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Perspective

The Enediyne Antibiotics

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Introduction

In 1987 the announcement of the structures of two new classes of antitumor antibiotics, the calicheamicins[1] and esperamicins,[2,3] aroused a great deal of interest in chemical and biological circles. The newly discovered compounds combined unprecedented molecular structures with striking biological activities. At the same time, a remarkable mechanism of action was proposed for the molecules to account for their phenomenal biological profiles.[1−3] The molecules contained a (*Z*)-1,5-diyn-3-ene unit embedded within their complex architectural frameworks, and it was proposed that these molecules delivered the enediyne portion within the minor groove of a target cell's DNA and then initiated a series of reactions leading to cycloaromatization of the enediyne and 1,4-benzenoid diradical formation (Figure 1).[4] The highly reactive 1,4-benzenoid diradical would then be perfectly positioned to strip hydrogen atoms from the sugar phosphate backbone of adjacent strands of DNA causing scission of the DNA double helix. The related natural product neocarzinostatin, containing a chromophore whose structure had been elucidated 2 years previously,[5] was now viewed with renewed interest because of its structural, biological, and mechanistic similarities with the newly discovered enediynes. The ensuing years have seen the emergence of further classes of enediyne antibiotics—the dynemicins in 1989,[6] kedarcidin in 1992,[7] and C-1027[8,9] and maduropeptin[10] in 1993. No doubt the coming years will reveal other, as yet undiscovered, related systems.

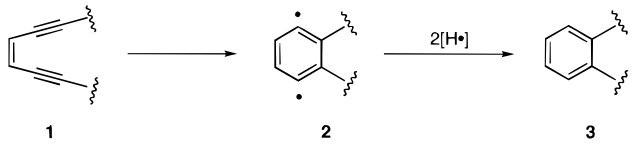

Figure 1. Cycloaromatization of a (*Z*)-1,5-diyn-3-ene system, a reaction first studied by Robert Bergman in the 1970s and which is key to the mechanism of action of the enediyne antibiotics.[4]

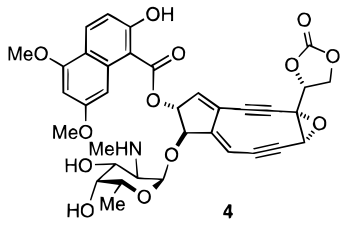

Figure 2. Structure of the neocarzinostatin chromophore (**4**).

Neocarzinostatin

Neocarzinostatin (NCS) first reported by Ishida *et al.* in 1965,[11] is a 1:1 noncovalently associated mixture of a protein component (NCS apoprotein) and a chromophoric molecule (NCS chromophore, Figure 2). The mixture was separated somewhat later into its component parts and eventually characterized structurally. The chromophoric component was shown in 1985 to have the novel bicyclic polyeneyne skeleton **4** by Edo *et al.*,[5] the apoprotein has been characterized as a 113 amino acid polypeptide based upon the gene base sequence[12] and apoprotein crystal structure,[13] the three-dimensional solution structure of intact neocarzinostatin has been determined by Hirama *et al.* using 2 D NMR techniques,[14] and the crystal structure of holo-NCS has been reported by the groups of Myers and Rees.[15] Until

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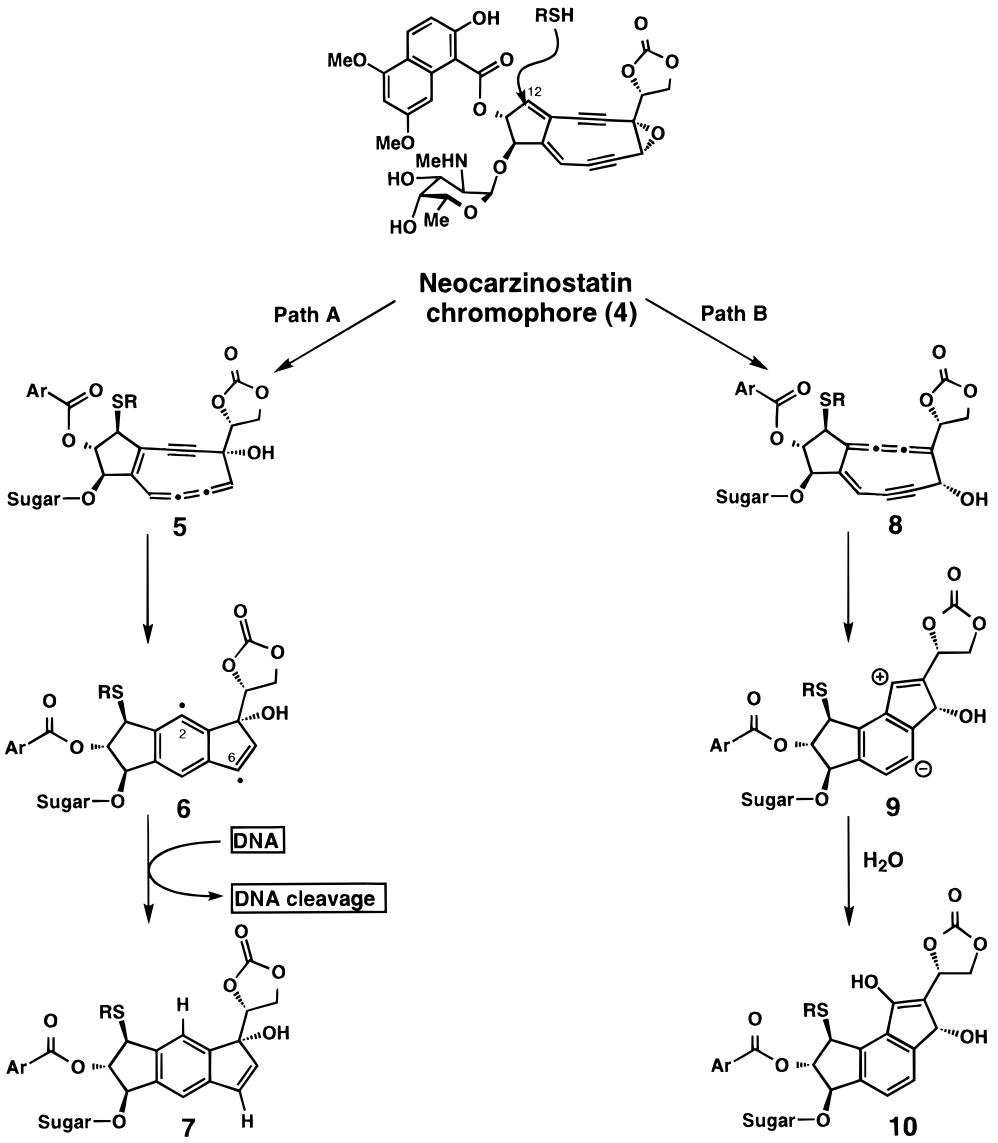


Figure 3. Triggering pathways for the neocarzinostatin chromophore (**4**).

recently it appeared that the mechanism-based biological activity of NCS resides primarily in the chromophore, while the apoprotein serves to both stabilize the chemically sensitive chromophore and act as a transporter. Recent studies, however, suggest that the apoprotein may also contribute actively to the cytotoxicity through selective proteolytic activity.[16] The NCS apoprotein binds tightly and specifically to NCS chromophore ($K_D = 1 \times 10^{-10}$ M)[17] and delivers the active chromophore to its target, DNA, by controlled release.[18] The biological activities of NCS include potent antitumor and antibacterial actions and are exerted by DNA cleavage. The DNA-damaging activity of the free NCS chromophore results primarily in single-strand DNA cuts and proceeds *via* an oxygen-dependent reaction.[19] Thiols[20] and UV radiation[21] greatly enhance the DNA-cleaving properties of the NCS chromophore.

