Nucleic Acids Research, Vol. 19, No. 21 **5965–5971**

A simple procedure for the preparation of protected 2′-O-methyl or 2′-O-ethyl ribonucleoside-3′-O-phosphoramidites

Ernst Wagner, Berndt Oberhauser, Armin Holzner[1], Helmut Brunar[1], Georg Issakides[1], Gotthold Schaffner, Matthew Cotten, Max Knollmüller[1] and Christian R.Noe*
Institute for Molecular Pathology, Dr. Bohr Gasse 7, A-1030 Vienna and [1]Institut für Organische Chemie, Technische Universität Wien, Getreidemarkt 9, A-1060 Vienna, Austria

Received July 1, 1991; Revised September 19; Accepted October 10, 1991

ABSTRACT

Protected 2′-O-methyl and 2′-O-ethyl ribonucleoside-3′-O-phosphoramidites were prepared via alkylation of the ribonucleosides at an early stage in the synthesis. Utilizing a strategy of minimal protection, the alkylation was performed with unprotected cytidine and adenosine, or with O^6-protected guanosine and N^3,5′-O-protected uridine using methyl or ethyl iodide and sodium hydride. In subsequent steps, the introduction of standard protective groups for oligonucleotide synthesis and the concomitant separation from 3′-O-alkylated isomers was accomplished. A modification of the phosphitylation procedure permitted facile isolation of the desired phosphoramidites which show high coupling efficiencies in oligomer assembly.

INTRODUCTION

2′-O-Methyl oligoribonucleotides are oligonucleotide analogs which exhibit high resistance to both DNA- and RNA-specific nucleases and form hybrids of high thermal stability with complementary RNA (1–7). These analogs, as well as the recently described 2′-O-allyl oligoribonucleotides (8,9), have proven to be valuable antisense compounds for studying snRNP-mediated pre-mRNA splicing and processing (10–15). We have demonstrated sequence-specific inhibition of histone pre-mRNA processing *in vitro* using 2′-O-methyl or 2′-O-ethyl oligoribonucleotide 19mers complementary to the 5′-end of the U7-snRNP-RNA. These compounds inhibited processing at a 300-fold lower concentration than that required using the corresponding DNA oligomer (16).

Considerable effort has been directed toward developing efficient alkylation reactions that yield 2′-O-alkylribonucleoside building blocks suitable for oligonucleotide assembly. Various alkylating agents such as dimethylsulfate (17), organostannous compounds (18), trimethylsulfoniumhydroxide (19,20) and most commonly diazomethane (21–25) have been used. To synthesize pyrimidine and adenosine 2′-O-methylribonucleotides, Inoue *et al.* (4) used methyl iodide/silver oxide to alkylate ribonucleoside that were 3′-5′-protected with the Markiewicz disiloxane reagent (26). The 2′-O-methyladenosine building block was synthesized in 7 steps starting from the 6-chloropurine nucleoside. In the case of guanosine, diazomethane was used to monomethylate the unprotected 2′,3′-cis-diol. In alternative procedures developed by Sproat *et al.* (6), efficient 2′-O-alkylation of 3′-5′-disiloxane-protected ribonucleosides was achieved using methyl iodide/BDDDP. By this method the adenosine amidite was synthesized in an 8-step procedure starting from the 6-chloropurine nucleoside, and the guanosine amidite in 12 steps from the 2-amino-6-chloropurine nucleoside. As described recently (9), these procedures were also adapted for the preparation of 2′-O-allyl ribonucleoside monomers utilizing allyl bromide/BDDDP or palladium(0)-catalyzed allylation.

To prepare 2′-O-methyl building blocks, we initially exploited the preferred alkylation by diazomethane at the 2′-O position to obtain the properly protected amidites in few steps starting from the ribonucleosides (27). Separation from 3′-O-methyl isomers was performed by vacuum-flash-chromatography (VFC, (28)). A major drawback of diazomethane alkylation, besides the inherent risks in the use of a toxic and potentially explosive reagent, is its restriction to the introduction of methyl groups.

In this paper we describe a simple and efficient procedure for the preparation of 2′-O-methylated or 2′-O-ethylated ribonucleoside building blocks for oligomer synthesis. Alkylation of the nucleosides is performed at an early step of the synthesis using methyl or ethyl iodide. The direct methylation of unprotected adenosine has already been described (29). Analogously, we alkylated cytidine without any protective groups, whereas with uridine or guanosine it was crucial to prevent N^3- or O^6-alkylation by protection. The selectivity of this reaction for the 2′-O position of the purine nucleosides is considerably higher than that seen using the diazomethane procedure. The ratio of the 2′-O-isomer to the 3′-O-isomer exceeded 4:1 for all four nucleosides. Because the alkylation reaction is carried out at an early stage of the synthesis, the 3′-O-isomers can easily be separated at subsequent reaction steps. Introduction of 5′- and base-protecting groups and phosphitylation leads to the formation of oligonucleotide building blocks in relatively few synthetic steps. As a result of the simple handling of the alkylating agents,

* To whom correspondence should be addressed

the use of cheap and easily accessible reagents, and the few synthetic steps, these procedures are useful for the preparation of 2′-O-alkylated nucleotides.

RESULTS AND DISCUSSION

The syntheses of the cytidine and adenosine building blocks started with the monomethylation of the unprotected ribonucleosides (**Fig. 1**).

The methylation of cytosine (**1**) was performed with methyl iodide (1.1 equiv.) in DMF after deprotonation with sodium hydride (1.6 equiv.). The 2′- and 3′-monomethyl isomers showed almost identical behavior on chromatography and were separated from small amounts of higher methylated side products by VFC. Treatment with benzoyl chloride in pyridine after transient protection (30) of the sugar hydroxyls with TMS chloride, and desilylation with aqueous-methanolic ammonia gave a mixture of the N^4-benzoylated O-methyl isomers **2a/5a** (81:19 as determined from 1′-H and the methoxy signals of the ^{1}H-NMR spectrum). After reaction with DMTrCl in pyridine the 5′-tritylated products could be separated by VFC to give 25% (based on **1**) of the 2′-isomer **3a** and 5% of the 3′-isomer **6a**. In order to characterize the 5′-unprotected isomers, **3a** and **6a** were detritylated to give pure **2a** and **5a**.

