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TOTAL SYNTHESIS AND ANTIMICROBIAL ACTIVITY EVALUATION OF NATURAL PRODUCTS AND THEIR ANALOGUES

BY

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Dedicated to

My Beloved Wife,

RANI,

AND

MY PARENTS

For All Your Support and Sacrifices…..

Take Risks In Your Life
IF You Win, You Can Lead!
IF You Loose, You Can Guide!

Swami Vivekananda…

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled *"TOTAL SYNTHESIS AND ANTIMICROBIAL ACTIVITY EVALUATION OF NATURAL PRODUCTS AND THEIR ANALOGUES"* which is being submitted to the **University of Milan, Milan** for the award of **Doctor of Philosophy in Chemistry** by **Rahul Dagadu Kaduskar** was carried out by him under my supervision at University of Milan, Milan. This work is original and has not been submitted in part or full, for any degree or diploma to this or any other University.

Dr. Stefania Mazzini

(Research Supervisor)

CANDIDATE'S DECLARATION

I hereby declare that the thesis entitled *"TOTAL SYNTHESIS AND ANTIMICROBIAL ACTIVITY EVALUATION OF NATURAL PRODUCTS AND THEIR ANALOGUES"* submitted for the award of degree of **Doctor of Philosophy** (Ph.D) in Chemistry, to the **University of Milan, Milan**. This work is original and has not been submitted in part or full, for any degree or diploma to this or any other university. This work was carried out by me at the DeFENS, Department of Food, Environmental and Nutritional Sciences, University of Milan, Milan, Italy.

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24th March 2017

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It has never been my intention to hurt anyone's feelings knowingly or unknowingly. In spite of my efforts, if I have caused any inconvenience to anyone, my apologies for the same.

At last but not the least, I thank whole heartedly, the omnipotent God.

Rahul Dagadu Kaduskar

CONTENTS

CHAPTER 1: GENERAL INTRODUCTION

ABBREVIATIONS

Ac	-	Acetyl
ACN	-	Acetonitrile
AcOH	-	Acetic acid
Ac_2O	-	Acetic anhydride
Ar	-	Aryl
Bn	-	Benzyl
Allyl	-	Allyl
Alloc	-	Allyloxycarbonyl
BnBr	-	Benzyl bromide
Brs	-	Broad singlet
Boc	-	*tert* -Butoxy carbonyl
$(Boc)_2O$	-	*Di-tert-butyl dicarbonate*
t-Bu	-	*tert-Butyl*
BuLi	-	Butyl Lithium
CAN	-	Ceric ammonium nitrate
Cat.	-	Catalytic/Catalyst
$CDCl_3$	-	Deuterated chloroform
COSY	-	Correlation spectroscopy
CH_2Cl_2	-	Dichloromethane
DBE	-	Double bond equivalent
d	-	Doublet
dd	-	Doublet of doublet
DCC	-	Dicyclohexylcarbodiimide
DDQ	-	2,3-Dichloro-5,6-dicyano-1,4-benzoquinone
DIBAL-H	-	Diisobutylaluminium hydride
DIPEA	-	Diisopropylethylamine
DMAP	-	*N, N'*Dimethylaminopyridine
DMF	-	*N, N'*Dimethylformamide
DMSO	-	Dimethyl sulfoxide
Et	-	Ethyl
Et_3N	-	Triethyl amine
EtOAc	-	Ethyl acetate

Et_2O	-	Diethyl ether
EtOH	-	Ethanol
g	-	grams
h	-	hours
HMBC	-	Heteronuclear Multiple Bond Correlation
HMPA	-	Hexamethylphosphoramide
HPLC	-	High performance liquid chromatography
HREI-MS	-	High resolution electrospray ionisation mass spectrum
Hz	-	Hertz
IBX	-	2-Iodoxybenzoic acid
LAH	-	Lithium aluminium hydride
LDA	-	Lithiumdiisopropylamide
LiHMDS	-	Lithium bis(trimethylsilyl)amide
m	-	Multiplate
Me	-	Methyl
mg	-	Miligram
MIC	-	Minimal inhibitory concentration
min	-	Minutes
MeOH	-	Methanol
mmol	-	mmol
MBC	-	Minimal bactericidal concentration
M.P	-	Melting point
MRSA	-	Methicillin resistant *Staphylococcus aureus*
MEM	-	2-Methoxyethoxymethyl
NaOEt	-	Sodium ethoxide
NCS	-	N-Chlorosuccnimide
NCE	-	New chemical entity
NBS	-	N -Bromosuccinimide
NIS	-	N-Iodosuccinimide
NMR	-	Nuclear magnetic resonance
NOESY	-	Nuclear overhauser effect spectroscopy
NMM	-	N-methyl morpholine
PCC	-	Pyridiniumchlorochromate
PPTS	-	Pyridinium-p-toluenesulfonate

Ph-NO	-	nitrosobenzene
PDC	-	Pyridiniumdichromate
PFP	-	Pentafluorophenol
PMB	-	Paramethoxybenzy
Ph	-	Phenyl
Pd/C	-	Palladium on carbon
ppm	-	Parts per million
p-TSA	-	*para* -Toluenesulfonic acid
Py	-	Pyridine
rt	-	Room temperature
s	-	Singlet
TBAF	-	Tetrabutylammonium fluoride
TBS	-	*tert* -Butyldimethylsilyl
TBDPS	-	*tert* -Butyldiphenylsilyl
THF	-	Tetrahydrofuran
TFA	-	Trifluoroacetic acid
TLC	-	Thin layer chromatography
TMSCl	-	Trimethylsilyl chloride

CHAPTER 1

GENERAL INTRODUCTION

1.1. NATURAL PRODUCTS:

Chemical substances biosynthesized in the living system i.e. plants, animals and microbes are called as natural products.

Natural products are mainly divided into two major classes:

1.1.1. PRIMARY METABOLITES:

Primary metabolites are the biochemical's which are utilized by the basic metabolic pathways required for life. They play enormous cellular functions such as absorption of nutrients, generation of energy, growth and development of organism. They have a broad species distribution which encompasses many phyla and more than one kingdom e.g. carbohydrates, lipids, amino acids and nucleic acids[1,2] which are the essential macromolecules of life.[3]

1.1.2. SECONDARY METABOLITES:

Contradictorily, the secondary metabolites are nonessential and are not vital for the survival of organism. Furthermore, secondary metabolites typically have a narrow species distribution. Secondary metabolites display a broad range of functions. These include pheromones; the social signaling molecules between the individuals of the same species, signaling molecules that magnetize and activate symbiotic organisms, agents which sequester, solubilize and transport nutrients (siderophores etc.) across cell membrane and defensive weapons like repellants, venoms, toxins etc. that are used against competitors, prey, and predators.[4] Secondary metabolites are not essential to survival but do increase the competitiveness of the producer[5] within its environment, because of their ability to modulate biochemical and signal transduction pathways. General structural classes of secondary metabolites include alkaloids, phenyl propanoids, polyketides, and terpenoids, peptides, fatty acids etc.

1.2. MEDICINES IN FOLKLORE:

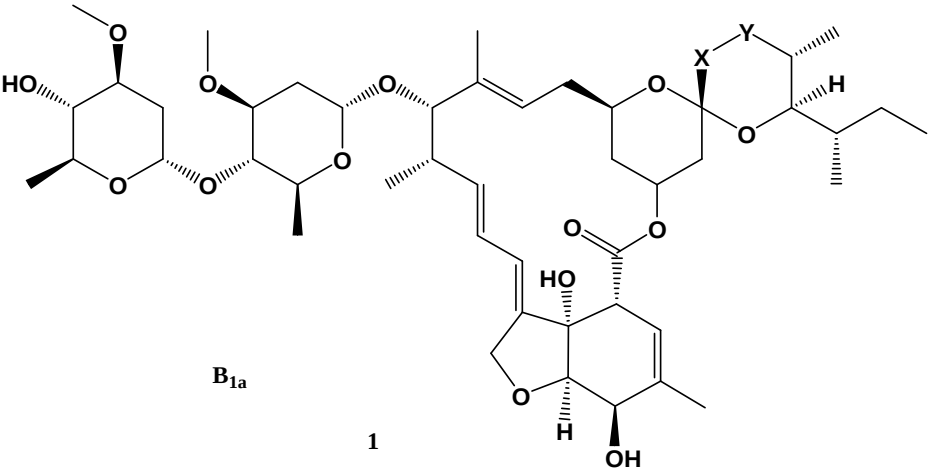
Natural products have been recognized since ancient ages as a source of medicine in the form of traditional medicines, remedies, potions, and oil for the treatment of human diseases. History of medicines dates back practically to the dawn of human civilization. The ancient civilizations of the Chinese, Indians and North Africans provide written evidence for the use of natural products as drugs.

Before the 20th century, crude and semi-pure extracts from plants, animals, microbes and minerals represented the only available medication to treat the illness. The earliest record of use of natural products as medicines was documented in 2600 B.C. The clay tablets in cuneiform from Mesopotamia depict the use of oils from *Cupressus sempervirens* (Cypress) and *Commiphora* species (myrrh) to treat cough, cold and inflammation, which are still being used. The Ebers papyrus (2900 B.C.) is an Egyptian pharmaceutical record, which documents over 700 plant based drugs ranging from gargles, pills, and infusions to ointments. The Chinese Materia Medica (1100 B.C.), Shennong Herbal (~ 100 B.C., 365 drugs) and the Tang Herbal (659 A. D., 850 drugs) are the documented records of the uses of natural products.[6] Ayurveda is the traditional healing modality of the Vedic culture from India. Ayurveda is said to be 2000 to 5000 years old which claims the use of plant extracts to cure different human diseases e.g. *Alghi maurorum* Medik (Camel thorns) aids in the treatment of anorexia, constipation, dermatosis, epistaxis, fever, leprosy, and obesity.[7] The Konkani peoples from south western India use to treat asthama by smoking the plants; whilst the Romans used to have the plant for nasal polyps.[8] The steam-produced charcoal of the fungus *Piptoporus betulinus* was used as an antiseptic and disinfectant.[9] The red algae *Chodrus crispus* and *Mastocarpus stellatus* were sources of beverages, which were popular as a folk cure for colds, sore throats, and chest infections including tuberculosis. The alga was also used for kidney trouble and burns when boiled with milk or water.[10, 11]

In the 20th century, the receptor theory of drug action revolutionized the thinking in the use of drugs. The effect of drug in the human body is mediated by the specific interaction of the drug molecules with biological macromolecules. This breakthrough led to the beginning of new era in the discovery of new drugs, as the extracts from the natural sources have replaced pure, isolated chemicals and since then it became the standard treatments for diseases. The isolation of morphine from sticky mixture of alkaloids (including codeine, morphine, noscapine, thebaine, and papaverine) by Friedrich Serturner from *Papaver somniferum* in 1806, and digoxin, a heart stimulant originating from flower *Digitalis lanata* were the classical example of isolation of bioactive ingredient and elucidation of their structures. The evolution in synthetic chemistry led to the chemical synthesis of these compounds, since then natural products have been extensively screened for their medical purposes.

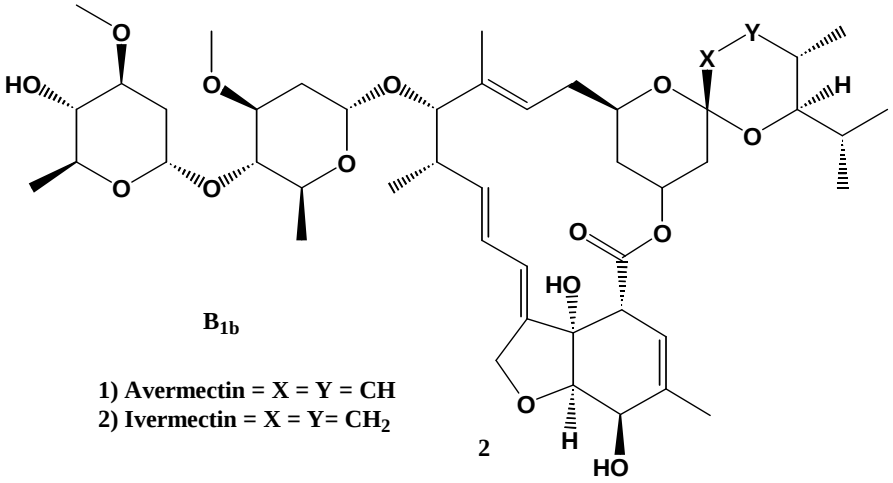
1.3. CRUCIAL ROLE OF NATURAL PRODUCTS IN DRUG DISCOVERY:

The reward of half of the 2015 Nobel Prize in medicine or physiology to Drs. Satoshi Omura and William C. Campbell for their discovery and development of antiparasitic **avermectin B_{1a}, B_{1b} (1)** and **ivermectin B_{1a}, B_{1b} (2)** complexes (figure-1), with the other half being awarded to Prof. Youyou Tu for her discovery and development of antimalerial drug **artemisinin (3)** (figure-2) highlights the imperative role of natural products in drug discovery**.**



B_{1a}

1



B_{1b}

1) Avermectin = X = Y = CH
2) Ivermectin = X = Y= CH_2

2

Figure-1: Avermectin/Ivermectin complex**.**

Nature's biosynthetic machinery continues to provide unique structural diversity in comparison to standard and/or combinatorial synthesis. The investigation of natural products brings out a variety of lead structures, which may be used as a template for the development of new therapeutic agents. Complex

natural scaffolds gains some more advantages over synthetic scaffolds; indeed, the following characteristics of the natural products make them the favorable leads in drug discovery:

1.3.1. CHEMICAL DIVERSITY:

It has been recognized that the natural products structures have the characteristic of high chemical and structural diversity. In fact, about 40% of the chemical scaffolds found in the natural products are absent in the today's medicinal chemistry. As a result of the natural selection process, natural products possess a unique and vast chemical diversity with optimal interaction with biological macromolecules.

The studies by Feher and Schmid[12] reveal that natural products typically have a greater number of chiral centers and native steric complexity, with bridgehead tetrahedral carbon atoms, rings and chiral centers than either synthetic drugs or combinatorial libraries.

Moreover, the drugs and combinatorial molecules bears significant number of nitrogen, sulphur, and halogen containing groups, whereas natural products contain more oxygen atoms.[12]

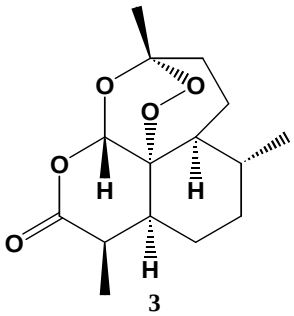
Natural products contains less number of aromatic ring atoms and heavy metal atoms compared to synthetic drugs and combinatorial libraries, nevertheless, the natural products incurs prominent molecular rigidity with more number of solvated hydrogen bond donors and acceptors.

Natural product libraries also have a broader distribution of physicochemical properties such as molecular weight, octanol-water distribution coefficient and ring diversity compared to synthetic counterparts. In fact, the currently marked drugs bears less than one fifth of the ring systems found in natural products.[12]

1.3.2. BIOCOMPATIBILITY:

Another important advantage with natural products is that they are biologically evolved. Moreover, the typical biosynthesis of natural products involves repeated interaction of biosynthetic intermediates with the modulating enzymes, and the actual biological function of many natural products comprises binding to the other macromolecules such as nucleic acids, proteins, enzymes, receptors, ion channels and carbohydrates etc. One can assume that the ability of natural products to bind with other macromolecules is biologically validated. It is a disregarded fact that most of the natural products carries an advanced binding structural motifs compared with synthetics, most likely due to the sterically complexity and typical spatial arrangement of atoms in natural products scaffolds.[13]

Pertaining to this chemo-diversity and bio-specificity, natural products have proven to be the richest source of lead compounds for new drug development.



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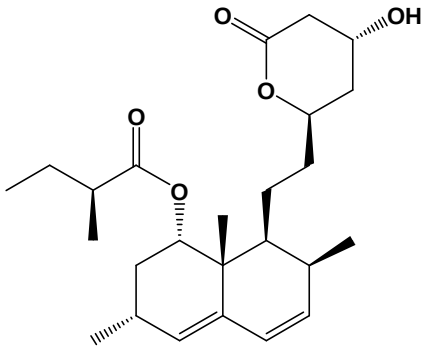
Artemesinin
Target: hemin
Antimalarial.



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Lovastatin
Target: HMG Co-A reductase, cholesterol lowering agent

Figure-2: Representative examples of natural product drugs, modulating different targets.

Indeed, 1355 New Chemical Entities (NCEs) have been reported in the years 1981-2010, of which 540 (40%) NCEs were either natural products or derivatives of natural products. In particular, 63 of the 99 (64%) small molecules anticancer drugs and 78 of the 104 (75%) antibiotics developed from 1981-2010 have emerged from natural products. The representative examples include, **artemisinin** (antimalarial) **(3)** and **lovastatin** (cholesterol lowering agent) **(4)** (figure-2).[14] Thus, the natural products can be sensed as a population of privileged structures selected by evolutionary pressures to interact with a wide variety of proteins and other biological targets to elicit specific biological response, an analogy based on the fact that natural products have become effective drugs in a wide variety of therapeutic indications.

1.4. HISTORICALLY IMPORTANT NATURAL PRODUCTS:

Natural products, including plants, animals and minerals have been the most successful source of potential drug leads. Indeed, clinical, pharmacological and chemical studies of these traditional medicines, which were predominantly derived from plants, were the basis of early medicine. The well known example to date would be the anti-inflammatory agent; acetylsalicylic acid (**aspirin**) **(5)** derived from the natural product, salicin isolated from the bark of willow tree *Salix alba* L.[15] **Morphine (6)** as mentioned earlier was a commercially important drug, first reported in 1803. The antimalarial drug **quinine (7)** isolated from the bark of *Cinchona succirubra*, has been approved by US FDA in 2004. Prior to this approval, it had been used for centuries to treat malaria, fever, indigestion, mouth and throat disease and cancer.[15] The L-histidine derived alkaloid, **Pilocarpine** (**8**), found in *Pilocarpus jaborandi* has been used as a drug in the treatment of chronic angle glaucoma and acute angle-closure glaucoma for over 100 years. In 1994, an oral formulation of **pilocarpin**e (**8**) was approved by FDA to treat dry mouth (xerostomia) which is a side effect of head and neck cancer radiation therapy. **Pilocarpine** (**8**) is also used as an stimulant for the sweat glands to measure the concentration of sodium and chloride ions in the sweat, which is used in the diagnosis of cystic fibrosis[16] **(**figure-3**).**

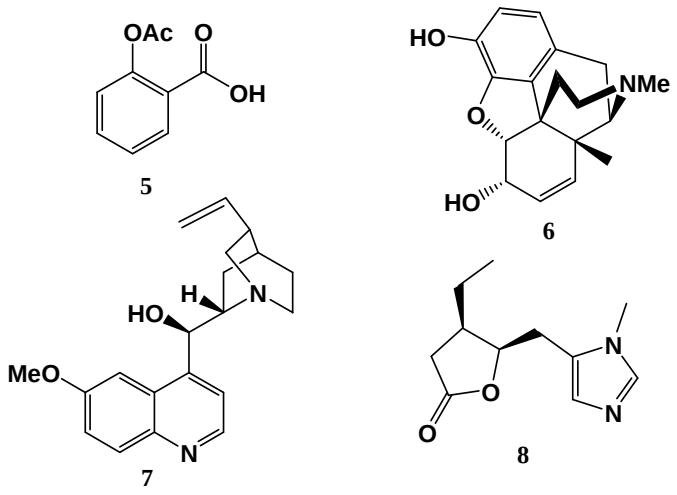


Figure -3: Acetylsalicylic acid **(5)**, Morphine **(6)**, Quinine **(7)** and Pilocarpine **(8)**.

1.5. SOURCES OF NATURAL PRODUCTS:

Origins of natural products can be broadly divided into four parts.

1.5.1. NATURAL PRODUCTS FROM MICROORGANISMS

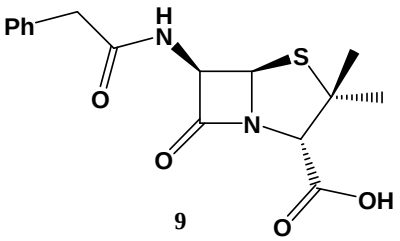
1.5.2. NATURAL PRODUCTS FROM PLANTS

1.5.3. NATURAL PRODUCTS FROM MARINE SOURCES

1.5.5. NATURAL PRODUCTS FROM ANIMALS

1.5.1. NATURAL PRODUCTS FROM MICROORGANISMS:

Microorganisms particularly fungi have been a part of human life for thousands of years. They have a capacity to produce a wide variety of bioactive complex organic molecules which are used in microbial warfare. Microbial secondary metabolites were unexplored as a source of potential drugs until the discovery of antibacterial filtrate "**penicillin G**" (**9**) by Fleming in 1928. Re-isolation and clinical studies by Chain, Florey, and co-workers in the early 1940s, followed by commercialization of synthetic penicillins revolutionized the drug discovery.[17a-f] Following the success of penicillin, industry and academic research groups assembled and screened large number of microbial cultures in order to discover new antibiotics. The outcome of research was enormous, which led to the discovery of structurally divergent antibiotics such as **chloramphenicol** (**10**), **cephalosporin C** (**11**), **chlortetracycline** (**12**), **streptomycin** (**13**), and **erythromycin** (**14**). All of these compounds, or derivatives thereof, are still in use as drugs today. The key breakthrough in drug discovery was the use of mechanism based screening for bioassay guided fractionation. One of the compounds identified in early 1970s using mechanism based screening methods was the HMG-CoA reductase inhibitor **mevastatin** (**15**) from *Penicillium citrium*. **Mevastatin** (**15**) was also reported as an antifungal agent from *P. brevicompactum.* Later **mevastatin** (**15**) and **lovastatin** (**3**) were found to be lead structures for a series of antilipidemic drugs collectively known as"statins"[18,19]



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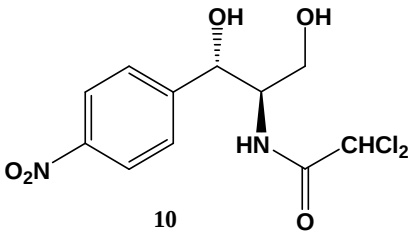

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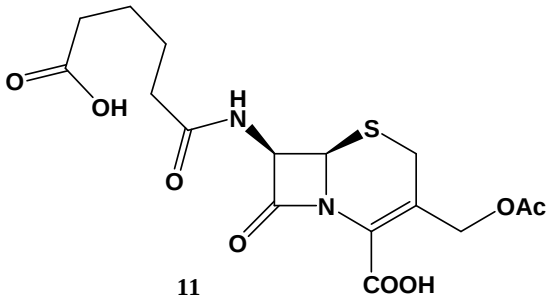

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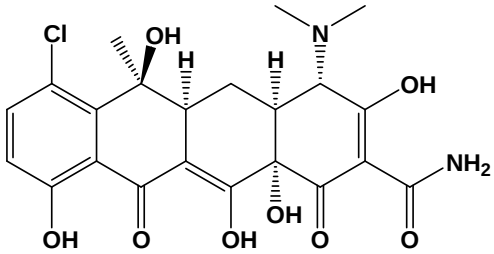

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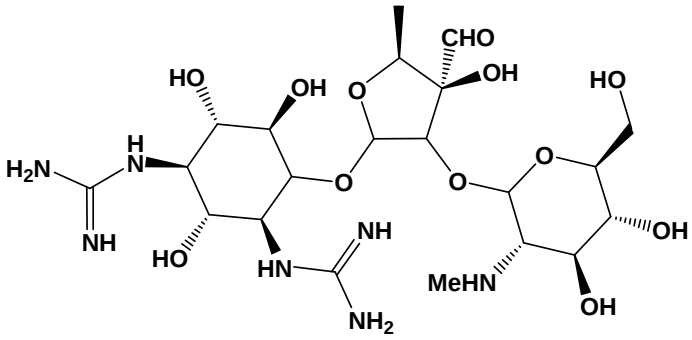

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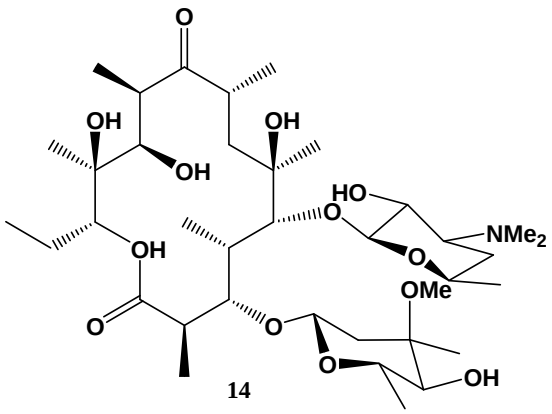

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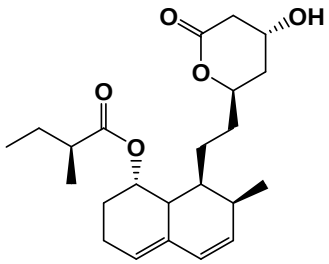

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Figure-4: Structures of Penicillin G (**9**), Chloramphenicol (**10**), Cephalosporin C (**11**), Chlortetracycline (**12**), Streptomycin (**13**), Erythromycin (**14**) and Mevastatin (**15**).

1.5.2. NATURAL PRODUCTS FROM PLANTS:

Human civilizations from different parts of the world have well documented the medicinal uses of plants from decades. Plants ecosystem have evolved and adapted over millions of years to withstand against insects, phytopathogenic microorganisms i.e. bacteria, fungi, and environmental changes. The wide distribution of plant species encompassing the world prepares them to produce unique, structurally diverse secondary metabolites. Their ethnopharmacological properties have been used as a primary source of medicines for early drug discovery.[21, 22] According to the World Health Organization (WHO), 80% of people still rely on plant-based traditional medicines for primary health care[22] and 80% of 122 plant derived drugs were related to their original ethnopharmacological purpose.[23] The knowledge associated with traditional medicine (complementary or alternative herbal products) has promoted further investigations of medicinal plants as a potential medicines and has led to the isolation of many natural products that have become well known pharmaceuticals.

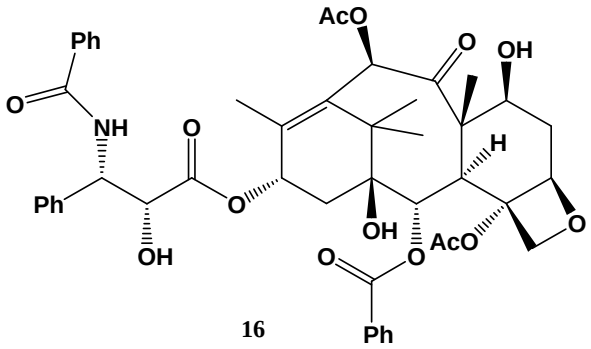

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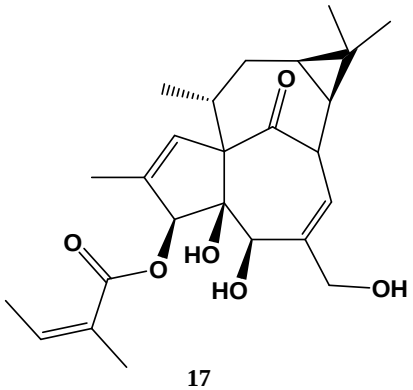
Figure-5: Paclitaxel (**16**).

The most widely used breast cancer drug is **paclitaxel** (Taxol ®) (**16**), isolated from the bark of *Taxus brevifolia* (pacific yew). In 1962 the US department of agriculture first collected the bark as part of their exploratory plant screening program at the National Cancer Institute (NCI). It has received the FDA approvals for several uses as a anticancer drug in 1992.[24] Another example of anticancer compound currently used in anticancer therapy is **ingenol-3-angelate** (**17**) a derivative of the polyhydroxy diterpenoid ingenol isolated from the sap of *Euphorbia peplus.*[25] A gel formulation of the drug has been approved by the US Food and Drug Administration (FDA) and by the European Medicines Agency (EMA) for the topical treatment of actinic keratosis.



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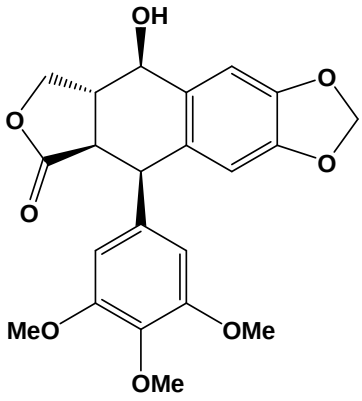

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Figure -6: Ingenol-3-angelate (**17**), Podophyllotoxin (**18**).

Podophyllotoxin (abbreviated as PPT, **18**) also known as podofilox, is a lignan extracted from the roots and rhizomes of *Podophyllum* species. It is used on the skin as a topical treatment of external genital warts, caused by some types of the human papilomavirus (HPV), and other warts. PPT and its synthetic derivatives display a wide selection in medical applications such as purgative, vesicant, antirheumatic, antiviral, and antitumor agents.[26]

1.5.3. NATURAL PRODUCTS FROM MARINE SOURCES:

The majority of the currently marketed drugs are of terrestrial origins. However, the mining of novel sources, such as the marine environment, which covers 70% of the earth's surface and represents the largest unexplored wealthy resource, have opened the avenues for chemical and biological novelties. A comparative analysis by Kong and co-workers showed that marine natural products are superior to the terrestrial one in terms of chemical diversity.[27] In the deep sea under extreme environmental conditions like high temperature (more than 400 °C in hydrothermal vent), extreme pH and pressure and in presence of high levels of abiotic chemicals such as H_2S, high densities of biologically diverse communities with distinct metabolism were observed. Hence, the marine environment represents a unique resource that encloses a massive biological diversity, which will potentially lead to unique biologically active compounds that we can translate into novel medicines.[28a]

Natural products from filter-feeding marine invertebrates and in particular, sponges, have proven to be a rich source of structurally unique pharmacologically active compounds, with over 16,000 molecules isolated so far with a continuing pace of discovery of hundreds of novel bioactive molecules per year. All classes of pharmaceuticals have been represented in this discovery process, including antiprotozoals,

pesticides, TGF-beta inhibitors and cationic channel blockers, anticancer, cytotoxic, antiviral, anti-inflammatory and antibacterial compounds. The important biosynthetic pathways found in sponges which give rise to these compounds include the terpenoids, fatty acid, polyketide, alkaloid, isoprenoid, and non-ribosomal protein synthase pathways.[28 b-c]

As of 2015, the following five marine-derived drugs are approved in the market[29a]

1) **Ziconotide** (Prialt; **19**) possesses a potent analgesic effect, which is mediated through a completely novel mechanism of action; it reversibly blocks N-type voltage-sensitive calcium channels, inhibiting the activity of a subset of neurons including pain-sensing primary nociceptors.[29b] It is synthetic form of ω-conotoxin peptide derived from the toxin of cone snail species *Conus magnus.* Ziconotide represents the first N type calcium-channel blocker and the first marine-derived peptide drug which hits the market.[30] Moreover, the discovery of this novel mechanism of action proved the key role of N-type calcium channels in signal-regulation innociceptive neurons, and further validated those channels as drug targets.[29b, 30] Interestingly, ziconotide is 1000-times more potent than morphine and it is not addictive in nature, which is common side effect of opiate based therapies.[31]

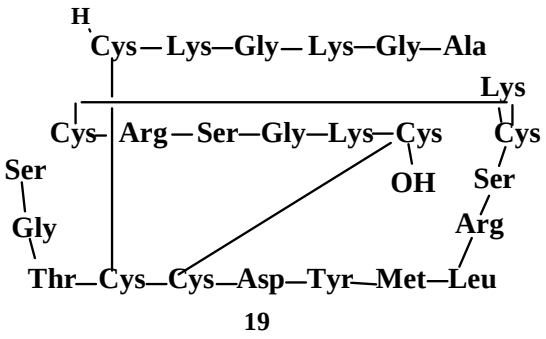

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Figure-7: Ziconotide (Prialt; **19**).

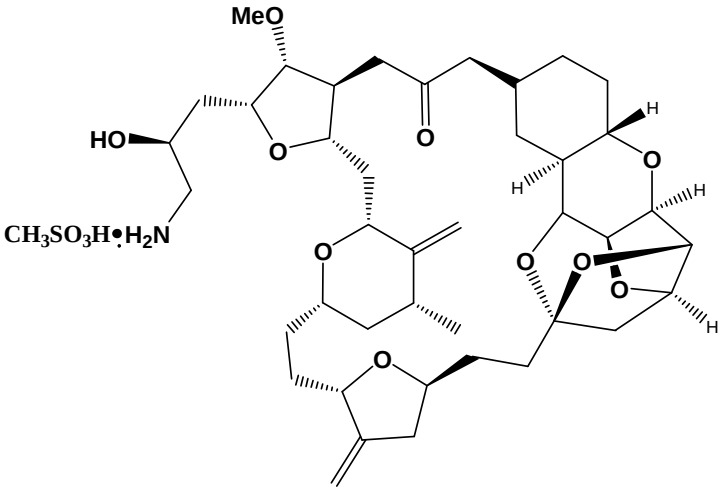
2) **Cytarabine**: FDA approved in 1969 as anticancer drug,

3) **Vidarabine**: FDA approved in 1976 as antiviral drug.

4) **Trabectedin** (Yondelis®) is the first marine derived anticancer agent to get approval in the EU and is recently approved (October-2015) by US FDA for the treatment of soft tissue sarcoma and relapsed ovarian cancer,[29a]

5) **Eribulin mesylate** (Halaven; **20**) was recently approved marine originated drug for the treatment of metastatic breast cancer and may be used off-label for certain solid tumors, including those from prostate cancer, and non-small lung cancer tumors. It acts by a novel microtubule-targeting mechanism, where it aggregates in tubulin and selectively blocks microtubule growth in a fashion that is discrete from other

antimitotic drugs.[32] Eribulin is a synthetic analogue of **halichondrin B** (**21**), which was isolated from several sponges, including *Halichondria okadai* (Japan); *Axinella* species from the western pacific, *Phakellia carteri* from the Eastern Indian Ocean. **Halochondrin B** (**21**) was originally discovered by Hirata and Uemura from the Meijo University in Nagoya, Japan in the waters of Miura Penisula, south of Tokyo.[33]



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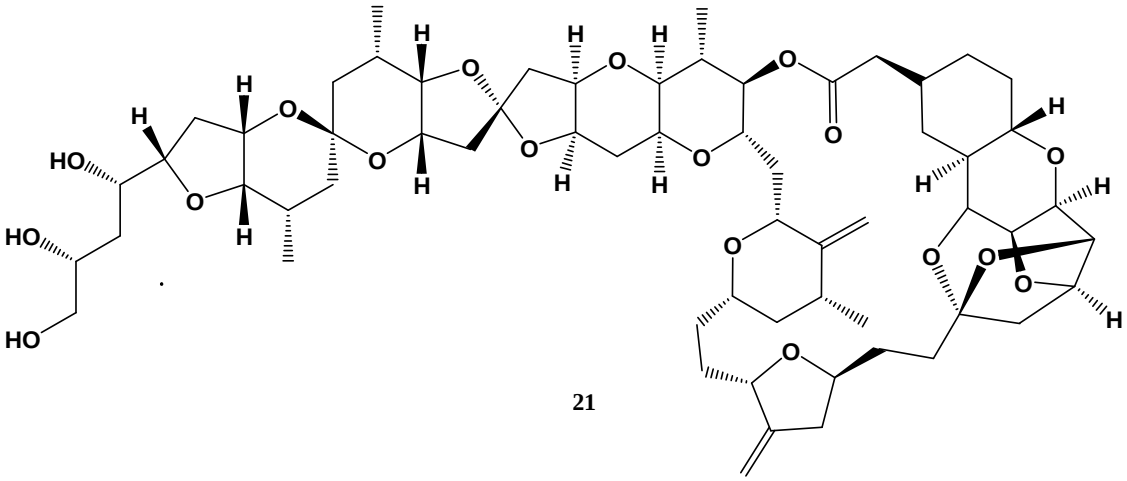

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Figure-8: Eribulin mesylate (Halaven; **20**), Halochondrin B (**21**).

There are about 12 marine natural products derived drugs on their way to hit the market, the examples include anticancer agent **bryostatin 1** (**25**), the dolastatin derivatives soblidotin (auristatin PE; TZT-1027) and the synthadotin (tasidotin; ILX-651).[29a]

1.5.4. NATURAL PRODUCTS FROM ANIMALS:

Animals often have been a source of new lead compounds. According to Van Oosten *et. al*. the UniProt protein database contains over 5,000 bioactive peptides derived from animal venoms and toxins, rendering this a significant resource for drug discovery and development.[34] The major components of most venom are peptides and proteins that are often protease-resistant due to their disulfide-rich architectures. Some of these toxins have become valuable as pharmacological tools and/or therapeutics due to their extremely high specificity and potency for particular molecular targets. There are currently six FDA-approved drugs derived from venom peptides or proteins. Venom-derived drugs have been approved for treatment of a wide range of pathophysiological conditions, including chronic pain, diabetes and hypertension. Numerous venom-derived peptides and proteins are in clinical trials or undergoing preclinical development for an even wider range of medical conditions including cancer, chronic pain, congestive heart failure, myocardial infarction, stroke and autoimmune diseases such as multiple sclerosis.[35]

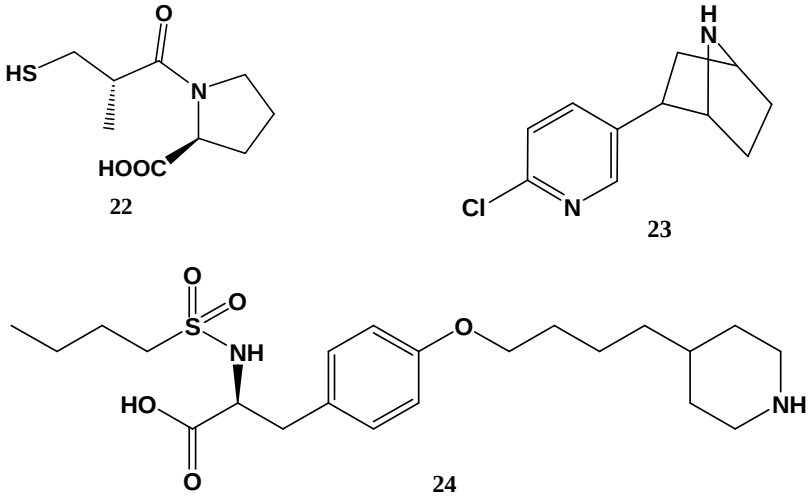

Figure-9: Captopril (**22**), Epibatidine (**23**), Tirofiban (**24**)

The era of venom based drug discovery has began in 1970s with the discovery of **captopril** (**22**) from the venom of Brazilian pit viper *Bothrops jaracaca.* **Captopril** (**22**) is an antihypertensive drug which is an inhibitor of angiotensin converting enzyme.[36] **Epibatidine** (**23**), obtained from the skin of an ecuadorian poisonous frog is ten times more potent than morphine.[37] **Tirofiban** (**24**) is an antiplatelet drug. **Tirofiban** (**24**) is a synthetic, non-peptide inhibitor acting at glycoprotein (GP) IIb/IIIa receptors in human platelets. It therefore constitutes an anticoagulant, specifically an inhibitor of platelet aggregation. It is a modified version of an anticoagulant found in the venom of the saw-scaled viper *Echis carinatus*.[38]

1.6. NATURAL PRODUCTS AND HIGH THROUGHPUT SCREENING:

Automated testing of large collection (libraries) of compounds against a specific biological target such as enzyme or receptors is called as High throughput screening (HTS). This technique is very useful in identifying natural products (and also synthetic) molecules as potential drug leads. The natural product libraries might be composed of crude extracts (10-100 compounds), semi-pure mixtures (5-10 compounds) or alternatively, single purified natural compounds. The latter follows the same screening process as that of synthetic pure libraries. However, in the first case, diversity of natural product library samples adds two additional levels of complexity to screening process. The first is that once a response for the sample is detected in screening, one or more rounds of chemical purification and biological assay might be necessary for indentifying the active principle of the mixture. The second hurdle is that the complexity of crude or semi-pure natural samples, and the chemical nature of many of the components found in them, often challenges the robustness of HTS technology. For example in case of the kinase assays, as the relative concentration of each component in a sample are not precisely known, the outcome of the HTS screening may vary depending on the concentration of the active component. Indeed, the highly abundant components can exhibit inhibition as result of non specific binding, perturbation of assay pH or other physicochemical properties. Furthermore, natural product samples may contain compounds that either fluoresce or absorb or at excitation or emission wavelengths of the fluorophore (e.g. fluorescein), or by light scattering of insoluble components, which therefore affect the readout of the assay.[39]

Effective strategy that helps to alleviate these disturbances, as well as shorten the time required for isolation of active ingredient is the implementation of purified or fractionated samples for screening, from the original crude extract.[40a-b] The samples produced by the fractionation process are less complex for use in screening. The result of this procedure is that the final isolation of the active component is simplified, typically requiring one additional purification step. Simplification of the mixture results in relative increase in concentration of minor component, thereby enhancing the opportunity to uncover the novel bioactive metabolites. HTS analysis of natural compounds libraries using mass spectrometry has been successfully used to obtain the significant results. For example electron spray ionization fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR) screening against RNA targets.[41] These and other developments make it likely that future lead generation through the screening of natural products need not be limited by HTS technology.

1.7. NATURAL PRODUCT BASED MOLECULAR LIBRARIES:

Taking into account the chemical diversity and biochemical specificity of the natural compounds, they should not be viewed as stand-alone approach distinct from combinatorial synthesis. Combination of both the approaches i.e. use of combinatorial synthesis for generation of natural product based libraries will be much more effective strategy. Number of strategies can be adopted through which the unique molecular diversity of natural products can be leveraged in the design of combinatorial libraries.

The **target oriented synthesis** can be adapted to elaborate the structural modifications on the existing bioactive natural scaffolds in a parallel, systemic fashion in order to improve its inherent biological activity or physicochemical properties (drug-like properties). This can be performed either by semi-synthetic modifications of the parent molecule, or by fully synthetic methods. This strategy was used by Waldmann *et al.,* who developed a potent, selective inhibitor of TIE2 receptor tyrosine kinase by parallel synthesis of a small (74 molecules) focused library based on nakijiquinone C.[42] Earlier in 1990s several natural product scaffolds have been effectively utilized such as yohimbine[43] and paclitaxel[44] for solid-phase synthesis of focused libraries. Ellman *et. al*. performed an elegant study on vancomycin; the biaryl template of vancomycin was combinatorially derivatized by split-and-mix library synthesis to discover analogues with improved selectivity for vancomycin- resistant bacterial strains[45a-b] (figure-10**)**.

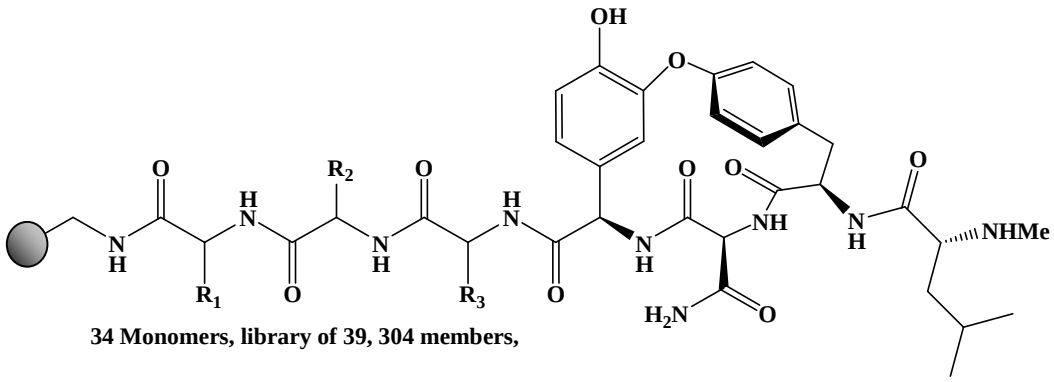

Figure-10: Screening of Vancomycin peptides for differential binding against D-Ala-D-Ala and D-Ala-D-Lactate.

Another distinct approach is the **diversity-oriented synthesis**, which can be pursued with privileged structural motifs of natural-product scaffolds to synthesize combinatorial libraries capable of binding a wide range of targets.[46] The diversity-oriented synthesis approach successfully produced high-quality screening libraries by solid-phase synthesis based on natural product benzopyran scaffolds.[47a-b] Later,

refinements have made it possible to rapidly synthesize diversity-oriented small-molecule microarray libraries to produce molecules that bind Hap3p, a subunit of the Hap2/3/4/5p transcription factor complex and HIV protease. The combinatorial generation of analogues is relatively straightforward, providing a concise route to the skeleton is available. For example the fumitremorgin[48] and fumiquinazoline alkaloids were synthetically assembled on solid phase from tryptophan,[49, 50] resulting in the discovery of unnatural analogues that are cell-cycle inhibitors and antagonists of the breast cancer resistance protein, a multidrug resistant transporter (figure-11).

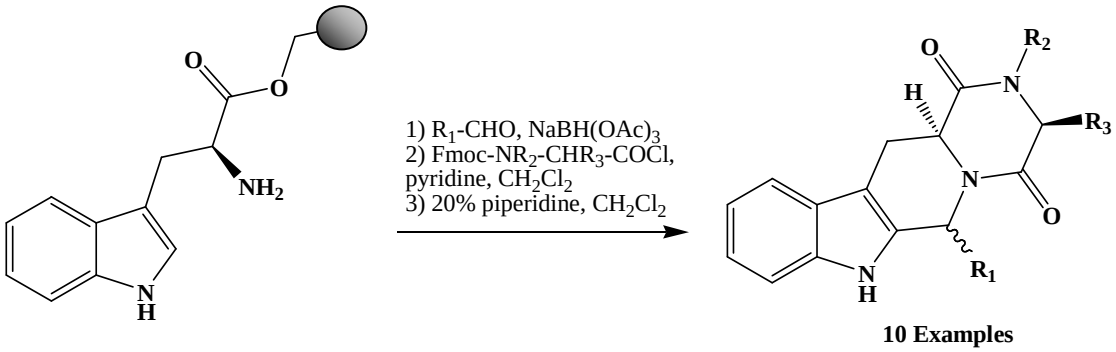

Figure-11: Assembling of natural products scaffolds from tryptophan.

Researchers have also demonstrated that it is possible to synthesize diversity-oriented natural products libraries by chemical recombination of complex fragments obtained by chemical degradation of diverse bioactive natural products.[51]

1.8. SYNTHESIS OF NATURAL PRODUCTS:

1.8.1. COMBINATORIAL BIOSYNTHESIS:

The advances in the synthetic organic chemistry have achieved the synthesis of complex natural compounds in recent years. However, the inherent structural complexity of the natural compounds limits the applications of current synthetic techniques for the modification of complex natural products to generate libraries with significant structural diversity. The combinatorial biosynthesis employs the use of genetic engineering to produce novel "unnatural" natural product analogs, generating the libraries with distinct chemical diversity and improved medicinal activity. The promising feature of the combinatorial biosynthesis is its ability to incorporate the variety of functional groups in the natural scaffolds which are difficult to introduce using traditional chemical synthesis.

Combinatorial biosynthesis usually involves three major strategies:

1.8.1.1. PRECURSOR DIRECTED COMBINATORIAL BIOSYNTHESIS

1.8.1.2. ENZYME LEVEL COMBINATORIAL BIOSYNTHESIS

1.8.1.3. PATHWAY LEVEL COMBINATORIAL BIOSYNTHESIS

1.8.1.1. PRECURSOR DIRECTED COMBINATORIAL BIOSYNTHESIS:

The biosynthetic machinery utilizes the diverse building blocks to generate a structural diversity in natural products. The precursor directed biosynthesis takes the advantage of enzyme ability to incorporate the miscellaneous non native substrates to produce various natural product analogs. The growing culture of the engineered microorganism is fed with different non natural organic molecules, which are incorporated in the biosynthetic pathway to produce the novel secondary metabolites.

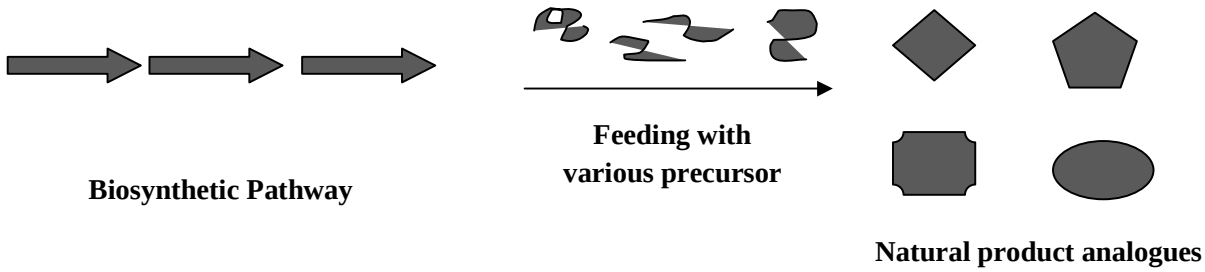

Figure-12: Precursor -directed biosynthesis.

