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REVIEW

Mode of action and biosynthesis of the azabicycle-containing natural products azinomycin and ficellomycin

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Only a handful of aziridine-containing natural products have been identified out of the more than 100,000 natural products characterized to date. Among this class of compounds, only the azinomycins (azinomycin A and B) and ficellomycin contain an unusual 1-azabicyclo[3.1.0]hexane ring system, which has been reported to be the reason for the DNA crosslinking abilities and cytotoxicity of these metabolites. Both families of natural products are produced by *Streptomyces* species, *Streptomyces sahachiroi* and *Streptomyces ficellus*, respectively. Up until recently, much of the work on these molecules has focused on the synthesis of these natural products or their corresponding analogs for *in vitro* investigations evaluating their DNA selectivity. While one of the most intriguing aspects of these natural products is their biosynthesis, progress made in this area was largely impeded by difficulties with obtaining a reliable culture method and securing a consistent source of these natural products. In this review, we will cover the discovery and biological activity of the azinomycins, their mode of action, related synthetic analogs and biosynthesis, and finish with a discussion on the less studied metabolite, ficellomycin.

1 Introduction

Since the early discovery of the beneficial effects of nitrogen mustards in the early 1900s, followed by cisplatin [$PtCl_2(NH_3)_2$] in the 1960s, and their subsequent clinical success, DNA alkylating agents have attracted considerable interest as potential cancer therapeutics.[1–7] The azinomycins and ficellomycin, for example, represent two closely related families of compounds, each possessing a 1-azabicyclo[3.1.0]hexane ring system, which has been reported to be the reason for the DNA crosslinking abilities and cytotoxicity of these metabolites. The azinomycins have an added electrophilic group, an epoxide moiety that also contributes toward its ability to crosslink DNA. Significant effort has been made to evaluate the mode of action of these compounds and their synthetic derivatives, particularly in the case of the azinomycins. While understanding of the biosynthesis of these agents has lagged considerably, it is fortuitous that both of these agents are produced by culturable and genetically amenable strains of bacteria, which not only facilitates the functional characterization of the natural product pathways, but also lends promise for subsequent genetic manipulation in the design of chemotherapeutic agents with enhanced stability and tumor selectivity that can be cheaply produced through fermentation.

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2 Discovery and biological activity of the azinomycins

Azinomycin B (Fig. 1) was originally isolated from *Streptomyces sahachiroi* in 1954 as carzinophilin.[8] It was re-isolated along with azinomycin A from *S. griseofuscus* in 1986[9] and found to exhibit potent *in vitro* cytotoxic activity and *in vivo* antitumor effects.[10] The molecular weights of azinomycins A, **1** and B, **2** were determined as 593 and 623, corresponding to molecular formulas of $C_{30}H_{33}N_3O_{10}$ and $C_{31}H_{33}N_3O_{11}$, respectively.[11] The fermentation broth of *S. griseofuscus* produces additional metabolites, **3**, **4** and **5**, each lacking the 1-azabicyclo[3.1.0] ring system. While it was originally concluded that these metabolites lacked anti-bacterial or anti-tumor activity,[11] further studies by Terashima and co-workers showed that naphthoate epoxyamide **3** exhibited a strong cytotoxic effect [IC_{50} = 0.0036 μg mL^{-1}] against a P388 murine leukemia cell line.[12] However, **3** showed weak activity *in vivo* against P388 leukemia. In a related study, the azinomycins showed potent cytotoxic activity against the leukemia cell line L5178Y (IC_{50} = 0.07 μg mL^{-1} for **1** and 0.11 μg mL^{-1} for **2**).[10] Azinomycin B showed strong anti-tumor activity against murine transplantable tumors with an effective range of 2–32 μg kg^{-1}. A maximum value of 32 μg kg^{-1} showed an increased life span (ILS) of 193%, which is comparable to mitomycin C that showed a 204% ILS at 1 mg kg^{-1} day^{-1} in P388 leukemia. Azinomycin A, on the other hand, showed antitumor activity against murine tumors but exhibited lower potency and a narrower antitumor spectrum than azinomycin B. An early-phase clinical investigation with azinomycin B gave favorable results in 36 cases of malignant neoplasms. Remarkable efficacy was observed in a case of squamous cell carcinoma (a form of skin cancer). Local administration of the agent led to rapid reduction of the ulcer surface and eventual disappearance of the tumor cells.[13]

The azinomycins have also shown bioactivity against a range of Gram-negative and Gram-positive bacteria. An antibacterial agar diffusion assay demonstrated that azinomycin A is more effective than azinomycin B.[9] Azinomycin A gave a minimum inhibitory concentration (MIC) of 6.25 μg mL^{-1}, compared to azinomycin B with an MIC of 50 μg mL^{-1} against some *S. aureus* strains. The MIC values were equivalent for a range of other microbial strains except in *Klebsiella pneumonia* ATCC 10031, where azinomycin A exhibited an MIC of 3.13 μg mL^{-1}

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Jennifer Foulke-Abel grew up in Texas, where she earned a B.A. in chemistry from Texas A&M University. She continued at Texas A&M and completed her Ph.D. in biological chemistry with Professor Watanabe, studying natural product biosynthesis and bacterial drug resistance mechanisms. In November 2010, she became a postdoctoral research fellow in the laboratory of Professor Craig Townsend at The Johns Hopkins University in Baltimore, MD.

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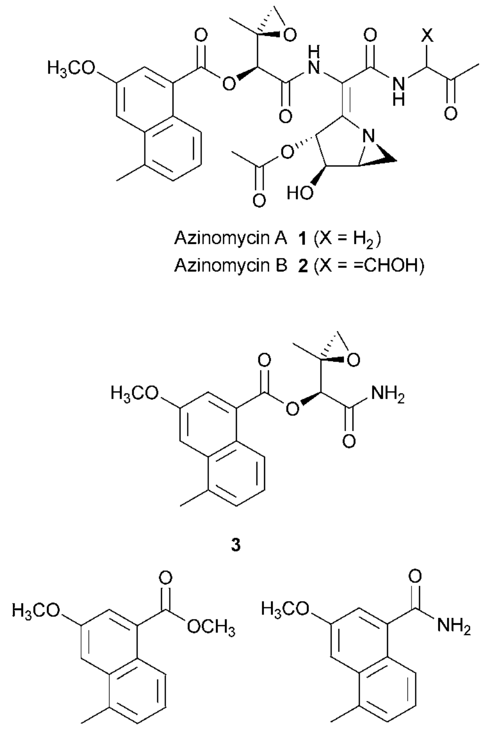
Shogo Mori was born and raised in Nagoya, Japan. He obtained a B.S. degree in Chemistry in 2008 from Jacksonville State University in Alabama. He then entered the graduate program in chemistry at Texas A&M University and began studying with Professor Coran Watanabe. His research interests reside in functional characterization of biosynthetic proteins and identification of putative immunosuppressive and anti-metastatic agents from marine extracts.

Coran Watanabe

Coran Watanabe was raised in Mililani on the island of Oahu, Hawaii. She received a B.S. in Chemistry from the University of Hawaii, Manoa, and obtained her Ph.D. degree with Professor Craig Townsend at The Johns Hopkins University. Her post-doctoral studies were carried out with Professor Peter G. Schultz, initially at the University of California, Berkeley, and subsequently at The Scripps Research Institute. She joined the faculty at Texas A&M University in 2002 as an assistant professor of chemistry, and was promoted to the rank of associate professor, with tenure, in 2008.



