AUTOMATED CHLORIDITE AND AMIDITE SYNTHESIS OF OLIGODEOXYRIBONUCLEOTIDES ON A LONG CHAIN SUPPORT USING AMIDINE PROTECTED PURINE NUCLEOSIDES*

Luboš ARNOLD[a], Zdeněk TOČÍK[a], Eliška BRADKOVÁ[a],
Zdeněk HOSTOMSKÝ[b], Václav PAČES[b] and Jiří SMRT[a]

[a] *Institute of Organic Chemistry and Biochemistry and*
[b] *Institute of Molecular Genetics, Czechoslovak Academy of Sciences, 166 10 Prague 6*

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Preparation of solid supports with long aliphatic spacer for the oligonucleotide synthesis is described. Automated synthesis of oligodeoxyribonucleotides using in situ prepared methylchlorophosphite intermediates from amidine protected purine nucleosides is described. Phosphoramidite synthesis of oligodeoxyribonucleotides mediated by 1-methylimidazole trifluoromethanesulfonate is described. Novel isolation of crude oligonucleotides by ethanol precipitation of potassium salts is described.

In the last paper from our Laboratory[1] a modified methylchlorophosphite method for the semi-automated synthesis of oligodeoxyribonucleotides was described. With aminopropylated silica gel[2] used as insoluble support in that paper consistent 67% yields of the first condensation step were observed. This support-induced steric effect could be overcome by introduction of a long alkylamine spacer[3]. We were therefore trying to improve the yield of the first condensation step by introduction of an aliphatic amide or ester group containing spacers by successive addition of succinic anhydride and 1,3-diaminopropane or 1,10-decanediol to aminopropylated support. (Analogous method has been recently described[4].) With supports of this type we were able to obtain better results in the first condensation step (80 – 85%) than with supports without the spacer but the condensation reaction still failed to consume completely (or nearly competely) the supported nucleoside. This phenomenon might be explained by lower accessibility of a part of the supported nucleoside. We have found by titration with perchloric acid in acetic acid that the silica gel or CPG (Controlled Pore Glass) supports of 500 Å* porosity, prepared by aminopropylation in aqueous ethanol[5], contain 160 – 170 μmol of amino functions and supports of 1 000 Å porosity 80 μmol of amino functions per 1 g. About one tenth of these amino

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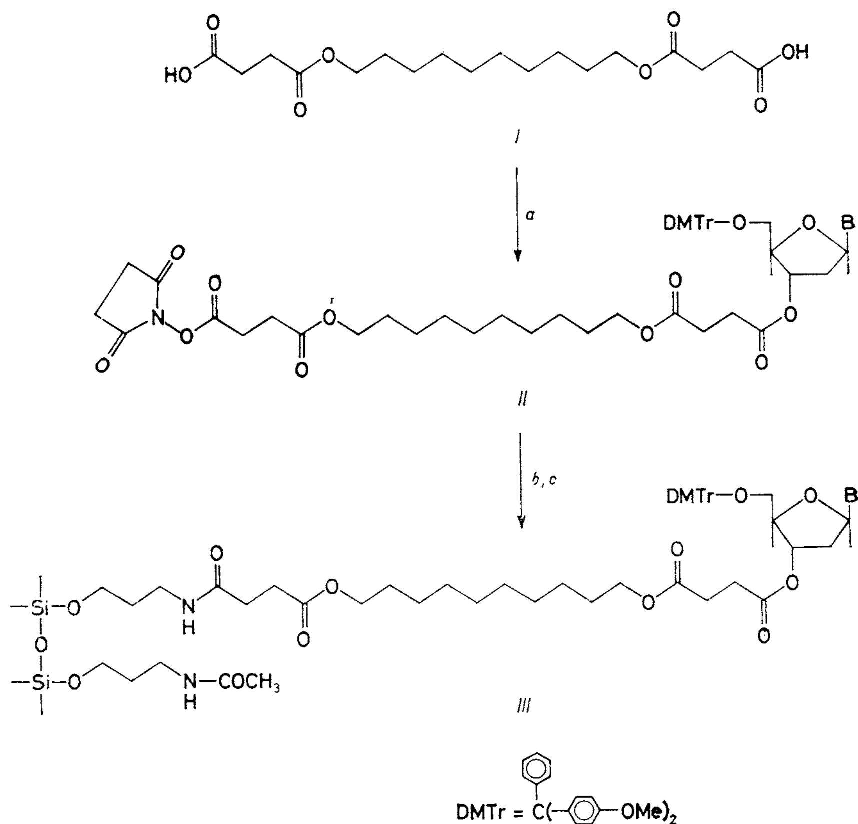
** 1 Å = 10^{-10} m.

