# $N^2$- and C-7-Substituted Actinomycin D Analogues: Synthesis, DNA-Binding Affinity, and Biochemical and Biological Properties. Structure–Activity Relationship

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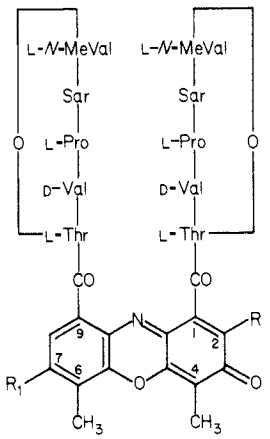
$N^2$-*n*-Alkyl- and ω-amino-*n*-alkylactinomycin D and 7-alkoxy-, 7-aralkoxy-, and 7-(acyloxy)actinomycin D were synthesized by modification of the parent actinomycin D molecule at the $N^2$ and C-7 positions of the phenoxazinone moiety. The intermediate for $N^2$ substitution was 2-deamino-2-chloroactinomycin D. For C-7 substitution, 7-hydroxyactinomycin D was used as the intermediate. Treatment of 2-deamino-2-chloroactinomycin D with an excess of the appropriate amine produced the $N^2$-substituted derivatives. Condensation of the required alkyl or acyl halides with 7-hydroxyactinomycin D, aided by solid anhydrous potassium carbonate, yielded the C-7-substituted analogues. Calf thymus DNA-binding affinity was determined by equilibrium binding and also by thermal denaturation of DNA techniques, inhibitory activity of nucleic acid synthesis was examined using P388 cells in vitro, cytotoxicity measurements to tumor cells in vitro employed human lymphoblastic leukemic cells (CCRF-CEM), and antitumor activity was assayed against P388 mouse leukemia in $CDF_1$ mice. Synthesis of a number of new analogues in each series and determination of the biophysical, biochemical, and biological properties established a more thorough structure–activity relationship in these analogues. These results establish that with the selection of ω-(*n*-alkylamino) groups at the $N^2$ site or *O*-*n*-alkyl or *O*-acyl groups at the C-7 site a variety of modifications can be carried out on the actinomycin molecule while preserving biological activity. $N^2$-3′-Amino-*n*-propyl- and $N^2$-10′-amino-*n*-decyl-actinomycin D, 7-methoxy- and 7-ethoxyactinomycin D, and the 7-*O*-(1′-adamantoyl) ester of 7-hydroxyactinomycin D were found to be the most effective antitumor agents in vivo and in vitro. They also strongly inhibit cellular RNA and DNA synthesis and, with the exception of the ester, retain high DNA-binding affinity.

Actinomycin D (AMD, 1), a chromopeptide antibiotic




| | | R | $R_1$ |
|---|---|---|---|
| 1 | AMD | $NH_2$ | H |
| 2 | 7-hydroxy-AMD | $NH_2$ | OH |
| 2a–f | 7-*O*-*n*-alkyl-AMD | $NH_2$ | *O*-*n*-alkyl |
| 2g–i | 7-*O*-aralkyl-AMD | $NH_2$ | *O*-aralkyl |
| 3a–d | 7-*O*-acyl-AMD | $NH_2$ | *O*-acyl |
| 4a–f | $N^2$-*n*-alkyl-AMD | NH-*n*-alkyl | H |
| 5a–e | $N^2$-ω-(*n*-alkylamino)-AMD | NH-ω-(*n*-alkylamino) | H |
| 6 | 2-chloro-AMD | Cl | H |
| 7 | 7-amino-AMD | $NH_2$ | $NH_2$ |
| 7a–f | 7-(alkylamino)- and 7-(aralkylamino)-AMD | $NH_2$ | NH-alkyl or -aralkyl |

whose 2-aminophenoxazin-3-one chromophore is attached to two cyclic pentapeptide lactones, is an established agent in molecular biological studies related to RNA metabolism due to its specific inhibition of RNA synthesis.[1-12] This biological activity of AMD is believed to originate from its precise ability to bind to dG-dC base pairs in double-helical DNA by intercalation of its chromophore and by hydrogen bonding and hydrophobic interactions of its peptide functions.[13-17]

Although AMD is used clinically and is known to cure two different tumors, i.e., Wilms' tumor[18] and gestational choriocarcinoma,[19] its spectrum of activity in man is relatively narrow.[20] Poor uptake of AMD by several tumors and its acute and cumulative toxicity due to lack of detoxification in, and insufficient elimination from, the human system are implicated in its narrow spectrum of activity and restricted use in patients.[20-24] The development of

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Table I. Physicochemical Properties of O⁷-Substituted 7-Hydroxyactinomycin D Analogues (R = $NH_2$)

| no. | $R_1$ | $R_f$[a] | yield, % | mol formula | $[\alpha]^{25}_D$ ($c$, $CHCl_3$),[b] deg | UV $\lambda_{max}$, nm ($\epsilon$)[c,d] |
|---|---|---|---|---|---|---|
| 2 | OH[d] | 0.38 | | $C_{62}H_{86}N_{12}O_{17}\cdot 2H_2O$ | −318 ± 10 (0.20) | 469 (23 000) |
| 2a | $OCH_3$ | 0.58 | 66 | $C_{63}H_{88}N_{12}O_{17}\cdot 2H_2O$ | −372 ± 10 (0.22) | 466 (21 000) |
| **2b** | $OC_2H_5$ | 0.59 | 58 | $C_{64}H_{90}N_{12}O_{17}\cdot 2H_2O$ | −388.5 ± 10 (0.22) | 467 (22 500) |
| 2c | O-$n$-$C_3H_7$ | 0.59 | 39 | $C_{65}H_{92}N_{12}O_{17}\cdot 2H_2O$ | −386 ± 15 (0.24) | 469 (20 100) |
| 2d | O-$n$-$C_4H_9$ | 0.60 | 66 | $C_{66}H_{94}N_{12}O_{17}\cdot 3H_2O$ | −400 ± 10 (0.25) | 465 (20 500) |
| 2e | O-$n$-$C_6H_{13}$ | 0.62 | 72 | $C_{68}H_{98}N_{12}O_{17}\cdot 4H_2O$ | −359 ± 10 (0.19) | 466 (19 800) |
| 2f | O-$n$-$C_7H_{15}$ | 0.62 | 58 | $C_{69}H_{100}N_{12}O_{17}\cdot 6H_2O$ | −350 ± 15 (0.22) | 466 (17 100) |
| 2g | O($p$-$NO_2$-Bzl) | 0.64 | 74 | $C_{69}H_{91}N_{13}O_{19}\cdot 3H_2O$ | −256 ± 15 (0.18) | 469 (19 200) |
| 2h | O($p$-Cl-Bzl) | 0.61 | 75 | $C_{69}H_{91}N_{12}O_{17}Cl$ | −336 ± 10 (0.18) | 460 (18 300) |
| 2i | O(3′,4′-$Cl_2$-Bzl) | 0.65 | 61 | $C_{69}H_{90}N_{12}O_{17}Cl_2$ | −240 ± 20 (0.17) | 459 (19 100) |
| 3a | $OCOC_6H_5$ | 0.21 | 21 | $C_{69}H_{90}N_{12}O_{18}\cdot 5H_2O$[e] | −210 ± 20 (0.1) | 459 (19 900) |
| 3b | $OCOC_{10}H_7$ ($\beta$-naphthyl) | 0.32 | 18 | $C_{73}H_{92}N_{12}O_{18}\cdot 3H_2O$ | −202 ± 18 (0.1) | 459 (15 300) |
| 3c | OCO(3′,4′-$Cl_2$-Bzl) | 0.23 | 26 | $C_{70}H_{90}N_{12}O_{18}Cl_2$ | −232 ± 16 (0.1) | 462 (11 900) |
| 3d | $OCOC_{10}H_{15}$ (1′-adamantyl) | 0.30 | 39 | $C_{73}H_{100}N_{12}O_{18}\cdot 2H_2O$ | −195 ± 25 (0.1) | 459 (12 100) |

