Synthetic Alkaloid Enzymology for Anti-Ageing Therapeutic Development

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***Abstract* — By establishing a precursor-directed combinatorial biosynthetic route involving promiscuous acid-CoA ligases and polyketide synthases (PKSs), and utilizing nitrogen-containing compounds as substrates, a wide range of alkaloids can be biosynthesized. 17 nitrogen-containing substrates were tested on three acyl-CoA ligases: a putative carnitine-CoA ligase (Ec1) from *Escherichia coli* strain K12 MG1655, a putative CoA ligase (Sc3) from *Streptomyces coelicolor* A3 (2), and a putative long chain fatty acid-CoA ligase (Cg2) from *Corynebacterium glutamicum* ATCC 13032. The substrate used for malonyl-CoA synthase (MCS) from *Rhozobium trifolii* was malonic acid. Type III PKS from *Oryza sativa* (OsPKS) was also used. OsPKS has a large tolerance over a wide range of starter acyl-CoAs and extender acyl-CoAs. Studies showed the great potential to utilize OsPKS to generate libraries of compounds with a polyketide backbone, such as pseudoalkaloids with a polyketide backbone. The formation of potential pseudoalkaloids was analysed using reverse-phase High Performance Liquid Chromatography (HPLC). Seven potential pseudoalkaloids were synthesised from three starter acyl-CoA thioesters, namely 2-quinolinecarboxyl-CoA, isoquinoline-1-carboxyl-CoA and benzylmalonyl-CoA, with extender malonyl-CoA. The expansion of the substrate library of OsPKS has promoted biosynthesis as an efficient means to develop compound libraries for drug screening. Pseudoalkaloids formed from two starter acyl-CoA thioesters – 2-quinolinecarboxyl-CoA and isoquinoline-1-carboxyl-CoA, with extender malonyl-CoA were tested for anti-ageing effects by measuring the mitochondrial respiration rate of *Caenorhabditis elegans* after an 8-day exposure to these pseudoalkaloids. Results showed that the basal respiration rate of *C. elegans* exposed to the pseudoalkaloids formed from isoquinoline-1-carboxyl-CoA with malonyl-CoA was significantly higher than the control. However, no significant increase was seen in the maximal respiration rate. Hence, more experimental data is needed to conclude on the anti-ageing properties of these pseudoalkaloids. Their chemical structures can be analyzed using Mass Spectrometry and Nuclear Magnetic Resonance to identify anti-ageing compounds.**

Keywords: alkaloid, C. elegans, acyl-CoA ligases, OsPKS, High Performance Liquid Chromatography, anti-ageing, mitochondrial respiration rate

I. Introduction

1.1 Intensive research in the field of anti-ageing by using C. elegans as an animal model

Ageing refers to post-maturational gradual deterioration of cells, leading to quicker death of an organism. (Bergamini *et al.* 2007) To understand ageing, a useful approach is to study its mechanisms in model organisms such as *C. elegans*, which has a rapid 3-day life-cycle from egg to adult, small size (1.5-mm-long adult) and can be easily manipulated in laboratory. (Riddle 1998) Resveratrol, a potential anti-ageing compound, increases mitochondrial resistance to oxidative stress, extending the lifespan of *C. elegans* (Ungvari *et al.* 2011*)* yet research show potential side effects like nephrotoxicity in resveratrol. Thus, discovery of more potent anti-ageing compounds than resveratrol to extend human lifespan is essential.

1.2 Alkaloids

Alkaloids are a diverse group of secondary metabolites in living organisms with a wide range of biosynthetic pathways and medicinal properties. (Roberts *et al.* 1998) Some alkaloids have psychotropic (psilocin) and/or stimulative (caffeine, heroin) biological effects (Figure 1).

