Review Article

THE CHEMISTRY, MECHANISM OF ACTION AND BIOLOGICAL PROPERTIES OF CC-1065, A POTENT ANTITUMOR ANTIBIOTIC

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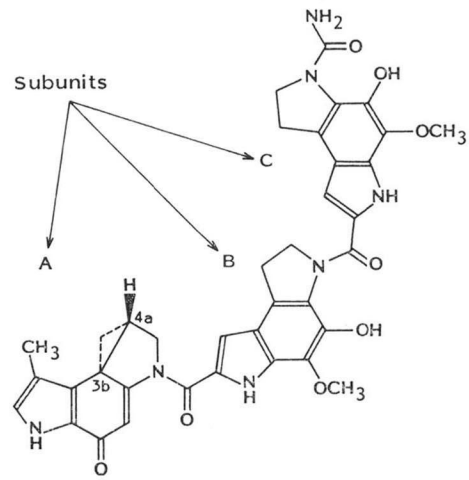
CC-1065 (NSC-298223) is an extremely potent antitumor antibiotic produced by *Streptomyces zelensis* NRRL 11,183. The microorganism producing this antibiotic was isolated by investigators at The Upjohn Company by screening soil cultures for agents displaying both cytotoxic activity against L1210 cells in culture and *in vivo* activity against P388 leukemia in mice[1~3]. The production of CC-1065, its *in vitro* biological activity, microbiological assays and taxonomy have been described[1], and more recently improved methods for production and isolation of this compound have also been published[3]. CC-1065 has also been isolated by scientists in the USSR from *Streptomyces canulus*[4]. Fermentation broths of *S. zelensis* produce only very modest amounts of CC-1065 (approximately 6~9 mg/liter)[3] and precautions need to be taken by laboratory workers involved in the isolation and handling of this potent antitumor agent.

The structure of CC-1065 (Fig. 1) was determined by X-ray crystallography[5,6]. Prior to isolation of a suitable crystalline form of CC-1065 for X-ray diffraction data collection, a chemical degradation product (**1**) obtained by reaction of CC-1065 with ethyl isocyanate in pyridine was crystallized and its structure determined by X-ray crystallography[7]. A closely related compound, PDE I (**2**), was previously isolated from a fermentation broth[8], but is nontoxic in mice at 200 mg/kg. This is in sharp contrast to CC-1065, which is toxic at a dose of 0.1 mg/kg[5]. Recently the products of alkaline and acidic hydrolysis of CC-1065 have been purified and characterized[9].

Fig. 1. Structure of CC-1065.



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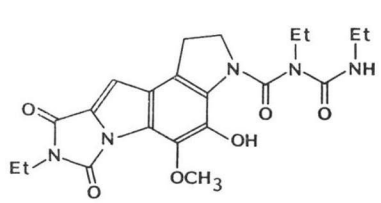
The CC-1065 molecule consists of 3 substituted benzodipyrrole moieties linked by amide bonds. The "A" subunit has a cyclopropyl ring containing the only asymmetric carbons in the molecule. While only the relative configuration of these carbons is available from X-ray data, the absolute configuration has been inferred as 3b*R*, 4a*S* by using DNA as a chiral reagent (*vide infra*)[10]. The "B" and "C" subunits are identical benzodipyrrole moieties. CC-1065 has a right handed twist along its length, due to the out-of-plane distortion of the amide groups between each subunit[6]. The physicochemical properties of CC-1065 have been described[3], and the tentative ^{13}C NMR assignments have been proposed[3,6] with revisions for the "A" subunit[10]. A comprehensive account of the ^{1}H and ^{13}C NMR assignments of CC-1065 and its acetic acid degradation product (**3**) will be presented in due course (SCAHILL, T. A.: Unpublished results).



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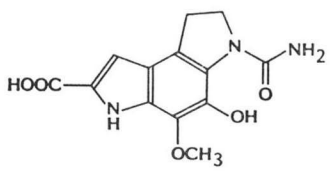

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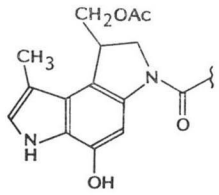

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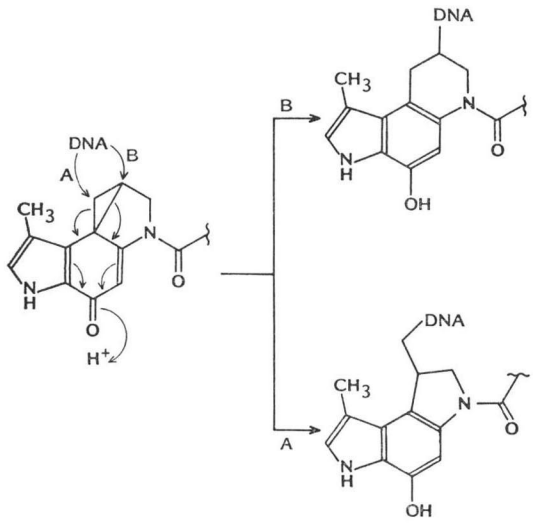
Fig. 2. Possible alkylation mechanisms for reaction of the "A" subunit of CC-1065 with DNA.

