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Phosphoramidites Derived from Tertiary Alcohols. Why Do They Sometimes Couple with Low Efficiency?

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Abstract. *tert*-Alkyl phosphoramidites are somewhat sterically hindered, but give phosphites in good yields with tetrazole catalysis when the coupling time with alcohols is prolonged. Low yields of phosphotriesters are caused by elimination of the tertiary alkyl group during the subsequent oxidation of the phosphite with iodine/water/pyridine, and can be avoided by the use of *tert*-butyl hydroperoxide as the oxidant.

One of the main methods to prepare DNA and RNA oligomers uses nucleoside phosphoramidites as the monomers, and phosphoramidite methods are often chosen to prepare a variety of other biologically interesting phosphomonoesters and phosphodiesters.[1] In this method, the phosphoramidite reacts with an alcohol in the presence of a catalyst, most often tetrazole, to give a phosphite, which subsequently is oxidized to a phosphotriester, usually with a mixture of iodine, water, and pyridine. Finally, protective groups are removed to give the desired phosphomonoester or phosphodiester. In the solid phase synthesis of DNA and RNA oligomers the 5'-hydroxy group is usually protected with dimethoxytrityl, and the yield of each coupling is estimated from the amount of dimethoxytrityl cations liberated after a coupling cycle. Provided the water content is kept low, such "DMT efficiencies" can be above 99%, which makes this method so attractive for the preparation of longer phosphodiester oligomers.

The phosphoramidite method has also been popular to prepare modified oligonucleotides for antisense purposes.[2] In connection with studies of sugar modified oligonucleotides we observed that 3'-*O*-phosphoramidites derived from 3'-*C*-alkyl nucleosides, e.g. **1** - **3** (**Fig. 1**), coupled with very low DMT efficiencies (< 5% to about 40%), even after repeated couplings and prolonged reaction times.[3-5] However, some bicyclic analogues gave varying results. Thus, **4** gave satisfactory DMT efficiencies after 2 x 12 min coupling (ca. 95%),[6] **5** gave medium (50%) and **6** quite low efficiency (30%).[7] Other groups have published similar results, e.g. Herdewijn et al. reported that **7** failed completely to couple,[8] whereas Leuman et al. obtained high DMT-efficiencies (>98% for 6 min couplings) for the bicyclic phosphoramidites **8**.[9] These phosphoramidites are all derived from tertiary alcohols and are expected to react more slowly than phosphoramidites derived from primary or secondary alcohols due to steric hindrance, but this effect is not large,[10] and does not explain the varying results described above. We show here that phosphoramidites derived from tertiary alcohols react efficiently with alcohols to give phosphites, but the phosphites are cleaved in the subsequent oxidation step by the usual oxidation reagent, iodine/water/pyridine, if elimination of the tertiary alkyl group is facile. Good yields of phosphotriesters can be obtained by simply replacing iodine/water/pyridine with *tert*-butyl hydroperoxide as the oxidant.

