DNA Intercalating Compounds as Potential Antitumor Agents. 2. Preparation and Properties of 7*H*-Pyridocarbazole Dimers

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In order to obtain antitumor agents, various 7*H*-pyridocarbazole dimers were prepared by quaternization of the pyridinic nitrogens of the different isomeric 7*H*-pyridocarbazole rings with halogenoamino alicyclic or aliphatic chains. The dimers interact with DNA more markedly than with the corresponding monomers, and the bisintercalation depends upon the nature, the flexibility, and the ionization state of the linking chains. They most often bisintercalate at pH 5 where the chain is protonated and monointercalate at pH 7.4. The apparent binding constants (K_{ap}) range from 10^8 to 10^9 M^{-1} at pH 5 and from 5×10^5 to 2×10^7 M^{-1} at pH 7.4. The bisintercalating dimers covered four DNA base pairs, whereas most of the monointercalating dimers covered two bases pairs. The antitumor activity against L1210 murine leukemia is strongly dependent on the position of attachment, the nature of the linking chain, and its rigidity. Three highly active dimers were obtained in the series of 7*H*-pyrido[4,3-*c*]carbazole dimers with rigid bis(ethylpiperidinyl) chains. On the other hand, two ellipticine dimers were prepared which were found completely inactive on L1210. These results show that in the series of 7*H*-pyridocarbazoles the process of dimerization leads to very active antitumor compounds.

As seen in the preceding paper,[1] a direct correlation between the DNA affinity of intercalating agents and their pharmacological properties has not been observed. Nevertheless, the probability of finding active derivatives is much higher among compounds exhibiting high affinity for DNA.[2] A rational approach in the search for antitumor compounds consists, therefore, in designing intercalating compounds having DNA binding affinities as high as possible. It is not expected, however, that simple chemical modifications of intercalating rings which have DNA binding constants, at their best, on the order of 10^6 M^{-1} could lead to molecules able to compete on DNA with proteins such as RNA polymerase or repressors which bind to DNA with binding constants close to 10^{12} M^{-1}.

We have already shown in our previous studies[3-6] that the dimerization of various intercalating moieties can lead to a very large increase in the DNA binding affinity. In theory, provided that the losses in entropic factors are not too large and the two rings of the dimer can intercalate into DNA, the free energy of interaction of each moiety may be additive. Thus, the DNA binding constant for the dimers could be as high as the square of that of the corresponding monomers.

To design the best bifunctional intercalators, planar compounds with the highest DNA binding affinities must first be selected. Secondly, a chain of proper length and conformation must be attached to the aromatic ring at the appropriate place.

In studies on model compounds, the importance of the nature and of the conformation of the linking chain has already been demonstrated.[7-13] NMR[7-10] and kinetic[11] studies have shown that these dimeric molecules tend to fold and stack their two rings on top of each other. Since the unfolding of these dimers is a necessary intermediate step in DNA binding, the ability of these compounds to react with DNA is dramatically dependent on the folding $\rightleftharpoons$ unfolding equilibrium of the dimer. Consequently, our approach was first to use intercalating moieties able to bind to DNA with high affinity, such as the 7*H*-pyridocarbazoles described in the preceding paper,[1] and secondly to use rigid linking chains to prevent intramolecular stacking as much as possible. In this respect, aminoalkyl chains containing a cyclic moiety able to bring rigidification were best suited for this kind of study: they increase the water solubility of the dimers and the DNA affinity through secondary electrostatic interactions between the amino groups of the chains and the DNA phosphate backbone. Furthermore, many derivatives, including the aminoalkyl groups, are of biological interest[14,15] as both antitumor and antiparasitic drugs. The selected chains are attached by quaternization of the pyridinic nitrogen to the aromatic ring. The presence of a positive charge on each ring is supposed to destabilize the folded structure. In addition, the influence of the position of chain attachment on the biological properties could be readily investigated by dimerization of the different isomeric pyridocarbazoles.

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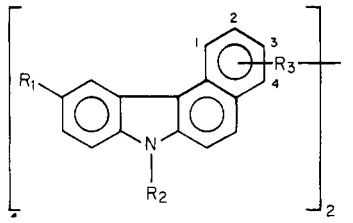
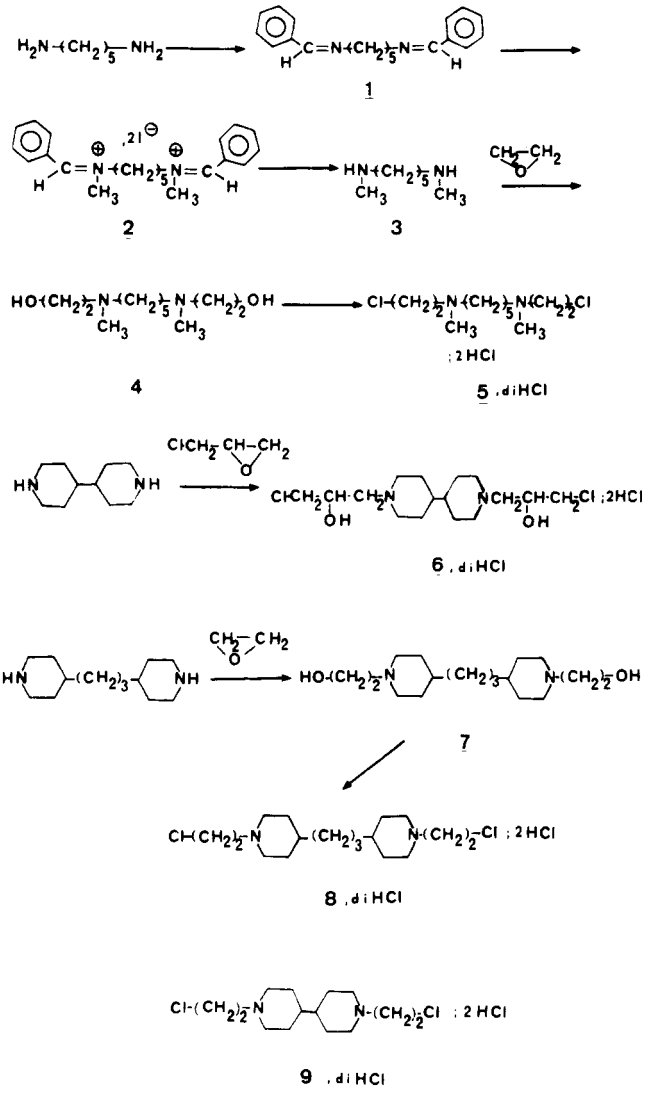
Table I. Physicochemical and DNA Properties of the 7*H*-Pyridocarbazole Dimers



