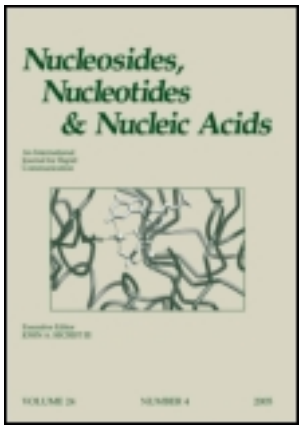

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Solution Stability and Degradation Pathway of Deoxyribonucleoside Phosphoramidites in Acetonitrile

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Solution Stability and Degradation Pathway of Deoxyribonucleoside Phosphoramidites in Acetonitrile

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ABSTRACT

The impuritiy profiles of acetonitrile solutions of the four standard *O*-cyanoethyl-*N,N*-diisopropyl-phosphoramidites of 5′-*O*-dimethoxytrityl (DMT) protected deoxyribonucleosides (dG^{ib}, dA^{bz}, dC^{bz}, T) were analyzed by HPLC-MS. The solution stability of the phosphoramidites decreases in the order T, dC > dA > dG. After five weeks storage under inert gas atmosphere the amidite purity was reduced by 2% (T, dC), 6% (dA), and 39% (dG), respectively. The main degradation pathways involve hydrolysis, elimination of acrylonitrile and autocatalytic acrylonitrile-induced formation of cyanoethyl phosphonoamidates. Consequently, the rate of degradation is reduced by reducing the water concentration in solution with molecular sieves and by lowering

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the amidite concentration. Acid-catalyzed hydrolysis could also be reduced by addition of small amounts of base.

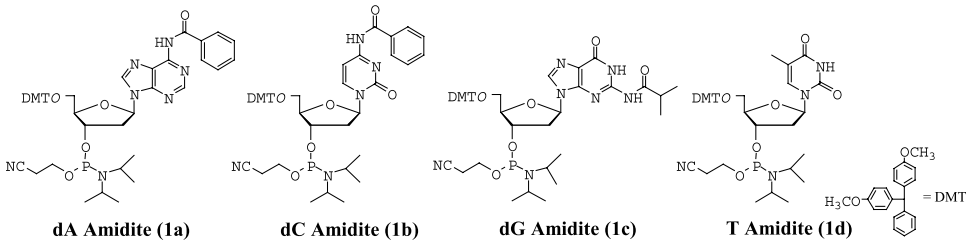
Key Words: Degradation pathway; Deoxyribonucleoside; Phosphoramidite; Stability.

INTRODUCTION

Oligodeoxyribonucleotides are synthesized through phosphoramidite chemistry on a polymeric support by sequential addition of nucleoside building blocks [*O*-Cyanoethyl-*N,N*-diisopropylphosphoramidites of protected nucleosides: dA^{bz} (**1a**), dC^{bz} (**1b**), dG^{ib} (**1c**), T (**1d**), bz = benzoyl, ib = isobutyryl] to a growing oligonucleotide chain which is covalently attached to the solid support (Scheme 1).[1–3] Prior to solid-phase synthesis the phosphoramidites are dissolved in anhydrous acetonitrile at concentrations of ca 0.2 M. Frequently, reagent solutions are prepared for multiple syntheses and used over a period of days. Due to the increasing demand for both large scale production and high throughput synthesis of oligonucleotides we investigated the stability of **1a–d** after dissolution in acetonitrile. In this report, we describe the formation of the major degradation products and provide a mechanistic rationale.

RESULTS AND DISCUSSION

O-Cyanoethyl-*N,N*-diisopropyl-phosphoramidites **1a–d** are starting materials in the synthesis of oligonucleotides and phosphorothioate oligodeoxyribonucleotides, a promising new class of therapeutic agents used to treat various diseases through antisense mechanisms of action.[4] The 5′-*O* protecting group is 4,4′-dimethoxytrityl (DMT), the protecting group for the exocyclic amino groups of adenine and cytosine are benzoyl and for guanine it is isobutyryl. Upon activation with a weak organic acid (1*H*-tetrazole or an alternative activating agent) phosphoramidites **1** couple to the 5′-hydroxyl function of a support-bound oligonucleotide.[5] To obtain high coupling efficiency throughout the synthesis, a molar excess of **1** is used. Typical synthesizer programs use the same volume of phosphoramidite solution for each coupling. Ideally, oligonucleotide synthesis is performed with a minimum molar excess of reagent at each coupling. Further reduction of the amidite excess through



Scheme 1. Chemical structure of deoxyribonucleoside phosphoramidites.

