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ENGINEERING OF POLYKETIDE BIOSYNTHETIC PATHWAYS FOR

BIOACTIVE MOLECULES

by

Siyuan Wang

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Biological Engineering

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ABSTRACT

Engineering of polyketide biosynthetic pathways for bioactive molecules

by

Siyuan Wang, Doctor of Philosophy

Utah State University, 2016

Major Professor: Jixun Zhan

Department: Biological Engineering

Polyketides are a large group of structurally diverse natural products that have shown a variety of biological activities. These molecules are synthesized by polyketide synthases (PKSs). PKSs are classified into three types based on their sequence, primary structure, and catalytic mechanism. Because of the bioactivities of polyketide natural products, this study is focused on the engineering of PKS pathways for efficient production of useful bioactive molecules or structural modification to create new molecules for drug development.

One goal of this research is to create an efficient method to produce pharmaceutically important molecules. Seven biosynthetic genes from plants and bacteria were used to establish a variety of complete biosynthetic pathways in *Escherichia coli* to make valuable plant natural products, including four

phenylpropanoid acids, three bioactive natural stilbenoids, and three natural curcuminoids. A curcumin analog dicafferolmethane was synthesized by removing a methyltransferase from the curcumin biosynthetic pathway. Furthermore, introduction of a fungal flavin-dependent halogenase into the resveratrol biosynthetic pathway yielded a novel chlorinated molecule 2-chloro-resveratrol. This demonstrated that biosynthetic enzymes from different sources can be recombined like legos to make various plant natural products, which is more efficient (2-3 days) than traditional extraction from plants (months to years). Phenylalanine ammonia-lyase (PAL) is a key enzyme involved in the first biosynthetic step of some plant phenylpropanoids. Based on the biosynthetic pathway of curcuminoids, a novel and efficient visible reporter assay was established for screening of phenylalanine ammonia-lyase (PAL) efficiency in *Escherichia coli*.

The other goal of this research is to characterize and engineer natural product biosynthetic pathways for new bioactive molecules. The biosynthetic gene cluster of the antibacterial compound dutomycin was discovered from *Streptomyces minoensis* NRRL B-5482 through genome sequencing. Confirmation of the involvement of this gene cluster in dutomycin biosynthesis and creation of a series of new molecules were successfully conducted by rationally modifying the biosynthetic pathway. More importantly, a new demethylated analog of dutomycin was found to have much higher

antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*.

(174 pages)

PUBLIC ABSTRACT

Engineering of polyketide biosynthetic pathways for bioactive molecules

by

Siyuan Wang, Doctor of Philosophy

Utah State University, 2016

Major Professor: Jixun Zhan

Department: Biological Engineering

This study is focused on engineering of natural product biosynthetic pathways for efficient production of pharmaceutically important molecules or generation of new bioactive molecules for drug development.

Plant natural products are an important source of therapeutics, such as paclitaxel (anticancer) and artemisinin (anti-malarial). Production of plant natural products relies on conventional plant cultivation and solvent extraction, which is time-consuming and cost-ineffective. This work built a biosynthetic platform in *Escherichia coli* using seven biosynthetic genes from plants and bacteria, which were used to make valuable compounds such as the strong antioxidant resveratrol and anti-inflammatory agent curcuminoids. Through different combinations of these genes, *E. coli* was engineered

to produce four phenylpropanoid acids (cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid), three bioactive natural stilbenoids (resveratrol, piceatannol and pinosylvin), and three natural curcuminoids (curcumin, bisdemethoxycurcumin and dicinnamoylmethane). “Unnatural” natural products including dicaffferolmethane and 2-chloro-resveratrol were also generated by modifying the existing pathways. Based on the color of curcuminoids, a novel and efficient visible reporter assay was established for screening of phenylalanine ammonia-lyase (PAL), an enzyme involved in the first biosynthetic step of many plant natural products.

Actinomycetes are a group of bacteria well-known for the production of antibiotics. A strong antibacterial agent, dutomycin, was discovered from *Streptomyces minoensis* NRRL B-5482. Through genome sequencing and targeted gene disruption, a type II polyketide biosynthetic gene cluster was found to be responsible for the assembly of dutomycin. Several key enzymes in the pathway were functionally characterized and a series of new analogs were generated, including a new compound with more promising antibacterial activity.

Metabolic engineering of natural product biosynthetic pathways showed its promise for creating and producing valuable compounds with chemical diversity for drug discovery. This research has biosynthesized 17 valuable molecules with medicinally relevant bioactivities. The results from this study provided important

platforms and technical basis for further engineering polyketide biosynthetic pathways to produce valuable bioactive molecules and generate novel analogs for bioactivity screening and drug development.

DEDICATION

I would like to dedicate my doctoral dissertation to my family. A special feeling of appreciate to my parents Dr. Hongzhi Wang and Xiaowen Tang for their support and encouragement.

To my fiancée, Dr. Chun Yang, and her family, Liping Yang and Sulian Zhou for many years spent waiting patiently and tireless support.

Siyuan Wang

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Siyuan Wang

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ABBREVIATIONS

ACP	Acyl carrier protein
ARO	Aromatase
AT	Acyl transferase
ATCC	American Type Culture Collection
CLF	chain length factor
CoA	Coenzyme A
COMT	Catechol-O-methyltransferase
CURS	curcumin synthase
CUS	curcuminoid synthase
CYC	cyclase
C3H	4-coumarate 3-hydroxylase
DCS	diketide-CoA synthase
ER	enoyl reductase
g	gram
GT	Glycosyltransferase
HCA	hydroxycinnamic acids
HPLC	high performance liquid chromatography
IPTG	isopropyl β -D-1-thiogalactopyranoside
KR	ketoreductase
KS	ketosynthase

L	liter
LB	Luria Bertani broth
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MS	malt soybean broth
MT	methyltransferase
NMR	nuclear magnetic resonance
NRRL	Northern Regional Research Laboratory
ORF	open reading frame
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PKSs	polyketide synthases
STS	stilbene synthase
TAL	tyrosine ammonia-lyase
USDA	United States Department of Agriculture
YM	yeast extract/malt extract
3-O-GT	3-O-glucosyltransferase
4CL	4-coumaroyl coenzyme A ligase

CHAPTER 1 INTRODUCTION

1.1 Background

Polyketides are a class of secondary metabolites produced by bacteria, fungi, and plants, such as rapamycin, erythromycin, and lovastatin. They draw more and more attention from scientists and engineers in the biomedical field due to their biological activities, potential for drug discovery, and commercial value. These natural products have displayed a variety of biological activities, such as antibacterial, anticancer, antifungal, antiparasitic, coccidiostats, and immunosuppressive properties. Engineering of polyketide biosynthetic pathways allows the generation of novel compounds that are difficult to be synthesized by other methods. Type I PKSs are multifunctional enzymes that are arranged into modules with different catalytic domains. Ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) are the essential domains for polyketide chain elongation. Other functional domains like ketoreductase (KR), enoyl reductase (ER), dehydratase (DH), and methyltransferase (MT) may be involved according to different polyketide structures. Type I PKSs are found in bacteria, fungi, and other organisms.

Type II PKSs are aggregates of small discrete proteins with different mono-functions. This group of PKSs is essentially involved in the biosynthesis of bacterial aromatic polyketides [1], such as oxytetracycline [2] and pradimicin [3-5]. The minimal PKS of type II PKSs is composed of KS_{α} , KS_{β} (chain length factor, CLF)

and ACP. The nascent polyketide chain synthesized by the minimal PKS is subjected to further modifications by KR, cyclase (CYC), methyltransferase (MT) and glycosyltransferase (GT). The polyketide backbone is generated by condensation of an acyl starter unit and multiple malonyl-CoA extender units. In addition to malonyl-CoA that is a common starter and extender unit, other CoA substrates such as fatty acyl-CoAs and benzoyl-CoA also act as starter units in aromatic polyketide biosynthesis [1-5]. This feature enables the structural diversity and unique biological activities of aromatic polyketides. Based on the polyphenolic ring system and their biosynthetic pathways, bacterial aromatic polyketides can be classified into different subfamilies, such as anthracyclines, angucyclines, aureolic acids, tetracyclines, tetracenomycins, pradimicin-type polyphenols, and benzoisochromanequinones [6].

Anthracyclines are ranked among the most effective anticancer drugs that have been developed [7]. The first two anthracyclines discovered were named rhodomycin and cinerubicin [8]. A few years later, daunorubicin (trade name Daunomycin) was isolated from *Streptomyces peucetius* in the 1950s by Farmitalia Research Laboratories as well [9]. Anthracyclines inhibit DNA and RNA synthesis and topoisomerase II enzyme to prevent the rapidly growth of cells [10-12]. Also, they can generate free oxygen radicals to damage the DNA, protein and cell membranes [10]. Hence, several anthracyclines, including daunorubicin, epirubicin, doxorubicin and aclacinomycin, are used in clinic for cancer chemotherapy. The main producer of

anthracycline polyketides in the nature is streptomycetes, which are well-known producers of structurally and functionally diverse molecules [13,14].

Dutomycin (Fig. 1) is an anthracycline that was first isolated from soil bacteria *Streptomyces sp.* 1725 from a soil sample collected in Yunnan, China [15]. This compound showed strong *in vitro* cytotoxic activity against leukemia P388 [15]. Dutomycin consists of a tetracyclic anthraquinone core structure and associated sugar moieties, which is similar to other anthracycline antibiotics such as polyketomycin, daunorubicin, epirubicin, doxorubicin and aclacinomycin. All of these compounds are generated by the corresponding producers through a type II polyketide biosynthetic pathway [16]. Dutomycin is structurally similar to polyketomycin from *Streptomyces sp.* MK277-AF1, which showed activity against Gram-positive bacteria including the multi-drug-resistant strain methicillin-resistant *Staphylococcus aureus* (MRSA) [17].

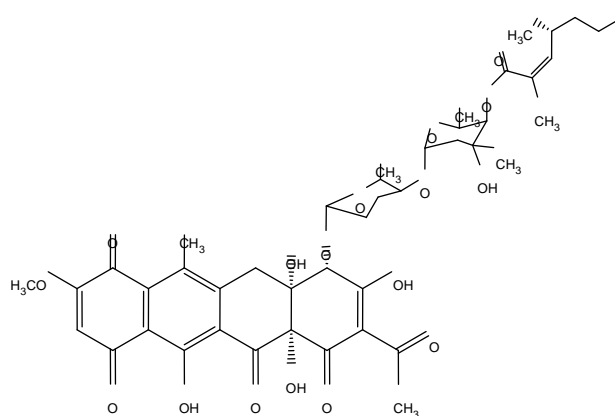


Fig. 1 The structure of dutomycin.

Type III PKSs are single enzymes can conduct substrate priming, polyketide

chain extension, and cyclization to form polyketide products. They are small dimeric proteins (40-45 kDa) that catalyze iterative condensation reactions with malonyl-CoA. In recent years, type III PKSs, especially plant type III PKSs, drew more and more attentions since their applications in medicine and agriculture. Plant type III PKSs have been extensively studied since the first type III PKS was discovered in the 1970s [18]. The major compounds produced by plant type III PKSs are flavonoids, stilbenoids and curcuminoids.

Stilbenoids (Fig. 2), containing a C6-C2-C6 structure, are a class of plant secondary metabolites which are often involved in injury control when the plant is threatened by bacteria or fungi [19]. These molecules have shown a variety of biological properties, such as antioxidant, antibacterial [20], antifungal [21], anticancer, anti-inflammatory [22] and anti-aging activities [23]. Their biosynthesis has been studied in plants [24], yeast and mammal cells [25]. Stilbene synthase (STS) is a type III PKS that plays an essential role in the biosynthesis of stilbenoids. In 2003, Becker J. et al. first reported the reconstruction of a biosynthetic pathway consisting of 4-coumaroyl coenzyme A ligase (4CL) and STS in *S. cerevisiae* to produce resveratrol [26]. When 5 mM *p*-coumaric acid was supplemented, resveratrol was produced by engineered strains of *S. cerevisiae* and *E. coli* at 6 mg/l and 16 mg/l, respectively [27]. Also, Watts et al. reconstituted the same biosynthetic pathway work in *E. coli* with a titer of >100 mg/l [28]. Further improvements were made since

then. For instance, Zhang et al. used a fusion protein of 4CL and STS to increase the yield of resveratrol in *S. cerevisiae* by about 20 folds [25]. Coincidentally, Lim et al. finally improved the titer to 2.3 g/l resveratrol with this precursor pathway by testing different STS genes from various plant species and optimizing the expression platform in *E. coli* [29].

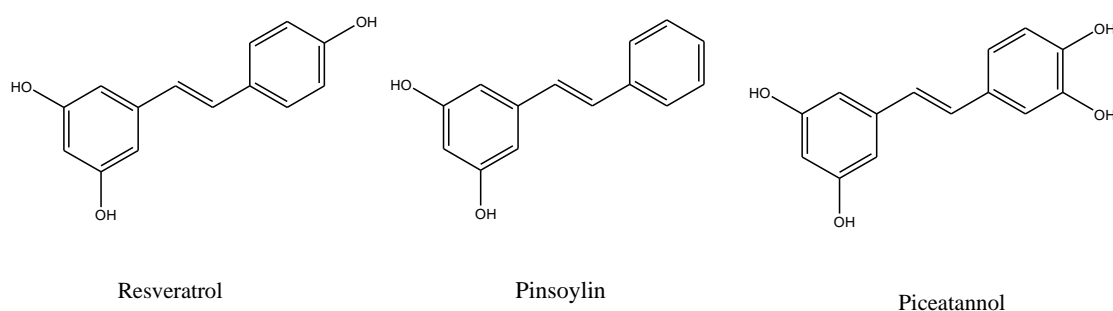


Fig. 2 Structures of three major natural stilbenoids.

Stilbenoid biosynthesis uses cinnamoyl-CoA or *p*-coumaroyl-CoA as the starter unit, meaning that it shares the early biosynthetic steps as in the biosynthesis of flavonoids and isoflavonoids described above. To achieve the total biosynthesis of stilbenoids, tyrosine ammonia-lyase and phenylalanine ammonia-lyase (TAL/PAL) and 4CL need to be added into the pathway, which will allow *in situ* supply of the desired CoA starter. Wang et al. reported a stepwise increase of resveratrol biosynthesis in *S. cerevisiae* to 3.44 mg/l from the amino acids in 2011 [30]. On the other hand, it is possible to modify the structure of stilbenoids through the introduction of some foreign enzymes to generate new derivatives. For example, a 3-*O*-glucosyltransferase (3-*O*-GT) was introduced to the resveratrol biosynthetic pathway to generate *trans*-resveratrol-3-*O*-glucoside or *trans*-piceid [31].

Curcuminoids are another group of plant phenylpropanoid metabolites, which consist of two aromatic rings connected with a 7 carbon chain with different chemical groups [32]. A representative member of this family is curcumin (Fig. 3). It is a natural plant polyphenol with yellow color, and was isolated from the herb *Curcuma longa* with other two derivatives demethoxycurcumin and bisdemethoxycurcumin [33]. Curcuminoids are used as food spice (curry) and food coloring agent (E100). In addition, they are the major bioactive components in turmeric that has been used in traditional medicine for centuries. Curcuminoids are well-known for their anti-tumor, anti-oxidant, anti-inflammatory, anti-allergic, and hepatoprotective activities [34-39]. The polyketide biosynthetic pathways of curcuminoids in different plants have been investigated. Kiat et al. studied the biosynthetic pathway of curcuminoids in turmeric using ^{13}C -labeled precursors [40]. To gain a better understanding of curcuminoid biosynthesis, two novel type III PKSs, diketide-CoA synthase (DCS) and curcumin synthase (CURS), were cloned from *C. longa* and subjected to enzymatic studies. It was revealed that DCS catalyzes the condensation of feruloyl-CoA and malonyl-CoA to form feruloyldiketide-CoA, which is subsequently used by CURS to form curcumin [41]. Two additional CURSs were discovered in the *C. longa* and named as CURS2 and CURS3. These two CURSs were found to have different substrate preference. CURS2 synthesizes curcumin and demethoxycurcumin, while CURS3 has the ability to produce curcumin, bisdemethoxycurcumin, and demethoxycurcumin [42].

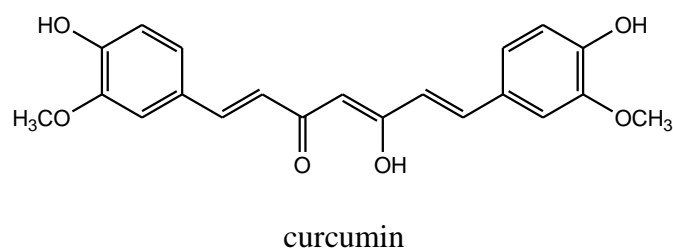


Fig. 3 The structure of curcumin.

There is another way to generate curcuminoids in nature. Curcuminoid synthase (CUS) was identified from *Oryza sativa*. Unlike the pathway in *C. longa* that requires collaborative actions of two type III PKSs, this single PKS has the ability to synthesize curcuminoids from malonyl-CoA and aromatic CoA esters such as 4-coumaroyl-CoA, cinnamoyl-CoA, and feruloyl-CoA [43]. The broad substrate spectrum makes it possible to synthesize derivatives with different functional groups and improved water solubility or bioactivity. Three curcuminoids including dicinnamoylmethane, bisdemethoxycurcumin and cinnamoyl-*p*-coumaroylmethane were produced by the engineered *E. coli* strain [44]. Seventeen curcumin analogs were produced in *E. coli* through precursor-directed biosynthesis [45].

1.2 Objectives

The main goal of my doctoral dissertation research was to engineer natural product biosynthetic pathways for *de novo* synthesis of existing or new bioactive molecules, including phenylpropanoid acids, stilbenoids, and curcuminoids. Additionally, I developed an *in vivo* reporter assay for phenylalanine ammonia-lyase. Lastly, I investigated the biosynthetic pathway of the antibacterial natural product

dutomycin via genome sequencing and gene disruption, and generated a promising antibacterial agent.

Specific objectives include:

1. Construct a platform for *de novo* biosynthesis of various plant natural products and their novel derivatives in *E. coli*.
2. Establish an *in vivo* reporter assay for phenylalanine ammonia-lyase.
3. Investigate the biosynthetic pathway of dutomycin and generate new antibacterial analogs.

1.3 A guide to the dissertation

This dissertation includes five chapters. Chapter 2 and Chapter 3 describe the engineering of a variety of plant natural product biosynthetic pathways into *Escherichia coli* to generate bioactive molecules, including phenylpropanoid acids, stilbenoids, and curcuminoids [46]. In Chapter 4, an *in vivo* novel and efficient visible reporter assay was established for screening of PAL efficiency in *Escherichia coli*, based on the color of a curcuminoid synthesized through this platform [47]. Finally, Chapter 5 focuses on the study of an unknown type II polyketide biosynthetic pathway that is responsible for the synthesis of dutomycin in *Streptomyces*. New compounds were generated through rational modification of the pathway [48]. Several scientific techniques were used in these studies, including molecular cloning, genetic

engineering, microorganism culturing, protein expression, natural products isolation, NMR and MS for structure determination, DNA sequence analysis, and others.

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CHAPTER 2 ENGINEERED PRODUCTION OF HYDROXYCINNAMIC ACIDS AND STILBENOIDS IN *E. COLI**

ABSTRACT

Plants produce a variety of natural products with promising biological activities, such as phenylpropanoids and stilbenoids. While these molecules are naturally assembled through dedicated plant metabolic pathways, combinatorial biosynthesis has become an attractive tool to generate desired molecules. In this chapter, we demonstrated that biosynthetic enzymes from different sources can be recombined like legos to make various molecules. Seven biosynthetic genes from plants and bacteria were used to establish a variety of complete biosynthetic pathways in *Escherichia coli* to make valuable compounds. Different combinations of these biosynthetic bricks were made to design rationally various natural product pathways, yielding four phenylpropanoid acids (cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid), three bioactive natural stilbenoids (resveratrol, piceatannol and pinosylvin).

2.1 Introduction

Plants are a major producer of structurally diverse and medicinally important molecules, such as resveratrol and curcumin. Resveratrol belongs to the family of stilbene natural products, which also include piceatannol, pinosylin and other

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stilbenoids. These natural products are present in several plants such as grapes and peanuts, but are not widely distributed in other common food sources. Interestingly, the slight structural differences of stilbenoids may lead to different functions and applications. For instance, resveratrol is widely used in the clinical and nutraceutical fields due to its protection effects against a series of diseases, such as Parkinson's, cancer, and heart disease. It also reduces serum lipids and possesses other beneficial effects. Recent studies demonstrated that resveratrol can significantly prolong life-span in non-vertebrate model organisms and short-lived vertebrates [1, 2]. Piceatannol is a hydroxylated derivative of resveratrol. It naturally occurs in various plants, including grapes, berries, passion fruit, white tea, and Japanese knotweed. It is also a metabolic product of resveratrol in the human body. This compound has shown strong anticancer, anti-aethrogenic, antioxidant, anti-inflammatory, antimicrobial and estrogenic activities, which is even more potent than resveratrol [3, 4]. Like resveratrol, piceatannol is also a bioactive component in wine, although at a much lower concentration [4]. Pinsoylin is a dehydroxylated derivative of resveratrol and present in the heartwood of Pinaceae. This compound is a pre-infectious fungitoxin and protects the wood from fungal infections. It showed stronger antifungal activity against *Candida albicans* and *Saccharomyces cerevisiae* than resveratrol [5]. These suggested that structural modifications of these molecules may lead to new bioactive molecules.

In this chapter, we established a library of biosynthetic bricks with five biosynthetic enzymes from plant, bacterial and fungal sources. These include phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), and 4-coumarate: CoA ligase (4CL), stilbene synthase (STS), 4-coumarate 3-hydroxylase (C3H). These enzymes were expressed or co-expressed in *E. coli* BL21 (DE3) in different combinations, resulting in the production of four phenylpropanoid acids including cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid). Introduction of the corresponding PKS led to the total biosynthesis of three bioactive natural stilbenoids (resveratrol, piceatannol and pinosylvin).

2.2 Materials and methods

2.2.1 General method

Products were analyzed and purified on an Agilent 1200 HPLC instrument. ESI-MS spectra were obtained on an Agilent 6130 quadrupole LC-MS. NMR spectra were recorded on a JEOL NMR instrument (300 MHz for ^1H NMR). The chemical shift (δ) values are given in parts per million (ppm). The coupling constants (J values) are reported in Hertz (Hz).

2.2.2 Strains, media and culture conditions

E. coli XL1-Blue was used for routine cloning and plasmid propagation. It was grown at 37°C on LB agar plates or in liquid LB medium supplemented with appropriate antibiotics (ampicillin, 50 $\mu\text{g}/\text{ml}$; kanamycin, 50 $\mu\text{g}/\text{ml}$; streptomycin, 50

µg/ml; chloramphenicol, 25µg/ml). *E. coli* BL21 (DE3) was used for protein expression and compound biosynthesis. *E. coli* BL21 (DE3) was grown at 37°C on LB agar plates or in liquid LB medium supplemented with appropriate antibiotics. Enzymes were expressed in *E. coli* BL21 (DE3) at 28°C.

2.2.3 Gene manipulation and plasmids construction

Restriction enzymes, Phusion® High-Fidelity DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). CloneJET™ PCR Cloning Kit was purchased from Fermentas (Glen Burnie, MD, USA), and the pET28a, pCDFDuet-1 and pACYCDuet-1 vectors were purchased from Novagen (Madison, WI, USA). The *4cl* cDNA (GenBank accession number U18675) was amplified from pUC-4CL1 (provided by Dr. Claudia Schmidt-Dannert, University of Minnesota). The *sts* gene (GenBank accession number AB027606) was amplified from pUC-STs (obtained from Addgene). The *tal* gene (GenBank accession number ABC88669) was cloned from the *S. espanaensis* NRRL 15764 genomic DNA. The *c3h* gene (GenBank accession number DQ357071) was amplified from *S. espanaensis* NRRL 15764 genomic DNA. The *comt* gene (GenBank accession number 166420) was cloned from iGEM part BBa_I742107 (provided by Dr. Charles Miller, Utah State University). The primers are shown in Table 1. These four PCR products were gel purified and ligated into the pJET1.2 cloning vector to yield pSW52, pSW63, pSW87, pSW96 and pSW98 respectively (Table 2). The genes were confirmed by digestion checks and sequencing.

Table 1. Primers used in Chapters 2 and 3.*

Primer	Sequence
4CL1-F	5'-AACATATGGCGCCACAAGAACAAGC-3'
4CL1-R	5'-AACTCGAGTCACAATCCATTTGCTAGTTTT-3'
STS-F	5'-AAGCTAGCATGGTGTCTGTGAGTGGAAT-3'
STS-R	5'-AAGGATCCTTATATGGCCATGCTGCGGA-3'
TAL-F	5'-AACCATGGTGACGCAGGTCGTG-3'
TAL-R	5'-AAGAATTCATCCGAAATCCTTCCCGT-3'
C3H-F	5'-AACATATGACCATCACGTCACCTGC-3'
C3H-R	5'-AACTCGAGTCAGGTGCCGGG-3'
COMT-F	5'-AACTGCAGATGGGTTAACAGGTGAAA-3'
COMT-R	5'-AAGCGGCCGCTTATTAAACCTTCTTAA-3'

* Restriction sites are italicized and the start/stop codons are shown in bold.

After sequencing, the sts gene was excised from pSW63 via NheI/BamHI and ligated to pET28a between the same sites to form pSW65. The tal gene was excised from pSW87 via NcoI/EcoRI and introduced to the pCDFDuet-1 vector to yield pSW95. The c3h gene was then excised from pSW96 and ligated to pSW95 between the NdeI and XhoI sites to yield pSW97. The comt gene was cut from pSW98 with NotI/PstI and ligated into pACYCDuet-1 vector to form pSW117 (Table 2).

Table 2. Plasmids used in Chapters 2 and 3.

Plasmid	Description	Reference
pUC-4cl1	The <i>4cl</i> cDNA from <i>A. thaliana</i>	(Watts et al., 2006)
pET16b-cus	The <i>cus</i> cDNA from <i>O. sativa</i>	(Katsuyama et al., 2008)
pUC-sts	The <i>sts</i> cDNA from <i>A. hypogaea</i>	Purchased from Addgene
pZJ54	<i>rdc2</i> in pET28a	(Zeng and Zhan, 2010)
pSW24	<i>cus</i> in pET28a	(Wang et al., 2013)
pSW54	<i>4cl</i> in pACYCDuet-1	(Wang et al., 2013)
pSW56	<i>pal</i> and <i>4cl</i> in pACYCDuet-1	(Wang et al., 2013)
pSW60	<i>pal</i> in pACYCDuet-1	(Wang et al., 2013)
pSW63	<i>sts</i> in pJET1.2	this work
pSW65	<i>sts</i> in pET28a	this work
pSW87	<i>tal</i> in pJET1.2	this work
pSW95	<i>tal</i> in pCDFDuet-1	this work
pSW96	<i>c3h</i> in pJET1.2	this work
pSW97	<i>c3h</i> and <i>tal</i> in pCDFDuet-1	this work
pSW98	<i>comt</i> in pJET1.2	this work
pSW99	<i>comt</i> and <i>4cl</i> in pACYCDuet-1	this work
pSW117	<i>comt</i> in pACYCDuet-1	this work
pSW121	<i>sts</i> and <i>tal</i> in pCDFDuet-1	this work

2.2.4 Biosynthesis of hydroxycinnamic acids and stilbenoids in *E. coli*

Corresponding plasmids were electroporated into *E. coli* BL21 (DE3). The resulting strains were grown in 50 ml of LB medium supplemented with appropriate antibiotic(s) at 37 °C with shaking at 250 rpm. Once the OD₆₀₀ reached 0.4-0.6, IPTG

was added at a final concentration of 200 μM , and the induced culture was maintained at 28°C with shaking at 250 rpm for an additional 5 h. Substrates (glucose and L-phenylalanine or L-tyrosine) were added, and the culture was incubated under the same conditions for an additional 48 h. For biotransformation, resveratrol or ferulic acid was added as the substrate. After fermentation, 1 ml of the culture was sampled and centrifuged at 14,000 rpm for 10 min. 100 μl of the supernatant was then analyzed by LC-MS.

