# A New Ionizable Chromophore of 1,4-Bis(alkylamino)benzo[g]phthalazine Which Interacts with DNA by Intercalation

M. Pons,*,† L. Campayo,‡ M. A. Martínez-Balbas,§ F. Azorin,§ P. Navarro,‡ and E. Giralt†

*Departament de Química Orgànica, Facultat de Química, Universitat de Barcelona, Martí i Franquès, 1-11, E-08028 Barcelona, Spain, Grup de Química Macromolecular, Centro de Investigación y Desarrollo, C.S.I.C., ETSEIB, Diagonal, 650, E-08028 Barcelona, Spain, and Instituto de Química Médica, Consejo Superior de Investigaciones Cientificas, Juan de la Cierva, 3, E-28006 Madrid, Spain.* *Received November 27, 1989*

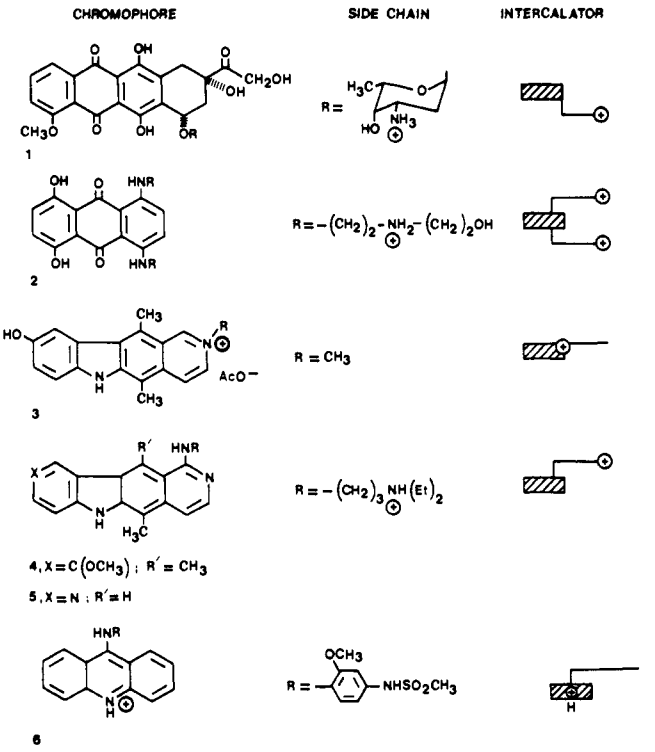
The tricyclic heteroaromatic nucleus of 1,4-bis(alkylamino)benzo[g]phthalazine can be protonated at physiological pH, depending on the nature of the side chains. The interaction of the 3-methoxypropyl derivative with calf thymus and closed, circular DNA has been studied with UV-vis spectroscopy and NMR. The effect of drug binding on the topology of closed, circular DNA was determined by topoisomerase-I catalyzed relaxation of the complex followed by gel electrophoresis. The results strongly support intercalative binding and suggest that this series of compounds are promising targets for anticancer activity evaluation.

## Introduction

The intercalation of planar aromatic molecules with the DNA double helix[1] is considered to be important in mutagenesis, carcinogenesis,[2] and the medicinal action of antibacterial,[3] antiparasitic,[4,5] and antineoplastic[6] drugs. Intercalators contain a planar chromophore with two to four fused aromatic rings, with an optimum of three, but they also ought to possess other features such as a proper quadrupole moment and groups available for hydrogen bonding.[7] A positive charge is generally needed for activity.[8,9] Scheme I illustrates several combinations of aromatic and cationic moieties found in known intercalators used in therapy. Adriamycin (1) and mitoxantrone (2) have cationic side chains linked to a neutral tricyclic aromatic system and show excellent antineoplastic activity[10,11] while 1,4-diaminoanthraquinone is inactive by itself. In 2-methyl-9-hydroxyellipticinium (3), used in the treatment of breast cancer,[12] the cationic moiety arises from the quaternized aromatic 2-nitrogen belonging to its own chromophore. Analogues with a nonquaternized, related chromophore but with an appended cationic side chain, such as 4 and 5, also exhibit high antitumor activities.[13,14] Amsacrine (6) has a basic chromophore of 9-aminoacridine, whose heteroaromatic ring is easily protonated (p$K_a$ 7.4)[15] and binds tightly to double-stranded DNA by intercalation as shown by helix-unwinding studies with closed, circular DNA[16] and by NMR.[17] In this class of drugs the side chains modulate the biological activity[18] and the basicity of the chromophore.[19] We have previously found that the tricyclic heteroaromatic nucleus of 1,4-bis(alkylamino)benzo[g]phthalazine (7) (Scheme II) containing two of the four conjugated nitrogens as part of the ring, has basic character.[20,21] The crystalline structure of the dimeric tetrachloro cobaltate salt 8 obtained by treatment of **7b**·HCl with $Co^{II}Cl_2$ in neutral media showed that the proton was clearly located at the ring nitrogen atoms, with the positive charge being shared among the four nitrogens, endocyclic and exocyclic.[22] The presumed contribution to structure 7 of the 2H- and 3H-tautomeric cations 7′ and 7″, which in solution may exist in fast equilibrium,[23] could explain the formation of the dimeric salt 8.

