Pergamon

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Bioorganic & Medicinal Chemistry Letters 13 (2003) 3537–3540

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

NBS–DMSO as a Nonaqueous Nonbasic Oxidation Reagent for the Synthesis of Oligonucleotides

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Received 17 October 2002; accepted 8 July 2003

Abstract—A new method for the oxidation of nucleoside phosphite triester into phosphate triester under nonbasic and nonaqueous conditions using NBS–DMSO in CH_3CN has been developed. The utility of this method for solution- and solid-phase synthesis of oligonucleotide is demonstrated.

Synthetic oligonucleotides are an emerging class of drug molecules that are assembled via phosphoramidite chemistry. The quality and yield of oligonucleotides made by this protocol depends on four key steps namely detritylation, coupling, oxidation and capping. Among these oxidation is a crucial step that converts an internucleosidic phosphite triester linkage P(III) to the corresponding phosphate triester linkage P(V).[1] The development of efficient methods for such oxidation is an important area of research particularly when therapeutic oligonucleotides will be required in large quantities.[2]

Currently, iodine in THF/water/pyridine (or lutidine) is used for oxidation in conventional oligonucleotide synthesis.[3] Although this method of oxidation is widely accepted for small-scale synthesis, it is not suitable for the large-scale synthesis of oligonucleotides required for antisense therapeutics. On large-scale, the presence of iodine, water and base compromises the quality and yield of the product and increases the overall cost. For example, controlled pore glass is a frequently used support that holds water tightly and complete removal of water before the coupling step requires excessive washings with acetonitrile. This increases the volume and cost of the solvent used. Presence of iodine in the reagent imparts a pale coloration to the oligonucleotide product that is not desirable for therapeutic applications. Lastly, exposure of aqueous base may not be appropriate for the base labile protecting groups, particularly with modified bases containing labile protecting groups.[4]

Due to these problems, we have focused our attention towards the development of reagents useful for oxidation under nonbasic and nonaqueous conditions. Several strategies including nitrogen dioxide (N_2O) in dichloromethane,[5] *m*-chloroperbenzoic acid (MCPBA) in dichloromethane,[6] *tert*-butyl hydroperoxide (TBHP)/toluene solution,[7] bis(trimethylsilyl)peroxide in dichloromethane or an acetonitrile dichloromethane mixture,[8] dimethyldioxirane in dichloromethane,[9] (1*S*)-(+)(10-camphorsulphonyl)oxaziridine (CSO)[10] in acetonitrile and ethyl(methyl)dioxirane in dichloromethane[11] have been reported in the literature. These methods have some limitations for large-scale applications such as toxicity of N_2O and MCPBA, and the undesired cleavage of 5′-*O*-dimethoxytrity group that results when *m*-chlorobenzoic acid is liberated in the MCPBA oxidation step. Reagents like TBHP in decane, bis(trimethylsilyl)peroxide and CSO are very expensive. Additionally, silylated peroxides and dimethyldioxirane are explosive reagents. Dimethyldioxirane is not available commercially on large-scale and undesirable oxidative modifications of thymidine,[12] uracil[12] and adenine bases have been reported with this reagent.[13]

These limitations with nonbasic and nonaqueous reagents reported in the literature encouraged us to investigate the applications of CCl_4-dimethyl sulfoxide

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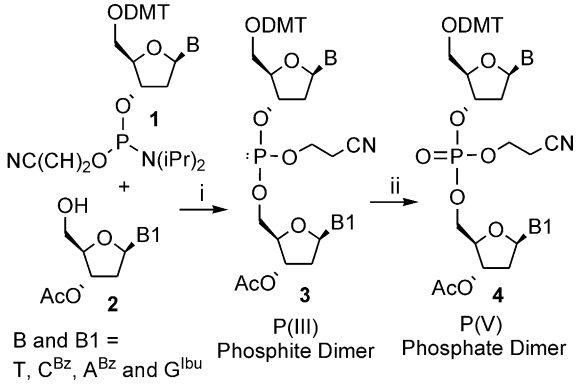
doi:10.1016/S0960-894X(03)00754-6

(DMSO) mixture as an oxidative reagent for oligonucleotide synthesis.[14] Volatility, toxicity and carcinogenicity issues have increasingly limited use of CCl_4 in the chemical industry. To avoid the use of halogenated compounds, herein we report the use of *N*-bromosuccinimide (NBS)–DMSO as a safer and green reagent[15] for the oxidation of oligonucleotide phosphite P(III) to their corresponding phosphate P(V).