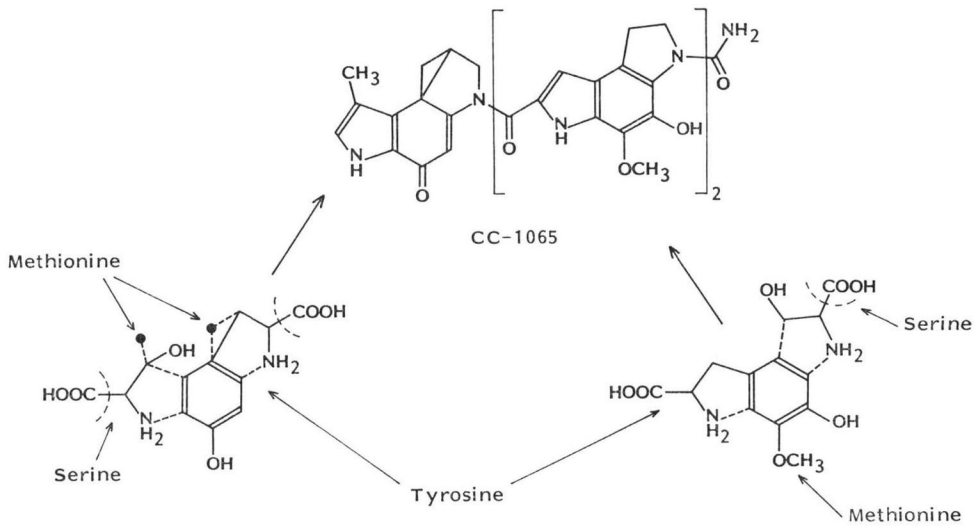


From a biological standpoint CC-1065 has several important chemical features which are crucial for its reaction with DNA. First, the "A" subunit has an alkylating capability (Fig. 2). Second, the right-handed twist along the length of the CC-1065 molecule suggests binding within one of the grooves of DNA in the "B" form. The distribution of hydrophobic and hydrophilic groups along the concave and convex edges of the drug molecule is probably important in facilitating binding of CC-1065 in the minor groove of DNA.

Biosynthesis of CC-1065

The biosynthesis of the antitumor antibiotic CC-1065 has been investigated by radioactive isotope techniques in combination with chemical degradation of CC-1065[11]. Tyrosine, DOPA, serine, and methionine (S-CH_3 group) have been shown to be precursors of CC-1065. Tyrosine is proposed to be a precursor of all three benzodipyrrole subunits, while DOPA appears to be solely incorporated into subunits "B" and "C". Serine is postulated to contribute 2-carbon units, with loss of C-1, to all three subunits of CC-1065. The S-CH_3 group of methionine probably contributes four C-1 units to CC-1065 of which one is incorporated with considerable loss of tritium, most probably into the cyclopropane ring of subunit "A". The precursors of CC-1065 and their postulated labeling patterns in CC-1065 are shown in Fig. 3.

Fig. 3. Proposed biosynthetic labeling pattern of precursors in CC-1065.

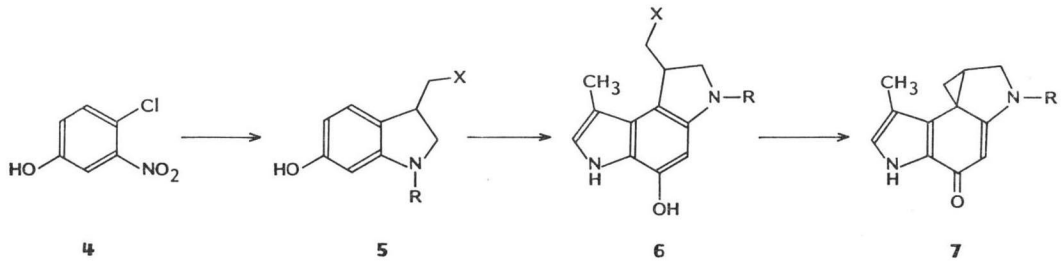


Synthesis of CC-1065

Both the remarkable potency of CC-1065 and its unique structure make this drug molecule an attractive target for synthesis. The development of synthetic methodology is of course a prerequisite for the production of analogs of CC-1065. Synthetic analogs of CC-1065 will be particularly important in attempts to determine the molecular basis for the potency of CC-1065 and also to provide possible means to circumvent the hepatotoxicity of CC-1065[12] while attempting to retain the antitumor activity.

Synthesis of the "A" Subunit

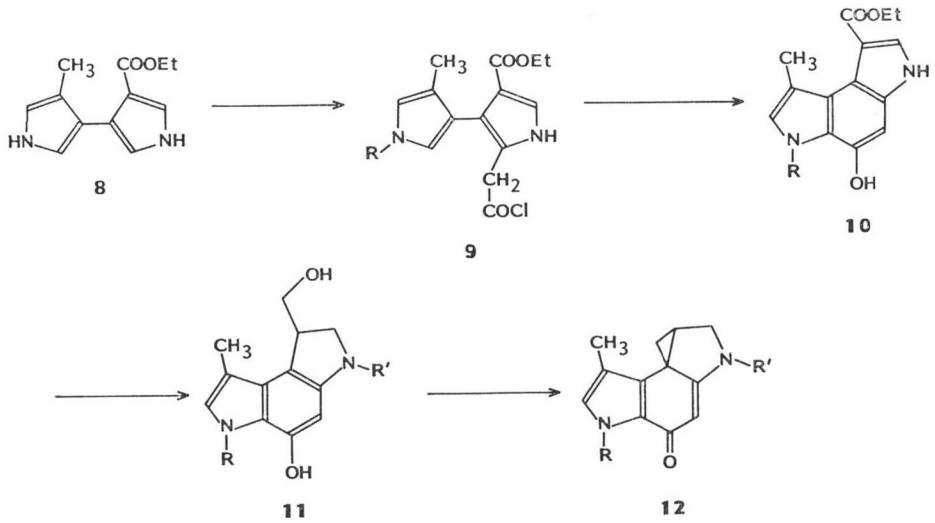
The "A" subunit containing the alkylating cyclopropane ring perhaps presents the greatest challenge to the synthetic chemist. Several synthetic strategies have been described which lead to the moiety. WIERENGA[13] was the first to publish a pathway in which 4-chloro-3-nitroanisole (**4**) was converted in a regiospecific synthesis to the 6-hydroxyindoline (**5**). The aromatic ring of **5** was then nitrated and reduced to the amine to direct the regiospecific introduction of the 8-methylindolic moiety of **6**. Through an intramolecular *para* alkylation reaction **6** was converted to cyclopropylspirocyclohexadienone (**7**), the "A" subunit of CC-1065.