The 1,4-bis(alkylamino)benzo[g]phthalazine system is structurally related to the terminal aromatic ring of mitoxantrone (2) and 1-alkylaminoellipticines 4 and 5. Furthermore, the p$K_a$ of the heterocyclic group depends on the nature of the side chains as in the 9-aminoacridine series. The p$K_a$ of **7a** (with cationic side chains) is 6.20 and that of **7b** and **7c** (with neutral chains) is 8.3. Con-

**Scheme I**

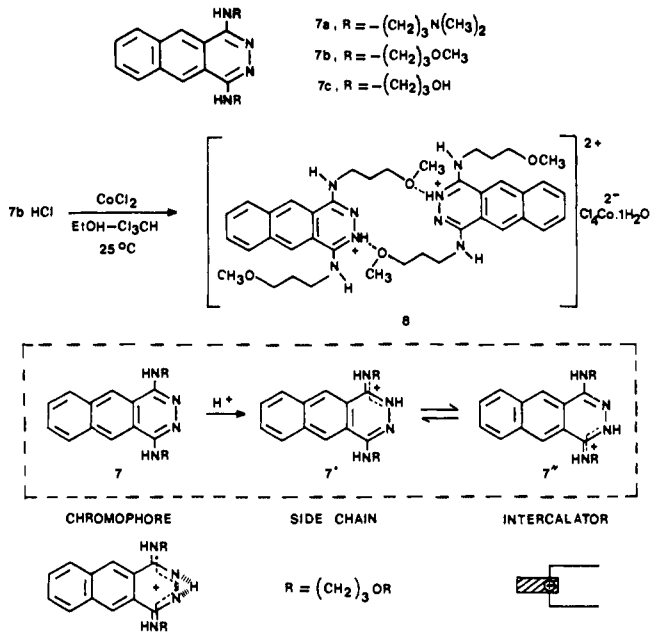



† Universitat de Barcelona.
‡ Instituto de Química Médica.
§ Centro de Investigación y Desarrollo.

(1) Wilson, W. D.; Jones, R. L. In *Intercalation Chemistry*; Whittingham, M. S.; Jacobson, A. J., Eds.; Academic Press: New York, 1981; Chapter 14.
(2) Waring, M. J. *Annu. Rev. Biochem.* **1981**, *50*, 159.
(3) Waring, M. J. In *The Molecular Basis of antibiotic Action*, 2nd ed.; Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H., Waring, M. J., Eds.; Wiley: London, 1981.
(4) Thompson, P. E.; Werbel, L. M. *Antimalarial Agents*; Academic Press: New York, 1972.
(5) Steck, E. A. In *The Chemotherapy of Protozoan Diseases*; U.S. Government Printing Office: Washington, DC, 1973; pp 23, 77-123, 177.
(6) Denny, W. A.; Baguley, B. C.; Cain, B. F.; Waring, M. J. In *Mechanism of Action of Anticancer Drugs*; Neidle, S., Waring, M. J., Eds.; McMillan: London, 1983.
(7) Miller, K. J.; Newlin, D. D. *Biopolymers* **1982**, *21*, 633.
(8) Lerman, L. S. *J. Mol. Biol.* **1961**, *3*, 18.
(9) Waring, M. J. *J. Mol. Biol.* **1970**, *54*, 247.
(10) Arcamone, F. *Doxorubicin Anticancer Antibiotics*; Academic Press: New York, 1981.
(11) Smith, I. E. *Cancer Treat. Rev.* **1983**, *10*, 103.

0022-2623/91/1834-0082$02.50/0 

**Scheme II**




sequently, **7b** and **7c** may be ionized at physiological pH, as occurs with amscarine **6**.