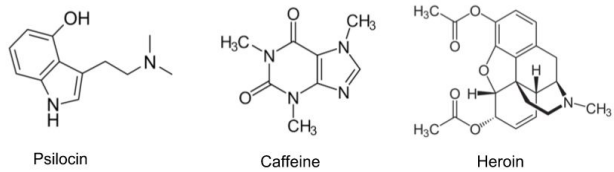

Figure 1: Examples of Alkaloids

Alkaloids are classified into three categories based on biosynthetic pathways and chemical structures - protoalkaloids, true alkaloids and pseudoalkaloids. Protoalkaloids and true alkaloids are amino acid derivatives with nitrogen present in the heterocyclic ring of true alkaloids but not in that of protoalkaloids. Pseudoalkaloids are not amino acid derivatives but are related to amino acid metabolic pathways and obtain a nitrogen atom through transamination. (Aniszewski 2007)

A major limitation of anti-ageing therapeutic development is developing novel and bioactive alkaloids for drug screening. Although chemical synthesis can be used to generate alkaloid analogues, their structural complexity and derivatives make it difficult to do so. (Keasling 2008)

1.3 Creation of pseudoalkaloids library using type III polyketide synthases (PKSs)

Type III PKSs are homodimeric enzymes, with broad substrate specificity (Jez *et al.* 2002), making them ideal for compound biosynthesis. An example is chalcone synthase from *Oryza sativa* (OsPKS). A diverse polyketide library is generated with OsPKS's tolerance over a wide range of starter and extender acyl-CoAs. OsPKS is utilised to produce compounds with a polyketide backbone, such as pseudoalkaloids. (Go *et al.* 2015) By establishing a precursor-directed combinatorial biosynthetic route involving acid-CoA ligases and OsPKS, and utilising nitrogen-containing substrates, pseudoalkaloids can be biosynthesised (Figure 2).

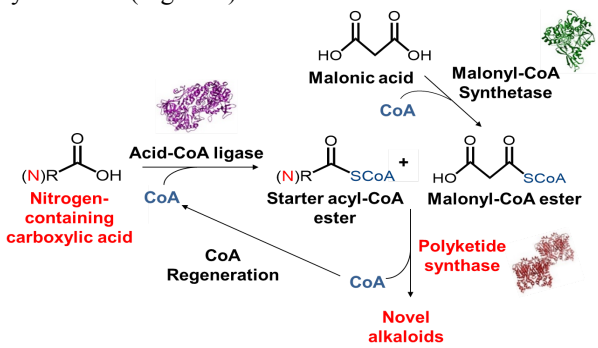

Figure 2: Precursor-directed biosynthesis of alkaloid compound libraries for drug screening.

1.4 Anti-ageing drug screening through Extracellular Flux Analysis

An Extracellular Flux Analyzer is used instead of conventional lifespan assays for anti-ageing drug screening which are time consuming and impractical to screen for a large compound library. (Gruber *et al.* 2009) Pharmacological agents such as electron transport chain (ETC) inhibitors are introduced to manipulate mitochondrial activity of *C. elegans.* (Miles 2003) Studies showed *C. elegans* subjected to anti-ageing treatment such as caloric restriction had a higher metabolic rate (Heilbronn *et al.* 2003) and hence, a higher oxygen consumption rate (OCR) than *C. elegans* fed on a normal diet. We hope to screen for potential anti-ageing drugs by measuring the OCR of *C. elegans* exposed to alkaloid compound libraries. This study serves as a primer towards anti-ageing drug development via synthetic enzymology.

II. Materials and Method

2.1 Acyl-CoA ligases and OsPKS

The four acyl-CoA ligases used are namely: MCS (GI: 3982573) from *R. trifolii,* Ec1 (GI: 221142682) from *E. coli* strain K12 MG1655, Sc3 (GI: 21218859) from *S. coelicolor* A3 (2), and Cg2 (GI: 499323704) from *C glutamicum* ATCC 13032. OsPKS is from *O. sativa* (GI: 115485731) and was a kind gift from Z. Chao Yin, Temasek Life Sciences Laboratory.

