# Final report

## Determining which of the halogenated small molecules from Asparagopsis is responsible for the observed activity in inhibiting methane production in cattle and sheep


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| Project code: | P.PSH.1264 |
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| Date published: | 11th May 2022 |

PUBLISHED BY
Meat & Livestock Australia Limited
PO Box 1961
NORTH SYDNEY NSW 2059

This is an MLA Donor Company funded project. Meat & Livestock Australia acknowledges the matching funds provided by the Australian Government to support the research and development detailed in this publication.

**Abstract**

This project is undertaken as basic R&D toward developing a commercially viable feed additive for sheep and cattle with acceptable methane emission reduction (MER) for the red meat industry. It is aimed at examining quantitatively the MER effect of halogenated compounds from *Asparagopsis* seaweeds and their nonhalogenated analogues as inhibitors of the Factor F430 containing enzymes in two methanogenic archaea: *Methanothermobacter marburgensis* (MM) and *Methanobrevibacter ruminantium* (MR). MM is a model organism that has been well studied and is well understood in terms of methanogenic pathways. MR is one of the principle methanogens in cattle rumen, responsible to ruminant methane emissions.

In stage I of the project, an *in vitro* assay using live organisms as well as an *enzyme* assay, under strictly controlled anaerobic conditions, using purified methyl CoM reductase enzyme complex (MCR) from MM, were established to test potentially active compounds. A total of 18 compounds were assayed with *in vitro* assays and 3 known compounds assayed with the *enzyme* MCR assay. It was confirmed that 3-NOP and 2-BES inhibit MCR but that bromoform is a weak inhibitor of the enzyme but a strong inhibitor in vitro, suggesting it inhibits methane production via a different mechanism.

The development of a defined feed additive for the mitigation of methane emissions from ruminant animals will have potential economic impacts on the red meat industry in terms of increased production and helping to meet the industry's net zero carbon emission by 2030 target (CN30). The long-term goal is to develop a cost-effective and commercially viable solution based on additives with well-defined mode-of-action.

# Executive summary

## Background

In Australia and also globally, the livestock sector is critical for food security with increasing consumer demand particularly on high quality protein sources such as red meat. Concurrently, heightened consumer awareness of substantial enteric methane emissions from livestock requires the industry to adopt sustainable technologies for methane emission reduction (MER). Feed additive technologies are central to the industry's viability with direct impacts on product quality/ competitiveness, animal health, as well as environmental compatibility.

The MER global market is estimated at US$2 billion per annum, according to the investment firm Jefferies (Scott, 2019). While several existing and emerging additive approaches have demonstrated positive MER effects, a well-defined, cost-effective and environmentally benign solution, with high MER suitable for diverse farming conditions, remains an unmet need.

The Australian Red Meat Industry has a carbon neutral by 2030 (CN30) target, and this project forms an important step toward this goal under the Emission Avoidance Partnership (EAP). It will investigate an emerging feed additive approach that promises to have positive and quantifiable MER impact with high potential of synergistic effects with existing feed additives. By taking the learnings on how naturally occurring methane inhibitors work and produce improved versions with targeted and quantifiable effects in MER, the long-term goal of the project is to fill a key knowledge gap in the cost-effectiveness of this emerging feed additive technology and deliver a viable solution to MER within the livestock emissions framework.

This report addresses the first-stage goals of this project toward a scalable and field-worthy feed additive solution based on methane reducing constituents of *Asparagopsis* seaweeds through understanding their specific modes of action and characterising reliably their MER effects. In this first stage, investigations focused on how naturally occurring methane inhibitors work with targeted and quantifiable effects in MER. Two key methanogenic bacteria, *Methanothermobacter marburgensis* (MM-a well understood methanogen model) and *Methanobrevibacter ruminantium* (MR-a methanogen in livestock), were investigated to establish first enzyme/in vitro MER models and test initial lead inhibitors. In vivo assays with MM and MR, and enzyme assays with $MCR_{Red1}$ isolated from MM were established to test both known and unknown inhibitors. Inhibitors tested included 10 coenzyme M mimics, 3 coenzyme B mimics, and 5 halogenated methane derivatives. Among these, halogenated derivatives were the most potent inhibitors of methanogenesis in vitro, and an $IC_{50}$ for bromoform is reported for the first time.

This first-stage success sets the groundwork for the 2nd stage goals of tracking down exactly which compounds in *Asparagopsis* seaweeds best inhibit methanogenesis and designing and testing rational compounds as mimics of nature for MER with well-defined mode of action. This will fill a key knowledge gap in the cost-effectiveness of this emerging feed additive technology and ascertain its emerging role in developing a livestock emissions framework.

## Objectives

The aims/objectives of this first stage were to:

1. **establish a continuous cultures of MM and MR:**
   both *Methanothermobacter marburgensis* (MM) and *Methanobrevibacter ruminantium* (MR) continuous cultures were established at small to medium scale;

2. **isolate and purify an active MCR enzyme:** MethylCoenzyme M Reductase (MCR) from *Methanothermobacter marburgensis* was isolated, purified in its "red1" form that demonstrated activity in the presence of freshly prepared substrates;
3. **demonstrate a robust MCR inhibition assay**:
   the MCR inhibition assay was established by synthesising known inhibitors and testing as positive controls for the active MCR from *Methanothermobacter marburgensis*;
4. **testing initial compounds from *Asparagopsis* seaweeds and their mimics:**
   Nine compounds, in addition to bromoform, from *Asparagopsis* were tested in whole cell assays and 5 showed complete inhibition at 5 mM. In addition, we synthesed 3 CoB mimics (no activity) and 9 CoM mimics, 5 of which showed modest activity

Overall, a robust testing/rapid screening platform is now in place to further test the other (~110) *Asparagopsis* compounds and any new leads and integrate with downstream pipelines of *in vivo* testing, optimisation, formulation, scaling, and field deployment.

## Methodology

A collection of chemical and biological methods were successfully applied to achieve the objectives. These include:
1) strictly anaerobic cell culturing,
2) enzyme isolation/purification/protein characterisation,
3) gas chromatography-mass spectrometry (GC-MS) for direct methane detection,
4) organic synthesis for making cofactors, substrates and inhibitors,
5) compound characterisation via spectroscopic techniques; and
6) computational analyses for structure-guided inhibitor design.

## Results/key findings

1) *Methanothermobacter marburgensis* and *Methanobrevibacter ruminantium* were successfully grown under strictly anaerobic conditions continuously over 1 year;
2) $MCR_{red1}$ was isolated and purified from *Methanothermobacter marburgensis* and shown to generate methane directly from Methyl-coenzyme M and Coenzyme B;
   a. Methyl-coenzyme M and Coenzyme B were successfully synthesised at 0.3 g scale.
3) In vitro assay for MCR inhibition was successfully established using head space analysis with positive controls and in vitro assays for MER was established for both *Methanothermobacter marburgensis* (MM) and *Methanobrevibacter ruminantium* (MR).
4) 18 potential inhibitors were tested, including 10 methyl-CoM mimics, 3 CoB mimics and 5 *Asparagopsis* compounds.
5) $IC_{50}$ values were calculated on known methanogenesis inhibitors 2-BES, 3-NOP and bromoform.

## Benefits to industry

A scalable and field-worthy compound as a feed additive, with defined mode of action (MOA) and reliable MER effects, would be highly valued by the red meat industry in maintaining its commercial viability and increasing production. In addition, it would enable the industry to pursue the Red Meat 2030 Vision of "doubling the value of red meat sales as the trusted source of the highest quality protein" while achieving its Carbon Neutral by 2030 target (CN30). The rapid reduction of methane emissions will also disproportionally slow global warming compared to reductions in $CO_2$ emissions.

## Future research and recommendations

With the groundwork set for finding and developing nature-mimicking compounds for defined MER effects, we strongly recommend proceeding with the second stage of this project:

**Aim 1)** Finding new chemical entities (NCEs), by designing and testing rationally nature-mimicking compounds, as potential feed additives with target-specific activity against the MCR enzyme;

**Aim 2)** Testing in vitro combinations of inhibitors for synergistic effects of MER with the potential of finding new targetable aspects of methanogenesis.

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# 1. Background

Ruminant livestock are responsible for production of a large proportion of Greenhouse gases, particularly methane (87–97 megatons/y; Chang et al, 2019) which contributes disproportionally to global warming and climate change because methane has 84x the heat absorption capacity of carbon dioxide but only lasts about a decade in the atmosphere, giving it a 20 year-global warming potential (GWP) of 56x compared to carbon dioxide. Methane levels have risen almost 4x on their long-term average and doubled since the 1950's so near term reductions in methane emission will have a disproportionally large effect on global warming. Methane is an end product of fermentation (methanogenesis) of plant material by the microbial ecosystem in the rumen. This ecosystem is very complex and involves thousands of species of microbes that interact with the feed, their host and each other. While this ecosystem is relatively poorly understood, it is clear that methanogenic archaea are the source of most if not all the methane produced by ruminants. Recently, it has been found by the CSIRO/MLA/James Cook University that adding the red seaweed *Asparagosis taxiformis* to feed can reduce methane emission of cattle to almost zero.[1, 2] While *A. taxiformis* represents a promising natural strategy for reducing methane emissions from ruminant livestock there are a number of potential issues:

1) Sourcing enough *A. taxifiormis* to feed Australia's sheep and cattle population (~130 million head) would be difficult
2) To date the genus *Asparagopsis* has been reported to contain 124 halogenated small molecules varying in molecular weight from 94 to 731. The effect of these compounds on the health of cattle is unknown. The bioaccumulation and metabolomics of all these compounds needs to be understood in the long term.
3) Many of the small molecules found in *Asparagopsis* seaweeds are themselves Greenhouse gases and/or known to be ozone depleting.

It makes sense therefore to screen all the compounds from *Asparagopsis* and related seaweeds (*Delissia, Ptilonia, Falkenbergia* and *Bonnemaisonia*) for their ability to inhibit methanogenesis and then develop a well-defined chemical feed additive based on these findings that can be fully characterized and understood from a mechanistic, metabolomic and environmental perspective. All known methanogens use the same enzymatic reaction catalysed by methyl-coenzyme M reductase (MCR) to reduce coenzyme M with coenzyme B to form methane. Halogenated methane analogues (HMAs) such as bromoform, chloroform bromochloromethane, dichloromethane trichloroacetaldehyde and 2-bromoethane sulfonate have been shown to have antimethanogenic properties.

It is widely believed that these halogenated compounds (e.g. bromochloromethane (BCM)) inhibit methane production by reacting with reduced vitamin $B_{12}$ which inhibits cobamide-dependent methanogenesis, most papers citing the work of Wood.[3] Our experience with the fermentation of methanobacteria suggests this might be incorrect and that the halogenated compounds in *Asparagopsis* actually inhibit Coenzyme M reductase (MCR), a cofactor $F_{430}$ containing enzyme found exclusively in methanogens that does not contain vitamin $B_{12}$ but rather a nickel cofactor. A more worrying aspect is if bromoform does inhibit vitamin $B_{12}$ dependant methyltransferases, then it will be potentially toxic to all the other bacteria in the rumen and, indeed, humans.

It is known that haloalkanes, such as 2-bromoethanesulfonate (2-BES), structural analogs to coenzyme-M, competitively inhibit MCR with an apparent $IC_{50}$ of 0.4 μM.[4] While BCM has been shown to be effective in reducing methane emission from cattle,[5] BCM was prohibited by the Australian Government in 2004 under the Ozone Protection and Synthetic Greenhouse Gas Management Act 1989.

Attempts to define the chemical entities responsible for reduction in methane emission from *A. taxiformis* have been largely unsuccessful, the crude biomass being more effective in methane suppression than any of the tested compounds.[6] However, bromoform was the most active compound found of the five compounds tested in batch fermentation assays.

We believe that one or more of the halogenated small molecules from *Asparagopsis* is responsible for the observed activity in inhibiting methane production in cattle and it is the aim of this project (in two stages, this report covering only stage 1) to determine which compounds are responsible. The first stage of this project was to establish the bioassays and test initial compounds.

The long-term goal is to use this knowledge to develop a veterinary solution to methane emission reduction either as a feed additive or medicine that is thoroughly understood and evaluated. A single, highly effective compound that could be added to feed will tackle the emission issue at scale.

# 2. Objectives

The key objective is to use small molecules to target specific steps in the methanogenesis pathway to find novel compounds, designed and developed with a rational, structure-guided approach with clues from natural products, that can specifically and safely inhibit methane production. Three key enzymes, namely 2-phospholactate transferase (2PLT), CoB-CoM heterodisulfide reductase (Hdr) and methyl coenzyme M reductase (MCR) have been identified as targets in the methanogenesis pathway. This first stage of the project targets just MCR, with two major advantages:

1) MCR catalyses the final step of the methane production cycle by generating methane directly from CoM-$CH_3$ and for the direct chemical step of methane production at the end of the cycle (unlike the genes that encode the earlier steps in methanogenesis with a large amount of variation across the methanogens),

2) MCR is the universally conserved enzyme of all methanogens and the gene from *Methanothermobacter marburgensis* is closely related to rumen specific methanogens such as *Methanobrevibacter ruminantium, Methanobrevibacter millerae, Methanothermobacter smithii, Methanobacterium formicicum* and *Methanothermobacter wolfeii*.

The objective of this stage was to isolate and purify MCR enzyme in its “red1” active form, scalable synthesis and purification of coenzyme B, development of MCR inhibition assay enzyme, and testing of initial inhibitor leads in vitro.

# 3. Methodology

## 3.1 Cell culture and storage

### 3.1.1 Small/medium scale culture

*Methanothermobacter marburgensis* (DSM 2133, referred to here as MM) was obtained from the German Collection of Microorganisms and Cell Cultures GmbH. Batch cultures were cultivated in either 18×150 mm anaerobic tubes filled with 5 mL of medium DSMZ 119 or 100 mL serum flasks filled with 40 mL of the same medium. Medium DSMZ 119 was prepared according to the protocol from the commercial supplier, with the following modifications: 1 mL of 0.1% w/v sodium resazurin solution was used instead of 0.5 mL; sludge fluid and fatty acid mixture were not included. The trace element solution was prepared according to the method by Balch et al (Balch et al., 1979) with the following modifications: $NiSO_4 \cdot 6H_2O$ was added at 0.1 g/L; $Na_2SeO_4$ was added at 0.2 g/L; $Na_2WO_2 \cdot 2H_2O$ was added at 0.1 g/L. Cultures were inoculated at a 1:20 ratio. $H_2$-$CO_2$ (80:20 vol/vol) was added after inoculation at a pressure of 26 psi. Cultures were then incubated at 65 °C with rigorous shaking and grown for 7 days. The headspace of cultures was exchanged for fresh $H_2$-$CO_2$ every second day.

*Methanobrevibacter ruminantium* (DSM 1093, referred to here as MR) was obtained from the CSIRO, Queensland. Cultures were grown under similar conditions as for MM, except for medium composition and incubation temperature. Medium for MR was prepared according to the procedure by Balch et al (Balch et al., 1979) with the following modifications: trace vitamins solution was not included, and the media was supplemented with $NH_4Cl$ (1 g/L), clarified rumen fluid (50 mL/L), 0.1% w/v sodium resazurin solution (1 mL/L) and coenzyme M (2 mg/L). The trace element solution was identical to that described for MM above. Cultures were incubated at 37 °C.

To quantify growth, a 1 mL aliquot of *M. marburgensis* and *M. ruminantium* were taken and centrifuged at a low speed (standard benchtop micro-centrifuge) for 30 minutes. A drop of media surrounding the small cell pellet was taken and deposited on a laboratory microscope slide. The slide was visualised under a microscope wherein the *M. marburgensis* and *M. ruminantium* cocci were observed clustered together. Due to the fluorescently active cofactor F420, the cells were observed to emit a blue fluorescence when the slide was irradiated with a 376 nm UV lamp.

Under the reported conditions, the methodology was efficient for the culture of both microorganisms.

### 3.1.2 Large scale culture

Batch cultures of MM and MR were grown on 1 L serum flasks containing 400 mL of the corresponding medium. All other conditions were identical to those for small/medium scale. Cultures were inoculated at a 1:20 ratio (20 mL) using small cultures grown for 1 week. $H_2$-$CO_2$ (80:20 vol/vol) was added after inoculation at a pressure of 26 psi. Cultures were then incubated at 65 °C (MM) or 37 °C (MR) with rigorous shaking and grown for 14 days. The headspace of cultures was exchanged for fresh $H_2$-$CO_2$ every second day. This methodology was efficient for the culture of both microorganisms. Under the current capacities, a volume of 1.6 L of cell culture can be obtained every week for MCR isolation.

### 3.1.3 Cell storage

For short term storage, cultures grown as indicated above can be stored without any further processing at 4 °C. These samples are viable for further culture propagation for at least one month. For long term storage, cell cultures are diluted 1:1 with anoxic 50% w/w glycerol in water and stored in 8 mL serum vials at −80 °C. These samples are viable for at least one year.

## 3.2 Cell lysis and protein purification

### 3.2.1 Cell harvest with activation and lysis

All solutions were prepared and all manipulations were performed under strictly anaerobic conditions in an LC-1 Glovebox maintained under nitrogen gas at < 1 ppm of oxygen. Before harvesting, the headspace of the cell cultures was purged with carbon monoxide for 5 minutes, followed by incubation at 37 °C for two hours (Zhou et al., 2013). The cells were then harvested in batch by centrifugation at 18,000 × *g*, 4 °C, 40 min. The cell pellet was resuspended in 1/100 of the original culture volume with 50 mM Tris-HCl pH 7.6 supplemented with 10 mM coenzyme M. The cell resuspension was treated with carbon monoxide and incubated at 37 °C for 1 hour. Cell lysis was performed by sonication on ice for 3 × 1 minute cycles with pulses of 1 second at 100% amplitude. Cell debris and membranes were removed by centrifugation at 100,000 × *g*, 4 °C, 40 min (Rospert et al., 1991. Duin et al., 2011). These conditions were efficient to maintain MCR in its active $MCR_{red1}$ form in the clarified supernatant.