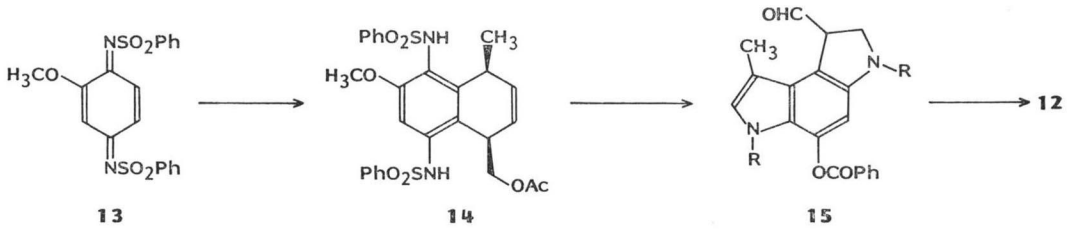
4 **5** **6** **7**

MAGNUS and GALLAGHER[14] have published a synthesis using the 3,3-bipyrrole (**8**) which was produced by sequential conjugate addition of *p*-tolylsulfonylmethyl isocyanide to ethyl sorbate. The

3,3-bipyrrole was then converted through a series of reactions into the acid chloride (**9**) which was immediately treated with $SnCl_4$ - CH_2Cl_2 at $-78°C$ to give the tricyclic phenol (**10**). Selective reduction of **10** leads to the formation of the alcohol (**11**) which was converted by the intramolecular MITSUNOBU reaction into the cyclopropylspirocyclo-hexadienone (**12**), the "A" subunit of CC-1065.



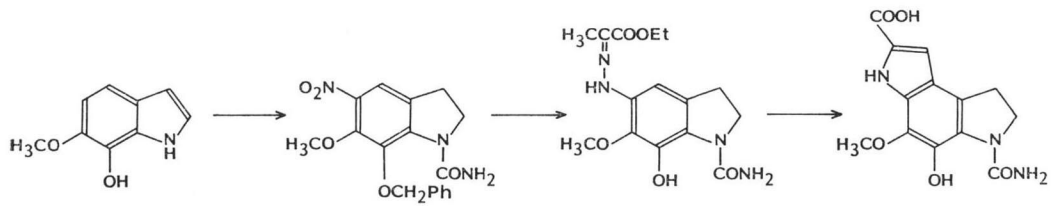
Most recently, KRAUS *et al.*[15] reacted the diene (**13**) regioselectively with 2-acetoxypento-1,3-diene, in a DIELS-ALDER reaction, to produce **14**. Necessary protecting group shuffling, ozonolysis and cyclization afforded **15** which was converted to **12** in an overall yield of 5% from **13**.



Another interesting attempt has been made to synthesize the "A" subunit of CC-1065 by SUNDBERG and NISHIGUCHI[16], utilized an intramolecular carbenoid photoaddition route which gave cyclopropane-6-spiroindol-7-one but not the cyclopropane-4-spiroindol-7-one of CC-1065.

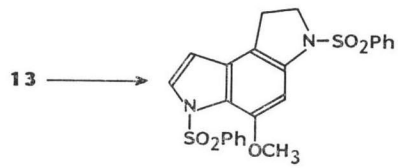
Synthesis of the "B" and "C" Subunits

At least five synthetic strategies leading to the identical "B" and "C" subunits of CC-1065 have been developed. Japanese workers in 1979 synthesized PDE-I[17], which is similar in structure to the "B" and "C" subunits of CC-1065 by converting 7-hydroxy-6-methoxy-indole (**16**) into 7-benzyloxy-1-carbamoyl-2,3-dihydro-6-methoxy-5-nitro indole (**17**). The nitro group of **17** was then reduced and converted into the hydrazone (**18**). The hydrazone was converted through FISHER cyclization followed by deesterification to **19**, the "B" and "C" subunits of CC-1065.



16 17 18 19

KRAUS and YUE[18] have synthesized the benzodipyrrole (**20**) in seven steps with a 29% overall yield from **13**, utilizing a synthetic route similar to the one they used to synthesize **12** reported before.



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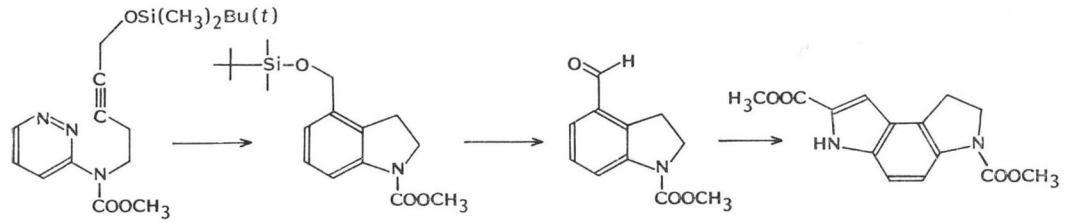
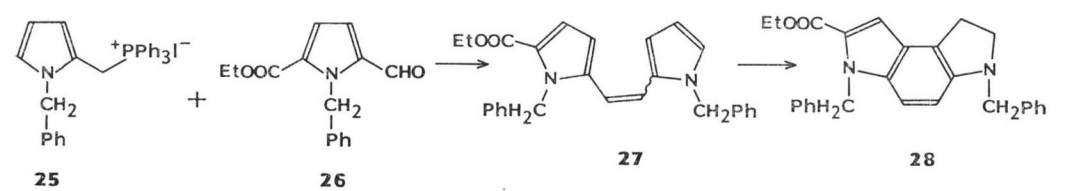
BOGER and COLEMAN[19] have reported a synthetic pathway in which an intramolecular DIELS-ALDER reaction of alkyne 1,2-diazine (**21**) gave the *tert*-butyldimethylsilyl-protected indoline (**22**). This was then deprotected and oxidized to the aldehyde (**23**) which was condensed with methylazidoacetate followed by mild thermolysis to give *N*-carbomethoxy-5-carbomethoxypyrrolo(3,2e)indoline (**24**). This strategy is now being applied toward the synthesis of the subunits of CC-1065.



21 22 23 24

RAWAL and CAVA[20] have developed a photochemical strategy leading towards the synthesis of CC-1065 and its analogues. The trimethylammonium iodide was converted to the phosphonium salt (**25**) and condensed with ethyl *N*-benzyl-5-formylpyrrole-2-carboxylate (**26**) through a WITTIG olefination reaction to give the light sensitive olefin (**27**). Oxidative photocyclization of **27** followed by a mild reduction gave the dideoxy-analog (**28**) of the CC-1065 "B" and "C" subunits. This procedure has been used to synthesize PDE-type units which contain other hetero-atoms and is currently being applied toward the synthesis of CC-1065 subunits.



