2-(6-Nitro-1-oxy-benzotriazol-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (NBTU): A new coupling reagent in oligonucleotide synthesis

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Received on September 5, 2000.

Abstract
A new coupling reagent 2-(6-nitro-1-oxy-benzotriazol-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (NBTU) has been developed for use in solution and solid-phase methods of oligonucleotide synthesis. The reagent is also applicable to anchor the leader nucleoside onto the Ica-CPG.

Keywords: Coupling reagent, oligonucleotide synthesis, H-phosphonate, phosphotriester.

1. Introduction
The applications of synthetic oligonucleotides and their analogs as antisense¹ and antigene² have been the major driving forces for research in the area of chemical synthesis of oligonucleotides. A variety of methods are now available to meet the increasing demand of synthetic oligonucleotides.¹ In all these methods, the crucial step is the internucleotide bond formation. Therefore, considerable research efforts have been expended in developing efficient coupling reagents.² Hashmi et al.³ from this laboratory have previously introduced 6-nitrobenzotriazol-1-ylxy-tris-dimethylaminophosphonium hexafluorophosphate (NBOP) as a stable and more efficient coupling reagent in the triester-based oligonucleotide synthesis. However, during the course of coupling reaction hexamethylphosphoramidate (HMPA) is released which is a cancer-suspect agent.⁴ In order to overcome this drawback, we replaced the hexamethylphosphonium cation of NBOP with a tetramethyluronium cation. We surmised that such a replacement would render both the molecule as well as the byproducts of the coupling reaction less harmful. This led to a new coupling reagent 2-(6-nitro-1-oxy-benzotriazol-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (NBTU). In this communication, we report the synthesis and application of NBTU in phosphotriester and H-phosphonate method of oligonucleotide synthesis.

The reagent was synthesized by suitable modification of the procedure reported for the synthesis of NBOP.⁵ Subsequently, with the help of detailed spectroscopic analysis including X-ray crystallography we have identified the correct structure for NBTU⁶ (Fig. 1).

¹Dedicated to Prof. S. C. Bhattacharyya.
²CDRI Communication No. 6052.
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2. Experimental

2.1. 2-(6-Nitro-1-oxy-benzotriazol-3-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (NBTU)

Tetramethylurea (99.85 mmol, 11.95 ml) was dissolved in dry toluene (25 ml) and a 20% (v/v) solution of phosgene in toluene (100 ml) was added to it very slowly with efficient stirring under ice-cold condition. The reaction mixture was then stirred for 30 min at room temperature and again cooled in ice-bath followed by the addition of anhydrous ether (350 ml). It was further stirred for 5 min and then filtered with careful exclusion of moisture and the precipitate was washed with anhydrous ether (2 x 50 ml). The precipitated chloride salt of tetramethyluronium chloride was then dissolved in dichloromethane (300 ml) and shaken with saturated aq. solution of KPF$_6$ (125 ml) for 15 min. The organic layer was then separated, washed with water, dried over anhydrous MgSO$_4$ and concentrated to yield a syrupy mass, which solidified on addition of ether. The product, 2-chloro-1,1,3,3-tetramethyluronium hexafluorophosphate, was filtered, washed with ether and dried over P$_2$O$_5$.

A solution of 6-nitro-1-hydroxybenzotriazole (42.77 mmol, 7.70 g) and triethylamine (42.77 mmol, 5.96 ml) in dichloromethane (50 ml) was added to a solution of 2-chloro-1,1,3,3-tetramethyluronium hexafluorophosphate (42.77 mmol, 12 g) in dichloromethane (100 ml) and the reaction mixture was stirred for 15 min. The precipitated NBTU was filtered and washed with dichloromethane.

Yield: 16.01 g (88%); m. p.: 188°C [decomp.]; FAB (m/z): 279 [M-PF$_6$]$^+$; $^{31}$P NMR: $\delta$ 144.48 (septet, $J = 710$, PF$_6$); $^1$H NMR: $\delta$ 3.07 (s, 6H, NMe$_2$); 3.42 (s, 6H, NMe$_2$); 8.06 (d, 1H, $J = 9.2$, H-4); 8.67 (dd, 1H, $J = 9.2$ and 2.0, H-5); 8.86 (d, 1H, $J = 1.9$, H-7); $^{13}$C NMR: $\delta$ 41.52 (NMe$_2$); 41.94 (NMe$_2$); 112.50 (C-7); 115.97 (C-4); 127.34 (C-5); 133.05 (C-4a); 135.94 (C-7a); 145.73 (C-6); 149.75 (C-8); Micro analysis (%): Found: C, 30.24; H, 3.48; N, 19.18; C$_{11}$H$_{15}$N$_6$O$_3$PF$_6$: Calc: C, 31.14; H, 3.56; N, 19.81.

