

Review

Biosynthesis of DNA-Alkylating Antitumor Natural Products

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Abstract: DNA-alkylating natural products play an important role in drug development due to their significant antitumor activities. They usually show high affinity with DNA through different mechanisms with the aid of their unique scaffold and highly active functional groups. Therefore, the biosynthesis of these natural products has been extensively studied, especially the construction of their pharmacophores. Meanwhile, their producing strains have evolved corresponding self-resistance strategies to protect themselves. To further promote the functional characterization of their biosynthetic pathways and lay the foundation for the discovery and rational design of DNA alkylating agents, we summarize herein the progress of research into DNA-alkylating antitumor natural products, including their biosynthesis, modes of action, and auto-resistance mechanisms.

Keywords: biosynthesis; DNA alkylating agents; antitumor natural products; resistance



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1. Introduction

Natural products (NPs) are an important source of pharmaceuticals due to their diverse bioactivities [1]. Since DNA is essential for living organisms, DNA-targeting NPs, which usually function as carcinogenesis or cancer treatment, constitute an indispensable family of bioactive NPs [2,3]. Although the genotoxic metabolite colibactin, produced by human gut bacteria, is shown to cause colorectal cancer by alkylating DNA to generate DNA mutation [4–6], some DNA-targeting NPs are applied in chemotherapy. They can interact with specific DNA duplex structures and cause DNA damage via different modes of action [7]. One of the mechanisms is the cleavage of DNA through inducing the production of radical DNA by redox reactions or nucleophilic addition. Broad anti-cancer antibiotic bleomycin (BLM) can be transformed to HOO-Fe(III)-BLM in the presence of Fe/O₂ to damage DNA [8]. The enediyne-containing NPs dynemicin A and calicheamicin can generate biradical intermediates to cleave DNA activated by reducing the quinone moiety and the attack of a thiol, respectively [9–12]. Some chemicals, such as streptozotocin, conduct the methylation of DNA [13,14]. Additionally, another family of DNA-targeting antitumor agents can alkylate DNA in situ with covalent bonds. They can directly react with DNA using highly active functional groups such as epoxide, cyclopropane, and aziridine to form bulky DNA adducts [15,16]. Furthermore, as a result of the potent cytotoxicity of DNA-alkylating NPs, it is preferable for their producers to possess resistant genes located in biosynthetic gene clusters (BGCs) to protect themselves. BGCs-associated self-resistance is mainly achieved through excision of the abnormal base, degradation of active functional groups, and the binding or transport of toxins [17].

The biosynthesis and resistance of radical-based DNA damage agents, including BLMs and enediynes, have already been well reviewed [18]; herein, we mainly discuss DNA-alkylating (except DNA-methylation) antitumor NPs, including their modes of action, BGC-associated self-resistance, and biosynthetic pathways, especially the construction of their highly active groups as a warhead.

2. Spirocyclopropane-Containing Cyclohexadienone Natural Products

The spirocyclopropylcyclohexadienone family, including yatakemycin (YTM, **1**), CC-1065 (**2**), and duocarmycin SA (**3**), all contain a highly active cyclopropane moiety and exhibit potent antitumor activities (Figure 1) [19–22]. Duocarmycin-based antibody-drug conjugates (ADC, SYD985 (**4**), and MDX-1203 (**5**)) have entered clinical trials for the treatment of specific cancers as prodrugs (Figure 1) [23,24]. They can selectively bind AT-rich regions in the DNA minor groove by non-covalent interaction, then form a covalent bond with DNA in which the cyclopropanol group is attacked by the N-3 of adenine (Figure 2A) [25]. The YTM-producer was first identified as protecting itself with DNA glycosylases YtkR2 through the base-excision repair mechanism (Figure 2A). The homologous enzyme C10R5 exhibited a similar function in the CC-1065-producing strain. [26] Because the cyclopropane warhead exhibits strong potency, additional self-protection of their hosts can also be achieved by the cleavage of this moiety. A GyrI-like protein was verified to hydrolyze the cyclopropane moiety in YTM and CC-1065 to facilitate detoxification (Figure 2B) [27–29].

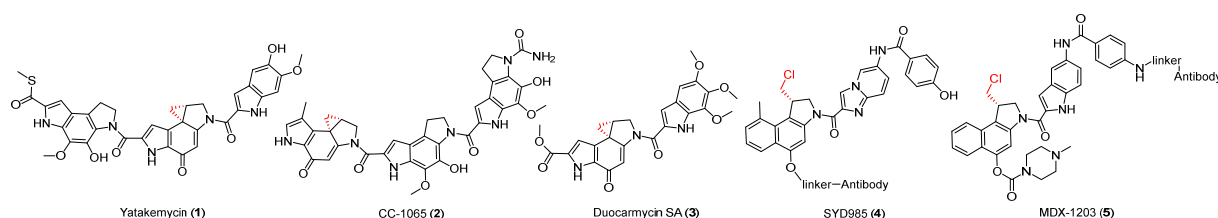


Figure 1. Chemical structures of spirocyclopropylcyclohexadienone family compounds.

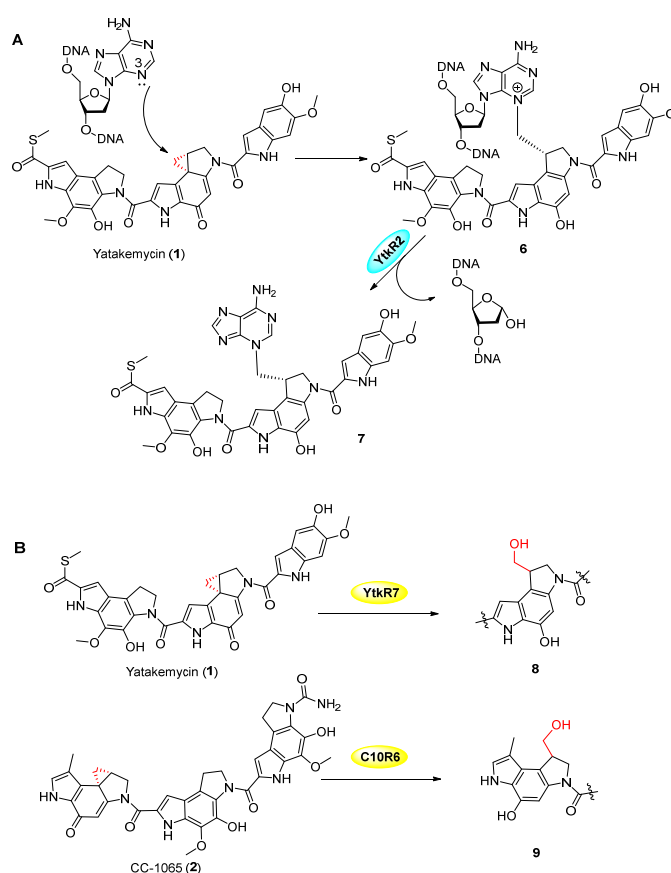


Figure 2. (A) DNA modification by YTM, and excision of DNA-drug complex by YtkR2. (B) Hydrolysis of the cyclopropyl moiety in YTM and CC-1065 by YtkR2 and C10R6, respectively.

