Structure of the Adduct Formed between 3-Aminocarbazole and the Apurinic Site Oligonucleotide Model d[Tp(Ap)pT]

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Structural elucidation of the adduct formed between 3-aminocarbazole and the model apurinic/apyrimidinic site-containing oligonucleotide d[Tp(Ap)pT] is described. One- and two-dimensional ^{1}H NMR spectroscopy and high-resolution mass spectrometry were employed to secure the structure. Formation of the adduct containing a pyrido[2,3-*c*]carbazole moiety involves base-catalyzed β-elimination of a phosphomonoester at the AP site and Michael addition followed by air-oxidative ring closure.

Introduction

It is well established that apurinic DNA is cleaved, via a β-elimination process, under basic, including amine, catalysis.[1] The postulated mechanism for primary amine mediated breakage involves formation of a Schiff base, which facilitates abstraction of the ribose proton in position 2′, leading to the phosphate bond scission.[1-3] The recent finding of efficient breakage of DNA apurinic sites by certain indolamines related to 9-aminoellipticine[4] led us to examine the chemical reaction of 3-aminocarbazole (**1**) with an apurinic site model, i.e., d[Tp(Ap)pT] **2**, recently synthesized in our group.[5]

Accordingly, we expected to isolate the corresponding Schiff base **3** (Scheme I), thereby providing an approach to link related indolamines to various oligonucleotide probes.

Results and Discussion

Reaction of 2 with 3-Aminocarbazole. The reaction of **1** (8 mM) with **2** (2 mM) was performed in water/methanol (1:4, v/v) at room temperature, and after completion of the reaction (2 days), an adduct was isolated by silica gel and DEAE-Sephadex chromatographies. However, neither the NMR spectrum nor mass spectral data of this adduct correspond to the expected structure **3**. In particular, no ethylenic AB system could be detected in the NMR spectrum and the (+) FAB spectrum of the isolated adduct showed a molecular weight 2 mass units lower than that required by structure **3**. Furthermore, alternative structures (Scheme I) such as **4** (which would result from 1,4-Michael addition on the α,β-unsaturated aldehyde) or **5** (from base-catalyzed prototropic shift resulting in cyclopentenone derivative formation)[2] were not in agreement with the observed spectral data. Therefore, additional ^{1}H NMR spectroscopy and mass spectrometry techniques were applied in order to determine the structure of the adduct. Analytical HPLC was used to monitor the course of the reaction by recording the UV absorption of the intermediates with a multichannel UV detector. This technique demonstrated the existence of two intermediates A and B, the first being formed upon pT abstraction (Figure 1). Therefore, the formation of the isolated adduct C could be depicted according to the following equation:

$$2 \rightarrow A + pT \rightarrow B \rightarrow C$$

The UV spectrum of the first intermediate A excluded the presence of the aminocarbazole chromophore and showed, upon subtraction of the absorbance of the Tp chromophore, a maximum at 222 nm, which strongly indicated formation of the α,β-unsaturated aldehyde through pT elimination. This datum is in accord with the reported UV spectrum of *trans*-2,3-dideoxy-D-glyceropent-2-enose.[6] The second intermediate B appears to incorporate the aminocarbazole chromophore on the basis of the UV absorption at 305 and 366 nm and should correspond to nucleophilic attack of the 3-aminocarbazole on the α,β-unsaturated aldehyde A. Furthermore, it was observed that the isolated adduct was formed upon evaporation of the solvent from B. The UV spectrum of the final adduct differs from its precursor B but retains a maximum in the 340–350-nm range.

High-Resolution Mass Spectrometry Studies. The final adduct was examined by positive- and negative-ion fast atom bombardment (FAB) mass spectrometry, and the electron ionization mass spectrum was determined after conversion to the volatile trimethylsilyl (TMS) derivative. The positive-ion FAB mass spectrum of the monosodium salt showed MH^+ and MNa^+ ions (intensity ratio 1:3) at m/z 583 and 605, respectively. The negative-ion mass spectrum taken of the triethylammonium salt showed the $(M - H)^-$ ion at m/z 581. Trimethylsilylation produced a derivative of molecular weight 942 (M = 942, M – CH_3 = 927, 5:1 intensity ratio), which had incorporated five TMS groups as determined by the mass spectrum of the [$^2H_9$]trimethylsilyl derivative (M = 987).