Azinomycin A **1** (X = H_2)
Azinomycin B **2** (X = =CHOH)

3

4 **5**

Fig. 1 Structures of azinomycin and related compounds isolated from *S. sahachiroi* and *S. griseofuscus*.

as opposed to azinomycin B with an MIC of 50 μg mL^{-1}. While it was initially reported that these natural products are inactive against yeast and fungi,[9] further studies by our group demonstrated the DNA-damaging effect of azinomycin B against yeast.[14] A number of investigations have begun to address the molecular mechanism of action of these natural products and will be discussed in the next section. Early investigations were hindered by the limited availability and poor chemical stabilities of the azinomycins. The high reactivity of the heterocycles in these molecules hindered early efforts to complete the total synthesis of azinomycin A, and total synthesis of azinomycin B has yet to be achieved. Since compound **3** exhibits strong cytotoxic activity against P388 murine leukemia,[12] it is thought to contain the necessary elements for cytotoxicity, acting as potent DNA alkylating agents, which has inspired a range of synthetic analogues.

3 Mode of action of the azinomycins and related synthetic analogs

Synthetic strategies and early mode-of-action studies on the azinomycins and their derivatives have been reviewed by Hodgkinson and Shipman.[15] A large number of activity studies commenced since this initial review. We, therefore, provide further examination of contributions dedicated to understanding the role of this intriguing antitumor natural product and its structurally and functionally related analogs.

3.1 Mode of action: Specificity towards DNA

In 1966, Terawaki and Greenberg first noted that azinomycin B preferentially inhibited DNA synthesis over RNA or protein synthesis in *Escherichia coli* B_0.[16] The DNA isolated from *E. coli* and *Bacillus subtilis* cultures treated with azinomycin B could not be irreversibly denatured and yielded fewer transformants,[17] indicating formation of interstrand cross-links (ISCs). Lown and Majumdar further investigated the ISC capabilities of azinomycin B by monitoring the change in ethidium fluorescence of treated DNA.[18] After heating, covalently cross-linked DNA exhibited greater ethidium fluorescence than untreated DNA, due to cross-links serving as annealing initiation sites. They also found that the presence of intercalated dye prior to azinomycin treatment inhibited cross-linking. The low pH-dependence of cross-linking and alkylation propensity suggested the DNA-reactive centers of azinomycin B were basic species requiring protonation to become actively electrophilic. The acidic environment available in tumor cells is a condition postulated to provide the observed specificity of azinomycin B in model systems.[8]

Azinomycin B was predicted to react selectively with guanine bases based on the ratio of crosslinking among DNA samples with varying G + C content,[18] but this was not confirmed until Armstrong and coworkers demonstrated alkylation at G residues of ^{32}P-end-labeled synthetic oligonucleotides.[19] Treatment of DNA with azinomycin B followed by piperidine cleavage of alkylated sites and denaturing polyacrylamide gel electrophoresis yielded scission fragments of bis-alkylated product. This corresponded to reaction at 5′-guanine bases involving a template-driven reaction at residues two base pairs away on the complementary DNA strand (Fig. 2A). Confirmation of guanine-N7 selectivity was achieved by replacement with 7-deazaguanine and inosine (Fig. 2B). Preference for 5′G placement indicated that azinomycin B reacts within the major groove of DNA.

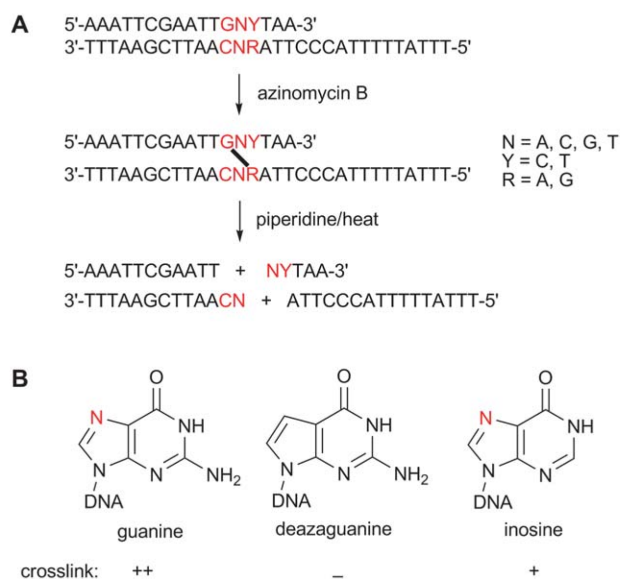


Fig. 2 DNA alkylation and strand breakage elicited by azinomycin: (A) Model oligonucleotides used to monitor cross-linking *via* depurination and base cleavage, (B) DNA base-pair substitutions and corresponding effect on cross-linking behavior.

Details on the cross-link chemistry of azinomycin B was provided by Saito *et al.* The self-complementary deoxy-oligonucleotide d(TAGCTA)$_2$ incubated with a 4-*O*-methyl-azinomycin derivative gave both monoalkylated and cross-linked product, implicating the aziridine and epoxide moieties in DNA alkylation.[20] An HPLC timecourse analysis of the reaction detected monoalkylation at the aziridine moiety but not at the epoxide. Monoalkylated azinomycin could be quickly converted to cross-linked material, indicating that the second reaction with the epoxide C21 is facilitated by initial alkylation at the aziridine C10, and the process is a two-step mechanism. Heating forced depurination, resulting in structures **6** and **7**, identified by HPLC purification and ion-spray mass spectrometry (Fig. 3). ^{1}H NMR and MS suggested the third species isolated after the abasic oligonucleotides eluted was the adenine-azinomycin-guanine adduct **8**.

Alcaro and Coleman optimized computational parameters to indicate that azinomycin A and B are preorganized for DNA binding and cross-linking.[21] Use of the modified AMBER* force field, coupled with results of Monte Carlo simulations based upon the monoalkylation and interstrand cross-linking work of Saito *et al.*, they found optimal interactions consistent with the specific DNA sequence cross-linking model.[22] Regarding sequence specificity, it was noted that thymine-based nucleobase triplets appeared to inhibit reactivity due to the steric effect of the C5-methyl group.[23]

The utility of a gel shift assay to assess DNA cross-linking was demonstrated by Hartley and co-workers using plasmid DNA and epoxyaziridine analogs as compared to cisplatin.[24] The Coleman group extended the methodology using azinomycin B to reveal a strong correlation between sequence nucleophilicity and ISC, defining an optimal recognition sequence of 5′-d(GCC)-3′ embedded in a 15-mer DNA duplex. Cross-link yields in excess of 90% were observed, which led to the conclusion that azinomycin targets 5′-purine residues situated two base pairs apart on complementary DNA strands.[25] An epoxyaziridine azinomycin analog **9** tested against a set of 15-mer DNA duplexes yielded a strikingly similar cross-linking preference (Fig. 4).[26] Sequence selectivity was shown to be dependent upon the stereochemistry of the epoxide in synthetic partial structures of the azinomycins. Studies examining the four epoxyamide stereoisomers yielded cross-linking and cytotoxic activity for only the natural (2*S*,3*S*)- and enantiomeric (2*R*,3*R*)-epoxyamides **10** and **11**.[27–29] The (2*R*,3*R*) enantiomer was further found to alkylate G residues at a 3′G rather than the usual 5′G favored by azinomycin, representing a completely inverted interaction.[28] Computational models were in agreement with the experimental outcomes. Despite variation in sequence selectivities, the enantiomers were found to have relatively similar cytotoxicities.[27,28]

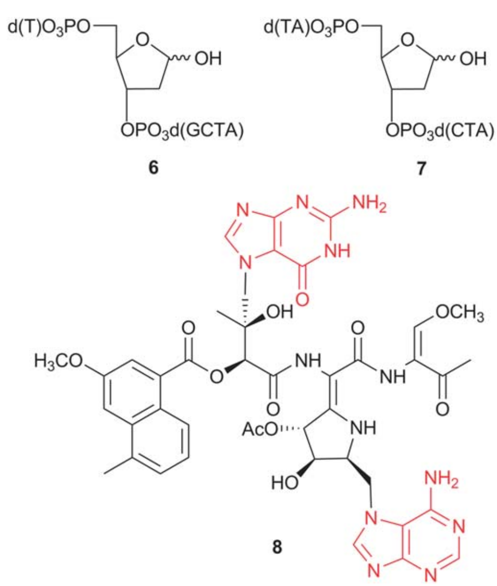


Fig. 3 Products generated as a result of heating and depurination of azinomycin-treated DNA.

