Synthetic calicheamicin mimics with novel initiation mechanisms: DNA cleavage, cytotoxicity, and apoptosis

KC Nicolaou*, EN Pitsinos, EA Theodorakis, H Saimoto and W Wrasidlo

Department of Chemistry, 10666 North Torrey Pines Road, The Scripps Research Institute, La Jolla, CA 92037, USA and Department of Chemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

Background: Calicheamicin γ_1^I is a bacterial product that is a prominent member of the enediyne class of antitumor antibiotics, and has been extensively studied. Calicheamicin γ_1^I binds to DNA, causing double-stranded breaks, and cells exposed to it eventually become apoptotic. It can now be made synthetically, and highly potent biological mimics have been designed. Such molecules have many potential clinical applications, but are complex to make. We therefore investigated whether simplified versions of these molecules are biologically active.

Results: We designed and synthesized a number of simple calicheamicin mimics and evaluated their biological activity. We also constructed mimics that are particularly suitable for conjugation to proteins, oligonucleotides, and other delivery systems. Several active mimics were found, and two in particular, which lack the trisulfide and oligosaccharide moieties of calicheamicin, had potent DNA-cleaving and cytotoxic activities. They caused chiefly single-stranded cuts in DNA, however, unlike the natural molecule, which causes double-stranded DNA cuts. Although they were able to induce apoptosis, they were less potent than the natural compound in this assay.

Conclusions: The simple enediyne mimics were less potent than calicheamicin γ_1^I, presumably because they lack the oligosaccharide DNA-binding domain. Nevertheless, considering their relatively primitive structures, they have remarkable biological properties. They may be useful biological tools and are potential leads for the development of chemotherapeutic agents. We propose that the ability of the enediynes to induce apoptosis is related to their ability to make double-stranded cuts in DNA.

Chemistry & Biology September 1994, **1**:57–66

Key words: apoptosis, calicheamicin, DNA cleavage, enediynes

Introduction

Calicheamicin γ_1^I (Fig. 1a) is the most potent naturally occurring member of the enediyne class of antitumor antibiotics [1]. This class of compounds was defined in 1987 when the structures of calicheamicin γ_1^I [2] and esperamicin A_1 [3] were elucidated. The previously known neocarzinostatin chromophore [4] was soon included in the classification, because it has a similar mode of action [5], and dynemicin A [6], kedarcidin chromophore [7] and C-1027 chromophore [8] were added later (Fig. 1a). The growing number of these antibiotics has been paralleled by an increasing interest in them from members of the chemical, biological and medical communities. Advances in the synthesis of these molecules, culminating in the total syntheses of calicheamicin γ_1^I [9,10], tri-O-methyl dynemicin A methyl ester [11], and a number of designed analogs [1,12] have paralleled advances in research related to their mechanism of action [1,13–19] and clinical development [1].

The molecular architecture of calicheamicin γ_1^I exemplifies the efficiency of design that is common to these compounds. The structure can be viewed as having three functional domains, each with distinct roles: first, the novel oligosaccharide chain, which allows the sequence-selective binding of the drug in the minor groove of double-stranded DNA [13]; second, the enediyne moiety (represented by calicheamicinone), which can undergo Bergman cycloaromatization (Fig. 2a) [20], generating benzenoid diradicals that can cleave double-stranded DNA through hydrogen atom abstraction; and third, the trisulfide group, which acts as a triggering device to initiate the cascade of reactions that leads to DNA damage (Fig. 2b) [21].

Encouraged by our previous successes in designing highly potent biological mimics of dynemicin A, such as PM-9 (Fig. 1b) [22], and of calicheamicin γ_1^I, such as calicheamicin θ_1^I (Fig. 1b) [23], we set out to design and synthesize simple biological mimics of calicheamicin γ_1^I with the goal of attaining optimum biological activity with minimum molecular complexity.

Results and discussion

Molecular design

Fig. 3a summarizes the possible structural modifications on the calicheamicinone core that were considered. We followed basic principles of organic chemistry to design potentially active compounds that have the following characteristics. First, they are chemically stable under neutral conditions but can undergo the Bergman cycloaromatization reaction upon suitable activation. Second, they are structurally simple and thus can be easily synthesized chemically. Third, they can be tethered to suitable delivery systems and other desirable moieties through

*Corresponding author.

 ISSN 1074–5521

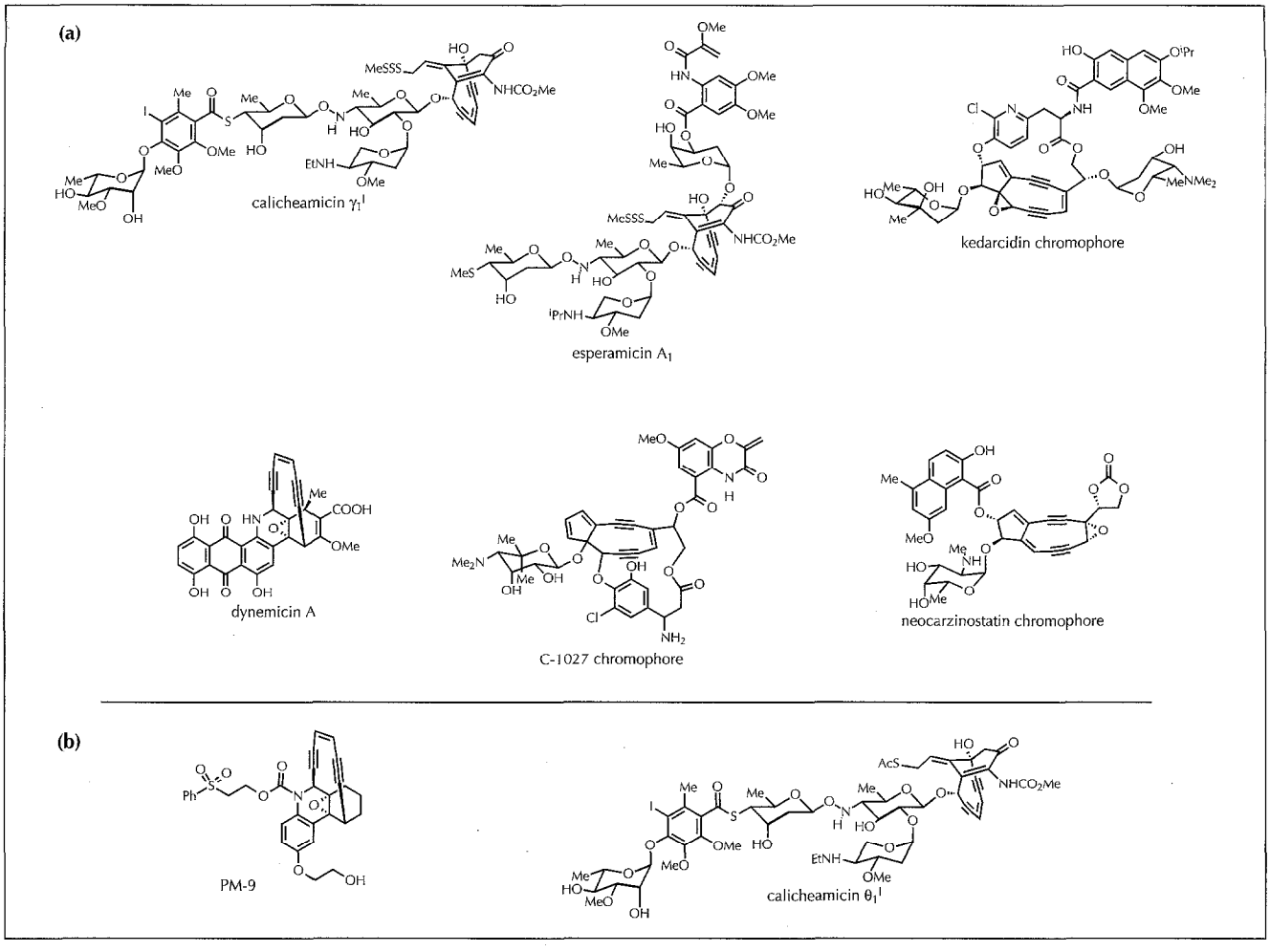

Fig. 1. Naturally occurring enediynes and designed mimics. **(a)** Naturally occurring representatives of the enediyne class of antitumor antibiotics. **(b)** Previously reported designed mimics of dynemicin A (PM-9), and calicheamicin γ_1^I (calicheamicin θ_1^I).

appropriate functionalities. Finally, they are equipped with suitable initiators that may be activated under mild chemical or biological conditions.

