

Tetracyclic Chromophoric Analogues of Actinomycin D: Synthesis, Structure Elucidation and Interconvertibility from One Form to Another, Antitumor Activity, and Structure-Activity Relationships

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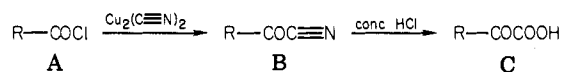
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Two different tetracyclic chromophoric analogues of actinomycin D have been synthesized by engaging two chromophoric DNA-binding functions in actinomycin D, i.e., 2-amino and 3-oxo, into either a 1,4-oxazin-2-one or an oxazole ring system. A third analogue has an extra quinone function at C-8 of the oxazole analogue. In all the analogues the chemical integrity of the peptide lactones of the parent antibiotic is kept intact, but their stereochemistry is altered. The analogues are designed as transport-modified prodrug forms of either the tricyclic active analogues of actinomycin D or actinomycin D itself. All analogues exhibit cytotoxicity that is several-fold less potent than AMD; they also have no binding affinity toward extracellular DNA. Nonetheless, the analogues of the first and the third series show improved antitumor activities (P388 leukemia, CDF₁ mice). In fact, two of these analogues having a phenyl substituent at the C-3 site of the oxazinone ring or the C-2 position of the 8-oxo-8*H*-oxazole ring exhibit the highest antitumor effects. Most of the analogues are active over a broader dose range than actinomycin D and are 6- to 16-fold less cytotoxic to human lymphoblastic leukemia (CCFR-CEM) cells in vitro. The analogues with the most pronounced antitumor activity are those that retain most elements in the peptide stereochemistry of actinomycin D and have a quinone function or demonstrate susceptibility of their chromophores to biotransformation.

Actinomycin D (AMD, **1a**; Chart I) a chromopeptide antibiotic, is one of the oldest antitumor drugs. It has sustained its importance over the decades as a clinically active agent.¹⁻⁴ The agent is curative in Wilm's tumor in children and choriocarcinoma in pregnant women; it is also effective in the treatment of testicular sarcoma, and recently, it has shown activity against Kaposi's sarcoma.⁵ Its limitations are its toxicity, narrow therapeutic index, and limited spectrum of antitumor activity.⁶ The high and cumulative toxicity of AMD appears to derive from the total lack of its metabolism in the patient system with the resulting long-term retention in the nuclei of cells in the host.⁷

Many investigations have been carried out in several laboratories, aimed primarily at enhancing the therapeutic index of this drug.⁸⁻¹⁴ For several years our laboratory has examined the structure-activity relationship in the actinomycin D analogues.¹⁵⁻²² During the course of these

Scheme I



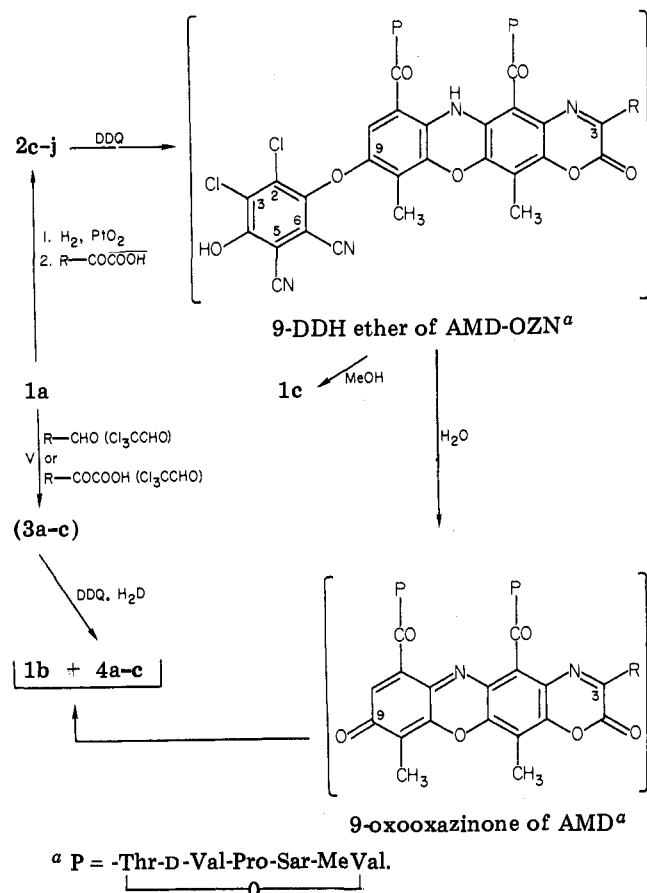
studies we synthesized several tetracyclic chromophoric analogues of AMD. These analogues demonstrated reduced in vitro cytotoxicity against human leukemic CCRF-CEM cells and enhanced in vivo activity in P388 leukemia in mice.^{15,20,23} Several of the new agents were designed as the transport-modified precursors of active forms of AMD, which could conceivably be biotransformed, preferentially inside the proliferating cells, to generate DNA active forms, e.g., actinomycin D or its active chromophoric analogues. As an extension of this project, we have recently synthesized several new tetracyclic 1,4-oxazinone (AMD-OZN, **2c-j**) and a series of newer tetracyclic 8-oxo-8*H*-oxazolo[4,5-*b*]phenoxazine analogues (AMD-OZL, **4a-c**). In the present paper we will report the syntheses and chemical and biological activities of these compounds as well as another set of oxazolo[4,5-*b*]phenoxazine analogues of AMD (**3a-d**).

