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Applications of green chemistry in the manufacture of oligonucleotide drugs*

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Abstract: We have modified the current phosphoramidite-based, solid-phase synthesis of antisense oligonucleotides to accommodate principles of green chemistry. In this article, we summarize key accomplishments that reduce or eliminate the use or generation of toxic materials, solvents, and reagents. Also discussed are methodologies that allow reuse of valuable materials such as amidites, solid-support, and protecting groups, thus improving the atom economy and cost-efficiency of oligonucleotide manufacture. Approaches to accident prevention and the use of safer reagents during oligonucleotide synthesis are also covered.

INTRODUCTION

Synthetic oligonucleotides are an emerging class of drug molecules with a broad spectrum of therapeutic application. With the successful launch of the first commercial antisense drug Vitravene™ in 1998, and ongoing clinical trials of a dozen other antisense oligonucleotides, there is anticipation of additional drugs reaching market in the near future [1]. Safe and cost-effective methods are needed for the manufacture of these drugs at commercial scales. To that end, we have made multiple modifications to our large-scale oligonucleotide manufacturing processes to accommodate some of the twelve principles of green chemistry [2]. Herein, we provide an overview of those accomplishments.

REPLACEMENT OF FISH-DERIVED NUCLEOSIDES WITH SYNTHETIC NUCLEOSIDES

The first generation of antisense drugs are 2'-deoxyoligonucleotides with uniform *O*,*O*-linked phosphorothioate diester backbones (Fig. 1). The key building blocks for assembly of 2'-deoxyoligonucleotides are 2'-deoxynucleosides, historically obtained via enzymolysis of DNA salt. The source DNA has been isolated exclusively from fish milt, mainly from salmon caught for human consumption. It is noteworthy that the milt from 100 metric tons of salmon would yield a meager 55 Kg of the four 2'-deoxynucleosides (T, dC, dA, and dG), in roughly equal amounts. Because of this dependence on marine DNA sources, a product ratio that buoys up prices of individual nucleosides, and a lengthy and labor-intensive isolation process, cost of 2'-deoxynucleosides has long been very high.

The seventh principle of green chemistry states that raw material feedstocks should be renewable, not depleting whenever technically and economically practicable. We, with others, have embarked on a mission to eliminate future impact of antisense drug production on salmon stocks and to reduce 2'-deoxynucleoside costs. Our work in this area has resulted in development of efficient 2'-deoxygenation protocols for conversion of readily available ribonucleosides to 2'-deoxynucleosides. The classic ribose deoxygenation protocols require toxic tin reagents. We have replaced tin with environmentally

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benign silyl reagents, without compromising reaction yields. In recent work by other groups, abundant and inexpensive D-glucose has been converted at very large scales by chemical and enzymatic steps to all four 2'-deoxynucleosides. Thus, large-scale 2'-deoxynucleoside supplies are now fully independent of marine DNA sources. The new routes to synthetic 2'-deoxynucleosides have and will continue to significantly reduce the cost of these key drug raw materials.

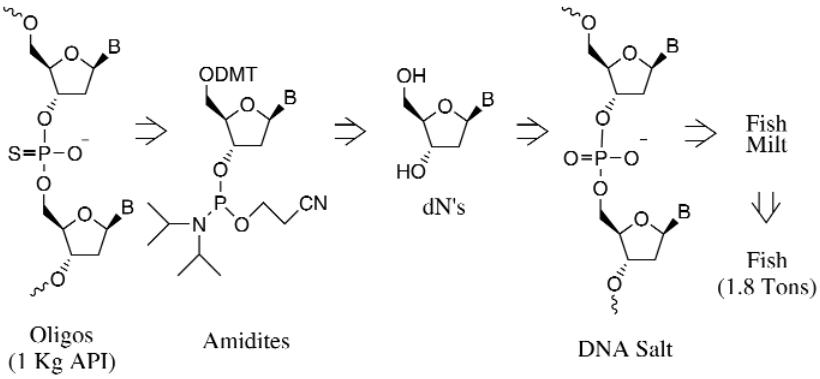


Fig. 1 Raw material pipeline for oligonucleotides.

ELIMINATION OF HALOGENATED SOLVENT AND WASTE FROM MANUFACTURING PROCESSES

Antisense oligonucleotides are made on solid supports via phosphoramidite coupling chemistry [3]. The quality and overall yields of oligonucleotides made by this protocol depend on four key synthetic steps (Scheme 1). A crucial step in each elongation cycle is removal of the acid-labile 5'-*O*-DMT (4,4'-dimethoxytrityl) protecting group with haloacetic acid, classically carried out in dichloromethane. Volatility, toxicity, and carcinogenicity issues have increasingly limited use of dichloromethane in the chemical industry [4]. In context of the fourth principle of green chemistry [2], we sought a replacement solvent with reduced toxicity that would preserve function and efficacy of the DMT deprotection reaction. An extensive search identified toluene as a green substitute for dichloromethane [5] that maintains quality and yield of product oligonucleotides. Furthermore, toluene is a widely accepted industrial solvent due to its lower vapor pressure and well-established environmental fate [6].