3. General procedure for coupling with NBTU

Triethylammonium salt of $N$-protected-5'-O-(4, 4'-dimethoxytrityl)-2'-deoxynucleoside-3'-P-(2-chlorophenyl) phosphate or $N$-protected of $N$-protected-5'-O-(4, 4'-dimethoxytrityl)-2'-deoxynucleoside-3'-P-methylphosphonate (0.24 mmol), 3'-OH protected and 5'-OH free nucleoside (0.2 mmol) and 1-methylimidazole (0.29 mmol, 22.9 µl) were dried by coevaporating with dry pyridine (2 x 3 ml) and dissolved in dry pyridine (3.0 ml). NBTU (0.36 mmol, 152.7 mg) was added to it and the reaction mixture stirred for 15 min. When complete, as monitored by TLC (5% MeOH in CHCl$_3$), the reaction mixture was concentrated under reduced pressure.
and the residue was taken in DCM. The organic layer was washed with aq. sodium bicarbonate solution and brine, dried over anhydrous sodium sulphate and concentrated under reduced pressure. The crude product was purified by flash silica gel column chromatography using stepwise gradient of 0-8% methanol in DCM. The fractions containing pure product were pooled and concentrated under reduced pressure. The residue was dissolved in a small amount of DCM and added dropwise with stirring to cold hexane (nearly 20 times the volume of DCM solution) to obtain the desired product as white powder.

3.1. Loading of protected 2'-deoxynucleoside-3'-succinate with NBTU

N-acyl-5'-O-(4, 4'-dimethoxytrityl)-2'-deoxynucleoside-3'-O-succinate (0.1 mmol) and triethylamine (0.2 mmol, 27.9 μl) were dissolved in 4:1 acetonitrile-DCM (2 ml) and the solution was then transferred to a vial containing lcaa-CPG (0.05 μmol of NH₂). The mixture was vortexed thoroughly and NBTU (0.2 mmol, 84.9 mg) was added. The reaction was quenched by adding 0.5 ml methanol to the reaction mixture. Then the CPG suspension was transferred to a sintered funnel and washed with methanol (3 x 2 ml) followed by ether (3 x 2 ml). Finally, the support, derivatized with nucleoside, was dried in vacuum and the loading assayed spectrophotometrically.

3.2. Solid-phase synthesis of T4 and T8 by H-phosphonate method

Solid-phase synthesis of oligonucleotides was performed in a glass reaction vessel of 5.0 ml capacity, fitted with a G-3 sintered filter disc. All the reaction steps during chain assembly, except oxidation, were carried out in an inert atmosphere maintained by bubbling anhydrous argon through the reactants, which also helped in efficient mixing of the reactants in the reaction vessel.

3.3. Application of NBTU in the synthesis of oligonucleotides via phosphotriester chemistry

The suitability of NBTU as a coupling reagent was explored using a dimer synthesis as a prototype. Conditions required for the optimal coupling yields, namely, solvent, reaction time and molar excess of the reagent were standardized using the 1+1 coupling reaction employing suitably protected phosphodiester and hydroxy component, taking out aliquots of the coupling reaction at 5-min interval up to a total period of 30 min. TLC was performed to monitor the progress of the coupling reaction. It was observed that the coupling reaction was complete within 10-15 min (disappearance of the hydroxy component on the RP-TLC). It was also found that best coupling results are obtained with only 1.5-fold molar excess of the reagent. This is a significant achievement considering the fact that many of the coupling reagents reported in the literature are used nearly in three-fold excess. Moreover, a second batch addition of the coupling reagent is invariably recommended in most of the earlier protocols while this is not required in the case of NBTU-mediated coupling. Having optimized the condition for coupling reaction, a few dinucleotides were synthesized in excellent yields using NBTU (Table I). The synthesis was also extended to include nonionic methylphosphonate dinucleotides via a solution phase method, in view of their importance as antisense oligos and also as probes in DNA–protein interaction studies. Furthermore, a pentamer and a hexamer were synthesized
using NBTU as a coupling reagent to prove its suitability in the solution phase method of oligonucleotide synthesis employing phosphotriester chemistry.

Syntheses of these oligomers were carried out by block condensation method in a 1+1; 2+2, 4+2 or 1+4 manner. At each stage coupling reaction proceeded uneventfully and in excellent yields (Table I). Since coupling reactions were quantitative, capping step was dispensed from the protocol. The coupling reaction with NBTU proceeded without any side reaction either with the hydroxy group or with the heterocyclic base moiety. This was deduced from an experiment in which suitably protected monomers having free 5'-OH were treated with NBTU under the condition used for coupling. After work up, the recovered product showed superimposable UV, NMR and FAB MS profiles with respect to the starting compound (data not shown) thus confirming that no side products are formed during NBTU-mediated coupling. After the synthesis was complete, the pentamer and the hexamer were deblocked by the standard procedure. Final compounds were purified by HPLC on a reversed-phase C-18 column. The major peak was found to correspond to the required product as confirmed by ESI mass spectroscopic studies.