The benzodipyrrole scaffold in CC-1065 was derived from serine, methionine, and tyrosine-derived DOPA, and was revealed by isotopic feeding experiments (Figure 3) [30,31]. Wu et al. proposed the possible biosynthetic pathway of CC-1065. Tyrosine was first oxidized to DOPA which underwent intramolecular cyclization to afford **10**. Next, **11** produced by the combination of serine and **10** was decarboxylated and cyclized to yield **13** which was further modified to form three different types of building blocks (**17**, **18**, and **19**). The assembly of these building blocks generated the final core structure **21** (Figure 4).

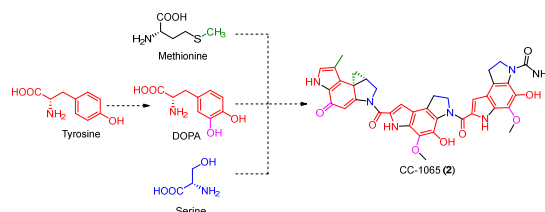


Figure 3. Isotopic labelling patterns with serine, methionine, and tyrosine.

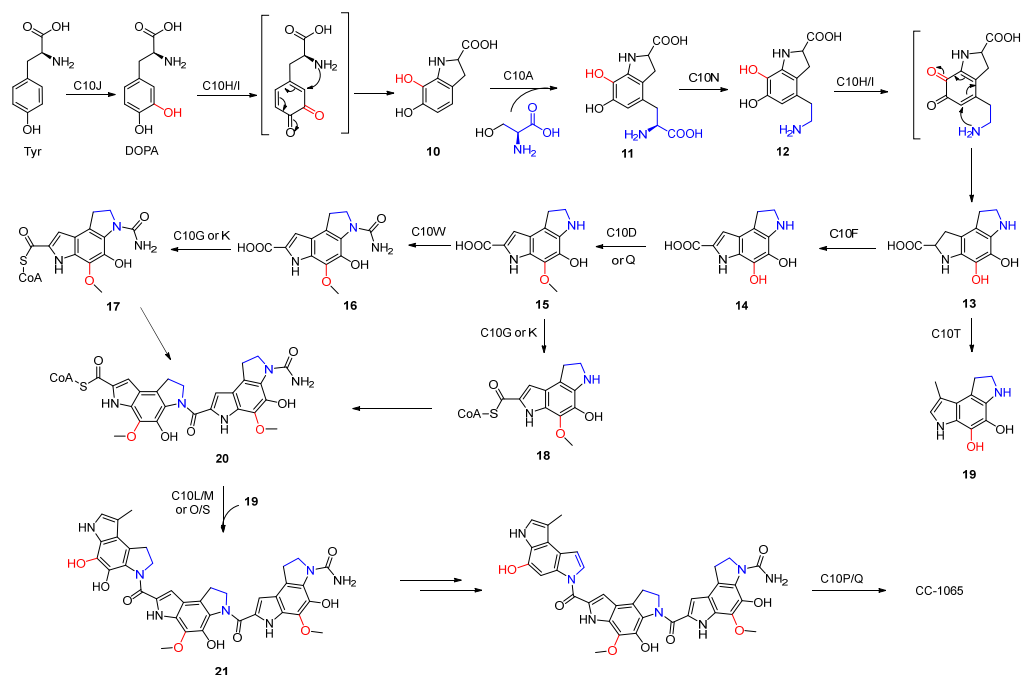


Figure 4. Proposed biosynthetic pathway of CC-1065.

Strategies for the incorporation of cyclopropane have long fascinated chemists, since it is an important synthetic building block and a common pharmacophoric group. The chemical synthesis of cyclopropane in this family of NPs was mainly achieved by nucleophilic cyclopropanation [32]. In the biosynthesis of CC-1065, Jin et al. reported that a two-component cyclopropanase system consisting of a HemN-like radical S-adenosylmethionine (SAM) enzyme C10P and a methyltransferase C10Q was responsible for generating the essential cyclopropane moiety involving a unique enzymatic mechanism (Figure 5) [33,34]. To explain in detail, the highly active SAM methylene radical attacks the C-11 position of **22** to generate the radical intermediate **23**, which subsequently abstracts hydrogen to yield the SAM-substrate adduct **24**. Following this, the deprotonation of the phenolic hydroxyl group in virtue of His-138 residue in C10Q induced S_N2 cyclopropanation to produce CC-1065 with S-adenosylhomocysteine (SAH) as the leaving group. Additionally, **24** could also be converted to **25** by non-enzymatic reaction with the release of SAH, and the following isomerization produces the methylated compound **26**.

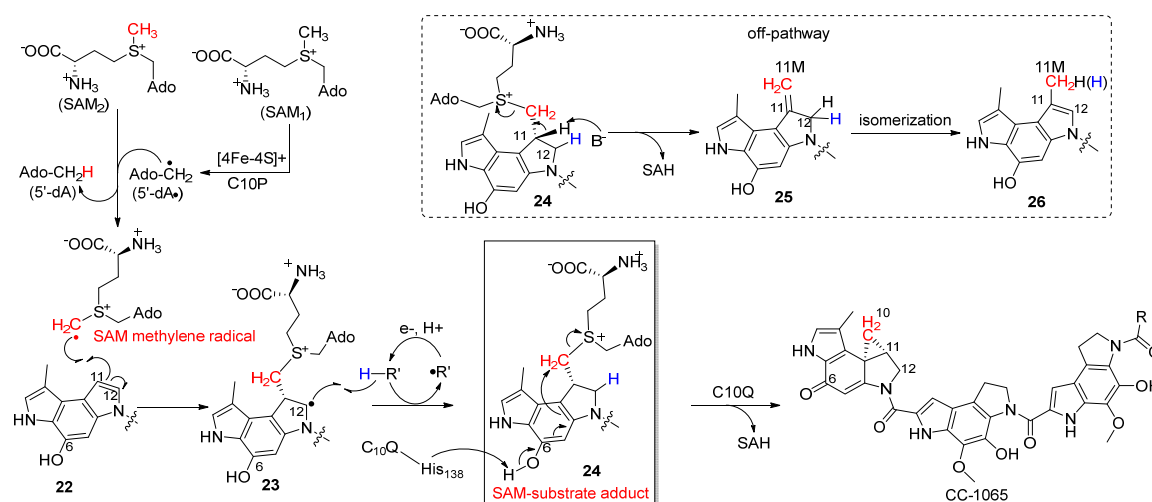


Figure 5. Proposed enzymatic mechanism of the cyclopropane moiety formation catalyzed by C10P and C10Q.

