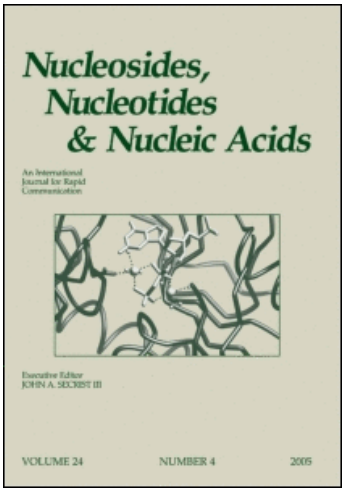

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Studies in the Solid Phase Synthesis of Oligo- and Poly-Ribonucleotides

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STUDIES IN THE SOLID PHASE SYNTHESIS OF OLIGO- AND POLY-RIBONUCLEOTIDES

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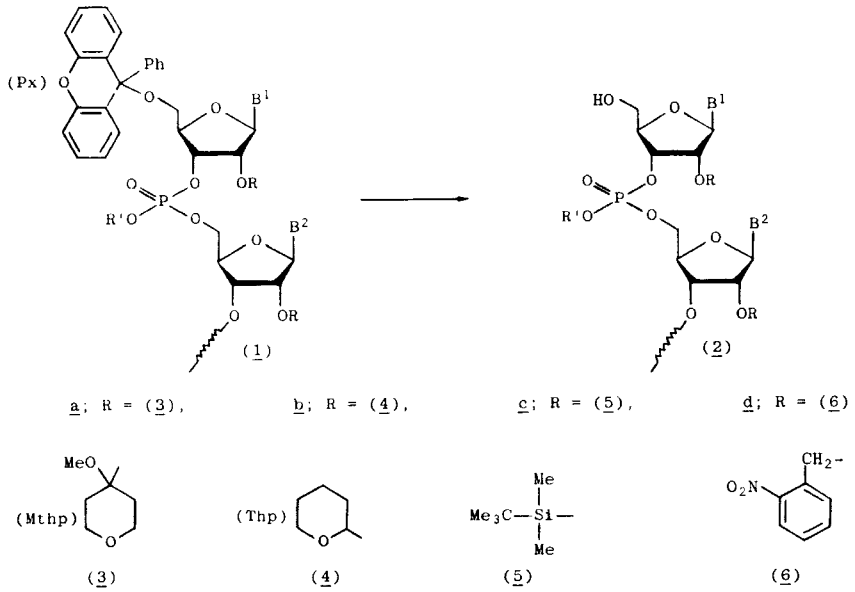
Abstract. The solid phase synthesis of the 3'-terminal 10-mer, 19-mer and 37-mer sequences of unmodified yeast $tRNA^{Ala}$ is described. The 2'- and 5'-hydroxy functions are protected, respectively, by the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl [Fpmp (11)] and 9-phenylxanthen-9-yl [Px] groups, and 2-cyanoethyl di-isopropylphosphoramidite building blocks are used.

The ready availability of chemically-synthesized deoxyribonucleic acid (DNA) sequences has, in the past decade or so, had a remarkable enabling effect on biological research. Due mainly to the discovery of ribozymes[1] and to some extent to other recent developments in biology, the availability of chemically-synthesized ribonucleic acid (RNA) sequences has also become a matter of considerable importance.

Much of the earlier successful work on the synthesis of oligo- and poly-ribonucleotides was carried out by the phosphotriester approach in solution[2,3]. This is an effective method and is especially useful when relatively large quantities of defined RNA sequences are required, say, for biophysical studies. On the other hand, if small quantities of RNA are needed for biological studies, synthesis in solution is both inconvenient and time-consuming. For this reason it has been important to develop a rapid synthesis of RNA, corresponding to DNA synthesis on a solid support. However, due to the added and crucial requirement of the protection of the 2'-hydroxy functions[4] in the RNA series, this has been far from a straightforward matter. As has been explained elsewhere[3], no really satisfactory alternative to an acid-labile modified trityl protecting group [such as 9-phenylxanthen-9-yl (Px)[5], as in (1), or

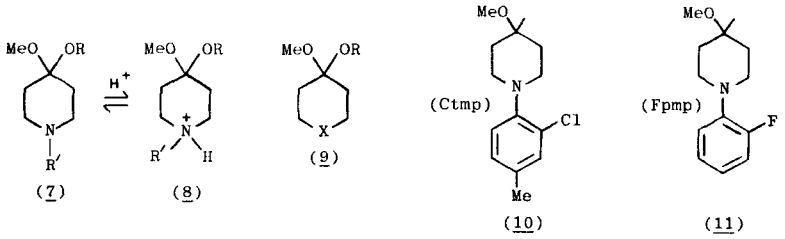


Scheme 1

4,4'-dimethoxytrityl (DMTr)[6] has so far been found to be really suitable for the protection of the 5'-terminal hydroxy functions in solid phase DNA or RNA synthesis. This introduced a possible difficulty as much of the previous work on the synthesis of oligoribonucleotides, albeit by the phosphotriester approach in solution[4], has involved the use of acid-labile acetal protecting groups, such as the Mthp (3)[7] [as in (1a)] and Thp (4)[8] [as in (1b)] groups for the protection of the 2'-hydroxy functions. Unfortunately, it does not appear to be possible to remove a 5'-terminal Px (or DMTr) group from a protected oligoribonucleotide sequence such as (1a) or (1b) without appreciable concomitant loss[9,10] of the acetal protecting groups from the 2'-hydroxy functions and consequent internucleotide cleavage[9,11].

