Organic Process Research & Development **2000**, *4*, 214−224

The Dimethoxytrityl Resin Product Anchored Sequential Synthesis Method (DMT PASS): A Conceptually Novel Approach to Oligonucleotide Synthesis

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Abstract:

Presented is a conceptually novel approach to oligonucleotide synthesis referred to herein as dimethoxytrityl resin product anchored sequential synthesis (DMT PASS). The DMT PASS process is characterized by the reaction of a 3′-protected nucleoside or oligonucleotide with an excess of a nucleotide 3′-phosphoramite or *H*-phosphonate which is bound to a dimethoxytrityl functionalized polystyrene resin. As a result, successfully coupled oligonucleotide product is then attached to the solid support, allowing for removal and potential recovery of starting materials. The protected oligonucleotide product is then released and subjected to an aqueous/organic extractive purification, which serves to remove monomeric impurities. The PASS process is anticipated to provide for the cost-effective manufacture of oligonucleotides on a scale which would allow for clinical development and pharmaceutical product commercialization. Herein we describe some aspects of our process development progress and discuss preliminary applications of the process to the synthesis of short oligodeoxyribonucleotide sequences, as well as a number of challenges confronting the PASS development effort.

Automated solid-phase synthesis allows for rapid preparation of small-scale amounts (0.1−10 μmol) of oligonucleotides at research scales.[1] The use of antisense oligonucleotides as a means for controlling gene expression and the potential for using oligonucleotides as pharmaceutical agents has necessitated the synthesis of gram to kilogram quantities. However, when challenged with cost-effectively providing larger quantities of modified oligonucleotides for drug development, this technology shows major limitations including lack of predictability in scale-up and high reagent and raw material costs. Considerable effort has been devoted to solid-phase oligonucleotide scale-up and has resulted, after many years of development, in the availability of 1, 10, and 100 mmol instruments. While these are significant achievements in scale, the adaptation of these instruments to a particular oligonucleotide compound remains laborious and costly. Furthermore, this development does not compare to the predictable and rapid scale-up of conventional synthetic organic processes.

An additional limitation of conventional solid-phase oligonucleotide synthesis at large scales lies in the contamination of the crude product by highly homologous failure sequences that arise from incomplete coupling, detritylation, and/or capping failures during synthesis. As a result, the purification of a crude oligonucleotide batch to a state acceptable for clinical studies is cumbersome, usually involving at least two chromatography (anion-exchange and reversed phase) steps. This downstream processing places serious limitations on scale-up and efficiency.

An ideal oligonucleotide manufacturing process would employ less expensive raw materials (e.g., nucleosides vs nucleoside phosphoramidites), allow for ready recovery and/or recycling of unconverted advanced synthetic intermediates, and resins, and yield a highly pure crude product that requires little or no scale-limiting chromatographic purification. We present herein a conceptually novel process which we feel could ultimately satisfy these criteria called the dimethoxytrityl resin product anchored sequential synthesis process (DMT PASS process).[2,3] We will describe some aspects of our process development progress and discuss preliminary applications of the process to the synthesis of short oligodeoxyribonucleotide sequences.

The DMT PASS is shown in Scheme 1 and summarized in Table 1. As can be seen, the approach reverses the traditional modality of solid-phase oligonucleotide synthesis. A suitably *N*-protected nucleoside[4] **4** is covalently bound to a 2% polystyrene resin support[5] at the 5′-position via a DMT functional linkage (**9**). A resin-bound nucleoside 3′-phosphoramidite **2** is then prepared, and in this manner, the reactive resin may then serve as the solid support for the subsequent coupling. By coupling a solution phase 3′-*O*-protected oligonucleotide substrate **3** to the resin-bound amidite, the advantages of traditional solid-phase oligonucle-

(1) Beaucage, S. L.; Iyer, R. P. *Tetrahedron* **1992**, *48*, 2223−2311. (b) *Oligonucleotides. Antisenese Inhibitors of Gene Expression*; Cohen, J. S., Ed.; CRC Press: Boca Raton, FL, 1989.

(2) The following abbreviations are used throughout this account: ACN, acetonitrile; AcCl, acetyl chloride; Abz, 4-acetoxybenzyl; TBS, *tert*-butyldimethylsilyl; TBDPS, *tert*-butyldiphenylsilyl; DCA, dichloroacetic acid; DCM, dichloromethane; DCI, 4,5-dicyanoimidazole; DMT PASS, dimethoxytrityl resin product anchored sequential synthesis; DMF, dimethylformamide; DIPA, diisopropylamine; DIPEA, diisopropylethylamine; EtOAc, ethyl acetate; HPLC, high-pressure liquid chromatography; LC/MS, liquid chromatography/mass spectrometric; MMT, monomethoxytrityl; NPE, 4-nitrophenethyl; TBAP, tetrabutylammonium periodate; v/v volume/volume.

(3) (a) Pieken, W. P.; Gold, L. "Method for solution phase synthesis of oligonucleotides and peptides." US Patent 5,874,532, Issued February 3, 1999. (b) Pieken, W.; Mcgee, D.; Settle, A.; Zhai, Y.; Huang, J. Method for solution phase synthesis of oligonucleotides. *PCT Int. Appl.*

(4) For the purposes of this account, unless otherwise specified, the following nucleosides were employed: thymidine (T), N^6-benzoyl-2′-deoxyadenosine (dA^{Bz}), N^2-isobutyryl-2′-deoxyguanosine (dG^{iBu}), and N^4-benzoyl-2′-deoxycytidine (dC^{Bz}). 3′-Protected monomers possessed a 3′-*O*-*tert*-butyldiphenylsilyl ether (e.g., 3′-*O*-TBDPS-dA^{Bz}).

(5) The polystyrene support employed in these studies was prepared according to the protocol of Farrall and Fréchet (Farrall, M. J.; Fréchet, J. M. J. *J. Org. Chem.* **1976**, *41*, 3877−3882).

 10.1021/op990095t CCC: $19.00 © 2000 American Chemical Society and The Royal Society of Chemistry
Published on Web 02/23/2000

