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SYNTHESIS OF OLIGONUCLEOTIDES WITH SEQUENCES IDENTICAL WITH OR ANALOGOUS TO THE 3'-END OF 16S RIBOSOMAL RNA OF *ESCHERICHIA COLI*: PREPARATION OF U-C-C-U-U-A AND A-C-C-U-C-C-U-U-A VIA THE MODIFIED PHOSPHOTRIESTER METHOD

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Abstract—The synthesis of oligoribonucleotides U-C-C-U-U-A and A-C-C-U-C-C-U-U-A, which are located at the 3'-terminus of 16S rRNA of *E. coli*, is described. The key-intermediates in the synthesis of these compounds are the fully-protected mononucleotides **5a–c**, which can be rapidly (2–4 min) functionalized by either of the two following specific deblocking procedures: (i) at the 3'-terminus with zinc in pyridine-2,4,6-triisopropylbenzenesulphonic acid and (ii) at the 5'-terminus with 0.5M hydrazine in pyridine-acetic acid. The fully-protected hexamer **17a** and nonamer **19**, prepared by utilizing these deblocking conditions, were completely deprotected by the action of fluoride ion, followed by treatment with base and acid to give the required oligonucleotides in high yield.

INTRODUCTION

The 3'-terminus of the 16S ribosomal RNA of *E. coli* has been the subject of numerous biological and biophysical studies.[1-8] Our approach in the elucidation of its function in protein synthesis is based on the chemical synthesis of oligoribonucleotide sequences identical with or analogous to the 3'-end of 16S rRNA, and subsequent testing of these compounds in biologically relevant systems. In this connection we reported on the synthesis, *via* phosphotriester intermediates, of hexaribonucleotide A-C-C-U-C-C and its *N*-methylated analogs m_2^6A-C-C-U-C-C and A-C-C-U-C-m_2^4C.[9,10] The practical difficulties encountered during the synthesis of these compounds, however, were such that a closer investigation of the synthetic methods was needed.

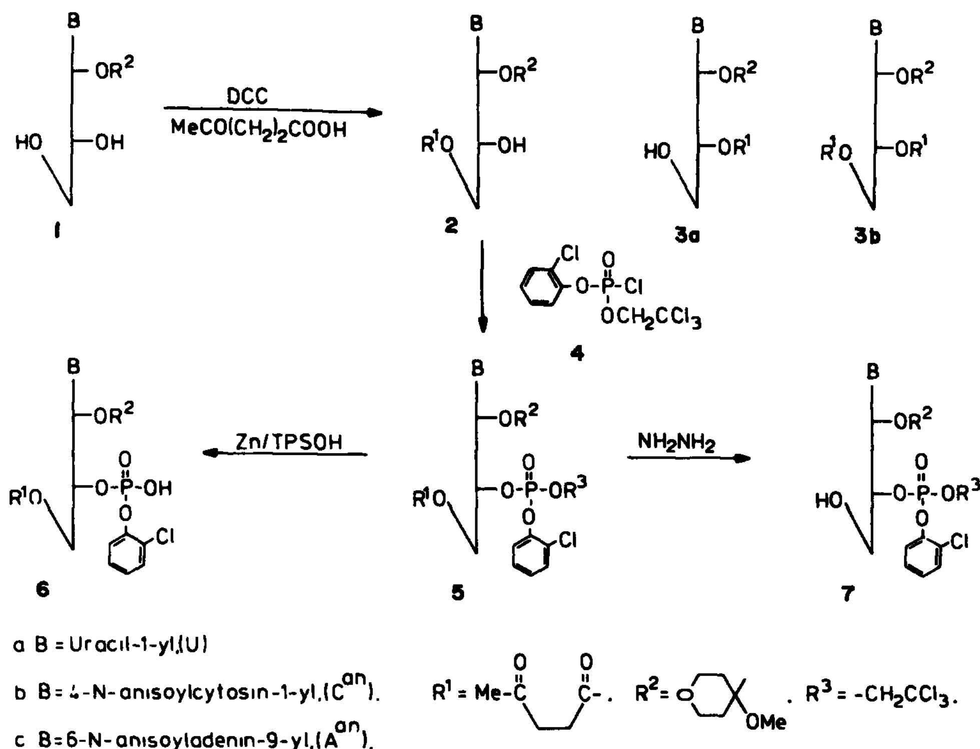
It was recognized that a successful method for synthesising oligoribonucleotides with defined sequence could only be obtained if at least two requirements were met; (i) the assemblage of oligonucleotide blocks should be efficient and unambiguous; (ii) the removal of a protective group at the 5'- and/or 3'-position of a growing oligonucleotide chain should be selective. The first requirement was met in a satisfactory way if we used the modified phosphotriester method,[11] which is based on the application of the monofunctional phosphorylating agent 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate: **4**, in combination with the activating agent 2,4,6-triisopropylbenzenesulphonyl 4-nitroimidazolide (**13b**, TPSNI).[10] Recently, we introduced levulinic acid (4-oxo-pentanoic acid) as a protective group for the 5'-hydroxy function[12] and showed that this group fully met the second requirement when applied to the synthesis of the tetradecaribonucleotide U-A-U-A-U-A-U-A-U-A-U-A-U-A.[13] The main advantage of levulinyl as protective group lies in its removal, from protected (oligo)nucleotides, under essentially neutral conditions:[14] 0.5M hydrazine in pyridine-acetic acid (4:1 v/v) for 2–4 min. Under these reaction conditions the half time of removal of the *N*-anisoyl group on cytidine is 10 h. All other protective groups used in the present study are even more stable to hydrazinolysis.[13] This indicates that levulinyl can be removed selectively in the presence of the other types of protective groups.

To determine further the scope of the present method we decided to extend our research on 16S ribosomal RNA of *E. coli* to the preparation of the heterosequences U-C-C-U-U-A and A-C-C-U-C-C-U-U-A. The nonaribonucleotide is the naturally occurring sequence of the 3'-terminus of the 16S rRNA. The synthesis of both oligonucleotides will be presented in this paper.

RESULTS AND DISCUSSION

The starting monomeric units, necessary for the preparation of the oligoribonucleotides, are the terminal nucleoside unit **14** (B = A^{an}),[9] which constitutes the 3'-end of the oligonucleotides to be synthesised, and the mononucleotides **5a–c** and **7a–c**.

