ONE STEP INTRODUCTION OF THE ACTIVATED CARBOXYL GROUPS WITH LONG ALIPHATIC ARM ONTO HYDROXYL GROUPS CONTAINING POLYMERS

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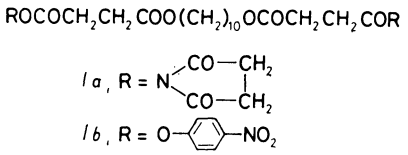
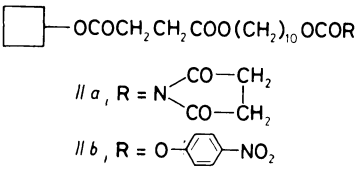
Bis-N-hydroxysuccinimide ester of 1,10-decanediol bis-succinate (*Ia*) reacts with hydroxyl groups bearing polymers (Spheron, cellulose, Sephacryl) affording activated carboxyl group containing derivatives *IIa*. Analogous 4-nitrophenyl group bearing Spheron derivative *IIb* was prepared starting from bis-4-nitrophenyl ester of 1,10-decanediol bis-succinate (*Ib*). 3′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-deoxythymidine 5′-(6-amino-1-hexyl)-phosphate (*V*) was developed as reagent to determine the extent of activated carboxyl groups. Attachment ranging from 3·1—7·3 μmol/g was achieved.

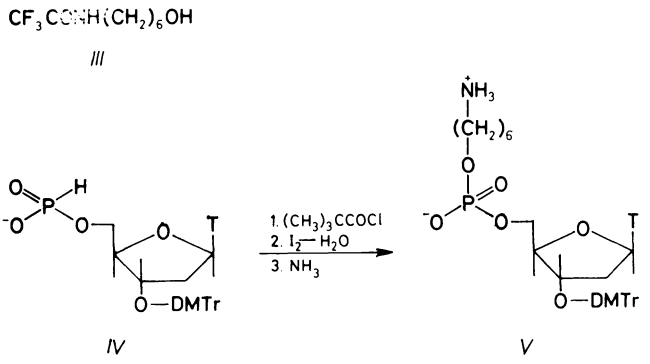
Immobilized DNA fragments have found broad application in biochemistry. Non-specific binding of polynucleotides onto nitrocellulose[1,2] or nylon membranes has been used extensively. Certain level of release from these supports were observed[3]. Covalent binding of DNA onto diazotized cellulose[3,4] or cyanogen bromide activated agarose[5,6] through the heterocyclic amino groups may affect the hybridization properties of the chain. From the chemical point of view, the only acceptable concept for a DNA fragment immobilization is single point covalent end-attachment[7–9]. This concept has been broadly discussed by Ghosh and Musso[8], who were studying the chemistry of covalent coupling of oligonucleotides to solid supports. They came to the conclusion that predominantly end-attachment can be achieved by the use of N-hydroxysuccinimide-activated esters, formed by treatment of vacuum-dried Sephacryl-carboxyl or CPG-carboxyl with N-hydroxysuccinimide and N,N′-dicyclohexylcarbodiimide. Single-point covalent end-attachment of DNA fragments onto hydrophilic supports via spacer arms might result in the extension of the oligonucleotide into the medium, which thereby approximates the conditions of interreactions in solution. In this paper we describe the direct introduction of N-hydroxysuccinimide-activated esters via spacer arm onto hydroxyl groups containing supports. This introduction* followed a method used previously for functionalization of

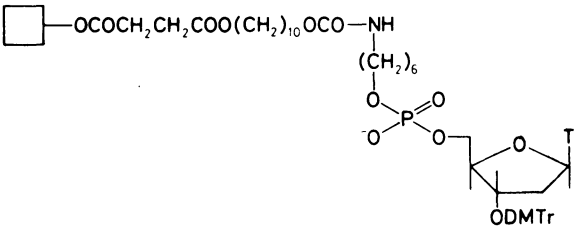
* Experiments using Fractosil-1000 bearing oligo-T, which has been synthesized on this support, were not successful due to the unappropriate properties of silica matrix for biochemical interreactions[11].

supports for oligonucleotide synthesis[10]. Bis-N-hydroxysuccinimide esters of 1,10-decanediol bis-succinate *Ia* reacted with cellulose, Spheron-1000 (glycol-methacrylate macroreticular gel), Sephacryl-500 and Sephacryl-1000 in the presence of N,N'-dicyclohexylcarbodiimide and 4-dimethylaminopyridine. With Spheron-1000 4-nitrophenyl ester (*Ib*) was used. The active esters obtained (*IIa*, *IIb*) were stored over silica gel blue and were found stable over a period of six months.









