Synthesis and use of disulfide-based H-phosphonate reagent for 3'- and/or 5'-oligonucleotide labelling via mercaptoalkyl linker

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Synthesis of new disulfide-based H-phosphonate reagent for solid-phase oligonucleotide functionalization with mercaptopropyl group at one or two termini is described. Using this single reagent, 3'- and 3', 5'-disulfide-linked oligonucleotides were synthesized, disulfide bonds cleaved with dithiothreitol and generated thiol groups labelled with iodoacetamidofluorescein. T$_{15}$ oligonucleotides containing one or two fluorescein residues at 3'- and 5'-ends were prepared in high yields.

Introduction. The need for methods and reagents to modify oligonucleotides has increased in an extraordinary rate due to new applications of oligonucleotide conjugates as potent research tools, diagnostic and therapeutic agents [1—5]. Oligonucleotides labelled with reporter groups, e. g. fluorescent dyes, are used in non-isotopic detection of nucleic acids, automated DNA sequencing, studies on nucleic acid structure and dynamics. A number of molecules, including intercalators, artificial nucleases, lipophilic carriers, peptides etc., has been covalently linked to oligonucleotides to improve their antisense activity by increasing cellular uptake, nuclease resistance or binding affinity.

A wide variety of methods for the preparation of oligonucleotide conjugates has been discussed in detail in comprehensive reviews and books [2, 6—9]. In principle, conjugation is based on the introduction of appropriate functional group into oligonucleotide, followed by its specific coupling with another molecule. Chemical approach to the preparation of labelled DNA involves incorporation of modified nucleotide or non-nucleotide reagent during oligonucleotide synthesis; once incorporated, these synthons are functionalized post-synthetically, although direct introduction of some reporter molecules during solid-phase synthesis is also possible. The most common labelling methods use aminoalkyl linker groups. Another popular approach is to employ the highly reactive thiol function introduced as mercaptoalkyl linker able to react with thiol-specific groups.

Certainly, the latter strategy seems to be more promising and flexible, as thiols may be used in greater variety of reactions than amines. The thiol is a group capable of selective derivatization in the presence of oligonucleotide functional groups, i. e. amines, phosphates and hydroxyls. Two types of thiol modification are available, to generate stable thiol ether linkage or easily cleavable disulfide bond. S-alkylation reaction, that is coupling of highly nucleophilic sulfhydryl group with molecules bearing electrophilic thiol-specific functions, yields oligonucleotide conjugated via thioether bond. Iodo(bromo)acetamides, aziridinyl sulfonamides, γ-bromo-α, β-unsaturated carbonyls, maleimides and less reactive acrylates are the functionalities observed to selectively react with aliphatic mercapto group [10].

Besides that, thiol group can also form disulfides with other thiols, usually after activation with 2,2'-dipyridyl disulfide or related reagent [11—14]. This approach is especially important for oligonucleotide coupling with peptides and proteins under mild conditions, although conjugation through thioether linkage was also used for this purpose [13—18]. An important feature of disulfide formation is that it is readily reversed by treatment with mercaptans like

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dithiothreitol. SH-oligonucleotides were reversibly bound to thiol-modified solid supports [12, 19], or to hydrophobic transport agents like thiocholesterol to be cleaved within the cell by cellular reducing agents releasing a free oligonucleotide [20]. S-S bond cleavage is widely used to generate thiol linkers from disulfide precursors [11, 13, 21—23]. It should be noted that disulfide formation is a common problem on storage of thiol oligonucleotides. On the other hand, circular and looped oligonucleotides were prepared by oxidative disulfide bond formation between mercaptoalkyl tether groups [23, 24]. An additional interesting property of mercapto group is its ability to bond readily to some metals, and thiol-modified oligonucleotides have been immobilized on metal electrode surfaces for use in hybridization assays, biosensors, etc. ([25] and references therein).

