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Pyridinium Trifluoroacetate/*N*-Methylimidazole as an Efficient Activator for Oligonucleotide Synthesis via the Phosphoramidite Method

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Abstract:

A new activator is reported for coupling phosphoramidites to a free 5′-hydroxyl group during oligonucleotide synthesis. Pyridinium trifluoroacetate/*N*-methyl imidazole is a remarkably efficient replacement for 1*H*-tetrazole in the solid-supported synthesis of oligonucleotides. This reagent is safe and inexpensive, is not moisture-sensitive, and is soluble in acetonitrile.

Introduction

In the past few years, oligonucleotides have become the focus of intense research regarding their use as antisense therapeutic agents.[1−5] Much of the research has been directed to development of phosphate diester backbone analogues that provide enhanced metabolic stability relative to naturally occurring DNA. Phosphorothioate oligodeoxyribonucleotides have emerged as promising analogues, showing promise as therapeutic agents for the antisense treatment of multiple diseases.[6−9] Overall successful development of these drugs for the market depends on their efficient synthesis in a safe environment.[10]

Phosphoramidite chemistry[11−13] is widely used for the synthesis of phosphorothioate oligonucleotides because of its potential for automation, high coupling efficiency, ease of site-specific thioate linkage incorporation, and ready scalability. Current state-of-the-art large-scale synthesizers[14] allow complete synthetic assembly of a uniformly modified phosphorothioate oligodeoxyribonucleotide 20-mer at 0.15 mol scale in 10 h. A critical step is the tetrazole-catalyzed coupling reaction of phosphoramidites with a free 5′-hydroxyl group of the growing oligonucleotide chain to form a phosphite triester intermediate. Successful synthesis depends, inter alia, on this coupling reaction being fast and near quantitative, without serious side reactions. Activation of phosphoramidites is usually achieved by reaction with 1*H*-tetrazole. While this activator is the accepted standard for small-scale synthesis, alternative activators have been investigated for several reasons.[15] For development of drugs in particular, 1*H*-tetrazole is not preferred, because of the large quantities involved. Tetrazole has the potential to explode[16] if melted and, in addition, is relatively expensive. Tetrazole solutions in acetonitrile must be warmed to make them homogeneous. This is particularly inconvenient for routine large-scale synthesis. The possibility of dry tetrazole residues forcefully decomposing in heated equipment is real. Thus, invention of new activation reagents, which are equally as effective as tetrazole, inexpensive, and safe to use, is highly desirable.

Screening of pyridinium salts resulted in the identification of pyridinium trifluoroacetate (PTFA) as the activator of choice. This activator is inexpensive, safe, not moisture-sensitive, and highly effective in solid-supported synthesis of oligonucleotides. Synthesis and analysis of an oligonucleotide phosphorothioate 19-mer with multiple 2′-*O*-methoxyethyl substitutions indicated that PTFA could be used for synthesis of second generation oligonucleotides as well.

Materials and Methods

Pyridinium hydrochloride, pyridinium hydrobromide, pyridinium triflate, pyridinium *p*-toluenesulfonate, pyridinium 3-nitrobenzenesulfonate, pyridinium trifluoroacetate were obtained from Fluka or Aldrich and used without further purification. Deoxyribonucleoside phosphoramidites and oligonucleotide synthesis reagents were obtained from Amersham-Pharmacia Biotech, Chem-Impex International, Aldrich, or Perkin-Elmer-Applied Biosystems. 2′-*O*-Methoxyethyl-5-methyluridine phosphoramidte was obtained from Amersham-Pharmacia Biotech. Phenylacetyl disulfide was purchased from Schweizerhall and Synchem. Derivatized

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polystyrene support bearing deoxyribonucleoside was obtained from Amersham-Pharmacia Biotech.

Synthesis of Oligonucleotides

A 20-mer phosphorothioate oligodeoxyribonucleotide [ISIS 5132; d(5′-TCC-CGC-CTG-TGA-CAT-GCA-TT)][17−19] was chosen for investigation. Sulfurization was performed with a 0.1 M solution of phenylacetyl disulfide (PADS) in 3-picoline−acetonitrile (1:1, v/v, 4 column volumes) for 60 s. Detritylation was performed in 3% dichloroacetic acid−toluene (v/v).

Synthesis on Oligopilot I

The initial screening of activators was performed in an Amersham-Pharmacia Biotech OligoPilot I synthesizer at 30 μmol scale using polystyrene Primer Support (90 μmol/g). 2.5 equiv of phosphoramidite per coupling (2 volumes of activator for 1 volume of amidite) were used. Coupling time was maintained at 3 min. Results with various candidate activators are shown in Table 1.

