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An Improved Process for the Manufacture of 5′-*O*-(4,4′-Dimethoxytrityl)-N^2-isobutyryl-2′-*O*-(2-methoxyethyl)guanosine

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Keywords: MOE G PNS, oligonucleotide precursor, DAPR alkylation, guanosine protection, design of experiments (DOE)

Abstract

A revised, optimized process for the manufacture of 5′-*O*-(4,4′-dimethoxytrityl)-N^2-isobutyryl-2′-*O*-(2-methoxyethyl)guanosine (MOE G PNS) that controls critical impurities to less than 0.2% was developed. The 2′-*O*-alkylation of 2,6-diaminopurine riboside (DAPR) with 1-bromo-2-methoxyethane (MOE-Br) in DMSO was examined using a

Design of Experiments (DOE) approach, which lead to the selection of LiOH as the optimal base choice. Equivalents of base and MOE-Br were optimized to control levels of the critical 3′-*O*-(2-methoxyethyl) impurity and residual DAPR. DMSO was removed with a solid-phase extraction using SP-207 resin, affording the desired 2′-*O*-(2-methoxyethyl)-2,6-diaminopurine riboside (MOE DAPR) in an aqueous solution in 53% yield. The methylbromide impurity commonly found in bulk sourced MOE-Br was controlled using a co-distillation with MEK, thereby controlling the resulting critical 2′-*O*-methyl nucleoside impurity. The aqueous solution was telescoped into the enzymatic conversion of MOE-DAPR to 2′-*O*-(2-methoxyethyl)guanosine (MOE G) using adenosine deaminase. MOE G purity is enhanced at this step due to enzymatic selectivity and crystallization from the reaction mixture, which together significantly reduce levels of the 3′ MOE G isomer. Installation of the isobutyryl and 4,4-dimethoxytrityl protecting groups proceeded similarly to published literature methods, but the work-up and isolation conditions were optimized. A series of extractions after isobutyrlation was used to control critical and non-critical impurities, and the final product was crystallized to upgrade purity. The improved process

was carried out at production-scale to afford 59 kg of MOE G PNS with no critical impurity over 0.2% in an overall yield of 26%.

ABBREVIATIONS

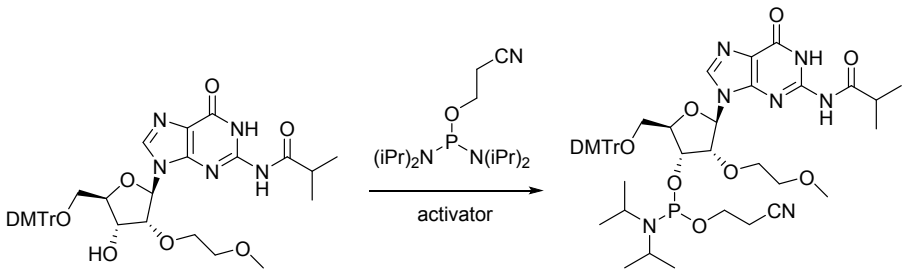
MOE, 2-methoxyethyl; DAPR, 2,6-diaminopurine riboside; PNS, protected nucleoside; DCI, 4,5-dicyanoimidazole.

Introduction

Oligonucleotides are a distinct and growing therapeutic platform with the ability to target genes linked to diseases that are considered undruggable by classic small molecule approaches.[1,2,3] Several nucleic-acid based drugs have been approved, and many more are in the late stages of clinical development. Nucleic-acid therapeutics are manufactured by solid-phase synthesis, where phosphoramidite monomers are coupled sequentially on a solid support, which is typically functionalized with a universal linker such as UnyLinker™ to allow easy removal of the desired sequence from the solid support.[4] Synthesis is typically carried out using a four step cycle comprising acid treatment to

remove a 5′-*O*-4,4′-dimethoxytrityl protecting group, attachment of a phosphoramidite to the support-bound oligonucleotide, conversion of the newly formed phosphite triester to either its phosphorothioate triester with a sulfurizing reagent or a phosphate triester with an oxidizing reagent, and finally acetylation of unreacted 5′-hydroxyls.[5] In this manner the desired sequence is constructed from the 3′ to 5′ terminus. [6,7] Several commercially approved drugs and many late-stage clinical candidates contain 2′-*O*-(2-methoxyethyl) modified (MOE) nucleosides. Historically, the most expensive and challenging MOE phosphoramidite to manufacture has been 2′-*O*-(2-methoxyethyl) guanosine phosphoramidite (MOE G phosphoramidite), **2**, which is produced by phosphitylating the corresponding 2′-*O*-(2-methoxyethyl) guanosine protected nucleoside (MOE G PNS) **1** using 2-cyanoethyl-*N*,*N*,*N*′,*N*′-tetraisopropylphosphorodiamidite (Phos reagent[8]) in the presence of weakly acidic activators such as tetrazole and DCI (**Scheme 1**). The synthesis of high purity phosphoramidites relies on the production of high purity protected nucleosides.[9]

Scheme 1. Phosphitylation of 2′-*O*-(2-Methoxyethyl) Guanosine Protected Nucleoside