Thus, the unique chemistry of sulfhydryl group makes thiol oligonucleotides very attractive for diverse applications, and at the same time the development of reagents and methods for their preparation and conjugation is still an important problem. Here we describe the synthesis and use of H-phosphonate reagent for solid-phase oligonucleotide functionalization with aliphatic thiol groups at both 3'- and/or 5'-termini.

Materials and Methods. 4,4'-Dimethoxytrityl chloride (DMTrCl), 5-(iodoacetamido)fluorescein (IAF) and 1,2,4-triazole (Tri) were purchased from «Fluka» (Switzerland), pivaloyl chloride (PivCl) and 1,4-dithiothreitol (DTT) from «Merck» (Germany). Reagents for gel electrophoresis were from «Sigma» (USA). Solvents were dried as follows: pyridine was distilled over ninhydrin and then refluxed over calcium hydride for 2 h and distilled; acetonitrile was distilled over phosphorus pentoxide and calcium hydride. TLC was performed on Kieselgel 60F$_{254}$ plates («Merck») using the following solvent systems: chloroform (A), chloroform-methanol 9:1 (B) and isopropanol — conc. NH$_3$ — water 7:2:1 (C). To detect nucleoside derivatives, the plates were sprayed with a mixture 95 % EtOH — acetic acid — anisaldehyde — conc. H$_2$SO$_4$ 9:0.1:0.5:0.5 (v/v) and heated at 110 °C. Nucleosides are revealed as blue to black spots [26]. SH-containing compounds were detected by spraying TLC plates with 0.5 % ethanol solution of 5,5'-dithiobis(2-nitrobenzoic acid) («Sigma») as yellow spots [27]. Thiols were also detected by exposing TLC plates to iodine vapors (white spots on dark background), although this reagent is less specific producing similar results with all compounds that could be oxidized by iodine, e. g. dialkyl sulfides.

Absorbance spectra were recorded on HP 8452 spectrophotometer («Hewlett-Packard», USA). ^{1}H

NMR spectra were obtained with Bruker AC-250 spectrometer (250 MHz) in CDCl$_3$ with TMS as internal standard. ^{31}P NMR spectra were recorded on the same device operating at 101 MHz using 85 % H$_3$PO$_4$ as external standard. Solid-phase oligonucleotide synthesis was performed on Applied Biosystems Model 380B automatic DNA synthesizer by H-phosphonate method [28] using reagents delivered by instrument supplier. Capping with isopropyl H-phosphonate has been employed [29]. Concentrations of H-phosphonates, including reagent (5), and pivaloyl chloride (PivCl) as condensing reagent in acetonitrile — pyridine (4:1) were 0.03 M and 0.15 M, respectively. Reverse phase HPLC was performed on Waters 600E chromatograph using μBondapak-C$_{18}$ column (3,9 × 150 mm, «Waters», USA) with a linear gradient of acetonitrile (0—30 % in 30 min) in 0,1 M TEAA buffer, pH 7.5 (flow rate 1 ml/min). Polyacrylamide gel electrophoresis (PAGE) was carried out on standard 20 % denaturing gel. Oligonucleotide bands were visualized under UV light (254 nm) using UV-shadowing technique, or at 365 nm for fluorescein conjugates.

Extinction coefficient ε_{260} for T$_{15}$ oligonucleotide calculated according to [30] was $1.22 \cdot 10^5$.

Synthesis of reagent. 3-A c e t y l m e r c a p t o p r o p a n o l-1 (1) and 3-m e r c a p t o p r o p a n o l-1 (2) were prepared according to [31].

3,3'-D i t h i o d i p r o p a n o l (3) was synthesized by adapting procedure described for the preparation of diethanoldisulfide [32]. To 2.20 g of 3-mercaptopropanol-1 (2, 2.5 mmol) 0.5 eq. of 30 % hydrogen peroxide was added dropwise with stirring and ice-cooling. The solution was allowed to stand overnight at room temperature until thiol had completely disappeared (test with thiol reagent). Water was removed under reduced pressure. 2.17 g of colourless oil was obtained (quantitative yield). Purity of (3) was confirmed by ^{1}H NMR: δ (ppm) 3.72 (t, 2H, CH$_2$O); 2.78 (t, 2H, CH$_2$S); 2.40—2.60 (br. s., 1H, OH); 1.92 (quint, 2H, CH$_2$ internal).