Starting with the natural mechanism of activation of calicheamicin γ_1^I (Fig. 2b), we envisaged a number of variants (structure I, Fig. 3b) that use the generation of nucleophiles centered on oxygen, nitrogen or sulfur to activate the Bergman reaction [24]. An interesting alternative in which the carbonyl group of the enone system was protected as a ketal (structure II, Fig. 3b) was considered as a compound that could be activated under acidic conditions [24,25]. Both structure I and structure II produce the same intermediate after activation (structure IV), and the rest of the reaction pathway is much the same as that of natural calicheamicin γ_1^I. We also designed a mimic with a different mode of activation, structure III (Fig. 3c), which relies on the known propensity of enamines to hydrolyze to carbonyl compounds, and which we expected to undergo activation as shown in Fig. 3c.

We chose the secondary propargylic alcohol (shown in Fig. 3a) at the position of R_3 as an appropriate handle for attachment of tethers, as it has this function in the natural product. Compounds 3 and 4 (Fig. 4a) were thus targeted for synthesis. Because chemotherapeutic agents are often targeted by conjugation to antibodies [26], we designed mimics that are suitably activated for the construction of non-symmetric disulfides at this position, for example compounds 9 and 10. Such groups could have two functions, being both reductively cleavable initiators and tethering devices.

Chemical synthesis

Figs. 4 and 5 summarize the structures of the novel compounds 1–7 and 9–15 and their chemical synthesis. To synthesize compounds 1–5, we used as starting material the optically active compound shown in Fig. 4 as compound 16, which is an advanced intermediate in our previously developed route [27] to calicheamicinone, shown as compound 8 in Fig. 4. Thus, selective benzoylation of this diol gave monobenzoate 17 as the major product accompanied by the corresponding dibenzoate 18 (Fig. 4a). Concomitant removal of both the ketal and silyl protecting groups from compounds 17 and 18 by aqueous acid treatment resulted in the formation of compounds 1 and 2, respectively. The *p*-nitrobenzoate 6 was synthesized in a similar fashion from compound 16.

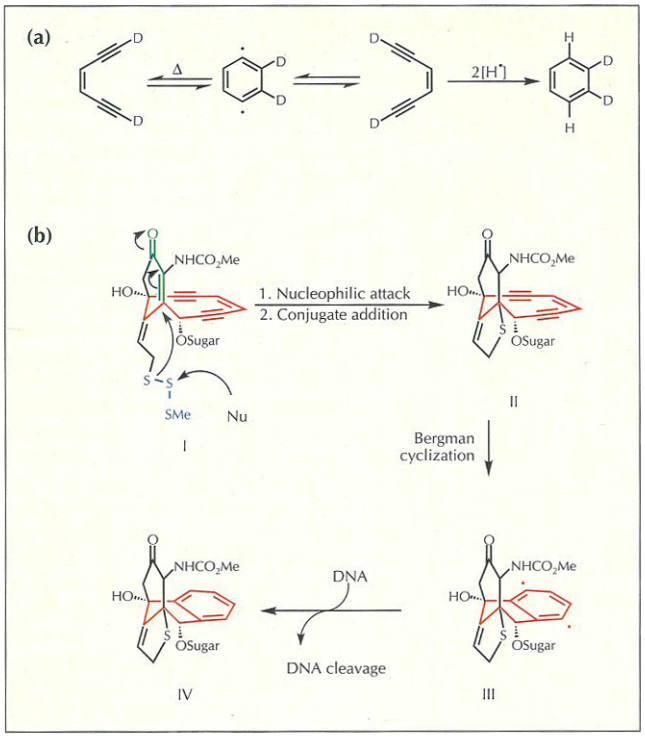

Fig. 2. **(a)** The Bergman cycloaromatization reaction. **(b)** The postulated mechanism of action of calicheamicin. The enone functionality is shown in green, the trisulfide moiety, or triggering device, in blue, and the 10-membered ring or benzenoid system in

The differentiation of the functional groups in compound 17 allowed the introduction of a tethering group at the propargylic position using glutaric anhydride under basic conditions to give compounds 20 and, after coupling with *p*-nitrophenol, 19. These were converted to enones 3 and 4, respectively, under acidic conditions. By conjugating desoxymethasone, a steroid involved in gene regulation, with compound 4 to give compound 5, we produced a compound that could be used to probe the biological effect of damaging a steroid receptor involved in gene regulation [28].

For compounds 7, 9 and 10, we started with the readily available thioacetate 21 [27] (Fig. 4b). This was converted to the free thiol with diisobutyl aluminum hydride (DIBAL) or sodium methoxide, which was reacted *in situ* with MeSSCOOMe [29] or 2,2'-dipyridyl disulfide (aldrithiol-2) [30] to give the methyl disulfide 22 or the 2-pyridyl disulfide 23, respectively. Deprotection of these intermediates (compounds 21–23) as above gave the expected enones 7 [31], 9, and 10, respectively.

Compound 24, another readily available intermediate of calicheamicin synthesis [27], was used as a branching point for the synthesis of a new series of designed calicheamicinones, shown as 11–14 in Fig. 4c. Thus, desilylation of compound 24 led to compound 11, whereas acetylation gave compound 25. Esterification with the appropriate chloroformate produced carbonmate 26, which oxidized smoothly to afford the sulfone derivative 27. On the other hand, mesylation of alcohol 24 followed by azide displacement gave the azide, 28, which was reduced and acetylated *in situ* [32] leading to acetamide 29. Deketalization of 25, 27, and 29 gave enones 12, 13, and 14, respectively (Fig. 4).

Compound 15, which contains the 2'-phenylsulfonylethyl carbonate group on the nitrogen as an activator, presented

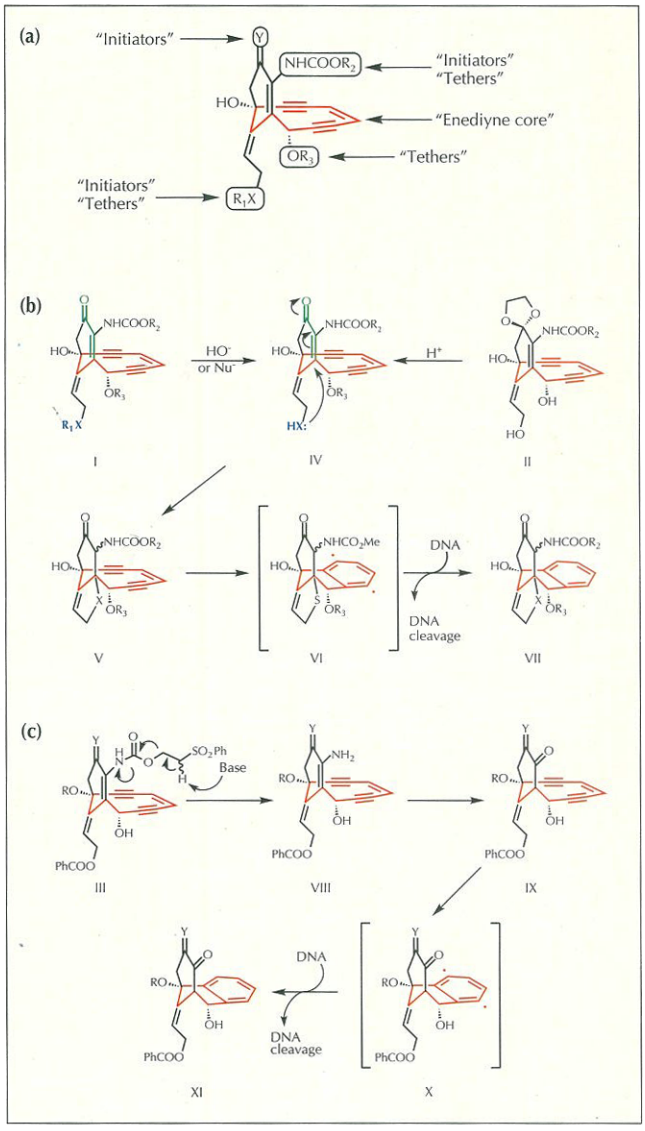

Fig. 3. The rationale for and postulated mechanism of action of designed enediynes **(a)** Rationale for molecular design of calicheamicin γ_1^I mimics. The enediyne core, or 10-membered ring, is shown in red, and the positions at which initiators or "tethers" can be attached are noted. **(b)** Postulated mechanism of action of designed enediyne structures I, II, and **(c)** III. The mechanism of action of enediynes with structure I is similar to that of the natural compounds (see Fig. 2b); nucleophilic attack at R_1 leads to conjugate addition (IV) and Bergman cyclization. For structure III, the nucleophilic attack is instead at the R_2 position, resulting in an enamine (VIII) which readily hydrolyses to a carbonyl (IX), activating the enediyne ring (structure X). The designed compounds 1–10 and 12–14 belong to class I, compound 11 belongs to class II and compound 15 belongs to class III. The enone functionality is shown in green, the triggering device in blue and the 10-membered ring, or benzenoid system,

