Nucleotides

Part LXII[1])

Pyridinium Salts – An Effective Class of Catalysts for Oligonucleotide Synthesis

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Various pyridinium salts (see **1**–**9**) have been tested as catalysts for the condensation step in the phosphoramidite approach of oligonucleotide synthesis. Pyridinium chloride (**1**) turned out to be the most effective activator, speeding up the condensation tremendously. Pyridinium bromide (**2**) and 4-methylbenzenesulfonate (**4**) can also be regarded as powerful substitutes for the commonly used 1*H*-tetrazole. The acidic pK_a of the pyridinium cation provides an optimal range for phosphoramidite activation, which is followed by a nucleophilic attack of the pyridine ring to give the *P*-pyridinio intermediate **11** as the most likely precursor of phosphite ester formation (*Scheme*). ^{31}P-NMR Studies support this proposal indirectly.

1. Introduction. – Over the years, the phosphoramidite approach [2–5] has been established as the method of choice for the synthesis of oligonucleotides. Most technologies in biochemistry and molecular biology developed recently (*e.g.* PCR [6], antisense approach [7]) rely mainly on chemically synthesized oligonucleotides of which high quality materials are in demand, and speed-up of their machine-aided synthesis would be very benefical. So far 1*H*-tetrazole is recommended as the most versatile activator of phosphoramidites [2], but also alternatives which are slightly more acidic or more soluble in MeCN, as *e.g.* 5-(ethylthio)-, 5-(4-nitrophenyl)-, and 5-(3-nitrophenyl)-1*H*-tetrazole [8–10] as well as, more recently, benzimidazolium triflate [11] and 1*H*-imidazole-4,5-dicarbonitrile [12], have been introduced as activators but have never replaced 1*H*-tetrazole. A combination of pyridinium chloride and 1-methyl-1*H*-imidazole was introduced as activating agent to oligonucleotide synthesis by *Gryaznov* and *Letsinger* [13][14], and it was suggested from ^{31}P-NMR data that the reactive intermediate generated is a P^{III} phosphoromonochloridite, but it seems to us, for thermodynamical reasons, more reasonable to postulate a pyridinium phosphite derivative as the active species. Nevertheless, it is surprising that the obviously high reactivity of this intermediate has not been applied more often to accelerate the condensation step, in general.

Our interest in the activator pyridinium chloride (**1**) resulted from the proposal of a highly selective *O*- over *N*-phosphitylation [13] [14] which could, however, not be established in our hands, working with highly reactive diethylphosphoramidites. By-products due to undesired *N*-substitution at the [2-(4-nitrophenyl)ethoxycarbonyl] (protected npeoc) moieties of cytidine and adenosine were observed. Since 1*H*-tetrazole worked

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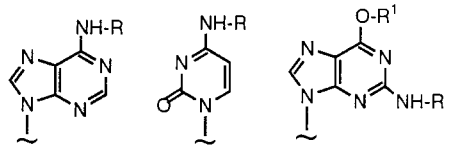
under the same conditions cleanly, the reaction time of the condensation step was reduced drastically with pyridinium chloride (**1**) and, fortunately, by-product formation could not been detected anymore by chromatographical means. Therefore, we decided to investigate pyridinium salts, in general, as cheap and highly effective substitutes for 1*H*-tetrazole as potential catalysts in phosphoramidite-based oligonucleotide syntheses.

2. Results. – To demonstrate the general applicability of pyridinium chloride (**1**) as effective activator, not only the standard acyl- but also the npe/npeoc- [15] and the phthaloyl-protected [1] phosphoramidites (see *Fig. 1*) were used as monomeric building blocks in these investigations, which were performed on standard commercially available LCAMA-CGP ((long-chain-alkyl)methylamine controlled-pore glass) and DBU-stable LCAMA-CPG [16] solid-support material (DBU = 1,8-diazabicyclo[5.4.0]-undec-7-ene).