1-O-(4,4'-d i m e t h o x y t r i t y l)-3,3'-d i-t h i o d i p r o p a n o l (4). 1.82 g of 3,3'-dithiodipropanol (3, 10 mmol) was evaporated three times with anhydrous pyridine and dissolved in the same solvent (10 ml). The solution of 1.19 g of dimethoxytrityl chloride (3.5 mmol) in anhydrous pyridine (10 ml) was added dropwise with stirring over 1 h. The mixture was kept at room temperature for additional hour, poured into 100 ml of 5 % NaHCO$_3$ and extracted with chloroform (3 × 30 ml). Combined organic layer was washed with 0.25 M TEAB (pH 7.5, 50 ml) and water, dried over Na$_2$SO$_4$ and evaporated. After coevaporation with toluene to

remove remaining pyridine, product was isolated by silica gel chromatography in the gradient 40—80 % $CHCl_3$ in hexane to give a light yellow oil after evaporation. Yield 1.20 g (2.48 mmol, 71 % based on DMTrCl). R_f 0.10 (system A); 0.68 (B). ^1H NMR: δ (ppm) 7.15—7.45 (m, 9H, Ar); 6.82 (d, 4H, Ar); 3.78 (s, 6H, $2CH_3O$); 3.72 (t, 2H, CH_2OH); 3.15 (t, 2H, CH_2ODMTr); 2.70—2.85 (m, 4H, $2CH_2S$); 1.85—2.05 (m, 4H, $2CH_2$ internal). Ca. 10 % of ditritylated dipropanoldisulfide were also isolated (R_f 0.61 in system A).

1-O-(4,4'-d i m e t h o x y t r i t y l)-3,3'-d i-t h i o d i p r o p a n o l-1'-O-(H-p h o s p h o n a-t e) (5). Standard procedure for nucleoside phosphitylation [28] was used with some modifications. 1,2,4-Triazole (1.86 g, 27 mmol) was evaporated twice with anhydrous acetonitrile and suspended in the same solvent (15 ml). N-methylmorpholine (5.5 ml, 50 mmol) and then phosphorus trichloride (718 μl, 8.25 mmol) were added with cooling (5—10 °C). The mixture was stirred for 30 min at this temperature. Then solution of monotritylated dithio-dipropanol (4, 0.80 g, 1.65 mmol in 10 ml of dry CH_3CN) was added dropwise over 20 min with cooling, and the reaction mixture was stirred for further 20 min at room temperature. TLC (system B) showed almost complete conversion of starting material into product with low mobility. The mixture was poured into 100 ml of 0.5 M TEAB (pH 7.5) and 10 min later extracted with chloroform (3 × 50 ml). Organic extract was washed with 0.25 M TEAB (2 × × 50 ml) and water (50 ml), dried over sodium sulfate and evaporated. Product (5) was isolated by flash silica gel chromatography. Column was washed with 5 % methanol in chloroform and then the desired H-phosphonate was eluted with 7—8 % MeOH in chloroform containing 1 % triethylamine. Eluate was washed with 0.25 M TEAB and water, dried over Na_2SO_4 and evaporated. Product was dried with oil pump. 0.72 g of viscous pale yellow oil was obtained (67 %). R_f 0.05 (system B). ^1H NMR: δ (ppm) 7.15—7.45 (m, 9H, Ar); 6.82 (d, 4H, Ar); 6.82 (d, 1H, H-P, J_{HP} 620 Hz); 3.93 (m, 2H, CH_2O-P); 3.78 (s, 6H, $2CH_3O$); 3.05—3.20 (m, 8H, CH_2ODMTr + + $3CH_2N$ (TEAH)); 2.70—2.90 (m, 4H, $2CH_2S$); 1.85—2.10 (m, 4H, $2CH_2$ internal); 1.31 (t, 9H, $3CH_3$ (TEAH)). Purity of reagent was > 95 %, according to ^{31}P NMR analysis ($CDCl_3$: δ_p 2.95).

