170. Synthesis and Characterization of DNA Fragments Bearing an Adenine Radiation Product: 7,8-Dihydroadenin-8-one

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The 7,8-dihydroadenin-8-one is one of the base derivatives formed by the action of ionizing radiation upon DNA. In order to investigate the mutagenic effects and the repair of DNA lesions induced by gamma rays, the synthesis of oligonucleotides bearing this damage has been performed by the phosphoramidite methodology. The preparation of the corresponding protected mononucleotide **6** (see *Scheme*) and its insertion into a DNA fragment are described. The modified oligonucleotide was purified by HPLC, characterized by DNA sequencing, enzymatic hydrolysis, and FAB mass spectrometry. In the experimental conditions used herein, no basic or acidic degradation was observed. In the DNA chain, the lesion is stable on piperidine heating under the usual DNA sequencing conditions.

Introduction. – The chemical modifications induced by the action of ionizing radiation upon genetic material are in great part responsible of the biological effects which follow irradiation: mutation, cellular lethality, carcinogenesis [1–6]. The DNA adenine fragment is partly transformed into the 7,8-dihydroadenin-8-one (= 6-amino-1,7-dihydro-8*H*-purin-8-one) moiety. Monoclonal antibodies have been elicited to determine and measure this DNA defect [7]. So far, the biological role played by this lesion has not been investigated. This is due to the fact that ionizing radiation produces a broad spectrum of DNA modifications in very small quantities. Consequently, oligonucleotides bearing a well defined DNA defect in a determined position in the sequence are very useful tools for radiobiologists investigating radiation-induced DNA damage.

In this article, we describe the total chemical synthesis of oligodeoxyribonucleotides bearing the 7,8-dihydroadenin-8-one moiety. The sequences have been chosen to facilitate the biological studies which are presently in progress. Characterization and purification of the oligonucleotides are also reported.

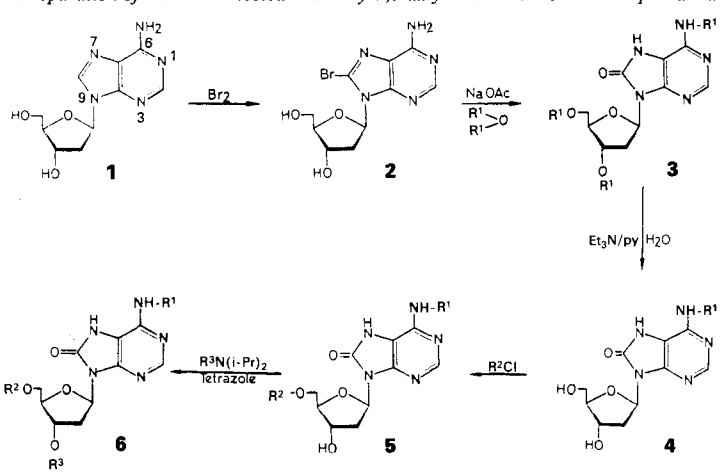
Results. – When DNA in aerated solution is submitted to the action of ionizing radiation, 7,8-dihydroadenin-8-one moiety, usually named 8-hydroxyadenine, in place of an adenine residue is one of the lesions directly formed. This radiation product is generated by the attack of the OH radical at C(8) of the purine ring (for the mechanism, see [8]). Inspection of the IR spectra 2′-deoxy-7,8-dihydroadenosin-8-one (ho^8A_d; **4**, R^1 = H) shows the presence of a main absorption band in the 1720-cm region; it can thus be concluded that the keto form is predominant [9]. This modified nucleoside is produced in low yield by gamma irradiation of aqueous solutions of 2′-deoxyadenosine (**1**), besides other radiation products. Therefore, irradiation cannot conveniently be used to prepare the modified nucleoside **4** (R^1 = H) needed for the chemical synthesis of the oligonucleo-

tide. A derivative thereof, **4** with $R^1 = C_6H_5OCH_2CO$, was synthesized from **1** in three steps (see *Scheme* below).

