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2-Deaminoactinomycin D, Synthesis and Interaction with Deoxyribonucleic Acid

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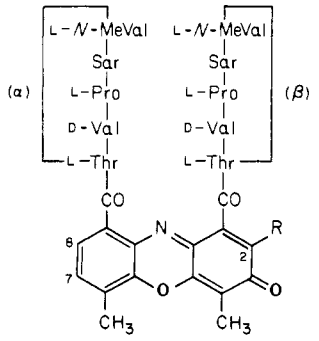
2-Deaminoactinomycin D has been synthesized and characterized. It binds to DNA by intercalation according to NMR, CD, thermal denaturation, and unwinding studies on the drug–DNA complex. Loss of the 2-amino group does not seriously affect binding parameters relative to actinomycin D; affinity for calf thymus DNA may even be increased, according to ΔT_m measurements. The unwinding of circular DNA caused by this compound is at least as large as that effected by actinomycin D and ethidium bromide. Nevertheless, 2-deaminoactinomycin D is less effective than actinomycin D in inhibiting nucleic acid syntheses in L1210 cell culture and in in vivo antitumor activity against P388 leukemia.

Actinomycin D (**1**), an antiobiotic with antitumor activity limited to a few specific tumors,[1-3] has been extensively studied, not only to effect modifications to improve its therapeutic index but also to clarify its mode of action as an effective drug. It has been established that actinomycin inhibits DNA-dependent RNA synthesis[4-8] and also, to a lesser degree, DNA synthesis, apparently by forming an intercalation complex with DNA.[9-11]



1, R = NH_2
2, R = OH
3, R = Cl
4, R = H

Derivatives of **1** in which the 2-amino group on the chromophore has been replaced by OH (**2**), Cl (**3**), or mono- or disubstituted amines (NRH or NR_2) have been nearly or completely inactive biologically.[12-16] It has been assumed therefore that the 2-amino group is required for DNA binding and biological activity.[17] However, the 2-deamino analogue **4** has not been prepared and studied. [The material referred to in early literature[12,16] as "de(s)aminoactinomycin" is actually described as 2-deamino-2-hydroxyactinomycin. The product reported by Moore et al.[13] might be 2-deamino-2-chloroactinomycin.] In this paper we are reporting the synthesis of **4** and certain characteristics of its complex with DNA.

Conversion of **1** to **3**, via intermediate **2**, was carried out according to reported procedures.[12,13,16] The product **3** showed an absorption spectrum almost identical with that of "chloroactinomycin C_3" reported by Brockmann[12] but different from the spectrum reported by Moore et al.[13] The latter group also indicated that the melting point of their product was 30 °C lower than ours. Movement on thin-layer chromatographic plates was also somewhat different from our results.

Although 2-deamino-2-chloroactinomycins C_2 and C_3 have been treated with a variety of amines to give *N*-alkyl analogues,[14,15] we found that displacement of the chlorine in **3** by catalytic hydrogenation proceeded very slowly. The course of the reaction was followed by monitoring the

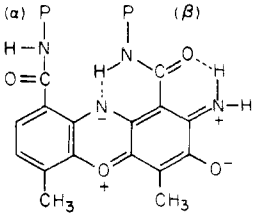
Table I. NMR Spectra of Actinomycin and Derivatives[a]

Group	Chemical shift, ppm		
	Compd 3	Compd 4	Compd 1[b]
-NH (Val)	8.66	8.58	8.10
	8.58	8.50	7.94
-CH (aromatic 8-H)	7.26	7.33	7.64
(aromatic 7-H)	7.45	7.47	7.37
=CH (quinoid 2-H)		7.02	
-NH (Thr)	6.74	6.81	7.84
	6.64	6.71	7.21
-CH (Pro)	6.31	6.24	6.03
	6.24	6.18	5.98

[a] 100-MHz spectrum of $CDCl_3$ solutions; chemical shifts in parts per million (δ) to low field from internal Me_4Si. [b] Data from ref 18 and Figure 1.

intensity of the signal, assigned to the resonance of the 2-H, at about 7 ppm in the NMR spectrum and by elemental chlorine analysis. Complete reaction was accomplished only after heating in the presence of an acid acceptor and a large excess of palladium black for many hours.

