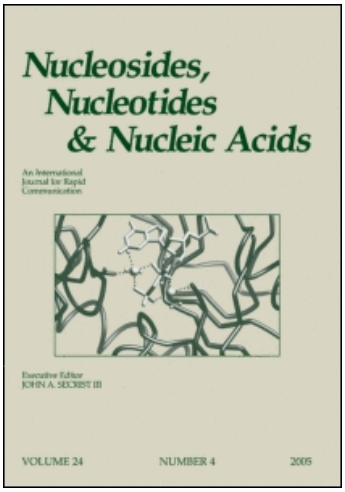

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Synthesis of Oligodeoxynucleotides Containing the C-Nucleoside and 2'-Deoxy-2'-Fluoro-*ara*-Nucleoside Moieties by the H-Phosphonate Method.[1,2]

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SYNTHESIS OF OLIGODEOXYNUCLEOTIDES CONTAINING THE C-NUCLEOSIDE AND 2'- DEOXY-2'-FLUORO-*ara*-NUCLEOSIDE MOIETIES BY THE H-PHOSPHONATE METHOD.[1,2]

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ABSTRACT: A module type, computer-controlled, multipurpose synthesizer displaying a novel device for the transport of liquids, was constructed and used in the synthesis of oligomers containing some C-nucleosides and 2'-deoxy-2'-fluoro-*ara*-nucleoside moieties. H-Phosphonate method was applied in terms of a further adjustment of construction features of the synthesizer versus chemistry of the process. Results of preliminary studies on the effects of the modified nucleosides on the stability of duplexes showed a clear tendency of destabilization of duplexes in the case of C-nucleosides while fluorinated nucleosides in most cases stabilize the formed duplexes.

INTRODUCTION

With most of the practical goals in the automated DNA synthesis virtually accomplished over the past decade, interest in preparation and study of oligomers featuring specific structural changes in the molecule has become greatly increased. Thus, oligonucleotides containing either modified monomeric units or altered phosphate backbone have attracted considerable attention, since such oligomers may have potential in antiviral and/or anticancer chemotherapy.[3,4]

These recent developments prompted us to utilize our modified C-nucleosides and 2'-deoxy-2'-fluoro-*ara*-nucleosides, close analogues of natural constituents of DNA, as monomeric units in oligonucleotide synthesis to study their biochemical and biophysical properties for eventual application to chemotherapeutic drug designs.[3,5]

Although the phosphoroamidite method[6,7] for oligonucleotide synthesis is currently considered superior as it gives somewhat higher stepwise yields and provides more uniform products especially with longer sequences, we chose the H-phosphonate approach[8-10] for our study. This approach on the other side appears to have important advantages: (**a**) the good stability of the monomer with ease recycling the excess of monomer, (**b**) "self-capping" coupling step and single oxidation step at the end of the elongation cycles, and (**c**) versatility in terms of the preparation of oligonucleotides with modified internucleotide linkages (*e.g.*, phosphorothioates, phosphoroamidates).

Most of currently available commercial synthesizers are constructed for phosphoroamidite method,[6,7] but only few of them originally developed for phosphoroamidite method are possible to modify for H-phosphonate mode[8,9] *e.g.*, 381A DNA synthesizer from Applied Biosystems.[11] We decided to construct a module type,[12] simple and fully automated multipurpose synthesizer which can be used not only for both phosphoroamidite and H-phosphonate methods using conventional protocols but also for new methods to be developed in the future for the synthesis of modified oligomers.

The development and adjustment of the synthetic work with our synthesizer is presented here along with the preparation of starting monomeric components and their use in solid-phase synthesis.

RESULTS AND DISCUSSION

Principle and Construction of a Novel Synthesizer.

The reasons for building our synthesizer were as follows:

a) Full control over all parameters of the synthesis (solvent delivery, type of solvent, reagents, reaction times, program details, etc.).

b) Delivery of precise amount of reagents.
Many commercial synthesizers require particular solvents and reagents (including nucleoside intermediates) specified by the manufacturers. None of our unnatural nucleosides are commercially available, and these nucleotide monomers usually require different parameters for synthesis than natural nucleotide monomers.

The synthesizer we constructed (Figure 1) is a through-flow system which utilizes suction of a motorized syringe pump located at the output side of the reaction column,

