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7-Substituted Actinomycin D Analogs. Chemical and Growth-Inhibitory Studies†

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The synthesis and biological activity of three 7-substituted actinomycin D derivatives are reported. Three such derivatives, 7-nitro-, 7-amino-, and 7-hydroxyactinomycin D, were synthesized via new methods which were first tested successfully with a chromophore model system. Of these, 7-nitro- and 7-aminoactinomycin D were assayed for growth inhibitory activity against mammalian cells (CCRF-CEM human lymphoblastic leukemia) in vitro and against the Ridgway osteogenic sarcoma and the L1210, P1534, and P388 murine leukemias in vivo. In these systems, the inhibitory activity of the 7-substituted analogs was comparable to actinomycin D. In two bacterial systems (*L. casei* and *L. arabinosus*) in vitro, on the other hand, these compounds showed inhibitory profiles which were distinctly different from actinomycin D. These studies demonstrate that substitution at the 7 position, which does not interfere with DNA binding, is capable of yielding experimental antitumor agents with significant activity against a variety of tumors.

Actinomycin D (AMD) is an established agent in clinical cancer chemotherapy.[1] It has been effective alone or with X-ray therapy in the treatment of several types of human cancer, most notably in Wilm's tumor[2] and choriocarcinoma.[3] AMD is one of a family of chromopeptide antibiotics, the first of which was reported by Waksman and Woodruff in 1940.[4]

A great deal of work has been done by a number of investigators on the chemistry, biological activity, binding specificity, pharmacology, and clinical antitumor activity of the actinomycins. Particularly notable work has been done in the laboratories of Waksman,[4] Brockmann,[5] Katz,[6] and Reich,[7] summarized recently in an excellent review by Meienhofer and Atherton.[8] A recent report describes the preliminary clinical pharmacology of AMD.[9] The naturally occurring actinomycins differ in one or several amino acid sites in the cyclic pentapeptide lactone rings, but all are biologically active, although at various levels of inhibition.[8] At the molecular level AMD is known to bind to the guanine residues in double helical DNA and to inhibit DNA-dependent RNA polymerase.[7]

An intercalation model for the interaction of AMD with deoxyguanosine residues in bihelical DNA has been proposed based on earlier work and X-ray crystallographic data.[10] The remarkable activity of the actinomycins, namely the absolute DNA–guanine specificity, inhibition of DNA-directed RNA polymerase, and inhibition of bacterial and mammalian cells in vitro and experimental rodent tumors in vivo, all at extremely low inhibitor concentrations, has prompted research to develop actinomycin analogs or derivatives as more useful clinical cancer chemotherapeutic agents. To date, only actinomycin D and actinomycin C [which is a mixture of actinomycins C_1 (or D), C_2, and C_3] have been shown to be effective in human cancer.[1,8]

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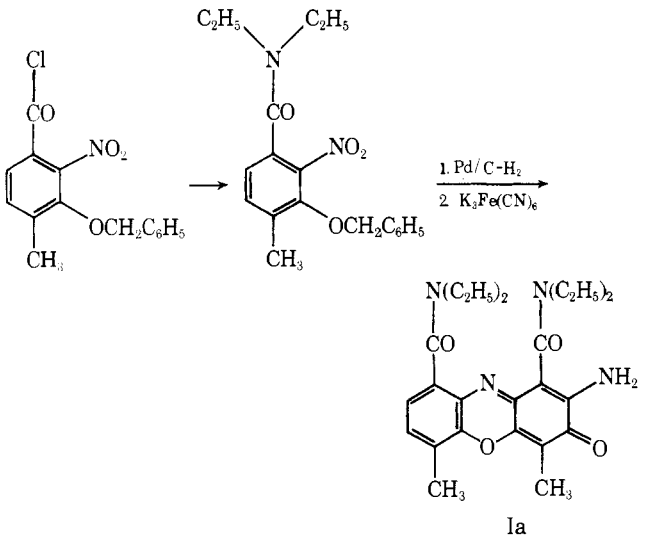
The clinical usefulness of AMD is limited by the failure of more human neoplasms to respond to the agent and by the toxicity of AMD to normal cells. Initially, in attempts to develop actinomycins with improved therapeutic indices or a broader range of antitumor properties, naturally occurring actinomycins were examined and approaches via biosynthesis and chemical synthesis were followed.[8] Some success was achieved by biosynthesis,[6] although the activity of AMD itself has not yet been surpassed. However, most chemical modifications or synthesis of analogs of the actinomycins have led to loss of activity due to the requirement of many structural features of the molecule for retention of DNA-binding specificity and inhibitory properties.[5]

The chemical approach was difficult since the DNA-binding properties and biologic activity of the actinomycin molecule require the intactness of the 2-amino group, the 3-quinoid oxo function, and the methyl groups at positions 4 and 6 of the chromophore, as well as the integrity of the cyclic pentapeptide lactones. We deduced on the basis of published information[7] that the DNA-binding capacity and DNA–guanine specificity of analogs of the molecule could be preserved only by appropriate substitution at position 7 (and perhaps at position 8) of the phenoxazinone chromophore. Following initial chemical studies on actinomycin chromophore model systems, we have prepared 7-nitro-AMD (IIb) and 7-amino-AMD (IIIb), which are comparable to AMD itself in DNA–guanine binding specificity and in the inhibition of human lymphoblastic (CCRF-CEM) cells in vitro and four transplantable mouse tumors (P388, L1210, and P1534 leukemias and Ridgway osteogenic sarcoma) in vivo. However, the two compounds exhibit different inhibitory profiles against two *Lactobacillus* systems in vitro.[11]