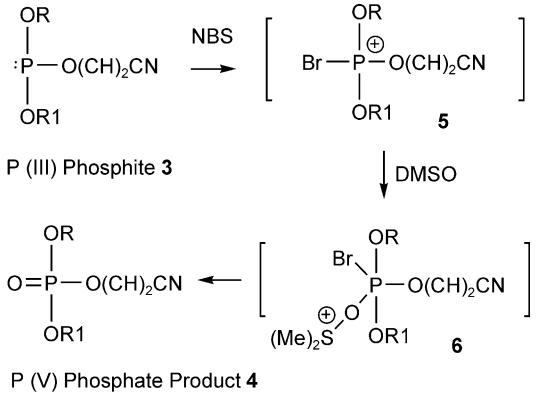
In order to develop appropriate conditions for the oxidation cycle required during solid-phase oligonucleotide synthesis, we first studied the NBS–DMSO mediated oxidation in solution phase using a dinucleoside phosphite triester **3** (Scheme 1). The synthesis of dimer **3** was accomplished by coupling of nucleoside phosphoramidite **1** with nucleoside **2** in the presence of 1*H*-tetrazole at room temperature for 5 min in anhydrous CH_3CN. Product **3** was isolated in good yields (89%) after chromatographic purification. Interestingly, the low cost of DMSO has allowed oxidation of various compounds on large-scale.[16] Similarly, NBS has been reported to be an effective reagent for the oxidation of a variety of functional groups.[17] In this study we utilised for the first time a combination of these two reagents for the oxidation of P(III) to P(V) species. After significant efforts, oxidation of **3** was successfully accomplished with NBS–DMSO in CH_3CN to furnish phosphate **4** in 90% isolated yield. The progress of the reaction was monitored by ^{31}P NMR spectroscopy indicating that **3** (δ 144.2) was oxidized completely to furnish **4** (δ 1.88). All of the possible sixteen dimers having different combinations of A^{Bz}, T, G^{ibu}, C^{Bz} were prepared following the NBS–DMSO protocol in >90% isolated yield. The UV, ^1H NMR and HPLC analysis indicated that the heterocyclic moiety and all protecting groups (DMTr, –COR, $–OCH_2CH_2CN$) remain unaffected under the oxidation conditions. The key to success was based on the use of the right amount of reagents (NBS–DMSO–CH_3CN; w/v/v 0.02/0.07/1), freshly crystallized NBS and anhydrous solvent. Reducing the amount of NBS–DMSO compromised the purity of the dimers (<90% HPLC).

The proposed mechanism for NBS–DMSO mediated oxidation of P(III) to P(V) is shown in Scheme 2. The first step appears to be halogenation of the P(III) species with NBS to furnish an intermediate **5** which undergoes addition of DMSO to provide an activated sulfonium ion **6**. This eliminates the dimethyl sulfide resulting in the formation of stable P(V) product **4**.

Next, the solution-phase conditions were applied to the solid-phase synthesis on an automated DNA synthesizer. Synthesis of d(CATGG) was undertaken because it represents all four natural bases. Initial experiments with similar concentrations of NBS–DMSO (w/v 0.02/0.07 in CH_3CN) furnished ~50% full-length product by HPLC in the crude mixture. The 2-fold increase in the concentrations and contact time of NBS–DMSO afforded better results with ~70% full-length product. The oligonucleotide synthesis was performed in triplicate on controlled pore glass (CPG) bound dG via a succinyl linker.[18] The modified synthesis cycle is shown in Table 1. Each monomer coupling was affected in the average yield of 95% as determined by the colourimetric method of released DMTr cation. The final oligonucleotide was cleaved from solid support by ammonium hydroxide treatment.[3] The ^{31}P NMR spectrum of the crude oligonucleotide product had no signal due to the trivalent phosphorus indicating quantitative oxidation. The



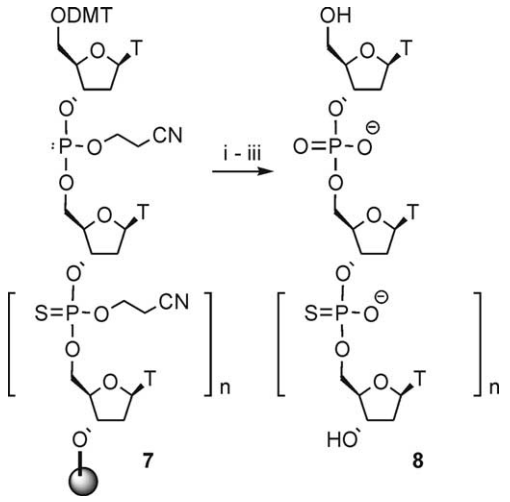
Scheme 1. Solution-phase synthesis of 16-dimers. Reagents and conditions: (i) 0.1 mmol **1**, 0.1 mmol **2**, CH_3CN, 1-H tetrazole, rt, 2 min, 85–90% **3**; (ii) 0.1 mmol **3**, 6 mL NBS/DMSO/CH_3CN (w/v/v 0.02/0.07/1), rt, 5 min, 90–95% **4**.



Scheme 2. Proposed mechanism for oxidation.

