$ ($C_{34}H_{49}N_2O$ requires 501.3845); (**2.53**) ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.27-1.43 (m, 16H), 1.55 (m, 2H), 1.66 (m, 2H), 2.27 (s, 3H), 2.51 (s, 3H), 2.65 (m, 4H), 2.82 (s, 6H), 4.82 (s, 2H) and 7.20-7.52 (m, 10H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.5, 19.6, 27.7, 29.48, 29.49, 29.52, 29.53, 29.6, 29.7, 29.71, 29.74, 29.77, 29.78, 30.3, 31.7, 36.1, 42.7, 75.3, 121.3, 125.7, 127.7, 128.1, 128.3, 128.5, 128.6, 137.8, 143.0, 145.2, 146.1, 147.6 and 158.7; mass spectrum (APCI), *m/z* 501.3844 $(M+H)^+$ ($C_{34}H_{49}N_2O$ requires 501.3845).



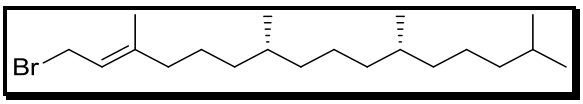
6-(Dimethylamino)-4,5-dimethyl-2-(12-phenyldodecyl)pyridin-3-ol (2.16). To a solution containing 14.0 mg (0.02 mmol) of **2.52** in 2 mL of MeOH was added 4.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue obtained was purified by column chromatography on a silica gel column (10 × 2 cm). Elution with 1:9 MeOH–$CHCl_3$ afforded **2.16** as a yellowish oil: yield 8.0 mg (70%); silica gel TLC R_f

0.50 (1:9 MeOH–$CHCl_3$); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 1.20-1.38 (m, 16H), 1.60 (m, 2H), 1.70 (m, 2H), 2.18 (s, 3H), 2.21 (s, 3H), 2.59 (m, 2H), 2.77 (s, 8H), 4.28 (br s, 1H) and 7.15-7.28 (m, 5H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.0, 18.7, 27.2, 28.6, 29.4, 29.6, 29.7, 29.73, 30.1, 31.6, 36.1, 43.0, 122.2, 125.6, 128.3, 128.4, 130.9, 138.9, 142.9, 144.6 and 155.8; mass spectrum (APCI), *m/z* 411.3384 $(M+H)^+$ ($C_{27}H_{43}N_2O$ requires 411.3375).

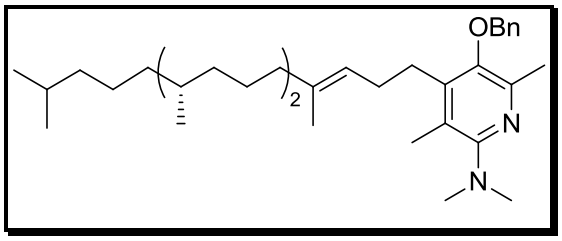


6-(Dimethylamino)-2,5-dimethyl-4-(12-phenyldodecyl)pyridin-3-ol (2.17). To a solution containing 62.0 mg (0.12 mmol) of **2.53** in 5 mL of MeOH was added 6.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue obtained was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:9 MeOH–$CHCl_3$ afforded **2.17** as a yellowish oil: yield 40.0 mg (79%); silica gel TLC R_f 0.54 (1:9 MeOH–$CHCl_3$) respectively; ^{1}H NMR (CD_3OD, 500 MHz) δ 1.19-1.28 (m, 16H), 1.29 (m, 2H), 1.38 (m, 2H), 1.49 (m, 2H), 2.10 (s, 3H), 2.26 (s, 3H), 2.46 (m, 2H), 2.57 (s, 6H), 4.85 (br s, 1H) and 6.99-7.12 (m, 5H); ^{13}C NMR (CD_3OD, 125 MHz) δ 14.1, 18.7, 28.0, 29.6, 30.3, 30.6, 30.67, 30.68, 30.7, 31.0, 32.7, 36.9, 43.3, 123.7, 126.5, 129.2,

129.3, 141.4, 142.1, 143.8, 146.7 and 156.3; mass spectrum (APCI), *m/z* 411.3370 $(M+H)^+$ ($C_{27}H_{43}N_2O$ requires 411.3375).

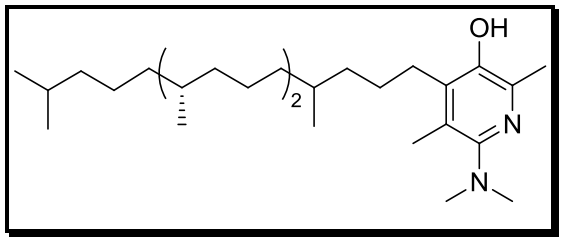


(7*R*,11*R*,*E*)-1-Bromo-3,7,11,15-tetramethylhexadec-2-ene (2.54).[122] To a flame dried two-necked round bottom flask, 500 mg (1.69 mmol) of natural phytol was taken and treated with 176 μL (1.85 mmol) of phosphorus tribromide at 0 °C. The reaction mixture was stirred for 2 h and then washed with satd $NaHCO_3$. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane afforded **2.54** as a light yellowish oil: yield 390 mg (64%); silica gel TLC R_f 0.50 (hexane); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.80-0.91 (m, 12H), 1.09-2.10 (m, 21H), 1.76 (s, 3H), 4.02 (d, 2H), 5.53 (t, 1H, J = 6.5 Hz).



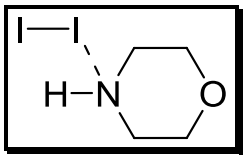
3-(Benzyloxy)-6-*N*,*N*-dimethylamino-4-((8*R*,12*R*)-4,8,12,16-tetramethylheptadecyl)-2,5-dimethylpyridine (2.55).[116] To a stirred solution containing 98.0 mg (0.36 mmol) of

2.35 and 50.0 mg of 3Å molecular sieves in 3 mL of anh THF was added 54 μL (0.36 mmol) of *N,N,N',N'* -tetramethylethylenediamine (TMEDA), 145 μL (0.36 mmol) of a 2.5 M solution of *n*-BuLi in hexane, followed by 143 mg (0.39 mmol) of **2.54** at 0 ^{o}C. After 15 min, 145 μL (0.36 mmol) of a 2.5 M solution of *n*-BuLi in hexane was added again. The reaction mixture was stirred at 0 ^{o}C for another 30 min, quenched with satd aq ammonium chloride and then extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (35 × 2 cm). Elution with 9:1 hexane–Et_2O afforded **2.55** as a yellowish oil: yield 40.0 mg (20%); silica gel TLC R_f 0.18 (9:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.80-0.91 (m, 12H), 1.02-1.41 (m, 22H), 1.51 (m, 2H), 1.92 (m, 2H), 2.24 (s, 3H), 2.45 (s, 3H), 2.65 (m, 2H), 2.76 (s, 6H), 4.78 (s, 2H), 5.18 (t, 1H, J = 7.2 Hz) and 7.21-7.52 (m, 5H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.6, 16.0, 19.6, 19.8, 19.9, 22.8, 22.9, 23.5, 24.7, 25.0, 25.5, 25.53, 28.0, 28.1, 32.9, 37.6, 39.5, 40.2, 42.7, 75.2, 123.3, 127.6, 127.7, 128.1, 128.6, 136.5, 137.7, 147.7 and 158.7; mass spectrum (APCI), *m/z* 549.4797 $(M+H)^+$ ($C_{37}H_{61}N_2O$ requires 549.4784).

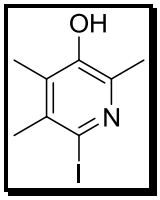


6-*N*,*N*-Dimethylamino)-2,5-dimethyl-4-((8*R*,12*R*)-4,8,12,16-tetramethylheptadecyl)pyridin-3-ol (2.18). To a solution containing 40.0 mg (0.07

mmol) of **2.55** in 5 mL of MeOH was added 8.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 ^{o}C under a H_2 atmosphere (1 bar) for overnight. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue obtained was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:9 MeOH–$CHCl_3$ afforded **2.18** as a yellowish oil: yield 29.0 mg (85%); silica gel TLC R_f 0.33 (1:9 MeOH–$CHCl_3$) respectively; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.80-0.93 (m, 15H), 1.01-1.48 (m, 24H), 1.52 (m, 2H), 2.22 (s, 3H), 2.39 (s, 3H), 2.58 (m, 2H), 2.72 (s, 6H) and 4.62 (br s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 14.1, 19.7, 19.79, 19.83, 19.85, 19.9, 22.8, 22.9, 24.6, 24.9, 26.1, 27.5, 28.1, 32.9, 32.93, 37.4, 37.5, 37.53, 37.6, 29.5, 43.1, 122.3, 138.6, 139.1, 144.7 and 155.7; mass spectrum (APCI), *m/z* 461.4472 $(M+H)^+$ ($C_{30}H_{57}N_2O$ requires 461.4471).



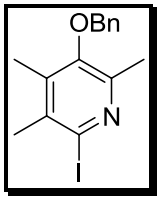
Morpholine–iodine complex (2.56).[124,125] To a homogenized solution of 60 mL (0.68 mol) of morpholine in 200 mL benzene was added 28.8 g (0.11 mol) of I_2. The mixture was stirred in dark for 3 h. The orange precipitate obtained was filtered, washed with benzene and Et_2O; and finally dried under vacuum in dark to afford **2.56** as an orange powder: yield 34.0 g (88%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.92-2.03 (br s, 1H), 3.21-3.24 (m, 4H) and 3.94-3.96 (m, 4H).



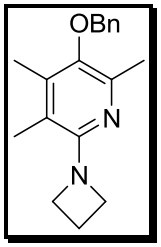
6-Iodo-2,4,5-trimethylpyridin-3-ol (2.57).[119,123] To a stirred solution of 20.0 g (97.3 mmol) of pyridoxine hydrochloride in 80 mL of thionyl chloride was added 800 μL of DMF. The reaction mixture was stirred at reflux for 2 h. The cooled reaction mixture was treated with 60 mL of Et_2O. The suspension was stirred for 1 h and then filtered and the precipitate was washed with 60 mL of Et_2O. The precipitate so obtained was dissolved in 84 mL of glacial acetic acid and 19.0 g (29.1 mmol) of zinc dust was added in three portions. The reaction mixture was stirred at reflux for 2 h. The cooled reaction mixture was filtered and washed with glacial acetic acid. The filtrate was concentrated under diminished pressure and then neutralized with 6 M NaOH. The formed precipitate was filtered and washed with small amount of brine. The orange precipitate obtained was dissolved in 10 M HCl and solid NaCl was added to salt out 12.0 g of crude 2,4,5-trimethylpyridin-3-ol.

To a mixture of 6.50 g (47.4 mmol) of crude 2,4,5-trimethylpyridin-3-ol and 19.6 g (142 mmol) of K_2CO_3 in 100 mL of distilled water was added 32.3 g (94.8 mmol) of **2.56** in eight portions over a period of 2 h. The reaction mixture was stirred for an additional 4 h in the dark and then washed with satd aq sodium thiosulfate. The crude product was extracted using CH_2Cl_2; the combined organic layer was washed with brine, dried ($MgSO_4$), and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 6 cm). Elution with CH_2Cl_2 and then with

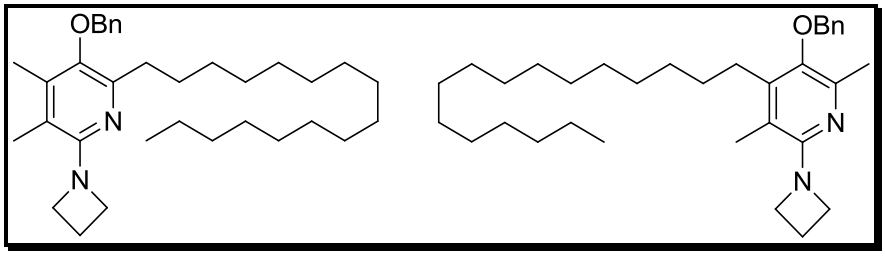
5:95 MeOH–CH_2Cl_2 afforded **2.57** as a colorless oil: yield 7.20 g (58%); silica gel TLC R_f 0.59 (1:9 MeOH–CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.22 (s, 3H), 2.34, (s, 3H), 2.40 (s, 3H) and 3.70 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.6, 18.9, 23.5, 113.8, 132.8, 135.6, 144.1 and 149.1; mass spectrum (EI+), *m/z* 262.9808 $(M)^+$ ($C_8H_{10}NOI$ requires 262.9807).



3-(Benzyloxy)-6-iodo-2,4,5-trimethylpyridine (2.58). To a solution of 1.40 g (5.32 mmol) of **2.57** in 50 mL dry DMF was added 2.94 g (21.3 mmol) of K_2CO_3 and 759 μL (6.38 mmol) of BnBr under an argon atmosphere. The reaction mixture was stirred at room temperature for 3 h and then the DMF was concentrated under diminished pressure. The residue was dissolved in 100 mL CH_2Cl_2 and washed with saturated $NaHCO_3$. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by elution with 19:1 hexane–EtOAc and finally with EtOAc afforded **2.58** as a colorless solid: yield 1.17 g (62%); mp 39-40 °C; silica gel TLC R_f 0.55 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.25 (s, 3H), 2.35 (s, 3H), 2.46 (s, 3H), 4.76 (s, 2H) and 7.34-7.45 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 19.4, 23.5, 75.1, 118.9, 128.0, 128.5, 128.7, 136.1, 136.6, 140.0, 151.4 and 152.1; mass spectrum (EI+), *m/z* 353.0280 $(M)^+$ ($C_{15}H_{16}NOI$ requires 353.0277).

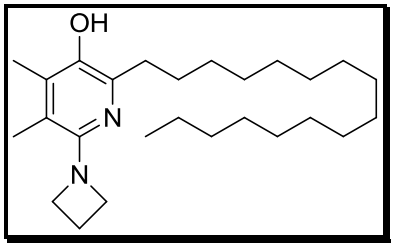


2-(Azetidin-1-yl)-5-(benzyloxy)-3,4,6-trimethylpyridine (2.59).[126] In a sealed tube containing 24.0 mg (0.03 mmol) of $Pd_2(dba)_3$, 46.0 mg (0.11 mmol) of 1,3-bis(2,6-diisopropylphenyl)imidazolium chloride, 827 mg (7.37 mmol) of KO*t*Bu, and 654 mg (6.03 mmol) of azetidine hydrochloride was added 474 mg (1.34 mmol) of **2.58** in 7 mL of dry dioxane. The reaction mixture was stirred at 90 °C for 3 h. The cooled reaction mixture was filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by elution with 95:5 hexane–Et_2O afforded **2.59** as a colorless oil: yield 268 mg (71%); silica gel TLC R_f 0.45 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.05 (s, 3H), 2.18 (s, 3H), 2.27 (quint, 2H, J = 7.6 Hz), 2.42 (s, 3H), 4.04 (t, 4H, J = 7.2 Hz), 4.72 (s, 2H) and 7.31-7.49 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.8, 14.2, 17.0, 19.4, 52.7, 75.0, 116.7, 128.0, 128.1, 128.7, 137.6, 140.0, 145.7, 146.0 and 156.9; mass spectrum (EI+), *m/z* 282.1736 $(M)^+$ ($C_{18}H_{22}N_2O$ requires 282.1732).



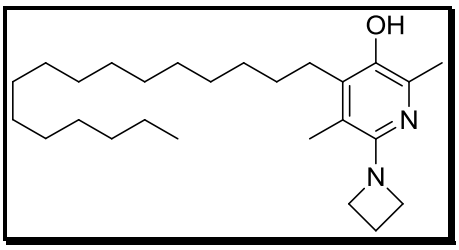
2-(Azetidin-1-yl)-5-(benzyloxy)-6-hexadecyl-3,4-dimethylpyridine (2.60) and 2-(Azetidin-1-yl)-5-(benzyloxy)-4-hexadecyl-3,6-dimethylpyridine (2.61).[120] To a stirred solution containing 109 mg (0.38 mmol) of **2.59** and 65.0 mg (0.57 mmol) of KO*t*Bu in 6 mL of anh THF at –78 °C was added 228 μL (0.57 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 165 μL (0.57 mmol) of 1-bromopentadecane was added dropwise. The reaction mixture was stirred at 0 °C for an additional 30 min, quenched with satd aq ammonium chloride and then extracted with 100 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a column of silica gel (30 × 2 cm). Elution with hexane followed by 96:4 hexane–Et_2O afforded **2.60** and **2.61** as yellowish oils: yields 43.0 mg (23%) and 74.0 mg (40%), respectively; silica gel TLC R_f 0.50 (3:2 hexane–Et_2O) and R_f 0.38 (3:2 hexane–Et_2O); (**2.60**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.90 (t, 3H, *J* = 7.2 Hz), 1.23-1.37 (m, 26H), 1.76 (quint, 2H, *J* = 7.6 Hz), 2.06 (s, 3H), 2.19 (s, 3H), 2.27 (quint, 2H, *J* = 7.6 Hz), 2.73 (t, 2H, *J* = 8.0 Hz), 4.05 (t, 4H, *J* = 7.6 Hz), 4.72 (s, 2H) and 7.32-7.50 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.9, 14.1, 14.3, 17.1, 22.8, 28.9, 29.5, 29.81, 29.87, 29.9, 32.08, 32.1, 52.7, 75.5, 116.4, 127.9, 128.1, 128.6, 137.8, 139.8, 145.7, 149.5 and 156.8; mass spectrum (APCI), *m/z* 493.4159 $(M+H)^+$ ($C_{33}H_{53}N_2O$ requires 493.4158);

(**2.61**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, *J* = 7.2 Hz), 1.24-1.34 (m, 26H), 1.48 (quint, 2H, *J* = 7.6 Hz), 2.07 (s, 3H), 2.27 (quint, 2H, *J* = 7.6 Hz), 2.44 (s, 3H), 2.61 (t, 2H, *J* = 8.0 Hz), 4.05 (t, 4H, *J* = 7.2 Hz), 4.75 (s, 2H) and 7.33 -7.50 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.9, 14.3, 17.0, 19.5, 22.9, 27.1, 29.5, 29.6, 29.76, 29.82, 29.83, 29.9, 30.3, 32.1, 52.7, 75.4, 116.2, 127.7, 128.0, 128.7, 137.9, 144.7, 145.85, 145.91 and 157.1; mass spectrum (APCI), *m/z* 493.4149 $(M+H)^+$ ($C_{33}H_{53}N_2O$ requires 493.4158).

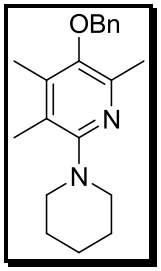


6-(Azetidin-1-yl)-2-hexadecyl-4,5-dimethylpyridin-3-ol (2.19). To a solution containing 85.0 mg (0.17 mmol) of **2.60** in 5 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 °C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.19** as a yellowish oil: yield 59.0 mg (85%); silica gel TLC R_f 0.43 (1:9 MeOH–$CHCl_3$) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 6.4 Hz), 1.19-1.38 (m, 26H), 1.67 (quint, 2H, *J* = 6.4 Hz), 2.01 (s, 3H), 2.09 (s, 3H), 2.22 (quint, 2H, *J* = 6.8 Hz), 2.61 (t, 2H, *J* = 7.2 Hz), 3.96 (t, 4H, *J* = 6.8 Hz) and 5.31 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.3, 14.1, 14.3, 17.1, 22.9, 28.4, 29.5, 29.8, 29.9, 32.1, 52.8, 116.9,

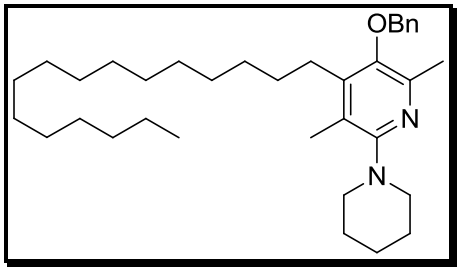
134.5, 142.8 and 154.5; mass spectrum (APCI), *m/z* 403.3700 $(M+H)^+$ ($C_{26}H_{47}N_2O$ requires 403.3688).



