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Improved Process for the Preparation of Nucleosidic Phosphoramidites Using a Safer and Cheaper Activator

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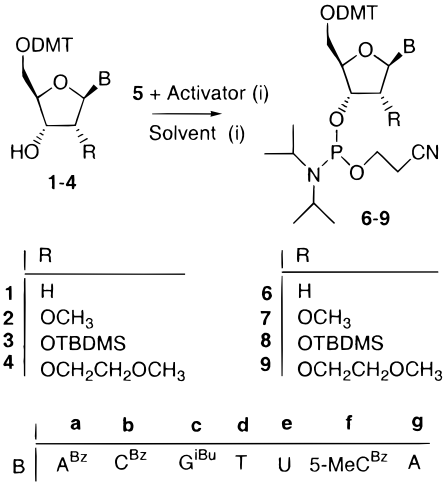
Abstract:

A new, simplified commercial process for the preparation of nucleosidic phosphoramidites, key raw materials for the automated solid-supported synthesis of oligonucleotide-based drugs, was developed. Phosphitylation of a variety of protected nucleosidic derivatives (1−4) with a small excess of 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropyl phosphoramidite (5, bis-reagent) and pyridinium trifluoroacetate (Py·TFA) as the activator in an appropriate solvent at room temperature formed 75−96% of desired nucleosidic phosphoramidite products in less than 2 h. An efficient nonaqueous work-up has been developed to further streamline the isolation of moisture-sensitive P(III) nucleosidic compounds. The key finding is the use of Py·TFA, which is effective, inexpensive, stable, less acidic (pK_a 5.2) than 1*H*-tetrazole, nontoxic, safe, and highly soluble in organic solvents. The reaction mechanism for phosphitylation with Py·TFA as an activator has also been studied. An improved, robust, and versatile process for the preparation of nucleotide phosphoramidites under very concentrated reaction conditions was developed to support commercial manufacture of oligonucleotide-based drugs.

Introduction

Over twenty synthetically prepared oligonucleotides are currently undergoing human clinical trials for the treatment of a variety of cancers and viral infections and a host of inflammatory disorders.[1] In 1998 FDA approved Vitravene, the first commercial antisense oligonucleotide drug, for the treatment of cytomegalovirus retinitis in HIV patients.[2] With the growing success of clinical programs and potential market demand for oligonucleotide-based drugs, it is important to develop a safe, environmentally clean and cost-efficient synthesis of these molecules. Due to the high coupling efficiency of phosphoramidite building-blocks (Scheme 1, **1**−**4**) and a rapid turnaround time, automated solid-support synthesis by means of phosphoramidite chemistry is the preferred method for the synthesis of the majority of oligonucleotides.[3] Though very efficient, this method of synthesis must be fully optimized for ton-scale annual oligonucleotide production, as the method requires expensive raw materials, solid-support, nucleosidic phosphoramidites, and dry acetonitrile.

Scheme 1. **Synthesis of phosphoramidites**



(i) See table 1-3 for experimental details and yield

Therefore, we have focused our attention on the various process improvements, including cost reduction of expensive raw materials. The nucleosidic phosphoramidite building-blocks alone contribute over 33% of the total raw material cost today and their scale-up remains difficult. Particularly, the phosphitylation step which employs 1*H*-tetrazole, a hazardous reagent in combination with expensive phosphorus reagents. Frequently, phosphitylation of protected nucleosides is performed with 2-cyanoethyl-*N*,*N*-diisopropylchlorophosphoramidite, an expensive reagent that is very reactive and does not require activation.[4] Additionally, this reagent does not keep well at room temperature, and has the potential to explode upon heating. As an alternative, the use of the less reactive and relatively cheaper reagent, 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphoramidite (Scheme 2, **5**, bis-reagent) has been reported.[5] However, since bis-reagent is not reactive enough, it requires the use of an in-situ activator, such as 1*H*-tetrazole. There are several inherent problems

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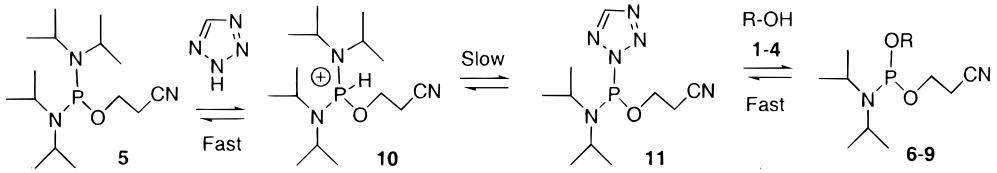
Scheme 2. **Mechanism[11] of activation with 1*H*-tetrazole**