Evaluation of the cellular effects of azinomycin B *in vivo* was shown in a series of assays employing *Saccharomyces cerevisiae* (Watanabe *et al.*).[14] Nuclear localization was observed by treating cells with azinomycin B, utilizing innate naphthoate fluorescence as a marker. In addition, the DNA shearing effects caused by azinomycin B bisalkylation and subsequent double-strand breakage were evident in yeast genomic DNA isolated from treated cells as opposed to control cells. GeneChip analysis of treated yeast further reflected the DNA targeting ability of the natural product as exhibited by the upregulation of DNA synthesis, repair and double-strand breakage response genes (Fig. 5A). Fluorescence-activated cell sorting of yeast treated with azinomycin B indicated a dominant shift to the S phase and a decrease in the G2/M phase population at concentrations of 10 and 100 μg mL^{-1} (Fig. 5B). The azinomycin epoxyamide, which is believed to function as a DNA monoalkylator, was recently shown to induce ASK1 and CASP3 activation toward commencement of apoptosis in THP-1 human myeloid leukemia cells.[30]

3.2 Role of non-covalent binding substituents (naphthoate)

Non-covalent interactions between azinomycin and duplex DNA have been evaluated to identify key structural determinants important for azinomycin activity. Studies have primarily focused on the effect of the naphthalene (presence/substitution) residue on DNA binding and cytotoxicity. Zang and Gates studied the effect of epoxide-containing truncated analog **10** on DNA binding and alkylation. Significant reactivity was retained, but DNA binding of non-epoxy analogs **12** and **13** directed by the naphthalene ring showed weak affinity (Fig. 6).[31] In

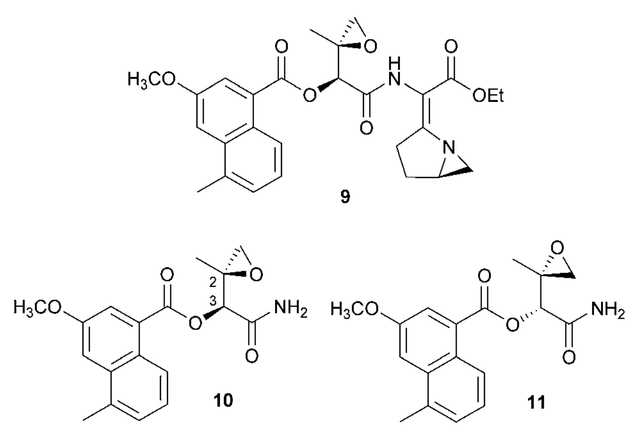


Fig. 4 Structures of epoxyamide analogs.

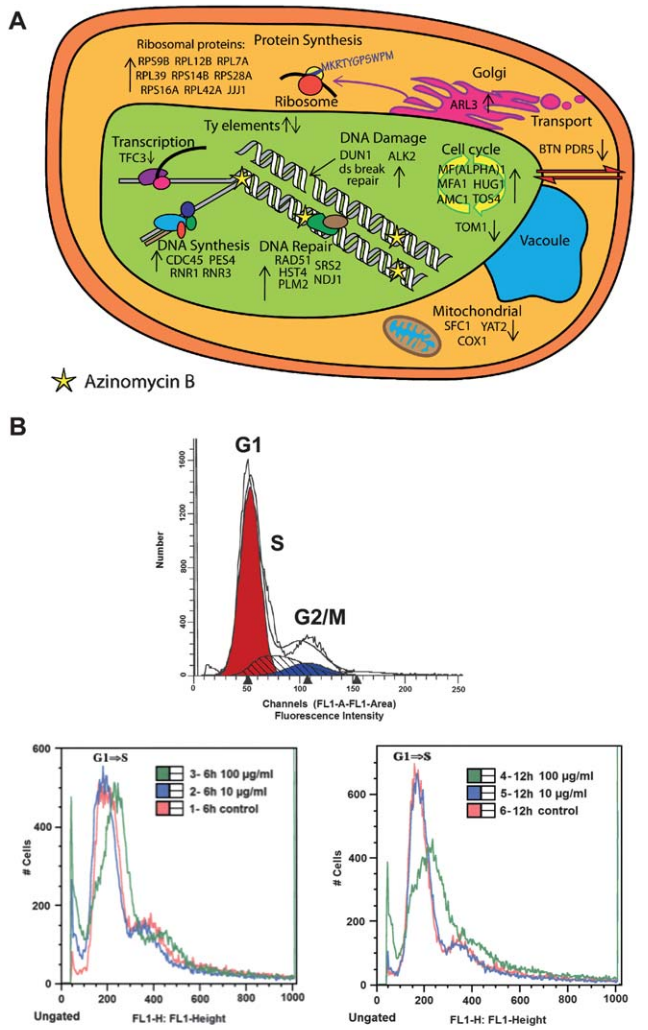
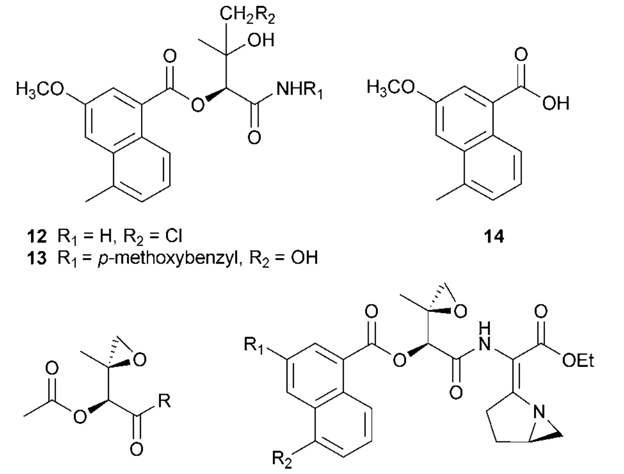


Fig. 5 Transcriptional and cell cycle effects exhibited by azinomycin B treatment: (A) Schematic overview summarizing Affymetrix GeneChip profile results; (B) Cell cycle effects: The top panel represents distribution of wild-type yeast cells, the bottom left panel shows the cell cycle profile of yeast cells treated with 0, 10, or 100 μg mL^{-1} of azinomycin B at 6 h, and the bottom right panel shows the cell cycle profile of yeast cells treated with 0, 10, or 100 μg mL^{-1} of azinomycin B at 12 h.

a subsequent study by Coleman and co-workers, the isolated naphthoate group **14** failed to effectively intercalate calf thymus DNA as measured by viscometry.[25] Yet epoxides **15** and **16** lacking the naphthoate moiety also failed to react with DNA, and were found to be biologically inactive.[32] The result of removing the C5′-methyl **17**, C3′-methyl ester **18**, or a combination of the two **19** from the naphthalene ring indicated that the C5′-methyl substituent of an azinomycin analog is essential for DNA association and subsequent ISC.[33]

Hodgkinson and coworkers found that simply substituting an ethoxy group at the methoxy position on the naphthalene ring reduces cytotoxicity by an average of 25-fold.[27] Complete replacement of the naphthalene ring with the DNA intercalator 2-quinoxaloyl abolished cytotoxic activity, but this may be attributed to poor uptake associated with low lipophilicity, a factor guiding the future utility of naphthoate replacement.