[a] $R_f$ on silica gel plates, EtOAc–acetone (2:1). [b] Concentrations denoted by $c$, in g/100 mL. [c] Using 1-cm path-length cells and a 50–100 μM solution in $CHCl_3$. Extinction values ($\epsilon$) are concentration dependent. [d] See ref 31. [e] Calcd: C, 55.86; H, 6.93; N, 11.33. Found: C, 55.76; H, 6.29; N, 10.77.

modified AMD analogues possessing a broader range of antitumor activity and reduced host toxicity is highly desirable.

In order to enhance the antitumor activity while simultaneously decreasing the high toxicity associated with AMD treatment, a large number of AMD derivatives have been prepared and evaluated for antitumor and antibacterial activities.[25-31] Structure–activity studies summarized in an excellent review by Meienhofer and Atherton[32] reveal that the intact pentapeptide lactones and the unsubstituted 2-amino, 3-oxo, and 4- and 6-methyl groups of the chromophore are necessary for optimal antibiotic activities. More recently, Moore et al.,[27] Mosher et al.,[28] Sinha et al.,[30] and our group[31] have shown that an unsubstituted 2-amino group is not essential for antitumor activity or DNA-binding efficiencies of the analogues. We have, moreover, reported that certain small substituents at the C-7 position of the chromophore[26] and even several bulky groups, e.g., aralkylamino, do not interfere adversely in the biological[29] and biochemical properties of AMD.[33] In this article we will report a systematic study of a series of new modifications on the $N^2$ and C-7 sites of the AMD molecule and their effect on DNA-binding affinity, nucleic acid synthesis, and growth-inhibitory properties in tumor cells in vitro and the antitumor activities in vivo. The final goal of this investigation is to establish a guideline for the design and synthesis of chromophore-substituted AMD analogues leading to clinically effective agents with reduced host toxicity and improved therapeutic activity.

**Synthesis and Physicochemical Properties.** We have synthesized four major classes of analogues for this purpose: (a) C-7-substituted analogues with either linear chain $n$-alkoxy (**2a–f**) or bulky aralkoxy groups (**2g–i**), (b) C-7-substituted acyloxy analogues (**3a–d**), (c) $N^2$-$n$-alkyl substituted analogues (**4a–f**), and (d) $N^2$-ω-amino-$n$-alkyl-AMD analogues (**5a–e**). We are also reporting additional data on a fifth class of compounds, the 7-(aralkylamino)-AMD analogues (**7a–e**), whose synthesis and cytotoxic and antitumor activities we previously reported.[29]

Synthesis of **2a–i** used 7-hydroxy-AMD[26] (**2**), $[\alpha]^{20}_D$ −318 ± 10°, $[\alpha]^{20}_{644}$ −294 ± 15° ($c$ 0.2, $CHCl_3$), as the starting material. The reaction with the appropriate alkyl halide in a dry acetone solution in the presence of anhydrous potassium carbonate gave the desired product, which was isolated readily.[34] Purification of the product of the reaction by silica gel thin-layer chromatography with ethyl acetate–acetone (2:1) gave the pure material in good yield (Table I). The resulting analogues (**2a–i**) showed strong negative specific rotations ($[\alpha]^{20}_D$ ($CHCl_3$), Table I) and long-wavelength absorption maxima at 465–470 nm similar to 7-hydroxy-AMD. However, in aqueous buffer, pH 7.6, these derivatives showed small hypsochromic shifts (2–4 nm) in their visible absorption maxima. In contrast to this, 7-hydroxy-AMD in the same buffer shows a very large bathochromic shift of the maximum to 560 nm.[14] Any minor contamination of 7-hydroxy-AMD in the solutions of **2a–i** can easily be monitored at this wavelength.

Analogues **3a–d** are acyl derivatives of 7-hydroxy-AMD (**2**) and were prepared by condensation of the required acyl halides with **2** under the same conditions described above for **2a–i**. Those derivatives which are esters of aromatic acids (**3a,b**) are labile in aqueous basic buffer (pH 7.8) and are hydrolyzed more readily to 7-hydroxy-AMD than **3c** or **3d**. In fact, **3d** is quite stable in pH 7.8 buffer medium at 37 °C for several days. The base lability is most pronounced in **3a** and least in **3d**. The progress of hydrolysis of **3a–c** can be easily followed by the extinction values of 7-hydroxy-AMD at the absorption maximum at 560 nm.

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Table II. Physicochemical Properties of $N^2$-Substituted Actinomycin D Analogues ($R_1$ = H)

| no. | R | mp, °C | yield, % | mol formula | $R_f$[a] | $[\alpha]^{25}D$, ($c$, $CHCl_3$),[b] deg | UV $\lambda_{max}$ nm ($\epsilon$)[c] |
|---|---|---|---|---|---|---|---|
| 1 | $NH_2$ | | | $C_{62}H_{86}N_{12}O_{16}\cdot 2H_2O$ | 0.65 | −363 ± 20 (0.2) | 442 (21 500) |
| 4a | $NH(CH_2)_2CH_3$ | 247-249 | 63 | $C_{65}H_{92}N_{12}O_{16}\cdot 2H_2O$ | 0.75 | −201 ± 10 (0.19) | 412 (20 300) |
| 4b | $NH(CH_2)_3CH_3$ | 232-233 | 76 | $C_{66}H_{94}N_{12}O_{16}\cdot 2H_2O$ | 0.76 | −243 ± 10 (0.18) | 417 (15 800) |
| 4c | $NH(CH_2)_4CH_3$ | 234-236 | 46 | $C_{67}H_{96}N_{12}O_{16}\cdot H_2O$ | 0.72 | −283 ± 15 (0.20) | 416 (15 200) |
| 4d | $NH(CH_2)_5CH_3$ | 254-258 | 54 | $C_{68}H_{98}N_{12}O_{16}\cdot 2H_2O$ | 0.70 | −275 ± 15 (0.17) | 420 (13 100) |
| 4e | $NH(CH_2)_8CH_3$ | 212-220 | 53 | $C_{71}H_{104}N_{12}O_{16}\cdot H_2O$ | 0.86 | −295 ± 20 (0.2) | 426 (12 800) |
| 4f | $NH(CH_2)_{10}CH_3$ | 232-236 | 19 | $C_{73}H_{108}N_{12}O_{16}\cdot 3H_2O$ | 0.88 | −310 ± 15 (0.2) | 426 (13 300) |
| 5a | $NH(CH_2)_3NH_2$ | 286-288 | 55 | $C_{65}H_{93}N_{13}O_{16}\cdot 2H_2O$ | 0.05 | −308 ± 20 (0.1) | 412 (10 100) |
| 5b | $NH(CH_2)_4NH_2$ | 234-236 | 63 | $C_{66}H_{95}N_{13}O_{16}\cdot 2H_2O$ | 0.12 | −312 ± 15 (0.1) | 410 (11 500) |
| 5c | $NH(CH_2)_5NH_2$ | 218-222 | 29 | $C_{67}H_{97}N_{13}O_{16}\cdot H_2O$ | 0.13 | −309 ± 10 (0.2) | 411 (9800) |
| 5d | $NH(CH_2)_8NH_2$ | 236-238 | 33 | $C_{70}H_{103}N_{13}O_{16}\cdot H_2O$ | 0.21 | −312 ± 15 (0.2) | 408 (7300) |
| 5e | $NH(CH_2)_{10}NH_2$ | 246-248 | 40 | $C_{72}H_{107}N_{13}O_{16}$ | 0.20 | −315 ± 20 (0.1) | 410 (7100) |

