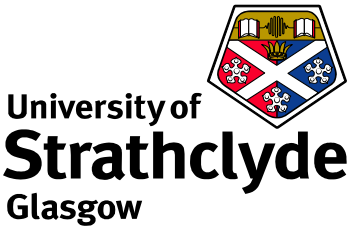
Total synthesis and semi-synthesis of lipid and protein components of *Mycobacterium tuberculosis*



A thesis submitted to the University of Strathclyde for
the Degree of Doctor of Philosophy
by

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DECLARATION OF AUTHENTICITY AND AUTHOR'S RIGHTS

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to my parents

Abstract

Acyl carrier protein (ACP) is an essential, universal and highly conserved cofactor in the biosynthesis of a number of key components in mammals, yeast, bacteria and plants. In metabolic pathways ACP carries out its function using the essential 4'-phosphopantetheine prosthetic group attached to a serine.

In *Mycobacterium tuberculosis* (*Mtb*), ACP plays a fundamental role in the biosynthesis of fatty acids linking insoluble intermediates by a thioester bond. Recently, a novel ACP-dependent cyclopropanating enzyme, PcaA, was found essential for the virulence and persistence of *Mtb*, and is considered a promising target in TB drug discovery. PcaA substrate is a long-chain fatty acid, *cis,cis*-diunsaturated α-meromycolic acid, linked to an acyl carrier protein (AcpM); this substrate has a fundamental function in the interaction with PcaA.

For biological and medicinal purposes the following achievements have been reached:

- synthesis of *cis,cis*-diunsaturated α-meromycolic acid. Two different retrosynthetic strategies have been used. A scrupulous study of side-reactions was conducted during all steps, leading to the identification and understanding of unexpected reactions.
- expression and purification of AcpM. The protein was expressed in *E. coli* and purified by Ni^{2+}-affinity column and hydrophobic interaction chromatography. The impurities of these purifications were identified.
- development of a novel methodology for the chemical site-selective thioesterification of proteins with fatty acids in aqueous solution. The methodology is based on the activation of fatty acids by the conversion into sodium acyl-5-sulfobenzimidazole (acyl–NaSBI), and on a catalytic system where sodium 5-sulfo-benzimidazole (NaSBI) is responsible for the chemo-selective activation of sulfhydryl compounds *via* base catalysis. The methodology was validated by reactions with model compounds using NMR spectroscopy, and by reactions with AcpM.

CONTENTS

CHAPTER 6

CHAPTER 7

INDEX OF FIGURES, SCHEMES AND TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

ABBREVIATIONS

ACP	Acyl carrier protein
AcpS	Holo-acyl carrier protein synthase
Aq	Aqueous
BaSBI	Barium 5-sulfo-benzimidazole
CoA	Coenzyme A
COSY	Correlation Spectroscopy
δ	Parts per million
DBU	1,8-Diazobicyclo[5.4.0]undec-7-ene
DHP	3,4-Dihydro-2H-pyran
DEPT	Distortionless Enhancement by Polarization Transfer
DMA	Dimethyl acetamide
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMI	1,3-Dimethyl-2-imidazolidinone
DMPU	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTT	1,2-Diamonipropionic acid
DTN	Ellman's reagent
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
EDTA	Ethylenediaminetetraacetic acid
Eq	Equivalent
ESI	Electrospray
FPLC	Fast protein liquid chromatography
GC	Gas Chromatography
HMDS	Hexamethyldisilazane
HMPA	Hexamethylphosphoramide
HMPT	Hexamethylphosphoric triamide

HPLC	High pressure liquid chromatography
HRMS	High resolution mass spectrometry
IPr	*N,N'*-bis(2,6-diisopropylphenyl)imidazol-2-ylidene
IPTG	Isopropyl-β-D-thiogalactopyranoside
KAPA	Potassium 3-aminopropylamide
KHMDS	Potassium bis(trimethylsilyl)amide
Kcal	Kilo calorie
KDa	Kilo Dalton
LAH	Lithium aluminium hydride
MALDI	Matrix-assisted laser desorption/ionization
MHz	Mega hertz
m.p.	Melting points
MCPBA	3-Chloroperbenzoic acid
MS	Mass spectrum
MW	Microwave
NaHMDS	Sodium bis(trimethylsilyl)amide
NAPA	Sodium 3-aminopropylamide
NaSBI	Sodium 5-sulfo-benzimidazole
NHC	*N*-heterocyclic carbenes
NHS	*N*-hydroxysulfosuccinimide
NMI	*N*-methylimidazole
NMP	*N*-heterocyclic carbenes
NMR	Nuclear magnetic resonance
OA	Oxidative addition
OP	Oxophoethane
PEPPSI	Pyridine-enhanced precatalyst preparation, stabilization, and initiation
PMA	Phosphomolybdic acid
PCC	Pyridinium chlorochromate
4'-PP	4'-Phosphopantetheine
PPTase	Phosphopantetheinyl transferase
PR_3	Phosphines

PTC	Phase transfer catalysis
RE	Reductive elimination
Rf	Retention factor
r.t.	Room temperature
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
THF	Tetrahydrofuran
THP	2-Tetrahydropyranyl
TLC	Thin Liquid Chromatography
TM	Transmetalation
TPPA	*NN'N"*-tris(tetramethylene)phosphoramide
TS	Transition states

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support, but especially to cheer me up during the difficulties, reminding me the passion for the research.

CHAPTER 1

1. GENERAL INTRODUCTION

1.1 TUBERCOLOSIS

Tuberculosis (TB) is an infectious disease caused by the bacillus *Mycobacterium tuberculosis* (*Mtb*). Recently, traces of the disease have been detected in a hominid fossil that dates back about 500,000 years ago.[1] In addition, genetic studies showed that the progenitor species, from which the *Mtb* clonal group evolved, is as old as 3 million years, suggesting that our remote hominid ancestors may well have already suffered from TB.[2, 3] However, despite the very ancient origins of *Mtb*, TB is nowadays a very current disease. TB is second only to HIV/AIDS as the leading infectious cause of death, killing nearly 2 million people each year.[4]

From 17th- to 19th-century, 20–30% of deaths in Europe were caused by TB.[5] The mortality due to this disease began to fall as living standards improved at the start of the 20th century, and, particularly, with the advent of antibiotics from the 1940s. For few decades, it seemed as though TB would be eradicated, at least among the wealthy, but several factors contributed to the re-emergence of the disease.[6] Nowadays, nearly 10 million of new TB cases are expected each year, which make this disease more widespread today than at any other time in history.[5]

Figure 1.1. Symbol chosen for "World TB Day 2007".[7] A flower that dispels petals through breath of wind, symbol of a very contagious disease; a grey flower just outlined, emblem of a disease not always well perceived and often forgotten.

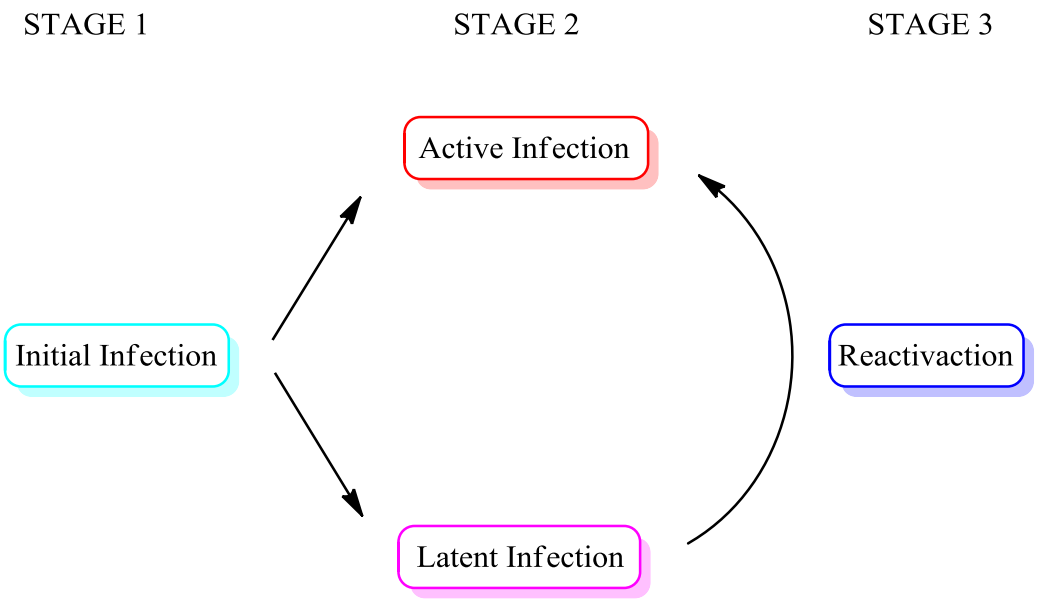


Figure 1.2. Schematic representation of the three stages of TB infection.

TB is a challenging disease, which is characterized by a complex infection that can be divided in three separate stages (Figure 1.2).[8] In the first stage of the *Mtb* infection, the mycobacterium is inhaled and resides within the lungs, where it infects phagocytic cells (macrophages and dendritic cells) or monocytes. *Mtb* has developed numerous strategies that allow it to survive and multiply within host cells. The second stage involves the acquired immunity, which relies on the host's immune response. In the case of immunocompromised host, the mycobacterium typically results in the establishment of an acute infection characterized by uncontrolled bacillary proliferation and dissemination of the organism to distal sites. Symptomatically, people become gradually weaker; develop fever, night sweats and cough. Conversely, if the infected host is immunocompetent, the host's immune system will typically resolve the initial infection, or alternatively, hold the infection in check using mechanisms that prevent further bacillary proliferation, limit the dissemination of the organism, and concentrate the immune response directly to sites of infection. Nevertheless these individuals continue to be persistently infected by bacterium, although they do not exhibit overt disease symptoms and are not infectious. The third stage of infection can appear months or years later; it is

characterized by reactivation of the bacterium from latency, and subsequent initiation of the acute infection in the host. The reactivation arises from conditions of host's immune status, which can be suppressed by factors such as HIV infection, steroid therapy, age and malnutrition. In particular, the increasing spread of HIV infection during the last three decades is doubtless an important factor that has contributed to the re-emergence of TB.[6, 9]

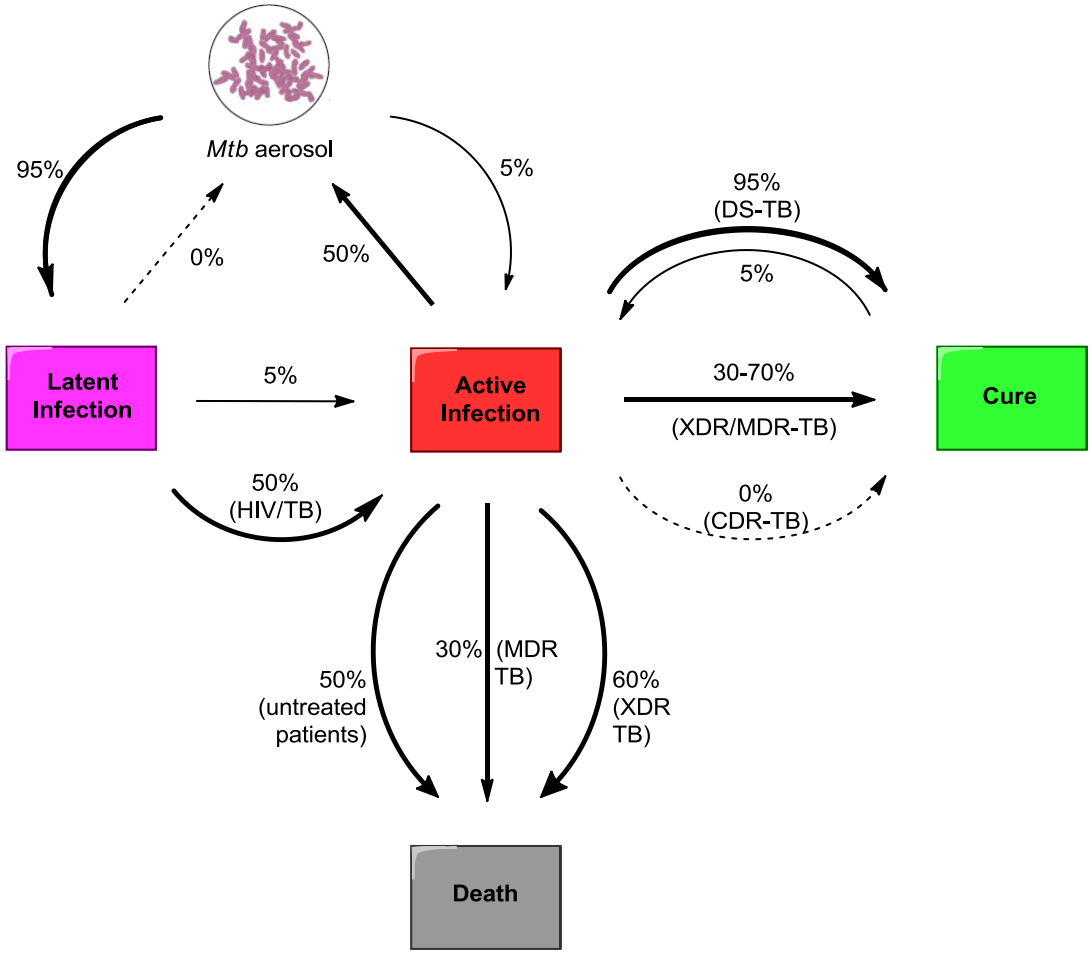


Figure 1.3. Stages of TB infection and related transmission, progression and cure of the disease. *Mtb* is generally in a non-infectious "latent" state, which can be activated by several factors such as the HIV co-infection. The infective "active" state can be lethal if not correctly treated, and the success of the therapy depends on the drug-suceptibility of TB infection (DS: drug-susceptible, MDR: multi-drug resistant, XDR: extensively drug-resistant CDR: completely drug-resistant). This Figure was modified from Koul *et al.*[10] and based on the data previously reported.[11, 12]

The stages of the *Mtb* infection, described above, are highly interrelated as shown in Figure 1.3. In particular, the ability of *Mtb* to exist in a latent state, which can reactivate, imposes a long treatment time since that, in this state, the bactericidal drug action is extremely low. The standard TB therapy is a treatment with a cocktail of drugs for at least six months, which is prescribed under DOTS (directly observed treatment, short-course). Specifically, the therapy consists of an "intensive phase" of treatment with four first-line drugs: isoniazid (INH), rifampin (RIF), pyrazinamide (PZA), and ethambutol (EMB) for 2 months, followed by a "continuation phase" with INH and RIF for further 4 months.[13] In order to allow the complete eradication of mycobacterium, the therapy must not be interrupted, despite the apparent disappearance of symptoms. Indeed, poor adherence to therapy by patients, as well as incorrect treatment, drug malabsorption and poor drug quality led to the emergence of drug-resistant strains.[14, 15]

1.2 DRUG-RESISTANT TUBERCULOSIS

As discussed in the previous section, several factors have led to the emergence of drug-resistant *Mtb* strains that are responsible for high mortality rates. In particular, these strains have been reported with an increasing frequency during the last 20 years.[6, 15] Importantly, in 2006, a report of 53 TB patients from South Africa, infected with drug resistant strains and with a high prevalence of human immunodeficiency virus (HIV) co-infection, showed an alarming case fatality rate of almost 100% with a median survival of 16 days.[16] These data were immediately presented by the scientific community to point out the alarming implications of new drug-resistant *Mtb* strains for global public health.[17-19] Specifically, *Mtb* strains resistant to at least INH and RIF, the two most powerful first-line TB drugs, are known as multidrug-resistant (MDR) strains (Figure 1.4).[20] MDR *Mtb* strains, with further resistances to any fluoroquinolone (class of antibiotics that has been demonstrated as indispensable in the treatment for MDR-TB)[21-23] and at least one of the three injectable second-line drugs (amikacin, kanamycin and capreomycin) are termed "extensively resistant" (XDR).[24, 25]

The worldwide proportion of patients with MDR-TB is 4–5% among all TB cases,[12, 26] whereas the prevalence of XDR-TB is 7–8% among all MDR-TB cases.[27, 28] The treatment of drug-resistant TB becomes more complicated as the antibiotic resistance profile of *Mtb* strains broadens, resulting in high mortality in the cases of MDR- and XDR-TB. However, the prudent use of combinations of drugs can increase the probability of successful treatment.[29, 30] Recent guidelines for the therapy of patients with MDR- and XDR-TB have been reported by World Health Organization (WHO).[31] The recommended therapy is based on individually designed regimens with at least four drugs to which the *Mtb* strains isolated are likely to be susceptible. Nevertheless, the costs, the limited efficacy, the poor tolerability and the possibility of toxic adverse events due to some of these drugs, and the long therapy duration (at least 2 years), often make this treatment impracticable, inefficacious or toxic.

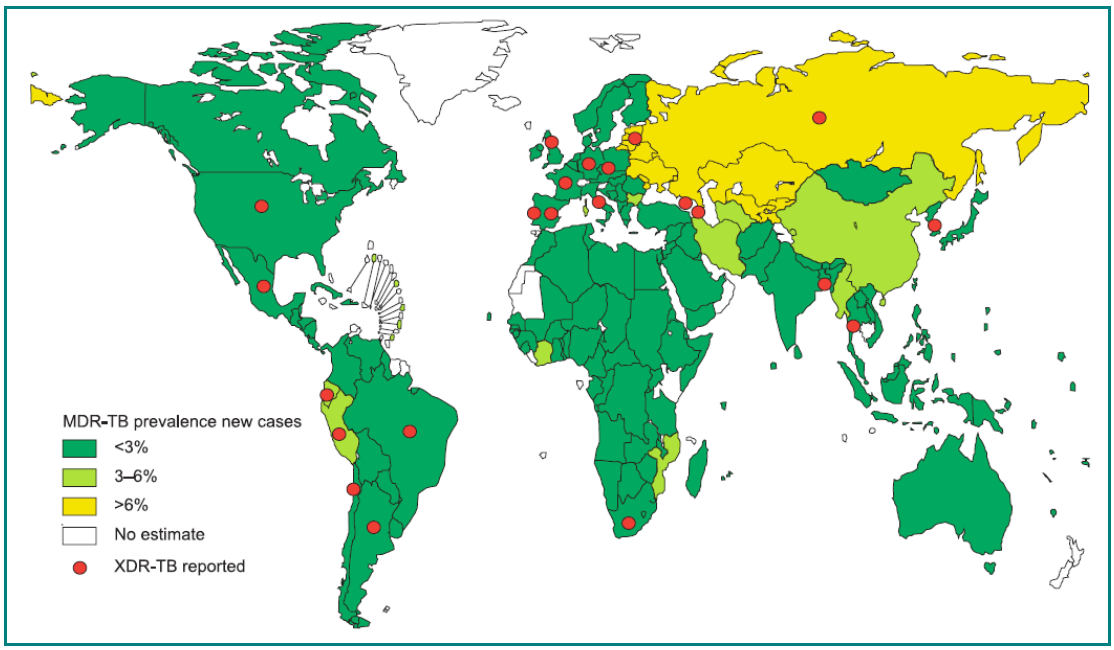


Figure 1.4. Distribution of MDR-TB and presence of XDR-TB in the world in 2006.[25] By the end of September 2009, at least one case of XDR-TB had been reported by 25 countries in the WHO European Region.

Even more remarkably, more recent studies have reported TB cases resistant to all drugs currently available to treat the disease, confirming the existence of completely drug-resistant TB strains (CDR-TB).[32, 33] The analysis of these strains by transmission electron microscopy showed bacilli with a cell wall that is extraordinarily thick in comparison with drug-susceptible bacilli; this modification is considered responsible for the drug-resistance of CDR strains.[34] Indeed, it is important to note that, despite drug-resistance is generally accompanied by a "cost" that leads to bacteria with minor virulence in comparison to the original strain,[35] low cost or no cost mutations have been reported in the case of *Mtb*.[12, 36] These findings highlight the urgent need to develop new drugs that may be efficacious against drug-resistant *Mtb* strains.

1.3 NOVEL TARGETS FOR NEW TB DRUGS

Despite several achievements on *Mtb* research have been reached in the last 20 years, the current chemotherapy is still based on half century old drugs.[13] The only drugs developed in the last decades (1980s) and used in therapy are fluoroquinolones, which are however second-line drugs. Despite this class of "new" drugs was initially considered essential in treating MDR-TB cases,[21-23] its use has been more recently reduced with the emergence of XDR *Mtb* strains.[37] In general, the current TB therapy shows several drawbacks, due to the long treatment duration, the costs, the limited efficacy, the poor tolerability and the possibility of toxic adverse events. It is essential and urgent to develop new drugs that may:[10]

- ✓ be cheap, potent and well-tolerated;
- ✓ shorten the current long therapy duration;
- ✓ be efficacious against drug-resistant strains;
- ✓ have no drug-drug interactions.

In particular, in order to increase the patient compliance and reduce the therapy duration, it is essential that the new dugs have a strong bactericidal activity against the mycobacterium in the latent stage. Moreover, in order to treat drug-resistant strains, it is essential that the new drugs inhibit new targets. To this end, it is encouraging to note that the availability of the genome sequence of *Mtb* since 1998,[38] has largely driven the identification of new TB targets in the last years.[39] In fact, several targets have been recently identified, validated and proved to be valuable for the development of new drugs (Figure 1.5).

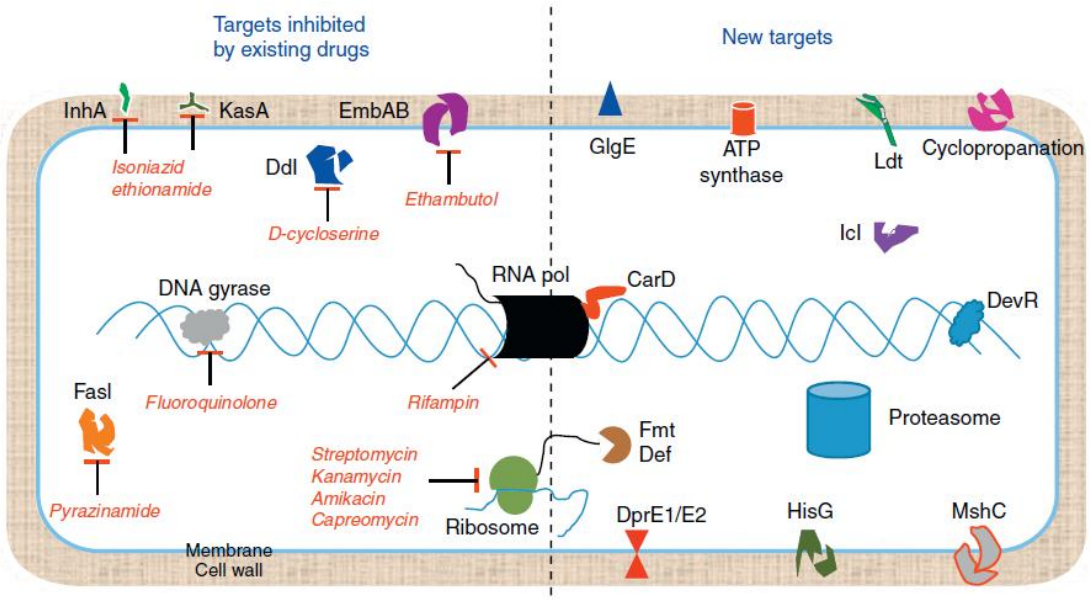


Figure 1.5. Scheme of *Mtb* illustrating targets inhibited by existing drugs (left) and new targets (right) as reported by Lamichhane.[39]

1.3.1 The *Mtb* cell wall

The mycobacterial cell envelope is composed of the plasma membrane, the periplasma, the cell wall and the outer layer.[40] The cell wall of *Mtb* has a unique composition of lipids and carbohydrates,[40, 41] which makes it an ideal and well-established target for the development of new TB drugs.[41, 42] Indeed, it is interesting to note that among the anti-TB agents used in therapy or under clinical trials,[10, 43] several compounds inhibit targets involved in the synthesis of components of cell wall. Specifically, three of the five first-line TB drugs, isoniazid, pyrazinamide and ethambutol, and other compounds such as thiolactomycin, triclosan, thiacetazone, ethionamide, isoxyl, PA-824, OPC-67683 and SQ109 are inhibitors of mycobacterial cell wall biosynthesis.[10, 42, 43] Indeed, it is known that the cell wall of *Mtb* is associated with its pathogenicity, virulence and drug-resistance. In particular, the distinctive thickness of the *Mtb* cell envelope contributes to unusually low

permeability to therapeutic agents, and to the drug-resistance in MDR, XDR and CDR strains.[34] For these reasons, drugs that inhibit targets involved in *Mtb* cell wall biosynthesis have generally potent bactericidal activity, and are important also to improve the efficiency of other antibiotics used in combination increasing the cell permeability.

The *Mtb* cell wall presents extraordinary high lipid content, containing characteristic C_{60}–C_{90} fatty acids called mycolic acids. These very-long-chain fatty acids are the major constituents of cell envelope and constitute 40–60% of the dry weight of the mycobacterium.[41] Mycolic acids are a family of compounds constituted from the "meromycolic" β-hydroxy fatty acids (up to C_{56}) with α-alkyl side chain ($C_{24–26}$), where the stereocentres in the α- and β- positions relative to the carboxylic group have R-configuration. In *Mtb*, four distinct structural classes of mycolic acids have been found: α-, hydroxy-, methoxy- and keto-mycolic acids,[44, 45] which are present as series of homologue compounds differing by two methylene units.[46-48] In particular, α-mycolic acid are *cis,cis*-dicyclopropyl fatty acids (Figure 1.6), which represent the most abundant forms of all mycolic acids (>70%), and the major homologue is shown in Figure 1.6.[49, 50]

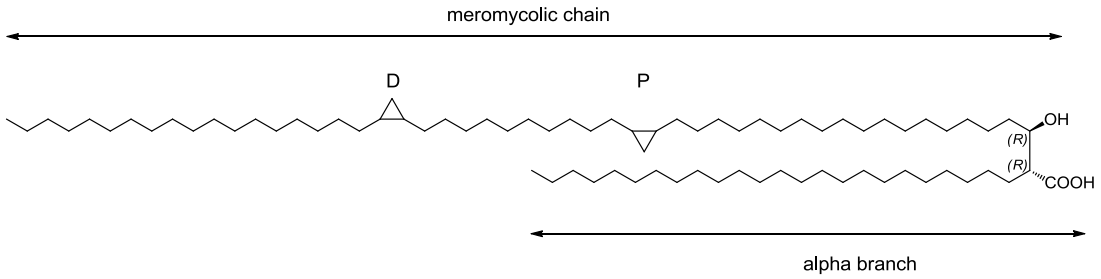


Figure 1.6. Structure of the most abundant form of α-mycolic acids in *Mtb*. It is constituted from the meromycolic β-hydroxy fatty acid (C_{52}) with a α-alkyl side chain (C_{26}) and containing two cyclopropane rings (D: distal, P: proximal)

1.3.2 Biosynthesis of mycolic acids

Although, in the last 30 years, an impressive number of works has investigated the biosynthetic pathways of mycolic acids in *Mtb*, only recently a clear and complete scheme it has been proposed.[49] The biosynthesis of mycolic acids occurs by numerous enzymes *via* metabolic pathways that remain to be clearly elucidated. However, it is generally accepted that the biosynthesis of mycolic acids can be virtually divided into three stages:

1) the synthesis and elongation of the precursors of the C_{26} α-branch and the meromycolic chain;
2) the introduction of functional modifications;
3) the condensation of the functionalised meromycolic acid and the C_{26} α-branch.

Specifically, the biosynthesis of mycolic acids starts from acetyl-CoA by the fatty acid synthase I (FAS-I). FAS-I is a large multi-domain protein that is capable of performing *de novo* synthesis of fatty acids corresponding to the precursor of the α-branch and to $C_{16–18}$ precursors of the meromycolic acid. The $C_{16–18}$ fatty acid intermediates synthesised by FAS-I are then linked to an acyl carrier protein (AcpM) and elongated by the fatty acid synthase II (FAS-II), a complex of dissociable enzymes, which synthesises the diunsaturated meromycolic chain. The diunsaturated meromycolic acid is generally considered the common precursor of the α-, methoxy-, and keto-mycolic acids, which are synthesised by different enzymes. The modified meromycolic acid is then condensed to the C_{26} α-branch by Pks13 and other enzymes.

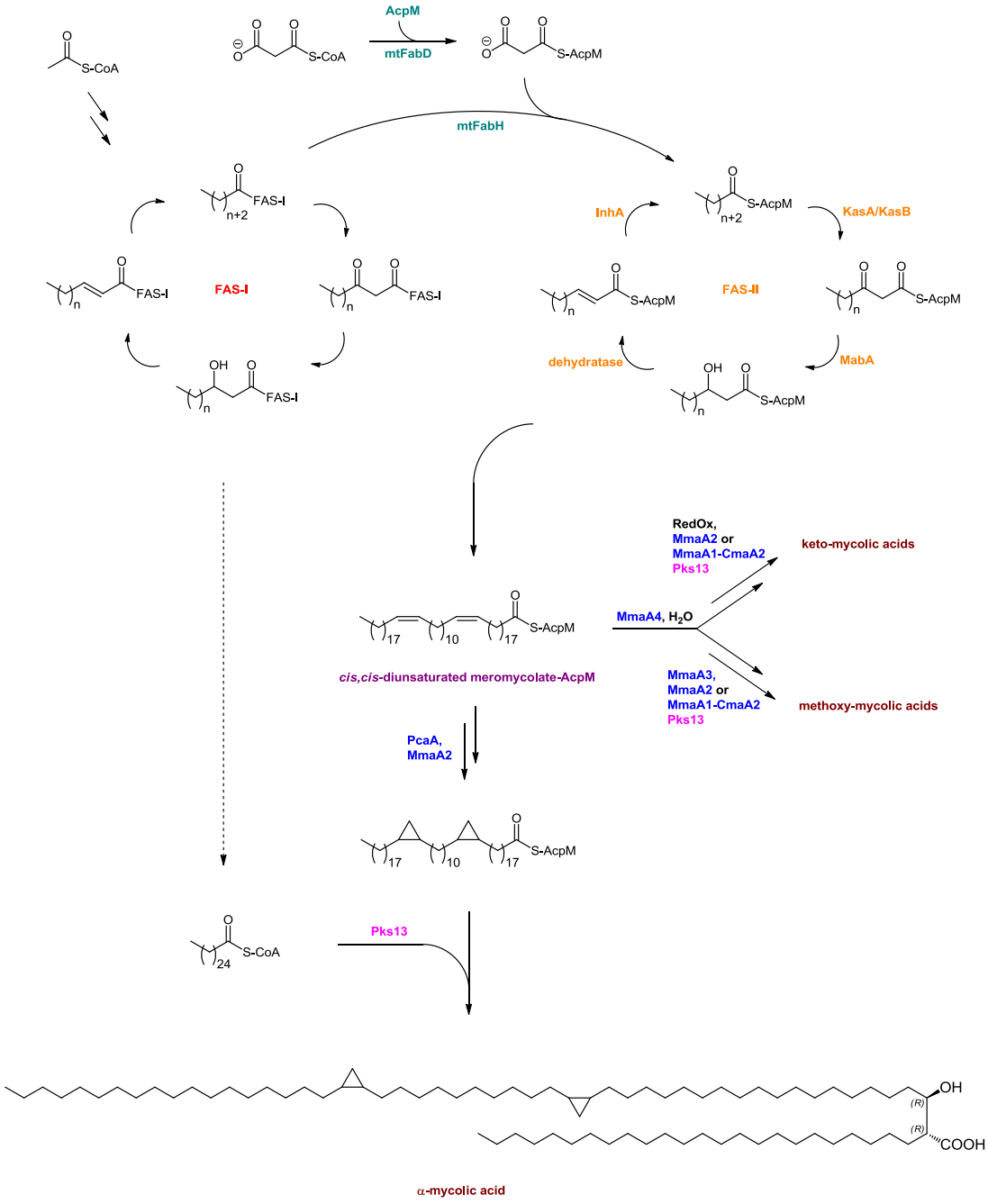


Figure 1.7. General scheme for the biosynthesis of mycolic acids in mycobacteria

1.3.3 PcaA: a promising target for TB drug discovery

Among the enzymes involved in the modification of meromycolic chain, the enzymes responsible for the formation of cyclopropane rings (cyclopropane mycolic acid synthases, CMASs) play a fundamental role in pathogenesis of the final mycolic acids.[51] In particular, in 2000, a key enzyme in the biosynthesis of cell wall lipids, PcaA, was identified and found responsible for the cyclopropanation of the proximal double bond of the α-meromycolic acid. Specifically, this enzyme catalyses *cis*-cyclopropanation at the proximal position of the unsaturated meromycolate, which is linked to a carrier protein (AcpM) *via* a thioester bond (Figure 1.8). The reaction is carried out using *S*-adenosyl methionine (SAM) as cofactor, which is converted in *S*-adenosylhomocysteine (SAH). Remarkably, in 2000, gene knockout studies showed that the deletion of *pcaA* could "switch off" mycobacterial persistence and virulence in a mouse model of infection, and that this enzyme was required to establish a lethal chronic TB infections.[52] Specifically, Glickman *et al.* proposed that the removal of such molecular functionality interfered with serpentine cording between the mycobacterial cell wall and the host immune system. For these reasons, this enzyme has been considered an attractive target in TB drug discovery.[53]

In contrast, knockout studies of other methyltransferases have given different results. Specifically, the selective loss of CmaA2 led to hypervirulence and hyperinflamatory innate immune activation in macrophages,[54] while the loss of MmaA4 results in excessive IL-12 production.[55] However, more recently, Glickman and co-workers have reported that the non-selective inhibition of methyltransferases using dioctylamine as a tool compound resulted in the loss of cyclopropanation, cell death, loss of acid fastness, and synergistic action with isoniazid and ciprofloxacin.[51] Prior studies from Alahari, Kremer and co-workers indicated that the antitubercular thiacetazone affects cyclopropanation and that MmaA4 is required for the activity of this second-line drug.[56, 57] These results strongly encourage the discovery of more potent non-selective PcaA inhibitors as promising TB drugs.[58]

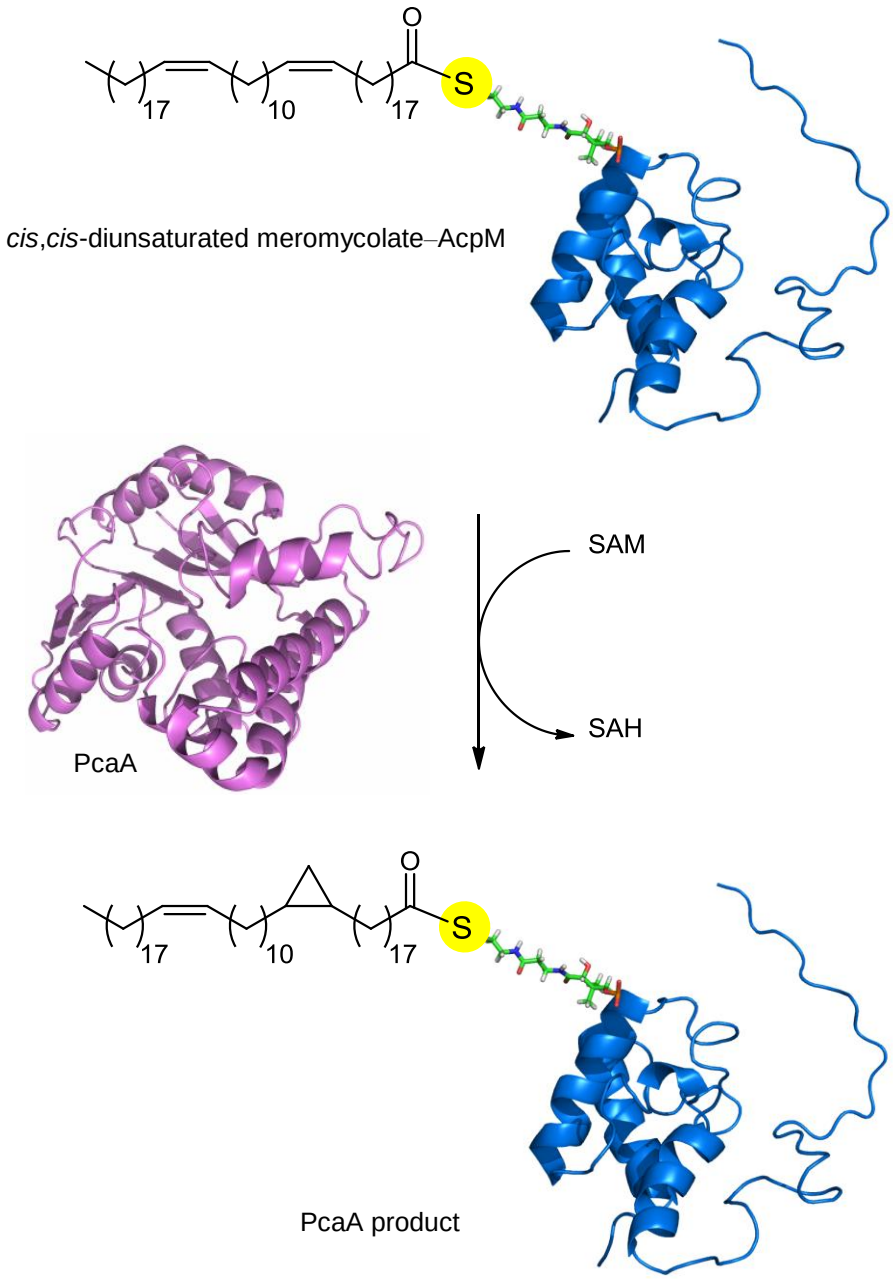


Figure 1.8. PcaA reaction.

In order to develop inhibitors for PcaA, firstly it is essential to develop a biochemical assay to quantity the reaction of this enzyme. Colourimetric[59] or fluorometric[60] methods to quantify SAH are known in literature. Nevertheless, to this end, it is important to use the native PcaA substrate, which allows essential interactions with the enzyme. In particular, the protein–protein interactions between PcaA and AcpM are essential for the enzymatic reaction and a simulation of these interactions is shown in Figure 1.9. The essential role of acyl carrier proteins in enzymatic reactions where they are involved is discussed in more detail in Chapter 4.

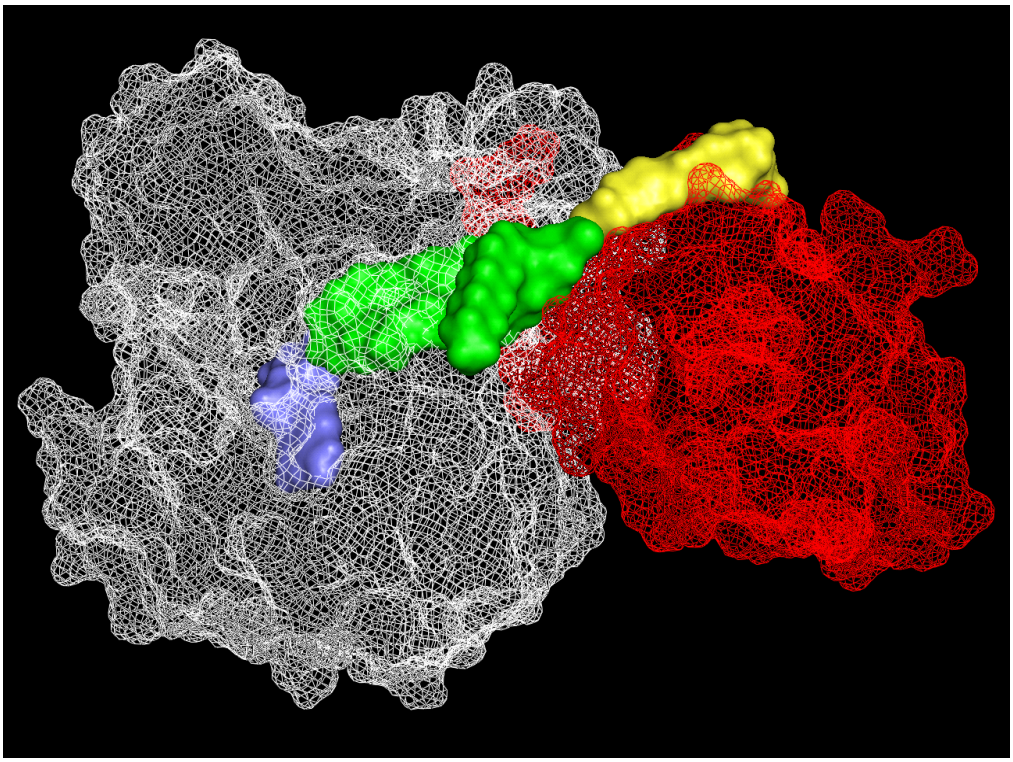


Figure 1.9. Interactions between PcaA (white), containing SAM (blue) and AcpM (red) bearing *cis-cis diunsaturated meromycolate* (green) *via* phosphopantetheine prosthetic group (yellow). The simulation was kindly performed by Dr. N. Anthony using Insight 2 and prepared by PyMol.[61]

1.4 AIMS

The overall aim of this thesis was to synthesise the PcaA substrate for future applications in TB drug discovery. The specific aims are shown in Figure 1.10 and were:

- the synthesis of *cis,cis*-diunsaturated α-meromycolic acid (*Chapters 2 & 3*);
- the expression and purification of AcpM (*Chapter 4*);
- the development of a novel methodology for the chemical site-selective thioesterification of proteins with fatty acids in aqueous solution (*Chapter 5*).

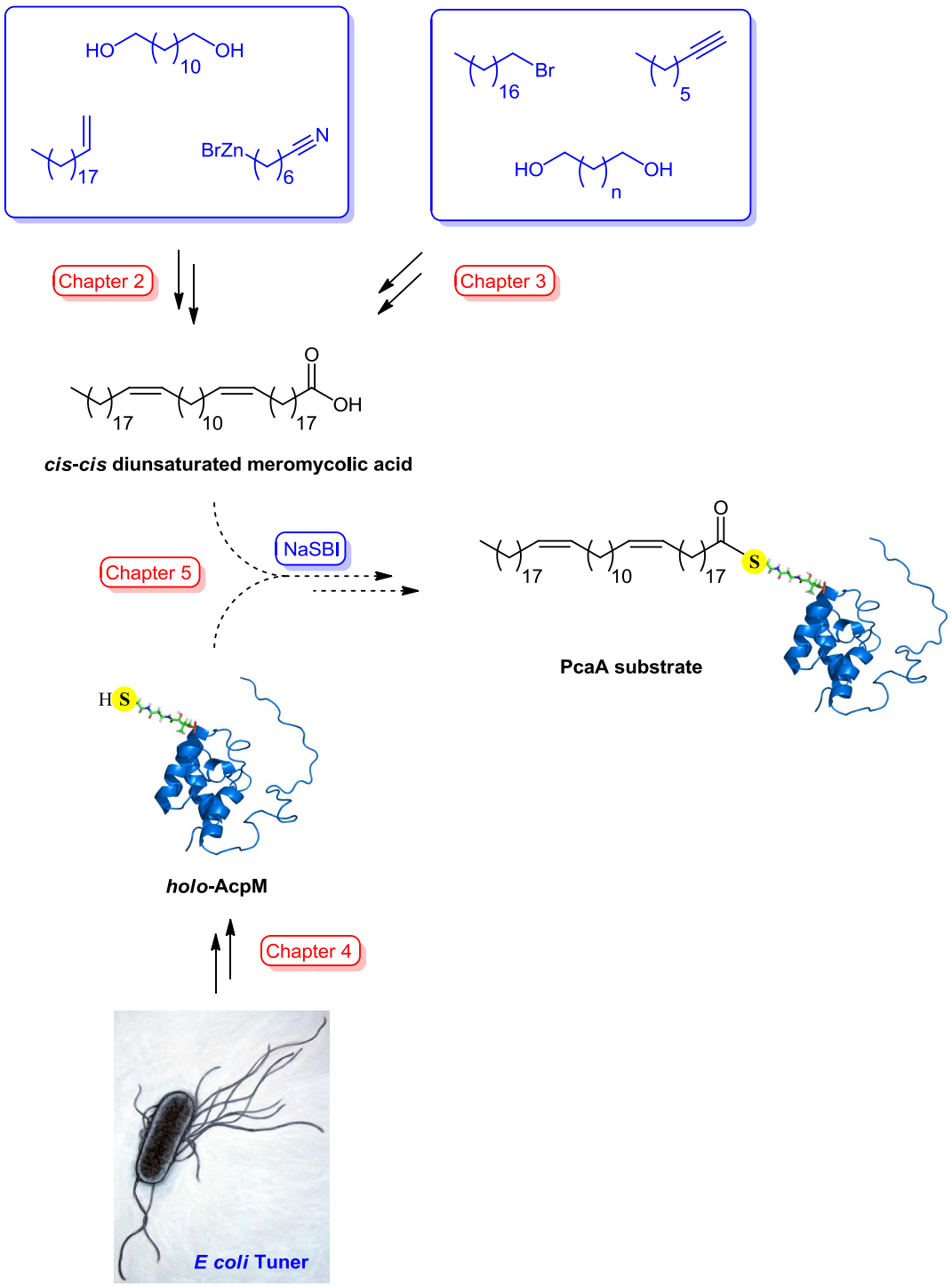


Figure 1.10. Schematic presentation of the key chapters of this thesis according to aims of this work.

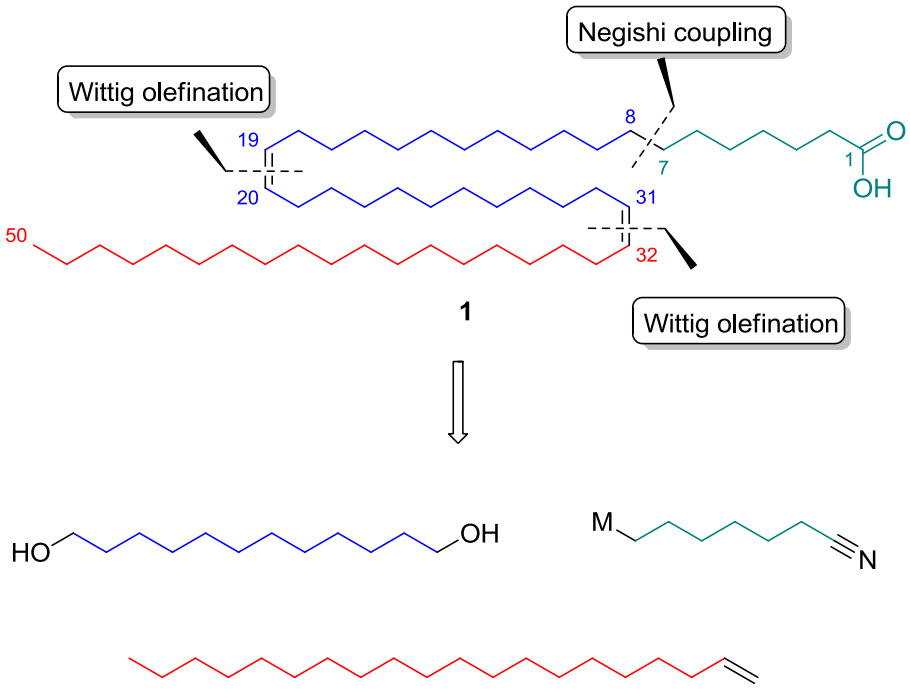
CHAPTER 2

2. SYNTHESIS OF THE *CIS*,*CIS*-DIUNSATURATED MEROMYCOLIC ACID

2.1 INDRODUCTION

2.1.1 The synthetic strategy for the *cis,cis*-diunsaturated meromycolic acid

The retrosynthetic analysis of *the cis,cis*-diunsaturated meromycolic acid **1** (Scheme 2.1) shows two *cis*-double bonds as points of disconnection for two stereoselective Wittig olefinations of three fragments: the C1−C19 fragment (proximal), the C20−C31 fragment (internal), the C32−C50 fragment (distal). The proximal fragment is an α,ω-disubstituted C_{19} alkane, too long for commercial availability and thus requires a further C−C disconnection of the carbon skeleton, which may be prepared by the Negishi coupling of a C_{12} compound. The same C_{12} compound could be derivatised to form the internal C20−C31 fragment.



Scheme 2.1. Schematic disconnective analysis of the *cis,cis*-diunsaturated meromycolic acid

It is important to emphasise that the sequence of transformations in synthetic strategies towards *cis*-olefins is limited as *cis*-*trans* isomerisation may occur during the synthetic pathway. Indeed, although this possibility is not often considered, several reaction conditions tend to convert the *cis*-olefin to the thermodynamically more stable *trans* isomer. Isomerisation of the C=C double bound occurs *via* photocatalysed[62] or thermal[63] processes, or mechanism catalysed by strong acids,[64, 65] nucleophilic or electrophlic reagents, such as phosphines[66] or iodine,[67] and inorganic salts such as Pd(0),[68] Pd(II),[69] and Ni(0).[70]

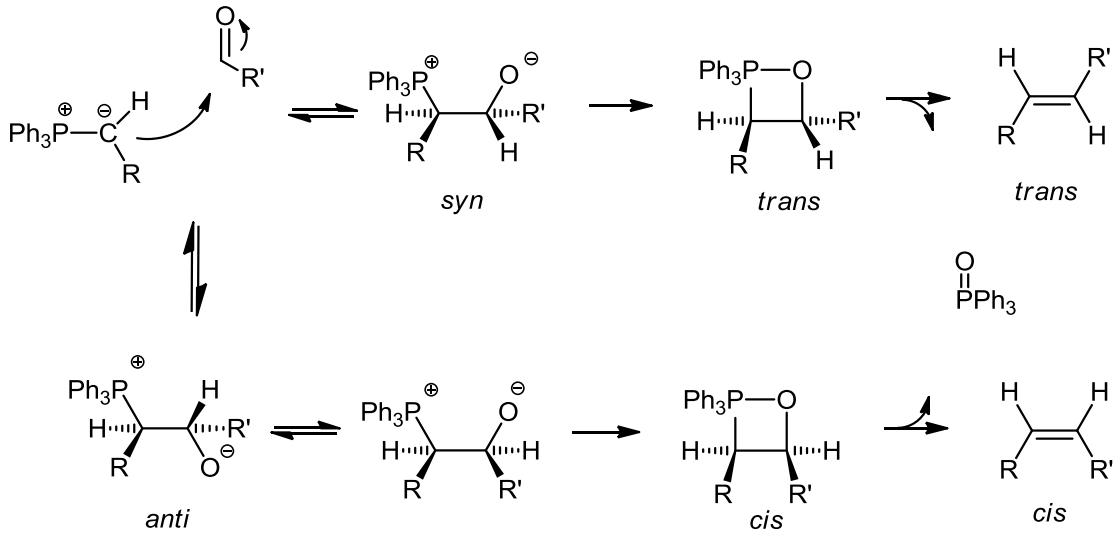
The synthetic strategy used to prepare the *cis*,*cis*-diunsaturated meromycolic acid is discussed in detail in chapter 2 and will be preceded by an overview of the two key reactions: the Wittig olefination and the Negishi coupling. The efficiency of the synthetic strategy, in terms of stereoselectivity and overall yield, will depend on the successful choice and application of the conditions for *cis*-olefination and alkyl−alkyl cross-coupling reaction.

2.1.2 Stereochemistry and mechanism of the Wittig reaction

The synthesis of alkenes is one of the most common and investigated reactions in organic chemistry. Compounds with double bonds, or those derived from them (*e.g.* epoxides and cyclopropane rings) are frequently found in nature and have attracted the attention of synthetic and medicinal chemists. Examples may be found human (prostaglandins), animals (sex pheromones), plants (terpenes) and bacteria (mycolic acids).

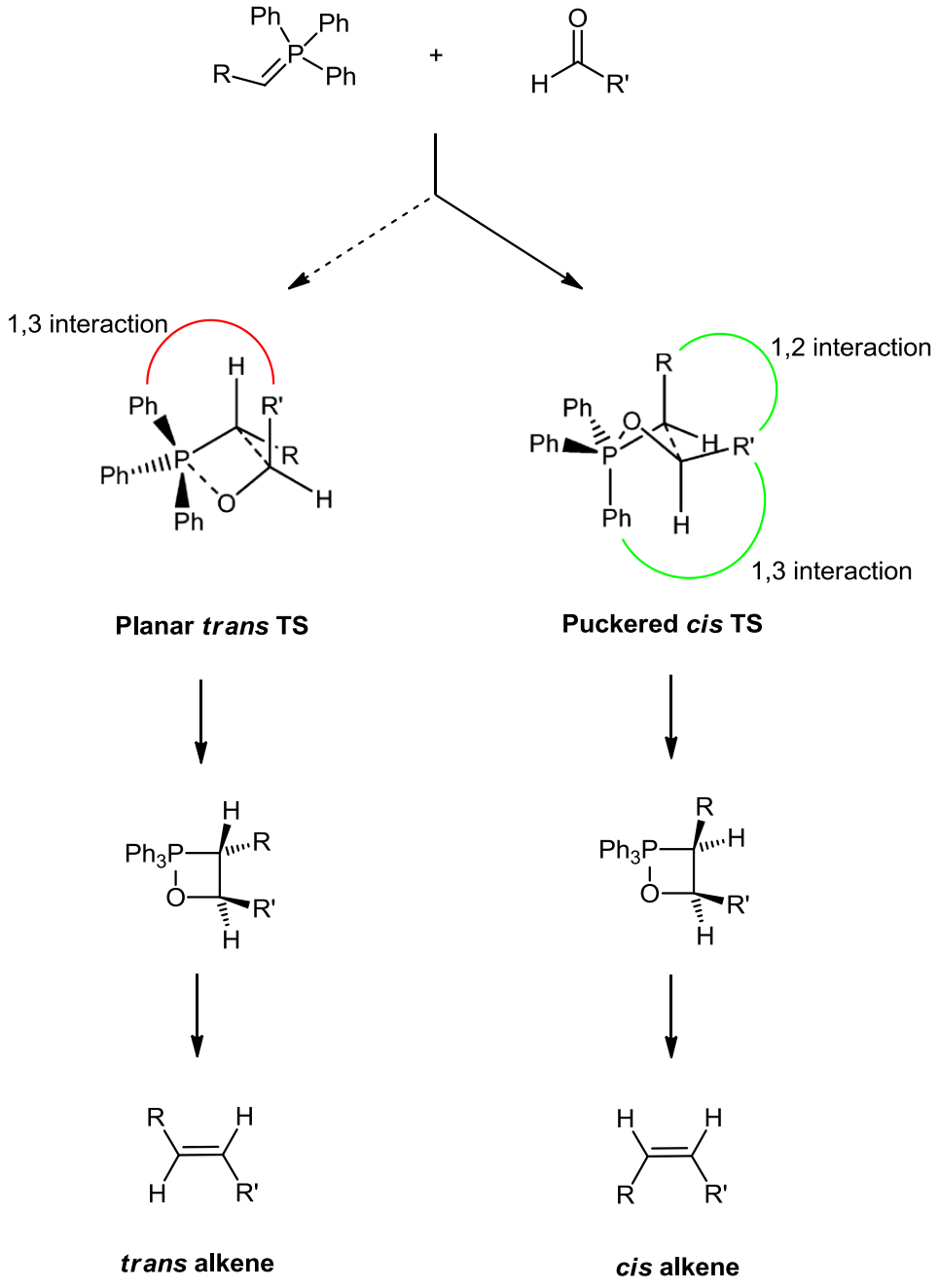
Since Wittig and co-workers discovered the method to obtain a double bond *via* the reaction of aldehydes or ketones with phosphonium ylides in the early 1950s,[71, 72] the olefin synthesis has changed radically. However, a clear explanation of this reaction, which permits the preparation of alkenes with unambiguous positioning of the double bond, was not evident. This stimulated chemists' to investigate possible reaction mechanisms and find ideal conditions to control stereoselectivity.

For many years it was believed that the Wittig reaction involved the formation of a betaine intermediate and that the stereoselectivity was linked to formation of these structures according to a *syn* or *anti* approach of the ylide on the carbonyl group (Scheme 2.2).[73]



Scheme 2.2. The old ionic mechanism (betaine intermediates assumed) for the Wittig reaction commonly accepted in 1970s and 1980s.

Several mechanistic investigations were published between 1970s and 1990s[74] and nowadays it is generally accepted that the Wittig reaction occurs by [2+2] cycloaddition mechanism between the ylide and the aldehyde (Scheme 2.3).[75]



Scheme 2.3. Interactions during the transition states for the Wittig reaction *via* [2+2] cycloaddition mechanism.

Recently, Aggarwal, Harvey and co-workers have published an elegant computational study on the Wittig reaction.[76] In this study for the reaction of non-stabilized ylides with aldehydes, the transition states (TSs) investigated are those postulated by Vedejs on the basis of experimental results.[77, 78] These TS structures have planar and puckered geometries, respectively for the formation of *trans* and *cis* alkenes (Figure 2.1).

In this model, the stereoselectivity is explained by an interplay of 1,2 and 1,3 steric interactions respectively between the ylide substituent and the aldehyde substituent, and between the aldehyde substituent and the phosphorus substituents. In the case of the reaction between ethylidene-triphenylphosphorane and acetaldehyde, the *cis* TS is 3.3 kcal/mol lower in energy than the corresponding TS leading to the *trans* oxaphosphoethane and this difference is in agreement with the experimental high *Z*-stereoselectivity of this reaction (Figure 2.1).

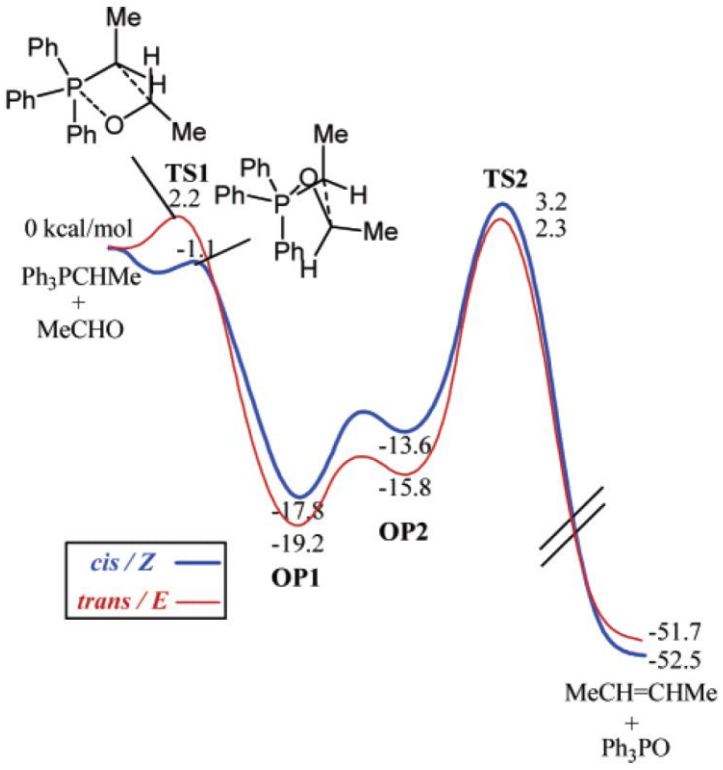


Figure 2.1. Comparison of energy during the Wittig reaction between ethylidene-triphenylphosphorane and acetaldehyde for *cis* and *trans* transition states as reported by Aggarwal, Harvey and co-workers.[76] (TS: transition state; OP: oxophosphoethane)

The stereoselectivity in the synthesis of non-conjugated alkenes is regulated by different factors including:

- Substituents to phosphorus;
- temperature of reaction;
- salt effect;
- solvent of reaction.

The role of substituents at phosphorus was investigated by Vedejs, showing that bulky groups, such as phenyl, lead to high *cis*-stereoselectivity, while small alkyl substituents favour the formation of *trans* alkenes.[77] This experimental investigation has been confirmed by the computational study of Aggarwal, Harvey and co-workers[76] and justified by the different 1,3 interaction between the aldehyde substituent and the phosphorus substituents.

Schlosser and co-workers showed the critical role of the temperature in *Z*-selectivity during the synthesis of non-conjugated alkenes for the Wittig reaction (Table 2.1).[79] The behavior can be explained by the difference in energy between the transition states involved in the synthesis of *trans* and *cis* isomers.

Table 2.1. Relationship between the temperature and the *Z:E* ratio during the Wittig reaction between the ylide generated from ethyltriphenylphosphonium bromide and sodium amide, and pentanal.[79]

Temperature	***Z*:*E* ratio**
25° C	87:17
0° C	90:10
−25° C	87:13
−50° C	94:6
−75° C	96:4
−100° C	97:3

The stereoselectivity of the Wittig reaction is also strongly influenced by the presence of lithium salts in solution. It has been reported by Maryanoff and co-workers that during the synthesis of non-conjugated alkenes, the *Z:E* ratio decreases as the concentration of lithium cation increases (Table 2.2).[80]

Table 2.2. Relationship between the concentration of Li^+ and the *Z:E* ratio during the Wittig reaction between the ylide generated from propylltriphenylphosphonium bromide and lithium hexamethyldisilazane, and pentanal at −78° C.[80]

Concentration of Li^+	***Z:E* ratio**
0.03 M	90: 10
0.07 M	85:15
0.25 M	80:20
0.31 M	82:18
0.90 M	78:22

In order to decrease this "lithium effect", highly stereoselective Wittig reactions were carried out using hexamethylphosphoramide, HMPA (sporadically abbreviated as HMPT, hexamethylphosphoric triamide), in solutions where lithium ions were present. This additive is an useful polar aprotic solvent, which has a highly polarized P=O double bond that captures lithium ions and consequently increases the *Z/E* ratio.[81] The same co-solvent can similarly form complexes with Na ions and for this property is generally employed in stereoselective Wittig reactions where sodium hexamethyldisilazane (NaHMDS) is used as a base (the effect of Na ions on the stereoselectivity is however less prominent in comparison to lithium ions).[82] Nevertheless, HMPA has been shown to induce nasal cancer in rats even at low concentration,[83] and it must be considered as a substance potentially carcinogenic to humans by inhalation.[84, 85]

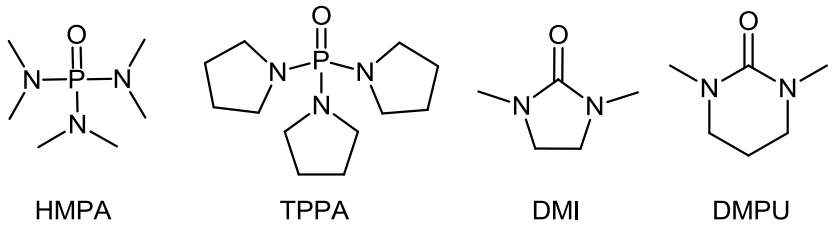


Figure 2.2. Some highly polar aprotic solvents used in salt-free conditions.

Useful alternatives in this sense are *NN'N''*-tris(tetramethylene)phosphoramide (TPPA), 1,3-dimethyl-2-imidazolidinone (DMI) and 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU) (Figure 2.2). Although TPPA has the highest known electron-donating power,[86] the high cost of this solvent limits its use, while the two tetrasubstituted ureas (DMI and DMPU) are widely used as co-solvents for their high dipole moment.[87] Nevertheless, the 2:1 mixture of THF and DMI has been shown not to be completely soluble at –30 ºC,[88] unlike a 1:2 mixture of DMPU with THF which has been reported to give clear and homogeneous solutions at –90 ºC.[89] These findings allow considering DMPU as the best alternative to HMPA in the Wittig reaction where the *Z* isomer is required.

2.1.3 Transition-metal-catalysed cross-coupling reactions

The construction of carbon−carbon bonds has always played a essential role in organic synthesis. Although several reactions allow C−C bond synthesis, the cross-coupling reaction, a reaction between an organometallic reagent and a carbon electrophile (Figure 2.3), has a fundamental relevance.[90, 91]

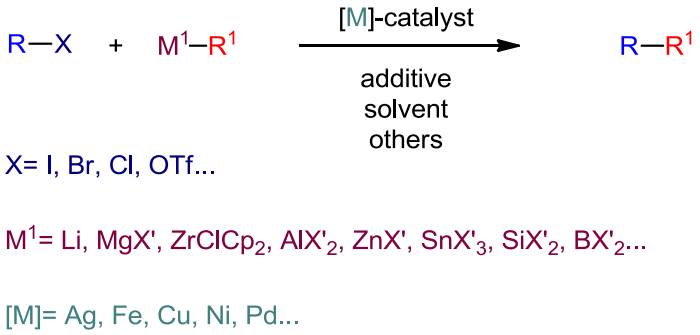


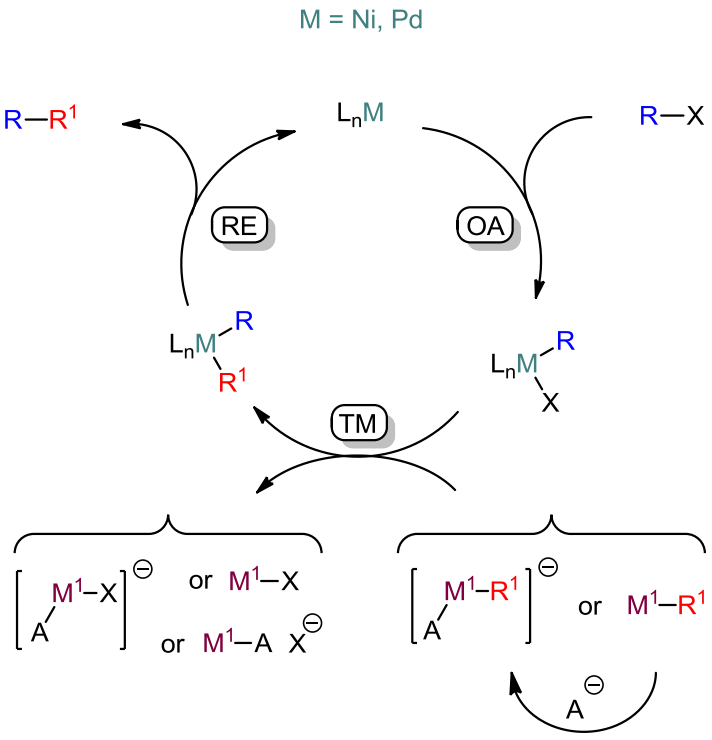
Figure 2.3. General scheme for transition-metal-catalysed cross-coupling reactions.

Initially, this coupling employed organolithium or Grignard reagents by catalysis of a variety of transition metal halides (generally silver[92], copper[93] or iron[94]). These harsh conditions, due to the reactivity of such organometallic reagents (Table 2.3), however, limited the presence of functional groups (although nowadays functionalised organo-lithium[95] and –magnesium[96, 97] intermediates are widely used in total synthesis despite their low stability). In addition, these catalysts led to the formation of homo coupling and disproportionation by-products, reducing the applicability of such transformations.[98]

Table 2.3. Typical metals used in organometallic reagents and their relative reactivity reported as the difference of Allred – Rochow electronegativity values between carbon and metal (adapted from Knochel *et al.*[99]).

M	$_{3}$Li	$_{12}$Mg	$_{40}$Zr	$_{13}$Al	$_{30}$Zn	$_{50}$Sn	$_{14}$Si	$_{5}$B
χ_C-χ_M	1.53	1.27	1.22	1.03	0.84	0.78	0.76	0.49
	← **Increasing reactivity of M−R reagents**							

Only in the 1970s with the application of, at first, nickel[100] and, subsequently, palladium as catalyst, the cross-coupling reactions began becoming common and essential transformations in laboratories of synthetic and medicinal chemistry. These named reactions couple, in presence of nickel or palladium, carbon electrophiles (generally halides) with organic derivatives of magnesium (Kumada-Tamao[100], Corriu[101]), boron (Suzuki-Miyaura)[102, 103], tin (Stille[104], Migita[105]), zinc (Negishi[106]), or silicon (Hiyama[107]). The mechanism involved in these reactions is similar both in the presence of palladium[108] and nickel[109], and it can be generalized as shown in Figure 2.4.



M^1:	**Named Reaction**:	**Additives (A^-)**
MgX'	Kumada-Tamao-Corriu	LiCl
ZnX'	Negishi	Br^-, Cl^-
SnX'_3	Stille	F^-, OH^-
BX'_2	Suzuki-Miyaura	OR^-, F^-, OAc^-, CO_3^-, PO_4^{2-}, Et_3N...
SiX_3	Hiyama	F^-

Figure 2.4. General catalytic cycle in palladium- or nickel-catalysed cross-coupling reactions that starts with oxidative addition (OA) of the electrophile to transition metal, followed by transmetalation (TM) step and final reductive elimination (RE).

The catalytic cycle starts with the oxidative addition of the electrophile to a coordinatively unsaturated low-valent transition metal to give an organonickel or organopalladium compound, to which the second organic group is transferred from organometallic nucleophile by transmetalation. The diorganometal species undergoes

reductive elimination *via* a concerted mechanism to yield the organic product and the low valent metal that restarts a new catalytic cycle.

It is important to emphasize that the all the named cross-coupling reactions mentioned above differ only in the transmetalation reaction, which represents the most peculiar and controversial step of the reaction.[108] The transmetalation may proceed *via* open or cyclic mechanism,[108] and although it depends on the reaction conditions, it is important to highlight that transmetalation takes place only if the organometallic reagent is sufficiently nucleophilic. The role and importance of additives in the activation of the organometallic species in cross-coupling reactions is a current theme of research.

In the study of reaction between organoelectrophiles and boranes it was Suzuki [102, 103] who observed that an additional base was required to accelerate the coupling. Nevertheless, the role of the base was not clear for two decades[110] until it was shown that hydroxide ion forms a hydroxyborate complex that permits the transmetalation.[111] However, more recently, experimental and computational studies of cross-coupling reactions with vinyl[112] and aryl[113-115] boronic acids have showed that transmetalation in Suzuki-Miyaura reaction may occur *via* alternative pathways. In particular, it was suggested that the transmetalation may proceed *via* initial replacement of the halide with the hydroxide/alkoxide ion to form a nucleophilic (oxo)palladium(II) complex that undergoes reaction with the neutral organoboron species.

In the Hiyama coupling the additive is a fluorine source that activates the silane *via* pentacoordinate silicon- ate complex.[116] Similarly to organosilanes, tin derivates undergo cross-coupling through the activation of a fluorine ion *via* a hypervalent fluorinated organotin species.[117] Moreover, it was reported that alkyl-, aryl-, and vinyltrichlorostannaned derivatives react in aqueous solution to afford cross-coupled products *via* hydroxide ion catalysis.[118]

Although in the cases previously described the importance of the activation of organometallic reagents in the transmetalation step is not-unexpected, due to low reactivity of the reagents and to low ionic character of the C−M bond (Table 2.3), it is somewhat surprising that also organozinc and Grignard-type reagents undergo transmetalation under catalysis of an additive. Knochel has recently shown that the

presence of lithium chloride facilitates the preparation of Grignard reagents, and results in the formation of a complex (RMgX·LiCl) with higher reactivity towards electrophiles.[119] These activated Grignard reagents, known as Knochel-type Grignard reagents, have been also used in Kamada-Tamao-Corriu reactions.[120, 121] Similarly, Knochel has also demonstrated that the use of lithium chloride in the preparation of organozinc derivates yields RZnX·LiCl complex with higher reactivity.[122] Subsequently it has been reported that the presence of lithium chloride is essential in Ni- or Pd-catalized cross-coupling reactions of organohalides, pseudohalides or CO_2 with organozinc compounds.[123-128]

The importance of lithium halide in the activation of organomagnesium and organozinc reagents in cross-coupling reactions is not completely clear. Knochel attributed the LiX-based reactivity to the breaking up of polymeric aggregates of these reagents by lithium halide salts. More recently, the need of an excess of lithium chloride for the activation of alkyl zinc bromide in the Negishi reaction was justified for the formation of a higher order zincate,[129, 130] due to the complex between a molecule of organozinc and two molecule of lithium halide, which was postulated as the reacting species.[131] The activation of organozinc reagents by lithium halide salts clarified the different reactivity observed using reagents prepared by different methods. In particular, commercially available organozinc reagents, prepared using Rieke's method,[132] are organometallics with higher reactivity for the presence of LiCl as by-product.[131]

The next section discusses the practical differences between the cross-coupling reactions mentioned previously, initially introducing the Suzuki-Muyaura reaction and then the Negishi reaction.

2.1.3.1 The Negishi reaction

Among the named cross-coupling reactions mentioned in the previous section, the Suzuki-Muyaura reaction is doubtless the most popular,[133-135] by *virtue* of the stability of organoboron reagents to heat, oxygen, and water, which permits very mild reaction conditions and user-friendly handling. Indeed, this reaction permits a very high tolerance to most functional groups, especially in comparison to the Kumada-Tamao-Corriu reaction, which should however be considered as the first option[135, 136]. Moreover, organoboranes are generally non-toxic compounds, especially in comparison to the very toxic organotin reagents, that permit green[137] aqueous and protein cross-couplings.[138] Additionally the wide commercial availability of boronic acids and esters have made it a common reaction in combinatorial and medicinal chemistry.

Conversely, the stability of organboran reagents, due to the covalent nature of C−B bond (Table 2.3), occasionally makes these organometallics poor transmetalating agents. Organozinc reagents, however, represent "a good compromise"[139] between reactivity in transmetalation and tolerance to functional groups. In recent years the interest and application of the Negishi reaction have been increased by the development of highly efficient preparations of organozinc regents possessing unmasked functional groups,[140] using direct insertion of zinc metal, activated by iodine[141] or lithium chloride[122], or through a halogen−zinc exchange reaction using dilithium tetra-*tert*-butylzincate,[142, 143] or *via* direct zincation by mixed-metal base[144, 145].

In the next section it will be discussed a transformation where organozinc reagents are generally more efficient than organoboranes: the cross-coupling reactions between unactivated sp^3 carbon centres.

