



Escherichia coli acyl-CoA dehydrogenase inhibitors and genetic engineering for short chain alkane production

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Abstract

The depletion of fossil fuels is an increasing problem, which drives the search for alternative, sustainable and cost-effective methods. Due to advancements in genetic engineering, genetically modified microbes (e.g. *Escherichia coli*) receive more attention as potential “fuel factories”. The "hot topic", in the field, is an alteration of the fatty acid metabolism. In 2013 the production of short-chain alkanes (in other words petrol) by genetically modified *E. coli was* reported. Up to date, the only genetic modification was utilized as a tool for microbial metabolism manipulation.

This project aims to build a library of potential chemical acyl-CoA dehydrogenase inhibitors and establish their potency. Pantothenic acid was used as a scaffold for total synthesis of inhibitors such as 2-pentynoyl-S-pantetheine. Unlike previous studies where protection group chemistry and hydroxybenzotriazole, N,N'-dicyclohexylcarbodiimide mediated amide coupling. A simple four-step synthesis was achieved with 1`,1`-carbonyldiimidazole as a coupling agent. The utilisation of this approach decreased the number of steps which resulted in higher yields, as well as reduced overall cost and toxicity of by-products. These factors are important if the proposed technique was to be used for industrial applications. During this study synthesis and purification of 28 S-acyl pantetheines was achieved.

The potency of the inhibitors was tested on overexpressed and purified FadE. *E. coli* protein was overexpressed in *E. coli* BL21 strain and purified by Styrene Maleic Acid-Lipid Particles technique and affinity chromatography. Protein activity was assessed with ferrocenium hexafluorophosphate assay. Synthesised pantetheines exhibit different levels of activity. 2-Pentynoyl-S-pantetheine proved to be the most potent compound with the half maximal inhibitory concentration of 0.04 mM.

The fatty acid metabolism regulator protein (FadR) is responsible for the regulation of fatty acid metabolism, to lift this regulation knockout with the λ-red technique was achieved. Two plasmids carrying genes for fatty acyl-ACP thioesterase, fatty acyl-CoA synthetase, fatty acyl-CoA reductase and fatty aldehyde decarbonylase were also developed.

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Abbreviations

AA-CoA – Acetoacetyl-CoA

ACADs – Acyl-CoA dehydrogenases

AccABCD – Acetyl CoA carboxylase

ACP – Acyl-Carrier Protein

ACPs – Acyl-ACP thioesterases

Acr – Fatty acyl-CoA reductase

AD – Anno Domini

adb – Alcohol dehydrogenase

Amp – Ampicillin

AMP – Adenosine Monophosphate

APS – ammonium persulphate

ATP – Adenosine Triphosphate

BCA – Bicinchoninic acid assay

Bcm – Billion Cubic Meters

BP – British Petroleum

BSA – Bovine Albumin Serum

cAMP – Cyclic Adenosine Monophosphate

CDI – 1`,1`Carbonyldiimidazole

CDP-ME – 4- Diphosphocytidyl-2-C-methylerythritol

CDP-MEP – 4-Diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate

CER1 – fatty aldehyde decarbonylase

CGSC – Coli Genetic Stock Centre

Chl – Chloramphenicol

CNG – Compressed Natural Gas

CoA – Coenzyme A

COSY – Correlation spectroscopy

CSP – Concentrated Solar Power

DCC – N, N'-Dicyclohexylcarbodiimide

DCM – Dichloromethane

DCU – Dicyclohexylurea

DDM – n-Dedecyl-β-D-maltopyranoside

DIPEA – N,N-Diisopropylethylamine

DMAP – 4-Dimethylaminopyridine

DMAPP – Dimethylallyl Pyrophosphate

DMF – Dimethylformamide

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic Acid

dNTPs – Deoxynucleotides

DXP – 1-Deoxy-D-Xylulose-5-Phosphate

DXS – 1-Deoxy-D-Xylulose-5-Phosphate Synthase

EDC – 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide

EDTA – Ethylenediaminetetraacetic acid

EIA – Energy Information Administration

EtOAc – Ethyl acetate

EU – European Union

FA – Fatty Acids

FabA – 3-hydroxyacyl-ACP dehydrase

FabB – 3-ketoacyl-ACP synthase

FabD – ACP transacylase

FabI – Enoyl-[ACP] reductase

FabZ – 3-hydroxyacyl-ACP dehydrase

FAD – Flavin Adenine Dinucleotide

FadA – 3-Ketoacyl-CoA Thiolase

FadD – Fatty acyl-CoA synthetase

FadG – 3-oxoacyl-[ACP] reductase

FadH – β-ketoacyl-(ACP) synthase III

FadL – Outer membrane transporter protein

FadR – Fatty Acid Metabolism Regulator Protein

FAEEs – Fatty Acid Ethyl Esters

FAMEs – Fatty Acid Methyl Esters

FAO – Food and Agriculture Organisation

FFAs – Free Fatty Acids

FPP – Farnesyl Pyrophosphate

G3P – Glyceraldehyde 3-Phosphate

Glu – Glutamate

GPP – Geranyl Pyrophosphate

GWh – Gigawatt hour

HMB-PP – (E)-4-hydroxy-3-methyl-but- 2-enyl pyrophosphate.

HMG-CoA - 3-Hydroxy-3-Methylglutaryl-Coa

HMPBPP – Hydroxymethylbutenyl Diphosphate

HOBt – Hydroxybenzotriazole

HPLC high-performance liquid chromatography ()

IAEA – International Atomic Energy Agency

IC_{50} – The half maximal inhibitory concentration

IEA – International Energy Agency

IPP – Isopentenyl Pyrophosphate

IPTG – Isopropyl-β-D-thiogalactopyranoside

Kan – Kanamycin

KDC – 2-Ketoacid Decarboxylase

kWh – Kilowatt Hour

LB – Luria Broth

LPG – Liquefied Petroleum Gas

mcl-PHA – Medium chain length Polyhydroxyalkanoate

Me_2CO – Acetone

MeCN – Acetonitrile

MECPP – 2,4-Cyclodiphosphate

MeOH – Methanol

MEP – 2- Methylerythritol-4-Phosphate

Mt – Million Tones

Mtoe – Million Tonnes of Oil Equivalent

MVA – Mevalonate

MVAP – Mevalonate-5-Phosphate

MVAPP – Mevalonate 5-Diphosphate

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NMR – Nuclear magnetic resonance

OTG – n-Octyl-1-thio-β-D-glucopyranoside

PA- Pantothenic Acid

PBS – Phosphate-buffered saline

PCR – Polymerase Chain Reaction

Pdc – Pyruvate dehydrogenase

Pgi – Phosphoglucose isomerase

PK – Pantothenate kinase

PMSF – Phenylmethylsulphonyl fluoride

pTSA – p-Toluenesulfonic acid

PyBOP – Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate

RP-HPLC – Reverse-phase high-performance liquid chromatography

RPM – Revolution Per Minute

RT – Room Temperature

SCA – Short Chain Alkane

SD – Standard Deviation

SDS-PAGE – Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SMALPs –Styrene Maleic Acid Lipid Particles

TAIR – *The Arabidopsis Information Resource*

TBS-T – Tris-buffered saline – 0.1% Tween

TEPES – Total Energy Supply

TesA - Acyl carrier protein thioesterase

TFA – Trifluoroacetic acid

THF – Tetrahydrofuran

TIPS – Triisopropylsilyl

TLC – Thin layer chromatography

TOF-MS – Time of flight- Mass spectrometry

TPES – Total Primary Energy Supply

Trt – Trimethylphenyl (Trityl)

TrtCl – Trimethylphenyl (trityl) chloride

UK – United Kingdom

USD – United States Dollar

WEC – World Energy Centre

WS/DGAT – The wax ester synthase/acyl coenzyme A (acyl-CoA): diacylglycerol Acyltransferase

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Chapter 1

Introduction

1.1 Conventional and renewable energy sources – overview

The first mention of steam-powered devices could be traced back to the first century AD and Heron of Alexandria. However, those early inventions were not powerful enough to be widely used.[1] Many researchers worked on the steam power over the centuries, but it was not until the late 17th century and James Watt`s engine, when steam power dominated many areas of life, from transportation to the industry.[2] The steam engine and many other discoveries led to a transition from hand methods to the factory-based system, which is now known as the industrial revolution.[2] The rapid development of industry required fuel; wood and coal became in high demand, and mining technologies started to develop.[3] Coal was not the only fossil fuel for which demand increased rapidly. The invention of the internal combustion engine[4] and oil refinery[5] in a mid-19th century helped to pave the way for the development of the modern car (early 20th century)[6] and consequently an increased demand for petroleum.

According to reports from the International Energy Agency (IEA), fossil fuels remain the primary energy supply (Figure1.1)[7]. Moreover, the Total Primary Energy Supply (TPES) is continuously growing. Over the last 40 years, it increased almost 2.5-fold to meet energy demand. Different organisations including, the World Energy Centre (WEC)[8], the Energy Information Administration (EIA)[9] and the European Union (EU)[10] released similar reports.

However, between 1973 and 2015 oil usage changed from 46% to 32%, which is a significant number (Figure 1.1).[7] There are a few factors which are affecting oil availability. Over the years deposits of fossil fuels are depleting, and extraction of crude oil becomes less cost-effective, more dangerous[11] and requires more complex technology.

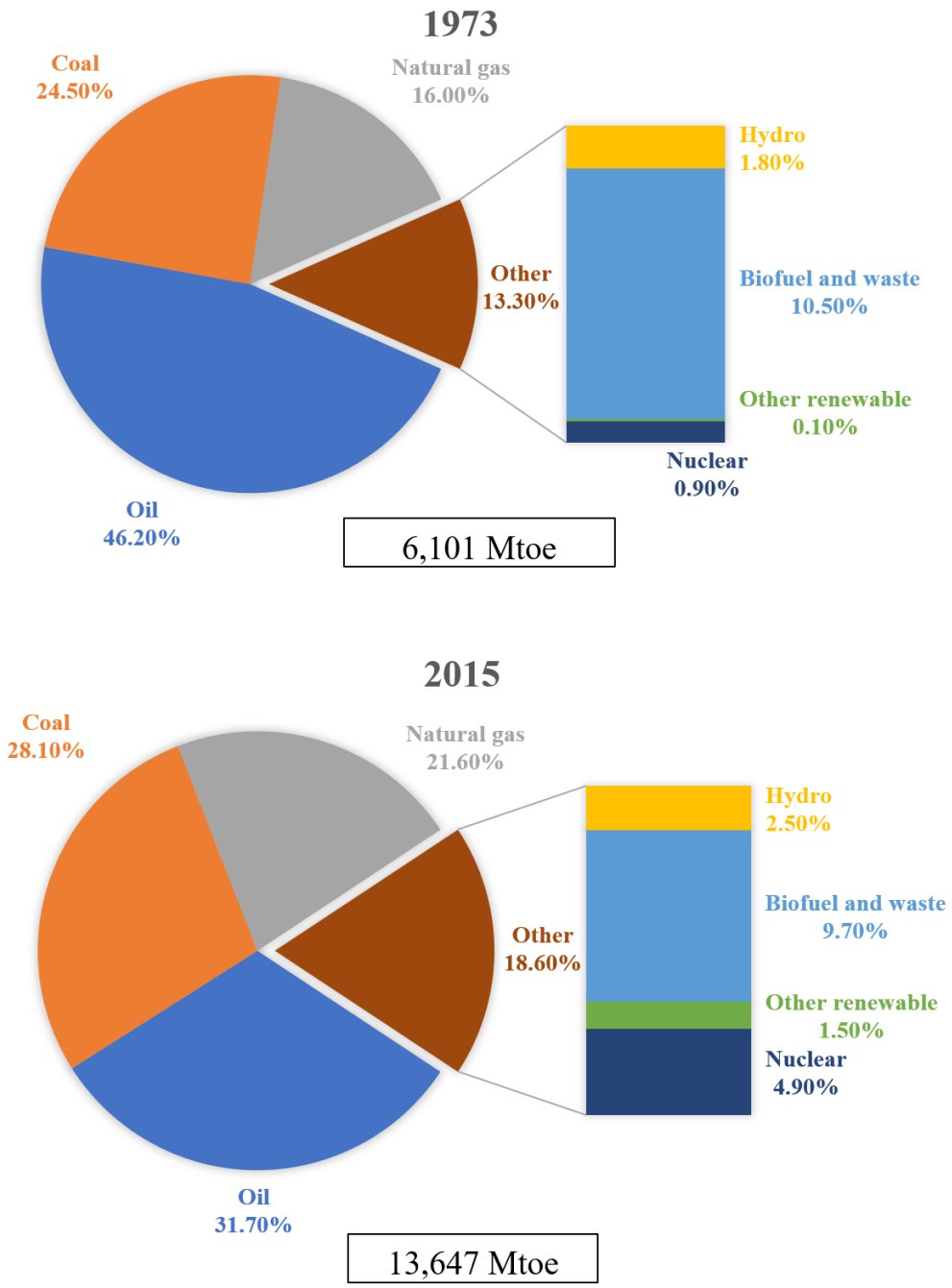


Figure 1.1 The World Total Primary Energy Supply (TPES) between 1973 and 2015. Other renewable includes: solar, geothermal and wind power. Tonne of oil equivalent is defined as the amount of energy released by burning one tonne of crude oil. Source: IEA[7]

Advanced technologies are required to drill deeper or look for new reservoirs, e.g., under the seafloor[12] or in Antarctica (currently any mining is prohibited)[13]. British Petroleum (BP), which is one of the oil and gas companies, predicts that with current production the global oil reserve will be sufficient for *ca.* 50 years.[14] With increased extraction resources will deplete faster, which

could become a source of international tension. The ongoing conflict between Russia and Ukraine over natural gas supply support this statement. Restricted access to natural resources will ensure prices rise.[15] As of 2018, out of the 195 countries in the world around 98 have oil reserves of different abundance.[16] However, only four countries hold over 50% of available crude oil (Figure 1.2). Thus oil supply (or other natural resources) could become a tool in foreign policy.[17,18]

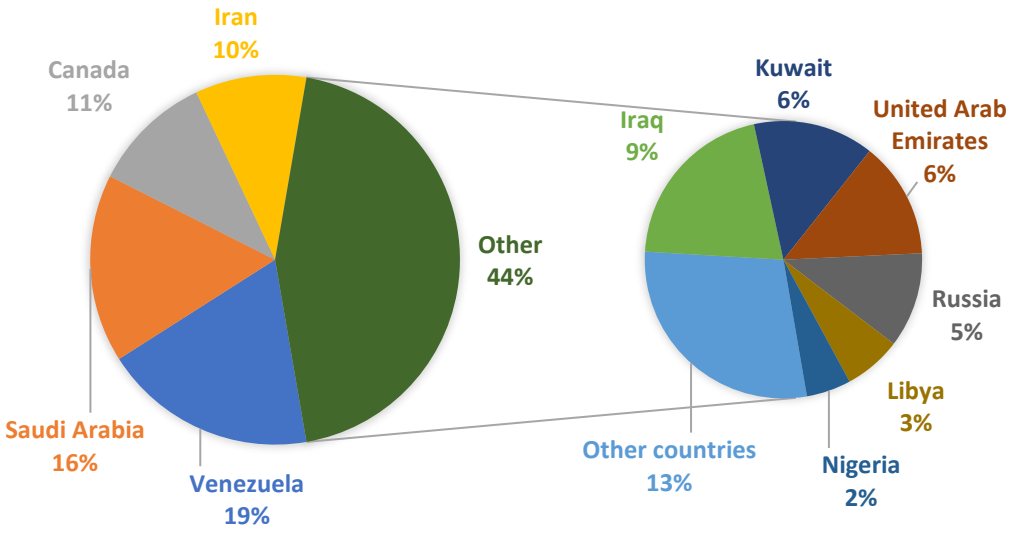


Figure 1.2 Crude oil reserve in 2018. Venezuela, Saudi Arabia, Canada and Iran hold over 50% of the available oil reservoir. Source EIA[9]

In the same period (1973 – 2015) reliance on coal and natural gas increased (Figure 1.1). It is not surprising that coal remains one of the significant energy supplies since it is affordable and widely distributed. Moreover, mining and processing technologies are well developed and considered safe.[19] All those reasons make coal a desirable source of energy for rapidly growing economies, such as China or India. Increasing awareness about environmental impact pushes research towards the development of new methods, and which are referred to as clean coal technology and aim to reduce pollution emission during coal processing.[20–22]

China is the leader in coal production and consumption and heavily relies on local coal deposits. In 2016, 3,242 million tonnes (Mt) of coal were extracted, which is only 125 Mt less than the next nine countries combined (Figure 3).[7] According to Tang et al. over last three decades coal dependence in China was increasing.[23] However, this trend is changing since 2014, and the Chinese government has been reporting a steady decrease in coal usage,[24] which could be related to the slowdown in economic growth.[25] Furthermore, in recent years, increasingly since the 2008 Olympic Games in Bejing, air pollution came under international attention and scrutiny. Since then China introduced new policies to remedy the issue. [26,27]

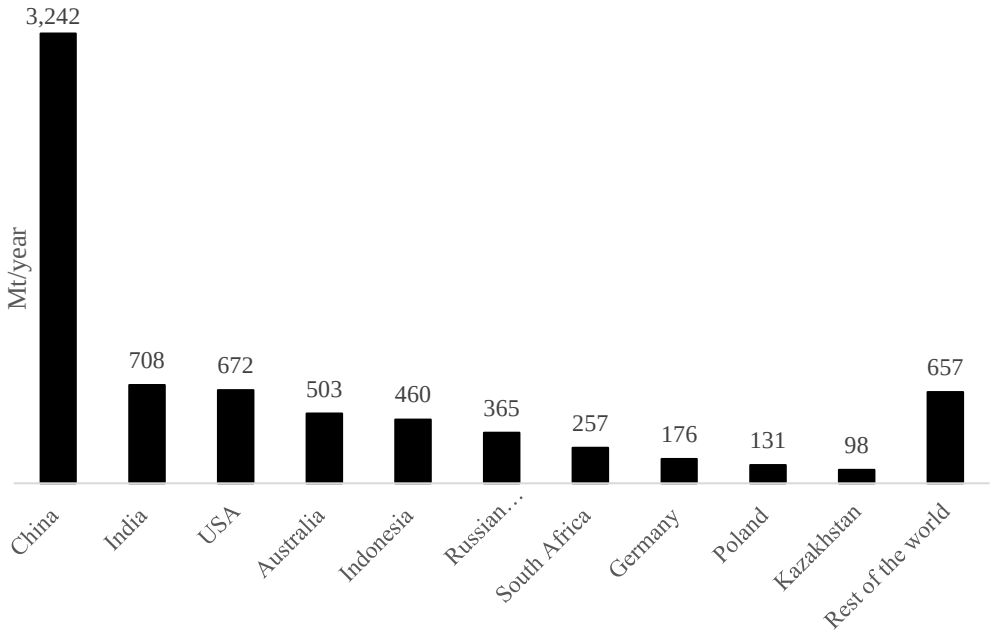


Figure 1.3 Major coal producers in 2016. Total mined coal expressed in Mt – millions of tonnes.

Another prominent carbon-based fossil fuel is natural gas, a mixture of different hydrocarbons primarily of methane, which can contain varying quantities of other alkanes, and a small percentage of carbon dioxide, nitrogen, hydrogen sulphide, or helium.[28] Due to its properties, it is the most efficient available non-renewable fossil fuel and is projected to further increase its share in global energy source, with the USA and Russia as lead suppliers (Figure 1.3).[7,8] Furthermore, natural gas processing releases fewer air pollutants, such as CO_2, various nitrogen oxides and SO_2. [29,30] In an era of increasing pollution and climate change, natural gas presents itself as a convenient and cleaner solution

(Figure 1.4). It is relatively inexpensive, and due to new technologies, accessible deposits are increasing. Unfortunately, some of the extraction technologies, i.e. gas extraction by horizontal drilling and hydraulic fracturing (fracking),[31] have a negative environmental impact, e.g. increase water pollution.[32] Additionally, public perception of fracking is unfavourable, and extraction might be difficult if local communities oppose it.[33,34]

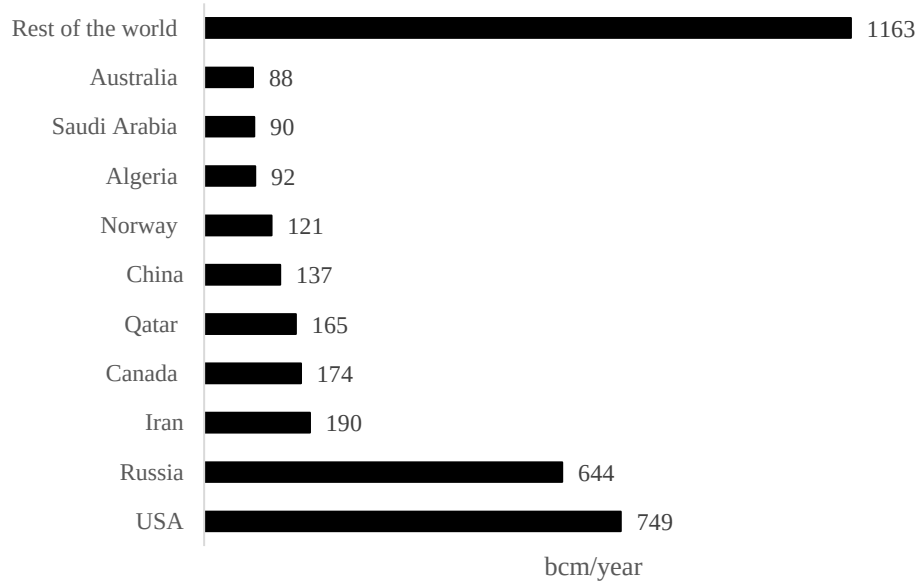


Figure 1.4 Major natural gas producers in 2016. Total mined coal expressed in bcm – billion cubic meters.[7]

Between 1973 and 2015, the use of nuclear energy increased by 4%.[7] In fact, over the past 60 years 613 nuclear power plants were opened out of which 441 are still operational, the peak in construction was in the 80s of 20th century.[35] Since then, accidents in Chernobyl (1986) and more recent reactor meltdown at Fukushima Daiichi plant (2011) resulted in nuclear power slowdown. Difficulties in disaster management, long-term environmental impact, as well as problematic waste storage, make the international community more cautious and apprehensive. Some countries (e.g. Germany) decided to gradually phase out nuclear reactors.[36] International Atomic Energy Agency (IAEA) predicts an increase in nuclear energy usage by the year 2050, as means satisfy energy consumption.[37]

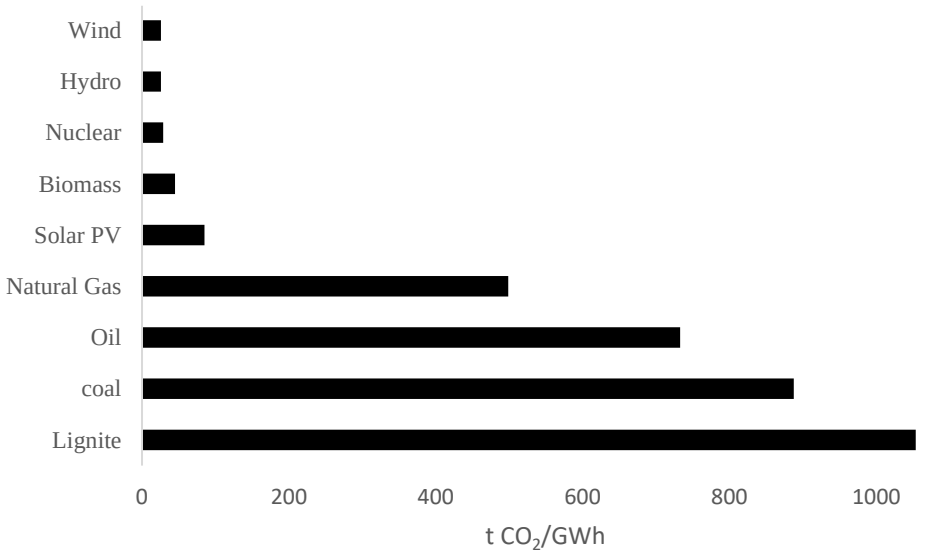


Figure 1.5 Tonnes of carbon dioxide emitted per GWh of energy produced, energy source comparison. Mean values from multiple independent studies.

Currently, conventional fuels (oil, coal, natural gas) provide *ca.* 80% of the World`s consumed energy (Fig 1.5)[8]. The production of one Gigawatt hour (GWh) of energy from carbon-based fuels, for example, brown coal generates *ca.* 1000 tonnes of CO_2, in comparison, the nuclear plant and hydro plant releases only 29 t and 26 t respectively (Figure 1.5).[38] According to estimates, in 2014 annual energy demand was 12.5 $x10^3$ Mtoe, which corresponds to 14.5 $x10^{13}$ GWh (1 Mtoe is equal to 0.012 GWh),[39] and is predicted to grow over the next decades. [40] Carbon dioxide is one of the greenhouse gasses that is responsible for global temperature increase. Even when absorbing capabilities of plants and ocean are taken into account, the CO_2 in the atmosphere is steadily growing since 1750 and has increased by 40%.[41,42]

The carbon footprint of non-conventional energy sources is significantly lower than the conventional one (Figure 1.5). Moreover, prices volatility, political instability, limited access to resources and climate change concerns drive the development and improvement of new technologies for renewable energy.[43] In 2017, over USD 279 billion was invested in this sector, a 2% increase compared to the previous year.[44] Hydropower, solar power, wind power and biomass supply majority of renewable energy.[45]

Currently, hydropower provides the majority of renewable energy with, China as a world leader. In fact, China`s hydro-energy production is larger than the next three countries combined.[8] There three main types of hydropower plants

are the following:

a) Impoundment which requires a dam and water reservoir
b) Diversion which harvests energy from the flowing river
c) Pumped storage where energy is obtained by transferring waters between reservoirs located on different elevation levels.

The most common type is the first.[46,47] Although hydropower plants emit significantly less CO_2 (Figure 1.5) are reliable, in many cases provide flood protection, and sometimes offer new trade routes, they are not without flaws.[48] Construction of the new dam results in flooding of areas of land and potentially destroys and changes the natural habitats of many animals, both terrestrial and aquatic. Moreover, in many cases, the local population has to be relocated and provided with new settlements, which in almost all cases generates strong opposition from local communities.[46,] Also, flooded areas can release methane, which is even more potent greenhouse gas than carbon dioxide.[49] One of the projects that brought great controversies is the Three Gorges Dam project in Yiling District, Yichang, Hubei province, China (currently the biggest of a kind in the world). To build the reservoir over one million people were resettled and the area slightly smaller than Singapore (over 600 km^2) was flooded.[50]

Not only hydropower is observing growth in improving technologies and capabilities. Solar and wind power technologies are continuously developing and have become less expensive. Solar power plants use the following systems:

a) Photovoltaic systems that require solar panels installed either on rooftops or the ground and directly convert sunlight to electricity [51]
b) Concentrated solar power (CSP) which uses sunlight to heat the liquid which is used to produce electricity *via* a turbine. [52]

One of the major drawbacks of solar technology is the efficiency, which currently, depending on a specific type of solar cells, varies between 3-22% and on average is around 15% for commercially available units. [53–55] However, it has improved to maximise the quantity of energy harvested from 1 m^2. To date, the highest recorded efficiency is 46%[56] for experimental technology, which shows that the efficiency limit is yet to be achieved. Another, but not unrelated issue,

is the energy storage. Improving capacity and length of energy storage will allow satisfying energy demand during the less sunny days or winter, while sun exposure is shorter and lower the price of 1 kWh.[57]

Hydro and solar power is not the only energy sectors with rapidly developing technology. Wind energy is increasing its shares in global energy supply, with some smaller countries achieving over 40% of their energy demand with wind turbines.[58] The European Union wind farms provide for almost 12% of energy, and according to recent reports, wind energy has overtaken coal as the second type of power and is predicted to increase its shares.[59] The appeal of wind technology lies not only in the availability of source but also in landscape management. Stronger and more steady winds over the seas are an advantage for wind farms located offshore, which in turn minimises aesthetic impact and land space demand.[60] Similar to solar cells, energy storage is an issue that has not been fully resolved and requires further research.[61] So far "the golden technology" has not been developed. Therefore, some researchers postulate the hybridisation of different technologies, e.g. solar and coal[62] or solar and wind[63] , to achieve more efficient and robust systems.

Another method to mitigate energy shortage is the recycling of biomass and waste as substrates for biofuel and energy production.[64,65] Known biological processes (e.g. fermentation) have been exploited and further developed for biofuel production.[66]. The most common criteria of differentiation between biofuels is a raw material or feedstock. New advancements in the field led to the development of so-called "biofuel generations". Thus terms such as first generation, second generation and third generation biofuels were coined. [67]

The first generation approach entitled the production of the bioethanol, biogas and biodiesel from the maze, vegetable oil, soybeans or cereals [68,69] To date ethanol remains the primary biofuel produced by the method[70,71]. Ethanol production is a multistep process that requires either starch- or saccharose-based substrate (Figure 1.6).[72] Regardless of feedstock, the raw material requires pre-treatment to disrupt cell walls. Bioethanol production from starch-based materials requires an additional step - hydrolysis.[73] Yeast, the organism used for fermentation, is not capable of processing complex carbohydrates, such as starch. Therefore, the hydrolysis of complex carbohydrates to simple six-carbon sugars

is required.[74] Fermentation product is further purified by physical separation methods to produce distilled alcohol. Bioethanol is used as an additive for conventional fuels, blended or stored accordingly. By-products of the process can be used as animal feed or for heat production [73].

Second generation biofuels mostly use non-edible lignocellulosic waste, such as rice straw and wood chip.[75] In principle the methodology involves fermentation, but substrate pretreatment is a limiting step.[76] The feedstock requires much more harsh pretreatment and processing towards simple hexoses. In the case of 1st generation pre-treatment destroyed cell walls and released desired carbohydrates, 2nd generation substrates require hydrolysis of the cellulose. Researchers have developed new enzymatic, chemical, physical and physicochemical techniques to decrease cost and time and increase efficiency.[77,78]

Third generation biofuels utilised algae as feedstock and were previously clustered together with the 2nd generation approach. Because of higher yields and a broader scope of genetic manipulation and achieved products, the use of algae is considered as a new group.[79] In comparison to higher plants, the advantage of microalgae lies in the more efficient conversion of sunlight energy. It is also worth to mention that due to suspension in water they have better access to nutrients and carbon dioxide.[80] Moreover, the combination with cellulolytic bacteria such as *Clostridium thermocellum*, which can directly hydrolyse cellulose can lead to even higher yields.[81] It has been postulated that use of algae could be a viable remedy for eutrophic waters, where overgrown microorganisms hinder or in many cases, prevent other vegetation.[82]

Lignocellulosic biomass is the most abundant material in the world[83]. Estimated lignocellulose production corresponds to 90% of global plant biomass. Despite the fact that out of total 200 x 10^9 tons of feedstock available annually, only 4 to 10 % is possibly accessible, it still is amount worth of consideration.[84] Currently, in many cases waste biomass is burned, adding to air pollution and

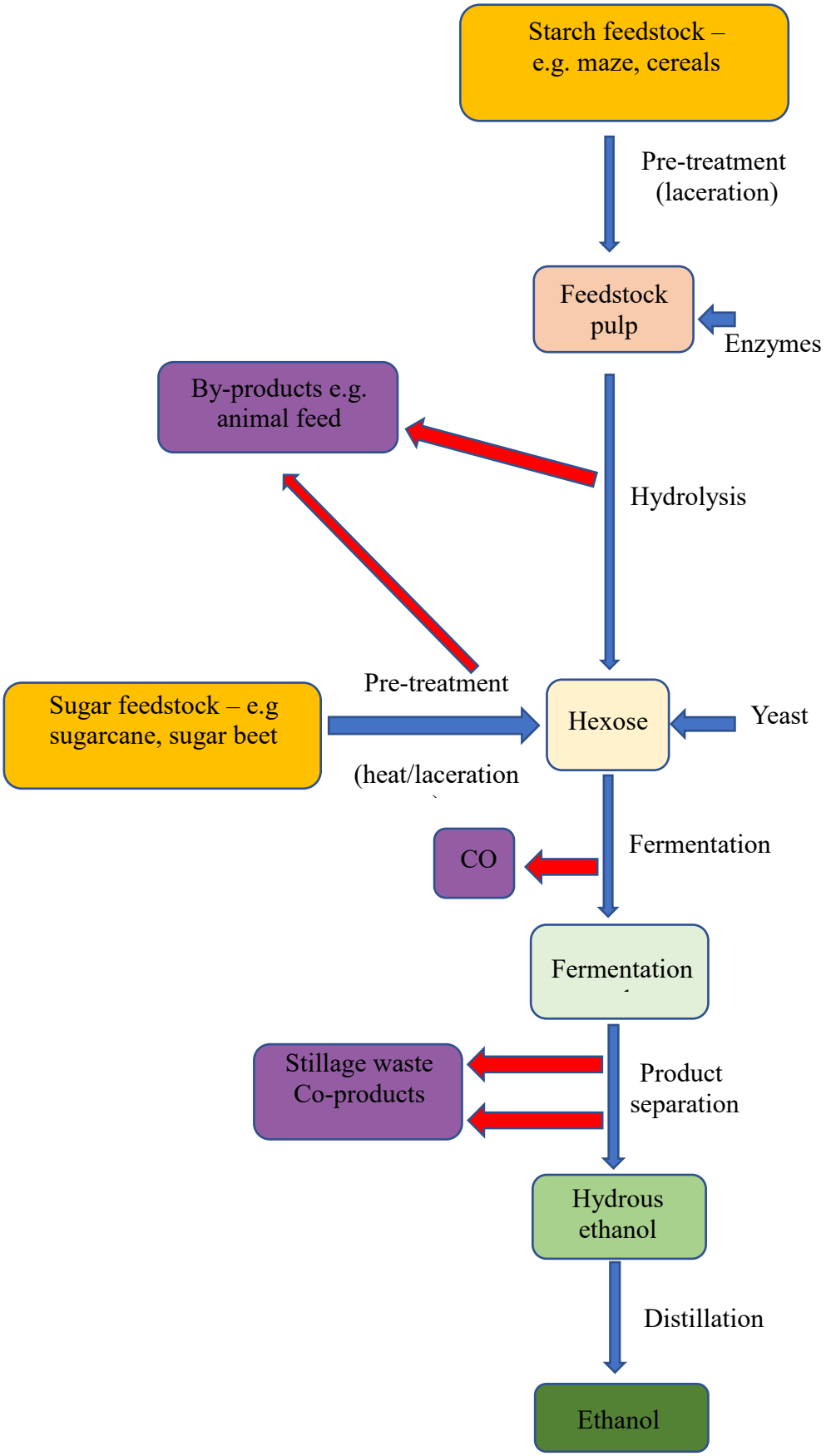


Figure 1.6 Simplified scheme for production of bioethanol from feedstock

causing international tension. The situation which prevails in South-East Asia, and combined with climate change and drought, affect population health and death rates.[85–87] Hence the development and improvement of lignocellulose waste processing methods could provide a viable alternative.

Initially, improvement of efficiency and broader feedstock variety were the main research targets. Although three generations of biofuel production prevail, they are not without disadvantages (Table 1.1). Processing is not the only problem that arises in biofuel production; it is also worth to mention the environmental impact.

Table 1 Fuel production comparative overview.

	Petroleum	**1st generation**	**2nd generation**	**3rd generation**
Feedstock	Crude oil	Vegetable oil, maize, sugarcane	Waste biomass	Algae
Product	CNG, LPG, Diesel petrol	Biodiesel, bioethanol, biogas	Bio-oil, ethanol, butanol	Butanol, ethanol, biodiesel
Advantages	Well established technologies	Easy access to feedstock, simple processing	Abundant substrate	Broder scope of products, higher yield
Disadvantages	Resources depletion, pollution, increasing cost of extraction	Food vs fuel case, land availability, deforestation	Issues with feedstock conversion, land availability	Expensive, technology, requires a large quantity of water and nutrients

Agriculture requires vast amounts of land. In fact, some areas have observed increased plant cultivation for biofuel production, so-called “biofuel hotspots”.[88] This trend is particularly strong in the tropical regions of the world, because of favourable conditions for oil palm growth.[89] Due to the monoculture character of plantations, there are rising concerns about biodiversity, deforestation and soil erosion.[90,91] In the last 60 years, the South East Asian island of Borneo, has experienced a rapid change in forest cover. Significantly,

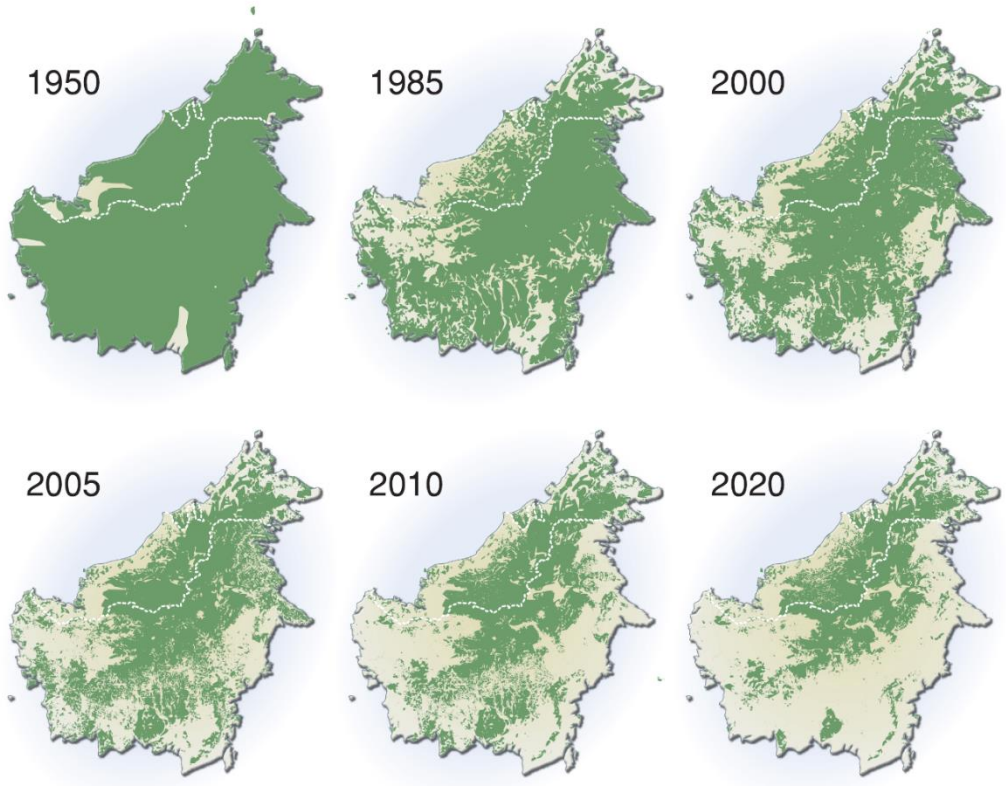


Figure 1.7 Change in Borneo`s forest cover between 1950-2010 and 2020 prediction. [92]

the forest area has been reduced by 50% (Figure 1.7).[92] Coincidentally, in many cases, it overlays with granted oil palm concessions rights. (Figure 1.8)[93,94]. Brazil and other South American countries, as well as equatorial Africa struggle with similar issues.[95] In 2017, the European Parliament released the report regarding the matter in Borneo, mainly Indonesia, to support discussion on palm oil import control with the long-term aim of limiting deforestation.[96] According to the report, oil palm plantations are responsible for 40% of deforestation in Indonesia. Borneo remains the main "bargaining chip" in palm oil dispute because 85% of world's a supply is provided by Malaysia and Indonesia, with the exception of the small country of Brunei, share most of the island. However, another study reports that oil palm plantations caused only 25% of islands deforestation.[97] In fact, more than one paper argues against hasty conclusions as the data is only partial and it is too early to see the full picture.[98–100]

Biofuel can be and is produced from food crops. This raises concerns about food security and the ethics of assigning crops for such purposes. The debate had begun during the 2007-2008 food crisis when food prices

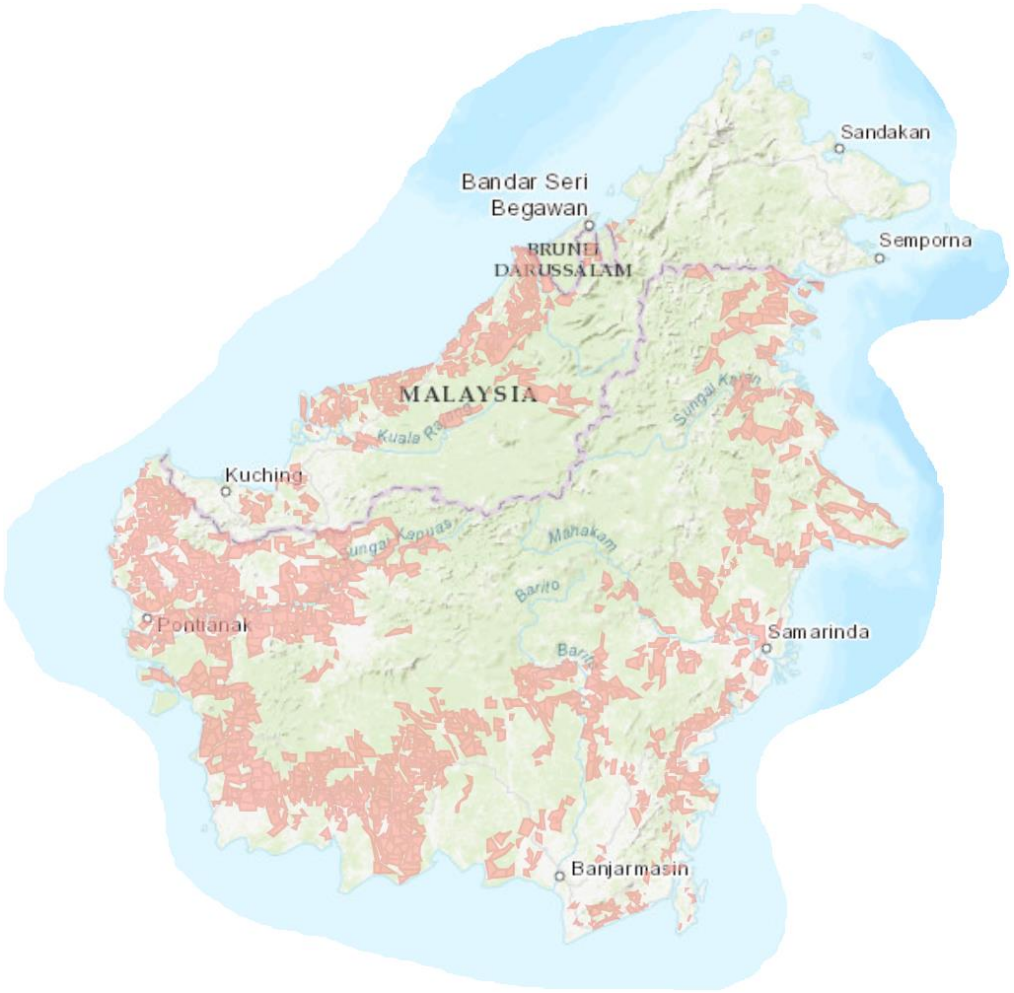


Figure 1.8 Oil palm concessions in Borneo. Data for 2018 [93]

increased significantly.[101–103] Food and Agriculture Organisation (FAO) estimates that in 2016 almost 11% of the population was malnourished, a 1 % increase in comparison to 2014.[104] According to United Nations, world hunger is caused by poverty trap (the poorest cannot afford nutritious food), war and conflicts, insufficient investment in agriculture, unstable markets and food wastage (around third of produced food is lost or wasted)[105,106] Although fuel production has not appeared on the list, the discussion on the topic prevails both in media and academia. The current state of research does not provide final answers, but a significant amount of "could, might, provides possibility, is a potential threat", terms which do not allow for definitive assessment.[107–110] The problem in question is a complex one. Due to factors that affect food prices and availability, the future outcomes are hard to predict, thus providing short and simple answer might not be possible. [111] The issue could be partially remedied

by second and third generation biofuels which do not require crops.[112]

Advancement of synthetic biology, engineering and genetic manipulation opened new routes and areas of research. Microbial fuel production is one of the most researched topics in modern science. There is a broad variety of techniques that are being developed which include but are not limited to: conversion of CO_2 to fuel, for example, direct CO_2 conversion by photosynthesising algae[113] or immobilisation on polymers and photosynthetic microbial hydrogen production[114], genetic engineering of various metabolic pathways.[115–117]

1.2 Microbial fuel production – current state of research

Regardless of the route towards biofuel production (Figure 1.9), the biomass requires saccharification. Thus. it is a limiting step in the process[118]. Multiple studies focus on this issue, and acidic hydrolysis and enzymatic saccharification have received the most of attention. The search for more versatile, robust and efficient enzymes is one of the major topics in the field.[119–121]

Another prominent challenges that limit industrial microbial production are cost efficiency and maintaining a high yield of the product with minimal effort. However, overcoming these obstacles is essential if microbial biofuel production is to compete with conventional fuels or bioethanol. For example, according to the UK's Department of Business, Energy and Industrial Strategy, in 2017 the petroleum demand equalled to 69.5 million tonnes.[122] Without appropriately efficient technologies covering even a small percentage of this demand will remain theoretical.

A common theme in microbial biofuel production is the modification of existing metabolic pathways in order to ultimately afford alcohols, fatty acids or alkanes. [123,124]

Typically, microorganisms for such purposes are chosen for the presence of specific metabolic pathways and different types of catalytic enzymes. For example, *Clostridium acetobutylicum* contains a pathway that allows for acetone, butanol and ethanol production, a pathway which is not present in *Escherichia coli.*[125] To date, at least 14 different microorganisms were reported to produce

various chemicals after genetic modifications and the introduction of new enzymes (Table 1.2)

Alcohols such as ethanol and butanol are the main products of microbial biofuel production. *Escherichia coli* often is an organism of choice, even when additional engineering is required. The popularity of *E.coli* is a result of the high efficiency of DNA transduction into cells, rapid growth, undemanding culture conditions, easy handling and a variety of non-pathogenic strains.[126]. There are significant differences between the production of the same compounds between different strains, due to different genetic modifications and organism predispositions.[127]

Table 1.2 Chemicals produced by an engineered microorganism.

Microorganism	Compound	Titer (g/L)
Bacillus subtilis	Isobutanol	3.83[128]
Bacillus subtilis	Propanol	3.83[128]
Caldicellulosiruptor bescii	Ethanol	0.70
Clostridium acetobutylicum	**Butanol**	**130**[129]
Clostridium acetobutylicum	Mixed alcohols	35[125]
Clostridium thermocellum	Isobutanol	5.4
Corynebacterium glutamicum	Isobutanol	413[130]
Cryptococcus vishniaccii	Lipids	7.8
Escherichia coli	Isobutanol	22
Escherichia coli	Short chain alkanes	0.59[131]
Escherichia coli	**Butanol**	**30**[132]
Escherichia coli	Propanol	10.8[133]
Escherichia coli	Ethanol	25
Escherichia coli	**Isopropanol**	**143**[134]
Pseudomonas putida	Butanol	0.05
Saccharomyces cerevisiae	Fatty acids	0.38
Saccharomyces cerevisiae	Isoprenoid based-biofuel	40
Synechococcus elongates	1, 3-propanediol	0.28
Synechococcus sp.	Limonene	0.04
Trichoderma reesei	Ethanol	10
Yarrowia lipolytica	**Fatty acids**	**55**[135]
Zymomonas mobilis	2, 3-butanediol	10

The introduction of new genes aimed to improve process efficiency and yields. Thus,is often a necessary process. Microorganisms that are lacking the metabolic pathways for a particular compounds synthesis can be modified with the appropriate genes, which would result in an artificial strain with desired properties.[136] Competing pathways that lower the quantity of biofuels or the

precursors (e.g. pyruvate, acyl-CoA), as well as the enzymes that interfere with the biofuel synthesis, can be removed with gene knockout approaches.[137,138] Moreover, the catalytic activity of the enzymes can be enhanced by manipulating its gene expression and mutations.[139]

Another limiting factor in microbial biofuel production is organism tolerance towards the produced compounds. *Pseudomonas putida* were reported to be most resistant to biofuel-like compounds,[140,141] Apart from organism survival, it has been noticed that higher product tolerance affects yield. Thus where the product was either removed from bioreactor or microorganism was modified with genes which enhanced tolerance to the product, higher yields were observed.[142,143]

Pyruvate or acetyl-CoA, which are the basic building block for the biofuel production can be obtained from the organic feedstock.[71] Majority of the research aims for carbon chain extension, which can be achieved by utilisation/modification of existing metabolic pathways (Figure 1.9). The most popular platforms for biofuel production are *E. coli* and yeast, which already contain parts of those pathways. Modern synthetic biology has provided tools that allow the introduction of new pathways in an organism which, without genetic manipulation, are unable to produce desired compounds. Although a wide scope of modification is available, differences, in for example transcription and translation of DNA between prokaryotes and eukaryotes, have to be considered while developing new strains (Figure 1.9). Over the last two decades, progress has been made in identifying critical enzymes and pathways essential for biofuel production.[127,144] Extensive research helped to increase understanding of metabolic pathways regulation, which in turn allowed for improved production rates.[145]

Every living organism has multiple metabolic pathways. Current research involves utilisation and modification, to a different extent, three metabolic processes: the ketoacid pathway, the isoprenoid pathway, and various stages of fatty acid metabolism.