All our initial chemical investigations used the actinomycin model **1f** in place of AMD, which is extremely expensive and is of limited supply. The model derivative¹¹ has the identical chromophore of AMD but lacks its pentapeptidolactone (P) groups; instead of these P groups **1f**

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Scheme II

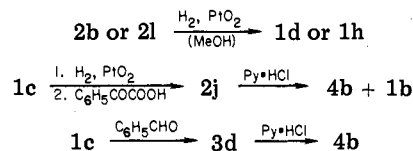


similar DDQ oxidation on the model oxazinone derivative **2k**, has been reported.¹¹ When the above oxidative reactions were applied on **3a-c**, the corresponding 8-oxo-8*H*-oxazolo[4,5-*b*]phenoxazine AMD analogues (**4a-c**) were formed. These analogues also could be prepared from the AMD-OZN analogues **2a,b,g** by the application of the same DDQ oxidation (Scheme II).

Formation of both oxazinone (**2**) and oxazole (**3**) analogues increases the electrophilicity of the original aromatic ring A in **1**; consequently, the ring A (in **2** and **3**) becomes prone to electrophilic substitution by DDQ. The primary product of substitution of DDQ at C-9 of a typical analogue **2b** is believed to be an intermediate hydroquinone ether (9-DDH ether of **2b**, Scheme II). A reaction with protic agents, e.g., MeOH or H₂O, displaces this ether substituent as 2,3-dichloro-5,6-dicyano-1,4-dihydroxybenzene (DDH). Thus, MeOH gives a 9-methoxy-substituted analogue (**2j**), which with a final loss of the protective 1,4-oxazinone ring is converted to 7-methoxy-AMD (**1c**).²⁰ A similar treatment of water on the intermediate 9-DDH ether analogue gives an transient analogue (most probably with a hydroxy substituent at C-9 of **2b**), which is promptly oxidized in air to the intermediate 9-oxooxazinone compound. In the actinomycin D derived analogues, this intermediate derivative was found to be unstable and was converted to a mixture of AMD-OZL analogue (**4b**) and 7-hydroxy-AMD (**1b**). However, a corresponding 9-oxooxazinone analogue with R = CH₃, derived from the same DDQ oxidation of the model analogue **2k**, is rather stable, and therefore it can be isolated as a crystalline, orange-red solid.¹¹ Transformation of this compound to the 7-hydroxy model (**1g**) derivative has been reported.¹¹

Oxidation of the oxazole analogues **3a-c** to **4a-c** follow a similar sequence of reactions; in this case the tetracyclic analogues are quite stable and do not rearrange to 7-

Scheme III



hydroxy or 7-alkoxy analogues. Thus, a DDQ reaction on **3b**, followed by a silica gel and water treatment, gives only **4b**; a methanol treatment produces **3d**. An authentic sample of **3d** was prepared by condensing **1c** with refluxing "benzaldehyde" in an oxygen-free atmosphere, which upon demethylation with the aid of pyridine hydrochloride in refluxing CHCl₃ solution (60–80 °C) generated the above **4b** (Scheme III).

As a rule, the protective oxazole ring in structures **3** and **4** is more stable than the corresponding oxazinone ring in **2**. For example, the pyridine hydrochloride demethylation of **2j** is followed by rearrangement of the oxazinone ring, resulting in a mixture of **4b** and **1b**. The same mixture of compounds, i.e., **4b** and **1b**, is isolated when **2b** is treated with DDQ and H₂O as mentioned above. The oxazinone ring in the intermediate 9-oxooxazinone undergoes transformation via either a contraction to an oxazole ring of **4b** or a rearrangement to the tricyclic chromophoric analogue, 7-hydroxy-AMD (**1b**).^{11,15}

The synthesis of the 7-hydroxy derivative of AMD (**1b**) from, for example, **2a** is always accompanied with the formation on some **4a**. When ¹⁴C- and ³H-labeled **2a** is used, where ¹⁴C is evenly distributed among the C-2, C-3, and 3-methyl residues only and tritium is restricted to the methyl groups in the pentapeptidolactone groups and to the methyl groups in the rings A and B, the conversion to **4a** resulted in the loss of one-third of the ¹⁴C label, and 7-hydroxy-AMD was free of any ¹⁴C label. It is worth mentioning that we did not plan the synthesis of the analogues **4a-c** as candidates for bioassay; we discovered them serendipitously from the material during the synthesis of 7-hydroxy-AMD (**1b**).

The compounds of structure **2** suffer a rupture of the protective oxazinone during catalytic hydrogenation; in contrast, the analogues of the **3** and **4** series are stable. For example, when **2b** or **2l** is hydrogenated in the presence of platinum, **1d** and **1h** are formed, respectively (Scheme III).