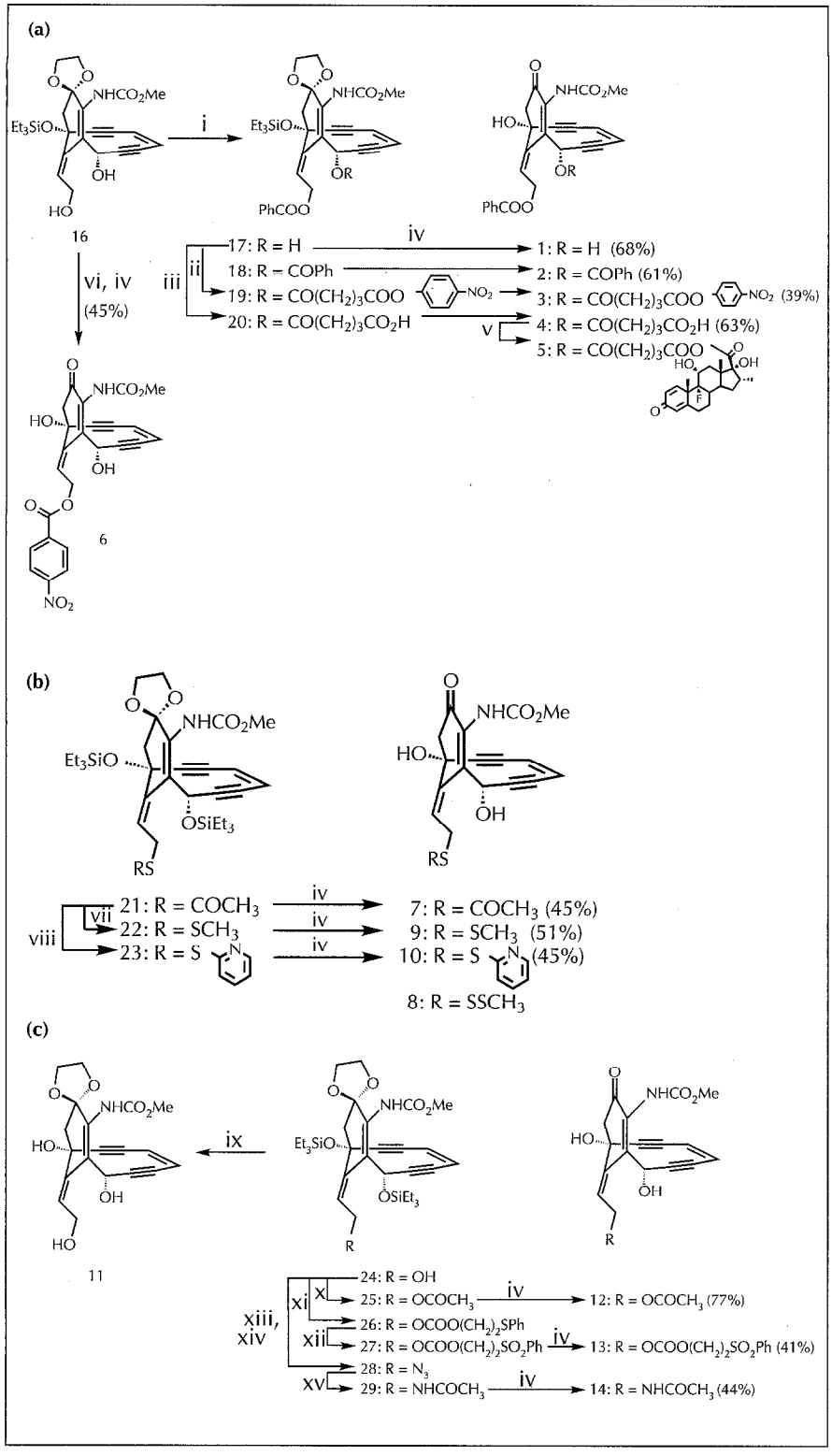

Fig. 4. **(a)** Synthesis of designed calicheamicin mimics 1–6. Starting with compound 16 [27], we produced compounds 6 and 17–20, and thence compounds 1–5, using the following conditions in the order shown: (i) 2.6 equivalent of BzCl, 6.0 equivalent of Pyr, CH_2Cl_2, 1.5 h, –15 °C, 68 % of 17 and 26 % of 18; (ii) 9.0 equivalent of glutaric anhydride, 12.0 equivalent of DMAP, CH_2Cl_2, 25 °C, 2.5 h; then 9.0 equivalent of DCC, 9.0 equivalent of *p*-nitrophenol, 2 h, 46 %; (iii) 1.6 equivalent of DMAP, 3.0 equivalent of glutaric anhydride, 25 °C, 6 h, 69 %; (iv) 5.5 equivalent of $TsOH{\cdot}H_2O$, aqueous THF, 25 °C; (v) 2.4 equivalent of desoxamethasone, 1.5 equivalent of DCC, 0.5 equivalent DMAP, CH_2Cl_2, 25 °C, 2 h, 53 %; (vi) 3.0 equivalent of Pyr, 0.5 equivalent of DMAP, 2.3 equivalent of *p*-nitrobenzoylchloride, CH_2Cl_2, –15 °C, 1.5 h, 66 %; **(b)** Synthesis of designed calicheamicin mimics 7, 9 and 10. Compound 21 [27] was used to produce compounds 22 and 23 using the following conditions in the order shown: (vii) 7.0 equivalent of DIBAL, CH_2Cl_2, –78 °C, 30 min; then 10.0 equivalent MeOH, –78→25 °C, 4.2 equivalent of MeSSCOOMe, 12 h, 70 %; (viii) 4.3 equivalent of NaOMe, MeOH, 0 °C, 3 h; then 5.0 equivalent of aldrithiol-2, 3 h, 66 %. Reaction (iv) (above) was then performed on compounds 21–23, giving compounds 7, 9 and 10. **(c)** Synthesis of compounds 11–14. Starting with compound 24, compounds 11, 25, 26 and 28 were constructed, and subsequently compounds 27 and 29, using the following conditions in the order shown: (ix) HF·Pyr, THF, 0→25 °C, 4 h, 96%; (x) 4 equivalent of Pyr, 0.5 equivalent of DMAP, 3.0 equivalent of Ac_2O, 0 °C, 2 h, 92 %; (xi) 7.0 equivalent of DMAP, 20 equivalent of $PhSCH_2CH_2OCOCl$, CH_2Cl_2, 0 °C, 4 h, 88 %; (xii) 4.0 equivalent of *m*CPBA, CH_2Cl_2, 25 °C, 2 h, 94 %; (xiii) 10.0 equivalent of Pyr, 5.0 equivalent of MsCl, 0.5 equivalent of DMAP, CH_2Cl_2, 0 °C, 3 h; (xiv) 10.0 equivalent of NaN_3, DMF, 50 °C, 53 %; (xv) AcSH, 25 °C, 2 h, 48 %. Compounds 25, 27 and 29 were then converted into compounds 12–14 using reaction (iv), above. Bz: PhCO, Pyr: pyridine, DMAP: 4-dimethylaminopyridine, DCC: 1,3-dicyclohexylcarbodiimide, *m*CPBA: *m*-chloroperbenzoic acid, DIBAL: diisobutylaluminum hydride.

a different problem and was made as outlined in Fig. 5. The readily available amine 30 [27] was converted to urethane 31 in 82 % yield, by treatment with oxalyl chloride and 2-phenylthioethanol in the presence of pyridine. Lactone 31 was then reduced sequentially with DIBAL and $NaBH_4$ leading to diol 33 via lactol 32 (63 % overall yield). Selective monobenzylation of the primary alcohol in diol 33 produced monobenzoate 34 (69 %), which was oxidized by *m*-chloroperbenzoic acid (*m*CPBA) to sulfone 35 in 100 % yield. Finally, deprotection under aqueous acid conditions generated the targeted enediyne 15 in 36 % yield.