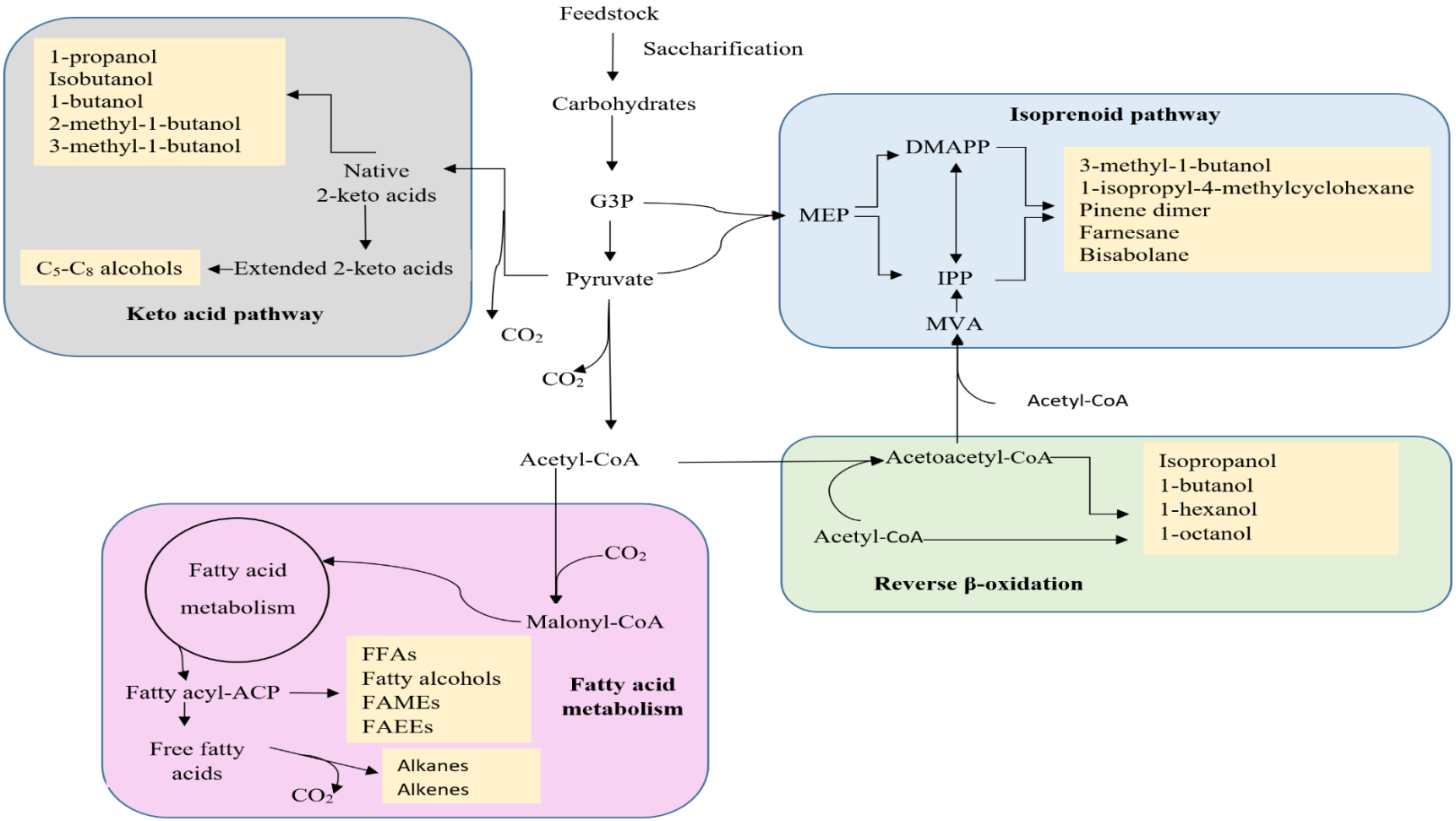


Figure 1.9 Overview of biofuel production strategies. ACP- acyl-carrier protein; FFAs-free fatty acids; FAMEs - fatty acid methyl esters; FAEEs-fatty acid ethyl esters; DMAPP-dimethylallyl pyrophosphate IPP-isopentenyl pyrophosphate; MEP-methylerythriol-4-phosphat. [71]

1.2.1 Keto acid route

This method utilises an intermediate of amino acid biosynthesis, 2-ketoacid. If the metabolic pathway is appropriately modified and decarboxylation occurs, the substrates can be converted to aldehydes, which after reduction can be transformed into alcohols or carboxylic acids (Figure 1.10).[146]

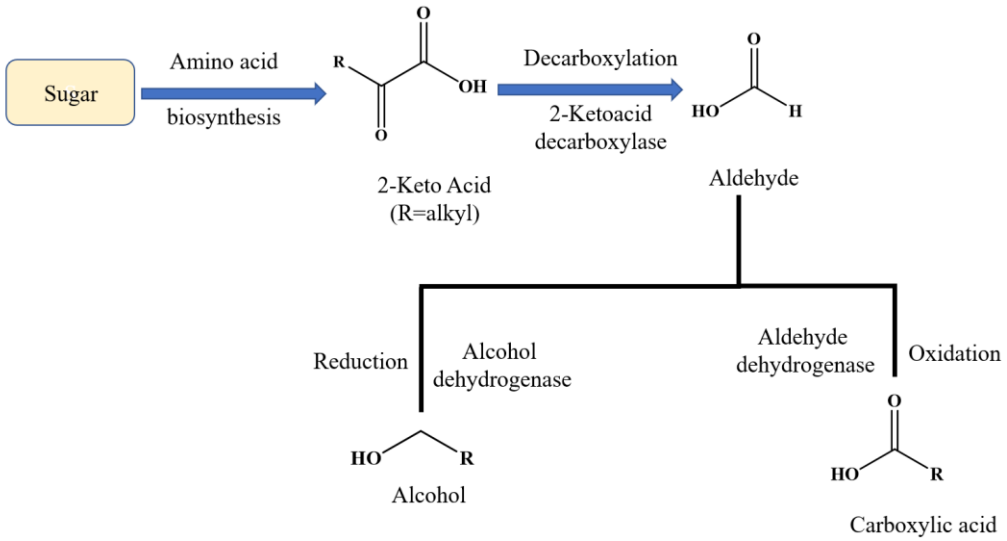


Figure 1.10 2-keto acid pathway for production of aliphatic alcohols and carboxylic acids.

Various compounds including four, five and six carbon alcohols were synthesised using this method. In one study, the production of 22 g/L of butanol was achieved, in which the biosynthetic pathway for valine was engineered.[147] The approach involved the introduction of 2-ketoacid decarboxylase (KDC) from *Lactococcus lactis* and alcohol dehydrogenase from *Saccharomyces cerevisiae* (Figure 1.10).[147,148] The first genetic engineering was applied to *E.coli.* Since then, reports of successful chemicals production *via* this pathway was reported in cyanobacteria,[149,150] yeast,[151,152153] *Bacillus subtilis,*[154] and *Clostridium thermocellum*[155]. Other alcohols were successfully synthesised as well, in which different amino acid pathways were exploited, such as isoleucine and leucine for C5-C8 compounds.[156–158] These studies suggest that synthesis of compounds with chains longer than five carbons do not occur naturally, due to lack of 2-keto intermediate. This issue was addressed with the *leuABCD* gene was introduced, a step which allowed to extend carbon chain.[159,160]

Another approach that involves a ketoacid pathway uses norvaline as a precursor for butanol. Introduction of norvaline biosynthesis into *E. coil* leads

to the production of 2-ketovalerate which was a precursor for butanol. In fact, it was observed that this approach increases yield.[161,162] Alternatively, the citramalate pathway, identified in *Leptospira interrogans* and *Methanocaldococcus jannaschii* can be implemented.[152] In which case, pyruvate is converted into 2-ketobutyrate in one step reaction and then further modified towards butanol.

Furthermore, the α-keto acid pathway is robust and can lead to the production of carboxylic acids if aldehyde dehydrogenase is introduced,[163,164] but is not only limited to it. Significantly, the use of lysine and glutamate as 2-ketoacid precursors could lead to the production of diols[165], diamines[166] or even dicarboxylic acids[167]. Besides various industrial applications, those compounds could be used as building blocks for polymers (e.g. nylon, polyester).

The advantage of this pathway lies in the fact that it incorporates and builds on amino acid biosynthesis, which is already present in the organism. In turn, it enhances the pathway compatibility with different organisms and increases the numbers of modifications that can be introduced.

1.2.2 Reverse β-oxidation

The reverse β-oxidation utilises the non-decarboxylative Claisen condensation pathway, which was first described in 1941.[168] This pathway involves two molecules of acetyl-CoA, which are condensed to yield acetoacetyl-CoA. In the next step, acetoacetyl-CoA is reduced to butyric acid by a β-reduction reaction. This condensation pathway elongates the carbon chain attached to Coenzyme A and uses short-chain acyl-CoAs as substrates, with acetyl-CoA being the compound most widely incorporated in the process. [169] Thus, β-reduction reactions can be achieved by change in direction of the β-oxidation cycle and was first described by Dellomonaco *et al.*[170] In natural conditions, the β-oxidation cycle is designed to shorten fatty acid carbon chain, but in the absence of fatty acids (FA) and the presence of another source of carbon (e.g. glucose), the pathway can be re-engineered to follow the opposite direction, i.e. instead of shortening, the carbon chain it will be elongated. Instead of β-oxidation, the occurring reactions will be β-reduction.

Fatty acid degradation cycle is heavily regulated and requires multiple

gene modifications to release gene repression. Deletion of *fadR* (fatty acid metabolism regulator protein) releases *fad* enzymes which are otherwise repressed in the absence of FAs in which case β-oxidation is inhibited.[171] Moreover, acetyl-CoA acetyltransferase (enzyme classified as thiolase) (Figure 1.11 step 1) was overexpressed to increase its activity. This enzyme in natural conditions catalyses the cleavage of 3-ketoacyl-CoA, however it also capable of conducting reverse reaction, which is condensation of acyl-CoA, an essential step for carbon chain elongation.[172] β-oxidation is also under the regulation of cyclic AMP (cAMP) receptor protein which is repressed in the presence of glucose, to release further repression c*rp* gene was replaced with the cAMP-independent mutant. Thus, the regulatory mechanism was bypassed. Also, the *arcA* gene was deleted to avoid repression of the operons responsible for the β-oxidation cycle. [170] The initial source of carbon (e.g. glucose) is converted *via* glycolysis, to acetyl-CoA, which enters the cycle (Figure 1.11). To terminate the reaction acyl-CoA thioesterase is essential to cleave thioester to final product and CoA. Choice of appropriate thioesterase allows for control and regulation of fatty acid chain lenght.[170]

Non-fatty acid carbon sources are a suitable substrate for this pathway, e.g. glucose and glycerol. Elongation of the carbon chain is achieved by multiple cycles and can be enhanced by overexpression of FadA. So far, the pathway was successfully introduced in *E. coli* [170,173] and yeast[174].

Reversal of the beta-oxidation cycle has been proved to be an efficient method of producing fatty acids. Cintolesi *et al.* argue that it can be a potent tool for the production of a variety of chemicals.[175] This pathway has at least two advantages: it allows incorporation of already existing functional groups in the product, and it introduces orthogonal control of the structures of substrates and molecules added to acyl-CoA substrate alike. This pathway is likely to become more versatile in the future. New enzymes are discovered, which in turn could improve the diversity of the elongated chain. In addition, different strategies are developed to fine-tune and control the number of extension cycles.[176] Although so far only n-alcohols and carboxylic acids were obtained, Clomberg *et al*. postulated, that due to its modularity and fine-tuning with synthetic biology, it could provide a robust platform for the production of diols, fatty acids or

alkane.[177]

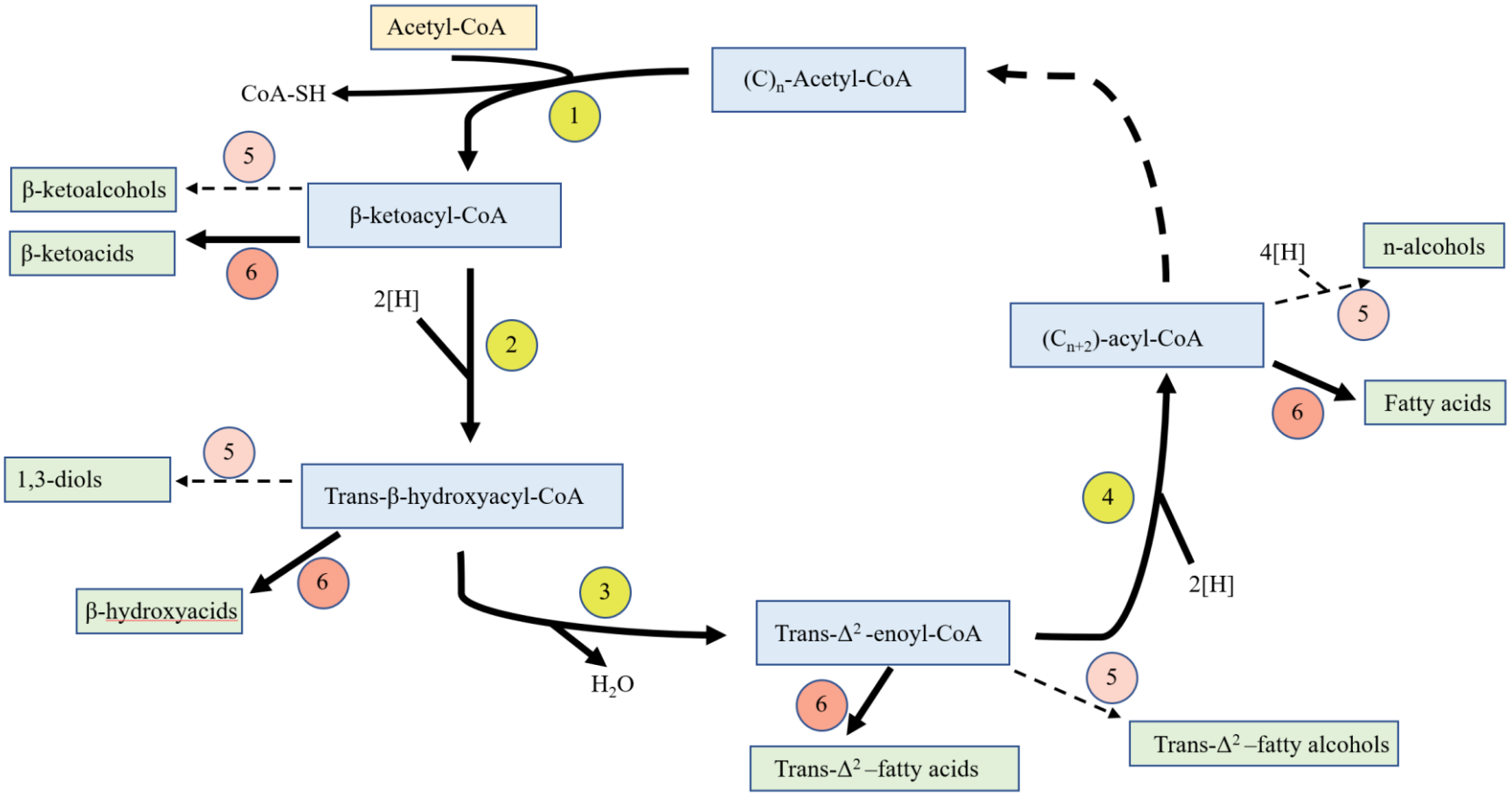


Figure 1.11 Enzymes involved in the reversal of the β-oxidation cycle (1) thiolase; (2) hydroxyacyl-CoA dehydrogenase; (3) enoyl-CoA hydratase; (4) enoyl-CoA reductase. Each turn of the cycle generates an acyl-CoA that is two carbons longer than the first acyl-CoA. Intermediates in the engineered pathway can be converted to different molecules using aldehyde-forming acyl-CoA reductases and alcohol dehydrogenases (5) and acyl-CoA thioesterase (6). Dashed line represents multiple steps

1.2.3 Isoprenoid route

The isoprenoids are also considered as potential source of biofuels, terpenes and terpenoids. This group of compounds contains many industrially useful compounds, including monoterpenes, sesquiterpenes, diterpenes, triterpenes, and carotenoids.[178] The appeal of this pathway lies in the versatility of terpene synthases, which provides means of generating a wide array of diverse products.[179,180] The two common precursors for this pathway are isoprenyl diphosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). In principle, carbon chain extension occurs through the assembly of IPP or DMAPP units to yield polyisoprenoid phosphate which can be converted to terpenes or terpenoids by terpene synthase.[181]

Synthesis with glyceraldehyde-3-phosphate (G3P) and pyruvate as substrates occurs in seven steps *via* the methylerythritol pathway, also known as a non-mevalonate pathway. The 1-deoxy-D-xylulose-5-phosphate (DXP) is assembled by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) (Figure 1.12)[182] DXP is converted to 2-methylerythritol-4-phosphate (MEP) by 1-deoxy-D-xylulose- 5-phosphate isomerase. MEP is cyclised in three steps which involve cytidylate, phosphorylation and cyclisation to obtain 2,4-cyclodiphosphate (MECPP) which is converted to hydroxymethylbutenyl diphosphate (HMPBPP). The last step involves the reduction of DMPP or IPP reductase.[183]

The mevalonate pathway utilizes acetyl-CoA as a substrate which is transformed in six consecutive steps to IPP.[184] Two molecules of Ac-CoA are condensed to acetoacetyl-CoA (AA-CoA) by AA-CoA thiolase. In the next step, another molecule of acyl-CoA is condensed with AA-CoA to yield 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), the reaction is catalysed by 3-hydroxy-3-methylglutaryl coenzyme A synthase. Mevalonate (MVA) is obtained by NADPH dependent reduction of HMG-CoA by reductase. MVA is then converted to mevalonate 5-diphosphate (MVAPP) by mevalonate kinase and phosphomevalonate kinase in two-step phosphorylation. In final step ATP-dependent decarboxylation converts MVAPP to IPP (diphosphomevalonate decarboxylase).[185]

Although the engineering of this pathway is still at an early stage, the isoprenoid pathway is a valuable asset in biofuel production since many terpenes

and terpenoids show diesel and jet-fuel like properties. Compounds such as pinene and limonene could be produced through geranyl pyrophosphate (GPP) for jet fuels and through farnesyl pyrophosphate (FPP) intermediate to yield farnesene and bisabolene which could potentially substitute diesel fuels.[176] Other identified products of this pathway with potential industrial applications are linalool, 1,8-cineole[186] and geraniol[187].

Overexpression of native enzymes in *E. coli* and *S. cerevisiae* has been used to increase the availability and quantity of precursors,[188] but the introduction of foreign genes into those organisms is also pursued.[189] Such strategies were employed for *E. coli* carotenoid production, and so far platforms utilising genes from *Erwinia uredovara*[190] and *Brevibacterium linens*[191] were successfully developed. More recent literature identified *Bacillus subtilis* ability to biosynthesise isopentenol from the IPP precursor.[192] Moreover, the *E.coli* strain was engineered to produce isoprenol and phenol by the introduction of a pyrophosphatase from *Bacillus subtilis*.[193] Production of farnesol and farnesene was achieved in *E. coli* and *S. cerevisiae* with the introduction of a yeast phosphatase and some plant terpene synthases.[194]

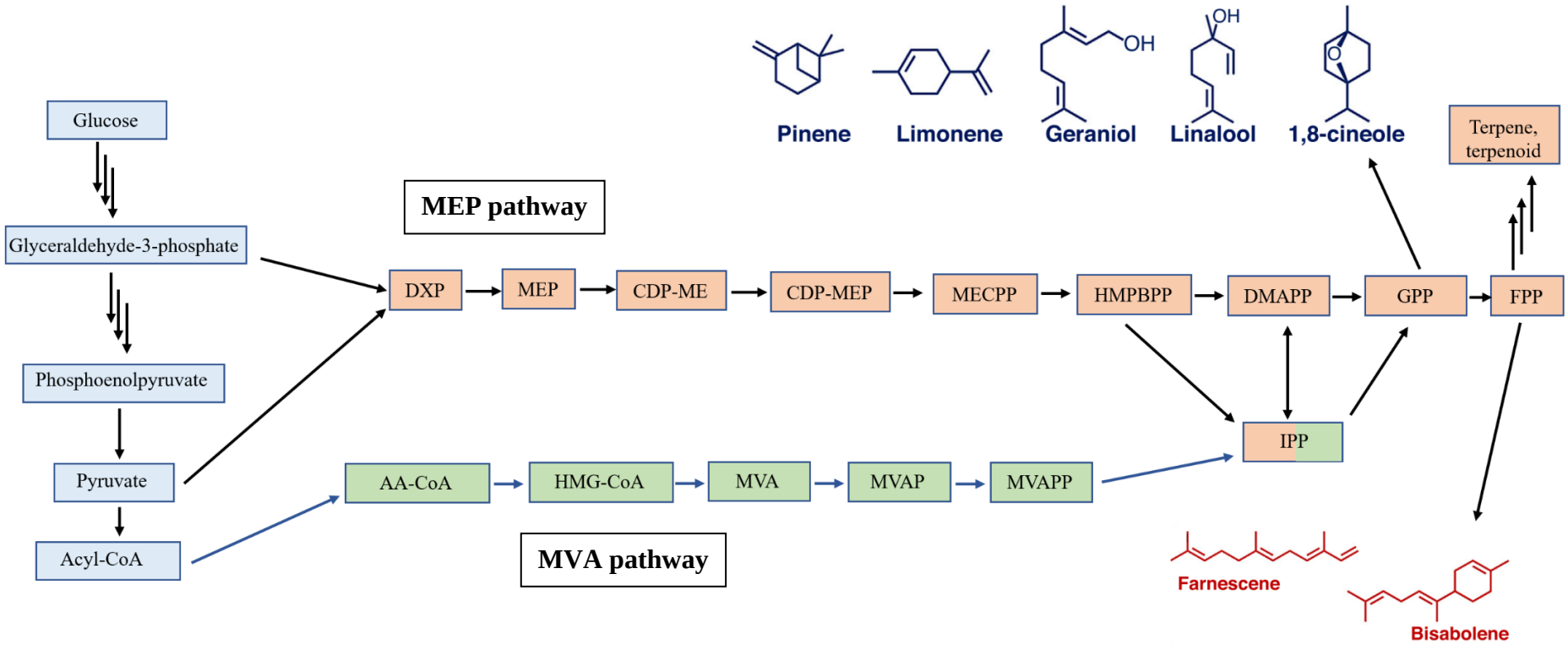


Figure 1.12 Isoprenoid pathways. Mevalonate pathway in light green. MEP pathway in orange. AA-CoA: acetoacetyl-CoA; MVA: mevalonate; MVAP: mevalonate-5-phosphate; MVAPP: mevalonate-diphosphate; MEP: 2-methylerythritol-4-phosphate; CDP-ME: 4- diphosphocytidyl-2-C-methylerythritol; CDP-MEP: 4-diphosphocytidyl-2-C-methyl-D-erythritol-2-phosphate; HMB-PP: (E)-4-hydroxy-3-methyl-but- 2-enyl pyrophosphate. Adapted from[177]

1.2.4 Fatty acid metabolism modification

Acyl-ACP is a vital product of the FA biosynthesis, which is further modified in cells as required (e.g. as membrane building blocks). In the production of fatty acid derived chemicals, FFAs are key intermediates. Thus acyl-ACP must be cleaved. This issue has been addressed in multiple studies on acyl-ACP thioesterases (ACPs). ACPs catalyse the hydrolysis of the thioester bond that links the acyl chain to the sulfhydryl group of ACP.[131,195–199] Most of the investigations focused on thioesterase overexpression and the introduction of enzymes from different organisms (e.g. plants[197] and bacteria[199]). Additionally, thioesterase substrate preference has been reported[200–202] which triggered a search for the more versatile enzyme.[203,204] Strategies that boost enzymes of fatty acids biosynthesis are also researched. Those include enzyme overexpression[205] and/or regulator gene (*fadR*) deletion[131]. Choi and Lee achieved short chain alkane formation (SCA) in *E. coli*, through the conversion of the fatty acid acyl-CoA to free fatty acid.[131] It was achieved by introduction of new enzymes into engineered microorganisms: fatty acyl-ACP thioesterase, fatty acyl-CoA synthetase, fatty acyl-CoA reductase and fatty aldehyde decarbonylase. The limiting step in the process is the inactivation of one of the acyl-CoA dehydrogenases (ACADs), specifically the short-chain acyl-CoA dehydrogenase (FadE) which is responsible for the first step of FA acid degradation. Therefore, for the pathway to work, FadE has to be inhibited, either by gene knock out or chemical inhibition.[131]

Through numerous genetic modifications production of various FFAs derived chemicals can be achieved. Inactivation of β-oxidation cycle (Figure 1.13) leads to the production of fatty alcohols, SCA, alkanes, fatty acid methyl and ethyl esters (FAME and FAEE). Depending on the introduced genes production of FFA and fatty aldehydes was also achieved. Production of FAEEs was achieved through wax ester synthase/acyl-CoA: diacylglycerol acyltransferase (WS/DGAT) (Figure 1.13) introduced from *Acinetobacter sp*. or *Marinobacter hydroxycarboniclasticus* [206,207], but the process requires further experimentation due to different substrate specificity. Promising results and yields for FAMEs production were achieved by introduction of O-methyltransferase (*Mycobacterium marinumi*) to *E. coil*[208] Although waxes and

oils with long-chain FA cannot be directly used as fuel, they can find applications in other branches of industry, such as food additives or lubricants.[144,206]

Given the versatility of the metabolism and an ability to modulate or manipulate the pathway for biofuel product, a detailed account of fatty acid metabolism is outlined below.

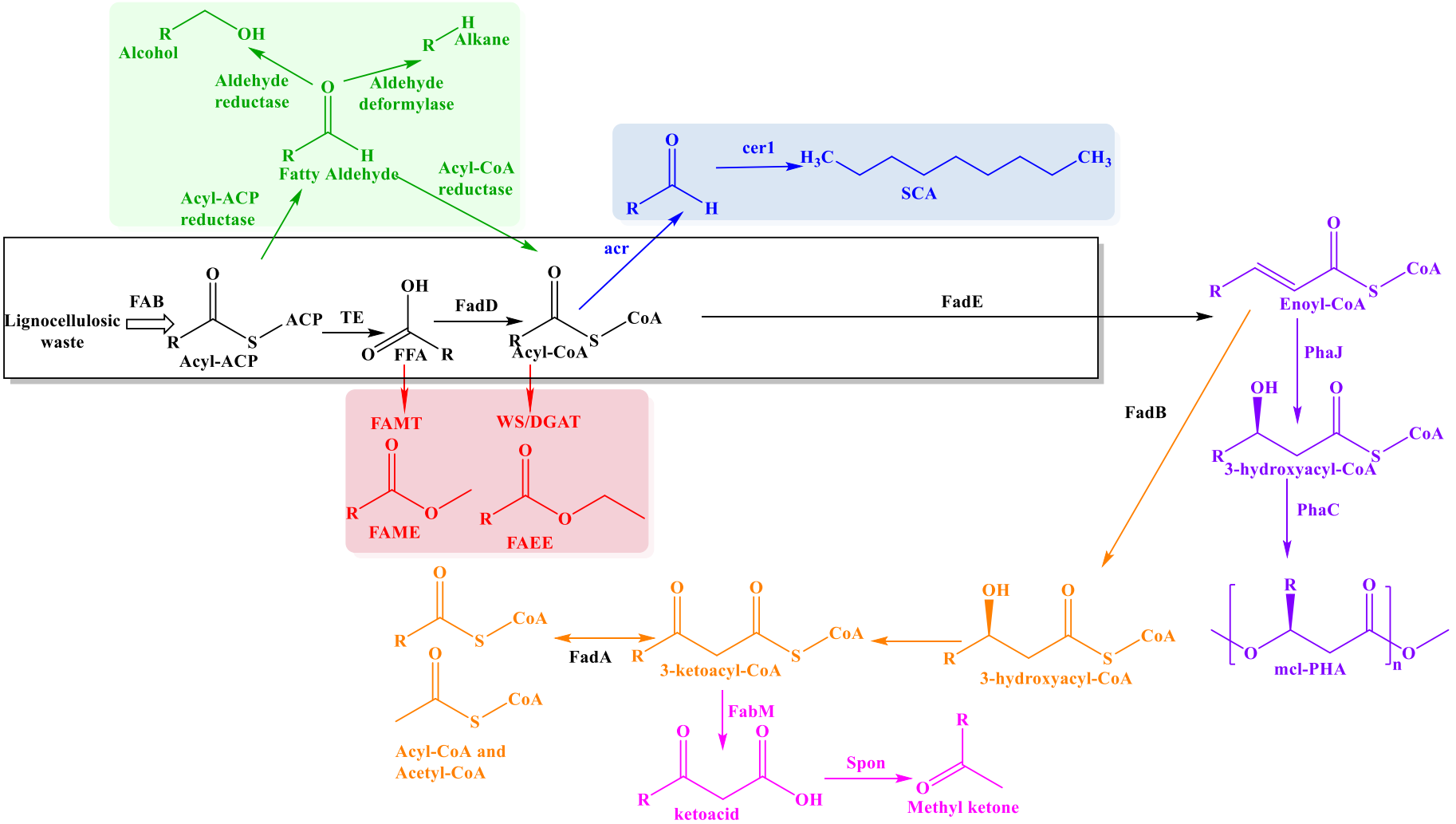


Figure 1.13 Overview of metabolic engineering of fatty acid metabolism leading to chemicals production. Pathways in coloured boxes achieved with FadE inhibition, if FadE and FadB inhibited dark blue pathway occurs. Inhibition of FadA could lead to pink pathway. Enzymes written in colour are foreign to *E. coli*

1.3 Fatty acid metabolism

1.3.1 Fatty acid biosynthesis

Free fatty acids (FFA) are present in large quantities in plant oils (e.g. palm oil, rapeseed). Thus, they seem to be a perfect carbon source for microbial biofuel production. However, for the reasons mentioned earlier alternative materials are required. Due to its abundance, accessibility and low costs, lignocellulosic waste (e.g. straw, wood chip) are one of the considered resources.[209,210] To date, *Saccharomyces cerevisiae* and *Escherichia coli* have received the most attention as a platform capable of fine chemicals production.[175,211–213] *E. coli* has the advantage of being able to grow on pentose sugars (xylose, arabinose)[214] which are one of the products of lignocellulosic degradation,[161] while yeast requires genetic modification. [215] In the abundance of sugars, and when required, biosynthesis of fatty acids is triggered (Figure 1.14). Fatty acids have a diverse function in the cell (membrane formation, ATP source, signalling molecules). Thus, their biosynthesis is essential and highly conserved among living organism.[216,217]

In *E.* coli the process begins with glucose transformation to pyruvate and then acetyl-CoA (Figure 1.14). Acetyl CoA carboxylase (AccABCD) converts acetyl-CoA to malonyl-CoA, then CoA is exchanged for acyl carrier protein (ACP) by malonyl-CoA catalysed by ACP transacylase (FabD). The ACP unit protects the fatty acid moiety from degradation. Malonyl-ACP then condenses with acetyl-CoA, which is catalysed by β-ketoacyl-(ACP) synthase III (FadH). The product reacts with acyl-ACP to give 3-ketoacyl-ACP (catalysed by 3-ketoacyl-ACP synthase, FabB), which is then reduced by 3-oxoacyl-[ACP] reductase (FadG). The next step involves 3-hydroxyacyl-ACP dehydrase (FabA, FabZ), which removes water from 3-hydroxyacyl-ACP. Then obtained enoyl-ACP is finally reduced to acyl-ACP by enoyl-[ACP] reductase (FabI). The newly biosynthesized acyl-ACP condensates with malonyl-ACP and follows another cycle. The cycle is terminated when the chain reaches required length.[217]

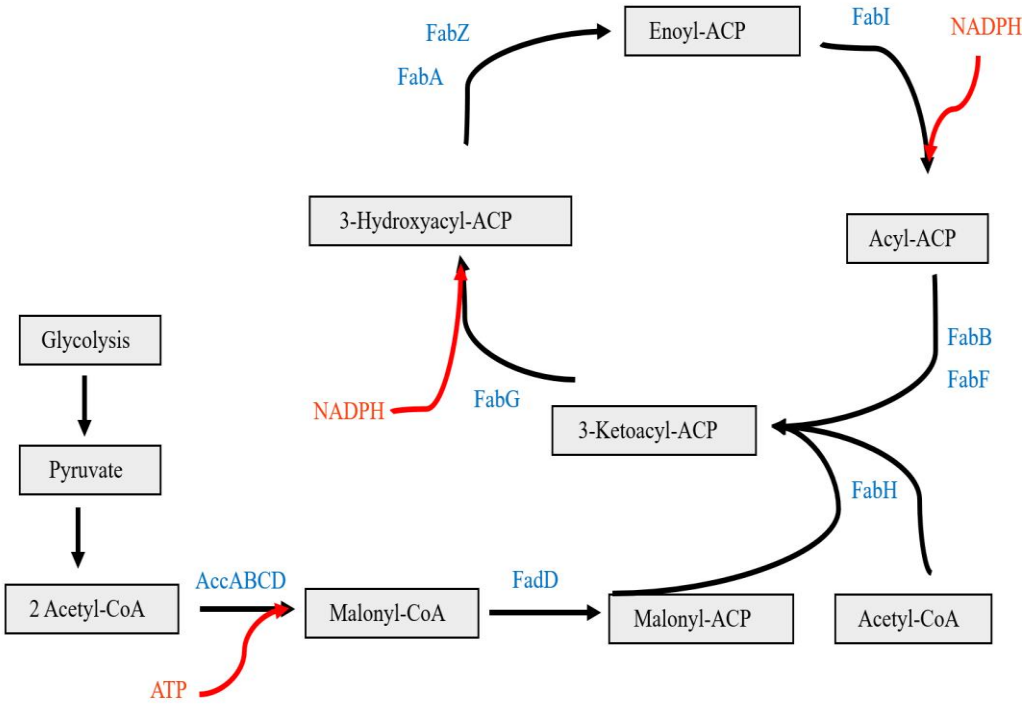


Figure 1.14 Fatty acid biosynthesis cycle - overview. FAB – fatty acid biosynthesis

1.3.2 β-oxidation cycle

The conversion of FFA to acyl-CoA esters is a vital step before FFAs can be metabolised (Figure 1.15). If fatty acids are from an external source, outer membrane transporter protein (FadL) coupled with acyl-CoA synthase (FadD) is required.[218,219] FadL is responsible for extracellular molecule transport to the periplasm. It is classified as a porin. Hearn *et al.* proposed a lateral diffusion mechanism of action for this transporter protein.[220] The hydrophobic compounds (long chain lipids) diffuse laterally through the outer membrane, a process that is possible via the hydrophobic passageway. The authors speculate that transport occurs through a stable lateral opening in the barrel wall. This mechanism allows bypassing the hydrophilic lipopolysaccharide layer of the outer membrane. In addition, the process is spontaneous and energetically favourable in comparison to an alternative aqueous channel mechanism. Interestingly in *E. coli* medium chain, fatty acids do not require FadL to pass through outer membrane.[220] FadD is responsible for acyl-CoA thioester formation at the expense of ATP. Thus, FFAs enters the cell as a coenzyme A thioester.[217] Pech-Canul *et al.* demonstrated that FadD could also utilise FFAs already present in cells, for

example from membrane degradation. If the *fadD* gene is deleted, the free fatty acids accumulate in the cytosol.[221] The next step is oxidation to enoyl-CoA (i.e. double bond is introduced at the β carbon). This reaction requires the reduction of a FAD to $FADH_2$ and is performed by acyl-CoA dehydrogenase (FadE). The following enzyme (FadB) performs two further steps of β-oxidation. First, it hydrates enoyl-CoA to 3-hydroxyacyl-CoA followed by oxidation to

3-ketoacyl-CoA. The last step catalysed by β-ketothiolase (FadA) yields acetyl-CoA and acyl-CoA (shorter by two carbons in comparison to the initial substrate). Acyl-CoA from the last step can enter another cycle.[222] In contrast to eukaryotic cells, *E. coli* has only one FadD with broad substrate specificity. Enhancement of FadD activity, i.e. oxidation of a saturated hydrocarbon to unsaturated hydrocarbon moieties, could be achieved by overexpression[131,223] or directed mutagenesis[224] In studies that utilise FFAs for biofuel production, *fadE* is a knockout target to avoid product degradation. [203,224,225]

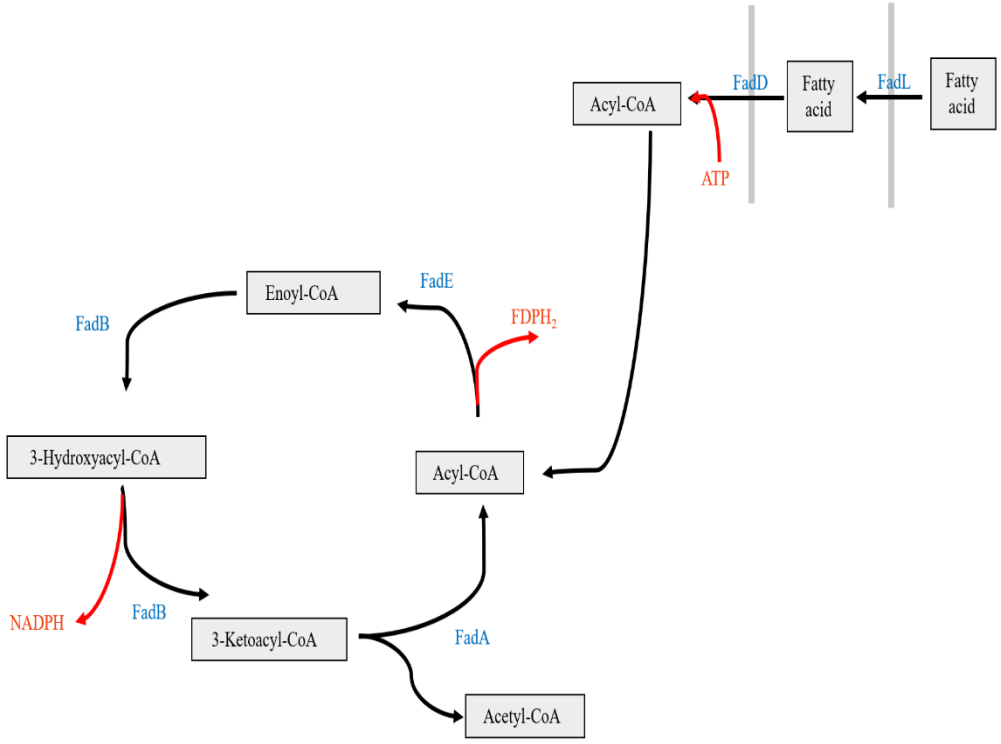


Figure 1.15 β-oxidation cycle – overview. Grey, vertical lines indicate cell membrane

1.3.3 FadR regulon

A key regulator of fatty acid metabolism in FadR protein (encoded by *fadR*) which is the fatty acid-responsive transcription factor (Figure 1.16). FadR acts as a molecular switch for the fatty-acid biosynthesis and degradation.[226] Acyl-CoA ester binds to FadR, which causes its release from the operator occurs. Thus repression of β-oxidation cycle enzymes genes (*fad*) is averted.[222,227] In nature, an external source of fatty acids or membrane degradation can cause genes release.[228] If glucose and FA are both present β-oxidations occurs only at a low

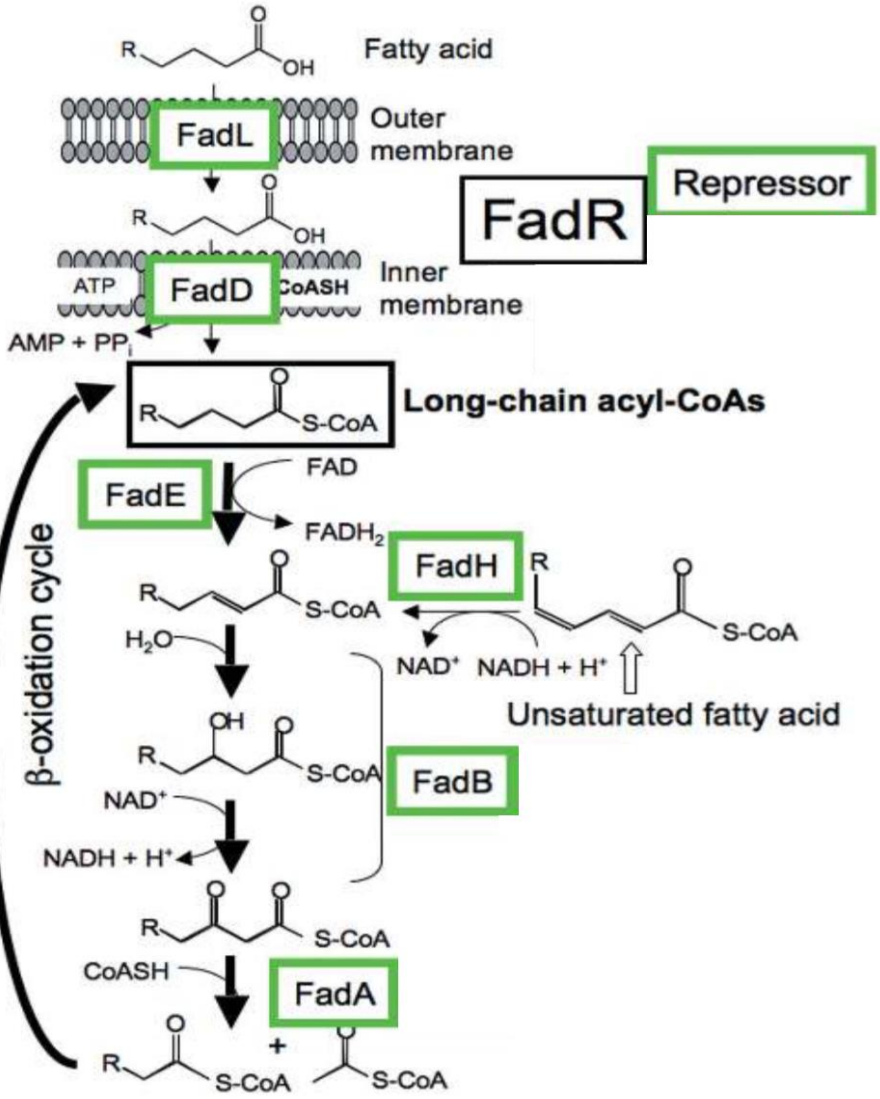


Figure 1.16 Fatty acid degradation in E. coli and its regulation through the FadR protein. Long-chain fatty acids are transported across the cell membrane *via* transport/acyl-activation mechanism involving an outer membrane protein, FadL, and inner membrane-associated FadD protein. [221]

level due to glucose being the preferred carbon source.[217] Apart from *fad* genes, FadR is also responsible for the activation of fatty acid synthesis through positive control of *fabA, fabB* and *fabHDG* genes.[229,230] In the study by Overath *et al.* all *fad* genes were active in *E.coli* mutant with no functional FadR, but at the same time concentration of unsaturated fatty acids decreased.[231]

1.3.4 Fatty acid metabolism manipulation and target of the chemical inhibition approach

Discovery of new genes and a better understanding of metabolic pathways allows for new methods of fine chemicals and biofuel production. Approaches with versatile strains of microbes as well as industrial-scale production are yet to be developed. One of the possible routes is chemical modulation of the enzymes to enable switching on/off enzyme activity depending on the inhibitor. For example, acyl-CoA dehydrogenase (FadE) is identified as one of the crucial enzymes in microbial biofuel production which targets fatty acid metabolism. In addition, the enzyme mechanism of action was already established and will be discussed in detail below and in Chapter 2. Therefore, its substrate could be the basis for inhibitor development.

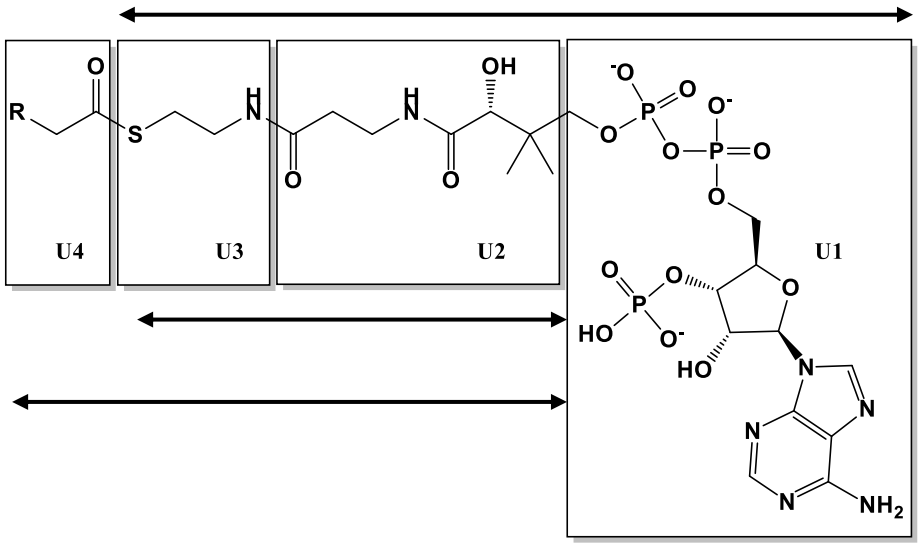


Figure 1.17 Structure of acyl-CoA. U1 - adenosine 3-phosphate 5-pyrophosphate unit, U2 – pantothenate unit (PA), U3- cysteamine unit, U4 - fatty acid unit, R – fatty acid chain

In fact, FadE can be inactivated by a variety of mechanism-based inhibitors, including 2-alkynoyl-CoA, 2-pentynoyl-CoA, 2-bromoalkyl-CoA and vinylacetyl-CoA.[232] The utilisation of this mechanism would allow for the on/off enzyme switch. Thus, β-oxidation activity could be maintained, and when desired, the production of target compounds could be initiated. 2-Pentynoyl-CoA (Figure 1.18) was reported to inactivate acyl-CoA dehydrogenase at a rate that is significantly higher than other related inhibitors. Thus it was chosen as a base for structural refinements.[233,234] The main disadvantage in the use of 2-pentynoyl-CoA as a chemical inhibitor is its high molecular weight, which makes it unlikely to penetrate the bacterial cell membrane (Figure 1.17 and 1.18). Fortunately, it is known that pantetheine derivatives are recognised by the enzyme and can compete with the substrate.[235] These analogues have a smaller size as they lack

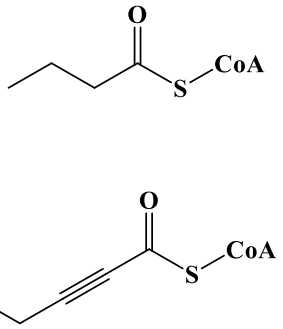


Figure 1.18 Structural comparison between FadE inhibitor 2-pentynoyl-CoA (B) and substrate butyryl-CoA (A)

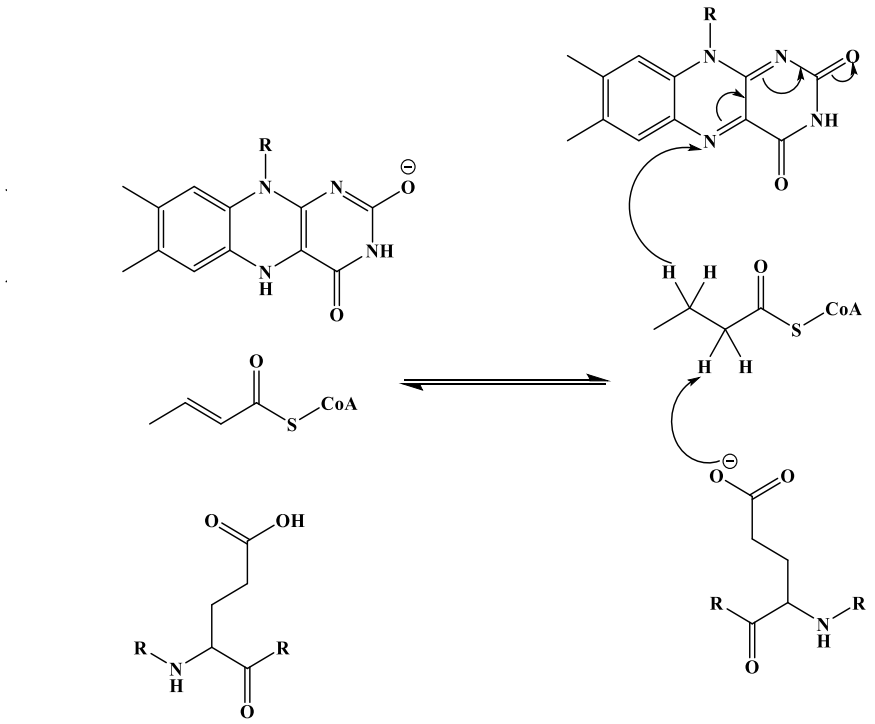


Figure 1.19 Acyl-CoA dehydrogenase (FadE) mechanism overview. Adapted from Ghisla and Thorpe.[238]

adenosine 3-phosphate 5-pyrophosphate unit (Figure 1.17 U1).

