"Reverse Chromophore" of Actinomycin D. Synthesis of 2-Substituted 7-Amino-8*H*-8-oxo-oxazolo[4,5-*b*]phenoxazine Ring System.

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The synthesis and physico-chemical properties of a new 7-amino-8*H*-8-oxo-oxazolo[4,5-*b*]-phenoxazine ring system (**8a** and **8b**) are reported. These tetracyclic heteroaromatic rings posses an extra oxazolo ring fused to the phenoxazinone chromophore observed in actinomycin D (**1b**). These tetracyclic compounds (**8a-8b**) possess a structure in which the orientation of the A and B rings in **1b** are "reversed". Since DNA binding and the resulting specificity of the antitumor antibiotic **1b** is believed to depend on the spatial orientation of P(α) and P(β) relative to the functions on the B ring of **1b**, these new compounds represent a novel approach for investigating AMD-DNA interactions.

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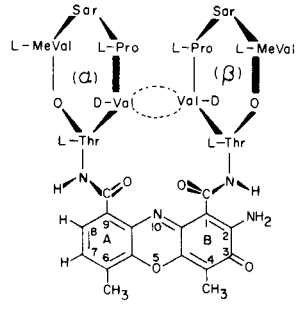
Actinomycin D (AMD, **1b**) Fig. 1 is a chromopeptide antibiotic (1,2) with antitumor properties (3-5). Clinically, the spectrum of antitumor activity of AMD is limited and its administration difficult due to its narrow therapeutic index (6). Extensive studies have been initiated to effect improvement in the therapeutic index of AMD (7-9) to understand its mode of action in biological systems (7-14), and to determine the precise mechanism of its binding to DNA (15-22).

In the structure of AMD (**1b**), there are two peptide lactone groups, P(α) and P(β), attached to the 9 position of ring A and to the 1 position of ring B in the tricyclic phenoxazinone (23) chromophore. The chromophore has 2-amino, 3-oxo, and 4- and 6- methyl groups. These functions are considered vital for the interaction of the AMD molecule with a double helical DNA (7,17,24,25). The formation *in vivo* of this AMD-DNA complex results in the inhibition of cellular RNA synthesis and this mechanism is the probable basis for the biological activity of AMD (10,26).

Recent work by these laboratories has shown that bulky substitutions at the 7 position of AMD do not appear to interfere with DNA binding properties, inhibition of cellular RNA synthesis, or the antitumor properties (27). This work has further demonstrated that other prodrug analogs of AMD possess improved therapeutic properties against selected transplantable murine tumors *in vivo* (28). These results, which are corroborated by similar recent work from other laboratories (9,25), suggest that, in contrast to other studies (29), actinomycin D (**1b**) can accomodate certain substitutions on the A ring of the chromophore and still retain the biological activity. This work implies that AMD analogs with a broader spectrum of antitumor activity can be achieved by appropriate modifications directed toward improving the cellular uptake and retention of the analogs in the tumor cells (30).

Figure 1



Actinomycin D (1b)

In AMD (**1b**) ring A is aromatic and ring B is a quinoidal. The peptide lactones, P(α) and P(β), in **1b** assume an unique conformation by virtue of well defined α,β-interannular hydrogen bonds (31). These hydrogen bondings are reported to exist in aqueous solution and in the crystalline state of actinomycin D (18). This conformation generates a highly defined spatial relationship between the peptide lactones and the chemical functions on the chromophore. An alteration of this spatial relationship in the AMD molecule might result in analogs with modified DNA binding properties and biological behavior.

Synthesis of a limited number of AMD derivatives that are approximately the "reverse" of AMD (**1b**) are being pursued in our laboratories. As a part of this program, the addition of an oxazole ring through the 2-amino and 3-oxo functions of the B ring in the chromophore of the model derivative, **1a**, (8) was accomplished (32). Subsequently, in order to regenerate the "lost" feature in the chromophore, new amino and quinone functions were introduced at the 7 and 8 positions, (**8a** and **8b**). The final structures (on rotating 180° around N_5-O_{10} axis)