Synthesis of the Protected Phosphoramidite **6**. The method described by *Ikehara* for bromination of 2′-deoxyadenosine (**1**) was improved [10] by stabilizing the pH of the mixture at 5 (addition of NaOAc) and optimizing the Br_2 concentration, time of reaction, and temperature. The product 8-bromo-2′-deoxyadenosine (**2**) was obtained in excellent yield (88%) after crystallization. It is known that treatment of **2** with NaOAc in the presence of AcOH or Ac_2O leads to the substitution of the Br by an OH group and also to acetylation [11] [12]. In order to introduce the phenoxyacetyl ($PhOCH_2CO$) group in place of the Ac group, **2** was heated with $(PhOCH_2CO)_2O$, NaOAc, and pyridine, thus avoiding N-glycosylic bond rupture. After workup and chromatography on silica gel, **3** was obtained in 72% yield. A difficult step was the selective hydrolysis of the $PhOCH_2CO$ groups. To avoid the elimination of the substituent at N^6, **3** was reacted with Et_3N/pyridine/H_2O to give **4** (60% yield). The structure of **4** was confirmed by ^{1}H-NMR and mass spectrometry. Subsequently, the 5′-OH function was selectively protected by a dimethoxytrityl group (→**5**, characterization by ^{1}H-NMR and FAB-MS) and transformed to the phosphoramidite **6** by the action of (2-cyanoethyl)bis[(diisopropyl)amino]phosphine in the presence of tetrazole and diisopropylamine according to a method described by *Barone* [13]. The high-purity product **6** required for the oligonucleotide assembly step was obtained by HPLC (silica gel, CH_2Cl_2/$(i\text{-}Pr)_2NH$/MeOH gradient) followed by precipitation in hexane at −70°.

Assembly of the Protected Nucleotides on Silica-Gel Support. The protected phosphoramidite nucleotides were assembled on silica-gel support and the oligonucleotides deprotected according to a method recently described [14]. The protected phosphoramidite **6** was condensed with a final yield quite similar to that obtained with the other mononucleotides. A variety of oligonucleotides bearing the 7,8-dihydroadenin-8-one moiety and of length ranging from 9-mer to 39-mer were prepared for biological purposes (see **7**–**13**, *Fig. 1*).

Scheme. *Preparation of the* N^6*-Protected 2′-Deoxy-7,8-dihydroadenosin-8-one Phosphoramidite* **6**



R^1 = $PhOCH_2CO$; R^2 = 4,4′-dimethoxytrityl; R^3 = $(NCCH_2CH_2O)[(i\text{-}Pr)_2N]P$

7 d (A-A-G-C-T-T-G-A-A-T-T-C-T-A-G-A-T-C-T-G-T-T-A-A-C)
8 d (A-A-G-C-T-T-G-A-A-T-T-C-T-X-G-A-T-C-T-G-T-T-A-A-C)
9 d (A-A-G-C-T-T-G-A-A-T-T-C-T-A-G-X-T-C-T-G-T-T-A-A-C)
10 d (A-A-G-C-T-T-G-A-X-T-T-C-T-A-G-A-T-C-T-G-T-T-A-A-C)
11 d (X-A-T-G-G-C-A-C-T-T-C-G-G-A-A)
12 d (C-G-G-X-T-A-T-C-C)
13 d (T-A-T-G-G-C-A-C-T-T-C-G-G-A-A-T-G-G-T-X-G-G-C-T-G-C-T-A-A-C-C-T-C-G-C-T-A-A-G)

Fig. 1. *Sequence of the chemically synthesized oligodeoxynucleotides* **7-13** *with and without the 2'-deoxy-7,8-dihydroadenosin-8-one moiety* ($ho^8A_d = X_d$) *in place of the non-modified nucleoside* A_d

Purification and Characterization of the Modified Oligonucleotides. The nucleotides were purified by HPLC on a *Partisil SAX* anion exchange column and desalted by dialysis against distilled H_2O. If further additional purification was necessary, this was performed on an *ODS* reverse-phase column. *Fig. 2* shows elution profiles obtained on these columns. The length and the sequence of products were confirmed by ^{32}P labeling with (gamma-^{32}P)adenosine triphosphate and polynucleotide kinase, followed by polyacrylamide gel electrophoresis [15]. The autoradiography of the crude mixtures shown in *Fig. 3* evidences the high quality of these chemical syntheses.