Harvey *et. al.*[52] developed a strategy combining the precursor directed biosynthesis with colony bioassay to rapidly discover new macrolide antibiotics. A culture of engineered *Escherichia coli* HYL3 strain is fed with a synthetic precursor that mimics the natural diketide intermediate in biosynthesis of macrolides to generate the library of alkynyl and alkenyl-substituted erythromycin analogues. *Escherichia coli* HYL3 strain contains a plasmids expressing module 2-6 of the mPKS (modular type -1 polyketide synthase) 6-deoxyerythronolide B synthase (DEBS), and proteins for sugar biosynthesis and glycosyl transfer. Subsequently, the precursors were incorporated into the corresponding glycosylated macrolides. The newly generated analogs were screened against the *Bacillus subtilis* in a single colony assay. Using this approach the authors have obtained equipotent and orthogonally functional analogs as a promising lead structures for antibiotic drug development.[52]

Precursor directed biosynthesis can also be applied to the natural products whose precursor are known but the characterization of the biosynthetic enzyme is not detailed. Although the technique has an immense advantage of producing the compounds biosynthetically, it has also got some shortcomings such as challenging fermentation process, inefficient incorporation of the precursors, and the scale.

1.8.1.2. ENZYME LEVEL COMBINATORIAL BIOSYNTHESIS:

1.8.1.2.1. SWAPPING OF ENTIRE DOMAINS, MODULES, AND SUBUNITS:

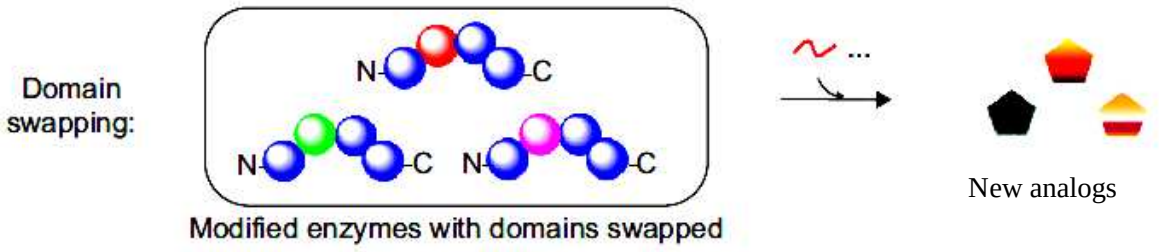

Figure-13: Domain swapping.

PKSs (polyketide synthetases) and NRPSs (non-ribosomal polyketide synthetases) have got the sequentially organized modules, each module comprised of a set of catalytic domains and subunits required to complete ne cycle of chain extension. These enzymes utilize the stepwise biosynthesis strategy for the synthesis of polyketides. These modular structures make them favorable choice for the combinatorial biosynthesis. The entire domain, modules or subunits are exchanged with other domains to generate a library of hybrid molecules. A large combinatorial library of 61 6-deoxyerythronolide B (6-DEB) analogs was constructed by substituting the AT (Acyl tranferase) domains and the β-carbon

processing domains of erythromycin mPKS with their respective counterparts from the rapamycin Mpks.[53] Module and domain sweeping of daptomycin synthetase (NRPs) led to fruitful combinatorial biosynthesis of analogs. Subunit and module exchanges between related cyclic lipopeptide A54145 and calcium dependent antibiotic (CDA), coupled with modification of glutamine (Gln) at position 12 and variations in lipid side chain, generated a combinatorial library of 72 compounds, many of which were active antibacterial agents.[54a-c]

1.8.1.2.2. SITE SPECIFIC MUTAGENESIS:

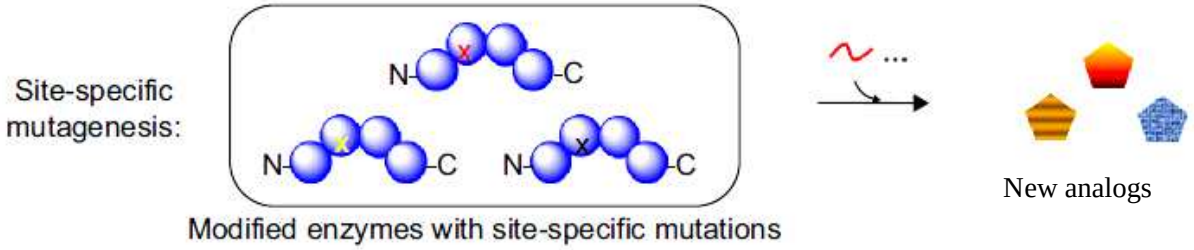

Figure-14: Site specific mutagenesis.

Most of the times due to denaturation of proteins native structure and thus of their function, very often domain sweeping leads to insoluble protein expression, impaired activities and reduced product yields. Moreover, the drastic structural changes may also affect the down streaming of intermediates from catalytic domain. Site specific mutagenesis enables one point change i.e. substitution of one amino acid residue at a time, which is less invasive and renders effective way for enzyme modulation. The selected module of monensin PKS was subjected to mutagenesis to disable the reductive domains such as ketoreductase (KR), dehydratases and enoylreductases (ER). Inactivation of the reductive domains led to a library of 22 premonensin redox derivatives.[55] The $ER2^0$-A, -B, -C derivatives exhibited increased antibacterial activity against *Pseudomonas aeruginosa* and also showed activity against *B. subtilis*, against which the native premonensin exhibited poor activity.[55] Introduction of S2107A mutation into the AT domain of sixth module of the mPKS 6-deoxyerythronolide B synthase (DEBS), the substrate specificity of AT switched to fluoromalonyl CoA from methylmalonyl CoA.[56] As the fluorine has a great impact on the physicochemical properties such has lipophilicity, acidity, basicity, and metabolic stability, this study has opened new avenues for the synthesis of fluorinated analogs of natural compounds.[57]

1.8.1.2.3. DIRECTED EVOLUTION:

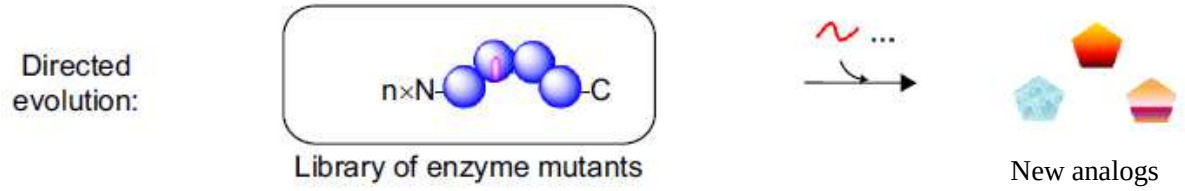

Figure-15: Directed evolution strategy.

Directed evolution, is another enzyme engineering approach which uses saturation mutagenesis. The microorganisms are randomly mutagenised to obtain chimeric variants which are having ability to produce structural analogues of the natural product. The saturation mutagenesis of the active site residues of A domain of AdmK gene involved in hybrid NRPS/PKS pathway was employed for the biosynthesis of andrimid derivatives.[58]

1.8.1.3. PATHWAY LEVEL COMBINATORIAL BIOSYNTHESIS:

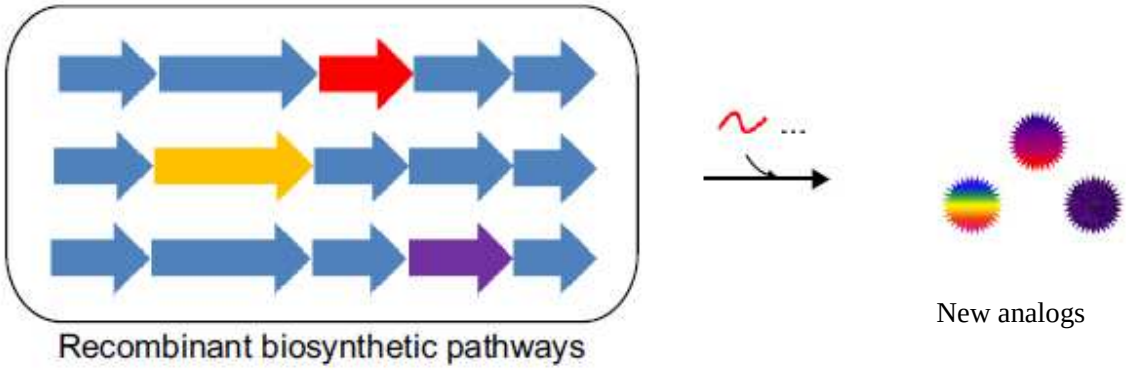

Figure-16: Pathway-level recombination.

In a well characterized host using modern techniques of genetic engineering it is possible to express heterologously biosynthetic genes from different species. In 1985, Hopwood *et. al.*[59] reported the first attempt to produce the hybrid antibiotic, mederrhodin A by interchanging and combining of genes from multiple species to generate combinatorial pathways. Since then, especially in the field of drug discovery hybrid pathways have been widely used for the production of natural products. For example, plant secondary metabolites triterpene saponins which are having versatile biological activity were heterologously expressed using biosynthesis approach in recombinant yeast strains. Recombinant strains expressing the combination of *β*-amyrin synthase, cytochrome P450 reductase, β-amyrin oxidases

CYP93E2 and CYP72A61v2 or the β-amyrin synthase, cytochrome P450 reductase, β-amyrin oxidases CYP716A12 and CYP72A68v2, were able to produce soyasapogenol B and gypsogenic acid respectively.[60]

(Figure-13 to 16 were taken from- *Ang. Drug Des. Devel. Ther*. **2015**, *9*, 823)

1.8.2. TOTAL SYNTHESIS:

To conduct the biological investigation of natural compounds and develop them further into a suitable lead compounds, it is mandatory to have sufficient quantities of the natural products. The quantity available from the natural sources limits the use of these compounds. Furthermore, large scale isolation of compounds from natural sources is a tedious process, which involves repeated extraction and purification steps. In fact, large amount of the natural resources will be destructed to obtain milligram quantities, which is economically and environmentally not viable. The most practical solution to this problem is the chemical synthesis of the natural compounds. But, the inbuilt structural complexity of the natural products makes it a tough task for the synthetic organic chemists. However, recent advances in organic synthesis made it possible to chemically synthesize a large variety of natural products. Researchers from academia and industries have developed enormous methodologies to achieve the total synthesis of diverse natural products, which are well documented in the literature.[61, 62] Chemical syntheses has empowered the uninterrupted and large-scale supply of rare bioactive scaffolds and subsequently eased the pharmacological evaluation (i.e. target identification, mechanism of action and developing preliminary structure activity relationship) of the compounds. The natural lead compounds were further simplified to more potent analogs based pharmacophoric models. For example, in 1960s a group of macrolide lactones called bryostatins were isolated from a species of bryozoan, *Bugula neritina*, by George Pettit.[63] In 1988 Wender *et. al.* proposed a pharmacophoric model of **bryostatin 1** (**25**), a potent inhibitor of protein kinase C.[64] This model was used to investigate the functional groups required for its biological activity. In the past years number of simplified analogues (e.g. **26, 27**) were synthesized which were found to be more potent and structurally less complex[65a-d] (figure-13).

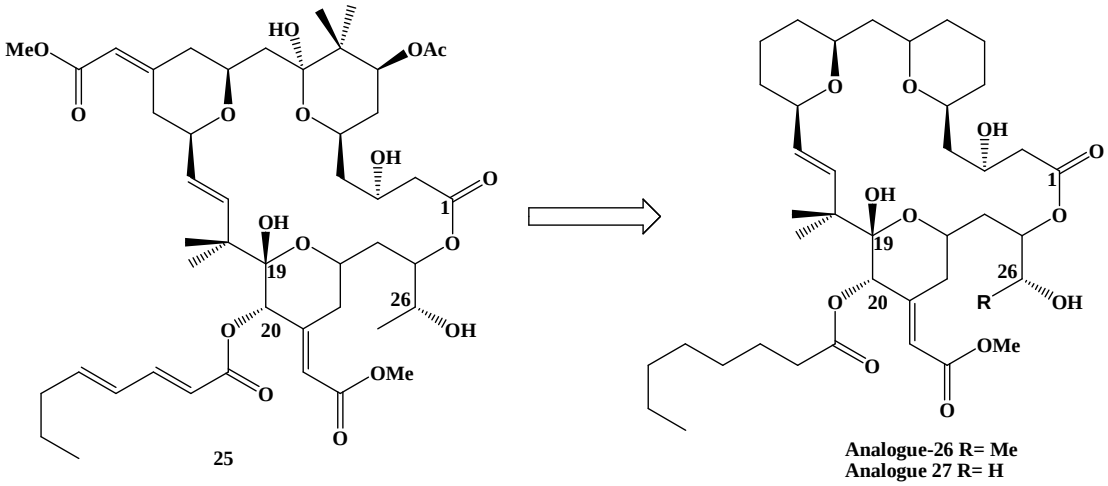

Figure-13: Total synthesis approach for simplifying the natural product.

1.8.3. MUTASYNTHESIS:

The term mutasynthesis was coined by Rinehart[66] in late 1970s but this approach was first described by Birch in 1963.[67] Mutasynthesis can be considered as an extension of precursor-directed biosynthesis. The genes responsible for the biosynthesis of particular precursor are silenced using random mutagenesis, thus the mutant strains obtained can only be able to produce the natural compound when fed with the precursor; usually the synthetic building blocks. Depending on the number of building blocks supplemented variety of new natural compounds can be produced.

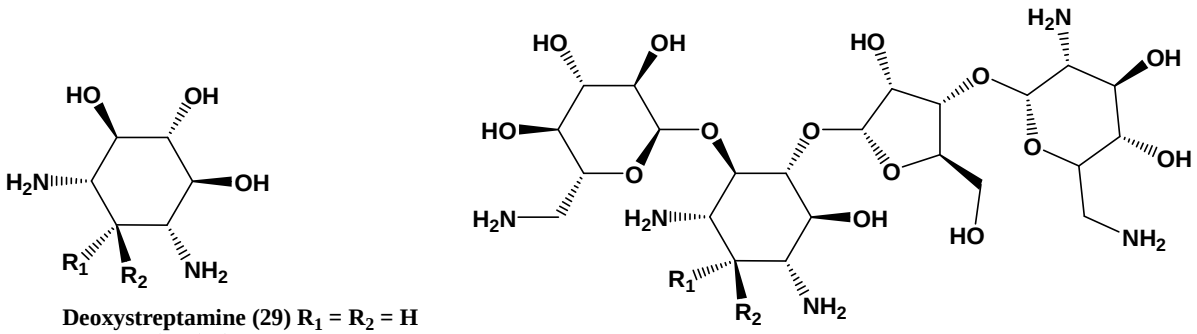

Figure-14: Mutasynthesis of neomycin analogue.

Mutasynthesis gains some advantages over precursor-directed biosynthesis mainly because of boycotting the substrate competition with natural precursor. One of the earliest applications of mutasynthesis was the

biosynthesis of unnatural analogues of the aminoglycosides-aminocyclitol antibiotic **neomycin B** (**28**). The random mutagenesis of antibiotic producer strain *Streptomyces fradiae* yielded a mutant which was only able to produce the antibiotic in presence of aminocyclitol precursor **deoxystreptamine** (**29**). The mutant strain was supplemented with synthetic precursor's **streptamine** (**30**) and **epistreptamine** (**31**), which were utilized in the biosynthetic pathways to produce novel neomycin analogues, **hybrimycin A2** (**32**) and **hybrimycin B2** (**33**).[68]

1.8.4. SEMI-SYNTHESIS:

Total synthesis of natural products offers a great advantage of obtaining the complex natural compounds in small quantities for initial biological studies. But, the large scale synthesis of structurally too-complex molecules remains a tough challenge, particularly low yields; number of steps and multiple protection-deprotections make the chemical synthesis less efficient and economically impracticable. One of the prominent solutions to this problem is semi-synthesis of natural products or advanced intermediates, which later can be converted into the target molecule by chemical synthesis. Semi-synthesis and derivatization of natural products plays a key role in natural product based drug discovery in almost all disease area.[69] Owing to intrinsic structural complexicity of natural molecules, chemical derivatization of compounds obtained from the natural sources to develop structure-activity relationships and prepare analogs with improved biological and physicochemical properties in the only feasible option.

Ground-breaking discoveries in the field of genomics and structural biology have deepened the understanding of natural product biosynthetic pathways. Genetic manipulations of these pathways are being utilized to generate unnatural natural products. In fact, this process is relatively slow and requires deep understanding of the latent biosynthetic pathways. Combination of microbial metabolic potential with biochemical and genetic knowledge can be applied to natural product biosynthesis by developing a semi-synthetic process by means of direct fermentation of engineered microorganisms. The direct fermentation is being used for the production of anticancer drug **epirubicin** (**34**, figure-15**)** and antiparasitic drug **ivermectin**[70a-c] (**1, 2,** figure-1).

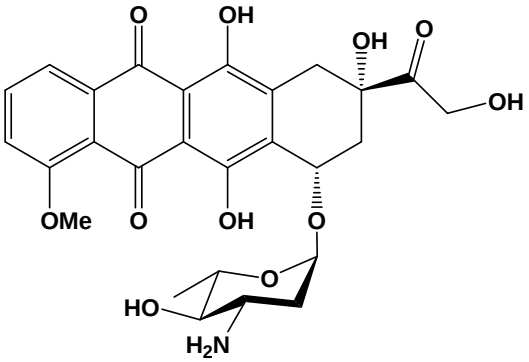

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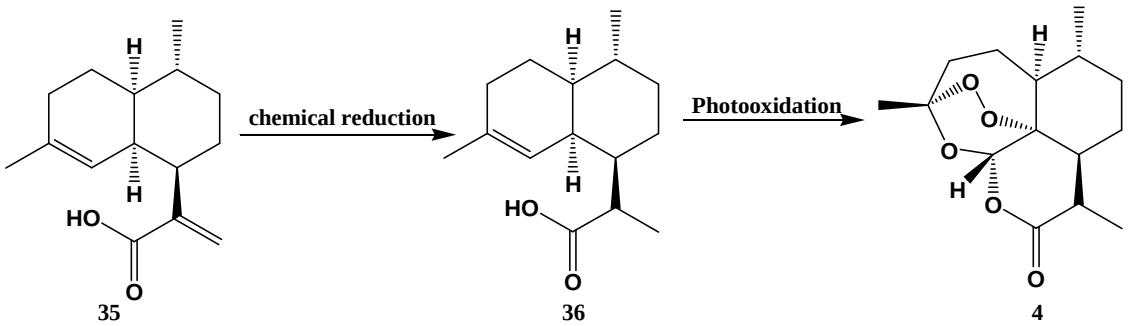
Figure-15: Structure of epirubicin (**34**)

Semi-synthesis of anti-malarial drug **artemisinin** (**4**) is another classical example of combination of chemical synthesis with metabolic engineering. Currently artemisinin-based therapy is the most effective treatment against the fatal malaria. Extraction of artemisinin from plant *Artemisia annua* and the chemical synthesis are not sufficient enough to supply the requisite quantity of artemisinin at low cost, needed for large-scale treatment in growing world. To circumvent the supply problem, the genes required for the production of the artemisinin precursor, **artemisinic acid** (**35**) have been heterologously expressed in an engineered strain of the yeast *Saccharomyces cerevisiae.* **Artemisinic acid** (**35**) obtained from the fermentation process was transformed in to **artemisinin** (**4**) using two simple chemical steps[71a-b] (figure-16).



35 **36** **4**

Figure-16: Production of artemisinin (**4**).

1.8.5. BIOMIMETIC SYNTHESIS:

Biomimetic synthesis is a branch of natural product synthesis which involves the synthetic sequence for a target molecule imitating the biosynthetic pathways. The term biomimetic synthesis was coined by Nobel laureate Sir Robert Robinson[72] in 1917. Biomimetic sequence utilizes series of reactions which are passing through intermediate structures which closely resembles to those occur during biosynthesis of target molecule from its innate source. It is a comprehensive term for both the testing of a proposed biosynthetic pathway by designing the reaction sequence parallel to the proposed one and also to study reaction sequence intended for synthetic goal designed to mimic one or more enzymatic transformations of the already established biosynthetic pathway. The results obtained from the biomimetic reactions have also proved the bio-catalytic role of enzyme in biosynthesis. For example, the reactions which require the drastic condition such as high temperature, specialized reagents or those who proceeds with poor selectivity are often cited as an evidence of *in vivo* enzyme catalysis.

Biomimetic synthesis has gained a promising application in the total synthesis on natural compounds. The reactions are elegant and usually are highly efficient in terms of stereo and regio selectivity. The earliest example of a biomimetic synthesis is Robinson's synthesis of the alkaloid **tropinone** (**38**) starting from succinaldehyde, methylamine and acetone dicarboxylic acid.[72]

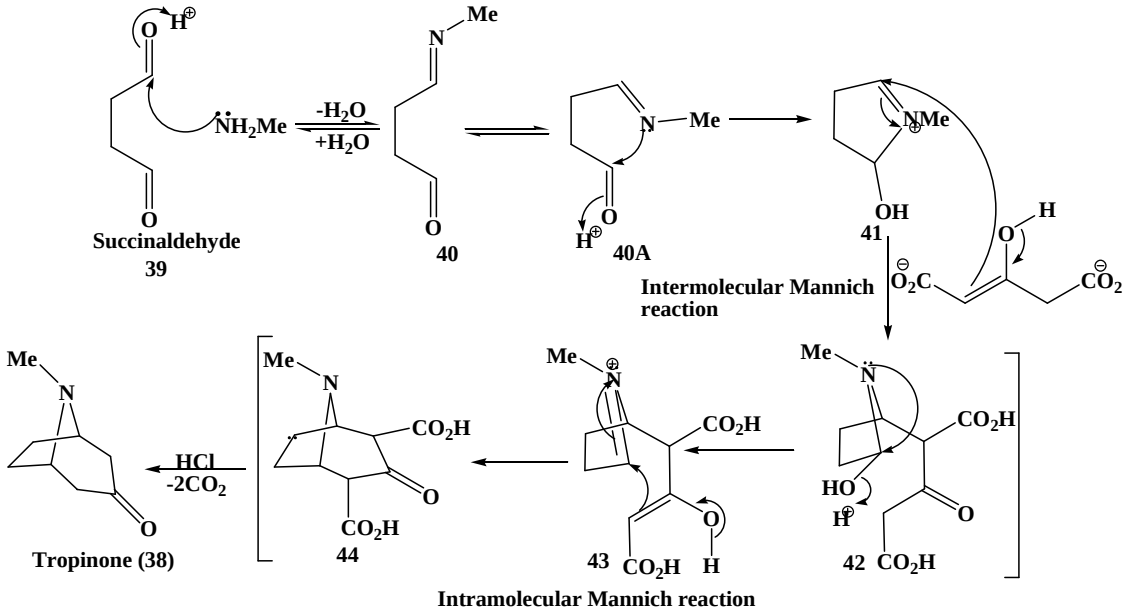


Figure-17: Biomimetic synthesis of tropinone (**38**)

In 1997 Corey *et. al.* published the elegant biomimetic synthesis of the tetracyclic marine sesterterpene **scalarenedial** (**45**). The synthetic strategy involves the concomitant formation of all four carbocyclic rings starting from a chiral oxirane, mimicking the cyclase catalyzed cyclisation of linear polyenes.[73]

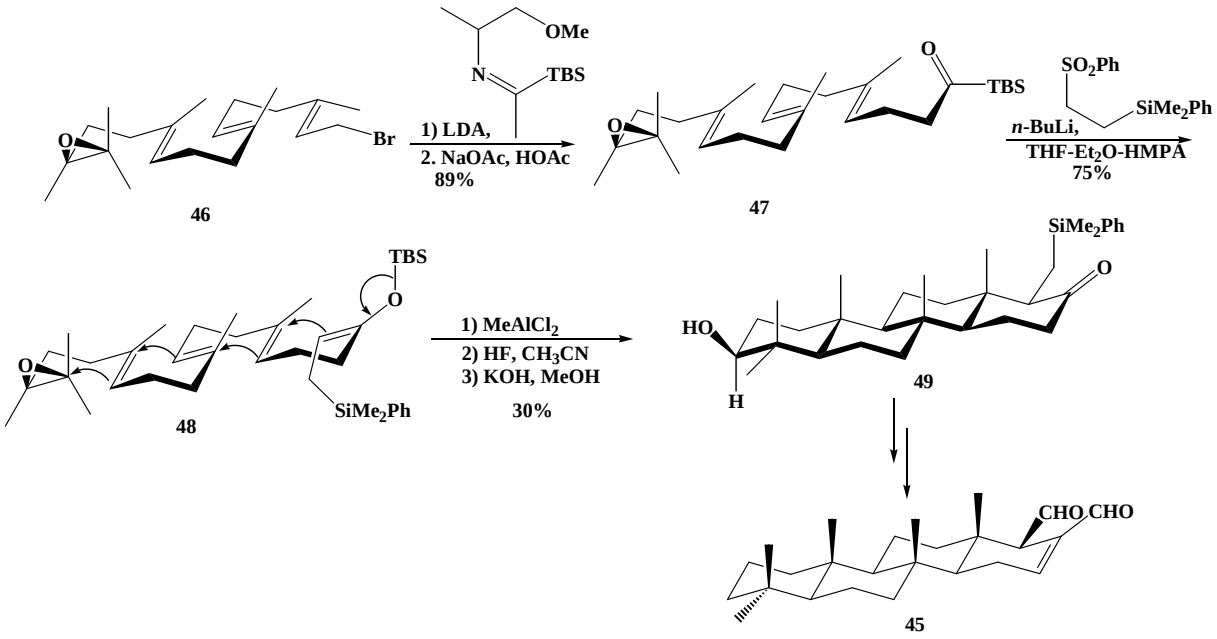


Figure-18: Corey's biomimetic synthesis of scalarenedial (**45**).

1.9. SUMMARY:

Mother Nature is serving the human kind via the elegant synthesis of privileged structures called as "natural products". The intrinsic chemical diversity offered by natural compounds make them the favorite choice in the search of highly specific and potent modulator of biological macromolecules. They could serve as promising leads to address poorly populated and underexplored chemical space. Biological screening of crude extracts obtained from natural sources finds difficulties mainly because of the complex nature of the extract. However, simplification of crude extracts using fractionation techniques furnish semi pure natural product fractions and the fractions obtained have enhanced concentration of minor (and may be active one) component, which makes the bio-assay screening simpler. Libraries generated from semi pure natural product fractions can be used directly in HTS screening reducing the time and money and efforts. Moreover, advanced approaches in separation and structure elucidation have further brought down the prime hurdles in screening mixture of complex molecules which has accelerated the pace of natural product based drug discovery. In depth understanding of evolutionary specificity and biocompatibility gained by secondary metabolites has delineated their role in mediating the cellular response. Total synthesis, semi-synthesis and biosynthesis of natural products have played a crucial role in contributions made by natural products in drug discovery and chemical biology. The genetic information being gathered from genomic mining of microbial sources will be the key in future to produce the novel skeleton of natural products. The advances in genomics, metabolic engineering and chemical synthesis hold great promise to exploit the unearthed chemical diversity in search of new drugs.

Advances in chemical synthesis has revolutionized the structural diversification and in turn modulation of biological activity of complex natural products to obtain more potent and structurally simpler analogs. Therefore many researchers from academia and industry have dedicated research programs to prepare compounds which mimic the unique structural properties of natural products.[74] One of the major hurdles in bringing the natural product based complex molecules to market is the "supply problem". However, the combination of synthetic chemistry and biosynthesis holds to meet the challenges of difficulties of material supply and structural modifications.[75] Highly efficient syntheses of complex natural products by Baran *et. al.* show the coupling of potent and appealing natural products using the skills of synthetic organic chemistry.[76] In today's scenario, it seems that there is no complex molecule which cannot be synthesized in the laboratory.

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CHAPTER-2

SCOPE OF THE THESIS

Concerns are being raised worldwide over ever-growing anti-microbial resistance (AMR). The evolutionary inevitable mechanisms in bacteria and other pathogens make them resistant to the current therapies. The innate capacity of microbes to mutate makes our drugs ineffective. In ability to treat the drug resistant microbial infections has already caused an estimated 700,000 deaths yearly and if left unchecked is predicted to cause ten million deaths annually and may cost up to US $ 100 trillion by 2050.[1]

Antibiotic resistance is a global threat irrespective of level of income of different countries as resistance originating in one part of the world spreads easily with the speed and volume of intercontinental travel today. The mixing of different microbial strains from different territories enables the sharing of genetic information among them creating new resistant strains. International human mobility and food trade accelerates the spread of AMR across different species from microbes in animals to those in humans.[2]

Any use of antibiotics even though appropriate contributes to the growing resistance, but abuse or overuse of antibiotics in few parts of the world has worsen the scenario which is enough to counter the achievements gained to combat AMR elsewhere.[3] The availability of antibiotics without prescription and even in many cases frequent unnecessary prescription practices facilitates the misuse of antimicrobials. In developed and developing countries excessive use of antibiotics is a major challenge but it is also a fact that in the poorer countries essential and effective life saving antibiotics are inaccessible. Poor supply of newer and relatively more expensive drugs and depriving efficacy of older and cheaper antibiotics also counts for increasing resistance.[4] The development of new drugs is a time taking process and requires high cost. Even though if new drugs are launched there are no strategies to minimize the unwanted use to maintain long term efficiency. An inefficient and costly diagnosis also affects the physician's ability to prescribe appropriate antibiotic or not to prescribe at all.[5]

Many of the modern healthcare treatments depend heavily on the use of antibiotics. In fact, most of the times post-surgery patients are treated with prophylactic antibiotics to reduce the risk of bacterial infections. If antibiotics do not stay in, workforce surgeries would become far more dangerous and will be risky to undertake. Modern cancer chemotherapy and organ transplants result in immunosupression making the patient more vulnerable to infection. Thus, without effective antibiotics it is impossible to imagine both and the chemotherapy will become life threatening.

The pace at which we are discovering the new antibiotics is much slower than the rising antibiotic resistance. The relentless increasing drug resistance has outstripped the discovery of new antimicrobial agents and there is urgent need for new antibiotic drugs with novel mechanism of action. The global health system will be paralyzed without effective antimicrobials, specifically the antibiotics.

To overcome AMR crisis, research in the following five key areas needs to be accelerated to conceive new antibiotic leads into effective drugs:[6]

1. In depth understanding of evolutionary mechanisms.
2. Molecular level knowledge of antibiotic mode of action and resistance.
3. Development of new chemical and genetic probes.
4. Amalgamation of structural biology into antibiotic discovery.
5. **Discovery of new antimicrobial chemical scaffolds.**

Along with the rise in antibiotic resistance, cancer is one of the leading causes of human illness and deaths. Despite of technological advances, cancer has become very prevalent and there is significant rise in number of new cancer cases. Plethora of antitumor agents has been developed based on detailed analysis of their structures and mechanism. Severe side effects and recurrence of tumors often reduce the efficacy of chemotherapeutic agents and limits their use. Despite of their severe toxicity, chemotherapy, irradiation and immunotherapy have become the standard line of treatment. Thus, there a constant need of newer anticancer agents being more specific and having reduced side effects. One of the key strategies to develop efficient anticancer agents is the study of anticancer agents derived from natural sources. The anticancer agents derived from nature have proven to be effective against a wide variety of cancers.[7] In fact, since 1930s to 2014 77% of the FDA approved anticancer drugs are either natural products, their derivatives or natural product inspired molecules.[8]

Since their discovery, natural products have served as a rich source of lead structures, predominantly as antibiotics and antitumor agents. The widespread presence of natural products as drugs may be attributed to the evolution of secondary metabolites as bioactive molecules which conferred the selectional advantages to the producer. Although they display relatively complex chemical scaffolds and rich functional groups, natural products are logical starting points for the discovery of new drugs. As mentioned earlier (chapter-1), total synthesis of natural compounds has a profound impact in discovery and development of novel bioactive molecules. The natural chemical scaffolds have become the inspiration for the development of modern medicines. Total synthesis is one of the very viable and economical options in the initial phase of developing new drug like molecules from natural resources.

The aim of my Ph.D work is the total synthesis of novel antimicrobial natural compounds, investigation of their mechanism of action and preparation of analogues to understand the pharmacophoric functional groups in the molecule.

In **chapter 3**, we describe the total synthesis approach to the antibiotic promysalin. Promysalin represents a structurally novel lead of antibiotic molecule. The structural features and species specific antibiotic activity of promysalin prompted us to undertake the total synthesis of this unprecedented chemical scaffold.

Chapter 4 deals with the mechanistic investigation of promysalin and synthesis of its analogues to study structure activity relationships. Promysalin and analogues were screened against number of gram + and gram - microorganisms. These results revealed that promysalin is a broad spectrum antibiotic. We also investigated the mechanism of action of promysalin and we found that promysalin exerts its antibacterial effect by disrupting the cell membrane.

Chapter 5 underscores my contribution towards the total synthesis of Leopolic acid A.

Chapter 6 highlights the total synthesis of resormycin. The developed synthesis in turn could be extended for the synthesis of androprostamine A and B. These natural compounds are peptides which show herbicidal, antifungal and anticancer activity. Pertaining to the promising biological activity of these structures, they can serve as leads for the development of new bioactive molecules.

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CHAPTER 3

TOTAL SYNTHESIS OF PROMYSALIN

3.1. MYRISTIC ACID METABOLITES:

Fatty acids play a significant role in the cell metabolism of living organisms. They act as a metabolic fuel; constitute important components of cell membrane, and also act as secondary messenger and gene regulator. Fatty acids are backbones of several natural products isolated mainly form microbial sources. Fatty acid metabolites display broad range of bioactivity such as antifungal, antibacterial, immunosuppressant, cytotoxic, anti-inflammatory etc. Myristic acid (**2**), one of the sufficiently hydrophobic fatty acids, is a biosynthetic precursor of a number of secondary metabolites, some of which are depicted in figure-1**.**

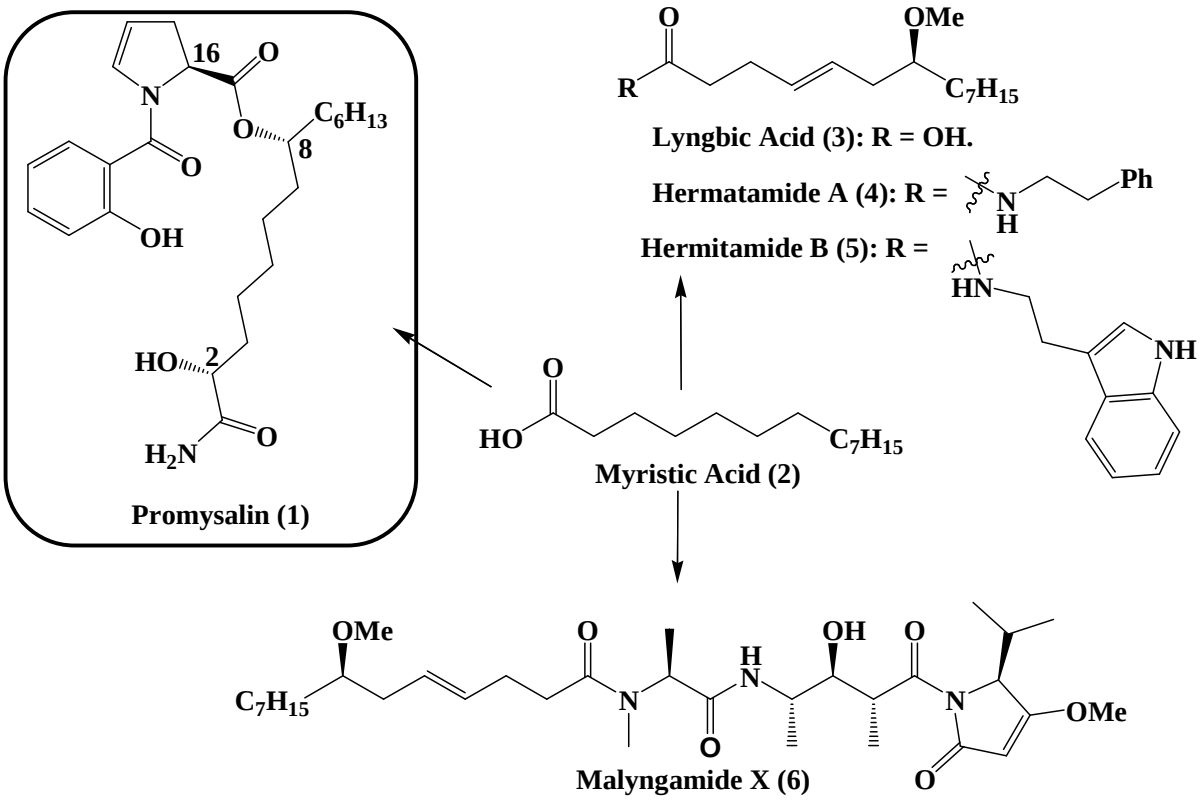


Figure-1: Natural products derived from myristic Acid.

Lyngbic acid (**3**) was isolated from the lipid extract of marine cyanobacteria *Lyngbya majuscula* (Moore et al. 1978).[1] Later Gerwick demonstrated that (**3**) has antibacterial activity against the gram positive

strains of *S. aureus* and *B. subtilis*. Moreover, (**3**) also obstructs the CqsS-mediated quorum sensing signalling pathways in *Vibrio harveyi*.[2] In 2000, Hermitamide A (**4**), and B (**5**), the two amide derivative of Lyngbic acid were reported by Gerwick *et al*. from the same cyanobacteria.[3] These compounds exhibited immunosuppressive activity and also block the voltage gated sodium ion channels.[4] In addition, they were found cytotoxic to neuro-2a neuroblastoma cells and also active in brine shrimp toxicity assay.[3] The malyngamides are a class of N-substituted amides of methoxy myristic acid and exhibit a wide range of bioactivities such as anti-inflammatory, anti-feedant, cytotoxicity to marine animals, anti-leukemic, anti-tumor, antifungal, anti-HIV, and most often antineoplastic activities.[5] The tripeptide Malyngamide X (**6**) displays remarkable antimalarial and antitubercular activity.[5]

Among the natural products containing a myristic moiety, a salicylate metabolite promysalin (**1**, Fig. 1), has recently aroused great interest in the field of antibiotics mainly because of its distinctive biological profile.

3.2. SYNTHESIS OF PROMYSALIN:

3.2.1. INTRODUCTION:

The narrow region of the soil immediately adjacent to the roots is described as rhizosphere. The rhizosphere microbiome is inhabited with a blend of neutral, beneficial and pathogenic (to plants and humans) microorganisms i.e. bacteria, viruses and fungi. *Pseudomonas* species are the most prevalent in rhizosphere and are extensively studied for their bio-control activity. Competition with other soil and rhizosphere microbes, especially phytopathogenic fungi, for space and vital resources led to the development of an evolutionary mechanism which enforced them to produce metabolites with specific bio-activities assisting survival of the producer. These metabolites possess diverse functions: they can act as biosurfactants,[6] antibiotics,[7] siderophores,[8] and virulence factors.[9] A plethora of new secondary metabolites has been identified via the characterization of the chemical compounds used in this bacterial "pathowars". These compounds have become the richest source of new molecular frameworks for the development of new leads in medicine and agriculture. *Pseudomonas* are well known producers of diverse class of antibiotics including phenazines, phloroglucinols, pyoluteorin, pyrrolonitrin, and cyclic lipopeptides.[10] Usually the production of secondary metabolites is triggered by environmental signals that activate the Gac/Rsm signal transduction pathways. The antagonism between two *Pseudomonas* species is usually reconciled by production of narrow spectrum bacteriocins or protein toxins.[11] For example the antagonism among two *Pseudomonas syringae* mediated by 3-methylarginine renders the unusual case of a secondary metabolite targeting other *Pseudomonas*.[12]

In 2011, Li. *et al.*[13] isolated a novel type of amphipathic metabolite, promysalin (**1**, Figure 1) biosynthesized by *Pseudomonas putida* RW10S1strain in the rice root rhizosphere. Promysalin displayed unusual intragenus antagonism towards other *Pseudomonas,* which is a typical characteristic of bacteriocins but an uncommon property of secondary metabolites. Promysalin was found to be a narrow spectrum antibiotic, which selectively inhibited the growth of other *Pseudomonas* strains and other gram negative bacteria. This molecule has attracted the attention because of its unique species specific bioactivity, especially against the multidrug resistant opportunistic human pathogen *Pseudomonas aeruginosa,* restraining the bacterial proliferation at sub-micromolar concentration.[13] Interestingly, the compound did not show any antibacterial effect against gram +ve equivalents. However, along with the narrow spectrum of antagonistic properties, promysalin contributes to the biofilm formation and also facilitates the swarming and surface colonization of the producer.

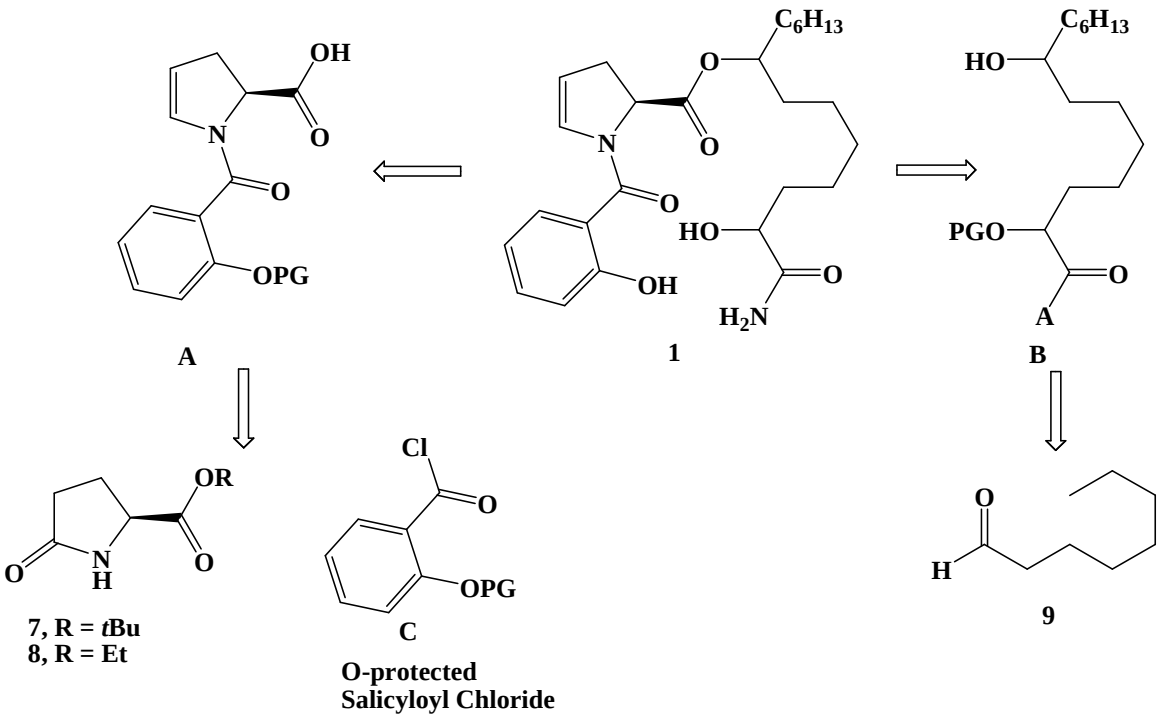
The molecule is assembled of salicylic acid, 2,8-dihydroxymyristamide and a 5-carboxy-2-pyrroline-moiety. The two unique moieties (2-pyrroline-5-carboxylic acid and 2.8-dihydroxymyristamide) are unprecedented in a secondary metabolite. The distinctive biological profile of the molecule suggests another physiological function for a salicylic acid derivative in bacteria. The role of salicylic acid as a signalling molecule in plant defence system against invading phytopathogens and its ability to interfere with bacterial quorum sensing deserves further investigation of promysalin.[13] Li. *et al.*[13] have proposed a biosynthetic pathways based on bioinformatics studies and characterization of intermediates from mutants. The structure of promysalin was confirmed using spectroscopic analysis. The recent studies by Wuest *et al.*[14] have revealed that promysalin is able to disperse established biofilms and obstruct pyoverdine synthesis. In quest of discovering new antibiotic scaffolds to cope with escalating antibiotic resistance, promysalin could serve as a promising lead molecule. Owing to tempting structural features and remarkable bioactivity, we decided to develop a stereoselective synthetic approach to **1**.

3.2.2. RETROSYNTHETIC ANALYSIS:

Scheme-1 outlines the retrosynthetic strategy developed towards promysalin. The target compound **1** could be easily accessed by esterification of the appropriately protected salicyldehydroproline core (**A)** with the dihydroxymyristamide framework (**B)**.

We envisioned that the fragment A could be efficiently obtained using Yu's[14] strategy of one pot reductive elimination of a lactam. The lactam in turn could be generated by aroylation of the appropriate L-pyroglutamate ester with suitably O-protected salicyloyl chloride. The stereoselective introduction of C-2 and C-8 alcohol functions in myristamide chain could be established using proline-catalyzed MacMillan asymmetric α-aminoxylation[15] of the corresponding aldehydes. As the absolute configuration

of C-2 and C-8 was not assigned, using this strategy one can easily access both the stereoisomers by employing the relevant proline catalyst.



Scheme 1: Retrosynthesis of promysalin. PG = Protecting Group.

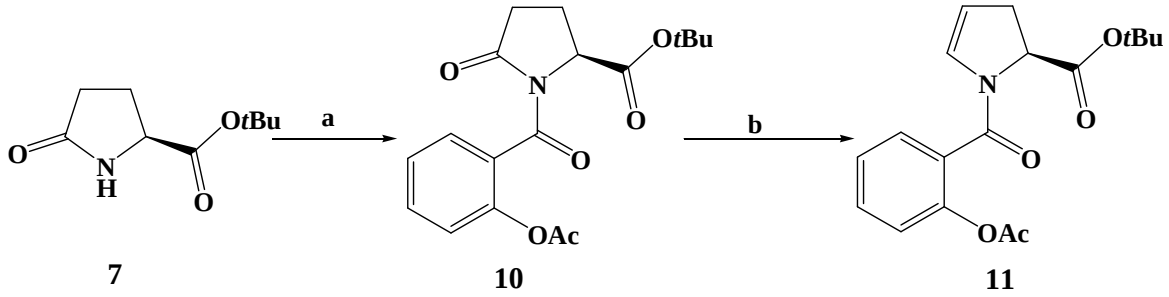
3.2.3. SYNTHESIS OF THE SALICYLDEHYDROPROLINE CORE:

Based on the proposed biosynthetic pathway, L-proline is the biosynthetic precursor of the dehydroproline moiety incorporated in the molecule. Assuming the (*S*)-configuration for natural amino acids, the (*S*)-configuration at C-16 was randomly chosen for the synthesis of an enantiopure distereoisomer.[13]

3.2.3.1. INITIAL APPROACH:

The synthesis of salicyldehydroproline core was commenced with the aroylation of L-pyroglutamic acid *tert*-butyl ester **7** with O-acetyl salicyloyl chloride. Compound **10** was prepared in moderate yield following the sequence reported in Scheme-2**.** Unfortunately, the next step i.e. the reduction of lactam using lithium triethylborohydride, followed by TFAA and DIPEA mediated in situ elimination of lactamol, was found to be quite troublesome.[14] Lactam **10** proved to be a tough substrate for reductive elimination. In fact, all the attempts with variation in temperature, solvents (THF, CH_2Cl_2, and toluene) and equivalents of reagents gave the enamide **11** in very poor yield, with concomitant formation of

products derived from the hydrolysis of the acetate group. The vulnerability of the acetate group under the reaction conditions enforced us to change the O-protecting groups of both pyroglutamate and salicylic acid cores.

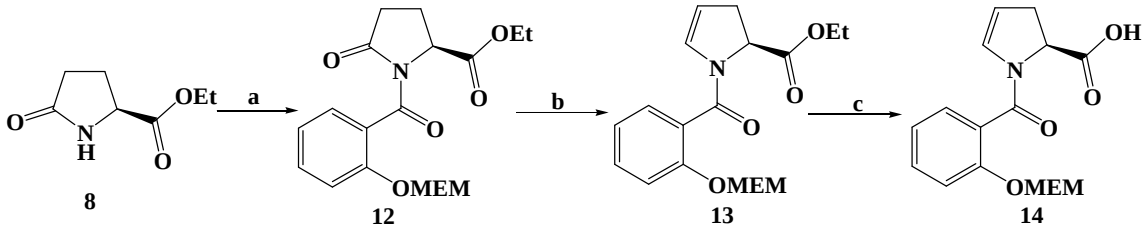


Scheme-2: Initial reductive elimination attempt. Reagents and conditions: a) o-acetyl salicyloyl chloride, NEt_3, Toluene, 80 °C, 5h, 53%, b) i) $LiBHEt_3$, PhMe, -78 °C, 1 h, ii) TFAA, DIPEA, Cat. DMAP, rt, 3h, 11%.

3.2.3.2. OPTIMIZATION OF REDUCTIVE ELIMINATION:

In an alternate strategy we decided to aroylate ethyl-L-pyroglutamate with variably protected salicyloyl chlorides using an optimized protocol prior to lactam reduction. The substitution of acetate with allyl, benzyl or p-methoxybenzyl did not give conclusive results in the reductive elimination step. In fact, in all the cases the starting material got decomposed. However, protection of salicylate with 2-methoxymethyl (MEM) produced encouraging results. The chemoselective reduction of lactam and base promoted subsequent *in situ* elimination went smoothly, afforded enamide in acceptable yield.

