Carbon-7 Substituted Actinomycin D Analogues as Improved Antitumor Agents: Synthesis and DNA-Binding and Biological Properties

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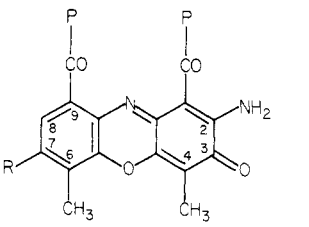
7-(2,3-Epoxypropoxy)actinomycin D has been synthesized along with its major companion product, 7-(2,3-dihydroxypropoxy)actinomycin D. They were characterized by UV–visible and CD spectra and by NMR studies. According to UV–visible absorptiometry, circular dichroism, and thermal denaturation studies, they bind to DNA in a manner that is comparable to actinomycin D. The analogues are, like actinomycin D, extremely cytotoxic to human lymphoblastic leukemic cells (CCRF-CEM) in vitro but are significantly less toxic than actinomycin D to normal CDF_1 mice in vivo. Unlike actinomycin, these analogues are metabolized in rats, and the metabolites are excreted in rat urine at a rapid rate. Compared to actinomycin D, the antitumor activity of the 7-(2,3-epoxypropoxy)actinomycin analogue against P-388 leukemia in mice is decidedly superior, and the therapeutic index is improved several fold.

Actinomycin D (AMD, **1b**) is a chromopeptide antibiotic



a, P = $N(C_2H_5)_2$
b, P = Thr-D-Val-Pro-Sar-MeVal–O–

1, R = H
2, R = OH
3, R = $OCH_2CH(O)CH_2$
4, R = $OCH_2CH(OH)CH_2OH$
5, R = OCH_3

whose potent activity in several tumors, including Wilm's tumor, gestational choriocarcinoma, and Kaposi's sarcoma, has been reported.[1,2] AMD (**1b**) at submicromolar concentrations strongly inhibits DNA-dependent RNA synthesis and, to a lesser extent, DNA synthesis. Its interaction with DNA has been extensively studied, and the details of the mechanism of binding to DNA has been proposed.[3-5] It has been assumed that the cytotoxicity of **1b** is due to its inhibition of RNA polymerase following the intercalative binding to DNA.[6] It is quite possible, however, that the distortions in helical DNA resulting from the strong noncovalent association with AMD may not be solely responsible for the observed biological effects. For example, Nakazawa et al. suggest that an intermediate free-radical form of AMD may be the active form that causes DNA damage and cell death.[7]

Furthermore, the proximal mechanism of biochemical action of **1b**, which is evident from the inhibition of RNA synthesis, may not be the principal mechanism of selective cytotoxicity of the agent at the pharmacological level. "For it is known that AMD is far more cytotoxic in those proliferating cells in which it inhibits DNA synthesis than in those of liver, kidney, muscle, etc., that are nonproliferating but are heavily dependent upon RNA synthesis for protein renewal".[8]

Another pharmacological behavior of AMD is that it is not metabolized in vivo. Absence of metabolic conversion or detoxification of AMD leads to its accumulation in the cell nuclei of the host organs and causes cumulative toxicity. This acute cumulative toxicity limits the wide clinical application of AMD.[9]

A primary goal in our synthesis of new pharmacologically active analogues of AMD is the maximization of its drug efficacy. To achieve this, we have attempted to increase the drug potency, by enhancing drug activity in the tumor cells and also by decreasing toxicity to the host.

As the first step, we did a systematic study of structure–antitumor activity relationship on the chromophore substituted actinomycin D analogues. We synthesized a variety and a series of AMD analogues with substitutions at the C-7 and N^2 positions and determined their biophysical, UV–visible absorptiometric, specific rotation, circular dichroic, and NMR properties. The purpose was to ensure that the integrity of the tricyclic phenoxazinone ring system as well as the conformation of the interannularly hydrogen-bonded pentapeptide lactone rings and the relationships between the chromophoric and the peptide lactones were unaltered from those of AMD. We also demonstrated that the analogues that retained the biological activity in terms of inhibition of cellular nucleic acid synthesis also exhibited proportionate extracellular DNA-binding affinity. In a limited number of analogues, we were able to establish that the DNA bindings were not only G–C base pair specific but also cooperative; i.e., the interaction of the chromophore and hydrogen bonding and hydrophobic interaction of the peptide lactone moieties with DNA were simultaneous. The experimental results established, among other things, that small alkoxy substituents, e.g., methoxy, ethoxy, and *n*-propoxy, at C-7 retained most of the above properties of AMD. Contrary to previous reports, our results clearly showed that the molecule of actinomycin D can accomodate a number of well-defined modifications at the C-7 and N^2 sites and still retain most of its physicochemical, biophysical, biological, and tumor-inhibitory properties (see ref 10 and the references quoted therein).

