PRECURSOR-DIRECTED IDENTIFICATION OF NATURAL PRODUCT SCAFFOLDS

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John MacMillan, Ph.D

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DEDICATION

I would like to thank my research mentor Dr. John MacMillan for his support and guidance. His instruction and dedication have furthered my development as a researcher and reshaped my understanding of science.

PRECURSOR-DIRECTED IDENFITICATION OF NATURAL PRODUCT SCAFFOLDS

by

DAVID ANDERSON BRUMLEY

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David Anderson Brumley M.S.

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Supervising Professor: John MacMillan Ph.D

The rapid generation of natural product analogs is a fundamental challenge in both the optimization of medicinal potency and exploration of novel biological activity. Previous work with *Streptomyces variabilis* has demonstrated that natural products that incorporate non-enzymatic transformation can be exploited for the rapid generation of analogs. Herein we demonstrate a general methodology to use fluorinated or isotopically labeled substrates to identify natural product frameworks prone to non-enzymatic pathways. As proof of principle our precursor directed methodology was used in the study of discoipyrrole A formation, the guided isolation of a novel iminoquinone and the detection of a novel ammosamide analog via incorporation of a carbon-based nucleophile.

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CHAPTER ONE
Introduction

The discovery and optimization of chemical diversity is a constant challenge in the development of novel therapeutics. Fundamentally serving as signaling molecules and defensive/offensive compounds, natural products (NPs) have significantly impacted the development of therapeutics and inspired innovative synthetic methodologies. [38] Through technological advances and the development of on-target screening platforms, the rapid identification of drug candidates from synthetic small molecule and NP libraries has become a reality. [44] [3] However, the generation of natural product analogs via structure activity studies (SAR) for optimization of the medically necessary potency, selectivity and PK/PD properties is a major bottleneck in drug discovery/development from NPs. The innate structural complexity of many biologically active NP leads confounds this process due to: 1) limited availability of a common intermediate, 2) lengthy synthetic sequences, 3) optimization of reaction conditions, 4) complex stereochemistry. [34]

The issue of stereochemicial complexity is exemplified by total synthetic studies of vinigrol -- spanning over two decades. This diterpene isolated from the fungi Virgaria nigraby Devaux *et. al.* in 1993 displayed both antihypertensive and antigoagulation effects. [50]Since that time efforts to synthesize this NP were hampered by the highly congested decahydro-1,5 butanonaphthalene core and 8 contiguous stereocenters. While the Baran group was able to develop a 23-step synthesis with remarkably high intermediate yields (gram scale) late stage reactions were notably challenging to optimize and resulted in only

3% over all yield. [37] Furthermore, any attempts at meaningful SAR studies would require integration of structural diversity early on in the synthesis and could potentially disrupt the desired stereochemistry.

The lack of a common intermediate in the synthesis of natural product analogs was a confounding factor in the SAR of the vinca alkaloids – vinblastine and vincristine. These plant-derived alkaloids continue to serve as two of the most efficacious combinational therapeutics in the treatment of a host of human illnesses including: testicular, ovarian, breast and neck cancers. [42] Early efforts to produce analogs relied on semisynthetic modifications leading to a limited number of products bearing minimal modulation the core structure. [32, 39] The Boger group successfully synthesized a series of vindoline analogs with significant structural changes, however these examples were only accessible via total synthesis – hampering the development of large libraries. [14, 28, 51] Boger and coworkers recently reported their intermediate based SAR methodology – notably influenced by vinca biosynthesis. [48] Their biomimetic approach allowed for the coupling of various vindoline and catharanthine subunits in a one-pot reaction. This study resulted in the discovery of a series of C-20' urea substituted analogs displaying picomolar activity (30 fold improvement) vs various tumor cell lines and nanomolar potency against vinblastine resistant lines (e.g. MCF-7 & HCT116/VM46). While the application of optimized synthetic methodology was effective in this example when one considers that the vinca alkaloids first entered the clinic in 1958 the pitfalls faced by the synthetic chemistry clearly hamper the development of new drugs. In light of these challenges alternative routs to generate chemical diversity around natural product scaffolds are necessary for the continued treatment of human disease.

NATURAL PRODUCT BIOSYNTHESIS

Natural products are evolutionary crafted signaling or defensive/offensive biomolecules that originate from complex cellular machinery (i.e. protein catalysts) -- imparting both stereochemical and structural complexity. [9, 17] The study of the natural production or biosynthesis (BS) of various natural products familes has resulted in the discovery of impactful scientific insights ranging from the ecological to the biomedical. [17-18]. While the final product of a series of biosynthetic step may represent an impactful drug candidate/theruputic, equally valuable are the insights gained by understanding the biomolecular processes and machinery involved

The Shikimate Pathway

The shikimate biosynthetic pathway allows for the production of aromatic amino acids (i.e. tyrosine, phenyl alanine and tryptophan) and their subsequent incorporation into a variety of primary and secondary metabolites. [31] Starting from the precursors phosphoenol pyruvate and erythrose phosphate a series of enzymatically mediated steps results in the formation of chorismate. Chorismate serves as a handle for the production of a variety of primary metabolites and biosynthetic precursors for secondary metabolism including: the aromatic amino acids, quinones, folates, alkaloids and flavonoids. The alkaloid family of natural products is derived from tryptophan, tyrosine or phenylalanine through their subsequent catabolism/modifications. Members of this NP family have been thoroughly studies due to their notable biological activities and chemical diversity with notable examples including: morphine, cocaine, nicotine, vinblastine and vincristine. [19]

Nonribosomal Polypeptide Biosynthesis

Nonribosomal polypeptides (NRPs) are a class of microbial natural products, which display a wide range of chemical diversity through the adaptation of macromolecular multifunctional enzymes or NRP synthases (NRPS). [15] Generally, an NRPS is composed of multiple modules serving to incorporate different building blocks into the growing peptide. Individual modules contain various domains with catalytically active residues – often times resembling core motifs. The (A) or adenylation domain functions to select and active the various substrates that constitute the primary sequence of the encoded product. While ribosomal based peptides are generally limited to the standard 20 amino acids, NRPS can access hundreds of modified amino acids. Once activated the substrate can be transported by the arm like peptidyl carrier protein (PCP) [6] to various catalytic sites within the module. This process is facilitated by the formation of a thioester bond between a conserved PCP serine residue and the activated substrate. Chain elongation can then occur in the (C) or condensation domain where amine, imino or hydroxyl groups of the activated substrate can then condense other building blocks. The adaptation of tailoring enzymes further contributes to NRP chemical diversity by catalyzing additional modifications to the peptide including: methylation, glycosylation, heterocyclizations, redox reactions and epimerizations. Classic examples of the NRPS family of natural products include: surfactin, gramicidin S and cyclosporin A. [15]

Terpenoid Biosynthesis

Terpenoid biosynthesis can be generalized as a series of enzyme mediated nucleophilic additions, rearrangements and cyclizations resulting in a wide range of products from similar substrates. [17] The fundamental building block of any terpanoid is the 5-carbon isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). The notably activated allylic position of DMAPP promotes the nucelophilic addition of IPP and results in the formation of a 10-carbon chain with a stabilized 3° carbocation. [16]This process can be iterated until the correct carbon chain length is reached. In the case of farnesyl diphosphate three unique products (i.e. pentalenene, epi-aristolochene and aristolochene) can be obtained based on the active enzyme. The biosynthesis of multiple products can be obtained from the same linear substrate due to: (1) the reactivity of the generated carbocation and (2) the enzymatic control of these reactions (e.g. rearrangements and cyclizations) at the active site. The biological success of this pathway is demonstrated by the discovery of almost 55,000 different terpenoid examples many of which have strong biomedical/biochemical impacts.[16]

Polyketide Biosynthesis

The polyketide family of natural products are produced by macromolecular enzymes known as polyketide synthaseses (PKSs) that have adapted multiple catalytic domains or modules. [46] Historically polyketides has been classified into three classes based on the catalytic behavior of their respective PKS. Regardless of mechanism, in general all PKSs

catalyze the sequential decaboxylative condensation of acyl CoA building blocks via a conserved ketoacyl synthase (KS) domain/subunit. Type I PKS are organized into a noniterative modular system where each model contains multiple catalytic domains responsible for the addition of one specific building block. [52] In the case of Type I and II acyl building blocks are activated acyl carrier proteins (APCs) which also transports through the growing polyketide intermediate down the assembly line. [6]Type I PKS are reasonable for the production of macrolides such as rapamycin and erythromycin A. Type II PKS work in an iterative fashion where the growing polyketide is passed through a single set of catalytic domains. These products are generally aromatic and/or polycyclic as demonstrated by tetracenomycin C. Type III PKS behave similarly to the iterative mechanism of Type II, however they lack APCs and directly activate substrates for incorporation. Products of Type III are generally mono or bicyclic aromatic molecules such as flavolin. [46]

These and related pathways are the genetically encoded sequence of events that lead to the structurally diverse, complex structures of natural products. Over the past 60 years a tremendous amount of progress on the chemical, enzymatic and genetic basis of these pathways has been made.

In addition to the enzyme driven transformations that our laboratory and others have been exploring are, there are growing examples of non-enzymatic transformations that are driven by the inherent reactivity of biosynthetic intermediates. These reactive intermediates include electrophilic substrates such as aldehydes, iminium species, *p*-quinione methides and nucleophilic species such anthranilates, amino acids and other reactive species (e.g. sulfur, nitrogen and oxygen containing species). These reactive intermediates participate in non-

enzymatic reactions with endogenous substrates or metabolites produced by other biosynthetic pathways, catabolism or primary metabolism. As we understand the role of these non-enzymatic pathways, we can look to harness this reactivity as a powerful approach to drug discovery/development through precursor-directed analog generation.

EXAMPLES OF NON-ENZYMATIC SYSTEMS & THEIR BIOLOGICAL IMPACTS

Our efforts to develop a novel discovery platform are justified based on increasing literature examples of natural products whose biosynthesis include one or more non-enzymatic transformations. The adaptation of these pathways has been demonstrated by a host of organisms ranging in complexity from simple microbes to higher order animals – often times tied to fundamental biological responses. Studies of these systems have shown that in many cases the existence of reactive intermediates participating in non-enzymatic reactions can be further exploited for the generation of novel analogs.

Oxazinin (A)

Work from the Schmidt lab and others have demonstrated that tunicates represent a niche for the discovery of novel NPs. [47]While ascidians have been shown to produce a variety of biologically active NPs, literature precedent suggests that many of NPs are produced by their symbiotic microbes. [10] The Schmidt's lab efforts to further explore the chemical capacity of these microbes resulted in the discovery of a novel fungal isolate belonging to the class Eurotiomycetes. [36] Bioassay guided fractionation of extracts from this filamentous fungus lead to the isolation of the structurally interesting NP oxazinin A (**3**) (Fig. 1).