It therefore soon became clear to us that, despite its otherwise very favourable properties[2,7] [achirality, insensitivity to light, lack of tendency to migrate, stability to base, easy and clean removability under conditions under which RNA is completely stable], we should have to find an alternative to the Mthp group (3) for the protection of the 2'-hydroxy functions in solid phase RNA synthesis. The main requirement

for this new protecting group would be that, in addition to retaining all the favourable properties of the Mthp group, it should also be stable to the acidic conditions required for the removal of the 5'-terminal Px (or DMTr) group. The t-butyldimethylsilyl (5)[12] [as in (1c)] and 2-nitrobenzyl (6)[13] [as in (1d)] groups have been used to protect the 2'-hydroxy functions in solid phase RNA synthesis. While both of these groups appear to be stable under the acidic conditions required for 5'-unblocking, each of them lacks at least one of the above favourable properties of the Mthp group. It therefore seemed that it would be necessary to design a new protecting group especially for this purpose.



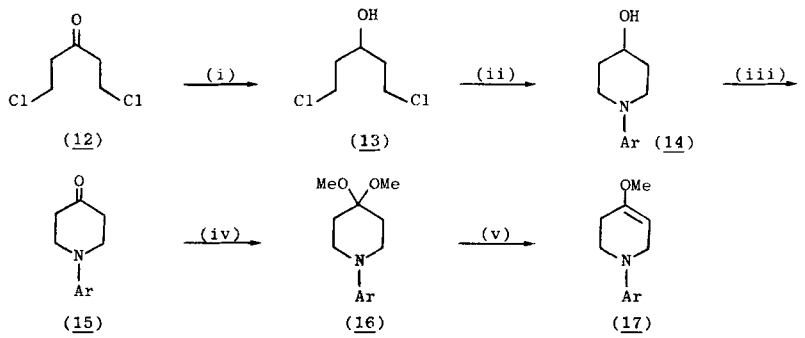
(7) (8) (9) (10) (11)

Acetal hydrolysis is subject to specific acid catalysis, and its rate is dependent on substrate concentration and on the concentration of hydrogen ions[14]. It occurred to us that it should be possible to design a piperidin-4-one acetal system (7), the rate of hydrolysis of which would be virtually pH independent in the pH range 0.5 - 2.5, but which would be similar to that of the corresponding tetrahydropyran-4-one acetal system [(9; X = O), i.e. Mthp protecting group] at pH 2. Earlier studies[15] on the comparative rates of hydrolysis of a tetrahydrothiopyran-4-one acetal (9; X = S) and the corresponding sulphone (9; X = SO_2) suggested to us that, due to the very large inductive effect of the positively charged nitrogen, the rate of hydrolysis of the conjugate acid (8) would be negligible compared with that of (7). If the piperidinyl protecting group is to undergo hydrolysis at pH 2 at a rate similar to that of the Mthp group[7,16], then it must be substantially unprotonated [as in (7)] at that pH. Thus it seemed reasonable to aim for a piperidinyl protecting group in which the tertiary amino function had a pK_a of *ca.* 2. Then if it could be assumed, as a first approximation, that the rate of hydrolysis of the conjugate acid form (8) in the pH range 0.5 - 2.5 was so slow that it could be neglected,

the observed rate of hydrolysis of the piperidone acetal system would be expected to be pH independent in that pH range.

As we were seeking a piperidinyl system with a weakly basic (i.e. $pK_a \sim 2$) tertiary amino function, we investigated the hydrolysis properties of 1-aryl-4-methoxypiperidin-4-yl [as in (7; R' = aryl)] protecting groups. After rejecting two unsatisfactory groups, we found that the 1-(2-chloro-4-methylphenyl)-4-methoxypiperidin-4-yl [Ctmp (10)][17,18] group had almost ideal hydrolysis properties (see below). We were fortunate to be so successful in this first study as we were rather severely limited in our choice of aryl substituents by the methods then available[19] for the synthesis of 1-arylpiperidin-4-ones (15). Furthermore, it soon became clear that the hydrolysis properties of 1-aryl-4-methoxypiperidin-4-yl protecting groups were critically dependent on aryl substitution. We then developed[20] a convenient general synthesis (Scheme 2) of 1-arylpiperidin-4-ols (14). The latter compounds were readily converted[21], by Moffatt oxidation[22], into the corresponding ketones (15) and thence, by a two-step process[20], into the required enol ether reagents (17). This enabled us to develop the 1-(2-fluorophenyl)-4-methoxypiperidin-4-yl [Fpmp, (11)] group[20] which we now believe to be the protecting group of choice for solid phase RNA synthesis, and which is used in the present study described below.