3. DNA-Alkylating Natural Products with Heterocyclic Propane as Pharmacophore

3.1. Pluramycins

As an important family of NPs, type II polyketides display various structurally diverse biological activities [35,36]. Anthracycline compounds such as daunomycin and nogalamycin exhibit antitumor activities by intercalating into grooves of DNA, while most of these compounds are unable to form a covalent bond with duplex DNA [37,38]. Nevertheless, pluramycin antibiotics including hedamycin (27) and altromycin B (28) (Figure 6A), which usually contain an epoxide moiety, can intercalate and alkylate DNA simultaneously. Similar to daunomycin, their anthraquinone ring was characterized as intercalating into DNA and binding saccharides in the minor or major groove, thereby contributing to the stabilization of the drug–DNA complex [39–41]. Furthermore, their epoxides could be opened via nucleophilic attack of N-7 of guanine, resulting in the formation of an adduct by covalent bond (Figure 6B).

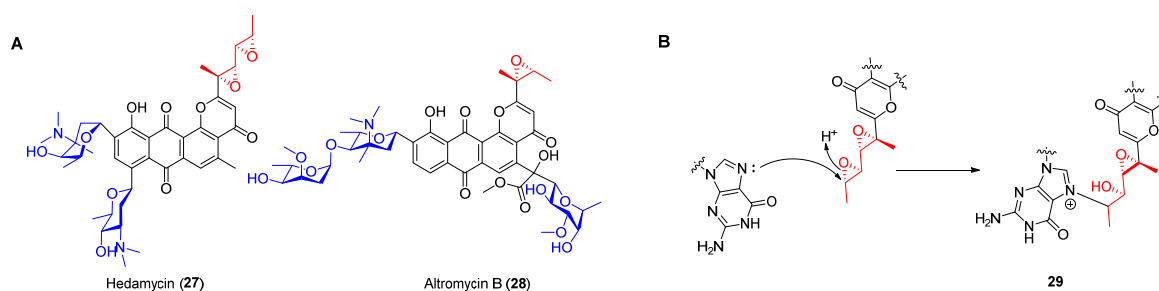


Figure 6. (A) Chemical structures of pluramycins. (B) Alkylation of DNA by hedamycin.

Biosynthetically, the epoxides in hedamycin were formed on its non-acetyl starter unit generated by two separate type I polyketide synthases (PKSs, HedT, and HedU). HedU was proposed to catalyze two rounds of chain elongation employing the acetyl starter unit provided by HedT (Figure 7) [42–44]. The obtained unsaturated 2,4-hexadienyl unit was then transferred to the downstream type II PKS to produce the aromatic precursor. The following oxidation of the C2-alkyl side in intermediate 30 afforded the epoxide intermediate 31 which was further modified by methyltransferase and two C-glycosyltransferases to yield hedamycin (Figure 7).

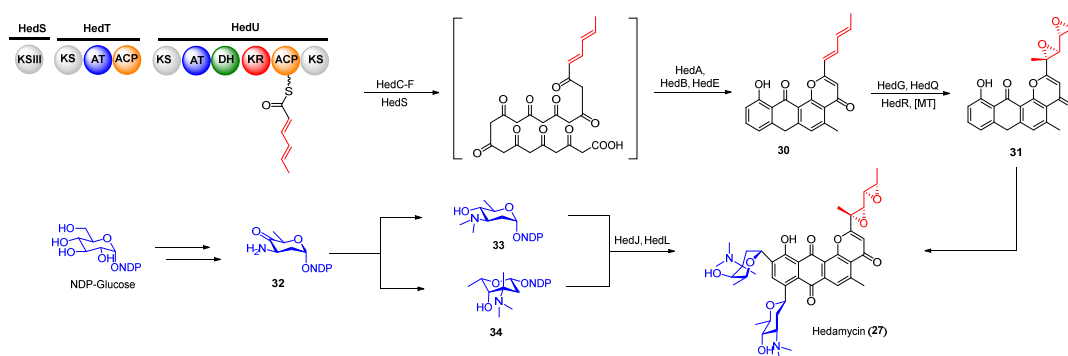


Figure 7. Proposed biosynthetic pathway of hedamycin. KS, ketosynthase; KR, ketoreductase; ACP, acyl carrier protein; DH, dehydratase; AT, acyl transferase.

Trioxacarcins (TXNs), firstly isolated from *Streptomyces bottropensis* NRRL 12051 in 1981, exhibit extraordinary antibacterial, antimalarial, and antitumor activities [45]. Among the TXNs, TXN A (35) showed the most potent antitumor activities with sub-nanomolar IC_{50} values against various cancer cell lines (Figure 8A). The unique fused spiro-epoxide of TXN A is essential for its bioactivities because the epoxide can react with N-7 of guanine to form a DNA–TXN A complex (Figure 8B). The crystal structure of this complex revealed that glycosyl groups at C-4 and C-13 were docked with the minor and major groove, respectively [46]. It also displayed an unexpected flipping out of the base at the intercalation site, which might be important for DNA–protein interaction. Recently, the study of the TXN analog LL-D49194 α 1 (36) showed that the deglycosylated compounds (37 and 38) exhibited more potent anticancer activities than 36, possibly suggesting a new mode of interaction with DNA (Figure 8A) [47]. Furthermore, the DNA–TXN complex (39) could be cleaved to yield guttingimycin (40) involving a self-resistance mechanism of an excising base (Figure 8B) [48,49]. Recently, four DNA glycosylases, TxnU2, TxnU4, LldU1, and LldU5, were reported to be responsible for excising the intercalated guanine adducts [50].