NMR Spectra. Portions of the NMR spectra of **1**, **3**, and **4** are shown in Figure 1. Table I lists the chemical shifts. Assignments were based on similarities with the spectrum of **1**,[18] on D_2O exchange experiments, and on the differential broadening of the aromatic protons. The broader aromatic signal has been assigned to the 7-H because of coupling to the 6-methyl protons. The signal at 7.02 ppm in Figure 1 (b), absent in the spectra of **1** and **3** [Figure 1 (a and c)], is assigned to the 2-H of the chromophore in **4**. In addition, there are other important differences between the spectra of **1** and **4**. In general, the spectra indicate that the two peptide lactone rings are more alike in their conformations in both **3** and **4** than in **1**. There is an accompanying significant downfield shift of the Val-NH signals. These facts indicate that while the peptide conformation seems to be altered, the twofold pseudosymmetry axis and the hydrogen bonds between the D-Val-NH of one ring and the D-Val-carbonyl of the other ring as found in **1**, established by x-ray analysis[10] and NMR spectral studies,[19] are retained on removal of the 2-NH_2. That the spectra of **3** and **4** are more similar to each other than to that of **1** may indicate that the 2-NH_2 has a function in stabilizing a particular peptide conformation. The large upfield shift of the β-Thr-NH proton resonance of **3** or **4**, compared to **1**, may provide a clue to the role of the 2-NH_2 group. Müller and Crothers[9] have suggested an important contribution of the resonance structure **5** to the specificity of actinomycin for G–C base pairs. As shown in **5**, the charge distribution predicted by this structure would favor hydrogen bond formation between the ring nitrogen and the β-Thr-NH, as well as between



5, P = peptide rings as in 1

the carbonyl oxygen (linking the β-pentapeptide lactone to the chromophore) and the 2-NH_2. Such structures are definitely observed in model compounds.[19] The amide group in actinomycin is not in the plane of the chromophore as shown in **5**; however, interactions analogous to those shown in **5** would provide a torque, tending to twist

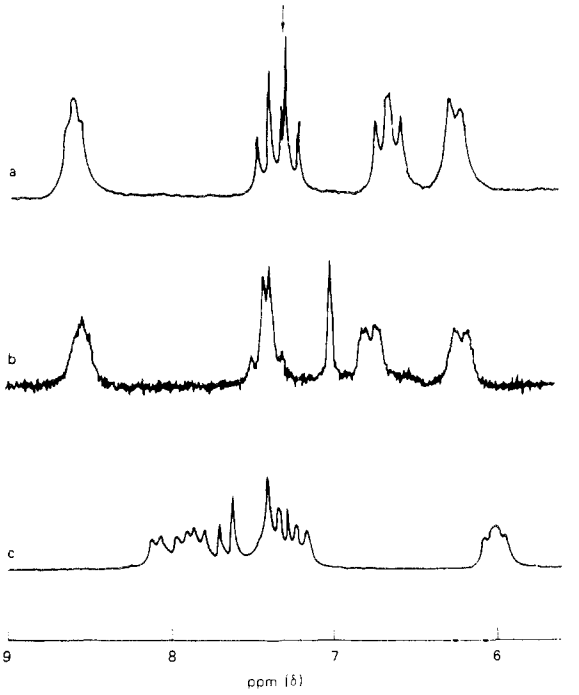


Figure 1. Portion of NMR spectra in $CDCl_3$ at ambient temperatures. Chemical shifts are in parts per million (δ) to low field from internal Me_4Si and were recorded using Varian XL-100-15 spectrophotometer: (a) 2-deamino-2-chloroactinomycin D (**3**) (the arrow indicates a small amount of benzene, present as impurity); (b) 2-deaminoactinomycin D (**4**); (c) actinomycin D (**1**).

the plane of the β-chromophore threonyl amide group closer to the phenoxazone plane, and would be expected to result in a downfield shift for the Thr-NH protons, with a larger shift for those in the ring adjacent to the -NH_2 on the chromophore. When the amino group is replaced by Cl or H, this resonance structure is no longer possible, and the Thr-NH signals shift upfield and are closer together.