With regard to the preparation of the enol ether reagent (17; Ar = $2\text{-}FC_6H_4$), required for the introduction of the Fpmp protecting group, the 1-arylpiperidin-4-ol derivative (14; Ar = $2\text{-}FC_6H_4$) was prepared[20] from 1,5-dichloropentan-3-ol[23] (13) and 2-fluoroaniline [Scheme 2, step (ii)] in 76% yield on a greater than 100g scale. The remaining three steps [(iii), (iv) and (v)], which have not yet been optimized, were effected[20] in yields of 67.5, 75 and 72%, respectively. Ribonucleoside building blocks, represented by (20), in which the 2'- and 5'-hydroxy functions were protected by Fpmp (11) and Px [as in (1)] groups, respectively, were required for solid phase RNA synthesis, and were prepared in four steps [Scheme 3] from appropriate N-acyl derivatives of adenosine (18; B = 21), cytidine (18; B = 22), and guanosine (18; B = 23), and from uridine (18; B = 24). The N-acyl protecting groups selected[24] were stable under the conditions used in the preparation of the building blocks [Scheme 3], but were readily removed in the course of the ammonolysis step which also led [see Scheme 6 below] to the

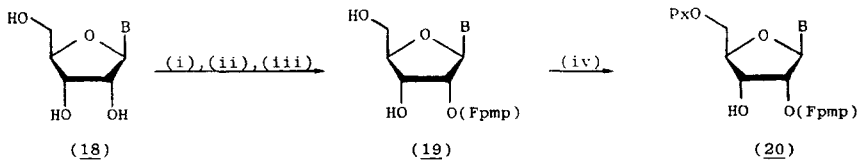


Reagents: (i) $NaBH_4$, EtOH, H_2O ; (ii) $ArNH_2$, K_2CO_3, NaI, Me_2NCHO, 100°C; (iii) $C_6H_{11}N{=}C{=}NC_6H_{11}$, CF_3CO_2H, C_5H_5N, Me_2SO, C_6H_6; (iv) $HC(OMe)_3$, $4\text{-}MeC_6H_4SO_3H$, MeOH, reflux; (v) $4\text{-}MeC_6H_4SO_3H$, 150°C.

Scheme 2

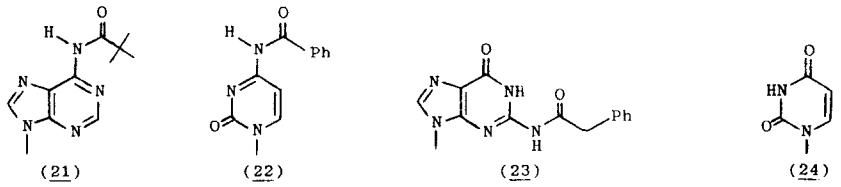
release of the synthetic 2'-protected RNA sequence from the solid support. The average overall yield for the four steps [Scheme 3, steps (i)-(iv)] was found to be almost 50% and, on average, only *ca.* 2 molecular equivalents of the enol ether reagent (17; Ar = $2\text{-}FC_6H_4$) were required in the crucial step [i.e. step (ii)] leading to the introduction of the Fpmp group (11).

It can be seen from Table 1 that the hydrolysis properties of the 2'-O-(Fpmp) and 2'-O-(Ctmp)[17] derivatives of uridine [(25b) and (25a), respectively] are very similar indeed. However, the Fpmp (11) is on average *ca.* 1.3 times more stable to acidic hydrolysis than the Ctmp protecting group (10) in the pH range 0.5 - 2.5. This is advantageous in that the use of the Fpmp would be expected to lead to even greater selectivity in the 5'-unblocking step than was previously obtained with the Ctmp group. From studies carried out with fully-protected dinucleoside phosphates[17,18], it can be estimated that the selectivity of the removal of a Px group [as in (1)] is *ca.* 300 times greater when the 2'-hydroxy function vicinal to the protected internucleotide linkage is blocked with a Ctmp (10) than it is when the 2'-hydroxy function is blocked with an Mthp group (3). It is noteworthy that the half-times of hydrolysis of the Fpmp and Ctmp derivatives of uridylyl-(3'→5')-uridine [(26b) and (26a), respectively] at pH 2 are more closely similar than those of (25b) and (25a). This suggests that the *conditions required*

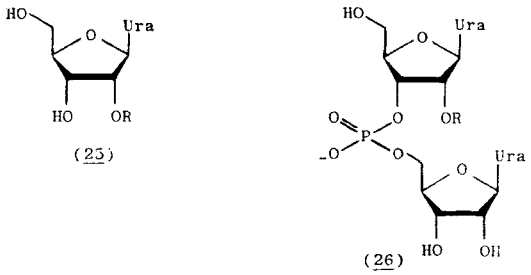


(18) (19) (20)

Reagents: (i) $Pr^i_2Si(Cl)OSi(Cl)Pr^i_2$, imidazole, MeCN; (ii) (17; Ar=2-FC_6H_4), CF_3CO_2H, CH_2Cl_2; (iii) Et_4NF, MeCN; (iv) 9-chloro-9-phenylxanthene (PxCl), C_5H_5N.