Model 5'-functionalization of nucleoside. 33 mg of reagent 5 (0.05 mmol) and 17 mg of 3'-O-acetylthymidine (0.06 mmol) were evaporated twice with anhydrous pyridine, dissolved in the same solvent (600 μl) and treated with pivaloyl chloride (24 μl, 0.2 mmol). 3 min later TLC (system B)

showed complete conversion of starting H-phospho-nate (R_f 0.05) into trityl-, nucleoside-containing coupling product (R_f 0.42) well separated from the slight excess of starting nucleoside (R_f 0.33). Iodine (25 mg, 0.1 mmol) in 400 μl of pyridine-water (20:1) was added, and 10 min later the complete oxidation of H-phosphonate diester into phosphodiester (trityl-, nucleoside-positive, R_f 0) was observed. The mixture was diluted with 0.25 M TEAB (1 ml) and aqueous solution of Na_2SO_3 was added dropwise carefully to neutralise excess iodine until its colour disappeared. No disulfide bond cleavage was observed. Further 2 ml of 0.25 M TEAB were added, and the mixture was extracted with chloroform (3 × 1 ml), extract was washed with 0.25 M TEAB and water (1 ml each) and evaporated. Disulfide-functionalized dT(Ac) containing a little amount of starting nucleoside was dissolved in 1 ml of $CHCl_3$-TEA (9:1), and 0.25 mmol of DTT was added. In 2 hours TLC showed the complete disappearance of starting trityl-positive material with R_f 0, to be converted into 2 new products of S-S bond cleavage: (a) DMTr-negative, nucleoside-positive, SH-positive, R_f 0 (systems A, B); 0.73 (C), and (b) DMTr-positive, nucleoside-negative, SH-positive, R_f 0.75 (A); 1.0 (B). Compound (b) was formed also as the only product upon treatment of 1,1'-O-bis-dimethoxytrityl-3,3'-dithiodipropanol (see above) with DTT, it was trityl-positive product of 1-O-dimethoxytrityl-3,3'-dithiodipropanol (4) cleavage with DTT, and, finally, the same compound was formed upon deacylation of $AcSCH_2CH_2CH_2ODMTr$ with KOH/EtOH. Therefore, compound (b) could be considered as $HSCH_2CH_2CH_2ODMTr$, whereas compound (a) with low TLC mobility could be 5'-mercaptopropylphosphate nucleoside derivative (6, $R = -dT(Ac)$).

Synthesis of oligonucleotide conjugates. I n t r o-d u c t i o n   o f   d i s u l f i d e   r e a g e n t   i n-t o   o l i g o n u c l e o t i d e s. The solution of disulfide H-phosphonate reagent (5) was attached to the spare port on the synthesizer. For thiol introduction at the 3'-terminus, reagent was directly coupled to thymidine-derivatized solid support (CPG) with coupling time extended to 5 min (coupling yield 92 %). Standard H-phosphonate synthesis of oligo-T_{15} chain was then performed, with average coupling yield 98.1 %. After sequence elongation was completed, the support containing 3'-disulfide-linked T_{15} was divided into 2 equal parts. The first one was used for the preparation of 3'-thiol oligomer, whereas from the other half of polymer 5',3'-dithiol derivative was obtained by repeating coupling with reagent (5) (coupling time 5 min, yield 95 %, as determined by detritylation of small weighed part of polymer). After

standard oxidation (2 % iodine in pyridine-water 98:2, 10 min) oligonucleotides were cleaved from the support (concentrated ammonia, room temperature overnight). 5'-DMTr-containing sequences were purified by Oligo-Pak cartridge (Milligen/Biosearch, USA) following manufacturer's protocol. Oligonucleotides were detritylated directly on the cartridge, eluted and evaporated. (Note that at this step both oligomers contained additional thymidine residue linked at 3'-end through disulfide tether). Normal T_{15} oligonucleotide was also synthesized by standard H-phosphonate method to be used as control.