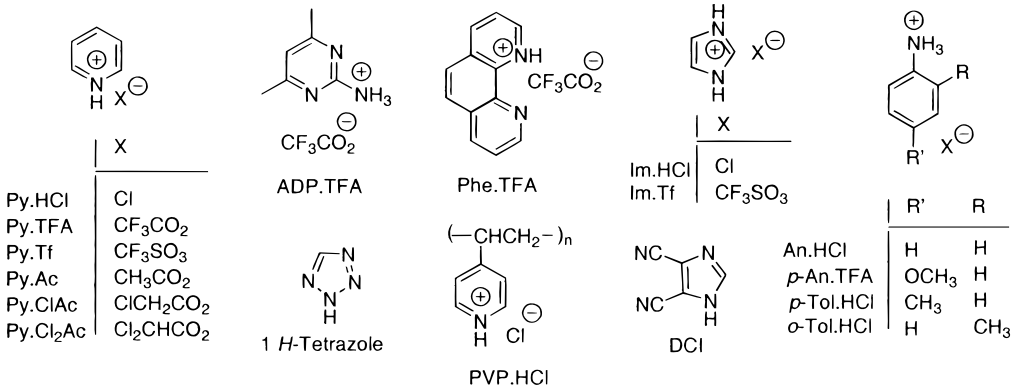


Figure 1. **General structure of activators studied.**

with the use of 1*H*-tetrazole, such as reported toxicity, an explosive nature, and high cost.[6] Also, 1*H*-tetrazole is acidic (pK_a 4.89) and is reported to cause unwanted detritylation and possibly depurination during automated synthesis.[7]

Recently, trimethylsilyl chloride,[8] 4-5-dicyanoimidazole (Figure 1, DCI)[9] and pyridinium hydrochloride[10] have been reported as a substitute for 1*H*-tetrazole during phosphitylation of nucleosides. In our scale-up studies of nucleosidic phosphoramidites, we have tried all of the above activators and found them to be unsatisfactory for a commercial process. As a result, our exhaustive search for an ideal activator has led us to pyridinium trifluoroacetate (Figure 1, Py·TFA) as a reagent of choice for phosphitylation of nucleosides. We believe that for phosphitylation reactions Py·TFA is an excellent activator that is cheap, stable at room temperature, less acidic than 1*H*-tetrazole and DCI, less toxic, safe in handling, and highly soluble in a range of organic solvents.

Herein, we describe for the first time a systematic search of an activator, its reaction mechanism, and its application to a variety of protected nucleosides. The study also describes the efforts made to find the most suitable process conditions from the viewpoint of commercial manufacturing/process development/scale-up considerations.

The seminal work of Dahl et al.[11] describing the mechanisms of activation with 1*H*-tetrazole during phosphitylation was a cornerstone in our selection criterion for an improved activator. In the proposed mechanism (Scheme 2), the first step in activation is the fast protonation of the bis-reagent **5** by 1*H*-tetrazole, followed by a second slower step where the conjugate base of tetrazole displaces the *N*,*N*-diisopropylamino group to furnish a very reactive tetrazolide intermediate, which reacts quickly with R−OH to yield a stable RO-phosphoramidite. On the basis of this mechanism, we elected to evaluate several compounds that fit the acidic and nucleophilic profile of 1*H*-tetrazole. The screening of best activator was based on safety, cost, pK_a, size, and ease of handling during phosphitylation reaction.

Safety Considerations. A nitrogen-rich heterocycle, such as 1*H*-tetrazole has been reported to be explosive upon heating or heavy impact.[6,12] Recently, DCI has been reported as a good substitute for 1*H*-tetrazole.[9] Although, DCI has a similar number of nitrogens, it is not an explosive compound.[13] However, we chose a pyridinium and an anilinium compound for our initial screening. Both with a single nitrogen atom, one being a cyclic and the other an exocyclic functionality. Furthermore, both the pyridinium and anilinium class of compound are safely used in chemical industries in very large quantities.

Cost Considerations. Alhough DCI was proven to be safe, we realized that the cost of the reagent was even higher than 1*H*-tetrazole.[14] Whereas pyridinium and anilinium compounds are one-tenth of the current cost of 1*H*-tetrazole. For example, most of the pyridinium and anilinium salts can be prepared efficiently and economically in a standard chemical industrial setup.

(6) (a) Stull, D. R. *Fundamentals of Fire and Explosion*; AIChE Monograph Series, No. 10; American Institute of Chemical Engineers: New York, 1977; Vol. 73, p 22. (b) See MSDS for T 7641 in Sigma database. (c) The bulk price for 1*H*-tetrazole is $700/kg from ChemImpex International, Chicago, IL.

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(12) BAM Fallhammer test with 1*H*-tetrazole created a large explosion at 50−60 J limiting impact energy of sample. The results were provided by Nexstar Pharmaceuticals, Boulder, CO, Report No. NE 1767RP.

(13) Nexstar Pharmaceuticals, Boulder, CO, performed BAM Fallhammer test with 4,5-dicyanoimidazole and reported no reaction at >60 J limiting impact energy of sample.

(14) The bulk price of 4,5-dicyanoimidazole is $2,200/kg from PrOligo, Boulder, CO.