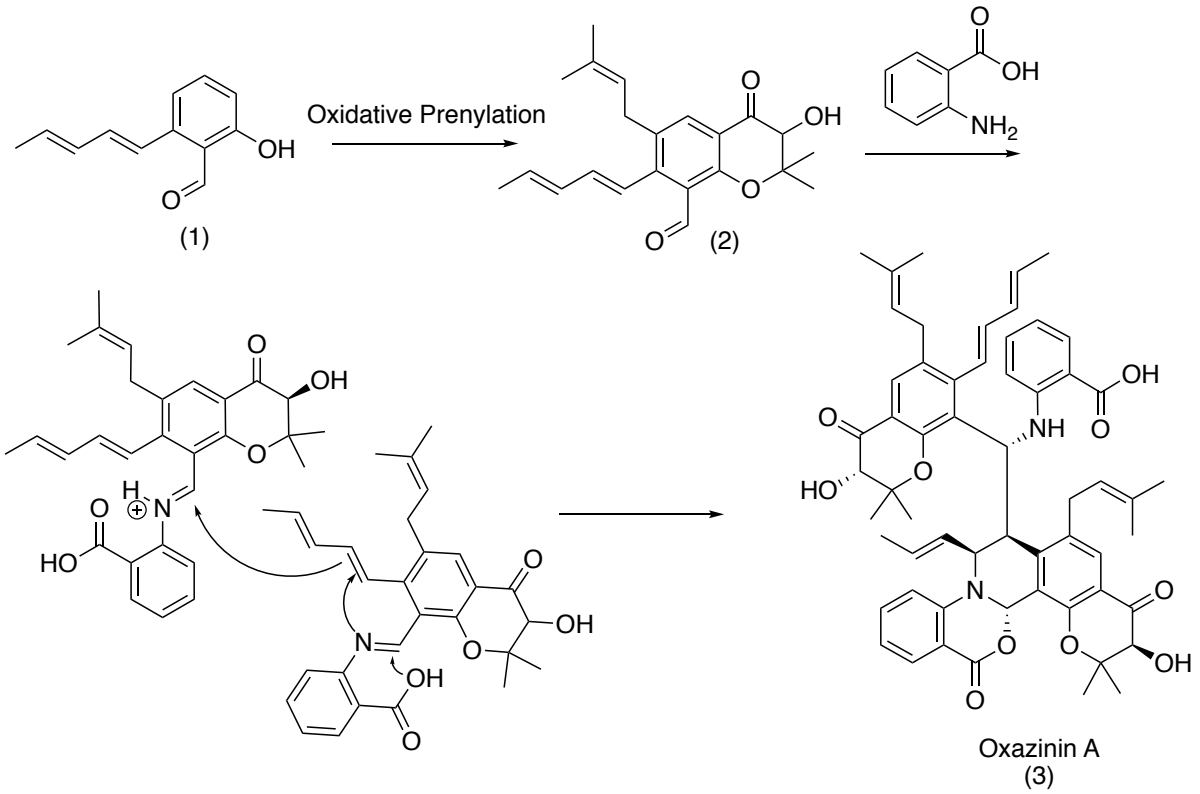


Figure 1. Proposed biosynthesis of **3** incorporating non-enzymatic chemistry.

While only showing modest biological activity against *Mycobacterium tuberculosis*, **3** is a structurally complex example of a NP. Oxazinin A is a racemic, polyketide dimer with an unusual pentacyclic moiety composed of benzoxazine, isoquinoline and pyran ring systems. Lin *et. al* theorized that following oxidative prenylation of **1** the addition of anthranillic acid to the aldehyde of **2** results in the formation of an activated aldimine. [35] Two equivalents of this intermediate can then undergo intramolecular cyclizations and dimerization resulting in the fully elaborated ring system of **3**. Anthranillic acid is an early intermediate in tryptophan metabolism [31] and can thus function as a readily available nucleophile – promoting the production of the aldamine. Given the planar nature of the aldimine intermediate any non-enzymatically mediated nucelophilic addition should result in a racemic product. These observations and final isolation

of **3** as a racemate lead the authors to conclude that the late stages of oxazinin biosynthesis were non-enzymatically driven.

Tridachinones

The tridachione and related family of metabolite assemblages 5, **6** & **4** have prompted investigations ranging from total syntheses to ecological studies due to their biological activities and structural complexities (Fig.2). [26, 49] Originally isolated by Ireland *et. al.* from the sacoglossan mollusk species *Tridachiella diomedea* and *Tridachia crispate* **9** and other members of this family **5** were hypothesized to originate from a linear polyene precursor **7** anchored to a γ- pyrone head group. [26] Given the unusual bicyclo[3.1.0]hexane group of **5** and related NPs it was theorized that these metabolites undergo a photochemically driven electrocyclization and rearrangement reactions. This hypothesis was later supported by *in vivo* ^{14}C incorporation studies conducted under ecologically relevant conditions using ambient sunlight. [27] These results indicated that the γ- pyrone head group likely functioned as a photosensitizer and gave rise to the question of the ecological function of these metabolites.

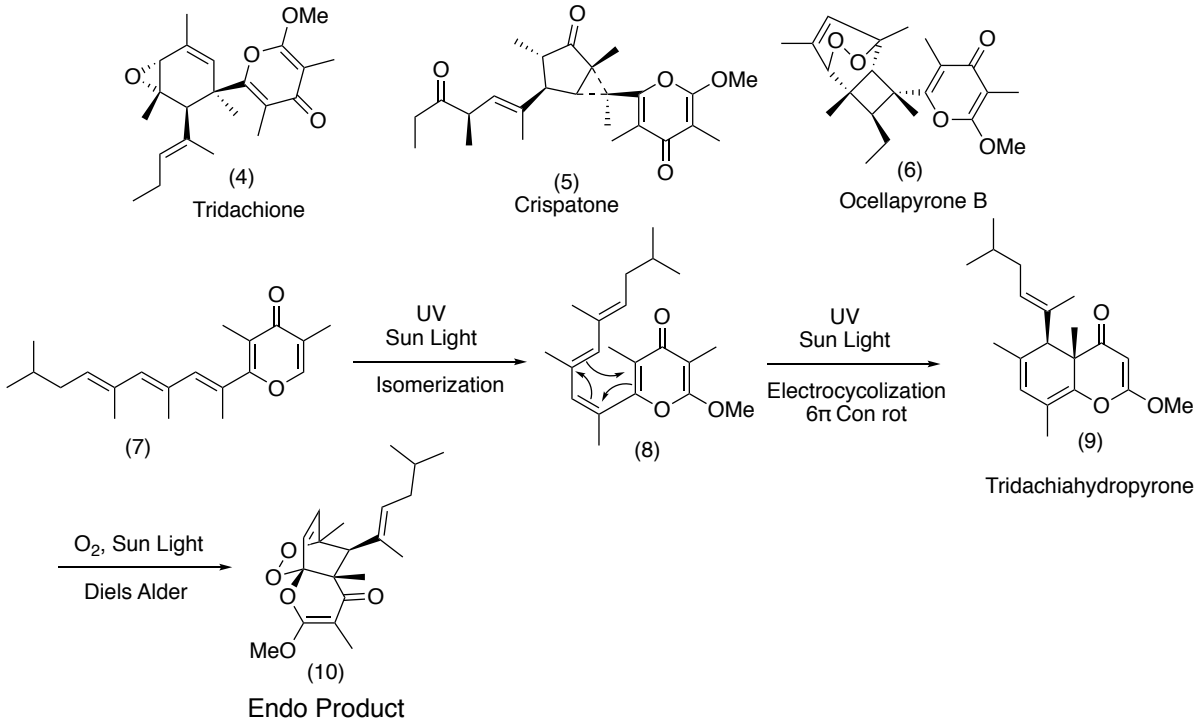


Figure 2. Proposed biosynthesis of **9** and **10** and structural representations of other members of the tridachione family of natural products.

The Mollusk species associated with the production of the Tridachione family and related metabolites are unique in the sense that they maintain no outer shell and that they sequester functional chloroplasts from siphonous algae through grazing. [20] Previous studies indicated that these metabolites are concentrated in mollusk tissues enriched with photosynthetically active chloroplast. [20, 27] These observations implied that the tridachione superfamily could function as natural sunscreens – protecting the animal from UV radiation.

The later discovery of unusually oxidized metabolites **5, 6** and **10** raised further questions about the rule of these NPs as syncs for reactive ROS species. [4] Prompted by these observations further biomimetic studies were conducted by Sharma *el. al* to verify the proposed biosynthetic

route from the linear precursor **7** to **9** and subsequent oxidation to **10**. [49] Their results indicated that the initial isomerization of **7** to **8** was spontaneous in ambient sunlight and likely driven by the elevation of 1,3 eclipsing interactions on the polyene chain. Furthermore, it was found that the proposed 6 π con-rotary cyclization was preferential under the same conditions and that the alternative dis-rotary pathway required non-biologically relevant temperatures. The final irradiation of 9 in the presence of molecular oxygen yielded exclusively the endo-adduct **10** through a [4+2] cycloaddition with singlet oxygen.

Jadomycins

The jadomycins are examples of structurally complex NPs discovered through the through the manipulation or induction of indigenous microbial stress responses. Initial work had established that various actinomycetes were capable of producing the antibiotic chloramphenicol (**12**) (Fig. 3). [1] Ayer *et. al.* later noted a reduction of **12** when *S. venezuelae* (strain ISP5230) was fermented in media supplemented with D-galactose and L-isoleucine as the sole respective carbon and nitrogen sources at elevated temperatures (32 °C). [2] Furthermore, under these conditions an undiscovered metabolite **17** was isolated from fermentation extracts. Structural characterization of **17** indicated the presence of a pentacyclic 8*H*-ben[b]oxazolo[3,2-*f*]-phenathridine backbone including both dihydropyridine and oxazole rings. [2] The induced production of **17** based on fermentation conditions prompted Doull *et. al.* to further explore metabolites associated strain ISP5230. Their fermentation conditions included the same as D-galactose and L-isoleucine supplemented media as Ayer *et. al.* ,but also utilized higher

temperatures (47 °C) and ethanol exposure (6% v/w). [11] These conditions lead to the discovery of the glycosylated jadomycin analog **18 --** containing an L-digitoxose moiety.

