Apurinic DNA Reactivity: Modelisation of Apurinic DNA Breakage with Phenylhydrazine and Formation of a Pyrazole Adduct

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Dedicated to Professor Norman H. Cromwell

We have elucidated the exact mechanism of apurinic oligodeoxynucleotide d(Tp[AP]pT) cleavage by phenylhydrazine and shown that the final isolated product is in fact a 3-substituted derivative of 1-phenylpyrazole.

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We recently synthesized the apurinic oligonucleotide d(Tp[AP]pT) **1** [1,2] as a model to study the mechanism of DNA cleavage under the influence of various aromatic amines such as 3-aminocarbazole [3] and 9-aminoellipticine [4].

Furthermore we were also interested in looking at the reactivity of phenylhydrazine with this model, as it has been shown by Livingston [5,6] that such aldehyde reagent gives rise also to DNA degradation at AP sites. The proposed mechanism involved a β-elimination process, formation of an intermediate α,β-unsaturated hydrazone and obtention of a compound which was postulated to be a 1-phenylpyrazole derivative. However this final compound was not isolated nor characterized and the only given structural proof was based on its uv absorption spectrum.

Therefore we decided on our AP model to reexamine the course of this reaction and to determine the structure of the final adduct in order to eventually open a new approach to link an heterocyclic moiety to the 3'-end of an oligonucleotide.

Using the same experimental conditions as those described by Livingston [6] the reaction of **1**, as its sodium salt (20 mg, 2.54 10^{-2} mmoles), with phenylhydrazine hydrochloride (73.6 mg, 50.9 10^{-2} mmoles) was performed in water (8 ml) at 37°. Analytical hplc was used to monitor the course of the reaction, recording with a multichannel uv detector the uv absorption spectra of the detected intermediates. It is thus possible to follow the kinetics of the reaction by plotting the concentration of each detected product against time [7].

Figure 1 presents the overall evolution of the reaction mixture. We observed first a very rapid formation of an intermediate **2** (R_T 16.70 min, λ max 269 nm) with concomitant disappearance of **1** (R_T 10.18 min, λ max 266 nm) within a few minutes (k_1 = 156 h^{-1}, $t_{1/2}$ = 0.26 min), but without significant formation of 5'-phosphate thymidine (pdT). This first observation could account for the formation of the phenylhydrazone derivative of **1**. We then detected another intermediate **3** and a final product **4**. Compound **3** was formed upon 5'-phosphate thymidine abstraction (R_T = 6.09 min, λ max 266 nm) from **2** (k_2 = 0.128 h^{-1}, t_{max}(**2**) = 2.74 min, C_{max}(**2**) = 3.55 mM). The uv spectrum of **3** (R_T 23.38 min) present a maximum at 330 nm which strongly indicated formation of an α,β-ethylenic phenylhydrazone in accord with the literature data [6]. This compound then disappears (k_3 = 0.139 h^{-1}, t_{max}(**3**) = 7.5 h, C_{max}(**3**) = 1.256 mM) to give a final adduct **4** (R_T = 19.08 min).