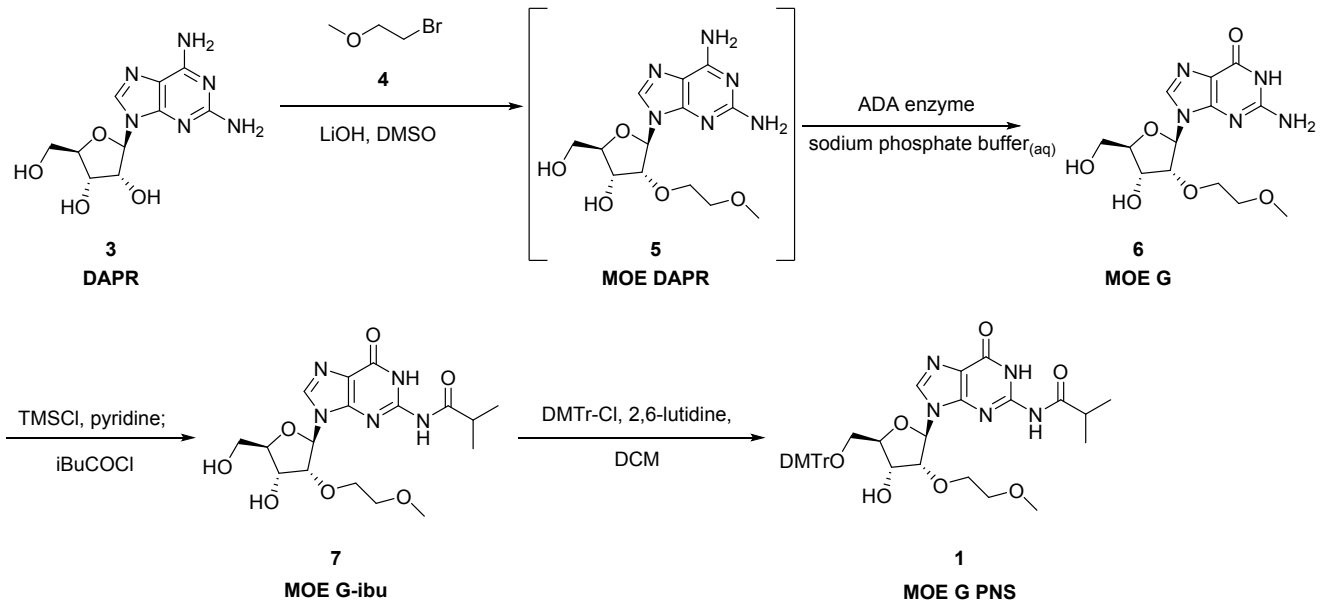
1 **2**

MOE G PNS **MOE G phosphoramidite**

Previously, we reported a process for the manufacture of MOE G PNS, **1**, where alkylation of the 2′ oxygen of 2,6-diaminopurine ribose was carried out using 1-bromo-2-methoxyethane and KOH in DMSO.[10] While the process was successfully used in a pilot plant setting, further scale-up resulted in uncontrolled and variable levels (0.11% - 1.22%) of an impurity containing a 2′-*O*-methyl in place of the desired 2′-*O*-(2-methoxyethyl), which we thought formed by reaction of methyl bromide originating as both a trace impurity in 1-bromo-2-methoxyethane (MOE-Br), and from degradation of MOE-Br during the alkylation reaction. After failed attempts to bring the 2′-*O*-methyl impurity under control at production scale, the need for material to support clinical studies drove us to set aside the MOE-Br/DMSO conditions in favor of a lower yielding aqueous alkylation process,

which used methoxyethyl mesylate and did not result in detectable methylation. Over the past several years we have carried out process development on the MOE-Br/DMSO process to enable control of the 2′-*O*-methyl impurity without chromatography. Herein we report an improved process for MOE G PNS using MOE-Br in DMSO, which has been successfully demonstrated at ca 60 kg batch scale. The overall route is the same as previously published (**Scheme 2**), however many changes have been made to the reaction conditions and workups.

Scheme 2. Preparation of 2′-*O*-(2-Methoxyethyl) Guanosine Protected Nucleoside (MOE G PNS)



Results and Discussion

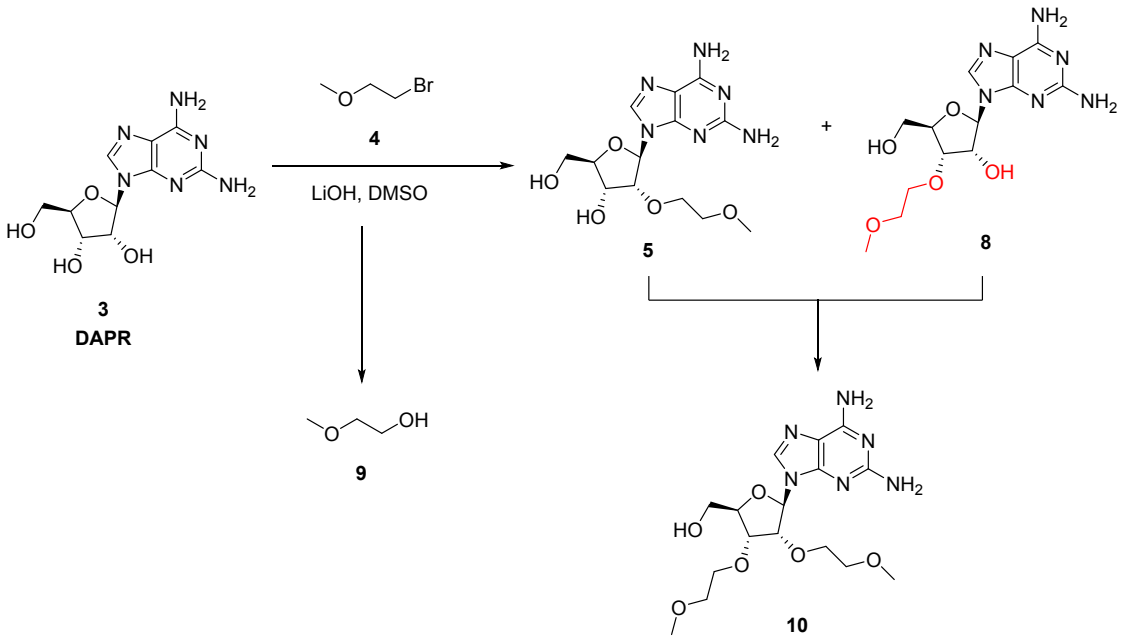
Attempts have been made to find a cost-effective route to MOE G PNS starting from guanosine; however, direct alkylation of guanosine leads to preferential O^6-alkylation.[11,12] Conversion of guanosine to 2,6-diaminopurine riboside (DAPR) prior to alkylation protects the nucleobase against alkylation. Once converted to DAPR, another difficulty is encountered while attempting to alkylate the 2′-hydroxyl selectively. That is, while the 2′-OH vs. 3′-OH selectivity favors the desired product, a significant amount of the 3′-MOE regioisomer impurity **8** is also formed. Note that the 3′-MOE regioisomer is a critical impurity because, if carried through to phosphoramidite, the 3′-MOE phosphoramidite impurity will compete with the parent phosphoramidite and result in a 3′-MOE oligonucleotide impurity that cannot be purified away from the desired parent oligonucleotide. Additionally, the reaction is complicated by over alkylation products. The 2′-hydroxyl proton of DAPR is more acidic than the 3′-hydroxyl (predicted pKa value for 2′-OH is 12.9 and 3′-OH is 14.2), but once the 2′-hydroxyl is alkylated, the pKa of the 3′-hydroxyl becomes closer to that of the 2′-hydroxyl of DAPR (predicted to be 13.4).