Table 1: **Synthesis of ISIS 5132 using various phosphoramidite coupling activators**

activator	% full length by CGE	% (n+1)-mer	crude yield [OD/μmol]
0.11 M Pyr·HCl	61.2	0.61	107
0.22 M Pyr·HCl	75.1	2.0	118
0.22 M Pyr·HCl + 0.11 M NMI	69.0	0.5	125
0.11 M Pyr triflate + 0.11 M NMI	64.7	2.9	118
0.22 M PPTS + 0.11 M NMI	58.3	2.8	92
0.22 M Pyr 3-nitrobenzene-sulfonate + 0.11 M NMI	36.6	ND	76
0.22 M Pyr·HBr + 0.11M NMI	51.3	NDa	98
0.11 M Pyr trifluoroacetate	73.2	0.5	96
0.22 M Pyr trifluoroacetate	68.0	3.9	119
0.22 M Pyr trifluoroacetate + 0.11 M NMI	75.6	0.5	127

[a] ND = not determined.

Table 2: **Automated synthesis cycle for the preparation of ISIS 5132 phosphorothioate**

step	reagents	equiv	time (min)
detritylation	dichloroacetic acid in toluene (3% v/v)	-	4
coupling	deoxyribonucleoside phosphoramidite (0.2 M) + PTFA/NMI in CH_3CN	1.75	5
sulfurization	phenylacetyl disulfide (PADS) (0.2 M) in 3-picoline/CH_3CN (1:1) (v/v) (1 colume volume)	6.25	1
capping	Cap A: 20% NMI in CH_3CN Cap B: 20% Ac_2O:30% Pyr:50% CH_3CN	-	1

Analysis of the above table shows that higher concentration of activators leads to higher levels of (n+1)-mer formation, activators such as PPTS, pyridinium 3-nitrobenzenesulfonate, and pyridinium hydrobromide are not suitable for oligomerization due to relatively low full-length product formation, and addition of NMI to activators reduces the formation of (n+1)-mer formation.

Synthesis on Oligopilot II

The above table clearly shows that pyridinium trifluoroacetate (PTFA) is far superior to other pyridinium salts screened. Hence, PTFA was selected for further optimization. The same 20-mer oligonucleotide (ISIS 5132) was synthesized on an Amersham-Pharmacia Biotech OligoPilot II at 180−190 μmol scale using polystyrene Primer Support (90 μmol/g). 1.75 equiv of phosphoramidite per coupling (2 volumes of activator for 1 volume of amidite) were used. The coupling cycle is shown in Table 2. At the end of synthesis, the oligonucleotide was cleaved from the resin, and base and phosphate-protecting groups were removed in

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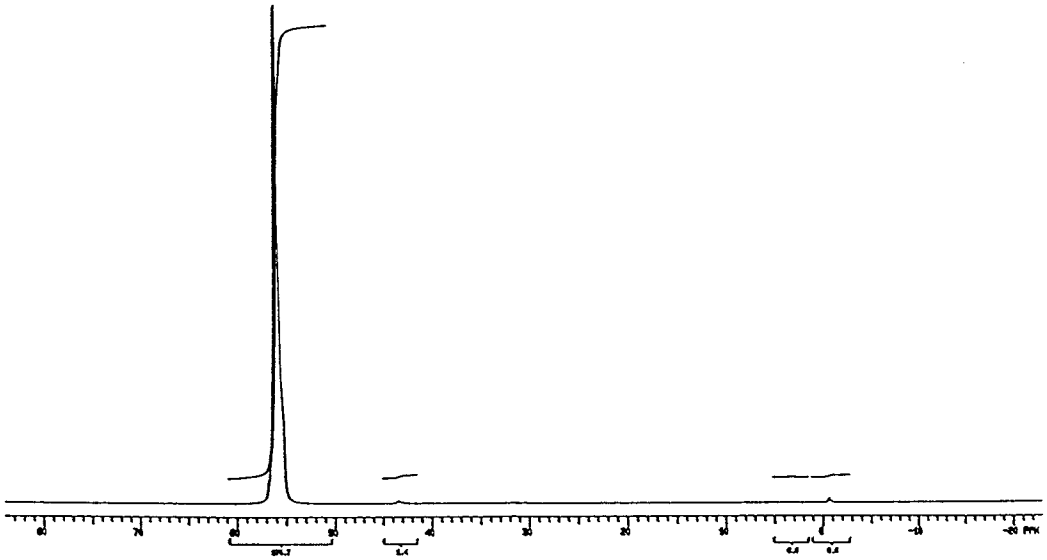