12 R_1 = H, R_2 = Cl
13 R_1 = *p*-methoxybenzyl, R_2 = OH
14

15 R = OBn
16 R = NH_2

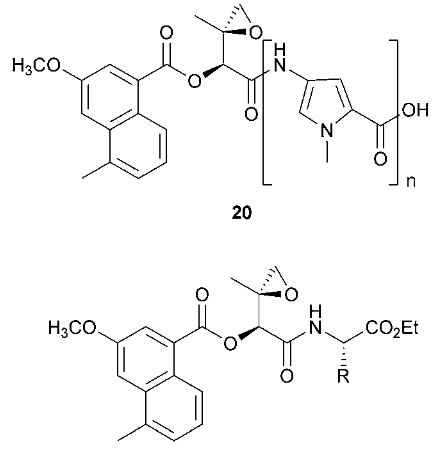
17 R_1 = OCH_3 R_2 = H
18 R_1 = H R_2 = CH_3
19 R_1 = H R_2 = H

Fig. 6 Synthetic analogs to evaluate the role of the naphthoate moiety on DNA binding.

3.3 Activity of epoxyamide, azabicycles, and related structural analogs

In addition to manipulations on the naphthoate structure, analog studies have concentrated on varying alkylating groups and linkers attached to a conserved naphthoate scaffold. These studies were largely inspired by the related metabolite epoxyamide **10**, also isolated from azinomycin-producing *Streptomyces* strains,[11] which exhibits DNA-alkylating and cytotoxic behaviors.[12,25] One of the first molecules was an epoxyamide–lexitropsin hybrid **20** that exhibited *in vitro* DNA-cleaving ability (Fig. 7).[34] Additional structures with an epoxyamide core and extended amide side chain **21** and **22** were observed to restore diminished cytotoxicity compared to the natural epoxyamide.[27]

Less attention has been paid to structures containing exclusively the azabicyclo[3.1.0]hexane ring, in part because of inherent instability. Miyashita and coworkers synthesized several 3,4-epoxypiperidine derivatives, *e.g.* **23–24**, which are postulated



20

21 R = H
22 R = $CH(CH_3)_2$

Fig. 7 Synthetic analogs utilizing a naphthoate epoxyamide scaffold.

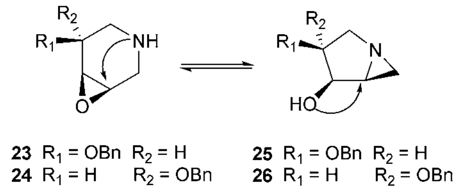


Fig. 8 Synthetic epoxypiperidine derivatives and their corresponding azabicycles.

to spontaneously undergo intramolecular substitution to form azabicycles **25–26** (Fig. 8).[35] Although testing revealed some activity in a DNA relaxation assay, the compounds were much less potent than other DNA alkylators. Numerous other azabicycle-containing substructures of azinomycin exhibited similar behavior in cell-based assays.[12,36,37] Ring-opening was cited as a major drawback to analogs based on the aziridinopyrrolidine system.

Epoxyaziridine analogs are especially attractive due to bisalkylating activity that is not afforded by simple epoxyamide structures. A nitrogen mustard analog **27** on a naphthamide core exhibited stable *in vitro* DNA unwinding as assessed by gel migration of supercoiled X174 DNA, but lacked the cytotoxicity of the epoxyamide (Fig. 9).[38] This is possibly due to poor uptake of the charged piperidine-mustard relative to the neutral epoxyamide. The nitrogen mustard concept was extended to the synthesis of a nitrogen mustard epoxyamide **28**, which gave appreciable DNA cross-linking behavior but remained a poor cytotoxic alternative to the natural epoxyamide.[39] Hashimoto and colleagues synthesized a C10 mesylate azinomycin analog **29** that proved especially effective against P338 murine leukemia (IC_{50} of 0.0032 μg mL^{-1}), but elicited general health deterioration in a mouse model of Sarcoma 180.[12] An additional analog **30** from the same study, containing benzyl substitution at C12 and C13, also showed promise as a more potent therapeutic. However, SDEM modeling suggested that loss of the right-hand side-chain negatively impacts anchoring of the drug for binding within the DNA major groove, and thus may not employ the expected mode of action against cells.[40] Congeners **31** and **32** lacking only the right-hand side-chain tested against P388 murine leukemia displayed toxicity levels (IC_{50} of 2.3 ng mL^{-1} and 3.6 ng mL^{-1} respectively) similar to that of the clinical anthracycline drug adriamycin (IC_{50} of 2 ng mL^{-1}). Multiple component condensation strategies applied toward generation of functional azinomycin analogues[41] gave three structures with simplified aziridine functionalities **33–35**. *In vitro* cytotoxicities against HCT116 human colon carcinoma were only 5-fold less than azinomycin B. Complete removal of the aziridinopyrrolidine moiety to yield "top-half" structures **36–37** of the azinomycins has been reported, but the substructures have yet to be tested in DNA alkylation experiments.[42]

A slightly different approach toward the use of engineered structures inspired by the azinomycins involves symmetrical dimers of the naphthalene–epoxyamide fragment. The bisepoxides feature linkers of either alkyl chain or rigid aromatic character (Fig. 10). The effects of such analogs are varied as measured by DNA cross-linking and cytotoxicity screens.[43–45] While aromatic linkers such as 1,3- and 1,4-diaminobenzene **38** and **39** provided modest ISC and *in vitro* cytotoxicities,[45] more desirable outcomes were observed using flexible tethers. In particular, a three-atom linker containing a central methylamino group **40** displayed enhanced cross-linking abilities of 2–4-fold more than all flexible alkyl linkers observed, and overall superiority in average cytotoxicity against 60 cancer cell lines.[44]