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Table I. Comparison of NMR Chemical Shifts in the Chromophore Ring Protons and C-7 Substituents in Actinomycin D and Analogues and Model Derivatives[a]

	chemical shift, ppm								
compd	7-H[b]	8-H[b]	2-NH_2[b]	4-CH_3[b]	6-CH_3[b]	ArOCH_2[c]	2-H[c]	3-H[c]	2-OH, 3-OH[c]
1b	7.53	7.63	7.08	2.24	2.67				
3b		7.21	7.09	2.24	2.41	3.70-4.14	3.49	2.62-2.87	
4b		7.26	7.08	2.24	2.45	3.72-4.13	3.53	2.65-2.90	4.16-4.24
1a	7.17	7.38	5.61	2.30	2.53				
3a		6.89	5.39	2.28	2.39	3.88-4.28	3.41	2.72-3.00	
4a		6.93	5.39	2.28	2.41	3.88-4.24	3.41	2.70-2.96	4.20-4.28

[a] 90-MHz spectrum of $CDCl_3$ solution, chemical shifts in parts per million (δ) to low field from internal tetramethylsilane. [b] These protons are present in the chromophoric rings of AMD and model analogues. [c] These protons are part of the substituent chains at C-7 of the synthetic analogues **3a,b** and **4a,b**.

We considered the synthesis and biological evaluation of the 7-(2,3-epoxypropoxy)actinomycin analogue, since the alkoxy substituent at C-7 of **3b** would be comparable in size to *n*-propoxy and would, consequently, be expected to retain the biochemical and biological properties of AMD. Also the epoxy group is potentially alkylating, which, once inside the cell, should be able to augment nuclear fragmentation and cell death. Furthermore, AMD is known to be active against a limited number of tumor lines. The tumor lines that are not responsive to AMD are known to be cross-resistant to other intercalating agents, e.g., adriamycin, but not to alkylating agents, e.g., cytoxan and L-PAM.[11] In order to broaden the spectrum of activity and efficacy, epoxy-substituted AMD appears to be the compound of choice. It should be noted that epoxy is only a labile alkylating function and is likely to be hydrolyzed and deactivated, especially by the action of hepatic microsomal hydrolytic enzymes. In that case, the resulting hydroxylated analogues might easily be liable to conjugation in the liver, thereby lessening the chance of cumulative toxicity. The present article deals with the synthesis, DNA-binding properties, in vivo and in vitro antitumor activity, metabolism and excretion of two analogues of AMD, **3b** and **4b**, one of which, 7-(2,3-epoxypropoxy)actinomycin D (**3b**), is highly active.

Chemistry. 7-(2,3-Epoxypropoxy)actinomycin D (**3b**) was prepared from 7-hydroxyactinomycin D (**2b**).[10] Our initial synthetic investigations were carried out on the model 7-hydroxy analogue (**2a**).[12] The chemical and spectral nature of these model analogues are very similar to the correspoding AMD analogues.

Mild reaction of epibromohydrin with **2a** in dry acetone at 50–55 °C in the presence of finely powdered anhydrous potassium carbonate yielded the alkylated products **3a** and **4a**. Similarly, AMD analogues **3b** and **4b** were obtained, starting from 7-hydroxyactinomycin (**2b**). Previous investigations from our laboratory have confirmed that alkylation of **2** occurs entirely on the 7-hydroxy group, which is phenolic and is relatively less hindered compared to the alternative (tautomeric) 3-hydroxy group.[10]

However, in the present case, alkylation of **2** with epibromohydrin yielded a major companion compound, **4**, along with the desired product, **3**. Identical derivatives were obtained both in the model and AMD series. The companion compounds were readily identified as the 7-(2,3-dihydroxypropoxy) analogues **4a** and **4b**, respectively, in these series. They could be generated, alternatively, from **3** via mild acid-catalyzed hydrolysis of the epoxide ring. All these analogues were characterized by TLC, HPLC, UV, and NMR properties and elemental analyses.

Spectral Properties. NMR. The structures of the model substituted compounds **3a** and **4a**, as well as AMD analogues **3b** and **4b**, could be easily verified by their NMR spectra. The assignments of the characteristic chemical shifts in the substituent chain at C-7 in **3a** and **3b** are in agreement with those reported for the epoxypropyl ether side chain in hexesterol.[13] Also, the NMR characteristics for the side-chain protons in model and AMD analogues are identical. In fact, the model analogues were of substantial help in assignment of appropriate chemical shifts in the complex and elaborate spectra of corresponding AMD analogues (See Table I).