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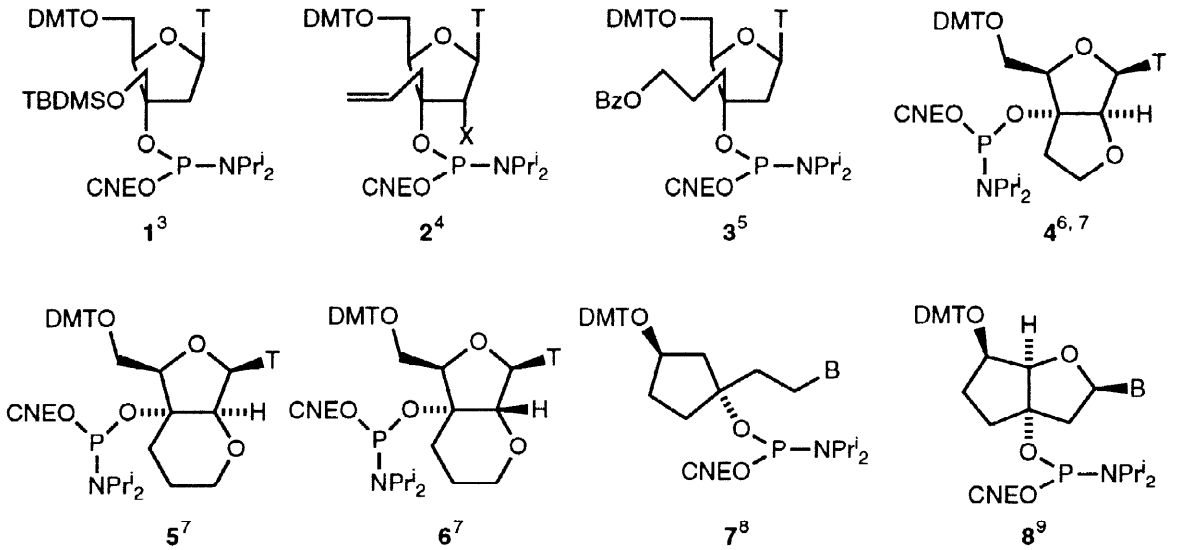

Fig. 1. Phosphoramidites derived from tertiary alcohols. Bz = benzoyl, CNE = 2-cyanoethyl, DMT = 4,4'-dimethoxytrityl, TBDMS = *tert*-butyldimethylsilyl, X = H or TBDMSO, T = 1-thyminyl, B = protected nucleobase.

Model experiments

tert-Butyl 2-cyanoethyl N,N-diisopropylphosphoramidite **9a**[11] and 3-(4,4'-dimethoxytrityloxy)-1,1-dimethylbutyl 2-cyanoethyl N,N-diisopropylphosphoramidite **10**[12] (**Fig. 2**) were prepared for ^{31}P NMR and solid support model experiments, respectively. The phosphoramidite **9a** (δP 137.5) was treated with 3-hydroxypropionitrile (2 eq) and tetrazole (10 eq) in acetonitrile to give the phosphite **9b** (δP 135.4) quantitatively in less than 2 min at rt. Oxidation with iodine/water/pyridine in THF (1 M I_2 in H_2O/Py/THF, 1/2/7 v/v/v) gave about 80% of a product (δP ca. -2.9) shown to be di-(2-cyanoethyl) phosphate **9c**.[13] Oxidation with *tert*-butyl hydroperoxide gave more than 95% of another product (δP -7.7) identified as *tert*-butyl di-(2-cyanoethyl) phosphate **9d**.[14] During the iodine oxidation, an intermediate was observed (δP -14.8), probably tetra-(2-cyanoethyl) pyrophosphate,[15] but no **9d** (less than 2%) was formed. This shows that iodine oxidation causes elimination of the tertiary alkyl group, probably at the iodophosphonium ion step (**Fig. 2**), and that the elimination is faster than the reaction of the iodophosphonium ion with water to give the oxidation product **9d**. The transient formation of tetra-(2-cyanoethyl) pyrophosphate is explained by the reaction of **9c** with its precursor.

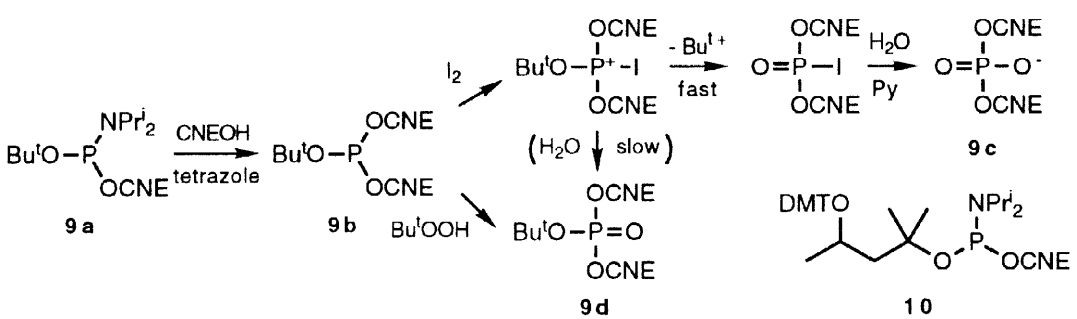

Fig. 2. Phosphoramidites **9a** and **10**, and proposed mechanism for the formation of **9c**.