groups are functionalized by bulky dimethoxytritylnucleoside derivative. On the other hand, the small molecules used during the successive elongation process had to cover the surface of the support more efficiently than the nucleoside derivative which resulted in a population of shorter and longer spacer chains. A part of these chains was functionalized by the nucleoside derivative at the end of the process and the rest of unfunctionalized capped chains may interfere in the first condensation step. Thus, we designed and prepared (Scheme 1) functionalized solid supports bearing a nucleoside attached over a long aliphatic chain to the matrix which is covered with short acetylated aminopropyl groups. The idea was to prepare a derivative of 5'-O-dimethoxytrityl-(N-protected)-2'-deoxyribonucleoside esterified on 3'-hydroxyl group with a long chain α,ω-dicarboxylic acid. The second carboxyl



SCHEME 1

a) N-Hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, 5'-O-dimethoxytrityl-(N-protected)-2'-deoxyribonucleoside, 4-dimethylaminopyridine; *b*) aminopropyl-trimethylsilyl CPG or silica gel; *c*) acetic anhydride, 4-dimethylaminopyridine

function should bear an activated ester. *p*-Nitrophenyl ester, generally used for functionalization of supports, could be prepared in case of dT and N-acyl protected nucleosides. In the presence of basic amidine protecting groups which are used in our Laboratory for dA and dG, *p*-nitrophenyl esters are not formed. Therefore, active esters with N-hydroxysuccinimide were selected. Reaction of aminopropylation support with that bulky nucleoside derivative would lead to functionalization of the best accessible amino groups on the matrix surface.

Every long chain α,ω-dicarboxylic acid could be used for our purpose. We have prepared a dicarboxylic acid containing two ester groups (*I*) by reaction of 1,10-decanediol with excess of succinic anhydride in the presence of 4-dimethylaminopyridine. A solution of this dicarboxylic acid (1 eq.) and 5'-O-dimethoxytrityl-(N-protected)-2'-deoxyribonucleoside (1·2 eq.) in pyridine was treated with N-hydroxysuccinimide (2·5 eq.), N,N'-dicyclohexylcarbodiimide (5 eq.) and 4-dimethylaminopyridine (2·5 eq.) for several days. N-Hydroxysuccinimide ester *II* was formed accompanied by bis-nucleoside derivative. After the removal of N,N'-dicyclohexylurea, this solution was used for functionalization of aminopropylated CPG and/or silica gel, suspended and degased in N,N-dimethylformamide. The washed material was treated with acetic anhydride and 4-dimethylaminopyridine (DMAP) to cap the remaining amino groups. The loading of CPG-500 Å amounted to 14 – 16 μmol/1 g, that of Fractosil-1 000 Å to 6 – 10 μmol/1 g. The yields of the first step in automated oligonucleotide synthesis carried out on these supports were satisfactory affording 90 – 95% by the chlorophosphite method and 95 – 99% by the phosphoramidite method.

