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Facile Methods to Recycle Nucleosides during Solid Phase Synthesis of Oligonucleotides

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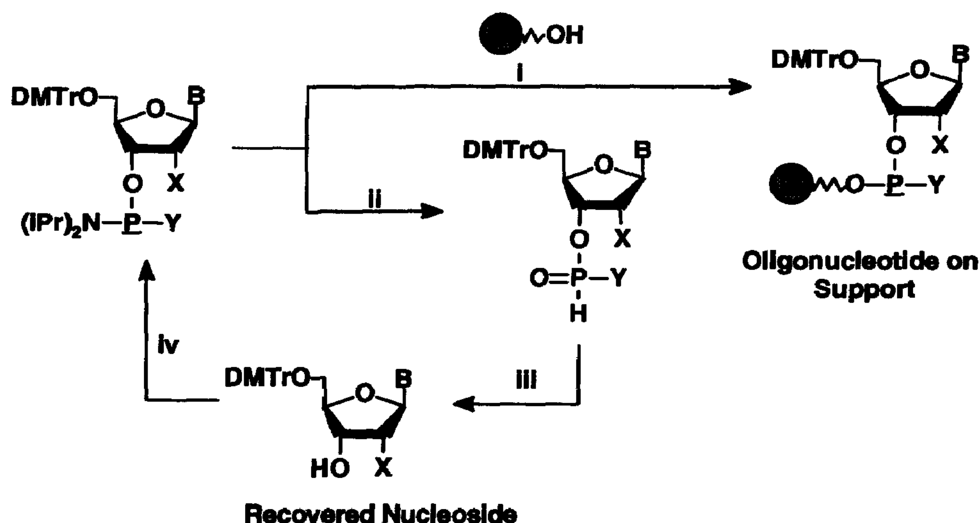
Abstract: Solid phase syntheses of oligonucleotides, using nucleoside phosphoramidites or methylphosphonamidites require a large excess of nucleoside monomers over the hydroxy functions of the growing oligonucleotide chain bound onto the solid phase. The outlined method allows to recover the excess nucleosides. All the protective groups on the sugar and the nucleobase of the monomers are maintained throughout the recycling process.

Automated solid phase synthesis methods for oligonucleotides, employing phosphoramidites[1] or methylphosphonamidites[2] have become the predominant source of synthetic oligonucleotides within recent years. Throughout the synthesis, water either being a contamination of the solvent or being adsorbed to the inner walls of the synthesizer, sequesters the phosphoramidites[3] or methylphosphonamidites of the nucleosides. Therefore, to obtain quantitative coupling yields, DNA-synthesizers generally consume 2.5 to 20 equivalents of nucleoside monomer per coupling reaction with respect to the immobilized coupling component. In multi-g-scale syntheses or, if valuable nucleoside analogs[4] are being used, recycling of the nucleosides is highly desirable.

Here, we report the recovery of fully protected nucleosides from DNA-synthesizer effluents, generally without need for chromatography.[5] Key starting material are the hydrolysates of the nucleoside phosphoramidites[3], or the methylphosphonamidites, the nucleoside 3'-cyanoethyl-H-phosphonates and nucleoside 3'-methyl-H-phosphonites, respectively. They are usually stable for at least three days in a solution of tetrazole in acetonitrile, but are prone to solvolysis in presence of base[6] or *Lewis acids*.[7] The removal of the phosphorus moieties appeared attractive, since it would render nucleosides with free 3'-hydroxy functions, which could readily be reconverted to phosphoramidites or methylphosphonamidites.

Thus, methyl-H-phosphonite residues[8] may selectively be hydrolyzed, mediated by fluoride ions yielding methylphosphinic acid (^{31}P ($CDCl_3$): 18,76 ppm). In turn, imidazole or pyridine-N-oxide, also known to catalyze nucleophilic displacements on pentavalent phosphorus appeared less effective. (Fig.1) In a typical experiment, 10 ml of a 0.01 M solution of TBAF in (THF/H_2O; 2:1, v/v) was added to the hydrolysate to afford conversion to the nucleoside within three hours at r.t.. The progress of the hydrolysis may best be monitored using tlc: (toluene/acetone; 1:1, v/v) and by ^{31}P-NMR with the disappearence of the starting material. After three hours, the reaction is

Fig. 1: Recycling of Nucleosides



I) Tetrazole, acetonitrile; II) Tetrazole, H_2O; III) for Y=Me: TBAF in THF/H_2O 9:1 (9:1, v/v), r.t. 3h ; for Y=OCNE: Imidazole/MeOH r.t. 3 days, or 0.1 M KF/MeOH,r.t. 2 days or 0.2 M KF/MeOH, r.t. 4 h; iv) amidite synthesis.