Chemical Studies. Our initial chemical investigation began with actinomycin chromophore model compounds (such as Ia), in which the pentapeptide lactone amide moieties at positions 1 and 9 were replaced by diethylcar-

boxamide groups. We have applied these results with chromophore model compounds to AMD itself and have obtained the 7-nitro (IIb), 7-amino (IIIb), and 7-hydroxy (VIb) analogs of AMD. The model compound Ia was synthesized from 3-benzyloxy-2-nitro-*p*-toluic acid *N,N*-diethylamide, which was prepared from 3-benzyloxy-2-nitro-*p*-toluyl chloride[12] and diethylamine. The *N,N*-diethylamide derivative was reductively debenzylated. Oxidation of the resulting intermediate aminophenol with potassium ferricyanide in a buffer gave Ia in high yield (Scheme I).

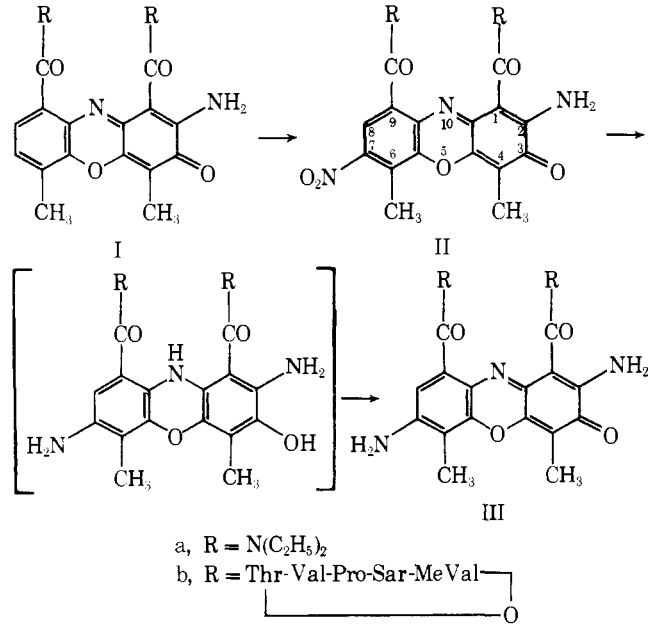
Scheme I. Synthesis of the Chromophore Model Compound



Ia

The model compound Ia was nitrated with a mixture of nitric and sulfuric acids. The 7-nitro derivative IIa which was thus obtained was then catalytically reduced to IIIa in almost quantitative yield. Application of these reactions to AMD gave the 7-nitro (IIb) and 7-amino (IIIb) analogs of AMD (Scheme II). In a preliminary communication,[14]

Scheme II. 7-Nitro and 7-Amino Analogs of AMD and the Chromophore Model Compound



I II III

a, R = $N(C_2H_5)_2$

b, R = Thr-Val-Pro-Sar-MeVal

Brockmann and coworkers have outlined the conversion of actinomycin C_2 to the 7-nitro compound by a three-step procedure but have not published experimental details; catalytic reduction of 7-nitroactinomycin C_2 gave 7-aminoactinomycin C_2.[14]

Both IIb and IIIb have prominent visible absorption bands and show optical rotation values which are comparable to actinomycin D and are consistent with the integrity of the pentapeptide lactone groups in these derivatives. Also, 7-nitro-AMD and 7-amino-AMD have been shown by several methods to bind to DNA with GC specificity in a manner qualitatively and quantitatively similar to that of AMD itself.[11]

7-Amino-AMD proved to be a fluorescent compound with an excitation maximum at 500 nm and emission in the red at 672 nm in aqueous buffer (pH 7). Interestingly, the 7-amino-AMD–DNA complex has an excitation optimum at 555 nm with an emission maximum at 655 nm. Thus, by use of different excitation wavelengths, one can distinguish between free and bound 7-amino-AMD, as we reported earlier[11] on the basis of uncorrected excitation and emission values. These data will be presented in a later publication.