Acyl-CoA dehydrogenase inhibitors interact with glutamate residue of the enzyme. [233,234] Uninterrupted enzyme catalyses introduction of a double bond between α and β carbons of hydrocarbon moiety. The FadE mechanism is based on E2 elimination and requires initiation by glutamate (Glu) residue and FAD as a cofactor. Depending on the enzyme the Glu residue can appear in a range of locations.[236] The enzyme follows a mechanism (Figure 1.19) described below. Thus, the hydrogen of the α-carbon is deprotonated by the Glu (functioning as a general base). As a result, the substrate is converted into unsaturated thioester. As hydrogen is transferred, carbonyl oxygen adjacent to N-1 nitrogen receives a negative charge.[236]

1.4 Aims and Objectives

Acyl-CoA thioesters are key intermediates required for short chain alkane production. FadE is a pivotal enzyme as it catalysis conversion of acyl-CoA to enoyl-CoA (first reaction of β-oxidation). Thus, it is beneficial to inactivate the enzyme to avoid acyl-CoA thioesters degradation. To the date, most attempts at FadE inhibition were based solely on genetic manipulation by gene deletion.[131,237–239] This approach leads to "one mutant-one product" strategy as well as to permanent metabolic pathway modification which might hinder bacterial growth and decrease the final yield of chemicals, which in the long run is not efficient. Moreover, if the broader spectrum of FFAs derived compounds are considered (not just SCA), not all engineered approaches require FadE inhibition.[144,237] Interestingly, many mechanism-based FadE inhibitors were reported.[232] Additionally contrary to other organisms FadE seems to be only acyl-CoA dehydrogenase present in *E.coli*, [229] which makes it even better target for chemical inhibition.

The primary aim of this project was a synthesis of a library of potential acyl-CoA dehydrogenase inhibitors. As mentioned before, derivatives of acyl-CoA (S-acyl pantetheines) are recognized by bacterial enzymes; therefore, they will be used as a basis to build further analogues.[232] 2-pentynoylpantetheine was reported as a potent and rapid FadE inhibitor and was used as a reference for further modifications and assays.[240]

We also aimed to test the potency of the synthesized compounds on overexpressed and purified acyl-CoA of *E. coli.* In addition, the purified enzyme was purified and characterised.

Simultaneously the *E. coli* strain overexpressing, modified fatty acyl-ACP thioesterase (TesA, *E. coli*), fatty acyl-CoA synthetase (FadD, *E. coli*), fatty acyl-CoA reductase (AcR, *Clostridium acetobutylicum*) and fatty aldehyde decarbonylase (Cer1, *Arabidopsis thaliana*) was developed.

Chapter 2

Materials and Methods

2.1 Materials and methods: chemical synthesis of S-acyl pantetheine analogues

2.1.1 Materials

All chemicals were purchased from Sigma-Aldrich, Acros Organics, VWR International, Merck-Millipore, Alfa Aesar, and used without further purification. Solvents used were either reagent or high-performance liquid chromatography (HPLC) grade and were purchased from Acros Organics, Fisher Scientific or Sigma-Aldrich. Anhydrous dichloromethane (DCM) and tetrahydrofuran (THF) were used as supplied by Acros Organics. Deuterated chloroform ($CDCl_3$) was purchased from Cambridge Isotope Laboratories Inc. and deuterated methanol (CD_3OD) were obtained from Sigma-Aldrich. Milli-Q water was used for chemical reactions, purification and biological assays. Solvents used for column chromatography were reagent grade. Silica gel was obtained from.

General Methods and Instrumentation

Unless otherwise stated all reactions were carried out at room temperature, moisture under a nitrogen atmosphere using oven-dried glassware. Reactions were monitored using one or more methods described below:

- Analytical TLC on commercially available pre-coated aluminium backed plates (Merck Kiesegel 60 F254). The visualisation was achieved using UV lamp (λ = 254 nm) and cerium molybdate stain (Hanessian`s stain – 12 g of ammonium molybdate and 0.5 g of ceric ammonium molybdate were dissolved in 235 m of Mill-Q water, and 15 mL of

concentrated sulphuric acid was slowly added to the mixture. The stain was stirred for 1 h and stored in 250 mL wide mouth jar).

- Liquid Chromatography-Mass Spectrometry (LC-MS) was performed on a Shimadzu Corp. UFLCXR system coupled to an Applied Biosystems API2000 LC/MS/MS mass spectrometer. Two columns at 40^{o}C were used.

Column one: Phenomenex Gemini-NX 3m-110A C18, 50x2 mm

Column two: Phenomenex Luna 3m (PFP2) 110A, 50x2 mm.

Flow rate 0.5mL/min UV detection at 220 (channel2) and 254 nm (channel 1). SHORT Gradient: Pre-equilibration run for one min at 5% B; then method run: 5 to 98% solvent B in 2 min, 98% B for 2 min, 98 to 5% B in 0.5 min then 5% for one min

- Analytical reverse-phase liquid chromatography (RP-HPLC) as described below.

Organic solvents were evaporated *in vacuo* at ≤ 40°C (water bath). Following work up all organic extracts were dried over anhydrous sodium sulphate, filtered under gravity and evaporated to dryness. If necessary lyophilization from water was achieved with Edwards Modulyo FD freeze drier.

RP-HPLC was performed on a Waters 510 twin pump using an Onyx Monolithic-C_{18} column (100 x 4.6 mm) at

RP-HPLC was carried out with a Waters 510 twin pump coupled with a 2487 dual wavelength detector (λ=216). Solvent A was 0.06% trifluoroacetic acid (TFA) in water, and Solvent B was 0.06% TFA in 90% aqueous acetonitrile. The linear elution gradient was 20–50 % B over 9 min at 3.0 mL/min.

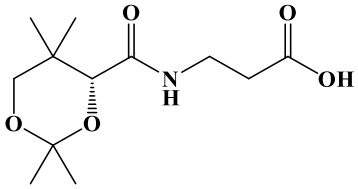
^{1}H-Nuclear Magnetic Resonance (NMR) spectra were obtained at 20°C on a Bruker AV400 operating at 400.13 MHz. Residual protons for solvents (δ) were reported in parts per million (ppm), referenced to $CDCl_3$ (δ_H = 7.26 ppm) and CD_3OD (δ_H = 3.31 ppm). The residual protons for solvents are the result of its contamination with non-deuterated solvent. In case of chloroform, it is $CHCl_3$ and for methanol CHD_2OD. Coupling constants (*J*) are given in Hz, and significant multiplicities are described as; singlet (s), doublet (d), triplet (t), the quartet (q), broad (br), multiplet (m).

^{1}H assignments were achieved using correlation spectroscopy (COSY). ^{13}C-NMR spectra were obtained at 20°C on a Bruker AV400 operating at 100.12 MHz. Chemical shifts (δ) were reported in parts per million (ppm), referenced to $CDCl_3$ (δ_H = 77.16 ppm) and CD_3OD (δ_H = 49.00 ppm).

Melting points (m.p.) were determined in a glass capillary using Gallenkamp 3A 3790 melting point apparatus and are uncorrected. Melting point was reported for pantothenic acid acetonide (2.28) due to its crystallisation. Other compounds synthesised during this study were obtained as oil or sticky solid which prevented the reliable measurements of melting point.

All SDS-PAGE gels were run and visualised with Bio-Rad Protein Electrophoresis and Blotting system. Activity assay was carried out with TECAN Infinite M200 apparatus.

2.1.2 Preparation of pantothenic acid acetonide (2.28)

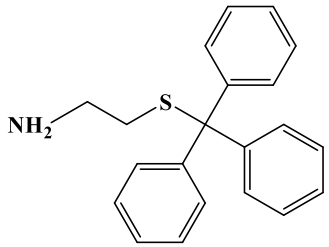


D-Pantothenic acid hemi calcium salt (10.00 g, 38.6 mmol), p-toluenesulfonic acid monohydrate (9.58 g, 55.6 mmol), and 10.00 g of 3 Å molecular sieves were suspended in 500 mL of reagent grade acetone. The flask was capped, and the suspension was stirred at room temperature for 12 h. After which 4.79 g of PTSA and 5.00 g 3 Å molecular sieves were added, the reaction was capped again and stirred for an additional 12 h. The thick slurry was filtered through Celite and washed with acetone (3 x 200 mL). The filtrate was concentrated to a viscous, pale yellow oil. The oil was dissolved in 200 mL of EtOAc, washed with brine (2 x 100 mL). The organic layer was dried with Na_2SO_4. The EtOAc was removed *in vacuo*. Compound **2.28** was recrystallized from EtOAc and hexane (5:1), dried under high vacuum to give the title compound as a white crystals (9.07 g, 83%); m.p 108.6° C; ^{1}H-NMR (400 MHz, CD_3OD): δ 4.15 (s, 1H), 3.81 – 3.52 (m, 2H), 3.52 – 3.39 (m, 2H), 2.55 (t, *J* = 6.7 Hz, 2H), 1.47 (s, 6H), 1.01 (d, *J* = 8.4 Hz, 6H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.11, 170.31, 99.09, 77.00, 71.35, 34.15, 34.03, 33.77, 32.96, 29.36, 21.97, 18.76 ppm **;** MS: *m/z*(+ESI) $C_{12}H_{22}NO_5^+$ cacld. 260.15, found 260.42

[M+H]+

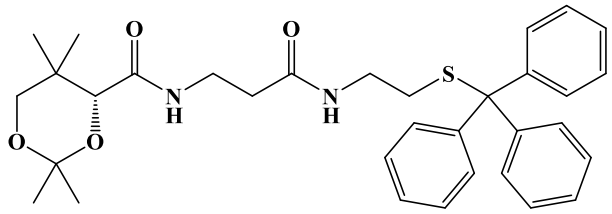
2.1.3 Synthesis of S-acylpantetheines

2-(tritylthio)than-1-amine (trityl cysteamine (2.23))



Trityl chloride (4.20 g, 15.1 mmol) was added to a stirred solution of cysteamine hydrochloride (1.50 g, 14.4 mmol,) and trifluoroacetic acid (2.22 mL, 28.7 mmol) in anhydrous DCM (30 mL,) equipped with a drying tube. The solution immediately turned a dark green colour. After 1 h, the reaction mixture was quenched with 1 M aqueous (aq.) NaOH (30 mL) – solution changed to clear. Dichloromethane (75 mL) and an additional 1 M NaOH (20 mL) were added. The organic and aqueous layers were separated, and the organic extract was washed with brine and dried over magnesium sulphate ($MgSO_4$), filtered and concentrated *in vacuo*. The crude yellow oil was purified by flash chromatography (3:7 Me_2CO: EtOAc) to give the title compound (**2.23**) as a pale yellow oil (2.87 **g**, 68%) C; ^{1}H-NMR (400 MHz, CD_3OD): δ 7.41 (m, 6H), 7.27 (m, 6H), 7.20 (m, 3H), 2.57 (t, J = 8 Hz, 2H), 2.33 (t, J = 8 Hz, 2H) ppm; MS: *m/z*(+ESI) $C_{21}H_{22}NS^+$ cacld. 320.14, found 320 [M+H]+

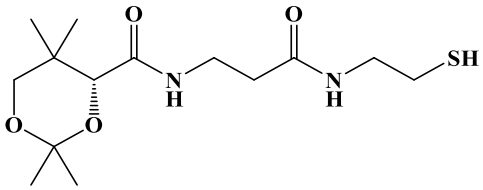
S-trityl pantetheine acetonide (2.31)



Pantothenic acid acetonide (0.80 g, 3.1 mmol, **(2)**), EDC (1.2 g, 6.1 mmol), HOBt (1.4 g, 9.3 mmol) and 2-tritylmercapto-ethylamine (1.49 g, 6.1 mmol, **(4)**) were combined and dissolved in anhydrous THF (30 mL). DIPEA was added (3.24 mL, 18.6 mmol) and the mixture was stirred for 48 h. Saturated aq. ammonium chloride (5 mL) was added to quench the reaction. THF was

evaporated and water were added (15 mL). The water phase was extracted with diethyl ether (3 x 25 mL). The combined organic phase was dried over $MgSO_4$, filtered and concentrated *in vacuo*. The residue was purified with flash chromatography (1:9, Hexane:EtOAc) to give title compound as a yellow oil (1.2 g, 71%); ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.14 (m, 15H), 4.08 (s, 1H), 3.77 – 3.37 (m, 3H), 3.21 – 2.95 (m, 2H), 1.47 (s, 3H), 1.42 (s, 6H), 1.04 (s, 3H), 0.96 (s, 3H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.48, 168.7, 129.59, 128.92, 127.93, 101.5, 74.63, 72.12, 66.96, 39.5, 38.91, 35.27, 28.27, 24.58, 21.85 ppm; MS: *m/z*(+ESI) $C_{33}H_{41}N_2O_4S^+$ cacld. 561.27, found 561.5 [M+H]+

Pantetheine acetonide (2.29)

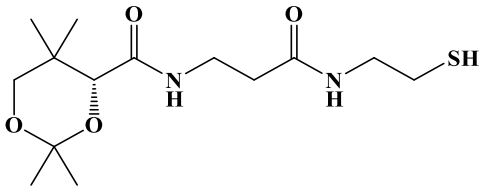


a) Compound **2.31** (23 mg, 0.041 mmol) was dissolved in MeOH (5 mL) and 0.1 M solution of iodine in MeOH (5 mL) was added. The reaction was monitored with TLC every 10 minutes. After 30 min, zinc metal was added to reduce iodine. The solution was filtered through Celite and evaporated *in vacuo* to give a pale-yellow oil. The product was dissolved in 10 mL of DCM and 1 M aq. sodium thiosulphate (5 mL) was added to reduce excess iodine. The organic layer was separated in a separation funnel, dried over $MgSO_4$, filtered and evaporated to dryness to yield a dark orange oil (80 % crude) The oil was purified by flash chromatography (7:3 EtOAc/Hexane). The obtained orange oil was suspended in 10 % MeCN in water and flashed through Biotage Isolute C18 reverse phase column. The column was washed with 40 mL of 10 % MeCN and then with 20 mL of 20-100% MeCN. The fraction collected between 30%-50% of MeCN were combined and evaporated in vacuo. The product was dried under N_2 flow and to give the title compound as a pale orange oil (15%). LC-MS 319.3, $[M+H]^+$).

b) Compound **2.31** (437.6 mg, 0.78 mmol) was dissolved in DCM (3 mL), TFA (3 mL) and TIPS (0.058 mL) were added. The reaction was stirred under N_2 for 3 hours. The reaction was monitored with TLC every 30 min until completion.

The reaction was quenched with sat.aq. NH_4Cl (3 mL). The product was extracted with DCM (3x10 mL) The organic layer was washed with 5 mL of brine, dried over Na_2SO_4, filtered and concentrated in vacuo. TLC and TOF-MS reviled compound decomposition.

Pantetheine acetonide (2.29)



a) Cysteamine hydrochloride approach

Compound **2.28** (3.69 g, 14.2 mmol) was dissolved in dry THF (70 mL), treated with 1,1-carbonyldiimidazole (CDI; 3.42 g, 21.34 mmol) and stirred for 1.5 h at room temperature. To this solution, cysteamine hydrochloride (1.64 g, 21.34 mmol) was added and stirred at room temperature for 36 h. The THF was concentrated in vacuo, and DCM was added (100 mL). The organic solution was washed with sat. aq NH_4Cl (1 × 50 mL) and brine (1 × 50 mL), dried over Na_2SO_4, filtered and concentrated to a pale-yellow oil. The oil was purified by flash chromatography (7:3 EtOAc/Hexane to 100% EtOAc). The solvent was removed *in vacuo.* Clear oil was obtained and dissolved in diethyl ether (20 mL). Equal volume of hexane was added and left overnight at room temperature for crystallisation to give title compound **2.29** as awhite sticky solid (3.46 g, 76%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.14 (s, 1H), 3.76 (d, *J* = 11.6 Hz,1H), 3.59 – 3.43 (m, 2H), 3.46 – 3.32 (m, 2H), 3.28 (d, *J* = 11.7 Hz, 1H), 2.62 (t, *J* = 6.8 Hz,2H), 2.46 (t, *J* = 6.7 Hz, 2H), 1.46 (d, *J* = 3.3 Hz, 6H), 1.00 (d, *J* = 9.2 Hz, 6H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.12, 170.25, 99.09, 77.41, 77.25, 77.09, 76.77, 71.38, 42.47, 36.14, 29.47, 24.53, 22.15, 18.92, 18.71 ppm; MS: *m/z*(+ESI) $C_{14}H_{27}N_2O_4S^+$ calcd. 319.17, found 319.30 [M+H]+

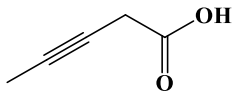
b) Cysteamine approach

The reaction was carried out as described in **a)** using cysteamine (1.65 g, 21.34 mmol). The oil was purified by flash chromatography (7:3 EtOAc/Hexane to 100% EtOAc). The solvent was removed *in vacuo.* DCM was removed *in vacuo* and obtained clear oil was dissolved in 20 mL of diethyl ether (20 mL).

Equal volume of hexane was added and left at room temperature for crystallisation yield compound **2.29** as white sticky solid (4.27 g, 94%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.14 (s, 1H), 3.76 (d, *J* = 11.6 Hz,1H), 3.59 – 3.43 (m, 2H), 3.46 – 3.32 (m, 2H), 3.28 (d, *J* = 11.7 Hz, 1H), 2.62 (t, *J* = 6.8 Hz,2H), 2.46 (t, *J* = 6.7 Hz, 2H), 1.46 (d, *J* = 3.3 Hz, 6H), 1.00 (d, *J* = 9.2 Hz, 6H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.12, 170.25, 99.09, 77.41, 77.25, 77.09, 76.77, 71.38, 42.47, 36.14, 29.47, 24.53, 22.15, 18.92, 18.71 ppm; MS: *m/z*(+ESI) $C_{14}H_{27}N_2O_4S^+$ calcd. 320.17, found 320.30 [M+H]+

Synthesis of unstable carboxylic acids

a) 3-pentynoic acid (2.43)



a) Jones oxidation

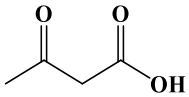
Chromium (VI) oxide (3 g, 30.00 mmol) was dissolved in 10 M H_2SO_4 (37.5 mL) to give Jones reagent. 3-penalty-1-oil was dissolved in acetone (50 mL). A solution of the alcohol was cooled down on ice to 0°C and Jones reagent was added dropwise over 2 h while the mixture was stirring. The reaction was kept on the ice. The reaction was then stirred for 2 h (temp. was kept at 0°C). Next acetone was evaporated under vacuum and mixture was diluted with 100 mL of water and extracted with diethyl ether (4x50 mL). Organic layers were combined, washed with water (50 mL). Volume was reduced by half and the solution was washed with 3 M NaOH (2x50 mL). Aqueous layers were combined and acidified with 37% HCl. Next compound was extracted with diethyl ether (3x50 mL). Dried over Na_2SO_4 and solvent was evaporated to yield a dark brown sticky solid (44.06 mg, 30%); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.91 (s, 1H), 3.31 (q, *J* = 2.6 Hz, 2H), 1.88 – 1.76 (m, 3H) ppm;

b) Synthesis with 4-Acetamido-2,2,6,6-tetramethyl-1-oxopiperidinium tetrafluoro- borate (Bobbitt`s salt) (2.44)

Bobbitt`s salt (5.89 g, 19.63 mmol) was dissolved in acetonitrile (32.13 mL) and water (3.57 mL) (9:1 ratio). 3-penalty-1-ol was added (750 mg, 8.93 mmol; conc. in solution 0.25 M). Flask was sealed and left stirring under

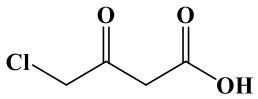
nitrogen for 24 h. Next day water (35 mL) was added to reaction mixture and compound was extracted with diethyl ether (5 x 50mL). Organic layers were combined and washed twice with 2 M HCl (45 mL). The organic layer was dried over anhydrous sodium sulphate and evaporated to dryness. Product was obtained as light yellow solid and used without further purification (599.8 mg, 68 %) ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.33 (q, *J* = 2.6 Hz, 2H), 1.86 (t, *J* = 2.6 Hz, 4H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.01, 79.95, 69.78, 25.84, 3.56 ppm

b) Acetoacetic acid (2.79)



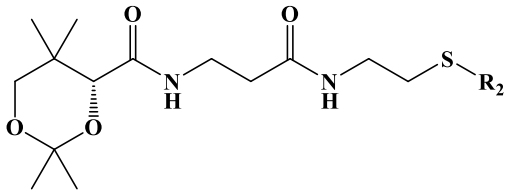
tert-Butyl acetoacetate (3 g, 19 mmol) was dissolved in DCM (5 mL), and the solution was cooled down to 0°C (ice bath). TFA (5 mL) was slowly added, and the reaction was kept on ice for 15 min. Afterwards, it was warmed to room temperature and stirred for an hour. Solvent was evaporated, and crude was left under high vacuum to give title compound as pale yellow oil, that was used for the next step without further purification (1.04 g, 54 %); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.11 (or s, 1H), 3.58 (s, 2H), 2.29 (s, 3H) ppm.

c) 4-chloroacetoacetic acid (2.80)

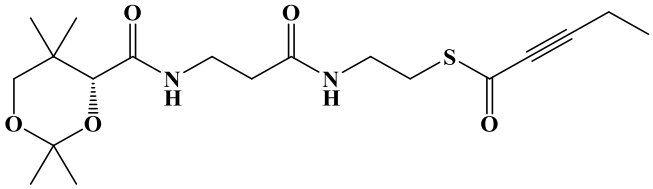


The reaction was carried out as described above (**b**)) using ethyl 4-chloroacetoacetate (3 g, 22 mmol). It yielded clear oil, which crystallised to give the title compound as pale pink crystals that were used without further purification (2.76 g, 93%); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 11.11 (s, 1H), 4.29 (s, 2H), 3.56 (s, 2H) ppm.

S-acyl pantetheine acetonides synthesis (2.31)



2-pent-S-pantetheine acetonide (2.37)



a) Steglich esterification

2-Pentynoic acid (140.2 mg, 1.43 mmol) was dissolved in anhydrous DCM (20 mL). DMAP (90 mg) and product from previous step (**2.28**) (0.50 g, 1.60 mmol) were added. Flask was placed in ice bath at 0˚C and DCC (330.1 mg, 1.60 mmol) was added to reaction, which was stirred at 0˚C for 5 min and at RT for further 12 h. Reaction was quenched with sat.aq. NH_4Cl (10 mL). Organic layer was washed with water (1x30 mL) and brine (1x15 mL), dried with Na_2SO_4. The crude material was purified by flash chromatography (Me_2CO 3:7 EtOAc) to give title compound as orange oil (39.25 mg, 28%) ^1H NMR (400 MHz, $CDCl_3$) δ 4.10 (d, *J* = 2.7 Hz, 2H), 3.76 – 3.66 (m, 2H), 3.65 – 3.44 (m, 4H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.12 – 3.04 (m, 2H), 2.96 (t, *J* = 6.9 Hz, 1H), 2.83 (t, *J* = 6.6 Hz, 2H), 2.77 (d, *J* = 7.4 Hz, 1H), 2.52 (m, *J* = 10.3, 6.7, 3.7 Hz, 3H), 2.43 (t, *J* = 6.0 Hz, 2H), 1.49 (s, 6H), 1.45 (d, *J* = 1.3 Hz, 6H), 1.21 (dt, *J* = 14.8, 7.4 Hz, 3H), 1.09 – 1.02 (m, 6H), 0.99 (d, *J* = 3.3 Hz, 6H) ppm; MS: *m/z*(+ESI) $C_{19}H_30NO_5S^+$ calcd. 399.19, found 739.33 which corresponds to acetonide deprotected adduct $[2M+Na]^+$ $C_{16}H_{26}N_2O_5SNa^+$ calcd. 739.32; found 739.33

b) Carbodiimide coupling

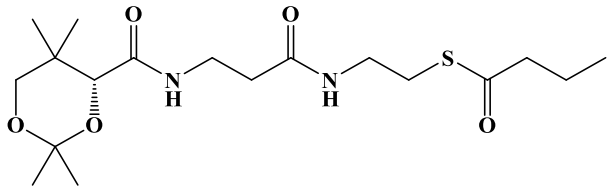
2-Pentynoic acid (110 mg, 1.12 mmol) was dissolved in DCM (20 mL). EDC (218.9 mg, 1.41 mmol), product form the previous step (**2.28**) (300 mg; 0.94 mmol) and DIPEA (0.327 mL, 1.87 mmol) were added to the flask and stirred at RT for for 12 h. Reaction was quenched with sat.aq. NH_4Cl (10 mL). Organic

layer was washed with water (1x30 mL) and brine (1x15 mL), dried with Na_2SO_4. The crude material was purified by flash chromatography (Me_2CO 3:7 EtOAc). Solvent was evaporated *in vacuo* to give title compound as orange oil (36.3 mg, 33%); ^{1}H NMR (400 MHz, Chloroform-*d*) δ 4.10 (d, *J* = 2.7 Hz, 2H), 3.76 – 3.66 (m, 2H), 3.65 – 3.44 (m, 4H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.12 – 3.04 (m, 2H), 2.96 (t, *J* = 6.9 Hz, 1H), 2.83 (t, *J* = 6.6 Hz, 2H), 2.77 (d, *J* = 7.4 Hz, 1H), 2.52 (ddd, *J* = 10.3, 6.7, 3.7 Hz, 3H), 2.43 (t, *J* = 6.0 Hz, 2H), 1.49 (s, 6H), 1.45 (d, *J* = 1.3 Hz, 6H), 1.21 (dt, *J* = 14.8, 7.4 Hz, 3H), 1.09 – 1.02 (m, 6H), 0.99 (d, *J* = 3.3 Hz, 6H); MS: *m/z*(+ESI) $C_{19}H_30NO_5S^+$ calcd. 399.19, found 739.33 which corresponds to acetonide deprotected adduct $[2M+Na]^+$ $C_{16}H_{26}N_2O_5SNa^+$ calcd. 739.32; found 739.33

c) DCC as coupling agent

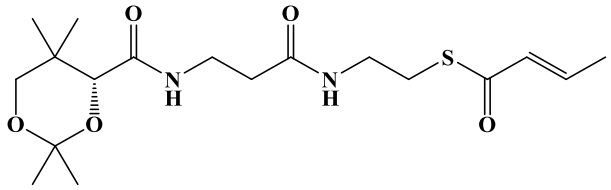
The 2-Pentynoic acid (368.86 mg, 3.76 mmol) was dissolved in DCM (10 mL). Flask was cooled down to 0 ˚C in an ice bath. DCC (775.80 mg, 3.76 mmol) was added to the reaction and stirred for 30 min on ice, a product from the previous step (**6**) (300.00 mg; 0.32 mmol) was added. The reaction was removed from ice and slowly warmed up to room temperature and stirred 12 h. Next, it was filtered through celiate and purified with flash chromatography (EtOAc:Hexane (4:1) to 100% EtOAc). The same reaction was repeated with 388.78 mg of EDC (1.88 mmol) to give title compound (**6**) as an orange sticky solid in both cases (185.1 mg, 49 %; 62.1 mg, 17% respectively). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.11 (s, 1H), 3.74 – 3.24 (m, 6H), 3.16 – 3.08 (m, 2H), 2.51 – 2.36 (m, 2H), 2.20 (d, *J* = 2.4 Hz, 1H), 1.49 (s, 3H), 1.45 (s, 3H), 1.32 – 1.19 (m, 3H), 1.07 (s, 3H), 1.00 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 193.4, 170.47, 168.7, 119.14, 101.5, 87.39, 74.63, 72, 39.5, 38.91, 36.4, 35.26, 29.00, 24.58, 22.27, 21.85, 14.00 ppm; MS: *m/z*(+ESI) $C_{19}H_{28}N_2O_5S^+$ cacld.399.20, found 399.32 [M+H]+

Butanoyl-S-pantetheine acetonide (2.45)



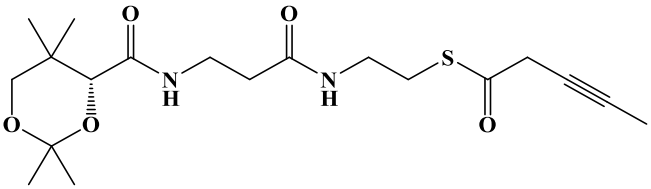
The reaction was carried out as described for **2.29** c**)** using butyric acid (331.3 mg, 3.76 mmol). To give title compound as white sticky solid (266.97 mg, 73%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (s, 1H), 3.77 – 3.37 (m, 5H), 3.31 (d, *J* = 11.7 Hz, 1H), 3.04 (t, *J* = 6.5 Hz, 2H), 2.58 (t, *J* = 7.4 Hz, 2H), 2.44 (t, *J* = 6.2 Hz, 2H), 1.72 (h, *J* = 7.4 Hz, 2H), 1.47 (d, *J* = 17.0 Hz, 6H), 1.08 – 0.90 (m, 9H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 197.71, 170.47, 168.7, 101.5, 74.63, 72, 44.95, 39.5, 38.915, 36.4, 35.26, 29.3, 24.58, 21.85, 18.37, 13.08 MS: *m/z*(+ESI) $C_{18}H_{32}N_2O_5S^+$ cacld.389.20, found 389.26 [M+H]+

Butenoyl-S-pantetheine acetonide (2.46)



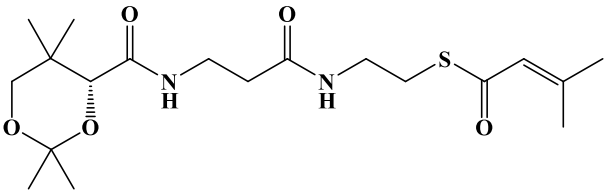
The reaction was carried out as described for **2.29** variant **c)** using crotonic acid (323.6 mg, 3.76 mmol). To give title compound as sticky white solid (189.30 mg, 52%), ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.96 (dq, *J* = 6.9, 15.4 Hz, 1H), 6.22 – 6.12 (m, 1H), 4.10 (s, 1H), 3.78 – 3.38 (m, 5H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.10 (t, *J* = 6.4 Hz, 2H), 2.44 (t, *J* = 6.2 Hz, 2H), 1.92 (dd, *J* = 1.7, 6.9 Hz, 3H), 1.46 (d, *J* = 17.3 Hz, 6H), 1.02 (d, *J* = 27.2 Hz, 6H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ 186.10, 170.48, 168.7, 132.48, 124.65, 101.5, 74.63, 72, 39.5, 38.92, 36.4, 35.26, 29, 24.58, 21.85, 17.54 ppm; MS: *m/z*(+ESI) $C_{18}H_{31}N_2O_5S^+$ cacld.387.20, found 387.32 [M+H]+

3-pentynoyl-S-pantetheine acetonide (2.47)



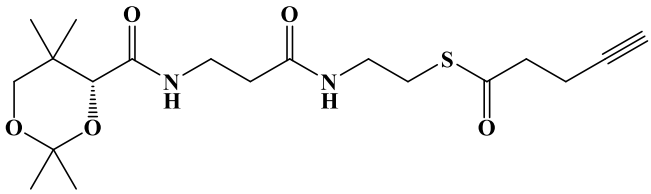
The reaction was carried out as described for **2.29** variant **c)** using **2.43** (368.8 mg, 3.76 mmol). To give the title compound as orange sticky solid (183.90 mg, 49 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (s, 1H), 3.77 – 3.35 (m, 6H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.06 (t, *J* = 6.5 Hz, 2H), 2.45 (t, *J* = 6.2 Hz, 2H), 1.91 – 1.84 (m, 3H), 1.46 (d, *J* = 16.6 Hz, 6H), 1.03 (d, *J* = 28.0 Hz, 6H) ppm; MS: *m/z*(+ESI) $C_{19}H_{28}N_2O_5S^+$ cacld.399.19, found 399.24 [M+H]+

3-methylbutenoyl-S-pantetheine acetonide (2.48)



The reaction was carried out as described for **2.29** variant **c)** using 3,3-dimethylacrylic acid (376.45 mg, 3.76 mmol). To give the title compound as orange sticky solid (180.40 mg, 49 %); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.86 (s, *J* = 6.9, 15.4 Hz, 1H), 6.22 – 6.12 (m, 1H), 4.10 (s, 1H), 3.78 – 3.38 (m, 5H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.10 (t, *J* = 6.4 Hz, 2H), 2.44 (t, *J* = 6.2 Hz, 2H), 1.92 (dd, *J* = 1.7, 6.9 Hz, 3H), 1.46 (d, *J* = 17.3 Hz, 6H), 1.39 (s, 3H), 1.02 (d, *J* = 27.2 Hz, 6H) ppm $C_{16}H_{29}N_2O_5S^+$ cacld. 361.47, found 361.63 [M+H]+

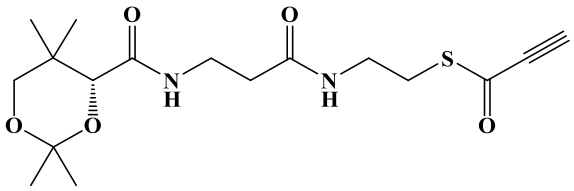
4-pentynoyl-S-pantetheine acetonide (2.49)



The reaction was carried out as described for **2.29** variant **c)** using 4-pentynoic acid (368.8 mg, 3.76 mmol). To give the title compound as orange sticky solid (195.53 mg, 52 %), ^{1}H NMR (400 MHz, $CDCl_3$) 6.43 (s, 1H), 4.10

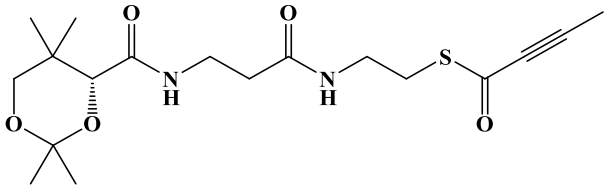
(s, 1H), 3.74 – 3.30 (m, 6H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.06 (t, *J* = 6.5 Hz, 2H), 2.45 (t, *J* = 6.2 Hz, 2H), 1.91 – 1.84 (m, 3H), 1.46 (d, *J* = 16.6 Hz, 6H), 1.03 (d, *J* = 28.0 Hz, 6H) $C_{19}H_{29}N_2O_5S^+$ cacld.399.20, found 399.31 [M+H]+

Propionoyl-S-pantetheine acetonide (2.50)



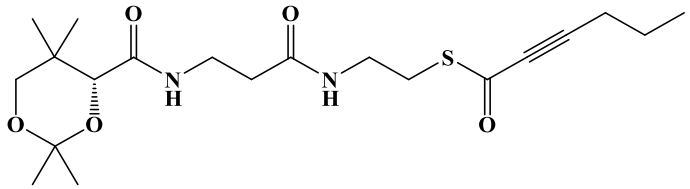
The reaction was carried out as described for **2.29** variant **c)** using propiolic acid (263.38 mg, 3.76 mmol). To give the title compound as orange sticky solid (115.2 mg, 33%^{1}H NMR (400 MHz, $CDCl_3$) δ 4.1) (s, 1H), 3.74 – 3.24 (m, 6H), 2.49 – 2.34 (m, 2H), 2.20 (d, *J* = 2.4 Hz, 1H), 1.49 (s, 3H), 1.45 (s, 3H), 1.32 – 1.19 (m, 3H), 1.07 (s, 3H), 1.00 (s, 3H); $C_{17}H_{27}N_2O_5S^+$ (162.60 mg, 33 %), cacld.371.16, found 371.20 [M+H]+

Butynol-S-pantetheine acetonide (2.51)



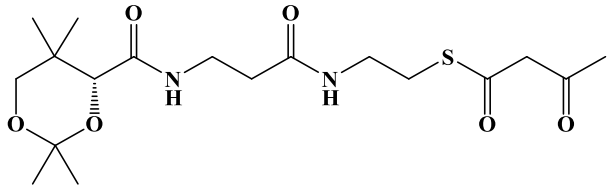
The reaction was carried out as described for **2.29** variant **c)** using 2-butynoic acid (316.10 mg, 3.76 mmol). To give title compound as orange oil solid (220.90 mg, 61 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (s, 1H), 3.77 – 3.36 (m, 5H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.12 (t, *J* = 6.6 Hz, 2H), 2.45 (t, *J* = 6.2 Hz, 2H), 2.08 (s, 3H), 1.48 (s, 3H), 1.44 (s, 3H), 1.06 (s, 3H), 0.99 (s, 3H); MS: *m/z*(+ESI) $C_{18}H_{29}N_2O_5S^+$ cacld. 385.17, found 385.28 [M+H]+

3-hexenoyl-S-pantetheine acetonide (2.52)



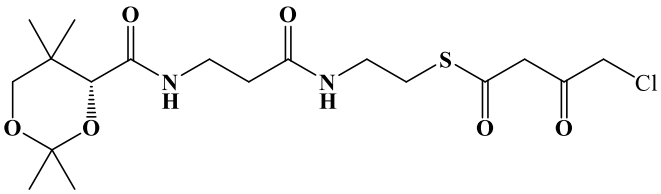
The reaction was carried out as described for **2.29** variant **c)** using 2-hexynoic acid (376.45 mg, 3.76 mmol). To give title compound as orange oil (189.50 mg, 54 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (s, 1H), 3.79 – 3.39 (m, 5H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.12 (t, *J* = 6.4 Hz, 2H), 2.51 – 2.33 (m, 4H), 1.47 (d, *J* = 16.4 Hz, 6H), 1.11 – 0.91 (m, 9H); MS: *m/z*(+ESI) $C_{20}H_{33}N_2O_5S^+$ cacld.413.20, found 413.55 [M+H]+

3-oxobutanoyl-S-pantetheine acetonide (2.53)



The reaction was carried out as described for **2.29** variant **c)** using acetoacetic acid (130.67 mg, 3.76 mmol). To give title compound as orange oil (162.60 mg, 45 %), ^{1}H NMR (400 MHz, $CDCl_3$) 4.12 (s, 1H), 3.74 – 3.30 (m, 6H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.16 (t, *J* = 6.5 Hz, 2H), 2.43 (t, *J* = 6.2 Hz, 2H), 1.86 – 1.82 (m, 3H), 1.46 (d, *J* = 16.6 Hz, 6H), 1.03 (d, *J* = 28.0 Hz, 6H) MS: *m/z*(+ESI) $C_{18}H_{31}N_2O_6S^+$ cacld. 403.11, found 403.40 [M+H]+

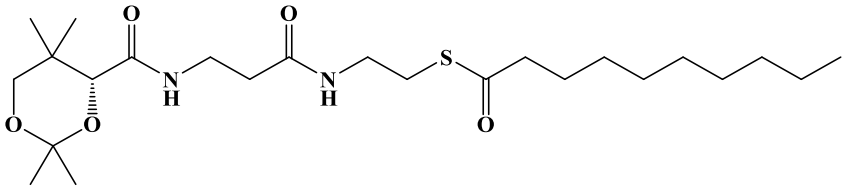
4-chloro-3-oxobutanoyl-S-pantetheine acetonide (2.54)



The reaction was carried out as described for **2.29** variant **c)** using 4-chloro acetoacetic acid (174.76 mg, 3.76 mmol). To give title compound as yellow oil (192.30 mg, 63 %), 4.34 ^{1}H NMR (400 MHz, $CDCl_3$) (s, 1H), 3.76 – 3.32 (m, 6H), 3.30 (d, *J* = 11.7 Hz, 1H), 3.16 (t, *J* = 6.5 Hz, 2H), 2.43 (t, *J* = 6.2

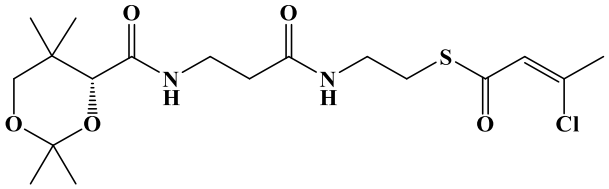
Hz, 2H), 1.92 – 1.84 (m, 3H), 1.46 (d, *J* = 16.6 Hz, 6H), 1.09 (d, *J* = 24.0 Hz, 6H) MS: *m/z*(+ESI) $C_{18}H_{30}ClN_2O_6S^+$cacld. 437.14, found 447.15 [M+H]+

Decanoyl-S-pantetheine acetonide (2.55)



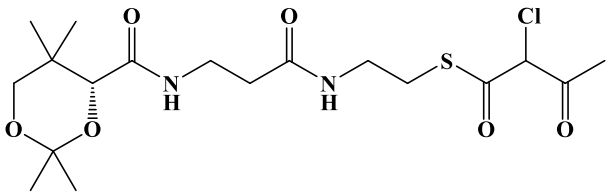
The reaction was carried out as described for **2.2.29** variant **c)** using decanoic (647.72 mg, 3.76 mmol). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.12 (s, 1H), 3.77 – 3.37 (m, 5H), 3.31 (d, *J* = 11.7 Hz, 1H), 3.04 (t, *J* = 6.5 Hz, 2H), 2.58 (t, *J* = 7.4 Hz, 4H), 2.44 (t, *J* = 6.2 Hz, 4H), 2.16 – 1.90 (m,6H), 1.72 (h, *J* = 7.4 Hz, 6H), 1.47 (d, *J* = 17.0 Hz, 6H), 1.08 – 0.90 (m, 9H) ppm; To give title compound as yellow oil (712.10 mg, 40 %), MS: *m/z*(+ESI) $C_{24}H_{45}N_2O_5S^+$ cacld. 473.69, found 473.73 [M+H]+

2-chlorobutenoyl-S-pantetheine acetonide (2.56)



The reaction was carried out as described for **2.29** variant **c)** using 3-chlorobutanol acid (433.9 mg, 3.20 mmol). To give title compound as yellow oil (234.60 mg, 56 %), ^{1}H NMR (400 MHz, $CDCl_3$) 7.06 (d, *J* = 5.9 Hz, 1H), 6.36 (s, 1H), 4.45 – 4.28 (m, 1H), 4.18 – 4.00 (m, 1H), 3.71 (d, *J* = 11.7 Hz, 1H), 3.65 – 3.38 (m, 2H), 3.29 (d, *J* = 11.7 Hz, 1H), 3.09 (t, *J* = 6.5 Hz, 2H), 2.46 (q, *J* = 6.2, 5.4 Hz, 2H), 2.18 (s, 2H), 2.16 – 1.90 (m, 2H), 1.45 (d, *J* = 15.7 Hz, 4H), 1.35 – 1.22 (m, 1H), 1.12 – 0.92 (m, 8H) MS: *m/z*(+ESI) $C_{18}H_{30}ClN_2O_5S^+$ cacld. 421.15, found 421.18 [M+H]+

2-chloro-3-oxobutanoyl-S-pantetheine acetonide (2.57)

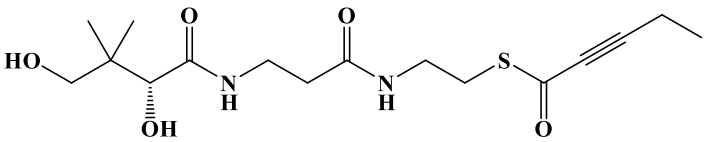


The reaction was carried out as described for **2.29** variant **c)** using 2-chloroacetoacetic acid (513.35 mg, 3.76 mmol). To give the title compound as yellow oil (212.46 mg, 53 %), ^{1}H NMR (400 MHz, $CDCl_3$) 6.49 (s, 1H), 4.09 (s, 1H), 3.77 – 3.66 (m, 1H), 3.66 – 3.35 (m, 4H), 3.29 (d, *J* = 11.7 Hz, 1H), 2.73 – 2.62 (m, 2H), 2.57 – 2.40 (m, 2H), 2.18 (s, 3H), 1.50 – 1.35 (m, 6H), 1.12 – 0.99 (m, 4H), 0.98 (s, 1H), 0.94 (s, 1H), MS: *m/z*(+ESI) $C_{18}H_{30}ClN_2O_6S$ cacld. 437.14, found 437.15 [M+H]+

Removal of an acetonide protection group (2.32)

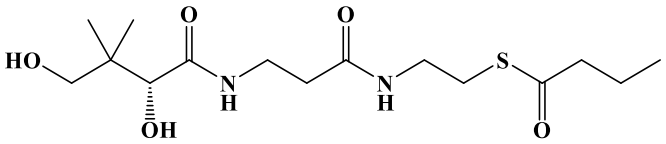
Compound **2.32s** were prepared by dissolving the corresponding acetonide protected compound in MeCN (2 mL) and added to 0.2M HCl (2 mL). The reaction was stirred for 2h. Next reaction mixture was added dropwise to the sat. $NaHCO_3$ (4 mL) and EtOAc (20 mL) (1:5) and compound was extracted. The aqueous layer was saturated with NaCl and extracted with 2x 20 mL EtOAc. Organic layers were combined and dried over Na_2SO_4 and evaporated to dryness.

2-pentynoyl-S-pantetheine (2.36)



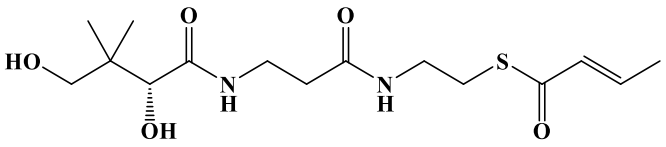
Compound **2.37** (152.6 mg, 0. 38 mmol) was prepared as described above. To give title compound **2.36** as an orange oil (129.7 mg, 85%) MS: *m/z*(+ESI) $C_{16}H_{27}N_2O_5S$ cacld. 359.16, found 359.21 [M+H]+

Butanoyl-S-pantetheine (2.58)



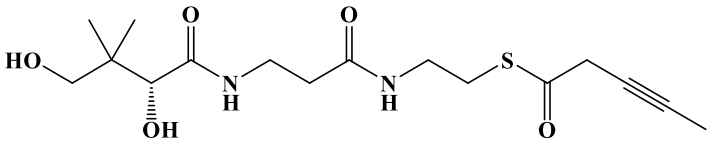
Compound **2.45 (**158.5 mg, 40.6 mmol) was prepared as described above. To give title compound **2.58** as white solid (139.2 mg, 88%), MS: *m/z*(+ESI) $C_{15}H_{29}N_2O_5S$ cacld. 349.17, found 349.20 [M+H]+

Butenoyl-S-pantetheine (2.59)



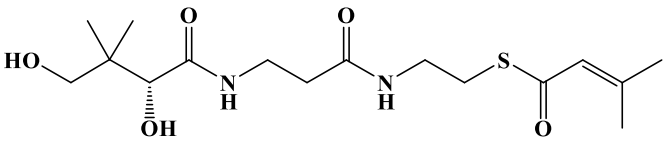
Compound **2.46 (**150.2 mg 0.39 mmol) was prepared as described above. To give title compound **2.58** as white solid (106.4 mg, 79%) MS: *m/z*(+ESI) $C_{15}H_{27}N_2O_5S^+$ cacld. 347.16, found 347.21 [M+H]+

3-pentynoyl-S-pantetheine (2.60)



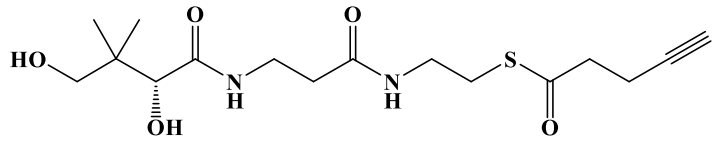
Compound **2.47 (**152.6 mg,) was prepared as described above. To give title compound **2.58** as yellow oil (116.6 mg, 85%) MS: *m/z*(+ESI) $C_{16}H_{27}N_2O_5S^+$ cacld. 359.16, found 359.18 [M+H]+

3-methylbutenoyl-S-pantetheine (2.61)



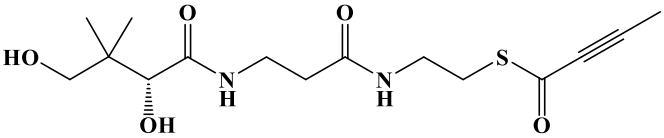
Compound **2.48 (**154.8 mg, 0.38) was prepared as described above. To give title compound **2.61** as clear oil (137.7 mg, 89%) MS: *m/z*(+ESI) $C_{16}H_{29}N_2O_5S^+$ cacld. 361.17, found 361.20 [M+H]+

4-pentynoyl-S-pantetheine (2.62)



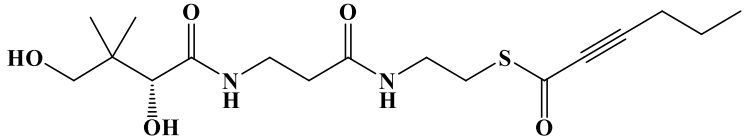
Compound **2.49 (**152.8 mg, 0.40 mmol) was prepared as described above. To give title compound **2.62** as yellow oil (139.1 mg, 91%) MS: *m/z*(+ESI) $C_{16}H_{27}N_2O_5S^+$ cacld. 359.17, found 359.20 [M+H]+

2-butynyl-S-pantetheine (2.63)



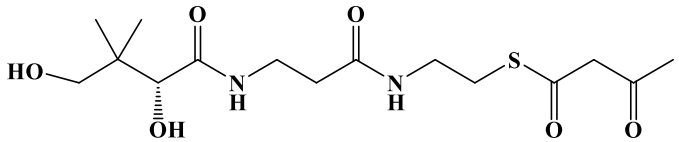
Compound **2.51 (**156.8 mg, 0.41 mmol) was prepared as described above. To give title compound **2.63** as yellow oil (128.6 mg, 82%) MS: *m/z*(+ESI) $C_{15}H_{25}N_2O_5S^+$ cacld. 345.14, found 345.18 [M+H]+

2-hexynoyl-S-pantetheine (2.64)



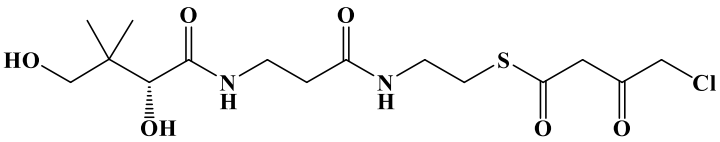
Compound **2.52 (**150.8 mg, 0.37 mmol) was prepared as described above. To give title compound **2.64** as yellow oil (123.2 mg, 82%) MS: *m/z*(+ESI) $C_{17}H_{29}N_2O_5S^+$ cacld. 373.48, found 373.51 [M+H]+

3-oxobutanoyl-S-pantetheine (2.65)



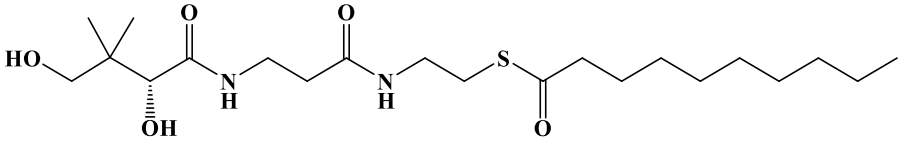
Compound **2.53 (**158.5 mg, 0.39 mmol) was prepared as described above. To give title compound **2.65** as clear oil (118.2 mg, 83%) MS: *m/z*(+ESI) $C_{15}H_{27}N_2O_6S^+$ cacld. 363.15, found 363.27 [M+H]+

4-chloro-3-oxobutanoyl-S-pantetheine (2.66)



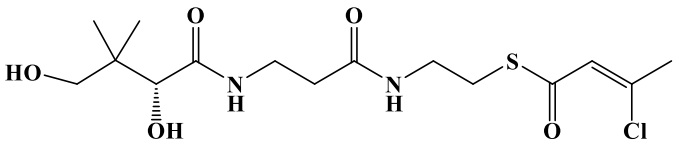
Compound **2.54 (**158.5 mg, 0.41 mmol) was prepared as described above. To give title compound **2.66** as a clear oil (123.8 mg, 86%) MS: *m/z*(+ESI) $C_{15}H_{26}ClN_2O_6S^+$ cacld. 397.11, found 397.8 [M+H]+

Decanoyl-S-pantetheine (2.67)



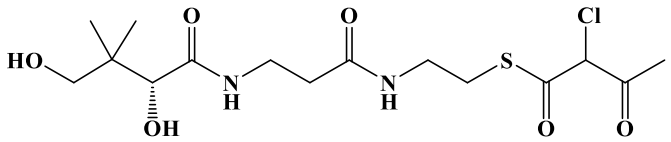
Compound **2.55 (**152.3 mg, 0.32 mmol) was prepared as described above. To give title compound **2.67** as a white solid (134.1 mg, 88%) MS: *m/z*(+ESI) $C_{21}H_{41}N_2O_5S^+$ cacld. 433.27, found 433.33 [M+H]+

2-chlorobutenoyl-S-pantetheine (2.68)



Compound **2.56 (**153.9 mg, 0.37 mmol) was prepared as described above. To give title compound **2.68** as a clear oil (123.1 mg, 80%) MS: *m/z*(+ESI) $C_{15}H_{26}ClN_2O_6S^+$ cacld. 381.12, found 381.17 [M+H]+

2-chloro-3-oxobutanoyl-S-pantetheine (2.59)



Compound **2.57 (**157.7 mg, 0.36 mmol) was prepared as described above. To give title compound **2.69** as a clear oil (130.9 mg, 83%) MS: *m/z*(+ESI) $C_{15}H_{26}ClN_2O_6S^+$ cacld. 397.11, found 397.20 [M+H]+

2.2 Materials and methods: Biological assays, cloning and protein purification

2.2.1 Materials and General Instrumentation

All chemical reagents were purchased from Sigma-Aldrich and Oxoid unless stated otherwise. Reagents and kits for biological assays were purchased from Thermo Scientific, Invitrogen, Promega, Novagen and Qiagen. 96-well UV-Star® microplates were purchased from Grenier. Tubes were supplied by Starsedt.