An unexpected effect that is difficult to explain is the fairly large influence of the 2-substituents on the chemical shift of the 8-proton in the aromatic ring of the chromophore. This shift may reflect a sizable change in the dihedral angle between the phenoxazone ring system and the chromophore threonine amide groups.

CD Spectra. The circular dichroism spectrum is an indicator of peptide conformation in actinomycin analogues, because it is the peptide lactone rings which confer optical activity on the almost planar chromophore.[20,21] The CD spectra of **3** and **4**, shown in Figure 2, confirm the conclusion drawn from the NMR spectra—that in these two analogues there is a very similar relationship between the peptides and the chromophore. The CD spectrum of **1** is also shown in Figure 2, but we have been unable to use it to analyze differences in conformation between **1** and **3** or **4** because the absorption spectra of these compounds are very different, especially in the visible region. In the far-UV, where the absorption spectra are similar, the CD spectra are reasonably similar. The *changes* in the CD spectra of **3** and **4** observed after adding calf thymus DNA [mole ratio of DNA (P)/drug = 10/1] are somewhat analogous to those for **1** (Figure 3). These difference CD spectra were obtained by subtracting the CD spectrum of each compound plus that of DNA from the spectrum of the DNA–drug complex. In each case a strong positive peak is induced in the region of 275 nm; in the spectrum of the drug alone, a negative peak appears in this same region. Above 350 nm the negative bands are enhanced in intensity and shift to longer wavelengths. The similarity

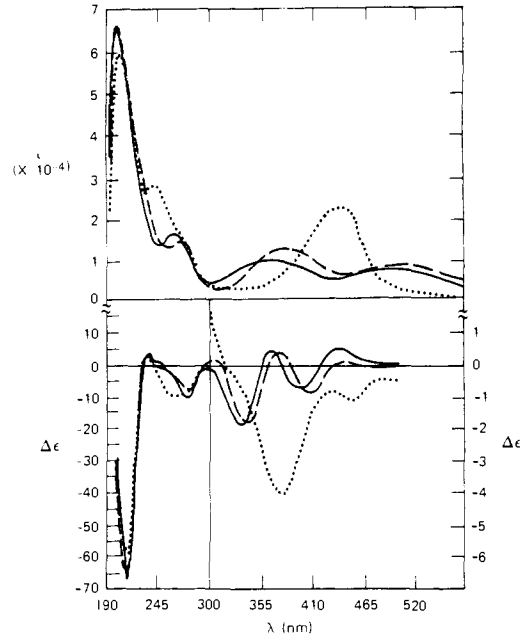


Figure 2. Absorption (top) and circular dichroism (bottom) spectra in 0.01 M phosphate buffer (pH 7) containing EDTA (10^{-5} M) and Me_2SO (5%): actinomycin D (···), 2.68×10^{-5} M; **3** (- - -), 9.32×10^{-6} M; **4** (—), 8.83×10^{-6} M.

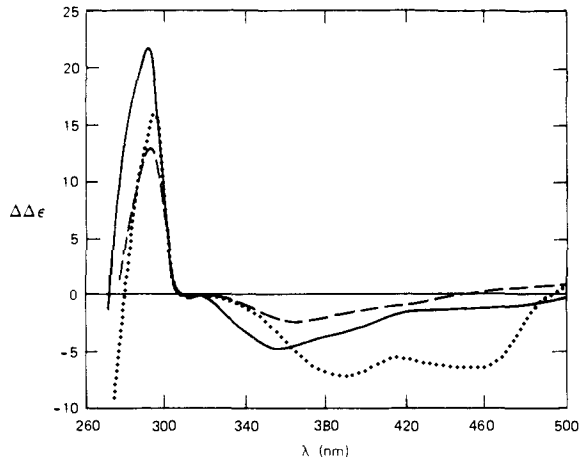


Figure 3. Difference CD spectra of DNA (P)–drug complex (ratio 10:1); the CD spectra of DNA and of drug were subtracted from that of the complex by computer. Solvent and symbols as in Figure 2. Concentration of drug in each case was 3×10^{-5} M.

in the induced dissymmetry indicated by the spectra of Figure 3 suggests similar DNA-binding modes for the three compounds.