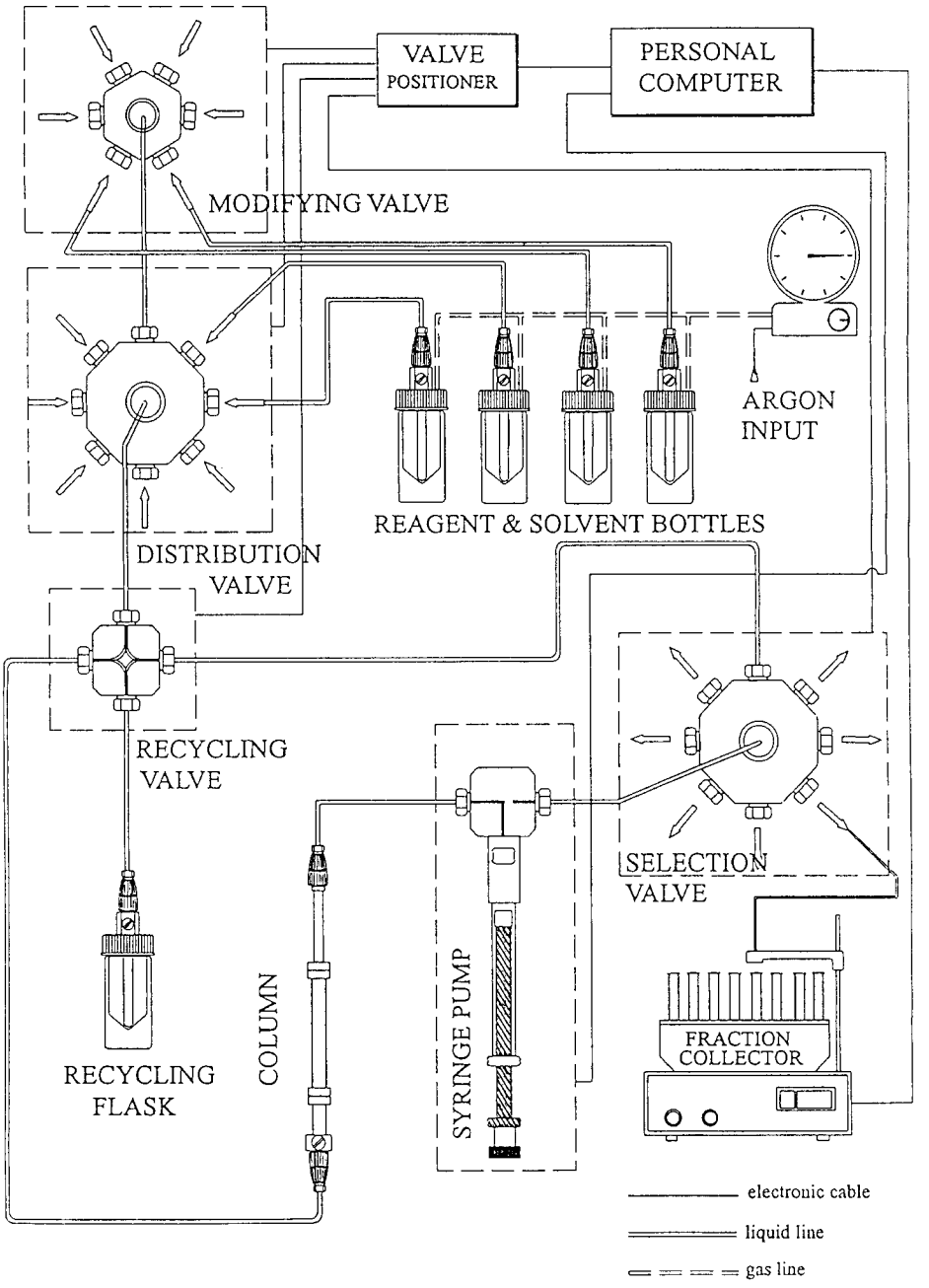

Figure 1. Multipurpose synthesizer - general arrangement.

whereby argon pressure has only an auxiliary role in balancing the pressure inside the system. This arrangement prevents the system from flow fluctuations, localized high pressure, bubble formation and wide range of flow rates can readily be achieved.[13] All the known commercial synthesizers employ either argon pressure or various mechanical pumps, or a combination of both to control the flow rate, with the pump inserted between the liquid reservoirs and the reaction column.

This system is also equipped with a recycling mechanism by which solvents and reactants can be used repeatedly. Recycling is important for large-scale synthesis, especially in the cases of unnatural monomers.

Delivery of liquids or recycling is accomplished by computer-controlled valves and syringes which, in connection with flexible software (the program not presented), allow for a modular concept of the whole system. The software design enables setting the parameters (time, volume, speed of syringe filling or dispensing, delay time, separating volume, number of loops, etc.) individually for each step in synthetic cycle. It also includes the procedure for hydrolytic cleavage of the produced oligomer from the support automatically. For this two hour procedure is used concentrated ammonium hydroxide from the modification reagent bottle, and collecting vial is connected to the spare position of the selection valve. The software program, moreover, is designed to be capable of controlling multiple syntheses if needed.[14]

Figure **1** shows the general arrangement: only four bottles are shown in this Figure, but actual number of reagent and solvent bottles connected for our purpose is 13. This universal system is easy transferable to any method of oligonucleotide synthesis currently available, although we optimized the H-phosphonate method only.

H-Phosphonate Strategy of Oligonucleotide Synthesis.

A series of natural and modified nucleosides was converted into their corresponding 3'-H-phosphonates *via* 4,4'-dimethoxytritylation and H-phosphonylation by the method of Takaku *et al.*[15] (Table **1**). The four basic nucleoside 3'-H-phosphonates were utilized in establishing general conditions for automated synthesis.

For the synthesis of modified oligomers, we prepared 3'-H-phosphonate derivatives of nucleosides listed in Figure **2**. Fluorinated nucleosides, 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-thymine (FMAU, **Ia**), -uracil (FAU, **IIa**), -5-iodouracil (FIAU, **IIIa**), as well as C-nucleosides, 5-(2'-deoxy-β-D-*erythro*-pentofuranosyl)-N^1-methyluracil (**IVa**), -N^3-

Table 1. Yields and basic characteristics of prepared modified nucleosides and nucleotides

Nucleoside		Yield of blocked nucleosides	Nucleoside loading of support [μmole/g]	Yield of purified nucleoside H-phosphonate ^{31}P NMR Data	(^{1}J)	Reference
FMAU	(Ia)	75%	36.5	89% 1.14 ppm	607.3 Hz	16-18
FAU	(IIa)	80%	30.5	89% 1.03 ppm	608.5 Hz	16-18
FIAU	(IIIa)	70%	32.7	57% 1.24 ppm	607.3 Hz	16-18
1-Me-ψ-dU	(IVa)	95%	30.5	88% 1.08 ppm,	598.7 Hz	20,21
3-Me-ψ-dU	(Va)	80%	31.5	68% 1.08 ppm,	599.4 Hz	22
1,3-Me$_2$-ψ-dU	(VIa)	85%	31.2	87% 1.11 ppm	598.1 Hz	20,21
ψ-dU	(VIIa)	84%	35.0	81% 1.05 ppm,	598.3 Hz	20,21

methyluracil (1-Me-ψ-dU, **Va**), -N^1,N^3-dimethyluracil (1,3-Me$_2$-ψ-dU, **VIa**) and -uracil (ψ-dU, **VIIa**) were prepared according to our published procedures. All compounds were protected at the 5' position by reaction with dimethoxytrityl chloride, the resulting fully protected derivatives **Ib** -**VIIb** were converted into the corresponding 3'-H-phosphonate triethylammonium salts by a slightly modified method[23] of Takaku et al.,[15] and purified on column of silica gel. The yields and NMR spectroscopic data are listed in Table **1**. Prepared nucleoside 3'-H-phosphonates were ^{31}P-NMR spectroscopically pure.

Assembly of the oligomer chains was mostly performed on a 0.5 μmol scale. The first unit was derivatized to the 3'-*O*-succinate which was further converted into the p-nitrophenyl ester, and then anchored on LCAA-controlled pore glass (CPG-500 Å).[24] The achieved loading amounted to 30-36 μmol/g (Table **1**), optimal value for 0.5-1 μmolar syntheses of short (10-30 bases) oligonucleotides.

The reactants and solvents are driven into the reaction column (Figure **1**) containing the loaded LCAA CPG through modifying valve, distribution valve and, if desired, recycling valve, by the suction of motorized syringe pump. After the coupling, the excess reagents and solvents are removed through the selection valve. Unreacted H-phosphonate may be driven

Table 2. Standard synthetic cycle with H-phosphonate method.