The ethylation of **1** was accomplished analogously using sodium hydride (1 equiv.) and ethyl iodide (20 equiv.) in DMF. After benzoylation the 2′-O-ethyl product **2b** was obtained in pure form by recrystallization from ethyl acetate in a yield of 24% (based on **1**) and was converted to the dimethoxytritylated product **3b**.

The direct alkylation of adenosine **7** was performed in a similar fashion as already described for the case of methylation (29). Subsequent treatment of the transiently TMS-protected monoalkylated products with phenoxyacetic anhydride yielded after desilylation a mixture of N^6-phenoxyacetyl-protected 2′- and 3′-O alkylated isomers **8** and **11.** In the case of methylation a selectivity in the products **8a/11a** of 84:16 was obtained; with ethylation a significantly higher selectivity (**8b/11b** 91:9) was found. Treatment of the isomeric mixtures with DMTrCl in pyridine gave the corresponding tritylated compounds that could be easily separated into the pure isomers by VFC. Thus, tritylated 2′-O-methyl-N^6-(phenoxyacetyl)adenosine **9a** was obtained in an overall yield of 25% (based on **7**). The yield of the corresponding ethylated product **9b** was low, mainly as a result of a non-optimized procedures for the introduction of phenoxyacetyl and dimethoxytrityl protective groups; the monoethylated products **8b/11b** were obtained in about 50%.

Base-protected 2′-O-alkylguanosines were synthesized by starting from O^6-nitrophenylethyl guanosine **13** (**Fig. 2**), since alkylation of unprotected, N^2-, or N^1 and N^2-protected guanosine preferentially occurred at the base moiety (4,31). The nitrophenylethyl (NPE) protective group (32) was chosen as it offers also a guanosine O^6-protection that is compatible with the oligonucleotide synthesis (33). Methylation of **13** was performed with sodium hydride and methyl iodide at low temperature (−50° to −15°C) in order to prevent the loss of the NPE group by elimination. After purification by VFC a crystalline mixture of the methyl ethers **14a/19a** was obtained in 48% yield with a ratio of 86:14 according to ^{1}H-NMR analysis. From this mixture the pure 2′-O-methyl isomer **14a** was isolated by recrystallization. For practical reasons both the pure isomer **14a** as well as the isomeric mixture **14a/19a**, obtained from chromatographic purification of the mother liquors of the recrystallization, were converted to the phenoxyacetylated compounds **15a/20a**. Further

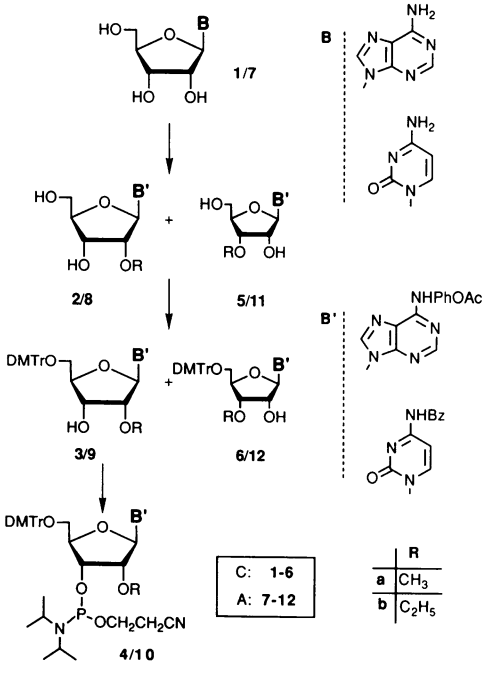

Figure 1. Syntheses of cytidine and adenosine building blocks.

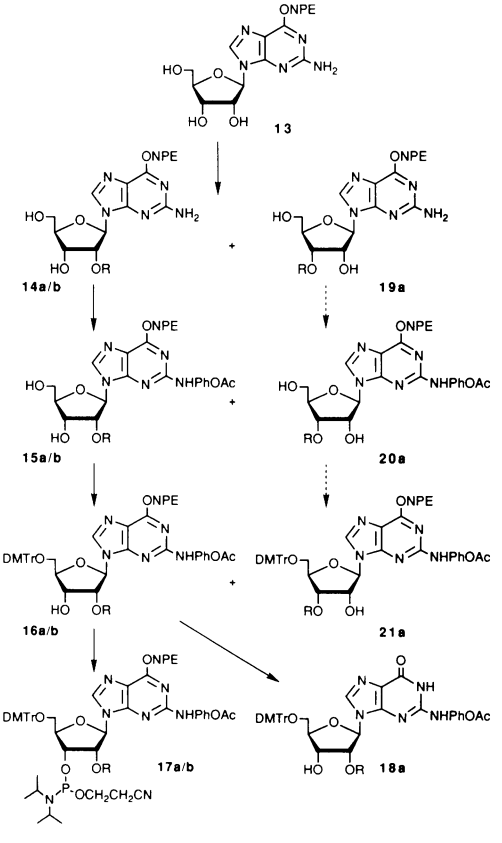

Figure 2. Synthesis of guanosine building block. **a**, R=methyl; **b**, R=ethyl.

treatment with DMTrCl allowed the efficient chromatographic separation of the 2'-O-methyl isomer **16a** from the 3'-O-methyl isomer **21a**. Removal of the NPE group with diazabicyclo(5.4.0) undec-7-en (DBU) in pyridine, neutralization with acetic acid and purification by chromatography gave **18a** in high yield.

The ethylation of **13** required a higher reaction temperature (0°C) as compared to the methylation. As a consequence of partial elimination of the NPE group the yield of 2'-O-isomer **14b** was significantly lower (13%). Conversion to **15b** and further to **16b** was accomplished as described for the methylated analogs.

