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Nucleosidic Phosphoramidite Synthesis via Phosphitylation: Activator Selection and Process Development

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Abstract:

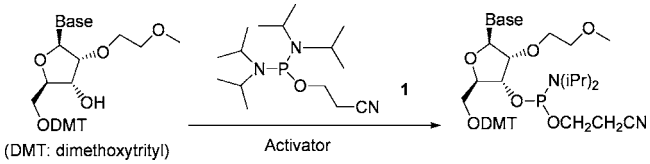
Nucleosidic phosphoramidites are key building blocks for the automated, solid supported syntheses of oligonucleotide-based drugs. A safe, industrially viable process for preparing nucleosidic phosphoramidites 5-Me-MOE-U and MOE-A has been developed and utilized on multikilogram scales. The optimization of this process is described in detail. Emphasis is placed on the search for activators to replace the hazardous 1*H*-tetrazole, the development of an extractive workup, and the advancement of a precipitation method to avoid both chromatographic purification and product foaming issues.

Introduction

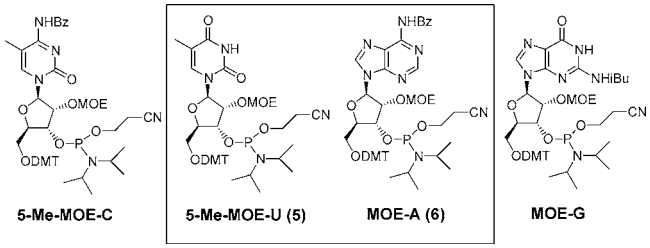
Numerous antisense oligonucleotide-based drugs are currently being evaluated in preclinical and clinical studies for the treatment of a variety of diseases including cancer, cardiovascular disease, diabetes, and inflammatory disorders.[1] A successful clinical evaluation and marketing strategy for such drugs requires that oligonucleotides become available in large quantities. Presently, clinical oligonucleotides are produced using solid-phase synthesis and employing nucleosidic phosphoramidites as the basic building blocks. However, synthesizing these nucleosidic phosphoramidites remains challenging, especially on large scale. With the growing success of clinical programs and the potential market demand, the development of efficient, robust syntheses of nucleosidic phosphoramidites is essential. Herein, we report a safe, scalable process for preparing nucleosidic phosphoramidites from nucleosides via phosphitylation (Scheme 1).

The chemically modified and structurally more complex oligonucleotides such as the 2′-*O*-methoxyethyl (MOE) derivatives have recently emerged as the second generation antisense drugs.[2] These compounds exhibit improved hybridization properties, more favorable nuclease resistance, and improved pharmacological profiles.[3] Given the medicinal importance of the 2′-*O*-MOE oligonucleotides, considerable efforts have been directed towards synthesizing the corresponding nucleosidic phosphoramidites. The processes we describe focus on two of the targets shown in Scheme 2. These targets are 5-Me-MOE-U amidite (**5**), a representative pyrimidine nucleosidic phosphoramidite class, and MOE-A amidite (**6**), a representative of the purine nucleosidic phosphoramidites.

Scheme 1. **Synthesis of nucleosidic phosphoramidites**



Scheme 2. **2′-*O*-MOE nucleosidic phosphoramidites**



Issues with the Initial Process.[4] Initially, the desired phosphoramidites were prepared by treating the appropriate nucleosides with 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropyl phosphorodiamidite (**1**),[5] 1*H*-tetrazole, and *N*-methylimidazole in DMF at ambient temperature. When the nucleosides were consumed, the reaction mixtures were diluted with DMF/H_2O, washed with hexanes, and extracted with hexanes/

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(1) (a) Holmlund, J. T. *Ann. N. Y. Acad. Sci*. **2003**, *1002*, 244. (b) Crooke, S. T. *Oncogene* **2000**, *19*, 6651. (c) Ravichandran, L. V.; Dean, N. M.; Marcysson, E. G. *Oligonucleotides*, **2004**, *14*, 49. (d) Yu, R. Z.; Su, J. Q.; Grundy, J. S.; Sewell, K. L.; Dorr, A.; Levin, A. A. *Antisense Nucleic Acid Drug Dev*. **2003**, *13*, 57.

(2) (a) Sewell, K. L.; Geary, R. S.; Baker, B. F.; Glover, J. M.; Mant, T. G. K.; Yu, R. Z.; Tami, J. A.; Dorr, F. A. *J. Pharmacol. Exp. Ther*. **2002**, *303*, 1334. (b) Garay, M.; Gaarde, W.; Monia, B. P.; Nero, P.; Cioffi, C. L. *Biochem. Pharmacol*. **2000**, *59*, 1033−1043. (c) Bost, F.; McKay, R.; Dean, N. M.; Potapova, O.; Mercola, D. *Methods Enzymol.* **2000**, *314* (Antisense Technology, Part B), 342−362.

(3) Altman, K.-H.; Dean, N. M.; Fabbro, D.; Frier, S. M.; Geiger, T.; Haner, R.; Husken, D.; Martin, P.; Monia, B. P.; Muler, M.; Natt, F.; Nicklin, P.; Phillips, J.; Pieles, U.; Sasmor, H.; Moser, H. H. *Chimia* **1995**, *50*, 168−176.

(4) Procedures of the initial process, see Experimental Section.

(5) Preparation of reagent **1** and its application in antisense oligonucleotide synthesis: (a) Barone, A. D.; Tang, J. Y.; Caruthers, M. H. *Nucleic Acids Res*. **1984**, *12*, 4051. (b) Kraszewski, A.; Norris, K. E. *Nucleic Acids Res*. **1987**, *18*, 177. (c) Song, Q.; Ross, B. WO 03/087130 A2.

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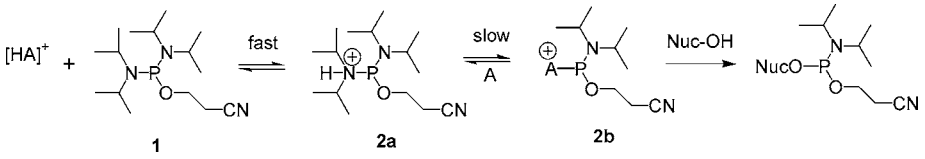
Scheme 3.[13] **Activation mechanism**



toluene. Those extracts were washed with water and subsequently concentrated to a foam to provide the corresponding nucleosidic phosphoramidites in 75−95% yields.