The mechanism by which the NCS chromophore exerts its DNA-damaging properties was first suggested in 1987.[22] According to this proposal (Figure 3, path A) the cascade of reactions leading to DNA damage is initiated by stereospecific nucleophilic attack at C(12). This triggering event is accompanied by rearrangement of the ring skeleton with epoxide opening and formation of cumulene **5** as shown in Figure 3. This highly strained and reactive intermediate then undergoes rapid cycloaromatization to form diradical **6**, which proceeds to attack DNA by hydrogen atom abstraction resulting in **7**. Corroboration of this scenario has been provided by using $HSCH_2CO_2Me$[22–25] and $NaBH_4$[25] as nucleophiles in *in vitro* experiments. The cumulene intermediate **5** has been observed by NMR at low temperature,[24] and the methyl thioglycolate adduct has been isolated and fully characterized, including its absolute configuration.[24] Evidence that the basic methylamino side chain on the sugar residue assists in the thiol addition at C(12) through base catalysis was provided by Myers who derivatized the amine as the corresponding nitrosamine; the NCS chromophore derivative thus produced was inert to thiols below 0 °C.[26] Valuable additional information has been provided by the three-dimensional solution structure of intact NCS[14]—the aminomethyl group of the sugar appendage is forced into close proximity to C(12) (4.3 Å) due to a salt bridge with Asp 33, suggesting that nucleophilic attack at C(12) may be assisted by this basic nitrogen, and together with additional steric hindrance at C(12) from the side chains of Ser 98, Asp 33, and Phe 52 and the positioning of the epoxide in a hydrophobic pocket away from an acid catalyst, this suggests how the apoprotein serves to

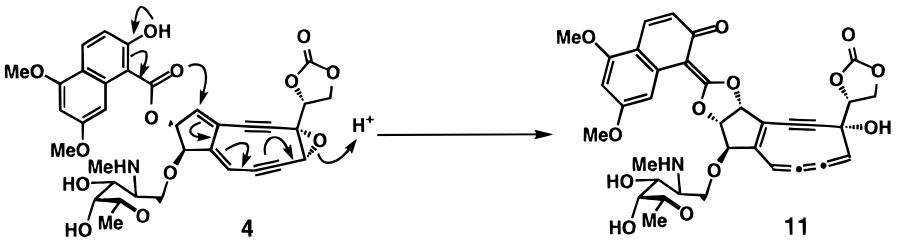


Figure 4. Disputed participation of the α-hydroxynaphthoate in the activation of neocarzinostatin chromophore (**4**).

stabilize the chromophore. Furthermore, the bicyclic core lies on a disulfide bridge which may stabilize the strained unsaturated system of the chromophore through a $HOMO_{dienediyne}-LUMO_{disulfide}$ interaction. This disulfide bridge is conserved in all chromoprotein antibiotics and may be a common stabilizing feature. A second, distinct, cycloaromatization pathway has been recently observed when the NCS chromophore is incubated with 2-mercaptoethanol in the presence of the apoprotein in which the zwitterionic intermediate **9** (Figure 3, path B) is indicated,[27,28] although this mechanism probably does not operate for the free chromophore. Since it is thought likely that dissociation of the NCS chromophore from the apoprotein and subsequent DNA binding precedes activation of the chromophore, the biological relevance of this second mechanism seems dubious, and Goldberg *et al*. have reported that this pathway is not responsible for DNA cleavage.[29]

It is generally accepted that the NCS chromophore intercalates into DNA *via* its naphthoate side chain, which positions the rest of the molecule within the minor groove.[30−32] Another role for the α-hydroxynaphthoate was suggested in the activation of the NCS chromophore involving participation of the hydroxy ester (at C(12)) in epoxide opening and cumulene formation (Figure 4).[33] However, this has been disputed by molecular modeling studies which indicate that a significant structural change of the NCS chromophore−DNA complex would have to occur for this proposal to be operative.[34]

Much work has gone into identifying the details of DNA damage by the NCS chromophore diradical **6**. It has been demonstrated that at least 80% of the DNA cleavage leads to the 5′-aldehyde of A and T residues selectively.[35] The chemistry leading to these breaks involves hydrogen atom abstraction from C(5′) of deoxyribose and reaction with molecular oxygen as outlined in Figure 5. Less than 20% of the strand breaks result from hydrogen atom abstraction at C(4′)[36−40] and C(1′)[37] (Figures 6 and 7). Goldberg has shown that the radical at C(2) of **6** is particularly susceptible to both internal and external quenching (up to 70% under physiological conditions),[41] accounting for the observation that the NCS chromophore effects primarily single-stranded DNA cuts by the C(6) radical at C(5′) of deoxyribose, whereas those double stranded lesions which are observed involve additional hydrogen abstraction by the C(2) radical from C(1′) or C(4′) of the deoxyribose on the complementary strand. Further insight into the interaction of the NCS chromophore with DNA has recently been provided with the observation that a thiol-independent cleavage mode is possible with single-stranded DNA bulges (regions where double-stranded structures are generated intramolecularly).[42] These findings imply that the DNA in these cases is an active participant in its own destruction, since DNAs containing point mutations which disrupt the bulge are not cleavage substrates.

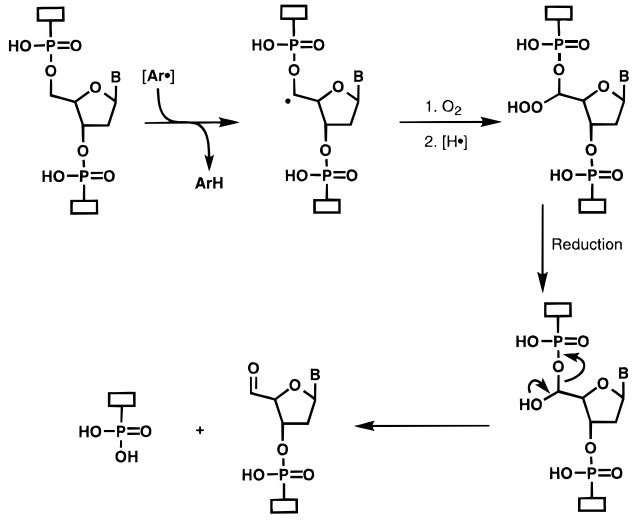


Figure 5. DNA cleavage by C(5′) hydrogen atom abstraction.

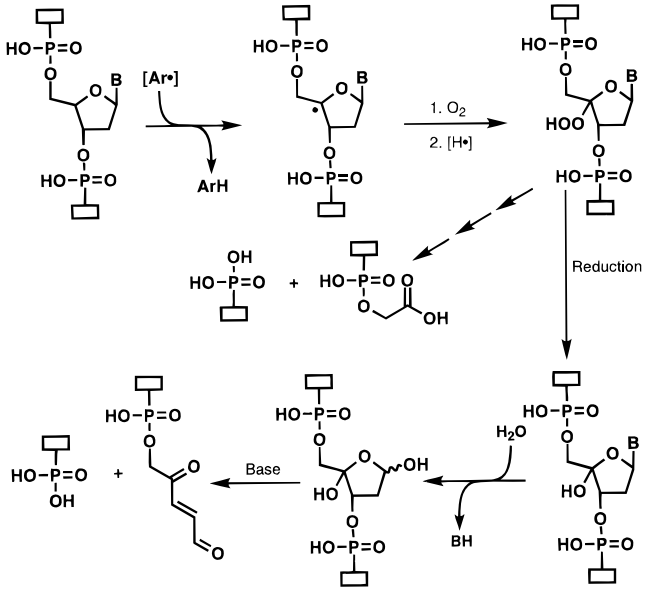


Figure 6. DNA cleavage by C(4′) hydrogen atom abstraction.

The Calicheamicins and Esperamicins

The calicheamicins (Table 1) are a family of enediyne antibiotics isolated from *Micromonospora echinospora* spp. *calichensis* and were identified in 1986[1] through a program aimed at identifying microbial fermentation products active in the biochemical induction assay (an assay exquisitely sensitive to certain DNA-damaging antitumor agents). Calicheamicin γ_1^I (**12**) is the most prominent member of this class of compounds. The

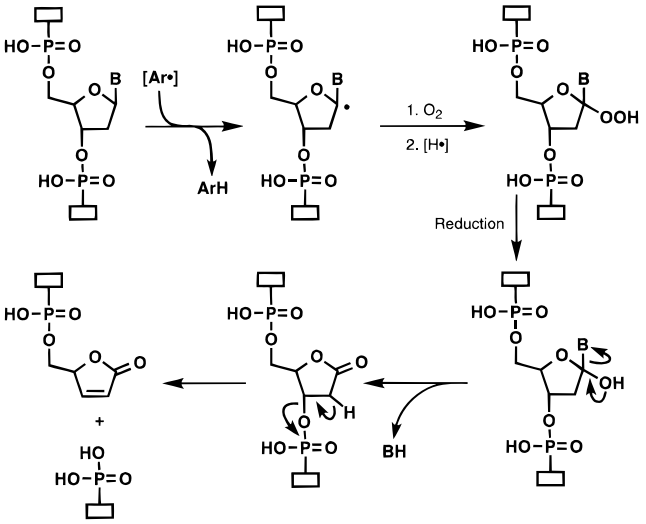
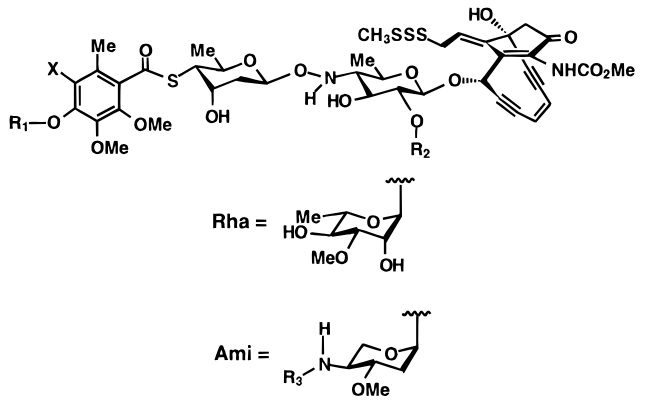


Figure 7. DNA cleavage by C(1′) hydrogen atom abstraction.

