(23) E. C. Miller, *Cancer Res.*, **38**, 1479 (1978).
(24) J. Althoff, F. W. Krueger, and U. Mehr, *J. Natl. Cancer Inst.*, **51**, 287 (1973).
(25) S. Dickhaus, G. Resznik, U. Green, and M. Ketkar, *Z. Krebsforsch.*, **91**, 189 (1978).
(26) F. W. Kruger, *Z. Krebsforsch.*, **76**, 145 (1971).
(27) M. E. Okada, T. Suzuki, J. Aoki, M. Iiyoshi, and T. Hashimoto, *GANN Monogr.*, **no. 17**, 161 (1975).
(28) J. S. Wishnok and M. C. Archer, *Br. J. Cancer*, **33**, 307 (1976).
(29) J. S. Wishnok, M. C. Archer, A. S. Edelman, and W. M. Rand, *Chem.-Biol. Interact.*, **20**, 43 (1978).
(30) W. Lijinsky, *New Sci.*, **27**, 216 (1977).
(31) W. Lijinsky, in "Structural Correlates of Carcinogenesis and Mutagenesis", I. M. Asher and C. Zervos, Eds., The Office of Science, Food and Drug Administration, 1978, p 148.
(32) P. C. Jurs, J. T. Chou, and M. Yuan, *J. Med. Chem.*, **22**, 476 (1979).
(33) A. J. Stuper, W. E. Brugger, and Peter C. Jurs, in "Chemometrics: Theory and Application", B. R. Kowalski, Ed., American Chemical Society, Washington, D.C., 1977.
(34) K. Lee, B. Gold, and S. S. Mirvish, *Mutat. Res.*, **48**, 131 (1977).
(35) J. McCann, E. Choi, E. Yamasaki, and B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.*, **72**, 5135 (1975).
(36) G. Del Re, *J. Chem. Soc.*, 4031 (1958).
(37) A. J. Hopfinger, "Conformational Properties of Macromolecules", Academic Press, New York, 1973.
(38) A. J. Stuper and P. C. Jurs, *J. Chem. Inf. Comp. Sci.*, **16**, 238 (1976).
(39) L. B. Kier and L. H. Hall, "Molecular Connectivity in Chemistry and Drug Research", Academic Press, New York, 1976.
(40) L. B. Kier, R. J. Simons, and L. H. Hall, *J. Pharm. Sci.*, **67**, 725 (1978).
(41) G. M. Singer, H. W. Taylor, and W. Lijinsky, *Chem.-Biol. Interact.*, **19**, 133 (1977).
(42) C. Nagata and A. Imamura, *Gann*, **61**, 169 (1970).
(43) B. Testa, J. C. Bunzil, and W. P. Purcell, *J. Theor. Biol.*, **70**, 339 (1978).
(44) B. Testa and D. Mihailova, *J. Med. Chem.*, **21**, 683 (1978).
(45) J. L. Marx, *Science*, **200**, 518, (1978).

Actinomycin D Oxazinones as Improved Antitumor Agents

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1,4-Oxazinone derivatives of the phenoxazinone chromophore in actinomycin D (AMD) have been synthesized by condensation of AMD with α-keto acids. By varying the starting α-keto acid, the substitutions on the oxazinone ring and, consequently, the lipophilicity of the molecule could be altered. These oxazinone derivatives revert to AMD in physiological media and it appears that these oxazinones are "depot" forms of AMD and possess physicochemical and DNA-binding properties which are significantly different from those of AMD. The oxazinones, which have bulky and lipophilic substituents at position 3, demonstrate more pronounced antitumor activity against P388 mouse leukemia and are less toxic than AMD.

Actinomycin D (AMD, **1**) is one of a family of chromopeptide antibiotics isolated from *Streptomyces* cultures.[2-4] The naturally occurring actinomycin antibiotics have a common 2-aminophenoxazin-3-one ring system with two cyclic pentapeptide lactones attached at the 1 and 9 positions and differ from one another in one or two amino acids in the peptide lactones.[5-12] The integrity and configuration of the peptide rings, as well as the functions at the C_2, C_3, C_4, and C_6 positions on the phenoxazine rings, have been shown to be important for deoxyribonucleic acid binding[13-19] and the expression of their antibiotic activity.[13-19]

AMD has been used in the treatment of human neoplasia and is one of the few agents possessing curative effects against two different tumors:[20] Wilms' tumor[21] and gestational choriocarcinoma.[22] Although AMD is effective at remarkably low doses, its spectrum of antitumor activity is relatively narrow and its clinical administration is complicated by the high toxicity of the drug.[23]

For the past few years this laboratory has carried out investigations on AMD and 7-substituted AMD analogues.[24,25] These studies have established that 7-nitro-AMD and 7-amino-AMD, in spite of poor antibacterial properties, are indeed comparable to AMD in four transplantable mouse tumor systems. Another AMD analogue, N^2-(γ-hydroxypropyl)-AMD, exhibiting similar behavior, has been reported by Meienhofer and Johnson.[26] These findings confirm that the antibacterial activity of AMD analogues, long used to indicate biological usefulness of such compounds, does not provide reliable predictive data for other bioassay systems.

The 3-methyloxazinone of actinomycin C_2 was synthesized by Brockmann[27] and reported to be a poor antibacterial agent,[28] but in this laboratory it was found that a comparable analogue, **2b**, is similar to AMD in activity against AMD-sensitive P388 murine leukemia.