Despite reasonable yields, the above procedure had a number of processing issues which made it impractical for pilot-plant operations. The major issue centered on the use of 1*H*-tetrazole as the activator for the relatively nonreactive phosphitylating agent **1**. 1*H*-tetrazole is classified as an explosive and must be stored in sealed magazines prior to use.[6] By Indiana State law, a Federal ATF[7] permit is required to purchase and hold the material. Thus, our first development goal was to eliminate this reagent from the synthesis.

A second problem with the original process was its lengthy and cumbersome workup protocol. A total of nine extractions were used to remove excess reagents and byproducts. Consequently, the workup volumes were very large, and the operation times were extensive, leading to potential decomposition of the phosphoramidites. Typically, the crude material from this procedure required further purification and manipulations to reach a suitable purity and form.

The third major issue with the procedure was the isolation of the desired product as an intractable foam. To our knowledge, none of the MOE-substituted nucleosidic phosphoramidites have been found to be crystalline entities, and many do not even form manageable amorphous solids. In our case, the final workup solution was concentrated to dryness, and the resulting foam was collapsed to fine particles via scraping with a spatula. Such operations are not viable on pilot-plant scale, and thus development of a reliable precipitation method was another major goal.

The process development of the nucleosidic phosphoramidite synthesis is further complicated by a number of other issues. Since the compounds are acid sensitive, they are subject to de-tritylation and de-purination reactions.[8] Since they are also base sensitive, hydrolysis reactions are also factors. Finally, the materials exhibit poor ARC[9] profiles with high risk of thermal liability. A hazards review by Lilly Chemical Hazards Laboratory indicated that the 5-Me-MOE-U (**5**) and MOE-A (**6**) phosphoramidites should not be exposed to temperatures above 45 and 35 °C, respectively.[10]

The above stated characteristics greatly limit the available workup and solvent-exchange conditions for these compounds and thus necessitate the development of highly efficient reaction conditions which provide very clean crude product as a starting point for isolation. With all of these issues in mind, we initiated the process development of the MOE nucleosides, while striving to rapidly determine practical reaction/isolation conditions which could facilitate the multikilogram production of phosphoramidites in our pilot plant.

Activator Selection. Literature surveys revealed several potential replacements for 1*H*-tetrazole as the activator for the phosphitylation chemistry. These included 4,5-dicyanoimidazole (DCI),[11] and various imidazolium, anilinium, and pyridinium salts.[12] Our lab trials on the phosphitylation of 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-N^4-benzoyl-2′-deoxy-5-methylcytidine (5-Me-dC) using DCI (0.8 equiv in DMF at ambient temperature for 5 h) afforded an 84% yield of the desired phosphoramidite, which was ∼5% lower than trials using 1*H*-tetrazole. A major drawback to using DCI was the need for a silica gel plug to remove the DCI residue. In the 1*H*-tetrazole case, an aqueous workup removes all activator residues, which is advantageous. Hence, we decided to focus on ammonium, pyridium, and azolium salts in our activator screening studies. These derivatives were widely available, had mild acidity, and were water soluble.

Ammonium Salts. The mechanisms described in the literature[12,13] indicate that the activator must be acidic to allow protonation of the diisopropylamine group of phosphitylating reagent **1** (Scheme 3). The rate-determining step in these mechanisms is the displacement of the diisopropyl amino group with the activator to form **2b**. A substitution reaction on this activated intermediate (**2b**) by the nucleoside (Nuc-OH) generates the nucleosidic phosphoramidite. An ideal activator thus not only requires mild acidity and good nucleophilicityy but should also serve as a good leaving group to complete the substitution reaction.

To better understand the requirement of the activator as a leaving group, we chose diisopropylammonium chloride as the activator, since the phosphitylation reaction itself produces one equivalent of diisopropylamine. Consequently, 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-N^4-benzoyl-2′-deoxy-5-methylcytidine (5-Me-dC nucleoside) was treated with 1 equiv of diisopropylamine hydrochloride and 1.5 equiv of phosphitylating reagent **1** in DMF at room temperature. Interestingly, the reaction went smoothly but required a longer reaction time than that of 1*H*-tetrazole. After 24 h, approximately 30% of the starting nucleoside remained in the reaction mixture. Longer reaction times increased con-

(6) (a) Stull, D. R. *Fundamentals of Fire and Explosion;* AIChE Monograph Series; 1977; Vol. 73(10), p 22. (b) See Material Safety Data Sheet in Sigma-Aldrich database: www.sigmaaldrich.com.
(7) ATF stands for Bureau of Alcohol, Tobacco and Fire Arms.
(8) Krotz, A. H.; Klopchin, P. G.; Walker, K. L.; Srivatsa, G. S.; Cole, D. L.; Ravikumar, V. T. *Tetrahedron Lett.* **1997**, *38*, 3875.
(9) ARC abbreviates as Accelerated Rate Calorimetry. The ARC data were accumulated by Mr. D. Cheatham of the Lilly Hazards Laboratory.
(10) Applicable to near atmospheric pressure and quantities of less than 500 kg.
(11) Vargeese, C.; Carter, J.; Yegge, J.; Krivjahsky, S.; Settle, A.; Kropp, E.; Peterson, K.; Pieken, W. *Nucleic Acids Res.* **1986**, *26*, 1046.
(12) (a) Hayakawa, Y.; Kawai, R.; Hirata, A.; Sugimoto, J.; Kataoka, M.; Sakakura, A.; Hirose, M.; Noyori, R. *J. Am. Chem. Soc.* **2001**, *123* (34), 8165−8176. (b) Sanghvi, Y.; Guo, Z.; Pfundheller, H.; Converso, A. *Org. Process Res. Dev.* **2000**, *4*, 175.
(13) Dahl, B. H.; Nielsen, J.; Dahl, O. *Nucleic Acids Res.* **1987**, *15*, 1729.