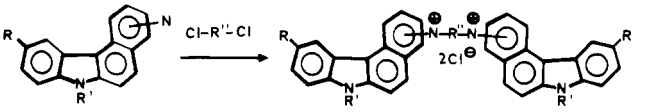
no.	R_1	R_2	R_3	mp, °C	formula[a]	slopes,[b] pH 5 1 + 2r	slopes, pH 5 1 + 4r	slopes, pH 7.4; 1 + 2r
10	OCH_3	H	2-$N^+(CH_2)_2$-1 piperidyl, Cl^-	>290	$C_{46}H_{50}N_6O_2Cl_2 \cdot 2H_2O \cdot 2HCl$	4.4	2.3	2.7
11	OCH_3	H	2-$N^+(CH_2)_2$-1 piperidyl-$(CH_2)_{1.5}$, Cl^-	>290	$C_{49}H_{56}N_6O_2Cl_2 \cdot 2H_2O \cdot 2HCl$	5.7	3.0	3.5
12	OCH_3	H	2-$N^+(CH_2)_2$, Br^-	>290	$C_{42}H_{44}N_6O_2Br_2 \cdot 3H_2O$	3.4	1.8	2.1
13	OCH_3	H	2-$N^+(CH_2)_2N(CH_3)(CH_2)_{2,5}$, AcO^-	>290	$C_{43}H_{48}N_6O_2Cl_2 \cdot 2H_2O \cdot 2HCl$	5.3	2.8	1.6
14	OCH_3	H	2-$N^+CH_2CH(OH)CH_2$-1 piperidyl, AcO^-	>290	$C_{48}H_{54}N_6O_4Cl_2 \cdot 3H_2O \cdot 2HCl$	4.4	2.3	2.4
15	OCH_3	CH_3	2-$N^+(CH_2)_2$-1-piperidyl, Cl^-	250	$C_{48}H_{54}N_6O_2Cl_2 \cdot 2.5H_2O \cdot 2HCl$	5.6	2.9	3.6
16	OH	H	2-$N^+(CH_2)_2$-1-piperidyl, Cl^-	>290	$C_{48}H_{46}N_6O_2Cl_2 \cdot 2H_2O \cdot 2HCl$	6.1	3.4	2.1
17	H	H	2-$N^+(CH_2)_2$-1-piperidyl, Cl^-	>290	$C_{44}H_{46}N_6Cl_2 \cdot 3H_2O \cdot 2HCl$	5.4	2.8	3.2
18	OCH_3	H	3-$N^+(CH_2)_2$-1-piperidyl, Cl^-	280	$C_{46}H_{50}N_6O_2Cl_2 \cdot 2H_2O \cdot 2HCl$	4.5[c]	2.4	1.3
19	OCH_3	CH_3	3-$N^+(CH_2)_2$-1-piperidyl, Cl^-	>290	$C_{48}H_{54}N_6O_2Cl_2 \cdot 2H_2O \cdot 2HCl$	5.3	2.7	*d*
20	OH	H	3-$N^+(CH_2)_2$-1-piperidyl, Cl^-	260	$C_{44}H_{46}N_6O_2Cl_2 \cdot 2.5H_2O \cdot 2HCl$	4.1[c]	2.1	1.7

[a] Analyses were all effected on C, H, N, Cl, except for compound **12**: C, H, N, Br. Analyses for the indicated elements were within ±0.4% of the theoretical values for the formulas provided. [b] Lengthening of sonicated calf thymus DNA. Slopes of the curves log $([\eta]/[\eta]_0)$ vs. log $(1 + 2r)$ and log $(1 + 4r)$, respectively. A bisintercalating drug is expected to give a slope value between 2.3 and 3 in the log $(1 + 4r)$ representation. See Experimental Section and text. [c] More H_2O-soluble lactate salts were used. [d] Precipitation of the dimer prevented any viscosimetric measure at this pH.

Scheme I



Scheme II



In this paper, we describe the synthesis, DNA binding properties, and antitumor activities of a series of 7*H*-pyridocarbazole dimers and of two ellipticine dimers. The DNA intercalating ability of the different dimers is discussed in relation to the position and the nature of the linking chain.

Chemistry. The synthesis of 7*H*-pyridocarbazoles was described in our preceding paper.[1] The linking chains 1,5-bis[*N*-methyl-*N*-(2-chloroethyl)amino]pentane (**5**) 1,1′-bis(3-chloro-2-hydroxypropyl)-4,4′-bipiperidine (**6**), and 1,3-bis(*N*-(2-chloroethyl)-4-piperidyl)propane (**8**) are prepared according to Scheme I.[15-17] These chains contain relatively unstable chloroalkylamino groups and are consequently stored as their hydrochlorides. The corresponding bases needed for the dimerization reactions are obtained by neutralization immediately before use.

The different dimers (Table I) were prepared by direct condensation of the corresponding 7*H*-pyridocarbazole with the appropriate dichloroalkyl chain in hot DMF (Scheme II). In this solvent, the expected dimers were only slightly soluble and consequently precipitated as relatively pure products during the reaction. Under the conditions used for the dimerization (7*H*-pyridocarbazole: linking chain, 2:1), very few monomeric species were obtained. The yields were greatly dependent on both the place of the pyridinic nitrogen in the heterocycle and the nature of the quaternizing chain.

As reported in the preceding paper, the series of 7*H*-pyrido[3,2-*c*]carbazole with the pyridinic nitrogen in

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Table II. Competition Experiments

compd	pH 5			pH 7.4		
	rf_{50}[a]	n[b]	K_{ap}	rf_{50}[a]	n[b]	K_{ap}
10	0.32	4	5×10^8	0.96	2	1×10^7
11	0.25	4	1×10^9	2.24	2	4×10^6
12	2.4	2	3×10^6	3	2	3×10^6
13	0.8	4	1×10^8	8.4	4	8×10^6
14	0.24	4	1×10^9	0.72	2	2×10^7
15	0.40	4	3×10^8	0.72	2	2×10^7
16	0.32	4	5×10^8	1.2	2	10^7
17	0.40	4	3×10^8	1.4	2	5×10^6
18	0.32	4	5×10^8	3.4	2	3×10^6
19	0.32	4	5×10^8	1.2	2	6×10^6
20	0.50	4	2×10^8	13	2	5×10^5
monomer[c]	9.5	2	1.8×10^6	20	2	3×10^5

[a] rf_{50} = ratio of total molar concentration of the studied dimer on the concentration of DNA expressed in base pairs necessary to displace 50% of DNA (calf thymus) bound ethidium dimer. [b] n = number of base pairs covered by one dimer. This number was determined by a competition experiment using ethidium bromide. See Experimental Section and text. [c] Monomer = 2-(β-ethylpiperidinyl)-7-methyl-10-methoxy-7*H*-pyrido[4,3-*c*]carbazolium chloride. See ref 1.

position 1 could not be quaternized. The quaternizations by the chloroethylpiperidyl containing chains **8** and **9** afforded high yields of pure dimers. The reactions using compounds **5** or **6** gave only moderate amounts of the expected products. Moreover, in this case, a subsequent purification by column chromatography on XAD_2 resin was necessary due to the presence of many contaminants, such as monomers, polymers, and chain-degradation products. In addition, several dimerization assays using aminoalkyl chains containing secondary amino groups were unsuccessful, because the polymerization of the chains happened to be faster than the quaternization process.