Instrumentation used for biological assays and cloning are listed below: Spectrophotometer (Novospec), Tecan Spark microplate reader, AKTA Prime protein purification system.

2.2.2 Growth media

2.2.2.1 Luria Broth (LB)

LB broth was prepared according to Green and Sambrook.[241] Ten grams of tryptone, 5 g yeast extract and 10 g of sodium chloride was per litre of broth. LB agar was prepared by the same method with 15 g of agar / L (1.5%).

Modified LB agar was prepared for independent ligation cloning. Media was prepared with 10 grams of tryptone, 5 g yeast extract, 15 g of agar and 100 g of sucrose (10%) per litre.

2.2.2.2 Antibiotic and isopropyl β-D-1thiogalactopyranoside (IPTG) concentration

Stock solutions were stored at -20 °C.[241] In this study ampicillin (Amp), kanamycin (Kan) and chloramphenicol (Chl) at 100 µg/mL, 50 µg/mL and 25 µg/mL working concentration respectively unless stated otherwise. The IPTG was prepared as a 0.5 M stock solution dissolved in dH_2O.

2.2.2.3 Growth conditions and bacterial strains used in this study

All liquid cultures were grown at 200 RPM, and bacterial agar plates were incubated aerobically. Growth temperature varied between strains (Table 2.1). Bacterial growth was monitored by optical density at λ=600 (OD_{600}) with a

spectrophotometer. All strains used in this study and its growth conditions are listed in Table 2.1.

Table 2.1 *E.coli* strains used in this study

Bacterial strain	Genotype/relevant information	[°C]	Source
	Escherichia coli		
MG1655	F^-, $lambda^-$, rph-1	37	CGSC
W3110	F^- $lambda^-$ IN(rrnD-rrnE)1 rph-1	37	CGSC
BL21 (DE3)	Recombinant protein expression strain	37; 22	Novagen
BL21 (DE3)/ pet21dFadE	*E.coli* strain expressing acyl-CoA dehydrogensae	22	This study
TOP10	Cloning strain		Invitrogen
Top10/pet21FadE			This study
MG1655/pKD46	pKD46, Amp^R	30	CGSC
W3110/pKD46	pkD46, Amp^R	30	This study
BW25141/pKD3	F-,*Δ(araDaraB)567*, *ΔlacZ4787*(::rrnB-3), *Δ(phoB-phoR)580*, λ^-, *galU95*, *ΔuidA3::pir*$^+$, *recA1*, *endA9*(del-ins)*::FRT*, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*, pKD3, Chl^R	37	CGSC
MG1655$^{\Delta\ FadR}$	ΔFadR::Chl^R	37	This study
W3110$^{\Delta\ FadR}$	ΔFadR::Chl^R	37	This study
DH5α/pCP20	F-, *Δ(argF-lac)169*, φ80d*lacZ58* (M15), *glnX44*(AS), λ^-, *rfbC1*, *gyrA96*(NalR), *recA1*, *endA1*, *spoT1*, *thiE1*, *hsdR17*, pCP20, Amp^R, Chl^R	30	CGSC
MG1655 Δ FadR/pCP20	ΔFadR::Chl^R, Amp^R	30	This study
W3110 Δ FadR/pCP20	ΔFadR::Chl^R, Amp^R	30	This study
MG1655 Δ FadR	Δ FadR	37	This study
W3110 Δ FadR	Δ FadR	37	This study
DH5α/pTrc-LIC	pTrc-LIC, Amp^R	37	Cheryl Arrowsmith

2.2.2.4 Long-term storage of bacterial strains

Overnight liquid culture was added (0.8 mL) with sterile 50% glycerol (0.2 mL) (v/v) in dH_2O, final concentration of glycerol was 10%. These were kept long-term in -80 °C in 2 mL cryotubes.

2.2.3 DNA manipulation

2.2.3.1 Primers and plasmids used in this study

Oligonucleotide primers were synthesized by Sigma-Aldrich. The sequence of restriction sites is underlined. All primers used in this study are presented in Table 2.2. All plasmids used and generated in this study are shown in Table 2.3.

Table 2.2 Primers used in this study

Primer name	Sequence (5`→3`) [f]	Role	Source
FadE_Fv	AACCGTCCATGGTGAT TTTGAGTATTCTG	Forward primer to amplify *fadE*	This study
FadE_Rv	AGCTAGAAGCTTCGC GGCTTCAACTTTCCG	Reverse primer to amplify *fadE*	This study
FadE_seqFR	AACCGAGTCTCATGG CTTTATATGCTTAT	Forward primer to sequence inserts *fadE*	This study
FadE_seqRV	AGCTAGATTTTGCCTC GGAATAGATTA	Reverse primer to sequence insert *fadE*	This study
MTesA`_Fv	CGACCATAGTTTGCAG GCGGACGTA	Forward primer to amplify *this* with mutation	Choi, Lee[242]
MTesA`_Rv	GCAAATACGTCCGCCT GCAAACTAT	Reverse primer *tes* with mutation	Choi, Lee[1]
TesA_Fv	TATACCATGGATGGC GGACACGTTATATTGA TTCT	Forward primer to amplify TesA	This study
TesA_Rv	TGTGTCTAGATTATGA GTCATGATTTACTAAA	Reverse primer TesA	This study
Tac_ TesA_Fv	TATAAAGCTTAAGCTG TGGTATGGCTGTCC	Forward primer to amplify TesA` with tac promoter	This study
Tac_ TesA_Rv	TATTAAGCTTTTATGA GTCATGATTTACTAAA	Reverse primer to amplify TesA` with tac promoter	This study
FadR_KO_Fv	TTTGTTTTGCTGTGTT ATGGAAATCTCACTGT GTAGGCTGGAGCTGC TTC	Forward primer for FadR KO	This study

FadR_KO_Rv	TTGAGGGGTTTGCTCT TTAAACGGAAGGGAA TGGGAATTAGCCATG GTCC	Reverse primer for FadR KO	This study
FadR_Cont_Fv	TTTGTTTTGCTGTGTT ATGGAAATC	Forward primer for FadR KO control	This study
FadR_Cont_Rv	TTGAGGGGTTTGCTCT TTAAACGGA	Reverse primer for FadR KO control	This study
AcR_Fv	AGGAAACAGACCATG ATGAAGCTTGAATTTA CATGTG	Forward primer for *acr* amplification	This study
AcR_Rv	CAAGCTTCGTCATCAT TAGATTTGTCTACTAA CCAAAAATG	Reverse primer for *acr* amplification	This study
FadD_Fv	TATACCATGGTTGAAG AAGGTTTGGCTTAAC	Forward primer for *fadD* amplification	This study
FaD_Rv	TGTGTCTAGATCAGGC TTTATTGTCCACTT	Reverse primer for *fadD* amplification	This study
Tac_FadD_Fv	TATATCTAGAAAGCTG TGGTATGGCTGTCC	Forward primer for *fadD* amplification with tac promoter	This study
Tac_ FaD_Rv	TATATCTAGACATAAC GGTTCTGGCAAATA	Reverse primer for *fadD* amplification with tac promoter	This study
Cer1_Fv	TATACCATGGATGGCC ACAAAACCAGGCGT	Forward primer for *cer1* amplification	This study
Cer1_Rv	TGTGTCTAGATTAATG ATGCGGAAGGAGTAG AGGC	Reverse primer for *cer1* amplification	This study

Table 2.3 Plasmids used in this study

Plasmid	Relevant characteristics	Source
pet21d(+)	IPTG induced His-tag protein expressing vector	Novagen
pet21d(+)FadE	FadE expressing vector	This study
pSF-Tac	IPTG induced non-tagged protein expression vector with tac promoter	Oxford Genomics
pTrc-LIC	Ligation independent cloning vector, IPTG induced non-tagged protein expression vector with the promoter	Cheryl Arrowsmith (Addgene plasmid # 62343)
pSF-TacCer1	A vector expressing Cer1 under tac promoter	This study
pSF-TacCer1FadD	A vector expressing Cer1 and FadD under tac promoter	This study
pSF-TacFadD	A vector expressing FadD under tac promoter	This study
pTrcAcR	A vector expressing FadD under trc promoter	This study
pSF-TacTesA`	A vector expressing Tes under tac promoter	This study
pTrcAcRtacTesA`	A vector expressing Acr under trc promoter and Tes under tac promoter	This study
pKD46	Recombinase producing vector	CGCS
pKD3	A vector containing chloramphenicol resistance cassette	CGCS
pCP20	Flippase expressing vector	CGCS

2.2.3.2 Extraction and purification of plasmids from E. coli

Isolation of plasmids from *E. coli* was carried out with Plasmid miniprep kit, according to the manufacturer's suggested protocol. Cells were harvested from 5 mL overnight culture by centrifugation (1 min at 13,000 RPM), re-suspended and lysed using provided reagents. The solution was left to incubate at RT for 5 min before the neutralisation buffer was added. The lysate was centrifuged for 10 min to separate the debris and liquid was separated from the precipitate. The lysate was loaded on filter-containing binding columns and then washed with reagents supplied by the manufacturer. The plasmid was eluted from the column with molecular biology grade water.

2.2.3.3 Gel electrophoresis protocol

Plasmids and DNA fragments obtained in this study were analysed by agarose gel electrophoresis with SYBR Safe DNA gel stain (Thermo Fisher). The agarose gel was prepared with 1x TAE buffer and 0.5% (w/v) agarose. The electrophoresis was run at 100 V for 60 min.

50 x TAE buffer

Tris base	242 g
EDTA disodium	18.61 g
Glacial acetic acid	57.1 mL
dH_2O	Up to 1 L
Before use dilute to 1x with d H_2O	

DNA molecular weight markers were obtained from NEB and used interchangeably depending on the size of the fragment. A 100bp DNA ladder was used for fragments between 100-1,500 bp. A 1kb ladder was used for fragments between 0.5-10.0 kb.

DNA extraction from the gel was carried out with a DNA extraction kit (Promega) following the protocol suggested by the manufacturer.

2.2.3.4 Polymerase Chain Reaction (PCR)

The PCR reaction was carried out to:

a) Amplify targeted genes
b) Validate performed genetic modification
c) Introduce restriction sites into the sequence
d) Generate DNA fragments with overlapping arms for λ-red recombinase KO

Amplification of target genes was performed with Phusion DNA polymerase (NEB); a high-fidelity enzyme was used. A 25µL total reaction volume consisted of 1x Phusion HF Buffer, 200µM dNTPs, 1µL of each of the following; 10µM forward primer and 10µM reverse primer, 0.5 units of Phusion DNA polymerase and 1-100ng DNA template.

To generate fragments Q5 High-fidelity polymerase was used Interestingly it was observed that use of Phusion High-Fidelity polymerase required 3% addition of DMSO for successful amplification which was not the case for Q5

High-Fidelity polymerase. PCR reactions were performed using a T3000 Thermocycler (Biometra). The standard conditions were as follows:

a) Generation of the fragments for FadR KO

1 cycle

1. 94°C at 5:00 (m:s)
2. 94°C at 0:15
3. 55°C at 0:30
4. 68°C at 2:00
5. 68°C at 7:00
6. 4°C at ∞

Steps 2-5 were repeated total of 30 times

b) PCR for other purposes

1 cycle

1. 95°C at 5:00 (m:s)
2. 95°C at 0:15
3. 65°C at 0:30
4. 72°C at 1:30
5. 72°C at 10:00
6. 4°C at ∞

2.2.3.5 DNA restriction digest

All restriction enzymes used were purchased from New England Biolabs and used according to the manufacturer's instructions. Restriction digests were performed with ~1µg DNA, 0.5µl of a restriction enzyme or enzymes, 1x CutSmart buffer, and made to a final volume of 50µL using sterile dH2O. The restriction digest was incubated for at least 30 min (fast digest with high-frequency enzymes) or until complete digestion was achieved ~3 h at 37 °C.

When only cut digest-ligation was carried out, vector plasmids were treated with Antarctic Phosphatase. Immediately after the restriction 1µL of Antarctic phosphatase enzyme, 6µL 10x AP Buffer and 3µL of sterile dH2O were added to reaction mixture. The was then incubated for another 30 min at 37 °C. Digests were analysed via gel electrophoresis, and when necessary,

desired fragments of DNA were cut out and underwent DNA extraction as described above

2.2.3.6 DNA ligation

T4 DNA ligase was used for all ligations. Any PCR products and digests were purified using a Gel/PCR Clean-up System (Promega) before the ligation. Ligation reaction mixtures contained 1x T4 DNA Ligase Buffer, 1µL T4 DNA Ligase, 50 ng of vector, 50-150 ng of insert/PCR product and brought to a total volume of 20 µL with dH_2O. The reaction was incubated at 16 °C for 16h. Before transformation into *E. coli,* competent cells ligation reactions were purified using a Gel/PCR Clean-up System (Promega).

2.2.3.7 HI-Fi DNA assembly

Arc and pTrc-LIC plasmid were assembled *via* HiFi DNA assembly cloning kit, following the recommended protocol. After obtaining PCR products with desired overhang regions, a reaction mixture containing was prepared 0.1 pmols of pTrc-LIC plasmid, 0.2pmols of *arc* fragment, 10 µL of NEBuilder HiFi DNA Assembly Master Mix, and sterile dH2O to the total volume of 20µL with. The reaction was then incubated at 50 °C for 15 min. Lastly, it was purified with Gel/PCR Clean-up System and transformed into *E.coli* and plated on LB agar supplemented with sucrose.

2.2.3.8 DNA concentration assessment and analysis

DNA concentration and purity were analysed using a nano spectrophotometer (Thermo Fisher). DNA sequencing was performed by SourceBioscience and was analysed using UniPro UGENE (www. http://ugene.net/)

2.2.3.9 Electrocompetent cells preparation

A 5 mL overnight culture of *E. coli* in LB broth was diluted 1 in 100 with fresh LB medium and incubated at 37 °C, 200 rpm until the culture had reached an OD600 0.5. The culture was then left in an ice-bath for 30 min. Cells were then harvested by centrifugation in a pre-chilled centrifuge (4 °C) at 5,000 RPM

for 10 min. Next, the cell pellet was resuspended in ice-cold sterile 10% glycerol (v/v) pelleted (5,000 RPM) and washed three times with ice-cold sterile 10% glycerol solution. After the final centrifugation, the supernatant was removed, and the cell pellet was gently re-suspended in 10% glycerol (1 mL of 10% glycerol per 400 mL of cell culture) and stored at -80 °C in 50 μL aliquots.

2.2.3.10 Transformation by electroporation

*E. coli electro*competent cells were gently thawed on ice and mixed with 2 μL of plasmid or DNA fragment. The mixture was then transferred to a 1mm electroporation cuvette (Bio-Rad). Electroporation was then carried out at 1.75kV, 25μF, 200 Ω (MicroPulser, Bio-Rad). Immediately after electroporation 1 mL of warm (preincubated at 30 °C) LB broth was added to the cuvette. The mixture was then incubated at 30 °C (for temperature sensitive plasmids) or 37°C for up to 3h before being plated on selective LB agar plates.

2.2.3.11 Deletion of *fadR* using one-step inactivation method[242,243]

2.2.3.11.1 fadR inactivation

λ-Red recombinase expressing plasmid pKD46 was electrocompetent E. coli MG1655 and W3110. Cells were grown on LB agar supplemented with Amp and grown at 30 °C. In apparel linear fragment of pKD3 was amplified with PCR. The obtained product was purified with Gel/PCR Clean-up System and digested with DpnI for 1 h, after which sampler was again purified.

A single colony of *E. coli* strain to harbour pKD46 plasmid and grown overnight in LB+Amp media. Next day the culture was diluted 1:100 and 50 mL culture was grown at 30 °C until OD_{600} = 0.1 was reached. Add this point L-arabinose from 1 M stock solution was added to the culture to a final concentration of 40 mM. Control without arabinose induction was also prepared. Cells allowed to grow until OD_{600}=0.5 and then the culture was centrifuged at 5000 rpm for 10 min. The cells were then chilled in an ice-water bath and resuspended in ice-cold 10% glycerol. Electrocompetent cells were prepared as described above was added. Then *E. coli*/pKD46 electrocompetent cells were transformed with the linear pKD3 fragment. After incubation at 37 ° C, cells well plated on LB agar supplemented with Chl grown at 37 C for 24 h.

Next colony PCR was performed to assess transformation and recombination product. Control PCR with the wild-type colony was also included. PCR product was resolved on 0.5% agarose gel with electrophoresis.

2.2.3.11.2 Removal of antibiotic resistance

A colony with *ΔfadR was* randomly picked and grown overnight at 37 °C after which cell were made electrocompetent as described before. Obtained cells were transformed with pCP20 plasmid and grown in LB media at 30 °C for 2 h and subsequently plated on LB agar plates supplemented with Amp, LB+Chl plates and grown at 30 °C for 24 h. After 24 h minimal growth was observed on LB+Amp plates. Therefore, they were grown for another 12 h to isolate bigger colonies. Amp is susceptible to satellite colonies, so it's more likely than larger colonies have pCP20 rather than smaller ones.

Colonies that have grown on LB+Amp were colony purified on nonselective LB media at 43°C and grown overnight.

Colonies were picked from the overnight plate and resuspended in 10 µL LB. The obtained solution was divided between plates:

a) LB+Amp and grown overnight at 30 °C
b) LB+ Chl and grown overnight at 37 °C
c) LB only and grown overnight at 37 °C

Colonies that shown growth on plate C but not on plates A and B were PCR screened for the presence of antibiotic resistance cassette.

Colonies that did not have antibiotic resistance cassette and *fadR* gene were made into glycerol stock.

2.2.4 Expression and purification of acyl-CoA dehydrogenase

Five mL of overnight culture of pet21-d(+)FadE in *E. coli* BL21 was used to inoculate 500 mL LB broth +Amp (100 µg/mL) in a 2L conical flask and incubated at 37 °C, 200 rpm to an OD_{600} = 0.4 The culture was then submerged into an ice bath and left for 30 min before the addition of 0.5 M IPTG to final concentration of 25 µM. Cultures were then incubated for a further 12 h at 22 °C, 200 rpm.

Bacterial cells were then harvested by centrifugation at 4 °C, 5,000 RPM for 30 min and stored on ice. The cell pellet was re-suspended in 30 mL lysis buffer:

Tris-HCl	20 mM
NaCl	300 mM
Glycerol	10% (v/v)
pH adjusted to 7.4 with HCl	

Cell cultures that were not immediately used were snap frozen using liquid nitrogen and stored in 30 mL aliquots using 50 mL falcon tubes and stored at -80 °C.

2.2.5 Cell lysis by sonification

Phenylmethanesulfonyl fluoride (PMSF) was prepared at a 100 mM stock solution in isopropanol and stored at 4 °C. If cell cultures were stored at -80 °C, they were defrosted in a water bath at room temperature until fully thawed, and then stored on ice. 1mM PMSF was added to the culture and then sonicated with a sonicator with an FB4220 tip. The cells were sonicated for a total process time of 8 min at an amplitude of 12 microns, with a pulse on time of 15 sec followed by a pulse off time of 30 sec.

Afforded cell lysate was centrifuged at 10,000 RPM for 30 min at 4 °C. The supernatant was then transferred into a 7.5 mL tube and centrifuged at 100,000 RPM for 60 min at 4 °C.

Obtained membrane pellet was then solubilised with a) detergent b) Styrene maleic acid polymer.

2.2.6 Nickel affinity chromatography preparation and use

Buffer A

Tris-HCl	20 mM
NaCl	150 mM
Glycerol	10% (v/v)
pH adjusted to 7.4 with HCl	

Buffer B

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	10 mM
pH adjusted to 7.4	

Buffer C

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	1 M
pH adjusted to 8	

Buffer D

NaH_2PO_4	50 mM
NaCl	300 mM
Imidazole	0 mM
pH adjusted to 8	

2.2.7 Preparation of HisTrap column

The expression vector pet21d(+) contains a hexahistidine (His6) tag at the C-terminal end of multiple cloning sites allowing purification by nickel affinity chromatography. This method was used for the DDM solubilized membrane. 5 mL HiTrap HP column. HisTrap column was prepared with a peristaltic pump. The column was washed with 25 mL of dH_2O followed by 25 mL Buffer D. Next protein was loaded on the column. The flow rate was 2.5 mL/min. The system was looped. Eight min were required for the whole sample to be pumped through the column. Total loading time of 24 min (3 cycles) was used. The column was rewashed with 25 mL of Buffer D.

2.2.7.1 Preparation of AKTA prime liquid chromatography system

A liquid chromatography handling system was used to create imidazole-containing buffers over a concentration gradient. The machine was set at a flow rate of 8.0 mL/min and of a pressure limit of 0.5. Both inlets of the AKTA prime were flushed with dH_20. Inlet A was then connected to BufferB, and inlet B was connected to Buffer C. Then both inlets were flushed with respective buffers. A concentration gradient programme was used with the following settings; a) length: 40 mL, b) set target: 100% inlet B, c) fraction size: 2 mL. Over a 40 mL of flow through there would be a decrease of buffer from inlet A; wash buffer, an increase of buffer from inlet B to a final concentration of 100%; elution buffer, and the flow through would then be collected in 2 mL fractions. Fractions were stored at 4 C , whilst the purity of protein isolated was analysed using SDS-PAGE. Then a loaded HiTrap column was fixed onto the AKTA prime, and increasing concentration of imidazole was pumped through the column at a flow rate of 1.5mL/min until a plateau in UV absorbance level was observed.

2.2.8 Membrane solubilisation

a) Detergent based solubilisation

The membrane obtained in the previous step was re-suspended in buffer A (10 mL) and split between cryotubes (each tube 0.5 mL). Three detergents were tested for protein solubilisation: DDM, Tween 20 and Triton-X at 20 mM concentration were tested.

Table 2.4 Protein solubilisation conditions

Detergent	Temp. [˚C]	Detergent	Tempe. [˚C]
DDM	22	TWEEN 20	4
DDM	4	Triton-X	22
TWEEN 20	22	Triton-X	4

The stock solution at 100 mM concentration was prepared for each detergent. Total sample volume was 1 mL. Samples were placed on an end-to-end rotator and left overnight at 4˚ or 22˚C. All samples were centrifuged at

34,000 RPM for 60 min, supernatant and pellet were resolved in 12% SDS-PAGE gel

b) SMALPs[244]

Preparation of styrene-maleic acid (SMA) copolymer (Figure 2.1)

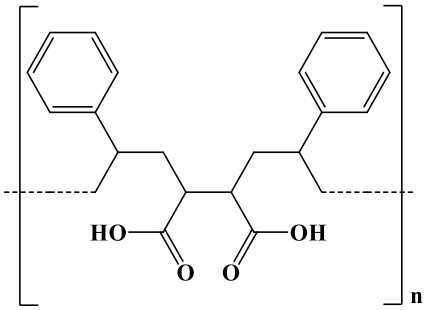


Figure 2.1 Styrene maleic acid polymer structure

Styrene-maleic acid anhydride (25 g) was suspended in 1 M sodium hydroxide (NaOH) (250 mL). Anti-bump granules (0.5 g) were added, and the reaction was brought to the boiling point and left under reflux for 2 h. Next solution was left aside and allowed to cool down to room temperature slowly. The reaction mixture was divided into 30 mL aliquots (50 mL "falcon" tubes) and conc. HCl was added dropwise until pH<5. The white precipitate was observed. Tubes were topped up to 45 mL with water and mixed well. Tubes were centrifuged at 10,000 RPM for 15 min at room temperature. The supernatant was discarded, and the pellet was re-suspended in water (up to 45 mL) and mixed thoroughly. Tubes were centrifuged at 10,000 RPM for 15 min at room temperature (washing step). This was repeated two more times. Pellet was resuspended in 0.6 M NaOH (10 mL per tube) and left overnight at 37°C with orbital shaking (200 RPM). Next day conc. HCl was added dropwise until pH<5. The washing step was repeated. Obtained pellet was re-suspended in 125 mL of 0.6 M NaOH and pH was adjusted to 8 (conc. HCl). The aqueous solution was freeze-dried to yield white solid (24 g), which was used for biological assays without further purification.

c) SMALPs solubilisation

The membrane pellet was resuspended in Buffer A to final membrane concentration of 30 mg/mL (wet weight of membrane pellet). SMA was added (conc. 2.5 %), and the tube was incubated at 30°C for an hour. Afterwards, the tube was centrifuged at 34,000 RPM for an hour and obtained supernatant was used for further analysis. The supernatant was loaded onto the TALON column with HisPur resin (Thermo Fisher) and mixed well with resin by pipetting. The column was put on an end-to-end rotator (at 4˚C) for 12 h. to settle down and Buffer A with 20 mM imidazole was loaded onto column (10 mL) and left to flow through – column wash. The sequence of buffer A with increasing concentration of imidazole (40 mM, 250 mM, 500 mM, 2 M) was loaded onto a column (1 mL for each concentration), and 1 mL fractions were collected.

2.2.9 Dialysis

Dialysis buffer

HEPES	50 mM
NaCl	300 mM
Glycerol	10 %
DTT	1 mM
pH adjusted to 7.2	

Purified fractions of acyl-CoA dehydrogenase were dialysed with buffer, using a sealed dialysis membrane (molecular weight cut off 6-8kD) (Spectra/Por1, Spectrum labs). The dialysis membrane was suspended in 1 L of dialysis buffer and was continually stirred at 25 rpm, 4°C for 2 h., after which buffer was exchanged for a fresh one and protein was dialysed for further 14 h. The sample was then removed from the dialysis membrane for further quantification and analysis.

2.2.10 Protein quantification

Protein was assessed with the Pierce protein BCA assay kit,according to protocol suggested by the supplier. In summary series of solutions with various BSA, the concentration was prepared (0-2000 µL). Standard solutions of the BSA and sample with unknown concentration were pipetted into separate 96 well microplate wells (25 µL) and to each 200 µL of WR reagent was added and

mixed thoroughly. The plate was covered and incubated at 37 C for 30 min. After 30 min the plate was cooled down to RT and absorbance was measured at 562 nm in Tecan M200 plate reader. Each sample was prepared in triplicate.

2.2.11 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

2.2.11.1 SDS-PAGE

Polyacrylamide gels were regularly used to assess the purity of FaD protein. SDS-PAGE gels (12% (w/v) were prepared as described below. After the addition of polymerising agents; 10% (w/v) ammonium persulphate (APS) and TEMED, the separating gel mixture was immediately poured into the gel casks, and 1 mL of isopropanol was added on top. When separating gel had set, isopropanol was removed and stacking gel added.

SDS-PAGE was run at 200 V for 60 min or until the loading dye reached the bottom of the gel. SDS-PAGE were stained with Coomassie blue staining for 1 hour at room temperature, and de-stained a minimum of 3 times for 20 min at room temperature, or overnight.

12% SDS-PAGE gel for 1 separating gel

dH_2O	1.6 mL
30% (w/v) acrylamide	2 mL
1.5M Tris-base [pH 8.8]	01.25 mL
10% (w/v) SDS	50 µL
10% ammonium persulfate (w/v)	50 µL
TEMED	7.5 µL

For 1-3 stacking gels

dH_2O	12.5 mL
30% (w/v) acrylamide	0.83 mL
0.5M Tris-base [pH 6.8]	0.62 mL
10% (w/v) SDS	50 µL
10% ammonium persulfate (w/v)	50 µL
TEMED	5 µL

Coomassie blue stain

Coomassie Brilliant Blue R250	1.2 g/mL
Ethanol	40% (v/v)
Acetic acid	10% (v/v)

Coomassie blue destain

dH_2O	50% (v/v)
Methanol	40% (v/v)
Acetic acid	10% (v/v)

Transfer buffer (Western blot)

Tris-base	3 g
Glycine	15 g
Methanol	20% (v/v)

Dissolve in dH_2O water and adjust the volume to 1 L

Transfer buffer (Western blot)

Tris-base	3 g
Glycine	15 g
Methanol	20% (v/v)

Dissolve in dH_2O water and adjust the volume to 1 L

20X TBS-T Buffer (Western Blot)

Tris-base	60.57 g
NaCl	84.66 g
Adjust ph to 7.6	

Dissolve in dH_2O water and adjust the volume to 1 L

2.2.11.2 Western blotting

Western blotting was carried out to determine the presence of a His-tagged protein. Protein samples were separated using 12% (w/v) polyacrylamide gels and transferred onto 0.4 μm nitrocellulose membrane (Thermo Fisher) in transfer buffer at 200 V for 60 min using an electrophoresis power supply (Bio-Rad). The membrane was initially blocked with 5% skimmed milk (Oxoid) at 4 C overnight. To detect His-tagged FadE a mice His_6 Antibody (Qiagen) was dissolved (1:4000) in the buffer and acted as the primary antibody and incubated at room temperature for 1 h. Subsequently, 1:4000 anti-mouse immunoglobulin G conjugated with horseradish peroxidase in TBS-T was used as a secondary antibody and incubated at the room temperature for 1 h. The ECL™ Western Blotting Detection Reagent was used as a substrate.

After incubation with the secondary antibody, the membrane was washed for a minimum of three times with TBS-T at room temperature. Reactive bands were detected using ECL™ Prime Western Blotting Detection Reagent; a peroxidase detection reagent and with exposure to photographic X-ray film.

2.2.12 Ferrocenium assay

HEPES (0.5 M) buffer at pH 7.4 was prepared, as well as solutions of 10 mM ferrocenium hexafluorophosphate solution salt in 10 mM HCl (standardised spectrophotometrically at 617 nm ε=410 $M^{-1}cm^{-1)}$ and 100 mM EDTA. Both prepared fresh each time due to compound decomposition. The assays were carried out at 25 ˚C. Each sample was run in triplicate and contained: 1 mM, 0.1% DDM (only for DDM solubilised protein), 100 mM HEPES buffer, 200 μM ferrocenium salt, 50 μM hexanoyl-CoA as substrate and protein. Absorbance was measured for 15 min at λ = 300. Total assay volume was 200μThree controls were prepared: one containing all named components

except protein and substrate (instead buffer was added) and second containing all components except protein and third containing all components without substrate.

2.2.13 Data analysis

All experiments were run in triplicate and repeated at least three times. Bars on the graphs represent the standard deviation (SD). For data analysis, Microsoft Excel 365, GraphPad Prism 6 and Unipro UGENE were used.

Chapter 3

Chemical Synthesis of the acyl-CoA dehydrogenase inhibitors

3.1 Acyl-CoA inhibition – research focus

The acyl-CoA dehydrogenase is a large family of enzymes with wide substrate specificity. Classification is based on the carbon chain length of fatty acid-CoA substrates and is divided into three main categories, short, medium and long-chain acyl-CoA.[245] The majority of research associated with these enzymes can be categorised into three groups:

a) genetic diseases – mutations in genes coding for these enzymes result in metabolic dysfunction which is linked to various conditions ranging from hypoglycaemia to sudden infant death syndrome, [246,247]
b) microbial metabolism engineering towards industrially useful compounds[131,173]
c) structural studies[245,248].

Following the extensive literature review, there appeared to be no published research on the chemical inhibition of acyl-CoA dehydrogenase for industrial applications. Existing literature identifies acyl-CoA and pantothenic acid analogues as acyl-CoA dehydrogenase inhibitors,[233,240] with some research focusing on antibiotic activity.[235] Coenzyme A biosynthesis has been determined as an antimicrobial drug target with pantothenate kinase (PK) identified as a crucial enzyme.[249,250] Thus, potential acyl-CoA inhibitors are to be used in industrial applications, and they had to be carefully considered to avoid bacterial growth suppression (Figure 3.1).

3.1.1 The rationale for inhibitor synthesis

The majority of studies focusing on acyl-CoA dehydrogenase uses various acyl-CoAs. However, if the synthesised compounds are to be used in an industrial context the size of these compounds (Figure 1.17) is a drawback. This

project aimed for development of A library of FadE inhibitors with different potency, which were to be supplemented to the growth media when needed. The compound has coenzyme A as a part of the structure, thus its size is a disadvantage. It is unlikely to penetrate through the cell membrane. Pantetheine derivatives are recognised by the enzyme and can compete with the substrate. those analogues are a smaller size and lack adenosine 3-phosphate 5-pyrophosphate. Additionally, pantothenic acid is used as a CoA building block and is readily recognized by bacteria. Therefore, a shorter version of the inhibitor with pantothenic acid as a scaffold was chosen for this study.

2-pentynoyl-CoA was reported as a strong FadE inhibitor, therefore, was chosen as a first compound to synthesize and base further modifications. We have not identified studies that used 2-pentyoylpantetheine as a potential inhibitor. To explore the influence of triple bond and chain length compounds **2.37**, **2.47**, **2.49**, **2.51** and **2.52** were synthesized. 3-pentynoylpantetheine was previously synthesised and used in inhibition studies, however, were not reported for *E. coli*. It is worth to mention that bulk of a FadE inhibition research was conducted in the 80s and 90s of twentieth-century.[251,252]

Additionally, compounds with double bond were previously reported to show inhibitor activity, but with lower inhibition levels than alkynoyl analogues. It was thought that depending on the context of research or need for microbially synthesized short-chain alkanes the irreversible and relatively potent inhibition of FadE might not be required. Therefore compounds **2.46** and **2.48** were synthesized. Alkenoyl pantetheines are also similar in structure to FadE reaction product. Therefore, it was hypothesized that as such it will be less toxic for bacteria and more readily recognized by the enzyme.

Halo-acyl-CoAs were reported to show inhibitory activity, however pantetheine variant was not synthesized and tested. Additionally reported data were either incomplete or did not offer any information on the compound potency.[253] Therefore compounds **2.54**, **2.56** and **2.57** were synthesised and tested.

Alkanoyl pantetheines such as compounds **2.45** and **2.55** were expected not to inhibit the enzyme and were synthesised as a control. In addition, butyryl-pantetheine was previously reported to substitute the enzyme substrate,[254]

therefore if synthesised in good yield could provide a cheaper alternative for expensive commercially available butyryl-CoA.

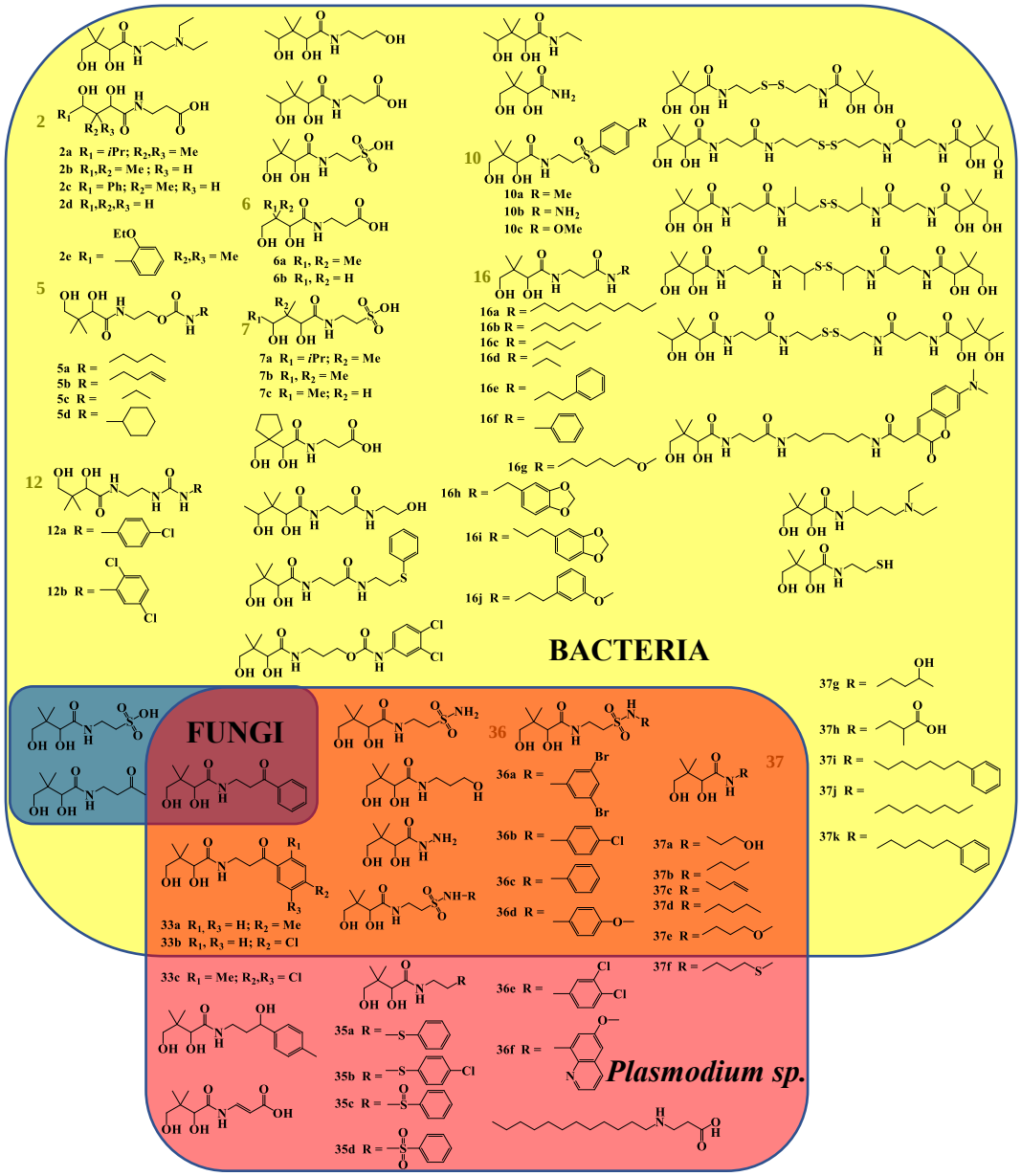


Figure 3.1 Chemical structures of pantothenic acid analogues reported in literature. Overlapping areas indicate activity against more than one organism. Stereochemistry not included in the figure. Adapted from Spry *et al.* [317,319]

3.2 Pantothenic acid analogues as enzymes inhibitors – mechanism of action

Chemical inhibitors of acyl-CoA dehydrogenase as growth inhibitors reported in the literature that are based on pantothenic acid generally do not utilize the full pantetheine structure, and present shorter chain and in many cases

do not have sulphur in the structure (Figure 3.1). Within the broad family of acyl-CoA dehydrogenases, the most researched and described is medium chain acyl-CoA dehydrogenase. Extensive research on the topic was conducted in the 1980s and 1990s, with current research on acyl-CoA dehydrogenases focusing more on structure elucidation and new acyl-CoAs discovery. The proposed mechanisms of inhibition seem to vary for different compounds; three of the mechanisms are discussed below (Figure 3.2).

The 3-Alkynoyl analogues (**2.1)** of pantetheine or CoA are thought to inactivate the short chain and medium chain ACADs through isomerisation to 2,3-allene (**2.3**) which leads to covalent reaction at the active site of the enzyme (Figure 3.2 A).

Interestingly, the inhibition with the triple bond located at β-carbon of the alkyl chain (**2.4**) leads to the generation of stable delocalized enolate (**2.5**) (Figure 3.2 B). However, the precise details of steps leading to this transformation remain obscure, despite structural studies indicating binding to the active site. At the same time, alkynoyl analogue inhibitors were found to be inactive for isovaleryl Acyl-CoA dehydrogenase, which indicates a certain level of selectivity.[233]

Apart from targeting the active site of the enzyme, some inhibitors can target the prosthetic group of ACADs, flavin adenine dinucleotide (FAD). An example of this mechanism is in hypoglycin A, a naturally occurring compound found in the fruits of Ackee tree and the seeds of box elder tree (*Acer negundo*).[255] An ingestion of corresponding fruits and seeds leads to Jamaican vomiting sickness, a reaction caused by the toxic metabolite, methylenecyclopropylacetyl-CoA. The proposed mechanism of action postulates formation of carbanion followed by interaction with the open ring and formation of adducts (**2.8**).[256] Although the starting point has been determined, reactions leading to ring opening and the final product (**2.9**) remain a topic of speculation. The involvement of radical species and adducts has been proposed by various research groups (Figure 3.2 C.[256,257]

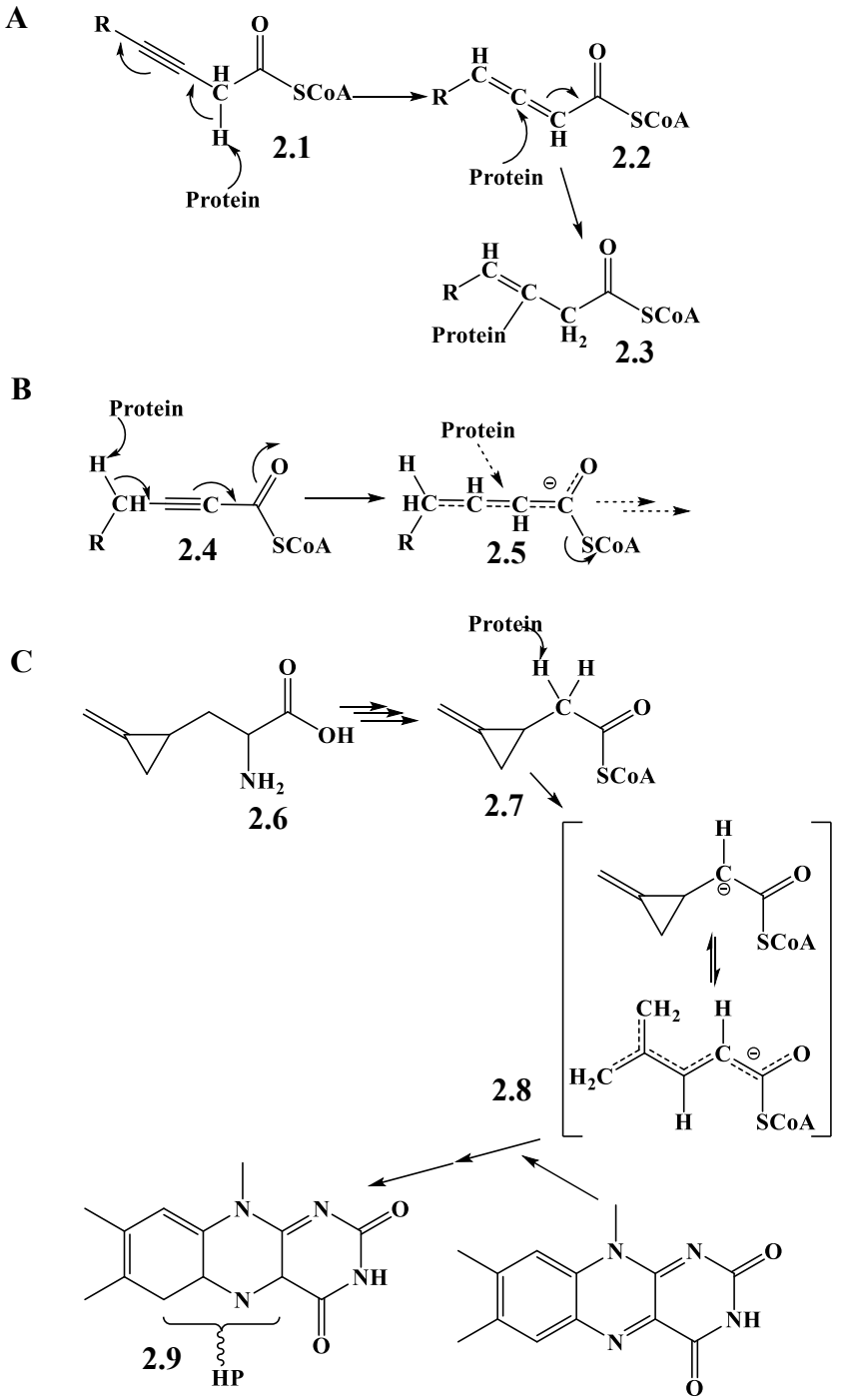


Figure 3.2 Mechanism of inhibition for A)3-alkynoyl-CoA B)2-alkynoyl-CoA C) hypoglicin. HP – product of hypoglicin. Adapted from Ghisla and Thorpe.[232]

Although most of the inhibitors discussed above showed irreversible mechanisms, a few examples of reversible inhibition have also been reported. Unsaturated thioesters have been identified as reversible inhibitors. The proposed mechanism of action involved five steps (Figure 3.3). The reaction begins with the binding of the analogue (**2.10**) with a free enzyme to form the Michalis complex (**2.11**). A glutamate residue in the active centre of the enzyme

binds the α-proton from the allen to form an enolate intermediate (**2.13**). In the next step, an activated compound forms an adduct with the flavin (at atom N-5 **2.14**). The stability of the reduced adducts varies between seconds to weeks, after which time it decays with the release of isomerised thioester and regeneration of the enzyme.[258] It is worth to mention that inhibition can be overcome by the presence of tightly binding substrate, in which case allene (**2.15)** will dissociate from the active centre of the enzyme.[232]

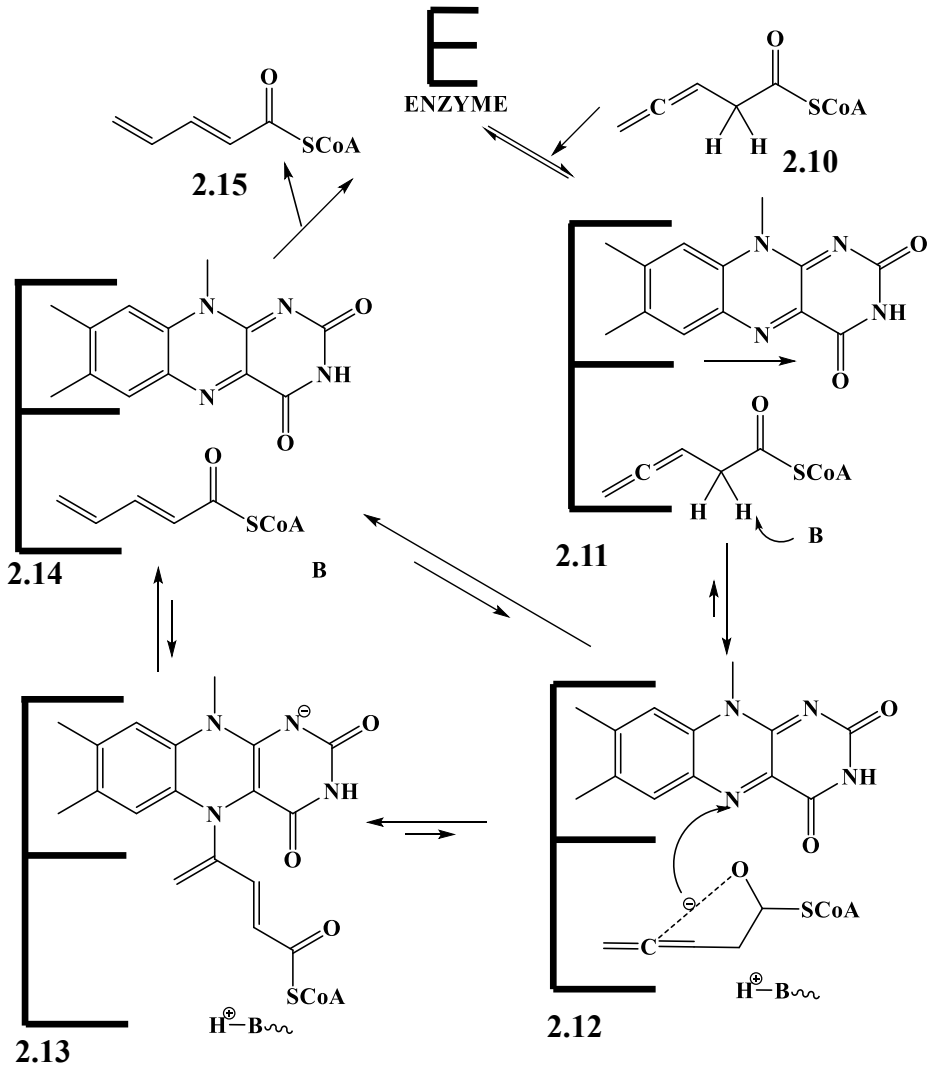


Figure 3.3 Mechanism of reversible inhibition. B with free pair of electrons indicates glutamate residue in the active centre of the enzyme. Adapted from Wenz *et al.*[253]

3.3 Reported synthesis of pantothenic acid derivatives

Pantothenic acid and pantothenic acid analogues were extensively researched. A brief summary of the proposed methods is discussed below.

