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Joining Forces: Fermentation and Organic Synthesis for the Production of Complex Heterocycles

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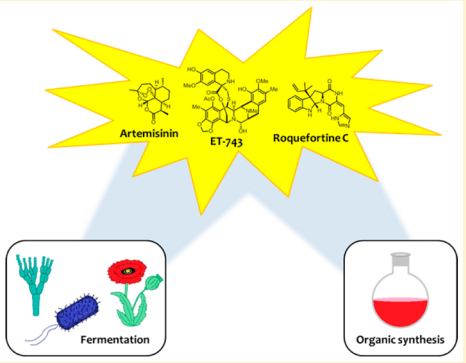
ABSTRACT: Commercial application of many promising heterocyclic natural products is limited by their natural abundance. While organic synthesis provides access to many natural products, total synthesis of numerous complex molecules is not economically feasible. In recent years, the combination of fermentation and organic synthesis has provided a new route for the production of complex heterocycles that are inaccessible by typical synthetic methods. This JOCSynopsis will review examples of how this union of disciplines has overcome obstacles in both academia and industry.



Heterocyclic natural products play an essential role in numerous industries, particularly pharmaceuticals. A majority of today's small molecule drugs and drug candidates contain at least one heterocyclic functionality.[1] Additionally, just over half of all small molecules approved as therapeutics between 1981 and 2014 are natural products or natural product based compounds.[2] However, the implementation of many promising biologically active compounds as pharmaceuticals is limited by natural product isolation. Many of these compounds are produced in very limited amounts by their natural source, and isolation of these compounds from their host organisms depletes natural supply. Such methods of production are not sustainable for clinical trials, much less commercialization. Historically, chemists have attempted to circumvent this problem through organic synthesis. While organic synthesis has allowed affordable access of natural products, total synthesis of many natural products is still not economically feasible for mass production.

Recent trends in the synthesis and production of heterocyclic molecules have shown increased interest in green synthetic processes. In particular, there has been a movement toward implementation of fermentation to solve problems commonly encountered in organic synthesis. Though much of fermentation in industry is employed for the production of food and alcoholic beverages, this technology can be expanded to produce advanced intermediates in the synthetic process. Microorganisms such as bacteria, yeast, and filamentous fungi can convert simple starting materials into stereochemically complex natural products. Fermentation limits the use of organic solvents and organic reagents, and in turn reduces waste products generated during the synthetic process. In addition, it allows for rapid and stereospecific production of large quantities of advanced intermediates. Natural products obtained by fermentation can be efficiently converted into new structures that would be difficult to obtain synthetically. The combination of fermentation and organic chemistry offers an alternative approach for the production and derivatization of biological compounds and could produce new therapeutic agents. Biosynthetic pathways offer complex scaffolds that can be used to synthesize other compounds that may exhibit novel biological properties and provide a new approach to drug development.

Natural products of bacterial or fungal origin are often amenable to production by fermentation. Besides sulfonamides and fluoroquinolones, the other major groups of antibiotics (penicillins, cephalosporins, tetracyclines, and macrolides) are all produced industrially by fermentation. The quintessential example of a fermented natural production is penicillin G (**1**), which is produced industrially by fermentation of *Penicillium notatum*. With the widespread availability of penicillin G following World War II, a number of bacterial strains developed resistance, and researchers sought to develop semisynthetic derivatives to combat bacterial resistance. (+)-6-Aminopenicillanic acid (6-APA, **2**), also found in *P. notatum* cultures,[3] was used as the base scaffold for new penicillin compounds (**3**–**7**), which are usually produced via acylation of 6-APA with the corresponding acid chloride or acid chloride hydrochloride (Scheme 1).[4] 6-APA can be prepared from the natural **1** via formation of the imino ether with PCl_3 in methanol (using silyl esters as protecting groups) followed by hydrolysis[4,5] or via deacylation enzymes.[6]

Cephalosporins, another class of beta-lactam antibiotics, are also produced industrially by combining fermentation and organic synthesis. The natural cephalosporins initially isolated

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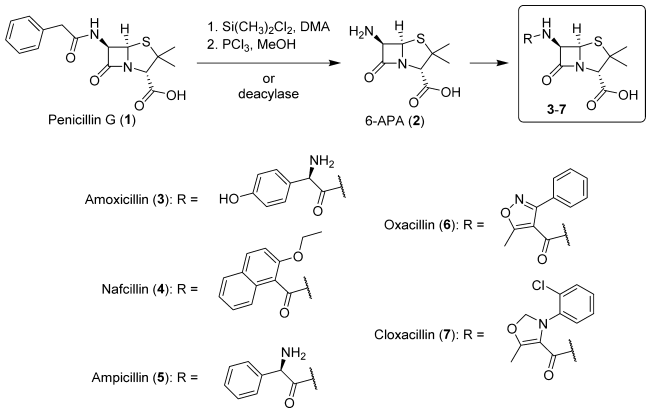
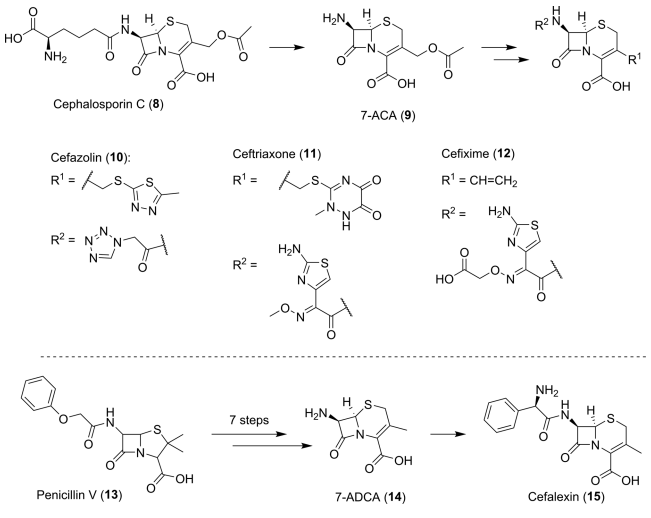
Scheme 1. Synthesis of Semisynthetic Penicillins 3−7