Both ellipticine dimers **22** and **23** (Table III) were synthesized like the 7*H*-pyridocarbazole dimers. The 1,1′-bis[(*N*,*N*-dimethylamino)ethyl]-4,4′-bipiperidine (**21**) was prepared by reaction of 1-(dimethylamino)-2-chloroethane on 4,4′-bipiperidine in toluene.[15]

Interactions with DNA. As discussed elsewhere,[3,6,11,18,19] the interaction of dimeric molecules with DNA is complex, due to the existence of several different modes of binding. Nevertheless, viscosimetric and fluorimetric competition experiments have already been successfully used to investigate some of the DNA binding parameters of compounds of this kind.[6,18]

Viscosimetric Studies. In order to determine whether the 7*H*-pyridocarbazole dimers were able to bisintercalate, we used viscosimetry to measure the extent to which they lengthened the helix of sonicated calf thymus DNA.[20] To study how the positive charge on the linking chain might influence the DNA binding properties of the dimer, these viscosimetric measurements were done under conditions where the nitrogens in the linking chain were fully (pH 5.0) or partially (pH 7.4) protonated.

As previously discussed[21] the relation between viscosity and length increase of DNA induced by intercalation is more complicated than expected. Theoretical treatment[22] demonstrates that, for short DNA pieces, if the DNA length increases by 3.4 Å upon intercalation the variation of viscosity must be:

$$([\eta]/[\eta]_0)^{1/3} = 1 + 2r$$

where $[\eta]$ and $[\eta]_0$ are, respectively, the intrinsic viscosities of the DNA in the presence and in the absence of the ligand, and r is the ratio of the molar concentration of bound molecules to the molar concentration of DNA expressed in nucleotides. Therefore, if $\log [\eta]/[\eta]_0$ is plotted vs. $\log (1 + 2r)$ a slope of 3 is expected.

However, for the classical intercalator ethidium bromide the slope of this plot was found significantly lower than 3 (2.3 to 2.5), although the real lengthening of the DNA helix as directly measured by electron microscopy is, as expected, 3.4 Å per intercalated molecule.[21] It was therefore concluded that viscosimetric studies allow only a semiquantitative measure of the length increase of DNA upon intercalation. Accordingly, a plot of $\log [\eta]/[\eta]_0$ vs. $\log (1 + 2r)$ is expected to give a slope between 2.3 and 3 for a monointercalation drug and a slope higher than 4 in the case of bisintercalation.

A better characterization of bisintercalating ligands can be obtained by plotting $\log [\eta]/[\eta]_0$ vs. $\log (1 + 4r)$: in this graphical representation, purely bisintercalating compounds must give a slope between 2.3 and 3. However, some bisintercalating compounds interact with DNA according to different modes with a mixture of mono- and bisintercalation.[6] In this case, intermediate slope values are expected.

Table I shows that, at pH 5, all but one of the dimers, **12**, give slopes in the $\log [\eta]/[\eta]_0$ vs. $\log (1 + 4r)$ plot which are typical of bisintercalating drugs. Compound **12**, with a C_{10} aliphatic chain, has a slope value of 1.8 in this representation which reflects a preferential monointercalating binding mode. The slope value of 2.1, for compound **20**, should be considered with reserve because the low solubility of this dimer could affect the viscosimetric measurements. However, this value may be regarded as an intermediate value between mono- and bisintercalation.

The bisintercalating character of compound **11** is verified by measuring the unwinding of supercoiled PM-2 DNA[20,23,24] at pH 5 (Figure 1). This assay determines the amount of bound drug required to convert the DNA to a form with maximal viscosity (equivalence point); the unwinding angle of the drug can be calculated from this value. Here the unwinding angle of dimer **11** was twice that of the monomer, 10-hydroxy-7*H*-pyrido[4,3-*c*]carbazolium methiodide.[1]

In contrast with the results obtained at pH 5, the dimers no longer bisintercalate at pH 7.4, where only two slope values (for compounds **11** and **15**) are significantly higher than 3, giving intermediate values between mono- and bisintercalation. This could be due, as already mentioned, to a mixture of mono- and bisintercalation. However, other hydrodynamic changes in the DNA structure might also lead to intermediate values. Further study needs to be done to establish the significance of these slopes.

The ability of a dimer to bisintercalate appears to be influenced not only by the length and chemical nature of its linking chain but also by the character of its aromatic ring system; these determinants appear to interact in a complex way. Among the different parameters of importance, the effect of the chain length was first studied.

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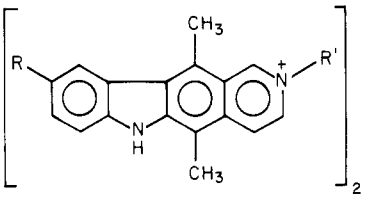
Table III. Physicochemical and DNA Binding Properties of 6*H*-Pyridocarbazole Dimers



no.	R	R'	mp, °C	formula[a]	slopes,[b] pH 5: 1 + 2r	pH 5 n	pH 5 K_{ap}	pH 7.4 n	pH 7.4 K_{ap}
22	OCH_3	-$(CH_2)_2$-1 piperidyl-, Cl^-	>290	$C_{50}H_{58}N_6O_2Cl_2 \cdot 3H_2O \cdot 2HCl$	3.4	4	2×10^8	2	10^6
23	OH	-$(CH_2)_2$-1 piperidyl-, Cl^-	>290	$C_{48}H_{54}N_6O_2Cl_2 \cdot 2HCl \cdot 2H_2O$	4.9	4	10^8	2	10^6

[a,b] See corresponding footnotes in Table I.

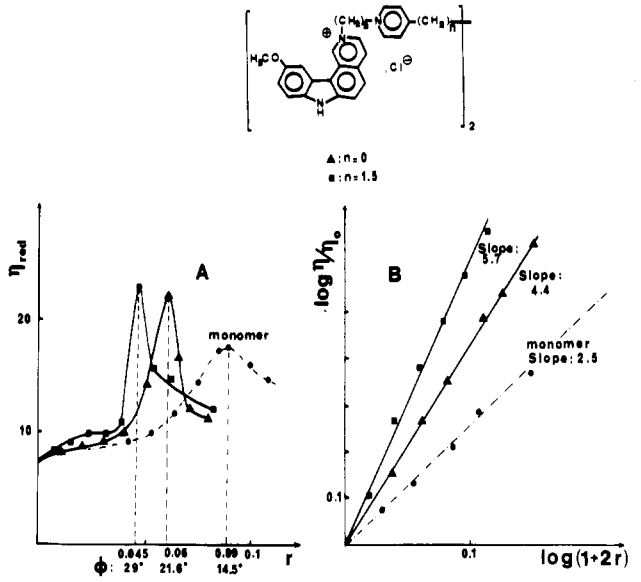


Figure 1. Unwinding and lengthening of DNA by 7*H*-pyridocarbazole dimers. The experiments were performed on compounds **10** and **11** at pH 5. For comparison purposes, the results obtained on a monomeric compound[1] (10-hydroxy-7*H*-pyrido[4,3-*c*]carbazolium methiodide) are presented. (A) unwinding of covalently closed circular DNA from PM2 phage. The reduced viscosity, η red, was measured as a function of r (number of bound molecules of the compound per nucleotide of DNA). The value of r which provided a maximum of viscosity (r_e) is equal to 0.06 in the case of compound **10** (▲) and 0.045 in the case of compound **11** (■), which leads to a DNA unwinding angle value (ϕ) of 21.6 and 29°; unwinding angles are based on a 26° unwinding angle for ethidium bromide. (B) Length increase of sonicated calf thymus DNA. The lengthening of the DNA helix is proportional to the slope of the function log (η/η_0) vs. log (1 + 2r), where η and η_0 are, respectively, the intrinsic viscosity of sonicated DNA in the presence and in the absence of the compound, and r is the ratio of the molar concentration of bound dimer to the molar concentration of DNA expressed in nucleotides. This slope is expected to be between 2.3 and 3 for a monointercalating agent and higher than 4 for a bisintercalating agent. Both experiments were performed in 0.1 M sodium acetate buffer, pH 5, and 0.1 M NaCl.