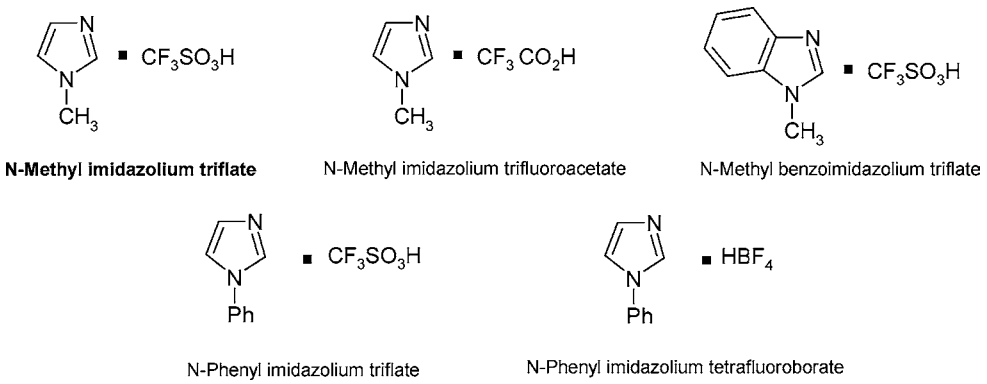
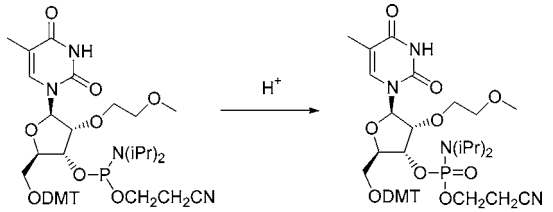


Figure 1. **Substituted imidazolium salts.**

Scheme 4. **Oxidation of 5-Me-MOE-U under acidic condition**



version, but led to higher levels of undetermined related substances, likely due to the instability of the phosphoramidite product over time. Similar reactions with 1*H*-tetrazole take 3−4 h to reach completion. This experiment showed that a better leaving group than diisopropylamine was required to increase the reaction rate.

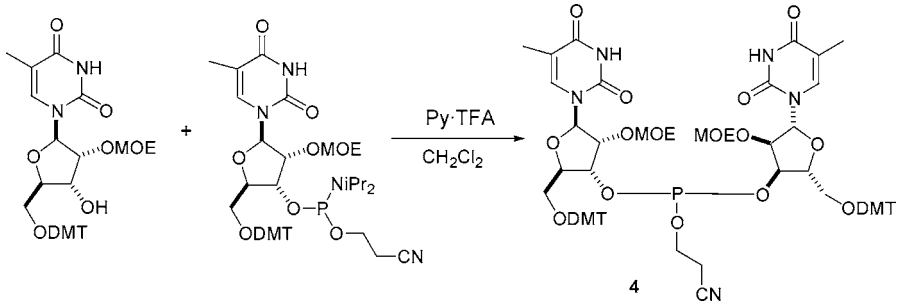
Pyridinium Salts. Based upon the availability, cost considerations, and literature precedent,[12b] pyridinium trifluoroacetate (Py·TFA) was the next activator tested in our 5-Me-MOE-U reactions. This salt worked well in a number of acceptable solvents (e.g., acetonitrile), with reasonable reaction rates (3−12 h). One problem observed when using Py·TFA as activator was residual pyridine in the crude product. Although the TFA salt was water soluble and thus easily removed during the aqueous workup, free pyridine was not readily washed from the system. We were unable to perform even mild acid washes due to product decomposition. For example, an aqueous wash of the crude 5-Me-MOE-U with a 1% solution of citric acid (2 × 5 vols) led to the formation (34%) of the phosphorus(V)-related impurity **3** (Scheme 4). Activated carbon (Darco, 20−40 mesh) treatment of the water-washed product decreased the pyridine amount to a minimum, but with a product yield loss of 20%. Therefore, any potential advantages of Py·TFA as activator were offset.

A second problem associated with Py·TFA was the formation of a dimer **4**[14] (Scheme 5). Phosphitylation of the 5-Me-MOE-U nucleoside with 1.2 equiv of reagent **1** and 1.0 equiv of Py·TFA in 5 vols of CH_2Cl_2 at room temperature yielded ∼30% of the dimer **4**. The mechanism of the dimer formation is similar to that of the 5-Me-MOE-U amidite formation. Py·TFA protonates the diisopropylamine group of the newly formed phosphoramidite product, and the dimer forms when another molecule of nucleoside displaces the activated amidite. Ideally, the activator should be active enough to trigger the protonation of the phosphitylating reagent without allowing subsequent protonation of the desired product. This requirement led us to investigate the azolium salts.

Azolium Salts. Figure 1 shows a number of different substituted imidazolium salts which could serve as potential activators for nucleosidic phosphitylation. *N*-Methyl benzimidazolium salts and *N*-phenylimidazolium (NPI) salts have been reported to be efficient promoters for the synthesis of oligodeoxyribonucleotides and oligoribonucleotides via the phosphoramidite approach.[12a] Our investigations on the use of these salts were not fruitful. Both reaction efficiency and chemical yield with *N*-methyl benzoimadazole triflate were acceptable, but the compound did not meet our requirement of being easily extractable by aqueous media. Laboratory trials using *N*-phenyl imidazolium triflate (NPI·Tf) and *N*-phenyl imidazolium tetrafluoroborate (NPI·HBF_4) afforded much slower reaction rates[15] and ∼10% lower yields than desired.

We also tested *N*-methyl imidazolium trifluoroacetate, a relatively cheap salt, which met our water solubility require-

Scheme 5. **Dimer formation with Py·TFA**



ment. This activator afforded good yields with acceptable reaction rates in the nucleosidic phosphitylation reaction. However, its hygroscopicity gave us reservations about its large-scale use.