Our initial studies were done on actinomycin chromophore model compounds in which the pentapeptide lactones P in **1** (Scheme I) were replaced by $N(C_2H_5)$ groups.[25] On the basis of studies on these model derivatives,[29] the 3-methyloxazinone of AMD (**2b**) was synthesized (Scheme I), and it was observed that **2b** could be converted almost quantitatively to AMD in the presence of aqueous ethanol at pH 7.3 or above. In addition, it was observed that the 3-phenyloxazinone of AMD (**2g**) required a higher pH above pH 7.6 or a longer time for conversion to AMD in aqueous ethanol. These observations suggested that the oxazinones of AMD may act as depot or prodrug forms of AMD, provided they behave similarly in biological systems, and that the nature of the substituents at the 3 position of the oxazinones might influence their biological activity.

Synthesis and Physicochemical Properties. The successful synthetic route for **2a** and **2g** is shown in Scheme I; the results are summarized in Table I. AMD (**1**) was reduced catalytically under 1 atm of hydrogen pressure, and the reduced derivative was condensed with α-keto acids in methanol. Air was scrupulously excluded from the reaction atmosphere. The products are stable in acid and are usually purified on silicic acid columns. They are easily differentiated from the starting material, AMD, by TLC, UV, and NMR analysis. It was also noted that the specific rotation changes noticeably upon for-

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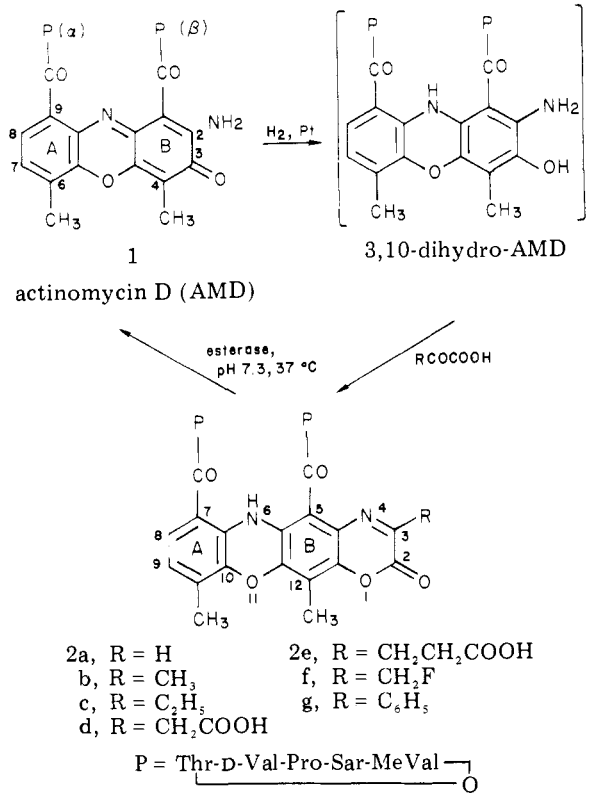
Table I

no.	α-keto acid	% yield	λ_{max}^{a} (ε), nm	$[\alpha]^{20}_{644}$ [b]	R_f [c]	formula
1			424 (21500), 442 (23000)	−300.4 ± 20° (c 0.10)	0.55	$C_{62}H_{86}N_{12}O_{16}\cdot 2H_2O$
2a	glyoxylic acid R = H, mp 55-52 °C	26.4	394 (7950), 501 (4700)	−100 ± 18° (c 0.10)	0.12	$C_{64}H_{86}N_{12}O_{17}\cdot 2H_2O$
2b	pyruvic acid R = CH_3, bp 165 °C	90	320 (8700), 393.0 (6200), 495 (7300)	−100 ± 18° (c 0.10)	0.12	$C_{65}H_{88}N_{12}O_{17}\cdot H_2O$
2c	2-ketobutyric acid R = CH_2CH_3, mp 32-34 °C	74.5	316 (9450), 488 (7050)	−100.6 ± 18° (c 0.18)	0.21	$C_{66}H_{90}N_{12}O_{17}\cdot 2H_2O$
2d	oxaloacetic acid R = CH_2COOH, mp 161 °C (dec)	68.2	320 (8700), 395 (6400), 500 (7100)	−103.5 ± 20° (c 0.11)	0.13	$C_{66}H_{88}N_{12}O_{19}$
2e	2-ketoglutaric acid R = CH_2CH_2COOH, mp 113-115 °C	76.6	351 (8700), 398 (9000), 509 (8160)	−104 ± 18° (c 0.11)	0.04	$C_{67}H_{90}N_{12}O_{19}\cdot 4H_2O$
2f	3-fluoropyruvic acid R = CH_2F, mp 145 °C (dec)	32.2	526 (6900)	−92.6 ± 12° (c 0.10)	0.12	$C_{65}H_{87}F_1N_{12}O_{17}\cdot H_2O$
2g	benzoylformic acid R = C_6H_5, mp 62-66 °C	58.5	349 (12770), 527 (6750)	−88 ± 12° (c 0.18)	0.30	$C_{71}H_{90}N_{12}O_{17}\cdot 2H_2O$

[a] Using 1-cm path length cells and a 30 μM solution of $CHCl_3$. [b] Solvent for specific rotation, $CHCl_3$; concentrations denoted by c in g/100 mL. [c] TLC solvent, 10% MeOH-EtOAc.

Scheme I



mation of oxazinone derivatives of AMD (Table I).