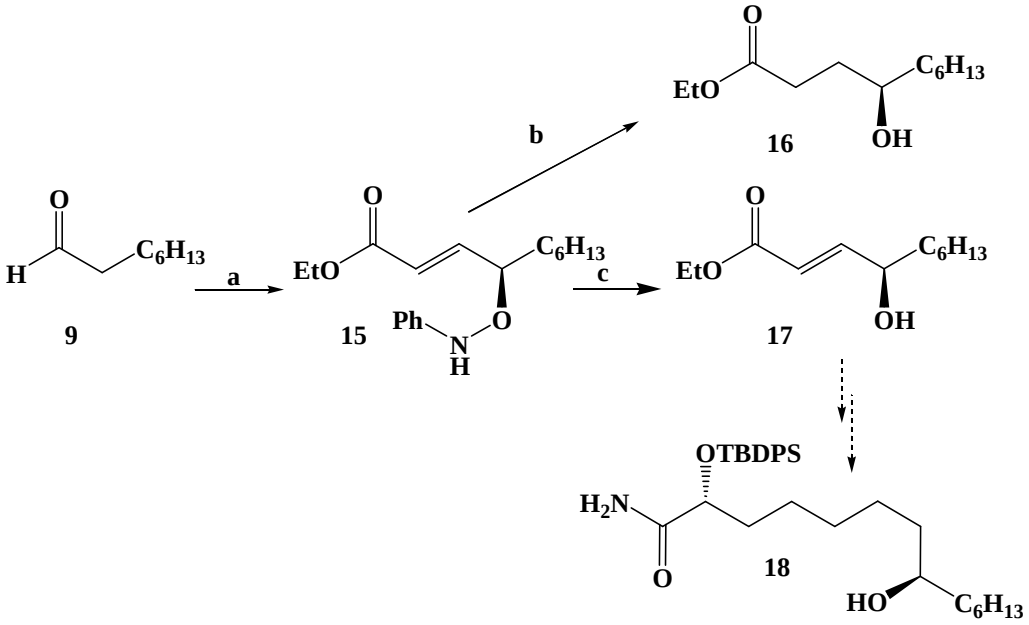
Having the optimized reaction conditions and protecting group in hand, we commenced the synthesis of pyrrolinecarboxylic acid **(-)-14** as shown in scheme 3. Treatment of ethyl (*S*)-pyroglutamate **8** with 2-((2-methoxyethoxy)methoxy)benzoic acid[16] using Ghosez reagent (1-chloro-*N*,*N*,2-trimethyl-1-propenylamine) and triethylamine in toluene[17] gave salicyloyl lactam **(+)-12** in 82% yield. The chemoselective reduction of pyroglutamate lactam **(+)-12** with $LiBHEt_3$ at -78 °C**,** followed by in situ dehydration of lactamol using TFAA and DIPEA[14] produced the salicyloyl enamide **(-)-13** in 62% yield**.** Lastly, the hydrolysis of ethyl ester using lithium hydroxide gave the desired intermediate **(-)-14.**



Scheme-3: Synthesis of salicyloyldehydroproline core. Reagents and conditions: a) i. 2-[(2-methoxyethoxy)methoxy]benzoic acid, 1-chloro-*N*,*N*,2-trimethyl-1-propenylamine, CH_2Cl_2, 0°C to rt, 1 h; ii. NEt_3, toluene, 80°C, 3 h, 82%; b) $LiBHEt_3$, toluene, -78 °C, 1 h, TFAA, DIPEA, cat. DMAP, -78 °C to rt, 3 h, 62%; c) LiOH, EtOH : H_2O, 0°C to rt, 5 h, 97%.

3.2.4. SYNTHESIS OF THE DIHYDROXYMYRISTAMIDE CHAIN:

After successful synthesis of the dehydroproline core, we focused our attention on the synthesis of dihydroxymyristamide chain (**B**). Initial approaches are summarized in scheme-4. Octanal **9** was subjected to proline catalyzed α-aminoxylation, followed by Horner–Wadsworth–Emmons olefination reaction (HWE reaction) and subsequent Pd/C catalyzed hydrogenation of crude aminooxy intermediate **15** to get the γ-hydroxy ester **16**. Unfortunately, this sequence resulted either in low yields or formation of complex mixtures. Alternatively, deprotection of **15** immediately after workup using $Cu(OAc)_2$ gave the *α*,*β*-unsaturated *γ*-hydroxy ester **17** in poor yield.



Scheme-4: Aminooxylation followed by HWE olefination strategy, Reagents and conditions: a) i. L-proline, PhNO, DMSO, rt, 2 h; ii) DBU, LiCl, HWE salt, CH_3CN, 0 °C to rt, 1 h; b) H_2, Pd/C, EtOAc, rt, 12 h, 17% (over two steps); c) $Cu(OAc)_2$, EtOH, rt, 12 h, 25-30% (over two steps).

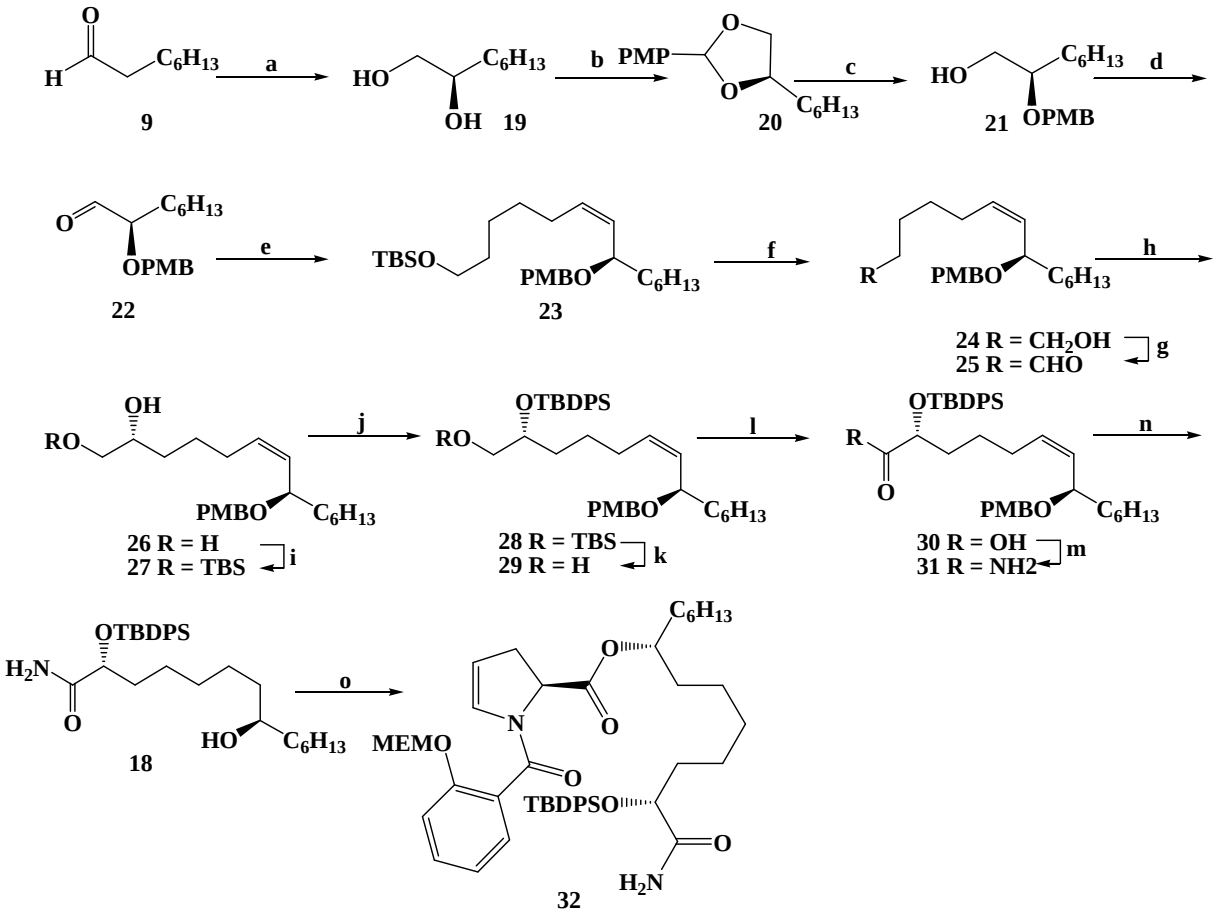
Several attempts were made to improve the outcome of this reaction (Table-1). Unfortunately we did not obtain either **16** or **17** in acceptable yields, and on consequence we abandoned this strategy.

Sr. No	Octanal (mmol)	Nitroso-benzene (mmol)	L-Proline (mmol)	Solvent	T (°C)	Product formed (% Yield)
1)	1	1.2	0.4	DMSO/CH_3CN	RT	**16**, 20% (After hydrogenation)
2)	1	1	0.1	CH_2Cl_2	0 °C	**17**, 30 % (Using $Cu(OAc)_2$
3)	2	1	0.1	DMSO	RT	Traces of **17** (Cs_2CO_3 is used as base.)
4)	1.2	1	0.1	DMSO	RT	Homodimerisation (Cs_2CO_3 is used as base.)
5)	2	1	0.1	DMSO/ACN	RT	Homodimerisation is the major side reaction.
6)	1	1	0.1	CH_2Cl_2	RT	**17**, 30 % (Using $Cu(OAc)_2$
7)	1.2	1	0.1	CH_2Cl_2	RT	**17**, 30 % (Using $Cu(OAc)_2$
8)	1.2	1	0.1	$CHCl_3$	+4 °C	**17**, 25% (Using $Cu(OAc)_2$
9)	1.2	1	0.4	$CHCl_3$	+4 °C	**17**, 27% (Using $Cu(OAc)_2$

Table-1: Optimization of aminoxylation followed by HWE Wittig and hydrogenation/ $Cu(OAc)_2$ reaction sequence.

Alternatively, instead of HWE olefination we decided to *in situ* reduce the aminooxyaldehyde to aminooxyalcohol. This approach for the synthesis of **18** from octanal is illustrated in scheme 5. L-proline catalyzed enantioselective α-oxyamination[15] of octanal **9** using nitrosobenze as a source of oxygen in $CHCl_3$ at +4 °C, and concomitant in situ reduction with $NaBH_4$ gave an oxyamino alcohol which was immediately deprotected upon workup using Zn/AcOH in EtOH to obtain diol **(+)-19** in good yield.

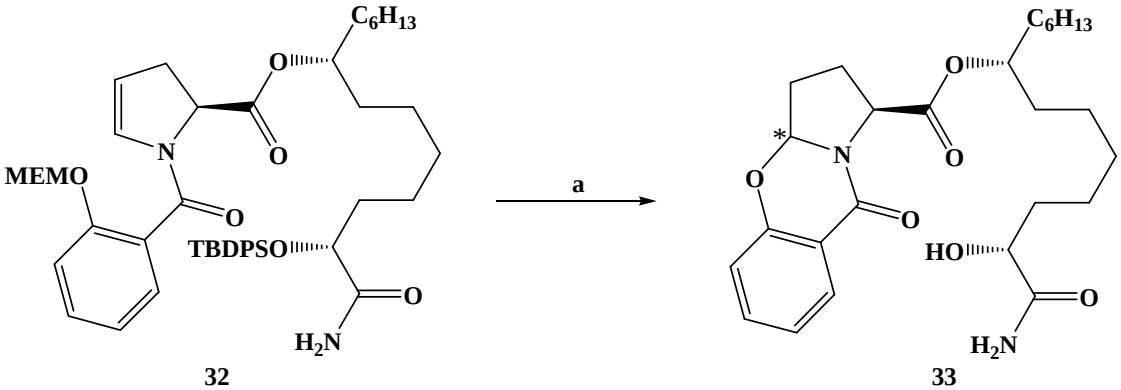
The absolute configuration of diol **(+)-19** was assigned to be (*R*), considering all literature reports[18] for aminoxylation reaction. Moreover, the specific optical rotation value of **(+)-19** ($[\alpha]_D^{23}$ = +14.1 (c 1.00, MeOH) was in agreement with the reported literature[19] value for antipode (S)-1,2 octanediol ($[\alpha]_D^{23}$ = -13.6 (*c* 1.00, MeOH, e.e. 97%), which affirms that we have synthesized the (*R*) enantiomer with more than 97% e.e.



Scheme 5: Synthesis of dihydroxymyristamide chain (B): Reagents and conditions: a) i. L-proline, PhNO, $CHCl_3$, +4 °C, 2 h, then $NaBH_4$, EtOH, 0 °C, 0.5 h; ii. Zn dust, EtOH : AcOH, rt, 1 h, 91% over two steps; b) PMB dimethylacetal, PPTS, CH_2Cl_2, 0 °C to rt, 2 h; c) DIBAL, CH_2Cl_2, -78 °C, 2 h, 72% over two steps; d) $(COCl)_2$, DMSO, NEt_3, CH_2Cl_2, -78 °C, 2 h, 94%; e) *n*-BuLi, $TBSO(CH_2)_6P^+Ph_3Br^-$, THF,-78°C to rt, 2 h, 74%; f) TBAF, THF, 0 °C to rt, 1 h, 88%; g) $(COCl)_2$, DMSO, NEt_3, CH_2Cl_2, -78°C, 1 h, 91%; h) i. L-proline, PhNO, $CHCl_3$, + 4°C, 2 h, then $NaBH_4$, EtOH, 0 °C, 0.5 h; ii. Zn dust, EtOH : AcOH, rt, 1 h, 77% over two steps; i) TBS-Cl, imidazole, CH_2Cl_2, rt, 6 h, 98%; j) TBDPS-Cl, imidazole, DMF, rt, 16 h, 98%; k) AcOH:THF:H_2O, rt, 36 h, 92%; l) TEMPO, bis(acetoxy)iodobenzene, $NaHCO_3$, ACN : H_2O, 0 °C, 4 h, 70%; m) HBTU, HOBT, NH_4Cl, DIPEA, DMF, 0 °C to rt, 1 h, 73%; n) H_2, Pd/C, MeOH, rt, 12 h, 73%; o) **(-)-14**, EDC, DMAP CH_2Cl_2, rt, 16 h, 50%.

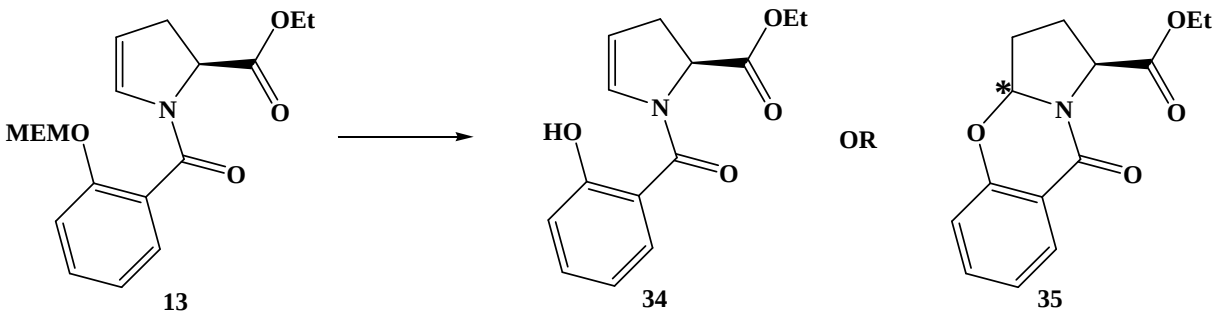
Pyridinium *P*-toluenesulfonate (PPTS) catalyzed protection of diol **(+)-19** with *p*-methoxybenzaldehyde afforded acetal **20**, which upon reductive opening with DIBAL furnished PMB-alcohol (-)-**21** in excellent yield.[20] Oxidation of primary alcohol using Swern[21] protocol confered aldehyde **(+)-22** in 96 % yield. Wittig olefination[22] of aldehyde **(+)-22** with *tert*-butyldimethylsilyloxybutyl (triphenyl) phosphonium

bromide smoothly facilitated the chain elongation to construct myristamide framework. Silyl ether **(+)-23** was cleanly deprotected on treatment with TBAF[23] at 0 °C offering the primary alcohol **(+)-24** in 88% yield. The enantiomeric purity and absolute configuration of alcohol **(+)-24** was assessed by measurement of optical rotation of corresponding (*R*)-tetradecane-1,8-diol ($[\alpha]_D^{23}$ = -0.63 (*c* 1.1, $CHCl_3$; lit.[24] for (*R*) enantiomer $[\alpha]_D^{23}$ = -0.48 (*c* 1.1, $CHCl_3$, derived from opening of an epoxide with 94% e.e.[25]); lit.[26] $[\alpha]_D^{23}$ = -0.6 (*c* 1.8, $CHCl_3$) which was obtained upon hydrogenation of **(+)-24**. Again, another sequence of Swern oxidation and successive aminoxylation reaction established stereoselectively the C-2 hydroxy to afford diol **(+)-26.** The (*R*) configuration at this carbon could be safely assumed on the basis of the mechanism of the reaction.[15] Attempted TEMPO[27] catalyzed selective oxidation of diol **(+)-26** to α-hydroxycarboxylic acid gave a complex mixture of compounds. After several futile attempts of direct oxidation and protection deprotection, the selective protection of primary and secondary alcohol as TBS and TBDPS ether respectively, followed by regioselective deprotection of primary silyl ether was found to be the optimal choice. This sequence enabled us to install at C-2 functionality inert under deprotection of the OH at C-8. Thus, diol (+)-**26** was treated sequentially with TBSCl and TBDPSCl, followed by selective desilylation of primary silyl ether using AcOH in THF/H_2O[28] to give alcohol **(-)-29** in excellent yield. Oxidation of this alcohol using Widlanski[29] protocol and subsequent amidation with NH_4Cl in DMF[30] provided myristamide (-)-**31**. Catalytic hydrogenation[31] of the double bond using Pd/C proceed smoothly with concomitant deprotection of PMB, furnishing the key alcohol intermediate **(-)-18** in 73% yield. Hassle free esterification of (-)-**14** with alcohol (-)-**18** using EDC and cat. DMAP gave fully protected promysalin **(-)-32**. Unfortunately, ester **(-)-32** was found to be highly sensitive to acidic condition. One-pot deprotection of MEM and TBDPS ethers under acidic condition led to the formation of the unusual cyclized product **33**, Scheme-6.



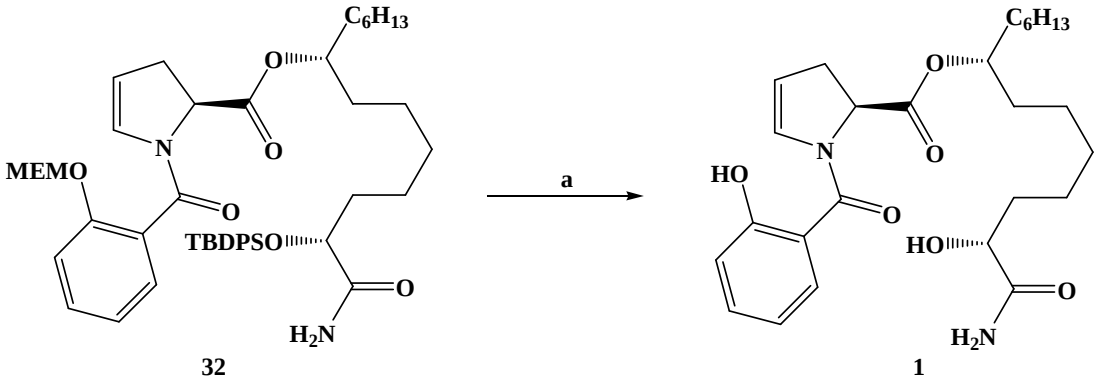
Scheme-6: Attempted global deprotection of MEM and TBDPS ether. a) $THF:H_2O$, 2N HCl, 0 °C to rt, 1h.

To circumvent this issue we went back to compound **(-)-13**, which was used as model substrate to optimize the MEM deprotection reaction. All attempts to remove 2-methoxyethoxymethyl ether with various reagents such as $ZnBr_2$,[32] PTSA,[33] CBr_4,[34] montmorillonite K-10,[35] TMS-OTf,[36] or ethylene glycol solvolysis,[37] were unsatisfactory and exclusively gave oxazinone **35** as a diastereomeric mixture. After several experiments, the MEM group was cleanly removed by treatment with $TiCl_4$ at -20 °C[38] solely obtaining phenol **(-)-34** without formation of any side products (Scheme-7).



Scheme-7: Optimization of MEM deprotection.

Pertaining to the sensitivity of dehydroproline core in acidic medium, the protecting groups were removed sequentially under mild, aprotic conditions. Thus, the careful treatment of **(-)-32** with $TiCl_4$ at -20 °C,[38] followed by TBAF mediated desilylation, gave promysalin (-)-**1** with absolute configuration 2*R*, 8*R*, 16*S* (scheme-8). Fortunately, the spectroscopic data of the synthesized compound completely matched those of the natural product, thus confirming that we had synthesized the right diastereomer.



Scheme-8: Completion of total synthesis. a) i. $TiCl_4$, -20 °C, 15 min; ii. TBAF, THF, 0 °C to rt, 2 h, 70% over two steps.

When we were in a final stage of this work, Wuest *et. al.*[14] published an efficient total synthesis of all four diastereomers of promysalin. The absolute configuration of natural compound was assigned as (2*R*, 8*R*, 16*S*) based on the biological screening.

Wuest *et al.* have synthesized the myristamide fragment in more efficient manner starting from 5-hexanoic acid over six steps in 45% overall yield. However, their strategy towards synthesis of salicyldehydroproline core seems to be less attractive, which needs seven steps from methyl salicylate to generate the dehydroproline acid.

The amalgamation of both the strategies would probably offer the best route to achieve the convergent synthesis of promysalin. The combined strategy in turn could be the best solution for the generation of structurally diverse analogues for further SAR studies and to shed light on its unknown mode of action.[14]

3.2.5. CONCLUSIONS:

In conclusion, we designed and executed the total synthetis of *Pseudomonas* antibiotic promysalin in longest linear sequence of 16 steps with 3% overall yield. Key steps of our approach encompass an organocatalytic asymmetric α-hydroxylation of carbonyl compounds employed to fabricate the myristamide framework, and Superhydride® mediated reductive elimination of lactam to obtain the salicyldehydroproline fragment. The straightforward and modular nature of the synthesis will give easy access to the preparation of analogues for biological activity evaluation.

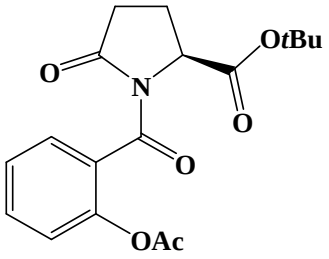
3.3. EXPERIMENTAL SECTION:

3.3.1. GENERAL INFORMATION:

All reagents and solvents were reagent grade or were purified by standard methods before use. Melting points were determined in open capillaries by a SMP3 apparatus and are uncorrected. ^{1}H spectra were recorded on Bruker AMX 300 MHz and Bruker AV600 spectrometers. TMS was used as an internal standard and the chemical shifts are reported in parts per million (δ). The peak patterns are indicated as follows: s, singlet; d, doublet; dd, doublet of doublet; t, triplet; m, multiplet; q, quartet. The coupling constants, *J* are reported in Hertz (Hz) and ^{13}C NMR spectra were recorded on Bruker AMX 300 MHz and Bruker AV600 spectrometers. Optical rotations were measured with a Perkin Elmer 241 polarimeter. IR spectra were recorded on a Perkin Elmer 1310 spectrophotometer and reported in wave numbers (cm^{-1}). The elemental analyses were recorded with a CARLO ERBA EA 1108 instrument. The accurate mass spectra were recorded using Bruker Daltonics model Autoflex III, accurate mass MALDI TOF/TOF MS/MS. Solvents were routinely distilled prior to use; anhydrous tetrahydrofuran (THF) and ether (Et_2O) were obtained by distillation from sodium-benzophenone ketyl; dry methylene chloride was obtained by distillation from phosphorus pentoxide. All reactions requiring anhydrous conditions were performed under a positive nitrogen flow and all glassware were oven dried and/or flame dried. Isolation and purification of the compounds were performed by flash column chromatography on silica gel 60 (230-400 mesh). Analytical thin-layer chromatography (TLC) was conducted on TLC plates (silica gel 60 F254, aluminium foil). Compounds on TLC plates were detected under UV light at 254 and 365 nm or were revealed spraying with 10 % phosphomolybdic acid (PMA) in ethanol.

3.3.2: EXPERIMENTAL PROCEDURES AND SPECTROSCOPIC DATA:

3.3.2.1. *tert-butyl (S)-1-(2-((2-acetoxy)benzoyl)-5-oxopyrrolidine-2-carboxylate.*



9

NEt_3 (2.3 mL, 16.62 mmol) followed by *O*-acetyl salicyloyl chloride (1.98 g, 9.98 mmol, in toluene 6 mL) were added dropwise to a stirred solution of *t*-butyl L-pyroglutamate (1.54 g, 8.31 in toluene 16 mL, 0.5 M) at 0 °C under N_2 atmosphere. The reaction mixture was warmed to room temperature and stirred at 80°C for 3 h. Sat. $NaHCO_3$ (10 mL) was added and the organic layer was separated. The aqueous layer was extracted with EtOAc (2 x 15 mL). The combined organic extracts were washed with brine (15 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The purification using flash column chromatography in 0-17% EtOAc : Hexane furnished lactam **9** as a white solid (1.55 g, 53%).

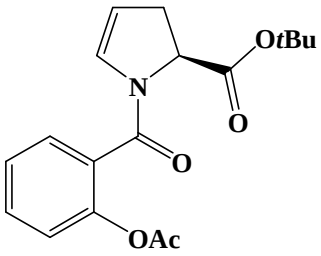
$\mathbf{R_f}$ (40% EtOAc/Hexane) = 0.6.

mp: 124-126 °C.

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.60 – 7.35 (2H, m); 7.33-7.09 (2H, m); 4.77 (1H, dd, J = 3.7, 9.5 Hz); 2.81 – 2.63 (1H, m); 2.62 – 2.32 (2H, m); 2.26 (3H, s); 2.19 – 2.02 (1H, m); 1.49 (9H, m).

^{13}C NMR (150 MHz, $CDCl_3$): δ 172.6, 169.4, 168.4, 166.2, 147.3, 131.3, 128.6, 127.6, 125.0, 122.3, 82.1, 58.3, 31.1, 27.5 (× 3C), 21.2, 20.5.

3.3.2.2. *tert-butyl (S)-1-(2-((2-acetoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate*.



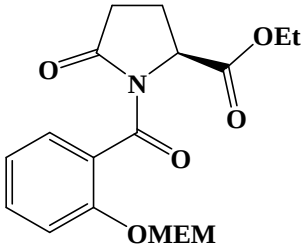
10

To a stirred solution of **9** (97 mg, 0.27 mmol) in dry toluene (1 mL) was added Superhydride® (0.33 mL, 0.33 mmol, 1M in THF) at -78°C under N_2 atmosphere. The solution was stirred for 1 h at -78°C, then DMAP (0.6 mg, 0.055 mmol) and DIPEA (0.26 mL, 1.59 mmol) were added, followed by very slow addition of TFAA (0.04 mL, 0.33 mmol). The reaction mixture was gradually warmed to room temperature and stirred for 3 h. Water (5 mL) was added. The aqueous layer was extracted with ethyl acetate (2 × 7 mL); the combined organic extracts were washed with brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using flash column chromatography with 0-10 % acetone : hexane to give **10** (10 mg, 11%) as a gummy solid.

R_f (20% acetone : hexane) = 0.4.

^{1}H NMR (300 MHz, $CDCl_3$): δ 7.58-7.39 (2H, m), 7.34 -7.23 (1H, m), 7.65 (1H, d, J = 7.8 Hz), 6.19-6.27 (1H, m), 5.11-5.00 (1H, m), 4.85 (dd, J = 3.6, 10.5 Hz, 1H), 3.19-2.98 (1H, m), 2.24-2.08 (1H, m), 2.30 (3H, s), 1.50 (9H, s).

3.3.2.3. *ethyl (S)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-5-oxopyrrolidine-2-carboxylate*.



12

Ghosez's reagent (1-chloro-*N*,*N*,2-trimethyl-1-propenylamine) (2.5 mL, 0.02 mol) was added dropwise to a solution of 2-((2-methoxyethoxy)methoxy)benzoic acid[16] (4.1 g, 0.02 mol) in dry dichloromethane (40 mL) at 0°C under N_2 atmosphere. The reaction mixture was stirred at room temperature for 1 h. The solvent was removed *in vacuo* and the residue was dried under vacuum and used in the next step without purification. In another 2-necked dry flask was placed ethyl L-pyroglutamate (1.4 g, 9.2 mmol) in dry toluene (15 mL) and the solution was cooled to 0°C. To this was added NEt_3 (3.2 mL, 0.02 mol) followed by dropwise addition of the crude acid chloride solution in toluene (15 mL). The reaction mixture was heated at 80 °C for 3 h, and then it was cooled to room temperature and quenched by addition of saturated aqueous $NaHCO_3$ (30 mL). The organic layer was separated and the aqueous layer was extracted with ethyl acetate (2 × 100 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification of the residue by flash column chromatography with 0-18% acetone : hexane gave **(+)-12** (2.75 g, 82%) as a yellow gummy solid.

$\mathbf{R_f}$ (20% acetone : hexane, double run) = 0.30.

$\mathbf{[\alpha]_D^{23}}$ = +30.9 (*c* 1.0, $CHCl_3$).

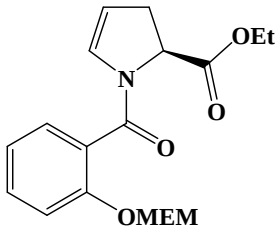
$\mathbf{\nu_{max}}$ (thin film) : 3045, 1760, 1700, 1620, 1510, 1470, 1340, 1280, 1250, 1210, 1120, 1010, 750, 710 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.40 (dd, *J* = 7.5, 7.5 Hz, 1H), 7.31 (d, *J* = 7.5 Hz, 1H), 7.21 (d, *J* = 7.5 Hz, 1H), 7.05 (dd, *J* = 7.5, 7.5 Hz, 1H), 5.27 (d, *J* = 7.0 Hz, AB, 1H), 5.23 (d, *J* = 7.0 Hz, AB, 1H), 4.91 (dd, *J* = 2.9, 9.5 Hz, 1H), 4.28 (q, *J* = 7.3 Hz, 2H), 3.80 (t, *J* = 4.5 Hz, 2H), 3.55 (t, *J* = 4.5 Hz, 2H), 3.38 (s, 3H), 2.73-2.64 (m, 1H), 2.55-2.50 (m, 1H), 2.50-2.45 (m, 1H), 2.2-2.10 (m, 1H), 1.33 (t, *J* = 7.3 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 172.8, 170.9, 167.8, 154.5, 131.8, 128.4, 125.8, 121.6, 114.5, 93.9, 71.6, 67.7, 61.7, 58.9, 58.2, 31.6, 21.7, 14.1 ppm.

Elemental analysis calcd (%) for $C_{18}H_{23}NO_7$: C 59.17, H 6.34, N 3.83; Found: C 59.38, H 6.32, N 3.84.

3.3.2.4. *ethyl (S)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



13

To a stirred solution of **(+)-12** (1.1 gm, 3.0 mmol) in dry toluene (22 mL) was added Superhydride ® (3.6 mL, 3.6 mmol, 1 M in THF) at -78°C under N_2 atmosphere. The solution was stirred for 1 h at -78°C, then DMAP (7 mg, 0.06 mmol) and DIPEA (2.8 mL, 0.02 mol) were added, followed by very slow addition of TFAA (0.5 mL, 3.6 mmol) over 5 min. The reaction mixture was gradually warmed to room temperature and stirred for 3 h. Water (30 mL) was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (2 × 75 mL); the combined organic extracts were washed with brine (15 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using flash column chromatography in 0-50 % ethyl acetate : hexane to give **(-)-13** (650 mg, 62%) as yellow oil.

$\mathbf{R_f}$ (35% ethyl acetate : hexane) = 0.30.

$\mathbf{[\alpha]_D^{23}}$ = -104.3 (*c* 1.00, $CHCl_3$).

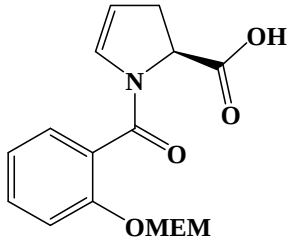
$\mathbf{\nu_{max}}$ (liquid film): 3050-2800 (br), 1760, 1660, 1640, 1620, 1510, 1470, 1425, 1250, 1210, 1110, 1045, 1000, 860, 780 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.41-7.34 (m, 2H,), 7.23 (d, *J* = 7.8 Hz, 1H), 7.07 (dd, *J* = 7.8, 7.8 Hz, 1H), 6.2 (m, 1H), 5.30 (s, 2H), 5.10-5.03 (m, 1H), 5.00 (dd, *J* = 3.6, 10.5 Hz, 1H), 4.27 (q, *J* = 7.2 Hz, 2H), 3.86-3.81 (m, 2H,), 3.57-3.52 (m, 2H), 3.37 (s, 3H), 3.18-3.13 (m, 1H,), 2.74-2.69 (m, 1H), 1.33 (t, *J* = 7.2 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 170.8, 164.9, 153.4, 131.2, 130.8, 128.8, 125.9, 122.1, 115.3, 108.5, 93.7, 71.5, 67.9, 61.3, 59.0, 58.0, 34.1, 14.1 ppm.

Elemental analysis calcd (%) for $C_{18}H_{23}NO_6$: C 61.88, H 6.64, N 4.01; found: C 62.01, H 6.62, N 4.00.

3.3.2.5**.** *(S)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid***.**



14

A solution of **(-)-13** (920 mg, 2.6 mmol) in EtOH (22.5 mL) was cooled to 0 °C. To this was added dropwise a solution of LiOH (166 mg, 3.9 mmol) in water (11.25 mL). After complete addition the reaction mixture was warmed to room temperature and stirred for 5 h. EtOH was removed *in vacuo*, the aqueous layer was washed with 40% ethyl acetate in diethyl ether (2 × 25 mL), cooled to 0 °C and acidified using 5% citric acid. The product was extracted using 5% methanol: dichloromethane (3 × 100 mL). The combined organic extracts were washed with brine (15 mL), dried over anhydrous Na_2SO_4 and concentrated to afford pyrrolinecarboxylic acid **(-)-14** (809 mg, 97%) as a pale yellow gummy solid.

$\mathbf{R_f}$ (5% methanol : dichloromethane) = 0.35.

$\mathbf{[\alpha]_D^{23}}$ = -97.6 (*c* 1.00, $CHCl_3$).

$\mathbf{\nu_{max}}$ (thin film) 3300-2900 (br), 1760, 1660, 1640, 1625, 1505, 1475, 1440, 1292, 1005, 760, 725 cm^{-1}.

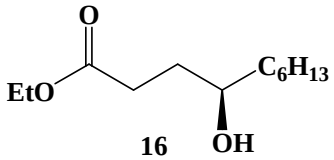
^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.38 (dd, *J* = 7.9, 7.9 Hz, 1H), 7.35 (d, *J* = 7.9 Hz, 1H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.05 (dd, *J* = 7.9, 7.9 Hz, 1H), 6.62 (brs, 1H), 6.09 (d, *J* = 2.2 Hz, 1H), 5.28-5.25 (m, 2H), 5.17-5.22 (m, 1H), 5.08 (dd, *J* = 4.9, 10.1 Hz, 1H), 3.80-3.76 (m, 2H), 3.52 (t, *J* = 4.7 Hz, 2H), 3.34 (s, 3H), 3.08-3.02 (m, 2H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 172.2, 167.1, 153.4, 131.8, 129.7, 128.8, 124.8, 122.1, 115.1, 111.2, 93.7, 71.5, 68.0, 58.9, 53.4, 32.9 ppm.

Elemental analysis calcd (%) for $C_{16}H_{19}NO_6$: C 59.81, H 5.96, N 4.36, found: C 59.99, H 5.94, N 4.35.

HRMS Accurate mass (ES+) calculated for $C_{16}H_{19}NO_6Na$ $(M + Na)^+$ 344.11046, Found: 344.11114.

3.3.2.6. *(R)-ethyl 4-hydroxydecanoate.*



16

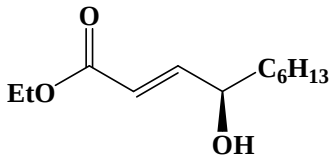
To a stirring solution of nitrosobenzene (100 mg, 0.933 mmol), L-proline (42.9 mg, 0.373 mmol), in DMSO (4 mL) under N_2 atmosphere, was added *n*-octanal (0.17 mL, 1.12 mmol) at room temperature. The reaction mixture was stirred at room temperature for 30 minutes, by this time the colour of the mixture changed from green to yellow. Reaction mixture was cooled to 0 °C; to this was added premixed mixture of DBU (0.41 mL, 2.79 mmol), LiCl (118 mg, 2.79 mmol), and triethyl phosphonoacetate in CH_3CN (4 mL). After complete addition the ice bath was removed and the reaction mixture was stirred at room temperature for 1 h. Ice pieces were added and the CH_3CN was removed *in vacuo*. The aqueous layer was extracted with ethyl acetate (2 x 10 mL), combined organic extracts were washed with brine (7 mL), dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo*; the crude aminooxy alcohol (350 mg) was dissolved in ethyl acetate (7 mL), Pd/C (35 mg, 10% by crude weight) was added. The RB flask was evacuated and flushed with H_2 (three times) and the reaction mixture was stirred overnight at room temperature under H_2 atmosphere. The reaction mixture was filtered through celite bed and the residue was washed with ethyl acetate (10 mL). *In vacuo* concentration followed by flash column chromatography furnished γ-hydroxy ester **16** (37 mg, 20%) as a yellow oil.

R_f (7% EtOAc : Hexane) = 0.35.

^{1}H NMR (200 MHz, $CDCl_3$) δ = 4.13 (q, *J* = 7.2 Hz, 2H), 3.67–3.52 (m, 1H), 2.50–2.39 (m, 2H), 1.99–1.61 (m, 4H), 1.56–1.29 (m, 8H), 1.29–1.22 (m, 3H), 0.92 (d, *J* = 4.8 Hz, 3H).

^{13}C NMR (126 MHz, $CDCl_3$) δ = 174.1, 80.9, 71.0, 60.2, 37.4, 32.1, 30.7, 29.5, 25.5, 22.5, 14.0, 13.9 ppm.

3.3.2.7. *(R, E)-ethyl 4-hydroxydec-2-enoate.*



17

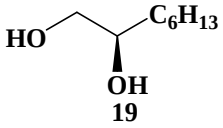
To a stirring solution of nitrosobenzene (83 mg, 0.781 mmol), L-proline (34 mg, 0.296 mmol), in dichloromethane (2.7 mL) under N_2 atmosphere, was added *n*-octanal (0.12 mL, 0.781 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1.25 h, by this time the colour of the mixture changes from green to yellow. To this was added premixed and pre-cooled mixture of DBU (0.41 mL, 2.79 mmol), LiCl (118 mg, 2.79 mmol), and triethyl phosphonoacetate in dichloromethane (2.7 mL). After complete addition the ice bath was removed and the reaction mixture was stirred at room temperature for 1 h. Ice pieces were added and the dichloromethane was removed *in vacuo.* The aqueous layer was extracted with ethyl acetate (2 x 10 mL), combined organic extracts were washed with brine (7 mL), dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo*; the crude aminooxy alcohol (285 mg) was dissolved in ethanol (9 mL), $Cu(OAc)_2$ (28 mg, 0.156 mmol) was added. The reaction mixture was stirred at room temperature for 12 h. EtOH was removed *in vacuo*, the residue was taken in water (15 mL) and the aqueous layer was extracted with diethyl ether (2 x 10 mL). The combined organic extracts were washed with brine (8 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The purification using flash column chromatography in 10% EtOAc : Hexane yielded alcohol **17** (46 mg, 26%) as dark yellow oil.

$\mathbf{R_f}$ (7% EtOAc : Hexane) = 0.40.

^{1}H NMR (200 MHz, $CDCl_3$) δ = 0.89 (t, J = 6.8 Hz, 3H), 1.21–1.41 (m, 11H), 1.56 (m, 2H), 1.99 (brs, 1H), 4.19 (q, J = 7.2 Hz, 2H), 4.30 (m, 1H), 6.01 (dd, J = 15.6, 1.5 Hz, 1H), 6.93 (dd, J = 15.6, 4.9 Hz, 1H).

^{13}C NMR (50 MHz, $CDCl_3$): δ 14.1, 14.2, 22.6, 25.2, 29.1, 31.7, 36.6, 60.3, 71.0, 120.0, 150.3, 166.4.

3.3.2.8. *(R)-octane-1,2-diol.*



19

A suspension of L-proline (322 mg, 2.8 mmol) in $CHCl_3$ (30 ml) was cooled to 4 °C and stirred for 15 min, then nitrosobenzene (3 g, 0.03 mol) was added in one portion. At this time the solution turned green. To this suspension was added octanal **9** (13.1 ml, 84.0 mmol) in one portion. The resulting solution was then stirred at 4 °C for 2 h and it turned yellow. The reaction mixture was then added dropwise to an ethanol (25 mL) suspension of $NaBH_4$ (1.05 g, 0.03 mol) at 0 °C. After 30 min, the reaction was treated with saturated aqueous $NaHCO_3$ (50 mL). The aqueous layer was extracted with dichloromethane (3 × 75 mL); the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. To a solution of the oxy-aniline adduct (14.6 g) in EtOH/AcOH (3:1, 156 mL), Zn dust (8.1 g, 0.12 mol) was added portionwise. The resulting suspension was stirred at room temperature for 1 h. The reaction mixture was filtered through a plug of celite and the residue was washed with ethanol (50 mL); the filtrate was concentrated *in vacuo* at a temperature <40 °C. The residue was dissolved in ethyl acetate (200 mL) and washed with saturated aqueous $NaHCO_3$ solution (100 mL), brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The resulting yellow oil was purified using flash column chromatography (eluent: 20-50% ethyl acetate : hexane) to afford (*R*)-octane-1,2-diol **(+)-19** (3.7 g, 91%) as an off-white sticky solid.

R_f (50% ethyl acetate : hexane) = 0.35.

$[\alpha]_D^{23}$ = +14.1 (*c* 1.00, MeOH).

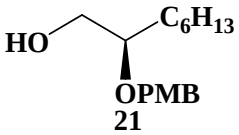
ν_{max} (liquid film) 3600-3300 (br), 3100, 1550, 1490, 1445, 1290, 1130, 950 cm^{-1}.

^{1}H NMR (600 MHz, $CDCl_3$) δ = 3.76-3.70 (m, 1H), 3.67 (dd, *J* = 3.2, 11.1 Hz, 1H), 3.45 (dd, *J* = 7.9, 11.1 Hz, 1H), 2.50-2.35 (m, 2H), 1.49-1.26 (m, 10H), 0.90 (t, *J* = 7.0 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$): δ = 72.3, 66.8, 33.2, 31.7, 29.3, 25.5, 22.6, 14.0 ppm.

Elemental analysis calcd (%) for $C_8H_{18}O_2$: C 65.71, H 12.41; found: C 65.50, H 12.44.

3.3.2.9. *(R)-2-(4-methoxybenzyloxy)octan-1-ol.*



21

A solution of (*R*)-octane-1,2-diol **(+)-19** (7.4 g, 0.05 mol), PPTS (254 mg, 1.1 mmol) in dry dichloromethane (126 mL) was cooled to 0 °C. 4-Methoxybenzaldehyde dimethylacetal (12.9 mL, 0.08 mol) was added dropwise. The reaction mixture was stirred at room temperature for 2 h under N_2 atmosphere. Excess NEt_3 (10 mL) was added, and then the reaction mixture was concentrated *in vacuo*. The product was filtered through a short pad of silica gel (neutralized with 5 % NEt_3 in hexane) in 1% ethyl acetate: hexane to afford (*R*)-4-hexyl-2-(4-methoxyphenyl)-1,3-dioxolane **20**. (16 g of crude compound, mixture of diastereomers.) as a yellow oil; R_f (5% ethyl acetate: hexane) = 0.55. Compound **20** was unstable, thus it was used immediately in the next step without further purification.

To a stirred solution of **20** (16.0 g, crude) in dry dichloromethane (300 mL), DIBAL (90.90 mL, 0.09 mol, 1 M in dichloromethane) was added dropwise at -78 °C under N_2 atmosphere and stirred for 2 h at -78 °C. The reaction mixture was gradually warmed to -20 °C over 1 h, methanol (5 mL) was added dropwise and the resulting solution was stirred for 5 min. Dilution with diethyl ether (150 mL) followed by addition of saturated aqueous solution of Rochelle's salt (150 mL) gave a thick suspension which was stirred vigorously until the two layers become clear. The organic layer was separated; and the aqueous layer was extracted with ethyl acetate (3 × 150 ml). The combined organic extracts were washed with brine (100 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography in 0-12% ethyl acetate: hexane gave **(-)-21** (9.7 g, 72% over two steps) as a pale yellow oil.

R_f (15% ethyl acetate : hexane) = 0.46.

$[\alpha]_D^{23}$ = -17.5 (*c* 1.00, $CHCl_3$).

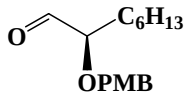
ν_{max} (liquid film) 3650-3300 (br), 1640, 1620, 1540, 1495, 1370, 1205, 1100, 850 cm^{-1}.

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.30 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 2H), 4.58 (d, *J* = 11.4 Hz, AB, 1H), 4.49 (d, *J* = 11.4 Hz, AB, 1H), 3.74-3.68 (s, 3H), 3.70 (m, 1H), 3.55-3.48 (m, 2H), 2.00 (brs, 1H), 1.70-1.62 (m, 1H), 1.55-1.48 (m, 1H), 1.39-1.25 (m, 8H), 0.91 (t, *J* = 7.2 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ =159.3, 130.6, 129.4 (× 2), 113.9 (× 2); 79.5, 71.2, 64.3, 55.3, 31.8, 30.8, 29.5, 25.4, 22.6, 14.1 ppm.

Elemental analysis calcd (%) for $C_{16}H_{26}O_3$: C 72.14, H 9.84; found: C 72.39, H 9.81.

3.3.2.10. *(R)-2-(4-methoxybenzyloxy)octanal.*



22

To a stirred solution of oxalyl chloride (8.8 mL, 0.10 mol) in dry dichloromethane (286 mL), was added DMSO (9.7 mL, 0.14 mol) in dry dichloromethane (143 mL) dropwise at -78 °C under N_2 atmosphere. Stirring was continued for 15 min, then **(-)-21** (9.1 g, 0.03 mol) in dry dichloromethane (143 mL) was added dropwise. After complete addition, the reaction mixture was stirred at -78 °C for 1 h, then NEt_3 (47 mL, 0.34 mol) was added dropwise. The reaction mixture was gradually warmed to 0 °C and stirred at this temperature until complete conversion was observed. The mixture was diluted with diethyl ether (50 mL) and poured in cold sat. $NaHCO_3$ (140 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether (3 × 100 mL). The combined organic extracts were washed with brine (75 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography in 0-5% ethyl acetate : hexane gave aldehyde **(+)-22** (8.5 g, 94%) as a colorless oil.

$\mathbf{R_f}$ (4% ethyl acetate : hexane) = 0.47.

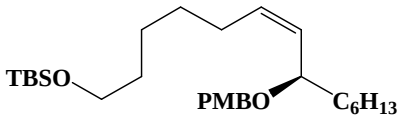
$\mathbf{[\alpha]_D^{23}}$ = +37.8 (*c* 0.75, MeOH);

^{1}H NMR (600 MHz, $CDCl_3$) δ = 9.63 (d, *J* = 2.3 Hz, 1H), 7.30 (d, *J* = 8.5 Hz, 2H), 6.91 (d, *J* = 8.5 Hz, 2H), 4.62 (d, *J* = 11.8 Hz, AB, 1H), 4.50 (d, *J* = 11.4 Hz, AB, 1H), 3.82 (s, 3H), 3.78-3.72 (m, 1H), 1.71-1.63 (m, 2H), 1.49-1.20 (m, 8H), 0.91 (t, *J* = 7.1 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 204.1, 159.5, 129.7 (×2), 129.4, 113.9 (×2), 83.2, 72.2, 55.2, 31.6, 30.0, 29.0, 24.7, 22.5, 14.0 ppm.

Elemental analysis calcd (%) for $C_{16}H_{24}O_3$: C 72.69, H 9.15; found: C 72.92, H 9.12.

3.3.2.11. *(R)-Z-8-(4-methoxybenzyloxy)tetradec-6-enyloxy)tert-butyldimethylsilane*.