According to the *CRC Handbook*,[16] *N*-methyl imidazole has infinite solubility in water. *N*-Methyl imidazolium triflate is not only highly water soluble but also has excellent solubility in polar organic solvents such as DMF and acetonitrile. Consequently, this salt was our next choice to test as an activator for our phosphitylation reactions. A small amount of *N*-methyl imidazolium triflate (NMI·Tf) was prepared using a literature example.[12b] The compound was tested in the 5-Me-MOE-U amidite formation and was found to perform better than 1*H*-tetrazole. The reaction activated with NMI·Tf consistently provided >90% chemical yield with product purity >98%. Careful ^{1}H NMR and HPLC analyses of our crude products were performed to ensure that all residual salt and free amine had been removed by the aqueous washes.

As confidence increased that this salt was the activator of choice for our chemistry, the literature preparation of *N*-methyl imidazolium triflate was quickly modified to replace diethyl ether with MTBE and to increase concentration. Using the adjusted conditions, we were able to produce over 27 kg of the pure salt to meet our longer-term production needs.

To date, NMI·Tf stands as the best activator among all those tested. This compound met all of our processing requirements and allowed us to proceed with the development of the nucleosidic phosphoramidites described in the following sections. Unless specifically indicated otherwise, NMI·Tf was employed as the activator in those studies.

Synthesis of 5-Me-MOE-U (5). With the activator identified, our attention turned to optimizing the reaction conditions and developing a dependable isolation/purification method. Because of the ready availability of the starting nucleoside, 5-Me-MOE-U was chosen to start our studies. However, the solubility characteristics of **5** indicated that a precipitative isolation would be difficult.

Our studies on activation with Py·TFA had demonstrated that solvent played an important role in the phosphitylation of 5-Me-MOE-U. For comparative purposes, CH_2Cl_2 was tested in the phosphitylation of 5-Me-MOE-U while using NMI·Tf as the activator. These trials were successful, producing high quality **5** in 90% yield. However, this solvent did not lend itself to precipitation methods for the 5-Me-MOE-U amidite, requiring vacuum distillation for product isolation as a foam.

Acetonitrile was tested next and was found to exhibit a number of advantages over other solvents. First, its high polarity afforded good solubility for all the substrates and reagents. The phosphitylation reactions on 5-Me-MOE-U were both fast and very clean relative to those using other solvents. Additionally, a solvent exchange could be affected easily by MTBE extraction of the crude product followed by aqueous extraction of acetonitrile (and other impurities) during workup. Further development of this extractive solvent exchange led to the very efficient system of MTBE with 1:1 DMF/H_2O washes for the workup protocol.

To this point, all development for the MOE nucleosides had been performed using small, laboratory-scale experiments (<1 g scale). On such scale, the reagents were typically combined with no concern for exothermic events. When a 5-g trial was performed in this manner, a 15−20 °C exotherm ensued, with concomitant decrease in overall product purity. This exotherm would likely be more significant on larger scale. With the ARC data indicating the MOE amidites to be unstable at only moderately elevated temperatures, exothermic events were unacceptable.

Subsequently, an experiment was performed whereby reagent **1** was dissolved in a small amount of acetonitrile, and that solution was added slowly to the 5-Me-MOE-U substrate. This method controlled the exotherm and afforded good-quality product. However, ^{31}P NMR analysis of the products indicated a significant impurity at 141 ppm.[17] LC/MS data indicated the new peak to have a mass of 1337 (vs the desired mass of 818). These data, along with the ^{31}P NMR results, supported the conclusion that dimer **4** was again being produced. The adjusted reaction conditions (i.e. slowly adding the reagent **1** to the MOE nucleoside), which had been invoked to control exothermic events, had led to the new problem of dimer formation. We also observed that the amount of dimer **4** formed (20−40 mol %) depended on the addition rate. This result suggested that the solution to the problem might simply be inverse addition of the reagents. Thus, an experiment was performed whereby the 5-Me-MOE-U starting material was dissolved in acetonitrile and added dropwise to a solution of reagent **1** and activator NMI·Tf. This protocol, which maintains an excess of **1** throughout the addition, both controlled the exotherm and eliminated the dimer **4** formation.

The above modifications to the reaction procedures resulted in large strides towards the goal of consistently obtaining good-quality 5-Me-MOE-U amidite. However, even that product quality was still not considered high enough to afford reproducible results in a precipitation method. Further investigation into this reaction was clearly needed.

A known culprit in phosphitylation reactions is adventitious water. Several advantages of choosing acetonitrile as reaction solvent were mentioned earlier. Another major advantage stems from the solvent's known azeotrope with water.[18] When the starting 5-Me-MOE-U nucleoside was dissolved in an excess (6 vols) of acetonitrile and then vacuum distilled to the desired reaction concentrations (4 vols) prior to any other reagent additions, a significant jump in product purity was realized. Consequently, the acetonitrile

(14) Dimer **4** was identified by ^{31}P NMR. The chemical shifts of 5-Me-MOE-U amidite and **4** are very different. 5-Me-MOE-U amidite absorbs at δ ~150 ppm, while **4** absorbs at δ ~140 ppm ($CDCl_3$).

(15) Reactions using NPI·Tf and NPI·HBF_4 required 16 h to go completion.

(16) *CRC Handbook of Chemistry and Physics*, 58th ed.; CRC PRESS: West Palm Beach, Florida, 1977; p 347.

(17) The ^{31}P NMR ($CDCl_3$) data show a single peak at 141 ppm, not two peaks. This fact indicated that the diastereomers that are normally produced around the phosphorous atom were not present and meant that the impurity might have two identical groups on the P atom.

(18) *Azeotrope Data-III*; Advances in Chemistry Series 116; Gould, R. F., Ed.; American Chemical Society: Washington, D.C., 1973. The binary azeotrope between water and acetonitrile forms at 81.6 °C and is 16.5% water in the distillate at atmospheric pressure.

azeotrope was incorporated into the procedure to control the solution water content to <0.1%.[19]

Stoichiometric Ratio. After finalizing reaction parameters such as solvent, concentration, and temperature, we turned our attention to determining the optimum ratio of nucleoside/reagent **1**/activator (NMI·Tf). Separation of the excess P(III) reagent **1** from amidite could be achieved using heptane/DMF/H_2O extractions, with **1** distributed in the heptane layer. However, since earlier ARC[9] studies had demonstrated that the amidite was sensitive at temperatures ≥45 °C, a solvent exchange from acetonitrile with heptane/DMF was not an option and was consequently abandoned.