[a] $R_f$ in solvent, EtOAc-acetone, 2:1 (silica gel plates). [b] Concentrations denoted by $c$ in g/100 mL. [c] Using 1-cm path-length cells and a 50-100 μM solution in $CHCl_3$. Extinction values ($\epsilon$) are concentration dependent.

Table III. Stabilization of Calf Thymus DNA against Thermal Denaturation ($\Delta T_m$) and Inhibition of Incorporation of Labeled Thymidine and Uridine by P388 Cells in Vitro in the Presence of Actinomycin D and Analogues (R = $NH_2$)

| compd | $R_1$ | $K_{app}$,[c] $M^{-1}$ | $B_{app}$[c] | $\Delta T_m$,[a] °C | drug concn, μM, for 50% inhibn of incorp[b]: thymidine $ED_{50}$ (DNA) | uridine $ED_{50}$ (RNA) |
|---|---|---|---|---|---|---|
| AMD | H | $23 \times 10^6$ | 0.108 | 7.1 ± 0.15 | 0.996 | 0.046 |
| 2 | OH | $32 \times 10^6$ | 0.100 | 6.7 ± 0.15 | 5.08 | 3.22 |
| 2a | $OCH_3$ | $7.7 \times 10^6$ | 0.110 | 5.5 ± 0.15 | 0.628 | 0.069 |
| 2b | $OC_2H_5$ | $6.9 \times 10^6$ | 0.097 | 5.9 ± 0.15 | 1.63 | 0.151 |
| 2c | $O(CH_2)_2CH_3$ | $2.1 \times 10^6$ | 0.078 | 3.5 ± 0.15 | 1.98 | 0.23 |
| 2d | $O(CH_2)_3CH_3$ | $1.1 \times 10^6$ | 0.055 | 2.7 ± 0.3 | 11.3 | 0.77 |
| 2e | $O(CH_2)_5CH_3$ | [d] | [d] | 1.0 ± 0.15 | [e] | [e] |
| 2f | $O(CH_2)_6CH_3$ | [d] | [d] | 0 | [e] | [e] |
| 2g | O(*p*-$NO_2$)Bzl | $0.1 \times 10^6$ | [d] | 0 | [e] | [e] |
| 2h | O(*p*-Cl)Bzl | $0.1 \times 10^6$ | [d] | 0 | [e] | [e] |
| 2i | O(3',4'-$Cl_2$)Bzl | $0.01 \times 10^6$ | [d] | 0 | [e] | [e] |
| 3a | $OCOC_6H_5$ | [d] | [d] | [f] | [e] | [e] |
| 3b | $OCOC_{10}H_7$ (β-naphthyl) | [d] | [d] | [f] | [e] | [e] |
| 3c | OCO(3',4'-$Cl_2$)Bzl | [d] | [d] | [f] | [e] | [e] |
| 3d | $OCOC_{10}H_{15}$ (1'-adamantyl) | [d] | [d] | [f] | 0.730 | 0.176 |

[a] $\Delta T_m$ = $T_m$ of DNA-drug complex minus $T_m$ of DNA (see Experimental Section). [b] Using viable P388 cells in growth medium. For details see Experimental Section. [c] $K_{app}$ = apparent binding constants to DNA base pairs determined from Scatchard plot of binding isotherms in 0.01 M phosphate, pH 7.0 buffer at 20 °C. $B_{app}$ = apparent number of binding sites of the drug per base pairs in DNA (see ref 14). [d] Not measurable. [e] Values are high and variable. Low solubility of the agents prevents accurate determination of data. [f] Not determined, due to partial hydrolysis of analogues to 7-hydroxy-AMD at high temperatures.

Compared to **2a–i**, the analogues **3a–d** possess low specific rotations [$[\alpha]^{20}_D$ ($CHCl_3$)] and extinction maxima [$\epsilon$ ($CHCl_3$)] (Table I). On the other hand, **3a–d** have an enhanced peak at 1725–1740 $cm^{-1}$ (ester C=O) in the infrared spectrum ($CHCl_3$). This peak is weaker in both **2a–i** and in AMD where it originates from the peptide lactones.

$N^2$-Substituted actinomycin D analogues have two kinds of substitution on the 2-$NH_2$ group of actinomycin D; **4a–f** carry *n*-alkyl chains of varying length, while **5a–e** have ω-amino-*n*-alkyl chains.[34] The number of methylene groups separating the 2-amino nitrogen and the terminal nitrogen is increased in the series **5a–e**. Starting from 2-deamino-2-chloroactinomycin D (**6**), reaction with the appropriate amine or diamine according to the procedure of Moore et al.[27] gave all the $N^2$-substituted analogues. The intermediate **6**, which was prepared by the two-step reaction reported previously by Moser et al.,[28] was homogeneous by thin-layer chromatography. The reaction was carried out at 45–50 °C using a large excess of amine or diamine.[30] The purity and homogeneity of each synthetic analogue was tested by TLC routinely in two solvent systems and by HPLC before ascertaining their physicochemical characteristics.

Analogues **4a–f** were distinguishable from **5a–e** by their (a) $R_f$ values in TLC, (b) specific rotations, and (c) visible absorption maxima (Table II). All the compounds in the series **4a–f** and **5a–e** show characteristic hypsochromic shifts in absorption maxima compared to AMD (**1**). Compounds **5a–e** have specific rotation values comparable to AMDs but higher than those of compounds **4a–e**. However, **5a–e** are more polar and have significantly lower $R_f$ values in thin-layer chromatography on silica gel with ethyl acetate–acetone (2:1).