Strategy	Protecting groups			
	R	R	R	R^1
Acyl	bz	bz	ibu	H
npe/npeoc	npeoc	npeoc	npeoc	npe
phthaloyl	phth	phth	phth	npe

Fig. 1. *Protection of nucleo-bases by acyl, npe/npeoc and phthaloyl protecting groups*

A series of oligodeoxyribonucleotide sequences up to a chain length of 22 nucleotide units (see *Table*) were synthesized under standard conditions without changing the flow rates of the normal cycles on the DNA/RNA synthesizer. A 0.5M solution of pyridinium chloride (**1**) in MeCN worked best in our hands, despite the fact that more concentrated solutions (up to 0.75M) can be prepared for solubility reasons. Neither a precipitation of the activator **1** on the solid support nor the undesired removal of the dimethoxytrityl protecting group was observed. The standard coupling time of 60 s in the presence of the activator 1*H*-tetrazole was reduced sequentially to practically no waiting time upon replacement of this activator by pyridinium chloride (**1**). The phosphoramidite/**1** mixture was simply pumped onto the column, and immediately after the column was filled, the reaction mixture was pumped off by a flush of Ar. This improved procedure worked very well for shorter sequences, but for standard routine oligodeoxyribonucleotide synthesis of chain lengths up to 22 units, a condensation step of *ca.* 6–12 s is recommended. The crude oligomers, as checked by reversed-phase HPLC, showed satisfactory purity and homogeneity (*Fig. 2*).

The most exciting improvement in speeding up the condensation was observed in oligo*ribo*nucleotide synthesis. The well-known lower reactivity of the ribonucleoside phosphoramidites, requiring 700–1200 s condensation time by use of 1*H*-tetrazole, could be enhanced considerably (70–280 s condensation time) on activation with

Table. *Oligodeoxyribonucleotide Sequences Synthesized by Pyridinium Chloride* (**1**) *Activation*

5′-Sequence-3′	Length	Coupling time [s]		
		acyl strategy	npeoc strategy	phthaloyl strategy
d(AAAAAA)	6	12	12	12
d(CCCCCC)	6	12	12	–
d(GGGT)	4	12	12	30
d(TTTTT)	5	–	0	–
d(TTTTTT)	6	–	3	–
d(GTTATT)	6	–	–	30
d(GCTGCATG)	8	–	–	24
d(TGACGTTATT)	10	–	–	30
d(CCTCCAATCTAG)	12	–	–	30
d(TGTAGTAGTGGT)	12	–	–	30
d(GTAAAACGACGGCCAGT)	17	12	–	–
d(TCAATGCTTGACTGCGTT)	18	6	9	–
d(CAACGGGACCCTGAAGAT)	18	9	9	–
d(AGCCTTTGGATTGAGCCAC)	19	9	9	–
d(GCCTCTGAACCTCTTCAGCA)	20			24
d(CTCAGACAGGGCAAAGTTCC)	20	9	9	–
d(GGCCGAGTCGATTTCAGACCA)	21	12	12	–
d(AACAATATCACCAGGGACGAGG)	22	12	12	–
d(GTTGGGTCCGAATATTTCAAGA)	22			24
d(TTACTAATCAGAATGTCTCTCA)	22			24

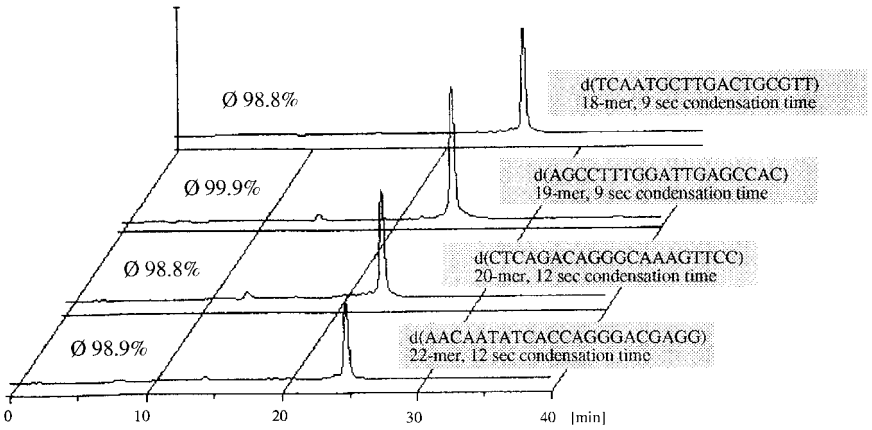


Fig. 2. RP-18 *HPLC of DNA sequences synthesized with pyridinium chloride* (**1**) via *the acyl approach*. Average stepwise yields 98.8–99.9%.