In the semi-automated modified methylchlorophosphite synthesis[1] we prepared 0·04 mol l^{-1} solutions of 5'-O-dimethoxytrityl-(N-protected)-2'-deoxyribonucleoside-3'-methylchlorophosphites in a syringe 10 min before the condensation step. The reason for this short reaction time was the observed low stability of N^2-isobutyryl-dG derivative. The utilization of the modified chlorophosphite method in a fully automated flow-through DNA-synthesizer (Syngen-1, constructed in this Institute) required stable stock solutions of active intermediates including the dG derivative. Operating on the assumption that the instability of the N^2-isobutyryl-dG methylchlorophosphite solution was caused by phosphitylation of O-6 on guanine ring, we tried to change the electron structure of guanine ring by replacement of the acyl protecting group by dimethylaminomethylene (amidine) group. By mixing equal volumes of 0·06 mol l^{-1} solution of 5'-O-dimethoxytrityl-N^2-dimethylaminomethylene-2'-deoxyguanosine in 0·5 mol l^{-1} 2,4,6-collidine in 1,2-dichloroethane and 0·05 mol l^{-1} solution of methyl phosphodichloridite in 1,2-dichloroethane we prepared the nucleoside methyl chlorophosphite. The solution was allowed to stand for 6 h to check its stability and then an automated synthesis of d-GGGGGT was carried out according to the reaction cycle Table I. The yields (dimethoxytrityl determination) were (%): 90, 99, 99, 98, 98.

The product of the synthesis was deblocked and removed from the support by means of pyridine–conc. ammonia mixture[6,7] and purified by preparative TLC[1]. HPLC of the isolated oligonucleotide showed one peak. The oligonucleotide was further treated successively by snake venom exonuclease and bacterial phosphatase (simultaneous treatment with both enzymes did not degrade the compound because of the presence of ammonium sulfate in phosphatase causing the aggregation of dG chains). The products of the degradation were separated by TLC and the UV absorbing bands characterized by UV spectra. The degradation mixture was also separated and characterized by HPLC. Both methods did not show any modified nucleosides and the method used for the synthesis was therefore considered to be safe. Analogously, d-T_6, d-C_5T and d-A_5T were synthesized and characterized (data not shown). Satisfactory results of these experiments showed that even longer chains could be produced by automated chlorophosphite synthesis. For the construction

TABLE I

Reaction cycle using modified chlorophosphite method (100 mg of Fractosil-1 000 with an aliphatic arm loaded with 0·7 μmol of nucleoside was used)

Step	Operation	Solvent/Reagent	Time s	Flow rate ml/min
1	Washing	nitromethane	90	3
2	Detritylation	3% trichloroacetic acid in 1,2-dichloroethane	70	3
3	Washing	nitromethane	50	3
4	Washing	acetonitrile	60	3
5	Condensation	nucleoside-chlorophosphite in 1,2-dichloroethane[a]	25	3
6	Condensation	nucleoside-chlorophosphite in 1,2-dichloroethane[a]	240	0·3
7	Washing	acetonitrile	100	0·3
8	Oxidation	0·1M-I_2 in THF-2,6--lutidine-water (7 : 1 : 2)	40	3
9	Washing	acetonitrile	20	3
10	Capping	0·8M-Ac_2O/0·25M DMAP in THF	30	3

[a] Reagents were prepared by mixing equal volumes of 0·06M solution of 5'-dimethoxytrityl--(N-protected)-2'-deoxyribonucleoside in 0·5M 2,4,6-collidine in 1,2-dichloroethane and 0·05M solution of $MeOPCl_2$ in 1,2-dichloroethane at room temperature under argon 1 h before starting the synthesis. N-Protection: Benzoyl (dC), dimethylaminoethylidene (dA), dimethylamino-methylene (dG). After the last cycle, steps 1—3 were performed.