Comparison by the *Maxam-Gilbert* sequencing procedure of normal and modified oligonucleotides having the same sequence but for the modified residue gives useful information. The intensity of the band of X_d (= ho^8A_d) is much lower at the lanes $G_d + A_d$ and much higher at the lanes $C_d + T_d$. That confirms that in the expected position, the base residue is different from adenine.

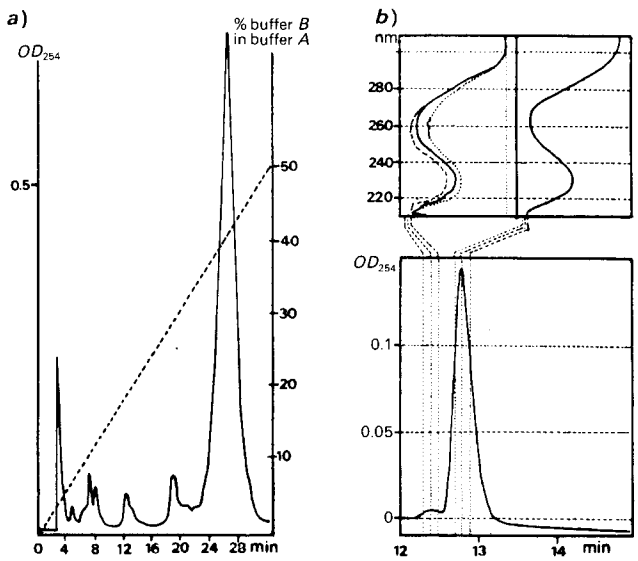


Fig. 2. *HPLC profile of oligonucleotide d(C-G-G-X-T-A-T-C-C)* (**12**). *a*) Crude reaction mixture on an anionic column (*Partisil 10 SAX*, 0.75 × 30 cm); linear gradient (30 min) of buffer *B* (0.4M KH_2PO_4 in H_2O/MeCN 8:2, pH 6.8) in buffer *A* (1 mM KH_2PO_4 in the same solvent); flow rate 4 ml/min. *b*) Purified product on a reverse-phase column (*Hypersil ODS*, 10 µm, 0.46 × 25 cm); gradient 3–10% MeCN in 0.05M NH_4OAc (pH 7) in 15 min; flow rate 1 ml/min. Spectra acquired from slope and apex clearly differentiate impure peaks.

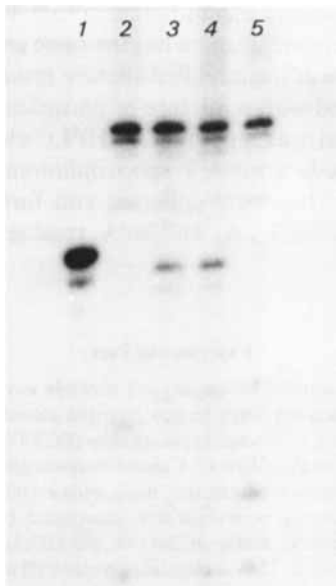


Fig. 3. *Autoradiogram of crude synthetic oligonucleotides* **7–10** *in denaturing conditions polyacrylamide gel electrophoresis. Lane 1:* 17-mers of reference; *Lanes 2–5:* 25-mers **7**, **8**, **9**, and **19**, resp.