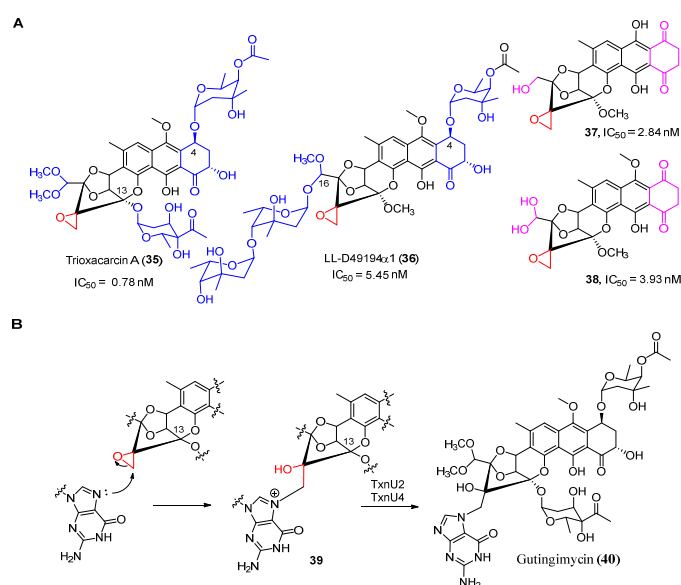


Figure 8. (A) Chemical structures of trioxacarcin A, LL-D49194 α 1 and their derivatives. (B) Alkylation of DNA by trioxacarcins.

Based on the work of Zhang et al., TXNs were also biosynthesized from anthraquinone-intermediate parimycin (47), similarly to the precursor of hedamycin, as revealed by gene-deletion experiments [45]. According to isotope-labelled precursor feeding experiments,

the unusual starter unit 2-methylbutyryl of TXNs was derived from L-isoleucine through transamination. After a series of modifications, including condensation with acetyl-CoA and decarboxylation, this starter unit was incorporated into the polyketide chain in virtue of KSIII (Figure 9). The formation and subsequent cyclization of the polyketide chain provided intermediate **46**, whose pyrone ring was formed by a CalC-like protein, TxnO9 [51]. The decarboxylated intermediate **47** underwent complex tailoring steps to afford intermediate **51** with a unique spiro-epoxide structure, but the specific enzymatic process and mechanism remained uncharacterized. Following that, the methylation of **51** at C-4 and C-13 yielded **52**, whose C4-sugar was finally acetylated by the membrane-bound O-acetyltransferase TxnB11 to form **35** (Figure 9) [52]. Unlike TXNs, the C-16 and C-4 of **45** were glycosylated and methylated to produce LL-D49194 α 1.

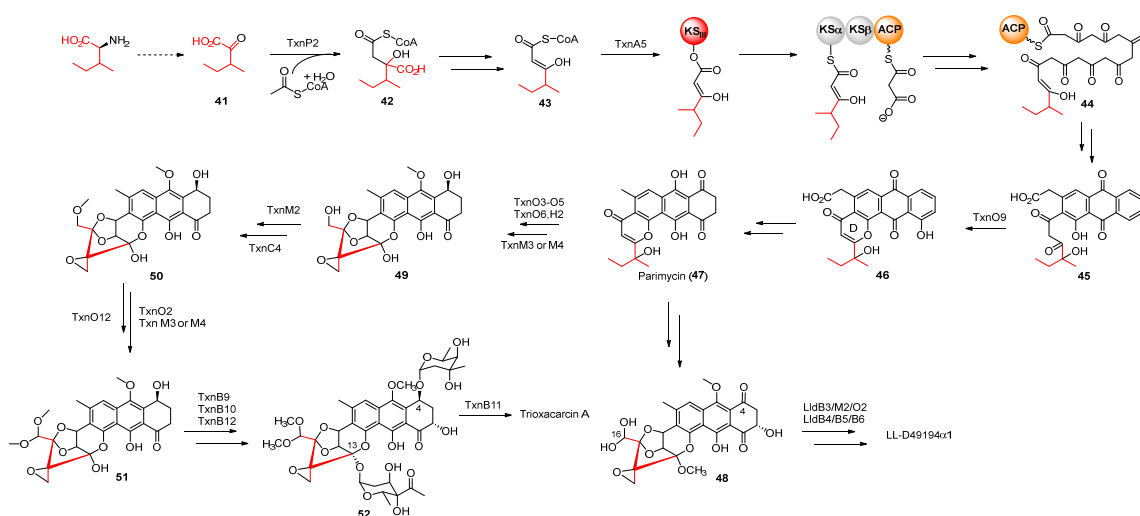


Figure 9. Proposed biosynthetic pathway of trioxacarcin A and LL-D49194 α 1. CLF, chain length factor.

3.2. Mitomycins

Mitomycins (MMs, such as MMA, B, and C) are antitumor NPs discovered in *Streptomyces*. They all contain the quinone backbone and a unique azabicyclo moiety (Figure 10A) [18,53]. Among these compounds, MMC has been used as a chemotherapeutic agent in the clinic for more than five decades. MMC can form inter-strand and intra-strand cross-linking with DNA at the selective sequence (5'-CG-3') and resides in the minor groove [54]. Other compounds of the mitomycin family, such as FR900482 and FR66979, also showed potent DNA cross-linking activity as well as bioactivities against cancer cell lines (Figure 10A). FR900482 was superior to MMC in both efficacy and safety [55].

The mode of action of MMs is well studied. Firstly, a reductive pathway is required to activate the quinone moiety of MMC by either enzymatic or chemical means to form the hydroquinone intermediate **60** [18,53]. Subsequent elimination of methanol in **60** affords **61** which undergoes tautomerization and the ring-open reaction of aziridine ring to yield **65** (Figure 10B). The N-2 of guanine attacks the C-1 position to generate the DNA-compound complex, then the departure of carbamate produces the iminium intermediate **69**, which is attached by the second guanine of DNA in the same way to form **71**. Furthermore, the first reductive activation could be inhibited by a FAD-dependent oxidoreductase MCRA (encoded by *mcrA*) which enables the oxidation of the hydroquinone form to the quinone form to confer self-resistance [56–59]. Although the alkylating mechanism of FR900482 is similar to that of MMC, it is activated by cleaving the N-O bond to form **59** (Figure 10B).