pyridinium chloride (**1**) during the synthesis of 9- to 20-mer oligoribonucleotides. The HPLC profile of a crude 2′-*O*-(4-methoxytetrahydro-2*H*-pyran-4-yl)nonaadenylate synthesized with 5′-*O*-(dansylethoxycarbonyl)-2′-*O*-(4-methoxytetrahydro-2*H*-pyran-4-yl)-N^6-[2-(4-nitrophenyl)ethoxycarbonyl]adenosine 3′-[2-(4-nitrophenyl)ethyl diisopropylphosphoramidite] (dnseoc strategy [17]) activated in the condensation step by 0.5M pyridinium chloride (**1**) for 70 s is represented in *Fig. 3*.

All synthesized oligonucleotides showed high purity, even as crude products, as shown by reversed-phase and anion-exchange-HPLC, polyacrylamide-gel capillary

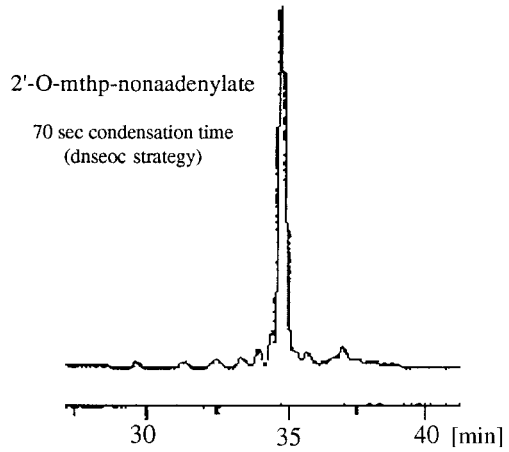


Fig. 3. *Reversed-phase HPLC of 2'-*O*-mthp-nonaadenylate synthesized with pyridinium chloride* (**1**) *by the dnseoc strategy*

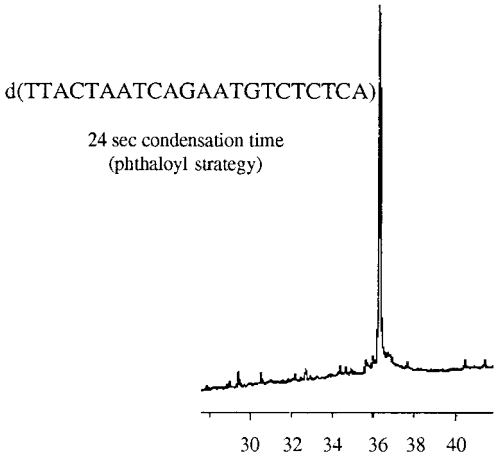


Fig. 4. *Capillary electrophoresis of a 22-mer synthesized by the phthaloyl strategy with* **1**

electrophoresis (PAGE; *Fig. 4*), and MS. Furthermore, the biological usability of the derived DNA sequences was demonstrated by employing them as specific sequences in PCR experiments [18]. The increased effectiveness of using pyridinium chloride (**1**) as the activator was demonstrated recently also by the synthesis of highly modified pyranosyl-RNA analogs [19] and high density of DNA arrays [20].

The excellent results obtained with pyridinium chloride (**1**) forced us to test also other pyridinium salts, *i.e.* **2**–**9** (*Fig. 5*), as activators of phosphoramidites. To avoid any precipitation of the catalyst on the column of the DNA synthesizer during the condensation step, 0.5M solutions of the appropriate pyridinium salt in MeCN were prepared and stored over molecular sieves for 24 h. Since some pyridinium salts, however, showed only a low solubility in MeCN, the supernatant of the suspension was

used in the experiment. Due to different concentrations, a direct comparison of the actual activity is only of qualitative nature but, nevertheless, the standard conditions of the condensation cycle were still applied. Pyridinium bromide (**2**), 3-nitrobenzenesulfonate (**3**), and 4-methylbenzenesulfonate (**4**) were chosen to check a potential influence of the anion whereas in the hydrochlorides **5**–**8** of 4-chloropyridine, 1-(4-pyridyl)pyridinium chloride, 2,6-di(*tert*-butyl)pyridine, and 2-(*tert*-butyl)pyridine, the acidity of the pyridinium cation should account for anticipated differences in activation. Pyridinium-3-sulfonate (**9**) may reveal special effects based on its betaine structure.