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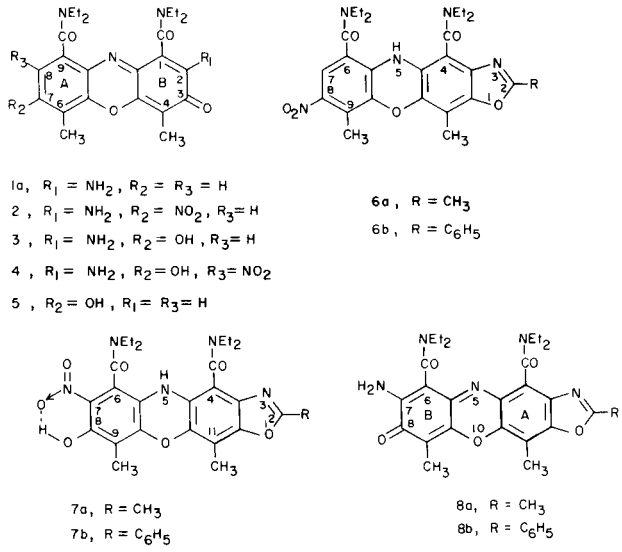

Figure 2

would restore the original AMD chromophoric functions, 2-amino and 3-oxo, with an oxazole ring attached to the original 7 and 8 positions in the AMD structure. Since bulky substitutions at the 7 position of AMD do not seriously interfere with either DNA-binding or biological activity (33), it is expected that the new oxazole ring fused on the 7 and 8 positions of AMD would not destroy DNA binding or biological properties of the product. However, the spatial relationship of the hydrogen-bonded peptides may be altered substantially. We term these molecules with the potentially altered spatial relationship between the peptides and the chromophore as the "reverse actinomycin" analogs.

Initial chemical investigations were based on the studies with the actinomycin chromophore model compound **1a** (Fig. 2), in which the pentapeptide lactone moieties at the 1 and 9 positions of AMD (**1b**) (Fig. 1) were replaced by diethylcarboxamide groups. The results of studies based on derivatives of the model compound are reported here.

Compound **3** obtained from **1a** by the reported procedure was nitrated to **4** with dilute nitrous acid in ethylacetate (34). A short reaction time of thirty minutes at 0° gave the desired product, **4**, with nitration going entirely at the 8-position. The proton magnetic resonance spectrum (Table I) showed the absence of the 8-proton and the presence of intact amino protons at the 2-position of **4**. However, when the above reaction time was increased to two hours a companion product was obtained by way of diazotization of the 2-amino group only. This intermediate 2-diazonium salt derivative exhibited an infrared peak at 4.7 μ, consistent with a $N{\equiv}N^+$ function on the 2-position of the chromophore (35) and underwent loss of a molecule of nitrogen on treatment with absolute ethanol. The resulting product which is 2-deamino-7-hydroxy model derivative (**5**) showed a loss of the 4.7 μ peak in the infrared. Nmr spectrum (Table I) of **5** recorded a gain of an extra hydrogen (τ 3.38 in deuteriochloroform) at the 2-position and the loss of 2 NH_2 proton.

The 2-amino and 3-oxo functions in **4**, (containing the nitro group at the 7 position, infrared peak at 6.5 μ) were rendered into an oxazole ring system of **7a** and **7b** by the previously reported methods (32). According to this procedure, acetaldehyde (*via* pyruvic acid) produced the oxazole derivative **7a** and benzaldehyde gave **7b**. Prior to this synthesis of **7a** and **7b**, a trial aldehyde condensation reaction was carried out on the 7-nitro-model derivative, **2a** (8), (6.5 μ, in the infrared) to ascertain the stability of an aromatic nitro group towards this reductive condensation reaction, which is believed to proceed *via* some mechanism involving reduction of the chromophoric quinone function (32,36). Compound **2a** produced oxazole derivatives **6a** and **6b** with nitro groups intact at position 8 under identical reaction conditions. This observation, in addition to demonstrating the innocuous nature of these reactions on aromatic nitro groups, gave additional evidence which supports the proposed mechanism of these aldehyde condensations, one that postulates a concerted intramolecular hydrogen shift during the formation of 5*H*-oxazolo[4,5-*b*]phenoxazine ring (**6a** or **6b** from **2**) (36). The suggested alternative mechanism of stepwise reduction of the chromophoric quinone to a phenol and condensation with aldehydes (36) is not validated by these results.