A variety of methods have been used to investigate ligand–DNA complex formation in solution.[3] Spectrophotometric methods allow the measurement of binding constants and cooperativity effects while the binding mode is most easily determined by NMR (see Table I in ref 17) or by the effect of the drug on the sedimentation velocity of linear DNA or the unwinding of closed circular DNA.[2] In this work, taking **7b** as a model, we have studied the binding of this new tricyclic ionizable chromophore by using spectroscopic methods as well as measurements of the topological consequences of its binding to closed circular DNA based on the use of electrophoresis to separate the topoisomers. Compounds **7a–c** showed citostatic activity against HeLa cells ($ED_{50}$ = 1.25, 10, and 25 μg/mL).[20] Related compounds that also contain the benzo[g]-phthalazine nucleus have recently been shown to possess antitumoral activity, both in vitro and in vivo as well as antitripanosomial and antitricomonicidal activity in vitro.[24]

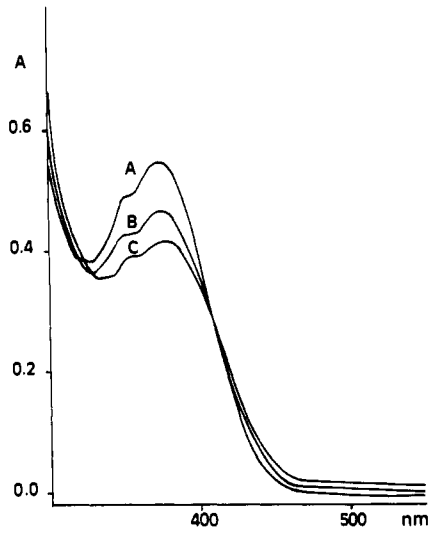



**Figure 1.** Binding of **7b** to calf thymus DNA. Spectra correspond to a 55 mol $L^{-1}$ solution of **7b** in phosphate buffer (10 mmol $L^{-1}$) containing 10 mmol $L^{-1}$ NaCl (pH = 6) at three different phosphate to drug ratios: A, 0; B, 3.3; C, 6.05. Isosbestic behavior was maintained up to a ratio of 6.05 or up to 15.6 if the spectra were corrected for light scattering.

**Table I.** $^1H$ NMR Chemical Shifts of **7b** in Different Solvents

| | DMSO-$d_6$[a] | DMSO-$d_6$[b] | $D_2O$[c] |
|---|---|---|---|
| H-5, H-10 | 8.80 | 9.42 | 8.70–8.80 |
| H-6, H-9 | 8.13 | 8.14 | 8.15 |
| H-7, H-8 | 7.71 | 7.82 | 7.90 |
| $CH_2$ (α) | 3.00 | 3.52 | 3.57 |
| $CH_2$ (β) | 1.91 | 2.00 | 2.05 |
| $CH_2$ (γ) | 3.48 | 3.50 | 3.63 |
| $OCH_3$ | 3.28 | 3.28 | 3.40 |

[a] Sample contains one drop of 1 M KOH. Chemical shifts in ppm downfield from TMS. [b] Sample contains one drop of concentrated $H_2SO_4$. [c] pH = 6. Extrapolated to infinite dilution. Reference is DSS.

## Spectroscopic Experiments

Addition of **7b** to a sample of sonicated calf thymus DNA in phosphate buffer (10 mmol $L^{-1}$, pH = 6.0) increases its melting temperature (i.e. the half-point of the thermal denaturation of double-helical DNA) from 65 to 70 °C with a **7b** to DNA ratio of 0.33 and to 76 °C when the ratio is increased to 0.66. This implies that **7b** stabilizes the double helix, a common feature of DNA-binding chemotherapeutic drugs.

Binding of **7b** to calf thymus DNA induces changes in the UV-visible spectra of the drug. At pH = 6.0 in phosphate buffer containing 10 mmol $L^{-1}$ NaCl, addition of DNA induces hypochromicity (up to a maximum of 42%) and a red shift of the absorption maximum, initially at 372 nm. An isosbestic point is apparent at ca. 420 nm, indicating a two-state equilibrium (Figure 1). Dipole interaction between two chromophores can induce hypochromic or hyperchromic effects. Stacking generally induces hypochromicity.[25]

At pH = 8 in TRIS buffer containing 200 mmol $L^{-1}$ NaCl (the same pH and ionic strength of the experiments with topoisomerase; see below), the maximum hypochromicity