23

To a stirred suspension of $TBSO(CH_2)_6P^+Ph_3Br^-$[22] (11.85 g, 0.021 mol) in anhydrous THF (76 mL) cooled to -78 °C, *n*-BuLi (12.35 mL, 0.02 mol, 1.6 M in hexane) was added. The brown red colored suspension obtained was stirred at -78 °C for 30 min under N_2 atmosphere. Compound **(+)-22** (4.0 g, 0.01mol) in anhydrous THF (76 mL) was added dropwise; on complete addition the solution turned pale yellow. The reaction mixture was stirred at -78 °C for 15 min, warmed to room temperature and stirred for 2 h. After addition of saturated NH_4Cl (100 mL), the product was extracted with diethyl ether (3 × 100 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification by flash column chromatography in 0-1% ethyl acetate: hexane yielded **(+)-23** (5.2 g, 74%) as a colorless oil.

$\mathbf{R_f}$ (1% ethyl acetate : hexane) = 0.36.

$\mathbf{[\alpha]_D^{23}}$ = +15.6 (*c* 1.00, $CHCl_3$).

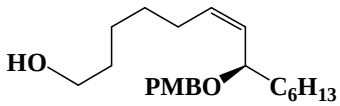
$\mathbf{\nu_{max}}$ (liquid film) 3050, 1570, 1550, 1535, 1480, 1290, 1110, 860, 760, 730 cm^{-1}.

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.24 (d, *J* = 8.5 Hz, 2H), 6.86 (d, *J* = 8.5 Hz, 2H), 5.59 (dt, *J* = 11.0, 7.3 Hz, 1H), 5.29 (dd, *J* = 11.0, 9.4 Hz, 1H), 4.49 (d, *J* = 11.6 Hz, AB, 1H), 4.24 (d, *J* = 11.6 Hz, AB, 1H), 4.12-4.02 (m, 1H), 3.80 (s, 3H), 3.60 (t, *J* = 6.4 Hz, 2H), 2.12-1.95 (m, 2H), 1.73-1.18 (m, 16H), 0.89 (m, 12 H), 0.05 (s, 6H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 158.9, 133.1, 131.2 (×2), 129.2 (×2), 113.6 (×2), 73.8, 69.4, 63.1, 55.2, 35.8, 32.7, 31.8, 29.6, 29.3, 27.8, 26.0 (×3), 25.5, 25.4, 22.6, 18.3, 14.1, -5.3 (×2) ppm.

Elemental analysis calcd (%) for $C_{28}H_{50}O_3Si$: C 72.67, H 10.89; found: C 72.40, H 10.92.

3.3.2.12. *(R)-8-(4-methoxybenzyloxy)tetradec-6-en-1-ol.*



24

A solution of TBS ether **(+)-23** (9.5 g, 0.02 mol) in anhydrous THF (82 mL) was cooled to 0 °C. TBAF (61 mL, 0.06 mol, 1M in THF) was added at 0 °C under N_2 atmosphere. The reaction mixture was warmed to room temperature and stirred for 1 h. Saturated NH_4Cl (125 mL) was added, and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (3 × 150 mL), and the combined organic extracts were washed with brine (80 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using flash column chromatography in 0-15% ethyl acetate : hexane to afford desired **(+)-24** (6.3 g, 88%) as pale yellow oil.

$\mathbf{R_f}$ (15% ethyl acetate : hexane) = 0.41.

$\mathbf{[\alpha]_D^{23}}$ = +20.0 (*c* 1.00, $CHCl_3$).

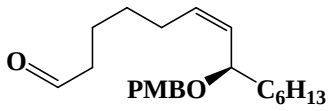
$\mathbf{\nu_{max}}$ (liquid film) 3600-3200 (br), 3090, 3020, 1640, 1530, 1480, 1320, 1290, 1280, 1105, 1050, 760 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.26 (d, *J* = 8.5 Hz, 2H), 6.88 (d, *J* = 8.5 Hz, 2H), 5.61 (dt, *J* = 11.0, 7.3 Hz, 1H), 5.31 (dd, *J* = 11.0, 9.5 Hz, 1H), 4.51 (d, *J* = 11.9 Hz, AB, 1H), 4.27 (d, *J* = 11.9 Hz, AB, 1H), 4.15-4.05 (m, 1H), 3.82 (s, 3H), 3.65 (t, *J* = 6.4 Hz, 2H), 2.15-2.00 (m, 2H), 1.75-1.50 (m, 4H), 1.48-1.30 (m, 12H), 0.88 (t, *J* = 7.0 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 158.9, 133.0, 131.2, 131.1, 129.2 (×2), 113.7 (×2), 73.8, 69.3, 62.8, 55.3, 35.7, 32.6, 31.8, 29.5, 29.3, 27.8, 25.4, 25.4, 22.6, 14.1 ppm.

Elemental analysis calcd (%) for $C_{22}H_{36}O_3$: C 75.82, H 10.41; found: C 75.60, H 10.43.

3.3.2.13. *(R)-8-(4-methoxybenzyloxy)tetradec-6-enal.*



25

To a stirred solution of oxalyl chloride (4.7 mL, 0.05 mol) in dry dichloromethane (150 mL), a solution of DMSO (5.1 mL, 0.07 mol) in dry dichloromethane (75 mL) was added dropwise at -78 °C under N_2 atmosphere and stirred for 15 min. Tetradecenol **(+)-24** (6.3 g, 0.02 mol) in dry dichloromethane (75 mL) was added dropwise. After complete addition, the reaction mixture was stirred at -78 °C for 1 h. After addition of NEt_3 (25 mL, 0.18 mol), the reaction mixture was gradually warmed to 0 °C and stirred at this temperature until complete conversion was observed. The mixture was diluted with diethyl ether (50 mL) and poured in cold sat. $NaHCO_3$ (80 mL), the organic layer was separated and aqueous layer was extracted with diethyl ether (3 × 150 mL). The combined organic extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography with 0-5% ethyl acetate: hexane gave **(+)-25** (5.7 g, 91%) as a colorless oil.

$\mathbf{R_f}$ (3% ethyl acetate : hexane) = 0.44.

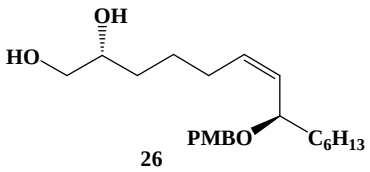
$\mathbf{[\alpha]_D^{23}}$ = +17.8 (*c* 1.00, MeOH).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.78 (s, 1H), 7.25 (d, *J* = 8.8 Hz, 2H), 6.88 (d, *J* = 8.8 Hz, 2H), 5.59 (dt, *J* = 11.0, 7.3 Hz, 1H), 5.34 (dd, *J* = 11.0, 9.5 Hz, 1H), 4.51 (d, *J* = 11.3 Hz, AB, 1H), 4.26 (d, *J* = 11.3 Hz, AB, 1H), 4.012-4.02 (m, 1H), 3.82 (s, 3H), 2.44 (t, *J* = 7.3 Hz, 2H), 2.12-1.98 (m, 2H), 1.73-1.56 (m, 4H), 1.49-1.35 (m, 4H), 1.32-1.18 (m, 6H), 0.89 (t, *J* = 7.0 Hz, 3H) ppm.

^{13}C NMR (75 MHz, MeOD) δ = 200.3, 159.4, 133.2, 130.7, 129.3, 129.1, 113.5, 113.3, 104.6, 73.4, 69.2, 54.4, 35.5, 32.3, 31.8, 29.4, 29.1, 27.6, 25.2, 24.1, 22.5, 13.2 ppm.

Elemental analysis calcd (%) for $C_{22}H_{34}O_3$: C 76.26, H 9.89; found: C 75.98, H 9.87.

3.3.2.14. *(2R,8R)-8-(4-methoxybenzyloxy)tetradec-6-ene-1,2-diol.*



26

A suspension of L-proline (57.6 mg, 0.5 mmol) in $CHCl_3$ (16.5 mL) was cooled to 4 °C and stirred for 15 min, then nitrosobenzene (525 mg, 5 mmol) was added in one portion. At this time the solution turned green. To this suspension was added a solution of tetradecenal **(+)-25** (3.4 g, 9.9 mmol) in $CHCl_3$ (16.5 mL) in one portion. The resulting solution was then stirred at 4 °C for 2 h. At this time the solution turned yellow. The reaction mixture was then added to an ethanol (50 mL) suspension of $NaBH_4$ (374 mg, 9.9 mmol) at 0 °C. After 30 min. the reaction was treated with saturated aqueous $NaHCO_3$ (125 mL). The aqueous layer was extracted with dichloromethane (3 × 150 mL). The combined organic extracts were washed with brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The oxy-aniline adduct (4.1 g) was dissolved in EtOH/AcOH (3:1, 30 mL) and Zn dust (1.2 g, 0.02 mol) was added portionwise. The resulting suspension was stirred at room temperature for 1 h. The reaction mixture was filtered through a plug of celite and the residue was washed with ethanol (25 mL). The filtrate was concentrated *in vacuo* at <40 °C. The residue was dissolved in ethyl acetate (200 mL) and washed with a saturated $NaHCO_3$ solution (100 mL), brine (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The resulting yellow oil was purified using flash column chromatography with 20% - 50% ethyl acetate : hexane to afford 1,2 diol **(+)-26** (2.78 g 77%) as a yellow oil.

$\mathbf{R_f}$ (40% ethyl acetate : hexane) = 0.36.

$\mathbf{[\alpha]_D^{23}}$ = +18.9 (*c* 1.00, $CHCl_3$).

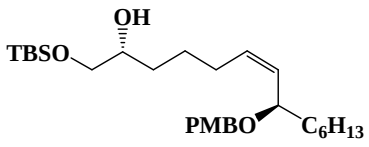
$\mathbf{\nu_{max}}$ (liquid film) 3600-3200 (br), 3090, 1640, 1540, 1482, 1090, 1070, 760, 730 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.23 (d, *J* = 8.5 Hz, 2H), 6.86 (d, *J* = 8.5 Hz, 2H), 5.56 (dt, *J* = 11.0, 7.3 Hz, 1H), 5.32 (dd, *J* = 11.0, 9.5 Hz, 1H), 4.49 (d, *J* = 11.4 Hz, AB, 1H), 4.25 (d, *J* = 11.4 Hz, AB, 1H), 4.12-4.02 (m, 1H); 3.79 (s, 3H), 3.71-3.53 (m, 2H), 3.45-3.35 (m, 1H), 2.88 (brs, 1H), 2.26 (brs, 1H), 2.15-1.95 (m, 2H), 1.73-1.15 (m, 14H), 0.87 (t, *J* = 7.0 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 159.1, 132.8, 131.6, 131.1, 129.4 (×2), 113.8 (×2), 73.9, 72.2, 69.5, 66.8, 55.4, 35.8, 32.8, 31.9, 29.4, 27.8, 25.8, 25.5, 22.7, 14.2 ppm.

Elemental analysis calcd (%) for $C_{22}H_{36}O_4$: C 72.49, H 9.95, found: C 72.70, H 9.94.

3.3.2.15. *(2R,8R)-Z-1-((tert-butyldimethylsilyl)oxy)-8-(4-methoxybenzyloxy)tetradec-6-en-2-ol.*



27

To a stirred solution of tetradecene-1,2 diol **(+)-26** (400 mg, 1.1 mmol) in dry dichloromethane (11 mL) was added imidazole (149 mg, 2.2 mmol), then TBSCl (215 mg, 1.4 mmol) was added portionwise at 0 °C. The reaction mixture was warmed to room temperature and stirred for 6 h, then it was quenched by addition of ice; the aqueous layer was extracted with dichloromethane (2 × 30 mL). The combined organic extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The residue was purified using flash column chromatography (0 - 8% ethyl acetate : petroleum ether) to afford mono TBS ether **(+)-27** (518 mg, 98%) as a colorless oil.

R_f (5% ethyl acetate : petroleum ether) = 0.35.

$[\alpha]_D^{23}$ = +14.3 (*c* 1.00, $CHCl_3$).

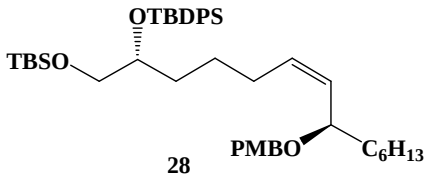
ν_{max} (liquid film) 3610, 3580-3400 (br), 1625, 1605, 1530, 1480, 1260, 1120, 1090, 1050, 850, 795 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.26 (d, *J* = 8.5 Hz, 2H), 6.88 (d, *J* = 8.5 Hz, 2H), 5.61 (dt, *J* = 11.0, 7.3 Hz, 1H), 5.33 (dd, *J* = 11.0, 9.5 Hz, 1H), 4.51 (d, *J* = 11.3 Hz, AB, 1H), 4.26 (d, *J* = 11.3 Hz, AB, 1H), 4.12-4.02 (m, 1H), 3.82 (s, 3H), 3.69-3.59 (m, 2H), 3.40 (dd, *J* = 8.5, 10.4 Hz, 1H), 2.17-1.96 (m, 2H), 1.74-1.11 (m, 14H), 0.92 (s, 9H), 0.89 (t, *J* = 7.0 Hz, 3H), 0.09 (s, 6H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 159.1, 132.9, 131.6, 131.2, 129.3 (×2), 113.8 (×2), 73.9, 71.7, 69.5, 67.3, 55.4, 35.9, 32.5, 32.0, 29.4, 27.9, 26.0 (×3), 25.9, 25.5, 22.7, 18.4, 14.2, -5.2, -5.3 ppm.

Elemental analysis calcd (%) for $C_{28}H_{50}O_4Si$: C 70.24, H 10.53; found: C 70.01, H 10.54.

3.3.2.16. *(R)-5-((R)-Z-6-((4-methoxybenzyl)oxy)dodec-4-en-1-yl)-2,2,8,8,9,9-hexamethyl-3,3-diphenyl-4,7-dioxa-3,8-disiladecane.*



28

A solution of mono TBS ether **(+)-27** (518 mg, 1.1 mmol), imidazole (220 mg, 3.2 mmol) in dry DMF (5.4 mL) was cooled to 0 °C. TBDPS-Cl (0.42 mL, 1.6 mmol) was added dropwise at 0 °C. After complete addition, the reaction mixture was stirred at room temperature under N_2 atmosphere for 12 h. TLC showed partial completion of reaction, thus other 302 mg (1.1 mmol of TBDPS-Cl were added and the mixture stirred for further 8 h. The reaction was quenched by addition of ice; the aqueous layer was extracted with diethyl ether (3 × 50 mL), then the combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using flash column chromatography with 0-3% ethyl acetate : petroleum ether to afford bis-silyl ether **(+)-28** (773 mg, 98%) as yellow oil.

R_f (2% ethyl acetate : hexane) = 0.47.

$[\alpha]_D^{23}$ = +5.5 (*c* 1.2, $CHCl_3$).

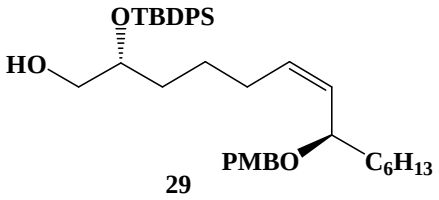
ν_{max} (liquid film) 3100, 1630, 1520, 1480, 1265, 1120, 850, 720 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.76-7.65 (m, 4H), 7.47-7.31 (m, 6H), 7.24 (d, *J* = 8.5 Hz, 2H), 6.87 (d, *J* = 8.5 Hz, 2H), 5.51 (dt, *J* = 11.0, 7.0 Hz, 1H), 5.27 (dd, *J* = 11.0, 9.5 Hz, 1H), 4.48 (d, *J* = 11.6 Hz, AB, 1H), 4.21 (d, *J* = 11.6 Hz, AB, 1H), 4.011-4.01 (m, 1H), 3.81 (s, 3H), 3.85-3.71 (m, 1H), 3.53-3.38 (m, 2H), 2.00-1.84 (m, 2H), 1.65-1.20 (m, 14H), 1.06 (s, 9H), 0.89 (t, *J* = 7.0 Hz, 3H), 0.83 (s, 9H), 0.06 (s, 3H), 0.01 (s, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 159.1, 136.0 (×6), 134.6, 134.5, 133.3, 131.3, 129.7, 129.4, 127.6 (×5), 113.7 (×2), 74.1, 73.6, 69.6, 66.3, 55.4, 35.9, 33.6, 32.0, 29.5, 28.1, 27.2 (×3), 26.0 (×3), 25.5, 24.8, 22.8, 19.5, 18.4, 14.3, -5.3 (×2) ppm.

Elemental analysis calcd (%) for $C_{44}H_{68}O_4Si_2$: C 73.69, H 9.56: found: C 73.50, H 9.55.

3.3.2.17. *(2R,8R)-Z-2-((tert-butyldiphenylsilyl)oxy)-8-((4-methoxybenzyl)oxy)tetradec-6-en-1-ol.*



29

A mixture of bis-silyl ether **(+)-28** (698 mg, 0.9 mmol) in AcOH/ THF/ H_2O 3: 1: 1 (36 mL) was stirred at room temperature for 36 h. The reaction was quenched by addition of saturated aq. K_2CO_3 (50 mL), followed by addition of solid K_2CO_3. The aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* Purification using flash column chromatography with 0-8% ethyl acetate : petroleum ether produced **(-)-29** (500 mg, 92%) as a yellow oil.

R_f (5% ethyl acetate : petroleum ether) = 0.56.

$[\alpha]_D^{23}$ = -18.07 (*c* 1.3, $CHCl_3$).

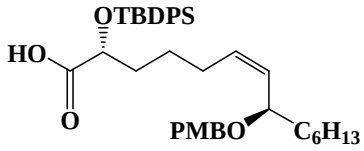
ν_{max} (liquid film) 3610, 3580-3300 (br), 1635, 1600, 1530, 1480, 1445, 1270, 1120, 840, 760, 730 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.75-7.58 (m, 4H), 7.49-7.32 (m, 6H), 7.21 (d, *J* = 8.6 Hz, 2H), 6.85 (d, *J* = 8.6 Hz, 2H), 5.41 (dt, *J* = 11.2, 7.4 Hz, 1H), 5.24 (dd, *J* = 11.2, 9.6 Hz, 1H), 4.44 (d, *J* = 11.4 Hz, AB, 1H), 4.18 (d, *J* = 11.4 Hz, AB, 1H), 4.07-3.95 (m, 1H), 3.79 (s, 3H,), 3.80-3.72 (m, 1H), 3.58-3.41 (m, 2H), 1.91-1.73 (m, 2H), 1.66-1.13 (m, 14H), 1.07 (s, 9H), 0.87 (t, *J* = 7.0 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 159.1, 136.0 (×2), 135.8 (×2), 134.0, 133.8, 132.7, 131.5, 131.2, 129.9 (×2), 129.3 (×2), 127.9 (×4), 113.8 (×2), 1C overlapped to the solvent signal, 74.0, 69.5, 66.0, 55.4, 35.8, 33.3, 31.9, 29.4, 27.8, 27.2 (×4), 25.5, 22.7, 19.5, 14.2 ppm.

Elemental analysis calcd (%) for $C_{38}H_{54}O_4Si$: C 75.70, H 9.03; found: C 75.42, H 9.00.

3.3.2.18. *(2R,8R)-Z-2-((tert-butyldiphenylsilyl)oxy)-8-((4-methoxybenzyl)oxy)tetradec-6-enoic acid.*



30

A suspension of **(-)-29** (340 mg, 0.6 mmol), $NaHCO_3$ (141 mg, 1.7 mmol) in acetonitrile / water (3.4 mL:3.4 mL) was cooled to 0 °C and stirred for 10 min. TEMPO (17 mg, 0.1 mmol), and bis(acetoxy)iodobenzene (451 mg, 1.4 mmol), were added in one portion and the solution was stirred at 0 °C for 4 h. Saturated aq. $NaHCO_3$ (20 mL) was added at 0 °C and the aqueous layer was extracted with ethyl acetate (2 × 50 mL). The combined organic extracts were washed with brine (15 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The crude oil was purified using flash column chromatography (0-20% ethyl acetate: petroleum ether) to furnish **(+)-30** (259 mg, 70%) as a colorless oil.

$\mathbf{R_f}$ (20% ethyl acetate : hexane) = 0.43.

$\mathbf{[\alpha]_D^{23}}$ = +5.9 (*c* 1.00, MeOH).

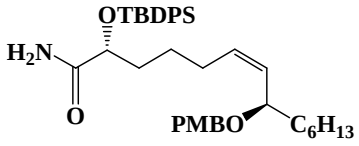
$\mathbf{\nu_{max}}$ (liquid film) 3100-2800 (br), 1730, 1630, 1605, 1525, 1480, 1445, 1280, 1260, 1125, 845, 760, 720 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.70-7.53 (m, 4H), 7.51-7.31 (m, 6H), 7.20 (d, *J* = 8.2 Hz, 2H), 6.85 (d, *J* = 8.2 Hz, 2H), 5.48-5.38 (m, 1H), 5.27 (m, 1H), 4.43 (d, *J* = 11.4 Hz, AB, 1H), 4.340-4.32 (m, 1H), 4.16 (d, *J* = 11.4 Hz, AB, 1H), 4.08-3.92 (m, 1H), 3.79 (s, 3H), 1.95-1.18 (m, 16H), 1.12 (s, 9H), 0.86 (t, *J* = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 174.8, 159.1, 135.8 (×4), 135.4, 132.9, 132.4, 131.8, 131.1, 130.5, 130.3, 129.3 (×2), 127.9 (×4), 113.8 (×2), 73.9, 72.9, 69.5, 55.4, 35.8, 34.3, 31.9, 29.8, 29.4, 27.4, 27.0 (×3), 25.4, 22.7, 19.4, 14.2 ppm.

Elemental analysis calcd (%) for $C_{38}H_{52}O_5Si$: C 73.98, H 8.50; found: C 74.22, H 8.52.

3.3.2.19. *(2R,8R)-Z-2-((tert-butyldiphenylsilyl)oxy)-8-((4-methoxybenzyl)oxy)tetradec-6-enoylamide.*



31

A solution of **(+)-30** (224 mg, 0.4 mmol), NH_4Cl (38 mg, 0.7 mmol) in dry DMF (5 mL) was cooled to 0 °C. HOBT (73 mg, 0.5 mmol) and HBTU (205 mg, 0.5 mmol) were added followed by DIPEA (0.23 mL, 1.4 mmol). The reaction mixture was warmed to room temperature and stirred for 1 h. Ice was added, and then the aqueous layer was extracted with ethyl acetate (2 × 25 mL). The combined organic extracts were washed with cold brine (3× 10 mL) and dried over Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified using flash column chromatography (0-30% ethyl acetate : petroleum ether) to furnish **(-)-31** (180 mg, 73%) as a white sticky solid.

$\mathbf{R_f}$ (25% ethyl acetate : hexane) = 0.45.

$\mathbf{[\alpha]_D^{23}}$ = -8.9 (*c* 1.4, MeOH).

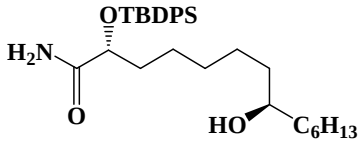
$\mathbf{\nu_{max}}$ (thin film) 3550, 3505, 3450, 3100, 1700, 1635, 1605, 1580, 1540, 1485, 1450, 1285, 1135, 850, 760 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.73-7.54 (m, 4H), 7.50-7.30 (m, 6H), 7.20 (d, *J* = 8.2 Hz, 2H), 6.85 (d, *J* = 8.2 Hz, 2H), 6.73 (brs, 1H), 5.78 (brs, 1H), 5.50-5.39 (m, 1H), 5.32-5.22 (m, 1H), 4.43 (d, *J* = 11.4 Hz, AB, 1H), 4.33.4.25 (m, 1H), 4.17 (d, *J* = 11.4 Hz, AB, 1H), 3.98 (m, 1H), 3.80 (s, 3H), 1.94-1.83 (m, 2H), 1.75-1.15 (m, 14H), 1.12 (s, 9H), 0.87 (t, *J* = 6.6 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 177.7, 160.5, 137.3 (×2), 137.1 (×2), 134.4, 134.0, 133.1, 132.7, 131.8 (×2), 130.8 (×2), 129.5 (×4), 115.3 (×2), 75.5 (×2), 71.0, 56.8, 37.3, 35.6, 33.4, 31.3, 30.9, 29.0, 28.6 (×3), 26.9, 25.1, 24.2, 20.8, 15.7 ppm.

Elemental analysis calcd (%) for $C_{38}H_{53}NO_4Si$: C 74.10, H 8.67, N 2.27; found: C 74.38, H 8.69, N 2.26.

3.3.2.20. *(2R,8R)-2-((tert-butyldiphenylsilyl)oxy)-8-hydroxytetradecanoylamide.*



18

To a solution of **(-)-31** (68 mg, 0.11 mmol) in methanol (6 mL) was added 10% Pd/C (20 mg). The suspension was evacuated under vacuum and flushed with H_2 gas (4 times). The reaction mixture was stirred under H_2 atmosphere for 12 h at room temperature, then filtered through a plug of celite and the residue was washed with ethyl acetate (10 mL). The filtrate was concentrated *in vacuo* and purified using flash column chromatography (0-40% ethyl acetate: petroleum ether to obtain **(-)-18** (40 mg, 73%) as a pale yellow oil.

R_f (30% ethyl acetate : hexane) = 0.35.

$[\alpha]_D^{23}$ = -10.1 (*c* 1.0, MeOH).

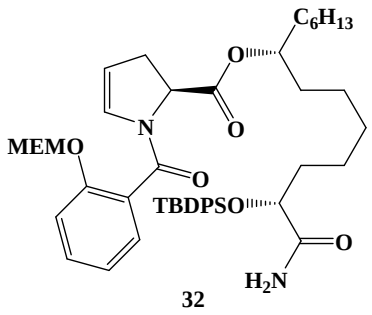
ν_{max} (thin film) 3550, 3505, 3450, 3100, 1710, 1604, 1575, 1450, 1299, 930, 790, 740 cm^{-1}.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.70-7.54 (m, 4H), 7.50-7.31 (m, 6H), 6.72 (brs, 1H), 5.67 (brs, 1H), 4.27 (t, *J* = 4.6 Hz, 1H), 3.60-3.50 (m, 1H), 1.94-1.55 (m, 2H), 1.48-1.15 (m, 19H), 1.12 (s, 9H), 0.89 (t, *J* = 6.6 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 176.6, 135.8 (×4), 133.1, 132.6, 130.3 (×2), 128.0 (×4), 74.4, 72.0, 37.5, 34.5, 32.0, 29.5, 27.2, 27.1 (×3), 25.7 (×2), 23.5, 22.8 (×2), 19.4, 14.1 ppm.

Elemental analysis calcd (%) for $C_{30}H_{47}NO_3Si$: C 72.38, H 9.52, N 2.80; found: C 72.19, H 9.54, N 2.79.

3.3.2.21. *(7R,13R)-14-amino-13-((tert-butyldiphenylsilyl)oxy)-14-oxotetradecan-7-yl (S)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



32

To a stirred solution of pyrrolinecarboxylic acid **(-)-14** (66 mg, 0.2 mmol) in dry dichloromethane (8 mL) was added EDC·HCl (52 mg, 0.3 mmol) and DMAP (5 mg, 0.04 mmol) at 0 °C. A solution of **(-)-18** (69 mg, 0.14 mmol) in dichloromethane (2 mL) was added dropwise at 0 °C. The reaction mixture was warmed to room temperature and stirred for 16 h, then it was poured into water (10 mL) and the organic layer was separated. The aqueous layer was extracted with dichloromethane (2 × 25 mL) and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo* and the residue was purified using flash column chromatography (FCC) with 0-40% ethyl acetate : hexane to furnish **(-)-32** (56 mg, 50%) as a pale yellow oil.

R_f (40% ethyl acetate : hexane) = 0.35.

$[\alpha]_D^{23}$ = -44.9 (*c* 1.0, MeOH).

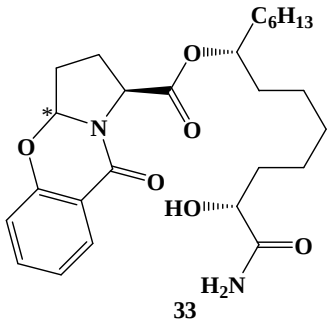
ν_{max} (liquid film) 3520, 3410, 3090, 1760, 1710, 1660, 1640, 1440, 1280, 1130, 1010, 920, 790 cm^{-1}.

^{1}H NMR (600 MHz, acetone-d_6) (mixture of conformers) δ = 7.74-7.65 (m, 4H), 7.53-7.41 (m, 7H), 7.31 (dd, *J* = 1.6, 7.6 Hz, 1H), 7.27 (d, *J* = 8.2 Hz, 1H), 7.14-7.08 (m, 1H), 6.97 (brs, 1H), 6.72 (brs, 1H), 6.22-6.18 (m, 1H), 5.30 (s, 2H), 5.114-5.10 (m, 1H), 4.95- 4.87 (m, 2H), 4.16 (t, *J* = 5.1 Hz, 1H), 3.80 (t, *J* = 4.8 Hz, 2H), 3.51 (t, *J* = 4.8 Hz, 2H), 3.27 (s, 3H), 3.20-3.12 (m, 1H), 2.70-2.55 (m, 1H), 2.20-1.92 (m, 2H), 1.67-1.11 (m, 18H), 1.11 (s, 9H), 0.87 (t, *J* = 7.3 Hz, 3H) ppm.

^{13}C NMR (150 MHz, acetone-d_6,) δ = 175.1, 170.5, 164.1, 153.5, 135.7 (×2), 135.6 (×2), 133.3, 132.8, 131.0, 130.7, 130.0, 129.5, 128.6, 127.8 (×4), 126.5, 121.8, 115.5, 108.1, 93.7, 74.5, 74.3, 71.4, 67.9, 58.1, 57.9, 34.5, 34.0, 34.0, 33.8, 31.5, 29.6, 29.5, 26.5 (×3), 25.0, 24.7, 23.4, 22.3, 18.9, 13.4 ppm.

Elemental analysis calcd (%) for $C_{46}H_{64}N_2O_8Si$: C 68.97, H 8.05, N 3.50; found: C 69.20, H 8.03, N 3.52.

3.3.2.22. *(7R,13R)-14-amino-13-hydroxy-14-oxotetradecan-7-yl (1S)-9-oxo-1,2,3,3a-tetrahydro-9H-benzo[e]pyrrolo[2,1-b][1,3]oxazine-1-carboxylate.*



33

2N HCl (0.7 mL) was added dropwise to stirred solution of MEM ether **(-)-32** (40 mg, 0.049 mmol) in THF (0.7 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1h. THF was removed *in vacuo*; the aqueous layer was extracted with ethyl acetate (2 x 7 mL). The combined organic extracts were washed with brine (6 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo.* The product was purified using flash column chromatography with 15-45 % EtOAc : Hexane to afford **(-)-33** as a colourless oil (9 mg, 35 %, more polar diastereomer) and 2.5 mg (11%, less polar diastereomer), stereochemistry not assigned.

Spectral data for the more polar diastereomer:

R_f (50 % EtOAc : hexane) = 0.32.

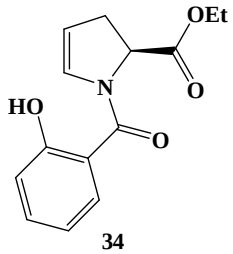
$[\alpha]_D^{23}$ = -54.0 (*c* 0.75, $CHCl_3$).

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.83 (d, *J* = 7.6 Hz, 1H), 7.44 (dd, *J* = 7.6, 7.6 Hz, 1H), 7.09 (dd, *J* = 7.6, 7.6 Hz, 1H), 6.99 (d, *J* = 7.6 Hz, 1H), 6.87 (s, 1H), 5.56 (dd, *J* = 6.2, 7.3 Hz, 1H), 5.48 (s, 1H), 4.99-4.93 (m, 1H), 4.60 (d, *J* = 8.9 Hz, 1H), 4.29-4.26 (m, 1H), 4.13-4.08 (m, 1H), 2.50-2.44 (m, 1H), 2.40-2.23 (m, 2H), 2.20-2.15 (m,1H), 1.84-1.04 (m, 20H), 0.85 (t, *J* = 7.3 Hz, 3H).

^{13}C NMR (150 MHz, $CDCl_3$) δ = 177.3, 170.6, 161.4, 157.8, 134.4, 127.8, 122.8, 118.9, 116.9, 88.5, 75.5, 71.0, 57.1, 34.4, 33.8, 33.7, 31.7, 30.2, 29.0, 27.7, 26.0, 25.3, 24.5, 24.0, 22.5, 14.0.

Elemental analysis. Calcd. for $C_{26}H_{38}N_2O_6$ C, 65.80; H, 8.07; N, 5.90. Found: C, 65.87; H, 8.09; N, 5.91.

3.3.2.23. *ethyl (S)-1-(2-hydroxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



34

To a stirred solution of ethyl (*S*)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-5-oxopyrrolidine-2-carboxylate **(-)-13** (35 mg, 0.10 mmol) in CH_2Cl_2 (0.6 ml, 15 M) was added $TiCl_4$ (0.2 mL, 0.2 mmol, 1M in CH_2Cl_2) at -20 °C under N_2 atmosphere. The reaction mixture was stirred at -20 °C for 10 min; then aqueous ammonia (1.2 mL) was added. The aqueous layer was extracted with ethyl acetate (2 x 5 mL), and the combined organic extracts were washed with brine (5 ml) and dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The purification using preparative TLC in 30 % EtOAc: Hexane, gave the title compound (-)-**34** as a colourless oil (20 mg, 76 %).

R_f (20 % EtOAc: hexane) = 0.3.

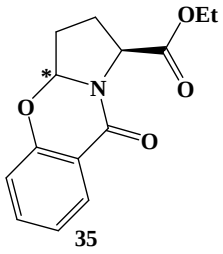
$[\alpha]_D^{23}$ = - 132.3 (*c* 1.00, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.76 (s, 1H), 7.46-7.34 (m, 2H), 7.00 (dd, *J* = 1.2, 8.3 Hz, 1H), 6.88 (ddd, *J* = 8.3, 8.3, 1.2 Hz, 1H), 6.84-6.78 (m, 1H), 5.30-5.24 (m, 1H), 5.02 (dd, *J* = 5.2, 11.4 Hz, 1H), 4.30-4.19 (m, 2H), 3.18-3.05 (m, 1H), 2.78-2.66 (m, 1H), 1.29 (t, *J* = 7.1 Hz, 3H).

^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.1, 167.8, 159.3, 133.7, 131.1, 128.5, 119.1, 118.2, 117.0, 110.9, 61.9, 59.5, 33.6, 14.3.

Elemental analysis Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79, N, 5.36. Found: C, 64.52; H, 5.78; N, 5.34.

3.3.2.24. *ethyl (1S)-9-oxo-1,2,3,3a-tetrahydro-9H-benzo[e]pyrrolo[2,1-b][1,3]oxazine-1-carboxylate.*



35

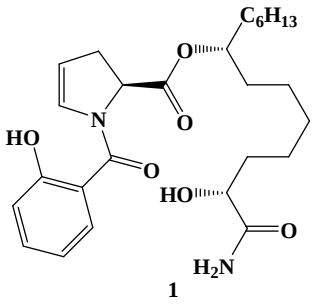
2N HCl (0.5 mL) was added dropwise to stirred solution of MEM ether (20 mg, 0.0572 mmol) in THF (0.5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1h. THF was removed *in vacuo*; the aqueous layer was extracted with ethyl acetate (2 X 5 mL). The combined organic extracts were washed with brine (4 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The product was purified using flash column chromatography with 25 % EtOAc: Hexane to afford **35** as yellow oil (8 mg, 35 %, major diastereomer, stereochemistry not assigned)

R_f (30 % EtOAc: Hexane) = 0.3.

^{1}H NMR (300 MHz, $CDCl_3$) δ = ^{1}H-NMR: 7.93 (dd, *J* = 1.8, 7.7 Hz, 1H), 7.45 (m, 1H), 7.11 (td, *J* = 1.3, 7.6 Hz, 1H), 6.99 (m, 1H), 5.64 (m, 1H), 4.65 (m,1H), 4.22 (m, 2H), 2.54 – 2.20 (m, 4H), 1.26 (t, *J* = 7.10, 3H)

^{13}C NMR (150 MHz, $CDCl_3$) δ = 171.2, 161.3, 158.0, 134.4, 128.3, 122.9, 119.5, 117.2, 88.5, 61.9, 56.9, 30.4, 26.3, 14.4.

3.3.2.25. *(7R,13R)-14-amino-13-hydroxy-14-oxotetradecan-7-yl (S)-1-(2-hydroxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



1

To a stirred solution of (-)-**32** (28 mg, 0.03 mmol) in dichloromethane (1 mL) was added $TiCl_4$ (0.13 mL, 0.13 mmol, 1 M in dichloromethane) at -20 °C. The reaction mixture was stirred at -20 °C for 10 min; then aqueous ammonia (2 mL) was added. The aqueous layer was extracted with ethyl acetate (2 × 30 mL), and the combined organic extracts were washed with brine (10 mL) and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo*. The crude compound (24 mg) was dissolved in dry THF (1 mL) and cooled to 0 °C. TBAF (0.1 mL, 0.1 mmol, 1 M in THF) was added dropwise. The reaction mixture was warmed to room temperature and stirred for 1 h, then saturated NH_4Cl (5 mL) was added. The aqueous layer was extracted with ethyl acetate (2 × 25 mL); and the combined organic extracts were washed with brine (10 mL) and dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified using preparative TLC in 5% methanol : dichloromethane to furnish **1** (10 mg, 70% over two steps) as colorless oil.

R_f (3% methanol : dichloromethane) 0.33.

$[\alpha]_D^{23}$ = -33.4 (*c* 1.00, $CHCl_3$).

^{1}H NMR (600 MHz, $CDCl_3$) δ = 9.53 (brs, 1H), 7.40-7.34 (m, 2H), 6.97 (d, *J* = 8.2 Hz, 1H), 6.89 (dd, *J* = 7.5, 7.5 Hz, 1H), 6.70 (brs, 1H), 6.59 (brs, 1H), 5.42 (brs, 1H), 5.36-5.26 (m, 1H), 5.00 (dd, *J* = 4.7, 11.3 Hz, 1H), 5.02-4.95 (m, 1H), 4.08 (dd, *J* = 3.6, 8.1 Hz, 1H), 3.60-3.40 (m, 1H), 3.12 (ddd, *J* = 17.1, 11.3, 2.5 Hz, 1H), 2.68 (m, 1H), 1.98-1.08 (m, 20H), 0.85 (t, *J* = 6.8 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 177.0, 171.3, 167.4, 158.0, 133.4, 130.8, 128.3, 119.4, 118.0, 117.8, 111.0, 76.0, 71.4, 59.3, 34.5, 34.3, 34.1, 33.6, 31.8, 29.2, 28.3, 25.5, 24.8, 24.5, 22.6, 14.1 ppm.

Elemental analysis calcd (%) for $C_{26}H_{38}N_2O_6$: C 65.80, H 8.07, N 5.90; found: C 66.02, H 8.05, N 5.91.

HRMS Accurate mass (ES+) calculated for $C_{26}H_{37}N_2O_6$ $(M-1)^+$ 473.26571, Found: 473.26487.

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CHAPTER-4

PART-A

BIOLOGICAL INVESTIGATION OF PROMYSALIN

4.1. INTRODUCTION:

Historically, the discovery of antibiotics and innovative developments in the field of antibiotics has saved numerous lives. The success of antibiotics is mainly attributed to their wide-spectrum activity which is amenable for the treatment of diverse bacterial infections. Recent studies by Cox. *et al.*[1] emphasize the prolonged effect of antibiotics on human physiology and health on early exposure, these findings underscore that some antimicrobials certainly damage the commensal host population. In fact, the perturbation of commensal microbiota by the use of oral antibiotics often allows the proliferation of pathogens, which results in elevated chances of gastrointestinal inflammations and allergies.[2] These findings prompted the researchers to expand their interest for the development of new chemical scaffolds with narrow-spectrum antibiotic activity.

Promysalin is reported as a narrow spectrum antibiotic with species-specific activity against *Pseudomonas aeruginosa*. It exhibits the species-specific activity also against other members of genus *Pseudomonas*,[3,4] without affecting the other gram-positive and gram-negative bacteria. Furthermore, promysalin attracted more attention because of its ability to inhibit the production of pyoverdine,[4] a siderophore which is often linked to the virulence. Due to its intrinsic specificity, promysalin could serve as an alternative to develop new anti-virulence therapy against opportunistic human pathogen *Pseudomonas aeruginosa*. Recently we have accomplished the total synthesis of promysalin;[5] herein, we discuss the biological investigation of promysalin. This work was done in collaboration with Prof. Diego Mora (Department of Food, Environmental and Nutritional Sciences, University of Milan)

4.2. PROMYSALIN- A BROAD SPECTRUM ANTIBIOTIC:

Promysalin was tested against several gram-positive and gram-negative bacteria using standard microdilution methods. The surprising results obtained (table-1), indicate that promysalin is not species specific antibiotic (as reported by Wuest *et. al*), having a broad-spectrum of activity against gram-positive and gram-negative bacteria including some well known human pathogens. The compound significantly inhibited the growth of *Pseudomonas aeruginosa* ATCC 10145 and ATCC 27853, *Staphylococcus aureus* ATCC 29213 and *Streptococcus thermophilus* DSM 201617^T with minimal inhibitory concentration

(MICs) of 16 µg/ml. Moreover, it is also active against several *Lactobacillus* species with MICs of 256 µg/ml. MICs of 64 µg/ml were measured against *Enterococcus durans* NCDO956, *E. faecalis* ATCC 29292, ATCC 19433 and LMG 19456, *E. faecium* ATCC 19434, *Staphylococcus epidermidis* ATCC 14990^T and *Streptococcus pyrogenes* ATCC 12344^T. The minimal bactericidal concentration (MBCs) values were determined for all the strains tested. The value ranges from 64 µg/ml for *S. thermophilus* and *S. pneumonia* to 256 or > 256 µg/ml for all the other bacterial strains tested. In conclusion, these findings reveal that promysalin is more active against streptococci and lactococci than toward lactobacilli.

Bacterial species	**Strains**	**MIC**	**MBC**
Escherichia coli	ATCC 25922	64	256
Pseudomonas aeruginosa	ATCC 10145	16	128
Pseudomonas aeruginosa	ATCC 27853	16	128
Enterococcus durans	NDCO 956	64	>256
Enterococcus faecalis	ATCC 29212	64	>256
Enterococcus faecalis	ATCC 19433	64	>256
Enterococcus faecalis	LMG 19456	64	>256
Enterococcus faecium	ATCC 19434	64	>256
Enterococcus italicus	DSM 15952	32	>256
Lactococcus cremoris	DSM 20069	32	32
Lactococcus garviae	DSM 20684	64	>256
Staphylococcus aureus	ATCC 25923	32	>256
Staphylococcus aureus	ATCC 29213	16	>256
Staphylococcus epidermidis	ATCC 14990^T	64	128
Streptococcus pneumoniae	ATCC 700669	32	64
Streptococcus pneumoniae	ATCC 49619	32	64
Streptococcus pneumoniae	Pen6	32	64
Streptococcus pyogenes	ATCC 12344^T	64	128
Streptococcus thermophilus	DSM 20617^T	16	64
Pediococcus acidilactici	PA-1	128	128
Lactobacillus acidophilus	DSM 20079	256	>256
Lactobacillus delbrueckii subsp. *bulgaricus*	ATCC 11842	256	>256
Lactobacillus casei	LMG 6904	256	>256
Lactobacillus helveticus	ATCC 15009^T	256	>256

Lactobacillus paracasei subsp. *Paracasei*	DSM 5622 ^{T}	256	>256
Lactobacillus plantarum	ATCC 4008	256	>256

Table 1: The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of promysalin against Gram-negative and Gram-positive bacteria.

The above results of the promysalin antibacterial activity against a wide range of gram-positive microorganisms are in contrast to the previous observations,[3] where promysalin was described as a species-specific antibiotic. The reason for this disparity in data could be attributed to the screening method used by Li. *et al*.[3] against promysalin sensitive bacteria, i.e. the agar diffusion test. We observed that the agar diffusion test (Figure-1) is not robust enough to detect the promysalin sensitive bacteria except for the sensitive strain *P. stutzeri* LMG 2333; on the contrary the microdilution was found to be a more effective and reliable method to identify promysalin-sensitive bacteria.

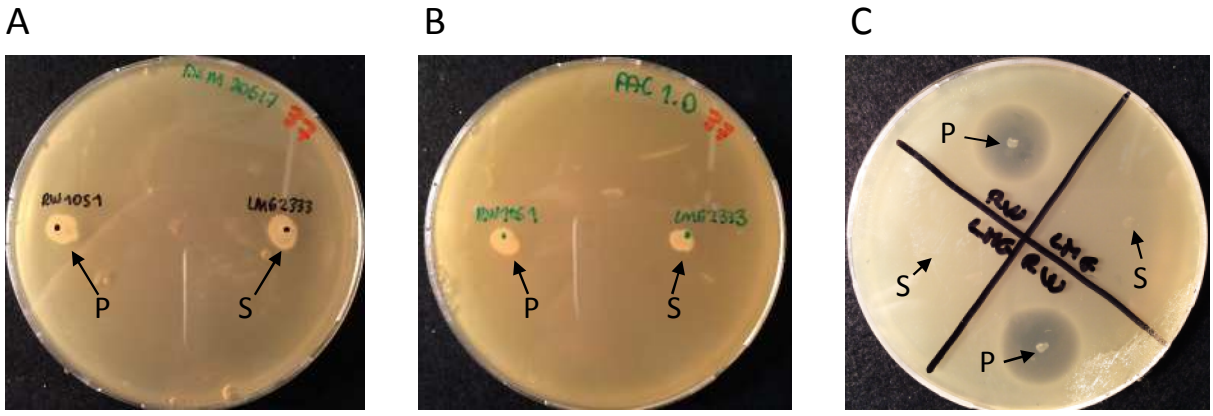


Figure1: Agar diffusion assay carried out using *P. putida* RW10S1 (promysalin producers), and *P. stutzeri* LMG 2333 (promysalin-sensitive) as reference strains[3] inoculated in TSB agar. Promysalin production and activity was tested against the soft agar overlay containing *Streptococcus thermophilus* DSM 20617^{T} **(A)**, *Pediococcus acidilactici* PAC1.0 **(B)** and *Pseudomonas stutzeri* LMG 2333 (**C**). P and S represent the growth of the promysalin-producer RW10S1, and the promysalin-sensitive strain, respectively, in TSB agar.

The probable explanation to the above conflicting results could be the following factors:

1) The effective concentration of promysalin released by the producer in the solid medium is lower than the MIC of sensitive strain.

2) The amphipathic nature of the promysalin might limit its solubility in aqueous medium which in turn interferes with the susceptibility test.
3) Promysalin was able to show its inhibitory effect against the gram-negative *Pseudomonas stutzeri* LMG 2333 in the agar diffusion test, whereas it was inactive against the gram-positive counterparts (figure-1). It could be hypothesized that promysalin might have a different mode of action against *Pseudomonas sp.* and gram-positive bacteria.

4.3. MECHANISM OF ACTION:

In order to understand the mode of action of promysalin against gram positive bacteria, *S. thermophilus* DSM 20617^T was used as a model organism. The cells were exposed to promysalin and the cell viability was measured using flow cytometry. Interestingly, when the cells were treated with 100 µg/ml of promysalin they rapidly lost their viability with significant increase in their propidium iodide fluorescence. Similar results were obtained with the biocide chlorhexidine at 100 µg/ml (figure-2) but with a faster rate.

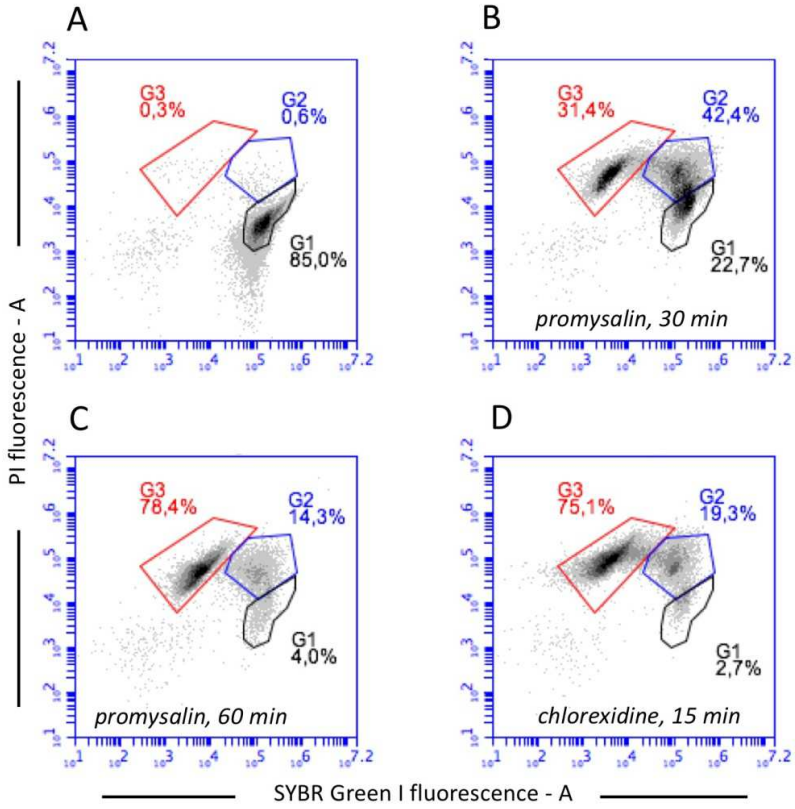


Figure-2: Effect of promysalin on cell viability of *S. thermophilus* DSM 20617^T. Flow cytometry density diagrams show SYBR Green I *vs* PI fluorescence of cells exposed to promysalin or chlorhexidine (100 µg/mL). A) Cells before exposure to antimicrobials; B) Cells after 30 min of exposure to promysalin. C)

Cells after 60 min exposure to promysalin and D) Cells after 15 min exposure to chlorhexidine. Viable cells are gated in G1, and viable cells with slightly damaged cell membranes are gated in G2. Dead cells with damaged membranes are gated in G3. The transition of cell populations from gate G1 to gate G3 is correlated to cell membrane damage.