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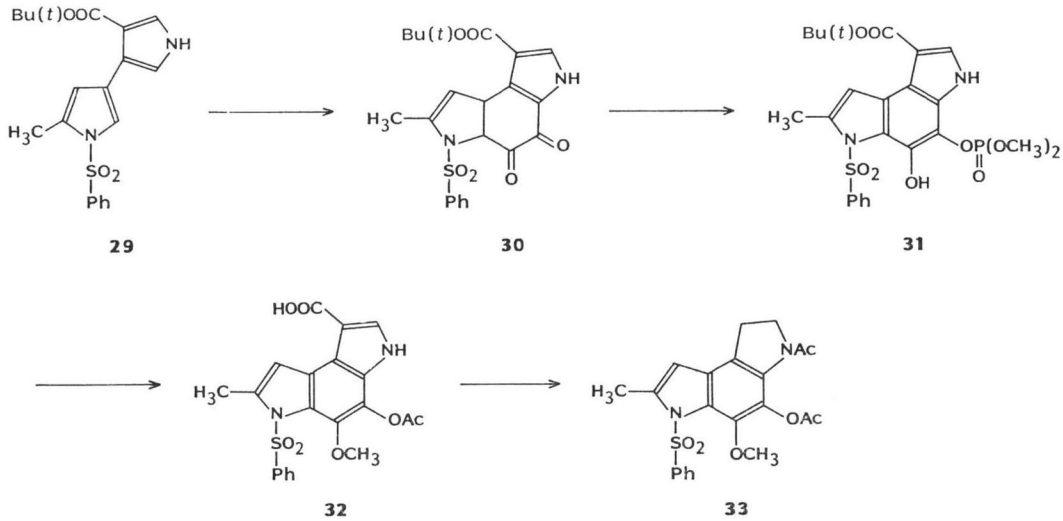
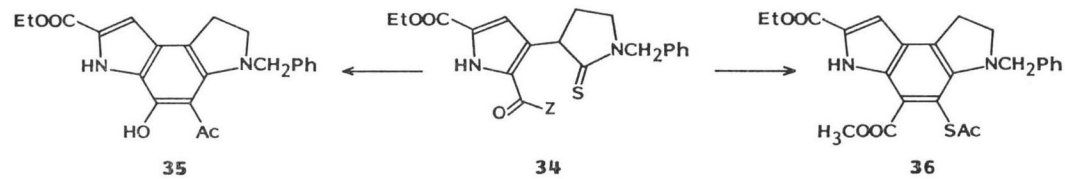
HALAZY and MAGNUS[21,22] have recently reported their strategy for the synthesis of the key features of the "B" and "C" subunits. The bipyrrole (**29**) was made by standard methods[14] and treated with oxalyl chloride to give the *o*-quinone (**30**). The reduction of **30** with $P(OCH_3)_3$ followed by hydrolysis gave the phenolic phosphate ester (**31**). Cyclic oxaphospholene was used for the *O*-methylation of **31** followed by alkaline conditions for the removal of the phosphate ester. The phenol was reprotected and the *tert*-butyl ester removed to give the acid (**32**). The acid was decarboxylated, the unsubstituted double bond was reduced, and the amine protected to give the diacetate (**33**).



SUNDBERG and PEARCE[23] have most recently described their strategy for the synthesis of several compounds related to the "B" and "C" subunits of CC-1065. The key intermediate in the synthesis was a 3-(3-pyrrolyl)thiopyrrolidone (**34**) which could cyclize by one of two possible routes depending on the electronic character of Z. If Z was an electron withdrawing group then the cyclization proceeded through an aldol-type condensation reaction but, if Z was a good leaving group the cyclization proceeded through an Eschenmoser sulfur extrusion type reaction. Both cyclization routes were clearly illustrated by the synthesis of **35** and **36** from **34**, the former by the aldol-type condensation route, where Z is $CH=C(OH)CH_3$ and the latter by the synthesis of **36**, by the Eschenmoser process, where Z is CHN_2.



Mechanism of Action of CC-1065

Intensive study has been directed at determining the mechanism of action of CC-1065. To date, experimental results point strongly to DNA as the cellular target for CC-1065. It has been established that, upon binding, CC-1065 exerts profound effects on various properties of the DNA-helix and its ability to function as a template for DNA and RNA polymerases. Furthermore, the DNA sequence

specificity of drug binding has been determined and the structure of the CC-1065-DNA adduct has been elucidated. Knowledge at the molecular level of the mechanism of action of CC-1065 can be used to postulate reasons for the remarkable biological potency of this agent.

DNA is the Cellular Target for CC-1065

Evidence that DNA is the cellular target for CC-1065 is found in results from experiments in which the interaction of CC-1065 with various biological macromolecules was examined by 1) difference circular dichromism (CD), 2) analysis of differential drug toxicity following premixing of the drug with various biopolymers, and 3) Sephadex chromatography and UV absorbance measurements.

CD measurements demonstrated that difference spectra could only be observed upon combining CC-1065 with double-stranded DNA[24], while little or no interaction between CC-1065 and RNA, or protein was observed. The ability of CC-1065 to bind to various DNA polymers was assessed with the assumption that the induced ellipticity in the CC-1065 electronic transition upon binding of the drug to DNA is directly proportional to the binding affinity of the drug for that DNA polymer[6,25]. Results (Table 1) demonstrate clearly that CC-1065 requires AT-containing, double-stranded DNA for binding[6,25].

Table 1. Circular dichroism binding results[25].

Polymer	ΔCD (mdeg llipticity) at 388 nm
Poly(dA·dT)	62
Poly(dA)·poly(dT)	52
Calf thymus DNA	50
T4 Phage DNA	49
Poly(dG·dC)	15
Heat denatured calf thymus DNA	5
Poly(dG)·poly(dC)	−2
Poly(dA)	−2
Poly(dT)	−3

Additional evidence that DNA is the cellular target for CC-1065 was obtained in studies in which CC-1065 potency (measured as the degree of drug-induced inhibition of L1210 cell growth) was assessed both before and after premixing of the drug with various biological macromolecules, including calf thymus DNA, yeast RNA and human serum albumin[24]. Results demonstrated that a strong reduction in CC-1065 potency occured upon premixing of the drug with calf thymus DNA. CC-1065 potency was reduced to a small degree upon premixing of the drug with high levels of albumin. This decrease in CC-1065 potency was apparently due to a weak, reversible interaction between the drug and protein[24]. Such non-covalent interactions are likely the basis for the weak inhibition of thymidine kinase activity by CC-1065[24].