(21) (22) (23) (24)



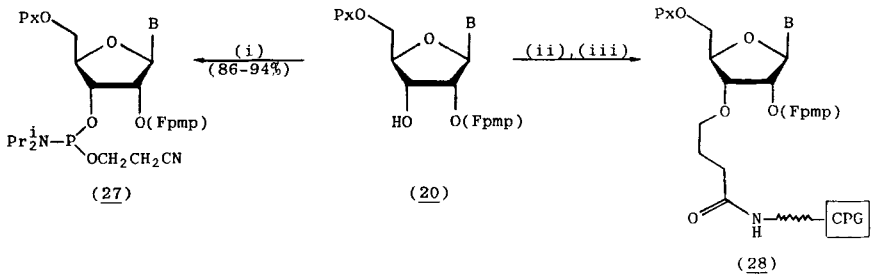
(25) (26)

Ura = uracil-1-yl; a, R = Ctmp (10); b, R = Fpmp (11)

Scheme 3

TABLE 1. Half-times of Hydrolysis of 2'-O-(Ctmp) and 2'-O-(Fpmp) Derivatives of Uridine [(25a) and (25b)] and Uridylyl-(3'→5')-uridine [(26a) and (26b)] at 25°C.

Substrate	pH	$t_{\frac{1}{2}}$(min)[17]	Substrate	pH	$t_{\frac{1}{2}}$(min)
(25a)	0.5	33.5	(25b)	0.5	41
(25a)	1.0	35.5	(25b)	1.0	44
(25a)	1.5	35	(25b)	1.5	50
(25a)	2.0	41	(25b)	2.0	52
(25a)	2.5	52	(25b)	2.5	70
(25a)	3.0	80	(25b)	3.0	166
(25a)	3.5	168	(25b)	3.5	334
(26a)	2.0	21.5	(26b)	2.0	23
(26a)	2.5	23.5	(26b)	2.5	31.5

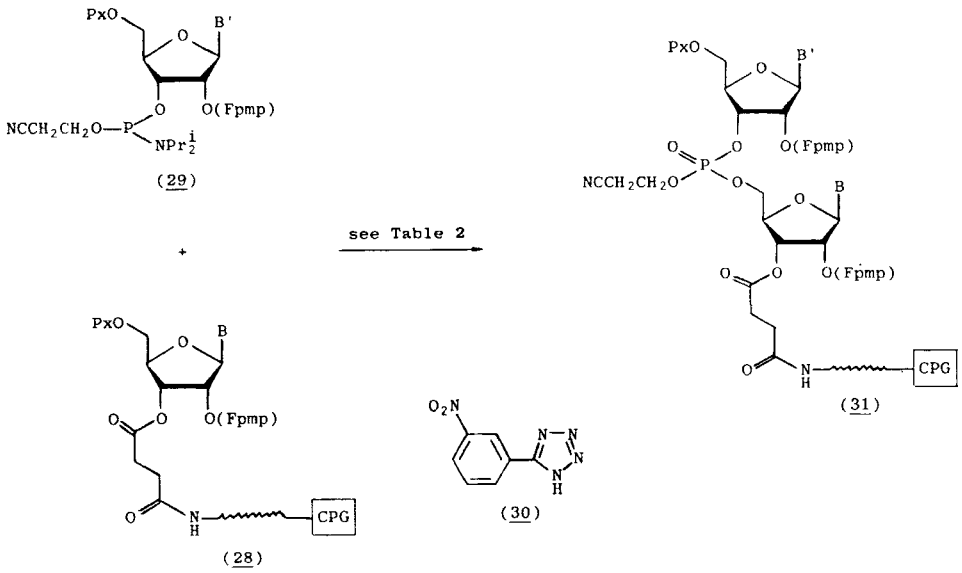


Reagents: (i) $NCCH_2CH_2O-P(Cl)NPr^i_2$, $EtNPr^i_2$, THF, RT; (ii) succinic anhydride, C_5H_5N; (iii) DCC, DMAP, functionalised controlled pore glass (CPG), DMF.

Scheme 4

for the removal of the Fpmp protecting groups from 2'-protected RNA sequences (see below) will, for all practical purposes, be the same as those required for the removal of Ctmp protecting groups. Finally, it can be seen from Table 1 that the ratio of the half-times of hydrolysis at pH 2.0 and pH 0.5 is *ca.* 1.27 for the Fpmp (11) and *ca.* 1.22 for the Ctmp (10) group. The ratio for the half-times of hydrolysis at pH 2.5 and pH 0.5 are slightly greater (1.7 and 1.55, respectively) but it is clear that, as intended (see above), the rate of removal of both of these protecting groups is almost pH independent in this pH range. In contrast, the Mthp group (3) would be expected[17] to undergo hydrolysis at a rate some two orders of magnitude faster at pH 0.5 than at pH 2.5.