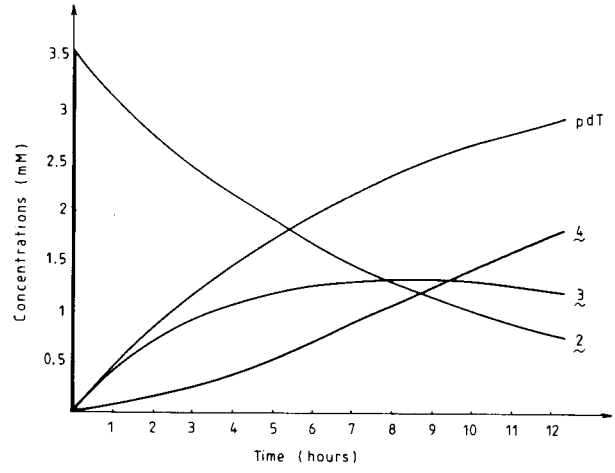
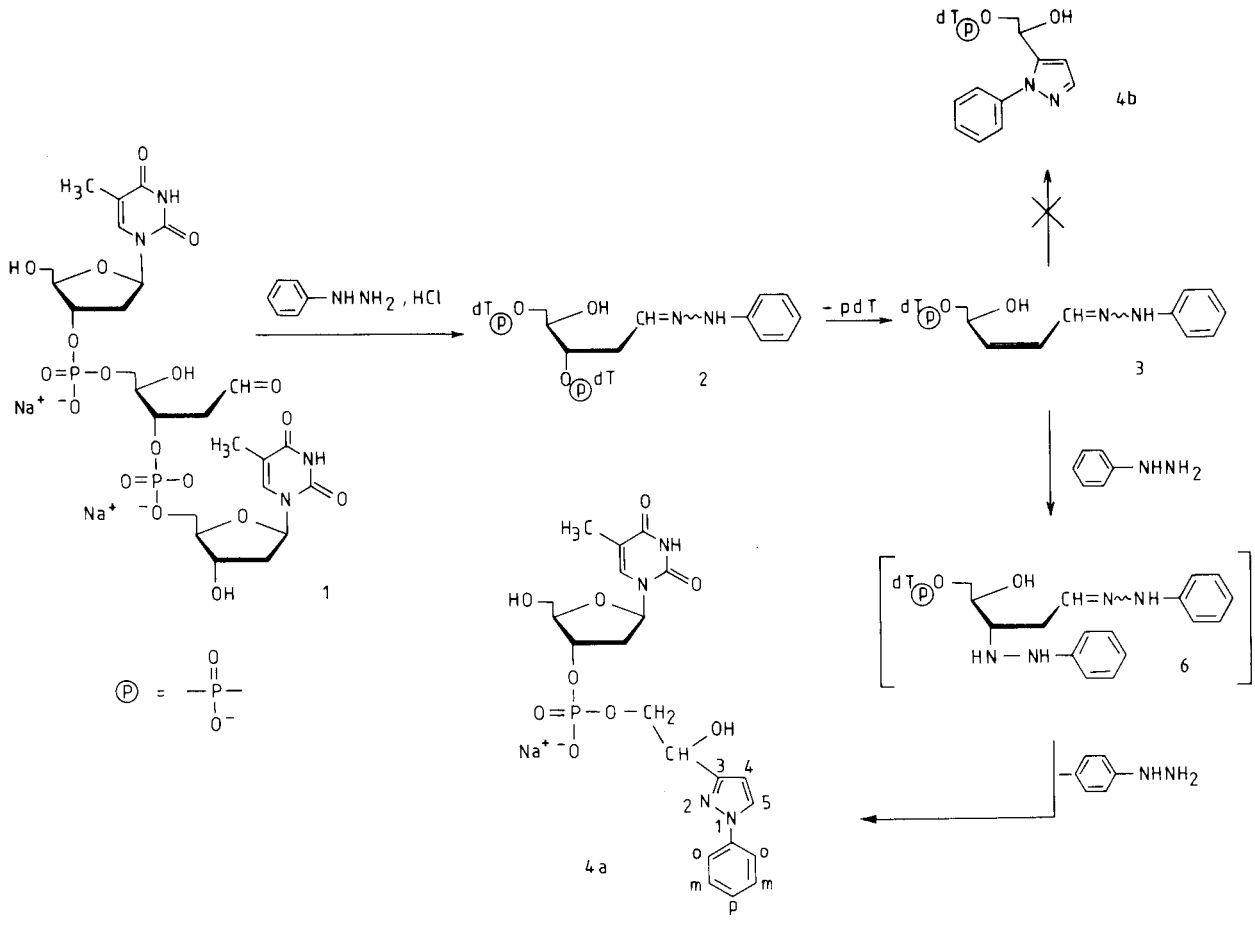


Figure 1. Kinetics of the reaction between d(Tp[AP]pT) and phenylhydrazine [7].

$$t_{max}(3) = \frac{\ln(k_3/k_2)}{k_3-k_2} \qquad C_{max}(3) = C_o(1)\ \frac{k_3}{k_2}\ \frac{k_3/k_2}{1-k_3/k_2}$$

Scheme 1



Therefore compound **4** was isolated by preparative hplc and analysed by mass spectrometry and ^1H nmr spectroscopy. The positive ion FAB mass spectrum of the ammonium salt of **4** showed MH^+ ion at m/z 509 which could be in accordance with a phenylpyrazole structure.

The ^1H nmr spectrum (360 MHz) of this compound was easily interpreted since all the protons related to the thymidine residue of the molecule and the connecting unit (CH_2CHOH chain) have been precedently assigned [3] (Table 1).

Table 1

NMR Data for Compound **4b**

Chemical Shifts of thymidine protons (TH).