Therefore, the 3′-hydroxyl of the desired 2′-MOE DAPR **5** competes in the alkylation reaction, resulting in the bisalkylated impurity **10** (Scheme 3). Note that impurity **10** is non-critical because if carried through to phosphoramidite, it would not have a free alcohol to be phosphitylated and therefore would not incorporate into the oligonucleotide. To complicate matters further, the base needed to carry out the reaction slowly hydrolyzes MOE-Br (**4**) to 2-methoxyethanol (**9**). Thus, insufficient MOE-Br leaves residual DAPR starting material **3**, which, if carried through to phosphoramidite results in an unreactive, non-critical, cyclic phosphite triester impurity due to the cis-diol functionality reacting twice with the Phos reagent during phosphitylation.

While the alkylation reaction is complex, preparing MOE G PNS (**1**) in this manner is the most efficient and cost-effective route we have found. Attempts at avoiding the selectivity complications using 3′,5′ protection strategies such as TIPDS or di-*t*-butylsilyl led to increased cost due to the two additional steps required and the cost of the silyl reagents with no discernable benefit in throughput or critical impurity control.[12–14]

We developed the MOE-Br/DMSO process and the control strategy with the goal of producing MOE G PNS with tightly controlled levels of critical impurities. Rather than hold each reaction step to a criterion of high purity, we developed the process to reduce or purge critical impurities throughout to ensure material of high quality at the MOE G PNS stage.

Scheme 3. Alkylation Products with Critical Modifications Relative to Parent in Red



To determine which reaction parameters affected the yield and levels of critical impurity **8**, the alkylation reaction conditions to produce intermediate **5** were reexamined using a

two level, five-factor, 16 run 2^{5-1} screening DOE. Equivalents of base and alkylbromide **4** were assumed to be important and were fixed in the DOE, while the volume of DMSO, temperature, base type, base addition rate, and alkylbromide addition rate were varied. (**Table 1**) Reaction progress was followed over time and the reaction profile assessed for maximum product formation, time to maximum product, time product remained within 2.5% of the maximum value (reaction plateau), and impurity levels at the time of maximum product formation (**Figure 1**). Analysis of the results indicated that the maximum product was formed when the reaction was carried out under more dilute conditions (15 vol DMSO) using LiOH, with reaction volume being slightly more impactful than the type of base. No other main effects or two factor interactions were observed. The time the reaction took to reach maximum product was governed by a complicated set of factors comprising type of base, temperature, base addition rate, and an aliased two-factor interaction, which is either reaction volume × type of base or temperature × bromide addition rate. Of these, base type was the predominant factor, with LiOH being favored. Analysis of the length of the reaction plateau showed that LiOH produced a longer, flatter reaction maximum, with a lower reaction temperature of 15 °C also contributing to a small

extent. The amount of residual DAPR starting material **3** at the point when the reaction reached its maximum level of product was unaffected by any of the parameters varied in the DOE. However, the amount of non-critical bis-alkylated impurity **10** was inversely affected by the reaction volume, and the critical 3′-MOE impurity **8** was affected by both the reaction volume and the type of base. When the reaction was concentrated, the level of 3′-MOE impurity **8** was lower, while the bis-alkylated impurity **10** was higher. Presumably, the more concentrated reaction conditions favor the continued conversion of 3′-MOE into the bis-alkylated species. The use of LiOH also favored lower levels of 3′-MOE impurity **8** over those formed with KOH. While higher volumes of solvent made a slight positive impact on maximum yield, the lower volume of solvent was ultimately selected to improve control of critical 3′-MOE impurity **8** and throughput. An optimization model built upon the DOE data for 5 volumes of DMSO suggested that the optimal balance of maximum yield, lowest 3′-MOE impurity **8**, and long plateau would be obtained using LiOH at 24 °C with the base and alkylbromide charged as single additions.

Table 1. DOE Variables

Variable	Type	Low	High
Vol DMSO	Numeric	5	15
Temperature (°C)	Numeric	15	25
base type	Categoric	LiOH	KOH
base addition rate	Categoric	All at once	Portions
alkylbromide addition rate	Categoric	All at once	Portions
Equiv. base	Constant	2.6	
Equiv. **4**	Constant	2.7	

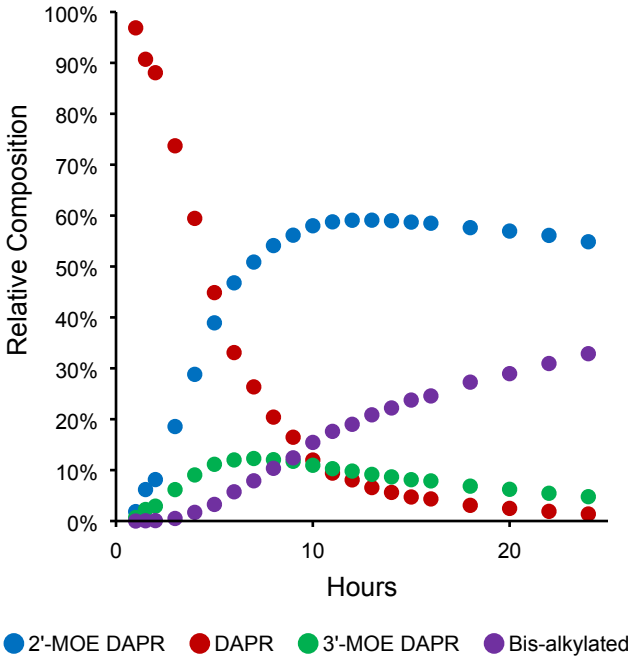


Figure 1. Example reaction profile from the DoE using 5 vol DMSO, LiOH, portion-wise addition of base, portion-wise addition of alkylbromide, 25 °C

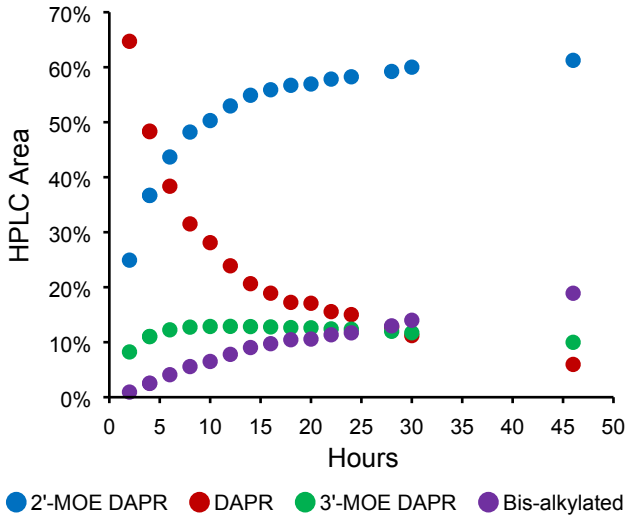


Figure 2. Reaction profile using 5 vol DMSO, 1.3 equiv. LiOH added all at once, 1.4 equiv. alkylbromide **4** added all at once, 25 °C