### 3.2.2 Ion exchange chromatography

The clarified cell lysate (7-8 mL) was loaded into a manually packed Q-Sepharose column (5 mL) equilibrated with buffer A (50 mM Tris-HCl pH 7.6 and 10 mM coenzyme M). After loading, the column was washed with buffer A (10 mL). The column was developed with a step gradient of NaCl in buffer A (10 mL each): 0.36 M, 0.4 M, 0.44 M, 0.48 M, 0.52 M, 0.6 M and 2 M (Duin et al., 2011). 1 mL fractions were collected and analyzed for $MCR_{red1}$ by UV-visible spectroscopy at 280 and 420 nm. MCR isoenzyme I typically eluted at 0.44 M NaCl. Fractions corresponding to MCR were concentrated to 1/10 of the original fraction volume by centrifugation at 1,000 × *g*, 10 min using a protein concentrator with a 100 KDa molecular weight cut-off. These conditions were efficient to isolate $MCRr_{ed1}$ in multiple preparations.

### 3.2.3 Protein quantification, gel analysis, and peptide sequencing

Protein concentration was determined using the BCA protein assay (Thermo Scientific, USA) on a microplate reader with BSA standards as per manufacturer's instructions. SDS-PAGE was performed using 10% TruPAGE precast gels (Sigma-Aldrich, USA). Peptide identification from SDS-PAGE protein bands was performed by the Australian Proteome Analysis Facility (Sydney, Australia) in accordance with APAF SOPs MS-057 (Reduction, alkylation and digestion for solid/liquid Samples), and MS-070 (QExactive Data Acquisition v1). Full details are reported in appendix 8.1

## 3.3 Synthesis of substrates and compounds

### 3.3.1 General methods

All reactions were performed under an inert atmosphere of nitrogen or argon. Ethyl 4-bromobutyrate (**3-BrCO2Et**, CAS: 2969-81-5, Oakwood Chemicals, Product Number (PN): 139400), methyl 4-bromobutyrate (**3-BeCO2Me**, CAS: 4897-84-1, Alfa Aeser, PN: L12636), ethyl 3-(methylthio)propionate (**3MeSCO2Et**, CAS: 13327-56-5, Combi-Blocks, PN: QF-2912), methyl 3-(methylthio)propionate (**3-MeSCO2Me**, CAS: 13327-56-5, Sigma Aldrich, PN: 103373), methyl 4-cyanobutanoate (**3-CNCO2Me,** CAS: 41126-15-2, Combi-Blocks, PN: QB-4663) and ethyl 3-cyanopropanoate (**2-CNCO2Et**, CAS: 10137-67-4, Combi-Blocks, PN: QK-7415) were commercially sourced and used without further purification. Anhydrous solvents (THF, $CH_2Cl_2$, $Et_2O$, Toluene, MeCN) were obtained from a solvent purification system and dried over 3Å MS (20% w/v) overnight before use. TLC was performed using 0.25 mm Merck Silica plates (60F-254) visualizing with short-wave UV light or stained with $KMnO_4$. Chloroform-*d* was purchased from Cambridge Isotope Laboratories, UK and stored over anhydrous $K_2CO_3$. All 1H and 13C NMR experiments were performed on a Bruker AVIIIHD 400 MHz NMR Spectrometer equipped with a BBFO SmartProbe (5mm). Chemical shifts were reported in ppm using residual $CHCl_3$ ($\delta_H$; 7.26 ppm, $\delta_C$; 77.16 ppm) or $(CH_3)_2CO$ ($\delta_H$; 2.05 ppm, $\delta_C$; 39.52 ppm) as an internal reference. Data are reported as: δ value, multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad) and integration. Ultra high purity methane (99.995% methane) was purchased from BOC Australia

### 3.3.2 Synthesis of methyl-CoM and CoB

#### 3.3.2.1 Methyl-CoM

Methyl-coenzyme M was synthesized following known literature procedures (Scheller et al., 2010) under strictly deoxygenated conditions. Coenzyme M sodium salt (400 mg, 2.44 mmol, 1.0 equiv) was dissolved in thoroughly degassed methanol (5 mL) and sodium methoxide (25% w/v in methanol, 0.99 equiv, 2.41 mmol) was added dropwise at 0 ˚C. After complete dissolution of coenzyme M, methyl iodide (161 mL, 2.58 mmol, 1.01 equiv) was added under vigorous stirring at room temperature and the reaction mixture was refluxed overnight under an inert atmosphere ($N_2$). After evaporation of the solvent the crude product was dissolved in water and precipitated with acetone to afford analytically pure S-methyl coenzyme M (367 mg, 85%). Spectra were consistent with the literature. (Scheller et al., 2010)

**$^1$H NMR (400 MHz, $D_2O$)** δ 3.23 – 3.15 (m, 2H), 2.92 – 2.83 (m, 2H), 2.17 (s, 3H).
**$^{13}$C NMR (100 MHz, $D_2O$)** δ 50.6, 27.6, 14.2.

#### 3.3.2.2 CoB

All precursors for CoB were synthesized following known literature procedures (Noll et al., 1987) starting from ethyl 7-bromoheptanoate (5 steps sequence, only last step reported here). A solution of O-phospho-L-threonine (400 mg, 2 mmol) and triethylamine (0.56 mL, 4 mmol) in 4 mL water was added under stirring to a solution of 7,7′-dithiobis(succinimido-oxyheptanoate) (362 mg, 0.7 mmol) in 18 mL tetrahydrofuran and 4 mL acetonitrile. After stirring at room temperature under nitrogen for 36 h, the solvents were removed under vacuum at 30 °C. The residue was diluted with water (5 mL) and washed with $CH_2Cl_2$ (10 mL). The aqueous layer was freeze-dried three times and used immediately without further purification. The crude residue was dissolved in water, and the pH adjusted to 9.5 with ammonia hydroxide. DTT was added in portions and the reaction stirred

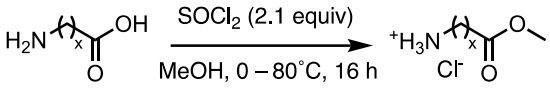
vigorously. After 30 minutes the reaction was frozen in liquid nitrogen and evaporated to dryness before purifying on anion-exchange resin (Q-Sepharose, $NH_4CO_3$ 0.1–1.0 M). Fractions (1 mL) were collected and assayed by Ellman's reagent and those containing the final product combined and freeze-dried to remove water and $NH_4CO_3$.

**$^1$H NMR (400 MHz, $D_2O$)** δ 4.65 – 4.53 (m, 1H), 4.15 – 4.09 (m, 1H), 2.68 (s, 1H), 2.55 (td, *J* = 7.2, 1.3 Hz, 2H), 2.36 (qd, *J* = 7.4, 1.3 Hz, 2H), 1.64 (d, *J* = 7.0 Hz, 2H), 1.61 (d, *J* = 7.2 Hz, 2H), 1.48 – 1.33 (m, 4H), 1.33 – 1.27 (m, 3H).

**$^{13}$C NMR (100 MHz, $D_2O$)** δ 177.1, 176.0, 72.1, 60.8, 35.6, 32.8, 27.7, 27.1, 25.2, 24.9, 23.7, 18.6.

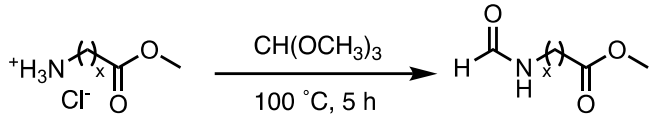
### 3.3.3 Synthesis of methyl-coenzyme M mimics

#### 3.3.3.1 General method A: methyl esters

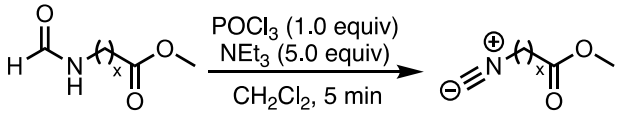



The amino acid (0.105 mol, 1.0 eq), was added to MeOH (50 mL) and the mixture cooled to 0 °C before the dropwise addition of thionyl chloride (16 mL, 0.221 mol, 2.1 eq) [caution: exothermic addition]. The reaction was allowed to warm to room temperature then heated at reflux overnight. The solution was concentrated in vacuo, treated with diethyl ether and the resulting crystals removed by filtration.

#### 3.3.3.2 General method B: Isonitriles




A 250 mL round bottom flask charged with 100 mL of $CH(OMe)_3$ (solvent) and amine 7 (10 mmol) was heated at 110°C for 5 hr. The solvent was removed under reduced pressure to afford the crude product 13 for the next step without further purification.




To the stirred solution of the formamide in dichloromethane (2M) was added triethylamine (5 equiv) at room temperature. Subsequently, phosphorus oxychloride (1.0 equiv) was added dropwise at 0 oC and the reaction mixture was stirred for 5 minutes. After completion of the reaction (indicated by TLC), the crude reaction mixture was loaded directly on a column (15 x 5 cm) packed with silica 100-200 mesh size (140 gm), and a layer of sand (1cm) was put on top of silica gel. Diethyl ether (100ml) was used as the mobile phase and fractions of 25ml were collected. The compound eluted within the first four fractions. Then, the polarity of eluent (25ml) was S6 increased by adding 25, 50, 75 % dichloromethane in diethyl ether. The solvents were evaporated under reduced pressure to afford the pure product.

**methyl 3-isocyanopropanoate**, brown oil, 56% Yield.

**$^1$H NMR (400 MHz, $CDCl_3$)** δ 3.75 (s, 3H), 3.73 – 3.64 (m, 2H), 2.74 (tt, *J* = 6.8, 2.1 Hz, 2H).

**$^{13}$C NMR (101 MHz, $CDCl_3$)** δ 170.0, 157.6 (t, *J* = 5.2 Hz), 52.4, 37.3 (t, *J* = 7.2 Hz), 34.1.

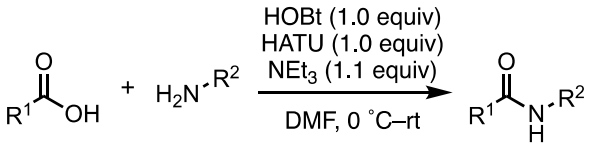
**methyl 4-isocyanobutanoate**, orange oil, 64% yield.

**$^1$H NMR (400 MHz, $CDCl_3$) δ** 3.70 (s, 3H), 3.54 – 3.45 (m, 2H), 2.51 (t, *J* = 7.1 Hz, 2H), 2.05 – 1.95 (m, 2H)

**$^{13}$C NMR (101 MHz, $CDCl_3$) δ** 172.7, 156.95 (t, *J* = 5.6 Hz), 52.0, 156.95 (t, *J* = 5.6 Hz), 30.3, 24.4

### 3.3.4 Synthesis of coenzyme B mimics

#### 3.3.4.1 General Method: Peptide Coupling




The acid (1 mmol) was dissolved in DMF (7 ml) at 0 ˚C and triethylamine (153 µL, 1.1 mmol, 1.1 equiv) was added dropwise. After complete addition HOBt (135 mg, 1.0 mmol, 1 equiv) and HATU (380 mg, 1.0 mmol, 1 equiv) were added. The reaction was stirred for 5 minutes before adding the amine (1 mmol, 1 equiv) in one portion and the reaction was left until completion by TLC. The reaction was poured into water (50 mL) and stirred for 30 minutes. The product was extracted with $CH_2Cl_2$ (50 mL) and washed with water (25 mL), HCl (1 M, 25 mL) brine (25 mL) and dried over sodium sulfate. The organic layer was concentrated to dryness to afford the pure amide. Analogues of Coenzyme B are reported in the literature (Wada et al., 2002. Koreishi et al., 2006. Zorana et al, 2014. Vudhgiri et al., 2018)

### 3.3.5 GC-MS/head-space analysis of methane production/head-space analysis

Methane in headspaces was detected using a Shimadzu GCMS-8040 with a split-splitless injector and a Restek MS-5A plot column (film thickness 20 µm, diameter 0.25 mm, 15 metres, part number: REST-19733) joined to a Rxi-1M trap column (3 metres, part number: REST-13301) using a PressFit Connector (part number: REST-22159). For sample analysis, head space was sampled with a 250 µL gas tight syringe with a tap, 200 µL of headspace was sampled, the tap shut and volume compressed to 100 µL. The column oven was heated to 200 °C; the temperature of the injector port and ion source were 200 °C. The carrier gas was helium with a flow rate of 3.25 mL $min^{-1}$ with a linear velocity of 90 cm $s^{-1}$. The retention time of methane was 2.64 min. Methane calibration was performed according to the literature (Aldridge et al., 2016).

### 3.3.6 GC-MS calibration

A calibration curve was constructed using the optimised method for methane detection by GC-MS. A culture tube was sealed, and the environment evacuated under high vacuum. This was connected to a cylinder of methane (BOC, 99.995% purity) with a pressure relief valve and methane was transferred into the culture tube. This process was repeated four times. Using gas tight syringes with taps accurate volumes of gas (20%–80% of the syringes volume) were sampled and analysed by GC-MS. The analysis was performed in triplicate and the area plotted using GraphPad Prism (Version 9.1.0)

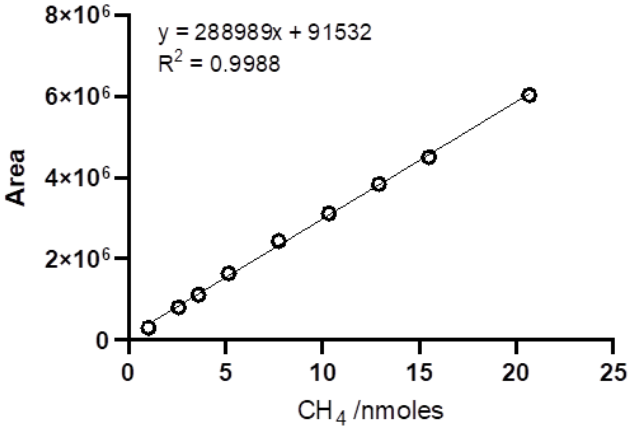
The nmoles of methane in each injection were determined using the expression:

$$n = \frac{P.V}{R.T}$$

Where n= moles, P= pressure (1 atm), V= Volume ($cm^3$), R = universal gas constant (82.057 $cm^3$ atm $K^{-1}$, $mol^{-1}$), T = temperature (K)

Calibration curve for methane by GC-MS.

| Line Statistics | |
|---|---|
| Slope | 288989 |
| Intercept | 91532 |
| Std. Error Slope | 2022 |
| Std Error Y-Intercept | 21822 |
| $R^2$ | 0.9988 |
| LOD | 0.25 nmol |
| LOQ | 0.76 nmol |




## 3.4 *In vitro* MER activity assay

*In vivo* MER assays were carried out following methane formation as indicated on section 3.4.1. Samples analysed consisted of 5 mL culture of either MM incubated at 65 °C for 12 hours or MR incubated at 37 °C for 72 hours (both prepared as indicated on section 3.1.1). Prior to inoculation, the inhibitor of interest was added as a 1,000X stock in DMSO.

## 3.5 *MCRred enzyme* MER activity assay

*MCR red enzyme* assays were carried out following the formation of methane from methyl-coenzyme M and coenzyme B (Bonacker et al., 1993). The anoxic 0.4 mL standard assay mixture in 3 mL serum bottles contained: 50 mM Tris-HCl pH 7.6, 10 mM methyl-CoM, 1 mM CoB, 0.3 mM vitamin B12, 15 mM DTT and 10-40 µL of purified $MCR_{red1}$. The inhibitors of interest (Tables 4.3-4.4) were added as a 100X stock (in 50 mM Tris-HCl pH 7.6, prepared from 1,000X DMSO stock) prior to $MCR_{red1}$ addition. The reaction was started by incubation at 65 °C. After 25 minutes, gas samples were withdrawn and analysed as indicated on section 3.4.1.

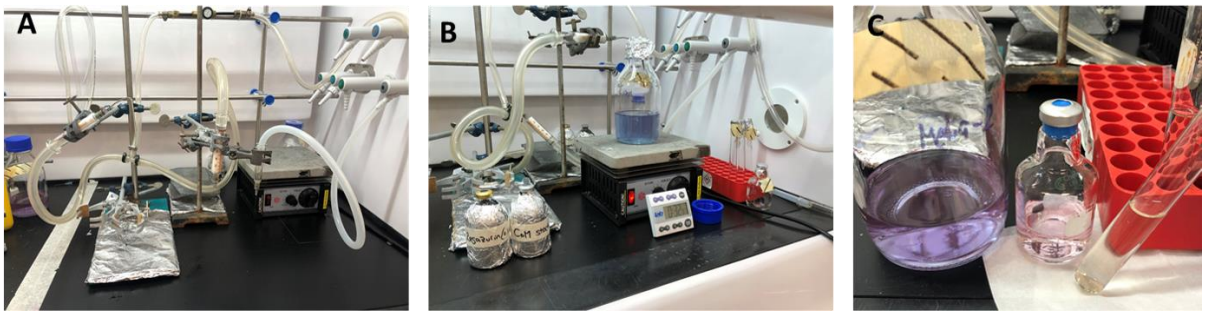
# 4. Results

## 4.1 Cell growth and viability studies at different scales

### 4.1.1 Small scale growth of the two organisms

Cell growth of MM and MR were carried out under strictly anaerobic conditions following general methods for cultivating methanogens (Wolfe 2011). This included the setup of a gassing station to carry out headspace exchange and media preparation (Fig. 4.1) based upon seminal studies by Balch and Hungate (Wolfe et al., 2011. Balch et al., 1979)

**Figure 4.1 Gassing station for headspace exchange and media preparation. A) Station set up. B) Media preparation under anoxic gas. C) Redox indicator resazurin was used to confirm anoxic conditions required for cell culture. Colour changes from blue to pink after boiling under anoxic gas, then colourless after addition of reducing agents L-Cys-HCl and $Na_2S$.**




For both organisms, cell growth was confirmed by multiple parameters, including visual inspection of turbidity (Fig. 4.2), $OD_{600}$ measurement, methane formation by headspace analysis (Fig. 4.3), and fluorescence microscopy (Fig. 4.4). MM presented a faster growth rate, compared with MR. For MM, cell growth was clearly confirmed after 12 hours of incubation at 65 °C (Fig. 4.5), while for MR cell growth was observed after 72 hours at 37 °C. Media composition for MM was also simpler compared to MR. A continuous supply of MM and MR was established at small scale. This was used as the source for further inoculation at large scale, as well as MER assays *in vitro*.