The marked retention of CC-1065 on Sephadex and the strong UV-absorbing properties (E_{365} = 48,000 M^{-1} cm^{-1})[3] of the drug allowed saturation binding experiments to be carried out. Results demonstrated that, after a 4-hour incubation at 37°C, binding levels occurred at approximately 1 drug/11 base pairs of DNA, while after prolonged incubations, levels of 1 drug/7 base pairs were achieved[25].

Properties of CC-1065-modified DNA

The binding of CC-1065 to DNA polymers has been found to result in profound stabilization of the double helix. This is illustrated by the results of experiments examining the effects of drug binding on 1) DNA-helix melting temperature, 2) DNA susceptibility to S1 nuclease digestion, and 3) the ability of ethidium bromide to intercalate and unwind supercoiled DNA. Results suggest that over-stabilization of the DNA-helix may be important in the mechanism of action of CC-1065[25].

The effect of CC-1065 on the thermal melting transition of DNA has been studied[25]. The results

Table 2. Effect of CC-1065 on the thermal melting transition of DNA[25].

DNA	CC-1065: DNA nucleotide	⊿Tm
Calf thymus DNA	1: 7	29
	1: 14	22
	1: 28	15
	1: 56	7
	1: 112	2.5
Poly(dA·dT)	1: 7	51

of these experiments (Table 2) demonstrated that CC-1065 binding resulted in a marked increase in DNA melting temperatures. For example, in the case of saturation binding to poly(dA·dT), an increase of 51°C in the melting temperature was observed.

In experiments carried out to determine the effect of CC-1065 binding on the sensitivity of DNA to digestion by S1 nuclease (which degrades single stranded DNA), it was determined that drug binding greatly inhibited the action of S1 nuclease[25]. This was interpreted to mean that CC-1065 suppresses local, transient DNA strand separation ("DNA breathing") in much the same way as was observed for the inhibition of thermally-induced DNA strand separation (see earlier discussion).

Studies using supercoiled DNA revealed that CC-1065 inhibited ethidium-induced unwinding as measured by alterations in electrophoretic mobility. This may have been due either to direct displacement of ethidium by CC-1065 or to a CC-1065-induced alteration in the DNA-helix such that subsequent intercalation by ethidium was discouraged. It was found that in additional experiments carried out at 37°C, CC-1065 itself was unable to alter the electrophoretic mobility of supercoiled DNA[25]. This was interpreted to mean that 1) CC-1065 does not bind to DNA by intercalation and 2) CC-1065 does not introduce strand breaks into the DNA at 37°C. However, treatment of the CC-1065-DNA complex to elevated temperatures (70°C and higher) resulted in the rapid formation of single-strand breaks, as determined by the conversion of Form I DNA to Form II DNA. At higher drug: nucleotide ratios, the appearance of Form III DNA could be detected upon thermal treatment of drug-bound DNA[25]. This was most likely due to the occurrence of two closely spaced single strand breaks on opposite strands, since additional experimentation (see later discussion) failed to detect evidence of double-strand breakage by single molecules of CC-1065.

Effects of CC-1065 on Macromolecular Synthesis and the Cell Cycle

CC-1065 effects on cellular macromolecule synthesis were examined in L1210 cells *in vitro* where it was demonstrated that CC-1065 dramatically inhibits DNA synthesis[25]. A 50% inhibition of cellular DNA synthesis was observed at a CC-1065 concentration of 7 pmol/ml. RNA synthesis was affected to a lesser degree and protein synthesis was the least affected. The inhibition of DNA synthesis by CC-1065 was examined *in vitro* using DNA polymerase-α[24]. Inhibition of DNA polymerase-α activity by CC-1065 was attributed to the ability of the drug to interact with the DNA template.

The profound effects of CC-1065 on DNA synthesis are somewhat surprising when considered in relation to studies on the cell cycle specificity of the drug where despite the potent inhibitory effects of CC-1065 on DNA synthesis, it was observed that S phase cells are the most resistant to the lethal effects of the drug[26]. Cells in G_2 and M phase were the most sensitive. It was speculated that the absence of a nuclear membrane in mitotic cells might allow more drug to reach the DNA[26]. The effects of CC-1065 on the progression of the cell cycle[26] revealed that CC-1065 blocked cells in G_2+M. No effect was noted in the transitions of M to G_1 or G_1 to S. Even though CC-1065 had a potent inhibitory effect on DNA synthesis, cells were able to pass slowly (50% of control rate) through S

phase at drug concentrations (1.4 nM CC-1065) which were sufficient to arrest the cell cycle at G_2 and M. At higher drug concentrations (7.1 nM CC-1065), blockage was observed in S phase. Conceivably, at low doses of CC-1065, only a few drug-DNA adducts were formed and the majority of scheduled DNA synthesis was able to proceed. However, the few adducts which did form were capable of arresting mitosis. At higher drug doses, enough CC-1065 adducts formed on the DNA template to block the bulk of DNA synthesis. This suggests that, in addition to its profound effects on DNA synthesis, CC-1065 has an enormous impact on mitotic events.

Structure of the CC-1065-DNA Adduct

The structure of the CC-1065-DNA adduct has been elucidated in a series of experiments. Results demonstrate that CC-1065 binds covalently to N3 of adenine with the resultant drug-adduct lying snugly cradled in the minor groove of DNA. The drug binds with defined stereochemistry and orientation in the minor groove and displays a 4~5 base pair-DNA sequence specificity.

CC-1065 Binds in the Minor Groove of DNA

Three lines of evidence indicate that CC-1065 binds within the minor groove of DNA:

1) Competitive binding experiments between CC-1065 and netropsin demonstrated that netropsin, which binds non-covalently in the minor groove of AT-rich regions of DNA[27], was able to inhibit subsequent binding by CC-1065[6]. Significantly, CC-1065 was eventually able to displace bound netropsin, but netropsin was unable to displace bound CC-1065. This indicated that CC-1065 might bind covalently to DNA[6]. Results of an additional competitive binding experiment using CC-1065 and anthramycin[25] were similar, although some differences were noted. Pre-incubation of the DNA with anthramycin (which binds covalently though N2 of guanine in the minor groove of DNA[28,29]) resulted in an inhibition of subsequent binding by CC-1065, but only at higher anthramycin binding ratios. This result suggested that, while anthramycin and CC-1065 both bind in the minor groove, the two drugs have different sequence specificities. This was confirmed in subsequent experiments[30,31].