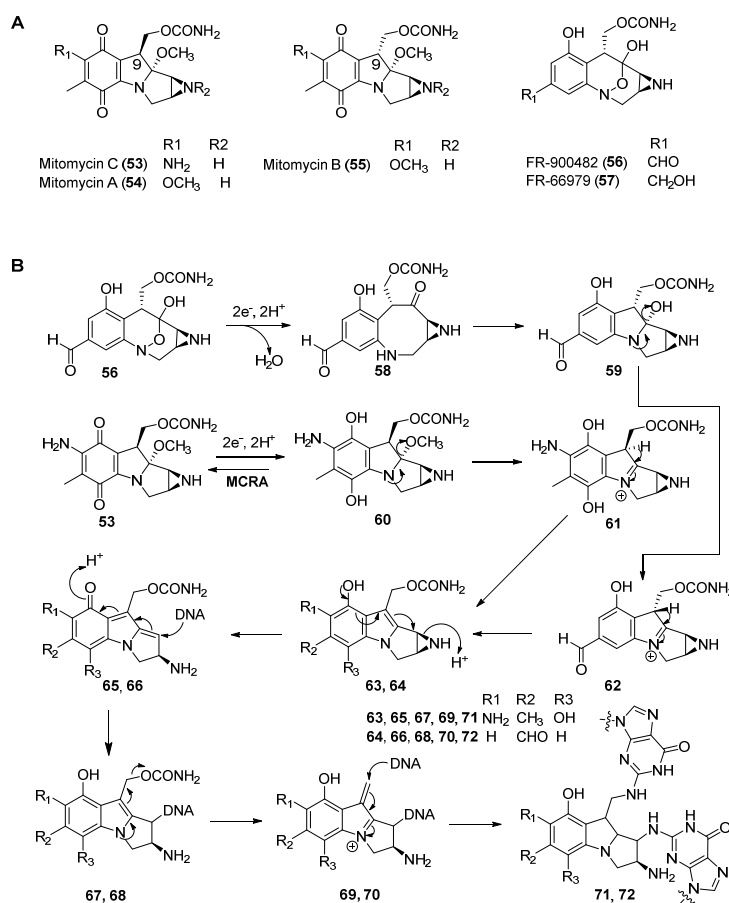


Figure 10. (A) Chemical structures of mitomycins. (B) Proposed mechanism of DNA cross-linking by mitomycin C and FR-900482.

Since these compounds possess excellent bioactivities and the common pharmacophoric group azabicyclo, their synthesis has attracted extensive attention. In chemical synthesis, the azabicyclo moiety of MMs is installed from benzazocane intermediates via intramolecular substitution [60], but their biosynthetic pathways are still not well elucidated. According to isotopic precursors feeding experiments conducted by Hornemann et al., the origins of the *O*-methyl group and the carbamate were methionine and L-citrulline, respectively, while the mitosane core was derived from 3-amino-5-hydroxybenzoic acid (AHBA, 81) and glucosamine [61,62]. The precursor AHBA was formed via the amino-shikimate pathway related to rifamycin and kanosamine biosynthesis [63,64]. After the formation of AHBA, it was firstly activated by acyl AMP-ligase MitE and was then loaded onto acyl carrier protein (ACP) MmcB (Figure 11). The glycosyltransferase MitB was verified to catalyze the glycosylation of AHBA-MmcB with UDP-GlcNAc [65–67]. Recently, Wang et al. traced all the ACP-channelled MM intermediates indicating that AHBA-MmcB-GlcNAc intermediate 85 should undergo the deacetylation by MitC to form 86 which was further transformed to 88 by MitF and MitD [68]. The epoxide intermediate might be cyclized to provide benzazocine 92. 92 then underwent oxidation and several uncovered modifications to generate hydroxyquinone intermediate 95 which was methylated to afford MMA, the direct precursor of MMC [69]. Sherman and co-workers also identified a methyltransferase MitM which methylated the nitrogen of aziridine in MMA rather than MMC to yield MMF (96) [70]. In addition, the epoxide of 88 could be opened by the nucleophilic attack to afford 89 which was the precursor of the MMs with α -C9. Moreover, the oxidation of the aniline amine in 89 facilitated forming the core structure of FR900482.

Previous isotope-labelled precursor feeding experiments revealed that the epoxy moiety, the azabicyclic fragment, and the terminal part in azinomycin B were derived from acetyl-CoA, valine, glutamic, and threonine, respectively (Figure 13) [79,80]. The feeding experiments with isotopically labelled substrates showed that 3-methyl-2-oxobutenoate (**105**) was incorporated into the azinomycin epoxide as the penultimate precursor (Figure 14A). The formation of **105** was achieved by oxidation, transamination as well as dehydration beginning with L-valine. Even so, the exact timing of forming epoxy amide remains unclear up to now [81].

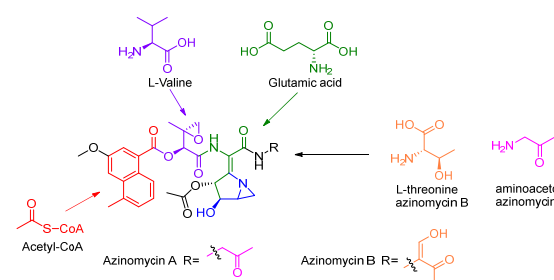


Figure 13. Origins of azinomycins revealed by isotopic labelling experiments.

