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Synthetic Enzymology and the Fountain of Youth: Repurposing Biology for Longevity

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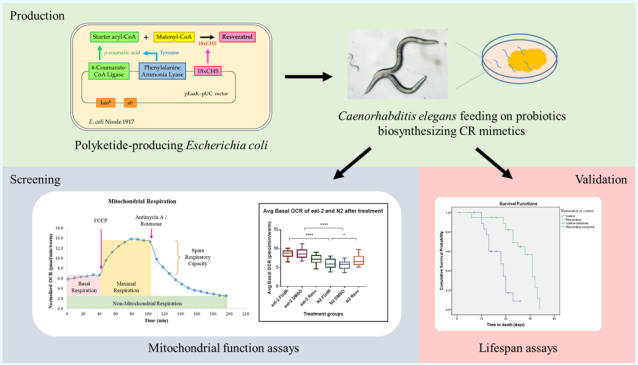
S *Supporting Information*

ABSTRACT: Caloric restriction (CR) is an intervention that can increase maximal lifespan in organisms, but its application to humans remains challenging. A more feasible approach to achieve lifespan extension is to develop CR mimetics that target biochemical pathways affected by CR. Recent studies in the engineering and structural characterization of polyketide synthases (PKSs) have facilitated their use as biocatalysts to produce novel polyketides. Here, we show that by establishing a combinatorial biosynthetic route in *Escherichia coli* and exploring the substrate promiscuity of a mutant PKS from alfalfa, 413 potential anti-ageing polyketides were biosynthesized. In this approach, novel acyl-coenzyme A (CoA) precursors generated by promiscuous acid-CoA ligases were utilized by PKS to generate polyketides which were then fed to *Caenorhabditis elegans* to study their potential efficacy in lifespan extension. It was found that CR mimetics like resveratrol can counter the age-associated decline in mitochondrial function and increase the lifespan of *C. elegans*. Using the mitochondrial respiration profile of *C. elegans* supplemented for 8 days with 50 μM resveratrol as a blueprint, we can screen our novel polyketides for potential CR mimetics with improved potency. This study highlights the utility of synthetic enzymology in the development of novel anti-ageing therapeutics.



1. INTRODUCTION

Caloric restriction (CR) is defined as a reduction of caloric intake by 30−40% of ad libitum consumption, without causing malnutrition.[1] CR can cause lifespan extension by triggering a shift from a physiological state of proliferation and growth, to repair and maintenance. Studies have shown that CR reduces oxidative damage, retards age-related functional decline such as deteriorations in DNA repair capacity, and causes a 30% increase in maximal lifespan of mammals.[2−4] Nevertheless, the amount and duration of CR necessary to extend lifespan is not practical in humans.[5] A feasible solution lies in developing a CR mimetic that can directly target biochemical pathways affected by CR and similarly achieve lifespan extension.

Natural products represent a good starting point for drug discovery, and there is great interest in synthesizing analogs of these compounds in order to explore the mechanism of action, and enhance bioactivity and bioavailability. Polyketides are functionally and structurally diverse secondary metabolites produced in bacteria, fungi, and plants. Many of these bioactive natural products have significant medical applications,[6] including their use as immunosuppressants,[7] antibiotics,[8] and potential anti-ageing therapeutics.[9] Resveratrol is hypothesized to be a CR mimetic[10,11] as it increases mitochondrial resistance to oxidative stress and extends the lifespan of *Caenorhabditis elegans*.[12] Although resveratrol confers some anti-ageing benefits, its application in humans is fraught with challenges. One problem of administering resveratrol in humans is its low solubility and high metabolism, resulting in low bioavailability.[13] Moreover, some research has suggested that resveratrol may have potential adverse effects like nephrotoxicity.[14,15] The corollary to these challenges requires a need to discover new CR mimetics that are more potent (increased bioavailability and efficacy) in bringing about lifespan extension in humans.

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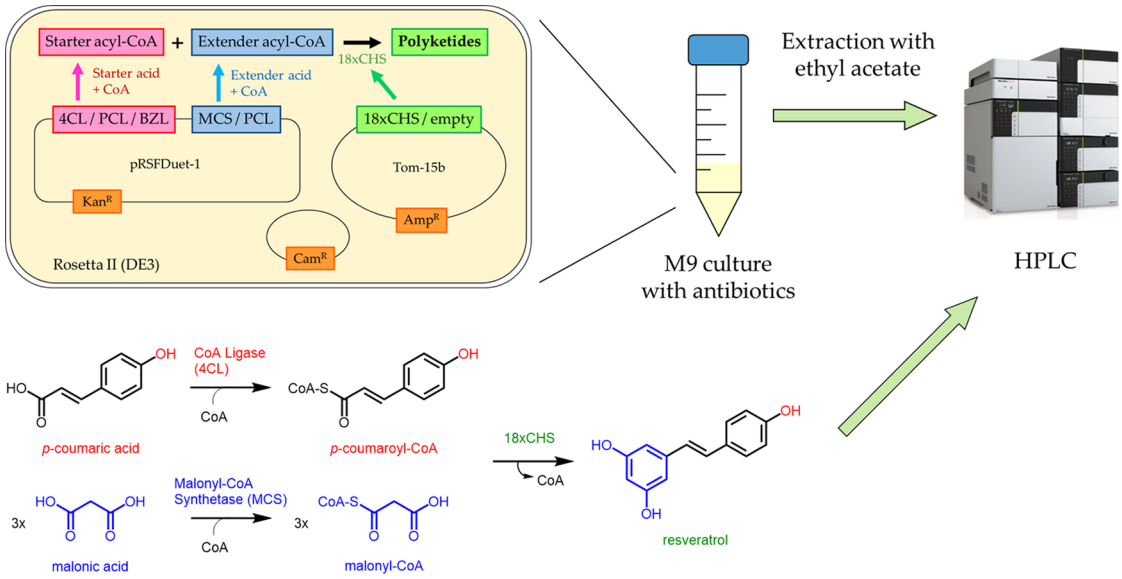

Figure 1. *E. coli* constructs generated for in vivo precursor-directed combinatorial biosynthesis of polyketides. Constructs containing the CoA ligases with an empty Tom-15b vector served as controls during the subsequent high-performance liquid chromatography (HPLC) analyses. When a starter acid such as *p*-coumaric acid and an extender acid such as malonic acid are introduced to the *E. coli* constructs, the respective CoA thioesters are formed, which are utilized by 18xCHS to form polyketides like resveratrol.

Polyketides are typically biosynthesized through successive decarboxylative condensations of coenzyme A (CoA)-derived precursors, into a complex polycyclic multi-carbon compound containing keto or hydroxyl groups.[16] Because of the chemical and structural complexity of polyketides and their derivatives, chemical synthesis is difficult. Current research in the engineering and structural characterization of polyketide synthases (PKSs) has facilitated their use as biocatalysts to generate novel polyketides, which can serve as potential drug leads.[17]

Type III PKSs are homodimeric and acyl-carrier protein-independent enzymes which can accomplish a series of decarboxylative condensations and cyclization reactions within a single active site. The relative simplicity, flexibility, and unusually broad substrate specificity of type III PKSs make them ideal candidates for engineering, facilitating the accessibility of bioactive polyketide libraries which are otherwise not easily available.[18,19] One of the most well-understood type III PKS is chalcone synthase (CHS), which is ubiquitous in higher plants and crucial for plant metabolism and defense.[18,20] Chalcone synthesis by CHS is initiated by the loading of *p*-coumaroyl-CoA onto the active site cysteine residue; CHS then catalyzes three iterative decarboxylative condensations of the extender substrate, malonyl-CoA, with the cysteine-bound starter substrate, to form a linear tetraketide intermediate. Subsequently, the intermediate undergoes an intramolecular C6 to C1 Claisen condensation and cyclizes into chalcone, which undergoes a further Michael-type ring closure to form naringenin.[20]

Unlike CHSs which are ubiquitous, stilbene synthases (STSs) are found in only a small subset of unrelated plants. Structure-based mutagenic transformation of alfalfa CHS into STS established that modulations in active-site residues enabled STSs to catalyze an intramolecular C2 to C7 aldol condensation, followed by decarboxylation and dehydration to produce a stilbene scaffold.[21] Eighteen distal amino acid residues in alfalfa CHS spanning from residue 98 to 268 were mutated, resulting in a mutant CHS with STS activity (18xCHS). The 18xCHS was able to produce resveratrol as the major product, instead of naringenin, when incubated with *p*-coumaroyl-CoA and malonyl-CoA.