In the case of chlorhexidine 75% of the cell population lost the viability in 15 min. However, similar effect was observed in 60 min when cells were exposed to promysalin, (figure-2). The significant loss of cell viability upon exposure of cells to promysalin and chlorhexidine was associated with the damage of cell membrane as it results in increased propidium iodide (PI) cell fluorescence with concomitant reduction of SYBR-green I cell fluorescence. In order to understand whether promysalin exerts the similar effect on gram-negative bacteria the same experiment was conducted with *E. coli* (Figure-3). To our delight, similar results were obtained to those previously obtained with *S. thermophilus* cells. In case of *E. coli* promysalin follows a different rate kinetics as the membrane damage was faster (evident after 20 min of exposure and almost complete in 90 min).

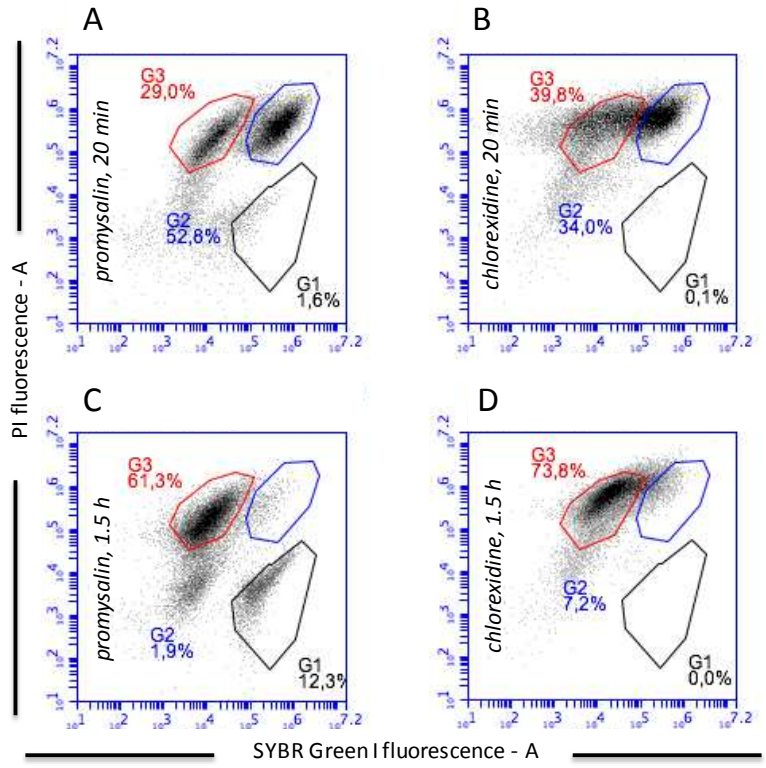


Figure-3: Effect of promysalin and chlorhexidine on *Escherichia coli* ATCC 25922 cell-membrane integrity. Flow cytometry density diagrams show SYBR Green I *vs* PI fluorescence of cells exposed to promysalin or chlorhexidine (100 and 200 µg/mL, respectively). A) and B) Cells after 20 min of exposure to antibacterials. C) and D) Cells after 1.5 h-exposure to antibacterials. Viable cells are gated in G1, and

viable cells with slightly damaged cell membranes are gated in G2. Dead cells with damaged membranes are gated in G3. The movement of cell populations from gate G1 to gate G3 is linked to cell membrane damage.

Chlorhexidine is a well known potent biocide, which exhibits its bactericidal effect by disrupting the cell membrane similar to antimicrobial peptides.[6] Benzalkonium chloride is another biocide which belongs to the class of quaternary ammonium compounds (QACs); like chlorhexidine it is a cationic surfactant which destructs the lipid bilayer of bacterial cell membrane.[7] Similar to chlorhexidine and promysalin, benzalkonium chloride induced the loss of membrane integrity on *S. thermophilus* and *E. coli* cells in 15 min, figure-4.

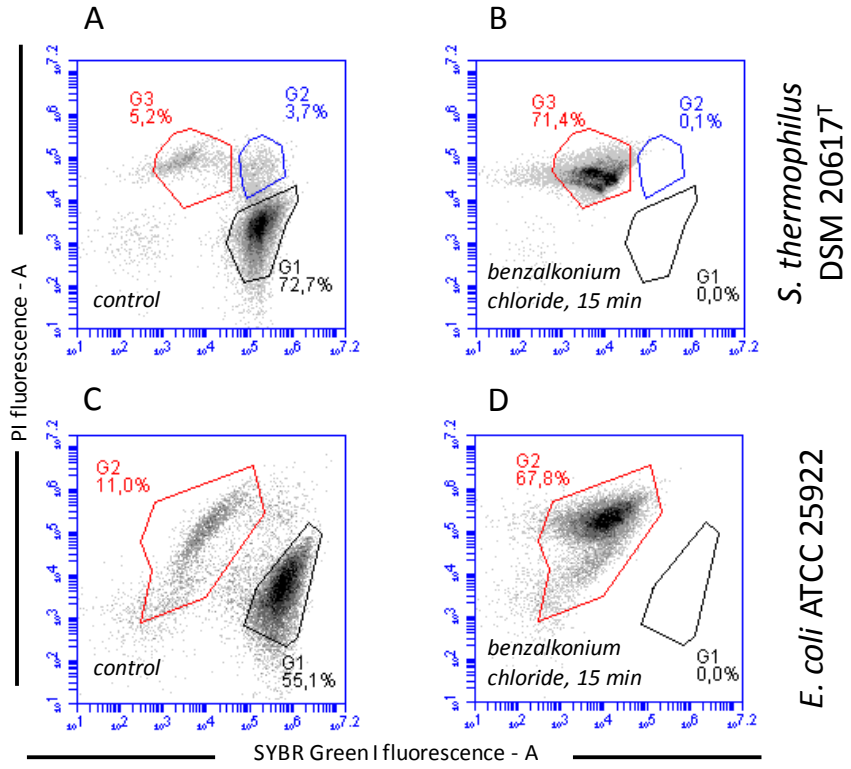


Figure-4: The effect of benzalkonium chloride on *Streptococcus thermophilus* DSM 20617^T and *Escherichia coli* ATCC 25922 cell membrane integrity. Flow cytometry density diagrams show SYBR Green I *vs* PI fluorescence of cells treated with promysalin or chlorhexidine (100 and 200 µg/mL, respectively). A) and C) Cells before exposure to benzalkonium chloride (100 µg/mL). B) and D) Cells after 15 min of exposure to benzalkonium chloride. Viable cells are gated in G1. Dead cells with damaged membranes are gated in G3. The transition of cell populations from gate G1 to gate G3 is correlated to cell membrane damage.

The amphipathic nature of promysalin is compatible with its potential to interact with the phospholipid bilayer. In this context, we therefore propose that promysalin, chlorhexidine and benzalkonium chloride could have similar mechanism of action.

4.4. PROMYSALIN-MEDIATED CELL MEMBRANE DISRUPTION AND RELEASE OF INTRACELLULAR COMPONENTS:

To understand the in detailed mechanism of action, we decided to verify whether the membrane damage is accompanied by release of intracellular material or not. We decided to use the fluorescence probe 5 (and 6-)carboxyfluorescein succinimidyl ester (cFSE) using the cFDASE precursor.[8] cFDASE can easily permeate the membrane and get cleaved by the intracellular esterases. The conjugation of cFSE with the aliphatic amine residues of intracellular proteins results in green fluorescent cell staining.[9] Thus, *S. thermophilus* DSM 20617^{T} cells were labeled with cFSE probe and exposed to promysalin and chlorhexidine. The cell membrane damage along with the leakage of cFSE fluorescence outside the cells was analyzed using flow cytometry and by standard fluorometer.

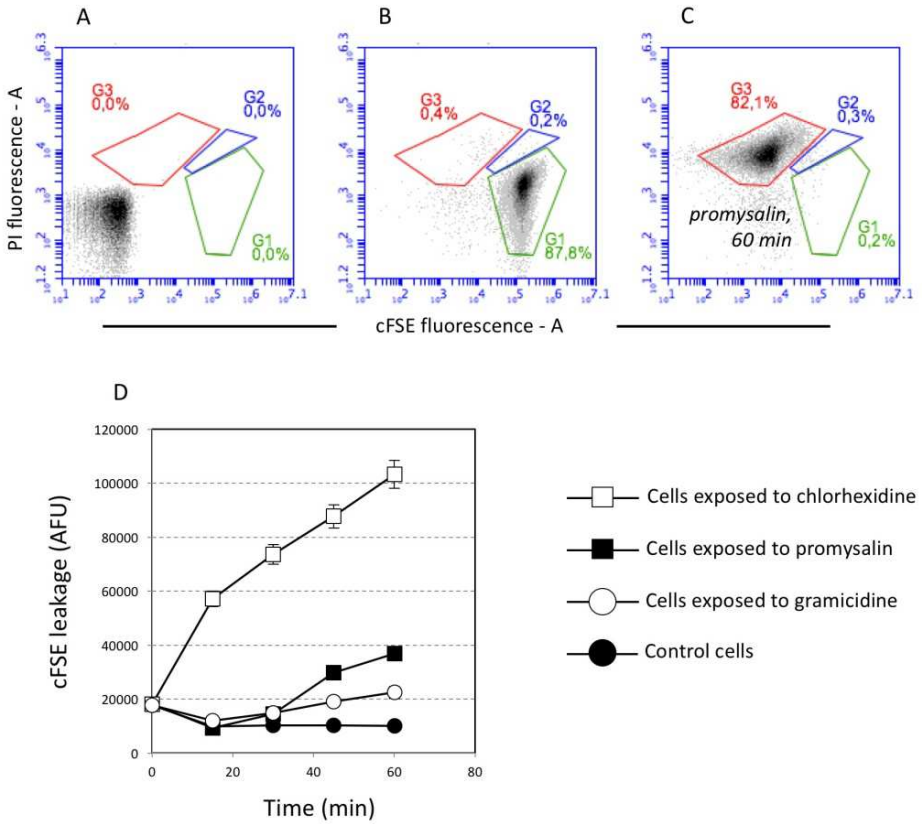


Figure-5: cell membrane damage and leakage of cFSE fluorescence outside the cell.

As depicted in figure-5, both promysalin and, more effectively, chlorhexidine promoted a significant reduction of cFSE fluorescence inside the cells and subsequent increase of cFSE fluorescence outside the cells. These results lead us to conclude that phospholipid bilayer disruption is followed by release of intracellular components.

These results indicate that the antibacterial effect of promysalin is linked to the disruption of the phospholipid membrane bilayer and not to the elevation of ion membrane permeability which is well characterized for the membrane uncoupling gramicidin.[10] Noteworthy, gramicidin did not show a membrane damage or a cFSE-fluorescence leakage in *S. thermophilus* DSM 20617^{T} (Figure-6).

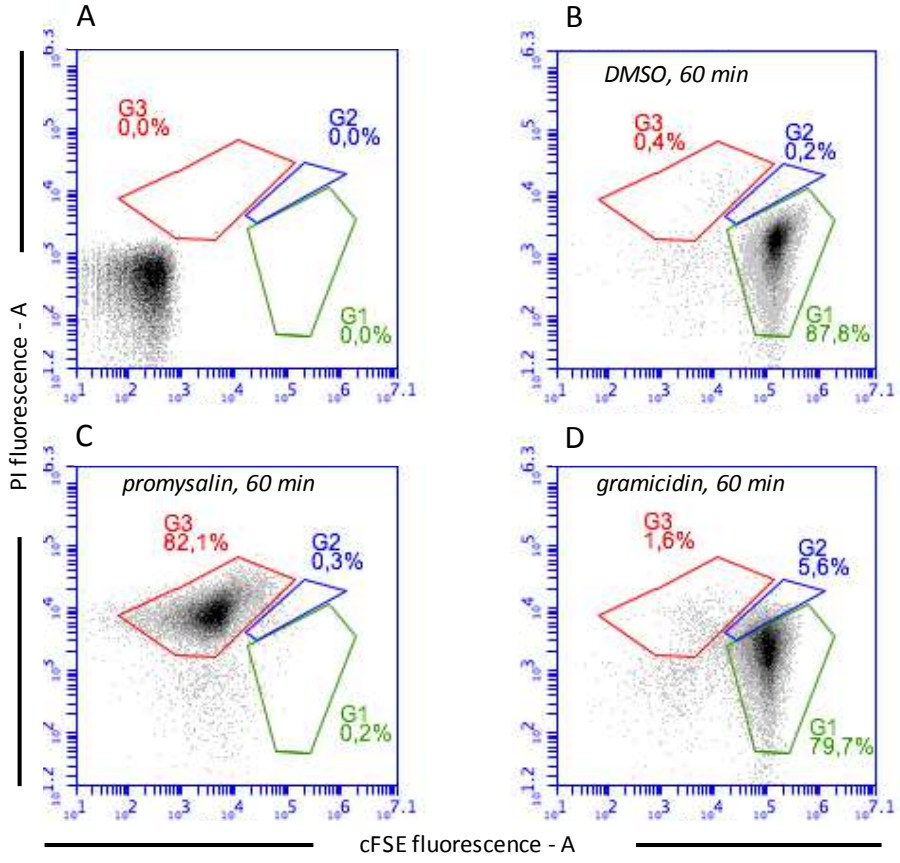


Figure-6: Effect of promysalin and gramicidin on *Streptococcus thermophilus* DSM 20617^{T} cell membrane integrity. Flow cytometry density diagrams show the cFSE *vs* PI fluorescence of cells exposed to promysalin or gramicidin (100 µg/mL and 100 mM, respectively). A) Cells before exposure to antimicrobials. B) Cells after 60 min of exposure to DMSO. C) Cells after 60 min of exposure to promysalin. D) Cells after 60 min of exposure to gramicidin. Viable cells are gated in G1. Dead cells with damaged membranes are gated in G3. The transition of cell populations from gate G1 to gate G3 is correlated to cell membrane damage.

4.5. EFFECT OF PROMYSALIN ON *SACCHAROMYCES CEREVISIAE*:

To expand the activity spectrum of promysalin towards eukaryotic microorganisms, we tested the compound against the cells of the yeast *Saccharomyces cerevisiae* BC1. A partial cell membrane damage as compared to the bisbiguanide biocide chlorhexidine was observed when the cells were exposed to promysalin, figure-7. Considering the partial cell membrane damage observed, promysalin was unable to inhibit completely the growth of *Saccharomyces cerevisiae* at the highest concentration tested (128 µg/ml). Instead, promysalin significantly delayed the lag phase of the growth by 8 h with respect to control (figure-8). This result underscores the potential of this molecule against yeast and fungi.

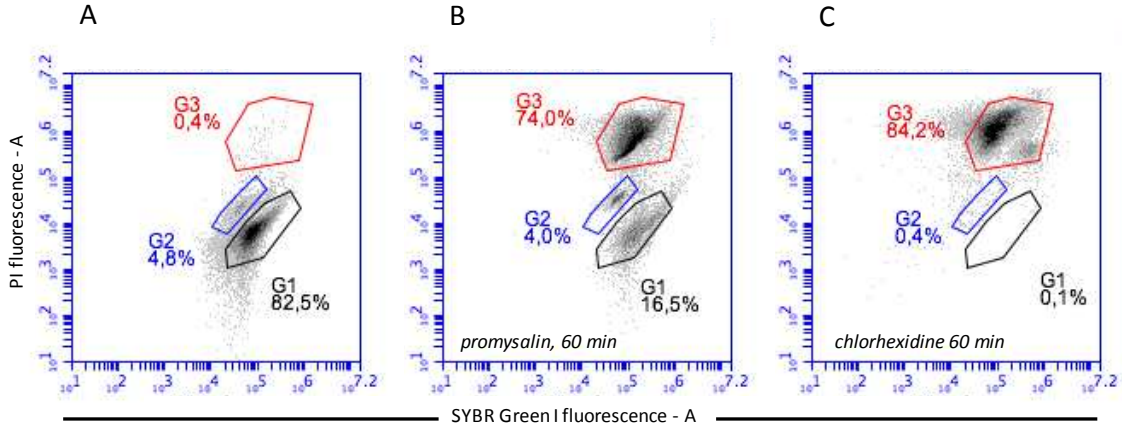


Figure-7: The effect of promysalin and chlorhexidine on *Saccharomyces cerevisiae* BC1 cell membrane integrity. Flow cytometry density diagrams show SYBR Green I *vs* PI fluorescence of cells exposed to promysalin and chlorhexidine (100 µg/mL). A) Cells before exposure to antimicrobials. B) Cells after 60 min of exposure to promysalin. C) Cells after 60 min of exposure to chlorhexidine. Viable cells are gated in G1. Dead cells with damaged membranes are gated in G3. The transition of cell populations from gate G1 to gate G3 is correlated to cell membrane damage.

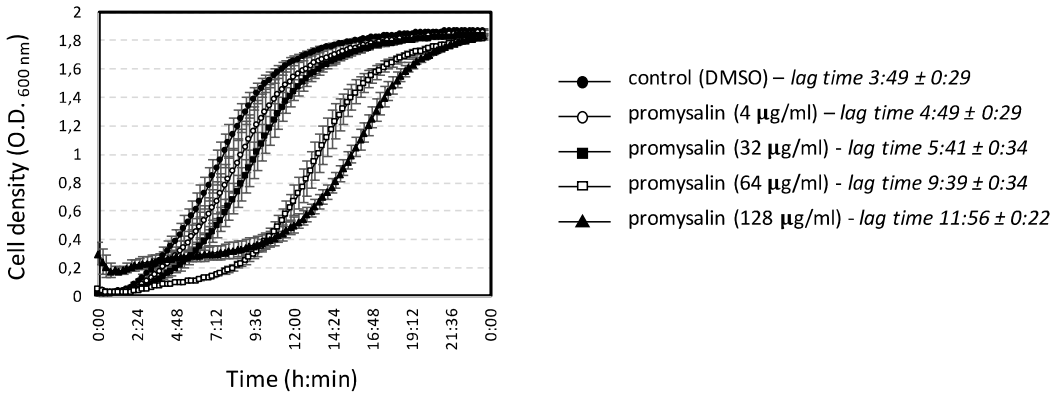


Figure-8: Growth of *Saccharomyces cerevisiae* BC1 with varying concentrations of promysalin. The calculated lag time (h:min) for each growth condition is indicated.

PART-B

PREPARATION OF ANALOGUES AND THEIR BIOLOGICAL STUDIES

The broad-spectrum bioactivity of the natural compound and its proposed mechanism of action prompted us to gain insights into the most vital structural features responsible for the activity. The diverted total synthesis of promysalin analogues by Wuest *et al.*[11] has demonstrated that the bioactivity of the molecule is sensitive to changes within its hydrogen bond network and also that the exact three-dimensional orientation of the molecule is mandatory for biological activity.[4] Recently, we reported an enantioselective convergent synthesis of promysalin.[5] The robust and modular nature of our synthetic route has enabled us to apply it easily for the synthesis of some representative analogs, figure-9.

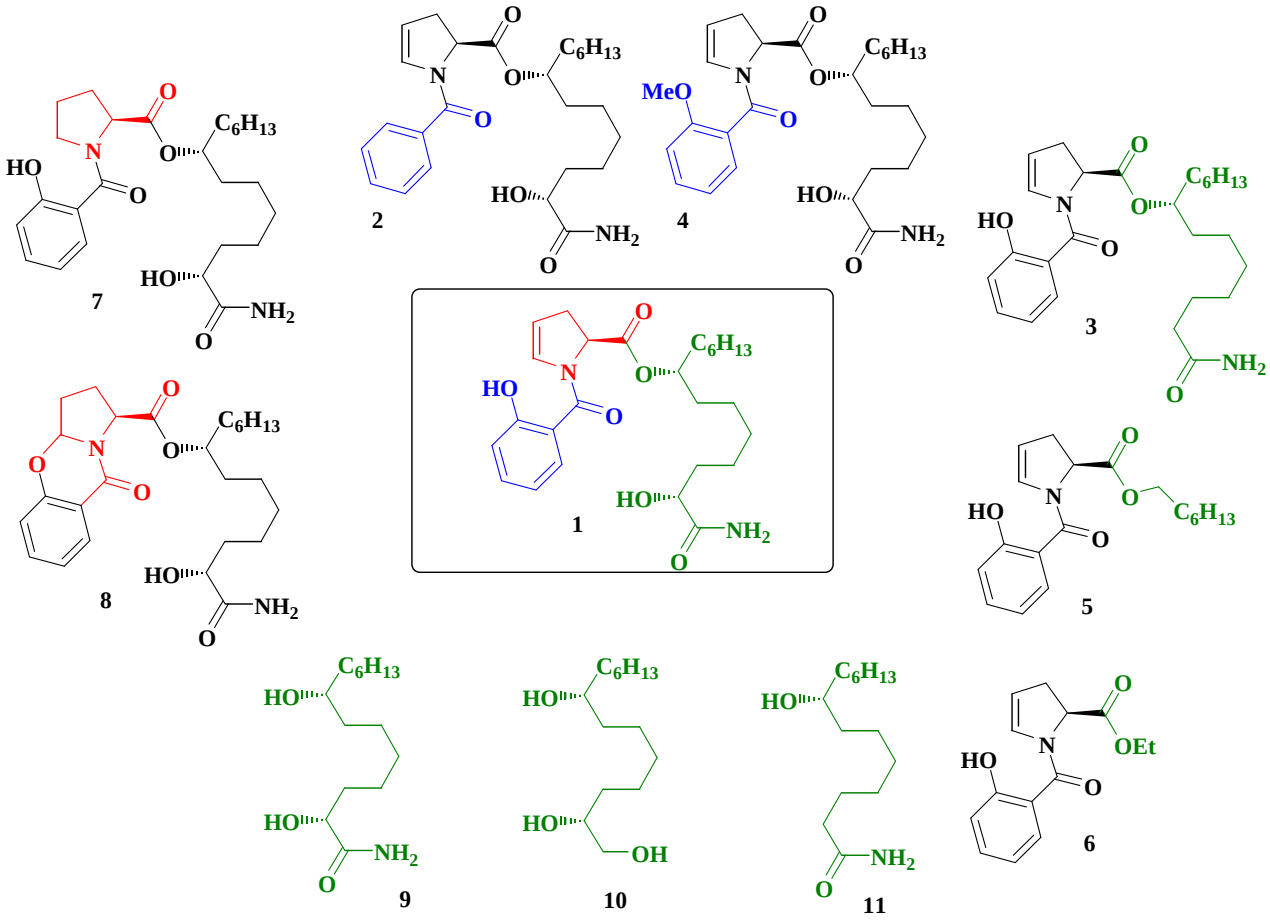


Figure-9: Structure of promysalin (**1**) and analogues **2-11.**

Our strategy was based on gradual modifications of three structural frameworks: the salicylate core, the dehydroproline fragment and the myristamide chain. To illuminate the role of the salicylate core, it was altered by removal of hydrogen bond donor phenolic OH (**2**) and introduction of a methoxy group (**4**). Whereas **3**, **5**, **6** were prepared to highlight the importance of the myristamide chain linked to dehydroproline system. The derivatives **9**, **10**, **11** were synthesized to test the antimicrobial potential of myristamide chain. Taking into account the susceptibility of promysalin to acidic conditions,[5] the enamide moiety was replaced with a proline fragment (compound **7**). The conformationally rigid cyclized analogue, having a tetrahydro-9-oxa-3a-aza-cyclopentanaphthalen-4-one ring (compound **8**) was obtained by treatment with TFA using literature procedure.[11]

The promysalin analogues were screened using microdilution assay and MIC and MBC values were determined against the following reference gram negative and gram positive strains: *Pseudomonas aeruginosa* ATCC 10145, *Staphylococcus aureus* ATCC 29213, *S. thermophilus* DSM 20617^T. The results are described in the table-2.

Compound	*Pseudomonas aeruginosa* ATCC 10145		*Staphylococcus aureus* ATCC 29213		*Streptococcus thermophilus* DSM20617^T	
	MIC	MBC	MIC	MBC	MIC	MBC
Promysalin	16	128	16	>256	16	64
2	128	>256	64	>256	64	128
3	32	512	32	>256	32	64
4	32	>256	32	>256	64	128
5	32	>256	>256	>256	128	>256
6	128	>256	>256	>256	256	>256
7	32	>256	>256	>256	64	128
8	128	>256	64	>256	64	64
9	128	>256	125	>256	128	>256
10	128	>256	>256	>256	128	>256
11	128	>256	>256	>256	128	>256

Table-2: Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values of promysalin and analogues against Gram-negative and Gram-positive bacteria.

The subtle changes in the salicylate moiety deteriorated the activity as the elimination of the phenolic group (cpd. **2**) led to 8-fold decreased antibacterial activity. However, the methyl ether **4** retained significant biological activity against both gram positive and gram negative organisms. The C-2 dehydroxy compound **3** partially retained the activity, most likely due to the chain flexibility allowing the amide carbonyl to participate in the hydrogen bonding interaction. Surprisingly, the heptyl ester **5** conserved its effect against *Pseudomonas aeruginosa* ATCC 10145 but failed to show significant inhibition of gram positive counterparts. As anticipated, further reduction of chain length, as in the ethyl ester **6,** did not give promising results. The proline derivative **7** maintains a modest activity; in contrast to the previous results by Wuest *et al.*[11] The structurally rigid oxazinone analogue **8** retained a moderate activity against *P. aeruginosa* ATCC 10145, *S. aureus* 29213, and *S. thermophilus* DSM 20617^{T}. Compounds **7** and **8** highlight the importance of the enamide moiety, as both the more flexible proline and the conformationally locked oxazinone analogues did not fully retain the activity. The myristamide fragments **9-11** did not show activity. In conclusion, analogs **2**, **3**, **4**, **7** retained the activity although with higher MIC compared to promysalin, MBC values lower than 256 μg/ml were observed only against *S. thermophilus* in case of the analogues **2**, **3**, **4**, **7** and **8**. These results imply that promysalin and analogues have a bacteriostatic rather than bactericidal effect against the other species tested. The salicylate fragment was found more essential for the antibacterial activity as small changes in this region led to significant loss of activity.

The analogs which retained MIC values below or equal to 128 μg/ml in *S. thermophilus* DSM 20617^{T} were also evaluated for their ability to induce cell membrane damage by flow cytometry using the SYBR Green I/PI double staining assay (figure-10). In addition, the viability of the cells treated with promysalin and its derivatives was also evaluated using standard plating method. Surprisingly, the benzoylenamide **3** in contrast to poor MIC displayed 88% membrane damage. The methyl ether **4** manifested a sounding 98% membrane disruption almost equal to promysalin. Pertaining to its conformational rigidity, the oxazinone analogue **8** gave moderate reduction in cell viability. The fully functionalized myristamide chain appears to be necessary to produce a remarkable antibacterial effect, a similar trend was observed in case of **5** and **6** as the ester derivatives induced diminished reduction of cell viability (15% and 35% respectively). Noteworthy, the C-2 dehydroxylation to give compound **3** had a huge impact, enabling only negligible cell membrane damage, thus again highlighting the importance of hydrogen bonding groups. The proline derivative **7** having the intact hydrogen bonding atoms shows 92% reduction irrespective of the change in orientation due to the loss of the double bond. In summary, the oxygen network in the parent molecule seems to play a crucial role and only modifications in the salicylate fragment are

tolerated and conserved significant activity. On the contrary, alterations in myristamide chain were found to be detrimental.

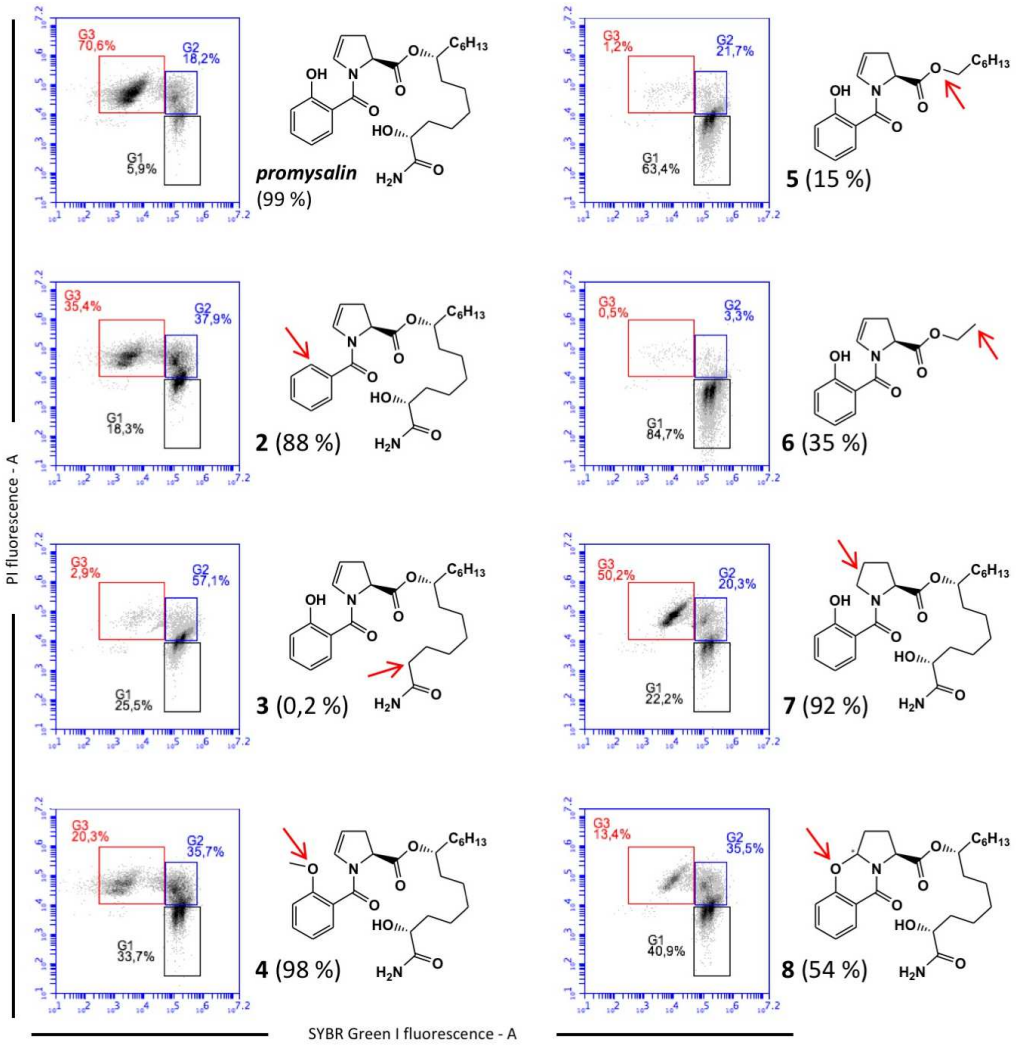


Figure-10: The effect of promysalin and analogs on *S. thermophilus* DSM 20617^{T} cell membrane integrity and cell viability. Flow cytometry density diagrams show the SYBR Green I *vs* PI fluorescence of cells exposed to promysalin or to the analogs **2-8** (100 µg/ml). Viable cells are gated in G1, viable cells with slightly damaged cell membrane are gated in G2. Dead cells with damaged membrane are gated in G3. The transition of cell population from gate G1 to gate G3 is related to the entity of cell membrane damage. The reduction of cell viability measured by standard plating was shown in parenthesis. The reduction of cell viability was calculated using as reference (100% of viability reduction) the bactericidal effect of chlorhexidine. The differences in structure between promysalin and the analogs are indicated by the red arrows.

4.6. SUMMARY:

In light of the results obtained, promysalin displays broad-spectrum antibacterial activity against gram-positive and gram negative bacteria, thus it cannot be considered as a species-specific narrow-spectrum molecule. Damage of the phospholipid layer seems to be the most probable mechanism of action of promysalin. The broad-spectrum activity together with ability of promysalin to disrupt the phospholipid bilayer of the microbial cell membrane makes it a biocide rather than an antibiotic. Thus, the use of promysalin to treat human infections, such as those caused by *Pseudomonas aeruginosa* as proposed by Wuest *et. al.*[11] needs a thorough investigation.

The representative analogues of promysalin were synthesized and evaluated for their antimicrobial activity. The results obtained suggest that subtle alteration of the functional groups in the natural compound led to loss of activity. The three dimensional architecture and the hydrogen bonding network is mandatory to have significant antimicrobial activity. The analogues were also evaluated for their membrane disruption potential against *S. thermophilus* DSM20617^{T}, which showed excellent to moderate reduction in cell viability.

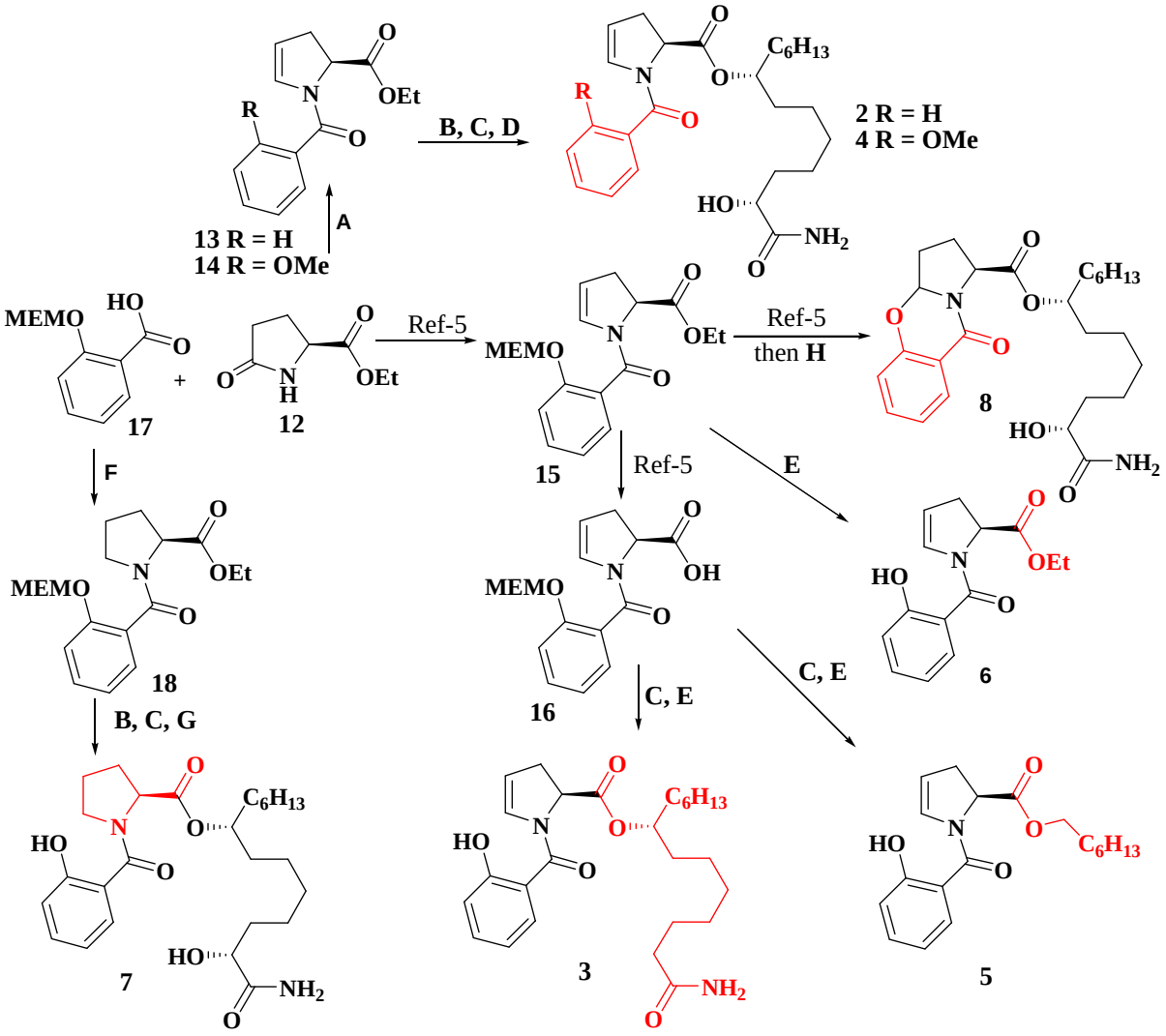
4.7. EXPERIMENTAL SECTION:

4.7.1. SYNTHESIS OF ANALOGUES:

4.7.1.1. GENERAL INFORMATION:

See chapter-3.3.1 for General information.

4.7.1.2. GENERAL PROCEDURES:



Scheme 1: Synthetic routes to compounds 2-8: a) i. aroyl chloride, NEt_3, 0 ºC to 80 ºC, 3h, ii. $LiBHEt_3$, toluene, -78 ºC, 1 h, then DIPEA, cat. DMAP, TFAA, -78 ºC to rt, 3 h; b) LiOH, EtOH : H_2O, 0 ºC to rt, 5 h; c) i) 2,4,6-trichlorobenzoyl chloride, NEt_3, THF, 0 ºC to rt, 2h, ii) alcohol, DMAP, toluene, 0 ºC to rt,

12 h; d) TBAF, THF, 0 ºC to rt, 1 h; e) $TiCl_4$, -20 ºC, 15 min.; f) L-proline ethyl ester.HCl, EDCI, HOBt; g) 1N HCl, THF, 0 ºC to rt; h) TFA, CH_2Cl_2, rt, 30 min.

4.7.1.2.1. GENERAL PROCEDURE A: ACYLATION OF ETHYL l-PYROGLUTAMATE.

NEt_3 (2 eq.), followed by acid chloride (1.2 eq.) were added dropwise to a stirred solution of ethyl L-pyroglutamate (1 eq.) in toluene (0.5 M) at 0 °C under N_2 atmosphere. The mixture was stirred at 80 °C for 3 h and cooled to room temperature. Sat. $NaHCO_3$ was added and the organic layer was separated. The aqueous layer was extracted with EtOAc (× 2). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The product was purified using flash column chromatography in 0-30% EtOAc/hexane.

4.7.1.2.2. GENERAL PROCEDURE B: REDUCTIVE ELIMINATION.

To a stirred solution of acylated pyroglutamate (1 eq.) in dry toluene (0.2 M) was added Superhydride® (lithium triethylborohydride) (1.2 eq., 1M in THF) at -78 °C under N_2 atmosphere. The mixture was stirred at -78 °C for 1h, then DMAP (0.1 eq.) and DIPEA (5.7 eq.) were added, followed by very slow addition of TFAA (1.2 eq.). The reaction mixture was gradually warmed to room temperature and stirred for 3 h. Water (× 10) was added and the organic layer was separated. The aqueous layer was extracted with ethyl acetate (× 2); the combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using flash column chromatography in 0-50 % ethyl acetate: hexane.

4.7.1.2.3. GENERAL PROCEDURE C: HYDROLYSIS OF THE ETHYL ESTER.

To a solution of ethyl ester (1 eq.) was added dropwise a solution of LiOH (1.5 eq.) in water (EtOH : H_2O 2:1, 0.08 M) at 0 °C. The reaction mixture was warmed to room temperature and stirred for 5 h. EtOH was removed *in vacuo*, the aqueous layer was washed with 40 % ethyl acetate in diethyl ether (× 2), cooled to 0 °C and acidified using 5% citric acid. The product was extracted using 5% CH_3OH : CH_2Cl_2 (× 3). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford carboxylic acid.

4.7.1.2.4. GENERAL PROCEDURE D: YAMAGOUCHI ESTERIFICATION.

NEt_3 (3 eq.) followed by 2,4,6-trichlorobenzoyl chloride (2 eq.) were added dropwise to a stirred solution of acid (1eq.) in THF (0.03 M) at 0 °C under N_2 atmosphere. The mixture was warmed to room temperature and stirred for 2h. THF was removed *in vacuo* and the residue was dissolved in toluene (0.03M). DMAP (3 eq.) followed by alcohol (0.8 eq.) in toluene were added at 0 °C under N_2 atmosphere.

The resulting suspension was stirred overnight at room temperature. EtOAc (× 15) was added, the organic layer was washed with sat. NH_4Cl, brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The product was purified by flash column chromatography 0-70 % EtOAc: hexane.

4.7.1.2.5. GENERAL PROCEDURE E: MEM DEPROTECTION

To a stirred solution of MEM ether (1 eq.) in CH_2Cl_2 (0.15 M) was added $TiCl_4$ (2 eq., 1M in CH_2Cl_2) at -20 °C under N_2 atmosphere. The reaction mixture was stirred at -20 °C for 10 min; then aqueous ammonia (20 times) was added. The aqueous layer was extracted with ethyl acetate (×2), and the combined organic extracts were washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo*. The product was purified using preparative TLC.

4.7.1.2.6. GENERAL PROCEDURE F: TBDPS DEPROTECTION.

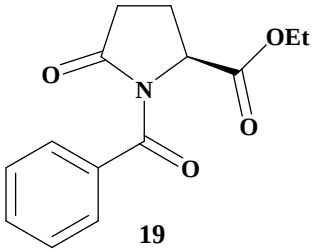
TBAF (3 eq. 1M in THF) was added dropwise to a stirred solution of silyl ether (1 eq.) in THF (0.2 M) at 0 °C. The reaction mixture was stirred at room temperature for 1h. Sat. NH_4Cl was added. The aqueous layer was extracted with ethyl acetate (× 2). The combined organic extracts were washed with brine, dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The product was purified using preparative TLC.

4.7.1.3. EXPERIMENTAL PROCEDURES:

Promysalin (compound **1**) was synthesized using literature procedure.[5]

4.7.1.3.1. SYNTHESIS OF COMPOUND 2.

4.7.1.3.1.1. *ethyl (S)-1-benzoyl-5-oxopyrrolidine-2-carboxylate.*



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Using general procedure A, ethyl L-pyroglutamate (500 mg, 3.28 mmol) yielded ethyl (*S*)-1-benzoyl-5-oxopyrrolidine-2-carboxylate (+)-**19** as a creamy solid (620 mg, 74 %);

R_f (25 % EtOAc: Hexane) = 0.25.

$[\alpha]_D^{23}$ = + 23.0 (*c* 1.00, $CHCl_3$).

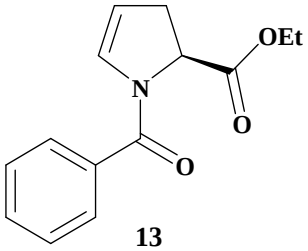
mp = 79-80 °C.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.70-7.63 (m, 2H), 7.58-7.50 (m, 1H), 7.46-7.37 (m, 2H), 4.89 (dd, *J* = 3.9, 8.9 Hz, 1H), 4.26 (q, *J* = 7.1 Hz, 2H), 2.83-2.68 (m, 1H), 2.66-2.38 (m, 1H), 2.23-2.09 (m, 1H), 1.30 (t, *J* = 7.1 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 173.7, 171.2, 170.6, 134.0, 132.5, 129.3 (×2), 128.1 (×2), 62.1, 59.0, 32.0, 22.1, 14.3 ppm.

Elemental Analysis Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: 64.25; H, 5.78; N, 5.37.

4.7.1.3.1.2. *Ethyl (2S)-1-benzoyl-2,3-dihydro-1H-pyrrole-2-carboxylate.*



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Using general procedure B, the ester (+)-**19** (478 mg, 1.83 mmol) yielded the title compound ethyl *(2S)-1-*benzoyl-2,3-dihydro-1H-pyrrole-2-carboxylate **(-)-13** as a colourless oil (200 mg, 44 %).

R_f (30 % EtOAc: Hexane) = 0.4.

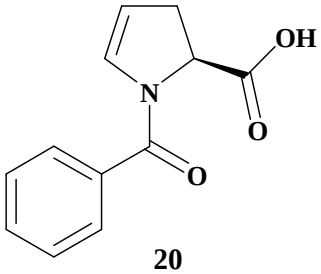
$[\alpha]_D^{23}$ = - 138.6 (*c* 1.00, $CHCl_3$).

^{1}H NMR (600 MHz, acetone-d_6) δ = 7.60-7.31 (m, 5H), 6.57 (s, 1H); 5.17 (s, 1H); 4.95 (dd, *J* = 5.1, 11.7 Hz, 1H), 4.28-4.12 (m, 2H), 3.20-3.12 (m, 1H), 2.70-2.63 (m, 1H), 1.35-1.19 (m, 3H) ppm.

^{13}C NMR (150 MHz, acetone-d_6) δ = 170.5, 166.0, 135.6, 130.6, 130.4, 128.4 (× 2), 127.6 (× 2), 108.4, 60.6, 58.4, 33.4, 13.5 ppm.

Elemental Analysis Calcd. for $C_{14}H_{15}NO_3$: C, 68.56; H, 6.16; N, 5.71. Found: C, 68.64; H, 6.15; N, 5.72.

4.7.1.3.1.3. *(S)-1-benzoyl-2,3-dihydro-1H-pyrrole-2-carboxylic acid.*



20

Using general procedure **C** the ester **(-)-13** (170 mg, 0.69 mmol) was hydrolyzed to give (*S*)-1-benzoyl-2,3-dihydro-1H-pyrrole-2-carboxylic acid **(-)-20** as a pale yellow solid (130 mg).

R_f (5 % MeOH : CH_2Cl_2) = 0.2.

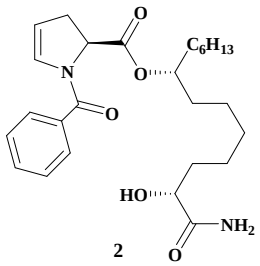
$[\alpha]_D^{23}$ = - 85.3 (*c* 1.20, $CHCl_3$).

^{1}H NMR (300 MHz, CD_3OD) δ = 7.62-7.55 (m, 2H), 7.52-7.42 (m, 3H), 6.51-6.47 (m, 1H), 5.26-5.21 (m, 1H); 4.84 (dd, *J* = 4.5, 11.2 Hz, 1H); 3.18-3.05 (m, 1H); 2.78-2.67 (m, 1H) ppm.

^{13}C NMR (150 MHz, CD_3OD) δ = 177.3, 168.0, 135.4, 130.7, 130.2, 130.0 (× 2), 128.4, 127.66 (× 2), 111.2, 60.6, 34.2, 26.5 ppm.

Compound (-)-**20** was used for the next step without further purification.

4.7.1.3.1.4. *(7R,13R)-14-amino-13-hydroxy-14-oxotetradecan-7-yl-(S)-1-benzoyl-2,3-dihydro-1H-pyrrole-2-carboxylate.*



2

NEt_3 (0.09 mL, 0.69 mmol) followed by 2,4,6-trichlorobenzoyl chloride (0.07 mL, 0.46 mmol) were added dropwise to a stirred solution of the above acid **(-)-20** (50 mg, 0.23 mmol) in THF (4.1 mL, 0.03 M) at 0 °C under N_2 atmosphere. The mixture was warmed to room temperature and stirred for 2 h. THF was removed *in vacuo* and the residue was dissolved in toluene (3 mL). DMAP (84 mg, 0.69 mmol) followed by (*2R,8R*)-2-((tert-butyldiphenylsilyl)oxy)-8-hydroxytetradecanamide[5] **(-)-29** (57 mg, 0.115 mmol) in toluene was added at 0 °C under N_2 atmosphere. The resulting suspension was stirred overnight at room temperature. EtOAc (8 mL) was added, the organic layer was washed with sat. NH_4Cl (5 mL), brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude silyl ether (54 mg, 0.076 mmol) was dissolved in THF (1 mL) and cooled to 0 °C; TBAF (0.22 mL, 0.23 mmol, 1M in THF) was added dropwise under N_2 atmosphere. The reaction mixture was stirred at room temperature for 1 h. Sat. NH_4Cl (7 mL) was added and the aqueous layer was extracted with ethyl acetate (2 × 7 mL) The combined organic extracts were washed with brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The product was purified preparative TLC in 6% MeOH: CH_2Cl_2, to afford the title compound (-)-**2** as colourless oil (28 mg, 52 % over two steps).

R_f (5 % MeOH: CH_2Cl_2) = 0.4.