Furthermore, the effects of the substituents at C-7 on the NMR chemical shifts of the vicinal proton in both the model and AMD chromophores are similar. The 8-H and 6-CH_3 signals are shifted upfield, as a result of alkoxy substitution at C-7, with no apparent effect on the 4-CH_3 proton shifts. The 2-NH_2 protons in the model analogues (**1a**, **3a**, and **4a**) behave differently from the corresonding AMD analogues (**1b**, **3b**, and **4b**). In AMD and analogues, the 2-NH_2 protons are hydrogen bonded with β-threonine-NH, as well as the actinocyl carbonyl oxygen, resulting in a downfield shift (7.08–7.09 ppm) compared with the 2-amino protons in the model analogues (5.61–5.39 ppm). This strong hydrogen bonding in AMD also stabilized the 2-NH_2 protons from the long-range inductive effect of 7-substitution in AMD analogues **3b** and **4b** but not in the model derivatives. In **1a**, **3a**, and **4a**, these protons are found to be shifted relatively upfield (from 5.61 to 5.39 ppm) as a consequence of the lack of the abovementioned hydrogen bonding. Another long-range effect of the presence of hydrogen bonding in the 2-NH_2 group is reflected in the chemical shift of the 8-proton in AMD analogues. In the model series **1a**, **3a**, and **4a**, the 8-proton is located upfield (0.25–0.33 ppm) relative to AMD and analogues (**1b**, **3b**, and **4b**). A similar observation was reported on the chemical shifts of this proton in actinomycin D and 2-deaminoactinomycin D, apparently for the same reason.[14]

It should be pointed out that the analogues **3b** and **4b** have a new center of chilarity at th C-2 carbon. Our reported study deals with the racemic mixture of each of these analogues.

CD Spectra. The circular dichroic spectra of **3b** and **4b** in Figure 1 show that **3b** and **4b** are almost identical in the electronic nature of the chromophores, as well as

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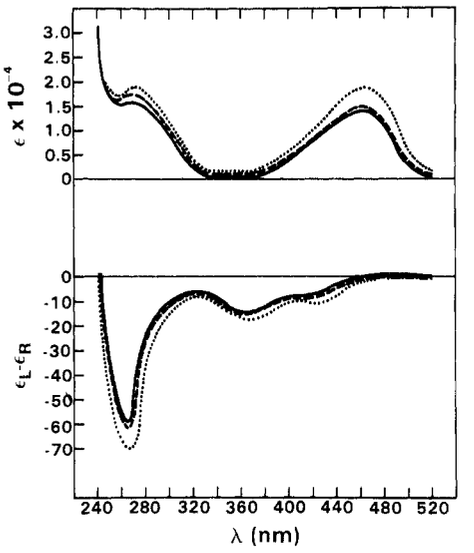

Figure 1. Absorption (top) and circular dichroism (bottom) spectra in 0.01 M phosphate buffer (pH 7) containing EDTA (10^{-5} M) and Me_2SO (5%): **3b** (···), **4b** (- - -), **5b** (—). Concentration of drug in each case was 1.0×10^{-5} M.

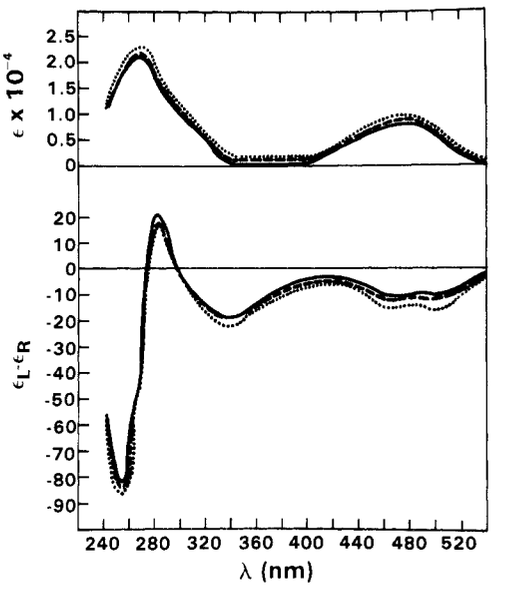

Figure 2. The absorption (top) and CD spectra of the DNA (P)–drug complex (ratio 10:1). Concentrations, buffer, and line types are as in Figure 1.