Table 1. Calicheamicin Family



calicheamicin	X	R_1	R_2	R_3
calicheamicin β_1^{Br}	Br	Rha	Ami	$CHMe_2$
calicheamicin γ_1^{Br}	Br	Rha	Ami	Et
calicheamicin α_1^{I}	I	H	Ami	Et
calicheamicin α_3^{I}	I	Rha	H	
calicheamicin β_1^{I}	I	Rha	Ami	$CHMe_2$
calicheamicin γ_1^{I} (**12**)	I	Rha	Ami	Et
calicheamicin δ_1^{I}	I	Rha	Ami	Me

iodine-containing calicheamicins were discovered when sodium iodide was added to the fermentation broth, resulting not only in the new compounds containing iodine rather than bromine but also in much improved yields.[43,44] The calicheamicins are active in the biochemical induction assay at concentrations below 1 pg/mL, extremely active against Gram-positive bacteria, and highly active against Gram-negative bacteria.[43,44] Most importantly, they exhibit extraordinary potency against murine tumors such as P338 and L1210 leukemias and solid neoplasms such as colon 26 and B-16 melanoma with optimum doses of 0.15−5 μg/kg.[45] These compounds are thought to exert their biological activities by damaging DNA. Indeed, the calicheamicins are highly potent DNA-cleaving agents giving rise primarily to sequence specific double-strand cuts.[46,47]

Calicheamicin γ_1^{I} (**12**) contains two distinct structural regions, each playing a specific role in its biological activity. The larger of the two consists of an extended sugar residue comprising four monosaccharide units and one hexasubstituted benzene ring which are joined together through a highly unusual series of glycosidic, thioester, and hydroxylamine linkages. The second structural region, the aglycon (termed calicheamicinone), contains a compact, highly functionalized bicyclic core housing a strained enediyne unit within a bridging 10-membered ring. The structure of the drug, including its absolute configuration, has been confirmed by total synthesis (Nicolaou *et al.*).[48,49]

The aryltetrasaccharide serves to deliver the drug to its target, binding tightly in the minor groove of double-helical DNA and displaying high specificity for sequences such as 5′-TCCT-3′ and 5-TTTT-3′ through significant hydrophobic interactions and other forces.[46,47,50−58] This binding is thought to be facilitated by substantial preorganization of the oligosaccharide into a rigid, extended conformation[53] with the unusual hydroxylamine linker providing distinctive torsion angles in the central region of the molecule necessary to complement the minor groove.[54] The DNA appears to distort upon binding in order to widen the minor groove and accommodate the drug (induced fit).[51,55−57] The specificity of binding appears to be associated with the hydrophobicity of the entire drug, however, and it would be wrong to assume that the sequence recognition involves contacts made only by the aglycon or the aryl oligosaccharide.[59−61] Molecular modeling calculations by Schreiber *et al.* suggest that a significant portion of the sequence selectivity for 5′-TCCT-3′ arises from a favorable interaction between the large and polarizable iodo substituent of the hexasubstituted aromatic ring and the exocyclic amino substituents of the two guanines in the 3′-AGGA-5′ tract.[62] Nicolaou *et al.* demonstrated the importance of the iodine in the DNA-binding affinity of the oligosaccharide by carrying out DNA footprinting experiments with synthetic oligosaccharide analogs.[61] Further studies suggest that the binding selectivity for pyrimidine-rich sequences results from the greater propensity of such sequences to distort and thus adapt to the particular shape of the drug.[63,64]

As mentioned above, the aglycon is a rigid, highly functionalized bicyclic core. An enediyne moiety is locked within a rigid 10-membered bridging ring awaiting activation to undergo the cycloaromatization reaction.[4] Also forming part of the aglycon is an allylic trisulfide, which serves as a trigger. Once the molecule is in the vicinity of DNA (it has yet to be proved that binding occurs first), a series of chemical events unfolds which eventually leads to DNA cleavage. A nucleophile (e.g. glutathione) attacks the central sulfur atom of the trisulfide group, causing the formation of a thiol which adds intramolecularly to the adjacent α,β-unsaturated ketone embedded within the framework of the aglycon (Figure 8). This reaction, converting a trigonal bridgehead position to a tetragonal center, causes a significant change in structural geometry which imposes a great deal of strain on the 10-membered enediyne ring. This strain is completely relieved by the enediyne undergoing the cycloaromatization reaction, generating the highly reactive 1,4-benzenoid diradical **15**. The calicheamicin diradical abstracts hydrogen atoms from duplex DNA at the C(5′) position of the cytidine in 5′-TCCT-3′ and the C(4′) position of the nucleotide three base pairs removed on the 3′ side of the complementary strand[50,65,66] leading to cleavage of both strands of DNA.[67] Carefully designed kinetics experiments by Townsend *et al.*[68−70] have revealed considerable information about the sulfur

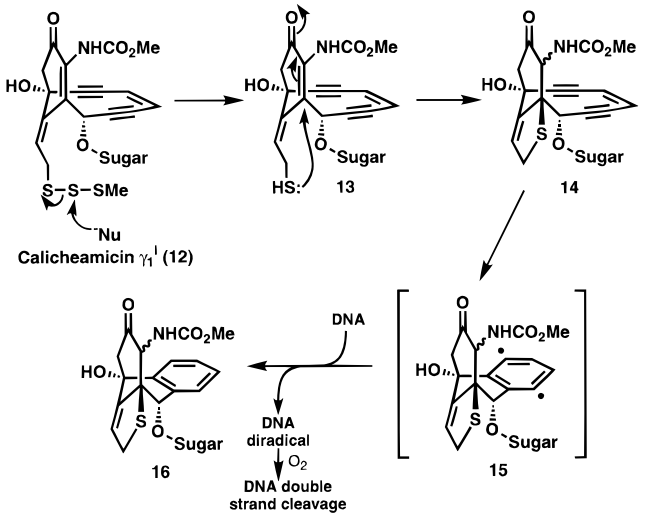


Figure 8. Mechanism by which calicheamicin cleaves DNA.

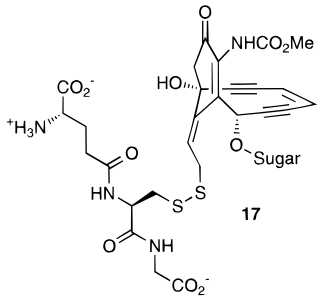
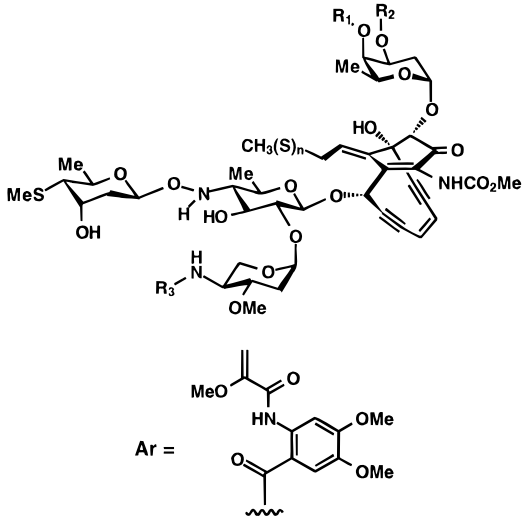


Figure 9. Calicheamicin−glutathione adduct **17**.