2.1.3.2 Alkyl−Alkyl cross-coupling reaction

Although between 1970s and 1990s several cross-coupling reactions of a wide variety of organo-electrophiles (such as aryl, alkenyl, benzyl, allyl and alkynyl halides) with various organometallic reagents have been successfully reported, such a transformation was considered for non-applicable to the cross-coupling of alkyl electrophiles bearing β-hydrogens with alkyl organometallics. Indeed, until first years of 1990s only unsuccessful alkyl−alkyl cross-couplings were reported.[146]

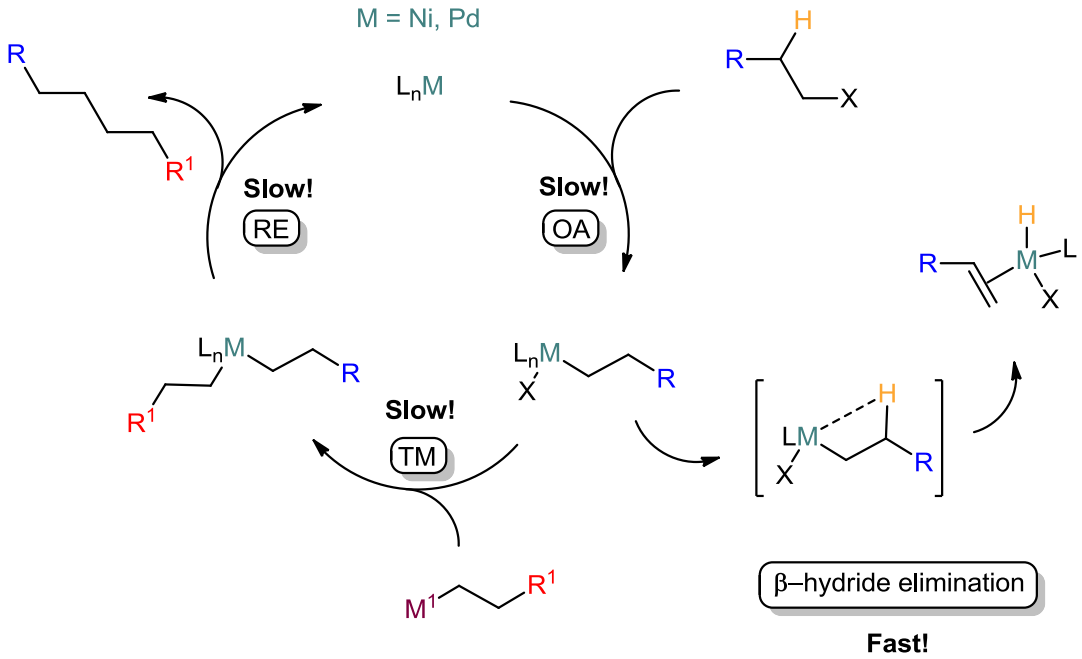
The difficulties met in alkyl−alkyl cross-couplings are due mainly to low reactivity of alkyl electrophiles and nucleophiles, which influences the catalytic cycle of such as transformation (Scheme 2.4) in four points:[147, 148]

1) slow oxidative addition of alky halides to the transition metal;

2) rapid β-hydride elimination from the alkyl–Pd(II) complex;

3) slow transmetalation by alkyl nucleophile;

4) slow reductive elimination.



Scheme 2.4. Catalytic cycle in alkyl−alkyl cross-coupling reactions.

The reluctance of haloalkanes to undergo oxidative addition, in comparison with the widely used allyl, alkenyl and aryl halides, is the initial issue (Figure 2.5). In this reaction the transition metal is considered the nucleophile and the organo halide the electrophile. Ligands attached to the transition metal, which increase the electron density, also increase the rate of the reaction.

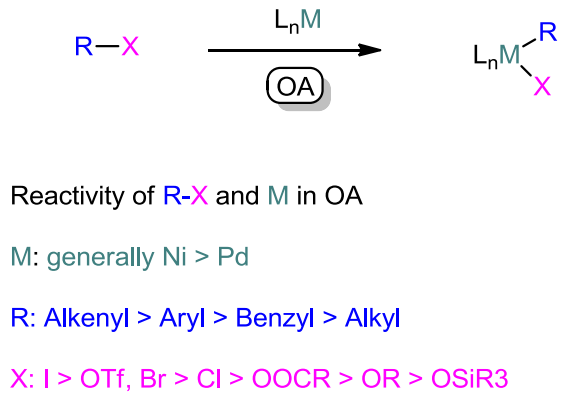
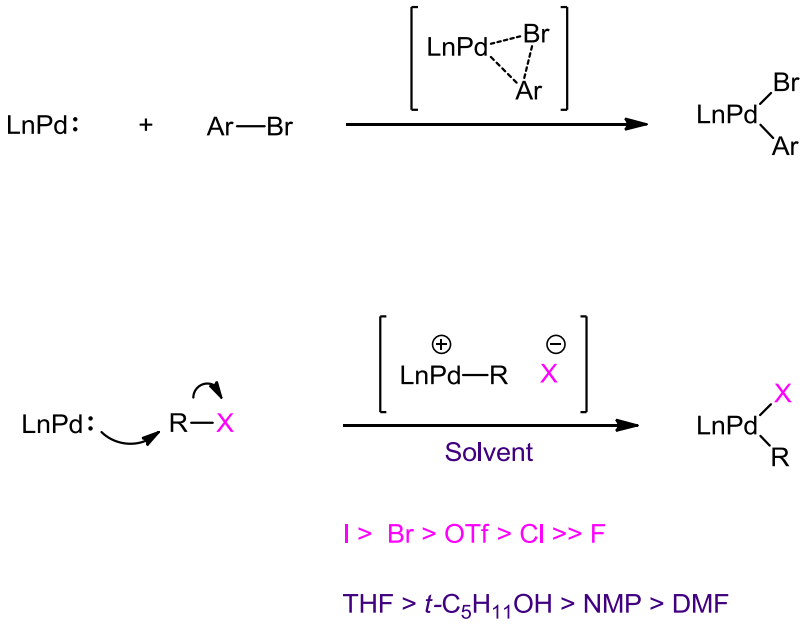


Figure 2.5. Approximate general order of the reactivity of electrophiles with transition metals during oxidative addition.

In the case of the oxidative addition of aryl halides to transition metals, it occurs smoothly *via* a concerted mechanism. Here, the reaction is facilitated by a π back-bonding interaction between the d orbital of metal and the Ar−X π^* orbital in the transition state. Conversely, a similar interaction is not possible in the oxidative addition of alkyl electrophiles to the transition metal and consequently the activation barrier is much higher.[149] Indeed Fu *et al.* showed that the oxidative addition for alkyl substrates occurs *via* a S_N2 mechanism, where the polarity of the solvent and the nature of leaving group have a substantial effect on the rate of the reaction.[150] The reactivity of the electrophile also depends on the leaving group and Fu *et al.* have calculated the rates of reaction for oxidative addition (Scheme 2.5). [108, 136, 150] These data justify the initial employment of iododerivates in alkyl−alkyl cross-coupling reactions.



Scheme 2.5. Mechanisms of the oxidative addition of aryl- and alkyl-electrophiles to palladium. For alkyl substrates the effect of the solvent and the leaving group on the rate of the reaction have been reported by Fu *et al*.[150]

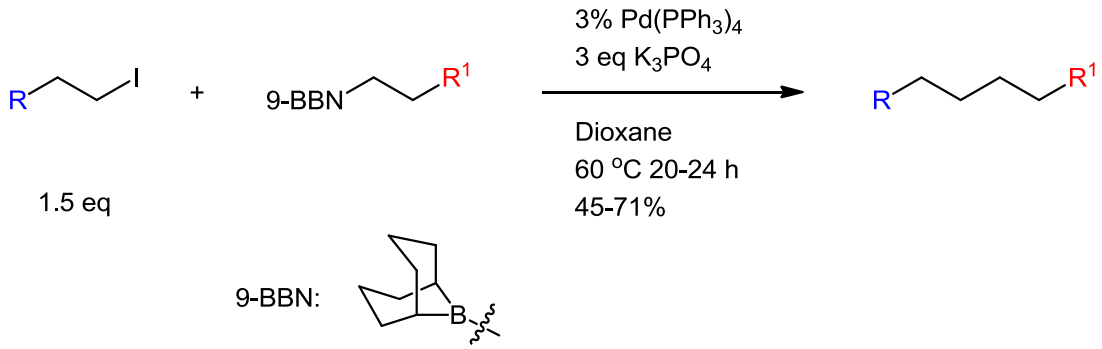
A second, and maybe more challenging issue, is the tendency of alkyl−Pd or -Ni derivates to undergo degradation *via* β-hydride elimination before undergoing transmetalation with the organometallic reagents. This concern is due to the high rate of β-hydride elimination, but also to the low reactivity of alkyl organometallics, which are poor transmetalating agents in comparison with unsaturated reagents.[151]

Finally, the reductive elimination of the coupling product formed from the dialkylmetal complex is slower in comparison with the same process from unsaturated substrates.

The reluctance of dialkyl−Ni and -Pd complexes to undergo reductive elimination is due to the high electron-donor ability of Csp^3 centres, which provide an electron-rich organometallic intermediate less prone to reductively eliminate the alkyl coupling product.[152]

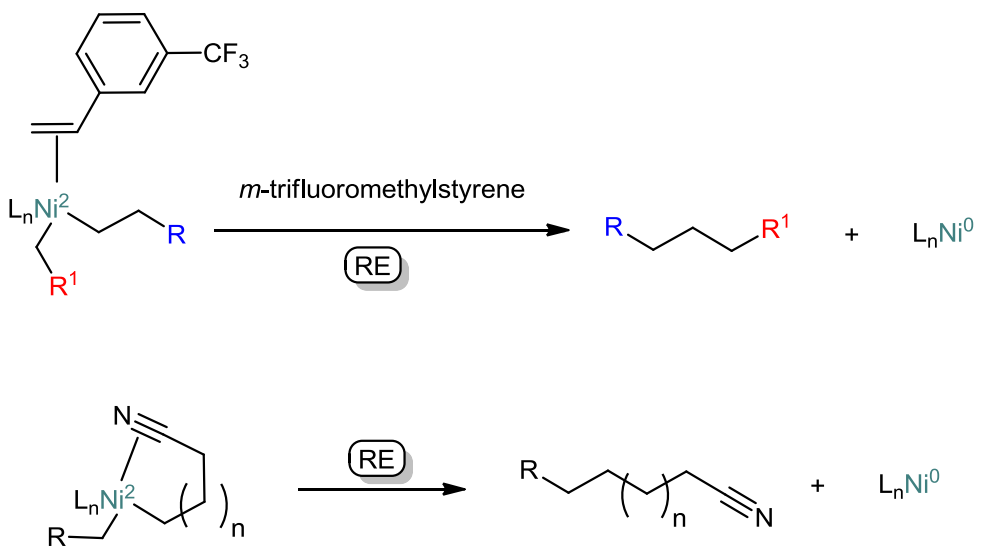
Despite these problems, in 1992 Suzuki *et al.* reported the first successful alkyl−alkyl cross-couplings between iodoalkanes and alkylboranes (*B*-alkyl-9-

BBN)[153, 154] in the presence of tetrakis(triphenylphosphine)palladium as catalyst (Scheme 2.6).[155]



Scheme 2.6. First successful alkyl−alkyl cross-couplings reported by Suzuki and co-workers.[155]

Although this achievement represents an important milestone, the yields reported for cross-couplings of alkyliodides were not high and under these conditions alkyl bromides failed to provide the expected coupling products. Successively, in order to overcome the low reactivity of alkyl electrophiles to undergo oxidative addition, nickel was preferred to palladium as transition metal by virtue of its higher reactivity.[156] In 1998 within the Knochel group, Giovannini *et al.*[157, 158] reported an efficient Ni-catalysed alkyl−alkyl cross coupling of alkyl iodide and diorganozinc compounds in presence of a cocatalyst to promote reductive elimination. In this protocol nickel catalyst affords a fast oxidative addition of alkyl iodide and the resulting organonickel complex smoothly undergoes transmetalation without degradation *via* *β*-hydride elimination. Finally the reductive elimination of the dialkylnickel complex is promoted by a π-acceptor ligand, such as *m*-trifluoromethylstyrene or acetophenone, which removes electron density from the nickel atom facilitating the synthesis of cross-coupling product and the regeneration of active catalyst (Scheme 2.7).[159]



Scheme 2.7. Reductive elimination of the dialkylnickel complex promoted by a π-acceptor ligand via an external promoter or via intramolecular coordination. [152, 160]

Interestingly, the same group showed that the co-catalyst is not necessary if a remote unsaturation (double bond, carbonyl group, cyano group) is present in the dialkylnickel complex, facilitating the cross-coupling reaction *via* intramolecular complexation (Scheme 2.7).[152, 160] In 2002, Knochel extended the same principle to the Ni-catalysed cross-coupling of alkyl iodides with less reactive and more functional-group-tolerant alkyl zinc iodide.[161] In this methodology, alkyl zinc iodides are activated by tetrabutylamonium iodide and the reductive elimination is promoted by 4-fluorostyrene.[162]

Although the application of nickel in alkyl−alkyl cross coupling was important and remains relevant,[109] it has recently been surpassed by palladium chemistry. In general, palladium catalysts offer advantages for the higher stability and lower reactivity in comparison with nickel-based systems, facilitating a wider tolerability of functional groups and a lower tendency to generate homocoupling side-products.[135, 148]

More recently, Fu and co-workers at the Massachusetts Institute of Technology and Organ and co-workers at York University have separately developed general and efficient protocols for alkyl−alkyl cross coupling reactions catalysed by bulky and

electron rich palladium species. These Pd-based catalysts contain sterically hindered and σ-donor ligands, such as phosphines (PR_3) and *N*-heterocyclic carbenes (NHC) (Figure 2.6), which show high catalytic activity for a wide range of cross-coupling reactions.[135, 163] Generally, ligands with strong σ-donor properties favour the oxidative addition step, while bulky groups favour the reductive elimination step.

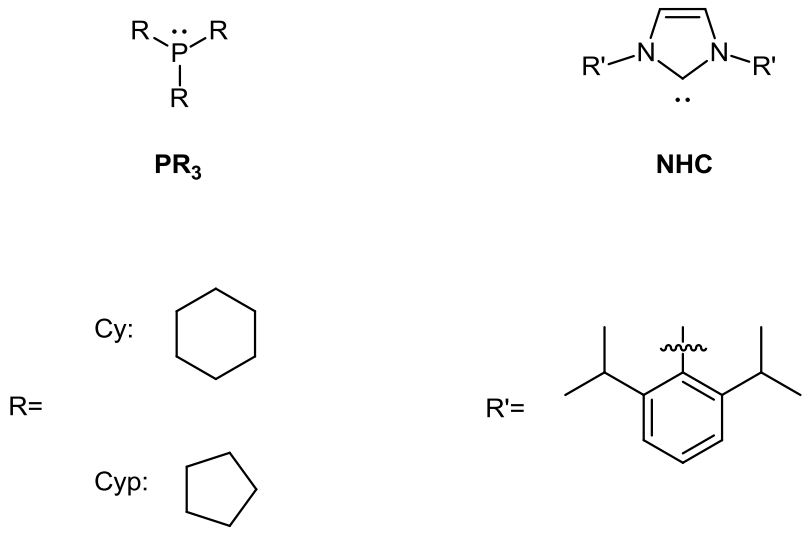
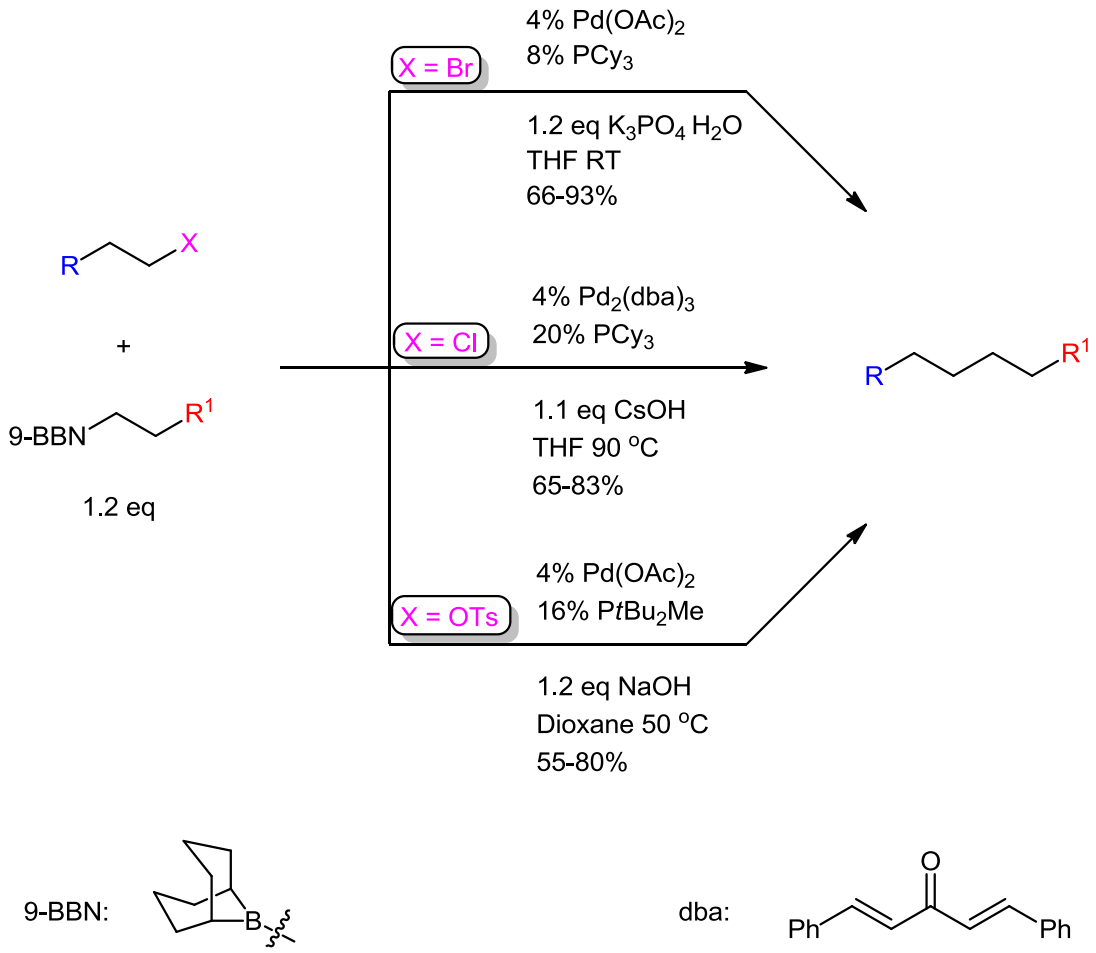


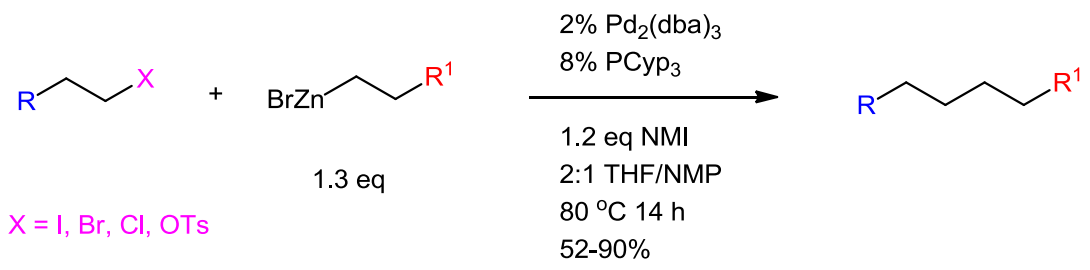
Figure 2.6. Examples of bulky and electrodonating ligands for Pd(0)Ln complexes.

Phosphine-type ligands were the first to be exploited in alkyl−alkyl cross-coupling reactions and were initially reported by Suzuki and co-workers in 1992.[155] However, only a decade later these ligands had been thoroughly investigated after the screening of different phosphines by Fu and co-workers. They reported that compounds with alkyl substituents at the phosphorus atom lead to ligands with higher σ-donating properties and more efficient in alkyl−alkyl cross-couplings in comparison with triphenylphosphine. In particular the Fu's group reported high-yield cross-couplings of primary bromides,[164] chlorides[165] and tosylates[166] with *B*-alkyl-9-BBN. In these protocols the reacting palladium species is generated *in situ* from air-stable palladium source: $Pd(OAc)_2$[167] or $Pd_2(dba)_3$,[168, 169] and phosphines: PCy_3 or P*t*-Bu_2Me.



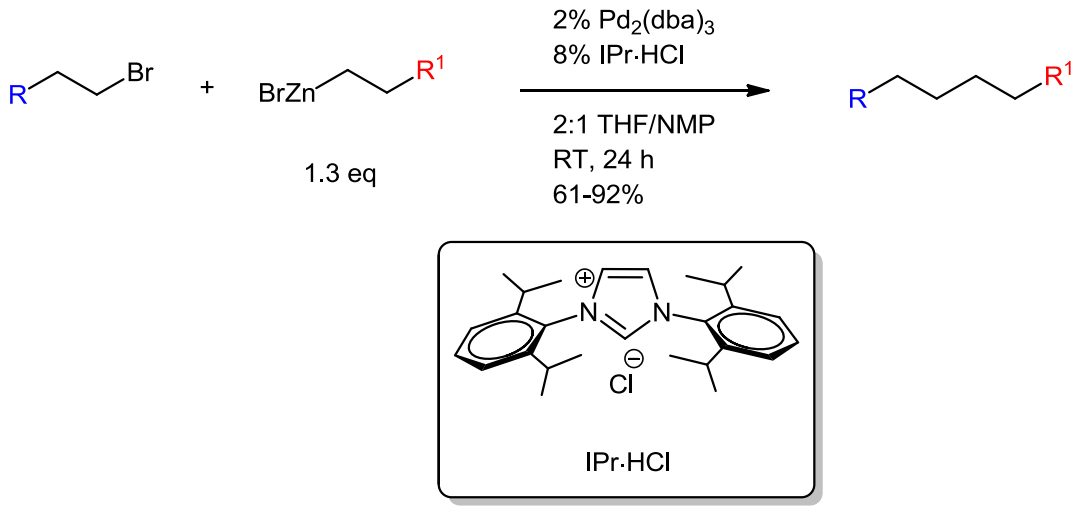
Scheme 2.8. Protocols of alkyl−alkyl Suzuki-type cross-coupling reactions developed by Fu and co-workers.

Successively, Fu and Zhou have reported the first general and efficient protocol for palladium-catalysed Negishi couplings of primary iodides, bromides, chlorides and tosylates. In this protocol the catalytic species is generated in situ from $Pd_2(dba)_3$ and $PCyp_3$ and the alkyl zinc bromide reagents are activated by *N*-methylimidazole (NMI).[170] It is important to note that in this work Zhou not only showed the essential role of the ligand, but also the importance of NMP as co-solvent and NMI as activating agent for alkyl zinc bromides.[170, 171]



Scheme 2.9. Palladium-catalysed alkyl−alkyl Negishi cross-coupling reported by Zhou and Fu in 2003.

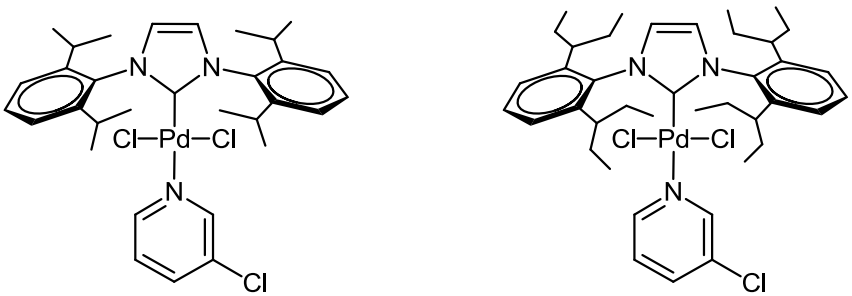
Contrary to phosphine ligands, *N*-heterocyclic carbenes (NHCs) have been developed as ligands in Pd-mediated cross-couplings and related transformations only in the last 15 years.[172] Although these compounds have been initially considered only "phosphine mimics", they have received increasing attention due to their superior performance compared to the more traditional phosphine ligands.[173, 174] These ligands have excellent σ-donor properties that permit facile oxidative addition even in challenging substrates, while their steric bulk facilitates the reductive elimination step. In addition the catalytic Pd−NHC complexes are characterized by a strong metal−ligand bond that contributes to the high stability of the active species at high temperatures, preventing the formation of black palladium and catalyst death.



Scheme 2.10. First alkyl−alkyl Negishi cross-coupling reaction utilizing a Pd−NHC catalyst.

Despite these advantages and the successful applications in a variety of Pd-catalysed reactions, NHC ligands realised only limited success in alkyl−alkyl cross-couplings until 2005,[165, 170, 175] when Organ and co-workers published an efficient protocol for the Negishi cross-coupling of alkyl bromides, where the catalytic species is generated *in situ* from $Pd_2(dba)_3$ or $Pd(OAc)_2$ and the NHC ligand *N*,*N*′-bis(2,6-diisopropylphenyl)imidazol-2-ylidene (IPr).[176, 177] The same group has contemporaneously reported a combined experimental and computational study of different NHC ligands, showing that IPr carbene has a superior efficiency in alkyl−alkyl cross-couplings, which is a consequence of a strong influence of the steric topography around the metal centre on catalytic cycle.[178] These conclusions were confirmed successively by Chass *et al.* showing that with IPr ligand, in contrast to other NHC ligands, has four hydrogen atoms from the isopropyl groups stabilize the alkyl−Pd^{II}−NHC complex by coordinatively saturating the metal and suppressing the β-hydride elimination.[179]

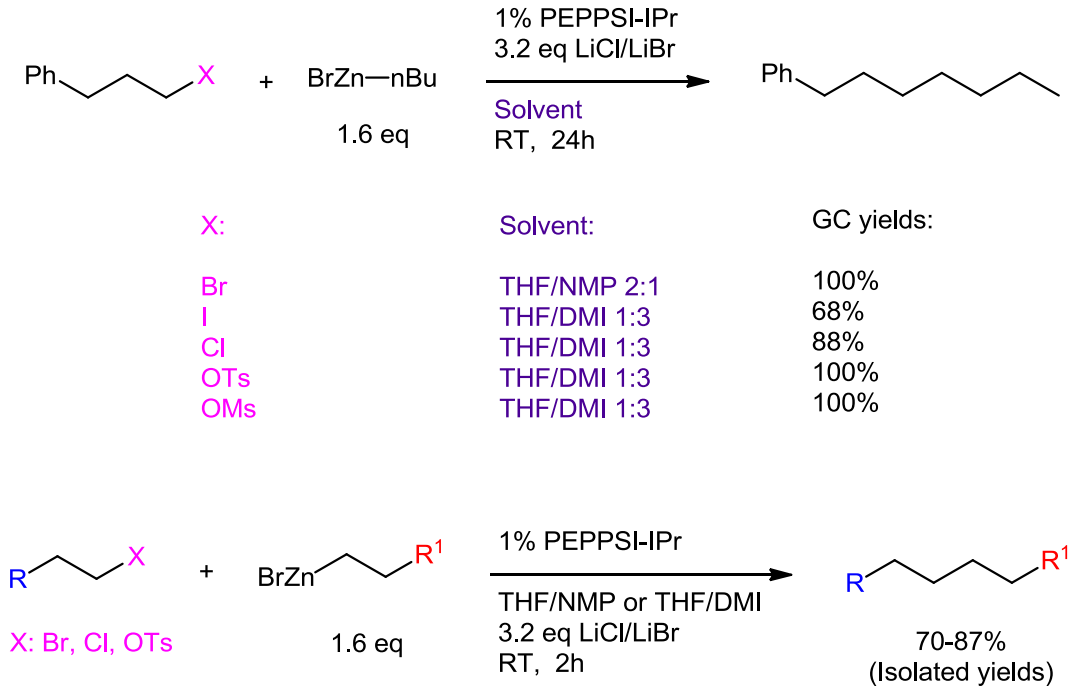
The application of the NHC-type ligands to cross-coupling reactions has become more popular since 2006, when Organ *et al.* reported a general protocol for cross-coupling reactions by novel air- and moisture-stable Pd−NHC complexes.[123, 180] These complexes, commercially available from Aldrich, are based on stable Pd(II) species bearing one NHC ligand, two anionic ligands (Cl), and a fourth ligand: 3-chloropyridine. The pyridine ligand is a "throw-away" ligand that plays a pivotal role at different stages of catalytic cycle, and by virtue of this effect these complexes were named PEPPSI (pyridine-enhanced precatalyst preparation, stabilization, and initiation).



PEPPSI-IPr PEPPSI-IPent

Figure 2.7. Examples of PEPPSI (pre)catalysts introduced by Organ and co-workers.

These precatalysts are easily converted to Pd(0) species *via* double transmetalation and reductive elimination.[180] Among this class of precatalysts (two examples are shown in Figure 2.7), PEPPSI-IPr was shown to be superior to the others for alkyl−alkyl Negishi couplings, due to the above-mentioned advantages of the active catalyst form (IPr). However, the use of the precatalyst was demonstrated to give extremely higher rates[180] in comparison with the protocol that generates the catalytic Pd−NHC species *in situ* from $Pd_2(dba)_3$ and the NHC ligand. Indeed it was reported that PEPPSI-IPr permits alkyl−alkyl cross-coupling reactions of iodides, bromides, chlorides, tosylates, and mesylates with alkylzinc halides in high isolated yields or quantitative GC conversions (Scheme 2.11).[123]



Scheme 2.11. Scheme 1. Palladium-catalysed alkyl−alkyl Negishi cross-coupling reported by Organ and co-workers.[123]

It is important to highlight that in this protocol, halides and pseudohalides undergo PEPPSI-catalysed Negishi cross-coupling reaction by a judicious choice of solvent. Indeed, alkylbromides are smoothly cross-coupled using THF/NMP 2:1 mixture, while alkylchlorides, iodides, tosylates and mesylates require a more polar solvent mixture with DMI. In particular, it is suggested that sulfonyl compounds are

converted to alkyl halides *in situ* before undergoing oxidative addition.[123] Nevertheless, Organ *et al.* did not provide a full explanation for the necessity of this solvent dependence. It is important to highlight that Organ *et al.* have also demonstrated that the use of different solvents for cross-coupling reactions of alkylbromides and chlorides permits interesting synthetic selectivity.

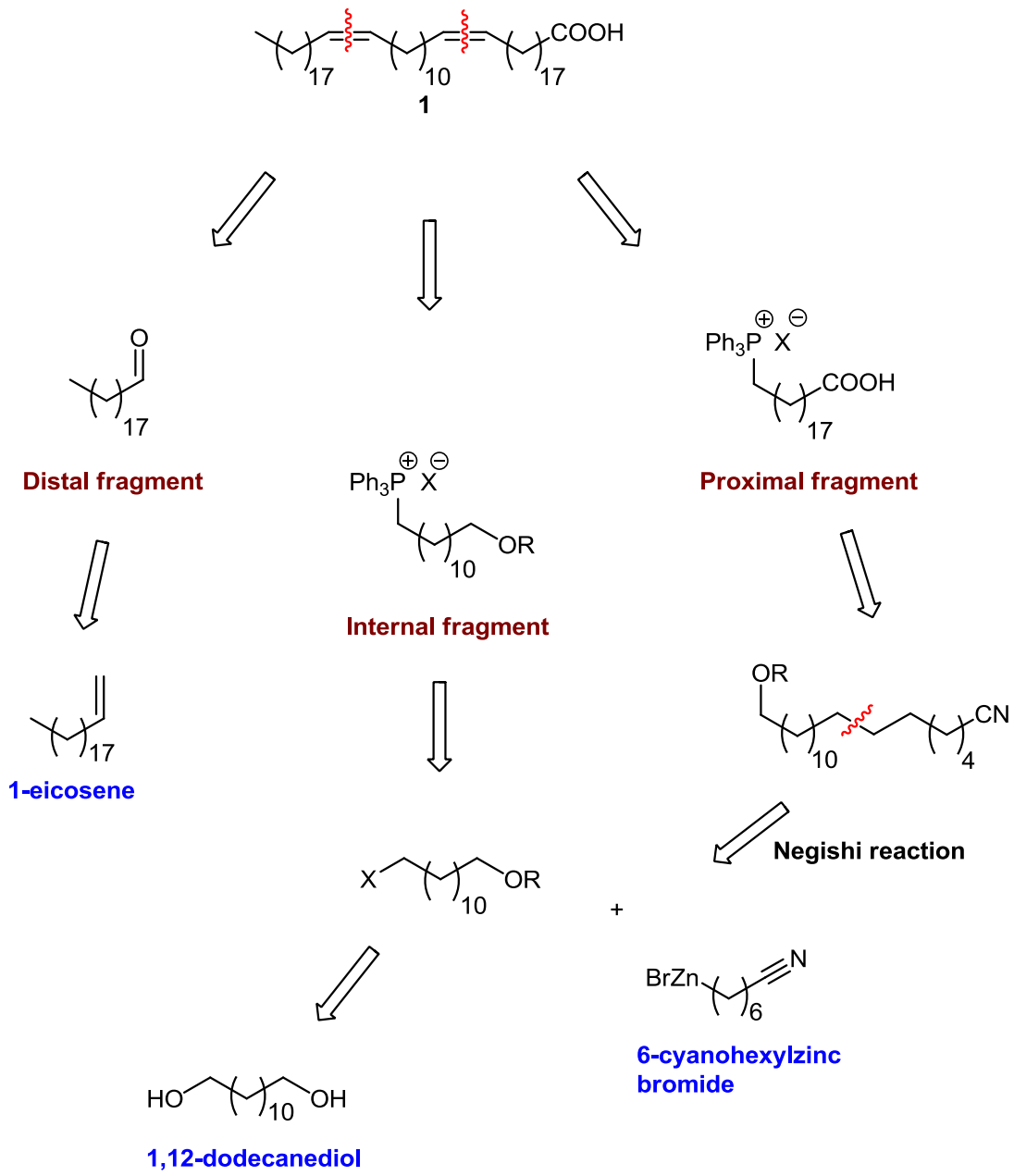
More recently, a novel PEPPSI catalyst, PEPPSI-IPent (Scheme 2.7), was found superior to other members of the same family for cross-couplings of sterically hindered and heterocyclic substrates,[181, 182] whereas PEPPSI-IPr continues to be the preferential catalyst for alkyl−alkyl Negishi[140, 179, 183] and Suzuki-Muyaura[111] cross-couplings.

2.2 RESULTS AND DISCUSSION

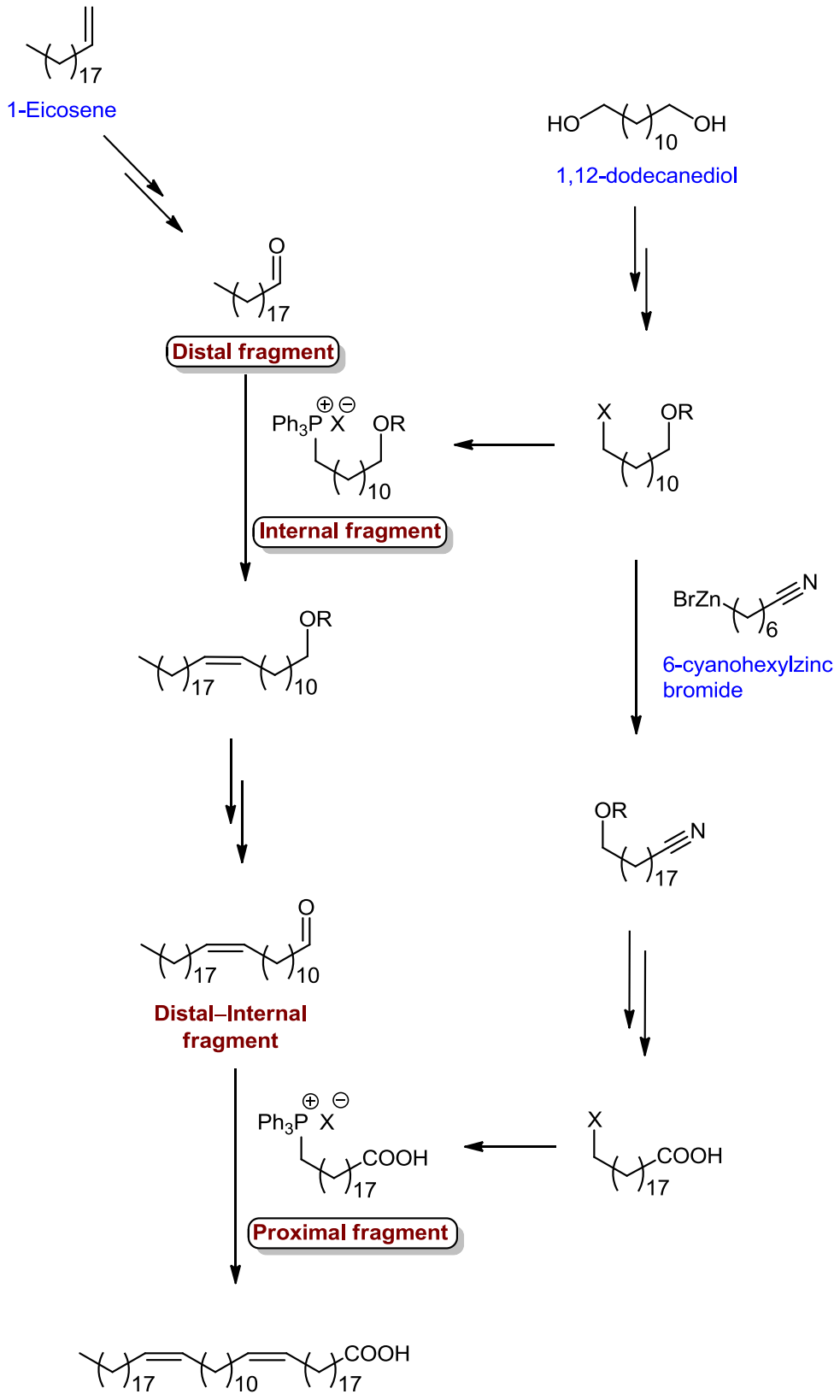
2.2.1 Synthetic strategy toward the *cis,cis*-diunsaturated meromycolic acid

According to our retrosynthetic analysis (Scheme 2.12), the synthesis of the *cis,cis*-diunsaturated meromycolic acid could be synthesised *via* Wittig reactions of three fragments from cheap and easily available starting materials *via* convergent strategy. The synthesis starts with the preparation of these three fragments: the distal fragment, 1-nonadecanal, was prepared from 1-eicosene (Scheme 2.13); the internal fragment, a C_{12} phosphonium salt, was prepared from 1,12-dodecanediol *via* halo-dodecanol derivate, which was used also for the preparation of the proximal fragment, a C_{19} phosphonium salt, by the Negishi reaction with 6-cyanohexyl zinc bromide and subsequent functional group interconversions.

The distal and the internal fragments were assembled *via* Wittig reaction to form the distal *cis* double bond of the diunsaturated meromycolic acid, which was derivatised to an aldehyde intermediate. Finally, this distal−internal fragment was coupled with the distal fragment to prepare the proximal *cis* double bond and achieve the target compound.



Scheme 2.12. Retrosynthetic analysis of the cis,cis-diunsaturated meromycolic acid.

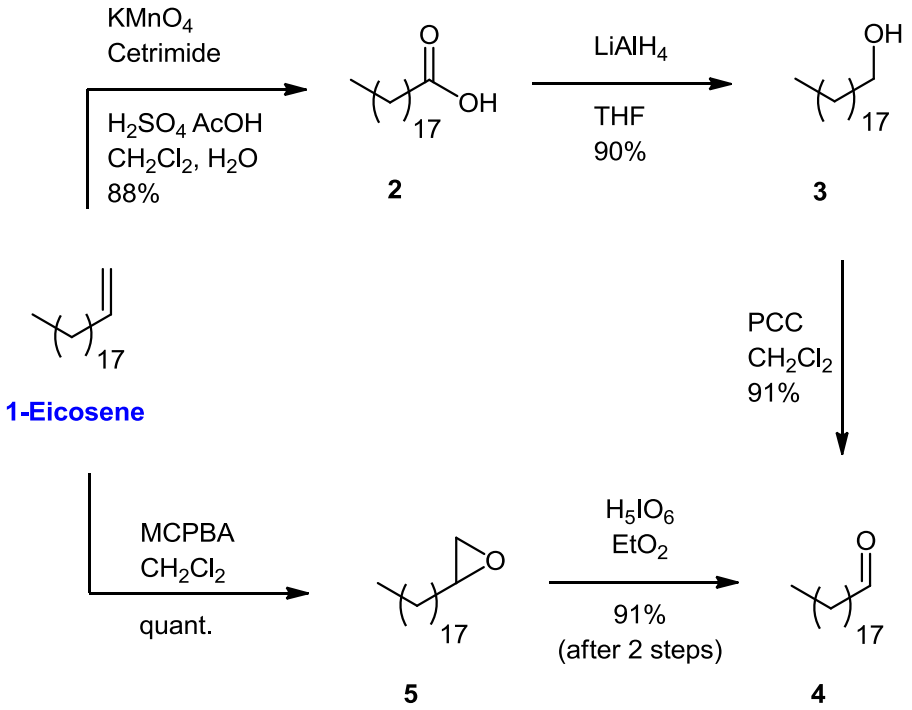


Scheme 2.13. Synthetic strategy toward the *cis,cis*-diunsaturated meromycolic acid *via* Wittig reactions.

2.2.2 Preparation of the distal fragment

2.2.2.1 Synthesis of 1-nonadecanal

The synthesis of nonadecanal was obtained from 1-eicosene using two different synthetic routes (Scheme 2.14). The first strategy included a series of oxidation-reduction reactions where the initial terminal alkene was subjected to oxidative cleavage by permanganate under phase-transfer conditions to give the corresponding carboxylic acid with one less carbon atom. [184]



Scheme 2.14. Synthesis of nonadecanal via two routes.

The acid **2** was then reduced by lithium aluminium hydride (LAH) to the corresponding primary alcohol in good yields. Nevertheless, frequently, especially in large scale, the reductions with LAH offer poor yields due to difficulties in work-up.

Indeed, during extractions, lithium aluminium salts form emulsions that are difficult to break. Although there are different methods to break these emulsions (*e.g.* Rochelle's salt), the procedure initially reported from Micovic and Mihailovic,[185] and nowadays often known as Fieser work-up,[186] which consists in the addition of water and aqueous solution of sodium hydroxide (see the synthesis of 1,14-tetradecanediol **34**), was found to be helpful.

The alcohol **3** was then oxidized to aldehyde using pyridinium chlorochromate (PCC).[187] The oxidation of 1-nonadecanol by PCC was conducted using anhydrous dichloromethane to avoid the formation of the aldehyde hydrate and the subsequent oxidation of this intermediate to carboxylic acid.[188] Purification of the reaction mixture was obtained simply by dilution and filtration through a silica gel pad to yield the aldehyde as a white solid. Attempts to catalyse the oxidation and increase reaction yields by addition of molecular sieves (3 Å) as reported by Antonakis *et al.*,[189, 190] were unsuccessful and gave similar yields.

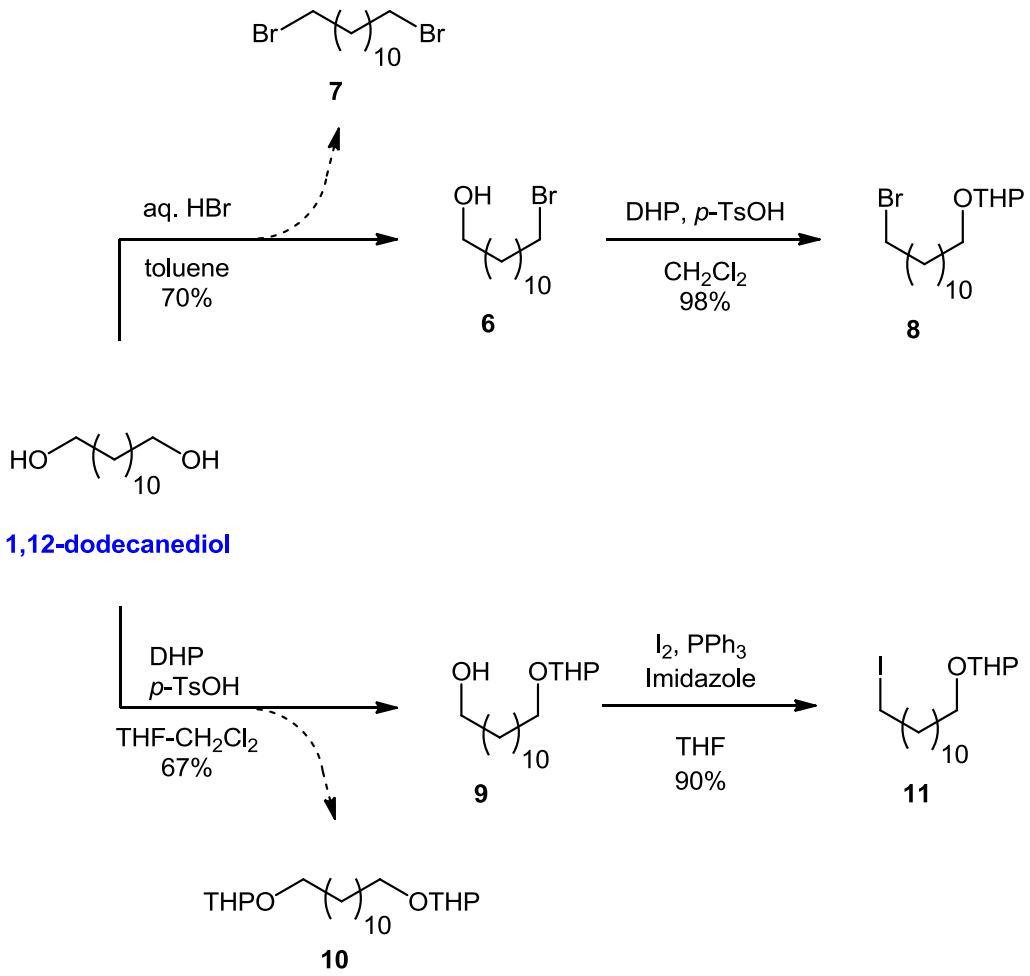
This route to nonadecanal offers almost quantitative conversions with no detectable by-products and without the need of chromatography purification. An alternative route to 1-nonadecanal was applied following the protocol of Coxon *et al.*[191] where the aldehyde was obtained from 1-eicosene *via* the epoxide intermediate **5** (Scheme 2.14). The epoxidation was obtained quantitatively by excess of 3-chloroperbenzoic acid (MCPBA).[192] The corresponding epoxide was subjected to oxidative cleavage by periodic acid to yield the nonadecanal in high yields.[193, 194] Parallel conversions of epoxide to aldehyde in anhydrous or aqueous diethyl ether gave similar yields.

Aldehydes are known for their instability and the tendency to be oxidised by atmospheric oxygen during storage. For these reasons, nonadecanal was generally used immediately or stored at −20 °C under nitrogen to prevent oxidation. Nevertheless, no oxidation was detected by NMR after storing of the aldehyde in a sealed vial at room temperature for two months.

2.2.3 Preparation of the internal fragment

2.2.3.1 Synthesis of 1-halo-12-(2'-tetrahydropyranyloxy)dodecanes

In order to synthesise the internal fragment of the *cis*,*cis*-diunsaturated meromycolic acid (Scheme 2.13), which will form after reaction with nonadecanal, the distal double bond, two C_{12} akyl halides were prepared. These intermediates will also conveniently be used to form the C_{19} fragment utilizing a Negishi coupling with a C_7 alkylzinc bromide.



Scheme 2.15. Synthesis of 1-halo-12-(2-tetrahydropyranyloxy)dodecanes from 1,12-dodecanediol.

These alkyl halides were synthesised from 1,12-dodecanediol (Scheme 2.15), using a 2-tetrahydropyranyl (THP) group to protect the remaining alcoholic moiety. The choice of this protecting group is due to its well-known stability in alkaline solution and its common use in the syntheses of phosphonium salts, Wittig olefinations and Negishi reactions. Conversely, the THP group is also generally labile to acidic conditions and its deprotection proceeds in high yields.

The synthesis of 1-bromo-12-(2-tetrahydropyranyloxy)dodecane was obtained *via* bromododecanol **6** and subsequent protection of hydroxyl group as THP ether. The bromination was accomplished by heating of a mixture of dodecanediol in toluene in presence of aqueous hydrobromic acid. Since HBr is a toxic gas, it was used (and highly recommended especially in large-scale reactions) a trap containing aqueous NaOH solution connected to the extremity of the condenser. Although the bromination using the Dean-Stark apparatus proceeded to a satisfying yield of 70%, it was noted that Amos Smith III and co-workers achieved 12-bromodecan-1-ol from the corresponding diol in 86% yield by continuous extraction of the product with heptane in order to avoid the formation of dibromo derivate.[195]

Finally the alcohol **6** was protected quantitatively by 3,4-dihydro-2*H*-pyran (DHP) in presence of a catalytic amount of *para*-toluenesulfonic acid (*p*-TsOH).

The synthesis of 1-iodo-12-(2-tetrahydropyranyloxy)dodecane was obtained *via* protection of a hydroxyl group of dodecanediol as THP ether and subsequent iodination of the remaining alcoholic group. The synthesis of alcohol **9** was obtained treating a solution of the diol in anhydrous tetrahydrofuran (THF) and dichloromethane with DHP and *p*-TsOH. The protection of the diol follows a statistical distribution that limits the yields and, in this case, excess DHP results in lower yields for the formation of diprotected derivate. Indeed Nishitani *et al.* reported the synthesis of compound **9** in yield of only 53% using 1.1 equivalents of DHP. In this work, instead, 1.0 equivalent of DHP was used and 1.4 equivalents of diol achieving the monoprotected dodecanol **9** in a satisfying yield of 67%. Nevertheless, it is important to report that in one occasion under these conditions the use of non-freshly-distilled THF resulted in a lower yield due probably to formation of by-products of DHP in presence of water and peroxides.

In the preparation of alkyliodide **11** the conversion of hydroxyl group into the iodo group was accomplished in high yields using triphenylphosphine, imidazole and iodine in anhydrous THF. Purification by flash chromatography afforded the iodide **11** as a colourless oil, which was stored under nitrogen in a dark container at −20 °C. Indeed, it is important to highlight that, as all alkyl iodides,[196] it is light and moisture sensitive, and storage upon exposure to light samples did develop a yellowish tinge caused by the formation of iodine.[197, 198] On the occasions this happened, a solution of the iodide in petroleum ether was washed with aqueous sodium thiosulfate solution to remove the iodine impurities and yield a colourless oil.

The purity of the iodide **11** is particularly important during the subsequent Negishi reaction, as the presence of iodine could affect the organozinc reagent and palladium catalyst.

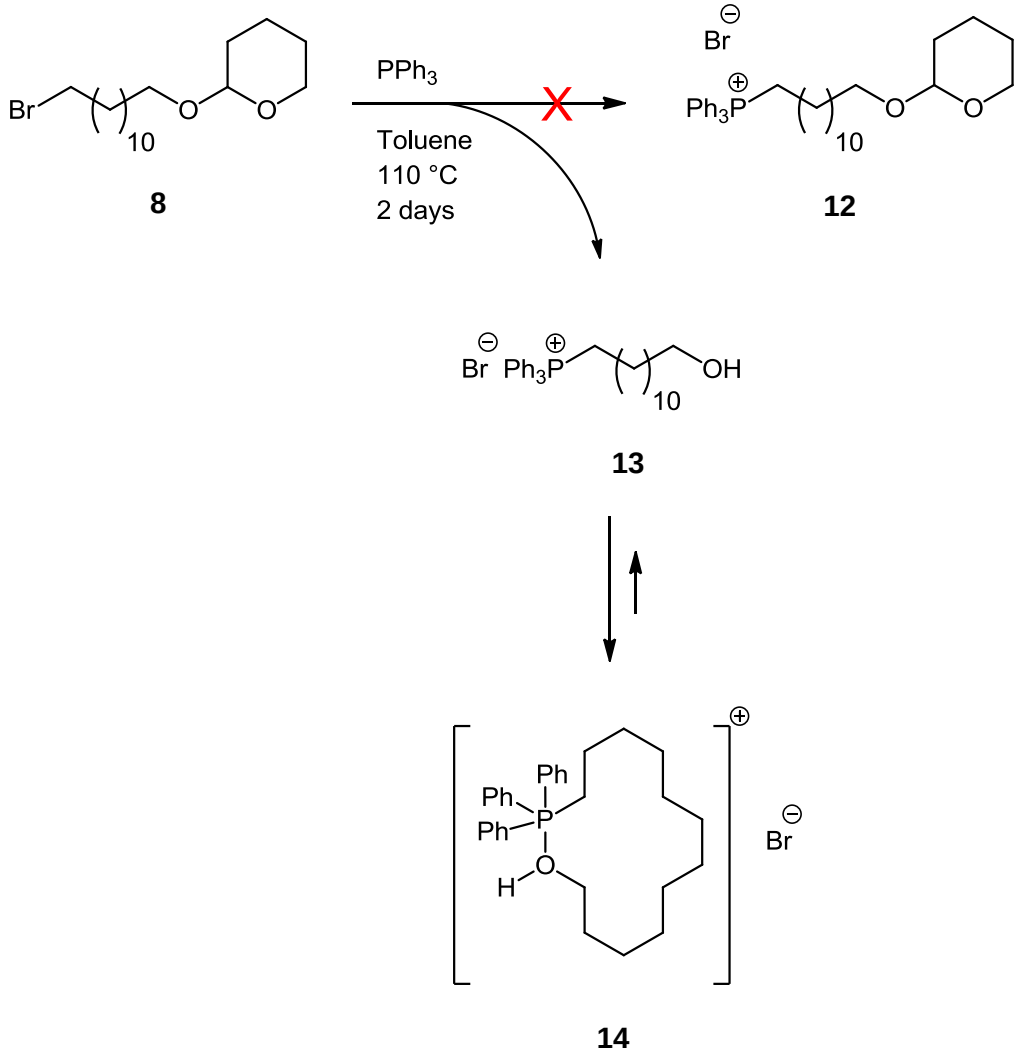
2.2.3.2 Synthesis of phosphonium halides and unexpected removal of the THP group

The organohalides **8** and **11** synthesised above were used to prepare the corresponding phosphonium salts. Generally the synthesis of phosphonium salts from primary alkyl halides proceeds smoothly *via* an S_N2 reaction of the soft phosphorus atom of triphenylphosphine with the methylene group adjacent to the halide.

The reaction of bromide **8** with triphenylphosphine in refluxing toluene gave, after cooling to room temperature, a yellow oil, which was insoluble in the reaction mixture. After removal of the supernatant, the resulting oil was re-dissolved in dichloromethane and precipitated by addition of diethyl ether to yield a sticky oil, which, nevertheless, did not correspond to the expected phosphonium bromide **12** (Scheme 2.16).

Proton NMR spectra indicated the presence of aromatic protons and a multiplet at 3.8 ppm, consistent with the successful formation of a phosphonium salt. However the expected peaks related to the THP group were absent, while two triplet-like signals were detected at 3.62 and 3.37 ppm (Figure 2.8b). High resolution mass analysis confirmed that the product corresponded to the phosphonium bromide but without THP group in the alcoholic moiety (Found/Calculated= 447.2829/447.2811 Da).

These results lead us to conclude that the sticky oil generated by the treatment of alkylbromide **8** with triphenylphosphine in refluxing toluene was the phosphonium salt **13** in equilibrium with the form **14** due to an intramolecular coordination of the hydroxyl group on phosphorus atom of phosphonium bromide (Scheme 2.16). This conclusion allows to attribute the triplet peak at 3.62 ppm to the methylene of the free hydroxyl moiety and the triplet-like peak at 3.37 ppm to methylene of the hydroxyl group when it is intramolecularly coordinated with the phosphonium moiety. It is important to report that proton NMR spectra of this phosphonium bromide in deuterated chloroform at higher concentrations showed a marked shift in comparison to the values previously mentioned (Figure 2.8c).



Scheme 2.16. Preparation of the phosphonium salt from the alkylbromide and unexpected removal of the THP group.

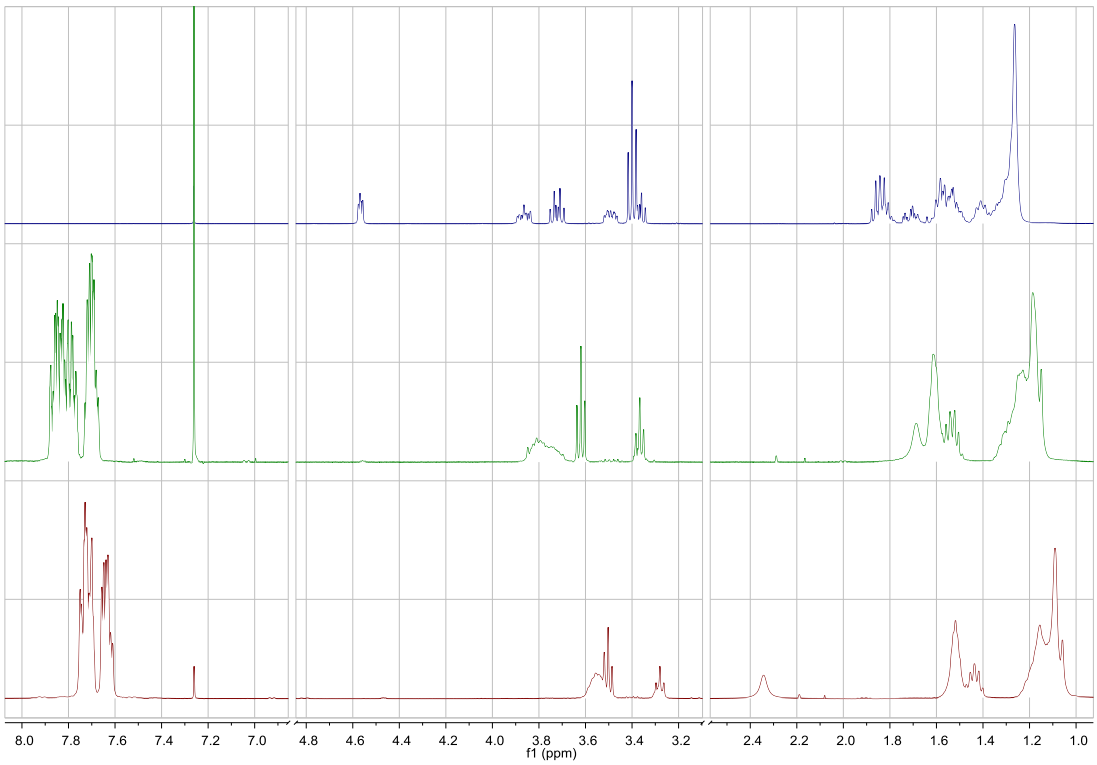


Figure 2.8. Proton NMR spectra of the attempt to convert the bromide **8** (a) to corresponding phosphoinum salt by treatment with triphenylphosphine in refluxing toluene. The resulting phosphonium bromide was purified by precipitation from diethyl ether, and checked by NMR in deuterated chloroform at concentration of ca. 10 mg/mL (b) and 50 mg/mL (c). All the spectra were recorded on JEOL 400 MHz spectrometer and calibrated using residual undeuterated solvent as an internal reference (δ 7.26).

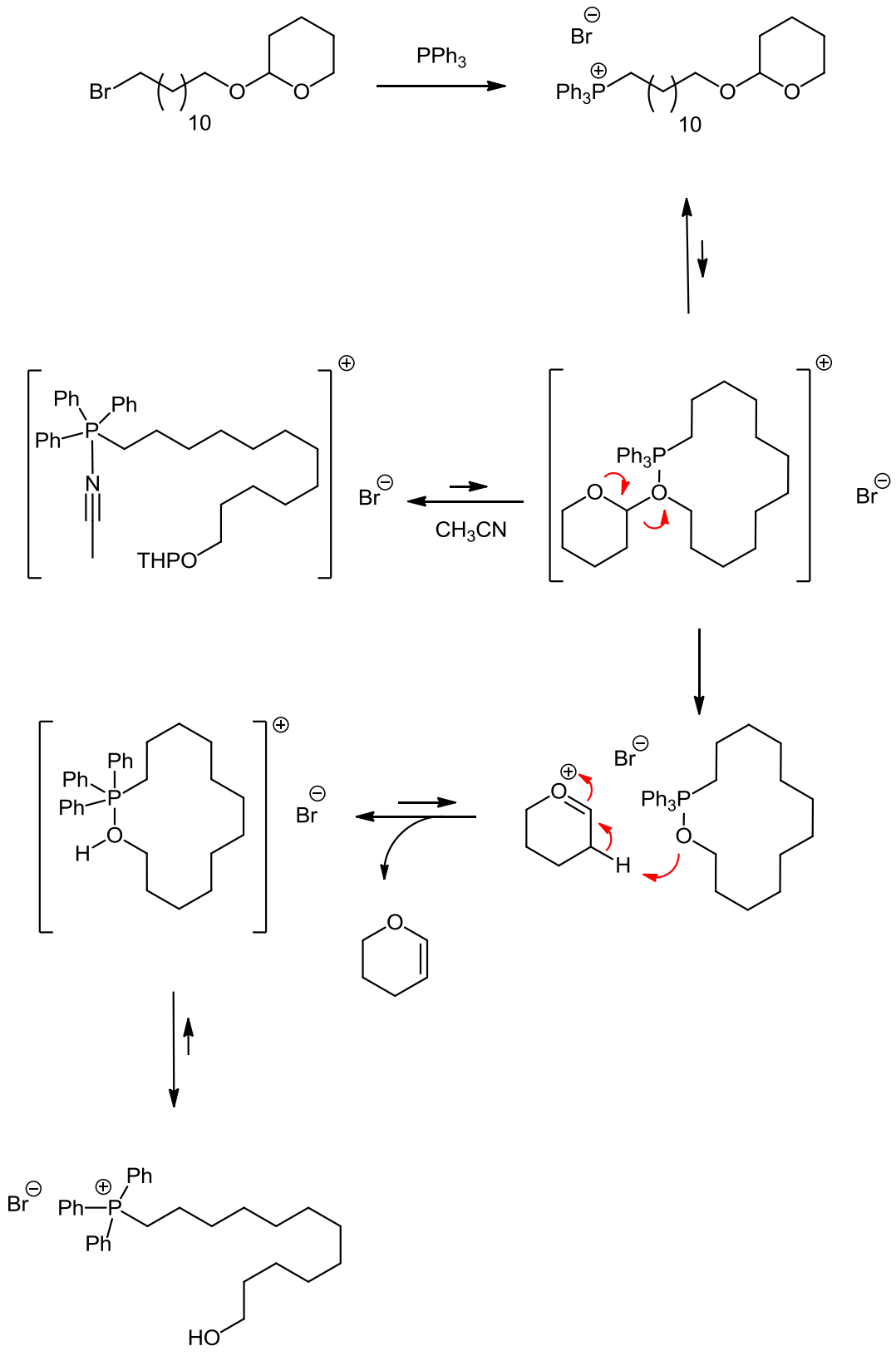
In order to synthesise the distal double bond, attempts were made to couple the phosphonium bromide **12** with the C_{19} aldhehyde, but afforded the expected alkene in low yields and unsatisfactory stereoselectivity (these reactions are reported and discussed in the next section). The presence of similar observations reported in the literature, led us to focus our attention on this side-reaction and conditions that may lead to the desired phosphonium salt **12**.

Initial attempts were made to reprotect the recovered phosphonium bromide **13** with excess DHP in presence of acid catalyst (*p*-TsOH or Amberlyst® 15) in anhydrous dichloromethane. Nevertheless, the protected phosphonium bromide **13** was isolated after column chromatography only in low yields (<10%). Moreover, the treatment of the resulting salt in refluxing toluene for 14 hours resulted again in the

complete deprotection of the THP group. In addition, it was noticed by NMR experiments that also the storage of the phosphonium salt **12** at room temperature caused an increase of the unprotected form within few weeks.

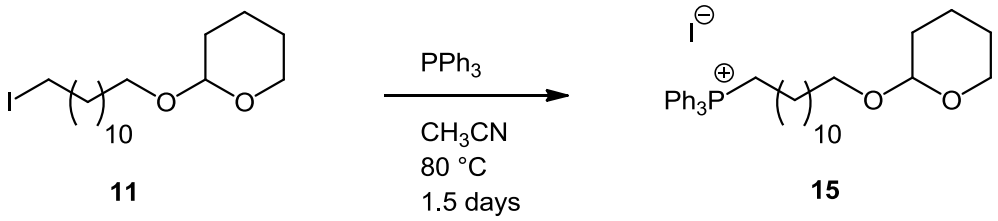
These observations support the hypothesis that this deprotection takes place only after the formation of the phosphonium moiety, which is responsible for the liability of the THP group (Scheme 2.17). In light of these postulations, our efforts focused on the study of the synthesis of phosphonium salt **12** under conditions that could prevent the deprotection of THP group during the reaction.

During this work, it was found that the use of acetonitrile as solvent decreased considerably the deprotection of THP group. We believe that this effect may be due to the coordinating affinity of nitrile group on phosphorus atom (Scheme 2.17). Conversely the presence of calcium carbonate, used previously in a similar reaction, did not reduce the formation of side-product.



Scheme 2.17. Plausible mechanism of the phosphonium-mediated deprotection of THP group and role of acetonitrile on the protection from this reaction.

Attempts to couple the alkyl bromide **8** with triphenylphosphine were however accomplished with a partial removal of THP group, due to the lability of the protecting group contained in the product. In order to increase the stability the THP in the formed phosphonium salt, the alkyl bromide **8** was replaced with the iodide **11** (Scheme 2.18), which has higher reactivity in the S_N2 reaction with triphenylphosphine and permits shorter reaction times. This would enable isolation of the freshly-synthesised product before it underwent degradation. Indeed the reaction of the alkyl iodide **11** with 1.05 equivalents of triphenylphosphine in refluxing acetonitrile for 36 hours (Scheme 2.18) yielded the expected product with high purity (Figure 2.9).



Scheme 2.18. Synthesis of phosphonium salt from iodide **11** and triphenylphosphine in acetonitrile.

Nevertheless, phosphonium iodide **15**, similarly to phosphonium bromide **12**, showed storage instability (Figure 2.9) and thus it was used in the Wittig reaction immediately after its preparation. The apparent instability of these phosphonium salts suggests that the previously-reported[199, 200] unexpected deprotection of a THP group during a Wittig reaction was probably due to a previous degradation of the protecting group contained in the phosphonium bromide rather than to the conditions of the olefination.

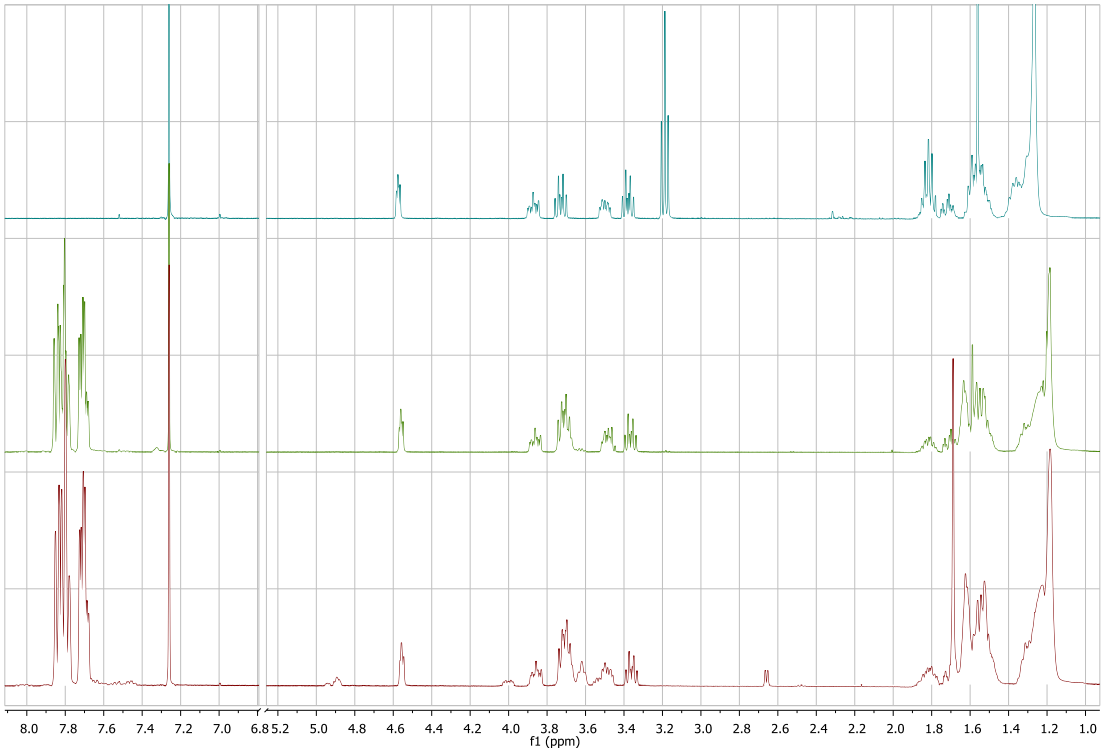


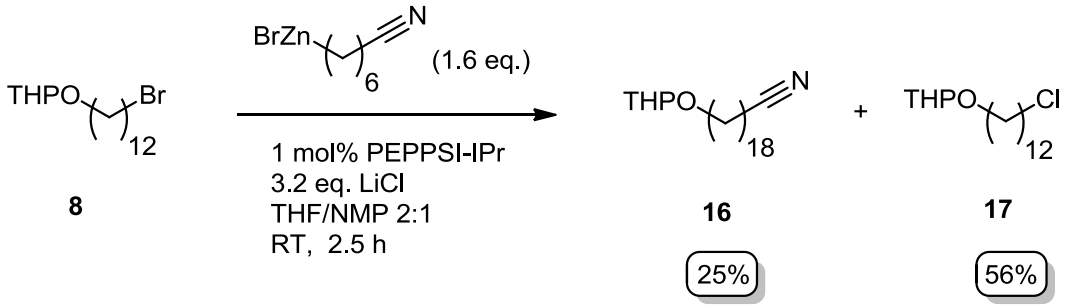
Figure 2.9. Proton NMR spectra of the conversion of the iodide **11** (a) to corresponding phosphoinum salt by treatment with triphenylphosphine in refluxing acetonitrile. The resulting phosphonium iodide was purified by precipitation from diethyl ether, and checked by NMR in deuterated chloroform (b). The phosphonium iodide stored at room temperature for some weeks showed to undergo degradation (c). All the spectra were recorded on JEOL 400 MHz spectrometer and calibrated using residual undeuterated solvent as an internal reference (δ 7.26).

2.2.4 Preparation of the proximal fragment

2.2.4.1 Synthesis of the C1−C19 fragment by PEPPSI-catalysed Negishi reaction

The proximal fragment (C1−C19) of the *cis,cis*-diunsaturated meromycolic acid is a C_{19} bifuntionalized chain that was synthesised by an alkyl−alkyl Negishi cross-coupling reaction using PEPPSI-IPr as a catalyst (Scheme 2.13). For this reaction, the bromide **8** or the iodide **11** were used as electrophile and 6-cyanohexylzinc bromide as organometallic reagent (mechanism for this transformation is shown in Scheme 2.20).

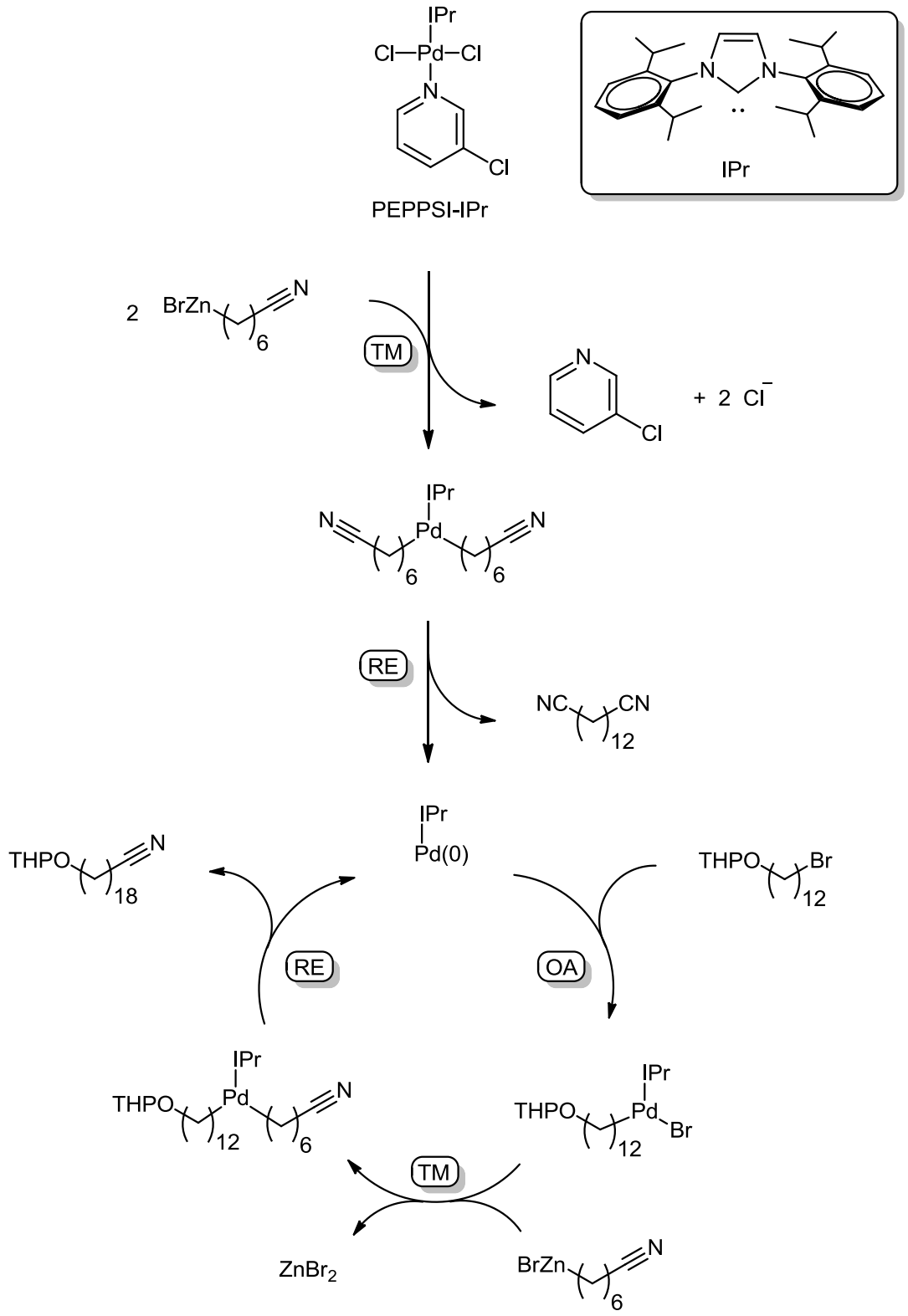
Initial attempts of cross-coupling between the bromide **8** and 1.6 eq. of 6-cyanohexylzinc bromide in the presence PEPPSI-IPr in anhydrous and degassed THF and NMP (Scheme 2.19), as reported by Organ *et al.*,[123] afforded the C_{19} product in poor yields (≤ 25%).