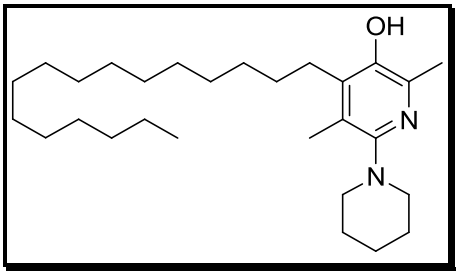
6-(Azetidin-1-yl)-4-hexadecyl-2,5-dimethylpyridin-3-ol (2.20). To a solution containing 149 mg (0.30 mmol) of **2.61** in 10 mL of MeOH was added 15.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 °C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 5:95 MeOH–$CHCl_3$ afforded **2.20** as a colorless solid: yield 114 mg (94%); mp 75-76 °C; silica gel TLC R_f 0.34 (1:9 MeOH–$CHCl_3$) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 6.8 Hz), 1.23-1.38 (m, 26H), 1.46 (quint, 2H, *J* = 7.6 Hz), 2.05 (s, 3H), 2.24 (quint, 2H, *J* = 7.2 Hz), 2.36 (s, 3H), 2.58 (t, 2H, *J* = 7.2 Hz), 3.97 (t, 4H, *J* = 7.2 Hz) and 5.33 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.3, 17.0, 18.9, 22.8, 26.6, 29.0, 29.5, 29.7, 29.75, 29.81, 29.85, 30.1, 32.1, 52.7, 116.8, 138.3, 138.5, 142.6 and 155.0; mass spectrum (APCI), *m/z* 403.3676 $(M+H)^+$ ($C_{26}H_{47}N_2O$ requires 403.3688).



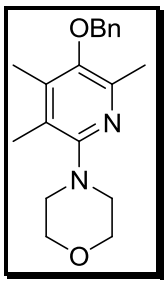
3-(Benzyloxy)-2,4,5-trimethyl-6-(piperidin-1-yl)pyridine (2.62).[126] In a sealed tube containing 15.0 mg (0.02 mmol) of $Pd_2(dba)_3$, 28.0 mg (0.07 mmol) of 1,3-bis(2,6-diisopropylphenyl)imidazolium chloride, 285 mg (2.54 mmol) of KO*t*Bu and 161 µL (1.63 mmol) of piperidine was added 301 mg (0.82 mmol) of **2.58** in 8 mL of dry dioxane. The reaction mixture was stirred at 90 °C for 3 h. The cooled reaction mixture was filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by elution with 6:1 hexane–Et_2O afforded **2.62** as a colorless solid: yield 201 mg (79%); mp: 103-105 °C; silica gel TLC R_f 0.38 (6:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.62 (m, 2H), 1.73 (m, 4H), 2.20 (s, 3H), 2.21 (s, 3H), 2.48 (s, 3H), 3.02 (t, 4H, J = 5.6 Hz), 4.76 (s, 2H) and 7.34-7.53 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.1, 14.6, 19.4, 24.7, 26.5, 51.8, 74.8, 122.7, 127.9, 128.1, 128.6, 137.5, 140.3, 146.1, 147.8 and 158.5; mass spectrum (EI+), *m/z* 310.2051 $(M)^+$ ($C_{20}H_{26}N_2O$ requires 310.2045).



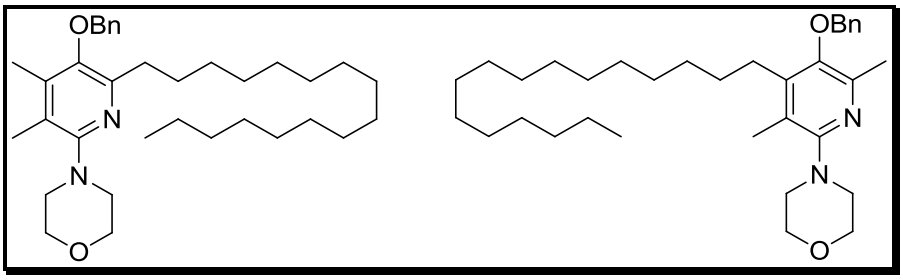
3-(Benzyloxy)-4-hexadecyl-2,5-dimethyl-6-(piperidin-1-yl)pyridine(2.63).[120] To a stirred solution containing 100 mg (0.32 mmol) of **2.62** and 72.0 mg (0.64 mmol) of KO*t*Bu in 7 mL of anh THF at –78 °C was added 256 μL (0.64 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 186 μL (0.64 mmol) of 1-bromopentadecane was added dropwise. The reaction mixture was stirred at 0 °C for an additional 30 min, quenched with satd aq ammonium chloride and then extracted with 100 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a column of silica gel (30 × 2 cm). Elution with hexane followed by 19:1 hexane–Et_2O afforded **2.63** as a yellowish oil: yields 71.0 mg (43%); silica gel TLC R_f 0.39 (9:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, J = 7.2 Hz), 1.20-1.51 (m, 26H), 1.49 (m, 2H), 1.59 (m, 2H), 1.69 (m, 4H), 2.21 (s, 3H), 2.46 (s, 3H), 2.60 (m, 2H), 3.01 (t, 4H, J = 5.2 Hz), 4.77 (s, 2H) and 7.33-7.49 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.3, 19.5, 22.8, 24.7, 26.6, 27.8, 29.49, 29.52, 29.54, 29.7, 29.8, 29.9, 30.3, 32.1, 51.9, 75.3, 122.3, 127.7, 128.1, 128.7, 137.7, 145.5, 146.3, 147.8 and 158.8; mass spectrum (FAB), *m/z* 521.4487 $(M+H)^+$ ($C_{35}H_{57}N_2O$ requires 521.4471).



4-Hexadecyl-2,5-dimethyl-6-(piperidin-1-yl)pyridin-3-ol (2.21). To a solution containing 33.0 mg (0.06 mmol) of **2.63** in 3 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 °C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 4:1 hexane–EtOAc afforded **2.21** as a colorless oil: yield 18.0 mg (66%); silica gel TLC R_f 0.36 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 6.8 Hz), 1.21-1.34 (m, 24H), 1.39 (m, 2H), 1.48 (m, 2H), 1.55 (m, 2H), 1.68 (m, 4H), 2.20 (s, 3H), 2.37 (s, 3H), 2.59 (m, 2H), 2.92 (t, 4H, J = 5.2 Hz) and 4.20 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.8, 14.3, 18.8, 22.8, 24.7, 26.6, 27.2, 28.6, 29.5, 29.7, 29.73, 29.81, 29.83, 29.9, 30.2, 32.1, 52.1, 122.8, 138.3, 138.8, 144.5 and 156.5; mass spectrum (FAB), *m/z* 431.4000 $(M+H)^+$ ($C_{28}H_{51}N_2O$ requires 431.4001).

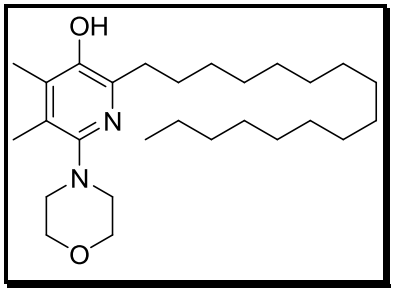


4-(5-(Benzyloxy)-3,4,6-trimethylpyridin-2-yl)morpholine (2.64).[126] In a sealed tube containing 16.0 mg (0.02 mmol) of $Pd_2(dba)_3$, 29.0 mg (0.07 mmol) of 1,3-bis(2,6-diisopropylphenyl)imidazolium chloride, 382 mg (3.40 mmol) of KO*t*Bu and 221 µL (2.55 mmol) of morpholine was added 301 mg (0.85 mmol) of **2.58** in 8 mL of dry dioxane. The reaction mixture was stirred at 90 °C for 3 h. The cooled reaction mixture was filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine. The organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by elution with 4:1 hexane–EtOAc afforded **2.64** as a colorless solid: yield 214 mg (81%); mp 88-89 °C; silica gel TLC R_f 0.37 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.19 (s, 3H), 2.20 (s, 3H), 2.47 (s, 3H), 3.08 (m, 4H), 3.86 (t, 4H), 4.75 (s, 2H) and 7.32-7.49 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.2, 14.5, 19.2, 50.9, 67.3, 74.8, 122.5, 127.9, 128.2, 128.6, 137.2, 141.0, 146.4, 148.2 and 156.7; mass spectrum (EI+), *m/z* 312.1837 $(M)^+$ ($C_{19}H_{24}N_2O_2$ requires 312.1838).

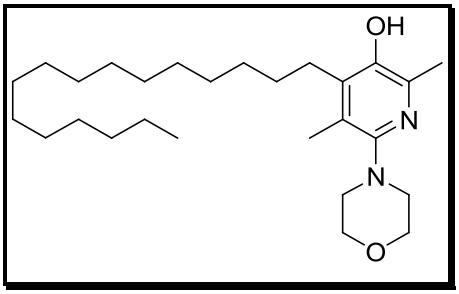


4-(5-(Benzyloxy)-6-hexadecyl-3,4-dimethylpyridin-2-yl)morpholine (2.65) and 4-(5-(Benzyloxy)-4-hexadecyl-3,6-dimethylpyridin-2-yl)morpholine (2.66).[120] To a stirred

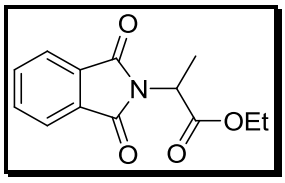
solution containing 100 mg (0.32 mmol) of **2.64** and 72.0 mg (0.64 mmol) of KO*t*Bu in 7 mL of anh THF at –78 °C was added 256 μL (0.64 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 186 μL (0.64 mmol) of 1-bromopentadecane was added dropwise. The reaction mixture was stirred at 0 °C for an additional 30 min, quenched with satd aq ammonium chloride and then extracted with 100 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a column of silica gel (30 × 2 cm). Elution with hexane followed by 19:1 hexane–Et_2O and then 9:1 hexane–Et_2O afforded **2.65** and **2.66** as yellowish oils: yields 24.0 mg (14%) and 70.0 mg (42%), respectively; silica gel TLC R_f 0.30 (4:1 hexane–Et_2O) and R_f 0.18 (4:1 hexane–Et_2O); (**2.65**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (t, 3H, *J* = 6.8 Hz), 1.19-1.37 (m, 24H), 1.71 (m, 2H), 1.84 (m, 2H), 2.17 (s, 3H), 2.19 (s, 3H), 2.74 (t, 2H, *J* = 7.2 Hz), 3.07 (m, 4H), 3.85 (m, 4H), 4.73 (s, 2H) and 7.32-7.49 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.3, 14.3, 14.6, 22.9, 25.8, 28.8, 29.5, 29.8, 29.82, 29.9, 32.0, 32.1, 51.0, 67.4, 68.1, 75.5, 122.1, 127.9, 128.2, 128.7, 137.5, 140.6, 148.0, 150.3 and 156.9; mass spectrum (FAB), *m/z* 523.4274 $(M+H)^+$ ($C_{34}H_{55}N_2O_2$ requires 523.4264); (**2.66**) ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, *J* = 6.8 Hz), 1.20-1.41 (m, 26H), 1.50 (m, 2H), 2.23 (s, 3H), 2.46 (s, 3H), 2.61 (m, 2H), 3.07 (m, 4H), 3.85 (m, 4H), 4.78 (s, 2H) and 7.32-7.51 (m, 5H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 14.2, 19.5, 22.8, 27.7, 29.5, 29.7, 29.76, 29.77, 29.8, 30.3, 32.0, 51.0, 67.4, 75.3, 121.9, 127.7, 128.1, 128.7, 137.6, 145.5, 146.7, 148.2 and 157.3; mass spectrum (FAB), *m/z* 523.4260 $(M+H)^+$ ($C_{34}H_{55}N_2O_2$ requires 523.4264).



2-Hexadecyl-4,5-dimethyl-6-morpholinopyridin-3-ol (2.22). To a solution containing 24.0 mg (0.04 mmol) of **2.65** in 2 mL of MeOH was added 5.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 °C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:9 MeOH–CH_2Cl_2 afforded **2.22** as a colorless oil: yield 13.0 mg (67%); silica gel TLC R_f 0.34 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.22-1.36 (m, 26H), 1.69 (m, 2H), 2.15 (s, 3H), 2.18 (s, 3H), 2.67 (t, 2H, *J* = 7.6 Hz), 3.02 (m, 4H), 3.84 (m, 4H) and 4.62 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 12.6, 14.3, 14.34, 22.9, 28.1, 29.5, 29.7, 29.8, 29.82, 29.9, 32.0, 32.1, 51.2, 67.5, 122.5, 142.7, 144.7, 148.4 and 154.4; mass spectrum (FAB), *m/z* 433.3770 $(M+H)^+$ ($C_{27}H_{49}N_2O_2$ requires 433.3794).

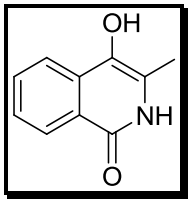


4-Hexadecyl-2,5-dimethyl-6-morpholinopyridin-3-ol (2.23). To a solution containing 70.0 mg (0.13 mmol) of **2.66** in 3 mL of MeOH was added 15.0 mg of 20% palladium hydroxide on carbon. The reaction mixture was stirred at 23 °C under a H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (10 × 2 cm). Elution with 1:9 MeOH–CH_2Cl_2 afforded **2.23** as a colorless oil: yield 56.0 mg (97%); silica gel TLC R_f 0.30 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, J = 6.8 Hz), 1.20-1.42 (m, 26H), 1.48 (m, 2H), 2.21 (s, 3H), 2.37 (s, 3H), 2.59 (m, 2H), 2.99 (m, 4H), 3.83 (t, 4H) and 4.60 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.2, 18.8, 22.8, 27.1, 28.6, 29.5, 29.6, 29.7, 29.77, 29.8, 29.81, 30.1, 32.0, 51.2, 67.5, 122.4, 138.4, 139.1, 144.9 and 154.8; mass spectrum (EI+), *m/z* 432.3715 $(M)^+$ ($C_{27}H_{48}N_2O_2$ requires 432.3716).

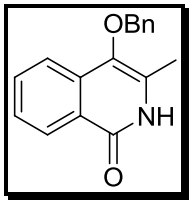


Ethyl 2-(1,3-dioxoisoindolin-2-yl)propanoate (2.67).[127,128] To a solution of 10.8 g (0.06 mol) of potassium phthalimide in 70 mL dry DMF was added 9.8 mL (0.08 mol) of 2-bromo propionate under an atmosphere of argon. The reaction mixture was stirred at room temperature for 2 h and then DMF was concentrated under diminished pressure. The residue obtained was dissolved in 300 mL CH_2Cl_2 and washed with water. The organic phase obtained was dried ($MgSO_4$) and concentrated under diminished pressure

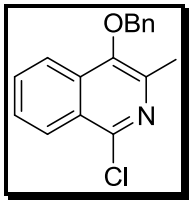
to afford **2.67** as a colorless solid: yield 14.0 g (98%); silica gel TLC R_f 0.48 (7:3 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.22 (t, 3H, J = 7.2 Hz), 1.68 (d, 3H, J = 7.6 Hz), 4.20 (dq, 2H, J = 7.2 Hz), 4.95 (q, 1H, J = 7.2 Hz) and 7.72-7.83 (m, 4H).



4-Hydroxy-3-methylisoquinolin-1(2H)-one (2.68).[127,128] In a sealed tube containing 14.0 g (0.06 mol) of **2.67** in 50 mL dry MeOH was added 50 mL of freshly prepared NaOMe, prepared by the addition of 2.57 g (0.11 mol) of sodium in 50 mL MeOH. The reaction mixture was stirred at 100 °C for 2 h. The yellow solution obtained was cooled and then concentrated under diminished pressure. The residue obtained was dissolved in minimum amount of water and then 1 N HCl was added dropwise until precipitation started (~pH = 3). The precipitate obtained was filtered, washed with Et_2O and dried in vacuum to afford **2.68** as a yellowish solid: yield 8.40 g (86%); silica gel TLC R_f 0.14 (5:95 MeOH–CH_2Cl_2); ^{1}H NMR (CD_3OD, 400 MHz) δ 2.30 (s, 3H), 7.48 (t, 1H, J = 7.6 Hz), 7.74 (t, 1H, J = 8 Hz), 7.94 (m, 1H) and 8.24 (m, 1H).

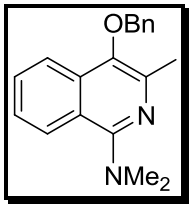


4-(Benzyloxy)-3-methylisoquinolin-1(2H)-one (2.69). To a solution of 8.40 g (0.05 mol) of **2.68** in 100 mL dry DMF was added 32.4 g (0.24 mol) of K_2CO_3 and 5.7 mL (0.05 mol) of BnBr under an atmosphere of argon. The reaction mixture was stirred at room temperature for 16 h and then DMF was concentrated under diminished pressure. The residue was dissolved in 200 mL CH_2Cl_2 and washed with satd $NaHCO_3$. The organic phase obtained was dried ($MgSO_4$) and concentrated under diminished pressure. The crude was purified by chromatography on a silica gel column (15 × 6 cm). Elution with hexane followed by 4:1 hexane–EtOAc and finally with EtOAc afforded **2.69** as a slightly yellowish-orange oil: yield 9.20 g (72%); silica gel TLC R_f 0.31 (5:95 MeOH–CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.59 (s, 3H), 4.98 (s, 2H), 7.32-7.59 (m, 6H), 7.72 (t, 1H, J = 7.2 Hz), 7.81 (d, 1H, J = 8.0 Hz), 8.44 (d, 1H, J = 8.0 Hz) and 11.87 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.0, 75.9, 120.9, 125.0, 126.1, 128.0, 128.3, 128.4, 128.8, 129.9, 132.7, 135.0, 135.2, 137.1 and 163.3; mass spectrum (APCI), *m/z* 266.1187 $(M+H)^+$ ($C_{17}H_{16}NO_2$ requires 266.1181).



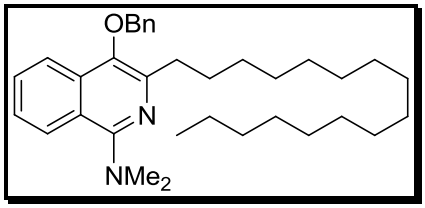
4-(Benzyloxy)-1-chloro-3-methylisoquinoline (2.70).[129] In a sealed tube containing 2.01 g (7.57 mmol) of **2.69** was added 706 μL of $POCl_3$. The reaction mixture was stirred at 90 °C for 1 h. Yellow solution obtained was cooled, diluted with 100 mL CH_2Cl_2 and then washed with cold water. The combined organic phase was dried ($MgSO_4$) and

concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 9:1 hexane–EtOAc afforded **2.70** as a colorless oil: yield 1.22 g (57%); silica gel TLC R_f 0.46 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.60 (s, 3H), 4.98 (s, 2H), 7.32-7.52 (m, 5H), 7.57 (t, 1H, *J* = 7.2 Hz), 7.68 (t, 1H, *J* = 7.6 Hz), 8.01 (d, 1H, *J* = 8.0 Hz) and 8.22 (d, 1H, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.7, 76.2, 121.5, 126.5, 126.6, 127.6, 128.0, 128.5, 128.7, 130.9, 133.3, 136.6, 143.2, 144.8 and 147.1; mass spectrum (APCI), *m/z* 284.0839 $(M+H)^+$ ($C_{17}H_{15}NOCl$ requires 284.0842).