2.2.5 HPLC analysis of the biosynthesized compounds

Analysis of p-coumaric acid, caffeic acid, and ferulic acid was conducted on an Agilent 1200 HPLC system with an Agilent Eclipse XDB-C18 column (5 μm , 250 mm \times 4.6 mm). The samples were eluted with acetonitrile-water (15:85, v/v) for 25 min under 300 nm at a flow rate of 1 ml/min. Cinnamic acid was analyzed on the same instrument, eluted with acetonitrile-water (30:70, v/v) for 25 min at 300 nm at a flow rate of 1 ml/min. Analysis of stilbenoids were conducted on the same equipment, eluted with a gradient of acetonitrile-water (20:80, v/v for 18 min, 20:80, v/v to 50:50 v/v from 18 min to 30 min, 50:50 v/v from 30 min to 40 min) at a flow rate of 1 ml/min. The products were detected at 330 nm. The titers of all the biosynthetic compounds were calculated based on the standard curves of the purified compounds or commercial samples.

2.2.6 Identification of the biosynthetic compounds

The biosynthesized compounds were characterized by LC-MS and a comparison with authentic samples. Resveratrol was also isolated from the fermentation broth and subjected to NMR analysis (Appendix B).

2.3 Results

2.3.1 Engineered biosynthesis of phenylpropanoid acids in *E. coli*

We have cloned a total of eight genes from various sources, including *pal* from *Trifolium pretense* (plant), *tal* from *Saccharothrix espanaensis* NRRL 15764 (bacterium), *4cl* from *Arabidopsis thaliana* (plant), *c3h* from *S. espanaensis* NRRL 15764 (bacterium), *comt* from *Medicago sativa* (plant), *sts* from *Arachis hypogaea* (plant). These genes constitute a tool box for combinatorial biosynthesis of a variety of molecules.

Using a biosynthetic approach, we have successfully produced four phenylpropanoid acids including cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid in *E. coli* BL21(DE3) in high yields when 3 mM L-phenylalanine and L-tyrosine was supplied. The compounds were characterized based on a comparison of the ESI-MS (Fig. 4), and retention time with those of authentic samples. As shown in Fig. 1, expression of *PAL* from *T. pretense* in *E. coli* yielded cinnamic acid as a dominant product (Fig 5 trace i) at 241.3 ± 2.8 mg/l. Similarly, expression of *TAL* from *S. espanaensis* NRRL 15764 generated *p*-coumaric acid from L-tyrosine in *E.*

coli at 160.5 ± 2.1 mg/l (Fig 5 trace ii). Co-expression of TAL with C3H from *S. espanaensis* NRRL 15764, which hydroxylates *p*-coumaric acid at C-3', led to the production of caffeic acid at 70.0 ± 0.9 mg/l (Fig 5 trace iii). Further addition of COMT from *M. sativa* into the system yielded ferulic acid at 28.8 ± 0.4 mg/l (Fig 5 trace iv).

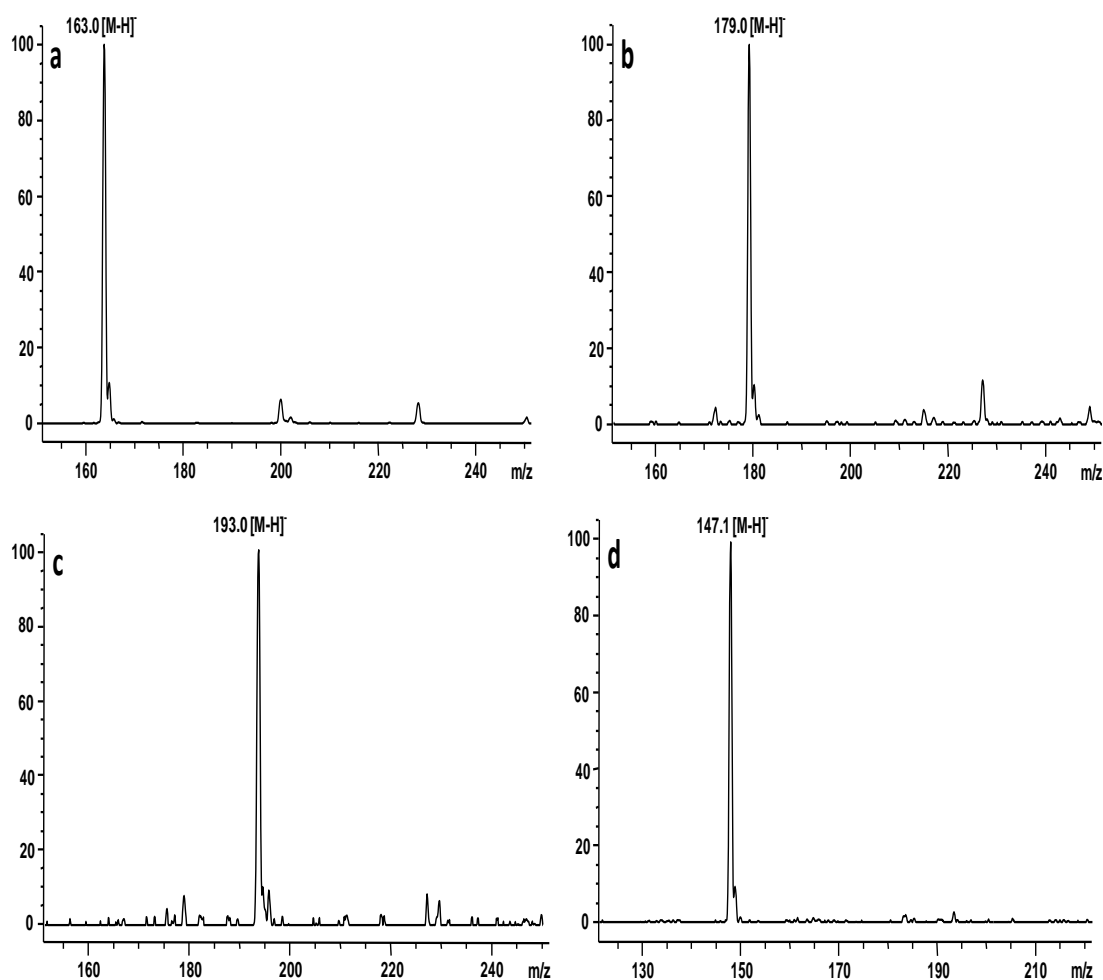


Fig. 4 ESI-MS analysis of hydroxycinnamic acids production. **a.** ESI-MS (-) spectrum of *p*-coumaric acid production from L-tyrosine; **b.** ESI-MS (-) spectrum of caffeic acid production from L-tyrosine; **c.** ESI-MS (-) spectrum of ferulic acid

production from L-tyrosine; **d.** ESI-MS (-) spectrum of cinnamic acid production from L-phenylalanine.

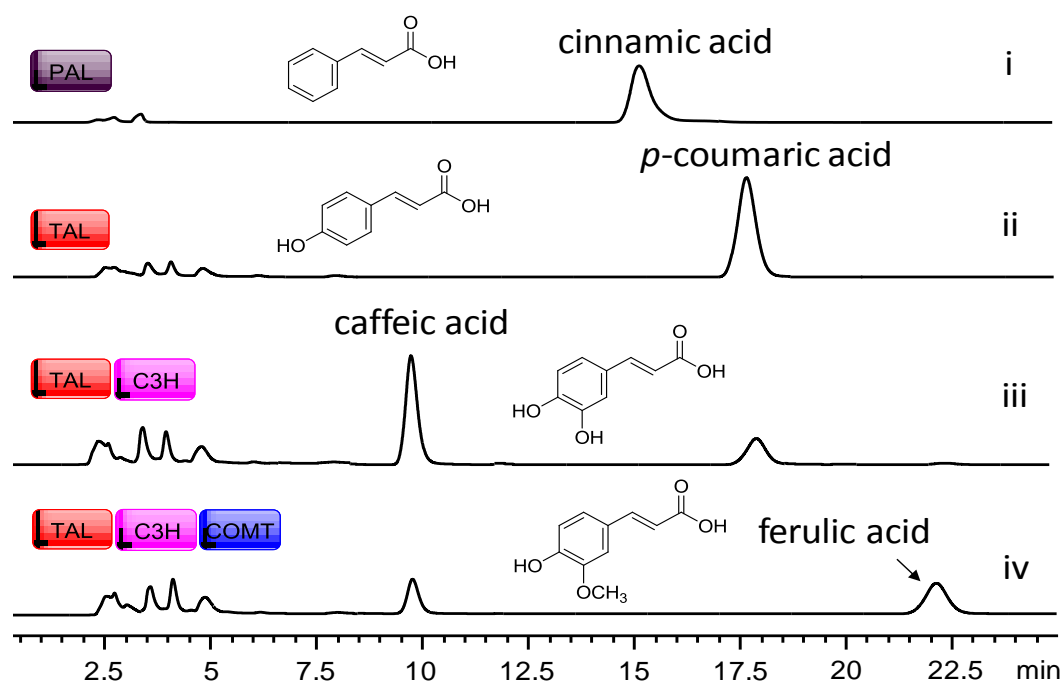


Fig. 5 Engineered biosynthesis of phenylpropanoid acids in *E. coli*. (i) HPLC analysis of the biosynthesis of cinnamic acid by *E. coli*/PAL; (ii) HPLC analysis of the biosynthesis of *p*-coumaric acid by *E. coli*/TAL; (iii) HPLC analysis of the biosynthesis of caffeic acid by *E. coli*/TAL-C3H; (iv) HPLC analysis of the biosynthesis of ferulic acid by *E. coli*/TAL-C3H-COMT. Mobile phase for trace i was 30% acetonitrile– water, and 15% acetonitrile–water was used for traces ii–iv. The products were detected at 300 nm.

2.3.2 Engineered biosynthesis of resveratrol in *E. coli*

Three enzymes including TAL, 4CL and STS are required to synthesize resveratrol from the amino acid precursor. To this end, we cloned these three genes into three expression vectors, including pET28a, pACYCDuet-1 and pCDFDuet-1, to yield three expression plasmids, pSW54 pSW65 and pSW95 (Table 2). These plasmids were co-expressed in *E.coli* BL21 (DE3). The engineered strain was grown in LB medium. 0.6 mM L-tyrosine and 4 g/l glucose were added into the induced culture as supplemented amino acid substrate and carbon source. As shown in Fig. 6, LC-MS analysis revealed that a major product at 26.5 min was produced, which was identified as resveratrol based on the ESI-MS (Fig. 6b), UV (Fig. 6c), and ¹H NMR spectra (Appendix B).

To optimize the production of resveratrol in the engineered *E. coli*, L-tyrosine and glucose were fed individually or together into the induced culture. As shown in Fig. 7a, when no nutrients were supplemented, the trace (trace ii) is same as that of the blank control *E. coli* BL21 (DE3) harboring the blank vectors (trace i). When glucose was added, resveratrol was produced by the engineered strain that harbors the corresponding biosynthetic genes. Thus, glucose is necessary for resveratrol biosynthesis, which provides the cells with a premium carbon source to supply the required polyketide biosynthetic precursor malonyl-CoA. By contrast, when L-tyrosine was supplemented, no resveratrol was detected, likely due to insufficient

supply of malonyl-CoA. When both glucose and L-tyrosine were supplied, the production of resveratrol was significantly improved. We next determined the optimal concentration of L-tyrosine and glucose. As shown in Fig. 7b, the titer of resveratrol increased with the increased concentrations of the substrates. The titer reached a maximum when 0.6 mM L-tyrosine and 4 g/l glucose were added. Further increase in the concentrations of these substrates did not lead to any improvements of resveratrol biosynthesis. However, the intermediate p-coumaric acid started to accumulate. The highest titer of resveratrol was determined to be 114.4 ± 3.5 mg/l.

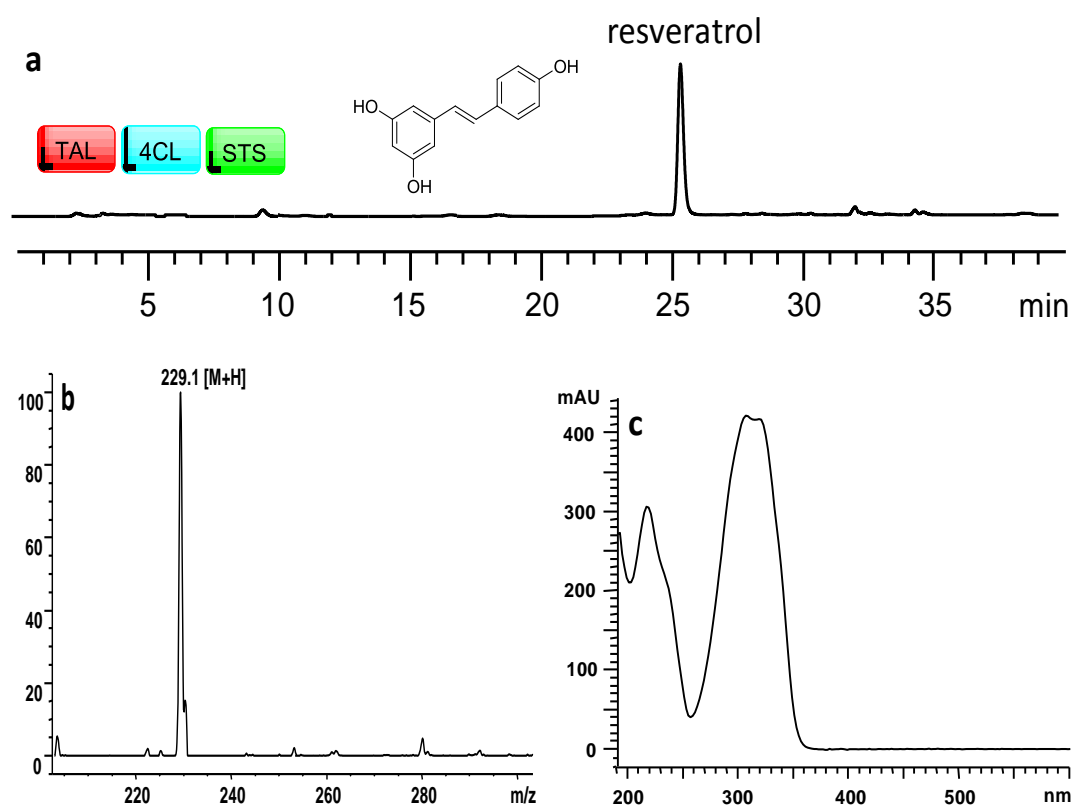


Fig. 6 LC-MS analysis of resveratrol production. a. the HPLC trace of resveratrol production from Tyrosine; b. ESI-MS (+) spectrum of resveratrol production from tyrosine; c. UV spectrum of resveratrol production from tyrosine.

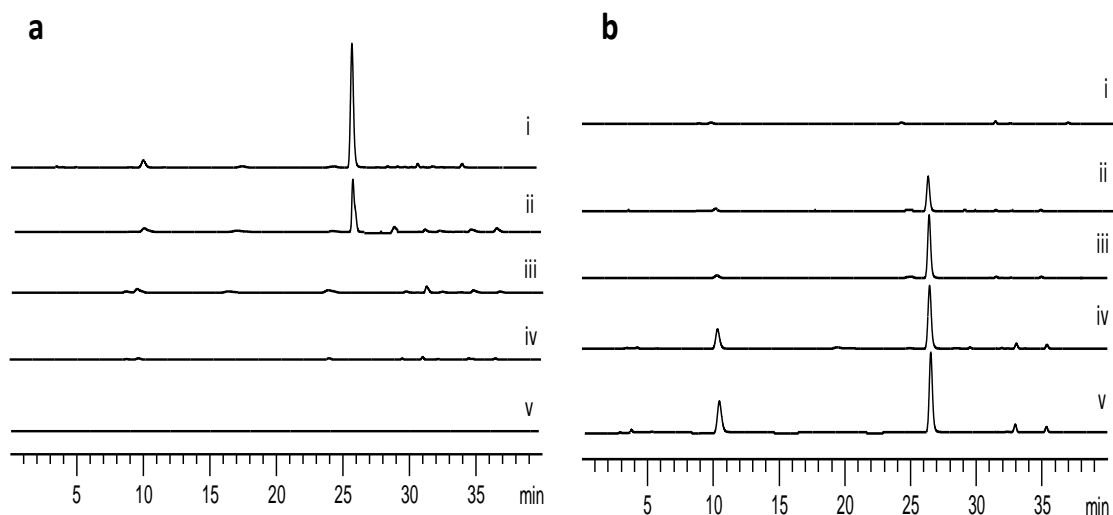


Fig. 7 Condition optimization for resveratrol production. a. different substrates condition for resveratrol production (i: Tyr, glucose feeding; ii: glucose feeding; iii: Tyr feeding; iv: none feeding control; v: blank vectors in BL21(DE3) with Tyr, glucose feeding control.); b. different substrates concentration (i: 0 mM Tyr, 0g/l glucose; ii: 0.3 mM Tyr, 2g/l glucose; iii: 0.6 mM Tyr, 4 g/l glucose; iv 1.5 mM Tyr, 10 g/l glucose; v: 3mM Tyr, 20 g/l glucose).

2.3.3 Engineered biosynthesis of piceatannol and pinosylvin in *E.coli*

Using the library of biosynthetic bricks constructed above, we next made two resveratrol analogs. Piceatannol is a natural resveratrol analog with an additional phenolic hydroxyl group at C-3'. To make this compound, we first employed a biotransformation approach using a C3H from *S. espanaensis* NRRL 15764. This enzyme was used to create caffeic acid and ferulic acid above, which indicated that it can introduce a hydroxyl group to p-coumaric acid at C-3'. However, it was unclear whether it could accept resveratrol as the substrate. To test whether this enzyme can

hydroxylate resveratrol at C-3', C3H was then individually expressed in *E. coli* BL21 (DE3). The IPTG-induced broth was incubated with resveratrol. LC-MS revealed that a major product at 15.5 min was generated by the engineered *E. coli* BL21 (DE3) strain, together with the substrate resveratrol at 26.5 min (trace ii, Fig. 8). The product was characterized as piceatannol by a comparison of the retention time, ESI-MS (Appendix B) spectra with those of the commercial standard of piceatannol. Conversion of resveratrol to piceatannol revealed the broad substrate specificity of this C3H. The titer of piceatannol from this biotransformation process was 65.4 ± 1.0 mg/l.

Total biosynthesis of piceatannol has never been reported. We next constructed a biosynthetic pathway of this natural product by introducing C3H into the resveratrol biosynthetic pathway. To this end, TAL, 4CL, C3H and STS were co-expressed in *E. coli* BL21 (DE3). LC-MS analysis revealed that a major product was synthesized from L-tyrosine. The retention time (trace iii, Fig. 8), UV and ESI-MS of this product were consistent with those of the authentic sample of piceatannol, confirming that a complete biosynthetic pathway of piceatannol was successfully constructed in *E. coli*. The titer of piceatannol from this total biosynthetic pathway was determined to be 21.5 ± 0.2 mg/l.

Pinosylvin is another natural resveratrol analog that lacks a hydroxyl group at C-4. The biosynthesis of this compound has never been studied. Based on its structure, we proposed that the biosynthetic pathway of this molecule is similar to that of resveratrol. To synthesize this compound, L-phenylalanine should be used as the starting amino acid precursor. Thus, we need a PAL to convert L-phenylalanine to cinnamic acid. However, successful biosynthesis of pinosylvin also requires the ability of 4CL and STS to accept “unnatural” substrates, meaning that 4CL can ligate cinnamic acid with CoA to yield cinnamoyl-CoA and STS can take cinnamoyl-CoA as the starter unit. To test this, we co-expressed PAL, 4CL and STS in *E. coli* BL21 (DE3). LC-MS analysis revealed that a major metabolite at 34.5 min was produced (trace iv, Fig. 8). The ESI-MS spectrum showed an ion peak $[M-H]^-$ at m/z 211 (Appendix B), suggesting that the molecular weight of this compound is 212. The UV spectrum (Appendix B) and retention time of this product were same as those of the commercial standard of pinosylvin. Therefore, we were able to for the first time establish the complete pinosylvin biosynthetic pathway in *E. coli*. The titer of pinosylvin was determined to be 13.3 ± 0.8 mg/l.

2.4 Conclusion

In this chapter, we established a library of biosynthetic bricks with five biosynthetic enzymes from different sources, including PAL, TAL, 4CL, STS and C3H. These enzymes were expressed or co-expressed in *E. coli* BL21 (DE3) in different combinations, resulting in the production of four phenylpropanoid acids

(cinnamic acid, p-coumaric acid, caffeic acid, and ferulic acid). Introduction of the corresponding PKS led to the total biosynthesis of three bioactive natural stilbenoids (resveratrol, piceatannol and pinosylvin). The results from this chapter provided important platforms and technical basis for further engineering polyketide biosynthetic pathways for producing valuable bioactive molecules.

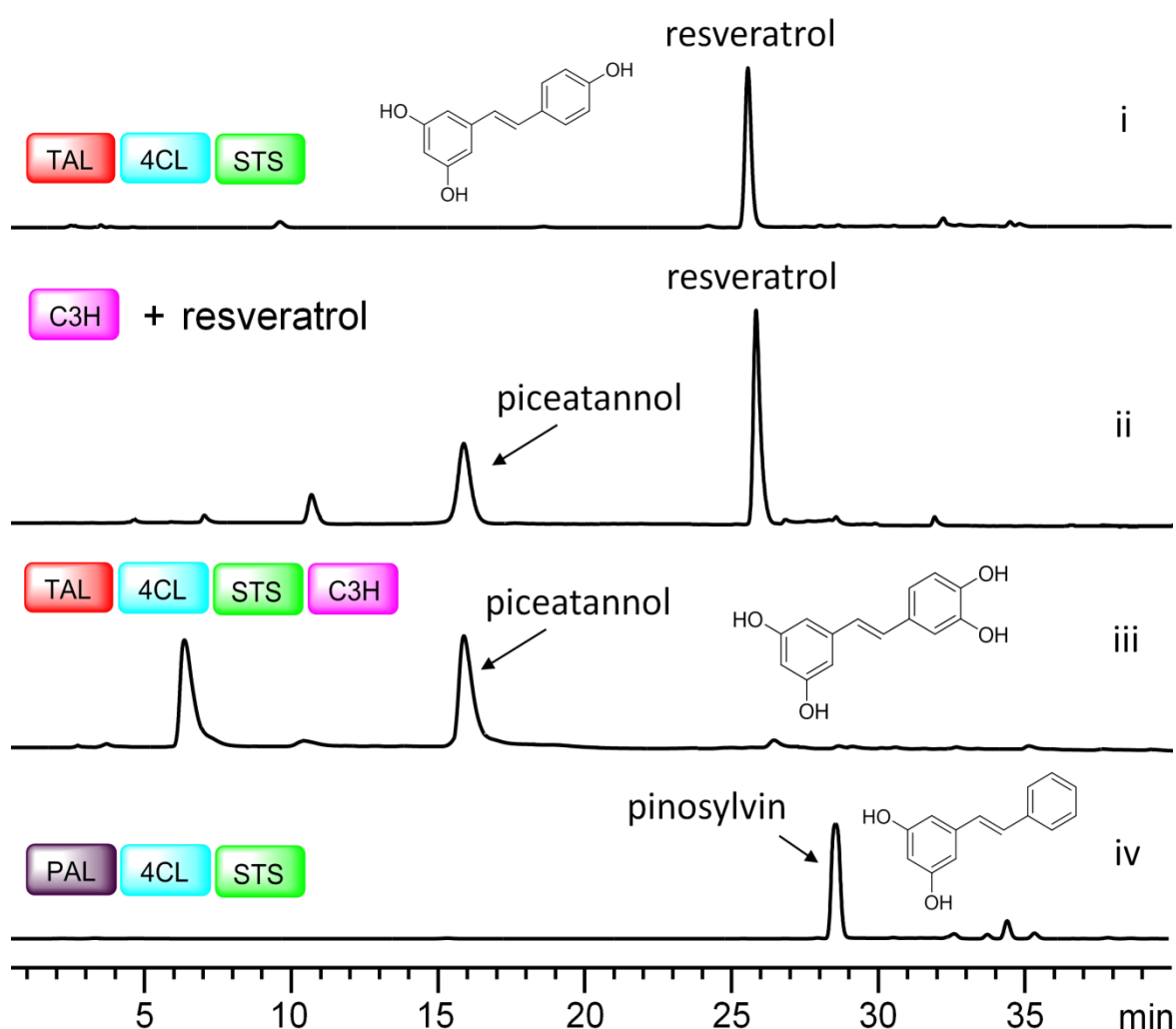


Fig. 8 Engineered biosynthesis of resveratrol analogs in *E. coli*. (i) HPLC analysis of the biosynthesis of resveratrol by *E. coli*/TAL-4CL-STS; (ii) Conversion of resveratrol to piceatannol by *E. coli*/C3H through the specific C-3' hydroxylation; (iii)

HPLC analysis of the biosynthesis of piceatannol by *E. coli*/TAL-4CL-STS-C3H; (iv) HPLC analysis of the biosynthesis of pinosylvin by *E. coli*/PAL-4CL-STS. The products were detected at 330 nm.

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CHAPTER 3 ENGINEERED PRODUCTION OF CURCUMINOID AND TWO UNNATURAL ANALOGS IN *E. COLI**

ABSTRACT

Curcuminoid, which is produced specifically by the expression of curcuminoid synthase (CUS) gene, has long been used as traditional Asian medicines because of its reported activities on anti-tumor, antioxidant and hepatoprotective. Hence, CUS gene is an attractive target for metabolic engineering. In this chapter, we modified the library of biosynthetic bricks mentioned in Chapter 2 with eight biosynthetic enzymes from plant, bacterial and fungal sources, which resulted in producing three natural curcuminoids (curcumin, bisdemethoxycurcumin and dicinnamoylmethane). Remarkably, a curcumin analog dicafferolmethane was synthesized by removing a methyltransferase from the curcumin biosynthetic pathway. Furthermore, introduction of a fungal flavin-dependent halogenase into the resveratrol biosynthetic pathway yielded a novel chlorinated molecule 2-chloro-resveratrol. This work thus provided a novel and efficient biosynthetic approach to create various bioactive molecules. Further expansion of the library of the biosynthetic bricks will provide a resource for rational design of various phenylpropanoids via the combinatorial biosynthesis approach.

3.1 Introduction

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Curcuminoids are another family of plant polyphenols, exemplified by curcumin, a major component in turmeric. Turmeric has long been used as a food spice (curry) and complementary and alternative medicine (CAM) in Asia [1-4]. These compounds possess various bioactivities and can be used for the treatment of different diseases such as allergy, asthma, cancer and Alzheimer's disease, while providing prevention against oxidative damage in normal cells [5-9].