Scheme 2.19. Synthesis of the nitrile **16** by PEPPSI-IPr catalysed Negishi reaction in presence of lithium chloride.

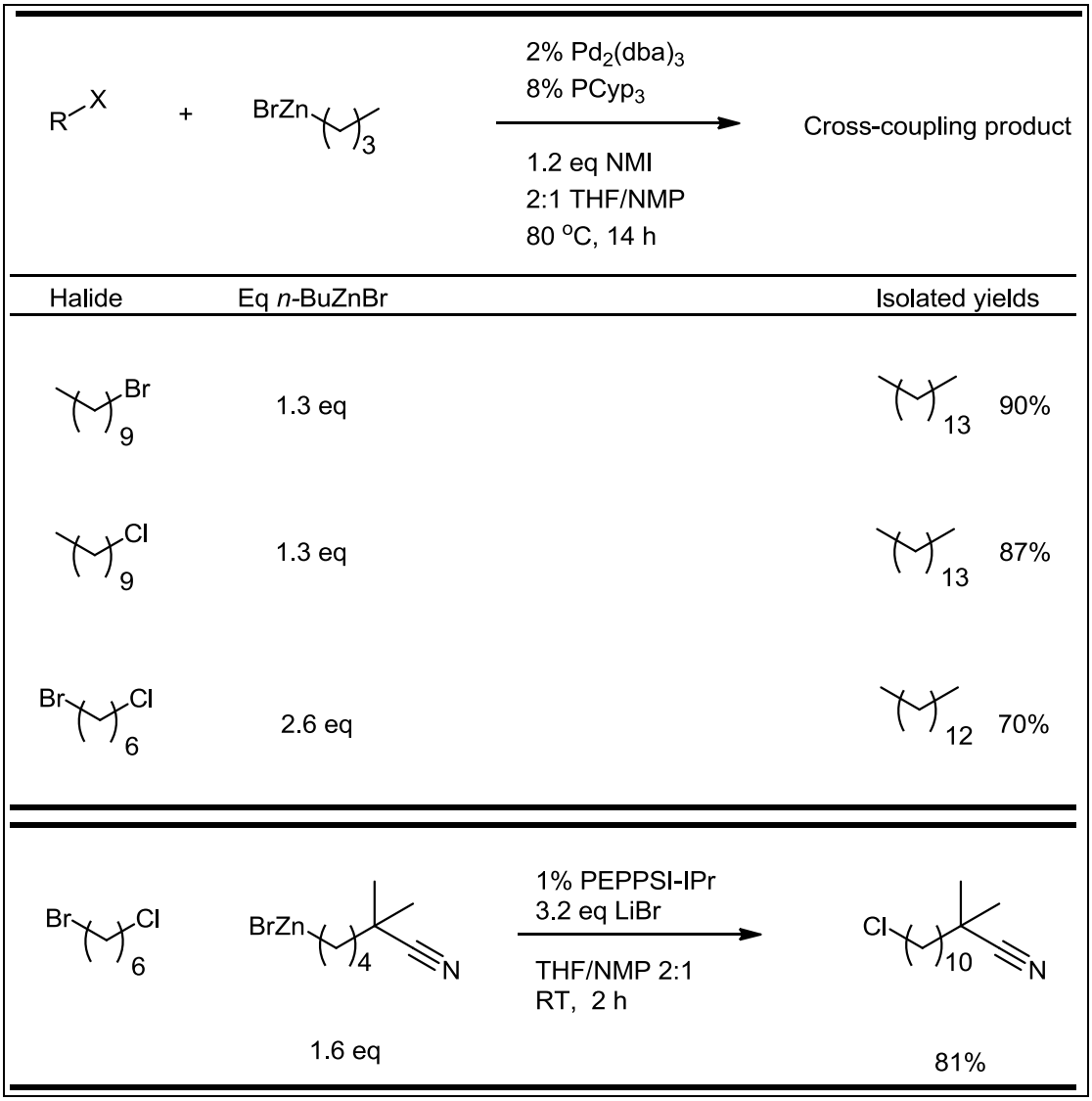
It is important to report that under these conditions, after work-up, no starting bromide was detected by NMR, but the chloride derivate **17** was isolated in high yield. The conversion of the starting alkylbromides to alkylchlorides was previously reported in Negishi cross-couplings by Zhou and Fu[170] and justified by the presence, in commercially available alkylzinc halides, of lithium chloride as by-product of the Rieke® preparation.[132]

In their protocol, Zhou and Fu reported that high yields of cross-coupling products may be isolated using indifferently both organobromides and -chlorides, and that in the case of 1-bromo-6-chlorohexane the mono-functionalization may not be achieved (Table 2.4). Conversely, in the protocol of Organ *et al.* the cross-coupling reactions of bromides and chlorides require two different conditions, reporting also the selective single cross-coupling of 1-bromo-6-chlorohexane.



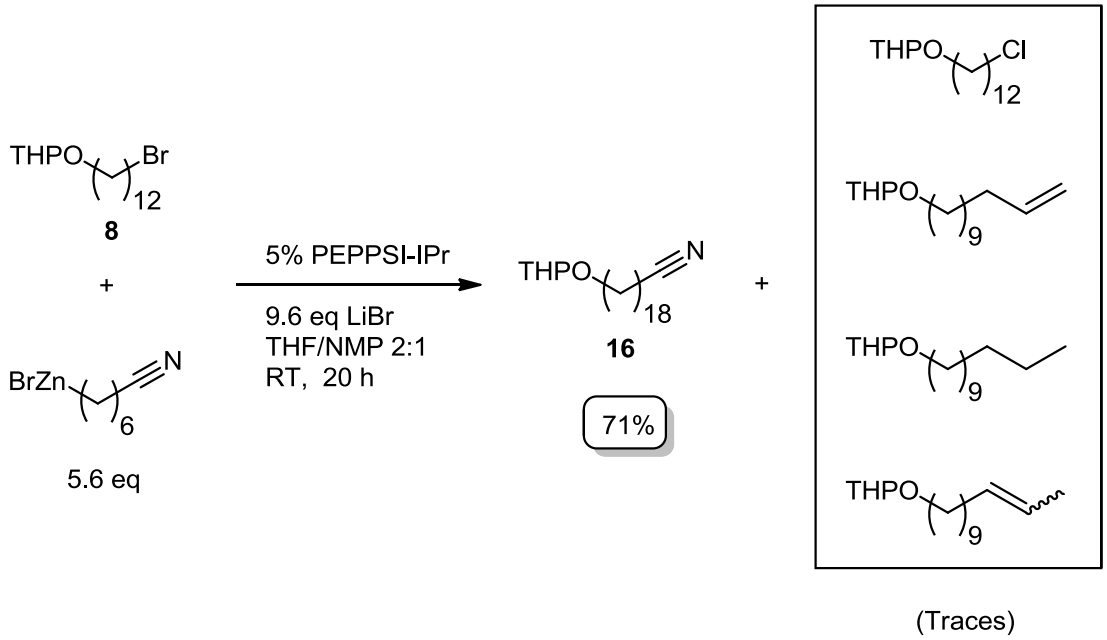
Scheme 2.20. Mechanistic scheme of the synthesis of the proximal fragment by PEPPSI-IPr-catalysed Negishi reaction.

Table 2.4. Reactivity and selectivity of alkylbromides and alkylchlorides in Negishi cross-coupling reaction using the protocols of Zhou-Fu and Organ *et al*.



Although it is generally known that the presence of lithium halide salts favours the transmetalation in the Negishi reaction, and that LiCl and LiBr are equally effective in the activation of organozinc reagents, these findings suggest that the unexpectedly fast transhalogenetation of the alkylbromide by lithium chloride may be the cause of the poor yields observed. Nevertheless the simple replacement of lithium chloride with lithium bromide failed to give high yields, showing that also other factors influence the final reaction yield. After an extensive investigation it was

found that the bromide **8** could undergo cross-coupling reaction in high yield if the catalyst and the alkylzinc bromide were used in large excess (Scheme 2.21).

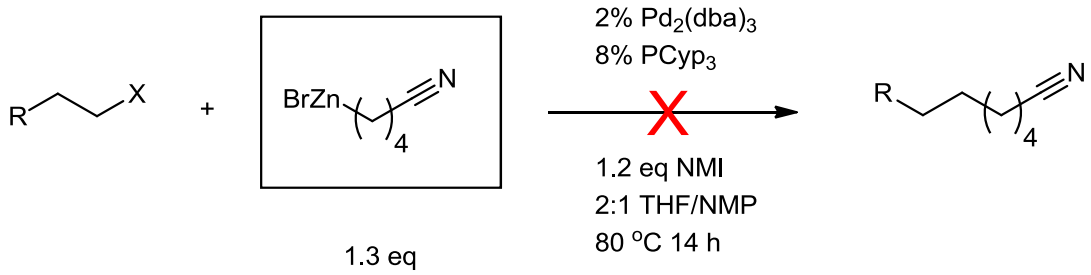


Scheme 2.21. Synthesis of the proximal fragment **16** performed under optimized conditions.

Under these conditions minor by-products were isolated and analysed by H-NMR and GC-MS[201], ascribable to transhalogenation and reduction[202] of the organobromide, or to β-hydride elimination of the organopalladium species.[179]

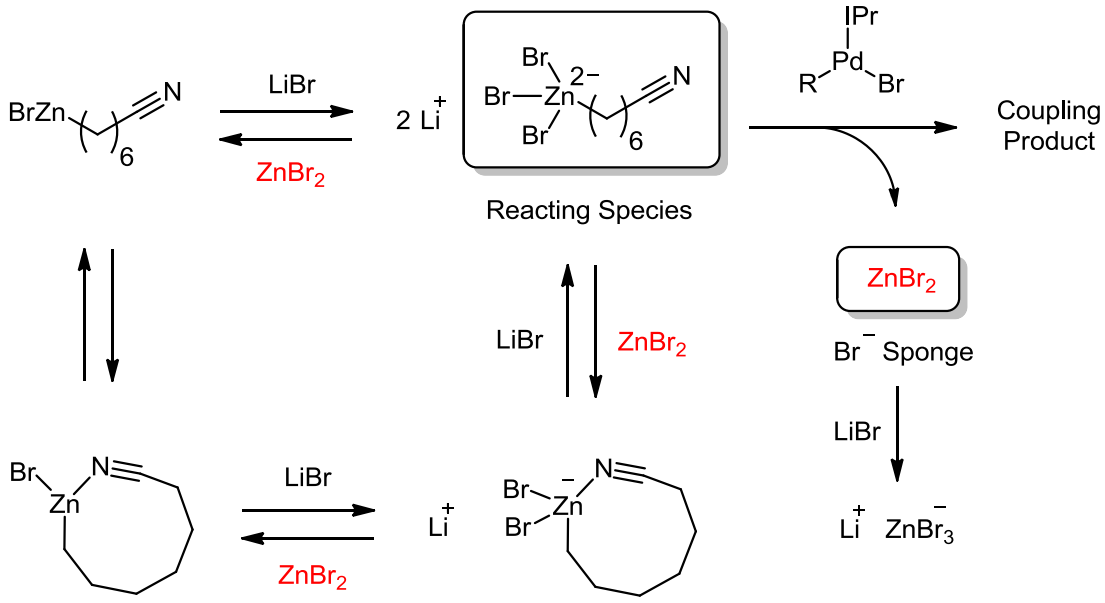
Nevertheless the reasons for the need of this modification to the protocol are not clear. Zhou reported in his PhD thesis[171] that some alkylzinc compounds failed to undergo cross-coupling reaction under the conditions published in 2003.[170] Moreover, among the very few examples of reluctant reagents, was reported 4-cyanobutylzinc bromide (Scheme 2.22). The analogy of this ω-cyanoalkylzinc bromide with the alkylzinc bromide used in our study has lead to the conclusion that the same pathway could be involved in the reaction.

Although no kinetic investigation was conducted for this reaction, it is plausible to suspect that the nitrile group may interfere in the transmetalation and the reductive elimination.



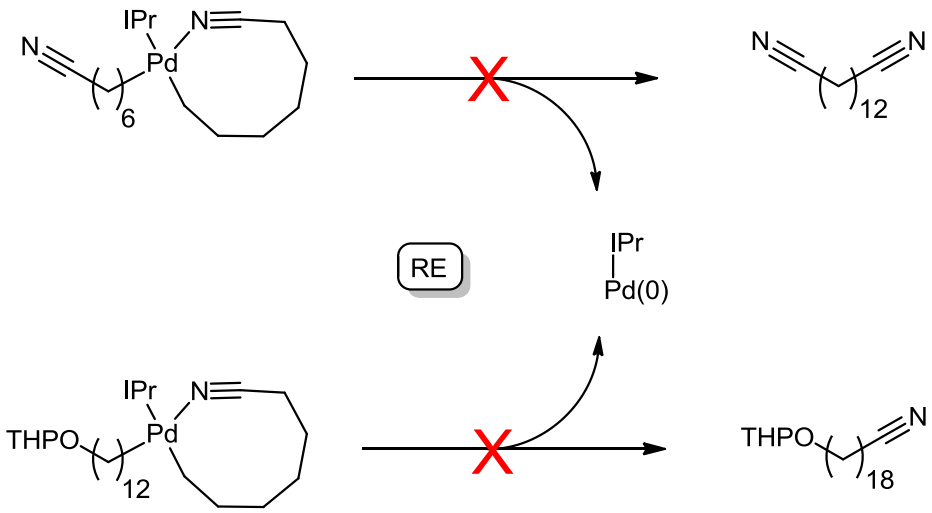
Scheme 2.22. Example of unsuccessful cross-coupling reaction using ω-cyanoalkylzinc bromide reported by Zhou. [171]

Despite that the transmetalation is considered a facile process in Negishi cross-coupling, recent publications have reported that it may be the rate-limiting step.[179, 203] Moreover Organ and co-workers have reported that the alkyl zinc halide reagents require activation to undergo transmetalation, suggesting that a higher-order zincate may be the reacting species. In this scenario an intramolecular coordination of the pendant nitrile group on the zinc atom could interfere on the reactivity or the activation of alkylzinc bromide (Scheme 2.23).



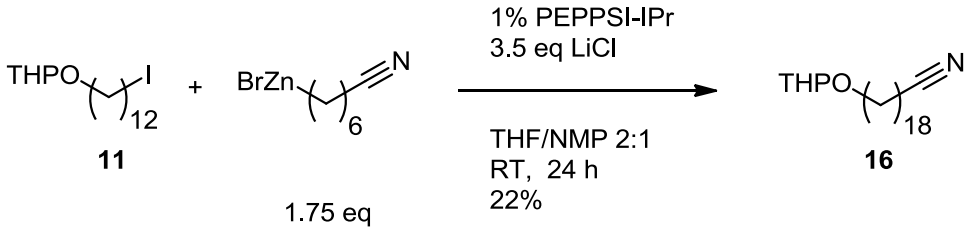
Scheme 2.23. Role of lithium bromide in the activation of 6-cyanohexylzinc bromide and the possible interference of the pendant nitrile group.

An additional effect of the nitrile group on the process of the reaction is the possible intramolecular coordination of this group to the Pd(II) centre (Scheme 2.24).[204-206] Recently, it was reported that acetonitrile may coordinate the diorganopalladium intermediate, increasing the electron density of the transition metal, and interfering with the reductive elimination step.[207] This possible intramolecular coordination would also affect the reduction of the Pd(II) precatalysts to Pd(0), which requires a double transmetalation and subsequent reductive elimination.[167]



Scheme 2.24. The intramolecular coordination of the nitrile group to the Pd(II) centre and influence on the reductive elimination steps in the activation of the precatalyst and in the synthesis of cross-coupling product.

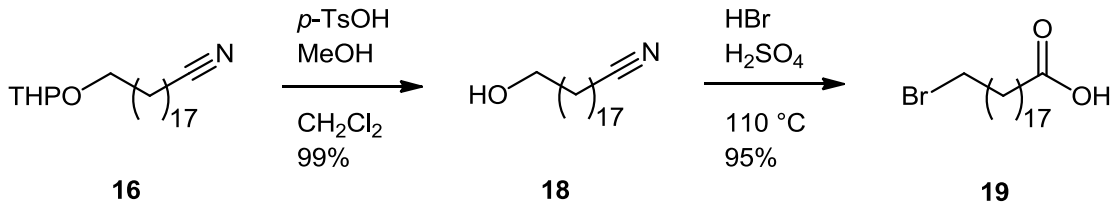
The nitrile **16** was also synthesised from the iodide **11** using the same catalyst (Scheme 2.25). Nevertheless, attempts of cross-coupling reaction between the iodide **11** and 6-cyanohexylzinc bromide in anhydrous degassed DMI and THF, in 3:1 ratio as reported by Organ and co-workers,[123] afforded the expect product in yields generally lower than those obtained with the alkylbromide. These differences of yields however, are not connected with the difference of reactivity of these organohalides towards the oxidative addition, since alkyl iodides are more reactive than bromide derivates. Nevertheless, the lower yields of the cross-coupling product obtained using alkyl iodide in place of bromide derivates were not totally unexpected. Indeed, to the best of our knowledge, only one example of alkyliodide was reported in Negishi cross-coupling reactions with PEPPSI catalysts, showing in a comparative study that the bromide derivates affords higher yields.[123] The explanation of this difference is not discussed, however, a more recent paper reported by Organ *et al.* has not excluded a deleterious effect of iodide.[131]



Scheme 2.25. Synthesis of the nitrile **16** via Negishi reaction of the iodide **11**.

2.2.4.2 Synthesis of the C_{19} alkyl bromide

In order to synthesise the proximal fragment of the *cis,cis*-diunsaturated meromycolic acid, a C_{19} phosphonium salt, the alkyl bromide **19** was prepared from nitrile **16** as shown in Scheme 2.26.



Scheme 2.26. Synthesis of the alkyl bromide **19**.

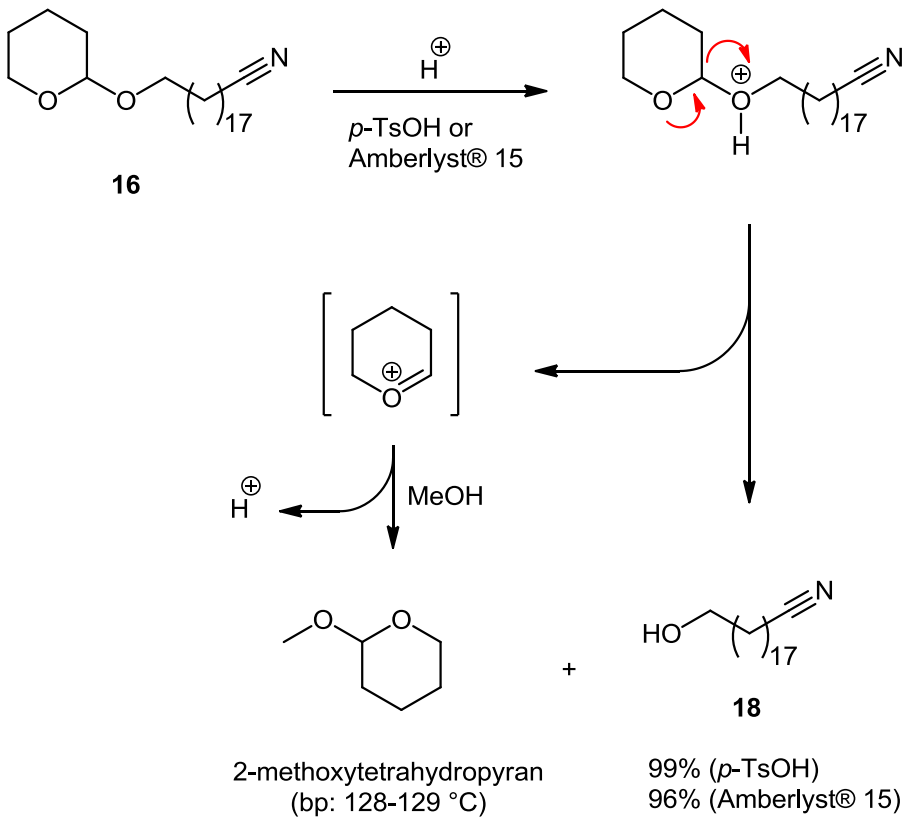
The tetrahydropyranyl group of nitrile **16** was removed by treatment with catalytic amounts of *p*-toluenesulfonic acid monohydrate in methanol.[208] Interestingly, the quantitative deprotection of the THP group took place after overnight stirring without affecting the nitrile moiety. It is important to highlight that the complete removal of the side-product of the reaction, 2-methoxytetrahydropyran (Scheme 2.27), may difficultly occur by simple extraction. Alternatively to a purification by crystallization or flash chromatography, it was found that this by-product (boiling point: 128–129 °C) may be easily removed by melting the crude product at 60 °C and keeping this at low pressure for a short time (70 mbar for 10 minutes). It is interesting to report that in this reaction technical grade methanol was used and that anhydrous solvent is not strictly required, since the presence of water forms 2-hydroxytetrahydropyran that is probably removed by extraction.

The deprotection of the THP group in the nitrile **16** was also conducted in the presence of Amberlyst® 15, as alternative to *p*-toluenesulfonic acid.[209] In this case the reaction was conducted in anhydrous methanol and afforded, after filtration and

evaporation, the expected product in 96% yield as light brownish solid. It is probable that the off-white colour is due to partial breaking of the resin during the stirring.

The cyanohydrin **18** was converted to the ω-bromo carboxylic acid in one step and in high yields by treatment with aqueous hydrobromic acid and concentrated sulfuric acid. The reaction was conducted at 110 °C overnight, using a trap connected to the extremity of condenser to neutralise the toxic HBr vapors. In these conditions the conversion to the expected product was quantitative and the extraction by organic solvents afforded the product in high purity without the need of further purification.

These results invitingly suggest the interesting possibility to synthesise the bromide **19** in one step directly from the nitrile **16**.



Scheme 2.27. Mechanism of deprotection of the THP ether in the presence of acids in methanol.

2.2.4.3 Synthesis of the C_{19} phosphonium salt by microwave irradiation

The synthesis of phosphonium salts from relative bromides is generally a slow transformation, which requires several hours or even days to reach completion. In order to drive slow reactions to completion in shorter times, the heating by microwave irradiation has been an increasingly popular approach since 1986, when two pioneering investigations sreported the possibility of using domestic "kitchen" microwave ovens in organic synthesis.[210, 211]

The first synthesis of phosphonium salts under microwave irradiation was published in 2000.[212] In this study the author reported the synthesis of some phosphonium salts from relative bromides and iodides, and triphenylphosphine, either neat or with xylene as the solvent. In all the cases the author reported that the use of a domestic microwave oven resulted in a remarkable rate acceleration in comparison to traditional means. Nevertheless these pioneering studies using domestic microwave oven, which were common in the late 1980s and 1990s, were difficult to reproduce, control in terms of temperature and pressure, and generally very dangerous for the flammability of organic solvents.

In the last ten years, the advent of microwave reactors[213] reduced the risks and permitted a better understanding of the real "microwave effect", through analytical and critical studies on the real benefits of microwave irradiation.[214]

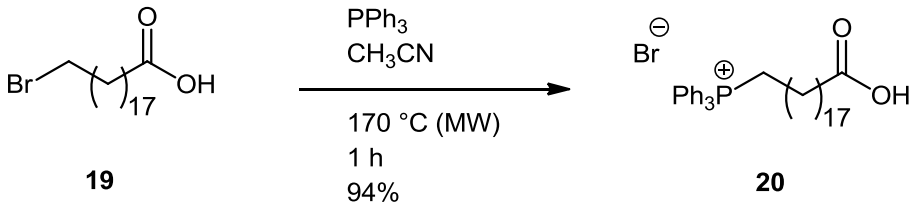
In 2004, Loupy and co-workers reported that, in the synthesis of phosphonium salts from benzyl-bromide, -chloride and -trimethylammonium chloride, the MW effect depended on the leaving group according to the following order $Br^- > Cl^- >> NMe_3$.[215]

More recently, in 2008, Kappe and co-workers published a critical revaluation about the supposed MW effect in four transformations: alkylation of triphenylphosphine and 1,2,4-triazole, Diels-Alder cycloaddition, direct amide bond formation.[216] The authors concluded that the effect previously reported was purely thermal, due to a formation of temperature gradients for an inefficient agitation, and not related to the microwave field. Indeed, ensuring an efficient agitation of the reaction mixture *via* magnetic stirring, no significant differences, in terms of

conversion and selectivity, were reported between experiments performed under oil bath or microwave conditions.

Although, in the last two years, some myths concerning the MW effect have been debunked, many advantages of using controlled MW reactors, in place of the traditional oil baths, remain indisputable.[217]

The bromide **19** was converted to the corresponding phosphonium salt using triphenyphosphine in acetonitrile under microwave irradiation (Scheme 2.28). Other attempts to reduce time or temperature of the reaction resulted in lower yieds.



Scheme 2.28. Synthesis of phosphonium salt **20** using triphenyphosphine in acetonitrile under microwave irradiation.

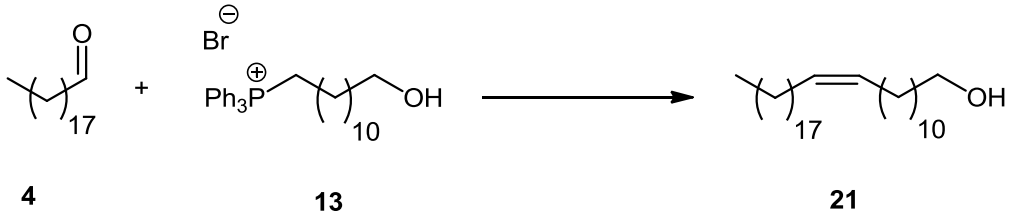
The reaction mixture was purified by evaporating the solvent and solubilising the crude material in a small quantity of anhydrous dichloromethane. The resulting oil was diluted with anhydrous toluene, heated to remove the halogenated solvent and cooled to yield the expected phosphonium salt in pure form as a beige solid.

2.2.5 Preparation of the distal−internal fragment

2.2.5.1 Synthesis of the distal *cis* double bond

The distal bond of the *cis,cis*-diunsaturated meromycolic acid was synthesised according to the Scheme 2.13 from nonadecanal and the C_{12} chain phosphonium salts described previously.

Initial efforts were focused on the synthesis of alkene **21** *via* a Wittig reaction using the phosphonium bromide **13** (Scheme 2.29). However, ω-hydroxyalkyl-phosphonium salts generate betaine-ylide intermediates that are known to afford the expected alkenols in low yields during the Wittig reaction.[218]



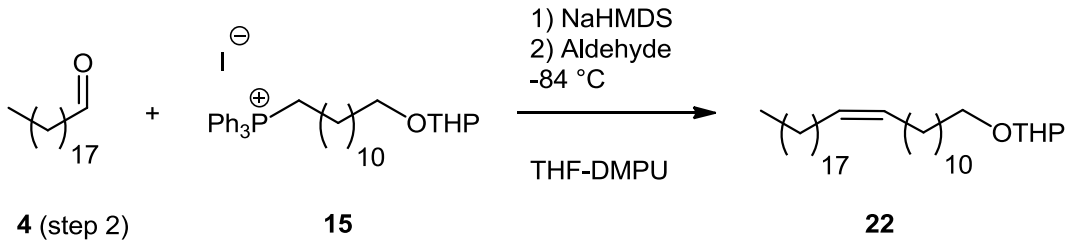
4 **13** **21**

Scheme 2.29. Synthesis of the distal cis double bond via a Wittig reaction using ω-hydroxyalkyl-phosphonium **13**.

Maryanoff and co-workers, during a study on the effect of nucleophilic groups in the phosphonium ylides, reported that Wittig reactions with (3-hydroxypropyl)triphenylphosphonium bromide or (4-hydroxybutyl)triphenylphosphonium bromide yielded little, if any, of the expected alkenol when sodium or potassium bis(trimethylsilyl)amide (NaHMDS or KHMDS) were employed as bases.[219] Although the reasons for the lack of reactions were not clear, they reported successful yields using lithium as a counter-ion of the base, suggesting the use of these bases for reactions with related phosphonium salts. To this end, the alkenol **21** was synthesised from the phosphonium bromide **13**, generating the related ylide using two equivalents of *n*-butyllithium. Unfortunately, the expected *cis*-olefin was afforded with 13% yield with an isomeric purity of 95%. After considering the presence of water in the phosphonium salt as possible factor for

reducing yield, the equivalents of base were increased. However this resulted in a drastic increase of the *trans* isomer. Specifically, the use of 7.8 equivalents of *n*-BuLi yielded *cis*-olefin with an isomeric purity of only 85%. Although it is not clear if the unsatisfactory yields are due to a degradation of hydroxyalkylphosphonium bromide under basic conditions *via* phospholane intermediate, it is important to report the poor solubility of phosphonium bromide in THF and the difficulties met in proceeding the reaction at temperatures lower than −78 °C. We therefore focused our attention on a more efficient and *cis*-selective synthesis of the target molecule using phosphonium iodide **15**.

The protection of hydroxyl group as tetrahydropyranyl ether in phosphonium iodide **15** solved the problems connected to poor solubility and suspected degradation, and permitted also lower temperatures and lithium-free conditions that enhanced the *cis*-isomeric purity (Scheme 2.30). Indeed phosphonium iodide **15** was treated with one equivalent of NaHMDS and the resulting ylide in DMPU and THF reacted with nonadecanal at −84 °C, yielding the expected *cis*-olefin in high yield and high stereoselectivity (>98%).

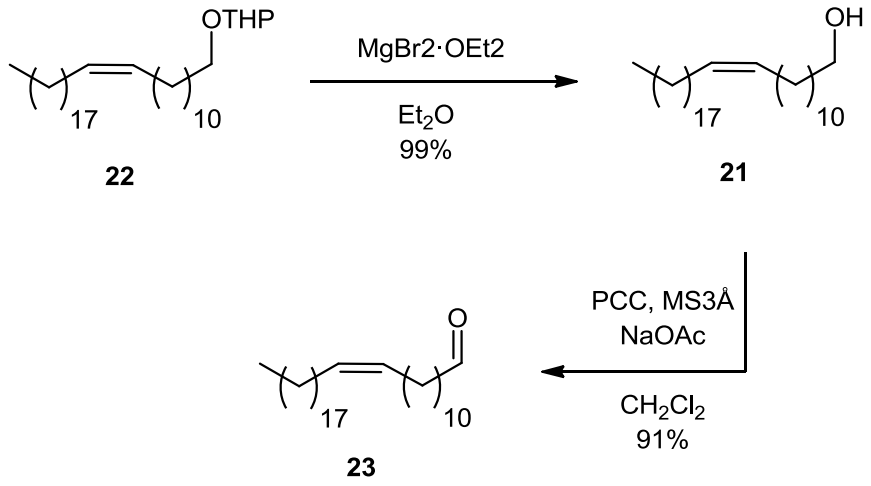


Scheme 2.30. Stereoselective synthesis of proximal double bond.

2.2.5.2 Synthesis of (Z)-hentriacont-12-enal

In order to synthesise the distal double bond of the *cis,cis*-diunsaturated meromycolic acid, the C_{31} aldehyde **23** was prepared from the alkene **22** (Scheme 2.31). Although there exist a number of procedures that permit the oxidative deprotection of the THP group to aldehyde, these generally fail to offer efficient conversion and employ reagents not compatible with the vinyl moiety.[220, 221] Therefore it was opted for a more efficient two-step strategy *via* deprotection of the THP group and subsequent oxidation of the generated hydroxyl group.

A myriad of different conditions are known for the deprotection of THP group,[222] but it is important to highlight that in acidic *media*, *cis-trans* isomerisation could occur and affect the stereochemical purity of the alkene. To this end, the deprotection of the alkene **22** was achieved in high yields using magnesium bromide ethyl etherate ($MgBr_2 \cdot OEt_2$),[223] which is a mild Lewis acid that does not affect the stereochemistry of the double bond.[224]



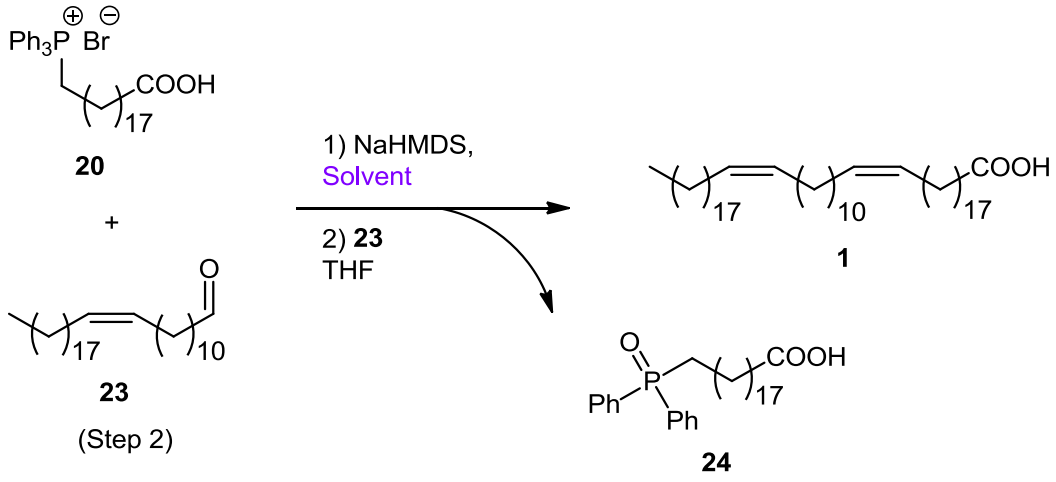
Scheme 2.31. Synthesis of (Z)-hentriacont-12-enal.

The resulting alcohol was finally converted to aldehyde using PCC in anhydrous dichloromethane. The oxidation was carried out in the presence of

molecular sieves as heterogeneous catalyst, as discussed previously for the preparation of nonadecanal. Since PCC possesses a slight acidity that may lead to *cis-trans* isomerisation the of alkene moiety, sodium acetate was added as a buffer to the reaction *medium*.

2.2.6 Synthesis of the *cis,cis*-diunsaturated meromycolic acid

The synthesis of the *cis,cis*-diunsaturated meromycolic acid was concluded with the Wittig reaction between the phosphonium salt **20** and the aldehyde **23**. Nevertheless, attempts to couple these two fragments, applying the protocol used previously for the synthesis of the distal double bond, failed to afford the desired alkene (Scheme 2.32). In fact, the reaction between the ylide, generated from the phosphonium salt **20** and NaHMDS in THF and DMPU, and the aldehyde **23**, did not yield the expected alkene. Unexpectedly, under these conditions the Wittig reaction yielded the diphenylphosphorylalkylcarboxylic acid **24** as major product (the term "phosphoryl" has to be preferred to commonly used "phosphonyl", as indicated in the IUPAC recommendations[225]).



Solvent	Yields
THF-DMPU	**24**: major product; **1**: 0%
THF	**24**: major product; **1**: 8%

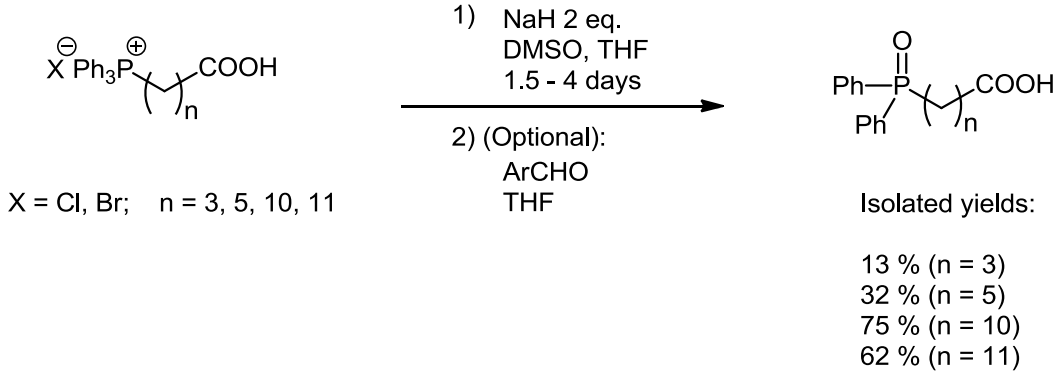
Scheme 2.32. Attempts to synthesise the *cis,cis*-diunsaturated meromycolic acid *via* the Wittig reaction in the presence of THF-DMPU or THF as solvent.

In order to prevent this side reaction during this reaction, some modifications to the previous protocol were made; in particular, the removal of DMPU as co-

solvent allowed the isolation of the desired product, which was validated by NMR analyses and high resolution mass spectroscopy. However, the poor yields obtained during this Wittig reaction lead us to study in more detail this unexpected side reaction.

2.2.6.1 Unexpected conversion of phosphonium salt to phosphoryl derivate during the Wittig reaction.

The conversion of phosphonium salts, containing long-chain ω-carboxyalkyl group, to corresponding phosphine oxides was previously reported by Narayanan and Berlin.[226] This procedure is based on treatment of phosphonium salts with two equivalents of sodium hydride in THF and DMSO for several days (Scheme 2.33).



Scheme 2.33. Conversion of phosphonium salts to corresponding phosphine oxides reported by Narayanan and Berlin.[226]

The authors reported also that the addition of aromatic aldehydes to the reaction mixture failed to afford the expected alkene. However in this work, the aldehyde was added only after some days from the addition of sodium hydride to the phosphonium salts and it does not demonstrate rigorously that this side-reaction is strictly competitive with the Wittig reaction. In addition, this study lacks of a reasonable mechanism or rational suggestions to avoid this side reaction. Despite this interesting work being published in a prestigious journal,[227] it has received limited attention, and

to the best of our knowledge, no successive study has investigated in more detail such reactions.

Successively to the work of Narayanan and Berlin, Maryanoff and co-workers have reported a detailed study of the effect of pendant nucleophilic groups in phosphonium salts on stereochemistry of the alkene product during the Wittig reaction.[219] Specifically, the presence of a pendant carboxy group in the ylide increases the *E* isomer formation during the Wittig reaction. This effect depends on the number of methylene units between the carboxylic group and the phosphonium moiety, and it is especially relevant in reactions with aromatic aldehydes.

In this work Maryanoff and co-workers focused their attention only on the effect of the carboxylic group on stereochemistry of the product, without considering a possible effect of this functional group on the yield of the isolated product. In fact, a critical re-examination of these data has highlighted the existence of a relationship between the structure of the starting ω-carboxyalkylphosphonium salts and the yields of the corresponding alkene products. Specifically, the plotting of the values reported by Maryanoff *et al.* for isolated yields of Wittig products highlighted the existence of a negative effect from the carboxyalkyl group of phosphonium salt on the olefination reaction (Figure 2.10). In particular this effect is more marked when there are more than 6 methylene units between the carboxylic group and the phosphonium moiety, and when the counter ion of carboxylate is less oxophilic such as potassium.

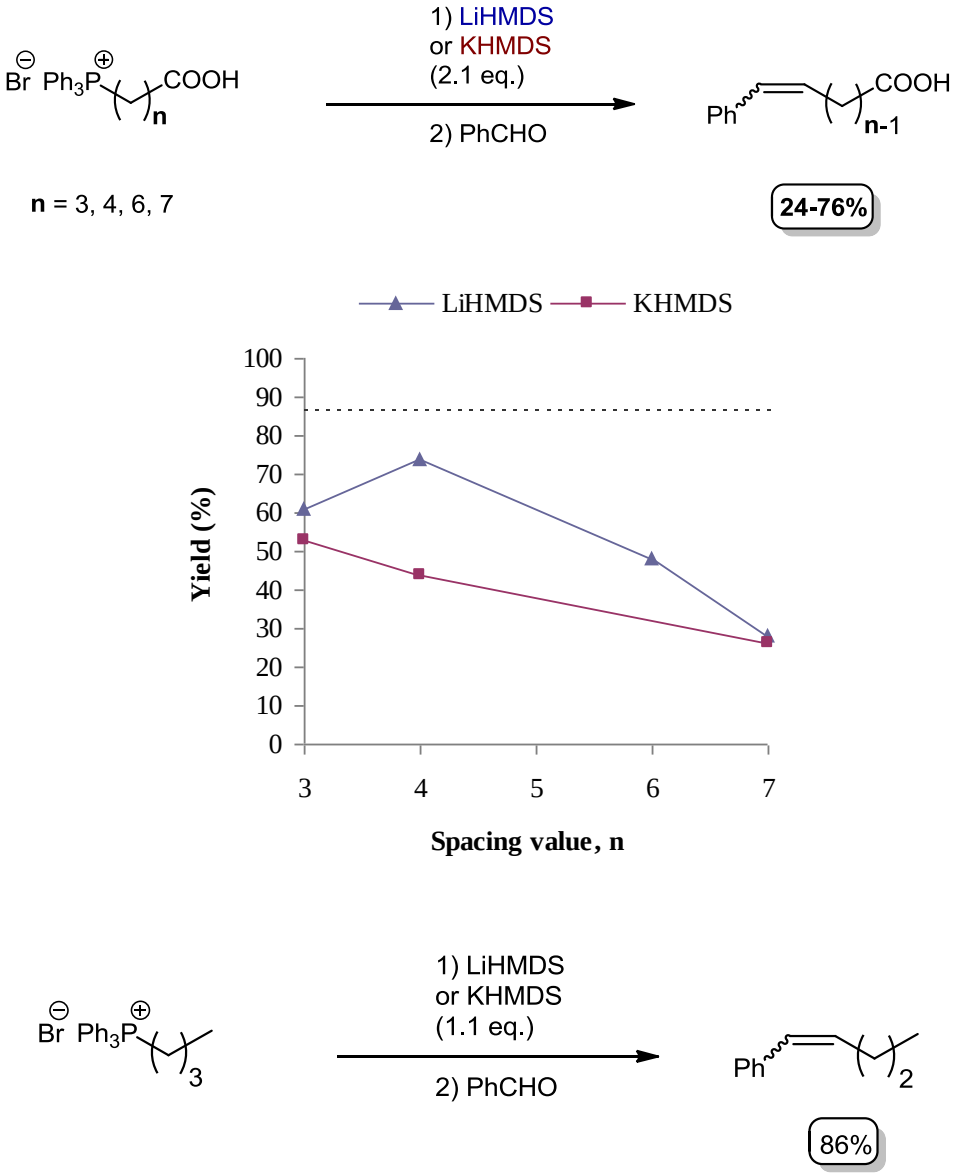
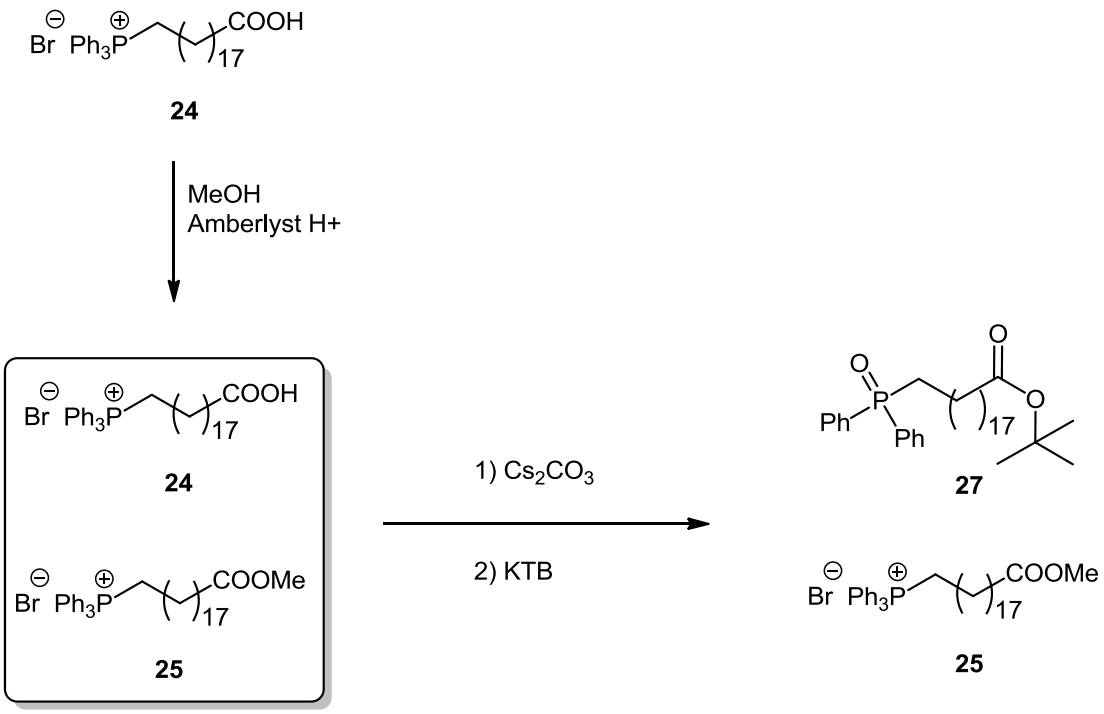


Figure 2.10. Effect of a pendant carboxylic group in phosphonium salts during Wittig reaction with benzaldehyde using lithium or potassium hexamethyldisilazane as base. The yields reported are extracted from Maryanoff *et al.*[219] and plotted with relation to the methylene units between phosphonium and carboxylic groups. The Wittig reaction with butyltriphenylphosphonium bromide reported in the same work was used for reference purposes and it is shown in the graph as horizontal dotted line.



Scheme 2.34. Preparation of a mixture of the ω-carboxyalkylphosphonium salt **24** and its methyl ester derivate **25** to monitor the instability under basic pH.

In order to check the stability of ω-carboxyalkylphosphonium salts, a mixture of the compound **24** and its methyl ester derivate was prepared by esterification with methanol and amberlyst (Scheme 2.34). This mixture was used to monitor the instability of the compound **24** and to correlate with the methyl ester analogue under basic conditions. The treatment with cesium carbonate resulted in the positional change of the triplet (3.36 ppm) related to the methylene group adjacent to the carboxy group, and of the multiplet (3.76 ppm) related to the methylene adjacent to the phosphonium group. After 14 hours, no other changes were detected and a stronger base, potassium tert-butoxide, was added. The addition of this base resulted in a rapid formation of a singlet at 7.36 ppm and a multiplet at 7.43-7.53 ppm, and in the disappearance of the triplet previously formed.

These results support strongly the mechanism reported in Scheme 2.35, where the first equivalent of base forms the phospholane intermediate. In the presence of a strong base, the phospholane intermediate undergoes degradation to form the phenyl anion, the phosphoryl group and a ketene intermediate. The phenyl anion is promptly

converted into benzene, whereas the ketene is converted into esters (or carboxylate) in the presence of alkoxides (or water). It is interesting to note that the triplet at 2.29 ppm, related to methyl ester did not change during the treatment with bases, showing the instability of 24 depends on the carboxylic moiety.

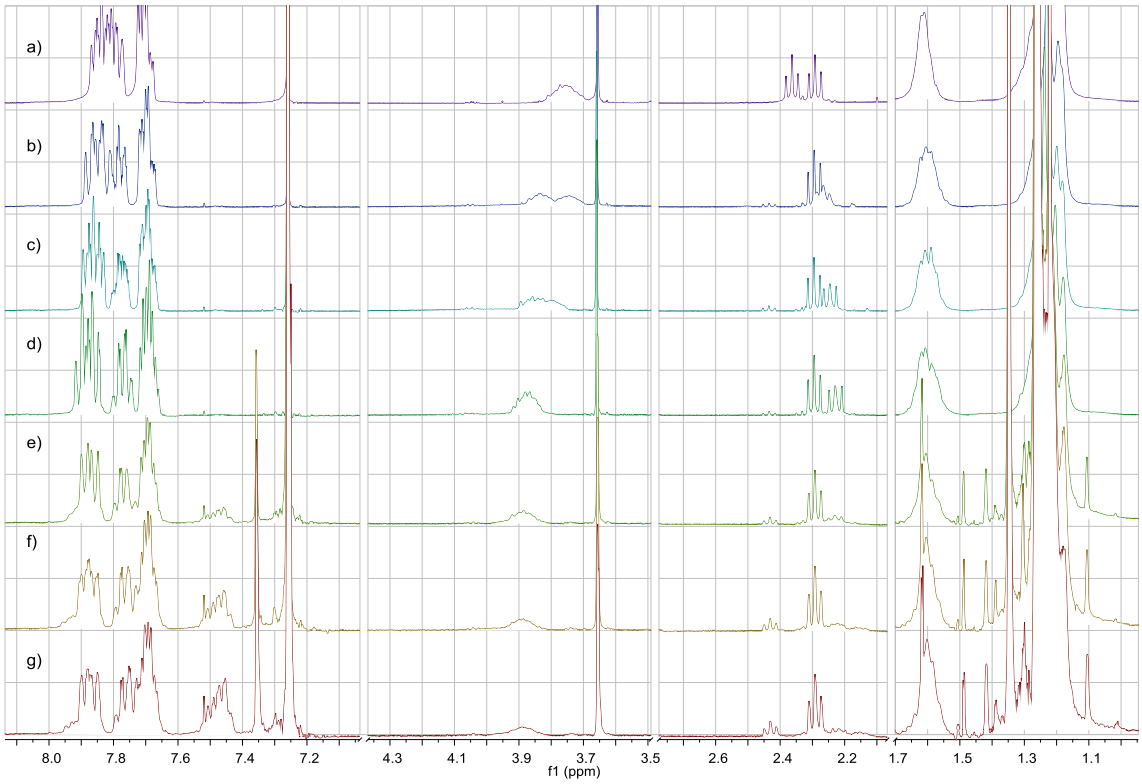
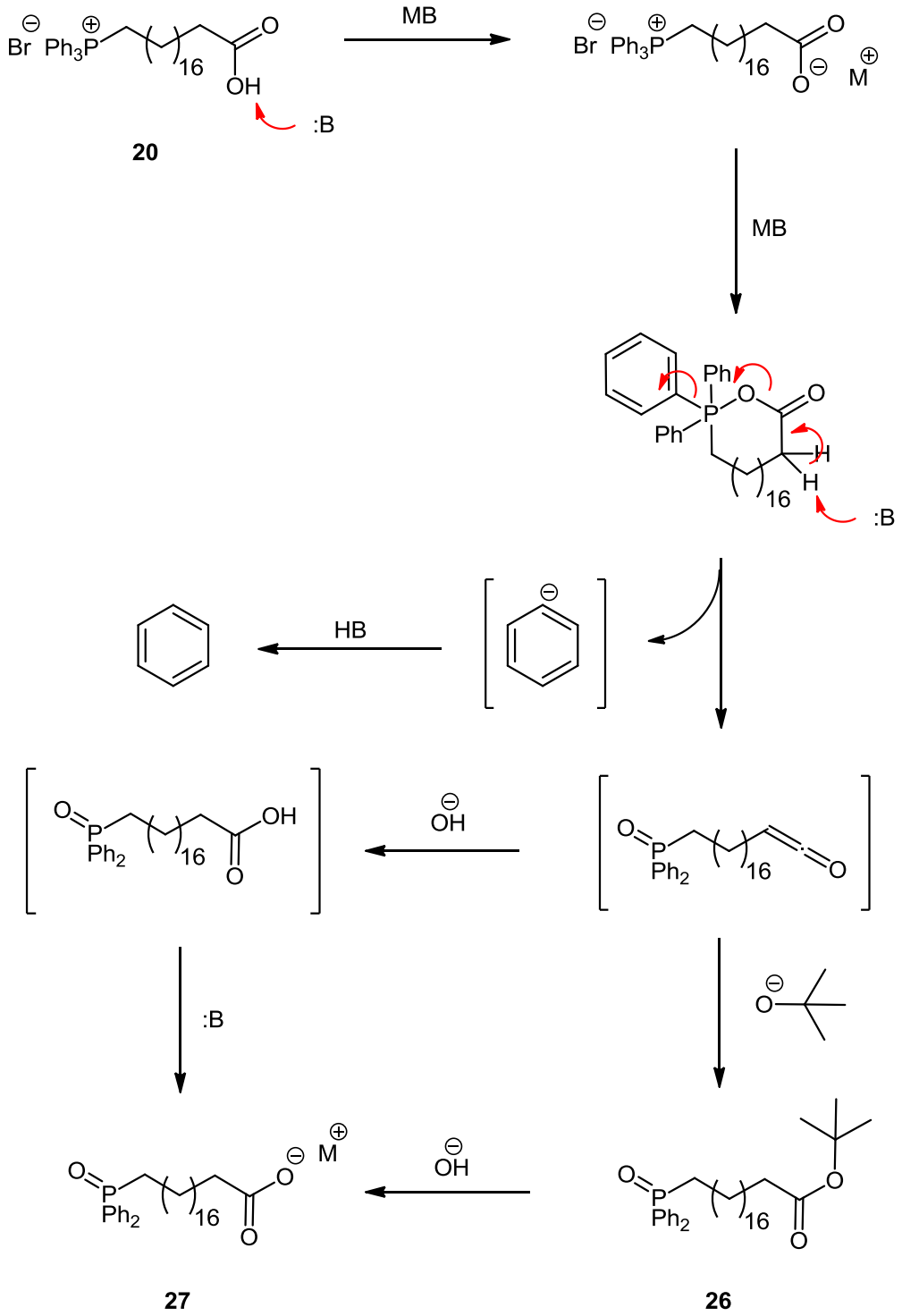


Figure 2.11. Instability of the compound 24 in the presence of potassium tert-butoxide. The mixture of compounds 24 and 25 (a) were treated with excess of cesium carbonate for 5 minutes (b), 3 hours (c) and 14 hours (d). Then an excess of potassium tert-butoxide was added (e) and monitored after 20 min (f) and 90 min (g).



Scheme 2.35. Proposed mechanism for the base-catalysed degradation of ω-carboxyalkylphosphonium salts

2.3 CONCLUSIONS AND FUTURE WORKS

In this Chapter, the *cis,cis*-diunsaturated meromycolic acid was synthesised using a convergent synthetic scheme from cheap and easily commercially available starting materials. The key steps in this strategy are the preparation of *cis* C=C bonds using stereoselective Wittig reactions, and the formation of a C–C bond by the Negishi reaction.

An alkyl–alkyl Negishi cross-coupling reaction was carried out using PEPPSI-IPr as catalyst. This reaction was studied in detail reporting high yields only using lithium bromide as additive, and alkyl zinc bromide and PEPPSI catalyst in large excess. In particular, the results reported in this thesis and recent literature findings support the hypothesis that the pendant nitrile group may be responsible for the low yields observed. Plausible mechanisms have been proposed, but further investigations are needed to confirm such conclusions. In general, the study of the effects of pendant groups on palladium-catalysed reactions represents an interesting non exploited research theme for future investigations.

Interestingly, the preparation of the phosphonium salt from 2-((12-bromododecyl)oxy)tetrahydro-2H-pyran and triphenylphosphine resulted in the unexpected removal of the THP group. Conversely, the reaction using the iodide derivate in acetonitrile showed to afford the desired product in high yields. Highly stereoselective Wittig reactions were carried out at –84 °C using DMPU as co-solvent.

The Wittig reaction for the synthesis of the proximal double bond of the *cis,cis*-diunsaturated meromycolic acid proceeded successively, although in low yields, only using THF as solvent and removing DMPU as co-solvent. In particular, it was note that low yields reported are due to the conversion of the starting ω-carboxyalkylphosphonium salt into diphenylphosphorylalkylcarboxylic acid. A meticulous study of similar Wittig reactions reported in literature showed that generally this reaction affords the desired product in low yields. Although, the conversion of ω-carboxyalkylphosphonium salt into diphenylphosphorylalkylcarboxylic acid using anhydrous bases was previously reported in literature, the present work shows that this reaction is fast and

competitive with the Wittig reaction. In addition, a plausible mechanism for this unexpected reaction was proposed on the base of experimental studies and literature results. Further studies are necessary to confirm the proposed mechanism and establish possible modifications that may reduce this side-reaction during the Wittig reaction.

The low yields obtained for the synthesis of the *cis,cis*-diunsaturated meromycolic acid have impeded the quantification of the geometrical purity. However, from proton and carbon NMR analysis, the sample appears constituted only by the Z isomers and no evidence of other geometrical impurities was detected. Further analyses are required and are currently in progress to establish the geometrical purity of the sample. In particular, it is interesting to report that during the preparation of this thesis a recent article has reported the quantification of the *Z:E* ratio by $^{1}H–^{13}C$-HSQC experiments.[228] Other approaches based on the use of the reversed-phase high-performance liquid chromatography are also currently considered.[229, 230]

CHAPTER 3

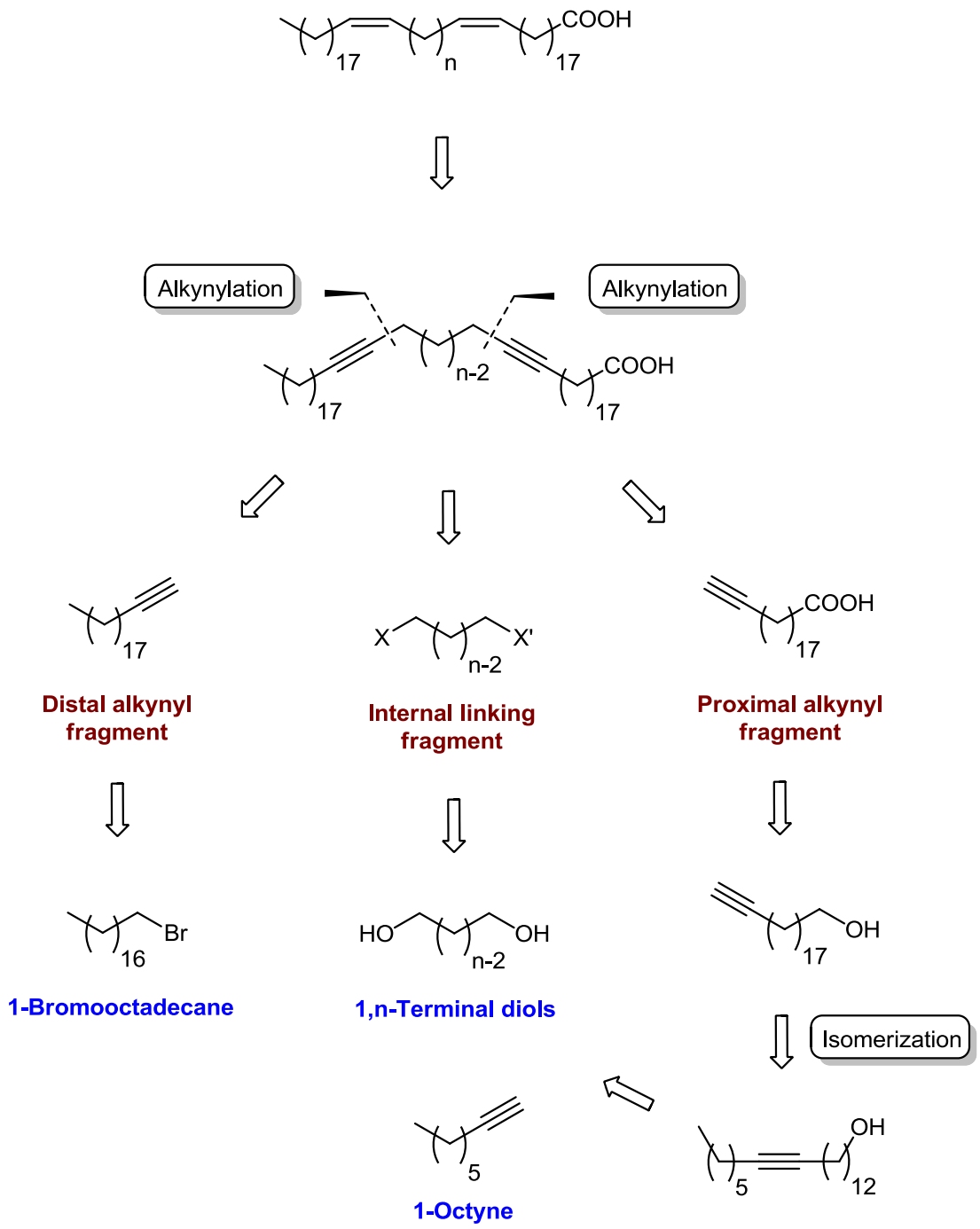
3. SYNTHESIS OF THE *CIS,CIS*-DIUNSATURATED MEROMYCOLIC ACID: A FLEXIBLE ALKYNE STRATEGY

3.1 INTRODUCTION

3.1.1 The alkyne strategy

Total synthesis is an art characterized by refinements and improvements that come one after the other with the intent of reaching ideality.[231-233] In the synthesis of the *cis,cis*-diunsaturated meromycolic acid *via* the strategy previously discussed, some unexpected side-reactions have limited the overall yield and stereoselectivity.

In order to synthesise the target compound in a larger scale and with higher stereoselectivity, a different synthetic route was devised. This strategy is based on the synthesis of the relative acetylenic fatty acid and stereoselective reduction of the alkynyl groups to *cis* double bonds. According to our retrosynthetic analysis the acetylenic meromycolic acid could be synthesised *via* alkyne-bromide coupling reactions of two alkynyl compounds, named proximal and distal alkynyl fragments, with organohalides, which constitute the internal fragment (Scheme 3.1). The internal fragment is prepared from terminal diols and the possibility of using substrates with different chain length permits an easy synthesis of different homologues of the diunsaturated meromycolic acid. Specifically, this chapter reports the preparation of these three fragments and the study of their assembly to afford the *cis,cis*-diunsaturated meromycolic acid.



Scheme 3.1. Alternative retrosynthetic analysis of the *cis*,*cis*-diunsaturated meromycolic acid: the alkyne strategy.

3.2 RESULTS AND DISCUSSION

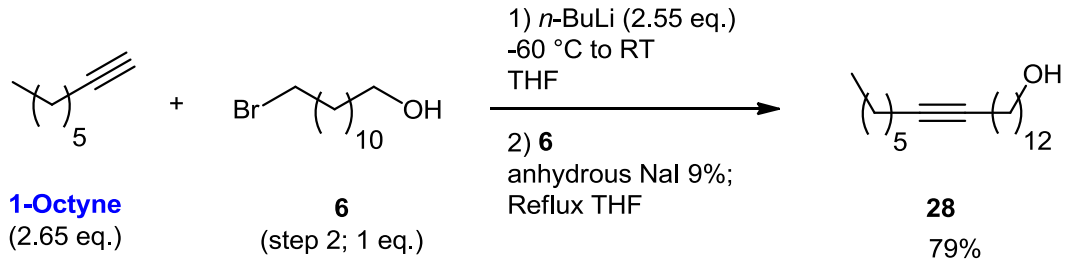
3.2.1 Synthesis of the proximal alkynyl fragment

3.2.1.1 The alkyne-bromide coupling reaction

We envisioned constructing the proximal alkynyl fragment from the isomerisation of an internal alkyne prepared *via* alkyne-bromide coupling of a cheap commercially available terminal alkyne, 1-octyne, and an organohalide prepared from dodecanediol (Scheme 3.2).

The alkyne-bromide coupling reaction requires the initial deprotonation of the terminal alkyne, which is generally performed by a strong base such as *n*-butyllithium, to form the corresponding alkynide that is coupled to the halide *via* S_N2 mechanism. The lithium alkyne derivate however reacts very slowly in THF and a more polar co-solvent is required to proceed satisfactorily.[234, 235] The co-solvent traditionally used in this reaction is HMPA,[236] but the carcinogenic properties of this solvent have more recently encouraged the replacement with safer alternatives. Alkylations of terminal alkynes with organohalides are reported to proceed in the presence of *N,N'*-dimethylated ureas such as DMI[237] and DMPU[238], while the acidity of DMSO limits the reaction only to relatively acidic stabilized alkynes.[239]

Despite the use of these co-solvents is common and often reported as essential in this reaction, recently it was reported a simple and high yielding protocol for the alkylation of alkynyllithiums with organohalides in refluxing THF and in absence of co-solvents.[240] Using these conditions it was possible to couple 1-octyne with 12-bromododecanol in high yields in the presence of anhydrous sodium iodide as catalyst (Scheme 3.2), where the iodide source permits a *in situ* conversion of the alkyl bromide to more reacting iodide derivate.



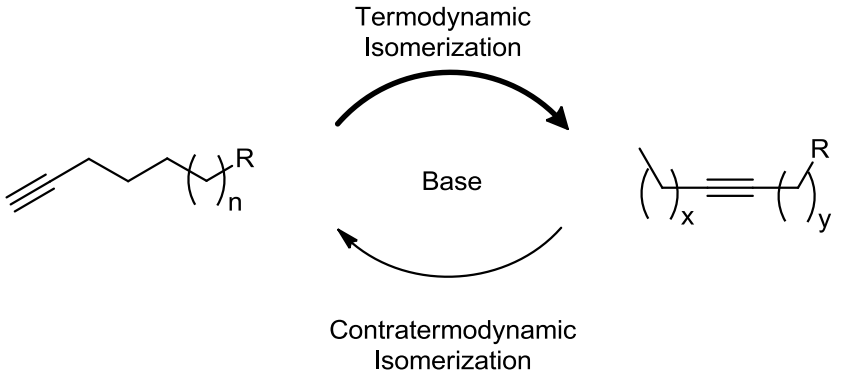
Scheme 3.2. Synthesis of internal alkyne **28** *via* the coupling of 1-octyne with 12-bromododecanol.

It is important to highlight that, despite the protocol mentioned above reported the coupling only if alcohol moieties were protected as tetrahydropyranyl ethers, we demonstrated that this protection is not necessary, by virtue of the low reactivity of lithium alkoxide intermediate generated *in situ* by one extra equivalent of base.

It is interesting to report that attempts to conduce the same reaction, generating the alkynyllithium by *n*-butyllithium or lithium diisopropylamide in THF, and adding 12-bromododecanol in DMPU yielded, after two days at room temperature, little, if any, of the desired product. In these reactions, after acidic work-up, it was possible to isolate starting materials in large quantities and minor impurities due probably to side reactions involving DMPU.

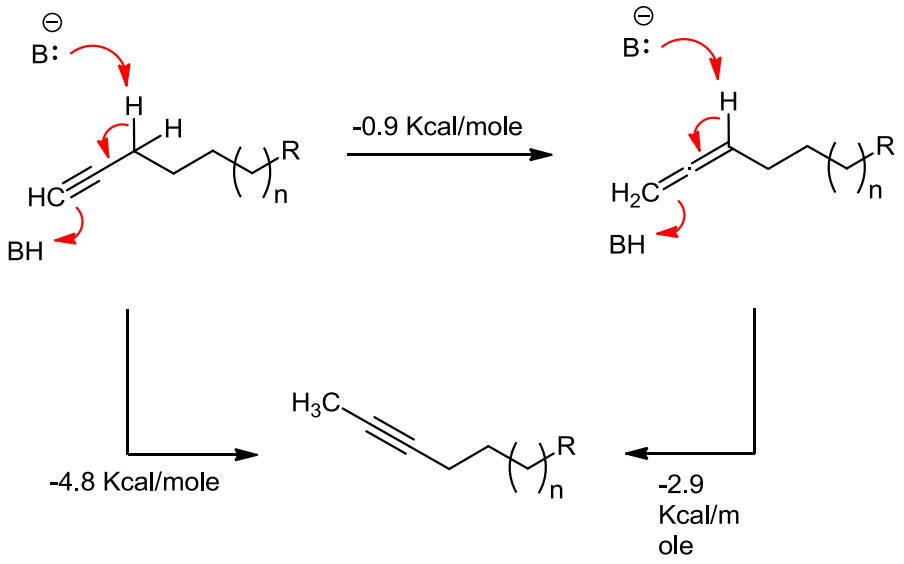
3.2.1.2 Synthesis and purification of terminal alkynes

The isomerisation of alkynes *via* migration of triple bond is a base-catalyzed process that was studied initially from Favorskii more that 120 years ago.[241] The migration of triple bond takes place through allene intermediates and is conducted by a wide range of bases.[242] This process may convert an internal alkyne to 1-alkyne or occur in reverse direction with the formation of the terminal alkyne (Scheme 3.3).



Scheme 3.3. Base-catalyzed isomerisation s of alkynes.

Initially, the most investigated isomerisation was the conversion of terminal alkynes to internal alkynes. In particular, the isomerisation of 1-alkynes to 2-alkynes is a relative fast process, which is driven by the formation of more thermodynamically stable isomers as shown in Scheme 3.3 and Scheme 3.4.[243] This isomerisation occurs smoothly by the use of weak bases, such as potassium hydroxide,[244] but also by stronger bases such as sodium amide.[245] In this last case it is important to report that sodium amide convert 1-alkynes to the thermodynamically more favoured 2-alkynes only if all the components are in solution. Indeed, strong bases may result in the formation of acetalydes, which may precipitate and shift the equilibrium in the direction of the 1-alkyne.