Thermal Denaturation of DNA. Formation of an intercalation complex generally increases the stability of the DNA helix relative to the denatured chain, thus increasing the thermal denaturation temperature as measured by the temperature at which the hypochromicity at 259 nm is reduced by half (T_m). The data in Table II show that actinomycin D, under conditions described in the Experimental Section, increases the T_m of calf thymus DNA by 7.1 °C. The ΔT_m value obtained for analogue **3** is 1.6 °C, indicating weaker DNA binding relative to **1**. The ΔT_m value for analogue **4** is 9.9 °C, indicating stronger complexing with DNA.

Nucleic Acid Synthesis Inhibition. The abilities of **2**, **3**, and **4** to inhibit synthesis of DNA and RNA in cultured L1210 cells have been determined and are compared with that of actinomycin in Table II. The very low values for **1** (ED_{50} 0.4 μM for DNA, 0.009 μM for RNA)

Table II. Effect of Actinomycin and Derivatives on T_m of DNA and on Nucleic Acid Syntheses

Compd	ΔT_m, °C[a]	Inhibn of nucleic acid syntheses,[b] ED_{50}, μM	
		DNA	RNA
1	7.1	0.4	0.009
2	0	>100	24
3	1.6	86	13
4	9.9	63	0.7

[a] ΔT_m = T_m of DNA-drug complex minus T_m of DNA. Concentration of drug, 5.2×10^{-6} M; of DNA (P), 5.2×10^{-5} M in 0.01 M phosphate buffer (pH 7), 10^{-5} M EDTA, and 5% Me_2SO. [b] Using actively growing L1210 cells in growth medium containing 1% Me_2SO.

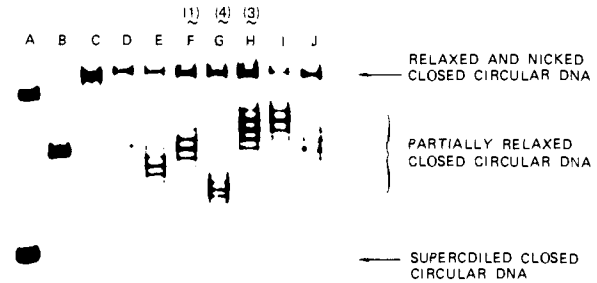


Figure 4. Electrophoretic patterns of plasmid DNA (pBR313) relaxed with *E. coli* ω enzyme in the presence of intercalating drugs. All experiments were done under identical conditions with 40×10^{-12} mol of drug and 1.10 μg of DNA, as described in the Experimental Section. Samples A–J are as follows. (A) The plasmid pBR313 as isolated from *E. coli* strain RR1. The preparation contains some pBR313 dimer. (B) The plasmid pBR313 treated with the restriction endonuclease *Eco* R1. Since the plasmid contains a single cleavage site for this nuclease, a linear molecule is formed. (C) The plasmid pBR313 treated with the *E. coli* ω enzyme. (D) A mixture of partially relaxed pBR313 closed-circular DNAs. (E) The plasmid pBR313 treated with the *E. coli* ω enzyme in the presence of ethidium bromide. (F) The same experiment as E but in the presence of actinomycin D (1). (G) The same experiment in the presence of 2-deaminoactinomycin D (4). (H) The same experiment in the presence of 2-deamino-2-chloroactinomycin D (3). (I) The same experiment in the presence of daunomycin. (J) Same as D.

confirm its strong inhibitory activity, particularly toward RNA synthesis. Although both **3** and **4** show less than $^1/_{100}$ of the potency of **1** as inhibitors of DNA synthesis, **4** is considerably more effective (ED_{50} 0.7 μM) than is **3** (ED_{50} 13 μM) in inhibiting RNA synthesis. Compound **2** is a much less effective inhibitor. The ratio of ED_{50} values for DNA/RNA synthesis for **4** is roughly comparable to the corresponding ratio for **1**.