DMTr = 4,4'-dimethoxytriphenylmethyl

For colorimetric determination of the content of active ester groups on the support, a reagent, 3'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxythymidine 5'-(6-amino-1-hexyl)-phosphate (*V*) was developed. This compound was synthesized by the reaction of 3'-O-(4,4'-dimethoxytriphenylmethyl)-2'-deoxythymidine 5'-H-phosphonate (*IV*) with 6-trifluoroacetamido-1-hexanol[12] (*III*) in the presence of pivaloyl chloride and iodine oxidation according to Garegg et al.[13]. The reagent *V* should react with the active esters bound to the support with the formation of amide bond containing product *VI*. The structure of the reagent was designed analogously to 5'-(6-amino-1--hexyl) phosphoryl derivatives of oligonucleotides.

As a solvent for determination of active ester loading of the supports, 50% aqueous formamide was selected in view of advantageous use of the same medium for oligonucleotide binding. Degased suspensions of the support *IIa* or *IIb* were reacted with compound *V* and triethylamine for two hours, the centrifuged pellet washed with 50% aqueous ethanol and the content of 4,4'-dimethoxytriphenylmethyl groups determined spectrophotometrically. In comparison with Ghosh[8] relatively high loading of active ester groups on support was observed, with cellulose 3·3 μmol/g, Spheron-1000 3·5 μmol/g, Sephacryl-500 3·24 μmol/g, Sephacryl-1000 4·72 μmol/g.

EXPERIMENTAL

Thin-layer chromatography (TLC) was performed on Silufol UV_{254} silica gel foils (Kavalier, Votice) in solvent systems S_1 (chloroform–methanol 8 : 2), S_2 (chloroform–methanol 9 : 1), S_3 (chloroform–methanol 7 : 3). Column chromatography was performed on Kieselgel 60 for flash-chromatography (Fluka).

Acetonitrile (HPLC, Fluka) and 1,2-dichloroethane (puriss. p.a., Fluka) were filtered through a column of aluminium oxide (activated, basic). Pyridine (puriss. p.a., Fluka) was refluxed over P_2O_5 for 8 h, distilled and filtered through aluminium oxide basic. Reagent grade formamide, pivaloyl chloride and phosphorus trichloride were distilled. Imidazole, dichloromethane and triethylamine puriss., p.a., (Fluka) were used directly. 4,4'-Dimethoxytriphenylmethyl chloride was prepared according to the published procedure[14]. Cellulose powder (Whatman, standard grade) and Spheron-1000 (Lachema, Brno) were dried at 20°C (1·3 Pa) for 6 h. Sephacryl was dried according to published procedure[8].

Preparation of Supports *IIa* and *IIb* with Long Spacer Containing Activated Carboxyl Groups

The suspension of the dry support (Spheron, Sephacryl, cellulose; 2 g) in N,N-dimethylformamide (15 ml) was degased by application of water aspirator vacuum, N-hydroxysuccinimide (0·40 g, 3·5 mmol), 4-dimethylaminopyridine (0·40 g, 3·3 mmol) and 1,10-decanediol bis-succinate[10] (0·37 g, 1 mmol) were added and the mixture shaken for 5 min. N,N'-Dicyclohexylcarbodiimide (1·50 g, 7·3 mmol) was then added and shaking was continued for 48 h. The solid was coolected, washed with N,N-dimethylformamide (4 % 20 ml), then with 1,2-dichloroethane (4 % 20 ml) and dried under diminished pressure to the constant weight. The products were stored in closed vials over silica gel blue (Fluka) at 4°C.

Spheron containing 4-nitrophenyl ester *IIb* was prepared analogously using 4-nitrophenol (0·40 g, 2·9 mmol).