The products **7a** and **7b** were obtained as the 7-nitro-8-hydroxy derivatives of the new oxazole substituted

Table I

Nmr and Ultraviolet Spectra of the Tricyclic Chromophore Derivatives

Compound	τ, Deuteriochloroform 8*H*	6*Me*	4*Me*	$2NH_2$	2*H*	*OH*	λ max chloroform nm ($\epsilon \times 10^{-3}$)
3	3.24	7.79	8.01	4.68		-0.07	460 (19.3), 291 inf (4.8)
4		7.56	7.77	4.47		-1.47	446 (23.5), 373 (16.9), 258 (15.6)
5	3.25	7.88	7.90		3.38	-0.05	484 (6.2), 430 inf (6.2), 335 inf (9.1) (9.4), 289 inf (9.9), 260 (16.0)

Table II

Uv Absorption Spectra of the Tetracyclic Derivatives

λ max chloroform nm (ϵ x 10^{-3})

Compound	
6a	305 (12.27) and 442 (8.81)
6b	287 (21.2), 382 (15.7) and 440 (13.0)
7a	310 (14.6), 350 (9.3) and 520 (6.3)
7b	289 (23.1), 301 (23.1), 320 (25.1), 392 (14.9) and 535 (15.0)
8a	247 (36.3, 430 (31.1) and 450 (33.61)
8b	248 (31.7), 294 (24.0), 440 (33.0) and 458 (40.0)

tetracyclic chromophore, and not as the probable alternative 8-quinone derivatives (compare with **8a** or **8b**). This was evident by the presence of 5-N*H* and 8-O*H* in the nmr spectrum of **7a** and **7b** (Table III). A strong hydrogen bonding between the phenolic 8-O*H* protons and the ortho-nitro oxygen in the 7-position may explain the stabilization of the structures of **7a** and **7b**. During the catalytic reduction of **7a** and **7b**, the nitro group was reduced and the resulting yellow aminophenols were readily oxidized to the red orthoaminoquinone derivatives **8a** or **8b**. The chromophores of **8a** and **8b** exhibited close similarity with the chromophores of **1a** and **1b**, as was evidenced by the characteristic double absorption maxima (Table II) in the visible absorption spectra (20,32).

The uv and nmr spectral data of the tricyclic phenoxazinone chromophore derivatives are given in Table I. Table II records the ultraviolet and Table III the nmr spectral data of the tetracyclic oxazolophenoxazinone derivatives. The data are in agreement with the assigned structures (36). An additional spectral feature worth mentioning is the visible absorption maximum of the 2-deamino derivative 5 (λ max 484 nm; ϵ, 6200) which appears at a longer wavelength (24 nm approximately) and exhibits a substantial reduction in the extinction value relative to the absorption characteristics of the corresponding parent derivative **3** (λ max 460 nm; ϵ, 19,300) (8). This observation is in agreement with the recently reported spectral properties of AMD (**1b**) and its 2-deamino derivative (25).

The nmr spectra of **8a** and **8b** in Table III are different from either **6a** and **6b** or **7a** and **7b** by virtue of the absence of 5-N*H* and 8-O*H* protons and low field shifts of 11-methyl protons. These observations confirm the continuous conjugation between the A and B rings in **8a** and **8b**. The assignment of τ values for the protons is in accordance with the structures and with the expected effect of the various substituents on these structures.

EXPERIMENTAL

Melting points were measured in Pyrex capillary tubes in a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.) and are uncorrected. Ir spectra were taken with a Perkin-Elmer Model 137B double-beam spectrophotometer, and nmr spectra were determined on a Varian A-60 spectrometer in deuteriochloroform with tetramethylsilane as the internal standard. Tlc's were done on Eastman silica gel chromagram sheets, with chloroform-acetone (4:1) as the developing solvent. Microanalyses were determined by Galbraith Laboratories, Knoxville, Tenn. Mass spectra were recorded using a AEI MS-9 and CEC 21-103 low resolution mass spectrometers; the sample was introduced at a probe temperature of 175°.

2-Amino-1,9-bis(*N,N*-diethylcarbamoyl)-7-hydroxy-4,6-dimethyl-8-nitro-3*H*-3-oxophenoxazine (**4**).