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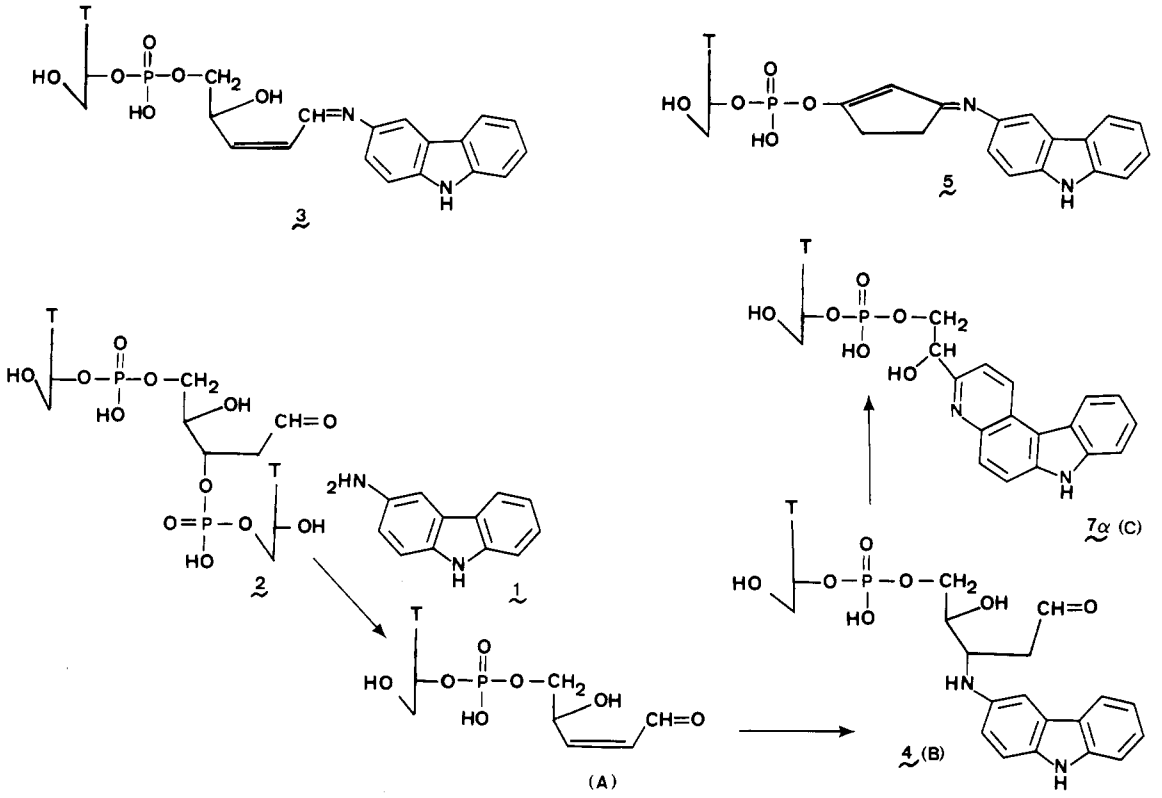
Scheme I



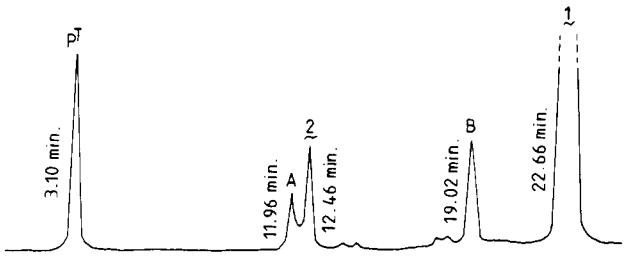


Figure 1. Chromatogram of the reaction mixture between 1 and 2 at t = 255 min.

These data rigorously establish the molecular weight of final adduct as 582.

The elemental composition was assigned as $C_{27}H_{27}N_4O_9P$, based on exact mass measurement of the MNa^+ adduct ion from FAB ionization as 605.1420 (theoretical 605.1414 for $C_{27}H_{27}N_4O_9PNa$). The negative-ion FAB spectrum of adduct C following hydrogen–deuterium exchange was determined as a means of unambiguously establishing the number of active hydrogen atoms.[7] The $(M - D)^-$ ion was found at m/z 585, therefore indicating the free acid to contain five exchangeable hydrogens and (by difference) 22 nonexchangeable hydrogen atoms.