As a rule, the AMD-OZL analogues (**4a,b**) are more soluble in water than the corresponding **2a,b** and **3a,b** analogues (See Experimental Section). The aqueous solubility of AMD is closely related to the unique conformation of the pentapeptidolactone rings P (both α and β) in **1a**. Like actinomycin D, the compounds **4a,b** are more soluble in water at 4 °C than at 20 °C and stick to the glass surface.^{32,33} In actinomycin D, the peptidolactones (P, α and β) are interannularly hydrogen bonded primarily between the two D-Val residues; P β is further hydrogen bonded to a chromophoric 2-amino hydrogen through the β -threonine residues.^{34,35} This unique conformation is due to the planar tricyclic chromophore of AMD. Most of this characteristic is preserved in the tetracyclic AMD analogues **4a-c**. This conformation of AMD contributes to its molar ellipticity in CD spectra and the G-C base-

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Table I. Properties of α -Keto Acids (R-COCOOH, Scheme I, C)

R	mp, °C	yield, %	anal.
o-ClC ₆ H ₄	76-78	66.5	C, H, Cl
m-ClC ₆ H ₄	61-63	64.5	C, H, Cl
p-ClC ₆ H ₄	67-69	60.2	C, H, Cl
2,4-Cl ₂ C ₆ H ₃	80-83	58.4	C, H, Cl
C ₆ F ₅ ^a	101-105	15.0	C, H, F
C ₆ H ₁₃ ^b	50-55 dec	60.0	C, H
C ₁₀ H ₇ ^c	80-85	61.8	C, H ^d

^a Pentafluorophenyl. ^b n-Hexyl. ^c 2-Naphthyl.
^d Calcd: C, 72.00; H, 4.03. Found: C, 71.39; H, 3.71.

pair-specific binding properties (Table VII). Therefore, as the planarity of the chromophore in **3a-d** is lost (in the same manner as in **2a-j**),²³ the $[\alpha]_D$ values and $[\theta]$ values at 350-370 nm in these analogues are greatly reduced⁴⁴ (Tables II and III). The chromophore of the compounds in the 4 series appears planar, but it is tetracyclic rather than tricyclic. This chromophore in 4 also lacks the important 2-amino function present in AMD. In fact 4 can be considered as a tetracyclic analogue of **1e** (2-deamino-actinomycin D).¹³ (Imagine the structure of 4, rotated

through 180° around the N₅-O₁₀ axis, oxazole at C-7 and C-8).^{10,25} The $[\alpha]_D$ and $[\theta]_{350-370}$ values in **4a** are intermediate between the high value in AMD and the relatively low values in either **2a** or **3a** (Tables II-IV), which indicates a minimal change in the peptide conformation.⁴⁴

NMR data in Table V supports these conclusions. The chemical shifts of the protons in **3b** (or **2b**, **2a**²³) suggest a shielding effect on the aromatic ring protons H₈ and H₇ in **3b** due to an increase in the electron density in ring A. The C-11 and C-9 methyls in **3b** exhibit closer chemical shifts compared to AMD because both A and B rings are aromatic. The chemical shifts of the D-Val NH and the β -threonine NH protons relate to the conformation of the chromophore and peptide lactones. The data in Table V show that the δ values of these protons in **3b** are shifted farthest from those in AMD. In **4b**, the δ values for the D-Val NH are about the same as in AMD; this is not true for the chemical shifts of the L-threonine NH. The change in conformation of the α - and β -threonine residues resulting from the lack of the 2-NH₂ function in **4b** may cause this shift.

These, along with the tetracyclic chromophore in analogues **2-4**, culminate in an observed loss of DNA binding

Table II. Physicochemical Properties of Actinomycin D Oxazinone (AMD-OZN) Analogues (2)

compd	R _f ^a	yield, %	$[\alpha]_{20}^{20}$, ^b deg (c)	UV λ_{max} , ^c nm (ϵ)	mol formula
2c	0.62	55	-96.0 ± 12 (0.16)	350 (10 200), 523 (5750)	C ₇₀ H ₈₉ N ₁₂ O ₁₇ Cl·H ₂ O
2d	0.61	72	-96.0 ± 18 (0.22)	350 (9800), 524 (5510)	C ₇₀ H ₈₉ N ₁₂ O ₁₇ Cl·H ₂ O
2e	0.62	76	-92.8 ± 16 (0.15)	351 (11 100), 523 (6290)	C ₇₀ H ₈₉ N ₁₂ O ₁₇ Cl·2H ₂ O
2f	0.67	82	-96.6 ± 16 (0.18)	350 (8500), 521 (4830)	C ₇₀ H ₈₅ N ₁₂ O ₁₇ Cl ₂ ·H ₂ O ^d
2g	0.29	11	-88 ± 12 (0.11)	350 (8100), 526 (4200)	C ₇₀ H ₈₅ N ₁₂ O ₁₇ F ₅
2h	0.43	32	-100 ± 16 (0.20)	320 (8100), 401 (95 800)	C ₇₀ H ₈₅ N ₁₂ O ₁₇ ^e
2i	0.42	22	-69 ± 12 (0.15)	320 (8100), 369 (6200), 498 (6100)	C ₇₄ H ₉₂ N ₁₂ O ₁₇
2j	0.31	11	-86 ± 10 (0.11)	350 (12 100), 526 (6800)	C ₇₁ H ₉₂ N ₁₂ O ₁₈ ·H ₂ O
1a ^f	0.55		-300 ± 20 (0.10)	424 (21 000), 442 (23 000)	C ₆₂ H ₈₆ N ₁₂ O ₁₅ ·2H ₂ O