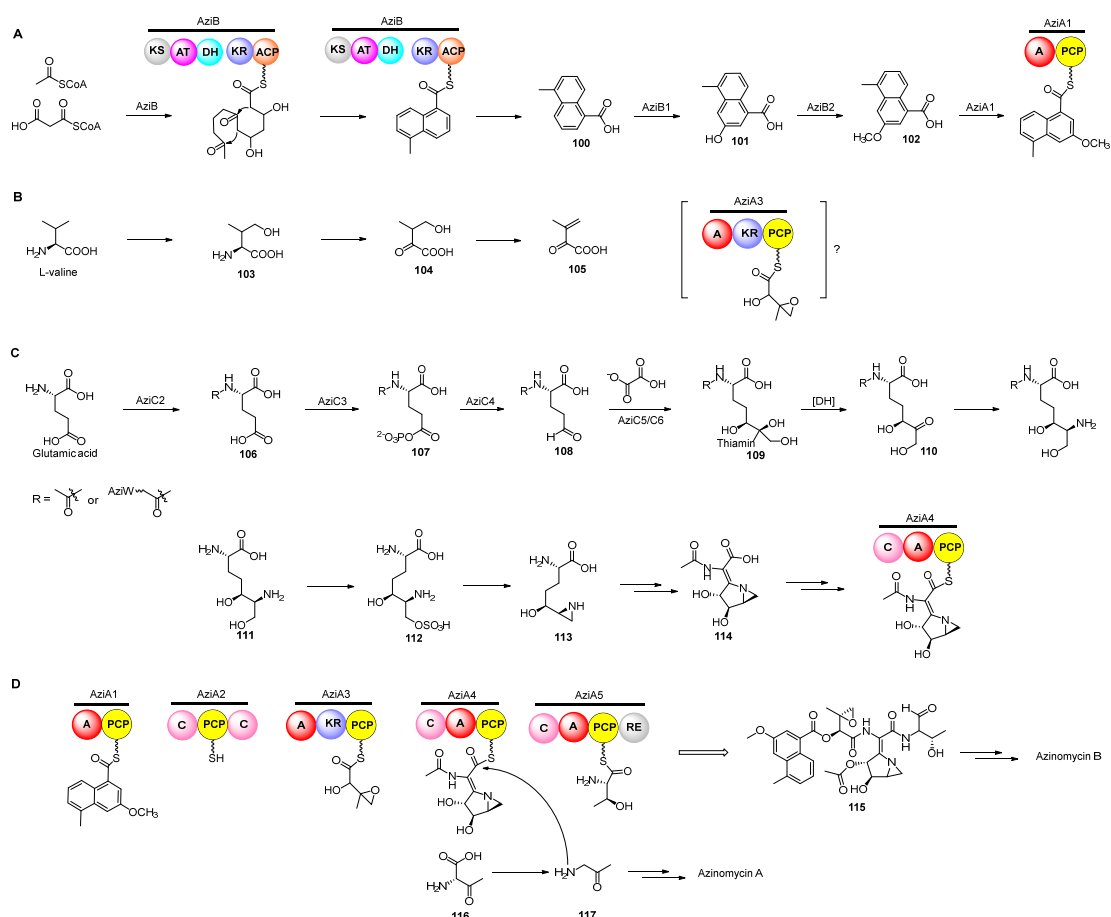


Figure 14. Proposed biosynthetic pathway of azinomycins. PCP, peptidyl carrier protein; A, adenylation; C, condensation; RE, reduction. Proposed pathway of constructing 3-methoxy-5-methyl-NPA moiety (A), epoxy intermediate (B), azabicyclic fragment (C), and incorporating building blocks (D).

In the biosynthesis of this class of non-ribosomal peptide-polyketide hybrid compounds, iterative type I PKS AzkB catalysed the formation of 5-methyl-NPA (**100**) which was further transformed to 3-methoxy-5-methyl-NPA (**102**) by a P450 hydroxylase AzkB1

and the *O*-methyltransferase AziB2 (Figure 14B) [82]. The first building block **102** was activated by the distinct adenylation (A) domain of the di-domain non-ribosomal peptide synthetase (NRPS) AziA1 to initiate the backbone formation of azinomycins [83,84].

The azabicyclic moiety was constructed from 3,4-epoxypiperidine derivatives via spontaneously intramolecular substitution in chemical synthesis [85]. Watanabe and co-workers unraveled the biosynthetic pathway of the azabicyclic fragment in azinomycin, wherein the glutamic acid was initially acetylated at the amino group by *N*-acetyltransferase AziC2 to form *N*-acetyl glutamate **106**. The *N*-acetyl glutamate kinase AziC3 subsequently phosphorylated the carboxyl to afford the *N*-acetyl-glutamyl 5-phosphate (**107**) which was subsequently reduced to *N*-acetyl-glutamate-5-semialdehyde (**108**) by an *N*-acetyl- γ -glutamate phosphate reductase AziC4 (Figure 14C), and the key two-carbon extension on aldehyde intermediate catalyzed by the transketolase AziC5/C6 afforded **110** which was further converted to the acetylated nonproteinogenic amino acid diamino-dihydroxy-heptanoic acid (DADH, **111**) by an aminotransferase (AziC1 or AziC7) [86–88]. Recently, Kurosawa et al. demonstrated that **111** could be further sulphated to **112** and the sulfate group in **112** was finally attacked by the ortho amino group to form the aziridine ring intermediate **113** which may be subsequently acetylated and cyclized to form the azabicyclic fragment **114** (Figure 14C) [89]. Additionally, glutamic acid might be firstly activated by the amino-group carrier protein (AmCP) and was further modified to produce DADH which was then introduced into the azabicyclic structure according to a recent study about the biosynthesis of vazabotide A [90]. Moreover, the enol in the final building block of azinomycin B was generated by the oxidation of L-threonine, while the decarboxylation of the intermediate **116** afforded the aminoacetone **117** in azinomycin A [91].

4. DNA-Alkylating Natural Products with Imine as Warheads

4.1. Pyrrolobenzodiazepines

Antitumor antibiotics pyrrolobenzodiazepines (PDBs), including anthramycin, sibiromycin, and tomaymycin, all contain three parts: anthranilate, diazepine, and hydropyrrole (Figure 15A) [92,93]. The imine in the diazepine can be attacked by N-2 of guanine to form a stable covalent bond resulting in inhibiting DNA synthesis (Figure 15C) [94]. Moreover, the crystal structure of the anthramycin-DNA complex indicates that the *S*-configuration of C-11a make it suitable for docking in the minor groove of DNA [95,96]. In addition, a PDB dimer, SJG-136 (**121**), which has completed the phase II clinical trial for treating leukemia and ovarian cancer, can form DNA inter-strand and intra-strand cross-linking of DNA (Figure 15B) [97,98].

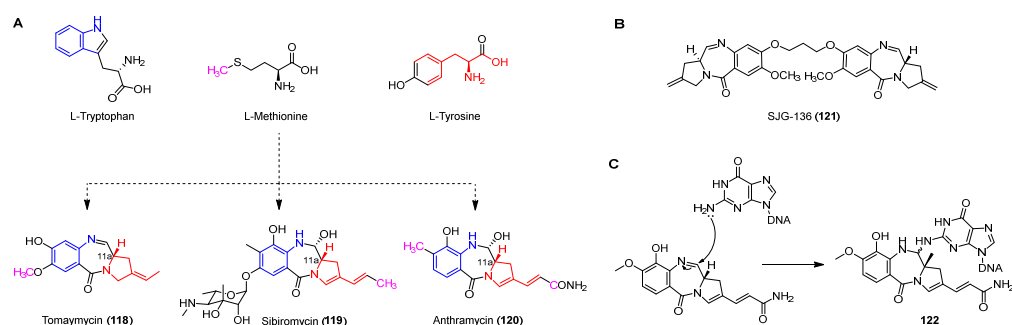


Figure 15. (A) Origins of pyrrolobenzodiazepines revealed by isotopic labeling experiments. (B) Chemical structure of SJG-136. (C) Proposed mechanism of DNA alkylating by pyrrolobenzodiazepines.

Based on previous feeding experiments, L-methionine, L-tyrosine, and L-tryptophan were supposed to be the biosynthetic precursors of pyrrolobenzodiazepines (Figure 15A) [99]. Like lincomycin biosynthesis, L-dopa from L-tyrosine is cleaved to yield semialdehyde intermediate **123**, followed by intramolecular cyclization to form dihydropyrrole interme-

Furthermore, a FAD-binding oxidoreductase NapU encoded in BGC of NDM was reported to activate and inactivate the matured prodrug by extracellular oxidation conferring self-protection [113]. Recently, a short-chain dehydrogenase NapW mediated the reduction of the hemiaminal pharmacophore, implicating another level of the self-resistance mechanism of the tetrahydroisoquinoline family [114].

