111. Chemical Synthesis of Oligonucleotides Containing N^6-Methyladenine Residues in the GATC Site

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To investigate by NMR the effects of adenine methylation, a set of oligodeoxynucleotides has been synthesized by phosphotriester or phosphoramidite procedures on silica-gel support. The preparation of fully protected 2'-deoxy-N^6-methyladenosine 3'-phosphate **4** and (2'-deoxy-N^6-methyladenosin-3'-*O*-yl)(methoxy)morpholinophosphine **5** is described. The large-scale chemical synthesis, purification by HPLC, and characterization by MS of these molecules containing N^6-methyladenine is reported.

Introduction. – Deoxyribonucleic acid may be methylated at the C(5) position of cytosine or at the 6-amino group of adenine [1] [2]. The nucleotide sequence d(GATC)[1]) is specifically methylated at adenine by dam methylase (EC 2.1.1.37) with a lag period behind replication. A methylated d(GATC) sequence and dam methylase [3] are thought to be involved in DNA strand replication during post-replicative mismatch repair and in the control of gene expression [4]. In order to perform NMR structural determination and biological studies of DNA repair, rather large quantities of synthetic oligonucleotides containing 2'-deoxy-N^6-methyladenosine (d(m^{6}A)[1])) residues in d(GATC) sequences were needed [5] [6].

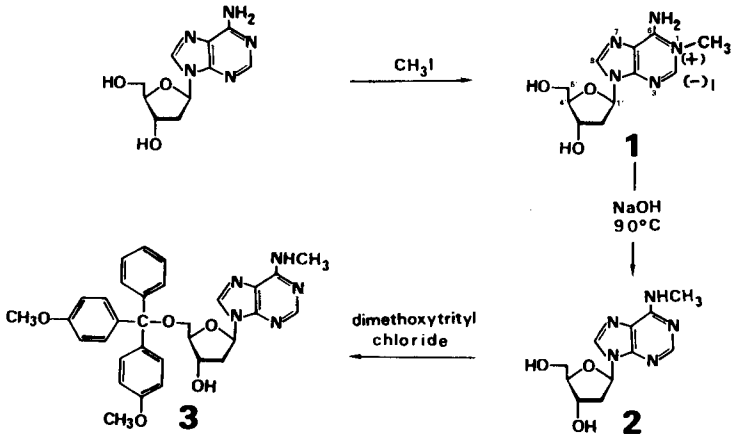
We wish to report their chemical synthesis by phosphoramidite and phosphotriester approaches. On extending oligonucleotide synthesis to unnatural bases, only a few studies have been reported [7] [8].

Results and Discussion. – The synthesis of the protected monomers of d(m^{6}A) needed in the oligonucleotide assembly involves the following steps: *1)* Preparation of 2'-deoxy-5'-*O*-(4,4'-dimethoxytrityl)-N^6-methyladenosine (**3**; used in the phosphotriester and phosphoramidite procedures), *2)* preparation of the 3'-(chlorophenyl phosphate) **4** derived from **3** (used in the phosphotriester procedure), and *3)* preparation of the (deoxyribonucleosid-3'-*O*-yl)(methoxy)morpholinophosphine **5** derived from **3** (used in the phosphoramidite procedure).

*2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N^6*-methyladenosine* (**3**; see *Scheme 1*). The salt 2'-deoxy-1-methyladenosin-1-ium iodide (**1**) was prepared according to the procedure previously described by *Jones* and *Robins* [9] by the action of MeI on deoxyadenosine at room temperature. The product was characterized by FAB-MS (fast atom bombardment MS). Compound **1** was converted at 90° in an alkaline medium into 2'-deoxy-N^6-methyl-

[1]) Abbreviations according to IUPAC/IUB; protecting groups: m = methyl, bz = benzoyl, ib = isobutyryl, [(MeO)$_2$Tr] = 4,4'-dimethoxytrityl.