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Table 1. Analytical characterization of compounds **2a-d** and **4a-d**.

Compound	Formula	Mass (calcd.)	Mass (found)[a]	^{31}P NMR[b]
2a	$C_{41}H_{39}N_6O_8P$	774.3	797.4 $[M + Na]^+$	8.5, 8.6
2b	$C_{40}H_{39}N_4O_9P$	750.3	773.3 $[M + Na]^+$, 749.1 $[M - H]^-$	8.6, 8.7
2c	$C_{38}H_{41}N_6O_9P$	756.3	757.3 $[M + H]^+$, 779.3 $[M + Na]^+$	8.7, 8.9
2d	$C_{34}H_{36}N_3O_9P$	661.2	684.3 $[M + Na]^+$, 660.1 $[M - H]^-$	8.6, 8.7
4a	$C_{47}H_{52}N_7O_7P$	857.4	880.4 $[M + Na]^+$, 856.3 $[M - H]^-$	32.0, 32.3
4b	$C_{46}H_{52}N_5O_8P$	833.4	856.4 $[M + Na]^+$, 832.4 $[M - H]^-$	32.3–32.9
4c	$C_{44}H_{54}N_7O_8P$	839.4	840.5 $[M + H]^+$, 862.4 $[M + Na]^+$, 838.3 $[M - H]^-$	32.3–33.7
4d	$C_{40}H_{49}N_4O_8P$	744.3	767.4 $[M + Na]^+$, 743.3 $[M - H]^-$	32.2–32.6

[a]Mass spectra were obtained in positive and/or negative mode.
[b]solvent: perdeuteroacetonitrile.
2a–d, see Refs. [6-8].
3a–d were purchased from Glen Research, Sterling, Virginia.
Preparation of **4a–d**. A solution of protected nucleoside phosphoramidite (DMT-dA^{bz}, DMT-dC^{bz}, DMT-dG^{ib}, DMT-T, 10 g), acrylonitrile (5 ml) and acetonitrile (50 ml) was heated at 60°C for 24 to 36 h under exclusion of moisture. The solution was evaporated and the residue was purified by flash-chromatography on silica using a gradient (ethyl acetate/0.1% triethylamine to ethyl acetate/methanol (9:1, v/v)/0.1% triethylamine. Yield 50 to 90%.

degradation of the phosphoramidite would lead to lower coupling efficiency resulting in an overall reduction of yield. Also, consideration needs to be given to the structure of the degradation products with respect to reactivity and potential impact on oligonucleotide quality.

Homogenous solutions of **1a–d** (0.2 M) in anhydrous acetonitrile (water content < 30 ppm) were prepared. To exclude absorption of moisture from the air the solutions were stored under a blanket of argon in stainless steel containers. Samples were removed at different time points and analyzed by HPLC/MS. The identity of the degradation products was confirmed by comparison of retention times and mass spectral data (molecular weight, fragmentation) with authentic reference material (Table 1).[6–8] A summary of structure assignments and quantitation of the degradation products by HPLC-UV (relative area-% at wavelength 260 nm) of the individual phosphoramidites is shown in Table 2. A representative RP-HPLC profile is shown in Fig. 1. The relative amount of intact phosphoramidite in solution as function of time is shown in Fig. 2. The proposed degradation pathway is shown in Scheme 2.

Phosphoramidites used in this study had initial HPLC UV-purities between 98.8 and 99.6 area-% (at wavelength 260 nm). Degradation of dG amidite **1c** proceeds significantly faster than degradation of dA, dC or T amidite. The rate of degradation decreases in the order dG > dA > dC ≈ T (Fig. 2). After 10 days the initial purity of dG amidite has been reduced by 7.5%, the main degradation products being CE-*H*-phosphonate (**2**), *H*-phosphonate (**3**) and CE-phosphonoamidate (**4**), combined accounting for 98% of the degradation products (Table 2). For comparison, over the same period of time the purity of dA, dC and T amidite was reduced only by

Table 2. (a) Composition of dA phosphoramidite **1a** solution as function of time (area-% by HPLC), (b) Composition of dC phosphoramidite **1b** solution as function of time (area-% by HPLC), (c) Composition of dG phosphoramidite **1c** solution as function of time (area-% by HPLC), (d) Composition of T phosphoramidite **1d** solution as function of time (area-% by HPLC).