Scheme 1. **DMT resin PASS cycle**

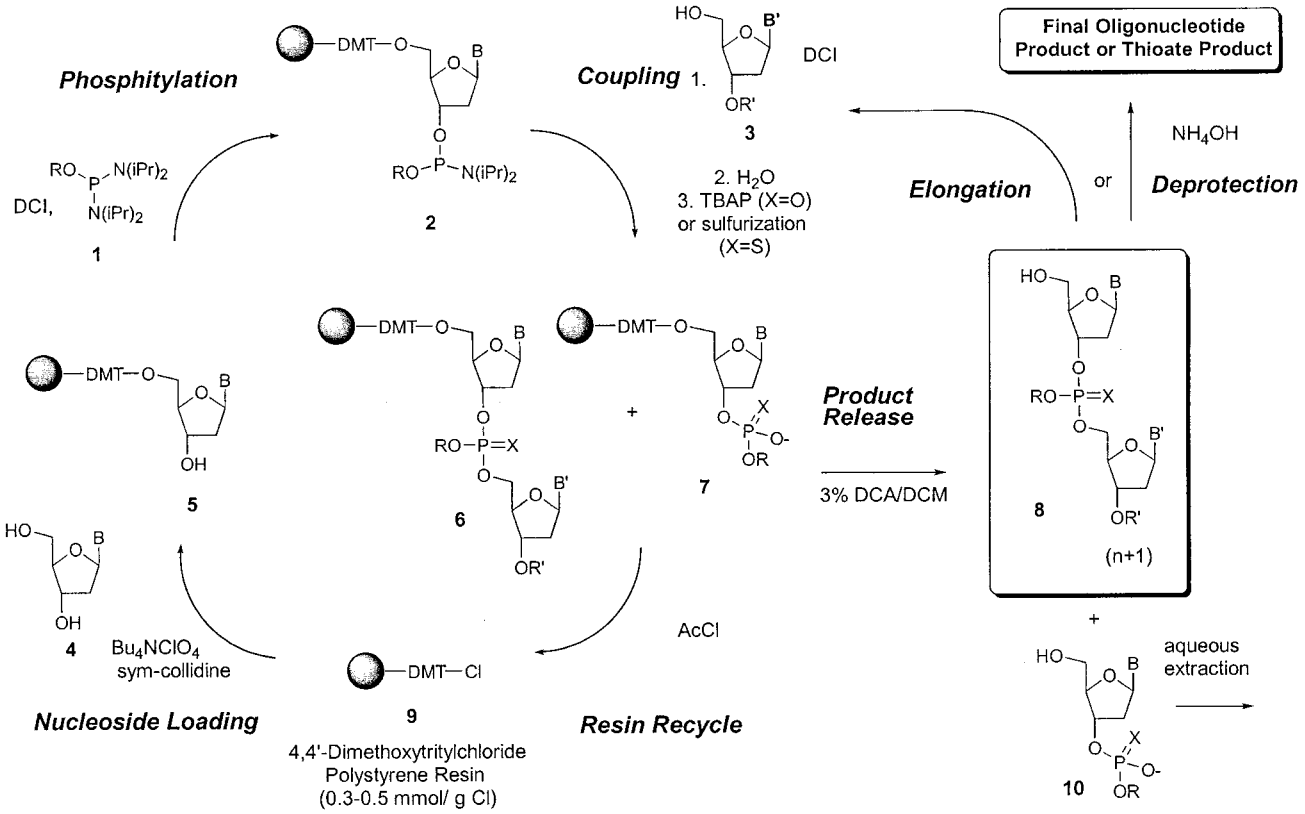


Table 1. **Summary of the DMT PASS process[2]**

	operation	procedure	comment
1	coupling	1.0−5.0 equiv amidite resin, 1.5 h reaction.	Extent of reaction monitored by HPLC.
2	quench	10% v/v H_2O (20 equiv) in ACN	Hydrolyzes excess resin-bound amidite monomer to the *H*-phosphonate diester.
3	oxidation	1.0 equiv TBAP in DCM, 0.5 h reaction	Oxidizes phosphite triester inter-nucleotide linkage to the phosphate triester and the hydrolzed amidite to the phosphate diester.
4	washes	10 mL/g resin (10x) alternating DCM/ACN	Failure sequences may be removed and recovered if necessary.
5	release	6 mL/g resin (5×) 3% DCA in DCM	Product is released directly into 0.2 M $NaHPO_4$ to minimize depurination.
6	oxidative wash and aqueous extractions	0.2 M Na_2HPO_4 (1×) 0.2 M $NaIO_4$ (1×) 0.2 M Na_2HPO_4 (3×)	Oxidizes and removes monomeric impurities.
7	drying	0.5 g Na_2SO_4/mL solvent	Followed by solvent removal to provide crude product.

otide synthesis, including facile removal of reagents and reactants from the growing oligo chain, are retained. However, in contrast to conventional solid-phase synthesis, this coupling step may be easily monitored in process, and should coupling fail, the valuable fully protected growing oligonucleotide intermediate **3** can be readily recovered. After a successful coupling sequence, release of the $n + 1$ oligonucleotide **8** from the DMT resin is accomplished. Purification of the growing chain after every cycle is achieved by simple aqueous/organic extraction, exploiting the differential solubility of the fully protected oligo **8** and the monomeric by-products (primarily diester **10**). It is noteworthy that, unlike crude products from solid-phase synthesis, oligonucleotides prepared according to this conceptual approach contain no highly homologous impurities or by-products. Additionally, the exhausted DMT functional resin can be recycled. It is conceivable that oligonucleotides prepared via the DMT PASS process may require no further chromatographic purification, and this feature, along with the recyclable nature of the resin, is anticipated to add significantly to the commercial viability of the process as applied to the large-scale synthesis of oligonucleotides.

In this account, aspects of the resin selection, preparation, and recycling will be discussed, followed by resin nucleoside loading and phosphitylation, as well as analytical characterization of the key nucleoside phosphoramidite resins. Next,

Table 2. **DMT polystyrene resin recycling**

entry	original nucleoside loading (mmol/g resin)	nucleoside loading after recycling (mmol/ g resin)	original amidite loading (mmol/g; as det'd by DIPA assay[8])	amidite loading after recycling (as det'd by DIPA assay)	original amidite loading (as det'd by coupling assay)	amidite loading after recycling (as det'd by coupling assay)
1	0.40	0.29	0.30	0.29	0.13	0.247
2	0.41	0.527	0.35	0.397	0.34	0.189
3	0.28	0.380	0.27	0.40	0.22	0.228
4	0.37	0.376	0.37	0.37	0.14	0.211
5	0.41	0.309	0.35	0.457	0.34	0.152

the coupling and oxidation steps will be delineated, followed by discussion of the oligodeoxyribonucleotide product release and extractive purification procedures. Analytical characterization and physical properties of DMT PASS intermediates will be discussed as will deprotection of these intermediates to afford short oligodeoxyribonucleotide products. Finally a summary of the problems and challenges of oligodeoxyribonucleotide synthesis via DMT PASS will be offered, as will a description of our efforts to address these challenges and insights into future PASS process development efforts.

Discussion

While polystyrene possesses some properties which complicate the PASS process (vida infra), the ready availability, durability, and predictability of the resin have resulted in the vast majority of our process development work to be conducted with polystyrene. While limited initial studies were carried out with commercially available (Novabiochem) MMT polystyrene resin, 2% cross-linked DMT polystyrene resin (**9**; Scheme 1, prepared according to the Farrell and Fréchet protocol) was found to have superior performance in our application.[6] We have produced 500 g lots of DMT resin **9** and have established the suitability of recycled resin in PASS cycles (vida infra*;* See Experimental Section for resin recycling procedure).

The nucleoside loading step consists of condensation of DMTCl polystyrene resin **9** and a solution-phase, base-protected nucleoside substrate. It employs tetrabutylammonium perchlorate and *sym*-collidine in DMF. The nucleoside loading of resin **5** (expressed in mmoles of nucleoside/gram of resin) is typically determined through nucleoside cleavage with 3% dichloroacetic acid/dichloromethane, followed by UV quantitation of the released nucleoside. Phosphitylation of the resin-bound nucleoside is carried out by treatment with either 2-cyanoethoxy-bis(diisopropylamino)phosphine (**1**, R = 2-cyanoethyl) or 2-(4-nitrophenethyloxy)-bis(diisopropylamino)phosphine (**1**, R = NPE) in the presence of 4,5-dicyanoimidazole (DCI).[7] Active amidite loading of resin **2** can be determined by a standard nucleoside coupling assay (see Experimental Section) or via FT/IR quantitation of released diisopropylamine upon treatment of a sample with excess DCI.[8] This two-step sequence for nucleoside loading and phosphitylation has been semi-automated by a modified program on an ABI 394 instrument and production of phosphitylated resin **2** (R = 2-cyanoethyl) in lots of hundreds of grams has been routinely conducted. The phosphoramidite resins are stable and, after drying, can be stored at −15 °C for months without any appreciable loss of activity.

Table 2 shows the results from several resin recycling runs. As can be seen, nucleoside loading and resin performance in coupling assays were comparable for virgin and recycled resins.

A substantial amount of PASS cycle optimization has been conducted, particularly regarding the reaction conditions for the coupling step. It has been found that coupling efficiency, as defined by the number of equivalents of resin **2** required to fully consume a model alcohol substrate, is dependent on a number of variables including solvent composition, amidite resin loading, alcohol (oligonucleotide) substrate concentration, and coupling time. Solvent composition, in particular, proved especially important. It is known that the formation of the polar intermediates involved in the activation of phosphoramidites by weak acids such as tetrazole or DCI is favored by the polar aprotic solvent acetonitrile.[9] However, the swelling properties of polystyrene in acetonitrile are poor, and we found it necessary to conduct the coupling step in an acetonitrile/dichloromethane mixture. Although resin-bound amidites showed improved coupling efficiency with an increasing fraction of acetonitrile in dichloromethane, when the acetonitrile concentration exceeds a certain value, the resin no longer is effectively swollen and the coupling efficiency is diminished. The optimal solvent composition was found to be approximately 70:30 acetonitrile/dichloromethane.