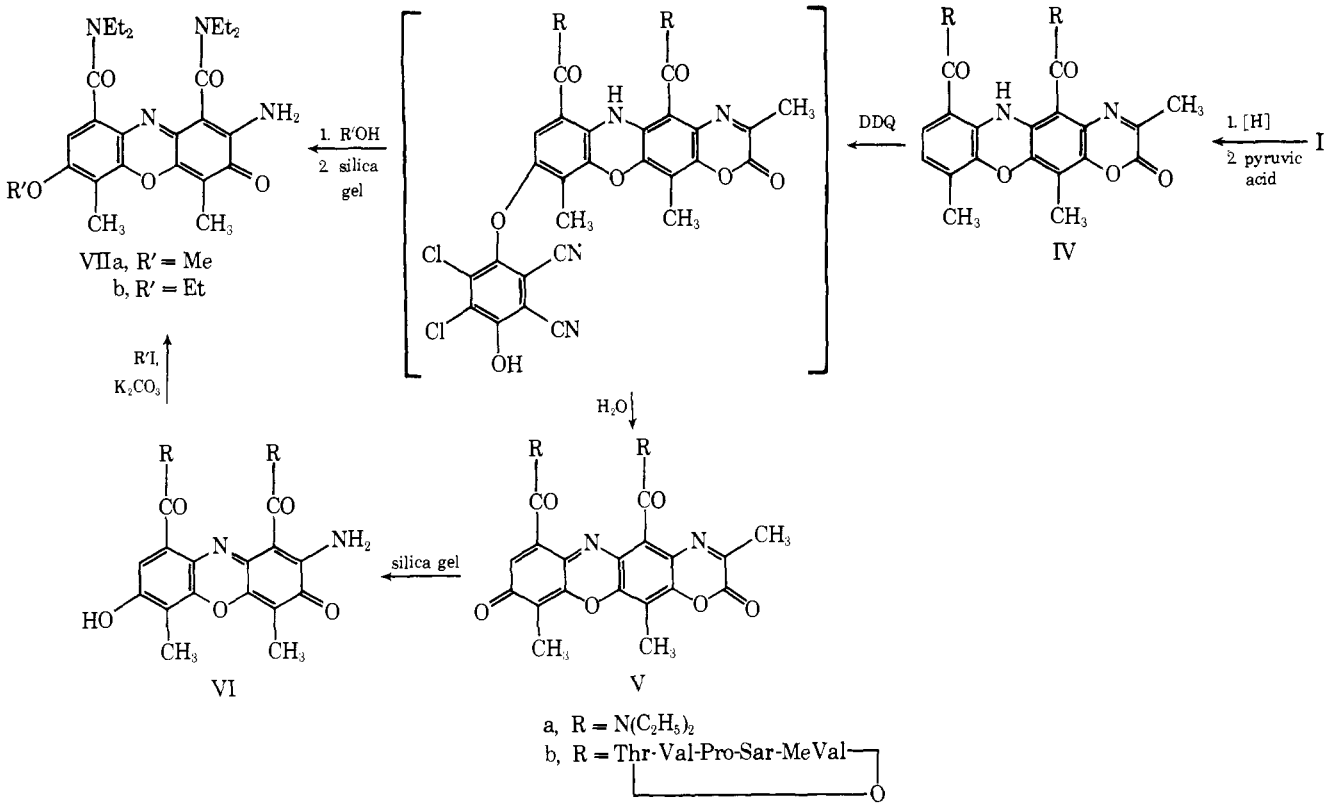
Synthesis of the 7-hydroxy model compound VIa and 7-hydroxy-AMD (VIb) required that the 2-amino and 3-oxo functions be protected before any electrophilic substitution on C_7 was attempted. The reported procedure of Brockmann for synthesis of oxazinone derivatives was quite satisfactory.[14] Reduction of the model compound Ia and coupling with pyruvic acid converted the 2-amino and 3-oxo functions of Ia into a protected oxazinone derivative (IVa). Deprotection and liberation of the chromophoric amino and oxo functions were readily attained with silica gel (Scheme III). Oxidation of IVa to Va was achieved by a novel method utilizing 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ). The primary reaction product is believed to be an intermediate hydroquinone ether; displacement with water introduces a hydroxy substituent, which is readily oxidized to the quinone form of Va. 2,3-Dichloro-5,6-dicyanohydroquinone (DDH) is also formed. Deprotection of Va with silica gel gave the 7-hydroxy model compound VIa.

In a preliminary experiment, application of this route to AMD (Ib) afforded the oxazinone derivative of AMD (IVb) and 7-hydroxy-AMD (VIb); these compounds were obtained in very small quantities, enough for chemical characterization but insufficient for biological evaluation. Brockmann has reported the synthesis of 7-hydroxy-AMC_2 by a different method;[14] our attempts to convert IVa into Va by the Brockmann method were unsuccessful.

The intermediate hydroquinone ether, on reaction with alcohols rather than water, undergoes electrophilic displacement with formation of 7-alkoxy-substituted AMD chromophore model compounds. The 7-methoxy- (VIIa) and 7-ethoxy- (VIIb) substituted model compounds have been prepared and characterized in this way.

Alternatively, both the methoxy (VIIa) and ethoxy (VIIb) chromophore model compounds were prepared by direct alkylation of VIa. The selectivity of this alkylation reaction depends on the pronounced phenolic nature of the 7-hydroxy group, the lesser reactivity of the (tautomeric) 3-hydroxy group (due to deactivation by the adjacent 2-amino group), and the reduced basicity of the 2-amino group; thus, in effect, only monoalkylation occurs and only at position 7. The alkylation is carefully controlled and is carried out with the alkyl iodide in dry acetone at ambient temperature in the presence of anhydrous potassium carbonate. The alkoxy derivative is obtained in nearly quantitative yield and shows a single spot on TLC.

Scheme III. 7-Hydroxy- and 7-Methoxy-Substituted Chromophore Model Compounds and 7-Hydroxy-AMD



Proof of the identity of the two methoxy and ethoxy derivatives prepared by the two methods was obtained by NMR. The NMR spectra of the two samples of VIIa prepared by the two methods were identical (Table I). These spectra show high-field shifts for the 8-H, 6-Me, and 2-NH_2 protons. Similar NMR results for VIIb are also reported in Table I.