^a R_f, EtOAc-acetone, 2:1 (silica gel plates). ^b In CHCl₃, concentration (c) in grams/100 mL. ^c In CHCl₃ solution (0.1-0.2 mM). Extinction values are concentration dependent. ^d Calcd: C, 57.66; H, 6.04; N, 11.53; Cl, 4.87. Found: C, 57.46; H, 6.11; N, 11.27; Cl, 4.34. ^e Calcd: C, 60.96; H, 7.11; N, 12.19. Found: C, 61.33; H, 7.01; N, 11.73. ^f Actinomycin.

Table III. Physicochemical Properties of Oxazolo Analogues of Actinomycin D (3)

compd	yield, %	R _f	UV λ_{max} , ^a nm (ϵ)	$[\alpha]_{25}^{25}$, ^b deg (c, CHCl ₃)	mol formula
3a	45	0.61	351 (7900), 389 (11 300)	-101 ± 20 (0.10)	C ₆₄ H ₈₈ N ₁₂ O ₁₆ ·2H ₂ O ^c
3b	31	0.66	387 (10 900)	-110 ± 20 (0.31)	C ₆₉ H ₉₀ N ₁₂ O ₁₆ ·H ₂ O
3c	15	0.70	379 (8100)	-90 ± 20 (0.15)	C ₆₄ H ₈₅ N ₁₂ O ₁₆ F ₅
3d	12	0.63	394 (12 100)	-110 ± 20 (0.11)	C ₇₀ H ₉₂ N ₁₂ O ₁₇ ·H ₂ O

^a Using 50-100 μ M solution in CHCl₃. ^b In a chloroform solution, concentration (c) in grams/mL. ^c Calcd: C, 58.36; H, 6.69; N, 12.77. Found: C, 58.75; H, 6.39; N, 11.97. R_f, TLC on silica gel plates using EtOAc-acetone, 2:1.

Table IV. Physicochemical Properties of 8-Oxo-8H-oxazolo Analogues (AMD-OZL) of Actinomycin D (4)

compd	yield, %	R _f ^a	$[\alpha]_D$, ^b deg (c, CHCl ₃)	UV λ_{max} , ^c nm (ϵ)	mol formula
4a	28	0.36	-242 ± 20 (0.11)	383 (17 300), 510 (11 700)	C ₆₄ H ₈₆ N ₁₂ O ₁₇ ·2H ₂ O
4b	55	0.41	-220 ± 20 (0.18)	387 (19 900), 498 (13 700)	C ₆₉ H ₈₈ N ₁₂ O ₁₇ ·3H ₂ O
4c	19	0.48	-201 ± 20 (0.18)	380 (17 900), 409 (12 300)	C ₆₉ H ₈₃ N ₁₂ O ₁₇ F ₅

^a R_f on silica gel plates using EtOAc-acetone, 2:1. ^b c, concentration in grams/mL. ^c In CHCl₃ solution (~80 μ M).

Table V. Comparison of NMR Chemical Shifts^a of Protons in 2-Phenyloxazole (3b) and 2-Phenyl-8-oxo-8H-oxazole (4b) Analogues of Actinomycin D with Chemical Shifts of Actinomycin D

proton (AMD, 1a)	δ	proton (3b)	δ	proton (4b)	δ
H ₇ ^b	7.37	H ₈	6.91		
H ₈ ^b	7.64	H ₇	7.06	H ₇	6.75
6-CH ₃ ^b	2.56	9-CH ₃	2.31	9-CH ₃	2.67
4-CH ₃ ^b	2.34	11-CH ₃	2.28	11-CH ₃	2.27
D-Val NH (α or β)	8.09, 7.94		9.11, 8.82		8.55, 8.48
L-Thr NH (α or β)	7.82, 7.20		8.47, 8.30		6.82, 6.73

^a CDCl₃ solution, 90-MHz spectrum, internal standard tetramethylsilane. ^b H₇, H₈, 6-CH₃, and 4-CH₃ in AMD (1a) \equiv H₈, H₇, 11-CH₃, and 9-CH₃, respectively, in 3b and 4b.

properties (Table VII) studied either by the difference spectral technique or by the thermal denaturation of DNA (ΔT_m) technique.^{9,16,17,20-23}

In this respect all these tetracyclic analogues, including 4a-c, differ from both AMD and 2-deamino-AMD. They do not seem to bind to DNA, whereas AMD and 2-deamino-AMD bind to DNA strongly by intercalation^{9,13} (Table VII).