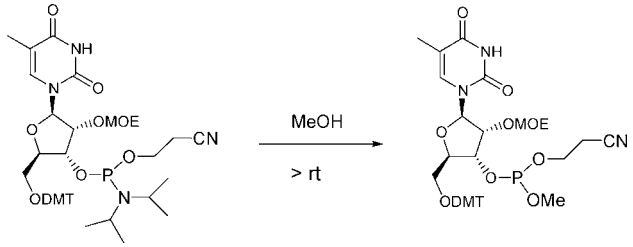
Control experiments had demonstrated that **1** was unstable at room temperature under the phosphitylation conditions. Furthermore, **1** is both moisture sensitive and tends to easily oxidize to P(V) compounds. We originally employed an excess amount (1.5 equiv) of reagent **1** to both promote reaction completion and prevent the formation of dimer **4**. With the difficulty of removing excess **1**, we decided to decrease the reagent level to 1.05 equiv.[20]

Initially, stoichiometric amounts of NMI·Tf were tested in conjunction with 1.1 equiv of reagent **1**. Subsequent experiments showed that 0.5 equiv of NMI·Tf was sufficient to attain reaction completion. However, the reaction time increased from 4 to 12 h when using the lowered activator amounts. Additionally, trials using 0.5 equiv of NMI·Tf typically had residual levels of **1** after workup. Adjusting the activator load to 0.75 equiv consumed essentially all reagent **1** with full conversion of the starting material to product. Therefore, the optimum ratio of nucleoside/**1**/activator was determined to be 1.00:1.05:0.75.

Extractive Purification. The impurities produced in the phosphitylation reaction include imidazole, imidazole triflate, diisopropylamine salts, low levels of **1**, and the P(V) impurities derived from both **1** and the product. The first three of these are easily removed by the aqueous extraction. However, separating the P(V) impurities from product by a traditional extractive workup was troublesome. We did determine that MTBE/DMF/H_2O extractions could remove the bulk of these P(V) impurities. The optimum ratio of DMF/H_2O is 1:1, with higher ratios causing unacceptable yield losses. Employing two such washes during the workup typically removed 80% of the P(V) impurities while losing < 5% of the product.

Precipitation. As stated previously, foaming is a major problem when isolating amidites on scale. Development of a suitable crystallization or precipitation method was required for our production levels. Solubility studies on the nucleosidic amidites had indicated that developing precipitation or crystallization methods would be very challenging. Antisolvents were limited to heptane, hexane, and water. Attempts to actually crystallize 5-Me-MOE-U under numerous conditions were unsuccessful.

Scheme 6. **Formation of *O*-methoxy-Derived byproduct**



A precipitation trial using MeOH/H_2O provided filterable solids with reduced P(V) impurity levels. However, the residual MeOH and H_2O were difficult to remove by the usual drying methods. Since these two solvents adversely affect the efficiency of the downstream chemistry (oligonucleotide synthesis), they needed to be eliminated. Additionally, a new byproduct formed when using this system. This byproduct, shown in Scheme 6, was identified by LC/MS as the methoxy derivative and was formed by MeOH displacement of diisopropylamine. Due to these issues, work on a MeOH−H_2O precipitation process was abandoned.

Our efforts next turned to finding a nonaqueous precipitation method. Adding the antisolvent hexane to MTBE solutions of **5** only led to glue-like mixtures of the product. Reverse addition of an MTBE solution of **5** to cold hexanes (−9 °C) was promising, providing filterable solids. Unfortunately, these solids oiled-out on the filter.

Another issue with the MTBE/hexane reverse addition method, was the large solvent volumes required to achieve precipitation. With the typical concentrations of **5** in MTBE after reaction workup, upwards of 100 vols of hexane were needed to induce precipitation. Further studies indicated that there was an optimum ratio of hexane/MTBE which would induce precipitation while preventing the filtered product from transforming into an oil. Modifying the process to include a vacuum distillation with simultaneous addition of hexane to maintain constant tank volume afforded the desired result. Using that process while maintaining a 20/1 hexane/MTBE ratio resulted in well-behaved, filterable solids.

A final processing challenge for **5** arose when oven drying the filtered solids. The material expanded to ∼3 times its initial volume while self-converting into a foam. Fortunately, a simple switch from hexane to heptane as the antisolvent solved this problem without adversely affecting the precipitation behavior of the product.

Synthesis of MOE-A Phosphoramidite (6). With the significant progress achieved on the 5-Me-MOE-U amidite process, our attention turned to the MOE-A amidite **6**. Much of the knowledge acquired during the process development of the 5-Me-MOE-U derivative was successfully applied to **6**. However, an initial laboratory trial using the identical protocol developed for 5-Me-MOE-U (acetonitrile, 1.1 equiv of phosphitylating reagent **1**, 0.75 equiv of activator salt) did not go to completion after stirring for 16 h. Obviously, further process development was needed to optimize the reaction conditions for the MOE A amidite.

A major difference between the "A" and "U" starting nucleosides was solubility. The N^6-benzoyl adenosine portion of the molecule afforded a decreased solubility profile for

(19) Water content was analyzed by the Karl-Fisher method.

(20) The purity of 2-cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (**1**) is required to be >98%. 2-Cyanoethyl *N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite used at the pilot plant was purchased from Digital Specialty Chemicals, Inc. Canada.

Table 1. **Effect of DMF/H_2O ratio for MTBE/DMF/H_2O extraction[a]**

	DMF:H_2O				
	0:0	1:5	1:2	1:1	2:1
remaining P(V)[22]	30.0%	9.3%	7.6%	4.6%	4.4%
recovery	100%	91%	89.4%	88.2%	85.5%

[a] Workup conditions: 15 vols MTBE/10 vols DMF−H_2O.

MOE-A versus that for MOE-U. Consequently, the compound did not fully dissolve when using the same concentration of acetonitrile employed for the 5-Me-MOE-U nucleoside.

