SYNTHESIS AND CHARACTERIZATION OF A SERIES OF 5H-BENZO[a]-PHENOXAZIN-5-ONE DERIVATIVES AS POTENTIAL ANTIVIRAL/ANTITUMOR AGENTS

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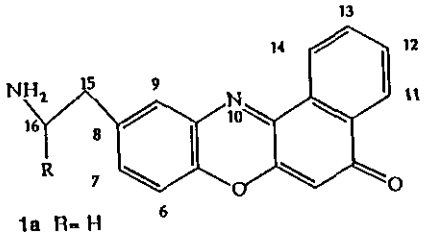
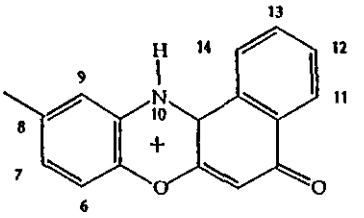
Abstract - A series of 1,2-benzo-3-phenoxazone also known as 5H-benzo[a]-phenoxazin-5-one (**1a-1c**) were synthesized and characterized by 1- and 2-D nmr, mass spectrometry and computer simulation on readily available, inexpensive software. It is proposed that these molecules intercalated in GC rich DNA regions and that internal hydrogen bonding caused by a molecule of water in the salt will help to anchor these actinomycin D-like antibiotic into these locations.

While studying the mechanism of reaction of tyramine with 1-nitroso-2-naphthol in presence of nitric acid, 1,2-benzo-8-(2-aminoethyl)-3-phenoxazone (BAP, **1a**) was produced.[1] **1a** is an analog of actinomycin D, a very potent antitumor agent which is also very toxic for human use.[2] Moreover, **1a** was selected by National Cancer Institute (NCI) of Bethesda, Maryland, for screening against HIV activity during its drug development program in 1988. The antiviral effect of **1a** was measured by plating susceptible human host cells, CEM-V and MT-2V with and without virus in microculture plated and adding concentrations of **1a**.[3] The data of this study has prompted us to synthesize and characterize by nmr and mass Spectrometry a series of compounds like **1a** (Structure **1**) by reacting various phenols with 1-nitroso-2-naphthol. By using this information, it is predicted that the intercalation of benzophenoxazones might take place in the minor groove of DNA duplexes.



1a R= H
1b R= CH_3
1c R= CO_2H

Structure 1



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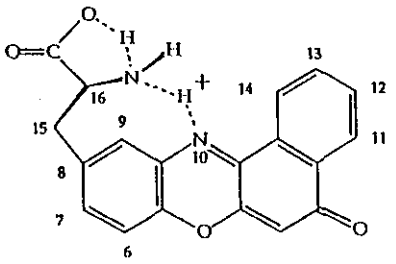

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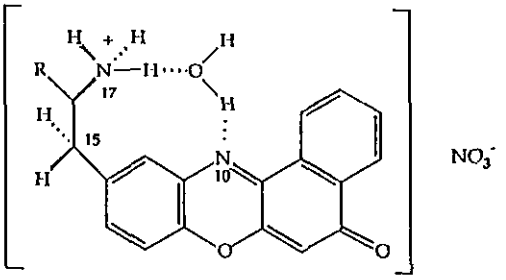


Structure 4

EXPERIMENTAL SECTION

It has been reported that most of the phenolic compounds except 3,4-dihydroxy phenolic compounds react with the reagent, 1-nitroso-2-naphthol.[4] Initially, this reagent was applied to assay tyramine in biological tissues and to prepare the complex.[5] This complex was further synthesized and characterized as 1,2-benzo-8-(2-aminoethyl)-3-phenoxazone (**1a**) by Bhansali and Kook.[1] During the synthesis of various 1,2-benzo-3-phenoxazone derivatives, it was shown that phenolic compounds having hydroxyl group(s) at different positions of aromatic ring and/or different aliphatic side chains produce similar to **1a** derivatives with variable solubility in different solvents. So, by using suitable solvent(s), the attempts have been made to synthesize a series of compounds like **1a** by reacting various phenols with 1-nitroso-2-naphthol.