To synthesize 5'-O-tritylated 2'-O-alkyl-uridines **25a** and **25b** (**Fig. 3**), it was necessary to protect the N^3-lactam group from alkylation, similar to the guanosine synthesis. Therefore the cyanoethyl group, which has been used for the protection of thymidine (33), was introduced at N^3 by treatment of 5'-O-tritylated uridine **22** with sodium hydride/acrylonitrile in DMF. Purification by VFC gave MMTr-protected **23a** or DMTr-protected **23b** in a yield of 45% or 55%, respectively. To prevent elimination of the cyanoethyl group during the methylation procedure, deprotonation of **23a** was performed with limiting amounts of sodium hydride (0.9 equiv.) at low temperature (−40°C), and upon addition of methyl iodide (1.9 equiv.) the reaction was allowed to warm up slowly to room temperature. The resulting 2'-O- and 3'-O-methyl isomers **24a** and **27a** could be easily separated by chromatography. The ratio of isomers **24a/27a**, as determined by yield, was about 81:19. As a side product dimethylated uridine **28a** was isolated. Deprotection of **24a** with potassium tert.-butylate gave 5'-O-MMTr-2'-O-methyluridine **25a**. The compound was further characterized by detritylation to 2'-O-methyl uridine **29a**. The ethylation of **23b** afforded **24b/27b** in an isomeric ratio of 83:17. Deprotection of **24b** from the cyanoethyl group gave 5'-O-DMTr-2'-O-ethyl uridine **25b**.

Phosphitylation of the protected alkylated nucleosides was accomplished as described (35) with a modified workup procedure (**Table 1**). The carefully dried precursor nucleoside was treated with (2-cyanoethoxy)-N,N-diisopropylaminochlorophosphine and diisopropylethylamine in THF. To avoid chromatographic purification, which in the case of the guanosine compound resulted in partial cleavage of the phenoxyacetyl protecting group, the reaction was quenched with an lipophilic secondary alcohol (iso-propanol or sec-butanol). Thus the excess of phosphitylating agent is converted to a highly soluble amidite which was easily removed by precipitation of the nucleoside

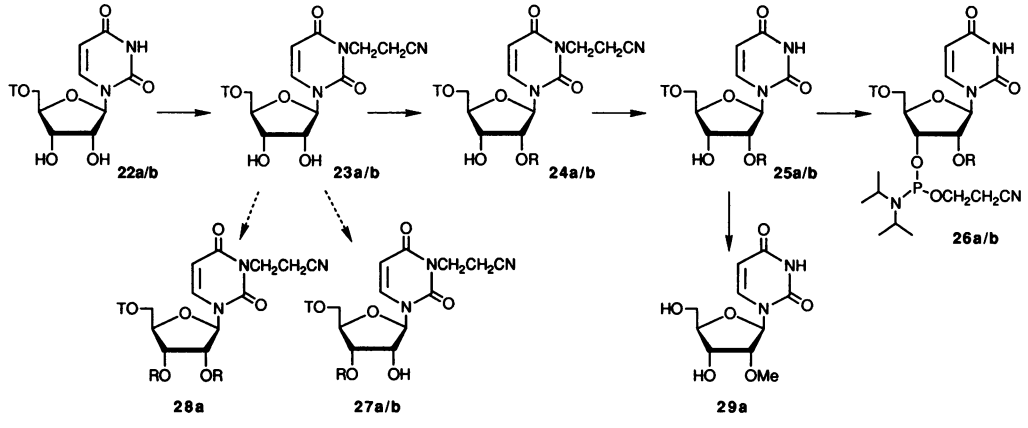

Figure 3. Synthesis of uridine building block. **a**, R=methyl, T=MMTr; **b**, R=ethyl, T=DMTr.

Table 1. Phosphitylation of protected 2'-O-methyl and 2'-O-ethylribonucleosides. TLC solvents: A, acetone/DCM 3:7; E, ethylacetate; D, DCM/ethylacetate 1:1. To avoid decomposition, TLC was developed without delay (less than 30 sec) after spotting the sample onto the plate.

		No.	mmol nucleotide	amine equiv.	chloroamidite equiv.	alcohol for quenching	yield	31 content	^{1}H-NMR 1'-H	^{1}H-NMR OMe	^{31}P-NMR		R_f
2'-OCH$_3$ **C**	DMTr	**4a**	5.12	2	1.4	MeOH	94%	n.d.	5.92/5.90	3.60	149.78/149.09		n.d.
2'-OCH$_3$ **C**	MMTr	**4a'**	3.16	3	1.2	i-PrOH	71%	3%	5.92	3.57	149.75/149.05	E	0.69/0.55
2'-OEt **C**	DMTr	**4b**	2.95	3	1.6	s-BuOH	96%	7%	5.89	-	149.64/148.77	D	0.51/0.61
2'-OCH$_3$ **A**	DMTr	**10a**	1.30	3	1.5	i-PrOH	93%	1%	6.11	3.46/3.44	149.88/149.57	D	0.63/0.56
2'-OEt **A**	DMTr	**10b**	0.68	2	1.5	i-PrOH	66%	2%	6.10	-	149.71/149.32	D	0.74/0.81
2'-OCH$_3$ **G^{NPE}**	DMTr	**17a**	0.28	2	1.4	MeOH	92%	n.d.	6.10	3.50	n.d.		n.d.
2'-OEt **G^{NPE}**	DMTr	**17b**	1.12	3	2.1	s-BuOH	93%	2%	6.02	-	149.71/149.49	D	0.87
2'-OCH$_3$ **G**	DMTr	**18a'**	1.28	3.6	2.4	i-PrOH	96%	2%	5.94	3.44/3.42	149.97/149.86	A	0.5
2'-OCH$_3$ **U**	MMTr	**26a**	1.30	3	1.5	i-PrOH	88%	3%	5.87	3.50/3.48	149.75/149.41	D	0.76/0.70
2'-OEt **U**	DMTr	**26b**	2.61	3	1.6	s-BuOH	97%	2%	5.85/5.83	-	149.57/149.18	E	0.83

amidite in petroleum ether. A small amount of N,N-diisopropyl-amino-O-cyanoethyl-H-phosphonate **31**, a hydrolysis product of the phosphitylating agent, coprecipitated with the amidite but did not cause any detectable decrease in subsequent coupling efficiency. The protected 2'-O-methyl- and 2'-O-ethylribonucleoside-3'-O-phosphoramidites have been successfully used for oligomer assembly (16). Employing standard methodology with an increased coupling time of 5 minutes, average coupling efficiencies of greater than 98% were obtained.