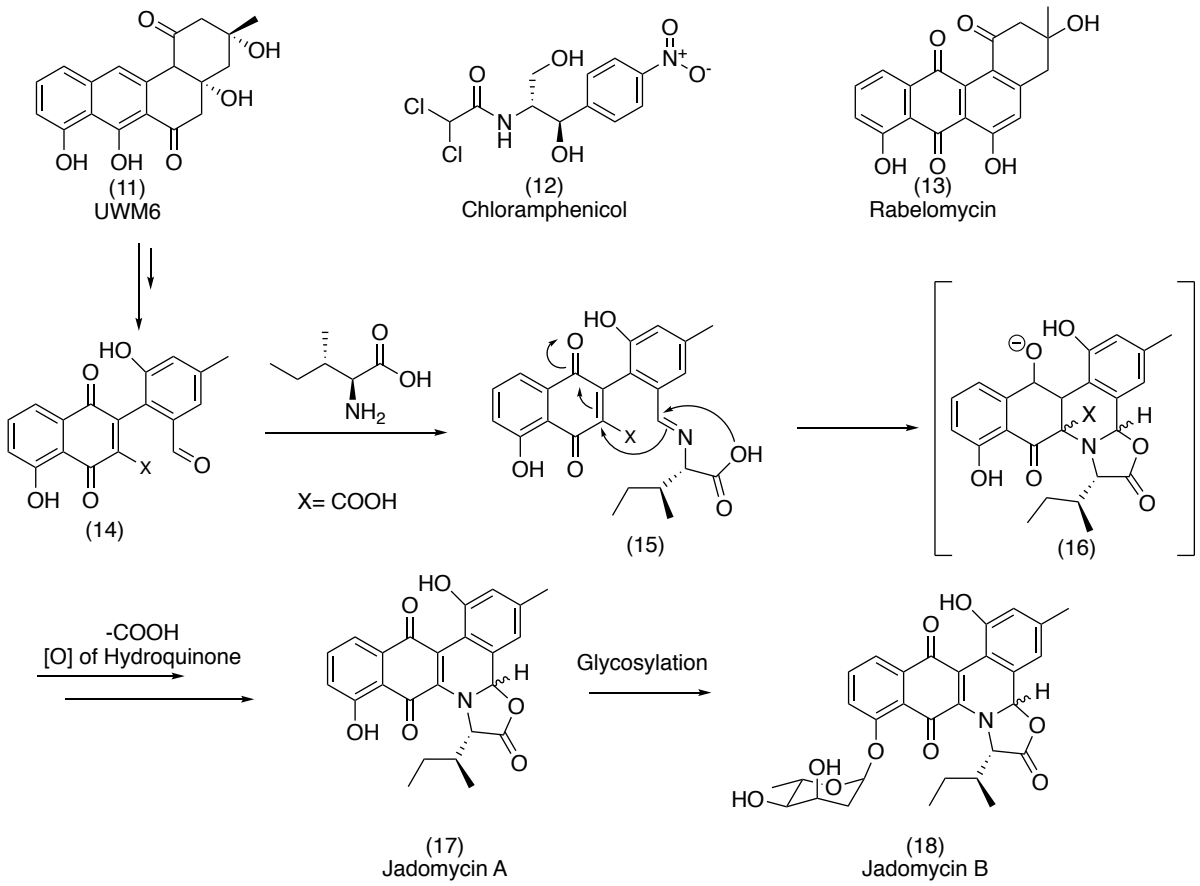


Figure 3. Proposed biosynthesis of **17** & **18**.

Though much of the biosynthetic pathway leading to the formation of the jadomycin core and subsequent glycosylation step are enzymatically driven there was strong evidence for the non-enzymatic incorporation of L-isoluecene. In their examination/optimization of fermentation conditions for the production of **18** Doull *et. al.* also explored alternative amino acid nitrogen sources. [12] During fermentation it was noted that amino acids substituted for L-isoluecene resulted in distinct pigment changes in *S. venezuelae* cultures – implying the formation of new NPs. Kulowski *et. al.* theorized that following the oxidative opening of **11** [33] leading to **14** could

readily undergo a series of non-enzymaticlly driven cyclizations. These reactions could be initiated by the nucleophilic addition of an amino acid to the alydehyde of **14**. The nitrogen of the resulting aldimine of **5** could then undergo Michael addition to the enone/quinone moiety forming a pentacyclic intermediate **16**. [29] This intermediate could then serve as a substrate for enzymatically-mediated chemistry leading to **17** & **18**.

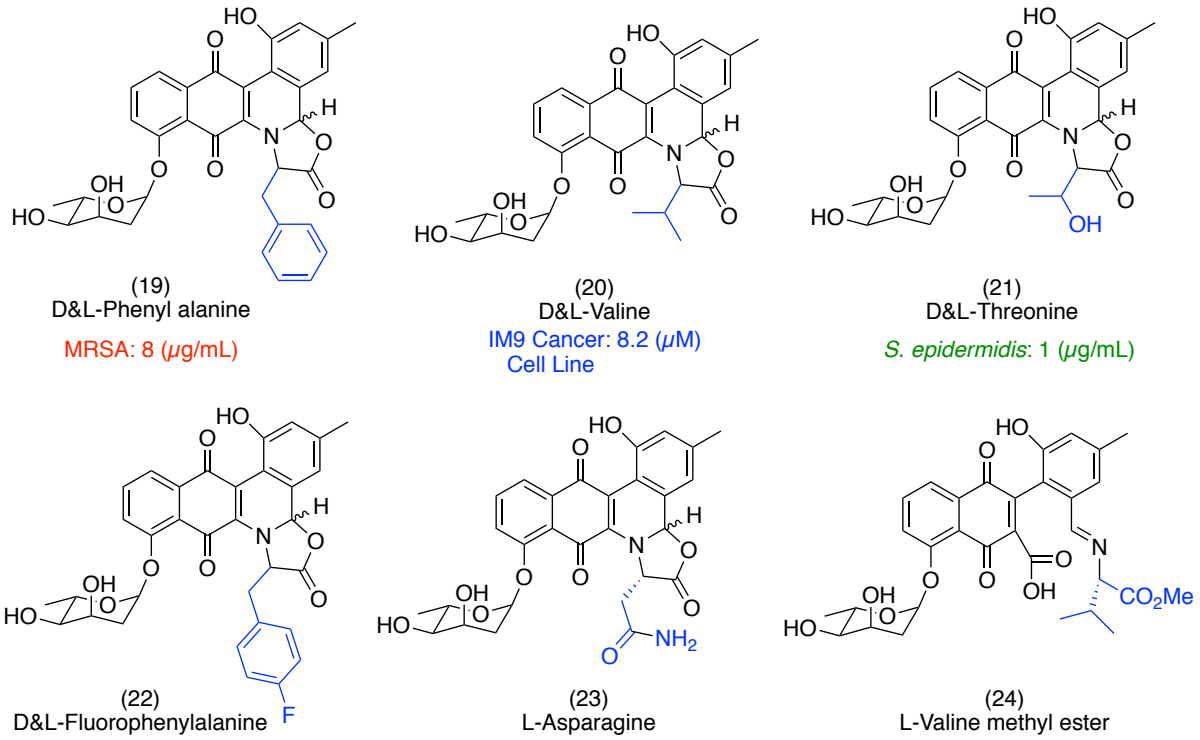


Figure 4. Structural activity relationship studies of jadomycin analogs **19 – 21** and additional analogs highlighting the structural diversity generated through precursor directed fermentations.

Natural products whose biosynthesis includes non-enzymatically derived reactive intermediates, such as the jadomycins, represent handles for SAR like analog generation. Doull *et. al.* first postulated that fermentation media supplemented variable amino acids could provide routes to alternative jadomycin analogs. [12] Jackman *et. al.* was among the first researchers to apply this concept and successfully isolate and characterize a number of jadomycin analogs (e.g. **19, 20** & **21**) (Fig. 4) incorporating canonical amino acids into the reactive intermediate **14**. [29]

Additional work sought to incorporate both non-natural & non-proteogenic/synthetic amino acids (e.g. **22** &**24**) via fermentation. [30] [13] The resulting jadomycin analogs displayed a wide range of biological activates and highlighted the importance of biological interrogation in the discovery/development of new therapeutics. [8, 53]

Ammosamides

The ammosamide family of NPs are examples of structurally intriguing marine metabolites that display a range of biological activities. Originally isolated by Hughes *et. al.* , extracts from the marine sediment-derived microbe *Streptomyces* sp. strain CNR-698 displayed activity versus colon carcinoma cancer cell line HCT-116. [24] Bioassay guided fractionation of these extracts resulted in the isolation of ammosamides A (**26**) and B (**27**)**.** Structurally characterization of **26** and **27** indicated the presence of a pyrroloquinoline core decorated with amine, chloride and amide functionalities. Hughes *et. al.* later attributed the observed HCT-116 potency of **27** to interactions with myosin. [23] Additionally, the ammosamides have been implemented as potential chemoprevative agents through inhibition of quinone reductase-2 (QR2). [45] Crystallographic, biophysical and biochemical studies of QR2 by Buryanovskyy *et. al.* and Calamini *et. al.* provided evidence that the endogenous activity of QR2 leads to the production of toxic reactive oxygen species. [5] [7] Furthermore, the inhibition of QR2 via small molecules (e.g. resveratrol) or RNAi knockdowns resulted in the up regulation of cellular antioxidant enzymes and increased survival in the K562 cell line. [7]Reports of QR2 inhibition by **27** prompted Reddy *et. al.* to further explore this biology through structural activity relationship

studies. [45] Their efforts resulted in the synthesis of a library of ammosamide analogs, resulting in a 15-fold increase in potency (nM) through methylation of the C-6 amine.