The above-mentioned natural products are assembled through well designed plant metabolic pathways. These compounds are traditionally extracted from the corresponding producing plants. Given the long growth period of plants, this is neither efficient nor cost-effective. Development of synthetic biology and metabolic engineering techniques provides a great opportunity to synthesize these molecules in microorganisms. Some progresses have been made in engineered biosynthesis of plant natural products such as coumarins [10, 11] in microorganisms. Resveratrol has also been produced in the yeast *Saccharomyces cerevisiae*, although the yield was only 6 mg/l [12, 13]. However, there is no report on total biosynthesis of piceatannol or pinsoylin. The biosynthesis of curcumin has also been studied. Two type III polyketide synthases (PKSs), diketide-CoA synthase (DCS) and curcumin synthase (CURS), are involved in the biosynthesis of curcuminoids in the herb *Curcuma longa* [14]. Compared to the collaborative actions of two type III PKSs in *C. longa*, one single type III PKS from *Oryza sativa*, curcuminoid synthase (CUS), was found to

synthesize curcuminoids. With supplementation of different carboxylate precursors, curcumin and several analogs were synthesized in *E. coli* [15]. However, total biosynthesis of curcumin from an amino acid precursor has not been reported.

In this chapter, we modified the library of biosynthetic bricks mentioned in chapter 2 with eight biosynthetic enzymes from plant, bacterial and fungal sources. These include phenylalanine ammonia-lyase (PAL), tyrosine ammonia-lyase (TAL), 4-coumarate:CoA ligase (4CL), stilbene synthase (STS), CUS, 4-coumarate 3-hydroxylase (C3H), caffeic acid 3-O-methyltransferase (COMT), and a flavin-dependent halogenase Rdc2. Introduction of the corresponding PKS led to three natural curcuminoids (curcumin, bisdemethoxycurcumin and dicinnamoylmethane). Remarkably, we also generated two novel molecules by constructing a new curcuminoid biosynthetic pathway and adding a fungal halogenase to the resveratrol pathway, respectively. This work thus demonstrated a highly efficient combinatorial biosynthesis approach to creating various plant phenylpropanoids in *E. coli*.

3.2 Materials and methods

3.2.1 General method

Products were analyzed and purified on an Agilent 1200 HPLC instrument. ESI-MS spectra were obtained on an Agilent 6130 quadrupole LC-MS. NMR spectra

were recorded on a JEOL NMR instrument (300 MHz for ^1H NMR). The chemical shift (δ) values are given in parts per million (ppm). The coupling constants (J values) are reported in Hertz (Hz).

3.2.2 Strains, media and culture conditions

E. coli XL1-Blue was used for routine cloning and plasmid propagation. It was grown at 37°C on LB agar plates or in liquid LB medium supplemented with appropriate antibiotics (ampicillin, 50 $\mu\text{g/ml}$; kanamycin, 50 $\mu\text{g/ml}$; streptomycin, 50 $\mu\text{g/ml}$; chloramphenicol, 25 $\mu\text{g/ml}$). *E. coli* BL21 (DE3) was used for protein expression and compound biosynthesis. *E. coli* BL21 (DE3) was grown at 37°C on LB agar plates or in liquid LB medium supplemented with appropriate antibiotics. Enzymes were expressed in *E. coli* BL21 (DE3) at 28°C.

3.2.3 Gene manipulation and plasmids construction

Based on the work in chapter 2, the *comt* gene was cut from pSW98 with NotI/PstI and ligated into pSW54 between the same sites, yielding pSW99. The *sts* was introduced to pSW95 to form pSW121 via the MefI/XhoI sites. Construction of pSW24, pSW54, pSW56, pSW60 and pZJ54 has been reported in our previous studies [16, 17].

3.2.4 Biosynthesis of hydroxycinnamic acids and stilbenoids in *E. coli*

Corresponding plasmids were electroporated into *E. coli* BL21 (DE3). The resulting strains were grown in 50 ml of LB medium supplemented with appropriate

antibiotic(s) at 37°C with shaking at 250 rpm. Once the OD600 reached 0.4-0.6, IPTG was added at a final concentration of 200 µM, and the induced culture was maintained at 28°C with shaking at 250 rpm for an additional 5 h. Substrates (glucose and L-phenylalanine or L-tyrosine) were added, and the culture was incubated under the same conditions for an additional 48 h. For biotransformation, resveratrol or ferulic acid was added as the substrate. After fermentation, 1 ml of the culture was sampled and centrifuged at 14,000 rpm for 10 min. 100 µl of the supernatant was then analyzed by LC-MS.

3.2.5 HPLC analysis of the biosynthesized compounds

Analysis of stilbenoids were conducted on the same equipment, eluted with a gradient of acetonitrile-water (20:80, v/v for 18 min, 20:80, v/v to 50:50 v/v from 18 min to 30 min, 50:50 v/v from 30 min to 40 min) at a flow rate of 1 ml/min. The products were detected at 330 nm. Curcuminoids were analyzed on the same equipment, eluted with a gradient of acetonitrile-water (from 10:90, v/v to 100:0, v/v over 30 min) at a flow rate of 1 ml/min. The products were detected at 420 nm. The titers of all the biosynthetic compounds were calculated based on the standard curves of the purified compounds or commercial samples.

3.2.6 Identification of the biosynthetic compounds

The biosynthesized compounds were characterized by LC-MS and a comparison with authentic samples. Curcumin, 2-chloro-resveratrol and dicafferolmethane were

also isolated from the fermentation broth and subjected to NMR analysis (Appendix C).

3.3 Results

3.3.1 Engineered biosynthesis of curcumin and two analogs in *E. coli*

The biosynthesis of curcuminoids has been previously studied. Curcuminoids including bisdemethoxycurcumin, dicinnamoylmethane and cinnamoyl-p-coumaroylmethane have been synthesized in *E. coli* that harbors PAL from the yeast *Rhodotorula rubra*, 4CL from *Lithospermum erythrorhizon* and CUS from *O. sativa*, in the presence of L-phenylalanine or L-tyrosine. Curcumin was also synthesized by CUS and 4CL in *E. coli* when ferulic acid was supplied as a substrate. However, total biosynthesis of curcumin from the amino acid precursor has not been achieved. We have co-expressed PAL from *T. pretense*, 4CL from *A. thaliana* and CUS from *O. sativa* in *E. coli* to generate the curcumin analog dicinnamoylmethane in our previous study (trace i, Fig. 9) [16]. This confirmed that CUS was functional in our system and it is possible to make curcuminoids directly from the amino acid precursor. We first replaced PAL with TAL in the dicinnamoylmethane biosynthetic pathway. Accordingly, co-expression of TAL, 4CL and CUS yielded a major product at 17.7 min (trace ii, Fig.9). The ion peak $[M-H]^-$ at m/z 307.0 in the ESI-MS spectrum (Fig. 10b) indicated the molecular weight of this product is 308. The retention time and UV spectrum (Fig. 10a) were consistent with those of the standard of

bisdemethoxycurcumin. Thus, we were able to construct a complete biosynthetic pathway for bisdemethoxycurcumin, which co-exists with curcumin in turmeric. This compound is a minor bioactive component of turmeric and a common impurity in the commercial product of curcumin. The titer of bisdemethoxycurcumin was determined to be 28.9 ± 1.0 mg/l. Our method thus provides an efficient way to produce this minor constituent of turmeric.

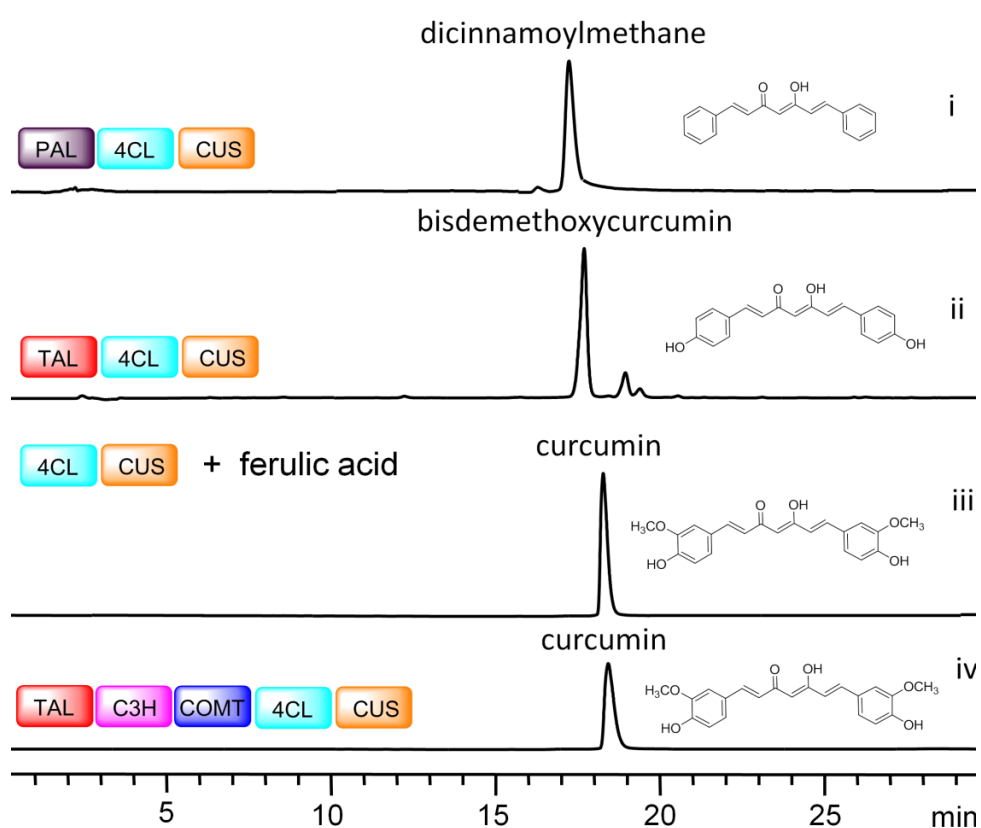


Fig. 9 Engineered biosynthesis of curcuminoids in *E. coli*. (i) HPLC analysis of the biosynthesis of dicinnamoylmethane by *E. coli*/PAL-4CL-CUS; (ii) HPLC analysis of the biosynthesis of bisdemethoxycurcumin by *E. coli*/TAL-4CL-CUS; (iii) HPLC analysis of the biosynthesis of curcumin by *E. coli*/4CL-CUS from ferulic acid; (iv)

HPLC analysis of the total biosynthesis of curcumin from L-tyrosine by *E. coli*/TAL-C3H-COMT-4CL-CUS. The products were detected at 420 nm.

We next attempted to establish a complete biosynthetic pathway of curcumin in *E. coli*. We first tested whether 4CL from *A. thaliana* can ligate ferulic acid with CoA to yield feruloyl-CoA, the starter unit for curcumin biosynthesis. The *cus* and *4cl* genes were respectively ligated to two expression vectors and co-expressed in *E. coli* BL21 (DE3). Ferulic acid was supplemented into the IPTG-induced culture of the engineered strain. HPLC analysis revealed a major product at 18.5 min (trace iii, Fig. 9). The molecular weight of the peak was determined to be 368 based on the quasimolecular ion peak $[M-H]^-$ at m/z 367.0 in the ESI-MS spectrum (Fig. 10d). The UV spectrum of this product (Fig. 10c) was same as that of the authentic sample of curcumin. The compound was isolated from the broth and subjected to 1H NMR analysis (Appendix C), which confirmed that this product was curcumin. Thus, we proposed that 4CL from *A. thaliana* could convert ferulic acid to feruloyl-CoA in our expression system. Because we have made ferulic acid using TAL, C3H and COMT in *E. coli*, we propose that co-expression of these enzymes with 4CL and CUS should yield curcumin. The five genes were then ligated to three expression vectors to yield pSW24, pSW97 and pSW99 (Table 2). Co-expression of these plasmids in *E. coli* cells yielded curcumin (trace iv, Fig. 9). Therefore, a total biosynthetic pathway of curcumin was established in *E. coli*. The titer of this compound was determined to be 0.6 ± 0.1 mg/l.

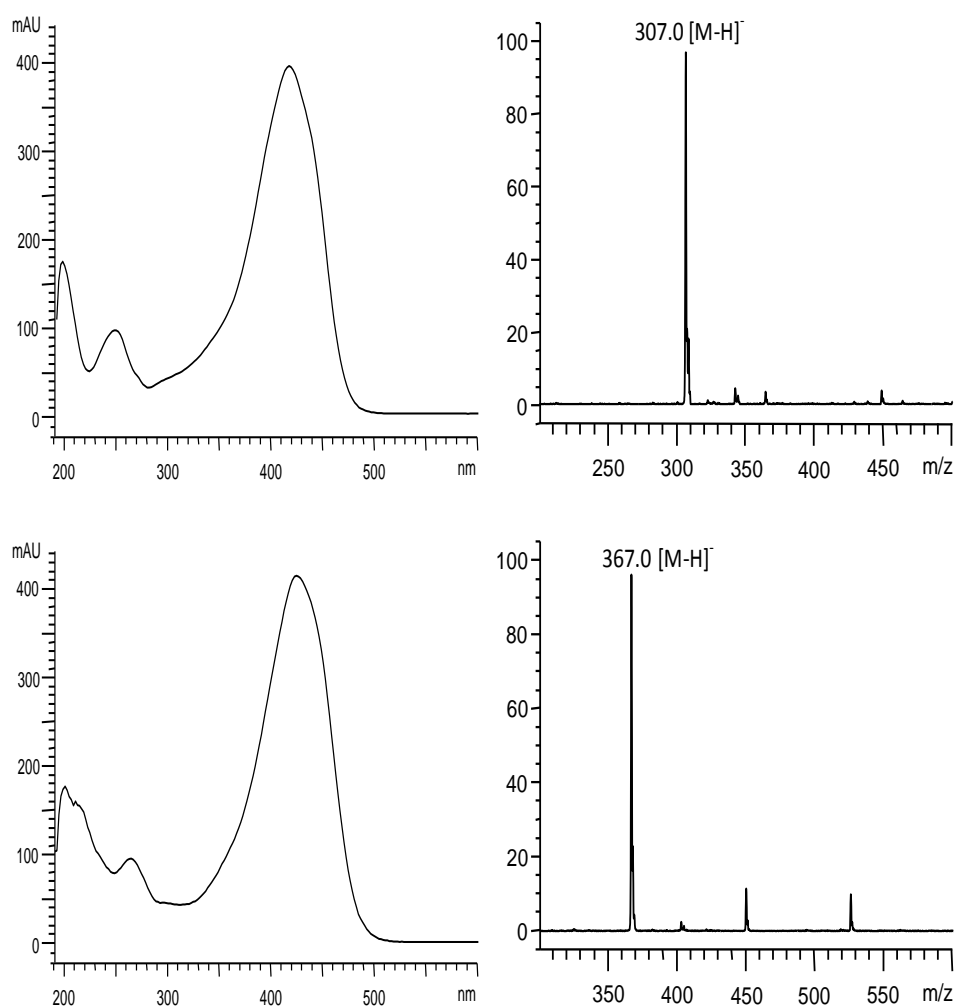


Fig. 10 LC-MS analyses of natural curcuminoids production. a. UV spectrum of bisdemethoxycurcumin production from tyrosine; b. ESI-MS (-) spectrum of bisdemethoxycurcumin production from Tyrosine; c. UV spectrum of curcumin production from tyrosine; d. ESI-MS (-) spectrum of curcumin production from tyrosine.

3.3.2 Engineered biosynthesis of novel resveratrol and curcumin analogs in *E. coli*

With the library of eight biosynthetic genes, we further expanded their uses for combinatorial biosynthesis of novel molecules. Because halogenated compounds

account for a quarter of the current drugs or those in the development pipeline, it will be of significant interest to generate halogenated molecules for bioactivity screening. To achieve this goal, we selected a fungal flavin-dependent halogenase Rdc2, which was characterized in our previous studies as the dedicated halogenase in radicicol biosynthesis [17]. This enzyme has broad-substrate specificity. As shown in trace i of Fig. 11, co-expression of TAL, 4CL, STS and Rdc2 in *E. coli* BL21 (DE3) yielded a new product at 30 min in addition to resveratrol. The molecular weight of this new product was found to be 262 based on the ion peaks $[M-H]^-$ at m/z 261 and 263 (3:1) and $[M+Cl]^-$ at m/z 297 (Fig. 12a). The ratio of 3:1 is a characteristic isotope pattern of monochlorinated molecule. Compared to resveratrol, a bathochromic shift was observed in the UV spectrum of the product (Fig. 12b). Thus, the product was deduced to be a chlorinated derivative of resveratrol. This compound was then purified and subjected to 1H NMR analysis (Appendix), based on which it was identified as 2-chloro-resveratrol. The titer was determined to be 7.0 ± 0.3 mg/l.

Commercially available curcumin product prepared from the herb *Curcuma longa* usually contains 77% curcumin, 17% demethoxycurcumin and 3% bisdemethoxycurcumin. However, no naturally occurring demethylated derivatives of curcumin are known. Given the poor water solubility of curcumin, demethylation of curcumin may represent an effective way to increase its polarity and water solubility. To this end, we constructed a new biosynthetic pathway with TAL, C3H, 4CL and

CUS so that a new demethylated curcumin analog can be generated with caffeoyl-CoA as the starter unit. These enzymes were co-expressed in *E. coli* BL21 (DE3). HPLC analysis revealed a dominant product at 14.8 min (trace ii, Fig. 11). Its molecular weight was determined to be 340 based on the $[M-H]^-$ ion peak at m/z 339.1 (Fig. 12c). It was 28 mass units less than curcumin, suggesting that it is a didemethylated analog of curcumin. This was supported by the UV spectrum of this compound, which is similar to that of curcumin. The product was then purified and subjected to NMR analysis (Appendix C), based on which it was characterized as dicafferolmethane. [18] The titer was determined to be 2.8 ± 0.5 mg/l.

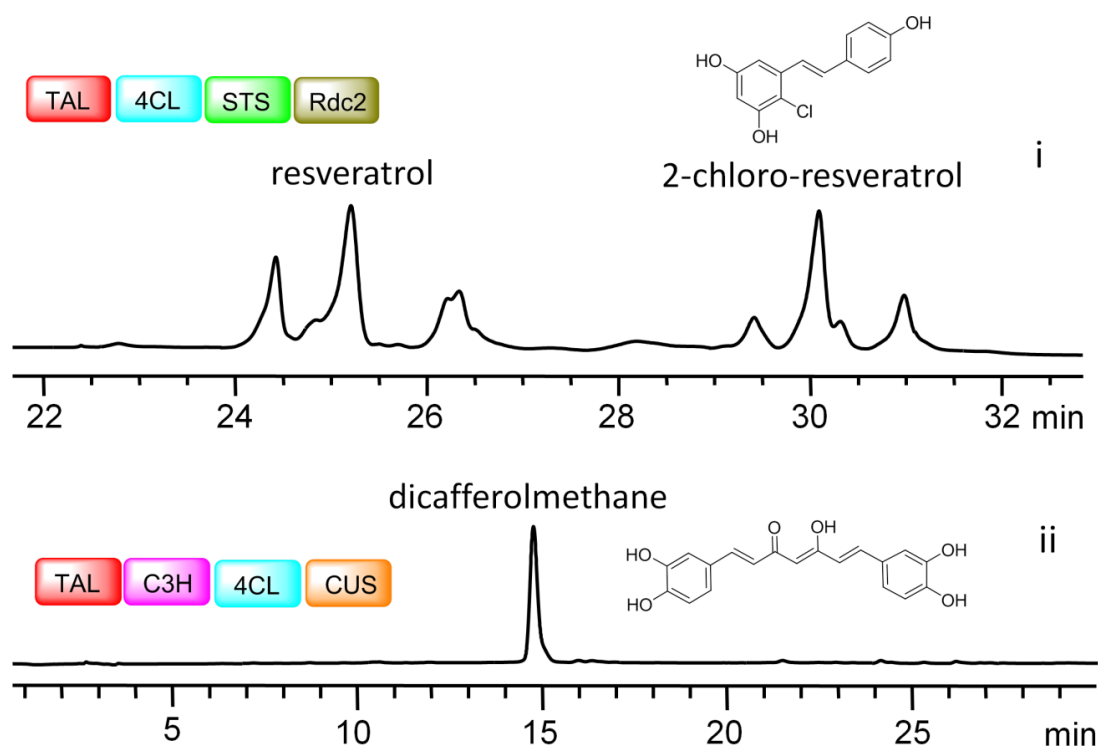


Fig. 11 Engineered biosynthesis of novel resveratrol and curcumin analogs in *E. coli*.

(i) HPLC analysis of the biosynthesis of 2-chloro-resveratrol by *E.*

coli/TAL-4CL-STS- Rdc2 at 330nm; (ii) HPLC analysis of the biosynthesis of dicaffeoylmethane by *E. coli*/TAL-C3H-4CL-CUS at 420 nm.

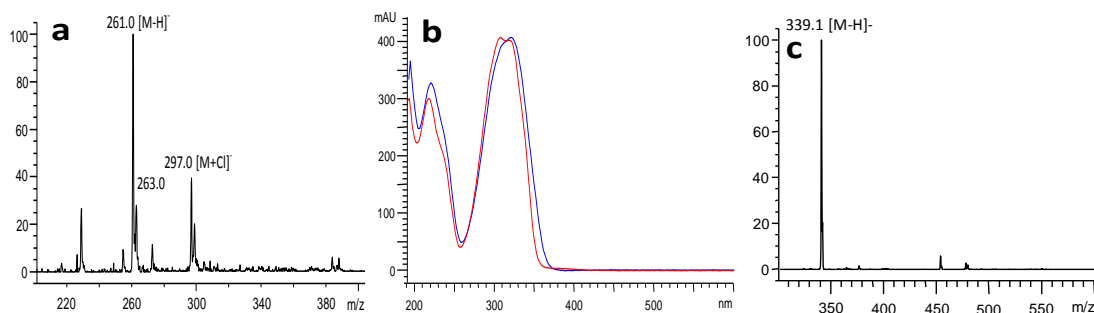


Fig. 12 LC-MS analyses of unnatural polyphenols production. **a.** ESI-MS (-) spectrum of halogenated resveratrol production from tyrosine; **b.** comparison of UV spectrum of halogenated resveratrol and resveratrol; **c.** ESI-MS (-) spectrum of dicaffeoylmethane production from tyrosine.

3.4 Conclusion

Natural products are a major source of new drugs. Numerous structures have been discovered from Nature, such as the well-known anticancer drug paclitaxel. The molecules are assembled through well designed biosynthetic pathways that consist of a series of collaborative biosynthetic enzymes. Plant natural products are traditionally extracted from the producing species. This process is inefficient since it takes long time from seeding to harvest. Although some natural product biosynthetic pathways from plants have been reported, direct engineering of these pathways in plants are technically challenging and time-consuming. Recent development in genome sequencing and synthetic biology has provided a unique opportunity to construct

artificial biosynthetic pathways in a heterologous host to generate plant natural products or novel structures. This work systematically demonstrated that a variety of biosynthetic pathways (Fig. 13) can be constructed based on eight biosynthetic enzymes to yield a number of biologically relevant molecules.

We made a small library of biosynthetic genes that were from different sources (plant, bacterial and fungal). Using a multiple-vector approach, these biosynthetic bricks can be combined in different ways to yield desired molecules. In phenylpropanoid biosynthesis, amino acids are first converted to various carboxylic acid precursors, which serve as the starter units for the following polyketide chain elongation. These phenylpropanoid acids include cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid. Some of these acids such as caffeic acid have also shown antioxidant activity [3]. In addition, these acids also show inhibitory activity on intestinal alpha-glucosidase activity, which delays the digestion of starch and disaccharides to absorbable monosaccharides and may help reduce postprandial hyperglycemia [19]. These compounds are synthesized from amino acids including L-phenylalanine and L-tyrosine. The simplest biosynthetic pathways constructed in this work were the production of *p*-coumaric acid (Fig. 13A, 160.5 ± 2.1 mg/l) and cinnamic acid (Fig. 13B, 241.3 ± 2.8 mg/l), which were synthesized from L-tyrosine by TAL and L-phenylalanine by PAL, respectively. While both enzymes showed high efficiency, the difference in the titer of these acids indicated that PAL might work

more efficiently than TAL in *E. coli*. *p*-Coumaric acid can be hydroxylated by C3H, as revealed by the production of caffeic acid at 70.0 ± 0.9 mg/l by *E. coli*/TAL-C3H (Fig. 13A). The titer of the final product decreased when an additional step was introduced to the pathway. This result was further supported by the production of ferulic acid (Fig. 13A) by *E. coli*/TAL-C3H-COMT at 28.8 ± 0.4 mg/l when the third enzyme COMT was introduced. Increasing the expression level of enzymes for the late steps may help increase the overall efficiency of such multi-step biosynthetic pathways.

With the pool of the eight biosynthetic enzymes, more complex biosynthetic pathways were constructed to generate a series of bioactive plant natural products. While resveratrol biosynthesis has been reconstituted in *E. coli* in previous studies, the total biosynthesis of piceatannol and pinosylvin was achieved for the first time in this work. The biosynthetic pathway of pinosylvin was rationally designed based on its structural similarity to resveratrol, and successfully established by taking advantage of the substrate flexibility of both 4CL and STS. The biosynthesis of piceatannol was constituted by the introduction of a C3H into the resveratrol biosynthetic pathway. This C3H represents a flexible hydroxylase with broad substrate specificity. It not only hydroxylates *p*-coumaric acid at C-3' to yield caffeic acid, but also converts resveratrol to piceatannol by the hydroxylation at the same position. A recent work reported that a non-P450 monooxygenase (HpaBC) from *E.*

coli can also hydroxylate resveratrol at the same position [20]. Production of pinosylvin and piceatannol provides in *E. coli* an efficient way to produce these plant natural products. More complex biosynthetic pathways were constructed using the pool of biosynthetic enzymes to yield a series of curcuminoids. Collaborative actions of five enzymes including TAL, 4CL, C3H, COMT and CUS in *E. coli* resulted in the total biosynthesis of the pharmaceutically important molecule curcumin.

We also demonstrated the feasibility of rational design of new molecules, either by removal or addition of an enzyme from a particular biosynthetic pathway. For example, removal of COMT from the curcumin biosynthetic pathway leaves the 8- and 8'-OH unmethylated, leading to the production of the curcumin analog dicafferolmethane. When a halogenase Rdc2 was added to the resveratrol biosynthetic pathway, a novel chlorinated analog of resveratrol was produced. This molecule has been previously chemically synthesized [18]. However, chemical preparation of chlorinated is limited by the lack of selection. In this constructed pathway, Rdc2 can specifically introduce a chlorine atom at C-2, further indicating that this enzyme has broad substrate specificity. 2-Chloro-resveratrol has shown stronger antioxidant and antimicrobial activity than resveratrol [18].

In summary, we used a simple set of eight biosynthetic enzymes to achieve the biosynthesis of 12 phenylpropanoids in *E. coli*. More structures can be easily

produced by varying the combinations of these enzymes. For example, co-expression of TAL, 4CL, C3H, STS and Rdc2 in *E. coli* is expected to generate 2-chloro-piceatannol. Functional expression of the biosynthetic enzymes in *E. coli* is required, while substrate flexibility of these enzymes is preferred as this will significantly increase the number of possible pathways that can be constructed. Further expansion of this library of biosynthetic “legos” will yield a huge resource to produce pharmaceutically important molecules in a fast-growing host or synthesize novel compounds for bioactivity screening.