An exploration of substrate and product profiles of 18xCHS across various acyl-CoA classes has not been systematically reported. Previous substrate specificity studies have demonstrated the production of unnatural polyketides by the promiscuous alfalfa CHS.[22] However, because of a lack of means to deliver novel acyl-CoA precursors, these studies were limited to structural derivatives of cognate substrates from the cinnamyl-CoA and malonyl-CoA families. Originating from the promiscuous alfalfa CHS scaffold and with the ability to produce the CR mimetic resveratrol, 18xCHS provided an excellent platform for the engineered biosynthesis of novel stilbenes and other polyketides with potential anti-ageing activities. Using precursor-directed combinatorial biosynthesis, unnatural acyl-CoA production by promiscuous CoA-ligases was coupled to polyketide biosynthesis by 18xCHS. By establishing the substrate specificities of 18xCHS, polyketide libraries can be synthesized and screened for anti-ageing properties.

The conventional way of anti-ageing drug screening is via lifespan assays. However, lifespan assays are time-consuming and impractical for screening a large library of bioactive compounds. This study aims to develop a medium throughput screening methodology by conducting mitochondrial function assays on *C. elegans* exposed to various compounds using an Extracellular Flux Analyzer. By periodically introducing pharmacological agents such as electron transport chain inhibitors to manipulate mitochondrial activity and respiratory function, the mitochondrial biology of *C. elegans* can be examined to establish a correlation between oxygen consumption rates (OCRs), CR mimetics, and lifespan extension.

2. RESULTS AND DISCUSSION

2.1. Establishing a Combinatorial Biosynthetic Route in *Escherichia coli*. Precursor-directed combinatorial biosynthesis is a useful means to generate polyketide analogs. As

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starter and extender CoA thioesters were either commercially unavailable or cost prohibitive, and the biosynthesis of CoA thioesters had to be coupled to PKS, 18xCHS, in order to generate polyketides (Figure 1). By supplying *p*-coumaric acid and malonic acid to the *E. coli* constructs, *p*-coumaroyl-CoA and malonyl-CoA were synthesized by 4-coumarate-CoA ligase (4CL) and malonyl-CoA synthetase (MCS), respectively. 18xCHS was validated to have resveratrol synthase activity when incubated with the biosynthesized *p*-coumaroyl-CoA and malonyl-CoA (Figure S1 in the Supporting Information). The resveratrol generated by 18xCHS was verified to have an experimental mass of 227.0712 in the negative MS mode (theoretical mass of 227.0714) (Figure S2 in the Supporting Information), and NMR analysis further established the identity of the compound (Figure S3 in the Supporting Information). Several minor peaks were also observed at retention times of 24.4, 35.0, and 40.4 min, indicating the potential biosynthesis of bisnoryangonin, *p*-coumaroyl triacetic acid lactone, naringenin, as well as other polyketides resulting from a different number of extension step or minor products derived from alternate intramolecular cyclization. This allowed us to set up the platform for precursor-directed combinatorial biosynthesis, which requires the utilization of different combinations of acyl-CoA derivatives for the generation of novel polyketides.

Through precursor-directed biosynthesis using different combinations of 69 starter CoA thioesters and 12 extender malonyl-CoA derivatives (Table S1 in the Supporting Information), the substrate profile of 18xCHS was established (Figure 2), and a catalog of polyketides was obtained based on

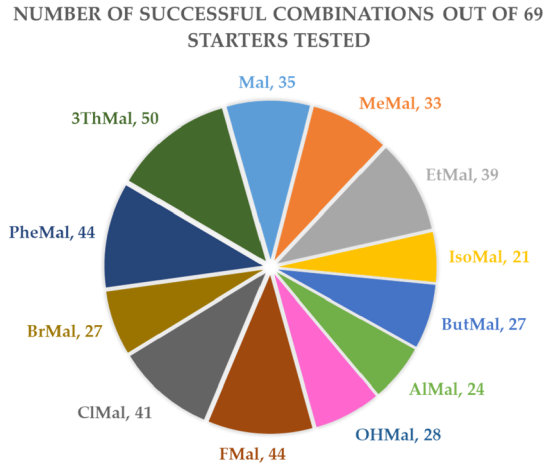

Figure 2. Substrate profile of 18xCHS using 69 starter CoA thioesters and 12 extender CoA thioesters; 413 out of 828 possible combinations (49.9%) gave rise to new polyketides. Extender acyl-CoAs are abbreviated as follows: malonyl-CoA (Mal), methylmalonyl-CoA (MeMal), ethylmalonyl-CoA (EtMal), isopropylmalonyl-CoA (IsoMal), butylmalonyl-CoA (ButMal), allylmalonyl-CoA (AlMal), hydroxymalonyl-CoA (OHMal), fluoromalonyl-CoA (FMal), chloromalonyl-CoA (ClMal) and bromomalonyl-CoA (BrMal), phenylmalonyl-CoA (PhMal), and 3-thiophenemalonyl-CoA (3ThMal).

HPLC analyses. 18xCHS was found to be promiscuous in substrate utilization, consistent with previously characterized alfalfa CHS.[22] 18xCHS was able to utilize unnatural starter and extender CoA thioesters as substrates for polyketide synthesis (Figure 3), thus highlighting the feasibility of precursor-directed combinatorial biosynthesis of polyketides. From the

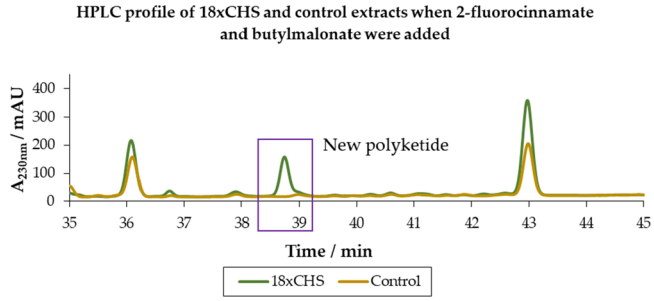

Figure 3. HPLC profile of organic extracts from *E. coli* constructs grown in M9 supplemented with 2-flurocinnamate and butylmalonate. Biosynthesized products in spent minimal medium containing either *E. coli* with CoA ligases + 18xCHS or *E. coli* with CoA ligases only (control construct) were extracted and subjected to HPLC analysis. An additional peak at retention time 38.7 min was observed in the extract containing the 18xCHS construct, but was not present in the extract containing the control construct, indicating that a new polyketide was biosynthesized when 2-fluorocinnamyl-CoA and butylmalonyl-CoA were supplemented to 18xCHS.

matrix of substrates used, at least 413 novel polyketides were generated.

2.2. Starter Acyl-CoA Substrate Preference. 18xCHS was able to use cinnamyl-CoA (Table S2 in the Supporting Information), phenylpropanoyl-CoA (Table S2 in the Supporting Information), benzoyl-CoA (Table S3 in the Supporting Information), phenylacetyl-CoA (Table S4 in the Supporting Information), bicyclic aromatic CoA (Table S4 in the Supporting Information), and saturated and unsaturated aliphatic CoA thioester derivatives (Table S5 in the Supporting Information) as starter substrates, in combination with various malonyl-CoA derivatives as the extender substrate (Figure 4). Interestingly, benzoyl-CoA derivatives were the most preferred starters (65.8% of the combinations led to new products formed), followed by unsaturated aliphatic CoA derivatives (61.7% of the combinations), phenylacetyl-CoA derivatives (51.4% of the combinations), cinnamyl-CoA derivatives (50% of the combinations), saturated aliphatic CoA derivatives (36.5% of the combinations), phenylpropanoyl-CoA derivatives (22.6% of the combinations), and bicyclic aromatic CoA derivatives (12.5% of the combinations).