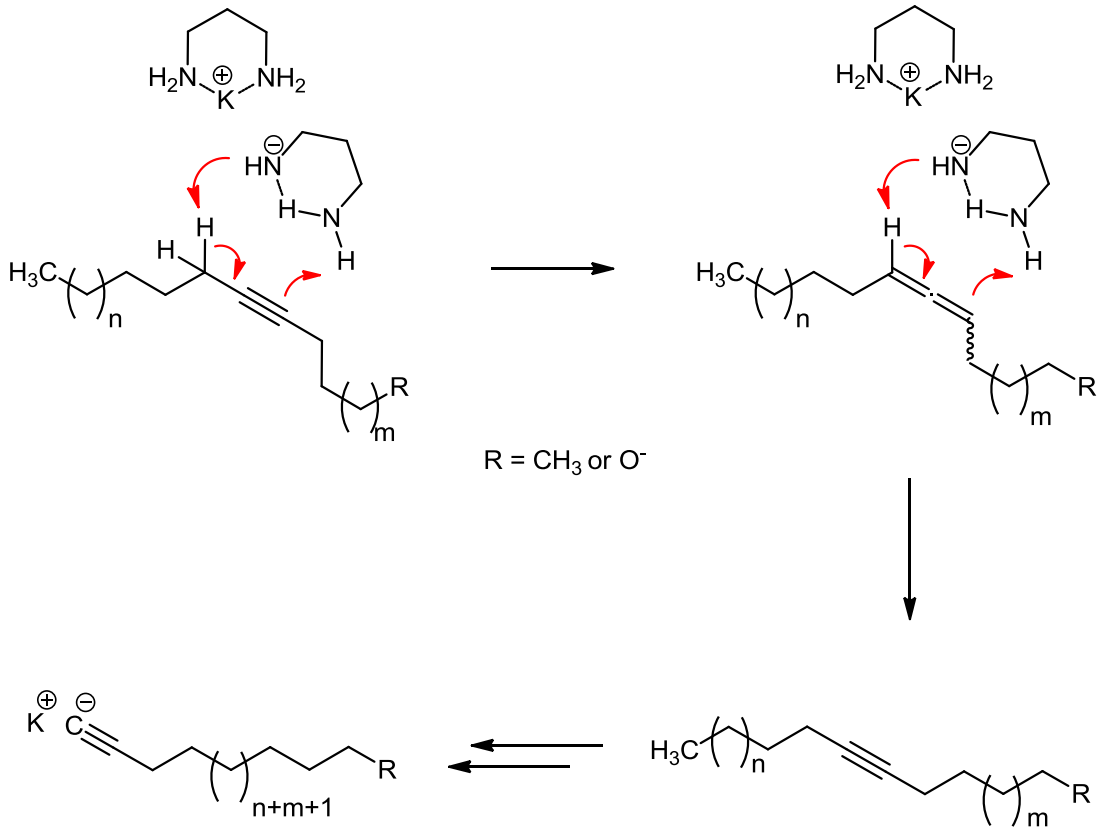


Scheme 3.4. Mechanism of isomerisation of 1-alkynes to 2-alkynes and approximate values of standard enthalpies of formation as reported in the literature. [243]

However, a high yielding multipositional isomerisation of internal alkynes to 1-alkynes was possible only in 1975 when Brown and Yamashita reported the preparation of potassium 3-aminopropylamide (KAPA) and its application in this reaction.[246] The authors suggested that this super base is responsible for the migration of triple bond *via* a "random-walk" process during which a series of allene−alkyne interconversions take place along the carbon chain until the terminal acetylide salt is formed (Scheme 3.5). The same authors reported subsequently that this extremely fast "zipper reaction" was also useful to prepare terminal alkynes from alkyn-1-ols, permitting an easy synthesis of α,ω-acetylenic alcohols.[247]

The preparation of KAPA, as reported by Brown and Yamashita, required however the use of potassium hydride, which is an extremely reactive and flammable base,[248, 249] and was also found to generate *in situ* a particularly troublesome foaming problem.[250] These drawbacks led several groups to investigate alternative bases for this reaction.[250-253] Several alternative protocols were proposed, including the preparation of sodium 3-aminopropylamide (NAPA) by sodium hydride;[250] the addition of diaminopropane to potassium or sodium amide in liquid ammonia followed by evaporation of ammonia;[251] the use of lithium to prepare lithium 3-

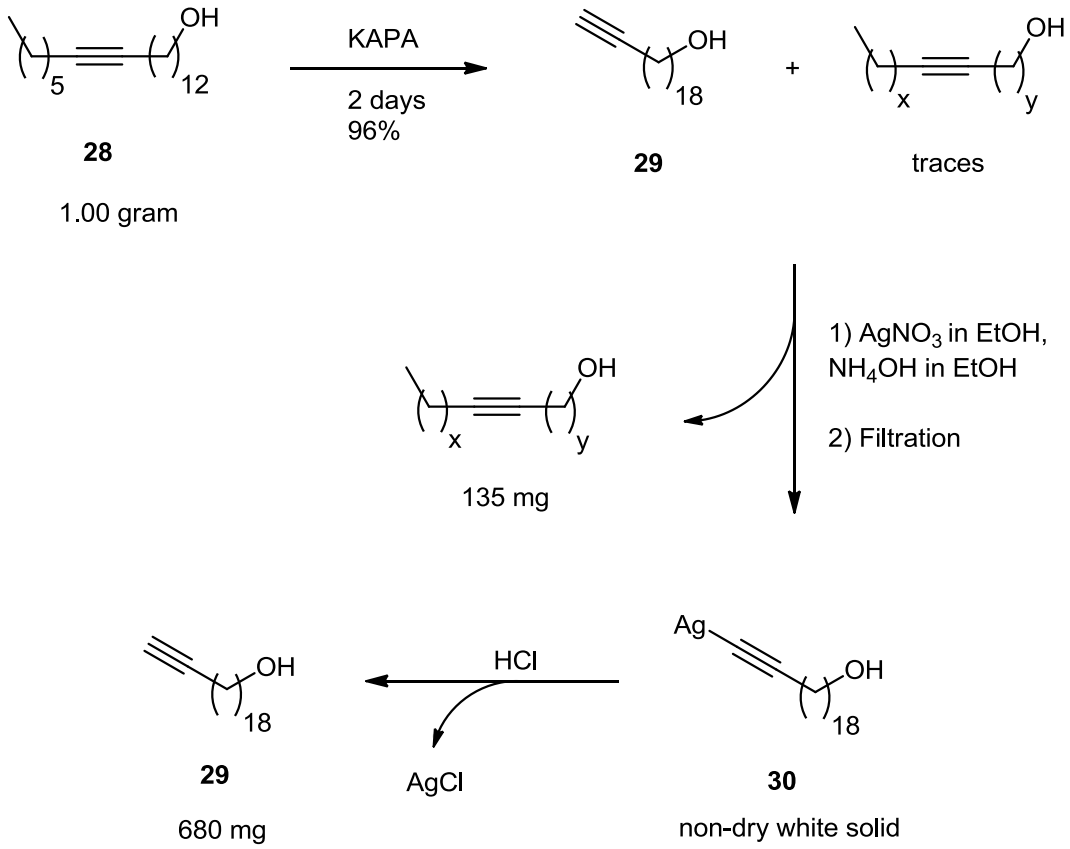
aminopropylamide and the subsequent ion exchange by potassium *tert*-butoxide.[252] Nevertheless, also these protocols present drawbacks due to the minor efficiency of sodium and mixed alkali metal 3-aminopropylamide in comparison to KAPA, or the use of a very inconvenient solvent such as liquid ammonia.



Scheme 3.5. Mechanism of KAPA-mediated allene−alkyne interconversion during the "random-walk" process of isomerisation to terminal alkyne.

We, therefore, opted to convert the internal alkyne **28** to terminal alkyne by the KAPA-mediated isomerisation , preparing this superbase through a different protocol previously reported by Kimmel and Becker.[253] This protocol, required the treatment of 1,3-diaminopropane with molten potassium in the presence of ferric nitrate under ultrasonic irradiation, since ultrasounds and ferric nitrate increase the rate of the heterogeneous reaction between the metal and the solvent. The mixture, heated to 90 °C for 1-2 hours, turned initially to blue and subsequently to green-brown. When all the potassium disappeared, a solution of internal alkyne in THF was added to

KAPA, the mixture became red-brown and was left stirring for two days. The time of reaction was longer than those reported initially by Brown, as suggested successively by Pabon and co-workers.[254] The reaction mixture was then quenched and extracted with diethyl ether to yield a crude material where the expected terminal alkyne was the major product (Scheme 3.6) as shown by proton NMR spectra (Figure 3.1)



Scheme 3.6. Synthesis of the terminal alkyne via KAPA-mediated isomerisation and purification via precipitation of the silver salt and successive reprotonation. The acetylide intermediates are known to be unstable and a complete evaporation of the solvent should be avoided.

The purification of the crude from isomerisation reactions is generally conducted by distillation. In the case of this long-chain alkyne, however, we opted to purify the terminal alkyne via formation of the relative silver salt. In fact, silver acetylides are promptly formed from terminal akynes in the presence of ammoniacal

silver nitrate in ethanol,[255] where precipitate as white solids differently to internal alkynes that remain in solution. The precipitated silver acetylides may be filtrated and reconverted to alkynes via acidification with strong acids. It is important to highlight that the filtration of silver acetylides must be conducted with extreme care, avoiding completely drying these solids, since they are known to be unstable and explosive decompositions have been previously reported.[256, 257]

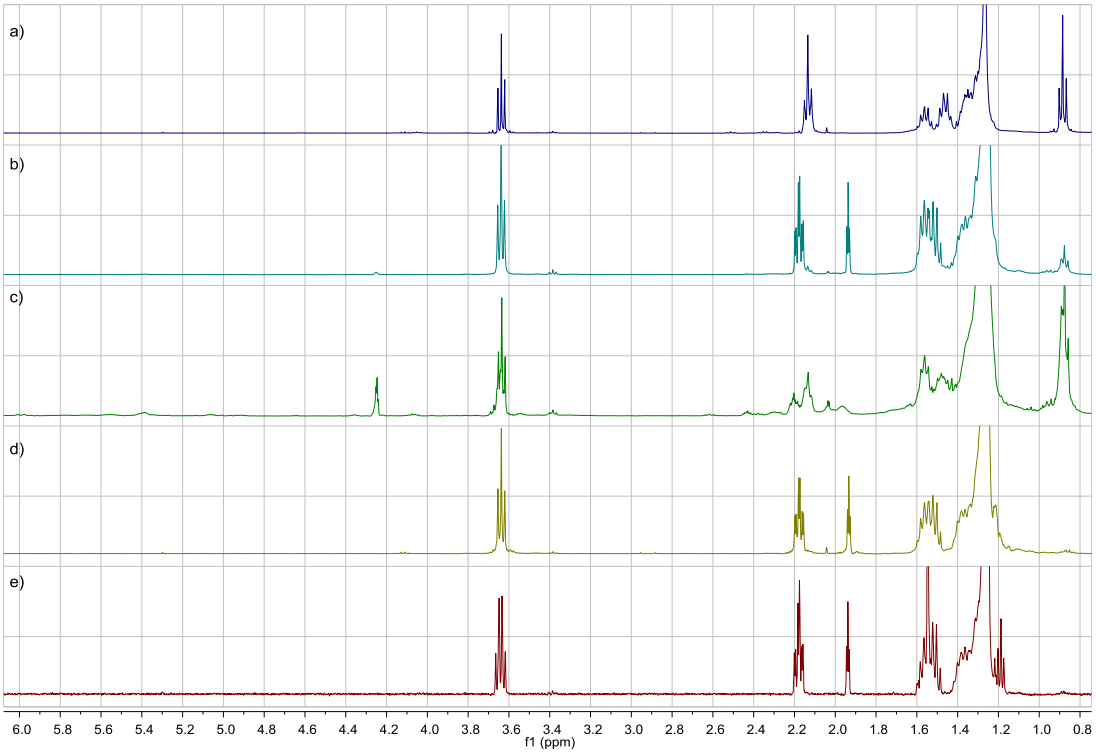


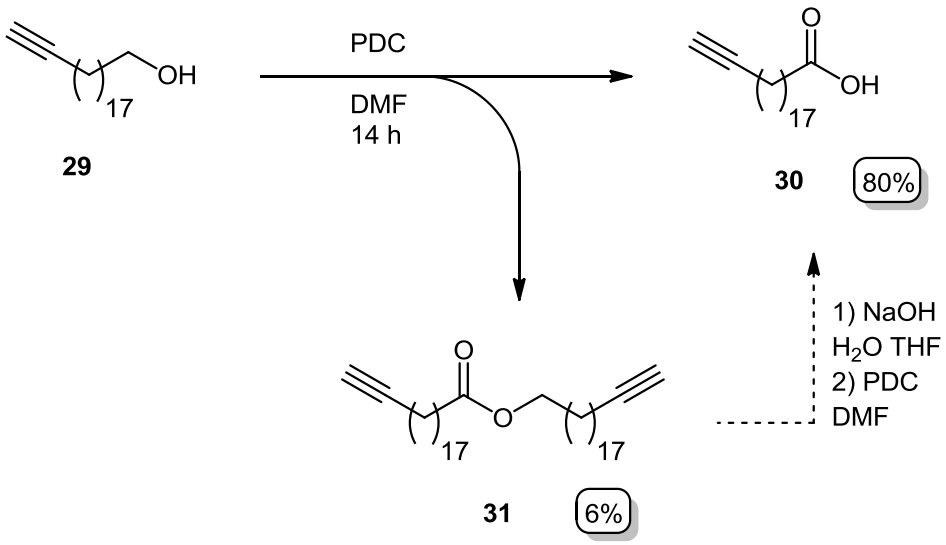
Figure 3.1. Proton NMR spectra of the conversion of internal alkyne to terminal acetylene via KAPA-mediated isomerisation and silver salt-mediated purification. The internal alkyne **30** (a) was treated with KAPA in 1,3-diaminopropane for 2 days. The reaction mixture was quenched and extracted with diethyl ether; the crude (b) was purified by treatement with ammoniacal silver nitrate in ethanol and successive filtration. The filtrate was removed (c), while the precipitated silver acetylide **30** was treated with hydrochloric acid to afford pure terminal alkyne **29** (d-e).

Silver acetylides are however extremely stable toward hydrolysis. This stability is related to several factors, such as the strength of the silver–sp carbon bond and the tendency to form polymeric structures. The hydrolysis of these compounds takes place only by strong acids such as HCl, HNO_3 or triflic acid.[257] The silver acetylide **30** was converted to alkyne in the presence of hydrochloric acid (*ca.* 10% aqueous solution), with concomitant formation of insoluble silver chloride. The terminal alkyne was extracted with diethyl ether to yield the compound **29** in pure form as a white solid.

It is important, however, to report that, in one occasion, the use of nitric acid (ca. 50% aqueous solution) for the conversion of the silver salt **30** to alkyne **29** on a gram scale resulted in a rapid decomposition with the evolution of a red gas and the impossibility to recover the terminal alkyne.

3.2.1.3 PDC-mediated oxidation of the alcoholic moiety to carboxylic acid

Oxidation of primary alcohols to carboxylic acids is a common transformation that may be conducted by a wide number of reagents.[258] In order to prepare the proximal alkynyl fragment, alcohol **29** was oxidised by pyridinium dichromate (PDC) in DMF (Scheme 3.7). The use of PDC in the oxidation reactions to carboxylic acids is generally known as "the method of Corey and Schimidt".[259] [260] This highly chemoselective oxidizing agent, which efficiently converts primary alcohols to aldehydes,[188] when employed in DMF as solvent, affords non-conjugated carboxylic acids in high yields.



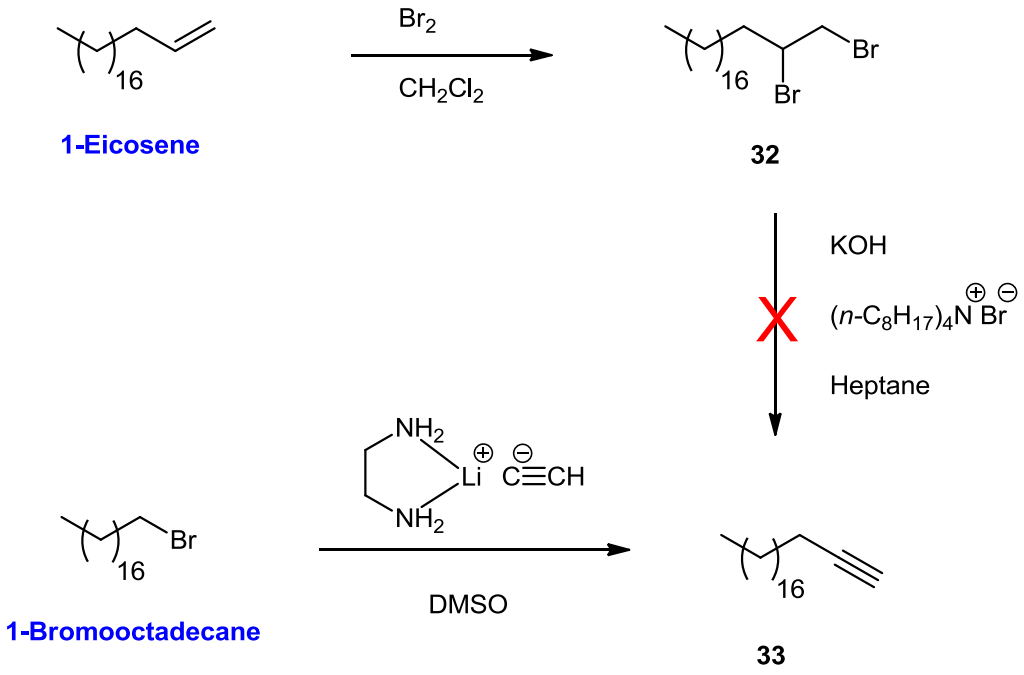
Scheme 3.7. Synthesis of the proximal alkynyl fragment the proximal alkynyl fragmentia *via* oxidation by PDC in DMF, and isolation of the dimer as major side-product.

It is interesting to note that, although often this oxidation is reported to take place in anhydrous conditions, in the presence of dry solvent and molecular sieves, in other cases it was reported to succeed in the presence of water.[260] Considering that the presence of water is responsible for the formation of aldehyde hydrate intermediates, which are subsequently oxidized to acid, we decided to treat alcohol

29 with PDC in non-anhydrous DMF. These conditions yielded the expected carboxylic acid in high yields and in the presence of the dimeric ester **31** as the only isolable side-product. The formation of a dimer was previously reported,[261] and it is probably due to the oxidation of hemiacetal formed by reaction of the starting alcohol with the intermediate aldehyde. This side-product was isolated only in very low amounts by *virtue* of the use of an excess of PDC and DMF. However, when the presence of the ester dimer was more significant, it was hydrolysed and oxidized again to complete the conversion into the desired acid.

3.2.2 Synthesis of the distal alkynyl fragment

In order to prepare the distal alkynyl fragment of the *cis,cis*-diunsaturated meromycolic acid, 1-eicosyne, two different strategies were followed using cheap and easily commercially available starting materials (Scheme 3.8). The first strategy pursued was a two-step route from 1-eicosene, consisting in bromination and dehydrobromination of the terminal olefin.



Scheme 3.8. Synthesis of 1-eicosyne.

The starting olefin was treated with a slight excess of bromine in stabilizer-free dichloromethane for 30 minutes, then the excess of brominating agents was neutralised and washed. The expected 1,2-dibromoeicosane was quantitatively formed in pure form as colourless oil. It is important to highlight that despite the presence of water in the solvent it has only a limited effect on the reaction, the common presence of amylene, used as stabilizer, could affect the purity of the product. In fact, amylene (1-pentene) under the conditions described would be converted in 1,2-dibromopentane, which due to high boiling point would be difficult

to remove. For these reasons dichloromethane was distilled from calcium hydride before use.

The dehydrobromination of 1,2-dibromoalkanes to 1-alkynes may be conducted by the use of a number of organic bases. Nevertheless, it was reported that the use of a weak base, such as potassium *tert*-butoxide in refluxing THF, yielded the desired alkynes only in poor-to-moderate yields, due to contamination of 2-alkynes.[262] As discussed previously, these thermodynamically favoured impurities, are a consequence of the migration of the triple bond.

We opted thus to dehydrohalogenate 1,2-dibromoeicosane *via* phase transfer catalysis (PTC). Unfortunately, when we tried to reproduce a previously-published procedure using powered potassium hydroxide and catalytic amounts of tetraoctylammonium bromide,[263] it was isolated a complex mixture of organohalides and unsaturated compounds (kinetic studies about the mechanism and intermediates of this PTC reaction can be found in literature[264-266]).

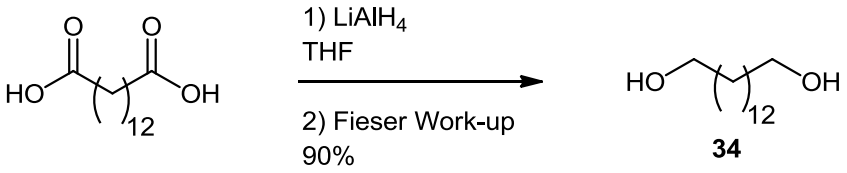
Therefore we turned our attention to an alternative route to 1-eicosyne, using 1-bromooctadecane as starting material. The preparation of terminal acetylenes is traditionally accomplished from alkyl bromide and sodium acetylide in liquid ammonia. The procedure however requires a very inconvenient solvent such as liquid ammonia, and it affords long-chain alkynes only at high temperatures and pressure.[267]

We decided to conduce the preparation of 1-eicosyne using 1-bromooctadecane and the ethylenediamine complex of lithium acetylide. This commercially available ethynylating agent has high stability by virtue of the complex with ethylenediamine, which avoids disproportionation of monolithium acetylide to acetlyne and dilithium acetylide.[268] This complexing agent contributes also to reactivity of aceylide as nucleophile and to solubility in organic solvents. The treatment of 1-bromooctadecane with 2.5 equivalents of lithium acetylide ethylenediamine complex afforded pure eicosyne as a white solid in an excellent yield.

3.2.3 Synthesis of the internal linking fragment

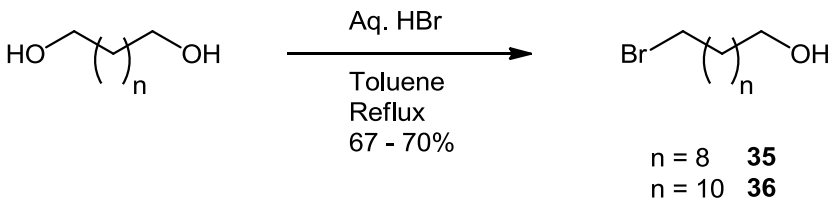
According to our retrosynthetic analysis (Scheme 3.1), the internal fragment has the role to link the proximal and the distal alkynyl fragments. To this end a series of α,ω-halohydrins were prepared from the corresponding α,ω-alkanediols. However, while 1,10-decanediol and 1,12-dodecanediol are easily commercially available in multigram-scale, 1,14-tetradecanediol is more expensive. For this reason tetradecanediol was prepared from tetradecanedioic acid by reduction with lithium aluminium hydride (Scheme 3.9).

As anticipated previously for the synthesis of 1-nonadecanol, these aluminium hydride reductions may generate grey emulsions which are difficult to break. In this case the work-up was conveniently conducted quenching the grey mixture by the Fieser[186] method (initially reported by Micovic and Mihailovic[185]). This allowed *n* grams of $LiAlH_4$ to be quenched the dropwise sequential addition of *n* ml of water, *n* ml of 15% aqueous solution of sodium hydroxide and 3*n* ml of water. The mixture was then stirred for 1 hour and allowed to settle overnight to produce a white granular precipitate easy to filter and wash.



Scheme 3.9. Synthesis of 1,14-tetradecanediol via reduction of tetradecanedioic acid.

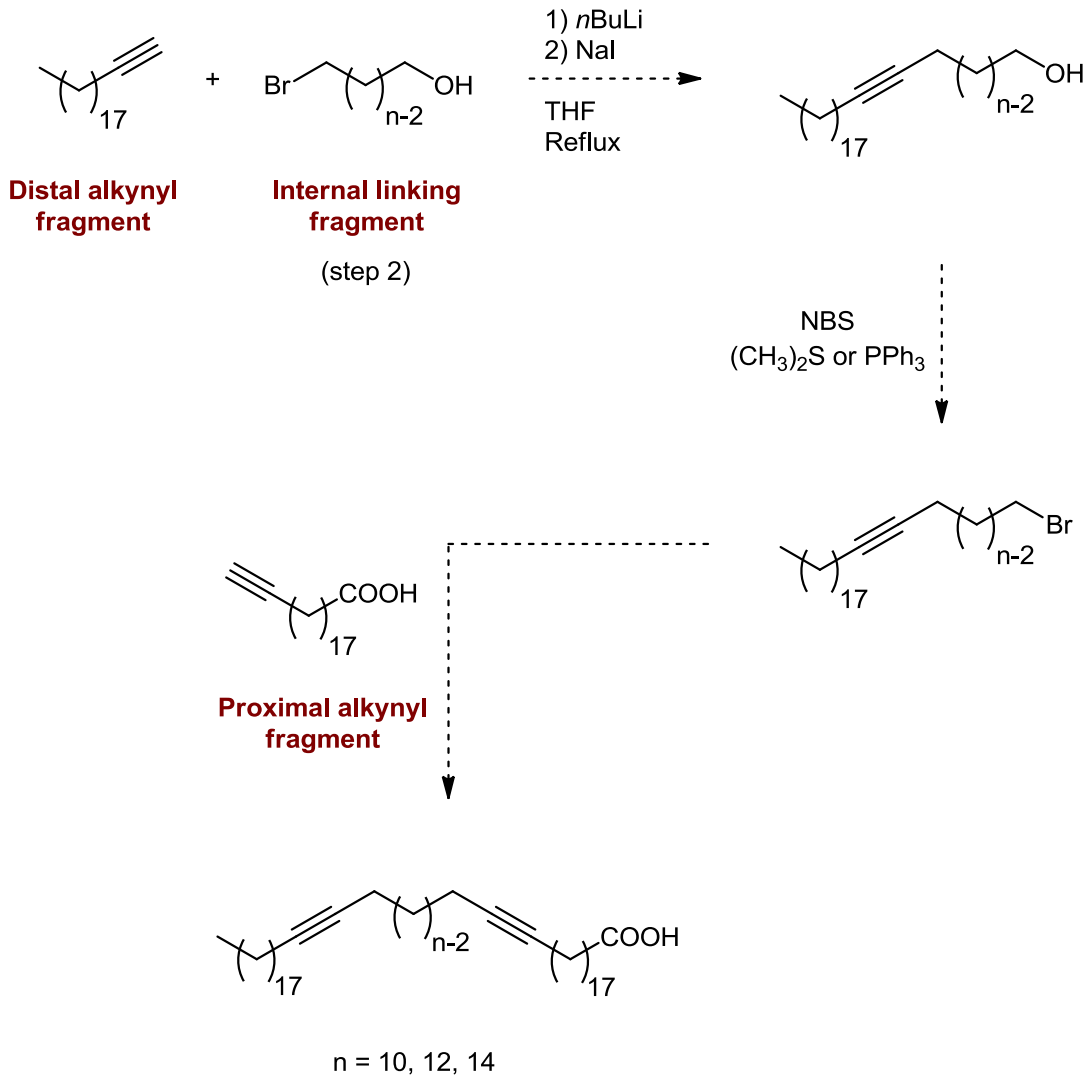
The α,ω-alkanediols were then converted to α,ω-halohydrins in toluene and in the presence of aqueous hydrobromic acid (Scheme 2.36). The yields for this transformation are high as discussed previously in the Chapter 2.



Scheme 2.36. Synthesis of α,ω-halohydrins by treatment of α,ω-alkanediols with aqueous hydrobromic acid.

3.2.4 Assembly of fragments

We envisioned assembling these three fragments (the proximal and the distal alkynyl fragments and the internal linking fragment) *via* alkyne-bromide coupling reactions according to the sequence shown in Scheme 3.10. This synthetic strategy starts with the coupling of the distal alkynyl fragment with the internal linking fragment, which are the easiest fragments to prepare. This coupling is expected to proceed smoothly through the conditions applied for the coupling of 1-octyne with 12-bromododecanol.



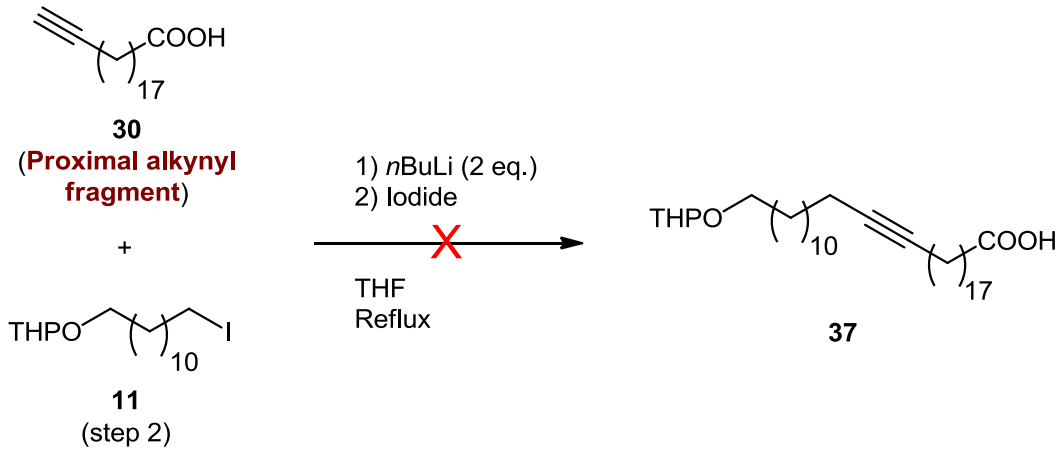
Scheme 3.10. Synthetic strategy for the preparation of acetylenic fatty acid via the assembly of three fragments.

The resulting alkynyl alcohol may then be converted in bromide. This transformation may proceed efficiently if the conditions used consider the presence of an alkyne group. Efficient methodologies for this conversion include the treatment of alcohol with *N*-bromosuccinimide (NBS) in the presence of dimethyl sulfide[269] or triphenylphosphine[270], and the Appel[271] reaction using tetrabromomethane and triphenylphosphine.[272]

The resulting bromide will then be used in the coupling with the proximal alkynyl fragment. The study of this reaction is the theme of the next section.

3.2.4.1 Coupling of the proximal alkynyl fragment with organohalides

The proximal akynyl fragment represents a challenging substrate for the alkyne-halide coupling reaction due to co-presence of the carboxylic group in the molecule. This reaction requires initially the conversion of the ω-acetylenic acid **30** to dilithium derivate and then the coupling with the organohalide. In order to study the reactivity of this alkyne, the iodide **11** was used as organohalide in this reaction (Scheme 3.11).

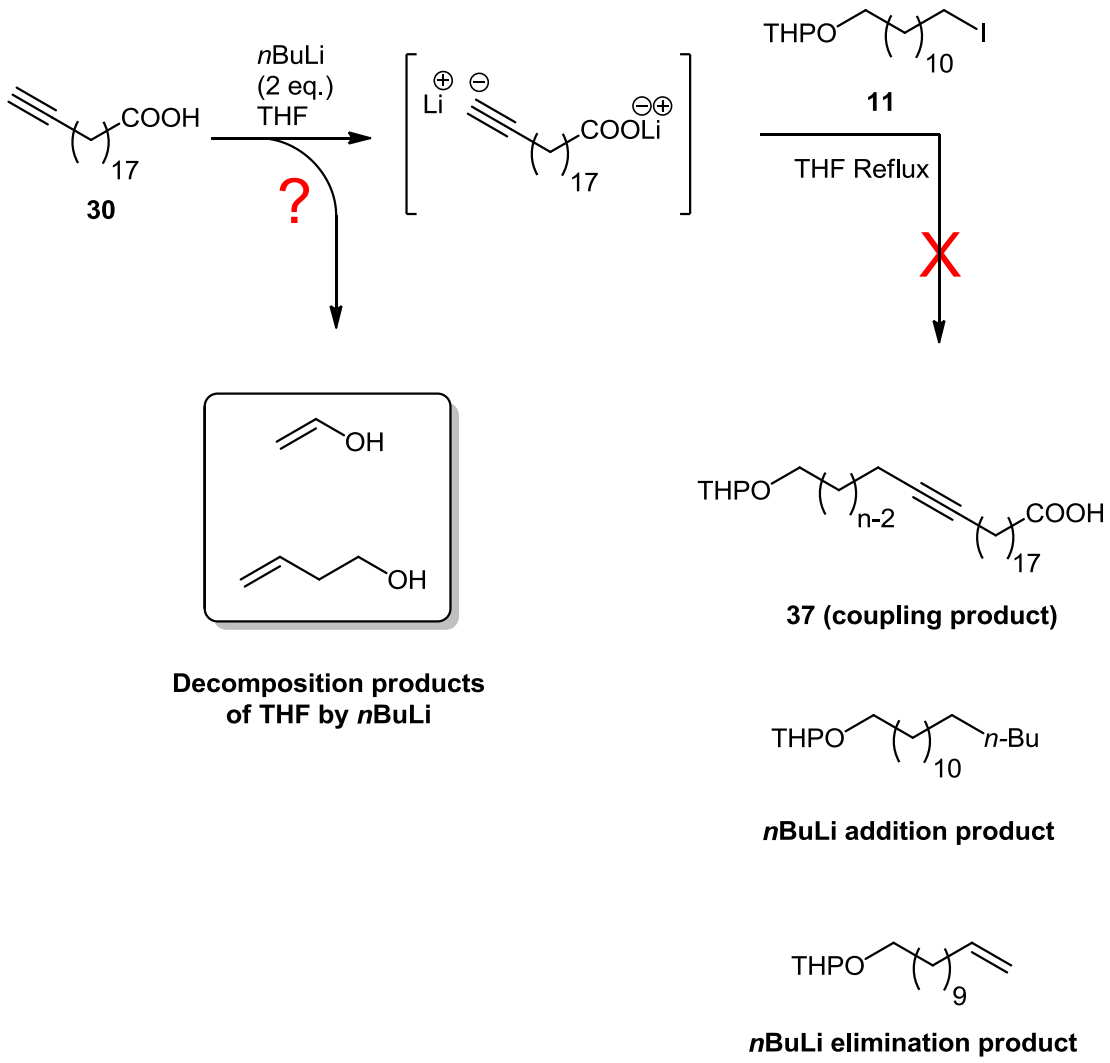


Scheme 3.11. Unsuccessful attempt to couple the proximal alkynyl fragment with an organohalide via lithium acetylide intermediate in refluxing THF.

Unfortunately, the attempt to conduct the dilithiation of the substrate **30** by two equivalents of *n*-butyllithium in THF and the following coupling with the halide **11** in refluxing THF was unsuccessful. In fact, the purification of reaction mixture allowed only the recovery of unreacted starting materials. In addition no side-product was detected from the crude reaction mixture by proton NMR experiments. In particular no evidence of the formation of *n*-butyl-lithium-mediated addition or elimination products from organoiodide was detected (Scheme 3.12).

Since the coupling between acetylide and iodide is supposed to proceed smoothly in refluxing THF, we believe that the formation of the dianion intermediate

may represent the key step in this reaction. The absence of side-products of the organoiodide demonstrates however that *n*-butyllithium was probably consumed at the moment of the addition of the halide.



Scheme 3.12. Possible side-products in the failed coupling between ω-acetylenic acid **30** and organohalides **11**.

Difficulties in the formation of di-lithium intermediates and in the subsequent reaction with electrophiles are reported in literature. Notably, Stanetty and co-workers showed that the preparation of dilithium *N*-Boc-aniline depended rigorously on several factors such as lithiation temperature, concentration, lithiating agent, and

solvent.[273] In particular the decomposition of tetrahydrofuran by *n*-butyllithium is a relevant side-process during the dilithiation of insoluble substrate. Despite tetrahydrofuran is commonly considered an inert solvent, its unstability in the presence of lithium alkanes is reported in the literature.[274-276] In particular the half-life of this solvent in the presence of *n*-butyllithium is 10 minutes at 35 °C.[276] Specifically, Clayden reported recently the mechanisms involved in the organolithium-mediated degradation of tetrahydrofuran to the lithium enolate of acetaldehyde and lithium but-3-en-1-oxide *via* deprotonation of the ring at the α- or β-position.[277]

From these results we concluded that the failed attempt to couple ω-acetylenic acid **30** and iodide **11** using *n*-butyllithium as base may be due to the low solubility of the monoanion intermediate in THF at low temperatures. In fact the addition of *n*-butyllithium to the ω-acetylenic acid **30** at −20 °C generated a white precipitate that we postulated to be the monoanion intermediate. The precipitation of this intermediate hampered the formation of the reacting dianion species and the subsequent coupling product. The remaining equivalent of *n*-butyllithium presumably decomposed reacting with THF before the addition of iodide **11**.

Therefore we turned our attention to an alternative procedure to couple these two fragments. Interestingly, it was found that a previous coupling reaction of various ω-acetylenic acids with organobromides on a mole scale was reported by Osbond *et al.* in 1961, using the ethyl Grignard reagent as base and cuprous cyanide as catalyst.[278]

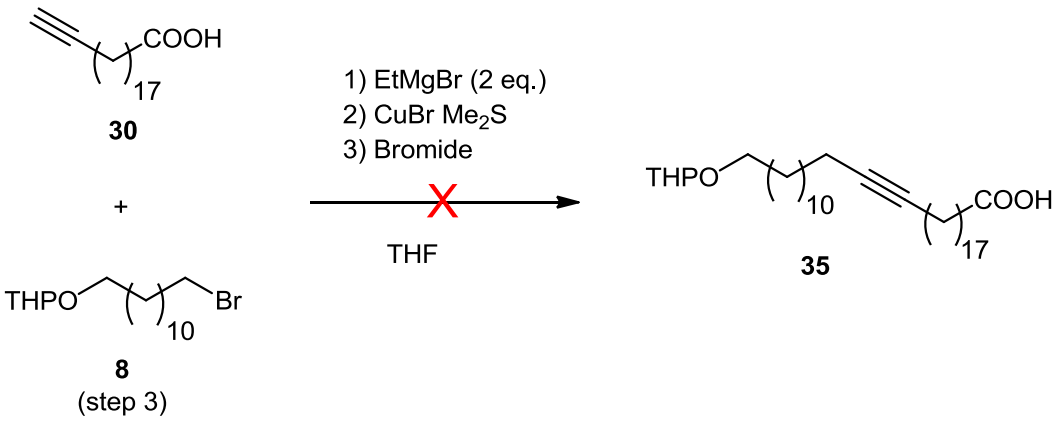
The synthetic applications of organocopper chemistry are ancient and precede the use of more modern transition metals such as palladium and nickel. In particular, the advent of copper in the catalysis of nucleophilic reactions of carbanions is dated back to 1941, when Kharasch and Tawney discovered that the presence of catalytic amounts of copper iodide could catalyze the chemoselective 1,4 addition of the hard centre of isobutylmagnesium bromide to benzophenone.[279] Since then, numerous studies have expanded the applicability and improved mechanistic understanding of processes involving this transition metal.[280]

Despite the contribution of organocopper chemistry to the field of total synthesis is decided, some transformations involving copper salts are challenging and

difficult to reproduce in small scale. The difficulties in these transformations are due to the tendency of organocopper derivate to undergo homocoupling *via* oxidative pathway. The oxidative dimerisation of copper acetylides under air exposition is known as Glaser reaction and it dates back to 1869.[281, 282]

The tendency of organocopper reagents to undergo oxidative dimerisation during cross-coupling reactions was investigated by Whitesides and House, who showed that this by-reaction takes place at low temperatures by different oxidant species such oxygen (from air), nitrobenzene and Cu(II) salts.[283, 284] It is important to highlight that the decomposition of organocopper reagents may be due also to significant amounts of Cu(II) salt impurities in air-sensitive Cu(I) catalyst. In order to avoid possible contaminations from Cu(II) salts, House proposed to use copper(I) bromide-dimethyl sulphide complex as convenient precursor of organocuprates.[285]

Nevertheless, in our hands, the coupling between the ω-acetylenic acid **30** with organobromides **8** using the ethyl Grignard reagent as base and copper(I) bromide-dimethyl sulphide complex as catalyst was unsuccessful (Scheme 3.13). Analytical experiments of the crude reaction mixture detected only unreacted starting materials.



Scheme 3.13. Unsuccessful attempt of a copper-catalyzed coupling reaction between the di-Grignard derivates of ω-acetylenic acid **30** with the organobromide **8**.

It is important to report, however, that differently to the preparation of the di-lithium intermediate, the treatment of ω-acetylenic acid **30** with the ethyl Grignard reagent yielded no precipitate, showing that the di-Grignard intermediate was

completely soluble in THF at room temperature. This behaviour excludes that the failed coupling may be due to problems of solubility. We believe instead that the possibility that some oxygen (from the air) may have reached the reaction mixture can not be excluded, in spite of our efforts to maintain an oxygen-free atmosphere. The application of the reaction on a sub-millimole scale had doubtless increased the susceptibility of organocopper reagent to oxidation by unwanted oxygen.

Alternatives to these reactions include the use of less basic base, such as LDA, or the application of Sonogashira conditions.[286] Further investigations were, however, interrupted due to the unexpected loss of *ca*. 5 g of the proximal alkynyl fragment due to the degradation of the silver acetylide under treatment with nitric acid.

3.3 CONCLUSIONS AND FUTURE WORKS

In this chapter, a convergent synthetic strategy for the *cis*,*cis*-diunsaturated meromycolic acid is proposed, based on the preparation of three fragments: two alkynyl fragments and an internal linking fragment. The key reactions in this strategy are the alkyne-bromide coupling reaction and the isomerisation of internal alkyne to terminal isomer. Attempts to optimise the alkyne-bromide coupling led to find that the use of *n*-butyl lithium as base deprotonates 1-octyne and allows the direct coupling with the non-protected bromo-dodecanol in refluxing THF. It was found that the isomerisation of the resulting alkyne proceeds in high yields using KAPA. The consequent purification by silver salt yielded the proximal alkynyl fragment in high yields and purity.

The distal alkynyl fragment and the internal linking fragments were prepared smoothly from cheap and easily commercially available starting material. The study of the assembly of these fragments was interrupted for the unexpected loss of proximal alkynyl fragment due to the degradation of the silver acetylide in the presence of concentrated nitric acid. However, this strategy remains promising and potentially important for large scale preparations of unsaturated meromycolates.

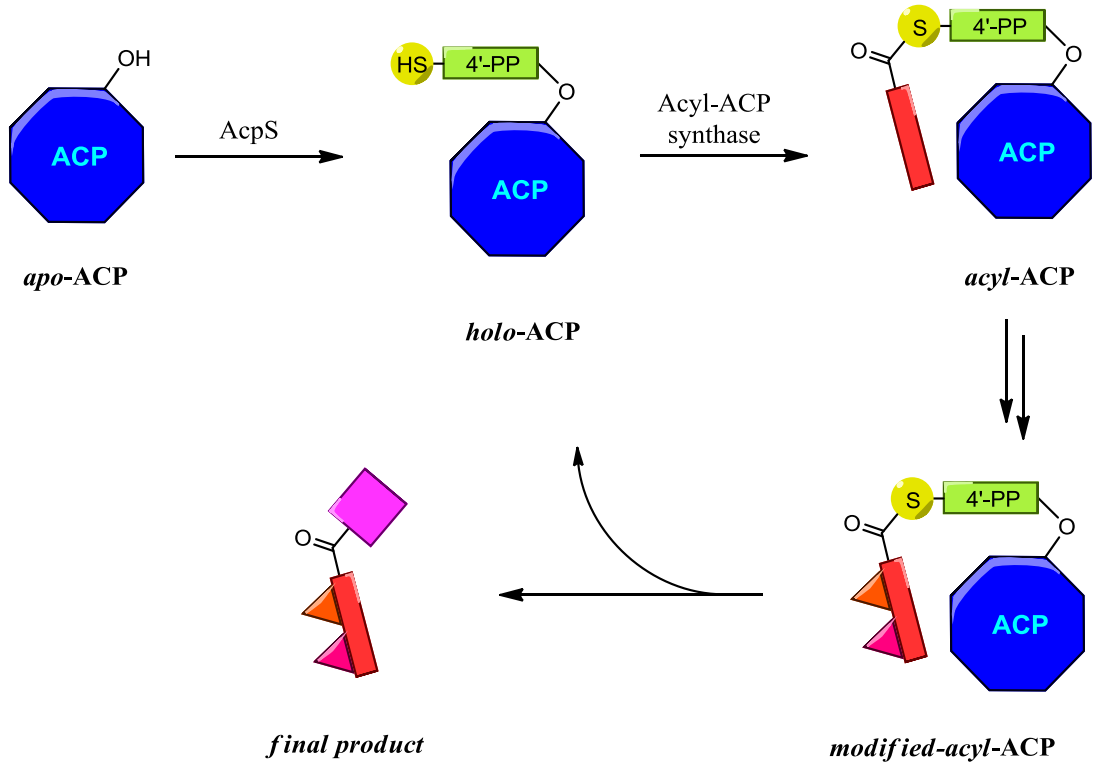
CHAPTER 4

4. EXPRESSION AND PURIFICATION OF MYCOBACTERIUM TUBERCULOSIS ACYL CARRIER PROTEIN

4.1 INTRODUCTION

4.1.1 Acyl carrier protein (ACP)

Acyl carrier protein (ACP) is an essential cofactor involved in the biosynthesis of a number of molecules that are key cellular components in mammals, yeast, bacteria and plants. ACP belongs to a broad family of small acidic proteins with a sequence that is generally constituted by 70-110 residues and is highly conserved among species.[287] ACPs (or ACP-like protein domains) play an essential role in the biosynthesis of fatty acids,[288-291] lipo-oligosaccharides,[292, 293] polyketides and nonribosomal peptides,[294] and in the post-translational acylation of proteins.[295]



Scheme 4.1. Cartoon representation of a typical metabolic pathway that involves ACP.

In metabolic pathways (Scheme 4.1), ACP carries out its functions using an essential prosthetic group, 4'-phosphopantetheine (4'-PP), attached to a conserved serine. ACP is initially expressed as *apo* protein and successively undergoes post-translational modification to form *holo*-ACP. This reaction occurs in the presence of coenzyme A (CoA) and is catalyzed by a phosphopantetheinyl transferase (PPTase),[296] termed *holo*-acyl carrier protein synthase (AcpS).[297]

The functional form of the protein, *holo*-ACP, contains a sulfhydryl group in the 4'-PP moiety, which represents the site of attachment of the acyl group. The acylation of *holo*-ACP is mediated by a broad family of enzymes termed acyl-acyl carrier protein synthase.[298]

The lipid of acylated protein (acyl-ACP) may thus be the substrate of a myriad of specific enzymes, and be the subject of further modifications, transferred to saccharides, peptides, proteins, or released to form fatty acids, phospholipids or polyketides. In these metabolic pathways ACPs allow the adequate solubility of the attached substrates and the prevention[299] of their degradation. Moreover, ACPs recognise the partner enzyme allowing the enzymatic reaction.

All the enzymatic reactions that involve ACP are allowed by its structure and high flexibility that allow to have dynamic conformation changes. In *virtue* by this structural feature, ACP interacts with a number of enzymes, alternatively sequestering and releasing its covalently attached acyl moiety to the active sites of partner enzymes.[287] The structure of ACP is essential for protein-protein interactions with partner enzymes. In particular, site-direct mutagenesis,[300-304] crystallographic[305, 306] and computational[307, 308] studies have shown that some residues of ACP are crucial and their substitution results in a drastic reduction of the enzymatic activity. In addition some residues have been shown to be important for maintaining the conformational stability of the protein.[300, 309]

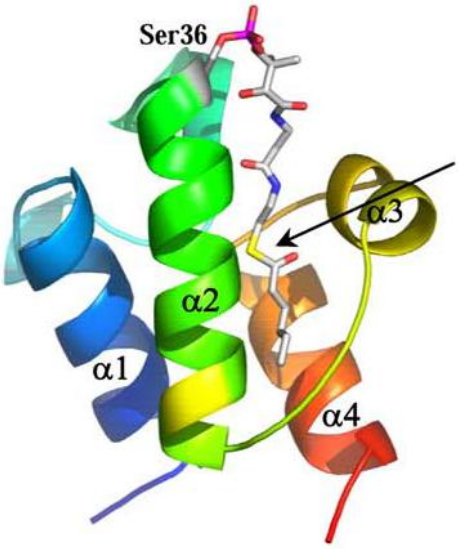


Figure 4.1. Crystallographic analysis of hexanoyl-ACP from *E. coli* as reported by Roujeinikova *et al.*[310]. The arrow indicates the thioester bond between the 4'-PP moiety and the acyl group.

The structures of ACPs determined so far by NMR or x-ray analyses have shown a common "ACP fold" consisting of a 4 helix bundle. This structure is characterized by three major α helices, where helix I is antiparallel to helices II and IV, whereas helix III is a short 3_{10} or 4_{13} (α) helical segment. Studies on ACPs with different partner enzymes have shown that the acidic residues in helix II are essential for protein-protein interactions and the catalytic activity (Figure 4.2).[300-308] NMR and crystal structures of acyl-ACPs have demonstrated the presence of a flexible hydrophobic binding pocket for the fatty acid, which allows interaction with hydrophobic residues in helices II and IV.[310-313]

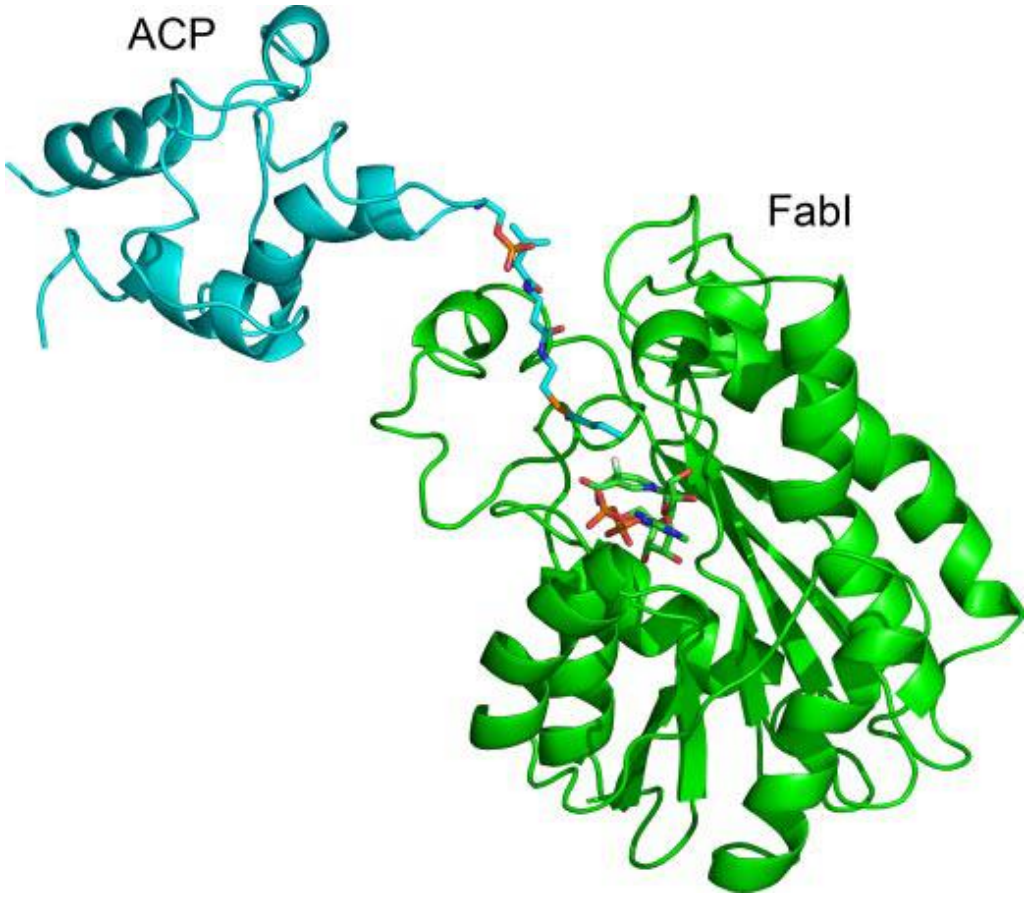


Figure 4.2. Protein-protein interaction between *E. coli* acyl-ACP and a partner enzyme (FabI), based on a combination of x-ray crystallography and molecular dynamics simulation as reported by Fafi *et al.*[305]. The structure shows the interaction between acidic residues in the ACP helix α2 and basic residues in FabI.

4.1.2 *Mycobacterium tuberculosis* acyl carrier protein (AcpM)

In 1998, Clifton Barry III and co-workers identified an acyl carrier protein (AcpM) in *Mycobacterium tuberculosis* by the isolation of upregulated hexacosanoyl-AcpM after treatment with isoniazid.[314] AcpM showed high homology with other ACPs and its function was supported by the location of *acpM* (Rv2244) in an operon that encodes enzymes involved in fatty acid biosynthesis (*fabD*, *kasA*, *kasB* and *accA*).[38, 314] In the same year, the same group confirmed the involvement of AcpM in the pathway to mycolic acids by *in vitro* experiments with cyclopropanating enzymes,[315] indicating that AcpM links growing mero acid during modification and synthesis.[315, 316] In 2001, AcpM was over expressed in *E. coli*, [317, 318] and in the following year, its solution structure was studied by NMR spectroscopy.[319]

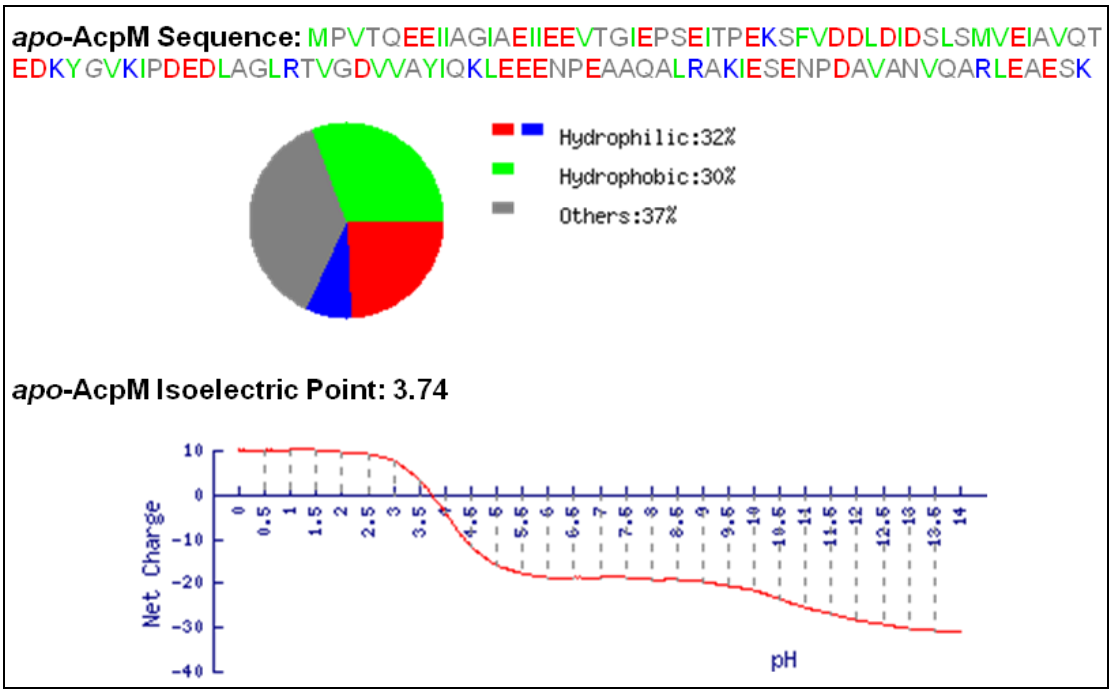


Figure 4.3. Properties of *apo*-AcpM calculated by online peptide property calculator (http://www.genscript.com). Acidic (D, E and C-terminal), basic, (R, K, H and N-terminal), hydrophobic uncharged (F, I, L, M, V, W and Y) residues are reported in red, blue and green respectively.

The sequence of AcpM is constituted by 115 residues (Figure 4.3) and shows a high degree of similarity with the other ACPs.[287, 319] The solution structure of AcpM has 4 α-helices and the superposition with other structures of the same family reveals the conserved "ACP fold".[319] Nevertheless, in contrast with the other bacterial ACPs, the protein from *M. tuberculosis* presents a unique carboxyl-terminal extension that has a relaxed, unfolded structure rich in hydrophobic residues. Although the function of this unique extension is not clear, it could accommodate the very long chain of the growing fatty acid. However, it is also plausible that this domain may have a role in the interaction with partner enzymes.[319]

Wong *et al.* conducted also a comparative study between the solution structures of *apo*- and *holo*-AcpM, reporting that the two forms have essentially the same structure (Figure 4.4). However, it was observed that the 4'-PP moiety creates a chemical shift perturbation in residues close to Ser-41, where the prosthetic group is covalently linked, and in the loop region between helices II and III. The authors have suggested that the prosthetic group could be in rapid equilibrium between two states, one where it is bound to the hydrophobic pocket and another where it is exposed to the solvent.[319]

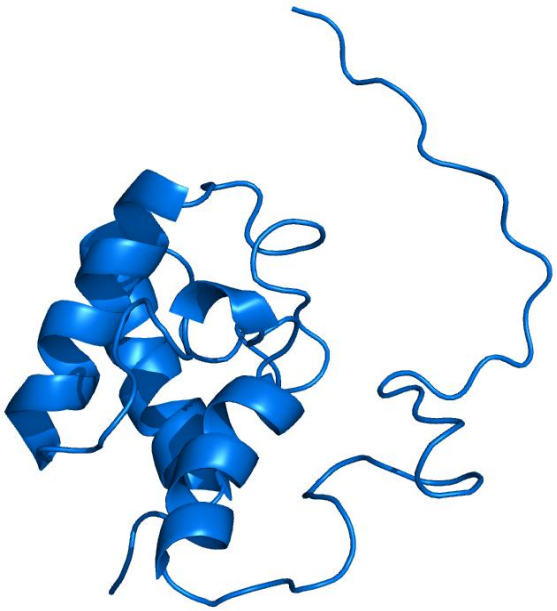


Figure 4.4. Solution structure of AcpM according to the study of Wong *et al.* [319]. (The figure was prepared by PyMol[61]).

4.1.3 Methodological considerations in the analysis of AcpM

During the last two decades, analysis and study of proteins and, more recently, proteomics,[320] have been established as new disciplines for the understanding of biological processes. The rise of this field of research was connected to the development of powerful techniques for protein identification and characterization. Mass spectrometry was, doubtless, the analytical method that allowed the solving of technical and challenging problems connected to proteomics.[321-323]

The study of ACPs by mass spectrometry represented one of the first challenges in protein analysis.[324] More recently, electrospray ionization (ESI) and surface-enhanced laser desorption/ionization (SELDI) mass spectroscopy were used in analysing AcpM.[317, 318] Despite nowadays the validation of the recombinant protein expression is generally based on the analysis by mass spectrometry,[325] sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an other analytical method that is historically and currently important.

SDS-PAGE was introduced in the late 1960s and is a technique widely used to analyse proteins.[326] It is based on the ability of SDS to denature and solubilise proteins forming SDS-protein complexes. These complexes, when put into an electric field, migrate towards the positive electrode (anode) for the presence of negatively charged sulfate groups. Since SDS binds generally to all protein samples in a constant weight ratio (*ca.* 1.4 g of SDS/g of protein),[327] the migration of SDS-protein samples under an electric field depends on the mass of the protein.

Nevertheless, not all the proteins rigorously respect the SDS/protein ratio and it complicates the analysis. In the case of the expression of AcpM in *E. coli* a mixture of *apo*-, *holo*- and palmitoyl-AcpM was obtained. It was reported that, despite these proteins have a similar molecular weight, it was possible to visualise three different bands. In fact, the presence of a phosphate group in the 4'-PP moiety results in a minor migration of *holo*-AcpM in comparison with *apo* form,[328] whereas the presence of a lipid in the protein leads to bind more SDS and, consequently, to have a lower apparent molecular weight.[329]

4.2 RESULTS AND DISCUSSION

4.2.1 Expression of AcpM in *E. coli*

In order to device a method to allow the selective thioesterification of *holo*-AcpM (Chapter 5), AcpM was overexpressed in *E. coli* and, subsequently purified. The overexpression of AcpM in *E. coli* was firstly reported in 2001 by two independent groups.[317, 318] They reported that the expression of AcpM yielded a mixture of *apo*-, *holo*- and acyl-AcpM in variable ratios. Schaeffer *et al.*[317] favoured the expression of *apo*-AcpM in *E. coli* BL21(DE3) cells using short induction time and treatment with phosphodiesterase. In addition, the authors reported that the expression of AcpM had a mildly toxic effect, decreasing the growth rates upon its induction, as reported previously for *E. coli apo*-ACP.[299] This toxic effect had probably lead Kremer *et al.*[318] to express AcpM in the *E. coli* C41(DE3) host strain,[330] which is effective in expressing toxic and membrane proteins.[330, 331]

In the present work we overexpressed AcpM in the *E. coli* Tuner (DE3) host strain using a pET vector. The Tuner strains are lacY deletion mutants of BL21, which allow a more uniform uptake of IPTG into all cells in the population, enhancing the production of recombinant proteins in soluble form. In fact, recombinant protein overexpression in *E. coli* frequently generates protein inclusion bodies instead of producing soluble and active proteins.[332] Tuner cells, under the control of the T7 promoter system and in the presence of IPTG, allow a slow expression ensuring that recombinant proteins fold correctly.

The pET28b::*acpM* plasmid was kindly provided by L. Kremer[318] and used in the transformation of competent *E. coli* cells by heat shock.[333] Successively, an overnight culture of *E. coli* DH5α cells was used to prepare purified pET28b::*acpM* plasmid by Wizard® Minipreps DNA Purification System.[334]

Overnight cultures of *E. coli* Tuner cells carrying the pET28b::*acpM* plasmid were inoculated into LB broth containing kanamycin, and incubated at 37 °C until mid-log phase (optical density at 600 nm = 0.5–0.7). Then the expression of AcpM

was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration = 1 mM) and the cultures were incubated at 16 °C overnight.

Cells were harvested, resuspended and lysed by French pressure cell press. The soluble fraction was purified by fast protein liquid chromatography (FPLC).

apo*-AcpM sequence from expression of pET28b::*acpM

GSSHHHHHHSSGLVPRGSH MPVTQEEIIAGIAEIIEEVTGIEPSEITPEKSFVD DLDIDSLSMVEIAVQTEDKYGVKIPDEDLAGLRTVGDVVAYIQKLEEENPEAA QALRAKIESENPDAVANVQARLEAESK

Chemical Formula:
$C_{630}H_{1008}N_{174}O_{217}S_2$

Molecular Weight:
14555.98

Isoelectric Point:
4.24

Hydrophilicity Analysis:

Peptide	Charge	Attribute
GSSHHHHHH SSGLVPRGSH MPVTQEEIIAG IAEIIEEVTGIE PSEITPEKSFV DDLDIDSLSM VEIAVQTEDK YGVKIPDEDL AGLRTVGDVV AYIQKLEEEN PEAAQALRA KIESENPDAV ANVQARLEAE SK	-11	acidic

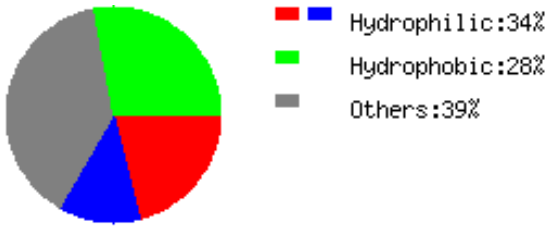


Figure 4.5. Sequence of *apo*-AcpM yielded from the expression of the pET28b::*acpM* plasmid. The native sequence is in violet and the thrombin recognition cleavage site (Leu-Val-Pro-Arg-Gly-Ser) is in pink. Properties of this protein are calculated by online peptide property calculator (http://www.genscript.com).

4.2.2 Purification of AcpM by Ni^{2+}-affinity chromatography

The AcpM protein obtained by the expression of pET28b::*acpM* contains a polyhistidine-tag (His-tag), which is constituted by six histidines (Figure 4.5). This short tag has a strong interaction with transition metal ions, and it allows a purification of the protein by Ni^{2+}-affinity chromatography.[335, 336] In fact, following to the French pressure cell press, the soluble fraction was purified by ÄKTA™ FPLC system using a Ni^{2+}-based column. The fractions eluted from this column were successively analysed by SDS-PAGE and electrospray ionization (ESI) mass spectrometry.

Specifically, SDS-PAGE analysis of purified fractions was carried out using both NuPAGE® 4–12% Bis-Tris gels and Novex® 18% Tris-Glycine gels, and run according to Laemmli[337]. In both cases, the gels showed two separate bands corresponding to *ca.* 15 KDa (Figure 4.6) indicating that the Ni^{2+}-affinity chromatography was successful. ESI analysis confirmed the SDS-PAGE results, indicating that the two bands corresponded to *apo*- and *holo*-AcpM consisted with the expected sequence (Figure 4.7). In addition, this technique has reported also a minor presence of the acylated forms, which probably showed similar apparent molecular weight on PAGE. SDS-PAGE analysis and ESI analysis have showed that the ratio between these protein forms was *apo*-AcpM > *holo*-AcpM >> acyl-AcpM, although tail fractions from Ni^{2+}-based column contained *holo*-AcpM as the major protein.

The expression of AcpM in *E. coli* yielded this carrier protein in acylated form, due to covalent bond with a series of fatty acids. Previous works reported that the expression of AcpM in *E. coli* yielded a mixture of acylated AcpM where the attached fatty acids were predominantly C16:0 and C18:0. In the present work, the identification of the fatty acids linked to AcpM was complicated by the low presence of this acylated form, in comparison to *apo*- and *holo*-AcpM, and by the tendency of these proteins to form adducts with a large number of sodium ions (Figure 4.7). A meticulous dialysis of the fractions containing acyl-AcpM allowed, surprisingly, to identify that a C16:1 fatty acid, presumably palmitoleic acid (*cis*-9-hexadecenoic),[338] was the lipid mostly linked to the carrier protein (Figure 4.8). Although the reasons

for this difference are not entirely clear, it could be due to the incubation at 16 °C. In fact, it is known that the fatty acid composition of *E. coli* changes as a function of growth temperature and that the proportion of unsaturated fatty acids increasing with lower growth temperature,[339, 340] in order to maintain an appropriate lipid fluidity.[341, 342]

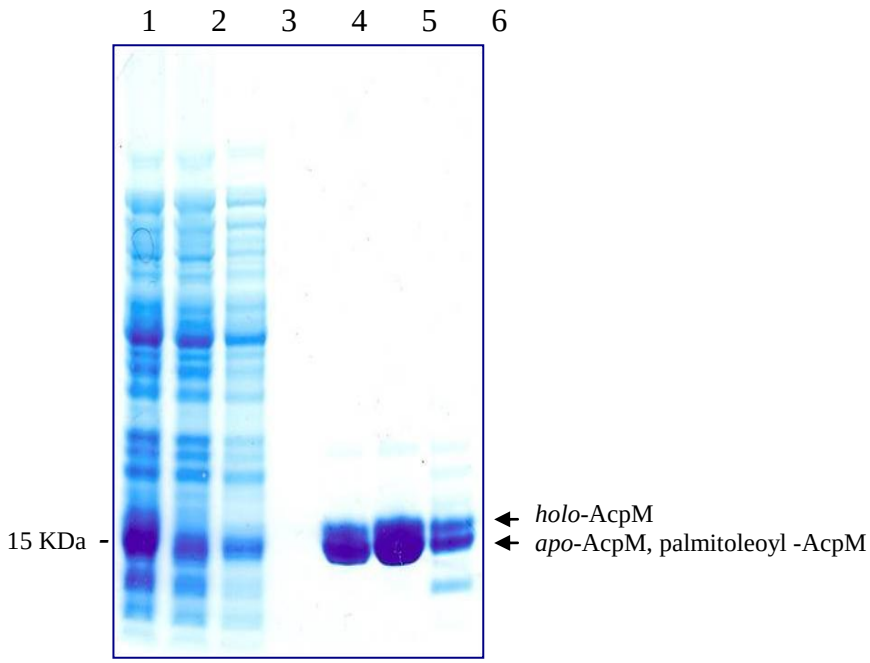


Figure 4.6. SDS-PAGE analysis of purification by Ni^{2+}-affinity chromatography using NuPAGE® 4–12% Bis-Tris gels under reducing conditions. The figure shows crude extract (lane 1), column flow through (lane 2), column wash (lane 3), washed column (lane 4), peak fraction (lane 5 & 6) and tail fraction (lane 7).

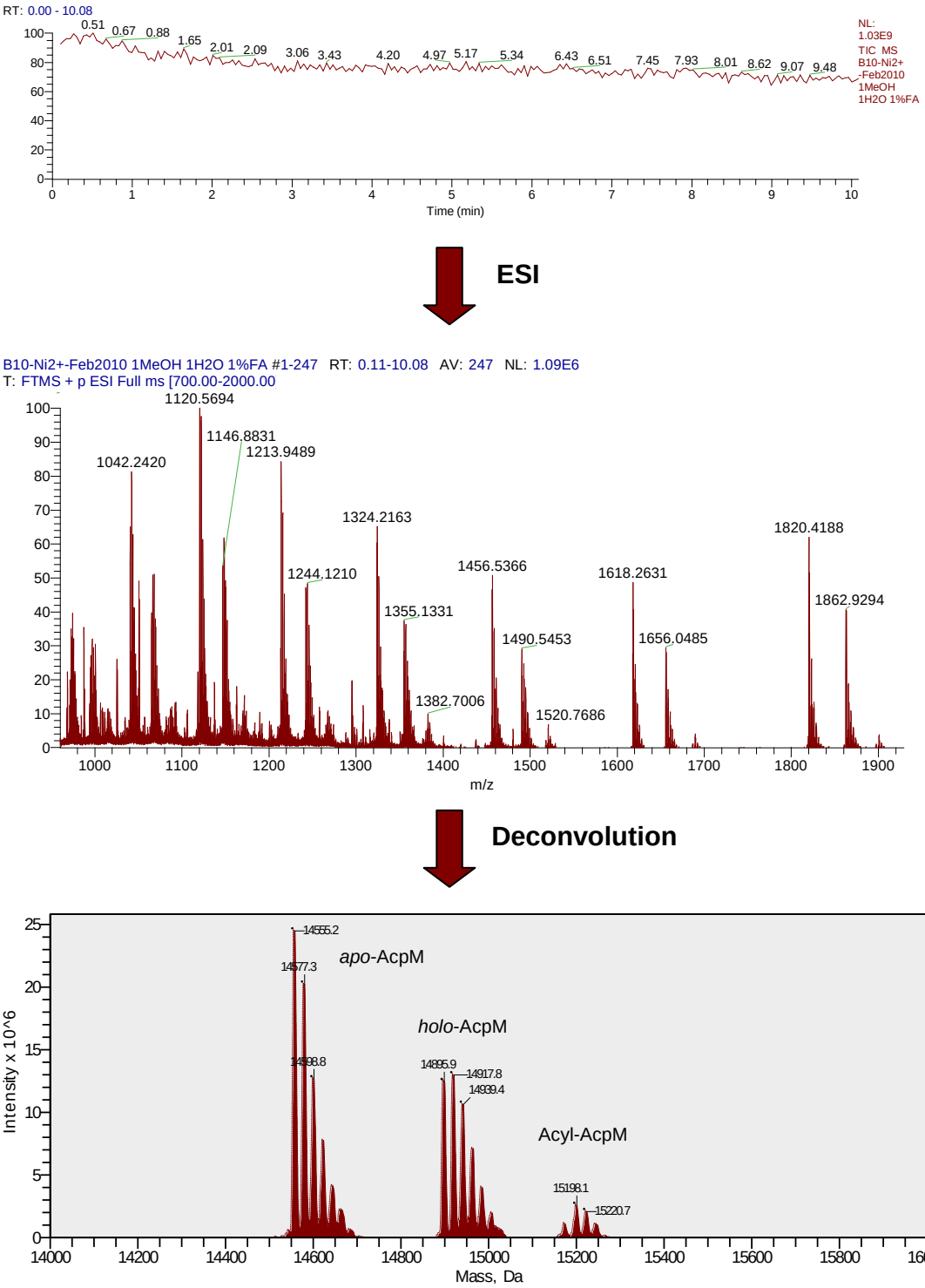


Figure 4.7. ESI analysis of the mixture of *apo*-AcpM (MW: 14555.3 Da), *holo*-AcpM (14895.9 Da) and acyl-AcpM after purification by Ni^{2+}-affinity chromatography. Peaks with difference of +22 Da are due to the exchange Na^{+}/H^{+}.

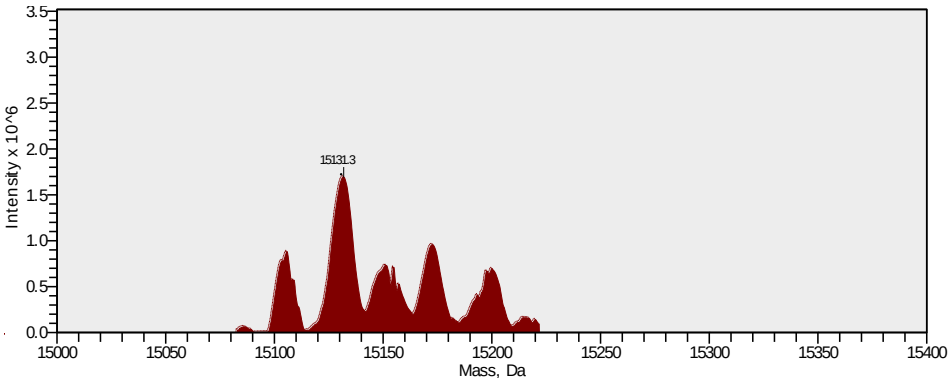


Figure 4.8. Deconvoluted spectrum of Acyl-AcpM. The major peak represents palmitoleoyl-AcpM (calculated mass: 15132 Da).

4.2.2.1 Yield of AcpM

Following Ni2+-affinity chromatography, protein quantification[343] by Bradford assay[344] showed that *ca.* 20 mg of AcpM were expressed and purified per liter of recombinant *E. coli* culture. It is important to report that attempts to optimise the conditions of expression of pET28b::*acpM*, using different temperatures and incubation times, proved fruitless as shown by SDS-PAGE analysis of the cellular lysate (data not shown).

4.2.2.2 Identification of protein impurities after the purification by Ni2+-affinity chromatography reveals a possible ACP–ribosomal protein interaction

Purity analysis of recombinant proteins has acquired an increasing importance during the last two decades. In particular, analysis of protein impurities is crucial in the preparation of biopharmaceuticals produced by recombinant DNA technology.[345-347] In fact, it is possible that, even after sophisticated purification steps, significant levels of host cell proteins remain present in the final purified product. Ni^{2+}-affinity chromatography represents one of the most powerful, selective and widely employed tools to purify recombinant proteins,[335, 336] and as reported in the previous section, this technique allowed an efficient purification of AcpM.

Nevertheless, on one occasion, during the purification of AcpM by Ni^{2+}-based column it was possible to isolate among tail fractions, one sample containing some protein impurities. SDS-PAGE analysis showed these impurities as bands between 10 and 20 KDa (Figure 4.9). ESI analysis showed that, in addition to *holo*-AcpM and *apo*-AcpM, were present other proteins having molecular weighs equal to 8875.3, 10137.4, 14363.4 and 16018.0 (Figure 4.10).

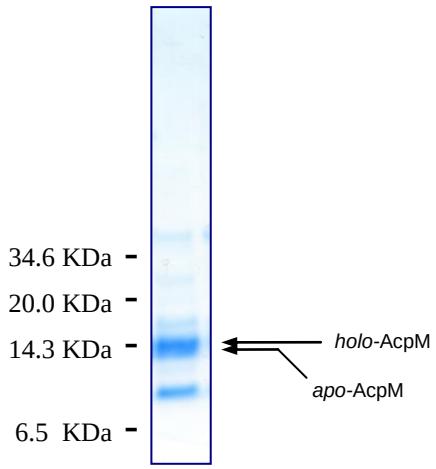


Figure 4.9. SDS-PAGE analysis of a tail fraction from Ni^{2+}-based column containing some protein impurities. Analysis was performed on Novex® 18% Tris-Glycine Gel.

Checking the detected mass values on the ExPASy server,[348, 349] it was possible to notice that they could correspond to the sequences of four ribosomal proteins: L28, S15, L17 and L13. The mass values detected were confronted with the values reported in literature by MALDI[350] and ESI[351] analysis. All the values detected in this work were found in the range of the values expected or previously reported (Table 4.1).