Table 1. Synthesis cycle

Step no.	Operation	Reagent	Time (min)
1	Washing	CH_3CN	0.5
2	Detritylation	3% $CHCl_2COOH$–CH_2Cl_2	1.8
3	Coupling	0.1 M Phosphoramidite–CH_3CN + 0.5 M 1*H*-tetrazole–CH_3CN	1.1
4	Washing	CH_3CN	0.2
5	Capping	Ac_2O/2,6-lutidine/ THF (1:1:2) + 6.5% DMAP–THF	0.4
6	Oxidation	NBS–DMSO–CH_3CN (W/V/V 0.04/0.14/1)	2



Scheme 3. Solid-phase synthesis of tetramer ($n=2$). Reagents and conditions: (i) NBS/DMSO/CH_3CN (w/v/v 0.04/0.14/1), rt, 2 min, CH_3CN wash; (ii) detritylation 3% $CHCl_2COOH$–CH_2Cl_2, 1.8 min; (iii) NH_4OH 55 °C.

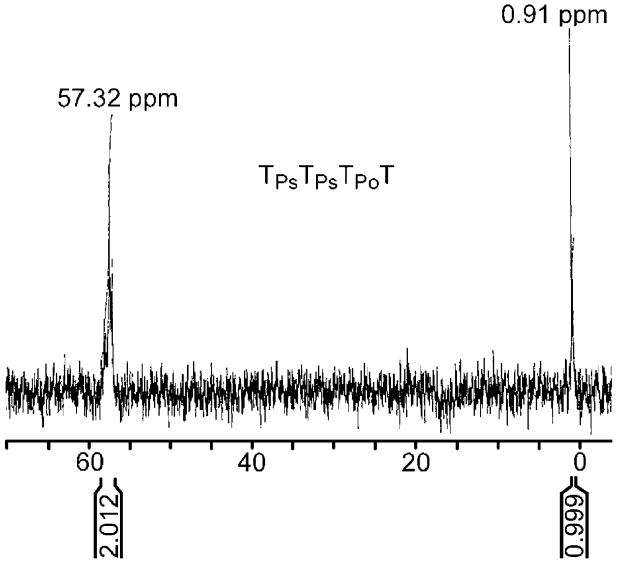


Figure 1. ^{31}P NMR of tetramer **8**.

integrity of the oligonucleotide was further confirmed by enzymatic hydrolysis of purified d(CATGG) which indicated the expected ratio of the natural bases.

The NBS–DMSO oxidation system was further evaluated for use in the synthesis of mixed backbone oligonucleotides due to their potential therapeutic applications.[19] A T-tetramer was chosen as a test sequence to demonstrate the capabilities of NBS–DMSO. Tetramer **7** containing a terminal P(III) linkage was synthesized following traditional phosphoramidite chemistry using Beaucage reagent for thiolation. Oxidation of **7** with the NBS–DMSO system on solid support using the conditions described in Table 1 provided **8** in excellent yield (Scheme 3). The purity of tetramer **8** was assessed by ^{31}P NMR spectrum (Fig. 1). The 1:2 ratio of the integration of PO (δ 0.91) and PS (δ 57.32) signals clearly indicated that there was no desulphurisation of phosphorothioate linkage during the NBS–DMSO based oxidative step. The LC–MS data (not shown) for **8** further confirmed the structure and purity of the product.

In summary, NBS–DMSO in CH_3CN is a useful reagent for the oxidation of nucleoside phosphite into phosphate under nonbasic and nonaqueous conditions. We demonstrated that this reagent could be used for synthesis of mixed backbone oligonucleotides in good yields and high purity both by solid as well as solution-phase synthesis. The easy accessibility, low-cost, safe handling and disposal of this new oxidation system is very attractive for the synthesis of oligonucleotides on a large scale.

Acknowledgements

One of the authors, M.C.U., is grateful to Isis Pharmaceuticals for financial assistance and gift of various chemicals and reagents. We are also thankful to Dr. Douglas Cole for his encouragement and support throughout this project.

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18. Oligonucleotides were synthesized on 5 μmol scale using an ABI-394 DNA synthesizer following a protocol described in Table 1. Doubling the ratio of NBS–DMSO (0.04/0.14) relative to the amount used for solution phase synthesis, improved the yield (95%) of tetramer **8** during solid-phase synthesis. The NBS–DMSO–CH_3CN solution was prepared freshly prior to the use. **General experimental conditions**: Commercial nucleoside loaded CPG (500 A, 50–60 umol/g) was used for all syntheses. When the synthesis was complete, the cleavage and deprotection were carried out by treatment with concentrated ammonium hydroxide solution (5 mL) at 55 °C for 12 h. CPG was removed by filtration, and ammonium hydroxide solution was dried by lyophilization. The crude product was analyzed by reverse-phase HPLC, ^{31}P NMR and MS (see ref 2 for more details).
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