Compound	Initial	Day 4	Day 7	Day 10	Day 18	Day 35
a						
dA amidite (***1a***)	99.58	99.18	99.01	98.87	98.17	93.56
CE-*H*-phosphonate (**2a**)	0.03	0.18	0.27	0.35	0.58	2.39
H-phosphonate (**3a**)	n.d.	0.03	0.05	0.12	0.30	2.58
CE-phosphonoamidate (**4a**)	n.d.	0.06	0.09	0.11	0.17	0.86
Sum of other impurities	0.39	0.55	0.58	0.55	0.78	0.61
b						
dC amidite (***1b***)	98.91	98.71	98.68	98.50	98.38	96.90
CE-*H*-phosphonate (**2b**)	0.11	0.27	0.32	0.39	0.51	0.56
H-phosphonate (**3b**)	< 0.01	0.01	0.02	0.04	0.11	0.66
CE-phosphonoamidate (**4b**)	n.d.	n.d.	n.d.	n.d.	0.04	0.13
Sum of other impurities	0.97	1.01	0.98	1.07	0.96	1.75
c						
dG amidite (***1c***)	99.02	97.30	95.39	91.53	81.49	59.96
CE-*H*-phosphonate (**2c**)	0.19	0.93	1.57	2.54	2.38	1.53
H-phosphonate (**3c**)	< 0.01	0.75	1.68	3.63	5.50	8.08
CE-phosphonoamidate (**4c**)	0.07	0.21	0.42	1.16	5.27	19.96
Sum of other impurities	0.71	0.81	0.94	1.14	5.36	10.47
d						
T amidite (***1d***)	98.79	98.66	98.6	98.38	98.48	97.23
CE-*H*-phosphonate (**2d**)	0.15	0.27	0.31	0.36	0.51	1.17
H-phosphonate (**3d**)	< 0.01	0.02	0.02	0.03	0.09	0.51
CE-phosphonoamidate (**4d**)	0.05	0.04	0.04	0.04	0.04	0.04
Sum of other impurities	1.00	1.01	1.03	1.19	0.88	1.05

n. d.: none detected.

0.7%, 0.4% and 0.4%, respectively. A common degradation pathway shown in Scheme 2 is found for the four phosphoramidites.

Formation of the Major Degradation Products of dG Amidite

In the context of oligonucleotide synthesis, the solution stability of **1a**, **1b** and **1d** in anhydrous acetonitrile is acceptable. Degradation of less than 1% starting material over a period of ten days will have little to no effect on the outcome of oligonucleotide synthesis. Hence, we focused our investigation on dG phosphoramidite **1c**. With degradation products being essentially identical in all four phosphoramidites the conclusions drawn here may be applied to the other phosphoramidites. Based on impurity profile analysis of phosphoramidite solutions stored for up to 35 days the following degradation pathway is evident. (Scheme 2) Degradation is initiated by the acid-catalyzed hydrolysis reaction of phosphoramidite **1** with water (water serves as its own activator) leading to formation of CE-*H*-phosphonate **2** and diisopropylamine. Figure 3A shows the initial rapid formation of degradation product **2** (2.5% after