(12) Paoletti, C.; Le Pecq, J. B.; Dat-Xoung, N.; Juret, P.; Garnier, H.; Amiel, J. L.; Rouesse, J. *J. Recent Results Cancer Res.* **1980**, *74*, 107.
(13) Lidereau, R.; Chermann, J. C.; Gruest, J.; Montagnier, L.; Ducrocq, C.; Rivalle, C.; Bisagni, E. *Bull. Cancer* **1980**, *67*, 1.
(14) Chermann, J. C.; Gruest, J.; Montaignier, L.; Wendling, F.; Tambourin, P.; Perin, J.; Pochon, F.; Ducrocq, C.; Rivalle, C.; Bissagni, E. *C. R. Hebd. Seances Acad. Sci. Ser. D.* **1977**, *285*, 945.
(15) Atwell, G. J.; Cain, B. F.; Denny, W. A. *J. Med. Chem.* **1977**, *20*, 1128.
(16) Waring, M. J. *Eur. J. Cancer* **1976**, *12*, 995.
(17) Feigon, J.; Denny, W. A.; Leupin, W.; Kearns, D. R. *J. Med. Chem.* **1984**, *27*, 450.
(18) Baguley, B. C.; Denny, W. A.; Atwell, G. H.; Cain, B. F. *J. Med. Chem.* **1981**, *24*, 520.
(19) Albert, A. In *Selective Toxicity*; J. Wiley and Sons: New York, 1973.
(20) Campayo, L.; Navarro, P. *Eur. J. Med. Chem.—Chim. Ther.* **1986**, *21*, 143.
(21) Amat, P. A.; Campayo, L.; Navarro, P.; Menéndez, M.; de Paz, J. L. G. 10th International Symposium on Medicinal Chemistry, Budapest, 1988; p 258.
(22) Campayo, L.; Navarro, P.; Cano, F. H.; Foces-Foces, C. *J. Chem. Soc. Perkin Trans. 2* **1987**, 569.
(23) Elguero, J.; Marzin, C.; Katritzky, A. R.; Linda, P. The tautomerism of heterocycles. In *Advances in Heterocyclic Chemistry*; Katritzky, A. R., Bouton, A. J., Eds.; Academic Press: New York, 1976; pp 81–163.
(24) Navarro, P.; Campayo, L.; Alvarez, I.; Escario, J. A. Spanish Patent No. 8901705, 1989.
(25) Leonard, N. J. *Acc. Chem. Res.* **1979**, *12*, 423.

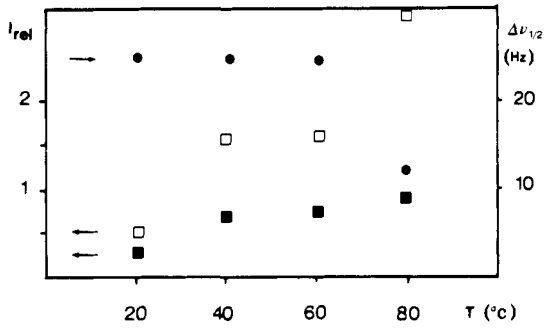



**Figure 2.** Temperature effect on the $^1H$ NMR spectra of **7b** (2.8 mmol $L^{-1}$) in the presence of DNA (14.0 mmol $L^{-1}$): filled squares, intensity of the $\gamma$-$CH_2$ signal normalized with respect to the intensity of DSS used as internal standard; open squares, intensity of the $OCH_3$ signal relative to DSS; filled circles, half-width of the $OCH_3$ signal. Spectra were run in $D_2O$ buffer (10 mmol $L^{-1}$ phosphate, 500 mmol $L^{-1}$ NaCl, pH = 6).

at the absorption maximum (376 nm) is 19% and an isosbestic point appears at 402 nm. Data were converted to values of $r$ (mol of drug bound per mol of nucleotide) and $c$ (the free-drug concentration), and the corresponding Scatchard plots were analyzed according to the model of McGhee and von Hippel[26] by nonlinear fitting of the data to eq 1 to obtain the binding constant to an isolated site and the apparent site size.

$$r/c = k(1 - nr)\left(\frac{1 - nr}{1 - (n - 1)r}\right)^{n-1} \quad (1)$$

The value of the binding constant is ca. $2 \times 10^4$ $mol^{-1}$ L and the differences between the two sets of conditions are smaller than the experimental error, probably because of the compensation of pH and ionic strength effects. This binding constant falls short of the values for strong binding (intercalation) of mitoxantrone, ethidium, or the acridines but is comparable with the reported value for chloroquine.[27] Nevertheless it should be noticed that **7b** is a monocation while mitoxantrone, with a comparable aromatic moiety, and chloroquine, with only two fused rings, are dications. The estimates for the size parameter, 4.6 and 5.8 at pH = 6 and 8, respectively, agree with the expected value of 4 for an intercalative drug subject to the neighbor-exclusion effect. The same range of values is also found in chloroquine and related drugs of low binding constant.[27]

The $^1H$ NMR chemical shifts of **7b** in $D_2O$ and DMSO-$d_6$ are shown in Table I. DNA causes strong broadening of the $^1H$ NMR signals from **7b**, especially in the aromatic part. Additionally, the reduced sensitivity suggests that the observed signals come only from a fraction of the drug present.

To test this hypothesis, a sample containing 2.8 mmol $L^{-1}$ **7b**, 14.0 mmol $L^{-1}$ DNA, and 500 mmol $L^{-1}$ NaCl (pH = 6) was heated to 79 °C. The intensities and line widths of the drug signals changed as shown in Figure 2. The changes are qualitatively the same for all the signals although quantitation is only possible for the aliphatic resonances that remain relatively sharp even at room temperature. The increase in the intensity between room temperature and 40 °C is not followed by a concomitant reduction in line width so that it corresponds to an increase in the area of the observed signal. The drastic reduction of the line width between 60 and 79 °C may be related to denaturation of DNA. These observations indicate that, at room temperature, the exchange between free and bound drug is slow on the NMR time scale and the fraction of the drug that is free or bound in a way that allows rapid exchange is only ca. 25% of the total. The remaining 75% is bound in a way that gives slow exchange and signals too broad to be detected.