Coupling of 5′-*O*-TBDPS-dT with polystyrene DMT-dT resin (1.5 equiv) and DCI (1.5 equiv) was shown to proceed optimally at nucleoside concentrations of roughly 0.1 M. At concentrations below this level, significantly reduced coupling yields were observed; therefore, oligonucleotide substrates (**3**, Scheme 1) must be soluble at or above this concentration in the 70:30 acetonitrile/dichloromethane mixture for successful PASS cycles.

(6) Typically, considerably larger excesses (up to 15 equiv vs 1−2 equiv) of MMT resin-bound amidites were required to accomplish full conversion of fully protected oligonucleotide substrates.

(7) Vargeese, C.; Carter, J.; Yegge, J.; Krivjansky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic Acids Res.* **1998**, *26*, 1046.

(8) Mihaichuk, J.; Tompkins, C.; Pieken, W. A Quick and Simple Method to Accurately Determine the Extent of Solid-Phase Reactions by Monitoring an Intermediate of a Non-Destructive Controlled Reaction, manuscript in preparation.

(9) (a) Dahl, B.; Neilsen, J.; Dahl, O. *Nucleic Acids Res.* **1987**, *15*, 1729−1743. (b) Berner, S.; Muehlegger, K.; Seliger, H. *Nucleic Acids Res.* **1989**, *17*, 853−865.

Table 3. **Oligonucleotide sequences produced using the DMT PASS process: cycle yields and product purity**

entry	product sequence[a] (equiv resin)	purity[b]	yield[c]	decyanoethylation (%)	deprotection purities[d] AE	CE
1	T-3′-3′-ATGTC-5′ (4)	83	88	6	82	82
2	3′-ACTG-5′ (3.2)	95	64		80	70
3	3′-ACTGCC-5′ (3.1)	91	76	9	67	74
4	3′-ACTGCCTA-5′ (3.4)	92	84	25		68
5	3′-ACTGCCTATTT-5′ (5.2)	72	15	66	61	64

[a] All nucleosides are 2′-deoxy. [b] Purity of protected oligonucleotide as determined by reverse phase HPLC inclusive of observed decyanoethylated species. [c] Yields determined by mass of isolated product. [d] Deprotection is performed using standard conditions: ammonium hydroxide at 55 °C (12 h), followed by treatment with tetrabutylammonium fluoride. Purities are determined by either anion-exchange HPLC (AE) or by capillary electrophoresis (CE).

Table 4. **Physical properties of some fully protected oligonucleotides: solubility and mass spectra**

entry	product sequence (protected)[a]	solubility, mg/mL ACN	DCM	7:3 ACN/DCM	mass spec MW of product (calcd/obsd)
1	5′-*O*-TBDPS-dT	46	>500		
2	T-3′-3′−A-5′				951.0/950.6
3	T-3′-3′-AT-5′	28	508	223	1308.3/1307.8
4	T-3′-3′-ATG-5′	21	0.3		1760.6/1760.8
5	T-3′-3′-ATGT-5′	12	35		2117.9/2118.1
6	T-3′-3′-ATGTC-5′	3.7	0.8		2564.2/2564.4
7	T-3′-3′-ATGTCT-5′	3.3	1.8	2.6	2921.5/2921.8
8	T-3′-3′-ATGTCTG-5′	4.5	1.8	2.4	3373.8/3372.8
9	T-3′-3′-ATGTCTGT-5′		0.2		3731.1/3730.8
10	3′-ACTG-5′	21	0.3		1173.8/1173.1
11	3′-ACTGCC-5′	5.5	15.3		1752.19/1751.6
12	3′-ACTGCCTA-5′	0.4	0		2369.59/2372.6
13	3′-ACTGCCTATT-5′	0	0		
14	3′-ACTGCCTATTT-5′	0	0		
15	3′-ACTGCCTAA-5′	0.2	0.6		

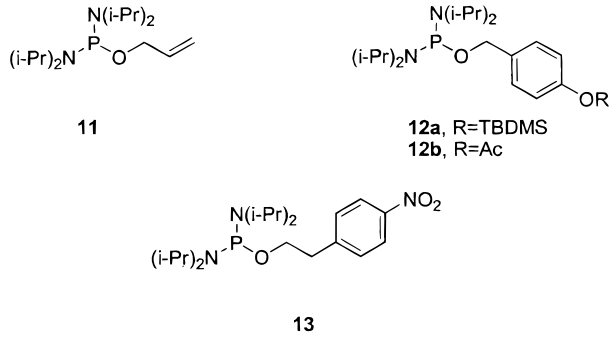
[a] All nucleosides are 2′deoxy.

Tetrabutylammonium periodate (TBAP) has proven to be an effective oxidant for the DMT PASS process. Stoichiometry studies reveal a molar ratio of 0.3−0.5 TBAP is all that is necessary to effect complete conversion of the P(III) intermediate. However, as will be discussed (vida infra) limiting the oxidation reaction time can reduce side reactions such as phosphate deprotection and premature cleavage of the product from the support.

Some results from the initial application of the DMT PASS process to the synthesis of short oligodeoxynucleotide sequences are summarized in Tables 3 and 4.

These preliminary experiments not only served to illustrate the concept of oligo synthesis via PASS but also exposed some shortcomings of the chemistry employed in DMT PASS process development studies to date. In particular, we found that fully protected oligos, once four to five nucleotides or more in length, became largely insoluble in the process

Scheme 2. **Alternative phosphitylation reagents**



solvents (dichloromethane, acetonitrile). Furthermore, LC/MS analysis of pentameric or longer oligodeoxyribonucleotide intermediates revealed the loss of cyanoethyl-protecting groups to a significant extent, which undoubtedly contributes to the diminished organic solubility. To address both of these issues, more robust and lipophilic alternatives to the 2-cyanoethyl group were considered. A number of phosphate-protecting groups have been studied, and Scheme 2 shows several reagents which were evaluated in the context of the DMT resin PASS process.[10]

Each of these alternative phosphate-protecting groups was eventually ruled out for different reasons. The commercially available phosphitylating reagent **11** (Aldrich) affords allyl-protected phosphate triester products;[11] however, trimeric and tetrameric substrates prepared with this modification did not display dramatically improved solubility relative to the analogous 2-cyanoethyl-protected sequences. The substituted benzyl phosphitylating reagents **12a** and **12b** were designed;[12] however, the 4-*tert*-butyldimethylsilyloxy benzyl phosphate substitution rendered oligos too hydrophobic to be soluble in acetonitrile solvent mixtures required for the coupling step. Acetoxybenzyl (Abz) phosphate-protected oligo intermediates derived from reagent **12b** were considerably more difficult to prepare than the corresponding 2-cyanoethyl intermediates. This was due in part to partial loss of the phosphate-protecting group upon oxidation with TBAP and to the fact that excess Abz-protected monomeric by-products could not be readily removed via extraction. This latter issue will be discussed further (vida infra).

Finally, limited success was realized with the Pfleiderer 4-nitrophenethyl (NPE) phosphitylating reagent **13**.[13] The pentameric, fully NPE-protected oligo sequence shown in Table 5 was prepared, although reversed phase HPLC purification at each step was required to adequately separate the growing oligo product from the NPE-substituted mononucleoside by-products. Although improved organic solubil-

(10) For a summary of phosphate protecting groups, see: Greene, T. W.; Wuts, P. G. M. In *Protective Groups in Organic Synthesis*, 3rd ed.; Wiley: New York, 1999.