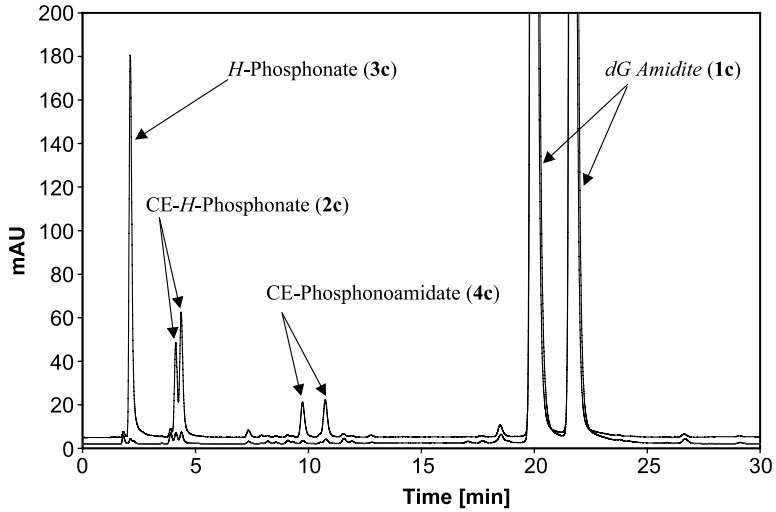

Figure 1. Representative HPLC profiles of amidite **1c** (Table 3, entry 1, lower trace) showing the formation of CE-*H*-phosphonate **2c**, *H*-phosphonate **3c** and CE-phosphonoamidate **4c**, respectively (Table 3, entry 2, upper trace) after 10 days of storage as solution in acetonitrile.

10 days). The water content of the individual phosphoramidites was as follows: dA (0.23%, w/w), dC (0.12%, w/w), dG (0.11%, w/w) and T (0.19%, w/w). Due to the ca 40- to 50-fold difference in molecular weight between phosphoramidites **1** and water, a water content of 0.1 to 0.2%, w/w, may cause 5 to 10% hydrolysis either upon storage or during oligonucleotide synthesis upon activation prior to coupling. Despite having the lowest water content **1c** degrades fastest, indicative of its intrinsic high reactivity at the phosphoramidite functionality.

After 10 days the water content is largely reduced and also the concentration of the liberated base (diisopropylamine) is high enough (ca 6 mol-%) to slow down the

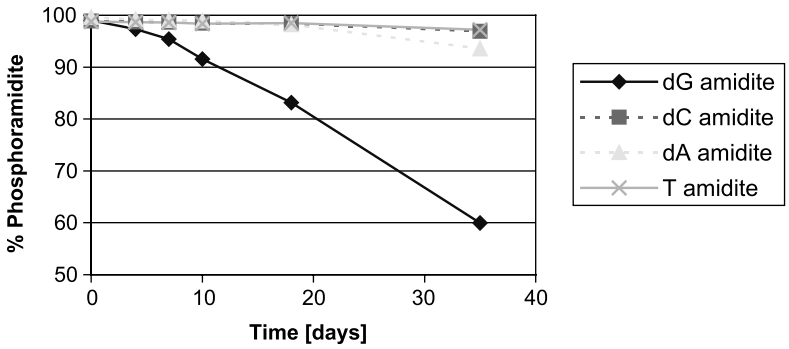

Figure 2. Stability of phosphoramidites **1a–d** in acetonitrile solution (0.2 M) at room temperature. (*View this art in color at www.dekker.com.*)

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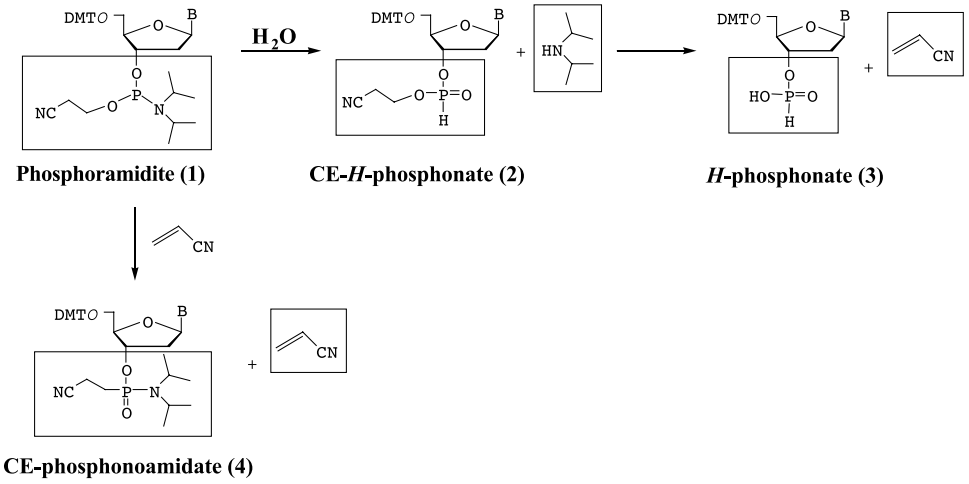

Scheme 2. Degradation pathway of deoxyribonucleoside phosphoramidites.