D i s u l f i d e   b o n d   c l e a v a g e. Disulfide-containing oligonucleotides were quantitatively reduced before labelling by treatment with 30 mM DTT (0.5 mg per 1 OD_{260} of oligonucleotide) in 10 mM Tris-HCl buffer (pH 8.0) overnight at 37 °C under argon. Cleavage was monitored by HPLC. Most of the DTT was removed by extraction with butanol, and the reduced thiol oligonucleotides were desalted at PD-10 cartridge («Pharmacia»).

O l i g o n u c l e o t i d e   l a b e l l i n g w i t h   f l u o r e s c e i n. 5 OD_{260} of 3'-thiol or 3',5'-dithiol oligonucleotide (0.04 mmol) were dissolved in 200 ml of 0.1 M sodium carbonate/bicarbonate buffer (pH 9.0). 20 (for 3'-thiol oligo-T_{15}) or 30 (for 3',5'-dithiol oligo-T_{15}) eq of 5-(iodoacetamido)fluorescein in 100 µl of freshly distilled DMF were added with agitation, and the reaction mixture was incubated under argon at room temperature overnight in the dark. Labelling reaction was monitored by reverse phase HPLC. If necessary, a further 5—10 eq of IAF could be added. The reaction mixture was diluted with 500 µl of water and passed through PD-10 gel filtration column to remove excess label. The desired product was eluted with 50 mM TEAB (pH 7.5) containing 5 % of ethanol, and eluate was evaporated. In the synthesis of 3'-labelled T_{15} oligonucleotide, some non-labelled starting oligomer was removed efficiently at Oligo-Pak cartridge following the protocol for DMTr-oligonucleotides isolation, and 3',5'-bis-fluorescein-T_{15} was purified by reverse phase HPLC. Purity of products was confirmed by PAGE and HPLC. 3'-Fluorescein-T_{15}: yield 78 %. UV/Vis: $A_{260}/A_{494} = 2.03$ (calculated ratio 1.96). 3',5'-bis-Fluorescein-T_{15}: yield 57 %; $A_{260}/A_{494} = 1.24$ (calculated = 1.15).

Results and Discussion. A number of methods for the preparation of thiol-modified oligonucleotides has been described [2, 6—9]. SH groups can be introduced into oligonucleotide using suitably protected nucleoside mercapto derivatives, e. g. 5'-mercapto-nucleosides for 5'-derivatization [33] or 5-mercapto-uridine for site-specific labelling [34]. Cystamine is

widely employed to prepare thiol oligonucleotides, as it has amino group to react with amine-specific entities, and cleavable disulfide bond generating thiol upon treatment with DTT. Cystamine linkers have been introduced at the internucleotide linkage via H-phosphonate approach [35], 5'-cystaminyl oligonucleotides have been synthesized to yield 5'-thiol derivatives [13, 16, 23], and 3'-thiol oligonucleotides were prepared by cleaving from the special support with cystamine with subsequent S-S bond cleavage [23]. More common approaches to the introduction of thiols into 3'- or 5'-terminus have been also developed. 3'-Thiol oligonucleotides are usually synthesized on specially designed disulfide-derivatized solid supports [11, 14, 18, 19, 22, 23]. Preparation of these supports is rather laborious procedure, although they are now commercially available. 5'-Thiol oligonucleotide functionalization is more simple, and corresponding reagents are also available. They are derivatives of S-protected mercaptoalcohols, namely S-trityl mercaptopropanol or -hexanol phosphoramidites [17, 36, 38] or H-phosphonates [37, 38]. These reagents can be introduced into oligonucleotides at the last coupling step of solid-phase synthesis, and then S-trityl group is removed by $AgNO_3/DTT$. This approach has been reported to work well with relatively short sequences (up to 12-mers), but yields decreased significantly for longer oligomers, especially with the use of amidite reagents [17, 37]. Low yields of thiol oligonucleotides were probably resulted from the chemical modification of 5'-thiol terminus during deprotection. So, there are several methods for oligonucleotide 5'- or 3'-end functionalization with thiol linker groups, but these approaches are usually not highly efficient, and moreover they are not applicable for the synthesis of oligonucleotides with thiols at both termini.