Table 1. **Studies for selection of activator**

entry	activator[d]	pk_a[e]	mol ratio **1**:act.:**5**	solvent[c]	reaction time (h)	% yield[a]	St.Mat→Pdt[b]
1	Py·HCl	5.23	1:1.4:1.4	CH_2C1_2	3	72	**1a→6a**
2	Py·TFA	5.23	1:1.2:1.2	CH_2C1_2	2	68	**1a→6a**
3	Py·Tf	5.23	1:1.2:1.0	CH_3CN	2	42	**1a→6a**
4	PVP·HCl	5.23	1:1.2:1.2	CH_2C1_2	2	14	**1c→6c**
5	Py·Ac[f]	5.23	1:1.2:1.2	CH_2C1_2	2	NR	**1b→6b**
6	Py·ClAc[f]	5.23	1:1.2:1.2	CH_2C1_2	2	SR	**1b→6b**
7	Py·$C1_2$Ac[f]	5.23	1:1.2:1.2	CH_2C1_2	2	89	**1b→6b**
8	1*H*-tetrazole	4.89	1:1.1:1.0	CH_2C1_2	3	94	**1d→6d**
9	DCI	5.20	1:1.2:1.2	CH_3CN	2	51	**1a→6a**
10	Im·HCl	6.99	1:1.2:1.2	CH_3CN	24	44	**1b→6b**
11	Im·Tf	6.99	1:1.2:1.2	CH_2C1_2	3	54	**1a→6a**
12	An·HCl	4.63	1:1.2:1.2	Mix.	48	NR	**1a→6a**
13	*p*-An·TFA	5.34	1:1.2:1.2	CH_2C1_2	48	NR	**1a→6a**
14	*p*-Tol·HCl	5.08	1:1.2:1.2	CH_2C1_2	18	NR	**1a→6a**
15	*o*-Tol·HCl	4.44	1:1.2:1.2	CH_2C1_2	18	NR	**1a→6a**
16	ADP·TFA	4.82	1:1.2:1.2	CH_2C1_2	0.5	71	**1a→6a**
17	Phe·TFA	4.84	1:1.2:1.2	CH_2C1_2	20	35	**1a→6a**
18	TMSCl		1:1.2:0.6	CH_2C1_2	3	41	**1d→6d**

[a] Isolated yield after flash column chromatography. [b] All products were characterized by ^{31}P NMR. [c] All solvents were anhydrous. [d] See Figure 1 for structure of activators. [e] Cited from The determination of Ionization Constants. In *A Laboratory Manual*, 3rd ed.; Albert, A., Serjeant, E. P., Eds.; Chapman and Hall Ltd.: New York, 1984. [f] Activator prepared in situ by addition of appropriate acid (ClAc = chloroacetic acid, $C1_2$Ac = dichloroacetic acid) to the pyridine solution, NR = no reaction, SR = slow reaction (<25% pdt. formation in 18 h), mix. = 1:1, v/v, CH_2Cl_2 and CH_3CN.

pK_a Considerations. On the basis of the earlier mechanism studies, we decided to screen four anilinium derivatives shown in Figure 1, where pK_a can be modulated on the basis of the substitution on the aromatic ring. Therefore, salts of *o*-toluidine (pK_a 4.44) and anisidine (pK_a 5.34) were considered for the study. Since the possibility of detritylation and depurination is pertinent between the pK_a 4.4−5.1 of anilinium derivatives, we added pyridinium salts (pK_a 5.23) to our studies, anticipating some reduction in undesirable reactions. To be completely safe, we included imidazolium salts (pK_a 6.99) as a neutral class of compound.

Size Considerations. Again referring to the mechanisms of the activation with 1*H*-tetrazole, we knew that nucleophilic participation of the activator was important during the reaction. Furthermore, DCI which is relatively bulkier than 1*H*-tetrazole also was believed to be reacting in a similar manner.[9] On the basis of these observations, we questioned the optimum size of the activator, and selected small, (imidazolium), medium (pyridinium and anilinium), and large (phenanthroline) ring systems for our studies (Figure 1), while of course maintaining the pK_a and cost and safety considerations.

Ease of Handling. This is yet another important criterion towards the selection of an activator for scale-up. Among various possible salts of pyridine and aniline, commercially accessible hydrochloride (HCl), trifluoroacetate (TFA), and trifluoromethane sulfonate (Tf) salts were selected. Among these three salts, TFA appeared to be the least hygroscopic in our initial screening. The possibility of preparing any of the above salts in situ prior to the reaction may further eliminate the handling of hygroscopic material in bulk for a manufacturing scenario.