The tumor-inhibitory activities of these tetracyclic analogues may depend on the biotransformation of the tetracyclic chromophores.³⁶ The chromophores of AMD-OZN (2) and AMD-OZL (4) have been found to be biolabile. Further, 4 has a quinone function that may participate in tumor growth inhibition.^{37,38}

Biological Activity. In vitro growth inhibition of the analogues was assayed against human lymphoblastic leukemia cells (CCRF-CEM) in log phase.³⁹ Results are shown in Table VI. The most cytotoxic compounds are the AMD-OZN analogues 2b,g and the AMD-OZL analogues 4a-c. However, compared to AMD, they are several-fold less cytotoxic to CEM-cells in vitro. These results could be due to the altered uptake and retention of the agents by the specific cells or to rates of extracellular and/or intracellular activation or deactivation.⁴⁰

In Vivo Antitumor Activity. These analogues were tested for antitumor activity against P388 lymphocytic leukemia in male CDF₁ hybrid mice (Table VI). The tumor was implanted intraperitoneally (ip) with 10⁶ cells. The drugs were administered once daily on days 1, 5, and 9, beginning 1 day after implantation. Compounds were tested over a range of doses, but only the optimal nontoxic doses are listed. In this system, analogues of the series 2 and 4 (Table IV), in general, showed improved biological activity. Of these, the two most active agents are 2b and 4b. They show superior activity (% ILS) and ability to produce long-term survivors. The same 2b analogue exhibited similar high activity against P388 in BDF₁ mice at a qd 1-4 schedule²³ and has recently shown very promising activity in B₁₆ melanoma.⁴¹

We investigated the effect of halogen substitution on the 2-phenyl ring or substituents like a long-chain alkyl or a larger aromatic naphthyl ring at the C-2 site of 2. We succeeded in increasing the lipophilicity of the agent in general (t_R values, HPLC) without enhancing the effectiveness as measured by % ILS. However, all of these analogues 2b-g (except 2h,i) and 4a-c analogues showed

high levels of activity over much broader dose ranges. This property differs distinctly from AMD, which has a remarkably narrow dose-response curve in man.⁴²

Conclusions

This paper reports the synthesis of three different tetracyclic chromophoric analogues with poor to high degrees of antitumor activity in murine leukemia. In all three different structural types, the sequence and composition of the amino acid and also the integrity of the lactone rings in the peptide parts are unchanged, but their conformations are altered. Those analogues that retain an optimal likeness of the peptide conformation of the parent actinomycin D are the most active biologically.

Experimental Section

Melting points were determined on a Thomas-Hoover melting point apparatus at a heating rate of 2 °C/min. Dry column chromatography was accomplished with silicic acid powder (Bio-Sil A 100-200 mesh, Bio-Rad Laboratories Inc.) or acid alumina (Woelm grade 1). Thin-layer chromatography was performed on silica gel plates (Brinkmann Instrument Inc.). For oxazinone analogues (2b), the silica gel plates were exposed to the vapor of concentrated HCl in an air-tight container for 20 min before application of the compound for TLC. This procedure helped to prevent the streaking of the compounds in the chromatogram. Solvent systems were (A) EtOAc-acetone (2:1), (B) dry *t*-BuOH-C₆H₆-C₆H₅N (85:12.5:2.5), and (C) Cifferri, the organic phase of the mixture EtOAc-MeOH-H₂O (20:1:20). High-performance liquid chromatography was carried out on a Varian Model 5020 gradient liquid chromatograph equipped with a CD-111L chromatography data system and fitted with a Varian reversed-phase C₁₈ column with isocratic solvent systems, CH₃CN-5 mM NH₄OAc buffer, pH 6.4 (68:32), flow rate 15 mL/min, with UV-visible variable- and fixed-wavelength dual detectors at 254, 440, 500, and 525 nm. IR spectra were obtained on a Perkin-Elmer Model 237 Infra Cord with KBr micropellets or in CHCl₃ solutions. UV-visible spectra were obtained on a Gilford 250 spectrophotometer, which, with the addition of a baseline reference compensator (Analog Multiplexer 5053) and thermo-programmer, auto four cell programmer, and thermoelectric cell holder 2577, were used to obtain thermal denaturation curves. Specific rotation values were determined in CHCl₃ solutions with a Cary 60 spectropolarimeter. NMR spectra were obtained in a JOEL FQ-90 MHz spectrometer equipped with Fourier transform. All elemental analyses were within ±0.4%, unless specified otherwise. The organic acids and some acid chlorides were purchased from Aldrich Chemical Co. for the synthesis of α -keto acids. [³H]Actinomycin D was purchased from Amersham, and [G-¹⁴C]pyruvic acid was purchased from New England Nuclear. Actinomycin D (NSC 3053, lot L554651-0-10) was generously provided by Dr. John Douros, Natural Products Branch, National Cancer Institute, Silver Spring, MD. Calf thymus DNA was purchased from Sigma Chemical Co.