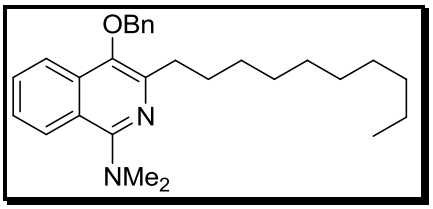
4-(Benzyloxy)-*N*,*N*,3-trimethylisoquinolin-1-amine (2.71).[126] In a sealed tube containing 21.0 mg (0.02 mmol) of $Pd_2(dba)_3$, 39.0 mg (0.09 mmol) of 1,3-bis(2,6-diisopropylphenyl)imidazolium chloride, 638 mg (5.68 mmol) of KO*t*Bu and 368 mg (4.52 mmol) of dimethylamine hydrochloride was added 322 mg (1.13 mmol) of **2.70** in 3.5 mL dry dioxane. The reaction mixture was stirred at 100 °C for 3 h. The reaction mixture was allowed to cool to room temperature, diluted with CH_2Cl_2, washed with water and then with brine. The combined organic phase was dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with CH_2Cl_2 followed by 95:5 CH_2Cl_2–EtOAc afforded **2.71** as yellowish oil: yield 262 mg (79%); silica gel TLC

R_f 0.35 (4:1 hexane–Et_2O); ^1H NMR ($CDCl_3$, 400 MHz) δ 2.57 (s, 3H), 3.08, (s, 6H), 4.96 (s, 2H), 7.33-7.58 (m, 7H), 8.00 (d, 1H, J = 8.4 Hz) and 8.14 (d, 1H, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 19.1, 43.4, 75.7, 121.4, 124.9, 126.5, 128.1, 128.2, 128.7, 129.5, 133.6, 137.6, 140.7, 142.6 and 157.8; mass spectrum (APCI), *m/z* 293.1655 $(M+H)^+$ ($C_{19}H_{21}N_2O$ requires 293.1654).



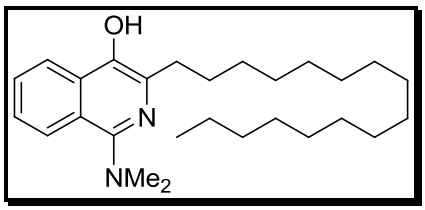
4-(Benzyloxy)-3-hexadecyl-*N*,*N*-dimethylisoquinolin-1-amine (2.72).[120] To a stirred solution containing 73.0 mg (0.25 mmol) of **2.71** and 78.0 mg (0.70 mmol) of KO*t*Bu in 3 mL of anh THF at –78 °C was added 172 μL (0.43 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 139 μL (0.48 mmol) of 1-bromopentadecane was added dropwise. The reaction mixture was stirred at 0 °C for an additional 30 min, quenched with satd aq ammonium chloride and then extracted with 100 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a column of silica gel (30 × 2 cm). Elution with hexane followed by 19:1 hexane–Et_2O and then 9:1 hexane–Et_2O afforded **2.72** as a yellowish oil: yield 40.0 mg (32%); silica gel TLC R_f 0.55 (4:1 hexane–Et_2O); ^1H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, J = 7.2 Hz), 1.22-1.43 (m, 26H), 1.79 (m, 2H), 2.89 (m, 2H), 3.07 (s, 6H), 4.96 (s, 2H), 7.31-7.59 (m, 7H), 8.00 (d, 1H, J = 8.4 Hz) and 8.14 (d, 1H, J

= 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 22.9, 28.9, 29.5, 29.8, 29.9, 31.8, 32.1, 43.4, 76.3, 121.3, 121.6, 124.9, 126.4, 128.0, 128.2, 128.7, 129.3, 133.6, 137.8, 142.3, 144.6 and 157.7; mass spectrum (APCI), *m/z* 503.4004 $(M+H)^+$ ($C_{34}H_{51}N_2O$ requires 503.4001).

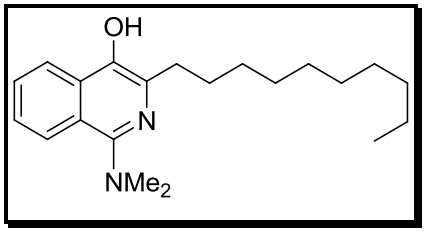


4-(Benzyloxy)-3-hexadecyl-*N*,*N*-dimethylisoquinolin-1-amine (2.73).[120] To a stirred solution containing 40.0 mg (0.14 mmol) of **2.71** and 31.0 mg (0.27 mmol) of KO*t*Bu in 2 mL of anh THF at –78 °C was added 170 μL (0.27 mmol) of a 1.6 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 30 min and then 39 μL (0.20 mmol) of 1-bromononane was added dropwise. The reaction mixture was stirred at 0 °C for an additional 30 min, quenched with satd aq ammonium chloride and then extracted with 50 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a column of silica gel (30 × 2 cm). Elution with hexane followed by 19:1 hexane–Et_2O followed by 9:1 hexane–Et_2O afforded **2.73** as a yellowish oil: yield 20.0 mg (35%); silica gel TLC R_f 0.53 (4:1 hexane–Et_2O); ^{1}H NMR δ ($CDCl_3$, 400 MHz) 0.84 (t, 3H, *J* = 6.0 Hz), 1.21-1.41 (m, 14H), 1.75 (m, 2H), 2.86 (m, 2H), 3.05 (s, 6H), 4.92 (s, 2H), 7.33-7.61 (m, 7H), 7.95 (d, 1H, *J* = 8.4 Hz) and 8.09 (d, 1H, *J* = 8.4 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 22.8, 29.0, 29.5, 29.8, 29.83, 32.1, 43.5, 76.3, 121.2,

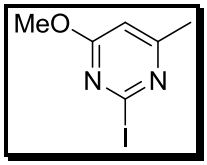
121.7, 124.9, 124.95, 125.0, 126.37, 126.39, 128.0, 128.2, 128.7, 137.7, 142.3 and 157.7; mass spectrum (APCI), *m/z* 419.3067 $(M+H)^+$ ($C_{28}H_{39}N_2O$ requires 419.3062).



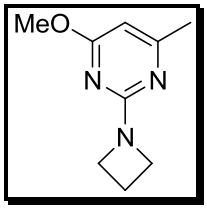
1-(Dimethylamino)-3-hexadecylisoquinolin-4-ol (2.24). To a solution containing 40.0 mg (0.08 mmol) of **2.72** in 7 mL of MeOH and 2 mL $CHCl_3$ was added 6.0 mg of 10% palladium on carbon. The reaction mixture was stirred at 23 °C under H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under reduced pressure. The crude products were purified on Phenomenex C_8 (2) reversed phase semi-preparative (LUNA 250 × 10 mm, 5 µm) HPLC column using a mobile phase consisting of aq 0.1% TFA and MeOH. A linear gradient of (99:1 0.1% aq TFA–MeOH → 0:100 0.1% aq TFA–MeOH) was employed over a period of 25 min at a flow rate of 3.5 mL/min. The combined fractions were concentrated under diminished pressure. Diluted with $CHCl_3$, dried ($MgSO_4$) and concentrated under reduced diminished to afforded **2.24** as a colorless oil: yield 4.0 mg (12%); silica gel TLC R_f 0.44 (1:19 MeOH–CH_2Cl_2) respectively; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.12-1.41 (m, 26H), 1.77 (m, 2H), 2.82 (m, 2H), 3.00 (s, 6H), 4.57 (br s, 1H), 7.46 (t, 1H, *J* = 7.6 Hz), 7.61 (t, 1H, *J* = 7.2 Hz), 8.05 (d, 1H, *J* = 8.0 Hz) and 8.13 (d, 1H, *J* = 8.4 Hz); mass spectrum (APCI), *m/z* 413.3529 $(M+H)^+$ ($C_{27}H_{45}N_2O$ requires 413.3532).



3-Decyl-1-(dimethylamino)isoquinolin-4-ol (2.25). To a solution containing 20.0 mg (0.04 mmol) of **2.73** in 3 mL of MeOH and 1 mL $CHCl_3$ was added 6.0 mg of 10% palladium on carbon. The reaction mixture was stirred at 23 °C under H_2 atmosphere (1 bar) for 30 min. The reaction mixture was filtered through Celite and the filtrate was concentrated under reduced pressure. The crude products were purified on Phenomenex C_8 (2) reversed phase semi-preparative (LUNA 250 × 10 mm, 5 µm) HPLC column using a mobile phase consisting of aq 0.1% TFA and MeOH. A linear gradient of (99:1 0.1% aq TFA–MeOH → 0:100 0.1% aq TFA–MeOH) was employed over a period of 25 min at a flow rate of 3.5 mL/min. The combined fractions were concentrated under diminished pressure. Diluted with $CHCl_3$, dried ($MgSO_4$) and concentrated under reduced pressure to afforded **2.25** as a colorless oil: yield 3.0 mg (23%); silica gel TLC R_f 0.51 (1:9 MeOH–CH_2Cl_2) respectively; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84 (t, 3H, J = 6.8 Hz), 1.13-1.43 (m, 14H), 1.75 (m, 2H), 2.82 (m, 2H), 3.02 (s, 6H), 4.54 (br s, 1H), 7.45 (t, 1H, J = 7.6 Hz), 7.59 (t, 1H, J = 7.2 Hz), 8.03 (d, 1H, J = 8.0 Hz) and 8.11 (d, 1H, J = 8.4 Hz); mass spectrum (APCI), *m/z* 329.2586 $(M+H)^+$ ($C_{21}H_{33}N_2O$ requires 329.2593).

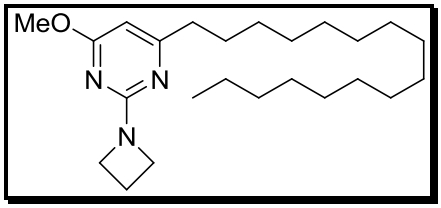


2-Iodo-4-methoxy-6-methylpyrimidine (2.74).[130] To a stirred solution containing 3.00 g (21.6 mmol) of 2-amino-4-methoxy-6-methylpyrimidine, 5.46 g (21.6 mmol) of iodine, 4.31 g (22.6 mmol) of CuI and 2.5 mL (30.9 mmol) of CH_2I_2 in 120 mL of anh THF was added 10.5 mL (78.2 mmol) of isoamylnitrite. The reaction mixture was stirred at reflux for 3 h. The reaction mixture was allowed to warm to room temperature and then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 5 cm). Elution with hexane followed by 95:5 hexane–Et_2O and then 80:20 hexane–Et_2O afforded **2.74** as a yellowish solid: yield 2.01 g (37%); mp 43-44 °C; silica gel TLC R_f 0.35 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.37 (s, 3H), 3.93 (s, 3H) and 6.50 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 23.7, 54.6, 106.5, 127.4, 169.0 and 169.1; mass spectrum (APCI), *m/z* 250.9675 $(M+H)^+$ ($C_6H_8N_2OI$ requires 250.9682).



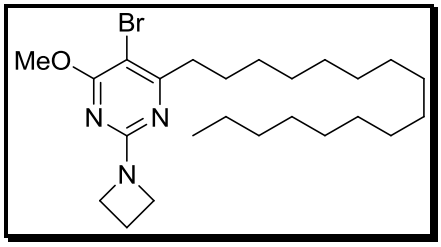
2-(Azetidin-1-yl)-4-methoxy-6-methylpyrimidine (2.75).[131] To a stirred solution containing 560 mg (5.98 mmol) of azetidine hydrochloride, 76.0 mg (0.39 mmol) of CuI,

and 3.90 g (11.9 mmol) of Cs_2CO_3 in 10 mL dry degassed DMF was added 1.00 g (3.99 mmol) of **2.74** and 95.0 mg (0.39 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline sequentially. The reaction mixture was stirred at 50 °C for 5 h. The mixture was allowed to warm to room temperature and then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 95:5 hexane–EtOAc and then 85:15 hexane–EtOAc afforded **2.75** as a yellowish oil: yield 515 mg (72%); silica gel TLC R_f 0.26 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.25 (s, 3H), 2.30 (quint, 2H, J = 8.0 Hz), 3.84 (s, 3H), 4.11 (t, 4H, J = 7.6 Hz) and 5.83 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.3, 24.1, 50.2, 53.0, 95.0, 163.2, 168.0 and 170.7; mass spectrum (APCI), *m/z* 180.1136 $(M+H)^+$ ($C_9H_{14}N_3O$ requires 180.1137).



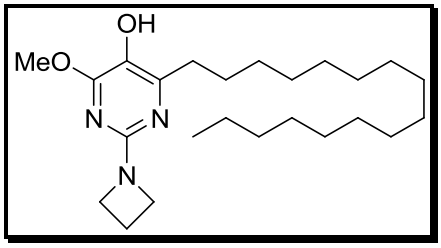
2-(Azetidin-1-yl)-4-methoxy-6-hexadecylpyrimidine (2.76).[118] To a stirred solution containing 261 mg (1.45 mmol) of **2.75** in 7 mL of anh THF at –78 °C was added 870 μL (2.17 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 15 min and then 300 μL (1.03 mmol) of 1-bromopentadecane was added. The reaction was stirred at 0 ^{o}C for another 30 min, then quenched with satd aq ammonium

chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 95:5 hexane–Et_2O afforded **2.76** as a yellowish solid: yield 142 mg (25%) and 87 mg (33%) starting material was recovered; mp 45-46 °C; silica gel TLC R_f 0.32 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, J = 7.2 Hz), 1.18-1.35 (m, 26H), 1.62 (quint, 2H, J = 7.2 Hz), 2.29 (quint, 2H, J = 7.2 Hz), 2.48 (t, 2H, J = 7.6 Hz), 3.82 (s, 3H), 4.10 (t, 4H, J = 7.6 Hz) and 5.83 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 16.3, 22.8, 28.7, 29.46, 29.5, 29.6, 29.7, 29.78, 29.8, 32.0, 37.9, 50.2, 52.9, 94.3, 163.3, 170.7 and 172.2; mass spectrum (APCI), *m/z* 390.3481 $(M+H)^+$ ($C_{24}H_{44}N_3O$ requires 390.3484).



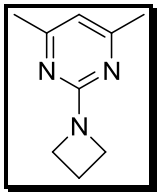
2-(Azetidin-1-yl)-5-bromo-4-methoxy-6-hexadecylpyrimidine (2.77).[118] To a stirred solution containing 106 mg (0.27 mmol) of **2.76** in 4 mL (1:1) CH_2Cl_2–acetonitrile was added 58.0 mg (0.33 mmol) of NBS under dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with 50 mL CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 95:5 hexane–Et_2O afforded **2.77** as a colorless solid: yield 121 mg (96%); mp 82-83 °C;

silica gel TLC R_f 0.55 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.19-1.37 (m, 26H), 1.64 (quint, 2H, *J* = 7.2 Hz), 2.32 (quint, 2H, *J* = 7.2 Hz), 2.69 (t, 2H, *J* = 7.6 Hz), 3.93 (s, 3H) and 4.10 (t, 4H, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 16.3, 22.8, 28.0, 29.5, 29.6, 29.7, 29.8, 29.9, 32.1, 37.0, 50.5, 54.3, 92.7, 161.2, 165.7 and 169.6; mass spectrum (APCI), *m/z* 468.2589 $(M+H)^+$ ($C_{24}H_{43}N_3OBr$ requires 468.2589).



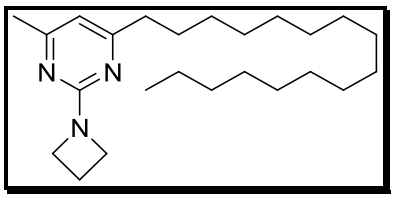
2-(Azetidin-1-yl)-4-methoxy-6-hexadecylpyrimidin-5-ol (2.26).[118] To a stirred solution containing 93.0 mg (0.19 mmol) of **2.77** in 2 mL of anh THF at –5 °C was added 30 μL (0.19 mmol) of TMEDA and 198 μL (0.49 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –5 °C for 15 min and then 66 μL (0.59 mmol) trimethoxyborane was added. The reaction was stirred for 30 min at room temperature followed by addition of 426 μL (4.35 mmol) of H_2O_2 (35% v/v). The reaction mixture was stirred for additional 30 min and poured into 20 mL water, neutralized with dilute aq HCl and then extracted with 100 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 90:10 hexane–EtOAc afforded **2.26** as a yellowish solid: yield 27.0 mg

(34%); mp 59-60 °C; silica gel TLC R_f 0.22 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.19-1.37 (m, 26H), 1.64 (quint, 2H, *J* = 7.2 Hz), 2.27 (quint, 2H, *J* = 7.2 Hz), 2.61 (t, 2H, *J* = 8.0 Hz), 3.92 (s, 3H), 4.04 (t, 4H, *J* = 7.6 Hz) and 4.61 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 16.3, 22.8, 28.1, 29.5, 29.7, 29.72, 29.8, 29.82, 29.9, 31.5, 32.1, 51.0, 53.6, 128.3, 155.2, 157.6 and 158.6; mass spectrum (APCI), *m/z* 406.3436 $(M+H)^+$ ($C_{24}H_{44}N_3O_2$ requires 406.3434).

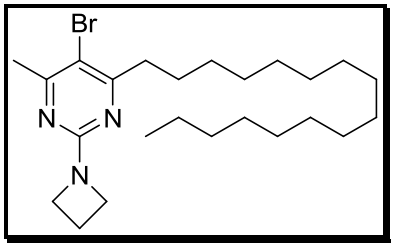


2-(Azetidin-1-yl)-4,6-dimethylpyrimidine (2.78).[131] To a stirred solution containing 655 mg (6.99 mmol) of azetidine hydrochloride, 133 mg (6.99 mmol) of CuI, and 3.42 g (10.5 mmol) of Cs_2CO_3 in 10 mL dry degassed DMF was added 500 mg (3.49 mmol) of 2-chloropyrimidine and 165 mg (6.99 mmol) 3,4,7,8-tetramethyl-1,10-phenanthroline sequentially. The reaction mixture was stirred at 50 °C for 4 h. The mixture was allowed to warm to room temperature and then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 4:1 hexane–EtOAc and then 1:1 hexane–EtOAc afforded **2.78** as yellowish solid: yield 372 mg (65%); mp 51-52 °C; silica gel TLC R_f 0.22 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.20 (s, 6H), 2.24 (t, 2H, *J* = 7.6 Hz), 4.05 (t, 4H, *J* = 7.2 Hz)

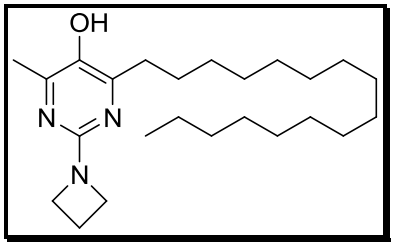
and 6.19 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 16.2, 23.9, 50.1, 109.1, 163.2 and 167.0; mass spectrum (FAB), *m/z* 164.1192 $(M+H)^+$ ($C_9H_{14}N_3$ requires 164.1188).



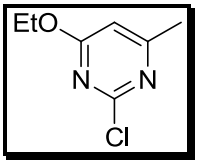
2-(Azetidin-1-yl)-4-hexadecyl-6-methylpyrimidine (2.79).[118] To a stirred solution containing 321 mg (1.96 mmol) of **2.78** in 10 mL of anh THF at –78 °C was added 1.02 mL (2.56 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 15 min and then 398 μL (1.37 mmol) of 1-bromopentadecane was added. The reaction was stirred at 0 °C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 96:4 hexane–EtOAc and then 90:10 hexane–EtOAc afforded **2.79** as a colorless solid: yield 307 mg (42%); mp 63-64 °C; silica gel TLC R_f 0.45 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (t, 3H, *J* = 6.8 Hz), 1.18-1.37 (m, 26H), 1.62 (quint, 2H, *J* = 7.6 Hz), 2.27 (s, 3H), 2.29 (quint, 2H, *J* = 7.6 Hz), 2.49 (t, 2H, *J* = 7.2 Hz), 4.11 (t, 4H, *J* = 7.2 Hz) and 6.24 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 16.4, 22.8, 24.2, 28.8, 29.46, 29.5, 29.6, 29.64, 29.75, 29.8, 32.0, 37.9, 50.3, 108.6, 163.4, 167.0 and 171.2; mass spectrum (FAB), *m/z* 374.3545 $(M+H)^+$ ($C_{24}H_{44}N_3$ requires 374.3535).



2-(Azetidin-1-yl)-5-bromo-4-hexadecyl-6-methylpyrimidine (2.80).[118] To a stirred solution containing 290 mg (0.77 mmol) of **2.79** in 5 mL CH_2Cl_2 was added 152 mg (0.85 mmol) of NBS under dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with 20 mL CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 96:4 hexane–EtOAc afforded **2.80** as a colorless solid: yield 338 mg (97%); mp 74-75 °C; silica gel TLC R_f 0.45 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.18-1.37 (m, 26H), 1.65 (quint, 2H, *J* = 7.6 Hz), 2.31 (quint, 2H, *J* = 7.6 Hz), 2.44 (s, 3H), 2.71 (t, 2H, *J* = 7.6 Hz) and 4.09 (t, 4H, *J* = 7.6 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 16.3, 22.8, 25.3, 27.8, 29.5, 29.6, 29.7, 29.8, 29.84, 32.1, 37.4, 50.5, 108.6, 161.3, 165.7 and 168.8; mass spectrum (FAB), *m/z* 454.2611 $(M+H)^+$ ($C_{24}H_{43}N_3O^{81}Br$ requires 454.2620).