from *Acremonium* fungi, cephalosporins P, N, and C, displayed antibiotic activity but not at the same potency of the penicillins. Analogous to 6-APA (**2**) for penicillin, hydrolysis of cephalosporin C (**8**) produced 7-aminocephalosporanic acid (7-ACA, **9**), which became the platform upon which more potent cephalosporin compounds were built.[7,8] Most commercial cephalosporins display varying functionalities on the C3 side arm of the β-lactam (**10**−**12** and **15**). These moieties are generated via synthetic manipulation of 7-aminodesacetoxycephalosporanic acid (7-ADCA, **14**) from penicillin V (**13**, Scheme 2)[9,10] or through transformation of the ester functionality of **9** (Schemes 2).[11−14]

Scheme 2. Semisynthesis of Cephalosporins 10−12 and 15



Fermentation has also contributed to the efficient production of vancomycin. Vancomycin is a commercial antibiotic used to treat a number of bacterial infections, particularly those resistant to penicillins. Its structure consists of an arylglycine-rich heptapeptide aglycon to which is appended an array of sugar residues. Since its isolation in 1956 from *Amycolatopsis orientalis,* several total syntheses of vancomycin have been completed, each requiring extraordinary efforts but giving low overall yields. The total synthesis of vancomycin illustrates the formidable power of organic synthesis. The challenge of these syntheses was the development of stereoselective methods for controlling the three stereochemical elements of the atropisomerism present in the molecule.[15−21]

Vancomycin was first prepared by fermentation by Eli Lilly in 1962,[22] and fermentation remains the most efficient, cost-effective method of production. In 1996, it was shown that vancomycin can be produced by *A. orientalis* in both batch and continuous culture.[23] Additionally, purification of vancomycin can be achieved through simple precipitation at pH > 7.8.[24]

Microbial Synthesis of Plant-Based Natural Products. Plant-based natural products make up a large proportion of current pharmaceuticals. In a number of cases, plant-based harvest is feasible; however, in many cases, plants may not be suitable for fermentation due to factors such as low isolation titers, poor extraction, or difficult purification due to many metabolites produced by the plant. Additionally, plant-based fermentation may not be sustainable, as it requires large amounts of space and because it depletes natural resources. Efforts to engineer the production of heterocyclic natural products in native plant hosts can be accompanied by adverse morphological effects, as plant metabolism is highly regulated.

With the recent advances in genome mining and characterization of biosynthetic pathways, researchers have been able to genetically engineer microorganisms to produce plant-based natural products. There are several advantages to transgenic production of plant metabolites: (1) the techniques used to manipulate genes and expression of proteins are well established for microorganisms such as yeast and bacteria, (2) these microorganisms tend to grow at faster rates, and (3) techniques for large scale fermentation are well established for these organisms. Efficient biosynthesis of complex molecules is still challenging, however, because it involves optimization of many enzyme-catalyzed reactions.

Genetically engineered yeast has been used in the production of the antimalarial drug artemisinin (**19**, Scheme 3). This tetracyclic sesquiterpene bearing a peroxide bridge was first isolated from the plant *Artemisia annua* by Youyou Tu in 1971, and artemisinin (**19**) as well as its semisynthetic derivatives were found to exhibit potent antimalarial activity against *Plasmodium falciparum* malaria.[25] The discovery of **19** has been one of the most important medical developments of the 20th century, earning Youyou Tu half of the 2015 Nobel Prize in Medicine.

Artemisinin (**19**) has been synthesized a number of times (Scheme 3), notably from (−)-isopulegol (**16**) by Schmid and Hofheinz (13 steps, ∼5% overall yield),[26] from cyclohexenone (**20**) by Zhu and Cook (9 steps, 7.6% overall yield),[27] and from (*R*)-(+)-pulegone (**22**) by Avery, Chong, and Jennings-White (9 steps, ∼3% overall yield).[28] The primary bottleneck of these total syntheses has been the construction of the peroxide bridge. In the synthesis by Schmid and Hofheinz, addition of singlet oxygen to compound **17** followed by addition of methanol is proposed to form hydroperoxide **18**, which undergoes acid-promoted ring closure to give artemisinin (**19**) in 30% yield.[26] The synthesis by Zhu and Cook features addition of singlet oxygen formed from ammonium molybdate-promoted decomposition of hydrogen peroxide to give a complex mixture of oxidation products, which upon addition of acid provided artemisinin (**19**) in 29−42% yield.[27] Construction of the peroxide bridge in the Avery synthesis is carried out by a one-pot ozonolysis, deprotection, and cyclization reaction sequence to give artemisinin (**19**) in 35% yield.[28]