The 1,2-benzo-8-(2-aminoethly)-3-phenoxazone nitrate (**1a**), $C_{18}H_{15}N_3O_5$ was prepared according to the method described by Udenfriend and Cooper.[5] One gram (5.774 mmol) of tyramine hydrochloride and 2 g (11.550 mmol) of 1 -nitroso-2-naphthol were dissolved in 250 ml of ethanol. To this were added 150 ml of 1 M nitric acid. The mixture was heated at 55° C for 3 h and cooled. Excess 1-niroso-2-naphthol was removed by chloroform extractions. Then, an equal volume of water was added, and crystals appeared. The compound was filtered and recrystallized from a mixture of 90% ethanol and 10% distilled water to afford 1a (0.250 g, 25.0%) mp 250-252°C. *Anal.* Calcd for $C_{18}H_{15}N_3O_5$: C, 61.17; H, 4.25; N, 11.89. Found: C, 60.97 H, 4.31; N, 11.93. The synthesis of 1,2-benzo-8-(2-aminoisopropyl)-3-phenoxazone nitrate (**1b**)$C_{19}H_{17}N_3O_5$ is as follows. One half gram (2.154 mmol) of *p*-hydroxyamphetamine hydrobromide and 1 g (5.775 mmol) of 1-nitroso-2-naphthol were dissolved in 125 ml of ethanol. To this were added 75 ml of 2M nitric acid and 100 mg (1.449 mmol) of sodium nitrite. The mixture was heated at 55-60°C for 2 h and cooled. Excess 1-nitroso-2-naphthol was extracted twice using 75 ml dichloroethane portion each. The product from aqueous ethanolic phase was separated by using two 25 ml each chloroform extractions. Upon adding crushed ice in chloroform extract, crystals appeared. The compound was filtered and recrystallized from a mixture of 90% ethanol and 10% distilled water gave a mp of 235°C. The yield was 0.150 g (30.0%) yield *Anal.* Calcd for $C_{19}H_{17}N_3O_5$ C, 57.43; H, 3.78; N, 10.58. Found: C, 57.54; H, 3.80; N, 10.61.

The synthesis of 1,2-benzo-8-alanyl-3-phenoxazone nitrate (**1c**)$C_{18}H_{15}O_7N_3$ is as follows. Four hundred mg (2.208 mmol) of tyrosine and 1.0 gm (5.775 mmol) of 1-nitroso-2-naphthol were dissolved in 150 ml of ethanol. To this mixture 250 ml of 1.2 N nitric acid solution was added. The mixture was heated at 60° C for 2 h and cooled. Excess 1-nitroso-2-naphthol was removed by three extractions each with 60 ml portions of ethylene dichloride. Reddish orange crystals appeared from the nitric acid solution at the bottom of the separatory funnel. The crystals were removed, filtered and dried. The yield was 0.225 g (56.25%) and the crystal gave a mp of 265°C. *Anal.* Calcd for $C_{18}H_{15}N_3O_7$: C. 57.43; H,3.78; N, 10.58. Found C, 57.41, H, 3.80; N, 10.56

The molecular weights and fragmentation patterns of phenoxazones (**1a-c**) were confirmed on a Finnigan 3300 instrument operating at 70 e V in the electron impact mode.

The ^{1}H nmr spectrum was obtained at 300.13 MHz on an IBM AF300 operating at 7.0 Tesla. The 128-32K FIDs were acquired in 5.11 s using a 10-μs pulse (30° flip angle) on a spectral window of 3205 Hz (10.7 ppm), giving a digital resolution of 0.196 Hz/pt. The final spectrum was exponentially weighted with a 0.3 Hz line-broadening factor.

The ^{1}H COSY (correlation spectroscopy) experiment[6] consisted of a 2K x 256W data matrix in which the spectral windows in both dimensions were equivalent (3206 Hz). The 256-2K FIDs, each consisting of 16 scans, were obtained in 0.319 s with a 1.0-s recycle delay using a $[90\text{-}(t_{1/2})\text{-}180\text{-}(t_{1/2})]_n$ pulse sequence. The F1 dimension was zero filled to 1K to create a square data array for symmetrization. Both dimensions were sine-bell weighed and symmetrized.

The ^{13}C nmr spectrum was obtained on the same IBM AF300 spectrometer at 75.5 MHz. The 512-32K FIDs were acquired in 0.85 s over a spectral window of 19,230 Hz (254.8 ppm) using a 1.5-μs pulse (30° flip angle) and a 1.0 s recycle time resulting in a digital resolution of 1.17 Hz/pt. The FID was exponentially weighted with a 3.0-Hz line-broadening factor. The proton decoupling was accomplished using a low-power waltz decoupler to minimize sample heating and line broadening. The ^{13}C DEPT (distortional enhancement by polarization transfer)[7] experiment was performed under the same conditions as the normal carbon spectrum using a 2.0-s recycle time which was optimized for ^1J(C---H) coupling of 159 Hz.