In Vivo Antitumor Activity. Preliminary tests on the antitumor properties of **4** against P388 lymphocytic leukemia in the mouse were kindly performed by Dr. Randall K. Johnson of the Division of Cancer Treatment, NCI. On the QD 1, 5, 9 schedule, **4** provided an increased life-span (ILS) for tumor-bearing mice of 92% at 15 mg/kg, the highest dose tested. The lowest dose at which activity was found was 1.94 mg/kg (ILS = 46%). No toxicity, as indicated by weight loss of treated animals, was evident at any dose of **4** tested. Actinomycin D was approximately as effective as **4** at a dose of 0.108 mg/kg (ILS = 100%) but showed better antitumor effects at higher, but toxic, doses.

Relaxation of Supercoiled Closed Circular DNA. Using a procedure similar to that of Keller,[22] we have qualitatively compared the unwinding produced by actinomycin D and its derivatives with that produced by ethidium bromide. This involves closed circular DNA that

is nicked and ligated with DNA-relaxing enzyme in the presence of the drug, followed by agarose gel electrophoresis. The experiment shown in Figure 4 demonstrates that the unwinding effected by 1 is slightly less than that produced by ethidium bromide (E). Using other techniques, Waring[23] and Wang[24] concluded that the unwinding of the DNA helix caused by actinomycin D was equal to or slightly less than that produced by ethidium bromide, upon intercalation between the base pairs of DNA.[25] At that time, the unwinding angle caused by ethidium bromide was considered to be 12°; subsequently this value has been determined to be 26 ± 2° at each intercalation site.[22,26] The unwinding produced by 4 is slightly greater than that of 1, while 3 produces considerably less unwinding under the same conditions. Daunomycin (I) provides a further comparison of unwinding angles; its unwinding angle is considerably smaller (about 10 ± 2°) than that of ethidium bromide or 1, in agreement with results determined by other methods.[11]

Conclusions

The structure of 2-deaminoactinomycin (4) has been established by NMR spectra and elemental analyses, with a suggested conformation similar to, but not identical with, that of actinomycin D (1). Measurements of the effects of 1 and 4 on the ΔT_m and the unwinding of DNA strongly support similar intercalative binding mechanisms; the higher values caused by 4 suggest that it may form a slightly more stable complex with DNA than does 1, perhaps by relieving some conformational restrictions caused by hydrogen bonding between cyclic peptide and 2-amino group in 1. The smaller ΔT_m, unwinding values, and CD effects caused by 3 are consistent with the theory that the 2-chloro group reduces the extent of intercalation because of steric effects. In the CD spectra, the DNA complexes of 1 and 4 show a similar pattern of enhanced peaks with slight shifts, relative to 1 and 4, respectively, indicating that the asymmetry induced by complexation is similar. The different electronic properties of the respective chromophores do not permit direct comparisons.

A question is raised when one considers the relatively poor antitumor activity and ability of 4 to inhibit nucleic acid synthesis (less that $^1/_{100}$ that of 1) vs. its apparently enhanced ability to bind to DNA. The answer may lie in the differences in the details of conformations of complexes of DNA with 1 and 4. The differences in peptide–lactone conformations between 1 and 4 may dictate differing dissociation rates in the presence of DNA or RNA polymerase.[9] Perhaps the intercalated 4 is more easily displaced by the advancing enzyme than is 1, resulting in greater inhibitory activity of 1. The relative inhibition of DNA vs. RNA synthesis (0.4 vs. 0.009 for 1 and 63 vs. 0.7 for 4) is maintained and may be due to specific interactions of the peptide with RNA polymerase.[9]

Experimental Section

Melting points, uncorrected, were determined on a Fisher-Johns apparatus. Dry-column chromatography was accomplished using Woelm silica gel, activity III, and thick-layer chromatography on 2-mm silica gel plates. Solvent systems for thin-layer chromatography: (A) *s*-BuOH–HCO_2H–H_2O (75:13.5:11.5) (B) EtOAc–acetone (2:1). Spectra were determined on the following instruments: NMR, on a Varian XL-100-15 spectrometer equipped with Fourier transform; CD, on a Durrum-Jasco ORD/UV spectrophotometer equipped with a Sproul Scientific SS-20 CD modification; UV–visible spectra and thermal denaturation curves, on a GCA-McPherson digital double-beam, ratio-recording spectrophotometer (EU-707-K), equipped with EU-707 SA automatic sample positioner, Lauda K-2/R constant temperature bath, Lauda R20 digital temperature regulator, and Brinkmann P-120 programmer.