EXPERIMENTAL SECTION

Abbreviations: BDDDP, 2-tert.-butylimino-2-diethyl-amino-1,3-dimethylperhydro-1,3,2-diazaphosphorin; DCM, dichloromethane; DMF, dimethylformamide; DMTr, dimethoxytrityl; DMTrCl, dimethoxytrityl chloride; LC, column chromatography; MMTr, monomethoxytrityl; NPE, 2-(4-nitrophenyl)ethyl; PhOAc, Phenoxyacetyl; RT, room temperature; TLC, thin layer chromatography; THF, tetrahydrofuran; TMS, trimethylsilyl; VFC, vacuum flash chromatography;

Ribonucleosides were obtained from Fluka (Switzerland) and dried (80°C/10^{-2} mbar / 20 hours) before use. Preparative liquid chromatography [LC, VFC (28)] and thin layer chromatography (TLC) were performed on Kieselgel 60 (Merck). LC and TLC of 5'-O-protected nucleosides were performed using solvents containing no stabilizing bases like pyridine or triethylamine, as the crude products still contained significant amounts of pyridine. The separations were completed within 15–30 min, and better separation properties were observed in the absence of additional bases. The purified trityl compounds may be stored in the presence of stabilizing amounts of triethylamine. NMR spectroscopy (^{1}H,^{13}C,^{31}P) was performed on a JEOL FX90Q, a Bruker AC-200, and a Bruker AC-250 spectrometer with variable temperature unit and QNP probehead; characteristic ^{1}H and ^{13}C signals of described compounds are shown in **Table 2.**

N^4-Benzoyl-5'-O-dimethoxytrityl-2'-O-methylcytidine 3a

A solution containing 40 g (0.164 mol) of cytidine in 650 ml of dry DMF was treated with 4.33 g (0.18 mol) sodium hydride for 45 min at 0°C. During a period of 4 hours 36.9 g (0.26 mol) methyl iodide as a 20% (w/v) solution in DMF was added in several portions and the reaction mixture was then allowed to warm up to RT. As the fraction of dimethylated products started to increase (after ca. 6 h, according to TLC analysis; DMF of

Table 2. ^{1}H and ^{13}C NMR of described compounds.

		^{1}H-NMR						^{13}C-NMR						
Comp.	R	1'	2'	3'	4'	5'	OCH_3	1'	2'	3'	4'	5'	OR	
2a	2'-OMe	5.83	3.87	4.12	3.98	3.9-3.7	3.48	88.78	83.38	67.11	84.55	58.96	58.09	-
2b	2'-OEt	5.87	-	-	-	-	-	87.56	80.97	65.89	82.88	57.95	64.55	13.98
3a	2'-OMe	6.02	-	-	-	-	3.70	87.99	83.51	67.42	82.46	60.58	58.22	-
3b	2'-OEt	5.84	-	-	-	-	-	90.06	82.74	68.66	83.49	62.01	67.00	15.45
5a	3'-OMe	5.76	4.33	-	4.08	3.9-3.6	3.34	91.31	72.24	77.01	82.54	59.46	57.38	-
6a	3'-OMe	5.98	4.47	4.02	4.31	3.6-3.4	3.44	92.24	73.92	78.01	81.50	61.64	58.27	-
8a	2'-OMe	5.95	-	-	4.3	4.1-3.6	3.45	88.53	82.80	69.73	87.40	62.30	58.30	-
8b[a]	2'-OEt	6.10	-	-	4.40	-	-	88.60	82.81	69.75	87.44	63.01	67.60	15.45
9a	2'-OMe	6.20	-	-	4.25	3.52	3.56	86.41	82.68	69.35	83.75	62.69	58.24	-
9b	2'-OEt	6.15	-	-	4.11	-	-	87.99	81.76	70.47	84.81	64.03	67.20	15.38
11a	3'-OMe	5.82	5.02	4.1*	4.36*	4.02/3.74	3.54	91.80	73.52	80.79	84.57	63.34	58.00	-
11b[a]	3'-OEt	5.90	-	-	-	-	-	-	-	-	-	-	-	-
12a	3'-OMe	6.04	4.9	-	-	3.6-3.3	3.50	89.27	73.59	79.97	81.89	63.12	58.13	-
14a	2'-OMe	5.75	4.65	4.55	4.32	3.95/3.74	3.35							
14b	2'-OEt	5.75	-	4.49	4.33	4.1-3.9	-	88.42	82.11	70.36	87.10	63.01	65.38	15.04
15a	2'-OMe	5.92	4.65	4.65	4.3	4.02/3.84	3.39	88.54	82.44	69.85	87.11	62.47	58.63	-
15b	3'-OEt	5.90	4.88	4.65	4.30	4.02/3.84	-	88.55	82.43	69.87	87.13	62.42	67.61	15.18
16a	2'-OMe	6.05	4.37	4.57	4.20	3.48	3.63	86.19	83.07	69.35	83.57	62.79	58.50	-
16b	2'-OEt	6.00	-	-	4.0	-	-	86.95	76.36	81.54	84.01	63.22	66.91	15.20
18a	2'-OMe	6.02	-	4.48	-	3.44	3.49	85.47	83.85	69.96	84.15	63.30	58.85	-
21a	3'-OMe	5.87	4.94	4.29*	4.03*	-	3.50	90.40	75.29	81.21	83.47	63.51	58.45	-
23a	2',3'-OH	5.92	-	-	-	3.51	-	90.43	75.26	69.84	83.66	62.09	-	-
23b	2',3'-OH	5.92	-	-	-	3.50	-	90.41	75.24	69.82	83.66	62.09	-	-
24a	2'-OMe	6.00	3.95	4.48	4.08	3.60	3.75	87.18	83.82	68.11	83.01	61.06	58.52	-
25a	2'-OMe	5.97	3.8	4.5*	4.0*	3.56	3.65	86.88	83.80	68.15	82.82	61.07	58.43	-
25b	2'-OEt	5.80	4.30*	3.94*	3.94*	-	-	87.39	82.44	69.50	83.87	62.96	67.01	15.41
27a	3'-OMe	5.91	-	4.03	-	3.5	3.44	90.13	73.35	78.27	80.70	61.98	57.96	-
28a	2',3'-OMe	5.95	-	-	-	3.5	3.66 3.46	88.25	80.69	76.36	81.75	60.83	58.31 58.07	-
29a	2'-OMe	6.00	4.25	-	-	3.74	3.30	86.15	82.90	68.43	85.33	60.57	57.60	-