Results and Discussion

A number of phosphitylation reactions were performed to arrive at the selection of best activator and the key results are summarized in Table 1. For the sake of clarity only the important experiments are presented in the Table. For example, a series of experiments were carried out to determine the optimum ratio of nucleoside **1**:bis-reagent **5**:activator, concluding that 1:1.2:1.2 was most desirable (data not shown). The foregoing phosphitylation reaction was found to be complete in 2 h at room temperature. It is believed that the acidic pK_a of some of the activators may cause the undesirable side reactions. To test this, an excess of bis-reagent and activator (Py·HCl) were used for a longer period of time (Table 1, entry 1). A reasonable 72% isolated yield of **6a** confirmed minimum side reactions.

With the knowledge of pyridinium salt working as an activator, we examined the influence of counterions or acid on the reaction. Various pyridinium salts were either purchased or made in situ (prior to experiment) and tested for their ability to function as activator (entries 2−7). In addition, polyvinyl pyridinium hydrochloride (PVP·HCl) was also tried with the hope that it could be recycled. The results indicate (entry 4) that the accessibility and acidity of counterions has a marked influence on the yield of each experiment. For example, PVP·HCl furnished a low yield, possibly due to the low accessibility of the resin-bound pyridinium salt. Reactions with Py·Ac (entry 5) failed to yield any product, and Py·ClAc (entry 6) reacted at a very slow rate, which may be due to counterions derived from weak acids. This assumption can be further strengthened by the good yields observed with the pyridine salt of the more acidic dichloroacetic acid (Py·$C1_2$Ac, entry 7). Interestingly, both pyridine and dichloroacetic acid are used separately as reagents for the automated synthesis of oligonucleotides on solid-support. There may be a clear advantage with use of Py·Cl_2Ac as an activator (prepared in situ), due to common storage and handling of raw materials, particularly in a scenario where both amidites and oligonucletides are made

at one site. Further development of Py·Cl_2Ac as an activator was not pursued for mainly two reasons. First, Py·Cl_2Ac is not a commercial product and second it is more hygroscopic than Py·TFA.

With the initial success of Py·TFA as an activator, we compared the results with the current results of using 1*H*-tetrazole (entry 8) which furnished 94% yield under optimized conditions. As another point of reference, we also compared the results with those of using DCI as an activator (entry 9) which gave modest yield in our hands. The high cost of the reagent and lower yield with DCI further discouraged us to use it as an activator for phosphitylation of nucleosides.

To check the influence of pK_a and the importance of the size of the activator, we tested Im·HCl and Im·Tf (entries 10, 11). Both reagents gave a modest yield, with much longer reaction times necessary for the completion of the reaction with Im·HCl. Clearly, a small and neutral molecule like imidazole salt as an activator may not be the best choice due to the high cost, lower yield, and their hygroscopic nature.

The effect of pK_a was further investigated by performing phosphitylation with four anilinium compounds having a pK_a between 4.44 and 5.34. Surprisingly, none of the anilinium activators worked, even after extended reaction times (entries 12−15). In all cases, unreacted starting material was recovered. Among the group of exocyclic protonated anilines, the use of 2-amino-4,6-dimethyl pyrimidine·TFA (ADP·TFA) as an activator (entry 16) gave 71% yield of the desired product. Although reaction with ADP·TFA was facile, its pK_a (4.8), which is similar to that of 1*H*-tetrazole, discouraged further development. Last, we also tried the sterically bulky activator 1,10-phenanthroline trifluoroacetate (Phe·TFA) and found it to be a poor activator (entry 17).

Among several activators in the literature TMS-Cl was also employed[8] in phosphitylation. Use of TMS-Cl in our hands furnished low yields (entry 18) and depurination in some experiments. Again, the air sensitivity and potential difficulties in handling TMS-Cl on large-scale under anhydrous conditions forced us to look for better alternatives.

The overall evaluation of the old and new activators in Table 1 suggested that Py·TFA was the best choice among the group and should be further examined. One of the biggest challenges of making a phosphitylation reaction work was to assume that the protected nucleoside and activator were highly soluble in organic solvents. The primary reason for the failure of some of the activators was due to poor solubility in appropriate solvents. In some cases, we noticed that a change of solvent could have a dramatic effect on the yield (Table 2). Since all of the first generation antisense oligonucleotides required phosphoramidite of 2′-deoxynucleosides (**6a**−**d**), we decided to further explore and select the best solvent for each reaction.