The synthesis of mononucleotides **5a** (B = U), **5c** (B = A^{an}), together with their respective nucleoside precursors **2a,c**, has been described.[13] Of these compounds only the uridine nucleoside derivative **2a** (B = U) could be isolated as a crystalline compound. The 4-*N*-anisoylcytidine derivatives **5b** and **7b** (B = C^{an}) were prepared similarly. Thus nucleoside **1b** (B = C^{an}), protected at the 2'-position with the acid-labile methoxytetrahydropyranyl group and on the exocyclic amino function with the base-labile *p*-anisoyl group, was acylated at the 5'-position with levulinic acid in the presence of dicyclohexylcarbodiimide (DCC), using 1,2-dimethylimidazole as a catalyst (Scheme 1). Under these conditions three different products, depending on the selectivity of the esterification reaction, may be formed, i.e. **2b**, **3a** and **3b** (B = C^{an}). Examination of the reaction mixture by TLC showed, apart from starting product **1c** (B = C^{an}), the presence of two products both of which had a R_f-value higher than starting product **1c** (B = C^{an}), and one with a higher R_f-value than the other. Isolation of the product with the highest R_f value afforded a homogeneous compound which could be converted fast and quantitatively with hydrazine into starting product **1c** (B = C^{an}). The other product could also be converted



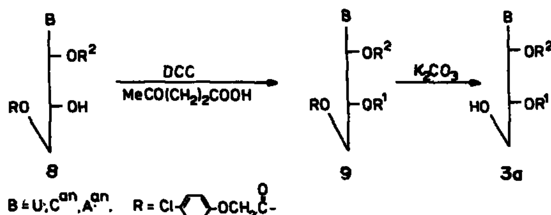
Scheme 1.

with hydrazine into starting product **1c** (B = C^{an}). However, both compounds failed, under mild conditions, to react with triphenylchloromethane. From these data we may conclude that the compound with the highest R_f-value is the di-acylated nucleoside **3b** (B = C^{an}) and the other the required 5'-protected derivative **2b** (B = C^{an}).

The formation of the di-acylated nucleoside **3b** (B = C^{an}) indicates that the levulinylation is not very selective and that, as a consequence, the required product **2b** (B = C^{an}) may be contaminated with the nucleoside **3a** (B = C^{an}).

The nucleosides **3a** (B = U, C^{an}, A^{an}, respectively), protected at the 3'-position with the levulinyl group, could be prepared unambiguously by acylation of derivatives **8** (B = U, C^{an}, A^{an}, respectively) with levulinic acid in the presence of DCC, followed by removal of the *p*-chlorophenoxyacetyl group with potassium carbonate (Scheme 2).

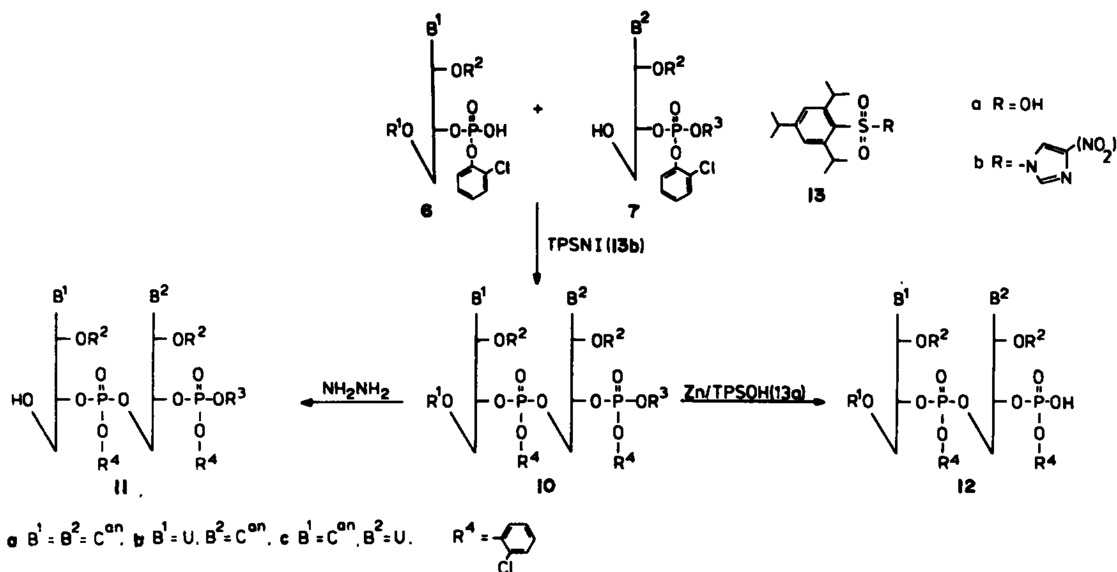
The above experiment also demonstrated that the rate of levulinylation of the 3'-hydroxy function was rather slow. For instance we found that, under the conditions employed for the levulinylation of nucleoside **1b** (B = C^{an}), only a small quantity (8–10%) of nucleoside **8** (B = C^{an}) was converted into the fully-protected nucleoside **9** (B = C^{an}). This low rate of levulinylation is in accordance with the quantity (10–12%) of di-acylated products **3b** (B = U, C^{an}, A^{an}, respectively) formed during the levulinylation of nucleosides **1a–b** (B = U, C^{an}, A^{an}). These findings indicate that the fast rate of levulinylation of the 5'-hydroxy function of nucleoside derivatives **1a–c** (B = U, C^{an}, A^{an}) will prevent the formation of the unwanted 3'-levunilyl derivatives **3a** (B = U, C^{an}, A^{an}, respectively). The latter is also supported by the fact that: (a) the required nucleosides **2a–c** (B = U, C^{an} or A^{an}) fail to react with tritylchloride in pyridine, while the nucleosides **3a** (B = U, C^{an}, A^{an}, respectively) under the same conditions, react smoothly with this reagent: (b) comparison of the ^{1}H NMR spectra of derivatives **3a** with those of compounds **2a–c** showed that the latter were not contaminated with the former derivatives. Thus, despite the fact that, under the levulinylation conditions employed (Scheme 1), concomitant 3'-levulinylation occurred, nucleoside **2b** (B = C^{an}) could be isolated as a homogeneous and colourless glass in reasonable yield (52%).



Scheme 2.

Phosphorylation of the latter compound **2b** with 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate **4** and 1-methylimidazole in acetonitrile solution (Scheme 1) afforded the fully-protected mononucleotide **5b** (B = C^{an}) as a homogeneous glass in a yield of (88%).