Armed with the information from the DOE, we turned our attention to the base and alkylbromide equivalents. Reducing the equivalents of LiOH to 1.3 and alkylbromide **4** to 1.4 did not change the reaction profile (10 hour time point **Figure 1** vs. 30 hour time point **Figure 2**); however, the reaction progression slowed considerably. Under these conditions the reaction has reached just above 60% product formation (not yet the maximum) after 48 hours with approximately 6% residual DAPR starting material **3**, 10% 3′-MOE impurity

8, and 19% bis-alkylated 10. While reducing the equivalents of reactants is generally desirable, these conditions are not ideal for several reasons. To ensure rugged control in the downstream steps, we wanted critical 3′-MOE impurity 8 levels to be as low as possible at the crude stage of the alkylation. Further, non-critical DAPR starting material 3 continues to react throughout the process and the resulting species are not easily removed; therefore, it would be beneficial to control 3 to low levels in the alkylation crude. Both impurities can be reduced by running the reaction past the product maximum and trading some yield for impurity control. Running the reaction to the point at which not more than 2.0% 3 remains typically produces levels of 3′-MOE impurity 8 and bis-alkylated impurity 10 around 5% and 33% respectively. To reach this point using reduced equivalents of base and alkylbromide would take more than 48 hours, which was deemed unacceptable in terms of throughput. After screening several pairings of base and alkylbromide equivalents, 2.15 equivalents of both LiOH and alkylbromide 4 were chosen as a reasonable balance between reagent usage and throughput, with the reaction reaching 2.0% 3 in approximately 18 hours while maintaining a long product plateau. Despite the yield loss (ca. 5%) associated with running the reaction past the point of

maximum product, the new conditions provide a doubling of the yield over the former aqueous alkylation process (~54% vs. 28%).

As mentioned previously, the quality of 1-bromo-2-methoxyethane (**4**) is critical. Bulk supplies of **4** can contain small amounts of alkylbromide impurities that can compete during the alkylation reaction (**Figure 3**). Most of these impurities can be controlled by an appropriate specification on 1-bromo-2-methoxyethane; however, bromomethane is a decomposition by-product of the reagent, which complicates control of the resulting 2′-*O*-Me nucleoside impurity. A simple co-distillation of alkylbromide **4** and MTBE in DMSO was sufficient to reduce 2′-*O*-Me impurity formation to very low levels (<0.05%). The co-distillation procedure was implemented at production scale successfully. However, when the same process was used in a subsequent campaign with a different nucleoside (adenosine), unexpectedly high levels of the corresponding 2′-*O*-Me impurity were generated. Because the co-distillation was expected to behave similarly regardless of nucleoside, the result brought into question control of the 2′-*O*-Me impurity by co-distillation. We therefore reexamined the co-distillation conditions and determined that

carrying out the alkylbromide/MTBE distillation in the absence of DMSO resulted in reproducible control of the 2′-*O*-Me impurity. The new conditions were demonstrated during a subsequent campaign to alkylate adenosine (data to be disclosed in a future publication). While the new co-distillation conditions have not formally been demonstrated for MOE G PNS, control of the 2′-*O*-Me impurity in the adenosine series indicates the procedure will be similarly successful in the synthesis of **1**.

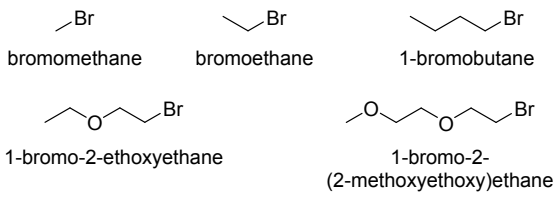


Figure 3. Critical impurities that may be present in bulk 1-bromo-2-methoxyethane

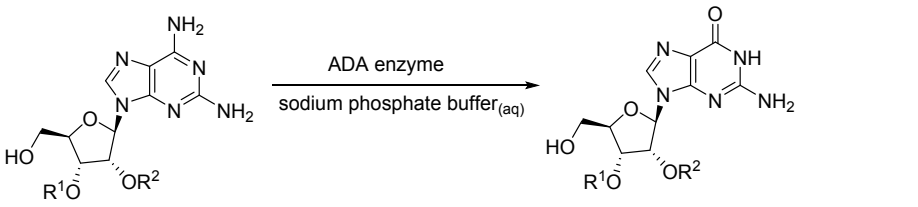
The next step in the process is enzymatic deamination of MOE DAPR **5** to MOE G **6** using adenosine deaminase. The enzyme can tolerate up to ca. 20% DMSO; however, even a small amount of DMSO impairs crystallization of **6** from the reaction mixture, which is a key component of the impurity control strategy. Therefore, a method to remove the DMSO prior to the enzymatic reaction was required. Attempts at biphasic aqueous

extractive removal of DMSO using brine and THF or n-butanol showed an unfavorable partitioning of MOE DAPR **5** into the aqueous phase. Organic solvent nanofiltration and counter current extractions both showed promise, but required substantial capital investment in specialized equipment, and so an alternative solid-phase extraction was developed. The solid phase chosen was brominated polystyrene matrix SP-207, which was placed in a column with a height to diameter ratio of 2:1. The 2:1 ratio provides enough depth of matrix to hold the product and remove DMSO, even without uniform loading. After capturing the crude mixture on the solid-phase, the resin is washed with 10% $NaOAc_{(aq)}$, which is critical in disrupting the DMSO binding. Once the DMSO is removed, the resin is rinsed with water to remove sodium acetate (a necessity for the following enzymatic reaction), and the product released from the resin using 80%-100% $methanol_{(aq)}$ with a recovery of about 94%. Methanol is removed under reduced pressure to afford the crude alkylation mixture in water, which is used directly in the next step.