A study for the selection of an appropriate solvent for phosphitylation of 2′-deoxynucleosides **1a**−**d** was undertaken with Py·TFA as the activator (Table 2). First, it was necessary to understand the maximum solubility of **1a**−**d** in key solvents, such as CH_3CN, CH_2Cl_2, and EtOAc

Table 2. **Studies for selection of solvent with Py·TFA as activator**

entry	solvent[c]	reaction time (h)	% yield[a]	St.Mat.→Pdt[b]
1	CH_3CN	2	90	**1a→6a**
2	CH_2Cl_2	2	68	**1a→6a**
3	CH_2Cl_2	1	93	**1b→6b**
4	CH_3CN	5	74	**1b→6b**
5	EtOAc	6	73	**1b→6b**
6	CH_3CN	20	80	**1c→6c**
7	EtOAc	3	66	**1c→6c**
8	CH_2Cl_2	3	74	**1c→6c**
9	CH_2Cl_2	3	88	**1d→6d**

[a−c] See Table 1 footnotes for details.

commonly utilized for phosphitylation. Second, knowledge of the solubility of both nucleoside and Py·TFA together at high concentration was important. Preliminary experiments indicated that Py·TFA alone was highly soluble (>1 M) in all of the three key solvents. However, solubility of each nucleoside (**1a**−**d**) varied considerably, depending on the solvent used, and may impact the yield of corresponding amidite (**6a**−**d**). For example, phosphitylation of **1a** in CH_3CN gave 90% yield of **6a** compared to only 68% in CH_2Cl_2 (entries 1, 2). On the contrary, conversion of **1b** to **6b** was most effective in CH_2Cl_2 (93% in 1 h), compared to that in CH_3CN and EtOAc, which also appears to be much slower (entries 3−5). Protected 2′-deoxyguanosine **1c** furnished **6c** in 80% yield in CH_3CN, albeit a longer reaction time was required (entry 6), whereas, conversion of **1c** → **6c** was complete in EtOAc and CH_2Cl_2 (entries 7, 8) in much less time (<3 h), although the yield was somewhat compromised. Last, phosphitylation of protected thymidine **1d** was found to be best carried out in CH_2Cl_2 (88%, entry 9) compared to other solvents (data not shown).

After the selection of Py·TFA as an activator (Table 1) and the selection of the appropriate solvent (Table 2), it was necessary to confirm that there were no side reactions, such as detritylation and depurination during phosphitylation under the optimum conditions described in Tables 1 and 2, we decided to check the outcome of less optimum (run-away) conditions. As an example, phosphitylation of **1b** was carried out with bis-reagent and Py·TFA as an activator in CH_2Cl_2 under reflux. After 5 h, TLC indicated no trace of detritylation of **1b** when co-spotted with a standard. Furthermore, there was no visible orange-red coloration in the reaction mixture that would have been an indication of free trityl cation. On this basis, we believe that Py·TFA is not acidic enough to remove the 5′-*O*-DMT group during phosphitylation reaction of protected nucleosides.

Among the four deoxynucleosides, **1a**−**d**, protected adenosine **1a** is known to be sensitive to acid-catalyzed depurination.[15] Therefore, phosphitylation of **1a** was carried out as described in Table 2 (entry 1). Upon completion of the reaction (by TLC), the solution was refluxed for 2 h and analyzed for presence of depurinated species. Complete absence of free or protected adenine species in the foregoing

(15) Zieloncka-Lis, E. *Nucleosides Nucleotides* **1989**, *8*, 383.

Table 3. **Phosphitylation of various nucleosides with Py·TFA activator**

entry	reaction time (h)	% yield[a]	St.Mat.→Pdt[b]
1	1	60	**1g→6g**
2	2	91	**1f→6f**
3	<1	75	**2a→7a**
4	2	94	**2b→7b**
5	2	86	**2c→7c**
6	2	88	**2e→7e**
7	<1	95	**3a→8a**
8	2	90	**3b→8b**
9	2	82	**3c→8c**
10	2	84	**3e→8e**
11	<1	96	**4a→9a**
12	2	94	**4c→9c**
13	3	95	**4d→9d**
14	2	92	**4f→9**

[a,b] See Table 1 footnotes for details.

experiment confirmed that the use of Py·TFA as an activator will not cause depurination of **1a** during phosphitylation.

With the successful use of Py·TFA as an activator for phosphitylation of 2′-deoxynucleosides, next we turned our attention to the application of this methodology for the synthesis of other important nucleosidic phosphoramidites.

Recently, Hayakawa et al. reported the use of the base-unprotected phosphoramidite of 2′-deoxyadenosine and its application in automated synthesis of oligonucleotides.[16] It was intriguing for us to see if our methodology was able to convert unprotected **1g** into **6g**. Phosphitylation of **1g** with Py·TFA as an activator was quick and gave **6g** in 60% isolated yield with a small amount of unidentified products (Table 3, entry 1). Incorporation of base- or sugar-modified 2′-deoxynucleoside residue into oligonucleotide is a well-established art.[17] One of these modifications, 5-methyl-2′-deoxycytidine (5-Me-dC, **1f**) was recently used in an antisense oligonucleotide that entered Phase I clinical trials.[18] The use of 5-MedC in place of unmodified 2′-deoxycytidine in an antisense oligonucleotide is anticipated to increase the affinity for target RNA.[19] Therefore, it was pertinent for us to undertake phosphitylation of 5-Me-dC. Reaction of **1f** with bis-reagent in the presence of Py·TFA in CH_2C1_2 gave **6f** in excellent yield (Table 3, entry 2).