Scheme 1. *Synthesis of Protected 2'-Deoxy-*N^6*-methyladenosine* **3**

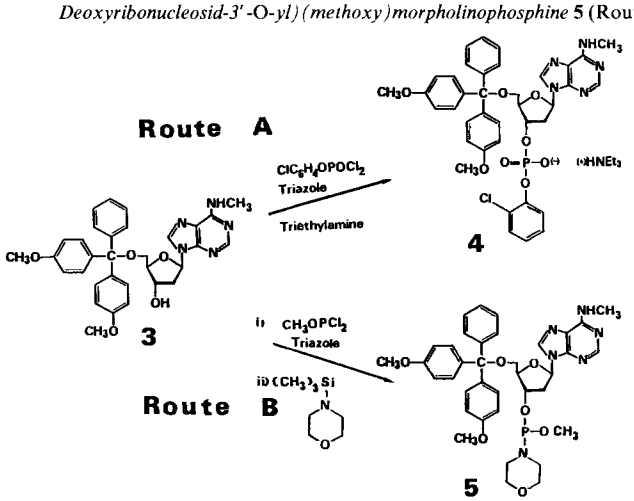


adenosine (**2**). The reaction is a *Dimroth* rearrangement and proceeds through the opening of the pyrimidine ring due to the attack at C(2) by the OH^- ion.

Attempts were made to protect the secondary amine function of **2** by benzoylation. They were unsuccessful, but this protection proved to be unnecessary in the subsequent synthetic steps. The 5'-OH function was selectively blocked by a $[(MeO)_2Tr]^{\oplus}$) using 4,4'-dimethoxytrityl chloride to give the desired product **3**.

*2'-Deoxy-5'-*O*-(4,4'-dimethoxytrityl)-*N^6*-methyladenosine 3'-[(2-Chlorophenyl)triethylammonium phosphate]* (**4**). Owing to the high stability in alkaline medium of 2'-deoxy-N^6-methyladenosine (**2**), 2-chlorophenyl was considered to be a suitable protecting group for the internucleotide phosphate. The phosphorylation method introduced by *Chattopadyaya* and *Reese* [10] for the natural nucleotides was adapted to give the desired phosphate **4** *(Scheme 2, Route A)*. When the condensation was complete, most of the

Scheme 2. *Synthesis of the Protected Deoxyribonucleoside (Chlorophenyl phosphate)* **4** (Route A) *and of the Deoxyribonucleosid-3'-*O*-yl)(methoxy)morpholinophosphine* **5** (Route B)



by-products were eliminated by washing the $CHCl_3$ solution of the reaction mixture with an aqueous $NaHCO_3$ solution. Purification was completed by prep. HPLC (silica gel *60 (Lichroprep)*; gradient (0–10%) of MeOH in CH_2Cl_2). Washing of the selected fraction with $Et_3NH_2CO_3$ gave the high-purity grade required for **4** in the further steps of the oligonucleotide assembly by the phosphotriester approach. Phosphate **4** was characterized by ^1H- and ^{31}P-NMR and FAB-MS *(Fig. 1)*.

[2'-Deoxy-5'-O-(4,4'dimethoxytrityl)-N^6-methyladenosin-3'-O-yl](methoxy)morpholinophosphine (**5**). The introduction of a methyl phosphoramidite moiety as the basic synthetic unit in place of the unstable chloro(methoxy)phosphine made the phosphite