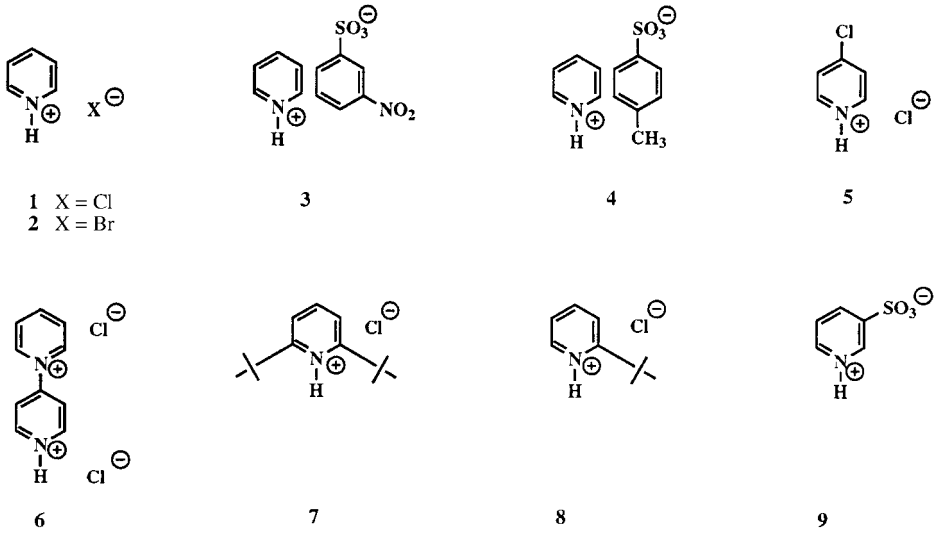


Fig. 5. *Tested pyridinium salts*

In a set of experiments, the pyridinium salts were checked for their abilities to accelerate the condensation step under standard conditions of oligodeoxyribonucleotide synthesis. First, all pyridinium salts were used in the synthesis of a d(T_4) oligonucleotide by a standard protocol on a *ABI-392* DNA synthesizer with a 60-s wait step during the condensation event. As a reference, the same synthesis was performed with the standard activator 1*H*-tetrazole. Compounds **5**, **6**, and **9** failed to show good condensation properties (*Fig. 6*). Among them, pyridinium-3-sulfonate (**9**) and 4-(pyridinio)pyridinium dichloride (**6**) did show no or only moderate ability to serve as a catalyst for the condensation of phosphoramidites, and 4-chloropyridinium chloride (**5**) catalyzed the reaction only gradually to give low yields, as seen by the presence of shorter oligomers, namely d(T_1), d(T_2), and d(T_3).

The other pyridinium salts, *i.e.*, **2**–**4**, **7**, and **8** showed enhanced condensation power compared to the standard activator 1*H*-tetrazole, as noticed from more detailed studies during the syntheses of d(T_4) by the same protocol but shorter waiting steps (12 and 3 s) during the condensation event. Under these conditions, all five pyridinium derivatives **2**–**4**, **7**, and **8** accelerated the condensation step, even when the condensation time was set as low as 3 s, to give a product showing a single HPLC peak with undetectable peaks of failure sequences (data not shown). It should be noted, that pyridinium