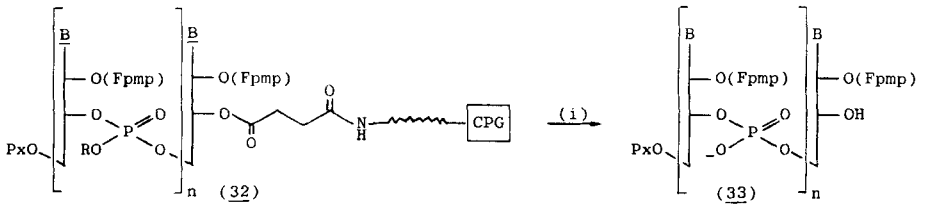
The above [see Scheme 3] 2'-O-(Fpmp)-5'-O-(Px) ribonucleoside building blocks (20) were used in solid phase synthesis in two ways. First, they were converted into the corresponding 2-cyanoethyl N,N-di-isopropylphosphoramidites[25] (27) in good (86-94%) yields by treatment with 2-cyanoethyl di-isopropylphosphoramidochloridite[25] and, secondly, they were loaded onto 500 Å functionalized controlled pore glass (CPG) by the two step procedure indicated [Scheme 4, steps (ii) and (iii)]. The loading achieved was generally *ca.* 30-35 μmol per gram. Solid phase synthesis [Scheme 5] was usually carried out on a 1.0 - 1.5 μmolar scale according to the protocol indicated in Table 2. The concentrations of phosphoramidite (29) and 5-(3-nitrophenyl)-1*H*-tetrazole[26] were both 0.1M. Iodine[27] and t-butyl hydroperoxide[28] were both found to be effective oxidizing agents [Table 2, step no. 5]. Each synthetic cycle



Scheme 5

TABLE 2. Protocol for Solid Phase RNA Synthesis

Step No.	Process	Reagent and/or Solvent	Time(min)
1	Detritylation	CF_3CO_2H-CH_2Cl_2 (1:99 v/v)	0.67
2	Washing	MeCN	3.5
3	Coupling	Phosphoramidite (29) [13-20 mol. equiv.], (30) [13-20 mol.equiv.], MeCN	2.5 - 5.0
4	Capping	Ac_2O, DMAP, 2,6-lutidine, THF	2.0
5	Oxidation	I_2, 2,6-lutidine, THF, water **or** $tBuO_2H$, $Me_2CHCH_2CMe_3$, CH_2Cl_2	2.25
6	Washing	MeCN	7.0



R = CH_2CH_2CN; N-acylated and unprotected base residues are represented by B and B, respectively.

Reagent: (i) concentrated aqueous ammonia (d 0.88), RT, 24 hr.

Scheme 6

took *ca.* 20 min. Trityl (i.e. 9-phenylxanthen-9-yl) cation assays suggested that the coupling efficiency was *ca.* 97% but the estimated efficiencies of the first two or three coupling steps were often lower.

After the completion of all of the coupling cycles, the products containing the desired sequence attached to the solid support (32) were treated (Scheme 6) with concentrated aqueous ammonia at room temperature for 24 hr. Under these conditions, the N-acyl protecting groups [as in (21), (22) and (23)] were removed from the base residues, the 2-cyanoethyl protecting groups were removed from the internucleotide linkages and the 2'-protected RNA sequence (33) was released from the solid support. Unlike t-butyldimethylsilyl[29] (5), Fpmp protecting groups are completely stable under the ammonolysis conditions, and the 2'-protected RNA (33) is stable to endonuclease attack. This is advantageous as it makes it possible to purify the latter material by a number of techniques (including gel filtration, polyacrylamide gel electrophoresis and liquid chromatography) with no risk of degradation. The 2'-O-(Fpmp) groups and the terminal 5'-O-Px group were then removed (see below) by treatment with 0.01 M hydrochloric acid (pH 2) at room temperature to give the pure unprotected RNA sequence.

In the past decade, the efficacy of the methods that we have developed for the synthesis of oligo- and poly-ribonucleotides have generally been tested by undertaking the synthesis of specific sequences of yeast $tRNA^{Ala}$ (Figure 1a). Thus we recently reported[30,31] the preparation of the 3'-terminal heptatriacontamer (37-mer) sequence of