$[\alpha]_D^{23}$ = -42.0 (*c* 0.45, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.57-7.36 (m, 5H), 6.85-6.72 (m, 1H), 6.53-6.46 (m, 1H), 5.20-5.14 (m, 1H), 5.13-5.01 (m,1H), 4.97 (dd, *J* = 4.1, 12.1 Hz, 1H), 4.27 (m, 1H), 4.11-4.02 (m, 1H), 3.50 (brs, 1H), 3.21-3.07 (m, 1H), 2.77-2.64 (m, 1H), 1.90-1.17 (m, 20H), 0.88 (t, *J* = 6.9 Hz, 3H) ppm.

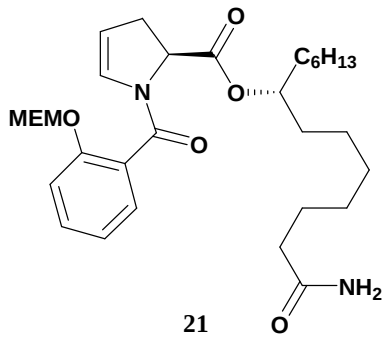
^{13}C NMR (75 MHz, $CDCl_3$) δ = 177.6, 170.7, 167.9, 134.8, 131.1, 130.9, 128.8 (× 2), 127.8 (× 2), 110.0, 75.3, 70.7, 58.7, 34.9, 34.2, 34.1, 33.7, 31.9, 29.3, 27.6, 25.7, 24.8, 24.3, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{26}H_{38}N_2O_5$: C, 68.10; H, 8.35; N, 6.11. Found: C, 68.01; H, 8.33; N, 6.12.

HRMS: Accurate mass (ES+) calculated for $C_{26}H_{38}N_2O_5Na$ $(M + Na)^+$ 481.26729, Found: 481.26861.

4.7.1.3.2. SYNTHESIS OF COMPOUND 3.

4.7.1.3.2.1 *(R)-14-amino-14-oxotetradecan-7-yl (S)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



21

Using general procedure D, (*S*)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid[5] **(-)-16** (50 mg, 0.15 mmol) was reacted with (*R*)-8-hydroxytetradecanamide (11, see below) (32 mg, 0.139 mmol) to give (*R*)-14-amino-14-oxotetradecan-7-yl (*S*)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate (-)-**21** (40 mg, 54%) as a colourless oil.

$\mathbf{R}_f$ (4 % MeOH : CH_2Cl_2) = 0.3.

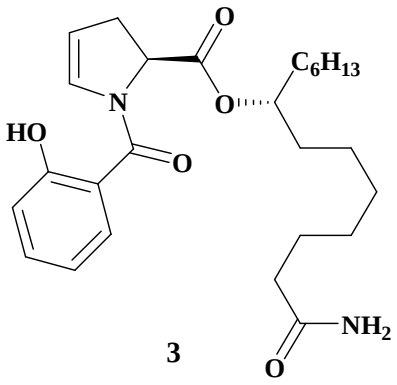
$\mathbf{[\alpha]_D^{23}}$ = -54.5 (*c* 0.75, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.41-7.31 (m, 2H), 7.22 (dd, *J* = 8.2, 1.0 Hz, 1H), 6.30 (brs, 1H), 6.21-6.14 (m, 1H), 5.28 (s, 2H), 5.09-4.91 (m, 3H), 3.84-3.78 (m, 2H), 3.57-3.50 (m, 2H), 3.36 (s, 3H), 3.20-3.07 (m, 1H), 2.73-2.63 (m,1H), 2.42-2.34 (m,1H), 2.19 (t, *J* = 7.5 Hz, 2H), 1.72-1.18 (m, 20H), 0.87 (t, *J* = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 176.3, 170.8, 165.3, 153.6, 131.6, 131.0, 128.9, 125.9, 122.4, 115.6, 109.0, 94.0, 75.5, 71.7, 68.2, 59.2, 58.2, 36.1, 34.7, 34.4, 31.9, 29.4, 28.9, 28.6, 25.6, 25.2, 24.8, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{30}H_{46}N_2O_7$: C, 65.91; H, 8.48; N, 5.12. Found: C, 65.83; H, 8.50; N, 5.10.

4.7.1.3.2.1. *(R)-14-amino-14-oxotetradecan-7-yl (S)-1-(2-hydroxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



3

Using general procedure E, compound **(-)-21** (85 mg, 0.159 mmol) was deprotected to afford the title compound (-)-**3** as a colourless oil (38 mg, 52 %).

$\mathbf{R}_f$ (2 % MeOH:CH_2Cl_2) = 0.4.

$[\alpha]_D^{23}$ = -50.1 (*c* 1.5, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.45-7.33 (m, 2H), 6.99 (dd, *J* = 8.2, 1.0 Hz, 1H), 6.89 (ddd, *J* = 8.2, 8.2, 1.0 Hz, 1H), 6.79 (brs, 1H), 5.67 (brs, 1H), 5.32-5.16 (m, 2H), 5.09-4.91 (m, 2H), 3.23-3.05 (m, 1H), 2.76-2.62 (m, 1H), 2.20 (t, *J* = 7.6 Hz, 2H), 1.74-1.11 (m, 20H), 0.86 (t, *J* = 6.9 Hz, 3H) ppm.

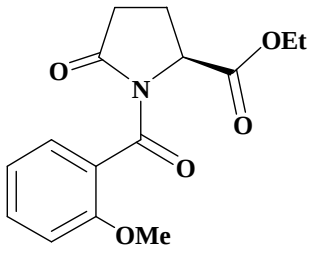
^{13}C NMR (75 MHz, $CDCl_3$) δ = 176.1, 171.1, 167.5, 158.8, 133.5, 131.1, 128.5, 119.2, 118.1, 117.5, 110.8, 76.1, 59.6, 36.1, 34.4, 34.2, 31.9, 29.3, 29.1, 29.0, 25.5 (× 3), 25.0, 22.7, 14.3 ppm.

Elemental Analysis Calcd. for $C_{26}H_{38}N_2O_5$: C, 68.10; H, 8.35; N, 6.11. Found: C, 68.02; H, 8.37; N, 6.11.

HRMS: Accurate mass (ES+) calculated for $C_{26}H_{38}N_2O_5Na$ $(M + Na)^+$ 481.26729, Found: 481.26829.

4.7.1.3.3. SYNTHESIS OF COMPOUND 4:

4.7.1.3.3.1. *ethyl (S)-1-(2-methoxybenzoyl)-5-oxopyrrolidine-2-carboxylate.*



22

Using general procedure **A,** ethyl L-pyroglutamate (500 mg, 3.28 mmol) afforded compound **(+)-22** as a white solid (723 mg, 77 %).

R_f (30 % EtOAc: Hexane) = 0.4.

M.P = 93-94 °C.

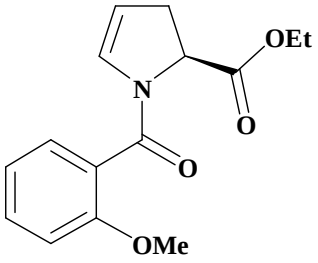
$[\alpha]_D^{23}$ = + 65.5 (*c* 1.00, $CHCl_3$).

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.44 (ddd, *J* = 8.4, 7.7, 1.7 Hz, 1H), 7.33 (dd, *J* = 7.7, 1.7 Hz, 1H), 7.01 (dd, *J* = 7.7, 7.7 Hz, 1H), 6.93 (d, *J* = 8.4 Hz, 1H); 4.92 (dd, *J* = 9.5, 2.8 Hz, 1H), 4.29 (q, *J* = 6.9 Hz, 2H), 3.83 (s, 3H), 2.73-2.66 (m, 1H) 2.57-2.52 (m, 1H), 2.47-2.40 (m, 1H), 2.19-2.13 (m, 1H), 1.34 (t, *J* = 7.1 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 173.1, 171.4, 168.3, 157.2, 132.3, 129.0, 125.3, 120.8, 111.3, 62.1, 58.6, 56.1, 53.8, 31.9, 22.1, 14.5 ppm.

Elemental Analysis Calcd. for $C_{15}H_{17}NO_5$ C, 61.85; H, 5.88; N, 4.81. Found: C, 61.95; H, 5.89; N, 4.80.

4.7.1.3.3.2. *ethyl (S)-1-(2-methoxybenzoyl)2,3-dihydro-1H-pyrrole-2-carboxylate.*



14

Using general procedure **B**, ethyl (*S*)-1-(2-methoxybenzoyl)-5-oxopyrrolidine-2-carboxylate **(+)-22** (723 mg, 2.48 mmol) afforded compound **(-)-14** as a clear oil (405 mg, 60 %).

$\mathbf{R}_f$ (30 % EtOAc: Hexane) = 0.5.

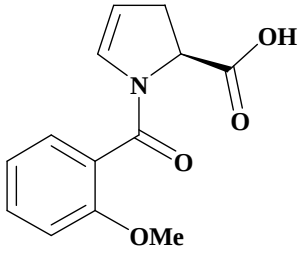
$\mathbf{[\alpha]_D^{23}}$ = - 117.8 (*c* 1.25, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.43-7.33 (m, 2H), 7.00 (ddd, *J* = 8.2, 8.2, 1.1 Hz, 1H), 6.94 (d, *J* = 8.2 Hz, 1H), 6.18-6.12 (m, 1H), 5.08-5.03 (m, 1H), 5.00 (dd, *J* = 5.02, 11.7 Hz, 1H), 4.34-4.19 (m, 2H), 3.84 (s, 3H), 3.20-3.05 (m, 1H), 2.76-2.65 (m, 1H), 1.32 (t, *J* = 7.1 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.2, 165.3, 156.1, 131.5, 131.0, 129.2, 125.1, 121.0, 111.6, 108.7, 61.6, 58.2, 56.0, 34.3, 14.3 ppm.

Elemental Analysis Calcd. for $C_{15}H_{17}NO_4$: C, 65.44; H, 6.22; N, 5.09. Found: C, 65.54; H, 6.21; N, 5.08.

4.7.1.3.3.3. *(S)-1-(2-methoxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid.*



23

Using general procedure **C**, compound **(-)-14** (250 mg, 0.908 mmol) was hydrolyzed to yield **(-)-23** ((*S*)-1-(2-methoxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid) as a yellow solid (185 mg, 82 %).

R_f (4 % MeOH : CH_2Cl_2) = 0.4.

M.P = 145-146 °C.

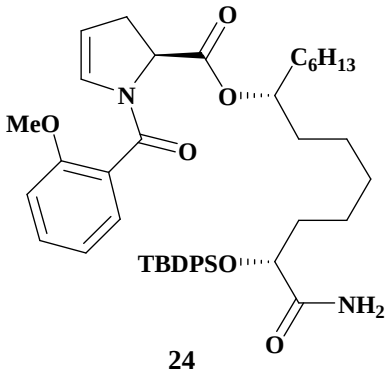
$[\alpha]_D^{23}$ = -82.8 (*c* 0.5, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.47-7.35 (m, 2H), 7.03 (ddd, *J* = 8.2, 8.2, 1.0 Hz, 1H), 6.96 (d, *J* = 8.2 Hz, 1H), 6.07-6.02 (m, 1H), 5.26-5.20 (m, 1H), 5.13 (dd, *J* = 4.3, 10.8 Hz, 1H), 3.84 (s, 3H), 3.28-3.16 (m, 1H), 3.11-2.96 (m, 1H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 173.1, 167.3, 156.0, 132.2, 130.0, 129.3, 124.0, 121.1, 111.6, 111.3, 59.1, 56.0, 33.2 ppm.

Elemental Analysis Calcd. for $C_{13}H_{13}NO_4$: C, 63.15; H, 5.30; N, 5.67. Found: C, 63.23; H, 5.29; N, 5.66.

4.7.1.3.3.4. (*7R,13R*)-14-amino-13-((tert-butyldiphenylsilyl)oxy)-14-oxotetradecan-7-yl-(*S*)-1-(2-methoxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.



24

Using general procedure **D**, the above acid **(-)-23** (35 mg, 0.14 mmol) was reacted with (2R,8R)-2-((tert-butyldiphenylsilyl)oxy)-8-hydroxytetradecanamide[5] **(-)-29** (56 mg, 0.112 mmol) to give (*7R,13R*)-14-amino-13-((tert-butyldiphenylsilyl)oxy)-14-oxotetradecan-7-yl-(*S*)-1-(2-methoxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate **(-)-24** as a pale yellow oil (42 mg, 50 %).

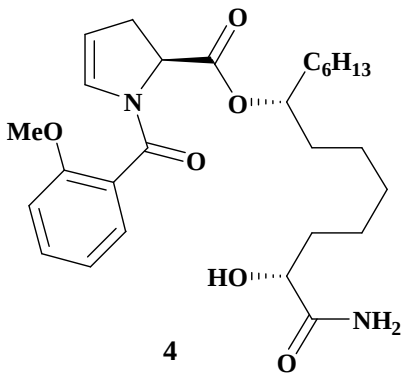
$\mathbf{R_f}$ (50 % EtOAc : hexane) = 0.3.

$\mathbf{[\alpha]_D^{23}}$ = -39.0 (*c* 0.4, $CHCl_3$).

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.67 (d, *J* = 7.0 Hz, 2H), 7.62 (d, *J* = 7.0 Hz, 2H), 7.50-7.34 (m, 8H), 7.00 (dd, *J* = 7.0, 7.0 Hz, 1H), 6.95 (d, *J* = 8.3 Hz, 1H), 6.74 (d, *J* = 4.5 Hz, 1H), 6.18-6.14 (m, 1H), 5.58-5.54 (m, 1H), 5.06-4.92 (m, 3H), 4.27 (dd, *J* = 3.9, 5.2 Hz, 1H), 3.85 (s, 3H), 3.17-3.10 (m, 1H), 2.71-2.66 (m, 1H), 1.70-1.12 (m, 29H); 0.98 (t, *J* = 7.1 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 176.2, 170.8, 164.9, 155.9, 135.7(× 2), 135.6(× 2), 133.0, 132.6, 131.2, 130.9, 130.2, 130.1, 127.9 (× 2), 127.8(× 2), 125.1, 120.8, 111.3, 108.3, 75.4, 74.2, 58.1, 55.7, 34.3, 34.2, 33.9, 33.8, 31.7, 29.7, 29.3, 29.2, 27.0 (× 2), 25.2, 24.8, 23.3, 22.6, 19.3, 14.0 ppm.

4.7.1.3.3.5. *(7R,13R)-14-amino-13-hydroxy-14-oxotetradecan-7-yl (S)-1-(2-methoxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



4

Compound **(-)-24** (30 mg, 0.041 mmol) was deprotected using general procedure **F** to give the title compound **4** as a colourless oil (19 mg, 99 %).

$\mathbf{R_f}$ (4 % MeOH : CH_2Cl_2) = 0.3.

$\mathbf{[\alpha]_D^{23}}$ = -32.8 (*c* 0.5, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.44-7.37 (m, 1H), 7.33 (dd, *J* = 1.8, 7.6 Hz, 1H), 7.03-6.97 (m, 1H), 6.95 (d, *J* = 8.2 Hz, 1H), 6.91 (brs, 1H), 6.18-6.12 (m, 1H), 5.14-4.99 (m, 3H), 4.96 (dd, *J* = 4.5, 11.6 Hz, 1H), 4.36 (d, *J* = 5.4 Hz, 1H), 4.11-4.01 (m, 1H), 3.83 (s, 3H), 3.21-3.07 (m, 1H), 2.73-2.62 (m, 1H), 1.91-1.17 (m, 20H), 0.88 (t, *J* = 6.7 Hz, 3H) ppm.

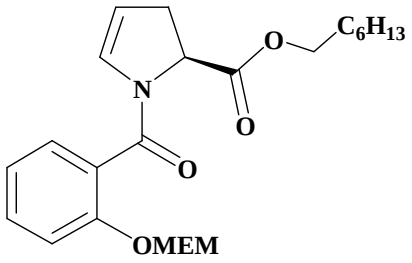
^{13}C NMR (75 MHz, $CDCl_3$) δ = 177.9, 170.7, 166.0, 156.0, 131.9, 130.8, 129.0, 124.3, 121.1, 111.6, 109.8, 75.1, 70.5, 58.2, 56.0, 35.0, 34.3, 33.7, 31.9, 29.9, 29.3, 27.5, 25.7, 24.8, 24.4, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{27}H_{40}N_2O_6$: C, 66.37; H, 8.25; N, 5.73. Found: C, 66.21; H, 8.26; N, 5.72.

HRMS: Accurate mass (ES+) calculated for $C_{27}H_{40}N_2O_6Na$ $(M + Na)^+$ 511.27786, Found: 511.28881.

4.7.1.3.4. SYNTHESIS OF COMPOUND 5:

4.7.1.3.4.1. *heptyl (S)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



25

Using general procedure **F**, compound (-)-**16** (*S*)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylic acid[5] (50 mg, 0.15 mmol) was reacted with *n*-heptanol (0.026 mL, 0.19 mmol) to give heptyl (*S*)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate **(-)-25** as a colourless oil (50 mg, 58 %).

R_f (30 % EtOAc: hexane) = 0.2.

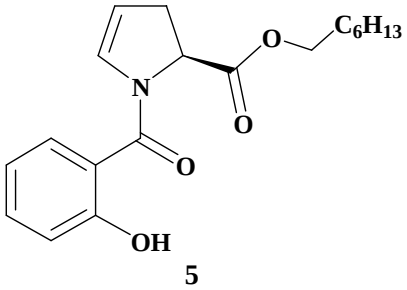
$[\alpha]_D^{23}$ = -85.7 (*c* 1.25, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.39-7.32 (m, 2H), 7.21 (dd, *J* = 1.2, 8.2 Hz, 1H), 7.04 (ddd, *J* = 1.2, 8.2, 8.2 Hz, 1H), 6.18-6.13 (m, 1H), 5.28 (s, 2H), 5.05-5.01 (m, 1H) 4.99 (dd, *J* = 5.0, 11.8 Hz, 1H), 4.23-4.12 (m, 2H), 3.83-3.76 (m, 2H), 3.56-3.48 (m, 2H), 3.35 (s, 3H), 3.19-3.03 (m, 1H), 2.74-2.63 (m, 1H), 1.71-1.60 (m, 2H), 1.42-1.18 (m, 8H), 0.86 (t, *J* = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.2, 165.1, 153.7, 131.4, 131.0, 129.1, 126.1, 122.3, 115.5, 108.7, 93.9, 71.7, 68.1, 65.8, 59.2, 58.1, 34.4, 31.9, 29.1, 28.8, 26.0, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{23}H_{33}NO_6$ C, 65.85; H, 7.93; N, 3.34. Found: C, 65.73; H, 7.91; N, 3.33.

4.7.1.3.4.1. *heptyl (S)-1-(2-hydroxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



5

Using general procedure **E** the above MEM ether **(-)-25** (25 mg, 0.04 mmol) was deprotected to give the title compound **5** as a colourless oil (14 mg, 99 %).

R_f (20 % EtOAc: hexane) = 0.4.

$[\alpha]_D^{23}$ = -112.7 (*c* 0.65, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.78 (s, 1H), 7.45-7.33 (m, 2H), 7.01 (dd, *J* = 1.2, 8.3 Hz, 1H), 6.88 (ddd, *J* = 1.2, 8.3, 8.3 Hz, 1H), 6.84-6.80 (m, 1H), 5.31-5.23 (m, 1H), 5.03 (dd, *J* = 5.3, 11.4 Hz, 1H), 4.27-4.09 (m, 2H), 3.20-3.04 (m, 1H), 2.78-2.67 (m, 1H), 1.72-1.61 (m, 2H), 1.40-1.16 (m, 8H), 0.87 (t, *J* = 7.1 Hz, 3H) ppm.

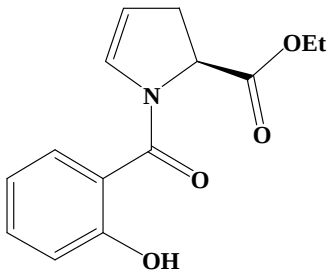
^{13}C NMR (75 MHz, $CDCl_3$) δ = 170.9, 167.6, 159.2, 134.1, 130.9, 128.3, 118.8, 118.0, 116.8, 110.6, 65.8, 59.3, 33.5, 31.7, 28.8, 28.5, 25.7, 22.5, 14.0 ppm.

Elemental Analysis Calcd. for $C_{19}H_{25}NO_4$: C, 68.86; H, 7,60; N, 4.23. Found: C, 68.68; H, 7,58; N, 4.24.

HRMS: Accurate mass (ES+) calculated for $C_{19}H_{25}NO_4Na$ $(M + Na)^+$ 354.16758, Found: 354.16798.

4.7.1.3.5. SYNTHESIS OF COMPOUND 6:

4.7.1.3.5.1. *ethyl (S)-1-(2-hydroxybenzoyl)-2,3-dihydro-1H-pyrrole-2-carboxylate.*



6

Using general procedure **E** ethyl (*S*)-1-(2-((2-methoxyethoxy)methoxy)benzoyl)-*2,3-dihydro-1H-pyrrole-2-carboxylate*[5] (-)-**15** (35 mg, 0.10 mmol) was deprotected to give the title compound (-)-**6** as a colourless oil (20 mg, 76 %).

$\mathbf{R_f}$ (20 % EtOAc : hexane) = 0.3.

$\mathbf{[\alpha]_D^{23}}$ = - 132.3 (*c* 1.00, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.76 (s, 1H), 7.46-7.34 (m, 2H), 7.00 (dd, *J* = 1.2, 8.3 Hz, 1H), 6.88 (ddd, *J* = 8.3, 8.3, 1.2 Hz, 1H), 6.84-6.78 (m, 1H) 5.30-5.24 (m, 1H), 5.02 (dd, *J* = 5.2, 11.4 Hz, 1H), 4.30-4.19 (m, 2H), 3.18-3.05 (m, 1H), 2.78-2.66 (1H, m), 1.29 (3H, t, *J* = 7.1 Hz) ppm.

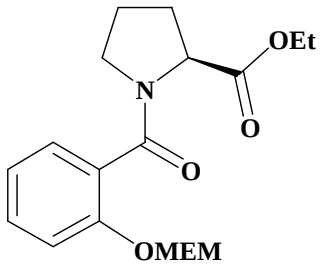
^{13}C NMR (75 MHz, $CDCl_3$) δ = 171.1, 167.8, 159.3, 133.7, 131.1, 128.5, 119.1, 118.2, 117.0, 110.9, 61.9, 59.5, 33.6, 14.3 ppm.

Elemental Analysis Calcd. for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79, N, 5.36. Found: C, 64.52; H, 5.78; N, 5.34.

HRMS: Accurate mass (ES+) calculated for $C_{14}H_{15}NO_4Na$ $(M + Na)^+$ 284.08933, Found: 284.08971.

4.7.1.3.6. SYNTHESIS OF COMPOUND 7:

4.7.1.3.6.1. *ethyl (2-((2-methoxyethoxy)methoxy)benzoyl)-L-prolinate.*



18

HOBt (125 mg, 0.93 mmol) and EDC·HCl (168 mg, 0.93 mmol) were added sequentially to a stirred solution of 2-((2-methoxyethoxy)methoxy)benzoic acid[5] **17** (150 mg, 0.66 mmol) and L-proline ethyl ester·HCl (143 mg, 0.80 mmol) in DMF (2 mL, 0.3 M) at 0 °C under N_2 atmosphere. DIPEA (0.54 mL, 3.31 mmol) was added dropwise and the reaction mixture was stirred at room temperature for 16 h. The reaction mixture was poured into ice cold water (15 mL) and the aqueous layer was extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with cold brine (2 × 5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. Purification using flash column chromatography in 2 % MeOH : CH_2Cl_2 afforded the compound **(-)-18** as a colourless oil (185 mg, 79 %).

R_f (2 % MeOH : CH_2Cl_2) = 0.2.

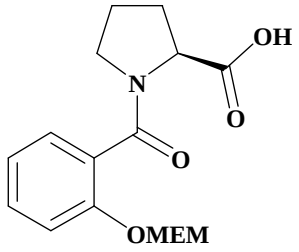
$[\alpha]_D^{23}$ = - 69.7 (*c* 1.2, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) mixture of rotamers (major) δ = 7.34-7.12 (m, 3H), 7.07-7.00 (m, 1H), 5.29 (s, 2H), 4.65 (dd, *J* = 4.3, 8.6 Hz, 1H), 4.23 (q, *J* = 6.9 Hz, 2H,), 3.86-3.76 (m, 2H), 3.57-3.51 (m, 2H), 3.36 (s, 3H), 3.38-3.25 (m, 2H), 2.37-1.78 (m, 4H), 1.31 (t, *J* = 6.9 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = (major) 172.2, 168.0, 153.1, 130.2, 128.0, 127.7, 122.2, 115.4, 94.0, 71.5, 67.9, 61.0, 59.0, 58.6, 48.2, 29.6, 24.7, 14.2 ppm.

Elemental Analysis Calcd. for $C_{18}H_{25}NO_6$: C, 61.52; H, 7.17; N, 3.99. Found: C, 61.39; H, 7.16; N, 4.00.

4.7.1.3.6.2. (2-((2-methoxyethoxy)methoxy)benzoyl)-L-proline.



26

Using general procedure C ester **(-)-18** (165 mg, 0.47 mmol) was hydrolyzed to (2-((2-methoxyethoxy)methoxy)benzoyl)-L-proline **(-)-26** as a colourless gummy mass (140 mg, 92 %).

R_f (10 % MeOH : CH_2Cl_2) = 0.3.

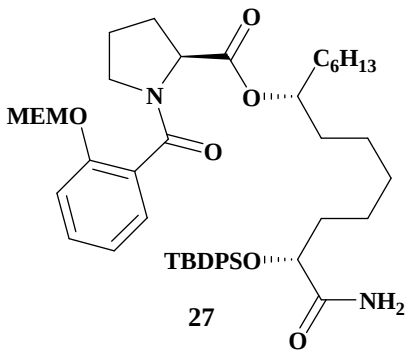
$[\alpha]_D^{23}$ = -79.3 (*c* 1.7, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) mixture of rotamers (major) δ = 7.41-7.17 (m, 3H), 7.06 (dd, *J* = 7.8, 7.8 Hz, 1H), 5.29 (s, 2H), 4.76 (dd, *J* = 4.2, 8.2 Hz, 1H) 3.83-3.77 (m, 2H), 3.57-3.52 (m, 2H), 3.37 (s, 3H), 3.40-3.32 (m, 2H), 2.55-2.36 (m, 1H), 2.26-1.80 (m, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = major: 170.2, 168.5, 153.2, 131.4, 128.0, 126.6, 122.5, 115.5, 114.8, 94.1, 71.7, 68.2, 59.7, 59.2, 49.0, 28.6, 24.8 ppm.

Elemental Analysis Calcd. for $C_{16}H_{21}NO_6$: C, 59.43; H, 6.55; N, 4.33. Found: C, 59.55; H, 6.54; N, 4.34.

4.7.1.3.6.3. *(7R,13R)-14-amino-13-((tert-butyldiphenylsilyl)oxy)-14-oxotetradecan-7-yl(2-((2-methoxyethoxy) methoxy)benzoyl)-L-prolinate.*



27

Using general procedure F, the above acid **(-)-26** was reacted with (*2R,8R*)-2-((tert-butyldiphenylsilyl)oxy)-8-hydroxytetradecanamide[5] **(-)-29** (61 mg, 0.12 mmol) to give (*7R,13R*)-14-amino-13-((tert-butyldiphenylsilyl)oxy)-14-oxotetradecan-7-yl-(2-((2-methoxyethoxy)methoxy)benzoyl)-L-prolinate **(-)-27** as a yellow sticky solid (58 mg, 59 %).

R_f (3 % MeOH : CH_2Cl_2) = 0.4.

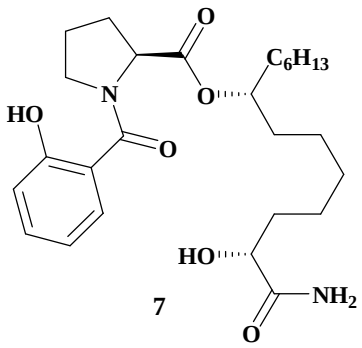
$[\alpha]_D^{23}$ = -25.1 (c 0.35, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) mixture of rotamers (major) δ = 7.69-6.98 (m, 14H), 6.72 (brs, 1H), 5.52 (brs, 1H), 5.29 (s, 2H), 4.70-4.58 (m,1H), 4.29-4.20 (m, 1H), 3.87-3.72 (m, 3H), 3.58-3.47 (m, 2H), 3.35 (s, 3H), 3.45-3.24 (m, 2H), 2.40-1.77 (m, 4H), 1.74-1.04 (m, 29H), 0.87 (t, *J* = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = (major) 176.4, 172.1, 167.8, 153.3, 136.0 (× 2), 135.8 (× 4), 133.2, 132.8, 130.7, 130.4, 128.2, 128.1 (× 4), 122.4, 115.2, 94.1, 75.3, 74.4, 71.8, 68.1, 59.2, 48.4, 34.5, 34.2, 32.0, 31.9, 31.5, 30.0, 29.9, 29.5, 29.4, 29.3, 27.3, 25.4, 25.0, 23.0, 22.8, 22.78, 19.5, 14.3 ppm.

Elemental Analysis Calcd. for $C_{46}H_{66}N_2O_8Si$: C, 68.79; H, 8.28; N, 3.49. Found: C, 68.67; H, 8.26; N, 3.48.

4.7.1.3.6.4. *(7R,13R)-14-amino-13-hydroxy-14-oxotetradecan-7-yl (2-hydroxybenzoyl)-L-prolinate.*



7

2N HCl (0.5 mL) was added dropwise to a stirred solution of the **(-)-27** (50 mg, 0.06 mmol) in THF (0.5 mL) at 0 °C. The reaction mixture was stirred at room temperature for 1h. THF was removed *in vacuo*; the aqueous layer was extracted with ethyl acetate (2 × 7 mL). The combined organic extracts were washed with brine (5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The product was purified using preparative TLC in 10% MeOH : CH_2Cl_2, to afford the title compound (-)-**7** as a pale yellow oil (25 mg, 75 %).

$\mathbf{R_f}$ (10 % MeOH : CH_2Cl_2) = 0.4.

$\mathbf{[\alpha]_D^{23}}$ = -13.0 (*c* 1.0, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 10.66 (1H, s); 7.55-7.44 (m, 1H), 7.40-7.30 (m, 1H), 6.96 (dd, *J* = 1.0, 8.2 Hz, 1H), 6.87 (dd, *J* = 8.2, 8.2 Hz, 1H), 6.59 (brs, 1H), 5.29 (brs, 1H), 4.98 (brs, 1H), 4.65 (dd, *J* = 5.5, 8.6 Hz, 1H), 4.15-4.06 (m, 1H), 3.95-3.74 (m, 2H), 3.40-3.31 (m, 1H), 2.43-2.21 (m, 1H), 2.17-1.89 (m, 3H), 1.83-1.07 (m, 20H), 0.87 (t, *J* = 6.7 Hz, 3H) ppm.

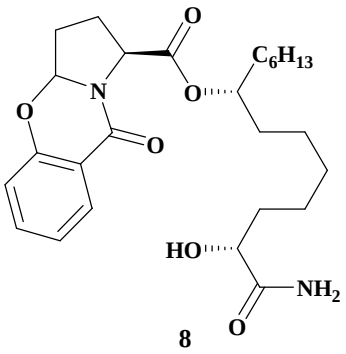
^{13}C NMR (75 MHz, $CDCl_3$) δ = 177.3, 172.4, 170.4, 159.1, 133.3, 128.2, 118.9, 117.95, 117.87, 75.5, 71.7, 60.7, 50.8, 34.6, 34.5, 34.4, 31.9, 29.4, 29.3, 28.6, 25.9, 25.6, 25.0, 24.7, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{26}H_{40}N_2O_6$: C, 65.52; H, 8.46; N, 5.88. Found: C, 65.63; H, 8.48; N, 5.89.

HRMS: Accurate mass (ES+) calculated for $C_{26}H_{40}N_2O_6Na$ $(M + Na)^+$ 499.27786, Found: 499.27697.

4.7.1.3.7. SYNTHESIS OF COMPOUND 8:

4.7.1.3.7.1. **(***7R,13R)-14-amino-13-hydroxy-14-oxotetradecan-7-yl-(1S)-9-oxo-1,2,3,3a-tetrahydro-9H-benzo[e]pyrrolo[2,1-b][1,3]oxazine-1-carboxylate*.

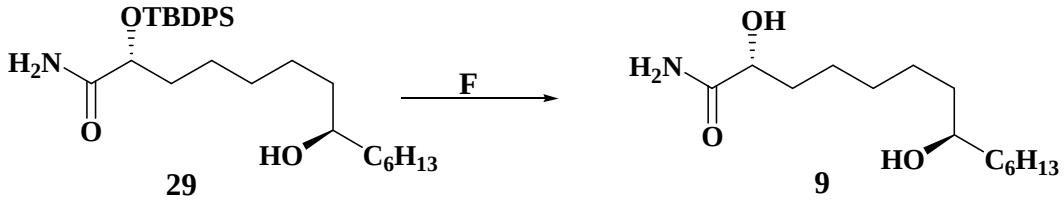


8

Compound **8** was synthesized as described in the Chapter-3, synthesis of Intermediate **33.**

4.7.1.3.8. SYNTHESIS OF COMPOUND 9:

4.7.1.3.8.1. *(2R,8R)-2,8-dihydroxytetradecanamide **9**.*



Using general procedure **F**, silyl ether (-)-**29**[5] (38 mg, 0.076 mmol) yielded the title compound (+)-**9** as an off white solid (12 mg, 63 %).

R_f (2 % MeOH : CH_2Cl_2) = 0.3.

M.P = 100-102 ºC.

$[\alpha]_D^{23}$ = +15.0 (*c* 0.5, $CHCl_3$).

^{1}H NMR (300 MHz, CH_3OH - d_4) δ = 3.97 (dd, *J* = 3.9, 7.7 Hz, 1H), 3.50 (brs, 1H), 1.83-1.67 (m, 1H), 1.66-1.52 (m, 1H), 1.50-1.22 (m, 20H), 0.90 (t, *J* = 7.0 Hz, 3H) ppm.

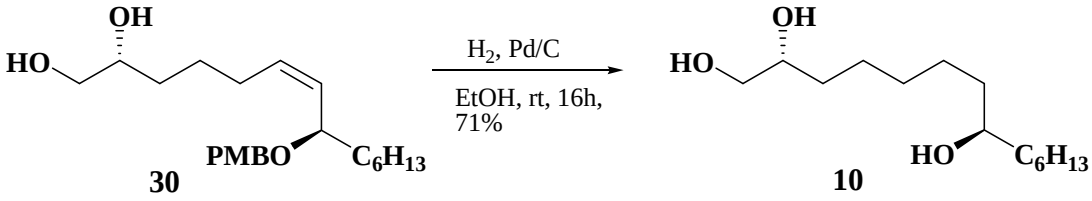
^{13}C NMR (75 MHz, CH_3OH - d_4) δ = 179.5, 71.5, 71.2, 37.3, 37.2, 34.4, 31.9, 29.4, 29.4, 25.6, 25.5, 24.9, 22.5, 13.2 ppm.

Elemental Analysis Calcd. for $C_{14}H_{29}NO_3$: C, 64.83; H, 11.27; N, 5.40. Found: C, 64.75; H, 11.29; N, 5.41.

HRMS: Accurate mass (ES+) calculated for $C_{14}H_{29}NO_3Na$ $(M + Na)^+$ 282.20396, Found: 282.20448**.**

4.7.1.3.9. SYNTHESIS OF COMPOUND 10:

4.7.1.3.9.1. *(2R, 8R)- 2,8 dihydroxytetradecanol.*



To a solution of diol **30**[5] (100 mg, 0.274 mmol) in ethanol (7 mL) was added 10% Pd/C (10 mg). The suspension was evacuated under vacuum and flushed with H_2 gas (4 times). The reaction mixture was stirred under H_2 atmosphere at room temperature for 12 h. The reaction mixture was filtered through a plug of celite and the residue was washed with ethyl acetate (2 × 5 mL). The filtrate was concentrated *in vacuo*, the concentrate was triturated with diethyl ether (5 mL) to obtain alcohol **10** (50 mg, 74 %) as white solid.

R_f (70 % ethyl acetate: hexane) = 0.2.

M.P = 103-104 °C.

$[\alpha]_D^{23}$ = +8.0 (*c* 0.5, MeOH).

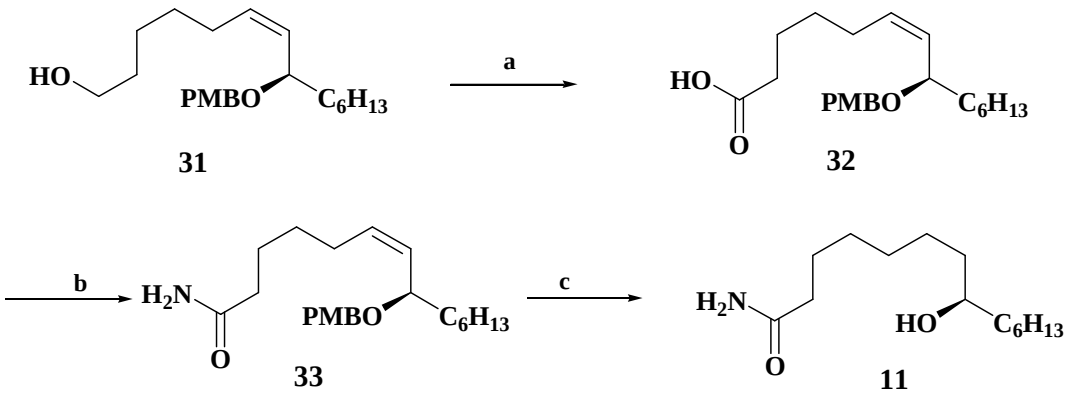
^{1}H NMR (300 MHz, 300 MHz, CH_3OH - d_4) δ = 3.62-3.34 (m, 4H), 1.60-1.22 (m, 20H), 0.90 (t, *J* = 6.9 Hz, 3H) ppm.

^{13}C NMR (75 MHz, CH_3OH - d_4) δ = 72.1, 71.2, 66.2, 37.3, 37.2, 33.2, 31.9, 29.7, 29.3, 25.6 (× 2), 25.5, 22.5, 13.2 ppm.

Elemental Analysis Calcd. for $C_{14}H_{30}O_3$: C, 68.25; H, 12.27. Found: C, 68.33; H, 12.29.

HRMS: Accurate mass (ES+) calculated for $C_{14}H_{30}O_3Na$ $(M + Na)^+$ 269.20872, Found: 269.20931.

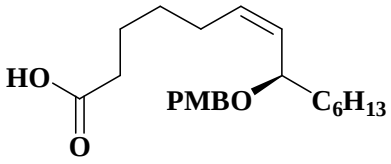
4.7.1.3.10. SYNTHESIS OF COMPOUND 11:



31 **32** **33** **11**

Scheme-2: Synthesis of 11; a) TEMPO, BAIB, $NaHCO_3$, ACN/ H_2O, 0 °C, 4h, 65%; b) HBTU, HOBT, NH_4Cl, DIPEA, DMF, 0 °C to rt, 1 h, 82%; c) H_2, Pd/C, EtOH, rt, 16 h, 71%.

4.7.1.3.10.1. *(R,Z)-8-(4-methoxybenzyloxy)tetradec-6-enoic acid.*



32

A suspension of alcohol **(+)-31**[5] (700 mg, 2.02 mmol), $NaHCO_3$ (509 mg, 6.06 mmol) in CH_3CN : H_2O (1 :1) (25 mL) was cooled to 0 °C and stirred for 10 min. TEMPO (63 mg, 0.40 mmol) and bis(acetoxy)iodobenzene (1.62 gm, 5.05 mmol), were added sequentially in one portion and the solution was stirred at 0 °C for 4 h in the dark. Saturated aq. $NaHCO_3$ (20 mL) was added at 0 °C and the aqueous layer was extracted with ethyl acetate (2 × 25 mL). The combined organic extracts were washed with brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The crude oil was purified using flash column chromatography with 0-30% ethyl acetate: petroleum ether) to furnish (*R,Z*)-8-(4-methoxybenzyloxy)tetradec-6-enoic acid **(+)-32** (475 mg, 65 %) as white translucent oil.

$\mathbf{R_f}$ (30 % ethyl acetate: hexane) = 0.25.

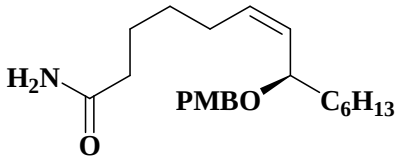
$\mathbf{[\alpha]_D^{23}}$ = +28.5 (*c* 1.00, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.24 (d, *J* = 9.1 Hz, 2H), 6.86 (d, *J* = 9.1 Hz, 2H), 5.58 (dt, *J* = 11.2, 7.4 Hz, 1H), 5.32 (dd, *J* = 11.2, 9.5 Hz, 1H), 4.50 (d, *J* = 12.3 Hz, 1H), 4.26 (d, *J* = 12.3 Hz, 1H), 4.11-4.02 (m, 1H), 3.80 (s, 3H), 2.35 (t, *J* = 7.3 Hz, 2H), 2.16-1.93 (m, 2H), 1.74-1.18 (m, 14H), 0.87 (t, *J* = 7.3 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 179.7, 159.2, 132.6, 131.9, 131.3, 129.5 (× 2), 113.9 (× 2), 74.0, 69.6, 55.5, 35.9, 34.1, 32.1, 29.5, 29.4, 27.7, 25.6, 24.6, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{22}H_{34}O_4$: C, 72.89; H, 9.45. Found: C, 72.77; H, 9.44.

4.7.1.3.10.2. *(R,Z)-8-(4-methoxybenzyloxy)tetradec-6-enamide.*



33

A solution of carboxylic acid **(+)-32** (475 mg, 1.31 mmol), NH_4Cl (282.3 mg, 5.27 mmol) in dry DMF (18 mL, 0.07 M) was cooled to 0 °C. HOBT (534 mg, 3.93 mmol) and HBTU (1.5 gm, 3.93 mmol) were added, followed by DIPEA (1.87 mL, 5.27 mmol). The reaction mixture was warmed to room temperature and stirred for 1 h. Ice pieces were added, and then the aqueous layer was extracted with ethyl acetate (2 × 20 mL). The combined organic extracts were washed with cold brine (3 × 15 mL) and dried over anhydrous Na_2SO_4. After removal of the solvent *in vacuo*, the residue was purified using flash column chromatography with 0-50% ethyl acetate: petroleum ether to furnish (*R,Z*)-8-(4-methoxybenzyloxy)tetradec-6-enamide **(+)-33** (389 mg, 82 %) as a colourless oil.

R_f (50 % ethyl acetate: hexane) = 0.33.

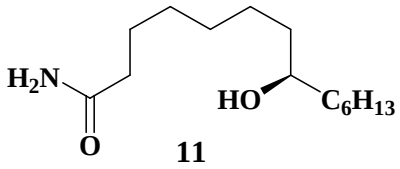
$[\alpha]_D^{23}$ = +17.4 (*c* 0.5, $CHCl_3$).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.24 (d, *J* = 8.9 Hz, 2H), 6.86 (d, *J* = 8.9 Hz, 2H), 5.58 (dt, *J* = 11.2, 7.4 Hz, 1H), 5.45 (brs, 2H), 5.31 (dd, *J* = 11.2, 9.3 Hz, 1H), 4.49 (d, *J* = 11.7 Hz, 1H), 4.26 (d, *J* = 11.7 Hz, 1H), 4.12-4.00 (m, 1H), 3.80 (s, 3H), 2.20 (t, *J* = 7.6 Hz, 2H,), 2.15-1.93 (m, 2H), 1.74-1.17 (m, 14H), 0.87 (t, *J* = 7.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 175.4, 159.2, 132.7, 131.8, 131.3, 129.5 (× 2), 113.9 (× 2), 74.0, 69.6, 55.5, 35.9, 35.8, 32.0, 29.5 (× 2), 27.7, 25.6, 25.4, 22.8, 14.3 ppm.

Elemental Analysis Calcd. for $C_{22}H_{35}NO_3$: C, 73.09; H, 9.76; N, 3.87. Found: C, 73.17; H, 9.73; N, 3.87.

4.7.1.3.10.3. *(R)-8-hydroxytetradecanamide.*



11

To a solution of **(+)-33** (389 mg, 1.083 mmol) in ethanol (30 mL) was added 10% Pd/C (200 mg). The suspension was evacuated under vacuum and flushed with H_2 gas (× 4). The reaction mixture was stirred under H_2 at room temperature for 12 h, then it was filtered through a plug of celite and the residue was washed with ethyl acetate (2 × 20 mL). The filtrate was concentrated *in vacuo;* the concentrate was triturated with diethyl ether (10 mL) to obtain (*R*)-8-hydroxytetradecanamide (+)-**11** (150 mg, 71 %) as a white solid.

R_f (50 % ethyl acetate: hexane) = 0.15.

M.P = 97-99 ºC.

$[\alpha]_D^{23}$ = +9.0 (*c* 1.0, $CHCl_3$).

^{1}H NMR (300 MHz, CD_3OD) δ = 3.49 (brs, 1H), 2.49-2.40 (m, 1H), 2.22-2.15 (s, 3H), 1.66-1.22 (m, 20H), 0.90 (t, *J* = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, CD_3OD) δ = 178.1, 71.2, 37.3, 35.3, 31.9, 29.6, 29.5, 29.4, 29.3, 29.1, 25.7, 25.6, 22.5, 13.3 ppm.

Elemental Analysis Calcd. for $C_{14}H_{29}NO_2$: C, 69.09; H, 12.01; N, 5.75. Found: C, 69.15; H, 12.03; N, 5.74.

HRMS: Accurate mass (ES+) calculated for $C_{14}H_{29}NO_2Na$ $(M + Na)^+$ 266.20905, Found: 266.20960.

4.7.2. MATERIALS AND METHODS RELATED TO MICROBIOLOGICAL ASSAYS:

4.7.2.1. MICROBIOLOGICAL MEDIA AND CULTURE CONDITION:

Bacteria strains and yeast were maintained at -80 °C and cultivated in M17 broth (Difco, Laboratories, Detroit, MI) for *Streptococcus* and *Lactococcus* species, and in MRS broth (Difco) for all *Lactobacillus*, *Pediococcus*, and *Enterococcus* species. *Staphylococcus* species were cultivated in Brain Heart Infusion broth (Difco). *Streptococcus pneumoniae* was routinely maintained in Trypticase Soy Broth (TSB) (Difco, Laboratories, Detroit, MI) with 3% (v/v) defibrinated horse blood in a 5% CO2 incubator. *E. coli* and *Pseudomonas* species were cultivated in TSB (Difco, Laboratories, Detroit, MI). Cultures were incubated at 30 °C and 37 °C for mesophilic and thermophilic species, respectively.

4.7.2.2. AGAR DIFFUSION ASSAY.

An agar diffusion assay was carried out using *P. putida* RW10S1 (promysalin producers), and *P. stutzeri* LMG 2333 (promysalin sensitive) as reference strains.[3] Strains RW10S1 and LMG 2333 were spotted on the surface of agar Trypticase Soy Broth (TSB) (Difco, Laboratories, Detroit, MI) and incubated for 10-18 h at 30 °C. After growth, the surface of the Petri plate was exposed to a saturated atmosphere of chloroform for 10 min, and an overlay containing a suspension of 10^7 CFU/mL of the target strain in the appropriate soft agar medium was poured onto the surface. The soft agar overlay, containing agar 7.5 g/L, was prepared in M17 medium (Difco, Laboratories, Detroit, MI) for *Streptococcus thermophilus* DSM 20617^T, and in MRS medium (Difco, Laboratories, Detroit, MI) for *Pediococcus acidilactici* DSM20284^T. After solidification, the plates were incubated at the appropriate temperature for 18 h, and the presence or the absence of an inhibition halo around the *P. putida* RW10S1 was verified.

4.7.2.3. EVALUATION OF MINIMAL INHIBITORY CONCENTRATION (MIC) AND MINIMAL BACTERICIDAL CONCENTRATION (MBC) OF PROMYSALIN AND DERIVATIVES AGAINST GRAM-POSITIVE AND GRAM-NEGATIVE BACTERIA.

Minimum inhibitory concentration (MIC) was determined using the broth microdilution method: after overnight growth on Mueller-Hinton broth-II (MHB-II) (Sigma-Aldrich, Milan, Italy) plates, strains were suspended in MHB-II to a standardized OD590 of 0.5. Three 10-fold dilutions were performed, and each cell suspension was inoculated in the presence of promysalin or its derivative analogues at the indicated concentrations. Determination of the minimum bactericidal concentration (MBC) was performed by subculturing 10 µL from each well without visible microbial growth. After 48 hours of incubation, the promysalin or analogue dilutions yielding three colonies or less were scored as the MBC for starting inocula of 10^5 CFU/mL. The experiments were performed in triplicate. MIC and MBC were performed

according to CLSI (Clinical and Laboratory Standards Institute) methods for dilution antimicrobial susceptibility tests for aerobic bacteria (approved standard, Wayne, PA, USA: CLSI; 2009).