3′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-deoxythymidine
5′-H-Phosphonate (Triethylammonium Salt) (*IV*)

The stirred solution of imidazole (3·89 g, 57 mmol) in dichloromethane (150 ml) was cooled down in ice bath, phosphorus trichloride (1·51 ml, 17·3 mmol) and triethylamine (8·43 ml, 60·5 mmol) were successively added and after 15 min stirring a solution of 3′-O-(4,4′-dimethoxytriphenylmethyl)-2′-deoxythymidine[15] (2·18 g, 4 mmol) in dichloromethane (60 ml) was added dropwise over a period of 30 min. TLC S_1, R_F 0·63 → 0·33. Water (25 ml) was added, the mixture evaporated, the oily residue dissolved in chloroform (100 ml) containing triethylamine (0·1 ml) and extracted with water (3 × 50 ml). The chloroform solution was dried over magnesium sulfate and evaporated. The residue was dissolved in chloroform (15 ml) and the solution added into stirred diethylether (750 ml). The solid was collected, washed with diethylether and dried under diminished pressure. Yield 2·4 g (84%). TLC S_1, R_F 0·33.

3′-O-(4,4′-Dimethoxytriphenylmethyl)-2′-deoxythymidine 5′-(6-Amino-1-hexyl)-phosphate (*V*)

Pivaloyl chloride (0·62 ml, 5 mmol) was added to the solution of 6-trifluoroacetamido-1-hexanol[12] (*III*; 0·256 g, 1·2 m mol) and the compound *IV* (0·71 g, 1 mmol) in a mixture of pyridine (5 ml) and acetonitrile (5 ml). After 1 min stirring TLC S_2, R_F 0·10 → 0·70. After another 10 min, 0·1M solution of iodine in a mixture of tetrahydrofuran and water (4 : 1, 11 ml) was added dropwise. After another 30 min, the mixture was partitioned between chloroform (30 ml) and 0·5M aqueous sodium thiosulfate (30 ml). The aqueous layer was extracted with chloroform (10 ml) and the combined chloroform extracts were, after addition of triethylamine (1·1 ml), evaporated. The residue was dissolved in 25% aqueous ammonia (5 ml) and the mixture heated in tightly stoppered flask to 60°C for 16 h. The reaction mixture was evaporated, the residue dissolved in a mixture of chloroform and triethylamine (99 : 1) and the solution applied on a column of silica gel (100 ml) equilibrated in the same solvent. Elution was performed with 100 ml portions of the starting solvent, containing successively 10, 20, 30, 40, and 50% of methanol. Appropriate fraction (TLC S_3, R_F 0·16) were evaporated, the remaining solid was triturated with chloroform (10 ml) and diethylether (300 ml) was added. The solid was collected, washed with diethylether and dried in vacuo. Yield 547 mg (68%), m.p. 176–176·5°C. For $C_{37}H_{45}N_3O_{10}P$ (721·7) calculated: 5·82% N, 4·25% P; found: 5·67% N, 4·55% P.

Determination of Active Ester Groups in *IIa*, *IIb*

A suspension of *IIa* or *IIb* (20 mg) in a solution of *V* (2 mg, 2·5 μmol) in 50% aqueous formamide (0·4 ml) was degased by application of water aspirator vacuum, triethylamine (1 μl, 7 μmol) was added and the mixture shaken for 2 h. Aqueous ethanol (1 : 1, 8 ml) was added, the solid centrifuged, the supernatant decanted and the pellet was washed with 50% aqueous ethanol. The pellet was suspended in a mixture of 60% aqueous perchloric acid and ethanol (1 : 1, 10 ml) centrifuged and the absorbance at 498 nm determined. The content of N-hydroxysuccinimide ester groups per 1 g of support found: 3·3 μmol (cellulose), 3·5 μmol (Spheron-1000), 3·24 μmol (Sephacryl-500) and 4·72 μmol (Sephacryl-1000). The content of 4-nitrophenyl ester groups on Spheron-1000 was found 3·11 μmol/g of support.

Analysis was performed in the Analytical Department (V. Pechanec, Head) of this Institute.

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Translated by the author (J.S.).

Notes added in proof: In formulae *Ia* and *IIa* for R=N... should read R= O—N...
In formula *I* for $ROCOCH_2CH_2$... should read $RCOCH_2CH_2$...
In formula *II* for ... $(CH_2)_{10}OCOR$ should read $(CH_2)_{10}OCOCH_2CH_2COR$.
In formula *VI* for ...$(CH_2)_{10}OCO$—... should read $(CH_2)_{10}OCOCH_2CH_2CO$...