**Figure 4.2 Confirmation of cell growth by visual inspection of turbidity**

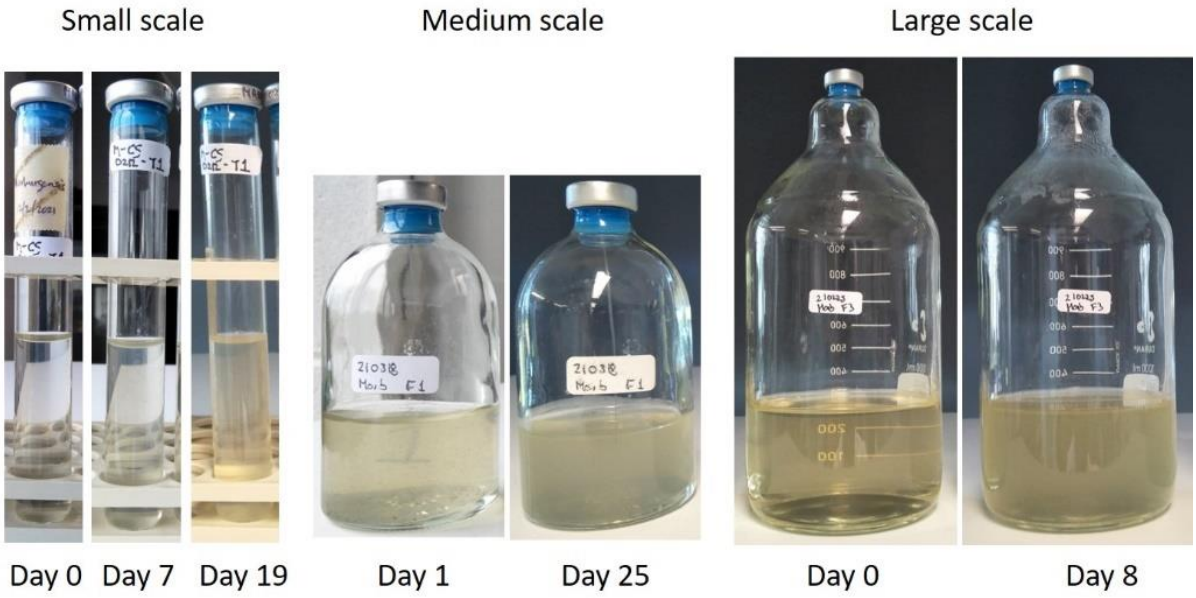
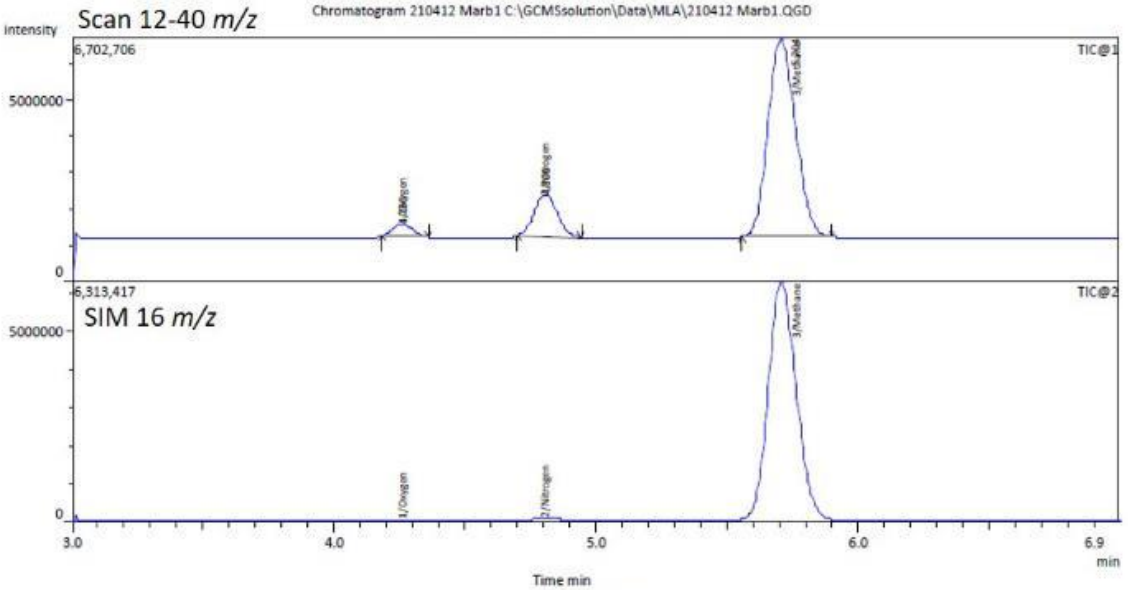



**Figure 4.3 GC-MS chromatogram of headspace sample of an actively growing culture of MM**

Sample Information

| | |
|---|---|
| Sample Name | : 210412 Marb1 |
| Sample ID | : 210412 Marb1 |
| Injection Volume | : 75.00 uL |
| Method File | : C:\GCMSsolution\Data\MLA\210319 140 SIM 40 Split 1 mLmin-1.qgm |
| Org Method File | : C:\GCMSsolution\Data\MLA\210319 140 SIM 40 Split 1 mLmin-1.qgm |
| Tuning File | : C:\GCMSsolution\System\Tune1_default.qgt |




Peak Report TIC

| Peak# | R.Time | I.Time | F.Time | Area | Area% | Name |
|---|---|---|---|---|---|---|
| 1 | 4.260 | 4.183 | 4.359 | 1766022 | 3.58 | Oxygen |
| 2 | 4.806 | 4.700 | 4.942 | 7055500 | 14.32 | Nitrogen |
| 3 | 5.704 | 5.558 | 5.899 | 40461266 | 82.10 | Methane |
| | | | | 49282788 | 100.00 | |

**Figure 4.4 Methanogens cells observed under fluorescent microscope. Fluorescence observed due to F420 cofactor. A) MM B) MR.**

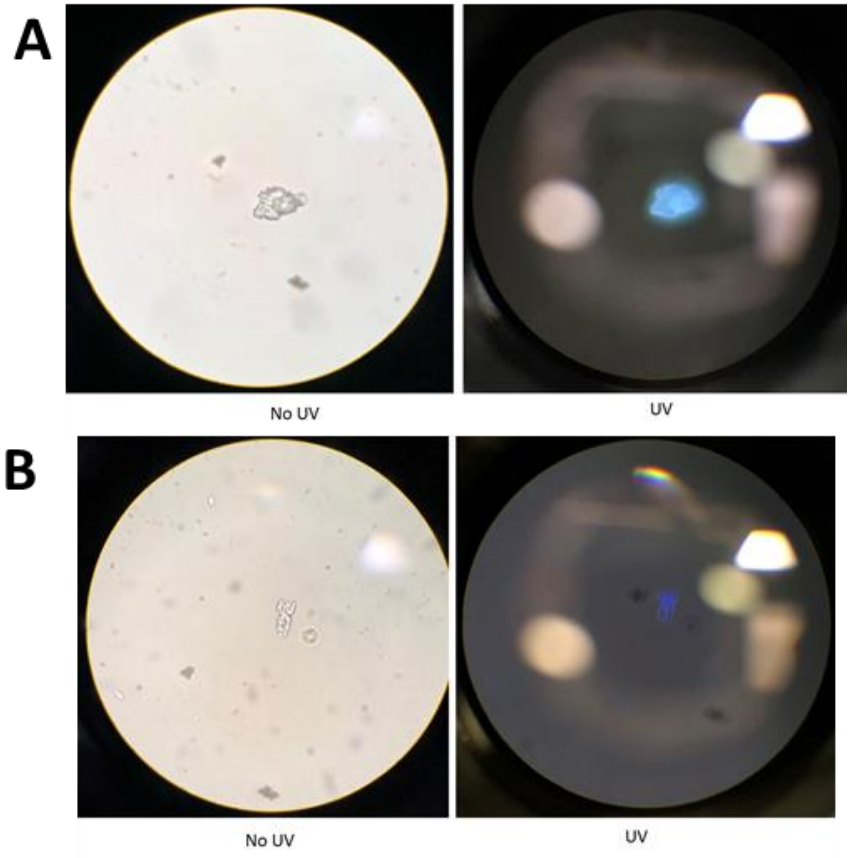
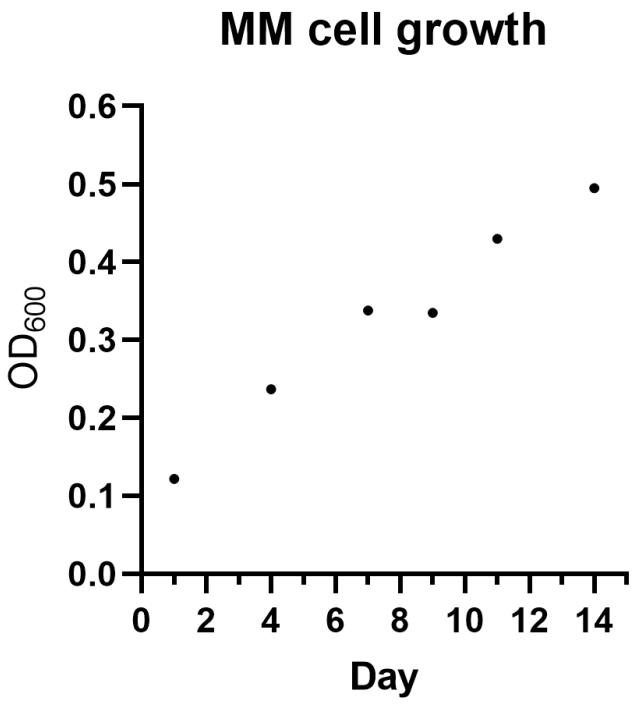



**Figure 4.5 Curve of cell growth for MM determined by $OD_{600}$ measurement.**



### 4.1.2 Large scale growth of the two organisms

Large scale growth of both MM and MR was also established and confirmed by the same parameters as small scale. However, due to the differences in technical demands and growth rate, only MM was cultured continuously at large scale and used for the isolation of $MCR_{red1}$. Current technical capacity allows the growth of 1.6 L of MM culture in seven days.

### 4.1.3 Cell storage and propagation

For long-term storage, cell cultures were diluted 1:1 with 50% glycerol at –80 °C. The addition of the reducing agent cysteine and the redox indicator resazurin allowed the confirmation of strictly anaerobic conditions required for the preservation of these organisms. Under these conditions, cells were confirmed to be viable for further propagation after one year of their preparation.

## 4.2 MCR isolation purification and activation

### 4.2.1 Cell lysis and anaerobic activation

The isolation of $MCR_{red1}$ was performed as previously reported by Duin et al (Duin et al., 2011), with some modifications. The main differences were related to the mode of MCR activation and the scale used for the procedure. An overview of the isolation process is presented in Figure 4.6. When MCR is isolated from *M. marburgensis* directly from its growing conditions (80% $H_2$/20% $CO_2$), the enzyme is mostly in an inactive form (Rospert et al., 1991. Thauer 2019). Activation of MCR to its red1 form has been typically achieved by treatment with 100% hydrogen prior to cell harvesting (Rospert et al, 1991). Here we made use of carbon monoxide, as this provides a faster and more sustained activation of MCR, and, unlike hydrogen, can activate MCR also after cell harvesting (Zhou et al., 2013). To minimize loss of enzymatic activity, CO treatment was performed both before and after cell harvesting. $MCR_{red1}$ is typically isolated starting from at least 10L cultures and harvest in a flow-through process (Duin et al., 2011). Here we made use of a smaller scale, starting with 1.6 L culture harvest in batch. Strictly anaerobic conditions were confirmed using the redox indicator resazurin in both the cell harvesting and cell lysis step (Fig. 4.7). Carbon monoxide treatment was effective in the activation of MCR to its red1 form, as methane formation was confirmed in the clarified cell lysate in *enzyme* assays. A typical procedure starting with 1.6 L of MM cell culture afforded 7-8 mL of clarified cell lysate with a protein concentration 2.5–2.8 mg/mL.

**Figure 4.6 Overview of the process for isolation of $MCR_{red1}$.**

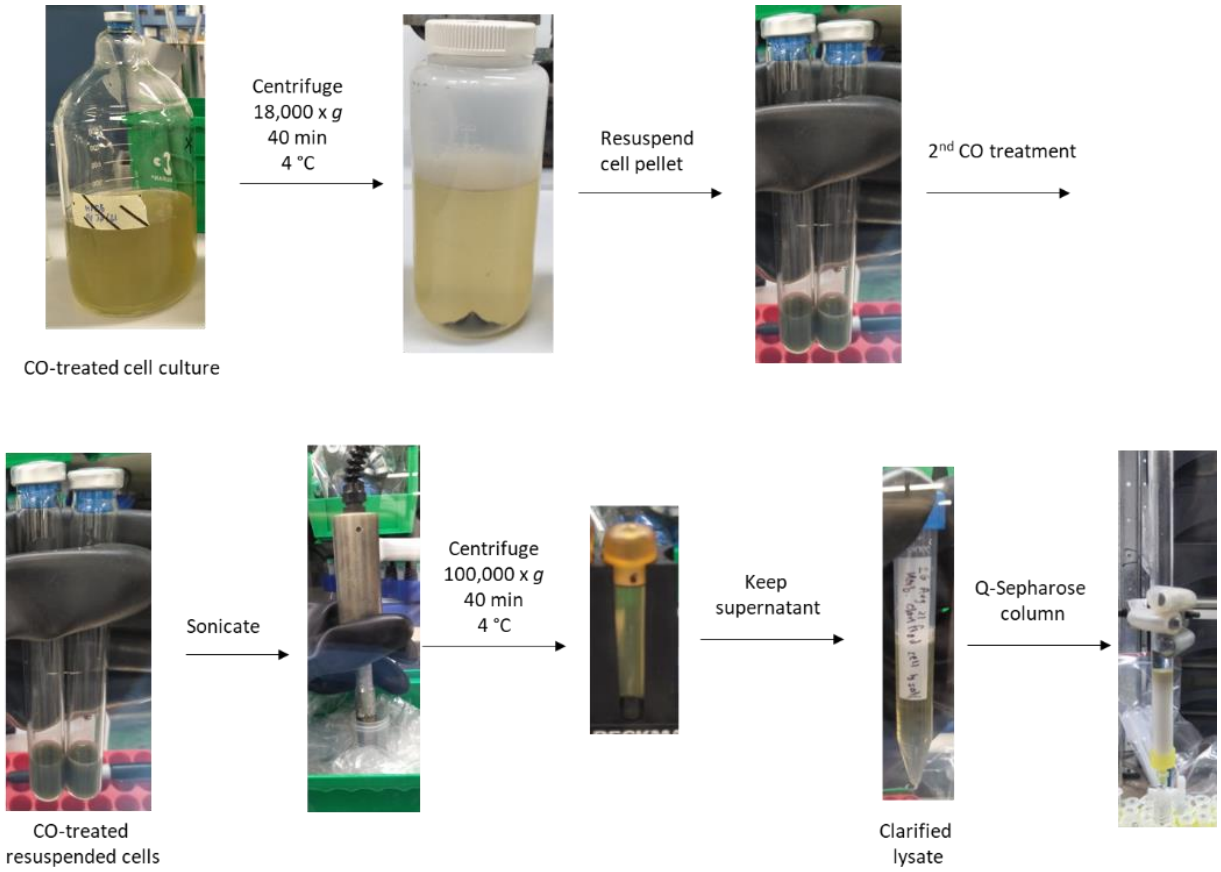
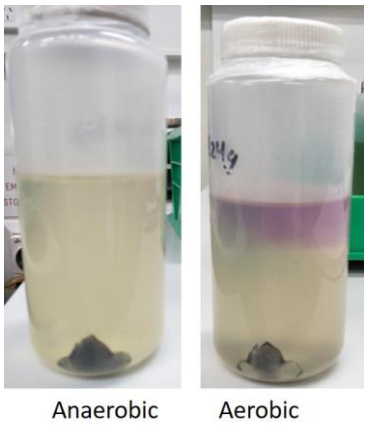


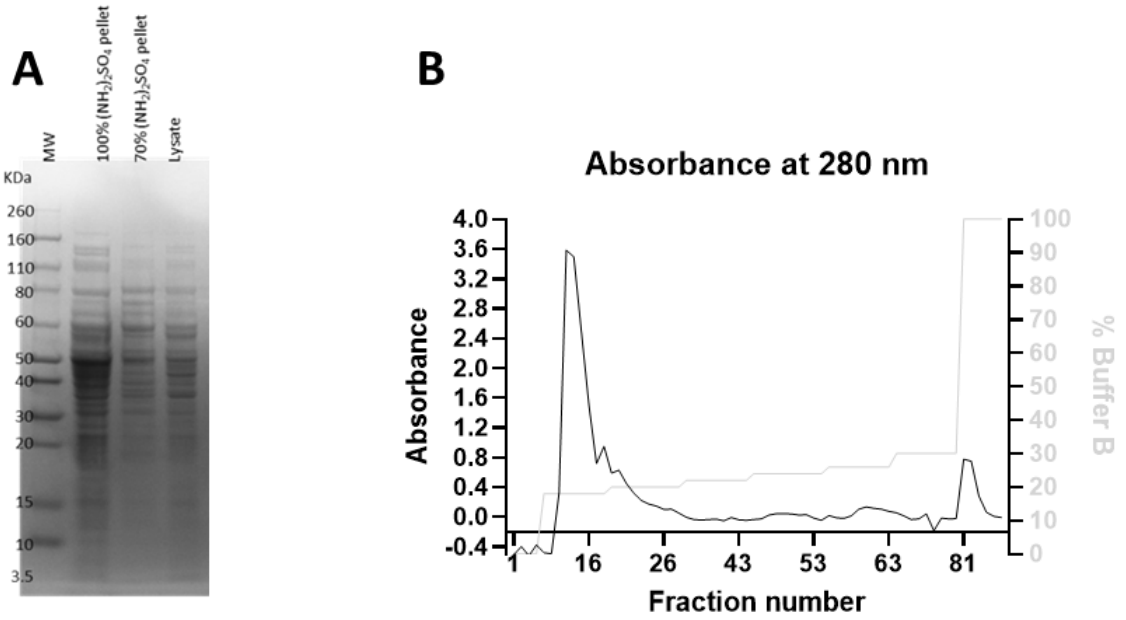

**Figure 4.7 Example of cell harvesting step under aerobic and anaerobic conditions. Resazurin was used as redox indicator (pink colour indicates presence of oxygen).**




### 4.2.2 IEC results

Two major differences were observed in the isolation of $MCR_{red1}$ compared with the reported literature after the biomass extraction process (Duin et al., 2011). First, the ammonium sulfate precipitation step prior to ion exchange column chromatography was omitted, as under our tested conditions this step was not effective in the purification of MCR as confirmed by SDS-PAGE analysis (Fig. 4.8). Second, a manually packed Q-Sepharose column with gravity flow was used instead of prepacked columns for FPLC. Anoxic conditions of the buffers system and the column were confirmed with resazurin as redox indicator (prior to MCR isolation).