When the oxazinones are formed, a change in visible absorption properties takes place. A bathochromic shift of up to 100 nm [compared to the absorption maximum of AMD (442 nm), either in chloroform or buffer] is observed, and the extinction coefficients of the maxima are reduced substantially. Figure 1 dramatizes the difference between the molar absorptivities of AMD and a typical oxazinone of AMD, **2g**; whereas AMD shows a maximum at 442 nm (ε 26 900), the oxazinones typically show a minimum at or near 442 nm (ε 5000–7000). Spectral properties provide a sensitive technique for the quantitative estimation of conversion of oxazinones into AMD at 442 nm.

During the characterization of AMD oxazinones, NMR chemical shift and specific rotation data suggested that

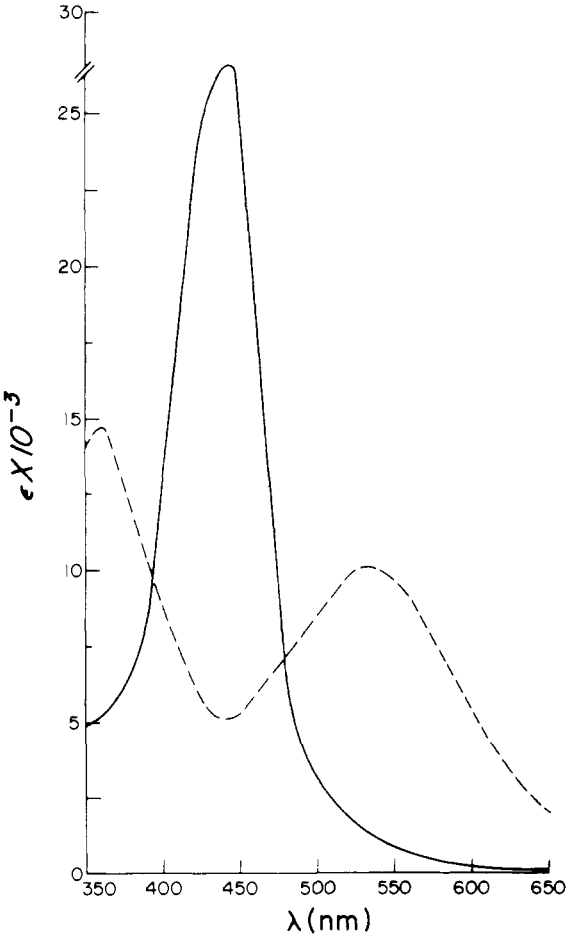


Figure 1. Absorption spectra of AMD (1; solid line) and the 3-phenyloxazinone of AMD (**2g**; broken line) in 0.16 M acetate buffer (pH 5.0) were obtained using 2 μM solutions of each in 10-cm path-length cells with a Cary 11 recording spectrophotometer.

the molecular conformation of the AMD oxazinones was unlike the conformation of AMD or of 7-substituted AMD analogues.[30,31] Four key factors are believed to be responsible for determining the molecular conformation of AMD:[30-40] (1) the fundamental conformation of the peptide lactone,[41,42] (2) the spatial relationship of the peptide lactone relative to the chromophore ring,[31-33] (3) the interannular hydrogen bonds between the NH of

Table II. Comparison of NMR Chemical Shifts[a] (τ Values) of Protons in 3-Methyloxazinone (2b), 3-Phenyloxazinone (2g) and AMD

proton	2b	2g	proton	AMD
H_9	3.25	3.18	H_7[b]	2.63
H_8	3.1	3.04	H_8[b]	2.36
12-Me	7.73	7.74	6-Me[b]	7.44
10-Me	7.81	7.81	4-Me[b]	7.76
6-NH	−0.85	−0.63	10-N	
D-Val NH (α or β)	−0.55	1.00		1.91
D-Val NH (α or β)	−0.55	1.29		2.06
Thr NH (α or β)	0.81	1.43		2.18
Thr NH (α or β)	1.22	1.80		2.80
Me-Val CH (α or β)	2.10	2.30		3.97
Me-Val CH (α or β)	2.31	2.46		4.02
β-Thr CH (α or β)	3.1–3.2	1.77		4.79
β-Thr CH (α or β)	3.1–3.2	1.77		4.85
N-Me of sarcosine	6.6–6.8	7.12		7.06 (3 H)
N-Me's of Me-Val, *N*-Me of sarcosine	6.6–6.8	6.9–7.04		7.10 (9 H)

[a] In $CDCl_3$ solvent, 100 MHz (Varian XL-100). [b] H_9, H_8 in oxazinone = H_7, H_8 in AMD, respectively. 12- and 10-Methyl in oxazinones = 4- and 6-methyl in AMD, respectively. 6-*N*H in oxazinones (**3**) are derived from 10-*N* of AMD (**4**), which does not carry a covalently bonded hydrogen (Figure 2).

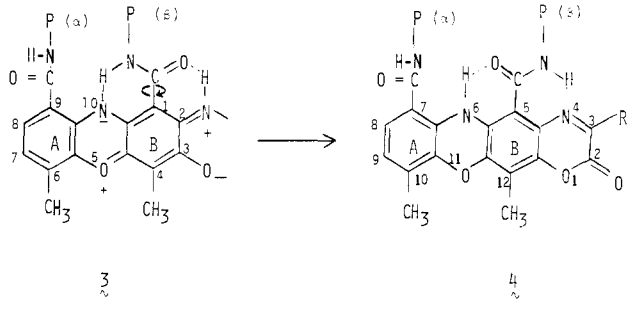


Figure 2. P = peptide rings as in Scheme I.