in the peptide conformations. In this respect, they are almost indistinguishable from the known analogue 7-methoxyactinomycin D (**5b**), whose structure and physical properties we have previously reported.[11] The absorption spectra of all these analogues in aqueous buffer are also almost superimposable (Figure 1). The differences in the extinction values in the absorption spectra between the analogues **3b** and **4b** and 7-methoxyactinomycin D (**5b**) are simultaneously exhibited in the CD spectra of the respective analogues. The absorption spectrum of the analogues above 240 nm is mainly due to the electronic configuration of the tricyclic chromophore. The CD spectrum in the same wevelength region is an indicator of peptide conformation in actinomycin analogues, because it is the peptide lactone ring that confers optical activity on the nearly planar chromophore.[15] The nature of the CD spectra (**3b**, **4b**, and **5b**) asserts that in these analogues there is an identical relationship between the chromophores and peptide lactones. This relationship is very important, because during binding to DNA, AMD and some analogues are known to rely on the cooperative role between the chromophore and the peptide lactones for their highly specific DNA-binding property.[16]

Any change in the configurations of the peptide lactones on binding to DNA is generally expressed in the CD spectra of the analogue–DNA complexes. Simultaneously, the change in the electronic configuration of the chromophore is exhibited by the bathochromic and hypochromic shifts of the visible absorption maximum of the chromophore.[15] The changes in the visible absorption spectra of **3b**, **4b**, and **5b** and also their CD spectra observed after adding calf thymus DNA [mole ratio of DNA (P)/analogue = 10:1] are identical (Figure 2).

These changes are closely parallel to those observed in this spectra of 7-nitro- and 7-aminoactinomycin D analogues reported by us previously.[15] We found that the analogues, i.e., 7-nitro- and 7-aminoactinomycin D, bind to DNA in exactly the same way as AMD, i.e., by intercalation of the chromophore and concomitant hydrogen and hydrophobic interactions of the peptide lactones. The similarity of these characteristics in the absorption and CD spectra of **3b**, **4b**, and 7-methoxyactinomycin D (**5b**) with those of AMD strongly suggests similar DNA-binding modes.

Thermal Denaturation of DNA. Thermal denaturation studies were carried out according to the procedure described by us previously.[10] The ΔT_m values in Table II give a measure of stabilization of DNA helical structure as a consequence of drug binding. These values are derived at high temperature (67–75 °C) and may not always correlate with the results of other DNA-binding studies (e.g., absorptiometric or circular dichroic). The ΔT_m data for the AMD analogues show that binding of all these analogues to the double helices of DNA effect an almost uniform stabilization, except for **3b**, which gives a very broad and sometimes erratic ΔT_m value, possibly as a result of alkylation and covalent binding and scission of the DNA chain. In **3b**, when it is bound to DNA, the active epoxy function may cause DNA fragmentation at the high temperature of the experiment. The compounds **1a**–**4a** do not appear to bind to DNA (either by T_m or by absorption spectra). The broad melting profile of the **3b**–DNA complex compared with the sharp and smooth profiles for the melting profiles of **1b**, **2b**, and **4b** complexes of DNA is an indirect evidence for covalently bound **3b** with DNA. Similar broad melting profiles for covalently bound DNA–drug complexes have been recorded.[17]

Biological Activity. The analogues were assayed for in vitro growth-inhibitory activity against human lymphoblastic leukemic cells (CCRF-CEM) in the log phase,[18] and the results are reported in Table II. The assay is highly sensitive for AMD and its analogues, and it provides relative cytotoxicity values for these agents.

Table II shows that **3b** is about sevenfold more cytotoxic and **4b** is about 1.5 times less cytotoxic than AMD. In comparison, the model analogues **3a** and **4a** are more than 1000-fold less toxic compared to AMD and its analogues. It would appear that the epoxy function alone does not

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Table II. Effect of Actinomycin D Analogues and Model Derivatives on T_m of DNA. In Vitro Growth-Inhibitory Activity

compd	T_m,[a] °C	in vitro (CCRF-CEM)[b] ID_{50}, ng/mL
1b	7.1 ± 0.15	60
2b	6.7 ± 0.15	950
3b	6.0–4.3[c]	9.0
4b	5.1 ± 0.15	85
1a, 2a, 4a	0	~10 × 10³[d]
3a	0	~9000

[a] ΔT_m = T_m of DNA-drug complex minus T_m of DNA. Concentration of drug, 1.4×10^{-4} M; of DNA (P), 1.4×10^{-3} M in 0.01 M phosphate buffer (pH 7.0), 5×10^{-3} M in EDTA and 5% dimethyl sulfoxide. [b] Using human lymphoblastic leukemia cells in log-phase growth. Compounds were dissolved in Me_2SO medium, final growth medium contained 1% Me_2SO. [c] The DNA-melting curves, i.e., change in the OD at 259 nm due to the separation of strands in the DNA double helix, was not smooth and uniform in this instance, indicating major changes in the DNA strands due to drug interaction during the experiment. This is quite different from the very uniform and smooth changes of OD at 259 nm observed with other analogues. [d] Highest dose tested.