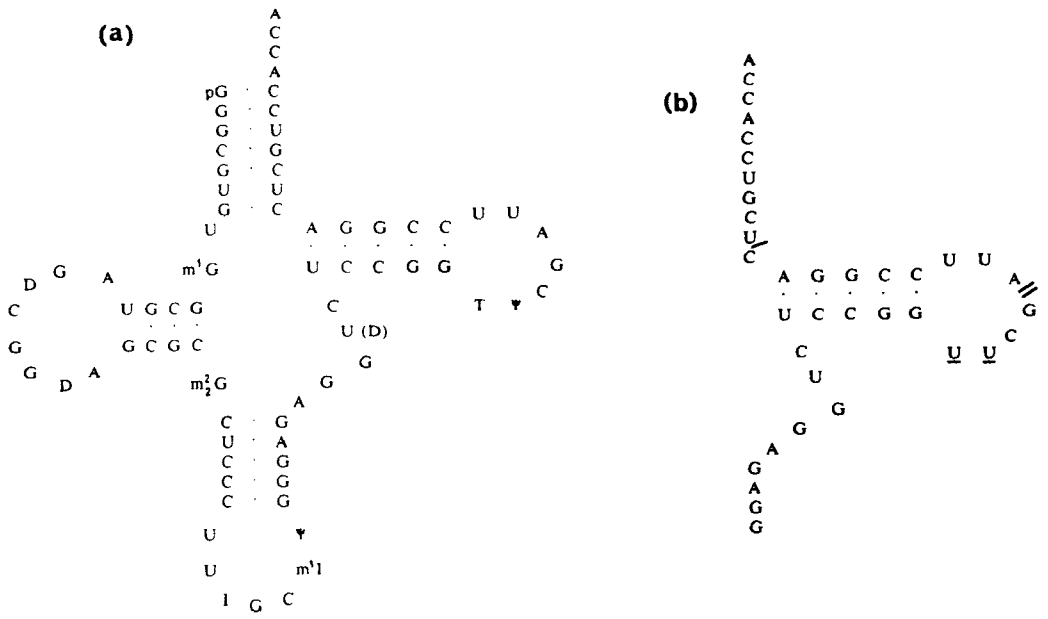


FIGURE 1. Nucleotide sequences of (a) yeast $tRNA^{Ala}$ and (b) the 3'-terminal unmodified heptatriacontamer sequence of yeast $tRNA^{Ala}$.

the tRNA molecule by the phosphotriester approach in solution; we have also prepared[32] the 3'-terminal decamer and nonadecamer sequences of yeast $tRNA^{Ala}$ by manual solid phase synthesis using the Px and Ctmp groups, respectively, for the protection of the 5'- and 2'-hydroxy functions. In the present study, we have undertaken the automated solid phase synthesis of the unmodified 3'-terminal heptatriacontamer (37-mer) sequence of yeast $tRNA^{Ala}$ (Figure 1b) using the Fpmp (11) group for the protection of the 2'-hydroxy functions as described above.

In Figure 1b, the unmodified heptatriacontamer sequence is divided by two lines (//) into the 3'-terminal nonadecamer (r[AUUCCGGACUCGUCCAC-CA]) and an octadecamer, and the nonadecamer is further divided by one line (/) into the 3'-terminal decamer (r[UCGUCCACCA]) and a nonamer. The underlined base residues in the heptatriacontamer sequence (Figure 1b) replace the modified base residues in the native tRNA molecule (Figure 1a). The preparation of the 3'-terminal decamer, nonadecamer and heptatriacontamer sequences has been undertaken. After the assembly of the three sequences, followed by ammonolysis [Scheme 6, step (i)],

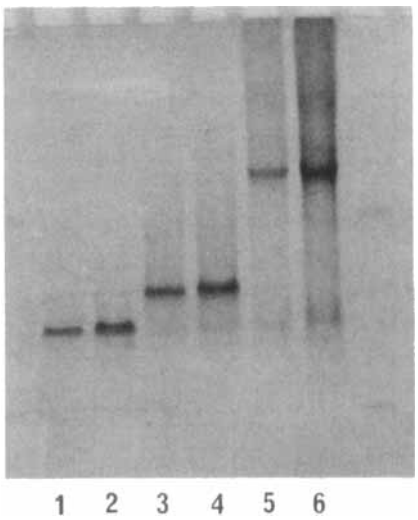


FIGURE 2. Lanes 1 and 2, crude Px-U'C'G'U'C'C'A'C'C'A'; Lanes 3 and 4, crude Px-A'U'U'C'C'G'G'A'C'-U'C'G'U'C'C'A'C'C'A'; lanes 5 and 6, crude Px-G'G'A'G'A'G'G'U'C'U'C'C'G'G'U'U'C'G'-A'U'U'C'C'G'G'A'C'U'C'G'U'C'C'A'C'C'A'.

the crude products were subjected to 20% polyacrylamide gel electrophoresis. A photograph of the resulting electrophoretogram, which was illuminated by ultraviolet light, is illustrated in Figure 2. Following a system of abbreviations proposed earlier[33], A', C', G' and U' represent 2'-protected [with the Fpmp (11) protecting group] adenosine, cytidine, guanosine and uridine residues, respectively. The decamer preparation was carried out on a 1.38 μmolar scale, and 60 [36 after fractionation on Sephadex G75] A_{260} units of crude material (Figure 2, lanes 1 and 2) were obtained. The nonadecamer preparation was carried out on a 1.48 μmolar scale, and 96 [40 after fractionation on Sephadex G75] A_{260} units of crude material (Figure 2, lanes 3 and 4) were obtained. Finally, the heptatriacontamer preparation was carried out on a 1.57 μmolar scale, and 213 [59 after fractionation on Sephadex G75] A_{260} units of crude material (Figure 2, lanes 5 and 6) were obtained. Lanes 1 and 2 differ only in that more material was applied to the gel in lane 2 than in lane 1. Similarly, more material was applied in lanes 4 and 6 than in lanes 3 and 5.