**Figure 4.8 Attempts to $MCR_{red1}$ isolation starting with ammonium sulfate precipitation step. A) SDS-PAGE analysis of cell lysate and pellets obtained by precipitation at 70% and 100%. B) Chromatogram for Q-Sepharose column of 100% pellet resolubilized.**




### 4.2.3 Protein fractionation and quantification

Fractions corresponding to MCR were identified by UV-Visible analysis at 280 nm (total protein) and 420 nm (specific for MCR cofactor 430 exposed to oxygen). Monitoring at 420 nm facilitated the identification of MCR, as the corresponding peak observed at 280 nm can be missed as background (Fig. 4.9). MCR typically eluted at a concentration of 0.48 M NaCl (24% buffer B) in a columned used for the first time. However, despite regenerating Q-Sepharose column after every purification, MCR could elute at lower concentrations around 0.44 M NaCl (22% buffer B) after a couple of times. Thus, the same Q-Sepharose column was reused for a maximum of five times. The identity of $MCR_{red1}$ was confirmed by *enzyme* assay, with methane formation *enzyme* confirmed for only this peak/fraction. In a typical procedure starting from 1.6 L of MM, isolated $MCR_{red1}$ was recovered as 100 µL of a 20 µg/µL solution approximately. An 18-fold enrichment was observed for $MCR_{red1}$ with respect to the initial clarified lysate, which was consistent with the 15-fold value reported in the literature (Ellermann et al., 1988).

**Figure 4.9 Example of a typical chromatogram for Q-Sepharose column of clarified lysate of MM. A) Absorbance at 280 nm. B) Absorbance at 420 nm.**

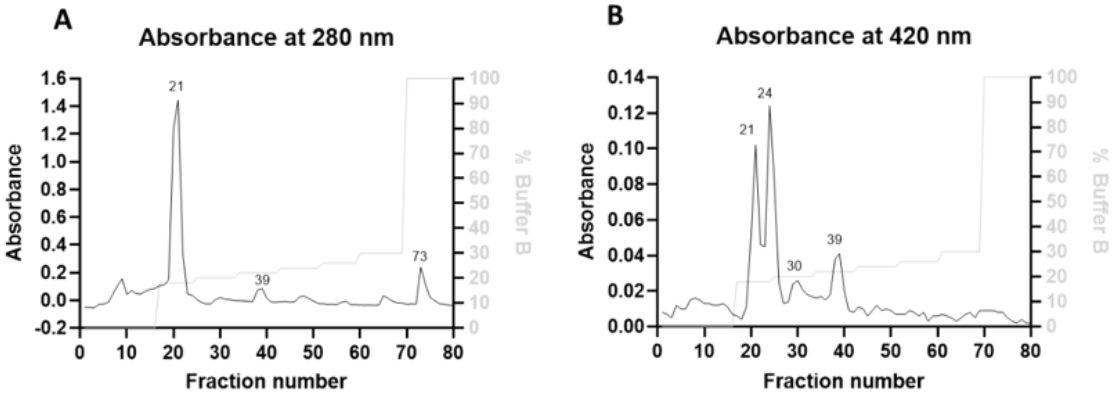


**Table 4.1 Elution of $MCR_{red1}$ from Q-Sepharose column used in consecutive times (column regenerated in between runs).**

| *Column number* | *Concentration of NaCl (M) for $MCR_{red1}$ elution* | *Fraction number in which $MCR_{red1}$ is observed.* |
|---|---|---|
| 1 | 0.52 | 61 |
| 2 | 0.48 | 57 |
| 3 | 0.48 | 56 |
| 4 | 0.44 | 40 |
| 5 | 0.44 | 35 |

### 4.2.4 Protein gel and sequence results to confirm—it is MCR?

The identity of $MCR_{red1}$ was further confirmed by SDS-PAGE analysis. Bands of approximately 60, 50 and 35 KDa were observed for the MCR fraction (Fig. 4.10), in accordance with the literature reports (Bonacker et al., 1993). These results were consistent among different batches of cell cultures, Q-Sepharose beads and buffers, confirming the reproducibility of the isolation process. MCR was also confirmed at the peptide level by mass spectrometry in SDS-PAGE excised gel bands (Table 4.2). Detailed information for this peptide identification is reported in appendix 8.1.

**Figure 4.10 SDS-PAGE of fractions from Q-Sepharose column shown in Figure 4.8**

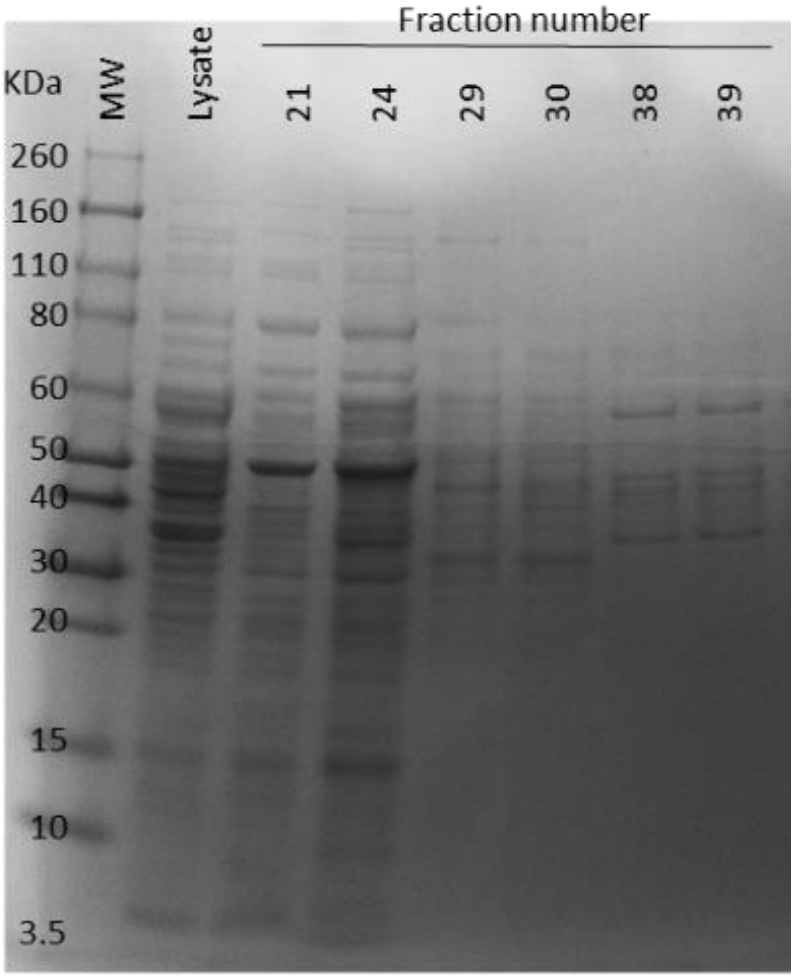


**Table 4.2 Selected peptide/proteins identified by LC-MS/MS from bands excised from SDS-PAGE gel**

| BAND | PROTEIN IDENTIFIED | PROTEIN SCORE | PROTEIN MATCHES | PROTEINS IDENTIFIED WITH HIGHER SCORE |
|---|---|---|---|---|
| 60 KDa | Methyl-coenzyme M reductase II subunit beta | 383 | 20 | 0 |
| 60 KDa | Type A flavoprotein FprA | 138 | 13 | 1 |
| 50 kDa | Type A flavoprotein FprA | 1177 | 83 | 0 |
| 50 kDa | Fructose-1,6-bisphosphate aldolase/phosphatase | 920 | 60 | 1 |
| 35 kDa | Tetrahydromethanopterin S-methyltransferase subunit H | 1109 | 49 | 0 |
| 35 kDa | Methyl-coenzyme M reductase I subunit gamma | 31 | 2 | 9 |

## 4.3 Synthesis of enzyme substrates and compounds

### 4.3.1 Synthesis of methyl CoM and CoB with improved scales

The synthesis of methyl-coenzyme M (Scheller et al., 2010) and coenzyme B (Noll et al., 1987) was successfully completed under strictly anoxic conditions following the methods reported in the literature. Detailed synthesis of methyl CoM is outlined in Section 3.3.1.1 and CoB is outline in Seciton 3.3.1.2. Both substrates were performed at a scale of approximately 300 mg. At least two different batches of substrates were successfully tested in *enzyme* assays, confirming the reproducibility of this method. Thoroughly scrubbed ultra-high purity argon was used for the synthesis of coenzymes to limit the oxidation side reaction.

Increasing the scale of the synthesis of methyl CoM required rigorous control of the reaction conditions to prevent the oxidation of the thioether that is difficult to remove without significant loss of methyl CoM. Extensive degassing of methanol and careful control of the sodium methoxide stoichiometry minimised oxidation of the thioether. The reaction was initiated at 0 ˚C to prevent a significant exotherm whilst adding sodium methoxide to CoM sodium salt. These processes contributed to an isolated analytically pure methyl CoM in an 85% yield.

To increase the scale of CoB, extensive purification of solvents and close monitoring of reaction times were required. Lengthened reactions times were required for larger scale syntheses of CoB with thoroughly degassed solvents essential for conversion. The pH of the reduction was carefully controlled to facilitate facile reduction of the disulfide. Further purification of CoB could be achieved by careful precipitation from thoroughly degassed methanol/diethyl ether and filtration under strictly anaerobic conditions.

### 4.3.2 Synthesis of methyl-coenzyme M/CoB mimics

Facile synthetic routes to the desired motifs have been developed that are scalable and robust, details regarding the synthesis of analogues can be seen in Section 3.3.3, enabling structural

analogues to be synthesised. The design philosophy was to look at the structure of compounds found in *Asparagopsis* and other known inhibitors (e.g., 3-NOP and 2-BES) and design non-halogenated analogues. The first round of inhibitors focussed on 3-bromopropenoic acid; a prominent compound found in *Asparagopsis taxiformis*. We envisaged that the carboxylic acid could mimic the sulfate of coenzyme M, and the bromo the *S*-methyl. We thus first made the methyl ester to facilitate passive membrane transport. To replace the bromine and look at the effect of different chain lengths we made another 9 compounds. Three mimics of CoB were tested. Testing of these 12 inhibitors is reported in section 4.7

## 4.4 Methane production by MCR *enzyme* and *in vitro assays*

### 4.4.1 *In vitro* assay

The methodology for methane detection by GC-MS was first tested with a methane standard to optimize conditions of analysis. These conditions were then further refined for the detection of methane in actively growing cultures of both MM and MR as indicated in section 3.5. In a typical analysis, the methane signal for a blank sample used as 100% (0% inhibition) reported as area under the curve was $7\times10^6$ for MM and $2\times10^5$ for MR.

### 4.4.2 Enzymatic assay using active MCR

*In vitro* assays were carried out under identical conditions to *in vitro* assay, with the only difference being the sample source. Samples were prepared according to literature procedures as indicated in section 3.6. In every batch of isolated $MCR_{red1}$, the corresponding cell lysate of origin was first tested as a reference to confirm the activate state and enrichment of MCR in its active form. In a typical procedure, 240 µL (600 µg) of clarified cell lysate produced a methane signal of $4\times10^4$ (area under the curve), while 24 µL (480 µg) of purified $MCR_{red1}$ produced a methane signal of $1\times10^5$.

## 4.5 $IC_{50}$ of positive controls 2-BES/3-NOP against MCR *enzyme* and *in vitro*

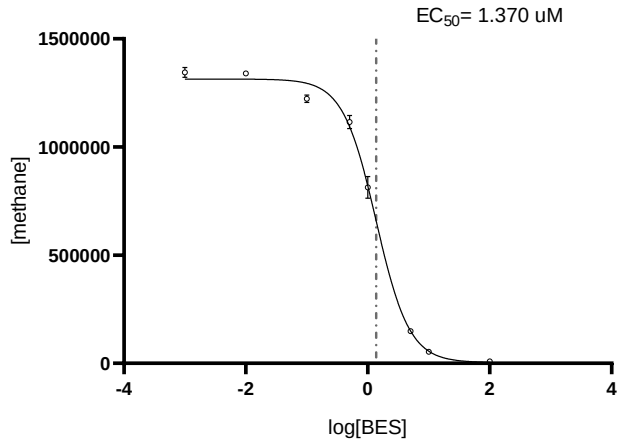
### 4.5.1 2-BES results

2-Bromoethanesulfonate (2-BES), a coenzyme M analogue, is one of the first specific inhibitors of MCR reported in the literature (Ellermann et al., 1989). The negative charged of 2-BES prevents its free diffusion through the cytoplasmic membrane of methanogens, making it a poor inhibitor of methanogenesis *in vitro* (Ungerfeld et al., 2004). An exception to this poor performance is MR. This organism requires coenzyme M as a vitamin and contains a coenzyme M transporter, which allows 2-BES to be carried across the cell membrane (Ungerfeld et al., 2004).

Consistent with this fact, at a concentration of 5 µM, 2-BES completely inhibited methane formation and cell growth *in vitro* in MR under our experimental conditions. With this *in vitro* assay in MR, an $IC_{50}$ value of 1.37 µM was determined (Fig. 4.11), closely matching the 0.8 µM value reported in the literature (Gräwert et al., 2014).

At a concentration of 5 µM 2-BES had no inhibitory effect in MM *in vitro* (Fig. 4.11), but at 10 µM *enzyme* produce 40% inhibition in methane formation (from $MCR_{red1}$ isolated from MM). Reported $IC_{50}$ values for $MCR_{red1}$ *enzyme* are 4 µM (isolated from MM, Duin et al., 2016) and 0.4 µM (isolated from MR, Gräwert et al., 2014). In the future, $MCR_{red1}$ isolated from MR could be also considered for *enzyme* assays.

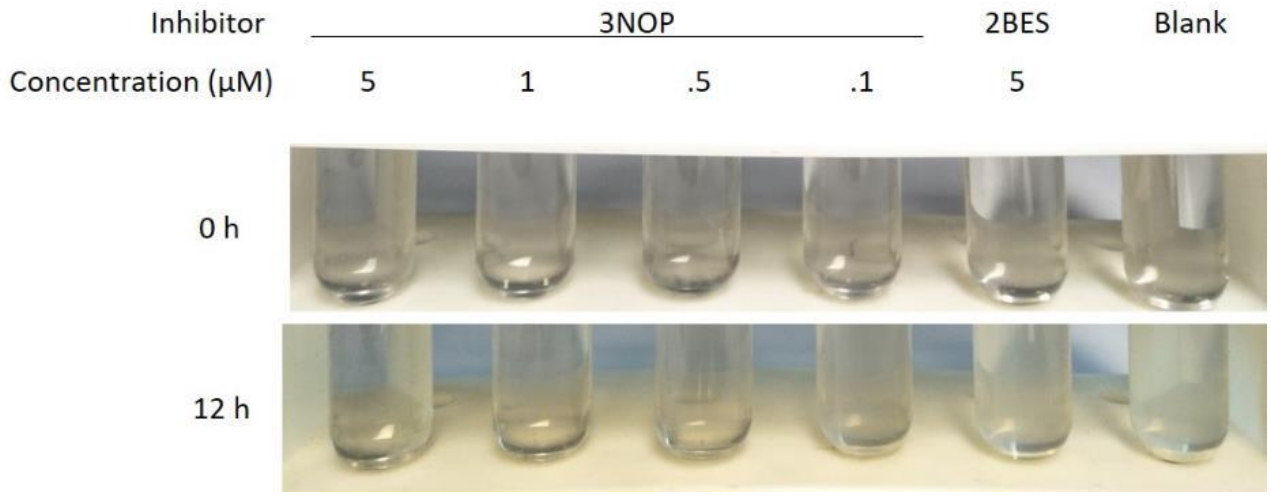
**Figure 4.11 Determination of $IC_{50}$ value for 2-BES *in vitro* (MR)**




### 4.5.2 3-NOP confirmation

3-nitrooxypropanol (3-NOP) is a small molecule recently reported as a specific MCR inhibitor (Duin et al., 2016). At a concentration of 5 µM, 3-NOP completely inhibited methane formation and cell growth *in vitro* in MM under our experimental conditions (Fig. 4.12). This is consistent with the literature, in which the same results are observed at a concentration of 10 µM in MM (Duin et al., 2016). An $IC_{50}$ of approximately 0.5 µM was calculated for 3-NOP *in vitro*. To the best of our knowledge, and $IC_{50}$ value for 3-NOP has not been previously reported. The results observed *in vitro* were also replicated in *enzyme* assays, with a methane inhibition of 87% observed at 10 µM concentration, and 12% at 1 µM concentration.