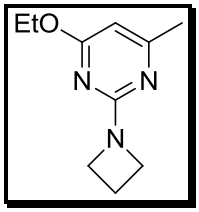


2-(Azetidin-1-yl)-4-hexadecyl-6-methylpyrimidin-5-ol (2.27).[131] To a stirred solution containing 57.0 mg (0.13 mmol) of **2.80** in 2 mL of anh THF at –5 °C was added 84 μL (0.75 mmol) of trimethoxyborane and 156 μL (0.39 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at 23 °C for 30 min followed by addition of 221 μL (3.25 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min and poured into 20 mL water, neutralized with dilute aq HCl and then extracted with 100 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane–EtOAc followed by 80:20 hexane–EtOAc afforded **2.27** as a yellowish oil: yield 28.0 mg (55%); silica gel TLC R_f 0.27 (3:2 hexane–EtOAc); ^{1}H NMR (CD_3OD, 400 MHz) δ 0.90 (t, 3H, J = 6.8 Hz), 1.27-1.32 (m, 26H), 1.64 (m, 2H), 2.25-2.34 (m, 5H), 2.65 (m, 2H), 4.04 (t, 4H, J = 7.6 Hz) and 4.28 (br s, 1H); ^{13}C NMR (CD_3OD, 100 MHz) δ 14.5, 17.0, 18.6, 23.8, 29.1, 30.5, 30.6, 30.7, 30.8, 30.81, 30.83, 32.8, 33.1, 52.2, 140.7, 157.6, 159.9 and 161.6; mass spectrum (FAB), *m/z* 390.3480 $(M+H)^+$ ($C_{24}H_{44}N_3O$ requires 390.3484).



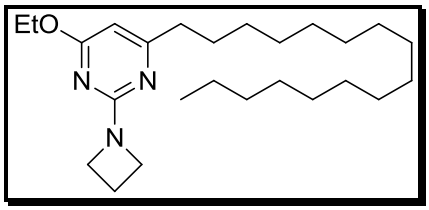
2-Chloro-4-ethoxy-6-methylpyrimidine (2.81). To a stirred solution containing 2.01 g (12.3 mmol) of 2,4-dichloro-6-methylpyrimidine in 40 mL of anh THF was added 927 mg (38.6 mmol) of NaH (60% suspension in oil) and 392 μL (12.9 mmol) of EtOH. The reaction mixture was stirred for 5 h at room temperature and then slowly poured into 200

mL of water. The crude was extracted with two 300-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 6 cm). Elution with 19:1 hexane–EtOAc afforded **2.81** as a colorless solid: yield 2.16 g (51%); mp 37-38 °C; silica gel TLC R_f 0.41 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.38 (t, 3H, J = 7.2 Hz), 2.42 (s, 3H), 4.42 (d, 2H, J = 7.2 Hz) and 6.46 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 23.8, 63.5, 105.7, 159.8, 169.8 and 170.9; mass spectrum (APCI), *m/z* 173.0477 $(M+H)^+$ ($C_7H_{10}N_2OCl$ requires 173.0482).



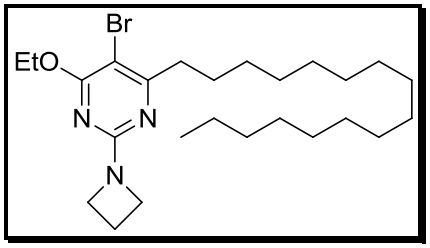
2-(Azetidin-1-yl)-4-ethoxy-6-methylpyrimidine (2.82).[131] To a round bottom flask containing 600 mg (3.48 mmol) of **2.81**, 489 mg (5.22 mmol) of azetidine hydrochloride, 131 mg (0.69 mmol) of CuI, 164 mg (0.69 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline and 2.83 g (8.70 mmol) of Cs_2CO_3 was added 15 mL dry degassed DMF. The reaction mixture was stirred at 50 °C for 3 h. The mixture was allowed to cool to room temperature and then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 19:1 hexane–EtOAc followed by 9:1 hexane–EtOAc afforded **2.82** as a colorless solid: yield 565 mg (84%);

mp 42-43 °C; silica gel TLC R_f 0.29 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.24 (t, 3H, *J* = 7.2 Hz), 2.16 (s, 3H), 2.20 (quint, 2H, *J* = 7.6 Hz), 4.01 (t, 4H, *J* = 7.6 Hz), 4.20 (q, 2H, *J* = 7.2 Hz) and 5.73 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.4, 16.1, 23.9, 49.9, 61.2, 95.0, 163.0, 167.7 and 170.1; mass spectrum (APCI), *m/z* 194.1289 $(M+H)^+$ ($C_{10}H_{16}N_3O$ requires 194.1293).

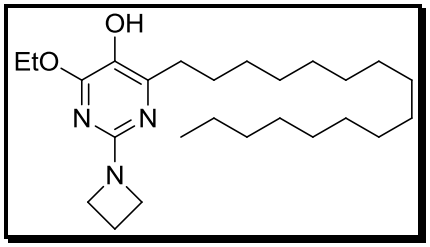


2-(Azetidin-1-yl)-4-ethoxy-6-hexadecylpyrimidine (2.83).[118] To a stirred solution containing 450 mg (2.32 mmol) of **2.82** in 20 mL of anh THF at –78 °C was added 1.02 mL (2.56 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 15 min and then 475 μL (1.63 mmol) of 1-bromopentadecane was added. The reaction was stirred at 0 °C for another 30 min, then quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 19:1 hexane–EtOAc afforded **2.83** as a colorless solid: yield 421 mg (45%); mp 40-41 °C; silica gel TLC R_f 0.42 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.84 (t, 3H, *J* = 6.8 Hz), 1.18-1.33 (m, 29H), 1.60 (quint, 2H, *J* = 6.8 Hz), 2.24 (quint, 2H, *J* = 7.6 Hz), 2.44 (t, 2H, *J* = 7.6 Hz), 4.05 (t, 4H, *J* = 7.6 Hz), 4.26 (q, 2H, *J* = 7.2 Hz) and 5.78 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.1, 14.5, 16.2, 22.7, 28.6, 29.39, 29.42, 29.55,

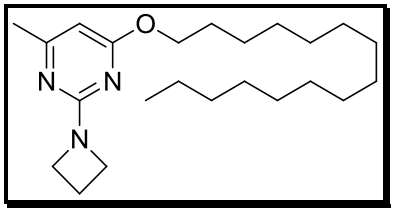
29.61, 29.7, 29.8, 32.0, 37.8, 50.1, 61.3, 94.4, 163.2, 170.21 and 172.0; mass spectrum (FAB), *m/z* 404.3632 $(M+H)^+$ ($C_{25}H_{46}N_3O$ requires 404.3641).



2-(Azetidin-1-yl)-5-bromo-4-ethoxy-6-hexadecylpyrimidine (2.84).[118] To a stirred solution containing 464 mg (1.15 mmol) of **2.83** in 10 mL CH_2Cl_2 was added 209 mg (1.17 mmol) of NBS under dark (round bottom flask was wrapped with aluminum foil). The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 96:4 hexane–EtOAc afforded **2.84** as a colorless solid: yield 522 mg (94%); mp 69-70 °C; silica gel TLC R_f 0.56 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 7.2 Hz), 1.18-1.40 (m, 29H), 1.64 (quint, 2H, *J* = 7.6 Hz), 2.29 (quint, 2H, *J* = 7.6 Hz), 2.69 (t, 2H, *J* = 7.6 Hz), 4.06 (t, 4H, *J* = 7.6 Hz) and 4.37 (q, 2H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 14.5, 16.2, 22.8, 27.9, 29.5, 29.56, 29.58, 29.7, 29.78, 29.83, 32.1, 37.0, 50.3, 62.8, 92.9, 161.1, 165.2 and 169.4; mass spectrum (FAB), *m/z* 482.2753 $(M+H)^+$ ($C_{25}H_{45}N_3OBr$ requires 482.2746).



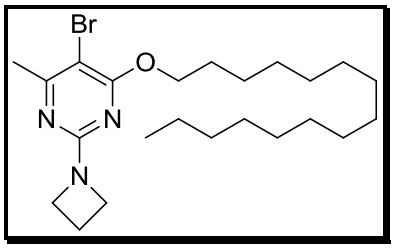
2-(Azetidin-1-yl)-4-ethoxy-6-hexadecylpyrimidin-5-ol (2.28).[118] To a stirred solution containing 400 mg (0.83 mmol) of **2.84** in 10 mL of anh THF at –5 °C was added 663 μL (1.66 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 278 μL (2.49 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by addition of 1.2 mL (18.3 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane–EtOAc afforded **2.28** as a colorless powder: yield 250 mg (72%); mp 79-80 °C; silica gel TLC R_f 0.33 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.19-1.39 (m, 29H), 1.63 (quint, 2H, J = 7.6 Hz), 2.26 (quint, 2H, J = 7.2 Hz), 2.61 (t, 2H, J = 7.6 Hz), 4.02 (t, 4H, J = 7.6 Hz), 4.37 (q, 2H, J = 7.2 Hz) and 4.89 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 14.7, 16.3, 22.8, 28.1, 29.5, 29.71, 29.73, 29.77, 29.81, 29.85, 31.5, 32.1, 50.9, 62.3, 128.3, 155.1, 157.6 and 158.3; mass spectrum (FAB), *m/z* 420.3578 $(M+H)^+$ ($C_{25}H_{46}N_3O_2$ requires 420.3590).



2-(Azetidin-1-yl)-4-methyl-6-(pentadecyloxy)pyrimidine (2.85).[131] To a stirred solution containing 1.01 g (6.13 mmol) of 2,4-dichloro-6-methylpyrimidine in 20 mL of anh THF was added 620 mg (25.8 mmol) of NaH (60% suspension in oil) and 1.47 g (6.44 mmol) of 1-pentadecanol. The reaction mixture was stirred for 24 h at room temperature and then slowly poured into 100 mL of water. The crude was extracted with two 200-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure to afford crude 790 mg of 2-chloro-4-methyl-6-(pentadecyloxy)pyrimidine.

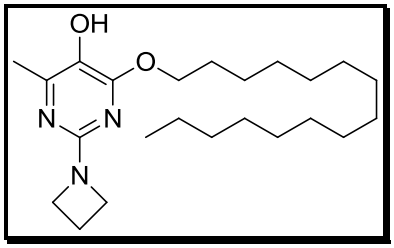
To a round bottom flask containing 350 mg (0.99 mmol) of crude 2-chloro-4-methyl-6-(pentadecyloxy)pyrimidine, 139 mg (1.49 mmol) of azetidine hydrochloride, 19.0 mg (0.09 mmol) of CuI, 23.0 mg (0.09 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline and 806 mg (2.48 mmol) of Cs_2CO_3 was added 15 mL dry degassed DMF. The reaction mixture was stirred at 50 °C for 5 h. The mixture was allowed to cool to room temperature and then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (15 × 3 cm). Elution with 19:1 hexane–EtOAc followed by 9:1 hexane–EtOAc afforded **2.85** as a colorless solid: yield 282 mg (76%); mp 40-41 °C; silica gel TLC R_f 0.27 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ

0.86 (t, 3H, *J* = 6.8 Hz), 1.21-1.38 (m, 24H), 1.70 (quint, 2H, *J* = 7.2 Hz), 2.24 (s, 3H), 2.29 (quint, 2H, *J* = 7.6 Hz), 4.09 (t, 4H, *J* = 7.6 Hz), 4.21 (t, 2H, *J* = 6.8 Hz) and 5.81 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 16.4, 22.9, 24.2, 26.2, 29.1, 29.5, 29.7, 29.76, 29.8, 29.9, 32.1, 50.3, 65.9, 95.3, 163.2, 168.0 and 170.6; mass spectrum (FAB), *m/z* 376.3317 $(M+H)^+$ ($C_{23}H_{42}N_3O$ requires 376.3328).

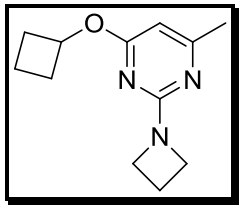


2-(Azetidin-1-yl)-5-bromo-4-methyl-6-(pentadecyloxy)pyrimidine (2.86).[118] To a stirred solution containing 145 mg (0.39 mmol) of **2.85** in 4 mL CH_2Cl_2 was added 72.0 mg (0.41 mmol) of NBS under dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 96:4 hexane–EtOAc afforded **2.86** as a colorless solid: yield 159 mg (90%); mp 71-72 °C; silica gel TLC R_f 0.53 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, *J* = 6.8 Hz), 1.21-1.47 (m, 24H), 1.75 (quint, 2H, *J* = 7.6 Hz), 2.30 (quint, 2H, *J* = 7.2 Hz), 2.40 (s, 3H), 4.07 (t, 4H, *J* = 7.6 Hz) and 4.30 (t, 2H, *J* = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 16.2, 22.8, 24.5, 26.1, 28.9, 29.45, 29.5, 29.7, 29.72, 29.8, 29.83, 32.1, 50.4, 67.1, 93.3,

161.0, 165.3 and 166.0; mass spectrum (FAB), *m/z* 454.2421 $(M+H)^+$ ($C_{23}H_{41}N_3OBr$ requires 454.2433).



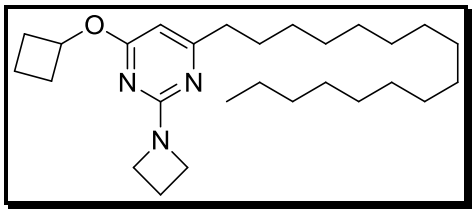
2-(Azetidin-1-yl)-4-methyl-6-(pentadecyloxy)pyrimidin-5-ol (2.29).[118] To a stirred solution containing 130 mg (0.28 mmol) of **2.86** in 3 mL of anh THF at –5 °C was added 229 μL (0.57 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 94 μL (0.84 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by addition of 419 μL (6.16 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane–EtOAc afforded **2.29** as a colorless powder: yield 66.0 mg (60%); mp 83-85 °C; silica gel TLC R_f 0.21 (3:2 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.86 (t, 3H, *J* = 6.8 Hz), 1.05-1.41 (m, 24H), 1.70 (quint, 2H, *J* = 6.8 Hz), 2.15-2.32 (m, 5H), 4.01 (t, 4H, *J* = 7.2 Hz), 4.30 (t, 2H, *J* = 6.8 Hz) and 5.11 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 16.3, 17.8, 22.8, 26.1, 29.0, 29.5, 29.7, 29.74, 29.8, 29.83, 32.1, 50.9, 66.6, 128.6, 151.1, 157.3 and 158.5; mass spectrum (FAB), *m/z* 392.3286 $(M+H)^+$ ($C_{23}H_{42}N_3O_2$ requires 392.3277).



2-(Azetidin-1-yl)-4-cyclobutoxy-6-methylpyrimidine (2.87).[131] To a stirred solution containing 1.01 g (6.13 mmol) of 2,4-dichloro-6-methylpyrimidine in 40 mL of anh THF was added 620 mg (25.8 mmol) of NaH (60% suspension in oil) and 504 µL (6.44 mmol) of 1-cyclobutanol. The reaction mixture was stirred for 24 h at room temperature and then slowly poured into 100 mL of water. The crude was extracted with two 200-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure to afford 810 mg crude 2-chloro-4-cyclobutoxy-6-methylpyrimidine.

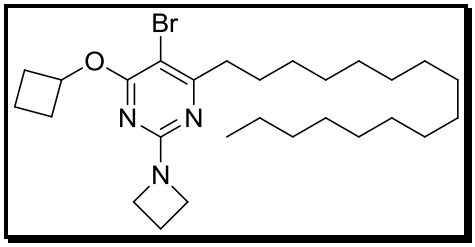
To a round bottom flask containing 350 mg (1.76 mmol) of crude 2-chloro-4-cyclobutoxy-6-methylpyrimidine, 247 mg (2.64 mmol) of azetidine hydrochloride, 34.0 mg (0.18 mmol) of CuI, 41.0 mg (0.18 mmol) of 3,4,7,8-tetramethyl-1,10-phenanthroline and 1.43 g (4.40 mmol) of Cs_2CO_3 was added 15 mL dry degassed DMF. The reaction mixture was stirred at 50 °C for 5 h. The mixture was allowed to cool to room temperature and then filtered through Celite and the Celite pad was washed with CH_2Cl_2. The combined organic phase was washed with water and then with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by flash chromatography on a silica gel column (20 × 3 cm). Elution with 19:1 hexane–EtOAc followd by 9:1 hexane–EtOAc afforded **2.87** as a colorless solid: yield 286 mg (74%); mp 60-61 °C; silica gel TLC R_f 0.22 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ

1.60 (m, 1H), 1.76 (m, 1H), 2.07 (m, 2H), 2.20 (s, 3H), 2.25 (quint, 2H, J = 7.6 Hz), 2.34 (m, 2H), 4.05 (t, 4H, J = 7.6 Hz), 5.01 (m, 1H) and 5.74 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.6, 16.2, 24.1, 30.7, 50.1, 70.0, 94.9, 163.1, 168.1 and 169.5; mass spectrum (APCI), *m/z* 220.1445 $(M+H)^+$ ($C_{12}H_{18}N_3O$ requires 220.1450).

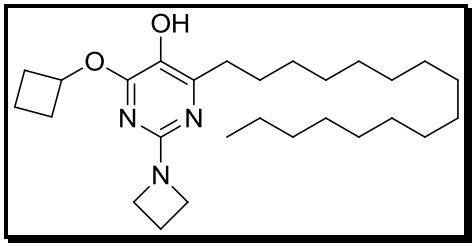


2-(Azetidin-1-yl)-4-cyclobutoxy-6-hexadecylpyrimidine (2.88).[118] To a stirred solution containing 180 mg (0.82 mmol) of **2.87** in 10 mL of anh THF at –78 °C was added 492 μL (1.23 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 20 min and then 214 μL (0.74 mmol) of 1-bromopentadecane was added. The reaction was stirred at 0 °C for 15 min and then at room temperature for another 30 min. The reaction mixture was quenched with satd aq ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with 19:1 hexane–Et_2O afforded **2.88** as a colorless solid: yield 130 mg (37%); mp 39-40 °C; silica gel TLC R_f 0.46 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (t, 3H, J = 7.2 Hz), 1.15-1.36 (m, 26H), 1.62 (m, 3H), 1.79 (m, 1H), 2.13 (m, 2H), 2.28 (quint, 2H, J = 7.6 Hz), 2.38 (m, 2H), 2.47 (t, 2H, J = 7.6 Hz), 4.08 (t, 4H, J = 7.6 Hz), 5.06 (m, 1H) and 5.77 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.2, 16.3, 22.8, 28.8, 29.46, 29.5, 29.6, 29.7,

29.8, 29.82, 30.8, 32.1, 37.9, 50.2, 70.1, 94.3, 163.3, 169.6 and 172.4; mass spectrum (FAB), *m/z* 430.3786 $(M+H)^+$ ($C_{27}H_{48}N_3O$ requires 430.3797).



2-(Azetidin-1-yl)-5-bromo-4-cyclobutoxy-6-hexadecylpyrimidine (2.89).[118] To a stirred solution containing 130 mg (0.30 mmol) of **2.88** in 10 mL CH_2Cl_2 was added 57.0 mg (0.32 mmol) of NBS under dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 19:1 hexane–EtOAc afforded **2.89** as a colorless solid: yield 141 mg (92%); mp 71-73 °C; silica gel TLC R_f 0.59 (6:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.19-1.42 (m, 26H), 1.65 (m, 3H), 1.84 (m, 1H), 2.19 (m, 2H), 2.30 (quint, 2H, *J* = 7.6 Hz), 2.41 (m, 2H), 2.69 (t, 2H, *J* = 7.6 Hz), 4.06 (t, 4H, *J* = 7.6 Hz) and 5.13 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.3, 16.2, 22.8, 28.0, 29.5, 29.6, 29.7, 29.8, 29.81, 29.9, 30.9, 32.1, 37.1, 50.4, 71.3, 92.7, 161.2, 164.7 and 169.5; mass spectrum (FAB), *m/z* 508.2897 $(M+H)^+$ ($C_{27}H_{47}N_3OBr$ requires 508.2902).



