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Rapid Automated Derivatization of Solid-Phase Supports For Oligonucleotide Synthesis Using Uronium or Phosphonium Coupling Reagents.

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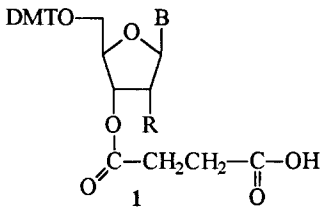
Abstract: Very fast (4 sec) attachment of nucleosides to CPG supports, via succinyl or QDA linkers, is possible with either uronium (HBTU, HATU, HBPyU, HBPipU) or phosphonium (BOP, PyBOP®, PyBroP, BroP) coupling reagents and automated derivatization of CPG and polystyrene supports just prior to oligonucleotide synthesis has been demonstrated. © 1997 Elsevier Science Ltd.

Automated solid-phase synthesis of oligonucleotides has become very common. However, faster and cheaper methods for generating large numbers of sequences are still required. In a previous paper,[1] we described how the total "on-instrument" time for synthesis can be reduced ~30-40%, without requiring other changes to reagents or procedures, by using hydroquinone-O,O'-diacetic acid (QDA) instead of succinic acid as the linker joining the first nucleoside to the solid-phase support. In this paper we would like to show how the time required to attach the first nucleoside to a solid-phase support can be drastically reduced. So much so, that it is feasible to construct instrumentation which utilize supports containing underivatized amino groups. Although such a strategy has long been available for peptide synthesis,[2] prederivatized supports for oligonucleotide synthesis have been used because fewer nucleosides are required. However, new high capacity synthesizers using multiwell plates[3] have greatly increased the difficulty and risk of error in placing the proper support in the correct position. A fast, automatable method for adding the first nucleoside to a universal support is therefore required.



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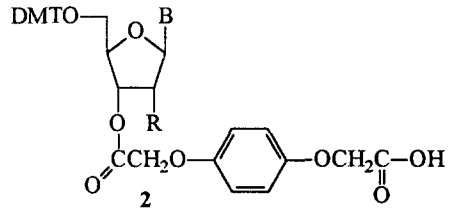

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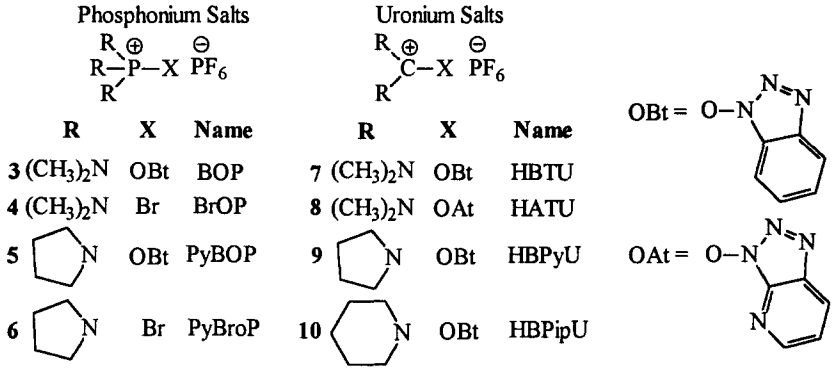
Derivatization requires formation of an amide linkage between a nucleoside-3'-O-carboxylate (either succinyl, **1**, or QDA, **2**) and an immobilized amino group. Typically, this is performed using a carbodiimide reagent with either a basic[4] (i.e. DEC and DMAP) or acidic[5] (i.e. DIC and HOBT) additive. However, these reactions are too slow (1-16 h) to be automated. A variety of reagents, based upon phosphonium or uronium salts, have been used in amide coupling reactions in solid-phase peptide synthesis. The phosphonium reagent, BOP,[6] was the first and it was followed by BroP[7] and the less hazardous PyBOP®,[8] and PyBroP[9] derivatives. Similar uronium reagents, beginning with HBTU[10] and followed by HBPyU,[11] HBPipU, and HATU[12] were also developed. Although, some of these reagents have been used to attach various linkers[13,14,15,16,17] to solid-phase supports, they have not been used to attach nucleosides such as **1** and **2** to CPG supports.

Structures of Phosphonium and Uronium Coupling Reagents



In our first experiments, we used the HBTU/HOBT (1:1) combination widely used in peptide synthesis.[18] Preliminary reactions showed best results in acetonitrile, although DMF, dichloromethane, or pyridine could also be used. Support derivatization with both succinylated or QDA derivatized nucleosides was performed manually. Either **1** or **2** (0.05 mmol), HBTU (0.05 mmol), HOBT (0.05 mmol), LCAA-CPG (250 mg, 101 μmol/g NH_2 groups), anhydrous DIEA (5 mmol) and anhydrous acetontrile (4.1 ml) were shaken at room temperature (10 min); the CPG was filtered off, washed (CH_2Cl_2), and dried. Nucleoside loading was determined by trityl analysis[4] (Table 1).

Table 1. Manual Derivatization of LCAA-CPG using HBTU/HOBT.

		Nucleoside Loading (μmol/g)	
B	**R**	**6** (QDA Linker)	**7** (Succinyl Linker)
A^{Bz}	H	53	63
C^{Bz}	H	55	57
G^{iBu}	H	43	57
T	H	50	63
U	O-*t*-butyldimethylsilyl	37	

The excellent nucleoside loadings, obtained in such a short time, along with the stability of the reagents, encouraged us to automate the derivatization on a PE/ABD 394 DNA synthesizer.[19] This allowed us to systematically evaluate the many available coupling reagents and additives. An optional wait step, allowed coupling reactions of precisely controlled times. However, to our surprise, the coupling reactions were exceptionally fast and no waiting period was required. The loadings obtained, when the reagents were flushed off the support immediately after being added (4 sec addition), are shown in Table 2.