Structural features of derivative C as determined by mass spectrometry are summarized in Figure 2. The negative-ion FAB spectrum showed principal fragment ions of m/z 125 as expected[8,9] for the thymine base and m/z 321 corresponding to the resonance-stabilized phosphate cleavage characteristic of oligonucleotides.[10,11] The

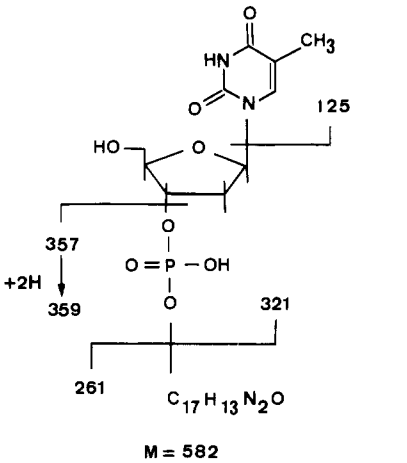


Figure 2. Ions from FAB mass spectrometry of the carbazole adduct.

positive-ion FAB mass spectrum exhibited no significant peaks outside of the molecular ion region, and so the MH^+ ion was subjected to collision-induced dissociation. This resulted in two major fragment ions, m/z 359 and 260 (intensity ratio 1:1), measured by tandem mass spectrometry.[8] As shown in Figure 2, m/z 359 is assigned as the protonated phosphate ion, a nucleotide ion often observed in desorption mass spectra.[8] The m/z 260 ion is related to the carbazole portion of the molecule.

The TMS derivative of adduct C produced an electron ionization mass spectrum supportive of the structure shown in Figure 2 but with little structural detail: m/z 147, 211, 299, 315, characteristic of all nucleotides;[12] the thymine base +58 ion at m/z 255;[12] and m/z 404, the

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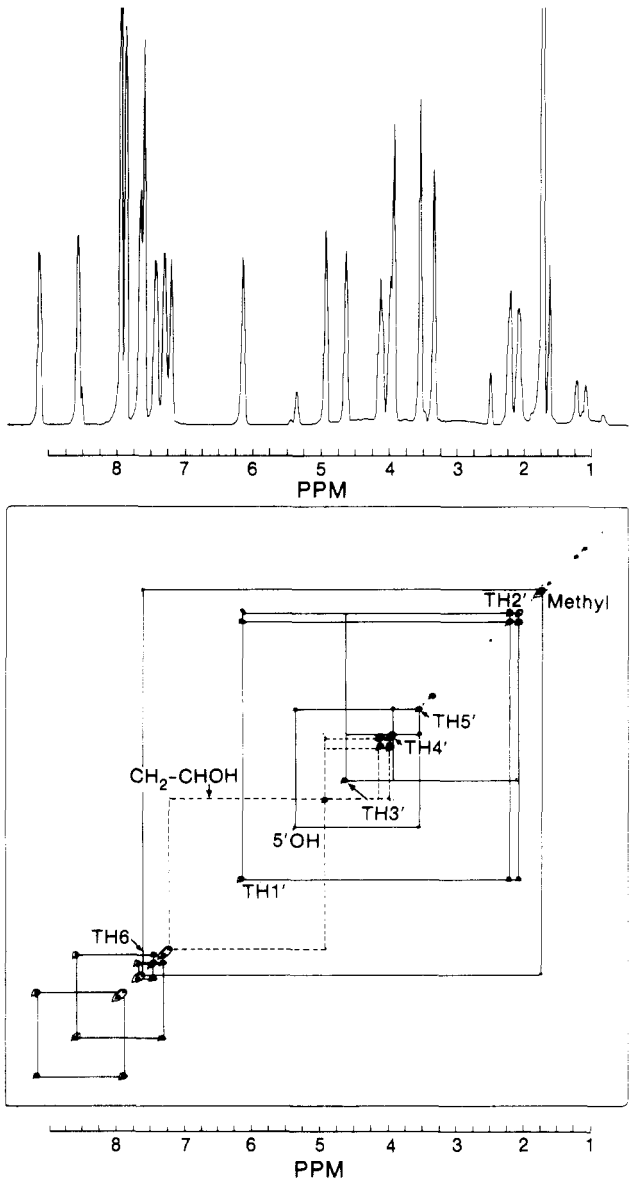

Figure 3. ^{1}H COSY 45 spectrum of the adduct at 20 °C showing the connectivities in the thymine, carbazole, and CH_2CHOH moieties. Experimental conditions: F_2 = 1K, initial t_1 = 2 μs, Δt_1 = 0.27 ms, sweep width 3800 Hz. A total of 32 fids were accumulated for each of the 256 experiments.