The semisynthesis of **19** from natural precursor artemisinic acid (**31**, Scheme 4) has been demonstrated by sequential oxidations[29] and by multistep reaction cascades;[30,31] however, like artemisinin (**19**), artemisinic acid (**31**) suffers from low

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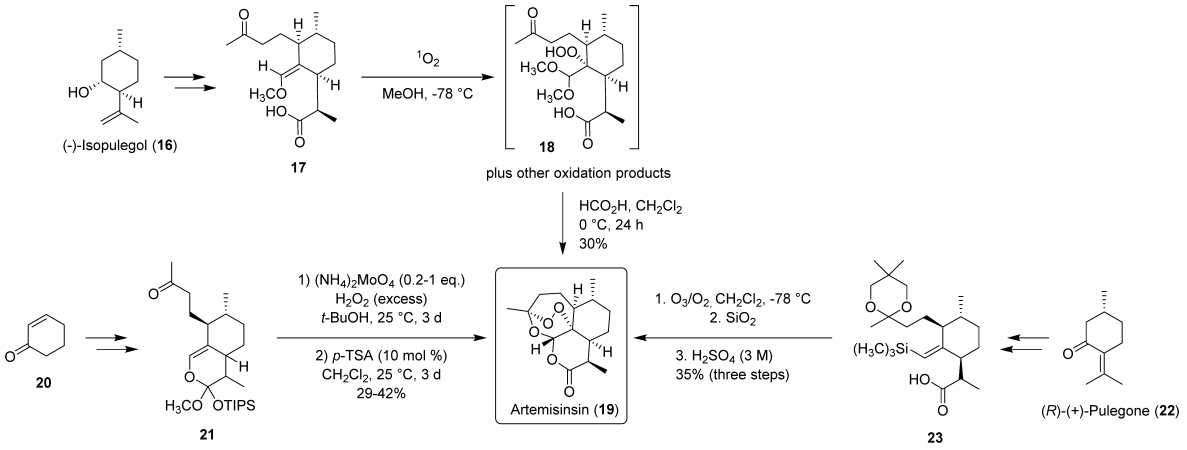
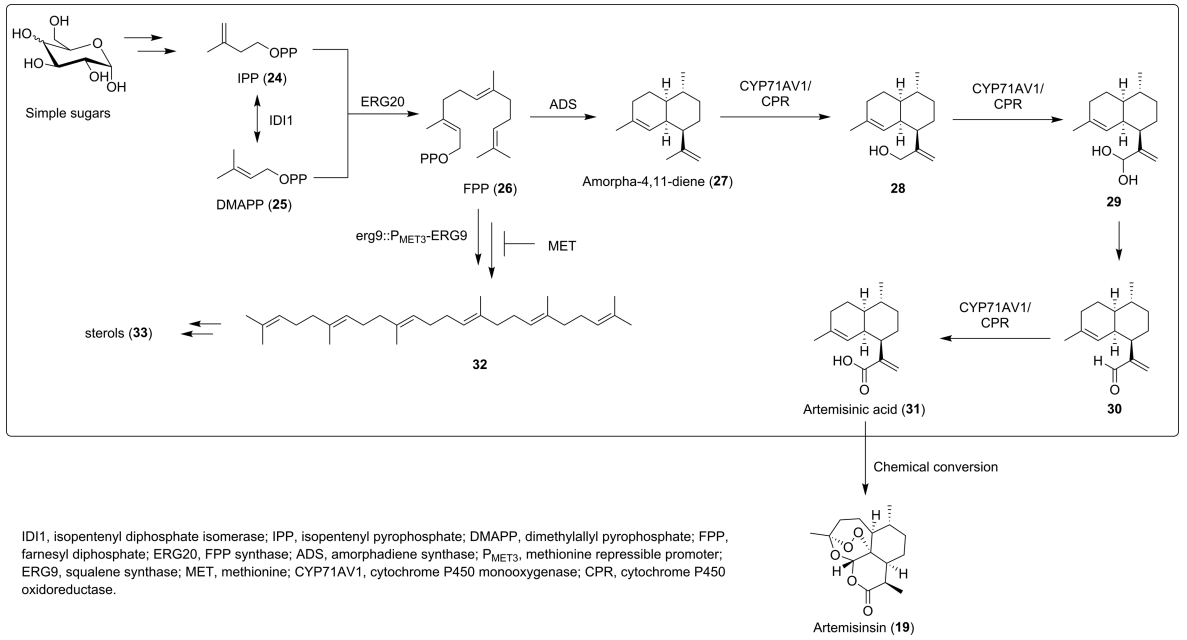
Scheme 3. Synthetic Approaches to Artemisinin (19)



Scheme 4. Engineering Pathway for the Biosynthesis of Artemisinic Acid (31) and Semisynthesis of Artemisinin (19) from Artemisinic Acid (31)



IDI1, isopentenyl diphosphate isomerase; IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl diphosphate; ERG20, FPP synthase; ADS, amorphadiene synthase; P_{MET3}, methionine repressible promoter; ERG9, squalene synthase; MET, methionine; CYP71AV1, cytochrome P450 monooxygenase; CPR, cytochrome P450 oxidoreductase.