The enzymatic deamination of DAPR intermediate is carried out using adenosine deaminase (ADA) in the presence of aqueous phosphate buffer. Reaction conditions

were optimized to balance conversion speed with amount of enzyme. We settled on an enzyme charge of 176 units of ADA per gram 2′-MOE DAPR, which afforded MOE G **6** in 9 hours in 84-90% yield. During the reaction, not only is 2′-MOE DAPR **5** deaminated to form the desired 2′-MOE G **6**, but other components in the crude alkylation mixture are also deaminated (**Scheme 4**). Of these, only 3′-MOE G **12** is critical. The other critical impurity observed in the crude reaction mixture is residual 2′-MOE DAPR **5**. Interestingly there are two characteristics of the reaction that we took advantage of in the control strategy of critical impurities **5** and **12**. First, the ADA enzyme selectively deaminates 2′-MOE DAPR **5** over 3′-MOE DAPR **8**. This is evinced by the enrichment of the 2′-MOE G isomer over the course of the reaction (ratio of 2′ to 3′ MOE DAPR at ~7:1 in the alkylation reaction mixture vs. the ratio of 2′ to 3′ MOE G at ~18:1 in the deamination crude). Secondly, 2′-MOE G **6** crystallizes selectively from the sodium phosphate buffer. Provided the reaction is run until not more than 1.0% of **5** remains, the crystallization allows for a simple workup by filtration, as the product precipitates from the reaction mixture to afford material with a ~180:1 ratio of **6** to **12** and residual **5** reduced to ~0.15% in ~81% (purity corrected) yield.

Scheme 4. Mixture of Compounds in the Deamination Reaction with Critical Impurities Demarcated in Red



5 R^1 = H, R^2 = $CH_2CH_2OCH_3$
3 R^1 = H, R^2 = H
8 R^1 = $CH_2CH_2OCH_3$, R^2 = H
10 R^1 = $CH_2CH_2OCH_3$, R^2 = $CH_2CH_2OCH_3$

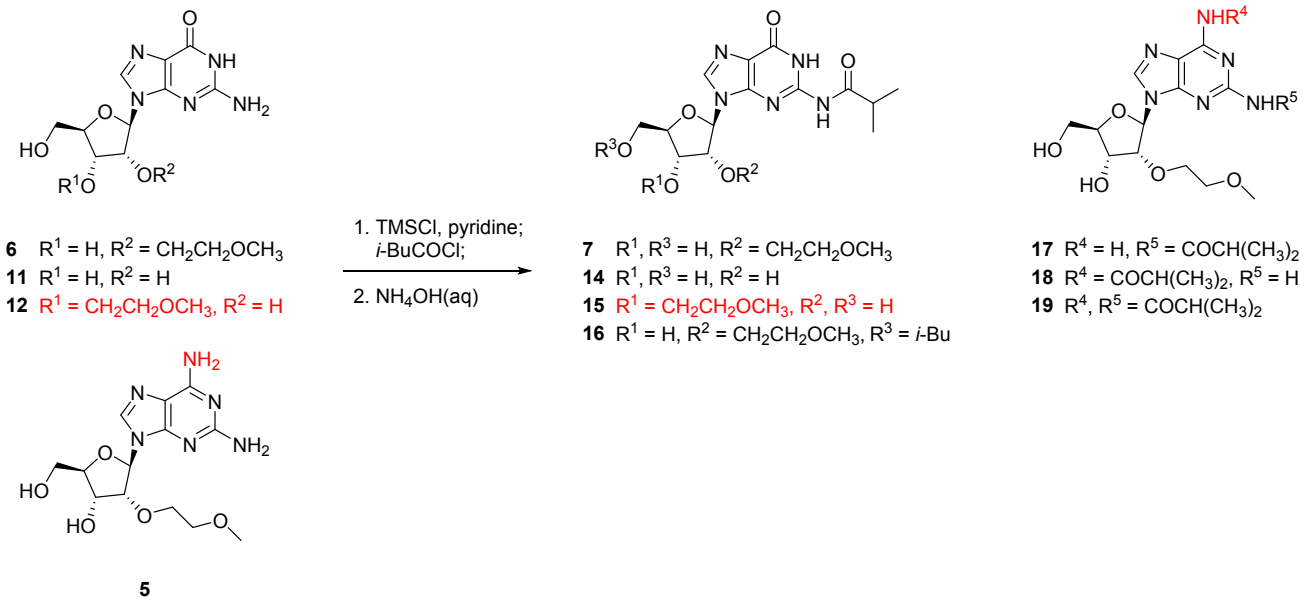
6 R^1 = H, R^2 = $CH_2CH_2OCH_3$
11 R^1 = H, R^2 = H
12 R^1 = $CH_2CH_2OCH_3$, R^2 = H
13 R^1 = $CH_2CH_2OCH_3$, R^2 = $CH_2CH_2OCH_3$

The next step in the process is to install the isobutyryl protecting group. The reaction is carried out with transient protection of the 3′ hydroxyl as the trimethylsilyl ether, followed by *N*-acylation using isobutyryl chloride.[15] The reaction is quenched with methanol and adjusted to neutral pH with ammonium hydroxide.

Under the previous aqueous alkylation conditions, the alkylation reaction was stopped near its maximum product formation to maximize throughput in a poor yielding process. To obtain the desired product 2′-MOE G^{ibu} **7** in an organic phase while keeping more polar impurities such as triol **14** in the aqueous phase, an extensive continuous extraction followed by a complicated series of back extractions were required. However,

because the alkylation in DMSO can be pushed further along the reaction pathway with minor yield loss (*vide supra*), there is much less impurity **14** (carried forward from unreacted **1**). This allows for the continuous extraction to be replaced with a simpler batch extraction workup. The acylation reaction is quenched with methanol and precipitated pyridine hydrochloride removed via filtration. The filtrate is solvent swapped to water, and the aqueous solution extracted with 3% methanol in DCM to extract the product and leave most of impurity **14** in the aqueous layer. Attempts to replace DCM with other solvents failed to identify a suitable alternative. The combined organic extract is washed with brine to reduce impurity **14** to undetectable levels. The organic layer is azeotropically dried, concentrated to 4 vol, and swapped to acetone to crystallize the desired product **7** in ~82% yield, further reducing the level of critical impurity **15** and removing trace amounts of the critical isobutyrylated products of 2′-MOE DAPR **5** carried through from the previous step (**17**, **18**, **19**).