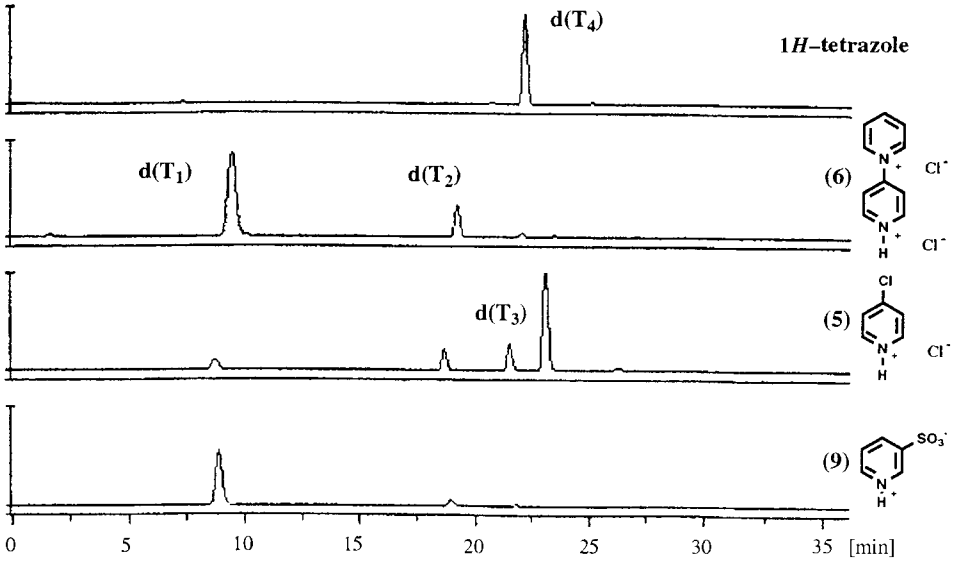


Fig. 6. *Comparison of 1*H*-tetrazole and pyridinium salts* **5**, **6** *and* **9** *as activators in the condensation step of oligodeoxyribonucleotides: reversed phase HPLC of the products*

3-nitrobenzenesulfonate (**3**) showed high catalytic properties, although present only in a very low concentration due to its low solubility in MeCN.

Finally, the pyridinium salts **2**–**4** and **7** were applied to the synthesis of a mixed oligodeoxyribonucleotide, *i.e.* d(CACCGACGGCGC), setting the condensation time to 12 s: the target 12-mer was formed in high purity (*Fig. 7*).

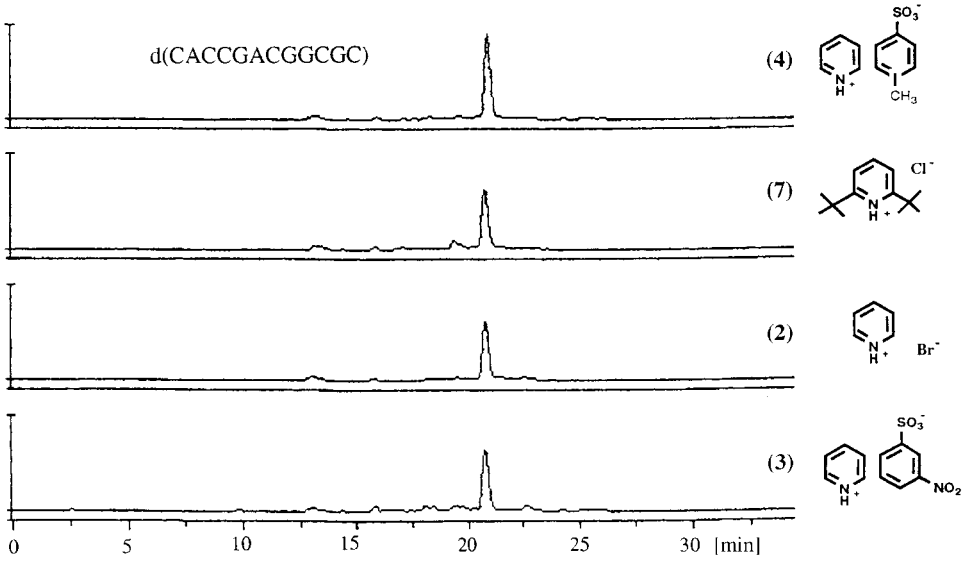


Fig. 7. *Synthesis of d(CACCGACGGCGC) with the pyridinium salts* **2**–**4**, *and* **7** *during a condensation step of 12 s: reversed-phase HPLC of the products*

Although the four pyridinium salts **2**–**4** and **7** exhibited almost equal activation properties, pyridinium bromide (**2**) and 4-methylbenzenesulfonate (**4**) provided the best results regarding purity and homogeneity of the products of which almost no truncated sequences were detectable. For solubility reasons, only pyridinium bromide (**2**) was investigated further in the syntheses of various mixed 21-mer sequences (12 s condensation time) which were checked in their crude form by HPLC (*Fig. 8*). Despite the shortened condensation procedure, no loss in performance was observed, and the quality of the derived oligomers was at least equal, if not higher, compared to those of the oligonucleotides synthesized with 1*H*-tetrazole. Additionally, the biological quality of the crude oligomers was checked by their use as perfect primers for PCR experiments [18].