2-(Azetidin-1-yl)-4-cyclobutoxy-6-hexadecylpyrimidin-5-ol (2.30).[118] To a stirred solution containing 141 mg (0.27 mmol) of **2.89** in 5 mL of anh THF at –5 °C was added 242 μL (0.60 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 100 μL (0.90 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by addition of 449 μL (6.60 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 95:5 hexane–EtOAc afforded **2.30** as a colorless powder: yield 72.0 mg (60%); mp 95-97 °C; silica gel TLC R_f 0.42 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, *J* = 7.2 Hz), 1.21-1.36 (m, 26H), 1.63 (m, 3H), 1.83 (m, 1H), 2.12 (m, 2H), 2.26 (quint, 2H, *J* = 7.2 Hz), 2.41 (m, 2H), 2.61 (t, 2H, *J* = 7.2 Hz), 4.01 (t, 4H, *J* = 7.2 Hz), 4.76 (br s, 1H) and 5.17 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.6, 14.3, 16.3, 22.8, 28.2, 29.5, 29.71, 29.73, 29.8, 29.81, 29.9, 31.0, 31.5, 32.1, 50.9, 70.8, 128.1, 155.2, 157.6 and 157.7; mass spectrum (FAB), *m/z* 446.3742 $(M+H)^+$ ($C_{27}H_{48}N_3O_2$ requires 446.3747)

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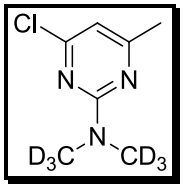
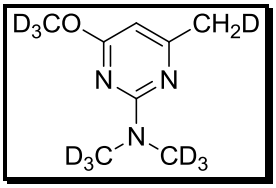
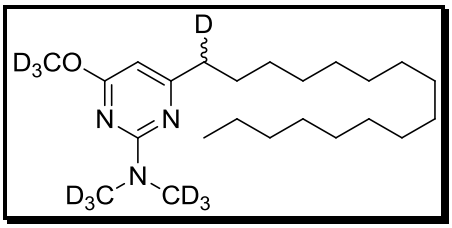


4-Chloro-6-methyl-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.90). To a stirred solution containing 500 mg (3.48 mmol) of 2-amino-4-chloro-6-methylpyrimidine and 435 µL (6.96 mmol) of methyl iodide-(d_3) in 10 mL of anh THF was added 417 mg (17.4 mmol) of NaH (60% suspension in oil) in two aliquots at 0 °C in the dark. The reaction mixture was slowly warmed to 23 °C, stirred for 5 h under dark and then slowly poured into 100 mL of water. The crude was extracted with two 200-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 9:1 hexane–EtOAc afforded **2.90** as a yellowish solid: yield 533 mg (86%); mp 29-30 °C; silica gel TLC R_f 0.51 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 500 MHz) δ 2.19 (s, 3H) and 6.23 (s, 1H); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 23.9, 36.0, 107.2, 160.5, 161.9 and 168.8; mass spectrum (APCI), *m/z* 178.1017 $(M+H)^+$ ($C_7H_5N_3{}^2H_6Cl$ requires 178.1018).

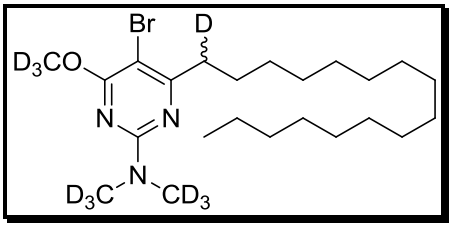


4-(Methoxy-d_3) -6-(methyl-d_1)-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.91). To a stirred solution containing 530 mg (2.98 mmol) of **2.90** in 10 mL of anh THF was added 430 mg (17.9 mmol) of NaH (60% suspension in oil) and 244 μL (5.96 mmol) of CD_3OD. The reaction mixture was stirred at reflux for 20 h and then allowed to cool to room temperature. The mixture was slowly poured into 200 mL of water and extracted with two 300-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with hexane followed by 97:3 hexane–EtOAc afforded **2.91** as a colorless oil: yield 350 mg (66%); silica gel TLC R_f 0.25 (7:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.23 (m, 2H) and 5.77 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.2, 36.0, 52.0, 93.8, 162.4, 167.8 and 170.3; mass spectrum (APCI), *m/z* 178.1762 $(M+H)^+$ ($C_8H_4N_3O^2H_{10}$ requires 178.1765).



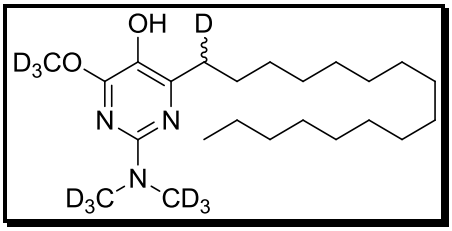
4-(Methoxy-d_3)-6-(1-hexadecyl-d_1)-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.92).[118] To a stirred solution containing 240 mg (1.36 mmol) of **2.91** in 15 mL of anh THF at –78 °C was added 817 μL (2.04 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 20 min and then 355 μL (1.22 mmol) of 1-bromopentadecane was added. The reaction was stirred at 0 ^{o}C for 15 min and then at room temperature for another 30 min. The reaction mixture was quenched with satd aq

ammonium chloride and extracted with 150 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (30 × 3 cm). Elution with 19:1 hexane–Et_2O afforded **2.92** as a colorless solid: yield 250 mg (47%); mp 45-46 °C; silica gel TLC R_f 0.58 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 6.8 Hz), 1.19-1.37 (m, 26H), 1.64 (m, 2H), 2.48 (q, 1H, J = 8.0 Hz) and 5.79 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.2, 22.8, 28.6, 29.4, 29.5, 29.52, 29.7, 29.73, 29.8, 29.9, 32.1, 36.0, 37.6, 38.0, 52.0, 93.2, 162.5, 170.4 and 171.9; mass spectrum (FAB), *m/z* 388.4117 $(M+H)^+$ ($C_{23}H_{34}N_3{}^2H_{10}O$ requires 388.4112).



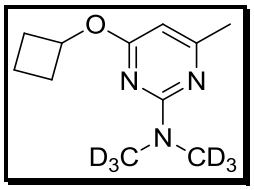
3-Bromo-4-(methoxy-d_3)-6-(1-hexadecyl-d_1)-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.93).[118] To a stirred solution containing 320 mg (0.83 mmol) of **2.92** in 10 mL CH_2Cl_2 was added 154 mg (0.87 mmol) of NBS under dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 19:1 hexane–EtOAc afforded **2.93** as a colorless solid: yield 159 mg (90%); mp 63-64 °C; silica gel TLC R_f 0.31 (19:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.19-1.40 (m, 26H), 1.66 (m, 2H) and 2.69 (q, 1H, J = 7.6 Hz); ^{13}C

NMR ($CDCl_3$, 100 MHz) δ 14.3, 22.8, 27.7, 29.5, 29.6, 29.63, 29.7, 29.8, 29.9, 32.1, 36.5, 36.9, 53.3, 91.3, 160.3, 165.2 and 169.2; mass spectrum (APCI), *m/z* 468.3208 $(M+H)^+$ ($C_{23}H_{33}N_3O^2H_{10}{}^{81}Br$ requires 468.3197).

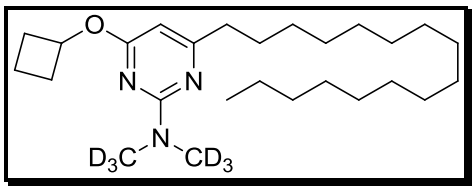


2-(*N*,*N*-dimethylamino-d_6)-4-(1-hexadecyl-d_1)-6-(methoxy-d_3)-pyrimidin-5-ol (2.31).[118] To a stirred solution containing 276 mg (0.59 mmol) of **2.93** in 10 mL of anh THF at –5 °C was added 473 μL (1.18 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 197 μL (1.77 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by addition of 883 μL (12.9 mmol) of H_2O_2 (50% v/v). The reaction mixture was stirred for additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with 95:5 hexane–EtOAc afforded **2.31** as a colorless powder: yield 150 mg (63%); mp 75-76 °C; silica gel TLC R_f 0.38 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.19-1.39 (m, 26H), 1.65 (m, 2H), 2.60 (m, 1H) and 4.50 (br s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 14.3, 22.8, 27.9, 29.5, 29.6, 29.7, 29.72, 29.8, 29.82, 29.9, 32.1, 54.4, 127.1,

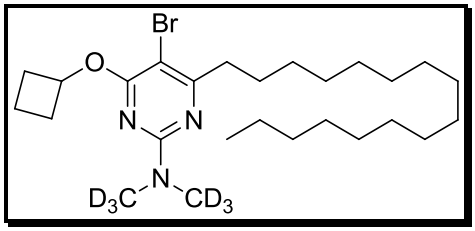
155.1, 156.1 and 158.2; mass spectrum (APCI), *m/z* 404.4067 $(M+H)^+$ ($C_{23}H_{34}N_3O_2{}^2H_{10}$ requires 404.4061).



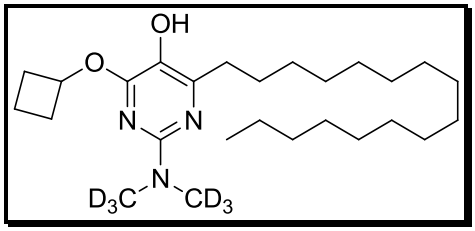
4-Cyclobutoxy-6-methyl-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.94). To a stirred solution containing 500 mg (2.81 mmol) of **2.90** in 10 mL of anh THF was added 405 mg (16.9 mmol) of NaH (60% suspension in oil) and 343 µL (4.38 mmol) of 1-cyclobutanol. The reaction mixture was stirred at reflux for 48 h and then allowed to cool to room temperature. The mixture was slowly poured into 100 mL of water and extracted with two 150-mL portions of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with 19:1 hexane–Et_2O afforded **2.94** as a colorless oil: yield 391 mg (65%); silica gel TLC R_f 0.36 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.62 (m, 1H), 1.78 (m, 1H), 2.10 (m, 2H), 2.21 (s, 3H), 2.38 (m, 2H), 5.08 (quint, 1H, J = 7.2 Hz) and 5.71 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.6, 24.2, 30.7, 36.0, 69.8, 93.9, 162.4, 167.9 and 169.2; mass spectrum (APCI), *m/z* 214.1832 $(M+H)^+$ ($C_{11}H_{12}N_3O^2H_6$ requires 214.1827).



4-Cyclobutoxy-6-hexadecyl-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.95).[118] To a stirred solution containing 391 mg (1.83 mmol) of **2.94** in 20 mL of anh THF at –78 °C was added 1.09 mL (2.74 mmol) of a 2.5 M solution of *n*-BuLi in hexane. The reaction mixture was stirred at –78 °C for 20 min and then 477 μL (1.64 mmol) of 1-bromopentadecane was added. The reaction was stirred at 0 °C for 15 min and then at room temperature for another 30 min. The reaction mixture was quenched with satd aq ammonium chloride and extracted with 300 mL of EtOAc. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (20 × 3 cm). Elution with 19:1 hexane–Et_2O afforded **2.95** as a colorless solid: yield 434 mg (56%); mp 39-40 °C; silica gel TLC R_f 0.58 (4:1 hexane–Et_2O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 7.2 Hz), 1.15-1.39 (m, 26H), 1.65 (m, 3H), 1.81 (m, 1H), 2.14 (m, 2H), 2.41 (m, 2H), 2.47 (m, 2H), 5.12 (quint, 1H, J = 7.2 Hz) and 5.74 (s, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.2, 22.8, 28.6, 29.48, 29.5, 29.6, 29.7, 29.8, 29.83, 30.8, 32.1, 36.0, 38.0, 69.8, 93.3, 162.4, 169.2 and 172.0; mass spectrum (APCI), *m/z* 424.4182 $(M+H)^+$ ($C_{26}H_{42}N_3O^2H_6$ requires 424.4174).



5-Bromo-4-cyclobutoxy-6-hexadecyl-(*N*,*N*-dimethylpyrimidin-2-amine-d_6) (2.96).[118] To a stirred solution containing 286 mg (0.67 mmol) of **2.95** in 5 mL CH_2Cl_2 was added 126 mg (0.71 mmol) of NBS under dark. The reaction mixture was stirred for 30 min at room temperature under dark, then diluted with 50 mL CH_2Cl_2, washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with hexane followed by 98:2 hexane–EtOAc afforded **2.96** as a colorless solid: yield 306 mg (91%); mp 57-59 °C; silica gel TLC R_f 0.66 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (t, 3H, *J* = 6.8 Hz), 1.21-1.41 (m, 26H), 1.68 (m, 3H), 1.84 (m, 1H), 2.22 (m, 2H), 2.45 (m, 2H), 2.70 (m, 2H) and 5.16 (quint, 1H, *J* = 7.2 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.3, 22.9, 27.8, 29.5, 29.6, 29.64, 29.8, 29.9, 30.8, 32.1, 36.2, 37.0, 71.1, 91.4, 160.3, 164.3 and 169.2; mass spectrum (APCI), *m/z* 502.3274 $(M+H)^+$ ($C_{26}H_{41}N_3OBr^2H_6$ requires 502.3279).



4-Cyclobutoxy-2-(*N*,*N*-dimethylamino-d_6)-6-hexadecylpyrimidin-5-ol (2.32).[118] To a stirred solution containing 270 mg (0.54 mmol) of **2.96** in 10 mL of anh THF at –5 °C was added 429 μL (1.07 mmol) of a 2.5 M solution of *n*-BuLi in hexane and 181 μL (1.62 mmol) of trimethoxyborane. The reaction mixture was stirred at 23 °C for 30 min followed by addition of 808 μL (11.9 mmol) of H_2O_2 (50% v/v). The reaction mixture

was stirred for additional 30 min, poured into 20 mL $NaHCO_3$ and then extracted with 100 mL of CH_2Cl_2. The combined organic phase was washed with brine, dried ($MgSO_4$) and concentrated under diminished pressure. The residue was purified by chromatography on a silica gel column (15 × 3 cm). Elution with 97:3 hexane–EtOAc afforded **2.32** as a colorless powder: yield 160 mg (67%); mp 72-73 °C; silica gel TLC R_f 0.53 (4:1 hexane–EtOAc); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.88 (t, 3H, J = 6.8 Hz), 1.14-1.44 (m, 26H), 1.68 (m, 3H), 1.83 (m, 1H), 2.14 (m, 2H), 2.43 (m, 2H), 2.61 (m, 2H), 4.58 (br s, 1H) and 5.19 (m, 1H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.7, 14.3, 22.8, 27.9, 29.5, 29.7, 29.72, 29.8, 29.82, 29.9, 31.0, 31.5, 32.1, 70.6, 127.0, 155.2, 156.2 and 157.2; mass spectrum (APCI), *m/z* 440.4119 $(M+H)^+$ ($C_{26}H_{42}N_3O_2{}^2H_6$ requires 440.4123).

Cell Lines and Culture Conditions

Human mitochondrial disease cell lines, Friedreich's ataxia lymphocytes (GM15850), and Leigh's syndrome lymphocytes (GM13740) were obtained from Coriell Cell Repositories (Camden, NJ). Lymphocytes were cultured in RPMI-1640 medium (Gibco, Life Technologies, Grand Island, NY) with 15% fetal calf serum, 2 mM glutamine (HyClone, South Logan, UT) and 1% penicillin–streptomycin antibiotic supplement (Cellgro, Manassas, VA). Cells were passaged every other day to maintain them in log phase growth and kept at a nominal concentration of 5-10×10^5 cell/mL. A CoQ_{10} deficient lymphocyte cell line (GM17932) was obtained from Coriell Cell Repositories. A nutrient sensitized screening strategy to identify CoQ_{10} analogues that function within the mitochondrial respiratory chain was used by growing the CoQ_{10}-deficient lymphocyte in galactose containing media to force energy production

predominantly through oxidative phosphorylation rather than glycolysis.[117,118,135,136] The lymphocytes were cultured in RPMI 1640 glucose free medium (Gibco, Grand Island, NY) supplemented with 25 mM galactose, 2 mM glutamine and 1% penicillin–streptomycin, and 10% dialyzed fetal bovine serum (FBS) (<0.5 µg/mL) (Gemini Bio-Product, West Sacramento, CA).

NADH Oxidase Activity

The effect of the exogenous CoQ_{10} analogues (Table 2.1) on the activities of complexes I, III and IV within the respiratory chain was evaluated using bovine heart mitochondria during the co-oxidation of their respective substrate (NADH) as described previously.[116,117] Briefly, a small scale preparation of bovine heart mitochondria was prepared as described by Smith.[140] Bovine heart submitochondrial particles (SMPs) were prepared as described by Matsuno-Yagi and stored in a buffer containing 0.25 M sucrose and 10 mM Tris-HCl, pH 7.4, at –80 °C.[141] SMPs were diluted to 0.5 mg/mL. Mitochondrial complexes I, III, and IV activity were assayed at 30 °C and monitored spectrophotometrically using a Beckman Coulter DU-530 (340 nm, $\varepsilon = 6.22\ mM^{-1}cm^{-1}$). NADH oxidase activity was determined in 50 mM Hepes buffer containing 5 mM $MgCl_2$, pH 7.5, in a total volume of 2.5 mL. The final mitochondrial protein concentration was 30 µg/mL. The initial rates of NADH oxidation were calculated from the linear portion of the traces. Data are reported as the mean of three independent experiments each run in triplicate.

Lipid Peroxidation Assay

Lipid peroxidation was measured by a quantitative FACS assay using the oxidation-sensitive fatty acid probe C_{11}-BODIPY$^{581/591}$ (Molecular Probe) as described.[116-118] The degree of probe oxidation was followed using flow cytometry. Briefly, FRDA lymphocytes (5×10^5 cell/mL) were plated (1 mL in 24-well plates), treated with the test compounds and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. The following day, cells were treated with 1 μM of C_{11}-BODIPY$^{581/591}$ probe in phenol red-free media and incubated at 37 °C in the dark for 30 min. Oxidative stress was induced with 5 mM DEM in phenol red-free RPMI-1640 media for 120 min. Cells were collected by centrifugation at 300 × g for 3 min and then washed with phosphate buffered saline (PBS). Cells were resuspended in phosphate buffered saline and were analyzed immediately by FACS (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ± 15 nm emission filter. The generation of lipid peroxide was detected as a result of the oxidation of the polyunsaturated butadienyl portion of the dye, resulting in a shift of the fluorescence emission peak from red to green. In each analysis, 10,000 events were recorded after cell debris were electronically gated out. Data are reported as means ± S.E.M. (n = 3). Results were expressed as a percentage of lipid peroxidation scavenging activity.

Reactive Oxygen Species (ROS) Assay

Quantitative analysis of intracellular ROS levels in FRDA lymphocytes, challenged with 5 mM diethyl maleate (DEM) in presence or absence of the test

compounds, was obtained by FACS analysis using a dichlorodihydrofluorescein diacetate probe (DCFH-DA), as described previously.[112-117] Briefly, 1 mL of FRDA lymphocytes (5×10^5 cells) was plated in a 24-well plate, treated with the test compounds and incubated at 37 °C for 16 h in a humidified atmosphere containing 5% CO_2 in air. Cells were treated with 5 mM diethyl maleate (DEM) for 80 min, collected by centrifugation at 300 × g for 3 min and then washed with phosphate buffered saline (Life Technologies). Cells were resuspended in PBS containing 20 mM glucose and incubated at 37 °C in the dark for 25 min with 10 µM DCFH-DA. Cells were collected by centrifugation at 300 × g for 3 min and then washed with PBS. The samples were analyzed immediately by flow cytometry (C6 Accuri, BD Biosciences, San Jose, CA), using a 488 nm excitation laser and the FL1-H channel 530 ±15 nm emission filter. The generation of ROS, mainly peroxides, was detected as a result of the oxidation of DCFH. In each analysis, 10,000 events were recorded after cell debris was electronically gated out. Results obtained were verified by running duplicates and repeating experiments in three independent runs. Results were expressed as a percentage of ROS scavenging activity.