3.5 Acknowledgments

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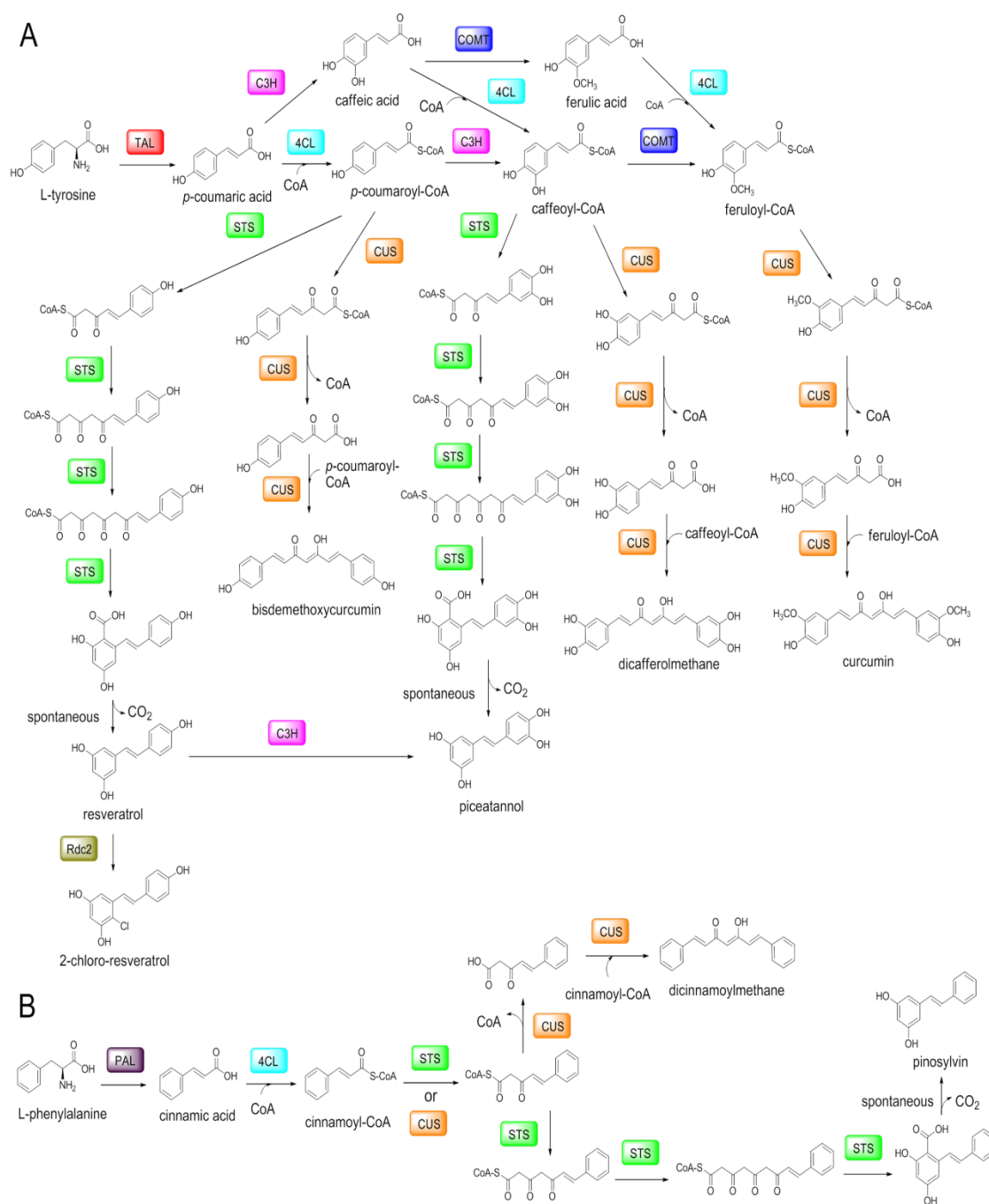


Fig. 13 Combinatorial biosynthesis of plant natural products and their analogs in *E. coli* from L-tyrosine (A) and L-phenylalanine (B) using biosynthetic bricks from different sources.

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CHAPTER 4 DESIGN AND APPLICATION OF AN IN VIVO REPORTER

ASSAY FOR PHENYLALANINE AMMONIA-LYASE *

ABSTRACT

Phenylalanine ammonia-lyase (PAL) is an important enzyme that links primary metabolism to secondary metabolism. Its efficiency is often a critical factor that affects the overall flux of a related metabolic pathway, the titer of the final products, and the efficacy of PAL-based therapies. Thus, PAL is a common target for metabolic engineering and it is of significant interest to screen efficient PALs for industrial and medical applications. In this study, a novel and efficient visible reporter assay for screening of PAL efficiency in *E. coli* was established based on a plant type III polyketide biosynthetic pathway. The candidate PALs were co-expressed with a 4-coumarate:CoA ligase 4CL1 from *Arabidopsis thaliana* and curcuminoid synthase (CUS) from *Oryza sativa* in *E. coli* BL21(DE3) to form a dicinnamoylmethane biosynthetic pathway. Taking advantage of the yellow color of the product, a microplate-based assay was designed to measure the titer of dicinnamoylmethane, which was validated by HPLC analysis. The different titers of the product reflect the overall performance (expression and enzymatic efficiency) of the individual PALs in *E. coli*. Using this system, we have screened three PALs (PAL1, PAL3 and PAL4) from *Trifolium pratense*, among which PAL1 showed the best performance in *E. coli*.

The engineered *E. coli* strain containing PAL1, 4CL1 and CUS led to the production

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of dicinnamoylmethane at a high level of 0.36g/l. Supplement of 2-fluoro-phenylalanine yielded two fluorinated dicinnamoylmethane derivatives, 6, 6'-difluoro-dicinnamoylmethane and 6-fluoro-dicinnamoylmethane, of which the latter is a new curcuminoid.

4.1 Introduction

Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the conversion of phenylalanine (Phe) to trans-cinnamic acid and ammonia through deamination. It is considered to be an essential linker between the primary metabolism and secondary metabolism [1, 2] as it is involved in both amino acid metabolism and natural product biosynthetic pathways. Because of its importance in different metabolic pathways, PAL has gained considerable significance in medical, industrial, and biotechnological applications [3]. Since its discovery in 1961[4], PAL has been extensively investigated. Many PAL genes have been discovered from plants and microorganisms and some of the structures have been solved [5, 6].

PAL is a potential agent in the treatment of phenylketonuria, an autosomal recessive genetic disorder that results in an inability to metabolize Phe and elevated levels of this amino acid in the bloodstream, which can cause severe mental retardation. Oral or subcutaneous administration of PAL led to substantial lowering of plasma and brain Phe levels [7-9]. With further improvement of the stability, this enzyme can be used in enzyme substitution therapy of phenylketonuria. PAL was

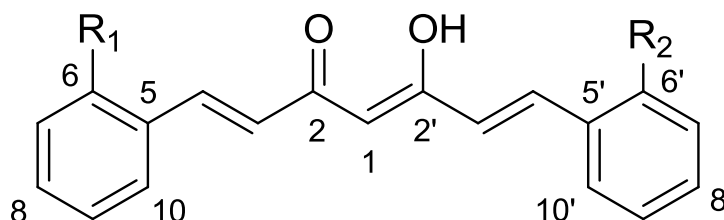
used in industry to produce L-Phe from trans-cinnamic acid by taking advantage of the reversal of the physiological reaction [10]. A PAL from *Rhodotorula glutinis* can directly convert trans-cinnamic acid methyl ester to L-Phe methyl ester, which eliminates the need for chemical esterification of L-Phe with methanol in aspartame synthesis [11]. PAL is also frequently observed in secondary metabolic pathways that lead to the formation of various natural products such as enterocin in the marine bacterium *Streptomyces maritimus* [12] and phenylpropanoids in plants [13] including flavonoids, isoflavonoids, coumarins, stilbenes, hydroxycinnamic acids, lignin, and other phenolic compounds. In addition to serving as regulatory or signaling molecules in plants [14], many of these natural products have also displayed promising biological activities such as antioxidant, antimicrobial and anti-inflammatory properties. PAL catalyzes the first biosynthetic step of phenylpropanoid products and plays a key regulatory role in controlling the biosynthesis of these natural products. Its efficiency is important for carbon flux into these pathways and directly influences the yields of the final products. Thus, PAL has become a critical metabolic engineering target for many natural product biosynthetic pathways.

Curcuminoids are a group of plant phenylpropanoid natural products, exemplified by curcumin, and have long been used as a traditional Asian medicine because of their anti-tumor, antioxidant, anti-inflammatory, anti-allergy and hepatoprotective activities [15-24]. They are also the major constituents in curry spice that is widely used in

various foods. Recently, curcuminoids have been found to be synthesized by type III polyketide synthases (PKSs) in plants. Two type III PKSs, diketide-CoA synthase (DCS) and curcumin synthase (CURS), are involved in the biosynthesis of curcuminoids in the herb *Curcuma longa*. DCS takes feruloyl-CoA and malonyl-CoA to synthesize feruloyldiketide-CoA, which is further converted into curcumin by CURS [25]. When other substrates such as 4-coumaroyl-CoA are taken, different curcuminoids can be formed. In contrast to the collaborative actions of two type III PKSs in *C. longa*, an individual type III PKS from *Oryza sativa*, curcuminoid synthase (CUS), was found to synthesize curcuminoids from aromatic CoA starters and malonyl-CoA. CUS has been functionally expressed in *Escherichia coli*. Its co-expression with PAL from *Rhodotorula rubra* and 4-coumarate: CoA ligase (4CL) from *Lithospermum erythrorhizon* has led to the production of plant-specific curcuminoids in *E. coli* [26]. The engineered strain was also utilized to produce unnatural curcuminoids by providing a variety of carboxylate precursors [27].

In spite of the importance of PAL, there is no efficient screening assay for this type of enzyme. The yellow color makes curcuminoids potential reporter molecules. In this study, we established a novel and efficient visible screening assay for efficient PALs in an *E. coli* system based on the curcuminoids biosynthetic pathway. The overall performance of a PAL is reflected in the titer of dicinnamoylmethane (**1**, Fig. 14) that can be easily detected by eyes and measured by a UV-Vis microplate reader.

Using this system, we have compared the efficiency of three PALs from *Trifolium pratense* and found that PAL1 showed the highest efficiency in *E. coli*. The collaborative work of PAL1 with 4CL1 from *Arabidopsis thaliana* and CUS from *O. sativa* led to the high-yield production of **1** in *E. coli*. Supplement of 2-fluoro-L-Phe into the fermentation broth yielded two fluorinated dicinnamoylmethane derivatives, 6-fluoro-dicinnamoylmethane (**2**, Fig. 14) and 6,6'-difluoro-dicinnamoylmethane (**3**, Fig. 14). **2** is a new compound.



Dicinnamoylmethane (**1**) $R_1 = R_2 = H$
 6-Fluoro-dicinnamoylmethane (**2**) $R_1 = F, R_2 = H$
 6,6'-Difluoro-dicinnamoylmethane (**3**) $R_1 = R_2 = F$

Fig. 14 Curcuminoids (**1-3**) synthesized in *E. coli*. **1** was synthesized from L-Phe.

4.2 Materials and methods

4.2.1 General method

Products were analyzed and purified on an Agilent 1200 HPLC instrument. ESI-MS spectra were acquired on an Agilent 6130 quadrupole LC-MS in the positive mode. NMR spectra were recorded in acetone-d₆ on a JEOL NMR instrument (300 MHz for ¹H NMR and 75 MHz for ¹³C NMR). The chemical shift (δ) values are given in parts per million (ppm). The coupling constants (J values) are reported in

Hertz (Hz). The titers of **1** were analyzed on a SpectraMax® 190 Absorbance Plate Reader (Molecular Devices, CA, USA) and an Agilent 1200 HPLC system.

4.2.2 Bacterial strains and materials

E. coli XL1-Blue was utilized for routine cloning and plasmid propagation. *E. coli* BL21 (DE3) was used for protein expression and the designed reporter assay. All *E. coli* strains were grown at 37 °C on LB agar plates or in liquid LB medium supplemented with appropriate antibiotics (ampicillin, 50µg/ml; kanamycin, 50µg/ml; chloramphenicol, 25µg/ml; Gold Biotechnology, MO, USA). Restriction enzymes, Phusion® High-Fidelity DNA polymerase, and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA, USA). CloneJET™ PCR Cloning Kit was from Fermentas (Glen Burnie, MD, USA), and the pET28a and pACYCDuet-1 vectors were purchased from Novagen (Madison, WI, USA). The plasmids including pMLS252-PAL1, pMLS252-PAL3 and pMLS252-PAL4 that contain the cDNAs of PALs were gifts from Dr. Mike Sullivan from USDA. The plasmids pUC-4CL1 (containing the cDNA of 4CL1 from *A. thaliana*) and pET16b-CUS (containing the cDNA of CUS from *O. sativa*) were respectively provided by Dr. Claudia Schmidt-Dannert at the University of Minnesota and Dr. Nobutaka Funa at the University of Shizuoka, Japan.

4.2.3 Gene amplification and plasmids construction

The cDNAs encoding PAL1 (GenBank accession number DQ073809), PAL3 (GenBank accession number DQ073808) and PAL4 (GenBank accession number DQ073811) were amplified with Phusion® High-Fidelity DNA polymerase from pMLS252-PAL1, pMLS252-PAL3 and pMLS252-PAL4, respectively. A 30-cycle polymerase chain reaction (PCR) program (60 s at 98 °C, 60 s at 68 °C and 150 s at 72 °C) was used. The 4CL1 cDNA (GenBank accession number U18675) was amplified from pUC-4CL1 using a 30-cycle PCR program (60 s at 98 °C, 60 s at 62 °C and 135 s at 72 °C). The primers are shown in Table 3. These four PCR products were gel-purified and ligated into the pJET1.2 cloning vector to yield pSW48, pSW49, pSW50 and pSW52 (Table 4). The genes were confirmed by digestion checks and sequencing.

The CUS gene (GenBank accession number Q8LIL0) was excised from pET16b-CUS with NdeI and BamHI and ligated into pET28a between the same sites, yielding pSW24. The 4CL1 gene was excised from pSW52 with NdeI and XhoI and ligated into pACYCDuet-1 between the same sites, yielding pSW54. The PAL1, PAL3 and PAL4 genes were excised with PstI and NotI from pSW48, pSW49 and pSW50, respectively, and ligated to pSW54 between the same sites to yield pSW56, pSW57 and pSW58 (Table 4).

Table 3. Primers used in Chapter 4.*

Primer	Sequence
PAL1-F	5'-AACTGCAGATGGAGGGAATTACCAATGG-3'
PAL1-R	5'-AAGCGGCCGCTCAACATATTGGAAGA-3'
PAL3-F	5'-AACTGCAGATGGAAGCAGTAGCAGCA-3'
PAL3-R	5'-AAGCGGCCGCTTAACAAATTGGAAGA-3'
PAL4-F	5'-AACTGCAGATGGAAGCAGAGCATCAGC-3'
PAL4-R	5'-AAGCGGCCGCTTAACATATTGGAAGAG-3'
4CL1-F	5'-AACATATGGCGCCACAAGAACAAGC-3'
4CL1-R	5'-AACTCGAGTCACAATCCATTTGCTAGTTTT-3'
PAL-RT-F	5'-CCATGTTTACCACTTCGCG-3'
PAL-RT-R	5'-AGAACAATAGAAGCTAAACCAGAAC-3'
16S-RT-F	5'-CTCCTACGGGAGGCAGCAG-3'
16S-RT-R	5'-GWATTACCGCGGCKGCTG-3'

* Restriction sites are italicized and the start/stop codons are shown in bold.

Table 4. Plasmids used in Chapter 4.

Plasmid	Description	Reference
pMLS252- <i>pal1</i>	The intron-free PAL1 gene from <i>T. pratense</i>	(Sullivan 2009)
pMLS252- <i>pal3</i>	The intron-free PAL3 gene from <i>T. pratense</i>	(Sullivan 2009)
pMLS252- <i>pal4</i>	The intron-free PAL4 gene from <i>T. pratense</i>	(Sullivan 2009)
pUC- <i>4cl1</i>	The 4CL1 cDNA from <i>A. thaliana</i>	(Watts et al. 2006)
pET16b- <i>cus</i>	The CUS cDNA from <i>O. sativa</i>	(Katsuyama et al. 2007)
pSW24	CUS in pET28a	this work
pSW48	PAL1 in pJET1.2	this work
pSW49	PAL3 in pJET1.2	this work
pSW50	PAL4 in pJET1.2	this work
pSW52	4CL1 in pJET1.2	this work
pSW54	4CL1 in pACYCDuet-1	this work
pSW56	PAL1 and 4CL1 in pACYCDuet-1	this work
pSW57	PAL3 and 4CL1 in pACYCDuet-1	this work
pSW58	PAL4 and 4CL1 in pACYCDuet-1	this work

4.2.4 Dicinnamoylmethane production in *E. coli*

A typical fermentation procedure is as follows. Engineered *E. coli* BL21 (DE3) strains were precultured overnight at 37 °C in 5 ml of LB medium supplemented with

50 µg/ml kanamycin and 25 µg/ml chloramphenicol. The preculture was transferred into 50 ml of LB medium with the same antibiotics and cultured at 37 °C until the OD₆₀₀ reached 0.4~0.6. IPTG was then added at a final concentration of 1 mM and the induced culture was maintained at 25 °C with shaking at 250 rpm for an additional 5 h. After that, 3 mM L-Phe, 40 g/l glucose and 25 g/l CaCO₃ were added, and the culture was incubated at 25 °C for an additional 60 h. The fermentation broth was harvested by centrifugation at 4,000 rpm for 10 min. The supernatant was adjusted to pH 3.0 with 6 M HCl and extracted with an equal volume of ethyl acetate. The cells were extracted with 10 ml of methanol by sonication. The extracts were concentrated by solvent evaporation and subjected to product analysis.

4.2.5 Microplate-based assay for dicinnamoylmethane biosynthesis in *E. coli*

To purify **1** for structural characterization and quantification of its production in the engineered *E. coli* strains, the extract of 100 ml of the fermentation broth of *E. coli* BL21 (DE3)/pSW24+pSW56 was separated on a silica gel 60 open column. The column was successively washed with a gradient of hexane/acetone (1:0, 9:1, 3:1, 1:1, and 0:1, v/v). The fraction eluted by hexane/acetone (9:1) was found to contain **1** and further separated on an Agilent 1200 HPLC using a SUPELCO Supelcosil™ LC-PAH column (5 µm, 250 mm × 10 mm), eluted with acetonitrile-water (70:30, v/v) at a flow rate of 3 ml/min for 45 min. The peak at 30 min was collected, yielding 12 mg of **1** in pure form. The compound was structurally characterized based on its ESI-MS and NMR data.

Dicinnamoylmethane (**1**): yellow powder. ^1H NMR (300 MHz, acetone- d_6): δ 7.71 (4H, d, $J = 5.9$ Hz, H-6, H-6', H-10, and H-10'), 7.70 (2H, d, $J = 15.1$ Hz, H-4 and H-4'), 7.44 (6H, m, H-7, H-7', H-8, H-8', H-9, and H-9'), 6.89 (2H, d, $J = 15.8$ Hz, H-3 and H-3'), 6.14 (1H, s, H-1). ^{13}C NMR (75 MHz, acetone- d_6): δ 184.4 (C-2 and C-2'), 141.2 (C-4 and C-4'), 136.1 (C-5 and C-5'), 131.1 (C-8 and C-8'), 129.9 (C-7, C-7', C-9, and C-9'), 129.1 (C-6, C-6', C-10, and C-10'), 125.2 (C-3 and C-3'), 102.6 (C-1).

The purified compound was dissolved in methanol and prepared as different concentrations by a serial dilution. The solutions of **1** was distributed into the wells in a 96-well UV-plate (Corning, NY, USA) and measured on a SpectraMax® 190 Absorbance Plate Reader at 390 nm to get the standard curve. For time course analysis, the fermentation broths were sampled at 12, 24, 36, 48, 60, and 72 h, respectively. The samples (1 ml each) were extracted as described above and the dried extracts were re-dissolved in 1 ml of methanol, from which 200 μl of each extract was subjected to titer analysis on the SpectraMax® 190 Absorbance Plate Reader.

4.2.6 HPLC analysis of dicinnamoylmethane biosynthesis in *E. coli*

A standard curve of **1** was established by injecting different amounts of the purified compound into HPLC and correlating the peak areas to the amounts. Product analysis was conducted using an Agilent 1200 HPLC system with an Agilent

Eclipse XDB-C18 column (5 μ m, 250 mm \times 4.6 mm). The samples were eluted with acetonitrile-water (70:30, v/v) at a flow rate of 1 ml/min for 30 min, and the product was detected at 390 nm. For time course analysis, 200 μ l of the same samples used for the microplate-based assay were analyzed by HPLC.

4.2.7 Quantitative real-time PCR analysis of PAL expression in *E. coli*

For quantitative real time-PCR (qRT-PCR) analysis of the expression of PAL1, PAL3 and PAL4, total RNA was extracted from *E. coli* BL21(DE3)/pSW24+pSW56, *E. coli* BL21(DE3)/pSW24+pSW57 and *E. coli* BL21(DE3)/pSW24+pSW58, respectively, using a Quick-RNA Miniprep kit (ZYMO Research, CA, USA). cDNAs were then synthesized by RT-PCR with a SuperScript III First-Strand Synthesis System (Invitrogen, CA, USA). The synthesized cDNAs served as the template for qRT-PCR. Reactions were run using the iTaq Universal SYBR Green Supermix (Bio-Rad, CA, USA) in 20- μ l reaction mixtures on a Bio-Rad iQ5 Real Time PCR Detection System (Bio-Rad, CA, USA). A pair of primers including PAL-RT-F and PAL-RT-R (Table 3) was used. 16S rDNA was chosen as the internal control with 16S-RT-F and 16S-RT-R as the primers (Table 3). Initial denaturation was done at 95 °C for 30 s, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s. Data were analyzed with auto Ct (threshold cycle) and auto baseline functions of the system and Ct values of the triplicate reactions were averaged. Δ Ct were determined as Ct (internal control) - CtPAL for each gene.

4.2.8 Purification and structural analysis of the new fluorinated dicinnamoylmethane derivative **2**

The fermentation procedure was same as described above excepted that 3 mM 2-fluoro-L-Phe was added instead of L-Phe after induction by 1 mM IPTG for 5 h at 25 °C. The culture was incubated at 25 °C for an additional 60 h. The extract was analyzed by LC-MS. For large-scale preparation of the new fluorinated dicinnamoylmethane derivative **2**, the fermentation was performed in 2 L of LB medium. The extract was first separated on a silica gel 60 open column, successively eluted with increasing concentrations of hexane/acetone (1:0, 9:1, 3:1, 1:1, and 0:1, v/v). The fractions containing **2** were lumped and further separated on a SUPELCO Supelcosil LC-PAH column (5 µm, 250 mm × 10 mm), eluted with acetonitrile-water (70:30, v/v) at a flow rate of 3 ml/min for 45 min purification. The peak at 35 min was collected and subjected to NMR analysis.

4.3 Results

4.3.1 Design of a reporter assay for PAL expression in *E. coli*

Dicinnamoylmethane (**1**) is a curcuminoid synthesized by the sequential actions of PAL, 4CL and CUS (Fig. 15a). PAL catalyzes nonoxidative deamination of L-Phe to yield trans-cinnamic acid, which is then ligated with CoA by 4CL to generate cinnamoyl-CoA. CUS is a type III PKS and it takes two units of cinnamoyl-CoA and one unit of malonyl-CoA to yield **1**. We chose to use **1** as the reporter molecule for in

vivo activity of PALs by taking advantage of its yellow color. To this end, we designed a reporter assay system consisting of two plasmids that harbor 4CL and CUS, respectively (Fig. 15b). Specifically, a 4CL from *A. thaliana*, named 4CL1, was cloned into pET28a to yield pSW24, while CUS from *O. sativa* was inserted into pACYCDuet-1 to afford pSW54. A candidate PAL gene can be ligated into pSW54 between PstI and NotI (or other available sites such as BamHI and EcoRI). All these three biosynthetic genes in pET28a and pACYCDuet-1 are under the control of separate T7 promoters. Co-expression of the resulting plasmids will build the necessary enzymatic platform for dicinnamoylmethane biosynthesis. The production of **1** will be visible because of its yellow color and the titer can be monitored by HPLC or UV-Vis microplate reader.

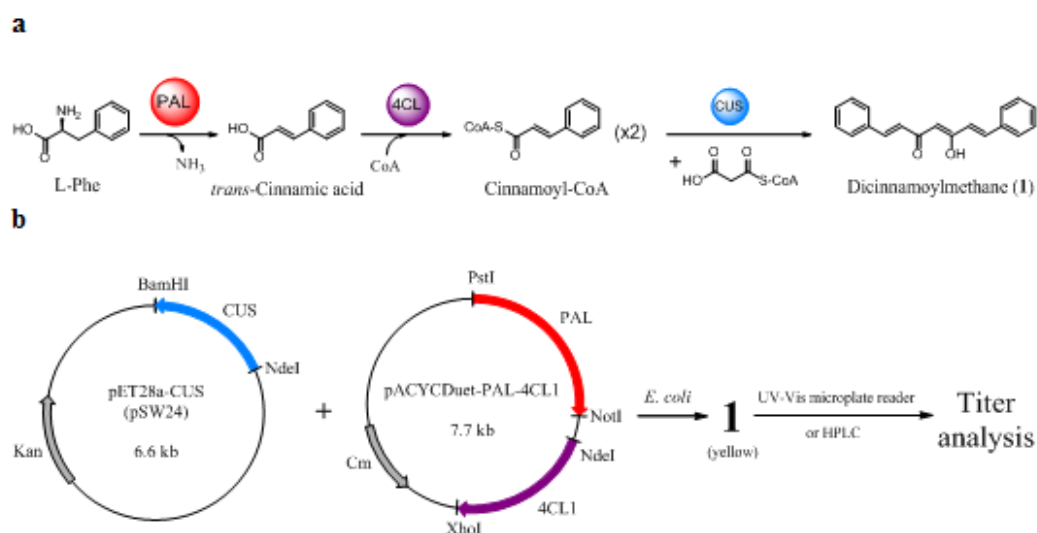


Fig. 15 Design of a reporter assay for PALs. **a**. Biosynthetic pathway of **1** from L-Phe by the sequential actions of PAL, 4CL and CUS. **b**. A reporter assay for screening of PALs in *E. coli*. The system consists of two plasmids. The first one contains CUS and

the second one has 4CL1. A candidate PAL gene will be ligated into the second vector between PstI and NotI. Co-expression of the two plasmids will lead to the biosynthesis of **1**, which is visible for its yellow color and can be analyzed by HPLC or UV-Vis microplate reader.

4.3.2 Examination of the expression of three PALs from red clover in *E. coli* using the designed *in vivo* reporter assay

To test the effectiveness of the designed *in vivo* reporter assay, we chose three plant PALs from *T. pretense*. The cDNAs encoding PAL1, PAL3 and PAL4 were individually introduced to pSW54 between the PstI and NotI sites to yield pSW56, pSW57 and pSW58 (Table 4), respectively. Color changes in the fermentation broths of *E. coli*/pSW24+pSW56, *E. coli*/pSW24+pSW57 and *E. coli*/pSW24+pSW58 were observed, compared to *E. coli*/pSW24+pSW54 that served as the negative control. LC-MS analysis of the extracts of these cultures revealed that *E. coli*/pSW24+pSW56, *E. coli*/pSW24+pSW57 and *E. coli*/pSW24+pSW58 produced the same yellow product **1** at 17.5 min, as shown in Fig. 16a. The ion peaks $[M+H]^+$ at m/z 277.0 and $[M+Na]^+$ at m/z 298.9 in the ESI-MS spectra suggested that **1** has a molecular weight of 276. This peak showed a maximum UV absorption at 390 nm, which is consistent with that for dicinnamoylmethane [26]. NMR analysis confirmed that the purified compound is dicinnamoylmethane [26]. These results suggested that all three tested PALs were functionally expressed in *E. coli* BL21 (DE3). We also found that more than 71% of **1** was in the *E. coli* cells.