**Figure 4.12 Testing of known inhibitors 3-NOP and 2-BES *in vitro* in MM cells.**



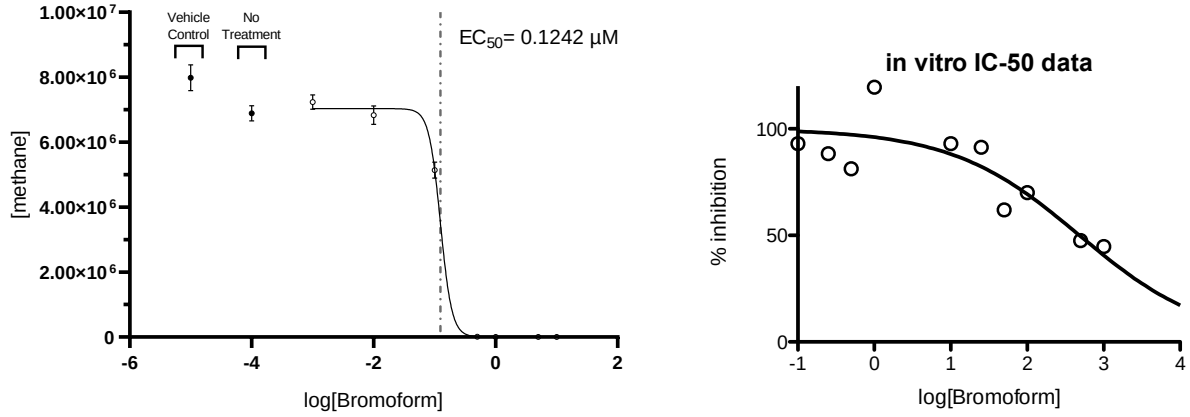

Together with the results obtained from known inhibitors 2-BES and 3-NOP demonstrated the utility of this method for screening other methane inhibitors.

## 4.6 IC50 of bromoform against MCR *enzyme* and *in vitro*

### 4.6.1 Results of bromoform *in vitro*

Bromoform is another compound known to inhibit methanogenesis (Honan et al., 2021. Roque et al., 2021). To the best of our knowledge, specific inhibition of MCR by bromoform, or $IC_{50}$ determination either *in vitro* or *enzyme* has not been previously reported. *In vivo* assays with MM provided an $IC_{50}$ of 0.12 µM for bromoform (Fig. 4.13). This value was lower than those observed for 3-NOP and 2-BES under similar conditions.

**Figure 4.13 Determination of *in vitro* and *enzyme* (MM) $IC_{50}$ value for bromoform.**



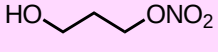
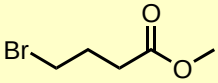
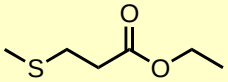

### 4.6.2 Results of bromoform *enzyme* against MCR

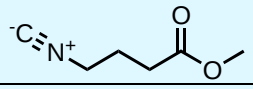
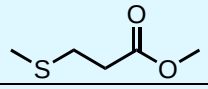
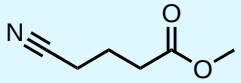
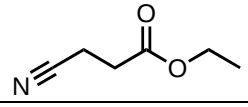
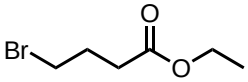
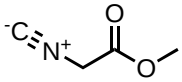
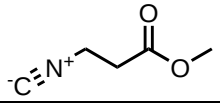
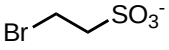
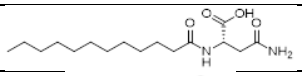
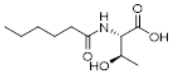
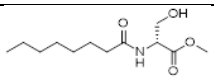
In contrast with the results observed *in vitro*, *enzyme* assays with bromoform showed no significant inhibition of methane formation at concentrations below 10 µM. Even at high concentrations of 500 µm and 1 mM, only 50% of inhibition was observed (Fig. 4.12). This suggests a number of possibilities: 1) that bromoform does not inhibit MCR; 2) that bromoform is actively transported into MM cells to produce a low apparent IC50 or that 3) bromoform inhibits a subset of MCR enzymes.

## 4.7 Testing of CoM and CoB mimics *in vitro*

After confirming the utility of *in vitro* assays with known methanogenesis inhibitors, several methyl-CoM and CoB mimics (both synthesized and commercially available) were tested under the same conditions (**Table 4.3**). Only two of the carboxylic acid esters tested resulted in a significant inhibition of methane production (**Table 4.3**, entries 2-3). In general, the *in vitro* testing allowed a rapid screening of inhibitors under near physiological conditions.

**Table 4.3. Percentage of methane inhibition by different small molecules in live MM cells.**

| Entry | Structure | Internal name | Reduction in methane[a] (%) |
|---|---|---|---|
| 1 |  | 3-NOP (control) | 99.9 ± 5.0 |
| 2 |  | 3-BrCO2Me | 14.4 ± 0.2 |
| 3 |  | 3-MeSCO2Et | 11.3 ± 0.2 |

| | | | |
|---|---|---|---|
| 4 |  | 3-NCCO2Me | 8.6 ± 0.1 |
| 5 |  | 3-MeSCO2Me | 8.5 ± 0.0 |
| 6 |  | 3-CNCO2Me | 6.5 ± 0.1 |
| 7 |  | 2-CNCO2Et | 0.0 ± 0.1 |
| 8 |  | 3-BrCO2Et | 0.0 ± 0.0 |
| 9 |  | 1-NCCO2Me | 0.0 ± 0.2 |
| 10 |  | 2-NCCO2Me | 0.0 ± 0.1 |
| 11 |  | 2-BES | 0.0 ± 0.00 |
| 12 |  | | 0.0 ± 0.0 |
| 13 |  | | 0.0 ± 0.0 |
| 14 |  | | 0.0 ± 0.0 |

## 4.8 Testing of halogenated compounds *in vitro*

Besides bromoform, other halogenated methane analogues are known to reduce methane formation (Roque et al., 2021). A few of these compounds, all of which occur in *Asparagopsis*, were tested *in vitro* in MM. At a concentration of 5 µM, all but two fully inhibited methane formation (Table 4.4).

**Table 4.4 *In vivo* testing of halogenated methane analogues in MM**

| Entry | Compound name | Concentration | Methane inhibition |
|---|---|---|---|
| 1 | Dibromomethane | 5 µM | 100% |
| 2 | Bromoiodomethane | 5 µM | 100% |
| 3 | Dibromochloromethane | 5 µM | 100% |
| 4 | Iodoform | 5 µM | 100% |
| 5 | Tetrabromomethane | 5 µM | 100% |
| 6 | 1,1,3,3-tetrabromoacetone | 5 µM | 25% |
| 7 | 1,3-dichloroacetone | 5 µM | 30% |

# 5. Conclusion

As stated earlier, the purpose of the project is to learn from Nature, by understanding how naturally halogenated alkanes inhibit enteric methanogenesis, and use that learning to target specific biosynthetic steps in the methanogenesis pathway in order to find novel compounds from a rational, structure-guided approach for economical and reliable MER. A cost-effective, safe to animals and safe for humans' solution to mitigating enteric methane production for the livestock sectors will help the industry enter into a carbon-neutral future with long-term sustainability. In this first stage important milestones have been met such as growing methanogens, establishing assays that specifically target key biosynthetic enzymes, testing preliminary lead compounds, and optimizing conditions for screening.

With the isolation of MCR in its active state, and the establishment of methanogenesis inhibition assays both in live cells and enzyme, only the testing of more compounds remains to be completed. For stage 2, the project will progress toward ultimately developing an efficacious feed additive through targeted pathways where the mechanism of action is well understood.

## 5.1 Key findings

- MM and MR culture established at small/medium/large scales;
- Robust activation protocols optimised;
- MCR was isolated in its active red 1 form as confirmed by ion-exchange chromatogram with SDS-PAGE and methane detection by GC in the corresponding enzyme assay;
- The synthesis and purification of coenzyme B was completed at close to gram-scale;
- MCR inhibition assay enzyme was developed and used to test known MCR inhibitors;
- In vitro assay established for screening more seaweed compounds and other inhibitors; and
- No new MER inhibitors better than 3-NOP or bromoform were discovered in part 1 of this project.

## 5.2 Benefits to industry

The establishment of the enzyme and in vitro assays using two important methanogens now sets the stage for finding and developing novel feed additives, by mimicking nature, that will have reliable and sustainable MER effects. This will increase the productivity of the Australian Red Meat industry with concurrent reduction of the environment impact due to methane emission in order to meet the CN30 targets.

# 6. Future research and recommendations

This stage has overcome a significant challenge of isolating and purifying the MCR enzyme in its active form for establishing an enzymatic assay. Given the extremely sensitive and unstable nature of the enzyme, the establishment of this enzyme assay enzyme firmly secures the fundamental groundwork toward future development on targeted feed additives with defined MOA against MCR.

In addition, in vitro screening assays have also been established for testing compounds against two methanogens: *Methanothermobacter marburgensis* (MM) and *Methanobrevibacter ruminantium* (MR). Direct measurement of methane production in the assay ensures that this key capability with enough throughput and reliability will benefit compound testing in general for the industry.

Initial testing of halogenated compounds from *Asparagopsis* seaweeds and a few mimics point to the relevance of MCR inhibition by some of the seaweed components. It also reveals that MCR inhibition is responsible for part of the effect but not all. This points to exciting new opportunities of finding and developing new modes of action for the seaweed compounds and their mimics.

With the groundwork established, recommendations are made for further stages of efforts:

1. Further optimise the MCR enzyme assay to include MR-MCR with higher throughput
2. Continue to screen in vitro, using MM and MR organisms, compounds from *Asparagopsis* seaweeds and validate the mode of action for high activity leads with the enzyme MCR assay
3. Rationally design and synthesise, using a structure-guided approach, mimics of the most active, naturally occurring compounds (e.g., compounds of 2-4 carbon units with polar groups at the ends) for testing in the established enzyme/in vitro pipeline against the MCR from MM, and more appropriately, MR
4. Use the pipeline to help find targeted leads from other sources of compounds
5. Use the pipeline to find other targetable aspects of methanogen biosynthesis
6. Investigate combination targeting strategies to enhance MER effects and reduce costs
7. Develop field-worthy applications of the most active and targeted mimics with defined MOA
8. Validate the effects of leads in the field against emission targets
9. Investigate synergistic effects of the targeted additive with other types of feed additives
10. Develop commercial viability of targeted feed additives either as singular agents or in combination with other agents or feed additives

In summary, a targeted approach to novel feed additives represents a new and valuable addition to existing strategies to sustainable feed-based innovation, given the recently established commercial path for a targeted feed additive for MER. Further development and adoption of this approach will be critical to maintaining the competitiveness of the Australian Red Meat industry and securing the long-term sustainability by meeting the emission reduction requirements within the CN30 framework.

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*Refer to the document instructions for completing this section.*

# 8. Appendix

## 8.1 Mass spectroscopy characterization of putative methyl-coenzyme M reductase subunits

### 8.1.1 Sample details

**Table X**: List of samples with APAF sample codes

| Sample Name | APAF Sample Code |
|---|---|
| MLA 35 | S0012653 |
| MLA 50 | S0012654 |
| MLA 60 | S0012655 |

Samples were received at room temperature and stored in the APAF-MS 4 °C fridge (FRE009). Samples consisted of Coomassie stained SDS-PAGE gel pieces in Milli Q water

### 8.1.2 Method details

Samples were analysed in accordance with APAF SOPs MS-057 (Reduction, alkylation and digestion for solid/liquid Samples), and MS-070 (QExactive Data Acquisition v1). Experimental details are briefly described below.

#### 8.1.2.1 Sample preparation

Gel pieces were destained in 300 µL ammonium bicarbonate (25 mM) in 50% (v/v) acetonitrile. After discarding the destaining buffer, proteins were sequentially reduced and alkylated using dithiothreitol (10 mM) and iodoacetamide (20 mM) respectively in ammonium bicarbonate solutions (50 mM). Reactions were performed at 37°C in the dark for 60 min each. Upon discarding the alkylation solution, 900 µL ammonium bicarbonate (50 mM) followed by 1 ng trypsin was added, and samples incubated overnight at 37°C. To extract and pool peptides, each digest solution was removed to a new microfuge tube and the gel slices treated with the following solutions sequentially for 30 min each: 150 µL 0.1% (v/v) formic acid/67% (v/v) acetonitrile; and 150 µL 100% acetonitrile. The pooled digest and peptide extraction solutions for each sample were then dried in a SpeedVac before resuspending in 200 µL of 0.1% (v/v) formic acid.

#### 8.1.2.2 Mass spectrometry

Instrument set-up:

Mass Spectrometer: Q-Exactive (Thermo Fisher Scientific)

NanoLC system: Easy nLC-1000 (Thermo Fisher Scientific)

Peptide trap column: Halo-C18, 160Å, 2.7 µm, 100 µm x 3.5 cm

Nano-LC column: Halo-C18, 160Å, 2.7 µm, 75 µm x 15 cm

Loading buffer: 99.9% water, 0.1% formic acid

Mobile phase A: 99.9% water, 0.1% formic acid

Mobile phase B: 99.9% acetonitrile, 0.1% formic acid

Data Dependent Acquisition (DDA) LC-MS/MS:

Peptide samples were injected (6 µL) onto the peptide trap column and washed with loading buffer for 10 minutes. The peptide trap was then switched in line with the analytical nano-LC column. Peptides were eluted from the trap onto the nano-LC column and separated with a linear gradient of 1% mobile phase B to 50% mobile phase B over 60 min at a flow rate of 300 nL/min, then held at 85% mobile phase B for 8 minutes prior to re-equilibration.

The column eluent was directed into the ionization source of the mass spectrometer operating in positive ion mode. Peptide precursors from 350 to 2,000 m/z were scanned at 35k resolution. The 10 most intense ions in the survey scan were fragmented by HCD using a normalized collision energy of 30 with a precursor isolation width of 3.0 m/z. Precursors with unassigned charge states or charge state +1 were excluded from MS/MS analysis. The MS method had a minimum signal requirement value of $2.5\times10^4$ for MS2 triggering, an AGC target value of $1\times10^6$, and a maximum ion injection time of 120 ms. MS2 scan resolution was set at 17.5k, an AGC target value of $2\times10^5$, and a maximum injection time of 60 ms. Dynamic exclusion was set to 10 seconds.

Sequence Database Searches

Sequence database searches were performed using the search program Mascot (version 2.4.1, Matrix Science). Peak lists derived from LC-MS/MS were searched against the SwissProt database (January 2021 release, 564,277 sequence entries) using the following parameters:

Enzyme Trypsin

Maximum Missed Cleavages 2

Fixed modifications None

Variable modifications Carbamidomethyl (C), Oxidation (M)

Peptide Mass Tolerance 5 ppm

Fragment Mass Tolerance 0.02 Da

Instrument type Default

Searches were performed using sequences from all taxonomies in the SwissProt database.

#### 8.1.2.3 Results

Result summaries can be found in the 210414_P31903_MLA35_summary.xlsx, 210414_P31903_MLA50_summary.xlsx and 210414_P31903_MLA60_summary.xlsx spreadsheets. Each spreadsheet summarises the results for one of the samples (MLA35, MLA50 and MLA60 respectively). There are three workbooks in each spreadsheet: i) a summary of the search parameters; ii) a summary of the proteins identified; and iii) a summary of the individual peptides identified for each protein.

Protein hits associated with methyl-coenzyme M reductase subunits are highlighted in yellow in the 'protein hits' worksheets. The MLA35 sample contained the gamma subunits of both methyl-coenzyme M reductase I and II. The MLA50 sample contained both beta and gamma subunits of methyl-coenzyme M reductase II; however peptide counts were very low, indicating that these

proteins were of very low abundance in this sample. The MLA60 sample contained beta subunits of both methyl-coenzyme M reductase I and II, and alpha subunits of methyl-coenzyme M reductase II.