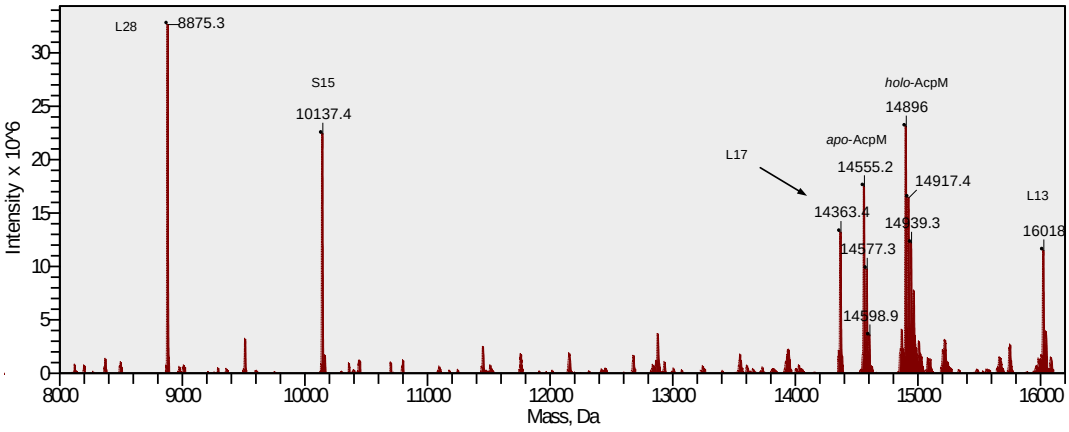


Figure 4.10. Deconvoluted spectrum showing protein impurities corresponding to ribosomal proteins.

Table 4.1. Comparison of the ESI values of the protein impurities found in this work, and the mass values calculated or previously reported for some ribosomal proteins.

E. coli ribosomal proteins	*Theoretical mass*	*Experimental MALDI mass*[350]	*Experimental ESI mass*[351]	*Exp. values detected in this work*
L28	8875.3	8875.3	8875.5	8875.4
S15	10137.6	10137.6	10137.1	10137.4
L17	14364.7	14364.7	14364.2	14364.4
L13	16018.6	16018.0	16018.5	16018.0

The analysis of the sequences of these ribosomal proteins, however, shows few consecutive histidine residues to support the hypothesis of a direct binding with the transition metal of the column. Conversely, ribosomal proteins are rich in lysine and arginine residues, and it suggests that the presence of these impurities could be due to electrostatic interactions with the acidic residues of AcpM.

- **50S ribosomal protein L28** (Chain: 2-78, pI: 11.42):
SRVCQVTGKRPVTGNNRSHALNATKRRFLPNLHSHRFWVESEKRFVTLRVSAKGMRVI DKKGIDTVLAELRARGEKY

- **30S ribosomal protein S15** (Chain: 2-89 pI: 10.40):
SLSTEATAKIVSEFGRDANDTGSTEVQVALLTAQINHLQGHFAEHKKDHHSRRGLLRMV SQRRKLLDYLKRKDVARYTQLIERLGLRR

- **50S ribosomal protein L17** (Chain: 1-127, pI: 11.05):
MRHRKSGRQLNRNSSHRQAMFRNMAGSLVRHEIIKTTLPKAKELRRVVEPLITLAKTDS VANRRLAFARTRDNEIVAKLFNELGPRFASRAGGYTRILKCGFRAGDNAPMAYIELVDR SEKAEAAAE

- **50S ribosomal protein L13** (Chain: 1-142, pI: 9.91):
MKTFTAKPETVKRDWYVVDATGKTLGRLATELARRLRGKHKAEYTPHVDTGDYIIVLNA DKVAVTGNKRTDKVYYHHTGHIGGIKQATFEEMIARRPERVIEIAVKGMLPKGPLGRAMF RKLKVYAGNEHNHAAQQPQVLDI

Figure 4.11. Sequences and isoelectric points (pI) of some *E. coli* ribosomal proteins.

In this context, the occasional and marginal presence of ribosomal proteins could be due to high concentration of proteins in the cellular lysate. In this case, the variation of ionic strength of buffer during the purification by Ni^{2+}-affinity chromatography could decrease the electrostatic interactions and remove these impurities.[352, 353]

It is interesting to note that the presence of ribosomal proteins, could be interpreted as a consequence of an interaction that is present also *in vivo*. In this *scenario*, the inhibition of cell growth due to overexpression of ACPs,[299, 317] could be connected with the putative interaction with ribosomal proteins. Although the interaction between ribosomal proteins and ACPs could have a relevant biological role, these effects have not been investigated in this thesis.

4.2.3 Purification of *holo*-AcpM by hydrophobic interaction chromatography

In order to purify *holo*-AcpM, the fractions from Ni^{2+}-affinity chromatography, containing in particular *apo*-AcpM as major protein, were purified by hydrophobic interaction chromatography using an octyl sepharose column (Figure 4.12). The protein sample was load using aqueous 1 M ammonium sulfate in order to increase the hydrophobic interactions between proteins and octyl groups of the column. The proteins were then eluted decreasing the ionic strength of the buffer. It is interesting to note that *holo*-AcpM was obtained as last fraction despite it does not contain a particular hydrophobic group. Rock and Garwin, in their study on hydrophobic chromatography of *E. coli* ACP reported that the activation of column matrix by CNBr could create positive charges that were principally responsible for the binding with acidic proteins.[354] However, personal communications from the manufacturer (Dr. Hui Xie, GE Healthcare) have excluded the presence of positive or partially positive charges in the column matrix.

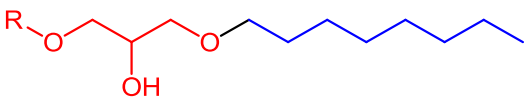


Figure 4.12. Type of ligand in Octyl Sepharose 4 Fast Flow.

It is important to report, however, that the purification by Octyl Sepharose 4 Fast Flow has shown only a mild binding capacity, which could be due to a low degree of substitution (Figure 4.13).[354] It has excluded the possibility to purify *holo*-AcpM in large scale by this methodology.

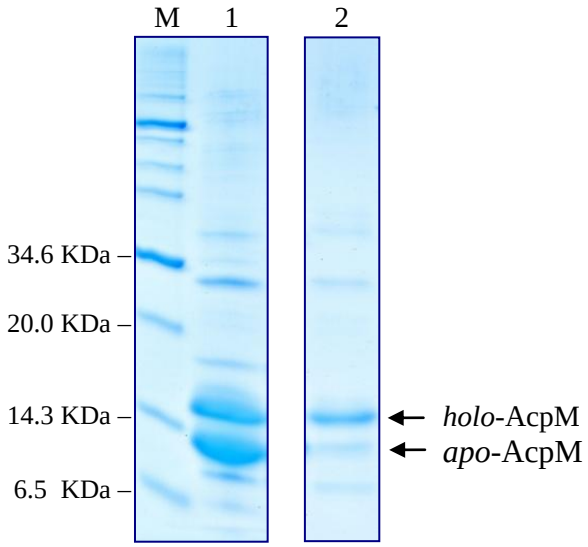


Figure 4.13. Purification of *holo*-AcpM by hydrophobic interaction chromatography. The figure shows the starting crude containing *apo*-AcpM and *holo*-AcpM (lane 1), the fraction after purification containing *holo*-AcpM as major protein (lane 2) and the molecular weight marker (lane M). Analysis was performed on Novex® 18% Tris-Glycine Gel.

4.2.4 Dialysis by centrifugal filter units: unexpected dimerisation of *holo*-AcpM

The removal of organic and inorganic impurities is essential in the ESI analysis. In the present work, the protein samples were dialysed using Microcon® centrifugal filter units with nominal molecular weight cut-off of 3 KDa. The dialysis and the consequent ESI analysis were generally efficient. Nevertheless, it is important to report that the desalting process for fractions containing *holo*-AcpM tended to result in the recovery of the corresponding disulfide dimer, as validated by SDS-PAGE (Figure 4.14) and ESI analysis (found/calculated mass values: 29788/29790 Da). It is important to note that, on SDS-PAGE, the dimer was promptly converted in *holo* form if loaded by reducing Laemmli buffer. The band corresponding to the dimer was visible on SDS-PAGE, only removing the reducing agent from the loading buffer (Figure 4.14).

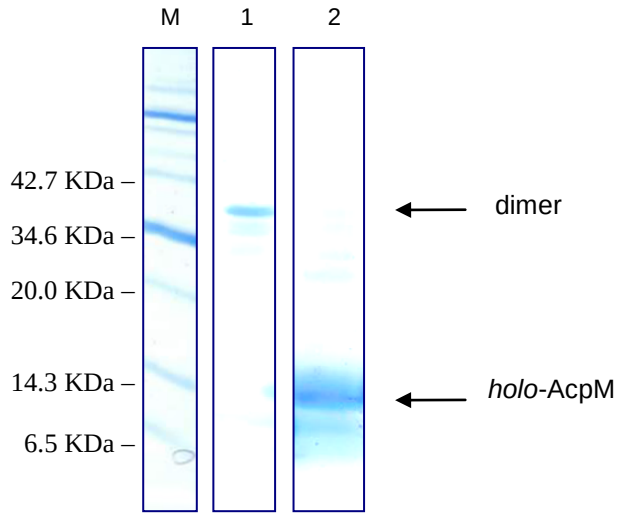


Figure 4.14. SDS-PAGE analysis of holo-AcpM after the dialysis by Microcon® centrifugal filter units (Lane 1) and subsequent treatment with DTT (Lane 2). The lane M reports the molecular weight marker, the lanes 1-2 were run in non-reducing loading buffer. Analysis was performed on Novex® 18% Tris-Glycine Gel.

In light of this unexpected dimerisation, the desalting process was generally carried out in the presence of 10–20 mM DTT at *ca.* 4 °C and avoiding to reduce the solvent to less than 50 μL. These modifications have allowed detection of *holo*-AcpM without the presence of the dimer.

The complete conversion of *holo*-AcpM to the corresponding dimer during the dialysis by centrifugal filter unit may be due to the increase of protein concentration that consequently increased also the rate of the dimerisation reaction. To the best of our knowledge, this drawback has not been highlighted or reported previously.

4.3 CONCLUSIONS AND FUTURE WORKS

In this chapter, *Mycobacterium tuberculosis* acyl carrier protein (AcpM) was overexpressed in *E. coli* Tuner (DE3) host strain using a pET vector. The purification of the cellular lysate by Ni^{2+}-affinity chromatography yielded three different forms of this protein: *apo*-AcpM, *holo*-AcpM and acyl-AcpM. The form acylated of this carrier protein was constituted predominantly by palmitoleic acid.

The purification by Ni^{2+}-affinity chromatography was efficient to separate the three forms of recombinant protein. This process allowed purification almost to homogeneity with the detection of some minor protein impurities, which were identified as *E. coli* ribosomal proteins. The putative interaction between AcpM and ribosomal proteins represents an interesting research theme for a future investigation.

The *holo* form was separated from the other two AcpM forms by hydrophobic interaction chromatography. Interestingly, the dialysis of *holo*-AcpM carried out by centrifugal filter units yielded the disulfide dimer as unique product. The dimer was then reconverted promptly to monomer by the treatment with DTT. These results suggest the attractive possibility to purify *holo*-AcpM from the other forms, through the conversion to the corresponding dimer, consequent size-exclusion chromatography and treatment with DTT.

CHAPTER 5

5. CHEMICAL SITE-SELECTIVE THIOESTERIFICATION OF PROTEINS WITH LONG-CHAIN FATTY ACIDS

5.1 INTRODUCTION

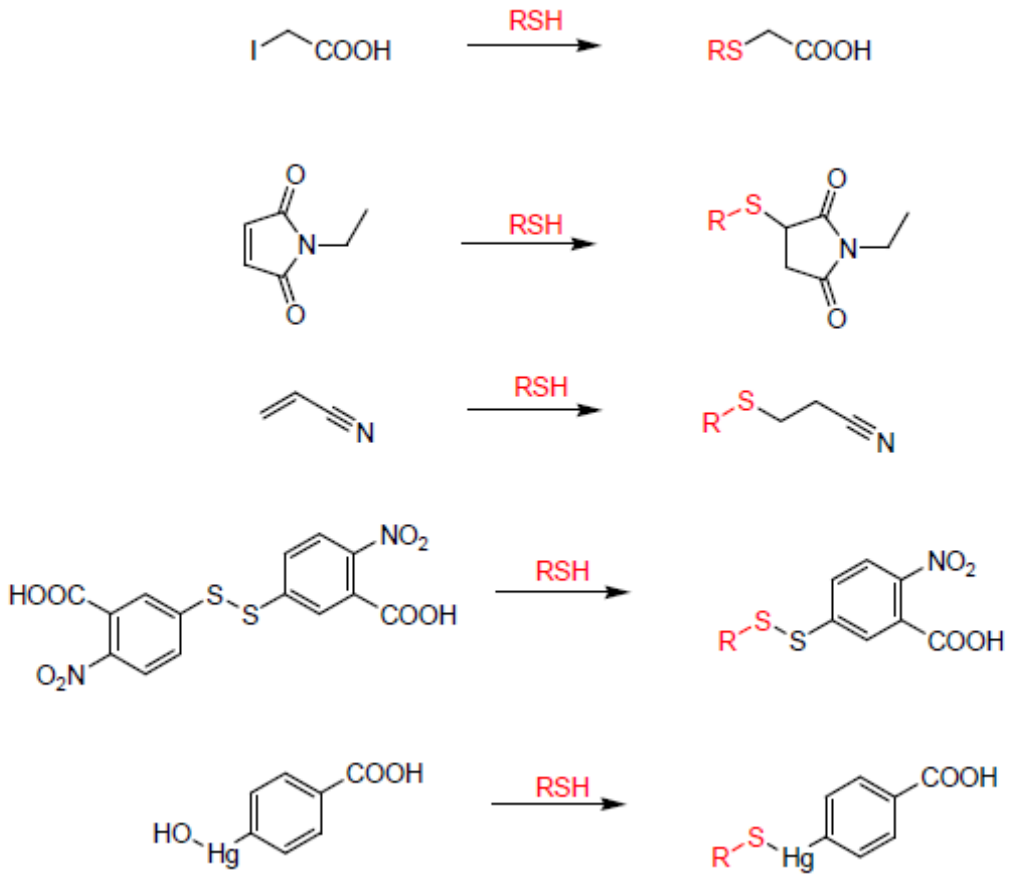
5.1.1 Semi-synthesis of proteins

In the last 40 years, there has been a growing interest in the chemical modification of proteins. Initially, this methodology represented the most powerful method to study enzymatic mechanisms and identify the residues involved in the catalytic activity of enzymes.[355] Nowadays, the chemical modification of proteins is a sophisticated method that finds application in various fields. This method allows the development of biocatalysts,[356, 357] drug delivery systems,[358] gene delivery therapeutics[359] and therapeutic protein conjugates. In particular, protein conjugates have been investigated for the therapeutic treatment against cancer,[360] HIV,[361, 362] malaria,[363] and pathogenic bacteria.[364] The discipline that allows the creation of novel protein entities *via* chemical modification of recombinant proteins is known as "protein semi-synthesis".[365-367]

Although the chemical modification of proteins is a powerful technique, it is characterized by challenging limitations. In fact, the conditions used during this process must consider the tendency of the substrate to undergo denaturation at acidic or basic pH values, at high temperatures and in the presence of organic solvents or surfactants.[368] Another important issue during this process is the need to modify a specific residue in spite of the presence of hundreds of competing groups with similar reactivity. In fact, protein chemistry does not permit the application of a synthetic strategy based on introduction and cleavage of protecting groups. A specific selectivity during the reaction with proteins can be achieved only by an accurate study of reagents and conditions.

5.1.1.2 Chemical modification of proteins at sulfur atom

In light of these challenging needs, the chemical modification of proteins has been based on rigid protocols and selective reagents.[369] Among the residues that undergo selective modification, the cysteine has attracted an increasing attention as an ideal point of attachment for the synthesis of bioconjugates.[370] This amino acid provides two remarkable features: a low natural abundance[371, 372] and its unique reactivity.



Scheme 5.1. Selective probes for cysteine residues.

Indeed, different compounds are known to react selectively with the sulfhydryl groups of a protein: iodoacetamide,[373] iodoacetate,[373, 374] *N*-ethylmaleimide,[375] acrylonitrile,[376] Ellman's reagent (DTNB)[377] and mercuribenzoate.[378] The selectivity of these reagents is based on fact that the sulfur atom of cysteine is a "soft" nucleophilic centre,[379] which has different reactivity in comparison to other nucleophilic residues of a protein. By *virtue* of this unique reactivity, cysteines react selectively with the sp^3 carbon of iodoacetamide or iodoacetate, the sp^2 carbon of maleimide or acrylonitrile, the sulfur atom of DTNB, and the heavy metal of mercuribenzoate (Scheme 5.1).

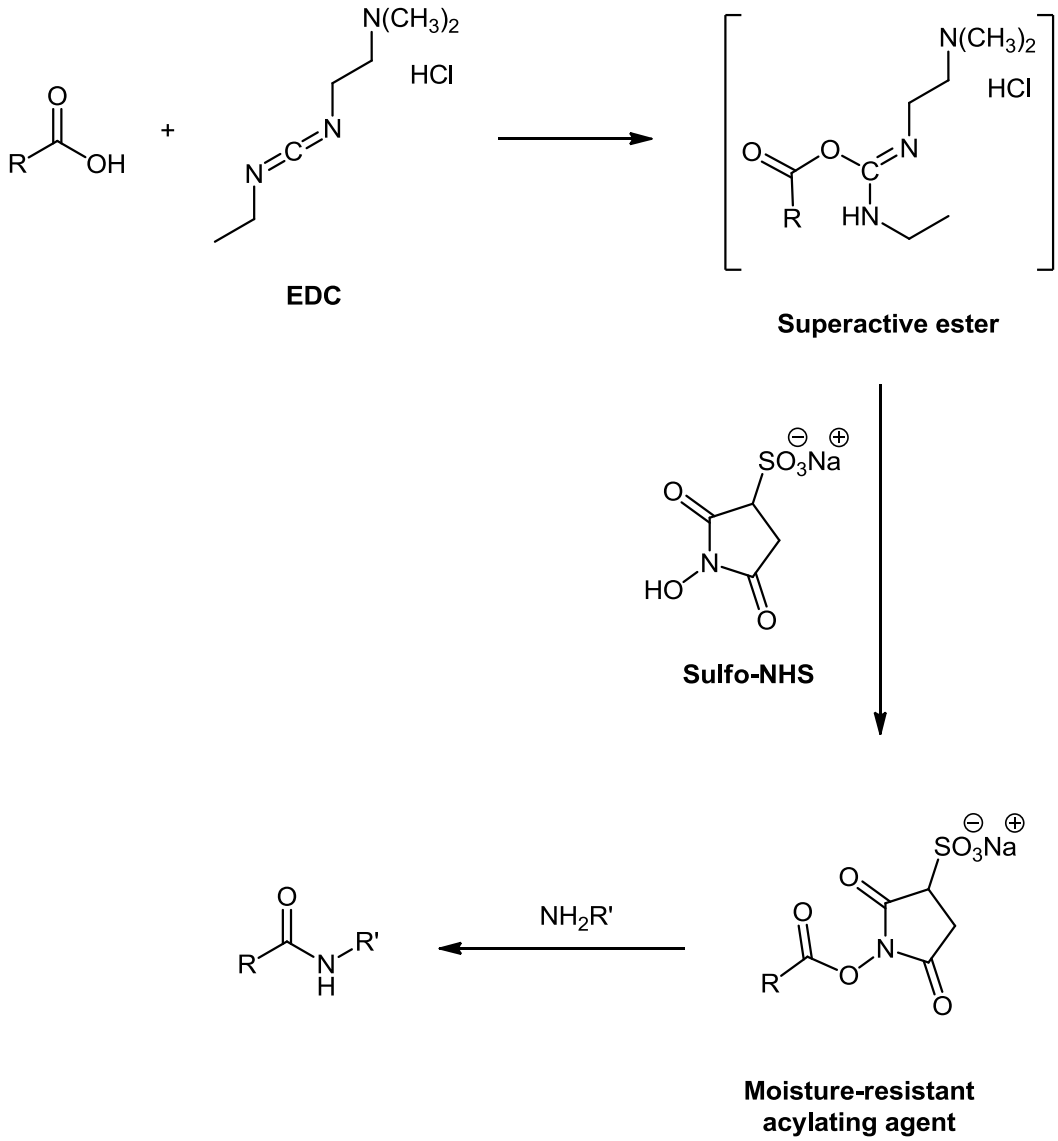
Conversely, lysines, tyrosines, histidines are "hard nucleophiles" and they are modified from carbonyl compounds such as acetic anhydride, diethylpyrocarbonate and acetylimidazole.[369] Unfortunately, this different reactivity between thiol groups and "hard nucleophiles" makes difficult a direct selective acylation of phosphopantetheine group of acyl carrier proteins.

5.1.2 Acylation of bioorganic compounds

The formation of amide, ester or thioester bonds plays a key role in the synthesis of a large number of bioorganic molecules and in particular during peptide synthesis. In general, during these transformations, the carboxylic group is initially converted in a reactive form, termed "superactive ester",[380] and successively left reacting with the nucleophile. A plethora of so-called "coupling reagents", have been developed to convert the -OH of the acid into a good leaving group.[381-383] Nevertheless such reagents or the resulting "superactive ester" are generally moisture sensitive and require the use of anhydrous organic solvents. Although the conditions involving coupling reagents are not applicable to the modification of proteins, the concept of "superactive esters" remains valid for the rational design of a successful strategy to form thioester bond in proteins.

5.1.2.1 Acylation of proteins

Acylation of proteins is often carried out *via* formation of active acylating ester by *N*-hydroxysulfosuccinimide (sulfo-NHS)[384] and *N*-hydroxysuccinimide (NHS)[385] (Figure 5.2). In these protocols, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) activates the carboxylic groups to form *O*-acylisourea intermediates, which in the presence of NHS and sulfo-NHS are converted into more stable acylating agents. In the absence of these additives, the aqueous reaction is not efficient since the superactive ester reacts very quickly with water resulting hydrolysis and regeneration of the carboxyl acid. In particular, Sulfo-NHS ester intermediates are active acylating species, which maintain a sufficient stability in water allowing the coupling with the proteins. In addition, the presence of a sulfonate group ensures a good solubility to these active esters. Unfortunately, it was reported that sulfo-NHS-based active ester reacts at pH 7.4 with histidine and lysine more quickly than with cysteine residues.[386] This reactivity excludes the application of this methodology for the formation of thioester bonds.



Scheme 5.2. Acylation based on the activation of the carboxylic acid by sulfo-NHS.

An interesting protocol for the formation of a thioester bond was reported by Cronan and Kalges.[387] In this work the authors reported a chemoselective thioesterification *E. coli holo*-ACP was carried out activating fatty acids as acyl imidazole derivates and in presence of imidazole buffer at pH 6-6.5 (Figure 5.1). Imidazole buffer allows a selective catalysis of thiol groups towards the thioesterification with acylimidazole.[388] Unfortunately, although successful chemo-

selective thioesterification were reported for short-chain fatty acids, reactions with C16-C18 fatty acids took place in poor and non-reproducible yields even in presence of detergents (Triton X-100 or Brij 35). The authors justified these results by "the variability in the dispersion of these insoluble compounds". This limitation connected to solubility of acylating agent excludes the application of this methodology for the formation of thioester bonds between proteins and long-chain acids. Moreover, to the best of our knowledge, there are no studies that support the possibility that the thioesterification may take place despite the presence of histidines in the protein.

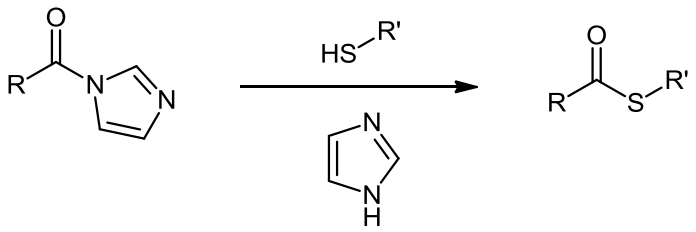


Figure 5.1. Thioesterification according to the method of Cronan and Kalges.[387]

5.2 RESULTS AND DISCUSSION

5.2.1 Rational design of a novel methodology for chemical site-selective thioesterification of proteins with long-chain fatty acids

The present chapter reports the development of a novel methodology for the thioesterification of proteins with long-chain fatty acids. This methodology was designed in order to allow the coupling between the diunsaturated meromycolic acids and the sulphur atom of the 4-phosphopantetheine moiety of *holo*-AcpM. Such coupling represents a challenging example of chemical modification of proteins and two important factors must be considered:

1. the carboxylic group of the fatty acid needs to be converted into an active form, which must have a moderate solubility and stability in aqueous buffer solution;

2. the conditions used need to favour the coupling of the fatty acid with the sulphur atom of the prosthetic group of AcpM and not with other nucleophilic centres of the protein.

In light to these considerations and previous reported studies, the strategy that was devised, was based on the conversion of fatty acids into active esters containing a permanent ionic charge, which could ensure adequate solubility and stability in aqueous buffer, while the chemoselectivity of the reaction was achieved by the concept of base catalysis. According to this strategy, fatty acids were converted into sodium acyl-5-sulfobenzimidazole (acyl–NaSBI) and the selective thioesterification was carried out in acidic solution and in the presence of sodium 5-sulfo-benzimidazole (NaSBI), a weak base with a calculated pK_a of 4.42±0.10 (value calculated using Advanced Chemistry Development (ACD/Labs) Software V11.01 for Solaris© 1994-2011 ACD/Labs). It was thought that a buffer prepared *in situ* from NaSBI and standard solution of hydrochloric acid could form a catalytic system where the pH values could be calculated according to the Henderson-Hasselbach equation. Specifically, the preparation of a solution of NaSBI in the presence of 0.15 equivalents of hydrochloric acid yields a solution with pH of *ca.* 5.2 where 85% of

NaSBI is in solution as free base, which can provide the base catalysis for a selective thioesterification of acyl–NaSBI (Figure 5.2).

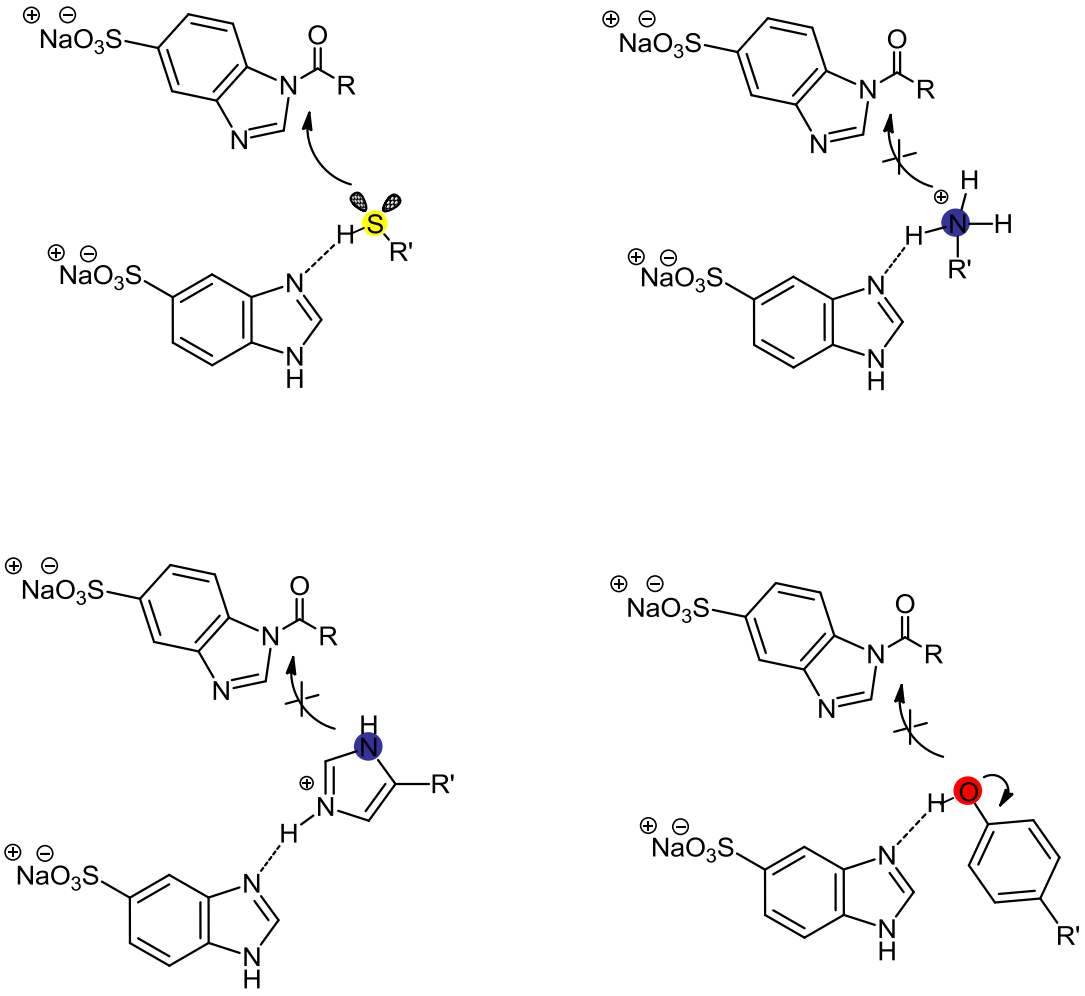


Figure 5.2. Concerted mechanism of acylation of sulfhydryl, lysyl, histidyl and tyrosyl residues by acyl-5-sulfobenzimidazole in presence of 5-sulfobenzimidazole.

Indeed, at this pH lysine residues (pKa of 10.4–10.5)[389, 390] are completely protonated, so that the nitrogen atoms of the residues have no external electrons to

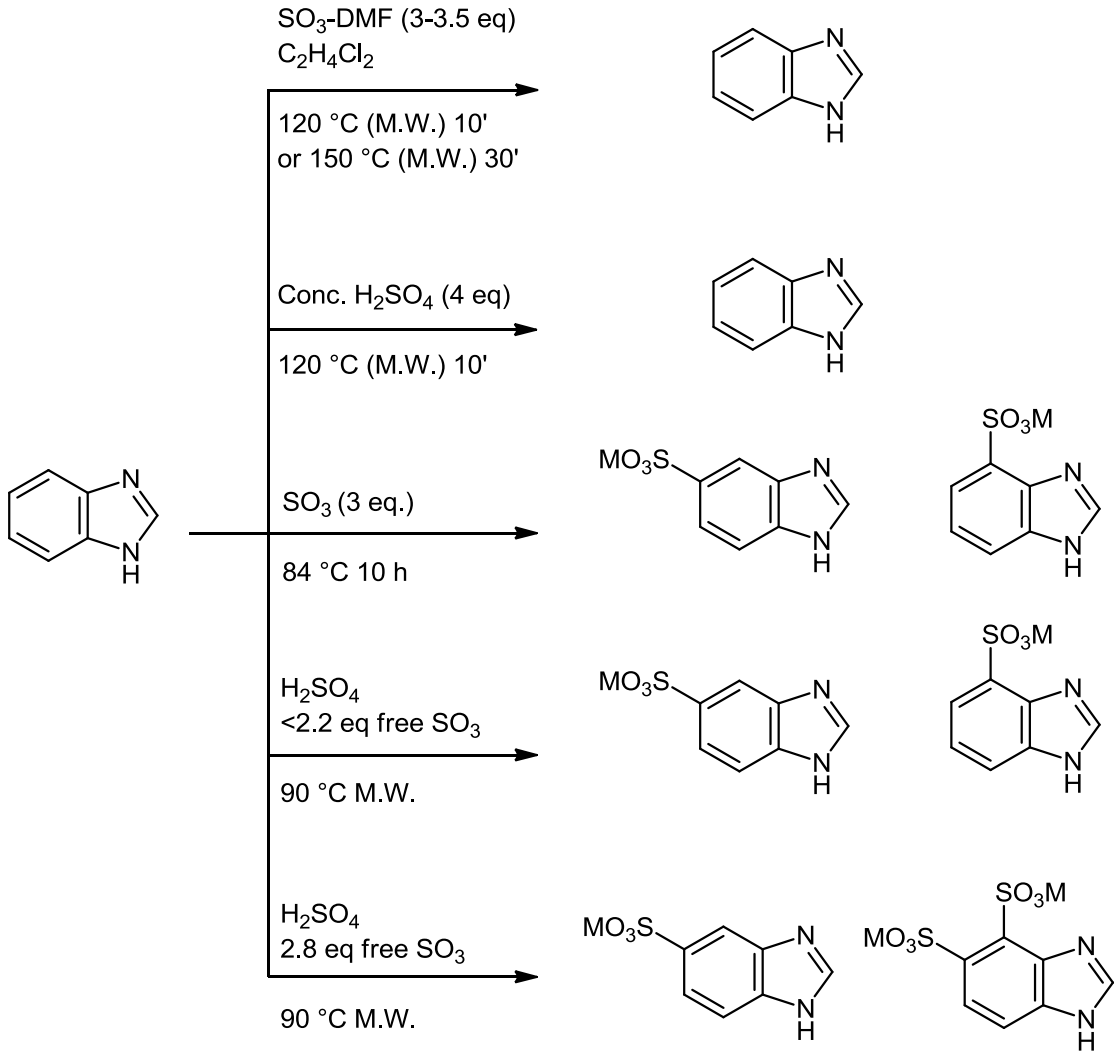
react with acylating agents, despite a possible H-bond with NaSBI. In fact, Jencks and Carriuolo showed that in the acylation of amines a base catalysis takes place even at pH of 6-7, encouraging the use of acidic buffer to avoid amide formation.

In contrast with the acylation of thiols and amines, it was reported that the acylation of tyrosines takes place without increase of rate constant for the presence of imidazole,[391] suggesting the use of base catalysis in the selective acylation of thiols.

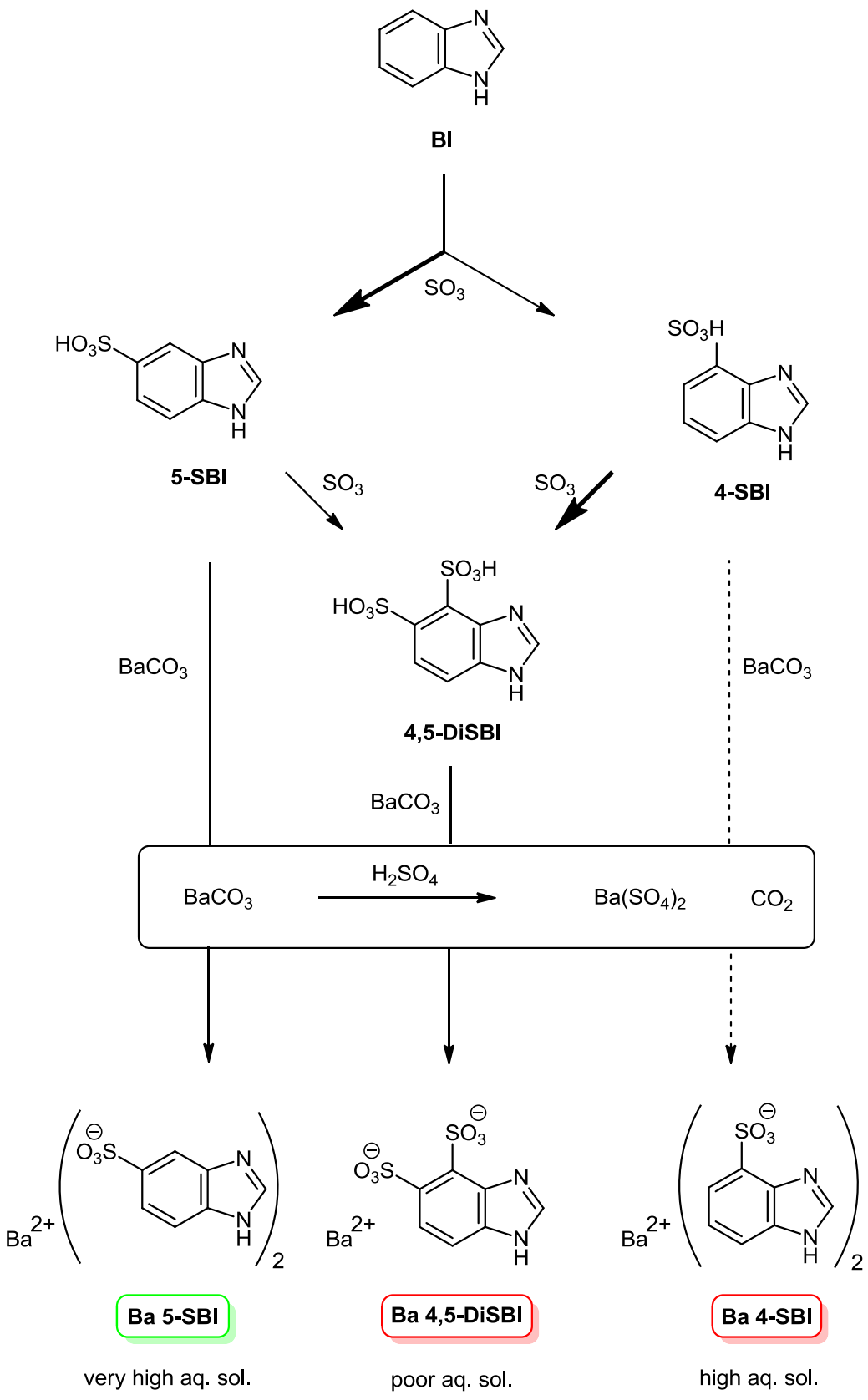
The chemoselectivity of thioesterification in presence of histidine residues is a crucial aspect. It is known that in reactions with acylating agents histidine residues are more reactive than sulfhydryl compounds at pH 7.4. Nevertheless, histidine residues have pK_a of 6.4–7.0 (as calculated for related model compounds)[390] and at pH 5.0–5.5 are predominantly in protonated form, yielding the imidazole ring less reactive.

5.2.2 Synthesis of barium 5-sulfobenzimidazole

The synthesis of NaSBI from benzimidazole was investigated using different sulfonating agents (Scheme 5.3). In general, the work-up consisted of the addition of water, to convert the sulfonating agent in sulphuric acid, and in the neutralization by barium carbonate. This useful inorganic base, in the presence of sulphuric acid, generates gas and insoluble salts, keeping in solution only the benzimidazole derivates in non protonated form as a barium salt.



Scheme 5.3. Reactions of benzimidazole (**BI**) with different sulfonating agents.



Scheme 5.4. Mechanism of sulfonation and purification of Ba 5-SBI.

Initial efforts were focused on the attempt to sulfonate benzimidazole using sulfur trioxide (SO_3) according to the conditions described by Bochkareva *et al.*[392]. The heterocyclic compound was then treated with three equivalents of SO_3 in dichloroethane at 84 °C for 10 hours. Nevertheless, the work-up with $BaCO_3$ generated two barium sulfobenzimidazoles corresponding to the sulfonation at position 5 and 4 in ratio 4:1 and in 91% yield. Surprisingly, the precipitation with a mixture of ethanol and acetone (1:1) as reported by Bochkareva *et al.*, yielded 5-sulfobenzimidazole and 4-sulfobenzimidazoles in ratio 2:1 and in 32% yield. Attempts were made to find ideal conditions to crystallise selectively the desired product. It was found that the addition of organic solvents (acetone, ethanol or tetrahydrofuran) to aqueous solution containing 4- and 5-sulfo benzimidazoles led principally to the precipitation of the unwanted isomer, whereas the desired product could be isolated after evaporation of the solvent. However, the yields of this purification were low, due to the similar solubility of the two isomers.

It is important to report that the reagent used in this SO_3-based reaction was supplied from Aldrich and contained a stabilizer in order to facilitate melting. Nevertheless, we have encountered serious problems in melting this reagent, which boils at 44.7 °C. However, the main disadvantages of using stabilized SO_3 are due to the fact that it is extremely reactive, hygroscopic and tend to react with halogenated solvents with explosive violence, forming toxic or irritating gases.[393]

The results obtained using SO_3 and its dangerous features, have led us to investigate new sulfonating reagents. In this context, a valid alternative is constituted by sulphur trioxide-dimethylformamide complex (SO_3–DMF), which is a stable user-friendly reagent. However, since dimethylformamide is a very weak base, SO_3–DMF is highly reactive, even below room temperature.[394] Nevertheless, attempts to sulfonate benzimidazole by excess of SO_3–DMF at high temperatures allowed only to recover the starting heterocyclic compound.

Since it is known that concentrated sulphuric acid is a suitable sulfonating agent under microwave irradiation,[395, 396] such conditions were investigated for benzimidazole. Nevertheless, the treatment of benzimidazole with 4 eq. of H_2SO_4 at 120 °C under M.W. irradiation for 10 minutes yielded only the starting material. In order to increase the M.W. effect on the reaction, the sulfonation was carried out in

the presence of 2.5 eq. of H_2SO_4 at fixed M.W. irradiation (100 W) for 1 minute. In these conditions rapid increases of temperature and pressure were obtained with final values of 220 °C and 20 Bar. The purification of the crude reaction mixture by $BaCO_3$ yielded a mixture of 5-sulfobenzimidazole, 4-sulfobenzimidazole and starting material.

The low reactivity of SO_3–DMF and sulphuric acid have lead us to turn our attention to use fuming sulphuric acid containing 30% free SO_3. Initial attempt to sulfonate benzimidazole using fuming sulphuric acid containing 1.1 eq. of free SO_3 resulted in the formation 5-sulfobenzimidazole and 4-sulfobenzimidazole, which were not separable by crystallization. It was noticed that the increase of sulfonating agent resulted in the formation of barium bisulfo-benzimidazole, which had a marked lower aqueous solubility in comparison to barium 5-sulfo-benzimidazole. Interestingly, it was noticed that the disulfonation occurred preferentially on the 4-sulfobenzimidazole. This observation lead us to find the ideal conditions to convert 4-sulfobenzimidazole into bisulfonate species, which could be neutralised as barium salt and promptly removed from the desired product reducing the temperature or adding organic solvents.

Parallel reactions were investigated using fuming sulphuric acid containing 30% free SO_3, quenching the reaction with barium carbonate and studying the compounds by NMR and mass analyses. Specifically, it was noticed that the disulfonation occurred only at high temperatures (160 °C) and that the use of a low number of equivalents of fuming sulphuric acid resulted in the presence of 4-sulfobenzimidazole, which could not be removed successively. Conversely, an excess of sulfonating agent (3.3 eq. of free SO_3) resulted in the isolation of bisulfonate species as major product. The ideal conditions were individuated when benzimidazole was heated in fuming sulphuric acid with 2.8 eq. of free SO_3 at 160 °C under microwave irradiation. The reaction mixture was diluted with dropwise addition of cold water and neutralised with portionwise addition of barium carbonate. Inorganic barium salts were removed by filtration and the filtrate was evaporated to yield barium 5-sulfobenzimidazole in high purity and in moderate yields.

It is important to report that the study of the conditions to sulfonate benzimidazole and to purify barium 5-sulfobenzimidazole was complicated by the

unexpected fact that ^{1}H-NMR analyses of this salt in deuterium oxide yielded different outputs in relation to the concentration of the sample (Figure 5.3). This remarkable concentration-dependent variation of ^{1}H-NMR chemical shifts of aromatic protons in some heterocyclic compounds was reported for the first time at the end of 1998 and has crucial implications for structure elucidation.[397]

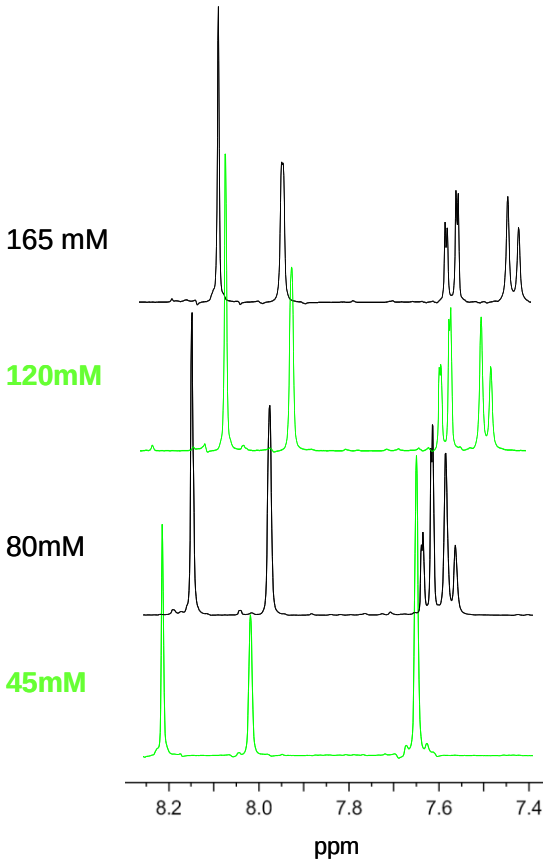
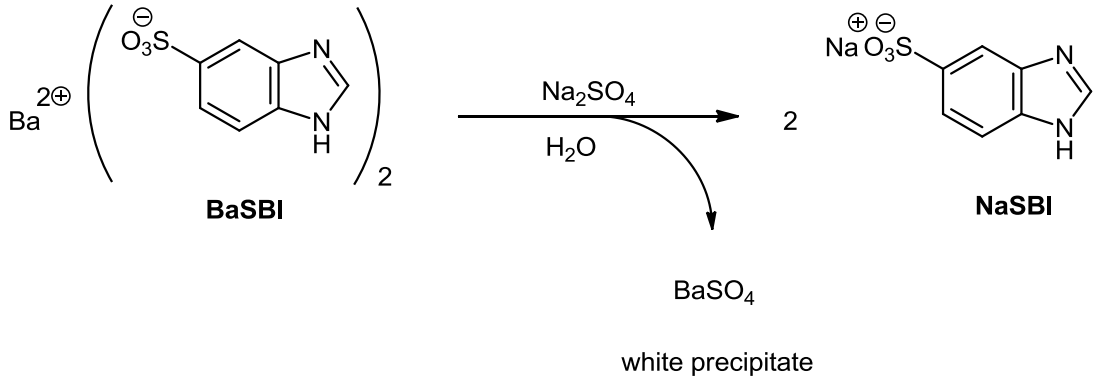


Figure 5.3. Concentration-dependent variation of ^{1}H-NMR chemical shifts of the aromatic protons in BaSBI.

5.2.3 Preparation and properties of NaSBI

Barium 5-sulfobenzimidazole obtained as reported in the previous section was solubilised in water and converted into a sodium salt by the addition of sodium sulfate (Scheme 5.5). The resulting barium sulfate is insoluble in water and was removed by filtration.



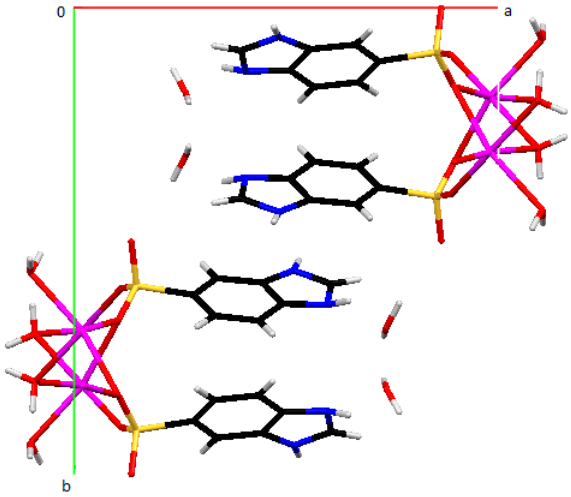
Scheme 5.5. Conversion of barium 5-sulfobenzimidazole into NaSBI by sodium sulfate.

It is important to report that NaSBI was found to be very hygroscopic, and it was generally thoroughly dried before its use in the synthesis of acyl–NaSBI. Conversely, in order to find a stable form, it was found that NaSBI could form a stable trihydrate (NaSBI·3H_2O) when it was crystallized from water. The structure of the NaSBI·3H_2O was validated by elemental analysis (Table 5.1) and X-ray crystallography (Figure 5.4).

Table 5.1. Elemental analysis of NaSBI·3H_2O.

Element	*C*	*H*	*N*
Expected (%)	30.66	4.04	10.22
Found (%)	30.48	3.94	10.07

a)



b)

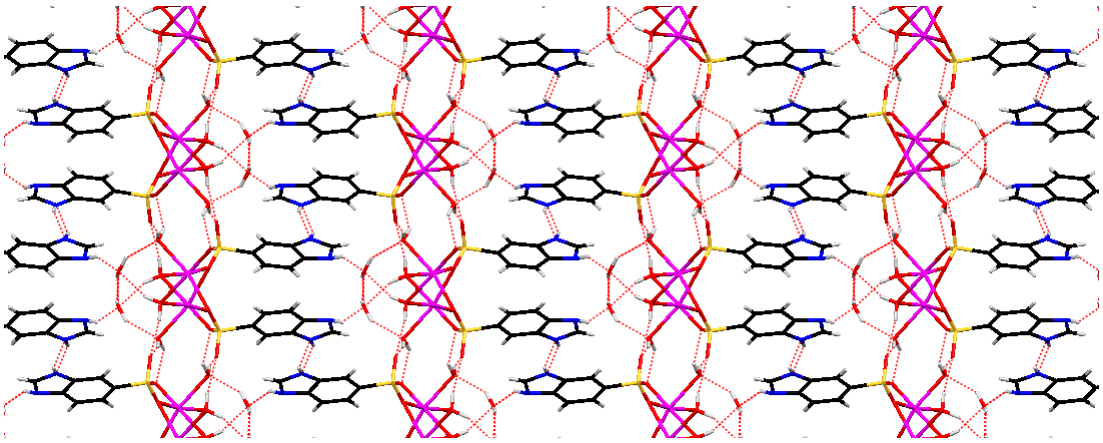
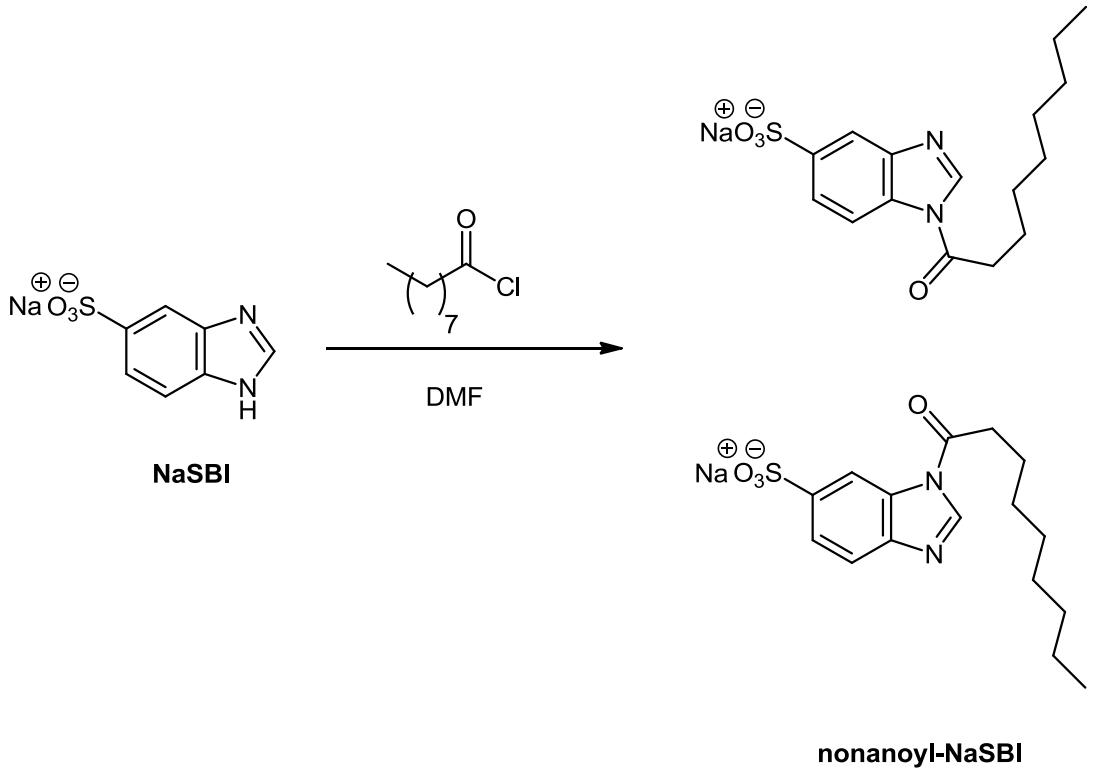


Figure 5.4. Crystal structure of NaSBI·3H_2O. The unit cell (a) and the hydrogen bonded network (b) are shown along the c-axis (hydrogen bonds are denoted with dashed red lines). The X-ray analysis was performed and processed by Dr. J-B Arlin.

NaSBI·3H2O was also used to find experimentally the pKa value of this benzimidazole derivate by the method of the half equivalent point. The value of pKa found was equal to 4.52, very close to that predicted.

5.2.4 Synthesis and purification of nonanoyl–NaSBI

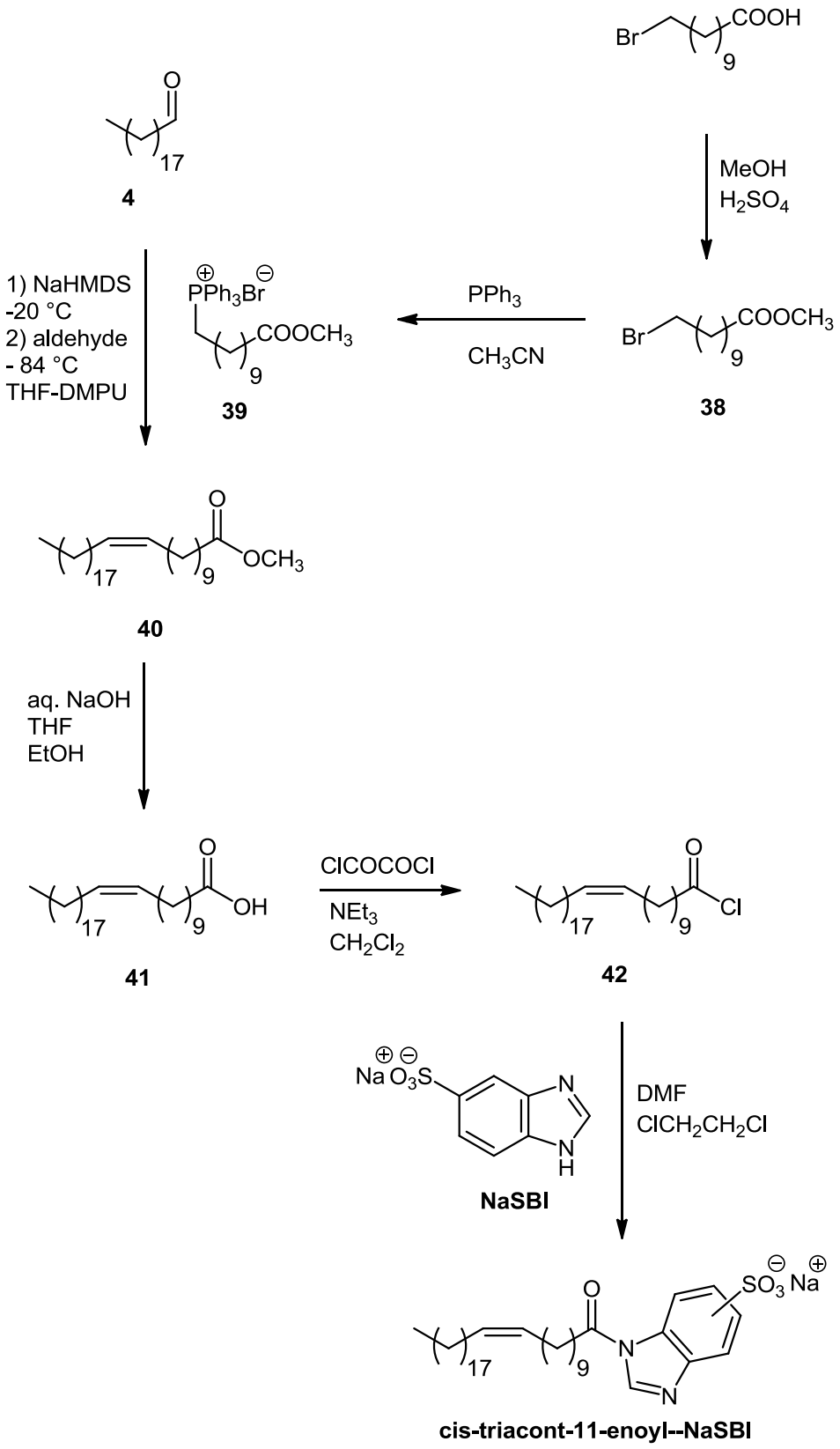
In order to study and confirm the putative chemo-selectivity of this protocol, two fatty acids were activated using the NaSBI concept. The formation of acyl–NaSBI bond was obtained treating the corresponding acyl chloride with NaSBI. Specifically, nonanoyl–NaSBI was prepared heating a mixture of nonanoyl chloride and NaSBI in anhydrous DMF. The crude reaction mixture was then purified by C18 flash chromatography showing that the presence of two isomers corresponding to 1-nonanoyl-benzimidazole sulfonated at position 5 or 6. These active esters were obtained in good to moderate yields, which were affected by the tendency of NaSBI to absorb water. In light of this drawback, NaSBI was thoroughly dried before use.



Scheme 5.6. Acylation of NaSBI by nonanoyl chloride in DMF.

5.2.5 Synthesis and purification of triacontenoyl–NaSBI

In order to study the reactivity and chemo-selectivity of the NaSBI-catalysed thioesterification of long-chain fatty acids, *cis*-triacont-11-enoic acid was synthesised and activated as acyl–NaSBI. The synthesis of this fatty acid was accomplished *via* Wittig-based strategy using transformations exposed in the Chapter 2. In particular, in light to the previously-reported base-catalysed degradation of ω-carboxyalkylphosphonium salts, 11-bromo-undecanoic acid was converted into methyl ester by the treatment with methanol in the presence of sulphuric acid. The resulting bromide **38** was converted into corresponding phosphonium bromide by the treatment with triphenylphosphine in acetonitrile under microwave irradiation. The phosphonium salt **39** was used in the Wittig reaction under *cis*-stereoselective conditions as discussed in the Chapter 2. The resulting alkene **40** was then hydrolysed using aqueous NaOH in THF/ethanol.



Scheme 5.7. Synthesis of *cis*-triacont-11-enoic acid and activation as acyl–NaSBI.

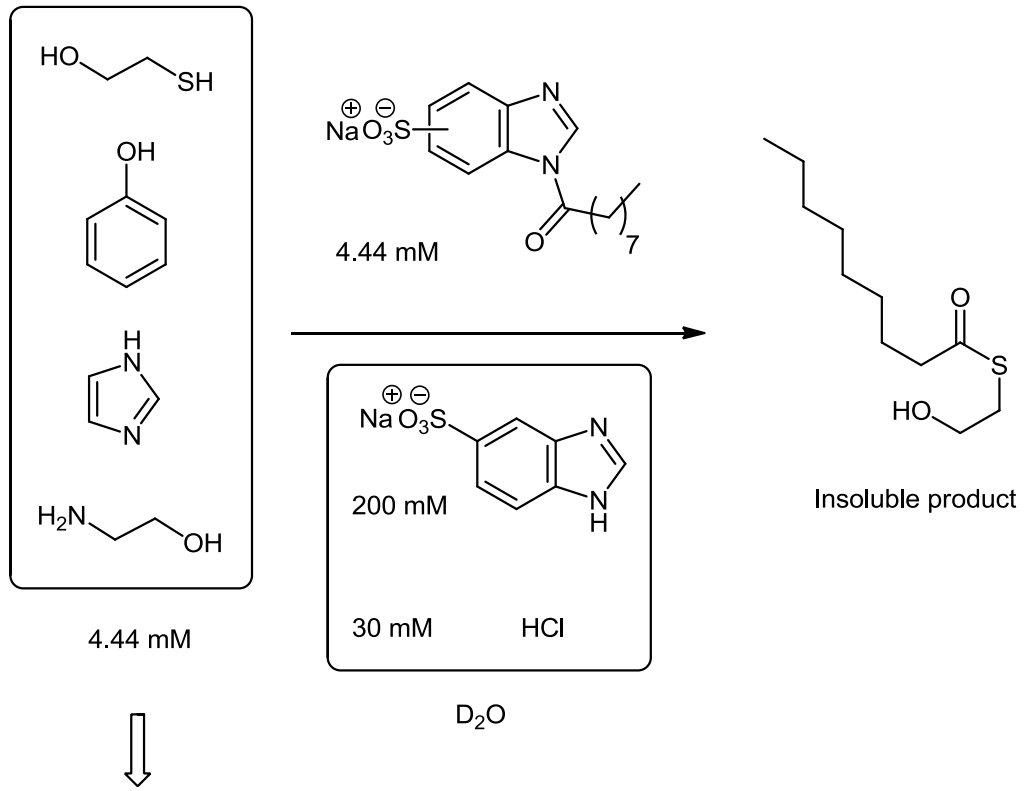
The C_{30} fatty acid **41** was then converted into acyl chloride by treatment with oxalyl chloride and triethylamine in anhydrous dichloromethane. Finally, the C_{30}-acyl–NaSBI was prepared from the acyl chloride **42** by treatment with NaSBI in anhydrous DMF and 1,2-dichloroethane. The purification of the crude of reaction was carried out by H_2O/AcOEt-petroleum ether extraction, where the product was recovered by filtration as white solid.

5.2.6 Chemo-selectivity of NaSBI-based thioesterification on model systems

The reactivity and chemo-selectivity of the NaSBI-based thioesterification of proteins was validated by NMR spectroscopy using model systems. Initially, the study was carried out using nonanoyl–NaSBI, which is a water soluble active ester. The reactivity of the two forms of nonanoyl–NaSBI was firstly investigated by NMR spectroscopy in deuterated water, where the two isomers showed similar half life time (*ca.* 24 hours).

The systems used to study reactivity and chemo-selectivity of the NaSBI-catalysed thioesterification included reactions of model compounds with acyl–NaSBI in deuterated water. Specifically, mercaptoethanol or thioglycerol were used to mimic the sulfhydryl residues of a protein, whereas phenol, imidazole and propylamine or ethanolamine were used to reproduce nucleophilic groups in tyrosine, histidine and lysine residues respectively.

Exploratory experiment was carried out using mercaptoethanol, phenol, ethanolamine and imidazole as model compounds in a solution containing NaSBI and HCl in D_2O (Scheme 5.8). Nonanoyl–NaSBI was added and the experiment was monitored by proton NMR experiments in presaturation. Successive analyses showed progressive selective decrease of the peaks relative to the active ester and mercaptoethanol, showing their involvement in the thioesterification. As expected, the resulting nonanethioate was not detected due to its very poor solubility in D_2O.



Scheme 5.8. Investigation of the reactivity and chemo-selectivity in the NaSBI-based catalytic system by NMR analysis of a model reaction carried out in D_2O.

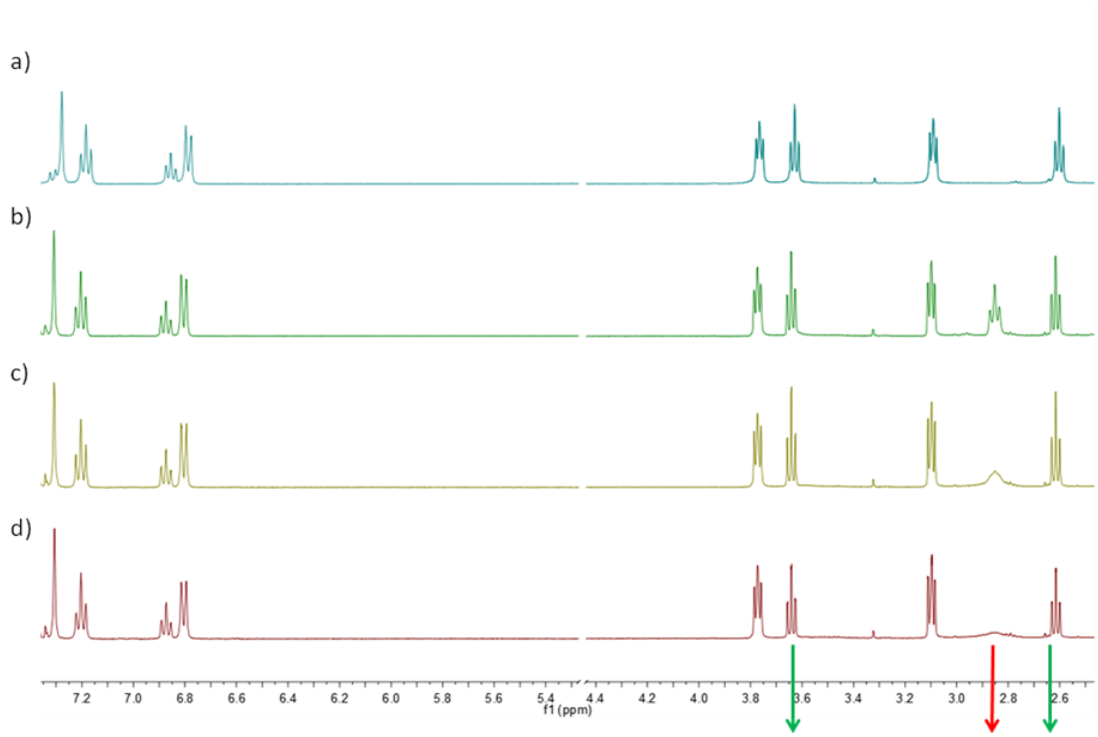
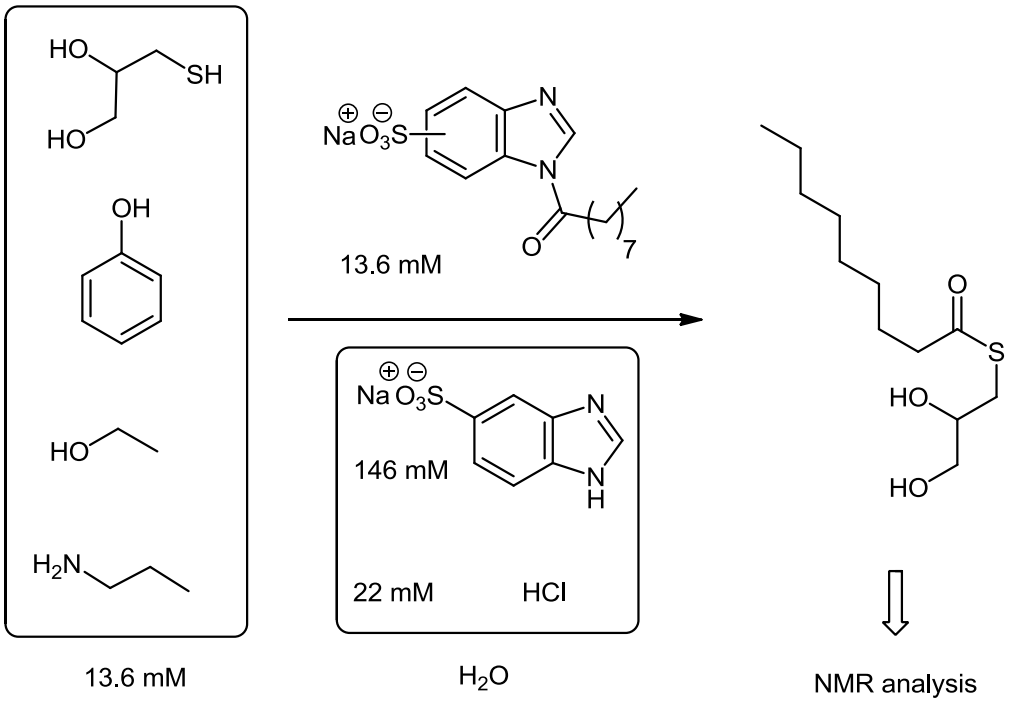


Figure 5.5. The reaction of nonanoyl–NaSBI with the model compounds (imidazole, phenol, aminoethanol, mercaptoethanol) in the presence of the NaSBI-based catalytic system was monitored by HNMR at pH *ca.* 5.3, at 25 °C in D_2O. The reaction mixture is reported before the addition of acylating agent and after 5', 50' and 2.5 hours. After the addition of acylating agent, the triplets corresponding to mercaptoethanol and nonanoyl–NaSBI decrease as shown by the green and red arrows respectively.

In order to validate the chemo-selectivity of the catalytic system, additional experiments were carried out using model compounds in water, from where the products were successively extracted in organic solvents and analysed in deuterated chloroform by NMR spectroscopy (Scheme 5.9). These experiments showed the selective formation of the thioester bond. Specifically, in a reaction of nonanoyl–NaSBI with thioglycerol, phenol, propylamine and ethanol in the presence of the NaSBI-based catalytic system, only the characteristic peaks of *S*-(2,3-dihydroxypropyl)-nonanethioate were detected by COSY experiment (Figure 5.6). No peaks related to the formation of ester or amide bonds were detected.[398, 399] The only impurity detected was the corresponding carboxylic acid due principally to impurities in the starting nonanoyl–NaSBI.



Scheme 5.9. Investigation of the chemo-selectivity in the NaSBI-based thioesterification in H_2O by NMR analysis of AcOEt extract.

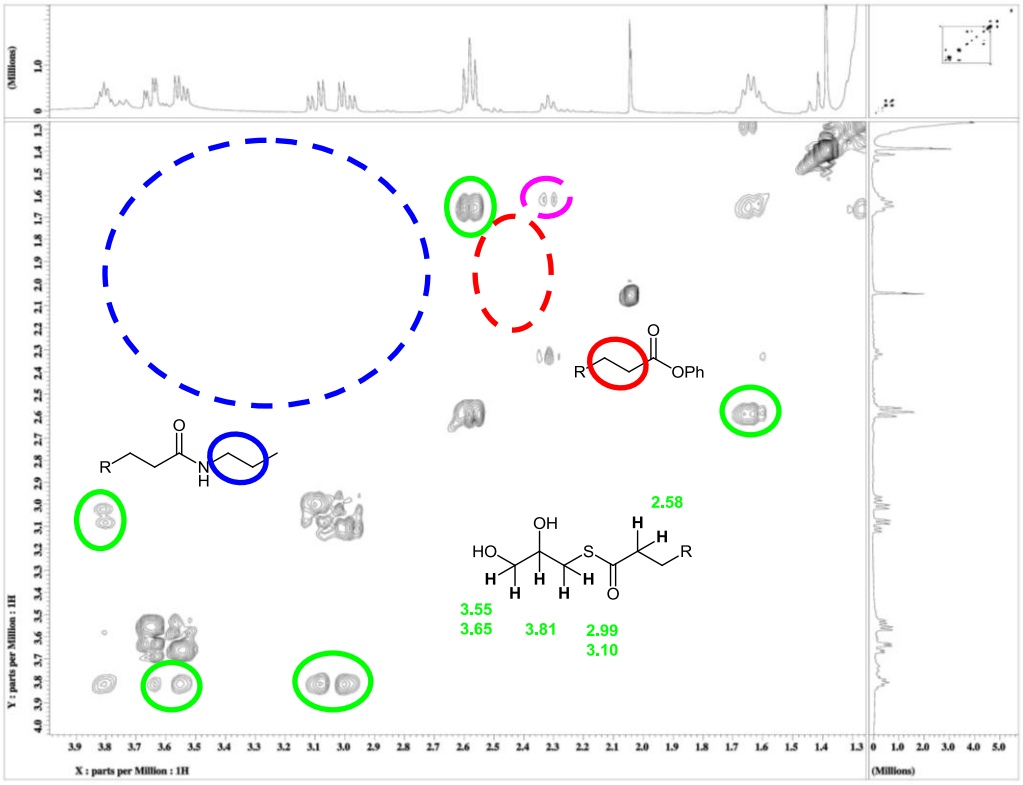


Figure 5.6. COSY experiment of the organic extract from the reaction of model compounds with nonanoyl–NaSBI in the presence of NaSBI at pH 5.27.

5.2.7 Study of the NaSBI-based thioesterification of long-chain fatty acids on model systems.

A challenging aspect of the chemical modification of proteins with fatty acids is the difficulty to work with lipids in aqueous solution. In fact, fatty acids have a very poor solubility in water and the use of tensioactives or co-solvents are required to carry out the reaction efficiently. As reported above, Cronan and Kalge reported unsuccessful attempts to carry out chemical modification of proteins with long chain fatty acids in the presence of non-ionic detergents (Triton X-100 or Brij 35).[387] Davis and co-workers reported an elegant procedure for the prenylation of proteins using 20% DMSO in aqueous solution.[400] Nevertheless, in reactions with geranylgeranyl, no conversion was obtained, due to the insolubility of the C_{20} substrate.

In order to increase the solubility of the acyl–NaSBI reagents, a variety of tensioactives and solvents were screened using model systems. In general, *cis*-triacont-11-enoic acid was used as referential long-chain fatty acid, activated as acyl–NaSBI and used in reactions with model compounds to quantify the reactivity under the conditions used. The presence of an ionic centre in this C_{30}-acyl–NaSBI makes this active ester a tensioactive with a hydrophile–lipophile balance (HLB) value >20.[401, 402] This property results in a solubilising activity of hydrophobic compounds in water and in the possibility to form o/w micelles using organic solvents. Nevertheless, attempts to use hexane or diethyl ether in low concentrations to form emulsions and solubilise the active ester were unsuccessful, resulting in low yields in reactions with model compounds.

Non-ionic tensioactives represent a good approach to solubilise activated long-fatty acids without compromising the stability of proteins in solution. The correct choice of the tensioactive depends on the HLB value, which should be around 13, and on the critical micelle concentration (CMC), which should be low to allow an efficient activity at low concentrations and a rapid dialysis.