of cow colostrum trypsin inhibitor gene[8] five oligonucleotides (1–5, Table II) 30 bases long were synthesized according to the reaction cycle Table I. Overall yields calculated comparing the dimethoxytrityl (DMTr) levels at 498 nm for the first and final cycles amounted to 20–50% showing step-wise coupling efficiency of 95–96%. After the standard deprotection with thiophenol and ammonia[16] the crude products were isolated by a novel method. The concentrated solutions were precipitated with ethanol in the presence of potassium acetate. The pellets after centrifugation were washed with ethanol, dissolved in water at 80°C, insoluble silica centrifuged off and the supernatants (30–60A_{260} units) lyophilized affording crude oligonucleotides (1·2–2·5 mg). Pure oligonucleotides were prepared by electrophoresis on 16% polyacrylamide gel under denaturing conditions. The chloridite method of oligonucleotide synthesis has an advantage of easily accessible reaction components. The lower coupling efficiency, however, allows its utilization for the synthesis of shorter chains.

Better performance along with the possibility to obtain longer chains was expected with the 2-cyanoethyl phosphoramidite chemistry[9], considered recently the most advanced chemistry for DNA synthesis. For this approach, synthons were prepared according to the published procedures[10]. For dA amidine protected derivative was used. In order to ensure a short condensation time we considered to use a more

TABLE II

Catalogue of oligonucleotides prepared by methylphosphochloridite (*1*–*5*) and phosphoramidite (*6*–*17*) method

(*1*)	AGCTTAAGGAGGTGAGCTCATGTTCCAGAAA
(*2*)	CCGCCGGACCTGTGCCAGCTGCCGCAMGCT
(*3*)	CGTGGTCCGTGCAAAGCTGCTCTGCTGCGT
(*4*)	TACTTCTACAACTCTACCTCTAACGCTTGC
(*5*)	GAACCGTTCACCTACGGTGGTTGCCAGGGT
(*6*)	AACAACAACAACTTCGAAACCACCGAAATG
(*7*)	TGCCTGCGTATCTGCGAACCGCCGCAGCAG
(*8*)	ACCGACAAAAGCCCGGGA
(*9*)	GCACAGGTCCGGCGGTTTCTGGAACATGAGCTCACCTCCTTA
(*10*)	TTTGCACGGACCACGAGCCTGCGGCAGCTG
(*11*)	AGAGTTGTAGAAGTAACGCAGCAGAGCAGC
(*12*)	GTAGGTGAACGGTTCGCAAGCGTTAGAGGT
(*13*)	GAAGTTGTTGTTGTTACCCTGGCAACCACC
(*14*)	GCAGATACGCAGGCACATTTCGGTGGTTTC
(*15*)	AGCTTCCCGGGCTTTTGTCGGTCTGCTGCGGCGGTTC
(*16*)	CGTGGTCCGTGCGTTGCTGCTCTGCTGCTG
(*17*)	AACGCACGGACCACGAGCCTGCGGCAGCTG

active mediator than the classical 1*H*-tetrazole. Recent information indicated 5-(4--nitrophenyl)-1*H*-tetrazole[10], 5-trifluoromethyl-1*H*-tetrazole and 1-methylimidazole hydrochloride[11] as possible candidates. Though the combination of the last two seemed to be acceptable (data not shown), both substances are highly hygroscopic and therefore not easy to handle. From the other salts of 1-methylimidazole with strong acids, the perchlorate and trifluoromethanesulfonate were found to be absolutely non-hygroscopic and possessed excellent solubility in acetonitrile. Both these neutral salts are able to mediate phosphoramidite condensation in less than 2 min reaction time (TLC). Automated test synthesis of short oligonucleotidic chains indicated better performane (over 99% per step) with 1-methylimidazole trifluoromethanesulfonate. This mediator was then used in synthesis of oligonucleotides of 30–48 nucleotides long (6–17, Table II), according to the reaction cycle Table III. Overall yields calculated comparing the DMTr levels at 498 nm for the first and final cycles amounted to 60–90% showing step-wise coupling efficiency over 98%.