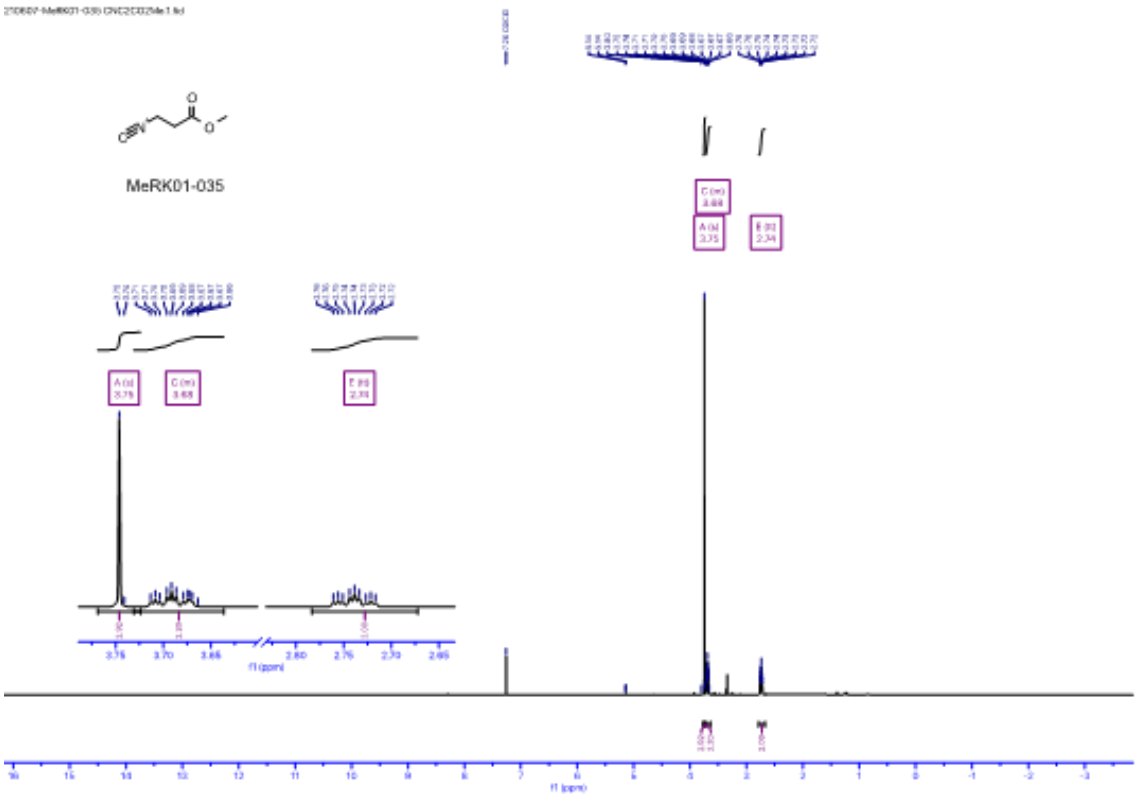
Please see Opinions and Interpretations for additional information

#### 8.1.2.4 Opinions and interpretations

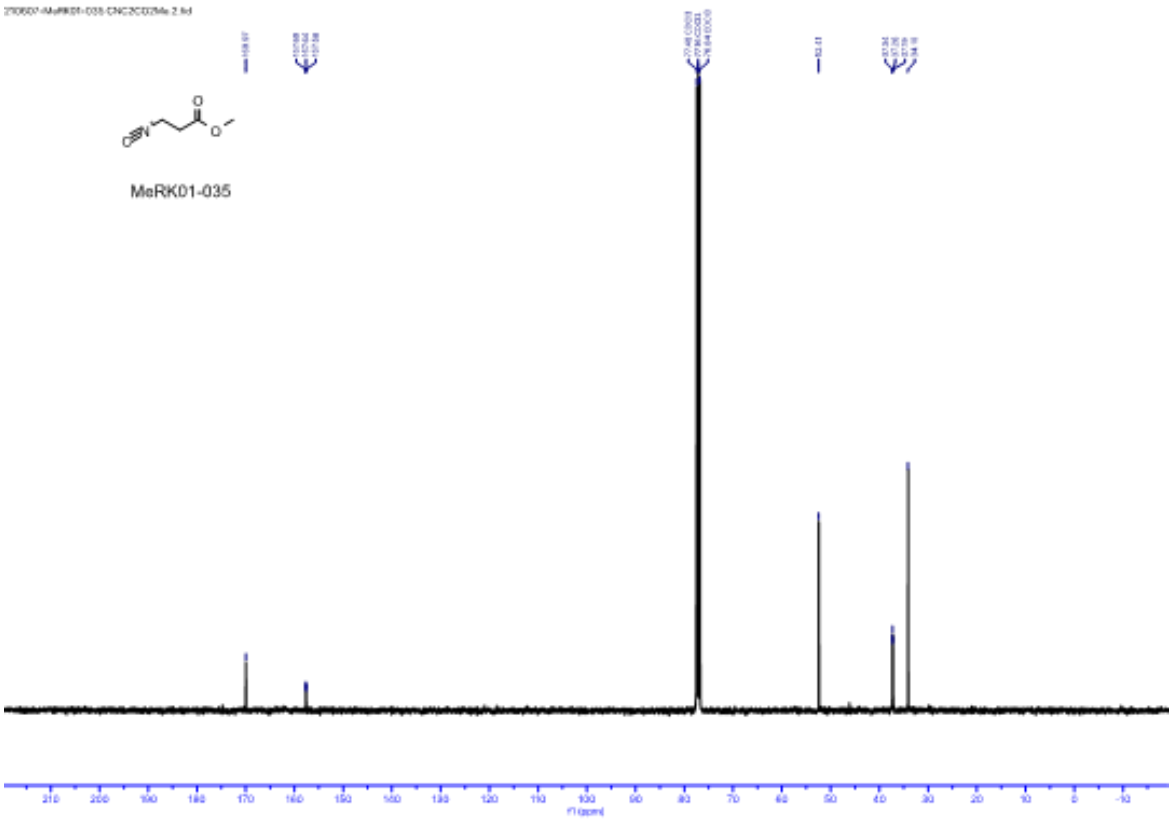
The searches were performed against all taxonomies in the SwissProt database. This means that the searches considered *Methanothermobacter marburgensis, Methanobacterium thermoautotrophicum* and other archaea. The hits for methyl-coenzyme M reductase proteins from different archaea can likely be explained by sequence homology, and when sequences are not unique to a particular protein, it is possible for the sequence database search engine to assign the protein identification to the wrong archaeon. The 'peptide hits' worksheets can help tease out the details of these protein identifications; for example they can reveal whether peptides that can distinguish Methanothermobacter marburgensis from Methanobacterium thermoautotrophicum have been identified.

(Opinion issued by Dr Gene Hart-Smith, Technology Manager, 18th May 2021.)

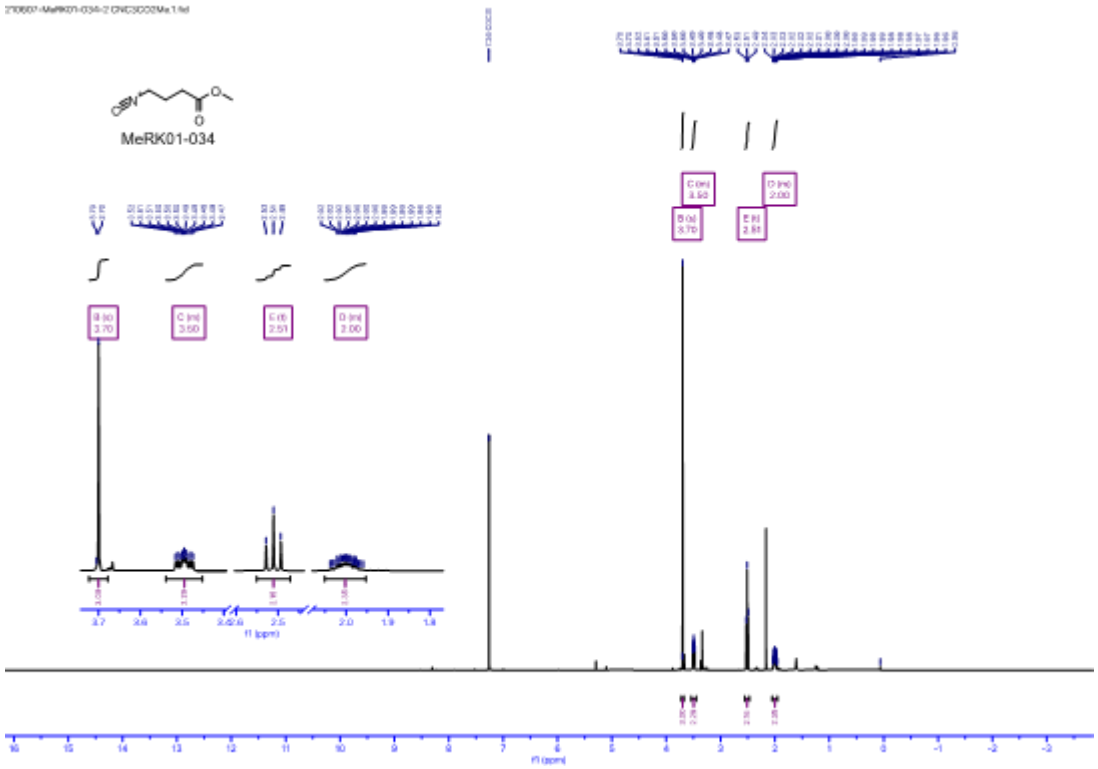
## 8.2 NMR spectra of synthesised compounds