DNA cleaving activity

The synthesized compounds were tested for their ability to cleave DNA under a variety of conditions (Fig. 6). Supercoiled DNA (ΦX174 or pBR322) was incubated with each compound in buffer solutions at different pH values and in the presence or absence of 2-mercapto-

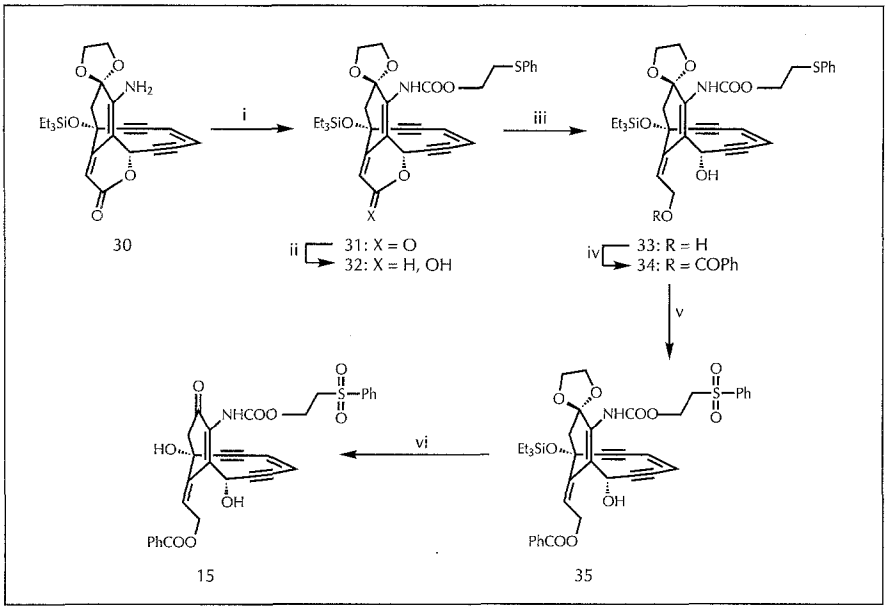

Fig. 5. Synthesis of designed calicheamicin mimic 15. Starting with compound 30 [27], compounds 31–35 were made, and compound 35 then converted to compound 15, by sequential application of the following conditions: (i) 10.0 equivalent of Pyr, 3.0 equivalent of $(COCl)_2$, CH_2Cl_2, 25 °C, 30 min; then 10.0 equivalent of Pyr, 10.0 equivalent of $PhSCH_2CH_2OH$, 2 h, 82 %; (ii) 2.2 equivalent of DIBAL, CH_2Cl_2, –78 °C, 30 min, 4:1 mixture of epimers, 95 %; (iii) 55.0 equivalent $NaBH_4$, MeOH, 0 °C, 2 h, 66 %; (iv) 5.0 equivalent Pyr, 2.4 equivalent of BzCl, 0 °C, 1 h, 60 %; (v) 5.0 equivalent of *m*CPBA, CH_2Cl_2, 25 °C, 1.5 h, 100 %. (v) 5.5 equivalent $TsOH{\cdot}H_2O$, aqueous THF, 25 °C, 24 h, 36 %.

ethanol. DNA damage was assayed by agarose gel electrophoresis. Compounds 6, 7–9, and 13 had the highest potencies in the DNA cleavage assay (data not shown for compound 13). Except for compounds 8 and 9, which require the presence of thiol, cleavage was observed under basic conditions in all cases. Some DNA damage was also observed with neutral and slightly acidic media. This effect may be caused by the local basicity or nucleophilicity of DNA, as has been suggested earlier for a series of dynemicin A mimics [22].

Table 1. Comparison of cytotoxicities of designed enediynes 6–8, calicheamicin γ_1^I, and θ_1^I and PM-9 against a panel of 17 transformed cell lines and four normal cell lines.

Cell type	6	8	calicheamicin γ_1^I	7	calicheamicin θ_1^I	PM-9
Normal cell lines						
CHO	1.1×10^{-6}	1.8×10^{-7}	1.2×10^{-9}	1.1×10^{-5}	4.3×10^{-9}	3.1×10^{-6}
HMEC	3.4×10^{-7}	1.1×10^{-6}	1.0×10^{-9}	2.4×10^{-6}	$<10^{-12}$	6.3×10^{-6}
NHDF	–	9.8×10^{-7}	1.0×10^{-9}	2.9×10^{-6}	$<10^{-12}$	5.0×10^{-6}
RPMI766	–	3.1×10^{-8}	1.6×10^{-9}	2.5×10^{-6}	$<10^{-12}$	–
Transformed cell lines						
BT-549	9.9×10^{-7}	5.3×10^{-8}	1.0×10^{-9}	4.5×10^{-6}	$<10^{-12}$	–
CAPAN1	1.2×10^{-6}	7.1×10^{-7}	1.3×10^{-9}	1.3×10^{-5}	$<10^{-12}$	3.1×10^{-9}
H322	1.7×10^{-6}	2.0×10^{-6}	1.7×10^{-8}	3.6×10^{-5}	1.0×10^{-9}	3.9×10^{-7}
HL-60	2.0×10^{-10}	5.1×10^{-10}	$<10^{-12}$	–	$<10^{-12}$	3.6×10^{-11}
HT-29	5.1×10^{-6}	3.6×10^{-6}	7.0×10^{-9}	1.2×10^{-5}	$<10^{-12}$	1.6×10^{-6}
M24-MET	4.1×10^{-7}	–	1.0×10^{-9}	–	$<10^{-12}$	–
MCF-7	2.6×10^{-6}	1.9×10^{-6}	4.1×10^{-8}	3.6×10^{-6}	1.2×10^{-11}	7.8×10^{-7}
MOLT-4	4.4×10^{-11}	1.3×10^{-12}	$<10^{-12}$	1.9×10^{-7}	$<10^{-12}$	$<10^{-12}$
OVCAR-3	4.8×10^{-6}	1.3×10^{-6}	2.2×10^{-9}	1.7×10^{-5}	$<10^{-12}$	7.8×10^{-7}
UCLA-P-3	4.2×10^{-6}	1.8×10^{-6}	1.7×10^{-9}	1.3×10^{-5}	$<10^{-12}$	9.8×10^{-8}
PC-3	3.4×10^{-8}	6.3×10^{-7}	4.4×10^{-9}	7.5×10^{-6}	$<10^{-12}$	–
SIHA	5.0×10^{-7}	1.6×10^{-6}	6.7×10^{-10}	1.4×10^{-6}	$<10^{-12}$	–
SK-MEL 28	1.8×10^{-6}	2.4×10^{-6}	1.6×10^{-9}	5.1×10^{-5}	$<10^{-12}$	–
U251	1.3×10^{-6}	7.4×10^{-8}	1.1×10^{-9}	4.1×10^{-5}	9.9×10^{-9}	–
786-0	1.4×10^{-5}	3.7×10^{-7}	1.0×10^{-8}	1.8×10^{-5}	$<10^{-12}$	–
L1210	2.7×10^{-8}	–	–	–	–	1.3×10^{-9}
SK-N-SH	7.8×10^{-9}	–	–	–	–	–

Cytotoxicities (IC_{50} values in mol l^{-1}) were determined by the sulforhodamine B assay [37]. Key: CHO, Chinese hamster ovary cells; HMEC, human mammary epithelial cells; NHDF, normal human dermal fibroblast; RPMI766, human peripheral blood lymphoblast; BT-549, human breast ductal carcinoma; CAPAN1, pancreatic carcinoma; H322, lung adenocarcinoma; HL-60, human promyelomic leukemia; HT-29, human colon adenocarcinoma; M24-MET, metastatic melanoma; MCF-7, breast carcinoma; MOLT-4, human T-cell carcinoma; OVCAR-3, human ovarian carcinoma; UCLA-P-3, lung adenocarcinoma; PC-3, human prostate carcinoma; SIHA, human squamous carcinoma, CERVIX; SKMEL 28, melanoma; U251, human CNS cancer; 786–0, human perirenal cell carcinoma; L1210, murine leukemia; SK-N-SH, neuroblastoma.