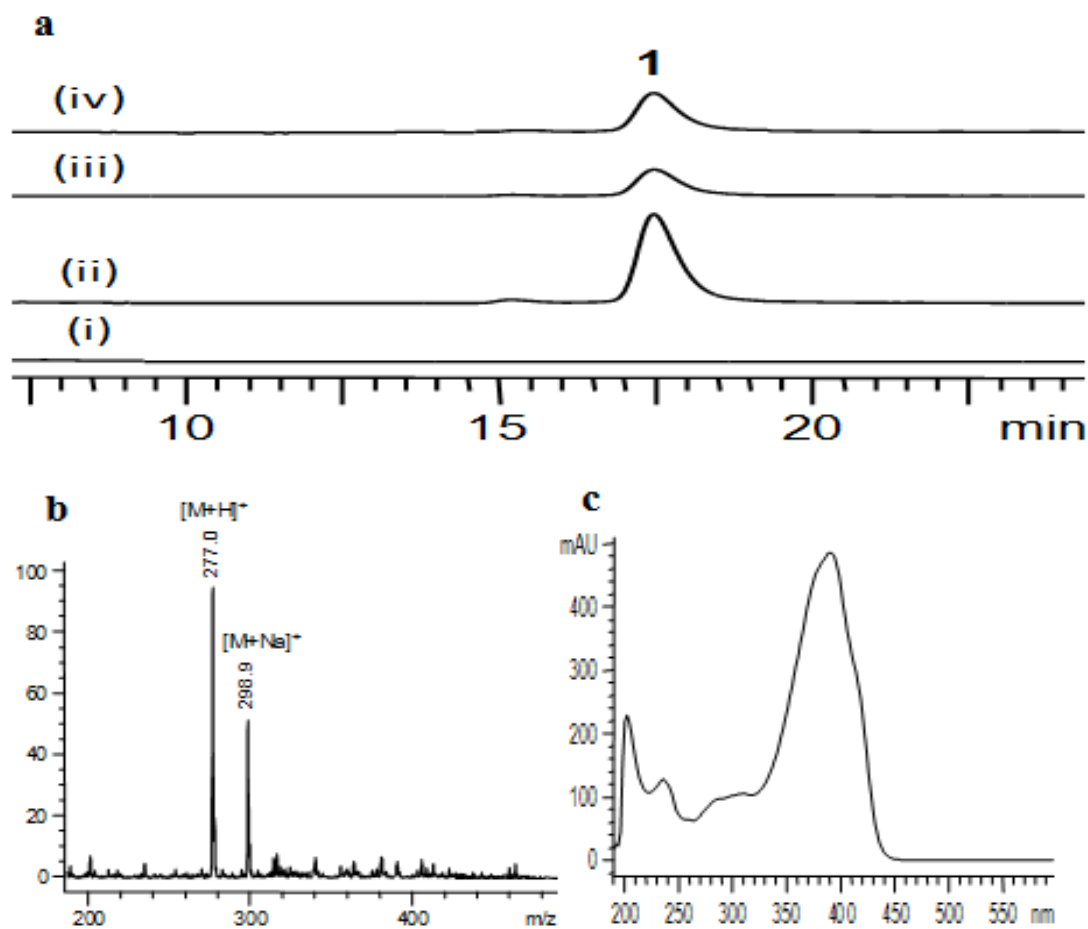


Fig. 16 HPLC-MS analysis of the production of **1** in engineered *E. coli*. **a.** HPLC traces of the methanol extract of the cell pellets of *E. coli*/pSW24+pSW54 (i), *E. coli*/pSW24+pSW56 (ii), *E. coli*/pSW24+pSW57 (iii) and *E. coli*/pSW24+pSW58 (iv); **b.** ESI-MS (+) spectrum of the peak at 17.5 min; **c.** UV absorption spectrum of the peak at 17.5 min.

4.3.3 Development of a quantitative microplate-based assay for the production of **1**

To differentiate the titers of **1** by different PALs, a microplate-based assay was developed. Briefly, 1 ml of the fermentation broth was pelleted by centrifugation. The supernatant was extracted with 1 ml of ethyl acetate and the cells were extracted with

500 μ l of methanol. The extracts were combined and dried under reduced pressure. The sample was then re-dissolved in methanol, distributed into a 96-well UV-plate and measured on a UV-Vis microplate reader at 390 nm. The concentrations were determined based on the standard curve of **1** (Fig. 17a). Using this assay, we conducted a time course analysis for *E. coli*/pSW24+pSW56, *E. coli*/pSW24+pSW57 and *E. coli*/pSW24+pSW58. After the addition of L-Phe, glucose and CaCO₃, the fermentation broths were sampled at 12, 24, 36, 48 60 and 72 h, and the titers of **1** are shown in Fig. 17b. It is apparent that *E. coli*/pSW24+pSW56 produced much higher amounts of **1** than *E. coli*/pSW24+pSW57 and *E. coli*/pSW24+pSW58 did, suggesting that the overall performance (expression and efficiency) of PAL1 is better than the other two PALs. The best titer of **1** by *E. coli*/pSW24+pSW56 that contains PAL1, 4CL1 and CUS reached 0.36 g/l after 72 h, while the titers by PAL3 and PAL4 were only 0.08 g/l and 0.11 g/l, respectively. To validate the microplate-based assay, the same samples were analyzed by HPLC (Fig. 17c) and the titers of **1** were determined based on the areas of peak **1**. Although there were occasional small differences between the HPLC and microplate reader measurements, which was likely due to the interfering compounds in the fermentation broth at different stages, overall the data from the microplate-based assay and HPLC analysis were consistent, indicating that this quantitative microplate-based assay was effective.

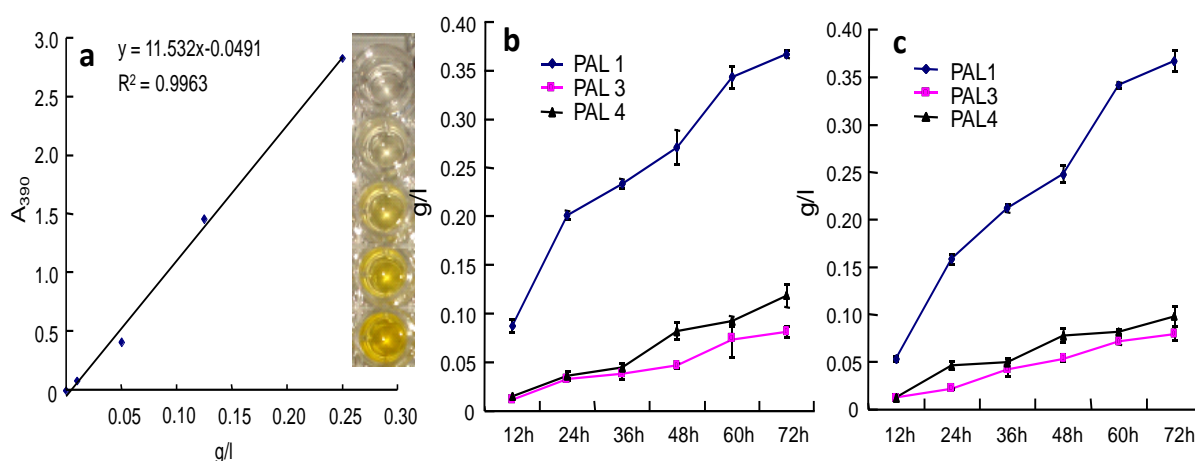


Fig. 17 Time course analysis of the production of dicinnamoylmethane by the co-expression of 4CL1 and CUS with PAL1, PAL3 or PAL4. **a.** Standard curve of dicinnamoylmethane on a UV-Vis microplate reader at 390 nm; **b.** Time course analysis by a UV-Vis microplate reader at 390 nm; **c.** Validation of the microplate-based assay by HPLC analysis at 390 nm.

4.3.4 qRT-PCR analysis of the expression of PAL1, PAL3 and PAL4 in *E. coli*

We also used qRT-PCR to compare the expression level of the three plant PALs in *E. coli*/pSW24+pSW56, *E. coli*/pSW24+pSW57 and *E. coli*/pSW24+pSW58, respectively. As shown in Table 5, the qRT-PCR results indicated that the expression level of PAL1 was higher than the other two, and the expression level of the PAL3 was the lowest among these three PALs. This is consistent with the analysis results of the titers of **1** in the corresponding *E. coli* strains, although the latter is the combined effects of both the expression level and catalytic activity of PALs.

Table 5. Relative expression level of three different plant PALs in *E. coli*

Enzyme	ΔCt
PAL1	-4.06 \pm 0.46
PAL3	-1.10 \pm 0.34
PAL4	-3.50 \pm 0.36

4.3.5 Precursor-directed biosynthesis of a new fluorinated dicinnamoylmethane derivative

With the designed reporter assay, we determined that PAL1 showed the best performance in *E. coli* among the three tested plant PALs. We then used the strain *E. coli*/pSW24+pSW56 that expressed PAL1, 4CL1 and CUS for the synthesis of new curcuminoids. Previous studies have shown that it is possible to synthesize new curcuminoids with the curcuminoids biosynthetic enzymes by feeding different precursors [27, 28]. To this end, we fed 2-fluoro-L-Phe into the induced culture of *E. coli*/pSW24+pSW56 instead of L-Phe. As shown in Fig. 18a, LC-MS analysis revealed that three major curcuminoids were produced. In addition to **1**, two new products **2** and **3** were synthesized. The ESI-MS spectra of **2** (Fig. 18b) revealed that it has a molecular weight of 294, suggesting that it is a monofluorinated derivative of **1**. The ESI-MS spectra of **3** (Fig. 18c) showed that this compound has a molecular weight of 312, suggesting that it has one more fluorine atom than **2** and is a difluorinated derivative of **1**. While 6, 6'-difluoro-dicinnamoylmethane has been previously reported [27], ¹³C NMR spectrum of **2** revealed that it has a quaternary carbon at C-6 instead of a CH in **1**. The chemical shift of C-6 is δ 163.3, suggesting that this position is fluorinated. Further 1D and 2D NMR analysis confirmed that it is

6-fluoro-dicinnamoylmethane, which is a new compound. The proton and carbon signals are given in Appendix D. These two fluorinated products resulted from the incorporation of one and two 2-fluoro-L-Phe units, respectively.

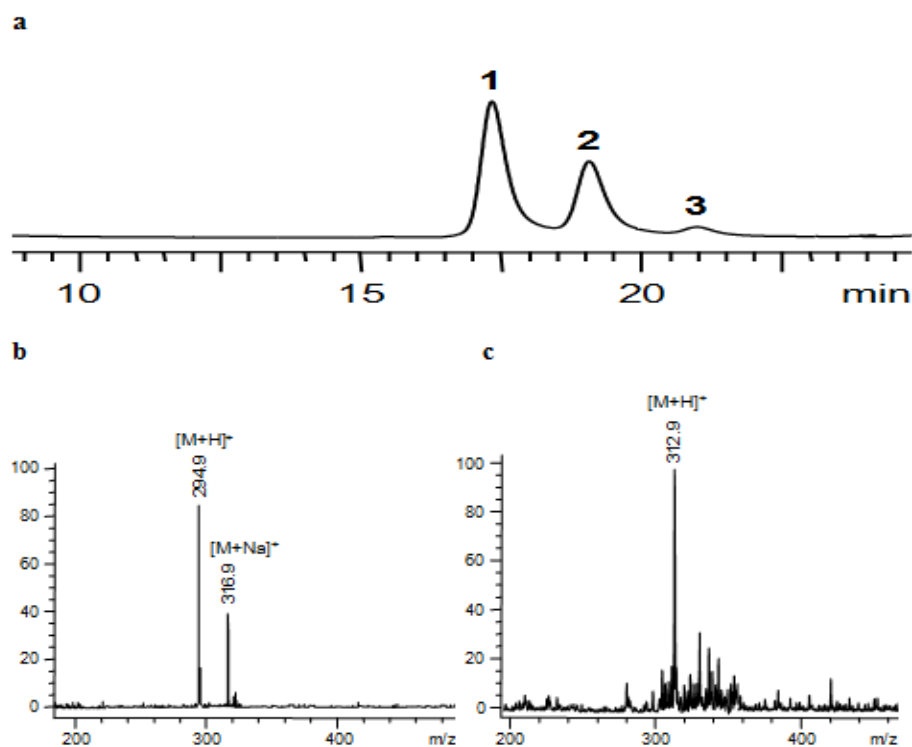


Fig. 18 LC-MS analysis of the production of a new fluorinated dicinnamoylmethane derivative **2** in *E. coli*. **a.** HPLC trace of the methanol extract of the cells of *E. coli*/pSW24+pSW56 supplemented with 2-fluoro-L-Phe; **b.** ESI-MS (+) spectrum of **2**; **c.** ESI-MS (+) spectrum of **3**.

4.4 Conclusion

PAL is a key enzyme involved in both primary and secondary metabolism, and has attracted more and more attention because of their applications in agriculture, medicine and industry. The efficiency of PAL has direct impacts on the efficacy of

the enzyme-based treatment or an industrial production process. Screening of efficient PALs is thus of significant interest. In this work, we developed a microplate-based reporter assay for screening of efficient PALs using the *E. coli* system, which is easily accessible. PAL is involved in the biosynthesis of many plant phenylpropanoids, among which curcuminoids are a group of yellow compounds synthesized from L-Phe by a type III polyketide biosynthetic pathway consisting of three enzymes. In this work, we developed a novel reporter assay for PALs using a two-plasmid screening system that expresses 4CL1 from *A. thaliana* and CUS from *O. sativa*. Together with any functional PAL, this system will yield **1**, which allows efficient and visible screening of PALs in *E. coli*. Further development of the microplate-based assay, which was validated by HPLC analysis, will facilitate high throughput screening for efficient PALs.

Biological reporter systems have been developed for different uses in research and industry. For example, a reporter assay has recently been developed for bacterial and mammalian cells based on the blue color of the non-ribosomal peptide indigoidine. Indigoidine is synthesized from two units of L-glutamine [29]. Accordingly, the designed system consisted of the *Streptomyces lavendulae* non-ribosomal peptide synthetase BpsA and the *Streptomyces verticillus* 4'-phosphopantetheinyl transferase Svp that activates the thiolation domain in BpsA [30]. While our work established a reporter assay in *E. coli*, it is possible to construct

similar screening system in other hosts such as yeasts and mammalian cells for different purposes with the help of synthetic biology techniques.

The tested three PALs were from red clover (*T. pratense*). PAL3 and PAL4 are nearly identical with 97% identity, and they share only 89% identity with PAL1. These enzymes are involved in the biosynthesis of phenylpropanoid compounds in the plant, and PAL3 and PAL4 were also reported to participate in pathogen defense response [31]. However, no biochemical studies on these enzymes have been performed and their catalytic activities remain unknown. We compared these three PALs in *E. coli* using the designed reporter assay and found that PAL1 showed the best overall performance, which is a combined effect of the expression level and catalytic efficiency. Furthermore, the qRT-PCR analysis confirmed that the expression level of PAL1 was the highest in *E. coli* among the three tested PALs. It is known that many molecular technology tools, such as RT-PCR, SDS-PAGE and western blotting, can be used to analyze protein expression at the transcriptional or translational level. However, these analyses are often time-consuming and more costly. The reporter assay we established provides a novel, visible and easy-to-assay approach for screening highly efficient PALs, which requires remarkably smaller volumes, lower costs and shorter time compared to traditional analytic methods. Furthermore, the results from this assay directly reveal the overall performance of PALs in *E. coli* instead of only reporting the expression level or catalytic efficiency.

With the successful screening of the most efficient enzyme from a group of plant PALs, this reporter assay can be further utilized to screen more efficient PALs from a larger library for the production of useful products. In this work, the titer of **1** reached 0.36 g/l in *E. coli*. Because more than 71% of the product is in the cells, the screening can be further simplified by focusing on the cells and discarding the supernatants after centrifugation. Precursor-directed biosynthesis of **2** and **3** revealed that PAL1 can deaminate 2-fluoro-L-Phe to yield the corresponding fluorinated trans-cinnamic acid, which is then processed by 4CL1 and CUS to generate the fluorinated curcuminoids. This confirmed that the three biosynthetic enzymes including PAL1, 4CL1 and CUS have relatively flexible substrate specificity, thus opening an opportunity to synthesize novel curcuminoids by feeding different precursors.

4.5 Acknowledgments

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CHAPTER 5 BIOSYNTHESIS AND ANTIBACTERIAL ACTIVITY OF DUTOMYCIN AND ANALOGS

ABSTRACT

Dutomycin, an anthracycline natural product, was isolated with its precursor POK-MD1 from *Streptomyces minoensis* NRRL B-5482. A putative dutomycin biosynthetic gene cluster was discovered by genome sequencing and its involvement in dutomycin biosynthesis was confirmed by disruption of the ketosynthase gene. The open reading frames were analyzed and annotated based on the sequence homology. Two polyketide synthases (PKSs) are present in the gene cluster, including a type II PKS and an iterative type I PKS. The minimal type II PKS (DutA, DutB and DutC) synthesizes the nascent poly- β -ketone chain, which is subjected to extensive modifications by tailoring enzymes such as oxygenases, methyltransferases and glycosyltransferases to afford POK-MD1. The type I PKS DuG repeatedly uses its active sites to create a 9-carbon triketide chain that is subsequently transferred to the second sugar moiety at 4''-OH of POK-MD1 to yield dutomycin. Using a heterologous recombination approach, we disrupted four genes including two methyltransferases (*dutMT1* and *dutMT2*) and two glycosyltransferases (*dutGT1* and *dutGT2*). Analysis of the metabolites of these mutants revealed the functions of these genes and yielded three new dutomycin analogs SW140, SW82 and SW75. Dutomycin, POK-MD1, SW82 and SW75 were subjected to the antibacterial assay. Dutomycin and its C6-demethylated derivative SW82 showed strong antimicrobial

activity against Gram-positive bacteria *Staphylococcus aureus* (SA) and methicillin-resistant *S. aureus* (MRSA). In contrast, POK-MD1 and SW75 have no activities at test concentrations up to 250 µg/ml, indicating the essential role of the 9-carbon polyketide chain attached to 4"-OH. While the MICs of dutomycin against SA and MRSA were determined to be 0.25 µg/ml, it is interesting that SW82 has stronger activity with the MICs 0.125 µg/ml against the same strains, indicating that the antibacterial activity of these molecules can be improved by biosynthetic structural modifications.

5.1 Introduction

Antibiotics play a critical role in treating bacterial infections and have saved millions of lives. The first antibiotic was discovered in 1928, and since then, more natural compounds with promising antibacterial activities have been identified and used as anti-infectious drugs such as tetracycline and kanamycin. However, extensive uses of antibiotics also led to the emergence of drug resistance. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) is considered a "superbug" because its ability to fight off treatment, including the antibiotic methicillin. Spread of these antibiotic-resistant bacterial pathogens poses a serious threat to public health. Therefore, there is an urgent need for new generation antibiotics that can directly combat MRSA and other pathogenic bacteria.

Natural products are an important source of new drugs. In fact, many clinically used antibiotics are natural products including tetracycline, penicillin, and vancomycin. Actinomycetes are a major group of producers of many antibiotics. Continuous search for new antimicrobial molecules from nature is critical for development of new antibiotics. On the other hand, engineered biosynthesis can complement the time-consuming and resource-intensive discovery process, and represents an attractive approach to creating molecular diversity for drug discovery. Novel “unnatural” natural products can be obtained through direct genetic modification of the biosynthetic pathway in the producing strains or combinatorial biosynthesis in a heterologous host. Polyketides are a large group of medicinally important natural products, exemplified by lovastatin (anti-cholesterol), oxytetracycline (antibacterial), pradimicin A (antifungal and antiviral), and erythromycin (antibacterial). These molecules are assembled by polyketide synthases (PKSs) and associated tailoring enzymes through stepwise condensations of simple carboxylic acid precursors and further structural decorations [1]. PKSs are classified into three types. Type I PKSs such as DEBS involved in erythromycin biosynthesis are modular enzymes that contain a series of catalytic domains [2]. Ketosynthase (KS), acyl transferase (AT) and acyl carrier protein (ACP) are essential domains for polyketide chain elongation. Reductive domains such as ketoreductase (KR), dehydratase (DH) and enoyl reductase (ER) are also often found in type I PKSs. Type II PKSs are discrete small enzymes that form multi-enzyme complexes to synthesize

the polyketide structures. KS, chain length factor and ACP are the minimal PKS to form the nascent poly- β -ketone chain, which is subjected to the modifications by tailoring enzymes such as KR, aromatase (ARO), cyclase (CYC), oxygenase, methyltransferase (MT) and glycosyltransferase (GT) to form diverse aromatic molecules [3]. Type III PKSs are single enzymes that form a dimeric structure to catalyze substrate loading, chain elongation, and cyclization [4]. PKSs are one of the most amenable systems for engineered biosynthesis because of their inherent genetic organization and ability to produce structurally and functionally diverse molecules [5].

Here, we report the isolation and identification of the anthracycline antibiotic dutomycin (**4**, Fig.19) and its precursor POK-MD1 (**5**, Fig.19) from *Streptomyces minoensis* NRRL B-5482. The dutomycin biosynthetic gene cluster was then discovered by genome sequencing and confirmed by disrupting the ketosynthase gene. Functions of several tailoring enzymes were identified by targeted gene disruption and three new dutomycin analogs (**6-8**, Fig.19) were generated. Compounds **4-7** were tested for their antibacterial activity and **6** was found to have better activity against SA and MRSA than the natural product **4**. This work not only provides useful information about the formation of dutomycin and the structure-activity relationship (SAR), but also yields a potent antibacterial agent for further structure and activity optimization.

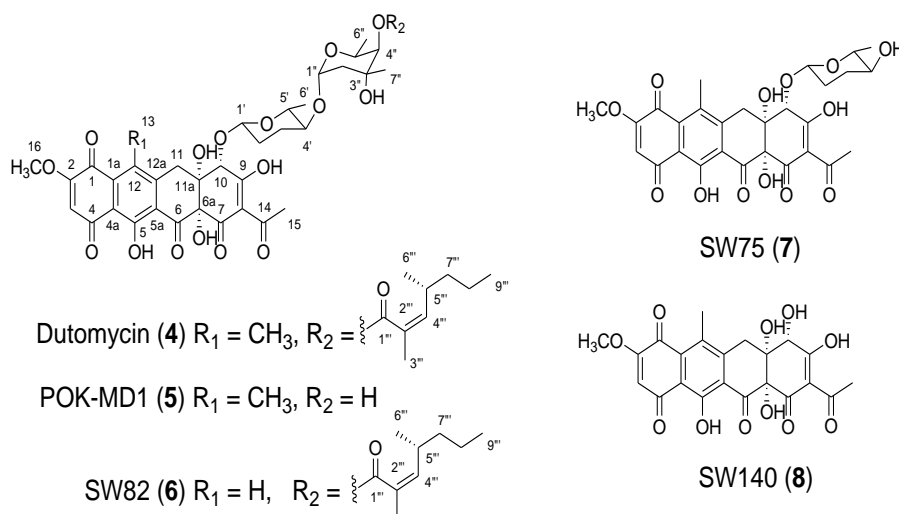


Fig. 19 Structures of dutomycin (**4**) and analogs **5-8**.

5.2 Materials and methods

5.2.1 General Equipment.

Products were analyzed and purified on an Agilent 1200 HPLC instrument with an Agilent Eclipse XDB-C18 column (5 μm , 250 \times 4.6 mm). The samples were eluted with acetonitrile-water (30:70, v/v to 95:5, v/v) at a flow rate of 1 ml/min for 30 min and detected at 460 nm. ESI-MS spectra were obtained on an Agilent 6130 quadrupole LC-MS in the negative mode. 1D and 2D NMR spectra were recorded on a JEOL ECX-300 NMR instrument (300 MHz for ^1H NMR and 75 MHz for ^{13}C NMR). The chemical shift (δ) values are given in parts per million (ppm). The coupling constants (J values) are reported in hertz (Hz).

5.2.2 Strains and Vectors.

S. minoensis NRRL B-5482 was obtained from the USDA Agricultural Research Service Culture Collection. *E. coli* ATCC 25922, *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* ATCC 33591 were purchased from the American Type Culture Center and used for the antibacterial assay. *E. coli* XL-1 Blue (Stratagene) and the pJET1.2 cloning vector (Fermentas) were used for routine subcloning. *E. coli* ET12567 (pUZ8002) and pKC1139 vector were used for the gene disruption experiments [20].

5.2.3 Media and Culture Conditions.

S. minoensis NRRL B-5482 and its mutants were maintained on YM (yeast extract-malt extract) agar plate at 28°C [21]. *S. aureus* and *E. coli* were cultured in Luria-Bertani (LB) broth at 37°C. MS [21], ISP4, and TSB media were used in the conjugation. Ampicillin (50µg/ml) and apramycin (50µg/ml) were added to the media appropriately for the cloning and conjugation.

5.2.4 Extraction and Sequencing of the Genomic DNA.

S. minoensis NRRL B-5482 was grown in 50 ml of YM medium at 28°C with shaking at 250 rpm for 5 days. The genomic DNA was extracted as previously reported. The genome was then sequenced using an Illumina MiSeq desktop sequencer and assembled with the short read de novo assembler Velvet. The genome sequence was analyzed with RAST (rapid annotation using subsystem technology).

The dutomycin gene cluster was deposited in GenBank under accession number KP710956.

5.2.5 Construction of Plasmids.

To disrupt three different *ks* fragments, *dutMT1*, *dutMT2*, *dutGT2* and *dutGT1* in *S. minoensis*, a homologous recombination single crossover approach was used. To find the type II KS involved in dutomycin biosynthesis, a ~0.6-kb fragment was amplified from the genomic DNA of *S. minoensis* using a pair of degenerated primers (KS α -F and KS α -R, Table S1) with a 30-cycle PCR program (15 s at 98°C, 15 s at 55°C, and 30 s at 72°C). The PCR product was ligated into pJET1.2 vector to yield pLS51, pLS52 and pLS53 based on the sequencing results. The three different *ks* fragments in these plasmids were then excised and ligated into pKC1139 between BglIII and BamHI to afford pLS54, pLS55 and pLS56, respectively. For inactivation of *dutMT1*, *dutMT2*, *dutGT2* and *dutGT1*, 0.6-kb fragments of these genes were amplified from the genomic DNA with primer pairs DutMT1-F/DutMT1-R, DutMT2-F/DutMT2-R, DutGT2-F/DutGT2-R, and DutGT1-F/DutGT1-R (Appendix E) using a 30-cycle PCR program (15 s at 98°C, 15 s at 60°C, and 30 s at 72°C) and ligated to pJET1.2 to yield pSW70, pSW82, pSW83 and pSW138, respectively. After the sequences were confirmed by sequencing, these gene fragments were introduced to pKC1139 to form pSW75, pSW85, pSW91 and pSW140 between HindIII and XbaI. All the plasmids used in this study are listed in Table 6.

Table 6. Plasmids used in Chapter 5.

Plasmid	Description	Reference
pLS51	<i>ks1</i> in pJET1.2	this work
pLS52	<i>ks2</i> in pJET1.2	this work
pLS53	<i>ks3</i> in pJET1.2	this work
pLS54	<i>ks1</i> in pKC1139	this work
pLS55	<i>ks2</i> in pKC1139	this work
pLS56	<i>ks3</i> in pKC1139	this work
pSW70	<i>dutGT2</i> fragment in pJET1.2	this work
pSW75	<i>dutGT2</i> fragment in pKC1139	this work
pSW82	<i>dutMT1</i> fragment in pJET1.2	this work
pSW83	<i>dutMT2</i> fragment in pJET1.2	this work
pSW85	<i>dutMT2</i> fragment in pKC1139	this work
pSW91	<i>dutMT1</i> fragment in pKC1139	this work
pSW138	<i>DutGT1</i> fragment in pJET1.2	this work
pSW140	<i>DutGT1</i> fragment in pKC1139	this work

5.2.6 Conjugation and Confirmation of the Mutants.

The gene disruption plasmids were introduced to *E. coli* ET12567 (pUZ8002) through chemical transformation. Plasmids were introduced into *S. minoensis* NRRL B-5482 by *E. coli*–*Streptomyces* conjugation, following the procedure as reported [22, 23]. The resulting *E. coli* strains were used as donors and *S. minoensis* NRRL B-5482 was the acceptor. The positive colonies were picked to TSB medium with 50 µg/ml of apramycin after 20 days of culture on MS plates. The colonies were grown in TSB medium at 28°C for 5 days and 50 µl of these cultures were spread on ISP4 plates with 50 µg/ml of apramycin. Colonies showing up at 37°C were considered as asrecombination strains, which were further cultured on YM plate with the 50 µg/ml of apramycin at 28°C for product checking. The mutants were confirmed by PCR analysis using genome- and pKC1139-specific primers (Appendix E) and a 30-cycle

PCR program (30 s at 98°C, 30 s at 58°C, and 60 s at 72°C). The PCR verification results are shown in Appendix E.