The ^{13}C/^{1}H heteronuclear correlation experiment (XHDEPTD = 2-D DEPT with F1 proton decoupling)[8] consisted of a 2K x 128W data matrix in which the spectral windows for the protons (SW_1) and carbon (SW_2) were the same as the 1D values. The 128-2K^{13}C FIDs, each consisting of 128 scans, were obtained in 0.59 s with a 2.0-s recycle time. The F1 dimension was zero filled to 256W, and both dimensions were square sine-bell weighted.

RESULTS AND DISCUSSION

Electron-impact mass spectrometry, EI/ms, does not normally produce a parent ion of a nitric acid salt of amines, such as compounds (**1a, 1b, and 1c**), since they will sublime as loosely associated ion pairs. However, it is possible to look at positively charged fragments of the nitrite anion as well as the cation of

the salts and its fragments using EI/ms. Table I shows a comparison of some of the characteristic fragment ions from EI/ms of compounds (**1a**, **1b**, and **1c**); all three compounds have a very strong fragment ion at m/z 261 in common. This ion is consistent with a fragmentation between carbons 15 and 16 in each of the three compounds yielding a positively charged species that is protonated and consistent with proposed structure (**2**).

The proton most likely resides on the basic nitrogen at position 10 in the fragment ion and would suggest that the acid proton in the cation of the salt would also reside primarily at position 10 for the mononitrate salt species since m/z 44 is the base peak for compound (**1b**) and that m/z 43, 42, and 41 intensities are consistent with the sequential loss of protons from m/z 44. This suggests the species $[CH_3\text{-}CH\text{-}NH_2]+$ and that it somehow retains the positive charge more often than the other half of the cation of the salty fragment at m/z 261 which is the base peak for compounds (**1a**) and (**1c**). Compounds (**1a**) and (**1b**) show values for the cations of their respective salts a m/z 291 and m/z 305 as expected. but compound (**1c**) does not and shows only a weak peak corresponding to the molecular formula for the free amino acid. This could possibly be due to the fact that **1c** exists as a cyclical hydrogen bonded Zwitter ion with a structure like **3** and any nitrate salt proton may be weakly held and lost prior to mass analysis. All three compounds show a strong value at m/z 30 consistent with $[NO]^+$ is probably responsible for the m/z 46 intensities showing the presence of nitrate in all three compounds.

The ^1H nmr spectra (Figure I) and the COSY contours (Figure II) of **1c** clearly shows the ABCD spin system of the H11-H14 and the ABM spin system of the H6,H7 and H9 are almost identical to those of **1a** which we previously reported. The singlet peak at 6.4 ppm in the ^1H nmr spectrum (Figure I) is a finger print for the benzo-3-phenoxazone ring system. Moreover, the presence of protons at 3.2 and 4.4 ppm are the methtlene and α-proton of the alanyl side chain respectively.

Exami nation of Table II, which contains the proton chemical shifts and J-coupling constants for **1a-c**, demonstrates the expected similarity of these benzophenoxazone rings. The J-coupling values were obtained through the use of a 7-spin simulation program (PANIC)[9] which did a non-linear, least-squares, iterative fit

TABLE I. Characteristics EIms Fragment Ions of 5H-Benzo[a]phenoxazin-5-one

m/z	1a	1b	1c
28	19.5 $[CH_2N]^+$	69.3 $[CH_2N]^+$	11.7 $[CO+]^+$
30	31.7 $[NO]^+$	57.1 $[NO \& CH_4N]^+$	37.2 $[NO]^+$
41		7.4 $[C_2H_3N]^+$	1.3
42		23.9 $[C_2H_4N]^+$	.9
43		22.7 $[C_2H_5N]^+$	1.7
44		100.0 $[C_2H_6N]^+$	46.1 $[CO_2]^+$
46	38.0 $[NO_2]^+$	4.3 $[NO_2]^+$	29.0 $[NO_2]^+$
260	24.3 $[C_{17}H_{10}NO_2]^+$	17.2 $[C_{17}H_{10}NO_2]^+$	3.0 $[C_{17}H_{10}NO_2]^+$
261	100.0 $[C_{17}H_{11}NO_2]^+$	56.8 $[C_{17}H_{11}NO_2]^+$	100.0 $[C_{17}H_{11}NO_2]^+$
290	3.3 $[C_{18}H_{14}N_2O_2]^+$		3.2 $[C_{18}H_{14}N_2O_2]^+$
291	6.4 $[C_{18}H_{15}N_2O_2]^+$		0.9 $[C_{18}H_{15}N_2O_2]^+$
292	1.5		0.4
305		0.4 $[C_{19}H_{17}N_2O_2]^+$	
334			0.2 $[C_{19}H_{14}N_2O_4]^+$