4.7.2.4. FLOW CYTOMETRY EVALUATION OF CELL MEMBRANE DAMAGE AND MEASUREMENT OF cFSE FLUORESCENCE CELL LEAKAGE.

To evaluate whether membrane damage was linked to cell leakage of intracellular components, microbial cells grown for 18 h in the appropriate medium in Petri dishes were collected and diluted in sterile filtered (0.2 µm) phosphate-buffered saline (PBS) (NaCl 8 g/L; KCl 0.2 g/L; Na_2HPO_4 1,44 g/L; KH_2PO_4 0.24 g/L; pH 7.4) to a final concentration of 10^8 events per mL. The cell suspension was diluted to 10^6 events/mL and then exposed to promysalin (100 µg/mL) or its derivative analogues (100 µg/mL), chlorhexidine (100 µg/mL) (Sigma-Aldrich) or benzalkonium chloride (100 µg/mL) (Sigma-Aldrich) at 37 °C. The cell suspension was also exposed to a DMSO control. At the time requested, a sample was collected and subjected to SYBR Green I/PI double staining and analysis by flow cytometry and, when necessary, to a standard plate count in the appropriate medium. In flow cytometry, particles/cells that pass through the beam will scatter light, which is detected as forward scatter (FSC) and side scatter (SSC). FSC correlates with cell size, cell shape and cell aggregates, whereas SSC depends on the density of the particles/cells (i.e., the number of cytoplasmic granules and membrane size). In this manner, cell populations can often be distinguished based on differences in their size and density. Cell suspensions were subjected to dual nucleic acid staining with cell permeate SYBR Green I (1X) and cell impermeant propidium iodide (PI) (5 µg/mL) (Sigma-Aldrich, Milan, Italy). SYBR Green I permeate the membrane of total cells and stains nucleic acids with green fluorescence. After incubation at room temperature for 15 min, the labeled cell suspensions were diluted to approximately 10^6 events per mL, and analyzed by flow cytometry. Cell suspensions that were prepared as described above were analyzed using a flow cytometer with the following threshold settings: FSC 5,000, SSC 4,000, and 20,000 total events collected. All parameters were collected as logarithmic signals, and a 488-nm laser was used to measure the FSC values. The rate of events in the flow was generally lower than 2,000 events/s. The obtained data were analyzed using BD AccuriTM C6 software 1.0 (BD Biosciences, Milan, Italy). Cell-membrane damage was carried out by applying double staining with SYBR Green I and PI. The SYBR Green I fluorescence intensity of stained cells was recovered in the FL1 channel (excitation 488 nm, emission filter 530/30 nm). PI fluorescence was recovered in the FL3 channel (excitation 488 nm, emission filter 670 nm long pass). PI penetrates only bacteria with damaged membranes, causing a reduction in SYBR Green I fluorescence when both dyes are present. Thus, live bacteria with intact cell membranes fluoresce bright green (defined as active fluorescent cells), bacteria with slightly damaged membranes exhibit both green

and red fluorescence (defined as slightly membrane damaged cells) and cells with broken membranes fluoresce red (defined as non-active fluorescent cells) (ISO 19344:2015; IDF 232:2015). Active fluorescent cells, damaged cells and non-active fluorescent cells were electronically gated in density plots of green fluorescence (FL1) versus red (FL3) fluorescence. Green and red fluorescence allowed for optimal distinction between stained microbial cells and instrument noise or sample background. Active fluorescent cells were gated in G1, cells with a slightly damaged membrane were gated in G2, and cells with broken membranes fluoresce red were gated in G3. To evaluate whether membrane damage was linked to cell leakage of intracellular components, microbial cells grown for 18 h in the appropriate medium in Petri dishes were collected and diluted in PBS to a final concentration of 10^8 per mL. The obtained cell suspension was supplemented with 4 µM cFDASE (Sigma-Aldrich, Milan, Italy), which is a precursor molecule of cFSE. The suspensions were incubated for 30 min at 37 °C. During this incubation, membrane-permeating cFDASE was cleaved by intracellular esterases, and the resulting cFSE molecules were conjugated to the aliphatic amines of intracellular proteins. After centrifugation at 15,000 x *g* for 1 min and washing with PBS solution, the cells were suspended in an equal volume PBS. To ensure that unconjugated and free probes were eliminated by the cells, we periodically monitored cell fluorescence by flow cytometry as described below. The stability of the cell fluorescence was assessed; stained cells kept on ice in PBS after staining maintained a stable fluorescence, indicating that no free cFSE was inside the cells. Cell suspensions, prepared as described above and diluted to 10^6 events/mL, were analyzed using a flow cytometer with the previously described threshold settings. The cFSE fluorescence intensity of stained cells was recovered in the FL1 channel (excitation 488 nm, emission filter 530/30, provided by BD Biosciences, Milan, Italy). The cFSE-labeled cell suspension was then exposed to promysalin (100 µg/mL) or its derivative analogues (100 µg/mL), chlorhexidine (100 µg/mL) (Sigma-Aldrich) or benzalkonium chloride (100 µg/mL) (Sigma-Aldrich) at 37 °C. As a control, the cFSE-labeled cell suspension was also exposed to a volume of DMSO solvent equal to that used for promysalin and its derivative analogues. At the time point, two samples of each cell suspension were collected: i) one sample was labeled with PI as described above, incubated at room temperature for 15 min, and analyzed by flow cytometry. The second sample was used to measure cFSE-fluorescence cell leakage. In flow cytometry, density plots of cFSE-green fluorescence (FL1) and FSC allowed for optimal distinction between cFSE-stained microbial cells and instrument noise or sample background. Active cells showing only cFSE fluorescence were gated in G1, and cells with a slightly or heavily damaged membrane showing cFSE and PI fluorescence were gated in G2 and G3. Electronic gates on the green fluorescence/FSC density plot were used to select the measured bacterial concentration expressed as a % equal to the number of events in the gate divided by the total events counted. The sample for the measurement of cFSE-fluorescence

cell leakage was centrifuged (13000 rpm, 2 min), and the cell-free supernatant transferred to a 96-microtiter plate for measurement of cFSE-fluorescence in a Victor 3 fluorometer (PerkinElmer). The fluorescence data were calculated as the average of three independent assays and expressed in arbitrary units of fluorescence ± the standard deviation.

4.7.2.6. EVALUATION OF GROWTH KINETIC PARAMETERS OF *SACCHAROMYCES CEREVISIAE* BC1 IN THE ABSENCE OF PROMYSALIN.

S. cerevisiae BC1 growth was monitored in 96-well plates that were filled using an automatic liquid handling system (EpMotion, Eppendorf, Italy) to a final volume of 200 µL in the presence and absence of promysalin at different concentrations (4-128 µg/mL). A set of promysalin solutions at different concentrations in DMSO was prepared to add the same volume to each well, regardless of the final promysalin concentration. The growth of *S. cerevisiae* BC1 in the presence of promysalin was compared to its growth in the presence of a DMSO control added to the medium. Microbial growth was monitored using a spectrophotometer (MicroWave RS2, Biotek, USA) programmed for 145 readings ($O.D._{600\ nm}$) every 10 min for 24 h at 37 °C. At the end of the incubation, the growth curve and lag time (h:min) were obtained using Gen5 software (Biotek, USA). The data were calculated as the average of three independent assays ± the standard deviation.

4.8. REFERENCES:

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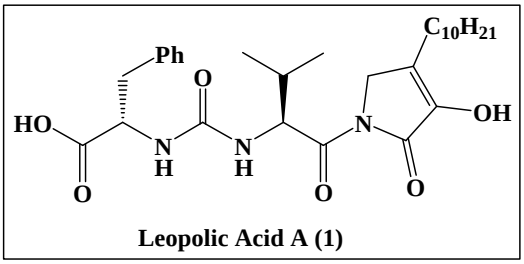
CHAPTER-5

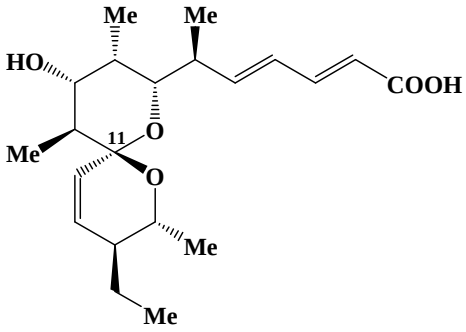
TOTAL SYNTHESIS OF LEOPOLIC ACID A

5.1. INTRODUCTION:

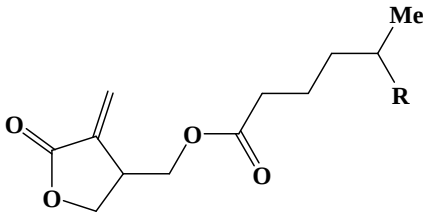
Plant associated actinomycetes have proven to be rich sources of biologically active secondary metabolites and therefore have attracted increasing attention in recent years. The secondary metabolites isolated from these sources represent a group of chemically diverse small molecules with wide range of bioactivity such as antibacterial, antifungal, cytotoxic and plant growth promoter. Few of these metabolites are shown in Figure-1.[1, 2]



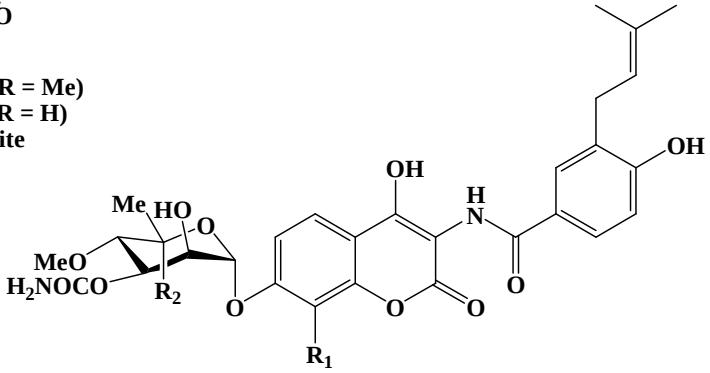
Leopolic Acid A (1)



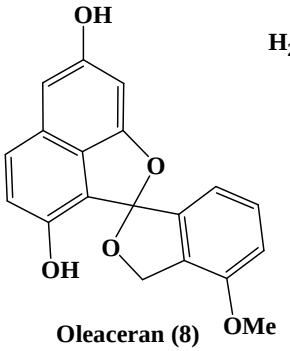
Pteridic acid A (2)
Pteridic acid B (3) (11-epimer)
Plant Grwoth promoter.



Cedarmycin A (4) (R = Me)
Cedarmycin B (5) (R = H)
Antifungal metabolite



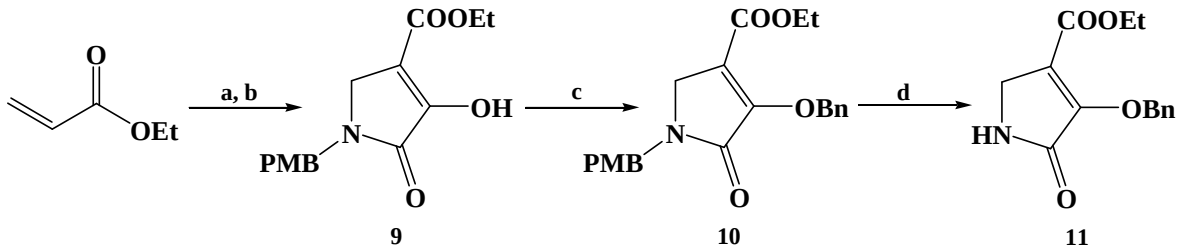
7'-Dimethylnonobiocin (6) (R_1 = H, R_2 = Me)
5"-Demethylnovobiocin (7) (R_1 = Me, R_2 = H)
Antibacterial antibiotic



Oleaceran (8)
Antifungal, cytotoxic

Figure-1: Plant associated actinomycetes metabolites.

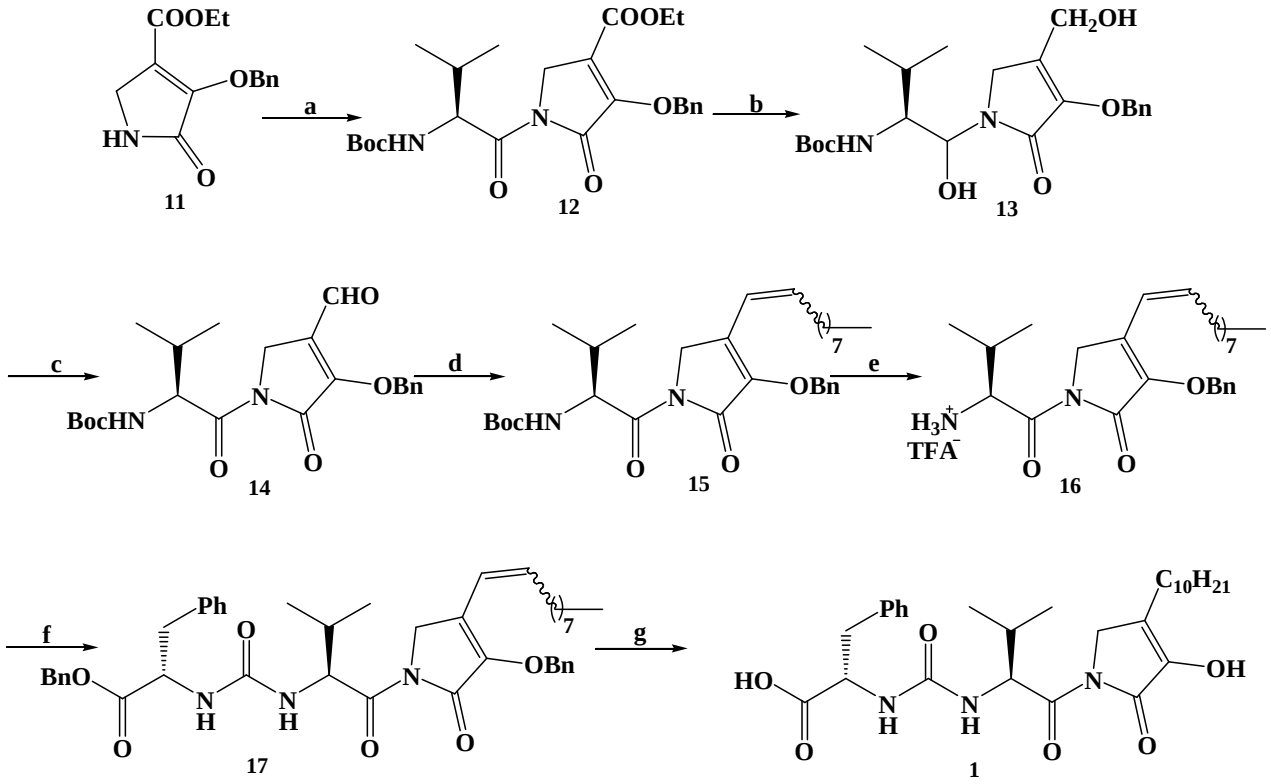
In 2012, through the chemical analysis of a terrestrial-derived Streptomyces sp. isolated from the rhizosphere of the plant *Juniperus excelsa*, Raju and coworkers[3] elucidated the structure of a new metabolite, leopolic acid A (**1**, Figure-1). Leopolic acid has unprecedented structural features consisting of an aliphatic side chain attached to a 2,3-pyrrolidinedione residue, which in turn, is connected to the ureido dipeptide L-Phe-L-Val. The compound showed antifungal and antibacterial activity against *Mucor hiemalis* and *Staphylococcus aureus* with a MIC of 32 and 16 μg/mL respectively. Recently, our research group has been engaged in the total synthesis of this intriguing metabolite. For this purpose, it has been developed the synthesis of the pyrrolidine-2,3-dione scaffold outlined in Scheme 1.[4] The pyrrolidine-2,3-dione **9** was obtained in good yield by the Michael addition of *p*-methoxybenzylamine on ethyl acrylate followed by Dieckmann cyclisation with diethyl oxalate. The alkylation of enolic group using K_2CO_3 and benzyl bromide in dry DMF produced O-benzyl lactam **10**, which was subsequently deprotected using CAN in CH_3CN : water to furnish PMB deprotected lactam **11** in good yields.



Scheme 1: Synthesis of pyrrolidine-2,3-dione. Reagents and Conditions: a) *p*-methoxybenzylamine, EtOH, rt, 12 h, 98 %; b) diethyl oxalate, NaOEt, EtOH, reflux, 3h, 83%; c) BnBr, K_2CO_3, DMF, 0 °C to rt, 1 h, 50 %; d) CAN, CH_3CN:H_2O, 0 °C to rt, 3 h, 73 %.

5.2. SYNTHESIS OF LEOPOLIC ACID A:

Part of my research activity was focused on the development of the synthetic pathway from the intermediate **11** onwards (scheme-2). The acylation of lactam **11** using *n*-BuLi and the pentafluorophenol ester of Boc-valine gave N-acyl lactam (+)-**12** in 73% yield.[5]



Scheme-2: Synthesis of leopolic acid A. Reagents and Conditions: a) 2-tert-butoxycarbonylamino-3-methylbutyric acid pentafluorophenyl ester, *n*-BuLi, THF, -78 °C, 0.5 h, 71%; b) DIBAL-H, CH_2Cl_2, -78 °C, 2 h, 30%; c) PCC, CH_2Cl_2, 0 °C to rt, 12 h, 46%; d) n-nonyltriphenylphosphonium bromide, *n*-BuLi, THF, -78 °C to 0 °C, 2 h; e) TFA, CH_2Cl_2, 0 °C to rt, 1 h; f) L-phenylalanine benzyl ester, DIPEA, triphosgene, CH_2Cl_2, rt, 1 h, 60% over three steps; g) H_2, Pd/C, EtOAc, rt, 2 h, 77%.

The selective reduction of ester to primary alcohol using equivalent amounts of LAH, $NaBH_4$, $LiBH_4$, Superhydride® did not gave the fruitful results. Finally, the treatment of lactam (+)-**12** with 3 equivalents of DIBAL-H in CH_2Cl_2 at -78 °C chemoselectively reduced the ester and exocyclic amide to yield the diol (-)-**13** in acceptable yield. The oxidation of diol (-)-**13** was found to be nontrivial as under mild acidic condition and at room temperature the lactamol underwent cleavage to the corresponding lactam with

concomitant oxidation of primary alcohol to aldehyde. The oxidation under Swern condition and with Dess-Martin periodinane did not give the oxidized product in good yields. The PCC promoted one pot oxidation of diol (-)-**13** furnished the aldehyde (+)-1**4** in moderate yield.

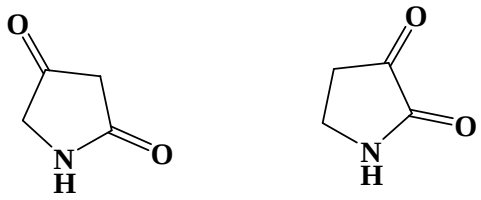
The Wittig olefination of the resultant aldehyde with *n*-nonyltriphenylphosphonium bromide and *n*-BuLi at -78 °C facilitated the installation of decyl chain to furnish **15** as mixture of *E/Z* isomers.

The intermediates **14** and **15** were susceptible to the mildest basic conditions and give rise to the hydrolysed products. Thus, the Boc deprotection of crude **15** followed by treatment with triphosgene and phenylalanine benzyl ester at room temperature produced urea **17**. Finally, the global deprotection using catalytic hydrogenation generated the target molecule leopolic acid A (+)-**1**. We published these results in *Beilstein J. Org. Chem.* **2016**, *12*, 1624-1628.

5.3. CONCLUSIONS:

In conclusion, the Leopolic acid synthesis was accomplished in 11 steps. Although the molecule structurally looks simple, the 2,3-pyrrolidinone core was quite troublesome to install and several attempts were made to find the right pathway. The main problem we faced was the instability of a number of intermediates containing the 2,3 pyrrolidinedione moiety. The NH-free pyrrolidinedione scaffold, for instance, was found to be stable only in the presence of the ester group in position 4. The instability of the majority of the compounds resulting from the 2,3-pyrrolidinone core might be the probable reason for the rare presence of this structure in the literature.

5.4. PRELIMINARY ANTIMICROBIAL ACTIVITY EVALUATION STUDIES ON 2,3-PYRROLIDINEDIONE CONTAINING MOLECULES:



Tetramic acids (18) **2,3-pyrrolidinone (19)**

Figure-2: Tetramic acid (**18**) and 2,3-pyrrolidinedione (**19**) scaffolds.

A literature survey revealed that more than one hundred compounds containing the 2,4-pyrrolidinedione ring system (**18**, tetramic acids) have been isolated from a variety of natural sources.[6] Most of these molecules have attracted considerable attention for their biological activities (i.e. antibacterial, antiviral, antifungal and anticancer). Compared to the numerous studies on bioactivities of tetramic acids, compounds with a 2,3-pyrrolidinedione skeleton (**19**) have been considerably less investigated. Even though this core can be considered as an attractive target, synthetic studies directed to find new biologically active compounds are very few.[7]

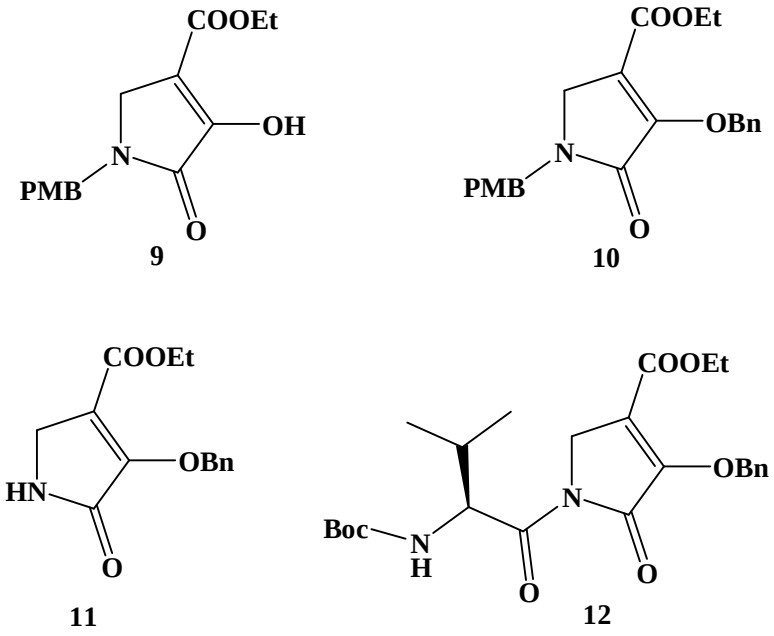


Figure-3: Structure of 2,3-pyrrolidinedione containing molecules tested.

Thus, while accomplishing the total synthesis of leopolic acid, we planned the biological screening of intermediates in order to investigate their antimicrobial and antifungal activities. In particular, in the perspective of a potential application for the development of new molecules for oral healthcare, we tested the antimicrobial activity of representative compounds (Figure-3) on *Streptococcus mutans* and *Candida albicans*. The results are shown in Table 1.

Derivative (concentration)	***S. mutans*** **inihibition (%)**	***C. albicans*** **inhibition (%)**
PBS + EtOH (10%) + DMSO 10% (negative control)	0.0	0.0
9(0.2%)[a]	**51.3**	10.7
9(1%)	25.6	45.5
10 (0.2%)	2.7	23.1
10 (1%)	0.0	32.3
11 (0.2%)	4.8	27.9
11(1%)	3.4	43.2
12(0.2%)	48.6	16.7
12 (1%)	**89.5**	**50.7**
CHX (0.2%) (positive control)	**93.6**	**49.9**

Table-1: MTT assay (data are expressed as % growth inhibition of microbial cells viable biomass after 1 h exposure to different concentrations of the tested derivatives).

A 0.2% solution of compound **9**, containing a free enolic OH, gave a 51.3% inhibition of *S. mutans* growth. On *C. albicans*, however, the inhibition rate did not reach 50%, even at the highest concentration tested (1%). The protection of the enolic OH by a benzyl group (**10**) totally deprived the compound of any antibacterial activity.

The effect of substituent on the nitrogen atom was also investigated. Removal of the PMB group from compound **10** to obtain compound **11** did not significantly change its antibacterial and antifungal activities. An unexpected gain in antibacterial activity was observed with the introduction of the protected amino acidic moiety to obtain compound **12**.

5.5. CONCLUSIONS:

In conclusion, based on the results obtained, the compounds having 2,3-pyrrolidinedione nucleus can be considered promising lead structures for the development of structurally simplified and novel antimicrobial agents.

We published these results in *Bioorg. Med. Chem. Lett.* **2016**, *26*, 1376-1380**.**

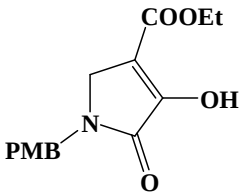
5.6. EXPERIMENTAL SECTION:

5.6.1. GENERAL INFORMATION:

As per chapter **3.3.1. GENERAL INFORMATION**.

5.6.2: EXPERIMENTAL PROCEDURES AND SPECTROSCOPIC DATA:

5.6.2.1. *ethyl 1-(4-methoxybenzyl)-2,5-dihydro-4-hydroxy-5-oxo-1H-pyrrole-3-carboxylate.*



9

Ethyl 3-(4-methoxybenzylamino)propanoate. A mixture of 4-methoxybenzylamine (10.00 g, 72.89 mmol) in ethanol (100 mL) and ethyl acrylate (6.27 g, 62.62 mmol) was stirred at room temperature for 12 h. After completion of reaction, the mixture was concentrated *in vacuo* to give ethyl 3-(4-methoxybenzylamino)propanoate (16.88 g, 98%) as a pale yellow liquid. $\mathbf{R_f}$ (75% EtOAc/ Hexane) = 0.5.

^{1}H-NMR ($CDCl_3$) δ = 7.23 (d, J = 8.5 Hz, 2H), 6.85 (d, J = 8.5 Hz, 2H), 4.13 (q, J = 7.2 Hz, 2H), 3.80 (s, 3H), 3.74 (s, 2H), 2.88 (t, J = 6.6 Hz, 2H), 2.52 (t, J = 6.6 Hz, 2H), 1.25 (t, J = 7.2 Hz, 3H) ppm.

A mixture of ethyl 3-(4-methoxybenzylamino) propanoate (15.00 g, 63.21 mmol), NaOEt (6.40 g, 94.21 mmol) and diethyl oxalate (10.99 g, 75.85 mmol) was refluxed for 3h. After removal of all the volatiles under vacuum, the solid residue obtained was dissolved in boiling water and filtered while hot. Neutralization of the filtrate with 2 N HCl gave a precipitate, which was filtered, washed with water and dried in vacuum to give the desired product **9** (18.01 g, 83%) as a white solid.

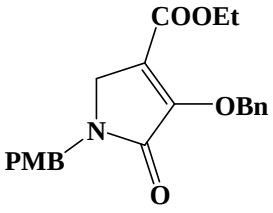
$\mathbf{R_f}$ = (10% MeOH : CH_2Cl_2) = 0.5.

m.p. = 132-133 °C.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.18 (d, J = 8.7 Hz, 2H), 6.86 (d, J = 8.7 Hz, 2H), 4.60 (s, 2H), 4.26 (s, 2H), 3.83 (s, 2H), 3.79 (s, 3H), 1.29 (t, J = 7.0 Hz, 3H) ppm.

Elemental Analysis calcd. (%) for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81. Found: C, 61.60; H, 5.90; N, 4.82.

5.6.2.1. *ethyl 1-(4-methoxybenzyl)-4-(benzyloxy)-2,5-dihydro-5-oxo-1H-pyrrole-3-carboxylate.*



10

To a stirred solution of **9** (15.02 g, 51.56 mmol) in dry DMF (150 mL) was added anhydrous K_2CO_3 (21.45 g, 154.0 mmol) at 0 °C and the reaction was stirred for 15 min, and then BnBr (9.52 g, 56.00 mmol) was added. The reaction mixture was allowed to warm up and stirred for further 1h. After completion of the reaction, the mixture was diluted with EtOAc and washed with a cold brine solution. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by flash column chromatography (25% EtOAc : Hexane) to afford **10** (10.03 g, 50%) as a white solid.

R_f (30% EtOAc : Hexane) = 0.5.

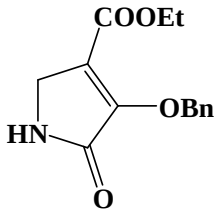
mp = 72-74 °C.

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.54-7.45 (m, 2H), 7.42-7.31 (m, 3H), 7.18 (d, *J* = 8.5 Hz, 2H), 6.88 (d, *J* = 8.5 Hz, 2H), 5.82 (s, 2H), 4.57 (s, 2H), 4.24 (q, *J* = 7.0 Hz, 2H), 3.86 (s, 2H), 3.82 (s, 3H), 1.30 (t, *J* = 7.0 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 164.9, 162.3, 159.3, 153.2, 136.7, 129.5 (× 2), 128.3 (× 2), 128.2, 128.0, 127.6 (× 2), 114.5, 114.2 (× 2), 72.5, 60.6, 55.2, 46.5, 46.2, 14.1ppm.

Elemental Analysis calcd. (%) for $C_{22}H_{23}NO_5$: C, 69.28; H, 6.08; N, 3.67. Found: C, 69.05; H, 6.10; N, 3.66.

5.6.2.2. *ethyl 4-(benzyloxy)-2,5-dihydro-5-oxo-1H-pyrrole-3-carboxylate.*



11

A stirred solution of **10** (5.00 g, 13.10 mmol) in ACN:Water (3:1) (40 mL) was added with Cerium ammonium nitrate (28.74 g, 52.43 mmol) at 0 °C, then it was stirred for 1h at room temperature. After the completion of reaction, the mixture was diluted with EtOAc and washed with water and brine solution. The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by flash column chromatography (50% EtOAc : Hexane) to afford **11** (2.50 g, 73%) as a white solid.

R_f (50% EtOAc : Hexane) = 0.3.

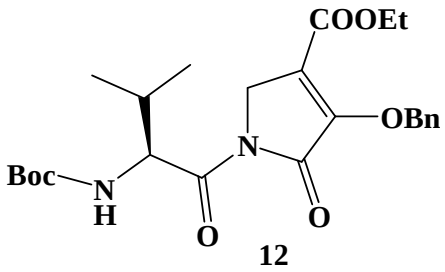
mp = 160-161 °C.

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.51-7.42 (m, 2H), 7.41-7.30 (m, 3H), 6.71 (brs, 1H), 5.77 (s, 2H), 4.29 (q, J = 7.0 Hz, 2H), 4.06 (s, 2H), 1.34 (t, J = 7.0 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 168.4, 162.4, 153.1, 136.5, 128.4 (× 2), 128.1, 127.6 (× 2), 116.8, 72.9, 60.7, 43.0, 14.1 ppm.

Elemental Analysis Calcd. (%) for $C_{14}H_{15}NO_4$: C, 64.36; H, 5.79; N, 5.36. Found: C, 64.60; H, 5.77; N, 5.35.

5.6.2.3 *4-Benzyloxy-1-(2-tert-butoxycarbonylamino-3-methylbutyryl)-5-oxo-2,5-dihydro-1H-pyrrole-3-carboxylic ethyl ester.*



12

To a stirred solution of **11** (1.01 g, 3.86 mmol) in anhydrous THF (30 mL) was added n-BuLi (1.6 M in hexane, 2.6 mL, 4.17 mmol) at -78 °C over 10 min. 2-*tert*-Butoxycarbonylamino-3-methyl-butyric acid pentafluorophenyl ester[5] (1.62 g, 4.25 mmol) in THF (2 mL) was then added, and the mixture was stirred for 30 min at the same temperature. The reaction was quenched by addition of sat. NH_4Cl and extracted with EtOAc (3 ×15 mL). The organic layer was dried over anhydrous Na_2SO_4, filtered and concentrated. The crude product was purified by flash column chromatography (12% EtOAc : Hexane) to afford **12** (6.05 g, 86%) as a colorless viscous oil.

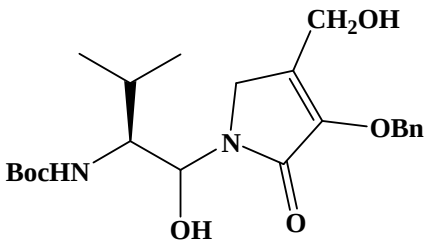
R_f (80% EtOAc : Hexane) = 0.5.

^{1}H NMR (600 MHz, $CDCl_3$) δ = 7.50-7.31 (m, 5H), 5.67 (s, 2H), 5.41 (m, 1H), 4.35 (AB, 1H), 4.31 (q, *J* = 7.0 Hz, 2H), 4.30 (AB, 1H), 4.14 (q, *J* = 7.0 Hz, 2nd rotamer, 2H), 2.15 (m, 1H), 1.46 (s, 9H), 1.34 (t, *J* = 7.0 Hz, 3H), 1.28 (t, *J* = 7.0 Hz, 2nd rotamer, 3H), 1.11 (d, *J* = 7.0 Hz, 3H), 0.84 (d, *J* = 7.0 Hz, 2nd rotamer, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) mixture of rotamers (major) δ = 172.5, 164.2, 161.7, 155.9, 150.8, 135.9, 128.6 (× 2), 128.5, 128.4 (× 2), 118.3, 79.8, 73.6, 61.3, 58.5, 44.6, 30.2, 28.2 (× 3), 19.8, 16.0, 14.1 ppm.

Elemental Analysis calcd. (%) for $C_{24}H_{32}N_2O_7$: C, 62.59; H. 7.00; N, 6.08. Found: C, 62.80; H. 7.02; N, 6.09.

5.6.2.4**.** *(1S, 1'S) {1-[(3-Benzyloxy-4-hydroxymethyl-2-oxo-2,5-dihydropyrrol-1-yl)-hydroxymethyl]-2-methyl-propyl}-carbamic acid tert-butyl ester.*



13

A solution of **12** (1.00 g, 2.17 mmol) in dry CH_2Cl_2 (20 mL) was added with diisobutylaluminium hydride (1.0 M in CH_2Cl_2, 5.42 mL, 5.42 mmol) at -78 °C and stirred for 2.5 h at the same temperature. After completion of reaction, the mixture was quenched by addition of sat. Rochelle salt (20 mL) and EtOAc (40 mL) was added. The thick slurry obtained was stirred vigorously till the two layers became transparent. The organic phase was separated and the aqueous phase was extracted with EtOAc (3 × 25 mL). The combined organic extracts were washed with brine (35 mL) and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo*. The crude product was purified by flash column chromatography (40% EtOAc : Hexane) to afford **13** (0.27 g, 30%) as a yellowish solid;

R_f (40% EtOAc : Hexane) = 0.3.

mp = 60-62 °C.

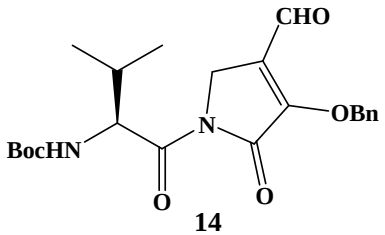
$[\alpha]_D^{23}$ = -0.52 (*c* 1.00, MeOH).

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.49-7.28 (m, 5H), 5.44 (dd, *J* = 4.6, 9.5 Hz, 1H), 5.25 (d, *J* = 11.3 Hz, 1H), 5.11 (d, *J* = 11.3 Hz, 1H), 4.62 (d, *J* = 11.0 Hz, 1H), 4.46 (d, *J* = 4.6 Hz, 1H), 4.31 (d, *J* = 14.5 Hz, 1H), 4.12 (d, *J* = 14.5 Hz, 1H), 4.04 (s, 2H), 3.77 (ddd, *J* = 3.5, 9.5, 11.0 Hz, 2H), 2.35 (brs, 1H), 2.28-2.15 (m, 1H), 1.33 (s, 9H), 0.97 (d, *J* = 6.7 Hz, 3H), 0.92 (d, *J* = 6.7 Hz, 3H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 168.3, 156.6, 143.4, 136.8, 133.9, 128.8 (× 2), 128.6, 128.4 (× 2), 79.8, 75.9, 73.2, 56.9, 56.2, 43.9, 28.5 (× 2), 28.4, 28.0, 20.3, 15.6 ppm.

Elemental Analysis calcd. (%) for $C_{22}H_{32}N_2O_6$, C 62.84, H 7.67, N 6.66%, Found C 62.93, H 7.65, N 6.67%.

5.6.2.5. *(1S) [1-(3-Benzyloxy-4-formyl-2-oxo-2,5-dihydropyrrole-1-carbonyl)-2-methyl-propyl]carbamic acid tert-butyl ester.*



14

To a stirred solution of compound **13** (0.22 g, 0.50 mmol) in CH_2Cl_2 (5 mL) PCC (1.12 g, 5.23 mmol) was added at 0 °C, then the solution was allowed to stir for 12 h at rt. After completion, the reaction mixture was filtered through a pad of celite and washed with CH_2Cl_2. The filtrate was concentrated *in vacuo*. The crude product was purified by flash column chromatography (20% EtOAc : Hexane) to afford **14** (0.100 g, 46%) as a colourless viscous oil.

R_f (20% EtOAc : Hexane) = 0.5.

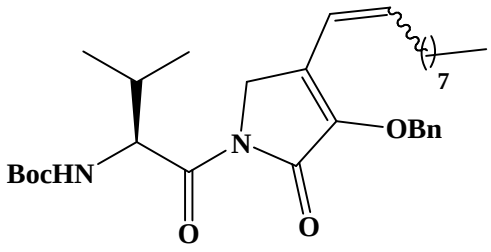
$[\alpha]_D^{23}$ = + 8.32 (*c* 1.00, MeOH).

^{1}H NMR (600 MHz, $CDCl_3$) δ = 10.14 (s, 1H), 7.51-7.35 (m, 5H), 5.73-5.60 (m, 2H), 5.39 (dd, *J* = 2.8, 9.5 Hz, 1H), 5.17 (d, *J* = 9.5 Hz, 1H), 4.39 (d, *J* = 18.9 Hz, 1H), 4.24 (d, *J* = 18.9 Hz, 1H), 2.19-2.05 (m, 1H), 1.44 (s, 9H), 1.09 (d, *J* = 7.1 Hz, 3H), 0.84 (d, *J* = 7.1 Hz, 3H) ppm.

^{13}C NMR (150 MHz, $CDCl_3$) δ = 185.3, 173.0, 164.6, 156.1, 153.8, 135.0, 129.3, 129.1, 128.4 (× 2), 125.0 (× 2), 80.1, 74.3, 58.7, 43.0, 30.5, 28.5 (× 3), 20.1, 16.3.

Elemental Analysis calcd. (%) for $C_{22}H_{28}N_2O_6$, C 63.45, H 6.78, N 6.73%. found: C 62.56, H 6.75, N 6.71%,

5.6.2.6. *(1S) [1-(3-Benzyloxy-4-dec-1-enyl-2-oxo-2,5-dihydro-pyrrole-1-carbonyl)-2-methylpropyl]carbamic acid tert-butyl ester.*

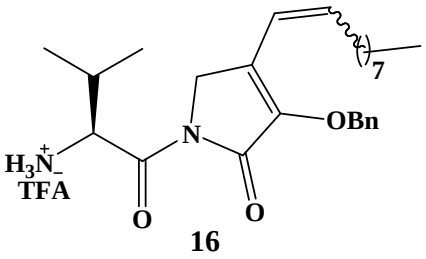


15

To an ice cold solution of (1-nonyl)triphenylphosphonium bromide (0.07 g, 0.14 mmol) in 3 mL of dry THF under nitrogen *n*-BuLi (1.6 M in hexane, 0.18 mL, 0.18 mmol) was added dropwise and the mixture was allowed stir for 45 min at the same temperature. The red solution was cooled to -78 °C and aldehyde **14** (0.03 g, 0.07 mmol) in anhydrous THF (2 mL) was added dropwise. The reaction was stirred for 1 h at -78 °C, warmed to 0 °C and quenched with sat. NH_4Cl (10 mL). The aqueous phase was extracted with EtOAc (2 × 15 mL). The combined organic extracts were washed with brine (10 mL) and dried over anhydrous Na_2SO_4, filtered and concentrated *in vacuo* to give crude **15** which was used for the next step without further purification and characterization.

$\mathbf{R_f}$ (10% EtOAc : Hexane) = 0.2.

5.6.2.7. *(1S) 1-(3-Benzyloxy-4-dec-1-enyl-2-oxo-2,5-dihydro-pyrrole-1-carbonyl)-2-methylpropyl ammonium trifluoro-acetate***.**

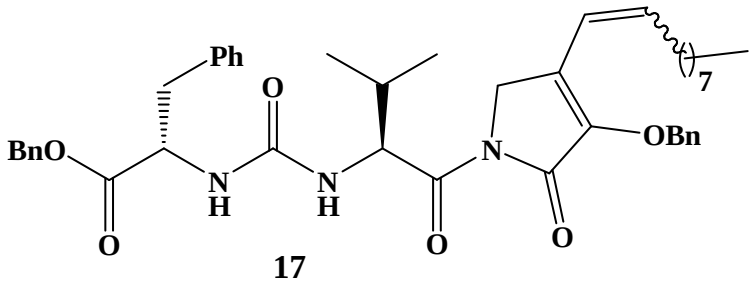


16

To a stirred solution of compound **15** (0.02 g, 0.04 mmol) in dry CH_2Cl_2 (1 mL) TFA (0.5 mL) was added at 0 °C. The reaction mixture was warmed to room temperature and stirred for 1 h. The reaction mixture was concentrated *in vacuo*. The residue was triturated with diethyl ether (3 ×5 mL) and dried to afford **16**. The crude product was used for the next step without further purification.

R_f (10% MeOH : CH_2Cl_2) = 0.2.

5.6.2.8. *(2S, 1'S) 2-{3-[1-(3-Benzyloxy-4-dec-1-enyl-2-oxo-2,5-dihydro-pyrrole-1-carbonyl)-2-methyl-propyl]ureido}-3-phenyl-propionic acid benzyl ester.*



17

A mixture of L-phenylalanine benzyl ester (0.02 g, 0.04 mmol) and diisopropylethylamine (0.01 g, 0.08 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise to a stirred solution of triphosgene (0.004 g, 0.013 mmol) in dry CH_2Cl_2 (2 mL) at rt. After 15 min. stirring, a solution of **16** (0.03 g, 0.05 mmol) and DIEA (0.02 mL, 0.13 mmol) in dry CH_2Cl_2 (2 mL) was added dropwise. The reaction mixture was stirred for 1 h at room temperature and the solvent was removed *in vacuo*. The residue was diluted with ethyl acetate (10 mL). The organic layer was washed with 10% aqueous $KHSO_4$ (5 mL), 5% aqueous $NaHCO_3$ (5 mL), brine (5 mL), dried over Na_2SO_4, filtered and concentrated *in vacuo*. The product was purified using PLC (35% EtOAc : Hexane) to give **17** (31 mg, 60% over three steps) as a white viscous liquid.

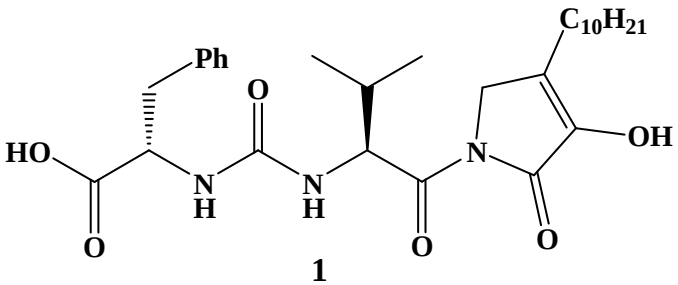
R_f (30% EtOAc : Hexane) = 0.4.

^{1}H NMR (300 MHz, $CDCl_3$) (mixture of stereoisomers) δ 7.53-6.88 (m,15H), 6.36 (d, *J* = 16.9 Hz, 1H), 6.15-5.97 (m, 2H), 5.82-5.20 (m, 4H), 5.16-4.77 (m, 3H), 4.02 (d, *J* = 19.1 Hz, 1H), 3.61 (d, *J* = 19.1 Hz, 1H), 3.20-2.90 (m, 2H), 2.21-2.07 (m, 3H), 1.78-1.63 (m, 2H), 1.52-0.64 (m, 19H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) (major stereoisomer) δ 174.0, 172.6, 165.7, 157.1, 141.3, 139.1, 137.0, 136.1, 135.4, 135.0, 129.7 (× 2), 128.8 (× 2), 128.75 (× 2), 128.7(× 2), 128.6 (× 2), 128.57 (× 2), 128.5 (× 2), 127.0, 119.5, 73.1, 67.1, 57.6, 53.8, 44.6, 39.3, 33.8, 32.1, 31.2, 29.6, 29.5, 29.46, 29.1, 22.9, 20.1, 16.6, 14.3 ppm.

Elemental Analysis calcd. (%) for $C_{43}H_{53}N_3O_6$, C 72.96, H 7.55, N 5.94%, found: C 72.83, H 7.56, N 5.95%.

5.6.2.9. *(2S, 1'S) 2-{3-[1-(4-Decyl-3-hydroxy-2-oxo-2,5-dihydro-pyrrole-1-carbonyl)-2-methyl-propyl]-ureido}-3-phenyl-propionic acid.*



1

To the solution of **17** (0.03 g, 0.04 mmol) in EtOAc (5 mL) was added a catalytic amount of 10% Pd/C (6 mg, 20% w/w). The reaction mixture was evacuated and flushed with H_2 gas (3 times), then stirred for 2h at rt under H_2 atmosphere. The reaction mixture was filtered through a celite pad and the pad was washed with EtOAc (3 ×5 mL). The filtrate was concentrated *in vacuo*. The crude product was purified by reverse phase PLC (25% H_2O : MeOH) to afford **1** (0.02 g, 77 %) as a clear oil.

R_f (25% H_2O : MeOH) = 0.4.

^{1}H NMR (600 MHz, DMSO-d_6) δ = 12.62 (s, 1H), 9.42 (s, 1H), 7.27 (dd, *J* = 7.6, 7.4 Hz, 2H), 7.21 (dd, *J* = 7.6, 7.6 Hz, 1H), 7.19 (d, *J* = 7.4 Hz, 2H), 6.47 (d, *J* = 9.2 Hz, 1H), 6.34 (d, *J* = 8.0 Hz, 1H), 5.37 (1H, dd, *J* = 9.2, 3.7 Hz, 1H), 4.28 (m, 1H), 4.10 (d, *J* = 18.0 Hz, 1H), 4.00 (d, *J* = 18.0 Hz, 1H), 2.99 (dd, *J* = 13.7, 5.1 Hz, 1H), 2.85 (dd, *J* = 13.7, 7.5 Hz, 1H), 2.31 (m, 2H), 2.00 (m, 1H), 1.54-1.10 (m, 16H), 0.91 (d, *J* = 6.9 Hz, 3H), 0.86 (t, *J* = 6.4 Hz, 3H), 0.76 (d, *J* = 6.9 Hz, 3H) ppm.

^{13}C NMR (150 MHz, DMSO-d_6) δ = 174.2, 172.7, 166.5, 157.9, 141.4, 138.0, 129.8 (× 2), 129.1, 128.6 (× 2), 126.8, 57.1, 54.6, 46.7, 38.0, 31.8, 30.3, 29.5 (× 2), 29.4 (× 2), 29.2, 27.3, 25.3, 22.6, 20.2, 16.8, 14.5 ppm.

Elemental Analysis calcd. (%) for $C_{29}H_{43}N_3O_6$, C 65.76, H 8.18, N 7.93%, Found: C 65.84, H 8.16, N 7.91%.

5.7. REFERENCES:

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2) Raju, R.; Gromyko, O.; Fedorenko, V.; Luzhetskyy, A.; Muller, R. *Org. Lett.* **2013**, *15(14)*, 3487.
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CHAPTER-6

TOTAL SYNTHESIS OF RESORMYCIN

6.1. INTRODUCTION:

In 1997, Takeuchi *et. al.* while screening *Streptomyces platensis* MJ953-SF5 isolated from the soil collected at Yokohama kawakawa, Japan, isolated a novel tripeptide cinnamic acid-containing metabolite, resormycin[1] (**1**). The molecule showed remarkable growth inhibition of monocotyledonous and dicotyledonous weeds. The metabolites of cinnamic acid such as 3,5-dihydroxy cinnamic acid,[2] raphanusols,[3] lespedazole[4] from plants are known for their plant growth regulatory activity but they are not reported for the herbicidal activity. Moreover, resormycin also inhibits the growth of phytopathogenic fungi.