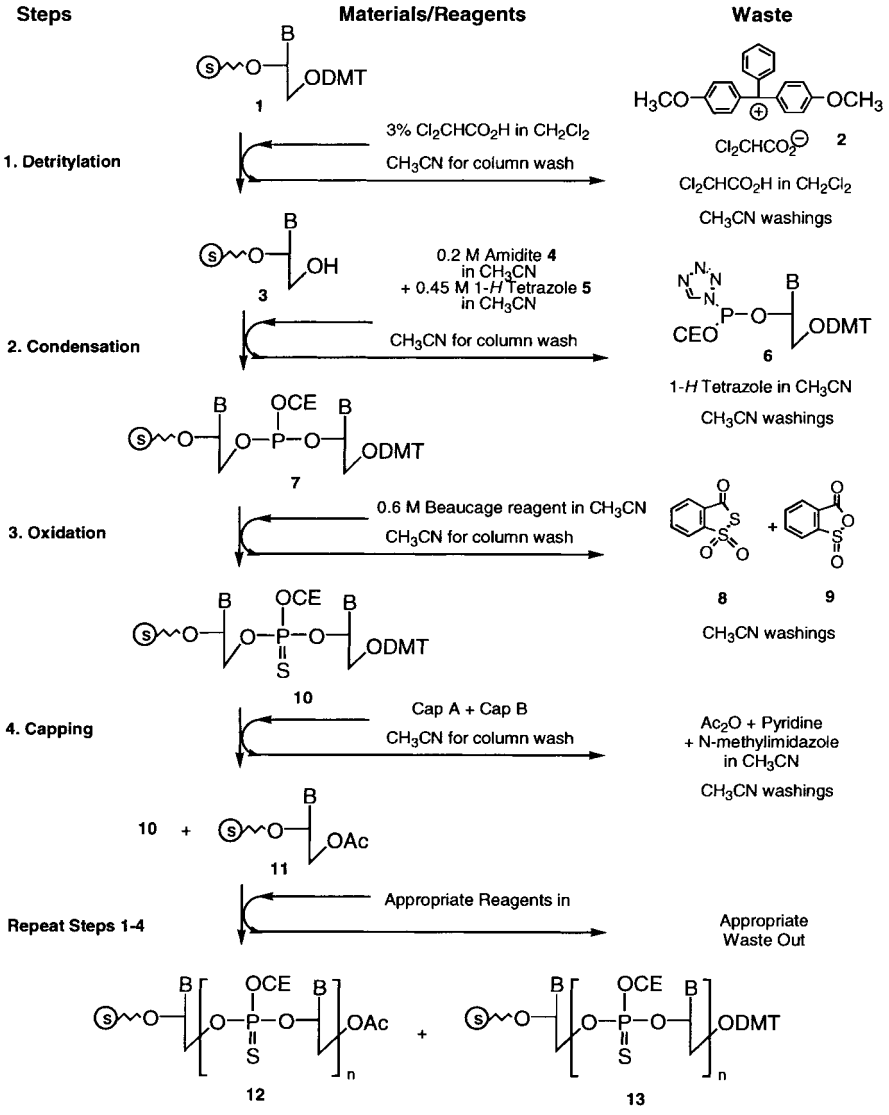
USE OF PYRIDINIUM TRIFLUOROACETATE AS A SAFER ACTIVATOR THAN 1*H*-TETRAZOLE

Another critical step in phosphoramidite coupling chemistry is 1*H*-tetrazole-mediated reaction of P^{III} phosphoramidites with the free 5'-hydroxyl groups of growing oligonucleotide chains to form phosphite triester intermediates (Scheme 1). Although 1*H*-tetrazole is widely used in oligonucleotide manufacture and no serious accidents have been reported thus far, its explosive properties must not be underestimated [7]. A large molar excess of 1*H*-tetrazole is employed in saturated anhydrous acetonitrile solution. It has been observed that 1*H*-tetrazole can crystallize from solution during the winter months and block the synthesizer feed lines, posing risk to a fully automated process. Furthermore, leaky containers and acetonitrile evaporation could leave solid tetrazole residues, the form in which the compound is unstable to impact. Our search for a safer coupling activator than 1*H*-tetrazole with excellent solubility in acetonitrile resulted in identification of pyridinium trifluoroacetate (PTFA) as reagent of choice in combination with *N*-methyl imidazole [8]. A number of antisense oligonucleotides were made to demonstrate that the synthesis proceeds efficiently without compromising quality or yield of products.