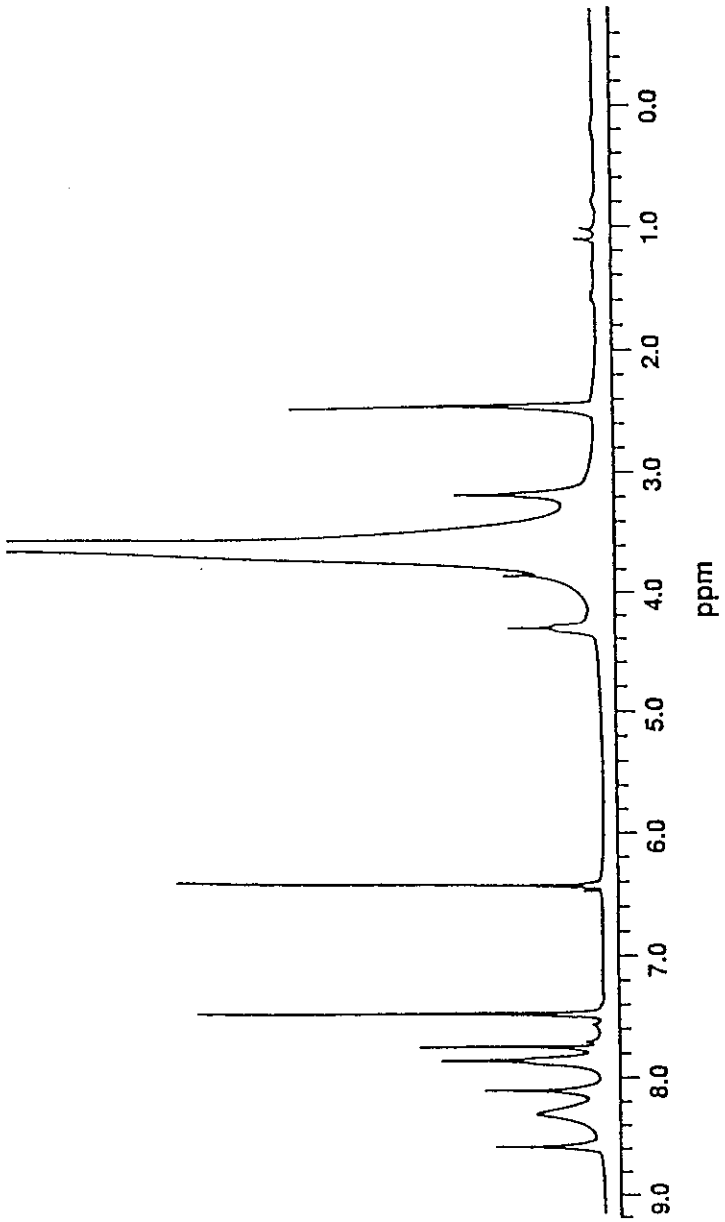