account for the extraordinarily high cytotoxicity of **3b**. The model analogue, **3a**, cannot function as a potent growth-inhibitory agent (ID_{50} = 9 μg/mL) when it is a derivative of **1a**, because **1a** has no DNA-binding related activity. This does not imply that the epoxy group in **3b** has no substantial contribution in the biological activity of this analogue. In fact, it does, and the final biological activity of **3b** is a combination of two important factors, the integrety of the active form of actinomycin and the substituent epoxy group that happens to be of the right size and is placed at the right position of AMD[10] in order to retain the DNA-binding property.

In Vivo Antitumor Activity. Studies on in vivo activity of AMD and AMD analogues were carried out with P-388 lymphocytic leukemia in CDF_1 hybrid male mice. The drugs were administered either daily for 4 successive days (qd 1–4) or on days 1, 5, and 9 (q4d 1, 5, 9) beginning 1 day after tumor implantation.[10,12]

Table III shows the results obtained on schedules q4d 1, 5, 9. The analogues **3b** and **4b** at their optimal nontoxic dose levels demonstrate, respectively, about a 2.5- and 1.5-fold increase in survival time over AMD (**1b**) or 7-hydroxyactinomycin D (**2b**). The compounds **1b** and **2b** are approximately equiactive in respect of % ILS, although **2b** needs an over 14-fold higher dose than AMD to effect the equiactivity. In fact, for optimum activity, all the analogues need several fold higher level doses than AMD; therefore, the homogeneity of all the test solutions were ascertained carefully by TLC and HPLC each time the agents were tested. In this test system, compound **3b** shows activity that is superior to AMD in many respects. Its dose–response curve is very broad, as opposed to AMD's dose–response curve, which is very narrow. Additionally, **3b** produces long-term survivors throughout this dose range. Furthermore, at optimal dose ranges of 1.2 and 1.8 mg/kg, it produces cure (tumor free) in three out of seven treated animals.

The therapeutic indexes of both **3b** and **4b** in qd 1–4 and q4d 1, 5, 9 schedules of treatment are calculated and expressed in Table IV. Both analogues are demonstrated to be transformed in vivo to highly polar metabolites, all

Table III. Effects of AMD and Chromophore Substituted AMD Analogues on CDF_1 Mice with P-388 Leukemia (Survival and Cure)[a]

compd	dose, mg/kg	MST,[b] days	% ILS[c]	cure[d]
no drug		11.0		0/15
1b	0.031	13	18	0/7
	0.062	16	45	0/7
	0.125	26	136	1/7
	0.250	17	55	0/7
	0.375	9		
2b	0.15	12	10	0/7
	0.30	17	55	0/7
	0.60	21	89	0/7
	1.2	25	127	0/7
	1.8	26	136	0/7
	3.0	15	37	0/7
3b	0.15	29	164	0/7
	0.30	40	263	1/7
	0.60	41	255	1/7
	1.20	43	291	3/7
	1.80	44	300	3/7
	2.40	26	136	2/7
4b	0.15	16	45	0/7
	0.30	20	82	2/7
	0.60	25	136	1/7
	1.20	32	191	0/7
	1.80	15	36	0/7

[a] Male CDF_1 (18–23 g) mice were inoculated intraperitoneally (ip) with 10^6 P-388 cells on day 0. Drugs were administered, also ip, in 10% dimethyl sulfoxide-saline on days 1, 5, and 9. Test solutions were kept at 0–4 °C, protected from light, and the stability and homogeneity were checked periodically by TLC and HPLC. [b] MST = median survival time. [c] % ILS = percent increase in life span. [d] Over 55 day survivors. Average of three experiments.

of these in the form of conjugates of **4b**. These and other unidentified polar metabolites are excreted at about two to three times the rate of AMD in rat urine. This process of in vivo metabolic transformation and fast excretion appears to play a major role in reducing the in vivo toxicity factor of **3b** and **4b**. A lack of these properties in AMD is known to cause acute and cumulative toxicity in patients. Details of these metabolic experiments will be communicated shortly.[19]

Furthermore, **3b** is an agent that has an alkylating function, i.e., the epoxy group, and also the elements of the actinomycin molecule that contribute to it DNA-binding affinity. Combination of these two attributes in the structure of **3b** may probably also contribute to its efficiency as a DNA alkylating agent in highly proliferating cells.