	TH_6	TH_1'	TH_2''	TH_2'	TH_3'	TH_4'	$TH_5',_5''$	CH_3-5
δ (ppm)	7.63	6.12	2.22	2.10	4.62	3.93	3.57	1.76

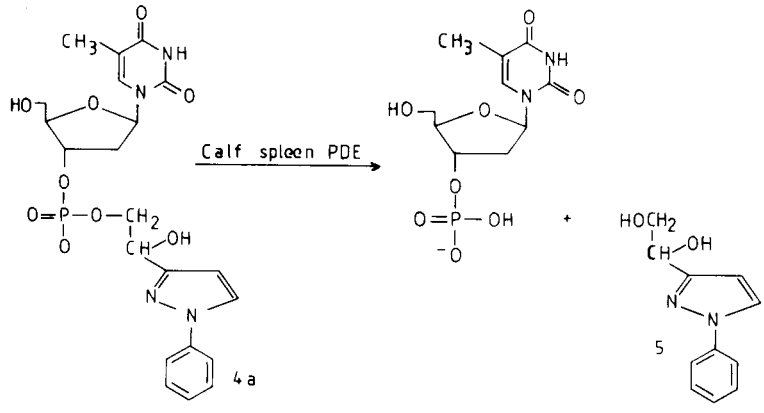
Chemical Shifts of *N*-phenylpyrazole protons (PH) and connecting residue protons.

	CH_2	CH(OH)	PH_4	PH_5	Ho	Hm	Hp
δ (ppm)	3.87	4.77	6.47	8.36	7.79	7.46	7.26

Two signals, respectively at 6.47 and 8.36 ppm with a coupling constant of J = 2.46 Hz, can be assigned to pyrazole ring protons. This observed coupling constant indicates that the isolated product is a 3-substituted pyrazole **4a** as it has been precedently shown in *N*-aromatic substituted pyrazoles that $2.6 > J_{45} > 2.3$ Hz and $1.9 > J_{34} > 1.5$ [8-10]. This result was quite surprising as cyclisation of the hydrazone **3** should result in the 5-substituted pyrazole **4b** as suggested by Livingston [6].

Examination of the phenyl proton signals (Table 1) corroborates this assignment. It has been precedently shown [8,9] in the 1-phenylpyrazole series that the phenyl protons resonate as a multiplet except if a substituent is adjacent to the *N*-phenyl group. In such case the corresponding phenyl signal is a singlet. The observed multiplicity of the phenyl signal protons (Table 1) is therefore also in agreement with the 3-substituted structure.

Scheme 2



It is then possible to assign the signal at 6.47 ppm to the H-4 pyrazole proton and the one at 8.36 ppm to the H-5 proton [6].

Structure **4a** is also in complete agreement with the uv data obtained as follows: enzymatic degradation of the isolated adduct by calf spleen phosphodiesterase (Scheme 2) gives rise to a dihydroxyethyl-*N*-phenylpyrazole **5** with a λ max 257 nm in ethanol. This data is closely related to the uv spectrum of 3-methyl-1-phenylpyrazole (λ max 257 nm) in the same solvent and differs strongly from that corresponding to the 5-methyl substituted isomer (λ max 241 nm) [11].

On the basis of all those data, the following reaction scheme can be proposed (Scheme 1) for DNA degradation with phenylhydrazine at an AP site. A rapid formation of the hydrazone **2** occurs which undergoes then β-elimination of pdT to afford the corresponding α,β-unsaturated hydrazone **3**. As the result of this last step, the apurinic oligonucleotide chain is cleaved.

As the final adduct is a 3-substituted 1-phenylpyrazole, the reaction must involve a 1,4-Michael addition of phenylhydrazine on compound **3** to form a disubstituted intermediate such as **6** followed by an oxidative ring closure process. One can notice that such intermediates have been isolated previously [12] but this mechanism does not completely preclude another hypothesis which implicate a hydrolysis of **3** to the corresponding α,β-unsaturated aldehyde followed by 1-4 addition [3].

The overall mechanism presented here is very close to the one observed on the same model **1** with 3-aminocarbazole where a pyrido[2,3-*c*]carbazole derivative was isolated [3,4]. In both cases there is first a 1,2-addition on the aldehydic function followed by the β-elimination process, hence the DNA cleavage. The α,β-unsaturated derivative thus formed is then subject to a 1,4-Michael addition followed by an oxidative ring closure to the corresponding heterocycle linked to the phosphate group of the 3'-nucleotide with a CH_2CHOH linkage.

In this work we have elucidated the exact mechanism of DNA breakage at an AP site with phenylhydrazine and determined the kinetic parameters of the various detected steps of this reaction. Furthermore elucidation of the final adduct structure opens a new potential approach to functionalize oligonucleotides at their 3'-end with heterocyclic moieties such as 1-phenylpyrazole.