**DNA-Binding Studies. (a) Equilibrium Binding Measurements.** Commercial calf thymus DNA (Sigma Chemical Co.) sheared by sonication and homogenized to an approximate[13] molecular weight of $1 \times 10^5$ to $5 \times 10^5$ was used in 0.01 M phosphate buffer (pH 7.0) at 20 °C. The binding constants ($B_{app}$ and $K_{app}$) were determined by spectral titration techniques and from the Scatchard plot of binding isotherms as described previously.[14]

The results in Table III show that the binding of **2a–d** to DNA takes place in varying degrees which is measurable. Binding occurs despite substitution with progressively longer-chain alkoxy groups up to *n*-butoxy. The strength of binding ($K_{app}$) and the number of drug molecules bound per DNA base pair ($B_{app}$) diminishes as the alkyl chain is lengthened.

With 7-(alkylamino)-substituted AMD analogues, binding to DNA is found to be very strong in spite of the

Table IV. Stabilization of Calf Thymus DNA against Thermal Denaturation ($\Delta T_m$) and Inhibition of Incorporation of Labeled Thymidine and Uridine by P388 Cells in Vitro in the Presence of Actinomycin D and Analogues ($R_1$ = H)

| compd | R | $\Delta T_m$,[a] °C | drug concn, μM, for 50% inhibn of incorp: thymidine $ED_{50}$ (DNA) | drug concn, μM, for 50% inhibn of incorp: uridine $ED_{50}$ (RNA) |
|---|---|---|---|---|
| AMD | $NH_2$ | 7.1 ± 0.15 | 0.996 | 0.046 |
| 4a | $NH(CH_2)_2CH_3$ | 1.5 ± 0.2 | >5.5 | 1.82 |
| 4b | $NH(CH_2)_3CH_3$ | 0.2 ± 0.15 | [c] | >6.0 |
| 4c | $NH(CH_2)_4CH_3$ | 0.0 | [c] | [c] |
| 4d | $NH(CH_2)_5CH_3$ | 0.0 | [c] | [c] |
| 4e | $NH(CH_2)_8CH_3$ | 0.0 | [c] | [c] |
| 4f | $NH(CH_2)_{10}CH_3$ | 0.0 | [c] | [c] |
| 5a | $NH(CH_2)_3NH_2$ | 6.2 ± 0.15 | 0.430 | 0.118 |
| 5b | $NH(CH_2)_4NH_2$ | 1.8 ± 0.15 | 3.30 | 0.346 |
| 5c | $NH(CH_2)_5NH_2$ | 1.1 ± 0.15 | >4.6 | 0.46 |
| 5d | $NH(CH_2)_8NH_2$ | 0.3 ± 0.15 | [c] | [c] |
| 5e | $NH(CH_2)_{10}NH_2$ | 6.6 ± 0.3 | 2.7 | 0.7 |

[a] $\Delta T_m$ = $T_m$ of DNA-drug complex minus $T_m$ of DNA (see Experimental Section). [b] Using viable P388 cells in growth medium. For details, see Experimental Section. [c] Not determined because of lack of DNA binding ($\Delta T_m \simeq 0$).

bulk of the substituents (**7a–e**)[29,35] (Table V).

Evidence from CD spectral studies corroborates these observations.[33] The probable mechanism of accommodation of these bulky groups in the DNA backbone is discussed in detail (see ref 33). For **2a–i**, it is obvious that beyond a certain length of alkoxy chain, i.e., *n*-butoxy, the affinity for DNA falls off rapidly and binding is not measurable by spectral titration (Table III). In the case of **7a–e**, the additional nitrogen atoms in the substituents at C-7 may interact with the neighboring phosphate groups in the DNA helix to give an extra measure of stabilization to the drug–DNA complex. In fact, the compound **7d** which has two additional basic nitrogen functions exhibits the strongest DNA-binding properties. In the comparable C-7 alkoxy analogues, **2e–i**, DNA-binding is not stabilized and the binding parameters are not determinable by our standard techniques.

**(b) Thermal Denaturation of DNA.** Thermal denaturation studies were carried out as described under Experimental Section.[36,37] The $\Delta T_m$ values (the difference of temperature at which 50% hyperchromicity at 259 nm in native and drug complexed DNA is induced) are recorded in Tables III–V. The values give a measure of stabilization of the DNA helical structure as a consequence of drug binding. In general, there is only an approximate correlation between the strength of binding of agents observed by $\Delta T_m$ (at 67–84 °C) and $K_{app}$ (at 20 °C) values. The $\Delta T_m$ data derived for **4a–f** and **5a–e** point out that the terminal ω-amino group in **5a–e** is very important for binding to DNA and, furthermore, the length of the alkyl chain between $N^2$ and the terminal amine is very critical for such binding. The interaction of the additional amino function in these analogues **5a–e** with the vicinal phosphate groups of the DNA double helix probably promotes stability of the DNA–drug complex. Molecular model building suggests that the chain length of the order of $-(CH_2)_3-$ and $-(CH_2)_{10}-$ could be optimal for the interaction of the terminal amine with the nearest and the next to the nearest phosphate groups relative to the site of drug binding to the G–C base pairs in DNA.

These DNA-binding studies, evaluated by equilibrium binding constants and $\Delta T_m$, reveal that binding is not seriously hindered by substitution of the C-7 site of AMD with aralkylamino (**7a–e**) or selected *n*-alkoxy groups (**2a,b**). This is also true for $N^2$ substitution, provided the straight-chain alkyl substituent carries an extra amino group at the terminus of the chain. For $N^2$-ω-amino-*n*-alkyl-AMD (**5a–e**) and for 7-alkoxy-AMD (**2a–d**) the length of the substituent chains are critical for binding. With 7-(acyloxy)-AMD analogues, **3a–e**, no binding was observed by spectral shift techniques, verifying the reported observation[13] that these derivatives may not have measurable DNA-binding affinity. Furthermore, these analogues are partially hydrolyzed to 7-hydroxy-AMD (**2**) at high temperature, even at pH 7.0, making any $\Delta T_m$ studies unnecessary.

**Biochemical Properties. (a) Stability in the Presence of Rat Serum (10%).** In the presence of 10% rat serum, some 7-(acyloxy) derivatives, e.g., **5a** and **5b**, were found to be hydrolyzed extensively to 7-hydroxy-AMD. In contrast, 7-*O*-(1′-adamantoyl)-AMD (**5d**) was very stable, suggesting that acyl groups derived from aromatic acids, e.g., benzoic or β-naphthoic, are hydrolyzed faster than the one derived from a bulky alicyclic acid, e.g., 1-adamantoic acid. $N^2$-3′-Aminopropyl-AMD (**5a**), in contrast to $N^2$-*n*-propyl-AMD (**4a**), appears to be converted to minute amounts of AMD in 24 h. Similarly, 7-methoxy-AMD (**2a**), but not 7-*n*-propoxy-AMD (**2e**), is converted to 7-hydroxy-AMD, although only in a very minute amount. 7-Amino-AMD (**7**), 7-hydroxy-AMD (**2**), and several 7-(alkylamino) analogues (**7a** and **7b**) were found to be perfectly stable. On the basis of these observations, the biological and biochemical properties of some agents and especially of the ester derivatives 7-(benzoyloxy)-AMD (**5a**) and 7-(β-naphthoyloxy)-AMD (**5b**) can be regarded as originating either partly or entirely from the products of biotransformation. A typical experiment of rat serum component dependent transformation of a few selected analogues is described under Experimental Section.

