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Large-Scale Synthesis of Antisense Oligonucleotides without Chlorinated Solvents

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Abstract:

It is demonstrated that mixed-sequence phosphorothioate oligodeoxyribonucleotides can be synthesized on scales from 1 μmol up to 80 mmol without using chlorinated solvents such as dichloromethane or dichloroethane, while preserving both high yield and purity of the product. A solution of dichloroacetic acid in organic solvents (e.g., toluene, xylenes, benzotrifluoride) cleanly and efficiently removes the 4,4′-dimethoxytrityl (DMTr) group from the 5′-terminus of the growing oligonucleotide chain during synthesis on solid support. We have therefore replaced hazardous dichloromethane, formerly used in oligonucleotide synthesis, as the solvent for DMTr-removal, with toluene.

Oligonucleotides are finding widespread application in diagnostics and molecular biology and as therapeutic agents. In recent years, DNA analogues, especially phosphorothioate oligonucleotides in which a nonbridging oxygen of the natural internucleotide phosphate group is replaced by sulfur, have emerged as potential drugs for treatment of diseases through an antisense mechanism of action.[1] With FDA approval of the first antisense oligonucleotide achieved[2] and several other oligonucleotides currently in human clinical trials, the development of economical large-scale oligonucleotide synthesis methods has become necesssary. In the standard synthesis of phosphorothioate oligodeoxyribonucleotides through phosphoramidite coupling,[3] removal of the acid-labile 5′-*O*-4,4′-dimethoxytrityl (DMTr)-protecting group from support-bound oligonucleotide plays a role in each cycle (Scheme 1) that is crucial to high product yield and quality.[4] Although several reagents have been developed for this purpose,[5] many have limited applicability to automated oligonucleotide synthesis on solid supports. The most commonly used reagents today are dilute solutions (2−15%) of an organic acid such as trichloroacetic acid (TCA, pK_a 0.8) or dichloroacetic acid (DCA, pK_a 1.5) in a halogenated solvent, typically dichloromethane. The high volatility (vapor pressure 380 mm at 22 °C) of dichloromethane and its high toxicity and carcinogenicity issues has resulted in stringent regulation by regulatory agencies.[6] As oligonucleotide synthesizers are now available for syntheses up to 0.2 mol scale,[7] the quantities of chlorinated waste generated have become quite large. Disposal of halogenated solvents is burdensome, and as a consequence solvent recycling may be preferred. In the context of developing synthesis methods for ton-scale manufacture of phosphorothioate oligonucleotides, we were interested in replacing dichloromethane as the deblocking reagent solvent with a less problematic solvent while preserving product yield and quality. We now report that several common organic solvents are as efficient in the deblocking step of oligonucleotide synthesis on solid support as hazardous halogenated solvents such as dichloromethane.

Results and Discussion

Since the introduction of small-scale solid-phase synthesizers (50 nmol to 10 μmol), removal of the acid labile DMTr-protecting group from the 5′-terminus of the growing chain during each synthesis cycle with a solution of an organic acid (dichloroacetic acid or trichloroacetic acid) in a halogenated solvent such as dichloromethane or dichloroethane has become standard procedure. On this synthesis scale, exposure to hazardous vapors is fairly limited, and the amount of chlorinated waste solvent generated is rather small. The advent of antisense technology spurred development of large-scale oligonucleotide synthesizers. Currently, oligonucleotide synthesizers are available for synthesis of 0.2 mol of oligonucleotide in one run. Even though the amount of deblock solution per oligonucleotide is reduced as a consequence of increased synthesis scale, the volume of deblock solution has increased, raising issues for handling

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(2) In August 1998, Isis Pharmaceuticals, Carlsbad, CA, and Ciba-Vision, a division of Novartis AG, Switzerland, received FDA approval for Vitravene (fomivirsen sodium injectible) for the treatment of cytomegalovirus (CMV) retinitis in patients with AIDS.