natural abundance, thus limiting this method for commercial use. Recently, the production of **31** was reported in engineered yeast *Saccharomyces cerevisiae* (Scheme 4).[32,33] An engineered mevalonate pathway was optimized for the production of artemisinic acid precursor amorpha-4,11-diene (**27**) through upregulation of farnesyl diphosphate (FPP, **26**) production and inhibition of sterol (**33**) biosynthesis. Artemisinic acid was then synthesized from **27** by cytochrome P450 monooxygenase CYP71AV1. Employment of a high-copy plasmid system increased production of **31** from 100 to 250 mg/L in shake-flask cultures and 1 g/L in bioreactors.[33]

The benzylisoquinoline alkaloids (BIA) make up a family of heterocyclic compounds with a large presence in the pharmaceutical industry. These compounds include a number of common analgesic compounds such as morphine (**41**, Scheme 5) and codeine (**40**, Scheme 5), as well as the muscle relaxant (+)-tubocurarine and antibacterial agents such as berberine (**39**, Scheme 5), palmatine, and magnoflorine (**43**, Figure 1). These alkaloids are produced by a number of flowering plant families, including the Papaveraceae, or poppy, family and are known to be derived from tyrosine (**34**) and share (*S*)-reticuline (***S*-38**, Scheme 5) as a precursor. Current production of opioids such as morphine (**41**) involves extraction of opium or poppy straw, which are the raw materials obtained from the poppy plant *Papaver somniferum*. While **41** is produced in high yields from the poppy plant, environmental factors such as weather and fertile soil can cause variations in the amount produced each year, making plant-based production an unsustainable practice. Additionally, many other alkaloids in this family are not produced in high levels.

Several publications describe the reconstitution of the BIA biosynthetic pathway in microorganisms. Minami et al. have developed a method for microbial production of BIAs (*S*)-scoulerine (**42**) and magnoflorine (**43**) (Figure 1). The BIA biosynthetic pathway was reconstituted in *Escherichia coli* for production of ***S*-38** from **35**. *S. cerevisiae* was used to reconstitute downstream enzymes as some enzymes needed to form BIAs from ***S*-38** are not expressed in bacteria in their active form. The combination of the two transgenic microbes

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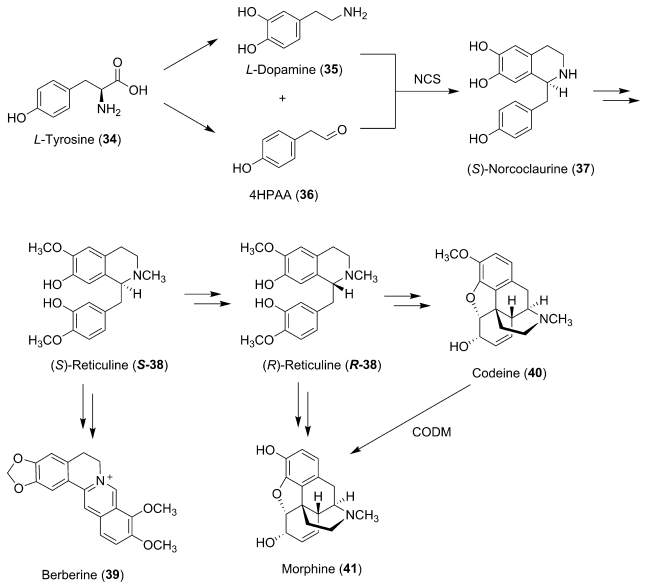
Scheme 5. Biosynthesis of Benzylisoquinoline Alkaloids



4HPAA, 4-hydroxyphenylacetaldehyde; NCS, norcoclaurine synthase.

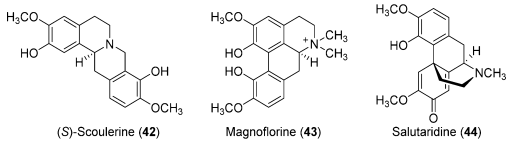


Figure 1. Benzylisoquinoline alkaloids produced by genetic engineering.

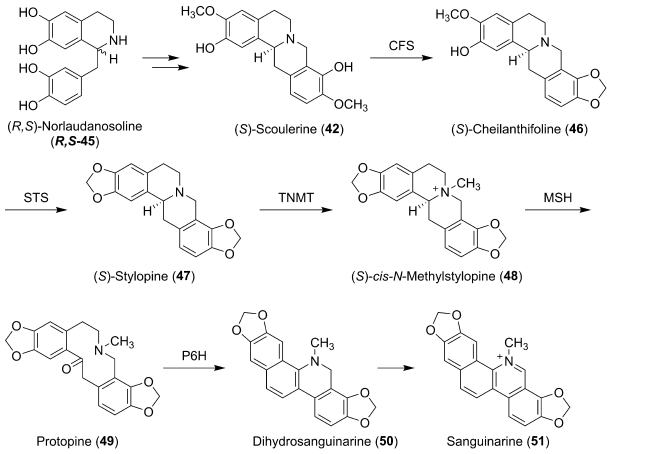
led to production of **42** and **43** in 8.3 and 7.3 mg/L, respectively.[34]