Phosphoramidite **10** when used in solid phase DNA syntheses (Biosearch 8750 DNA Synthesizer, standard conditions apart from extended coupling times) gave similar results. The DMT efficiency was ca. 95% for the incorporation of **10** once in a DNA sequence with 24 min coupling time and oxidation with *tert*-butyl hydroperoxide; the efficiency was reduced to ca. 5% when iodine/water/pyridine was used as the oxidant, because the tertiary alkyl group containing the DMT group was largely eliminated during the oxidation. Oxidation with the Beaucage reagent[16] gave phosphorothioates with ca. 95% efficiencies as expected. The main product from the attempted coupling of **10** to a tetramer DNA sequence 5'-d(ATGC), followed by iodine/water/pyridine oxidation, was shown by MALDI TOF to be the 5'-phosphorylated tetramer (found: 1251.2, calc. 1252.2).

Coupling experiments with tertiary nucleoside monomers

Monomer **1** was selected for solid phase syntheses of a nonamer DNA analogue, 5'-d(GTG ATA TGC), where the T monomer was substituted with **1** one or three times. One coupling of **1** with *tert*-butyl hydroperoxide oxidation gave ca. 85% efficiency after 16 min, and 93-94% after 24 min coupling time; the next two couplings with **1** were somewhat less efficient (ca. 80% after 24 min coupling) for unknown reasons. With iodine/water/pyridine oxidation the efficiency was varying from batch to batch of **1** between 5% and 20%, probably due to varying small amounts of impurities in **1**. A crude, deprotected nonamer prepared using **1** as the fifth monomer with iodine oxidation (15 % efficiency) was examined by MALDI TOF MS and capillary electrophoresis (CE). Apart from the nonamer peak (found: 2786, calc. 2783.2), a major peak was found corresponding to 5'-p-d(ATGC) (found: 1254.6, calc. 1252.2). The two major peaks in CE were shown by spiking with authentic samples to arise from the nonamer and the phosphorylated tetramer. This shows that the tertiary nucleoside had been largely eliminated and a phosphate group added.[17] The nonamer was prepared using **1** three times with 24 min coupling time and *tert*-butyl hydroperoxide oxidation throughout to give 5'-d(GT*G AT*A T*GC) in an isolated yield, after deblocking with aq. $MeNH_2$ and $Et_3N{\cdot}3HF$, followed by butanol precipitation, of 35% and a purity of ca. 60% according to CE.

Discussion and Conclusion

The above results show that the reason for low DMT efficiencies of many phosphoramidites derived from tertiary alcohols is that the usual oxidation reagent, iodine/water/pyridine, causes elimination of the tertiary alkyl group. The elimination of a tertiary carbocation from the iodophosphonium intermediate (**Fig. 2**) is considerably faster than hydrolysis to the stable trialkyl phosphate when the carbocation can obtain a planar structure. The propensity of bicyclic, bridgehead phosphoramidites like **4**, **5**, **6**, and **8** to give elimination products depends on how easily the derived carbocations can attain planarity. Thus **4** and **8** couple efficiently under standard conditions because a planar, bridgehead carbocation is high in energy and therefore not formed, whereas **5** and **6** give lower DMT efficiencies because they more easily can form a nearly planar carbocation.[18] The ease of elimination of non-constrained tertiary alkyl groups from tertiary alkoxyphosphonium structures invoked here is a general phenomenon.[19] Elimination can be avoided by using oxidation agents which do not form phosphonium intermediates, like *tert*-butylhydroperoxide, nitrogen dioxide, or sulfur reagents.[20] With this exchange of oxidation reagent, and a prolonged coupling time or the use of a better catalyst than tetrazole, it should now be possible to prepare modified DNA or RNA sequences from any tertiary alkyl phosphoramidite in good yields.