chemistry involved in the thiol-induced conversion of **12** into **13**, indicating complex chemistry involving the formation of intermediate mixed disulfides and trisulfides. The rate-determining step in the process appears to be thiolate formation from **13**, with subsequent rapid 1,4-addition to give the dihydrothiophene **14**, which has been observed at low temperature by NMR and is estimated to cycloaromatize at 37 °C with a half-life of 4.5 ± 1.5 s and a free energy of activation from **14** → **15** of 19.3 ± 0.2 kcal/mol.[68] It has been suggested that the ethylamino side chain of the E ring of the calicheamicin γ_1^I oligosaccharide plays a role as a general-base catalyst in the rate-limiting thiolate formation step,[47] although recent evidence suggests that this is not the case and that the amine simply enhances the affinity of the drug for DNA through an energetically favorable ionic interaction.[70] Kinetics measurements[71] suggest a complex series of interactions with DNA leading to DNA cleavage. First, calicheamicin γ_1^I (**12**) binds to DNA; DNA-bound **12** reacts with glutathione to form **17** (Figure 9) which dissociates from the DNA and reacts with free glutathione to form **13** and then **14**; the product(s) of the latter reaction (probably **14**) bind to DNA; DNA-bound **14** rearranges to the diradical **15**, which then abstracts hydrogen atoms from the ribose backbone of DNA. Comparison of the dissociation rate of **14** from DNA (~3 s^{-1} at 25 °C) with the half-life of **14** then suggests that the sequence selectivity of DNA cleavage by calicheamicin γ_1^I is controlled by thermodynamic rather than kinetic factors.[72]

The esperamicins (Table 2) are a subclass of naturally occurring enediynes with extremely high activities as broad-spectrum antibiotics and antitumor agents. They were isolated from the fermentation broth of *Actinomadura verrucosospora* and first reported in 1985.[73] The producing organism was collected at Pto Esperanza in Argentina, from which the series derives its name. Their structural elucidation was reported 2 years later[2] simultaneously with the calicheamicins, to which they are closely related, sharing an almost identical bicyclic enediyne core (esperamicin contains an additional hydroxyl group) and having sugar appendages with similarly unusual structural motifs. The esperamicins, together with the calicheamicins, are among the most powerful antitumor agents known, exhibiting powerful activity against a number of murine tumor models such as P388, B16, and M5076 at injected doses in the 0.1 μg/kg range.[74] The esperamicins are thought to exert their biological action through cleavage of DNA in an almost identical manner to the calicheamicins. Esperamicin A_1 (**18**), however, exhibits less sequence selectivity than calicheamicin γ_1^I (**12**) and shows a preference for T > C > A > G. As in the case of calicheamicin γ_1^I (**12**), the DNA-cleaving ability of the esperamicins is significantly enhanced by thiols, resulting in a mixture of single- and double-stranded cuts. It has been demonstrated that C(5′) and C(4′) hydrogen abstractions from DNA are the major chemical events initiated by the esperamicins. Esperamicin A_1 (**18**) itself effects almost exclusive single-stranded DNA cuts, and it appears that this is due to the fucosyl anthranilate side chain inhibiting the C(4′) hydrogen atom abstraction[75] by intercalating into the duplex DNA within the minor groove.[76]

Table 2. Esperamicin Family

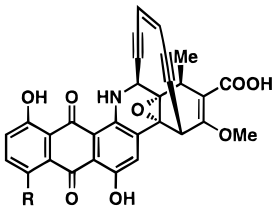


esperamicin	*n*	R_1	R_2	R_3
esperamicin A_1 (**18**)	3	H	Ar	$CHMe_2$
esperamicin A_{1b}	3	H	Ar	Et
esperamicin A_{1c}	3	H	Ar	Me
esperamicin P	4	H	Ar	$CHMe_2$
esperamicin A_2	3	Ar	H	$CHMe_2$
esperamicin A_{2b}	3	Ar	H	Et
esperamicin A_{2c}	3	Ar	H	Me

The Dynemicins

Dynemicin A (**19**), the first member of the dynemicin class of enediyne antibiotics to be discovered, is a violet-colored solid isolated from the fermentation broth of *Micromonospora chersina*. The structure was first reported by in 1989[6] and immediately attracted atten-

Table 3. Dynemicins



compd no.	name	R
19	dynemicin A	OH
20	deoxydynemicin A	H

tion due to its novelty, combining an anthraquinone (reminiscent of the anthracycline antibiotics) with a 10-membered bridging enediyne ring. The X-ray structure of dynemicin A (**19**) shows that the anthraquinone portion of the molecule is puckered rather than flat.[6] Dynemicin A (**19**) exhibits high potency against a variety of cancer cell lines and significantly prolongs the life span of mice inoculated with P388 leukemia and B16 melanoma cells.[6] Furthermore, dynemicin A (**19**) and its derivatives exhibit promising *in vivo* antibacterial activity with low toxicity. Subsequently, a second member of this family, deoxydynemicin A (**20**, Table 3), a bioactive compound with a similar profile to dynemicin A (**19**), was isolated from *Micromonospora globosa* MG331-hF6.[77] The absolute configuration of dynemicin A (**19**) has been confirmed by total synthesis.[78]

Biosynthetic studies indicate that dynemicin A (**19**) is biosynthesized from two heptaketide chains, which form the bicyclic enediyne core and the anthraquinone moiety, respectively.[79] Both are formed from seven head-to-tail-coupled acetate units, while the carboxyl group is derived from one carbon of an acetate unit and the *O*-Me group from methionine. Synthetic studies have resulted in the total synthesis of the di- and tri-*O*-methyldynemicin A methyl ester derivatives,[80] and enantioselective[78] and racemic[81] syntheses of dynemicin A (**19**).

Dynemicin A (**19**) cleaves duplex DNA, causing both single- and double-stranded cuts.[82−88] The potency as a DNA-cleaving agent is significantly enhanced by thiols[83] and by visible light irradiation,[84] and it preferentially attacks the 3′ side of purine bases such as 5′-AG, 5′-GC, 5′-GT, and 5-AT with clear selection for G and, to a lesser extent, A.[83,84] Intercalators and minor groove binders interfere with DNA cleavage, suggesting both intercalation and minor groove binding for this agent.[83]

It is suggested that intercalation of the anthraquinone portion of dynemicin A (**19**) into the target DNA *via* the minor groove is the first step in a series of events leading to DNA damage by dynemicin A.[83] This intercalation is accompanied by a local distortion of the DNA double helix in order to accommodate the drug,[89,90] with the molecule recognizing conformationally flexible regions of DNA and acting as a "molecular wedge".[91] The anthraquinone then undergoes bioreduction (Figure 10) to give the anthraquinol **21**. The electron-rich anthraquinol is then able to open the epoxide moiety by electron push as shown, perhaps being assisted by transfer of the acidic phenolic proton to the neighboring basic nitrogen atom to generate a quinone methide intermediate (**22**). This is then either trapped by a nucleophile such as H_2O (path A) or protonated (path B), resulting in an overall *cis* opening of the epoxide to give **23** and **27**, respectively. Opening the epoxide introduces a great deal of strain into the system which is rapidly relieved by the molecule undergoing the cycloaromatization reaction to generate a 1,4-benzenoid diradical species (**24**/**28**) which strips hydrogen atoms from the DNA, resulting in its cleavage. Both **26** and **29** have been identified as reaction products indicating that, in path A, a reoxidation step is involved (e.g. **25** → **26**). It is also possible that epoxide opening is initiated by electron push from the nitrogen atom rather than the phenol, and this will be discussed in a later section. In either case, epoxide opening is the trigger for cycloaromatization, diradical formation and DNA damage.[92−94]

The Chromoprotein Enediyne Antibiotics

The extensive body of knowledge relating to neocarzinostatin has already been mentioned. It is now becoming clear, however, that there is a substantial family of closely related enediyne antibiotics, a number of which had been isolated several years ago but remained structurally uncharacterized. They share the common properties of being noncovalently associated complexes between unstable chromophores and stabilizing proteins, they possess DNA-cleaving properties (both single and double stranded) associated with the chromophore, and they are potent antitumor agents. The distinctiveness of the various complexes is demonstrated, however, by a general specificity of binding of a particular chromophore to its own apoprotein. The revelation of the structure of the kedarcidin chromophore (**30**) in 1992[7] thus heralded the arrival of this new class of chromoprotein enediyne antibiotics and was followed shortly afterward by C-1027[8,9] and maduropeptin.[10] It seems likely that actinoxanthin[92,93] and macromomycin/auromomycin[97−102] are set to join them.