Scheme 5. Mixture of Compounds in the Isobutyryl Protection Reaction



6 R^1 = H, R^2 = $CH_2CH_2OCH_3$
11 R^1 = H, R^2 = H
12 R^1 = $CH_2CH_2OCH_3$, R^2 = H

5

7 R^1, R^3 = H, R^2 = $CH_2CH_2OCH_3$
14 R^1, R^3 = H, R^2 = H
15 R^1 = $CH_2CH_2OCH_3$, R^2, R^3 = H
16 R^1 = H, R^2 = $CH_2CH_2OCH_3$, R^3 = *i*-Bu

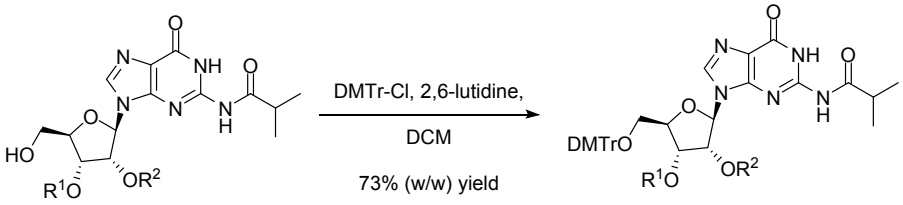
17 R^4 = H, R^5 = $COCH(CH_3)_2$
18 R^4 = $COCH(CH_3)_2$, R^5 = H
19 R^4, R^5 = $COCH(CH_3)_2$

The final step in the process is to install the dimethoxytrityl protecting group. The reaction and workup were modified slightly from the previously mentioned published procedure. At this point in the synthesis, it becomes difficult to upgrade purity, so emphasis is placed on clean conversion to **1**. Based on previous work, 2,6-lutidine was preferred over pyridine, since it increased the regioselectivity of the tritylation (5′ over 3′ hydroxyl). Running the trityl protection with 2,6-lutidine proceeded smoothly over 30 minutes to give **1** in excellent yield and purity, and so no further optimization of the reaction conditions was performed. However, the workup was extensively modified. It

was previously reported that the reaction was quenched with methanol, vacuum distilled to a residue, dissolved and treated with a series of extractions, purified by silica gel chromatography followed by another series of extractions to remove residual lutidine, before again evaporating to a residue, and triturating with hexane to induce formation of an amorphous solid, which could be isolated by filtration. While this process was viable at low kg scales, we wanted a rugged process capable of being more easily scaled.

The methanol quench was maintained, but no concentration to a residue was employed. Rather, sodium bicarbonate washes were carried out on the DCM/MeOH solution directly (reducing residual levels of **7** to less than 0.1%). The column chromatography step was removed and replaced by precipitation and recrystallization from DCM/cyclohexane to afford crystalline MOE G PNS **1** in ~73% (w/w) yield. To our knowledge, this is the first report of crystalline MOE G PNS. Attempts to replace DCM in the crystallization with other solvents failed to provide a suitable alternative, so to simplify the process, DCM was chosen as the reaction solvent.

Scheme 6. Mixture of Compounds in the DMTr Protection Reaction



7 R^1 = H, R^2 = $CH_2CH_2OCH_3$
15 R^1 = $CH_2CH_2OCH_3$, R^2 = H

1 R^1 = H, R^2 = $CH_2CH_2OCH_3$
17 R^1 = $CH_2CH_2OCH_3$, R^2 = H

The improved process was demonstrated at production scale starting with 91.2 kg 2,6-DAPR to afford 58.8 kg MOE G PNS (assay corrected) with all critical impurities below 0.15%. This represents an overall yield of ~26%, which is a 153% increase over material produced by the former unpublished aqueous alkylation process.

Experimental Section

Materials and Instrumentation. Except for 1-bromo-2-methoxyethane, commercially available starting materials were used as received without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 300 MHz spectrometer at ambient temperature (298 K) using tetramethylsilane as an internal standard. The multiplicities of ^{1}H NMR signals are indicated as follows: s = singlet, d = doublet, t = triplet, m =

multiplet, br = broad, or combinations of thereof. Coupling constants (J) are reported to the nearest 0.1 Hz.

*5′-O-(4,4′-dimethoxytrityl)-N²-isobutyryl-2′-O-(2-methoxyethyl)guanosine (MOE G PNS) (**1**).* Into a 2000 L reactor at 20-35 °C under nitrogen was charged DCM (714 L), 47.6 kg **7** (a/a% purity corrected: 47.1 kg, 115 mol), 2,6-lutidine (49.1 kg, 458 mol), and DMTr-Cl (42.8 kg, 126 mol). The reaction mixture was stirred at 20-35 °C for 30 minutes at which point unreacted **7** was undetectable by HPLC. The reaction was cooled to 10-15 °C, quenched with methanol (3.1 L, 97 mol) at 10-15 °C, and the organic layer washed three times with 10% sodium bicarbonate(aq) ($NaHCO_3$: 9.52 kg, water: 95 L) at 20-35 °C and once with sat. NaCl(aq) (NaCl: 28.6 kg, water: 95 L). The organic layer was dried with sodium sulfate (47.6 kg, 335 mol), filtered, and the filtered sodium sulphate rinsed with DCM (95L). To the combined filtrate and rinse was charged cyclohexane (1428 L) and the material was precipitated by removing the DCM by vacuum distillation (<45 °C). The mixture was cooled to 20-35 °C and filtered. The resulting crude solid was charged into a reactor containing DCM (476 L) and stirred for