2.2 Substrates for acyl-CoA ligases and MCS

The substrate for MCS was malonic acid. The 17 nitrogen-containing carboxylic acids tested on three other CoA ligases (Appendix) were purchased from Sigma-Aldrich Co. (St. Louis, MO)

2.3 Determination of substrate profiles for acyl-CoA ligases

Purified enzymes were assayed by enzyme-coupled spectrophotometric assays, using UV-2550 spectrophotometer (Shimadzu). The acyl-CoA ligases' activity was monitored by observing the oxidation of NADH to NAD^+ at 340 nm (Figure 3).

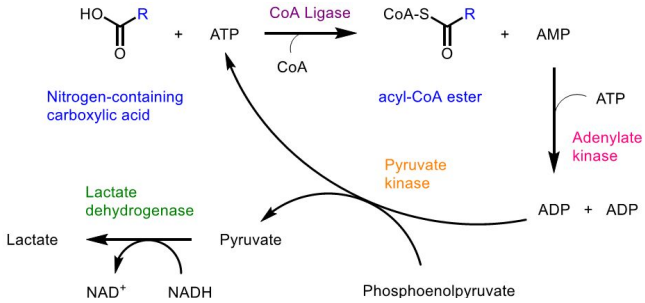

Figure 3: Diagrammatic representation of the enzyme reactions involved in the assay.

2.4 In vivo biosynthesis of pseudoalkaloids

The biosynthesis of pseudoalkaloids was adapted from Katsuyama's method. (Katsuyama *et al.* 2007) To establish a combinatorial biosynthetic route in *E. coli*, MCS which generates extender CoA thioester was subcloned into multiple cloning site 1 (MCS1) of a pRSFDuet-1 vector (Novagen). To produce starter CoA thioester, Ec1, Sc3 and Cg2 were subcloned separately into MCS2 region of the same vector. The pRSFDuet-1 vector was then co-transformed with the Tom-15b vector containing OsPKS into *E. coli Rosetta II (DE3)* strain (Novagen) (Figure 4). An *E. coli* construct without OsPKS gene is a control for subsequent detection of novel alkaloids.

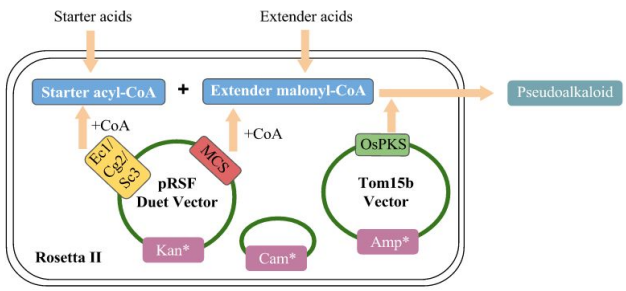

Figure 4: Host cell E. Coli Rosetta II containing pRSF-Duet 1 construct and Tom15b construct containing the acyl-CoA ligases and OsPKS, respectively.

The *E. coli* constructs were grown in a 10-mL LB culture containing 100 µg/mL ampicillin, 34 µg/mL chloramphenicol, and 30 µg/mL kanamycin at 25 °C. Once the optical density at 600 nm has reached 0.5, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce enzymes expression overnight at 25 °C. The cells were harvested by centrifugation and resuspended in 10 mL of M9 media containing 2% (v/v) glucose, 0.1 mM IPTG, 1 mM starter carboxylic acid, and 3 mM of malonic acid. The culture was incubated at 25 °C for 48 hours for the *in vivo* production of acyl-CoA thioesters and pseudoalkaloids. The cells were harvested and discarded. The supernatant was acidified to pH 3.0 with 6 M hydrochloric acid (HCl). The products were extracted with ethyl acetate, which was then removed with a vacuum concentrator. The dried sample was re-dissolved in 100 µL dimethyl sulfoxide (DMSO) for HPLC analysis.

2.5 Analysis of potential pseudoalkaloids using HPLC

The analysis of potential pseudoalkaloids formation was carried out using HPLC. An Atlantis® T3 Analytical C18 (4 mL, Waters) reverse-phase HPLC column, which was first equilibrated with 10% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water at a flow rate of 1 mL/min for 15 min, was used. 10 µL of sample was loaded in the column and the mobile phase was changed to 50% acetonitrile, 0.1% TFA in water under a linear gradient over 40 min at flow rate of 1 mL/min. For the next 5 min, a linear gradient to 100% acetonitrile containing 0.1% TFA was conducted. The eluted compounds were detected by measuring the absorbance at 259 nm. Chromatogram peaks (minimum height of 20 mAU) present in construct extracts contain OsPKS but are absent in control constructs extracts, indicate alkaloid biosynthesis.