Kedarcidin. Kedarcidin was first reported in 1991 as the fermentation product of a novel actinomycete strain obtained from soils collected in India.[103] It exhibits potent *in vivo* antitumor activity similar to that of the other enediyne antibiotics and pronounced activity against Gram-positive bacteria. Kedarcidin was separated relatively easily by reversed-phase HPLC into the apoprotein and chromophore components and is found to be a varying complex depending upon fermentation conditions.[7]

The kedarcidin apoprotein exists as three main variants:[7] the major variant consists of 114 amino acid residues and a further two minor variants lack one or both of the first two amino acids (an alanine and a serine) of the major variant. The solution conformation of the apoprotein has been determined,[104] revealing a four-stranded antiparallel β-sheet, a three-stranded antiparallel β-sheet, and two two-stranded antiparallel β-sheets. The tertiary structure is very similar to that of the related apoproteins of neocarzinostatin, macromomycin, and actinoxanthin. Similarly, three kedarcidin chromophores have been identified having molecular weights of 1029, 1015, and 1001. Unlike the 1:1 apoprotein−chromophore ratio observed with neocarzinostatin, this ratio varies from 1:1 to 18:1 for kedarcidin. Using pilot scale fermentations (1000 L) with fish emulsions in the production media, a complex contain-

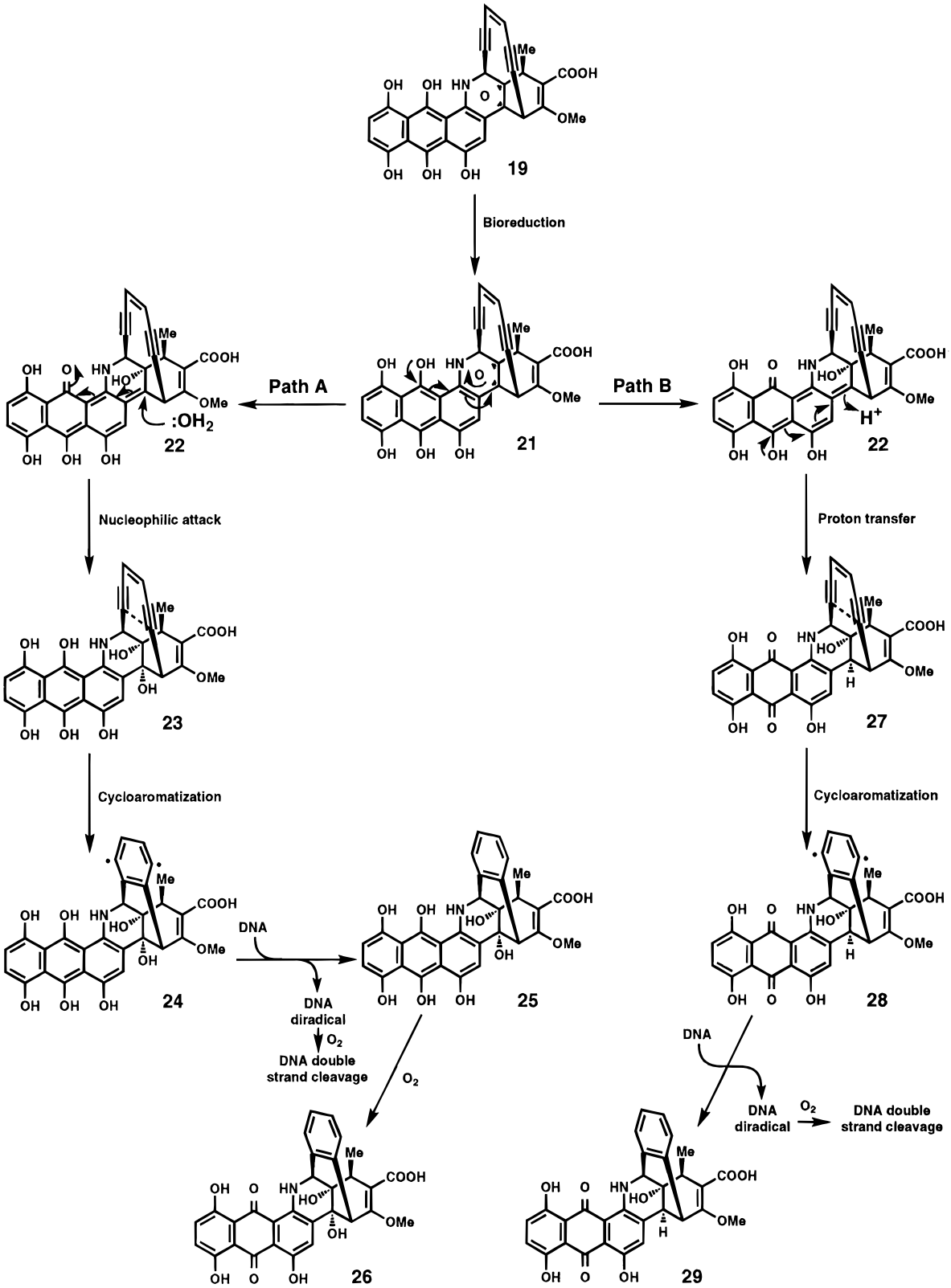


Figure 10. Triggering of dynemicin A.

ing only the chromophore of molecular weight 1029 was produced, and this was used for structural characterization of the chromophore. The kedarcidin chromophore (form I, Figure 11) (**30**) bears a striking resemblance to the neocarzinostatin chromophore (**4**). It is a highly unstable molecule, and organic solutions of it rapidly darken upon concentration. For the first time observed in an enediyne antibiotic, the enediyne unit is contained within a highly strained nine-membered ring which is "locked" by an allylic epoxide forming part of a fused bicyclic system. As with the other enediyne antibiotics, there is an assortment of sugar[105] and aromatic appendages; there is also a peptidic linkage associated with a macrocyclic structure.

The DNA-damaging properties of kedarcidin reside principally in the chromophore, resulting in highly sequence specific single-stranded cuts.[106] The principal DNA recognition sequence is 5′-TCCTN-3′, similar to

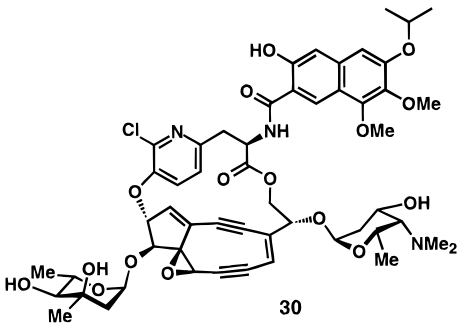


Figure 11. Structure of the kedarcidin chromophore (**30**).

calicheamicin γ_1^I (**12**), raising intriguing questions as to why two such structurally dissimilar molecules should recognize the same sequence. The cleavage chemistry requires reducing agents and oxygen, similar to the other enediyne antibiotics, and is enhanced by the presence of thiols. In contrast to calicheamicin and esperamicin, however, DNA cleavage by the kedarcidin chromophore is inhibited by the addition of divalent ions such as Ca^{2+} and Mg^{2+} which chelate with the 2-hydroxynaphthoate moiety, and NMR experiments implicate this moiety as being involved in binding to DNA.[106] The mechanism of activation of the kedarcidin chromophore is thought to be similar to that of the neocarzinostatin chromophore, with nucleophilic addition at C(12) initiating epoxide opening (Figure 12).[7] The change in structural geometry then facilitates the cycloaromatization reaction, leading to 1,4-benzenoid diradical formation, hydrogen abstraction from DNA, DNA strand cleavage, and cell death.

Despite the observation that the DNA cleaving properties of kedarcidin reside primarily in the chromophore, cytotoxicity assays using the human colon cancer cell line HCT 116 showed that the chromophore and apoprotein exhibit similar IC_{50} values of 10^{-9} M, suggesting that the apoprotein contributes actively to the cytotoxicity of kedarcidin. This led to the finding[16] that the kedarcidin apoprotein, a highly acidic polypeptide, exhibits selective proteolytic activity against peptides which are most opposite in net charge such as histones (the proteins around which chromosomal DNA is coiled to form chromatin). Preliminary experiments indicate that the apoproteins of other chromoprotein antitumor antibiotics such as neocarzinostatin[16] and macromomycin[98] also exhibit proteolytic activity, suggesting that this dual DNA-cleaving/proteolytic mechanism for attacking chromatin, the packaged genetic material of a target cell, is a common feature of all these chromoprotein enediyne antibiotics.