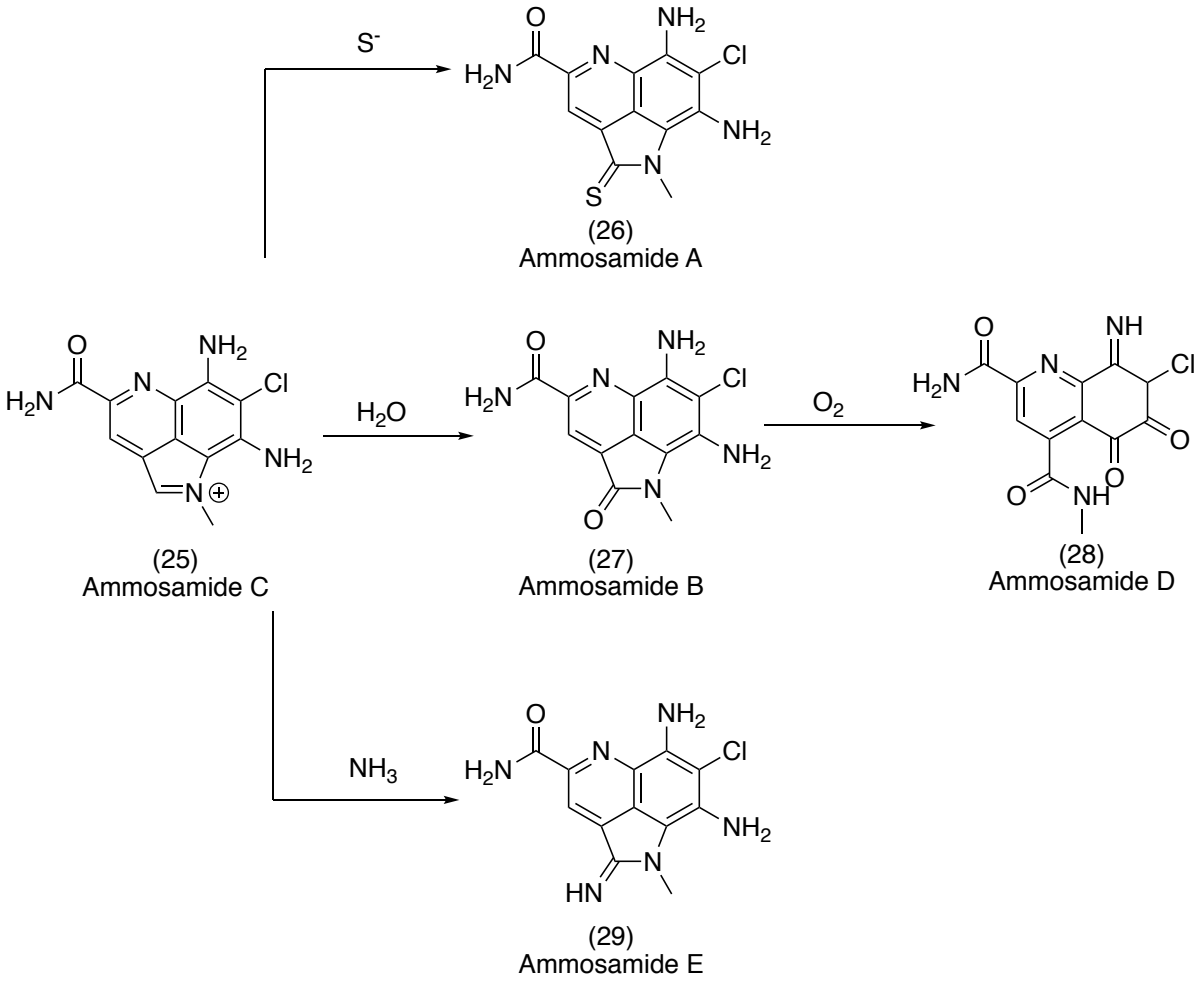


Figure 5. Proposed biosynthesis of the ammosamide family of natural products.

Studies in the MacMillan laboratory on a marine-derived *Streptomyces variabilis* (strain SNA-020) led to the proposal that late stage of ammosamide biosynthesis are mediated by non-enzymatic chemistry. In particular, discovery of ammosamide E (**29**) raised further questions about the generation of the unusual amidine moiety (Fig. 5). [43] Initial efforts to stimulate production of **26** and **27** by supplementing fermentation media with excess tryptophan, the expected precursor to ammosamide biosynthesis, unexpectedly lead to the production of a new

metabolite ammosamide F (**30**) (Fig. 6) which incorporated anthranilic acid at C-2 of the ammosamide framework to give rise to the amidine.

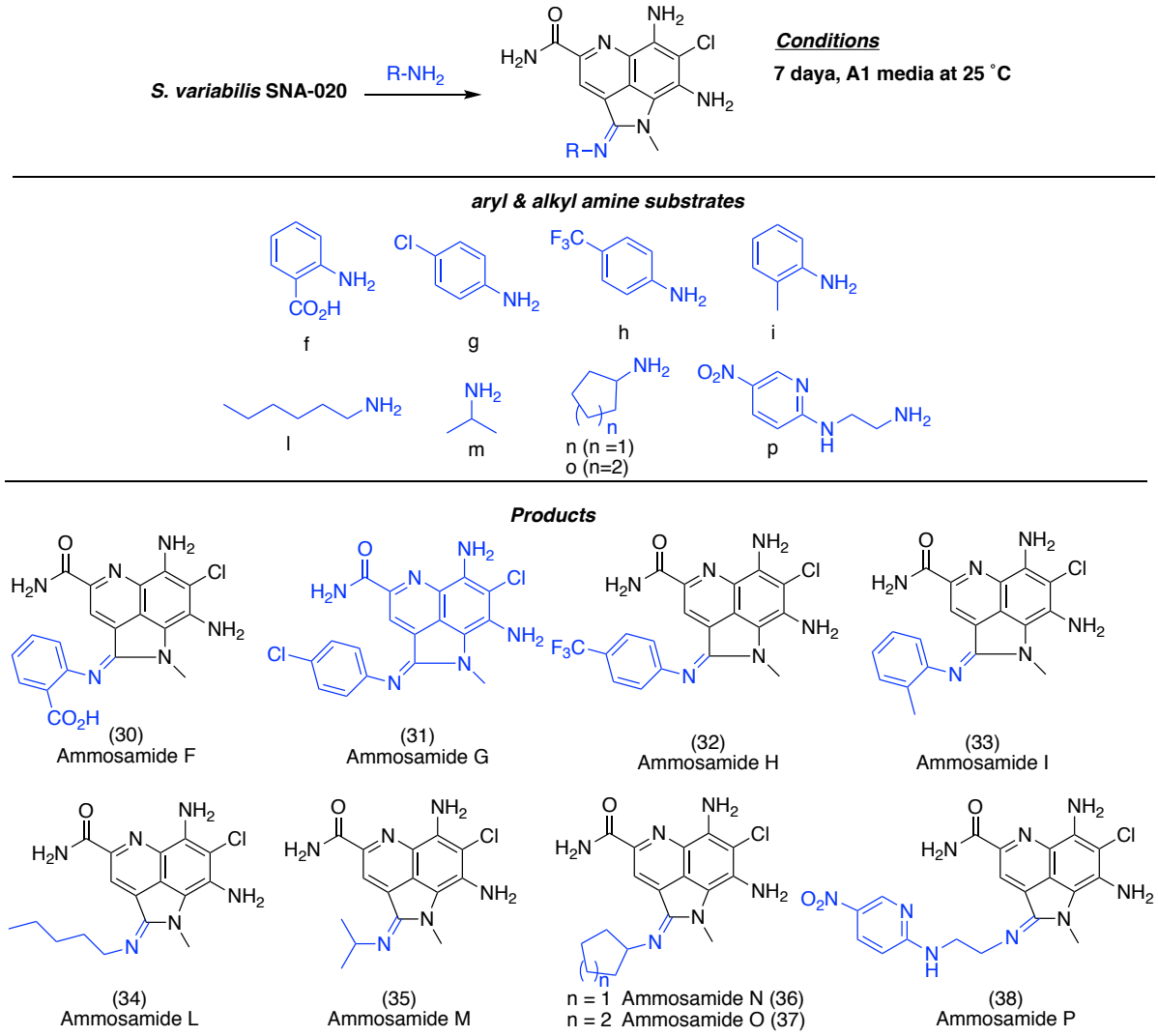


Figure 6. The precursor directed approach utilized the reactive iminium intermediate **25** produced by SNA020 as a handle for SAR like analog generation (top). Various alkyl and aryl amines were dosed into SNA020 liquid cultures and allowed to ferment for 7 days (middle). This process resulted in various amidine ammosamide analogs for screening efforts (bottom).

In light of the apparent "molecular plasticity" of the ammosamides and LC-MS data indicating the presence of *N*-methyliminium species **25,** Pan *et. al.* proposed that this family of

NPs could be derived from non-enzymatic reactions. The existence of **25** was further validated by the total synthetic efforts of Hughes *et. al.* [22] Their work found that the methylation of the thiol of **26** followed by sodium borohydride reduction resulted in an iminium product **25.** [22] The product mass was consistent with that observed in fermentation extracts by Pan *et. al.*. To further explore the participation **25** in non-enzymatic reactions Pan *et. al.* prepared **25** via a semi-synethic route [22] and reacted the product with 4-chloroaniline in fermentation media at ambient temperatures – resulting in **31**. Product **31** could also be produced in "spent" fermentation media containing (i.e. media free of exogenous enzymes) non-synthetic **25.** These results lead to the theory that **25** produced during fermentation could react with various nucleophiles (e.g. oxygen, sulfur, nitrogen containing species) within the media -- giving rise to the analogs **26-29.** [43] These results prompted the additional SNA-020 fermentations with a host of aryl and alkyl amine precursors – generating a library of amidine analogs.[43]

The ammosamides exemplify the capacity for analog generation/SAR studies through the manipulation of biosynthetic pathways incorporating non-enzymatic chemistry. Exploitation of reactive intermediates in the form of electrophilic species, such as iminium ions in the case of ammosamide C, provided a route to generating chemical diversity. While Cushman *et. al.* was able to produce a series of analogs and enhance QR2 inhibition through methylation of the C-6 amine they were unable to explore C-2 functionality – due to limitations in synthetic methodology. [45] Furthermore, efforts by Pan *et. al.* to semi-synthetically convert the thioamide **26** to analog **31** under numerous reaction conditions resulted in mixtures of unwanted products, decomposition of starting materials or a lack of reaction. The application of the precursor

directed approach lead to the successful generation of 20 ammosamide analogs in good yields, with relative full reduction of the indigenous NPs **26** – **29**

When assayed versus a panel of non-small cell lung cancer (NSCLC) cell lines in addition to QR2 inhibition a notable increase in activity was observed as demonstrated by the highlighted ammosamide analogs (Fig. 7). Additionally, their SAR data alluded to the importance of lipophilic alkyl amines for biological activity as demonstrated by analogs **39** & **40**. The success of the ammosamide system and other examples necessitates the discovery of additional biosynthetic systems utilizing non-enzymatic chemistry.