3.4 Pantolactone ring opening

Pantothenic acid (PA) **(2.19)** [259] was first discovered in 1933, and synthetic pantothenate **(2.19)** [260] was obtained, and its activity was described as early as the 1940s. The synthesis of analogues with potential antimicrobial properties was also attempted, and the activity was tested against yeast.[261,262] The synthetic approach involves the reaction of pantolactone (**2.17**) with ethyl ester (**2.18**) to form a pantothenic acid (Figure 3.4).

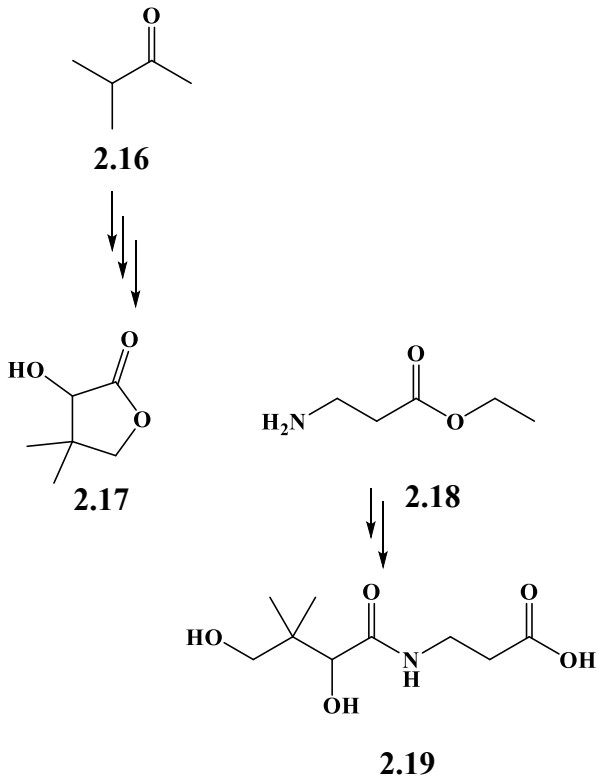


Figure 3.4 Total synthesis of pantothenic acid proposed by Stiller *et al.*[258]

One of the first total synthesis of PA was reported by Stiller *et al.* His approach involved five-steps reaction which required the condensation of isobutyryl aldehyde (**2.16**) to achieve pantolactone (**2.17**). The racemic mixture was then resolved by forming a quinine salt, to yield (D)-pantolactone. The lactone (**2.17**) was then coupled with β-alanine ethyl ester. [263] Since then, several

methods involving different alkaloids to resolve the racemic mixture and different β-alanine esters were proposed towards pantothenate synthesis.[264]

Approaches that propose base-initiated nucleophilic ring opening were previously reported to generate pantothenic acid analogues.[265,266] However, long reaction time and risk of racemisation are significant drawbacks in the process. More recently, microwave-assisted synthesis with triethylamine non-nucleophilic base as the base was proposed by Burkart *et al.*, and yield between 42 and 82% was achieved with significantly shorter reaction time (30 min)[267].

With regards to methods that directly couple pantolactone with β-alanine derivatives, there are several drawbacks of such procedure. This approach relies heavily on β-alanine. Additionally, undesired side reactions and low yields of β-alanine derivatisation lower the variability of the potential analogues. Moreover, pantolactone is a stable compound. Thus harsh conditions are required for its opening and presence of α-hydroxyl group increases the risk of racemisation.[266,268]

Pantothenic acid is a good starting point for its analogues synthesis. To achieve a synthesis of acyl-CoA dehydrogenase inhibitors introduction of units containing nitrogen and sulphur is necessary. An extensive literature review revealed that cysteamine is the most viable compound to utilize in S-loaded pantothenic acid analogues (pantetheines). A brief summary of the method is discussed below.

3.5 Synthesis of pantothenic acid analogues

3.6 Protecting groups chemistry for pantothenic acid analogues synthesis

In 2004, Mandel *et al.* proposed a synthesis that divided the target molecule into three units, cysteamine (M1), β-alanine (M2) and pantoic acid (M3) (Figure 3.5).[266]

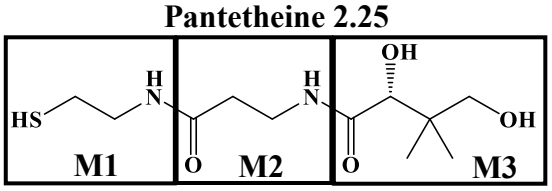


Figure 3.5 Division of the pantetheine into synthetic modules proposed by Mandel *et al.*

This approach allows for the modification of each unit separately, which not only increases the variability of potential analogues but also decreases the time of analogue generation (Figure 3.6). The first step of the synthesis is a D-pantolactone reduction to (R)-3,3-dimethylbutane-1,2,4-triol (**2.20**) with lithium aluminium hydride ($LiAlH_4$).

The compounds proposed as building blocks contain more than one functional group. Hence, 6-membered cyclic p-anisaldehyde acetal is required. This will ensure that the remaining exposed hydroxyl will be subjected to Severn oxidation followed by chlorite oxidation to yield the required protected pantoic acid (**2.21)**. In parallel the cysteamine was protected reacted with trityl chloride. (**2.23).** The introduction of trityl protection group masks sulfhydryl group and ensures that competing for the reaction of thioesterification will not occur. In the next step compound, **2.23** was coupled with Fmoc-β-alanine (**2.24**). Amide coupling was achieved by carbodiimide conditions. The use DIPEA in the reaction is important for two reasons: it is a poor nucleophile; thus, the risk of amine deactivation is eliminated, and it ensures basic conditions to maintain protection group (**2.25)**, which is removed under acidic conditions. Before the next step, N-deprotection was required. Deprotected amide (**2.25)** was then coupled with **2.21** through carbodiimide conditions. In the final step deprotection with iodine was achieved to yield pantetheine (**2.26**) (Figure 3.6).

The drawbacks of this approach are multiple steps, which lowered the overall yield and small scale of the reaction.

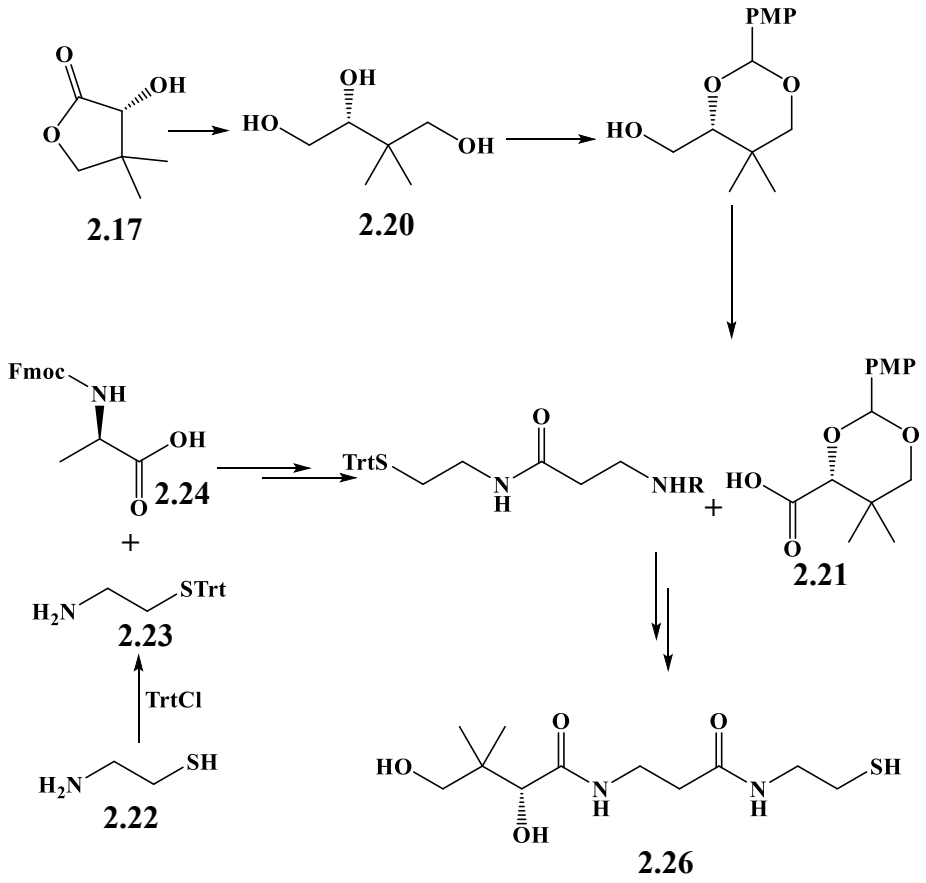


Figure 3.6 Synthesis of pantetheine proposed by Mendel *et al*.

The method that utilises the acetonide group for masking the hydroxyl groups of pantothenic acid was also reported by different research teams.[269,270] Protected pantothenate (**2.28)** was obtained through the reaction of D-calcium pantothenate (**2.27**) in acetone under acidic conditions (pTSA).[271] This was followed by PyBOP-mediated coupling with cysteamine hydrochloride under basic conditions (DIPEA). A variety of analogues was synthesized with EDC as coupling reagent and DIPEA. In contrast, to previous method deprotection was achieved with aqueous acetic acid (Figure 3.7).

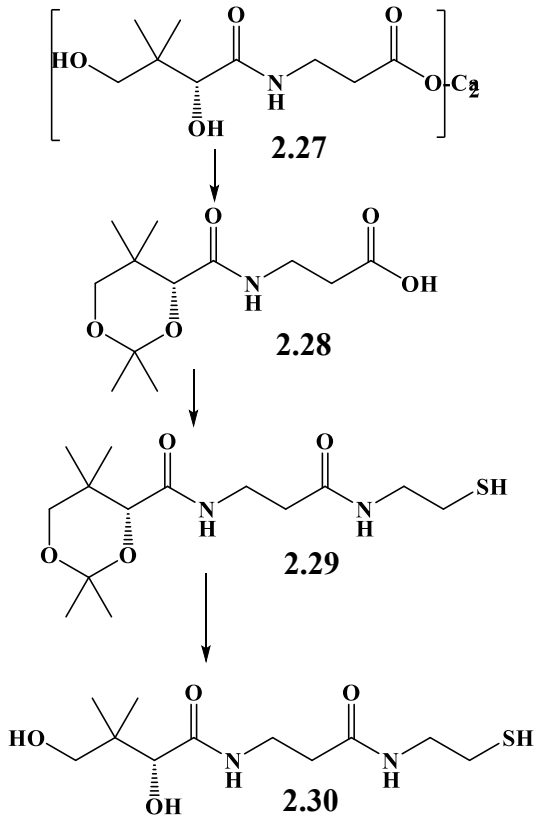


Figure 3.7 Synthesis of pantetheine proposed by Agarwal *et.al*

Reported methods offer good conditions for pantetheine synthesis. However, the introduction of protection groups requires additional steps, which is time-consuming and often generates toxic waste. In addition, previously reported synthesis is considered in drug design or research context. Thus, the scale of synthesis is small and upscaling possibilities have not been explored. Therefore, other methods were also explored.

3.6.1.1 Carbonyl diimidazole in pantothenic acid analogues synthesis

In 1960 Paul and Anderson reported 1,1`-carbonyl diimidazole (CDI) as a good coupling agent for amide formation.[272] Since then CDI has been used in peptide synthesis, it was also reported to be useful in CoA thioester formation.[273] This reagent has the potential to be used in large-scale reactions due to its relatively safe handling, being inexpensive and available in large quantities. Additionally, the reaction by-products are mostly non-toxic and can be easily removed with aqueous work up.[274] Coupling is possible *via* acid anhydride

formation. This method is therefore considered potentially useful for the pantetheine and thioester formation steps.

3.7 Total synthesis of S-acyl pantetheines

3.7.1 General consideration for the synthesis of S-acyl loaded pantetheines.

Previously described mechanism of inhibition shows that modified acyl-chain of the S-loaded pantetheine is required for activity.

Careful literature review allowed the identification of potential synthetic routes. Additionally, pantothenic acid (**2.19)** or more precisely pantothenic acid acetonide (**2.28**) was identified as a good starting material. The acid has two kinds of functional groups: carboxyl (-COOH) and two hydroxyls (-OH). The hydroxyl group can react with the activated carboxyl group and form an ester. Additionally, this reaction is more favourable than amide formation. Thus, the acetonide protection group was introduced to mask –OH groups. Numerous studies suggest HOBt, DIPEA and DCC or EDC for amide formation.[266,269] Two functional groups are present in cysteamine: amine (-NH_2) and the sulfhydryl (-SH), which after carboxyl activation, could lead to an amide and a thioester respectively. Even though the formation of the amide is preferred to that of a thioester, the crude reaction product could contain both, which in turn will make purification more demanding and will decrease the reaction yield. Additionally –SH group is prone to reaction with atmospheric oxygen to form disulphides, thus making storage more demanding if the group is unprotected.

To decide the most favourable conditions of synthesis different reaction conditions were tested (Figure 3.8). Initially, the introduction of the trityl protection group was proposed and EDC or DCC as a coupling agent with DIPEA as a base (Figure 3.8 A). Those conditions were tested but dismissed due to low yield, tedious purification and the need for protecting groups, which introduces additional steps. The carbonyldiimidazole route was therefore considered for pantetheine formation and DCC for S-acyl pantetheine formation (Figure 3.8 B).

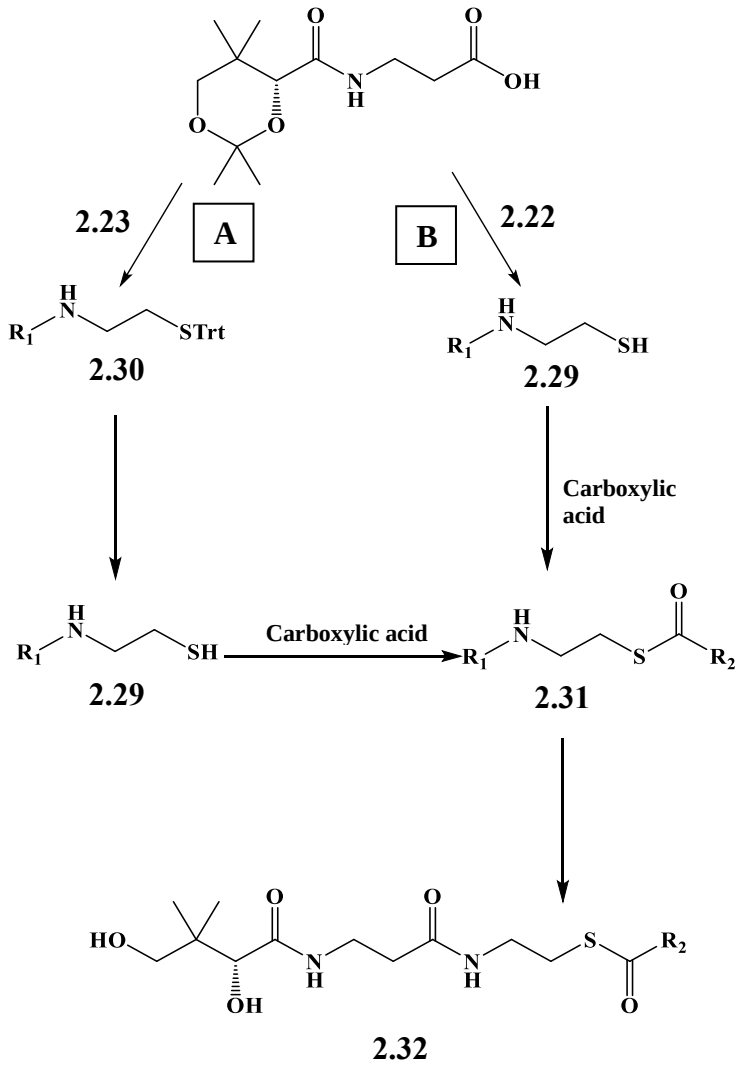


Figure 3.8 Synthetic routes for S-acyl pantetheines. R_1-pantothenic acid acetonide moiety, R_2 -carboxylic acid moiety. Route A- through protection group chemistry. Route B – synthesis without protection roups.

Detailed conditions and total synthesis of S-acyl pantetheines is reported and discussed below. The method was developed based on a synthesis of 2-pentynoyl-S-pantetheine.

3.7.2 Synthesis of pantothenic acid acetonide

Synthesis of pantothenic acid *via* β-lactone ring opening and condensation with β-alanine is established and well-developed method, due to its disadvantage, such as the possibility of racemization, harsh reaction condition and tedious purification, a method with calcium pantothenate D-pantothenic acid hemicalcium salt was preferred. Moreover, it was advantageous due to the

introduction of hydroxyl group protection. Details of synthesis are described below.

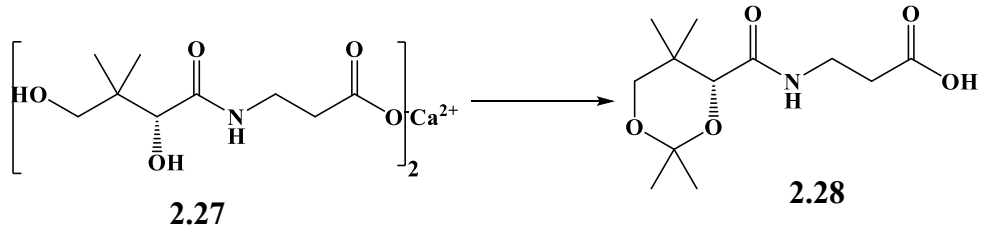


Figure 3.9 Synthesis of pantothenic acid acetonide.

Different methods for pantothenate acetonide formation have been reported.[269,275] For example use of Amberlite IR-120 (Plus) column and camphor sulfonic acid with DMF as a solvent.[276] However, the method described by Gaudelli *et al.* (Figure 3.9) offered easier work up and inexpensive substrates, therefore it was used in this study.[275] Additionally, this method allowed for acidification that removed calcium ion and the introduction of acetonide in one step. The 1,3-diol group was protected as acetonide to prevent reaction of the free hydroxyl groups with the activated carboxylic acids in later synthetic steps. The acetonide group is convenient because it is stable to the conditions used for the trityl deprotection of the thiol group used further downstream. As a result, protecting groups can be removed one at a time. Thus, it allows for "orthogonal" conditions of deprotection of diol and thiol. Compound **2.27** and acetone were reacted in the presence of p-toluenesulfonic acid (pTSA) and molecular sieve. Initially, after the addition of pTSA and molecular sieve to the reaction, a yield of only 47% was achieved after 12 h. When a further 12 h stirring was allowed, the yield improved significantly to 83%. The obtained product was used for further reactions.

3.8 Synthesis of S-acylpantetheines with trityl protected the 2-mercaptoethanol amine

An extensive literature review revealed that the synthesis of pantothenic acid analogues with protection groups chemistry showed good result.[266,269] Therefore, initially thiol protection with trityl was attempted. Reaction conditions were previously proposed by Mandel *et al.*, [266]

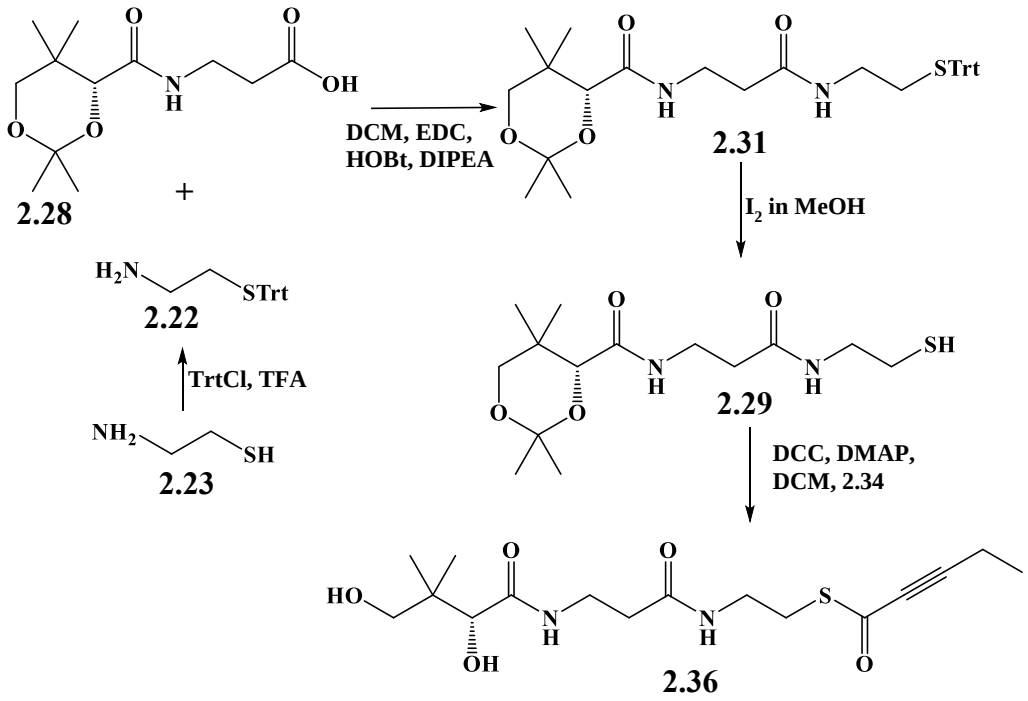


Figure 3.10 Synthesis of S-acyl pantetheines with trityl protection group

For the synthesis an appropriately prepared 2-tritylmercaptoethyamine **2.22** is required. The S-trityl protection was achieved by reaction of cysteamine **2.23** with trimethylphenyl (trityl) chloride under acidic conditions (TFA). The desired compound was obtained in good yield (68%) (Figure 3.10).

Further based on the research of Mandel *et al.* coupling of pantothenate acetonide **2.28** with thiol protected cysteamine **2.22** was attempted. The formation of amide was expected. EDC served as a coupling reagent for the reaction (activation of the carboxyl group). DIPEA ensured basic conditions and the utilisation of anhydrous THF reduced the side reaction with water present in the mixture. Compound **2.31** was obtained with a 46% yield.

The next step involved deprotection of the trityl group of the compound **2.31** without the removal of acetonide. This condition is crucial; otherwise further synthesis could be hindered by the formation ester (Figure 3.11).

Aforementioned "orthogonal protection" is useful in this case. For deprotection, a solution of iodine in methanol was used to yield the desired product **2.29**. The compound of molecular mass of 318.16 was expected to form. Major impurities were present in LC-MS along with the desired compound **(**m/z found 319.3, [M+H]+). The reaction was performed on the very small scale, attempts to upscale reaction required tedious, two-step work up. Firstly, flash chromatography was performed followed by reverse phase chromatography. Compound **2.29** was obtained at a low yield (15%).

Due to time-consuming work up for reaction described above reaction, deprotection with TFA: DCM (1:1) was also tested. However, it resulted in compound decomposition.

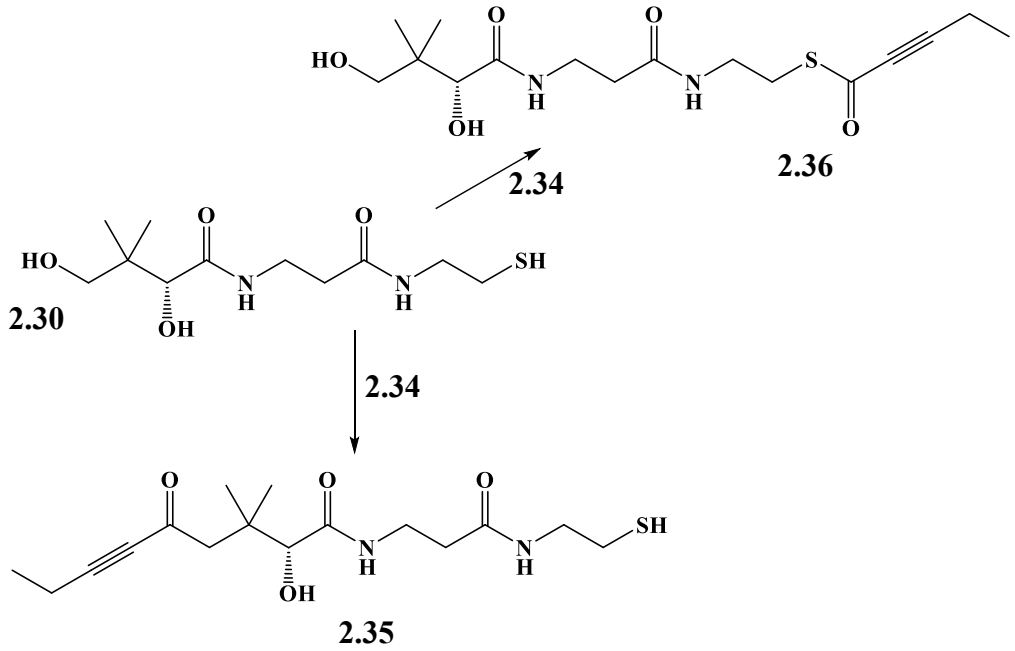


Figure 3.11 Competing reaction to thioester formation.

The final step to synthesise **2.36** was achieved *via* Steglich esterification.[277] serving as a base. Compound **2.36** was achieved at a low yield (28%). Detailed procedure reported in point **2.1.3** (Figure 3.11).

A tedious, time-consuming work up and low yields of final compounds lead to evaluation of the alternative method. This strategy would entitle the synthesis of S-acyl pantetheines without protecting the group.

3.9 Synthesis of S-acyl pantetheines without trityl protecting group

Based on the research of Gaudelli *et al.*[275] the coupling of pantothenate acetonide with unprotected cysteamine or cysteamine hydrochloride was proposed.

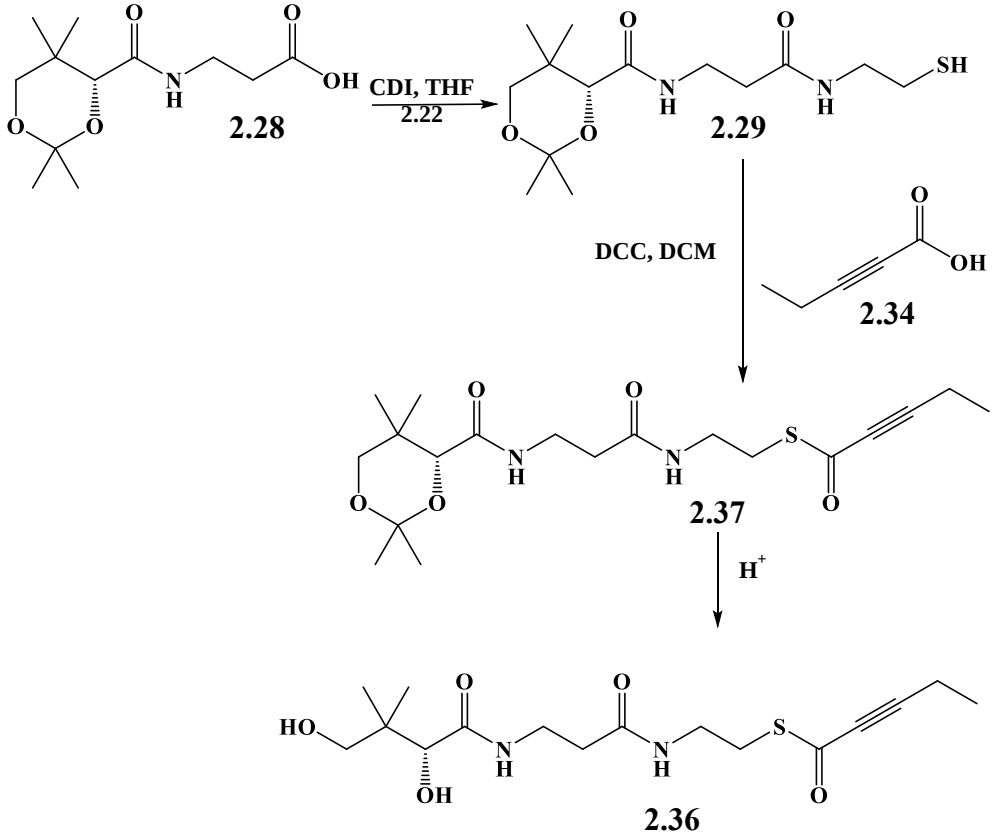


Figure 3.12 Total synthesis of 2-pentynoyl-S-pantetheine without trityl protecting group

3.9.1.1 Pantetheine acetonide synthesis

The key intermediate of pantetheine synthesis (Figure 3.12, **2.29**) was prepared following the procedure previously reported.[275,278] The original paper proposes pantetheine synthesis with cysteamine hydrochloride and 1`,1`-carbonyldiimidaole (CDI). CDI can facilitate amide bond formation with amines and amine hydrochloride salts.[279,280] Further research revealed that it was also used to obtain thioester in a reaction that is performed in aqueous conditions,[281] Due to acetonide instability in the presence of water, the use of aqueous media was not considered.

Although the original method suggests the use of cysteamine

hydrochloride,[275] the free cysteamine initially considered. Due to better accessibility of free amine group, a 12 h reaction time and simple workup were sufficient. When the hydrochloride salt was used, 36 hours were required to afford the desired compound without any thioester impurities. It is known that thioesters have poor stability in basic conditions.[282]. Thus, it was proposed that extended reaction time leads to thioester hydrolysis and the only amide was observed as the final product. For cysteamine hydrochloride, the yield is lower (*ca.* 80%) than in the case of cysteamine (*ca.* 90%) and the purification is more time-consuming. Reactions with both compounds are easy to upscale and can be used alternately. Interestingly, the pantetheine synthesis was achieved without additional protecting groups to mask thiol. Purification steps are straight forward and yield good results. The utilisation of anhydrous THF reduced the side reaction with water present in the mixture. This reaction involves the transacylation of acids. Mechanism of carboxylic acid activation with CDI (**2.38**) is shown in Figure 3.13. The reaction of carboxylic acid activation involves the initial formation of carboxy imidazole (**2.39)** and imidazole (**2.40)**. However, both species easily and quickly react together to form activated acyl imidazole (**2.41)**. The formation of the CO_2 is a driving force in this reaction.[283,279] Compared to the use of acid chloride reaction, it is safer and easier to handle and offers a wider scope in synthesis.

Compound **2.29** is subsequently used in the next steps of S-acyl pantetheine synthesis.

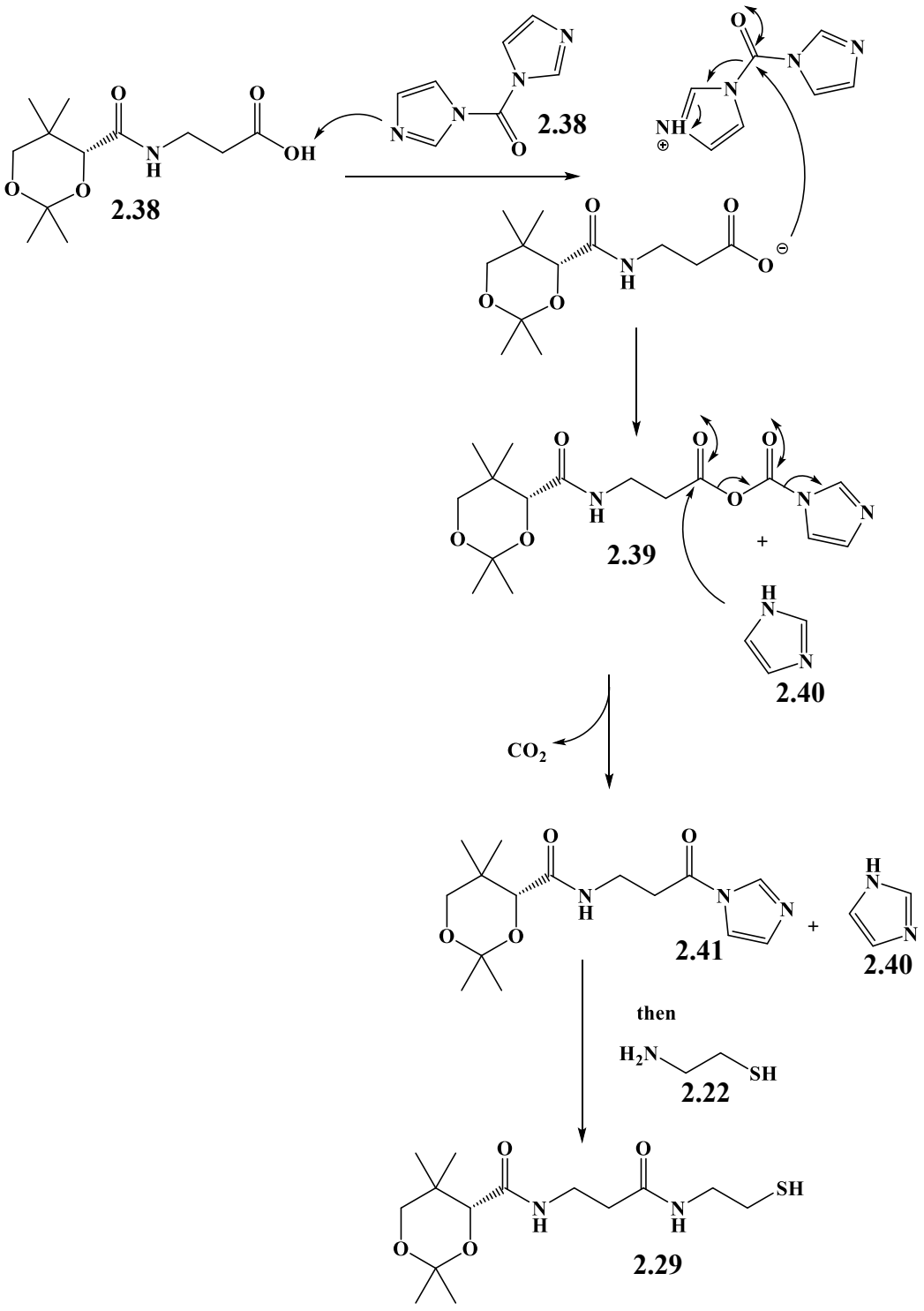


Figure 3.13 Activation of a carboxylic acid with CDI to yield active acyl imidazole. Adapted from Montalbetti and Falique.[271]

3.9.1.2 S-loaded pantetheine synthesis

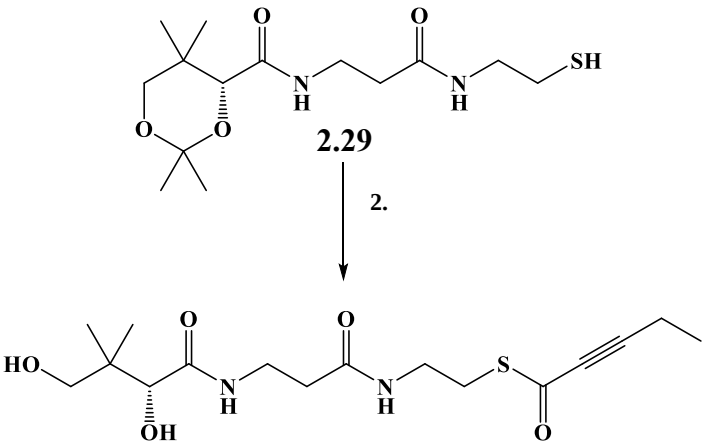


Figure 3.14 Acetonide group protected S-loaded pantetheine (2.29) and deprotection towards free S-loaded pantetheine

For the formation of the first thioester, three different conditions were tested, it was determined that DCC coupling yielded the best results and was used for the synthesis of the required S-loaded pantetheine analogues.

a) Steglich esterification[277]

The reaction was performed as described in the literature with *N*,*N'*-dicyclohexylcarbodiimide (DCC) as a coupling agent and 4-dimethylamino pyridine (DMAP) as a base. However, purification was tedious, and the obtained yield was low, 28%. Additionally, LC-MS and NMR suggested that the acetonide protection group was removed.

b) Carbodiimide and DIPEA coupling

Alternatively, HOBt and EDC with DIPEA as a base were considered as reaction conditions. Carbodiimides are widely used in organic chemistry.[284,285] Reaction yield was 33 %. As in the case of Steglich esterification acetonide protection group was removed.

c) DCC as coupling agent[286]

In 2010, Meier *et al.* reported pantetheine thioester formation (Figure 3.14). The method gave 40-70% yield, depending on the coupled compound. It was also determined that longer activation of carboxylic acid resulted in higher

yields. The reaction requires 4 eq. of DCC, reduction of its quantity to 2 eq. results in significantly lower yield.

The formation of unprotected S-acyl pantetheine has not preferred the route of reaction. However, the removal of the acetal protection group is not surprising. Acetonide is unstable and easily removed in aqueous acidic conditions. Post reaction works up with aqueous saturated ammonium chloride, which is mildly acidic, introduced conditions favourable for deprotection. If the same carbodiimide conditions were applied, but reaction quenching was omitted, and the reaction mixture was evaporated *in vacuo* and submitted to purification with column chromatography, the deprotection would not occur. This was demonstrated by for example Agarwal *et al.*[269] In addition introduction of acidic conditions could be partially responsible for low yields if partial hydrolysis of formed ester occurred.

Carbodiimides are most typically used for amide formation (an example of which was shown in **2.1.3**). However, since 1979 they are also used for ester formation.[277] Use of DCC presents side reactions such as rearrangement of the O-acylisourea to inactive N-acyl urea or coupling of an activated carboxylic acid with another carboxylate. Those side reactions could be prevented by the addition of catalytic amounts of nucleophile such DMAP. Alternatively, the use of HOBt also lowers the chances of N-acyl urea formation. [287,288]

In addition, a by-product of DCC is dicyclohexylurea (DCU), traces of which can be difficult to remove. Introduction of reaction additives can result in by-products, which could make purification more time consuming, which occurred when Steglich esterification and carbodiimide with additives reactions were discussed.in case of HOBt and its variants safety must be taken into consideration as these compounds show explosive properties. Another possibility of overcoming side reactions is cooling down the reaction mature to 0°C.[279,289] It is also postulated that the addition of an excess of DCC allows for rapid carboxylic acid activation and lowers chances of anhydride formation. DCU can be removed from the reaction mixture with celiate filtration or precipitation in EtOAc at -20 °C. Hence after careful consideration, synthesis of S-loaded pantetheines according to conditions proposed by Meier *et al.* was chosen, and remaining compounds were synthesized *via* this method.

3.9.2 Synthesis of unstable carboxylic acids

Not all carboxylic acids are commercially available. However, they can be synthesised from corresponding esters or alcohols. Here we report the synthesis of 3-pentynoic acid and acetoacetic acid and 4-chloro acetoacetic acid.

3.9.2.1 Synthesis of 3-pentanoic acid

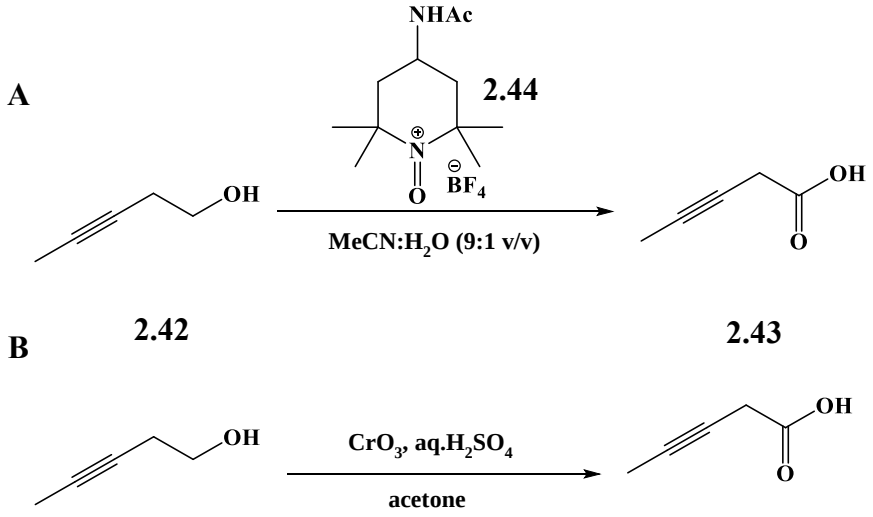


Figure 3.15 Synthetic approach to 3-pentynoic acid **2.43.** Conditions for A-Bobbitt`s salt, B-Jones oxidation

The 3-pentynoic acid is not commercially available therefore 3-pentanol **2.42** was oxidised to the carboxylic acid. Oxidation of alcohol is possible with chromium (VI) oxide (CrO_3) in an acidic environment, also known as Jones oxidation.[290] This reaction is hazardous, CrO_3 is known to be cancerogenic, and by-products of the reaction are toxic. Additionally, work up is tedious and only a 30 % yield was obtained with multiple impurities. Thus, more environmentally friendly, oxoammonium salt **2.44** (also known as Bobbitt`s salt) was considered (Figure 3.15).[291] Hence, **2.42** was treated with **2.44** to afford **2.43** in good yield (69 %) efficiency and good purity. In addition to a good yield, Bobbitt`s salt offers easy work up and waste disposal. The drawback is a high quantity of salt required, as stoichiometric equivalents are needed.

3.9.2.2 Synthesis of acetoacetic acid (2.78) and 4-chloroacetoacetic acid (2.79)

The synthesis was achieved *via* hydrolysis of the corresponding esters with TFA. [292] Hydrolysis of *tert*-butyl acetoacetate was performed with a 54% yield and ethyl 4-chloroacetoacetate with a 94% yield. The obtained carboxylic acids were used in synthesis without further purification.

3.9.3 Removal of the acetonide protection group

Removal of acetonide (Figure 3.16) protecting group was done in one step (0.2 M HCl) [293] and did not require further purification after work up. In addition, the product was obtained in good yield >80%

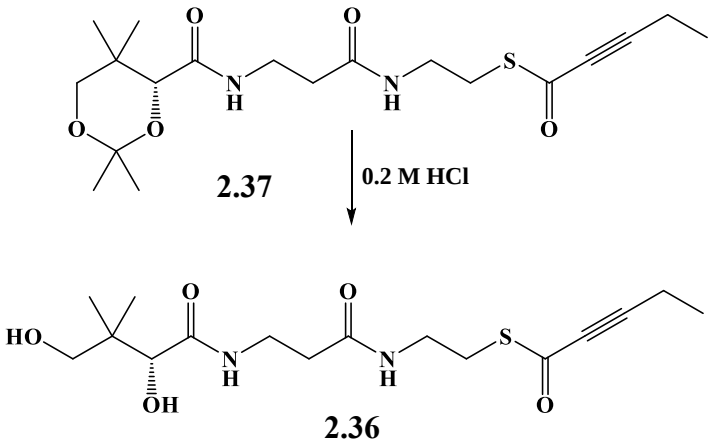


Figure 3.16 Deprotection of 2-pentynoic-S-pantetheine acetonide

3.10 Conclusion

In conclusion, this chapter reports the synthesis of S-loaded pantetheines which will be used as acyl-CoA inhibitors in the subsequent microbiology studies. The synthesis was achieved by CDI mediated amide formation, followed by thioesterification with carboxylic acid (Figure 3.17).

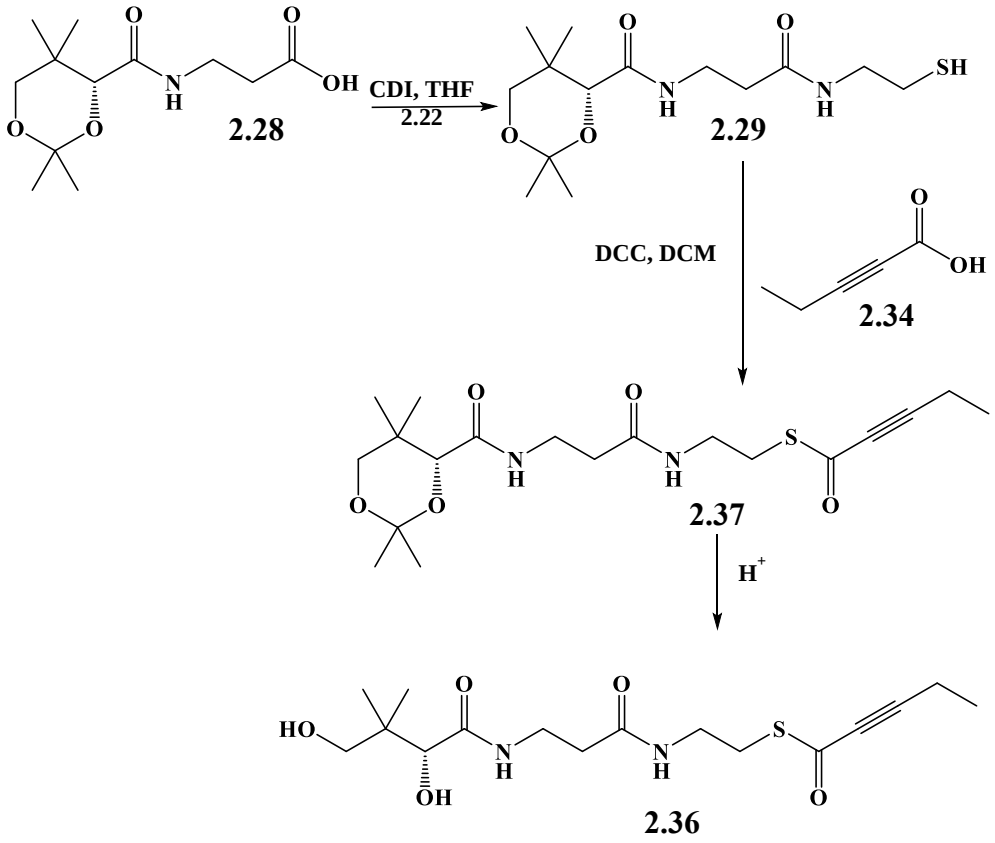


Figure 3.17 Total synthesis of 2-pentynoyl-S-pantetheine without trityl protecting group

The method was developed with the synthesis of 2-pentynoyl-S-pantetheine. Total of twenty-seven compounds was synthesized using the following method (Table 3.1 and Table 3.2). To ensure that reaction reactions are a repeatable synthesis of all compounds were attempted at least twice. Compounds **2.45**, **2.46**, **2.47**, **2.53**, **2.56** (and respective acetonide deprotected variants) were previously synthesised. However only compounds **2.47** and **2.60** were previously tested as FadE inhibitors.

Table 3.1 Acetonide protected compounds synthesized during this study part I, with corresponding yields. The red asterisk indicates compounds previously synthesised.