2) As measured by difference CD, CC-1065 was able to react with bacteriophage T4 DNA (65% glucosylated in the major groove) to almost the same extent (Table 1) as with calf thymus DNA (no major groove modifications)[25]. This result indicated that CC-1065 was unlikely to bind in the major groove of DNA.

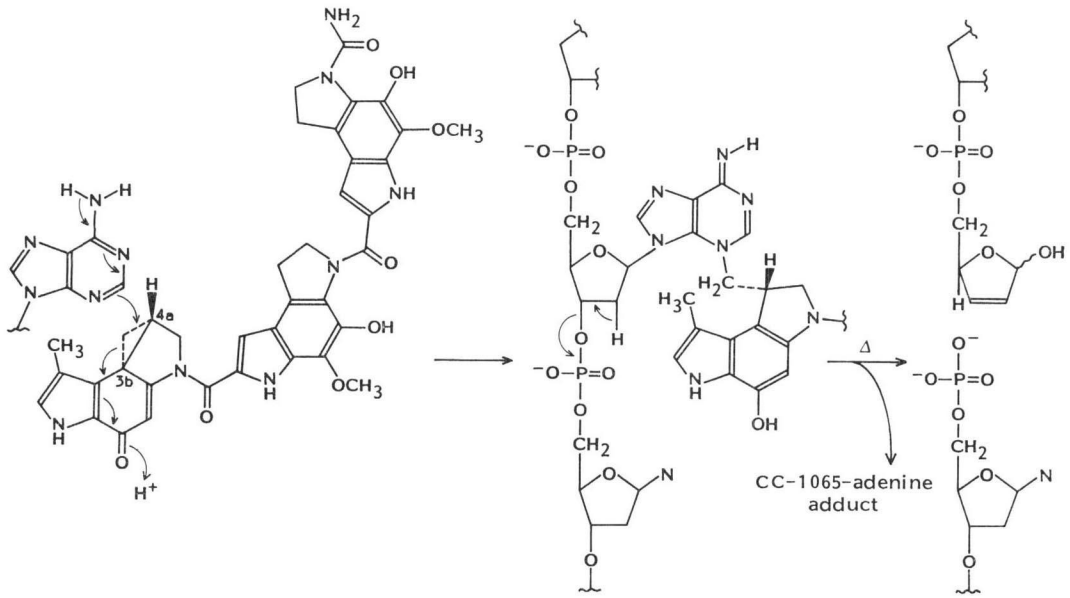
3) Results of alkylation inhibition studies[25] demonstrated that the binding of CC-1065 to DNA produced an inhibition of alkylation by methylnitrosourea and ethylnitrosourea at sites in the minor groove. No such inhibition of alkylation of sites in the major groove was detected. Similar results were obtained in experiments using CC-1065 to inhibit alkylation by methyl methane sulfonate[25].

While the results of these studies do not rule out the possibility that CC-1065 may interact to a small degree with the major groove, the evidence overwhelmingly points to the minor groove as the predominant (if not the exclusive) site of CC-1065 binding by DNA.

CC-1065 Binds Covalently to N3 of Adenine

Initial results from binding experiments using synthetic DNA polymers (Table 1) illustrated the requirement for either adenine or thymine in the reaction of CC-1065 with DNA[6,25]. The key role of adenine was demonstrated by the combined results of several experiments. First, thermal treatment of CC-1065-DNA adducts resulted in the release of a chemically modified CC-1065 chromophore. Up to 85% of this chromophore could be recovered by butanol extraction. The possibility that the

Fig. 4. Reaction of CC-1065 with DNA to form the CC-1065-(N3-adenine)-DNA adduct and the consequences of thermal treatment of CC-1065-DNA adducts.



butanol-solubilized material was linked to a DNA base moiety was examined by using DNA labeled with tritiated deoxyadenine, deoxyguanine, deoxycytidine or thymidine to produce, in separate experiments, labeled DNA samples modified by CC-1065. Heating of these CC-1065-([^{3}H]base)-DNA samples followed by butanol extraction led to only background levels of radioactivity extractable into the butanol, except in the case of the ([^{3}H]adenine)-DNA sample where a 4.3-fold increase of extractable tritium over control was found[10]. Since 1) almost all of the CC-1065 chromophore was released from the DNA by thermal treatment and 2) butanol extraction could recover tritium only in the case of ([^{3}H]adenine)-DNA, it was concluded that the reaction of CC-1065 with DNA involved a covalent linkage of the drug molecule to adenine. The nature of this covalent linkage was examined. The butanol-soluble material was analyzed by ^{13}C and ^{1}H NMR and was identified as the CC-1065-(N3-adenine) adduct depicted in Fig. 4[10]. This result is consistent with earlier data indicating that CC-1065 bound in the minor groove of DNA. This result also demonstrates that alkylation by CC-1065 occurs *via* pathway A in Fig. 2.

Relationship of the CC-1065-directed DNA Strand Break to the Adenine Covalent Binding Site

Thermal treatment (100°C, 30 minutes) of CC-1065-DNA adducts results in the formation of single strand breaks and apurinic (AP) sites on DNA[9,30]. In order to obtain information on the site and possible mechanism for the CC-1065-induced DNA strand break, the relationship of the position of the strand breakage to the AP site was investigated[30]. Examination of Maxam-Gilbert sequencing gels[30] in which 5′-^{32}P singly end-labeled DNA restriction fragments were used gave ambiguous information on the site for CC-1065-induced strand breakage[30]. Firm evidence for the position of thermal cleavage of DNA by the CC-1065-DNA adduct comes from experiments using DNA restriction fragments ^{32}P-labeled on the 3′-terminus rather than the 5′-end. In this case, the CC-1065-induced DNA strand break produced a radiolabeled DNA species which exactly co-migrated with an A band

in the Maxam-Gilbert sequencing lanes. Thus, the CC-1065-induced breakage of DNA yielded a 3′-^{32}P end-labeled DNA fragment (Fig. 4) with a 5′-phosphate terminus. This 5′-phosphate end lies on the 3′-side of the CC-1065-modified deoxyadenosine site (Fig. 4). The identity of this 5′-phosphate group was recently confirmed in experiments[33] using DEAE-cellulose chromatography[34] on DNA fragments released from treatment of CC-1065-poly(dA·dT) adducts following successive thermal and AP-endonuclease II treatment. The inability to label the 3′-terminus of the thermal cleavage product by *Escherichia coli* DNA polymerase I suggests that this 3′-terminus is a modified deoxyribose[33]. These results are consistent with a single thermally-induced β-elimination occurring on the 3′-side of the adenine to which CC-1065 is covalently bound (Fig. 4).