Starting from this compound **5b**, the first type of protective group (i.e. levulinic could be removed in the presence of the second type (i.e. 2,2,2-trichloroethyl) and the other types of protecting groups and vice versa, making chain-extension in the 5'- and 3'-direction possible. Thus, the partially-protected mononucleotide **7b** (B = C^{an}) was obtained in high yield (91%) by treating **5b**

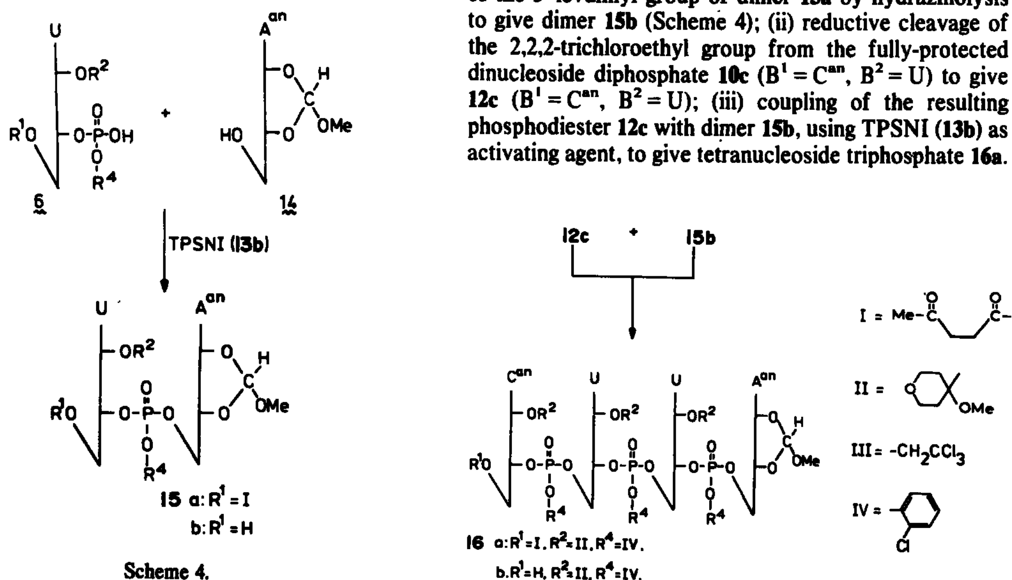


Scheme 3.

(B = C^{an}) with 0.5M hydrazine in pyridine-acetic acid (4:1, v/v) for 2 min at 20°. On the other hand, treatment of **5b** (B = C^{an}) with zinc in pyridine, in the presence of 2,4,6-triisopropylbenzenesulphonic acid (**13a**, TPSOH), afforded phosphodiester **6b** (B = C^{an}) in quantitative yield (Scheme 1). The same deblocking procedures could be performed successfully on the uridine and adenosine derivatives **5a** (B = U) and **5c** (B = A^{an}) to give the required phosphodiesters **6a** (B = U) and **6c** (B = A^{an}), respectively (see Experimental).

The phosphodiester derivatives of type **6** (B^1 = C^{an} or U) served as the key intermediates for the synthesis of three dinucleoside diphosphates (Scheme 3) **10a** (B^1 = B^2 = C^{an}), **10b** (B^1 = U, B^2 = C^{an}) and **10c** (B^1 = C^{an}, B^2 = U), and also one dinucleoside monophosphate **15a** (Scheme 4). Thus a solution of a fully-protected mononucleotide of type **5** (B = C^{an} or U, 1 mmole) and TPSOH (**13a**, 0.2–0.3 mmole) in pyridine (10 ml) was treated with activated zinc (*ca.* 10 mmole).[10] The deblocking of the trichloroethyl group proceeded rapidly and was complete in 2–3 min, as shown by TLC analysis (System A, R_f *ca.* 0.5→0). Excess zinc was then filtered off and the filtrate diluted with chloroform and extracted with an aqueous M triethylammonium bicarbonate buffer (TEAB, pH 7.5). In this way, the zinc ions were drawn into the aqueous layer and the triethylammonium salt of the phosphodiester of type **6** (B^1 = C^{an} or U) was obtained as the sole nucleotide in quantitative yield. The phosphodiester of type **6** (B^1 = C^{an} or U) thus obtained was dried by repeated coevaporation with pyridine, and reacted together with condensing agent TPSNI (**13b**) and a 5′-hydroxy mononucleotide of type **7** (B^2 = C^{an} or U), to give, after work-up and chromatography of the crude reaction mixture, the dinucleoside diphosphates **10a–c** in good yields (78–84%). In the same way (Scheme 4), the dinucleoside monophosphate **15a** was obtained, in 84% yield, by condensing the uridine phosphodiester **6** with the 5′-hydroxy adenoside derivative **14**.

Assemblage of four dimers proceeded in the 5′-direction via a repeating three-step process: (i) removal of the 5′-levulinyl group of dimer **15a** by hydrazinolysis to give dimer **15b** (Scheme 4); (ii) reductive cleavage of the 2,2,2-trichloroethyl group from the fully-protected dinucleoside diphosphate **10c** (B^1 = C^{an}, B^2 = U) to give **12c** (B^1 = C^{an}, B^2 = U); (iii) coupling of the resulting phosphodiester **12c** with dimer **15b**, using TPSNI (**13b**) as activating agent, to give tetranucleoside triphosphate **16a**.



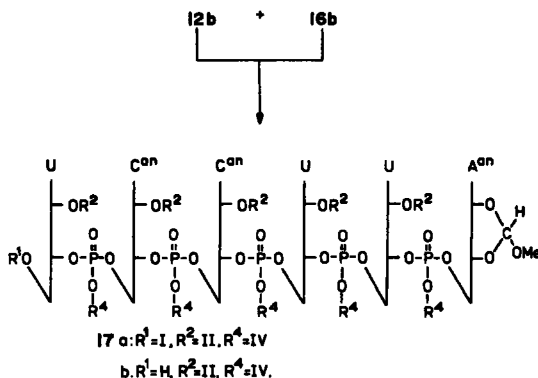
Scheme 4.