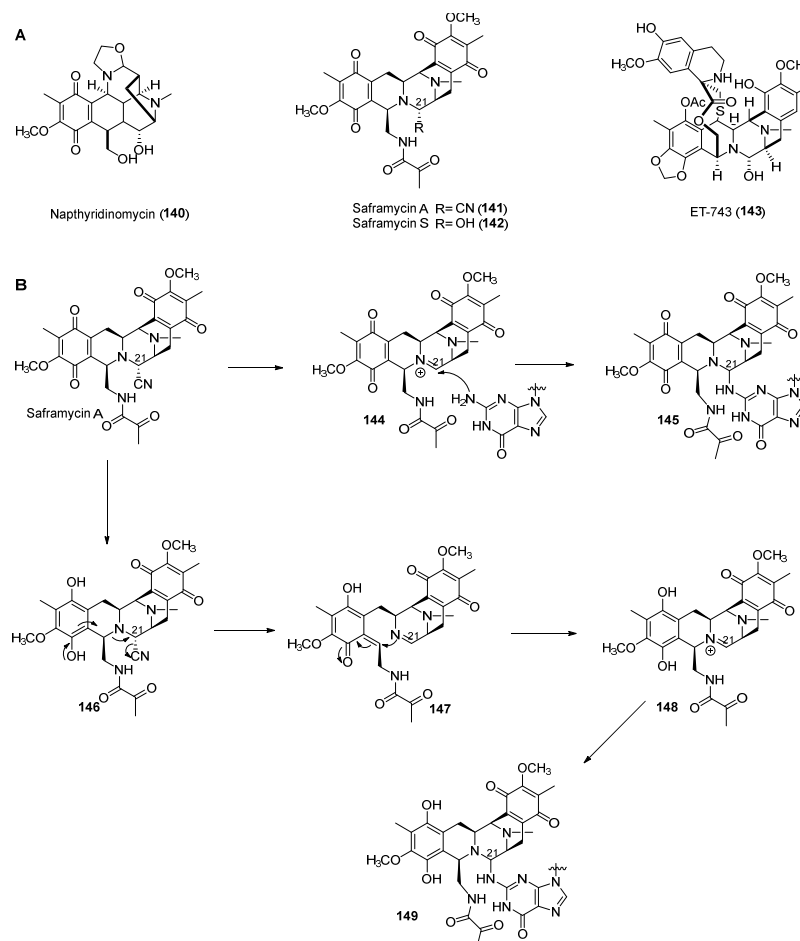


Figure 17. (A) Chemical structures of tetrahydroisoquinoline family. (B) Proposed mechanism of DNA alkylation by saframycin A.

Isotope-labeled precursor feeding experiments have revealed that the skeleton structure of SFMs is derived from tyrosine, alanine, glycine, and methionine (Figure 18) [115,116]. L-tyrosine undergoes C-methylation, oxidation, and O-methylation to afford the precursor 3-hydroxy-5-methyl-O-methyltyrosine (152) (Figure 19A) [117,118]. In 2010, Oikawa and co-workers reconstituted the formation of the core structure in vitro, revealing that two Pictet–Spengler (PS) reactions were involved in this process. Specifically, 152 was firstly activated and uploaded onto PCP of NRPS SfmC, assembling with intermediate 153 produced by NRPSs SfmA and SfmB via the first PS reaction. Following this, 154 formed by the subsequent reduction underwent a second PS reaction and reduction to generate 155 (Figure 19B) [119,120]. The following reduction and intramolecular cyclization of 155 yielded 156 which was then oxidized and methylated to generate 157. Subsequently, 157 was transported outside the cell in company with its fatty acid chain and was removed by the membrane-anchored protein SfmE to produce 158. Finally, SfmCy2 catalyzed the extracellular deamination of 158 to form 142, indicating a prodrug maturation process (Figure 20) [121].

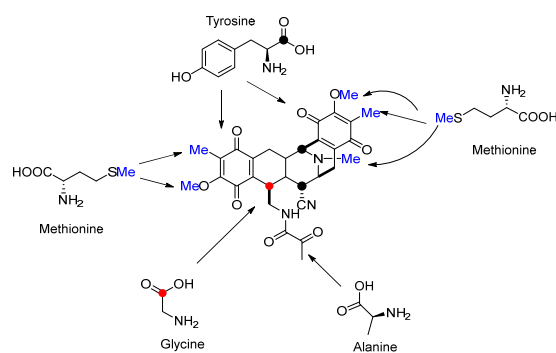


Figure 18. Origins of saframycins revealed by isotopic precursors labeling experiments.

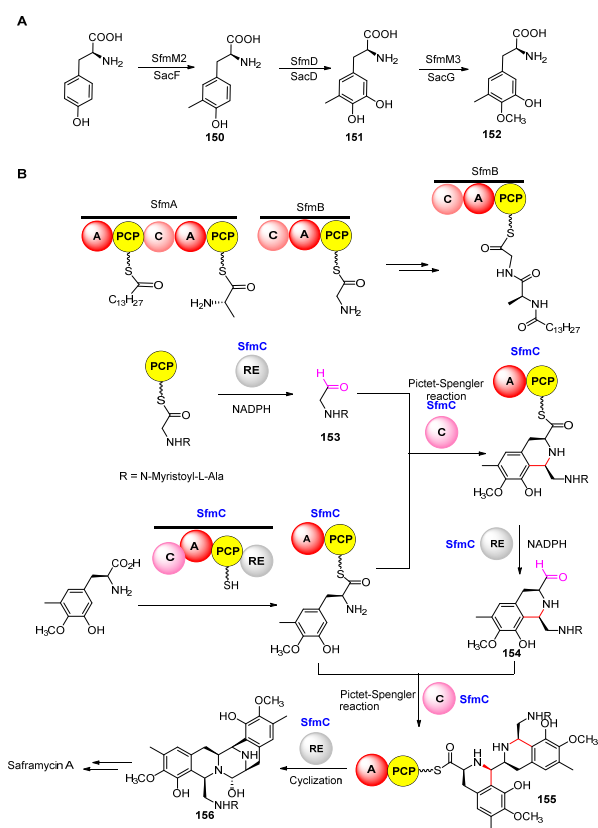


Figure 19. Proposed biosynthetic pathway of quinone moiety (A) and forming the skeleton of saframycins (B).