(11) Hawakawa, Y.; Wakabayashi, S.; Kato, H.; Noyori, R. *J. Am. Chem. Soc.* **1990**, *112*, 1691−1696.

(12) Le, T. X. H.; Hill, K.; Pieken, W. The 4-*O*-TBDMS-benzyloxy (TBSB) Group and 4-*O*-Acetyloxybenzyloxy (AB) Group: Versatile New Protecting Groups for Nucleotide Phosphate Protection, manuscript in preparation.

(13) Schirmeister, H.; Pfleiderer, W. *Helv. Chim. Acta* **1994**, *77*, 11. For a review on the use of the 4-nitrophenethyl protecting group in oligonucleotide chemistry, see: Pfleiderer, W.; Himmelsbach, F.; Charubala, R.; Schirmeister, H.; Beiter, A.; Schulz, B.; Trichtinger, T. *Nucleosides Nucleotides* **1985**, *4*, 81.

Table 5. **Oligonucleotide sequences produced using the DMT PASS process incorporating 2-(4-nitrophenyl)-ethyl (NPE) phosphate protection: cycle yields and product purity**

product sequence[a]	crude yield (%)	purity (%)[b]	purified yield (%)
5′-TBDPS-T-3′-3′-A-5′	92.4	70.6	62.9
5′-TBDPS-T-3′-3′-AT-5′	82.7	69.9	37
5′-TBDPS-T-3′-3′-ATG-5′	121	79.2	81.7
5′-TBDPS-T-3′-3′-ATGT-5′	66	83.1	44.9

[a] All nucleosides are 2′deoxy. [b] Purity of fully protected intermediate as determined by RP HPLC analysis.

ity did result, two problems led us to rule out this protecting group option: reagent synthesis and monomer solubility. NPE nucleoside phosphoramidites have typically been prepared from crude **13**, followed by chromatographic purification that accomplishes separation of the desired amidite from trace impurities deriving from the crude phosphitylating reagent. To prevent contamination of the oligo product by unanticipated products resulting from reagent impurities, the PASS process requires the use of extremely pure phosphitylating reagent. While purification of **13** was accomplished by chromatography on deactivated alumina, the purification yields were low, and scaled synthesis of pure **13** was expected to be expensive and difficult. Additionally, increased organic solubility of the monomeric by-products (for example, **10** (R = NPE)) modified by the NPE group complicated the use of this phosphate-protecting group.

A critical aspect of the PASS method is that any excess amidite resin **2** employed during the coupling step to drive complete conversion of the solution-phase growing substrate **3** simply affords the charged mononucleoside phosphate diester by-product **10** upon acid treatment of the resin (see Scheme 1). For a scaleable manufacturing process, excess monomer **10** needs to be readily separated from the hydrophobic $n + 1$ oligo product **8** via straightforward liquid/liquid extraction (or a similarly facile method). However, when the phosphate diester by-product is substituted by a group substantially more hydrophobic than the 2-cyanoethyl group (such as NPE, 4-acetoxybenzyl, or 4-TBSO-benzyl) the tendency of monomer **10** to also partition into the organic phase was observed. The design of a phosphate-protecting group which facilitates improved organic solubility and stability of fully protected oligodeoxyribonucleotides yet allows for aqueous extraction of monomeric by-products (for example **10**) remains one of the fundamental development challenges of the amidite DMT resin PASS process.

The organic solubility of fully protected oligodeoxyribonucleotides might also be improved by the protection of thymidine residues via N^3-acylation[14] or the introduction of a second nucleobase protecting group on guanine residues.[15] Such protection would be anticipated to improve organic solubility while at the same time preventing possible side reactions resulting from the nucleobase imide funtionality.

(14) Sekine, M.; Fujii, M.; Nagai, H.; Hata, T. *Synthesis* **1987**, 1119.
(15) For example, see: Kamimura, T.; Tsuchiya, M.; Koura, K.; Sekine, M.; Hata, T. *Tetrahedron Lett.* **1983**, *24*, 2775−2778.

Table 6. **Solubility comparison (mg/mL solvent) of thymidine dimer, trimer, and tetramer in dichloromethane (DCM) and acetonitrile (ACN) with and without N^3-benzoyl thymdine protection**

	N^3-benzoyl thymidine		unprotected thymidine	
product sequence[a]	DCM	ACN	DCM	ACN
5′-TBDPS-T-3′-3′-T	>500	>500	>500	>500
5′-TBDPS-T-3′-3′-TT	>500	>500	21	29
5′-TBDPS-T-3′-3′-TTT	>500	290	0.4	

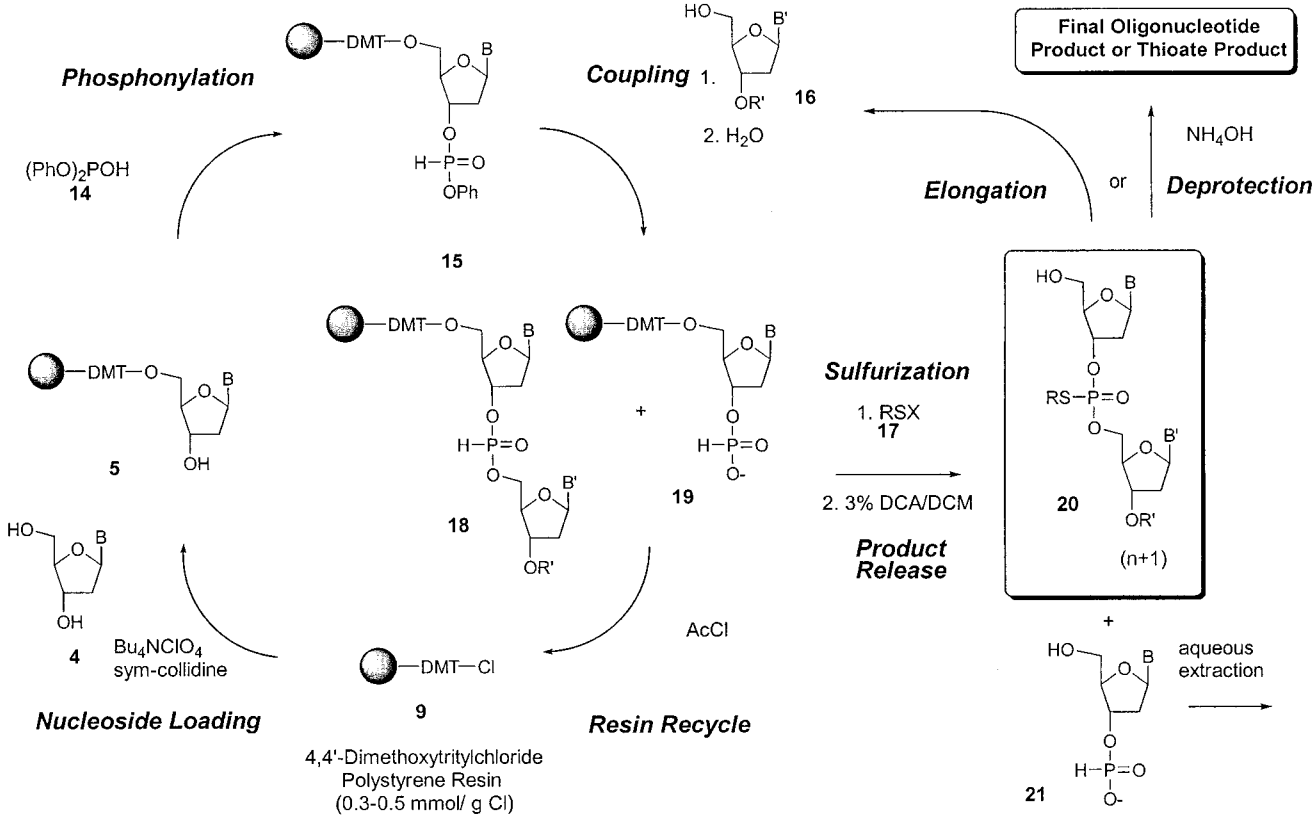
[a] All nucleosides are 2′deoxy, and all internucleotide linkages are protected as 2-cyanoethyl triesters.