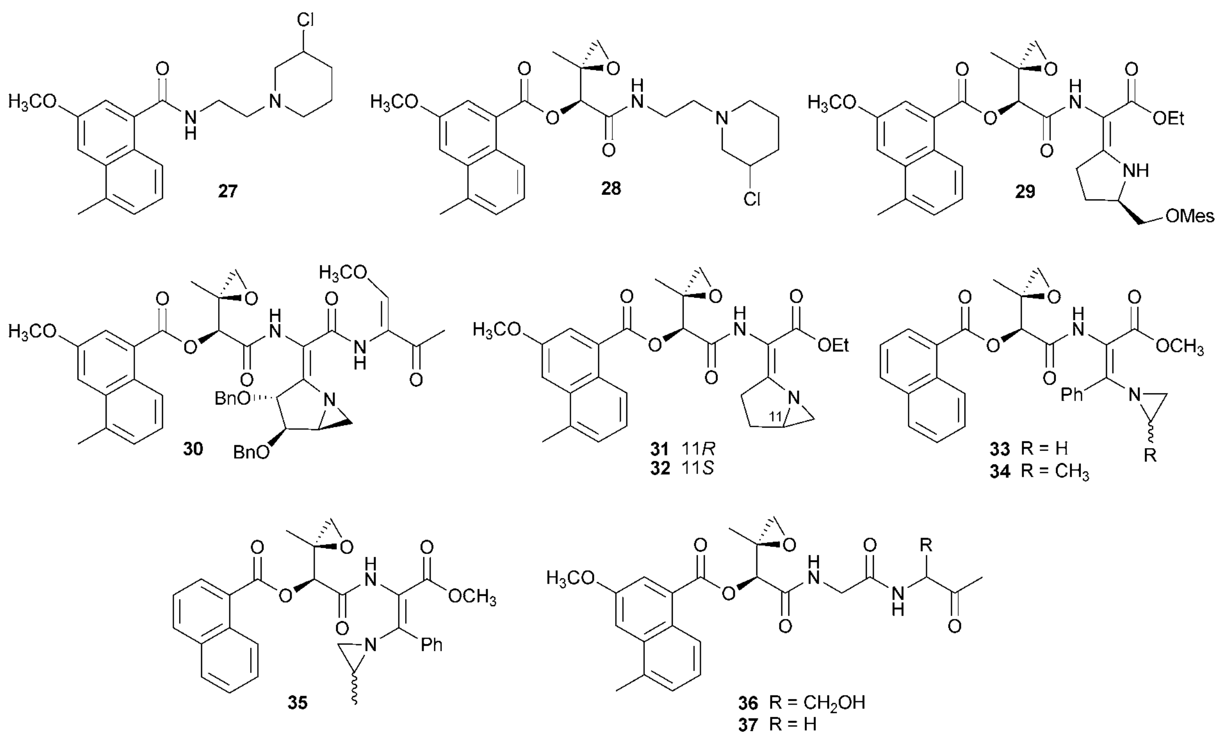


Fig. 9 Synthetic naphthoate derivatives incorporating nitrogen mustards and other DNA reactive groups.

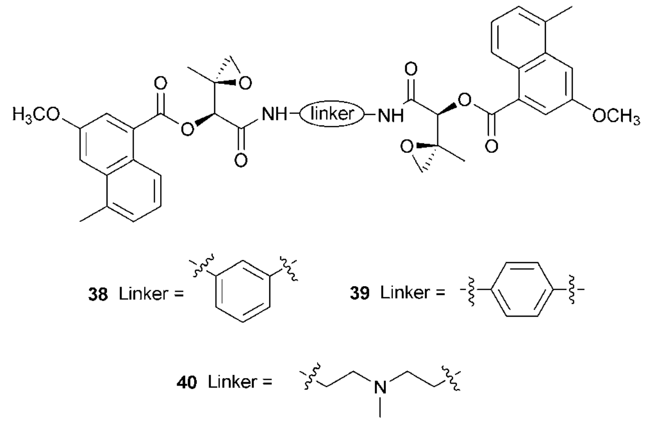


Fig. 10 Dimeric epoxides linked by spacers of varying alkyl or aromatic character.

Valuable information has been gleaned from azinomycin B mode of action and substructure analysis. SAR (structure activity relationship) studies on azinomycin and its structurally and functionally related agents to identify compounds with enhanced relative stability, cell membrane permeability, and selective DNA targeting ability bring us closer to understanding the tenets of the antitumor behavior of azinomycin B, and how to best exploit this knowledge in the quest for innovative treatments.

4 Biosynthesis of the azinomycins

The biosynthesis of the azinomycins is a fascinating unsolved problem. The structures of azinomycin A and B (Fig. 11) suggest a hybrid biosynthetic origin. The naphthoate would conceivably be polyketide-synthase-derived, the aziridine alkaloid-derived, while the skeletal backbone of the molecule including the epoxide and enol portions of the molecule would be predicted to arise from condensation of amino-acid-like substrates orchestrated by modular NRPS enzymes. A variety of biosynthetic enzymes for tailoring reactions, *e.g.*, oxidation, reduction, methylation and acetylation are also likely to participate.

The polyketide origin of the molecule was initially confirmed through isotopic-labeling experiments as detected by enhanced ^{13}C-NMR resonance spectroscopy by Lowden *et al.*[46] Exogenous feeding of [1-^{13}C]-, [2-^{13}C]-, or [1,2-^{13}C]-acetate to cultures of *S. sahachiroi* resulted in incorporation of label within the azinomycin naphthoate (Fig. 12A), a result consistent with polyketide synthase-mediated biosynthesis derived from activated acetyl and malonyl-CoA units. Feeding of doubly-enriched acetate gave the expected incorporation of label and coupling pattern

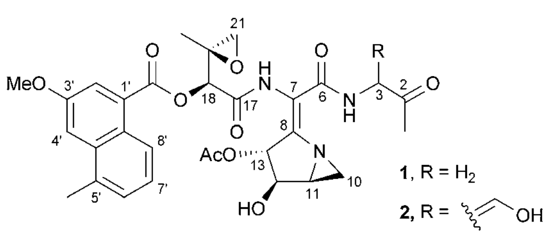


Fig. 11 Structures of azinomycins A and B with numbered atom positions.

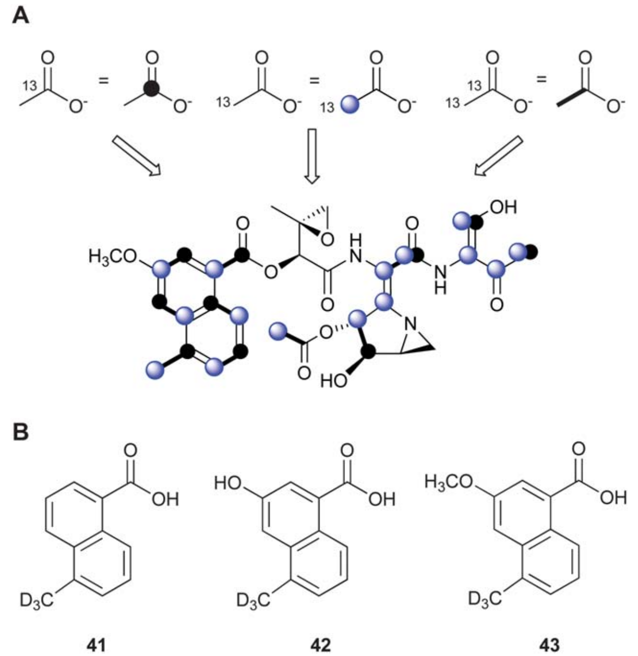


Fig. 12 Isotopic labeling studies performed on the azinomycin naphthoate: (A) Schematic diagram illustrating isotopic labeling patterns observed with [1-^{13}C]-, [2-^{13}C]-, or [1,2-^{13}C]-acetate to cultures of *S. sahachiroi*; (B) Structures of deuterated naphthoic acids.

within the acetyl decoration at C13, supporting the involvement of acetyl-CoA. Additional incorporation due to metabolic scrambling of acetate in the Krebs cycle showed up in C1–C4 of the keto-enol chain, which was proposed to be derived from threonine, which is in turn derived from oxaloacetate. Coupled labeling also appeared between the pairs C6–C7 and C12–C13, again attributed to scrambling of acetate to form α-ketoglutarate, the precursor of glutamate, glutamine, arginine, and proline, any of which would give conceivable building blocks of the azabicyclo[3.1.0]hexane ring. Supplying the culture medium with synthetic deuterated naphthoic acids **41–43** demonstrated significant uptake of all three C3′ variants (Fig. 12B) into the azinomycin structure as detected by ^{2}H-NMR, consistent with the naphthoate being fully functionalized prior to introduction to the rest of the molecule.[47]

Full reconstitution of the azinomycin biosynthetic pathway was achieved with cell-free extracts of *S. sahachiroi*. The method enabled identification of the substrates and cofactors needed to support biosynthesis of the natural product (Watanabe *et al.*).[48] Sensitivity of the assay was achieved by use of radioactive precursors to track the relative amount of azinomycin B produced by the cell-free system. Cofactors NADPH and SAM were found essential for biosynthesis. Cerulenin, an inhibitor of FAS/PKS enzymes, reduced naphthoate synthesis. Cytochrome P450 oxidases were targeted using miconazole, metyrapone, and chloramphenicol, eliciting decreased production of both naphthoate and azinomycin B. Incubations with ^{14}C-labeled amino acid building blocks demonstrated reasonable incorporation of valine, threonine, glycine, and ornithine, consistent with proposed biosynthetic origins based on structure analysis.