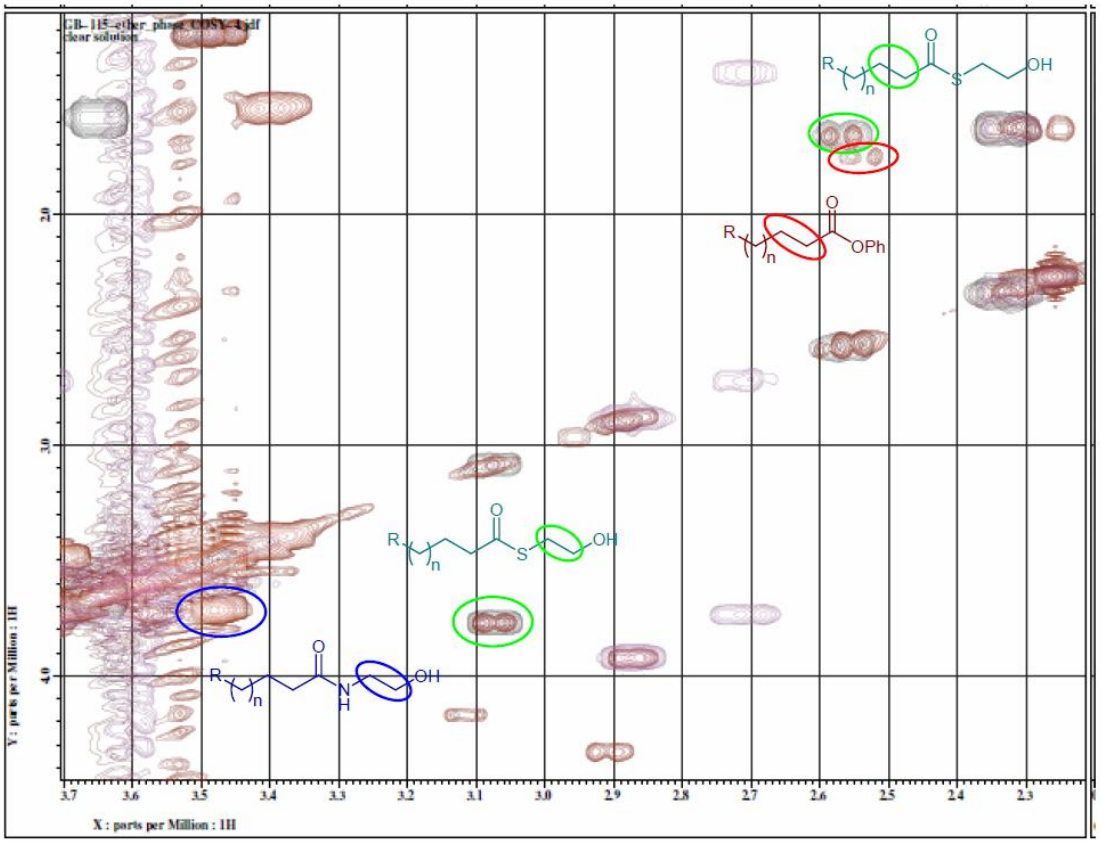


Figure 5.7. Exploratory study of the reactivity of the C_{30}-acyl–NaSBI with model compounds and in the presence of additives by the comparison of COSY experiments of organic extracted. Spectrum in black is due to the NaSBI-based acylation in the presence of 1-pentanol, spectrum in violet to Brij-56, whereas spectrum in red is given by the use of Triton X-100. The reaction with Brij-56 is performed in alkaline solution showing that at higher pH the chemoselectivity is poor. The use of 1-pentanol was found useful in the reactions with long-chain active ester.

Brij-56 (Polyoxyethylene (10) cetyl ether) is an ideal detergent, having values of HLB and CMC equal to 12.9 and 35 μM respectively;[403] In addition, polyoxyethylene-detergents are generally 'mild' towards proteins and enzymes.[404, 405] Indeed, the use of Brij-56 at concentration of 730μM has permitted an almost completely solubilisation of the active C_{30} fatty acid (at mM concentrations).

The ability of these tensioactives to solubilise the acylating agent led us to study also the reactivity of this reagent in the presence of these detergents (Figure

5.7). Nevertheless a preliminary attempt of thioesterification of this acylating agent under these conditions was unsuccessful, recovering not reacted active fatty acid. Previously Cronan and Kalge reported a decrease of rate of thioesterification of short fatty acids using Brij-35.[387] However this behaviour was not rationalized and to the best of our knowledge there is no study of reactivity of active fatty acid in correlation of the detergent. Possible explanation of the low reactivity of reagents in Brij-56 could be due to the position of reacting groups (activated carbonyls) within the micelles. Although the shape of Brij detergents in water is ellipsoidal, the co-presence of ionic detergents constituted from acyl–NaSBI, forms a mixed surfactant system implying a more spherical shape, where polyoxyethylene chains shield the ionic repulsion between anionic charges.[406, 407] It is plausible that acyl–NaSBI reagents may be less reacting in the presence of non-ionic detergents, due to the fact that the reacting carbonyl groups are trapped from polyoxyethylene chains of non ionic tensioactives (Figure 5.8).

These findings led us to focus our attention on the search of an ideal co-solvent. During this study a variety of solvents were screened, using the sodium octadecanoate as model for the NaSBI-based active esters (Figure 5.9). Among these solvents, propan-1-ol was found very efficient in the process of solubilisation of C_{30}-acyl–NaSBI in presence of the co-solvent at concentration of 1-2% v/v. In contrast with non ionic detergents, 1-pentanol allowed a high reactivity in reaction with model compounds, due probably to the stabilization of the micelles (Figure 5.10).

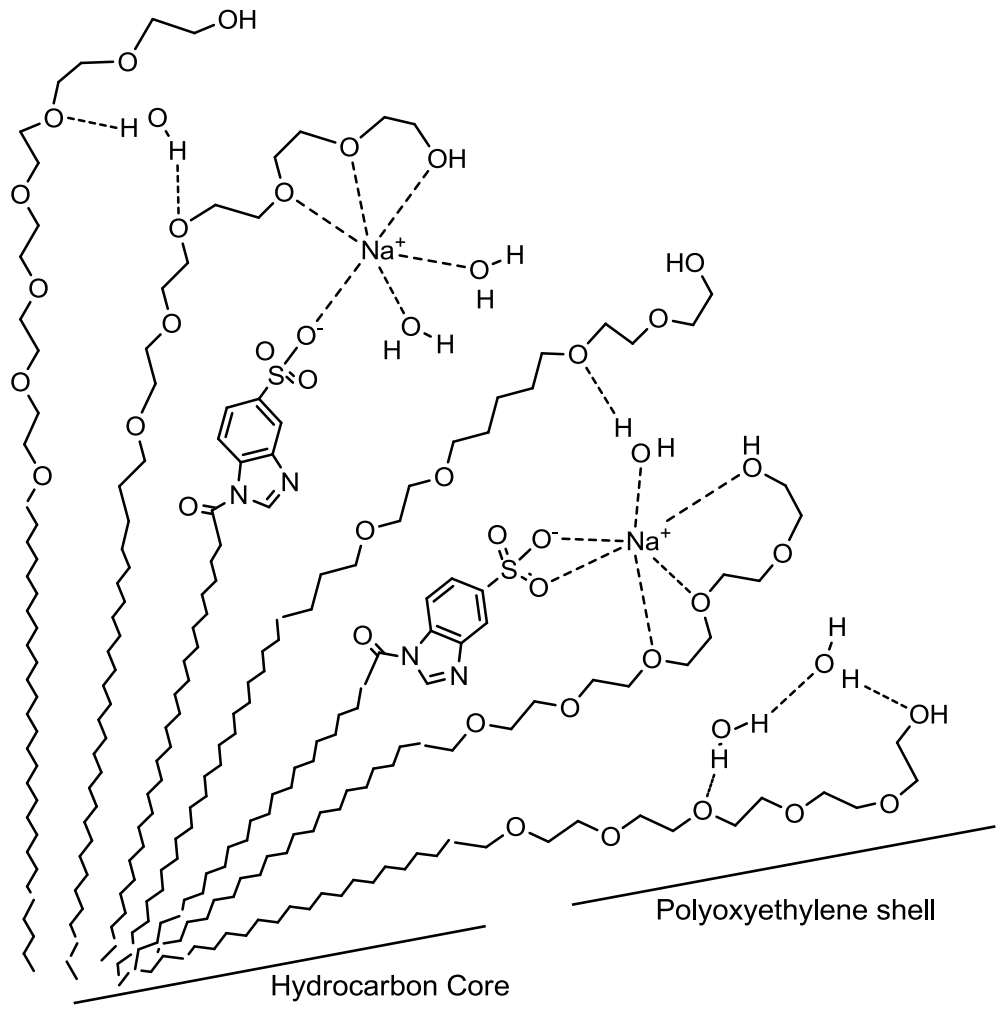


Figure 5.8. Schematic representation of a possible disposition of a non-ionic detergent within a mixed surfactant system with acyl–NaSBI. This Figure was prepared according to the interactions previously theorised within non-ionic/ionic mixed surfactant systems.[408]

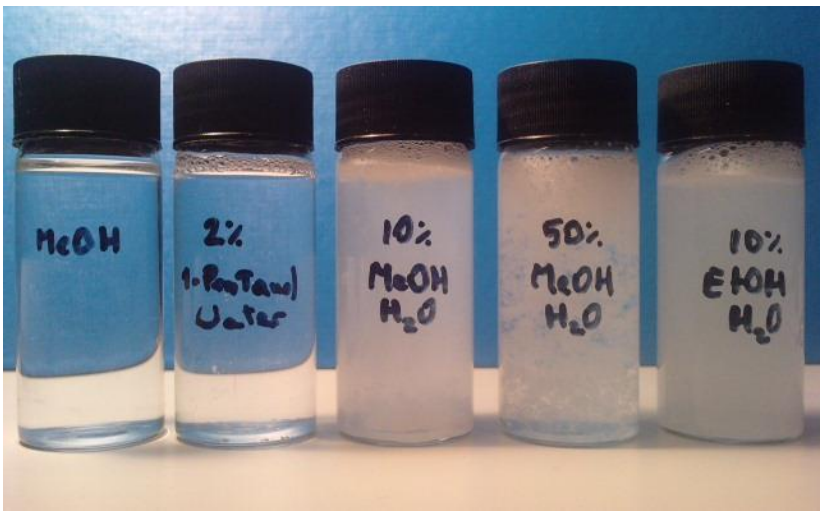


Figure 5.9. Examples of tests for the solubility of sodium octadecanoate in different solvent systems at 11 mM concentration.

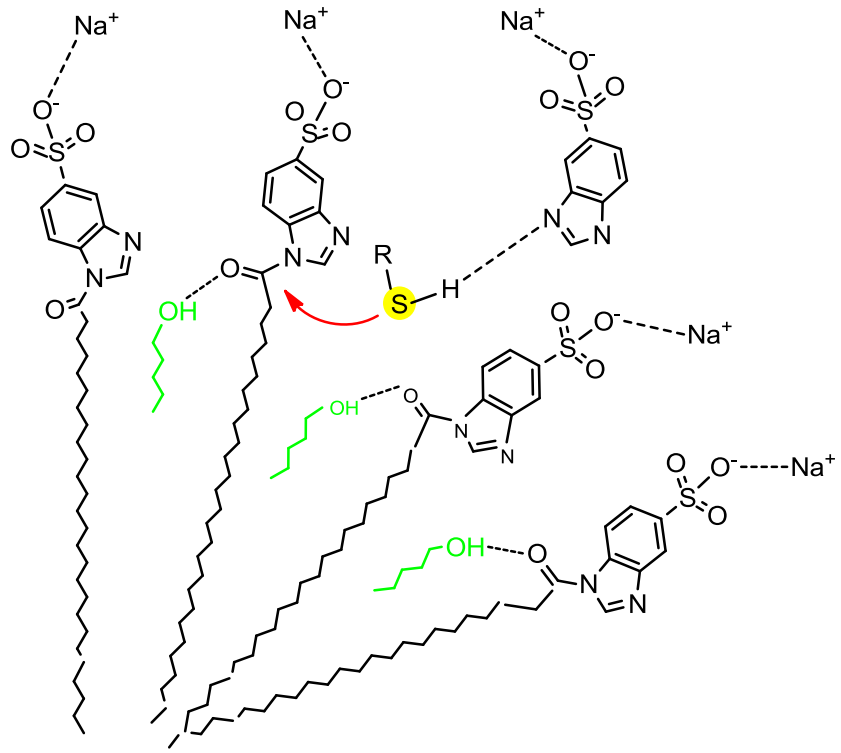


Figure 5.10. Putative role of 1-pentanol in the solubilisation of acyl–NaSBI.

5.2.8 Chemo-selective thioesterification of holo-AcpM

These promising results prompted validation of this catalytic system on a protein substrate. In order to validate the chemo-selectivity of thioesterification on protein substrates by the NaSBI-based system, a sample containing *apo*-AcpM and *holo*-AcpM was used under the condition used in model reactions. As discussed in the Chapter 4, *holo*-AcpM contains the phosphopantetheine moiety, but also histidine, tyrosine and lysine residues, whereas *apo*-AcpM is devoid of sulfhydryl groups. Thus, the reaction in presence of both *holo*-AcpM and *apo*-AcpM is an ideal method to validate the chemoselectivity of the methodology proposed. Specifically, an experiment with nonanoyl–NaSBI and in the presence of 200 mM of NaSBI and 30 mM of hydrochloric acid was carried out using as substrate a mixture of *holo*-AcpM and *apo*-AcpM in 3:2 ratio. Four parallel reactions were carried out, where the concentrations of the acylating agent used were equal to 13, 39, 118 and 353 eq. in comparison to *holo*-AcpM.

After 9 hours, the reactions were dialysed and checked by ESI. In these reactions, increasing concentrations of acylating agent led to the acylation of *holo*-AcpM and *apo*-AcpM, and to the formation of mono-, di- and tri-acylated proteins. However, it is important to note that *holo*-AcpM and *apo*-AcpM show different reactivity and that with low concentrations of acylating agents (Figure 5.12.b), it is possible to convert selectively *holo*-AcpM (MW: 14894.9 Da) to nonanoyl–*holo*-AcpM (MW: 15036 Da as expected). Since that the difference between *holo*- and *apo*-AcpM is the presence of the 4'-phosphopantetheine prosthetic group, it is plausible that in the first acylation the C9 chain is linked to the thiol group to form a thioester bond. A concentration 3 times higher of acylating agent led to the partial formation of nonanoyl–*apo*-AcpM (Figure 5.12.c), whereas a higher concentration (4.63 mM) led to the formation of di- and tri-acylated derivates (Figure 5.12.d). The reaction mixture of the acylation in the presence of an even higher concentration (13.9 mM) of nonanoyl–NaSBI gave a poor ESI output, probably due to the precipitation of the pluri-acylated proteins.

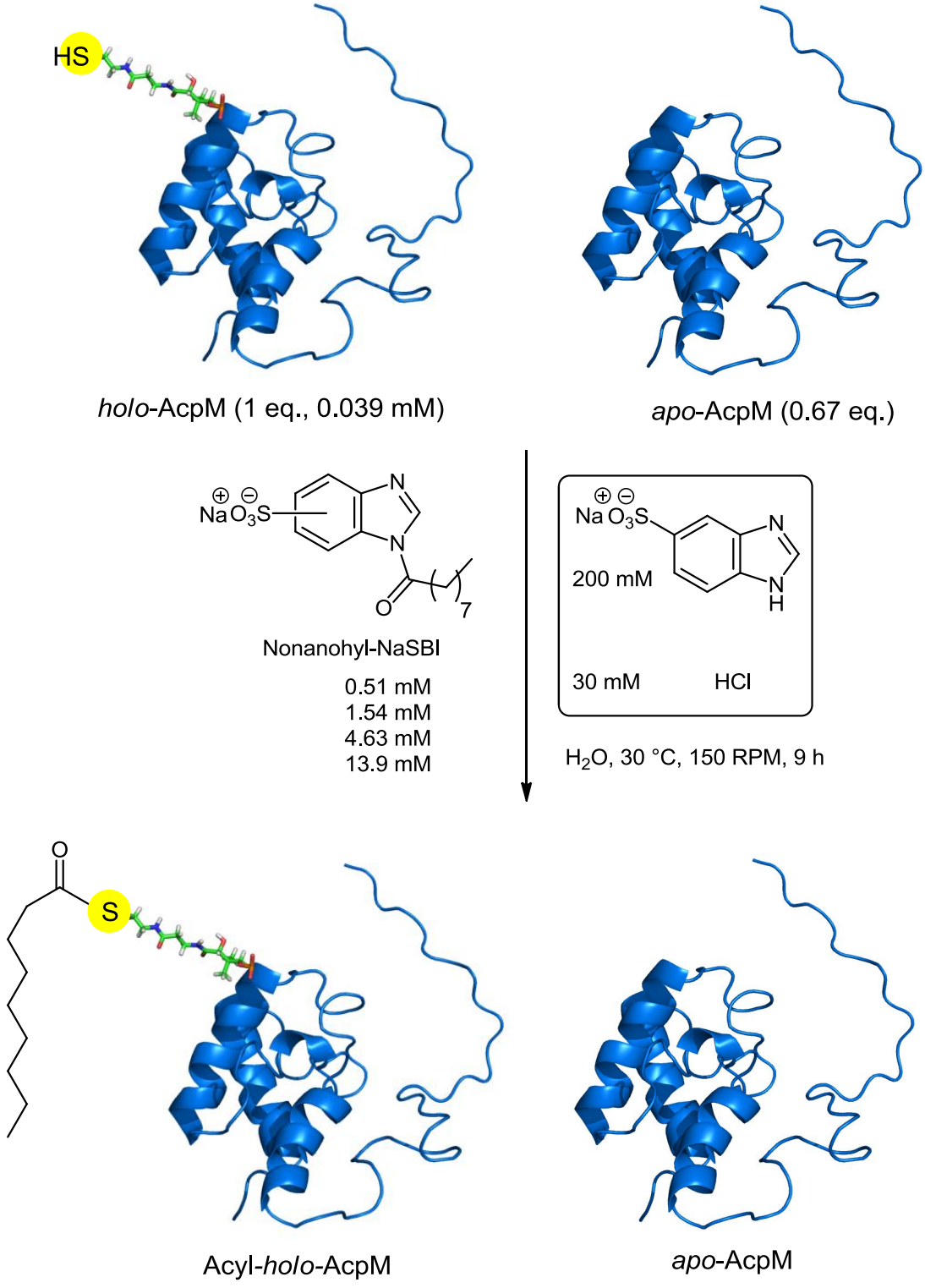


Figure 5.11. Chemo-selective thioesterification of *holo*-AcpM.

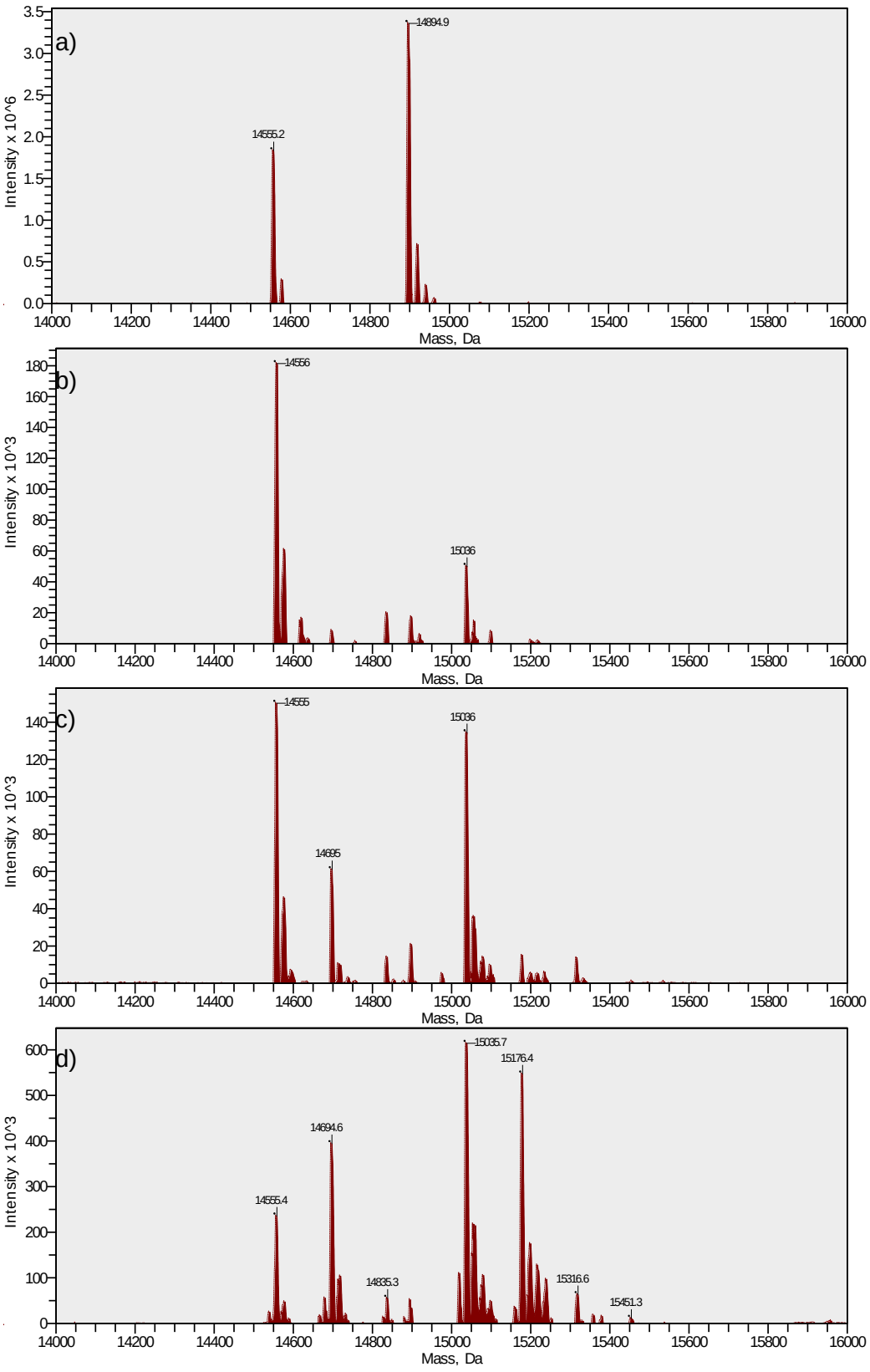


Figure 5.12. Deconvoluted ESI spectra of the reactions of thioesterification with *holo*-AcpM (14895–14896 Da, 1 eq.) *and apo*-AcpM (14555–14556 Da, 0.67 eq.). Starting proteins (a) were acylated with 13 eq. (b), 39 eq. (c) or 118 eq. (d) of nonanoyl–NaSBI.

5.3 CONCLUSIONS AND FUTURE WORKS

In this chapter, it was reported the development of a novel methodology for the thioesterification of proteins with long chain fatty acids. This method is based on the activation of fatty acids by the conversion into sodium acyl-5-sulfobenzimidazole (acyl–NaSBI), and on a catalytic system where sodium 5-sulfo-benzimidazole (NaSBI) is responsible for the chemo-selective activation of sulfhydryl compounds *via* base catalysis at acidic pH values.

NaSBI was prepared by sulfonation of benzimidazole with fuming sulphuric acid under microwave irradiations. The work-up was based on barium carbonate and the barium sulfobenzimidazole was converted to the related sodium salt. The proposed synthetic protocol improves the purity of the product in comparison with the method reported in literature. The desired product was characterised by NMR, reporting remarkable concentration-dependent ^{1}H NMR spectra. The X-ray analysis and the calculation of pKa were performed. The conversion of fatty acids into acyl–NaSBI was obtained smoothly from related acyl chloride derivates.

The chemoselectivity of thioesterification was validated by reactions with model compounds using NMR spectroscopy, and by reactions with AcpM. However, further studies are necessary to investigate the ideal conditions in terms of temperature, concentration, pH and time.

For the thioesterification of long-chain fatty acids 1-pentanol was found ideal to solubilise the active ester. Further studies will be important to demonstrate the possibility to couple the meromycolic acid to AcpM. It is possible also to anticipate that this methodology will find applications in coupling of insoluble probes to proteins or in synthesis of biocatalysts.

CHAPTER 6

6. MATERIALS AND METHODS

6.1 SYNTHETIC EXPERIMENTAL SESSION

6.1.1 General methods

Unless otherwise noted, air- and moisture-sensitive reactions were carried out in oven-dried (>110 ºC) glassware capped with rubber septa under a positive pressure of dry nitrogen from a manifold or balloon. Likewise, air- and moisture-sensitive reagents, solvents, and solutions were transferred via syringe or stainless steel cannula under a dry inert atmosphere (oxygen-free nitrogen). Stirring was achieved using oven-dried (>110 ºC) Teflon™-coated magnetic stir bars cooled under a stream of dry nitrogen. Reaction temperatures refer to the external temperature or to the temperature of the bath in which the reaction vessel was partially immersed. Room temperature indicates an ambient indoor temperature in the range of 18-24 ºC. Elevated temperatures were maintained using a silicone oil bath equilibrated with constant current flow through a wire heating element or actively controlled with a thermostat device. Low temperatures refer to the bath temperatures where the flash was immersed. Temperatures of 0 ºC and –84 ºC were achieved with an ice/water slurry or a N_2(liq.)/EtOAc mixture, respectively. Concentration of organic solutions *in vacuo* was achieved using a rotary evaporator equipped with a membrane vacuum pump. Residual solvents were removed from samples under high vacuum line under a constant reduced pressure of <5 mbar. Yields refer to chromatographically and spectroscopically (1H NMR) homogeneous materials, unless otherwise stated.

6.1.1.1 Reagents and solvents

All commercial reagents and solvents were purchased from Sigma-Aldrich/Fluka, Alfa Aesar, or Fisher Scientific/Acros Organics and used without further purification.

Ethylmagnesium bromide in THF was purchased from Acros Organics (Catalogue No: 210471000, Lot No: A0273541).

Anhydrous 1-methyl-2-pyrrolidinone (NMP) and 1,3-dimethyl-2-imidazolidinone (DMI) were purchased from Sigma-Aldrich and degassed under nitrogen. Anhydrous 1,3-dimethyl-3,4,5,6-tetrahydro-2(1*H*)-pyrimidinone (DMPU) was purchased from Sigma-Aldrich (over molecular sieve), Anhydrous tetrahydrofuran (THF) was distilled from sodium/benzophenone under nitrogen prior to use, or occasionally purchased from Sigma-Aldrich (inhibitor-free, Catalogue number: 401757, Lot No: 01749BJ, 32896CK, 57696CK, 33296EJ). Anhydrous diethyl ether was distilled from sodium/benzophenone under nitrogen prior to use, or occasionally purchased from Sigma-Aldrich (inhibitor-free). Anhydrous dichloromethane was distilled from calcium hydride under nitrogen and stored over molecular sieve. Anhydrous acetonitrile (CH_3CN), methanol (CH_3OH), dichloroethane, toluene were purchased from Sigma-Aldrich.

Concentrations and purity of purchased reagents and solvents refer to analysis certificate of the specific lot number.

6.1.1.2 NMR

Proton nuclear magnetic resonance (1H NMR) spectra were recorded on JEOL 400 spectrometer. Proton chemical shifts are expressed in parts per million (δ scale) and are calibrated using residual undeuterated solvent as an internal reference (CHCl3: δ 7.26). Data for 1H NMR spectra are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration). Multiplicities are reported as follows: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet and/or multiple resonances, br = broad, app = apparent, or combinations thereof. Carbon nuclear magnetic resonance (13C NMR) spectra were recorded at 101 MHz. Carbon chemical shifts are expressed in parts per million (δ scale) and are referenced from the carbon resonances of the solvent (CDCl3: δ 77.16).

6.1.1.3 Mass Spectrometry

Electrospray ionization mass spectrometry was conducted using a Finnigan LCQ PECA system and the mass spectrometer operated in the positive- and negative-ion detection mode.

6.1.1.4 Chromatography

Flash column chromatography was performed in glass columns according to the method of Still and co-workers,[409] or by SP4 Biotage MPLC apparatus using silica gel 0.040-0.063 mm as a particle size (Merck or Fisher).

Analytical TLC was performed using silica gel 60 F_{254} aluminium sheets purchased from Merck. TLC plates were generally run for *ca.* 7 cm and visualized by exposure to ultraviolet light (UV) and/or exposure to an ethanolic solution of phosphomolybdic acid (PMA) or an aqueous solution of potassium permanganate ($KMnO_4$).

6.1.1.5 M.W. apparatus

The M.W. reactions were performed in a Biotage Initiator apparatus, time and temperature are reported in every reaction.

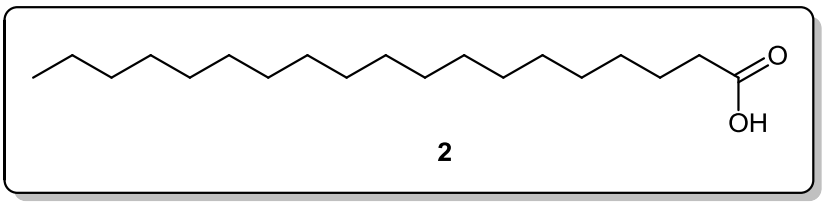
6.1.1.6 Melting point apparatus

The melting points were recorded in a SMP1 Stuart Scientific Melting Point apparatus and they were not corrected.

6.1.2 Experimental session of Chapter 2

6.1.2.1 Synthetic methods

Synthesis of nonadecanoic acid



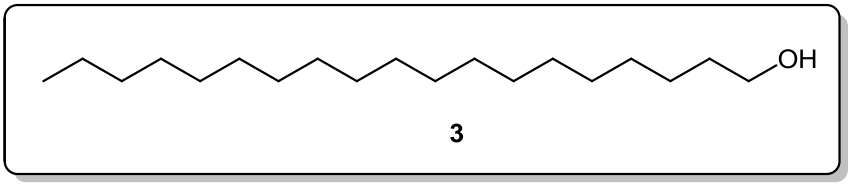
2

A 2 L three-neck round-bottom flask fitted with a mechanical stirrer was charged with cold (ice bath) water (500 mL). Under stirring concentrated sulphuric acid (29.5 mL) was added dropwise, followed by dichloromethane (500 mL), hexadecyltrimethylammonium bromide (3 g, 8.23 mmol, 0.1 eq.), 1-eicosene (25 g, 89.1 mmol, 1 eq. purchased from Fluka with purity: 91.4%), AcOH (10 mL). To the resulting vigorously stirred mixture was added $KMnO_4$ (40 g, 253 mmol, 2.84 eq.) over 3.5 hours and the reaction was stirred at room temperature for 18 hours. The reaction mixture was then cooled to 0 °C before being quenched with sodium sulfite (40 g), the organic layer was separated and the aqueous layer was extracted with dichloromethane (2 × 200 mL). The combined organic layers were concentrated *in vacuo* to 200 mL and cooled to 0 °C to give a white precipitate that was filtrated. The filtrate was concentrated to smaller volume and cooled (0 °C) to give a white solid that was filtered and combined to the previous precipitate. The crystals were washed with cold Dichloromethane and evaporated *in vacuo* to afford the title compound **2** (21.0 g, 79%) as white solid.

m.p.: 67–68 °C

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 2.35 (t, *J* = 7.5 Hz, 2H), 1.63 (app. p, *J* = 7.4 Hz, 2H), 1.36 – 1.17 (m, 30H), 0.88 (t, *J* = 6.8 Hz, 3H).

Synthesis of nonadecan-1-ol



3

To a stirred cooled (ice-bath) solution of acid **2** (5.40 g, 18.1 mmol, 1 eq.) in anhydrous THF (150 mL) was added lithium aluminium hydride (2.06 g, 54.3 mmol, 3 eq.) in small portions under nitrogen. The mixture was stirred at room temperature for 1 hour and at 45 °C for 30 minutes. The reaction mixture was then cooled to 0 °C and quenched with dropwise addition of water and 10% aqueous HCl solution. The mixture was diluted with diethyl ether and washed with water and brine. The organic phase was dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a crude mixture that was crystallized from refluxing methanol to afford the title compound **3** (4.63 g, 90%) as white solid.

[Note: Frequently, especially in large scale, lithium aluminium salts form aluminium emulsion that are difficult to break. In presence of these emulsions the Fieser work-up is suggested (see sytnthesis of 1,14-tetradecenediol)].

m.p.: 62–63 °C

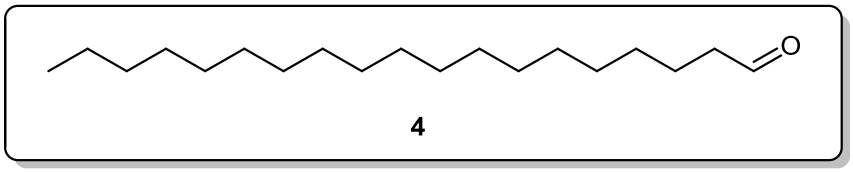
^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.64 (td, *J* = 6.6, 5.1 Hz, 2H), 1.65 – 1.49 (m, 2H), 1.37 – 1.21 (m, 30H), 1.18 (t, *J* = 5.4 Hz, 1H), 0.88 (t, *J* = 6.8 Hz, 3H).

Or **^{1}H NMR** (400 MHz, CHLOROFORM-D) δ 3.64 (t, *J* = 6.6 Hz, 2H), 1.56 (app. p, *J* = 6.7 Hz, 2H), 1.38 – 1.17 (m, 30H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 63.22, 32.94, 32.08, 29.85, 29.81, 29.76, 29.59, 29.52, 25.89, 22.84, 14.26.

FTIR (neat): 3275, 2915, 2847, 1473, 1462, 1061, 729, 719 cm^{-1}

Synthesis of nonadecanal



4

To a stirred suspension of PCC (4.02 g, 18.66 mmol, 2eq.) in anhydrous dichloromethane (40 mL) was added a solution of alcohol **3** (2.65 g, 9.33 mmol, 1 eq.) in anhydrous dichloromethane (50 mL) under nitrogen. The mixture was stirred at room temperature for 3 h, then it was passed through a short plug of silica gel and eluting with Dichloromethane to an off-white solid that was purified by flash chromatography (4% diethyl ether in petroleum ether b.p. 40–60 °C) afford the title compound **4** as white solid (2.39 g, 91%). The aldehyde **4** was used immediately or stored at −20 °C under nitrogen.

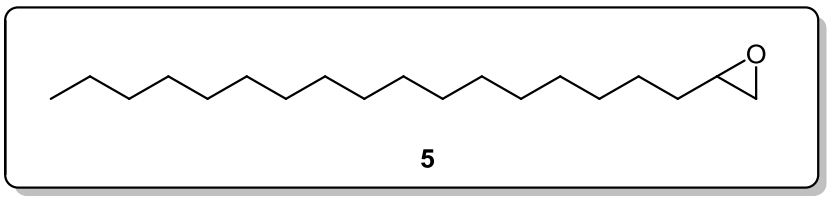
m.p.: 62–64 °C.

Analytical TLC: *Rf* = 0.55 (12% Et_2O in petroleum ether b.p. 60–80 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 9.76 (t, *J* = 1.9 Hz, 1H), 2.42 (td, *J* = 7.4, 1.9 Hz, 2H), 1.62 (p, *J* = 7.2 Hz, 2H), 1.34 – 1.21 (m, 30H), 0.88 (t, *J* = 6.9 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 203.14, 44.08, 32.08, 29.84, 29.58, 29.52, 29.33, 22.85, 22.25, 14.28.

Synthesis of 2-heptadecyloxirane



5

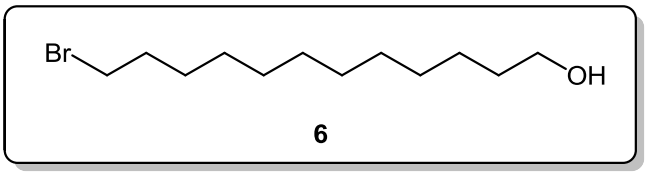
To a solution of 1-eicosene (9.0 g, 32.7 mmol, 1 eq., purchased from Fluka with purity: 91.4%) in 1,2-dichloroethane (135 mL) was added *m*-chloroperbenzoic acid (7.74 g, 44.94 mmol, 1.4 eq.) and stirred at room temperature for 6 hours. The reaction mixture was then washed with saturated aqueous $NaHCO_3$ solution (200 mL), water (2 × 200 mL) and brine (200 mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **5** as white solid (9.48 g, 105%). The resulting epoxide was chromatographically homogeneous by TLC analysis and was used directly without further purification.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 2.95 – 2.86 (m, 1H), 2.75 (t, *J* = 4.4 Hz, 1H), 2.46 (dd, *J* = 4.9, 2.7 Hz, 1H), 1.56 – 1.20 (m, 32H), 0.88 (t, *J* = 6.7 Hz, 3H).

Alternative synthesis of nonadecanal from epoxide 5

To a solution of epoxide **5** (one third of the crude epoxide obtained above, 3.16 g) in diethyl ether (60 mL) was added periodic acid (3.81 g) and the mixture was stirred at room temperature for 1.5 hours. The reaction mixture was then washed with saturated aqueous $NaHCO_3$ solution (2 × 50 mL), water (2 × 50 mL) and brine (50 mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **4** (2.76 g, 91% after 2 steps) as a white solid.

Synthesis of 12-bromododecan-1-ol



6

To a suspension of 1,12-dodecanediol (19.0 g, 93.9 mmol) in toluene (150 mL) was added 48% aqueous hydrobromic acid (18 mL) and the mixture was gently refluxed using a Dean-Stark apparatus with the condenser connected with a trap of

aqueous $NaHCO_3$ solution to neutralise HBr vapours. After 30 hours the neutral reaction mixture was evaporated *in vacuo* to give a low melting solid that was purified by flash chromatography (petroleum ether to remove the expected di-bromo derivate, then 50% diethyl ether in petroleum ether b.p. 40–60 °C) to afford the title compound **6** (17.5 g, 70%) as a white solid.

m.p.: 29–30 °C

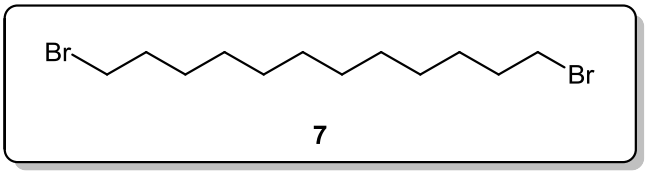
Analytical TLC: *Rf* = 0.39 (50% Et_2O in petroleum ether b.p. 60–80 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.63 (t, *J* = 6.6 Hz), 3.40 (t, *J* = 6.9 Hz), 1.84 (app. p, *J* = 7.2 Hz), 1.56 (app. p, *J* = 7.2 Hz), 1.45 – 1.21 (m).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 63.20, 34.19, 32.96, 32.93, 29.70, 29.65, 29.64, 29.55, 28.89, 28.30, 25.86.

FTIR (neat): 3303, 2917, 2848, 1462, 1051, 1027, 726 cm^{-1}

Synthesis of 1,12-dibromododecane



7

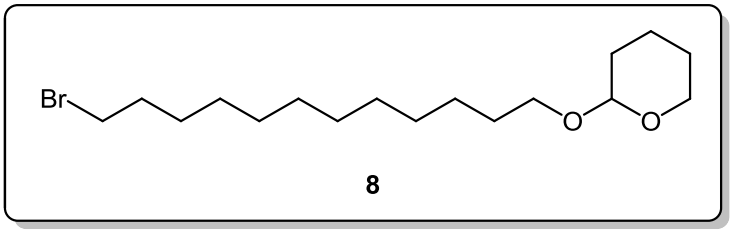
During the synthesis of bromide **6** 1-12,dibromododecane was isolated as a minor product. This by-product was purified by flash chromatography and yielded as a white solid.

m.p.: 38–39 °C

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.41 (t, *J* = 6.8 Hz, 2H), 1.85 (app. p, *J* = 7.2 Hz, 2H), 1.46 – 1.37 (m, 2H), 1.35 – 1.23 (m, 6H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 34.21, 32.97, 29.61, 29.55, 28.89, 28.30.

Synthesis of 2-((12-bromododecyl)oxy)tetrahydro-2H-pyran



8

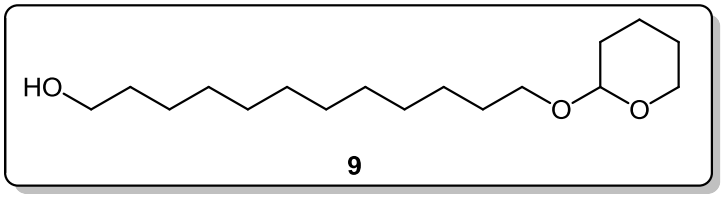
To a cooled (ice-bath) solution of **6** (17 g, 64.1 mmol, 1 eq.) and 3,4-dihydro-2*H*-pyran (8.8 mL, 96.2 mmol, 1.5 eq.) in Dichloromethane (30 mL) was added *p*-toluenesulfonic acid monohydrate (12.2 mg, 0.064 mmol, 0.001 eq.) and stirred at room temperature. After 2 hours the reaction mixture was washed with water (30 mL), the aqueous phase was extracted with dichloromethane (40 mL) and the combined organic layers were dried ($MgSO_4$), filtered and evaporated *in vacuo.* The crude product was purified by flash chromatography (20% diethyl ether in petroleum ether b.p. 40–60 °C) to afford the title compound **8** (21.9 g, 98%) as a colourless oil which solidified when stored at −20 °C.

Analytical TLC: *Rf* = 0.37 (10% Et_2O in petroleum ether b.p. 40–60 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.57 (dd, *J* = 4.3, 2.8 Hz, 1H), 3.86 (ddd, *J* = 11.1, 7.3, 3.4 Hz, 1H), 3.72 (dt, *J* = 9.5, 6.9 Hz, 1H), 3.54 – 3.45 (m, 1H), 3.43 – 3.31 (m, 3H), 1.91 – 1.76 (m, 3H), 1.76 – 1.65 (m, 1H), 1.65 – 1.46 (m, 6H), 1.46 – 1.21 (m, 16H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 98.87, 67.71, 62.35, 34.01, 32.90, 30.85, 29.82, 29.61, 28.83, 28.24, 26.31, 25.59, 19.76.

Synthesis of 2-((12-bromododecyl)oxy)tetrahydro-2H-pyran



9

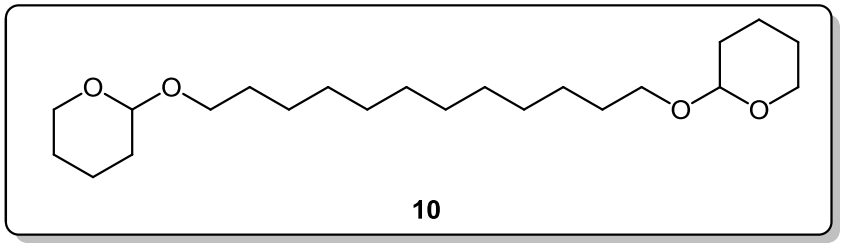
To a solution of 1,12-dodecanediol (15.0 g, 74.1 mmol, 1.4 eq.) in anhydrous THF (230 mL) and anhydrous dichloromethane (40 mL) were added *p*-toluenesulfonic acid monohydrate (101 mg, 0.01 eq.) and, dropwise, 3,4-dihydro-2*H*-pyran (4.83 mL, 53.0 mmol, 1 eq.). The solution was stirred at room temperature for 19 hours and then diluted with dichloromethane (*ca*. 80 mL), washed with water (1 × 50 mL and 1 × 100 mL) and brine (100 mL) and dried ($MgSO_4$) and filtered. To recover excess starting diol the organic solution was reduced *in vacuo* to 150 mL volume and diluted with Et_2O (*ca*. 80 mL), The suspension was filtered to give a white solid that washed with Et_2O and dried to yield 1,12-dodecanediol (4.58 g, 22.6 mmol) as a white solid. The combined organic solutions were evaporated *in vacuo* to yield a white solid (14.8 g) that was purified by flash chromatography (20% EtOAc in petroleum ether b.p. 40–60 °C) to afford the title compound **9** as colourless oil (10.2 g, 67%) and 1,12-bistetrahydropyranyloxydodecane (3.20 g) as colourless oil.

Analytical TLC: *Rf* = 0.31 (50% Et_2O in petroleum ether b.p. 40–60 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.59 – 4.52 (m, 1H), 3.85 (ddd, *J* = 11.1, 7.5, 3.3 Hz, 1H), 3.71 (dt, *J* = 9.5, 6.9 Hz, 1H), 3.61 (t, *J* = 6.7 Hz, 2H), 3.52 – 3.44 (m, 1H), 3.36 (dt, *J* = 9.5, 6.7 Hz, 1H), 1.86 – 1.76 (m, 1H), 1.74 – 1.46 (m, 10H), 1.36 – 1.23 (m, 15H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 98.94, 67.81, 63.11, 62.44, 32.90, 30.88, 29.84, 29.67, 29.58, 29.54, 26.33, 25.85, 25.60, 19.79.

Synthesis of 1,12-bis((tetrahydro-2H-pyran-2-yl)oxy)dodecane



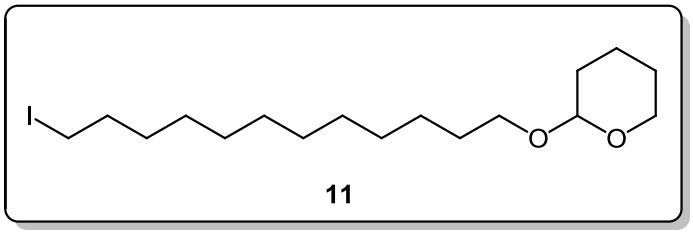
10

During the synthesis of alcohol **9** the diprotected alcohol **10** was isolated as a minor product. This by-product was purified by flash chromatography and yielded as colourless oil (3.20 g, 8.64 mmol).

Analytical TLC: *Rf* = 0.84 (50% Et_2O in petroleum ether b.p. 40–60 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.57 (dd, *J* = 4.2, 2.8 Hz, 2H), 3.87 (ddd, *J* = 11.2, 7.3, 3.4 Hz, 2H), 3.73 (dt, *J* = 9.5, 6.9 Hz, 2H), 3.54 – 3.45 (m, 2H), 3.38 (dt, *J* = 9.5, 6.7 Hz, 2H), 1.87 – 1.78 (m, 2H), 1.75 – 1.67 (m, 2H), 1.63 – 1.48 (m, 12H), 1.38 – 1.22 (m, 16H).

Synthesis of 2-((12-iododododecyl)oxy)tetrahydro-2H-pyran



11

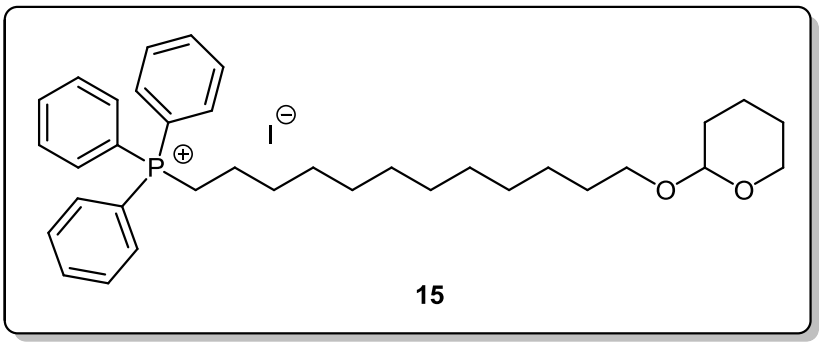
To a cooled (ice-bath) solution of **9** (4.56 g, 15.92 mmol, 1 eq.), triphenylphosphine (4.59 g, 17.51 mmol, 1.1 eq.) and imidazole (2.17 g, 31.84 mmol, 2.0 eq.) in anhydrous THF (120 mL) was added iodine (4.44 g, 17.51 mmol, 1.1 eq.) over 30 minutes, during which time it assumed a permanent brown colour (due to the excess of iodine). The reaction mixture was stirred at room temperature for 30

min and then diluted with Et_2O (*ca*. 200 mL) and quenched with 1 M aqueous sodium thiosulfate solution (100 mL). The colourless organic solution was collected, washed with water (2 × 100 mL), brine (1 × 100 mL), dried ($MgSO_4$), filtered and reduced *in vacuo*. To remove triphenylphosphine oxide the crude material was suspended in petroleum ether b.p. 40–60 °C (200 ml), cooled to 0 °C and filtered to give a white solid (7.0 g). The filtrate was evaporated *in vacuo* to yield a colourless oil that was purified by flash chromatography (7% Et_2O in petroleum ether b.p. 40–60 °C) to afford the title compound **11** (5.67 g, 90%) as a colourless oil which solidified when stored at −20 °C.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.57 (dd, *J* = 4.3, 2.7 Hz, 1H), 3.93 – 3.82 (m, 1H), 3.73 (dt, *J* = 9.5, 6.9 Hz, 1H), 3.55 – 3.44 (m, 1H), 3.38 (dt, *J* = 9.6, 6.7 Hz, 1H), 3.19 (t, *J* = 7.0 Hz, 2H), 1.88 – 1.77 (m, 3H), 1.76 – 1.66 (m, 1H), 1.62 – 1.49 (m, 6H), 1.42 – 1.23 (m, 16H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 98.97, 67.81, 62.47, 33.70, 30.92, 30.63, 29.88, 29.66, 29.53, 28.67, 26.36, 25.64, 19.84, 7.46.

Synthesis of triphenyl(12-((tetrahydro-2H-pyran-2-yl)oxy)dodecyl)phosphonium iodide



15

A solution of iodide **11** (1.74 g, 4.38 mmol) and triphenylphosphine (1.16 g, 4.42 mmol) in anhydrous CH_3CN (50 mL) was gently refluxed at 80 °C for 36 hours. The solution was then reduced *in vacuo* and the resulting oil was re-dissolved in the smallest volume of anhydrous dichloromethane (*ca.* 5mL) and followed by anhydrous diethyl ether (*ca.* 40 mL) to precipitate the phosphonium salt as colourless

oil. The mixture was left to settle, by gravity, for at least 1 hour. The upper clear Et_2O/CH_2Cl_2 solution containing starting iodide and triphenylphosphine was then removed from the precipitated oil by pipette. The precipitated oil was evaporated *in vacuo* and purified other two times by the previous procedure. Finally, the precipitated oil was washed with anhydrous diethyl ether (*ca.* 30 mL) and evaporated *in vacuo* to afford the title compound **15** (2.71 g, 94%) as colourless sticky oil.

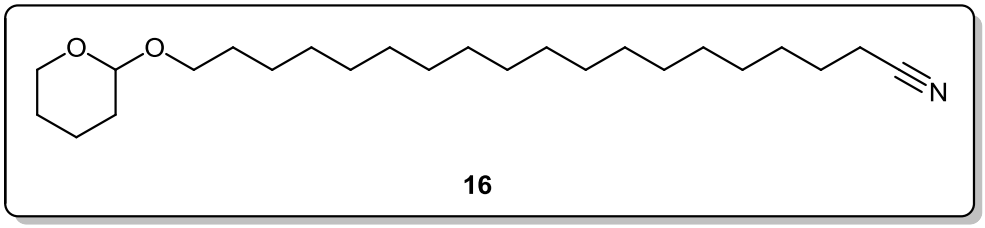
[Note: In the purification of phosphonium salt an high quantity of dichloromethane, or a premature careless removal of turbid Et_2O/CH_2Cl_2 solution could result in a partial loss of product].

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.88 – 7.75 (m, 9H), 7.76 – 7.65 (m, 6H), 4.56 (dd, *J* = 4.8, 2.6 Hz, 1H), 3.94 – 3.79 (m, 1H), 3.79 – 3.63 (m, 3H), 3.54 – 3.43 (m, 1H), 3.37 (dt, *J* = 9.7, 7.0 Hz, 1H), 1.90 – 1.76 (m, 1H), 1.76 – 1.45 (m, 11H), 1.39 – 1.11 (m, 14H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 135.17, 133.69 (d, *J* = 9.8 Hz), 130.61 (d, *J* = 12.4 Hz), 118.20 (d, *J* = 86.2 Hz), 98.94, 67.72, 62.46, 30.83, 30.55, 30.40, 29.76, 29.50, 29.18, 26.22, 25.52, 23.34, 22.83, 22.64, 19.78.

FTIR (neat): 2914, 2846, 1465, 1065, 723 cm^{-1}

Synthesis of 19-((tetrahydro-2H-pyran-2-yl)oxy)nonadecanenitrile



16

An oven-dried, nitrogen-flushed 250 mL three-neck round-bottom flask was charged with bromide **8** (1.09 g, 3.12 mmol, 1 eq.) and PEPPSI-IPr catalyst (106 mg, 5 mol%) under nitrogen. To the previous mixture were added, by syringe, 20 mL of 1.5 M degassed solution of lithium bromide prepared from ultra dry salt (5g, supplied from AlfaAesar in ampoule under argon) in degassed anhydrous NMP (39 mL),

finally was added 6-cyanohexylzinc bromide solution (40 mL, 17.6 mmol, 5.6 eq., in THF) and the reaction mixture was stirred for 20 hours. The reaction was then diluted with diethyl ether/petroleum ether (100 mL) and washed with 250 mM aqueous Na_3EDTA solution (3 × 150 mL; prepared from EDTA, 29.2 g, NaOH, 12 g, and water, 400 mL), water (150 mL) and brine (150 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a yellow solid (2.10 g) that was purified by flash chromatography (13% diethyl ether in petroleum ether b.p. 40–60 °C) to afford the title compound **16** (840 mg, 71%) as white solid.

m.p.: 42–44 °C

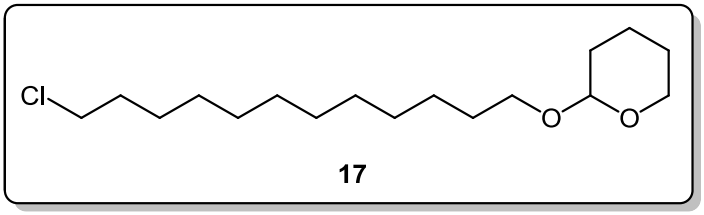
^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.56 (dd, *J* = 4.4, 2.7 Hz, 1H), 3.86 (ddd, *J* = 11.1, 7.3, 3.4 Hz, 1H), 3.72 (dt, *J* = 9.6, 6.9 Hz, 1H), 3.53 – 3.44 (m, 1H), 3.37 (dt, *J* = 9.5, 6.7 Hz, 1H), 2.32 (t, *J* = 7.1 Hz, 2H), 1.89 – 1.76 (m, 1H), 1.75 – 1.47 (m, 9H), 1.47 – 1.38 (m, 2H), 1.38 – 1.15 (m, 26H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 119.99, 98.96, 67.81, 62.46, 30.91, 29.87, 29.78, 29.62, 29.42, 28.88, 28.78, 26.36, 25.62, 25.49, 19.83, 17.24.

Alternative synthesis of nitrile **16** from iodide **11**:

An oven-dried three-neck round-bottom flask was charged with PEPPSI-IPr catalyst (89 mg, 1 mol%) and purged with nitrogen. To this flask were added 53 mL of 300 mM solution of lithium chloride in 1,3-dimethyl-2-imidazolidinone (DMI), prepared from anhydrous LiCl (860 mg, 20.3 mmol) and anhydrous and degassed DMI (68 ml, ca. 72 g) under nitrogen. To this solution were added 6-cyanohexylzinc bromide (18 mL, 7.92 mmol, 1.75 eq., solution in THF) and iodide **11** (1.79 g, 4.52 mmol). Workup and purification as reported above.

Synthesis of 2-((12-chlorododecyl)oxy)tetrahydro-2H-pyran



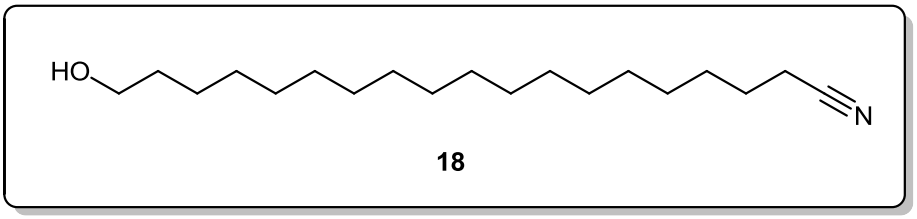
17

Occasionally, during the synthesis of nitrile **16**, the chloride **17** was isolated as product of transhalogenation of starting halide. This by-product was purified by flash chromatography and yielded as colourless oil.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.61 – 4.52 (m, 1H), 3.86 (ddd, *J* = 11.1, 7.4, 3.4 Hz, 1H), 3.72 (dt, *J* = 9.6, 6.9 Hz, 1H), 3.57 – 3.44 (m, 3H), 3.37 (dt, *J* = 9.6, 6.7 Hz, 1H), 1.87 – 1.66 (m, 4H), 1.63 – 1.46 (m, 6H), 1.46 – 1.21 (m, 17H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 98.93, 67.78, 62.43, 45.26, 32.75, 30.89, 29.85, 29.65, 29.57, 28.99, 26.98, 26.33, 25.61, 19.80.

Synthesis of 19-hydroxynonadecanenitrile



18

To a solution of **16** (800 mg, 2.11 mmol) in dichloromethane (6 mL) and MeOH (70 mL) was added *p*-toluenesulfonic acid monohydrate (20 mg, 0.105 mmol, 5%) and stirred at room temperature until TLC analysis indicated complete consumption of starting material (a overnight stirring was required). The reaction mixture was then quenched with $NaHCO_3$ (35 mg) and evaporated *in vacuo* to give a white solid (854 mg) that was dissolved in 20% THF in petroleum ether b.p. 60–80 °C (250 mL) and washed with water (3 × 150 mL) and brine (150 mL). The organic phase was dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a solid,

which was heated at 60 °C (to give a colourless oil) and kept at low pressure (70 mbar) for 10 minutes to remove low boiling point by-products and to afford the title compound **18** (615 mg, 99%) as a white solid.

m.p.: 54–55 °C

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.63 (t, *J* = 6.6 Hz, 2H), 2.33 (t, *J* = 7.1 Hz, 2H), 1.65 (app p, *J* = 7.4 Hz, 2H), 1.56 (app p, *J* = 7.2 Hz, 2H), 1.49 – 1.21 (m, 28H).

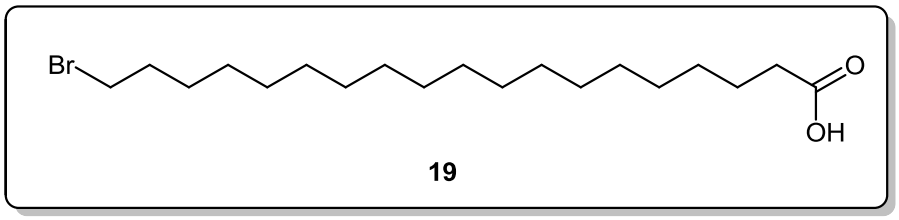
^{13}C NMR (101 MHz, CHLOROFORM-D) δ 120.01, 63.22, 32.94, 29.79, 29.76, 29.63, 29.57, 29.43, 28.90, 28.80, 25.87, 25.50, 17.26.

FTIR (neat): 3302, 2913, 2845, 2246, 1462, 1064, 723 cm^{-1}

Alternative Synthesis of nitrile **18** from iodide **16**:

To a solution of **16** (351 mg, 0.925 mmol) in anhydrous diethyl ether (5 mL) and anhydrous MeOH (50 mL) was added Amberlyst® 15 (1.0 g) and mildly stirred for three days. The reaction mixture was then filtered and evaporated as mentioned above to afford title compound **18** (263 mg, 96%) as a light brownish solid.

Synthesis of 19-bromononadecanoic acid



19

A 100 mL round-bottom flask was charged with nitrile **18** (237 mg, 0.802 mmol), and, in a well-ventilated fume hood, 48% aqueous hydrobromic acid (29 g) was added, followed by dropwise addition of concentrated sulfuric acid (6 g). The mixture was heated at 110 °C using a condenser connected with a trap of aqueous $NaHCO_3$ solution to neutralise HBr vapours. After 19 hours the mixture was diluted

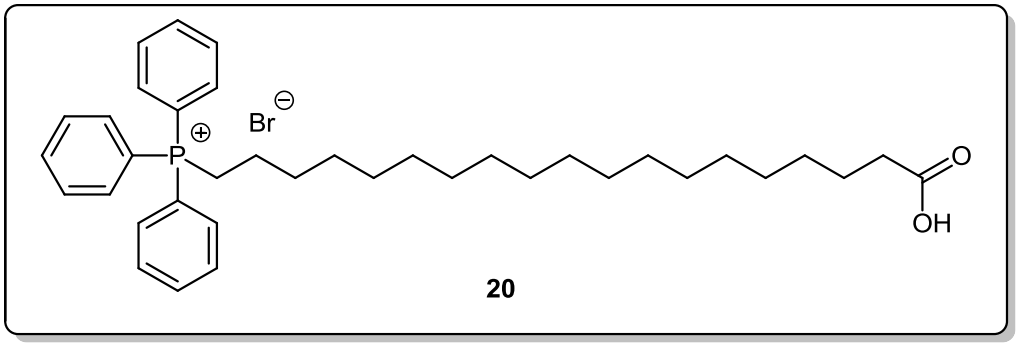
with Na_2SO_4 solution to pH 0.5-0.8 and extracted with diethyl ether (2 × 150 mL). The combined organic layers were washed with water (2 × 200 mL) and brine (150 mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **19** (288 mg, 95%) as an off-white solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.41 (t, *J* = 6.9 Hz, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 1.85 (app p, *J* = 7.2 Hz, 2H), 1.63 (app p, *J* = 7.5 Hz, 2H), 1.50 – 1.20 (m, 28H).

^{3}C NMR (101 MHz, CHLOROFORM-D) δ 179.36, 34.23, 34.05, 33.00, 29.81, 29.74, 29.69, 29.59, 29.39, 29.21, 28.92, 28.33, 24.84.

FTIR (neat): 2915, 2847, 1709, 1462, 895, 730, 719 cm^{-1}

Synthesis of (18-carboxyoctadecyl)triphenylphosphonium bromide



20

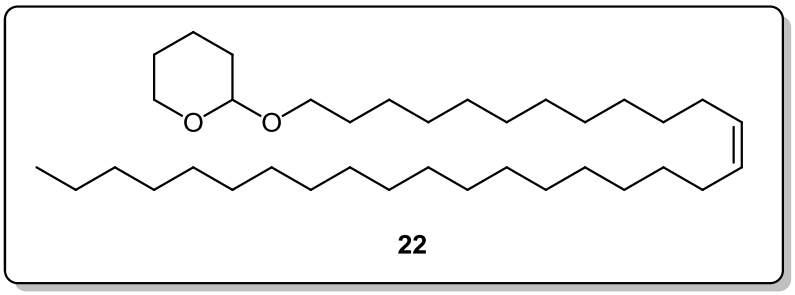
A 2-5 mL MW vial was charged with bromide **19** (632 mg, 1.67 mmol, 1 eq.), triphenylphosphine (459 mg, 1.75 mmol, 1.05 eq.) and anhydrous CH_3CN (5 mL), purged with nitrogen and heated at 170 °C under microwave irradiation for 1 hour. The reaction mixture was then cooled to room temperature and evaporated *in vacuo* to give a sticky orange oil that was dissolved in anhydrous dichloromethane (15 mL) and concentrated *in vacuo* to give an oil (*ca.* 1 mL volume). To the resulting oil was added anhydrous toluene (15 mL) and the mixture was heated to reflux (110 °C for 30 minutes) and then cooled slowly to 0 °C to give a precipitate. The supernatant was removed and the precipitate was suspended in anhydrous toluene (15 mL), heated (110 °C for 30 min), cooled down (0 °C for 1 h) to give a solid that was filtered and

kept under high vacuum to afford the title compound **20** (999 mg, 94%) as a beige solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.88 – 7.75 (m, 9H), 7.70 (td, *J* = 7.6, 3.4 Hz, 6H), 3.83 – 3.65 (m, 2H), 2.36 (t, *J* = 7.5 Hz, 2H), 1.68 – 1.52 (m, 6H), 1.36 – 1.14 (m, 26H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 177.61, 135.11 (d, *J* = 2.9 Hz), 133.83 (d, *J* = 10.0 Hz), 130.61 (d, *J* = 12.4 Hz), 118.57 (d, *J* = 85.7 Hz), 34.30, 30.64, 30.49, 29.58, 29.53, 29.51, 29.47, 29.37, 29.30, 29.14, 24.92, 23.10, 22.81, 22.77, 22.60.

Synthesis of (Z)-2-(hentriacont-12-en-1-yloxy)tetrahydro-2H-pyran



22

In a tared 250 ml one-neck a solution of phosphonium salt **15** in anhydrous dichloromethane was rigorously dried and then weighed (5.67 g, 8.61 mmol, 1.2 eq.). The flask was connected with a three-neck adaptor and purged with nitrogen. The sticky oil was solubilised in anhydrous THF (60 mL) to yield a light yellow solution, which was cooled to −20 °C. To this stirred solution was added dropwise 1M solution of NaHMDS in THF (8.6 mL, 8.6 mmol, 1.2 eq.). The solution turned immediately to orange and was warmed and stirred at room temperature for 30 minutes. The resulting red-orange solution was cooled to −25 °C, then was added anhydrous DMPU (18 mL) and the solution was cooled to −84 °C. To this solution was added dropwise a solution of aldehyde **4** (2.02 g, 7.17 mmol, 1 eq.) in anhydrous THF (22 mL) over 90 minutes and keeping constantly the temperature of the reaction close to −84 °C. The reaction was stirred at this temperature for *ca.* 1 hour, then the reaction system was allowed to warm slowly to room temperature and stirred for

further 3 hours. The solution was then quenched with 0.5 M solution of ammonium chloride (*ca.* 50 mL) and extracted with petroleum ether (*ca.* 150 mL). The organic phase was washed with 0.5 M solution of ammonium chloride (3 × 120 mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a white semi-solid (5.5 g). The crude was purified by flash chromatography (2% diethyl ether in petroleum ether b.p. 40–60 °C) to afford the title compound **22** as white solid (2.00 g, 52%).

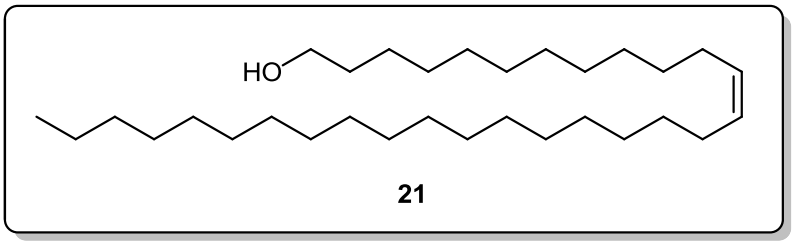
Analytical TLC: *Rf* = 0.31 (4% Et_2O in petroleum ether b.p. 40–60 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 5.42 – 5.27 (m, 2H), 4.57 (dd, *J* = 4.2, 2.8 Hz, 1H), 3.86 (ddd, *J* = 11.2, 7.5, 3.5 Hz, 1H), 3.72 (dt, *J* = 9.6, 6.9 Hz, 1H), 3.54 – 3.44 (m, 1H), 3.37 (dt, *J* = 9.6, 6.7 Hz, 1H), 2.00 (app q, *J* = 6.3 Hz, 4H), 1.89 – 1.77 (m, 1H), 1.75 – 1.66 (m, 1H), 1.64 – 1.46 (m, 6H), 1.38 – 1.19 (m, 48H), 0.87 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 130.01, 98.94, 67.82, 62.42, 32.08, 30.92, 29.86, 29.66, 29.52, 29.47, 27.35, 26.40, 25.66, 22.84, 19.83, 14.26.

FTIR (neat): 2915, 2849, 1471, 1036, 716 cm^{-1}

Synthesis of (Z)-hentriacont-12-en-1-ol



21

To a vigorously stirred solution of alkene **13** (1.95 g, 3.65 mmol, 1 eq.) in anhydrous diethyl ether (45 mL) was added magnesium bromide ethyl etherate (3.30 g, 12.78 mmol, 3.5 eq.) under nitrogen and the initial slightly yellow solution was stirred for 6.7 hours, during which time a white precipitate formed. The reaction mixture was diluted with diethyl ether (100 mL), quenched with saturated aqueous

NH_4Cl solution (150 mL), washed with water (2 ×100 mL), brine (100 mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a pale yellow solid (1.76 g). The crude product was purified by flash chromatography (20% diethyl ether in petroleum ether b.p. 40–60 °C, *Rf*: 0.25) to afford the title compound **21** (1.64 g, 99%) as a white solid.

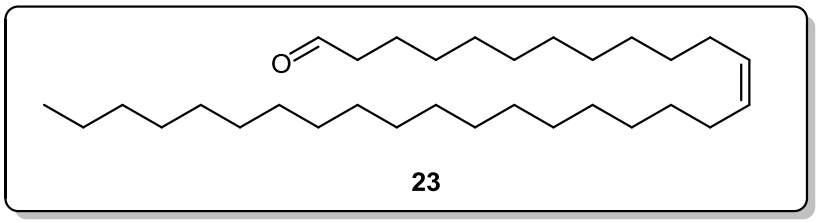
Analytical TLC: *Rf* = 0.25 (20% Et_2O in petroleum ether b.p. 40–60 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 5.35 (app t, *J* = 4.8 Hz, 2H), 3.64 (app q, *J* = 6.2 Hz, 2H), 2.00 (app q, *J* = 6.3 Hz, 4H), 1.62 – 1.52 (m, 2H), 1.40 – 1.20 (m, 48H), 1.18 (t, *J* = 5.4 Hz, 1H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 130.05, 63.24, 32.95, 32.08, 29.86, 29.59, 29.52, 29.47, 27.36, 25.89, 22.85, 14.28.

FTIR (neat): 3425, 2914, 2848, 1471, 1058, 715 cm^{-1}

Synthesis of (Z)-hentriacont-12-enal



23

To a vigorously stirred mixture of PCC (1.65 g, 7.63 mmol, 2 eq.), molecular sieves 3Å (1.91 g, powder) and anhydrous NaOAc (188 mg, 2.29 mmol, 0.6 eq.) in anhydrous Dichloromethane (60 mL) was added alcohol **21** (1.72 g, 3.82 mmol, 1 eq.) under nitrogen. The resulting dark orange slurry suspension was stirred vigorously at room temperature for 3 hours before it was diluted with petroleum ether (40 mL) and passed through a short plug of silica gel and eluting with Dichloromethane to give an off-white crude (*ca.* 1.6 g) that was purified by flash chromatography (4% diethyl ether in petroleum ether b.p. 40–60 °C) to afford the title compound **23** (1.55 g, 91%)

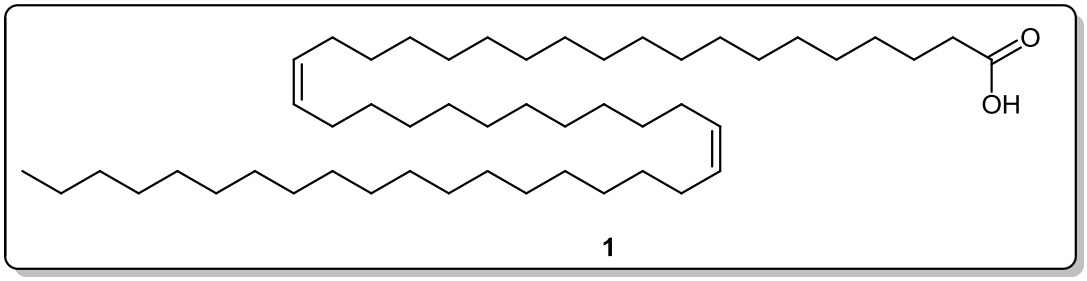
as a white solid. The aldehyde **23** was used immediately or stored at −20 °C under nitrogen.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 9.76 (t, *J* = 1.8 Hz, 1H), 5.42 – 5.28 (m, 2H), 2.42 (td, *J* = 7.4, 1.8 Hz, 2H), 2.01 (app q, *J* = 6.3 Hz, 4H), 1.62 (p, *J* = 7.2 Hz, 2H), 1.37 – 1.22 (m, 46H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 203.03, 130.09, 130.00, 44.08, 32.09, 29.92, 29.86, 29.82, 29.72, 29.71, 29.68, 29.58, 29.52, 29.48, 29.45, 29.33, 27.36, 22.85, 22.25, 14.27.

FTIR (neat): 2912, 2848, 1712, 1471, 715 cm^{-1}

Synthesis of (19Z,31Z)-pentaconta-19,31-dienoic acid



1

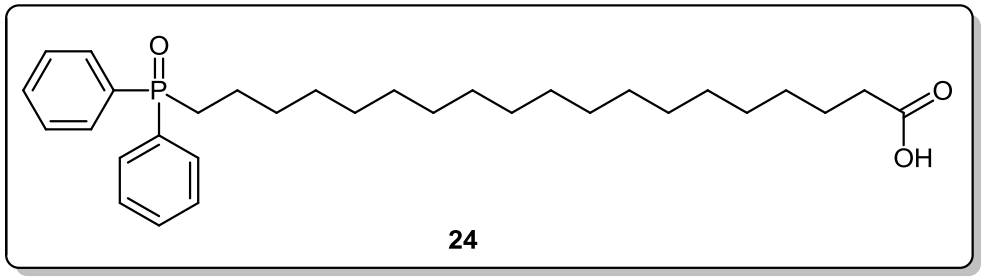
To a stirred suspension of phosphonium bromide **20** (218 mg, 0.341 mmol, 1.0 eq.) in anhydrous THF (20 mL) was added dropwise sodium hexamethyldisilazane in THF (1 M, 0.78 mL, 0.78 mmol, 2.2 eq.) at room temperature. The phosphonium bromide started to solubilise after the first equivalent of base, after the complete addition of base the solution became orange. This orange solution was cooled to −84 °C and a solution of aldehyde **23** (159 mg, 0.354 mmol, 1 eq.) in anhydrous THF (10 mL) was added dropwise over 30 minutes, keeping constantly the temperature of the reaction close to −84 °C. The mixture was stirred at *ca.* −80 °C for 30 minutes. Then the reaction system was allowed to warm slowly to room temperature and stirred for further 2 hours. The solution was then quenched with a dropwise addition of aqueous solution of ammonium sulfate at pH 3 (prepared by the addition of concentrated sulphuric acid) and extracted with diethyl ether − petroleum ether (ratio 6:1). The organic phase was washed with aqueous solution of ammonium sulfate at pH 3 (2 × 150 mL), dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a white solid (290 mg). The crude was purified by flash chromatography (10% diethyl ether and 0.1% AcOH in petroleum ether b.p. 40–60 °C) to afford the title compound **1** (20 mg, 8%) as white solid.