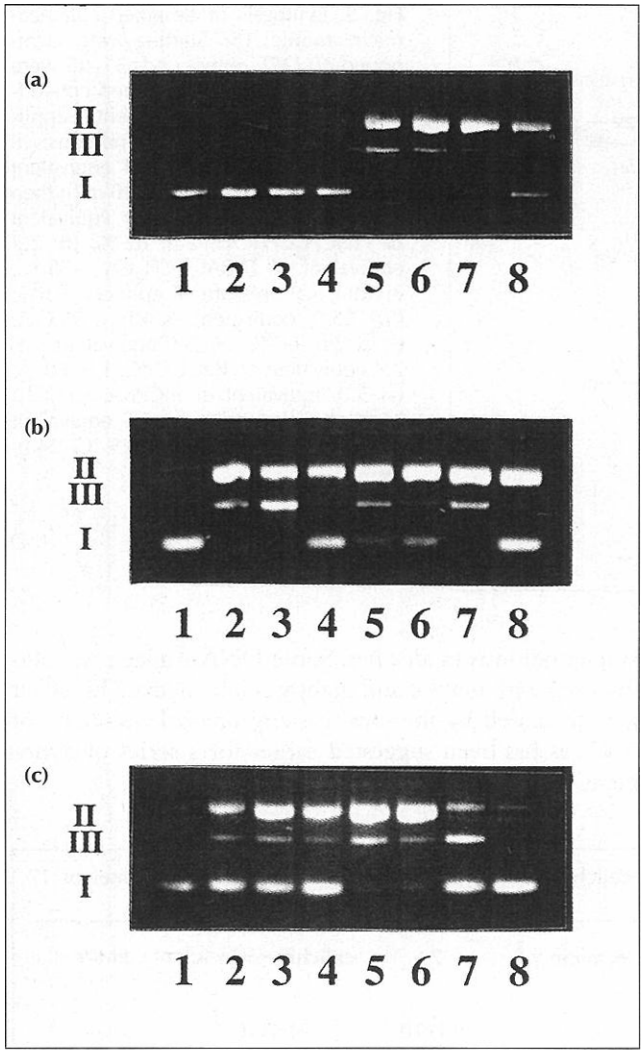

Fig. 6. DNA cleavage activity of designed calicheamicin mimics. Supercoiled DNA runs at the position noted as I, nicked DNA at position II, linear DNA (the result of double-strand cleavage) at position III. **(a)** Cleavage activity of compound 6. Lanes 1–4 show supercoiled ϕX174RFI DNA controls at pH 6.0, 7.0, 7.5, and 8.5. Lanes 5–8 show supercoiled DNA plus compound 6 (1 mM) at pH 8.5, 7.5, 7.0, and 6.0. **(b)** Cleavage activities of compounds 1, 2, 6, 7, 12, and 15. Lane 1, supercoiled ϕX174RFI DNA (control); lanes 2 and 3, DNA plus compounds 1 and 6, respectively (5 mM); lane 4, DNA plus compound 2 (10 mM); lanes 5 and 6, DNA plus compounds 12 and 7, respectively (5 mM); lane 7, DNA plus compound 15 (10 mM); lane 8, nicked DNA (RFII standard). **(c)** Activation of compounds 8 and 9 by mercaptoethanol. Cleavage of supercoiled pBR322 DNA with the dynemicin mimic PM-9 (see Fig. 1b), calicheamicin θ_1^I (Fig. 1b), and compounds 6–9 was assayed under the conditions noted. Lane 1, supercoiled DNA, pH 8.5 (control); in lane 2, PM-9 (5 mM, pH 8.5) was added; in lane 3, compound 8 (5 mM, 2-mercaptoethanol 19 mM, pH 7.0); in lane 4, compound 9 (5 mM, 2-mercaptoethanol 19 mM, pH 7.0); in lane 5, compound 6 (5 mM, pH 8.5); in lane 6, compound 7 (5 mM, pH 8.5); and in lane 7, calicheamicin θ_1^I (0.5 μM, pH 8.5). Lane 8 contained supercoiled DNA in 2-mercaptoethanol 19 mM, pH 7.0 as a control.

The most significant observation in these DNA cleavage studies is the predominance of single-stranded DNA breaks for the aglycones (except for compound 6), in contrast to the double-stranded breaks caused by the natural calicheamicins [31]. The dibenzoylated derivative, compound 2, is less potent in DNA cleavage compared with compound 1 (Fig. 6b). This finding indicates that the propargylic hydroxyl might be important in the hydrolysis of the allylic benzoate (an effect previously observed by Danishefsky and colleagues [24]), the event that leads to the Bergman activation cascade. The relative efficiencies of compounds 1, 2, 6, 7, and 12 in cleaving DNA correlate well with their molecular structure and reflect the expectations of the molecular design (Fig. 6b). Thus, the more labile *p*-nitrobenzoate, compound 6, damages supercoiled DNA to a greater extent than benzoate 1 or acetate 12 do under the same conditions (Fig. 6b). This compound also cleaves DNA with progressively higher efficiency at increasing pH values (Fig. 6a), as expected from the higher rates of hydrolysis that lead to activation. The disulfide-containing compound 9 cleaves DNA only in the presence of added thiol (Fig. 6c), as expected from its propensity to cleave under the influence of the thiol nucleophile. Surprisingly, compound 35 (Fig. 5) failed to cleave DNA at a range of pH values (pH 6–8.5) (data not shown). Given the lability of the 2'-phenylsulfone ethyl carbamate moiety under basic conditions [22], this can only be attributed to a relatively high and unexpected stability of either the postulated enamine intermediate VIII or ketone IX (Y = OCH_2CH_2O, Fig. 3b; similar observations are reported in [33]). The DNA cleaving activity of compound 15 may result from its activation through benzoate hydrolysis rather than enamine-induced triggering.

Cytotoxicity

Cytotoxicity studies were carried out with the synthesized compounds using a broad spectrum of cell lines, ranging from the drug resistant SK-Mel-28 melanoma to the highly sensitive Molt-4 leukemia cells, to assess antineoplastic activity and cell-type selectivity. A number of these compounds, such as 6 and 9, exhibited high cytotoxicities, with the most potent ones being comparable with calicheamicinone (compound 8, Fig. 4b) but considerably less potent than calicheamicins γ_1^I and θ_1^I (Table 1). Fig. 7 shows the IC_{50} values of several synthesized compounds reflecting their potencies against transformed and normal cells. It is noteworthy that the relative potencies of these compounds correlate well with their ability to cleave supercoiled DNA. Plausible explanations for the low cytotoxicity of the enediyne–desoxymethasone conjugate 5 may be that the steroid conjugate is not recognized by the glucocorticoid receptor or that the glucocorticoid receptor–DNA complex does not properly position the enediyne moiety on the target for effective DNA cleavage.

It is clear from these data that although compounds with cytotoxicities comparable with calicheamicinone (compound 8) can be obtained by significantly simplifying the structure of the enediyne moiety, the effect of the oligosaccharide portion of calicheamicin cannot be matched by any of the designed simplifications. Comparing the potencies of compound 8 with