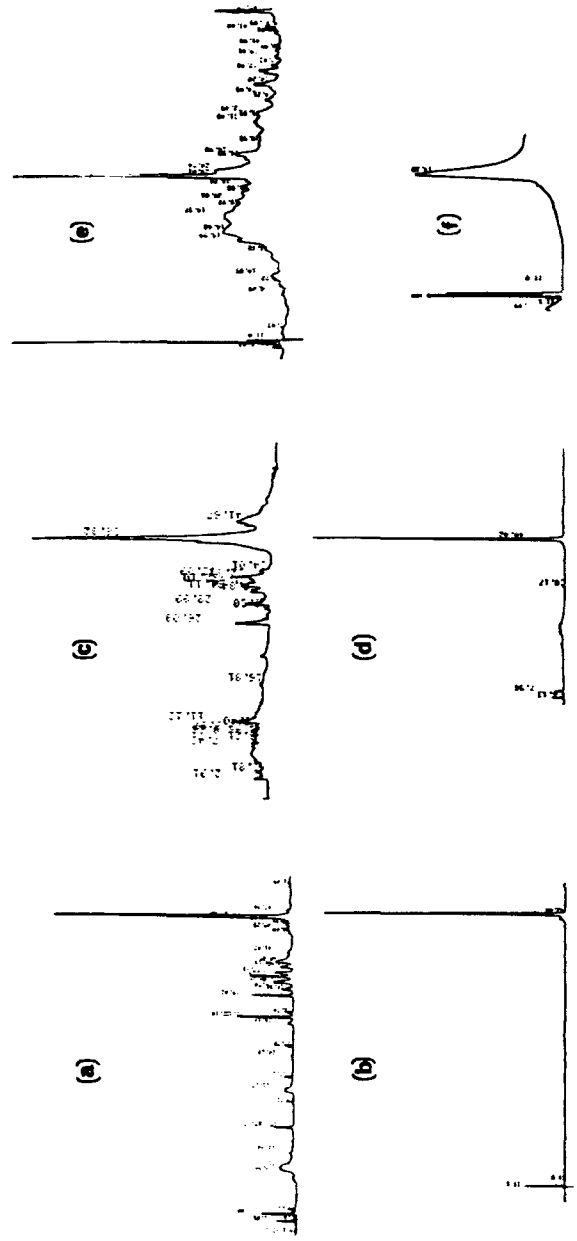


FIGURE 3. L.c. profiles (on Jones APEX ODS 5μ column, eluted with 0.1M-triethylammonium acetate–acetonitrile) of crude and purified Px-U'C'G'U'C'C'A'C'C'A' [(a) and (b), respectively], crude and purified Px-A'U'U'C'C'G'G'A'C'U'C'G'U'C'C'A'C'C'A' [(c) and (d), respectively], crude and purified Px-G'G'A'G'A'G'G'U'C'U'C'C'G'G'U'U'C'G'-A'U'U'C'C'G'G'A'C'U'C'G'U'C'C'A'C'C'A' [(e) and (f), respectively].

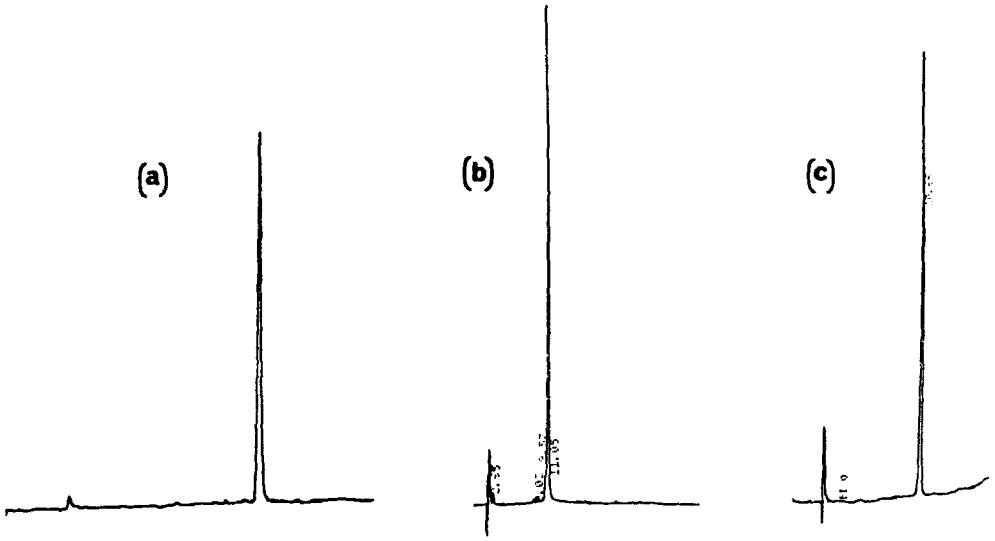


FIGURE 4. *L.c. profiles (on Jones APEX ODS 5μ column, eluted with 0.1M-triethylammonium acetate-acetonitrile) of (a) purified r[UCGUCCACCA], (b) purified r[AUUCCGGACUCGUCCACCA], and (c) purified r[GGAGAGGUCUCCGGUUCGAUUCCGGACUCGUCCACCA].*

The crude partially-protected RNA sequences were then purified by liquid chromatography (l.c.) on a reverse phase column which was eluted with appropriate mixtures of 0.1M-triethylammonium acetate and acetonitrile. The elution profiles of the **crude** decamer, nonadecamer and heptatriacontamer sequences are illustrated in Figures 3(a), 3(c) and 3(e), respectively; the elution profiles of the **purified** decamer, nonadecamer and heptatriacontamer sequences are illustrated in Figures 3(b), 3(d) and 3(f), respectively. As indicated above, such 2'-protected RNA sequences can be stored indefinitely without any danger of digestion by contaminating endonucleases and then readily converted by acidic hydrolysis under mild conditions [0.01M-hydrochloric acid (pH 2), RT, 20 hr] to fully-unblocked RNA without the cleavage or migration of the internucleotide linkages occurring to a detectable extent.