Additional work by the Smolke group involved the engineering of *S. cerevisiae* for the production of ***S*-38** from precursor (*S*)-norcoclaurine (**37**) as well as the production of sanguinarine and berberine intermediates from ***S*-38**. Morphine precursor salutaridine (**44**, Figure 1) was also produced via a two-step isomerization of ***S*-38** to ***R*-38** followed by oxidation with human cytochrome P450 CYP2D6.[35] Further work by the Smolke group demonstrated production of protoberberine alkaloids **42**, **46**, **47**, and **48**, protopine (**49**), and benzophenanthridine alkaloids **50** and **51** in the sanguinarine biosynthetic pathway, achieving high titers for each of the metabolites (Scheme 6).[36] As a proof of concept, Smolke et al. have also engineered yeast to produce thebaine and hydrocodone starting from sugar.[37]

Microbial production of natural BIAs could provide more efficient routes to less abundant natural BIAs such as tubocurarine as well as semisynthetic opioids such as hydrocodone, hydromorphone, and oxycodone. While the titers of opioid alkaloids are not yet sufficient for commercial production, the research offers other possibilities such as synthetic production of superior drugs and drugs with more favorable pharmacokinetic properties. While major challenges remain, this approach has already proven feasible.

Knowledge of metabolites produced by organisms can be used for creative solutions to natural products that are not easily obtained. Reconstituting biosynthetic pathways may not be necessary if precursors or related compounds are naturally produced by microorganisms amenable to fermentation.

Scheme 6. Production of Protoberberine Alkaloids 42, 46, 47, and 48, Protopine (49), and Benzophenanthridine Alkaloids 50 and 51 in *S. cerevisiae*



CFS, cheilanthifoline synthase; STS, stylopine synthase; TNMT, tetrahydroprotoberberine *N*-methyltransferase; MSH, *cis*-*N*-methylstylopine 14-hydroxylase; P6H, protopine 6-hydroxylase.

Ecteinascidin 743 (ET-743, **62**, Scheme 7) is a tetrahydroisoquinoline compound produced by Caribbean marine tunicate *Ecteinascidia turbinata*[38] and is the first marine compound to be approved for the treatment of cancer. ET-743 was first reported in 1969, and following structure elucidation in 1986,[39] it was found to be very similar to safracin compounds produced by *Pseudomonas fluorescens*.[40,41] Preclinical and early clinical trials were completed using ET-743 obtained from aquaculture, but it was clear that this method of production would not be sustainable should the compound be approved for commercial use.[42] Fermentation of cyanosafracin B (**52**) on kilogram scale has allowed for the efficient semisynthesis of **62** from **52** to be developed (Scheme 7).[43] Though the total synthesis of **62** was completed in 2002,[44] semisynthesis still remains the most efficient method of production. ET-743 has been approved for the treatment of sarcoma and ovarian cancer by the European Union's EMEA, for the treatment of ovarian cancer in the Philippines, and for malignant soft tissue tumors in Japan. It is currently in clinical trials in the United States for the treatment of a number of cancers.

Mutasynthesis as a Substitute for Late-Stage Modification. Despite the great potency of many heterocyclic natural products, unfavorable solubility, bioavailability, and pharmacokinetics often require drug candidates to be derivatized prior to commercialization. Recent breakthroughs in late stage modification have enabled derivatization of many complex molecules; however, a number of these methods are not general for heterocyclic compounds. Many total syntheses of heterocyclic natural product analogs require introduction of new functional groups at the beginning of the synthetic route and thus require many additional steps to generate synthetic derivatives.

Mutasynthesis can be used to efficiently prepare derivatives of heterocyclic natural products. In this process, microorganisms lack the machinery to generate a biosynthetic precursor of a natural product, and only when the precursor is supplied by the researcher can the natural product be generated. These mutant strains were initially developed through random mutagenesis; however, with the advent of genome mining and thorough characterization of biosynthetic pathways, targeted gene disruptions have allowed for the facile