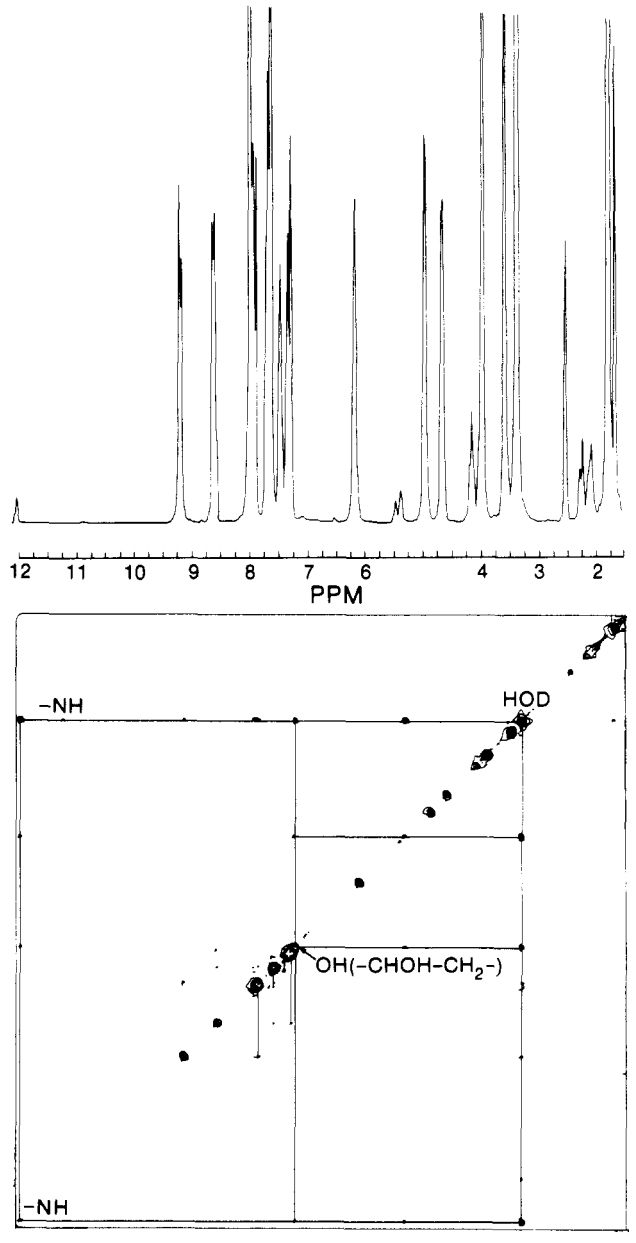

Figure 4. NOESY spectrum for the adduct at 20 °C. Experimental conditions: F_2 = 1K, initial t_1 = 3 μs, Δt_1 = 0.22 ms, sweep width 4700 MHz, mixing time 0.5 s. A total of 48 fids were accumulated for each of the 256 experiments. Small differences in 1D spectra between Figures 3 and 4 are due to slightly different conditions of concentration and temperature.

bis(silyl) analogue of m/z 260 and its daughter ion m/z 389 from loss of CH_3.

The overall data from mass spectrometry indicate no modification in the thymidine phosphate portion of the molecule and suggest incorporation of the aminocarbazole nucleus, along with all five carbons but only one oxygen from the apurinic sugar moiety. The phosphate-substituted structural unit as shown in Figure 2 is required by difference from the molecular composition to be $C_{17}H_{13}N_2O$ and, therefore, to contain 12 degrees of insaturation, or 3 more than 3-aminocarbazole.