In our view, a major obstacle preventing more detailed analysis of the pathway (*e.g.* extensive isotopic labeling studies or genetic studies) was erratic production of azinomycin B by *S. sahachiroi* following available literature procedures. Significant effort was thus invested to establish an optimal fermentation system capable of providing consistent azinomycin B production. Final conditions implemented use of dehydrated agar plates for "stressed" spore production, a two-stage starter culture, and subsequent fermentation with minimal medium (Watanabe *et al.*).[49] Optimized culture conditions enabled investigation of the origin of the enol or terminal fragment of azinomycin B with labeled threonine and other synthesized substrates (Fig. 13). Supplementation of the culture with [U-^{13}C]-threonine produced azinomycin B, site-specifically labeled at the C1–C4 positions. Additional postulated enol intermediates (β-ketoamino acid **44**, β-hydroxyamino aldehyde **45**, and β-ketoaminoaldehyde **46**) were each synthesized in ^{13}C-labeled form and evaluated to identify the most advanced precursor to be accepted by the NRPS biosynthetic machinery. All three of the more advanced precursors failed to incorporate above background. This is suggestive that threonine is the substrate accessed by an adenylation domain for initial incorporation, and undergoes subsequent oxidation and reduction steps (of which the specific order is unknown) to provide the final keto–enol moiety of azinomycin B (Watanabe *et al.*).

Azinomycin A, in contrast, a metabolite produced in minute quantities by *S. sahachiroi*, lacks the enol fragment, leaving a terminal ketoamide. Thus, we suspected that the origin of azinomycin A C1–C3 arose from aminoacetone **50**, a metabolic precursor derived from either glycine **47** or threonine **48** *via* action of a 2-amino-3-ketobutyrate coenzyme A ligase or L-threonine 3-dehydrogenase, followed by spontaneous decarboxylation (Fig. 14). Cultures provided universally-labeled threonine, [1-^{13}C]- or [2-^{13}C]-glycine or [2-^{13}C]-aminoacetone were compared for relative incorporation into azinomycin A. While minimal threonine- and glycine-derived ^{13}C-labels were observed in azinomycin A, aminoacetone was incorporated to a significant extent, 26.3% (Watanabe *et al.*).[50] A draft genome sequence of *S. sahachiroi* by our lab has also been obtained, revealing homologs of the enzymes necessary for aminoacetone synthesis.[50]

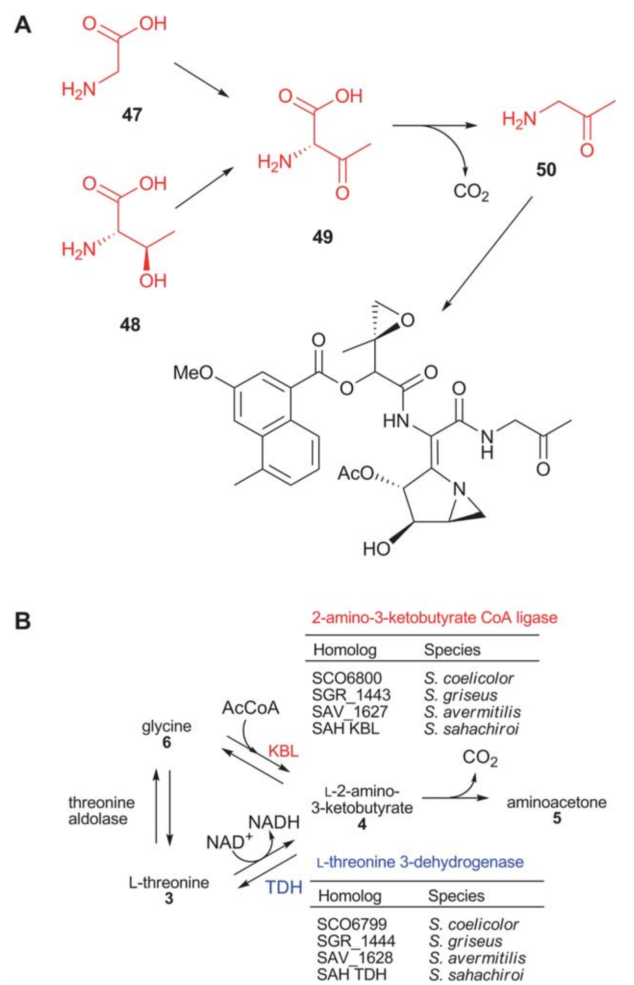


Fig. 14 Illustration of the molecular origin of azinomycin A: (A) Proposed biosynthetic route for the formation of aminoacetone and its subsequent site-specific incorporation into azinomycin A; (B) Biosynthetic pathways facilitating conversion of glycine and threonine to L-2-amino-3-ketobutyrate, which undergoes decarboxylation to give aminoacetone.

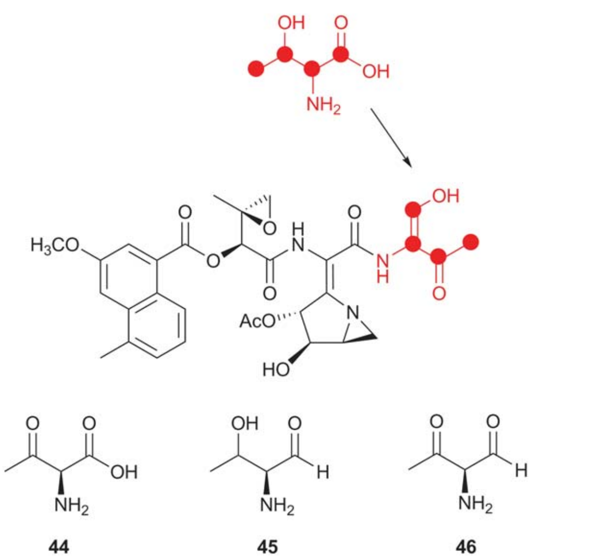


Fig. 13 Illustration of the biosynthetic origin of the enol fragment of azinomycin B including the labeling pattern observed with [U-^{13}C]-threonine and the structures of other tested synthetic analogues.