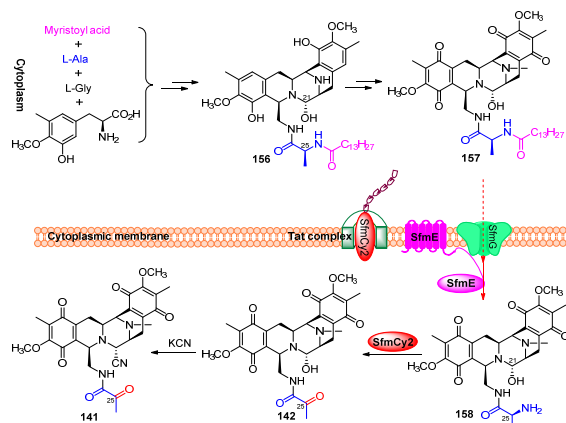


Figure 20. Post-modification of saframycins.

5. Others

5.1. Leinamycin

Antitumor agent leinamycin (LNM, **159**) is a hybrid peptide-polyketide NP and contains a unique 1,3-dioxo-1,2-dithiolane moiety, which is essential for its anticancer activity (Figure 21). The alkylation of DNA involves a rearrangement reaction in which LNM is initially activated via the attack of thiol to form the sulfenic acid intermediate **165**, which also enabled, triggering oxidative DNA cleavage via generating unstable hydrodisulfide intermediate (RSSH) (Figure 21) [122–125]. The oxathiolane intermediate **165** could be produced by breaking the S-S bond of LNM via thiol attack or hydrolysis. The C6-C7 alkene of **165** then attacks the electrophilic sulfur of the oxathiolanone group to generate an episulfonium ion intermediate, **166**, following an intermolecular nucleophilic attack with the N7 of guanine residues in duplex DNA to yield the DNA–drug adduct **168**. Unlike the reduction-mediated alkylation of DNA by LNM, LNM E1(**160**) could be transformed to its episulfonium ion intermediate **167** through an oxidative reaction catalyzed by reactive oxygen species (Figure 21) [126].

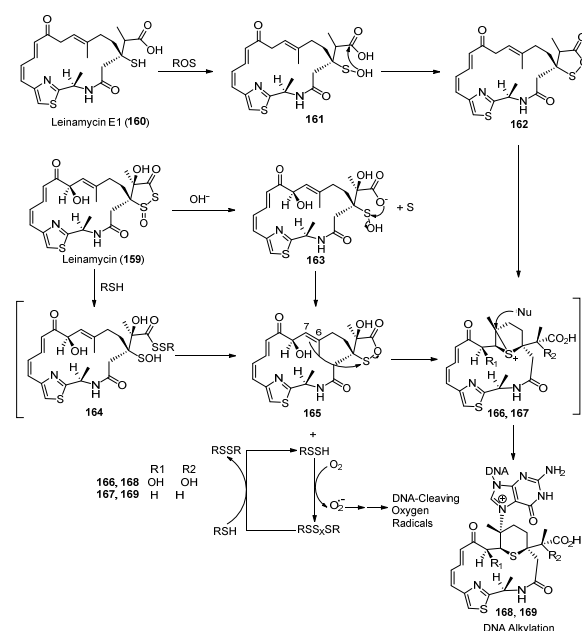


Figure 21. Proposed mechanisms of DNA alkylating by leinamycin and leinamycin E1.

The skeleton of LNM was biosynthesized by hybrid NRPS-PKS assembly lines [127]. The LnmQ, LnmP, and NRPS module of LnmI are responsible for the unique thiazole-containing starter unit. The polyketide chain of LNM is elongated by two AT-less type I PKSs, LnmI and LnmJ, as well as a *trans*-AT enzyme LnmG [128–132]. A β -branched C3 unit derived from methylmalonyl-CoA was then installed by a set of proteins including a free-standing ACP (LnmL), a bifunctional AT-decarboxylase (LnmK), an HMGS homolog (LnmM), and an ECH homolog (LnmF) (Figure 22) [133,134]. Module 8 in LnmJ, containing a domain of unknown function domain (DUF), added an L-cysteine into C-3 of intermediate **170** to produce intermediate **171**. The PLP-dependent cysteine lyase domain (SH) can catalyze the cleavage of the C-S bond to yield **172**, which is cyclized to release the chain to generate LNM E1 (Figure 22) [135]. Recently, Meng et al. demonstrated that LnmJ-SH domain directly installed a -SSH group into the LNM polyketide scaffold via cleavage of the C-S bond linking the thiocysteine to form LNM E (**173**), which might be further transferred to LNM by a set of uncharacterized enzymes [136,137].

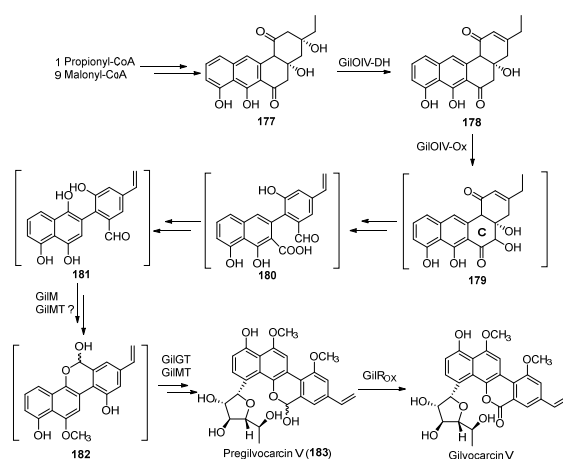


Figure 24. Proposed biosynthetic pathway of gilvocarcin V.

6. Perspective

Since DNA-alkylating NPs exhibit potent antitumor activity, their biosynthesis has received extensive attention. Obviously, the highly active functional groups including epoxide, cyclopropane, aziridine, and imine in their chemical structures play an important role in alkylating DNA to form the DNA–drug adduct. Elucidation of their biosynthetic pathways not only facilitates the discovery of new NPs with these biological active groups by genome mining but also is valuable for engineering NPs and drug design [147]. The unprecedented enzymology involved in their biosynthetic pathways can exert a positive influence on the development of biocatalysts as well. As a result of their strong DNA-alkylating activities, their producers have to confer self-resistance strategies to avoid damaging themselves, mainly through the cleavage of the DNA–drug complex or modification of the functional groups. Therefore, resistant gene-guided genome mining also contributes to discovering new DNA-alkylating antibiotics [148]. In addition, other moieties which contribute to affinity and reactivity with DNA are also indispensable for their ability to alkylate DNA. The modification of these moieties may enhance their activity or sensitivity and facilitate linking the NPs to antibodies via chemical synthesis.

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