DMF is certainly one of the most common solvents employed in the nucleoside phosphitylation literature, and the MOE-A nucleoside was quite soluble in this solvent. Reactions using DMF performed well. Our processing goal was to maintain the azeotropic drying capabilities of acetonitrile while keeping the amount of DMF in the system to a minimum. Towards that end, a set of experiments was designed to determine the best combination of DMF and acetonitrile for this reaction. The ending conditions utilized 2 vols of DMF to dissolve the starting material and 8 vols of acetonitrile for the azeotropic drying. When this initial acetonitrile was removed, the remaining DMF solution of the starting material was added slowly to an acetonitrile solution (2 vols) of **1** and the activator salt. This protocol resulted in very clean MOE-A amidite reaction profiles after 16 h.

A reaction which employed the DMF/acetonitrile conditions using 1.00 equiv of activator and 1.25 equiv of phosphitylating reagent, exhibited an impurity at ~140 ppm[21] in the ^{31}P NMR spectrum. A subsequent experiment where unadulterated reaction samples were checked by ^{31}P NMR showed the impurity was forming during the reaction, not during workup. Returning to a full equivalent of the activator suppressed these impurities.

Extractive Workup. The MTBE/DMF/H_2O workup procedure, developed earlier for the 5-Me-MOE-U amidite, was successfully applied to compound **6** with only minor modifications. To determine the best extraction method to remove P(V) impurities, we started with crude material containing 30% of the P(V) species. We then varied the ratio of DMF/H_2O in the extractions and analyzed the products for the amount of residual P(V) impurities. The results, summarized below in Table 1, indicate that the use of 1:1 DMF/H_2O afforded the best balance of product recovery and impurity rejection for the MOE-A amidite.

Precipitation. Attempts to crystallize MOE-A utilizing binary solvent systems such as CH_2Cl_2/hexane, acetone/water, acetonitrile/MTBE, acetonitrile/hexanes, EtOAc/hexane, acetone/heptane, and toluene/heptane afforded thick oils or gummy solids. Accordingly, we turned our attention to the development of a feasible precipitation method comparable to that developed for the 5-Me-MOE-U amidite.

Fortunately, those same solubility characteristics which had caused problems with the MOE-A starting material were found to be advantageous for isolation of the MOE-A product. We rapidly determined precipitation conditions using an EtOAc/hexanes system with a reverse addition protocol. Thus, adding an EtOAc solution of the product to stirring hexanes resulted in the immediate formation of solids. This method also reduced the levels of P(V) impurities to an acceptable level. The only drawback of the EtOAc/hexanes system was the large solvent volumes. The minimum hexanes/EtOAc ratio needed for successful precipitation of MOE-A amidite was 50:1, making scale-up difficult.

Further investigations revealed MTBE be a good replacement for EtOAc. Using a 5:1 ratio of hexanes (or heptane) to EtOAc, and employing a reverse addition protocol, resulted in easily filtered MOE-A amidite. This solvent system efficiently removed the P(III) and P(V) impurity species, providing crude products of >98% purity.

Conclusions

In summary, we have achieved our goal of developing a safe, reliable process for the synthesis of nucleosidic phosphoramidites 5-Me-MOE-U and MOE-A. We identified a cheap, water-soluble activator to replace the hazardous 1*H*-tetrazole typically used in these reactions. Significantly, dependable precipitation and isolation conditions were developed for the traditionally problematic phosphoramidite products. The finely tuned process affords products of such purity as to avoid the typical chromatographic purifications common in the literature. Finally, these two key nucleosidic phophoramidites have been successfully tested on pilot-plant scale. The results represent a step forward in the synthesis of these key building blocks for the oligonucleotide-based drug candidates.

Experimental Section

General. All reagents and solvents were purchased and used without further purification. ^1H and ^{13}C NMR spectra were recorded respectively at 500 and 125 MHz on a Varian Inova spectrometer with the solvents as internal standard (δ_H: $CDCl_3$ 7.26 ppm; δ_C: $CDCl_3$ 77.0 ppm). *J* values are given in Hz. ^{31}P NMR spectra were recorded at 202 MHz on a Varian Inova spectrometer with H_3PO_4 as an external standard (δ_P: H_3PO_4 0.00 ppm). Product purities and in-process controls were measured by HPLC employing a Discovery HS C18 column (7.5 cm × 4.6 mm i.d., 3 um, s/n: 40962-03), plus a 2 cm × 4.0 mm, 3 um guard, at a column temperature of 30 °C and UV wavelength of 260 nm. The gradient elution was 60% acetonitrile/10 mM NH_4-OAc ramping to 90% acetonitrle/10 mM NH_4OAc over 15 min at flow rate of 1.0 mL/min, then kept at 90% acetonitrle/10 mM NH_4OAc for 8 min.

Initial Process Procedure.[22−23] *[5′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-O-(2-methoxyethyl)-5-methyluridin-3′-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (5).* 5′-*O*-

(21) The impurity was assumed to be dimer, with a similar chemical shift as **4** (^{31}P NMR).

(22) Mole percentage of the remaining P(V) impurities is determined by the sum of integration of all P(V) peaks which is <60 ppm in ^{31}P NMR ($CDCl_3$).

(23) The initial process was operated in 50-L three-necked flasks.

(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-5-methyluridine (1237 g, 2.0 mol) was dissolved in anhydrous DMF (2.5 L). The solution was co-evaporated with toluene (200 mL) at 50 °C under reduced pressure. After cooling to room temperature, 2-cyanoethyl tetraisopropylphosphorodiamidite (900 g, 3.0 mol) and tetrazole (70 g, 1.0 mol) were added. The mixture was shaken until all tetrazole was dissolved, and *N*-methylimidazole (20 mL) was added. After stirring at room temperature for 5 h, triethylamine (300 mL) was added. The resulting mixture was diluted with DMF (3.5 L) and water (600 mL) and then was extracted with hexane (3 × 3 L). The aqueous layer was further diluted with water (1.6 L) and extracted with a mixture of toluene (12 L) and hexanes (9 L). The two layers were separated, and the upper layer was washed with DMF/water (7:3, v/v, 3 × 3 L) and water (3 × 3 L). The upper layer was dried (Na_2SO_4), filtered, and concentrated in vacuo. The residue was co-evaporated with acetonitrile (2 × 2 L) under reduced pressure and dried to a constant weight (25 °C, 0.1 mmHg, 40 h) to give the product as an off-white foam.

