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Aflatoxin and Deconstruction of Type I, Iterative Polyketide Synthase Function

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Introduction

In late 1969 I became one of the few Americans in Ian Scott's research group then recently arrived at Yale from the UK. I was the first graduate student to work on a new challenge to study the biosynthesis of vitamin B₁₂. All around me was the intense and main group effort in indole alkaloid biosynthesis and lesser-known investigations of fungal polyketides such as 6-methylsalicylic acid (6-MSA), patulin and tropolones. In a group meeting I learned about a complex and dazzlingly rearranged family of fungal toxins, the aflatoxins. I was inspired by the brilliant series of papers by George Büchi and his group describing elucidation of the structures, their total synthesis and, finally, in 1968 a *tour-de-force* in classical radiochemical incorporation and systematic degradation of aflatoxin B₁ (**1**) to determine the sites of carbon label from acetate (Scheme 1).^{1, 2}

At around this time the first commercial FT-NMR spectrometers became available and we were able to benefit from this time saving technical leap in our investigation of vitamin B₁₂. It was soon recognized³ that ¹³C-enriched carbons could be more than simply mapped from precursor to product and, indeed, unprecedented insight could be gained in “bond labeling,” or “paired isotope,” experiments (¹³C–¹³C, ¹³C–²H, ¹³C–¹⁸O, ¹³C–¹⁵N) to monitor polyketide folding patterns, carbon skeleton rearrangements, atom shifts, changes in oxidation state, hydrogen, oxygen or nitrogen retention or loss using ¹³C as a reporter nucleus. I still regard the review in NPR by John Vederas as the seminal reference for these methods.⁴ As a graduate student, we connected chlorophyll biosynthesis to vitamin B₁₂ through the last common intermediate, uroporphyrinogen III,⁵ as well as from methionine.⁶ I left the States to take up an International Exchange Postdoctoral Fellowship for further study with Duilio Arigoni at the ETH in Zürich. There I carried out a synthesis of chiral methyl groups using two linked electrocyclic reactions⁷ and showed that the seven “extra” methyl groups introduced on the corrin macrocycle occurred by intact transfer from [¹³C,²H₃]methionine in an early application of paired isotope methods.⁸

Upon arriving at Hopkins in 1976, the bicentennial year, I resolved to tackle the aflatoxin problem with synthetic precursor molecules labeled with stable isotopes to ask specific mechanistic questions. This work has been reviewed^{9, 10} and only a few selected studies will be briefly summarized here. I should say at the outset we were greatly helped along the way by a series of mutants blocked in the aflatoxin biosynthetic pathway that had been generated by Joan Bennett (then at Tulane) and Louise Lee (USDA, New Orleans),¹¹ which were used

by Pieter Steyn and his group at the CSIR in South Africa and others to map the incorporation of doubly ^{13}C -labelled acetate into each known metabolite.¹² John and Tom also contributed to this problem.^{13–16} The anthraquinone and xanthone pigments that accumulated in these blocked mutants could be ordered on paper and subsequently by experiment to reveal first creation of the characteristic dihydrobisfuran present in versicolorin A (**4**) from averufin (**3**), and, second, what turned out to be the complex cleavage of **4** to first the xanthone **5**, two ordered *O*-methylations to **6** and **7**, and, finally, oxidative ring scission, rearrangement and decarboxylation to the coumarin core of aflatoxin B₁ (**1**) itself (Scheme 1).