**Table II.** $^1H$ NMR Chemical Shifts (ppm from DSS) of **7b** in Aqueous Solutions under Different Conditions of Temperature, Ionic Strength, and Concentration

| | 25 °C, no NaCl | | 0.5 M NaCl, 0.5[a] | |
|---|---|---|---|---|
| | 1[a] | 0.2[a] | 25 °C | 80 °C |
| H-5, H-10 | 8.22 | 8.56 | 7.87 | 8.58 |
| H-6, H-9 | 7.78 | 7.85 | 7.56 | 7.83 |
| H-7, H-8 | 7.93 | 8.10 | 7.61 | 8.08 |
| $CH_2$ ($\alpha$) | 3.44 | 3.53 | 3.30 | 3.55 |
| $CH_2$ ($\beta$) | 2.05 | 2.05 | 2.01 | 2.07 |
| $CH_2$ ($\gamma$) | 3.67 | 3.65 | 3.66 | 3.66 |
| $OCH_3$ | 3.44 | 3.40 | 3.45 | 3.40 |

[a] Concentration of **7b** in mg $mL^{-1}$.

The chemical shifts of **7b** measured in the presence of DNA are downfield from those measured in a sample of the same concentration but without DNA. This effect has to be interpreted in terms of reduced autostacking of the drug due to the lower concentration of free **7b** resulting from binding to DNA. Table II shows the concentration dependence of the chemical shifts of **7b** in $D_2O$ as well as the effects of temperature and ionic strength. These results indicate strong aggregation of **7b** in aqueous solution induced by hydrophobic interactions and stacking of the aromatic ring of the drug. This behavior is typical of many intercalative drugs.[28-30]

Plots of $(\Delta\delta/[\mathbf{7b}]_0)^{1/2}$ vs $\Delta\delta$ (were $[\mathbf{7b}]_0$ is the total concentration of **7b** and $\Delta\delta$ is the difference between the observed chemical shift and the value extrapolated to infinite dilution in $D_2O$) are linear. With the equation of Dimicoli and Hélène,[31] the $x$-axis intercept ($x_0 = 2\Delta\delta_{dimer}$) gives the chemical shift difference between the free molecule and the stacked dimer. The slope is $(K/x_0)^{1/2}$, where $K$ is the self-association constant and its value is 205 ± 20 $mol^{-1}$ L.

The crystal structure[22] of the salt of **7b** with $CoCl_4^{2-}$ provides a model for the aggregates found in solution. In the crystal the aromatic rings are stacked and the long aliphatic chains are packed perpendicular to the plane of the rings. This disposition locates the $\gamma$ methylene protons and the terminal methoxy groups near the plane of the aromatic ring of the *next* molecule in the stack. This structure is compatible with the observation of upfield shifts in the aromatic protons and the $\alpha$ methylene protons and downfield shifts in the $\gamma$ methylene and methoxy protons.

## Experiments with Polyadenilic Acid

**7b** can bind to polyadenilic acid (poly A) and the exchange is also slow. Increasing the temperature destroys the complex and causes an increase in the intensity of the signal from the free drug that reaches a maximum at 40 °C. The slowly exchanging component represents ca. 50% of the total drug at a **7b** to poly A ratio of 1:5 and room

(26) McGhee, J. D.; Von Hippel, P. H. *J. Mol. Biol.* **1974**, *86*, 469.
(27) Cohen, S. N.; Yielding, K. L. *J. Biol. Chem.* **1965**, *240*, 3123.
(28) Barbet, J.; Roques, B. P.; Combrisson, S.; Le Pecq, J. B. *Biochemistry* **1976**, *15*, 2642.
(29) Gaugain, B.; Markovits, J.; LePecq, J. B.; Roques, B. P. *Biochemistry* **1981**, *20*, 3035.
(30) Gabbay, E. J.; Sanford, K.; Baxter, C. S.; Kapicak, L. *Biochemistry* **1973**, *12*, 4021.
(31) Dimicoli, J. L.; Hélène, C. *J. Am. Chem. Soc.* **1973**, *95*, 1036.