C-1027. The antibiotic C-1027, first reported in 1988,[107] was isolated from a culture filtrate of *Streptomyces globisporus* C-1027 and shown to consist of an extremely labile nonprotein chromophore (**34**, Figure 13) tightly bound noncovalently to a 110 amino acid residue apoprotein with a 1:1 stoichiometry.[8,9,107−112] This new antibiotic displays extremely potent antineoplastic activity against a panel of transplantable tumors such as leukemia L1210, P388, and ascites hepatoma H22, and its cytotoxic effect is much stronger even than that of neocarzinostatin.[110] The primary cause of the cytotoxicity appears to be due to DNA cleavage brought about by the chromophore. The nature of the DNA cleavage is somewhat different from the previously described enediyne antibiotics since the antibiotic (and similarly the isolated chromophore) efficiently cleaves DNA in a double-stranded manner even in the absence of thiol compounds or reducing agents. Furthermore, the sites of cleavage in the two DNA strands are two base pairs

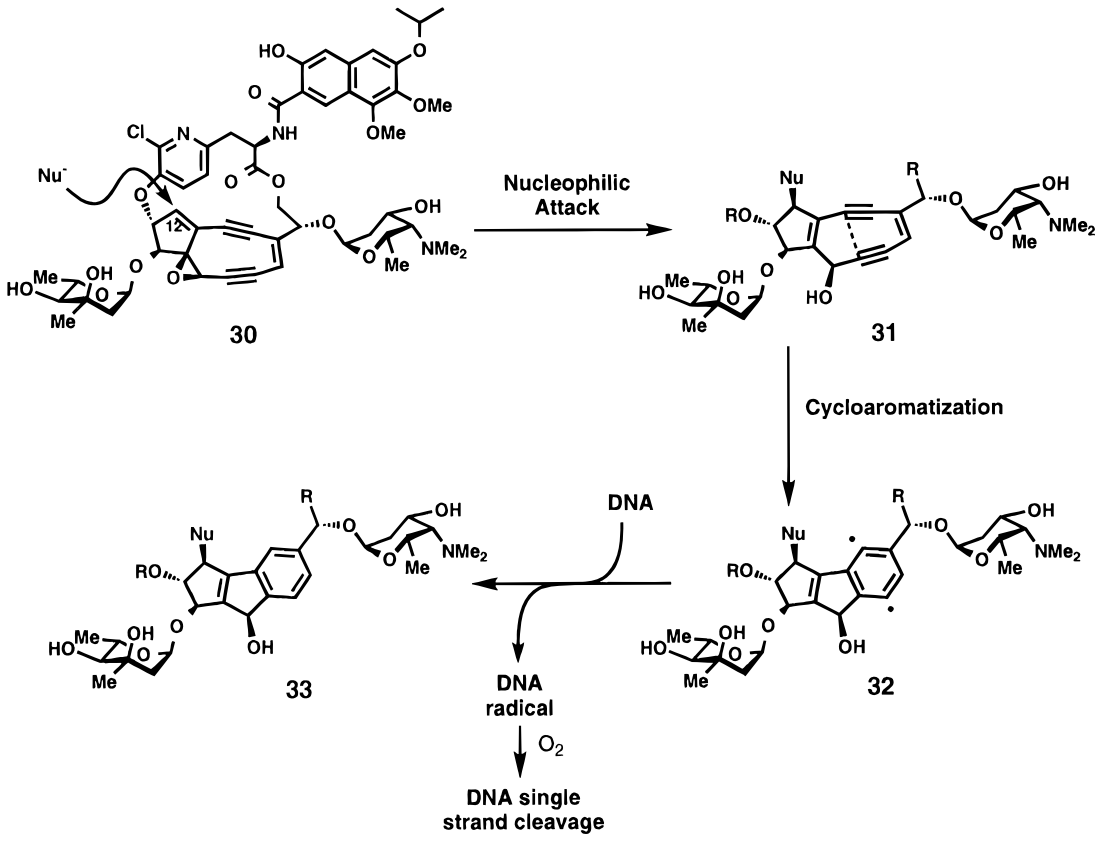


Figure 12. Proposed mechanism of action of the kedarcidin chromophore (**30**).

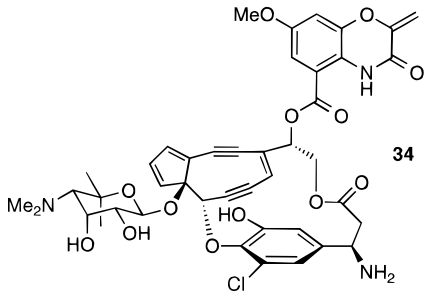
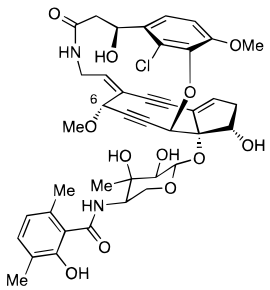


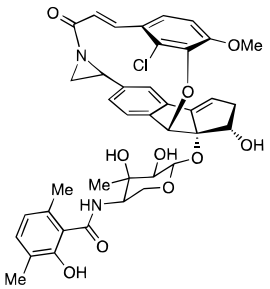
Figure 13. Structure of the C-1027 chromophore (**34**).

apart (rather than three base pairs apart as observed for other double-strand cleavers previously described) and are specific for sequences such as 5′-TA<u>T</u>-3′/3′-<u>A</u>TA-5′ and 5′-AG<u>A</u>-3′/3′-<u>T</u>CT-5′ in the two strands.[112,114] The DNA cleavage chemistry is primarily C(4′) hydrogen atom abstraction from deoxyribose. Unlike the other enediyne antibiotics, cycloaromatization of the C-1027 chromophore has been demonstrated to have a kinetic isotope effect with respect to hydrogen atom abstraction, indicating that this, rather than biradical formation, is the rate-limiting step.[114] The chromophore is readily extracted from the apoprotein by organic solvents such as methanol and was shown through careful NMR studies to have the structure **34**, in which the enediyne unit is contained within a strained nine-membered ring forming part of a bicyclic system resembling the core structures of NCS and kedarcidin chromophores.[8,9] Unlike all the previously characterized enediyne antibiotics, however, the system contains no triggering mechanism and is already primed to undergo the cycloaromatization reaction and produce the DNA-damaging 1,4-benzenoid diradical species. The chromophore is clearly demonstrated to be stabilized by its association with the apoprotein since the DNA-cleaving properties of intact C-1027 are retained for extended periods, whereas those of the isolated chromophore rapidly decay under similar conditions ($t_{1/2}$ = 10 h at ambient temperatures).[111,112] A study of the interaction between the chromophore and apoprotein of C-1027 reveals a deep hydrophobic pocket in the apoprotein which is thought to bind the benzoxazine side chain of the chromophore and may be partly responsible for the tight and specific binding of the chromophore to the apoprotein.[112,115] Molecular modeling experiments of the apoprotein−chromophore complex[115] suggest the most probable stereochemistry of the chromophore (8*R*,9*S*,13*R*,17*R*) at these previously unassigned stereocenters as well as the origin of the stabilization of the chromoprotein by the apoprotein. As with neocarzinostatin, an interaction between a disulfide bridge (Cys 36 and Cys45) and the chromophore acetylene bonds appears to contribute to the stabilization of the chromophore. The acetylenic bonds also appear to be stabilized by hydrophobic interactions with the bottom of the hydrophobic pocket, van der Waals contact with Pro 76, and $\pi-\pi$ stacking with the benzene moiety of the chromophore. In this molecular dynamics simulation, the distance between the termini of the enediyne (C2−C7) of the apoprotein-bound chromophore is longer than that of the unbound chromophore (3.16 *vs* 3.10 Å).

Structurally, C-1027 has some features in common with other chromoprotein antibiotics currently awaiting full structural characterization. Auromomycin, the holoprotein of macromomycin, is an antitumor chromoprotein antibiotic which has been isolated from *Streptomyces macromyceticus*.[97−102] It displays potent cytotoxicity against a range of tumor cell lines, and DNA cleavage is implicated in its mechanism of action. Degradation studies on the auromomycin chromophore indicate that it contains a benzoxazine side chain identical to that found in C-1027.[102] Similarly, actinoxanthin[95,96] is an antitumor chromoprotein antibiotic isolated from *Actinomyces globisporus* No. 1131 in 1957. The actinoxanthin apoprotein has been shown to have a high degree of sequence homology (95%) with the C-1027 apoprotein.[112]



35: Methanol adduct of maduropeptin chromophore



36: Cycloaromatization product of maduropeptin chromphore

Figure 14. Artefacts of the maduropeptin chromophore.

Maduropeptin. Maduropeptin, isolated from *Actinomadura madurae* in 1990, consists of a 1:1 complex of an acidic, water soluble carrier protein (32 kDa) and a nine-membered ring enediyne chromophore. It exhibits potent inhibitory activity against Gram-positive bacteria and tumor cells and strong *in vivo* antitumor activity in P388 leukemia and B16 melanoma implanted mice.[116] Maduropeptin apoprotein represents a new protein class, showing no sequence homology to the related chromoproteins neocarzinostatin, auromomycin, actinoxanthin, C-1027, or kedarcidin which are all in the 11.5 kDa range and show ∼40% sequence homology. The chromophore is tightly bound to the protein and, while its exact structure is uncertain, a number of artifacts such as **35** (Figure 14) resulting from methanol addition at C(6) during the isolation process have been characterized, revealing close similarity to the C-1027 chromophore (**34**).[10] The cycloaromatized product (**36**) contains an aziridine ring, indicating either that this is opened by methanolysis during the isolation procedure or that aziridine (and enediyne) formation forms part of the triggering process. Dehydration also appears to occur during activation.