D-valine (α,β) and the carbonyl of D-valine (α,β), respectively, in the peptide lactones,[35-38] and (4) the planarity of the tricyclic chromophore ring.

In AMD, one of the protons of the 2-NH_2 group is shown to be hydrogen bonded to the actinocyl carbonyl—resulting in a specific spatial relationship of the peptide lactones to the chromophore,[43,44] as shown in **3** (Figure 2). Consequently, the peptide lactones do have a favored conformation as a result of this hydrogen bonding. This is confirmed by the observation that the 2-deamino-2-chloro-AMD and 2-deamino-AMD analogues, which lack this hydrogen bonding, have altered circular dichroism[44] and compared to AMD they also exhibit reduced specific rotation values[19,26] (also, see Table I).

Reagents which disrupt the interannular hydrogen bonds in AMD are also known to induce large changes in the ORD spectra and in the properties specific rotation.[39,40] This implies that the interannular hydrogen bonds also play an important role in stabilizing the conformation of the peptide lactones in AMD.[35-38] From these observations it can be inferred that the lowering of specific rotation values for the AMD oxazinones **2a–g** is probably due to a disruption of interannular hydrogen bonds as a result of the loss of 2-amino protons in AMD (Figure 2).

There are other factors which affect the structure of the oxazinones **4** (Figure 2). The relatively planar conjugated chromophore of AMD (**3**) becomes nonconjugated and nonplanar (**4**) when an oxazinone is formed. In addition, the A and B rings in **4** become aromatic and are separated by a new dihydrooxazine ring (Figure 2). These findings are supported by the chemical shifts of various protons in the methyl- (**2b**) and phenyloxazinones of AMD (**2g**). A comparison of some NMR chemical shifts of oxazinones **2b** and **2g** with AMD is presented in Table II. [Due to different numbering systems used in oxazinones **4**, H_9 and H_8 are the same as H_7 and H_8, respectively, in AMD (**3**), and the methyl groups at C-12 and C-10 in **4** are the same as those at C-4 and C-6 in AMD (**3**).]

Several key observations can be made from the NMR spectra of the oxazinones. Protons H_9 and H_8 are shifted upfield due to an overall increase in the electron density of the A ring in **4**.[25] The C-12 and C-10 methyls have closer chemical shifts compared to AMD because both the A and B rings (**4**) are now aromatic.[25] The shift toward lower field (0.8–1.5 ppm) of the D-Val N*H* (α,β) may be a combination of an alteration of spatial conformation as a result of the disruption of the interannular hydrogen bonds between peptide lactones. Some changes are also seen in the chemical shifts of the methine protons of methylvaline (α,β) and threonine (α,β), along with the *N*-methyl groups of methylvaline and sarcosine (Table II). Based on the above observations, it is concluded that the peptide moieties in the oxazinones exist in an altered conformation relative to AMD and 7-substituted AMD analogues[46] (Table I).

Biochemical Properties. On the basis of present knowledge about the structural requirements of AMD for interaction with DNA, these oxazinones are not expected to interact readily with DNA because of (1) altered geometry due to change from a tricyclic (**3**) to a tetracyclic (**4**) chromophore, (2) blocking of the 2-amino and 3-quinone functions,[18,19,26,44] and (3) substantial alteration in the conformation of the peptide lactone rings. In fact, no observable binding was exhibited when the oxazinones **2a–g** were mixed with calf thymus DNA for 5 min either in 0.01 M phosphate buffer (pH 7.0) at 20 °C or in 0.15 M NaCl–0.01 M phosphate buffer (pH 7.3) at 37 °C. However, binding is observed by difference spectra (Figure 3) after 30 min, but the characteristics of the spectra are identical with those of DNA-bound AMD.[31] It was concluded that the binding observed was due to the product of hydrolysis (AMD) and not due to the oxazinone per se. This conclusion is further supported by the isolation and characterization of AMD from these solutions.

The antitumor properties of the oxazinones **2a–g** appear to be due to their conversion to AMD. In biological systems the oxazinones are found to be readily hydrolyzed to AMD. This can be demonstrated either in an aqueous buffer (pH 7.3–7.9) or in the presence of serum esterase (pH 7.3 at 37 °C). A typical experiment, described in the Experimental Section, using rat serum esterase in sa-

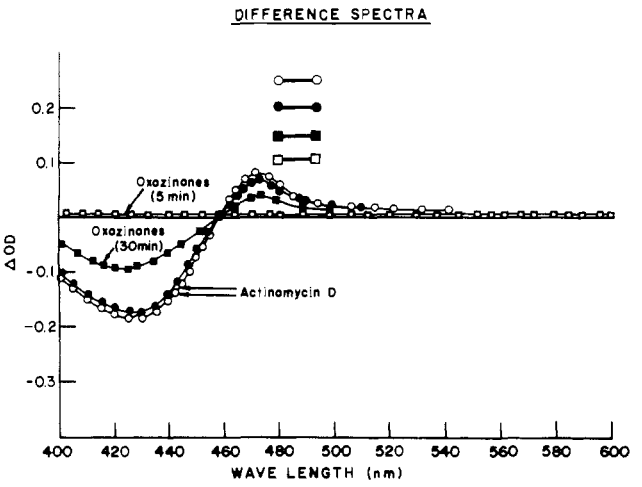


Figure 3. Difference absorption spectra of the oxazinones in the absence and in the presence of DNA. DNA binding of AMD and oxazinones (**2a–g**) in 0.01 M phosphate buffer, pH 7.0, at 20 °C and 0.15 M NaCl–0.01 M sodium phosphate buffer, pH 7.3, at 37 °C (times: 5 and 30 min). For details see Experimental Section.