2.6 Anti-ageing application using Seahorse Extracellular Flux Analyser

N2 *C. elegans* was a kind gift from Dr Takao Inoue, National University of Singapore. Young *C. elegans* were grown on Nematode Growth Media (NGM) plates with *E. coli OP50* strain as the food source to the adult stage. Adult *C. elegans* were classified into three groups and exposed to the respective conditions for eight days - thymidylate synthase inhibitor 5-fluoro-2'-deoxyuridine (FUdR) + *E. coli* OP50, FUdR + control construct, and FUdR + OsPKS construct. For OsPKS and control groups, the respective combinations of starter and extender acids, antibiotics, and IPTG were incorporated into NGM for CoA thioester and alkaloids biosynthesis. *C. elegans* were exposed to alkaloids when they feed on the *E. coli* constructs. FUdR was added to induce complete sterility within five hours by preventing eggs from hatching. (Mitchell *et al.* 1979)

The medium used is M9 of pH 7.4. After measuring basal respiration, 75 µL of 20 µM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and 75 µL of a mixture of 10 µM rotenone and 20 µM antimycin A were injected sequentially to each well. Maximal OCR is measured after adding FCCP. Rotenone and antimycin A were added to disrupt mitochondria ETC. The OCR measured after treatment is non-mitochondrial respiration. Biosynthesized pseudoalkaloids were tested for potential anti-ageing effects by analysing the basal and maximal OCR of *C. elegans.*

III. Results and Discussion

3.1 Investigation of potential substrates for acyl-CoA ligases

At 340 nm, NADH show strong UV absorption while NAD^+ does not. NADH depletion reflected by a fall in UV absorption, indicates the carboxylic acid tested is a suitable substrate.

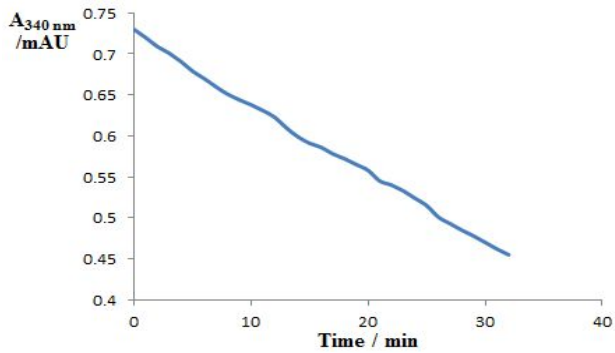

Figure 5: UV absorption at 340 nm when 3-Aminobenzoic acid is tested on Ec1.

The UV absorption level dropped from 0.73 to 0.47 mAU over 30 minutes (Figure 5). NADH oxidised to NAD^+ due to enzymatic reactions (Figure 3). 3-Aminobenzoic acid is a suitable substrate for Ec1. The other 16 acids were found to be suitable substrates for the respective CoA ligases used.

3.2 Precursor-directed combinatorial biosynthesis of novel pseudoalkaloids catalysed by OsPKS

The *E. Coli Rosetta II (DE3)* host cell expression system was used for pseudoalkaloids formation. Three out of 17 combinations - isoquinoline-1-carboxyl-CoA (Figure 6), 2-quinolinecarboxyl-CoA, and benzylmalonyl-CoA (Appendix), with malonyl-CoA as the extender have shown new pseudoalkaloids formation.