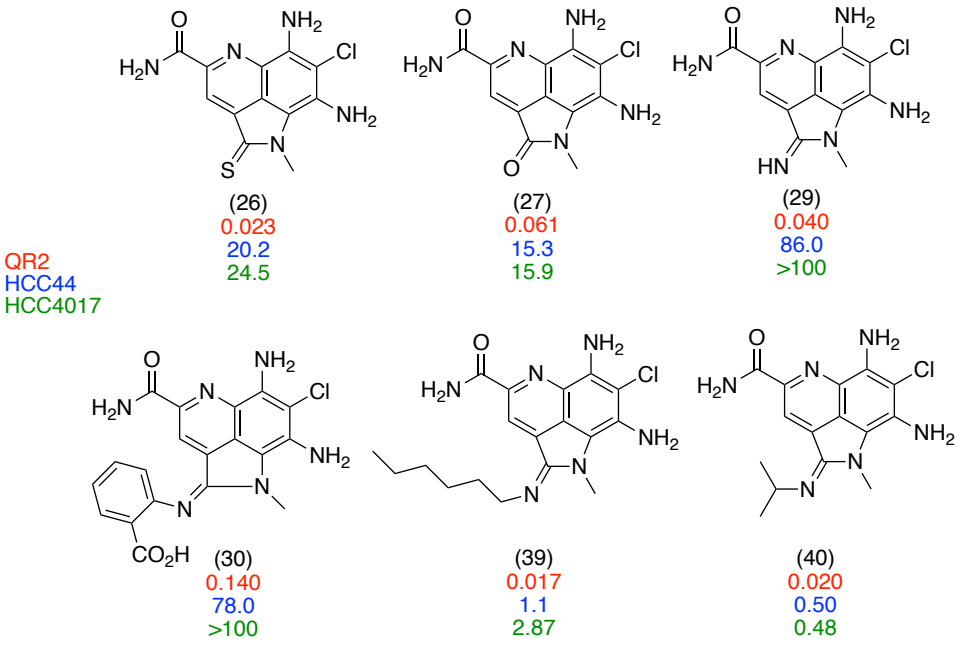


Figure 7. Amidine analog IC_{50} (μM) cytotoxicity results versus two NSCLC lines (HCC44 & HCC4017 and inhibition of QR2 (μM)

CHAPTER TWO
Precursor-Directed Identification of Natural Product Scaffolds

INTRODUCTION

Premise for The Discovery of Non-enzymatic Systems

Microbe-derived natural products, have been the source of nearly all classes of antibiotics, the original statin drugs and a variety of cancer therapeutics. [41] Over the last decade there has been a renaissance of microbial natural products (NP) driven by the chemical exploration of new microbial resources (e.g. marine systems, and anaerobic fermentations) [40] coupled with innovations in genome mining [25], analytical technologies and high content phenotypic screens. [44] These innovations have enhanced the discovery of novel NPs and our understanding of microbial chemical diversity.

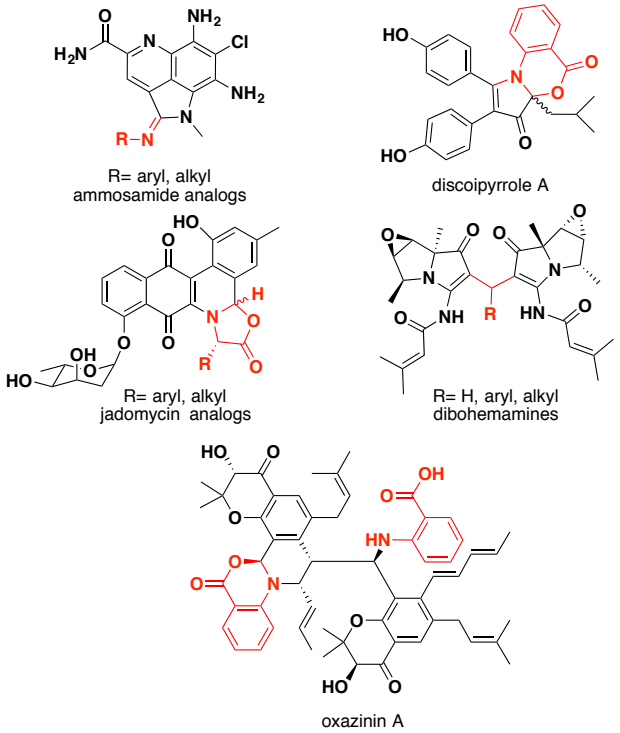


Figure 8. Examples of various natural products utilizing non-enymatic chemistry in their biosynthesis

In our efforts to discover novel NPs from a collection of marine-derived bacteria using information-rich phenotypic screens, [44] we identified three families of natural products, the discoipyrroles, [21] ammosamides [24, 43] and dibohemamines (Fig. 8), that are potent cytotoxins against non-small cell lung cancer (NSCLC) cell lines. Interrogation

of the chemistry of these families led to the discovery that they all incorporate one or more non-enzymatic transformations en route to the final compound.[43] For the ammosamide family, a non-enzymatic nucleophilic addition of a nitrogen, sulfur or nitrogen based nucleophile to an *N*-methyl iminium ion gives rise to a corresponding lactone, thiolactone or amidine.

Isotopic Substrate Discovery Platform

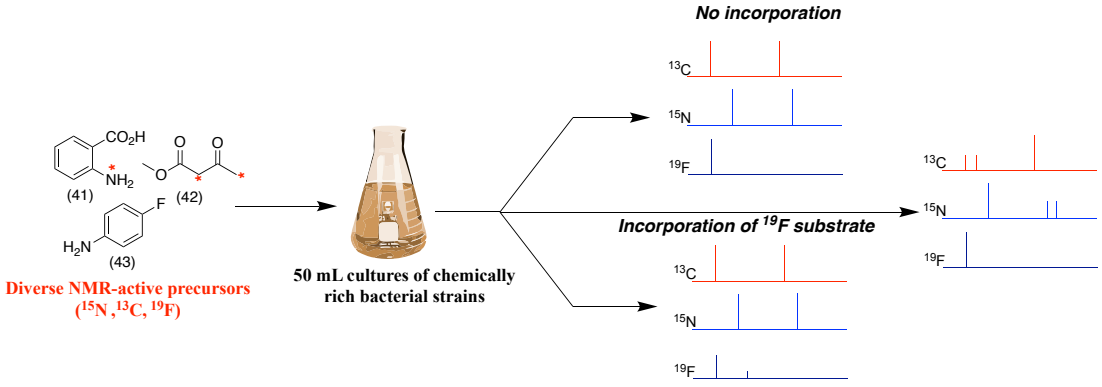


Figure 9. Over view of the use of isotopic labels to identify novel biosynthetic pathways utilizing non-enzymatic chemistry.

The development of a discovery platform to identify natural products scaffolds that can undergo non-enzymatic reactions would be a powerful tool in the natural product discovery arsenal. A few key features needed for an unbiased platform to be successful include: (1) sensitivity of detection, (2) moderate throughput, and (3) the ability to selectively identify compounds generated from non-enzymatic transformations over endogenous production. We have focused on a NMR-based strategy that takes advantage of NMR active isotope labels, such as ^{13}C, ^{15}N and ^{19}F and has the added ability to multiplex (Fig. 9). Due to the low natural abundance of these isotopes, feeding enriched precursors will allow us to identify potential non-enzymatic transformations.

As shown in Figure 9, we have focused on nitrogen and carbon based substrates that can act as nucleophiles at the pH of our microbial fermentations. ^{15}N-Anthranilic acid (**41**), a metabolite of tryptophan catabolism, was selected because it has been non-enzymatically incorporated in a number of the examples and is easily accessible with the ^{15}N label. Methyl 3-oxobutanoate-2,4-$^{13}C_2$ (**42**) was selected as our carbon-based nucleophile, while 4-fluoroaniline (**43**) was selected as our fluorine probe. We were particularly interested in utilizing **43** as fluorine is extremely rare in natural products and ^{19}F NMR is nearly as sensitive as ^{1}H NMR, and maintains a wide spectral width ranging from -220 to 550 ppm, thus providing an ideal probe for non-enzymatic incorporations.

RESULTS

^{15}N-Anthracitic Acid Incorporation

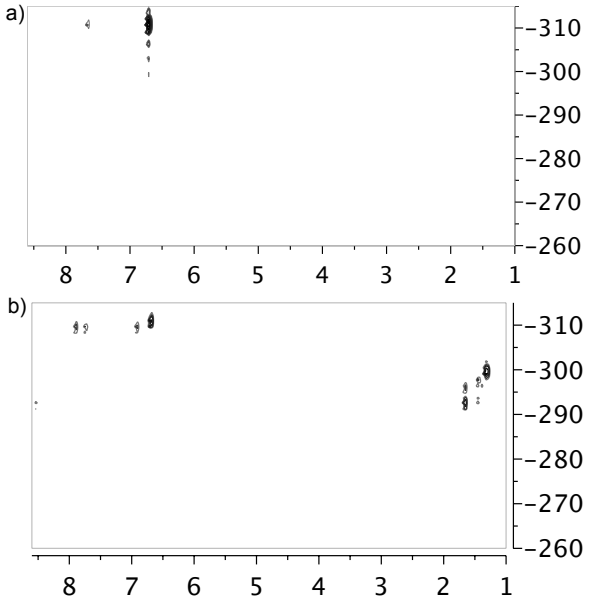


Figure 10. (a) ^{1}H-^{15}N HMBC of ^{15}N-Anthranilic acid (**41**). (b)^{1}H-^{15}N HMBC of extracts from SNA-048 fermented with **41**.

Examination of cultures that had been supplemented with ^{15}N-anthranilic acid (0.5 – 1.0 g/L) were carried out using ^{1}H – ^{15}N HMBC due to the greater sensitivity of indirect detection of ^{15}N. As proof of concept, one of the strains we utilized for this study was the discoipyrrole A producing *Bacillus hunanensis* strain SNA-048, which we have previously shown to arise through non-enzymatic incorporation of anthranilic acid. Analysis of the ^{1}H – ^{15}N HMBC NMR data for anthranilic acid alone shows correlations from the ^{15}N at δ -311.0 ppm to aromatic protons at d_H at 6.6 and 7.6 ppm (Fig. 10). Analysis of the ethyl acetate extract of SNA-048 displayed new correlations in the ^{1}H – ^{15}N HMBC with dramatic shift of the ^{15}N from δ_N -311.0 ppm to a series of signals at δ_N -292.0, -295.0 and -300.0 ppm. The signal at δ_N -292.0 ppm shows HMBC correlations to aliphatic protons at δ 1.7 and 1.5 ppm, which are indicative of the methylene protons on the isobutyl side-chain of discoipyrrole A.