Figure 1. **^{31}P NMR of the oligonucleotide synthesized.**

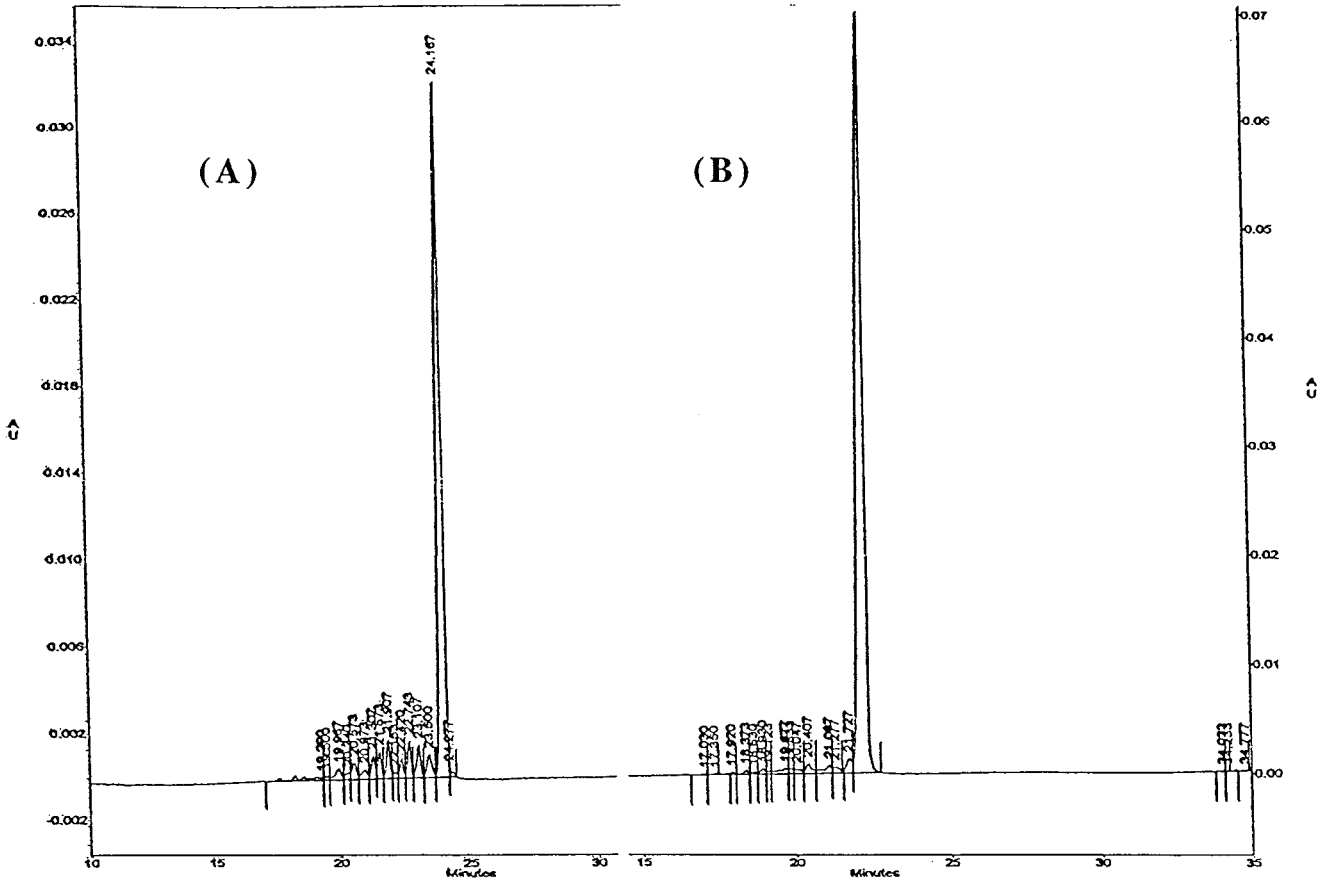

Figure 2. **Capillary gel electrophoresis (CGE) analysis of crude (A) and reversed phase HPLC purified (B) oligonucleotide synthesized on OligoPilot II synthesizer.**

30% aqueous ammonium hydroxide at 60 °C for 16 h. The crude product was purified by reversed phase HPLC,[20] and all DMT-bearing species were collected and analyzed. Figure 1 shows the ^{31}P NMR analysis of the crude oligonucleotide. Figures 2 and 3 show capillary gel electrophoresis (CGE)[21] and SAX HPLC of the purified oligonucleotide, respectively. Table 3 shows the results of using PTFA under different conditions.

Results and Discussion

During our extensive investigations on efficient, cost-effective, and environmentally safe routes for the synthesis

(20) HPLC: C column (Waters Nova Pak) 3.9 × 300 mm, flow rate 1.0 mL/min, CH_3CN (A), H_2O, gradient: 0−15 min; 2 to 98% A, 15 to 25 min: 98% A, $\lambda = 254$ nm.

(21) Capillary gel electrophoreses were performed using a Beckman P/ACE system 5000. Samples were electrophoretically injected into the capillary (100 mm i.d., total length 47 cm, effective length 40 cm) by applying a voltage of 5 kV for 2 s. Oligodeoxynucleotides were detected at 265 nm.