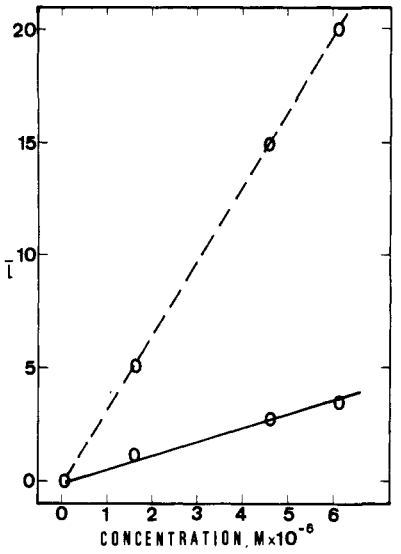



**Figure 3.** The average linking number difference ($L$) of the distributions of topoisomers generated by topoisomerase-I treatment of plasmid DNA in the presence of **7b** (solid line) or ethidium bromide (dashed line) is shown as a function of the concentration of the drug at which relaxation was performed.

temperature. Poly A induced hypochromicity (8%) is much smaller than in the case of DNA under the same conditions of buffer and concentration.

An essential difference between the two systems is indicated by the observation of multiple resonances for the aromatic protons in the $^1$H NMR spectra of **7b** in the presence of poly A at room temperature. These signals merge into the usual peak pattern on heating. Multiple resonances were also observed in the presence of denatured DNA at 80 °C. These observations imply the existence of at least two different types of binding to polyadenilic acid, both giving slow exchange, but with very different local mobility. Reported correlation times for fluoroquine bound to DNA are shorter than those found for the same molecule bound to polyadenilic acid.[32] Modified intercalation models have been suggested[33,34] to account for strong binding of acridines to single-stranded RNA and denatured DNA.

## Effects of Drug Binding on the Topology of Covalently Closed, Circular DNA

By modifying the helical repeat of the DNA molecule, intercalating molecules promote removal and reversal of the supercoiling of covalently closed, circular DNA.[2,35,36] To study the effects of drug binding on the topology of closed, circular DNA, native, as well as relaxed pBR322 were electrophoresed through agarose gels run in the presence of **7b** (data not shown). Binding of the drug resulted in a relaxation of native, negatively supercoiled pBR322 DNA. Relaxed pBR322 becomes positively supercoiled when analyzed under the same conditions. These results show that **7b** actually induces a positive superhelical twist rather than to simply loosen the helical structure of DNA. Quantitation of the effect of drug intercalation on the superhelical state of DNA with topoisomerase-I in the presence of different concentrations of **7b**. After relaxation, the drug was removed by phenol extraction. The topoisomers generated were then removed by phenol extraction. The topoisomers generated were then resolved by agarose gel electrophoresis and the mean of the linking number difference of each topoisomer distribution was determined. Figure 3 shows the average differences in linking number as a function of the drug to DNA ratio at which relaxation was carried out. The result of a control experiment with ethidium bromide under the same experimental conditions is included as a comparison. In both cases there is a linear dependence of the observed unwinding with the drug to DNA ratio. The change in the number of superhelical turns induced by ethidium bromide is 6 times that caused by **7b**. This quatitative difference is mainly accounted for by the fact that the binding constant of ethidium under the high ionic strength conditions of the topoisomerase reaction[37] is estimated to be 8 times that of **7b**. Therefore the unwinding angle of **7b**, i.e. the degree of unwinding of the DNA duplex as each drug molecule is bound, falls near the one of ethidium[38] and other intercalators,[2] leaving little doubt about the intercalative binding of the benzo[g]phthalazine chromophore to DNA.

## Conclusions

We have demonstrated that **7b**, a member of a new family of ionizable chromophores which contain the nucleus of 1,4-diaminobenzo[g]phthalazine and show cytostatic acivity, binds to DNA by intercalation, causing a positive superhelicoidal twist in closed, circular DNA with an unwinding angle comparable to that of ethidium bromide. This binding caused the expected hypochromicity in the UV spectra and an stabilization of DNA with respect to thermal denaturation. Bound and free drug molecules exchange slowly on the time scale of NMR and therefore only the signals from the free drug could be observed. Work is in progress to further characterize the binding by NMR with synthetic oligonucleotides.

## Experimental Section

**Sonication of DNA.** Calf thymus DNA (Sigma) was dissolved at concentrations between 4 and 6 mg mL$^{-1}$ in phosphate buffer (10 mmol L$^{-1}$, pH = 6.0) containing 0.2 mmol L$^{-1}$ EDTA and sonicated in an ice bath for 5 min in 30-s bursts with 30-s delays to prevent heating of the sample. The sample was centrifuged and the concentration was determined spectrophotometrically.

**Melting of DNA.** Melting temperatures were determined at 256 nm on sonicated DNA at a concentration of 88 μmol L$^{-1}$ in phosphate buffer (10 mmol L$^{-1}$, pH = 6) with 0.2 mmol L$^{-1}$ of EDTA. The maximum hyperchromicity was 44%. Samples containing **7b** were measured at 261 and 273 nm, where maximum changes could be detected. Drifts due to changes in the spectrum of **7b** could be minimized by recording the spectra on the second-derivative mode.