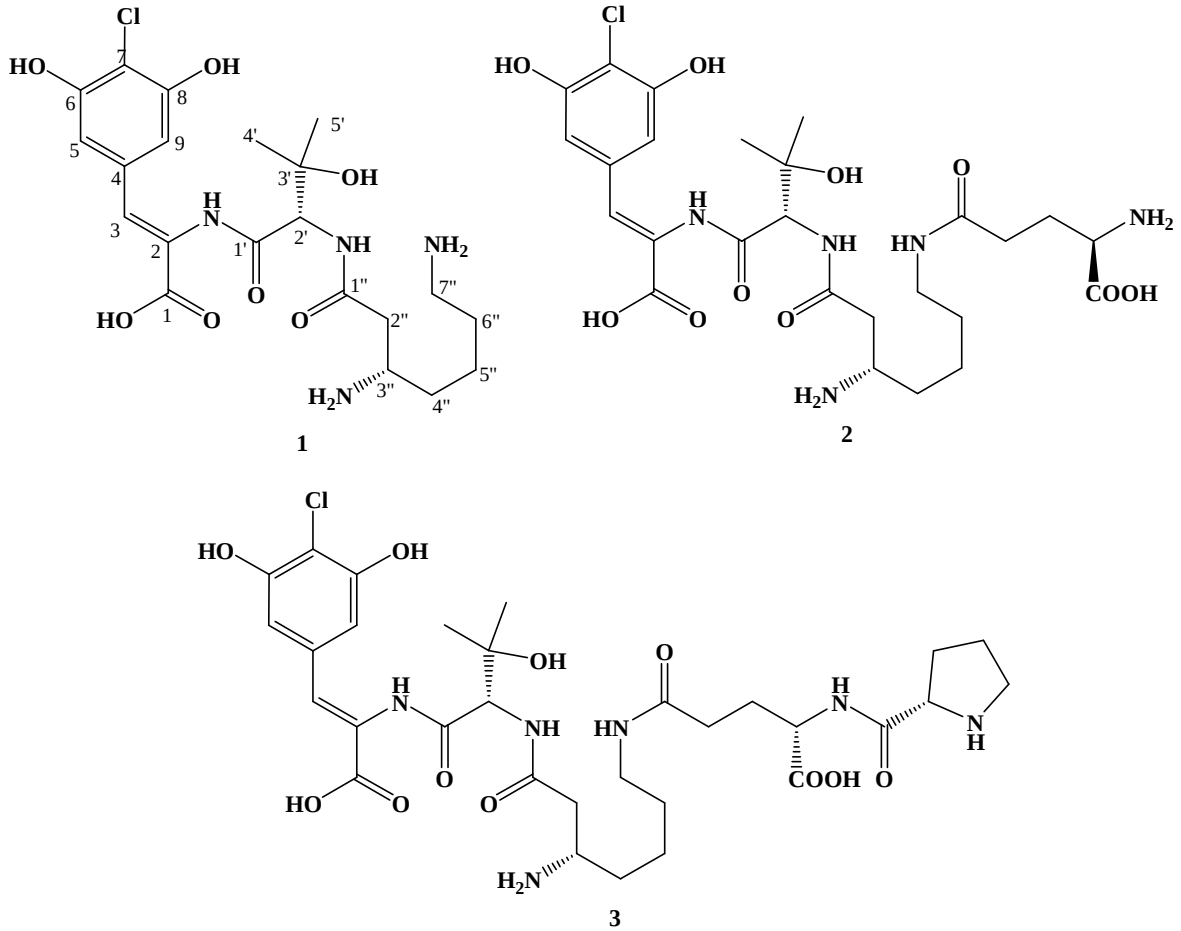


Figure-1: Structures of Resormycin (**1**), Androprostamine A (**2**), Androprostamine B (**3**).

The molecule is composed of three rare unnatural amino acid residues, hydroxyvaline, *β*-homolysine and an unusual chlorinated resorcyl-2,3-dehydro propenoic amino acid at C terminus.[5] The structure of the molecule was elucidated by spectroscopic techniques. The resorcyl fragment is attached to the di-peptide unit via a *Z*-alkene. In 2015, Momose *et. al.* isolated two novel peptide metabolites androprostamine A (**2**) and B (**3**) produced by *Streptomyces Sp.* MK932-CF8.[6] The compounds **2** and **3** display anti-prostate cancer activity by inhibiting the function of androgen receptor. The prostate cancer is very prevalent among men in western countries and is a second leading reason for male fatalities in USA.[7] Furthermore, in developing countries changes in life style and food habits have marked significant increase of prostate cancer cases. The androprostamine A (**2**) and B (**3**) share the tripeptide backbone core with resormycin.

The intriguing biological profile of resormycin spanning from herbicidal, fungicidal to anticancer activity and the distinctive structural architect makes it an interesting synthetic target, which could serve as lead structure for the development of new active compounds.

To explore the therapeutic potential of resormycin (**1**), we planned to develop a convergent synthetic approach, which in principle could be modulated for the generation of analogues to perform structure-activity relationship (SAR) studies. The synthetic strategy developed for resormycin can also be extended for the synthesis of androprostamine A (**2**) and B (**3**). The strong interest in these molecules is confirmed by the recent synthesis of resormycin (**1**) and androprostamine A (**2**) by Shibasaki *et. al.*[8] reported in the literature during the last part of this work.

6.2. SHIBASAKI APPROACH:

The Shibasaki strategy (Figure-2) is based on the use of Horner-Wadsworth-Emmons (HWE) olefination as a key reaction to install the 2,3-dehydroamino acid moiety, but the olefination step proceeds with poor yield and *E/Z* selectivity. The synthesis of resormycin is achieved in 10 steps with overall yield of 3%. The synthetic route involves some unstable intermediates, poor yielding synthetic steps and it also lacks use of orthogonal protecting groups which is essential to develop substantial structure activity relationship studies.

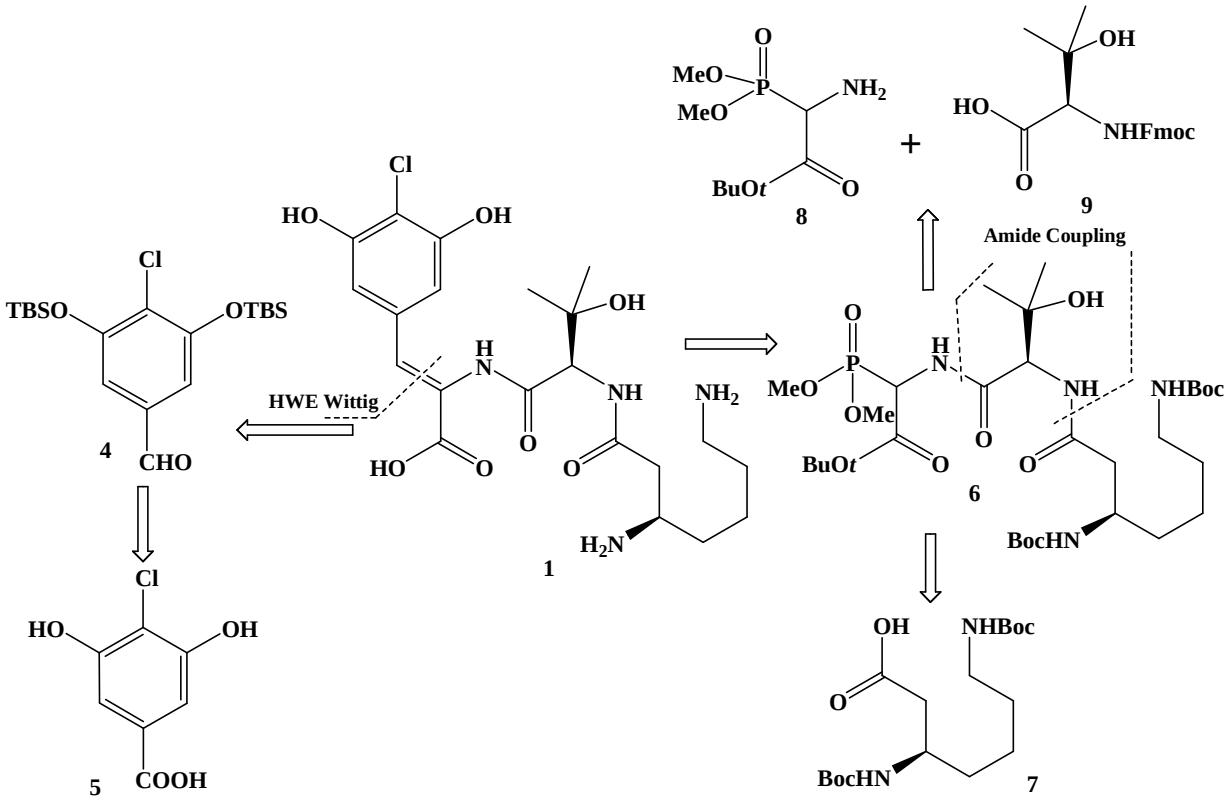


Figure-2: Shibasaki approach.

6.3. PRESENT PLAN:

Our retrosynthetic plan is depicted in Figure-3. We envisioned that the *Z*-olefin could be obtained by *Z*-selective elimination of alcohol **10.** The advantage of this method over HWE olefination is to selectively obtain the *Z*-olefin without the formation of undesired *E*-isomer under neutral condition. The aldol condensation of the *N*-(diphenylmethylene)glycine *tert*-butyl ester with appropriately protected aldehyde is expected to furnish amino alcohol **11**. The amino alcohol in turn could be produced starting from 4-chloro-3,5-dihydroxybenzoic acid. The acid (-)-**12** could be synthesized by coupling of NHS ester of protected β-homolysine with the hydroxyvaline **13**. The di-alloc protected NHS ester can be smoothly obtained starting from β-homolysine **14**.

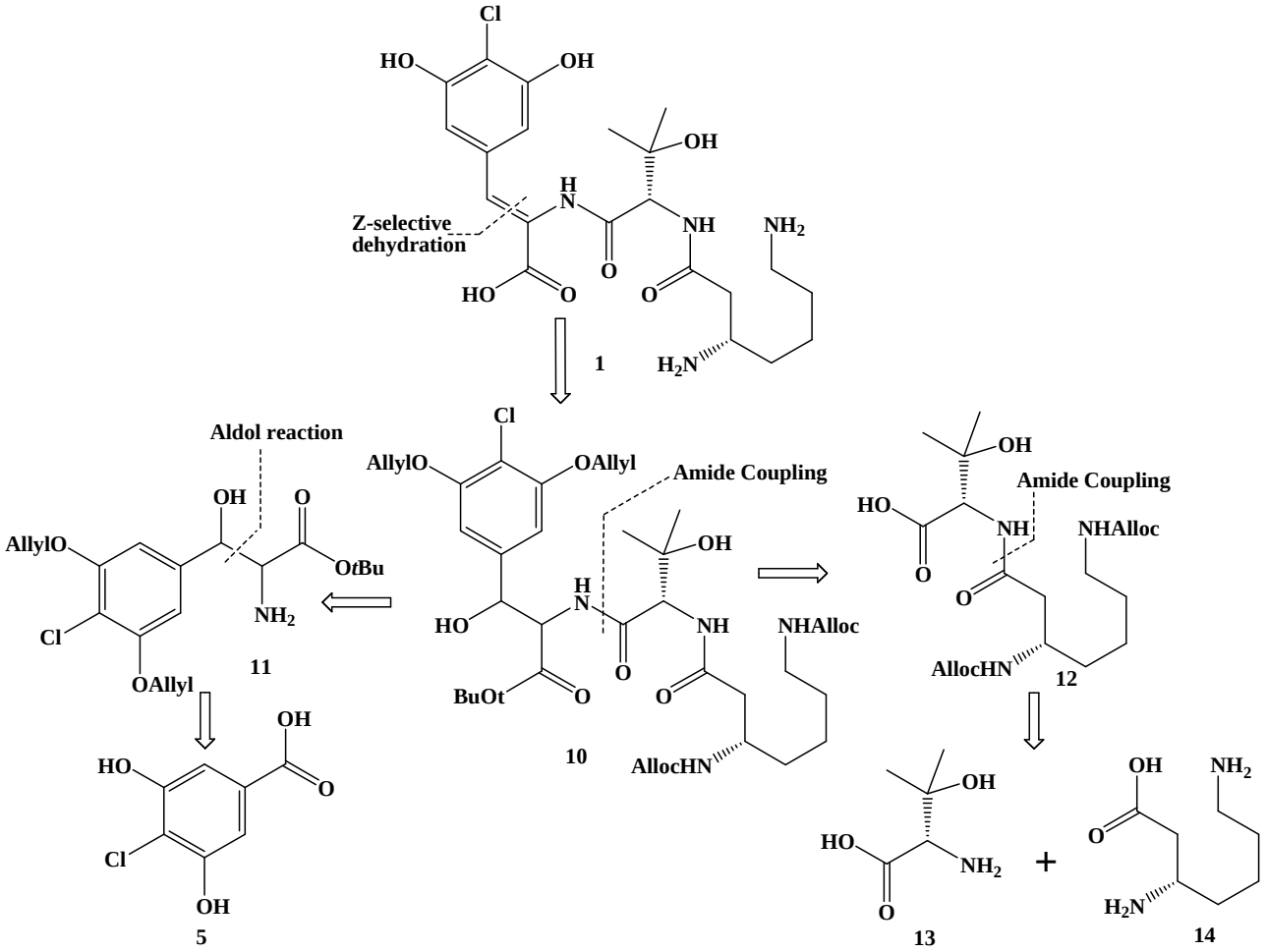
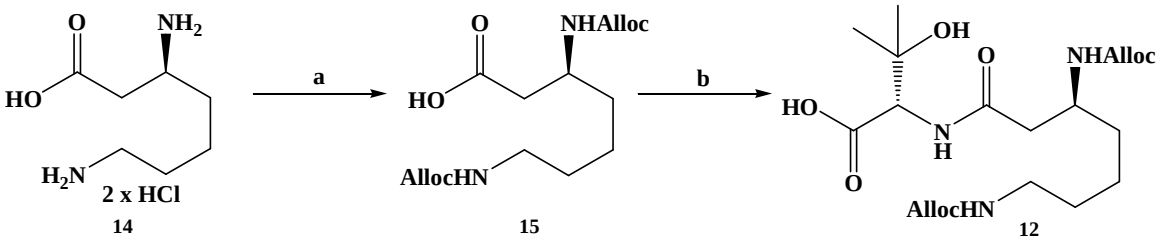


Figure-3: Our retrosynthetic approach to resormycin.

6.4. SYNTHESIS OF DIPEPTIDE 12:

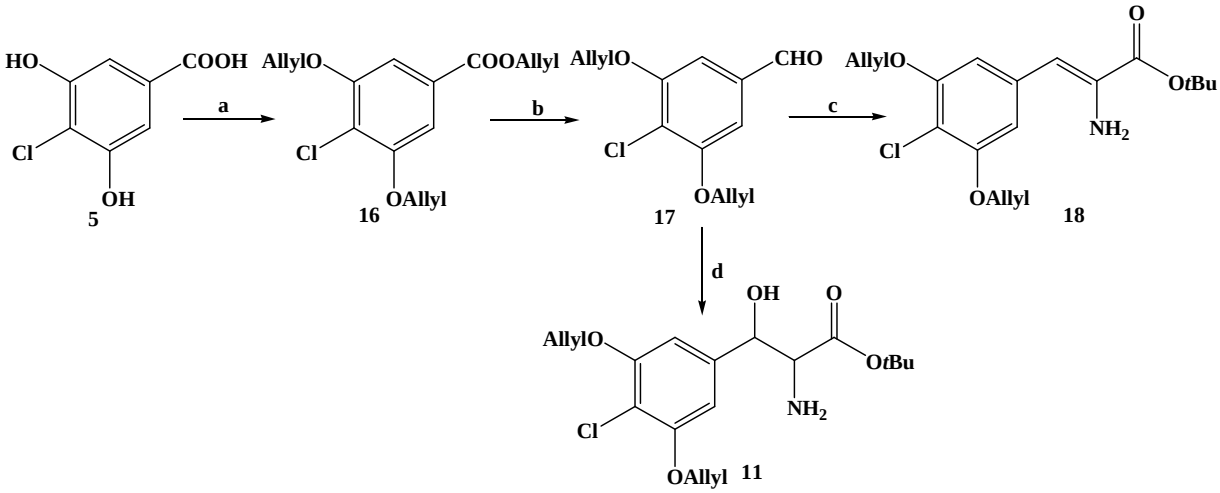
The shortest approach for the synthesis of dipeptide (-)-**12** is the amide coupling of NHS ester of di-Alloc-*β*-homolysine with the hydroxyvaline **13.** Thus, the di-alloc protection of *β*-homolysine **14** using allyl chloroformate and K_2CO_3 in H_2O afforded di-Alloc-β-homolysine (-)-**15** in 73% yield.[9] (Scheme 1). The treatment of compound **(-)-15** with N-hydroxysuccinimide and EDC·HCl in DMF cleanly furnished NHS ester.[10] The NHS ester obtained was pure enough and was used immediately upon water workup. The coupling of NHS ester with hydroxyvaline in dioxane: water using NEt_3 produced acid (-)-**12** in 86% yield over two steps.[6] The acid was pure enough and was used for the next step without purification.



Scheme-1: Synthesis of dipeptide acid; Reagents and condition: a) Allyl chloroformate, K_2CO_3, H_2O, 0 °C to rt, 16 h, 73%; b) i) NHS, EDC-HCl, DMF, rt 16 h, ii) (*S*)-2-amino-3-hydroxy-3-methylbutanoic acid, NEt_3, dioxane : H_2O (2:1), rt, 2 h, 86% over two steps.

6.5. SYNTHESIS OF THE ARYL CORE:

The synthesis of the aryl core is depicted in scheme-2. The shortest and easiest approach for the synthesis of aldehyde **17** was the regioselective chlorination of 3,5-dihydroxybenzaldehyde at C-4 position. Initially several attempts using NCS/PTSA/NaCl,[11] NaOCl/KOH,[12] NCS/AcOH,[13] NCS/MeOH,[14] NaOCl/piperidine[15] were tried for the selective chlorination of 3,5-dihydroxybenzaldehyde to give 4-chloro-3,5-dihydroxybenzaldehyde but the results were not encouraging in all the cases either chlorination went exclusively at C-2 position or there was no product formation at all. The chlorination using NaOCl/KOH[12] gave formation of multiple spots on TLC. The chlorination of 3,5-bis(allyloxy) benzaldehyde also did not gave the regioselective C-4 chlorination. After several futile attempts we decided to begin with the synthesis of aldehyde **17** starting from commercially available 4-chloro-3,5-dihydroxy benzoic acid. The triallyation of acid **5** using K_2CO_3/DMF[16] at room temperature gave the ester **16** in 86% yield. The ester **16** was subjected to reduction using LAH at 0 °C in dry diethyl ether, but the reaction gave incomplete conversion with formation of impurities. The treatment of ester **16** with DIBAL-H in CH_2Cl_2 at -78 °C cleanly furnished the benzyl alcohol which was used without further purification. The crude benzyl alcohol was oxidized using PCC/NaOAc system in CH_2Cl_2 giving the aldehyde **17** in 80% yield over two steps.[17] Having the aldehyde **17** in hand we decided to use the Zu's protocol[18] i.e. the condensation of aldehyde **17** with glycine tertiary butyl ester using Cs_2CO_3 in NMP at 100 °C which selectively gave the title enamine **18** with *Z*-stereochemistry.

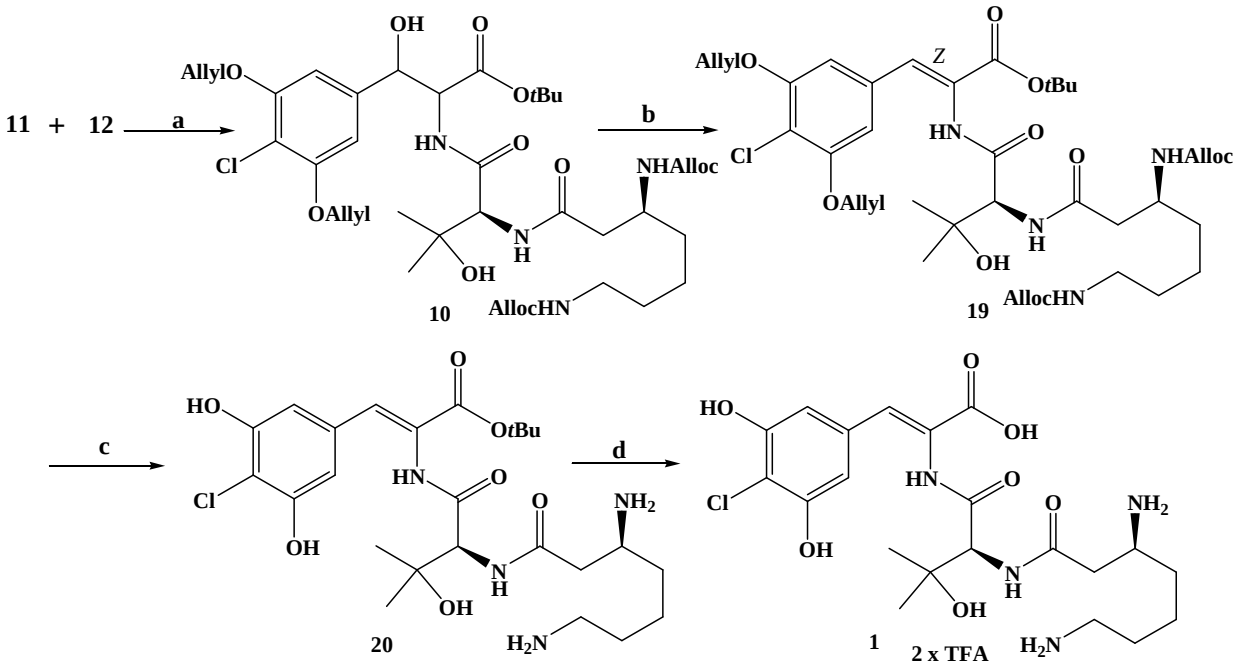


Scheme-2: Synthesis of aryl core; Reagents and condition: a) Allyl bromide, K_2CO_3, DMF, 0 °C to rt, 2 h, 86 %; b) i) DIBAL-H, CH_2Cl_2, -78 °C, 1 h, ii) PCC, NaOAc, 10 °C- rt , 2 h, 80% over two steps c) Cs_2CO_3, glycine tert-butyl ester hydrochloride, NMP,100 °C, 6 h. 51%; d) i) DIPA, *n*-BuLi, THF, 0 °C, 30 min, then *N*-(Diphenylmethylene)glycine-*tert*-butyl ester in THF, -78 °C, 30 min; ii) TMS-Cl, -78 °C-rt over 1h, then Cat. $ZnCl_2$ and **17** in THF, rt, 2 h, iii) 10 % citric acid, rt, 16 h, 81%.

Unfortunately, the coupling of enamine **18** with acid (-)-**12** using HOBt/EDCI, HATU, HBTU, Ghosez reagent, oxalyl chloride and mix anhydride using ethyl chloroformate did not give the formation of amide product. The most plausible explanation for the unreactivity of enamine **18** under standard coupling conditions may be the poor nucleophilicity of the amine. The unsatisfactory results made us to change our synthetic strategy. Alternatively, we planned to convert the aldehyde **17** into corresponding amino alcohol, which can be readily coupled to the carboxylic acid counter parts. Thus, LDA was generated using DIPA and *n*-BuLi in THF at 0 °C, and was added to the THF solution of *N*-(diphenylmethylene)glycine *tert*-butyl ester at -78 °C. The anion was quenched to generate the TMS enolate, which was condensed with the aldehyde **17** in presence of cat. $ZnCl_2$ to furnish the O-TMS- *N*-(diphenylmethylene)glycine *tert*-butyl ester imine intermediate. The intermediate obtained was cleaved using 10% citric acid to produce amino alcohol **11** in excellent yields.[29]

The amide coupling of amino alcohol **11** and acid **12** using HBTU/DIPEA and HBTU/NMM gave the amide **10** in 50% and 61% yield, respectively (scheme 3). The *O*-acylation was the major side reaction with DIPEA and NMM as base. The best results were obtained when DMAP was employed as a base furnishing the amide **10** in 73% yield. The stereospecific elimination of alcohol **10** using DAST/pyridine[20] and DAST/NEt_3 combination did not give good results. The O-DAST adduct formed did not undergo elimination at room temperature. The replacement of DAST with Martin sulfurane led to the formation of

Z-alkene **19** in 60% yields.[21] The one pot-deprotection of allyl and alloc groups using $Pd(PPh_3)_4$ catalyst in combination with N-methyl aniline and dimedone[22] as an allyl scavenger gave the partially deprotected compound. The palladium catalyzed hydrostanylation of alloc and allyl groups using $SnBu_3H$ and AcOH in CH_2Cl_2 results in clean deprotection to furnish diamine **20** in 60% yield.[23]



Scheme-3: Synthesis of resormycin; Reagents and condition: a) HBTU, DMAP, DMF, 0 °C for 2 h then at rt for 1 h. 73%, b) Martin sulfurane, CH_2Cl_2, 0 °C to rt, 16 h, 60 %; c) $SnBu_3H$, AcOH, $PdCl_2(PPh_3)_2$, CH_2Cl_2, rt, 12 h, 66 %; d) TFA, CH_2Cl_2, 0 °C to rt, 2 h, 97%.

The diamine **20** was treated carefully with TFA in CH_2Cl_2 at 0 °C to furnish the title compound resormycin.

6.6. CONCLUSIONS:

In conclusion, we have designed and accomplished the total synthesis of resormycin in longest linear sequence of 8 steps with overall yield of 16%. The key steps in our synthetic approach include the late stage stereospecific dehydration of alcohol to install the *Z*-olefin and palladium catalyzed one-pot deprotection of allyl and alloc groups. The current synthetic strategy gains some advantages over the Shibasaki approach[8] in terms of improved yields and orthogonality of protecting groups. The orthogonal nature and simplified synthetic approach makes it amenable for easy modifications of synthetic routes to generate the diverse analogues for biological investigation.

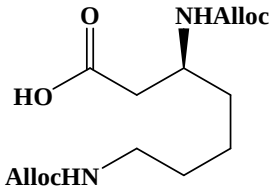
6.7. EXPERIMENTAL SECTION

6.7.1. GENERAL INFORMATION:

As per chapter 3.3.1**. GENERAL INFORMATION.**

6.7.2: EXPERIMENTAL PROCEDURES AND SPECTROSCOPIC DATA:

6.7.2.1. *(S)-3,7-bis-allyloxycarbonylaminoheptanoic acid.*



15

To a solution of *β*-homolysinedihydrochloride (320 mg, 1.37 mmol) in H_2O (2 mL) at 0 °C, a cold solution of K_2CO_3 (948 mg, 6.86 mmol) in H_2O (2 mL) followed by allyl chloroformate (0.36 mL, 3.432 mmol) were added dropwise. After complete addition, reaction mixture was stirred at room temperature for 16 h. The reaction mixture was cooled to 0 °C and acidified using 2N HCl and the aqueous layer was extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with brine (7 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The purification using flash column chromatography in 7-10% MeOH : CH_2Cl_2 yielded compound (-)-**15** (330 mg, 73%) as a colorless solid.

R_f (10 % MeOH : CH_2Cl_2) = 0.4.

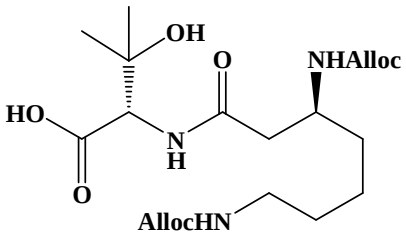
mp = 77-78 °C.

$[\alpha]_D^{23}$ = -12.8 (*c* 1.15, $CHCl_3$)

^{1}H NMR (300 MHz, CD_3OD) δ = 5.95-5.85 (m, 2H), 5.28 (dd, *J* = 17.1, 1.4 Hz, 2H), 5.16 (dd, *J* = 10.5, 1.4 Hz, 2H), 4.53-4.48 (m, 4H), 4.02-3.84 (m, 1H), 3.09 (t, *J* = 6.7 Hz, 2H), 2.44 (dd, *J* = 6.9, 2.0 Hz, 2H), 1.66-1.27 (m, 6H) ppm.

^{13}C NMR (75 MHz, CD_3OD) δ = 175.2, 158.8, 158.2, 134.6, 134.5, 117.3 (× 2C), 66.2 (× 2C), 49.4, 41.5, 40.9, 35.3, 30.6, 24.2 ppm.

6.7.2.2. *(S)-2-((S)-3,7-bis(((allyloxy)carbonyl)amino)heptanamido)-3-hydroxy-3-methylbutanoic acid.*



12

To a stirred solution of compound (-)-**15** (508 mg, 1.548 mmol) in dry DMF (5.1 mL, 0.3 M), N-hydroxysuccinimide (409 mg, 3.562 mmol) and EDC·HCl (682 mg, 3.562 mmol) were sequentially added under N_2 atmosphere. The clear solution obtained was stirred overnight at room temperature. The reaction mixture was poured in ice water (30 mL) and the aqueous layer was extracted with ethyl acetate (2 × 10 mL). The combined organic extracts were washed with cold brine (5 × 5 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to give NHS ester (785 mg). The crude NHS ester was used further without purification. To a solution of (*S*)-2-amino-3-hydroxy-3-methylbutanoic acid (269 mg, 2.02 mmol) in dioxane: H_2O (26.6 mL, 1:1 ratio) was added NEt_3 (0.64 mL, 4.60 mmol) followed by dropwise addition of solution of above NHS ester (785 mg, 1.84 mmol) in dioxane (13.4 mL). The resultant solution was stirred at room temperature for 2 h. The solvent was removed *in vacuo* and diluted with aq. $NaHCO_3$ (5 mL) and the aqueous layer was washed with ethyl acetate (2 ×10 mL). The aqueous layer was cooled to 0 °C and acidified using 2N HCl, then it was extracted with ethyl acetate (2 × 20 mL), washed with brine (10 mL) and dried over anhydrous Na_2SO_4. The solvent was concentrated *in vacuo* to yield (-)-**12** (595 mg, 86%) as a pale yellow solid.

R_f (10 % MeOH : CH_2Cl_2) = 0.1.

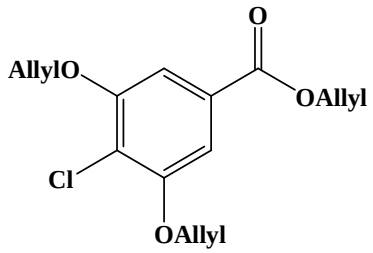
mp = 63-65 °C.

$[\alpha]_D^{23}$ = -18.4 (*c* 1.0, $CHCl_3$).

^{1}H NMR (300 MHz, CD_3OD) δ = 6.00-5.83 (m, 2H), 5.28 (dd, *J* = 17.4, 1.8 Hz, 2H), 5.20-5.13 (m, 2H), 4.53-4.47 (m, 4H), 4.42 (s, 1H), 3.95-3.91 (m, 1H), 3.09 (t, *J* = 6.4 Hz, 2H), 2.45 (dd, *J* = 6.9, 2.6, 2H), 1.68-1.29 (m, 6H), 1.29 (s, 3H), 1.26 (s, 3H) ppm.

^{13}C NMR (75 MHz, CD_3OD) δ = 178.2, 173.3, 158.7, 158.3, 134.6, 134.5, 117.5, 117.4, 73.4, 66.4, 66.2, 62.7, 49.9, 42.8, 41.5, 35.5, 30.6, 27.8, 26.1, 24.1 ppm.

6.7.2.3. *allyl 3,5-bis(allyloxy)-4-chlorobenzoate.*



16

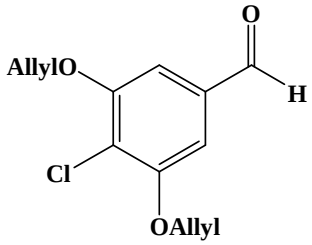
A suspension of 4-chloro-3,5-dihydroxy benzoic acid **5** (0.50 gm, 2.65 mmol) and anhydrous K_2CO_3 (2.50 gm, 18.55 mmol) in dry DMF (5 mL, 0.5 M) was stirred for 10 minutes and cooled to 0 °C. Ally bromide (0.80 ml, 9.28 mmol) was added dropwise at 0 °C and the mixture was stirred for 1 h at room temperature. Then, the reaction mixture was poured in ice water (25 mL) and the aqueous layer was extracted with diethyl ether (2 × 10 mL); the combined organic extracts were washed with brine (5 mL) and dried over anhydrous Na_2SO_4. The solvent was removed *in vacuo*. Purification using flash column chromatography in 3% EtOAc : petroleum ether afforded compound **16** (650 mg, 79 %) as a colorless oil.

R_f (5 % EtOAc : petroleum ether) = 0.3.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.29 (s, 2H), 6.18-5.94 (m, 3H), 5.53-5.28 (m, 6H), 4.82 (dt, J = 5.9, 1.3 Hz, 2H), 4.67 (dt, J = 5.2, 1.6 Hz, 2H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 165.7, 155.0 (×2), 132.4 (×2), 132.2, 128.9, 118.7, 118.3 (×3), 107.3 (×2), 70.1 (×2), 66.1 ppm.

6.7.2.4. *3,5-bis(allyloxy)-4-chlorobenzaldehyde.*



17

DIBAL-H (4.30 mL, 1 M in CH_2Cl_2, 4.30 mmol) was added dropwise at -78 °C under N_2 atmosphere to a stirred solution of allyl 3,5-bis(allyloxy)-4-chlorobenzoate **16** (532 mg, 1.72 mmol) in CH_2Cl_2 (11.4 mL, 0.15 M). After complete addition, the reaction mixture was stirred at -78 °C for 1 h. A solution of aq. Saturated Rochelle's salt (1 mL) was added followed by 2N HCl (5 mL), the reaction mixture was stirred vigorously till two layers became clear. The organic layer was separated and the aqueous layer was extracted with dichloromethane (10 mL); the combined organic extracts were washed with brine (6 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford 418 mg of benzyl alcohol as colorless oil.

To a stirring suspension of PCC (709 mg, 3.29 mmol) and NaOAc (67 mg, 0.82 mmol) in dry dichloromethane (3 mL) cooled to 10 °C, benzyl alcohol (418 mg, 1.64 mmol) in dichloromethane (5 mL) was added dropwise. The reaction mixture was stirred 2 h at room temperature. *In vacuo* concentration followed by flash column chromatography furnished the 3,5-bis(allyloxy)-4-chlorobenzaldehyde **17** (346 mg, 80 % over two steps) as a white solid.

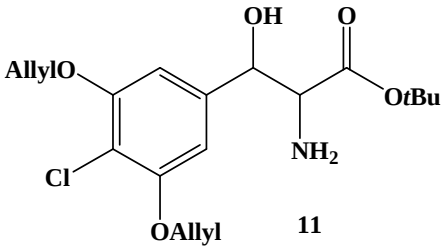
R_f (5 % EtOAc : petroleum ether) = 0.2.

mp = 88-89 °C

^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.88 (s, 1H), 7.09 (s, 2H), 6.15-5.99 (m, 2H), 5.55-5.43 (m, 2H), 5.37-5.31 (m, 2H), 4.71-4.67 (m, 4H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) δ = 191.2, 156.1 (× 2C), 135.2, 132.3 (× 2C), 118.6 (×3C), 107.0 (× 2C), 70.3 (× 2C) ppm.

6.7.2.4. *tert-butyl 2-amino-3-(3,5-bis(allyloxy)-4-chlorophenyl)-3-hydroxypropanoate.*



11

LDA generation: *n*-BuLi (1.3 mL, 1.6 M, 1.37 mmol) was added dropwise to a stirred solution of diisopropyl amine (0.20 mL, 1.43 mmol) in anhydrous THF (4 mL) at 0 °C under N_2 atmosphere. The pale yellow solution obtained was stirred at 0 °C for 30 minute.

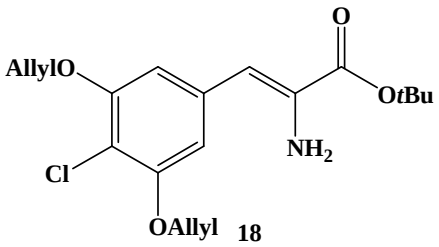
The above generated LDA solution was added dropwise to a solution of tert-butyl 2-(diphenylmethyleneamino)acetate (0.37 gm, 1.25 mmol) in THF (4 mL) cooled to -78°C under N_2 atmosphere. The wine red solution obtained was stirred at -78 °C for 30 minutes. TMS-Cl (0.47 mL, 3.75 mmol) was added dropwise (color changes from wine red to pale yellow); after complete addition the reaction mixture was warmed to room temperature over 1 h. The yellow solution obtained was added dropwise to a stirred solution of **17** (0.316 g, 1.25 mmol) and $ZnCl_2$ (0.16 mL, 0.16 mmol) in dry THF (4 mL) at room temperature and stirred for 2 h. The reaction mixture was cooled to 0 °C and quenched by dropwise addition of 10% citric acid (6 mL), after complete addition, the reaction mixture was stirred overnight at room temperature. THF was removed *in vacuo* and the aqueous layer was cooled to 0 °C and basified using saturated aq. $NaHCO_3$ and extracted with ethyl acetate (2 × 25 mL). The combined organic extracts were washed with brine (15 mL) and dried over anhydrous Na_2SO_4 and the solvent was removed *in vacuo*. The crude was purified using flash column chromatography in 0.5% MeOH: CH_2Cl_2 to give **11** (0.393 gm, 81%) as a colorless oil.

R_f (2 % MeOH: CH_2Cl_2) = 0.25.

^{1}H NMR (300 MHz, $CDCl_3$) major stereoisomer δ = 6.61 (s, 2H), 6.15-5.98 (m, 2H), 5.551-5.42 (m, 2H), 5.34-5.26 (m, 2H), 4.72 (d, *J* = 4.9 Hz, 1H), 4.65-4.58 (m, 4H), 3.49 (d, *J* = 4.9 Hz, 1H), 1.40 (s, 9H) ppm.

^{13}C NMR (75 MHz, $CDCl_3$) major stereoisomer δ = 172.2, 155.2 (x 2C), 140.7, 132.7 (x 2C), 118.0 (x 3C), 104.5 (x 2C), 82.2, 74.2, 70.0 (x 2C), 60.9, 28.0 (x 3C) ppm.

6.7.2.4. *tert-butyl (Z)-2-amino-3-(3,5-bis(allyloxy)-2-chlorophenyl)acrylate.*



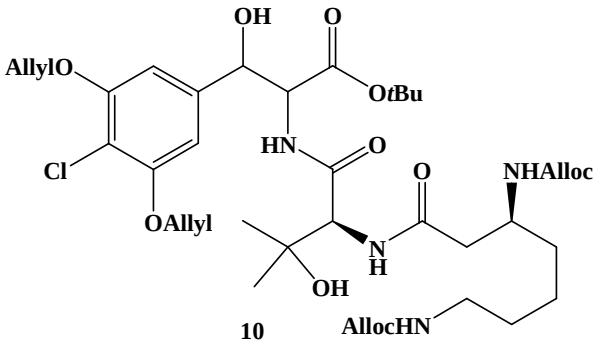
18

To a stirring suspension of glycine *tert*-butyl ester hydrochloride (29.8 mg, 0.178 mmol) and Cs_2CO_3 (76.8 mg, 0.236 mmol) in N-methylpyrrolidinone (0.2 mL), a solution of 3,5-bis(allyloxy)-4-chlorobenzaldehyde **17** (30 mg, 0.118 mmol) in N-methyl pyrrolidinone (0.6 mL) was added dropwise. The reaction mixture was stirred at 100 °C for 5 h. The reaction mixture was poured in cold water (2 mL) and the aqueous layer was extracted with ethyl acetate (2 × 5 mL). The combined organic extracts were washed with brine (10 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using flash column chromatography in 3% ethyl acetate: hexane to afford enamine **18** (22 mg, 51%) as colorless oil.

R_f (5 % EtOAc : Hexane) = 0.3.

^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.26 (s, 1H), 6.35 (s, 2H), 5.99-5.83 (m, 2H), 5.39-5.26 (m, 2H), 5.26-5.15 (m, 2H), 4.44-4.39 (m, 4H), 1.39 (s, 9H) ppm.

6.7.2.5. *tert-butyl(5S,9S)-9-(((allyloxy)carbonyl)amino)-2-((3,5-bis(allyloxy)-4-chlorophenyl)(hydroxy)methyl)-5-(2-hydroxypropan-2-yl)-4,7,15-trioxo-16-oxa-3,6,14-triazanonadec-18-enoate.*



10

A stirred solution of acid **12** (201 mg, 0.453 mmol) and amino alcohol **11** (190 mg 0.498 mmol) in dry DMF (4.5 mL, 0.1 M) was cooled to 0 °C. HBTU (188.8 mg, 1.583 mmol) and DMAP (60.8 mg, 0.498 mmol) were sequentially added at 0 °C under N_2 atmosphere. The reaction mixture was stirred at 0 °C for 2 h and then 1 h at room temperature. The reaction mixture was poured into cold water (30 mL) and the aqueous layer was extracted with ethyl acetate (2 x 15 mL). The combined organic extracts were washed with brine (10 mL), dried over anhydrous Na_2SO_4 and concentrated *in vacuo*. The residue was purified using column chromatography in 1.5 to 2.5 % MeOH : CH_2Cl_2 to afford amide **10** (270 mg, 73%, mixture of rotamers and conformers) as a white solid.

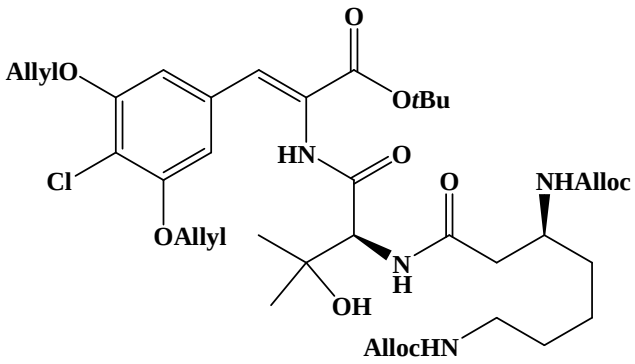
R_f (3 % MeOH : CH_2Cl_2) = 0.25.

mp = 61-62 °C

^{1}H NMR (300 MHz, CD_3OD) Major diastereomer δ = 6.77 (s, 2H), 6.17-6.00 (m, 2H), 5.98-5.83 (m, 2H), 5.54-5.43 (m, 2H), 5.34-5.11 (m, 6H), 4.74-4.37 (m, 10 H), 3.98-3.80 (m, 1H), 3.15-3.05 (m, 2H), 2.41 (d, *J* = 7.0 Hz, , 2H), 1.60-1.39 (m, 15H) ppm.

^{13}C NMR (75 MHz, CD_3OD) Major diastereomer δ = 173.2, 172.5, 172.2, 170.6, 170.4, 156.3 (×2), 142.4, 134.4 (×4), 117.6 (×4), 117.4, 105.3 (×2), 83.2, 73.6, 72.9, 70.7 (×4), 66.2, 61.2, 60.4, 50.0, 42.4, 41.5, 35.2, 30.5, 28.3 (×5) ppm.

6.7.2.6. *tert-butyl (5S,9S)-9-(((allyloxy)carbonyl)amino)-2-((Z)-3,5-bis(allyloxy)-4-chlorobenzylidene)-5-(2-hydroxypropan-2-yl)-4,7,15-trioxo-16-oxa-3,6,14-triazanonadec-18-enoate.*



19

To a stirred solution of alcohol **10** (30 mg, 0.037 mmol) in anhydrous CH_2Cl_2 (0.6 mL) was added dropwise at 0 °C a solution of Martin sulfurane (49.8 mg, 0.074 mmol) in anhydrous CH_2Cl_2 (0.4 mL) under N_2 atmosphere. The reaction mixture was stirred at room temperature for 3 h. Another 0.074 mmol of Martin sulfurane were added and the reaction mixture was stirred at room temperature for 12 h. The reaction mixture was concentrated *in vacuo* and the crude material was purified using flash column chromatography in 1.5 % of MeOH in CH_2Cl_2 to furnish enamide **19** (17.5 mg, 60%) as a white solid.

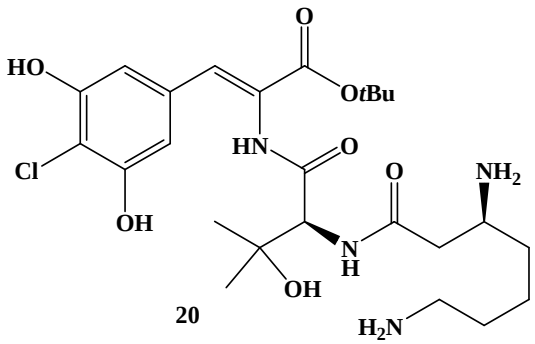
R_f (2 % MeOH : CH_2Cl_2) = 0.20.

mp = 72-73 °C

^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.09 (s, 1H), 7.22 (s, 1H), 6.71 (s, 2H), 6.65 (1H, d, J = 6.4 Hz), 6.16 – 5.98 (m, 2H), 5.94 – 5.75 (m, 2H), 5.53- 5.41 (m, 3H), 5.35-5.12 (m, 6H), 4.95-4.79 (m, 1H), 4.72-4.43 (m, 8H), 4.38 (1H, d, J = 6.4 Hz), 3.98- 3.67 (m, 2H), 3.20-2.97 (m, 2H), 2.56-2.38 (m, 2H), 1.58-1.30 (m, 6H), 1.53 (s, 9H), 1.25 (s, 3H), 1.22 (s, 3H) ppm.

^{13}C NMR (150 MHz, DMSO-d_6) δ = 171.6, 170.0, 164.1, 156.5, 156.0, 155.2 (× 2), 132.9, 132.6 (× 4), 132.1, 125.8, 117.9 (× 4), 117.6, 107.5 (× 2), 82.9, 71.8, 70.0 (× 3), 65.5, 60.0, 48.8, 40.6, 40.2, 33.7, 29.3, 28.0 (× 3), 27.5, 25.6, 22.9.

6.7.2.7. *tert-butyl-(Z)-3-(4-chloro-3,5-dihydroxyphenyl)-2-((S)-2-((S)-3,7-diaminoheptanamido)-3-hydroxy-3-methylbutanamido)acrylate.*



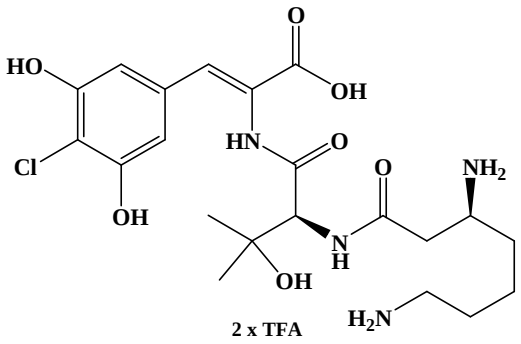
20

To a stirred solution of enamide **19** (23 mg, 0.029 mmol), AcOH (0.02 mL, 0.348 mmol) and $PdCl_2(PPh_3)_2$ (2 mg, 0.0029 mmol) in dry CH_2Cl_2 (0.8 mL, 0.035 M) under N_2 atmosphere $SnBu_3H$ (0.045 mL, 0.17 mmol) was added in one portion. The reaction proceeded with fast evolution of CO_2 and slight warming. The reaction mixture was stirred at room temperature for 2 h and again $SnBu_3H$ (0.045 mL, 0.17 mmol) was added in one portion. The reaction mixture was stirred overnight at room temperature, then it was concentrated *in vacuo* to dryness and the solid obtained was purified using reverse phase column chromatography in 2 % aq. NH_3 in MeOH : H_2O (1:1) to yield diamine **20** (10 mg, 66 %) as a yellow solid.

R_f (3 % NH_3 in (MeOH : H_2O, 1:1) = 0.2.

^{1}H NMR (300 MHz, CH_3OH-d_4) δ = 7.07 (s, 1H), 6.46 (s, 2H), 4.52 (s, 1H), 3.01-2.86 (m, 1H), 2.74 (t, *J* = 5.7 Hz, 2H), 2.37 (d, *J* = 6.6 Hz, 2H), 1.51 (s, 9H), 1.6-1.36 (6H, m), 1.37 (s, 3H), 1.34 (s, 3H) ppm.

6.7.2.8. *(Z)-3-(2-chloro-3,5-dihydroxyphenyl)-2-((S)-2-((S)-3,7-diaminoheptanamido)-3-hydroxy-3-methylbutanamido)acrylic acid. TFA salt.*



1

A solution of diamine **20** (10 mg) in CH_2Cl_2 (0.5 mL) was cooled to 0 °C, TFA (0.2 mL) was added dropwise and the reaction mixture was stirred at room temperature for 2 h. The reaction mixture was concentrated to dryness and the residue was triturated with diethyl ether to afford the title compound **1** (13 mg, 97%) as a white solid.

R_f (5 % NH_3 in (MeOH : H_2O, 1:1)) = 0.8.

^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.42 (brs, 1H), 8.38 (1H, d, J = 9.6 Hz), 8.02-7.60 (brs, 6H), 7.03 (s, 1H); 6.73 (s, 2H), 4.51 (1H, d, J = 9.6 Hz), 3.45-3.30 (m, 1H), 2.75 (t, J = 7.2 Hz, 2H), 2.68 (dd, J = 6.0; 15.6 Hz, 1H), 2.54 (dd, J = 6.0, 15.6 Hz, 1H), 1.63- 1.42 (m, 4H), 1.41- 1.29 (m, 2H), 1.22 (s, 6H) ppm.

^{13}C NMR (75 MHz, DMSO-d_6) δ = 169.8, 169.6, 166.2, 154.0 (x 2 C), 132.2, 131.0, 126.7, 108.8 (x 2 C), 108.5, 71.4, 60.5, 47.7, 38.3, 37.3, 31.2, 27.1, 26.4, 26.1, 21.3 ppm.

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