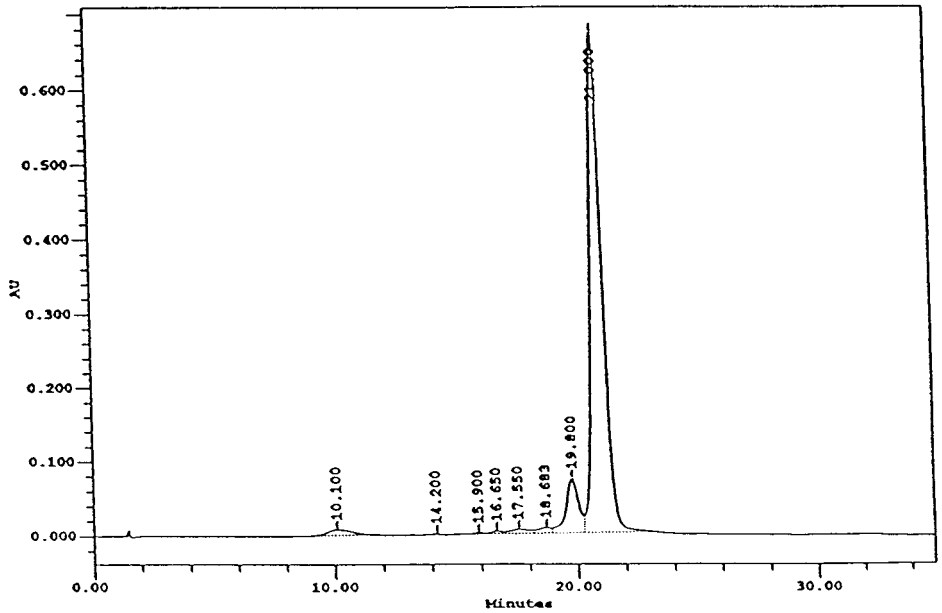

Figure 3. **SAX HPLC analysis of the purified oligonucleotide.**

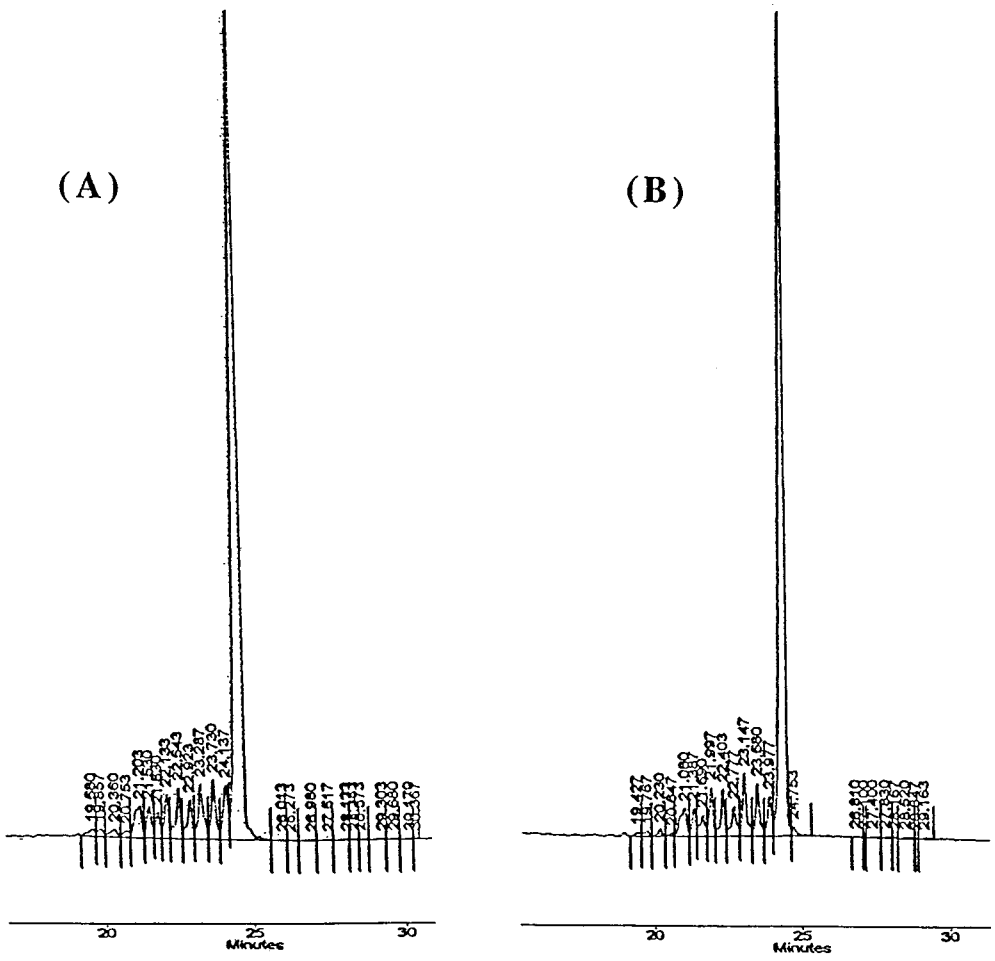

Figure 4. **CGE analysis of crude $(TGG)_6T$ synthesized using 1*H*-tetrazole (A) and PTFA as activator (B).**