Among the cinnamyl-CoA derivatives, meta-substituted and para-substituted derivatives were most preferred (62.5 and 60.7% of the combinations, respectively, led to new products formed), compared to ortho-substituted (25% of the combinations) and aliphatic chain-substituted (45.8% of the combinations) derivatives. This suggests that the presence of substituents at the ortho position may affect the interaction of the starter unit with the active-site residues of 18xCHS, causing ortho-substituted cinnamyl-CoA derivatives to be utilized less efficiently. This is consistent with previous work on alfalfa CHS by Jez and co-workers, which reported that the catalytic efficiency of alfalfa CHS is lower when 2-hydroxycinnamyl-CoA is used, compared to 3-hydroxycinnamyl-CoA and 4-hydroxycinnamyl-CoA.[22] Interestingly, 2-hydroxyphenylacetyl-CoA (reacted with 4 out of 12 extenders) was also less readily utilized by 18xCHS compared to 4-hydroxyphenylacetyl-CoA (reacted with 8 out of 12 extenders), which is structurally similar to, but shorter than *p*-coumaroyl-CoA by a carbon unit.

When substituent groups are present on the extender acyl-CoA substrate, cinnamyl-CoA derivatives are no longer strongly preferred. Instead, smaller and more flexible starters

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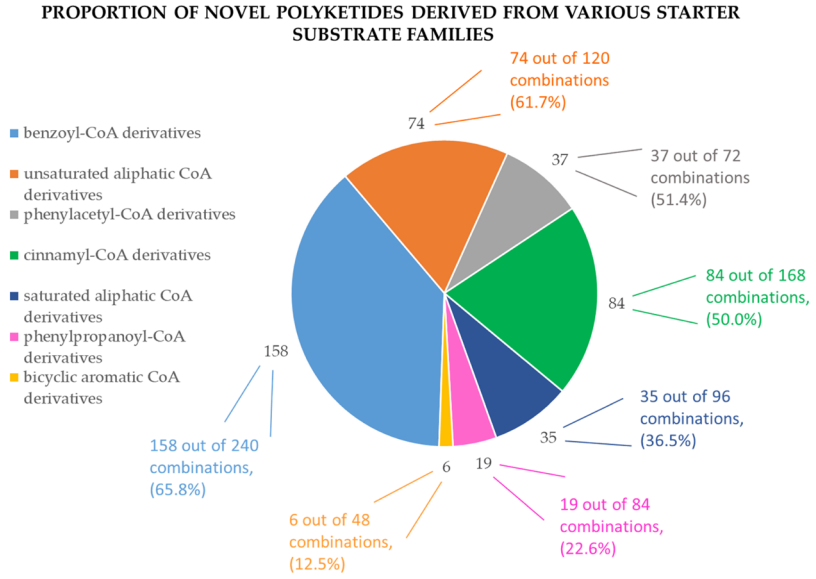

Figure 4. Substrate profile of 18xCHS based on the seven starter substrate families. Benzoyl-CoA derivatives were the most preferred starters while bicyclic aromatic CoA derivatives were least preferred by 18xCHS. A total of at least 413 novel polyketides were biosynthesized.

like benzoyl-CoA derivatives and unsaturated aliphatic CoA derivatives are more easily accepted by 18xCHS, together with unnatural malonyl-CoA derivatives to form unique polyketides. Most benzoyl-CoA derivatives were utilized as starters, regardless of the position of the substituent groups (ortho position: 63.9% of the combinations led to new products formed, meta position: 62.5% of the combinations, para position: 58.3% of the combinations), suggesting that the benzoyl-CoA backbone is small enough to participate in reactions with bulky extenders. For unsaturated aliphatic CoA derivatives, smaller starters with a butenoyl-CoA backbone were most preferred (70.8% of the combinations led to new products formed), followed by those with a pentenoyl-CoA backbone (62.5% of the combinations), and those with a hexenoyl-CoA backbone (41.7% of the combinations). This is consistent with previous studies on *Scutellaria baicalensis* CHS, which proposed that bulkier starter substrates contributed to unfavorable steric effects when additional substituent groups were present in the extender acyl-CoA used.[23]

Surprisingly, other than derivatives of the cognate substrates, 18xCHS could also utilize chemically distinct starter acyl-CoAs beyond its canonical substrate pool. In particular, we have shown for the first time that 18xCHS could accept bicyclic aromatic CoA thioesters together with malonyl-CoA derivatives to form novel polyketides (6 out of 48 combinations led to new products formed). This substrate promiscuity can be attributed to substrate mimicry, illustrating how slight substrate resemblance to the cognate substrates can play a role in starter unit recognition in 18xCHS and subsequently, the biosynthesis of novel polyketides (Figure 5).

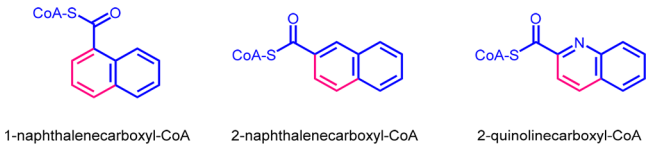

Figure 5. Structural mimicry of bicyclic aromatic CoA thioesters. The mimicry to phenylacetyl-CoA (for 1-naphthalenecarboxyl-CoA) and cinnamyl-CoA (for 2-naphthalenecarboxyl-CoA and 2-quinolinecarboxyl-CoA) is highlighted in blue.

2.3. Extender Acyl-CoA Substrate Preference. The repertoire of novel polyketides biosynthesized from the 69 × 12 assays also provided insights into the extender substrate preference of 18xCHS. By collating the number of starter acyl-CoAs that reacted with each extender substrate (Figure 2), the order of preference for extender acyl-CoA substrates for 18xCHS was established: 3-thiophenemalonyl-CoA > phenylmalonyl-CoA = fluoromalonyl-CoA > chloromalonyl-CoA > ethylmalonyl-CoA > malonyl-CoA > methylmalonyl-CoA > hydroxymalonyl-CoA > butylmalonyl-CoA = bromomalonyl-CoA > allylmalonyl-CoA > isopropylmalonyl-CoA. It was likely that the extender substrate preference of 18xCHS was dependent on the stability of the carbanion intermediate generated during catalysis.

When the extender acyl-CoA enters the active site of 18xCHS, it is decarboxylated to form a carbanion intermediate which can attack the starter unit or elongate polyketide bound to the catalytic cysteine residue in the active site. This carbanion intermediate is stabilized by a resonance effect in all the malonyl-CoA derivatives, aided by the nearby His303 and Asn336 residues (numbering in alfalfa CHS). Certain substituent groups in the malonyl-CoA derivatives can affect the degree of stabilization of the carbanion intermediate. For instance, 3-thiophenemalonyl-CoA and phenylmalonyl-CoA were strongly preferred compared to the other extenders (Figure 2), perhaps because of the presence of the aromatic ring which can confer additional stability via resonance effects (Figure 6).