[5′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-O-(2-methoxyethyl)-N⁶-benzoyladenosin-3′-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (6). 5′-*O*-(4,4′-Dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-N^6-benzoyladenosine (1098 g, 1.5 mol) was dissolved in anhydrous DMF (3 L). The resulting solution was coevaporated with toluene (300 mL) at 50 °C under reduced pressure. After cooling to ambient temperature, 2-cyanoethyl tetraisopropylphosphorodiamidite (680 g, 2.26 mol) and tetrazole (78.8 g, 1.24 mol) were added. This mixture was shaken until all tetrazole was dissolved and then *N*-methylimidazole (30 mL) was added. After stirring at ambient temperature for 5 h, triethylamine (300 mL) was added. The reaction mixture was diluted with DMF (1 L) and water (400 mL), and then extracted with hexanes (3 × 3 L). The aqueous layer was further diluted with water (1.4 L) and extracted with a mixture of toluene (9 L) and hexanes (6 L). The two layers were separated and the upper layer was washed with DMF/water (60:40, v/v; 3 × 3 L) and water (3 × 2 L). The organic layer was dried (Na_2SO_4), filtered and evaporated to a sticky foam. The residue was coevaporated with acetonitrile (2.5 L) under reduced pressure and dried to a constant weight (25 °C, 0.1 mmHg, 40 h) to give the product as an off-white foam.

Improved Pilot-Plant Procedures. *[5′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-O-(2-methoxyethyl)-5-methyluridin-3′-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (**5**).* A 30-gal glass-lined reactor was charged with (5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-5-methyluridine (5.00 kg, 8.08 mol) and acetonitrile (30 L). The resulting solution was concentrated via vacuum distillation (54−31 mmHg) to ∼15 L at 20−25 °C (vapor temperature) until the water content was <0.05% by Karl Fischer titration. The tank contents were filtered through a 5 μ cartridge filter[24] into a portable 8-gal vessel. The original tank was rinsed with 3 L of dry acetonitrile, and this rinse was also transferred through the line to the 8-gal vessel. The end solution was held under nitrogen for further processing.

Acetonitrile (10 L), NMI·Tf (1.41 kg, 6.07 mol), and 2-cyanoethyl tetraisopropylphosphorodiamidite (2.56 kg, 8.49 mol) were subsequently charged to a separate 30-gal glass-lined reactor. The vacuum-dried, acetonitrile solution of (5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-5-methyluridine from above was added to this second reactor over 30−60 min, keeping the tank temperature at 20−25 °C. The resulting mixture was stirred at 20−25 °C for 12−20 h until the starting material was <0.1% by HPLC. The tank contents were then transferred to a 50-gal glass-lined reactor, diluted with MTBE (75 L), washed with water (25 L), 1:1 (v/v) DMF:H_2O (2 × 50 L), water (25 L), and 13% brine (25 L). The MTBE layer was dried with Na_2SO_4 (10 kg) and filtered into a clean 30-gal glass-lined reactor, and the filter cake rinsed with MTBE (20 L). This product solution was then concentrated via vacuum distillation to a volume of 17 L while maintaining the liquid temperature at <35 °C.

Heptane (150 L) was charged to a 50-gal glass-lined reactor and held for use during the following precipitation procedure. Heptane (125 L) was charged to a second 50-gal glass-lined reactor which was set up for vacuum reflux/distillation. Sufficient vacuum and heat were applied to achieve reflux of the heptane at a liquid temperature of 20−25 °C. Once the heptane began to reflux, the reactor was switched to distillation mode. Next, the simultaneous co-addition of fresh heptane from the first 50-gal reactor and the MTBE solution of crude **5** from the 30-gal reactor was initiated (at a ratio of ∼8:1 heptane:product−MTBE solution) while maintaining a constant 125-L volume in the precipitation reactor. (Note: The product begins to precipitate immediately with its addition to the heptane solution). After the addition of the product solution was complete, the distillation was continued until the solution volume was reduced to 50 L. Additional heptane was then added to adjust the volume of the slurry to 125 L. Heating was stopped, and the resulting precipitate was allowed to stir at ambient temperature for 1.5 h.

The precipitate was vacuum filtered, and the solids were rinsed with hexane (3 × 25 L) and then allowed to pull dry. The filter cake was removed to a tared tray and vacuum-dried at 35 °C to constant weight. A total of 7.61 kg of white solids was recovered (93.2%) with an HPLC purity of 99.1% as a 1:1 mixture of a pair of diastereomers at the phosphorus center: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.88−8.94 (br, 1), 7.69 (d, 1 × 0.5, J = 1.1), 7.62 (d, 1 × 0.5, J = 1.1), 7.44−7.40 (m, 2), 7.33−7.22 (m, 7), 6.85−6.81 (m, 4), 6.08 (d, 1 × 0.5, J = 5.0), 6.03 (d, 1 × 0.5, J = 4.5), 4.54 (ddd, 1 × 0.5, J = 10.0, 5.0, 5.0), 4.48 (ddd, 1 × 0.5, J = 11.0, 5.0, 5.0), 4.30−4.28 (m, 1), 4.23−4.20 (m, 1), 3.98−3.50 (m, 9), 3.79 (s, 6), 3.33 (s, 3), 3.35−3.28 (m, 1), 2.65 (dd, 1, J = 6.0, 6.0), 2.38 (dd, 1, J = 6.0, 6.0), 1.34 (d, 3, J = 3.2), 1.18−1.16 (m, 9), 1.01 (d, 3, J = 7.0); ^{13}C NMR (125 MHz, $CDCl_3$) δ (164.1, 164.0), 158.7, (150.6, 150.5), (144.3, 144.2), 135.7, 135.4, (135.30, 135.2), (130.30, 130.26), (130.19, 130.17), (128.4, 128.2), (127.98, 127.95), 127.2,

(24) Filtration is required to remove unreactive solid particles contained in the custom-made raw material (5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-5-methyluridine. This unit of operation could be ignored if a high-quality raw material is employed.