Table 1. Data relating to the synthesis of the fully-protected nonaribonucleotide **19**

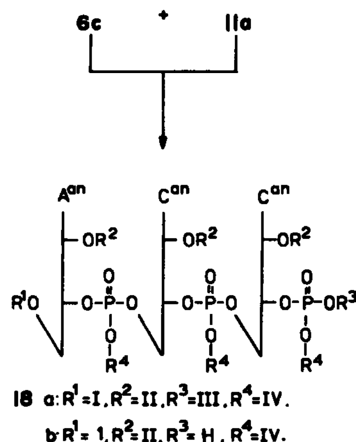
3'-Phosph. component No.	3'-Phosph. component mmole	5'-Hydroxy component No.	5'-Hydroxy component mmole	TPSNI mmole	Reaction time (days)	Product No.	Product yield[a]	R_f values A	R_f values B	Removal of levulinyl group: time (min)	Removal of levulinyl group: Product No.	Removal of levulinyl group: Product yield	Removal of levulinyl group: R_f values[b] A	Removal of levulinyl group: R_f values[b] B
5b	1.15	**7b**	1.0	1.2	1	**10a**	78%	0.66	0.71	2	**11a**	91%	0.57[c]	0.65[c]
													0.60	0.68
5a	0.72	**7b**	0.6	0.75	1	**10b**	80%	0.50	0.60					
5b	0.92	**7a**	0.8	1.0	1	**10c**	84%	0.53	0.66					
5a	1.15	**7c**	1.0	1.2	1	**15a**	82%	0.43	0.56	2	**15b**	92%	0.33[c]	0.46[c]
													0.37	0.49
10c	0.78	**15b**	0.63	0.70 + 0.40	1½	**16a**	64%	0.40	0.53	4	**16b**	83%	0.32	0.48
10b	0.48	**16b**	0.32	0.40 + 0.20	1½	**17a**	73%	0.37	0.51	4	**17b**	70%	0.32	0.45
5c	0.8	**11a**	0.6	0.75 + 0.20	1½	**18a**	68%	0.59	0.69					
18a	0.24	**17b**	0.12	0.18 + 0.12	2½	**19**	63%	0.46	0.57					

[a]Based on the 5'-hydroxy component; [b]On silica-gel plates in system A and B, respectively; [c]Pair of diastereoisomers.

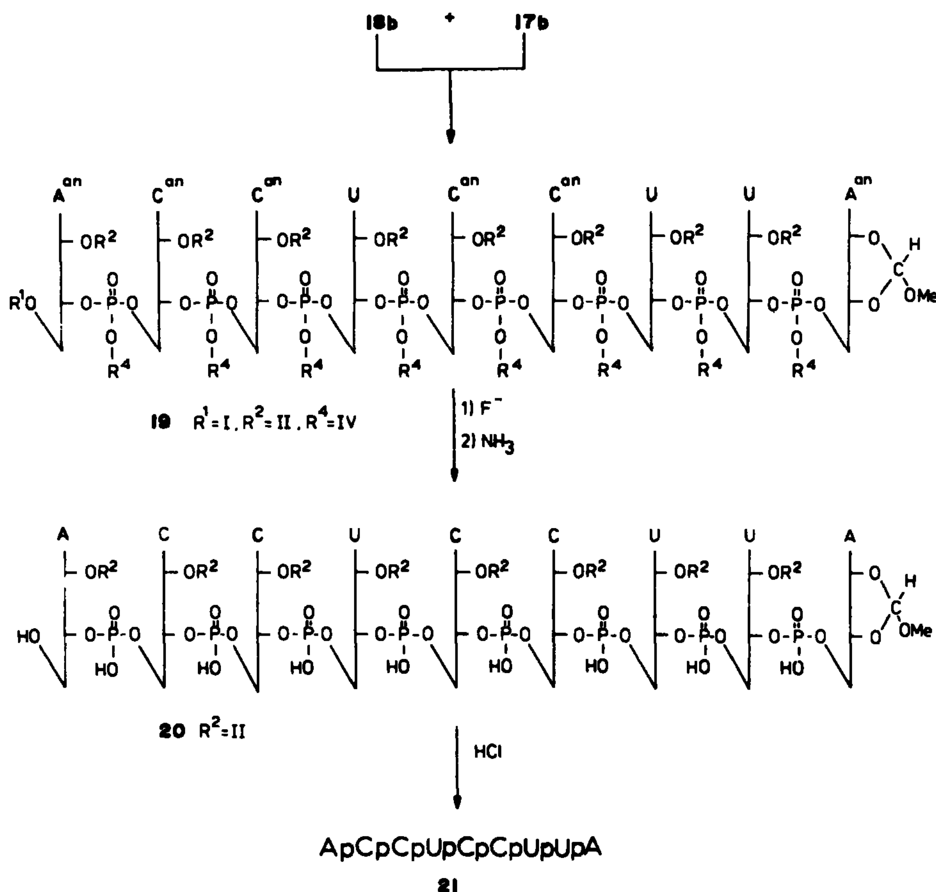
Data pertaining to the execution of this three-step process are summarized in Table 1. In the same way, the fully-protected hexanucleoside pentaphosphate **17a** was obtained by repeating the three-step procedure on tetranucleotide **16a** and dinucleotide **10b** ($B^1 = U$, $B^2 = C^{an}$). Yields and other relevant data of this three-step process are summarized in Table 1.



The final condensation-cycle was performed starting from the fully-protected trimer **18a** and hexamer **17a**. Thus, the latter product (**17a**, 0.23 mmole) was dissolved in pyridine (2.3 ml) and a molar hydrazine-hydrate solution in pyridine-acetic acid (3:2, v/v, 2.3 ml) was added. After 4 min, the reaction was quenched by the addition of an excess of pentane-2,4-dione and the reaction mixture was worked-up and chromatographed to give the partially-protected hexanucleotide **17b** in 70% yield. The fully-protected trinucleoside triphosphate **18a** was prepared by condensing **6c** ($B = A^{an}$), obtained by zinc

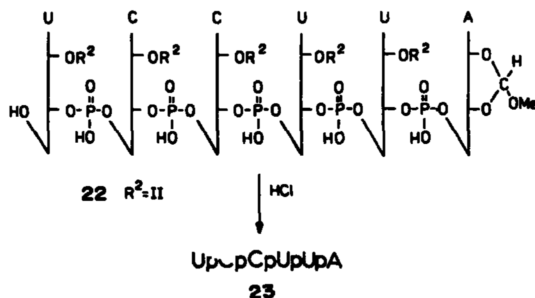


treatment of **5c** ($B = A^{an}$), with the hydrazinolysis product **11a** derived from dimer **10a** ($B^1 = B^2 = C^{an}$) under the influence of TPSNI (**13b**). Yields and other relevant data are recorded in Table 1. A solution of the fully-protected trimer **18a** (0.24 mmole) and TPSOH (**13a**, 0.06 mmole) in pyridine (2 ml) was treated with activated zinc (*ca.* 3 mmole). Filtration after 4 min and work-up of the reaction mixture, as described above, afforded the corresponding phosphodiester derivative **18b**. The latter (**18b**, 0.24 mmole) was condensed with the partially-protected hexamer **17b** (0.12 mmole) and TPSNI (0.18 mmole) in pyridine (2 ml). TLC after 24 h



showed still some starting material **17b**. More TPSNI (0.12 mmole) was added and, after a further 36 h, the reaction mixture was worked-up and purified to give the fully-protected nonamer **19** in 63% yield (based on hexamer **17b**).