5.2.7 Extraction, Analysis and Purification of Compounds.

S. minoensis NRRL B-5482 and its knockout mutants were grown on YM agar plates (with or without apramycin). The cultures were extracted three times with an equal volume of ethyl acetate. The extracts were then dried under reduced pressure and re-dissolved in methanol for LC-MS analysis. To isolate compounds **4** and **5**, 1 l of culture of wild type *S. minoensis* NRRL B-5482 was extracted and subjected to silica gel 60 column chromatography, eluted with 100:0, 75:25, 50:50, 25:75, 0:100 % CHCl₃-MeOH (v/v) to yield 5 fractions. The fraction (75:25) was found to contain **4**. It was further separated by HPLC with an Agilent Eclipse XDB-C18 column (5 μm, 250 × 4.6 mm) and eluted with a gradient of acetonitrile-water (70:30, v/v to 75:25, v/v in 20 min) at 1 ml/min. The peak at 17 min was collected to yield 48.7 mg of **4** in pure form. Similarly, the fraction (50:50) containing **5** was separated by the same HPLC instrument, eluted with acetonitrile-water from 30:70, v/v to 35:65, v/v in 15 min at 1 ml/min, to yield 6.2 mg of **5**. A total of 38.9 mg of **6** was isolated from 1 l of *S. minoensis*-ΔMT1 culture using the same procedure as for **4**. For the purification of **7**, the extract of 1 l of *S. minoensis*-ΔGT2 culture was separated on a MCI column, eluted with 0:100, 25:75, 50:50, 75:25, 100:0% MeOH-H₂O (v/v) to yield 5 fractions. The 100% MeOH fraction was found to contain **7** which was further separated by HPLC using an Agilent Eclipse XDB-C18 column (5 μm, 250 × 4.6 mm). The sample

was eluted with acetonitrile-water 30:70 (v/v) at 1 ml/min for 15 min, and the peak at 12 min was collected to yield 15.4 mg of **7** in pure form. All these compounds were subjected to NMR and MS analyses. The major product **8** in *S. minoensis*- Δ DutGT1 was identified by MS and a comparison with the methanolysis product of **4**.

5.2.8 Minimum inhibition concentration test.

E. coli XL-1 Blue, *S. aureus* ATCC 25923 and methicillin-resistant *S. aureus* ATCC 33591 were incubated in LB broth with the test samples at different concentrations (64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, and 0.064 μ g/ml) at 37°C in 96-well plates, and the growth was analyzed after 20 hours to determine the MICs.

5.3 Results

5.3.1 Isolation and Identification of Two Anthracyclines from *S. minoensis* NRRL B-5482.

HPLC analysis of the ethyl acetate extract of the culture of *S. minoensis* NRRL B-5482 on YM agar at 460 nm showed a major product at 25.6 min (**4**) and a minor metabolite at 10.4 min (**5**), respectively (trace i, Fig. 20). Both compounds shared the typical UV absorptions of anthracycline antibiotics (Appendix E), suggesting that they might be structurally related analogs. ESI-MS spectrum of **4** showed the [M-H]⁻ ion peak at m/z 854.2 (Appendix E), suggesting that it has a molecular weight of 854, which is the same as dutomycin, an anticancer agent previously isolated from *Streptomyces* sp. 1725. The ¹H and ¹³C NMR data are consistent with the reported

data, which confirmed that **4** is dutomycin (Fig. 19). ESI-MS analysis revealed that the molecular weight of **5** is 716 based on the $[M-H]^-$ peak at m/z 715.1 (Fig. S2), suggesting that **5** is a dutomycin derivative that lacks the C-4'' side chain. This compound was previously found in the Δ pokMT1 mutant of the polyketomycin-producing strain *Streptomyces diastatochromogenes* Tü6028, and was named POK-MD1. However, this compound was not structurally characterized. Thus, **5** was subjected to a complete NMR analysis. The ^{13}C NMR spectrum of **5** showed 35 carbon signals, which are 9 carbons than **4**. A comparison of the spectra with those of **4** revealed that the carbon signals of the 9-carbon side chain at C-4'' were missing. Accordingly, **5** was characterized as POK-MD1 shown in Fig. 19. The ^1H and ^{13}C NMR signals were assigned and are shown in Tables 7 and 8, respectively.

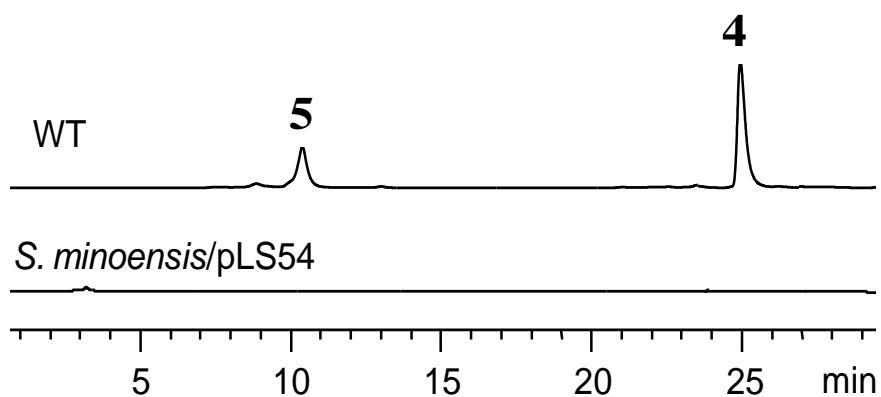


Fig. 20 HPLC analysis of the metabolites of wide type (WT) *S. minoensis* NRRL-B5482 and *S. minoensis* NRRL-B5482/pLS54 mutant at 460 nm.

Table 7. ^{13}C NMR data (75 MHz) for **4-7** (**4** and **6** in CDCl_3 , **5** in CD_3OD , and **7** in $\text{DMSO-}d_6$).

Position	4	5	6	7
1a	132.9	133.9	133.9	132.0
1	181.5	182.8	179.1	181.0
2	161.3	162.7	160.7	161.7
3	108.8	109.6	110.6	108.4
4	190.4	189.5	189.8	190.7
4a	113.8	114.9	113.6	111.3
5	162.5	163.3	163.7	160.6
5a	123.5	124.5	123.3	123.2
6	192.6	192.2	190.5	193.5
6a	81.2	83.4	81.5	82.2
7	195.2	195.3	195.4	196.5
8	110.8	112.6	110.7	112.8
9	191.2	192.2	191.7	191.5
10	73.9	73.2	73.5	74.4
11a	75.7	75.6	75.7	75.8
11	35.0	35.5	36.8	34.1
12a	150.9	152.8	150.1	151.0
12	132.4	133.8	120.1	131.5
13	16.8	17.0	-	16.1
14	200.9	200.5	201.3	198.5
15	26.7	27.7	25.9	26.3
16	57.1	57.7	57.1	57.1
1'	102.2	103.5	102.2	101.5
2'	29.5	30.6	29.5	30.2
3'	30.3	31.3	30.3	30.7
4'	80.1	81.3	80.1	70.2
5'	74.5	74.7	74.6	75.2
6'	17.4	17.9	18.1	16.9
1''	100.3	101.3	100.4	
2''	37.0	37.1	37.1	
3''	68.9	70.6	69.0	
4''	74.2	75.2	74.2	
5''	62.8	64.5	62.9	
6''	16.8	17.2	16.8	
7''	25.9	27.0	25.9	
1'''	167.4		167.5	
2'''	125.2		125.3	
3'''	21.1		21.1	
4'''	150.1		150.1	
5'''	33.5		33.5	
6'''	20.7		20.8	
7'''	39.8		39.8	
8'''	20.8		20.9	

9'''	14.4	14.4
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Table 8. ¹H NMR data (300 MHz) for **4-7** (**4** and **6** in CDCl₃, **5** in CD₃OD, and **7** in DMSO-*d*₆).

Position	4	5	6	7
3	6.11 (1H, s)	6.26 (1H, s)	6.17 (1H, s)	6.36 (1H, s)
5-OH	14.17 (1H, s)	-	13.50(1H, s)	14.10 (1H, s)
10	4.43 (1H, brs)	4.46 (1H, brs)	4.46 (1H, brs)	4.24 (1H, brs)
11	3.78 (1H, d, 17.5)	3.78 (1H, d, 17.5)	3.75 (1H, d, 17.5)	3.58 (1H, d, 17.5)
	3.01 (1H, m)	3.03 (1H, m)	3.05 (1H, m)	2.95 (1H, d, 17.8)
12	-	-	7.53 (1H, s)	-
13	2.57 (3H, s)	2.60 (3H, s)	-	2.48 (3H, s)
15	2.70 (3H, s)	2.65 (3H, s)	2.70 (3H, s)	2.58 (3H, s)
16	3.93 (3H, s)	3.93 (3H, s)	3.95 (3H, s)	3.90 (3H, s)
1'	4.66 (1H, d, 8.2)	4.67 (1H, brs)	4.71 (1H, d, 8.2)	4.52 (1H, d, 8.2)
2'	1.57 (1H, m)	1.57 (1H, m)	1.56 (1H, m)	1.45 (1H, m)
	2.18 (1H, m)	2.12 (1H, m)	2.18 (1H, m)	1.90 (1H, m)
3'	2.11 (1H, m)	2.12 (1H, m)	2.12 (1H, m)	1.90 (1H, m)
	1.63 (1H, m)	1.61 (1H, m)	1.66 (1H, m)	1.25 (1H, m)
4'	3.11 (1H, m)	3.10 (1H, m)	3.13 (1H, m)	2.83 (1H, brs)
5'	3.02 (1H, m)	2.95 (1H, m)	3.04 (1H, m)	2.83 (1H, brs)
6'	0.58 (3H, d, 5.5)	0.56 (3H, m)	0.68 (3H, d, 5.5)	0.42 (3H, d, 4.5)
1''	4.97 (1H, d, 3.1)	4.95 (overlapped)	4.99 (1H, d, 2.3)	
2''	1.88 (1H, m)	1.85 (1H, m)	1.86 (1H, m)	
	1.60 (1H, m)	1.60 (1H, m)	1.60 (1H, m)	
4''	4.76 (1H, s)	4.86 (overlapped)	4.76 (1H, s)	
5''	4.38 (1H, q, 6.5)	4.30 (1H, q, 6.3)	4.38 (1H, q, 6.5)	
6''	1.07 (3H, d, 6.9)	1.19 (3H, d, 6.9)	1.07 (3H, d, 6.9)	
7''	1.07 (3H, s)	1.14 (3H, s)	1.08 (3H, s)	
3'''	1.91 (3h, s)		1.92 (3H, s)	
4'''	5.70 (1H, dd, 1.0, 11.3)		5.71 (1H, d, 10.3)	
5'''	3.07 (1H, m)		3.10 (1H, m)	
6'''	0.96 (3H, d, 6.5)		0.97 (3H, d, 6.6)	
7'''	1.25 (2H, m)		1.26 (2H, m)	
8'''	1.25 (2H, m)		1.26 (2H, m)	
9'''	0.86 (3H, t, 6.5)		0.87 (3H, t, 6.5)	

4 and **5** are two anthracyclines that feature a tetracyclic quinone core structure and two sugar moieties, β-D-amicetose and α-L-axenose. The structures of **4** and **5** differ in the C-4'' side chain. We proposed that **5** serves as a biosynthetic intermediate in dutomycin biosynthesis. A time course analysis of the production of **4** and **5** was conducted (Appendix E). The titer of **5** reached the highest (10.0±0.7 mg/l) at 4 days

and then decreased with longer culture time, while the production of **4** kept increasing over the time. After 10 days, the production of **5** cannot be detected. This observation supports that **5** is a precursor of **4**. The titer of **4** reached 82.5 ± 1.8 mg/l at 10 days.

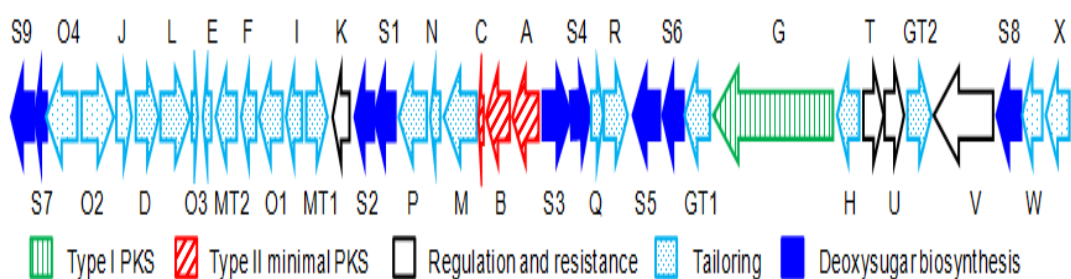
5.3.2 Identification of a Ketosynthase Gene Involved in Dutomycin Biosynthesis.

The structures of **4** and **5** indicated that they are synthesized through a type II polyketide biosynthetic pathway. We used a pair of degenerated primers, KS α -F (5'-TSGCSTGCTTCGAYGCSATC-3') and KS α -R (5'-TGGAANCCGCCGAABCCGCT-3') [6] to amplify the KS gene fragment from the genome of *S. minoensis* NRRL B-5482. These primers were used to amplify the conserved region of KS genes. A 0.6-kb fragment was obtained and ligated to pJET1.2. Sequencing of ten correct clones revealed three different KS genes that are homologous to reported KS genes in GenBank. These genes were named *ks1*, *ks2* and *ks3*, indicating that there are multiple type II polyketide biosynthetic gene clusters in this strain. To identify which *ks* gene is involved in dutomycin biosynthesis, we ligated these three fragments into temperature-sensitive *E. coli-Streptomyces* shuttle vector pKC1139 to yield the corresponding plasmids pLS54, pLS55 and pLS56 (Table 6). These plasmids were transferred into *S. minoensis* NRRL B-5482 through conjugation. Analysis of the products of the mutants revealed that the production of **4** and **5** was abolished in *S. minoensis* NRRL B-5482/pLS54 (trace ii, Fig.20), while the other two mutants still produced the two compounds (data not shown). Thus, *ks1* was identified to be involved in dutomycin biosynthesis.

5.3.3 Genome Sequencing and Discovery of the Dutomycin Biosynthetic Gene Cluster.

To locate the dutomycin (*dut*) biosynthetic gene cluster and better understand this strain, the genomic DNA of *S. minoensis* NRRL B-5482 was extracted and sequenced, yielding approximately 10.4 Mb of sequence data (GC content of 70.1%). We then searched the sequencing data using *ksI* that was found to be involved in dutomycin biosynthesis. A gene cluster covering 47.5-kb regions, which has 39 open reading frames (ORFs) (Fig. 21A), were identified. It was deposited into GenBank under accession number KP710956. Based on sequence homology search through BLAST, the putative functions of these ORFs are given in Table 4. Many of the genes are homologous to the previously reported polyketetomycin biosynthetic genes. Based on the putative functions of these genes, a dutomycin biosynthetic pathway is proposed in Fig. 21B.

a



b

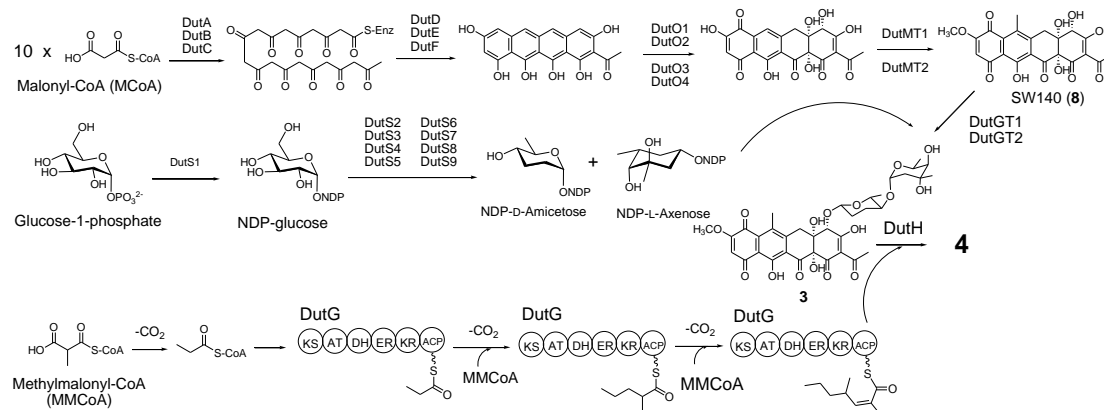


Fig. 21 The biosynthetic gene cluster of dutomycin (*dut*) (A) and the proposed biosynthetic pathway (B).

DutA, B and C are the KS, CLF, and ACP, respectively. They form the minimal PKS that generates the nascent poly- β -ketone chain from 10 units of malonyl-CoA. The *ks1* fragment amplified for the gene disruption above is a part of *dutA*. Specific primers including DutA-Check1 and DutA-Check2 were then designed (Appendix E). Together with the vector-specific primers M13-47 and RM-V, the disruption of *ksI* gene in *S. minoensis* NRRL B-5482/pLS54 was confirmed by PCR (Appendix E), further supporting that *ksI/dutA* is involved in dutomycin biosynthesis.

The *dut* biosynthetic gene cluster contains a variety of tailoring enzymes. The decaetide chain synthesized by the *dut* minimal PKS was then cyclized by the immediate tailoring enzymes including DutD (aromatase), DutE (cyclase) and DutF (cyclase) to form the tetracyclic structure. Further decorations are done by

additional tailoring enzymes such as oxygenases (DutO1-O4) and methyltransferases (DutMT1 and DutMT2) to afford the aglycone **8**. Nine deoxysugar biosynthetic genes were found in the *dut* gene cluster and named *dutS1-S9*. These genes are proposed to synthesize the two NDP-deoxysugars, including NDP-D-amicetose and NDP-L-axenose from glucose-1-phosphate [7]. Two GTs, DutGT1 and DutGT2, then transfer the sugar moieties to 10-OH of **8** to **5**. DutG is an iterative highly reducing type I PKS. It consists of six domains, including KS, AT, DH, ER, KR and ACP. Analysis of the sequence of the AT domain showed the GHSXG motif. The previous research reported that "X" in malonyl-specific ATs is usually a branched hydrophobic amino acid such as valine (V) or isoleucine (I), while ATs that have a less bulky residue such as glutamine (Q) or methionine (M) at this position select other substrates including methylmalonate [2, 8, 9]. DutG-AT has a GHSMG motif, suggesting that it selects a substrate other than malonyl-CoA. Based on the structure of C-4" side chain of **4**, it is apparent that the AT should take malonyl-CoA as the substrate. This was confirmed by the presence of the motif of VASH. It has been previously reported that a conserved [YVW] ASH motif is present in methylmalonyl-specific ATs, while a [HTVY] AFH motif is found in malonyl-specific ATs [10]. DutG selectively and repeatedly uses its reduction domains to generate a triketide chain from three units of methylmalonyl-CoA. An ACP shuttle-type acyltransferase (DutH) then transfers the triketide chain to the 10-OH of **5** to yield the final structure of **4**. Two SARP family regulator genes are

found in the *dut* gene cluster, *dutK* and *dutV*. The gene cluster also contains two ABC transporter genes *dutT* and *dutU*, which are likely involved in transporting the final product out of the cells.

5.3.4 Inactivation of Two Putative MTs (DutMT1 and DutMT2) Involved in Decorating the Aglycone and Isolation of a New Demethylated Derivative of Dutomycin.

DutMT1 and DutMT2 are two MTs that are supposed to be involved in the synthesis of the aglycone **8**. To understand their roles in dutomycin biosynthesis and create new analogs, we disrupted these two genes in *S. minoensis* NRRL B-5482 using the same approach for *ks1-ks3* described above. To this end, a fragment of *dutMT1* or *dutMT2* was amplified and ligated to pKC1139 to yield the corresponding disruption plasmid pSW91 and pSW85, respectively. Integration of pSW91 into the genome of *S. minoensis* NRRL B-5482 was screened by the apramycin resistance. An apramycin-resistant mutant was subjected to PCR analysis. As shown in Appendix E, ~1.0 kb gene fragments were amplified from the mutant using primer pairs M13-47/DutMT1-Check1 (lane 3) and RM-V/DutMT1-Check2 (lane 4), while no products were obtained from the wild type using the same primers. This result confirmed that pSW91 successfully inserted into *dutMT1* to yield the desired Δ DutMT1 mutant. The products of this mutant were analyzed by LC-MS. As shown in Fig. 22, the production of **4** in *S. minoensis* NRRL B-5482- Δ DutMT1 was not detected, while a major product **6** at 26.8 min was produced. We then isolated **6** from

a scaled-up culture of this mutant for structural characterization. ESI-MS analysis revealed that the molecular weight of **6** is 840 according to the $[M-H]^-$ ion peak at m/z 839.1 (Fig. S2), which is 14 mass units smaller than **4**. The ^{13}C NMR spectrum of **6** showed 43 carbons, one carbon less than **4**. A comparison of the NMR spectra of **4** and **6** confirmed that a methyl group was missing in **6**, while a new aromatic proton signal was found. The only methyl group attached to an aromatic ring is 13-CH₃. Therefore, **6** is likely the 12-demethylated derivative of **4**. This was supported by the HMBC correlations of 12-H at δ 7.53 to C-1 (δ 179.1), C-11 (δ 36.8), C-4a (δ 113.6) and C-5a (δ 123.3) (Fig. 24). Thus, the structure of **6** was determined to be 12-demethyldutomycin. It is a new compound and named as SW91. The carbon and proton signals were assigned based on 2D NMR and a comparison with **4**, and are shown in Tables 7 and 8, respectively. The titer of **6** was determined to be 66.697 ± 1.0 mg/l. Identification of **6** revealed that DutMT1 is a C-MT that attaches a methyl group to C-12.

The disruption plasmid pSW85 was also introduced into the dutomycin-producing strain for single crossover to yield *S. minoensis* NRRL B-5482- Δ DutMT2. The correct mutant was confirmed by PCR analysis (Appendix E). Sequence analysis predicted that DutMT2 is a putative O-MT. However, HPLC analysis of the products of *S. minoensis* NRRL B-5482- Δ DutMT2 revealed that this mutant did not produce any anthracycline metabolites including **4** at all (trace not

shown), suggesting that disruption of DutMT2 has shut down the entire *dut* biosynthetic pathway. A similar phenomenon was observed in the previous study on the polyketomycin biosynthetic gene cluster, where disruption of PokMT3 also abolished the production of any polyketomycin-related products in *S. diastatochromogenes* Tü6028 [11]. DutMT2 shares 95% similarity and 93% identity with PokMT3. BLAST analysis also revealed that DutMT2 is homologous to TcmN (51% identity and 65% similarity), a bifunctional protein that can catalyze the regiospecific cyclization and *O*-methylation of the tetracenomycin [12]. However, DutMT2 (350 aa) is much shorter than TcmN (494 aa), and lacks the N-terminal cyclization domain. Thus, it is unlikely to participate in the cyclization process. The putative function of DutMT2 is the 2-*O*-MT. However, given the fact that both inactivation of DutMT2 in *S. minoensis* NRRL B-5482 and PokMT3 in *S. diastatochromogenes* Tü6028 completely disrupted the corresponding biosynthetic pathway, this type of MT may also play an essential role in coordinating the collaborative actions of other biosynthetic enzymes.

5.3.5 Inactivation of DutGT1 and DutGT2 and Characterization of Two New Dutomycin Analogs.

Deoxysugars are frequently present in bioactive molecules such as erythromycin, doxorubicin and pradimicin A. Some of these sugar moieties are found to be essential in the biological activities. Thus, identification of enzymes responsible for transferring these sugars is of interest. There are two putative GTs in the *dut*

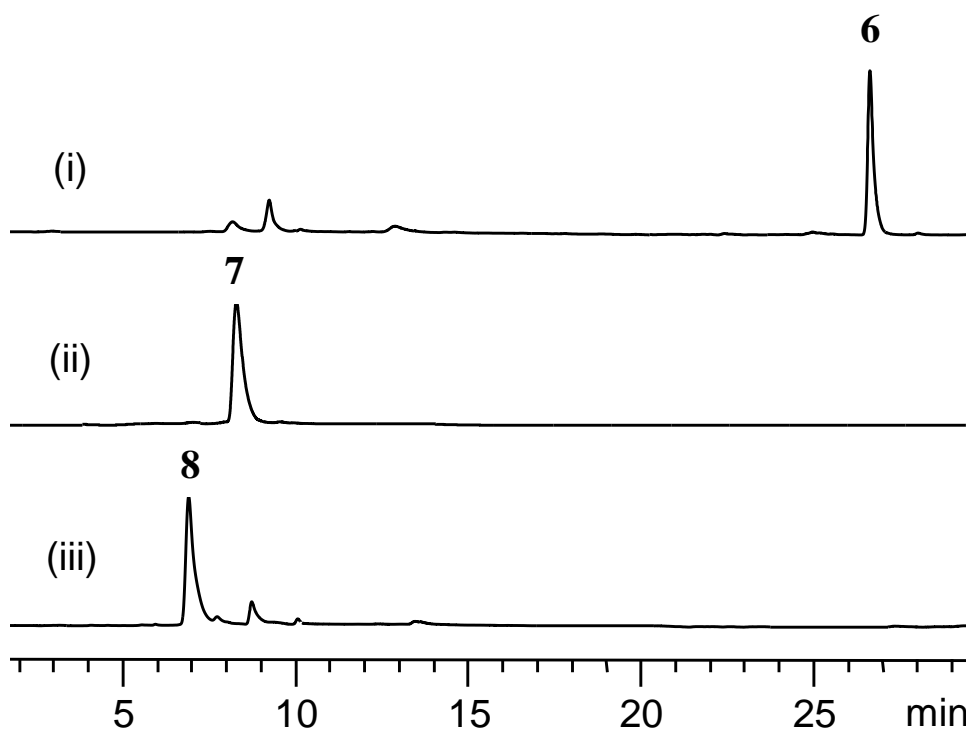


Fig. 22 HPLC analysis of the mutants of *S. minoensis* NRRL-B5482 at 460 nm. Trace i: Δ DutMT1; trace ii: Δ DutGT2; trace iii: Δ DutGT1.

biosynthetic gene cluster, likely involved in introduction of the two sugar moieties to the aglycone. In order to distinguish the roles of these two GTs and understand their roles, the *dutGT2* gene was disrupted using the same single crossover approach. The correct Δ DutGT2 mutant was verified by PCR (Appendix E). LC-MS analysis of the ethyl acetate of the culture of this mutant revealed that **4** was not produced, but a new product **7** accumulated as the major metabolite at 8.2 min (trace ii, Fig. 23). ESI-MS spectrum of **4** showed a $[M-H]^-$ ion peak at m/z 571.1, indicating that its molecular weight is 572. This corresponds to the structure of the aglycone with D-amictose, which is the first sugar moiety in **4**. To confirm the structure, **7** was isolated from 1 l

of the culture. The purified compound was subjected to 1D and 2D NMR analysis. A comparison of the NMR data of **7** and **4** revealed that the second sugar moiety and attached C-4" side chain were missing in this new compound, confirming that its structure only has the D-amictose moiety. The spin system of H-1'/H₂-2'/H₂-3'/H-4'/H-5'/H₃-6' of this sugar moiety was observed in the ¹H-¹H COSY spectrum (Fig. 24). Accordingly, the structure of **7** can be established as shown in Fig. 19 and was named as SW82. The proton and carbon signals were assigned based on the 1D and 2D NMR spectra and a comparison with the data of **4**. The assigned signals of **7** are listed in Tables 7 and 8, respectively. The titer of **7** was determined to be 34.8±0.2 mg/l. Identification of **7** indicated that the function of DutGT2 is the GT responsible for transferring the second sugar (L-axenose) moiety.