Evaluation of 4-fluoroaniline as a ^{19}F NMR Probe

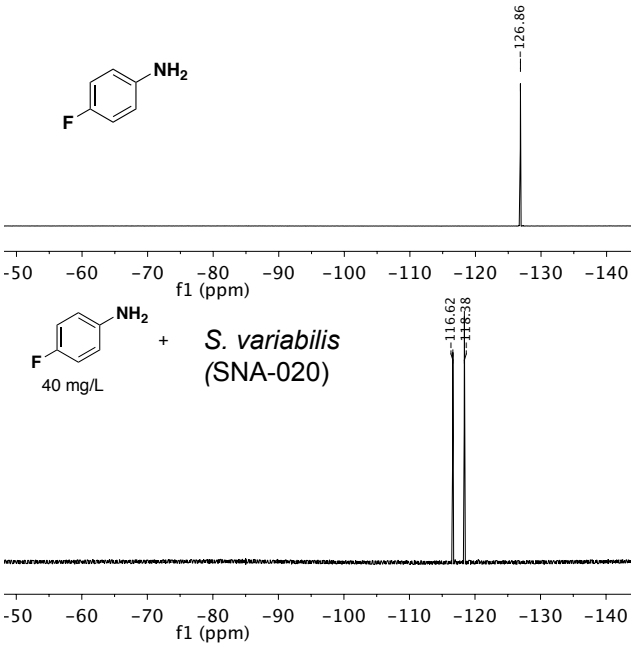


Figure 11. ^{19}F NMR spectrum of **43** (top). ^{19}F NMR spectrum of extracts from SNA-020 fermented with **43** (bottom).

We viewed 4-fluoroaniline (**43**) as the ideal probe for these studies due to the fact that fluorine is extremely rare in natural products, making it likely that any observed ^{19}F signal (s) would be derived from **43** or an incorporated product. Additionally, ^{19}F NMR is nearly as sensitive as ^{1}H NMR (0.83 relative sensitivity) making screening of extracts feasible in a short experiment, allowing for high throughput analysis.

We were unsure if reactions that take place on the nitrogen atom of **43** via non-enzymatic nucleophilic addition would result in a significant NMR shift change of the ^{19}F. Using a similar protocol as described above, a series of microbial cultures were supplemented with **43** (0.5 – 1.0 g/L), placed in shakers for five days, extracted and analyzed by ^{19}F NMR. One of the cultures, from *S. variabilis* strain SNA-020, showed the complete disappearance of **43 (**-126.8 ppm), and gain of two new signals at -116.6 and -118.4 ppm (Fig. 11). ^{19}F NMR guided purification led to the isolation of a novel the iminoquinone (**44**) containing two 4-fluoroaniline moieties.

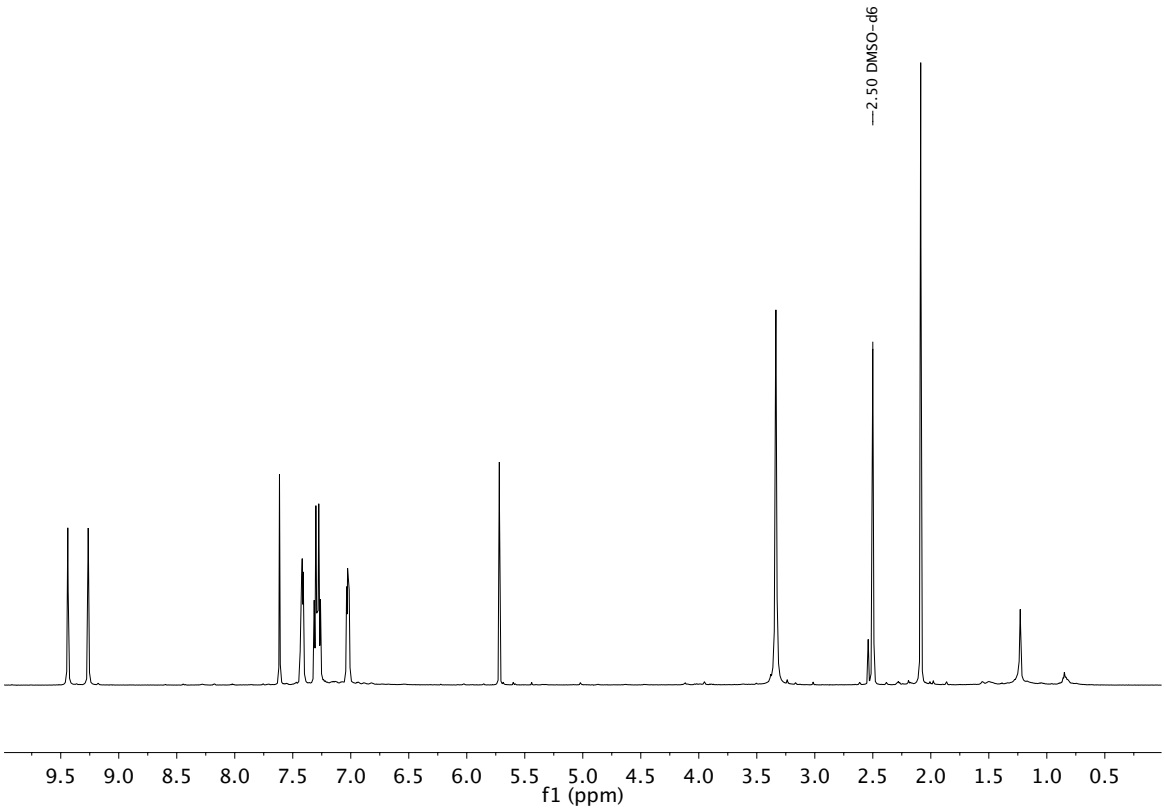


Figure 12. ^{1}H NMR spectrum of **44** collected in DMSO-d6 (600 MHz)

The molecular formula of **44** could be determined as $C_{20}H_{15}F_2N_3O_2$ by HRESIMS at *m/z* 368.1358 $[M + H]^+$ (calcd for $C_{20}H_{15}F_2N_3O_2$). Analysis of the ^{13}C NMR spectrum and HSQC data for **44** (Table 1) revealed nine quaternary carbons (two carbonyl and seven aromatic). The remaining 11 carbon signals were associated with one methyl and 10 aromatic methine carbons.

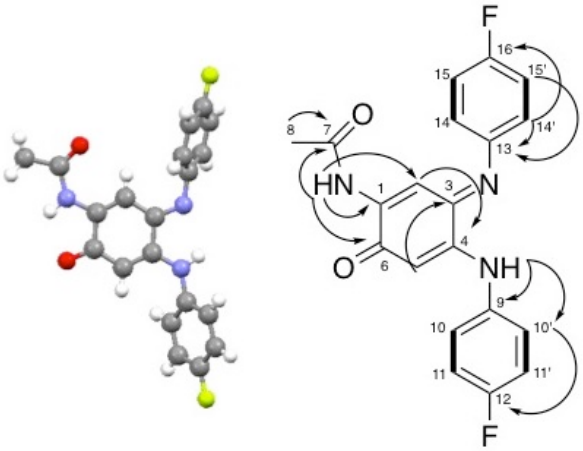


Figure 13. Crystal structure of **44** (left). Structural elucidation of **44** based on HSQC, HMBC (arrows) and COSY (bold lines) experiments (right)

Analysis of the ^{1}H NMR spectrum indicated the incorporation of two 4-

fluoroaniline subunits based on the observed proton resonances at: δ_H 7.42 (2H, dd, J = 8.1), 7.30 (2H, t, J = 8.7), 7.28 (2H, t, J = 6.4) and 7.03 (2H, dd, J = 8.7, 5.1) (Fig 12). This determination was consistent with the two signals observed in ^{19}F NMR spectrum (δ_F 116.62 & 118.38) (Fig. 11). Based on reported ^{13}C-^{19}F one and two bond coupling constants the fluorine substituted quaternary C-12 (δ_C 159.5, J_{CF} = 244.0) and methine C-11 (δ_C 116.1, J_{CF} = 22.3)/ C-11' (δ_C 116.1, J_{CF} = 22.3) carbons were assigned (Fig. 13). COSY correlations between H-11/H-11' (δ_H 7.28, 2H, t, J = 6.4) and H-10/H10' (δ_H 7.42, 2H, dd, J = 8.1) established the C-10/C-10' (δ_C 126.7, J_{CF} =8.2) carbons and was further supported by HMBC correlations from H-10/H-10' to C-12. HMBC correlations from both H-10/H-10' and H-11/H-11' to the C-9 (δ_C 134.5 , J_{CF} = 2.7) implicated this position as the quaternary amino carbon. HMBC correlations to both C-9 and C-10/C-10' from the notably deshielded exchangeable proton δ_H 9.26 (1H, s) confirmed this theory and solidified the first aniline system. Utilizing similar ^{13}C-^{19}F coupling arguments and 2D correlations the second aniline ring (C13-C16) could be assigned.

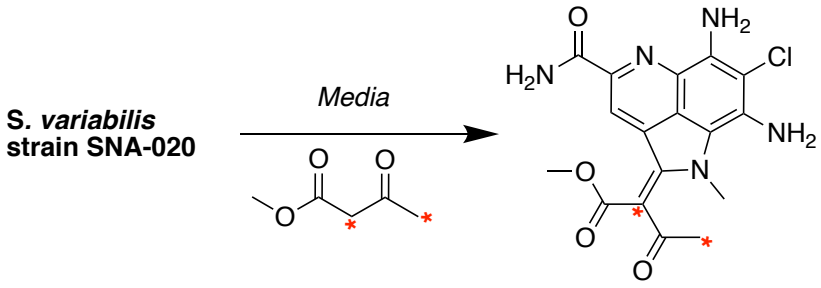
no.	44			
	δH, mult. (J in Hz)	δC (J_{FC} in Hz)	COSY	HMBC
1		137.0, C		
2	7.62, s	103.2, CH		C-4
3		154.8, C		
4		149.3, C		
5	5.72, s	97.0, CH		C-3
6		179.5, C		
7		170.6, C		
8	2.09, s	24.3, CH3		C-7
9		134.5, C, (2.7)		
10	7.42, dd, (8.1)	126.7 CH, (8.2)	H-11	C-12
10'	7.42, dd (8.1)	126.7, CH, (8.2)		
11	7.28, t (6.4)	116.1, CH, (22.3)	H-10	C-9
11'	7.28, t (6.4)	116.1, CH, (22.3)		
12		159.5, C, (244)		
13		145.3, C, (2.7)		
14	7.03, dd (8.7, 5.1)	122.1, CH, (8.2)	H-15	C-13,C16
14'	7.03, dd (8.7, 5.1)	122.1, CH, (8.2)		
15	7.30, t, (8.7)	116.1, CH, (21.3)		
15'	7.30, t, (8.7)	116.1, CH, (21.3)	H-14	C-13
16		159.8, C, (242)		
NH	9.26			C-9, C-10
NH	9.44			C-1, C-2, C-6, C-7

Table 1. Table of 2D NMR data for the structural elucidation of **44** collected in DMSO-d6 (600 MHz). Carbon assignments based on ^{13}C and HSQC spectra.