Alkaline deblocking of (substituted) phenyl groups from oligonucleotide phosphotriester intermediates, bearing labile ester functions (e.g. levulinyl) on sugar hydroxyls, has been shown to lead to the formation of impure oligonucleotides containing unnatural internucleotide linkages.[13,16] Fluoride ion, however, is able to hydrolyse the phosphotriester moieties of fully-protected oligonucleotides selectively in the presence of all other protective groups.[13,17] Because all hydroxy functions remain protected during this deblocking process, neighbouring group participation of free hydroxy functions is excluded and oligonucleotides with solely $3' \rightarrow 5'$ internucleotide linkages are obtained. Therefore, the fully-protected nonanucleotide **19** was thus deprotected as follows. Treatment of **19** with 0.05M tetrabutyl-ammonium fluoride (TBAF) in tetrahydrofuran-pyridine-water (8:1:1 v/v/v) for 16 h at 20° completely removed the 2-chlorophenyl protective groups. The resulting compound, which contains solely phosphodiester linkages, was then treated with 25% aqueous ammonia for 24 h at 50°, to cleave the levulinyl group and the *N*-protecting anisoyl groups, to give Amthp–Cmthp–Cmthp–Umthp–Cmthp–Cmthp–umthp–Umthp–Amm **20**.[20] In the same way the fully-protected hexanucleotide **17a** was partially deprotected to give Umthp–Cmthp–Cmthp–Umthp–Umthp–Amm[20] **22**. HPLC analysis (gradient program I) of the latter partially-protected hexanucleotide and the partially-protected nonanucleotide **20** showed only minor impurities due to internucleotide cleavage during phosphotriester deblocking[13] (Fig. 1). The oligonucleotides were purified by DEAE-Sephadex chromatography (Fig. 2) and, finally, treated with aqueous HCL (0.01N, pH 2.0) at 20° for 2 h. After neutralization of the reaction mixture with dilute ammonia the products were lyophilized to give U–C–C–U–U–A **23** and A–C–C–U–C–C–U–U–A **21** as fluffy colourless solids in 83% and 72% yield, respectively (determined spectrophotometrically). Analysis of these compounds by TLC (system E) and HPLC (program I) revealed no impurities.



The presence of only $3' \rightarrow 5'$ internucleotide linkages in the thus obtained completely deblocked products was established by complete digestion of the oligonucleotides with snake venom phosphodiesterase, Ribonuclease A

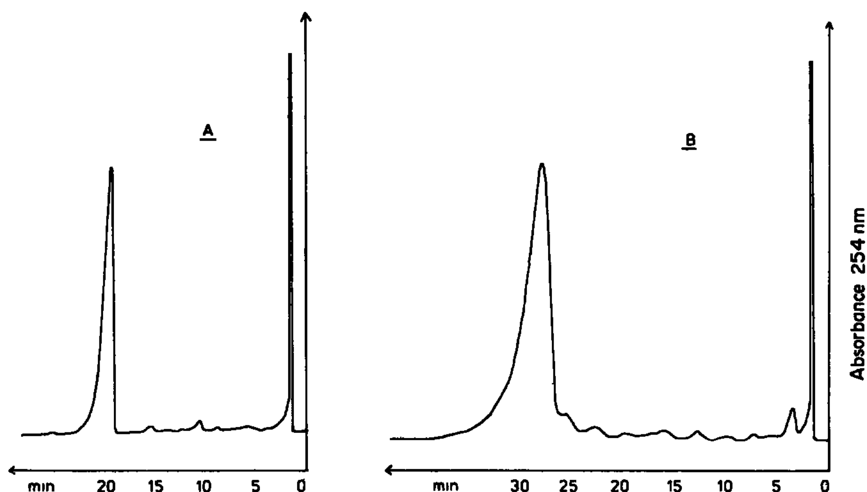


Fig. 1. HPLC analysis (gradient program I) of crude partially-protected hexanucleotide 22(A) and nonanucleotide 20(B) obtained after removal of all base labile protecting roups.

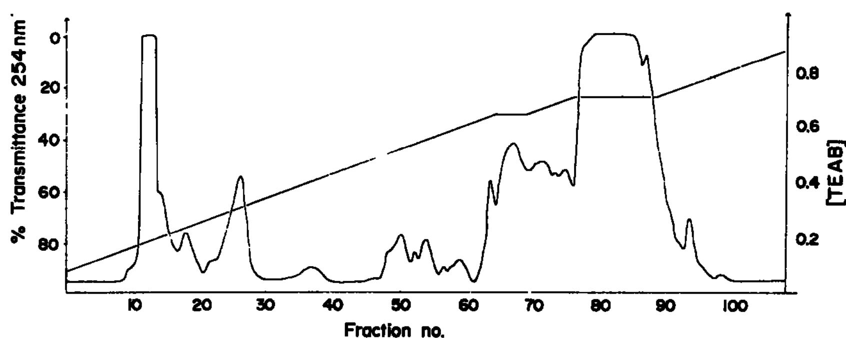


Fig. 2. Purification of the partially-protected nonanucleotide 20 by DEAE-Sephadex chromatography.

and Taka-diastase T_2 to the expected products in the correct ratios (Table 2). Moreover, the correct sequence of the nonaribonucleotide was unambiguously ascertained by treatment of this compound 21 with snake venom phosphodiesterase and following the course of the reaction with HPLC.[18] Experimental details on the sequence analysis of the nonanucleotide will be published elsewhere.[19]

CONCLUSIONS

The results described in this paper show that the modified phosphotriester method in combination with levulinyl as protective group for the 5′-position provides an excellent methodology for the synthesis of longer oligoribonucleotides. The efficiency of the several condensation steps is generally high and does not fall off rapidly with increasing chain-lengths. Furthermore, the

Table 2. Enzymic digestions of UCCUUA and ACCUCCUUA[a]

Compound	Enzyme		Molar ratios of digestion products								
			A-Cp	Ap	Up	Cp	pA	pU	pC	A	U
UCCUUA	venom	calcd.	—	—	—	—	1	2	2	—	1
		found					1.02	1.94	2.05		1.00
UCCUUA	RNase	calcd.	—	—	3	2	—	—	—	1	—
		found			2.86	2.02				1.11	
ACCUCCUUA	venom	calcd.	—	—	—	—	1	3	4	1	
		found					0.97	3.11	3.93	1.00	
ACCUCCUUA	RNase	calcd.	1	—	3	3	—	—	—	1	
		found	0.98		2.93	3.04				1.05	
ACCUCCUUA	T_2	calcd.	—	1	3	4	—	—	—	1	
		found		0.96	2.90	4.06				1.08	

[a]For details see experimental part and Refs. 9 and 18.

levulinyl group can be removed selectively from longer oligonucleotides. These two properties indicate that the present method should be suitable for the synthesis of oligoribonucleotides with chainlengths of 15–20 nucleotide units. The synthesis of oligoribonucleotides with sequences containing the nucleoside guanosine, is currently under investigation.