TABLE III

Reaction cycle using 2-cyanoethyl-N,N-diisopropylphosphoramidite approach mediated by 1-methylimidazole trifluoromethanesulfonate (100 mg of Fractosil-1 000 with aliphatic arm loaded with 0·7 µmol of nucleoside was used)

Step	Operation	Solvent/Reagent	Time s	Flow rate ml/min
1	Washing	nitromethane	70	4
2	Detritylation	3% TCA in 1,2-dichloroethane	70	4
3	Sashing	nitromethane	35	4
4	Washing	acetonitrile	70	4
5	Activation	0·3M phosphoramidite in acetonitrile	2	4
6	Activation	1M mediator in acetonitrile	2	4
7	Activation	repeat steps 5 and 6 three times		
8	Transfer to the support	acetonitrile	21	4
9	Condensation	acetonitrile	200	0·4
10	Oxidation	0·1M-I_2 in THF–2,6-lutidine–water (7 : 1 : 2)	40	4
11	Washing	acetonitrile	15	4
12	Capping	0·25M Ac_2O/0·25M DMAP in THF	35	4

After the last elongation cycle steps 1—3 were repeated. N-Protection: Benzoyl (dC), dimethylaminoethylidene (dA), isobutyryl (dG).

The products were isolated and purified by the same procedures as in chloridite synthesis. The sequencing was carried out on the whole gene assembled from oligonucleotides[8].

EXPERIMENTAL

Thin-layer chromatography (TLC) was performed on ready-for-use Silufol UV_{254} silica gel foils (Kavalier Glasworks, Votice) in solvent system S, chloroform–methanol (9 : 1). Column chromatography was performed on macroporous silica gel (Service Laboratory of the Institute).

Acetonitrile (HPLC, Fluka) was distilled from P_2O_5. Nitromethane (Fluka) was stored over 4 Å molecular sieves for 10 days, filtered through a column of aluminium oxide (activated, basic) and distilled. 1,2-Dichloroethane was distilled from P_2O_5, stored over molecular sieves and filtered before use (G_3). Dichloromethane was filtered through a column of aluminum oxide (activated, basic).

CPG 500 (Serva) and Fractosil 1 000 (Merck) were used. 2'-Deoxynucleosides were products of Pharma-Waldhof. 5'-O-Dimethoxytrityl derivatives of dT and N^4-benzoyl-dC were prepared according to the described procedures[12,13]. Protected 2'-deoxynucleoside-3'-(2-cyanoethyl)-N,N-diisopropylphosphoramidites were prepared according to the described procedure[9], except for dichloromethane being used instead of tetrahydrofuran for the phosphitylation.

5'-O-Dimethoxytrityl-N^6-N,N-dimethylaminoethylidene-2'-deoxyadenosine

The title compound was prepared according to ref.[14] by the following modified procedure. 2'-Deoxyadenosine (5·02 g; 20 mmol) was successively evaporated with pyridine (100 ml) and toluene (50 ml), suspended in a mixture of methanol (150 ml) and N,N-dimethylacetamide dimethyl acetal (10 ml, containing 10% methanol) and the mixture stirred till disappearance of the starting material. After 70 h (R_F-S 0·05 → 0·15), water (4 ml) was added and the volatile material removed in vacuo (13 Pa, 25°C, rotatory evaporator with dry ice condenser). The residue was evaporated with pyridine (2 × 20 ml) and dissolved in pyridine (150 ml). Dimethoxytrityl chloride (7·45 g; 22 mmol) was added, the mixture briefly shaken and then allowed to stand overnight. R_F-S 0·15 → 0·33. Methanol (5 ml) was added and, after 10 min, the mixture partitioned between 5% aqueous potassium hydrogen carbonate (300 ml) and chloroform (400 ml). The chloroform layer was extracted with water (100 ml), dried (Na_2SO_4) and evaporated. The residue was evaporated with two 50 ml portions of toluene, and dissolved in chloroform (40 ml). The solution was added dropwise to a stirred mixture of diethylether (100 ml) and petroleum ether (100 ml). The supernatant was discarded and the residue chromatographed on a silica gel column (250 g) using the following solvent systems: chloroform–triethylamine (99 : 1; 300 ml), chloroform–methanol–triethylamine (98 : 1 : 1; 300 ml), chloroform–methanol–triethylamine (97 : 2 : 1; 300 ml) and chloroform–methanol–triethylamine (96 : 3 : 1; 300 ml). Appropriate fractions R_F-S 0·33 were evaporated, the residue was dissolved in benzene (100 ml) and the solution lyophilized. Yield, 10·58 g; (85%).