During PASS process development efforts, we have observed that the introduction of unprotected thymidine residues in particular diminishes the organic solubility of fully protected oligodeoxyribonucleotides. Substantially improved organic solubility for 2-cyanoethyl-protected oligothymidylate sequences was accomplished by the use of N^3-benzoyl-protected thymidine. Table 6 compares the solubilities of thymdine dimer, trimer, and tetramer sequences with and without N^3-thymidine protection. N^3-Benzoyl protection of thymidine was found to increase the solubility of a thymidine tetramer by over a factor of 1000 relative to the N^3-unprotected tetramer. While the average cycle yield using N^3-benzoyl thymidine approximated a typical PASS yield, the initial product purity was considerably lower due to the presence of significant amounts of monomeric phosphate diester. Due to the increased organic solubility of this impurity, the usual extractive purification (0.2 M sodium phosphate) was ineffective. However, by extracting with 15% acetonitrile in water, final product purities exceeding 90% were realized, and we anticipate N^3-benzoylation of thymidine will offer practical benefits in future PASS process development efforts.

In addition to product solubility challenges, another complication that arose during the application of the PASS process to oligodeoxyribonucleotide synthesis was that observed coupling yields for purine nucleoside resin amidites were consistently lower than the corresponding pyrimidine steps. N^2-isobutyryl guanosine resin was particularly problematic. A systematic study of the coupling step leading to the tetrameric intermediate TATG was carried out,and it was found that significant quantities of product were released prematurely during the coupling and oxidation steps and the subsequent washes. In fact in one cycle, as much as 59% of the desired product was found in the eluent of the post-oxidation resin washing. Control experiments revealed that cleavage of dG from the resin upon treatment with TBAP solutions in acetonitrile was a function of both TBAP concentration and reaction time. Yields were optimized by limiting the oxidation time to 20 min and by minimizing the number of post-oxidation solvent washes. Alternatives to TBAP are currently being evaluated.

The introduction of adenosine residues has also posed some problems. During these cycles N^6-benzoyladenine was observed by HPLC to be a significant impurity in the crude product (∼5%). The depurination could be eliminated if,

***Scheme 3H*-Phosphonate DMT PASS**



during the acid-promoted release of the product from the resin, the eluent is transferred directly into a solution of 0.2 M Na_2HPO_4 (pH = 7.4).

While some challenges remain, clearly the DMT PASS process employing phosphoramidite coupling chemistry is potentially viable; however, we were intrigued by the prospect of applying *H*-phosphonate methodology to the process. Application of solution_phase *H*-phosphonate technology in large-scale oligonucleotide synthesis has received considerable attention of late,[16] and we felt some potential advantages might also be offered by *H*-phosphonates in the DMT PASS method. Oligonucleotide synthesis using *H*-phosphonate chemistry has typically consisted of reaction of an nucleoside *H*-phosphonate monoester with an activator (such as pivaloyl chloride or dipentafluorophenyl carbonate)[17] to form a mixed anhydride which is then coupled to the free 5′-hydroxyl group of a otherwise protected nucleoside. The rate of this condensation reaction is usually comparable to that observed with nucleoside phosphoramites, and it has been applied to the automated synthesis of oligonucleotides.[18] While applicable to oligonucleotide synthesis on CPG, this approach was not anticipated to be ideal for the PASS process since the longer coupling times observed with polystyrene-bound nucleotides would allow competitive reaction of the activator with the 5′-hydroxyl groups of the valuable growing oligonucleotide chain.

Recently it has been reported that the transesterification of aryl nucleoside *H*-phosphonate diesters provides a convenient method for the formation of internucleotide bonds.[19] Since no activator is required in this case the number of potential side reactions is minimized. Scheme 3 shows how transesterification of a resin-bound aryl H-phosphonate can be used as the key coupling step in the PASS process. A number of potential advantages compared to amidite chemistry exist including the range of potential phosphonylating reagents and the flexibility of reaction conditions in the coupling step. Finally, due to the differential reactivity of *H*-phosphonate diesters (e.g., **18)** and monoesters such as **19** toward electrophilic oxidation and sulfurization reagents (e.g., **17**),[20] selective preparation of protected phosphorothioate or phosphate triester linkages without derivatization of the monomeric by-products by the hydrophobic phosphate-protecting group should be possible. As a result, no limitations would exist on the structure (hydrophobicity) of the phosphate-protecting group and extractive partitioning of oligonucleotide product **20** and excess monomer **21** will be facile. This represents perhaps the most compelling advantage

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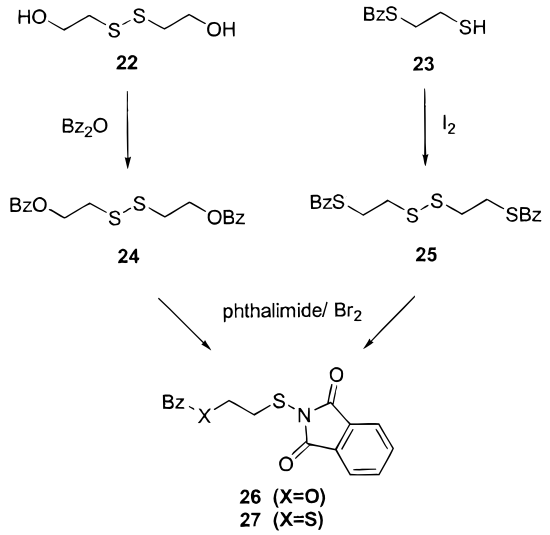
of the *H*-phosphonate option relative to the previously described phosphoramidite PASS process. Considering the above advantages, we have begun to evaluate *H*-phosphonate chemistry in the context of the DMT PASS process development effort and report here our preliminary findings.

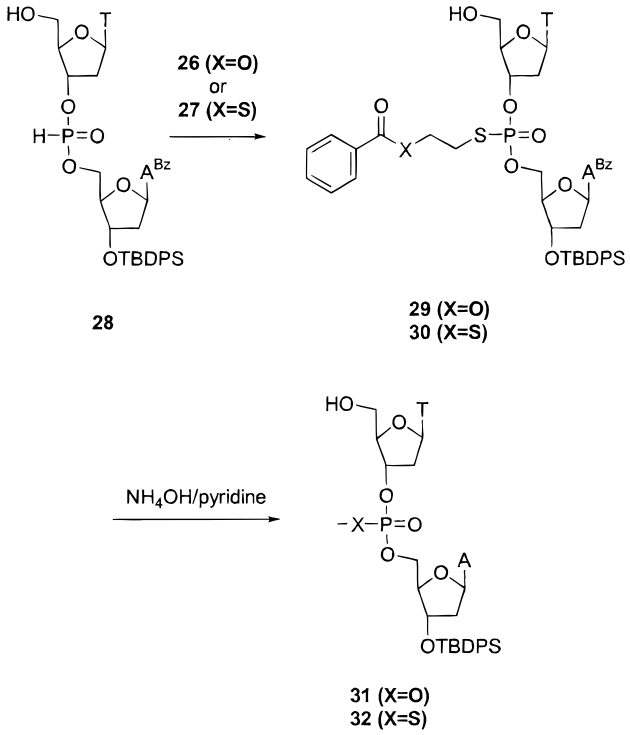
The majority of our work on the *H*-phosphonate DMT PASS process has focused on attempts to utilize aryl *H*-phosphonate transesterification for the formation of internucleotide linkages. The ready availability of inexpensive diphenyl phosphite (roughly $50/kilo), the lack of requirement of an activator and ambient reaction temperatures suggested that this methodology would be ideal in our application. In initial attempts to prepare phenyl *H*-phosphonate resin bound reactants, we simply treated nucleoside resin with excess diphenyl phosphite in a 1:1 pyridine/dichloromethane mixture. Uncharacterized resin-bound aryl *H*-phosphonates thus prepared were found to affect clean conversion of solution phase reactants, requiring somewhat longer reaction times than amidite resins, but affording clean *H*-phosphonate diester products in reasonable yields (70% unoptimized). The success of these experiments prompted pursuit of further examination of the phenyl *H*-phosphonate nucleoside resins in DMT PASS cycles.