The core structure of **44** was elucidated based on HMBC correlations from the exchangeable protons at δ_H 9.44 (1H, s) and 9.26 (1H, s) in combination with crystal diffraction data (Fig 13). Observed HMBCs from the amide proton (δ_H 9.44) to both C-1 (δ_C 137.0) and C-2 (δ_C 103.2) aromatic carbons in addition to correlations from this proton to the distinctive carbonyl C-6 (δ_C 179.5) strongly supported the presence of an tri-substituted

iminoquinone core. The structure determined by NMR data was further confirmed by an X-ray crystal structure (Fig. 13).

Evaluation of a ^{13}C Enriched Carbon Nucleophile



(45)

Figure 14. Inoculation of SNA-020 cultures with **42** resulted in the formation of ammosamide analog **45**

In order to explore the utility of carbon based nucelophiles we first sought to synthesis a variety of 1,3 diones, however when none of the tested substrates showed incorporation we decided to utilized 1,1,1-trifluoropentane-2,4-dione. Based on the acceptable ^{19}F NMR shift differentials observed in **44** as compared to **43** we thought that a trifluoromethyl group could serve as a handle for the detection of incorporation. As a proof of principle semi-synthetic ammosamide C was prepared and reacted with 1,1,1-trifluoropentane-2,4-dione. The isolated product ^{19}F NMR spectrum displayed a differential signal δ_f -73.4 ppm as compared to the unreacted substrate at δ_f -76.4 ppm (Fig.19) . When 1,1,1-trifluoropentane-2,4-dione fed into a culture of SNA-020 the resulting extract ^{19}F NMR spectrum showed a signal at δ_f -73.5 ppm – consistent with the synthetic product. However, attempts to isolate this analog resulted in unexpected decomposition.

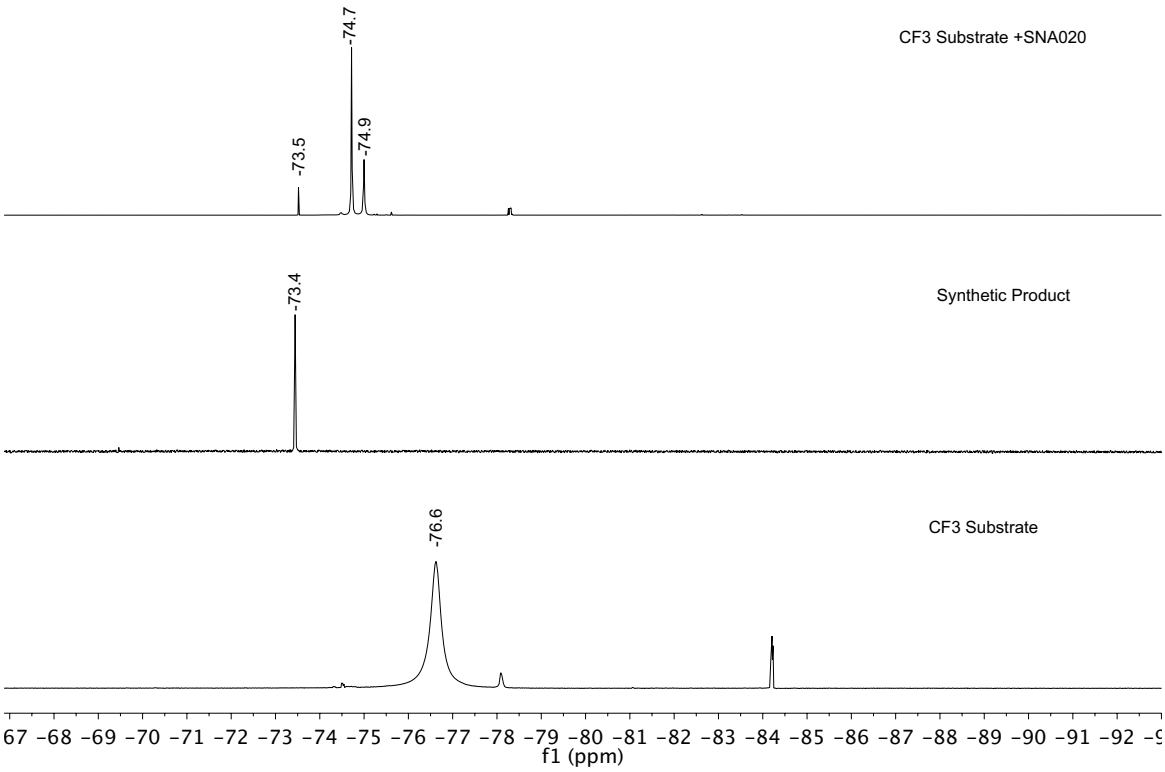


Figure 15. Comparison of ^{19}F NMR spectra of unreacted 1,1,1-trifluoropentane-2,4-dione (CF3), a potential ammosamide analog incorporating 1,1,1-trifluoropentane-2,4-dione and extracts from a SNA-020 culture fermented with 1,1,1-trifluoropentane-2,4-dione.

Encouraged by the potential incorporation of a carbon nucleophile into the ammosamide core we synthesized **42** (^{13}C enrichment at two positions)**. 42** was partly selected based on pKa (15.7) ensuring the presence of a nucleophile (i.e. enolate) un der aqueous fermentation conditions – this observation consistent with the ^{1}H NMR spectrum of **42**). Furthermore, **42** strongly resembles fundamental acyl building blocks utilized in the biosynthesis of various classes of natural products (e.g. PKs, NRPS, and RiPPs) and could potentially serve as a probe for multiple classes of intermediates. **42** was fed into a culture of SNA-020 and extracted following fermentation. LC-UV-MS analysis of the resulting extracts indicated the presence of an ammosamide analog with a $[M+2]^+$ ion at *m/z* 392 consistent with our

predicted product (Fig.16). Due to the low abundance of this analog the feeding experiment was repeated with commercially available non-enriched **42** and the resulting analog was isolated for characterization.

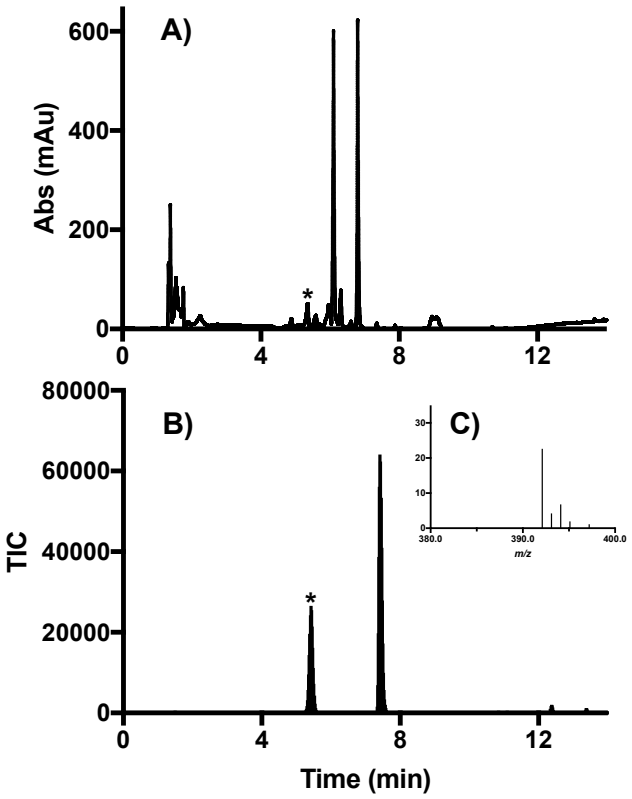


Figure 16. (a) Chromatogram of SNA-020 extracts from **42** fermentation study collected at 254 nm. (b) Ion extraction for $[M+2]^+$ product ion at *m/z* 392. (c) Mass spectrum of ions eluting at (*) peak.

5'(δ_H 3.48, 3H, s) methyl singlets – consistent with the expected resonances of **42** methyl and O-methyl groups. Furthermore, H-1' and H-5'showed

The ^{1}H NMR spectrum of the ammosamide analog **45** displayed three down field singlets at δ_H 8.76, 7.43 and 7.23 (Fig. 18) that were consistent with two aryl amines and amide functional groups in other analogs (Table 2) (Fig. 17). The expected aromatic H-3 proton was assigned to the singlet at δ_H 8.35 based on its attachment to a carbon at δ_C 120.1. The successful incorporation of **42** was first confirmed due to the observed H-1' (δ_H 2.34, 3H, s) and H-

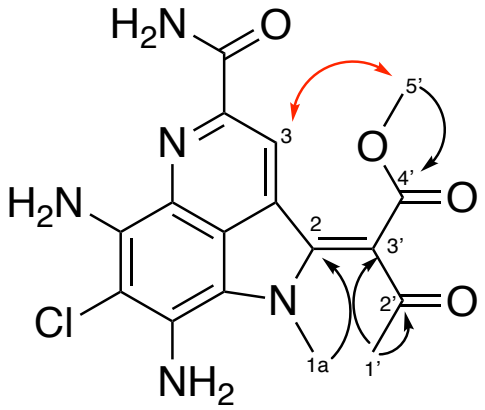


Figure 17. Critial HMBCs for conformation of the incorporation of **42** into the ammosamide core.

HMBC correlations to the ketone C-2' (δ_C 190.0) and ester C-4' (δ_C 168.0) carbonyls respectively. The attachment of this fragment to the ammosamide could then be confirmed based on correlations from H-1a (δ_H 3.73, 3H, s) to C-2 (δ_C 111.0) and H-1' to C-3' (δ_C 90.0). The *E* configuration of **45** was confirmed based on 1D NOE irradiations and observed signal enhancement between H-3 and H-1'.