acid-catalyzed hydrolysis reaction (see below). The presence of base leads to elimination of acrylonitrile of CE-*H*-phosphonate **2**. Comparing Fig. 3A and 3B it becomes apparent that the maximum rate of formation of *H*-phosphonate **3** is near day 10, when the concentration of CE-*H*-phosphonate **2** is near the maximum. Concurrent with the formation of **3** is the formation of acrylonitrile. Reaction of acrylonitrile with phosphoramidite **1** leads to formation of phosphonoamidate **4** (Fig. 3C). In this reaction, acrylonitrile is both a reagent and a reaction product. Therefore, once started, this reaction continues until the phosphoramidite is consumed making **4c** the major degradation product. The amount and concentration of acrylonitrile present is determined by the extent of the initial hydrolysis reaction. One may therefore expect to decrease the rate of phosphoramidite degradation by slowing down the rate of hydrolysis. This can be accomplished by reducing the concentration of the reactants (phosphoramidite, water) or by the addition of an inhibitor (a base) in order to reduce the acidity of the medium. (Table 3) A reduction of the phosphoramidite concentration (and water concentration introduced by the amidite) slows degradation significantly (entries 2, 3, 4). After 10 days at a phosphoramidite concentration of 0.05 M we observe ca 3% degradation as compared to 8% at a concentration of 0.2 M. Additional drying (aceotropically, vacuum) of the phosphoramidite had no effect on the solution stability (entries 5 and 6). However, the addition of molecular sieves (3Å) to phosphoramidite solutions provided significant benefit in terms of solution stability (entry 7). The addition of triethylamine leads to a kinetic stabilization of the amidite solution. As little as 5 mol-% with respect to the phosphoramidite reduces degradation by more than 60% after a period of 10 days, providing similar protection in terms of solution degradation as the addition of molecular sieves. To provide further evidence for the proposed mechanism additional water was added to the phosphoramidite solution (entries 11, 12, 13, 14). Interestingly, the extent of hydrolysis after 10 days is only slightly increased in the sample to which 38 mol-% (with respect to **1c**) water was added. This supports the earlier notion that diisopropylamine formed during the

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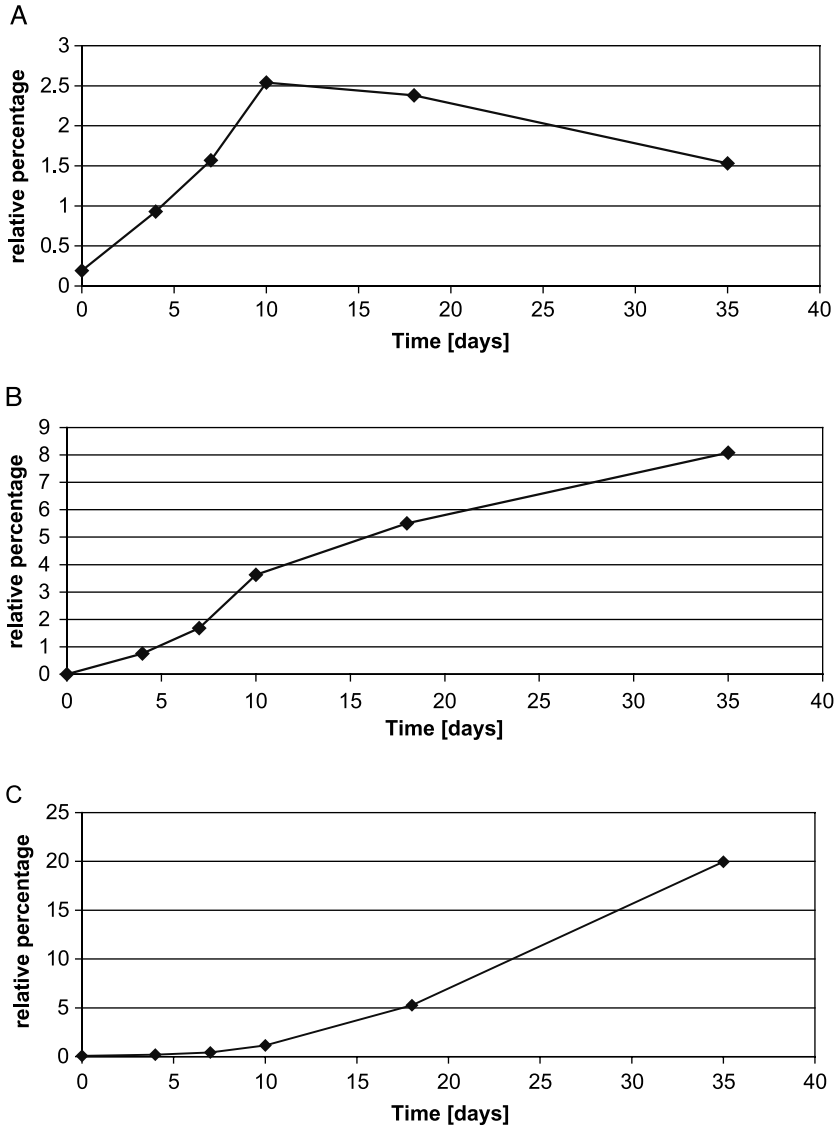