Preservation of Mitochondrial Membrane Potential ($\Delta\psi_m$)

Mitochondrial membrane potential of FRDA lymphocytes was assessed using the fluorescence probe Mitotracker TMRM (tetramethylrhodamine methyl ester; Molecular Probes, Portland, OR) as described previously.[114-118] TMRM is a lipophilic potentiometric dye which partitions between the mitochondria and cytosol in proportion to the negative membrane potential across the inner mitochondrial membrane, in

accordance with the Nernst equation.[135] Therefore, the accumulation of dye in the mitochondria and the intensity of the signal is a direct function of mitochondrial potential. Mitochondrial depolarization then causes the redistribution of dye from mitochondria into the cytosol, causing a change in signal intensity. The detection of mitochondrial depolarization using TMRM was accomplished by flow cytometry as described before.[116-118] Briefly, FRDA lymphocytes cells (5×10^5 cells) were pre-treated with or without the test compounds for 16 h. The cells were treated with 5 mM DEM for 120 min, collected by centrifugation at 300 × g for 3 min and washed with phosphate buffered saline. The cells were resuspended in PBS containing 20 mM glucose and incubated at 37 °C in the dark for 15 min with 250 nM TMRM. Cells were collected by centrifugation at 300 × g for 3 min and washed with phosphate buffered saline. Cells were resuspended in phosphate buffered saline supplemented with 20 mM glucose and were analyzed immediately by FACS (FACS Caliber flow cytometer, Becton-Dickinson) using a 488 nm excitation laser and the FL2-H channel. For each analysis 10,000 events were recorded and the percentage of cells exhibiting a high level of TMRM uptake, which reflects normal mitochondrial membrane potential, was determined and analyzed using CellQuest software (BD Biosciences). The results obtained were verified in three independent experiments. FCCP (carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone), a mitochondrial uncoupler, was used to produce a negative control. The results were verified by repeating the experiments in duplicate.

Cellular ATP Concentration Assay

The intracellular ATP content was determined by a bioluminescence assay measuring the light output from the luciferin-luciferase reaction as described previously.[117,118] Briefly, CoQ_{10} deficient lymphocytes (2×10^5 cell/mL) were plated (1 mL in 24-well plates) in glucose-free media supplemented with galactose and treated with the test compounds at final concentrations of 5, 10 and 25 µM, and then incubated at 37 °C for 48 h in a humidified atmosphere containing 5% CO_2 in air. Wells were mixed and cells in each well were transferred (100 µL) to 96-well microtiter black-walled cell culture plates (Costar, Corning, NY). The total intracellular ATP level was measured in a luminator (Clarity™ luminescence microplate reader) using an ATP Bioluminescence Assay Kit (ViaLight-Plus ATP monitoring reagent kit, Lonza, Walkersville, MD) following the manufacturer's protocol. The total ATP level was expressed as a percentage of untreated control. Data are reported as the mean of at least three independent runs.

Cytoprotection (Trypan Blue Exclusion Assay)

The cytoprotection conferred by the pyridinol derivatives was determined in six lymphocytes using the trypan blue exclusion method.[115-117] This method is used to determine the number of viable cells present in cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude trypan blue, whereas dead cells are not capable of excluding trypan blue. Briefly, lymphocytes were seeded at a density of 5×10^5 cells per mL and treated with different concentrations of the test

compounds. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air for 16 h. Oxidative stress was then induced by 5 mM diethyl maleate (DEM) treatment for 6 h. Cell viability was assessed microscopically by the use of a hemocytometer. The number of cells that absorbed the dye and those that excluded the dye were counted, from which the percentage of nonviable cell number over total cell number was calculated. Cytoprotection by the test compounds was assessed with respect to the untreated controls. Cells not treated with DEM had >90% cell viability whereas DEM treatment reduced cell viability to <20%. The cell viability was expressed relative to the vehicle control (DMSO only) group (n = 3).

CHAPTER 3

PHARMACOKINETIC AND PHARMACOLOGICAL EVALUATION OF PYRIDINOL AND PYRIMIDINOL ANALOGUES

3.1. Introduction

The traditional method for drug discovery involves the preparation of test compounds followed by appropriate *in vitro* and *in vivo* biological evaluation. The resulting lead compounds are further evaluated for their metabolic and pharmacokinetic properties, and their toxic effects. Often, such projects are impacted due to the discovery of adverse effects of the lead compounds or their metabolites late in the discovery process.[142] Currently, evaluation of drug metabolism, toxicity and pharmacokinetic characteristics are determined at an early stage. Information regarding the metabolic labilities of candidate drugs, the nature of their metabolites and their elimination route are important in choosing an optimized drug candidate for animal models, so as to avoid failures late in the drug discovery process.[143,144]

There are several ways in which a potential drug can be administered; for convenience and compliance the oral route is usually preferred. A drug taken orally is absorbed through the gut wall and then passes through the liver. After surviving liver enzymes (first pass) the drug candidate proceeds to the target organs/receptors via circulation in the blood. The amount of drug in the blood/plasma circulation, and ultimately in the target organ(s), defines its oral bioavailability (Figure 3.1).[144]

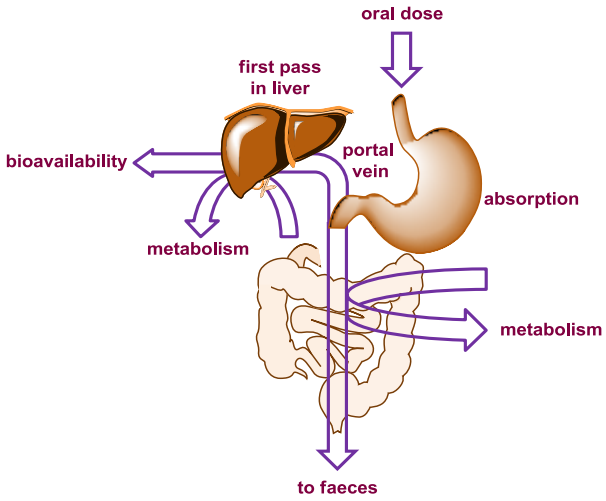


Figure 3.1. Schematic of Route Followed by a Drug After Oral Dosage. Adapted From Ref. 144.

The xenobiotics (foreign compounds) in the liver after oral absorption are metabolized by oxidative enzymes which eventually affect their half life and oral bioavailability. These enzymes are classified into two categories, namely phase I and phase II (Figure 3.2).[145,146] Phase I enzymes alter the molecule by adding a functional group or by exposing a functional group (e.g., reduction of ketones or aldehydes to the corresponding alcohols; reduction of azo or nitro compounds to the corresponding amines). Cytochrome P450 (CYP), xanthine oxidase (XO), aldehyde oxidase (AOX), monoamine oxidase (MAO), hydrolases and flavin-containing monooxygenase (FMO) are phase I enzymes. Uridine 5'-diphospho-glucuronosyltransferase/UDP-glucoronosyltransferase (UGT), glutathione *S*-transferase (GST), sulfotransferase (SULT), and *N*-acetyltransferase (NAT) represent examples of phase II enzymes. Phase II enzymes detoxify the xenobiotics by conjugation reactions, such as glutathione conjugation, sulfation and glucoronidation.[147] Detoxification can be done solely by phase

I or phase II enzymes, or by a series of reactions involving functionalization by phase I enzymes, followed by conjugation by phase II enzymes.[148]

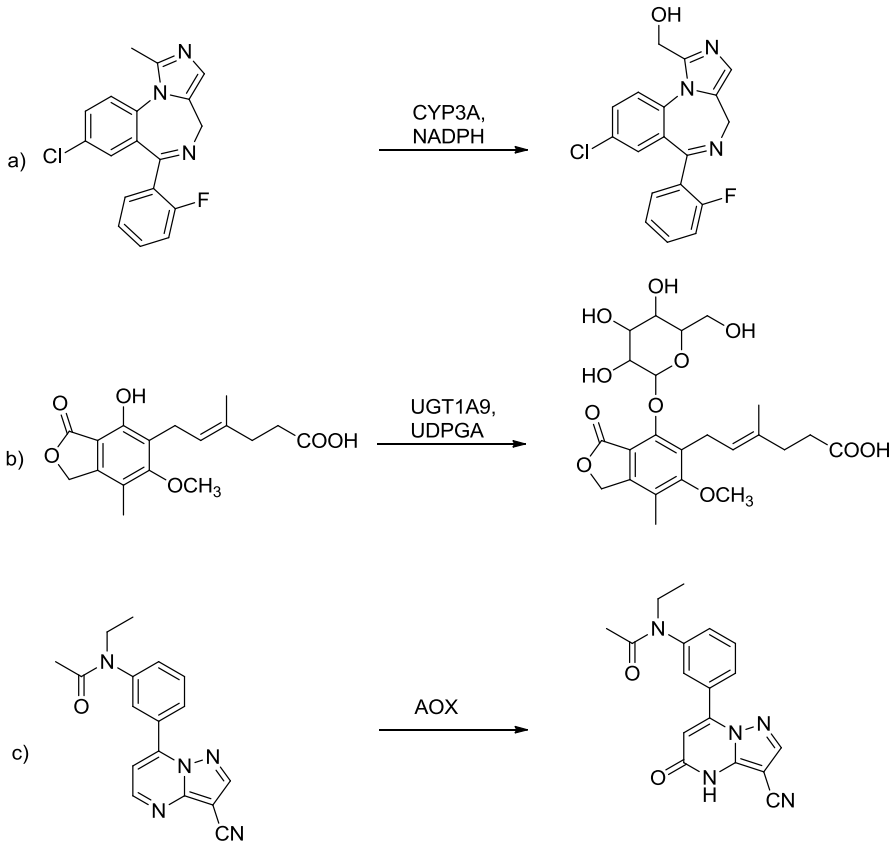


Figure 3.2. Metabolites Generated by CYP, UGT and AOX.[148]

A study in 2002 involving of the top 200 prescribed drugs revealed that 73% of the drugs were eliminated by hepatic clearance, 25% were eliminated through the kidneys and the remaining 2% by biliary clearance (Figure 3.3).[149] Cytochrome P450 enzymes were responsible for the drugs eliminated in the liver, reflecting the importance of cytochrome P450 in drug metabolism.[148] Cytochrome P450 enzymes, which include 57 different species, are present mostly in human liver, but they are also expressed in the kidney, intestines, adrenal glands and other tissues.[150,151] UGT and cytochrome P450

enzymes are located in the endoplasmic reticulum (ER) as they are membrane bound proteins and are isolated as a liver microsomal fraction. Soluble enzymes such as AOX, XO and SULT are found in the cytosol, which is referred to as the S9 fraction.[152]

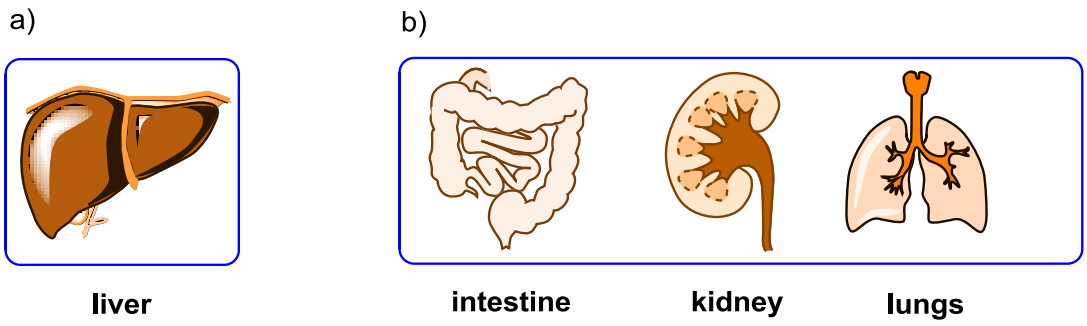


Figure 3.3. Organs Involved in Drug Metabolism (a) Major Drug Metabolizing Organ (b) Minor Drug Metabolizing Organs.

The catalytic cycle of cytochrome P450-mediated metabolism was first proposed in 1968 and over the years much refinement of the mechanism has been realized. Cytochrome P450-mediated biotransformations are mostly oxidative in nature. The enzymes involved are thereby referred to as mixed-function oxidases or monooxygenases.[153,154] The biotransformation of xenobiotics (RH) to oxidized metabolites (ROH) is represented by the following equation:[155,156]

$$NADPH + H^+ + O_2 + RH \longrightarrow NADP^+ + H_2O + ROH$$

Cytochrome P450 induced metabolism mainly includes heteroatom oxygenation (*N*-oxygenation), *C*-hydroxylation (aromatic/aliphatic), epoxide formation, *S*-oxidation, *O*-dealkylation, heteroatom release (*N*-dealkylation) and 1,2-migration reactions. The first step of the catalytic cycle involves binding of oxidized iron (Fe^{3+}) and substrate. In

the next step, one electron is transferred from NADPH-dependent cytochrome P450 reductase (flavoprotein or iron-sulfur protein) to the cytochrome P450–substrate complex which reduces the ferric ion cofactor (Fe^{3+}) to the ferrous (Fe^{2+}) form. The reduced cytochrome P450–substrate complex binds to molecular oxygen; it is believed that the complex so formed undergoes one electron reduction either by cytochrome P450 reductase–NADPH and/or cytochrome b_5 reductase–NADPH to generate a peroxide dianion–P450 (Fe^{3+})–substrate complex. At stage 5, a water molecule is released from the intermediate to give the activated oxygen–P450–substrate complex. The activated oxygen in the complex is transferred to substrate as it is highly electron deficient and a potent oxidizing agent. The oxidized substrate (ROH) is liberated from the enzyme complex and regenerates the oxidized form which is the resting state of cytochrome P450 (Figure 3.4).[157,159]

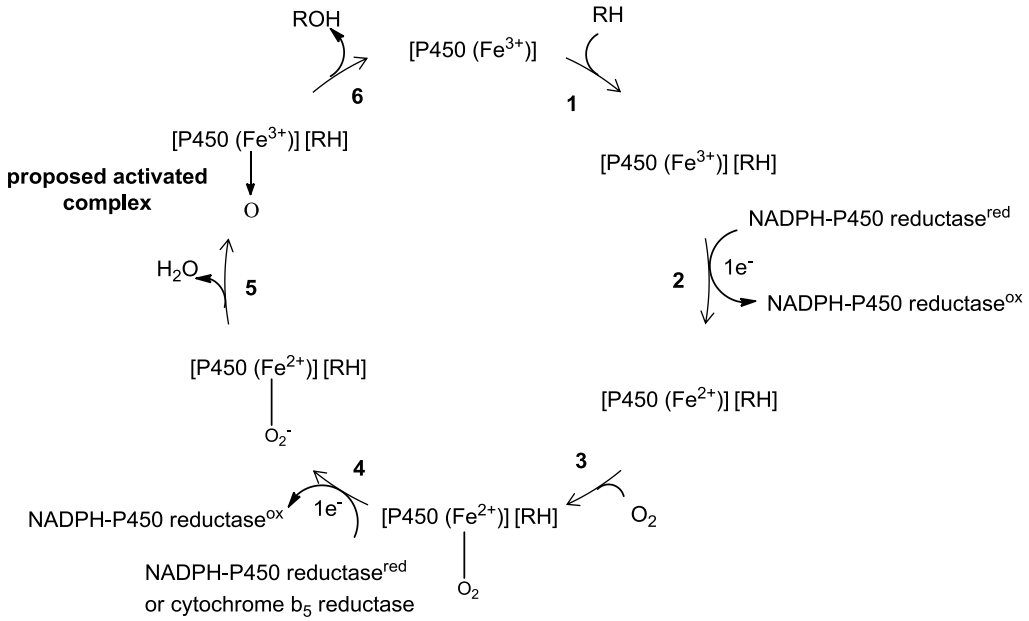
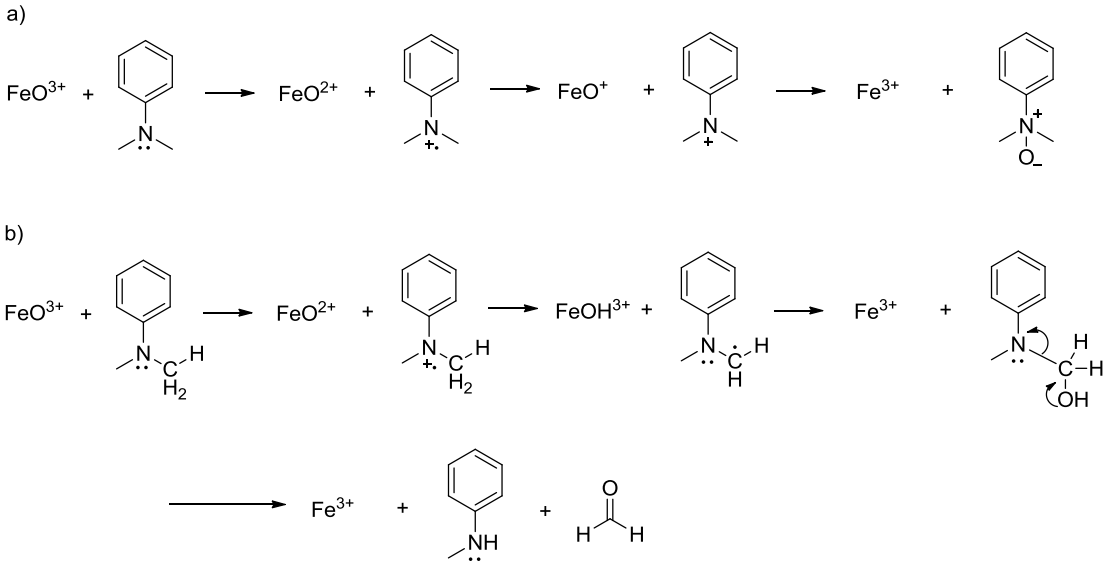
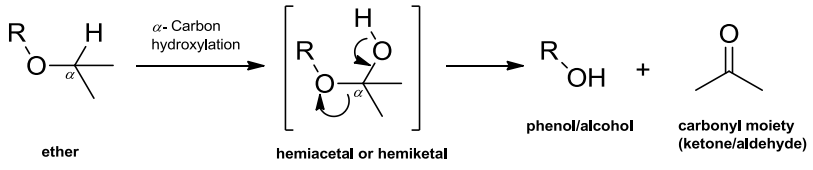


Figure 3.4. General Catalytic Cycle for a Cytochrome P450-mediated Oxidative Biotransformation. Adapted From Ref. 158 and 159.

Hence drug discovery and lead identification can be reinforced by assessing the metabolic fate of orally administered drugs using simple microsomal incubation experiments. Accordingly, *in vitro* microsomal studies were designed and carried out using bovine liver microsomes to screen available pyridinol and pyrimidinol analogues of our multifunctional radical quenchers (MRQs) for their metabolic stability. The exocyclic heteroatom present in these analogues can undergo the following transformations:[160,161]



Scheme 3.1. (a) Oxygenation of Heteroatom N by two Subsequent 1e$^-$ Transfer/Oxygen Rebound. (b) 1-Electron Transfer, Proton Abstraction and Oxygen Rebound Steps Occurring During *N*-dealkylation.



Scheme 3.2. Cytochrome P450-mediated Oxidation of Carbon-Oxygen Bond.[159,162]

3.2. Results and Discussion

Potential drug candidates are expected to exhibit pharmacokinetic parameters consistent with reasonable bioavailability. The metabolic fate of many orally administered drugs is often a function of clearance in the liver.[85,86] Accordingly, *in vitro* microsomal incubations were performed on available pyridinol, pyrimidinol and quinone analogues of our MRQs to assess their percentage recovery after incubation with bovine liver microsomes. The structures of the pyridinol analogues evaluated are shown in Figure 3.5.