of oligonucleotides for the market, we have identified toluene as a suitable replacement[22] for dichloromethane in the detritylation step and phenylacetyl disulfide (PADS) in 3-picoline−acetonitrile (1:1) (v/v) as an alternative[23] to Beaucage reagent[24,25] in the sulfurization step. These reagents have become standard for our manufacture of antisense drugs.

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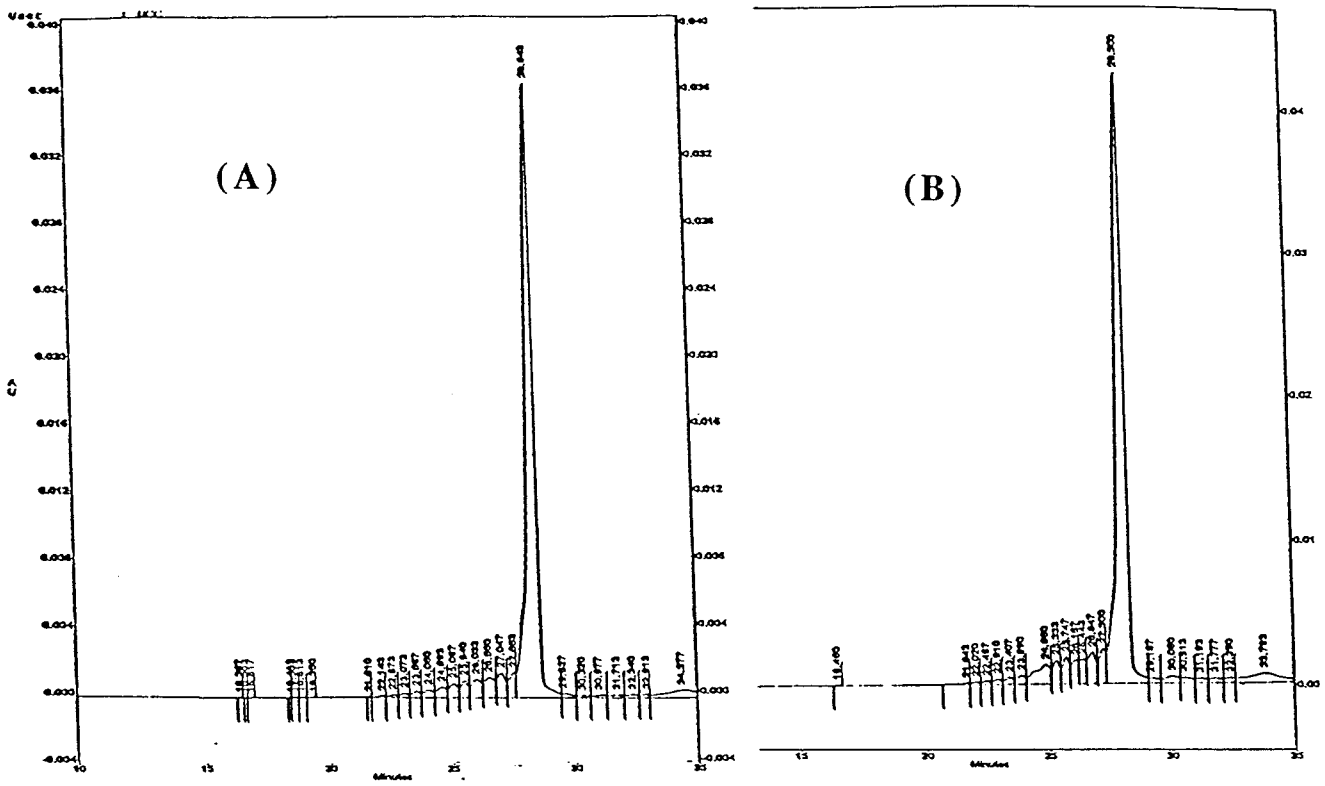

Figure 5. **CGE analysis of crude $U_{19}T$ synthesized using 1*H*-tetrazole (A) and PTFA as activator (B).**

Table 3: **Synthesis of ISIS 5132 using pyridinium trifluoroacetate**

activator	% full length by CGE	% (n+1)-mer	crude yield [OD/μmol]
0.22 M PTFA + 0.11 M NMI	81	1.4	103
0.12 M PTFA + 0.06 M NMI	74	0.65	116

In addition, the new activator reported here, because of its performance at phosphoramidite 1.75 equiv will become a standard reagent. The above synthetic cycle (Table 3) reflects these conditions, under which pyridinium trifluoroacetate/*N*-methylimidazole has been found to be an efficient activator for the synthesis of phosphorothioate oligonucleotides.