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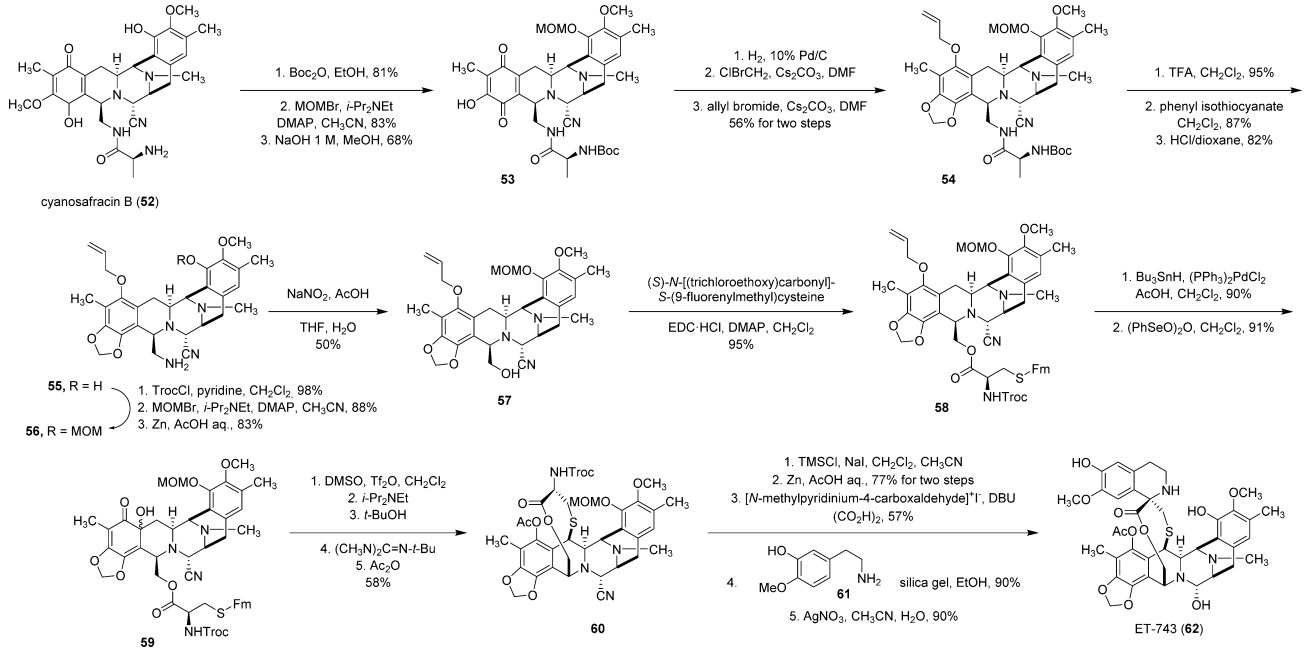
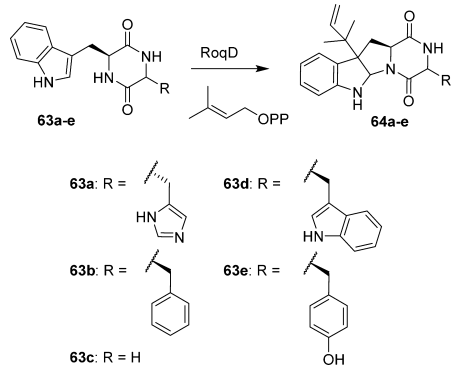
Scheme 7. Semisynthesis of ET-743 (62) from Cyanosafracin B (52)



production of mutant strains. Natural product analogues can be easily synthesized through mutasynthesis by simple addition of unnatural precursors. Mutasynthesis can be extremely beneficial for the inclusion of moieties like halogens, which seldom occur in natural products but are highly prevalent in pharmaceuticals. For example, RNAi-mediated knockdown of tryptophan decarboxylase (TDC) in tryptamine biosynthesis has been implemented to generate halogenated monoterpene indole alkaloids.[45] Stereoisomers of heterocyclic natural products can also be generated far more efficiently by mutasynthesis, as is evidenced in the production of roquefortine D analogues **64a–e** by Driessen, Overkleeft et al. (Scheme 8).[46]

Scheme 8. Synthesis of Roquefortine D Analogues 64a–e by Mutasynthesis



Commercial application of mutasynthesis is seen in the production of doramectin (**66**), an antiparasitic veterinary drug. Avermectin (**65**, Figure 2), whose discovery was awarded half of the Nobel Prize in Medicine in 2015, is a macrolide produced naturally by *Streptomyces avermitilis*. In a search for novel, more potent avermectin derivatives, researchers at Pfizer developed a mutant strain of *S. avermitilis* that blocked production of branched chain carboxylic acids. Supplementing the fermentation culture with various carboxylic acids led to a

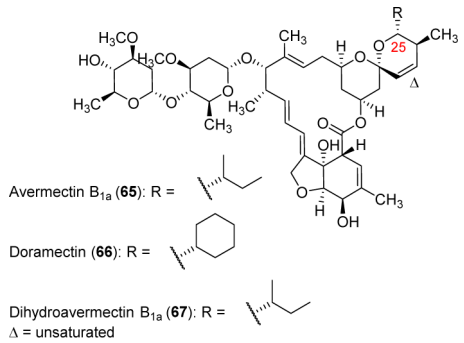


Figure 2. Avermectin B_{1a} (**65**), doramectin (**66**), and dihydroavermectin B_{1a} (**67**).

number of C25 analogues.[47] Doramectin (**66**), an avermectin analogue synthesized via supplementation with cyclohexanoic acid, was found to have superior plasma concentrations and a longer half-life as compared to dihydroavermectin B_{1a} (**67**), the main component of semisynthetic avermectin analogue ivermectin, while still possessing comparable, and in some cases superior, activity against a number of parasites.[48]

Chemobiosynthesis is a particular kind of mutasynthesis that involves disabling a particular enzymatic function in multienzyme polyketide synthases. The first instance of chemobiosynthesis was implemented in the synthesis of erythromycin analogues **69a–d** (Scheme 9). 6-Deoxyerythronolide B (6-dEB, **69a**) is a precursor to erythromycin, and it is synthesized by polyketide synthase 6-deoxyerythronolide B synthase (DEBS). These proteins are composed of several modules, each containing domains of varying enzymatic functions. The polyketide chain is constructed by passing it from one module to the next via transacylations of terminal thiols. The Khosla laboratory was able to produce 6-dEB analogues by introducing a point mutant to the ketosynthase of module 1 in DEBS, rendering this module ineffective. *N*-Acetylcysteamine thioesters **68a–d** were shown to be transformed by module 2 and subsequently incorporated in 6-dEB analogues **69a–d**.[49] Point