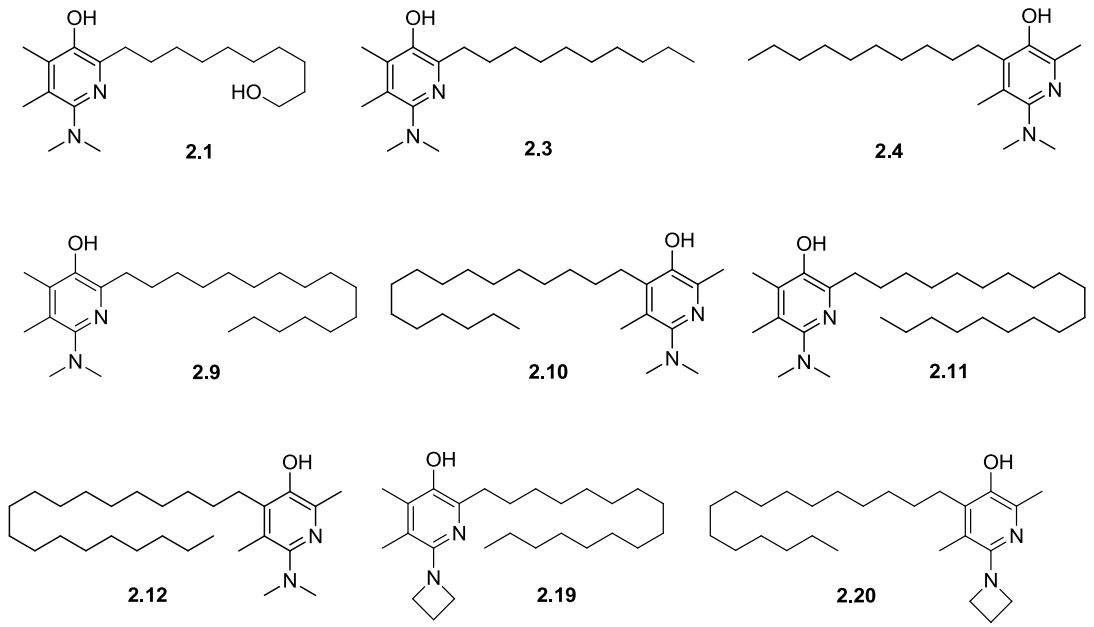


Figure 3.5. Structures of the Pyridinol Analogues Evaluated for Metabolic Stability in Bovine Liver Microsomes.

Reversed phase HPLC analysis afforded the results summarized in Figures 3.6, 3.8 and 3.10. Compound **2.1**, having a side chain the same as that found in idebenone,

was substantially degraded following 30 minutes of microsomal incubation (~60% degradation), consistent with an earlier metabolic study of idebenone, in which oxidation of the side chain was found.[163] The omission of the side chain OH group from compound **2.1** (affording **2.3**) resulted in a substantial improvement in metabolic stability (~25% degradation). Increasing the side chain length (from 10 to 16 carbon atoms) afforded compounds (**2.9** and **2.10**) having about the same microsomal stability (~20-25% degradation).

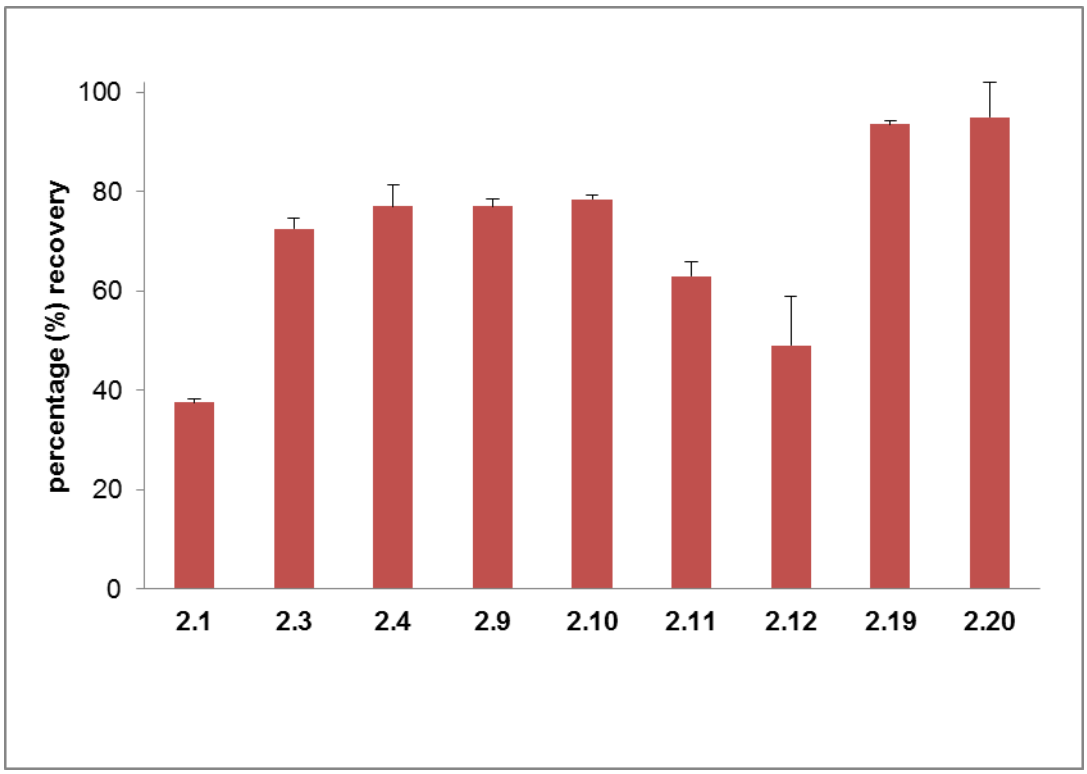


Figure 3.6. Microsomal Stability of Compounds Expressed as Percent of Compound Recovered After Reaction With Activated Microsomes for 30 Minutes. Microsomal Stability Values Represent Means ± SD. The Microsomal Incubation Assay for Compounds **2.11** and **2.12** was Performed by Dr. Yana Chen.

Isomeric compounds **2.11** and **2.12** having 19 carbon atom side chains, were less stable in this assay with 50-65% recovery. The exocyclic *N*,*N*-dimethylamino groups in compounds **2.9** and **2.10** are susceptible to oxidative demethylation by liver microsomes. This is consistent with the observed 70-80% recovery of compounds **2.3**, **2.4**, **2.9** and **2.10** after a 30-minute incubation. The effects of alteration of the *N*,*N*-dimethylated amino group, providing compounds **2.19** and **2.20**, was also studied under the same conditions. The azetidine analogues (**2.19** and **2.20**) were recovered in good yields (~95%) after a 30-minute microsomal incubation. Compounds **2.19** and **2.20** appear to possess sufficient metabolic stability to enable their study *in vivo* using animal disease models.

In order to diversify the study, compounds with different heterocyclic cores were included for evaluation in bovine liver microsomes. The structures of the pyridinol, pyrimidinol and quinone analogues included in the study are shown in Figure 3.7. The primary aim of the study was to identify sites in chosen compounds prone to oxidative damage from CYP450 and then design metabolically more stable compounds on the basis of the results obtained in the microsomal incubation assays that can further be evaluated in animal disease models.

Pyridinol analogues **2.8** and **2.23** showed good recovery. The lead compound, **2.2**, showed only 60% recovery, presumably due to the presence of two susceptible oxidative sites (–OMe and $–NMe_2$). The structurally modified analogues (**2.26** and **2.28**) similar to lead compound **2.2** in pyrimidinol series were recovered in good yields (80% and 77%, respectively) after a 30-minute microsomal incubation. Compound **3.1**, which had a modified chain with one oxygen atom in the middle of the chain, did not show any

improvement over compound **2.2** and was recovered only in 61% yield, again due to the presence of two sites susceptible to oxidative transformation. Improved recovery (82%) was observed for compound **3.2**, with one less labile site (replacement of –OMe with –Me) as compared to **2.2**.

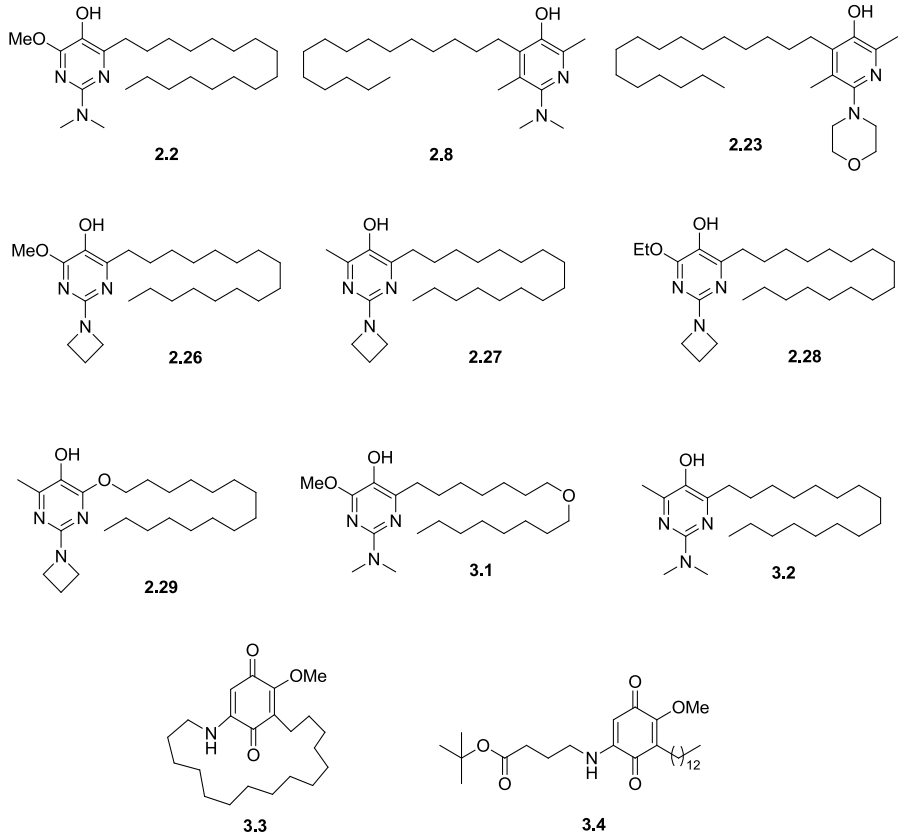


Figure 3.7. Structures of the Compounds Evaluated for Oxidative Metabolism in Bovine Liver Microsomes. Compounds **2.2**, **3.1**, **3.2**, **3.3** and **3.4** Were not Prepared as Part of This Thesis, but Were Included in the Assay. Compounds **2.2** and **3.2** Were Synthesized by Dr. Pablo M. Arce, Compound **3.1** was Synthesized by Cameron Cripe, Whereas Compounds **3.3** and **3.4** Were Synthesized by Dr. Manikandadas M. Madathil.

Compound **2.27**, having an exocyclic azetidine substituent in place of the *N*,*N*-dimethyl amino group (compound **2.2**), underwent only 13% degradation after a 30-min microsomal incubation. Compound **2.29** was designed to have a long chain and alkoxy

ether linkage that could be introduced in just one step, thereby avoiding the synthetic step involving *n*-BuLi which generally proceeds in low yield. As expected, **2.29** showed better recovery (80%) as compared to **2.2** but this modification drastically affected the efficacy of the compound in biochemical and biological assays (data not shown). Recovery for quinone analogues **3.3** and **3.4** following microsomal treatment were low (57% and 75%, respectively), which might have been anticipated based on to the presence of the –OMe group (Figure 3.8).

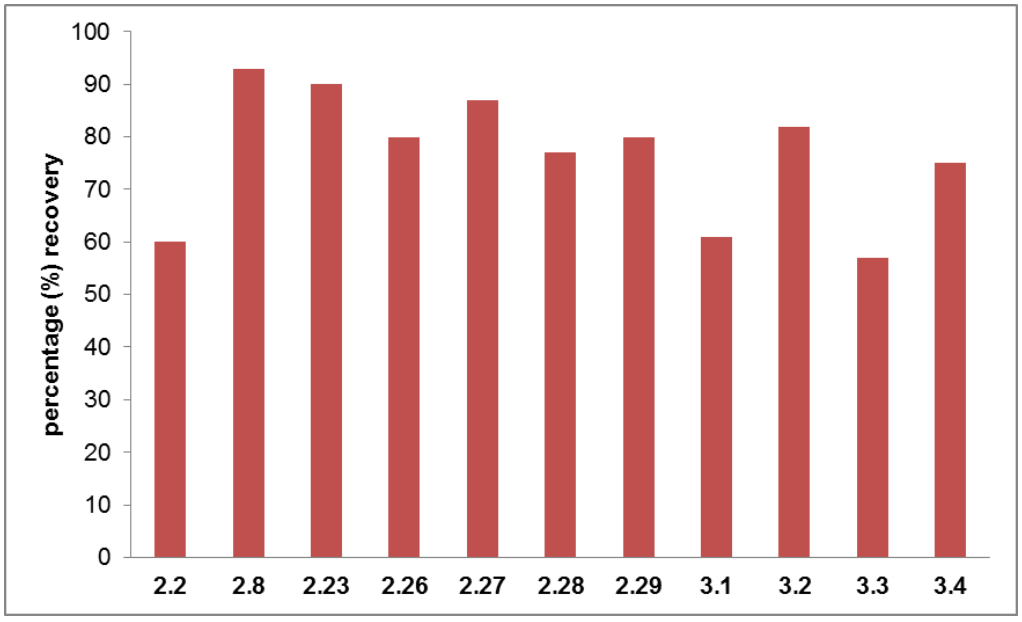


Figure 3.8. Microsomal Stability of Compounds Expressed as Percent of Compound Recovered After Treatment With Activated Microsomes. The Microsomal Incubation Assay for Compounds **2.28** and **2.29** was Performed by Dr. Yana Chen.

In further evaluation of pyrimidinol analogues in bovine liver microsomes, pyrimidine (**2.2**) was included as a negative control, as it was unstable metabolically presumably due to oxidation of –OMe and $–NMe_2$ functionalities. Pyridinol **2.20** was

again included as a positive control, since it was the most stable compound and had shown the highest recovery (95%) with another batch of bovine liver microsomes. After the preliminary screening of a number of compounds and identification of plausible sites of oxidation by CYP450, new analogues were designed, synthesized and evaluated biochemically. New pyrimidinol analogues included deuterated pyrimidinol **2.31** and pyrimidinols with a cyclic ether (**2.30** and **2.32**). These compounds were evaluated for their metabolic labilities to treatment with bovine liver microsomes (Figure 3.9).

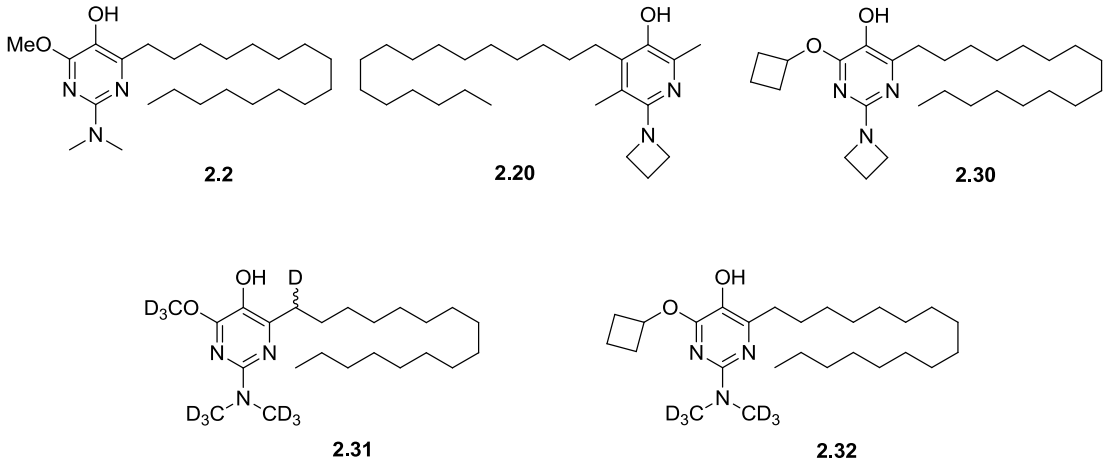


Figure 3.9. Structures of the Analogues Evaluated for Oxidative Lability in Bovine Liver Microsomes, Employing a New Microsome Preparation.

Compound **2.2**, having a methoxy group, was substantially degraded within 30 minutes of microsomal incubation (~48% degradation), reflecting a slightly lesser recovery than the earlier metabolic study of compound **2.2** (Figure 3.8). The azetidine analogue (**2.20**) was also recovered in only 77% yield, as compared to 95% yield under the same experimental conditions, but with a different batch of bovine liver microsomes. The deuterated analogue **2.31** showed substantial improvement in stability as compared

to its counterpart, compound **2.2**. The recovery of compound **2.31** after 30 minutes of microsomal incubation was comparable to the recovery observed for compound **2.20** (75% and 77%, respectively). The stability and hence the recovery of the pyrimidinol was further improved with the introduction of a cyclic ether (compounds **2.30** and **2.32**) instead a linear ether linkage to the heterocyclic core. Compounds **2.30** and **2.32** were degraded only to the extent of ~10% after a 30-minute microsomal incubation (Figure 3.10). In compound **2.31** all the C-H bonds were replaced with C-D bonds for –OMe and $–NMe_2$ and that resulted in a metabolically more stable compound as compared to **2.2**. It can be explained on the basis of kinetic isotopic effect ($\sim k_D/k_H$) due to which C-D bonds are more stable than C-H bonds.[164] Replacement of –OMe group with cyclic ether as in **2.30** and **2.32** increased the steric bulk which could be the reason for the better recovery (90% and 93%, respectively) observed for these two compounds.

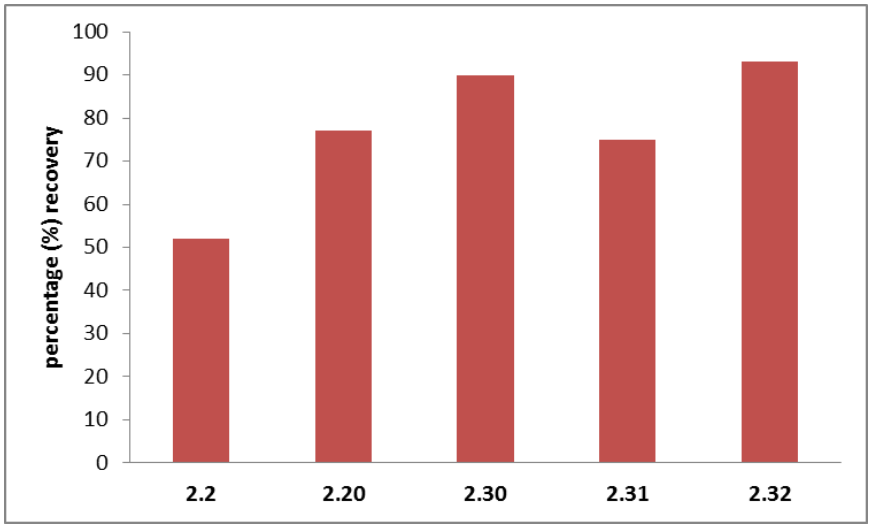


Figure 3.10. Microsomal Stability of Compounds Expressed as Percent of Compound Recovered After Reaction With Activated Microsomes. Microsomal Incubation Assay for Compounds **2.30** and **2.32** was Performed by Dr. Yana Chen.

Chromatogram obtained from the reversed phase HPLC for assessing the percentage recovery are shown in Figures 3.11 to 3.17. HPLC profile of the compound **2.2** with elution at 15.4 min. is shown in Figure 3.11. Internal standard, fluorene, was eluted at 9.6 min. (Figure 3.12). HPLC profiles for compound **2.2** after 30 minutes of deactivated and activated microsomal incubation are shown in Figures 3.13 and 3.14 respectively. Figure 3.15 shows the HPLC profile of compound **2.31** with elution at 15.2 minutes. Compound **2.31** HPLC profiles after 30 minutes of deactivated and activated microsomal incubation are shown in Figures 3.16 and 3.17, respectively.