Synthesis of 3-Substituted Actinomycin D Oxazinones. Procedure for 3-(2-Naphthyl)-10,12-dimethyl-2H,6H-oxazino[3,2-*b*]phenoxazin-2-one 5,7-Bis[carbonyl-L-threonyl-D-valyl-L-propylsarcosyl-L-N-methylvaline-(threonine hydroxyl)] Lactone (2b). General Method. AMD (1a; 75 mg, 0.06 mmol) was reduced with PtO₂ and hydrogen in methanol (25 mL). The reddish yellow color of the reaction mixture was discharged, and at this stage the reduced reaction mixture was filtered into a second flask filled with nitrogen and containing a solution of 2-naphthoyleformic acid (C, R = C₁₀H₇, Scheme I) in MeOH (25 mL). This reaction mixture was stirred for 5 h at ambient temperature, always maintaining the nitrogen atmosphere; at the end of reaction, the volume was reduced to about 1 mL under vacuum, and the concentrate was dissolved in ethyl acetate (50 mL) and washed with water (5 × 10 mL) until it was acid free (pH above 5). The residue from the dried (Na₂SO₄) and evaporated ethyl acetate fraction was chromatographed on silicic acid with CHCl₃-acetone (2:1 and 1:1). The process yielded pure AMD-OZN compound 2i in the 1:1 CHCl₃-acetone fraction: yield 18 mg; R_f (TLC), t_R (HPLC), UV absorption, and specific rotation values and microchemical analysis (C, H, and N) of this AMD-

(36) We have observed that the analogues 2b and 4b are metabolized, in part, to 7-hydroxyactinomycin D in the presence of mitochondria-free rat liver homogenates in vitro. Under identical conditions, no such conversion of 3b could be detected. The biochemical and biological properties of 7-hydroxy-AMD and the conversion of 2b analogues to AMD in the presence of rat plasma have been reported by us previously.^{16,20,23} Additional pharmacological properties of selected 2b and 4b analogues are being communicated.

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(40) In a subsequent communication we will report the results of our recent investigations on the molecular pharmacological behavior of two selected analogues, one in the 2b series and the other in the 4b series. These results should validate many of the premises that are stated here.

(41) Our experimental findings have been corroborated recently by parallel studies carried out by the Drug Evaluation Branch, NCI. We are in process of reporting these and other results in the subsequent communication.

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OZN are described in Tables II and VI.

Synthesis of 2-Phenyl-9,11-dimethyl-5H-oxazole[4,5-*b*]-phenoxazine 4,6-Bis[carbonyl-L-threonyl-D-valyl-L-prolyl-sarcosyl-L-N-methylvaline-(threonine hydroxyl)] Lactone (3b). General Method. A solution of AMD (50 mg, 0.04 mmol) in methanol-water (10:1, 50 mL) was allowed to react with chloral (0.5 mL, bp 97–99 °C) at ambient temperature, and during this period the optical rotation changed from a negative to a positive value and became steady at the end of 30–32 h. The change in the Cotton effect in AMD is a consequence of the disruption of the strong hydrogen bonding between the 2-amino hydrogen and the β -peptide lactone group.^{29,30} After this treatment, benzaldehyde (4 mL, bp 279 °C) was added, and the mixture was heated under N₂ at 50–55 °C (18 mm) until all the chloral, water, and methanol were removed. The residual mixture was then stirred under N₂ at 180 °C for 7 h until TLC or HPLC showed no trace of AMD. The reaction mixture was evaporated at 60 °C (4 mm) to remove all unreacted benzaldehyde. The residue which contained some benzoic acid was triturated with NaHCO₃, and after TLC (solvent B, R_f 0.51) a solid (16.3 mg, 31%) was obtained. Tables III and VI give the physicochemical characteristics and microchemical analytical data for this and the other oxazole analogues in the 3 series.

Synthesis of 2-Substituted Actinomycin D 8-Oxo-8H-oxazole (AMD-OZL) Analogues. Synthesis of 2-Phenyl-9,11-dimethyl-8-oxoxazol[4,5-*b*]phenoxazine 4,6-Bis[carbonyl-L-threonyl-D-valyl-L-prolyl-sarcosyl-L-N-methylvaline-(threonine hydroxyl)] Lactone (4b). General Procedure. A solution of 3b (140 mg, 0.1 mmol) in *p*-dioxane (15 mL) and DDQ (150 mg) was refluxed for 3 h under N₂. DDQ was added in 3 equal portions of 50 mg, first at the beginning of the reaction and then at the end of the first and the second hours. At the end of reaction, the dioxane was evaporated under reduced pressure, and the red-brown solid was chromatographed on silica gel TLC plates in solvent system B. The bands represented DDQ, R_f 0.67; the reaction product, R_f 0.3–0.35, which in all probability is a 8-DDH ether of 3 (R = C₆H₅; R₁ = 2,3-dichloro-5,6-dicyano-4-hydroxyphenoxy).¹¹ The third fluorescence band at or near the origin, R_f 0.02–0.07, was identified as that of DDH (2,3-dichloro-5,6-dicyano-1,4-dihydroxybenzene).²⁴ The middle band, after extraction with benzene and dioxane, gave a flaky, dark brown solid: yield 100 mg (65%). IR showed an additional band for C≡N at 4.85 μ m. A portion of this intermediate phenoxy ether (60 mg) was stirred with dioxane-water (10 mL, 95:5) for 24 h. After evaporation, the residue was chromatographed on Woelm alumina and then on silicic acid, eluting first with Cifferri and then with solvent A. The combined fractions in solvent A showed only one spot on TLC (solvent A), R_f 0.41; yield 45 mg (55%) of purple solid. See Tables IV–VI for detailed properties of this and other AMD-OZL (4) analogues. Another portion (35 mg) of the compound from the above purple band, R_f 0.3–0.35, was refluxed in dry methanol (5 mL) for 3 h. After chromatographic purification, a reddish-brown solid of another compound, 3d, R_f 0.63 (TLC, solvent A), was obtained: yield 25 mg (50%). See Table III for the physicochemical data of this compound. The yields in Table III are based on either the fusion of α -keto acids or the condensation the aldehydes (Scheme II).