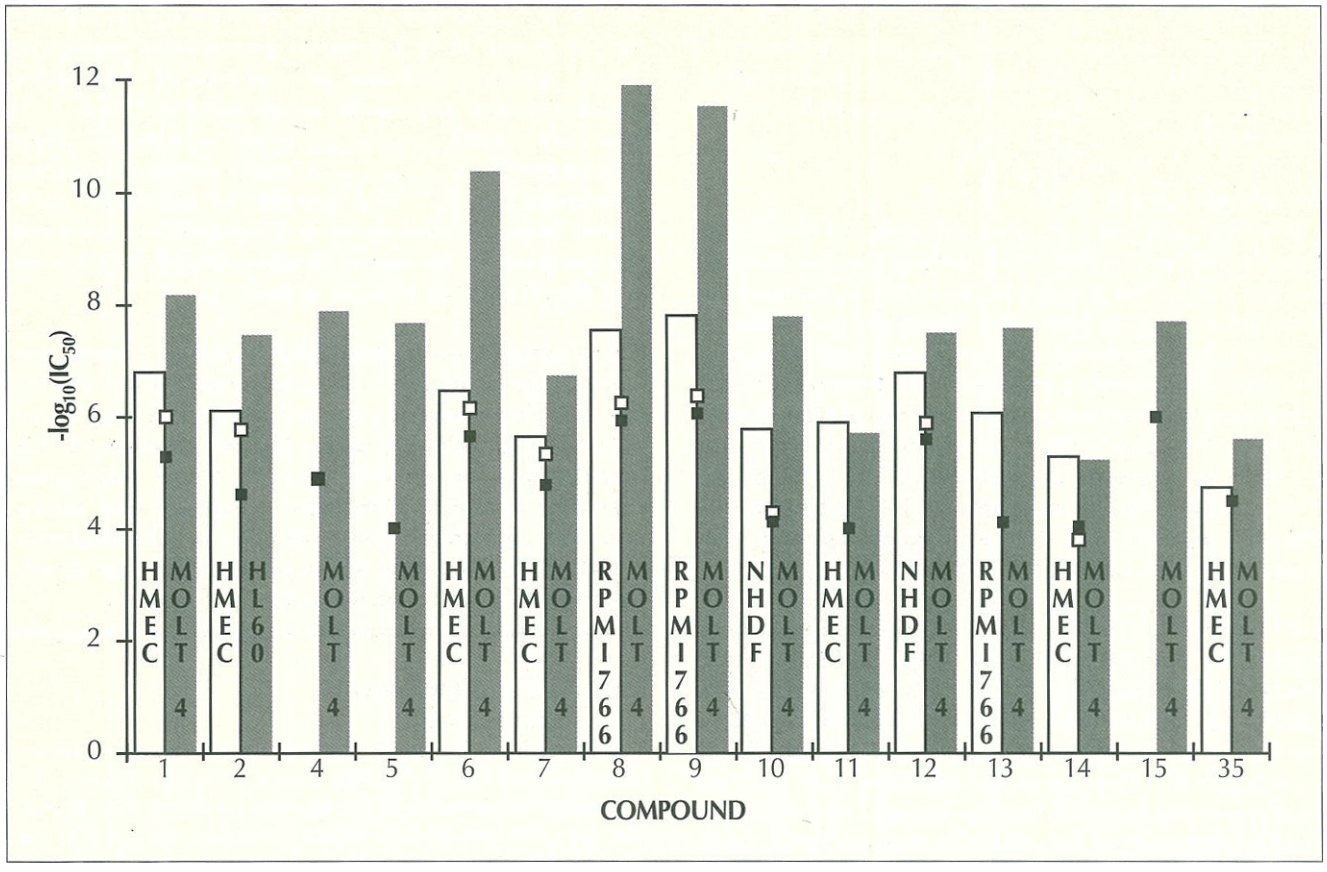

Fig. 7. Comparison of cytotoxicities (IC_{50} values in mol l^{-1}) of designed enediynes 1, 2, 4–15, and 35 against transformed and normal cells. Bars represent the cytotoxicities of each compound against the most sensitive transformed (gray) and normal (white) cell line (indicated inside the bars). Squares indicate the average cytotoxicities for each compound for the transformed (■, 17 different cell lines) and normal (□, 4 different cell lines) cells. Cytotoxicities were determined by the sulforhodamine B assay [37]. Key: HMEC, human mammary epithelial cells; NHDF, normal human dermal fibroblast; RPMI766, human peripheral blood lymphoblast; HL-60, human promyelomic leukemia; MOLT-4, human T-cell carcinoma.

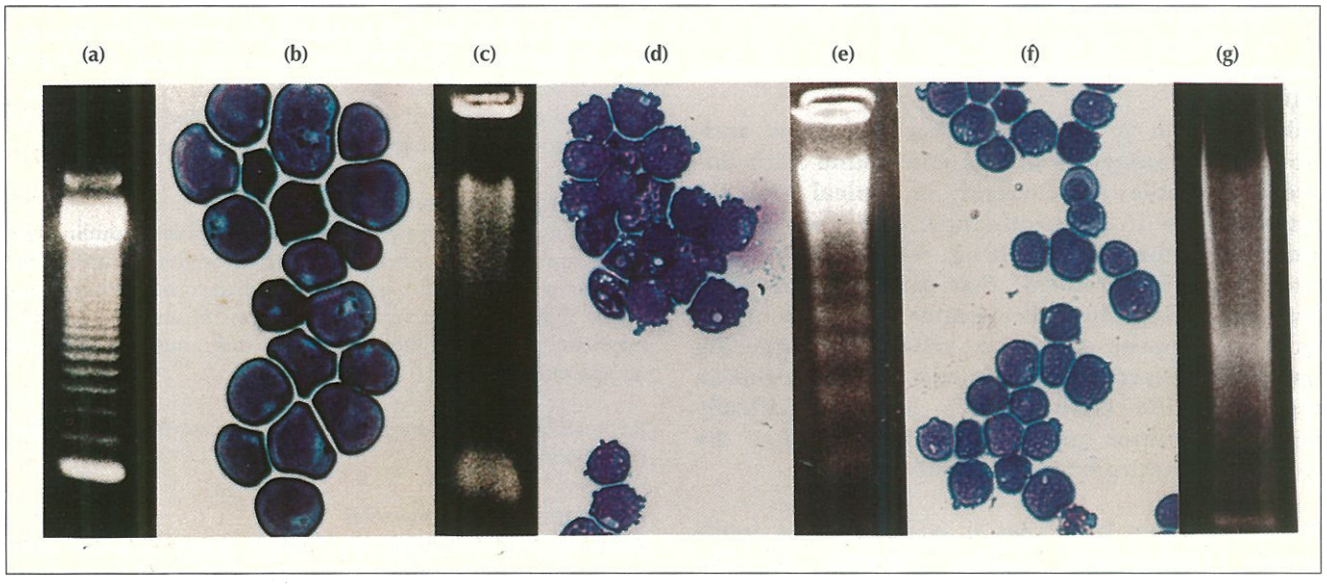

Fig. 8. Apoptotic morphology and nuclear DNA degradation induced by calicheamicin θ_1^I and the designed mimic compound 6. **(a)** Molecular weight markers consisting of multiples of a 123 base-pair fragment (Gibco BRL). **(b)** Untreated MOLT-4 leukemia cells. **(c)** DNA extracted from untreated MOLT-4 cells. **(d)** MOLT-4 cells incubated with 10^{-7} M of calicheamicin θ_1^I. **(e)** DNA extracted from MOLT-4 cells exposed to 10^{-7} M of calicheamicin θ_1^I. **(f)** MOLT-4 cells incubated with 10^{-7} M of compound 6. **(g)** DNA extracted from MOLT-4 cells exposed to 10^{-7} M of compound 6.

calicheamicin γ_1^I and compound 7 with calicheamicin θ_1^I (Table 1), it is reasonable to project that attachment of compound 6 to the oligosaccharide portion of calicheamicin or other carriers should increase its potency and/or selectivity.

Mechanism of action

The strong correlation between the ability of the synthesized enediynes to cause DNA cleavage and cytotoxicity suggests that DNA damage is the cause of cell death by these compounds. To learn more about the mechanism of action of these enediynes, cells were examined for signs of apoptosis [34] following exposure to compound 6, which was chosen as a representative of this class. Upon exposure of Molt-4 leukemia cells to compound 6, however, only moderate apoptosis (< 20 % of the cells at 10^{-7} M) was observed (Fig. 8f and g), in contrast to the strong apoptosis observed after the cells were exposed to the same concentration of calicheamicin θ_1^I (Fig. 8d and e).

It is noteworthy that all the calicheamicinone mimics synthesized and tested showed predominantly single-stranded DNA cuts (see Fig. 6). This observation is in contrast to calicheamicins γ_1^I and θ_1^I, both of which are known to induce predominantly double-stranded DNA damage [23,35]. This difference correlates well with the observed cytotoxicity of these compounds and their ability to cause apoptosis, the molecules with the DNA-binding domain having significantly higher potencies in both assays. Thus, it seems reasonable to suggest that, at least in the case of the enediyne type of DNA damaging agents, the propensity of a compound to induce apoptosis is proportional to its ability to cause double strand cuts of the genetic material. It seems likely that the inability of the cell to repair such drastic damage leads to the initiation of apoptosis, whereas the cell can presumably repair single-stranded cuts relatively easily, preventing cell death.

Significance

Calicheamicin γ_1^I is a promising antitumor antibiotic, and conjugates of this compound with different antibodies are currently in clinical trials for potential use in chemotherapy. The core of the molecule is the enediyne ring, which can be activated to form diradicals that can cleave double-stranded DNA. The other important parts of the molecule appear to be a trisulfide triggering group, which activates the enediyne group, and an oligosaccharide chain, which binds specifically along the minor groove of DNA, so that the molecule is appropriately targeted.