Sequence Specificity of CC-1065

The observation that CC-1065-DNA adducts were associated with the production of single-strand breaks in DNA at elevated temperatures[24] led to the formulation of a strategy to examine the DNA sequence specificity of CC-1065[30]. In these experiments, aliquots of singly ^{32}P end-labeled DNA restriction fragments were subjected either to Maxam-Gilbert sequencing reactions[32] or to incubation with various doses of CC-1065 followed by thermal treatment. In all, DNA restriction fragments consisting of over 1,000 bases were examined for CC-1065 binding sequences. A compilation of the sequences of these and other CC-1065 binding sites revealed the DNA sequence specificity of CC-1065. Two consensus sequences, 5′-PuNTTA* and 5′-AAAAA*, were identified in which CC-1065 bound covalently to A*[30].

Characterization of a Defined CC-1065-oligodeoxynucleotide Adduct

The previously described sequence specificity studies provided insight into a DNA sequence (5′-CGGAGTTAGGGGCG) which should bind one molecule of CC-1065 in an unambiguous manner. This sequence, which contains the CC-1065 adenine binding site within the sequence 5′-TTA, was chemically synthesized together with the complementary strand[35]. CC-1065 reacted with the oligoduplex to produce an adduct that maintained the B-DNA form and had a final CD spectrum similar to those of the CC-1065 complexes formed with calf thymus DNA. The above 14mer was 5′ end-labeled with ^{32}P, annealed with its complementary strand, reacted with CC-1065 and heated. Drug-mediated strand breakage was evaluated on a Maxam-Gilbert sequencing gel. A single DNA strand break occurred in the labeled strands to produce a DNA fragment that migrated as an 8.5mer; subsequent piperidine treatment produced a DNA fragment that migrated as a 7mer. These were the sizes expected from the known binding of CC-1065 at adenine in 5′-TTA of the 14mer sequence[35].

Orientation of CC-1065 in the Minor Groove of DNA and Absolute Stereochemistry of the Cyclopropane Ring of CC-1065

CPK models of the CC-1065-(N3-adenine)-DNA adduct predict that the drug molecule should cover about 5 base pairs[36]. However, since only the relative stereochemistry at C-3b and C-4a is known from X-ray crystallography[6], the polarity of the drug molecule in the minor groove in reference to the covalent binding site could not be predicted. The sequence specificity data confirm the CPK model prediction that CC-1065 should span about 5 base pairs, since this is the extent of the DNA sequence specificity (*i.e.*, 5′-PuNTTA* and 5′-AAAAA* where the drug binds covalently to A*). Furthermore, the strict orientation of the sequence specificity to the 5′-side of the adenine covalent binding site in both subclasses of drug binding sites was interpreted as being indicative of the polarity of

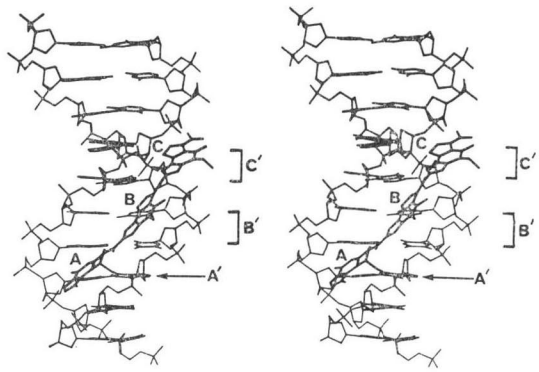
drug binding in the minor groove[10,30]. The CPK model of the CC-1065-(N3-adenine)-DNA adduct can be accommodated to agree with the polarity of drug binding only if the stereochemistry at the C-4a position of the CC-1065-DNA adduct molecule is *S*. Therefore, the absolute stereochemistry of CC-1065 must be 3b*R*,4a*S*[10,30]. This is illustrated in Fig. 1.

Model of the CC-1065-(N3-adenine)-DNA Adduct and Its Use to Rationalize the Chemical and Biological Consequences of CC-1065 Binding to DNA

An Evans and Sutherland computer graphics system was used to prepare a pair of stereo-drawings of the CC-1065-(N3-adenine)-DNA adduct which are shown in Fig. 5[10,30]. The stereo-drawings of the CC-1065-DNA adduct reveal a remarkably snug fit of the drug molecule within the minor groove of DNA. CC-1065 spans the 5 base pair region specified by the CC-1065 DNA sequence specificity. A careful examination of CPK models reveal that the floor of the minor groove is in close proximity to the inside edge of the CC-1065 molecule[36]. The width and depth of the minor groove appear just sufficient for CC-1065 to be accommodated without disruption of the helix or protrusion of the drug molecule outside of the minor groove. The closeness of the fit of CC-1065 within the minor groove of DNA is predictive of the considerable DNA sequence specificity exhibited by CC-1065. It has been suggested that a layer of water molecules could bridge the hydrophilic substituents on the outside edge of CC-1065 with phosphate groups on DNA[30]. This would provide additional stabilizing interactions that would inhibit both DNA melting and intercalation of other agents in the vicinity of the CC-1065-binding site. While, hydrogen bonding may facilitate stabilization of the CC-1065-DNA adduct, it is probably not involved in the original binding mechanism.

Fig. 5. Stereo-drawings of the CC-1065-(N3-adenine)-DNA adduct.

Key: A, B and C are the subunits of CC-1065; A′—adenine covalent binding site for CC-1065; B′—pair of highly conserved bases immediately to 5′-side of the adenine covalent binding site; C′—pair of less well conserved bases at the 5′-end of CC-1065 binding site. The base sequence from top to bottom is 5′-CGGAGTTAGG.