Although it is conceivable that oligo-*H*-phosphonate substrates might be carried through successive PASS cycles with a single oxidation step at the end, we suspected solubility problems would again emerge.[21] This strategy would also limit the application of the method to sequences with all thioate or all phosphodiester linkages. Alternatively, oxidation or sulfurization at each step (analogous to the amidite PASS variation) would be anticipated to provide benefits to both substrate stability and solubility properties. The electrophilic sulfurization reagents described by Reese[22] seemed ideal for application in the *H*-phosphonate PASS process and, after establishing the viability of the approach by sulfurization of a resin-bound dinucleotide *H*-phosphonate diester with commercially available *N*-(phenylthio)phthalimide, we prepared reagents **26** and **27**, shown in Scheme 4. These reagents, based on the work of Weisler and Caruthers,[23] were expected to afford phosphorothioate triester derivatives which could be deprotected by treatment with ammonium hydroxide. Furthermore, the structural flexibility with respect to the nature of the ester portion is attractive, as refinement of the physical properties of fully protected oligonucleotides will likely be desirable in future development studies. Solution-phase model experiments with *H*-phosphonate diester **28** (Scheme 5) showed electrophilic sulfurization reagents **26** and **27** led cleanly and efficiently to the expected phosphorothioate triesters. However, upon ammonium hydroxide deprotection, benzoate derivative **29** afforded exclusively phosphate diester **31**, rather than the expected phosphorothioate diester **32**. This serendipitous discovery complements the methodology employing oximate

Scheme 4. **Preparation of sulfurization reagents**



Scheme 5. **Thiophosphate triester precursors to phosphate diesters or phosphorothioate diesters**



salt conversion of aryl phosphorothioate triesters to phosphate diesters. As expected, the thiobenzoate intermediate **30** led to phosphorothioate **32** upon treatment with ammonium hydroxide although, under these deprotection conditions, contamination by 3−5% phosphate diester **31** resulted, and continued evaluation of alternative deprotection conditions is warranted. However, we are pleased that oligonucleotide sequences with either diester, phosphorothioate, or both should be possible via the *H*-phosphonate DMT PASS process.

Two noteworthy control experiments were conducted (Scheme 6). The reactivity of the excess resin-bound aryl *H*-phosphonate toward the sulfurization reagent needed to be established, as did the reactivity of *H*-phosphonate

(21) In fact, a tetrameric *H*-phosphonate substrate was prepared by successive application of the phenyl *H*-phosphonate resin DMT PASS method, and this compound did show a tendency to "oil out" in the extractive purification step.

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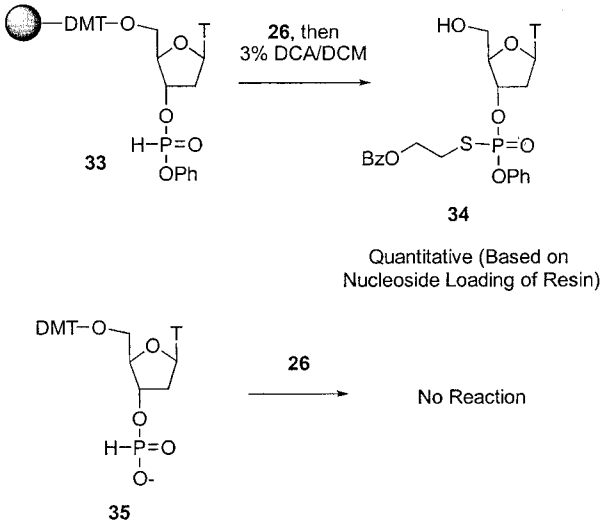
Scheme 6. **Sulfurization reagent control experiments**



monoester substrates. As it turns out, sulfurization of the thymidine phenyl *H*-phosphonate resin **33** via reagent **26**, followed by cleavage, recovery, and quantitation of the stable thiophosphate triester product **34** provides an excellent method for determination of phenyl *H*-phosphonate resin loading. In the above example, quantitative recovery (based on nucleoside resin loading) of the triester **34** was realized, indicating complete phosphonylation of the resin. Experiments validating this method as an approach to *H*-phosphonate resin-loading determination are underway.

Additionally, exposure of triethylammonium DMT nucleoside *H*-phosphonate substrate **35**[24] to reagent **26** resulted in no sulfurization of **35**. The implication is that, provided efficient hydrolysis of excess monomer from coupling steps prior to electrophilic sulfurization can be carried out, selective introduction of the phosphate-protecting group exclusively to the growing oligonucleotide product is possible. As discussed above, this selectivity can be exploited as a means of refining the physical properties of the fully protected oligonucleotide products without impacting the ability to readily separate the excess monomer via extraction.

Table 7 shows a proposed Aryl *H*-phosphonate PASS cycle that could be applied to the synthesis of oligonucleotide sequences. We are encouraged by the preliminary progress of this recent direction in the PASS development program and will report applications of this method to the synthesis of oligonucleotide sequences in due course.

Conclusions

The PASS process for oligodeoxyribonucleotide synthesis has been shown to be a promising, conceptually novel alternative to traditional synthesis on solid support or to solution-phase methods for large-scale production. Strengths of the process lie in that large excesses of expensive nucleoside phosphoramidite reagents are not required and that failure sequences are removed and recovered by a simple washing procedure. Removal of monomeric impurities by aqueous extraction and, if necessary, trituration has been shown to be an adequate means of providing fairly pure short oligodeoxyribonucleotide products without chromatography. Furthermore, the DMT PASS method is a scalable manufacturing process which operates at ambient temperatures and will be amenable to automation.

However, several challenges remain before PASS can be considered a viable alternative to existing methods for oligonucleotide synthesis. Due to the observed instability of the traditional 2-cyanoethyl phosphate-protecting group to the conditions of the PASS process, other alternatives must be considered. While some headway has been made in this regard, phosphoramidite PASS is met with the paradox that the phosphate-protecting group must impart good solubility to the oligonucleotide product while the monomeric byproducts must maintain aqueous solubility for facile removal by extraction. Preliminary experimentation with variations of *H*-phosphonate chemistry leave us optimistic about circumventing some of the challenges posed by the phosphoramidite-based method.

Equally important is that the currently achievable cycle yields do not compare favorably with those attainable by means of traditional solid-support methods and improved product purities must be realized. Optimization in these regards will be necessary before PASS can reach its full potential as a method for oligonucleotide synthesis.

Experimental Section

General. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded using a Varian-Gemini 300 operating at 300, 75, or 121 MHz, respectively. Anhydrous solvents and raw materials were obtained from commercial suppliers and used without further purification. Protected nucleoside monomers were obtained from Proligo LLC (Hamburg) and were rendered anhydrous prior to reactions by coevaporation of pyridine. N^3-Benzoylthymidine was synthesized according to literature procedure of Hata et al.[14] Reversed phase HPLC analyses were performed using a Waters 486 UV detector at 260 nm, run on a Jupiter C-18 300 Å 250 × 4.60 mm, 5 μm column. All samples are run using a gradient elution starting at 10% ACN in 0.1 M ammonium acetate and increasing to 97% ACN over a 29 min period. The final solvent mixture is then held for 15 min. Analytical anion-exchange chromatography was carried out on a 4 × 250 mm Dionex DNAPac PA-100 column, employing a linear gradient from 0 to 40% 1.0 M triethylammonium bicarbonate buffer in water over 60 min. Mass spectra were obtained from either NeXstar Pharmaceuticals, Inc., Boulder, CO, or M-Scan, West Chester, PA, using a ESI/MS or LC/MS procedure.