Among the halogen-substituted malonyl-CoA derivatives, the fluoro-substituted malonyl-CoA derivative has the greatest inductive effect because of the high electronegativity of the fluorine atom. Thus, the carbanion intermediate is greatly stabilized and fluoromalonyl-CoA is the preferred extender, compared to chloromalonyl-CoA and bromomalonyl-CoA. This is a finding similar to the extender acyl-CoA substrate preference of a type III PKS from *Oryza sativa*, where fluoromalonyl-CoA is one of the most preferred extender compared to the other 11 that were tested.[24]

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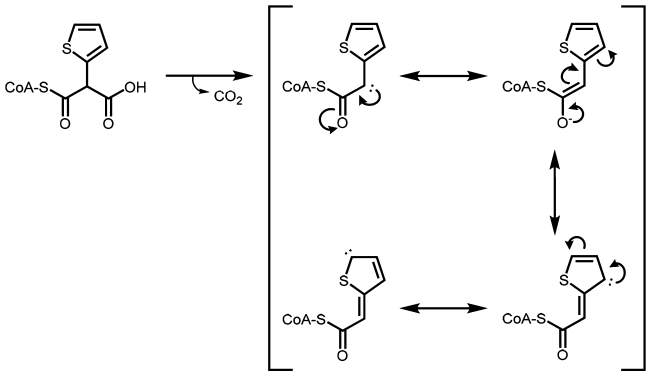

Figure 6. Resonance structures depicting the stabilization of the carbanion of 3-thiophenemalonyl-CoA after decarboxylation.

Compared to the aromatic malonyl-CoA derivatives, the aliphatic derivatives such as malonyl-CoA, methylmalonyl-CoA, ethylmalonyl-CoA, isopropylmalonyl-CoA, butylmalonyl-CoA, and allylmalonyl-CoA generated a range of novel polyketides with 21−39 starters out of the 69 starter substrates tested. However, the order of preference was not manifested as a significant trend. Taken together, by determining the substrate preference of 18xCHS, a library of novel polyketides with potential anti-ageing utility can be biosynthesized.

2.4. Mass Spectrometry Profiling of Novel Polyketides. The substrate profile of 18xCHS was confirmed by determining the mass of the polyketide products using mass spectrometry (MS). Selected combinations of starters and extenders were used to generate polyketides, which were then purified by HPLC and analyzed by MS. Most of the masses of the selected polyketides identified in this study matched their corresponding expected masses, and some of the potential chemical structures of the compounds were proposed (Table S6 in the Supporting Information). The exact identities of these products, however, require further validation by NMR analyses. In some instances, cyclization of the polyketide intermediate may not have occurred and the active-site cysteine-bound intermediate is hydrolyzed to form the corresponding acid, which may undergo decarboxylation to form a novel polyketide (Figure 7). In other cases, some of these polyketides could be truncated side products generated from the 18xCHS reaction, which include polyketides with fewer polyketide extension steps, or those that were biosynthesized through a lactonization, aldol or Claisen condensation of the linear polyketide intermediate (Figure S4 in the Supporting Information).

Many of the starter and extender substrates used in this research study have not been explored in previous studies, and the product library biosynthesized is expected to comprise novel polyketides with chalcone, stilbene, and lactone scaffolds. It has been shown that MS analysis provides an idea of the identity of the new products from precursor-directed combinatorial biosynthesis. These polyketides can then be further characterized by NMR to determine their chemical structure and properties. By obtaining a profile of novel polyketides, the compound library can subsequently be applied to drug screening efforts, such as the development of anti-ageing therapeutics.

2.5. Lifespan Assays Using Resveratrol or Novel Polyketides. Studies on resveratrol have revealed the compound to be a potential CR mimetic.[10] When 50 μM resveratrol was introduced to *C. elegans*, a significant lifespan extension was observed when compared to controls fed with dimethyl sulfoxide (DMSO) (Figure 8A). *C. elegans eat-2* mutants have a pharyngeal pumping defect, which results in compromised food intake[25] and consequently chronic CR. Similar to resveratrol-treated *C. elegans*, *eat-2* mutants have a longer lifespan compared to their wild-type counterparts (Figure 8B), suggesting an association between resveratrol and CR. Nevertheless, studies have shown that resveratrol is rapidly metabolized when ingested, and it has a broad spectrum of activity in various cellular components.[10,15] Hence, issues of bioavailability and adverse effects warrant the search for other CR mimetics that are more potent than resveratrol.

Of the 413 novel polyketides, 24 were tested for anti-ageing properties in a *C. elegans* lifespan assay (Table S7 in the Supporting Information). Most of the tested combinations had no significant effects on the lifespan of wild-type N2 *C. elegans* except for the products derived from 3-chlorocinnamyl-CoA + malonyl-CoA and 3-(3′-chloro-4′-methoxy)phenylpropanoyl-CoA + methylmalonyl-CoA which had toxic effects and led to a shorter lifespan compared to the control (Figure 9). Interestingly, worms treated with 3-(3′-chloro-4′-methoxy)-phenylpropanoic acid, methylmalonic acid, and the control *E. coli* construct had a mean lifespan of 26.5 days. This suggests that the acids used or the CoA esters produced could have some effects on the lifespan of wild-type *C. elegans*. More studies are needed to verify the biological effects of these compounds.

Although the 18xCHS strain can produce resveratrol when supplemented with *p*-coumaric acid and malonic acid, a significant lifespan extension effect was not observed. This is due to the low concentration of resveratrol biosynthesized by the *E. coli* constructs growing on the nematode growth media (NGM) agar plates (approximately 1 μM from HPLC analysis). Even though the concentration of novel polyketides being biosynthesized is low, we hope to identify anti-ageing compounds that can work well even at low doses. This may

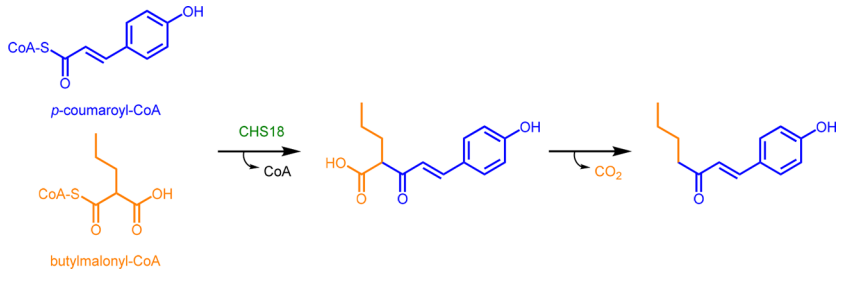

Figure 7. Potential polyketide formed from one unit of *p*-coumaroyl-CoA and butylmalonyl-CoA without cyclization.

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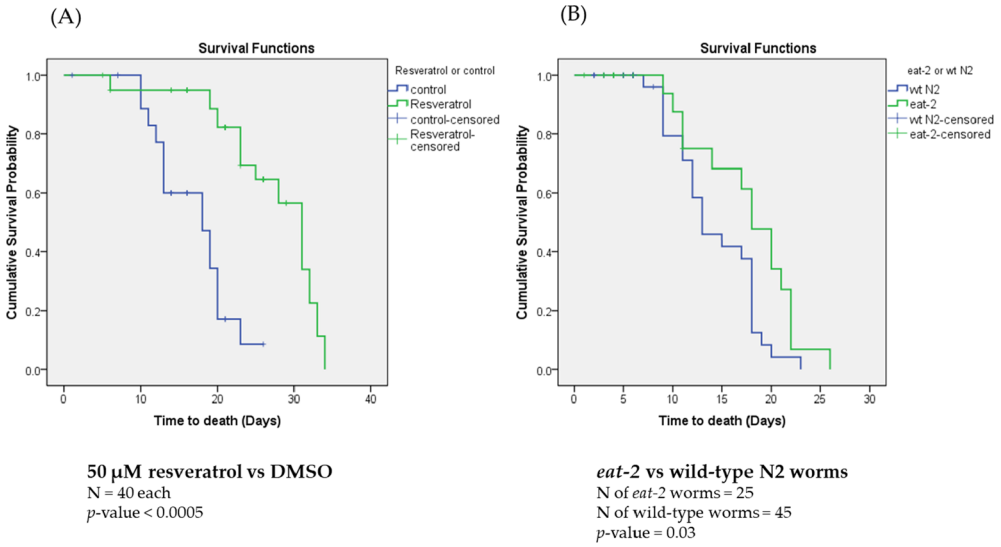

Figure 8. (A) Survival plots of *C. elegans* fed with 50 μM commercially available resveratrol (green) or 0.1% DMSO (blue). Worms exposed to 50 μM resveratrol had a significantly longer mean lifespan (27.4 days) compared to the DMSO control (17.1 days). (B) Survival plots of wild-type N2 (blue) and *eat-2* (green) *C. elegans*. *eat-2* mutants had a significantly longer mean lifespan (17.6 days) compared to the wild-type worms (14.3 days). The *p*-values were calculated using log rank test, and a *p*-value $\leq$ 0.05 is considered to be statistically significant.