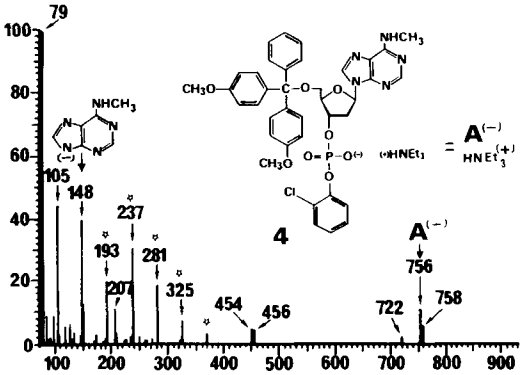


Fig. 1. *FAB-MS of* **4**

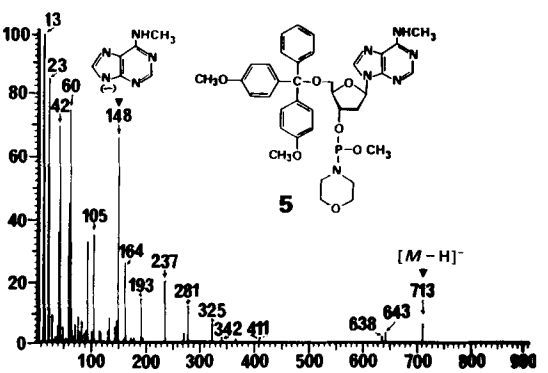


Fig. 2. *FAB-MS of* **5**

approach in solid-phase synthesis easier [11] [12]. The monomeric 2'-deoxy-N^6-methyladenosine-3'-*O*-yl phosphoramidite **5** was obtained by reacting successively methoxybis(triazolyl)phosphine with **3** and then with (trimethylsilyl)morpholine in THF according to the procedure proposed by *Fourrey* and *Varenne* [13] *(Scheme 2, Route B)*. The product **5** was purified by solvent precipitation, and its purity was checked by ^{31}P-NMR spectroscopy. It appeared as a mixture of two diastereoisomers. Small amounts (< 5%) of side-products due to the hydrolysis of the desired product could be evidenced. These traces of impurities did not disturb the assembly of the nucleotides on a silica-gel support. The crude product obtained by solvent precipitation proved to be very satisfactory in subsequent steps of the oligonucleotide phosphoramidite synthesis. The FAB-MS of **5** is shown in *Fig. 2*.

Table 1. *One Cycle of Operation for the Nucleotides Synthesis*[a])

Step	Reagent or solvent	Volume [ml]	Reaction time [min]
1. Wash	MeCN	7.5	3
2. Detritylation	3% Cl_3CCOOH in dichloroethane	5	2
3. Wash	Anh. MeCN	10	4
4. Condensation	Coupling mixture[b])	1	15[c])

[a]) The synthesis was carried out in a short stainless-steel column (4.7 × 42 mm) fitted to the automated synthesizer. Solvent flow-rate was 2.5 ml·min^{-1}.

[b]) The dry mononucleotide (50 mg, 55 μmol, 3 equiv.) was dissolved in anh. *N*-methylimidazole/MeCN 15:85 (*v/v*; 0.5 ml) and 2-chloromesitylene (100 mg, 110 μmol, 6 equiv.) in MeCN (0.5 ml). The coupling mixture was circulated through the silica-gel support at a flow rate of 0.6 ml·min^{-1}.

[c]) Recycle mode.

Phosphotriester Assembly and Deprotection. To perform the assembly of the protected monomeric units, we used the *Efimov* coupling procedure [14] on a silica-gel support. The long chain alkylamino controlled pore glass *(CPG, Pierce Chemical Company)* was loaded with the first nucleoside [15] [16]. The usual base-protecting groups (d(bzA), d(ibG) and d(bzC)[1])) were employed for the normal monomer units. The 2′-deoxy-N^6-methyladenosine 3′-[(2-chlorophenyl) triethylammonium phosphate] (see **4**) was included in the assembly cycle *(Table 1)* in place of a N^6-benzoylated 2′-deoxyadenosine 3′-[(4-chlorophenyl) triethylammonium phosphate] unit. Removal of the [$(MeO)_2$Tr] group was carried out using 3% Cl_3CCOOH acid in dichloroethane. During the deprotection step, the bright orange colour of the released $(MeO)_2Tr^+$ ion served to estimate the efficiency of the condensation steps and the time required for the acid deprotection. The colouring remained almost constant during all the deprotection steps, and no difference could be observed when the 2′-deoxy-N^6-methyladenosine monomer unit was used. Hexa- to decaoligonucleotides *(Table 2)* were prepared in large quantities for NMR studies, but even larger DNA fragments could be obtained in this way.

Table 2. *Synthesis of Oligonucleotides*

Nucleoside loading	d(c)-Silica gel, 65 μmol/g	d(G)-Silica gel, 68 μmol/g
Sequence	d(GGATCC)	d(GCGm^{6}ATCATGG)
	d(GGm^{6}ATCC)	d(GCGATCATGG)
	d(GGATATCC)	d(GCGATCm^{6}ATGG)
	d(GGm^{6}ATATCC)	d(CGCGTm^{6}ACGCG)
	d(CCATGATCGC)	

The chlorophenyl phosphate-protecting groups were eliminated by treatment with oximate ion [17]. The oligonucleotides were cleaved from the support by conc. NH_3 at room temperature. As shown by ^{1}H-NMR studies, isobutyryl groups of guanine moieties needed at least heating for two days in conc. NH_3 at 50° to be eliminated entirely.