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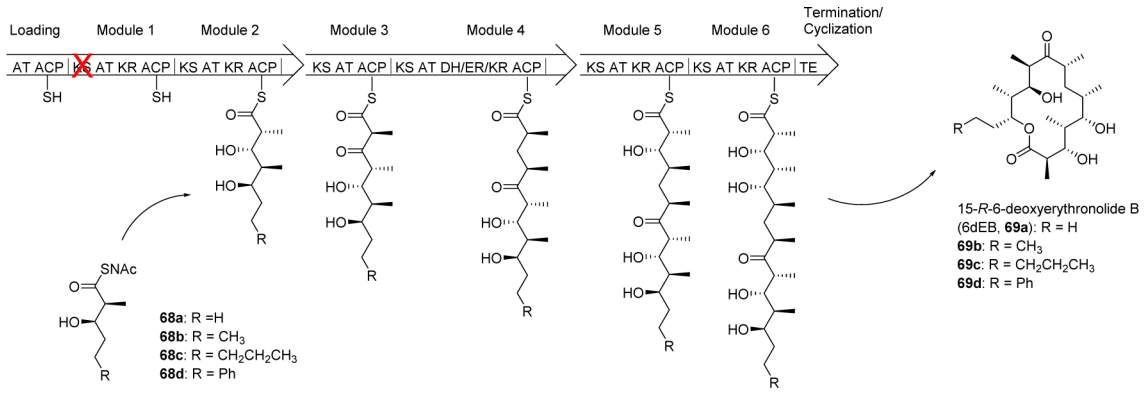
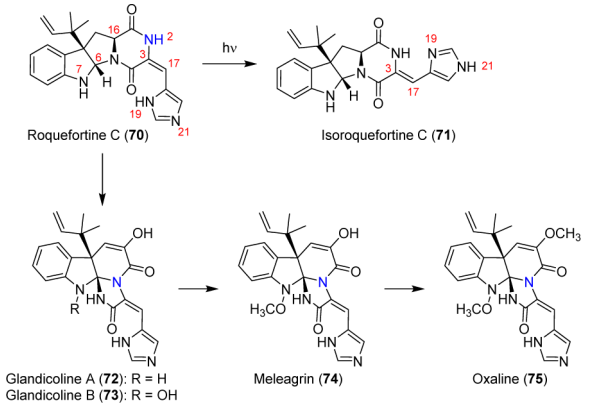
Scheme 9. Chemobiosynthesis of Erythromycin Derivatives 69a–d via Point Mutant to the Ketosynthase of Module 1



mutations have also been introduced into the DEBS loading module and have shown incorporation of nonchiral acyl thioesters into 6dEB analogues.[50]

Saga of the Roquefortines. *Penicillium roqueforti*, a fungus found in Roquefort cheese and a number of other blue-veined cheeses, produces a variety of complex and structurally interesting compounds including the roquefortine family. Roquefortine C (**70**, Scheme 10) is the most investigated

Scheme 10. Roquefortine C (70) and Related Compounds



member of the family and exhibits a very rare and photochemically unstable *E*-configuration of its C3–C17 double bond. This configuration was established by comparison to its photoisomer isoroquefortine (**71**), which has the *Z*-configuration.[51] Isoroquefortine has never been found in Nature and exhibits very different biological properties. As a consequence of the difference in stereochemistry, N19 of the imidazole is a hydrogen-bond donor in **70** and a hydrogen-bond acceptor in **71**. As a result, **70** exhibits a strong Fe–N interaction with hepatic cytochrome P450 and microperoxidase8 due to the available lone pair on N21, thus inhibiting hemeproteins.[52]

In addition to its unique chemical structure, **70** has acquired interest in recent years due to its role as a biosynthetic precursor to biologically active triazaspiro indole alkaloids glandicoline B (**73**), meleagrin (**74**), and oxaline (**75**). These compounds have shown a range of interesting biological properties, including antimicrobial activity,[53] antifouling activity,[54] and inhibition of tubulin polymerization in T lymphocyte cells.[55]

The mechanism proposed for the conversion of **70** into the triazaspiro structures was of interest to our research group. Radiolabeling studies in the 1980s were the first to suggest a biosynthetic pathway containing **70** and **75**.[56] This proposed path, which remained the accepted biosynthetic pathway for these metabolites until 2013, began with enzymatic hydroxylation of **70** at C16, followed by rearrangement to give **72**. The mechanism was thought to occur through the following steps: conversion of the hemiaminal to an open 9-membered dicarbonyl species, dehydrogenation at the C6 and N7 positions, nucleophilic attack of C6 by N2 to give **72**, oxidation at N7, and methylation to give **75**. A report that two diastereomers of 16-hydroxyroquefortine were characterized from a *Penicillium crustosum* species supported this mechanism.[57] It was proposed that enzymatic hydroxylation occurred in either a nonspecific fashion or through specific oxidation followed by isomerization to form both stereoisomers.