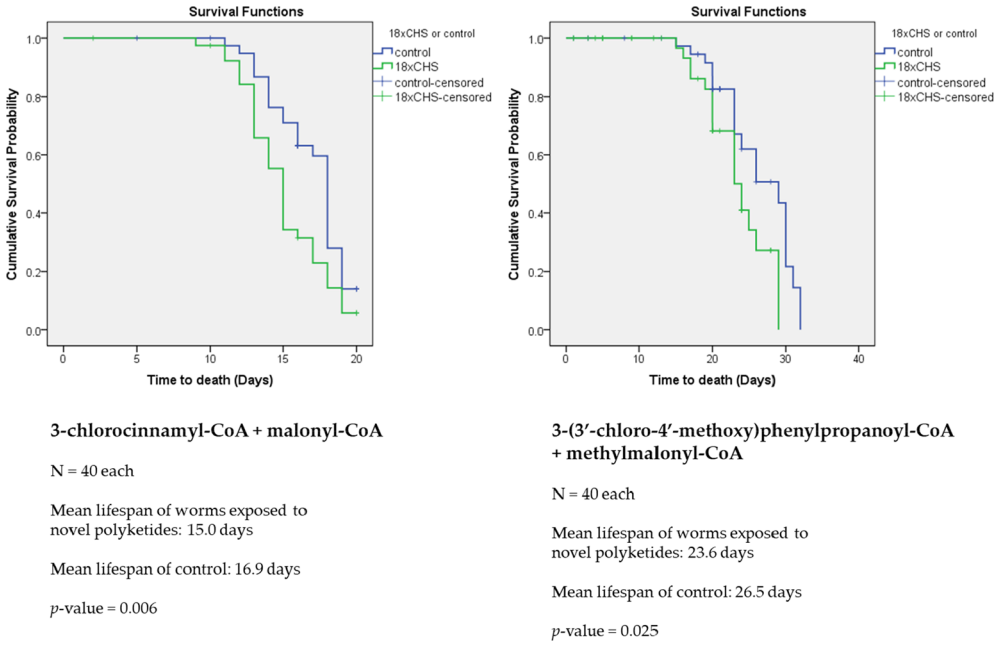

Figure 9. Survival plots of N2 *C. elegans* fed with either 18xCHS *E. coli* strain (green) or control *E. coli* strain (blue) supplemented with 3-chlorocinnamic acid + malonic acid (left) or 3-(3′-chloro-4′-methoxy)phenylpropanoic acid + methylmalonic acid (right). Worms exposed to the novel polyketides produced from these two combinations of starter and extender acids had a significantly shorter mean lifespan compared to the control which is exposed to the respective carboxylic acids and CoA esters only.

lead to the development of an anti-ageing drug lead that has minimal adverse effects common with resveratrol treatment.

2.6. *C. elegans* Respiration Assays Using Resveratrol or Novel Polyketides. Despite the utility of lifespan assays, they are rather time-consuming as the average lifespan of *C. elegans* is 3 weeks. Hence, there is a need to develop a higher throughput method to screen our library of compounds for anti-ageing properties. Since CR is known to promote longevity by modulating oxidative metabolism,[26] it would be logical to look at the mitochondrial activity of *C. elegans* when they were exposed to potential CR mimetics. Mitochondrial function assays were developed and optimized in order to establish a correlation between OCRs, mitochondrial activity, and longevity.

It was observed that *C. elegans* exposed to 8 days of 50 μM resveratrol have a higher average basal OCR, higher average maximal OCR, and higher spare respiratory capacity (Figure 10) when compared to their controls. This suggested that exposure to a potential CR mimetic for at least 8 days can counteract the age-related decline in the mitochondrial function in *C. elegans* by increasing the average basal respiration, average maximal respiration, and spare respiratory capacity. The increase in spare respiratory capacity was

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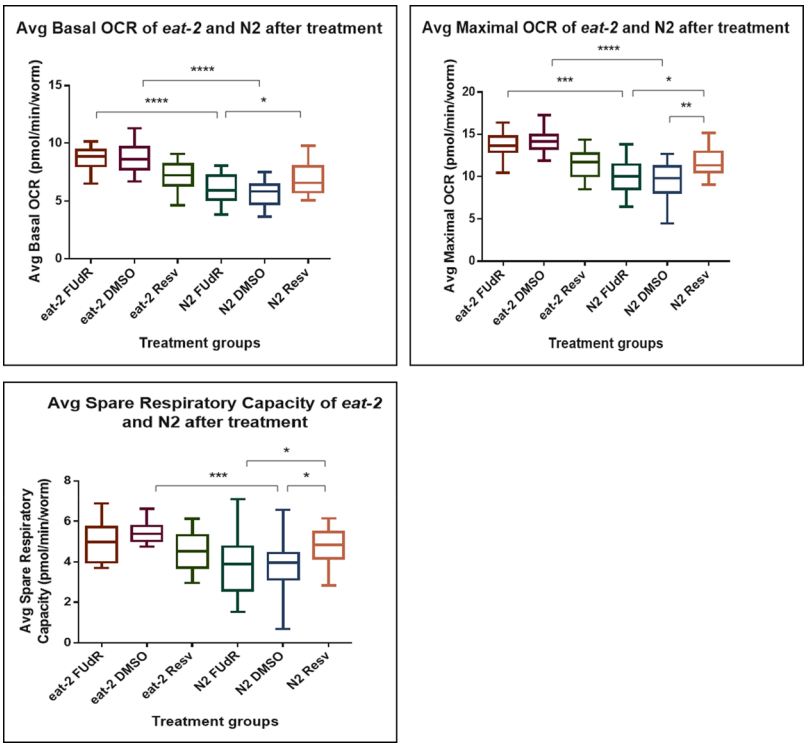

Figure 10. Wild-type N2 worms exposed to 8 days of 50 μM resveratrol had a significantly higher basal OCR, maximal OCR, and spare respiratory capacity compared to N2 worms exposed to 5-fluoro-2′-deoxyuridine (FUdR) or FUdR + 0.1% DMSO. Consistent with results using the CR mimetic resveratrol, *eat-2* mutants undergo chronic CR and have a higher basal respiration, maximal respiration, and spare respiratory capacity compared to wild-type *C. elegans*. Introducing 50 μM resveratrol to *eat-2* mutants does not improve their mitochondrial function further. FUdR: worms exposed to FUdR treatment only; DMSO: worms exposed to FUdR + 0.1% DMSO; Resv: worms exposed to FUdR + 50 μM resveratrol. *p-value $\leq$ 0.05; **p-value $\leq$ 0.01; ***p-value $\leq$ 0.001; ****p-value $\leq$ 0.0001.