Biological Evaluation. The 7-substituted actinomycin analogs have been investigated for inhibitory properties in several systems at the Sidney Farber Cancer Center. Inhibitory data in vitro are presented in Table II. In both the *Lactobacillus fermenti*–thiamine and the *Lactobacillus arabinosus*–pantothenate systems,[13] 7-nitro-AMD (IIb) is approximately as active as AMD (Ib). However, 7-amino-AMD (IIIb) is only about one-tenth as active as Ib in the *L. fermenti* system and is essentially inactive against *L. arabinosus*. Inhibitory data reported for the comparable analogs of actinomycin C_2 against *Bacillus subtilis*[14] compare favorably with our results. In mammalian cell culture assay against CCRF-CEM human lymphoblastic leukemic cells,[15] all three compounds showed comparable activity.

Data on inhibition of transplantable mouse tumors are given in Table III. Against the Ridgway osteogenic sarcoma[16] at the 150 μg/kg/day dose level, all three compounds produced long-term survivors without detectable tumor; on day 185 after the start of therapy, four of six mice treated with actinomycin D, three of six mice treated with 7-nitro-AMD, and two of six mice treated with 7-amino-AMD were alive. These survivors were sacrificed on day 185 after the start of therapy. Median survival times are given in Table III. No tumors were found at autopsy in the surviving mice and, presumably, the survivors would have lived substan-

Table I. NMR Spectra

	τ, $CDCl_3$					τ, Me_2SO-d_6				
Compd	7-H	8-H	6-Me	4-Me	2-NH_2	7-H	8-H	6-Me	4-Me	2-NH_2
Ia	2.83[a]	2.62[a]	7.47	7.77	4.40	2.85[a]	2.57[a]	7.50	7.86	3.55
IIa		2.10	7.26	7.76	4.17					
IIIa		3.37	7.78	7.75	4.79					
VIa		3.24	8.03	7.79	4.68		3.20	7.70	7.86	3.90
VIIa[b]		3.10	7.63	7.76	4.62		3.08	7.72	7.87	3.89
VIIa[c]		3.11	7.63	7.76	4.61		3.09	7.72	7.86	3.89
VIIb[b]		3.10	7.64	7.76	4.62		3.08	7.70	7.87	3.87
VIIb[c]		3.10	7.64	7.76	4.62		3.08	7.70	7.86	3.87
	9-H	8-H	10-Me	12-Me						
IVa	3.43[a]	3.25[a]	7.81	7.78						
Va		3.18	7.84	7.43						

[a] Center of a doublet. [b] By hydroquinone displacement (method B). [c] By direct alkylation of VIa (method A).

Table II. Inhibitory Activity of Actinomycin D, 7-Nitroactinomycin D, and 7-Aminoactinomycin D vs. Bacterial and Mammalian Cells in Vitro

Compd	ID_{50}, μg/ml		
	L. arabinosus–pantothenate (0.01 μg/ml)	*L. fermenti*–thiamine (0.01 μg/ml)	CCRF-CEM cells
Actinomycin D	0.008	0.006	0.065
7-Nitroactinomycin D (IIb)	0.007	0.0075	0.080
7-Aminoactinomycin D (IIIb)	0.3	0.072	0.085

tially longer without tumor. At the lower dose levels tested in this experiment, AMD was more effective than the two analogs.[11]

The results of evaluation of 7-nitro-AMD, 7-amino-AMD, and AMD against three mouse leukemias are also presented in Table III. Compounds were tested over a dose–response curve including toxicity at the higher dose levels, but results are presented for simplicity only at the optimal nontoxic dose levels for each compound. Against the L1210 leukemia, a tumor moderately well inhibited by AMD, and the P1534 leukemia, which does not respond as well to AMD, all three compounds are roughly equivalent. Against the P388 leukemia, the two analogs are somewhat more effective than AMD. Each of these three experiments was done twice with reproducible results. Evaluation of several naturally occurring and biosynthetically prepared actinomycins in these four and other transplantable mouse tumor systems has been reported.[16,17]

Discussion

Analysis of the biologic data makes it clear (a) that these two 7-substituted analogs of actinomycin D (IIb and IIIb) are comparable to AMD (Ib) in four transplantable mouse tumor systems; (b) that all three compounds are comparable in mammalian cell culture assay in vitro; and (c) that the antibacterial assay systems used by other investigators[8,14,18] do not necessarily provide good predictive data for other bioassay systems.‡

Overall, the most important aspect of this investigation is that we have established that substitution of the AMD molecule at position 7 provides analogs with biochemical activity, DNA-binding properties, DNA-binding specificity, inhibitory properties in vitro, and experimental antitumor activity in vivo comparable to the corresponding properties of AMD itself.

Experimental Section

Melting points (uncorrected) were determined in Pyrex capillary tubes by means of a Mel-Temp apparatus (Laboratory Devices, Inc., Cambridge, Mass.) at a heating rate of 2°/min. Column chromatography was performed on silica gel powder (Baker no. 3405, 60–200 mesh) and acidic alumina (Woelm, grade I). Acidic silica gel powder was made by exposing Baker no. 3405 silica gel powder to the vapor from concentrated hydrochloric acid for 20 hr in an airtight container. Sephadex LH-20, particle size 25–100 μ (Pharmacia Fine Chemicals), was used in the actinomycin D work. Thin-layer chromatography was performed on silica gel sheets (Eastman 6060, with fluorescent indicator, and Eastman 6061, without fluorescent indicator). The sheets were treated for 0.5 hr with HCl vapor for work on base-labile material. Solvents used were $CHCl_3$–acetone (4:1) and Ciferri[8] (organic phase of the mixture of EtOAc–MeOH–H_2O (20:1:20)). Ir spectra were obtained using a Perkin-Elmer Model 137B Infracord in KBr or KCl pellets, and uv spectra were determined with a Cary Model 11 spectrophotometer. NMR spectra were obtained using a Varian Model A-60 spectrometer with Me_4Si as an internal (and occasionally external) standard. Optical rotations were determined in a Cary 60 recording spectropolarimeter. Fluorescence was measured in a Perkin-Elmer Model MPF-4 fluorescence spectrophotometer with corrected spectra accessory; wavelengths reported are corrected. Unless otherwise specified, the solvents were dried over molecular sieves. Where analyses are specified only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of theoretical values. Elemental analyses were determined by Galbraith Microanalytical Laboratories, Tenn., and by Werby Laboratories, Boston, Mass. Actinomycin D, batch no. 3008-30B, kindly provided by Lederle Laboratories Division, American Cyanamid Co., and prepared by Dr. Nestor Bohonos and coworkers, was used in the reactions.