Table III. In Vitro (CCRF-CEM) and in Vivo (P388) Growth Inhibitory Activities

		P388[b]		
compd	ID_{50}[a]	dose[c]	% ILS[d]	no. of long-term survivs[e]
AMD	10	75	149	0/5
2a	24	150	169	0/5
2b	12	150	155	0/5
2c	12	150	204	1/5
2d	12	150	140	0/5
2e	28	300	159	0/5
2f	55	600	282	2/5
2g	110	600	300	2/5

[a] Concentration of compound in ng/mL required for 50% inhibition at 48 h of human leukemic lymphoblasts (CCRF-CEM) cells in suspension culture. [b] Male BDF_1 mice were inoculated ip with 10^6 P388 leukemic cells. Compounds were administered ip in physiological saline solution 1 day after tumor implantation and were given for a total of 4 days (qd 1-4). [c] Optimal nontoxic doses are given in (μg/kg)/injection × 4. [d] Percent increase in median survival time of treated mice as compared with controls. [e] Survival greater than 50 days; some up to 307 days (tumor free when sacrificed).

line–phosphate buffer shows that the oxazinones **2a–g** are converted in 8 h almost exclusively to AMD. This is substantiated by the fact that the products of their hydrolysis show characteristic DNA-binding properties and other physicochemical properties identical with those of AMD. Therefore, the biological activity of the oxazinones **2a–g** appears to be due to their conversion to AMD in biological systems. This conclusion implies that the oxazinones **2a–g** are not biologically active in themselves but may be regarded as prodrug or depot forms of AMD.

Biological Activity. Human leukemic lymphoblasts in continuous spinner culture (CCRF-CEM cells)[49] are highly sensitive to AMD and its derivatives [the 50% inhibitory dose (ID_{50}) for these compounds is in nanograms per milliliter]. The assay that determines the 50% inhibitory dose (ID_{50}) provides relative cytotoxicity values for the agents, and, in this assay system, 3-(fluoromethyl)oxazinone (**2f**) and 3-phenyloxazinone (**2g**) appear to be five- and tenfold less toxic, respectively, than AMD (Table III). The oxazinone derivatives, 3-methyl (**2b**), 3-ethyl (**2c**), and 3-(carboxymethyl) (**2d**), are about equitoxic with AMD, and the 3-(carboxyethyl)- (**2e**) and 3-unsubstituted (**2a**) oxazinone derivatives of AMD are about half as toxic as AMD. (The assay system is not sensitive enough to clearly distinguish between ID_{50} values of 12 and 24 ng/mL, and the real ID_{50} values may lie anywhere within this range.) Therefore, within the limits of experimental error, the toxicities of the agents (**2a–e**) are comparable with AMD; however, **2f** and **2g** have significantly lower toxicity compared to AMD in this assay system.

Table IV. In Vivo Antitumor Activity of 3-Ethyl- (2c), 3-(Fluoromethyl)- (2f), and 3-Phenyloxazinone (2g) Analogues of Actinomycin D against P388 Murine Leukemia[a]

compd	dosage range, (μg/kg)/inj	MST, days	% ILS (surv)
untreated control		11.5	
AMD	50	28	141 (0/5)
	75	30	160 (0/5)
	150	8	−31 (0/5)[b]
3-ethyloxazinone AMD (2c)	75	29	152 (0/5)
	150	35	204 (1/5)
	300	26	126 (0/5)[c]
3-(fluoromethyl)-oxazinone AMD (2f)	75	28	143 (0/5)
	150	29	152 (0/5)
	225	30	160 (0/5)
	300	42	265 (0/5)
	450	37	222 (0/5)
	600	44	282 (2/5)
3-phenyloxazinone AMD (2g)	75	26	126 (0/5)
	150	31	169 (0/5)
	300	29	152 (1/5)
	375	39	239 (1/5)
	450	45	291 (1/5)
	600	46	300 (2/5)

[a] Male BDF_1 mice were inoculated ip with 10^6 P388 leukemic cells. Compounds were administered ip in physiological saline solution 1 day after tumor implantation and were given for a total of 4 days (qd 1-4). MST = median survival time in days. % ILS = median percent increase in life span of treated/untreated mice. Surv = number of surviving mice/total mice at day 50. The data presented are averaged from two or more experiments. All doses were nontoxic, except as indicated in footnotes *b* and *c* below. [b] All treated mice died of drug toxicity before control mice. [c] Substantial weight loss in treated mice (>20%) due to drug toxicity at this dose level.

The in vivo experiments with P388 tumor bearing mice corroborate the fact that **2f** and **2g** indeed show substantially reduced toxicity (Table III). It appears that an electronegative or bulky substituent on the 3 position of AMD oxazinones reduces toxicity to mammalian cells in vivo (Table IV) and in vitro (Table III).

Studies of in vivo activity of the oxazinones (**2a–g**) were conducted with the P388 lymphocytic leukemia in BDF_1 mice.[50,51] In the assay system, the tumor was implanted intraperitoneally (ip) in BDF_1 male mice with 10^6 cells. The drugs were injected ip daily for 4 successive days, starting 1 day after implantation. Compounds were tested over a dose range including those doses which are toxic. For simplicity, the results in Table III are presented only at the optimal nontoxic dose for each compound.