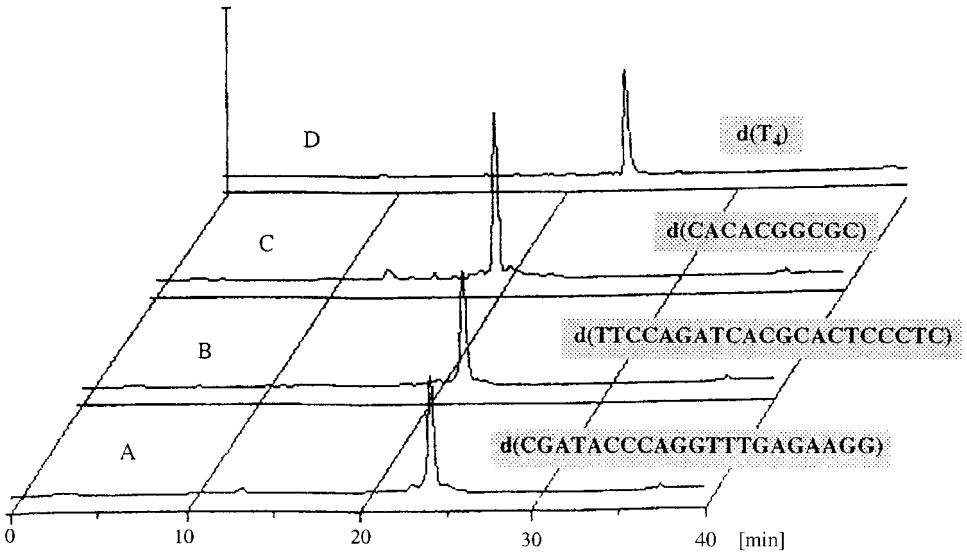
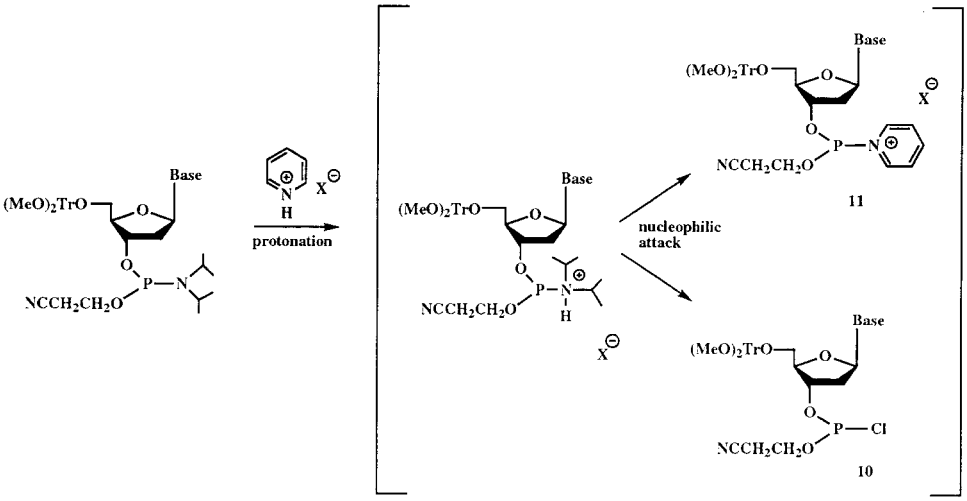


Fig. 8. *DNA Sequences synthesized with pyridinium bromide* (**2**), *and 12* (A and B), *6* (C), *and 3 s* (D) *condensation time: reversed-phase HPLC of the products*

Finally, we were interested in the mechanism of action of the pyridinium salts. *Gryaznow* and *Letsinger* [13] proposed that the activation during the condensation step proceeds *via* a phosphorochloridite species **10** (*Scheme*). A simple alternative would be the generation of a highly reactive pyridinium-ion intermediate **11**.