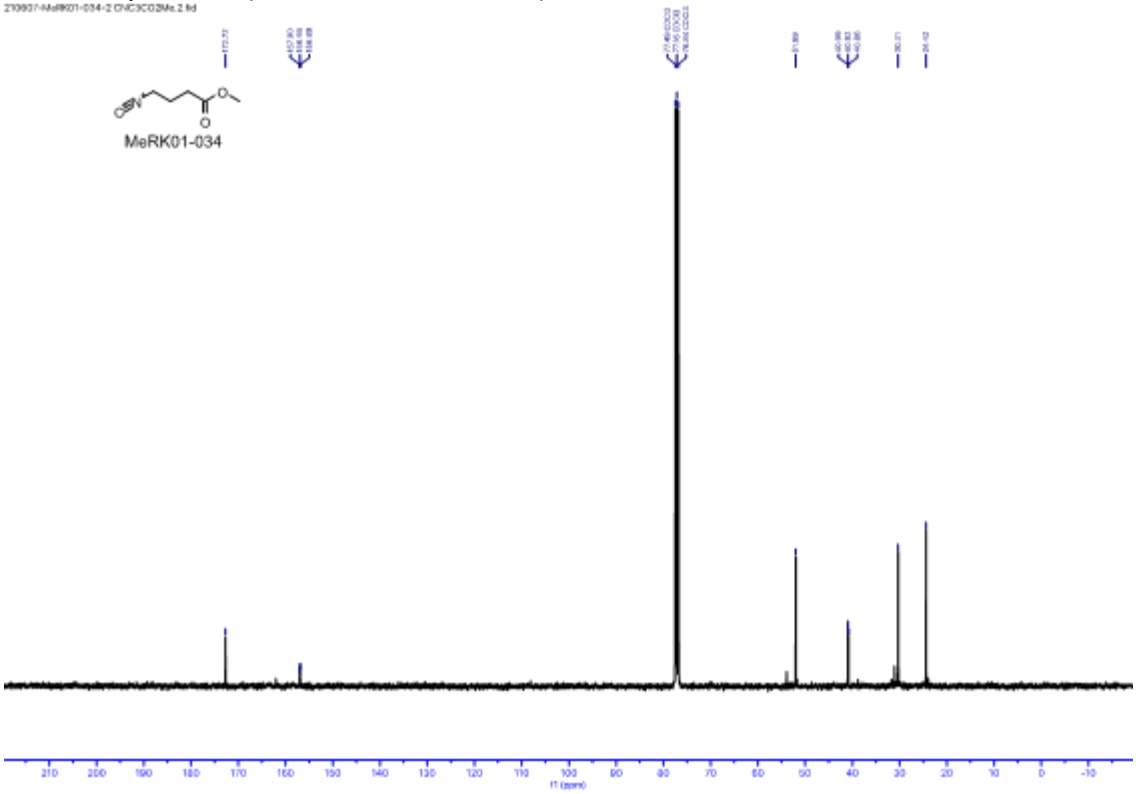

$^{1}$H NMR spectrum ($CDCl_3$, 400 MHz, 298K) of **2-NCCO2Me**



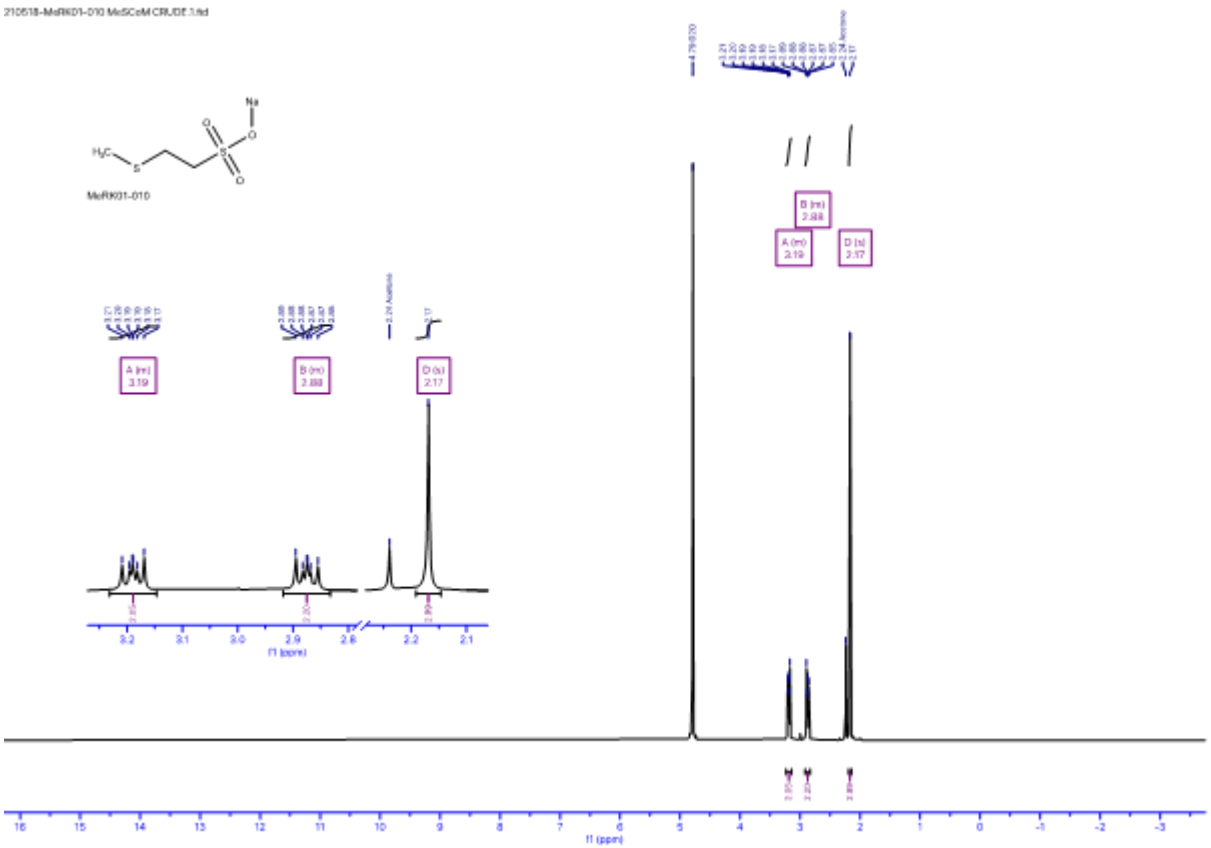

$^{13}$C NMR spectrum ($CDCl_3$, 100 MHz, 298K) of **2-NCCO2Me**



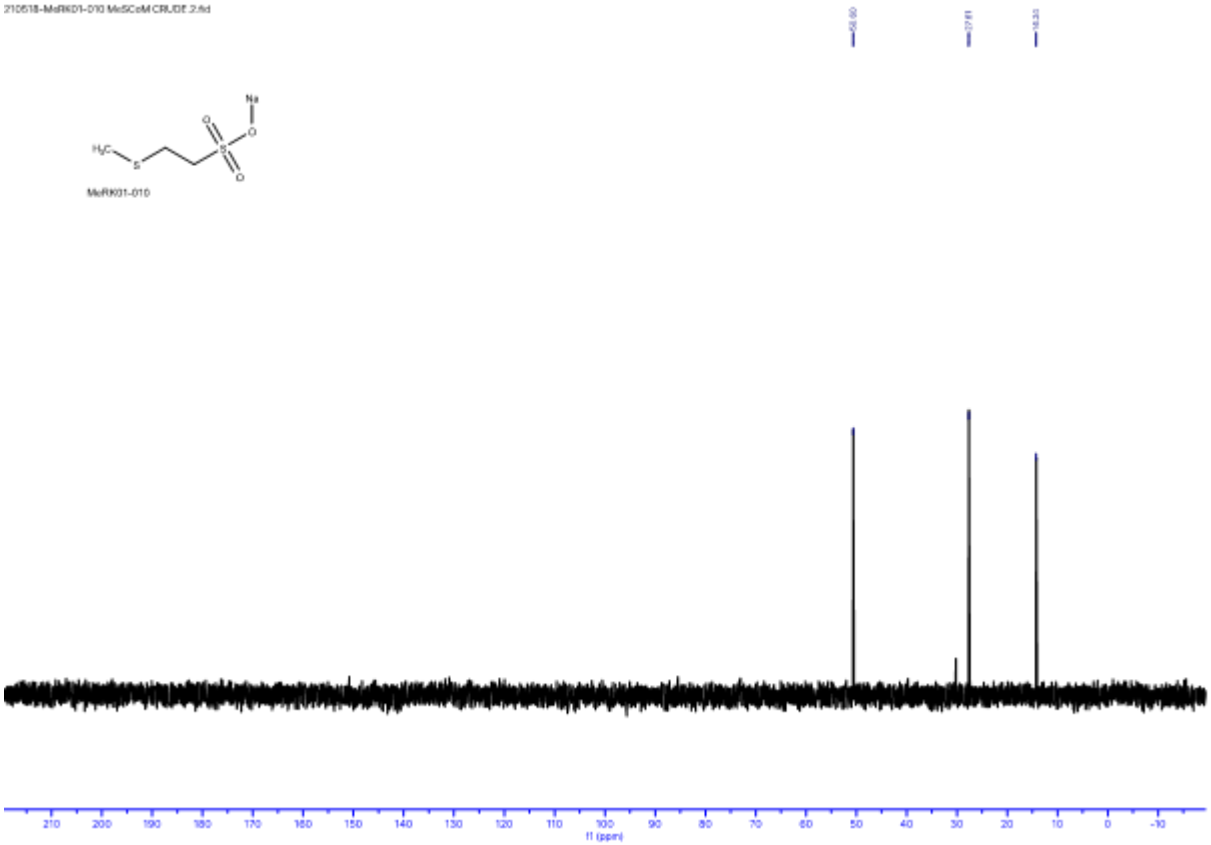

$^{1}$H NMR spectrum ($CDCl_3$, 400 MHz, 298K) of **3-NCCO2Me**



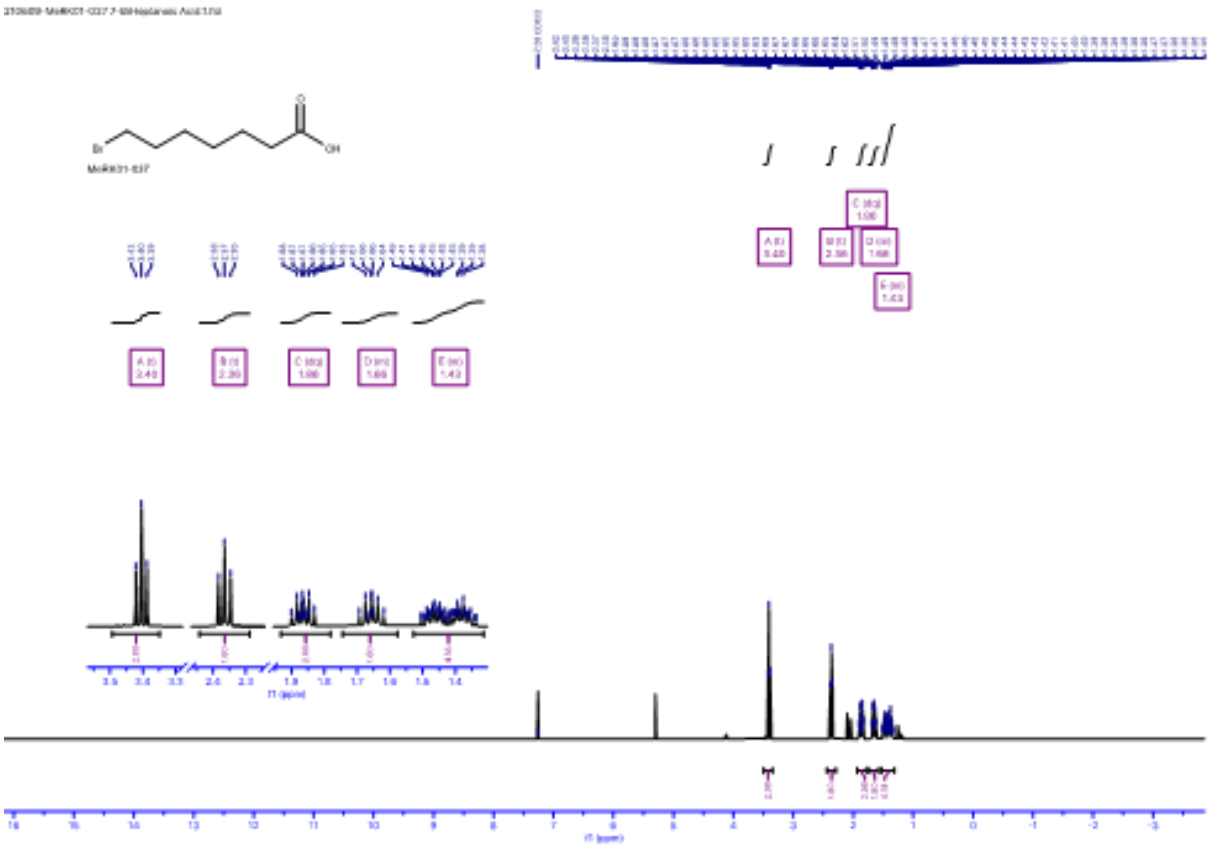

$^{13}$C NMR spectrum ($CDCl_3$, 100 MHz, 298K) of **3-NCCO2Me**



$^{1}$H NMR spectrum ($D_2O$, 400 MHz, 298K) of Methyl CoM



$^{13}$C NMR spectrum ($D_2O$, 100 MHz, 298K) of Methyl CoM



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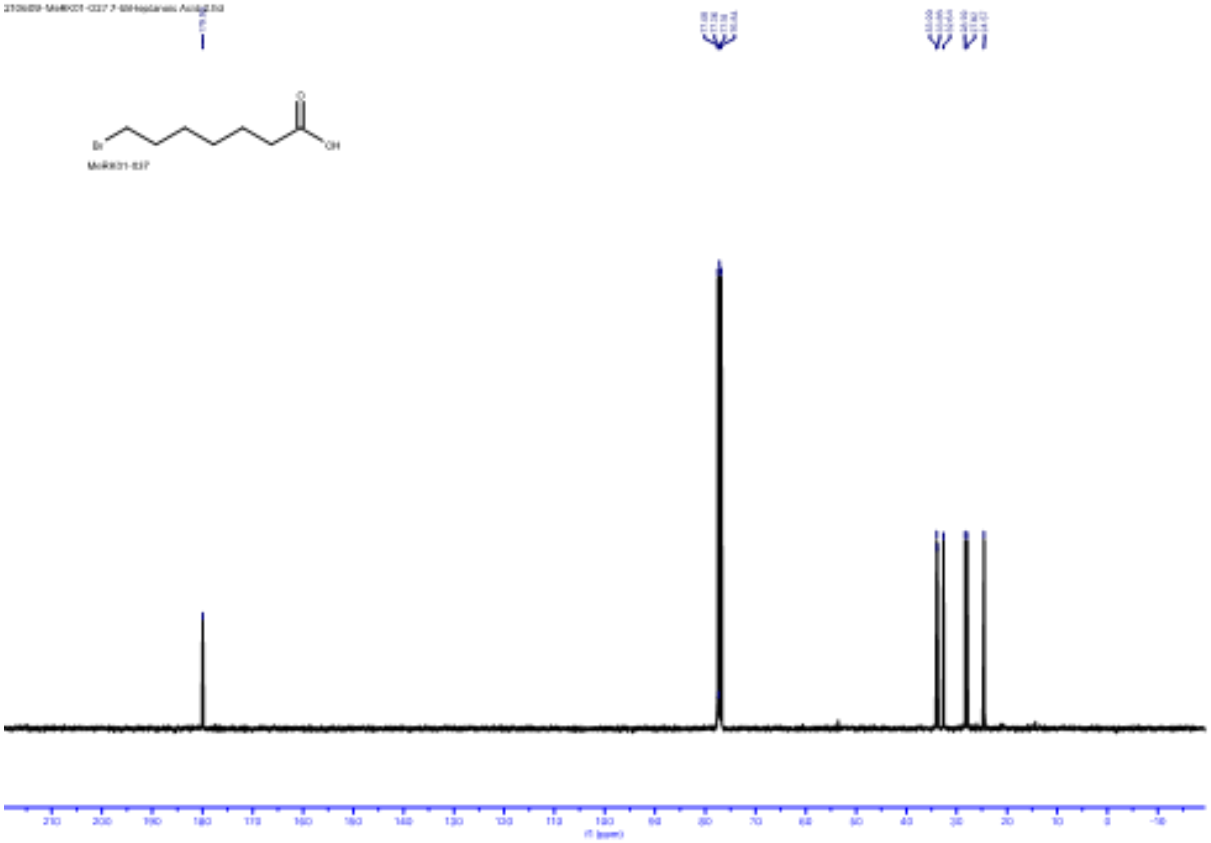

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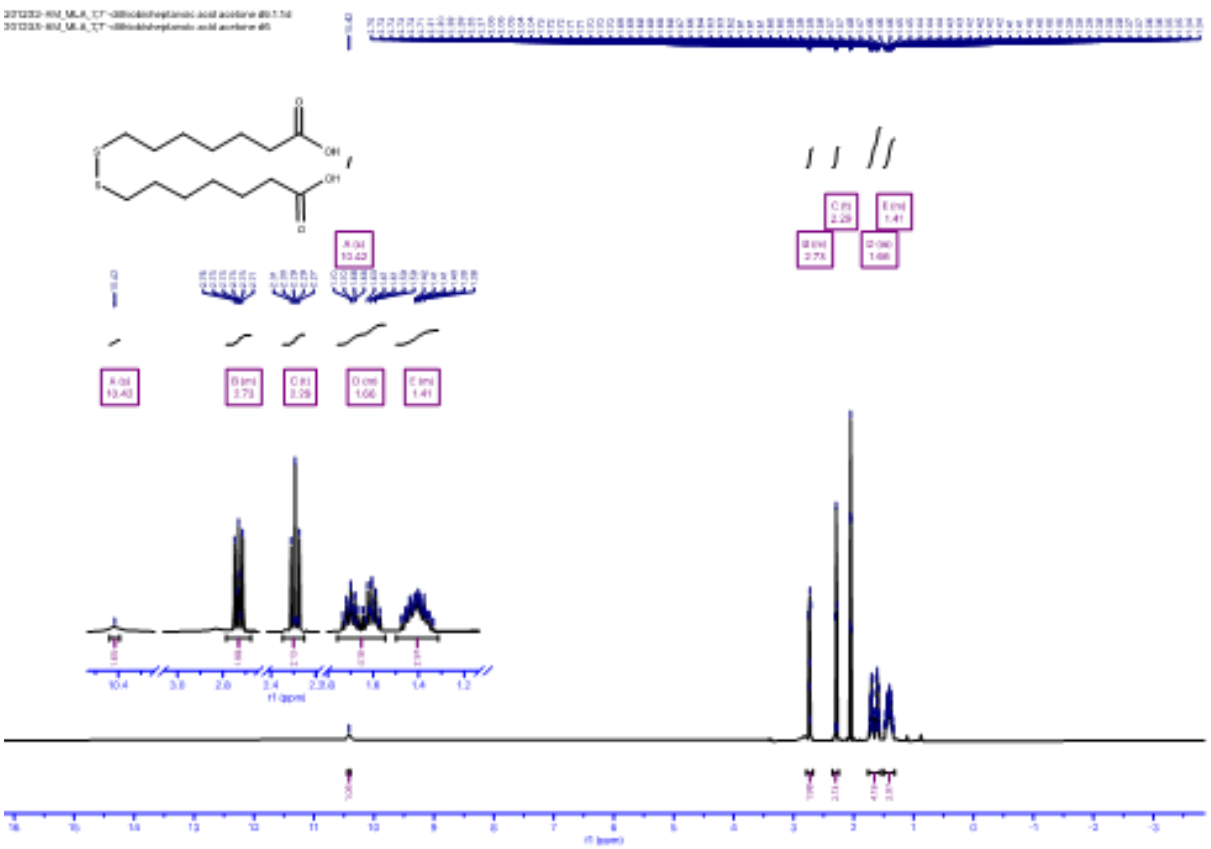
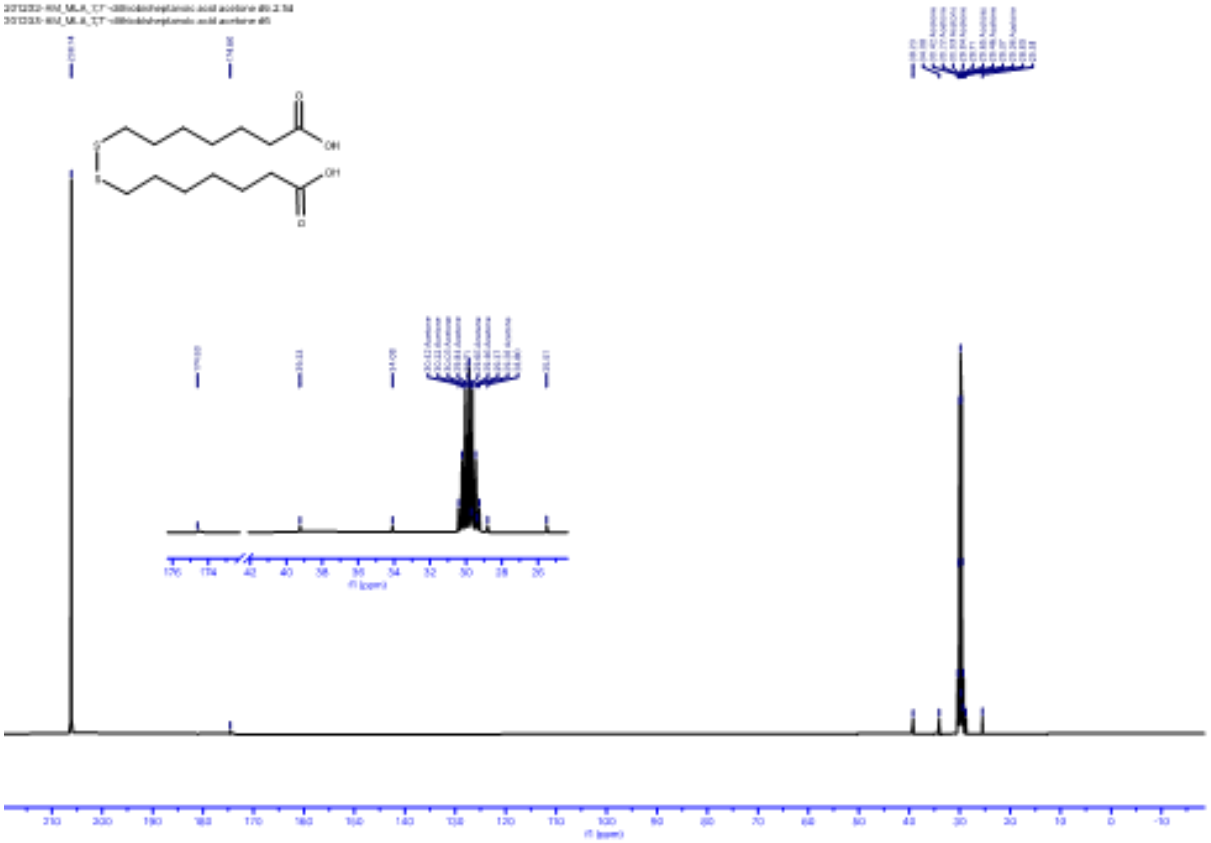
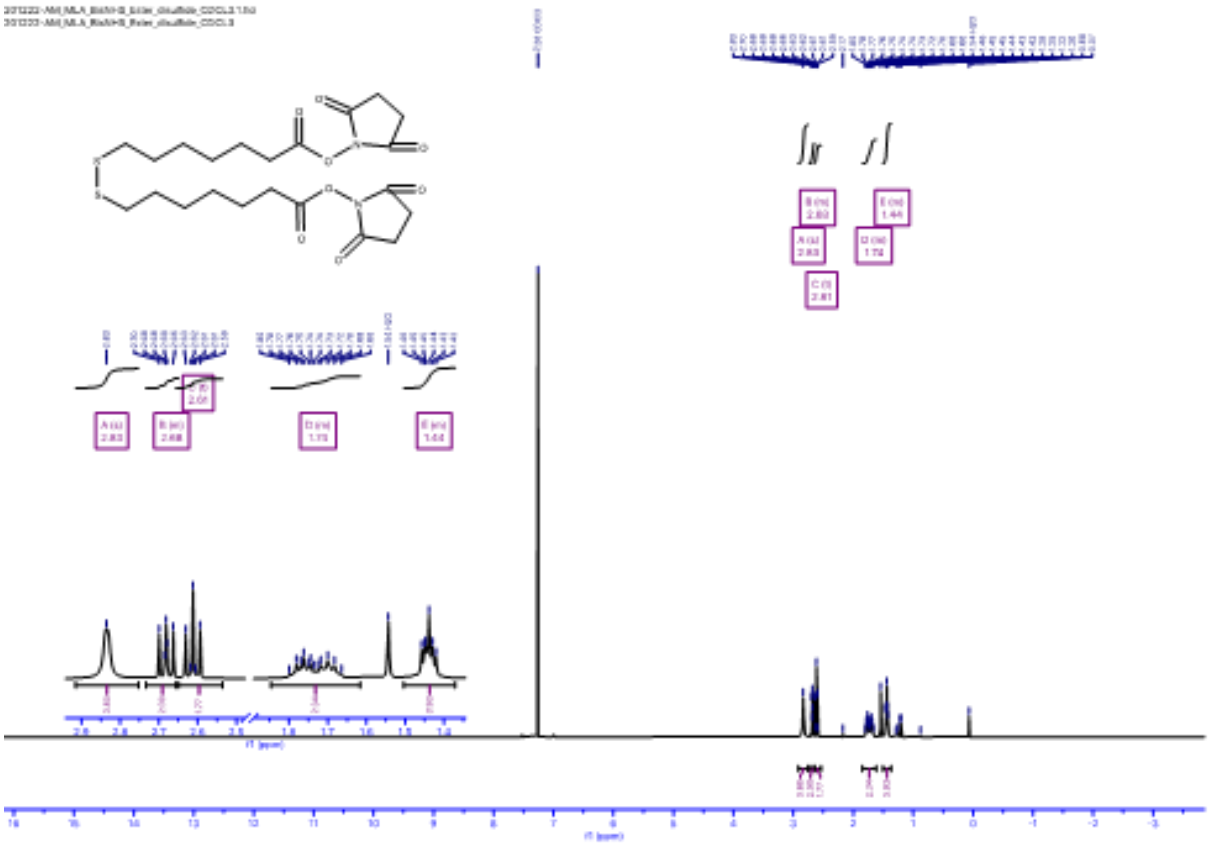