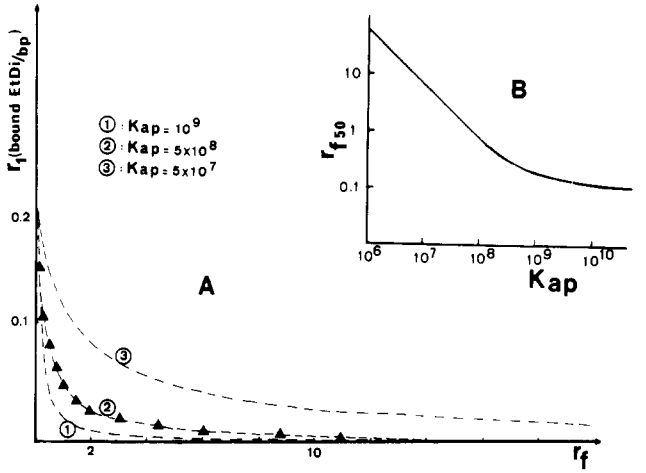


Figure 2. Displacement of the ethidium dimer (EtDi), bound to calf thymus DNA, by competition with 7*H*-pyridocarbazole dimer **10** in 0.2 M sodium acetate buffer, pH 5. (A) The concentration of bound ethidium dimer per DNA base pair (r_1) is deduced from fluorescence measurements (λ_{exc} = 540 nm; λ_{em} = 610 nm), and rf is the ratio of the total molar concentration of the competing ligand to the molar concentration of DNA base pairs, B (B = 1.6×10^{-6} M). The experimental values of r_1 (▲) are fitted with the theoretical curves computed for different values of the two parameters n, number of base pairs covered by one dimer, and K_{ap}, apparent DNA binding constant of the dimer. The value of n was previously determined by competition with ethidium bromide. Theoretical curves were then drawn for different values of K_{ap}. In the case of compound **10**, the fit was obtained for $K_{ap} = 5 \times 10^8$ M^{-1}. In this experiment, the binding of ethidium dimer to DNA is independent of pH, in the range from pH 5 to 7.4. In this range of pH, n = 4 and $K_{ap} = 2 \times 10^8$ M^{-1} for the ethidium dimer. (B) Relation between rf_{50} (ratio of total molar concentration of the studied dimer to the concentration of DNA base pairs necessary to displace 50% of the DNA-bound ethidium dimer) and K_{ap} (apparent affinity constant of the studied dimer), as calculated from the Mc Ghee and Von Hippel equations.

In the 9-aminoacridine series[25] it was observed that an alkyl chain length longer than about 7 Å enabled bisintercalation, whereas in the 2-methoxy-6-chloro-9-aminoacridine series[3] an aminoalkyl chain longer than 10 Å was apparently required for bisintercalation of the dimers.

In the present work it appears that the length of the chain is not the only limiting factor, since the presence of a positively charged nitrogen in the chain seems to be required for bisintercalation. Compound **12** with a simple alkyl chain does not bisintercalate at either pH, while other compounds bisintercalate only in acidic pH where the amino groups of the chain are protonated.

Determination of the DNA Binding Constant. As for our viscometric studies, DNA binding measurements were performed in two different buffers: 0.2 M sodium acetate buffer, pH 5, and 0.1 M NaCl, 0.1 M Tris-HCl buffer, pH 7.4.

To measure the DNA binding affinity of the dimers, we used the fluorimetric assay,[6] which is based upon the competition with ethidium dimer, whose binding affinity is the same at pH 5 and 7.4. We subsequently fit the experimental curves to those calculated from the Mc Ghee and Von Hippel equations[26] (Figure 2). In this kind of

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competition experiment, the rf_{50} or C_{50} values (respectively, ratio of total molar concentration of the displacing ligand on the concentration of DNA expressed in base pairs, and micromolar concentration of the displacing ligand necessary to displace 50% of the DNA bound ethidium dimer) are usually reported and discussed in terms of structure–activity relationships.[18]

We first measured the rf_{50} values in our series (Table II) by competition with a monomeric standard [2-(β-ethylpiperidinyl)-7-methyl-10-methoxy-7*H*-pyrido[4,3-*c*]-carbazolium chloride].[1]

At either pH, the rf_{50} is much lower for the dimers than for the monomer; e.g., compound **15**, the dimeric analogue of the monomeric standard, presents rf_{50} values 23 and 28 times lower at pH 5 and 7.4, respectively. Qualitatively, the most important feature of these results is the large decrease in DNA affinity, evidenced by a large increase in the rf_{50}, when the pH is raised from 5 to 7.4. According to the above discussion of the viscosimetric studies, titration of the amino group of the linking chain probably accounts for this large change. This is consistent with the lack of pH dependence of the rf_{50} value for **12**, which does not contain any amino group on its linking chain.

From these competition experiments, the DNA binding constants of the dimers can, in principle, be deduced as shown by Gaugain et al.[6] However, this method requires the experimental DNA displacing curve of the fluorescent ethidium dimer to be fit with a curve computed from the Mc Ghee and Von Hippel equations.[26] This computation requires both the DNA binding constant of the dimer (K_{ap}) and the number (n) of base pairs covered on DNA by the dimer. If the value of n is not known, the fitting process is no longer reliable. In addition, it has been shown[3,6,18] that different modes of DNA binding can occur with dimers corresponding to different values of n.

Furthermore, it has been demonstrated that some acridine dimers can bind to DNA as if $n = 3$.[25] It is therefore necessary to determine independently the value of n. This determination can be performed using competition experiments with ethidium bromide. When the DNA binding constant of a competing dimer is much larger than that of the ethidium monomer (at least 50-fold), the dimer displaces stoichiometrically ethidium bromide from DNA. The displacement curve only depends on the value of n and not on the binding constant of the displacing dimer. This allows an accurate determination of the number (n) for the dimer as shown in Figure 3.

This is no longer possible when both binding constants of displacing dimer and ethidium monomer are in the same range. Nevertheless, given the latter case, this ambiguity can still be resolved in the following way. From the competition with ethidium monomer one obtains several possible values of the binding constant corresponding to the possible values of n, but it has been found that only one of the possible paired values of K and n will give a good fit for the competition of the studied dimer with the ethidium dimer.

These two independent competition experiments with ethidium monomer, on the one hand, and ethidium dimer, on the other, have enabled us to determine the apparent value of the DNA binding affinity (K_{ap}) and of the number of base pairs covered by the dimer (n). These values are shown in Table II.