Recently, we have shown[26] that during oligonucleotide synthesis a small percentage of higher molecular weight products, referred to as "longmers" are formed in an activator-, base-, and contact time-dependent manner. Since undesired detritylation is a prerequisite for longmer formation, the problem is exacerbated in a dG-rich sequence (order of detritylation rates is dG > dA > dC > T). To investigate the extent of longmer formation using PTFA as activator in comparison to tetrazole, the model phosphorothioate sequence d(5′-TGG-TGG-TGG-TGG-TGG-TGG-T) was synthesized on an Amersham-Pharmacia Biotech OligoPilot I at 30 μmol scale using polystyrene Primer Support (90 μmol/g). 2.5 equiv of phosphoramidite per coupling (2 volumes of activator for 1 volume of amidite) were used. Coupling time was maintained at 3 min. The results are shown in Table 4. Figure 4 shows the CGE analysis of the crude oligonucleotide.

Table 4: **Comparison of longmer formation**

activator	% (n+1)-mer
1*H*-tetrazole	1.0
0.22 M PTFA + 0.11 M NMI	1.2

Synthesis of 2′-*O*-Methoxyethyl Oligoribonucleotide

Following up the growing preclinical and clinical success of oligodeoxyribonucleotide phosphorothioate antisense drugs, several second-generation oligonucleotide modifications are being investigated. Among evaluated sugar 2′-hydroxyl modifications, the 2′-*O*-methoxyethyl ether has emerged as a clear choice due to its attractive biophysical and pharmacological properties.[27−29] Several oligonucleotides containing this modification are presently being evaluated in pre-clinical studies. As an initial investigation, we explored utility of the new coupling activator PTFA in the synthesis of phosphorothioate 19-mer $U_{19}T$ where U represents 2′-*O*-

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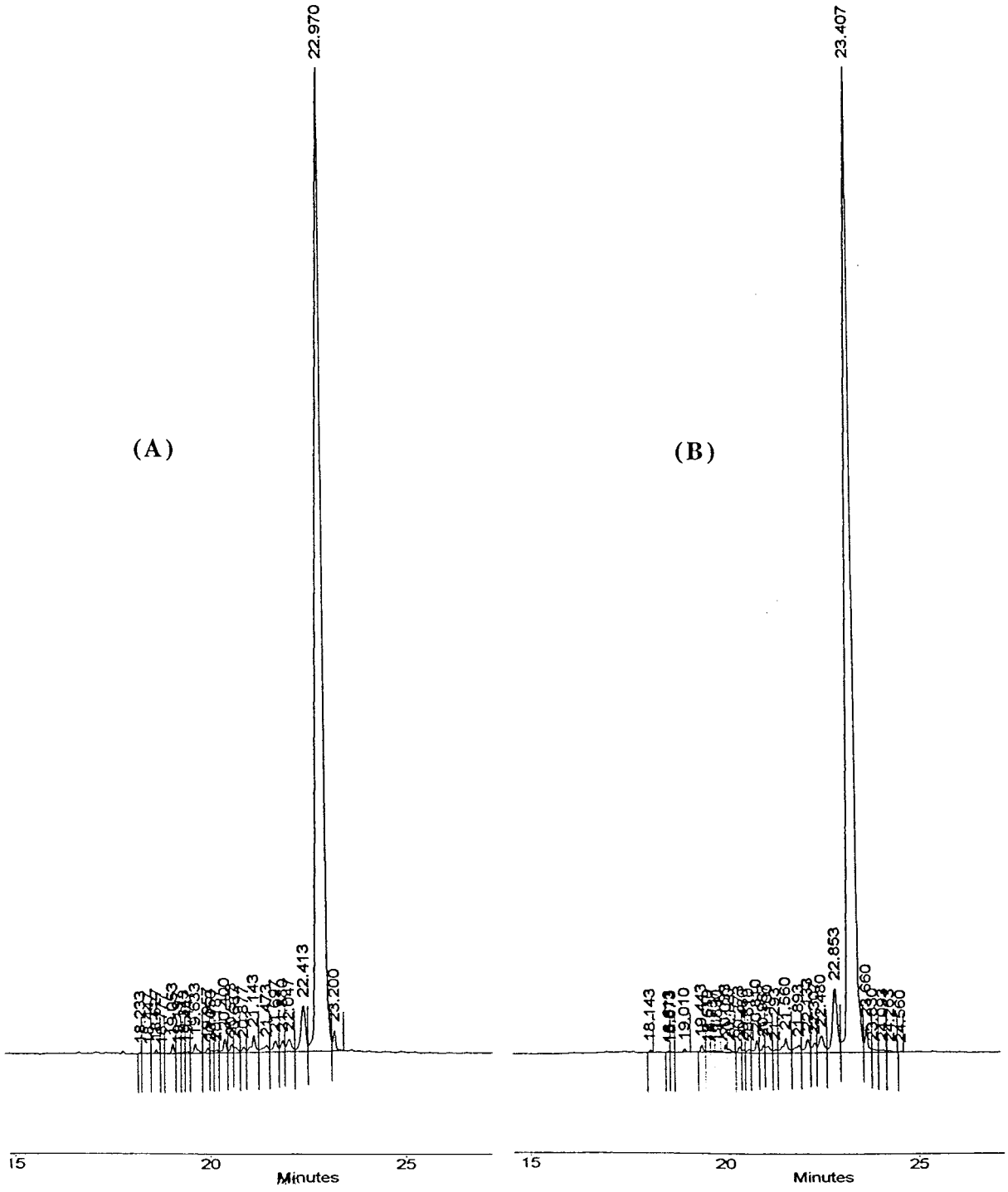