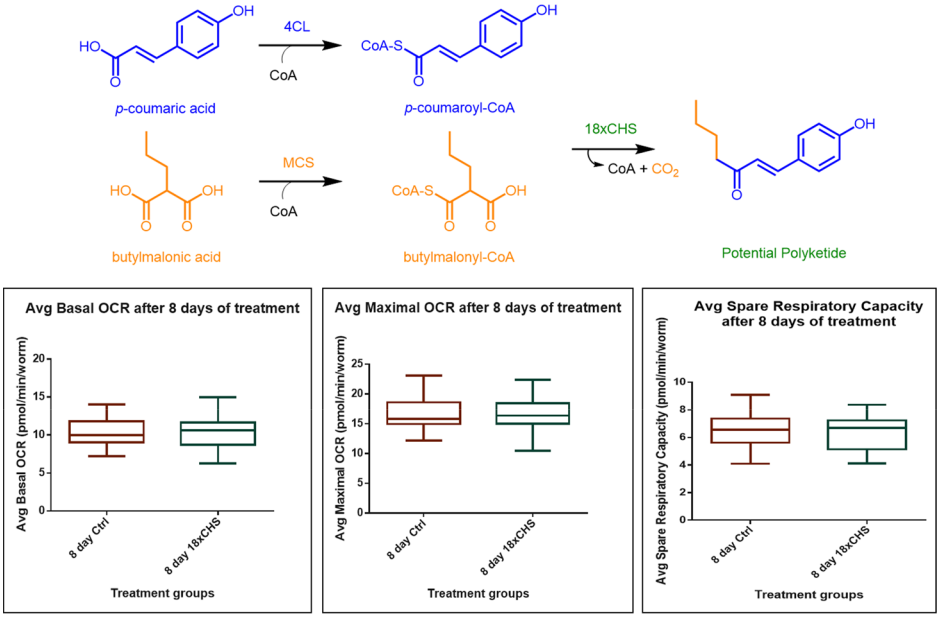

Figure 11. Butylmalonic acid and *p*-coumaric acid were incorporated into NGM agar together with either the control (Ctrl) or 18xCHS *E. coli* construct. *C. elegans* exposed to novel polyketides and CoA esters produced by the 18xCHS *E. coli* construct (contains CoA ligases + 18xCHS) do not have a higher basal respiration, maximal respiration, and spare respiratory capacity compared to *C. elegans* exposed to CoA esters only (control construct contains CoA ligases only).

indicative of improvements in the ability of the mitochondria to cope with changes in ATP demand.

Our findings supported a recent study which also observed an increase in the *C. elegans* basal mitochondrial respiration rate when the worms were exposed to resveratrol.[9] A likely hypothesis for this intriguing observation is that resveratrol can mimic a CR state, which causes metabolism to shift away from glycolysis toward respiration in order to maintain body

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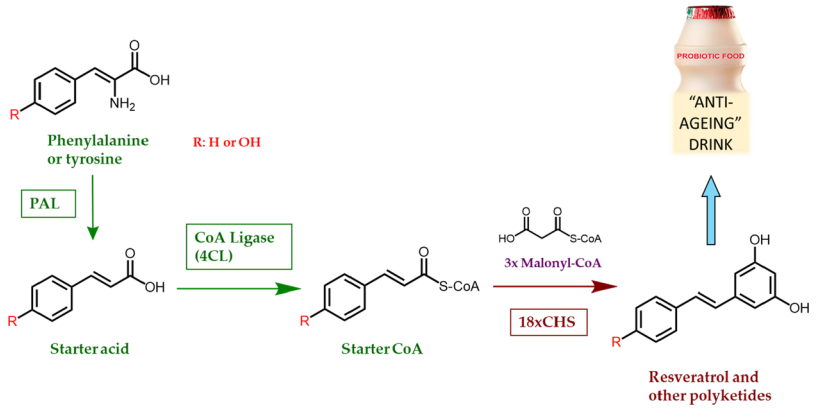

Figure 12. Combinatorial biosynthesis of resveratrol or other anti-ageing polyketides in *E. coli* Nissle.

function.[27,28] This eventually results in elevated electron transport, respiration, and ROS production. Low exposure to a greater oxidative stress triggers a secondary adaptive response called mitohormesis in the host's defence system, leading to better stress resistance and lifespan extension.[26,29]

In addition, *C. elegans eat-2* mutants exhibited a higher basal OCR, maximal OCR, and spare respiratory capacity compared to wild-type worms (Figure 10). This is consistent with the effects of the CR mimetic resveratrol on mitochondrial function, highlighting the potential of mitochondrial function assays in screening for CR mimetics. Nevertheless, subjecting *eat-2* mutants to resveratrol treatment did not result in further increases in basal OCR, maximal OCR, and spare respiratory capacity. This suggested a strain on the mitochondrial function of the *eat-2* mutants which were already experiencing chronic CR; subjecting the mutants to further CR mimetic exposures could have detrimental effects on mitochondrial function. Indeed, *eat-2* mutants exposed to 50 μM resveratrol treatment exhibited a shorter mean lifespan (15.9 days) as compared to *eat-2* mutants which were exposed to 0.1% DMSO (20.0 days) (p-value = 0.006).

CR in *C. elegans* has been demonstrated to induce catalase enzyme activity and improve oxidative stress resistance, resulting in a longer lifespan.[26] Sufficient evidence has pointed to the fact that resveratrol can mimic some aspects of CR, and can extend the lifespan of model organisms like *C. elegans*, although the detailed mechanism of action is still unknown. This study, along with research by other researchers,[28,30,31] suggests that lifespan, mitochondrial function, CR, and CR mimetics are intricately linked.

As a proof of concept, a few novel polyketides from our compound library were tested in mitochondrial function assays, but so far, none of them significantly improved the mitochondrial function of wild-type *C. elegans*. For instance, when butylmalonic acid and *p*-coumaric acid were incorporated into the NGM agar together with either the control or the 18xCHS *E. coli* construct, N2 worms exposed to the novel polyketides, butylmalonyl-CoA, and *p*-coumaroyl-CoA did not have a significantly higher basal respiration, maximal respiration, and spare respiratory capacity compared to worms exposed to the CoA esters only (Figure 11). Interestingly, the toxic polyketides derived from 3-chlorocinnamyl-CoA + malonyl-CoA and 3-(3′-chloro-4′-methoxy)-phenylpropanoyl-CoA + methylmalonyl-CoA did not have any effect on the mitochondrial respiration of *C. elegans*, suggesting that other pathways are affected by the toxicity instead.

Taken together, the development of mitochondrial function assays allows us to screen our libraries for anti-ageing compounds more efficiently without having to conduct lengthy lifespan assays. The anti-ageing properties of selected drug hits can subsequently be validated by conventional lifespan assays and other functional assays.

2.7. Synthetic Enzymology in a Probiotic System. Once a promising anti-ageing drug with minimal adverse effects is developed, we can either deploy the drug directly in humans or incorporate the combinatorial biosynthetic pathway (of the drug) into a probiotic strain such as *E. coli* Nissle 1917. Thus, other than preventing diseases of the gastrointestinal tract, the probiotic Nissle strain can lead to a sustainable biosynthesis of anti-ageing drugs within the human host. Although more research and development is required, we have taken the first steps toward using probiotics in anti-ageing drug delivery: we have constructed a combinatorial biosynthetic pathway involving phenylalanine ammonia lyase (PAL) as an enzyme upstream of the acid-CoA ligases and 18xCHS. PAL is able to deaminate endogenous phenylalanine and tyrosine to cinnamic acid and *p*-coumaric acid, respectively, while 4CL can make use of these carboxylic acids to generate starter acyl-CoAs for 18xCHS for the biosynthesis of potential anti-ageing compounds (Figure 12). As a proof of concept, our engineered *E. coli* Nissle strain is capable of producing resveratrol at 4.1 mg/L (Figure 13), a concentration comparable to that found in red wine (average 1.9 ± 1.7 mg/L).[32] Nutraceuticals are food or formulations of food that provide health or medical benefits such as the prevention or treatment of diseases.[33] This engineered strain of probiotic *E. coli* Nissle presents a potential vehicle for delivery of anti-ageing compounds to the human body without the need to take drugs orally or intravenously.