Conversion of Compound 3d to Compound 4b. A mixture 20 mg of the above 3d analogue and pyridine hydrochloride⁴³ (200 mg) in chloroform (20 mL) was refluxed for 18 h. The residue upon evaporation was dissolved in dichloromethane, washed successively with 1 N HCl and 0.5 M NaHCO₃, and dried over Na₂SO₄. After TLC (solvent A) and crystallization from methanol, purple solid (16 mg, 85%; R_f 0.41, TLC solvent A), identical with 4b, was recovered (Scheme III).

Catalytic Reduction of Compounds 2b and 2l to Compounds 1d and 1h. A solution of the above tetracyclic analogue 2b (45 mg, 0.03 mmol) in methanol (35 mL) was hydrogenated in the presence PtO₂. As the pale green colored reduction mixture was being filtered, the filtrate started turning deep yellow in contact with air, showing the generation of the tricyclic phenoxazinone chromophore of AMD. Evaporation and purification of the residue on TLC (solvent A) gave a major band (R_f 0.76)

of 1d, mp 218–220 dec; $[\alpha]_D^{20}$ –295 \pm 20° (c 0.1, CHCl₃); UV λ_{max} 420 nm (ϵ 12 800); yield upon elution with MeOH, 40 mg (87%). All attempts to remove the 1-(methoxycarbonyl)benzyl substituent chain on the N² site of 1d by hydrogenolysis were unsuccessful, probably because of the presence of the methoxycarbonyl group. Anal. (C₇₁H₉₄N₁₂O₁₈·H₂O) C, H, N.

Compound 1h was generated as above to form 2l: mp 152 °C; UV λ_{max} (CHCl₃) 424 nm (ϵ 14 800); R_f 0.61 (TLC, solvent A) for 1h. For 2l: mp 163–167; UV λ_{max} (CHCl₃) 502 nm (ϵ 11 200); R_f 0.39 (solvent A). Anal. for 1h (C₃₃H₃₈N₄O₆) C, H, N. Anal. for 2l (C₃₂H₃₄N₄O₅) C, H, N.

Conversion of Compound 2b to a Mixture of Compounds 4b and 1b via DDQ Oxidation. 2b·2H₂O²³ (135 mg, 0.1 mmol) and DDQ (70 mg, 0.3 mmol) in 25 mL of MeOH were refluxed for 1 h under N₂. The mixture was evaporated to dryness, and the residue was dissolved in CH₂Cl₂ (50 mL) and filtered to remove the precipitated DDH and DDQ. The filtrate was passed through an acidic alumina column, which freed it from the residual DDH and DDQ; the eluent in CH₂Cl₂, which contained the intermediate 9-oxoxazinone analogue (Scheme II) and showed consistent streakings on TLC (solvents A–C), without further purification was treated with 10 g of silica gel powder in 50% EtOH (30 mL) for 2 h. The suspension, after filtration and concentration, was banded on TLC (solvent B). Bands of 4b, R_f 0.59, and 1b, R_f 0.21 (no trace of starting 2b), were extracted in MeOH and were subjected to HPLC on a reversed phase C₁₈ column (CH₃CN–5 mM NH₄OAc, pH 6.4, 68:32; 1.5 mL/min). Fractions corresponding to a *t*_R of 2.7 (7-hydroxy-AMD, 1b; yield 36%) and 5.6 min (4b; yield 33%) were collected. These fractions were purified once more by HPLC to obtain samples of high purity and homogeneity.

Solubility of Actinomycin D and Analogues 2b, 3b, and 4b in Distilled Water at 20 and 4 °C. The solubility of the above analogues, which were determined from their OD values at their respective λ_{max} (nanometers), are as follows: solubility of AMD, 0.9 mg/mL at 20 °C and 20.5 mg/mL at 4 °C; solubility of 4b, 1.7 mg/mL at 20 °C and 11 mg/mL at 4 °C; solubility of 3b, 0.3 mg/mL at 20 °C and 1.2 mg/mL at 4 °C; solubility of 2b, 0.6 mg/mL at 20 °C and 2.2 mg/mL at 4 °C.

DNA-Drug Binding by Difference Spectral and Thermal Denaturation of DNA (ΔT_m) Techniques. These experiments were performed according to the methods described by us previously.^{16,20,26} Results are shown in Table VII.⁴⁴

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(44) Absorption and circular dichroic spectra (solvent 0.01 M phosphate, 10⁻⁴M EDTA, pH 7.0) of AMD (1a), 7-hydroxy-AMD (1b), 4a, and 3a:

	λ_{max} , nm (ϵ)	λ_{max} , nm ($[\theta]$)
1a	442 (21 000)	450 (–5000), 370 (–15 000)
1b	550 (12 000)	360 (–14 500)
4a	515 (15 700), 360 (21 300)	358 (–9 500)
3a	415 (10 500)	350 (–6 500)

The absorption maxima of AMD analogues above 240 nm originate from the chromophores (tricyclic or tetracyclic), but the CD ellipticities in the same regions are the direct derivatives of the peptide conformations. The molar ellipticity ($[\theta]$) values at 350–370 nm may imply either a retention (as in 7-hydroxy-AMD) or a gradual loss (as in 4b to 3b) in the conformation of the peptide moieties. The analogues show additional ellipticities at 250–260 nm (–ve), 235–245 nm (+ve), and 210–215 nm (–ve) with little discernible change from analogue to analogue.¹⁸ The only reliable indicator of peptide conformation in the analogues in CD spectrum is the value of $[\theta]$ at 350–370 nm.