Phosphoramidite Assembly and Deprotection. A typical reaction cycle involved: *a)* removal of the terminal 5′-[$(MeO)_2$Tr] protecting group by Cl_3CCOOH, *b)* internucleotide coupling with a 10-fold excess of the protected (deoxyribonucleosid-3′-*O*-yl)-(methoxy)morpholinophosphine unit in the presence of tetrazole as an activating agent, *c)* oxidation of the intermediate phosphite by I_2, and *d)* extensive washing procedures by MeCN between steps *a)*, *b)*, and *c)*. The efficiency of the coupling steps was estimated by the measurement of the $(MeO)_2Tr^+$ ion after each deblocking step. The yields appeared to be quantitative, and no decrease of the reaction efficiency was observed when the (2′-deoxy-N^6-methyladenosin-3′-*O*-yl)morpholinophosphine derivative was introduced in place of the corresponding N^6-benzoyl-2′-deoxyadenosine derivative.

The internucleotidic phosphate groups were deprotected by treatment with triethylammonium thiophenoxide in THF at room temperature [18]. The oligonucleotide was then cleaved from the silica-gel support by conc. NH_3, and the heterocyclic bases were deprotected by heating at 50° in NH_3 for two days.

HPLC Purification. The oligonucleotides were purified by HPLC on a *Partisil 10 SAX* anion-exchange column. When necessary, the oligonucleotides were further separated by HPLC on a *C18* reverse-phase column. They were desalted by dialysis against

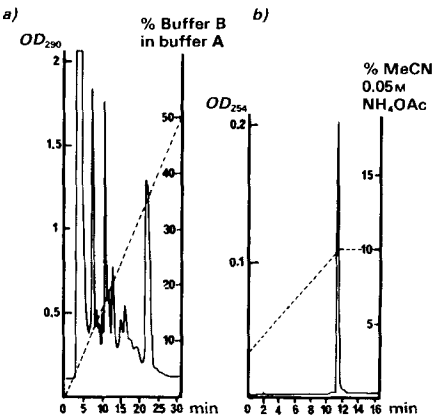


Fig. 3. *HPLC profiles of oligonucleotide d(GGm⁶ATCC). a)* Crude mixture on *Partisil 10 SAX* (0.75 × 30 cm) with a 30-min linear gradient (0–50%) of buffer B (0.4M KH_2PO_4 in H_2O/MeCN 80:20 *(v/v)*, pH 6.8) in buffer A (0.04M KH_2PO_4 in the same solvent) at 2 ml·min^{-1}. *b)* Purified product on an *Alltech Econosphere* (5 μm) *C18* column (0.47 × 15 cm) using a linear gradient (3–15%) of MeCN in 0.05M NH_4OAc (pH 7.0) at 1 ml·min^{-1}.

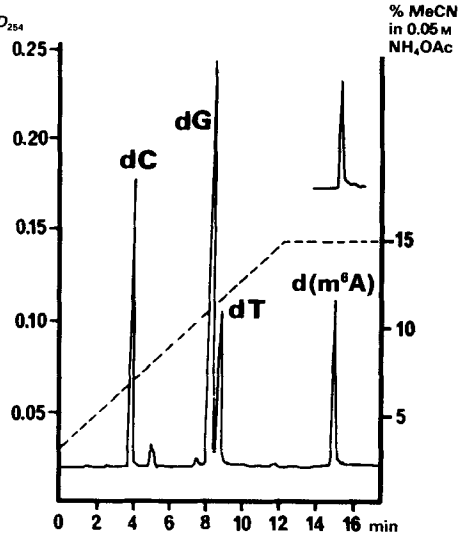


Fig. 4. *HPLC analysis of the enzymatic digestion of the hexamer d(GGm⁶ATCC) on a* Alltech Econosphere (5 μm) C18 *column* (0.47 × 15 cm). Eluent: 12-min linear gradient (3–15%) of MeCN in 50mM NH_4OAc (pH 7.0), flow rate 1 ml·min^{-1}. Lower part: dC, dG, dT, and d(m⁶A) indicate the peaks corresponding to 2′-deoxycytidine, 2′-deoxyguanosine, thymidine, and 2′-deoxy-N^6-methyladenosine, respectively. Upper part: Reversed-phase HPLC of 2′-deoxy-N^6-methyladenosine.

distilled H_2O. *Fig. 3* shows elution profiles obtained by *Partisil-10-SAX* and *C18*-reversed-phase HPLC.

