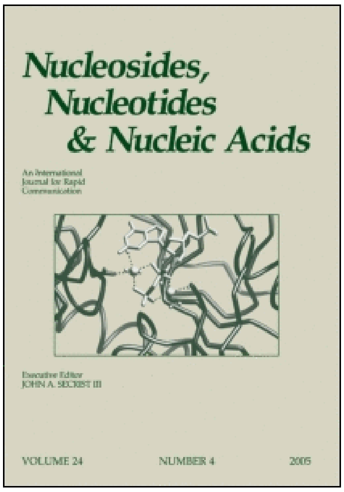

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RNA-SYNTHESIS USING THE H-PHOSPHONATE APPROACH AND AN IMPROVED PROTECTING GROUP STRATEGY

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Abstract: An improved version of the H-phosphonate approach to RNA-synthesis is presented and the studies that led to alterations in the protecting group strategy are discussed.

We have after the introduction of the H-phosphonate approach[1], and particularly since its use for RNA synthesis[2], systematically studied different aspects of the method with the aim to improve on it. A number of these studies have focused at optimising the protecting group strategy.

A key study was that on the stability of the *t*-butyldimethylsilyl (TBDMS) group when used as protection for the 2'-hydroxyl functions.[3] We found that this silyl protection is not completely stable under ammonolytic conditions that were used as standard at the time. At 55 °C up to about 70 % of the TBDMS groups are lost in conc. ammonia (Fig 1a) after 24h, and this is also accompanied by significant cleavage of internucleotidic linkages. By mixing 3 parts of the conc. ammonia with one part of ethanol a considerably improvement is noticed. The TBDMS group is about 8-10 times more stable under these conditions. When comparing ammonolysis at 55° and at room temperature (22-23°C) one can see that the loss of silyl protection becomes rather insignificant at the lower temperature, especially in conc. ammonia(aq)-ethanol (3:1) in which the TBDMS is again more stable than in ammonia alone (about 5-7 times).

In conc. ammonia(aq)-ethanol (3:1) at room temperature the half-life for 2'-O-*t*-butyldimethylsilyluridine 3'-(uridine 5'-phosphate) ($U^{Si}pU$) is

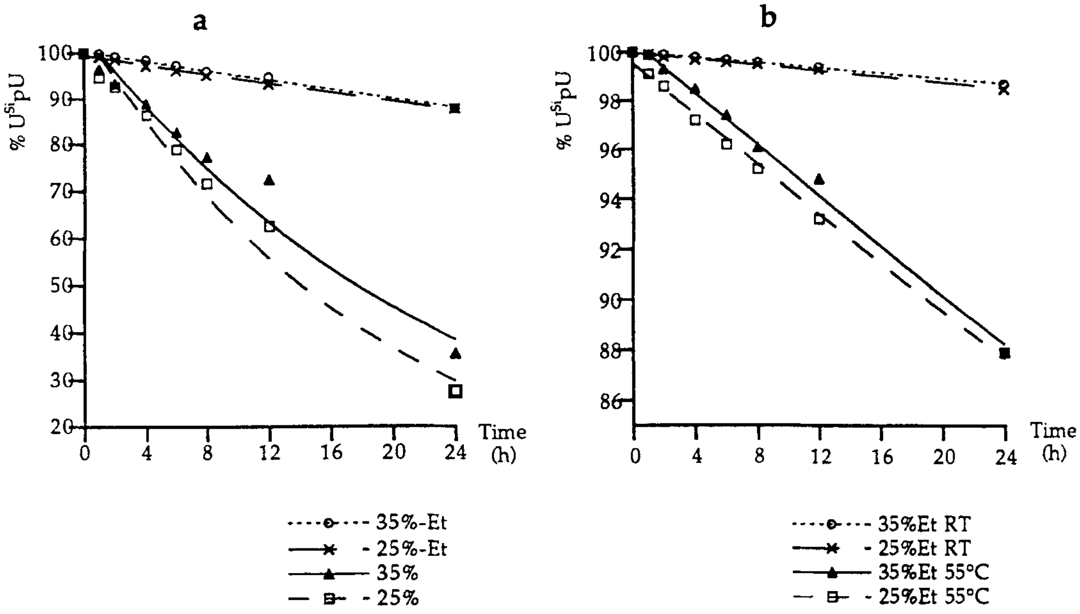

Figure 1. Disappearence of 2'-O-*t*-butyldimethylsilyluridine 3'-(uridine 5'-phosphate) (U^{Si}pU) with time during different ammonolysis conditions (Plot of data from ref. 3); 35%-Et = 35% ammonia (aq)-ethanol (3:1), 25%-Et = 25% ammonia (aq)-ethanol (3:1), 35% = 35% ammonia (aq), 25% = 25% ammonia (aq). (*a*) Ammonlysis at 55 °C in conc ammonia (aq) or conc ammonia (aq)-ethanol (3:1) (*b*) Ammonolysis in conc. ammonia-ethanol (3:1) at room temperature (RT) compared to at 55 °C.