**(b) Inhibition of DNA and RNA Synthesis in P388 Leukemic Cells In Vitro.** The abilities of a number of synthetic AMD analogues to inhibit synthesis of DNA and RNA in cultured P388 cells have been determined[38] and are compared with that of AMD (**1**) (Tables III–V). The very low values for AMD ($ED_{50}$ = 0.996 μM for DNA and 0.046 μM for RNA) exhibit its strong inhibitory activity, particularly toward RNA synthesis. Whereas the C-7-substituted derivatives with small substituents, e.g., 7-amino-AMD (**7**) and 7-methoxy-AMD (**2a**), are about equipotent, 7-hydroxy-AMD (**2**) is several fold less potent than AMD in spite of its very strong DNA binding affinity. In the latter case, poor influx of the anionic species of the drug may be a contributing factor.[14] 7-Ethoxy-AMD (**2b**) and 7-*n*-propoxy-AMD (**2c**) are less potent than 7-methoxy-AMD (**2a**), and the potencies in this series drop sharply as the length of the substituent chain is increased. One 7-(acyloxy) derivative (**3d**) shows remarkable activity, although no binding to extracellular calf thymus DNA is evident, suggesting a probable intracellular conversion to

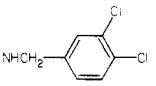
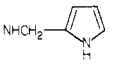
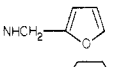
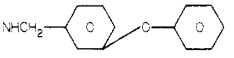
(35) S. K. Sengupta, M. S. Amdhavarao, and W. R. Beltz, "Abstracts of Papers", 175th National Meeting of the American Chemical Society, Anaheim, CA, Mar 13–17, 1978, American Chemical Society, Washington, DC, 1978, Abstr MEDI 51.

(36) W. O. Foye, O. Vajragupta, and S. K. Sengupta, *J. Pharm. Sci.*, submitted.

(37) S. K. Sengupta and Y. Kogan, unreported results from these laboratories.

(38) W. D. Meriwether and N. R. Bachur, *Cancer Res.*, **32**, 1137 (1972).

Table V. Stabilization of Calf Thymus DNA against Thermal Denaturation ($\Delta T_m$) and Inhibition of Incorporation of Labeled Thymidine and Uridine by P388 Cells in Vitro in the Presence of Actinomycin D and Analogues (R = $NH_2$)

| compd | $R_1$ | $K_{app}$,[c] $M^{-1}$ | $B_{app}$[c] | $\Delta T_m$,[a] °C | drug concn, μM, for 50% inhibn of incorp: thymidine $ED_{50}$ (DNA) | drug concn, μM, for 50% inhibn of incorp: uridine $ED_{50}$ (RNA) |
|---|---|---|---|---|---|---|
| AMD | H | $2.3 \times 10^7$ | 0.108 | 7.1 ± 0.15 | 0.996 | 0.046 |
| 7 (7-$NH_2$-AMD) | $NH_2$ | $2.3 \times 10^7$ | 0.121 | 8.2 ± 0.1 | 1.23 | 0.082 |
| 7a | $NHCH_2C_6H_5$ | $5.8 \times 10^6$ | 0.049 | 6.6 ± 0.2 | 2.70 | 1.4 |
| 7b |  | $2.6 \times 10^7$ | 0.058 | 7.0 ± 0.15 | 0.418 | 0.096 |
| 7c | $NH(CH_2)(CH_2)_6CH_3$ | d | d | 1.1 ± 0.25 | d | d |
| 7d |  | $9.2 \times 10^7$ | 0.055 | 15.8 ± 0.2 | 0.370 | 0.184 |
| 7e |  | $3.8 \times 10^6$ | 0.055 | 5.8 ± 0.3 | 2.45 | 0.81 |
| 7f |  | $8.8 \times 10^6$ | 0.055 | 5.3 ± 0.3 | 1.5 | 0.70 |

[a] $\Delta T_m$ = $T_m$ of DNA-drug complex minus $T_m$ of DNA (see Experimental Section). [b] Using viable P388 cells in growth medium. For details, see Experimental Section. [c] $K_{app}$ = apparent binding constants to DNA base pairs determined from Scatchard plot of binding isotherms in 0.01 M phosphate, pH 7.0 buffer at 20 °C. $B_{app}$ = apparent number of binding sites of the drug per base pairs in DNA. [d] Solubility too low for determination of data.

Table VI. In Vivo and In Vitro Antitumor Activity of O⁷-Substituted 7-Hydroxyactinomycin D Analogues (R = $NH_2$)

| compd | $R_1$ | in vivo P388[a] assay: optimum dose, μg/kg | in vivo P388[a] assay: MST,[b] days | % ILS[c] | in vitro (CCRF-CEM)[d] $ID_{50}$, ng/mL |
|---|---|---|---|---|---|
| no drug (control) | | | 10.5 | | |
| 1 (AMD) | H | 125 | 24 | 129 | 60 |
| 2 | OH | 1200 | 24 | 129 | 950 |
| 2a | $OCH_3$ | 600 | 19 | 81 | 160 |
| 2b | $OC_2H_5$ | 450 | 20 | 91 | 170 |
| 2c | $O(CH)_2CH_3$ | 750 | 16 | 52 | 310 |
| 2d | $O(CH_2)_3CH_3$ | 750 | 14 | 33 | 665 |
| 2e | $O(CH_2)_5CH_3$ | 900[e] | 13 | 24 | 2500 |
| 2f | $O(CH_2)_6CH_3$ | 900[e] | 13 | 24 | 3000 |
| 2g | O(*p*-$NO_2$)Bzl | 600 | 14 | 33 | 500 |
| 2h | O(*p*-Cl)Bzl | 450 | 16 | 52 | 500 |
| 2i | O(3',4'-$Cl_2$)Bzl | 500 | 19 | 81 | 1250 |
| 3a | $OCOC_6H_5$ | 750 | 20 | 91 | 2500 |
| 3b | $OCOC_{10}H_7$ (β-naphthyl) | 900 | 18 | 71 | 3000 |
| 3c | OCO(3',4'-$Cl_2$)Bzl | 500 | 21 | 100 | 650 |
| 3d | $OCOC_{10}H_{15}$ (1'-adamantyl) | 500 | 28 | 167 | 200 |

[a] $10^6$ P388 cells implanted intraperitoneally on day 0 into groups of seven $CDF_1$ male mice. Drugs were administered, also ip, in 5% dimethyl sulfoxide-saline on days 1, 5, and 9. [b] MST = median survival time. [c] % ILS = (treated − control/control) × 100. Average of two or more experiments. [d] Human lymphoblastic leukemic cells. [e] Highest dose tested.

an active component. Of the $N^2$-substituted analogues, **5a** is the most active, about one-half as potent as AMD. Compound **5e**, which appears to interact with extracellular DNA almost as strongly as **5a**, is not nearly as good an inhibitor as **5a**. Either differential influx or a metabolic activation of the drugs by the cells, or both, may contribute to such a difference. Consistent with the loss of their DNA-binding affinity, the $N^2$-alkyl-AMD derivatives **4a** and **4b** are not active. Compounds **4a–f** are not as potent as the $N^2$-ω-amino-*n*-alkyl-AMD analogues **5a–c** in inhibiting intracellular nucleic acid synthesis. The 7-(alkylamino) analogues (**7a–f**), which have strong calf thymus DNA binding properties, are not nearly as active in inhibiting RNA synthesis. Interestingly, some exhibit more potency in inhibition of DNA synthesis than AMD. This is probably true for the two best DNA-binding agents, **7b** and **7d**. In addition to the processes of cell uptake and biotransformation, a favorable intracellular retention of these agents or their metabolites can cause such a difference. In the case of 7-methoxy-AMD[37] which behaves in a similar manner, it was found (using 7-[$^3$H]methoxy-AMD) that this agent is retained in CEM cell lines at more elevated concentration than AMD for a period of at least 1 h. The biochemical properties of these agents, although derived from AMD, appear to be transformed somewhat, causing the observed quantitative and qualitative differences.