The overall yields of purified products were between 32 and 54%. The yields were determined by the UV absorbance of the pure compound (from HPLC and dialysis) at 260 nm, and are based on the amount of the first nucleoside bound to the silica gel. The isolated yield of pure product varied from 190 to 310 OD_{260} per 0.3 g of silica-gel support used.

Characterization. The chain-lengths of the products obtained were checked by polyacrylamide gel electrophoresis after ^{32}P-labelling with [^{32}P]ATP and T_4 polynucleotide kinase. The sequences were confirmed for the decanucleotides by the *Maxam* and *Gilbert* methods [19]. The N^6-methyladenine residues gave the same band patterns as those obtained with adenine residues. The sequence found in each case was as expected.

An aliquot of the oligonucleotides containing the N^6-methyladenine unit was treated by a mixture of snake phosphodiesterase and alkaline phosphatase. The hydrolysis products of the enzymatic reactions were separated by HPLC on a *C18*-reverse-phase column and 2′-deoxy-N^6-methyladenosine was characterized by its retention time *(Fig. 4)*. Product peaks were collected, freeze dried, checked by MS analysis, and shown to be identical to dT, dC, dG, dA, and d(m⁶A).

MS was performed under pyrolytic conditions to confirm the presence of the intact N^6-methyladenine moiety in the modified oligomers *(Fig. 5)*.

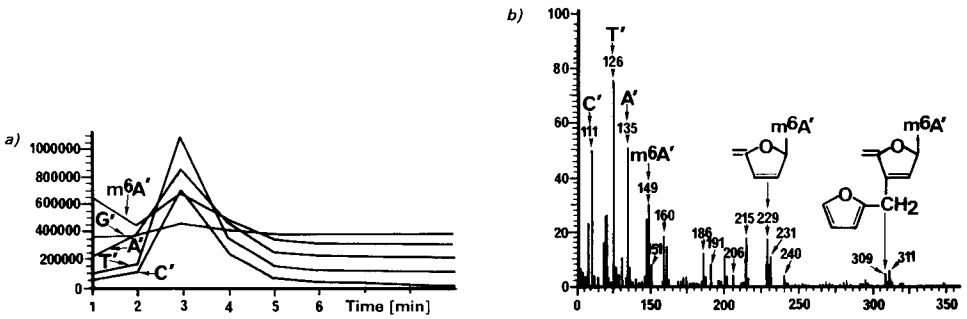


Fig. 5. *Pyrolytic MS of the octanucleotide d(GGm⁶ATATCC). a)* Intensities of different ions against time during pyrolysis at 250°. *b)* MS at 250° after 3 min of pyrolysis: A' = adenine, m⁶A' = N^6-methyladenine, C' = cytosine, G' = guanine, and T' = thymine.

A careful analysis by NMR spectroscopy at 500 MHz has been performed for the self-complementary oligonucleotides d(GGm⁶ATCC) [6] and d(GGm⁶ATATCC) [20]. Most of the protons have been assigned at various temperatures by decoupling experiments and nuclear *Overhauser* effect (NOE) measurements. Work on the other oligonucleotides is in progress.

Experimental Part

General. Some of the materials and general methods have been described in [21]. UV spectra: *Beckman 5230 UV* spectrophotometer: λ_{max} (ε) in nm. ^{1}H-NMR (250 MHz) and ^{31}P-NMR spectra (81 MHz): *Bruker WM 250* and *WM 200*, respectively; δ in ppm and J in Hz. MS: *Kratos MS 50* mass spectrometer for pyrolysis MS [22] and for positive- or negative-ion FAB-MS [23]; B = pyrimidine or purine base.