With the initiation of Phase I clinical trials[20] with Angiozyme, the first synthetic ribozyme molecule, and increasing demand for large-scale synthesis of RNA,[21] it was necessary to have improved methods for the preparation of ribophosphoramidites (**8a−c,e**). In addition to the first-generation of 2′-deoxyphosphorothioate antisense oligonucleotides, several second-generation antisense oligonucleotides have entered Phase I clinical trials.[22] Most of these oligonucleotides contain a 2′-modification that provides increased binding affinity for target RNA and enhanced stability in vivo.[23] To make larger quantities of these oligonucleotides, one requires 2′-substituted amidites, such as **7** and **9**. Therefore we elected to extend the Py·TFA activator methodology to (i) 2′-*O*-TBDMS-, (ii) 2′-*O*-Me-, and (iii) 2′-*O*-$(CH_2)_2OCH_3$–substituted nucleosides.

The data shown in Tables 1 and 2 demonstrate that unmodified 2′-deoxynucleoside phosphoramidites can be easily synthesized under optimal conditions described in this report (See Experimental Section). Almost identical conditions were then successfully applied to the transformation of 2′-*O*-Me nucleosides **2** → **7** in ∼75−94% yield, and 2′-*O*-methoxyethyl nucleosides **4** → **9** in ∼92−96% yield (Table 3). The phosphitylation conditions using Py·TFA as an activator were further extended to the corresponding ribonucleosides protected with 2′-*O*-TBDMS group. Thus, ribonucleosides (**3**) were converted into amidites **8** in excellent yield (82−95%, entries 7−10, Table 3). It is noteworthy that all of the reactions described in Table 3 were carried out in CH_2C1_2 as a solvent and found to be complete in under 3 h at room temperature, thus making it efficient and convenient for further scale-up work.

Scale-up Trials and Interpretation. With the phosphitylation procedures fully optimized, the scale-up trials were performed on 10 mmol scale with 2′-deoxynucleosides (**1**). The conventional method[4] of phosphitylation involves considerable skill in work-up after the reaction, isolation, and purification of amidites. Furthermore there are problems associated with storage and handling of reactive P(III) phosphoramidites,[24] such as compounds **6**−**9**. Therefore we have attempted to develop a universal process for phosphitylation which is simpler and works with a variety of nucleosides. A detailed procedure is described in the Experimental Section, and the highlights are summarized below. The simplicity of this procedure lies in eliminating the traditional aqueous work-up step for two reasons. First, any exposure of water to phosphoramidite is bound to hydrolyze a portion of the product to *H*-phosphonate, resulting in lower yield. Second, removal of traces of water is not only difficult but also cost- and labor-intensive. The excess of the reagents and byproducts are conveniently separated from the product by short silica gel flash column chromatography. Since the byproducts are inert and nonreactive, their coexistence with the product during chromatography is not an issue. In addition, the presence of traces of "free" pyridine avoids accidental cleavage of 5′-*O*-DMT group from amidites (**6**−**9**). This nonaqueous work-up

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(24) Phosphoramidites are stored as free-flowing powder under a blanket of inert atmosphere in glass bottles at room temperature (16−25 °C). These compounds must be used within 2−4 weeks after dissolution in acetonitrile. Particularly, the shelf life of dG amidite **1c** is shortest (∼2 weeks) among other 2′-deoxyamidites (**1**). All amidites are hygroscopic and should be handled appropriately.

Scheme 3. **Proposed mechanism for activation with Py·TFA**

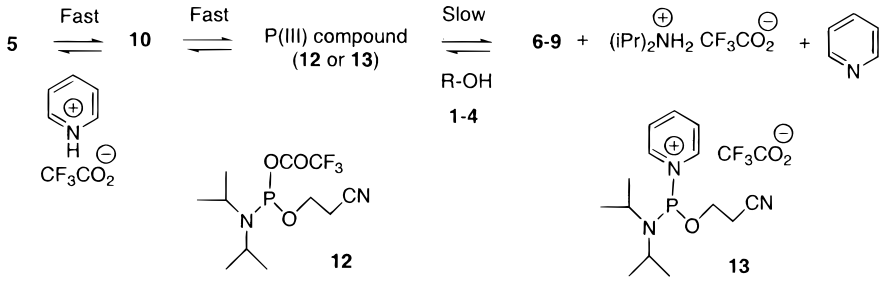

Table 4. **Summary of NMR results**

entry	compounds	chemical shift (ppm)
	^{31}P NMR recorded in CD_3CN	
1	**5**	125.8
2	**5** + Py·TFA	125.8, 158.8
3	**5** + Py·TFA + **1d**	151.0, 151.2
4	**5** + **1d**	125.8
5	**5** + **1d** + Add Py·TFA	125.8, 151.0, 151.2
	^{15}N NMR recorded in CD_3CN	
6	pyridine	−69.0
7	pyridine + TFA	−148.0
8	pyridine + TFA + **5**	−68.5
9	pyridine + TFA + **5** + **1d**	−71.7

process was repeated three times, affording the expected yield and purity for all compounds tested (Tables 2−3). Further scale-up demonstration at 1 mol scale is in progress[25] with four 2′-deoxynucleosides (**1**) required for the first generation of antisense drugs.