**Biological Activity.** In vitro growth inhibitory activities of the analogues were assayed against human lymphoblastic leukemic cells (CCRF–CEM),[39] and the results are reported[31] in Tables VI and VII. CCRF-CEM cells are highly sensitive to AMD and several of the synthetic analogues. The assay that determines the 50% inhibitory dose ($ID_{50}$) provided relative cytotoxicity values for the synthetic analogues compared to AMD.

The results show that AMD is highly cytotoxic to CEM cells and to some extent the analogues 7-methoxy-AMD

(39) G. E. Foley and H. Lazarus, *Biochem. Pharmacol.*, 16, 659 (1967).

Table VII. In Vivo and In Vitro Antitumor Activity of $N^2$-Substituted Actinomycin D Analogues ($R_1$ = H)

| compd | R | in vivo (P388) assay[a] optimum dose, μg/kg | MST,[b] days | % ILS[c] | in vitro (CCRF-CEM)[d] $ID_{50}$, ng/mL |
|---|---|---|---|---|---|
| no drug (control) | | | 11.0 | | |
| 1 (AMD) | $NH_2$ | 125 | 26 | 136 | 60 |
| **4a** | $NH(CH_2)_2CH_3$ | 1200 | 17 | 55 | 450 |
| **4b** | $NH(CH_2)_3CH_3$ | 900 | 15 | 36 | 950 |
| **4c** | $NH(CH_2)_4CH_3$ | 1150[e] | 13 | 18 | 1 600 |
| **4d** | $NH(CH_2)_5CH_3$ | 1200[e] | 11 | 0 | 3 000 |
| **4e** | $NH(CH_2)_8CH_3$ | 1800[e] | 10 | | 11 000 |
| **4f** | $NH(CH_2)_{10}CH_3$ | 1150[e] | 10 | | >15 000 |
| 5a | $NH(CH_2)_3NH_2$ | 1200 | 30 | 172 | 300 |
| **5b** | $NH(CH_2)_4NH_2$ | 1800[e] | 23 | 109 | 600 |
| **5c** | $NH(CH_2)_5NH_2$ | 1800[e] | 18 | 64 | 850 |
| **5d** | $NH(CH_2)_8NH_2$ | 1800[e] | 14 | 27 | 310 |
| **5e** | $NH(CH_2)_{10}NH_2$ | 1800 | 18 | 64 | 280 |

[a] $10^6$ P388 cells implanted intraperitoneally on day 0 into groups of seven $CDF_1$ male mice. Compounds were administered ip in 5% dimethyl sulfoxide–saline on days 1, 5, and 9; control groups received vehicles only. [b] Median survival time. [c] % ILS = (treated − control/control) × 100. Average of two or more experiments. [d] Human lymphoblastic leukemia cells in suspension culture. [e] Highest dose tested.

(**2a**), 7-ethoxy-AMD (**2b**), and 7-*n*-propoxy-AMD (**2c**), the $N^2$-substituted derivatives **5a** and **5e**, and 7-*O*-(1′-adamantoyloxy)-AMD (**3d**) demonstrate this extremely potent cytotoxicity.

In general, there is a correlation of in vitro inhibition of nucleic acid synthesis in P388 cells and the growth inhibitory activity of these compounds against CEM cells in vitro. Where correlation is not found, biotransformation of the agent or altered cellular transport may explain the observation. For compounds **5a** and **7b**, cellular biotransformation has already been observed and will be reported in a later communication.

The assay also demonstrates that the cytotoxic activities of $N^2$-substituted analogues (**4a–f**) decrease progressively with the higher alkyl homologues, while the activities alter substantially in the ω-amino-*n*-alkyl analogues (**5a–e**) as the length of the methylene chain separating the two nitrogen functions varies. Of the ester derivatives (**3a–d**), all but **3d** are considerably less toxic than AMD. Of the series, only **3d** remains stable in 10% rat serum in buffered saline medium (see Experimental Section).

These analogues were also tested for antitumor activity against P388 lymphocytic leukemia in $CDF_1$ hybrid mice[30,40] (Tables VI and VII). The tumor was implanted intraperitoneally (ip) with $10^6$ cells. The drugs were administered once daily on days 1, 5, and 9, beginning 1 day after implantation. Compounds were tested over a range of doses, but only the optimal nontoxic doses are listed. In this system many of the analogues have activity which is modest to poor compared to AMD. Out of these, only the $N^2$-substituted analogue **5a** and the 7-(acyloxy) derivative **3d** showed very high activity, although at higher dose levels compared to AMD. In fact, for optimum activity most of the agents required dose levels which are several fold higher than the AMD dose; therefore, the homogeneity of each of the agents was carefully checked by TLC, on heavily loaded thin-layer chromatograms employing two different solvent systems, and also by HPLC in the case of the most active agents.

The antitumor data listed in Tables VI and VII provide important information regarding the structure-related in vivo tumor inhibitory activity for four different series of analogues. The data reveal that $N^2$-alkyl analogues (**4a–f**) are, in general, less effective agents than the corresponding $N^2$-ω-amino-*n*-alkyl analogues (**5a–e**) and that there is a definite relationship between activity and the nature and chain length of substituents. The antitumor activity was more pronounced in those analogues which carried short alkyl amine chains (**5a** and **5b**) than in those with longer chains (**5c–e**), despite the showing by **5e** of high in vitro CCRF-CEM cytotoxicity. Of the 7-(acyloxy) analogues, **3d** was more active than **3a–c** and AMD. Activity was not particularly pronounced in the series (**2a–i**) and was further diminished by increasing length and bulk of substitution.

The biological activities of 7-(alkylamino) and 7-(aralkylamino) substituted analogues have been reported previously.[29] Those agents with good DNA binding affinity showed favorable antitumor activity.

In all the cases, the differential activities of these agents in various systems reported here may be associated with several factors, e.g., cellular uptake and retention, drug distribution, plasma half-life, metabolism, rates of renal and biliary excretion, and pharmacodynamic properties associated with in vivo activation and detoxification.