2'-Deoxy-1-methyladenosin-1-ium Iodide (**1**) was obtained in 90% yield following [9]. UV (H_2O): 257 (14600); min. 242 (10100). ^{1}H-NMR (250 MHz, (D_6)DMSO): 8.4 (*s*, H–C(2), H–C(8)); 6.40 (*dd*, J = 6.5, 6.8, H–C(1')); 4.57 (*m*, J = 4, 4.5, 6.5, H–C(3')); 4.05 (*m*, J = 4.5, 4.5, 5, H–C(4')); 3.8 (*s*, CH_3–C(1)); 3.35 (*m*, J = 4.5, 5, 12.8, 2 H–C(5')); 2.70 (*m*, J = 6.5, 6.5, 14.5, H–C(2')); 2.40 (*m*, J = 4, 6.8, 14.5, H–C(2')). FAB-MS (glycerol matrix, pos. ions): 358 (14, (MH + glycerol)$^+$), 266 (85, MH$^+$), 150 (100, BH_2^+). FAB-MS (glycerol matrix, neg. ions): 127 (100, I^-).

2'-Deoxy-N⁶-methyladenosine (**2**). According to [9], 5.2 g (77%) of **2** were obtained as a white solid which was recrystallized from hot MeOH, m.p. 205–206° ([9]: m.p. 206–208°). UV (H_2O): 265 (15400), min. 229 (1800). ^{1}H-NMR (250 MHz, (D_6)DMSO): 8.2, 8.3 (2*s*, H–C(1), H–C(8)); 6.36 (*dd*, J = 6, 7.5, H–C(1')); 4.41 (*m*, J = 2.5, 3, 6, H–C(3')); 3.9 (*m*, J = 2.5, 4.5, 4.8, H–C(4')); 3.6 (*m*, J = 4.5, 4.8, 12, 2 H–C(5')); 3.42 (*s*, CH_3NH–C(6)); 2.73 (*m*, J = 6, 7.5, 13.2, H–C(2')); 2.26 (*m*, J = 3, 6, 13.2, H–C(2')). EI-MS: 265 (17, M^+), 235 (($M - CH_2O$)$^+$), 176 ($BH^+ - CH{=}CH_2$), 149 (100, BH^+), 120 (($B - CH_2{=}CH$)$^+$). FAB-MS (neg. ions): (15, (M – H)$^-$), 148 (48, B^-). HR FAB-MS (pos. ions): 266.1240 (MH$^+$, $C_{11}H_5N_5O_3^+$, calc. 266.1253). Anal. calc. for $C_{11}H_{15}N_5O_3$ (265.12): C 49.78, H 5.78, N 26.40; found: C 49.58, H 5.66, N 26.33.

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N⁶-methyladenosine (**3**). At r.t., 250 mg of **2** were added to 4,4'-dimethoxytrityl chloride (480 mg) in dry pyridine (TLC control: silica gel, $CHCl_3$/MeOH 9:1). After 60 min, H_2O (12.5 ml) was added, the mixture extracted with AcOEt (3 × 5 ml), and the org. phase dried over Na_2SO_4 and evaporated. Purification by HPLC (*LiChroprep* silica gel *60* (1 × 30 cm), linear gradient (0–5%) of MeOH in CH_2Cl_2) gave 0.293 g (55%) of pure **3**, m.p. 116–117°. UV (MeOH): 271 (14400), 236 (26200), min. 256 (13600). ^{1}H-NMR (250 MHz, CD_3OD): 8.19, 8.14 (2*s*, H–C(2), H–C(8)); 6.41 (*t*, J = 6.5, H–C(1')); 4.64 (*m*, J = 4, 4.5, 6.5, H–C(3')); 4.12 (*d*, *t*, J = 4, 2 H–C(5')); 2.90 (*m*, J = 6.5, 6.5, 13.5, 1 H–C(2')); 2.50 (*m*, J = 6.5, 4.5, 13.5, H–C(2')). HR FAB-MS (pos. ions): 568.2546 (MH$^+$, $C_{32}H_{34}N_5O_5^+$, calc. 568.2560). FAB-MS (*PEG 200* matrix, pos. ions): 568 ((M + H)$^+$), 303 ((MeO)$_2$Tr$^+$), 150 ((BH + H)$^+$). FAB-MS (glycerol matrix, neg. ions): 566 ((M – H)$^-$), 148 (B^-). Anal. calc. for $C_{32}H_{33}N_5O_5$ (567.25): C 67.71, H 5.86, N 12.34; found: C 67.54, H 5.92, N 12.42.