EXPERIMENTAL

All solvents were dried as described previously[15] and stored over molecular sieves. 1-Methylimidazole, 1,2-dimethylimidazole and levulinic acid were obtained from Aldrich and distilled before use. Evaporations were carried out under reduced pressure (15 nm or 0.5 mm Hg) at bath temperatures below 40°.

UV spectra were measured with a Cary C14 recording spectrophotometer. ^{1}H NMR spectra were measured at 100 MHz with a Joel JNMPS 100 spectrometer; chemical shifts are given in ppm (δ) relative to TMS as internal reference.

The high-performance liquid chromatographic system used in this study has been described elsewhere.[18] Isocratic elution of monocleotides was effected with buffer A (0.005M KH_2PO_4, pH 4.5). Buffer B is composed of: 1.0M KCl, 0.05M KH_2PO_4, pH 4.5. Gradient elution was performed by building up a linear gradient, starting with buffer A and applying, at t = 0 min (gradient program I) or at t = 15 min (program II), 1.5% of buffer B per min. The latter program was used to obtain a complete separation of a mixture of several mononucleotides and oligonucleotides.

S&S DC Fertigfolien F1500 LS254 were developed in solvent system A (chloroform-methanol (92:8 v/v) and B (chloroform-methanol (88:12 v/v). Merck DC Alufolien Cellulose F254 plates were developed in solvent system. C (aq. M NH_4OAc-EtOH (3:7 v/v), D (aq. M NH_4OAc-EtOH (4:6 v/v) and E (aq. M NH_4OAc-EtOH (6:4 v/v). Merck Kieselgel H and Macherey Nagel Kieselgel MN were used for adsorption chromatography.

5′-O-Levulinyl-2′-O-(methoxytetrahydropyranyl)-4-N-p-anisoylcytidine **2b**. To a cooled, ice-water bath, solution of 2′-*O*-(methoxytetrahydropyranyl)-4-*N*-*p*-anisoylcytidine[15] (9.8 g, 20 mmole), 1,2-dimethylimidazole (1 ml, 10 mmole) and levulinic acid (6.8 g, 60 mmole) in dioxan (40 ml), dimethylformamide (10 ml) and 2,6-lutidine (6 ml) was added during 1 h a solution of dicyclohexylcarbodiimide (12.3 g, 60 mmole) in dioxan (60 ml). The precipitate of dicyclohexylurea was then filtered off and the filtrate diluted with chloroform (150 ml). The organic solution was washed with 2% aqueous sodium bicarbonate (150 ml) and the aqueous layer reextracted with chloroform (2 × 100 ml). The combined organic layers were dried ($MgSO_4$), concentrated to an oil and triturated with petroleum-ether (40–60°, 150 ml). The solid thus obtained was redissolved in chloroform (10 ml) and applied to a column (25 cm × 14 cm^2) of Kieselgel MN (120 g), suspended in chloroform. The column was washed with chloroform and then eluted with chloroform-methanol (97.5:2.5 v/v) to give **2b** (B = C^{an}) as a colourless glass (6.1 g, 52%) which could not be crystallized; R_f 0.38 (system A) 0.47 (system B). NMR ($CDCl_3$) 8.12 (H_6, d, J 7.5 Hz); 7.60 (H_5, d, J 7.5 Hz); 6.21 (H_1, d, J 4 Hz); 2.62 and 2.80 (CH_2CH_2, t, J 6 Hz) and 2.22 (CH_3, s).

5′-O-Levulinyl-2′-O-(methoxytetrahydropyranyl-4-N-p-anisoylcytidine 3′-O-(2,2,2-trichloroethyl-2-chlorophenyl)phosphate **5b**. To a cooled, ice-water bath, solution of the above obtained material (**2b**, B = C^{an}, 5.9 g, 10 mmole) in acetonitrile (50 ml) and 1-methylimidazole (2.0 ml, 25 mmole) was added dropwise during 5 min a solution of 2,2,2-trichloroethyl 2-chlorophenyl phosphorochloridate (**4**, 5.02 g, 14 mmole) in acetonitrile (10 ml). TLC (system A) after addition showed almost complete conversion of **2b** into **5b**. The reaction was stopped by the addition of a molar phosphate buffer (pH 6, 20 ml). The organic solvents were evaporated and the residue partitioned between chloroform (150 ml) and water (100 ml). The organic layer was dried ($MgSO_4$) and concentrated to an oil, which was triturated with petroleum-ether (40–60°, 2 × 100 ml). A solution of the thus obtained precipitate in chloroform (5 ml) was brought onto a column (20 cm × 14 cm^2) of Kieselgel MN (100 g), suspended in chloroform. The column was eluted with chloroform-methanol (98:2 v/v) and the appropriate fractions were collected and concentrated to a small volume (ca. 10 ml), which was added dropwise, with stirring, to diisopropyl ether (200 ml). The precipitate of **5b** was filtered off and dried *in vacuo* (P_2O_5) at 40°. Yield 8.0 g (88%); R_f 0.62 (system A) 0.67 (system B). UV (95% EtOH) λ_{max} 290 (ϵ 27,600) λ_{min} 237 nm (ϵ 7,100). NMR ($CDCl_3$) of a mixture of diastereoisomers: 8.8 (N-H, s); 8.10 (H_6, d, J 8 Hz); 7.53 (H_5, d, J 8 Hz); 7.2 (2-chlorophenyl, m); 6.31 ($H_{1'}$, d, J 7 Hz); 4.81, 4.77 (CH_2CCl_3, d, J 7 Hz); 2.78 and 2.59 (CH_2,CH_2, t, J 5.5 Hz) and 2.19 (CH_3, s).