The results in Table III indicate that the AMD oxazinones **2a**, **2b**, **2d**, and **2e** are at least equiactive with AMD in terms of percent ILS. The oxazinones **2f**, which has a fluoromethyl substituent, and **2g**, which has a phenyl substituent at the 3 position, are about twice as effective as AMD. In terms of the number of long-term survivors,

2f and **2g** are the most effective agents (two survivors for longer than 50 days, out of five mice tested); **2c** also demonstrated enhanced activity (one long-term survivor). On the other hand, AMD and other oxazinones, **2a**, **2b**, **2d**, and **2e**, fail to give long-term survivors. One survivor in each group (**2c**, **2f**, and **2g**) was sacrificed on the 307th day, and no trace of tumor was evident.

Oxazinones **2g** and **2f** were titrated at selected close dose ranges. The results are as follows (Table IV): at 150 μg/kg qd 1–4 **2g** showed an ILS of 169% and at 300 μg/kg **2g** exhibited an ILS of 152% with one out of five mice surviving 50 days, as compared to AMD which showed an ILS of 160% with no long-term survivors at the optimum dose level of 75 μg/kg qd 1–4. At 375 μg/kg qd 1–4 and upwards, **2g** showed significantly improved activity both in terms of percent ILS and in the number of *long*-term survivors (Table IV). The dose–response curve of AMD to P388 is steep; the ILS is 141% at 50 μg/kg, and it peaks at 75 μg/kg [ILS = 160% (average of 149–169%)]. Toxicity is frequently shown at 150 μg/kg. In contrast, **2g** starts showing a favorable response at 75–150 μg/kg (ILS = 126–169%) and maintains the effective response over a broader dose range through 300–450 μg/kg (ILS = 152–291%), gradually peaking at 600 μg/kg (ILS = 300%), and finally shows toxicity at approximately 1200 μg/kg. AMD shows toxicity at twice its optimum dose, whereas **2g** starts showing activity equivalent to AMD optimum activity at 150 μg/kg but does not show any toxicity up to a dose of 600 μg/kg. The data indicate that **2g** and **2f** are effective as antitumor agents.

These results demonstrate that a bulky lipophilic or electronegative substituent, e.g., phenyl or fluoromethyl at the 3 position of an oxazinone, reduces the toxicity and enhances the biological antitumor activity. In all these analogues, a reversion to AMD in biological media is observed.

Conclusion. It has been demonstrated that synthetically modified forms of actinomycin D, in which a removable oxazinone ring protects the principal chromophore-binding functions, are capable of regenerating AMD under physiological conditions. These AMD oxazinones are less toxic to mammalian cells in culture and to animals bearing P388 leukemia but are superior to AMD as antitumor agents. These results suggest that an effective antitumor prodrug form or AMD has been prepared and that there exists a real potential for developing additional analogues with improved antitumor properties and lower cytotoxicity.

Experimental Section

Infrared spectra were taken in a Beckman Acculab Model 4230 spectrophotometer. UV and visible spectra were recorded with Cary Model 11 and 15 spectrophotometers. Spectrophotometric studies for DNA binding were determined with Zeiss PMQ III and Gilford spectrophotometers. Specific rotations were measured in $CHCl_3$ solution with Cary-60 and JASCO-5UV/ORD spectropolarimeters. TLC's were done on Analtech silica gel plates with 10% MeOH–EtOAc as developing solvent, unless otherwise stated. NMR spectra were taken in Varian T-60A or 100-MHz Varian XL-100 FT spectrometers. Silicic acid was purchased from Bio-Rad Laboratories, Rockville Center, N.Y., and α-keto acids were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Microanalyses were performed by Galbraith Laboratories, Knoxville, Tenn. Actinomycin D (batch no. 3008A-30B), kindly provided by Dr. John B. Douros, Natural Products Branch, National Cancer Institute, Bethesda, Md., was used in these reactions.

General Method for the Synthesis of 3-Substituted Actinomycin D Oxazinones. Procedure for 2c: 3-Ethyl-10,12-dimethyl-2*H*,6*H*-oxazino[3,2-*b*]phenoxazin-2-one 5,7-Bis[carbonyl-L-threonyl-D-valyl-L-prolylsarcosyl-L-*N*-methylvaline-(*threonine hydroxyl*)] Lactone. AMD (1; 50 mg, 0.04 mmol) was reduced with PtO_2 and hydrogen in 25 mL of methanol. The colorless reduction mixture was filtered under nitrogen with the filtrate being added directly to 0.5 mL of 2-ketobutyric acid previously placed in a suction flask flushed with nitrogen. After 3 h of stirring at room temperature, the red solution was concentrated under vacuum almost to dryness. Ethanol (10 mL) and water (50 mL) were added, and the solution was extracted with ethyl acetate (3 × 30 mL) and washed with water (5 × 20 mL) until the last wash was pH 5 or higher. The extract was dried (Na_2SO_4) and evaporated. The residue was chromatographed on silicic acid, first with Ciferri solvent (top layer of EtOAc–MeOH–H_2O, 20:1:20) to remove the impurities including unreacted AMD and then with $CHCl_3$–acetone (1:1). The process yielded the pure **2c**, 38.5 mg. Experimental conditions and characterization of **2c** and other oxazinones are described in Table I.