Table 1

Base:	X:	Y:	Medium:	Yield [%]:
A^{bz}	H	OCNE	imidazole/MeOH	71[a],76[b*]
A^{bz}	H	Me	TBAF/H2O	97
U	OFpmp	OCNE	KF/MeOH[11]	75
T	H	OCNE	imidazole/MeOH	quant[b*]
T	H	Me	TBAF/H2O	73
C^{TBPAC}	H	OCNE	KF/MeOH[10]	72
C^{bz}	OFpmp	OCNE	KF/MeOH[12]	80
C^{bz}	H	OCNE	imidazole/MeOH	95[a*]
C^{bz}	H	Me	TBAF/H2O	81
G^{ib}	H	OCNE	imidazole/MeOH	73[a*]
G^{ib}	H	Me	TBAF/H2O	89

*) Yield was based on the assumption of a quantitative coupling yield and the amidite equivalents given in ref.13, a) and b) refer to the syntheses in ref. 13. CNE: cyanoethyl; Fpmp: N-(2-fluorophenyl)-4-methoxy-piperid-4-yl, TBPAC: 4-tert. butylphenoxyacetyl

quenched using 100 µl of (THF/pyridine/H_2O/I_2; 75:20:2:3, w/w/w/w)[9] to avoid rephosphitylation. The crude material is evaporated to dryness, taken up in 100 ml of ethyl acetate and subjected to

standard aqueous work up of a two fold extraction with saturated aqueous bicarbonate, then with brine. The combined organic phases were dried over sodium sulfate, concentrated to 5 ml and precipitated into 250 ml of n-pentane. Desiccation followed *in vacuo* over P_4O_{10}/KOH. Hydrolytic cleavage of nucleoside 3'-cyanoethyl-H-phosphonates[3b] appeared inconvenient, since both alcohols of the diester could act as leaving groups. Further hydrolysis of the resulting monoesters, one of which a nucleoside 3'-H-phosphonate, would then be difficult in presence of the other protective groups. Our method is a transesterification onto a solvent alcohol under conditions compatible with nucleoside protective groups. As catalysts, we chose bases with a strength ranging from pKa=6.9 to pKa=8.3 e.g. such as imidazole to N-methylmorpholine. Weaker bases caused the reaction to be very sluggish, while bases stronger than N-methylmorpholine lead to an increased formation of nucleoside 3'-H-phosphonate by the β-elimination of the cyanoethyl group.[3a]

Thus, imidazole catalyzed dephosphonylations were allowed to stand for three days at r. t.. The reactions were quenched by adding first 20 ml of water then 2 ml idodine solution. The product was resuspended in ethyl acetate, the imidazole was extracted using citrate buffer pH-3-4 and brine successively. Subsequently, the product was recovered from the organic layer by precipitation into n-pentane.

The fluoride mediated transesterifications are favorable especially for nucleoside analogs with labile protective groups such as the 4-tert.butyl phenoxyacetyl group (TBPAC)[10] and the 4-(2-fluorophenyl) 1-methoxy piperid-1-yl group (Fpmp).[11,12] Thus, the transesterifications were carried out for two days using 0.1 M KF in methanol and quenched with sulfur. The aqueous work up may proceed in either previously described way.

On-line recycling was performed during the synthesis of the oligodeoxyribonucleotide with (5'-AGAGAGAGAGATCG-3'), synthesized using a 8800 DNA synthesizer (Millipore) equipped with a 16-position electric actuator (*Valco*) to divert the reactor effluent into reservoirs for waste solvents, oxidizer, detritylation solutions (trityl yields) and the various nucleosides. Each tank designated for the collection of nucleosides contained 100 ml of the transestereification solution made of 20 g of imidazole per liter of methanol. The DNA-synthesis was then performed using 5.5 eq. of A-, 5.5 eq. of C-, 5 eq. of T- and 8.0 eq. of G-amidite per coupling. The amidite hydrolysate solutions were diverted into the appropriate transesterification solutions and recovered as described before[13].