3-Benzyloxy-2-nitro-*p*-toluic Acid *N,N*-Diethylcarboxamide. To an ice-cold (0–5°) solution of 150 g (0.49 mol) of 3-benzyloxy-2-nitro-*p*-toluyl chloride[12] in 2 l. of benzene and 500 ml of ether was added 128 g (1.75 mol) of diethylamine, and the mixture was left overnight at +4°. The separated solid was collected, and the filtrate was evaporated to dryness. The tan residue, mp 64–65°, was crystallized from a 4:1 mixture of ether and petroleum ether (bp 30–60°). A colorless crystalline solid (156 g, 90%), mp 65–66°, was obtained: λ_{max}^{EtOH} (ε) 224 (25900), 289 nm (1660). Anal. ($C_{19}H_{22}N_2O_4$) C, H, N.

2-Amino-1,9-bis(*N,N*-diethylcarbamoyl)-4,6-dimethyl-3*H*-phenoxazin-3-one (Ia). A solution of 3-benzyloxy-2-nitro-*p*-toluic acid *N,N*-diethylamide (80 g, 117 mmol) in 400 ml of methanol was hydrogenated at 40–50 psi in an Adam's apparatus with 4 g of 5% Pd/C. The mixture was filtered through Celite, and the filtrate was immediately poured into a solution of potassium ferricyanide (231 g, 701 mmol, in 1.25 l. of 0.2 *M* sodium phosphate buffer, pH 7.0). The pH of the reaction mixture went down initially to about 5 and was quickly adjusted to 7 by addition of 7.5 *N* NaOH. After stirring for 16 hr at room temperature, the mixture was filtered and the filter cake was washed with hot ethanol. The combined filtrate and washing were extracted with benzene. Evaporation of the benzene left a residue which was dried azeotropically with benzene. The residue was dissolved in 400 ml of benzene, and the solution was diluted to 1200 ml with heptane and left at 4° for crystallization: dark red needles; 47.50 g (92%); mp 198–199°; λ_{max}^{EtOH} (ε) 224 (25960), 418 infl, and 433 nm (28850). Anal. ($C_{24}H_{30}N_4O_4$) C, H, N.

2-Amino-1,9-bis(*N,N*-diethylcarbamoyl)-4,6-dimethyl-7-nitro-3*H*-phenoxazin-3-one (IIa). Ia (12 g, 27.4 mmol) was dissolved throughly in cold (0°) concentrated sulfuric acid (100 ml) and was nitrated by dropwise addition of a solution of 1.6 ml of fuming nitric acid in 8 ml of sulfuric acid.[19] After stirring for 1.5 hr at 0–5°, the mixture was poured into a well-stirred mixture of 50 ml each of ice water and chloroform. The chloroform layer was separated, washed with saturated sodium bicarbonate (freshly prepared solution) and brine, then dried, and evaporated. Crystallization of the residue from tetrahydrofuran and heptane (5:1) gave a dark brown solid: mp 269–271° (7.97 g, 60%); λ_{max}^{EtOH} (ε) 220 sh (31130), 226 sh (30100), 309 (10510), 430 (26120), and 444 nm (26730). Anal. ($C_{24}H_{29}N_5O_6$) C, H, N.

2,7-Diamino-1,9-bis(*N,N*-diethylcarbamoyl)-4,6-dimethyl-3*H*-phenoxazin-3-one (IIIa). A. An ethanolic solution of IIa (1.1 g, 2.3 mmol, in 100 ml of absolute ethanol) was reduced catalytically at atmospheric pressure with platinum oxide (100 mg) and hydrogen for 6 hr under efficient shaking with a vibrator. Removal of the catalyst and evaporation of the solvent gave 1 g (97%) of homogeneous (by TLC) dark red solid. Crystallization from benzene with charcoal yielded a red solid (80% recovery): mp 268°; λ_{max}^{EtOH} (ε) 246 (28800), 283 infl, and 519 nm (31500): $\lambda_{max}^{CHCl_3}$ 490 nm (34530). Anal. ($C_{24}H_{31}N_5O_4$) C, H, N.

B. A mixture of IIa (100 mg, 0.2 mmol), Pd/C (75 mg), and 95% hydrazine hydrate (1 ml) in ethanol (50 ml) was refluxed for 1.5 hr, filtered, and evaporated to yield red solid (85 mg, 89%), identical with that described in procedure A.