Operation	Solvent/Reagent	Volume [mL]	Time [s]
1. Deblocking	2,5% Dichloroacetic acid in dichloroethane	6.6	150
2. Washing	Dichloroethane	3.0	70
	Pyridine-MeCN (1:1)	2.0	48
3. Coupling	0.1 M Nucleoside 3'-H-phosphonate in pyridine	.075	76
	0.5 M Adamantanecarbonyl chloride in acetonitrile	.075	
4. Washing	Pyridine-MeCN (1:1)	2.0	48
	Dichloroethane	3.0	70

The first cycle is preceded by washing with dichloroethane and in the final one instead of coupling step is oxidation[28] (2% I_2 in THF-pyridine-H_2O 49:49:2/15 min).

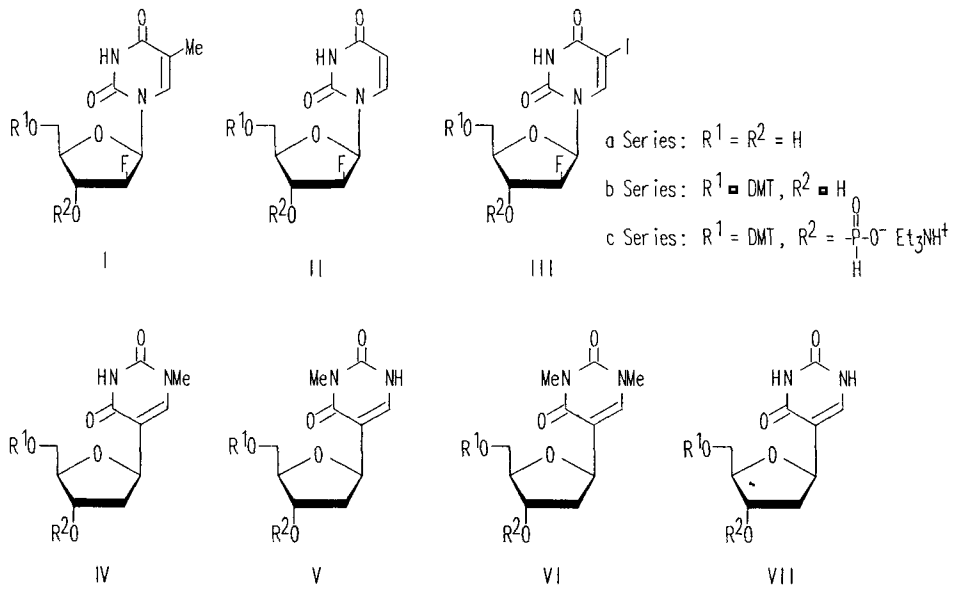

Figure 2. Modified nucleosides incorporated into oligomers.

to recycling flask through recycling valve. We used this approach only during the synthesis of fully modified homopolymers and after the standard work-up and chromatography on silica gel the monomers were identical to unused monomers according to TLC and ^{31}P NMR.

First experiments were done with pivaloyl chloride as condensing agent, later adamantanecarbonyl chloride[25] was found easier to handle and to give slightly higher and more reproducible coupling results in our system.

A typical protocol for the synthesis of oligomers is shown in Table 2.
The best results were obtained with the molar ratio 1 : 5 of the H-phosphonate and the coupling agent at 0.1M concentration of the H-phosphonate. At lower concentrations (20-90mM), the coupling yields, according to released dimethoxytrityl cation, were inconsistent and low (5-90%). Related problems on the coupling efficiency at lower concentrations of reactants were mentioned by Takaku *et al.*[15]

The key chemical step in the H-phosphonate method of oligonucleotide synthesis is very complex.[26,27] The active species is acyl phosphonate mixed anhydride. The main side reaction to avoid, among others, is the formation of bis(acyl)phosphite. The longer the premixing time of reactants before the coupling reaction starts, the more amount of the bis(acyl)phosphite forms. Despite the finding, the way to circumvent the side-reaction is not simple. Consequently, an obvious main goal to achieve is to reduce the ambiguity of the coupling step, namely, to delive- the right amounts of non-premixed components to the reaction column, rather than merely increase the amounts of reactants (as it is usually done elsewhere when yields are not satisfactory).

We therefore removed the tubing between the distributing valve outlet and reaction column which was placed directly on the top of the valve so that virtually zero dead-volume between the delivery device and the solid support was secured, and the premixing time minimized. Figure 3 provides the details of this simple, small-scale arrangement bypassing the recycling feature. This set-up shows the positions on modifying valve and distribution valve: three washing solvents (WASH1-3), five options for monomer (N1-5), and five positions for reagents - the deblocking reagent (DEBL), the condensing reagent (COND), the capping reagent (CAP), the oxidation solution (OXID), and the modifying reagent (MOD).

We also made further attempts to suppress premixing by designing a novel pattern for delivery of the monomer and condensing reagents to the reaction column. Thus, a "segmental" approach was employed in that pulses (separating volumes) of solvent (a 1:1 pyridine-acetonitrile mixture) are inserted between the alternating pulses of monomer and