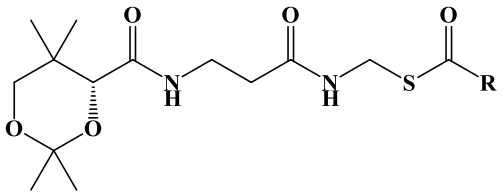
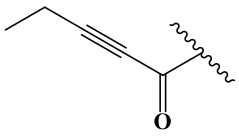
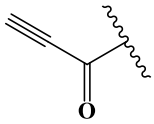
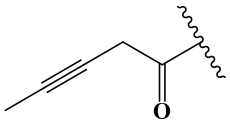
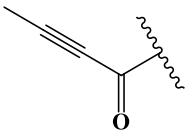
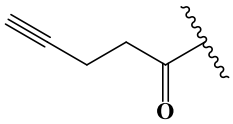
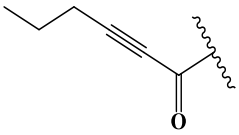
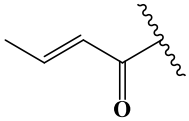
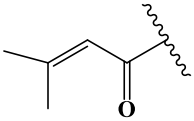
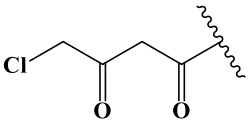
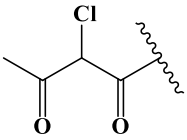
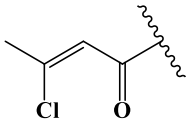
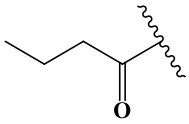
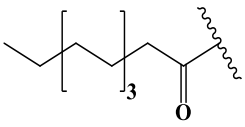
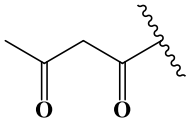
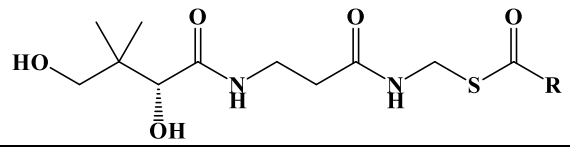
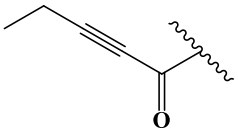
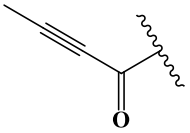
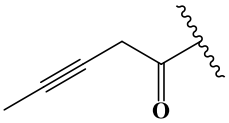
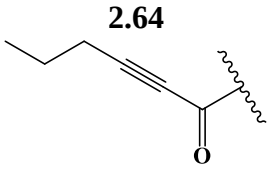
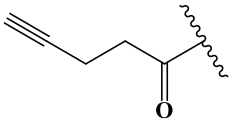
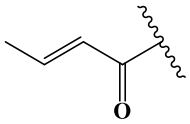
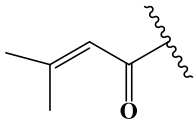
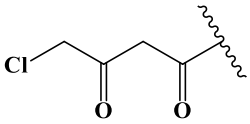
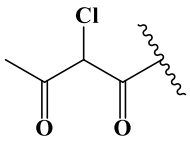
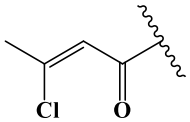
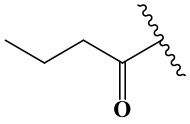
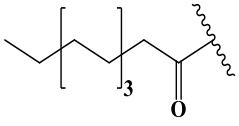
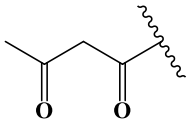
Reaction Yield [%]			
			
Alkynoyl structural analogues			
2.37	 49%	**2.50**	 33%
2.47*	 49%	**2.51**	 61%
2.49	 52%	**2.52**	 54%
Alkenoyl structural analogues			
2.46*	 52%	**2.48**	 49%
Structural analogues with chlorine			
2.54	 63%	**2.57**  53%	
2.56*	 56%		
Alkanoyl structural analogues			
2.45*	 73%	**2.55**  40%	
2.53*	 45%		

Table 3.2 Acetonide deprotected compounds synthesized during this study part II, with similar yields. The red asterisk indicates compounds previously synthesised.

Reaction Yield [%]			
			
Alkynoyl structural analogues			
2.36	 85%	**2.63**	 82%
2.60*	 82%	**2.64**	 82%
2.62	 91%		
Alkenoyl structural analogues			
2.59*	 79%	**2.61**	 89%
Structural analogues with chlorine			
2.66	 86%	**2.69**	 83%
2.68*	 80%		
Alkanoyl structural analogues			
2.58*	 88%	**2.67**	 88%
2.65*	 83%		

The synthetic approach was based on research by Agarwal *et al.*,[269] Gudelli *et al.*[275] and Mandel *et al.*[266] However the synthesis was modified and the number of steps was reduced. Mandel *et. Al* suggested modular synthesis, which required to use protection groups and required more steps as it used β-alanine and β-pantolactone for synthesis, which as explained in 3.6 requires harsh conditions. Therefore, following research of Gaudelli *et al.* pantothenic acid hemicalcium salt was used as starting material. It was observed that longer reaction times and an additional 0.5 eq. of PTSA increase the yield.

First, the protection groups chemistry was attempted because pantothenic acid contains carboxylic and hydroxyl functional groups. Therefore, it was reasonable to involve trityl as a protection group. Trytyl seems to be an unusual choice for thiol protection, as it is mostly used for amine protection. However, it was previously reported to give very good yields.[266] The most popular reagents for peptide bond formations are HOBt, EDC/DCC or PyBOP just to name a few.[269,277,294] Implementing principles of modular synthesis proposed by Mandel *et al.* HOBt and EDC/DCC were used for coupling of cysteamine and pantothenic acid. The synthesis was successful and in good yield (71%).

The first two steps of synthesis provided good yields and relatively easy purification. Unfortunately, removal of the protection group and further synthesis proved to be tedious and gave low yields, which was in contradiction to published literature. Multiple attempts and conditions change did not improve the reaction. Thus, a different synthetic approach was tested. After extensive literature research 1`,1`-carbonyl diimidazole (CDI) meet the criteria, it was inexpensive, available in large quantities and by-products were easily disposable.[278,280,295] Pantothenic acid and cysteamine coupling gave good yields 76-94% depending on reaction conditions, easy purification and can be prepared in large quantities.

CDI can facilitate reaction formation of thioester as well as amide, however, despite promising reports in literature[278,280], the formation of acyl-S-pantetheines was achieved in a lower yield than expected and sample required HPLC purification. Meier *et al.* reported the pantetheine thioester formation. Yields of 40-70% were achieved, depending on the coupled carboxylic acid. The longer activation of carboxylic acid resulted in higher yields. The drawback of

the method is a high quantity of DCC for reaction to provide good results. The reaction requires 4 eq. of DCC, reduction of its quantity to 2 eq. results in significantly lower yield.

Other coupling methods for example Stiglich esterification,[277] HOBt/EDC coupling were also tested and produced lower yields with more tedious and longer purification. In this case in contradiction to protocols described in the literature,[279,294,296] reverse phases chromatography combined with HPLC was required. Therefore, DCC was favoured as a coupling reagent. Easier purification allowed to obtain pure acetonide protected analogue, which was an advantage in the next step of the synthesis. Deprotected compounds were ready to use and sufficiently pure after simple after reaction workup (Extraction with ethyl acetate and evaporated to dryness).

In comparison to previously reported methods, synthesis proposed in this study (Figure 3.17) is relatively inexpensive and easy to upscale. Majority of reported results were obtained on a small scale (≥100 mg of starting material).[269,297–300] Additionally, it requires only four steps, similar to the total synthesis proposed by Agarwal *et al*. However, reported quantities are low 12 mg to 120 mg depending on the synthesised compound. If we consider the industrial application of synthesised compounds in bioreactors for biofuel production this amount could be a drawback which will increase production costs.

Chapter 4

Escherichia coli short-chain acyl-CoA dehydrogenase enzyme characterisation

4.1 Reported inhibitors of short-chain acyl-CoA

The bulk of research regarding acyl-CoA dehydrogenase inhibitors was conducted in the 80s and 90s.[301] Among them the alkynoyl derivatives of CoA are best described.[233] This chapter aimed to extract and purify *E.coli* short-chain acyl-CoA dehydrogenase and test its activity *in vivo* in a ferrocenium assay. In addition, the inhibition activity of compounds synthesised in Chapter 2 is assessed.

4.1.1 Construction and purification of recombinant E. coli short-chain acyl-CoA dehydrogenase

The primers for *fadE* amplification were designed (Table 2.2) to include 17 bp from the start and the end of *fadE* and restriction sites - NcoI for forwarding primer (-CCATGG-) and HindIII for reverse primer (-AACGTT-). Usually, primers with more than 12 bp are long enough to avoid random binding to the sequence. The selected vector (pET21-d(+)) (Figure 4.1) also contains the restriction sites described above. This allows cutting amplicon and vector to create" sticky ends" required for ligation. The utilisation of two enzymes ensures

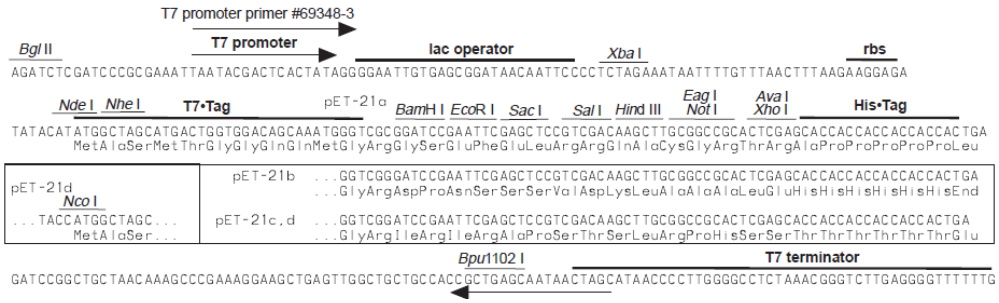


Figure 4.1 Clonning/expression region pET21d(+) vectors. Source: Novagen

that the insert is put into the plasmid in the right orientation.

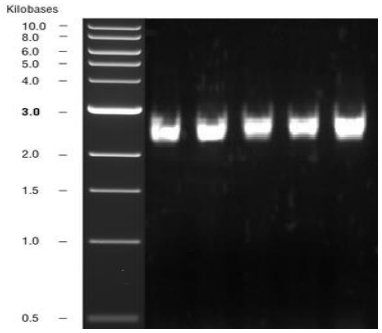


Figure 4.2 Five PCR amplification rreactions results with FadE_Fr and FadE_Rv

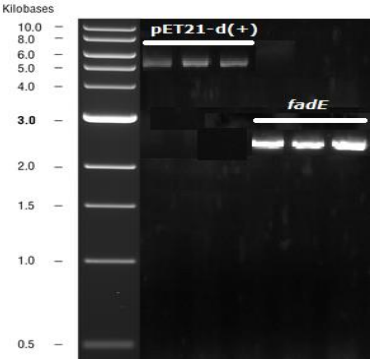


Figure 4.3 Restriction digest visualisation before bands were cut.

PCR with chosen primers yielded good results (Figure 4.2). All five samples have the amplicon of 2.5 kb size which corresponds to the *fadE* gene in *E.coli*. Nanodrop revealed that the sample has a high concentration of DNA (271 ng/µL). To obtain "sticky ends" both *fadE* and pET21-d(+) vector was digested with NcoI and HindIII restriction enzymes (Figure 4.3). Diagnostic electrophoresis showed that neither vector nor *fadE* was lost during the process

Transformation yielded *E.coli* Top10_pet21d(+)(FadE) strain. The plasmid carries Amp resistance. Thus cells without this plasmid should not grow on medium with Amp since TOP10 strain does not have antibiotic resistance itself. The presence of a *fadE* insert was confirmed with the diagnostic digest.

Sanger sequencing was chosen to determine if the insert is indeed *fadE*. As all sequencing methods, it generates sequence data in the form of independent reads, which then must be assembled together to form a complete sequence. The average read for Sanger sequencing is between 800 and 1000 kb. In addition, sequencing of the first few bases (15-40 bp), as well as bases after 900 kb, has poor quality.[302] pET21d(+) vector has a T7 promoter and T7 termination sites. Therefore primers T7_FR and T7_RV were chosen to avoid poor quality of sequencing for bases on the beginning and the end of the sequence. Since on average 900 bp are sequenced for each primer. Also, primers FadE_Fr and FadE_Rv (which allowed sequencing both ends of the gene) and FadE_seqFR, FadE_seqRV (which allowed to sequence the middle of the insert) were included. The utilisation of multiple primers led to the sequencing of the

entire *fadE* gene (consists of 2.5 kbp). Sequencing confirmed the presence of the desired insert in the introduced plasmid.

4.2 Recombinant acyl-CoA overexpression optimisation

The plasmid pet21dFadE was transformed into *E.coli* BL21(DE) strain, which was chosen due to high plasmid uptake, high protein expression level and easy induction. Multiple colony growths confirmed that this strain has high plasmid uptake.

Optimisation of the protein expression revealed significant differences in expression levels depending on temperature and time (Figure 4.5)

For cultures that IPTG has not added the band which corresponds to the

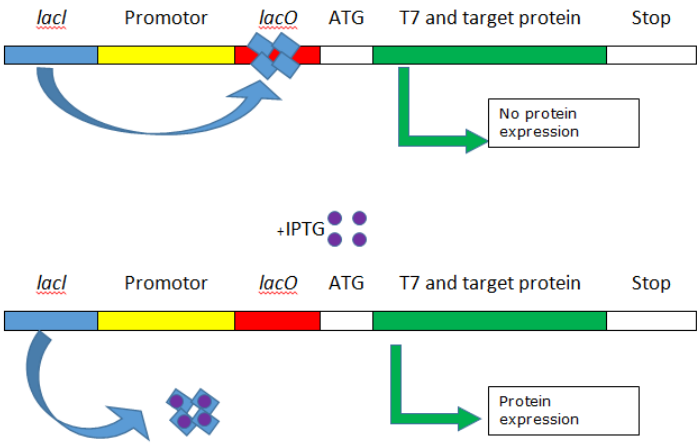


Figure 4.4 Expression of the protein of interest with isopropyl β-D-1-thiogalacto- pyranoside (IPTG)

molecular weight of FadE is not present the experiment. That shows that expression of the protein from the *fadE* present on pet21d(+) plasmid is triggered by IPTG. It is possible due to the presence of the operon (Figure 4.4).

At 37˚C there were no changes in protein yield between induction times. Conversely, lowering temperature increased protein expression with time. The expression levels at 30˚C were higher. However further protein analysis revealed that after membrane disruption overexpressed protein is precipitating from solution. Therefore, to improve protein solubility temperature and IPTG concentration were lowered to 22°C and 0.25 mM respectively. This is consistent with published literature, which reports better expression with lower

temperature and inducer concertation.[303]

Since the protein of interest was expected to be bound to the membrane, protein extract was fractioned by ultracentrifugation. The process yielded membrane as a brown pellet. SDS-PAGE confirmed the presence of protein in the membrane fraction. In addition, Western blot confirmed that the expressed protein was His-tagged. The vector used for cloning includes His-Tag on the C–terminal end of the protein, thus binding to anti-His-tag antibodies confirms its expression. *E. coli* BL23(DE3) was also transformed with an empty pet21 d(+) vector (Figure 4.5). Obtained strain was induced with IPTG; Western blot analysis showed no bands.

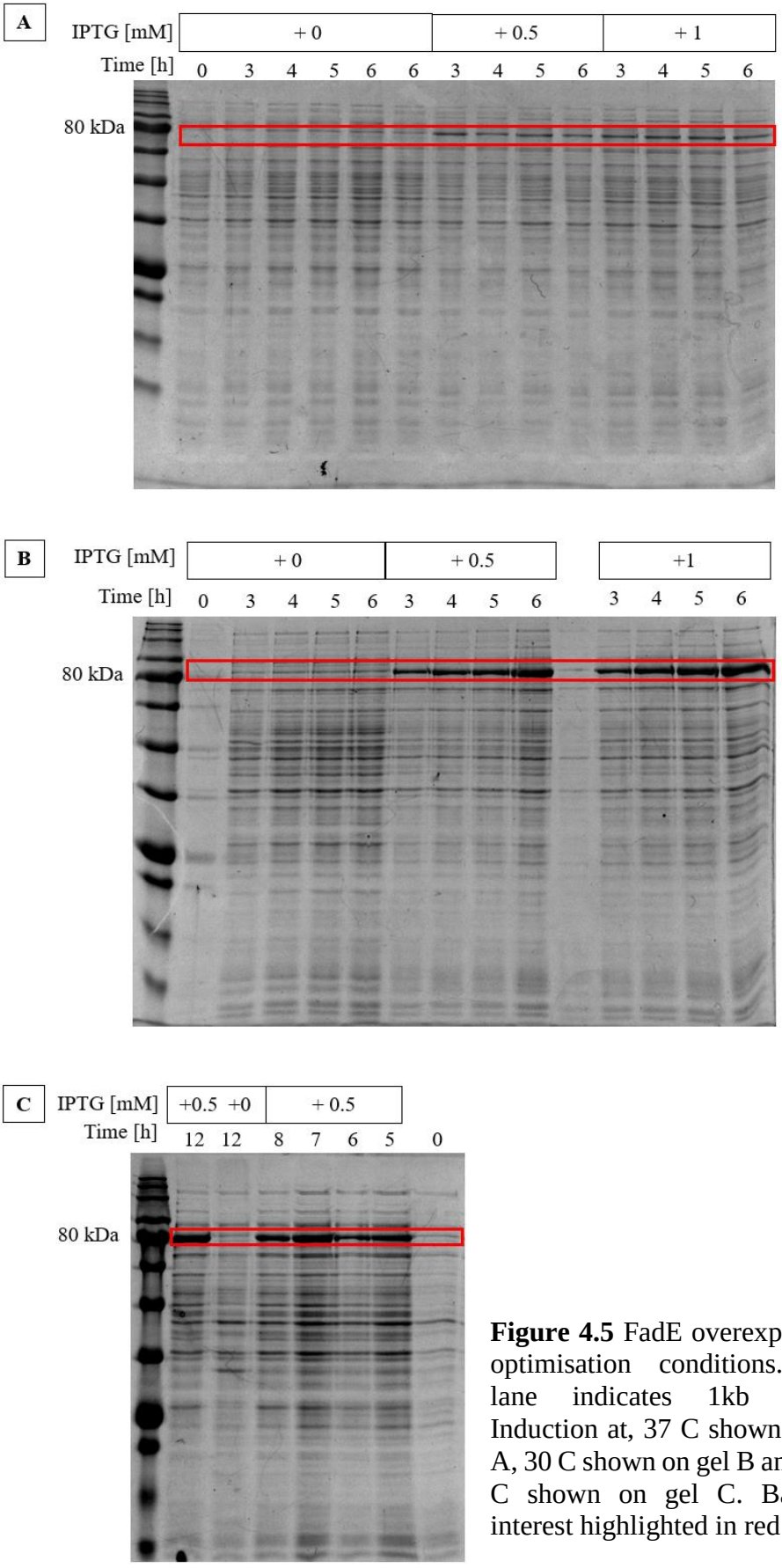


Figure 4.5 FadE overexpression optimisation conditions. First lane indicates 1kb ladder. Induction at, 37 C shown on gel A, 30 C shown on gel B and at 22 C shown on gel C. Band of interest highlighted in red frame.

4.3 Protein purification

Acyl-CoA dehydrogenase is typically inner membrane protein, therefore for further analysis, it had solubilized. It is a critical step, and it aims for extraction protein of interest from the membrane in its active form. In general membrane proteins require a hydrophobic environment to maintain activity.[304] Therefore membrane protein purification is not easy. The most popular and widely researched methodology involved the use of detergents. The process is based on the formation of membrane fragments with proteins and encapsulated in detergent, leading to micelle formation.[305,306] Unfortunately there is no "golden method" and solubilisation conditions have to be determined for each protein.

Use of detergents is an older method. In recent years, polymer-based membrane protein purification has gained more attention and number of publication on the topic is increasing rapidly, from one article published in 2004 to more than 20 published articles only in the first half of 2018.[307] Method allows for protein extraction, while the native environment is maintained. Up to date Styrene Maleic Acid (SMA) polymer is most widely used. SMA copolymers form a membrane nano-disks called SMA lipid particles (SMALPs).[308] Both methods were tested for FadE purification.

4.3.1 Membrane solubilisation

Four detergents were tested: octyl-β-D-glucopyranoside (ODG), Triton X, TWEEN and Dodecyl-β-D-maltopyranoside (DDM). Each detergent was tested at 20 mM concentration and at two different temperatures 4°C and 22°C. The DDM was chosen as a detergent for membrane solubilization as it was most effective (Figure 4.5). SDS-PAGE and Western blotting confirmed that solubilized protein is FadE. Solubilisation at 4°C showed the most promising results. It is possible that higher temperature results in protein degradation.

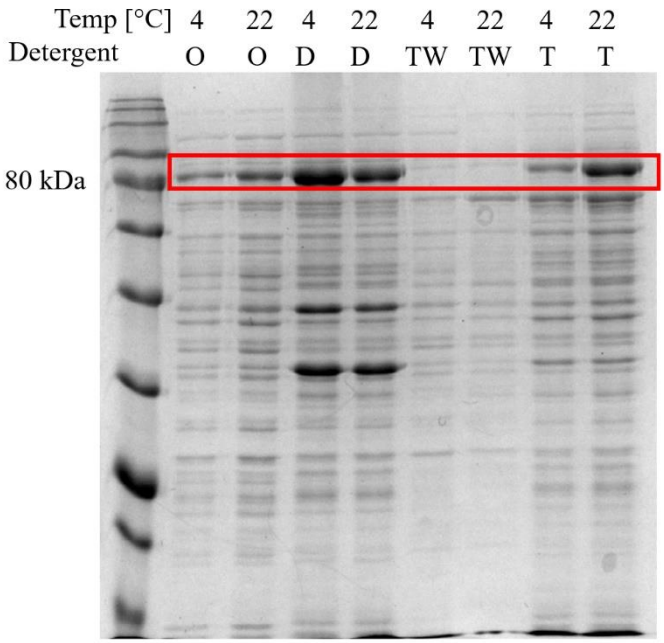


Figure 4.5 Solubilisation results for overexpressed protein. First lane indicates 1kb ladder. O-octyl-β-D-glucopyronoside (ODG), D-Dodecyl-β-D-maltopyr - anoside (DDM); Tw – Tween 20 T-Triton X. Protein of interest in red frame.

4.3.2 Affinity chromatography

The DDM solubilized protein was purified with affinity chromatography was performed. As mentioned before, the expressed protein has His-Tag attached; therefore, purification with nickel (Ni) is possible. In addition to protein overexpression, solubilisation, the small-scale purification was performed for *E. coli* BL21(DE) with empty pet21d(+) and E.coli BL21(DE) pet21d(+)FadE without IPTG induction. The results showed low levels of protein expression and minimal binding to the nickel column for the strain with empty pet21d(+) plasmid, which confirms that the His-tag is attached to the overexpressed protein. Nevertheless, histidine is present in the proteins. Thus non-specific binding to the column can occur. In comparison, the fractions collected for pet21d(+)FadE IPTG induced strain showed higher expression. In addition, the band that corresponds to FadE is much more distinct.

Initially, for detergent solubilized FadE purification was attempted with Talon columns, which relied on gravity aided flow for protein purification. FadE protein was eluted from the column with the imidazole gradient. However, impurities were still detected with SDS-PAGE, and protein was present also in

the flow-through fraction. This could be due to the small capacity of the column, as only 1 mL of resin was used. It is also possible that His-Tag requires a longer time to bind to the resin. Therefore, purification with HisTrap column and AKTA prime purification system was conducted (Exact procedure described in **2.2.6**)

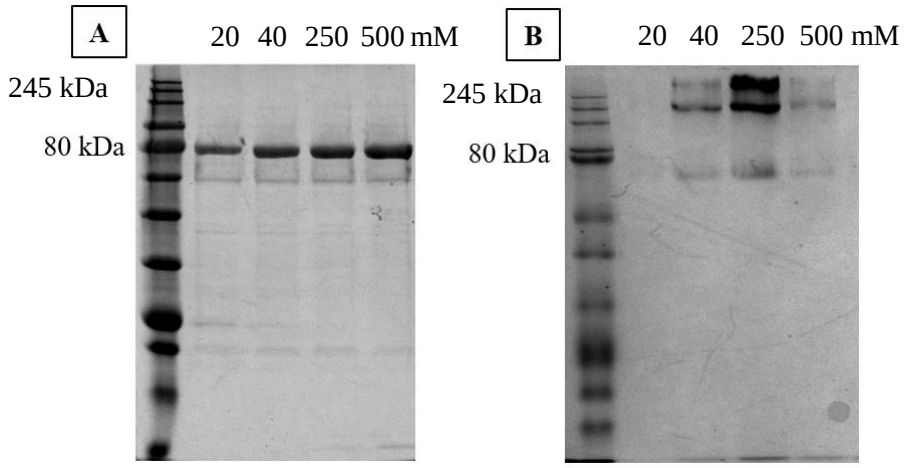


Figure 4.6 SDS-PAGE gel of purified FadE protein. A-DDM solubilized protein, B-SMALP solubilized protein. Numbers above the lanes correspond to concentration of imidazole in elution buffer.

All methods were tested to allow for FadE solubilisation. Purification of DDM-solubilised protein was more time consuming, as it required the AKTA prime system with an imidazole gradient. Minor impurities were still present in collected fractions. The SMALPs required less time to complete the protocol and yielded good results with gravity columns. The highest molecular weight of the bands for the protein ladder was 245 kDa. Samples purified with SMALPs showed the presence of the band with higher molecular weight. Therefore, Western blotting with anti-His-tag antibodies was conducted and revealed the presence of FadE. Therefore, it is reasonable to assume that SDS-PAGE for DDM solubilized protein, showed a single band corresponding to FadE monomer, and protein fully denatured. In the case of SMALP samples, bands corresponding to tetramer and trimer were present, which suggests that the protein was not fully denatured (Figure 4.6). Membrane proteins have a tendency to aggregate if heated to the boiling point. Therefore, samples were initially incubated in a hot water bath (56°C) for 2 min. These conditions were sufficient to unfold protein solubilized with detergent. However, in the case of

SMALPs harsher treatment was required. It was confirmed with an experiment where samples were incubated at the same temperature but for a longer period (6 min). The results showed a single band corresponding to a protein of interest, which was confirmed with western blotting.

4.3.3 Protein concentration determination

In protein research, Lowry and Bradford's assays are widely used to detect protein concentration, and in many cases are the assay of choice. However, due to the presence of styrene in SMA structure, protein purified with SMALPs is not suitable for quantification with assays. Protein concentration was determined with the bicinchoninic acid assay (BCA).[309] Concentration of the protein was estimated based on the calibration curve (Figure 4.7). The concentration was calculated from based on linear regression equation y = 0.0004979 x [X] + 0.003143 and was equal to 1.03 mg/mL.

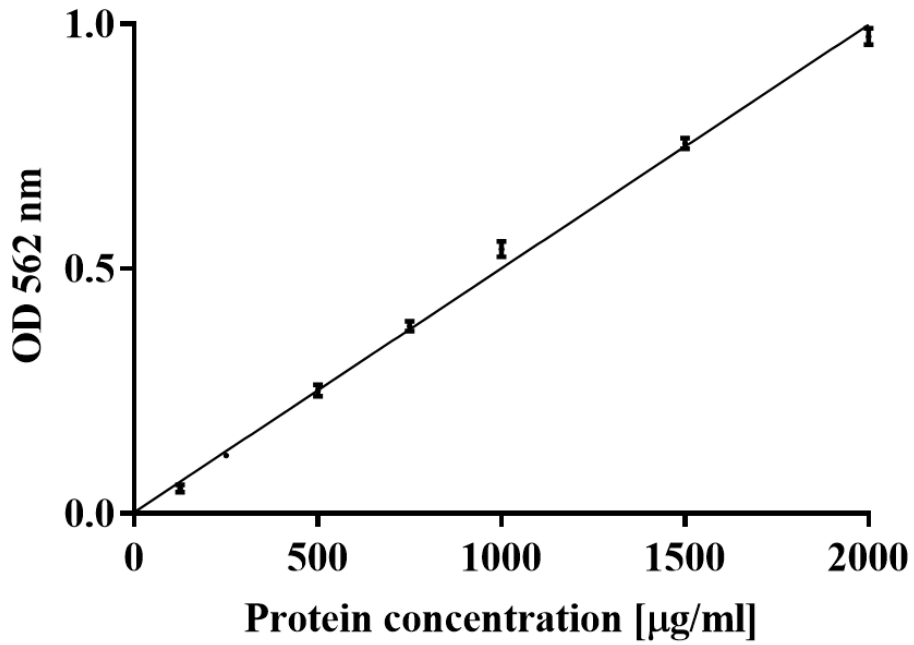


Figure 4.7 Calibration curve for BCA assay. Bars represent standard deviation.

4.4 Basic kinetic characterisation of E. coli short-chain acyl-CoA dehydrogenase

Traditionally, enzyme kinetics and enzyme characterisation were carried

out with a spectrophotometer in cuvets and water bath to ensure a stable temperature. Newer spectrophotometers allowed for automatic absorbance measurements at designated time intervals. Recently, microplate readers are increasingly employed, due to the fact that they allow for high-throughput screening, reduce the amount of time as well as help to maintain constant and stable conditions as they are customisable with thermostats, humidity cassettes invertors and gas control modules.[310,311]

Majority of biochemical reactions are too slow to sustain life, thus to ensure speed and efficiency, a catalyst is required – an enzyme. By lowering the activation energy, enzymes allow for an increase in reaction rate. The reaction is possible due to enzymes ability to activate the substrate, stabilise intermediates or providing an alternative reaction route.[312] In most cases enzyme kinetics can be described by the Michaelis-Menten and Briggs-Haldane model, although it is a historical model proposed over a century ago it is still valid and widely used. It proposes a two-step reaction mechanism. First, an enzyme-substrate complex is formed, followed by the conversion of substrate into product. The model assumes that enzyme activity will increase linearly with substrate concertation until the enzyme is saturated and further addition of substrate will not affect the reaction rate, and the plateau is reached. Michaelis-Menten model allows predicting the maximum rate of enzyme activity (V_{max}) and K_m which indicates substrate concentration at which the reaction rate is equal to the half of V_{max}. To help pinpoint V_{max} and K_m values historically double reciprocal plot (Lineweaver-Burk plot) was often used.[313]

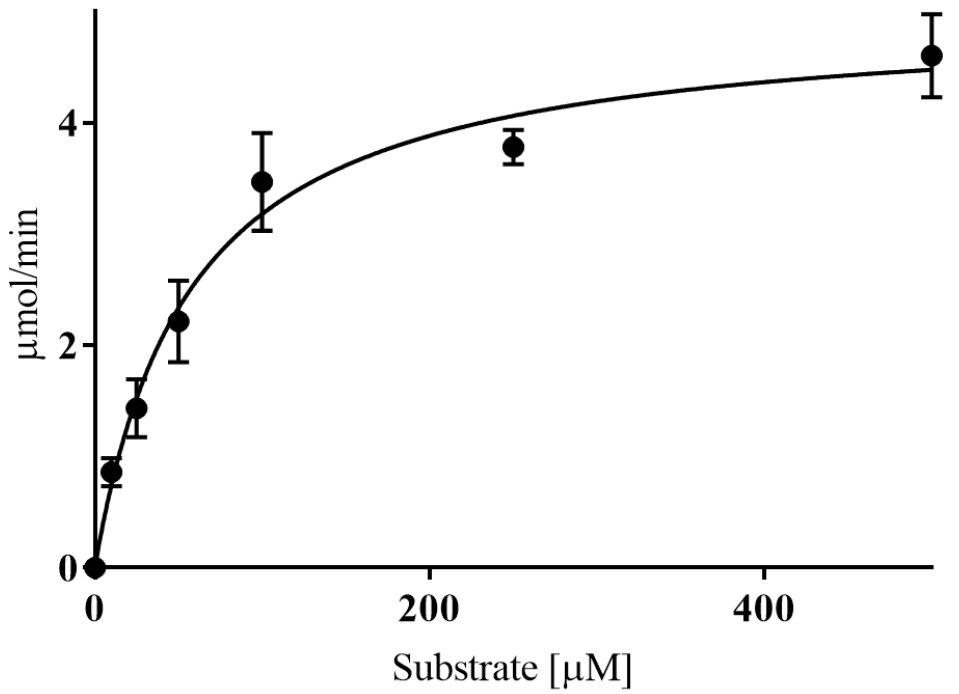


Figure 4.8 Plot of initial reaction rates of the ferrocenium assay as a function of substrate concentration. The assay was performed with 200 um ferrocenium in 100 mM HEPES buffer, pH 7.6 at 25 C and was initiated with the addition of 30 nM acyl-CoA dehydrogenase.

Enzyme activity was assessed with ferrocenium assay which utilizes ferrocenium ion as an electron acceptor instead of a FAD. This assay was first described in 1990 and since then was successfully applied for the study of different acyl-CoA dehydrogenases (Table 4.1).[314]

The enzyme kinetic values were determined with changing the concentration of substrate (butyryl-CoA) and fitting obtained data to the Michaelis–Menten equation using the GraphPad Prism software. K_m was determined to be at 56.6 +/- 1.25 µM and V_{max} at 4.98 +/- 0.82 µM/min (Figure 4.8). The activity of short-chain acyl-CoA dehydrogenase was previously reported, however for organisms other than *Escherichia coli* (Table 4.1). The enzyme was extensive research for rat (*Rattus norvegicus*) and first characterised for *Megasphera elsdenii,*[315] genus of ruminal bacteria belonging to *Clostridia*.[316] It is difficult to determine how comparable those values are since different assays were applied and the assay sensitivity could be different. However, Lehman *et al.* postulated that results of assay utilizing ferrocenium

assay and assay electron transferring flavoprotein with electron acceptor are comparable.[314]

Table 4.1 Comparison of K_m values of short-chain acyl-CoA dehydrogenase for the various organism. Adapted from BRENDA enzyme database.[301]

K_m value [µM]	**Organism**	**Assay**
0.6	*Rattus norvegicus*	DCIP/PMS assay
2	*Rattus norvegicus*	Ferrocenium assay
2.4	*Oryctolagus cuniculus*	DCIP/PMS assay
3	*Bos taurus*	DCIP/PMS assay
5	*Megasphera elsdenii*	DCIP/PMS assay
6	*Clostridium acetobutylicum*	DCIPP assay
9	*Bos taurus*	PMS assay
10	*Clostridium difficile*	Ferrocenium assay
11.2	*Rattus norvegicus*	Electron Transfer Flavoprotein (ETF) assay
12.9	*Homo sapiens*	ETF assay
14	*Bos Taurus*	ETF assay
15	*Megasphera elsdenii*	DCPIP assay
1.7	*Sus scrofa*	ETF assay
20	*Sus scrofa*	PMS reduction assay
55	*Homo sapiens*	Ferrocenium assay
56.6	***Escherichia coli***	**Ferrocenium assay (This study)**
125.3	*Pseudomonas putida*	Ferrocenium assay

K_m values reported in Table 4.1 were reported for the same substrate butyryl-CoA. It is widely reported in the literature that the enzyme has different specificity between organism, and that location of the glutamate residue is not

always the same, which in turn can affect enzyme activity.[236] The difference between values for *Rattus norvegicus* were reported for two different assays. However, the 2 μM activity was reported for the enzyme with an introduced double mutation (E368G/G247E) which affected glutamate residue.[301] This further supports the claim that differences are due to the enzyme active centre location. Moreover, data presented for *Sus scrofa* were reported for two different activity assay and are comparable. The original study, which developed ferrocenium activity assay, the experiments with ETF and DCPIP assays were also conducted and reported results were comparable.[314]

Obtained results obtained results showed that K_m values for *E. coli* acyl-CoA dehydrogenase activity are much higher for the majority of the organisms assessed with the exception of *H.sapiens* and *P.putida* enzymes.

4.4.1 Bacteriostatic effect of synthesized compounds

Variety of compounds with potential inhibitory activity were synthesized and tested against recombinant *E. coli*_short-chain acyl-CoA dehydrogenase. The tested compounds are designed to disrupt fatty acid metabolism and affect bacterial growth. To assess the bacteriostatic effect the overnight bacterial growth with interval turbidimetric measurements were carried out. This assay utilizes the fact turbidity of the bacterial culture is increasing with the number of bacterial cells. Light passing through the culture is scattered by the bacteria, thus increase in turbidity is proportional to bacterial growth. At 610 nm the background absorption is minimal and negligible. This assay was carried out with a Tecan microplate reader, which allows or controlled and stable growth conditions, and allows for the continuous overnight experiment. Assays were performed continuously for 16 h with measurements taken every 15 min. All compounds were tested at a concentration of 2 mM.

To establish a growth inhibition study, a colony of *E.coli* and *P.putida* were grown aerobically overnight in lysogeny broth (LB) in shaking incubator at 200 rpm at 37 C for *E. coli* and at 30 ^{o}C *P. putida*. The overnight culture was diluted with fresh LB media to an absorbance of 0.05 at 610 nm. The freshly prepared bacterial culture was then used to prepare samples containing test

compounds at the desired concentration. The aliquots were transferred into 96-well microtiter plate and incubated at 37 ºC (*E. coli*) and at 30 ºC (*P. putida*) for an overnight assay (Figure 4.9).

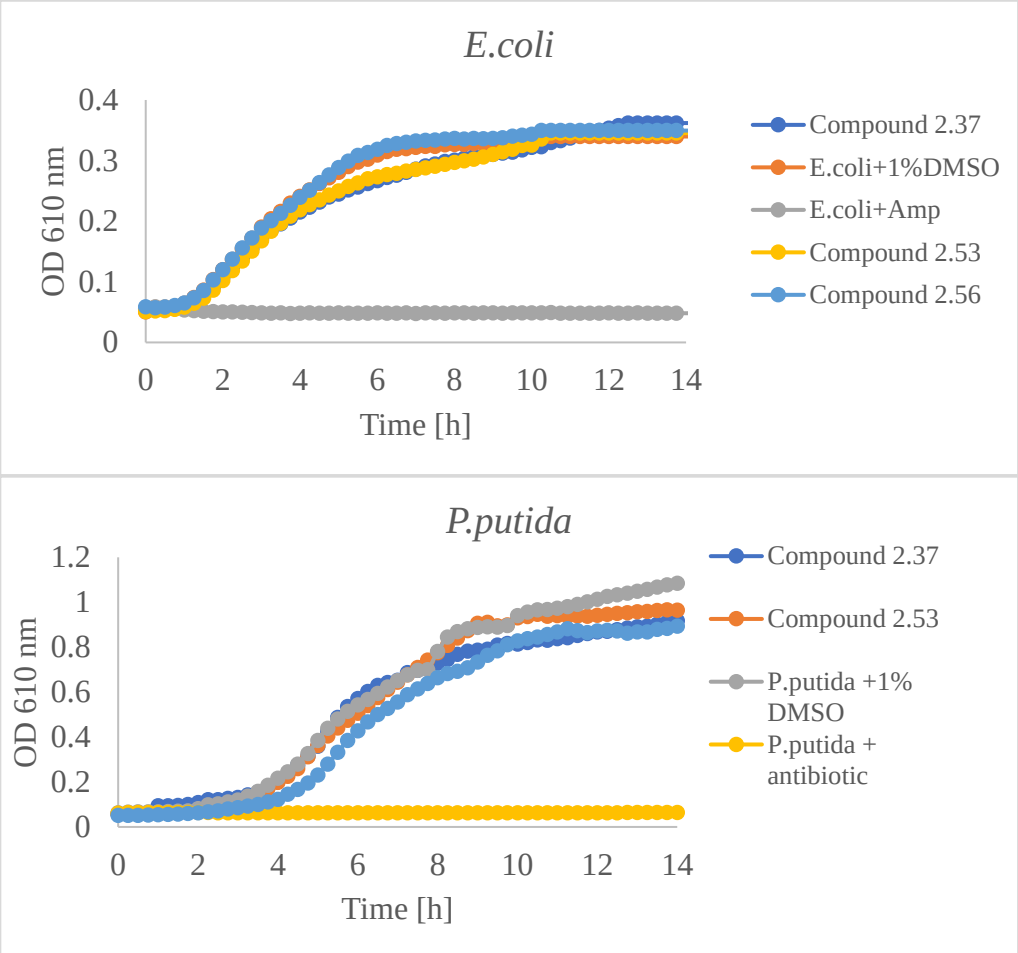
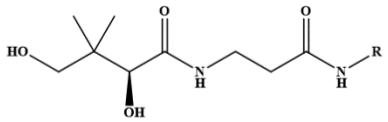


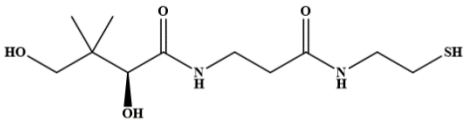
Figure 4.9 Growth curve of *E.coli* and *P.putida*. Growth of bacteria was measured in the absence (1% DMSO control) and presence of compounds **2.37**, **2.53** and **2.56** at concentration range of 2 mM. Respective cultures with antibiotic were also used as a control.

All compounds were tested against for inhibition growth; Figure 4.9 presents an example of each group to maintain clarity of the figure. Few trends were observed. In the case of *E. coli c*ompounds with a triple and double bond present at either α or β carbon of aliphatic chain showed a less steep and longer exponential phase before reaching a plateau. This indirectly indicates that these pantetheines pass through bacterial membranes and affect bacterial metabolism. Available literature reports plethora (Figure 3.1) of pantothenamides compounds which inhibit bacterial growth.[317] Strategies to use those compounds

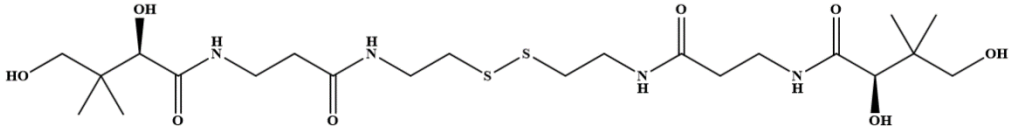
as new antimicrobials against gram-positive bacteria were also proposed.[318,319] Mercer *et al.*[235] offered an evaluation of antimicrobial activity against *E.coli* of selection of pantothenamides (Figure 3.1 and 4.10). Different studies conducted in between the 50s and 70s explored antimicrobial activity of pantethine derivatives and pantetheine analogues where the sulfhydryl group was replaced with different sulphur containing groups.[317] However, due to the lack of promising results further research was not conducted. To the best of our knowledge antimicrobial activity of S-acyl pantetheines was not reported in the current literature. Thus, it is proposed that the observed effect on bacterial growth could be a result of the disruption of lipid metabolism. However, compounds were tested at relatively high concentration, and might not meet "good drug" requirements, therefore further study might be required.[320]



Pantothenamide



Pantetheine



Pantethine

Figure 4.10 Pantothenamide, pantetheine, pantethine structure comparison

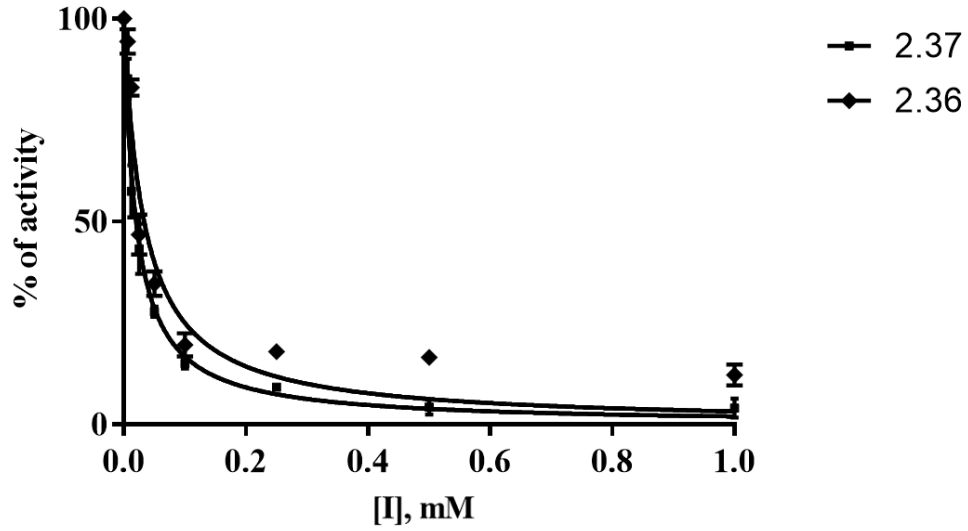
Additionally, synthesised compounds were also tested against *P. putida* which is reported to be a good platform for petrol- like biofuel production for its high tolerance to produced biofuels and solvents, including short-chain alkanes (e.g hexane).[141,321,322] Researchers identified at least two adaptive mechanisms that allow for increased chemicals tolerance in *P. putida.* First the ratio of cis- to trans- unsaturated fatty acids in the outer membrane, which makes membrane less fluid and more rigid and second removal of solvents from the bacterial cell

via membrane efflux pump. The pump is energy dependent which selectively removes organic solvents from the cell. Interestingly this pump is only activated by solvents and shows no response to antibiotics or heavy metals.[141,324,313]

4.4.2 Short-chain acyl-CoA dehydrogenase inhibitors screening

As mentioned before, no studies of *E. coli* acyl-CoA have been reported up to date; this includes inhibition studies. The enzyme inactivation is achieved through the mechanism described in Chapter 2. It is difficult to compare activity test results due to different assays used to determine inhibition activity, in addition, some studies focused on the mechanistic aspect of inhibition and did not provide exact inhibition data. Interestingly values can also vary for the same organism, and assay employed. Example of such variation can be reported for 3-pentynoyl-CoA where different studies for *M. elsdenii* reported complete enzyme inactivation at 0.31 mM and 0.62 mM. [254,315] These values are higher than the one obtained in this study for *E.coli*.

Compounds **2.49** and **2.50** showed no inhibition activity. For 4-pentynoylpantetheine (**2.49**) lack of activity can be explained by the fact that critical α and β-carbons are unhindered and the reaction can occur without issues. It was reported that 2-pentynoyl derivatives of CoA utilise β-carbon for enzyme inhibition,[233], therefore, lack of activity of propionyl pantetheine can be the result of the too short aliphatic chain, where β-carbon is not present. Therefore interaction and subsequent enzyme inhibition cannot occur. All existing literature suggests that alkynoyl analogues are irreversible inhibitors. Which, similarly to genetic knockout, offers a permanent modification of existing metabolic pathways. In contrast, alkanoyl analogues were reported to exhibit reversible inhibition which could be more useful in an industrial context and offer "on-off" switch. Dose-response curves for most active compounds presented in Figures 4.11-4.13.



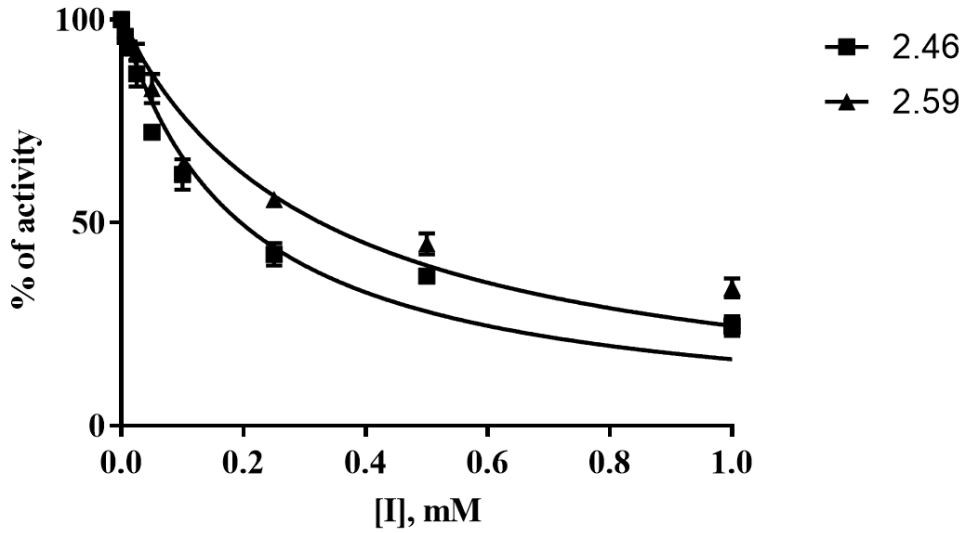


Figure 4.11 Dose-response curve. Activity calculated as % of activity. Results obtained with ferrocenium assay according to the conditions described in materials and methods section. Bars represent standard error. Substrate concentration 50 mM, T = 25° C, incubation time 10 min, without preincubation. Reaction was by addition of 30 nM acyl-CoA dehydrogenase.