5'-O-Dimethoxytrityl-N^2-N,N-dimethylaminomethylene-2'-deoxyguanosine

The title compound was prepared according to ref.[15] by the following modified procedure. 2'-Deoxyguanosine (2·67 g; 10 mmol) was evaporated with two 80 ml portions of pyridine and with toluene (50 ml), suspended in a mixture of N,N-dimethylformamide (45 ml) and N,N-dimethylformamide dimethyl acetal (4 ml; 37 mmol) and the mixture stirred for 18 h. R_F-S 0·03 →

→ 0·1. The resulting solution (or crystalline slurry) was evaporated at 13 Pa/25°C (rotatory evaporator equipped with dry ice condenser) and the residue evaporated with pyridine. Pyridine (50 ml) and dimethoxytrityl chloride (3·73 g; 11 mmol) were added, the mixture shaken for 30 min and then allowed to stand overnight. R_F-S 0·10 → 0·2. The mixture was diluted with ethanol (50 ml) and partitioned between chloroform (150 ml) and water (50 ml). The chloroform layer was washed with water, diluted with toluene (100 ml) and evaporated. The residue was evaporated with toluene (50 ml), dissolved in a mixture of chloroform (32 ml) and ethanol (8 ml) and the solution injected into vigorously stirred ether (450 ml). The solid was collected, washed with ether (4 × 50 ml) and dried under diminished pressure. Yield 5·75 g (92%). The product contains traces of 3′,5′-bis-dimethoxytrityl derivative (R_F-S 0·45) and dimethoxytrityl alcohol (R_F-S 0·80) which do not interfere with its use in preparation of phosphorochloridate.

1,10-Decanediol bis-succinate (*I*)

A suspension of 1,10-decanediol (17·4 g; 100 mmol), 4-dimethylaminopyridine (1·22 g; 10 mmol) and succinic anhydride (30 g; 300 mmol) in pyridine (150 ml) was stirred for 2 h and the clear solution kept at room tempearture for 5 days. The solvent was evaporated, the residue acidified with conc. aqueous hydrochloric acid, cooled down and extracted with chloroform (150 ml). The chloroform extract was washed with water (100 ml), dried ($MgSO_4$) and evaporated. The crystalline residue was triturated with cyclohexane at 90°C, the suspension cooled down, the solid collected and washed with cyclohexane and finally with petroleum ether and dried under diminished pressure. Yield, 35·5 g (95%); m.p. 94–95°C. For $C_{18}H_{30}O_8$ (374·4) calculated: 57·74% C, 8·08% H; found: 57·56% C, 7·94% H.

Preparation of Solid Supports Bearing 5′-O-Dimethoxytrityl--(N-protected)-2′-Deoxyribonucleosides Attached over an Aliphatic Arm (*III*)