Then we examined the possible occurrence of structures **6** and **7** (Chart I) which could result from ring closure of either **3** or **4**. Heterocycles of this type have been described previously,[13] arising from condensation of 3-aminocarbazole derivatives with acetylacetone in acidic medium (Combes–Beyer reaction) or via a Skraup condensation. In such a hypothesis, formation of the already mentioned m/z 260 ion in the positive-ion FAB mass spectrum can be understood on the basis of 3′-phosphate thymidine β-elimination.

Chart I

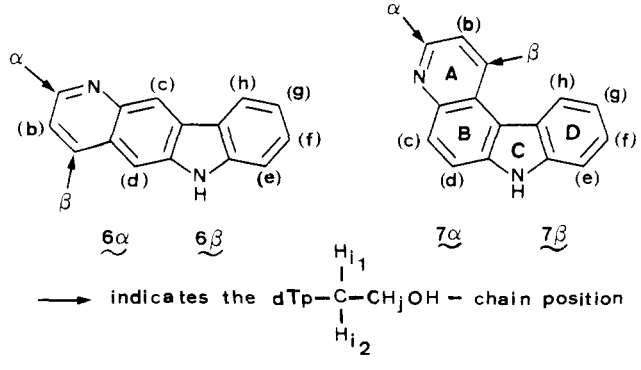

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Table I. Chemical Shifts and Coupling Constants of the Protons in the Adduct

Chemical Shifts of Thymine Protons (ppm)

TH6	TH1′	TH2′1	TH2′2	TH3′	TH4′	TH5′1	TH5′2	CH_3
7.60	6.14	2.22	2.09	4.62	3.93	3.55	3.55	1.73

Chemical Shifts of the Protons of Pyridocarbazole and Connecting Residue (ppm)

i_1	i_2	j	OH	a	b	c[b]	d[b]	e	f	g	h
4.12	3.98	4.92	7.27	9.16	7.88	7.956	7.944	7.66	7.44	7.31	8.58

Coupling Constants (Hertz) for the Thymidine Residue

$6,CH_3$	1′,2′1	1′,2′2	2′1,2′2	2′1,3′	2′2,3′	3′,4′	4′,5′1	4′,5′2	5′1,5′2	5′,P
1.3	7.1	6.1	13.8	6.3	2.9	1.8	5	3.8	11	6.5

Coupling Constants (Hertz) for Pyridocarbazole and Connecting Residue

i_1,i_2[c]	i_1,j[c]	i_2,j[a]	j,OH	a,b	a,c	b,c	c,d	e,f	f,g	g,h[c]	e,h	e,g
11.5	6.9	3	3.8	8.4	0.9	1.1	8.5	8.1	7	7.1	1.1	1.5

[a] Protons are designated in Chart I. [b] Assignment of c and d may be reversed. [c] Estimated values. Second-order effects are significant in these cases.

High-Resolution One- and Two-Dimensional ^1H NMR Spectroscopy Studies. Analysis by the application of COSY[14,15] (see Figure 3) and NOESY[16,17] (see Figure 4) provided through-bond and through-space connectivities between protons, respectively. Coupling constants between adjacent protons were estimated from the 2D *J*-resolved spectrum[15,18] (Figure 5).

The ^1H resonances from the dTp moiety are readily assigned from the 2D COSY results. In addition, the four signals appearing at 7.66, 7.44, 7.31, and 8.58 ppm can be attributed to the protons e, f, g, h of the D ring in the ABCD ring system as designated in either structures **6** or **7** (Chart I) by comparison with the reported ^1H NMR spectrum of carbazole.[19] Furthermore, the COSY spectrum indicates that the resonances at 3.98, 4.12, 4.92, and 7.27 ppm can be attributed to a CH_2CHOH moiety, which appears to be a connecting unit. The two signals at 7.945 and 7.951 ppm are barely resolvable. However, examination of the *J*-resolved spectrum (Figure 5) reveals a coupling constant value of 8.5 Hz so that they can be assigned to the ortho protons c and d in ring B, thus eliminating structures **6**.