Although the stereo-diagrams do not directly provide a single rational basis to explain the extreme cytotoxic potency of CC-1065, at least three possibilities can be suggested. The remarkable DNA sequence specificity of CC-1065 might lead to selective reactions with a critical target within DNA. However, recent studies with analogs of CC-1065 with lower DNA sequence specificity do not support this theory. Alternatively, CC-1065 might alter DNA-helix or chromatin structure and affect gene expression at critical sites some distance from the actual drug-binding position. Finally, the CC-1065-DNA adduct may represent an intractable lesion to DNA-repair enzyme processes or one that results in detrimental repair and/or the production of a lethal lesion in DNA. Some of these possibilities are presently being explored in our laboratories.

Antitumor Activity

CC-1065 was tested for antitumor activity against a panel of transplantable murine tumors under the auspices of the U.S. National Cancer Institute[37,38]. The spectrum and degree of antitumor efficacy

in murine models were fairly limited[3]. Confirmed activity meeting the NCI "good-activity" criterion (DN-2) was noted only against the ip-implanted B16 melanoma. Confirmed activity meeting the NCI "minimum-activity" criterion (MC-1) was observed against the sc-implanted CD8F1 mammary carcinoma, the ip-implanted colon 26 carcinoma, ip P388 leukemia, and ip L1210 leukemia. CC-1065 given ip was inactive upon initial or confirmation testing against the sc CX-1 colon xenograft, sc colon 38 carcinoma, sc LX-1 lung xenograft, iv Lewis lung carcinoma, and the sc MX-1 breast xenograft. Thus CC-1065 had significant activity only in models where both tumor cells and drug were injected ip.

Increases in life span (ILS) of 81 and 59% were attained in two experiments with daily $\times 9$ ip treatment of mice bearing ip-implanted B16 melanoma. Optimal doses were 33 and 12.5 μg/kg/day, respectively. No long-term survivors were observed. Against the ip P388 leukemia, ILS's of 111 and 70% and no long-term survivors were noted at optimal doses of 30 and 180 μg/kg/day, ip, days 1, 5, 9. In schedule-dependency testing in mice bearing ip-implanted P388, the days 1, 5, 9 schedule was marginally more active than the day 1 only schedule. ILS's were somewhat lower with days $1 \sim 9$ treatment.

BHUYAN *et al.* tested CC-1065 using the *in vitro* human tumor cloning assay[38]. With 1 hour exposure to 0.1 ng/ml, a 70% decrease in tumor-colony forming units (T-CFU) compared to control plates was noted in 1/9 tumors from lung cancer patients, 1/2 pancreatic tumors, 1/1 small bowel tumor, and 1/2 adenocarcinomas of unknown origin. Using $>50\%$ decrease as the criterion, CC-1065 was active against tumor explants from patients with cancer of the breast (3/9), ovary (2/8), pancreas (1/2), multiple myeloma (2/2), stomach (1/1), testis (1/1), small bowel (1/1), and adenocarcinoma of unknown origin (1/2). CC-1065 was not significantly active ($<50\%$ decrease in T-CFU) against tumor explants from one or more patients with melanoma, leukemia, esophageal cancer, non-Hodgkin's lymphoma, colon cancer or sarcoma.

Toxicology

Administration of CC-1065 to non-tumor-bearing mice revealed an unusual delayed form of hepatotoxicity occurring at the doses which prolonged life in tumor-bearing mice[12]. Toxic deaths were delayed *ca.* 50 days after a single iv dose of 12.5 μg/kg and as much as 70 days after 10 μg/kg was given ip. Mouse intravenous LD_{50}'s were 9 μg/kg, single dose, and 0.3 μg/kg/day, five daily doses. Intraperitoneal LD_{50}'s were variable between experiments, ranging from $0.53 \sim 6.90$ μg/kg, single dose. Mice treated with high doses iv died within 12 days with frank hepatic necrosis, whereas delayed deaths at lower doses were associated with ultrastructural changes in hepatic mitochondria. This suggested that separate mechanisms of hepatotoxicity were operative at high and low dose ranges. In lower dose ip-treated mice (moribund at day 30), extensive visceral adhesions involving the alimentary canal and the liver were observed. In this respect, CC-1065 appeared to be similar to VM-26 and various antileukemic phthalanilides, which caused a chemical form of peritonitis when administered ip[39,40].

Attempts to prevent the delayed toxicity of CC-1065 in the mouse by treatment with WR-2721, *N*-acetylcysteine, and agents inducing cytochrome P-450 metabolism were unsuccessful. No effect on LD_{50}'s or times of death were noted. Intravenous administration of lethal doses of CC-1065 to rabbits resulted in a pattern of toxicity similar to that seen in mice. Some deaths were delayed beyond 20 days and gross and microscopic evidence of liver damage was observed.

Conclusions

CC-1065 is a structurally and biosynthetically unique antitumor antibiotic that is believed to exert its potent antitumor activity by binding covalently to DNA through N3 of adenine. Although the spectrum and degree of antitumor efficacy in murine models is fairly limited, CC-1065 is one of the most potent compounds on a molar basis to be evaluated for antitumor activity. CC-1065 causes an unusual delayed form of hepatotoxicity at doses which prolong life in tumor-bearing animals.

Several synthetic strategies have been developed to produce the alkylating "A" subunit of CC-1065, which is required for antitumor activity. Based upon one of these synthetic schemes[13], analogs of CC-1065 have recently been made available for antitumor and cytotoxicity evaluations[41,42]. These analogs, which retain the "A" subunit but have modified "B" and "C" subunits, maintain the high level of biological potency in antitumor models while having reduced hepatotoxicity.

CC-1065 is one of a number of potent antitumor antibiotics which bind covalently in the minor groove of DNA without major distortion of the helix. The anthramycins are the other major group of compounds which bind covalently to DNA through a minor groove bindings site, in this case N2 of guanine[28,29]. Whether the potent biological effects of drugs that bind covalently in the minor groove are mediated through a common mechanism or not is unknown. We suggest that a common feature of CC-1065 and the anthramycins is the ability to form DNA adducts which may prove difficult to repair because of the nondistortive and stabilizing effects of drug binding.

The DNA adduct formed with CC-1065 is comparatively well characterized in a three dimensional sense. The 4~5 base pair sequence specificity can be rationalized by the computer-generated stereodiagrams of the CC-1065-DNA adduct. Our knowledge of the sequence specificity of CC-1065 has allowed us to synthesize oligodeoxynucleotides of defined sequence which form unique adducts with CC-1065. Our present work is concerned with the preparation of defined CC-1065-adducts in much longer pieces of DNA for structural and biochemical studies.

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