Evidence of a bifurcated pathway for azinomycin A and B biosynthesis modulated by the relative availability of precursors was indicated by unlabeled substrate feeding of aminoacetone, which provided an increased amount of azinomycin A relative to azinomycin B (Watanabe *et al.*).[50]

A series of ^{13}C-labeled epoxyvaline precursors were utilized to study the biosynthetic timing of valine incorporation in azinomycin and the related metabolite naphthoate epoxyamide. These studies revealed that much of the valine tailoring occurs prior to being loaded onto the NRPS machinery. Of the nine [1-^{13}C]-valine derivatives examined, incorporation levels suggest a pathway that begins with oxidation of L-valine **51** to γ-hydroxyvaline **52**, followed by transamination to give an α-keto acid **53** and dehydration to form 3-methyl-2-oxobutenoic acid **54** (Fig. 15, Watanabe *et al.*).[51] The exact timing of epoxidation is unclear as the instability of epoxide precursors in aqueous media

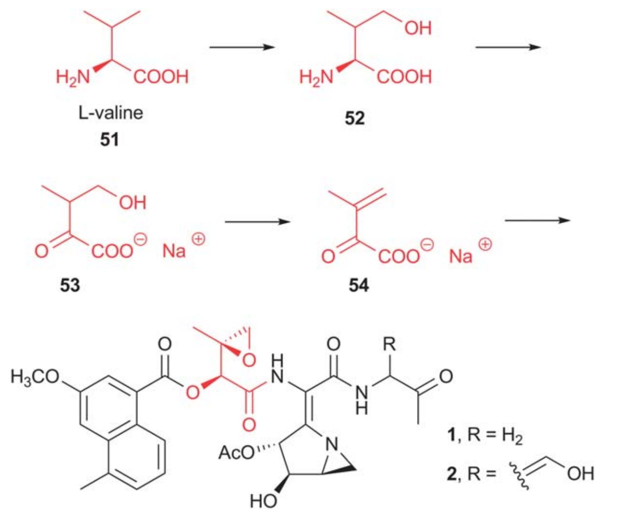


Fig. 15 Evaluation of the molecular origin of the epoxide unit: compounds **48–51** were each site-specifically incorporated into the azinomycin epoxide.

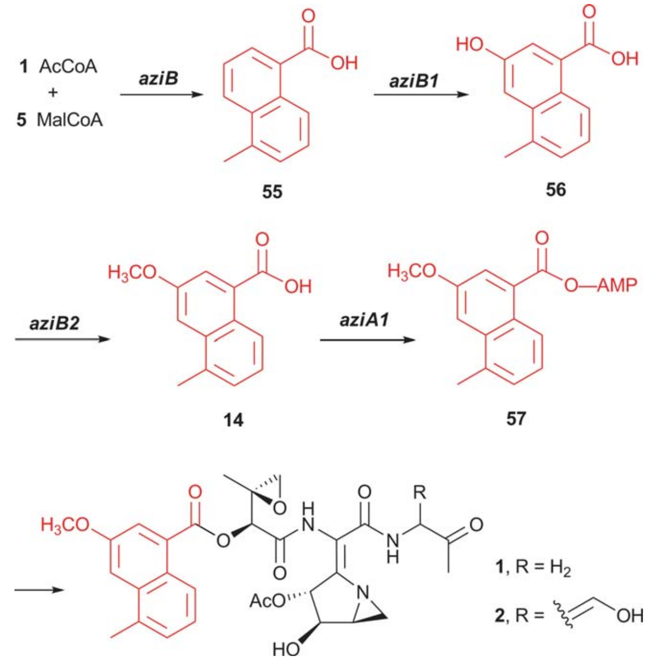


Fig. 16 Functional assignment of azinomycin pathway genes: *aziB*, *aziB1*, *aziB2*, *aziA1*.

may have contributed to the lack of observed incorporation in feeding experiments.

PCR screening of an *S. sahachiroi* genomic library enabled identification of a type I iterative PKS capable of naphthoic acid (NPA) production and a proposed azinomycin B biosynthetic gene cluster by Zhao and co-workers.[52] The results are consistent with our genome sequencing data and gene disruption experiments, with possible ambiguity regarding the specific ends of the gene cluster. Genome sequences were generated by a combination of Solexa/Illumina sequencing and 454 pyrosequencing which resulted in ~2 billion total base calls, corresponding to 32.7 million total reads. Sequence assembly was achieved with Velvet in collaboration with Andrew Su's bioinformatics group at GNF (Genomics Institute of the Novartis Research Foundation). Bioinformatic analysis provided annotation of genes encoding proteins associated with the naphthoate PKS, three NRPS modules, two multi-domain acyl ligase modules, and proteins related to possible azabicycle assembly and molecular tailoring. Verification of the pathway linkage to azinomycin biosynthesis was achieved by heterologous expression of *aziB*, the gene encoding the PKS, and detection of 5-methylnaphthoic acid secreted in the fermentation media (Fig. 16). Additionally, the roles of a cytochrome P450 hydroxylase (*aziB1*) and a SAM-dependent *O*-methyltransferase (*aziB2*) in naphthoic acid tailoring were demonstrated in the *Streptomyces albus* heterologous expression system, producing 3-methoxy-5-methyl-NPA as predicted.[52] Subsequent *in vitro* reconstitution of *aziB1* and *aziB2* overexpressed in *E. coli* presented further confirmation of hydroxylation and methyltransferase activities, while a standard ATP-[^{32}P]-PP$_i$ exchange assay, AziA1, was found to activate 3-methoxy-5-methyl-NPA, 5-methyl-NPA, and 3-hydroxy-5-methyl-NPA (Fig. 16).[53] A coupled PP$_i$ release experiment demonstrated AziA1 had 3–5-fold higher preference for the most advanced substrate, 3-methoxy-5-methyl-NPA.

Additional NPA analogs were tolerated by AziA1 with a range of catalytic efficiencies to suggest some promiscuity, but failure to activate 6-methylsalicylic acid or orsellenic acid reveals the naphthalene ring may be a necessary structural component for substrate recognition by this type I iterative PKS.

We have also identified and characterized a resistance protein, AziR.[54] The immunity protein exhibits a protective effect against azinomycin B when heterologously expressed in *S. lividans* and *E. coli* (Fig. 17). The protein, dubbed AziR for azinomycin resistance, exhibits homology to aminoglycoside phosphotransferases but behaves as an azinomycin-binding protein. While AziR confers resistance to azinomycin B, it is inactive against aminoglycoside antibiotics and other DNA alkylators. A nucleic acid staining assay indicates the protein enhances cell survival, and also prevents DNA damage effects normally observed following azinomycin treatment. Moreover, while *E. coli* treated with azinomycin B results in substantial upregulation of ribonucleotide reductase (a DNA damage response), corroborating our previous transcriptional profiling experiments in yeast, as anticipated transcription of the gene remained virtually unchanged when AziR was overexpressed in *E. coli*. Knowledge of azinomycin resistance proteins, such as AziR, and their protective mechanisms may in the future aid engineered biosynthesis of functionally useful azinomycin analogs.

The enzymology of epoxyvaline, azabicycle, and threonine/aminoacetone formation and incorporation remains to be explored *via* genetic manipulation and enzymatic reconstitution, and is a major effort by our group. Bioinformatic analysis certainly indicates the complexity of these pathways, as in the case of azabicyclo[3.1.0]hexane construction. Elucidation of the mechanism of azinomycin biosynthesis will represent a *tour de force* to reveal the intricacies of the chemistry involved in the construction of this highly functionalized antibiotic.