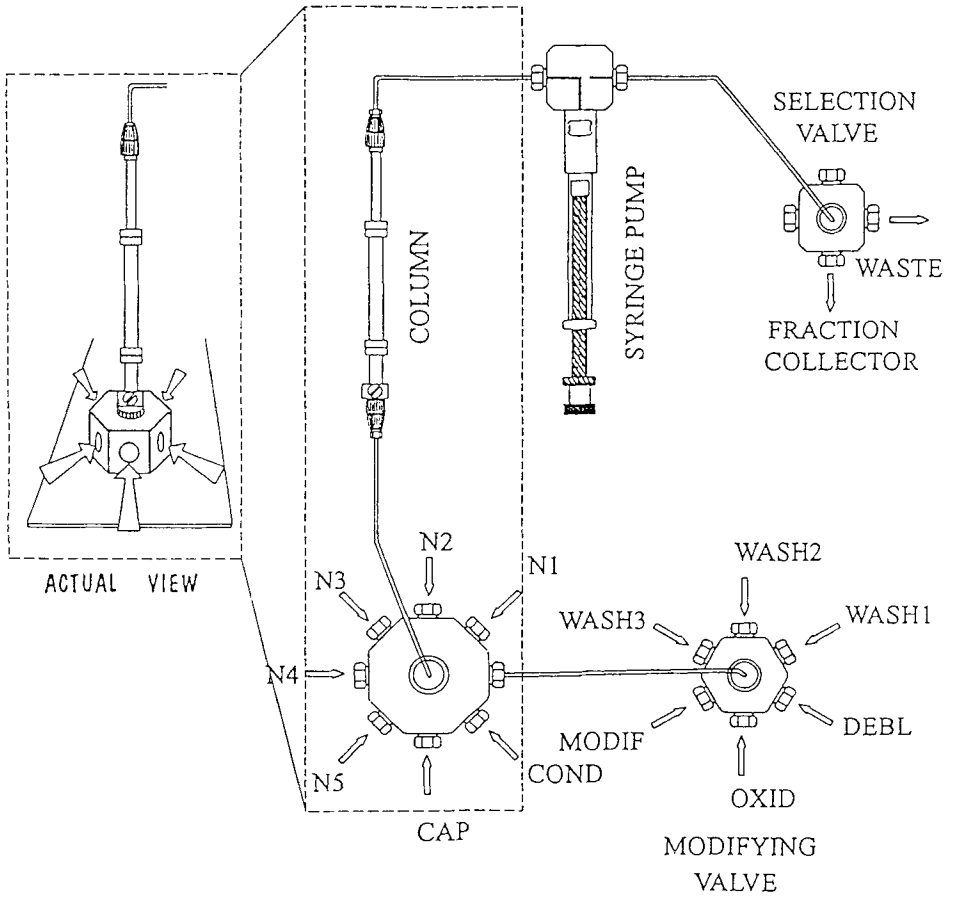

Figure 3. Synthesizer configuration for the H-phosphonate procedure.

coupling agent as they are driven to the column (step 3 in Table 2). In comparative experiments in the syntheses of homooligomers $d(T)_{10}$, $d(G)_{10}$ and $d(C)_{10}$ the "single segment" (monomer -> cond.agent -> solvent) and "double segment" (monomer -> solvent -> cond.agent -> solvent) variants provided 1.5 -2.0% higher average coupling efficiency than "standard segment" (monomer -> cond.agent). There was a marginal improvement by "single segment" over "double segment" methods (up to 0.5 %).

These results are consistent with the assumed suppression of preactivation. When pyridine was replaced by quinoline as the base and nucleophilic catalyst, as suggested by Efimov *et al.*[27] to decrease the rate of unwanted bis(acyl)phosphite formation, we observed in the synthesis of testing 10-mers no change or slightly worse performance in coupling

Table 3. Yields of oligonucleotides

Oligonucleotide		Length	Yield per step	Overall yield
$d(T)_{11}$		11	98.5%	69%
$d(T)_{30}$		30	98%	31%
$d(A\text{-}T)_5$		10	97%	58%
$d(G)_{11}$		10	97%	51%
$d(A)_{11}$		11	97.7%	61%
$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)$	N = 1-Me-ψ-dU	11	94.2%	33%
$d(T_5\text{-}N\text{-}T_5)$	N = 1-Me-ψ-dU	11	95%	41%
$d(N\text{-}T_4\text{-}N\text{-}T_5)$	N = 1-Me-ψ-dU	11	94.5%	31%
$d(N)_{10}$	N = 1-Me-ψ-dU	10	93%	21%
$d(A\text{-}N)_5$	N = 1-Me-ψ-dU	10	94%	27%
$d(N\text{-}A)_5$	N =1 ,3-Me_2-ψ-dU	10	94.1%	37%
$d(N)_{10}$	N = 1,3-Me_2-ψ-dU	10	93.5%	26%
$d(T_5\text{-}N\text{-}T_5)$	N = 1,3-Me_2-ψ-dU	11	94.8%	41%
$d(A\text{-}N)_5$	N = 1,3-Me_2-ψ-dU	10	93.7%	25%
$d(A\text{-}N)_5$	N = 3-Me-ψ-dU	10	93.4%	26%
$d(N)_{10}$	N = 3-Me-ψ-dU	10	93.5%	21%
$d(T_5\text{-}N\text{-}T_5)$	N = 3-Me-ψ-dU	11	94%	33%
$d(N)_{10}$	N = ψ-dU	10	93.2%	31%
$d(A\text{-}N)_5$	N = ψ-dU	10	93%	30%
$d(T_5\text{-}N\text{-}T_5)$	N = ψ-dU	11	95%	38%
$d(T_5\text{-}N\text{-}T_5)$	N = FAU	11	94.7%	39%
$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)$	N = FAU	11	94%	28%
$d(T_5\text{-}N\text{-}T_5)$	N = FIAU	11	95%	39%
$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)$	N = FIAU	11	93.8%	31%
$d(T_5\text{-}N\text{-}T_5)$	N = FMAU	11	94.8%	45%
$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)$	N = FMAU	11	94.2%	34%
$d(A\text{-}N)_5$	N = FMAU	10	92%	20%
$d(N)_{11}$	N = FMAU	11	93.3%	32%

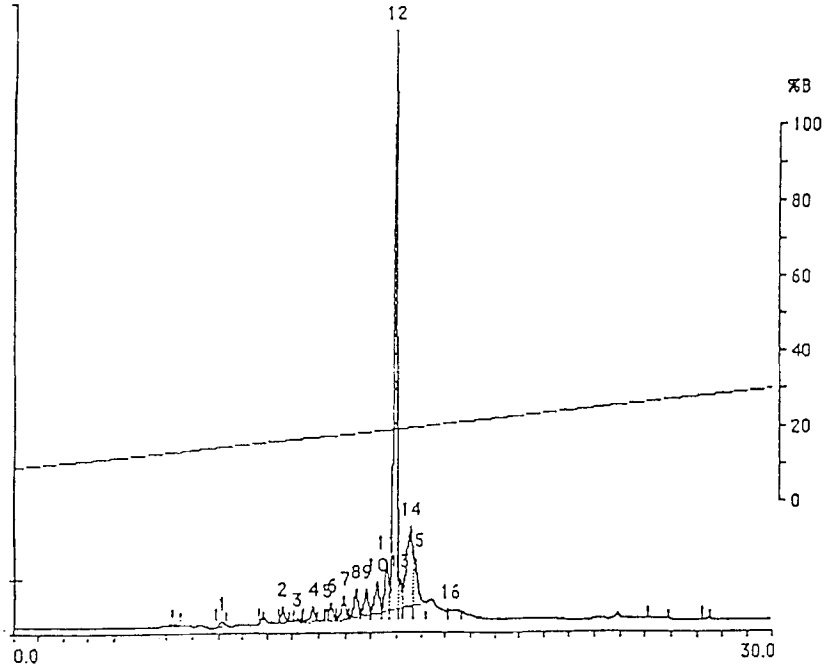

Figure 4. HPLC profile (crude mixture) of decamer of 2'-deoxy-1-methyl-ψ-uridine (1-Me-ψ-dU)$_{10}$; linear gradient A --> 30% B (30 min), flow rate 2 mL/min; A = 0.1 M TEAB (pH 7.5), B = 70% MeCN/H_2O.

yields with all types of segmental patterns. These results indicate that after the above-mentioned arrangements were made, further deactivation of the coupling reaction was unfavorable.