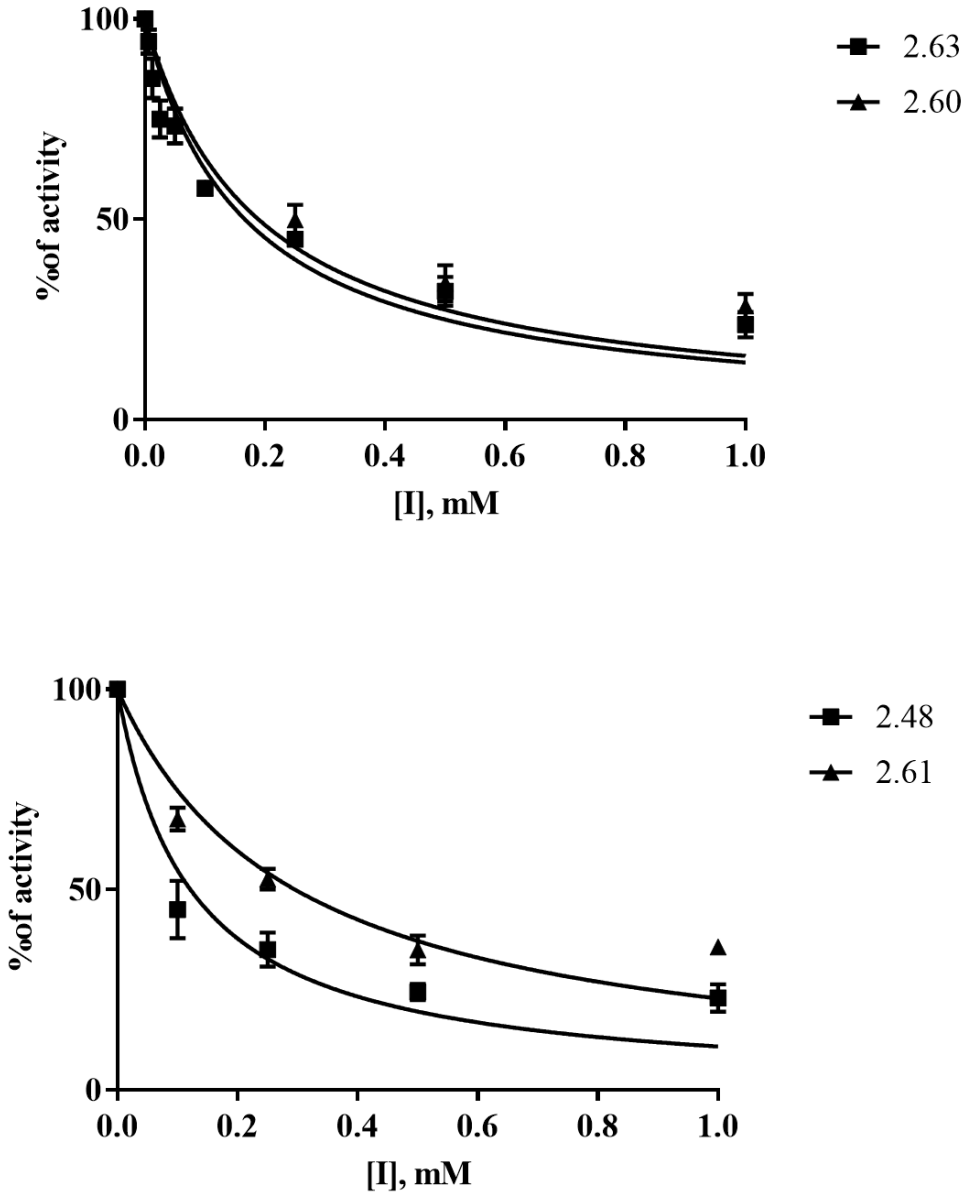


Figure 4.12 Dose-response curve. Data calculated as % of activity. Results obtained with ferrocenium assay according to the conditions described in materials and methods section. Bars represent standard error. Substrate concentration 50 mM, T = 25° C, incubation time 10 min, without preincubation. Reaction was by addition of 30 nM acyl-CoA dehydrogenase.

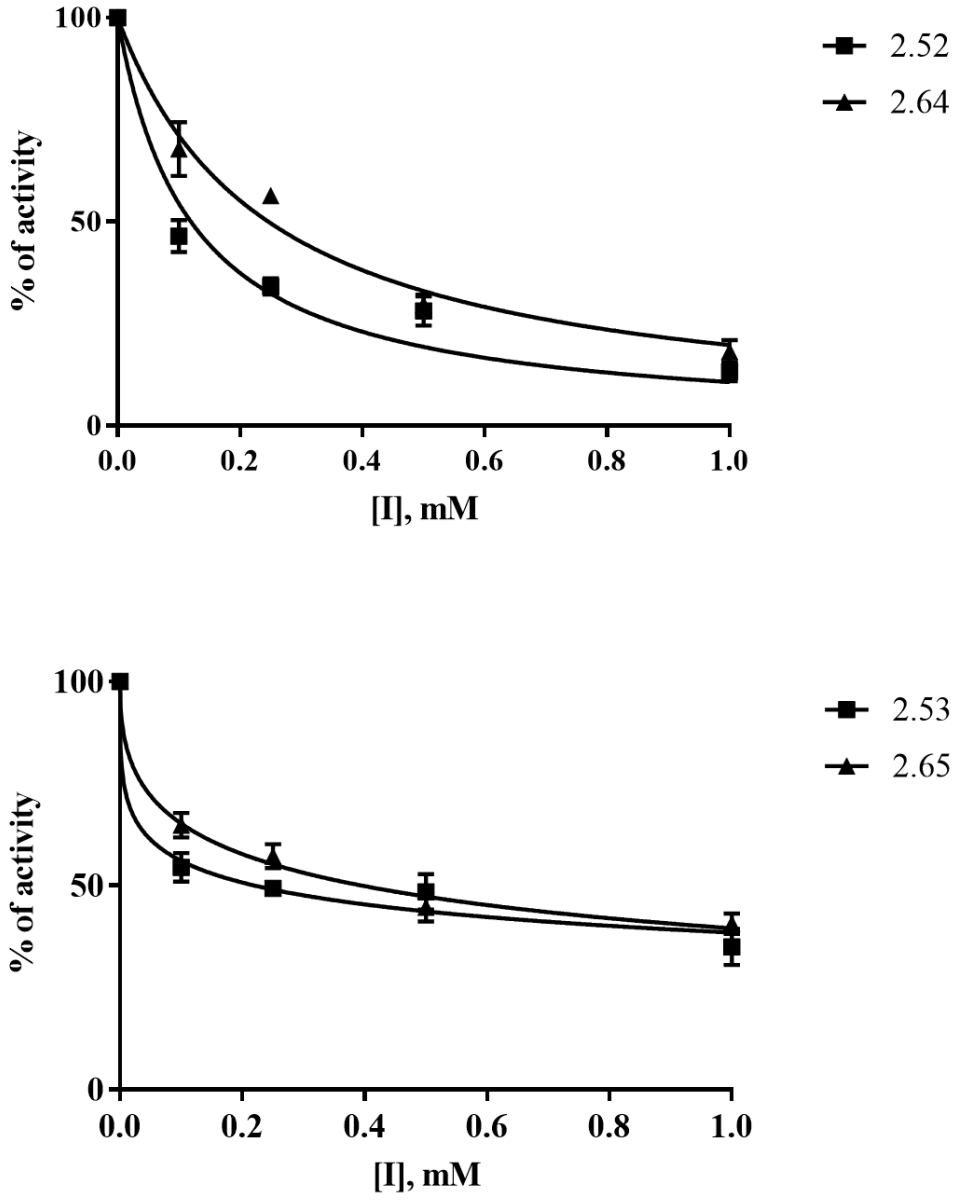


Figure 4.13 Dose-response curve. Activity calculated as % of activity. Results obtained with ferrocenium assay according to the conditions described in materials and methods section. Bars represent standard error. Substrate concentration 50 mM, T = 25° C, incubation time 10 min, without preincubation. Reaction was by addition of 30 nM acyl-CoA dehydrogenase.

Dose-response curves were created with GraphPad software and were analysed as non-linear fit. The inhibition activity was established as % of the activity of the uninhibited enzyme. Where 100 % was the activity of the enzyme when there was no inhibitor added. The activity was measured in triplicate for each inhibitor, 0% was established as no enzyme activity. Therefore, the data were normalized. The model assumes non-linear regression with the least squares fitting model. If more data points were available, the Hill slope (slope factor would be determined from it. However, each graph has between five to nine points, therefore this model assumed that the Hill slope was -1.0. The exponential model was considered since it is used to model data sets in which growth/decrease begins slowly and then accelerates rapidly, or where decrease begins rapidly and then slows down to get closer to zero. The data did not seem to follow the sigmoidal curve and plateaus were difficult to define the chosen model was preferred.

Not all the compounds follow the same curve. This could be due to the different structure-activity relationship, which will be further discussed later in the chapter. Alkynoyl pantetheines (**2.37**, **2.36**; **2.47**, **2.60**; **2.52**, **2.64**) have steeper dose-response curves, and it applies both to acetonide protected and deprotected forms. Interestingly differences between these two forms are smaller for alkynoyl compounds. This type of compound irreversibly binds to the enzyme.

Alkenoyl pantetheines were reported to reversibly bind to the active site, moreover, they are similar in structure to the enzyme`s product, which naturally can dissociate from the active site. Therefore, the less steep slope for compounds **2.48**, **2.61**; **2.46**, **2.59** could be explained by constant association and dissociation with the active site of the enzyme. Which allows for more interaction with the substrate.

Table 4.2 Structures of synthesised acetonide protected compounds with IC_{50} values. The most potent inhibitor highlighted in red.

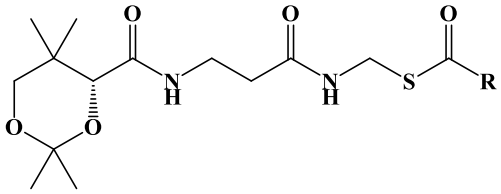
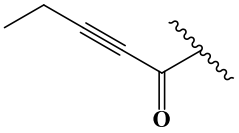
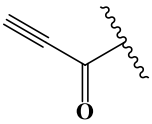
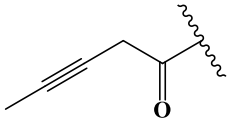
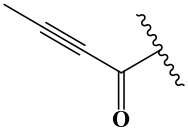
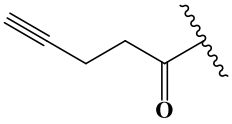
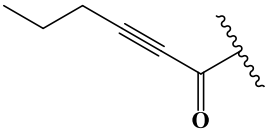
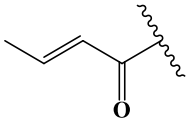
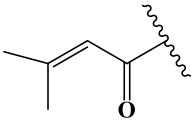
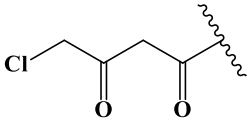
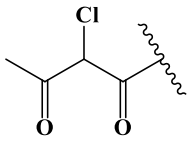
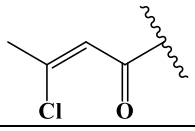
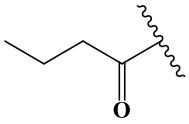
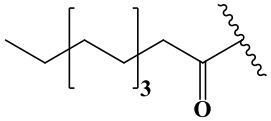
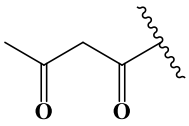
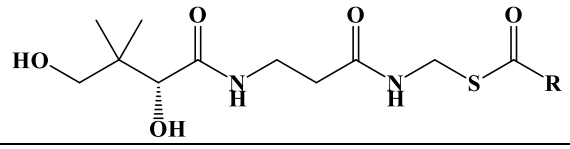
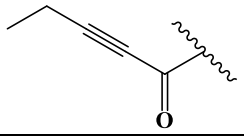
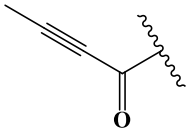
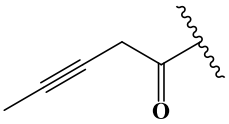
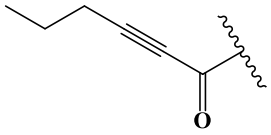
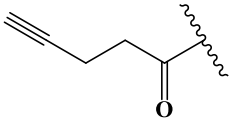
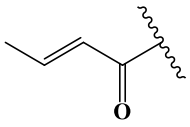
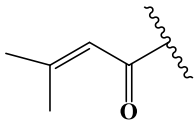
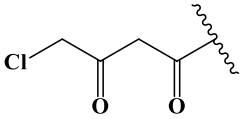
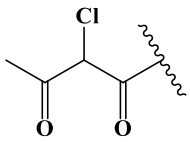
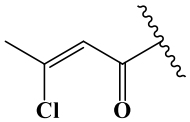
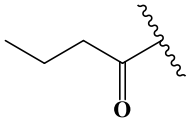
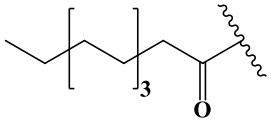
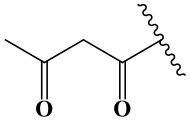
IC_{50} values [mM]					
					
Alkynoyl structural analogues					
2.37		**0.02 +/- 0.002**	**2.50**		NA
2.47		0.07 +/- 0.007	**2.51**		0.04 +/- 0.005
2.49		NA	**2.52**		0.12 +/- 0.006
Alkenoyl structural analogues					
2.46		0.19 +/- 0.01	**2.48**		0.13 +/- 0.02
Structural analogues with chlorine					
2.54		0.34 +/- 0.04	**2.57**		0.80 +/- 0.03
2.56		0.43 +/- 0.008			
Alkanoyl structural analogues					
2.45		NA	**2.55**		NA
2.53		0.31 +/- 0.03			

Table 4.3 Structures of synthesised acetonide deprotected compounds with IC_{50} values. The most potent inhibitor highlighted in red.

		IC_{50} values [mM]			
					
Alkynoyl structural analogues					
2.36		**0.04 +/- 0.01**	**2.63**		0.07 +/- 0.009
2.60		0.18 +/- 0.06	**2.64**		0.23 +/- 0.09
2.62		NA			
Alkenoyl structural analogues					
2.59		0.32 +/- 0.05	**2.61**		0.28 +/- 0.03
Structural analogues with chlorine					
2.66		0.49 +/- 0.08	**2.69**		0.91 +/- 0.05
2.68		0.51 +/- 0.04			
Alkanoyl structural analogues					
2.58		NA	**2.67**		NA
2.65		0.46 +/- 0.11			

Synthesised analogues can be divided into four groups:

- Alkynoyl structural analogues
- Alkenoyl structural analogues
- Structural analogues with chloride
- Alkanoyl structural analogues

The most potent analogue was 2-pentynoylpantetheine (0.02 mM). Incubation with this compound (**2.36**) resulted in rapid inactivation of the enzyme. The α -carbon of acyl-chain of pantetheine is important for FadE activity. The hydrogen of the α-carbon of the enzyme substrate (acyl-CoA) is deprotonated by glutamate residue, which in this case acts as a base. The reaction leads to the formation of unsaturated thioester. [236] When the triple bond is located at α-carbon the reaction of deprotonation is interrupted. The protein interacts with β- carbon and leads to the formation of the delocalised enolate, which binds to the active site, blocking substrate`s access to the active site (Figure 3.2 B). 2-hexynoylpantatheine (**2.52**) showed 6-fold lower activity than compound **2.36.** This compound has a carbon chain longer by an additional -CH_3 group. It is reasonable to assume that the longer carbon chain hinders the interaction with the active site. This seems to be supported by the fact that compound with four carbon chain (**2.51**) has IC_{50} value similar to 2-pentynoyl analogue (0.04 mM and 0.02 mM respectively). Compound **2.47** which has only slightly lower activity (0.06 mM) is a 3-alkenoyl pantetheine, which allows for deprotonation of α-carbon to initiate the reaction. However, the presence of a triple bond at β-carbon hinders interaction with cofactor FAD (Figure 1.9). This analogue was reported to inactivate the acyl-CoA dehydrogenase through isomerisation to 2,3-allene (*via* enolate), which leads to strong binding with the enzyme (Figure 3.2 A). Thus, preventing the substrate`s interaction with the active site. Compound **2.62** does not hinder α or β carbons of the acyl chain of pantetheine, therefore it does not affect the enzyme`s interaction with a compound or the substrate. No inhibition was observed. In the case of compound **2.64** despite the presence of a triple bond at α- carbon no activity was observed. It is speculated that carbon chain (only three carbons) is too short and is not recognized by the enzyme.

Synthesised alkenoyl analogues (**2.46** and **2.48**) showed activity lower to alkynoly analogues. However, it must be mentioned that the activity of acyl-CoA involves the introduction of a double bond between the second and third carbon of the carbon chain. The product of the enzymatic reaction was also reported to inhibit the enzyme through negative feedback, alkanoyl derivatives were reported to be competitive inhibitors. Synthesised compounds with contain the double bond, mimic product of the enzyme. Therefore, it is possible that compounds enter and exits the active site. In addition, it was previously reported that this type of compounds are reversible inhibitors[232], and their activity can be overcome with substrate concentration.

The study by Fendrich *et al.*[315] also reported enzyme inactivation by 3-chlorobutenoylpantetheine (**2.68**). However, no values were reported. This study identified the IC_{50} value of compound **2.68** 0.51+/-0.04. It was proposed that inactivation occurs when a proton is removed from the α-carbon and chloride is eliminated, which results in alkene formation. It is also worth to mention that chloride release during such transformation, could potentially lead to compound`s bacteriostatic properties and could affect bacterial growth. However, growth studies showed that bacterial growth is a comparable inhibitor untreated culture.

Acetoacetyl-CoA (**2.53)** was reported to show 60 % inhibition at 0.5 mM concentration in *Megasphera elsdeni* and suggested competitive inhibition.[325] Two other studies report inhibition effect in *Bos taurus* and *Rattus norvegicus* however exact values have not been reported.[314,315] Inhibition activity of acetoacetyl pantetheine was reported in this study (0.31 mM) and to the best of our knowledge has not been reported previously. Compound **2.53** has oxygen present at β-carbon, which hinders the role of FAD as a cofactor thus affecting the activity of the enzyme.

Compounds **2.58** and **2.57** have a structure similar to the substrate and did not show any inhibition activity.

All compounds were synthesised in two forms, with the acetonide protection group and without it. Interestingly compounds with masked hydroxyl groups showed better activity (by 2-fold). It is proposed that acetonide protected variants are more hydrophobic which allows for easier interaction with

membrane protein such as acyl-CoA dehydrogenase.

4.5 Conclusion

In conclusion, this chapter summarises the inhibitory activity of the synthesized compounds. 2-pentynoyl pantetheine (0.02 mM) presented the highest levels of activity with fast inactivation time. Shaller et al. and Rao et al. showed independently that 2-pentynoyl-CoA inactivates acyl-CoA dehydrogenase of *Paracoccus denitrificans* and human respectively.[233,234] Human acyl-CoA dehydrogenase was reported to have an IC_{50} value equal to 0.05 mM and *P. denitrificans* 0.03 mM. In this study IC_{50} from *E. coli* was established at 0.04 mM and is comparable to existing data3-Pentynoyl pantetheine showed lower activity (0.07 mM), although still significantly higher than other compounds. Alkenoyl analogues and compounds with chloride moiety present similar levels of activity. Compounds with unhindered α and/or β carbon do not show any inhibitory activity. Although the alkynoyl compound present excellent inhibitory activity, it is worth to consider that milder inhibitors could be more useful in an industrial context. Further studies are required to fully understand the influence of the carbon chain on inhibition activity.

Chapter 5

Towards development of an *Escherichia coli* short-chain alkanes producing strain

Growing concern about energy supply safety and by extension concern about the economy is driving a search for new and sustainable power sources. One alternate supply in the production of microbial biofuels, which are not only economically viable but also meet the criteria of being environmentally friendly. Examples of research into microbial biofuels were discussed in Chapter 1. Lipids metabolism is a potential target of research. Fatty acids are energy-rich compounds, researchers have made further attempts to modify metabolic pathways have been extensively researched. However, upscaling production and development of cost-efficient technology is yet to be achieved and applied in industry. The advantage of microorganisms in biofuel production lies in the potential diversity of the final compound or fuel. The summary of the scope of compounds is briefly described below. Good yields of biogas and alcohols were achieved *via* microbial fermentation.[326]

Photosynthetic microorganism such as microalgae is developed to convert carbon dioxide and water to biohydrogen and alcohol directly or alternatively as a source of feedstock for microbial or chemical processing to yield industrially useful compounds. An additional advantage of this approach is there is no need for a cultivation area, which could be an answer to the food vs fuel argument. However, technology is in the early stages of development and is a distant future.[327,328]

E. coli is widely used in academic and industrial context and is one of the first if not the first organism to be successfully modified towards the production of biochemicals. Initial research aimed for bioethanol production.[329]

Two wild-type genes from *Zymomonas mobilis* for pyruvate dehydrogenase (*pdc*) and alcohol dehydrogenase (*adb*) were introduced into *E.coli*, and the native gene for fumarate reductase was deleted. The new strain named KO11 produced ethanol at 95% of the theoretical yield. However, ethanol resistance at 3.5% and requirements for specific media supplements resulted in increased production costs.[330] Further research allowed to increase ethanol tolerance to 10 % and decrease nutritional demand.[329] Finally strain LY168 capable of yielding 45g of ethanol per litre in 48 h was developed.[331] Research into ethanol-producing strain showed how important metabolic pathways regulation is in the industrial microbial production of biochemicals. *Pdc* and *adb* genes in strain KO11 were regulated by the *pfl* promoter and complex transcriptional control. To generate strain LY168, these genes were integrated into the chromosome o aid expression.[332]

In 2016 Huerta-Beristain *et al.* achieved production of lactate and acetate through Entner-Doudoroff and pentose phosphate pathways. This research used *E.coli* KO11 as a base for modification and diversion of the glycolytic pathway, which was achieved by the deletion of phosphoglucose isomerase (*pgi*). The initial strain was submitted for further modification, and strain KO11 PPAL was developed. The strain was described to be able to divert the majority of carbon from pyruvate to ethanol production.[333] Different strains of ethanol producing strains were also reported such as SZ420 which was developed by deletion of the competing fermentation pathways in *E.coli*, leaving ethanol as the main product of fermentation.[334] Strains that utilise xylose instead of glucose as substrate was also developed, which is an important development considering that xylose is the main product of hemicellulose degradation.[335,336]

Ethanol is not the only alcohol researched as a substitute for current fuels. Butanol is considered to have better properties, such as higher energy density, lower vapour pressure, and is less hygroscopic, and thus is considered better fuel than ethanol. In addition, butanol properties are similar to petrol, which makes it compatible with petrol based technologies.[337] These properties give butanol the potential to substitute petrol directly. Butanol production was first described *Clostridia* bacteria and reported to be produced by acetone-butanol-ethanol fermentation.[125,129] Much of the research is aimed towards exploitation of

pathways established in *Clostridia*. However complex metabolic regulation and demanding growth conditions, present difficulties in genetic modification. It is worth mentioning that *E. coli* strains have also been developed, due to the fact that its metabolism is well understood, and genetic manipulation is relatively easy, making *E. coli* an attractive target. As mentioned before butanol production was initially achieved *via* pathways present in *Clostridia* and majority of research attempts to engineer clostridial pathways into other microorganisms including *E. coli*.[158,338]

5.1 Fatty acid metabolism regulator protein (FadR) deletion

Fatty acid metabolism regulator protein was identified in previous studies as one of the key enzymes involved in microbial fatty acids production.[131,239] FadR stimulates fatty acid biosynthesis and at the same time represses fatty acid degradation, *via* inhibition of *fadD* transcription which in turn impairs the use of FFAs for acyl-CoA dependent alkane synthesis (Figure 1.16). Therefore, a FadR knockout of regulon is required to release essential enzymes.

A *fadR* gene knockout was achieved by λ Red recombinase. A study by Choi and Lee utilized Cre-Lox recombination which utilised a similar FLT-FRP recombination method. Authors of this paper do not specify why different recombinase was chosen. A choice between FLT-FRT and Cre-Lox was reported to be more important in case of mammalian genome engineering as initially a reported FLP was thermolabile and is not suitable for use above 30 °C, although thermostable variants have been being since developed.[339,340] A choice of Cre-Lox was also not dictated by dual recombinase-mediated cassette exchange (dRMCE) as the gene was flanked by two loxP sites, lox 71 and lox66. dRMCE allows for the exchange of sequences flanked by single loxP and FRT sites.[341] Therefore we speculate that the preference of Cre-Lox was dictated by plasmid availability, as the majority of used plasmids was reported as lab stock. Since plasmids for FLP-FRT recombination were more readily available and successful gene knockouts were reported we decided to choose this system.[243]

The FLP-FRT gene deletion method requires a recombinase called flippase (FLP). This enzyme is combined with its recognition target called the FLP recognition target (FRT). Through PCR reaction a 34-base pair (bp) FRT sequence is introduced to the gene of interest as flanking motifs. In the presence of expressed FLP, the gene flanked by FRT motifs is then excised from the genome (Figure 5.1).

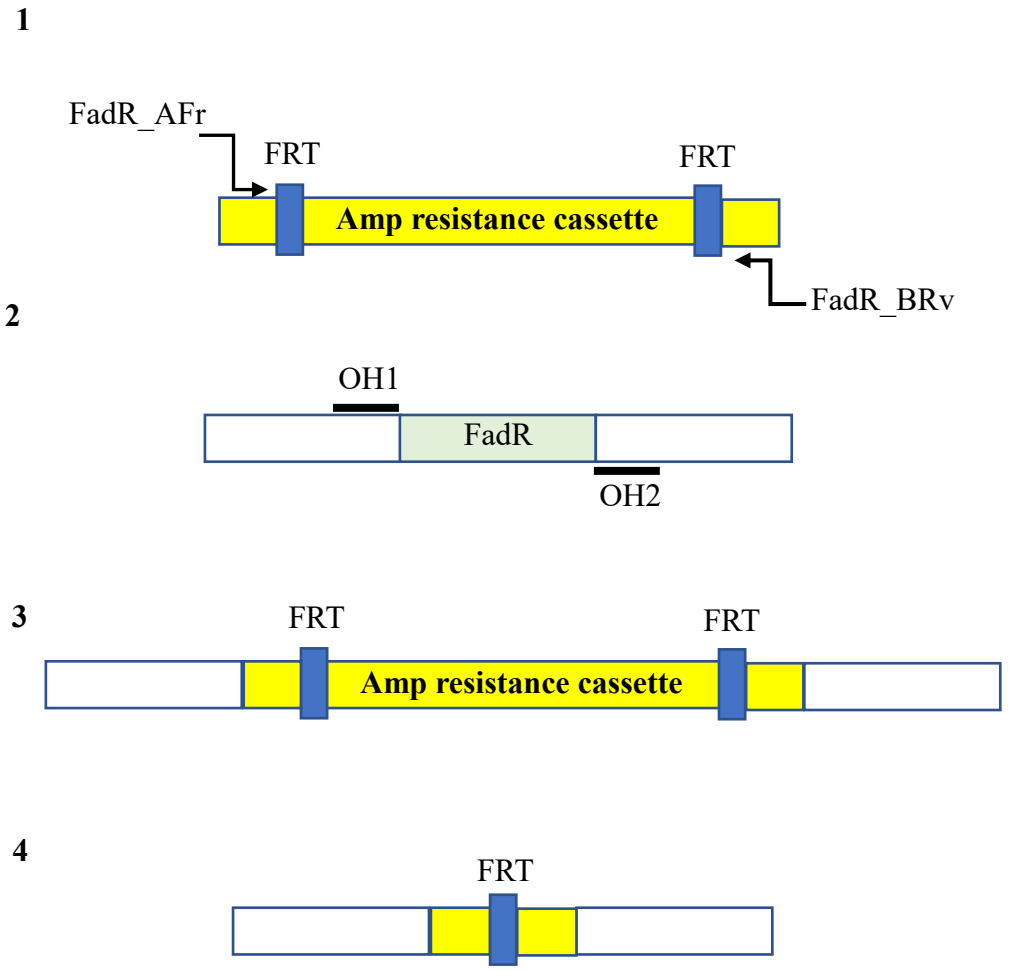


Figure 5.1 Gene knockout principle using FLP. OH1 and OH2 are homology extensions and FadR_AFr and FadR_BRv refer to primers used to amplify antibiotic resistance gene.

The first step of the process requires PCR amplification of an antibiotic resistance gene. Plasmid pkD3 which carries chloramphenicol resistance was used. The designed primers FadR_AFr and FadR_BRv contained extensions homologous to the sequence proceeding the FadR gene. Sequences of used primers can be found in Table 2.2 Chapter 2. Next, the *E.coli* harbouring recombinase expressing plasmid (pKD46) was transformed with the amplified

ampicillin resistance gene. The original paper suggests the use of 50 bp or 36 bp long homology arms,[243] further research shows shorter homology extensions give good results. In this study we used primers with 30 bp homology arms upstream and downstream from the gene of interest, we also tested 50 bp. However, these primers were very long (over 100 bp including FRT sequence), and we were not able to amplify the chloramphenicol cassette. We concluded that T_m of these primers (83.7 °C and 86.2 °C) is too high to produce viable results and shorter primers were used. In comparison, the T_m of 30 bp were 74.4°C and 74.1°C. If chloramphenicol cassette was successfully amplified and subsequently transformed into *E.coli* direct colony PCR showed a band at around 1.1 kb bp (Figure 5.2). In case of unsuccessful recombination, a control PCR would reveal a band around 700 bp which corresponds to the FadR gene.

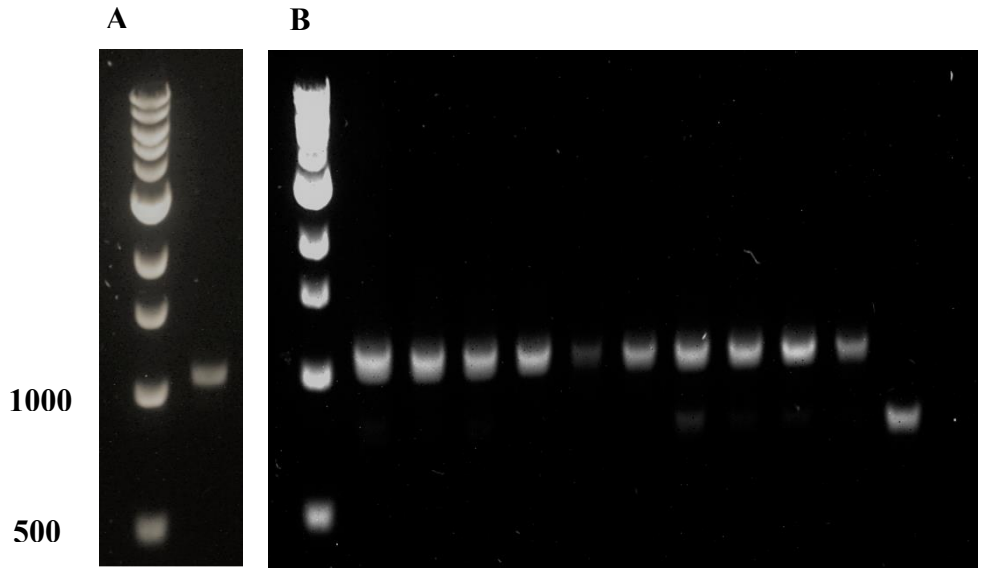


Figure 5.2 Results of PCR screeening for antibiotic cassette. A - cassete amplification from pKD3 plasmid; B-results of recombination for 10 randomly selected colonies (lanes 2-11). Lane 12 shows the PCR results with control primers for wild type *E.coli*

After selecting antibiotic resistant colonies, the resistance cassette was removed with the helper plasmid (pCP20). Antibiotic resistance was cured of the strain at 43 °C Loss of ampicillin resistance was then confirmed on ampicillin-containing plates and *via* direct colony PCR. Out of 10 tested colonies six lost antibiotic resistance towards ampicillin and chloramphenicol. Monk *et al.* identified seven *E.coli* strains widely used in research and

industry.[342] Reports of differences in physiology and genome can be found in literature, which can have implications for downstream applications and efficiency of biochemicals production. [342] Despite *E. coli* MG1655 and W3110 being closely related, strain W3110 has been shown to have stop codon at position 33 in *rpoS* which is not found in MG1655. RpoS is a global regulator of the stress response in *E.coli*; the aforementioned mutation was shown to decrease its activity, which can have an effect on overall expression.[343,344] Differences between these two platforms were previously reported for example for xylitol production.[345] Therefore in this study strains MG1655 and W3110 were simultaneously researched, and strains ΔFadR_MG1655 and ΔFadR_W3110 were developed *via* methodology described above.

5.2 Construction of plasmids overexpressing fatty aldehyde decarboxylase, fatty acid-CoA ligase (ptacCER1ptacFadD) and fatty acyl-CoA reductase and acyl-ACP thioesterase (ptrcArcpTacTesA)

E. coli does not possess all the enzymes required for short-chain alkane production. Therefore additional genes are required. Additionally, expressed genes have to be under the control of strong promoters that can be inducible. Therefore promoter *tac* and *trc* were introduced *via* helper plasmids. Both are stronger promoters than *lac* and can be induced with IPTG. The trc and *tac* promoters are a hybrid of the *lac* and *trp* promoters. In bacterial promoter two parts can be identified, the '-35' region and the '-10' region. The sigma factor of RNA polymerase is bound by these two fragments and is responsible for the initiation of transcription of the gene. The tac promoter is a hybrid of regions from two different promoters, the '-35' region of the *trp* promoter was fused together with the '-10' region of the lac promoter. The create *trc* promoter 1 bp insertion was made into the 16 bp sequence between -35 and -10 sequences of the *tac* promoter, to obtain of 17 bp between the -35 and -10 signals. This promoter includes the lac operator sequence lacO, which can be bound by the Lac repressor lacI. For inducible gene expression from the trc promoter, the lac repressor (LacI) is necessary. The repressor binds to the operator DNA with high specificity and inhibits gene expression until an inducer like IPTG is added to the medium. The inducer binds to the repressor, causing a change in its shape.

Thus altered, the repressor LacI is unable to bind to the operator, allowing RNA polymerase (RNAP) to transcribe the following genes and thereby leading to high levels of the encoded proteins.

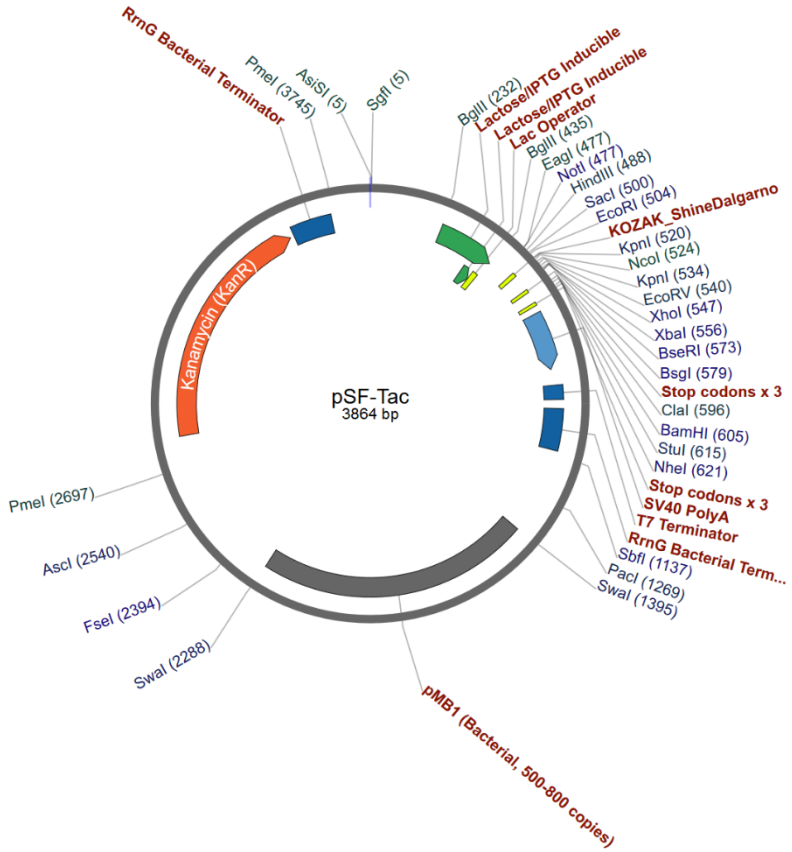


Figure 5.3 Plasmid pSF-Tac structure (Oxford Genetics)[346]

E. coli native acyl-ACP thioesterase shows preference towards long chain fatty acids, since this study aimed for production of short-chain alkanes a thioesterase capable of hydrolysing short chains was required. Previous studies showed that the introduction of a mutation in which Leucine 109 is changed into Proline enhances substrate range, and hydrolytic activity can be observed for short and long chains.[200] To introduce this mutation an *E.coli* thioesterase was amplified with two sets of primers. Tes_Fv , MTes_Fv; MTes_Rv Tes_XbaI_Rv. Thus, two fragments were created first 357 bp and containing a mutated base (cytosine instead of thiamine in position 329) and a shorter fragment of 245 bp. Both PCR products were purified, combined and amplified with primers Tes_Fr and Tes_Rv giving 572 bp long fragment corresponding to thioesterase (Figure 5.2).

Obtained fragment of 572 bp was digested with NcoI and XbaI and ligated into pSF-Tac (Figure 5.3) plasmid digested with the same enzymes.

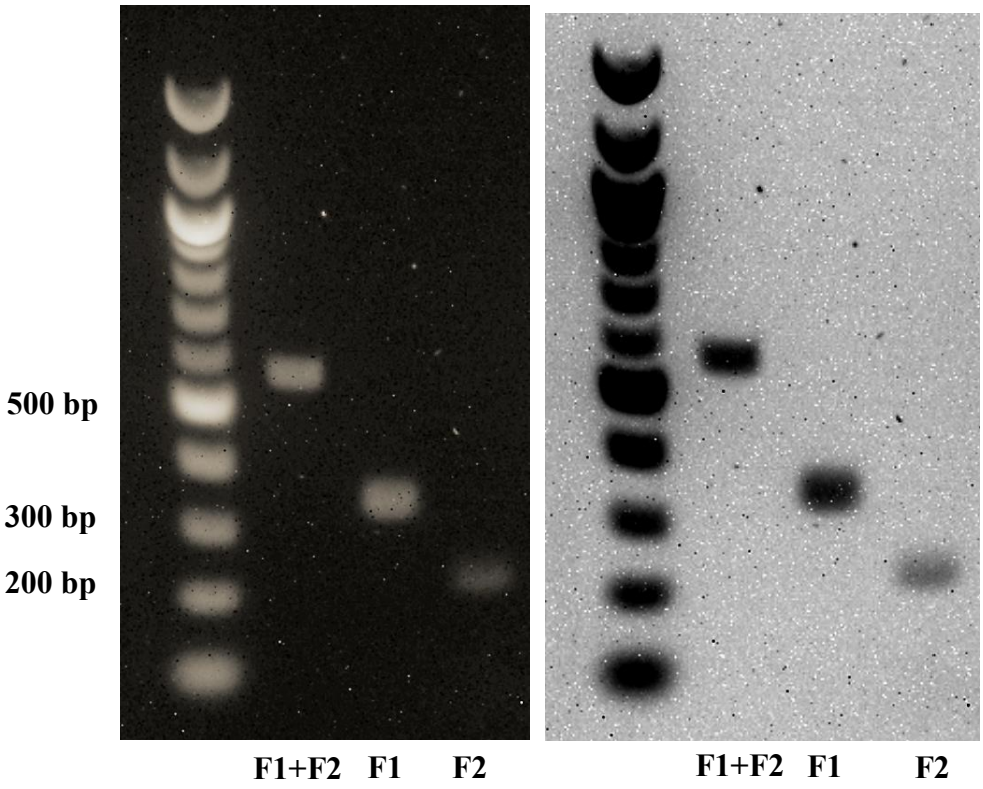


Figure 5.4 PCR amplification of mutated thioesterase. First lie indicates 100kb ladder. For better visualisation the inverse colours image is presented on right-hand side.

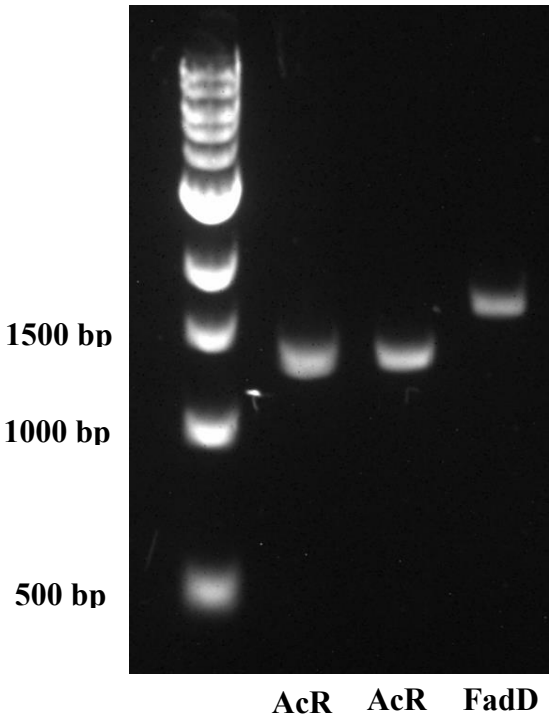


Figure 5.5 Acr and FadD amplification results

Therefore, plasmid pSF-tacTesA plasmid was obtained and was used for further studies. Fatty acyl-reductase (Arc) was amplified from genomic DNA of *Clostridium acetobutylicum* (ATCC 824). *Arc* is a 1,356 bp long gene (Figure 5.5). This enzyme catalysis reaction of reduction of fatty acyl-CoA to a fatty aldehyde.

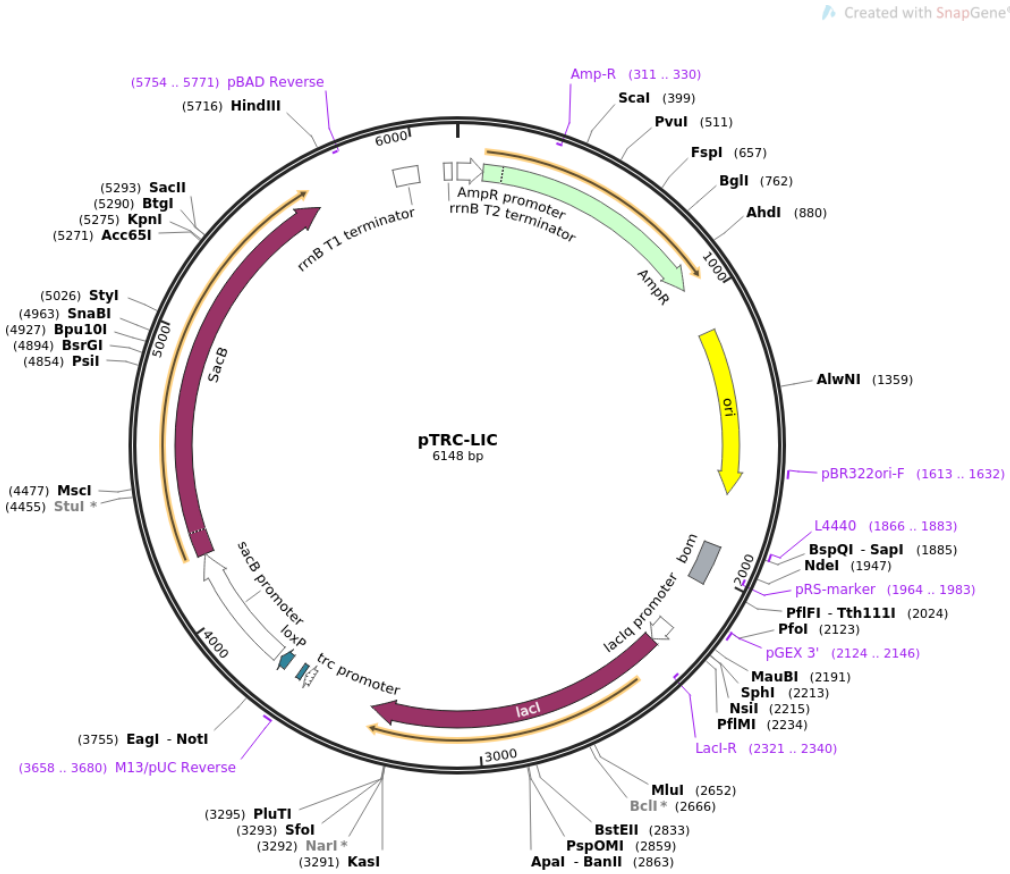


Figure 5.6 Plasmid pTRC-LIC was a gift from Cheryl Arrowsmith (Addgene plasmid # 62343)[347]

One of the solutions for short-chain alkanes production is the the introduction of acyl-CoA reductase, which transforms fatty acid aldehydes into SCA. However, the transformation of FFAs released by thioesterase into fatty acyl-CoA is necessary and can be achieved by FadD overexpression. cDNA sequence of acyl-CoA reductase (CER1) was obtained from TAIR and artificially synthesised by Eurofins. The synthetic gene was amplified with Cer1_Fv and Cer1_Rv (Table 2.2) digested with respective enzymes and ligated with NcoI and XbaI digested pSF-tac plasmid. FadD gene was amplified with FadD_NcoIFr and FadD_XbaIRv primers digested and ligated with the pSF-tac plasmid. Next pSF-tackFadD plasmid was used as a template and FadD with tac

promoter was amplified. Obtained fragment was digested with XbaI and ligated with the pSF-tacCER1 plasmid to give pSF-tacCER1tacFadD overexpressing plasmid. In the same manner, ptrcArcptacTes plasmid pSF-tacTesA was amplified digested with BamHI and ligated with pTrcArc.

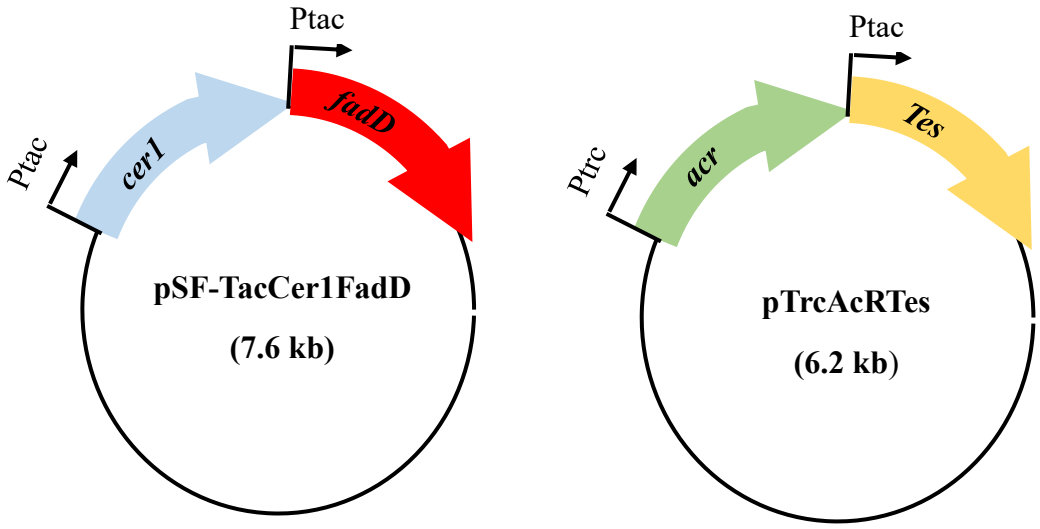


Figure 5.7 Plasmids constructed via genetic engineering

All developed plasmids gene modifications were sequenced with Sanger sequencing to confirm successful modification. In the future plasmids will be transformed into *E.coli* BL21(DE3) and expression of the proteins will be tested.

Chapter 6

Conclusion and Future work

6.1 General conclusion

Last few decades brought growing concern about energy security and fuel availability. Energy is currently mainly obtained from non-renewable resources. It is hard to estimate how long oil supply reserves will last. Various studies were conducted with vague conclusions which leave a place for speculations. Miller and Sorell suggest that current resource stock will be sufficient to satisfy the demand for at least the next 50 years.[348] It is only natural that oil production will decrease with time, and researchers predict that it will occur as soon as 2040.[11] Therefore, the need for alternative, sustainable resources and green strategies is more crucial than ever before. Biological processes start to have an increasing contribution to finding a solution. Simultaneously to ongoing research on renewable energy (wind-, solar-, hydro-energy), extensive research on microbial biofuel production is also conducted, with new microbial platforms emerging almost every year. Production of so-called "biofuels" started with bioethanol and biodiesel. Unfortunately, research is still in “proof of concept” stage and systems with efficiency and yield capable of industrial applications are yet to be developed. Currently, only processes are known for centuries, such as fermentation and distillation give efficiency and yields high enough to find industrial applications.[66]

Research by Choi and Lee, who successfully produced SCA through conversion of the fatty acid acyl-CoA (acyl carrier protein (ACP)) to free fatty acid by introduction of new enzymes into engineered *E.coli* (fatty acyl-ACP thioesterase, fatty acyl-CoA synthetase, fatty acyl-CoA reductase and fatty aldehyde decarboxylase) was the inspiration for this project.[242]

Acyl-CoA dehydrogenase (FadE) was reported to be a crucial enzyme in the microbial short chain alkane production, which are the main ingredient of

petrol.[242] In *E.coli*, FadE is an inner membrane homotetrameric protein with a unit mass of 89,224 Da, and catalyses the dehydrogenation of acyl-CoAs to 2-enoyl-CoAs, the first step of fatty acid degradation (β-oxidation cycle). [229] The re-engineering of microbial metabolic pathways has been successfully exploited. To the best of our knowledge, all advanced approaches relied solely on genetic engineering.

This study proposed combining genetic engineering and enzyme chemical inhibition. It is hypothesized that this approach will allow the development of an on/off switch for production of desired chemicals, which in broader perspective will allow the creation of a more robust and versatile strain capable of producing more than one type of chemicals.

One of the aims of the project is to build a sub-library of potential FadE inhibitors and establish their potency. As mentioned before, derivatives of acyl-CoA (S-acyl pantetheines) are recognized by bacterial enzymes; therefore, they will be used as a basis to build further analogues.[232] 2-Pentynoylpantetheine (Figure 44) was reported as a potent and rapid FadE inhibitor and was used as a reference for further modifications and assays.[240]

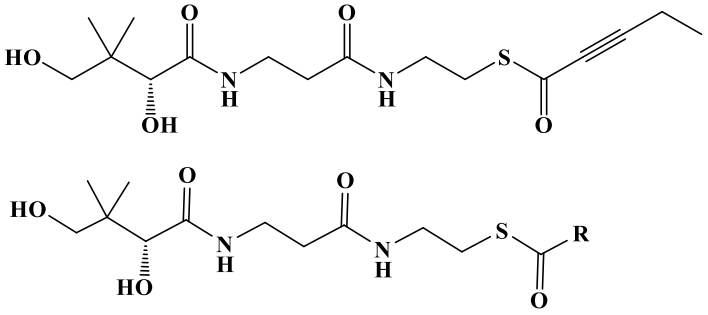


Figure 6.1 Structure 2-pentynoyl pantetheine (1st structure) and the general structure of pantetheine.