We have shown here that it is possible to make simple biologically active mimics of the calicheamicin structure that lack the trisulfide and oligosaccharide moieties. These relatively simple designed enediynes can still cleave DNA, and still show significant cytotoxicity. As we have removed the targeting oligosaccharide group, however, they would be expected to have relatively low affinity for DNA and to lack specific positioning along the minor groove. Perhaps as a result of this, these mimics cause primarily single-strand DNA cuts and are weaker inducers of apoptosis than calicheamicin γ_1^I and a previously designed mimic, calicheamicin θ_1^I. The properties of these molecules indicate that there is a correlation between the propensity to cause double-strand DNA cuts and the ability to initiate apoptosis.

These simple molecules may serve as convenient 'molecular warheads' for attachment onto targeting delivery systems such as antibodies and other proteins, oligonucleotides, oligosaccharides, DNA intercalators or groove binders. Such conjugates may find useful applications in biology and medicine. In addition, these simple yet biologically active molecules may serve as leads for the development of novel chemotherapeutic agents.

Materials and methods

DNA cleavage assay

The supercoiled plasmid ϕX174RFI (100 μM per base pair) was incubated for 8 h at 37 °C with the test compounds at the concentrations shown in the figure legends and analyzed by electrophoresis (1 % agarose gel, ethidium bromide stain). Buffer was 5 mM Tris-HCl, pH 8.5 unless otherwise noted. Compound 6 was also analysed at pH 6.0, 7.0 and 7.5 in various buffers. Plasmid pBR322 (100 μM per base pair) was treated similarly, but incubated for 4 h instead of 8 h.

Apoptosis assay

Solutions of calicheamicin θ_1^I or compound 6 in DMSO (20 mM) were diluted in culture medium to the appropriate concentrations and added to cell cultures. MOLT-4 cells (10^5) were incubated with 10^{-7} M final concentrations of calicheamicin θ_1^I or the mimic, compound 6, for apoptosis morphology assays, and with 10^{-6} M final concentrations for DNA degradation assays. After incubation for 4 h at 37 °C, cells were harvested and processed as previously described [36] to visualize apoptotic morphology and DNA degradation.

Preparation of compounds

Compounds were prepared by chemical synthesis as outlined in Figs 4 and 5 using standard laboratory procedures, purified by chromatography on silica gel, and fully characterized by spectroscopic techniques (infrared spectroscopy, ^1H and ^{13}C nuclear magnetic resonance, high-resolution mass spectroscopy and optical rotations).

Representative procedures and data are described below for compound 6.

Preparation of compound 6

Pyridine (25 μl, 0.3 mmol) and DMAP (6 mg, 0.5 μmol) were added to a solution of diol 16 (see Fig. 4; 50 mg, 0.1 mmol) in CH_2Cl_2 (10 ml) at −15 °C. *p*-Nitrobenzoyl chloride (26 mM in CH_2Cl_2) was added dropwise and in two portions (0.13 + 0.1 mmol, 1 h interval) until almost all starting material was consumed and then the reaction was quenched by MeOH (0.5 ml). After standard aqueous workup, the reaction

mixture was purified by flash column chromatography (50 → 100 % Et_2O/petroleum ether) to give the fully protected mono-*p*-nitrobenzoate (42 mg, 66 %). Small amounts of dibenzoate (4 mg, 0.5 %) and unreacted diol 16 (8 mg, 30 %) were also isolated.

Mono-p-nitrobenzoate

Foam; R_f=0.47 (Et_2O); $[\alpha]^{25}_D$–216 (*c* 1.40, CH_2Cl_2); IR (neat) ν_{max} 3326, 2956, 2931, 2877, 1723, 1606, 1528, 1501, 1461, 1343, 1274 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ8.27 (d, *J*=8.9 Hz, 2 H, *Ar*), 8.23 (d, *J*=8.9 Hz, 2 H, *Ar*), 6.50 (b s, 1 H, N*H*COOCH$_3$), 6.35 (dd, *J*=5.4, 7.6 Hz, 1 H, C=C*H*CH$_2$), 5.89 (d, *J*=9.5 Hz, 1 H, C*H*=CHC≡CCH), 5.84 (dd, *J*=1.0, 9.5 Hz, 1 H, CH=CHC≡CCH), 5.71 (d, *J*=4.8 Hz, 1 H, C*H*OH), 5.34 (dd, *J*=5.1, 13.8 Hz, 1 H, C*H*HOCOAr), 5.20 (b m, 1 H, CH*H*OCOAr), 4.03–3.91 (m, 4 H, ethylene ketal), 3.77 (s, 3H, NHCOOC*H*$_3$), 2.55 (d, *J*=14.0 Hz, 1 H, C*H*H-eq), 2.25 (d, *J*=14.0 Hz, 1 H, CH*H*-ax), 1.00 (t, *J*=7.9 Hz, 9 H, Si(CH$_2$C*H*$_3$)$_3$), 0.83–0.70 (m, 6 H, Si(C*H*$_2$CH$_3$)3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.6, 155.1, 150.3, 140.2, 136.1, 130.8, 123.4, 104.8, 99.9, 86.3, 85.6, 65.6, 65.4, 65.2, 63.6, 53.4, 50.4, 29.7, 7.1, 6.1; FAB HRMS (NBA/NaI) *m/e* 659.2050, ($M+Na^+$) calculated for $C_{32}H_{36}N_2O_{10}Si$: 659.2037.

A solution of the protected form of monobenzoate 6 (42 mg, 66 μmol) in THF (1.4 ml) and H_2O (4 drops) was treated with TsOH·H_2O (30 mg, 0.15 mmol) and the mixture was stirred at ambient temperature for 36 h. The reaction mixture was diluted with CH_2Cl_2, passed through a short column of Na_2SO_4 and purified by preparative TLC (20 % acetone in CH_2Cl_2) to give enone 6 (14 mg, 45 %): colorless solid; R_f = 0.63 (20 % acetone in CH_2Cl_2); $[\alpha]^{25}_D$ –910 (*c* 0.12, CH_2Cl_2); IR (neat) ν_{max} 3358, 2919, 1719, 1679, 1525, 1344, 1274 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) δ 8.29–8.22 (m, 4 H, *Ar*), 7.00 (b s, 1 H, N*H*COOCH$_3$), 6.43 (dd, *J*=4.3, 8.1 Hz, 1 H, C=C*H*CH$_2$), 6.01 (b d, *J*=4.7 Hz, 1 H, C*H*OH), 5.93 (dd, *J*=1.37, 9.5 Hz, 1 H, CH=C*H*C≡CCH), 5.89 (d, *J*=9.5 Hz, 1 H, C*H*=CHC≡CCH), 5.36 (dd, *J*=4.2, 14.2 Hz, 1 H, C*H*HOCO*Ar*), 5.19 (dd, *J*=8.1, 14.2 Hz, 1 H, CH*H*OCO*Ar*), 3.80 (s, 3 H, NHCOOC*H*$_3$), 3.65 (b s, 1 H, O*H*), 3.23 (d, *J*=17.0 Hz, 1 H, C*H*H-eq), 3.02 (b s, 1 H, O*H*), 2.80 (d, *J*=17.0 Hz, CH*H*-ax); ^{13}C NMR (125 MHz, $CDCl_3$) δ191.0, 164.6, 154.8, 150.6, 137.7, 135.5, 130.9, 130.2, 128.3, 124.6, 123.7, 123.5, 99.9, 99.6, 88.0, 85.2, 72.3, 53.6, 51.7, 29.7; FAB HRMS (NBA/CsI) *m/e* 611.0055, ($M+Cs^+$) calculated for $C_{24}H_{18}N_2O_9$: 611.0067.

Supplementary material available

Preparation procedures and physicochemical data for compounds 2, 3, 5, 11, 14, 17–19, 28, and 29.

Acknowledgements: We thank Drs Dee H. Huang and Gary Siuzdak for NMR and mass spectroscopic assistance, respectively, and Robert Merlock for assistance in biological assays. We also thank Dr Tianhu Li for helpful discussions. This work was supported by the NIH, The Scripps Research Institute, Merck Sharp and Dohme, Glaxo Inc., Pfizer, Inc. and Schering Plough.