**UV–Vis Measurements.** Binding experiments were performed with unsonicated DNA under two sets of conditions (A and B) modeling those of the NMR and the topoisomerase experiments. In each case DNA was dialyzed against the corresponding buffer and samples of different **7b** to DNA ratios and constant concentration of drug were prepared by mixing a solution of **7b** with a large excess of DNA and a DNA-free solution of **7b** of the same concentration. The conditions were as follows: (A) 10 mmol L$^{-1}$ phosphate, 10 mmol L$^{-1}$ NaCl, 1 mmol L$^{-1}$ EDTA, pH = 6; maximum DNA excess, 36; drug concentration, 55 mol L$^{-1}$; (B) 10 mmol L$^{-1}$ TRIS, 200 mmol L$^{-1}$ NaCl 1 mmol L$^{-1}$ EDTA, pH = 8; maximum DNA excess, 214; drug concentration, 45 μmol L$^{-1}$.

**NMR Experiments.** The NMR experiments were performed at pH = 6, well below the p$K_a$ value of **7b** (8.3 in $H_2O$–ethanol

(32) Bolton, P. H.; Mirau, P. A.; Shafer, R.; James, T. L. *Biopolymers* 1981, *20*, 435.
(33) Dourlent, M.; Hélène, C. *Eur. J. Biochem.* 1971, *23*, 86.
(34) Blake, A.; Peacocke, A. R. *Biopolymers* 1968, *6*, 1225.
(35) Bauer, W. R.; Vinograd, J. *J. Mol. Biol.* 1968, *33*, 141.
(36) Kitchen, S. E.; Wang, Y. H.; Baumstark, A. L.; Wilson, W. D.; Boykin, D. W. *J. Med. Chem.* 1985, *28*, 940.
(37) LePecq, J. B.; Paoletti, C. *J. Mol. Biol.* 1967, *27*, 87.
(38) Wang, J. C. *J. Mol. Biol.* 1974, *89*, 783.

(9:1)) in order to prevent artifactual shifts due to DNA-induced $pK_a$ changes.[39] DNA (from calf thymus) was sonicated to reduce the viscosity of the solutions.

Aliquots (0.5 mL) of a stock sonicated DNA solution were freeze-dried, treated with $D_2O$ to exchange the residual water, and relyophilized. A solution of **7b** in $D_2O$ was diluted so that the required amount of product was present in 0.5 mL of solution and this was used to dissolve the DNA. Accuracy of this protocol was ensured by checking the weights of the solutions.

Spectra were run on a Varian XL-200 instrument with presaturation of the residual water peak and the chemical shifts were measured with respect to internal DSS.

**Relaxation Experiments.** Plasmids were propagated in *Escherichia coli* strain HB101. Plasmid DNA was prepared by conventional procedures.[40] Topoisomerase-I (BRL) treatment in the presence of **7b** or ethidium bromide was performed as described by Peck and Wang.[41] After relaxation, the drug was removed by phenol extraction and the DNAs were precipitated with ethanol and redissolved in 10 mmol $L^{-1}$ TRIS, 1 mmol $L^{-1}$ EDTA, pH = 7.5, and the distributions of topoisomers were resolved by one-dimensional gel electrophoresis. Purified DNAs were electrophoresed through a 1% agarose–TBE gel containing 1.25 $\mu g\ mL^{-1}$ of the intercalator chloroquine phosphate (SIGMA). Electrophoresis was carried out for 20 h at 1.6 V $cm^{-1}$. After electrophoresis, gels were stained with 0.5 $\mu g\ mL^{-1}$ ethidium bromide and photographed under UV light. Densitometric scans of the negatives were carried out in a Joyce-Loebl densitometer.

**Acknowledgment.** Financial support from the Comisión Interministerial para la Ciencia y la Tecnologia (CICYT Grant No. BT 86-0018) is gratefully acknowledged.

**Registry No.** **7a**, 103344-05-4; **7b**, 103344-04-3; **7c**, 103344-03-2.

(39) Giralt, E.; Pons, M.; Andreu, D. *Bioorg. Chem.* **1985**, *13*, 171.
(40) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning. A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY, 1982.
(41) Peck, L.; Wang, J. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 6206.

# Nicotinamide Ethers: Novel Inhibitors of Calcium-Independent Phosphodiesterase and [$^3$H]Rolipram Binding

Fredric J. Vinick,* Nicholas A. Saccomano, B. Kenneth Koe, Jann A. Nielsen, Ian H. Williams, Peter F. Thadeio, Stanley Jung, Morgan Meltz, Jonathan Johnson, Jr., Lorraine A. Lebel, Lorena L. Russo, and David Helweg

*Pfizer Central Research, Groton, Connecticut 06340.* *Received May 8, 1990*

The synthesis and biological properties of a series of nicotinamide ethers are described. These compounds, structurally novel calcium-independent phosphodiesterase inhibitors, also inhibit the binding of [$^3$H]rolipram to rat brain membranes and reverse reserpine-induced hypothermia in the mouse. Several compounds exhibited potent in vivo activity comparable to the standard agent, rolipram.