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Scheme 1

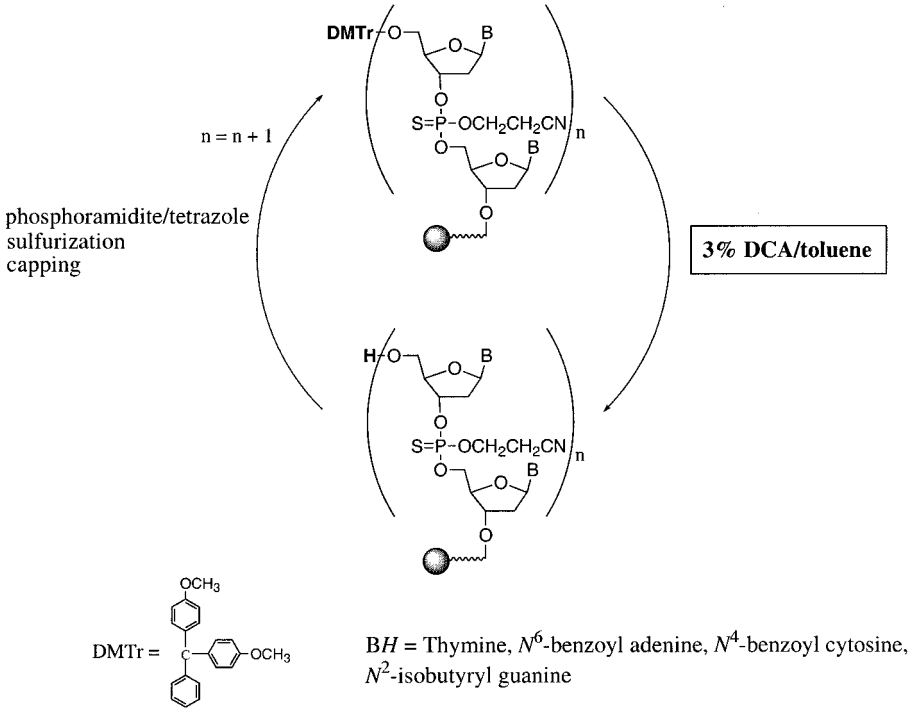

Table 1. **Phosphorothioate oligodeoxyribonucleotides 1−4 used in this study**

compound	sequence	biological target	clinical indication
ISIS 5132/CGP 69846A, **1**	5′-PS-d(TCCCGCCTGTGACATGCATT)	c-*raf* kinase	anti-cancer
ISIS 2503, **2**	5′-PS-d(TCCGTCATCGCTCCTCAGGG)	Ha-*ras*	anti-cancer
ISIS 3521/CGP 64128, **3**	5′-PS-d(GTTCTCGCTGGTGAGTTTCA)	PKC-α	anti-cancer
ISIS 2302, **4**	5′-PS-d(GCCCAAGCTGGCATCCGTCA)	ICAM-1	anti-inflammatory

and disposal of large volumes of halogenated solvents. In our ongoing cost-cutting efforts we wanted to replace dichloromethane with a solvent that is less harmful, recyclable, cheaper, and equally efficient for removing DMTr-protecting groups. A series of experiments with test sequences on a 1 μmol scale indicated that organic solvents such as benzene, toluene, xylenes, chlorobenzene, and benzotrifluoride[8] containing DCA (0.36 M) rapidly and efficiently remove DMTr groups from 5′-*O*-DMTr-protected nucleotides bound to controlled pore glass (CPG) solid support.[9] As there was no apparent benefit in using hazardous benzene or chlorobenzene, these solvents were not considered further. Reactor design and solid support are crucially important for successful large-scale oligonucleotide synthesis. In contrast to small-scale synthesizers (e.g., ABI 394 RNA/DNA Synthesizer) which typically use silica-based CPG solid support, large-scale synthesis (>10 mmol) is most commonly performed in packed-bed column reactors using polystyrene-based polymers as solid support. Therefore, four mixed-base phosphorothioate oligodeoxyribonucleotides [ISIS 5132/CGP69846A (**1**), ISIS 2503 (**2**), ISIS 3521/CGP 64128 (**3**), and ISIS 2302 (**4**), see Table 1] currently under evaluation as antisense therapeutic agents in clinical trials were synthesized on an OligoPilot II oligonucleotide synthesizer (Amersham Pharmacia Biotech, Uppsala, Sweden) closely resembling the production-scale synthesizer (OligoProcess). Polystyrene-based solid support (Primer support, Amersham Pharmacia Biotech) containing the 3′-terminal nucleoside of the sequence to be synthesized was tightly packed in a steel column (volume 6 mL). The synthesis scale range was between 150 and 170 μmol, depending on the nucleoside loading of the solid support (Primer support-dA: 96 μmol/g, Primer support-dG: 91 μmol/g, Primer support-T: 88 μmol/g). Details of the synthesis cycle are given in Table 2. Deblock solution (3% dichloroacetic acid/solvent, 50 mL) was passed through the column for 4 min. Removal of DMTr groups was followed spectrophotometrically. It is noteworthy that more than one column volume of deblock solution must be passed through the synthesis column before the orange color of the DMTr cation is first observed in the DMTr eluent. The first fractions of deblock solution that pass through the synthesis column are largely depleted of acid (through reaction with the DMTr-protected oligonucleotide and acid adsorption to the solid support and oligonucleotide, Scheme 2) and contain a significant amount of DMTr groups.[10] The occurrence of orange color (DMTr cation) is