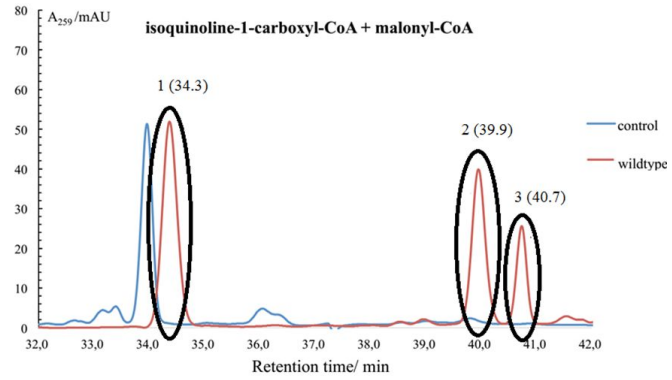

Figure 6: HPLC Chromatogram of potential pseudoalkaloids formed by isoquinoline-1-carboxyl-CoA with malonyl-CoA.

Three red peaks found at retention times of 34.3 min, 39.3 min and 40.7 min, reflect potential pseudoalkaloids formed. Mass spectrometry and nuclear magnetic resonance can be used to determine their structures. The expansion of OsPKS substrate library promotes biosynthesis to be more effective for drug screening, as compared to chemical synthesis.

3.3 Investigation of the anti-ageing effects of pseudoalkaloids on C. elegans

Pseudoalkaloids formed from isoquinoline-1-carboxyl-CoA with malonyl-CoA and 2-quinolinecarboxyl-CoA with malonyl-CoA (Appendix) were tested for anti-ageing effects.

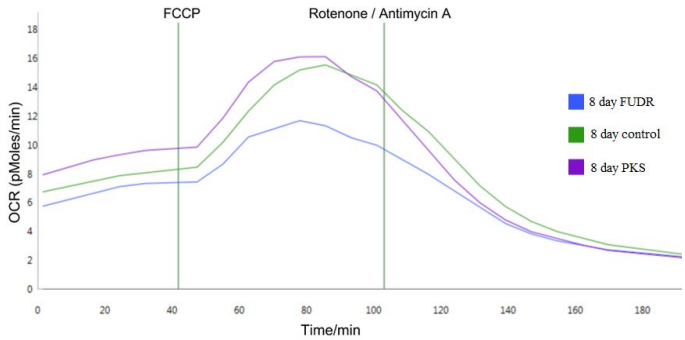

Figure 7: Mitochondrial Respiration of C. elegans tested with pseudoalkaloids formed from starter isoquinoline-1-carboxyl-CoA with extender malonyl-CoA.

An anti-ageing compound should increase both basal and maximal OCR. (Das *et al.* 2013) In the initial 40 minutes, the basal OCR for *C. elegans* exposed to pseudoalkaloids formed from starter isoquinoline-1-carboxyl-CoA with extender malonyl-CoA is constantly higher of approximately 1.3 pMoles/min than that of the control but there is no significant increase in the maximal OCR. At approximately 92 minutes, OCR for *C. elegans* exposed to alkaloids produced decreased below that of the control (Figure 7). Anti-ageing properties of these pseudoalkaloids are uncertain. More replicates are required to conclude if the OCR differences are significant.

IV. Conclusion

OsPKS' substrate library has been expanded using nitrogen-containing acyl-CoA thioesters to produce pseudoalkaloids. Seven potential pseudoalkaloids have been synthesised from three starters – 2-quinolinecarboxyl-CoA, isoquinoline-1-carboxyl-CoA and benzylmalonyl-CoA, with extender malonyl-CoA.

Future research can aim to enlarge the novel pseudoalkaloids library by altering enzymatic activity of OsPKS to produce new products.

Pseudoalkaloids formed from 2-quinolinecarboxyl-CoA and isoquinoline-1-carboxyl-CoA, with extender malonyl-CoA were tested for anti-ageing effects but no significant effects were identified for the former starter while slight effects were observed for the latter. The experiment could be repeated to obtain more accurate results for anti-ageing properties. The discovery of new anti-ageing compounds would form the basis for formulating anti-ageing supplements for the extension of human lifespan.

V. Acknowledge

We would like to thank our supervisor Prof. Yew Wen Shan for giving us the opportunity to do this research program in his laboratory and his continuous support throughout the project. We would also like to extend our gratitude to our further mentor Cheung Wing Ngar Vivian and Lim Yan Ping for their constant guidance and support during the project. Furthermore, we would like to thank our fellow laboratory mates who have helped us in one-way or another.