A solution of N-hydroxysuccinimide (1·15 g; 10 mmol), N,N′-dicyclohexylcarbodiimide (4·12 g; 20 mmol) and 4-dimethylaminopyridine (1·22 g; 10 mmol) and methanol (80 µl; 2 mmol) in pyridine (20 ml) was kept for 20 h at 20°C to remove traces of succinic anhydride. N,N′-Dicyclohexylurea was filtered off and washed with pyridine (10 ml). The combined filtrates were evaporated (13 Pa) and the residue evaporated with two 10 ml portions of pyridine. The residue was dissolved in pyridine (15 ml) and the solution divided to four parts. Every part was placed into one of four 10 ml centrifugation tubes, each of them containing different 5′-O-dimethoxytrityl-(N-protected)--2′-deoxyribonucleoside (1·2 mmol) and the compound *I* (1 mmol) in pyridine (3 ml). The stoppered tubes were shaken for 0·5 h and then kept at room temperature for 4–5 days. TLC showed quantitative transformation of the protected nucleoside derivatives in N-hydroxysuccinimide esters *II* (R_F-S 0·90, pyrimidines; and 0·86, purines). N,N′-Dicyclohexylurea was centrifuged off and the supernatants added to suspensions of aminopropyltrimethylsilyl[5] CPG 10, 500 Å (4 g) in N,N-dimethylformamide (12 ml), degased previously at 130 Pa. The mixtures were shaken tor 20 h, the supports collected, washed with three 30 ml portions of N,N-dimethylformamide, fhree 30 ml portions of pyridine, five 30 ml portions of methanol and five 30 ml portions of tetrahydrofuran. To the wet supports tetrahydrofuran (15 ml), 4-dimethylaminopyridine (915 mg 7·7 mmol) and acetic anhydride (1·4 ml; 15 mmol) were added, the suspensions shaken for 1 h, the supports collected, washed with six 30 ml portions of methanol, three 30 ml portions of ether and dried under diminished pressure to afford functionalized CPG (*III*). For 1 g CPG 500 Å contained 16·2 µmol dT, 14·2 µmol dC, 15·5 µmol dA and 15·5 µmol dG. Using the same procedure aminopropyltrimethylsilyl Fractosil-1 000 was functionalized to 7·2 µmol (dT, dC), 7·15 µmol (dA) and 6 µmol (dG) for 1 g.

1-Methylimidazole Trifluoromethanesulfonate

A solution of 1-methylimidazole (7·948 ml; 100 mmol) in acetonitrile (30 ml) was cooled down in ethanol–dry ice mixture. To the half-solidified mixture trifluoromethanesulfonic acid (8·787 ml; 100 mmol) was added, the mixture briefly shaken and evaporated (130 Pa). The residue was evaporated with two 5 ml portions of toluene and then heated in a 140°C warm oil bath in vacuo 13 Pa for 2 h. After cooling down the crystalline solid (m.p. 102—5°) was dissolved in acetonitrile (100 ml) to afford the mediator solution.

1-Methylimidazole Perchlorate

A solution of 1-methylimidazole (795 μl; 10 mmol) in aqueous 1·163 mol l^{-1} perchloric acid (8·6 ml) was evaporated and the residue evaporated with ethanol (30 ml). The residue was dissolved in ethanol at 70°C and the solution kept at 0°C for 20 h. The substance was collected, washed with ethanol (20 ml; 0°C) and dried under diminshed pressure at 50°C. Yield, 1·3 g (71%), m.p. 154—157°C.

Isolation of Potassium Salts of Crude Oligonucleotides

After completion of the automated synthesis, the methyl[16] or 2-cyanoethyl[9] and N-protecting groups[16] were removed, the support filtered off and washed with two 0·5 ml portions of conc. aqueous ammonia. The filtrate was heated in an open flask to 50°C for 30 min to remove excess of ammonia, ethanolic solution of 1M potassium acetate (0·4 ml) added, the solution cooled in ice and then the rest of ammonia removed on a rotatory evaporator by rotating at 130 Pa without external heating. After 30 min the heating bath (25—30°C) was applied and the solution concentrated to about 1 ml volume. The solution was transferred to a 10 ml centrifugation tube, diluted with ethanol (8 ml) and stored at —20°C for 18 h. The solid was centrifuged off and washed with three 8 ml portions of ethanol. Sterile water (2·5 ml) was added, the tube immersed in 90°C warm water bath for 5 min. The pellet dissolves under these conditions. (In cases of guanine-rich oligonucleotides conc. aqueous ammonia (20 μl) should be added.) The turbid mixture was centrifuged and the supernatant checked for A_{260} and lyophilized. About 1 mg of solid material was recovered for every 25 A_{260} units.

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