Stepwise yields of 97-98.5% were achieved (by the "single segment or "double segment" fashion) in the synthesis of unmodified oligonucleotides using a 15 molar excess of the monomer over the anchored unit. Sequences containing modified units afforded 3-5% lower yields per step. The isolated yields of prepared oligonucleotides after standard workup and reversed phase HPLC purification were 20-50% (Table 3). From an initial 0.5 μmol of 3'-bound nucleoside we were able to purify approximately 10-20 A_{260} of each oligonucleotide. For synthesis with the fluorine-containing units the reaction cycle was modified to provide a 22.5 fold molar excess of monomer in order to achieve reasonable yields. The step-wise coupling yields of the C-nucleosides were 93-95% and with fluoro nucleosides, 92 -95%. The reversed-phase HPLC profiles of unnatural nucleoside-containing oligomers (10-11 units in

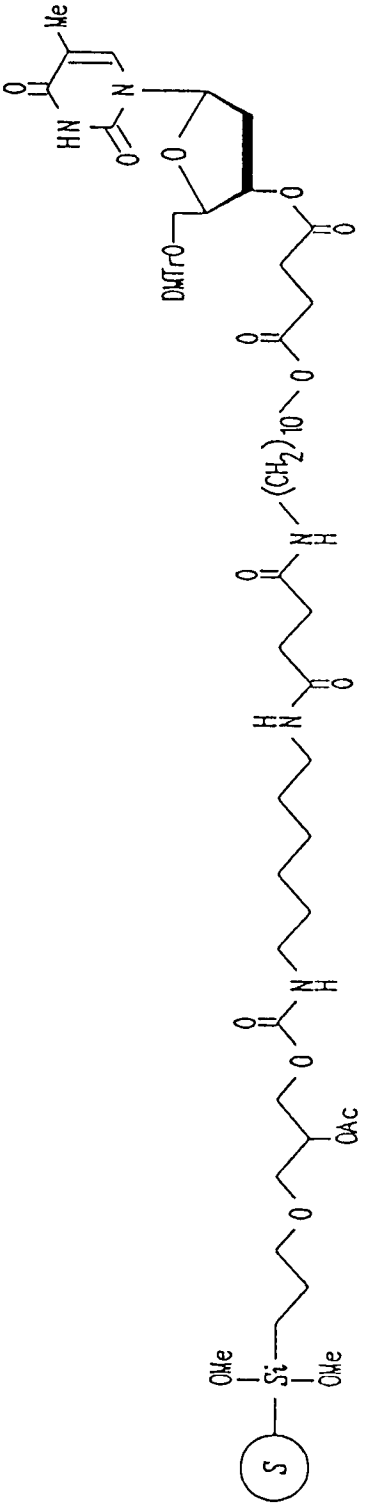

Figure 5. Solid support (extra-long-arm)

length) provided well-distinguished predominant peaks. The main collected peak was found to be homogeneous by RP HPLC. A second isolation using RP HPLC was required only in some cases. Figure 4 shows an example of HPLC profile.

Incorporation of the modified units was verified by examination of the nucleoside mixture composition after treatment of the oligomer with nuclease P1 and bacterial alkaline phosphatase. After RP HPLC was obtained, expected elution profile and nucleoside composition (calculated from the peak area integrals and nucleoside extinction coefficients) were consistent with the synthesized sequences.

The Role of Solid Support

We faced problems with the efficiency in the first coupling steps especially with natural purine monomers. The yield often dropped to ca. 90%. Since the importance of the length and type of spacer in coupling reactions,[29] we attempted to use another spacer. The 1,10-decanediol bis-succinate arm was attached to aminopropylated CPG-1000 Å by the method of Smrt *et al.*[30] Using this derivatized CPG and with prolonged coupling time, we prepared supports derivatized with 5'-O-dimethoxytrityl-N^2-dimethylaminomethylene-2'-deoxyguanosine (9.4 /mol/g) and 5'-dimethoxytrityl-N^6-dimethylaminoethylidene-2'-deoxy-adenosine (12 /mol/g). The first coupling reaction with protected nucleoside-3'-H-phosphonate occurred efficiently giving the first coupling in 96% yield (up 3-5% from the standard LCAA type), and subsequent steps in 97% yields. In a similar manner, the dT support (11.5 μmol/g) was prepared and compared with the novel, extra-long arm dT support (14.2 μmol/g, Figure 5), which was obtained by coupling the same activated decandiol-type intermediate as above with the LCAA-CPG instead of aminopropylated CPG. The yields of the first elongation steps with regular dT support (97.1%) and especially with extra-long arm dT support (97.7%) were again 2-4% better than with LCAA type (all tests with "single-segment" protocol). It appears that these supports may be beneficial in the synthesis of oligonucleotides containing rare monomers.