A molecule like **3b** can be a valuable probe for many biochemical and biological studies. It has a DNA-binding property that is closely parallel to AMD at ambient temperature. It also can bind covalently to DNA at higher temperatures (37 °C or higher) or with a longer period of incubation (~20 h).[19] We are in the process of determining the sequence of bases in DNA at or around the binding site of **3b**.

Experimental Section

Melting points were obtained with a Thomas-Hoover melting point apparatus at a heating rate of 2 °C/min. Column chromatography was accomplished with silica gel powder (Baker No.

(19) S. K. Sengupta et al., unpublished results.

Table IV. Comparison of AMD with Chromophore Substituted AMD Analogues vs. P-388 Leukemia[a]

drug	MED[b]		MTD[c]		therapeutic index: MTD/MED	
	3	4	3	4	3	4
1b	0.0625	0.05	0.25	0.15	4	3
2b	0.275	0.2	4.2	2.0	15	10
3b	0.05	0.0375	3.6	1.6	72	43
4b	0.15	0.125	1.8	1.4	12	6

[a] Drugs administered ip once daily either for 4 successive days (qd 1-4) or on days 1, 5, and 9 (q4d 1, 5, 9), starting 1 day after tumor implantation. [b] MED (minimum effective dose) is the dose (milligrams/kilogram) providing an increase in life span of 40% over control in P-388 tumor-bearing mice. [c] MTD (maximum tolerated dose) is the lethal dose (milligrams/kilogram) for 10% normal CDF_1 male mice (18-23 g); animals observed for deaths during 21 days (LD_{10} = 21 days). Values were calculated from a plot of log dose vs. percent mortality. Average of two to three experiments.

3405, 60–20 mesh) or acid alumina (Woelm grade 1). Limited gel-filtration work was done on Sephadex LH-20, particle size 25–100 μm (Pharmacia Fine Chemicals). Thin-layer chromatography was performed on silica gel plates (E. M. Laboratories, Inc.). Solvent systems used were (A) butanol–formic acid–H_2O (75:13:12), (B) EtOAc–acetone (3:1), and (C) Cifferri, the organic phase of the mixture EtOAc–MeOH–H_2O (20:1:20). High-performance liquid chromatography was carried out on a Varian Model 5020 gradient liquid chromatograph equipped with CD-111L chromatography data system and fitted with Varian reversed-phase C_{18} column with isocratic solvent systems, CH_3CN–5 mM NH_4OAc buffer, pH 6.4 (68:32 or 62:38), pressure 80–140 atm, flow rate 1.5 mL/min, with UV–visible variable and fixed wavelength dual detectors at 254 and 466 nm. Spectra were determined on the following instruments: IR spectra were obtained with a Perkin-Elmer Model 237 Infra Cord with KBr micropellets or chloroform solutions; UV–visible spectra were obtained on a Gilford 250 spectrophotometer, which, with the addition of a base-line reference compensator (Analog Multiplexer 6064) and thermoprogrammer, auto four cell programmer and thermoelectric cell holder 2577, was used to obtain thermal denaturation curves; NMR spectra were obtained on a JOEL FQ-90-90MHZ spectrometer equipped with Fourier transform; and CD spectra were obtained on a Cary 61 spectrophotometer. All elemental analyses were within ±0.4% of the theoretical values. Actinomycin D, batch no. NCS 3053, lot L554651-0-10, was generously provided by Dr. John Douros, Natural Products Branch, National Cancer Institute, Silver Spring, MD. Calf thymus DNA type 1 was purchased from Sigma Chemical Co.

7-(2,3-Epoxypropoxy)- (3b) and 7-(2,3-Dihydroxypropoxy)actinomycin D (4b). General Method. 7-Hydroxyactinomycin D (**2b**; 17 mg, 0.013 mmol) and powdered anhydrous potassium carbonate (8.5 mg) were placed in a tube containing a magnetic stir bar. After addition of dry acetone (4.2 mL) and epibromohydrin (170 μL), the tube was purged with N_2 and stoppered. The mixture was stirred at 50–55 °C for 16 h; the color of the solution turned from purple to orange when the reaction was complete. Filtration, followed by evaporation under N_2, gave an oily residue, which was chromatographed on a silica gel plate (solvent system B). Two major bands were obtained. The faster moving band (R_f 0.39, TLC, solvent B) upon elution with acetone yielded 3.7 mg (20.2%) of pure red solid (**3b**): mp 265–267 °C dec; UV λ_{max} ($CHCl_3$) 464 nm (ε 20100); $[\alpha]^{22}_D$ −406 ± 20° (*c* 0.1, $CHCl_3$); HPLC t_R 15.3 min vs. 17.6 min for 7-methoxyactinomycin D. Anal. ($C_{65}H_{90}N_{12}O_{18}\cdot 2H_2O$) C, H, N.