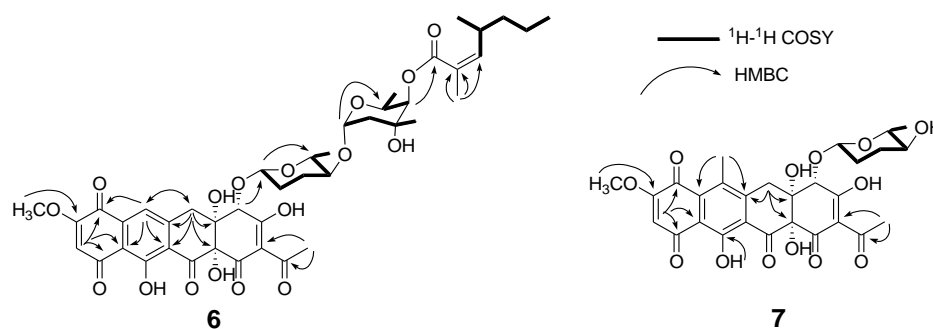


Fig. 23 Selected ¹H-¹H COSY and HMBC spectra for **6** and **7**.

The *dutGT1* gene was disrupted using the same single crossover approach as well. The correct Δ DutGT1 mutant was verified by PCR. LC-MS analysis of the ethyl acetate of the culture of this mutant revealed that **4** was not produced, but a major product **8** at 7.0 min was produced (trace iii, Fig. 23). The ESI-MS spectrum of **8**

showed a $[M-H]^-$ ion peak at m/z 457.0 (Appendix E), suggesting that the molecular weight of this compound is 458, which corresponds to the aglycone intermediate in dutomycin biosynthesis (Fig.20B). The identity of **8** was confirmed by a comparison of the methanolysis product of **4** (Appendix E). DutGT1 is thus confirmed to be responsible for introducing the first sugar moiety to the aglycone.

5.3.6 Minimum inhibition concentration test for dutomycin and its novel derivatives.

In our initial screening for antibacterial microbial extracts, the ethyl acetate extract of *S. minoensis* NRRL B-5482 was tested against three bacterial strains including Gram-negative bacterium *E. coli* ATCC 25922 and the Gram-positive bacterial strains *S. aureus* (SA) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) ATCC 33591. This extract was active against SA and MRSA, with MIC values of 500 and 1000 $\mu\text{g/ml}$, respectively. In contrast, it did not show any activity to *E. coli* (Table 9). After purifying compounds **4-7** from the wild type or mutants, we tested these molecules against the same panel of bacterial strains. **4** showed significant antibacterial activity against the Gram-positive strains, but was not active against the Gram-negative strain *E. coli*. This indicated that **4** specifically targets Gram-positive bacteria. The MIC values of **4** for both SA and MRSA were determined to be 0.25 $\mu\text{g/ml}$, indicating that this compound is a highly active antibacterial agent. Anthracyclines are ranked among the most effective anticancer drugs ever developed [13]. The first anthracyclines discovered were rhodomycin and cinerubicin [14]. Daunorubicin (trade name Daunomycin) was later isolated from *Streptomyces peucetius*. [15] Anthracyclines can

inhibit DNA and RNA synthesis and topoisomerase II enzyme to prevent the rapidly growth of cells.[16-18] They were also found to generate free oxygen radicals to damage the DNA, protein and cell membranes. [16] Hence, many anthracyclines, such as daunorubicin, epirubicin, doxorubicin and aclacinomycin, are used in clinic as cancer therapeutics. **4** was previously discovered as an antitumor compound [19] and showed strong *in vitro* cytotoxicity against leukemia P388 cells. [19] This is the first time it was found to be a potent antibacterial agent. Its natural analog polyketomycin from other actinomycetes has also shown similar antibacterial activity against Gram-positive bacteria. This suggests that this family of anthracyclines represents promising lead compounds for new antibiotics development.

The activity of **5-7** was also tested. However, this compound has no obvious antibacterial activity and the MICs were 250 µg/ml, which is 1,000-fold higher than **4**. The MICs of **6** against SA and MRSA were determined to be 0.125 µg/ml, while those of **7** were 250 µg/ml (Table 9). These antibacterial testing results of four compounds revealed some important SAR information. The presence of the C-4" side chain synthesized by the type I PKS DutG in **4** is essential, as removal of this side chain led to almost complete loss of the antibacterial activity. The same phenomenon was observed for **7**, which lacks both the L-axenose moiety and the attached C-4" polyketide chain, further confirming that the important role of this functional role. **6** is a demethylated derivative of **4**. Its MICs were only 50% of those of **4**, indicating that

removal of the 13-CH₃ can significantly increase the antibacterial activity. The activity of **8** was not tested as it lacks the sugar moieties and C-4" side chain, it is expected that this compound has no antibacterial activity.

Table 9. MICs (µg/ml) of **4-7** against three bacterial strains.

	<i>E. coli</i>	SA	MRSA
Crude extract	----	500	1000
4	>250	0.25	0.25
5	>250	250	250
6	>250	0.125	0.125
7	>250	250	250

5.4 Conclusion

In this study, dutomycin and its precursor POK-MD1 was isolated from *S.minoensis* NRRL B-5482 and structurally characterized. The dutomycin biosynthetic gene cluster was identified through genome sequencing and gene disruption, and the genes were annotated with putative functions based on sequence homology. Three new dutomycin analogs were generated through gene disruption. Antibacterial studies on these molecules revealed that the C-4" side chain plays an essential role in the biological activity on dutomycin. Removal the CH₃-13 significantly improved the antibacterial activity, suggesting that it is possible to create new dutomycin analogs through engineered biosynthesis and the antibacterial activity may be further optimized by additional structural modifications to yield novel antibiotics to combat Gram-positive pathogens including those developed with drug-resistance.

5.5 Acknowledgments

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5.6 References

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CHAPTER 6 SUMMARY AND ENGINEERING VALUE

6.1 Summary

In summary, this research has engineered polyketide biosynthetic pathways via two different approaches to make valuable bioactive molecules. Through heterologous expression, we built a very flexible yet efficient biosynthetic platform for the production of various plant natural products in *E. coli*. Enzymes from different sources were used as biosynthetic bricks that can be rationally recombined as legos for desired products. A variety of bioactive molecules naturally from plants have been produced in engineered *E. coli* strains within 2-3 days. Manipulation of these constructed pathways by removal or addition of biosynthetic enzymes led to novel “unnatural” molecules. A visible reporter assay was designed to facilitate the screening of efficient PALs, which are involved in the first biosynthetic steps of many plant phenylpropanoids. In addition to construction of artificial pathways in heterologous hosts, natural product biosynthetic pathways can also be modified through gene disruption in the original producing strain to yield new bioactive compounds. The biosynthetic gene cluster of dutomycin, a natural antibacterial and anticancer molecule, was discovered through genome sequencing and targeted gene disruption. Several enzymes in this pathway were functionally characterized. Moreover, a new demethylated analog of dutomycin was generated, which showed a

much higher antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* than the original natural product.

6.2 Engineering value

6.2.1 Engineered production of hydroxycinnamic acids and stilbenoids in *E. coli*

Hydroxycinnamic acids (HCAs) are important phytochemicals possessing significant biological properties such as management of oxidative stress-related diseases. Stilbenoids, containing a C6-C2-C6 core structure, are a class of plant secondary metabolites often involved in injury therapy when the plant is threatened by bacteria or fungi. These molecules have shown a variety of biological properties, such as antioxidant, antibacterial, antifungal, anticancer, anti-inflammatory and anti-aging activities. In Chapter 2, we produced resveratrol from L-tyrosine in a yield of 114 mg/l by engineering an artificial biosynthetic pathway into *E. coli* and optimizing the fermentation conditions. This yield is much higher than those previously reported. Furthermore, piceatannol is a hydroxylated derivative with better biological activities, but the application of this compound is limited by low yield in plants, which explains the high price of this compound. In Chapter 2, we made it possible to produce piceatannol in the *E. coli* system with the platform we established. The engineered *E. coli* strain that harbors C3H can efficiently convert the relatively inexpensive compound resveratrol (\$1.05/mg) to piceatannol (\$40/mg) for a yield of 65 mg/l in 2

days. This research thus provides a new method for large scale production of piceatannol.

6.2.2 Engineered biosynthesis of curcuminoids and two “unnatural” molecules in *E. coli*.

Curcuminoids are used as food spice (curry) and food coloring agent (E100). In addition, they are the major bioactive components in turmeric that have been used in Asian traditional medicine for centuries. Curcuminoids are well-known for their anti-tumor, anti-oxidant, anti-inflammatory, anti-allergic, and hepatoprotective activities. In Chapter 3, we modified the platform established in Chapter 2 with more genes from plants and successfully produced three different natural curcuminoids. While curcuminoids are present as a mixture in nature, this method allowed the production of a single curcuminoid in *E. coli*. That simplifies the downstream processing and purification of the three different curcuminoids that have similar physical and chemical properties. Also in Chapter 3, two novel unnatural compounds were generated by *E. coli* via genetic modification of the pathways. This demonstrated a highly efficient combinatorial biosynthesis approach to creating various plant phenylpropanoids in *E. coli*.

6.2.3 Design and application of an *in vivo* reporter assay for phenylalanine ammonia-lyase

Phenylalanine ammonia-lyase (PAL) is an important enzyme that links primary metabolism to secondary metabolism. Its efficiency is often a critical factor that affects the overall flux of a related metabolic pathway, the titer of the final products, and the efficacy of PAL-based therapies. In Chapter 4, a novel and efficient visible reporter assay for screening of PAL efficiency in *E. coli* was established based on the curcuminoid polyketide biosynthetic pathway. Taking advantage of the yellow color of the product, a microplate-based assay was designed to measure the titer of dicinnamoylmethane, which was validated by HPLC analysis. The different titers of the product reflect the overall performance (expression level and enzymatic activity) of the individual PALs in *E. coli*. This provides a convenient way to screen efficient PALs for various pathways including plant phenylpropanoid biosynthetic pathways studied in this dissertation.

6.2.4 Biosynthesis and antibacterial activity of dutomycin and analogs

In this study, dutomycin and its precursor POK-MD1 were isolated from *S. minoensis* NRRL B-5482 and structurally characterized. The dutomycin biosynthetic gene cluster was identified through genome sequencing and gene disruption. The genes were annotated with putative functions based on sequence homology. Three new dutomycin analogs were generated through gene disruption. Antibacterial studies on these molecules revealed that the C-4" side chain plays an essential role in the biological activity on dutomycin. Removal of the CH₃-13 significantly improved the antibacterial activity, suggesting that it is possible to create new dutomycin analogs

through engineered biosynthesis. Antibacterial activity may be further optimized by additional structural modifications to yield novel antibiotics for combating Gram-positive pathogens, including those developed with drug-resistance. All these studies open the door for further engineering, bioactivity screening, improvement, and clinical use of the novel antibiotic, dutomycin.

APPENDICES

APPENDIX A

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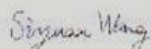
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Fee _____

Signed 

Date: 08/15/2015

Name: Siyuan Wang
Address: 1109 N 1600 E, Logan, UT 84341
Phone: 435-512-7447

Dear : Micah Rasmussen

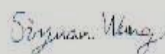
I am in the process of preparing my dissertation Department of Biological Engineering at Utah State University. I hope to complete in the Fall of 2015.

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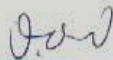
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Thank you for your cooperation.
Siyuan Wang

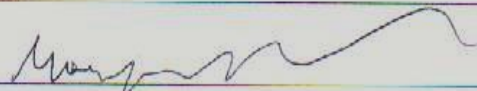


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(Description: Wang, S., Zhang, S., Xiao, A., Rasmussen, M., Skidmore, C., Zhan, J.* (2015): Metabolic engineering of Escherichia coli for the biosynthesis of various phenylpropanoid derivatives. *Metabolic Engineering* 29, 153-159).

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Signed



APPENDIX B

Resveratrol: $^1\text{H NMR}$ ($\text{DMSO-}d_6$) δ 9.53 (1H, s), 9.18 (2H, s), 7.39 (2H, d, $J = 8.6$), 6.94 (1H, d, $J = 16.5$), 6.80 (1H, d, $J = 16.5$), 6.75 (2H, d, $J = 8.6$), 6.38 (2H, d, $J = 1.3$), 6.12 (1H, d, $J = 1.3$).

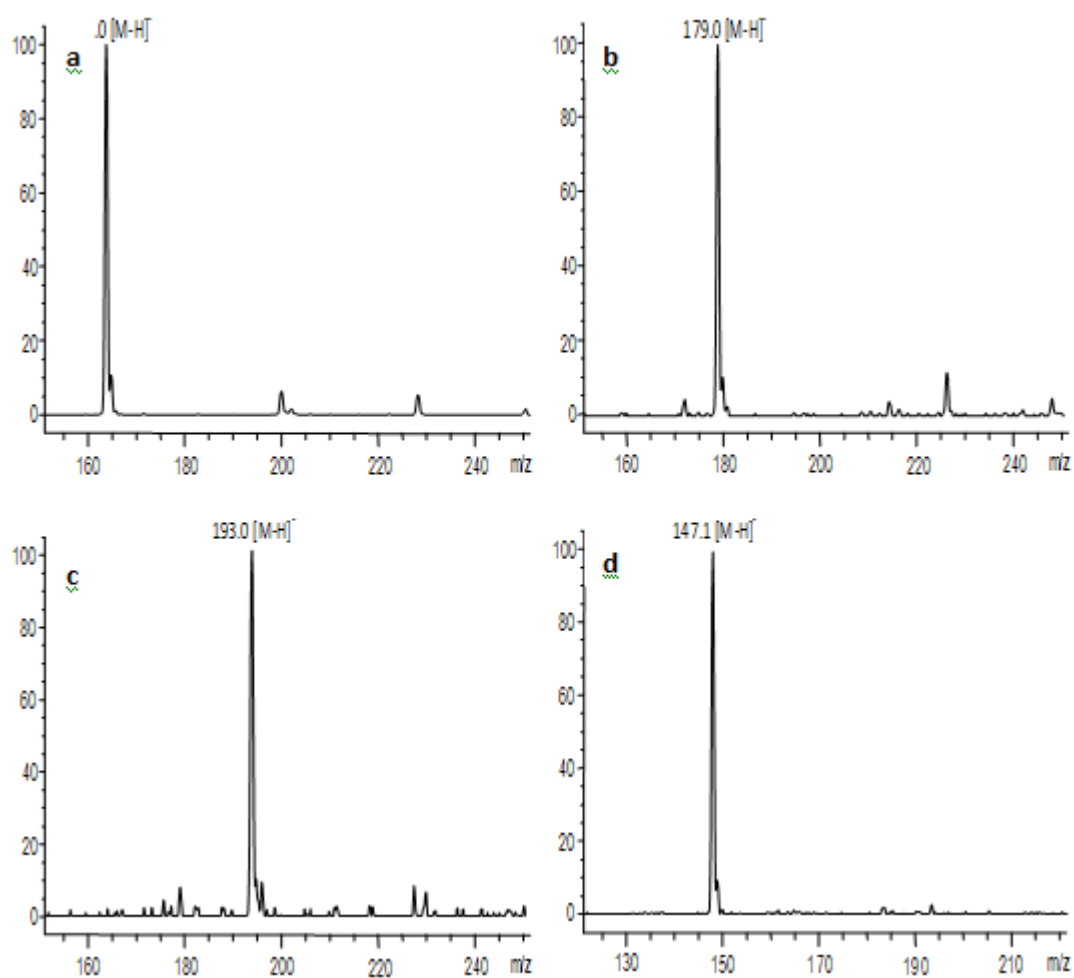


Fig.B1. ESI-MS analyses of hydroxycinnamic acids production. **a.** ESI-MS (-) spectrum of p-coumaric acid production from tyrosine; **b.** ESI-MS (-) spectrum of caffeic acid production from tyrosine; **c.** ESI-MS (-) spectrum of ferulic acid

production from tyrosine; **d.** ESI-MS (-) spectrum of cinnamic acid production from phenylalanine.

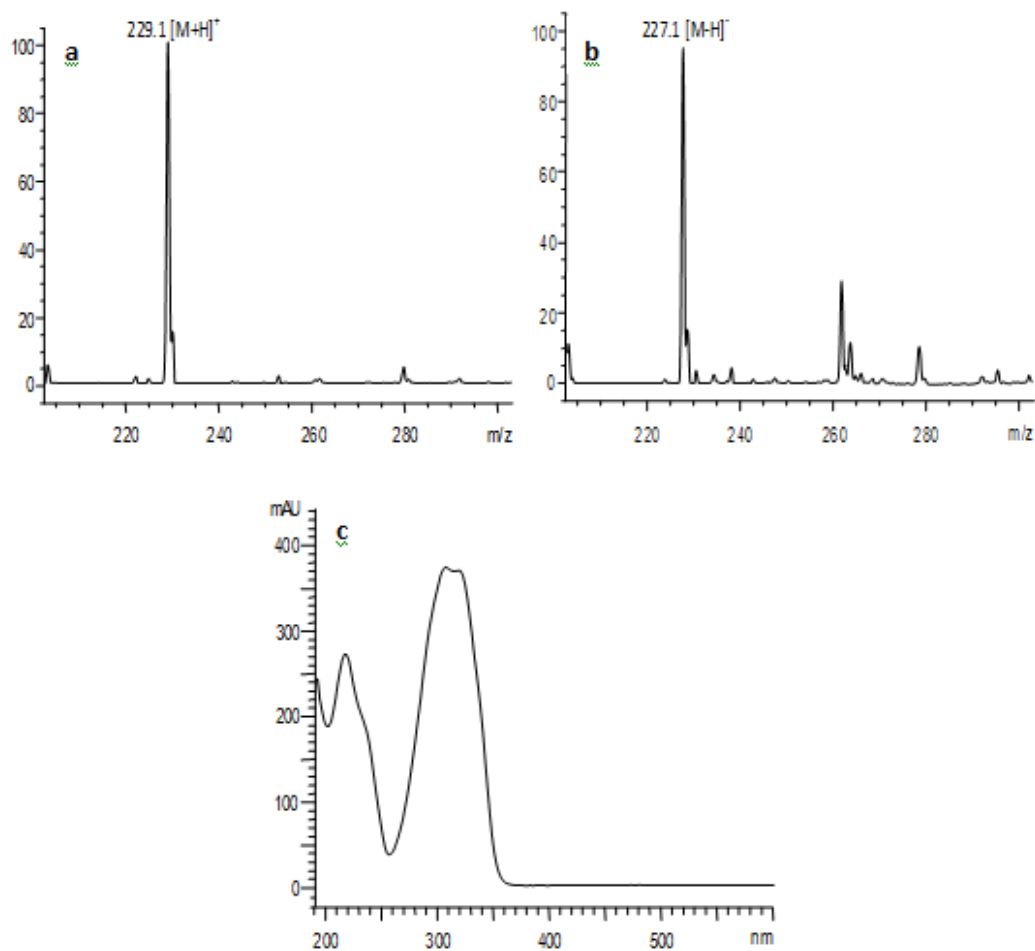


Fig.B2. LC-MS analyses of resveratrol production. **a.** ESI-MS (+) spectrum of resveratrol production from tyrosine; **b.** ESI-MS (-) spectrum of resveratrol production from tyrosine; **c.** UV spectrum of resveratrol production from tyrosine.

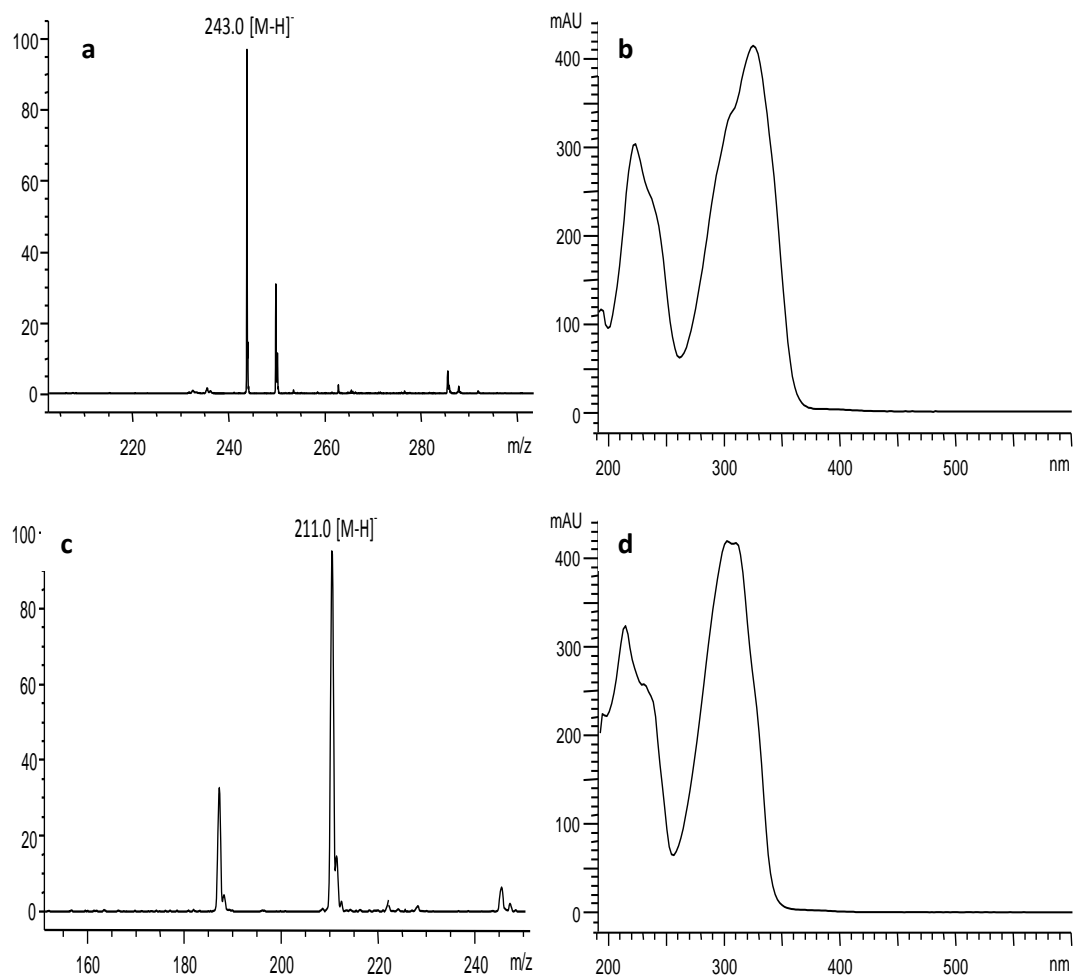


Fig. B3. LC-MS analyses of natural stilbenoids production. **a.** ESI-MS (-) spectrum of piceatannol production from tyrosine; **b.** UV spectrum of piceatannol production from tyrosine; **c.** ESI-MS (-) spectrum of pinosylvin production from phenylalanine; **d.** UV spectrum of pinosylvin production from phenylalanine.

APPENDIX C

Curcumin¹H NMR (acetone-*d*₆) δ 7.60 (2H, d, *J* = 15.4), 7.33 (2H, d, *J* = 2.0), 7.17 (2H, dd, *J* = 8.2, 2.0), 6.88 (2H, d, *J* = 8.2), 6.70 (2H, d, *J* = 16.5), 5.97 (1H, s).

2-Chloro- resveratrol: ¹H NMR (CD₃OD) δ 7.38 (2H, d, *J* = 8.6), 7.27 (1H, d, *J* = 16.1), 6.95 (1H, d, *J* = 16.1), 6.79 (2H, d, *J* = 8.6), 6.67 (1H, d, *J* = 2.8), 6.33 (1H, d, *J* = 2.8).

Dicafferolmethane: ¹H NMR (CD₃OD) δ 7.50 (2H, d, *J* = 15.7), 7.07 (2H, brs), 7.00 (2H, d, *J* = 7.5), 6.79 (2H, d, *J* = 7.5), 6.54 (2H, d, *J* = 15.7), 5.94 (1H, s).

APPENDIX D

Table D1. ^1H (300 MHz) and ^{13}C (75 MHz) NMR data for **2** (acetone- d_6 , δ in

Position	ppm, J in Hz)	
	^1H	^{13}C
1	6.17 (1H, s)	103.3
2	---	182.9
3	6.90 (1H, d, $J = 16.0$)	129.1
4	7.70 (1H, d, $J = 15.9$)	136.1
5	---	123.9
6	---	163.3
7	7.22 (1H, dd, $J = 2.5, 8.2$)	116.8
8	7.75–7.78 (1H, m)	117.1
9	7.28 (1H, t, $J = 7.7$)	125.8
10	7.81–7.83 (1H, m)	131.9
2'	---	184.9
3'	6.94 (1H, d, $J = 16.0$)	129.2
4'	7.77 (1H, d, $J = 16.1$)	140.7
5'	---	124.2
6'	7.70 (1H, dd, $J = 2.5, 7.7$)	130.6
7'	7.41–7.44 (1H, m)	130.7
8'	7.37–7.41(1H, m)	129.9
9'	7.41–7.44 (1H, m)	130.7
10'	7.70 (1H, dd, $J = 2.5, 7.7$)	130.6

Table D2. Time course analysis of the production of **1** by different PALs through UV-Vis microplate reader and HPLC analyses. The yields were calculated from three independent experiments and are presented as mean \pm SD (g/l).