Our intention was to achieve a simple technique for obtaining 3'- and/or 5'-thiol labelled oligonucleotides using single reagent. As many methods for 3'- and 5'-labelling described above briefly are based on the cleavage of disulfide precursors, it was clear that properly designed disulfide-containing functionalizing reagent could be able to label any end of oligonucleotide. Cystamine has been used for 3'- and 5'-labelling, however we would like to create a reagent for direct functionalization during solid-phase synthesis. We decided that such a reagent has to be bifunctional, like cystamine, but with hydroxy groups instead of amines, containing one DMTr-protected hydroxyl to allow for chain elongation and one phosphorylating moiety for reagent introduction as P-component during oligonucleotide synthesis; these two units should be connected with cleavable disulfide

linker. Dialcohol disulfides were an obvious choice for our purpose, and reagent resulted from tritylation and phosphorylation of two hydroxy groups linked via S-S bond would be introduced at 3'- or 5'-end of oligonucleotide to obtain 3'- or 5'-thiol derivatives after internal disulfide cleavage. Reagent under design was based on the same principle as oligonucleotide phosphorylating agent derived from the symmetric sulfodiethanol, with one hydroxyl being protected with DMTr group and another phosphorylated; it can be added at 3'- or 5'-end of oligonucleotide, and during ammonia deprotection central sulfonyl linkage is β-eliminated to produce 3'- or 5'-phosphate (or both) [39].

Our first attempts to prepare oligonucleotide functionalizing reagent based on the easily available diethanoldisulfide failed: when O-monotritylated intermediate was phosphorylated with tris(triazolyl)phosphine, starting material disappeared, but significant cleavage of the product (R_f ca. 0.05 in system B) was observed during its isolation with formation of two compounds able to be oxidized with iodine, probably the result of S-S bond cleavage or another redox process. This reaction was not further investigated, as it was found in the literature that 2-mercaptoethyl phosphates are anyway unstable under basic conditions cleaving ethylmercapto group to yield free phosphates. Nevertheless, it was demonstrated that mercaptopropyl and -hexyl phosphates were completely stable [21, 36].

As a result, we have prepared thiol-introducing H-phosphonate reagent (5) based on 3,3'-dithiodipropanol (dipropanoldisulfide). Our first report on this reagent was presented at the conference in 1993 [40], however its synthesis and use still has not been described in detail. Recently, a similar phosphoramidite reagent based on 6,6'-dithiodihexanol has been used to prepare circular oligonucleotides by oxidizing 5'-, 3'-terminal thiol groups [24]; to our best knowledge, its synthesis was not yet reported too.

The preparation of the reagent (5) (Scheme 1) started from the synthesis of 3,3'-dithiodipropanol (3). S-acetylmercaptopropanol (1) was obtained by the addition of thiolacetic acid to allyl alcohol in the presence of benzoyl peroxide, and deacylated with 10 % NaOH to give 3-mercaptopropanol (2), according to [31]. Its subsequent oxidation with calculated amount of hydrogen peroxide resulted in quantitative yield of the desired dipropanoldisulfide (3). The latter was tritylated with DMTrCl in pyridine (yield 71 %); this method was found to be more efficient than previously described tritylation in the presence of DMAP and TEA (yield 48 %) [14].