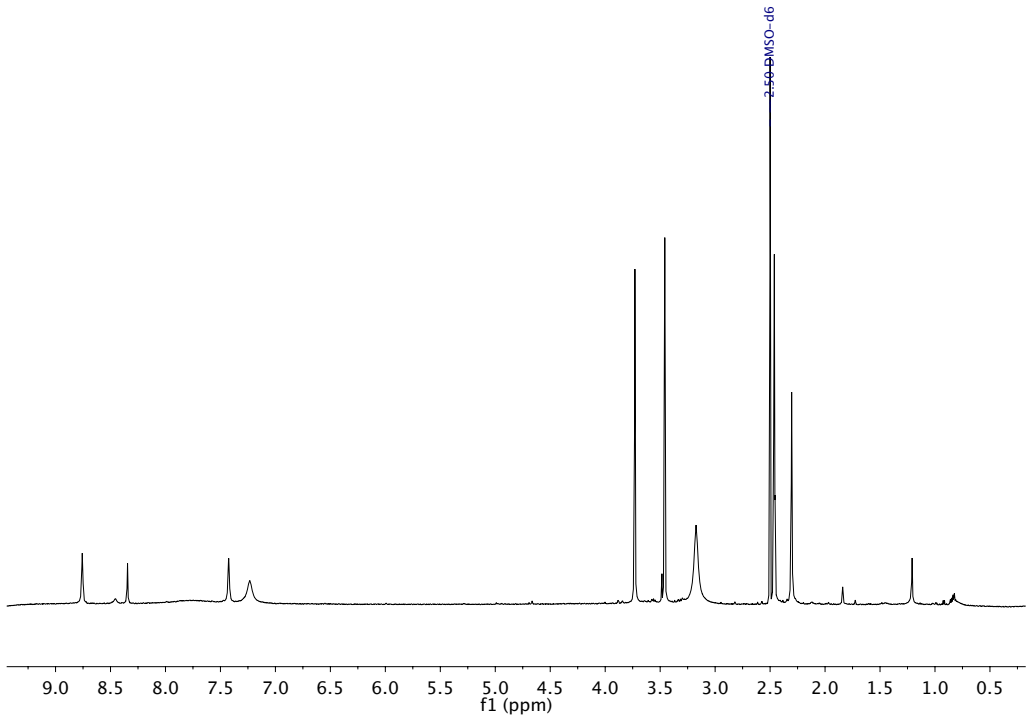


Figure 18. ^{1}H NMR spectrum of **45** collected in DMSO-d6 (600 MHz).

no.	45		
	δH, mult. (J in Hz)	δC	HMBC
1a	3.73, s	37.7, CH3	C-2
1'	2.34, s	29.4, CH3	C-2', C3'
2		111.0, C	
2'		190.0, C	
3	8.37, s	120.1, CH	
3'		90.0, C	
4'		168.0, C	
5'	3.48, s	49.2, CH3	C-4'
NH2	8.76, s		
NH2	7.43, s		
CONH2	7.23, bs		

Table 2. 1 & 2D NMR for the structural elucidation of **45** collected in DMSO-d6 (600 MHz). Carbon assignments were made based on HSQC and HMBC correlations.

EXPERIMENTAL

Isolation of a Novel Iminoquinone (44)

Following the standard culturing procedure a 1 liter culture of SNA-020 was pelleted via centrifugation and the aqueous layer was exhaustively extracted with ethyl acetate. The dried organic layer was adsorbed onto celite via rotary evaporation and purified via automated reverse phase fraction (65 gram C-18; Isco) over a 30 minute gradient from 20 to 100% aqueous acetonitrile. The ^{19}F NMR spectra of pooled fractions were collected and used to guide the further isolation of **44** at each purification step. Pooled fractions were adsorbed onto silica and purified via normal phase automated fractionation (16 gram silica; Isco) over a 30 minute gradient from 10 to 100% ethyl acetate in hexanes. The pooled NMR active fraction was solubilized in acetone and recrystallized over two days via slow evaporation. Collected crystals were submitted for X-Ray crystallography.

Isolation of Ammosamide Analog (45)

A one liter culture of SNA-020 fermented with **42** for 7 days was pelleted via centrifugation and extracted with ethyl acetate. The dried extracts were dry loaded onto celite and purified via reverse phase automatic fractionation (65 gram C-18; Isco) for 30 min over a gradient from 10 to 80% aqueous methanol. The pooled fractions containing **45** were suspended in methanol and purified via preparative reverse phase HPLC (Evo 18 ;C-18) over a 30 min gradient from 10 to 40% aqueous acetonitrile yielding purified **45**.

Synthesis of Methyl 3-oxobutanoate-2,4-$^{13}C_2$

In a oven dried 25 mL round bottom flask 1.0 eq (24.1 mMol) of sodium acetate-2-^{13}C was dissolved into 3.0 eq of dimethyl sulfate (72.3 mMol; 6.9 mL) and was heated to 165 C° over 3 hours. The reaction was dried en vacuo leaving behind pure methyl acetate-2-^{13}C (92% yield). In a dry pressure tubed hexane washed potassium hydride (1.3 eq) was suspended in 44.4 mL of THF (AH) under N_2 atmosphere. The methyl acetate-2-^{13}C product was suspended in THF and added to the pressure tube and reacted at 78 C° over 2 hours. The reaction was allowed to cool and quenched with minium amounts of D.I water. The pH was adjusted to ~6 with 1 N HCl.the aqueous layer was extracted thrice with 50 mL of diethyl ether and the organic layers were dried over magnesium sulfate. The condensed extracts contained pure **42** (37% yield).

Synthesis of 1-phenylbutane-1,3-dione-2-^{13}C

A 50 mL flame dried round bottom flask was charged with 5.0 eq (4.2 mMol) of sodium hydride and placed under N_2 atmosphere. 20 mL of dry THF was added followed by

a solution of acetophenone-^{13}C (1.0 eq; 4.2mMol) diluted in 1.0 mL of dry THF. The reaction was stirred for 30 min to allow for formation of the enolate and 4.0 equivalents of dry ethyl acetate was added to the flask drop wise. The reaction was followed by TLC and quenched with 1N HCl after 1 hour. The reaction was extracted twice with ethyl acetate; the organic layers were then adsorbed onto silica for normal phase automated purification. The desired product (90% yield) was purified over a 20 minute gradient from 10 to 50% ethyl acetate in hexanes on a 12 gram silica column.

Synthesis of N,N-dimethyl-3-oxobutanamide-N-^{15}N

In a dry pressure tube 1.0 eq (1.4 mMol) of $H_2{}^{15}NMe_2$ • HCl salt, 1.0 eq (1.4 mMol) of 2,2-dimethyl-4*H*-1,3-dioxin-4-one and 1.0 eq of triethyl amine were dissolved in 4 mL of MeC under argon atmosphere. The tube was fitted with a pressure cap and microwaved for 10 min at 180 °C. The reaction was then adsorbed onto celite for automated reverse phase purification. The desired product (60% yield) was isolated on a 65 gram C-18 column via a 25 min gradient from 10 to 100 percent aqueous methanol.

Microbial Sources, Cultivation and Characterization

In our proof of principle studies MacMillan Lab two soil microbe isolates were selected for precursor fermentation studies based on their inclusion of non-enzymatic chemistry in their biosynthesis of secondary metabolites (i.e. ammosamides & discolpyrrole). Strain SNA-048 was derived from a sediment sample collected at Galveston Bay, TX and selected using humic acid based media. The marine derived bacterium, strain SNA020, was isolated from a sediment sample collected at a depth of 1 meter near the prop roots of a mangrove tree in

Sweetings Cay, Bahamas (N 26º 33'27", W 77º 51'15''). Isolates were cultivated from sediments dried for 24 hours in an incubator at 35 ºC and the resulting sediment stamped onto a solid A1 media plate A1 media composed of: starch (10 g/L), peptone (3g/L), yeast extract (4 g/L), H2O and containing rifampicin (10 mg/L) and cycloheximide (50 mg/L). A colony of SNA-020 was selected from the plate after four weeks and re-streaked on a new A1 agar plate. SNA-048 and SNA-020 were phylogenetically characterized based on PCR (16S rRNA; FC27 & RC1492 primers) analysis of extracted genomic DNA. The resulting partial sequences from SNA-048 and SNA-020 were compared to online databases using Basic Logistical Alignment Search and determined to be *Bacillus hunanensis* and *Streptomycese variabilis* respectively.

Microbial culturing for Feeding Studies

Microbial isolates were cultured in 20 x 2.8 L Fernbach flasks or 150 mL Erlenmeyer flask containing 1 L or 50 mL (respectively) of a seawater based medium (10 g starch, 4 g yeast extract, 2 g peptone, 1 g CaCO3, 40 mg Fe2(SO4)3•4H2O, 100mg KBr). Experimentally, strains were allowed to grow to reach stationary phase (1-4 days) or initially dosed with 100 – 1000 mg/L of isotopically labeled substrate as a solution in DMSO and placed on a shaker at 200 rpm for an additional 2-6 days. Cultures were subsequently pelleted via centrifugation, extracted with ethyl acetate and analyzed by NMR spectroscopy.

DISCUSSION

Overall, we believe this is a promising approach to identify natural product scaffolds that have reactive, electrophilic intermediates that can be harnessed to generate molecular diversity, and if active, to rapidly carry out structure activity studies. Our initial surveys into this chemistry have focused on methods of detection and the types of nucleophiles that are amenable to use in fermentation media. There are still a number of questions that need to be addressed about the broader applicability of this approach. We will seek to further explore the limit of detection for incorporation of NMR active probes as this will have in impact on the throughput of analyzed strains. Additionally, we will further explore the fermentation scale (i.e. volume of bacterial fermentation) necessary for integration with high-throughput biological screening platforms.

In order to best optimize the scale ability of this technology one must consider the cost of synthesizing or purchasing the probe of choice and the limits of detection for the isotope of interest. Synthetic **42** was prepared from (2-^{13}C)acetate via Claisen condensation resulting in yields of 37% at the cost of $147/gram. However, given the limited sensitivity of ^{13}C NMR the target intermediate would have to be produced in high concentrations and incorporate a significant amount of the probed in order to show a definitive signal. The application of ^{15}N-anthranilic acid was also promising given the wide spectral range of the ^{15}N spectrum and the observed differential due to incorporation into DP. While ^{15}N NMR is notably insensitive we were able to take advantage of proton detected 2D NMR ^{15}N-^{1}H HMBC pulse sequences to detect shift changes. The primary evaluation of **41** as an ^{15}N probe was the relatively high cost at $935/gram. Our initial experiments with 4-fluoroaniline as a

^{19}F based probe were promising given that unbiased detection of probe incorporated products be detected and fractionated via NMR. Furthermore, 4-fluoroaniline was notably inexpensive costing only $0.66/gram making this substrate a promising tool for future studies.

Fermentation scale is a point of consideration that impacts both the feasibility of high-throughput screens and the economy of enriched materials (i.e. cost per fermentation). In our studies as a proof of principle 1 liter cultures were used at substrate concentrations of ~ 1 g/L. While larger scale fermentations do result in higher yields the limitations of fermentation space, the use of large quantities of solvent for extractions and high substrate economy make 50 mL scale experiments more attractive. Smaller scale feeding studies would require a probe enabling detection even at low yields – either by virtue of natural abundance or specialized pulse sequences. The feasibility coupling of 50 mL scale fermentations with an easily detected (i.e. ^{19}F containing substrates) and cost effective probe will be further explored.

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