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Table VI. HPLC Retention Times and Antitumor Activity of Tetracyclic Chromophoric Analogues of Actinomycin D and Compounds 2-4

compd	t_R (HPLC), ^a min	in vitro (CCRF-CEM) ^b ID ₅₀ , ng/mL	in vivo P388 assay			
			opt dose, μg/kg	MST, ^c days	% ILS ^d	cure ^e
control (no drug)				11.0		0/20
1a ^f	6.4	40	125	26	136	1/7
2b	8.9	440	600	45	309	2/7
2c	10.7	1 800	1200	30	172	1/7
2d	11.9	1 600	600	29	164	0/7
2e	11.0	1 600	600	29	164	0/7
2f	13.8	3 200	1200	28	155	0/7
2g	15.8	640	1800	31.5	186	1/7
2h	16.7	2 080	1200	25	127	0/7
2i	7.7	1 000	1200	26	136	0/7
3a	17.0	2 900	1200	21	91	0/7
3b	19.7	9 000	900	23	109	0/7
3c	22.7	12 500	1800	19	72	0/7
4a	5.3	230	450	31	182	1/7
4b	7.6	310	750	44	300	2/7
4c	11.2	630	900	30	172	1/7

^a HPLC system: Varian Model 5020; C₁₈ column; solvent CH₃CN-5 mM NH₄OAc, pH 6.4, 62:38; 1.5 mL/min. ^b Human lymphoblastic leukemia cells in log-phase growth and in a suspension culture. Compounds were dissolved in Me₂SO medium, final growth medium contained 5% Me₂SO. ^c 10⁶ P388 cells implanted intraperitoneally on day 0 into a group of seven CDF₁ male mice. Drugs were administered, also intraperitoneally, in 10% Me₂SO-saline on days 1, 5, and 9. Test solutions were kept at 0-4 °C, protected from light. ^d MST = median survival time. ^e % ILS = percent increase in life span. ^f Over 60-day survivors. Average of two experiments. (Homogeneity of each test solution needs to be ascertained carefully by HPLC before the agents are tested.) ^f Actinomycin D.

Table VII. Comparison of DNA-Binding Properties of Actinomycin D, 7-Hydroxyactinomycin D (1b), and the Tetracyclic Chromophoric Analogues of Actinomycin D, 3 and 4

compd	ΔT_m , ^a °C	diff absorption spectra, ^b	diff CD spectra, ^c
		$-\Delta\epsilon_{\max}$ (nm)	$-\Delta[\theta]_{\max}$ (nm)
1a ^d	7.1 ± 0.15	8240 (425)	40 000 (390)
1b ^e	6.7 ± 0.15	9380 (550)	31 000 (485)
3a	0.0 ± 0.3	nil	nil
4a	1.0 ± 0.3	nil	nil

^a $\Delta T_m = T_m$ of DNA-drug complex minus T_m of purified calf-thymus DNA.²⁰ ^b Diff. Absorption Spectra is the calculated absorption spectra obtained by subtracting the spectrum of the DNA-bound analogue from the spectrum of the free analogue, measured at 340-600 nm.¹⁶ ^c Diff. CD Spectra is the calculated CD Spectra obtained as the difference of the DNA-bound from the combined individual spectra of analogues and DNA at 340-600 nm.²⁶ Medium: 0.01 M phosphate containing 10⁻⁴ EDTA, buffer pH 7.0. ^d Actinomycin D. ^e 7-Hydroxyactinomycin D.

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Registry No. 1b, 21478-73-9; 1d, 87039-90-5; 1h, 87039-91-6; 2b, 70570-56-8; 2c, 87039-75-6; 2d, 87039-76-7; 2e, 87039-77-8; 2f, 87039-78-9; 2g, 87039-79-0; 2h, 87039-80-3; 2i, 87039-81-4; 2j, 87050-08-6; 2l, 87039-89-2; 3a, 87039-82-5; 3b, 87039-83-6; 3c, 87039-84-7; 3d, 87039-85-8; 4a, 87039-86-9; 4b, 87039-87-0; 4c, 87039-88-1; *o*-ClC₆H₄COCOOH, 26118-14-9; *m*-ClC₆H₄COCOOH, 26767-07-7; *p*-ClC₆H₄COCOOH, 7099-88-9; 2,4-Cl₂C₆H₃COCOOH, 32375-56-7; C₆F₅COCOOH, 72331-54-5; C₆H₁₃COCOOH, 328-51-8; C₁₀H₇COCOOH, 14289-45-3; benzaldehyde, 100-52-7.