Interestingly, 1*H*-tetrazole is also commonly used in synthesis of nucleoside phosphoramidites (Fig. 1). We were pleased to discover that pyridinium trifluoroacetate is also effective in phosphitylation reactions [9], thus further minimizing potential for accidents in the synthesis of process raw materials.

IMPROVING ATOM EFFICIENCY BY RECOVERY OF 4,4-DIMETHOXYTRITYL PROTECTING GROUPS AND EXCESS AMIDITES

Although phosphoramidite chemistry operates at very high coupling efficiency (~99%), it has the disadvantage of creating two high-molecular-weight waste products, **2** and **6** (Scheme 1). The DMT group used in 5'-hydroxyl protection comprises 35% of total nucleoside phosphoramidite weight and is released as cation **2** in the waste stream. Amidites **6** are used in a 0.5- to 0.7-fold molar excess that is also present in the waste stream.



Scheme 1 Key oligonucleotide synthesis steps with standard reagents.

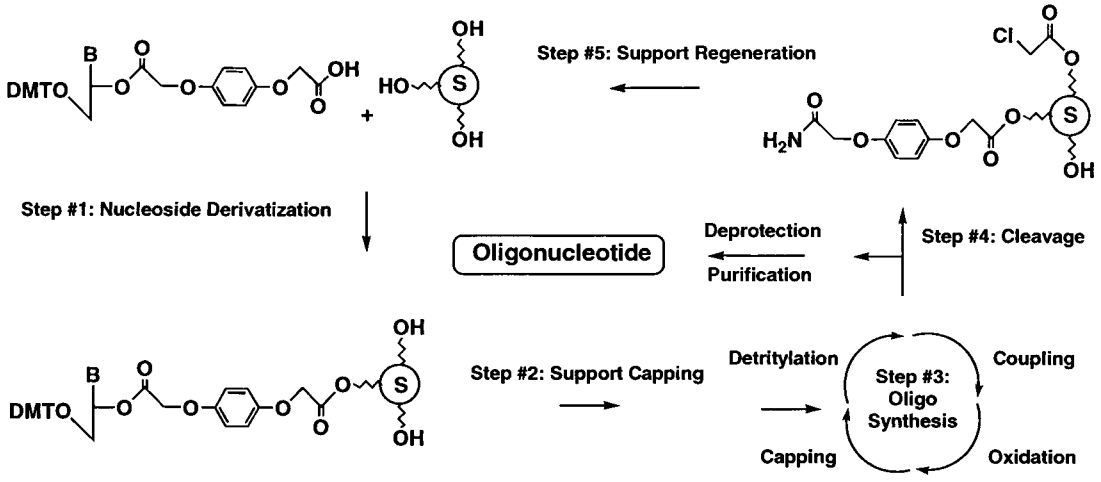
From an environmental perspective and increasingly from the economic point of view, it is important to consider how many starting material atoms end up in drugs and how many are waste. The concept of E-factor and the eighth principle of green chemistry were successfully applied to both of these high-molecular-weight oligonucleotide synthesis waste products. We have developed a simple two-step protocol for complete capture and recovery of the DMT group [10]. The DMT cation is first neutralized with NaOH to generate DMT alcohol that then undergoes efficient chlorination with acetyl chloride to furnish DMT chloride. The regenerated DMT chloride is satisfactory for reuse in the preparation of 5'-*O*-protected nucleosides.

An efficient process for capture and reuse of the excess amidite has been reported that operates without cleaving the costly 3'-oxygen-phosphorus bond [11]. Similarly, our three-step protocol involves conversion of activated amidite in the waste stream to the more stable *H*-phosphonate, halogenation with tris(2,4,6-tribromophenoxy) dichlorophosphorane, and *in situ* treatment with diisopropylamine to furnish the amidite in high yield. This method is also applicable to recovery of the more expensive dinucleoside phosphorothioate (dimer) amidites useful in solution-phase oligonucleotide synthesis [12].