3. CONCLUSIONS

In summary, a major limitation in anti-ageing therapeutic development lies in the production of novel and bioactive lead compounds for screening. By establishing a combinatorial biosynthetic route in *E. coli* and exploring the substrate promiscuity of 18xCHS, 413 potential anti-ageing compounds were biosynthesized. Resveratrol is postulated to be a CR mimetic and has been shown to extend the lifespan of several model organisms. Using the OCR profile of worms exposed to 8 days of 50 μM resveratrol as a blueprint, libraries of novel polyketides can be screened to identify potential CR mimetics. A molecular screening platform where compound libraries are screened in *C. elegans*-based mitochondrial function assays can

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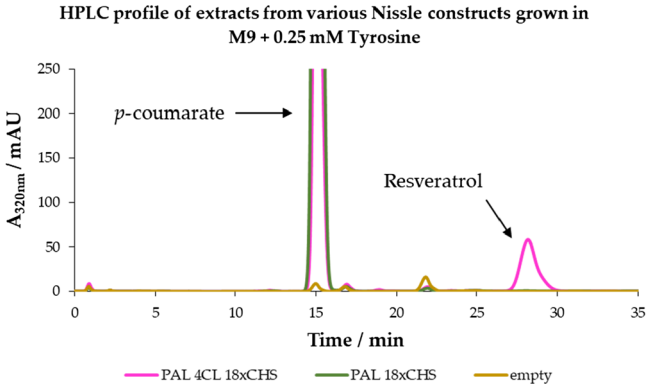

Figure 13. HPLC profile of organic extracts from *E. coli* Nissle constructs grown in M9 supplemented with 0.25 mM tyrosine. A peak corresponding to *p*-coumarate is present in extracts from Nissle constructs containing PAL and absent in the extract from the Nissle construct with an empty vector, indicating that PAL is active. In addition, resveratrol is produced by the Nissle construct containing PAL, 4CL, and 18xCHS.

thus be developed. We believe that these studies will augment current understanding toward polyketide compound library biosynthesis and provide an additional tool for anti-ageing therapeutic development.

4. MATERIALS AND METHODS

4.1. Reagents. The carboxylic acid substrates for acid-CoA ligases were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA), Tokyo Chemical Industry Co. (Tokyo, Japan), Extrasynthese Co. (Genay Cedex, France), and Lier Chemical Co. (Sichuan, People's Republic of China). A total of 81 carboxylic acids were utilized as precursors for acyl-CoA thioester biosynthesis catalyzed by four acyl-CoA ligases.[34] The acids were from the malonate, cinnamate, phenylpropanoate, benzoate, phenylacetate, naphthalene, quinoline, saturated aliphatic, and unsaturated aliphatic families.

A total of 12 malonate-type acids were used as extender substrates for the precursor-directed combinatorial biosynthesis—malonic acid, methylmalonic acid, ethylmalonic acid, isopropylmalonic acid, butylmalonic acid, allylmalonic acid, hydroxymalonic acid, fluoromalonic acid, chloromalonic acid, bromomalonic acid, phenylmalonic acid, and 3-thiophenemalonic acid. Starter substrates were composed of 69 carboxylic acids—(cinnamate type) cinnamic acid, 2-fluorocinnamic acid, 3-fluorocinnamic acid, 4-fluorocinnamic acid, α-fluorocinnamic acid, 3-chlorocinnamic acid, 3-chloro-4-methoxycinnamic acid, 4-chlorocinnamic acid, 2-hydroxycinnamic acid, 4-hydroxycinnamic acid (also known as *p*-coumaric acid), 3-methoxy-4-hydroxycinnamic acid, 4-methoxycinnamic acid, 4-methylcinnamic acid, α-methylcinnamic acid; (phenylpropanoate type) 3-phenylpropanoic acid, 3-(3′-chloro)phenylpropanoic acid, 3-(3′-chloro-4′-methoxy)phenylpropanoic acid, 3-(3′,4′-dihydroxy)phenylpropanoic acid, 3-(3′-methoxy)-phenylpropanoic acid, 3-(4′-methoxy)phenylpropanoic acid, 3-(4′-fluoro)phenylpropanoic acid; (benzoate type) benzoic acid, 2-fluorobenzoic acid, 3-fluorobenzoic acid, 4-fluorobenzoic acid, 2,6-difluorobenzoic acid, 2-chlorobenzoic acid, 3-chlorobenzoic acid, 4-chlorobenzoic acid, 2-bromobenzoic acid, 3-bromobenzoic acid, 4-bromobenzoic acid, 2-iodobenzoic acid, 2-hydroxybenzoic acid, 2,3-dihydroxybenzoic acid, 2,4-dihydroxybenzoic acid, 2,5-dihydroxybenzoic acid, 2-methoxybenzoic acid, 2-methylbenzoic acid, 3-aminobenzoic acid, 4-aminobenzoic acid; (phenylacetate type) phenylacetic acid, 2-hydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 4-methoxyphenylacetic acid, phenoxyacetic acid, phenylpyruvic acid; (naphthalene and quinoline type) 1-naphthalenecarboxylic acid, 2-naphthalenecarboxylic acid, 2-quinolinecarboxylic acid, 3-quinolinecarboxylic acid; (saturated aliphatic type) propanoic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic acid, decanoic acid; and (unsaturated aliphatic type) 2-butenoic acid, 2-methyl-2-butenoic acid, 3-methyl-2-butenoic acid, 3-butenoic acid, 2-pentenoic acid, 3-pentenoic acid, 4-pentenoic acid, 3-methyl-4-pentenoic acid, 3-hexenoic acid, and 5-hexenoic acid. The chemical structures of all substrates tested are depicted in Table S1 in the Supporting Information.

4.2. Establishing a Combinatorial Biosynthetic Route in *E. coli*. The type III PKS used in this research study is a mutant CHS with STS activity from *Medicago sativa* that was previously created by Austin et al. in 2004. Alfalfa CHS (GI: 166363) was cloned from *M. sativa* cDNA library into a modified pET-15b vector (Tom-15b vector) containing ten histidine tags at the N-terminus of the protein. PCR-mediated mutagenesis was conducted with reference to the paper published by Austin et al. (2004) in order to replicate the same mutations in alfalfa CHS, resulting in 18xCHS which has STS activity. In particular, the following sites were mutated—D98A, V100L, V101A, V102M, T131S, S133T, G134T, V135P, M137L, Y158V, M159G, M160V, Y161F, Q163H, L268K, K269G, D270A, and G273D.[21]

The cloning of the four acyl-CoA ligases were previously reported.[34] They are MCS (GI: 3982573) from *Rhizobium trifolii*, phenylacetate-CoA ligase (PCL) (GI: 1102907) from *Streptomyces coelicolor* A3(2), benzoate-CoA ligase (BZL) (GI: 1040685) from *Rhodopseudomonas palustri*, and 4-coumarate-CoA ligase (4CL) (GI: 12229632) from *Nicotiana tabacum*. In order to establish a combinatorial biosynthetic route in *E. coli*, three CoA ligases (4CL, PCL, and BZL) involved in the generation of starter CoA thioesters were subcloned into the multiple cloning site 1 of a pRSFDuet-1 vector (Novagen) separately. To produce the extender CoA thioesters, MCS and PCL were each subcloned into the MCS2 region of the same vector. Thereafter, the constructed plasmid was co-transformed with the Tom-15b vector containing 18xCHS into *E. coli* Rosetta II (DE3) strain (Novagen). Depending on the identity of the starter and extender acids, *E. coli* harboring the appropriate combination of CoA ligases was used for the in vivo precursor-directed combinatorial biosynthesis of polyketides (see Table S1 in the Supporting Information). An *E. coli* construct without the 18xCHS gene was also prepared to serve as a control for the subsequent detection of novel polyketides. A matrix of 69 starter acids and 12 extender acids were separately introduced to the engineered *E. coli* host cells, giving a possible combination of 828 substrate profiles in total.