3.4. Application of NBTU in the solid-phase H-phosphonate synthesis of oligonucleotides

Earlier it has been shown that (benzotriazol-1-yloxy)carbonyl or phosphonium type of condensing reagents and activated monomers produced by these reagents usually do not undergo side reactions in H-phosphonate chemistry. Encouraged by this observation and our own results, we have synthesized two oligomers, a tetramer (T4) and an octamer (T8), on 0.2 and 2.0 μmol scales by the solid-phase H-phosphonate chemistry. The solid-phase synthesis was carried out manually and the coupling efficiency was >95% in each coupling step as determined by the trityl assay.

The oligomers, T4 and T8, were also synthesized by the presently most popular phosphoramidite method on automated DNA synthesizer to compare our methodology. These syntheses were carried out on 0.2 μmol scale employing DMT-off protocol. The oligonucleotides synthesized by both the methods were deblocked from the solid support by treatment with con-
A NEW COUPLING REAGENT

Table II
Comparative data of T4 and T8 synthesized by two methods

<table>
<thead>
<tr>
<th>Oligomers</th>
<th>Method</th>
<th>O. D. units</th>
<th>Rt (min)</th>
<th>FAB MS [M-H]</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>T4</td>
<td>H-Phosphonate</td>
<td>3.9</td>
<td>13.2</td>
<td>1154</td>
<td></td>
</tr>
<tr>
<td>T4</td>
<td>Phosphoramidite</td>
<td>4.3</td>
<td>13.2</td>
<td>1154</td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>H-Phosphonate</td>
<td>9.6</td>
<td>14.7</td>
<td>2370</td>
<td></td>
</tr>
<tr>
<td>T8</td>
<td>Phosphoramidite</td>
<td>10.2</td>
<td>14.7</td>
<td>2370</td>
<td></td>
</tr>
</tbody>
</table>

Centrated ammonia. The completely deblocked oligonucleotides were purified to homogeneity by the reversed-phase HPLC and characterized by negative ion FAB mass spectroscopy. Comparison of the data for the synthesis of T4 and T8 by either method (Table II) suggests that the H-phosphonate method employing NBTU as a coupling reagent is almost equally potent as the popular phosphoramidite method.

3.5. Application of NBTU in loading of 2'-deoxynucleosides on solid support

Gupta et al. have employed a commercially available condensing reagent O-(1H-benzotriazol-1-yl)-N, N', N', N'-tetramethyluronium tetrafluoroborate (TBTU) or triphenylphosphine (TPP) along with 2, 2'-dithio-bis-(5-nitropyridine) (DTNP) for the functionalization of lcaa-CPG. Because of the structural similarities between TBTU and NBTU it was considered desirable to investigate the loading of the leader nucleoside on to lcaa-CPG employing NBTU. The hemisuccinate derivatives of appropriately protected deoxyribonucleosides were anchored on the lcaa-CPG by reacting with NBTU. The extent of loading was assayed spectrophotometrically. It was observed that the loading of the leader nucleoside on to lcaa-CPG attains an optimum value (25 μmol/g) within a minute and there is no considerable increase in loading value on extending the reaction time even up to 30 min. The loading betters the TPP/DTNP procedure and gives about 20 μmol nucleoside/g of solid support in 5 min in our hands.

In order to ensure that no modification of the nucleic bases occurred during the functionalization of polymer support, a small quantity of each of the derivatized support and commercially available supports were subjected to aqueous ammonia treatment in sealed vials at room temperature for 30 min. The released 5'-O-DMT-N-protected-2'-deoxynucleosides were found identical to the corresponding nucleosides released from the commercial supports as well as the synthetic 5'-O-DMT-N-protected-2'-deoxynucleosides.

4. Conclusion

It may be concluded from our studies that NBTU is a promising reagent in the chemical synthesis of oligonucleotides. The usefulness of the reagent lies mainly in its nonhygroscopic nature, extended shelf life and completely free from side reactions either with reactants or with products. The suitability of the reagent in H-phosphonate approach and phosphotriester approach makes it extremely significant for the chemical synthesis of oligonucleotides. As an obvious extension of the present study it is tempting to speculate that the reagent NBTU may prove effective in the H-phosphonate methodology employing the base unprotected mono-
mers. With the introduction of NBTU as a versatile coupling reagent, it has opened new possibilities for an efficient synthesis of natural and modified oligonucleotides. NBTU is also very effective in rapidly anchoring the leader nucleoside on to the iea-CPG support via succinyl linker without modifying the nucleobases.

Acknowledgement

Grateful acknowledgement is made to Regional Sophisticated Instrument Centre (RSIC), Lucknow, and National Parasite Bank, CDRI, for providing the spectral data and DNA synthesizer facility, respectively. TKS thanks the Council of Scientific and Industrial Research (CSIR) for financial assistance.

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