Figure 3. (A) Formation of CE-*H*-phosphonate **2c** of dG amidite **1c** over time; (B) Formation of *H*-phosphonate **3c** of dG amidite **1c** over time; (C) Formation of CE-phosphonoamidate **4c** of dG amidite **1c** over time. (*View this art in color at www.dekker.com.*)

hydrolysis reaction protects unreacted amidite from further hydrolysis. However, removal of water is the preferred mode of stabilization as any residual water reacts rapidly with the phosphoramidite upon activation with 1*H*-tetrazole (**1** acts as its own drying agent!) prior to the coupling reaction of oligonucleotide synthesis. Depending on the phosphoramidite excess used the coupling efficiency may be reduced, resulting in lower yield.

Table 3. Stability of dG amidite **1c** in acetonitrile solution under various conditions (area-% by HPLC).

Entry	Experimental conditions	HPLC analysis (day 10) 1c	2c	3c	4c	other
1	Control (day 0)	99.0	0.20	0.06	0.09	0.65
2	0.2 M	91.0	2.65	4.36	1.13	0.86
3	0.1 M	94.4	2.33	2.16	0.37	0.74
4	0.05 M	96.1	1.92	1.16	0.18	0.64
5	Amidite vacuum dried (4 micron, 2 days)	90.4	3.63	3.98	1.08	0.91
6	Amidite dried aceotropically (benzene)[a]	89.9	4.19	4.04	1.02	0.85
7	As entry 2, mol sieves 3Å[b]	95.9	0.98	1.22	0.49	1.41[c]
8	As entry 2, triethylamine (0.4 mg, 0.2 mol-%)	90.1	3.61	4.31	1.17	0.81
9	As entry 2, triethylamine (2 mg, 1 mol-%)	92.1	2.67	3.48	0.98	0.77
10	As entry 2, triethylamine (10 mg, 5 mol-%)	96.3	0.62	1.69	0.68	0.71
11	As entry 2, + water (3.5 mg, 10 mol-%)	88.6	4.39	4.99	1.23	0.79
12	As entry 2, + water (7 mg, 19 mol-%)	87.0	4.88	5.73	1.45	0.94
13	As entry 2, + water (10.5 mg, 29 mol-%)	86.8	5.14	5.80	1.37	0.89
14	As entry 2, + water (14 mg, 38 mol-%)	85.2	5.66	6.60	1.58	0.96

[a]A solution of **1c** (3.0 g) in benzene (50 ml) was evaporated to dryness at 30°C, then kept under vacuum (4 microns) for 2 days.
[b]Molsieves (1.0 g) was added to a solution of **1c** (1.68 g, 2 mmol) in acetonitrile (10 ml).
[c]A slight increase of trialkyl phosphite triester species is observed.

CONCLUSION

In conclusion, we have shown that standard phosphoramidites **1a–d** used in oligonucleotide synthesis degrade in solution through a mechanism that involves acid-catalyzed hydrolysis leading to CE-*H*-phosphonate **2**, base-catalyzed elimination of acrylonitrile of **2** leading to *H*-phosphonate **3** and an autocatalytic addition/elimination Arbuzov-type reaction leading to CE-phosphonoamidate **4**. The rate of degradation decreases in the order dG > dA > dC ≈ T. Consistent with the proposed degradation mechanism amidite solutions may be stabilized by dilution, by addition of molecular sieves or by addition of small amounts of base (e. g. triethylamine). Removal of moisture is certainly the preferred method. Synthetic methods and isolation procedures providing phosphoramidites with significantly lower (less than 0.01%) water content are desirable.

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