DNA Binding Studies with Oxazinones 2a–g. Difference spectra (Figure 3) were obtained manually with a Zeiss Model PMQ III spectrophotometer (5-cm semimicro cells) by recording the spectrum of a solution containing either AMD or AMD oxazinones **2a–g** and calf thymus DNA against a reference solution containing only the same concentration of AMD (5 μM).[31] The concentration of DNA was 100 μM. Difference spectra containing AMD: (○–○) in 0.01 M phosphate buffer (pH 7.0), temperature 20 °C, time 5 min; (●–●) in 0.15 M NaCl–0.01 M phosphate buffer (pH 7.3), temperature 37 °C, time 5 min. Difference spectra containing oxazinones **2a–g**: (□–□) in both the conditions mentioned above; (■–■) the difference spectrum of **2a–g** in 0.15 M NaCl–0.01 M sodium phosphate buffer (pH 7.3), temperature 37 °C, time 30 min.

Hydrolysis of Oxazinones 2a–g with Rat Serum Esterase and Isolation of AMD (1). Stock solutions (0.5 mM) of AMD (1) and the AMD oxazinones **2a–g** were prepared in saline–phosphate buffer (0.15 M NaCl, 0.01 M sodium phosphate, pH 6.0) at 37 °C. One milliliter of each stock solution, 8.0 mL of 0.15 M NaCl–0.01 M sodium phosphate buffer (pH 7.5), and 1.0 mL of fresh rat serum (final pH 7.3) were placed in stoppered centrifuge tubes and incubated in a 37 °C bath. After 8 h of incubation, the samples were extracted with ethyl acetate (3 × 10 mL). The extract was dried with Na_2SO_4, concentrated to 0.5 mL, spotted on a silica gel TLC plate, and developed in 2-butanol–formic acid–H_2O (75:13:12). All the samples contained only AMD, R_f 0.66. Samples **2d** and **2e** also showed some traces of unconverted material, R_f 0.13 and 0.12. In addition, the TLC's showed residues at the origin due to contamination by some serum component.

The remainder of the ethyl acetate extract was dried by aspiration, and the residue was dissolved in 0.15 M NaCl–0.01 M phosphate buffer (pH 7.3) (λ_{max} 442 nm). Calf thymus DNA was added. A bathochromic shift to λ_{max} 465 nm, with hypochromicity, suggestive of AMD binding to DNA, was observed for all the hydrolyzed samples.[30]

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References and Notes

(1) Present address: Department of Obstetrics and Gynecology, Boston University School of Medicine, Boston, Mass. 02118.
(2) (a) S. A. Waksman and H. B. Woodruff, *Proc. Soc. Exp. Biol. Med.*, **45**, 609 (1940). (b) H. Brockmann, *Fortschr. Chem. Org. Naturst.*, 18, 1 (1960).

(3) H. Umezawa, in "Index of Antibiotics from Actinomycetes" H. Umezawa, Ed., University Park Press, State College, Pa., 1967, pp 2–4, 91–101, and 155.
(4) S. A. Waksman, in "Actinomycin, Nature, Formation and Activities" S. A. Waksman, Ed., Wiley-Interscience, New York, 1968, pp 1–13.
(5) H. Brockmann and H. Muxfeldt, *Chem. Ber.*, **91**, 1242 (1958).
(6) H. Brockmann and H. Manegold, *Hoppe-Seyler's Z. Physiol. Chem.*, **343**, 86 (1965).
(7) H. Brockmann and B. Franck, *Angew. Chem.*, **68**, 68 (1956).
(8) E. Bullock and A. W. Johnson, *J. Chem. Soc.*, 3280 (1957).
(9) H. Kersten and W. Kersten, in "Inhibitors of Nucleic Acid Synthesis", Springer-Verlag, New York, 1974, pp 40–67.
(10) H. Brockmann and H. Lackner, *Chem. Ber.*, **101**, 1312 (1968).
(11) H. Brockmann and H. Lackner, *Chem. Ber.*, **101**, 2231 (1968).
(12) H. Brockmann, *Ann. N.Y. Acad. Sci.*, **89**, 323 (1960).
(13) H. Brockmann and J. H. Manegold, *Chem. Ber.*, **100**, 3814 (1967).
(14) H. Brockmann and P. Boldt, *Chem. Ber.*, **101**, 1940 (1968).
(15) H. Brockmann and F. Seela, *Tetrahedron Lett.*, 1489 (1971).
(16) H. Brockmann and F. Seela, *Tetrahedron Lett.*, 4803 (1965).
(17) H. Brockmann and F. Seela, *Tetrahedron Lett.*, 161 (1968).
(18) W. Müller, *Naturwissenschaften*, **49**, 156 (1962).
(19) J. Meienhofer and E. Atherton, in "Structure–Activity Relationships among the Semisynthetic Antibiotics", D. Perlman, Ed., Academic Press, New York, 1977, pp 427–529.
(20) E. Frei III, *Cancer Chemother. Rep.*, **58**, 49 (1974).
(21) S. Farber, *J. Am. Med. Assoc.*, **198**, 826 (1966).
(22) J. L. Lewis, Jr., *Cancer*, **30**, 1517 (1972).
(23) S. Perry, *Cancer Chemother. Rep.*, **58**, 117 (1974).
(24) S. K. Sengupta, S. K. Tinter, P. G. Ramsey, and E. J. Modest, *Fed. Proc., Fed. Am. Soc. Exp. Biol.*, **30**, 342 (1971).
(25) S. K. Sengupta, S. K. Tinter, H. Lazarus, B. L. Brown, and E. J. Modest, *J. Med. Chem.*, **18**, 1175 (1975).
(26) S. Moore, M. Kondo, M. Copeland, J. Meienhofer and R. K. Johnson, *J. Med. Chem.*, **18**, 1098 (1975).
(27) H. Brockmann, W. Müller, and H. Peterssen-Börstel, *Tetrahedron Lett.*, 3531 (1966).
(28) H. Brockmann, *Cancer Chemother. Rep.*, **58**, 9 (1974).
(29) S. K. Sengupta, H. Lazarus and L. M. Parker, *Proc. 5th Int. Symp. Med. Chem.*, IUPAC, Paris, July 9–22, 1976, abstr. 090.
(30) Y. C. Chiao, K. G. Rao, J. W. Hook III, T. R. Krugh, and S. K. Sengupta, *Biopolymers*, accepted for publication.
(31) S. K. Sengupta and D. Schaer, *Biochim. Biophys. Acta*, **521**, 89 (1978).
(32) H. Lackner, *Angew. Chem., Int. Ed. Engl.*, 14, 375 (1975).
(33) P. K. Ponnuswamy, R. F. McGuire, and H. A. Scherga, *Int. J. Pept. Protein Res.*, **5**, 73 (1973).
(34) H. Lackner, *Chem. Ber.*, **104**, 3653 (1971).
(35) D. E. Dorman and F. A. Bovey, *J. Org. Chem.*, **38**, 2379 (1973).
(36) H. Lackner, *Tetrahedron Lett.*, 1921 (1975).
(37) H. M. Sobell, S. C. Jain, T. D. Sakore, and C. E. Nordman, *Nature (London), New Biol.*, **231**, 200 (1971).
(38) P. DeSantis, R. Rizzo, and G. Ughetto, *Biopolymers*, 11, 279 (1972).
(39) F. Ascoli, P. DeSantis, and M. Savino, *Nature (London)*, **227**, 1273 (1970).
(40) F. Ascoli, P. DeSantis, M. Lener, and M. Savino, *Biopolymers*, 11, 1173 (1972).
(41) C. W. Mosher and L. Goodman, *J. Org. Chem.*, **37**, 2928 (1972).
(42) H. Lackner, in "Chemistry and Biology of Peptides", J. Meienhofer, Ed., Ann Arbor Science Publishers, Ann Arbor, Mich., 1972, pp 147–157.
(43) W. Müller and D. M. Crothers, *J. Mol. Biol.*, **35**, 251 (1968).
(44) C. W. Mosher, K. F. Kuhlman, D. G. Kleid, and D. W. Henry, *J. Med. Chem.*, **20**, 1055 (1977).
(45) B. H. Arison and K. Hoogsteen, *Biochemistry*, **9**, 3976 (1970).
(46) S. K. Sengupta and M. S. Madhavarao, *Biochemistry*, submitted.
(47) N. S. Angerman, T. A. Victor, C. L. Bell, and S. S. Danyluk, *Biochemistry*, 11, 2402 (1972).
(48) S. K. Sengupta and W. R. Beltz, unpublished data from these laboratories.
(49) G. E. Foley and H. Lazarus, *Biochem. Pharmacol.*, **16**, 659 (1967).
(50) A. Goldin and R. K. Johnson, *Cancer Chemother. Rep.*, **58**, 63 (1974).
(51) J. M. Venditti and B. Abbott, *Lloydia*, **30**, 332 (1967).