The fully-unblocked RNA sequences (r[UCGUCCACCA], r[AUUCCGGACUCGUCCACCA] and r[GGAGAGGUCUCCGGUUCGAUUCCGGACUCGUCCACCA]) were obtained by removing the acid-labile protecting groups from the corresponding purified partially-protected oligo- and poly-ribonucleotides (Px-U'C'G'-

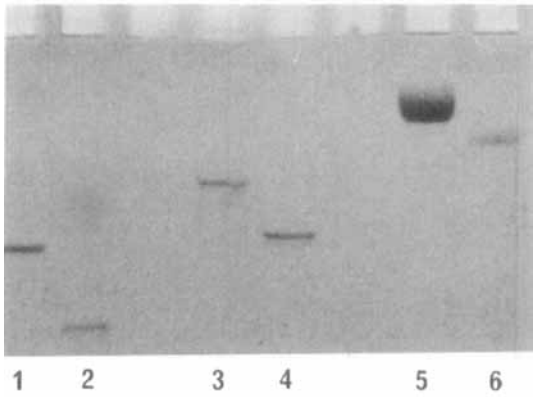


FIGURE 5. Lane 1, purified Px-U'C'G'U'C'C'-A'C'C'A'; lane 2, purified r[UCG-UCCACCA]; lane 3, purified Px-A'-U'U'C'C'G'G'A'C'U'C'G'U'C'C'A'C'-C'A'; lane 4, purified r[AUUCCGG-ACUCGUCCACCA]; lane 5, purified Px-G'G'A'G'A'G'G'U'C'U'C'C'G'G'-U'U'C'G'A'U'U'C'C'G'G'A'C'U'C'G'-U'C'C'A'C'C'A'; lane 6, purified r[GGAGAGGUCUCCGGUUCGAUUCCGGACUCG-UCCACCA].

U'C'C'A'C'C'A', Px-A'U'U'C'C'G'G'A'C'U'C'G'U'C'C'A'C'C'A', and Px-G'G'A'G'A'G'G'U'C'U'C'C'G'G'U'U'C'G'A'U'U'C'C'G'G'A'C'U'C'G'U'C'C'A'C'-C'A'), illustrated in Figures 3(b), 3(d) and 3(f), respectively. The l.c. elution profiles of these fully-unblocked 3'-terminal decamer, nonadecamer and heptatriacontamer sequences of yeast $tRNA^{Ala}$ are illustrated in Figures 4(a), 4(b) and 4(c), respectively. When the latter three RNA sequences were digested with appropriate phosphodiesterases and alkaline phosphatase, the four ribonucleoside constituents were obtained in the expected relative proportions within the limits of experimental error. Finally, in Figure 5, a photograph of an ultraviolet light illuminated polyacrylamide gel electrophoretogram of, in each case l.c. purified, partially-protected 3'-terminal decamer (lane 1), fully-unblocked 3'-terminal decamer (lane 2), partially-protected 3'-terminal nonadecamer (lane 3), fully-unblocked 3'-terminal nonadecamer (lane 4), partially-protected 3'-terminal heptatriacontamer (lane 5) and fully-unblocked 3'-terminal heptatriacontamer (lane 6) is illustrated.

The electrophoretic mobilities of the fully-unblocked decamer and nonadecamer sequences (lanes 2 and 4) were identical to the mobilities of the authentic oligoribonucleotides, synthesized[31] by the phosphotriester approach in solution.

We believe that these preliminary studies on the use of the Fpmp group (11) for the protection of the 2'-hydroxy functions in oligo- and poly-ribonucleotide synthesis are very encouraging indeed. There is, as yet, no evidence that the molecular weight of synthetic RNA available by this approach is being limited by lack of protecting group selectivity (i.e. by concomitant Fpmp removal) during the acid-promoted 5'-unblocking step [Table 2, step no. 1]. The purity of the crude partially-protected RNA [Figure 2 and Figures 3(a), 3(c) and 3(e)] does appear to decrease with increasing molecular weight. This may possibly be due to the occurrence of irreversible base modification reactions and, for this reason, we are undertaking the synthesis of the 3'-terminal heptatriacontamer sequence and the whole $tRNA^{Ala}$ molecule using guanosine and uridine building blocks in which the base residues are, respectively, doubly- and singly-protected[30]. It is also possible that, in the long term, the phosphoramidite approach[34] may not prove to be the method of choice for the chemical synthesis of RNA sequences of high molecular weight using the present protecting group strategy.

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