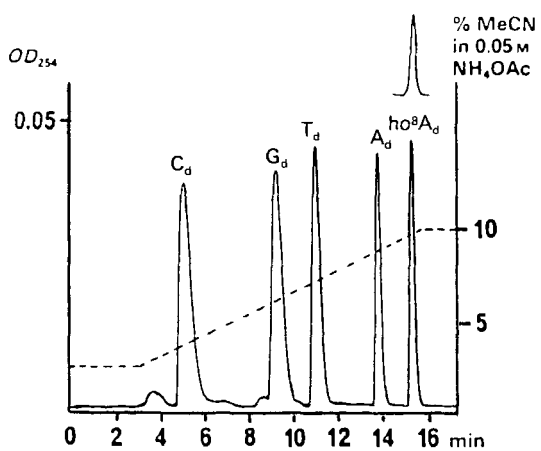


Fig. 4. *Analytical HPLC of the enzymatic digestion product of d(C-G-G-X-T-A-T-C-C)* (**12**) *on a* Hypersil ODS *column* (0.46 × 25 cm). Linear gradient of 3–10% MeCN in 0.05M NH_4OAc (pH 7); flow rate 1 ml/min. Lower part: C_d, G_d, T_d, A_d, and ho^8A_d indicate the peaks corresponding to 2′-deoxycytidine, 2′-deoxyguanosine, thymidine, 2′-deoxyadenosine and 2′-deoxy-7,8-dihydroadenosin-8-one. Upper part: HPLC of synthetic ho^8A_d under the same conditions.

The modified base 7,8-dihydroadenin-8-one cannot be directly identified by MS under pyrolytic conditions because guanine has the same empirical formula.

To verify the actual nature of the modified adenine residue in the chain, the modified oligonucleotide **12** was treated with a mixture of phosphodiesterase and alkaline phosphatase. The enzymatic digest was separated by HPLC chromatography *(Fig. 4)*. The peaks were detected by a diode array UV spectrophotometer and identified from the shape of their UV spectra. They were collected and further analysed by MS which confirmed the presence of C_d, G_d, T_d, A_d, and ho^8A_d residues.

Experimental Part

General. Solvents were dried or distilled before use, and tetrazole was sublimated under reduced pressure. (2-Cyanoethyl)bis[(*N*,*N*-diisopropyl)amino]phosphine was prepared according to [18]. Snake-venom phosphodiesterase (EC 3.1.4.1, *Crotallus durissus*) and alkaline phosphatase (EC 3.1.3.1, calf intestine) were products from *Boehringer*. TLC: precoated silica gel *60 F_{254} (Merck)*. Column chromatography: silica gel *60H (Merck)*. HPLC: prep. mode for the purification of synthetic products, anal. mode using a different purification technique to acquire pure compounds. M.p.: *Gallenkamp* melting-point apparatus; uncorrected. UV spectra: *Beckman-DU-8B* spectrophotometer; λ_{max} (ε) in nm. NMR spectra: *Bruker AC200* (^{1}H; 200 MHz) or *WM250* (^{31}P; 81.7 MHz) spectrometers; chemical shifts values in ppm rel. to TMS as internal reference (^{1}H) or to 85% H_3PO_4 as external standard (^{31}P). Pyrolysis MS [16] and positive or negative-ion fast-atom-bombardment (FAB) MS [17]: *Kratos MS 50* mass spectrometer (B = base, pac = phenoxyacetic group, dr = deoxyribose).

8-Bromo-2′-deoxyadenosine (**2**) was synthesized according to [10] with an 88% yield. TLC (MeOH/$CHCl_3$ 2:8): R_f 0.70. M.p. 202° ([10]: 200°). UV (H_2O, pH 6.9): 265 (15900); min. 229 (4700). ^{1}H-NMR (200 MHz, (D_6)DMSO): 7.37 (*s*, H–C(2)); 6.60 (*s*, NH_2–C(6)); 5.55 (*q*, *J* = 8.5, 6.5, H–C(1′)); 4.58 (*s*, OH–C(3′)); 4.62 (*s*, OH–C(5′)); 3.74 (*m*, *J* = 4.3, 6.8, H–C(3′)); 3.14 (*m*, *J* = 4.3, 4.8, 6.4, H–C(4′)); 2.86, 2.80 (2*m*, *J* = –9.7, 4.8, 6.4, CH_2(5′)); 2.53, 1.45 (2*m*, *J* = –13.1, 6.8, 8.5, CH_2(2′)). EI-MS: 299 ([*M* – CH_2O]$^{+\cdot}$), 240 ([BH + $CHCH_2$]$^+$), 215 and 213 (100, BH^+), 188 and 186 ([BH – HCN]$^+$), 135 ([213 – Br]$^+$), 117 (dr^+).