Chemical shifts [ppm] of characteristic signals; full NMR-spectroscopic data are available from the authors upon request; [a]: determined from a mixture **8b/11b**.

a TLC sample was removed in high vacuum at RT) the reaction mixture was filtered from precipitated sodium iodide and evaporated *in vacuo*. LC of the residue (chloroform/methanol 5:1) gave 22.2 g of a crude mixture of 2'- and 3'-O-methyl-cytidine (still containing some sodium iodide; TLC: chloroform/methanol 3:2., R_f=0.3 for both isomers). After coevaporation with benzene/pyridine 20.8 g of the product mixture were dissolved in 250 ml of dry pyridine and treated with 34.9 g (0.32 mol) of trimethylchlorosilane for 1 hour at RT. After addition of 13.5 g (0.096 mol) of benzoyl chloride the reaction was kept at RT overnight. The mixture was poured onto 300 ml water, diluted with 300 ml of methanol and then 250 ml of 25% aqueous ammonia was added. After 20 min the solution was extracted with DCM several times. The combined organic phases after drying with sodium sulfate, filtration and evaporation gave 32 g of a mixture containing **2a/5a** (ratio 81:19; TLC: DCM/methanol 10/1, R_f=0.2, for both isomers). This mixture, after coevaporation with benzene/pyridine was dissolved in dry pyridine and treated with 33 g (0.0976 mol) of dimethoxytrityl chloride for 2 hours at RT. The mixture was concentrated *in vacuo* and partitioned between DCM and aqueous sodium bicarbonate. The aqueous phase was extracted with DCM several times; the combined DCM extracts were dried with sodium sulfate and evaporated. LC of the residue (still containing small amounts of pyridine; 1200 g silica gel, eluent: DCM, then DCM/acetone 8:1) yielded 25.3 g (25%) **3a** and 5.1 g (5%) **6a** as slightly yellow solids (TLC: DCM/acetone 7:3, R_f=0.28 for **3a**, R_f=0.24 for **6a**).

N^4-Benzoyl-2'-O-methylcytidine **2a**: was obtained in pure form by detritylation of **3a** [2% trichloroacetic acid in DCM, 5 min/RT] followed by extraction with water, evaporation of aqueous phase and purification of the residue by LC (DCM/acetone first 4:1, then 3:2), 94% yield]. **2a**: white foam;TLC: DCM/acetone 1:1, R_f=0.32.

N^4-Benzoyl-3'-O-methylcytidine **5a** was characterized as the product obtained by detritylation of **6a** [2% hydrochloric acid in DCM/ether (50/1); purification by LC (DCM/acetone 2:1), precipitation from ethylacetate/methanol; 90% yield]. **5a**: white foam;TLC: DCM/acetone 1:1, R_f=0.43.

N^4-Benzoyl-2'-O-ethylcytidine 2b

Compound **2b** was obtained as described for **3a** except that 45 g (0.185 mol) of cytidine was treated with 0.185 mol of sodium hydride and 40.52 g (3.825 mol) of ethyl iodide. LC purification gave 25.5 g of isomeric mixture containing monoethylated products as colorless foam. Subsequent reaction with 32.8 g (0.301 mol) of trimethylchlorosilane and 19.8 g (0.140 mol) of benzoyl chloride gave a crude product which after crystallization from ethylacetate and drying yielded 16.6 g (24%) pure **2b** with a melting point of 193–194°C. TLC: DCM/isopropanol 9/1, R_f=0.34.

N^4-Benzoyl-5'-O-dimethoxytrityl-2'-O-ethylcytidine 3b

A solution containing 7.30 g (19.4 mmol) of **2b** in dry pyridine was treated with 9.88 g (29.2 mmol) of dimethoxytrityl chloride and worked up according to the tritylation procedure for **3a.** Purification by LC (DCM/Isopropanol 30:1) gave 7.43 g (56.6%) of pure **3b** as a pale yellow solid. TLC: DCM/isopropanol 9/1, R_f=0.55.

5'-O-Dimethoxytrityl-2'-O-methyl-N^6-(phenoxyacetyl) adenosine 9a

A solution containing 50 g (0.187 mol) of adenosine in 700 ml of dry DMF was cooled to 0°C and treated with 5.34 g (0.224 mol) of sodium hydride for 45 min at 0°C. During a period of 4 hours 42.2 g (0.30 mol) of methyl iodide as a 20% (w/v) solution in DMF, was added in several portions the reaction mixture was then allowed to warm up to RT. As the fraction of dimethylated products started to increase (according to TLC analysis; DMF of a TLC sample was removed in high vacuum at RT) the reaction mixture was filtered from precipitated sodium iodide and evaporated *in vacuo*. VFC of the residue (chloroform/methanol gradient 19:1 to 5:1) gave 42 g of a crude mixture of 2'- and 3'-O-methyl-adenosine (still containing some sodium iodide; TLC: chloroform/ethanol 3:1, R_f=0.3 for both isomers). After coevaporation with benzene/pyridine the product mixture was dissolved in 500 ml of dry pyridine and treated with 64.8 g (0.60 mol) of trimethylchlorosilane for 2 hours at RT. After addition of 51.2 g (0.179 mol) of phenoxyacetic anhydride the reaction was kept at RT overnight. The mixture was poured into 1000 ml of water and diluted with 400 ml of methanol. After 40 min about 25 g of solid sodium bicarbonate was added to the resulting clear solution, the mixture was stirred for a further 10 min and was then extracted with DCM several times. The combined organic phases were dried with sodium sulfate, filtered and evaporated *in vacuo*. After coevaporation with pyridine/benzene the crude product **8a/11a** (45 g, ratio 86:14) was dissolved in dry pyridine and treated with 53.2 g (0.157 mol) of dimethoxytrityl chloride for 2 hours at RT. The mixture was concentrated *in vacuo* and partitioned between DCM and aqueous sodium bicarbonate. The aqueous phase was extracted with DCM several times; the combined DCM extracts were dried with sodium sulfate and evaporated *in vacuo*. VFC of the residue (DCM then DCM/acetone 8:1) yielded 32.8 g (25%) of **9a** and 3.3 g (2.5%) of **12a** as colorless solids (TLC: DCM/acetone 7:3, **9a**: R_f=0.52,**12a**: R_f=0.48).