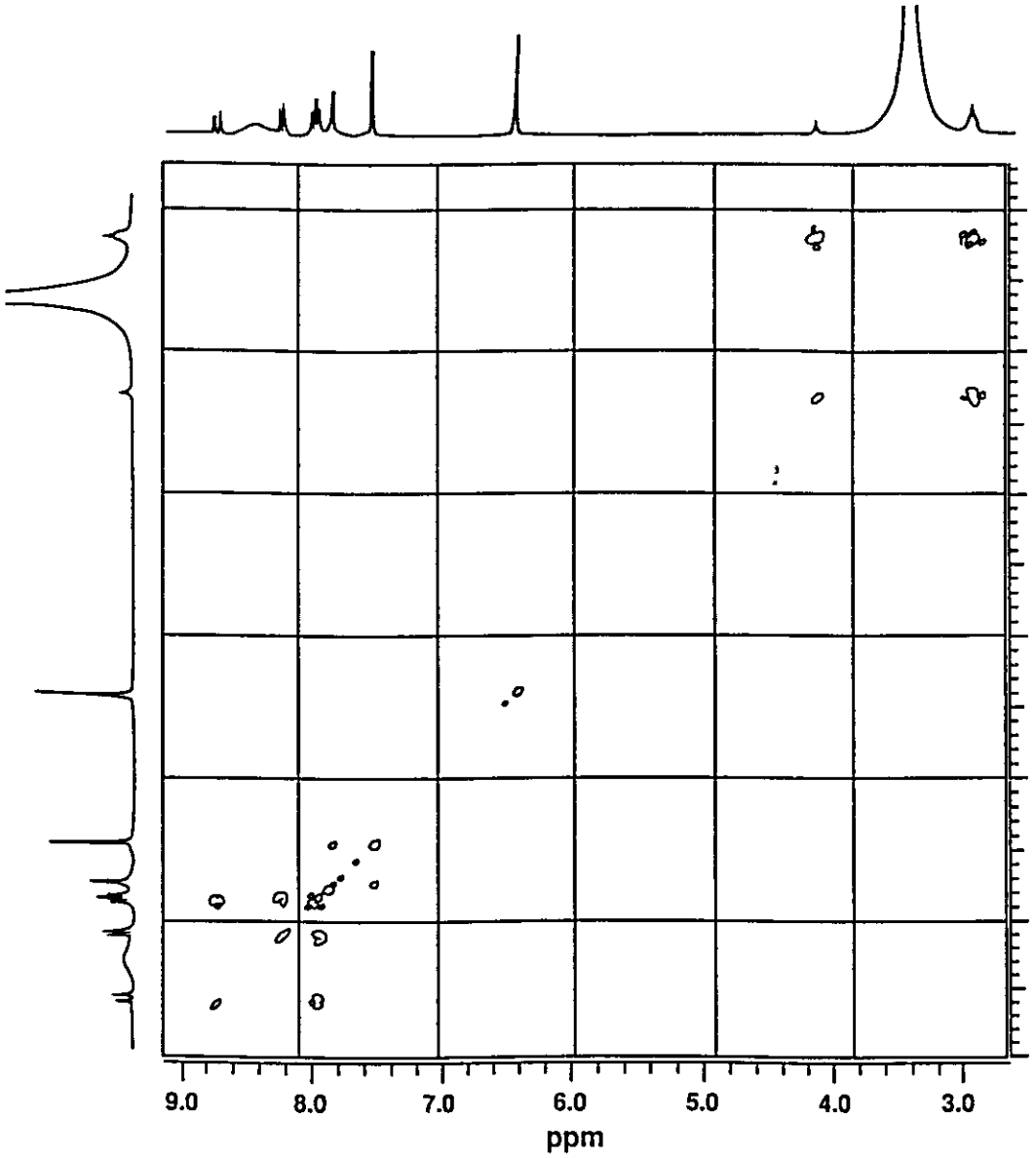