References

1. Nicolaou, K.C. & Dai, W.M. (1991). Chemistry and biology of enediyne anticancer antibiotics. *Angew. Chem.* **30**, 1387–1530.
2. Lee, M.D., *et al.*, & Borders, D.B. (1987). Calicheamicins, a novel family of antitumor antibiotics. 2. Chemistry and structure of calicheamicin γ_1^I. *J. Am. Chem. Soc.* **109**, 3466–3468.
3. Golik, J., *et al.*, & Doyle, T.W. (1987). Esperamicins, a novel class of potent antitumor antibiotics. 3. Structures of esperamicins A1, A2 and A1b. *J. Am. Chem. Soc.* **109**, 3462–3464.
4. Edo, K., *et al.*, & Ishida, N. (1985). The structure of neocarzinostatin chromophore possessing a novel bicyclo[7.3.0]dodecadiyne system. *Tetrahedron Lett.* **26**, 331–334.
5. Goldberg, I.H. (1991). Mechanism of neocarzinostatin action: role of DNA microstructure in determination of chemistry of bistranded oxidative damage. *Accounts Chem. Res.* **24**, 191–198.
6. Konishi, M., *et al.*, & Clardy, J. (1989). Dynemicin A, a novel antibiotic with the anthraquinone and 1,5-diyn-3-ene subunit. *J. Antibiot. (Tokyo)* **42**, 1449–1452.
7. Leet, J.E., *et al.*, & Matson, J.A. (1992). Kedarcidin, a new chromoprotein antitumor antibiotic: structure elucidation of kedarcidin chromophore. *J. Am. Chem. Soc.* **114**, 7946–7948.
8. Yoshida, K., Minami, Y., Azuma, R., Saeki, M. & Otani, T. (1993). Structure and cycloaromatization of a novel enediyne, C-1027 chromophore. *Tetrahedron Lett.* **34**, 2637–2640.
9. Nicolaou, K.C., *et al.*, & Saimoto, H. (1992). Total synthesis of calicheamicin γ_1^I. *J. Am. Chem. Soc.* **114**, 10082–10084.
10. Hitchcock, S.A., Boyer, S.H., Moyer, M.Y.C., Olson, S.H. & Danishefsky, S.J. (1994). A convergent total synthesis of calicheamicin γ_1^I. *Angew. Chem.* **33**, 858–862.
11. Taunton, J., Wood, J.L. & Schreiber, S.L. (1993). Total syntheses of di- and tri-O-methyl dynemicin A methyl esters. *J. Am. Chem. Soc.* **115**, 10378–10379.
12. Nicolaou, K.C., Smith, A.L. & Yue, E.W. (1993). Chemistry and biology of natural and designed enediynes. *Proc. Natl. Acad. Sci. USA* **90**, 5881–5888.
13. Dedon, P.C. & Goldberg, I.H. (1992). Free-radical mechanisms involved in the formation of sequence-dependent bistranded DNA lesions by the antitumor antibiotics bleomycin, neocarzinostatin, and calicheamicin. *Chem. Res. Toxicol.* **5**, 311–332.
14. Lee, M.D., Ellestad, G.A. & Borders, D.B. (1991). Calicheamicins: discovery, structure, chemistry, and interaction with DNA. *Accounts Chem. Res.* **24**, 235–243.
15. Uesugi, M. & Sugiura, Y. (1993). New insights into sequence recognition process of esperamicin A1 and calicheamicin γ_1^I: origin of their selectivities and 'induced fit' mechanism. *Biochemistry* **32**, 4622–4627.
16. Myers, A.G., Cohen, S.B. & Kwon, B.M. (1994). DNA cleavage by neocarzinostatin chromophore. Establishing the intermediacy of chromophore-derived cumulene and biradical species and their role in sequence-specific cleavage. *J. Am. Chem. Soc.* **116**, 1670–1682.
17. Ichikawa, A., Kuboya, T., Aoyama, T. & Sugiura, Y. (1992). Activation of DNA cleavage by dynemicin A in a B-Z conformational junction. *Biochemistry* **31**, 6784–6787.
18. Zein, N., *et al.*, & Casazza, A.M. (1993). Kedarcidin chromophore: an enediyne that cleaves DNA in a sequence-specific manner. *Proc. Natl. Acad. Sci. USA* **90**, 2822–2826.
19. Sugiura, Y. & Matsumoto, T. (1993). Some characteristics of DNA strand scission by macromolecular antitumor antibiotic C-1027 containing a novel enediyne chromophore. *Biochemistry* **32**, 5548–5553.
20. Bergman, R.G. (1973). Reactive 1,4-dehydroaromatics. *Accounts Chem. Res.* **6**, 25–31.
21. Myers, A.G., Cohen, S.B. & Mog Kwon, B. (1994). A study of the reaction of calicheamicin γ_1^I with glutathione in the presence of double-stranded DNA. *J. Am. Chem. Soc.* **116**, 1255–1271.
22. Nicolaou, K.C., Dai, W.M., Tsay, S.C., Estevez, V.A. & Wrasidlo, W. (1992). Designed enediynes: a new class of DNA-cleaving molecules with potent and selective anticancer activity. *Science* **256**, 1172–1178.
23. Nicolaou, K.C., Li, T., Nakada, M., Hummel, C.W., Hiatt, A. & Wrasidlo, W. (1993). Calicheamicin θ_1^I: a rationally designed and totally synthetic molecule with extremely potent and selective DNA cleaving properties and apoptosis inducing activity. *Angew. Chem.* **33**, 183–186.
24. Haseltine, J.N., Danishefsky, S.J. & Schulte, G. (1989). Experimental modeling of the priming mechanism of the calicheamicin/esperamicin antibiotics: actuation by the addition of intramolecular nucleophiles to the bridgehead double bond. *J. Am. Chem. Soc.* **111**, 7638–7640.
25. Haseltine, J.N., *et al.*, & Schulte, G.K. (1991). Total synthesis of calicheamicinone: New arrangements for actuation of the reductive cycloaromatization of aglycon congeners. *J. Am. Chem. Soc.* **113**, 3850–3866.
26. Hinman, L.M., Hamann, P.R., Wallace, R., Menendez, A.T., Durr, F.E. & Upeslacis, J. (1993). Preparation and characterization of monoclonal antibody conjugates of the calicheamicins: a novel and potent family of antitumor antibiotics. *Cancer Res.* **53**, 3336–3342.
27. Smith, A.L., Hwang, C.K., Pitsinos, E., Scarlato, G.R. & Nicolaou, K.C. (1992). Enantioselective total synthesis of (–)-calicheamicinone. *J. Am. Chem. Soc.* **114**, 3134–3136.

28. Yamamoto, K.R. (1985). Hormone-dependent transitional enhancement and its implications for mechanisms of multifactor gene regulation. In *Molecular development biology; expressing foreign genes, 43rd symposium of society for developmental biology.* (Bagorad, L. & Adelman, G., eds), pp. 131–148, Alan Liss, New York.
29. Brois, S.J. & Pilot, J.F. (1970). A new pathway to unsymmetrical disulfides. The thiol-induced fragmentation of sulfonyl thiocarbonates. *J. Am. Chem. Soc.* **92**, 7629–7631.
30. Carlsson, J., Drevin, H. & Axén, R. (1978). Protein thiolation and reversible protein–protein conjugation. *Biochem. J.* **73**, 723–737.
31. Aiyor, J., *et al.*, & Crothers, D.M. (1994). On the DNA recognition role of the carbohydrate sector in calicheamicin: a comparison of DNA cleaving capacity of enantiomeric calicheamicinones. *Angew. Chem.* **33**, 855–858.
32. Rosen, T., Ligo, I.M. & Chu, D.T.W. (1988). A convenient and highly chemoselective method for the reductive acetylation of azides. *J. Org. Chem.* **53**, 1580–1582.
33. Semmelhack, M.F., Gallagher, J.J., Minami, T. & Date, T. (1993). The enol-keto trigger in initiating arene diradical formation in calicheamicin/esperamicin analogs. *J. Am. Chem. Soc.* **115**, 11618–11619.
34. Ellis, R.E., Yuan, J. & Horvitz, H.R. (1991). Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.* 7, 663–698.
35. Dedon, P.C., Salzberg, A.A. & Xu, J.H. (1993). Exclusive production of bistranded DNA damage by calicheamicin. *Biochemistry* **32**, 3617–3622.
36. Nicolaou, K.C., Stabila, P., Azad, B.E., Wrasidlo, W. & Hiatt, A. (1993). Cell-specific regulation of apoptosis by designed enediynes. *Proc. Natl. Acad. Sci. USA* **90**, 3142–3146.
37. Skehan, P., *et al.*, & Boyd, M.R. (1990). New colorimetric cytotoxicity assays for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**, 1107–1112.

Received: **16 Jun 1994**; revisions requested: **13 Jul 1994**; revisions received: **29 Jul 1994**; accepted: **2 Aug 1994**.