Oligonucleotide duplex stability

In order to study systematically the effects of modified nucleosides on the stability of duplex, we prepared several sequences containing C- and 2'-fluoro-arabinosyl nucleoside units modified at single, adjacent double, alternating double, or several consecutive positions. The melting characteristics of hybrids with the corresponding complementray sequences are

Table 4. Melting characteristics of the oligonucleotides containing 2'-fluoro-pyrimidine nucleosides and C-nucleosides as substitute for thymidine

	System[a]	Oligomer[b]	Modified nucleoside	Tm (°C)	ΔTm[d]
1	A	dT_{11}/dA_{11}		23.0	-
	B			31.0	-
	C			35.3	-
2	C	$d(T_5\text{-}N\text{-}T_5)/dA_{11}$	N = 1-Me-ψ-dU	33.7	-1.6
3	B	$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)/dA_{11}$	N = 1-Me-ψ-dU	21.4	-9.6
	C			22.7	-12.6
4	A	$d(N\text{-}T_4\text{-}N\text{-}T_5)/dA_{11}$	N = 1-Me-ψ-dU	c	c
	C			31.4	-3.9
5	A,B,C	d(AN-AN-AN-AN-AN)	N = 1-Me-ψ-dU	c	c
6	A,B,C	$d(N)_{10}/dA_{11}$	N = 1-Me-ψ-dU	c	c
7	A	$d(T_5\text{-}N\text{-}T_5)/dA_{11}$	N = 1,3-Me_2-ψ-dU	7.9	-15.1
	C			20.0	-15.3
8	B	$d(T_5\text{-}N\text{-}T_5)/dA_{11}$	N = FAU	31.0	0
9	B	$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)/dA_{11}$	N = FAU	23.1	-7.9
10	B	$d(T_5\text{-}N\text{-}T_5)/dA_{11}$	N = FIAU	33.8	+2.8
11	B	$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)/dA_{11}$	N = FIAU	33.5	+2.5
12	B	$d(T_5\text{-}N\text{-}T_5)/dA_{11}$	N = FMAU	33.1	+2.1
13	B	$d(T_4\text{-}N\text{-}T\text{-}N\text{-}T_4)/dA_{11}$	N = FMAU	30.6	-0.4
14	A	$d(N)_{11}/dA_{11}$	N = FMAU	33.0	+10.0
	B			43.2	+12.2
	C			52.0	+16.7
15	B	d(AN-AN-AN-AN-AN)	N = FMAU	32.0	-

[a] Final concentration of buffers in 360 μL cuvette
A: 10 mM HEPES pH 6.9, 100 mM NaCl
B: 10 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl (medium salt)
C: 10 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 1 M NaCl (high salt)
[b] Concentration of oligomer is 5 μM in one strand
[c] no cooperative melting
[d] Δ Tm is the diference in Tm between the modified system and unmodified duplex dA_{11}/dT_{11} in the same buffer

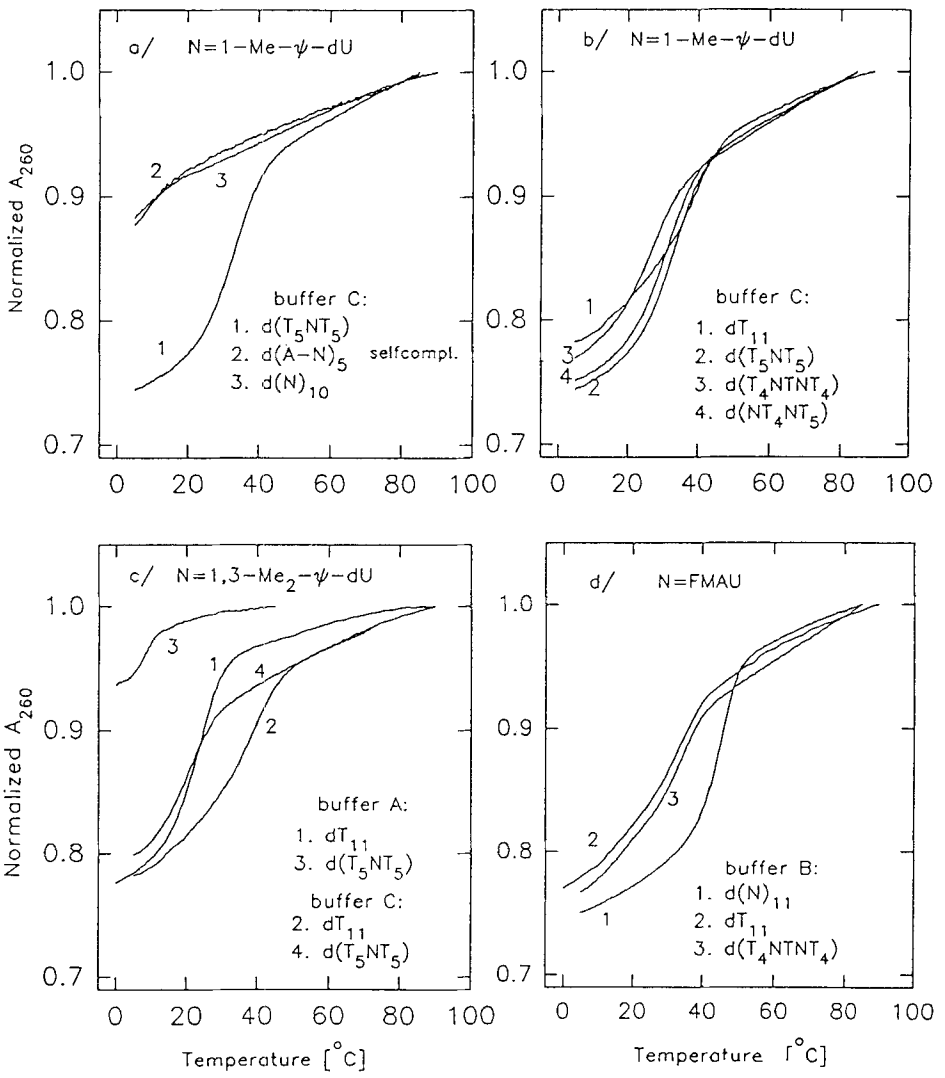

Figure 6. Melting profiles of oligonucleotides containing 1-Me-ψ-dU, 1,3-Me_2-ψ-dU, and FMAU. Sequences were hybridized with dA_{11} [except for self-complementary $d(A\text{-}N)_5$] at 5 μM concentration each.