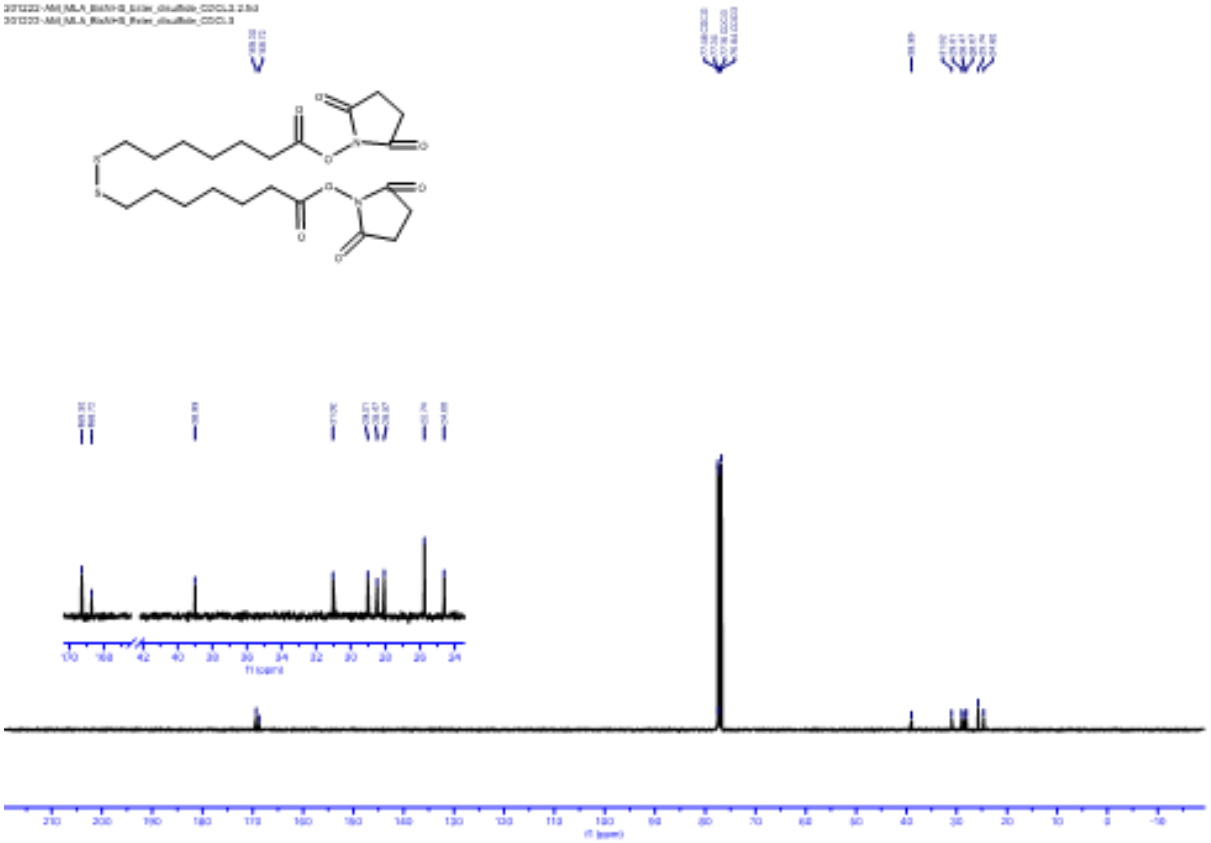
$^{1}$H NMR spectrum (Acetone-$d_6$, 400 MHz, 298K) of the disulfide acid



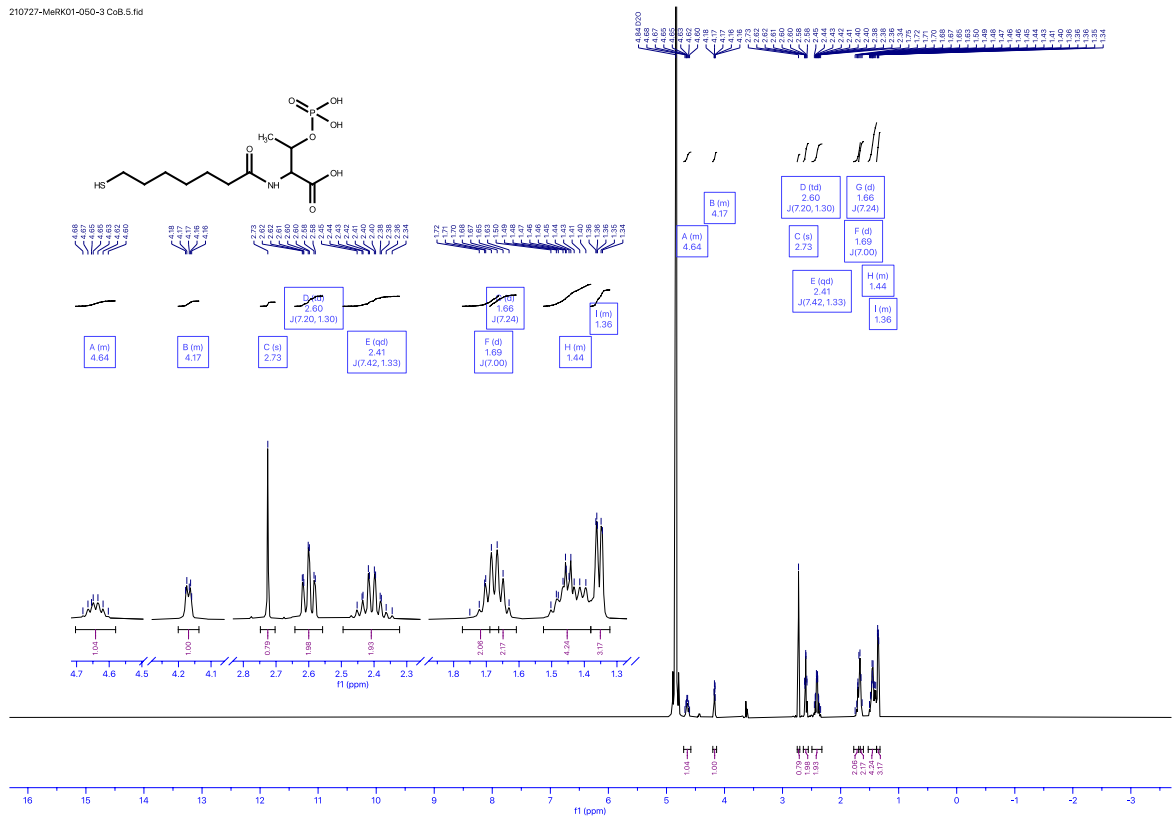
$^{13}$C NMR spectrum (Acetone-$d_6$, 400 MHz, 298K) of the disulfide acid



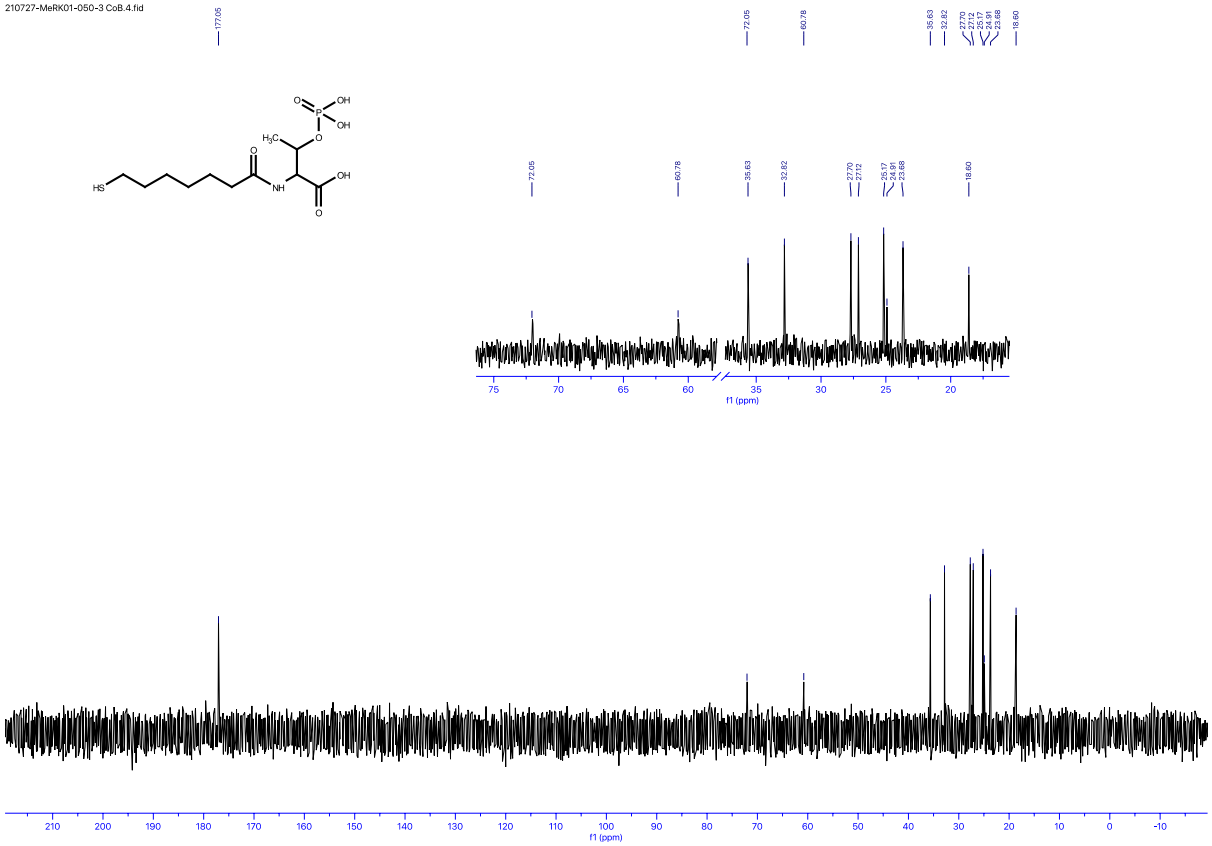
$^{1}H$ NMR spectrum ($CDCl_3$, 400 MHz, 298K) of the disulfide NHS ester



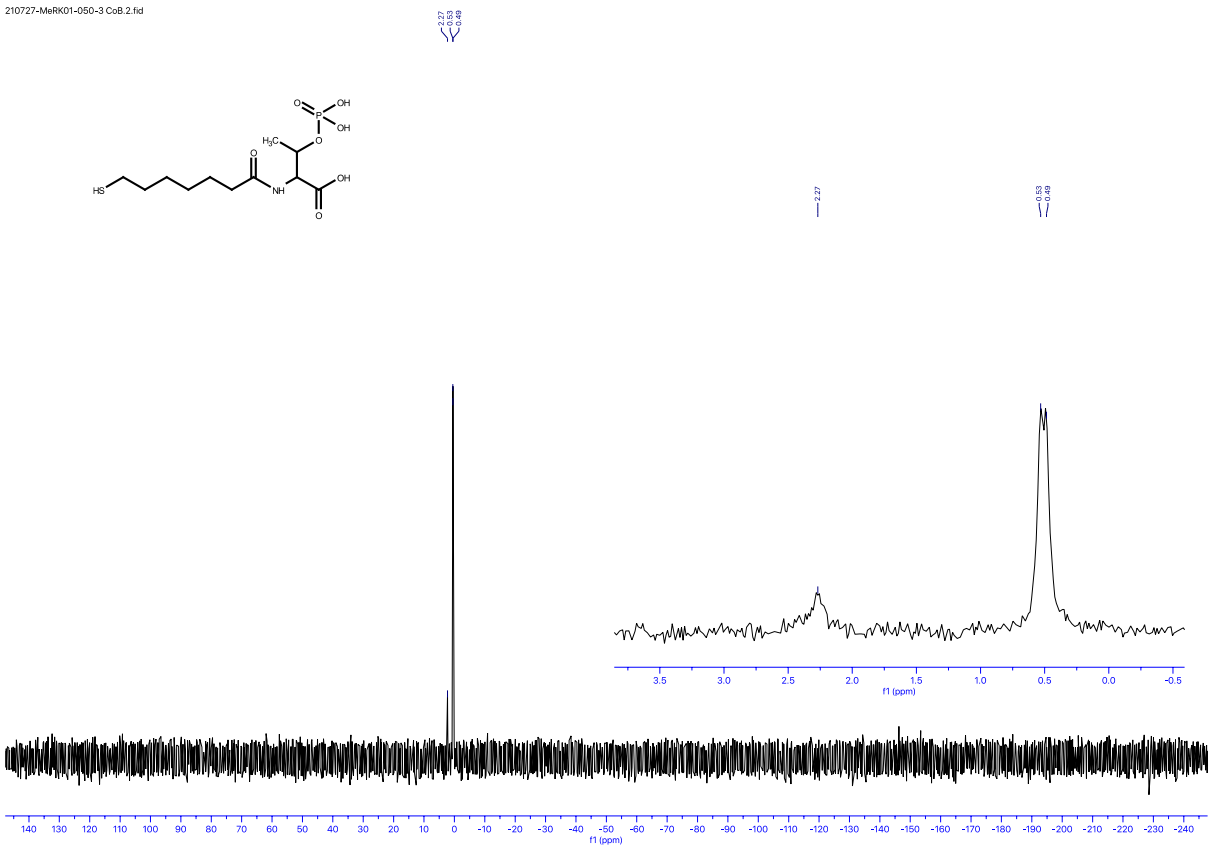
$^{13}C$ NMR spectrum ($CDCl_3$, 100 MHz, 298K) of the disulfide NHS ester




$^{1}$H NMR spectrum ($D_2O$, 400 MHz, 298K) of CoB




$^{13}$C NMR spectrum ($D_2O$, 400 MHz, 298K) of CoB




$^{31}$P NMR spectrum ($D_2O$, 162 MHz, 298K) of CoB

## 8.3 Milestone table from objectives

| MILESTONE | Scheduled | Completed | Notes |
|---|---|---|---|
| 1. Contract execution. Project initiation meeting to agree and confirm scope | 31/8/20 | 31/8/20 | Completed on time |
| 2. Deliverable:<br>Milestone report submitted and approved by MLA detailing:<br>Attempts to grow *Methanothermobacter marburgensis* and/or *Methanobrevibacter ruminantium* | 10/12/20 | 15/12/20 | *M. ruminatium* was successfully grown. *M. marburgensis* did not arrive before Xmas break and delivery in early January was refused. A new sample was ordered and arrive in February and then grown successfully |
| 3. Deliverables include<br>3a: active MRCred1 enzyme,<br><br>3b: 5 synthetic CoM and/or CoB mimics and<br>3c: a Milestone report submitted and approved by MLA detailing:<br>• Isolate and purify active MCR-red1 enzyme, | <br><br><br><br>30/5/21 | <br>30/7/21<br><br>30/6/21<br>30/6/21<br><br>30/5/21 | Active MRCred1 was isolated from M. marburgensis; Delayed.<br>9 synthetic CoM mimics were synthesised and tested. CoB mimics were moved to milestone 4 |

| | | | |
|---|---|---|---|
| including:<br>○ Extraction of biomass; ammonium sulfate cuts; ion exchange chromatography<br>○ Gel electrophoresis and analysis<br>○ Activity assay<br>• Synthesis of methyl-coenzyme M, coenzyme B, and 5 structure-guided inhibitors<br>• Recommendations for further R&D and delivery of phase 2 application including other enzymes in the methanogenic cycle and plans for a phase 3 application including animal trials (if applicable) | | 30/6/21<br>21/12/21<br><br>30/6/21 | Achieved on time<br><br>MeCoM was synthesised and CoB was move to milestone 4.<br>Recommendations for stage 2, was to develop a feed additive through targeted pathways where the mechanism of action is well understood (MERiL 1) |
| 4. Deliverables include<br>4a: a functional MCR-red1 methane bioassay and<br>4b: a Milestone report submitted and approved by MLA detailing:<br>• Development of MCR-red1 methane bioassay and initial testing of inhibitors of methanogenisis, including:<br>○ Establish a continuous supply of microbe<br>○ Develop methane inhibition assay in vitro using head-space analysis and GC-MS<br>4c: Develop methane inhibition assay in vivo<br>4d: Recommendations for further R&D and delivery of phase 2 application including animal trials (if applicable). | 30/9/21 | 21/12/21<br>21/12/21<br><br><br><br>30/6/21<br><br>21/12/21<br><br><br>30/6/21 | delivered<br>delivered<br><br><br><br>This was moved to milestone 3; delivered<br>Delivered<br><br><br>Moved to milestone 3 and delivered early<br><br>Move to milestone 5 |
| 5. Final report submitted and approved by MLA detailing:<br>• Testing inhibitors, including:<br>○ Test known positive (e.g. BCM, bromoform, BES, 3-NOP) and negatives<br>Test CoM and CoB mimics and calculate $IC_{50}$ values | 30/11/21 | (4/3/22) | |
| Maximum end date | 30/5/22 | (4/3/22) | |