At pH 5, all the bisintercalating dimers give n values equal to 4, whereas **12**, a nonbisintercalating dimer, exhibits an n value of 2. In contrast, at pH 7.4, only compound **13** covers four base pairs, while all the other dimers cover two base pairs. This behavior is consistent with the previously observed change in the mode of DNA binding at the two pH values. It is interesting to notice that compound **13** has a flexible aminoalkyl chain. Further studies are in progress to investigate a possible relation between this feature and its different binding behavior. For these compounds the only values found for n are either 2 or 4. This could be related to the length, rigidity, and charge of the linking chain.

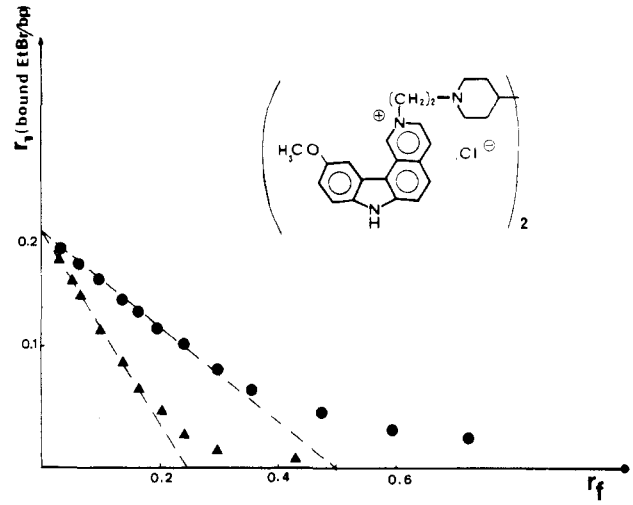

Figure 3. Displacement of ethidium bromide (EtBr) bound to calf thymus DNA, by competition with **10** at pH 5 and 7.4 and determination of n. The concentration of bound ethidium bromide per DNA base pair (r_1) is deduced from fluorescence measurements (λ_{exc} = 546 nm; λ_{em} = 590 nm) and rf is the ratio of the total molar concentration of the competing ligand to the molar concentration of DNA base pairs, B (B = 2.7×10^{-6} M). The total concentration of ethidium bromide is 2.6×10^{-6} M. When K_{ap} of the dimer is equal to/or greater than 10^7 M^{-1}, ethidium bromide is displaced stoichiometrically. The intersection of the linear part of the plot with the abscissa axis gives the value $1/n$, n being the number of base pairs covered by one dimer. In the case of **10**, at pH 5 (▲) n was found equal to 4 and at pH 7.4 (●) n was found equal to 2.

The K_{ap} values of the dimers are higher at pH 5 than at 7.4, except for compound **12** which has no nitrogen in its linking chain and which displays the same DNA affinity at either pH. At pH 5, where the dimers bisintercalate, the affinities remain in the range of 10^8 to 10^9 M^{-1}. At pH 7.4, the K_{ap} values of the dimers range from 5×10^5 to 2×10^7 M^{-1}. Therefore, most of the dimers have K_{ap} values 100 to 1000 times higher than those of the corresponding monomers. Similar results were obtained with other dimers on natural DNAs.[11] The binding affinity of the dimeric compounds is less than the maximum theoretical values obtained by summing up the free energy of interaction each subunit. This can be expected for a variety of reasons, as already discussed.[11] Finally, these results show that in these series the presence of positively charged nitrogen in the linking chain strongly influences the DNA binding affinities and the mode of DNA binding of the dimers. In contrast, no variation of the DNA binding affinity and mode of binding is observed with pH in the case of the ethidium dimer.[6]

It is interesting to point out that the displacement of one ligand on DNA by another one is dependent on both the binding affinity and the number of covered base pairs. The decrease in DNA binding affinity of these dimers does not affect their ability to displace the ethidium dimer very much (see values of rf_{50} in Table II), because of the con-

(26) J. D. Mc Ghee and P. H. Von Hippel, *J. Mol. Biol.*, 86, 469 (1974); see erratum, *ibid.*, 103, 679 (1976).

Table IV. Antitumor Properties on L1210 Murine Leukemia of 7H-Pyridocarbazole and 6H-Pyridocarbazole Dimers

no.	MTD,[a] mg/kg	opt dose[b]	L1210 T/C,[c] %
10	50	0.25	216
11	10	0.5	ns
12	10	0.5	124
13	10	0.5	ns
14	10	0.5	ns
15	25	0.8	155 (4)
16	50	0.4	157
17	5	0.5	121
18	100	0.2	130
19	15	0.5	ns
20	100	0.5	ns
22	100	0.5	ns
23	100	0.5	ns

[a] MTD = maximum tolerated dose. Determined by the method detailed in the Experimental Section. [b] Optimum intraperitoneal (ip) dose expressed as a fraction of the MTD in the antitumor assay. L1210 inoculum, 10^5 cells ip. One single dose was administered 24 h after tumor implantation. [c] % T/C = (treated survival/control) 100. All the results are highly significant. Number of 45-day survivors is provided in parentheses; ns signifies not significant.

comitant change in the number of covered base pairs and DNA binding modes. These factors are of importance if the biological activity of these derivatives is related to their ability to displace a natural ligand on DNA.

The ellipticine dimers (Table III), linked to the same rigid bis(ethylpiperidinyl) chain, also show a change in their mode of DNA binding with pH but show a larger decrease in K_{ap} at high pH. Thus, at pH 7.4, the DNA binding affinities of ellipticine dimers approximately equal those of the corresponding monomers.[27] This probably results from steric inhibition resulting from the proximity of the sugar–phosphate backbone of DNA and the position of attachment of the linking chain on ellipticine. This is apparent in the ellipticine ICpG minihelical complex structure.[28]

Antitumor Activity. L1210 murine leukemia was used for antitumor evaluations because of its good predicting value for activity in human cancer.[29] The acute toxicity of the compounds was measured by the usual procedure. The results are reported in Table IV. In contrast to their corresponding monomers,[1] several dimers exhibit a strong antitumor activity. For example, the dimeric compounds **10**, **15**, and **16** were the most potent antitumor dimers; nonetheless, their corresponding monomers were completely inactive or very slightly active, as shown in a preliminary communication.[30] A substantial percentage of cured animals is obtained with compound **15**. In addition, the therapeutic index of these dimers is remarkably high, since at doses corresponding to one-fiftieth of the maximum infralethal dose a significant antitumor effect is still observed (Figure 4).

The most striking observations in this dimer series are: (a) Only the series of dimers with chains attached at position 2 of the pyridocarbazole ring are active. (b) In this series only the derivatives with a completely rigid chain (compounds **10**, **15**, and **16**) are active. An apparently slight enhancement of chain's flexibility (compound

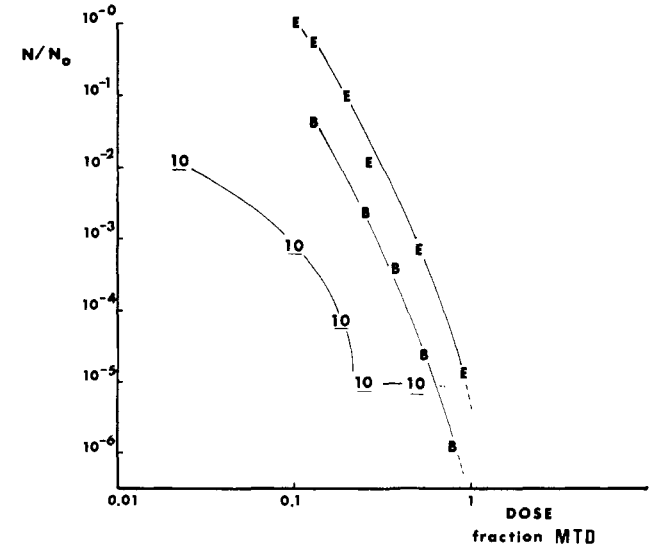

Figure 4. Compared dose–effect curves for endoxan (E), BCNU (B), and 7H-pyridocarbazole dimer **10** in the L1210 assay. N and N_0 are the number of L1210 cells in the treated and in the control animals, respectively. Doses are expressed as a fraction of the maximum tolerated dose.