DEVELOPMENT OF REUSABLE SOLID-SUPPORT CHEMISTRIES FOR OLIGONUCLEOTIDE SYNTHESIS

Solid-support selection has significant impact not only on synthesis efficiency and product cost but also on the environment. Currently, our manufacturing process uses HL-30™, a polystyrene bead support loaded at 90 mmol/g. The HL-30 bead has several limiting characteristics: (a) it is nonbiodegradable, (b) it can be used only once before disposal, (c) it contributes ~40% of raw material costs, and (d) it is a single-source raw material. In view of these limitations, we have developed new methodologies for effective regeneration of spent solid supports and their reuse in oligonucleotide synthesis.

The reusable solid-support technology is based on use of a Q-linker™ (hydroquinone diacetic acid) spacer arm between the 3'-end of the first nucleoside and a hydroxyl- functionalized support (Scheme 2). Details of the chemistry have been published elsewhere [13]. In summary, the method allows used support to be quickly rederivatized with protected nucleoside and reused, without opening and recharging the synthesis column. The solid-support bed may be used up to six times in this manner



Scheme 2 Reusable solid-support chemistry.

without compromise in the quality and yield of oligonucleotide product. The environmental impact and costs of solid-support waste disposal are thus sharply reduced.

In recent years, much research has gone into creation of biodegradable polymers and chemical synthesis solid supports with higher loading capacities. To minimize waste and increase synthesis throughput, Merckogel™ has been developed as a macroporous polyvinylacetate biodegradable support for oligonucleotide synthesis. Preliminary results indicate that oligonucleotides can be made in good yield and quality on such supports at very high loadings up to 400 mmol/g. At still higher levels, yield is compromised [14].

OVERALL REDUCTION IN SOLVENT CONSUMPTION AND ITS IMPACT ON THE WASTE STREAM

The first principle of green chemistry states that it is better to prevent waste production than to treat waste or clean it up after it has been created [2]. Although solvent volumes are relatively large in solid-phase synthesis, we have taken a parsimonious view and developed very efficient synthesis and purification protocols that minimize solvent consumption. A decade ago, our first oligonucleotide for clinical trials was synthesized in a stirred-bed Milligen 8800 reactor. The reactor design required very large solvent volume to product mass ratios, on the order of 17 L per gram of crude oligonucleotide. We soon established that a packed-bed reactor design could sharply reduce solvent consumption. Among packed-bed reactor designs including the LSB PCOS-2™ centrifugal synthesizer, we found that the Pharmacia OligoProcess™ axial flow reactor was most effective in reducing solvent usage. The OligoProcess reactor is essentially a large steel preparative chromatography column. Gratifyingly, this fixed-bed design uses only 2.7 L of solvent to make 1 g of crude oligonucleotide, thus achieving six-fold reduction in reaction solvent consumption. We now manufacture all GMP antisense drugs on this highly efficient synthesizer. The selection of an axial flow fixed-bed reactor design over the stirred-bed reactor not only reduced solvent consumption sharply, but allowed a 1000-fold increase our production capacity in the original physical facility.

PROCEDURE FOR SAFER CLEAVAGE OF PROTECTING GROUPS

The importance of safety in the chemical industry cannot be overstated. In our oligonucleotide manufacturing process, all steps but one are carried out at room temperature. In light of the twelfth principle of green chemistry, we have focused on improving safety of this step, carried out under pressure at elevated temperatures. Cleavage of oligonucleotide from solid-support and heterocyclic base deprotection is accomplished by treatment with concentrated ammonium hydroxide at 55° C in a sealed pressure vessel for 12 h. Deprotection at ambient temperature and pressure would clearly reduce the risk of operator injury. Therefore, a systematic study of deprotection rate was carried out using phosphorothioate oligonucleotide drugs [15]. The results indicated that all protecting groups are completely removed in 120 h at 20 °C, including the difficult guanine isobutyryl protecting group. Ambient temperature and pressure deprotection is a safer approach where extended reaction time is acceptable.

USE OF WATER INSTEAD OF ORGANIC SOLVENT FOR CHROMATOGRAPHY

GMP antisense oligonucleotide intermediates are routinely purified using reverse phase (RP) chromatography. This method yields oligonucleotides of high purity and is readily scalable to multi-hundred kilogram per year levels. Because some antisense drugs have potential market annual requirements in the multi-ton range, we have also developed efficient chromatographic protocols that operate entirely without organic solvent and its attendant hazards for use in very large scale facilities. A detailed account of purification by these anion exchange chromatography methods can be found elsewhere [16].

CONCLUSIONS

As we design new chemical syntheses and improve the manufacture of GMP oligonucleotides, decisions about whether hazardous substances will be used, whether toxic materials must be handled, whether hazardous waste will require special disposal, and the overall environmental issues associated with these processes must be seriously considered. Green synthesis protocols for oligonucleotide manufacture will yield less costly drug products when all direct and indirect costs are accounted.

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