General Procedure, Nucleoside Resin Preparation. DMTCl polystyrene resin (theoretical loading = 0.9 mmol/g, 1.0 equiv chloride)[5] and TBAP (1 equiv) are combined in an oven-dried round-bottomed flask. The flask is evacuated, then purged with and inert atmosphere (either Ar or N_2; repeat five times). A solution of 1.5 equiv *sym*-collidine in dichloromethane is added via syringe. The appropriate nucleoside (2.0 equiv), as a solution in 4:1 DMF:pyridine (2.5 mL/g nucleoside), is added via syringe. The reaction is agitated on an orbital shaker table at 200 rpm for 4 h, then quenched by addition of methanol (1 mL/g resin). After 15

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Table 7. **Summary of the DMT PASS process using an aryl *H*-phosphonate coupling procedure**

	operation	procedure	comment
1	nucleoside resin phosphonylation	20 equiv diphenyl phosphite, 1:1 pyridine/DCM	Phosphonylation of resin-bound nucleoside has been shown to be quantitative.
2	wash	10 mL/g resin, DCM (6×)	Removes excess diphenyl phosphite.
3	coupling	2−3 equiv resin, 1:1 pyridine/DCM	Extent of reaction monitored by HPLC; 24−48 h reaction time.
4	wash	10 mL/g resin, DCM (6×)	failure sequences may be removed and recovered if necessary.
5	hydrolysis	20 equiv H_2O relative to resin-bound nucleoside, pyridine	Hydrolyzes excess resin bound aryl *H*-phoshphonate monomer to the *H*-phosphonate monoester.
6	wash	10 mL/g resin, pyridine (2×) 10 mL/g resin, DCM (4×)	Removes pyridine prior to release step.
7	thioalkylation	1.5 equiv reagent **26** or **27** relative to anticipated product, 5 equiv DIPEA, DCM	Thioalkylation of resin-bound product; charged *H*-phosphonate monoesters do not react.
8	wash	10 mL/g resin, DCM (6×)	Removes excess sulfurizing reagent.
9	release	6 mL/g resin (5×) 3% DCA in DCM	Product is released directly into 0.2 M $NaHPO_4$ to minimize depurination.
10	aqueous extractions	0.2 M Na_2HPO_4 (2×), 0.5 M $NaIO_4$ (1×), sat. $NaHCO_3$ (1×), brine (1×)	Removes *H*-phosphonate monoester impurites.
11	drying	0.5 g Na_2SO_4/mL solvent	Followed by solvent removal to provide crude product.

min, the reaction is decanted into a fritted funnel. The resin is washed with DMF (3 × 10 mL/g resin), pyridine (3 × 10 mL/g resin), DCM (8 × 10 mL/g resin), and ACN (3 × 10 mL/g resin). The resin is then transferred to a round-bottomed flask, using ACN to wash as much resin as possible from the fritted funnel into the flask. The solvent is then removed in vacuo, and the resin is dried until the loss of weight upon further drying is <1%.

General Procedure, Nucleoside Resin-Loading Quantitiation. A standard absorption curve for the appropriate nucleoside is prepared by making up five samples of the nucleoside. The samples are prepared by accurately weighing (±0.1 mg) 1−25 mg into 10 mL volumetric flasks. Each sample is then dissolved into 5 mL 1:1 ACN/DI H_2O, and diluted to volume. The UV absorbance of each sample is measured at 260 nm at a 10-fold dilution, using a 1 cm path-length cell referenced against a 1:1 ACN/DI H_2O solution. The absorbance versus quantity nucleoside (in mg) is plotted and adjusted for the 10-fold dilution, and a linear regression analysis to fit the data to a standard curve is performed.

Three 15−25 mg samples of nucleoside resin are accurately weighed out (±0.1 mg) into silanized 1.5 mL ultracentrifuge tubes. The recommended procedure is to vary the weight by ±3 mg about a central value. Deblock solution (3% dichloroacetic acid in DCM) is then added to each centrifuge tube, and the reaction is agitated for 15 min. An aliquot (100 μL) is drawn from each centrifuge tube and placed in a 10 mL volumetric flask containing 5 mL of 1:1 ACN/DI H_2O solution. The samples are then diluted to volume with 1:1 ACN/DI H_2O solution. The UV absorbance of each sample is measured at 260 nm, using a 1 cm path-length cell referenced against a 1:1 ACN/DI H_2O solution. The loading obtained from each sample is calculated by means of the standard curve. The standard deviation between the samples is considered acceptable at <10% of the average loading.

General Procedure, Phosphoramidite Resin Preparation: All equivalents are based on the nucleoside loading on resin, as determined by detritylation and quantitative analysis by HPLC or UV/VIS (see above). The nucleoside-derivatized resin, along with the DCI (2.2 equivalents) as a dry powder, is pump/purged with Ar three times in an oven-dried round-bottom flask. A minimum amount of DCM is added (approximately 3−4 mL/g resin, enough to completely swell the resin). The phosphitylating reagent (either 2-cyanoethyl derivative **1** (R = 2-cyanoethyl) or NPE derivative **13**, 2.0 equiv) is added via syringe, and the reaction mixture is agitated for 2 h. The resin is then filtered from the reaction mixture in a fritted funnel and washed with DCM (6 × 10 mL/g resin) and ACN (4 × 10 mL/g resin). The resin is dried in vacuo for a minimum of 12 h prior to coupling.

Standard Resin-Coupling Assay. A 0.25 g sample of phosphoramidite resin is treated with 0.6 mL of a standard solution prepared by diluting 1.5 equiv (based on resin nucleoside loading, as determined above) of 5′-TBDPS-dT and 2.0 equiv of DCI to 3 mL with 70% (v/v) ACN/DCM. The suspension is agitated on an orbital shaker, and 10 μL aliquots of the supernatant liquid are sampled every 30 min. These aliquots are diluted with acetonitrile (990 μL). Reversed phase HPLC analysis of the samples are conducted and absorbances compared to a standard sample prepared by diluting 10 μL of the standard solution with acetonitrile (990 μL). The extent of conversion of the 5′-TBDPS-dT is determined, and the amount consumed enables the amidite resin-loading to be calculated and expressed in mmol amidite/g resin. An alternative analytical approach to involves FT-IR quantitation of the diisopropyl−DCI complex released upon treatment with an excess of DCI.[8]

General Procedure, PASS Cycle (See Table 1). In a fritted reactor, the dry phosphoramidite resin is pump/purged with argon three times. A minimum amount of DCM is added (approximately 3−4 mL/g resin, enough to completely swell the resin), followed by 2.2 equiv of DCI as a 1.0 M solution in ACN. The DCM/ACN ratio should be approximately 2:1. The oligomer to be coupled (1.0 equiv), as a solution in 2:1 DCM/ACN (0.2 M or the best concentration possible), is added via syringe. The reaction is agitated for 3 h with monitoring of the supernatant liquid by HPLC. If any oligonucleotide reactant remains, additional amidite resin is added until all of the substrate is consumed. The mixture is then treated with 10% water (v/v) in ACN (20 equiv water). After 30 min, 1.0 equiv of TBAP is added as a dry solid, and the reaction is allowed to agitate for an additional 20 min. The resin is then filtered from the reaction mixture and washed with DCM (4 × 10 mL/g resin), ACN (4 × 10 mL/g resin), and DCM (4 × 10 mL/g resin). The resin is then washed with 3% DCA in DCM (4 × 10 mL/g resin) followed by DCM (4 × 10 mL/g resin) and ACN (2 × 10 mL/g resin). The eluent from these washes is collected directly into 0.2 M Na_2HPO_4 (pH = 7.5). The combined organic layers are then washed with 0.2 M Na_2HPO_4 (pH = 7.5) until the pH of the aqueous fractions remains 7.5. The organic fraction is dried over Na_2SO_4, filtered, and concentrated in vacuo. The product is analyzed for purity by RP HPLC, and the yield is determined by mass balance relative to the starting oligomer.