Other on-line methods for the recovery of nucleosides from their cyanoethyl-H-phosphonates are oxidative work up with sulfur to give phosphorothioates or the β-elimination, favorably accomplished on a weakly basic ion exchanger in anhydrous free base form, to yield nucleoside-3'-H-phosphonates.

We have demonstrated simple high yielding methods for the recovery of fully protected nucleosides from DNA-synthesizer effluents. The possible options of transesterification, oxidative work up and ß-elimination allow to tailor methods to the individual needs of the synthesis facility.

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8) To 0.25 mmol of a nucleoside methylphosphonamidite or cyanoethylphosphoramidite in 5 ml acetonitrile was added 105 mg (1.5 mmol) of tetrazole and 10 µl of distilled water. Immediately the methylphosphinate or the cyanoethyl-H-phosphonate was formed. The obtained solutions are identical to the reactor effluents after internucleotide couplings.
5'-Dimethoxytrityl N^2-isobutyryl deoxyguanosine 3'-methylphosphinate: ^{31}P-NMR ($CDCl_3$): 35.22, 35.97 ppm; 5'-dimethoxytrityl N^4-benzoyl deoxycytosine 3'-methylphosphinate: ^{31}P-NMR ($CDCl_3$): 35.72, 34.94 ppm; 5'-dimethoxytrityl N^6-benzoyl deoxyadenosine 3'-methylphosphinate: ^{31}P-NMR ($CDCl_3$): 35.52, 34.73 ppm; 5'-dimethoxytrityl thymidine 3'-methylphosphinate: 35.62, 34.88 ppm.

9) In case of labile analogs, the transesterification may be quenched with sulfur instead of with aqueous iodine/pyridine systems.

10) **5'-Dimethoxytrityl-N^4- (4-tert. butyl phenoxyacetyl) deoxycytidine from the phosphoroamidite by *Millipore:*** ^{1}H-NMR ($CDCl_3$) [ppm]: 9.05: NH; 8.25: cytosine; 7.36-7.12: 9 H of DMTr, 2H of 4-tert. butyl phenoxyacetyl, 1H of cytosine; 6.83-6.77: 2H of DMTr, 2H of 4-tert. butyl phenoxyacetyl; 6.2: 1'; 4.52: 4-tert. butyl phenoxyacetyl, 4.45: 3'; 4.08: 4'; 3.72: DMTr; 3.4: 5'; 2.66, 2.23: 2'; 1.23: 4-tert. butyl phenoxyacetyl.

11) **5'-Dimethoxytrityl 2'-Fpmp uridine from the phosphoroamidite by *Cruachem*:** ^{1}H-NMR ($CDCl_3$) [ppm]: 7.75: uracil; 7.45-7.15: DMTr; 7.1-6.75: m, 4H of Fpmp, 4H of DMTr; 6,15: 1'; 5,2: uracil; 4.74, 4.33, 4,15: m, ribose; 3.76: DMTr; 3.38: m, 2H, 5'; 3.12: s, 3H, CH_3 Fpmp; 3.2-2.8, 4H, Fpmp; 2.15-1.8: m, 4H, Fpmp.

12) **5'-Dimethoxytrityl 2'-Fpmp N^4-benzoyl cytidine from the phosphoroamidite by *Cruachem*:** ^{1}H-NMR ($CDCl_3$) [ppm]: 8.28: cytosine; 7.82: benzoyl; 7.6-7.23: Bz, DMTr, cytosine; 7.03-6.77: 4H, DMTr, 4H, Fpmp; 4.66: 1'; 4.38, 4.17: ribose, 3.74: DMTr; 3.48: 5'; 3.25-2.85: s, 3H, CH3, Fpmp, m, 4H, Fpmp; 2.18-1.95: m, 4H, Fpmp.

13) The following quantities of nucleosides were isolated from a synthesis using a) 2.12g b) 1g of CPG-support with a loading of 38 µmol/g (Millipore).: 5'-dimethoxytrityl N^6-benzoyl deoxyadenosine: a) 1.42g, b) 0.38g; 5'-dimethoxytrityl N^2-isobutytyl deoxyguanosine: a) 1.8g; 5- 5'-dimethoxttrityl-N^4-benzoyl deoxycytidine: a) 0.14g ; 5'-dimethoxytrityl thymidine: b) 0.13g.

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