7-Nitroactinomycin D (IIb). Actinomycin D (800 mg, 0.64 mmol) dissolved in 15 ml of concentrated sulfuric acid was nitrat-

‡The growth-inhibitory properties of actinomycins and actinomycin analogs and derivatives, reported in ref 8, 14, 18, were evaluated on the basis of bacteriostatic activity, primarily against *B. subtilis*. Inhibition of mammalian cells in culture had not previously been reported, to the best of our knowledge, prior to ref 11.

Table III. Increase in Median Survival Time of Tumor-Bearing Mice Treated with 7-Substituted Actinomycin D Analogs[a]

	P388 leukemia		L1210 leukemia		P1534 leukemia		Ridgway osteogenic sarcoma	
	Optimal dose, μg/kg/inj	% ILS[b]	Optimal dose, μg/kg/inj	% ILS	Optimal dose, μg/kg/inj	% ILS	Optimal dose, μg/kg/inj	% ILS
Actinomycin D	75	118	75	44	50	30	150	>503[c]
7-Nitroactinomycin D (IIb)	300	154	450	55	100	20	150	>354[d]
7-Aminoactinomycin D (IIIb)	150	164	300	44	200	20	150	258[e]

[a]Leukemias were implanted ip in BDF_1 mice: 10^5 cells for L1210 and 10^6 cells for P388 and P1534 leukemias. The Ridgway osteogenic sarcoma was implanted im in AKD_2/F_1 mice. Compounds were administered ip daily for 4 successive days starting 1 day after implantation of the leukemias or on the day (day 17) of visualization of the sarcoma. There were five mice per group for the leukemias and six mice per group for the sarcoma. Optimal nontoxic doses are given in μg/kg/injection. [b]% ILS = percent increase in median survival time of treated compared with control animals calculated from the day of tumor implantation. [c]Four of six mice still alive and without palpable tumor when sacrificed on day 185 after the start of therapy. [d]Three of six mice still alive and without palpable tumor when sacrificed on day 185 after the start of therapy. [e]Two of six mice still alive and without palpable tumor when sacrificed on day 185 after the start of therapy.

ed as for IIa (0.2 ml of fuming nitric acid and 4 ml of concentrated sulfuric acid cooled to 0–3°). The residue from the chloroform extract was crystallized from a 1:1 mixture of ethanol and chloroform and was further purified by passage through a 150-cm long, 2.2 cm internal diameter Sephadex LH-20 column with 95% ethanol. Upon concentration, a crop of 465 mg (56%) of brown solid, mp 252–253° dec, was obtained: $[\alpha]^{20}_{644}$ −225 ± 12° (*c* 0.033, $CHCl_3$); λ_{max}^{EtOH} (ε) 310 (8230), 436–443 sh (22700), and 449 nm (22700); $\lambda_{max}^{CHCl_3}$ 314 (8060), 438 (23900), 445 nm (24300). Anal. ($C_{62}H_{85}N_{13}O_{18}$) C, H, N.

7-Aminoactinomycin D (IIIb). An ethanolic solution of pure IIb (200 mg, 0.15 mmol) was reduced catalytically with platinum oxide, as for IIIa. After removal of the catalyst, the purple-red filtrate upon concentration and cooling gave 153 mg (78%) of pure IIIb as deep purple plates: mp 252–253° dec; λ_{max}^{EtOH} (ε) 239 (34650), 287 infl, 313 infl, 529 nm (23700); $\lambda_{max}^{CHCl_3}$ 308 (6980), 501 nm (26700); $[\alpha]^{20}_{644}$ −348 ± 18° (*c* 0.033, $CHCl_3$). Anal. ($C_{62}H_{87}N_{13}O_{16}$) C, H, N.

5,7-Bis(*N*,*N*-Diethylcarbamoyl)-3,10,12-trimethyl-2*H*,6*H*-oxazino[3,2-*b*]phenoxazin-2-one (IVa). A solution of Ia (2.19 g, 5 mmol) in 200 ml of methanol was reduced catalytically with hydrogen and platinum oxide (300 mg) and the solution was filtered under nitrogen pressure into a flask containing pyruvic acid (8 ml), also kept under nitrogen. In a few minutes a red solid separated. The volume of methanol was reduced to ca. 30 ml, and the mixture was left at 4° for 20 hr. The red solid, 2.3 g (95%), mp 237–238°, was washed with ether and water and was crystallized from methanol: mp 237–238°; single red fluorescent spot on TLC (acidic silica gel, $CHCl_3$–actone, 4:1), R_f 0.92; λ_{max}^{EtOH} (ε) 383 (12100), 450 nm (5345); ν_{KBr} 1710 cm^{-1} (oxazinone carbonyl). Anal. ($C_{27}H_{32}N_4O_5$) C, H, N.

5,7-Bis(*N*,*N*-diethylcarbamoyl)-3,10,12-trimethyl-2*H*,9*H*-oxazino[3,2-*b*]phenoxazine-2,9-dione (Va). In a typical run, a mixture of IVa (123 mg, 0.25 mmol) in 20 ml of ethanol and DDQ (120 mg, 0.75 mmol) in 10 ml of ethanol was stirred under nitrogen for 4 hr. The resulting red solution was evaporated to dryness and the residue was taken up in methylene chloride and filtered to remove most of the precipitated DDH (2,3-dichloro-5,6-dicyanohydroquinone) and DDQ. The filtrate was passed through an acidic Woelm alumina column with CH_2Cl_2. The residual DDQ and DDH were absorbed on the column, and the eluent was evaporated to dryness. The residue was crystallized from a 1:1 mixture of ether and petroleum ether (bp 30–60°). Va was obtained as an orange-red solid: 93.1 mg (73.5%); mp 225–227°; $\lambda_{max}^{CHCl_3}$ (ε) 260 (21030), 267 (21410), 297 (19900), 353 (16900), 368 (16600), 447 nm (13200); ir in KCl shows a new carbonyl peak at 1761 cm^{-1}. The product is unstable in an aqueous or ethanolic solution. Anal. ($C_{27}H_{30}N_4O_6$) C, H, N.