The Role of Cations in Oxidative Rearrangements

A set of selectively labeled averufin probes was synthesized to examine dihydrobisfuran formation as summarized in Scheme 2. The starting two carbons (circled, Scheme 2A) are lost (ultimately as acetate) and the oxidation state of C1' changes from that of an alcohol to an aldehyde (Scheme 2B). Hence, the chain branching step is oxidative. In a critical experiment ^{18}O label at C1' (*) remains bonded to C5' and emerges uniquely as the carbonyl oxygen in versiconal acetate (**8**, Scheme 2C).¹⁷ In Scheme 2D a mechanism was proposed to account for these observations in which rearrangement is initiated by oxidation at C2'. In a further constraint, however, neither of the C2' alcohols, nidurfin (**9**) or pseudonidurufin (**10**), is active in the biosynthesis.¹⁷ Therefore, a radical or a cation generated at C2' *during* the oxidation is invoked for this key step. Each of these potential rearrangement pathways was examined in chemical model studies. Among several corroborating experiments, the 6,8-dimethoxy-2'-iodo-averufin diastereomers **11** and **12** were prepared. The *endo*-iodide **12** (X = I) whose migrating and departing groups are held orthogonal to each other under either cation-generating or radical-generating conditions gave no chain-branched products. In contrast, when *exo*-iodide **11** (X = I) was treated with silver trifluoroacetate, ready transformation to 6,8-dimethoxyversicolorone (**13**) took place. In a telling extension of this experiment, when the more nucleophilic silver acetate was used, in addition to chain-branched products, the proposed intermediate of 1,2-migration in Scheme 2D was stereospecifically trapped as **14**. Now, when the *exo*-iodide **11** (X = I) was treated under a series of radical-generating conditions, only reduction (**11**, X = H) or trapping of the radical by oxygen and reduction (**11**, X = OH) was observed.^{18, 19} With all the caveats of model systems, we take the observation of carbon skeleton rearrangement as evidence for a cation intermediate whether by electron transfer or other means. Nidurufin (**9**) is a shunt product of normal oxidation. The role of cations in cytochrome P450-catalyzed reactions remains controversial.^{20, 21} Stereochemical and radical clock experiments can be cited in their favor as I believe this and other natural product oxidative rearrangement steps will further support.

Oxidative Aryl Activation/De aromatization

From the late 1980's it would require additional studies with isotopically-labelled precursors coupled critically with experiments using a new cell-free extract (CFE) protocol to make progress on the transformation of versicolorin A (**4**) to demethylsterigmatocystin (DMST, **5**). The final skeletal reorganization of *O*-methylsterigmatocystin (OMST, **7**) to aflatoxin B₁

(1) appears to be catalyzed by a single P450.²² While we have made progress with this remarkable multistep process, it remains unsolved in its important respects.²³

In this period purified Ver-1 (AflM) was shown to set the absolute stereochemistry of the dihydrobisfuran ring, which is essential for the carcinogenic effects of aflatoxin to be manifest as a DNA-damaging agent.²⁴ By reverse genetics techniques its encoding gene was found in the emerging aflatoxin cluster.²⁵ In a timely and important advance for us, it was discovered how to produce a CFE from *Aspergillus parasiticus* that could reconstitute the *entire* pathway from the first stable intermediate, norsolorinic acid (2, Scheme 1), to aflatoxin B₁ (1).²⁶ Diafiltration was key in which small molecules and soluble cofactors could be removed to provide a clean background for biochemical experiments. Among the many run with this useful system was the conversion of versicolorin A (4) to OMST (7) in D₂O and d₇-glucose. Unexpectedly high levels of deuterium incorporation were observed in the A-ring of the isolated OMST (Scheme 3A. C8, 18%; C9, 64%; C10, 45%; all $\pm$ 3%).²⁶ This puzzling result could be rationalized finally several years later in an unanticipated sequence of *oxidation–reduction–oxidation* reactions as illustrated in Scheme 3B.²⁷ This proposed strategy of epoxidative activation or oxidative dearomatization more generally has been recognized in close parallels in other related natural products.^{28–31}

Hexanoyl Starter

Contemporaneous with the radiochemical incorporation experiments of Büchi, Holker examined the labeling pattern from [1-¹⁴C]acetate in sterigmatocystin (6), but found in the two bisfuran sites that the extent of labeling was *ca.* 10% lower than the xanthone core (*cf.* Scheme 1). At the time this discordant observation could not be explained and remained a haunting loose end.³² Thinking many years later that Holker's finding might be an unprecedented example of a hexanoyl starter unit, we tested the incorporation of [1-¹³C]hexanoate into averufin (3) in a whole-cell experiment. We fully expected rapid β -oxidation of this short fatty acid and trivial secondary incorporation as weakly labeled acetate. Astonishingly a 3–4% specific incorporation was, in fact, found superimposed on a roughly 0.5%/site random incorporation as acetate.³³ Later as the *N*-acetylcysteamine thioester (SNAC), hexanoate gave a impressive 22% specific incorporation.³⁴ While highly suggestive, these results in themselves did not strictly prove a hexanoyl starter. Isolation of the aflatoxin gene cluster and the adjacent encoding genes for HexA, HexB and PksA (having no reductase or dehydratase domains) made its role as the starter clear. In a technically difficult experiment, we were able to partially purify and characterize the HexA/HexB/PksA $\alpha_2\beta_2\gamma_2$ complex (*ca.* 1.3 kDa) capable of synthesizing norsolorinic acid anthrone.³⁵