Monotritylated 3,3'-dithiodipropanol (4) has been previously attached to solid support via succinate linkage for the preparation of 3'-thiol oligonucleotides solely [14, 23]. In our study, this intermediate was phosphitylated with PTri₃ to produce H-phosphonate reagent (5) for solid-phase synthesis. No product cleavage was observed in this case, as was with diethanoldisulfide derivative. As ^{31}P NMR demonstrated, reagent was completely stable: no additional signals were observed in the spectrum after one year storage at −18 °C. So, possible in theory self-oxidation of monoester P-H bond of reagent (5) by its S-S group didnot occur. Possible as well sulfurization of diester P-H bonds (including intramolecular reaction of 3'-linked disulfide) during solid-phase synthesis was not specially studied; however, this process seems to be unlikely to proceed under normal synthetic conditions, as dialkyl disulfides are not active sulfurizing agents since their S-S bond is not activated by electron-acceptor substituents. This type of side reaction has never been reported for phosphoramidite oligonucleotide synthesis on disulfide-containing supports, although P(III) species are much more susceptible to sulfurization with disulfides than tetracoordinated H-phosphonates. Clear HPLC profiles of reaction mixtures from the syntheses of thiol oligoucleotides and their conjugates could probably confirm this conclusion to a certain degree (see below).

To test the efficasy of H-phosphonate reagent for thiol group introduction into oligonucleotides, model labelling of nucleoside (3'-O-acetylthymidine) was first carried out in the solution, according to Scheme 2, a. Nucleoside was phosphitylated with (5) using pivaloyl chloride as condensing reagent, and hydrophosphoryl group of coupling product was oxidized smoothly with iodine in aqueous pyridine to give phosphodiester containing disulfide linkage. The latter was cleaved by DTT in the presence of base (TEA) to result in the formation of nucleoside functionalized with mercapto group (6, $R = -dT(Ac)$). This product was not however specially isolated and studied since model reaction was performed just to demonstrate that nucleoside could be converted to thiol-containing product by coupling with H-phosphonate reagent (5) followed by iodine oxidation and DTT treatment.

For oligonucleotide functionalization, proposed reagent can be added as P-component at the first and/or last coupling cycle of solid-phase synthesis, according to normal H-phosphonate synthetic protocol (Scheme 2). 5'-Thiol oligonucleotides (6) are prepared through the addition of reagent (5) at last H-phosphonate coupling step after the assembly of

Scheme 1

$$CH_2=CH-CH_2OH + CH_3COSH \xrightarrow{Bz_2O_2} CH_3COSCH_2CH_2CH_2OH \xrightarrow{10\% \ NaOH}$$

1

$$\longrightarrow HSCH_2CH_2CH_2OH \xrightarrow{H_2O_2} HOCH_2CH_2CH_2SSCH_2CH_2CH_2OH \longrightarrow$$

2 **3**

$$\xrightarrow[Py]{DMTrCl} DMTrOCH_2CH_2CH_2SSCH_2CH_2CH_2OH \xrightarrow{PTri_3}$$

4

$$\longrightarrow DMTrOCH_2CH_2CH_2SSCH_2CH_2CH_2O-\underset{\underset{H}{|}}{\overset{\overset{O}{\|}}{P}}-O^- \ TEAH^+$$

5

Scheme 2

a) 5'- functionalization:

$$5 + HO-R \xrightarrow{(i), \ (ii), \ (iii)} 5'- \ HSCH_2CH_2CH_2O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-OR$$

6

R - nucleoside or oligonucleotide

(i) PivCl / Py; (ii) I_2 / H_2O; (iii) DTT

b) 3'- functionalization:

$$5 + HO-P \xrightarrow{(i), \ (ii), \ (iii), \ (iv)} oligonucleotide\text{-}3'\text{-}O-\underset{\underset{O^-}{|}}{\overset{\overset{O}{\|}}{P}}-OCH_2CH_2CH_2SH$$