(117.9, 117.4), (113.24, 113.20), (87.6, 87.1), (87.1, 86.9), (81.8, 81.2), (72.4, 72.2), (70.7, 70.6), (70.4, 70.3), (70.0, 70.0), (62.7, 61.9), (58.97, 58.84), (57.9, 57.8), 55.3, (43.34, 43.23), (43.20, 43.12), (24.62, 24.57), (20.45, 20.40), (20.22, 20.17), (11.69, 11.63); ^{31}P NMR (202 MHz, CD_2Cl_2) δ (152.6, 152.6).

*[5′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-O-(2-methoxyethyl)-N_6-benzoyladenosin-3′-O-yl]-2-cyanoethyl-N,N-diisopropylphosphoramidite (**6**).* A solution of 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-N_6-benzoyladenosine (2.50 kg, 3.42 mol) in DMF (5 L) and acetonitrile (1 L) was prepared in a 22-L round-bottom Buchi flask. The flask was place on a Buchi rota-vapor, and its contents were concentrated in vacuo at 20−35 °C (vapor temperature) while dry acetonitrile (17 L) was simultaneously added at a sufficient rate to maintain a constant flask volume. (This vacuum distillation was continued until the water content of the final solution was <0.04% by Karl Fischer analysis.)

A second 22-L Buchi flask was charged with identical amounts of starting material and solvents as those above and the resulting solution subjected to the same vacuum distillation conditions. The resulting two portions of dried starting materials (5.00 kg, 6.83 mol total) were held under nitrogen for further processing.

To a 30-gal glass-lined reactor at ambient temperature was charged acetonitrile (10 L), NMI·Tf (1.59 kg, 6.84 mol), and 2-cyanoethyl tetraisopropylphosphorodiamidite (2.57 kg, 8.53 mol). The two dry DMF/acetonitrile solutions of 5′-*O*-(4,4′-dimethoxytriphenylmethyl)-2′-*O*-(2-methoxyethyl)-N^6-benzoyladenosine from above were combined and added over 30−60 min to the 30-gal tank. The tank temperature was maintained between 20 and 25 °C during this addition. The reaction mixture was stirred at 20−25 °C for 19 h until the starting material was <0.1% by HPLC. The ending mixture was transferred to a 50-gal glass-lined reactor, diluted with MTBE (75 L), washed sequentially with water (25 L), 1:1 DMF/H_2O (2 × 50 L), water (50 L), and brine (50 L). The resulting MTBE solution was dried with Na_2SO_4 (10 kg) and filtered into a clean 30-gal glass-lined reactor. This filtrate solution was concentrated to 20 L by vacuum distillation while maintaining the tank temperature below 25 °C. MTBE (50 L) was added to this product solution which was again concentrated to a volume of 20 L. This MTBE dilution and distillation sequence was repeated two more times, with the final concentration stopping at 40 L total volume.

The MTBE solution containing the crude, concentrated product was added over 30−60 min to stirring hexane (95 L) in a second 50-gal glass-lined reactor. The resulting precipitate was stirred at ambient temperature for 1 h and then filtered. The solids were washed with hexanes (3 × 13 L) and pulled dry. The filter cake was removed to a tared tray and dried under vacuum at 35 °C to constant weight. A total of 7.31 kg of white solids was recovered (95.0%) with a HPLC purity of 98.9% as a 1:1 mixture of a pair of diastereomers at the phosphorus center: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.95 (br, s, 1), 8.75 (s, 1 × 0.5), 8.72 (s, 1 × 0.5), 8.27 (s, 1 × 0.5), 8.23 (s, 1 × 0.5), 8.02−8.01 (m, 2), 7.62−7.59 (m, 1), 7.55−7.51 (m, 2), 7.45−7.40 (m, 2), 7.35−7.20 (m, 7), 6.82−6.75 (m, 4), 6.18 (d, 1 × 0.5, J = 5.5), 6.18 (d, 1 × 0.5, J = 5.5), 4.95 (dd, 1 × 0.5, J = 5.5, 5.0), 4.91 (dd, 1 × 0.5, J = 5.5, 5.0), 4.66 (ddd, 1 × 0.5, J = 10.5, 5.0, 5.0), 4.61 (ddd, 1 × 0.5, J = 11.0, 4.1, 4.1), 4.45−4.42 (m, 1 × 0.5), 4.38−4.35 (m, 1 × 0.5), 4.00−3.46 (m, 15), 3.35−3.32 (m, 1), 3.24 (s, 3), 2.67−2.63 (m, 1), 2.40−2.37 (m, 1), 1.24−1.16 (m, 9), 1.08 (d, 3, J = 7.0); ^{13}C NMR (125 MHz, $CDCl_3$) δ 164.5, 158.6, 152.7, 151.8, 149.5, (144.53, 144.46), (142.3, 142.2), 135.7, 135.6, 133.7, 132.7, 130.2, 130.1, 128.9, 128.3, 128.2, 127.8, 126.9, (117.8, 117.4), 113.1, (87.19, 87.09), (86.64, 86.54), (81.17, 80.69), (72.1, 72.0), (71.5, 71.4), (70.9, 70.8), (70.4, 70.1), (63.1, 62.7), (59.0, 58.9), (58.0, 57.9), 55.2, (43.4, 43.3), (43.2, 43.1), (24.62, 24.59), (20.43, 20.38), (20.17, 20.11); ^{31}P NMR (202 MHz, CD_2Cl_2) δ (152.9, 152.6).

Preparation of *N*-Methylimidazole Triflate Salt. To a 30-gal glass-lined reactor was charged 5.32 kg of 1-methylimidazole and 36 L of methylene chloride. The solution was stirred at room temperature for 30 min before the addition of 9.16 kg of triflic acid; 36 L of MTBE was then added for crystallization. The resulting mixture was stirred at room temperature for 30 min and filtered. The solids were rinsed with 18 L of MTBE, and dried to provide 13.7 kg (96.6%) of the desired salt.

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