"Designed" Enediynes

The disclosure in 1987 of the structures of the calicheamicins and esperamicins, together with their unique and intriguing mode of action, stimulated considerable activity in the laboratories of many synthetic and theoretical chemists. The triggering of the bicyclic enediyne core of calicheamicin causes a change in geometry of the molecule resulting in the termini of the enediyne unit being forced together and thus imposing further strain upon the system. This is accompanied by rapid cycloaromatization. It therefore occurred to Nicolaou *et al.* that, in the absence of other factors affecting strain, it might be possible to predict the reactivity of a cyclic enediyne system toward cycloaromatization from the distance *c*...*d* between the ends of the 1,5-diyn-3-ene system (**37**, Figure 15), and they

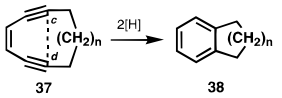


Figure 15. The cycloaromatization of cyclic enediynes studied by Nicolaou *et al*.

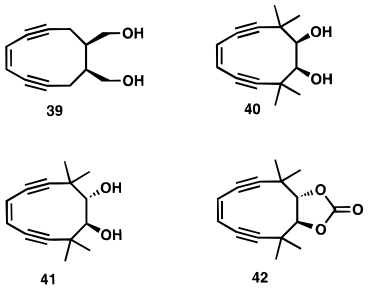


Figure 16. First generation "designed" enediynes (Nicolaou *et al*.).

undertook the study of a series of monocyclic enediynes of varying ring size (**37**, $n = 1-8$).[117]

The parent series of 10−16-membered ring enediynes **37** were readily prepared. The 10-membered ring enediyne **37** ($n = 2$) readily underwent the Bergman cycloaromatization reaction at room temperature with a half-life of 18 h, whereas the larger ring enediynes **37** ($n = 3-8$) were found to be stable. By contrast, the nine-membered ring **37** ($n = 1$) could not be prepared, although products formally arising from a Bergman reaction were identified. Comparison of the distances *c*...*d* between the termini of the enediyne moiety of these systems and the ease with which they underwent the Bergman reaction showed a clear trend in which a decreased *c*...*d* distance reflected, in addition to a closer intimacy between the acetylenic groups, an increased ring torsion and hence an increased tendency to undergo the Bergman reaction in order to relieve the strain. For these simple systems a critical upper limit for the *c*...*d* distance of around 3.2−3.3 Å appeared to be required for the Bergman reaction to occur at a measurable rate at ambient temperatures. Thus, while this empirical *c*...*d* distance rule is not strictly applicable to complex systems such as those found in calicheamicin or dynemicin where geometrical constraints prevent cycloaromatization prior to triggering, it does provide a convenient means of assessing the likely stability of many systems towards cycloaromatization. More sophisticated calculations and kinetic experiments have subsequently demonstrated[118,119] that the crucial factor in determining the ease with which a particular system undergoes the Bergman reaction is the relative strain energies of the ground and transition states for the reaction.

Since the simple 10-membered ring enediyne **37** ($n = 2$) underwent the Bergman reaction at physiological temperatures, the DNA-cleaving action of the calicheamicins and esperamicins was mimicked by using simple systems such as these. The diol **39** was designed in order to endow the molecule with some degree of water solubility and also to provide for the option of attachment to delivery systems (Figure 16).[117] It was correctly predicted from the calculated *c*...*d* distance of 3.20 Å that this molecule would be sufficiently stable for isolation and handling at ambient temperatures but would undergo the Bergman reaction at physiological temperature at a sufficient rate to cause DNA cleavage.

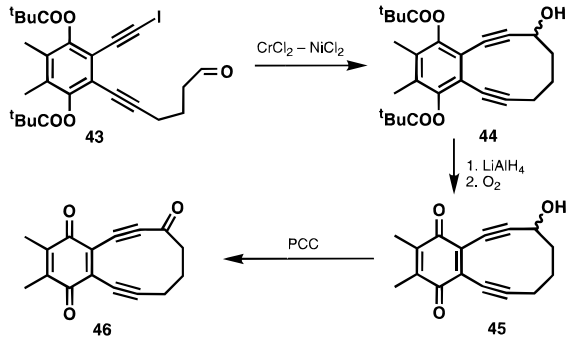


Figure 17. Redox controlled enediyne systems (Nicolaou *et al*.).

Thus enediyne **39** caused significant cleavage of phage ΦX174 double-stranded supercoiled DNA in the absence of any additives at concentrations as low as 10 μM at 37 °C, with the extent of cleavage being dependent upon concentration, incubation time, and temperature. The cleavage data is therefore consistent with a Bergman cyclization of **39** leading to a diradical species which abstracts hydrogen atoms from DNA in a mechanistic mode similar to the one proposed for the calicheamicins and esperamicins. The thermally reactive diols **40** and **41** were subsequently prepared and similarly demonstrated to effect DNA cleavage.[120] By contrast, the conformationally locked and thermally stable derivative **42** failed to cleave DNA. Under basic conditions, however, compound **42** became active *via* the release of diol **41**, thus exhibiting both DNA cleaving and cytotoxic properties.

Since the naturally occurring enediyne antibiotics are triggered to exert their biological actions by bioreductive processes, Nicolaou *et al*. designed the system shown in Figure 17 in order to control the Bergman cyclization by a hydroquinone ↔ quinone redox process.[121] It was postulated that a hydroquinone such as **44**, prepared *via* an intramolecular Nozaki-type coupling of the iodoalkyne **43**, should be rather more stable toward cycloaromatization than the corresponding quinone **45** due to its lower activation energy for the process. This was borne out by the measurement of the half-lives of these compounds: **44** ($t_{1/2}$ = 74 h at 110 °C), **45** ($t_{1/2}$ = 2.6 h at 55 °C), **46** ($t_{1/2}$ = 32 min at 55 °C).[122] Furthermore, interaction of compounds **44**−**46** with phage ΦX174 DNA at pH 7.4 and 37 °C revealed **44** has no DNA-cleaving activity whereas **45** and **46** showed significant DNA-damaging properties.

Dynemicin Analogs. When the structure of dynemicin **A** (**19**) was revealed in 1989,[6] the combination of its intriguing novelty, potent antitumor and antibiotic properties, and low toxicity by comparison with other enediyne antibiotics such as calicheamicin γ_1^I (**12**) were sufficient to ensure the attention of a scientific community already fascinated by its forebears.

Nicolaou *et al*. were first to identify and study the features of the natural product essential for its activation through the design and synthesis of model systems.[122] The dynemicin model **47** (see Figure 18) was thermally stable toward cycloaromatization. It was, however, readily triggered by acid-catalyzed epoxide opening, resulting in spontaneous cycloaromatization by analogy with Figure 10. By contrast, the deprotected amine **48** proved to be very labile and rapidly underwent epoxide opening/cycloaromatization at ambient temper-

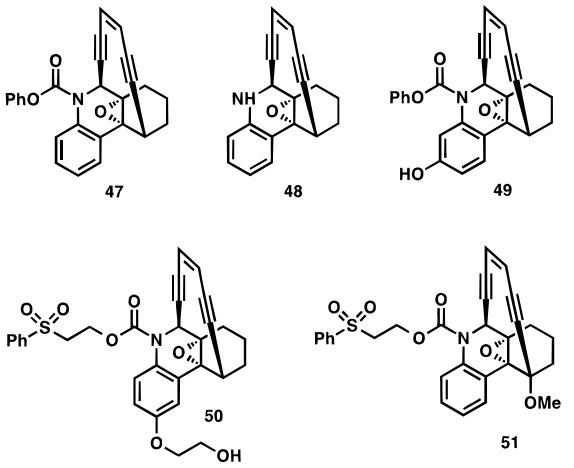


Figure 18. "Designed" enediynes related to dynemicin A (Nicolaou *et al.*).

atures in the absence of cofactors. The reactivity of **48** and its ability to cause double-stranded DNA breaks supports the notion of electron push from the freed nitrogen participating in the formation of an *o*-iminoquinone methide species during epoxide opening. This suggests that a similar contribution from the nitrogen in the dynemicin A cascade (Figure 10) may, at least in part, account for the triggering of the natural product following bioreduction. The anthraquinone portion of dynemicin A can thus be envisaged as acting as a "lock" for the epoxide by withdrawing electron density away from the nitrogen atom and hence preventing epoxide opening in a manner similar to the way the carbamate acts as a "lock" for **47**. Similarly, the phenolic system **49** was found to readily cycloaromatize and cleave DNA as a result of electron push from the phenol opening the epoxide. These findings allowed the design of the 2-(phenylsulfonyl)ethyl carbamate **50**. The facile manner in which the nitrogen protecting group could be removed under mildly basic conditions, even undergoing slow release at physiological pH, resulted in the possibility of it acting as a prodrug for the corresponding unstable and cytotoxic free amine.[123] This compound had an IC_{50} of 2.0×10^{-14} M against the MOLT-4 leukemia cell line making it one of the most potent *in vitro* antitumor agents which has been reported to date.