HRMS (ES–, *m*/*z*): $[M-H]^-$= 727.7344

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 5.41 – 5.29 (m, 4H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.01 (app dd, *J* = 12.1, 6.5 Hz, 8H), 1.69 – 1.58 (m, 2H), 1.37 – 1.18 (m, 76H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 178.69, 130.08, 130.02, 33.93, 32.08, 29.93, 29.86, 29.82, 29.72, 29.71, 29.68, 29.59, 29.52, 29.48, 29.46, 29.40, 29.22, 27.37, 24.85, 22.85, 14.27.

Synthesis of 19-(diphenylphosphoryl)nonadecanoic acid



24

During the Wittig reactions for the synthesis of (19Z,31Z)-pentaconta-19,31-dienoic acid, the phosphoryl derivate **24** was generally isolated as the major product. This unexpected product was purified by flash chromatography (1% MeOH and 1% AcOH in dichloromethane) and crystallized. Specifically, impure diphenylphosphoryl derivate (150 mg) was dissolved in dichloromethane (2 mL) and precipitated with petroleum ether (12 mL) to afford the title compound **24** as white solid (140 mg).

Analytical TLC: *Rf* = 0.41 % (5% MeOH and 1% AcOH in dichloromethane); UV-active, single spot (PMA).

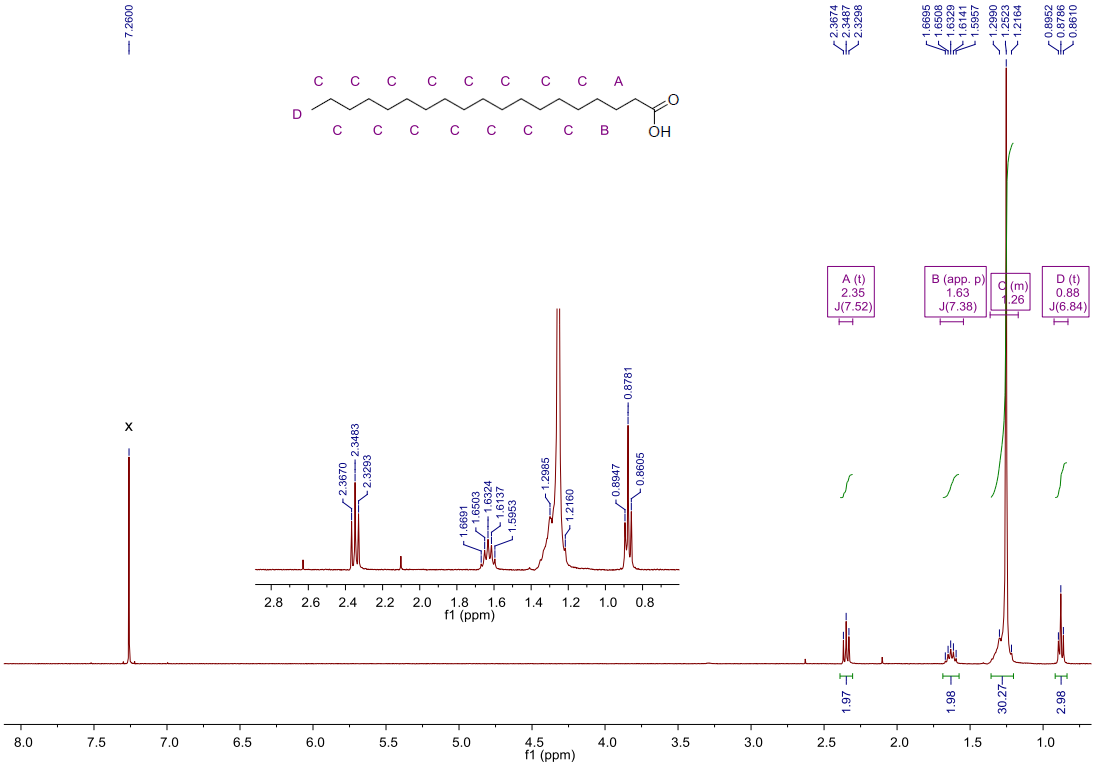
HRMS (ES–, *m*/*z*): $[M+H]^+$= 499.3356

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.78 – 7.69 (m, 4H), 7.55 – 7.43 (m, 6H), 2.35 – 2.23 (m, 4H), 1.68 – 1.54 (m, 4H), 1.43 – 1.17 (m, 7H).

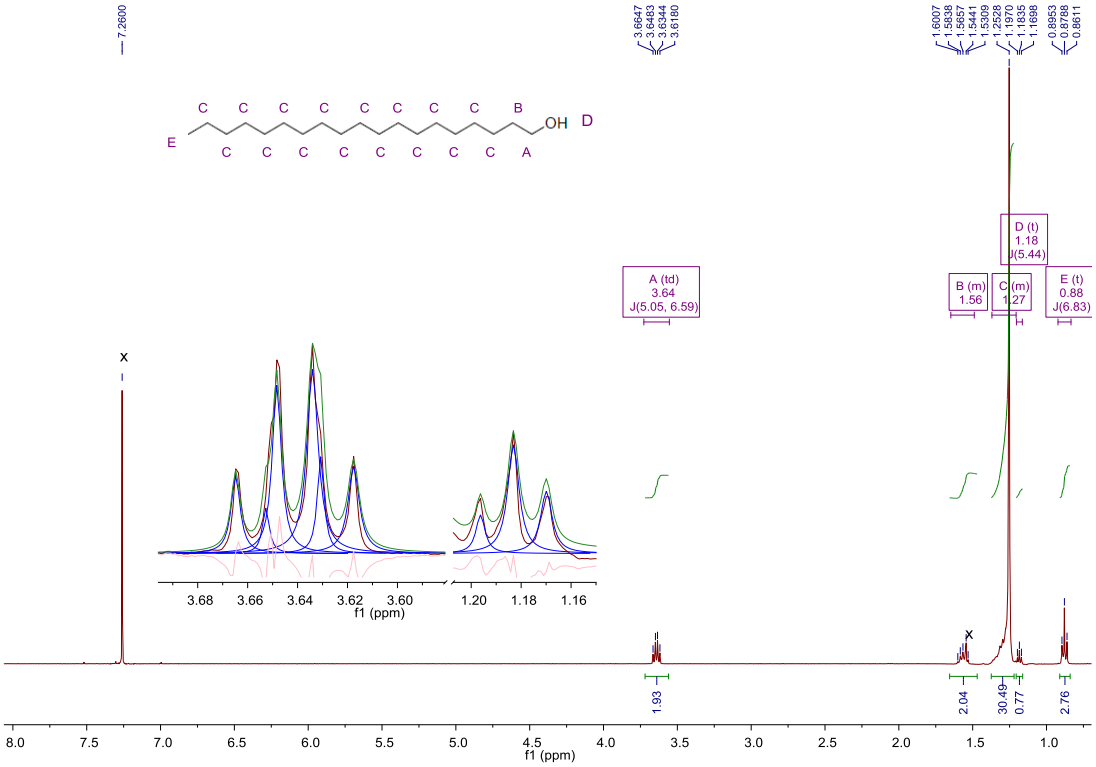
FTIR (neat): 2917, 2847, 1744, 1469, 1165, 718 cm^{-1}

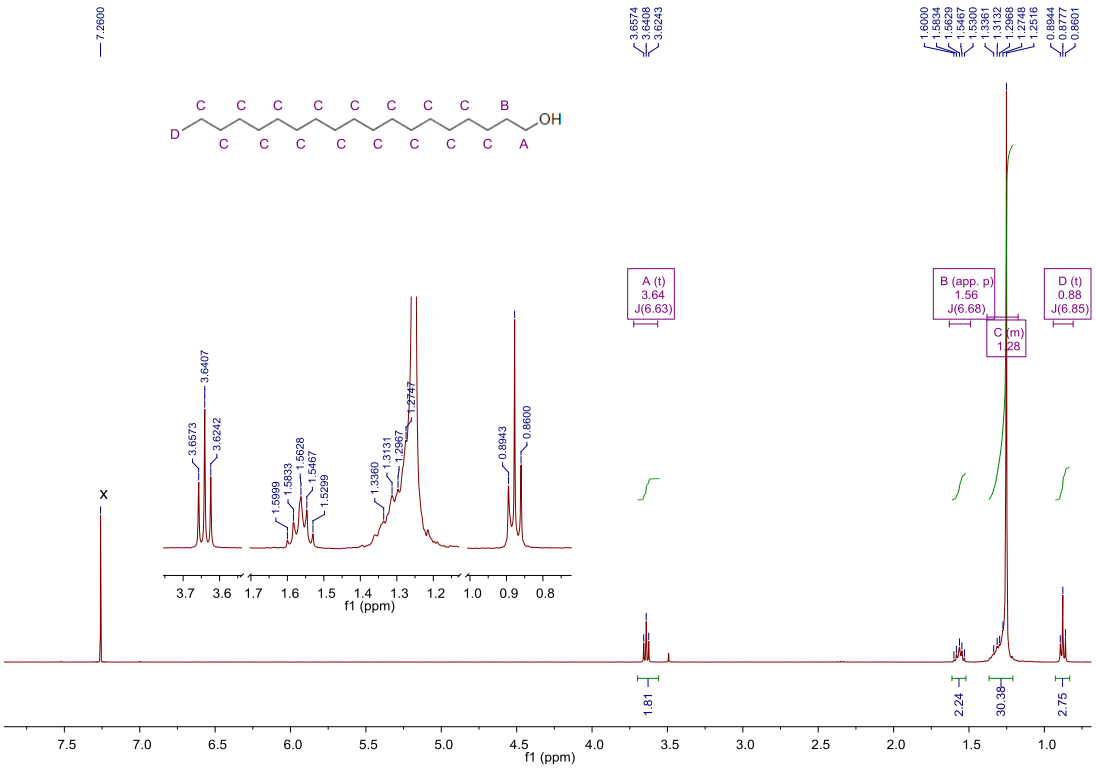
6.1.2.2 Selected NMR Spectra

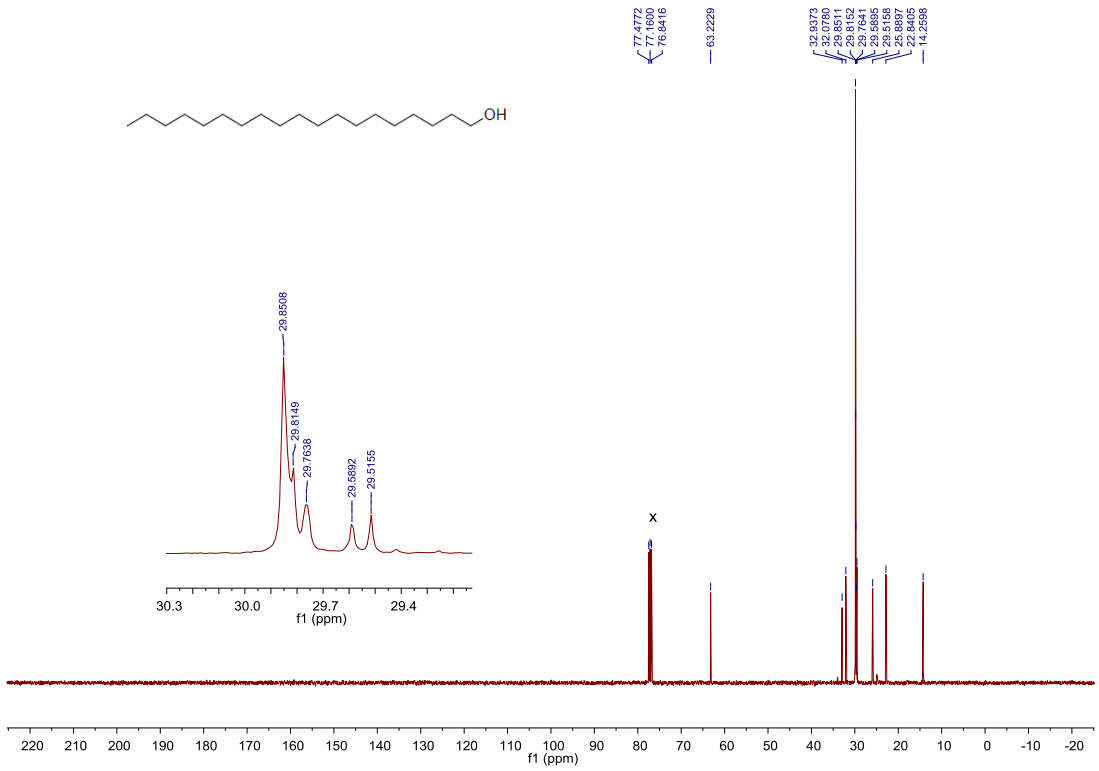
NMR Spectra of compound 2



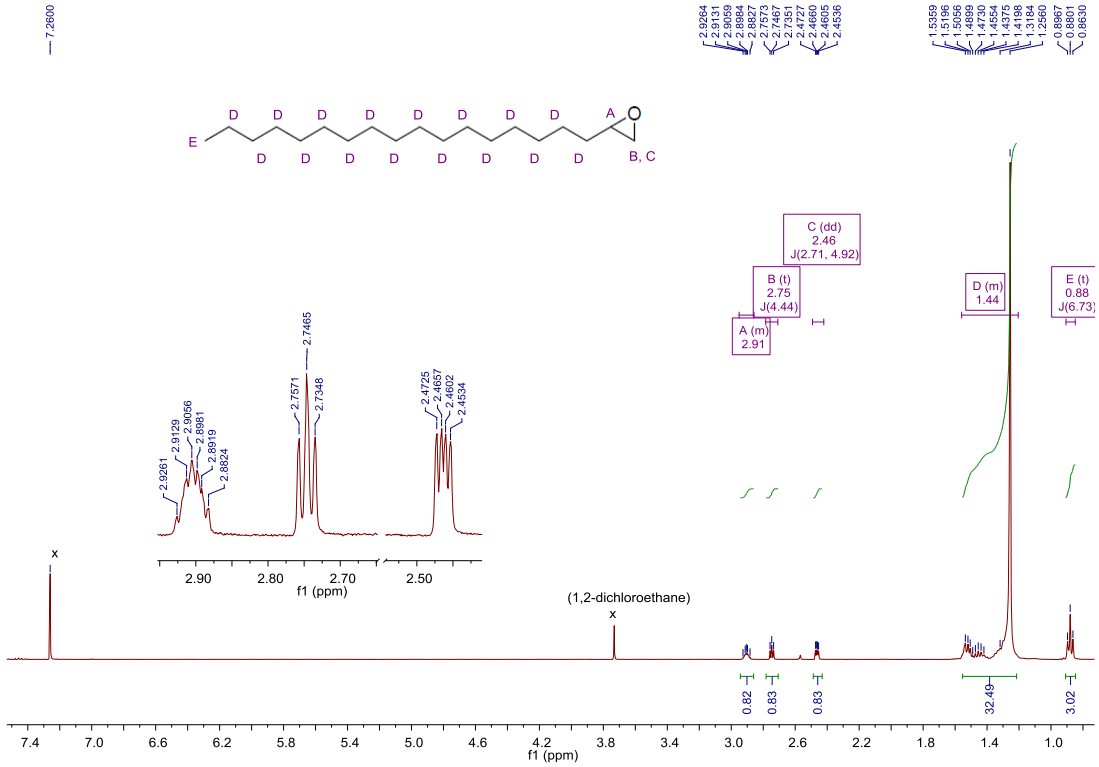
NMR Spectra of compound 3



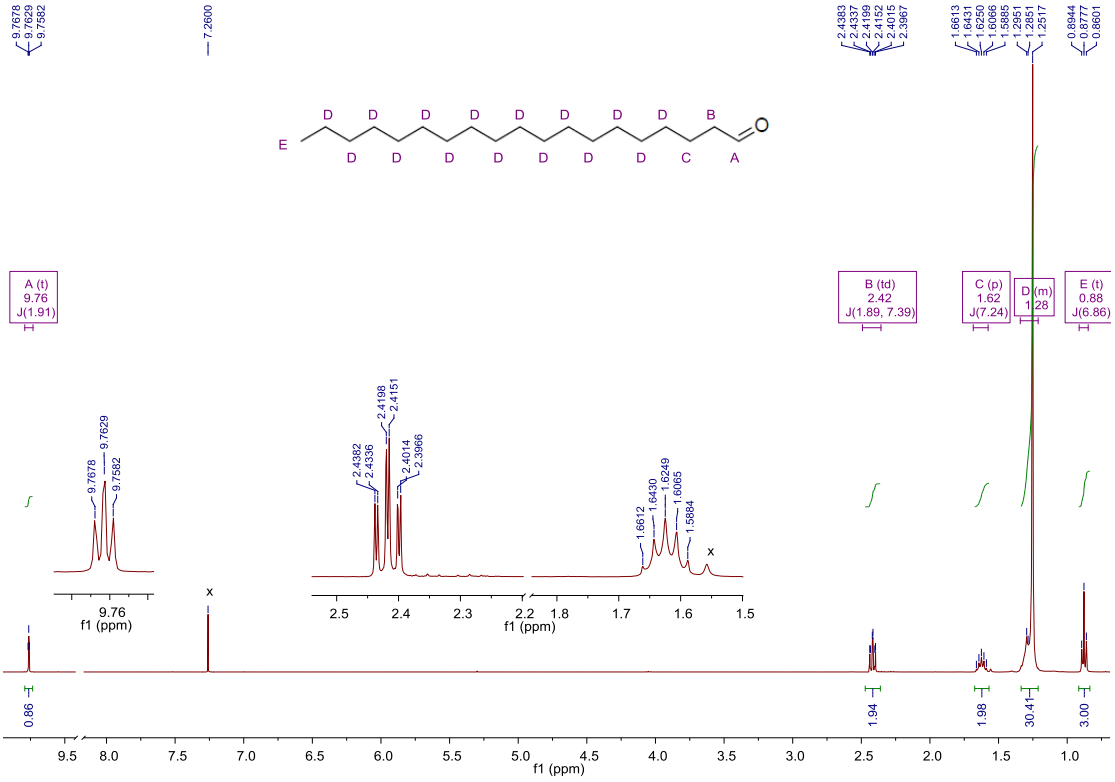


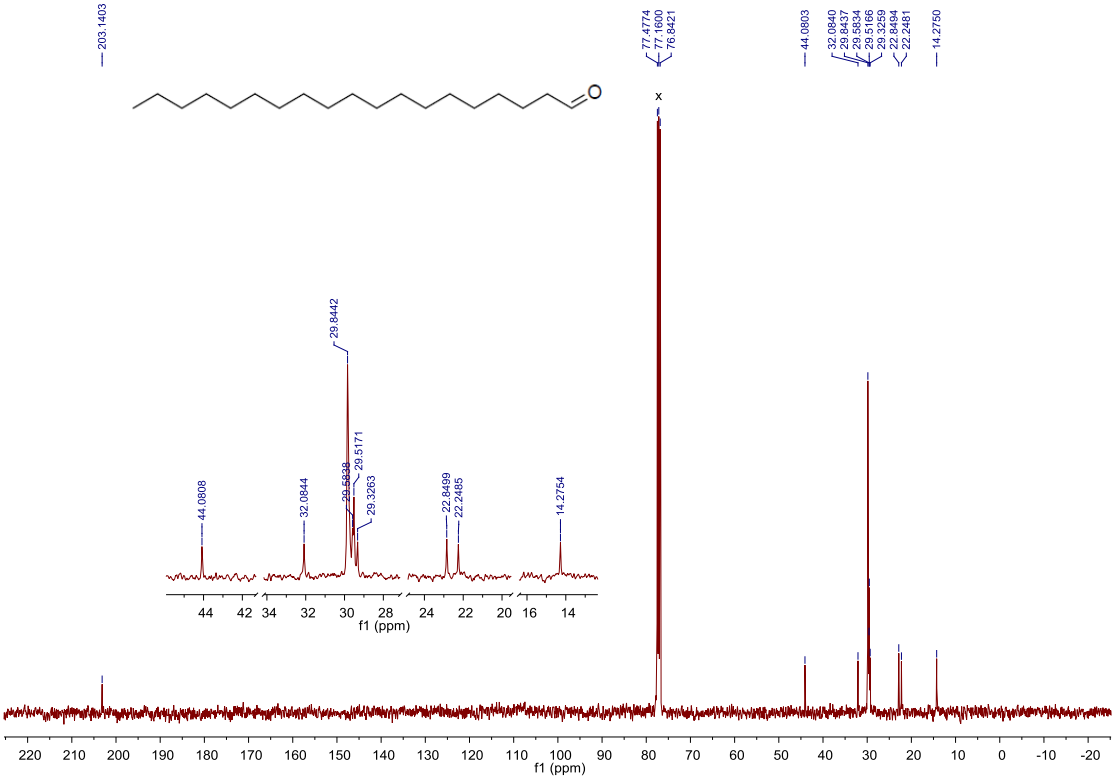

OH
77.4772
77.1600
76.8416
63.2229
32.9373
32.0780
29.8511
29.8152
29.7644
29.7341
29.5695
29.5158
25.8897
22.8405
14.2598
29.8508
29.8149
29.7638
29.5892
29.5155
30.3
30.0
29.7
29.4
f1 (ppm)
x
220 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0 -10 -20
f1 (ppm)