2'-O-Methyl-N^6-(phenoxyacetyl)adenosine **8a**: The crude product containing **8a/11a**, obtained according to the procedure given above, was purified by LC (400 g silica gel; DCM/acetone 4:1) to give 5.5 g (7.05% based on **7**) of **8a**, with given analytical data, and 14.1 g (18.1% based on **7**) of mixture of **8a/11a** (TLC: DCM/methanol=9:1, **8a:** R_f=0.50; **11a**: R_f=0.48).

3'-O-Methyl-N^6-(phenoxyacetyl)adenosine **11a** was characterized as the product obtained by detritylation of **12a** [2% hydrochloric acid in DCM/ether (50:1), −5°C for 1 min; purification by VFC (DCM/acetone 3.5:1), 92% yield]. **11a**: white foam;TLC: DCM/methanol 9:1, R_f=0.48.

5'-O-Dimethoxytrityl-2'-O-ethyl-N^6-(phenoxyacetyl) adenosine 9b

Compound 9b was synthesized according to the procedure used for preparation of **9a**, except that 50 g (187 mmol) of adenosine was treated with 5.40 g (225 mmol) sodium hydride and 81.3 g (525 mmol) of ethyl iodide for 4 hours at 0°C. The following LC purification gave 30 g of isomeric mixture containing monoethylated products as colorless foam. Subsequent reaction with 44.1 g (406 mmol) of trimethylchlorosilane and 58.4 g (203 mmol) of phenoxyacetic anhydride gave a crude product which upon partial purification by VFC (DCM/THF 1:1) and drying *in vacuo* gave 19.7 g (25%) of a mixture containing **8b/11b** (ratio

10:1) as colorless foam. Tritylation of 19.6 g of **8b/11b** in 100 ml of dry pyridine with 23.2 g (69 mmol) of dimethoxytrityl chloride, as described for **9a**, and VFC purification (DCM/THF gradient from 12:1 to 6:1) gave 4.8 g of **9b** (3.5% overall yield from adenosine) as white foam. **9b**: TLC: DCM/isopropanol 16/1, R_f=0.42. (3'-regioisomer **12b**,not isolated, R_f=0.46).

2'-O-Ethyl-(phenoxyacetyl)adenosine **8b**: TLC: DCM/isopropanol 9/1, R_f=0.34.

2'-O-Methyl-O^6-[2-(4-nitrophenyl)ethyl]guanosine 14a

After coevaporation with DMF/benzene 8.1 g **13** (32) was dissolved in 130 ml of dry DMF and treated with 1.6 equiv. of sodium hydride for 45 min at −50°C. During a 5 h period 8 equiv. of methyl iodide was added and the reaction mixture allowed to warm up to −15°C. Ammonium chloride was then added and the mixture was evaporated *in vacuo*. The resulting residue was purified by VLC (chloroform/methanol 80:1) to yield 4.0 g (48%) of a mixture containing **14a/19a** (86:14). 1.8 g (22%) of colorless crystals (Fp. = 109 − 111°C, NMR) of pure **14a** (less than 1% **19a**) was obtained by recrystallization from benzene/chloroform. The mother liquor was evaporated and purified by LC (DCM/isopropanol 30:1) to give 1.7 g (20.4%) crystals containing a mixture of **14a/19a** (82:18). TLC: DCM/isopropanol 5:1, R_f=0.56 (**14a**), R_f=0.61 (**19a**).

2'-O-Ethyl-O^6-[2-(4-nitrophenyl)ethyl]guanosine 14b

A solution containing 3.15 g of dry **13** (32) in 30 ml DMF was treated with 0.9 equiv. of sodium hydride at −15°C for 1 h. Then 8 equiv. of ethyl iodide was added during a period of 2 h at −15°C and the solution was subsequently stirred at 0°C for 3 h. The workup was performed as described for **14a**, with 0.44 g (13%) of white foam **14b** being obtained by LC (DCM/isopropanol 16:1). TLC: DCM/isopropanol 9:1, R_f=0.37 (**14b**), R_f=0.46 (3'-regioisomer **19b**, not isolated).

2'-O-Methyl-O^6-[2-(4-nitrophenyl)ethyl]-N^2-(phenoxyacetyl) guanosine 15a

After drying by coevaporation with pyridine/benzene, 1.5 g of **14a** was dissolved in 150 ml of pyridine and 5 equiv. of trimethylchlorosilane added at RT over a duration of 30 min. After 2 h 1.4 equiv. of phenoxyacetic anhydride was added at RT and the reaction mixture stirred over night. The reaction was then quenched by addition of 250 ml of aqueous methanol (50%) and 10 ml of conc. aqueous ammonia, after 10 min the product was extracted with DCM. Following drying (sodium sulfate) of the combined DCM phases and evaporation *in vacuo*, 1.85 g of colorless foam **15a** was obtained (NMR). Analogously 1.7 g of a mixture of **14a/19a** (82:18) was phenoxyacetylated to give 1.8 g (81%) of a mixture of the two isomers **15a/20a**. TLC: chloroform/methanol 15:1, R_f=0.36 (**15a**), R_f=0.38 (**20a**).

2'-O-Ethyl-O^6-[2-(4-nitrophenyl)ethyl]-N^2-(phenoxyacetyl) guanosine 15b

Phenoxyacetylation of 0.44 g of **14b** was accomplished as described for **15a**. The crude product was purified by LC (DCM/isopropanol 10:1) to yield 0.47 g (82%) of **15b** as colorless foam. TLC: DCM/isopropanol 9:1, R_f=0.45.

5'-O-Dimethoxytrityl-2'-O-methyl-O^6-[2-(4-nitrophenyl) ethyl]-N^2-(phenoxyacetyl)guanosine 16a

Compound **15a** (3.0 g of a mixture containing 0.3 g of regioisomer **20a**) was dried by coevaporation with benzene/pyridine and dissolved in 350 ml of pyridine. After addition of 1.2 equiv. of dimethoxytrityl chloride the solution was stirred for 2 h at RT and then evaporated *in vacuo*. The residue was taken up in aqueous bicarbonate and extracted with DCM. The combined organic phases were dried (sodium sulfate), filtered and evaporated *in vacuo*. The residue was separated by LC (toluene/isopropanol 30:1) to give 4.1 g (81%) of **16a** and 0.3 g (6%) of **21a** as pale yellow foams. TLC: toluene/isopropanol 9:1, R_f=0.24 (**16a**), R_f=0.28 (**21a**).