The pyridinium salts **1**–**4** offer different anions (chloride, bromide, 3-nitrobenzenesulfonate, and 4-methylbenzenesulfonate) from which a special effect of the coordinating anion on the ability to promote the condensation reaction should be detected. The pyridinium salts **5**–**9** represent more or less variations of the pyridine skeleton itself, providing eventually information on how simple substituents at the heterocyclic ring system influence the behavior towards phosphoramidite condensation. We conclude from our experiments that the reactive species, generated after protonation of the phosphoramidite, is a pyridinium-ion intermediate **11**, since the most promising pyridinium salts in oligonucleotide synthesis did not show any dependency on the coordinating anion. Thus, the bromide **2**, the 3-nitrobenzenesulfonate **3**, and the

Scheme



4-methylbenzenesulfonate **4** revealed almost the same catalytic properties, although their anions have different nucleophilicities. The common features of these salts can only be explained properly by assuming that the protonated phosphoramidite is attacked by the pyridine moiety and not by the coordinating anion. This is also supported by the fact that the chlorides **5** and **6** did not give rise to product formation due to a substantial reduction of nucleophilicity by the electron-withdrawing substituent in 4-position of the pyridine ring. On the other hand, however, the sterically hindered *tert*-butylpyridine derivatives **7** and **8** generated DNA sequences of high purity, although their nucleophilicity seems to be reduced due to the bulky substituents in 2- and 6-position.

To obtain more direct information, we tried to monitor the activation step by ^{31}P-NMR, adding pyridinium chloride (**1**), bromide (**2**), and 4-methylbenzenesulfonate (**4**) and 2,6-di(*tert*-butyl)pyridinium chloride (**7**) to a standard phosphoramidite solution in MeCN in an NMR tube. For an easy comparison with published data [14], the standard 5′-*O*-(dimethoxytrityl)thymidine 3′-(2-cyanoethyl diisopropylphosporamidite) was used. In the case of chloride **1**, bromide **2**, and 4-methylbenzenesulfonate **4**, the phosphoramidite peak of the educt almost immediately disappeared. The ^{31}P-NMR spectra showed resonances in the range of 10–20 ppm indicating a very fast reaction to a P^{III}–O species since no signals supporting the generation of a phosphorochloridite intermediate were observed. Furthermore, addition of 2,6-di(*tert*-butyl)pyridinium chloride (**7**) to the phosphoramidite solution indicated a much slower activation, the phosphoramidite resonance of the educt at 151.7 and 151.8 ppm being nearly unaffected after 10 min due to the expected steric hindrance by the bulky pyridine moiety. However, even extended waiting did not support the formation of a phosphorochloridite; instead the typical signals between 10–20 ppm of the phosphite ester function began to increase.

Although it was not possible to establish the activation of a phosphoramidite with a pyridinium salt directly by spectroscopic means, it is most probable that the protonated

phosphoramidite species undergoes a nucleophilic attack by the pyridine moiety to give a pyridinium-type intermediate.

3. Conclusion. – Among the pyridinium salts tested so far, the pyridinium chloride (**1**), bromide (**2**) and 4-methylbenzenesulfonate (**4**) showed almost identical activity in speeding up the condensation step during oligonucleotide synthesis by the phosphoramidite approach. These pyridinium salts are recommended as cheap and easy-to-handle substitutes for the commonly used 1*H*-tetrazole activator. No loss of performance by alleged detritylation during the condensation step was observed due to a somewhat decreased acidity of the pyridinium cation and an increased nucleophilicity of the pyridine ring, providing perfect features for phosphoramidite coupling reactions in MeCN.

Experimental Part

General. ^{31}P-NMR: *Jeol-JMGX-400-MHz spectrometer*; δ in ppm rel. to H_3PO_4. HPLC: *Merck Hitachi L-6200, D-2000* chromatointegrator, detection at 260 nm (*Uvikon 730 SLC*, Fa. *Kontron*); column *RP-18 Lichrospher* (125 × 4 mm 5 μm, *Merck*); flow rate 1 ml/min; mobile phase: eluent *A:* 0.1N (Et_3NH)OAc (pH 7), eluent *B:* MeCN; 30 min 2.5–20% *B* in *A*.

1. *Activator Solutions.* Pyridinium salts **1**–**9** were dissolved in anh. MeCN to give a final concentration of 0.5M. Due to the low solubilities of **3**, **5**, **6**, and **9**, their supernatants were used for the experiments. The activator solns. were stored for 24 h over activated molecular sieves 4 Å before use.

2. *Oligonucleotide Synthesis.* Oligonucleotide syntheses were performed on an *ABI 392* DNA/RNA synthesizer using standard acyl- (*Glen Research*), npe/npeoc-, and phthaloyl-protected phosphoramidites. A standard protocol, as provided by the manufacturer, was used for DNA synthesis, with the necessary changes concerning the waiting time during the condensation step.

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