2′-Deoxy-7,8-dihydro-3′-O,5′-O,N^6*-tris(phenoxyacetyl)adenosin-8-one* (**3**). To dry **2** (3 g, 9.1 mmol) in anh. pyridine (75 ml), anh. NaOAc (2.25 g, 16.5 mmol) and ($PhOCH_2CO)_2O$ (15 g, 51 mmol) were added. The mixture was refluxed for 10 h with stirring (TLC ($CHCl_3$/MeOH) 95:5 monitoring). H_2O (5 ml) was added and the mixture left at r.t. for 10 min. The mixture was evaporated, the oily residue dissolved in CH_2Cl_2 (100 ml), 5% aq. $NaHCO_3$ soln. (3 × 100 ml) added, the product extracted with CH_2Cl_2, and the combined org. layer washed with H_2O (100 ml), dried (Na_2SO_4), and evaporated. The residue was purified by column chromatography (120 g SiO_2, 5 × 12 cm, MeOH/$CHCl_3$ 1.5:100), yielding dry **3** (4.42 g, 72%). TLC ($CHCl_3$/MeOH 9:1): R_f 0.79. M.p. 99°. ^{1}H-NMR (200 MHz, (D_6)acetone): 8.37 (*s*, H–C(2)); 7.36–6.85 (*m*, arom. H); 6.33 (*t*, H–C(1′)); 5.69 (*m*, H–C(3′)); 4.84 (*s*, PhOCH_2); 4.83 (*s*, PhOCH_2); 4.70 (*s*, PhOCH_2); 4.54 (*m*, 1 H–C(5′)); 4.36 (*m*, 1 H–C(5′)); 4.25 (*m*, H–C(4′)); 3.50 (*m*, 1 H–C(2′)); 2.45 (*m*, 1 H–C(2′)). EI-MS: 669 (M^+), 286 ([Bpac + 2 H]$^+$), 285 ([Bpac + H]$^+$), 152 ([B + 2 H]$^+$), 151 ([B + H$^+$]).

2′-Deoxy-7,8-dihydro-N^6*-(phenoxyacetyl)adenosin-8-one* (**4**). A soln. of **3** (1 g, 1.5 mmol) in 50 ml of Et_3N/pyridine/H_2O 1:1:3 was stirred for 1.30 h (TLC ($CHCl_3$/MeOH 9:1) monitoring). The solvent was evaporated, H_2O (2 × 100 ml) added, and the resultant extracted with CH_2Cl_2 (2 × 100 ml). The combined extract was dried (Na_2SO_4), filtered, and evaporated and the residue purified by column chromatography (80 g SiO_2, 5 × 10 cm, MeOH/$CHCl_3$ 2:100) giving 360 mg (60%) of pure **4**. TLC ($CHCl_3$/MeOH 9:1): R_f 0.44. M.p. 102°. UV (MeOH): 287 (12970); min. 254 (2990). ^{1}H-NMR (200 MHz, (D_6)acetone): 8.54 (*s*, H–C(2)); 7.53 – 7.02 (*m*, arom. H); 6.55 (*q*, H–C(1′)); 5.10 (*s*, PhOCH_2); 4.78 (*m*, H–C(3′)); 4.15 (*m*, H–C(4′)); 3.90 (*m*, 1 H–C(5′)); 3.75 (*m*, 1 H–C(5′)); 3.16 (*m*, 1 H–C(2′)); 2.25 (*m*, 1 H–C(2′)). EI-MS: 401 (M^+), 371 ([*M* – CH_2]$^+$), 312 ([Bpac + $CHCH_2$]$^+$), 285 (79, [Bpac + H]$^+$), 151 ([B + H]$^+$).