Method	Time	PAL1	PAL3	PAL4
HPLC	12 h	0.087 \pm 0.007	0.011 \pm 0.001	0.014 \pm 0.001
	24 h	0.201 \pm 0.005	0.032 \pm 0.001	0.036 \pm 0.004
	36 h	0.233 \pm 0.005	0.038 \pm 0.006	0.044 \pm 0.004
	48 h	0.271 \pm 0.018	0.047 \pm 0.003	0.082 \pm 0.008
	60 h	0.343 \pm 0.012	0.073 \pm 0.019	0.092 \pm 0.005
	72 h	0.367 \pm 0.004	0.081 \pm 0.006	0.118 \pm 0.012
UV-Vis Microplate reader	12 h	0.053 \pm 0.003	0.012 \pm 0.001	0.012 \pm 0.001
	24 h	0.159 \pm 0.005	0.022 \pm 0.001	0.046 \pm 0.004
	36 h	0.212 \pm 0.004	0.042 \pm 0.007	0.049 \pm 0.004
	48 h	0.248 \pm 0.009	0.053 \pm 0.003	0.078 \pm 0.008
	60 h	0.342 \pm 0.003	0.072 \pm 0.004	0.082 \pm 0.002
	72 h	0.367 \pm 0.011	0.080 \pm 0.008	0.105 \pm 0.011

APPENDIX E

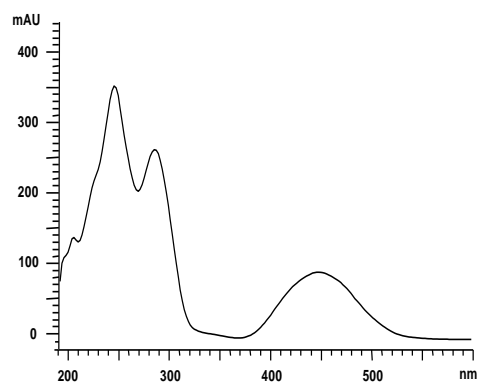
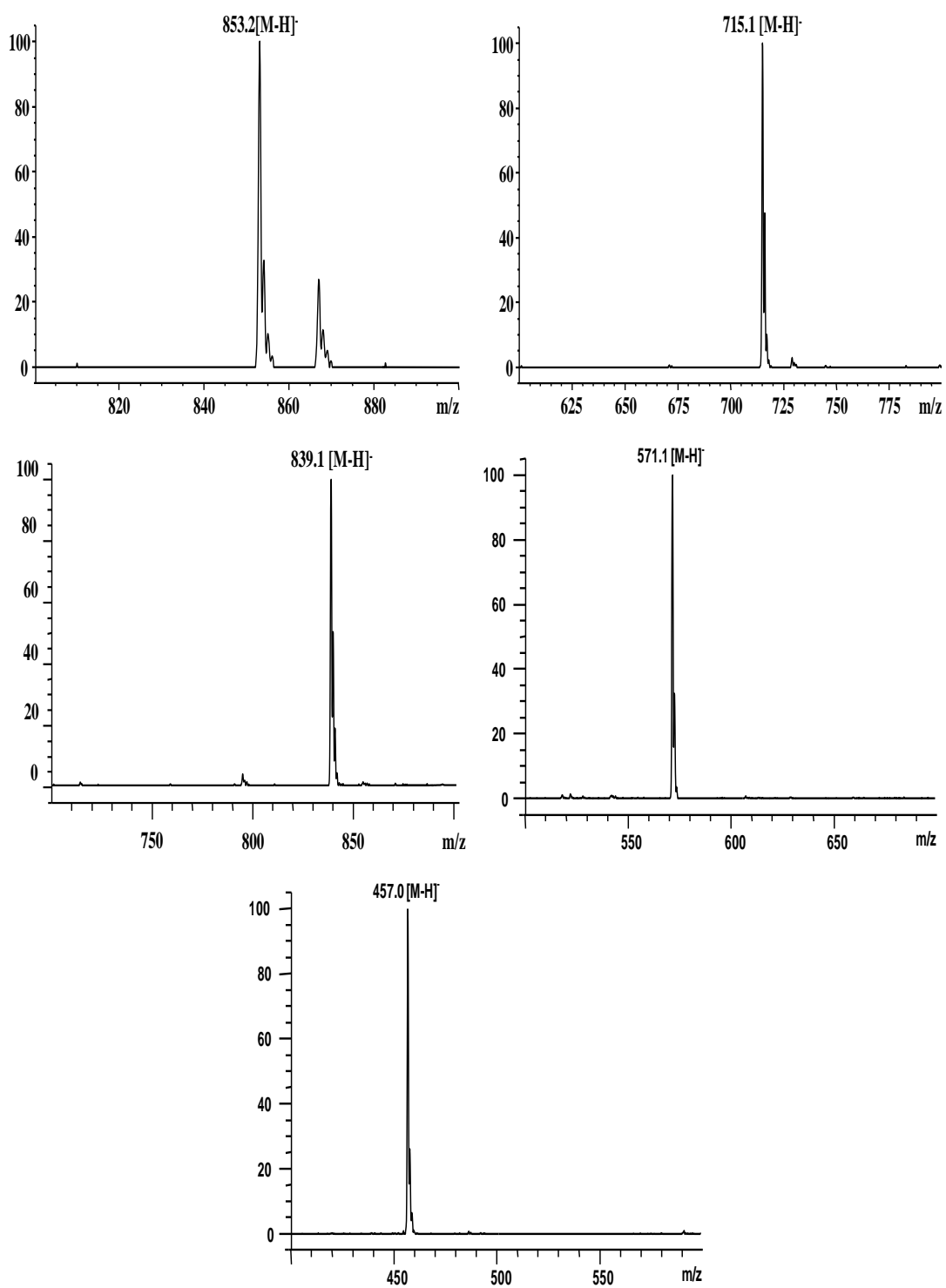


Fig. E1. Typical UV absorptions of anthracycline antibiotics.

Fig. E2 The ESI-MS analysis of **4-8**

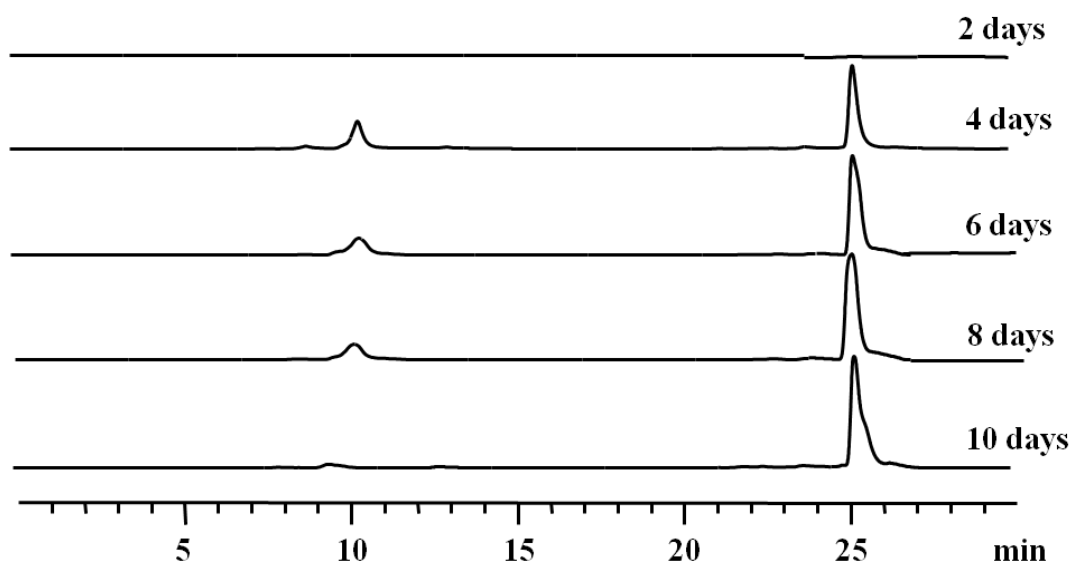
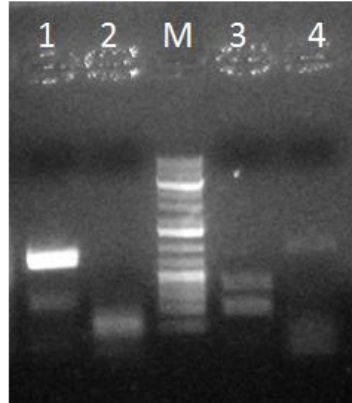


Fig E3 Time course of *Streptomyces minoensis* NRRL-B5482 production.

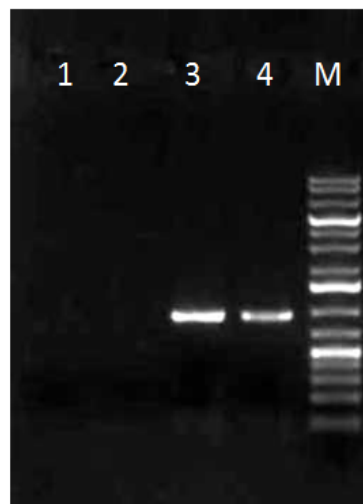
Table E1. Primers used in this study.*

Primer	Sequence
Type II KS-F	5'-TSGCSTGCTTCGAYGCSATC-3'
Type II KS-R	5'-TGGAANCCGCCGAABCCGCT-3'
DutMT1-F	5'- AAAAGCTT CCTGCGCGACATGGTCCTGT-3'
DutMT1-R	5'- AATCTAGAC GACCAGGTTCTCGATCACG-3'
DutMT2-F	5'- AAAAGCTT GCTCTACCGGATGCTGCGCT-3'
DutMT2-R	5'- AATCTAGACTT CAGGACGTACGCGTCGC-3'
DutGT2-F	5'- AAAAGCTT ACGGCATCCTGGACGAGCAT-3'
DutGT2-R	5'- AATCTAGATT CGGCTGGGCGACGATCAA-3'
DutGT1-F	5'- AAAAGCTT GATCCGCGCGCTGTA -3'
DutGT1-R	5'- AATCTAGA ATCTCTGTGCAGATCGGTGA -3'
DutKS-Check1	5'-GTCTCCACGGGCTGTACCTC-3'
DutKS-Check2	5'-ATCAGCCGGCTGGGCAGATG-3'
DutMT1-Check1	5'- ATGACAGCTCCCGCTCTCGAA -3'
DutMT1-Check2	5'- ATTTATTGGCGTCATCAGGTTGT -3'
DutMT2-Check1	5'- AGCCTCCGAAGAGGCTCGG -3'
DutMT2-Check2	5'- TTCTGCCAGATCGAGCGCCT -3'
DutGT2-Check1	5'-AGATCAAGCACAGTCCGGA-3'
DutGT2-Check2	5'-AAGCGACTCTGGAAGAGG-3'
DutGT1-Check1	5'-ATGGTCGACGAGTACGT-3'
DutGT1-Check2	5'- TGACGATGTTCGATGGCCAA-3'
M13-47	5'-CGCCAGGGTTTTCCAGTCACGAC-3'
RM-V	5'-GAGCGGATAACAATTTACACAGG-3'

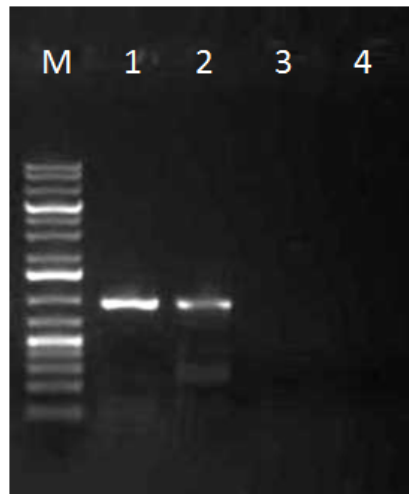
* Restriction sites are shown in bold.



Line 1: PCR by M13-47 KS1 check1 primers, *S. minoensis*- Δ KS1 as template;
 Line 2: PCR by M13-47 KS1 check1 primers, *S. minoensis* wild type as template;
 M: 1kb plus DNA ladder;
 Line3: PCR by RM-V KS1 check2 primers, *S. minoensis* wild type as template;
 Line4: PCR by RM-V KS1 check2 primers, *S. minoensis*- Δ KS1 as template.

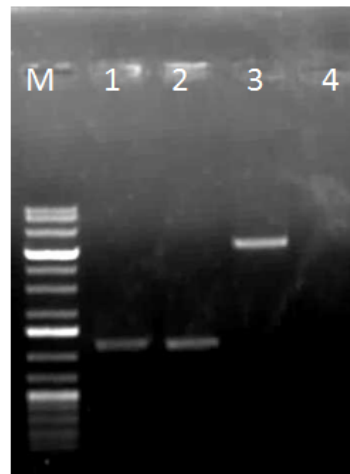


Line 1: PCR by M13-47 MT1 check1 primers, *S. minoensis* wild type as template;
 Line 2: PCR by RM-V MT1 check2 primers, *S. minoensis* wild type as template;
 Line3: PCR by M13-47 MT1 check1 primers, *S. minoensis* - Δ MT1 as template;
 Line4: PCR by RM-V MT1 check2 primers, *S. minoensis* - Δ MT1 as template;
 M: 1kb plus DNA ladder.



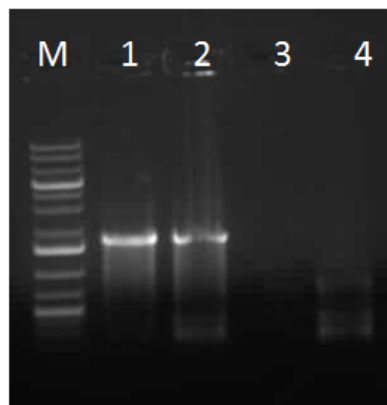
M: 1kb plus DNA ladder;

- Line 1: PCR by M13-47 MT2 check1 primers, *S. minoensis* - Δ MT2 as template;
 Line 2: PCR by RM-V MT2 check2 primers, *S. minoensis* - Δ MT2 as template;
 Line 3: PCR by M13-47 MT2 check1 primers, *S. minoensis* wild type as template;
 Line 4: PCR by RM-V MT2 check2 primers, *S. minoensis* wild type as template.



M: 1kb plus DNA ladder;

- Line 1: PCR by M13-47 GT1 check1 primers, *S. minoensis* - Δ GT1 as template;
 Line 2: PCR by RM-V GT1 check2 primers, *S. minoensis* - Δ GT1 as template;
 Line 3: PCR by M13-47 GT1 check1 primers, *S. minoensis* wild type as template;
 Line 4: PCR by RM-V GT1 check2 primers, *S. minoensis* wild type as template.



M: 1kb plus DNA ladder;

Line 1: PCR by M13-47 GT2 check1 primers, *S. minoensis* - Δ GT2 as template;

Line 2: PCR by RM-V GT2 check2 primers, *S. minoensis* - Δ GT2 as template;

Line 3: PCR by M13-47 GT2 check1 primers, *S. minoensis* wild type as template;

Line 4: PCR by RM-V GT2 check2 primers, *S. minoensis* wild type as template.

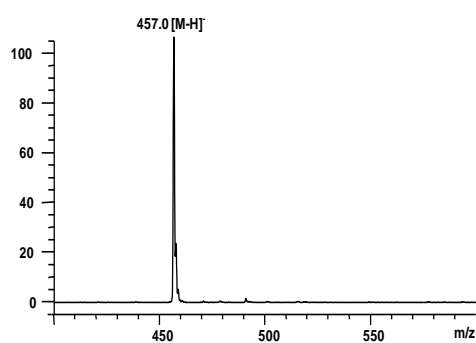
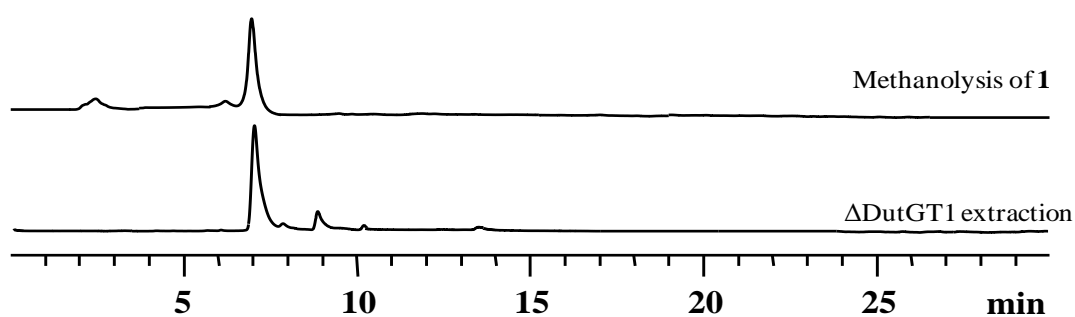
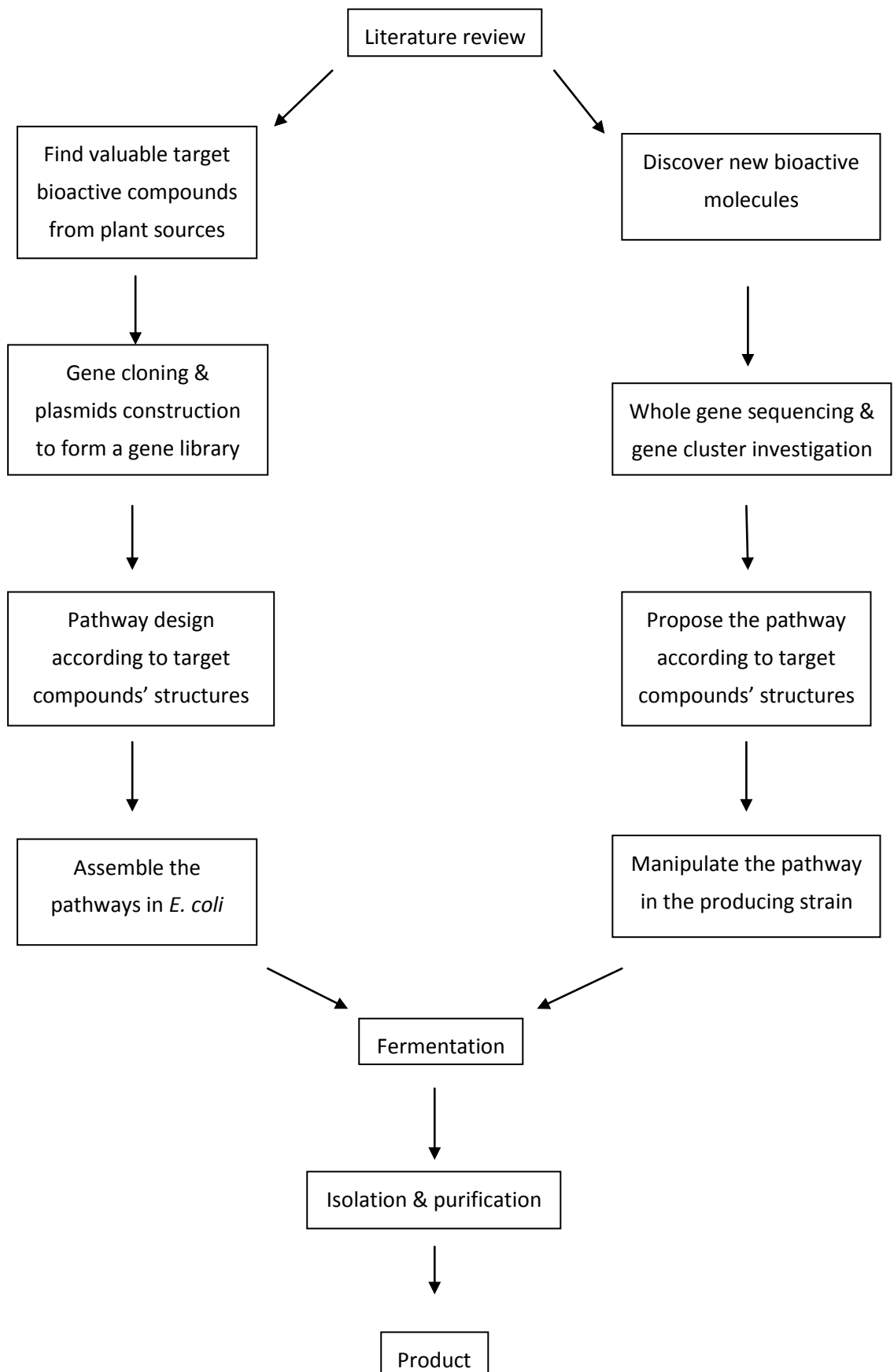


Fig. E8 The identity of **8** was confirmed by a comparison of the methanolysis product of **4**.

APPENDIX F ENGINEERING DESIGN



Engineering design components in Chapters 2 and 3: Biosynthetic enzymes from different sources were used to design artificial metabolic pathways to produce various molecules. A library of biosynthetic bricks was constructed and various biosynthetic pathways were assembled in *E. coli* using these biosynthetic bricks to generate four phenylpropanoid acids (cinnamic acid, *p*-coumaric acid, caffeic acid, and ferulic acid), three bioactive natural stilbenoids (resveratrol, piceatannol and pinosylvin), and three natural curcuminoids (curcumin, bisdemethoxycurcumin and dicinnamoylmethane). The yields were improved by optimizing the substrate concentrations.

Engineering design components in Chapter 4: Taking advantage of the yellow color of dicinnamoylmethane, a novel and visible microplate-based assay was designed for screening highly efficient PALs. This method requires remarkably smaller volumes, lower costs and shorter time compared to traditional analytic methods. This assay can be widely used in the industry or academia to search novel PALs for production improvement or therapeutics development.

Engineering design components in Chapter 5: The dutomycin biosynthetic gene cluster was discovered by genome sequencing and confirmed by gene disruption. Three new dutomycin analogs were generated by manipulating the pathway. This work thus provides a biosynthetic approach to creating new antibacterial agents.

CURRICULUM VITAE

SIYUAN WANG

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Logan, UT 84341

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Email: siyuan.wang@aggiemail.usu.edu

SUMMARY OF QUALIFICATIONS

Solid background in synthetic biology and natural product chemistry
Professional in LC-MS and NMR analysis and instruments maintenance
Extensive research experience in synthetic biology and actively published in top journals
1+ years experience as lab manager and safety officer

EDUCATION

Ph.D. in Biological Engineering Expected 2015
Utah State University, Logan, UT
Dissertation: Engineering of polyketide biosynthetic pathways for bioactive molecules. Adviser: Jixun Zhan
Cumulative GPA: 3.83/4.0

B.Sc. in Biological Technology (Emphasis on Pharmaceuticals) Jul 2011
Nanjing Normal University, Nanjing, China

PROFESSIONAL EXPERIENCE

Research Assistant Jan 2012 - present
Metabolic Engineering Laboratory
Utah State University, Logan, UT
Congressionally Directed Medical Research Programs of the Department of Defense, USA

- Demonstrated biosynthetic enzymes from different sources can be recombined like legos to make various bioactive molecules as design.
- Seven biosynthetic genes from plants and bacteria were used to establish a variety of complete biosynthetic pathways in *E. coli* to make valuable compounds.
- Made different combinations of biosynthetic bricks to design various natural product biosynthetic pathways, yielding four phenylpropanoid acids, three bioactive natural stilbenoids, three natural curcuminoids and two unnatural compounds.
- Established a novel and efficient visible reporter assay for screening of PAL efficiency in *E. coli* based on the curcuminoids polyketide

biosynthetic pathway.

National Institutes of Health grants

- Discovered the biosynthetic gene cluster for the antibacterial compound dutomycin.
- Confirmed the involvement of this gene cluster in dutomycin biosynthesis and created a series of new molecules by rationally modifying the biosynthetic pathway.
- Created a new demethylated analog of dutomycin that has much higher antibacterial activity against *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus*.

Lab Manager & Safety Officer

May 2014 –present

Research Laboratory for Metabolic Engineering

Utah State University, Logan, UT

- Check inventory and inspect lab safety.

Teaching Assistant

Jan 2013 – May 2013

Department of Biological Engineering

Utah State University, Logan, UT

- Teach and guided the lab session of Metabolic Engineering course.

Undergraduate Research Assistant

Sep 2009 - June 2011

College of Life Science

Nanjing Normal University Nanjing, China

Biotransformation of puerarin

- Modified puerarin by wild type strain biotransformation.

SKILLS

Molecular Biology

Plasmid construction, PCR, Electrophoresis, DNA Purification, RNA extraction, Fusion Expression, SDS-PAGE, Western Blot Analysis, q-PCR, primer design and gene sequencing analysis.

Instrument Analysis

HPLC, LC-MS, GC-MS, NMR, TLC, FT-IR.

Tools

Agilent chemistry station, Endnote, Primer Premier, DNAMAN, Origin Lab.

Computer Skills

C Programming, MatLab.

PUBLICATIONS

1. Zhang, S.#, **Wang, S.** #, Zhan, J.* (2015): Engineered biosynthesis of medicinally important plant natural products in microorganisms. *Current Topics in Medicinal Chemistry* (accepted). **IF=3.402**
2. **Wang, S.**, Zhang, S., Xiao, A., Rasmussen, M., Skidmore, C., Zhan, J.* (2015): Metabolic engineering of *Escherichia coli* for the biosynthesis of various phenylpropanoid derivatives. *Metabolic Engineering* 29, 153-159. **IF=8.258**
3. Zhang, S., **Wang, S.**, Zhang, Q., Chang, C., Zhan, J.* (2015): Three new antibacterial derivatives of cephalosporin P1 and isocephalosporin P1. *Bioorganic & Medicinal Chemistry Letters* 25(9) 1920-1924. **IF=2.331**
4. **Wang, S.**, Liu, G.,* Zhang, W., Cai, N., Cheng, C., Ji, Y., Sun, L., Zhan, J.*, Yuan, S.* (2014): Efficient glycosylation of puerarin by an organic solvent-tolerant strain of *Lysinibacillus fusiformis*. *Enzyme and Microbial Technology* 57, 42-47. **IF=2.966**
5. Mei, J.,# Wang, L.,# **Wang, S.**, Zhan, J.* (2014): Synthesis of two new hydroxylated derivatives of spironolactone by microbial transformation. *Bioorganic & Medicinal Chemistry Letters* 24(14), 3023-3025. (# : equal contribution) **IF=2.331**
6. **Wang, S.**, Zhang, S., Zhou, T., Zeng, J., Zhan, J.* (2013): Design and application of an *in vivo* reporter assay for phenylalanine ammonia-lyase. *Applied Microbiology and Biotechnology* 97, 7877–7885. **IF=3.811**
7. Yu, D.,# Xu, F.,# Valiente, J., **Wang, S.**, Zhan, J.* (2013): An indigoidine biosynthetic gene cluster from *Streptomyces chromofuscus* ATCC 49982 contains an unusual IndB homologue. *Journal of Industrial Microbiology and Biotechnology* 40(1), 159–168 **IF=2.505**
8. **Wang, S.**, Zhang, S., Zhou, T., Zhan, J.* (2013): Three new resorcylic acid derivatives from *Sporotrichum laxum*. *Bioorganic & Medicinal Chemistry Letters* 23(21), 5806–5809. **IF=2.331**
9. Yu, D.,# Xu, F.,# Zi, J., **Wang, S.**, Gage, D., Zeng, J., Zhan, J.* (2013): Engineered production of fungal anticancer cyclooligomer depsipeptides in *Saccharomyces cerevisiae*. *Metabolic Engineering* 18, 60–68. **IF=8.258**
10. Yu, D.,# Xu, F.,# Zhang, S.,# Shao, L., **Wang, S.**, Zhan, J.* (2013): Characterization of a methyltransferase involved in herboxidiene biosynthesis. *Bioorganic & Medicinal Chemistry Letters* 23(20), 5667–5670. **IF=2.331**
11. Liu, G., Sun, L., **Wang, S.**, Chen, C., Guo, T., Ji, Y & Yuan, S. * (2011): Hydroxylation modification and free radical scavenging activity of puerarin-7-O-fructoside. *Folia Microbiologica*, 56(4), 305-331. **IF=1.145**
12. Yu, C., Xu, H., Huang, G., Chen, T., Liu, G., Chai, N., **Wang, S.**, & Yuan, S. * (2010): Permeabilization of *Microbacterium oxydans* shifts

the conversion of puerarin from puerarin-7-O-glucoside to puerarin-7-O-fructoside. *Applied Microbiology and Biotechnology*, 86(3), 863-870. **IF=3.811**

PRESENTATIONS

1. "Engineered biosynthesis of plant polyphenols in *Escherichia coli*," Institute of Biological Engineering Annual Conference, March 2014.
2. "Design and application of an *in vivo* reporter assay for phenylalanine ammonia-lyase," Hansen Life Science Retreat, September 2013.
3. "Towards engineered biosynthesis of curcuminoids in lactic acid bacteria," Institute of Biological Engineering western regional conference, October 2013.
4. "Efficient glycosylation of puerarin by an organic solvent-tolerant strain of *Lysinibacillus fusiformis*," Intermountain Graduate Research Symposium, April 2012.

PROFESSIONAL MEMBERSHIP

Institute of Biological Engineering student membership

GRANTS AND AWARDS

Outstanding Engineering Graduate Scholar of Utah State University
2015

Second prize of podium presentation of IBE western regional conference
2012

RELEVANT COURSES

- Metabolic Engineering
- Synthetic Biological Engineering
- Biology System Modeling
- Natural Product Chemistry
- Biochemistry Engineering
- Supervised Teaching