The organization and analysis of the data base associated with this investigation were carried out using the PROPHET system, a unique national computer resource sponsored by the NIH.[27] Information about PROPHET, including how to apply for access, can be obtained from the Director, Chemical/Biological Information-Handling Program, Division of Research Resources, National Institutes of Health, Bethesda, Md. 20014.

The *Escherichia coli* ω enzyme preparation was provided by J. C. Wang and R. Depew. The strain pBR313 (RR1) was provided by H. Boyer. Photography of the ethidium bromide stained gel was done using a long wavelength transilluminator (Utraviolet Products) and a Minolta SLR with Kodax Plus-X film, through UV haze and 0–2 filters.

Calf thymus DNA, purchased from Miles Laboratories, was dissolved in 0.01 M phosphate buffer, pH 7.0, to give an approximately 4×10^{-3} M solution, which was filtered through a prewashed Celite pad on a sintered glass filter; the exact concentration was determined by measuring absorbance at 259 mm (ϵ 6800). Aliquots were stored frozen and thawed just prior to use.

Thermal Denaturation Values. An approximately 2.6×10^{-4} M drug solution was made by dissolving the necessary weight of drug in 0.5 mL of dimethyl sulfoxide and diluting to 5.0 mL with 0.01 M phosphate buffer, pH 7.0. An exact, calculated volume of this drug solution was mixed with 5.0 mL of 1.04×10^{-4} M DNA (molarity based on P) and 0.1 mL of 1×10^{-3} M EDTA in 0.01 M phosphate buffer and then diluted to 10.0 mL with 10% Me_2SO in 0.01 M buffer solution. Thus, concentrations in the final solution were 1×10^{-5} M EDTA, 5.2×10^{-6} M drug, and 5.2×10^{-5} M DNA (P) in 0.01 M phosphate buffer containing 5% Me_2SO.

Four solutions were placed in four cuvettes of 1-cm path length in a jacketed holder at room temperature. The temperature was rapidly raised to 50 °C and the solutions were allowed to reach thermal equilibrium (15–30 min). Any bubbles that formed on cuvette walls were shaken out. The temperature program, set at 18 °C/h, was started and the absorbance of each solution and the temperature in cell holder jacket were recorded, until absorbance values became almost constant. The T_m value was calculated by a computer program that includes corrections for volume expansion, temperature lag, and baseline. ΔT_m = T_m of drug–DNA complex – T_m of DNA solution.

L1210 Nucleic Acid Synthesis Inhibition Assay. This assay was performed as described previously[28] except that the compounds to be tested were dissolved in a calculated amount of Me_2SO and then diluted with the growth medium so that the final concentration of Me_2SO in the cell–drug mixture was 1%.

Closed Circular DNA. The plasmid-containing *E. coli* strain pBR313 (RR1) (mol wt 5.8×10^6 daltons) constructed and described by Bolivar et al.[29] was a convenient source of closed circular DNA. The bacteria was grown and a cleared lysate prepared according to the procedure of Kupersztoch-Portnoy et al.[30] Following ethanol precipitation of the DNAs, plasmid DNA was isolated by gel filtration on agarose A50.[29] Although the preparation contained several plasmid species, cleavage with *Eco* RI endonuclease[30] produced a single DNA fragment.