VI. References

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Appendix

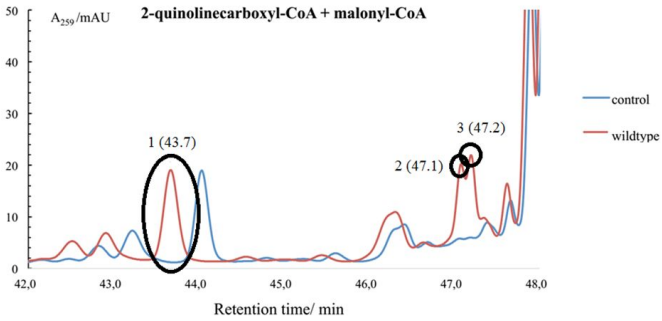

Figure A-1: HPLC Chromatogram of potential pseudoalkaloids formed by 2-quinolinecarboxyl-CoA with malonyl-CoA.

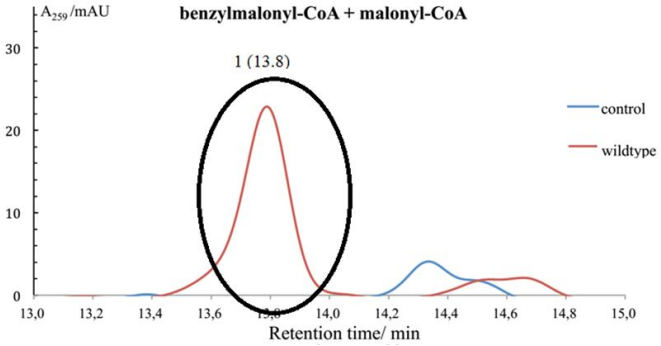

Figure A-2: HPLC Chromatogram of potential pseudoalkaloids formed by benzylmalonyl-CoA with malonyl-CoA.

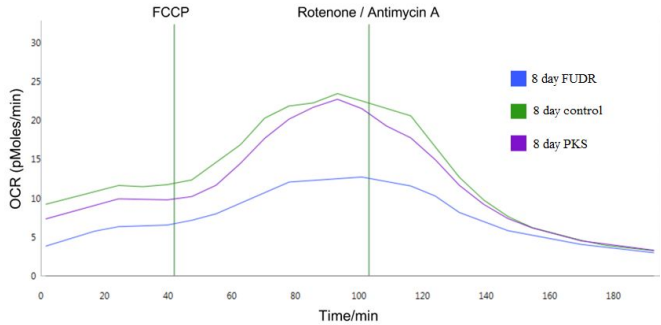

Figure A-3: Mitochondrial Respiration of C. elegans tested with pseudoalkaloids formed from starter 2-quinolinecarboxyl-CoA with extender malonyl-CoA.

Table 1: List of carboxylic acids and acyl-CoA ligase that catalyses the formation of the respective acyl-CoA thioester.

Carboxylic Acid	Acyl-CoA ligase
3-Aminobenzoic acid	Cg2
	Ec1
4-Aminobenzoic acid	Cg2
	Ec1
Pyridine-2-carboxylic acid	Cg2
Pyridine-3-carboxylic acid	Cg2
	Ec1
2-Chloropyridine-3-carboxylic acid	Cg2
Pyridine-4-carboxylic acid	Cg2
Pyrazinecarboxylic acid	Cg2
2-Quinolinecarboxylic acid	Cg2
	Ec1
	Sc3
2-Quinoxalinecarboxylic acid	Ec1
3-Quinolinecarboxylic acid	Ec1
4-Quinolinecarboxylic acid	Cg2
Isoquinoline-1-carboxylic acid	Cg2
	Sc3
Tyrosine	Ec1
Glutamic acid	Ec1
Cysteine	Ec1
	Sc3
Benzylmalonic acid	Sc3
Cyclopentylmalonic acid	Cg2