Figure 6. **CGE analysis of purified ISIS 5132 phosphate diester synthesized using 1*H*-tetrazole (A) and PTFA as activator (B).**

methoxyethyl-5-methyluridine. The synthesis was performed on an Amersham-Pharmacia Biotech OligoPilot I at 30 μmol scale using polystyrene Primer Support (90 μmol/g). 2.5 equiv of phosphoramidite per coupling (2 volumes of activator for 1 volume of amidite) were used. Coupling time was maintained at 6 min. Sulfurization was performed with 0.1 M solution of phenylacetyl disulfide (PADS) in 3-picoline−acetonitrile (1:1, v/v, 4 column volumes) for 60 s. Detritylation was performed in 3% dichloroacetic acid: toluene (v/v). Results are shown in Table 5. Figure 5 shows CGE analysis of the crude oligonucleotide.

Table 5: **Synthesis of 2′-*O*-methoxyethyl oligonucleotide**

activator	% full length by CGE	crude yield [OD/μmol]
1*H*-tetrazole	72	89
0.22 M PTFA + 0.11 M NMI	69	87

Synthesis of Oligodeoxyribonucleotide Phosphodiesters

Having demonstrated that PTFA/NMI is an efficient activator for the synthesis of phosphorothioates, we wanted

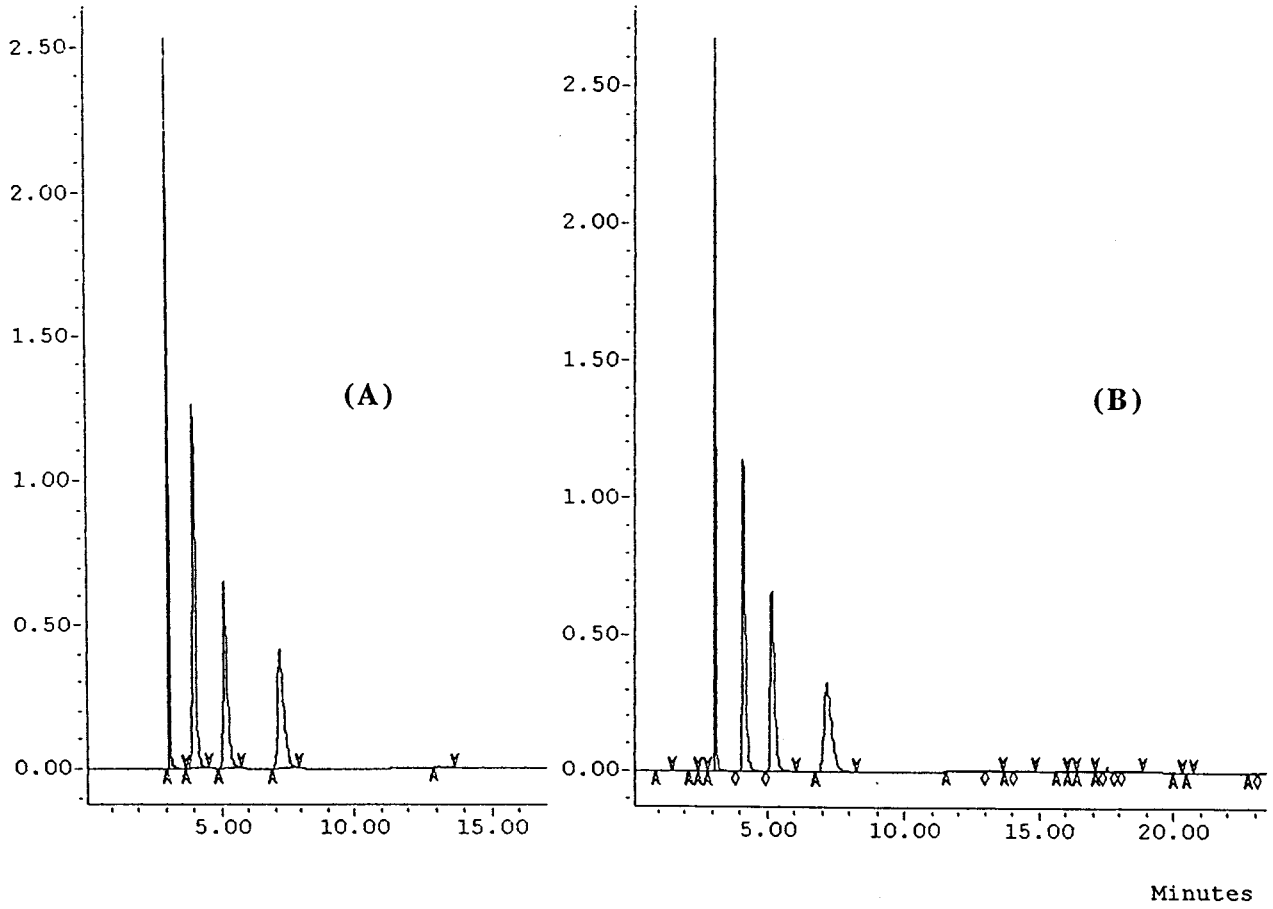

Figure 7. **Reversed phase HPLC chromatogram of digested ISIS 5132 phosphate diester synthesized using 1*H*-tetrazole (A) and PTFA as activator (B).**

to confirm that it is equally efficient for the synthesis of phosphodiesters. The phosphodiester analogue of ISIS 5132 d(5′-TCC−CGC−CTG-TGA-CAT-GCA-TT) was synthesized on 1 μmol scale using an ABI 390Z DNA/RNA synthesizer using controlled-pore glass as solid support. The standard coupling cycle as suggested by the vendor was used for the synthesis. Iodine was used for oxidation of the intermediate phosphites to form phosphate linkages. At the end of synthesis, the oligonucleotide was cleaved from the support, the base and phosphate-protecting groups were removed by incubating in 30% aqueous ammonium hydroxide at 60 °C for 16 h. The crude product was purified by reversed phase HPLC, and all DMT-bearing species were collected, detritylated, and analyzed. Figure 6 shows the capillary gel electrophoresis (CGE) analysis of the purified oligonucleotide. Also shown in Figure 6 is the analysis of ISIS 5132 phosphodiester oligomer synthesized using 1*H*-tetrazole as control.

Enzymatic Analysis of the Oligonucleotide Synthesized