Relaxation of Superhelical Closed Circular DNA in the Presence of Drugs. To a solution containing 1.10 μg of plasmid DNA (pBR313) in 28 μL of potassium phosphate buffer (80 mM, pH 7.6) containing $MgCl_2$ (3 mM) and EDTA (1 mM), 40×10^{-12} mol of drug was added. After this solution had been incubated at 37 °C for 10 min, 2 μL of solution containing approximately 300 units of *E. coli* ω enzyme[31] was added and incubation continued for 30 min. The reaction was terminated by the addition of 20 μL of 0.05 M EDTA followed by 20 μL of distilled phenol, freshly extracted with phosphate buffer. The sample, after two extractions with phenol and two with chloroform, was subjected to electrophoresis through a 0.4-cm slab gel of 0.7% agarose, as described previously.[32,33] The slab gel, after electrophoresis, was stained with ethidium bromide and then photographed while exposed to UV light (long wavelength UV transilluminator). The Boltzmann distribution of bands produced by the partially relaxed closed circular DNAs[34] was compared for each drug.

2-Deamino-2-chloroactinomycin D (3). Conversion[12,13,16] of 1.0 g of **2** yielded, after removal of chloranil, 1.14 g of crude 3 that showed several impurities on TLC. Purification on a 300-g silica

dry column, using ethyl acetate–methanol (2:1) as eluent, showed a broad red band on the column. The center portion of this band was cut out and extracted with acetone, and after removal of the solvent, the residue was precipitated from benzene solution with hexane to give 145 mg of red solid. The outer portions of the red band were similarly extracted and further purified on thick-layer chromatographic plates to give 368 mg of product: mp 262–263 °C; total yield, 415 mg (51%); TLC R_f 0.87 (A), 0.44 (B); UV max (CH_3OH) 260 nm (ϵ 14900), 273 (infl) (11850), 375 (14600), 483 (7480); UV max (0.01 M phosphate, pH 7, containing 10^{-5} M EDTA, 5% Me_2SO) 197 nm (ϵ 50429), 264 (15450), 381 (12553), 509 (8154). Anal. ($C_{62}H_{84}N_{11}O_{16}Cl \cdot 5H_2O$) C, H, N, Cl.

2-Deaminoactinomycin D (4). A solution of 118 mg of **3** in 10 mL of ethanol containing 120 μL of triethylamine was hydrogenated (1 atm) over 250 mg of Pd black at 75–85 °C for 32 h. Catalyst was removed from the colorless solution by filtering through Celite into a stirred solution of 100 mg of $K_3Fe(CN)_6$ in 3 mL of phosphate buffer, pH 7; after 15 min, the mixture was diluted with water and extracted several times with ethyl acetate. The extracts, washed and dried, were concentrated to give 95 mg of red solid residue, which was dissolved in ethyl acetate and precipitated with hexane, yielding 65 mg (57%) of product: mp 250–252 °C; TLC R_f 0.85 (A), 0.46 (B); UV max (CH_3OH) 257 nm (infl) (ϵ 16500), 372 (9940), 477 (6240); UV max (0.01 M phosphate, pH 7, containing 10^{-5} M EDTA, 5% Me_2SO) 196 nm (ϵ 49830), 261 (15860), 357 (9620), 504 (7360). Anal. ($C_{62}H_{85}N_{11}O_{16} \cdot 4H_2O$) C, H, N (% Cl found to be 0.0%).

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Synthesis and Antiallergic Activity of 2-Hydroxy-3-nitro-1,4-naphthoquinones[1]

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A selection of novel 2-hydroxy-3-nitro-1,4-naphthoquinones are shown to be potent inhibitors of rat passive cutaneous anaphylaxis (PCA) and to have highest potency with alkyl substitution at both C-6 and C-7. The most potent compounds were **7c** and **7e** which produced a 50% inhibition in the rat PCA test at doses of about 10 μM/kg following subcutaneous administration and showed activity after oral administration. Related 4-hydroxy-3-nitro-2(1*H*)-naphthalenones had no effect on rat PCA in doses up to 500 μM/kg.

2-Nitroindan-1,3-diones, **1**, and notably the 5,6-dimethyl derivative[2] have shown potent inhibition of the rat IgE mediated passive cutaneous anaphylaxis reaction in the rat (rat PCA).[3] Some of these compounds, moreover, will inhibit this reaction following oral administration. Our investigations into the limiting structural requirements for activity in the PCA test have led to other active classes of 2-nitro-1,3-dicarbonyl compounds of type **2** in which X may represent any one of the heteroatoms, oxygen,[4a] nitrogen,[4b] or sulfur.[4c]