An *E. coli* Nissle 1917 Δ*alr* Δ*dadX* strain which requires 50 μg/mL D-alanine for growth and an *alr*+ plasmid (pEaaK) for complementation was previously described.[35] PAL (GenBank: X51513.1) from *Rhodosporidium toruloides*, 4CL, and 18xCHS were cloned into the pEaaK vector under a constitutive J23119 promoter, TTGACAGCTAGCTCAGTCCTAGGTATAATGCTAGC. *E. coli* Nissle 1917 Δ*alr* Δ*dadX* strain complemented with the pEaaK vector construct was grown in LB or M9 minimal medium in the absence of exogenous D-alanine.

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4.3. In Vivo Precursor-Directed Combinatorial Biosynthesis of Polyketides and HPLC Analysis. The *E. coli* host cells harboring the biosynthetic genes were first grown in LB containing 30 μg/mL kanamycin, 34 μg/mL chloramphenicol, and 100 μg/mL ampicillin at 25 °C. When OD_{600nm} reached 0.6, protein expression was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 16 h. The cells were subsequently pelleted and resuspended in M9 minimal medium containing antibiotics, 0.1 mM IPTG, 1 mM starter acid, and 1 mM extender acid, and incubated at 25 °C for a further 72 h. The supernatant was acidified to pH 3.0 with 6 M HCl and extracted with ethyl acetate thrice. The organic solvent was subsequently removed by a vacuum concentrator, and the residue was dissolved in 100 μL DMSO for HPLC analysis.

The Atlantis analytical C18 reverse-phase column (Waters) was first equilibrated with 10% acetonitrile, 0.1% trifluoroacetic acid (TFA) in water at a flow rate of 1 mL/min for 15 min. The sample (10 μL) was then loaded onto the column, and the mobile phase was changed to 50% acetonitrile, 0.1% TFA in water under a linear gradient over a period of 40 min at a 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 230, 280, and 320 nm. Chromatogram peaks that were present (minimum absorbance of 20 mAU) in the extracts of constructs containing 18xCHS but absent in the extracts of control constructs indicate the occurrence of polyketide biosynthesis.

4.4. MS Profiling of Novel Polyketides. HPLC−MS/MS analysis of the purified polyketides was carried out on QTOF 6550 with iFunnel and turbo ion spray, connected to UHPLC 1290 (Agilent, Singapore). The Phenomenex Synergy-Polar C18, with a 2.1 mm × 50 mm column and a 3 μm particle size (Phenomenex, US), was first equilibrated with 5% acetonitrile, 0.2% formic acid in water for 1 min at a flow rate of 600 μL/min. The samples were dissolved in 100 μL acetonitrile, and 2 μL was loaded for the analysis. Separation of the polyketides was performed with a gradient from 5% acetonitrile, 0.2% formic acid in water to 90% acetonitrile, 0.2% formic acid in water over 5 min; 90% acetonitrile, 0.2% formic acid in water was then maintained for another 1 min, with the column temperature set at 40 °C throughout the run. The mass spectrometer was set to an MS scan range of 100−1400 m/z at 1 scan/s, and the three most intense precursor ions were selected for fragmentation at a fixed collision energy of 35. Data were recorded with MassHunter acquisition B6.0 (Agilent) and analyzed with MassHunter Qualitative Analysis software version 6 (Agilent). After MS detection, NMR analyses of selected compounds were carried out by the Nuclear Magnetic Resonance Laboratory (Department of Chemistry, National University of Singapore).

4.5. Lifespan Assays Using Resveratrol or Novel Polyketides. Wild-type *C. elegans* N2 strain and *eat-2* mutant strain were used in lifespan assays. *C. elegans* were maintained according to Stiernagle (2006) with minimal changes.[36] Resveratrol (50 μM, TCI Co.) or DMSO (as a control) was incorporated into 5 cm NGM agar plates by another lab member to ensure blinding of the assays. NGM plates used for lifespan assays also contained 90 μM FUdR (Sigma-Aldrich Co.) to ensure batch synchrony by preventing cell division and growth in newly hatched *C. elegans*.[37] Cultures of *E. coli* OP50 strain were grown in LB overnight at 25 °C and concentrated 10-fold by centrifugation and resuspension in a smaller volume of LB. OP50 was then spotted onto NGM agar plates as a food source for *C. elegans* and allowed to dry overnight at room temperature. *C. elegans* were cultured at 20 °C on the various NGM agar plates containing a lawn of *E. coli* OP50. The worms were examined daily and scored as dead if no response was observed when they were gently prodded with a platinum wire. *C. elegans* that were lost because of crawling on the walls of the agar plates were omitted from analysis. Survival analysis was conducted using the log rank test in the IBM SPSS Statistics 22 program.

For lifespan assays using novel polyketides, selected combinations of 0.5 mM starter acids and 1 mM extender acids together with 90 μM FUdR, 30 μg/mL kanamycin, 34 μg/mL chloramphenicol, 100 μg/mL ampicillin, and 0.1 mM IPTG were incorporated into NGM agar plates. The corresponding CoA ligases + 18xCHS *E. coli* constructs and constructs without 18xCHS were grown in LB with antibiotics overnight at 25 °C by another lab member to ensure blinding of the lifespan studies. Protein expression was induced by 0.1 mM IPTG for 3 h at 25 °C, and 0.5 mM starter acids and 1 mM extender acids were subsequently introduced to the culture. After incubation at 25 °C for another 3 h, the *E. coli* culture was concentrated 20-fold, seeded onto the respective NGM plates, and allowed to dry overnight at room temperature as the food source which can expose *C. elegans* to the biosynthesized polyketides concurrently. The lifespans of wild-type N2 *C. elegans* exposed to the CoA thioesters and novel polyketides were compared to the lifespans of N2 worms exposed to CoA thioesters only, using the log rank test.

4.6. *C. elegans* Respiration Assays Using Resveratrol or Novel Polyketides. Oxygen consumption was measured using the Seahorse XFe24 Extracellular Flux Analyzer (Seahorse Bioscience). *C. elegans* were recovered from NGM plates using M9 minimal medium and washed four times to eliminate eggs and residual bacteria.[38] The worms were transferred to 24-well seahorse islet plates (10−25 worms in 675 μL M9 minimal medium per well) and covered with an islet capture screen. Basal oxygen consumption was measured for six cycles before performing carbonylcyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP) treatment at a final concentration of 20 μM. Maximal oxygen consumption was subsequently measured for eight cycles. The Kruskal−Wallis test in the GraphPad Prism 6 program was used to determine the significance of differences in medians. Subsequently, Dunn's multiple comparisons test was used to determine if the medians varied significantly between any two selected groups.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b01620.

Structure and names of the 81 carboxylic acids used; biosynthesis of resveratrol by 18xCHS; substrate profile consisting of the various carboxylic acids utilized by the modular coupling of acid-CoA ligases and 18xCHS; some of the potential structures of the biosynthesized polyketides; different modes of cyclization of the linear polyketide intermediate to form novel polyketides; and the results of the lifespan assays conducted for 24 combinations of starters and extenders (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ACP, acyl carrier protein; BZL, benzoate-CoA ligase; CHS, chalcone synthase; 4CL, 4-coumarate-CoA ligase; CoA, coenzyme A; CR, caloric restriction; DMSO, dimethyl sulfoxide; ETC, electron transport chain; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; FUdR, 5-fluoro-2′-deoxyuridine; MCS, malonyl-CoA synthetase; NGM, nematode growth medium; OCR, oxygen consumption rate; PAL, phenylalanine ammonia lyase; PCL, phenylacetate-CoA ligase; PKS, polyketide synthase; ROS, reactive oxygen species; STS, stilbene synthase; TFA, trifluoroacetic acid

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