2′-O-(Methoxytetrahydropyranyl)-4-N-p-anisoylcytidine 3′-O-(2,2,2-trichloroethyl 2-chlorophenyl)phosphate **7b**. A molar solution of hydrazine hydrate in pyridine-acetic acid (3:2 v/v, 20 ml) was added to a solution of the above obtained fully-protected cytidine derivative (**5b**, 3.64 g, 4 mmole) in pyridine (20 ml). After 2 min at 20°, pentane-2,4-dione (4 ml, 40 mmole) was added and the reaction flask was immersed into an ice-water bath. After another 2 min, the reaction mixture was added to chloroform (150 ml) and water (200 ml). The organic layer was separated and washed with 10% aqueous sodium bicarbonate (150 ml) and water (150 ml). The dried ($MgSO_4$) chloroform layer was evaporated to an oil and triturated with petroleum-ether (40–60°, 250 ml). The precipitate was redissolved in chloroform (5 ml) and applied to a column (15 cm × 8 cm^2) of Kieselgel MN (40 g). The column was washed with chloroform and then eluted with chloroform-methanol (98:2 v/v). The appropriate fractions were concentrated to a small volume (*ca.* 5 ml), which was added dropwise, with stirring, to petroleum-ether (40–60°, 200 ml). The precipitate of **7b** was filtered off and dried *in vacuo* (KOH) at 20°. Yield 3.03 g (93%); R_f 0.53 (system A) 0.60 (system B). (Anal.: $C_{31}H_{34}Cl_4N_3O_{12}P$ (813.40); Calc.: C, 45.78; H, 4.21; N, 5.16. Found: C, 45.90; H, 4.26; N, 5.07). UV (95% EtOH) λ_{max} 289 (ϵ 28.000) λ_{min} 237 nm (ϵ 7,200). NMR ($CDCl_3$) of a mixture of diastereoisomers: 8.19 (H_6, d, J 7.5 Hz); 7.57 (H_5, d, J 7.5 Hz); 7.3 (2-chlorophenyl, m); 6.03 (H_1', d, J 7.5 Hz) and 4.76, 4.71 (CH_2CCl_3, d, J 7 Hz).

2′-O-(Methoxytetrahydropyranyl)uridine 3′-O (2,2,2-trichloroethyl 2-chlorophenyl)-phosphate **7a**. In exactly the same way as described above for the synthesis of **7b** (B = C^{an}), the fully-protected uridine derivative **5d** (3.11 g, 4 mmole) was converted into **7a**. Yield 2.55 g (94%); R_f 0.40 (system A) 0.49 (system B). Anal.: $C_{23}H_{27}Cl_4N_2O_{11}P$ (680.28); Calc.: C, 40.61: H, 4.00; N, 4.12. Found: C, 40.68; H, 4.09; N, 4.08). UV (95% EtOH) λ_{max} 260 (ϵ 10,200) λ_{min} 230 nm (ϵ 3,100). NMR ($CDCl_3$) of a mixture of diastereoisomers: 9.9 (N-H, s); 7.79 H_6, d, J 8.5 Hz); 7.3 (2-chlorophenyl, m); 6.03, 6.01 ($H_{1'}$, d, J 7 Hz); 5.80 (H_5, d, J 8.5 Hz) and 4.80, 4.76 (CH_2CCl_3, d, J 6.5 Hz).

Preparation of 3′-O-levulinyl-2′-O-(methoxytetrahydropyranyl)nucleosides **3a** *(B = U, C^{an} or A^{an}*. To a stirred solution of 5′-*O*-*p*-chlorophenoxyacetyl-2′-*O*-(methoxytetrahydropyranyl) uridine[21] (**8a**, B = U; 0.53 g, 1 mmole), 1,2-dimethylimidazole (0.05 ml, 0.5 mmole) and levulinic acid (0.4 g, 3 mmole) in dioxan (2 ml), dimethylformamide (0.5 ml) and 2,6-lutidine (0.3 ml) was added, at 20°, a solution of DCC (0.61 g, 3 mmole) in dioxan (3 ml). Analysis of the reaction mixture, after 24 h, still indicated the presence of starting material (*ca.* 40%). After a total reaction time of 40 h, the precipitated dicyclohexylurea was filtered off and the filtrate was diluted with chloroform (15 ml). The organic layer was washed with aqueous sodium bicarbonate (7 ml) and the aqueous layer re-extracted with chloroform (2 × 5 ml). The combined organic layers were dried ($MgSO_4$) and concentrated to an oil. The latter was redissolved in chloroform (1 ml) and applied onto a column (10 × 1 cm^2) of Kieselgel MN (10 g) suspended in chloroform. The column was washed with chloroform and then eluted with chloroform-methanol (98:2, v/v) to give **9** (B = U) as a homogeneous (TLC system A) glass (0.35 g, 55%).

The above material (0.35 g) was dissolved in dry dioxan (10 ml) and methanol (30 ml) and methanolic potassium carbonate solution (0.1 M, 5 ml) was added. After 4 min at 20°, the reaction mixture was neutralized with aqueous 1M KH_2PO_4 (3.5 ml), concentrated and the residue partitioned between chloroform (50 ml) and water (25 ml) which was brought onto a column (10 × 1 cm^2) of Kieselgel H (10 g) suspended in chloroform–

methanol (97:3, v/v). Elution of the column with the same solvent system afforded the required derivative **3a** (B = U) as a homogeneous (TLC system A) glass (0.23 g, 90%).

Hydrazinolysis of nucleoside **3a** (B = U), under exactly the same conditions as described for the synthesis of nucleoside derivative **7b** ($B = C^{an}$), gave solely, after isolation, a nucleoside which was identical (TLC analysis, m.p.) with starting product **1a** (= U). Furthermore, ^{1}H NMR ($CDCl_3$) of compound **3a** (B = U) showed *inter alia* the presence of a doublet at 5.30 ppm (J 4.5 Hz) which indicates that one of the hydroxy functions of nucleoside **3a** is acylated at the 3′-position. In the same way, the other two nucleoside derivatives **3a** ($B = C^{an}$ or A^{an}), derived from the corresponding nucleosides **8b**[10] ($B = C^{an}$) and **8c**[22] ($B = A^{an}$), were prepared and afterwards subjected to hydrazinolysis and analysis by ^{1}H NMR spectroscopy. In both cases, the results thus obtained were the same as those for the nucleoside derivative **3a** (B = U).

Tritylation of nucleosides **3a** *(B = U, C^{an} or A^{an}) and* **2** *(B = U, C^{an} or A^{an}).* To a solution of nucleoside **3a** (B = U; 89 mg, 0.2 mmole) in dry pyridine (0.5 ml) was added at once triphenylchloromethane[23] (112 mg, 0.4 mmole). Analysis (TLC system A) of the reaction mixture, after 2 h at 20°, showed complete tritylation of starting product. Work-up and purification of the reaction mixture afforded a homogeneous glass (124 mg, 90%), which gave a positive ceric sulfate spray test.[24]. Tritylation of nucleosides **2** ($B = U, C^{an}$ or A^{an}), under the conditions described above for the trytilation of nucleoside **3a** ($B = U, C^{an}$ or A^{an}), failed completely as shown by TLC analysis and the ceric sulfate spray test.