(8) Benzotrifluoride has been suggested for replacement of dichloromethane in a series of organic transformations: Ogawa, A.; Curran, D. P. *J. Org. Chem.* **1997**, *62*, 450−451.

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Table 2. **Synthesis parameters of cycle used on OligoPilot II Synthesizer**

step	reagent	volume (mL)	time (min)
DMTr removal	3% dichloroacetic acid/solvent	50	4
coupling	phosphoramidite (0.2M), 1*H*-tetrazole (0.45 M)	1.6, 5	5
sulfurization	3*H*-1,2-benzodithiol-3-one-1,1-dioxide[a] (0.5 M)	4.5	0.5
	or phenylacetyl disulfide (0.2 M)[b]	6	2
capping	Ac_2O/pyridine/acetonitrile, NMI/acetonitrile	1.7, 1.7	0.5

[a] Reference 11a. [b] Reference 11b.

Scheme 2

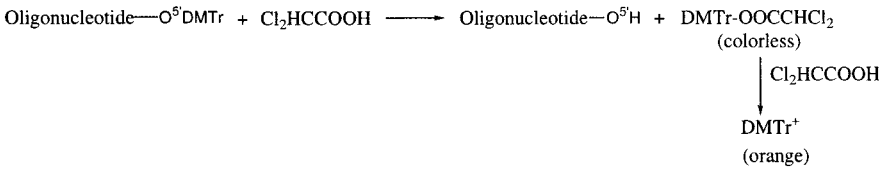

Table 3. **Analytical data of phosphorothioate oligonucleotides 1−4 synthesized using different deblock solutions[a]**

			Crude		HPLC-purified	
PS oligonucleotide	deblock solvent	crude yield [OD/μmol]	full length content (area-%)[b]	PO content[c,d]	full length content (area-%)[b]	relative ($n-1$) cont.[b]
1	CH_2Cl_2	125	72	0.6	94	2.4
	toluene	124	69	0.8	95	2.4
	xylene	125	72	0.5	95	2.4
	benzotrifluoride	127	69	0.8	93	2.3
2	CH_2Cl_2	123	73	0.5	93	2.9
	toluene	121	79	0.3	92	3.5
	xylene	123	79	0.7	92	3.5
	benzotrifluoride	124	73	0.4	89	3.3
3	CH_2Cl_2	133	73	0.6	92	5.0
	toluene	129	74	0.7	90	4.5
4	CH_2Cl_2	148	75	0.3	92	4.3
	toluene	148	73	0.2	92	3.6
	toluene (rec.)	148	73	0.3	91	4.2