EXPERIMENTAL

Because compound **4b** was charged and hygroscopic, elementary analysis was not performed; however the mass of the compound was given by its FAB + spectrum.

The mass spectrum was recorded in the positive mode on a JEOL JMS-DX300 mass spectrophotometer using glycerol as the matrix.

High performance liquid chromatographic analysis were carried out on a Radial-Pak Bondapak C_{18} (10 μm) cartridge in a Waters Z module. A Water U6K injector, two 6000A pumps, a M 720 solvent programmer, and a M 730 microprocessor-controlled data system were employed. The uv spectra were recorded using a Pye Unicam PU 4021 multichannel detector and a Pye-Unicam PU 4850 video chromatography control center. A linear gradient of 0-22% acetonitrile in 0.1 *M* ammonium acetate (*p*H 5.9) was applied in 25 minutes at a flow rate of 3 ml min^{-1} (gradient I). For the enzymatic hydrolysis experiment a linear gradient of 0-20% acetonitrile in 0.1 *M* ammonium acetate (*p*H 5.9) was applied in 20 minutes at the same flow rate (gradient II). Preparative hplc was run on the same column. Isocratic elution with 10% acetonitrile (0.1 *M* ammonium bicarbonate (*p*H 7.5) was applied for 10 minutes at a flow rate of 3 ml min^{-1}.

Calf spleen phosphodiesterase was purchased from Boehringer Mannheim.

Proton nuclear magnetic resonance (^{1}H-nmr) was recorded at 30° on a Bruker WB UM 360. The nmr sample was made up in DMSO-d_6 + 5% deuterium oxide. The shifts are given in ppm from internal tetramethylsilane.

Reaction of the Apurinic Model **1** with Phenylhydrazine.

A solution of **1** (20 mg, 2.54 10^{-2} mmoles) with phenylhydrazine hydrochloride (73.6 mg, 50.9 10^{-2} mmoles) in water (8 ml) was kept at 37°. At different reaction times, aliquotes were withdrawn and analyzed by hplc (gradient I). At 20 hours the final product (R_T 19.08 min) was purified by

hplc (7 injections). The combined appropriate fractions (hplc purity better than 98%) were evaporated under reduced pressure and the residue was treated with Dowex 50W (Na^+ form). After lyophilization, compound **4a** was obtained (5.5 mg sodium salt) in 41% yield. The ^{1}H-nmr spectral data of compound **4a** are reported in the Table.

Enzymatic Hydrolysis of Compound **4b** by Calf Spleen Phosphodiesterase.

To a solution of compound **4b** (1.04 10^{-4} mmoles) in water (50 μl) was added a 0.125 *M* ammonium acetate *p*H 7, 0.0025 *M* EDTA, 0.0625% Tween 80 buffer solution (670 μl) and calf spleen phosphodiesterase (stock solution, 20 μl). The resulting solution was maintained at 37°. After 16 hours reaction, hplc analysis (gradient II) of the hydrolysate indicated complete degradation of compound **4b** (R_T 13.97 min, λ max 258 nm) into 3'-phosphate thymidine (R_T 4.50 min, λ max 266 nm) and compound **5** (R_T 15.78 min λ max 255 nm). The solution was diluted with 1 *M* triethylammonium bicarbonate buffer solution *p*H 7.5 (2 ml) and applied on a C_{18} Sep-Pak cartridge (Waters Associates). After washing with water (10 ml), compound **5** was eluted with ethanol (5 ml).

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REFERENCES AND NOTES

[1] This "pseudo" trinucleotide (see Scheme 1) contains an apurinic (or apyrimidinic) site which is shown by [AP]. On the drawings, this AP site is represented by its open chain structure rather than with its tautomeric ring closed form, because it is the reactive species.

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[7] Concentrations of compounds **1** and pdT were correlated with the hplc peak areas at 254 nm assuming that molar absorption coefficient of pdT is half of absorption coefficient of **1** at the same wavelength. For compound **4**, concentration was correlated on the basis that final concentration of this compound was the same that initial concentration of **1**: Co(**1**) (stoechiometry 1:1). Rate constants k_1, k_2, k_3, were calculated according to the mechanism shown in Scheme 1. As $k_1 >> k_2$ and $k_1 >> k_3$, C_{max}(**2**) was found to be approximatively equal to Co(**1**). In case of compound **3** C_{max}(**3**) was calculated according to the following equations:

equation

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