In previous papers[1,2] we described catechol ether 2-imidazolidinone derivatives which constitute a very interesting class of calcium-independent phosphodiesterase (CaIPDE) inhibitors. Not only are these agents selective for the particular form of the enzyme responsible for hydrolysis of brain cAMP but they also potently inhibit [$^3$H]rolipram binding.[3] Rolipram is known to inhibit CaIPDE and elevate brain levels of cAMP.[4-8] It also increases norepinephrine (NE) synthesis and metabolism.[9] Increased neuronal cAMP concentrations and NE turnover may overcome some of the neurochemical perturbations associated with depression. Rolipram is active in animal models which detect antidepressant drugs, such as reserpine-induced hypothermia,[10] and has been investigated clinically.[11-14]

We now report a new class of compounds, structurally distinct from rolipram and other catechol ethers, that selectively inhibit CaIPDE and [$^3$H]rolipram binding. These novel agents, the 2-(aryloxy)alkoxynicotinamides were derived from the structure of another known inhibitor of calcium-independent phosphodiesterase, nitraquazone.[15]

Our rationale was to mimic the properties of nitraquazone with a ring-opened structure. We felt that the N-1

(1) Koe, B. K.; Lebel, L. A.; Nielsen, J. A.; Russo, L. L.; Saccomano, N. A.; Vinick, F. J.; Williams, I. H. *Drug Dev. Res.*, in press.
(2) Saccomano, N. A.; Vinick, F. J.; Koe, B. K.; Nielsen, J. A.; Whalen, W. M.; Meltz, M.; Phillips, D.; Thadeio, P. F.; Jung, S.; Chapin, D. S.; Lebel, L. A.; Russo, L. L.; Helweg, D. A.; Johnson, J. L., Jr.; Ives, J. L.; Williams, I. H. *J. Med. Chem.*, in press.
(3) Schneider, H. H.; Schmeichen, R.; Brezinski, M.; Seidler, J. *Eur. J. Pharmacol.* **1986**, *127*, 105.
(4) Davis, C. *Biochim. Biophys. Acta* **1985**, *797*, 354.
(5) Schwabe, U.; Miyake, M.; Ohga, Y.; Daly, J. W. *Mol. Pharmacol.* **1976**, *12*, 900.
(6) Nemoz, G.; Prigent, A.-F.; Moueqqit, M.; Fougier, S.; Macovschi, O.; Pacheco, H. *Biochem. Pharmacol.* **1985**, *34*, 2997.
(7) Randt, C. T.; Judge, M. E.; Bonnet, K. A.; Quatermain, D. *Pharmacol. Biochem. Behav.* **1982**, *17*, 677.
(8) Schneider, H. H. *Biochem. Pharmacol.* **1984**, *33*, 1690.
(9) Kehr, W.; Debus, G.; Neumeister, R. *J. Neural Trans.* **1985**, *63*, 1.
(10) Wachtel, H. *Neuropharmacol.* **1983**, *22*, 267.
(11) Zeller, E.; Stief, H.-J.; Pflug, B.; Sastre-y-Hernandez, M. *Pharmacopsychiatry* **1984**, *17*, 188.
(12) Eckmann, F.; Fichte, K.; Meye, U.; Sastre-y-Hernandez, M. *Curr. Ther. Res.* **1988**, *43*, 291.
(13) Hebenstreit, G. F.; Fellerer, K.; Fichte, K.; Fischer, G.; Geyer, N.; Meya, U.; Sastre-y-Hernandez, M.; Schoeny, W.; Schratzer, M.; Soukop, W.; Trampitsch, E.; Varosanec, S.; Zawada, E.; Zoechling, R. *Pharmacopsychiatry* **1989**, *22*, 156.
(14) Horowski, R.; Sastre-y-Hernandez, M. *Curr. Ther. Res.* **1985**, *38*, 23.
(15) Glaser, T.; Traber, J. *Agents Actions* **1984**, *15*, 341.
(16) Lugnier, C.; Stierle, A.; Beretz, A.; Schoeffter, P.; Lebec, A.; Wermuth, C.-G.; Cazenave, J.-P.; Stoclet, J.-C. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 954.
(17) Frossard, N.; Landry, Y.; Pauli, G.; Ruckstuhl, M. *Br. J. Pharmacol.* **1987**, *73*, 933.

0022-2623/91/1834-0086$02.50/0 