[a] Dichloroacetic acid/deblock solvent. [b]CGE. [c] ^{31}P NMR. [d]Sulfurizing agent for **1**−**3**: 3*H*-1,2-benzodithiol-3-one-1,1-dioxide,[11a] for **4**: phenylacetyl disulfide.[11b]

directly associated with the elution of dichloroacetic acid. Quantitative analysis of the detritylation step is typically performed by measuring either UV absorption or conductivity. Conductivity-based DMTr yields were obtained when halogenated solvents such as dichloromethane, chlorobenzene, or benzotrifluoride were used. In contrast, no conductivity-based detritylation yield was obtained in solvents such as benzene, toluene, or xylenes. The efficiency of the syntheses and the quality of the oligonucleotide product were judged by the yield, the full-length content of crude and purified oligonucleotide product, as well as by the relative amount of ($n-1$)-mer formed [determined by capillary gel electrophoresis (CGE)]. For purification, we used a C_{18} reversed phase HPLC method that allowed facile separation of the 5′-*O*-DMTr-on oligonucleotide from capped failure sequences. The total DMTr-on peak was collected without further fractionation. The results of CGE analysis are summarized in Table 3. Full-length contents of 73 ± 4% are typical when DCA (0.36 M) in dichloromethane is used for DMTr removal. Purification allows for separation of shorter DMTr-off failure sequences, thus increasing the full-length content to 93 ± 2% at good yield and reducing relative ($n-1$)-mer content to 2.4−5.0%, depending upon the sequence. The low relative ($n-1$)-mer contents of the DMTr-on fraction in HPLC-purified oligonucleotide product demonstrate very clearly that DMTr removal with DCA in aromatic solvents is as efficient as it is with DCA in dichloromethane. The phosphodiester content, as determined by ^{31}P NMR spectroscopy, was also within limits typically observed. Analytical results for oligonucleotides **1** and **2**

(10) In a synthesis of ISIS 5132, both colorless and orange-colored DMTr eluates were collected separately. The two fractions obtained for each coupling step were diluted with 3% DCA in toluene, and the DMTr content was quantitated spectrophotometrically at 510 nm. It was found that 40 to 60% of the total DMTr groups are eluted in the "colorless" DMTr eluent.

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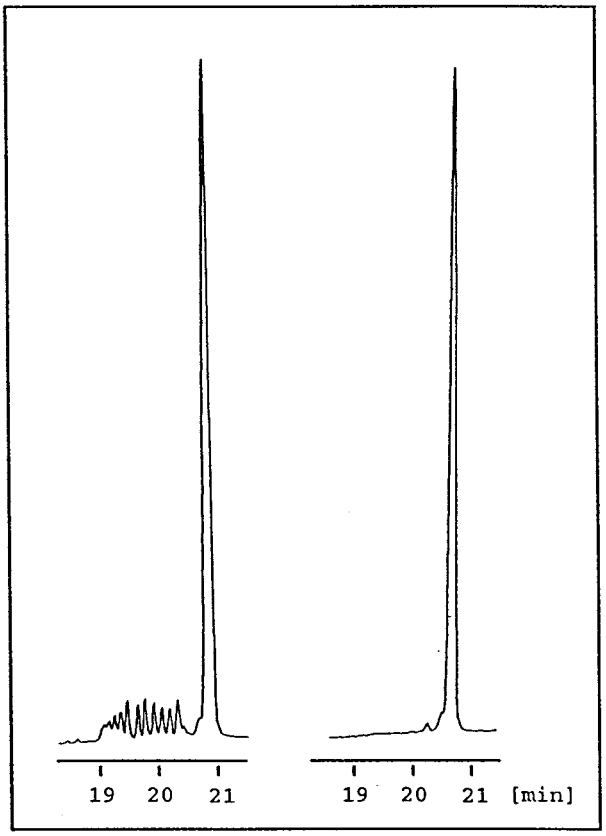

Figure 1. **CGE traces of crude (left) and RP-HPLC purified (right) phosphorothioate oligonucleotide 4 using 3% dichloroacetic acid/toluene for detritylation.**

synthesized with 3% DCA in toluene, xylenes, or benzotrifluoride were identical, within variation observed when 3% DCA in dichloromethane was used. Considering cost, boiling point (recycling), and viscosity (potential pressure build-up during large scale synthesis) issues, we found toluene to be the best alternative to dichloromethane for DMTr removal. Subsequent syntheses of **3** and **4** using 3% DCA/toluene solution confirmed earlier conclusions for other sequences.