11) led to a complete loss of biological activity on L1210.[31] In addition, we tested the piperidine-containing chain **21** on the same L1210 assay. This chain was found completely inactive on this system.

Conclusion

The main objective of this work was to obtain antitumor agents by preparing dimeric intercalating compounds of high DNA affinity.[32] For compounds of this type, the basic assumption of such an approach is that a high DNA binding affinity is required for antitumor activity but that this characteristic alone is not sufficient for biological activity. Indeed, we observed that dimerization led to some highly active antitumor compounds but that there was no direct correlation between DNA binding affinity and antitumor activity. Structural parameters of the dimers, such as the position of attachment of the linking chain on the aromatic ring, and the nature and rigidity of the spacer seem to strongly affect the antitumor activity. The understanding of these findings would require a detailed investigation of the mechanisms of these new derivatives at the molecular and cellular level.

Experimental Section

Melting points were determined on a Kofler apparatus and were not corrected. Structures of the products were confirmed by NMR spectra obtained on a Brüker 270-MHz spectrometer. The purity of the dimers was monitored by high-performance liquid chromatography on a Waters apparatus on a μ-Bondapak C_{18} column; the eluent was methanol/0.1 M ammonium acetate buffer, pH 6, 80:20. Where analyses are indicated only by the symbols of elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

N,N'-Dimethylcadaverine (**3**) was synthesized by the method of C. M. Bruneau and J. Lesec,[16] by quaternization of the Schiff base (**1**) of cadaverine (1,5-diaminopentane) and benzaldehyde and hydrolysis of the resulting quaternary salt **2**. **3** was obtained as a colorless liquid: 65% yield; bp 74 °C (10 mmHg).

1,5-Bis[*N*-methyl-*N*-(2-hydroxyethyl)amino]pentane (4). Ethylene oxide was bubbled at 25–30 °C in a solution of 13.8 g (0.106 mol) of dimethylcadaverine (**3**) in 80 mL of methanol until

(27) Results not shown.
(28) S. C. Jain, K. K. Bhandary, and H. M. Sobell, *J. Mol. Biol.*, **135**, 813 (1979).
(29) C. G. Zubrod, *Proc. Natl. Acad. Sci. U.S.A.*, **69**, 1042 (1972).
(30) B. P. Roques, D. Pelaprat, J. Le Guen, G. Porcher, C. Gosse, and J. B. Le Pecq, *Biochem. Pharmacol.*, **28**, 1811 (1979).
(31) C. Paoletti, J. B. Le Pecq, Nguyen-Dat-Xuong, P. Lesca, and P. Lecointe, *Curr. Chemother., Proc. Int. Congr. Chemother., 10th, 1977*, **2**, 1195 (1978).
(32) B. P. Roques, J. B. Le Pecq, D. Pelaprat, and I. Le Guen, French Patent, 78.23.801 (1978).

9.5 g (0.215 mol) was absorbed: 5.4 g (25%) of **4**, bp 119–121 °C (0.05 mmHg) was obtained by distillation of the reaction mixture.

1,5-Bis[*N*-methyl-*N*-(2-chloroethyl)amino]pentane Dihydrochloride (5·2HCl). Thionyl chloride (3.2 mL) diluted in 15 mL of $CHCl_3$ was added dropwise to a solution of 3 g (13.8 mmol) of **4** in 15 mL of $CHCl_3$. The mixture was then refluxed for 4 h and 15 mL of water was added after 150 min. The reaction was cooled to 0 °C, and the resulting solid was filtered, washed with cold $CHCl_3$, and thoroughly dried in vacuo. Recrystallization from 2-propanol gave 1.8 g (40%) of **5**·2HCl mp >260 °C. Anal. ($C_{11}H_{24}N_2Cl_2$·2HCl) C, H, N, Cl.

1,1′-Bis(3-chloro-2-hydroxypropyl)-4,4′-bipiperidine Dihydrochloride (6·2HCl). Epichlorhydrin (5 g, 53.5 mmol) was added at 30 °C to a suspension of 4.5 g (26.6 mmol) of bipiperidine in 100 mL of EtOH. The mixture was stirred for 1.5 h, and the resulting solid was filtered and dried in vacuo: yield 5.56 g. This solid was dissolved in 100 mL of methanol and 12 mL of 10 N HCl. The solution was filtered and concentrated, giving 4.1 g (38%) of **6**·2 HCl, mp >260 °C.

1,3-Bis[*N*-(2-hydroxyethyl)-4-piperidyl]propane (7). 1,3-Bis(4-piperidyl)propane (50 g, 240 mmol) was dissolved in 125 mL of methanol at 25–30 °C, and ethylene oxide was bubbled until 21 g (480 mmol) was absorbed. The methanol was evaporated and rectification gave 28 g (39%) of **7**: bp 200 °C (0.05 mmHg); mp 92 °C. Anal. ($C_{17}H_{34}N_2O_2$) C, H, N.

1,3-Bis[*N*-(2-chloroethyl)-4-piperidyl]propane Dihydrochloride (8·2HCl). Thionyl chloride (11 mL) in 20 mL of chloroform was added dropwise to 18 g of **7** in 100 mL of chloroform. The mixture was refluxed for 4 h and 100 mL of chloroform was added after 2 h. The reaction was then cooled to 0 °C, and the resulting solid was filtered, washed with chloroform, and thoroughly dried in vacuo. Recrystallization from 2-propanol gave 20.1 g (82%) of **8**·2 HCl, mp 224 °C. Anal. ($C_{17}H_{32}N_2Cl_4$·2HCl) C, H, N, Cl.