Rediscovery of Non-Reducing PKSs

In contrast to the luminous successes taking place with modular PKS systems (see Viewpoint of John Vederas), beginning in the early 1990's we entered a long, dark period attempting to express PksA in a variety of hosts with the intention to study the full-length protein in much the way 6-MSAS, the classical paradigm of fungal PKSs, had been studied.³⁶ A decade later we were utterly unsuccessful, apart from the heightened

excitement provided by the CFE characterization of the HexA/B•PksA complex noted above. But so too came the cold realization that, even if we could obtain *holo*-PksA in useful amounts, it was questionable we could progress beyond what was known from the heroic experiments with 6-MSAS. In truth, fungal iterative PKS remained poorly understood, but not for lack of effort. Progress was blocked by high technical hurdles and the fundamental, and perhaps not fully appreciated, experimental limitations imposed by iterative catalysis itself. Help was about to arrive in a completely unexpected way.

At that time we were also struggling with two nonribosomal peptide synthetases (NRPSs). We had great difficulty expressing soluble, functional domains for biochemical analysis. Being thwarted at the bench on two fronts stimulated a creative period that resulted in the adenylation (A) domain prediction protocol for amino acid substrates selectively activated by NRPSs.³⁷ Shortly thereafter the Udwy–Merski Algorithm (UMA) was formulated, which combines conventional sequence alignment with secondary structure prediction and local relative hydrophobicity to give a score that correlates to compactly folded domains or intervening linker regions. While the operating assumptions are simple, the output has proved powerful.^{38, 39}

When UMA was applied to PksA, it partitioned the protein into six distinct domains (the ACP is less well-defined because some comparison sequences contain tandem ACP domains) while up to this instant sequence alignments identified only four: a β -ketoacylsynthase (KS), malonylacyl transferase (MAT), acyl-carrier protein (ACP) and a TE (Fig. 1). But there was also an anomalously long *N*-terminal domain and a second apparent domain inserted between the MAT and ACP. Were these structural, or did they have function? We speculated at the time based solely on the perceived need to control otherwise indiscriminant reactivity of a hypothetical poly- β -ketone intermediate that the new internal domain was a “product template” (PT) domain.³⁸ Unlike modular or “assembly line” synthesis where the linear process can be interrupted or altered and the resulting truncated products identified, iterative catalysis uses and re-uses a smaller set of catalytic domains in a generally defined, or “programmed,” manner to create a specific product. But with no free intermediates, 20 or more reactions take place in a “black box” that become individually invisible in the complete PKS. Inactivation of a domain during iterative catalysis stops *all* synthesis and next to nothing can be learned. A different strategy was required to deduce the functions, if any, of the two newly recognized domains and of the enzyme as a whole. We elected a systematic dissection and reconstitution approach to examine domains individually, pairwise to *n* at a time, or “deconstruction.”^{40, 41}

Patient variation of cut sites guided by UMA soon yielded soluble, functional mono-, di- and tridomains from PksA and other NR-PKSs at a high success rate. A decade of failed experiments in yeast and fungal expression systems was easily overcome in *E. coli* to give catalytically autonomous parts that reassembled in solution to faithfully recapitulate native synthetic function. First, the unusually long *N*-terminal domain was found to be a “starter unit transacylase” (SAT) domain and the origin of the classically observed “starter unit effect,”^{42, 43} that ordinarily brings acetyl CoA onto the PKS to initiate fungal polyketide elongation, or, as in the case of PksA, an allied specialized FAS transfers a hexanoyl unit to the SAT.⁴⁴ It is now recognized that other PKSs can load the SAT of an NR-PKS as well.⁴⁵

By the summer of 2006 PksA had been fully deconstructed in preliminary experiments and the division of labor among SAT, PT and KS, MAT, ACP and the TE was understood. These findings were first reported at the memorable inaugural “Directing Biosynthesis” meeting organized by the late Joe Spencer in Cambridge that September. Seizing on the flood of new genome sequence information in fungi, Russell Cox codified domain identities and organization into what we commonly refer to now as non-reducing (NR-PKS), partially-reducing (PR-PKS) and highly-reducing (HR-PKS).⁴⁶ The field was exploding.