The slower moving band (R_f 0.28, TLC, solvent system B) yielded 7.4 mg (42.8%) of orange solid **4b**: mp 285 °C dec; UV λ_{max} ($CHCl_3$) 464 nm (ε 19470); $[\alpha]^{22}_D$ −308 ± 20° (*c* 0.1, $CHCl_3$); HPLC t_R 7.5 min vs. 9.3 min for AMD (CH_3CN–5 mM NH_4OAc, 62:38, 1.5 mL/min). Anal. ($C_{65}H_{92}N_{12}O_{19}\cdot 3H_2O$) C, H, N.

Compound 3a: mp 225–227 °C; UV λ_{max} ($CHCl_3$) 466 nm (ε 22100); TLC R_f 0.62 (solvent system B); yield 41%. Anal. ($C_{27}H_{34}N_4O_6$) C, H, N.

Compound 4a: mp 198–202 °C; UV λ_{max} ($CHCl_3$) 467 nm (ε 18500); TLC R_f 0.43 (solvent system B); yield 30%. Anal. ($C_{27}H_{36}N_4O_7$) C, H, N.

Conversion of Compound 3b to 4b. Compound **3b** (2 mg) in 1 mL of tetrahydrofurane was treated with 50 μL of 25% aqueous perchloric acid and let stand at 22 °C for 3 h. The solution was diluted with water (1 mL) and extracted with ethyl acetate (5 × 5 mL). The washed and dried extract was applied on TLC (solvent B), which separated compound **4b**, R_f 0.29 (65%), from 15% of compound **3b**, R_f 0.40, which remained unconverted. Compared with authentic samples by TLC solvent systems A–C, HPLC, and IR (KBr).

Compound 3b: R_f 0.32 (solvent system A), 0.28 (solvent system C).

Compound 4b: R_f 0.49 (solvent system A), 0.13 (solvent system C).

Thermal Denaturation of DNA vs. DNA–Drug Complexes. These experiments were performed according to the methods described by us previously.[10]

Biological Experiments. Determination of LD_{10} Values. Tumor-free CDF_1 male mice weighing 18–23 g were given a broad range of doses of drugs in 10% dimethyl sulfoxide–saline on either days 1, 5, and 9 or on 4 successive days. Drugs were administered intraperitoneally (ip).

The maximum tolerated doses of AMD and analogues that caused deaths in only 10% of the tested animals in 21 days were recorded as MTD. The results are an average of two experiments in each. The MTD values obtained following schedules q4d 1, 5, 9 (three injections) and qd 1–4 (four injections) are recorded in Table IV. These doses are the same as LD_{10} (lethal dose of 10% of tumor-free animals in 21 days).

The results show that on a q4d 1, 5, 9 schedule, MTD doses are 14-fold for **3b**, 7-fold for **4b**, and 17-fold for **2b** of the MTD dose for AMD. Similarly, on the qd 1–4 schedule, **3b** is 11-fold, **4b** is 9-fold, and **2b** is 13-fold less toxic than AMD.

In an additional experiment, two animals at LD_{10} (or MTD) dose levels were sacrificed on day 14, and the organ sections from these animals were evaluated for gross pathology in the following way. Organs, e.g., spleen, liver, kidney, large and small intestine, heart, lung, pancreas, and adrenals, were removed and fixed in 10% natural buffer formalin. They were kept immersed for 2 weeks, tissues were dehydrated through graded alcohols (70, 90, 95, and 100%), cleared in cedarwood oil, and embedded in paraffin. Sections were cut in 7 μm and mounted with gelatin on microscopoe slides. After drying, sections were stained with Mayer's hematoxylin and eosin and examined with light microscopy.

The sections of spleen showed haematopoiesis indicative of bone-marrow toxicity for all the analogues, as well as AMD. However, in other organs, e.g., liver, kidney, large and small intestines, heart, lung, pancreas, or adrenals, no observable toxic effects were in evidence.

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Studies on the Synthesis of Chemotherapeutics. 12.[1] Synthesis and Antitumor Activity of *N*-Phthalidyl-5-fluorouracil Derivatives[2]

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Several *N*-phthalidyl-5-fluorouracil derivatives were synthesized for preliminary antitumor evaluation. N_1-Phthalidyl- and N_3-phthalidyl-5-fluorouracils (**6** and **10**) showed a significant antitumor activity against experimental solid tumors with a good blood level of 5-fluorouracil in mice, even for oral administration.