about 7-8 weeks which means that only a fraction of a percent of TBDMS is lost for up to 12-16 h. Rate constants for disappearence of U^{Si}pU under different ammonolysis conditions are given in the Table.

In the above mentioned study[3] we concluded that more labile N-acyl groups would be necessary in order to obtain an efficient synthesis of longer RNA-fragments. This has since been introduced[4] and is now general practice, especially together with amidite chemistry. An additional advantage with using conc. ammonia(aq)-ethanol (3:1) is that the conditions shows a selectivity towards N-deprotection relative to loss of TBDMS-groups. The N-protection is removed only about 1.5 times slower in conc. ammonia (*aq*)-ethanol (3:1)[5] compared to in ammonia alone (*cf* TBDMS up to 7 times more stable).

Table. Kinetic parameters for disappearance of 2'-O-*t*-butyldimethyl-silyluridine 3'-(uridine 5'-phosphate) ($U^{Si}pU$) due to loss of TBDMS-protection under different ammonolytic conditions (obtained from first order plots of data from ref. 3)

	55 °C		RT (22-23°C)	
ammonolysis cond.	k (10^{-5} s^{-1})	$\tau_{1/2}$	k (10^{-7} s^{-1})	$\tau_{1/2}$
25% (aq)	1.5	13-17 h	12	1-2 weeks
35% (aq)	1.2		6.9	
25% (aq) - EtOH (3:1)	0.15	5-6 days	1.7	7-8 weeks
35% (aq) - EtOH (3:1)	0.15		1.5	

The base-protection we use is propionyl for cytidine (C^{Pr}, butyryl for adenosine (A^{Bu}) and phenoxyacetyl for guanosine (G^{PhOAc}).[5] We have chosen the base protection so that all can be removed in about the same time using conditions under which the 2'-silyl protection is virtually unaffected and all oligomer is released from the support. The groups are also chosen for their ease of introduction and because inexpensive commercially available reagents can be used. Half-lives in conc. ammonia (*aq*)-ethanol (3:1) are 5 min for C^{Pr}, 7 min for A^{Bu} and 7 min for G^{PhOAc},[5] which should ensure complete deprotection in 2-4 h but we regularly use 8-12 h to make certain all acyl groups are removed (and often overnight for convenience since the conditions are not detrimental during this time).

Optimisation of our approach is based upon the use of 5'-protection that is removed with acid. This is common for most methods of oligonucleotide synthesis but is particularly important with H-phosphonates since the diesters are much more stable in acid than in base[6]. The alternative of choosing other groups for 2'-protection has been evaluated but so far with results less successful than with TBDMS.[7] The use of acetal protection together with the H-phosphonate method gave considerable fragmentation of the oligomers.[7] An improvement can be expected if using the recently reported milder acetal deprotection[8], but the result is likely to still be worse than with TBDMS-protection since a significant part of the fragmentation seems to be due to cleavage of H-phosphonate linkages during the detritylation step.[7]

For functionalisation of the solid support we have adopted the use of nucleoside succinates that have *o*-chlorobenzoyl protection on the secondary hydroxyl that is not bound to the linker.[9] This ensures complete cleavage of the oligomer from the support during the time required for N-deprotection and has the additional advantage that the o-chlorobenzoyl protection and subsequent succinoylation is conveniently done in a one-pot procedure.[9]

The conditions for final removal of *t*-butyldimethylsilyl groups are also improved by using triethylamine trihydrofluoride (TEA, 3HF), a reagent recently introduced as a more efficient alternative to tetrabutylammonium fluoride (TBAF) for deprotection of RNA.[10] The reagent is also less moisture-sensitive[11] and thus more reliable than TBAF. Our approach to RNA-synthesis now has a set of protecting groups and deprotection conditions that are matched to a larger extent than before. The method in this latest version has already been applied to synthesis of a number of oligoribonucleotides and mixed ribo-deoxyribo oligonucleotides that have been used in structural studies.

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