## Conclusions

Actinomycin D is a unique antibiotic which is extremely effective against tumor cells in culture and is known to act in the cellular apparatus with well-defined specificity. It is used clinically and has curative activity in two tumors. However, its application in patients is severely limited because of its high systemic toxicity resulting from absence of metabolism, poor urinary and fecal elimination, and consequent accumulation in the nucleated cells of the host tissue. In contradiction to many previous reports, some recent work based on analogue synthesis has shown that certain modification of the actinomycin molecule does not reduce, and in fact may enhance, the antitumor activity of this antibiotic.[26,29,30] Our present report is a result of a systematic investigation on the structural modification and biological evaluation of synthetic analogues of AMD. Of these, the most effective agents are $N^2$-ω-*n*-(alkylamino)-AMD, short-chain 7-alkoxy-AMD, and the 1-adamantoyl ester of 7-hydroxy-AMD. The results clearly show that the molecule of AMD can accommodate a number of well-defined modifications at the C-7 and $N^2$ sites and still retain most of its biological, biochemical, and tumor-inhibitory properties. In a subsequent communication, we will report how this information can be used to produce agents having both higher antitumor activity and

(40) R. I. Green, N. H. Greenberg, M. M. MacDonald, A. M. Schumaker, and B. J. Abbot, *Cancer Chemother. Rep., Part 3*, 3(2), 1 (1972).

considerably lower toxicity in in vivo systems.

## 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 using silica gel powder (Baker No. 3405, 60–200 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 (2:1); 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 using 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, 440, 470, and 505 nm. Spectra were determined on the following instruments: IR spectra were obtained using 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 Varian XL-100-15 spectrometer equipped with Fourier transform; optical rotations were determined on a Cary 60 recording spectrophotometer; and CD spectra were obtained on a Cary 61 spectrophotometer. All elemental analyses were within ±0.4% of the theoretical values except where indicated. 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 and the nucleosides were purchased from Sigma Chemical Co. [$^3$H]Thymidine and [2-$^{14}$C]uridine were obtained from New England Nuclear. The various alkylamines, alkyldiamines, alkyl halides, and aliphatic and aromatic acids were obtained from Aldrich Chemical Co.

**7-Methoxyactinomycin D. General Procedure.** To 20 mg (0.0157 mmol) of 7-hydroxyactinomycin D (**2**) [prepared according to the published procedure],[26] dissolved in 8.0 mL of dry acetone, was added 8 mg of finely ground anhydrous potassium carbonate. The blue reaction mixture was stirred at room temperature under nitrogen and was kept protected from exposure to light. To this was added 1.1 mg of methyl iodide (5 equiv), and the mixture was stirred for 18 h; the color of the solution turned to light orange when the reaction was complete. The organic phase was filtered from the inorganic salts and was evaporated to dryness. The residue was dissolved in ether (75 mL), filtered free of all residual inorganic salts, and concentrated with the aid of a nitrogen jet to ~5 mL. Crystallization after the addition of 2.0 mL of ligroin (bp 90 °C) yielded 19.5 mg of solid, which was further purified on a thick-layer silica gel plate (solvent B) to afford 11.8 mg (homogeneous by TLC and HPLC): UV $\lambda_{max}$ ($CHCl_3$) 466 nm (ε 21 000); UV $\lambda_{max}$ (10 mM phosphate, pH 7.0) 460 nm (ε 15 570); NMR ($CDCl_3$), τ 2.6 (s, 1 H, 8-H), 2.7 (s, 2 H, 2-$NH_2$), 7.82 (s, 3 H, 6-$CH_3$), 7.75 (s, 3 H, 4-$CH_3$), 6.27 (s, 3 H, 7-$OCH_3$), compared with values for actinomycin D of τ 2.3 (d, 1 H, 8-H), 2.63 (d, 1 H, 7-H), 2.7 (br s, 2 H, 2-$NH_2$), 7.44 (s, 3 H, 6-$CH_3$), 7.76 (s, 3 H, 4-$CH_3$).

**7-*O*-(1′-Adamantylcarbonyl)-7-hydroxyactinomycin D. General Procedure.** A typical procedure is described for the synthesis of esters of 7-hydroxy-AMD. A solution of 7-hydroxy-AMD (15 mg, 0.0116 mmol) in minimum volume of dry acetone (~1 mL) was allowed to react with 1-adamantanecarboxylic acid chloride (3.46 mg, 0.0173 mmol) in the presence of anhydrous potassium carbonate (10 mg) under nitrogen atmosphere at ambient temperature for 4 h. TLC (solvent B) showed incomplete reaction, and the reaction was allowed to proceed for another 3–4 h until the reaction appeared to be complete. The orange reaction mixture was filtered to remove inorganic salts, the filtrate was evaporated, and the residue was crystallized from a 2:1 mixture of ether and petroleum ether (bp 30–60 °C). The solid, 11.6 mg (67%), mp 189–93 °C, was further purified on TLC (solvent B) to yield 9.8 mg (57%) of pure red solid: mp 191–193 °C; UV $\lambda_{max}$ ($CHCl_3$) 285 nm (infl), 459 (ε 12 100).

**$N^2$-(3′-Aminopropyl)actinomycin D. General Method.** 2-Deamino-2-chloroactinomycin D,[28] 1.1 mg [$R_f$ 0.45 (B); UV $\lambda_{max}$ ($CH_3OH$) 261 nm (ε 15 000), 373 (14 700), 483 (7500)], dissolved in 0.4 mL of methylene chloride and 10 μL (140 equiv) of 1,3-propanediamine (over 99.9% pure by GLC) was stirred at 45–50 °C under $N_2$ for 3 h, when TLC (solvent B) showed the almost complete disappearance of starting 2-deamino-2-chloroactinomycin. The reaction mixture was diluted with 5 mL of methylene chloride and extracted with three 1-mL portions of water. The organic solvent was removed under reduced pressure and the residue was chromatographed on a silica gel plate with solvent B. The major orange spot, $R_f$ 0.05, was separated from very minor yellow spots, $R_f$ 0.38, 0.45. The orange band was eluted with acetone to yield 0.6 mg (53%) of pure red solid: TLC (B) $R_f$ 0.05 and homogeneous; HPLC $t_R$ 1.8 min vs. 9.3 min for AMD ($CH_3CN$–5 mM $NH_4OAc$, 62:38; 1.5 mL/min).

**Elevation of DNA-Melting Temperature ($\Delta T_m$).** DNA-melting temperature determinations were made following the general procedure of Henry et al.[41] The DNA used was homogenized calf thymus DNA, Type 1 (Sigma), average molecular weight 100 000–500 000, in 0.01 M phosphate buffer containing $5 \times 10^{-3}$ M EDTA, pH 7.0. Drug and DNA nucleotide concentrations were $7 \times 10^{-4}$ and $7 \times 10^{-3}$ M, respectively. The changes in the 259-nm DNA peak were monitored, and the absorbance was read at 0.25, 0.5, and 1.0 °C intervals. The melting temperature of uncomplexed DNA under these conditions is 67 ± 0.15 °C.