Role of Py·TFA in the Activation of Bis-reagent 5 (Scheme 3). The mechanism of phosphitylation using 1*H*-tetrazole has been thoroughly investigated, first by Dahl[11] in 1987 and later by Seliger[26] in 1989. Both of them concluded that 1*H*-tetrazole acts as a weak acid and a nucleophile during the phosphitylation reaction. In the proposed mechanism, the first step is fast protonation of **5,** furnishing **10**, followed by a slower displacement of the *N*,*N*-diisopropylamino group by tetrazole, providing **11**, which reacts rapidly with 3′-hydroxyl group in **1**−**4** giving phosphitylated product **6**−**9** (Scheme 2).

Having selected Py·TFA as an activator for phosphitylation of nucleosides, it would be useful to understand the reaction mechanism. The main question is whether Py·TFA can act as both an acid and a nucleophile like 1*H*-tetrazole. Therefore, the role of Py·TFA in the activation step was investigated by ^{31}P and ^{15}N NMR experiments. The first set of experiments was performed with bis-reagent **5** (entries 1−5, Table 4). The ^{31}P NMR chemical shift of bis-reagent **5** was at 125.8 ppm, which is close to the shift reported in the literature.[27] The activator Py·TFA was then added to the solution of **5**. The ^{31}P NMR of the former solution was recorded after 5 min. A new signal appeared at 158.8 ppm, in addition to the original signal of 125.8 ppm (entry 2). We attribute the signal at 158.8 ppm to a reactive P(III) species that appears to be stable and formed quickly. Next, addition of nucleoside **1d** to the mixture collapses the signal at 158.8 ppm and results in the formation of 3′-*O*-phosphitylated **6d** with characteristic diastereoisomeric shift at 151.0 and 151.2 ppm (entry 3). In a controlled experiment bis-reagent **5** and nucleoside **1d** were dissolved in CD_3CN, and ^{31}P NMR was recorded over a period of 3 h, indicating no change in the 125.8 ppm shift, thus confirming the fact that bis-reagent **5** must be protonated or activated before it can phosphitylate. The necessity of activation was further demonstrated when Py·TFA was added to the mixture of **5** and **1d** and ^{31}P recorded again: two new peaks were observed at 151.0 and l51.2 ppm (entry 5). The new peaks were due to formation of **6d**.

In another experiment, bis-reagent **5** was treated with excess of TFA alone instead of Py·TFA, and ^{31}P NMR was recorded (data not shown). In this experiment, first the color of the solution changed from clear to dark in 5 min, and there were no peaks in the 120−140 ppm region.

The ^{31}P experimental data show that a reactive P(III) intermediate is formed via protonation of **5**, which is consistent with the mechanism proposed for 1*H*-tetrazole. The next question is whether the ^{31}P signal at 158.8 ppm is due to trifluoroacetyl anion reacting with **10**, furnishing **12** or pyridine reacting with **10** to form a pyridinium-type intermediate **13**. The formation of a reactive intermediate such as **13** was recently proposed[28] during oligonucleotide synthesis using pyridinium salts. To provide evidence for the role of pyridine in the activation of **5** and the support of the formation of **13**, we elected to perform ^{15}N NMR experiments with labeled pyridine.

The ^{15}N-labeled pyridine alone has a resonance peak of −69.0 ppm (entry 6, Table 4). Addition of TFA (1 equiv) to a solution of labeled pyridine (1 equiv) in CD_3CN gave Py·TFA (^{15}N) in situ, with a new resonance peak at −148.0 ppm. Addition of bis-reagent **5** (1 equiv) to the above solution collapsed the −148.0 resonance with appearance of a peak at −68.5 ppm (entry 8). Next, addition of nucleoside **1d** (0.95 eq.) to the mixture of **5** and Py·TFA (^{15}N) resulted in a resonance at −71.7 ppm. The ^{15}N experiment data, indicates that addition of Py·TFA to **5** results in formation of a relatively stable intermediate unlike **13** with presence of "free" pyridine in solution. It is noteworthy that free pyridine peak does not change upon addition of the nucleoside **1d**.

On the basis of the NMR data, we postulate that Py·TFA participates in the phosphitylation reaction as an activator

(25) The phosphitylation scale-up work is carried out by an outside company with a license to manufacture phosphoramidites in bulk quantities.
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in the following manner. Addition of Py·TFA to **5** generates a stable and reactive P(III) type intermediate via protonation of **5** in a reversible manner. The presence of free pyridine indicates that the reactive intermediate is different than structure **13**. Since there is no NMR data on the acyl type P(III) derivatives, we could not assign the signal at 158.8 ppm to structure **12**. Further mechanistic studies are in progress to determine the structure of the active intermediate.