NMR Spectra of compound 5

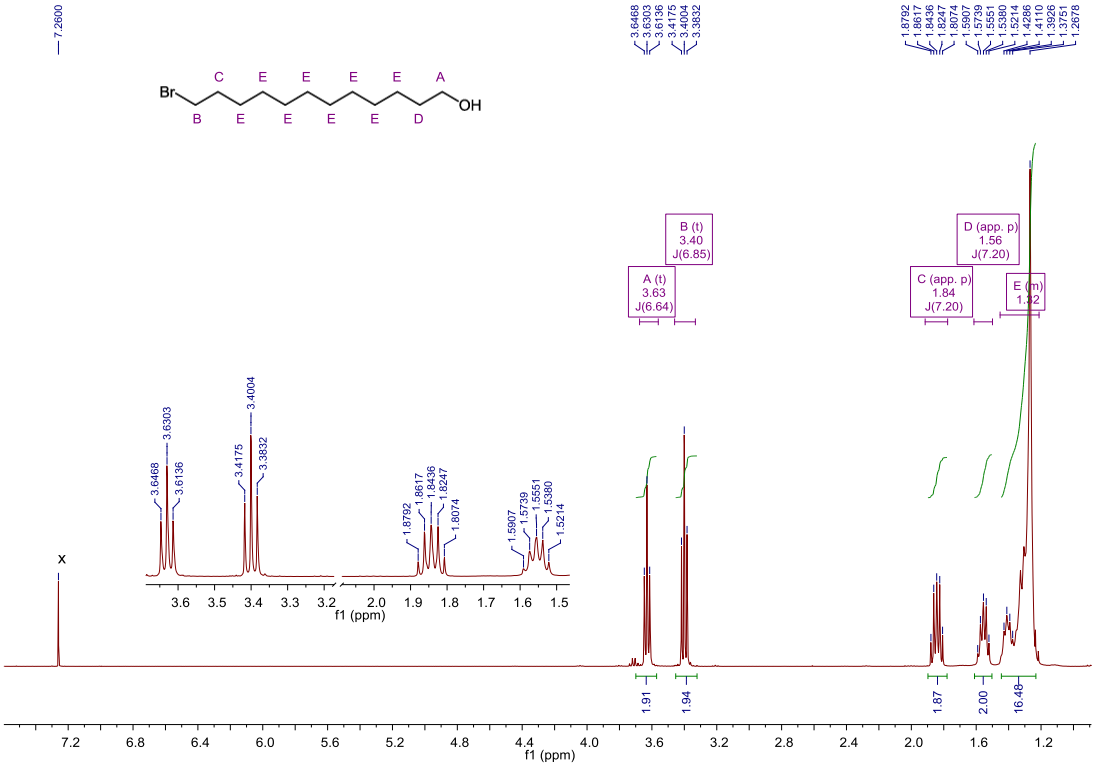


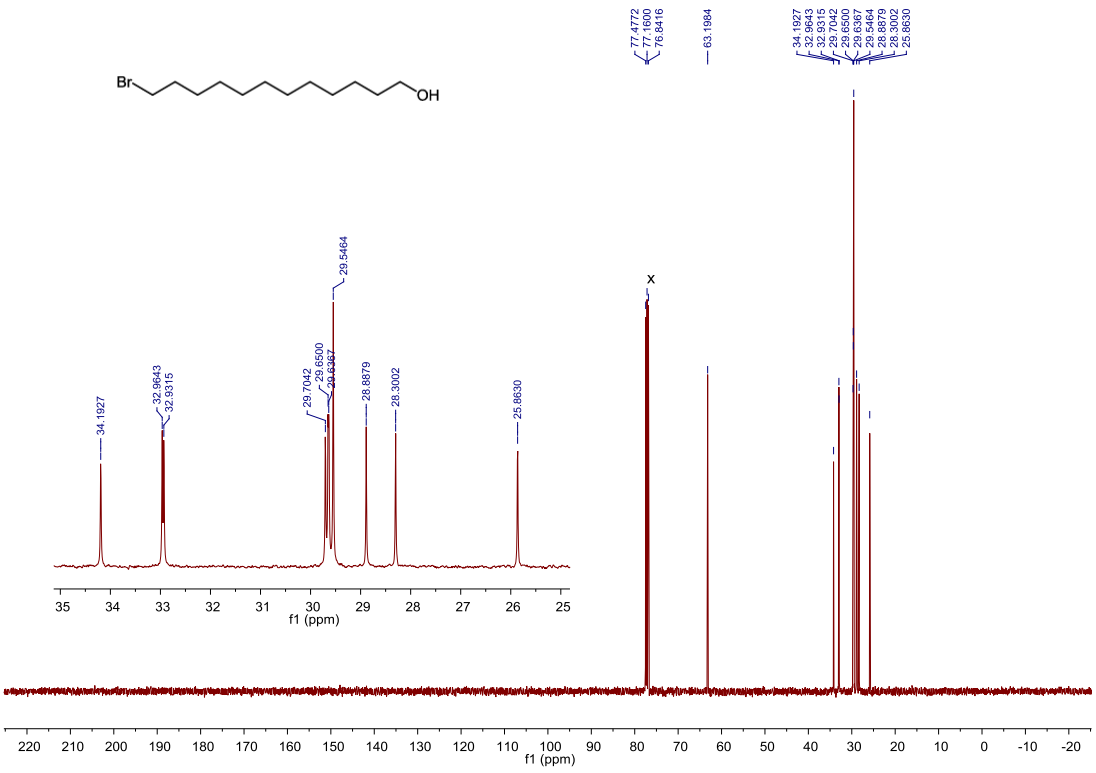
NMR Spectra of compound 4



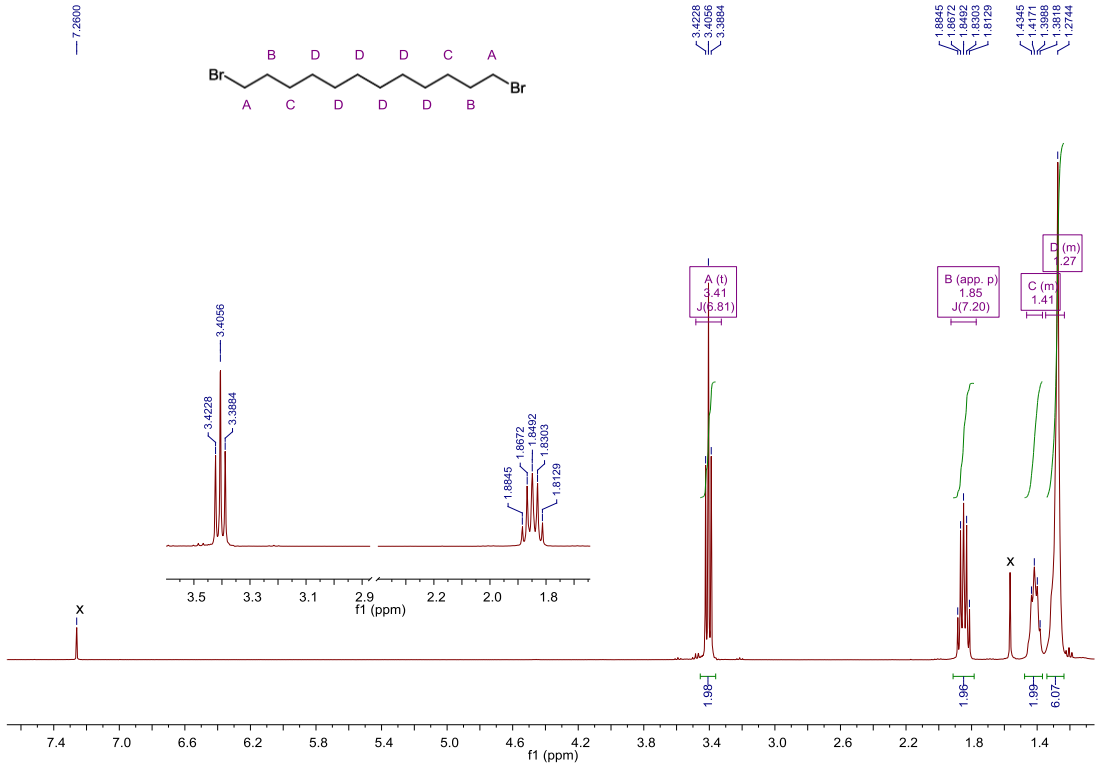


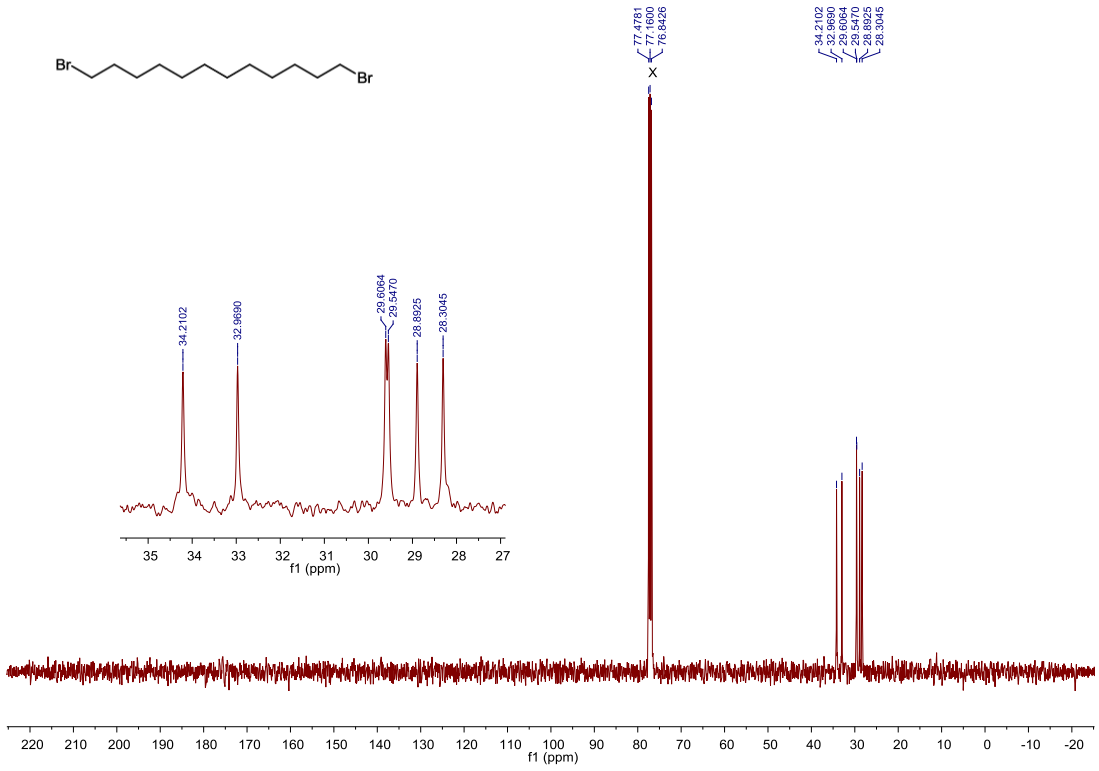
NMR Spectra of compound 6



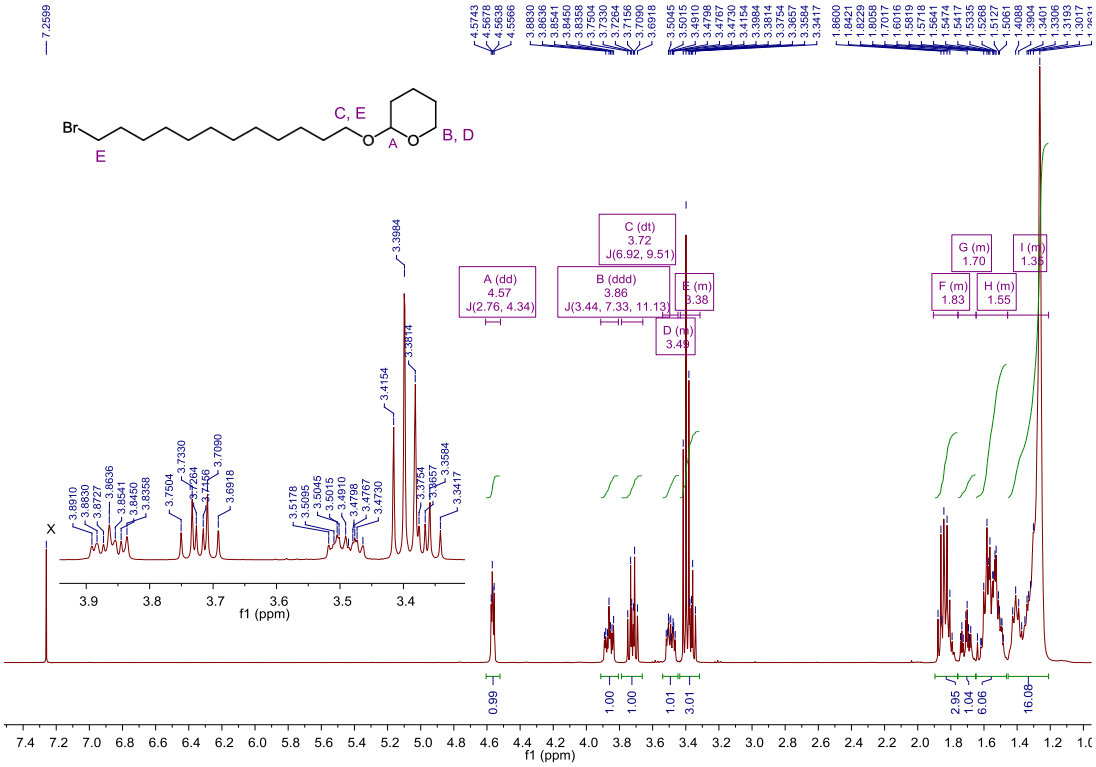


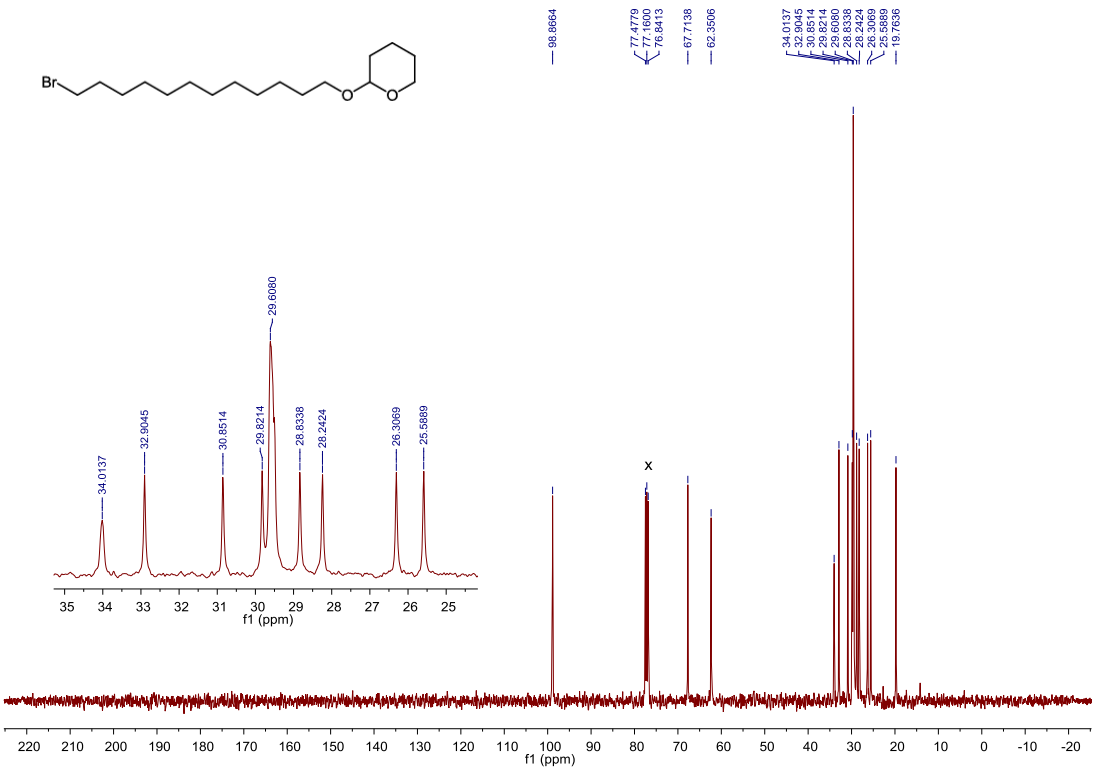
NMR Spectra of compound 7



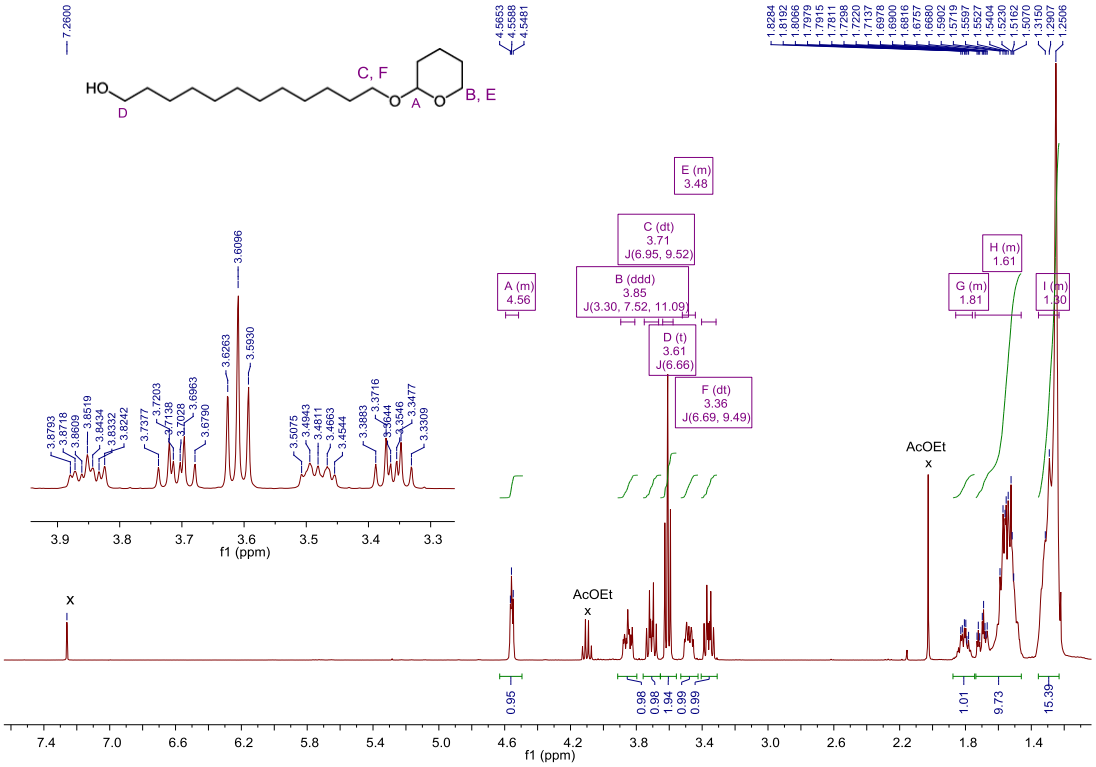


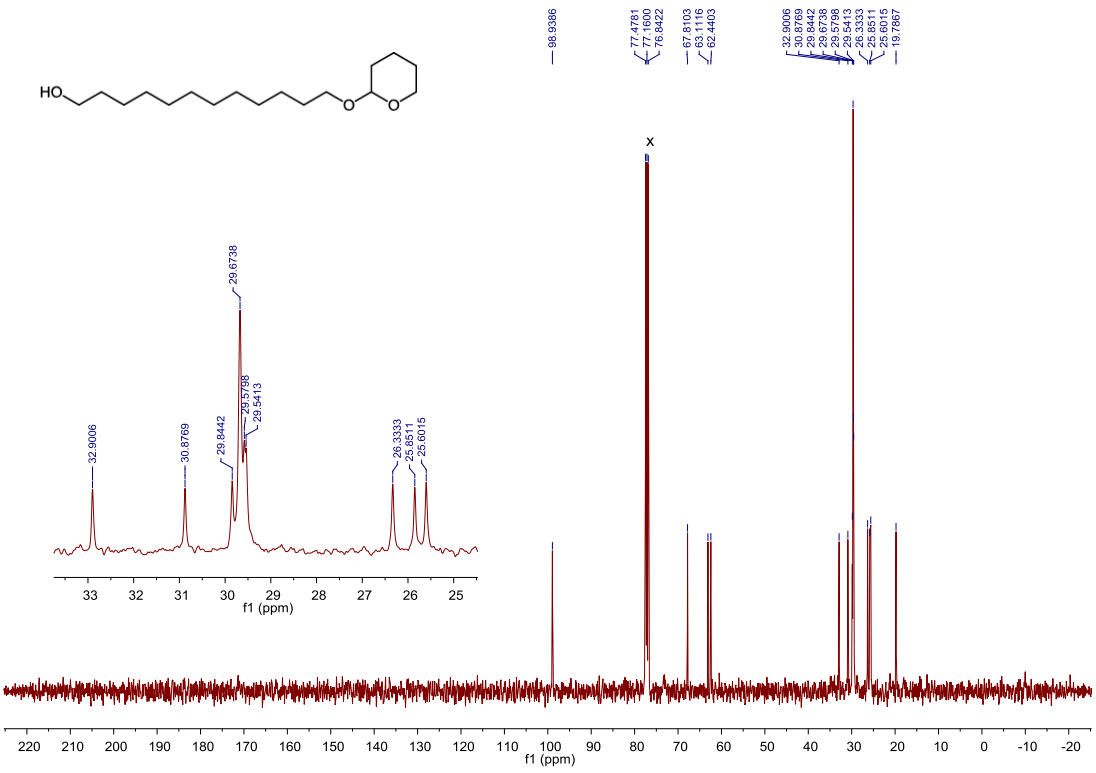
NMR Spectra of compound 8



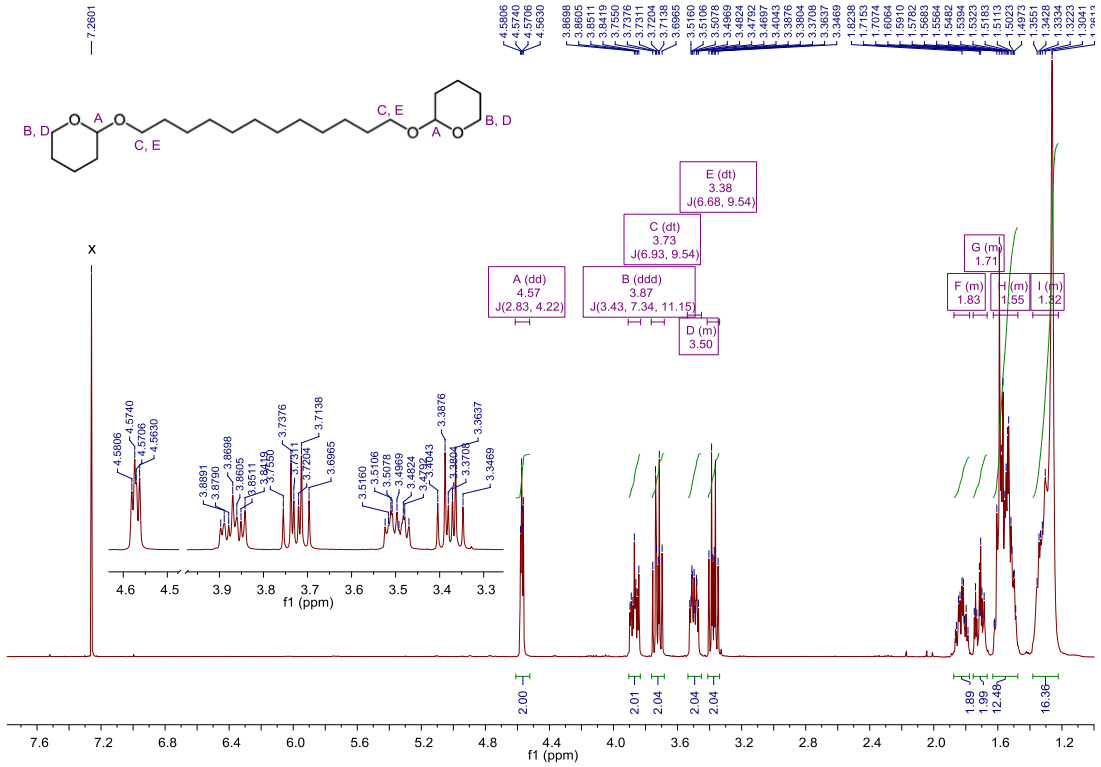


NMR Spectra of compound 9

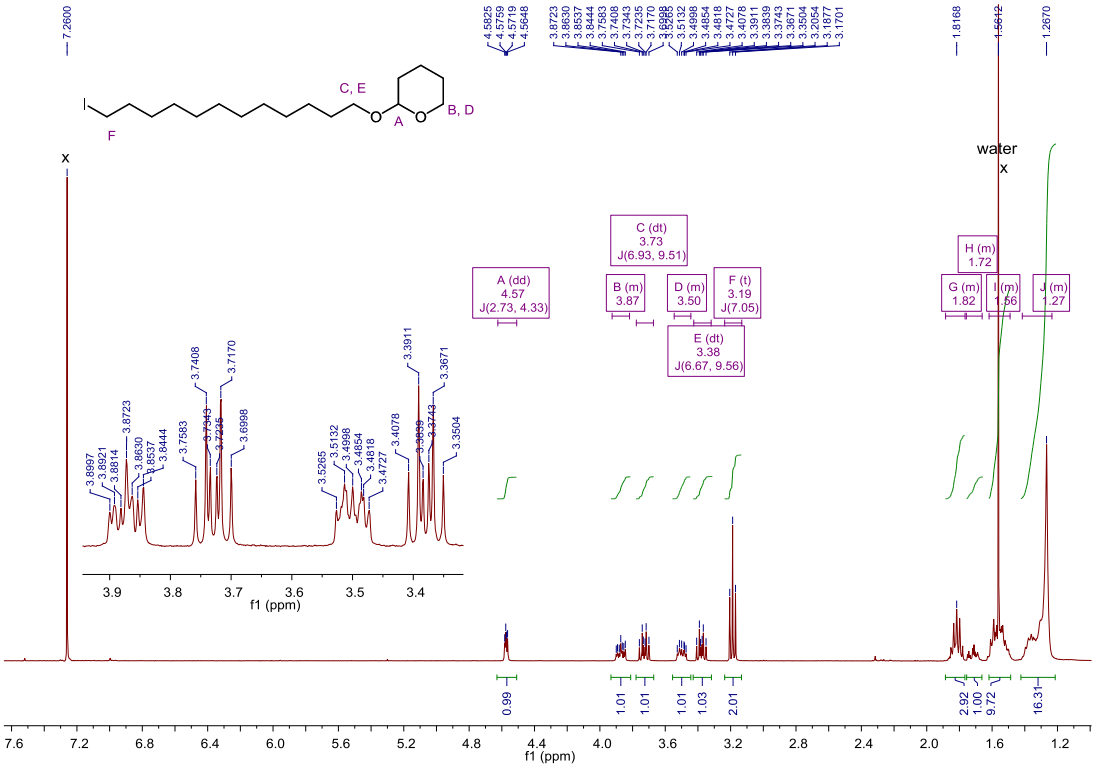


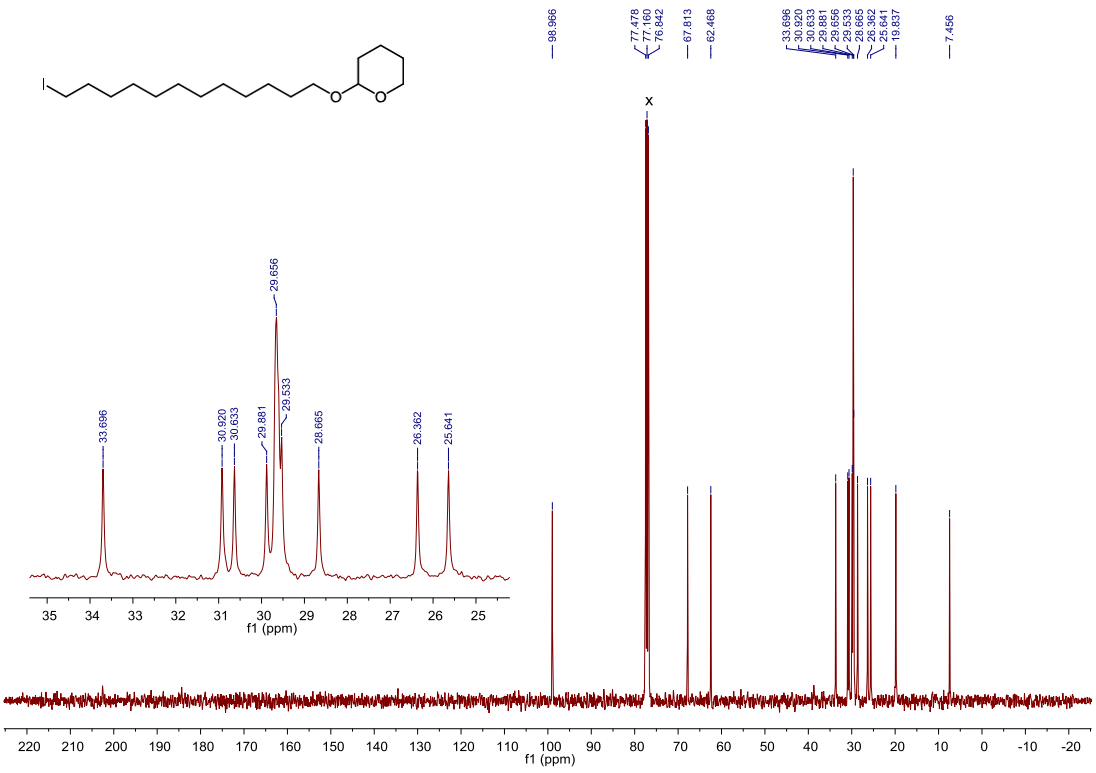


NMR Spectra of compound 10

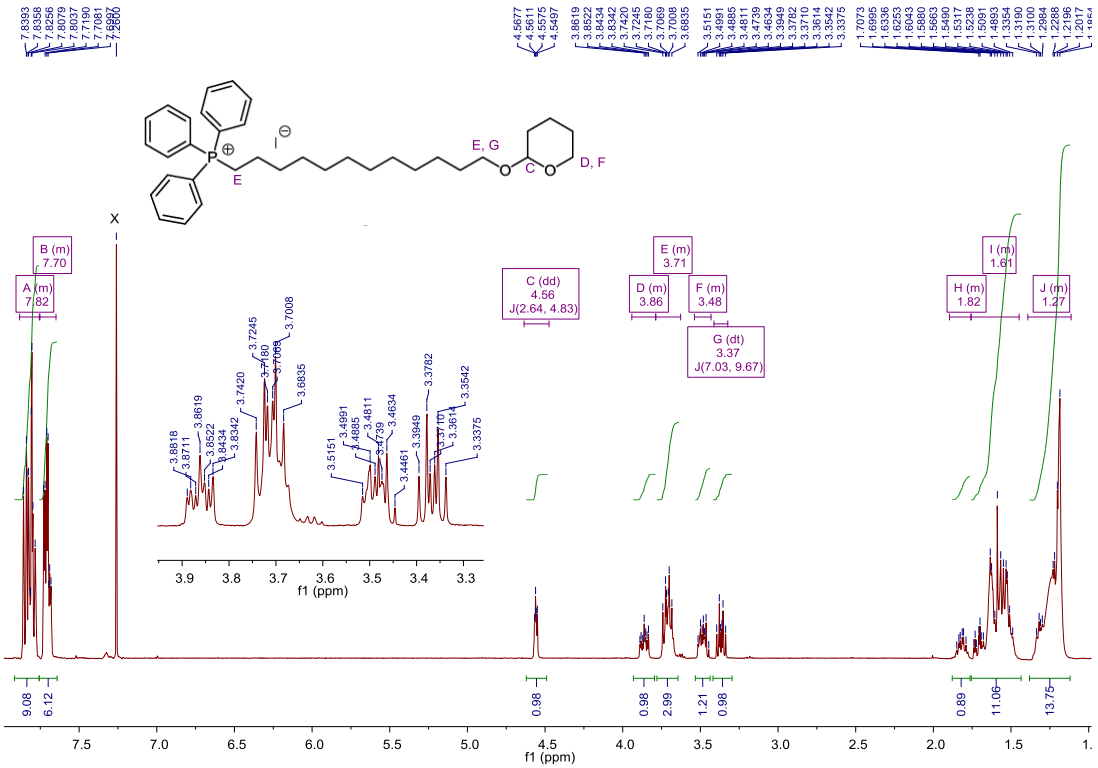


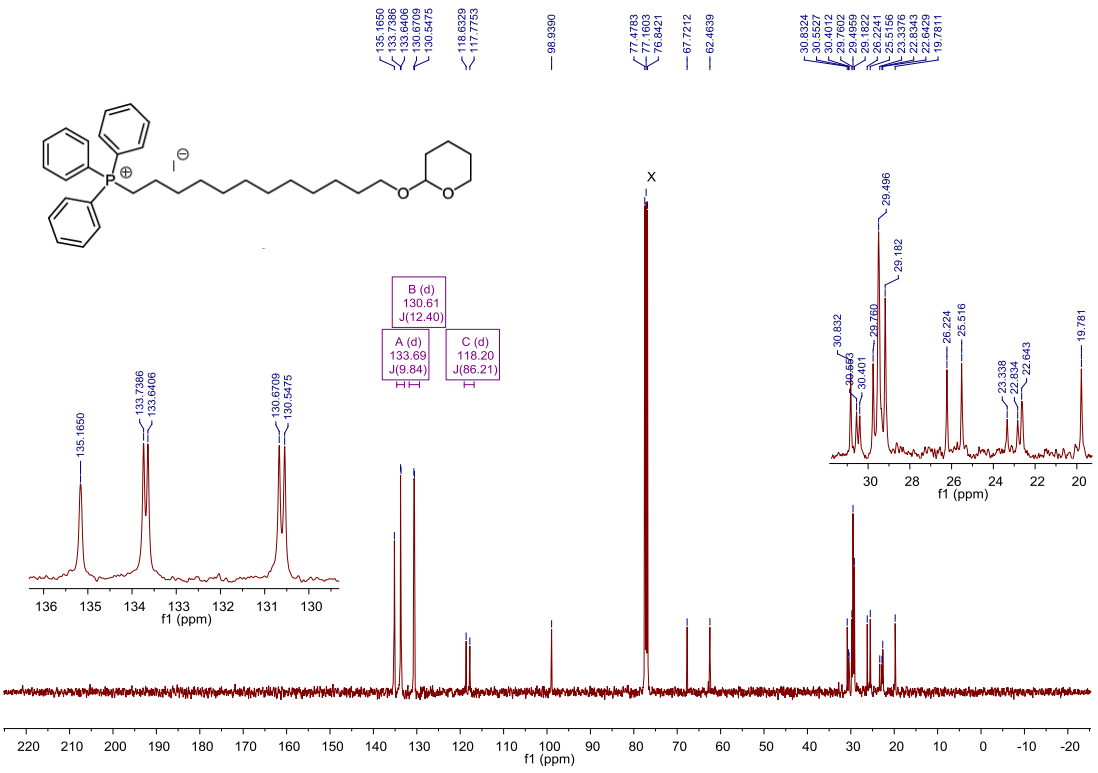
NMR Spectra of compound 11



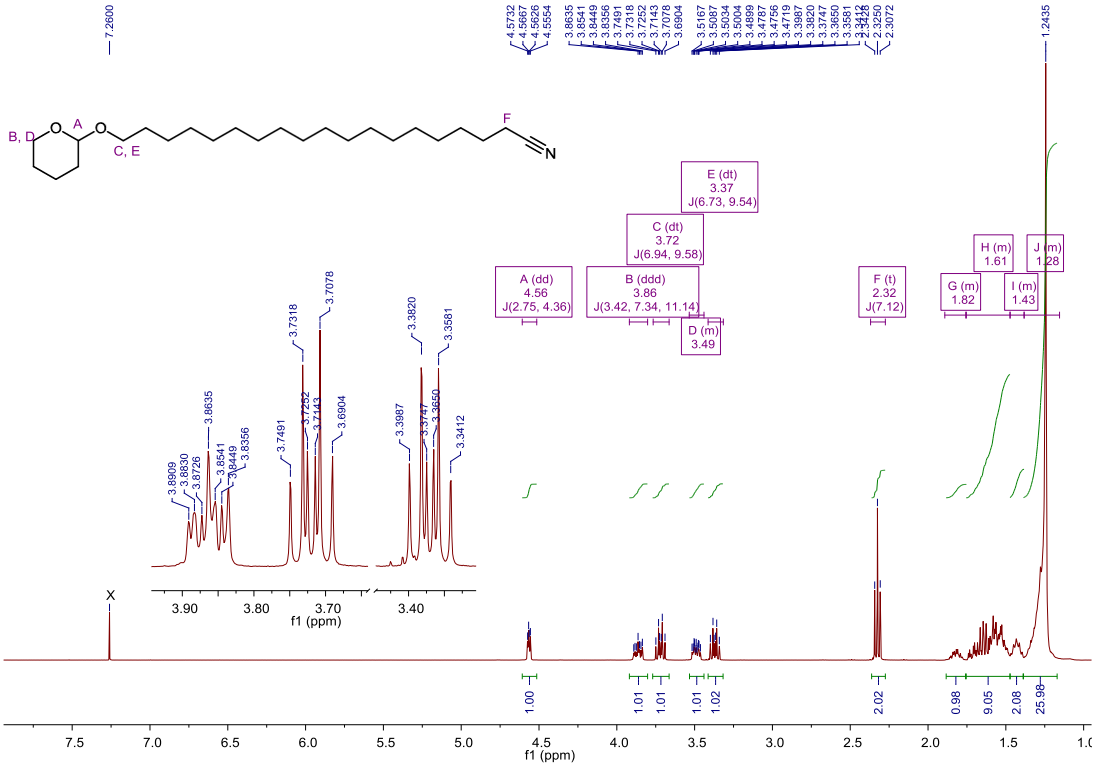


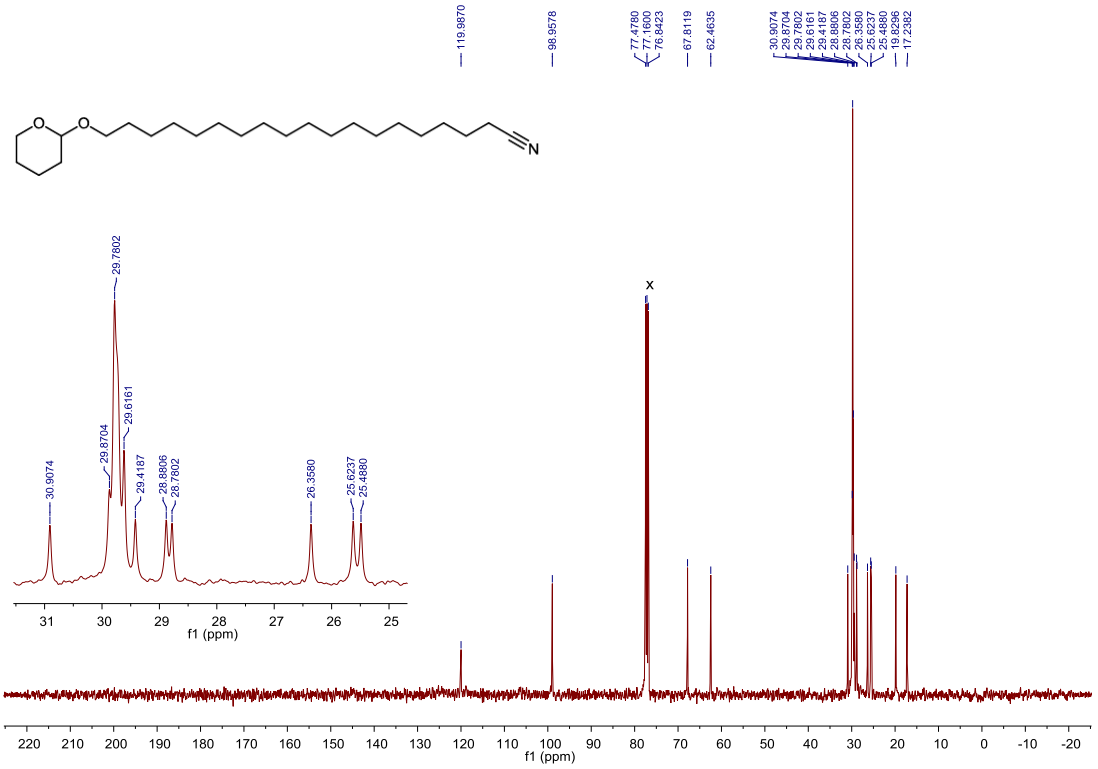
NMR Spectra of compound 15



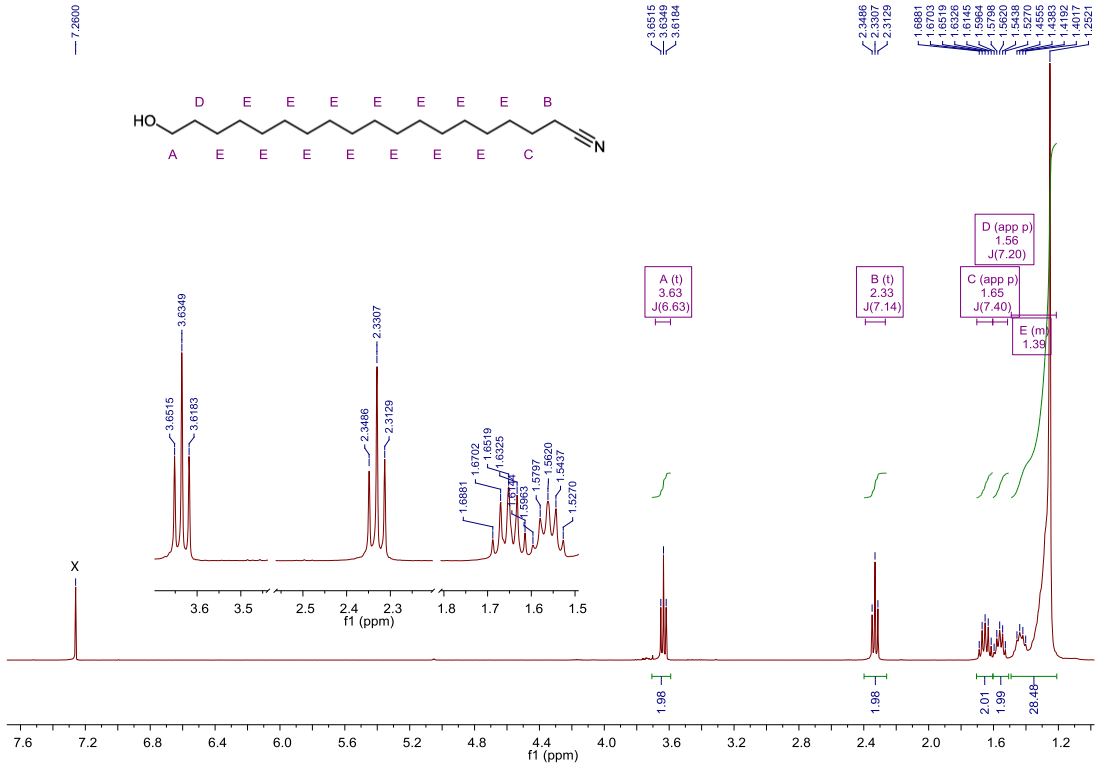


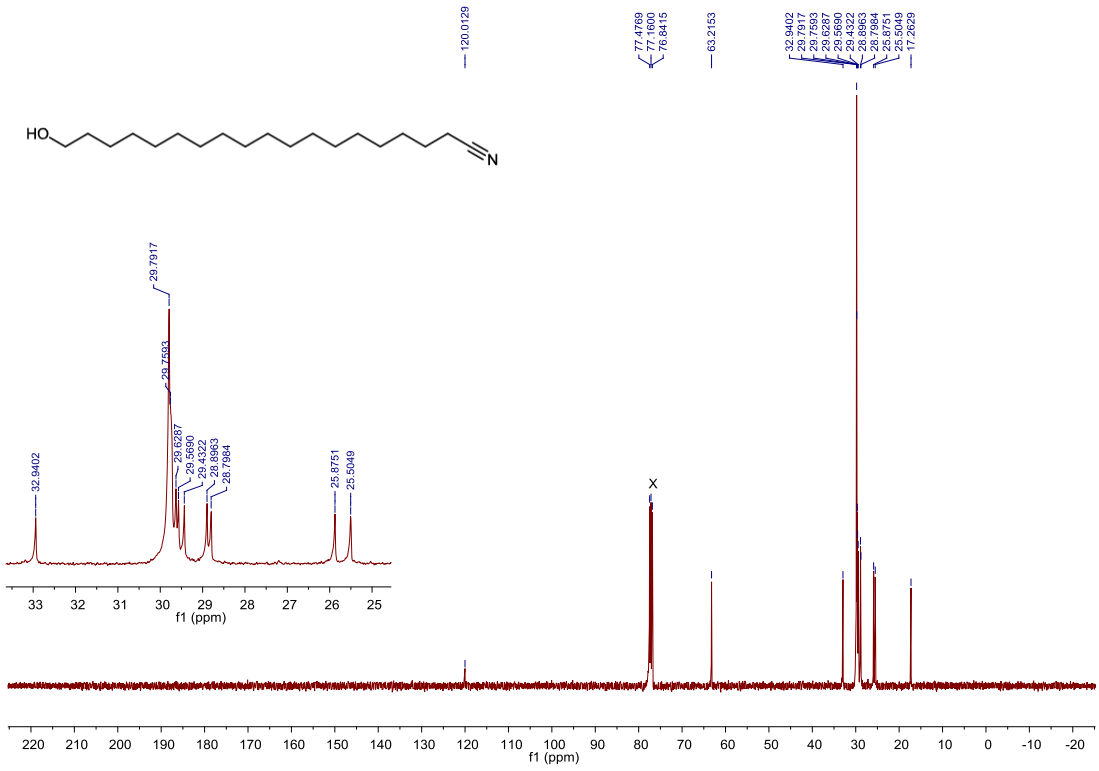
NMR Spectra of compound 16



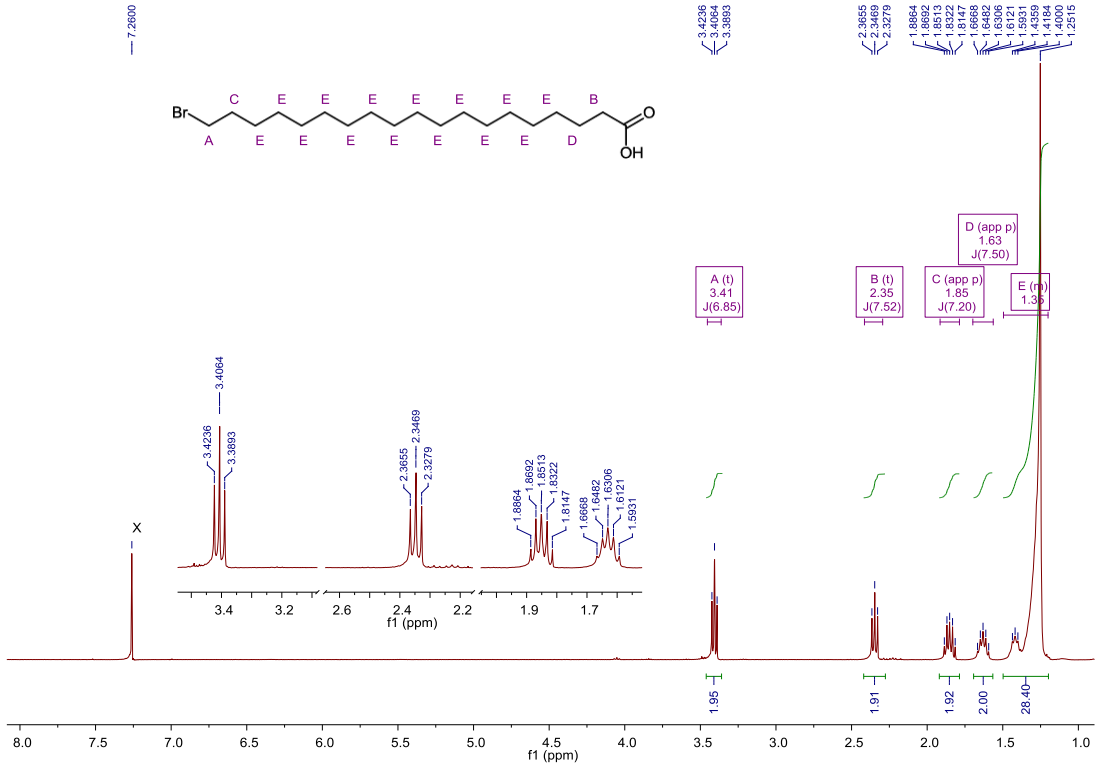


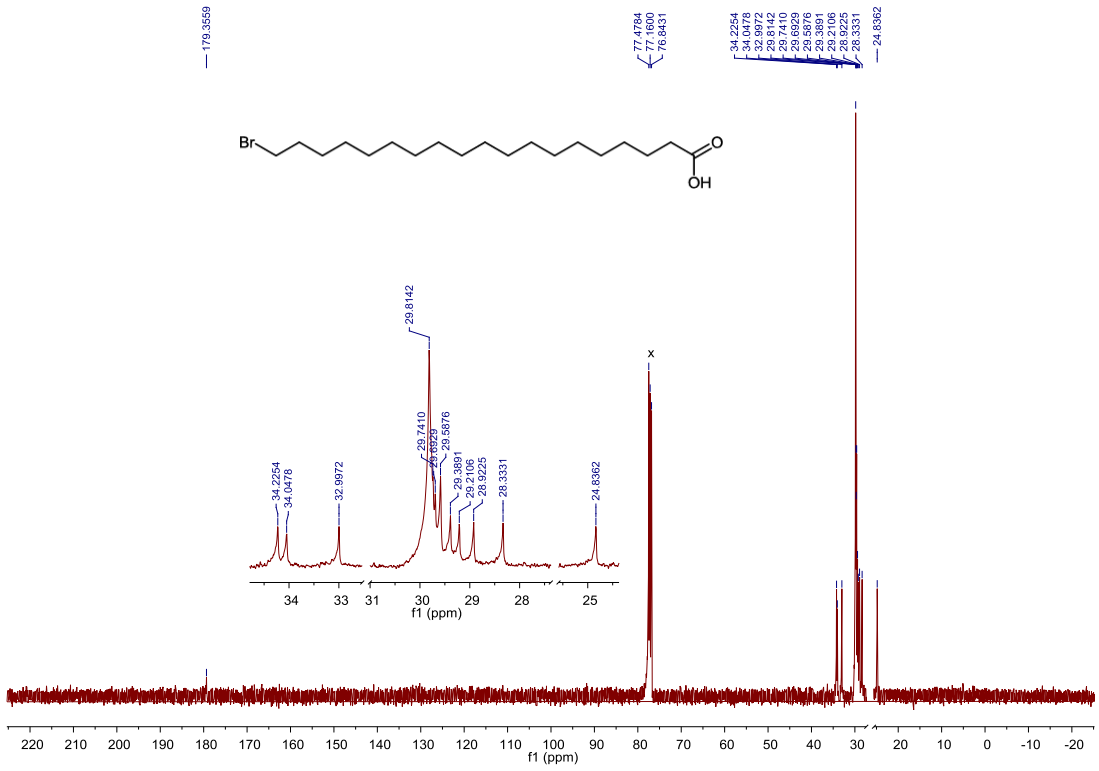
NMR Spectra of compound 18



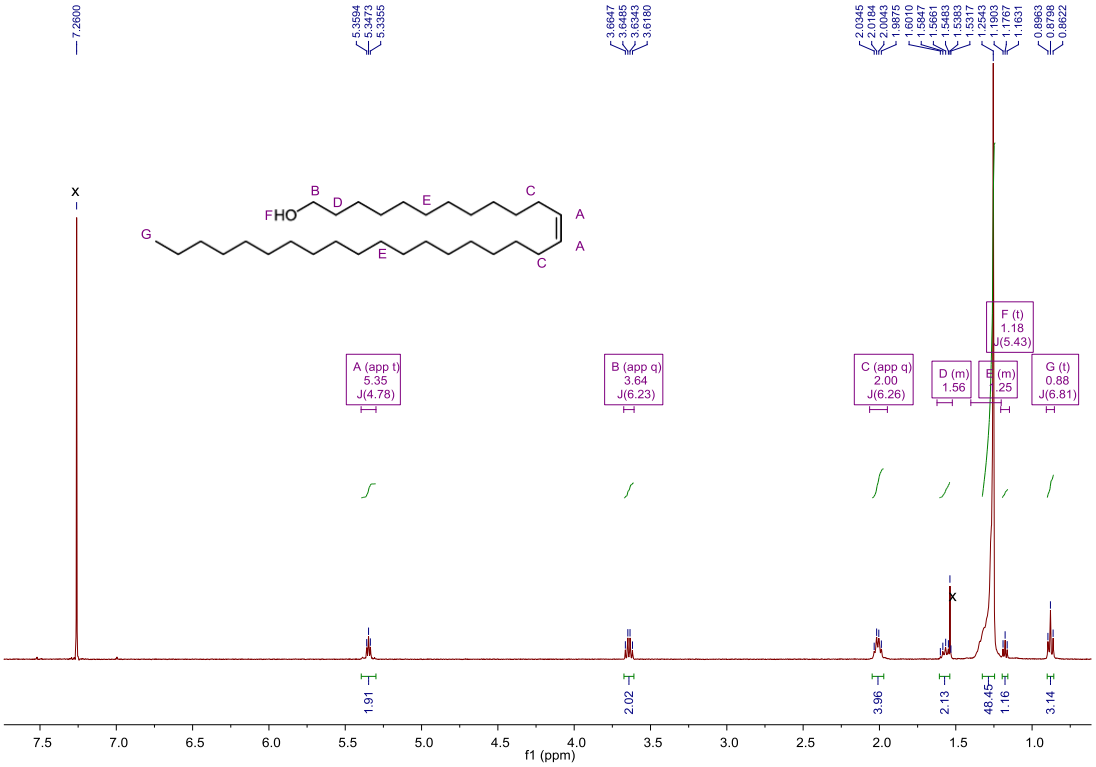


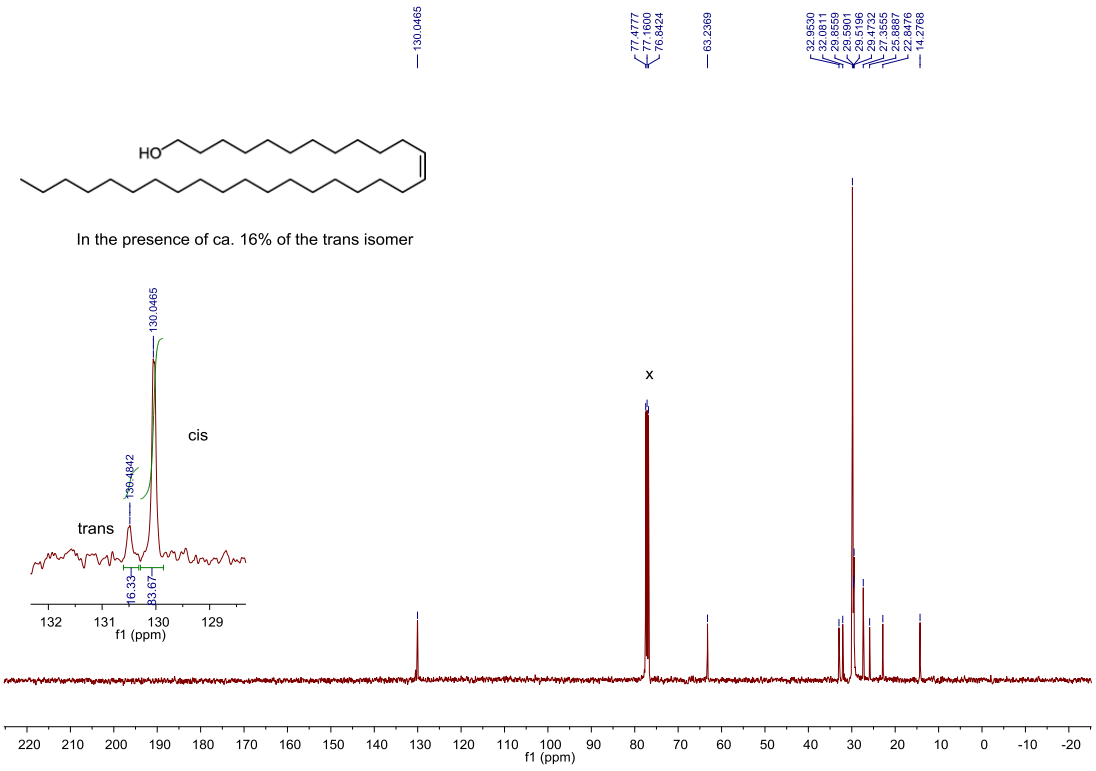
NMR Spectra of compound 19



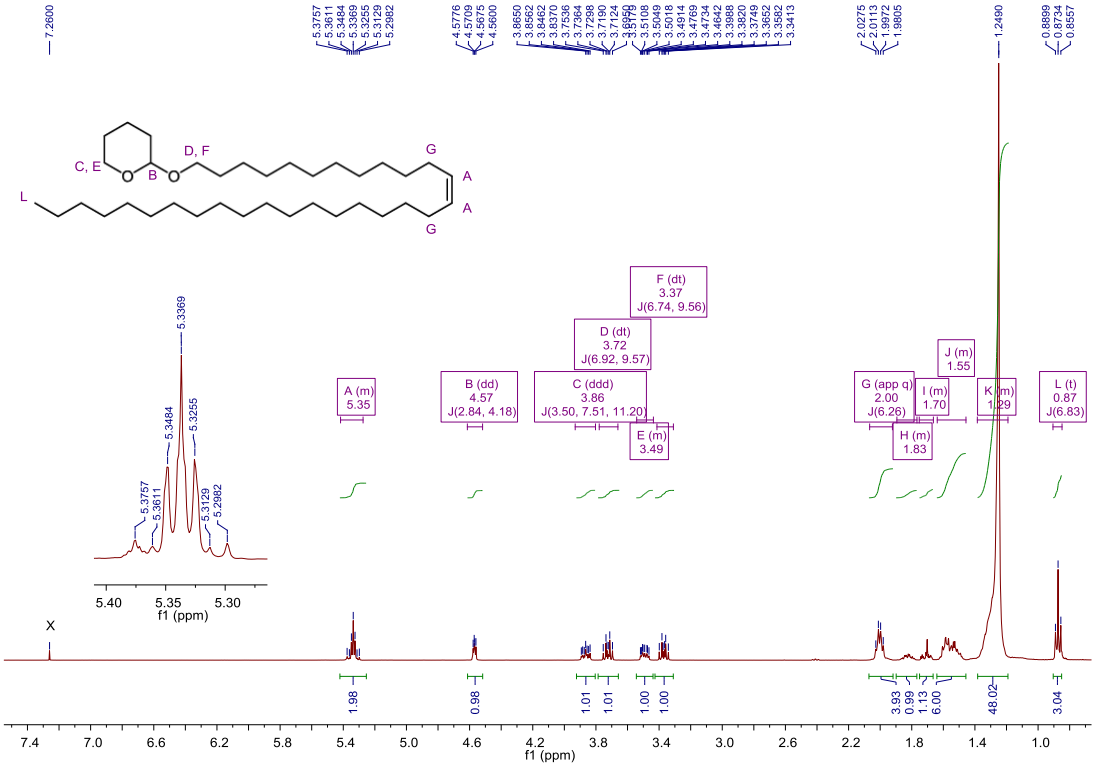


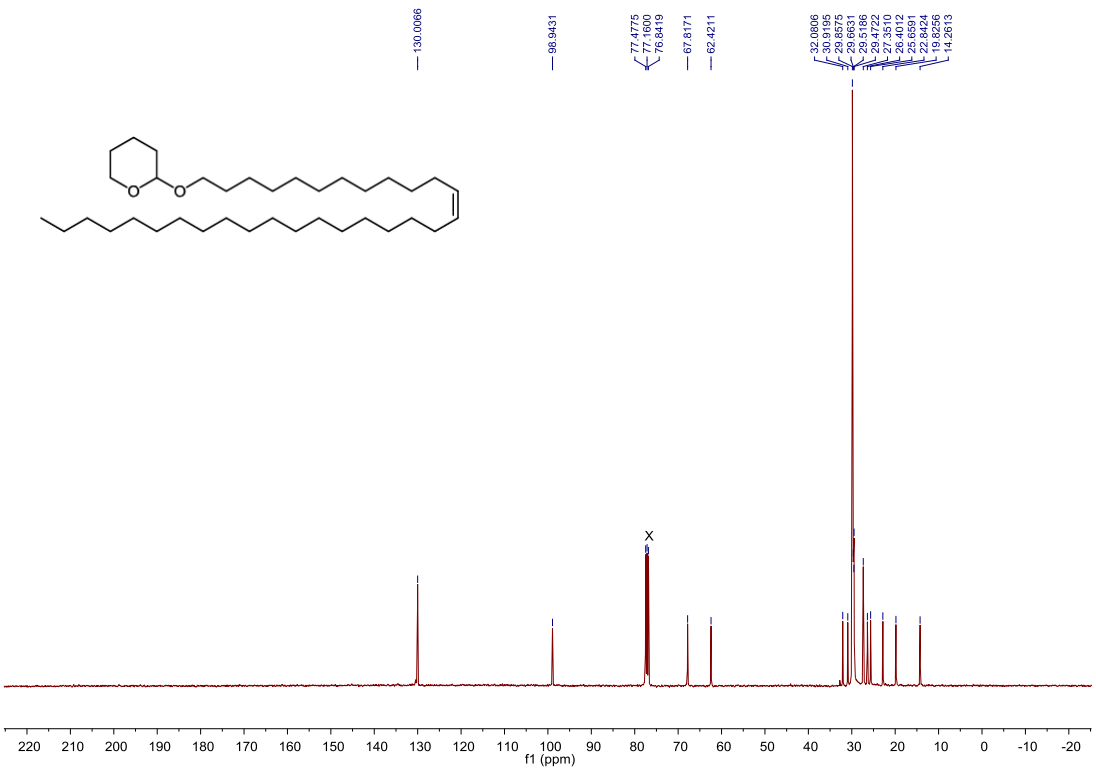
NMR Spectra of compound 21



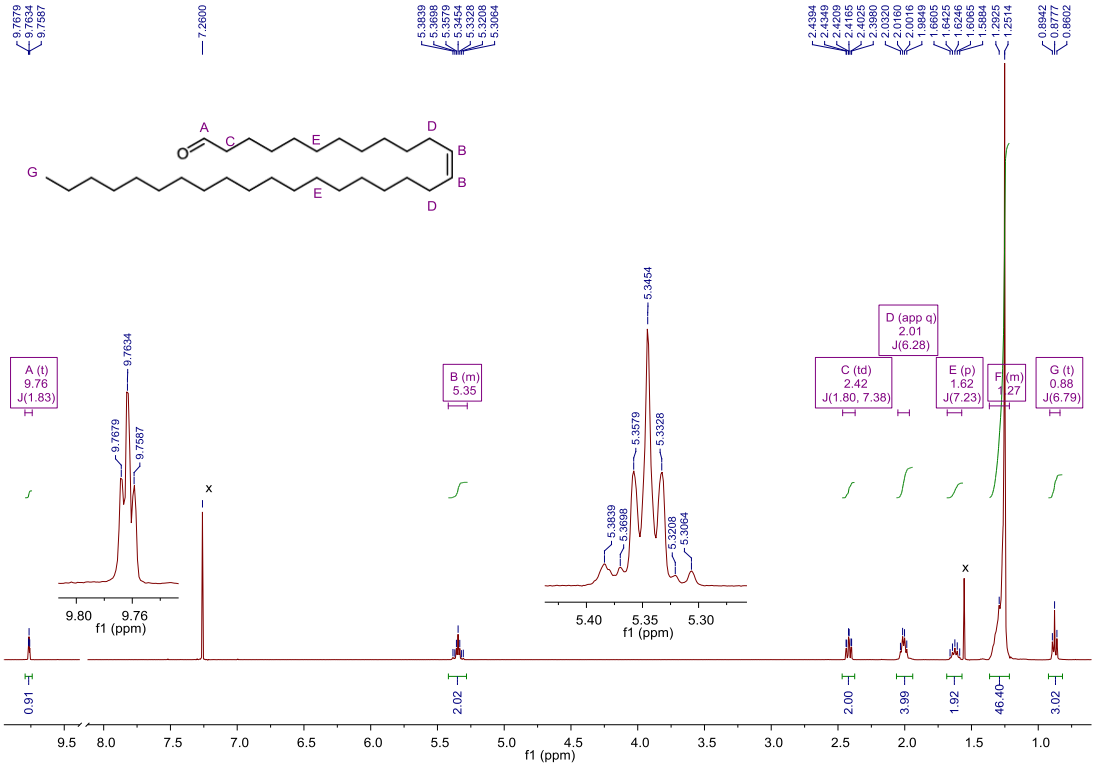


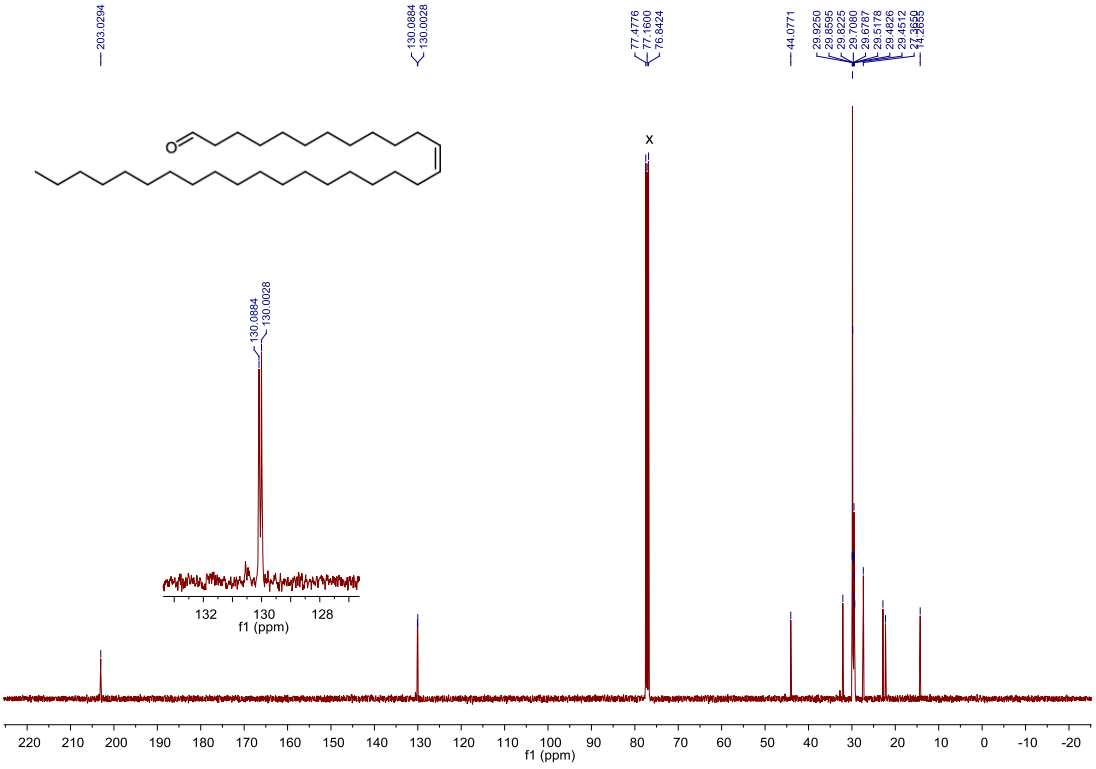
NMR Spectra of compound 22



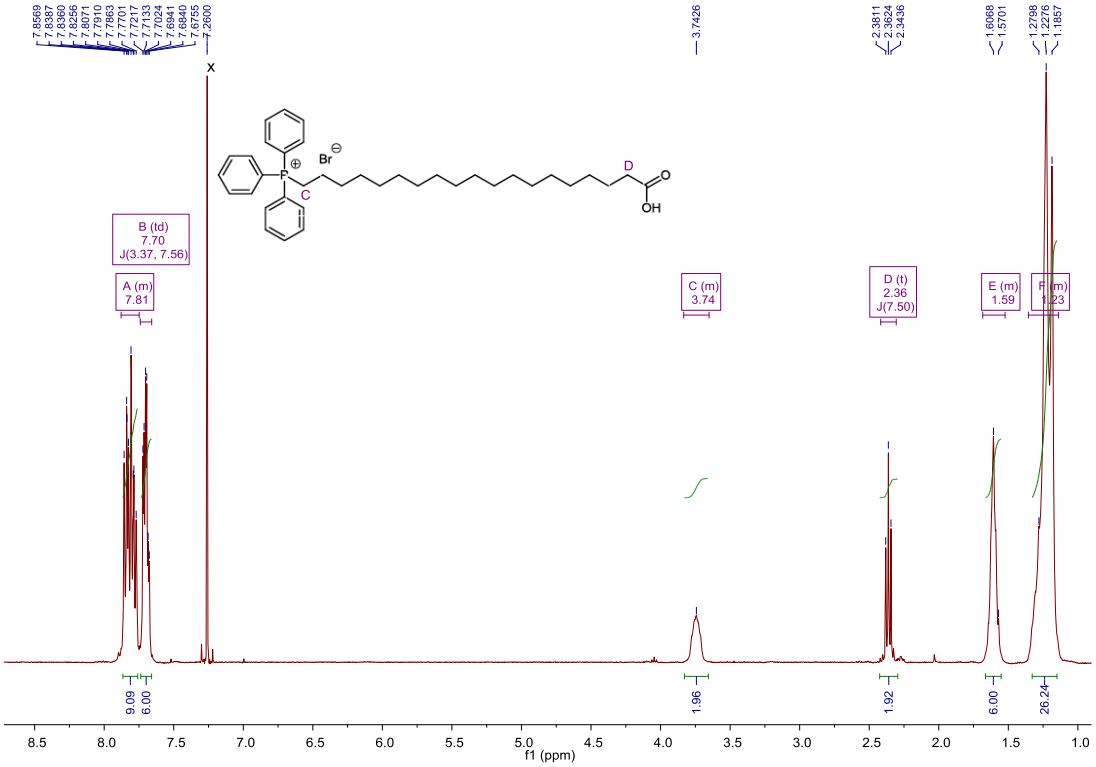


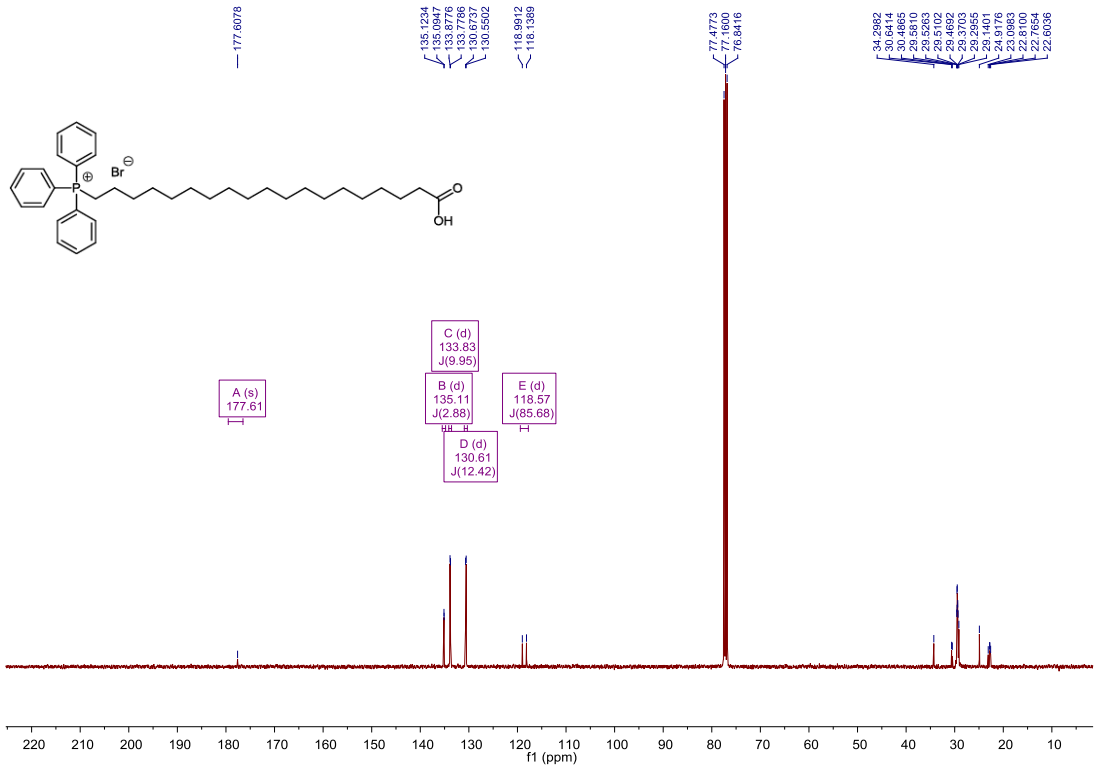
NMR Spectra of compound 23



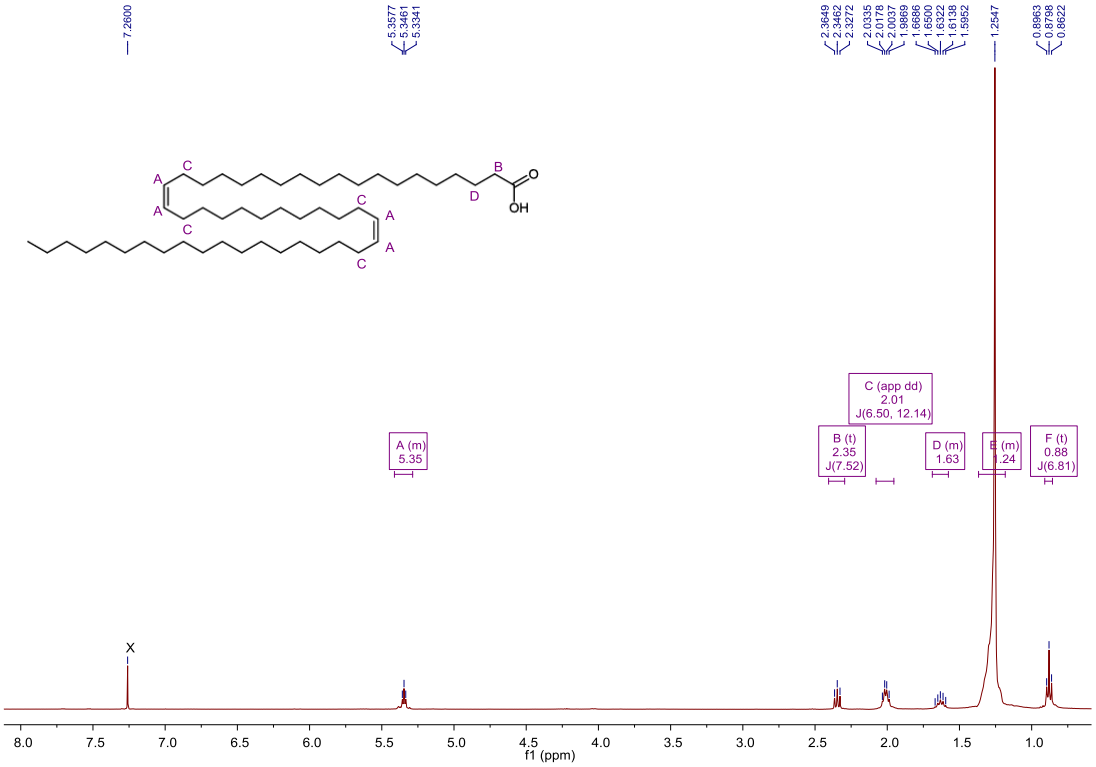


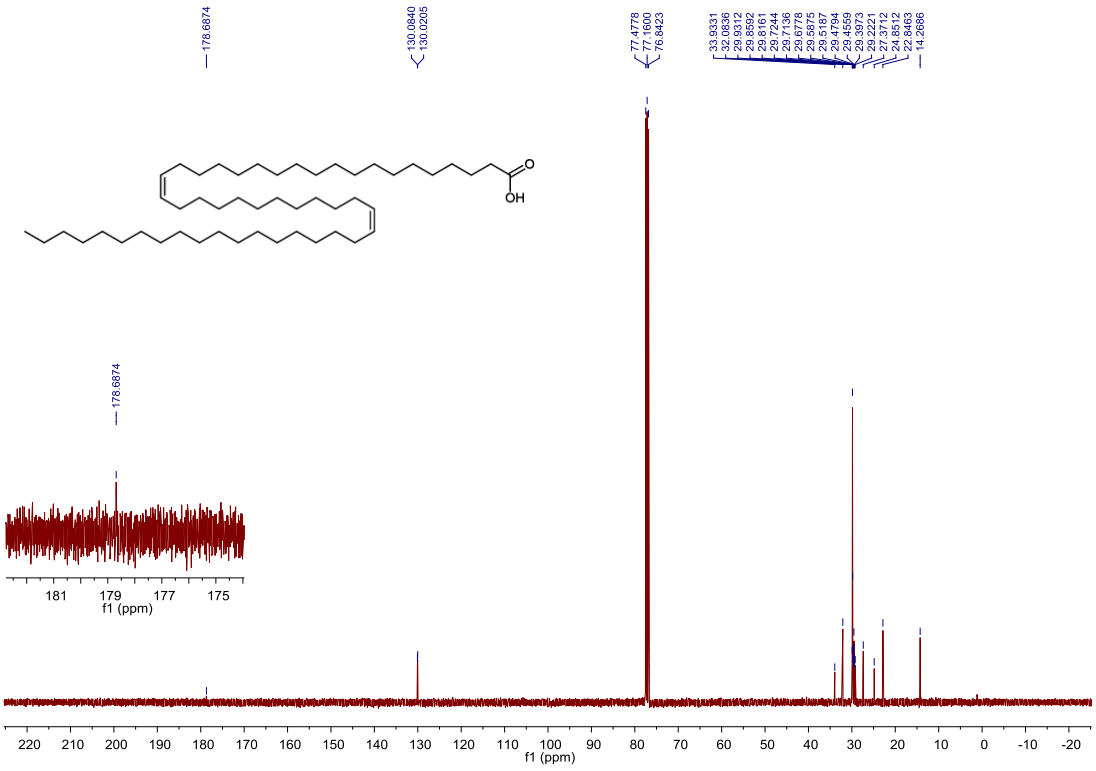
NMR Spectra of compound 20



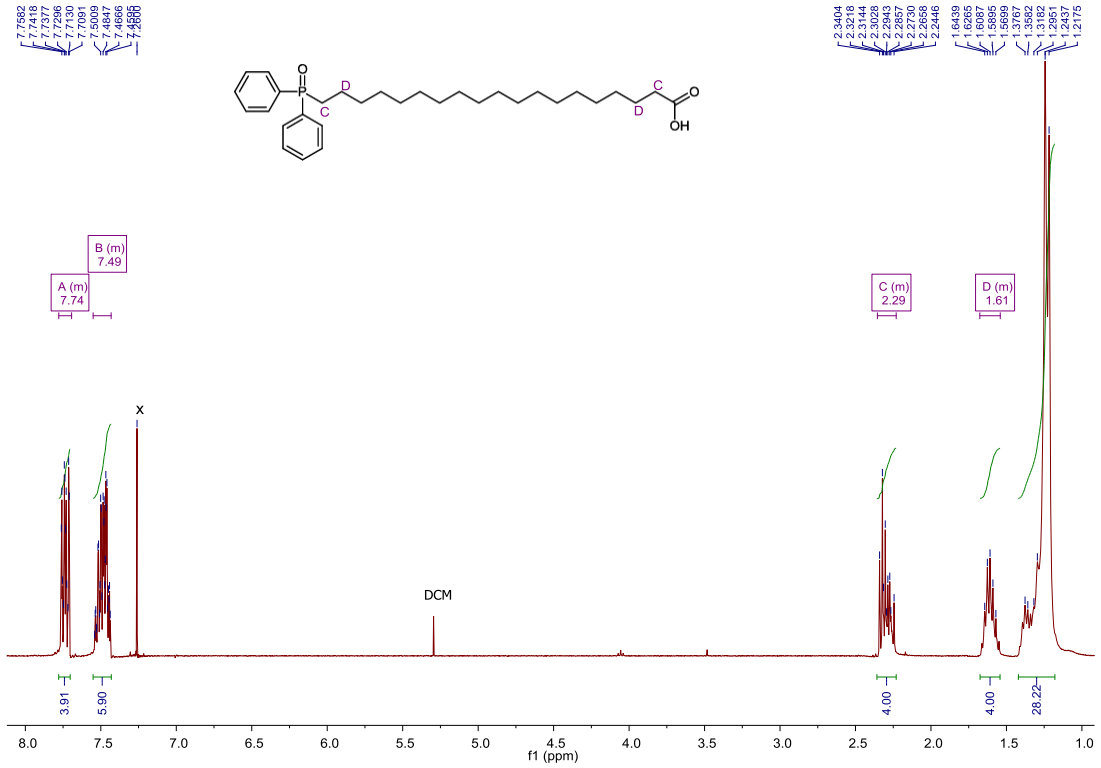


NMR Spectra of compound 1





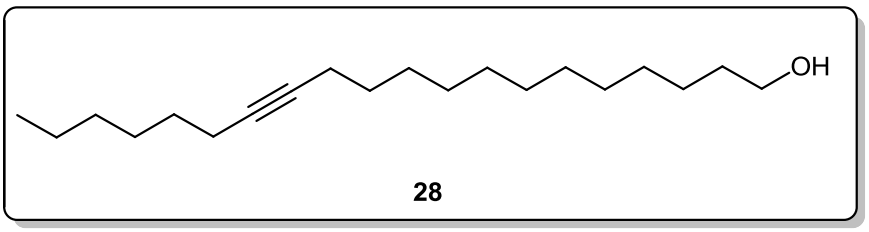
NMR Spectra of compound 24



6.1.3 Experimental session of Chapter 3

6.1.3.1 Synthetic methods

Synthesis of icos-13-yn-1-ol



28

A solution of 1-octyne (7.05 g, 63.97 mmol, 1 eq.) in anhydrous THF (40 mL) was cooled to −60 °C. To this stirred solution was added dropwise a solution of *n*-butyllithium in hexanes (2.56 M, 24ml, 61.44 mmol, 2.55 eq.) and small bubbles of gas were observed to evolve. Then the solution was allowed to warm to 0 °C over 30 minutes, stirred at this temperature for 30 minutes and at room temperature for further 30 minutes. A separate sealed tube was charged with bromide **6** (6.40 g, 24.13 mmol, 1 eq.) and anhydrous sodium iodide (325 mg, 2.35 mmol, 9 mol%) in anhydrous THF (35 mL) and heated briefly to form *in situ* the iodide derivate. This mixture of organohalides was added dropwise to the white suspension of lithium acetylide at 0 °C. The reaction mixture was refluxed for 4 days before being quenched with dropwise addition of aqueous solution of ammonium chloride at 0 °C and extracted with diethyl ether. The organic phase washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a yellow solid (7.8 g). The crude was purified by flash chromatography (25% diethyl ether in petroleum ether b.p. 40–60 °C) to afford the title compound **28** as white solid (5.60 g, 79%).

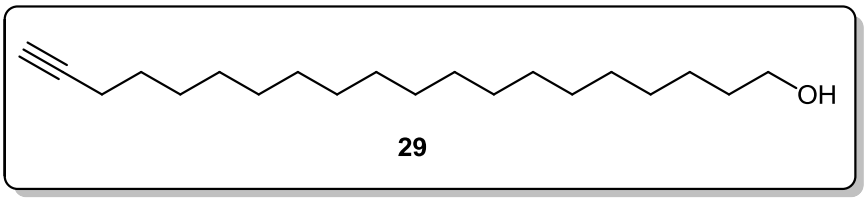
Analytical TLC: *Rf* = 0.45 (50% Et_2O in petroleum ether b.p. 60–80 °C); non-UV-active, single spot (PMA).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.63 (t, *J* = 6.7 Hz, 2H), 2.13 (t, *J* = 7.0 Hz, 4H), 1.62 – 1.51 (m, 2H), 1.52 – 1.42 (m, 4H), 1.42 – 1.21 (m, 24H), 0.88 (t, *J* = 6.9 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 80.39, 63.25, 32.96, 31.53, 29.75, 29.73, 29.70, 29.58, 29.32, 29.29, 29.01, 28.69, 25.88, 22.73, 18.91, 14.21.

FTIR (neat): 3286, 2929, 2917, 2846, 1458, 1072, 1054, 724 cm^{-1}

Synthesis of icos-19-yn-1-ol



29

Potassium chunks (98% in mineral oil, Acros Organics) are immersed in hexane, cut into 0.5 cm pieces, then washed with hexane and quickly weighed into a tared beaker of hexane. A vial was charged with potassium (1.16 g), ferric nitrate (3 mg) and 1,3-diaminopropane (20 mL, dried over molecular sieves 3 Å), capped and purged with nitrogen. The mixture was immersed in a ultrasound bath and heated to ca. 90 °C for ca. 2 hours. Occasionally, it was reduced the increasing internal pressure due to the evolution of hydrogen. The mixture turned initially to blue and subsequently to green-brown. When the molten potassium disappeared completely, the mixture was cooled to 0 °C and a solution of internal alkyne **28** (1.00 g, 3.40 mmol) in anhydrous THF (2 mL) was added. The mixture became red-brown and was left stirring at room temperature for two days. After this time the dark brown mixture was cooled to 0 °C and quenched with cold aqueous solution of ammonium chloride and extracted with diethyl ether. The organic phase was washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a yellow solid (960 mg). The crude was dissolved in ethanol (30 mL) and to this solution was added a solution of silver nitrate (1.7 g) in ethanol (120 mL), followed by a solution of NH_4OH (3.4 mL, concentrated aqueous solution) in ethanol (6.5 mL). The white precipitate was filtered and washed with further ethanol [CAUTION: considerable care had to be taken with the filtration of silver acetylides to avoid drying these solids potentially explosive when completely dry]. The resulting filter cake was

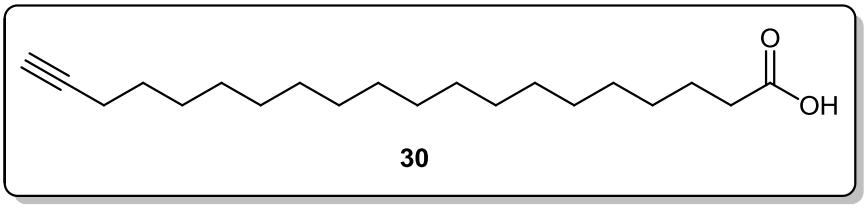
treated with 10% aqueous hydrochloric acid solution and extracted with diethyl ether. The organic phase was washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **29** (680 mg, 68%) as a white solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.63 (t, *J* = 6.7 Hz, 2H), 2.17 (td, *J* = 7.1, 2.6 Hz, 2H), 1.93 (t, *J* = 2.6 Hz, 1H), 1.60 – 1.46 (m, 4H), 1.43 – 1.22 (m, 30H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 84.98, 68.16, 63.26, 32.97, 29.82, 29.76, 29.66, 29.59, 29.27, 28.92, 28.65, 25.89, 18.55.

FTIR (neat): 3286, 2916, 2848, 1462, 1072, 719 cm^{-1}

Synthesis of icos-19-ynoic acid



30

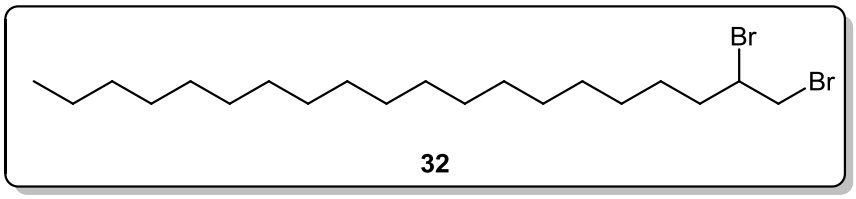
To a stirred solution of alcohol **29** (640 mg, 2.17 mmol, 1 eq.) in DMF (15 ml) was added PDC (4.10 g, 10.9 mmol, 5 eq.) and the resulting dark red slurry mixture was stirred at room temperature until TLC analysis indicated complete consumption of starting material. After *ca.* 14 hours, aqueous ammonium chloride solution was added and the mixture was extracted with diethyl ether. The organic phase was washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a white solid (620 mg). The crude was purified by flash chromatography (20% diethyl ether and 1% acetic acid in petroleum ether b.p. 40–60 °C) to afford the title compound **30** (534 mg, 80%) as a white solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 2.35 (t, *J* = 7.5 Hz, 2H), 2.18 (td, *J* = 7.1, 2.7 Hz, 2H), 1.94 (t, *J* = 2.7 Hz, 1H), 1.69 – 1.59 (m, 2H), 1.57 – 1.47 (m, 2H), 1.43 – 1.21 (m, 26H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 179.75, 84.98, 77.48, 77.16, 76.84, 68.16, 34.11, 29.81, 29.80, 29.78, 29.76, 29.74, 29.66, 29.58, 29.39, 29.26, 29.21, 28.92, 28.65, 24.83, 18.55.

FTIR (neat): 3282, 2915, 2848, 1693, 1471, 915, 717 cm^{-1}

Synthesis of 1,2-dibromoicosane



32

To a stirred solution of 1-eicosene (91.4%, 8.67 g, 30.9 mmol, 1 eq.) in amylene-free dichloromethane (45 mL) was added dropwise a solution of bromine (5.43g, 34.0 mmol, 1.1 eq.) in dichloromethane (5 mL) at 0 °C. the red solution was stirred at room temperature for 30 minutes before being quenched with thiosulfate solution. The colourless organic solution was diluted with dichlorometane, washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **32** (12.9 g) as colourless oil that solidified at 0 °C.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 4.17 (ddd, *J* = 13.2, 9.4, 3.8 Hz, 1H), 3.85 (dd, *J* = 10.2, 4.4 Hz, 1H), 3.63 (t, *J* = 10.0 Hz, 1H), 2.22 – 2.07 (m, 1H), 1.86 – 1.71 (m, 1H), 1.64 – 1.48 (m, 1H), 1.48 – 1.17 (m, 34H), 0.88 (t, *J* = 6.8 Hz, 3H).

Synthesis of icos-1-yne

33

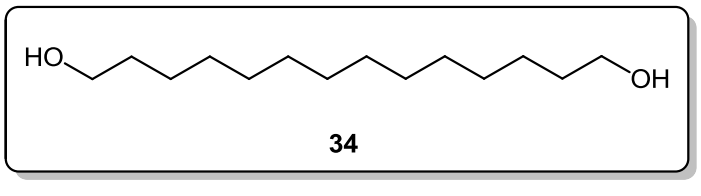
To a stirred mixture of lithium acetylide ethylenediamine complex (10 g, 108.6 mmol, 2.5 eq.) in anhydrous DMSO (40 mL) was added portionwise 1-bromooctadecane (14.5 g, 43.35 mmol, 1 eq.) at *ca.* 15 °C. The mixture was stirred at this temperature for 1.5 hours and at 30 °C overnight before being quenched with aqueous ammonium chloride solution and extracted with petroleum ether. The organic phase was washed with water, dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **33** (10.9 g, 90%) as a white solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 2.18 (td, *J* = 7.1, 2.6 Hz, 2H), 1.93 (t, *J* = 2.7 Hz, 1H), 1.57 – 1.47 (m, 2H), 1.44 – 1.21 (m, 32H), 0.88 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 84.96, 68.16, 32.09, 29.86, 29.82, 29.77, 29.67, 29.53, 29.28, 28.93, 28.67, 22.85, 18.56, 14.27.

FTIR (neat): 3287, 2915, 2847, 1472, 1462, 719 cm^{-1}

Synthesis of tetradecane-1,14-diol



34

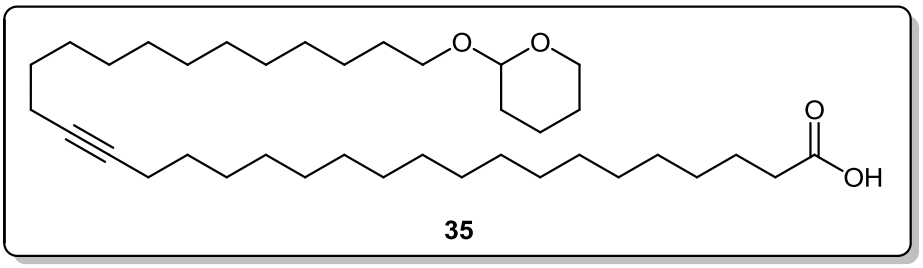
A mixture of lithium aluminium hydride (5.88 g, 154.82 mmol, 8eq.) in anhydrous THF (100 mL) was prepared at 0 °C and under nitrogen. To this stirred mixture was added dropwise a solution of tetradecanedioic acid (5.0 g, 19.36 mmol,

1 eq.) in anhydrous THF (100 mL) at 0 °C and stirred at this temperature for 30 minutes and at 40 °C for 3 hours. The resulting grey mixture was then cooled down, diluted with anhydrous diethyl ether (50 mL) and heated at *ca.* 40 °C for 1 hour. After this time the mixture was cooled to 0 °C and the reaction was quenched by the sequential dropwise addition of water (6 mL), 15% aqueous solution of sodium hydroxide (6 mL) and water (18 mL). The mixture was stirred for 1 hour and allowed to settle overnight to produce a white granular precipitate that was filtered and washed with diethyl ether and ethyl acetate. The combined organic solutions were dried ($MgSO_4$), filtered and evaporated *in vacuo* to afford the title compound **34** (4.0 g, 90%) as a white solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.64 (t, *J* = 6.6 Hz, 4H), 1.63 – 1.50 (m, 4H), 1.39 – 1.25 (m, 20H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 63.24, 32.94, 29.75, 29.73, 29.70, 29.56, 25.87.

Synthesis of 32-((tetrahydro-2H-pyran-2-yl)oxy)dotriacont-19-ynoic acid



35

Failed attempt A:

To a stirred solution of ω-acetylenic acid **30** (430 mg, 1.39 mmol, 1 eq.) in anhydrous THF (20 mL) was added dropwise a solution of *n*-butyllithium in hexanes (2.56 M, 1.2 mL, 3.07 mmol) to give a white precipitate. The resulting suspension was stirred at 0 °C for 1 hour and 45 minutes, and then at room temperature for 30 minutes. The mixture was then cooled to 0 °C before adding a solution of iodide **11** (900 mg, 2.27 mmol, 1.63 eq.) in anhydrous THF (6 mL). The reaction mixture was

refluxed for 13 hours before being quenched with dropwise addition of aqueous solution of ammonium chloride at 0 °C and extracted with diethyl ether. The organic phase was washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a pale yellow solid. The purification of the crude by flash chromatography yields starting materials in high yields but no desired product.

Failed attempt B:

A 50 mL 3-neck-flask was charged with ethylmagnesium bromide in THF under nitrogen. A solution of ω-acetylenic acid **30** (180 mg, 0.584 mmol, 1 eq.) in anhydrous THF (10 mL) was added dropwise to the ethyl Grignard reagent by 30 minutes at 16 °C. The light yellow clear solution was stirred at 35 °C for 90 minutes. The resulting clear solution was then cooled to *ca.* 18 °C before adding copper(I) bromide dimethylsulfide complex (18 mg, 0.0875 mmol, 15 mol%) under nitrogen. The resulting yellow solution was stirred for 30 minutes before adding bromide **8** in anhydrous THF (5 mL). The reaction mixture was stirred at room temperature for 1 hour and at 40 °C for two days during which time the clear solution became light beige in colour. The reaction mixture was quenched with dropwise addition of aqueous solution of ammonium chloride at 0 °C and extracted with diethyl ether. The organic phase was washed with water and brine, dried ($MgSO_4$), filtered and evaporated *in vacuo* to give a crude mixture, containing starting materials in high yields but no desired product.

6.2 EXPERIMENTAL SESSION OF CHAPTER 4

6.2.1 DNA Materials and methods

6.2.1.1 Sterilisation

Growth media and all chemical solutions were sterilised by autoclaving at 121 °C for 20 minutes unless otherwise stated. Stock solutions of antibiotics, heat-labile solutions and occasional solutions were filter-sterilised through a 0.22μm pore membrane.

6.2.1.2 Media for culturing E. coli

LB broth: tryptone 10 g, 5 g yeast extract, 10 g NaCl per 1 L distilled water; pH was checked and occasionally adjusted to 7.3 with 0.1M NaOH.

LB agar: 20g technical agar, tryptone 10 g, 5 g yeast extract, 10 g NaCl per 1 L distilled water

6.2.1.3 Preservation of strains

Glycerol stocks were used to preserve *E. coli* strains. Overnight cultures of *E. coli* strains were grown in standard media with antibiotic selection, when appropriate. The overnight culture was then diluted with sterile 80% glycerol (50% glycerol final concentration). Stocks were frozen at –20 °C for short-term or –80 °C for long-term storage. To retrieve the strains, a scraping from the stock was taken aseptically and spread onto an LB agar plates for single colonies.

6.2.1.4 Preparation of competent *E. coli* cells using $CaCl_2$

E. coli strain was spread onto LB agar plate and incubated at 37 °C overnight. A single colony was inoculated into LB broth (5 mL) incubated overnight at 37 °C with shaking at 250 rpm. The overnight culture was used to inoculate an appropriate volume of LB broth (generally *ca.* 400 mL in 2-liter flask for large-scale preparations) using a dilution factor of 1:100. The flask was incubated at 37 °C with shaking at 250 rpm, until exponential growth was reached (*ca.* 2-3 hours) indicated by an optical density at 600nm (OD_{600}) between 0.4 and 0.6. Cells were harvested by centrifugation at 3000 g, 4 °C for 10 minutes and the supernatant discarded. The pellet was gently resuspended in *ca.* 50 mL ice-cold 100 mM $CaCl_2$ containing 10% glycerol and incubated on ice for 1 hour. The cells were pelleted at 3000 g for 10 minutes at 4 °C, the supernatant discarded and the pellet resuspended gently in *ca.* 5 mL ice-cold 100 mM $CaCl_2$ containing 10% glycerol and left at 0 °C for 10 minutes. The cells were divided into 100µl aliquots, quick frozen in liquid nitrogen and stored at –80 °C. Aliquots were subsequently thawed on ice when required for transformation.

6.2.1.5 Transformation of *E. coli* strains by heat shock

Heat-shock transformation consists in rapid variations of temperature (0 °C → 42 °C → 0 °C), which allow to the plasmid to cross outer membrane (by releasing lipids and proteins, and creating pores on cell surface) and inner membrane (lowering the membrane potential) of the competent cell.[333]

Plasmid DNA (2 µL; *ca.* 100 ng) was added and mixed gently with an aliquot (200 µL) of competent cells. The suspension was incubated on ice for 20 minutes before incubation at 42 °C for 1 minute, followed by incubation at 0 °C for 2 minutes. LB broth (1ml) was added and incubated statically at 37 °C for 60–90 minutes to express the antibiotic resistance. The resulting culture (100 µL) was spread onto LB agar plates containing the appropriate antibiotic. The plates were incubated at 37 °C overnight and stored at 4 °C.

6.2.1.6 Purification of plasmid DNA

An overnight culture of *E. coli* DH5α cells containing the pET28b::*acpM* plasmid was used to prepare purified plasmid by Wizard® Plus Minipreps DNA Purification System (Promega). This procedure is used to extract plasmid DNA from bacterial cells and is based on the alkaline lysis procedure, which denatures nucleic acids and proteins, and on the neutralization by potassium acetate, which renatures correctly only plasmids.[334]

6.2.1.7 Expression of recombinant protein

An overnight culture of *E. coli* Tuner cells carrying the pET28b::*acpM* plasmid was inoculated into LB broth (*ca.* 400 mL in 2-liter flask and using a dilution factor of 1:100) containing 25 μg/mL kanamycin and incubated at 37 °C with shaking at 250 rpm until exponential growth was reached indicated by an optical density at 600nm (OD_{600}) between 0.5 and 0.7 (*ca.* 3–4 hours). Then the expression of AcpM was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (final concentration = 1 mM) and the cultures were incubated at 16 °C for 16–20 hours.

The cultures (OD_{600} = 1.0–1.5) were then harvested by centrifugation at 3000 g for 20 minutes at 4 °C and the supernatant discarded. The cell pellets were resuspended in tris buffer pH 7.5 in the presence of complete mini protease inhibitor tablets (Roche) and stored at –20 °C.

6.2.1.8 Lysis of *E. coli* cell pellets

A French pressure cell press was used to lyse *E. coli* cell suspensions. The cell suspension was processed 3 times through a mini-cell French press. The lysate were separated into insoluble and soluble fractions by centrifugation at 4000 g, 4 °C for 10 minutes.

6.2.2 Protein materials and methods

6.2.2.1 Purification by FPLC

All the FPLC purifications were performed on ÄKTA™ FPLC system using prepacked Hi-Trap Ni+ affinity columns (GE Healthcare). All solutions were filtered through 0.45 µm membrane before use.

6.2.2.1.1 Ni^{2+}-affinity chromatography

Ni^{2+}-affinity chromatography was performed using HisTrap FF (1 mL)

Binding buffer: 20 mM KH_2/ K_2HPO_4 pH 7.4, 500mM NaCl, 20 mM imidazole.
Elution buffer: 20 mM KH_2/ K_2HPO_4 pH 7.4, 500mM NaCl, 500 mM imidazole.

6.2.2.1.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography using Octyl Sepharose 4 Fast Flow (1 mL):

Matrix: highly cross-linked 4% agarose
Type of ligand Octyl: **R-O-CH2-CH(OH)-CH_2-O-$(CH_2)_7$ -CH_3**
Bead form: rigid, spherical, macro porous,
Mean particle size 90 µm (diameter)
Particle size range: 45–165 µm
Degree of substitution: approx. 5 µmol octyl/mL

Binding buffer: 1.5 M $(NH_4)_2SO_4$, 50 mM KH_2PO_4/ K_2HPO_4 pH 7.4
Elution buffer: 50 mM KH_2PO_4/ K_2HPO_4 pH 7.4
Washing buffer: 20% isopropanol
Storage buffer: 20% ethanol

6.2.2.2 SDS-PAGE

SDS-PAGE analyses were performed in XCell *SureLock*™ Mini-Cell System (Invitrogen) using pre-cast gels (Invitrogen) under reducing and non-reducing conditions. Samples were mixed with sample loading buffers and heated at 80 °C for 5 minutes immediately before the analysis.

Successively to the analysis the gels were soaked in Coomassie Blue stain for *ca.* 15 hours at room temperature with gentle shaking. Then the gels were soaked in de-staining solution 3–4 times during a 6–8 hour period.[410]

Sample loading buffer (Laemmli loading dye) 3X stock:
1M Tris-Cl pH 6.8 (2.4 ml), 20% SDS (3 ml), glycerol (3 ml), β-mercaptoethanol (1.6 ml), bromophenol blue (6 mg).

Non-reducing sample loading buffer 3X stock:
1M Tris-Cl pH 6.8 (2.4 ml), 20% SDS (3 ml), glycerol (3 ml), ethanol (1.6 ml), bromophenol blue (6 mg).

Coomassie Blue stain: dH_2O, methanol and glacial acetic acid mixed to a ratio of 4:5:1, respectively. Coomassie blue (1 g/L) was added and solubilised overnight in a dark container, stirring.

De-staining solution: As above without coomassie blue.

Protein Markers: Broad Range (2-212 KDa) Marker (New England Biolabs) and Precision Plus Protein Standards (10-250 KDa) (Biorad).

NuPAGE® Novex® 4–12% Bis-Tris Gel 1.0 mm, 10 well (Invitrogen)

Electrophoresis analysis was carried out on *ca.* 20 μL samples at 180 V in NuPAGE MES SDS running buffer (50 mM Tris base, 50 mM MES, 0.1% w/v SDS, 1 mM EDTA, pH 7.3) until the dye front reached the end of the gel (45 – 60 minutes).

Novex® 18% Tris-Glycine Gel 1.5 mm, 10 well (Invitrogen)

Electrophoresis analysis was carried out on *ca.* 35 μL samples at 125 V in Novex® Tris-Glycine Native Running Buffer (Invitrogen) until the bromophenol blue dye was just off (1.75 – 2 hours).

6.2.2.3 Dialysis and buffer exchange

Protein samples were dialysed using Microcon® centrifugal filter units (Millipore) with nominal molecular weight cut-off of 3 KDa. In a general procedure, a 0.5 mL protein sample was pipetted into the Microcon® reservoir and spinned at 14000 g for *ca.* 20 minutes at *ca.* 4 °C. Then new aqueous buffer was pipetted into the sample reservoir (*ca.* 300 μL) and spinned at 14000 g for *ca.* 20 minutes at 4 °C. The previous process was repeated at least 5 times, avoiding generally to reduce the solvent to less than 50 μL. The sample reservoir was finally placed upside down in a new vial and spinned at 1000 g 3 minutes. Desalting or buffer exchange processes involving concentrated samples of *holo*-AcpM were generally carried out in the presence of 10–20 mM DTT in order to avoid to form disulfide dimers.

6.2.2.3 ESI analysis

ESI analysis were performed on LTQ/Orbitrap mass spectrometer equipped with a high resolution FT mass analyser (R = 100,000) externally calibrated at 3 ppm.

Positive-mode ionisation was accomplished at a capillary temperature of 275 °C, a capillary voltage of 25 V and a source voltage of 4.5 kV. MS data were processed using Xcalibur 2.1 and then deconvoluted using Promass 2.8.

The samples were dialysed and diluted with methanol/formic acid (final concentration: 1% HCOOH in MeOH/H_2O 1:1 v/v) and were injected at a rare of 5 μL/min for 10 minute run. Protein samples in acetonitrile/water containing acetic acid yielded lower sensitivity levels.[411]

6.3 EXPERIMENTAL SESSION OF CHAPTER 5

6.3.1 Synthetic and semi-synthetic methods in NaSBI-based reactions

The methods used for reactions and analyses mentioned in this Chapter are related to those described in the previous sections.

6.3. 1.1 Synthesis of BaSBI

A 20mL MW vial was charged with benzimidazole (3.06 g, 25.9 mmol, 1 eq.) followed by a dropwise addition of fuming sulphuric acid containing 30% free SO_3 (10 mL, 2.8 eq. of free SO_3). The solution was heated at 160 °C for 3 hours under MW irradiation. After this time, the solution was cooled down and diluted with dropwise addition of cold water (80 mL) barium carbonate was added portionwise (*ca.* 50 g) until the solution was neutral. The mixture was filtered to remove inorganic salts and the filtrate consisted of NaSBI in the presence of *ca.* 7% of barium 4,5-DiSBI. The impurity was removed adding to the aqueous solution (70 mL) acetone (140 mL) and leaving the resulting mixture at 0 °C. The precipitate was filtered to give a clear solution that was evaporated to afford the desired barium salt in pure form as white solid (2.54 g, 36%).

^{1}H NMR analyses were found concentration dependent and related spectra are shown in Chapter 5.

FTIR (neat): 1157, 1086, 1031, 724 cm^{-1}

6.3. 1.2 Synthesis of NaSBI

A 50 mL Falkon tube was charged with BaSBI (2.49 g, 4.68 mmol) and dissolved in water (30 mL). To this solution, aqueous solution of sodium sulfate decahydrate (1.51 g in 20 mL) was added dropwise to converted the barium salt to

sodium analogue. The suspension was left to settle for some hours before centrifuging at 2000 g for 2 minutes. The clear solution was evaporated to afford NaSBI as white hygroscopic solid.

^{1}H NMR analyses were found concentration-dependent and equivalent to those reported for the related barium salt (Chapter 5).

^{13}C NMR (101 MHz, D_2O; Internal Standard: 1,4-dioxane) δ 144.33, 137.37, 120.33, 115.34, 113.65.

The salt sample (*ca.* 50 mg) was recrystallised from distillated water (1 mL) by slow evaporation (1 week) at room temperature.

X-ray analysis: A single crystal was isolated and used for single crystal X-ray diffraction. Crystal structures were measured at 123 K with graphite monochromated $Mo_{k\alpha}$ radiation (λ = 0.71073 Å) using a Bruker Appex II CCD instrument. All non hydrogen atoms were refined anisotropically. All structures were refined to converge against F^2 using the SHELXL-97 program. The unit cell of (name of the salt) was found to be monoclinic with space group $P2_1/c$. The unit cell dimensions were as follow: a= 13.045(7), b= 14.370(7), c = 5.936(3); α=90, β =90.11(2), γ=90. The R-factor (over observed data) being 8.58%. From the single crystal structure obtained, the desired salt can be clearly identified. Showing that it adopts a trihydrate form (Chapter 5).

CHN analysis: as reported in the Chapter 5.

6.3. 1.3 Synthesis of nonanoyl–NaSBI

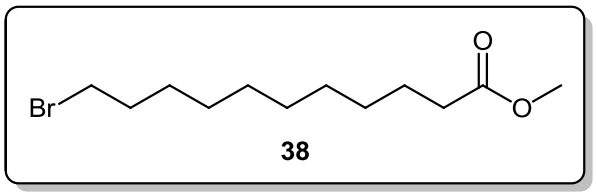
To a solution of NaSBI (400 mg, 1.82, 2 eq.) in anhydrous DMF (2.5 mL) was added Nonanoyl chloride (140 μL, 134 mg, 0.757 mmol, 1 eq.) and the mixture was stirred at 40 °C for 4 hours. Then the reaction mixture was washed with petroleum ether to remove the hydrolysed acid (*ca*. 63 mg).The crude reaction mixture was purified by flash chromatography using C–18 column. The product was elueted with 30% ethanol in water to give to isomers with the following ^{1}H NMR spectra:

^{1}H NMR (400 MHz, D2O) δ 8.84 (t, J = 2.6 Hz, 1H), 8.60 (s, 1H), 7.87 – 7.79 (m, 2H), 3.12 (t, J = 7.2 Hz, 2H), 1.77 (dd, J = 14.7, 7.3 Hz, 2H), 1.46 – 1.36 (m, 2H), 1.35 – 1.19 (m, 8H), 0.81 (t, J = 6.8 Hz, 3H).

1H NMR (400 MHz, D2O) δ 8.87 (s, 1H), 8.32 (d, J = 8.7 Hz, 1H), 8.16 (s, 1H), 7.89 (dd, J = 8.6, 1.4 Hz, 1H), 3.15 (t, J = 7.3 Hz, 2H), 1.81 (p, J = 7.3 Hz, 2H), 1.47 – 1.38 (m, 2H), 1.36 – 1.20 (m, 8H), 0.83 (t, J = 6.8 Hz, 3H).

6.3. 1.3 Synthesis of cis-triacont-11-enoyl–NaSBI

Synthesis of methyl 11-bromoundecanoate



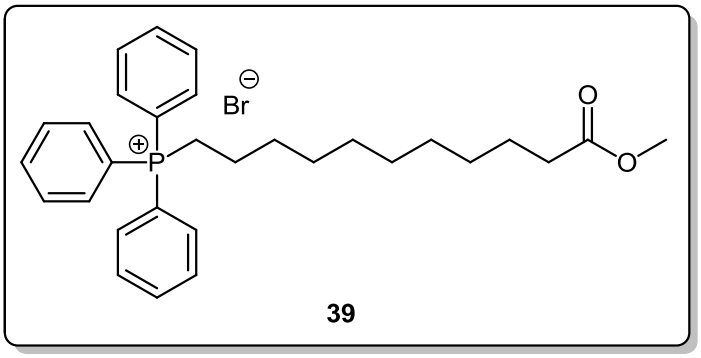
38

To a solution of 11-bromoundecanoate (4.00 g, 15.08 mmol, 1 eq.) in methanol (64 mL) and diethyl ether (6 mL) was added sulphuric acid (400 mg) and the solution was stirred at room temperature until TLC analysis (20% diethyl ether in petroleum ether) indicated complete consumption of starting material (4 hours). The reaction mixture was diluted with diethyl ether–petroleum ether (150 mL, 1:1) and washed with saturated aqueous $NaHCO_3$ solution (150 mL). The organic layer was separated and the aqueous layer was extracted with diethyl ether–petroleum ether (150 mL, 1:1). The combined organic layers were washed saturated aqueous $NaHCO_3$ solution (2 ×100 mL), dried ($MgSO_4$), filtered and evaporated in vacuo to afford the title compound **38** (3.96 g, 94%) as a colourless oil.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 3.63 (s, 3H), 3.37 (t, *J* = 6.8 Hz, 2H), 2.26 (t, *J* = 7.5 Hz, 2H), 1.88 – 1.75 (m, 2H), 1.63 – 1.53 (m, 2H), 1.44 – 1.33 (m, 1H), 1.31 – 1.21 (m, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 174.32, 51.49, 34.13, 34.05, 32.88, 29.40, 29.36, 29.25, 29.16, 28.78, 28.20, 24.98.

Synthesis of (11-methoxy-11-oxoundecyl)triphenylphosphonium bromide

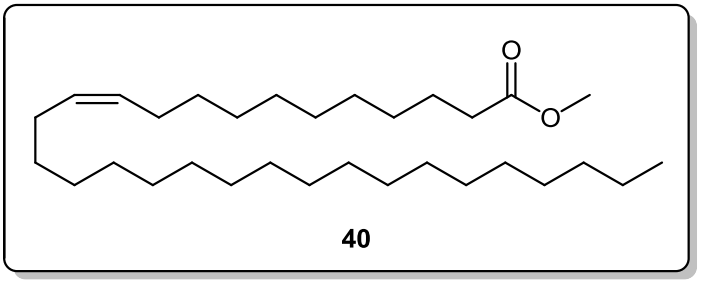


39

A 2-5 mL MW vial was charged with bromide **38** (3.75 g, 13.43 mmol, 1 eq.), triphenylphosphine (3.70 g, 14.10 mmol, 1.05 eq.) and anhydrous CH_3CN (5 mL), purged with nitrogen and heated under microwave irradiation for 20 minutes at 140 °C and for further 85 minutes at 160 °C. The reaction mixture was then cooled to room temperature and concentrated *in vacuo* to give an oil (8.05 g) that was washed with anhydrous diethyl ether (15 mL). The resulting oil was evaporated *in vacuo* dissolved in anhydrous dichloromethane (20 mL). To the resulting solution was added anhydrous petroleum ether (30 mL), the upper layer was removed and the precipitated oil was washed with anhydrous petroleum ether (2 × 30 mL). The oil was dissolved in anhydrous dichloromethane (10 mL), precipitated with anhydrous pentane (70 mL) and left at –20 °C for 3 hours. Then the clear supernatant was removed and the oil evaporated *in vacuo* to afford the title compound **38** (7.51 g, 100%) as a clear sticky oil.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 7.89 – 7.76 (m, 9H), 7.73 – 7.66 (m, 6H), 3.91 – 3.76 (m, 2H), 3.64 (s, 3H), 2.27 (t, *J* = 7.5 Hz, 2H), 1.67 – 1.51 (m, 6H), 1.31 – 1.14 (m, 10H).

Synthesis of (Z)-methyl triacont-11-enoate



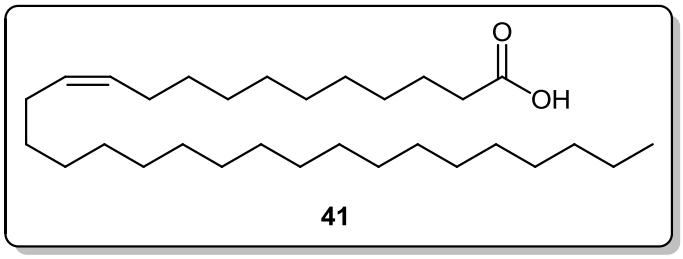
40

In 250 mL one-neck round-bottom flask fitted, phosphonium bromide **39** (3.65 g, 6.74 mmol, 1.2 eq.) was thoroughly dried before being connected with a two-neck adaptor and purged with nitrogen. The sticky oil was dissolved in anhydrous THF (40 mL) and cooled to –20 °C, to the resulting solution was added dropwise 1 M solution of NaHMDS in THF (7.0 mL, 7.0 mmol, 1.25 eq.). The solution turned immediately to orange and was warmed and stirred at room temperature for 30 minutes. To the resulting red-orange solution was added anhydrous DMPU (18 mL) and the solution was cooled to −84 °C for 30 minutes. To this solution was added dropwise a solution of aldehyde **4** (1.585 g, 5.62 mmol, 1 eq.) in anhydrous THF (15 mL) over 60 minutes, keeping constantly the temperature of the reaction close to −84 °C. The reaction was stirred at this temperature for *ca.* 1 hour at *ca*. –80 °C, then the reaction system was allowed to warm slowly to room temperature and stirred for further 2 hours. The solution was then quenched with 1 M solution of ammonium chloride (*ca.* 200 mL) and extracted with petroleum ether (*ca.* 200 mL). The organic phase was washed with water (2 × 250 mL) and brine (2 × 200 mL), concentrated *in vacuo* to give a 200 mL solution that was kept at –20 °C for *ca.* 2 hours. The resulting mixture was filtered to remove triphenylphosphine oxide. The filtrate was concentrated *in vacuo* to give a 50 mL solution that was filtered evaporated *in vacuo* to give a white low melting solid (2.86 g). The crude reaction mixture was purified by flash chromatography (2% diethyl ether in petroleum ether) to afford the title compound **40** (760 mg, 29%) as a white solid.

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 5.40 – 5.28 (m, 2H), 3.66 (s, 3H), 2.30 (t, *J* = 7.6 Hz, 2H), 2.00 (dd, *J* = 12.3, 6.5 Hz, 4H), 1.67 – 1.55 (m, 3H), 1.26 (d, *J* = 6.9 Hz, 44H), 0.87 (t, *J* = 6.8 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 174.51, 130.08, 129.98, 51.60, 34.26, 32.08, 29.92, 29.91, 29.86, 29.82, 29.72, 29.63, 29.58, 29.52, 29.48, 29.42, 29.41, 29.30, 27.35, 25.10, 22.85, 14.29.

Synthesis of (Z)-triacont-11-enoic acid



41

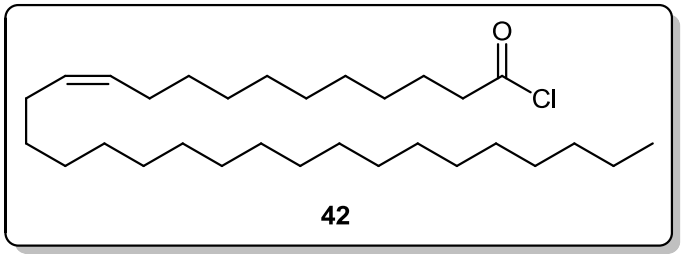
To a stirred solution of methyl ester **40** (414 mg, 0.891 mmol, 1 eq.) in ethanol (8 mL) and THF (7 mL) was added 2 M solution of sodium hydroxide (15 mL) and the reaction mixture was stirred for 1 day, during which time a white precipitate formed. The reaction mixture was diluted with petroleum ether (50 mL) and vigorously agitated in the presence of HCl solution (30 mL) until both layers were completely clear and the aqueous phase was at pH 2–3. The organic layer was separated, washed with aqueous HCl solution (pH 2–3) and dried (MgSO4), filtered and evaporated in vacuo to afford the title compound **41** (383 mg, 95%) as a white solid.

m.p.: 55–54 °C

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 5.42 – 5.28 (m, 2H), 2.35 (t, *J* = 7.5 Hz, 2H), 2.07 – 1.95 (m, 4H), 1.63 (dd, *J* = 14.5, 7.2 Hz, 2H), 1.26 (d, *J* = 8.4 Hz, 44H), 0.88 (t, *J* = 6.7 Hz, 3H).

^{13}C NMR (101 MHz, CHLOROFORM-D) δ 180.04, 129.98, 100.05, 34.15, 32.08, 29.86, 29.73, 29.64, 29.56, 29.53, 29.48, 29.43, 29.39, 29.21, 27.35, 24.81, 22.85, 14.29.

Synthesis of (Z)-triacont-11-enoyl chloride



42

A solution of fatty acid **41** (208 mg, 0.614 mmol, 1 eq.) in anhydrous dichloromethane (10 mL) containing anhydrous triethylamine (49 mg, 0.4845 mmol, 1.05 eq.) was cooled to 0 °C. To this stirred solution was added oxalyl chloride (176 mg, 1.384 mmol, 3 eq.) in anhydrous dichloromethane (5 mL). The reaction mixture was stirred for 30 minutes. The reaction mixture was diluted with petroleum ether and filtered to afford the title compound **42** as a white solid white solid (188 mg).

^{1}H NMR (400 MHz, CHLOROFORM-D) δ 5.40 – 5.30 (m, 2H), 2.88 (t, *J* = 7.3 Hz, 2H), 2.07 – 1.96 (m, 4H), 1.75 – 1.65 (m, 2H), 1.36 – 1.22 (m, 44H), 0.88 (t, *J* = 6.6 Hz, 3H).

The dissolution of this solid in methanol yielded the methyl ester **40**.

Synthesis of (Z)-triacont-11-enoyl–NaSBI

To a solution of NaSBI (176 mg, 0.801, 2 eq.) in anhydrous DMF (1 mL) contained in a 2–5 mL M.W. vial was added a suspension of acyl chloride **42** in anhydrous 1,2-dichloroethane (1 mL) and petroleum ether (1 mL). The vial was sealed and the reaction mixture was heated at 70 °C for 5 hours. The reaction mixture was washed with water, petroleum ether/AcOEt and filtered. This purification was repeated 3 times to afford the title compound **42** as a white solid.

^{1}H NMR (400 MHz, DMSO-D6) δ 8.94 (s, 1H), 8.10 (d, *J* = 8.5 Hz, 1H), 7.87 (s, 1H), 7.67 (d, *J* = 8.6 Hz, 1H), 5.37 – 5.27 (m, 2H), 3.15 (t, *J* = 7.4 Hz, 2H), 2.03 – 1.93 (m, 4H), 1.75 – 1.65 (m, 2H), 1.33 – 1.19 (m, 44H), 0.88 – 0.82 (m, 3H).

6.3.4 Study of the chemoselectivity of the NaSBI-based thioesterification in D_2O by NMR monitoring.

A solution of NaSBI·$3H_2O$ (34.3 mg, 0.125 mmol) was prepared using 18.8 μL of 1 M HCl (prepared diluting aqueous 20.2% HCl with D_2O), 471 μL of D_2O and 10 μL of a solution of model compounds containing: mercaptoethanol (19.45 μL, 0.278 mmol), phenol (26.1 mg, 0.278 mmol), aminoethanol (16.8 μL, 0.278 mmol) and imidazole (18.9 mg, 0.278 mmol) in 1.0 mL of D_2O.

A NMR tube was charged with the resulting solution and used to run a proton NMR experiment in presaturation. Then a solution of nonanoyl–NaSBI (1.0 mg, 2.78 μmol) in D_2O (125 μL) was added to the previous solution in the NMR tube. Successive proton NMR experiments were carried out in presaturation.

6.3. 1.5 Study of the chemoselectivity of the NaSBI-based thioesterification by NMR analysis of the products

A solution of NaSBI·$3H_2O$ (289 mg, 1.05 mmol) was prepared using 3.0 mL of water, 158 μL of 1 M HCl and 4.0 mL of a solution of model compounds containing: thioglycerol (204 μL, 2.44 mmol), phenol (228 mg, 2.44 mmol), propylamine (200 μL, 2.44 mmol) and ethanol (143 μL, 2.44 mmol) in 100 mL of H_2O. Nonanoyl–NaSBI (70.4 mg, 0.195 mmol). The reaction was shaken for 2 hours at room temperature and extracted with ethyl acetate. The organic phase was evaporated *in vacuo* to yield colourless oil, which was checked by NMR spectroscopy showing that dihydroxypropyl-nonanethioate was the major product. The only impurity detected was related to the fatty acid, which was contained in the starting nonanoyl–NaSBI or due to partial hydrolysis of the reagent.

6.3. 1.4 Study of the chemoselectivity of the NaSBI-based thioesterification in protein samples

Four parallel reactions were performed using in every reaction:

- a mixture of *holo*-AcpM and *apo*-AcpM (40 μL, 4 mg/mL, 0.26 mM, 1.0 and 0.67 eq. respectively);
- NaSBI-based catalytic system (40 μL, NaSBI: 800 mM, HCl: 120 mM);
- Aqueous solution of nonanoyl–NaSBI (80 μL) to yield the following final concentrations: 0.51 mM, 1.54 mM, 4.63 mM, 13.9 mM.

The reactions were shaken at 150 RPM at 30 °C for 9 hours. The reactions were checked by ESI as described in Chapter 4.

CHAPTER 7

7. REFERENCES

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