General procedure for the preparation of the fully-protected oligonucleotides. The 5′-O-levulinyl(oligo)nucleotide 3′-O-(2,2,2-trichloroethyl 2-chlorophenyl)-phosphate (1 mmole) and TPSOH (0.2–0.4 mmole) were dissolved in pyridine (10 ml) and activated zinc[10] (*ca.* 10 mmole) was added. The suspension was stirred magnetically. The course of the reaction was followed by monitoring the evolvement of heat. After 45–60 sec the temperature of the reaction mixture raised sharply to 30–40° and, after 3 min, the mixture was filtered to remove excess zinc. TLC (system A) of the filtrate showed that complete conversion to base-line material had occurred in all experiments. The filtrate was diluted with chloroform (100 ml) and washed with 1 M TEAB (pH 7.5, 50 ml) and 0.1M TEAB (50 ml). In case of mononucleotide **6a** (B = U), the aqueous solution was reextracted with chloroform-pyridine (9:1 v/v, 2 × 40 ml). The organic layer was concentrated to an oil and transferred to a small flask, containing already the 5′-hydroxy nucleoside (tide). The mixture was dried by repeated coevaporation with anhydrous pyridine (3 × 10 ml). TPSNI was added to the resulting viscous oil and the sealed reaction mixture was kept in the dark. After 16 h at 20°, the course of the reaction was checked with TLC (system A and B). More TPSNI, if necessary, was then added and the reaction mixture was kept in the dark for a further period as indicated in Table 1. Ice-water was then added and the reaction mixture was partitioned between chloroform (50 ml) and 5% aqueous sodium bicarbonate (50 ml). The organic layer was dried ($MgSO_4$) and concentrated to an oil. Trituration with petroleum-ether (40–60°) afforded a yellow to light brown coloured solid, which was redissolved in chloroform-methanol (97–95.5 : 3–4.5 v/v) and applied to a column of Kieselgel H (*ca.* 30 g per g of crude material), suspended in the same solvent. Elution of the column with the same solvent mixture and precipitation of the concentrated pure fractions from petroleum-ether (40–60°) afforded the fully-protected oligonucleotides as stable white solids in good to excellent yields. Data pertaining to the synthesis and analysis of these compounds are given in Table 1.

Removal of the levulinyl group from the fully-protected oligonucleotides. Removal of the levulinyl group was performed under analogous conditions as described for the synthesis of **7b**. Tetranucleotides and higher oligonucleotides, however, were treated with 10 equivalents of hydrazine instead of 5. The products were purified on a column of Kieselgel MN (5–10 g per g of crude material). Elution of the column was effected with chloroform-methanol (98–96.5 : 2–3.5 v/v). The partially-protected oligonucleotides were obtained as homogeneous white solids after precipitation of the concentrated pure fractions from petroleum-ether (40–60°). Conditions of removal, yields and analytical data are given in Table 1.

Deblocking of the fully-protected hexanucleotide **17a** *and nonanucleotide* **19**. To a solution of **17a** (56 mg, 16 μmol) or **19** (55 mg, 10 μmol) in tetrahydrofuran (4 ml) and pyridine (0.5 ml) was added an 0.5M aqueous tetrabutylammonium fluoride solution (pH 7.5, 0.5 ml, 3 equiv. per phosphotriester moiety). After 16 h at 20°, water (5 ml) and Dowex 50W cation-exchange resin (100–200 mesh pyridinium form, 2 g) were added. The resin was filtered off and a few drops of 1M TEAB buffer (pH 7.5) were added to the filtrate. The solution was concentrated to an oil, which was dissolved in 25% aqueous ammonia (10 ml). The reaction vessel was sealed and kept at 50°. After 24 h, the solution was concentrated till neutral and, after analysis by HPLC (gradient program I, Fig. 1), brought onto a column (20 cm × 2 cm²) of DEAE-Sephadex A_{25} (HCO_3-form), suspended in 0.1M TEAB. The column was eluted with a linear gradient of 0.1M → 0.6M TEAB for the hexanucleotide **22** and a linear gradient of 0.1 → 0.8M TEAB for the nonanucleotide **20** (Fig. 2). Fractions of 5 ml were collected. Those fractions in the main peak, which were found to be pure, when analyzed by HPLC (program I), were collected, evaporated down to dryness and coevaporated with water (3 × 50 ml). The products were then lyophilized. Sterile water was used throughout the whole process of purification and isolation. The yields of the oligonucleotides, determined (spectrophotometrically) as the percentage of the total amount of nucleoside and nucleotide material eluted from the DEAE-Sephadex column, were 83% for the hexanucleotide and 72% for the nonanucleotide.

Part of the purified hexanucleotide Umthp–Cmthp–Cmthp–Umthp–Umthp–Amm (**22**) and nonanucleotide Amthp–Cmthp–Cmthp–Umthp–Cmthp–Cmthp–Umthp–Umthp–Amm **20** (100 OD^{260} units of each) was dissolved in 0.01N HCL (0.5 ml) and the pH adjusted to 2.0 by the addition of 0.1N HCl. After 2 h at 20°, the solution was neutralized (pH 8) with 0.5 M ammonia and, after a further 10 min, lyophilized. The fully unprotected oligonucleotides U–C–C–U–U–A **23** and A–C–C–U–C–C–U–U–A **21** were found, by TLC (system E) and HPLC (program I) to be pure; R_f 0.27 and 0.12, respectively (TLC, system E).

Enzymic hydrolysis of U–C–C–U–U–A and A–C–C–U–C–U–U–A. (a) Venom phosphodiesterase: a solution of the oligonucleotide (2 OD units) in a buffer (0.05 ml) containing 25 mM Tris-HCl (pH 9.0), 5 mM $MgCl_2$ and 7 μg snake-venom phosphodiesterase (Crotalus terr. terr. Boehringer) was incubated at 37° for 2 h.

(b) RNase: the oligonucleotide (2 OD units) was incubated with 20 μg Pancreatic Ribonuclease (Merck) in 0.2 M Tris-HCl buffer (pH 8.0, 0.05 ml) at 37° for 2 h.

(c) Taka-diastase T_2: the oligonucleotide (2 OD units) was incubated with Taka-diastase T_2 (Calbiochem, 2 μl of a solution containing 200 U/ml 0.1M NaCl) in 0.1M NH_4OAc buffer (pH 4.5, 0.05 ml) at 37° for 2 h.

The enzymic digests were analyzed qualitatively by TLC (system C and D) and quantitatively by HPLC (gradient program II). It was found that complete digestion of the oligonucleotides to the expected products had occurred in all experiments. Analytical data are collected in Table 2.

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