2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-7,8-dihydro-N^6*-(phenoxyacetyl)adenosin-8-one* (**5**). To a stirred soln. of **4** (300 mg, 0.75 mmol) in dry pyridine at 4° was added 4,4′-dimethoxytrityl chloride (270 mg, 0.82 mmol). Stirring was continued overnight (TLC (MeOH/$CHCl_3$ 5:95) monitoring) until complete conversion to a major trityl spot (R_f 0.84). MeOH (2 ml) was added, and after stirring for 5 min, the soln. was evaporated, 5% aq. $NaHCO_3$ soln. (2 × 50 ml) added, this soln. extracted with CH_2Cl_2 (2 × 50 ml) and the combined org. layer washed with H_2O (100

ml), dried (Na_2SO_4), and evaporated. The oily foam was purified by column chromatography (60 g SiO_2, 3 × 15 cm) using a step gradient of 0–3% MeOH in CH_2Cl_2 to give 180 mg (35%) of **5**. M.p. 157°. UV (MeCN): 288 (13950); min. 258 (6060). ^{1}H-NMR (200 MHz, (D_6)acetone): 8.30 (*s*, H–C(2)); 7.60–7.10 (*m*, arom. H); 6.50 (*t*, H–C(1′)); 5.01 (*s*, PhOCH_2); 4.84 (*m*, 1 H–C(3′)); 4.51 (*d*, OH–C(3′)); 4.21 (*m*, H–C(4′)); 3.81 (*s*, $(MeO)_2$Tr); 3.49 (*m*, H–C(5′)); 3.41 (*m*, H–C(5′)); 3.33 (*m*, H–C(2′)); 2.35 (*m*, H–C(2′)). FAB-MS (neg. ions. PEG matrix): 702 (23, [*M* – H]$^-$), 610 ([*M* – $C_6H_5O_2$]$^-$), 568 ([*M* – pac]$^-$), 400 ([*M* – $(MeO)_2$Tr]$^-$), 284 ([Bpac]$^-$), 151 ([BH]$^-$), 150 (B^-).

*2′-Deoxy-5′-*O*-(4,4′-dimethoxytrityl)-7,8-dihydro-*N^6*-(phenoxyacetyl)adenosine-8-one 3′[(2-Cyanoethyl)* N,N*-Diisopropylphosphoramidite]* (**6**). To dry **5** (150 mg, 0.21 mmol) in anh. CH_2Cl_2 (2 ml) under dry Ar, dry (i-Pr)$_2$NH (0.1 mmol), anh. tetrazole (7.46 mg, 0.1 mmol) in 1 ml of CH_2Cl_2 and (2-cyanoethyl)bis[(*N,N*-diisopropyl)amino]phosphine (69 mg, 0.23 mmol) were added by syringe. After 30 min at 20° (TLC (MeOH/$CHCl_3$ 1:9) monitoring), diisopropylammonium tetrazolate was filtered off, and solvents were evaporated. The oily residue was dissolved in AcOEt (30 ml), shaken with aq. $NaHCO_3$ soln. (50 ml), and washed with H_2O (2 × 50 ml), and the combined extract dried (Na_2SO_4) and evaporated. The resulting dry foam in solvent *A* (CH_2Cl_2/hexane/(i-Pr)$_2$NH 60:40:1) was purified by HPLC (1 × 65 cm, silica, *Partisil* 10 µm; *Varian-5020* liquid chromatograph, variable-wavelength UV/VIS detector; equilibration with solvent *A*) with a 30-min linear gradient from 0–100% solvent *B* (CH_2Cl_2/MeOH/(i-Pr)$_2$NH 90:5:5) in solvent *A* at a flow rate of 4 ml/min. The resulting white foam was dissolved in CH_2Cl_2 (5 ml) and precipitated into stirred hexane (200 ml, –78°). Filtration and drying *in vacuo* over anh. P_2O_5 gave 180 mg (58%) of **6**, stored under dry Ar. M.p. 150°. UV(MeCN): 286 (9000); min. 260 (5400). ^{1}H-NMR (200 MHz, (D_6)acetone; 2 diastereoisomers): 8.37 (*s*, 0.5 H, H–C(2), isomer I); 8.36 (*s*, 0.5 H, H–C(2), isomer II); 7.61–6.86 (*m*, arom. H); 6.52 (2 *t*, 2 H, H–C(1′), isomers I and II); 5.0 (*s*, 2 H, PhOCH_2); 4.31 (*m*, 1 H, H–C(4′)); 4.30–3.70 (*m*, 2 H(CH_2(5′)); 3.85 (*s*, 6 H, (*Me*O)$_2$Tr); 3.48 (*m*, 1 H, H–C(2′)); 2.86 and 2.76 (2*t*,2 H, CH_2CH_2CN, isomers I and II); 2.57 (*m*, 1 H, H–C(2′)); 1.34 (*m*, CH_3 of i-Pr). ^{31}P-NMR (81.7 MHz, (D_5)pyridine): 149.4, 149.5. HR-FAB-MS: 902.3590 ([*M* – H]$^-$, $C_{48}H_{35}N_7O_9P^-$, calc. 902.3642). FAB-MS (neg. ions matrix PEG): 902 (88, [*M* – H]$^-$), 810 ([*M* – $C_6H_5O_2$]$^-$), 768 ([*M* – pac]$^-$), 600 ([*M* – $(MeO)_2$Tr]$^-$), 284 (Bpac$^-$), 150 (B^-).