Figure 1 - ^{1}H Nmr Spectrum of 5H-Benzo[a]phenoxazin-5-one

Figure 2 -'H COSY Contour Plot of 5H-Benzo[a]phenoxazin-5-one



of the experimental spectra.

The complete assignments of the protons and carbon chemical shifts (Table III) were obtained by the heteronuclear 2D nmr experiment (not shown) which correlates ^{13}C with their directly bonded ^{1}H. This is a good experiment for the assignment of the proton spectrum via the carbon heteronuclear correlation, especially in cases where highly overlapped and/or second-order regions are present in a proton spectrum of a molecule, For example, the H6 and H7 are very close in chemical shift due to similar diamagnetic shielding effects (σs); but C6 and C7 are extremely well separated (116 ppm versus 132.1 ppm) due to very different and strong paramagnetic shielding effects (σp) which greatly influence carbon but not protons in this aromatic π-system. Here again our assignments were identical to those found for **1a**.

The assignments of the alkyl side chains are straight forward since their chemical shifts in either proton or carbon spectra do no overlap with the phenoxazone rings. The mass spectra does demonstrate that the primary amino group (N17) is oriented towards the N10 position. In DMSO-d_6 and in the solid state there appears to be a favored orientation to hydrogen-bond between the two nitrogens. This would explain the diastereotropic nature of the methylenic protons of H15 in compounds (**1b**) and(**1c**). In the absence of hydrogen bonding or orientation of the side chain, these protons would appear to be identical in chemical shift (enantiotropic) since they would be free to move about in space randomly without much steric hinderance like **1a**. The fact that we have geminal couplings (8, 13.5 Hz) and two separate vicinal coupling (4.5, 5.7 Hz) and 6.3, 8.2 Hz) to nearest neighbor protons furthers the argument about hydrogen bonding and steric hinderance in **1b** and **1c**, respectively. Computer simulation of this geometry was fruitless because the gap between the N10 and N17 is too large for hydrogen bonding (>5 angstroms).[10] But, if a molecule of water is positioned between these nitrogens, as in structure (**4**), then the nmr and mass spec fragmentation data can be explained without much controversy. As a valuable note, the presence of water molecules and hydrogen bonding on the surface and in the interior of biopolymers and antibiotics should not be overlooked in trying to determine 3-D structure and binding activity.

These facts may help us to understand orientation of aminophenoxazones and the mode of action in the

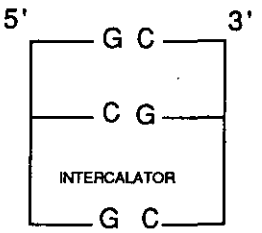
Table II H-1-Nmr Chemical Shifts (ppm vs TMS) and J Coupling Constants (Hz) of 5H-Benzo[a]phenoxazin-5-one

Position	1a	$^{3,4}J$	1b	$^{3,4}J$	1c	$^{3,4}J$
17	-	-	1.18	7.4	-	-
15	2.96	8.4, 7.0	2.85, 3.03	5.7, 8.2, 13.5	3.16, 3.22	4.5, 6.32, 8.0
16	3.15	8.4, 7.0	3.34	5.7, 8.2	4.33	4.5, 6.2
4	6.41	-	6.40	-	6.43	-
6	7.45	8.4	7.49	-	7.47	-
11	8.59	7.4, 1.4	8.59	7.4	8.59	1.3, 1.7, 7.9
14	8.12	7.6, 1.6	8.12	7.6	8.15	1.3, 2.3, 7.2
9	7.76	1.4	7.75	-	7.78	-
1	-	-	-	-	-	-
6a	-	-	-	-	-	-
12	7.88	7.6, 7.4, 1.6	7.88	7.6, 7.4, 1.6	7.88	1.3, 7.9, 9.1
13	7.85	7.6, 7.6, 1.4	7.84	7.6, 8.3	7.81	7.2, 9.1
7	7.48	8.4, 1.4	7.49	-	7.47	-

Table III C-13 Nmr Chemical shifts (ppm vs TMS) of 5H-Benzo[a]phenoxazin-5-one

Position	1a	1b	1c
17	-	17.8	170.5
15	32.9	39.5	35.1
16	39.9	43.1	53.1
4	106.4	106.5	106.6
6	116.0	116.1	116.2
11	124.1	124.3	124.3
14	125.2	125.3	125.4
9	129.4	130.1	130.5
1	130.7	130.9	130.8
6a	131.5	131.6	131.6
12	132.2	132.2	132.2
13	132.1	132.5	132.5
7	132.5	133.0	133.3
9a	142.4	142.7	143.0
1a	146.8	147.1	147.1
4a	151.0	151.3	151.3
3	182.6	182.8	182.9

active site of a DNA. It is known that the phenoxazones like actinomycin D can intercalate between b-DNA base-pairs in GC dinucleotide rich regions and can participate in a slowly reversible hydrogen bonding situations with the bases (structure 5).[11,12] This interaction causes a widening of the gap between adjacent base-pairs by 2 angstrom or more. This re-orients the sugar-phosphate backbone and may cause unwinding of the base-pairs further down the helix.[13] Like actinomycin D, this may occur in an asymmetric manner to the 5' and 3' strains of the DNA duplex. The amino group in the side-chain can help further anchor the group in the active site through hydrogen bonding with adjacent or nearest-neighbor base-pairs and/or electrostatic/ionic interactions with the sugar-phosphate backbone assisting in the further unwinding of the helix around the active site. This is left to future studies of this class of compounds.



Structure 5

ACKNOWLEDGMENTS

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