Dimerization with 1,1′-Bis(2-chloroethyl)-4,4′-bipiperidine (9): 10, 15–20, 22, and 23 Dihydrochlorides. **9** (0.7 equiv) in 1 mL of DMF was added to 1 equiv (100 mg) of the corresponding pyridocarbazole in 3 mL of DMF. The mixture was stirred overnight, the DMF was concentrated, and the resulting solid was filtered, washed thoroughly with ether, and dried in vacuo. This solid was then dissolved in 15 mL of methanol and 0.1 mL of water; 0.2 mL of 2.2 N HCl in methanol was added, and the methanol was evaporated in vacuo. The resulting dihydrochloride was thoroughly washed with ether and dried over sodium carbonate at 80 °C. **10**·2HCl: 69% yield; mp >290 °C. Anal. ($C_{46}H_{50}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl. **15**·2HCl: 50% yield; mp > 290 °C. Anal. ($C_{44}H_{46}N_6O_2Cl_2$·$2.5H_2O$·2HCl) C, H, N, Cl. **16**·2HCl: 66% yield; mp >290 °C. Anal. ($C_{44}H_{46}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl. **17**·2HCl: 60% yield; mp > 290 °C. Anal. ($C_{44}H_{46}N_6Cl_2$·$3H_2O$·2HCl) C, H, N, Cl. **18**·2HCl: 57% yield; mp >290 °C. Anal. ($C_{46}H_{50}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl. **19**·2HCl: 56% yield; mp > 290 °C. Anal. ($C_{48}H_{54}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl. **20**·2HCl: 69% yield; mp >290 °C. Anal. ($C_{44}H_{46}N_6O_2Cl_2$·$2.5H_2O$·2HCl) C, H, N, Cl. **22**·2HCl: 70% yield; mp >290 °C. Anal. ($C_{50}H_{58}N_6O_2Cl_2$·$3H_2O$·2HCl) C, H, N, Cl. **23**·2HCl: 79% yield; mp >290 °C. Anal. ($C_{48}H_{54}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl. Dimerization with 1,10-dibromodecane was performed as described for dimerization with **9**. **12** was obtained: 47% yield; mp >290 °C (47%). Anal. ($C_{42}H_{44}N_4O_2Br$·$3H_2O$) C, H, N, O, Br.

Dimerization with 5: 13·2HCl. **5**·2HCl (60 mg) was dissolved in 5 mL of water, and the solution was basified with 0.2 mL of 10 N NaOH, and extracted with 5 mL of chloroform. The organic phase was dried over calcium chloride and poured into a warm (85 °C) solution of 100 mg (2 equiv) of 10-methoxy-7*H*-pyrido[4,3-*c*]carbazole in 3 mL of DMF. The mixture was stirred overnight at 85 °C, the DMF was evaporated, and the residue (49 mg) was purified by a column chromatography on XAD_2 resin (MeOH–ammonium acetate buffer, 0.1 M, pH 5, 50:60), which gave 32 mg (25%) of pure **13**, mp >290 °C. This compound was then dissolved in 50 mL of water and poured on a 5-mL column of IRA 400 resin. The solution of the dihydrochloride was evaporated. The residue was dissolved in 10 mL of methanol, and 0.1 mL of water and 0.05 mL of 2.2 N HCl in methanol were added. The methanol was then evaporated, and the solid was washed thoroughly with ether and dried in vacuo over sodium carbonate at 80 °C. **13**·2HCl: 23% yield; mp 290 °C. Anal. ($C_{43}H_{48}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl.

Dimerization with 6: 14·2HCl. A solution of 85 mg (0.2 mmol) of **6** in 1 mL of DMF and 5 mL of methanol was added dropwise to a hot (105 °C) solution of 100 mg of 10-methoxy-7*H*-pyrido[4,3-*c*]carbazole in 3 mL of DMF. The mixture was stirred overnight at 105 °C, evaporated to 2 mL, and cooled to 5–10 °C; 2 mL of $(Me)_2CO$ was then added and 68 mg of crude **14** was obtained as an orange powder. This solid was chromatographed on a Servachrom XAD-2 (100 μm) resin column (MeOH–ammonium acetate buffer, 0.1 M, pH 6, 30:70 to 60:40). This gave 44 mg of **14**: 25% yield; mp >290 °C. This solid was transformed into its dihydrochloride by the same way as **13**. **14**·2HCl: mp >290 °C. Anal. ($C_{48}H_{54}N_6O_4Cl_2$·$3H_2O$·2HCl) C, H, N, Cl.

Dimerization with 8: 11·2HCl. **8**·2HCl (60 mg) was dissolved in 5 mL of water. The solution was basified with 0.2 mL of 10 N NaOH and extracted with dichloromethane. The organic phase was dried over calcium chloride and added dropwise to a hot (80 °C) solution of 100 mg (0.5 mM) of 10-methoxy-7*H*-pyrido[4,3-*c*]carbazole in 3 mL of DMF. The mixture was stirred overnight and cooled to 20 °C, and the resulting precipitate was filtered and thoroughly washed with ether: 82 mg (49%) of **11** was obtained, mp >290 °C. This solid was transformed into its dihydrochloride by the same way as **10**. **11**·2HCl: 49% yield; mp >290 °C. Anal. ($C_{49}H_{56}N_6O_2Cl_2$·$2H_2O$·2HCl) C, H, N, Cl.

1,1′-Bis[(*N*,*N*-dimethylamino)ethyl]-4,4′-bipiperidine Dihydrochloride (21·2HCl). Dimethylamino-2-chloroethane (15 g, 140 mmol) was dissolved in 20 mL of toluene and 9.6 g (57 mmol) of 4,4′-bipiperidine was added. The mixture was then heated and refluxed overnight, cooled, and filtered, and the filtrate was evaporated in vacuo. The resulting oil was diluted in ethyl acetate (30 mL) and acidified by HCl in ethyl acetate, until no more precipitation occurred. The solid was filtered. **21**·2HCl: 20% yield; mp >290 °C. Anal. ($C_{18}H_{38}N_4$·4HCl·$1H_2O$) C, H, N, Cl.

Fluorometric Determination of the Apparent Binding Constants by Competition with Ethidium Bromide and Ethidium Dimer. Competition with Ethidium Dimer. A photon counting spectrofluorimeter built in this laboratory was used.[33] Both excitation and emission wavelengths could be selected through a monochromator. Excitation light was provided by a Xenon lamp. The fluorescence of ethidium dimer synthesized in this laboratory[10] was excited at 540 nm ($\Delta\lambda$ = 10 nm). Emission was recorded at 610 nm ($\Delta\lambda$ = 10 nm). Measurements were done in two buffers at 20 °C: 0.2 M sodium acetate buffer, pH 5; 0.1 M NaCl, 0.1 M Tris-HCl buffer, pH 7.4.

Ethidium dimer (6.4×10^{-7} M) and calf thymus DNA (base pairs concentration: $B = 1.6 \times 10^{-6}$ M) (Sigma highly polymerized type 1, repurified by phenol extraction as previously described[34]) were equilibrated for 24 h before measurements with different concentrations of the competing drug. The concentration of bound ethidium dimer per base pair (r_1) was deduced from the fluorescent measurements as described by Gaugain et al.[6] and plotted vs. *rf* (the ratio of the total molar concentration of the competing ligand to the molar concentration of DNA base pairs); rf_{50} was defined as the *rf* value corresponding to a 50% displacement of DNA-bound ethidium dimer. The complete displacement curve could be computed as described by Gaugain et al.[6] for different binding constants, once a definite value of *n* (number of base pairs coved by one dimer) had been assumed and the computed curves had been compared to the experimental one. The *n* value was determined by competition with ethidium bromide.

Competition with Ethidium Bromide. This was performed in the same way, with ethidium bromide (2.6×10^{-6} M) and calf thymus DNA (base pairs concentration: $B = 2.7 \times 10^{-6}$ M): λ excitation = 546 nm; λ emission = 590 nm.