The results showed that all eight coupling reagents produced excellent nucleoside loadings.The absence of HOBT and the presence of only one equivalent of DIEA did not seem to seriously affect the nucleoside loadings obtained. The order of reactivity was HATU > PyBOP ≈ HBTU > BOP > BroP ≈ HBPipU ≈ HBPyU > PyBroP. When an equimolar amount of the acylation catalyst, DMAP,[20] was added to the coupling reagent only a small increase in nucleoside loading occurred.

Table 2. Automated derivatization of LCAA-CPG using various coupling reagents.

	Nucleoside Loading (μmol/g)	
Coupling Reagent	**without HOBT or DMAP**	**with DMAP (1 eq.)**
HATU	50.7	51.7
HBTU	49.5	49.8
HBPyU	45.3	52.6
HBPipU	45.5	44.1
BOP	47.8	49.6
PyBOP	49.6	49.8
PyBroP	43.9	47.7
BroP	45.7	49.4

Similar tests were also conducted with other bases and two other solid-phase supports; highly crossed-linked aminomethylpolystyrene (PS, 36 μmol/g), and amino-polyethylene glycol/polystyrene (Tentagel, 180 μmol/g). The results (Table 3) confirmed that the above conditions produced good nucleoside loadings with other nucleosides as well as with the PS support. However, very high loadings on the Tentagel resin were not obtained, even with an extended wait during the coupling. This was probably a result of the low reagent concentration and slow diffusion into the Tentagel matrix.

Table 3. Automated Derivatization of CPG, PS, and Tentagel Supports Using HBTU/DMAP.

Nucleoside	Support	Wait Time	Loading (μmol/g)
dC^{Bz}	CPG	0 sec	52.7
dG^{iBu}	CPG	0 sec	47.5
T	CPG	0 sec	45.4
dC^{Bz}	Tentagel	0 sec	32.9
dC^{Bz}	Tentagel	600 sec	82.5
T	Tentagel	0 sec	36.2
T	Tentagel	600 sec	83.1
T	PS	0 sec	30.0

All eight of the coupling reagents tested provided a dramatic improvement in the speed of the derivatization reaction for CPG and PS supports. Although, BOP/HOBT,[13] TBTU/HOBT,[14] HBTU,[15] TBTU,[16] and TOTU[17] have occasionally been used to manually derivatize other solid-phase supports, the remarkable speed of this reaction has not been exploited (0.5-16 h long reactions). With the exception of BOP and BroP, which liberate carcinogenic hexamethylphosphoramide, these stable, nonhygroscopic reagents hold great promise for replacing carbodiimide reagents in either conventional "batch" or automated *in-situ* derivatization reactions. Underivatized amino supports are also superior to the other "universal" supports[13b, 21] which have been developed because they are less expensive and allow faster cleavage, without modifications to deprotection conditions, especially when combined with the QDA linker.

Hopefully, new synthesizers and operating software,[22] which will be able to produce multiple rounds of oligonucleotides without human intervention or preselection of the required supports, will be developed using

this fast derivatization process. The few additional minutes required for the support derivatization step are more than compensated for by the reduced cleavage time, when QDA linkers are used instead of succinyl linkers. Therefore, these two improvements to the support chemistry should enable development of a new generation of automated very high throughput DNA synthesizers.

Acknowledgement. We would like to thank Alex Andrus (PE/ABD) for generous donation of the underivatized PS support and University Technologies International, Inc. (UTI) for intellectual property protection.

Abbreviations used. BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexfluorophosphate; BroP, bromotris(dimethylamino)phosphonium hexafluorophosphate; DEC, 1-(3-dimethylaminopropyl)ethylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; HATU, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HBPipU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-bis(pentamethylene)uronium hexafluorophosphate; HBPyU, *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-bis(tetramethylene)uronium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBT, N-hydroxybenzotriazole; LCAA-CPG, long chain alkylamine controlled pore glass; PE/ABD, Perkin-Elmer Applied Biosystems Division; PyBOP®, benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate; PyBroP, bromotripyrrolidinophosphonium hexafluorophosphate; QDA, hydroquinone-O,O'-diacetic acid; TBTU, 2-(1*H*-benzotrizol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TOTU, *O*-[(ethoxycarbonyl)cyanomethyleneamino]-1,1,3,3-tetramethyluronium tetrafluoroborate.

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19. A "Begin" procedure was created, which: 1, rinsed and flushed the column with acetonitrile and Ar (3x); 2, simultaneously delivered a solution of 6, B= A^{bz}, R =H, and DIEA (1:1, 0.05M) and a solution of coupling reagent (0.05M) to the synthesis column (4.0 sec); 3, flushed and rinsed the column with Ar and acetonitrile (3x); and 4, primed the phosphoramidite and tetrazole lines. The support (~ 12 mg) was accurately weighed into the synthesis column, so nucleoside loadings could be determined from the trityl color released from the first detritylation. Immediately after completion of the "Begin" procedure, an unmodified oligonucleotide synthesis program began.
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22. There are sufficient base positions on an eight port PE/ABD 394 to allow automated derivatization of up to three nucleosides per synthesizer and facilities with at least two synthesizers could use this method, if software to deliver different bases to different columns was available. Otherwise with existing software, each column must begin with the same nucleoside.

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