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11. **9a** was prepared from *tert*-butyl alcohol and 2-cyanoethyl N,N-diisopropylphosphoramidochloridite, 85%, bp 85-87 ^{o}C at 0.3 mbar, δ_P ($CDCl_3$) 138.4 ppm, FAB$^+$MS found 275.2 ($M+H^+$, calc. 275.2).
12. **10** was prepared from 2-methyl-2,4-pentandiol via protection with dimethoxytrityl chloride followed by reaction with 2-cyanoethyl N,N-diisopropylphosphoramidochloridite, oil, 50% after column chromatography, δ_P ($CDCl_3$) 138.2 ppm.
13. The isolated product **9c**: NMR (D_2O) δ_P -1.0, δ_H 4.00 (dt, $^3J_{PH}$ 6.7, $^3J_{HH}$ 6.0, 4H, OCH_2), 2.75 (t, $^3J_{HH}$ 6.0, 4H, CH_2CN). FAB$^-$MS found 203.0 (M^-, calc. 203.0).
14. The isolated product **9d**: NMR ($CDCl_3$) δ_P -7.2, δ_H 4.25 (dt, $^3J_{PH}$ 8.0, $^3J_{HH}$ 6.2, 4H, OCH_2), 2.77 (t, $^3J_{HH}$ 6.2, 4H, CH_2CN), 1.55 (s, 9H, Bu^t). FAB$^+$MS found 261.1 ($M+H^+$, calc. 261.1), 205.0 ($M+H^+$ - isobutene, calc. 205.0).
15. Compare δ_P -13 for tetraethyl pyrophosphate, R. A. Y. Jones and A. R. Katritzky, *Angew. Chem.* **1962**, *74*, 60-68.
16. R. P. Iyer, L. R. Phillips, W. Egan, J. B. Regan, and S. L. Beaucage, *J. Org. Chem.* **1990**, *55*, 4693-4699.
17. Attempted couplings of **7** likewise gave products terminated with a 5'-phosphate group, P. Herdewijn, personal communication.
18. Molecular modelling experiments were performed in either HyperChem^{TM} Rel. 4.5 for Windows NT using a MM$^+$ force field, or Sybyl^{TM} Rel. 6.3 for Silicon Graphics using Tripos Force Field with MOPAC derived charges for geometry optimisations and molecular dynamic studies. The modelling experiments showed a connection between the planarity of the carbocation (described as the angle between C-3" and the plane defined by C-2', C-3', and C-4') eliminated from the iodophosphonium intermediate, and the coupling yield. A perfect planar carbocation will have an angle of 0^o and a perfect pyramidal ion an angle of 60^o. A high degree of planarity was calculated for the carbocations formed from the nucleotides that gave low coupling yields (**1**(11^o), **2** (10^o), **3** (7^o), **6** (18^o), and **7** (8^o)), and an almost pyramidal carbocation from those which showed good coupling results (**4** (60^o) and **8** (55^o)). Nucleotide **5** (41^o) gave medium coupling efficiency corresponding to an intermediate angle.
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20. Benzyl phosphoramidites, widely used to prepare phosphorylated peptides, suffer the same problems as tertiary alkyl phosphoramidites because a benzyl cation is easily formed. The solution found was likewise to oxidize with *tert*-butylhydroperoxide or peracids, although the reason was not fully disclosed; see e.g. W. Bannwarth and A. Trzeciak, *Helv. Chim. Acta* **1987**, *70*, 175-186; J. W. Perich and R. B. Johns, *Tetrahedron Lett.* **1987**, *28*, 101-102.