A solution of **3** (430 mg., 0.1 mmole) in ethyl acetate (6 ml.) and glacial acetic acid (2.5 ml.) was treated with 5% aqueous sodium nitrite (28 ml.) at 0° for 30 minutes. The reaction mixture was extracted with ethyl acetate and the extract washed with water until it was acid free, dried (sodium sulfate) and evaporated. The brown solid was first crystallized from ligroin (b.p. 30°-60°) [330 mg. (70%), R_f 0.57 (major spot] and was then chromatographed over silica gel in chloroform. A homogenous purple solid was obtained (250 mg., 42%, R_f 0.57, m.p. 232-234°); ir (potassium bromide): 6.5 μ (nitro) and no 4.7 μ (N≡N$^+$) peak.

Anal. Calcd. for $C_{24}H_{29}N_5O_7$: C, 57.70; H, 5.85; N, 14.02. Found: C, 57.72; H, 5.93, N, 13.83. Mol. wt. Calcd. 499. Found 499 (Mass Spectrometer).

1,9-Bis(*N,N*-diethylcarbamoyl)-7-hydroxy-4,6-dimethyl-3*H*-3-oxophenoxazine (**5**).

In an identical run as above, the reaction was allowed to proceed for 2 hours instead of 30 minutes and a new dark brown solid 125 mg. (21%) (in addition to 250 mg. of **4**) was obtained; ir (potassium chloride): 4.7 μ (-N≡N$^+$ group). A 50 mg. portion of this dark brown solid was refluxed for 2 hours in ethanol (20 ml.), and the ethanol was evaporated. The residue was

Table III

Nmr Spectral Data of the Tetracyclic Derivatives τ, Deuteriochloroform

Compound	5-*NH*	7-*H*	7-*NH*$_2$	2-*CH*$_3$	9-*CH*$_3$	11-*CH*$_3$	8-O*H*
6a	2.29	2.34		7.43	7.52	7.66	
6b	2.10	2.38		(a)	7.54	7.59	
7a	3.32			7.43	7.80	7.61	-1.47
7b	2.93			(a)	7.80	7.52	-1.45
8a			4.53	7.35	7.75	7.36	
8b			4.52	(a)	7.74	7.29	

(a) Proton peaks at 1.4-1.6 τ (m, 2*H*) and 2.15-2.3 τ (m, 3*H*) appear in these spectra and account for 5 protons of 2-phenyl groups.

chromatographed over silica gel in a chloroform-acetone mixture (1:1) to give 33 mg. of **5** (m.p. 294°-295°) which showed absence of 4.7 μ (-N≡N^+) and 6.5 μ (nitro) peaks of **4** in the ir (potassium chloride) spectra. This compound appeared as a fluorescent spot on tlc, R_f 0.44. Nmr shows a new 2*H* peak at 3.38 τ and the absence of 2-NH_2 protons of **3**.

Anal. Calcd. for $C_{24}H_{29}N_3O_5$: C, 65.59; H, 6.65; N, 9.56. Found: C, 65.98; H, 6.67; N, 9.36.

4,6-Bis(*N,N*-diethylcarbamoyl)-2,9,11-trimethyl-8-nitro-5*H*-oxazolo[4,5-*b*]phenoxazine (**6a**).

A solution of **2** (121 mg., 0.25 mmole) in methanol (15 ml.) was refluxed 10 hours following the addition of 1 ml. of pyruvic acid. The mixture was evaporated and a chloroform solution (20 ml.) of the residue was washed several times with water (5 x 5 ml.) till the wash was neutral, dried with sodium sulfate and evaporated. Recrystallization from ether gave an orange red solid (99 mg., 78%); m.p. 250-251°, R_f 0.74, 6.5 μ (nitro) peak in ir (potassium chloride).

Anal. Calcd. for $C_{26}H_{31}N_5O_6$: C, 61.28; H, 6.13; N, 13.75. Found: C, 61.18; H, 6.16; N, 13.73

4,6-Bis(*N,N*-diethylcarbamoyl)-9,11-dimethyl-8-nitro-2-phenyl-5*H*-oxazolo[4,5-*b*]phenoxazine (**6b**).