15 minutes to dissolve. To the solution was added cyclohexane (762 L) and the mixture was stirred at 20-35 °C until solid formation was observed (ca. 75 minutes). Upon formation of solid, the slurry was maintained at 20-35 °C for 6 hr, then filtered, and the filtered solid rinsed with 38% DCM in cyclohexane (DCM: 90 L, cyclohexane: 143 L). The solid was dried under vacuum at 45-50 °C until the water content was less than 1.00% (ca. 16 hr.) to afford MOE G PNS, **1** (62.2 kg, 87 mol), with an assay of 95.5% and a purity of 98.96% a/a% in a 72.7% yield (assay corrected). Impurity profile (a/a%): 2′-*O*-methyl G PNS (0.06%), 3′-MOE G PNS **17** (0.05%) ^{1}H NMR (300 MHz, $CDCl_3$) δ 11.99 (s, 1H), 7.90 (s, 1H), 7.84 (s, 1H), 7.58 – 7.48 (m, 2H), 7.45 – 7.34 (m, 4H), 7.33 – 7.16 (m, 4H), 6.87 – 6.73 (m, 4H), 5.88 (d, *J* = 7.0 Hz, 1H), 4.91 (dd, *J* = 7.1, 4.9 Hz, 1H), 4.49 (dt, *J* = 4.8, 2.4 Hz, 1H), 4.26 – 4.19 (m, 1H), 3.88 (ddd, *J* = 11.3, 4.2, 2.4 Hz, 1H), 3.83 (d, *J* = 2.6 Hz, 1H), 3.78 (s, 3H), 3.76 (s, 3H), 3.69 (ddd, *J* = 11.2, 8.0, 2.5 Hz, 1H), 3.63 – 3.49 (m, 2H), 3.46 (ddd, *J* = 10.6, 4.2, 2.5 Hz, 1H), 3.35 (s, 3H), 3.13 (dd, *J* = 10.7, 3.3 Hz, 1H), 1.67 (hept, *J* = 6.8 Hz, 1H), 0.92 (d, *J* = 6.9 Hz, 3H), 0.73 (d, *J* = 6.9 Hz, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 178.38, 158.76, 155.51, 148.26, 147.17, 145.03,

139.02, 136.10, 135.71, 130.02, 128.06, 127.15, 122.43, 113.28, 86.43, 86.27, 84.51, 81.47, 77.24, 71.78, 70.03, 69.44, 63.78, 59.03, 55.28, 36.07, 18.57, 18.49.

*Co-distillation of 1-bromo-2-methoxyethane (**4**) to remove MeBr*

Methyl tert-butyl ether (32.4 Kg) and 2-methoxyethyl bromide (24 Kg) were charged to a reactor at 20-30 °C. The mixture was stirred for 15 minutes and then MTBE and any MeBr impurity present was distilled under vacuum (300-350 mm Hg) at 20-30 °C to 0.9-1.2 vol remaining in reactor. Note that temperatures above 30 °C at 500 mm Hg will evaporate 2-methoxyethyl bromide and should be avoided.

*2′-O-(2-methoxyethyl)-2,6-diaminopurine riboside (**5**)*. DMSO (295 L), 2,6-diaminopurine riboside **3** (91.2 kg, 323 mol), and lithium hydroxide monohydrate (29.1 kg, 694 mol) were charged to a 2000 L reactor at 20-35 °C under nitrogen and stirred for 30 min. The mixture was cooled to 15-20 °C and maintained at that temperature while 330 kg of a solution of 2-methoxyethyl bromide **4** (29.47 w/w%) in DMSO was charged over 75 minutes. The temperature was raised to 20-35 °C and maintained at that temperature until residual **3** < 2.0% a/a% by HPLC (1.58% in approximately 19 hr). The reaction was

quenched and diluted with chilled water (1368 L, 15-20 °C) over 190 min. The pH of the mixture was adjusted to 7.1 with 5N HCl(aq) (30 L). Sodium acetate (20.5 kg) was added to the crude mixture and the pH was again adjusted to 7.1 with 5N HCl(aq) (0.912 L). Crude reaction profile (a/a%): alkylated product **5** (56.6%), residual **3** (1.6%), 3′-MOE impurity **8** (5.3%), and bis-alkylated impurity **10** (33.2%).

*Solid-phase extraction of DMSO from crude 2′-O-(2-methoxyethyl)-2,6-diaminopurine riboside (**5**)*. SP-207 resin from Mitsubishi Chemical Corporation manufactured by Resindion Srl (14 vol relative to **3**, 1272 L) was charged to a 1500 L column (2:1 m depth:diameter). At this scale, the flow rate of all equilibration and sodium acetate containing solutions through the resin should be not more than 20 L/min (linear velocity ~25 m/min). The resin was conditioned with 5% methanol(aq) (70 L methanol, 1330 L water, 1.1 CV), water (1780 L, 1.4 CV), and 10% sodium acetate(aq) (254.4 kg NaOAc, 2544 L water, 2 CV). Half of the crude solution of **5** (929 kg) was charged to the column. The loaded column was washed with 10% sodium acetate(aq) (254.4 kg NaOAc, 2544 L water, 2 CV). Note that most of the DMSO was contained in the first CV (~16% assay)

with the remainder in the second CV (~2.5% assay). The residual sodium acetate was washed from the resin with water (2544 L, 2 CV). Once the sodium acetate is removed, the flow rate of the following washes can be increased to a linear velocity of ~50 m/min or 40 L/min at this scale. The alkylated product **5** was liberated from the resin with 80% MeOH(aq) (2036 L MeOH, 508 L water, 2 CV). The resin is rinsed with MeOH (2544 L, 2 CV) to remove any residual material. The equilibration, capture, and release process was repeated for the second half of the crude reaction mixture. Note that after the final MeOH wash, the resin can be stored in MeOH and reused (demonstrated up to 25 times in the lab with a similar resin). The washes containing 80% MeOH and **5** from the two capture release operations were combined and the volume was reduced to ~20% of the original volume via vacuum distillation keeping the solution below 50 °C. The resulting aqueous solution of 2′-MOE-DAPR **5** (438.3 kg, 13.4% w/w, 58.8 kg **5**, 173 mol, 53.4% assay yield) was used directly in the next step.

*2′-O-(2-methoxyethyl)guanosine (**6**)*. The *ca* 13%w/w aqueous solution of 2′-MOE-DAPR **5** (58.8 kg assayed, 173 mol) was charged to a 1000 L reaction vessel and

stirred at 25-30 °C. Sodium phosphate (9.47 kg, 58 mol) was charged into the solution and the pH was adjusted to 7.3 using a solution of sodium dihydrogen phosphate (8.17 kg, 68 mol) in water (59 L). In a second vessel, sodium phosphate (4.53 kg, 28 mol) was dissolved in water (118 L), and the pH was adjusted to 7.3 using a solution of sodium dihydrogen phosphate (3.12 kg, 26 mol) in water (22.8 L). To this second buffered solution was charged adenosine deaminase ADA-101 from Codexis (470 g, 22 units/mg activity, 176 units enzyme/g 2′-MOE-DAPR **5**). The enzyme solution was cooled and maintained at 5-10 °C for 90 min, and then charged into the solution of **5**, which was held at 25-30 °C. The reaction mass was maintained at 25-30 °C and a pH of 7.3, adjusting the pH as necessary using a solution of sodium dihydrogen phosphate (25 kg) in water (37 L). After 8 hours of maintenance, seed crystals of **6** (94 g) were added to the reaction, and the reaction was maintained at 25-30 °C and a pH of 7.3 until the residual level of **5** was not more than 1.0% (another 12.25 hr, 20.25 hr in total). The reaction mass was cooled to 15-20 °C and maintained for 35 min, before collecting the 2′-*O*-(2-methoxyethyl)guanosine product by filtration. The filter cake was washed with water (118 L), slurried in acetone (118 L), filtered, and transferred into a hot air dryer