General Procedure for Oligonucleotide Synthesis. Condensation. Each synthesis was carried out with 0.2 mmol (10 mg) of modified (by chemical transformation), controlled pore glass support in a short *Teflon* column (4 × 4 mm) on a DNA synthesizer (*Applied Biosystems*) using the cyanoethyl *N,N*-diisopropyl phosphoramidite method at a flow rate of 2.3 ml/min for optimal coupling efficiency.

Cleavage from Support, Deprotection, and Purification. Cleavage of the oligonucleotides and deprotection of the phosphate groups with aq. NH_3 soln. (5 × 200 µl) were carried out on the solid support according to previously described procedures [14]. HPLC purifications were performed on an anion-exchange column (0.75 × 30 cm, *Partisil SAX 10* µm) using a 1–200 mM linear gradient of KH_2PO_4 buffer (pH 6.8) with 20% MeCN over 30 min *(Fig. 2a)*. The pure products were desalted prior to use by dialysis against distilled H_2O (4 × 500 ml). If further additional purification was necessary, this was performed on a reverse-phase column (0.46 × 25 cm, *Hypersil ODS* 10 µm, *Société Française de chromato colonne*) under experimental conditions described in *Fig. 2b*. To evaluate crude-product syntheses and chain length, the 5′-end oligonucleotides were labeled with (gamma-^{32}P)ATP by T_4 polynucleotide kinase and checked by 20% polyacrylamide gel electrophoresis. The sequences were confirmed by a modified *Maxam-Gilbert* method for sequencing oligonucleotides [15].

Enzymatic Hydrolysis. The oligonucleotides (0.5 A_{260} unit) containing 2′-deoxy-7,8-dihydroadenosin-*8-one* were digested overnight with snake-venom phosphodiesterase (5 µg, 5 µl) at 37° in 0.1M *Tris*-$MgCl_2$ buffer (pH 7.8) and with alkaline phosphatase (5 µg, 1 h, 37°). To analyse the nucleosides, a rapid qualitative separation was performed by HPLC on a *Hypersil-ODS* reverse-phase column (0.46 × 25 cm) using a 3–10% linear gradient of MeCN in 50 mM NH_4OAc (pH 7) over 16 min at 1 ml/min. Product peaks (C_d, G_d, T_d, A_d, ho^8A_d) were collected, freeze-dried, and checked by MS to be identical to the 2′-deoxycytidine, 2′-deoxyguanosine, thymidine, 2′-deoxyadenosine, and authentic 2′-deoxy-7,8-dihydroadenosin-8-one [9].

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