2-Amino-1,9-bis(*N*,*N*-diethylcarbamoyl)-4,6-dimethyl-7-hydroxy-3*H*-phenoxazin-3-one (VIa). A solution of 790 mg (1.56 mmol) of Va in 25 ml of chloroform was stirred with a suspension of 40 g of silica gel powder in 250 ml of 95% ethanol for 4 hr in a nitrogen atmosphere. The suspension was filtered and the silica gel powder was washed with chloroform. The combined extract was evaporated to dryness, and the residue was crystallized from ether to give orange solid (640 mg, 87%): mp 261–262°; $\lambda_{max}^{CHCl_3}$ (ε) 291 (infl), 460 nm (19300). Anal. ($C_{24}H_{30}N_4O_5$) C, H, N.

In another experiment, when 75% aqueous methanol and 30 g of silica gel were used (pH 7.2–7.8) for 4 hr under identical conditions, 85% of VIa was isolated after purification.

2-Amino-1,9-bis(*N*,*N*-diethylcarbamoyl)-4,6-dimethyl-7-methoxy-3*H*-phenoxazin-3-one (VIIa). A. From VIa. A solution of VIa (22.7 mg, 50 mmol) in dry acetone (10 ml) was allowed to react with 2 drops of methyl iodide in the presence of anhydrous potassium carbonate (8 mg) in a nitrogen atmosphere at ambient temperature for 22 hr. The orange reaction mixture was filtered to remove inorganic salts, and the filtrate was evaporated. The residue was extracted with a 4:1 mixture of ether and petroleum ether (bp 30–60°). The solid, VIIa, was further purified by crystallization from the latter solvent: 21.5 mg (85%); mp 223–225°; $\lambda_{max}^{CHCl_3}$ (ε) 290 (infl), 459 nm (24800). Anal. ($C_{25}H_{32}N_4O_5$) C, H, N.

B. From IVa. A solution of 500 mg (1 mmol) of IVa and 454 mg (2 mmol) of recrystallized DDQ in dry benzene was stirred for 2 hr; evaporation gave a black, gummy residue. There was no separation of DDH on trituration of the gum with CH_2Cl_2, as was noted in previous reactions when alcohols were used as the reaction medium instead of benzene. The residue is presumed to be an addition product of the chromophore with the hydroquinone (DDH) linked through an ether function.[20] Treatment of the gum with absolute methanol for 20 min disrupted the hydroquinone ether and separated DDH as a brown solid (350 mg). The methanol-soluble material was recovered and chromatographed through alumina with CH_2Cl_2 to give Va (233 mg, 46%, mp 225–227°) and VIIa (50 mg, 10%, mp 222–225°): $\lambda_{max}^{CHCl_3}$ (ε) 292 (inf), 457 nm (25400). The latter product was identical by melting point comparison, TLC behavior, and ir, uv, and especially NMR spectra with material obtained by procedure A.

2-Amino-1,9-bis(*N*,*N*-diethylcarbamoyl)-4,6-dimethyl-7-ethoxy-3*H*-phenoxazin-3-one (VIIb). A. From VIa. In a manner similar to that described for VIIa, 45.4 mg (0.1 mmol) of VIa and 10 drops of ethyl iodide gave after purification 24.5 mg (50%) of product: mp 217–219°; $\lambda_{max}^{CHCl_3}$ (ε) 292 (infl), 461 nm (29670). This material was identical with an authentic sample, obtained as described below.

B. From IVa. A solution of DDQ (454 mg, 2 mmol) in 20 ml of absolute ethanol was added to IVa (492 mg, 1 mmol) in absolute ethanol (100 ml) and the mixture was stirred under nitrogen for 4 hr. DDQ and DDH were separated and the residue, after evaporation of the ethanol solvent, was taken up in CH_2Cl_2 and passed through an acidic alumina column with this solvent. The first band contained 394 mg (78%) of Va. The next two bands consisted of mixtures of Va (R_f 0.90) and another major yellow material (R_f 0.35–0.63) as evidenced by TLC (silica gel, $CHCl_3$–acetone); this mixture could not be further separated by alumina chromatography. The mixture was then dissolved in 50% aqueous ethanol and stirred over silica gel. The suspension was filtered, the filtrate was concentrated and extracted with $CHCl_3$, and the extract was passed through a silica gel column. The faster moving band yielded 31.5 mg (6.5%) of pure VIIb [R_f 0.61 (silica gel, $CHCl_3$–acetone); mp 217–219°; $\lambda_{max}^{CHCl_3}$ (ε) 290 (infl), 461 nm (29600)]; 24.2 mg (4.8%) of VIa [R_f 0.40 (silica gel, $CHCl_3$–acetone)] was recovered

from subsequent fractions. The visible and infrared absorption spectra and, particularly, the NMR chemical shifts of 6-methyl, 8-aromatic, and 2-amino protons are all consistent with the 7-ethoxy chromophore; the material was identical with that obtained by procedure A. Anal. ($C_{26}H_{34}N_4O_5$) C, H, N.