Conclusions

This new simplified approach may become the method of choice for the preparation of not only nucleosidic phosphoramidites but also for other molecules of interest because of the relative ease of work-up, the high selectivity of activation, and the mildness of the activator and byproducts resulting therefrom. The use of Py·TFA in place of 1*H*-tetrazole brings cost-effectiveness and an ideal safety profile to the process for larger scale manufacture. Furthermore, ease of preparation, storage, and handling of Py·TFA makes it very attractive for a commercial manufacturing site.

Recently, Beier and Pfleiderer[28] have reported on the use of pyridinium salts, particularly the hydrochloride salt, as activators during solid-phase oligonucleotide synthesis using phosphoramidite chemistry. However, we believe that it would be very difficult to handle Py·HCl at a commercial site, mainly due to the very hygroscopic nature of the compound. With the success of Py·TFA as an activator for phosphitylation of monomeric compounds, it was obvious for us to extend the evaluation as activator for oligomerization using phosphoramidite chemistry on solid-support. Indeed, Py·TFA also works as an activator for oligonucleotide synthesis, however, under modified conditions. A detailed account of this process is accepted for publication elsewhere.[29] Additionally, the use of *N*-methylaniline TFA as an activator during oligonucleotide synthesis has been reported by Forrey and Varenne.[30]

Experimental Section

General. All reactions were performed under a positive pressure of argon; solvent concentration was accomplished with a Buchi rotary evaporator at ~25 mmHg pressure using a vacuum pump. The use of water aspirator may lead to partial hydrolysis of phosphoramidites. Commercial grade anhydrous solvents and reagents were used without further purification or drying. All of the final compounds (**6**−**9**) were characterized by ^{1}H and ^{31}P NMR, and data were collected on a Varian Unity 400 MHz spectrometer. The chemical shift values are expressed in parts per million (ppm) δ values, relative to TMS as the internal standard. The detection of nucleosidic compounds was on TLC (60F-254, E M Reagents) by UV light and with 10% sulfuric acid in methanol spray followed by heating. The starting materials **1a**−**d** and **4a**, **c**, **d**, **f** were purchased from Reliable Biopharmaceuticals, St. Louis, MO; **2a**−**c**, **e** and **3a**−**c**, **e** were purchased from ChemGenes, Waltham, MA. The bis-reagent **5** was purchased from ChemImpex International, Chicago, IL. Most of the activators were purchased from Aldrich Chemical Co., Milwaukee, WI. or prepared in situ by mixing 1 mol equiv of acid and appropriate base.

Typical Procedure for the Preparation of Phosphoramidite (6−9). To a stirred solution of nucleoside (**1**−**4**, 10 mmol) in dry CH_2Cl_2 (25 mL) was added bis-reagent (**5**, 3.62 g, 12 mmol) at ambient temperature. Py·TFA (2.32 g, 12 mmol) was added to the reaction mixture, and the solution stirred for 1−3 h, unless noted otherwise in Tables 1−3. Upon complete consumption of the starting material (TLC), the entire reaction mixture was transferred directly onto the top of a short silica gel column (ICN 32/62, 60 Å, 100 g, 5 × 30 cm). The product was eluted with 60−80% EtOAc: hexanes (1% triethylamine) mixture based on the polarity of the amidite. The appropriate fractions were collected and pooled, and the solvent was evaporated to furnish the respective amidite as a mixture of diastereoisomers, free of any solvents (see Tables 1−3 for yield). The overall purity of amidites (**6**−**9**) was determined by ^{31}P NMR[31] and HPLC[32] methods (>97%). Additionally, these amidites were used in synthesizing authentic oligonucleotide sequences, and the coupling efficiency (>99.8%) was comparable to the commercial amidites.[33]

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(31) ^{31}P NMR data: **6a**, 149.32, 149.43; **6b**, 149.32, 149.88; **6c**, 148.39, 149.15; **6d**, 149.14, 149.57; **7a**, 150.94, 151.67; **7b**, 150.77, 151.35; **7c**, 150.71, 150.95; **7e**, 150.86, 151.39; **8a**, 150.60, 151.05; **8b**, 149.85, 150.72; **8c**, 149.43, 150.37; **8e**, 150.22, 150.61; **9a**, 149.66, 151.59; **9f**, 150.76, 150.82; **9c**, 150.23, 150.82; **9d**, 150.69, 150.83; **6f**, 149.14, 149.67; **6g**, 149.26, 149.92.
(32) HPLC conditions: reverse phase NovaPak C-18 column, 3.9 × 150 mm, 4 μm particle size, mobile phase A (250 mM TEAA):B (acetonitrile), 50/50.
(33) All commercial 2′-deoxyphosphoramidites **6** were purchased from Amersham Pharmacia Biotech, Piscataway, NJ.