A suspension of **2** (1 g.) in benzaldehyde (5 ml.) was refluxed gently for 2 hours (bath temperature 185°) under nitrogen. Benzaldehyde was evaporated under *vacuo* and the residue was crystallized stepwise, once from ethanol (10 ml.), once from ligroin (5 ml.) (b.p. 60-90°), and finally, once from benzene (5 ml). A brick red solid of **6b** was obtained, (1.17 g., (99.1%); ir (potassium chloride): 6.5 μ (nitro peak); m.p. 249-250°; R_f 0.86).

Anal. Calcd. for $C_{31}H_{33}N_5O_6$: C, 65.20; H, 5.82; N, 12.25. Found: C, 65.28; H, 5.94; N, 12.27.

4,6-Bis (*N,N*-diethylcarbamoyl)-8-hydroxy-2,9,11-trimethyl-7-nitro-5*H*-oxazolo[4,5-*b*]phenoxazine (**7a**).

A solution of **4** (100 mg., 2 mmole) in methanol (20 ml.) containing pyruvic acid (2 ml.) was refluxed under nitrogen for 22 hours. The reaction mixture was cooled, the solvent evaporated, and the residue extracted with chloroform (25 ml.). The chloroform extract was washed with water (4 x 10 ml.) (to remove unreacted pyruvic acid), dried with sodium sulfate, and evaporated. The residue was crystallized from a 1:1 mixture of ethylacetate and petroleum ether (b.p. 30-60°). Chromatography over silica gel in chloroform gave 53 mg. (50%) of red-brown solid (R_f 0.66, m.p. 231-232°).

Anal. Calcd. for $C_{20}H_{31}N_5O_7$: C, 59.41; H, 5.94; N, 13.33. Found: C, 59.37; H, 5.71; N, 13.23. Mol. wt. Calcd. 525. Found 525. (Mass Spectrum).

4,6-Bis(*N,N*-diethylcarbamoyl)-8-hydroxy-9,11-dimethyl-7-nitro-2-phenyl-5*H*-oxazolo[4,5-*b*]phenoxazine (**7b**).

A suspension of **4** (50 mg.) in benzaldehyde (3 ml.) was stirred at a bath temperature of 180° under nitrogen. After 5 hours the reaction mixture was cooled to room temperature, evaporated under vacuum and the residue was extracted with chloroform. The chloroform extract was washed with a freshly prepared sodium bicarbonate solution to free it from a trace amount of benzoic acid which was formed by the oxidation of benzaldehyde. The extract was dried (sodium sulfate) and evaporated. The crude residue (45 mg.) was chromatographed twice over silica gel in chloroform. Only 22 mg. (40%) of red-brown solid R_f 0.92, m.p. 254-260° was obtained.

Anal. Calcd. for $C_{31}H_{33}N_5O_7$: C, 63.36; H, 5.66; N, 11.92. Found: C, 63.63; H, 5.74; N, 11.69. Mol. wt. Calcd. 587. Found: 587. (Mass spectrum).

7-Amino-4,6-Bis(*N,N*-diethylcarbamoyl)-9,11-dimethyl-8*H*-8-oxo-2-phenyloxazolo[4,5-*b*]phenoxazine (**8b**).

Solution of **7b** (60 mg.) in ethanol (15 ml.) was hydrogenated (platinum oxide, 20 mg.) for 45 minutes. The initial purple color of the solution was discharged and the resulting light a yellow solution was filtered. In contact with air the filtrate turned red and furnished a brick-red residue on evaporation. The brick-red solid was chromatographed over silica gel in a 1:1 chloroform acetone mixture. Pure red solid, 45 mg. (80%), R_f 0.45, m.p. 298-300° was obtained.

Anal. Calcd. for $C_{31}H_{33}N_5O_5$: C, 67.01; H, 5.98; N, 12.60. Found: C, 66.85; H, 5.93; N, 12.51.

7-Amino-4,6-Bis(*N,N*-diethylcarbamoyl)-2,9,11-trimethyl-8*H*-8-oxo-oxazolo[4,5-*b*]phenoxazine (**8a**).

When 30 mg. of **7a** was reduced catalytically with platinum and hydrogen (as in above experiment) 24 mg. of **8a** R_f 0.22 m.p. 294-295° was obtained.

Anal. Calcd. for $C_{26}H_{31}N_5O_5$: C, 63.27; H, 6.33; 14.19. Found: C, 63.20; H, 6.40; N, 14.09.

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