(45-50 °C) to remove residual acetone and water (water < 0.5%) to afford the title compound as a crystalline solid (48.4 kg, 139 mol) with 98.3% a/a purity in an 80.6% yield (HPLC a/a% purity corrected). Impurity profile (a/a%): DAPR **3** (0.14%), guanosine **11** (1.11%), 3′-MOE G **12** (0.20%). Residual **5** and impurities **8** (3′-MOE DAPR), **10** (bis-alkylated DAPR), and **13** (bis-alkylated G) were all undetected.

*N^2-isobutyryl-2′-O-(2-methoxyethyl)guanosine (**7**)*. Into a 1000 L reactor at 20-35 °C under nitrogen was charged pyridine (241 L) and **5** (48.2 kg, 142 mol). The solution was cooled to 10-15 °C for 30 min and trimethylsilyl chloride (122.4 kg, 1130 mol) was charged to the reactor keeping the reaction mass at 10-15 °C during the 2 hr. addition. The temperature was increased to 20-30 °C and maintained for 5 hours. The reaction mass was again cooled to 10-15 °C for 45 min and isobutyryl chloride (22.4 kg, 210 mol) was charged to the reactor keeping the reaction mass at 10-15 °C during the 1 hr. addition. The temperature was increased to 20-30 °C and maintained for 6 hours. The reaction mass was cooled to 5-10 °C for 1 hr. and quenched with methanol (9.6 L, 237 mol) while maintaining the temperature below 10 °C during the 2.5 hr. addition. The

reaction was filtered, and the reactor and filter cake rinsed with acetone (193 L). The filtrate, wash, and water (96 L) were charged back into the reactor at 20-35 °C. The pH was adjusted to 7.0 with ammonium hydroxide (39.1 L) over 2.5 hours. The reaction mass was maintained at 20-35 °C for 2 hr with nitrogen bubbling. The volume was reduced to 20% original volume by vacuum distillation over 14 hr. while keeping the temperature below 60 °C. The resulting concentrated mixture was cooled to 30-35 °C. The vacuum distillation was repeated twice more adding water (241 L) to the concentrated mixture each time. After the three distillation cycles, water (272 L) was added such that the total volume reached 10 vol. The solution was cooled to 20-35 °C and sodium chloride (120.5 kg) was charged to make a saturated solution. The brine solution was extracted three times with 3% MeOH in DCM (17.4 L MeOH, 561 L DCM) and the organic layers combined (2146 L). To the combined organic layers was charged DCM (482 L) and the organic solution was washed with saturated sodium chloride(aq) (41.9 kg NaCl, 145 L water) until the level of **14** was less than 0.1% (one wash for this campaign). The organic layer was concentrated to 20% of original volume with a check that water content was less than 1.0%. The solvent was exchanged to acetone using

two additions of acetone (241 L) followed by a vacuum distillation to 20% original volume after each addition. After the second distillation, additional acetone (482 L) was charged to the solution. The solution was stirred for 30 min, filtered, and the filter cake washed with acetone (241 L). The resulting solid was dried in a vacuum tray drier keeping the temperature below 30 °C for *ca* 50 hr. Because the water content was >1.0% (4.0%), acetone (224 L) and the isolated solid were charged to a reactor and stirred for 20 min. at 20-35 °C. The slurry was evaporated to dryness under vacuum keeping the temperature below 35 °C and held for an hour under vacuum to afford the title compound (47.7 kg, 115 mol) in 82.7% yield (HPLC a/a% purity corrected) with a purity of 99.0% HPLC a/a%. Impurity profile (a/a%): 2′-MOE G **6** (0.36%), 3′-MOE G^{ibu} **15** (0.07%),

ASSOCIATED CONTENT

Supporting Information.

MOE G PNS - supporting information.docx containing details of DoE, analytical methods, and further characterization data (PDF)

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Notes

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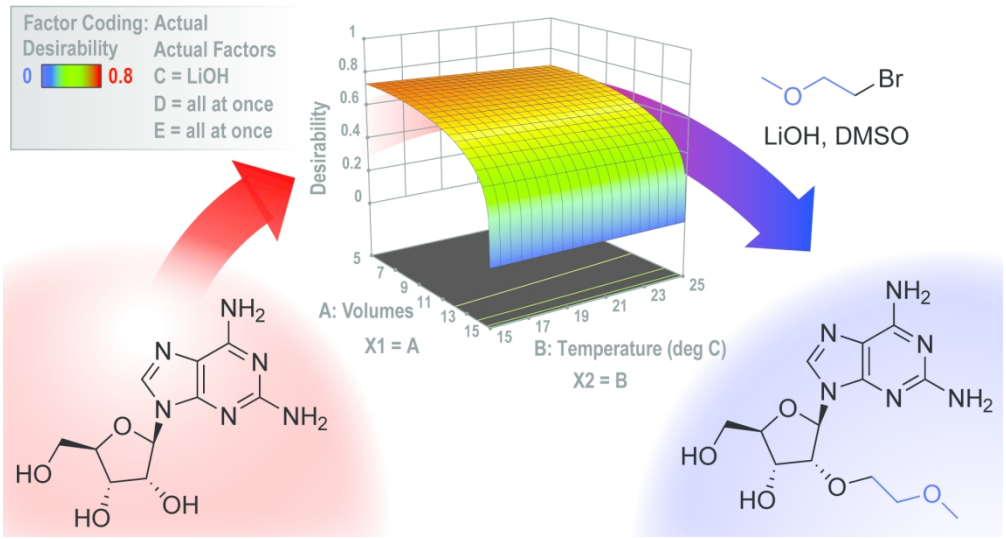
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Desirability
0
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Actual Factors
C = LiOH
D = all at once
E = all at once
Desirability
A: Volumes
X1 = A
B: Temperature (deg C)
X2 = B
Br
LiOH, DMSO
NH2
HO
OH

TOC graphic