Table 4 shows the IC_{50} values determined for enediyne **50** with 21 different cell lines.[123] There are significant differences in cytotoxicities, ranging from 10^{-6} M for the highly resistant melanoma cell lines to 10^{-14} M for the highly sensitive leukemia cell line. Particularly important is the high cytotoxicity against the multiple-drug-resistant TCAF-DAX cell line ($IC_{50} = 1.7 \times 10^{-9}$ M). Another striking feature is its relatively low cytotoxicity against a number of nontransformed cell lines, giving good therapeutic indices *in vitro* against the more sensitive tumor cell lines such as the pancreatic carcinoma and leukemia cell lines. This is a key issue in developing cytotoxic antitumor drugs, and preliminary *in vivo* studies with animals infected with leukemia and solid tumors have shown encouraging results.[124] In order to confirm that the remarkable cytotoxicity of enediyne **50** is indeed due to DNA damage, MOLT-4 leukemia cells were treated with ethidium bromide, which intercalated into the DNA, rendering it fluorescent. Exposure of these cells to enediyne **50** at a concentration of 10^{-5} M led to rapid

Table 4. Cytotoxicity of Designed Enediyne **50** against a Panel of 21 Tumor Cell Lines and 4 Normal Cell Lines

cell type	cell line	IC_{50} (M)
Tumor Cell Lines		
melanoma	SK-Mel-28	3.1×10^{-6}
melanoma	M-14	1.6×10^{-6}
melanoma	M-21	1.6×10^{-6}
colon carcinoma	HT-29	1.6×10^{-6}
ovarian carcinoma	Ovcar-3	7.8×10^{-7}
ovarian carcinoma	Ovcar-4	7.8×10^{-7}
astrocytoma	U-87 UG	7.8×10^{-7}
glioblastoma	U-251 MG	3.9×10^{-7}
breast carcinoma	MCF-7	7.8×10^{-7}
lung carcinoma	H-322	3.9×10^{-7}
lung carcinoma	H-358	2.0×10^{-7}
lung carcinoma	H-522	9.8×10^{-8}
lung carcinoma	UCLA P-3	9.8×10^{-8}
pancreatic carcinoma	Capan-1	3.1×10^{-9}
T-cell leukemia	TCAF	1.1×10^{-9}
T-cell leukemia[a]	TCAF-DAX	1.7×10^{-9}
myeloma	RPMI-8226	7.7×10^{-9}
mouse leukemia	P-388	4.6×10^{-9}
mouse leukemia	L-1210	1.3×10^{-9}
promyelocytic leukemia	HL-60	3.6×10^{-11}
T-cell leukemia	Molt-4	2.0×10^{-14}
Normal Cell Lines		
bone marrow	HNBM	5.0×10^{-5}
human mammary epithelial cells	HMEC	6.3×10^{-6}
normal human dermal fibroblast	NHDF	5.0×10^{-6}
Chinese hamster ovary	CHO	3.1×10^{-6}

[a] Multiple drug resistant cell line.

DNA strand breakage as determined by fluorimetry, resulting in 95% destruction after 4 h at 37 °C. Cell death showed an approximately 2 h delay relative to DNA strand breakage, implicating DNA damage as the direct cause of cell death in these experiments. It was also shown that enediyne **50** severely impaired the ability of MOLT-4 leukemia cells to synthesize DNA (inhibition of [^{3}H]thymidine uptake), RNA (inhibition of [^{3}H]-uracil uptake), and protein (inhibition of [^{3}H]-leucine uptake).

Treatment of MOLT-4 cells with enediyne **50** under appropriate conditions followed by observation of cell morphology and cell death revealed the phenomenon of programmed cell death (apoptosis) as the prevailing cause of cell destruction.[125] Furthermore, competition experiments using enediynes with relatively low toxicities resulted in the identification of certain inhibitors of apoptosis. Specifically, the methoxy enediyne **51**, which displayed diminished tendency to undergo the Bergman reaction, inhibited the cytotoxic action of compound **50**. Thus, when MOLT-4 cells were preincubated with enediyne **51** at 10^{-4} M for 1 h prior to treatment with the cytotoxic compound **50**, a dramatic reduction by a factor of 10^5 was observed in the cytotoxicity of **50**. Similar reductions by factors of 10^2–10^4 were observed in the cytotoxicities of the naturally occurring enediynes dynemicin A (**19**) and calicheamicin γ_1^I (**12**). Particularly intriguing was the observation that **51** inhibits apoptotic morphology of cell death by powerful inducers of apoptosis such as actinomycin D and cycloheximide, although cell viability was not affected in these cases.

Future Directions

The studies described above indicate how the mechanistic and synthetic challenges resulting from the discovery of the enediyne antibiotics have been approached. This had led to the development of "designed

enediynes" such as the dynemicin A models that show promise both *in vitro* and *in vivo* as antitumor agents. The ability to prepare these complex natural products by total synthesis has also provided the opportunity to investigate structure−activity relationships of the natural enediynes themselves. For example, the synthetic calicheamicin θ_1^I containing a thioacetate triggering device in place of the trisulfide is considerably more potent that any previously known enediyne (natural or synthetic).[126] However, perhaps ironically, the very fact that these complex natural products have finally succumbed to total synthesis has resulted in a noticeable decline over the past couple of years in publications from the synthetic community who are always looking for fresh pastures in which to pursuade the funding agencies to provide research grants. It may therefore require a higher level of interest from the pharmaceutical industry in order to develop the designed enediyne from good *in vitro* tools into drug candidates.

Among the natural products themselves, there has been considerable interest as anticancer agents in the pharmaceutical industry, and a number of clinical trials have been carried out. Esperamicin A_1 (**18**) was reported to be in phase II clinical trials. Calicheamicin γ_1^I (**12**), by contrast, is too toxic for therapeutic use. Calicheamicin−antibody immunoconjugates offer great promise, however, with products reported to be in phase I trials against ovarian cancer and others in preclinical trials against acute myeloid leukemia and colorectal cancer. The most studied systems relate to neocarzinostatin, perhaps because this has been available for the longest period of time. This compound has been shown to possess antitumor activity in patients with liver cancer, bladder cancer, stomach cancer, and leukemia as well as in various animal tumors.[127] SMANCS is a product of conjugation of neocarzinostatin with poly[styrene-*co*-(maleic acid)] which has shown high tumor-targetting efficiency and good antitumor activity in animal models following oral administration.[128,129] Immunoconjugates of neocarzinostatin such as A7-NCS have undergone clinical evaluation,[130] showing increased survival times when administered to postoperative cancer patients (both with and without metastases) when compared with other chemotherapies. These novel natural products, with their unprecedented modes of action, are therefore clearly more than a scientific curiosity, and it remains to be seen whether enediynes, either natural or designed, will become useful additions to the arsenal of chemotherapies available to clinicians for the treatment of cancer.

Biographies

Adrian L. Smith received his Ph.D. in 1989 from the University of Cambridge, England, working with Dr. Andrew B. Holmes on nitrone cycloaddition chemistry. He then worked on enediynes until 1992 with Prof. K. C. Nicolaou at the Scripps Research Institute, where he held a Fulbright Scholarship and NATO Fellowship. He is currently a Senior Research Chemist at Merck Sharp & Dohme at Terlings Park where he is in charge of combinatorial chemistry.

K. C. Nicolaou received his Ph.D. in 1972 from University College London, working with Professors F. Sondheimer and P. J. Garratt. After postdoctoral appointments at Columbia (1972−1973, Professor T. J. Katz) and Harvard (1973−1976, Professor E. J. Corey) he joined the faculty at the University of Pennsylvania. In 1989 he accepted joint appointments at the University of California, San Diego, where he is Professor of Chemistry, and The Scripps Research Institute, where he is the Darlene Shiley Professor of Chemistry and Chairman of the Department of Chemistry. His research interests focus on chemical synthesis, molecular design, molecular recognition, and the biological action of molecules.

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