Synthesis, Base-Catalyzed Hydrolytic Reactivity, and Anticancer Evaluation of *O*-Aryl Phosphorodiamidates as a Novel Class of Pro(phosphorodiamidic acid mustards)

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Bis(2-chloroethyl)phosphoramidic dichloride [$MP(O)Cl_2$, M = $N(CH_2CH_2Cl)_2$] has been used as the starting material for the synthesis of *O*-aryl phosphorodiamidates having the general structure MP(O)(NHR)OAr: **9**, R = H, Ar = 4-$NO_2C_6H_4$; **10**, R = H, Ar = C_6F_5; **11**, R = C_6H_5, Ar = C_6F_5; **12**, R = 4-MeC_6H_4, Ar = C_6F_5; and **13**, R = 4-$EtOC_6H_4$, Ar = C_6F_5. The phosphorodiamidic chloride precursor to **13** (**14**) was also isolated. Kinetics for the base-catalyzed hydrolysis of compounds **9**–**13** were investigated by UV and NMR methods and are considered in connection with service of these compounds as pro(phosphorodiamidic acid mustards) [MP(O)(NHR)OAr → MP(O)(NHR)OH] via an E1cB mechanism involving the intermediacy of a mustard-bearing metaphosphorodiimide [MP(O)=NR]. Anticancer screening tests against L1210 lymphoid leukemia in mice indicated that **9**–**14** are inactive; similar negative results were obtained with the KB cell culture, except in the case of **14** which was marginally active.

The cytotoxicity of nornitrogen mustard [$HN(CH_2CH_2Cl)_2$] results from DNA cross-linking by a sequence of two alkylation reactions, each involving aziridinium ion formation followed by nucleophilic ring opening.[1] Bis-alkylating activity of nitrogen mustards can be predictively moderated by delocalization of the electron pair on nitrogen, and the synthesis[2] of resonance-stabilized phosphorylated mustards [$>P(O)N(CH_2CH_2Cl)_2 \leftrightarrow >P(O^-)=N^+(CH_2CH_2Cl)_2$] capable of enzymatic "activation" (P–N bond hydrolysis) in tumors represents one of the first prodrug[3] approaches to selective cytotoxicity against cancer cells. The subsequent emergence of cyclophosphamide (**1**) as a phosphorylated mustard having clinical utility was followed by studies which have discounted the originally intended mechanism of action[4] and instead support enzymatic oxidations leading to the preferential generation

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