General Procedure, Resin Recycling. Exhausted polystyrene resin is stored in methanol following release of the oligonucleotide product. To recycle, the resin is filtered and washed with 3% DCA in DCM (2 × 3 mL/g resin), DCM (4 × 3 mL/g resin), 9:1 acetone:water (2 × 3 mL/g resin), acetone (1 × 3 mL/g resin), and toluene (2 × 3 mL/g resin). The resin is rinsed from the filter with toluene and dried in vacuo. The resin is then chlorinated using acetyl chloride in toluene at 60 °C overnight as described by Farrell and Fréchet.[5]

Preparation of 2-(4-Nitrophenyl)-ethyl (NPE) Phosphate-Protected Oligos via the PASS process. PASS coupling cycles with NPE amidite resin were conducted using the general procedure outlined above, except preparative reversed phase purification of the NPE-protected $n + 1$ oligo was necessary after each cycle. Preparative reversed phase purification was performed by adsorbing the crude oligomer onto C-18 125 Å bulk substrate with ACN, application of the C-18 with the oligomer to a C-18 pad and elution with 4:1 H_2O/ACN, gradually increasing the solvent strength to 2:3 H_2O/ACN in a stepwise fashion.

Synthesis of Sulfurizing Reagents. Reagents **26** and **27** were synthesized from the corresponding disulfides using literature procedures.[22]

Di-[(2-benzoyloxy)ethyl] Disulfide (24). Benzoyl chloride (64.5 mL, 0.56 mol, 2.1 equiv) was added dropwise over 1 h to a solution of 2-hydroxyethyl disulfide **22** (40.86 g, 0.27 mol, 1.0 equiv) and triethylamine (88.0 mL, 0.66 mol, 2.5 equiv) in methylene chloride cooled to 0 °C. After the addition was complete, the solution was allowed to warm to room temperature and stirred overnight. The solution was washed with Na_2CO_3 (2×), $NaHCO_3$ (2×) and brine, dried over Na_2SO_4 and concentrated to provide 96.98 g (100%) di-(2-benzoyloxyethyl) disulfide (**24**) as a light yellow liquid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.02 (d, J = 7.6 Hz, 4H), 7.54 (m, 2H), 7.41 (t, J = 7.0 Hz, 4H), 4.59 (t, J = 6.4 Hz, 4H), 3.08 (t, J = 6.4 Hz, 4). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.25, 133.07, 129.76, 129.60, 128.33, 62.68, 37.31. TLC R_f = 0.72 (2:1 hexanes/ethyl acetate).

Di-[2-(*S*-benzoylmercapto)ethyl] Disulfide (25). Iodine (21.0 g, 82 mmol, 0.7 equiv) was added to a solution of ethanedithiol monobenzoate **23**[22] (20.0 g, 0.12 mol, 1.0 equiv) in dichloromethane (600 mL). The solution was allowed to stir at room temperature for 2 days. Ethyl acetate was added and the solution washed with saturated Na_2SO_3 (3×) and brine. The organic phase was dried over Na_2SO_4 and concentrated to provide 15.08 g (76%) di-(2-benzoylmercaptoethyl) disulfide as a light yellow oil. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.94 (d, J = 7.0 Hz, 4H), 7.56 (m, 2H), 7.43 (t, J = 7.6 Hz, 4H), 3.42 (m, 4H), 2.99 (m, 4H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 191.16, 136.71, 133.49, 128.59, 127.22, 37.95, 28.47. TLC R_f = 0.69 (5:1 hexanes/ethyl acetate).

2-[(2-Benzoyloxy)ethyl]sulfanyl-1*H*-isoindole-1,3(2*H*)-dione (26). Bromine (16 mL, 0.31 mol, 1.15 equiv) was added dropwise over 1.5 h to a 0 °C mixture of di-(2-benzoyloxyethyl) disulfide (96.98 g, 0.27 mol, 1.0 equiv) and phthalimide (74.8 g, 0.51 mol, 1.9 equiv) in 1:1 pyridine/acetonitrile (450 mL). Overhead stirring is required for efficient mixing. After bromine addition was complete, the mixture was stirred an additional 30 min. Water (500 mL) was added, and the solution was stored at 6 °C overnight. The thick precipitate was isolated by filtration and washed with water (3 × 300 mL). The isolated material was dried in a vacuum desiccator overnight to provide 128.7 g (77%) of 2-(2-benzoyloxyethyl)sulfanyl-1*H*-isoindole-1,3(2*H*)-dione (**26**) as a fluffy off-white solid. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.86 (m, 4H), 7.73 (m, 2H), 7.47 (m, 1H), 7.30 (m, 2H), 4.57 (t, J = 6.2 Hz, 2H), 3.24 (t, J = 6.2 Hz, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 168.11, 166.01, 134.58, 132.92, 131.87,

129.44, 128.19, 123.85, 123.48, 63.55, 37.27. TLC $R_f = 0.41$ (2:1 hexanes/ethyl acetate).

2-[2-(*S*-Benzoylmercapto)ethyl]sulfanyl-1*H*-isoindole-1,3(2*H*)-dione (27): Synthesized according to the procedure used for **26** above and isolated in 51% yield. ^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.94 (m, 2H), 7.86 (m, 2H), 7.78 (m, 2H), 7.54 (m, 1H), 7.46 (m, 2H), 3.31 (m, 2H), 3.10 (m, 2H). ^{13}C NMR ($CDCl_3$, 75 MHz) δ 190.93, 168.39, 136.50, 134.63, 133.52, 131.95, 128.55, 127.16, 123.94, 39.01, 28.37. TLC $R_f = 0.23$ (5:1 hexanes/ethyl acetate).

Solution-Phase Thioalkylation and Deprotection. TA *H*-phosphonate dimer **28** (>98% purity) was synthesized in 53% yield via the *H*-phosphonate PASS process (vida infra) with purification by flash chromatography (SiO_2, 95:5 DCM/MeOH). ^{31}P NMR ($CDCl_3$) δ 9.3, 8.4 (diastereomers). Thioalkylation of **28** using reagents **26** and **27** was carried out using conditions reported in the literature.[25]

Dimer 29. *N*,*N*-diisopropylethylamine (92 μL, 0.52 mmol, 5.0 equiv) was added to a solution of dimer **28** (93 mg, 0.11 mmol, 1.0 equiv) and 2-(2-benzoyloxyethyl)sulfanyl-1*H*-isoindole-1,3(2*H*)-dione **26** (36 mg, 0.11 mmol, 1.0 equiv) in dichloromethane (0.52 mL). The solution was stirred at room temperature for 20 min, diluted with ethyl acetate, washed with $NaHCO_3$ and brine, dried over Na_2SO_4, and concentrated. The crude protected thioate was determined to be 91% pure by RP HPLC (no starting material present). ^{31}P NMR ($CDCl_3$) δ 29.8, 28.9 (diastereomers).

Dimer 30: synthesized according to the procedure above using reagent **27**. Crude product was isolated in 92% purity as determined by RP HPLC. ^{31}P NMR ($CDCl_3$) δ 30.0, 29.0 (diastereomers).

Deprotection of 2-(Benzoyloxy)ethyl and 2-(*S*-Benzoylmercapto)ethyl Protecting Groups; Dimer 31. Dimer **29** (28.6 mg) was treated with 0.8 mL 1:1 pyridine/NH_4OH and heated to 40 °C for 12 h. Removal of solvents under reduced pressure provided phosphodiester **31** in 75% crude purity. ^{31}P NMR (D_2O) δ −0.2.

Dimer 32: synthesized according to the procedure above. ^{31}P NMR (D_2O) δ 57.0, 56.6 (diastereomers).

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Acknowledgment

The authors acknowledge the helpful assistance of Greg Husar, Kim Frender, Jeffery Heyes, and Ryan Flansburg.

Received for review November 3, 1999.

OP990095T