5'-O-Dimethoxytrityl-2'-O-ethyl-O^6-[2-(4-nitrophenyl)ethyl-]-N^2-(phenoxyacetyl)guanosine 16b

Compound **16b** was prepared from 0.47 g of **15b** according to the procedure given for **16a**. LC purification (DCM/acetone 15:1) of the crude product gave 483 mg (68%) of pure **16b** as a yellow powder. TLC: DCM/isopropanol 9:1, R_f=0.60.

5'-O-Dimethoxytrityl-2'-O-methyl-N^2-(phenoxyacetyl) guanosine 18a

A solution containing 150 mg of **16a** in 5 ml of a 0.5 M 1,8-diazabicyclo(5.4.0)undec-7-ene (DBU) in pyridine was stirred for 2 h at RT. Then 1.5 ml of 1 M aqueous acetic acid was added slowly, the mixture diluted with water and the aqueous phase extracted with DCM. The combined organic phases were dried (sodium sulfate) and evaporated *in vacuo*. VFC of the residue (DCM/acetone/triethylamine 100:10:2) gave 95 mg (76%) of **18a** as a colorless oil. TLC: Chloroform/Methanol=4.8/0.3, R_f=0.38.

N^3-(2-Cyanoethyl)-5'-O-monomethoxytrityluridine 23a

After coevaporation with DMF/benzene 7 g of **22a** (prepared according to published procedures(34)) was dissolved in 100 ml of dry DMF, cooled to 0°C and treated with 1 equiv. of sodium hydride for 1 h. During 12 h 20 equiv. of acrylonitrile were added and the reaction mixture heated to 80°C. The reaction was then quenched by pouring onto water/DCM. The crude product obtained by evaporation of the DCM extract was purified by VFC (DCM/acetone 30:1) to yield 3.5 g (45%) of colorless foam **23a**. TLC: DCM/acetone=33:2, R_f=0.42.

N^3-(2-Cyanoethyl)-5'-O-dimethoxytrityluridine 23b

Compound **23b** (24.0 g) was obtained analogously to **23a** from 40 g of **22b** (prepared according to published procedures(34)) in 55% yield as colorless solid. **23b**: TLC: DCM/isopropanol 9:1, R_f=0.51.

N^3-(2-Cyanoethyl)-2'-O-methyl-5'-O-monomethoxytrityl-uridine 24a

A solution containing 600 mg of **23a** in 10 ml dry DMF was cooled to −40°C and treated with 0.9 equiv. of NaH for 45 min. Then 1.9 equiv. methyl iodide was added and during a period of 3 h the temperature raised to RT. The reaction was quenched by addition of ammonium chloride and evaporated. The residue was dissolved in water and extracted with DCM. After drying (sodium sulfate) and evaporation of the combined organic phases *in vacuo*, the reaction products were separated by LC (ether) to give 200 mg (33%) of **24a**, 48 mg (8%) of 3'-O-methyl product **27a** and 80 mg (13%) of dimethylated product **28a** as colorless foams. TLC: DCM/acetone=3.2:1.8, R_f=0.80 (**24a**), R_f=0.74 (**27a**), R_f=0.87 (**28a**).

2'-O-Methyl-5'-O-monomethoxytrityluridine 25a

A solution containing 100 mg of **24a** in DCM was treated with 2 equiv. of KOt-Bu for 30 min and then poured onto aqueous sodium bicarbonate. After extraction with DCM the organic phase was dried (sodium sulfate) evaporated *in vacuo* and the crude product purified by VFC (DCM/acetone 9:1) to yield 80 mg (85%) of **25a** as a colorless solid. TLC: DCM/acetone=3:2, R_f=0.41.

5'-O-Dimethoxytrityl-2'-O-ethyluridine 25b

A DMF solution containing 32.4 g of **23b** was treated with 1 equiv. of NaH for 1 h at −45°C. Then 1.6 equiv. of ethyl iodide was added and the temperature raised to RT during a period of 3 h. After workup and chromatography as described for **24a** 8.1 g of 2'-O-ethyl isomer **24b** and 1.6 g 3'-O-ethyl isomer **27b** were obtained. The N^3-deprotection of 24b (as described for **25a**) and purification by LC (DCM/acetone 12:1) gave 6.6 g (20% calc. from **23b**) of an off-white foam **25b**. TLC: DCM/isopropanol 9:1, R_f=0.63.

2'-O-Methyluridine 29a

A solution containing 8 g of **25a** in 100 ml of methanol was treated with 5 ml of 10% methanolic HCl at RT for 5 min. The reaction mixture was evaporated *in vacuo* and the residue partitioned between ether and water. The organic phase was washed twice with water and the combined aqueous phases evaporated *in vacuo*. The product was dried by evaporation with benzene and pyridine to give 3.27 g (93%) **29a** as colorless foam. TLC: Dichloromethane/Methanol=3/1. R_f=0.37.

Phosphitylation

After drying by coevaporation with pyridine, toluene and THF, 1−3 mmol of the protected nucleoside **3a**, **3a'** (1), **3b**, **9a**, **9b**, **16a**, **16b**, **18a**, **25a** or **25b** was dissolved in 25 ml THF (in the case of **3a**, **16a** dry DCM was used) and 2−3.6 equiv. of N,N-diisopropylethylamine (Hünig's base) added under nitrogen. 1.2 to 2.4 equiv. of (2-cyanoethoxy)-N,N-diisopropylaminochlorophosphine was subsequently added during a period of 5 min at RT. The reaction was then stirred for 2 h and monitored by TLC (the phosphoramidite gives a yellow color with ninhydrin reagent). If starting material was still present an additional 0.3 equiv. of phosphitylation agent was added. Any excess reagent was quenched by the addition of 3−5 equiv. of isopropanol (sec-butanol or methanol) and stirred for an additional hour. Then the solvent was partially removed by evaporation *in vacuo* and degased ethylacetate (50 ml) added. The solution was extracted twice with degased 1 M aqueous sodium bicarbonate (100 ml) under argon at 0°C, dried with sodium sulfate and evaporated. The residue was coevaporated twice with toluene and dissolved in 3−5 ml of degased toluene (pyrimidines) or ethylacetate (purines). The solution was added dropwise to 200 ml dry petroleum ether (bp. 40−60°C) and the colorless precipitate collected by filtration under nitrogen. After thorough washing with hexane, the phosphoramidite was dried *in vacuo* and dissolved in dry acetonitrile to give a 100 mM solution which was directly used for oligonucleotide synthesis. The content of the coprecipitated cyanoethyl-N,N-diisopropyl H-phosphonate **31** was judged by ^1H and ^{31}P-NMR and did not affect coupling efficiency (**Table 1**). The solution was stored at −20°C over 3 ü molecular sieve and could be kept for several months without detectable decomposition.