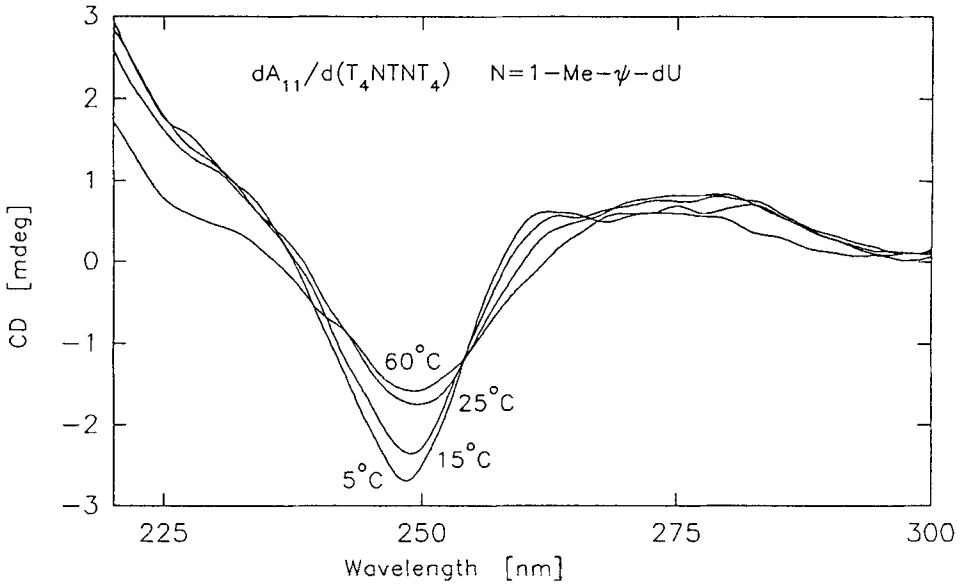

Figure 7. CD spectra of the double helix $dA_{11}/d(T_4NTNT_4)$ N = 1-Me-ψ-dU measured in buffer B (medium salt) at 5 - 60 °C at 5 μM concentrations.

shown in Table 4. Melting curves were measured in different buffers at different ionic strength. Some examples of melting profiles are given in Figure 6. Experiments in the HEPES buffer at pH 6.9 without magnesium cations show much less stable duplex or no duplex formation at all especially in the case of oligonucleotides containing C-nucleosides. Divalent cation remarkably stabilized the modified duplex, *e.g.*, model 14A *vs* 14B or 7A *vs* 7C.

Rather strong interaction was found in the case of duplex $(FMAU)_{11}/dA_{11}$ (14A-C). It is interesting to note, that similar results were observed by Eckstein *et al.*[31] with 2'-fluorothymidine (2'-F-ribo analogue) containing duplex $d(Tf_{11}T)dA_{12}$. The ΔTm observed for $(FMAU)_{11}/dA_{11}$ was 12 °C while the ΔTm value reported for $d(Tf_{11}T)dA_{12}$ was 16 °C. This duplex stabilization caused by fluorine substitution at the 2' position of both in the "up" (*arabino*) and "down" (*ribo*) configuration in the sugar moiety is interesting.

The relationships between the duplex stability, number and location of modified units in the oligomers do not appear to be straightforward. Introduction of one FMAU unit resulted stabilization (12B), two alternating FMAU units led to slight destabilization (13B). The same tendency was found in a model for the study of EcoRV restriction endonuclease.[31]

Influence of changes in both sugar and base part of nucleoside unit (FAU, FIAU) is even more complex. Stabilization by one or two FIAU units is near the same, 2.8 °C resp. 2.5 °C. On the contrary, incorporation of one FAU unit has no effect on the melting temperature, two isolated FAU units caused notable destabilization.

A large depression of Tm was found for duplexes with C-nucleoside containing oligomers **2-7**. This is very intriguing, because 1-methyl-2'-deoxy-ψ-uridine (Me-ψ-dU) is isosteric as well as isolelectric to thymidine (both are 1,5-disubstituted uracils with identical pKa), and somehow the replacement of a C-N glycosyl bond with a C-C linkage is influencing the structure of oligonucleotides. From these preliminary studies, the importance of the position of modified unit is clearly visible (2C, 3C and 4C). Introduction of one or two Me-ψ-dU units in the middle of sequence (2-3C) led to Tm despression, whereas two modifications in the middle and at the 5'-end (4C) caused Tm depression only half as much.

CD spectra of a oligonucleotide duplex containing C-nucleoside is shown in Figure 7. Melting temperature of this model duplex $dA_{11}/d(T_4\text{-N-T-N-}T_4)$ N = 1-Me-ψ-dU is 22.7 °C in buffer with 1M NaCl. At each temperature CD spectra are consistent with "B DNA" conformation.

EXPERIMENTAL

General. Pyridine, acetonitrile (both HPLC grade) and 1,2-dichloroethane were purchased from Baker, and all were dried prior to use in synthesis by passing through a column of activated basic alumina (Brockman I). For column chromatography, silica gel G60 (63-230 mesh; EM Science) was used. TLC was carried out on DC-Alufolien Kieselgel 60 F_{254} sheets (Merck). Pivaloyl chloride and adamantanecarbonyl chloride were from Aldrich. For preparation of solid supports, CPG-10-1000A (Fluka) and LCAA-CPG 500A (Sigma) were used. N,N-dibutylformamide dimethylacetal was prepared according to Sproat and Gait.32 Nuclease P1 from Penicillium citrinum and bacterial alkaline phosphatase were from Sigma. 31P NMR spectra were recorded on JEOL FX90Q spectrometer in pyridine-d_5; chemical shifts are given in ppm, relative to 85% H_3PO_4 as external standard; coupling constants ^1J are in Hz.

Spectral analyses and melting temperature experiments were performed using a Gilford Response spectrophotometer equipped with a six-position Peltier thermocell. Sample solutions (equimolar mixture of the complementary oligonucleotides) were placed in 360 µl

quartz cuvettes (10mm pathlength) with teflon stoppers. Buffer solution of the appropriate ionic strength was placed in a reference cuvette occupying first position in the holder. The composition of buffers were:

A: 10 mM HEPES pH 6.9, 100 mM NaCl

B: 10 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl (medium salt)

C: 10 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 1 M NaCl (high salt)

The absorbance at 260 nm vs temperature was measured at 0.5°C intervals and Tm determined as the maximum of the first derivative of the melting curve.

The CD spectra of modified oligonucleotides were measured on a JASCO J- 710 spectropolarimeter interfaced to an IBM AT computer. The same buffer was used as in the measuring of Tm. The instrument was calibrated with d_{10}- camphorsulfonic acid. The sample chamber was flushed with nitrogen and cell path length was 1 mm.

Reversed phase HPLC was performed on a Rainin HPX chromatograph with Knauer variable wavelength detector and Rainin Dynamax C18 columns.

Modified nucleosides **Ia-VIIa** were synthesized in our laboratory according to our published procedures (see Table **1**).