Viscosimetry. This was performed in the way described in the preceding paper.[1] Log $([\eta]/[\eta]_0)$ was plotted either as a function of log $(1 + 2r)$ or log $(1 + 4r)$, where $[\eta]$ and $[\eta]_0$ are the intrinsic viscosities of DNA measured in the presence and in the absence of bound agents, respectively, and *r* is the number of

(33) C. Paoletti, Thèse Doctorat ès Sciences, University of Paris (1972).

(34) G. Aubin, P. Chenaille, H. Lamonthezie, and C. Paoletti, *Biochim. Biophys. Acta*, **72**, 456 (1963).

bound agents per nucleotide on DNA. Log $([\eta]/[\eta]_0)$ is first plotted as a function of log $(1 + 2r)$. If monointercalation occurs, the slope of the curve is expected to be between 2.3 and 3. When a slope higher than 4 is obtained, log $([\eta]/[\eta]_0)$ is plotted vs. log $(1 + 4r)$. If bisintercalation is the dominant process, the slope value is between 2.3 and 3.

Biological Testing. L1210 was used to evaluate the antitumor activity of the dimers in the same way as described in the preceding paper.[1]

Acknowledgment. This work has been done under the auspices of EORTC Screening and Pharmacology Group. The authors are very grateful to T. M. Fasy for helpful discussions and A. Bouju for typing the manuscript. The technical assistance of J. Couprie and J. Morizet is gratefully acknowledged. This research was supported by grants from the Institut National de la Santé et de la Recherche Médicale, Centre National de la Recherche Scientifique, Délégation Générale à la Recherche Scientifique et Technique, Université René Descartes (Paris V), and Université Pierre et Marie Curie (Paris VI).

Nucleoside Conjugates as Potential Antitumor Agents. 3. Synthesis and Antitumor Activity of 1-(β-D-Arabinofuranosyl)cytosine Conjugates of Corticosteroids[1]

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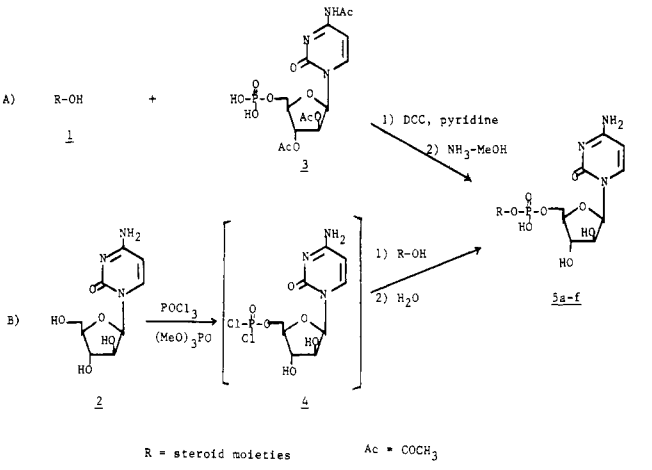
Department of Neurosurgery, Roswell Park Memorial Institute, Buffalo, New York 14263. *Received March 24, 1980*

Six 5′-(steroid-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosines have been prepared and evaluated against L1210 lymphoid leukemia in culture and in mice ($C_3D_2F_1/J$). These include the *ara*-C conjugates of 11-deoxycorticosterone (**5a**), corticosterone (**5b**), cortexolone (**5c**), fludrocortisone (**5d**), 6α-methylprednisolone (**5e**), and dexamethasone (**5f**). When the optimum dosage of *ara*-C [38 (μmol/kg)/day × 5] was given to mice bearing L1210, the ILS value found was 89%. A simple mixture of each steroid and *ara*-C gave ILS values that were on the whole significantly less than that of the parent nucleoside. However, of six conjugates, all but two (**5d** and **5f**) were more active than *ara*-C at their optimal doses. Both corticosterone- (**5b**) and cortexolone-*p*-*ara*-C (**5c**) were especially effective at the respective optimal doses of 76.7 and 115 (μmol/kg)/day × 5. These gave ILS values of 200% each. All of the conjugates were demonstrated to be enzymatically hydrolyzed to the corresponding steroid and *ara*-CMP, and the latter was further shown to be hydrolyzed to *ara*-C by phosphodiesterase I, 5′-nucleotidase, and acid phosphatase. However, they were shown to be resistant to hydrolysis by alkaline phosphatase.

As a result of our continuing efforts to develop more effective nucleoside derivatives for the treatment of malignant tumors, we have recently reported the synthesis and antitumor activity of 1-(β-D-arabinofuranosyl)cytosine (*ara*-C)[2] conjugates of cortisol and cortisone[3] and of prednisolone and prednisone.[4] The greater antitumor activities demonstrated for these conjugates as compared to those of *ara*-C alone or in combination with the steroid against L1210 lymphoid leukemia in mice have prompted us to synthesize and test conjugates of other available corticosteroids. The present report describes methods of syntheses and antitumor properties of six new *ara*-C conjugates of corticosteroids. Three of these steroids (11-deoxycorticosterone, corticosterone, and cortexolone) are naturally occurring and the other three (fludrocortisone, 6α-methylprednisolone, and dexamethasone) are synthetic corticoids.

Scheme I



Chemistry. The *ara*-C conjugates (**5a–f**) were prepeared by two methods (Scheme I). The first method (A) was the condensation of Ac_3-*ara*-CMP (**3**) with 2 molar equiv of steroid (**1**) in the presence of DCC and pyridine at room temperature for 2 days.[3,4] After the acetyl groups were removed in 2 N NH_3–MeOH, the conjugates were separated on a DE-52 (acetate) column using a HOAc gradient (0–2.0 N). The yields varied according to the steroid used, ranging from a high with the use of 11-deoxycorticosterone to lower relatively similar values for the others (Table I). In an attempt to improve the yield, the following conditions were varied: molar ratio of Ac_3-*ara*-CMP, steroid, and DCC, reaction temperature, time, and solvents. None of these increased the yield and

(1) This work has been previously presented. See Hong, C. I.; Nechaev, A.; West, C. R. In "Abstracts of Papers", 178th National Meeting of the American Chemical Society, Washington, D.C., Sept. 1979; American Chemical Society: Washington, D.C., 1979; Abstract MEDI 43.

(2) Abbreviations used are: *ara*-C, 1-(β-D-arabinofuranosyl)cytosine; *ara*-CMP, 1-(β-D-arabinofuranosyl)cytosine 5′-monophosphate; Ac_3-*ara*-CMP, N^4,2′,3′-triacetyl-1-(β-D-arabinofuranosyl)cytosine 5′-monophosphate; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DOC-*p*-*ara*-C, 5′-(11-deoxycorticosterone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosine; corticosterone-*p*-*ara*-C, 5′-(corticosterone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosine; cortexolone-*p*-*ara*-C, 5′-(cortexolone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosine; fludrocortisone-*p*-*ara*-C, 5′-(fludrocortisone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosine; MePred-*p*-*ara*-C, 5′-(6α-methylprednisolone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosine; DXM-*p*-*ara*-C, 5′-(dexamethasone-21-phosphoryl)-1-(β-D-arabinofuranosyl)cytosine; ip, intraperitoneally.

(3) Hong, C. I.; Nechaev, A.; West, C. R. *Biochem. Biophys. Res. Commun.* **1979**, *88*, 1223.

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