In comparison to previously reported strategies for pantetheines (Figure 6.1) synthesis,[266,269,275,277] was developed a quick method to obtain desired products in four steps reaction (Figure 6.2). In addition, the overall cost of used reagent was lowered, and the quantity and toxicity of reaction by-products were lower than previously reported. To achieve a stepwise synthesis of pantetheine analogues using this strategy, three building blocks were used the pantothenic acid (**2.28)**, the cysteamine or cysteamine hydrochloride (**2.23**) and various carboxylic acids were either synthesized or purchased from a commercial source.

The synthetic strategies and the outcome are summarised below.

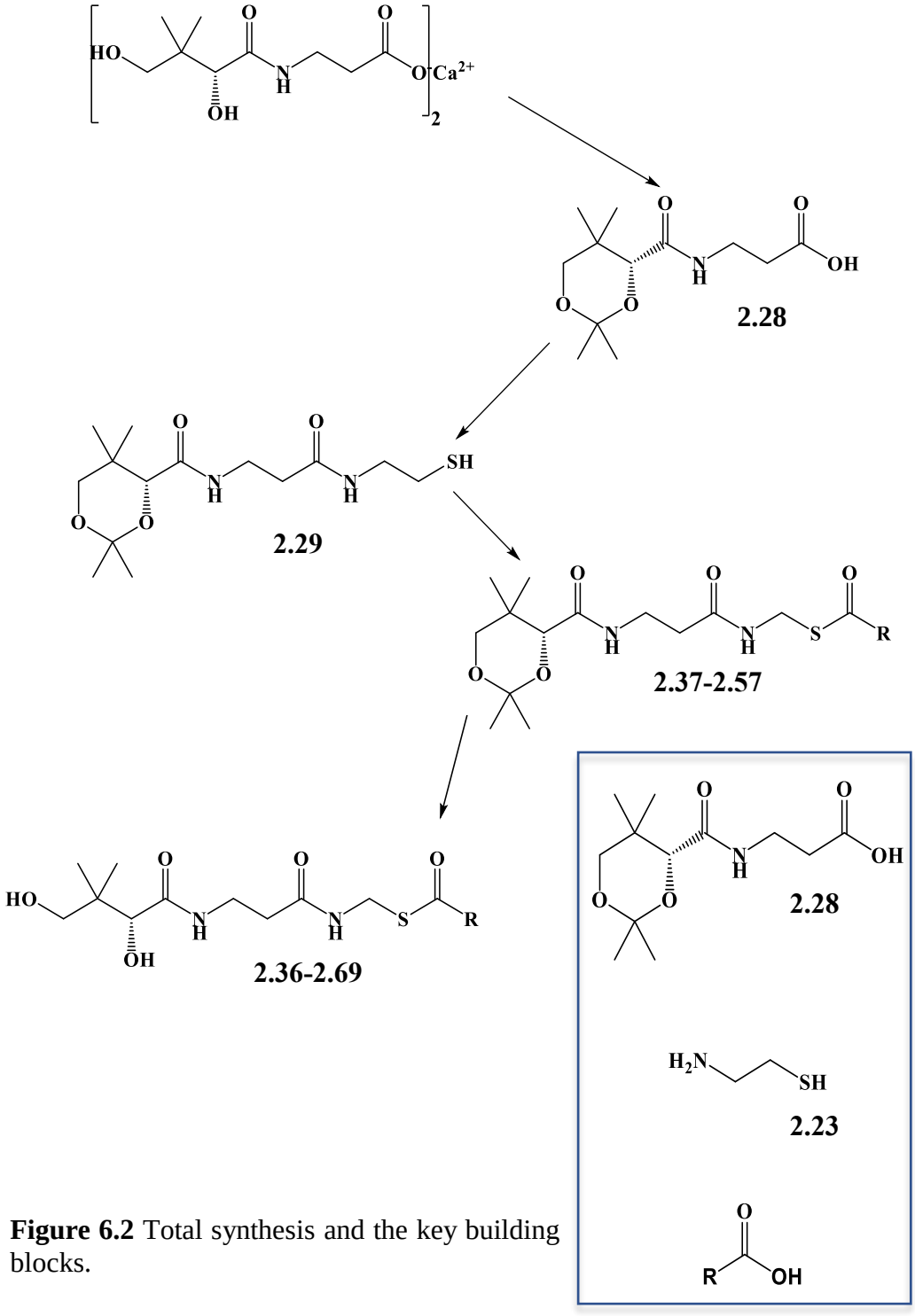


Figure 6.2 Total synthesis and the key building blocks.

The synthesis was achieved in four steps and is a modification of the methods proposed by Gaudelli *et al.*[275], Meier *et al.* [286], and Hermkens *et al.*[293] The preparation of pantothenic acid acetonide **2.28** (Figure 6.2) was achieved using the method previously reported by Gaudelli *et al.*[275] (Figure 6.2).

Commercially available pantothenic acid hemicalcium salt was transformed to pantothenic acid acetonide *via* acetonide protection with acetone with PTSA as a catalyst. The product was obtained as white crystals with an 83% yield.

The preparation of pantetheine **2.29** (Figure 6.2) was achieved using method previously reported by Gaudelli *et al.*[275] (Figure 3.9). The previously obtained **2.28** was reacted with cysteamine **2.23**. Amide formation was catalysed by 1`,1`-carbonyldiimidaole (CDI). The product was obtained as a white solid with a 90 % yield.

The syntheses of other S-acyl pantetheines analogues **2.37-2.57** (Table 6.1) were achieved according to conditions proposed by Meier *et al.*[275] The method utilises DCC as a coupling agent to catalyse the reaction of thioesterification. The method gave 40-70% yield, depending on the coupled compound. It was also determined that longer activation of carboxylic acid resulted in higher yields. The reaction requires 4 eq. of DCC, reduction of its quantity to 2 eq. resulted in a significantly lower yield.

The removal of acetonide protecting group to yield S-acyl pantetheine **2.36-2.69** was done in one step according to the method proposed by Hermkens *et al.* [293] (0.2 M HCl) and did not require further purification after work up. In addition, the product was obtained in good yield >80% (Table 6.2).

The potency of the synthesized compounds was tested on a purified acyl-CoA of *E. coli.*

Table 6.1 S-acyl pantetheine acetonide analogues with a respective yield

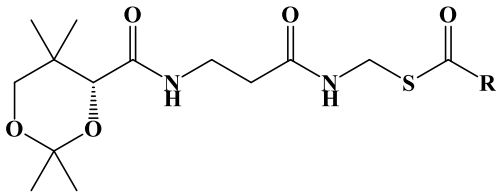
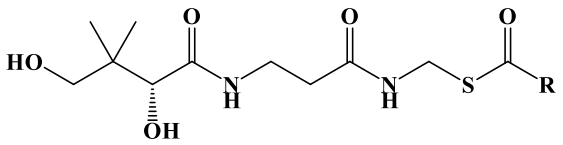
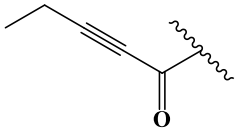
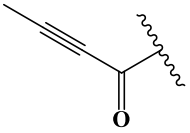
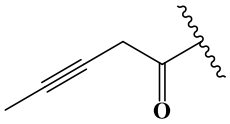
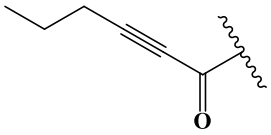
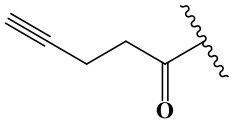
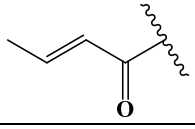
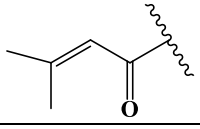
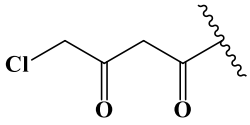
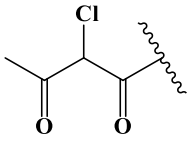
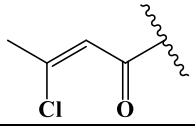
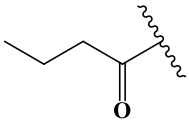
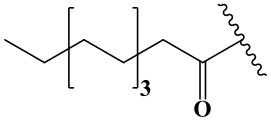
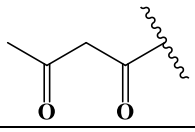
Reaction Yield [%]			
			
Alkynoyl structural analogues			
2.37	49%	**2.50**	33%
2.47*	49%	**2.51**	61%
2.49	52%	**2.52**	54%
Alkenoyl structural analogues			
2.46*	52%	**2.48**	49%
Structural analogues with chlorine			
2.54	63%	**2.57**	53%
2.56*	56%		
Alkanoyl structural analogues			
2.45*	73%	**2.55**	40%
2.53*	45%		

Table 6.2 S-acyl pantetheine acetonide analogues with a respective yield

Reaction Yield [%]					
					
Alkynoyl structural analogues					
2.36		85%	**2.63**		82%
2.60		82%	**2.64**		82%
2.62		91%			
Alkenoyl structural analogues					
2.59		79%	**2.61**		89%
Structural analogues with chlorine					
2.66		86%	**2.69**		83%
2.68		80%			
Alkanoyl structural analogues					
2.58		88%	**2.67**		88%
2.65		83%			

In parallel, the *E. coli* strain overexpressing modified fatty acyl-ACP thioesterase (Tes, *E. coli*), fatty acyl-CoA synthetase (FadD, *E. coli*), fatty acyl-CoA reductase (Arc, *Clostridium acetobutylicum*) and fatty aldehyde decarbonylase (Cer1, *Arabidopsis thaliana*) was constructed.

Thus, Chapter 4 reported the inhibitory activity of S-acyl pantetheines. During this study, several acyl-CoA dehydrogenase inhibitors with various levels of inhibition were identified. Within the group of inhibitors, compounds with triple bond present within the structure proved to be the most potent. The most potent inhibitor was 2-pentynoyl pantetheine, and the obtained results are in agreement with previous studies.[233,349] According to literature those inhibitors are irreversible.[234] Other inhibitors were also evaluated, such as compounds with double bond or chloride. Those compounds showed lower activity in the comparison to alkynoyl derivatives. However, they offer the appealing possibility of inhibition reversal. Therefore, in the industrial application context, this could be a viable option.

6.1.1 Short-chain acyl-CoA dehydrogenase inhibitors screening

Table 6.3 S-acyl pantetheine acetonide analogues with IC_{50} values

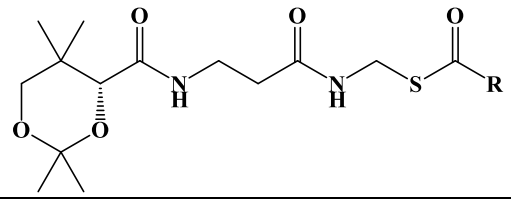
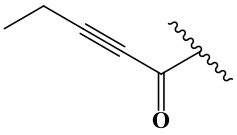
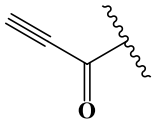
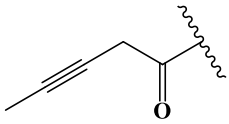
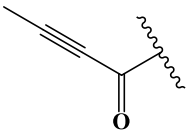
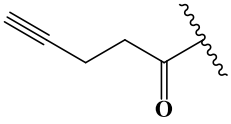
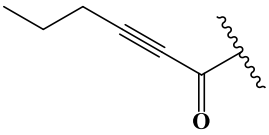
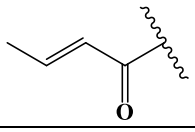
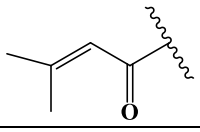
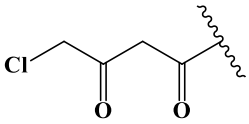
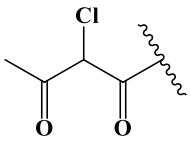
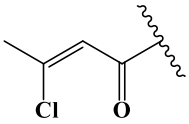
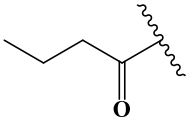
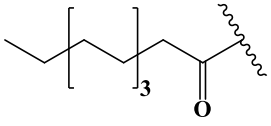
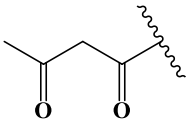
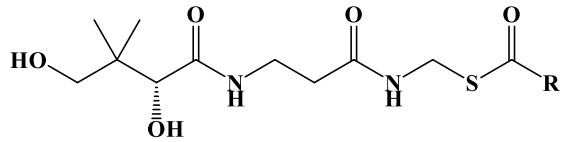
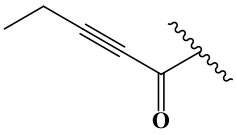
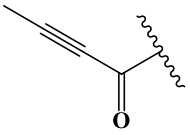
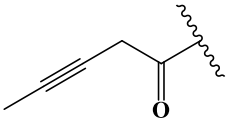
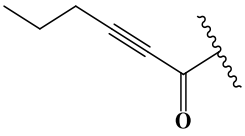
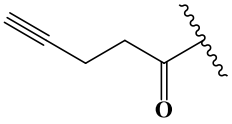
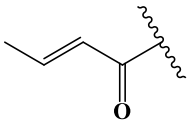
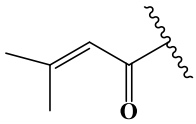
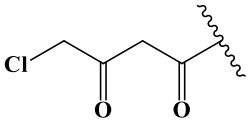
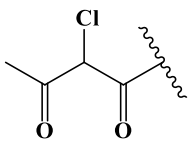
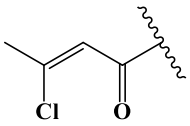
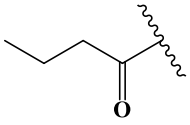
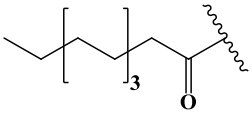
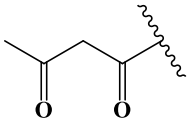
IC_{50} values [mM]					
					
Alkynoyl structural analogues					
2.37		**0.02 +/- 0.002**	**2.50**		NS
2.47		0.06 +/- 0.007	**2.51**		0.04 +/- 0.005
2.49		NA	**2.52**		0.12 +/- 0.006
Alkenoyl structural analogues					
2.46		0.19 +/- 0.01	**2.48**		0.13 +/- 0.02
Structural analogues with chlorine					
2.54		0.34 +/- 0.04	**2.57** 		0.80 +/- 0.03
2.56		0.43 +/- 0.008			
Alkanoyl structural analogues					
2.45		NA	**2.55** 		NA
2.53		0.31 +/- 0.03			

Table 6.4 Structures of synthesised compounds with IC_{50} values

IC_{50} values [mM]					
					
Alkynoyl structural analogues					
2.36		**0.03 +/- 0.01**	**2.63**		0.05 +/- 0.009
2.60		0.18 +/- 0.06	**2.64**		0.23 +/- 0.09
2.62		NA			
Alkenoyl structural analogues					
2.59		0.32 +/- 0.05	**2.61**		0.28 +/- 0.03
Structural analogues with chlorine					
2.66		0.49 +/- 0.08	**2.69**		0.91 +/- 0.05
2.68		0.51 +/- 0.04			
Alkanoyl structural analogues					
2.58		NA	**2.67**		NA
2.65		0.46 +/- 0.11			

The weaker activity of the double bonded derivatives could be explained by the fact that they are similar in structure to the FadE catalytic product, 2-enoyl acyl-CoA. In fact, in natural conditions, one of the controls of the FadE function is product negative feedback.

The fact that high quantities of the compound are needed is not necessarily a drawback. The synthesis was proved to be straight forward and

relatively inexpensive and is therefore scalable. Additionally, synthesized compounds are not used as drugs and dose is not as important as in the case of drug development. Of course, the environmental impact must be considered if industrial quantities were to be processed, as well as waste disposal from synthesis and bioreactors used for biochemicals production.

In addition, during this study kinetic values of K_m and V_{max} were elucidated which were not reported previously for *E.coli* acyl-CoA dehydrogenase. The obtained values are higher than reported in the literature. However, these data are obtained for organisms other than *E. coli.* and use a variety of methods, therefore a direct comparison is difficult.

For protein purification, the SMALPs technique was used for the first time for the *E. coli* FadE protein. After optimisation of the process, it offered a relatively quick and recyclable method of protein purification.

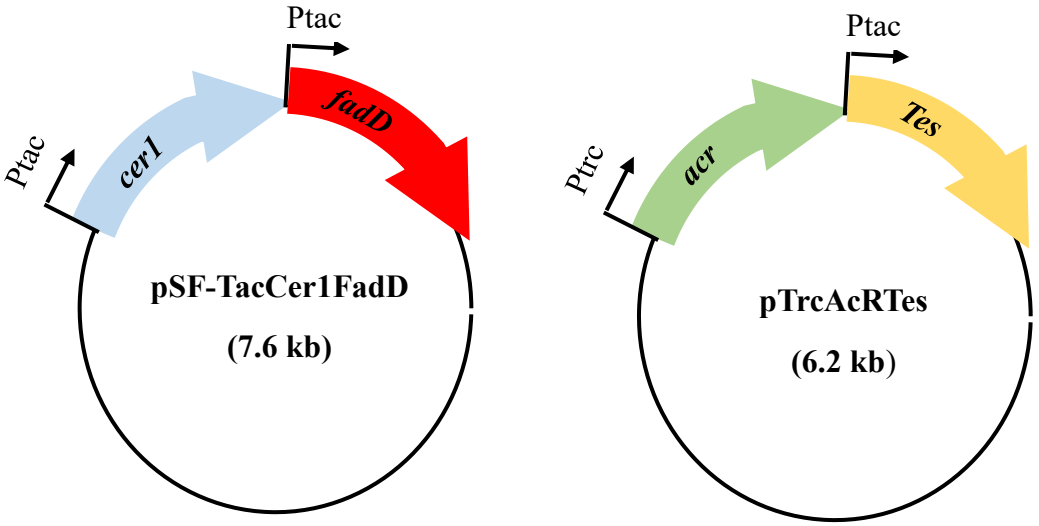


Figure 5.7 Plasmids constructed in this study

Thus, Chapter 5 outlined the development of *E. coli* strain that could be capable of short chain alkane production if supplemented with S-acyl pantetheine as an inhibitor to disrupt fatty acid metabolism and redirect key intermediate fatty acyl-CoAs towards new engineered pathway.

The fatty acid metabolism is controlled by FadR; it was previously reported that removing FadR control enhances the yield of SCA.[242] This was achieved by the genetic knockout. One-step inactivation of FadR with the λ-red mediated system was successfully achieved.[243] In addition, two plasmids were

developed (Figure 5.7), one carrying fatty acyl decarbonylase (Cer1, *Arabidopsis thaliana*) and fatty acyl-CoA synthase under the control of *tac* promoter. The second plasmid carried the fatty acyl-ACP thioesterase (Tes) under control of a *tac* promoter and fatty acyl-CoA reductase (*Clostridium acetobutylicum)* under *trc* promoter. The introduce enzymes are designed to transform fatty acyl-ACP, generated by the fatty acid biosynthetic pathway (Figure 4) to short-chain alkanes. The function of each gene is outlined in Figure 6.3.

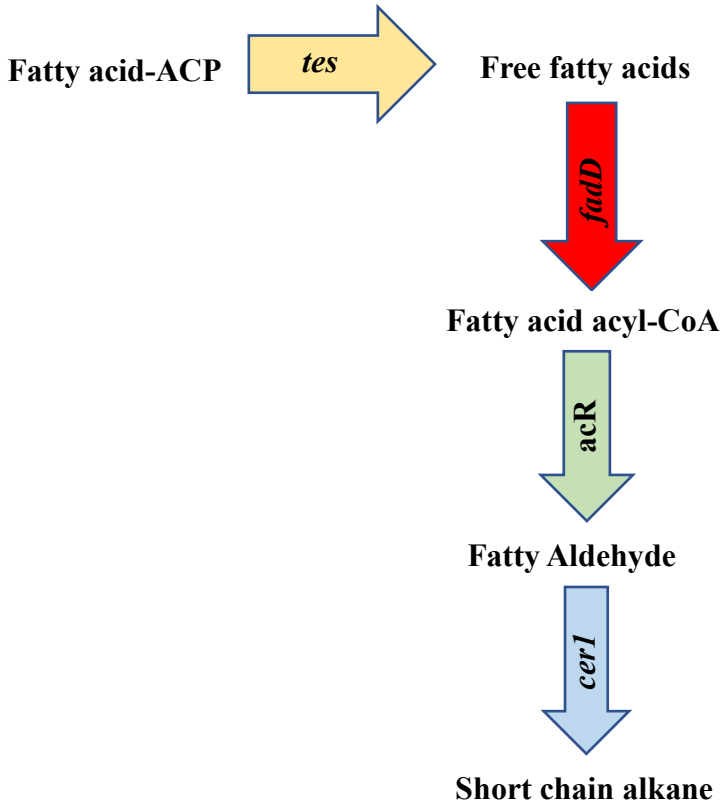


Figure 6.3 Metabolic pathway towards short chain alkane production

6.2 Future work

6.2.1 Microbial short chain alkane production

In the immediate future, the compatibility of the synthesized FadE inhibitors and engineered strains for SCA production will be evaluated.

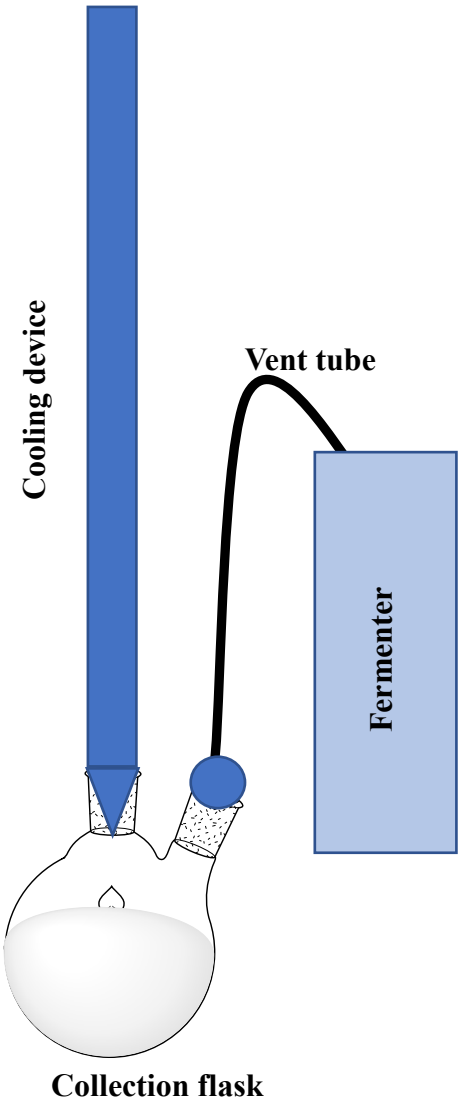


Figure 6.4 Schematic representation of device setup for SCA production test.

For such purposes, a fermenter connected to the cooling device such as a condenser (Figure 6.4) is proposed. Short chain alkanes are volatile, and condensation is necessary. Otherwise, the final product could be lost due to evaporation. If the reaction proves to be successful, volatile products will be analysed by Gas Chromatography-Mass Spectrometry (GC-MS).

6.2.2 Chemical inhibition of enzymes

To date, most attempts at inhibiting the enzymes have been achieved solely through genetic manipulation of the bacterial genome. It would also be interesting to explore the possibility of chemical inhibition of more than one enzyme. Instead, a mixture of chemical inhibitors of FadR and FadE could be synthesised and introduced to the system. Alternatively, an inhibitor with dual activity could be developed. The FadR protein is inhibited by binding of long-chain acyl CoA molecules to its hydrophobic cavity situated within the C-terminal helices bundle, which results in a conformational change of the N-terminal domain, thereby affecting dimerization and DNA-binding abilities of the protein.[327] Octynoyl-CoA was previously reported to inhibit FadR activity and could serve as a base for structural modifications of potential inhibitors. [350]

Previous studies revealed that pantetheine analogues which do not contain cysteamine moiety show antibiotic-like properties.[317] In future this class of compounds could be further developed to achieve topic and attempt a synthesis of inhibitors with no antimicrobial activity, i.e. synthesize more enzyme specific compounds since structures reported so far target CoA biosynthesis

6.2.3 Metabolism regulation

FadR is not the only regulatory protein in *E. coli*. For good anaerobic growth ArcA, a global transcription factor required. It was also reported that it relates to fatty acid metabolism regulation and is a major transcription factor in the process. The same study suggested that ArcA directly represses the expression of FadR regulon genes during anaerobic growth.[351] It would be interesting to see if the manipulation of this gene would affect the yields of

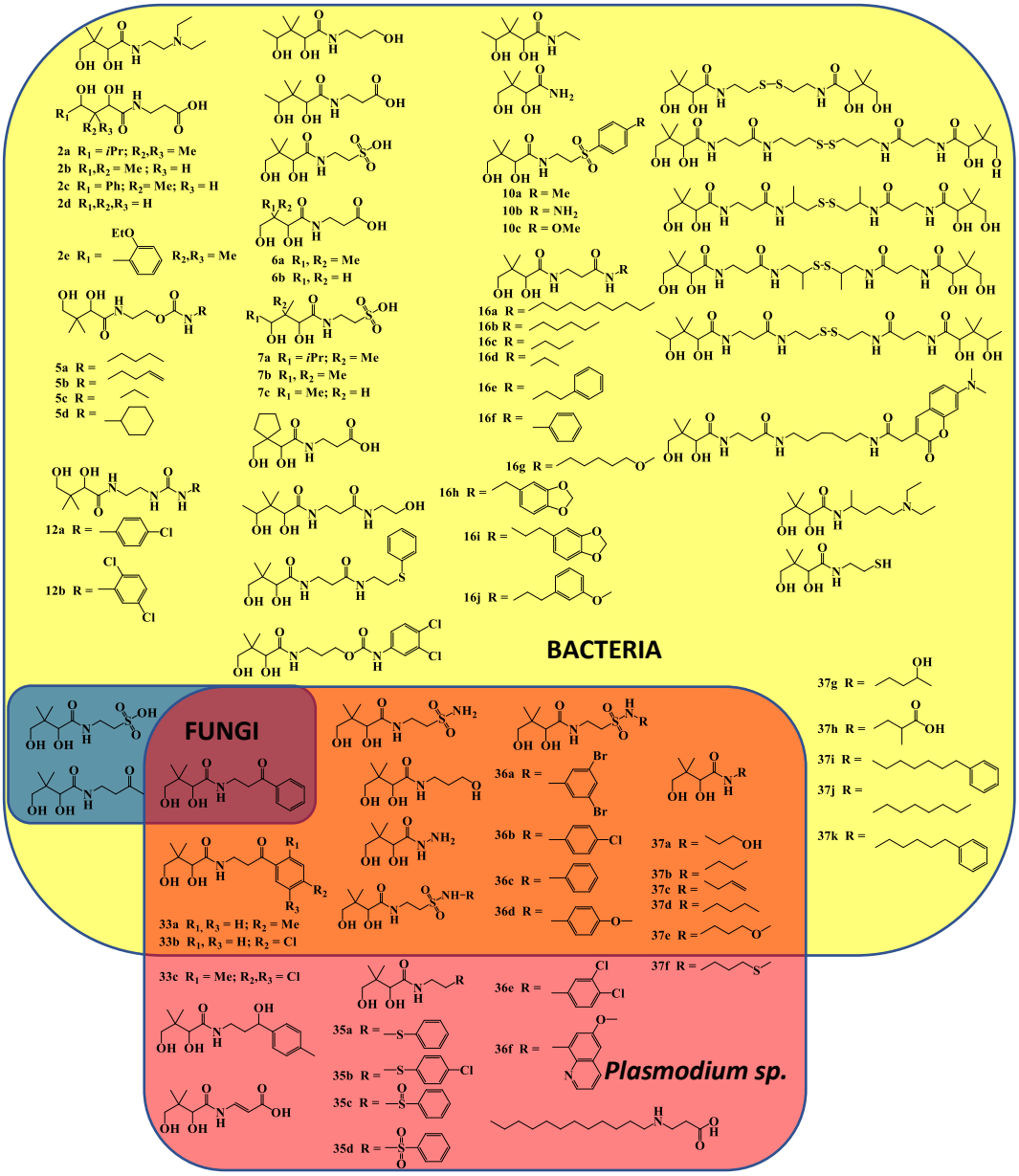


Figure 3.1 Chemical structures of pantothenic acid analogues reported in literature. Overlapping areas indicate activity against more than one organism. Stereochemistry not included in the figure. Adapted from Spry *et al.* [317,319]

alkane production. This could be important in terms of sustaining efficient production.

6.2.4 Alternative enzymes for biochemical production

Different genes can also be introduced to produce other fatty acid-based compounds, for example, free fatty acid esters. Additionally, microbial platforms, with some genetic adjustments such as *fadD* deletion for short-chain alkanes production, or introduction of the *Acinetobacter sp*. *adp1* wax ester synthase and the *E. coli* mutant alcohol dehydrogenase for short-chain fatty esters production.[326] In future, demand for alkane-based fuels will increase. Currently, methods for production of jet fuel are heavily researched.[352] With the introduction of enzymes from, for example, cyanobacteria production of longer alkanes could be possible.[353]

6.2.5 Other microorganisms as a platform

One of the prevailing problems of microbial biofuel production is the tolerance of the bacterium to the produced compounds.[354] *Pseudomonas putida* was previously reported to exhibit higher tolerance to solvent-like compounds.[355] This presents exciting opportunities for more robust strains, either by engineering of *P. putida* itself or by identification of the resistance gene and its transduction into *E. coli*. Therefore, if the proposed technology proves to e successful in *E. coli*, future work will also involve the study of *P. putida* and application of chemical and genetic inhibition towards industrially useful chemicals

Appendix

DNA sequencing results

Sequence of cloned FadE gene (2445 bp)

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pKD3, complete sequence (2569 bp)

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***Arc* C.acetobutylicum cloned from genomic DNA (1930 bp)**

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CER1 gene synthesised by Eurofins, optimised for *E.coli* expression (1740 bp)

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GTC GAC GGC TCA CGT CTG GCA GCG GCC GTT GTT ATC AAC AGC GTC CCC
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TAC ACC ATT GCG TCA GCT CTG TGT CAA CGT GGT GTT CAG GTA TCA ACG
CTC CGT TTA GAT GAG TAC GAG AAA ATT CGC AGC TGT GTC CCG CAG GAA
TGT CGT GAT CAC CTG GTG TAT CTG ACC AGT GAA GCA CTG AGC TCA AAT
AAG GGG TTT TGG GTG AAG GTG TGG CTG GTG GGA GAA GGT ACT ACC CGC
GAA GAA CAG GAA AAG GCG ACC AAA GGT ACG CTG TTC ATT CCT TTT AGT
CAG TTT CCC CTG AAA CAG CTT CGT CGC GAC TGC ATT TAC CAT ACC ACT
CCA GCG CTG ATT GTG CCG AAA AGC CTG GTG AAC GTG CAT TCG TGC GAA
AAC TGG CTG CCA CGC AAA GCC ATG TCC GCT ACG CGT GTT GCT GGG ATT
CTG CAT GCG TTG GAA GGC TGG GAA ATG CAC GAA TGT GGT ACA TCG CTG
TTG CTG AGC GAT TTA GAC CAA GTG TGG GAA GCG TGC TTG TCG CAT GGG
TTT CAA CCG TTA CTT CTG CCA CAT CAT TAA TCT AGA

FadD gene cloned from E.coli (1690 bp)

ATGTCAGGCTTTATTGTCCACTTTGCCGCGCGCTTCGTCACGTAATT
CTCGTCGCAAAATTTTTCCGACGTTAGATTTCGGTAACTCATCACGA
AACTCCACCAGCTTCGGTACTTTGTATCCCGTGAGCTGACGGCGGC
AAAAAGTCACCAGTGACTCTTCGGTAAGCGATGGATCTTTTTTCACT
ACGAAGATTTTCACCGCTTCACCACTGGAGCCGGAAGGTACGCCAA
CAGCCGCGACTTCCTGTACGCCAGGATGCTGCATGACGACATCTTC
AATCTCGTTGGGATAGACGTTAAAACCGGAAACCAGAATCATGTCT
TTTTTACGATCGACAATGCGCAGGAATCCTTCTTCATCCATTACCGC
GATGTCGCCGGTGTGTAACCAGCCATTTTTGATGATTTCATCGGTAG
CATCGGGACGCTGCCAGTAACCCAGCATCACCTGCGGTCCTTTGAC
ACAAAGCTCACCCGGTTGACCTGGTGGTACTTCATTATCATCATCAT
CCACCAGTTTGGCTTCCGTCGACGGCACCGGCAAACCGATGCTACC
ACTATGATAATCAATATCATATGGGTTAACGCTGACCAGCGGCGCA
CACTCGGTAAGGCCATAGCCTTCCAGCAGATACTGTCCGGTCAGTT
TCACCCAACGCTCTGCCACCACTTGCTGCACTGGCATCCCACCGCCT
GCGGAAAGATGCAGACTGGAGAAATCCAGCTGCTGGAACTCTTTAT
TGTTCAGCAACGCATTGAACAAGGTGTTAACGCCCGTGATAGCGGT
AAACGGATATTTCGCTAACTCTTTTACCAACCCTGGAATATCGCGCG
GGTTAGTGATAAGCAGGTTCTGCCCACCCAGTTCGATAAACAGCAG
GCAGTTAATGGTCAGGGCAAAAATGTGATACAGCGGCAGCGCCGTC
ACCACCAGCTCTTTGCCCGGATGCAACAGCGGACCATAGGTCGCGT
TAACCTGTTCCAGGTTCGCCAGCATATTGCGGTGAGTCAGCATCGC
GCCTTTCGCCACACCAGTGGTGCCGCCGGTGTATTGCAGAAAAGCT
AAATCTTCCGGCACCAGTTCGGGTTTGACGTACTGCATCCGGTAGC
CGTTATGCAGTGCGCTACGAAATGAAATGGCATCTGGCAGATGGTA
TTTCGGCACCAAACGCTTGATGTATTTAACAACGAAATTGACTACC
GTGCCTTTTGCCGTAGATAGCTGATCGCCCATACGGGTCAGAATTA
CGTGCTGAACGGCGGTTTTATCAACCACTTTTTCCAGTGTGTGAGCA
AAGTTAGACACGATAACAATCGCCGATGCGCCGCTATCGTTAAGCT
GATGCTCAAGCTCACGCGGGGTATACAACGGGTTAACGTTTACGAC
GATCATCCCGGCACGCAAAATGCCAAACAGCGCCACCGGATATTGC
AATAAATTAGGCATCATCAACGCAACGCGATCGCCTTTCTTCAGCC
CCAACCCTTGTTGCAAATAAGCGGCAAACGCGCGACTGCGTTCTTC
CAGCTTGCGGAAGGTCATTACCTCCCCCATATTCACAAACGCAGGT
TGATCGGCGTAGCGCGCGACCGACTGCTCAAACATATCTACCAGAG
ATTGATAACGGTCAGGGTTGATCTCCGTCGGAACGTCCGCGGGATA
ACGGTTAAGCCAAACCTTCTTCAA

TesA gene cloned from E.coli (572 bp)

ATGTTATGAGTCATGATTTACTAAAGGCTGCAACTGCTTCGCCATCC
AGTCGGCAATAAACGGCTGGGCGTCGCGGTTGGGATGAATACCGTC
ATCCTGCATCCATTGTGGCTTGAGGTAGACCTCTTCCATAAAAAAG
GGCAGCAGCGGAACATCAAACTCTTTGGCGAGTTTGGGGTAAATGG
CGCTAAAGGCTTCATTATAACGGCGACCATAGTTTGCAGGCAGACG
TATTTGCATTAACAATGGTTCAGCGTTGGCGGCTTTGACATCCTGCA
AAATCTGGCGCAGCGTTTGCTCGGTTTGCTGTGGCTGAAAACCACG
CAAACCGTCATTGCCGCCCAGTTCAACCAGCACCCAACGCGGCTGA

TGCTGTTTCAGCAGAGCCGGAAGGCGCGCCAGTCCTTGTTGCGAGG
TGTCGCCGCTGATGCTGGCATTAACTACCGACGTTTTACTCTGCCAC
TTATCATTCAACAAGGCAGGCCAGGCCGCGCTGGCAGACATTCGAT
ACCCGGCGCTCAGGCTATCACCCAGAATCAATAACGTGTCCGCTGC
GGCGGCACGGAAGGTTAACAGGACCAGGAACAGGAAGGGCAAATG
CCAGCGGAAAACATTGTTGAAGTTCATCAT

pTac*Cer1*+pTac*fadD* (3426 bp)

ATGAAGCTGTGGTATGGCTGTCCAGGTCGTAAATCACTGCATAATT
CGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTTGCGCC
GACATCATAACGGTTCTGGCCCATGG ATG GCG ACG AAA CCT GGC GTT
TTA ACC GAT TGG CCG TGG ACT CCG CTT GGC AGC TTC AAA TAC ATC GTG
ATT GCG CCG TGG GCC GTT CAC TCA ACC TAC CGG TTT GTG ACG GAT GAT
CCT GAG AAA CGT GAT TTA GGG TAC TTT CTG GTC TTT CCG TTT CTG CTC
TTT CGC ATT CTG CAC AAC CAA GTC TGG ATC TCG TTG AGT CGC TAC TAT
ACC AGC TCC GGC AAA CGC CGT ATT GTC GAC AAA GGC ATT GAC TTC AAC
CAA GTC GAT CGT GAG ACT AAT TGG GAT GAT CAG ATC CTC TTC AAT GGC
GTG CTG TTT TAC ATC GGC ATT AAT CTG CTG CCA GAG GCG AAA CAG CTG
CCT TGG TGG CGT ACG GAT GGT GTT CTG ATG GCA GCT CTC ATT CAT ACG
GGC CCG GTG GAA TTT CTG TAT TAT TGG TTA CAC AAA GCG CTG CAT CAT
CAT TTC CTG TAC TCT CGT TAT CAC AGC CAT CAT CAC TCC TCC ATT GTT
ACC GAA CCA ATC ACG AGT GTA ATT CAT CCC TTC GCT GAG CAT ATT GCG
TAT TTC ATT CTG TTC GCA ATT CCG CTG TTG ACT ACC CTG CTG ACG AAA
ACA GCG TCT ATC ATT AGT TTC GCC GGC TAT ATT ATT TAC ATC GAC TTT
ATG AAC AAT ATG GGT CAT TGC AAT TTT GAA TTA ATC CCG AAA CGG TTA
TTC CAC CTG TTT CCG CCG CTT AAG TTC TTG TGC TAT ACC CCT TCT TAT
CAT TCC TTA CAC CAC ACC CAG TTT CGG ACC AAC TAT AGC TTG TTT ATG
CCG CTG TAC GAT TAC ATC TAT GGT ACT ATG GAT GAA AGC ACC GAT ACA
CTC TAT GAG AAA ACC TTG GAA CGC GGT GAT GAC ATT GTT GAC GTC GTG
CAT TTA ACC CAT CTG ACC ACA CCG GAG TCT ATC TAT CAC TTG CGC ATT
GGT CTG GCA TCT TTC GCA AGC TAT CCG TTT GCC TAC CGC TGG TTT ATG
CGC CTT CTG TGG CCA TTT ACA TCC CTT TCG ATG ATC TTC ACG TTG TTC
TAT GCC CGC CTC TTC GTA GCC GAA CGC AAC AGC TTT AAC AAA CTT AAT
CTG CAA TCG TGG GTT ATC CCG CGC TAT AAT CTC CAG TAT CTT CTC AAA
TGG CGC AAA GAA GCT ATC AAC AAC ATG ATC GAG AAA GCC ATC CTG GAA
GCG GAT AAG AAA GGC GTG AAA GTC TTA TCT CTG GGT CTG ATG AAC CAG
GGA GAA GAG CTG AAC CGT AAT GGA GAA GTA TAT ATT CAC AAT CAC CCC
GAC ATG AAG GTA CGC CTG GTC GAC GGC TCA CGT CTG GCA GCG GCC GTT
GTT ATC AAC AGC GTC CCC AAA GCA ACT ACC AGT GTT GTA ATG ACG GGC
AAT CTG ACA AAG GTG GCC TAC ACC ATT GCG TCA GCT CTG TGT CAA CGT
GGT GTT CAG GTA TCA ACG CTC CGT TTA GAT GAG TAC GAG AAA ATT CGC
AGC TGT GTC CCG CAG GAA TGT CGT GAT CAC CTG GTG TAT CTG ACC AGT
GAA GCA CTG AGC TCA AAT AAG GGG TTT TGG GTG AAG GTG TGG CTG GTG
GGA GAA GGT ACT ACC CGC GAA GAA CAG GAA AAG GCG ACC AAA GGT
ACG CTG TTC ATT CCT TTT AGT CAG TTT CCC CTG AAA CAG CTT CGT CGC
GAC TGC ATT TAC CAT ACC ACT CCA GCG CTG ATT GTG CCG AAA AGC CTG
GTG AAC GTG CAT TCG TGC GAA AAC TGG CTG CCA CGC AAA GCC ATG TCC
GCT ACG CGT GTT GCT GGG ATT CTG CAT GCG TTG GAA GGC TGG GAA ATG

CAC GAA TGT GGT ACA TCG CTG TTG CTG AGC GAT TTA GAC CAA GTG TGG
GAA GCG TGC TTG TCG
CATGGGTTTCAACCGTTACTTCTGCCACATCATTAATCTAGAAAGCTGTGGT
ATGGCTGTCCAGGTCGTAAATCACTGCATAATTCGTGTCGCTCAAG
GCGCACTCCCGTTCTGGATAATGTTTTTTGCGCCGACATCATAACGG
TTCTGGCGTCGCCGGTGTGTAACCAGCCATTTTTGATGATTTCATCG
GTAGCATCGGGACGCTGCCAGTAACCCAGCATCACCTGCGGTCCTT
TGACACAAAGCTCACCCGGTTGACCTGGTGGTACTTCATTATCATCA
TCATCCACCAGTTTGGCTTCCGTCGACGGCACCGGCAAACCGATGC
TACCACTATGATAATCAATATCATATGGGTTAACGCTGACCAGCGG
CGCACACTCGGTAAGGCCATAGCCTTCCAGCAGATACTGTCCGGTC
AGTTTCACCCAACGCTCTGCCACCACTTGCTGCACTGGCATCCCACC
GCCTGCGGAAAGATGCAGACTGGAGAAATCCAGCTGCTGGAACTCT
TTATTGTTCAGCAACGCATTGAACAAGGTGTTAACGCCCGTGATAG
CGGTAAACGGATATTTCGCTAACTCTTTTACCAACCCTGGAATATCG
CGCGGGTTAGTGATAAGCAGGTTCTGCCCACCCAGTTCGATAAACA
GCAGGCAGTTAATGGTCAGGGCAAAAATGTGATACAGCGGCAGCG
CCGTCACCACCAGCTCTTTGCCCGGATGCAACAGCGGACCATAGGT
CGCGTTAACCTGTTCCAGGTTCGCCAGCATATTGCGGTGAGTCAGC
ATCGCGCCTTTCGCCACACCAGTGGTGCCGCCGGTGTATTGCAGAA
AAGCTAAATCTTCCGGCACCAGTTCGGGTTTGACGTACTGCATCCG
GTAGCCGTTATGCAGTGCGCTACGAAATGAAATGGCATCTGGCAGA
TGGTATTTCGGCACCAAACGCTTGATGTATTTAACAACGAAATTGA
CTACCGTGCCTTTTGCCGTAGATAGCTGATCGCCCATACGGGTCAG
AATTACGTGCTGAACGGCGGTTTTATCAACCACTTTTTCCAGTGTGT
GAGCAAAGTTAGACACGATAACAATCGCCGATGCGCCGCTATCGTT
AAGCTGATGCTCAAGCTCACGCGGGGTATACAACGGGTTAACGTTT
ACGACGATCATCCCGGCACGCAAAATGCCAAACAGCGCCACCGGA
TATTGCAATAAATTAGGCATCATCAACGCAACGCGATCGCCTTTCTT
CAGCCCCAACCCTTGTTGCAAATAAGCGGCAAACGCGCGACTGCGT
TCTTCCAGCTTGCGGAAGGTCATTACCTCCCCCATATTCACAAACGC
AGGTTGATCGGCGTAGCGCGCGACCGACTGCTCAAACATATCTACC
AGAGATTGATAACGGTCAGGGTTGATCTCCGTCGGAACGTCCGCGG
GATAACGGTTAAGCCAAACCTTCTTCAATGA

pTrcA*rc*pTac*TesA* (1930 bp)
ATGGTTTGACAGCTTATCATCGACTGCACGGTGCACCAATGCTTCTG
GCGTCAGGCAGCCATCGGAAGCTGTGGTATGGCTGTGCAGGTCGTA
AATCACTGCATAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGAT
AATGTTTTTTGCGCCGACATCATAACGGTTCTGGCAAATGAAGCTTG
AATTTACATGTGGCGAAGAGATAACTATTAAGGAAGTTAATAAATT
AAAGCTTGAACTTATGACAAGCGGTAAAGGTGATGTAGAGAAGGA
ATTAGAAAGGCTTAAAAAGAATAAAAATAAAGTTCATGATATAAA
AACCGAGGAAACAATTGACTTGCTTGATAGATGTGCAAAATTATGG
CTTGATGACAATTATTCAAAAAAGCATATTGAAACATTAGCACAGA
TAACAAACCAAAGTTTTGAATTAGTAAATTATGAGTTTAAGAGTAT
GATGCAAATGCTTCTTCGTGAAAATATACAAAATACTATTAAGAGG
GAACTTGGAGAGGTAGATATTTTAGATTCATGGCACAAAACAAATT
ATGGCTATGAGCATAGACAGCCAAGAGGGATTATATTTCATAATAT
TTCAGGAAATGCTTTCGTGGTTATACCAATGAGCATATCCATGGGTC

TTTTGTCAAAAAACTGTAATTTAGTTAAGGTATCTAAAGATGAACC
ATATTTTGCGTATGCATTTTATAAAAGTCTATGTGAGTTAGATGAAA
CTGTGAAAGATAGGCTATCCGTTTTGTACTTTGATAGTAGTAAAACT
GAAATCTATGACACAATCGTTAAAAATTCAGATGGTGTTATACACT
GGGGTGGAGAGTATTCTTCAAGAATAATAGGTGAAATGTGCGCAAA
GTATCAAAAACACATGATAATGCATGGAGCAAAAATAAGTTTTGAA
GTAGTTGATAAATGTGATGACTTAAATAAAGTATCAGAGGAGATAG
CAAAGGATATAATATGTTGGGAACAGAAGGCTTGTTTATCACCTAG
GGTAGTATTTCTAAATGATAAGATAGATGTAGATGAATTTTCACATT
GTCTTTCGGCTTCTCTTAGAAAAATCACAGAGGTTATACCTAAGGC
ATATTTAAGTGAATGGAATTCAGTTAAGACAATACAAGACAGACAA
TACTGTACTCTTAAATATGGAATGAAAAATGAAAGAAGATTTAAAG
TTTATTCTTCCTTCAATGCTGATTATACAGTAATTCTATCAGATGGA
ATGCCTGAAAAAGAAGATATAAACAAATGTTTTTATAGATTTATAT
TTGTATGTCCTTATTCAAGTAAAAGCGATGTTGTAAATTACGTCTCT
GAAAACTTAAAGGATTATCTTCAGACTATGGGATATAGCGGCAATG
ACGAAGAATTCATAGAAAAAATGACTTTACTTGGTGTTAGCATAGT
AACTAAACCTGGAAAGATGTCTTTACATGAGCCGGGAACTTCTCAT
GACGGAATACACAATCTTAAGGAGTTTACATTTTTGGTTAGTAGAC
AAATCTAAAAGCTGTGGTATGGCTGTCCAGGTCGTAAATCACTGCA
TAATTCGTGTCGCTCAAGGCGCACTCCCGTTCTGGATAATGTTTTTT
GCGCCGACATCATAACGGTTCTGGCCCA TGG ATG GCG ACG AAA CCT
GGCGTTTTACCGATTGGCCGTTATGAGTCATGATTTACTAAAGGCTGCA
ACTGCTTCGCCATCCAGTCGGCAATAAACGGCTGGGCGTCGCGGTT
GGGATGAATACCGTCATCCTGCATCCATTGTGGCTTGAGGTAGACC
TCTTCCATAAAAAAGGGCAGCAGCGGAACATCAAACTCTTTGGCGA
GTTTGGGGTAAATGGCGCTAAAGGCTTCATTATAACGGCGACCATA
GTTTGCAGGCAGACGTATTTGCATTAACAATGGTTCAGCGTTGGCG
GCTTTGACATCCTGCAAAATCTGGCGCAGCGTTTGCTCGGTTTGCTG
TGGCTGAAAACCACGCAAACCGTCATTGCCGCCCAGTTCAACCAGC
ACCCAACGCGGCTGATGCTGTTTCAGCAGAGCCGGAAGGCGCGCCA
GTCCTTGTTGCGAGGTGTCGCCGCTGATGCTGGCATTAACTACCGAC
GTTTTACTCTGCCACTTATCATTCAACAAGGCAGGCCAGGCCGCGCT
GGCAGACATTCGATACCCGGCGCTCAGGCTATCACCCAGAATCAAT
AACGTGTCCGCTGCGGGATGA

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