Preparation of solid supports anchoring modified nucleoside units. For functionalization of solid support we used 3'-O-succinic derivatives of the nucleosides prepared according to the literature procedure.[24] Yields of these 3'-O-succinates varied from 74 to 94% (R_f values on TLC EtOAc:Me_2CO:EtOH:H_2O 4:1:1:1 was *ca.* 0.6 for all these derivatives). The succinate was treated with 1 equivalent of *p*-nitrophenol in pyridine and 2.5 equivalents of DCC in dioxane, the mixture was allowed to react overnight at room temperature, and then filtered. To the filtrate was added LCAA-CPG 500A (500 mg) in DMF (0.5 mL) and Et_3N (0.1 mL). The suspension was swirled several times, kept overnight at room temperature, placed on a column (6.5 x 120 mm), and the column was washed successively with DMF (200 mL), MeOH (200 mL), and Et_2O (50 mL), and dried in vacuo. The dried CPG was treated with 2.5 mL of acetylation mixture [prepared by adding Ac_2O (3 mL) and 4-dimethylamino-pyridine (150 mg) in pyridine (45 mL)] overnight at room temperature for capping, filtered, washed with MeOH (300 mL) and Et_2O (50 mL), and dried in vacuo.

Preparation of supports with alternative decanediol spacer. 5'-O-DMTr-N^2-dimethylamino-methylene-2'-dGuo,[33,34] 5'-O-DMTr-N^2-dimethylaminoethylene-2'-dGuo[33,34] and 5'-O-DMTr-Thd were prepared according to published procedures. Supports with these

nucleosides were prepared according the method of Smrt *et al.*[11] using aminopropylated CPG-10-1000A. Loading was 9.4 /mol/g, 12.4 /mol/g, and 11.5 /mol/g respectively. In a similar manner support with extra-long arm was prepared from long chain aminoalkyl CPG 500A (Sigma) and 5'-O-DMTr-Thd (Figure **5**, 14.2 /mol/g loading). Coupling time for attachment of the long-arm nucleoside intermediate to the CPG was prolonged to 40 h for all four cases.

Preparation of nucleoside 3'-O-(H-phosphonates) Compounds **Ia-VIIa** were converted into their corresponding 5'-O-dimethoxytrityl derivatives by treatment with 4,4'-dimethoxytrityl chloride (1.2 equiv.) in dry pyridine (10 mL per 1 mmol of nucleoside) at room temperature (usually overnight) as monitored by TLC ($CHCl_3$-EtOH, 9:1 v/v). The reaction was quenched by addition of MeOH, and the mixture was partitioned between saturated aq. $NaHCO_3$ and $CHCl_3$ (100 mL/500 mL per 10 mmol). The organic phase was washed with 1M triethylammonium bicarbonate (TEAB), and concentrated in vacuo. Column chromatography on silica gel using $CHCl_3$-EtOH (1 -> 5% with 1% Et_3N) as the eluent afforded the 5'-O-dimethoxytritylated derivatives **Ib-VIIb** in reasonable yields (Table **1**) Then, the 5'-protected nucleosides **Ib-VIIb** was dried by co-evaporation with anhydrous pyridine (3 x), re-dissolved in pyridine (10 mL per 1 mmol), and the solution was cooled to 0 °C, to which was added tris-(1,1,1,3,3,3-hexafluoro-2-propyl)phosphite[15] (1.2 equiv.) and Et_3N (0.1 equiv.). After 2 h at room temperature TEAB (2 M; 30 mL/1mmol) was added, and after 20 min, the mixture was extracted (3 x) with $CHCl_3$ containing 1% Et_3N. After concentration of the organic layer, the residue was chromatographed on a silica gel column using the $CHCl_3$-1% Et_3N system with a 1 -> 5% MeOH gradient. Yields of the colorless foam and the ^{31}P NMR characteristics of product **Ic-VIIc** are given in Table **1**.

Deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine were N,O-blocked according to standard procedures.[33] Procedure of phosphonylation was the same as for modified nucleosides. Yields and the ^{31}P NMR characteristics of products were identical as published.

Automated oligonucleotide synthesis by the H-phosphonate method. Most syntheses were carried out on 0.5 /mol scale with a 15-molar excess of monomer. All solvents used in the synthetic cycle (Table **2**) were stored under argon. The starting monomers were dissolved in pyridine to make 0.1M solution. Oxidation was performed on the column as a part of the last program block; the iodine solution was prepared freshly before use.

After synthesis, the support was washed with MeOH (30 mL) and Et_2O (20 mL), oligomer was released from support and deblocked as described,[24] and purified by semipreparative HPLC on the reversed phase C18 Dynamax-300A (5/) column using gradient systems with (A) 0.1M TEAA or TEAB and (B) 70% MeCN-H_2O.

Characterization of oligonucleotides. The appropriate oligonucleotide (2 A_{260} units) in 100 µl of 20 mM NaAc pH 5.5, 5 mM $ZnCl_2$ was digested by 2 U of nuclease P_1 from *Penicillium citrinum* for 3 h at 37 °C. The pH of the reaction mixture was raised by the addition of 10 µl of 1M sodium glycinate buffer pH 8 and then 2 U of alkaline phosphatase was added. After 2 h incubation at 37 °C aliquots were examined by analytical RP HPLC.

CONCLUSION

We herein present the modified H-phosphonate methodology for the synthesis of oligonucleotides containing C-nucleosides and 2'-fluorinated analogues of natural deoxynucleosides. The proposed protocol can be easily adapted to the synthesis of oligonucleotides containing many other modified nucleosides. We have also demonstrated that the technical construction of the synthesizer and carefully optimized synthetic cycle could avoid undesirable side reactions in H-phosphonate methodology. Simple changes in delivery of reactants can obviously avoid, to certain extent, indiscriminatory use of the high molar excesses of monomers which is of importance especially with the rare modified nucleosidic units.

From the results of physico-chemical properties of synthesized oligonucleotides it is evident that the influence of sugar- or base-modified units on the melting characteristics is complex. More detailed study is necessary to provide information about possible structural alteration of duplex and to better understand the influence of carbohydrate conformation on oligonucleotide chain and duplex stability. Further structural studies with these new modified oligonucleotides are presently under way.

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12. Synthesizer consists of all commercially available parts: Hamilton selection valves and Omnifit solvent delivery system.

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14. Software can adequately control up to 52 independent automatic syringes and 316 independent automactic valves through two daisy chains. The program is also capable of controling a series of solenoid valves for argon gas, fraction collectter, etc.

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