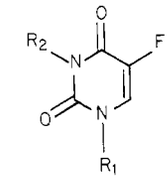
5-Fluorouracil (5-FU, **1**)[3] and its masked form, N_1-(2-tetrahydrofuryl)-5-fluorouracil (tegafur, **2**)[4] have been widely used in cancer chemotherapy, but they have been shown to possess a high toxicity and poor tumor affinity. Chemical modification of **1** and **2** has been extensively studied to develop more effective antitumor substances with fewer toxic side effects.[5]

In connection with our synthetic work on chemotherapeutics, we previously reported a novel synthesis of **2**[6] and a synthesis of numerous *N*-acyl- and *N*-(alkoxycarbonyl)-5-fluorouracil derivatives (**3**).[7] Of these, N_1-acetyl-N_3-*o*-toluyl-5-fluorouracil (**4**) appeared to be the most promising antitumor agent. Incidentally, in recent advances of prodrug formation of therapeutic agents, talampicillin, a phthalidyl ester of ampicillin, was found to show an improved bioavailability with an approximately twice as high serum concentration of ampicillin as the lead compound.[8]

These findings stimulated us to intend the further development of new 5-fluorouracil derivatives by the introduction of a phthalidyl group into the N_1 and/or N_3 position of **1**, **2**, and *N*-acyl-5-fluorouracils (**9** and **13**). In this paper, we report the synthesis and preliminary evaluation of the antitumor activity of novel *N*-phthalidyl-5-fluorouracil derivatives (**5**) (Chart I). Among these compounds, N_1- and N_3-phthalidyl-5-fluorouracil (**6** and **10**) appeared to retain the same level of antitumor activity toward solid experimental tumors as that of **2** following oral administration.

Chemistry. For the preparation of N_1-phthalidyl-5-fluorouracil (**6**), N_3-benzoyl-5-fluorouracil (**7**)[7] was reacted with 3-bromophthalide[9] in the presence of sodium hydride in DMF to give N_3-benzoyl-N_1-phthalidyl-5-fluorouracil (**8**). Solvolysis of **8** under a protic condition (AcOH–EtOH) afforded the desired compound **6**. In a similar way, the reaction of N_1-acetyl-5-fluorouracil (**9**)[5m] with 3-bromophthalide, followed by hydrolysis of the resulting N_1-acetyl-N_3-phthalidyl-5-fluorouracil (**11**) under an acidic condition (0.05 N HCl-EtOH), gave N_3-phthalidyl-5-fluorouracil (**10**). Compound **11** was alternatively prepared by acetylation of **10** with acetic anhydride.

Chart I



1, $R_1 = R_2 = H$
2, R_1 = i, $R_2 = H$
3, R_1, R_2 = H, acyl, i, or alkoxycarbonyl
4, $R_1 = COCH_3$, R_2 = *o*-toluyl
5, R_1, R_2 = H, acyl, i, or phthalidyl
phthalidyl = 1,3-dihydro-3-oxo-1-isobenzofuranyl (ii)

i

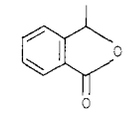


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Table I. *N*-Phthalidyl-5-fluorouracil Derivatives[a]

no.	method[b]	yield %	mp, °C (recrystn solvent)	formula[c]
6	C	10	292–296 dec	$C_{12}H_7FN_2O_4$
	D	54	(MeOH–AcOEt)	
8	A	59	207–210 (AcOEt)	$C_{19}H_{11}FN_2O_5$
10	E	50[d]	234–237 (MeOH)	$C_{12}H_7FN_2O_4$
11	A	72	187–190 (AcOEt)	$C_{14}H_9FN_2O_5$
12a	C	77[e]	255–258 (AcOEt)	$C_{20}H_{11}FN_2O_6$
12b			249–252 (AcOEt)	$C_{20}H_{11}FN_2O_6$
14	A	74	154–157 (AcOEt)	$C_{16}H_{13}FN_2O_5$
15	B	75	199–202 (AcOEt)	$C_{20}H_{13}FN_2O_5$

[a] All compounds were obtained as colorless crystals. [b] Capital letters refer to synthetic method A–E under Experimental Section. [c] Analyzed values for C, H, and N; analytical results were within ±0.4% of the theoretical values. [d] Yield from compound **9**. [e] Total yield of diastereomers (**12a** and **12b**).

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(1) Part 11: T. Kametani, K. Kigasawa, M. Hiiragi, K. Wakisaka, S. Haga, H. Sugi, K. Tanigawa, Y. Suzuki, K. Fukawa, O. Irino, O. Saita, and S. Yamabe, *Heterocycles*, **16**, 1205 (1981).

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