Similarly, protons a and b resonating at 9.16 and 7.88 ppm are coupled to each other with a coupling constant value of 8.4 Hz. The chemical shift 9.16 ppm can be assigned either to proton ortho (deshielding ortho effect[20]) or to proton para (deshielding para[20] and angular effect[13,21,22]). However, many examples of pyridine derivatives, including polycyclic derivatives, exhibit an ortho coupling constant value between protons ortho and meta to nitrogen less than 6 Hz.[21,23] This appears to be a general rule.[20,24]

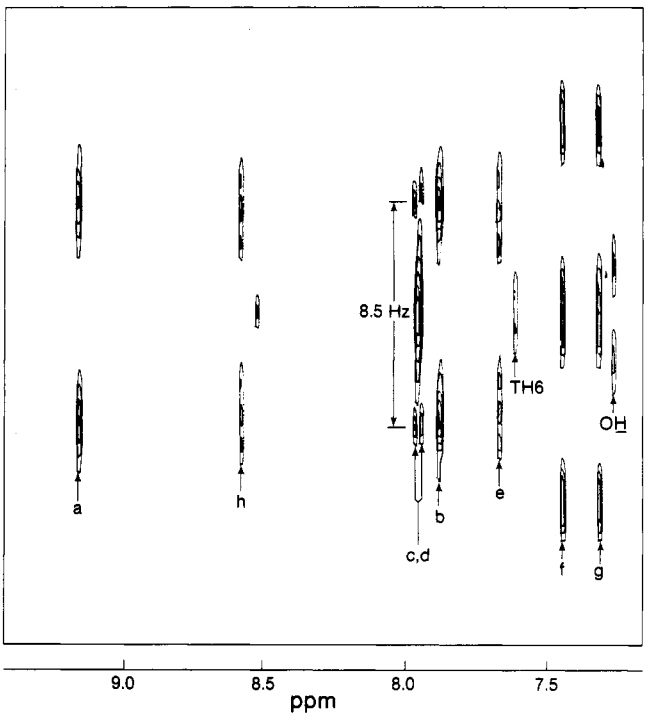

Figure 5. Two-dimensional *J*-resolved spectrum for the adduct at 20 °C. Experimental conditions: F_2 = 4K, initial t_1 = 3 μs, Δt_1 = 14 ms, sweep width for F_2 4600 Hz, sweep width for F_1 18 Hz. A total of 80 fids were accumulated for each of the 64 experiments.

Therefore, one can conclude that the connecting unit is adjacent to nitrogen and structure 7α is in better accord with the NMR data than 7β and, therefore, 7α represents the structure of the adduct.[25,26]

The number of exchangeable protons in the adduct is determined from NOESY experiments to be five,[27] in agreement with the results obtained from mass spectrometry. The ^1H chemical shifts and coupling constants of the adduct are summarized in Table I.

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(24) Reference 23, p 305.
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(26) In the original manuscript, we tentatively assigned structure 6β to adduct C on the basis of absence of NOE effect between protons a and c (or d) and lack of apparent coupling constant between protons c and d. Following the comment of one of the two reviewers, examination of the aromatic portion of the 2D J-resolved spectrum indicated an ortho coupling constant between protons c and d.
(27) Macura, S.; Wuthrich, K.; Ernst, R. R. *J. Magn. Reson.* **1982**, *47*, 351.

Conclusion

In light of the data, the overall mechanism of the reaction can be depicted as follows (Scheme I): (a) In the first step, formation of the unsaturated aldehyde A occurs via a β-elimination process. As a consequence, the DNA model is cleaved at the Ap center with elimination of a phosphomonoester, which is a good leaving group. (b) Subsequently, 1,4-addition of the 3-aminocarbazole to the α,β-unsaturated aldehyde system occurs to give the compound 4. (c) Oxidative ring closure ensues upon evaporation of the solvent to give the final adduct of pyrido[2,3-*c*]carbazole structure **7**α. This step requires protonation of the nitrogen atom and air oxidation of the intermediate dihydropyridine ring.

In conclusion, we have shown that reaction of 3-aminocarbazole on the apurinic model d[Tp(Ap)pT] gives rise, as expected, to a β-elimination cleavage process on the Ap-oligonucleotide chain but the resulting ring-closed adduct is a derivative of pyrido[2,3-*c*]carbazole. This facile ring closure provides an adaptable means of introduction of various nitrogen heterocyclic structures into DNA fragments.