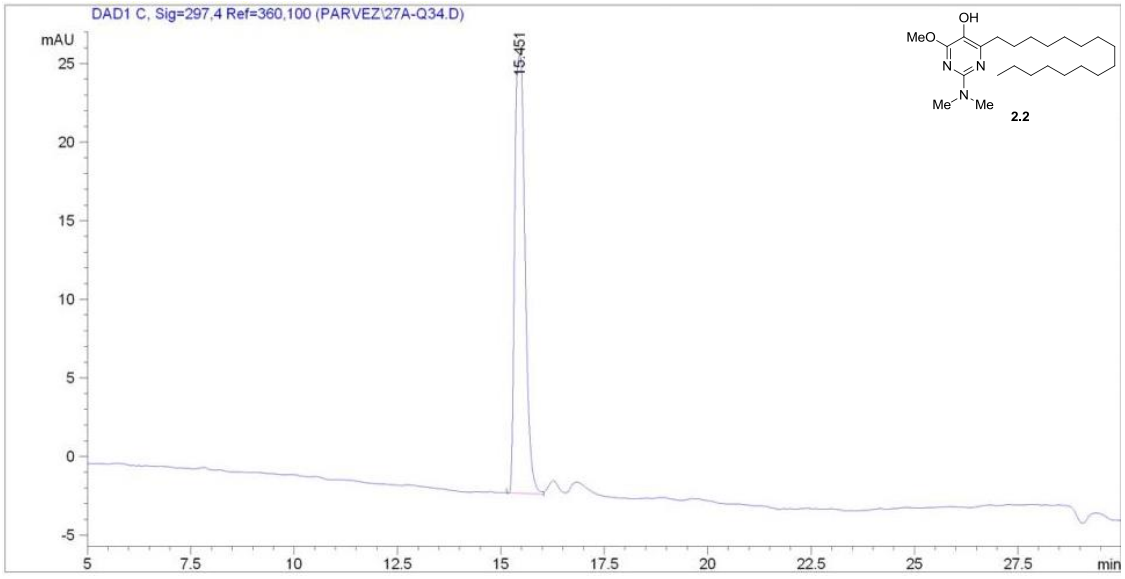


Figure 3.11. HPLC Profile for Compound **2.2** (λ_{max} 297 nm).

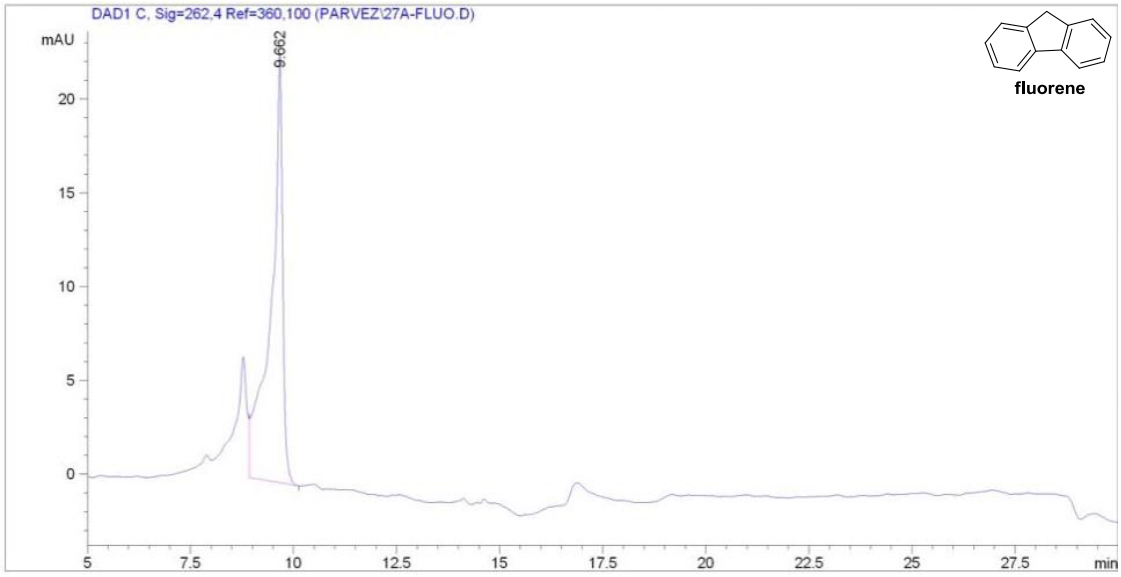


Figure 3.12. HPLC Profile for Internal Standard Fluorene (λ_{max} 262 nm).

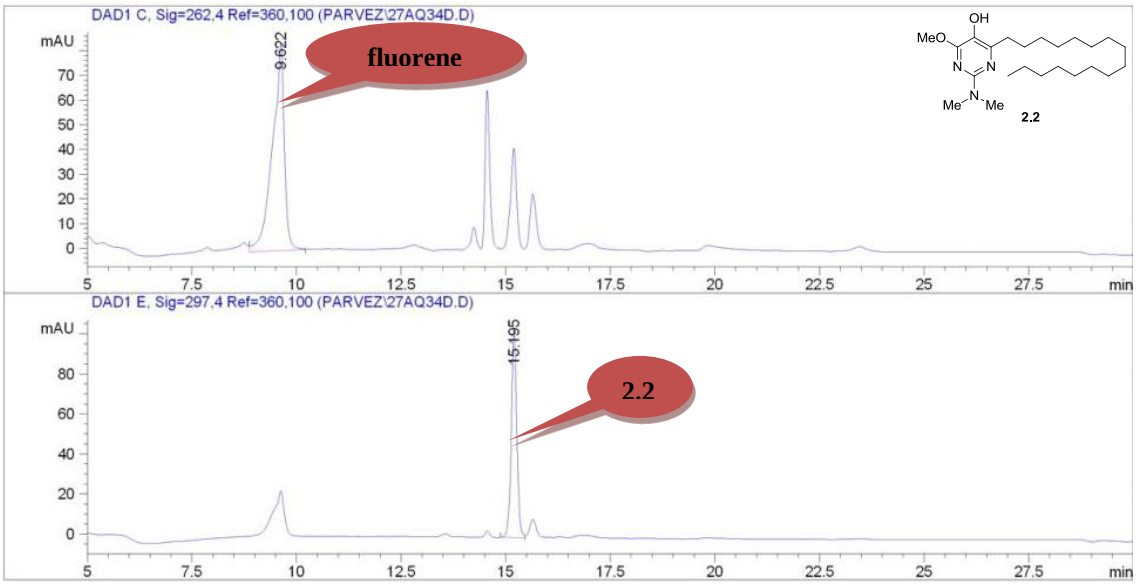


Figure 3.13. HPLC Profile for Compound **2.2** After a 30-minute Incubation in Deactivated Bovine Liver Microsomes.

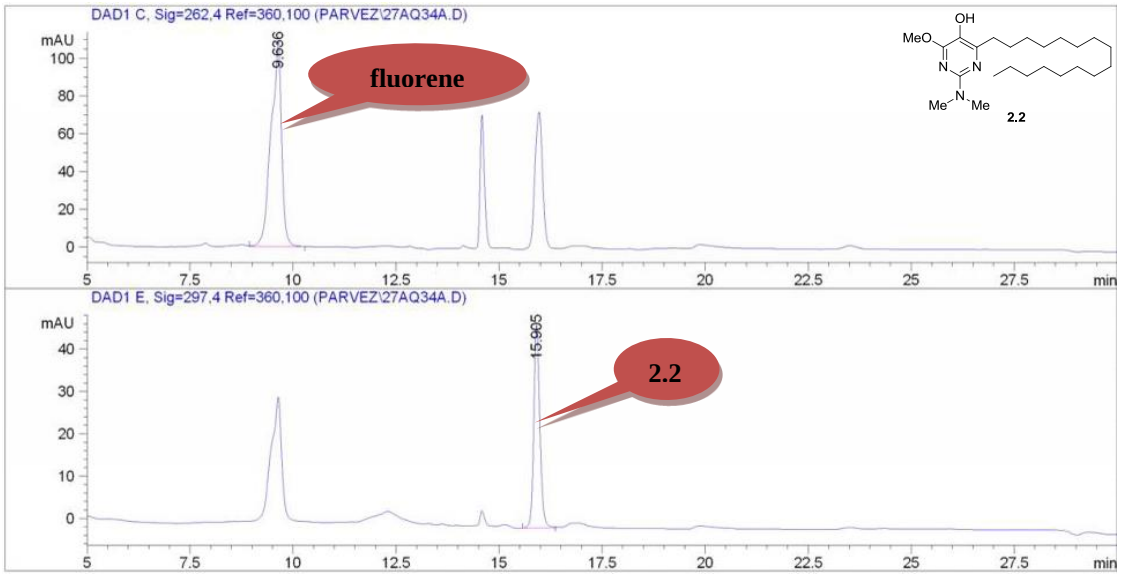


Figure 3.14. HPLC Profile for Compound **2.2** After a 30-minute Incubation in Activated Bovine Liver Microsomes.

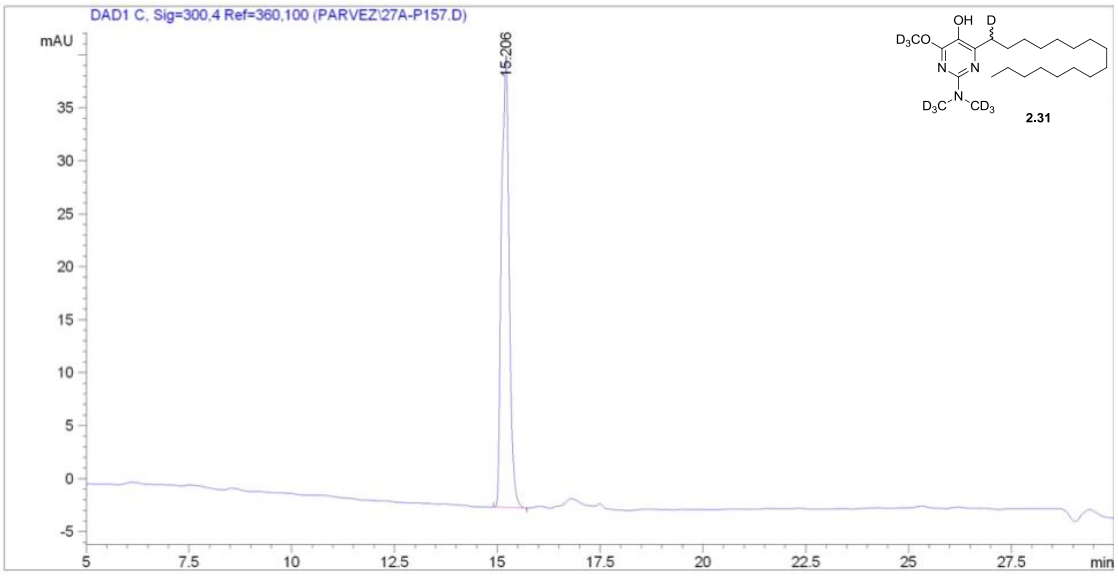


Figure 3.15. HPLC Profile for Compound **2.31** (λ_{max} 300 nm).

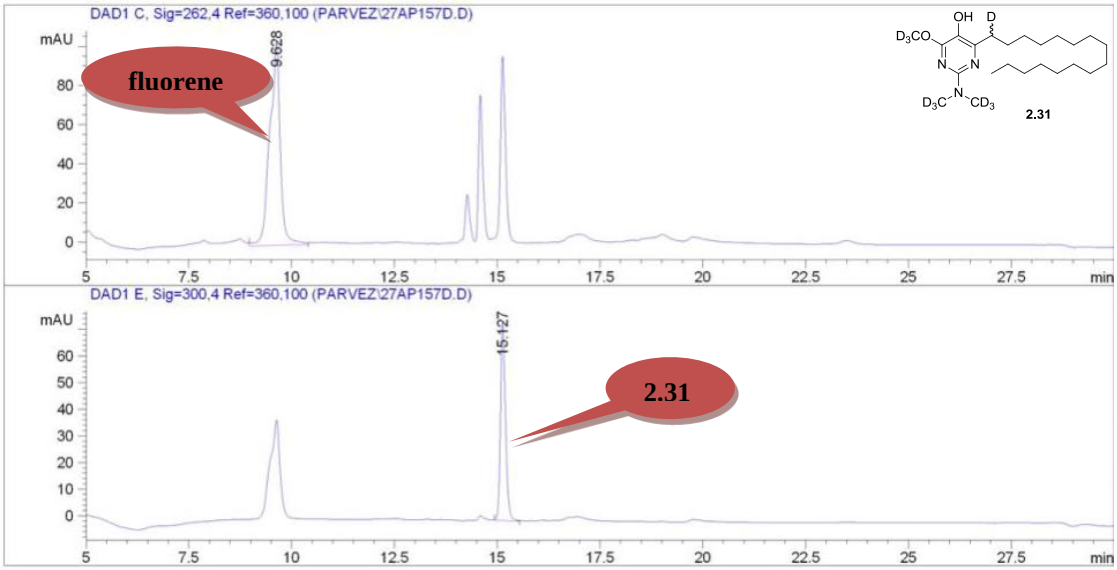


Figure 3.16. HPLC Profile for Compound **2.31** After a 30-minute Incubation in Deactivated Bovine Liver Microsomes.

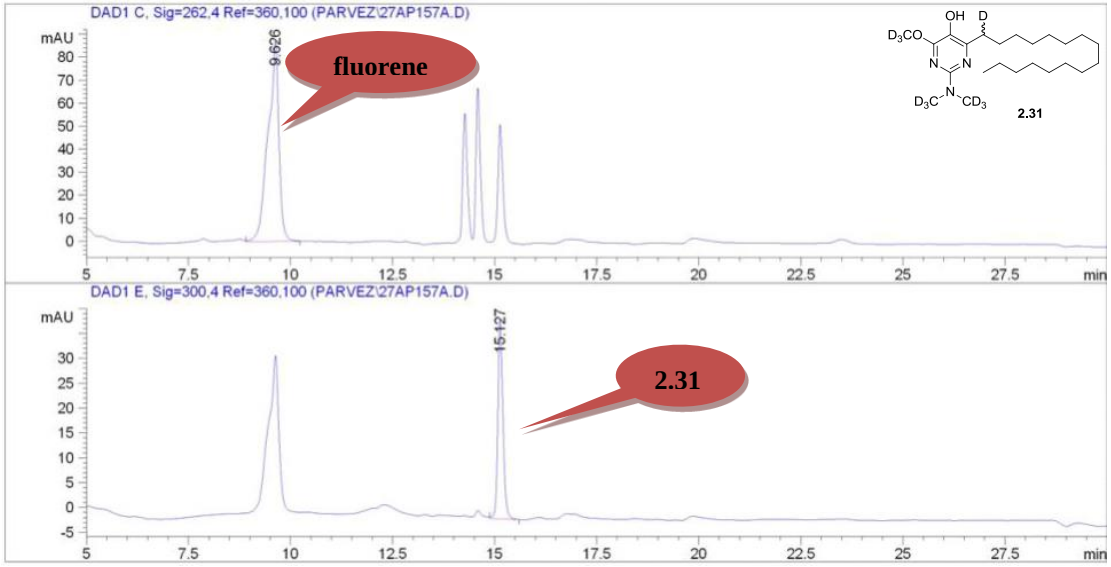


Figure 3.17. HPLC Profile for Compound **2.31** After a 30-minute Incubation in Activated Bovine Liver Microsomes.

3.3. Experimental

3.3.1. Microsomal Enzyme Preparation

Bovine liver microsomes were prepared from liver of a freshly slaughtered animal as previously reported, with some modifications.[165] Briefly, liver tissues were diced into small pieces and then washed with isotonic sucrose buffer (0.25 M sucrose, 10 mM Tris-HCl, 0.5 mM EDTA, pH 7.8). The diced tissue was passed through a precooled meat grinder and mixed with three-fold ice cold sucrose buffer supplemented with a mixture of protease inhibitors. The suspension was homogenized in a Waring blender for 25 sec at high speed. At this stage, the pH of the suspension was adjusted to 7.4 with 1 M Tris base. The homogenate was centrifuged for 20 min at 1200 × g to remove cell debris. The supernatant suspension was homogenized in a tight fitting Teflon-glass Potter-Elvejhem homogenizer and then centrifuged twice at 10,000 × g for 20 min, collecting the supernatant each time to remove mitochondria. The floating fat layer was carefully removed by filtering the supernatant through layers of cheesecloth. The supernatant was centrifuged at 150,000 × g for 30 min (Beckman-Coulter ultracentrifuge, XL-100K -01, SW 55 Ti rotor). The pellet (microsomal fraction) was suspended in 0.25 M sucrose buffer containing 10 mM Tris-HCl, pH 7.4, with 20% (v/v) glycerol, and centrifuged once more at 150,000 × g. The pellet was resuspended in sucrose buffer with 20% (v/v) glycerol. The protein concentration after resuspension was approximately 20 mg/mL, as determined by BCA protein assay (Pierce Chemical) using bovine serum albumin as a standard. Aliquots of microsomal suspensions were stored at –80 °C (Figure 3.18).

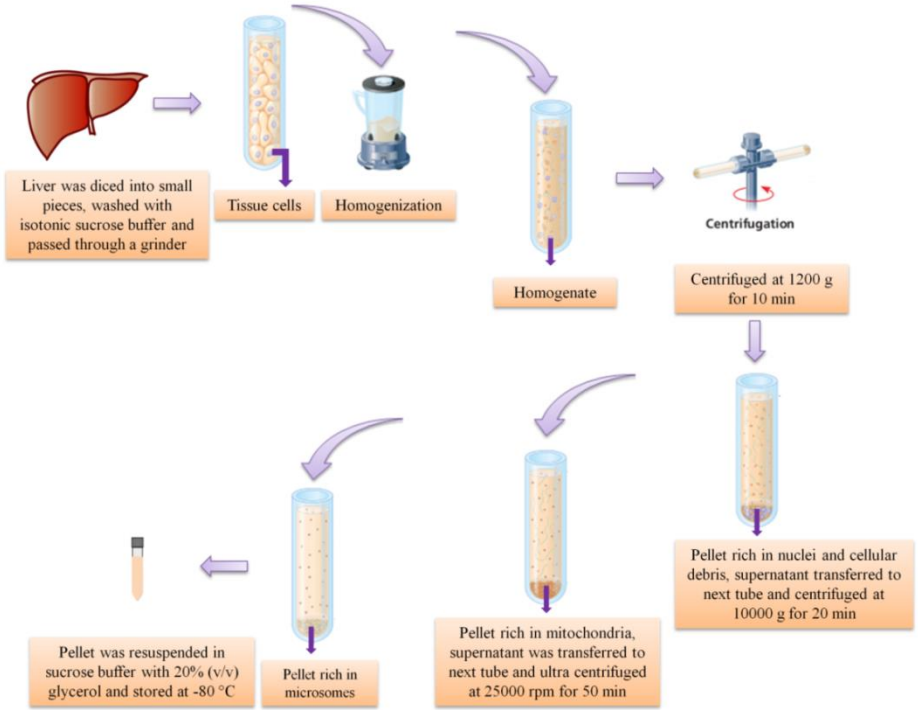


Figure 3.18. Schematic Diagram of Microsomal Preparation From Bovine Liver. Adapted From Ref. 166.

3.3.2. Microsomal Stability Assay

In vitro metabolic stability was determined in bovine liver microsomes at a protein concentration of 1 mg/mL in 50 mM phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$ in a final incubation volume of 0.5 mL. Each test compound was added to a final concentration of 25 µM. This mixture was pre-warmed to 37 °C prior to starting the reaction by the addition of β-NADPH to 1 mM final concentration. After incubation for 30 min at 37 °C, the reaction was quenched by the addition of 1 mL of propanol, vortexed for 2 min and centrifuged at 15,000 × g for 20 min to pellet the precipitated protein. The resulting supernatant was removed by pipetting and then concentrated under diminished pressure. A parallel incubation of the test compound with deactivated

microsomes lacking β-NADPH and quenched immediately with propanol served as a control and was run for each test agent to detect microsome-independent degradation. The sample was reconstituted in 130 µL MeOH and centrifuged again at 15,000 × g for 3 min. The supernatant was removed and 4 µM fluorene was added as an internal standard prior to HPLC analysis. HPLC analyses were performed on a reversed phase Zorbax SB-Phenyl reversed phase analytical (150 × 4.6 mm, 5 µm) HPLC column using a mobile phase consisting of MeOH/H_2O. A linear gradient of (50:50 MeOH/H_2O→ 100:0 MeOH/H_2O) was employed over a period of 14 min at a flow rate of 1 mL/min. Metabolic stability was expressed as percent of control remaining. The experiments were carried out in duplicate to verify the results for some of the compounds.

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APPENDIX A

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