Since recycling of solvents may become an issue of ton-scale production of oligonucleotides, we collected the deblock solutions after each DMTr removal. A simple distillation allowed us to recover toluene of high purity that was reused as deblock solvent in a subsequent synthesis of **4**. The analytical data of oligonucleotides synthesized with "new" or "recycled" toluene are identical (Table 3).

Next, we scaled up the synthesis for **4** another 500-fold using 3% DCA in DCM or 3% DCA in toluene to 80 mmol scale (OligoProcess). For large-scale synthesis it is crucial to optimize the deblocking step to minimize depurination as a yield-limiting side reaction. Detritylation rates decrease in the order DMTr-dG^{ib} > DMTr-dA^{bz} > DMTr-dC^{bz} > DMTr-T, and also, as the chain length of the support-bound oligonucleotide increases, longer acid treatments are necessary. To account for the individual DMTr removal times we used spectrophotometric monitoring with feedback to keep acid exposure time at a minimum. Again, we find equivalency of the crude (full length content 71.4% using DCM, 74.1% using toluene) and purified products (96.5 and 96.3%, respectively). CGE traces of crude and purified oligonucleotide **4** are shown in Figure 1.

Conclusions

As is evident from the data presented in this paper, a solution of dichloroacetic acid in toluene (or xylenes or benzotrifluoride) allows the removal of 4,4′-dimethoxytrityl groups from support-bound oligonucleotides to give antisense oligonucleotides **1**−**4** in high yield and purity. Multiple analytical methods established equivalence of **1**−**4** synthesized with dichloroacetic acid in toluene as delock reagent with oligonucleotides synthesized with the standard solution of dichloroacetic acid in dichloromethane. In conclusion, we have shown that hazardous dichloromethane can be replaced as the solvent for deblocking in solid-phase synthesis by toluene, without compromising the yield or the quality of oligonucleotide products.

Experimental Section

5′-*O*-Dimethoxytrityl-3′-*N*,*N*-diisopropylamino-*O*-(2-cyanoethyl) phosphoramidites (T, dA^{bz}, dC^{bz}, dG^{ib}) (Amersham Pharmacia Biotech, Uppsala, Sweden), 1*H*-tetrazole (American International Chemical, Natick, MA), 3*H*-1,2-benzodithiol-one-1,1-dioxide (R.I. Chemical, Orange, CA), acetonitrile anhydrous (J. T. Baker, Phillipsburg, NJ) and toluene (Gallade, Escondido, CA) were used as received. ^{31}P NMR spectra were recorded on a Unity-400 spectrometer (Varian, Inc., Palo Alto, CA) operating at 161.9 MHz. Capillary gel electrophoresis was performed on a eCAP ssDNA 100 Gel Capillary (47 cm) on a P/ACE System 5000 using Tris/borate/7 M urea buffer (all Beckman), running voltage 14.1 kV, temperature 40 °C. Analysis and purification of oligonucleotides by reversed-phase high performance liquid chromatography (RP-HPLC) was performed on a Waters NovaPak C_{18} column (3.9 × 300 mm) using a Waters HPLC system (600E system controller, 996 photodiode array detector, 717 autosampler). For analysis an acetonitrile (A)/0.1 M triethylammonium acetate gradient was used: 5 to 35% A from 0 to 10 min, then 35 to 40% A from 10 to 20 min, then 40 to 95% A from 20 to 25 min, flow rate = 1.0 mL/min; for DMTr-on/DMTr-off purification: 5 to 27% A from 0 to 1 min, 27% A from 1 to 8 min, 27 to 50% A from 8 to 9 min, 9 to 26 min at 50%, flow rate = 1.0 mL/min, t_R(DMTr-off) 10−11 min, t_R(DMTr-on) 14−16 min. The DMTr-on fraction was collected, evaporated in vacuo, and redissolved in 50 μL of water, and the DMTr group was removed with dilute acetic acid (25%).

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