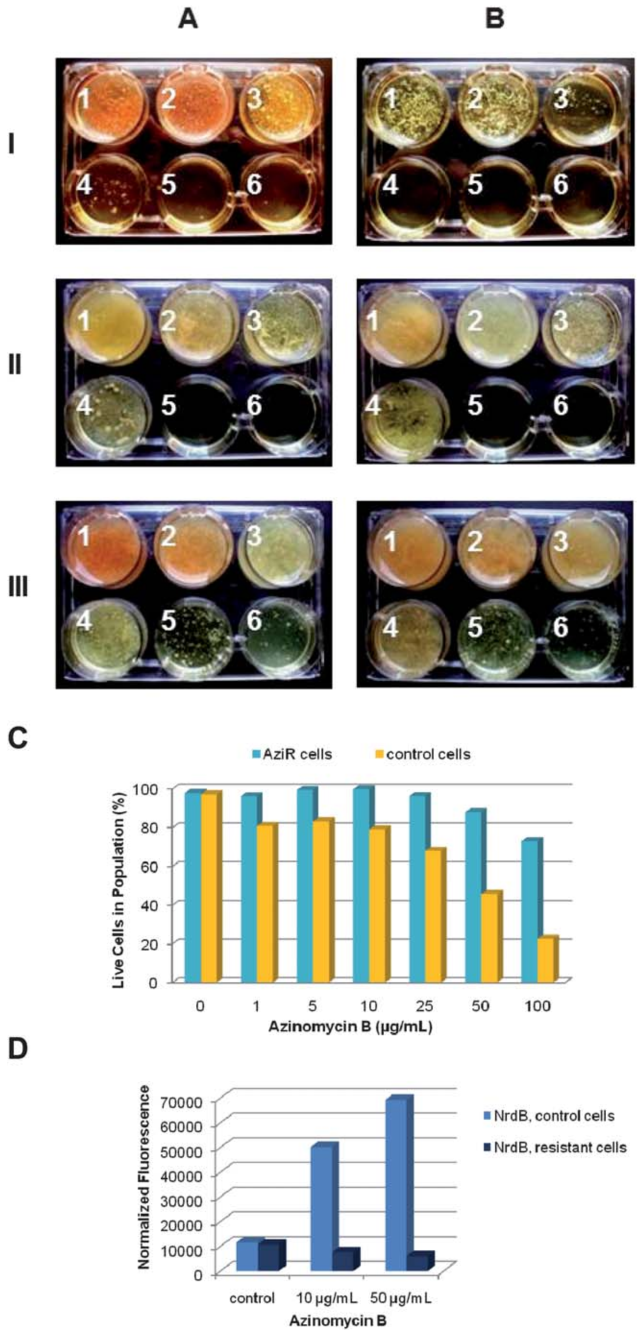


Fig. 17 Functional characterization of aziR: (A) AziR overexpressed in *S. lividans*; (B) A control plasmid transformed in *S. lividans*. Cells were treated as follows: Row I, azinomycin B at (1) 0, ethanol control; (2) 1; (3) 2.5; (4) 5; (5) 10; and (6) 15 µg mL^{-1}. Row II, methyl methanesulfonate at (1) 0; (2) 0.01; (3) 0.025; (4) 0.05; (5) 0.075 and (6) 0.1% v/v. Row III, streptomycin sulfate at (1) 0; (2) 1; (3) 2.5; (4) 5; (5) 10 and (6) 20 µg mL^{-1}; (C) Demonstration of the protective effect of AziR in *E. coli*; (D) RT-PCR results, demonstrating the effects of azinomycin B on ribonucleotide reductase expression in *E. coli* overexpressing AziR *vs.* control cells.

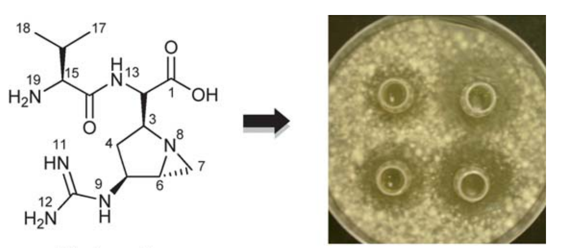
5 The structurally related natural product, ficellomycin

5.1 Bioactivity and mode of action of ficellomycin

Ficellomycin **58** (Fig. 18) represents the second azabicycle-containing natural product to be isolated to date, and was identified from the fermentation broth of *Streptomyces ficellus* by Upjohn Research Laboratories. The compound exhibited potent *in vitro* activity against *Staphylococcus aureus* including strains resistant to penicillin, streptomycin, neomycin, macrolides and the lincosaminide antibiotics. The antibiotic was also effective *in vivo*, where ficellomycin exhibited a CD_{50} of *ca.* 7.6 mg kg^{-1} against mice infected with *Staphylococcus aureus*.[56] The structure of ficellomycin was elucidated as valyl-2-[4-guanidyl-1-azabicyclo[3.1.0]hexan-2-yl]glycine by NMR, MS, and derivatization studies in 1988.[57] Chemically, the metabolite is stable in aqueous solutions at pH 8–10, but is slowly transformed to an inactive metabolite under acidic conditions.

The promising anti-bacterial properties of ficellomycin prompted further investigation. The mode of action of ficellomycin was evaluated through pulse-chase experiments by Reusser and co-workers at Upjohn Research Laboratories.[58] The results revealed that ficellomycin selectively impairs semi-conservative DNA replication in *E. coli* deficient in DNA polymerase I and rendered permeable to nucleotides by toluene treatment. Specifically, this antibiotic causes the accumulation of 34S DNA species, likely as a result of DNA alkylation. Further investigation indicated that ficellomycin neither inhibits steps occurring during the early initiation of replication, nor inhibits the polymerases, but instead causes the formation of deficient 34s DNA fragments. These fragments lack the ability to integrate into larger DNA pieces during the maturation and assembly process of the bacterial chromosome.

Ficellomycin inhibits a variety of bacteria *in vitro*. However, the minimum inhibitory concentration (MIC) of its fermentation broth amounts to >1000 µg mL^{-1} against most organisms. Ficellomycin was also inactive against a variety of fungi (except *Penicillium oxalicum*) and viruses *in vitro*.[58] For these reasons the natural product has not garnered much attention as a pharmaceutical agent. However, with the advent of combinatorial biosynthesis and chemoenzymatic methods, it may be possible to genetically engineer the ficellomycin producer, leading to structural modifications and improvement of its structure–activity relationship (SAR).



Ficellomycin
58

Fig. 18 The structure of ficellomycin and the effect of *S. ficellus* culture broths on the growth of *P. oxalicum* on solid medium.[55]

5.2 Biosynthesis of ficellomycin

The striking structural similarity between ficellomycin and azinomycin B has captured the attention of the biosynthetic research community. Ficellomycin, with its dipeptide motif, is comprised of one valine residue and an unusual amino acid, which houses the azabicyclic ring system. We have recently obtained a draft genome sequence of *S. ficellus* from which a putative gene cluster has been localized and fosmid clones containing the DNA region have been isolated. Solexa/Illumina paired-end sequencing of *S. ficellus* produced 644 million base calls (approximately 17 million reads) and the sequences were also assembled using Velvet. The *S. ficellus* strain is readily amenable to genetic manipulation, and gene disruption experiments are underway. Given the shared architectural characteristics of azinomycin and ficellomycin, it will be interesting to cross-compare their biosynthetic pathways and determine whether these similarities are also shared at the level of their formation.

6 Conclusion

The azinomycins and ficellomycins may lead to the discovery of a new generation of anti-cancer agents with better clinical performance than current DNA alkylating agents on the market today, such as cisplatin. The side-effects associated with cisplatin treatment are severe, and may include toxicity to the kidney and nervous system, hearing difficulties, nausea, vomiting, *etc.* Ficellomycin and azinomycin analogs could offer clinical advantage over cisplatin and other similar treatment regimes if they display activity against cancers with intrinsic or acquired resistance to current chemotherapeutic agents, display reduced toxic side-effects, display better selectivity to cancer cells *versus* normal cells, or can be administered orally.

Notwithstanding the anti-cancer potential of the azinomycins and ficellomycin, the biosynthetic machinery of these agents, especially that of the unusual azabicyclo[3.1.0]hexane ring system, presents an intriguing mechanistic problem. Evaluating the biosynthesis of these agents and elucidating the chemistry at a mechanistic level is a major effort by our research group. The culturability and genetic amenability of the azinomycin and ficellomycin host strains bodes well for future genetic engineering efforts in the generation of a new class of chemotherapeutic agents that can be produced in a cost-effective manner through fermentation.

7 Acknowledgements

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