Behaviour and Engineering of NR-PKSs

In collaboration with Neil Kelleher’s lab, some key insights into NR-PKS function were made. First, during PksA catalysis the steady-state population of the ACP contains hexanoyl starter units, malonyl extenders (and acetyl, presumably by decarboxylation; MS artifact?). In lesser detectable amounts were ions corresponding to the fully mature polyketide (I believe the first direct observation of the fundamental poly- β -ketone intermediate proposed by Birch,⁴⁷ if not before; Scheme 4A) and to one and two dehydrations, which almost certainly reflect one and two cyclizations as shown in Scheme 4B.⁴⁰ Although the PT domain does predestine the polyketide intermediate to a particular cyclization, the product itself is formed in the TE domain in a final thio-Claisen or Dieckmann condensation. In retrospect the “product template” domain should perhaps be more correctly called a “cyclization template”, or CT domain!

In deeper analysis with the Kelleher group, as before we observed no intermediate length polyketide chain extension products could be detected, even under conditions contrived to induce their accumulation. Polyketide elongation is extremely rapid. This makes intuitive sense since the intermediate is pluripotent in its reactivity and the role of the PT/CT domain to capture and limit that intrinsic reactivity is essential to successful overall synthesis of a discrete product. All domains are maintained in a virtually empty state (in marked contrast to modular PKSs⁴⁸) while the MAT domain is saturated with malonyl extender units. This observation also makes sense to sustain highly processive synthesis. There appears to exist communication/cooperativity among the KS and MAT, and the SAT domains where KS is held in a vacant state until loaded with the correct starter at which point polyketide extension is swift.⁴⁹

With Sheryl Tsai and members of her group we have been able to obtain informative high-resolution crystal structures of the PksA PT and TE domains, the two cyclization catalysts.^{50, 51} Palmitate fortuitously was bound in the large reaction chamber of the PT placing 16 precyclization-like carbons into the deepest hydrophobic extreme of the active site and extending out to the ring-forming machinery itself. Using these 16 carbons as a guide to binding the mature 20-carbon PksA polyketide intermediate, molecular modeling and energy minimization afforded a remarkable picture of dual hydrogen bonds to localized water molecules in the protein to activate the electrophilic carbonyl regiospecifically for cyclization/aromatization and provide a rationale of two successive ring formations.

The innate ability of iterative domain active sites to accommodate intermediates of widely varying size suggests they might be especially amenable for modified or engineered

synthesis. From a matrix of six NR-PKSs enzyme components we have found that ACPs are substantially interchangeable; maintaining SAT–KS pairs is beneficial to overall synthetic efficiency; chain length is largely, but not exclusively, determined by the KS with occasional participation by the PT; PT active sites are unexpectedly accommodating with respect to the chain lengths of the polyketide intermediates presented to them, yet the regiochemistry of their cyclizations is tightly controlled; and, as noted above, the TE is crucial to success. Enzyme-directed cyclizations must outpace spontaneous reaction and TE-mediated editing,^{52, 53} but “rules” have emerged from these studies where rapid combinatorial experiments can be used to quickly survey potential heterodomain combinations for assembly into single PKS chimeras for more efficient synthesis.⁵³

Outlook

Astonishing progress has been made in the last decade to understand iterative, Type I polyketide synthesis with major contributions by my fellow Viewpoint authors. Structural information by EM and X-ray diffraction will figure prominently in the future. But in these highly mobile macromolecular machines, dynamics information from mechanistic and kinetic analyses will also be essential to that understanding beyond static structural information. Both of these realms of investigation will inform attempts to engineer these sophisticated catalysts for synthetic purposes and address the largely unsolved issue of “programming.” We have already seen in MS experiments that domain•domain interactions, some surely triggered by the presence or absence of substrate or intermediate, underlie the rates of internal events. These subtle but important effects to overall synthesis and fidelity are fertile ground for biophysical measurements. Finally the importance of higher order protein complexes with auxiliary enzymes to programming is little explored. Compare, for example, the role of the MbtH superfamily to A domain activation in NRPSs.⁵⁴

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Figure 1.
UMA treatment of PksA

Scheme 1.

Scheme 2.