3,10,12-Trimethyl-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] (IVb). Ib (200 mg, 0.16 mmol) was reduced with PtO_2 in 100 ml of methanol. Under nitrogen, the reduction mixture was filtered to remove catalyst, with the filtrate adding directly to 2 ml of pyruvic acid previously placed in the suction flask. After 4.5 hr, the red solution was concentrated under vacuum to ca. 5 ml; ethanol (40 ml) and water (100 ml) were added and the solution was extracted twice with ethyl acetate (200 ml) and washed three times with 100-ml portions of water. The extract was dried over Na_2SO_4 (anhydrous). Filtered and evaporated, the residue was then passed through a Sephadex LH-20 column in 95% ethanol; fractions which contained mainly IVb and were relatively free of starting material, as evidenced by TLC (acidic silica gel, Ciferri solvent), were rechromatographed on Sephadex LH-20. Very careful fractionation and addition of ether to the fractions yielded crystals of IVb·H_2O (190 mg, 90%): mp 238–240°; $\lambda_{max}^{CHCl_3}$ (ϵ) 319 (8700), 397 (6400), 456 nm (8700); $[\alpha]^{20}_{644}$ −101 ± 12° (c 0.10, $CHCl_3$). Anal. ($C_{65}H_{88}N_{12}O_{17}\cdot H_2O$) C, H, N.

7-Hydroxyactinomycin D (VIb). IVb (66 mg, 0.05 mmol) in 5 ml of absolute ethanol was stirred with 35 mg (0.154 mmol) of DDQ in 1 ml of absolute ethanol for 2.5 hr under nitrogen and the mixture was evaporated to dryness. The residue was chromatographed on 5 g of acidic alumina and the first eluate, in CH_2Cl_2, and the second eluate, in ethyl acetate, were evaporated to yield 34.2 mg (50%) of Vb. This material, without further purification, was subjected to cleavage with 20 ml of 50% ethanol and 3 g of silica gel, as follows. The mixture was stirred for 1 hr, filtered, and concentrated. The concentrate was extracted with ethyl acetate, the extract evaporated, and the residue was chromatographed in 95% ethanol through Sephadex LH-20. Careful fractionation gave relatively pure VIb·$2H_2O$ (11.5 mg, 20% on the basis of Vb): mp 255–257° dec; $\lambda_{max}^{CHCl_3}$ (ϵ) 469 nm (23000); $[\alpha]^{20}_{644\pm10}$ −214 ± 20° (c 0.021, $CHCl_3$). Anal. ($C_{62}H_{86}N_{12}O_{17}\cdot 2H_2O$) C, H, N.

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Synthesis and Evaluation of [Des-Asp^1]angiotensin I as a Precursor for [Des-Asp^1]angiotensin II ("Angiotensin III")

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The nonapeptide [des-Asp^1]angiotensin I (IV), synthesized by Merrifield's solid-phase procedure, was tested as a possible substrate for the converting enzymes from porcine lung and plasma. IV, [des-Asp^1]angiotensin II (III), [des-(Asp^1,Arg^2)]angiotensin II (V), [des-(Asp^1,Arg^2,Val^3)]angiotensin II (VI), [Sar^1,Ile^8]angiotensin II (VII), and [des-Asp^1,Ile^8]angiotensin II (VIII) possessed 0.5, 20, 2, 0, <0.1, and <0.01% of the inotropic activity (rabbit atria), 1, 15, 5, 0, 3, and 0% secretory activity of the cat adrenal medulla, and 0.0, 150, 1, 0.5, 3, and 10% of the adrenal steroidogenic activity of angiotensin II, respectively. When tested for their antagonistic activity in the above tissues, only VII and VIII were found to inhibit responses to angiotensin II. The pA_2 values for VII and VIII were 8.31 and 10.0 in the adrenal cortex and 9.31 and 9.16 in the adrenal medulla, respectively. All these peptides were also tested as product inhibitors for the plasma and lung converting enzymes. With the plasma enzyme, the ID_{50} values were II, 1.6×10^{-4} *M*; III, 5×10^{-5} *M*; V, 1.2×10^{-4} *M*; VI, 5×10^{-4} *M*; VII, 5×10^{-5} *M*; VIII, 5×10^{-4} *M*. Thus, IV is a good substrate for converting enzymes from lung and plasma while all other compounds were inhibitors of these enzymes. The most potent inhibitors of converting enzyme were III followed by VII and VIII. With the exception of II and III, all the other analogs had very low intrinsic activities, per se. These results suggest (a) an alternate pathway for the formation of the heptapeptide III, viz., by the action of converting enzyme on the nonapeptide IV, and (b) that III may also be acting as inhibitor of the converting enzyme by the feedback mechanism.

It is a widely accepted concept that (a) converting enzyme (dipeptidylcarboxypeptidase), particularly in lung, converts the decapeptide angiotensin I (I) into the octapeptide angiotensin II and (b) angiotensin II acts on vascular smooth muscle to produce vasoconstriction and on the adrenal cortex to stimulate aldosterone biosynthesis. However, recently it has been shown that the C-terminal heptapeptide "angiotensin III" (III) is also responsible for the adrenal cortical stimulation.[1-4] There are two possible pathways by which the heptapeptide III could be formed