ACKNOWLEDGEMENTS

We appreciate the critical reading of this manuscript by Lisa Ballou and Stephen Phillips and we acknowledge the skillful preparation of figures by Hannes Tkadletz.

REFERENCES

1. Inoue, H., Hayase, Y., Asaka, M., Imura, A., Iwai, S., Miura, K. and Ohtsuka, E. (1985) *Nucl. Acids Res. Symp Ser.* **16**, 165−168.
2. Shibahara, S., Mukai, S., Nishihara, T., Inoue, H., Ohtsuka, E. and Morisawa, H. (1987) *Nucl. Acids Res.* **15**, 4403−4415.
3. Inoue, H., Hayase, Y., Iwai, S. and Ohtsuka, E. (1987) *FEBS Lett.* **215**, 327−330.
4. Inoue, H., Hayase, Y., Imura, A., Iwai, S., Miura, K. and Ohtsuka, E. (1987) *Nucl. Acids Res.* **15**, 6131−6148.
5. Sproat, B., Lamond, A., Beijer, B., Neuner, P. and Ryder, U. (1989) *Nucl. Acids Res.* **17**, 3373−3386.
6. Sproat, B., Beijer, B. and Iribarren, A. (1990) *Nucl. Acids Res.* **18**, 41−49.
7. Beijer, B., Sulston, I., Sproat, B., Rider, P., Lamond, A. and Neuner, P. (1990) *Nucl. Acids Res.* **18**, 5143−5151.
8. Iribarren, A., Sproat, B., Neuner, P., Sulston, I., Ryder, U. and Lamond, A. (1990) *Proc. Nat. Acad. Sci. USA* **87**, 7747−7751.
9. Sproat, B. S., Iribarren, A., Garcia, R.G. and Beijer, B. (1991) *Nucl. Acids Res.* **19**, 733−738.
10. Lamond, A., Sproat, B., Ryder, U. and Hamm, J. (1989) *Cell* **58**, 383−390.
11. Blencowe, B., Sproat, B., Ryder, U., Barabino, S. and Lamond, A. (1989) *Cell* **59**, 531−539.
12. Barabino, S., Sproat, B., Ryder, U., Blencowe, B. and Lamond, A., (1989) *EMBO J.* **8**, 4171−4178.
13. Barabino, S., Blencowe, B., Ryder, U., Sproat, B. and Lamond, A., (1990) *Cell* **63**, 293−302.
14. Ryder, U., Sproat, B., and Lamond A. (1990) *Nucl. Acids Res.* **18**, 7373−7379.
15. Carmo-Fonseca, M., Tollervey, D., Pepperkok, R., Barabino, S., Merdes, A., Brunner, C., Zamore, P., Green, M., Hurt, E. and Lamond, A. (1991) *EMBO J.* **10**, 195−206.
16. Cotten, M., Oberhauser, B., Brunar, H., Holzner, A., Issakides, G., Noe, C.R., Schaffner, G., Wagner, E. and Birnstiel, M.L. (1991) *Nucleic Acids Res.* **19**, 2629−2635.
17. Hisanaga, Y., Tanabe, T., Yamauchi, K. and Kinoshita, M. (1981) *Bull.Chem.Soc.Jpn.* **54**, 1569−1570.
18. Wagner, D., Verheyden, J.P.H. and Moffatt, J.G. (1974) *J.Org.Chem.* **39**, 24.
19. Yamauchi, T., Nakagima, T. and Kinoshita, M. (1980) *J.Org.Chem.* **45**, 3865−3868.
20. Yamauchi, T., Nakagima, T. and Kinoshita, M. (1986) *Bull.Chem.Soc.Jpn.* **59**, 2947−2949.
21. Martin, D.M.G., Reese, C.B. and Stephenson, G.F. (1968) *Biochemistry* **7**, 1406.
22. Khawja, T.A. and Robins, R.K. (1966) *J.Am.Chem.Soc.* **88**, 3640.
23. Robins, M.J., Naik, S.R. and Lee, A.S.K. (1974) *J.Org.Chem.* **39**, 1891.
24. Ekborg, G. and Garegg, P.J. (1980) *J.Carbohydr., Nucleosides, Nucleotides* **7**, 57−61.
25. Nyilas, A. and Chattopadhyaya (1986) *Acta.Chem.Scand.B.* **40**, 826−830.
26. Markiewicz, W.T. (1979) *J.Chem.Res. (S)* **1979**, 24−25.
27. Noe, C.R., Issakides, G., Oberhauser, B., Wagner, E., Brunar, H., Holzner, A. and Knollmüller M., in preparation.
28. Noe, C.R., Knollmüller, M., Göstl, G. and Gärtner, P. (1991) *Monatsh.Chem.*, in press.
29. Yano,J., Kan,L.S. and Ts'O, P.O.P. (1980) *Biochim.Biophys.Acta* **629**, 178−183.
30. Ti, G.S., Gaffney, B.I. and Jones, R.A. (1982) *J.Am.Chem.Soc.* **104**, 1316−1319.
31. Issakides, G. (1991) thesis, Technische Universität Wien.
32. Himmelsbach, F., Schulz, B.S., Trichtinger, T., Charubala, R. and Pfleiderer, W. (1984) *Tetrahedron* **40**, 59−72.
33. Mag, M. and Engels, J.W. (1988) *Nucl.Acids Res.* **16**, 3525.
34. Schaller, H., Weinmann, G., Lerch, B., and Khorana, H.G., *J.Am.Chem.Soc.* (1963), 3821−27.
35. Sinha, N.D., Biernat, J., and Köster, H. (1984) *Nucl.Acids Res.* **12**, 4539−4557.