**Hydrolysis with Rat Serum Esterases.** Stock solutions (0.5 mM) of AMD (**1**), 7-hydroxy-AMD (**2**), 7-amino-AMD (**7**), 7-methoxy-AMD (**2a**), 7-(*n*-propyloxy)-AMD (**2c**), 7-(benzoyloxy)-AMD (**3a**), 7-(β-naphthoyloxy)-AMD (**3b**), 7-(1′-adamantoyloxy)-AMD (**3d**), $N^2$-*n*-propyl-AMD (**4a**), $N^2$-3′-aminopropyl-AMD (**5a**), 7-[(3′,4′-dichlorobenzyl)amino]-AMD (**7b**), and 7-(benzylamino)-AMD (**7a**) were prepared in saline–phosphate buffer (0.15 M NaCl, 0.01 M sodium phosphate, pH 7.6). To 9 mL of each solution in culture tubes, 1 mL of fresh rat serum was added, and the samples were incubated in a shaking water bath for 24 h at 37 °C. The bath was kept covered to protect the samples from light. After incubation the samples were extracted with ethyl acetate (3 × 10 mL), and the extracts were dried (anhydrous $Na_2SO_4$), evaporated, redissolved in ethyl acetate, filtered, concentrated under $N_2$ to 0.5 mL, spotted on silica gel TLC plates, developed both in (A) 2-butanol–formic acid–water (75:13:12) and (B) EtOAc–acetone (2:1), and estimated by absorption spectroscopy in $CHCl_3$. The results show that 7-(benzoyloxy)-AMD (**3a**) and 7-(β-naphthoyloxy)-AMD (**3b**) are almost completely hydrolyzed to 7-hydroxy-AMD (**2**). In contrast, 7-(1′-adamantoyloxy)-AMD (**3d**) was more than 90% unhydrolyzed. 7-Methoxy-AMD (**2a**) also showed 5–10% conversion to 7-hydroxy-AMD, while 7-(*n*-propyloxy)-AMD remained practically unchanged. $N^2$-(3′-Aminopropyl)-AMD (**5a**) showed less than 2% conversion to AMD as determined by HPLC. AMD (**1**), 7-hydroxy-AMD (**2**), 7-amino-AMD (**7**), and $N^2$-*n*-propyl-AMD (**4a**) were essentially unchanged, except for negligible contamination of a faster moving common fluorescent spot originating from the medium. Compounds **7a** and **7b** were also unchanged. (Recovery from TLC plates was over 80%, with greater than 85% reproducibility.)

**P388 Leukemia Cell Lines.** P388/S cells were obtained from Randall K. Johnson of Arthur D. Little, Cambridge, MA, and were maintained by serial ip transplantation in DBA/2 mice. Ascitic cells were collected 7 or 8 days after transplant and suspended in Eagle's MEM containing fetal bovine serum (heat inactivated) after erythrocytes were removed by mild centrifugation. The average cell doubling time was less than 20 h.

**P388 Nucleic Acid Synthesis Inhibition Assay.** P388 cells in log phase were harvested by mild centrifugation (700 rpm) for 10 min in Eagle's MEM, pH 7.2, at ambient temperature, and freed from erythrocytes by washing twice with 3 volumes of the

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same medium (10 to 15 mL). The washed cells, which were over 95% viable and over 99% free from erythrocytes, were then suspended in a volume of medium calculated to give a cell density of $5 \times 10^5$ cells/mL. The final medium contained dialyzed, heat-inactivated fetal bovine serum (10%), 100 units/mL penicillin, and 100 μg/mL streptomycin supplemented with 100 μg/mL glutamine and was readjusted to pH 7.2 ± 0.1 with 10 mM Hepes buffer.

Aliquots (1.0 mL) of P388 cell suspension were added to individual siliconized tubes and were incubated with test compounds dissolved in 0.1 mL of the MEM containing 10% fetal calf serum and 20 mM Hepes buffer, pH 7.2. Actinomycin D and analogues were assayed initially at concentrations of 39.1, 15.63, 6.26, 2.5, 1.0, 0.4, 0.16, 0.064, and 0.016 μM. The appropriate concentrations exhibiting $ED_{50}$ were repeated. The samples were preincubated at 37 °C for 2 h in a shaking water bath under an atmosphere of 5% $CO_2$–95% oxygen. Control P388 cells containing no drug were included in each assay, as was actinomycin D as an internal standard. After this initial incubation, either 0.5 μCi of [$^3$H]-thymidine (20.2 Ci/mmol) or [2-$^{14}$C]uridine (18.1 mCi/mmol), both from New England Nuclear, Boston, MA, was added in 0.005 mL volume. The cells were then incubated for an additional 1 h; after this incubation, the assay solutions were made 10% (w/v) with TCA, chilled to 0 °C, and allowed to stand for 2 h at 0 °C. The resulting precipitates were collected on 1.2-μm millipore filters, which were prewet with 20% TCA and were washed thoroughly with 10% TCA. The filters were placed in scintillation vials with 10 mL of Liquiscint scintillation mixture (National Diagnostic, NJ) and allowed to stand for 16 h before measuring their radioactivity in a Packard Tri-Carb Model 3375 scintillation spectrometer.

All initial experiments were done in duplicate, and repeat experiments were in triplicate. The percent synthesis of DNA or RNA in test samples was calculated relative to the synthesis in the controls. The percentage of inhibition closest above and below 50% was plotted; by extrapolation the concentrations for 50% inhibition of either RNA or DNA were determined [$ED_{50}$ (RNA) and $ED_{50}$ (DNA)].

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# New Antiarrhythmic Agents. 7. 2,3-Diaminopropionanilides

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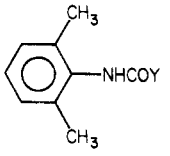
A series of 2,3-diaminopropionanilides was synthesized by acylation of mono- and disubstituted aniline derivatives with 2,3-dibromopropionyl chloride and subsequent amination with the appropriate secondary amines. The target compounds were evaluated in mice for antiarrhythmic efficacy against chloroform-induced tachycardia and for central nervous system toxicity. Several of the active agents were found to have much higher antiarrhythmic potencies than lidocaine, but they were also toxic. Evaluation of the target compounds for local anesthetic activity in the form of sciatic nerve block in rats showed that most compounds had durations of block similar to that of lidocaine; none exhibited the long duration of block seen with etidocaine.

Within the framework of a search for new orally active antiarrhythmic agents, we have described the synthesis and the pharmacological properties of primary amine analogues[2-4] of lidocaine (1). One representative, tocainide




1, Y = $CH_2N(C_2H_5)_2$
2, Y = $CH(CH_3)NH_2$

(2), had appropriate pharmacologic[2,5] and pharmacokinetic[6] parameters and was selected for clinical trials. Based on the relatively low potency of these primary amine analogues in general, and of tocainide specifically, we decided to search for more potent compounds with increased selectivity for antiarrhythmic vs. toxic effects. A series of primary and tertiary analogues of lidocaine with increased lipophilicity was synthesized,[7] yielding potent and selective agents as well as quantitative structure–activity relationships showing the usual positive correlation between partition coefficients and potency. In a parallel attempt at increasing potency, we decided to test aminoanilides with an additional amino group in the aminoacyl moiety. A series of 2,3-diaminopropionanilides carrying one alkyl, oxoalkyl, acyl, or aryl substituent on the aromatic ring had previously been synthesized[8] and evaluated for local anesthetic effects.[9] More recently the synthesis and local

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