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N^6-methyladenosine 3'-[(2-Chlorophenyl)triethylammonium Phosphate] (**4**). Freshly distilled 2-chlorophenyl phosphodichloridate (0.17 ml) in MeCN (1.1 ml) was added by syringe to 1,2,4-triazole (0.2 g) under dry Ar. Et_3N (0.3 ml) was added and the mixture kept at r.t. with stirring for 15 min. Then, **3** (250 mg), dried by coevaporation with pyridine and dissolved in pyridine (2 ml), was added dropwise. After 40 min, the mixture was treated with Et_3N (0.4 ml), H_2O (0.15 ml), and pyridine (0.8 ml) for 10 min. The org. phase was washed with $NaHCO_3$ soln. (30 ml) and the product extracted 3× with $CHCl_3$ (10 ml). The $CHCl_3$ phase was washed, dried over Na_2SO_4, and evaporated. Purification by HPLC (*LiChroprep* silica gel *60*, gradient (0–10%) of MeOH in CH_2Cl_2) gave a dry foam (235 mg, 62%). The salt **4** softened at 72° and decomposed on further heating. UV (MeOH): 271 (14300), 240 (16700), min. 254 (10800). ^{1}H-NMR (250 MHz, (D_6)acetone): 8.17, 8.08 (2*s*, H–C(2), H–C(8)); 6.47 (*dd*, *J* = 6, 8, H–C(1')); 5.21 (*m*, H–C(3')); 4.42 (*m*, 2 H–C(5')); 3.11 (*q*, *J* = 7.5, $(CH_3CH_2)_3NH^+$); 3.09 (*m*, H–C(2')); 2.72 (*m*, H–C(2')); 1.28 (*t*, *J* = 7.5, $(CH_3CH_2)_3NH^+$). ^{31}P-NMR (81 MHz, CD_3OD, 85% H_3PO_4 extt. ref.): −5.35 (*s*). HR FAB-MS (neg. ions): 756.1991 (A^-, $C_{38}H_{36}N_5O_8PCl^-$, see *Fig. 1*, calc. 756.1989). FAB-MS (*PEG 200* matrix, neg. ions): 756 ($(M - H)^-$), 148 (B^-), 454 ($(M - (MeO)_2Tr)^-$), 207 ($(ClPhOPO_3H)^-$), 97 ($(PO_4H_2^-$), 79 (PO_3^-). FAB-MS (*PEG 200* matrix, pos. ions): 758 ($(M + H)^+$), 456 ($(M - (MeO)_2Tr)^+$), 303 ($(MeO)_2Tr^+$).

[2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-N^6-methyladenosine-3'-O-yl](methoxy)morpholinophosphine (**5**). Under dry Ar, 270 mg (3.8 mmol) of dry 1,2,4-triazole and 0.67 ml (3.9 mmol) of Et(i-Pr)$_2$N were dissolved in 5 ml of dry THF. The mixture was cooled to −78°, and 0.2 ml (1.93 mmol) of dichloro(methoxy)phosphine was added. After 10 min, **3** (1 g, 1.8 mmol) in 4 ml of THF was added by syringe, and the mixture was kept for 15 min at −78°. Then, *N*-(trimethylsilyl)morpholine (0.85 ml, 5.3 mmol) was added, and the soln. was allowed to warm to r.t. The mixture was extracted with 100 ml of AcOEt, the org. phase washed twice with 5% aq. $NaHCO_3$ soln. (2 × 100 ml), dried over Na_2SO_4 and evaporated, and the residue taken up in 2 ml of toluene and added dropwise to 100 ml of hexane at −80°. The precipitate (750 mg) was quickly collected and kept in a sealed vial, m.p. 98–101° (dec.). UV (MeOH): 270 (14300), 239 (18400), min. 231 (18000), 254 (11000). ^{31}P-NMR (81 MHz, CD_3CN, 85% H_3PO_4 extt. ref.): 141.1, 141.8. FAB-MS (*PEG 200* matrix, neg. ions): 713, ($(M - H)^-$), 164 ($(CH_2OP(O)Morph.)^-$), 148 (B^-).

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