To investigate this mechanism, however, a sufficient supply of **70** was needed. The total synthesis of **70**, published by our research group in 2008,[58] presented several challenges and could not produce sufficient material for detailed mechanistic investigation. As **70** is produced by several fungi, we looked into fermentation as a source of **70**. As such, a procedure was developed by our group for the large scale fermentation of **70** from *P. crustosum*. This method allowed for the production of **70** at 125 mg/L for a fraction of the cost of total synthesis.[59]

As we were concluding our work in the development of the roquefortine C fermentation procedure, a major breakthrough in the elucidation of the biosynthetic pathway of roquefortine C derived metabolites was made by the Vreeken laboratory (Scheme 11).[60] Vreeken et al. observed several new metabolites in *Penicillium chrysogenum*, including novel indole nitrone roquefortine L (**76**). Due to their identical molecular weights, **76** had been previously misidentified as **72**, which was not found by Vreeken et al. in any cultures of *P. chrysogenum*. Roquefortines M (**77**) and N (**78**) were observed to form from the degradation of **76** in acidic aqueous conditions. Through a series of gene silencing studies, **76** was found to play a vital role in the biosynthesis of related metabolites **73** and **74** in *P. chrysogenum*.

Based on this work, it is clear that **76** is an important intermediate in the conversion of **70** to the triazaspirocyclic indole alkaloids. Our research group is currently investigating the oxidation of **70** to **76** as indole nitrones are uncommon in natural products. Through a collaboration with the Sherman research group, we have shown that this oxidation can be accomplished by flavin monooxygenase OxaD from *Penicillium oxalicum* (Scheme 12). A number of semisynthetic derivatives of **70** were generated, and OxaD was shown to tolerate substitution on the indole benzene ring and the imidazole as

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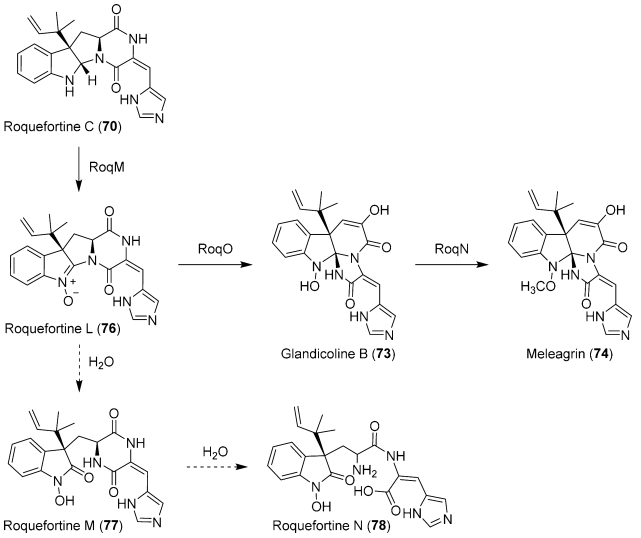
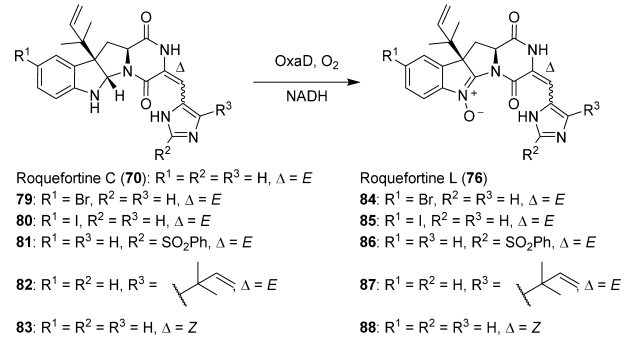
Scheme 11. Biosynthesis of Glandicoline B (73) and Meleagrin (74) from Roquefortine L (76) and Roquefortine C (70)



Scheme 12. *N*-Oxidation of Roquefortine C (70), Roquefortine E (82), and Semisynthetic Derivatives 79−81 and 83 by OxaD



well as isomerization about the C3−C17 double bond.[61] Concurrently, we have been able to develop special conditions to perform the oxidation chemically using a variety of electrophilic oxidizing reagents.[62]

Our results have shown that the combination of fermentation and organic synthesis can not only produce new heterocycles for use in the pharmaceutical industry but also achieve a better understanding of biosynthetic pathways and the discovery of novel mechanistic transformations.

CONCLUSION

Although the production of heterocyclic natural products by fermentation is a well-investigated field, as is organic synthesis, there are few examples of combining both disciplines. The merging of both fields could result in new research directions and innovative ideas, advancing knowledge and fostering creativity in both areas. Despite its many benefits, the field of fermentation is not without limitations. The implementation of fermentation for industrial purposes is currently limited by microbial output and cost. Currently, many genetically engineered systems are limited to biosynthetic precursors and other closely related natural products as opposed to simple, inexpensive starting materials. Additionally, many biosynthetic pathways of natural products have yet to be identified or characterized, precluding their eligibility for genetic engineering. As evidenced by the work described in this review, the combination of fermentation and organic synthesis provides a new approach to heterocyclic compounds. Production of organic compounds has becoming more and more interdisciplinary, and we must begin to embrace emerging technology for efficient methods of producing complex heterocyclic compounds.

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Notes

The authors declare no competing financial interest.

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Madeleine M. Joullié obtained a B.S. in Chemistry from Simmons College in 1949, after which she earned a Ph.D. from the University of Pennsylvania in 1953 under the guidance of Professor Allan R. Day. She then joined the faculty at the University of Pennsylvania, where she was one of the first female professors to earn tenure in Chemistry at a major university in the U.S. Her laboratory has focused on the chemistry of cyclopeptide alkaloids and the roquefortine and didemnin families of natural products as well as the development of compounds for the visualization of latent fingerprints as a forensic tool for law enforcement.

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