Experimental Section

High-performance liquid chromatographic analyses were carried out on a Radial-Pak C_{18} (10-μm) cartridge in a Waters Z Module. A Waters U6K injector, two 6000 A pumps, a M720 solvent programmer, a 440 UV detector operating at 254 nm, and a M 730 microprocessor-controlled data system were employed. UV spectra were recorded with a Pye Unicam PU 4021 multichannel detector and a Pye-Unicam PU 4850 video chromatography control center. A gradient (concave curve profile 8 Waters) of 2–25% acetonitrile/0.1 M ammonium acetate (pH 5.9) was applied in 16 min at 3 mL min^{-1}. Preparative thin-layer chromatography was performed by using Merck Kieselgel $60F_{254}$.

Mass spectra were determined with a MAT 731 instrument. Fast atom bombardment mass spectra were determined with an Ion Tech 11N saddle field-type ion source with a neutral Xe beam of 6-keV energy. Glycerol was used as the FAB matrix. Exact mass measurements were performed by peak matching at resolution 10 000. Collisional activation mass spectra carried out on ions generated by FAB were obtained at the Midwest Center for Mass Spectrometry, University of Nebraska, with a Kratos MS-50 triple analyzer mass spectrometer as described elsewhere.[28] Electron ionization spectra were determined at 70-eV ionizing energy following sample introduction by direct probe. Deuterium exchange experiments prior to FAB ionization were carried out as earlier described[7] following solution of the sample in a 1:1 solution of ^2H_2O (99.8 atom % ^2H) and [*hydroxy*-$^2H_3$]glycerol (98% atom % ^2H). Both deuteriated solvents were from Merck Isotopes, St. Louis, MO. The trimethylsilyl derivative of product C was prepared by heating approximately 20 μg of the vacuum-dried material in a 20-μL mixture of *N,O*-bis(trimethylsilyl)-acetamide, trimethylchlorosilane, and pyridine (100:1:10) for 1 h at 100 °C (reagents from Pierce Chemical Co., Rockford, IL). [$^2H_9$]Trimethylsilyl derivatives were prepared by a similar procedure, using *N,O*-bis([$^2H_9$]trimethylsilyl)acetamide and [$^2H_9$]-trimethylchlorosilane purchased from Merck Isotopes, St. Louis, MO.

The ^1H NMR spectra were recorded at 20 °C on a Bruker WH 400 cryospectrometer equipped with an Aspect 2000 data system. A solution of 5.4 mg of the adduct in 0.4 mL of DMSO-d_6 (Sigma, 99.9% D) was degassed and treated with a molecular sieve to remove H_2O. In the NOESY experiment, the mixing time was varied randomly by 15% to eliminate the effect of *J*-coupling.[20] The experimental conditions used are given in the legends to the figures.

Materials. Compound **2** was prepared according to the reported method.[5]

Reaction of the Apurinic Model 2 with Aminocarbazole (1). A solution of **1** (80.1 mg, 0.44 mmol) and **2** (43.25 mg, 0.055 mmol) in MeOH/H_2O (4:1, 55.5 mL) was kept at room temperature for 2 days. The reaction was monitored by HPLC. In this reaction, only formation of B but not **7**α was detected. The reaction mixture was diluted with water (30 mL) and then extracted with diethyl ether (6 × 50 mL). The aqueous layer was filtered, evaporated in vacuo, and chromatographed on a DEAE-Sephadex A 25 (HCO_3^- form) column (2 × 24 cm). The column was eluted with a triethylammonium bicarbonate buffer (pH 7.5, linear gradient from 0 to 0.4 M over 2000 mL) and fractions of 10 mL were collected. The appropriate fractions (HPLC purity better than 80%) were combined and evaporated. The residue was further subjected to preparative TLC ($MeOH/CH_2Cl_2$, 3:7, developed three times) to give 17.1 mg (45.3%) of **7**α as the triethylammonium salt. The residue was then treated with Dowex 50W (Na^+ form). The adduct **7**α was obtained after lyophilization as its sodium salt: 12.8 mg, 38.5% yield; HPLC purity over 99%.

In another reaction where **1** and **2** were used at equimolar concentrations (10 mM) but at 50 °C, both B and **7**α were detected, and the mixture was kept until the transformation of B into **7**α was completed.

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Registry No. **1**, 6377-12-4; **2**, 104105-86-4; **7**α·NEt_3, 110142-92-2; **7**α·Na, 110142-93-3.

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