Since side reactions are known to occur at the O^6 position of guanosine during coupling, it was important to test whether PTFA/NMI does any modification to the nucleobases. For this purpose, ISIS 5132 phosphate diester oligonucleotide that was synthesized above was subjected to enzymatic digestion to its nucleoside components. Figure 7 shows the HPLC analysis of the digested DNA. Also shown in Figure 7 is the analysis of the digested oligomer synthesized using 1*H*-tetrazole as control.

Mechanism of Activation

To determine the active species involved in the activation of phosphoramidites,[30,31] we synthesized *N*-methylimidazolium trifluoroacetate and used it as an activator in the synthesis of ISIS 5132. However, this experiment gave poor results (low trityl assay) indicating that *N*-methylimidazolium trifluoroacetate is not involved as an activator during the coupling step. In addition, Table 1 shows that pyridinium trifluoroacetate alone functions as a reasonably effective activator. Use of 0.11 M PTFA affords lower crude yield, while 0.22 M PTFA affords a higher percentage of longmers. PTFA (0.22 M), however, in combination with 0.11 M *N*-methylimidazole, substantially reduces (n+1)-mer levels while giving the expected crude yield. Thus, pyridinium trifluoroacetate appears to act as the activator, while *N*-methylimidazole increases basicity of the medium resulting in reduced detritylation and attendant longmer formation.[26] Also, *N*-methylimidazole is known to increase nucleophilicity of the attacking 5′-hydroxyl group, leading to increased yield.

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Conclusions

We have shown that phosphoramidite coupling in oligonucleotide synthesis proceeds rapidly and with no observable increase in formation of longmers when pyridinium trifluoroacetate/*N*-methylimidazole is used as the coupling activator. In summary, this activator meets all stringent criteria for use in the development of oligonucleotide drugs.[32] We highly recommend the use of this activator for the future synthesis of oligonucleotides and their analogues.

(32) Cost of the new activator is <$75/kg, whereas 1*H*-tetrazole is ~$950/kg at 100 kg scale.

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