7

P - polymer support

(i) PivCl / Py; (ii) oligonucleotide synthesis; (iii) I_2 / H_2O; (iv) DTT

desired oligonucleotide chain was completed. To prepare 3'-modified oligonucleotides (7), H-phosphonate reagent has to be introduced at the first addition to any nucleoside support, followed by the normal synthesis of target sequence. Post-synthetic reducing with DTT cleaves S-S bond leaving a thiol group at 5'- or 3'-terminus. In such a way, oligonucleotides labelled at both 3'- and 5'-ends could be also easily prepared.

We have used H-phosphonate (5) to synthesize a 15-mer oligothymidylate containing one or two thiol groups at 3'- and 5'-termini on DNA synthesizer by conventional H-phosphonate approach. Coupling time

for (5) was increased to 5 min, as is usually recommended for functionalizing reagents containing long alkyl chains. In this case, the yields were 92 and 95 % for the first and last coupling step, respectively, as determined by DMTr cation release, whereas common nucleoside H-phosphonates were coupled for 2 min with average yield about 98 %. After final oxidation with aqueous iodine, oligonucleotides were cleaved from the polymer support with concentrated NH_4OH. Ammonolysis was carried out overnight at room temperature, as only thymidines were present in the sequence; otherwise common deblocking (e. g. at 55 °C for at least 6 h) should be performed. Disulfide

bond cleavage could be performed simultaneously, by adding DTT to ammonia solution [19]. In our case, S-S bonds were kept during oligonucleotide purification to avoid the oxidative dimerization of SH-functionalized oligonucleotides [11, 12, 36]. 5'-O-DMTr protecting groups were not removed to simplify the isolation of product. As HPLC analysis of crude reaction mixtures showed good chromatographic patterns (Fig. 1, a), no special attempts were made to purify oligomers by gel electrophoresis or HPLC. DMTr- and disulfide-containing oligonucleotides were purified using Oligo-Pak cartridges. Purification is based there on the principle of reverse phase chromatography: the desired oligomer with hydrophobic trityl group is retained by the support while the failure sequences without DMTr residue, as well as deprotection by-products, are removed from the reaction mixture by washing to deliver an oligomer of good purity sufficient for most applications. Detritylation of oligonucleotides was performed directly on the Oligo-Pak support after removing side products. All oligonucleotides were of good quality after Oligo-Pak isolation, and no additional purification was carried out. Then 3'- and 5'-disulfide linkages in purified oligonucleotides were quantitatively cleaved with DTT to produce the corresponding thiol derivatives. The latter should be stored (if necessary) in the presence of a little amount of DTT (ca. 5—10 mM) to avoid dimerization. DTT can be removed before conjugation by butanol or ethylacetate extraction. In principle, it is not essential to purify 5'-thiol oligonucleotides at this step, as only SH-containing oligomer will react with labelling reagent and conjugation product could be easily purified. But in the synthesis of 3'-thiol oligonucleotides failure sequences also contain 3'-SH groups, and purification of starting oligomer would be desirable. Nevertheless, our thiol oligomers were of good purity, as HPLC showed, and additional purification seemed to be unnecessary. On reverse phase HPLC, thiol oligonucleotides had somewhat higher retention time than non-modified T_{15}, but lower than corresponding disulfide precursors (Fig. 1, b).

To demonstrate the utility of our approach for oligonucleotide labelling, we have synthesized T_{15} oligonucleotides bearing one or two fluorescein residues at 3'- and both 3'- and 5'-ends for the studies on fluorescence polarization. The coupling step involves reaction of oligonucleotide bearing a nucleophilic thiol linker(s) with dye reagent containing electrophilic thiol-specific iodoacetamide group (Scheme 3).

Labelling reactions were performed in sodium carbonate/bicarbonate buffer (pH 9) containing ca. 30 % DMF (where IAF was dissolved). Labelling was
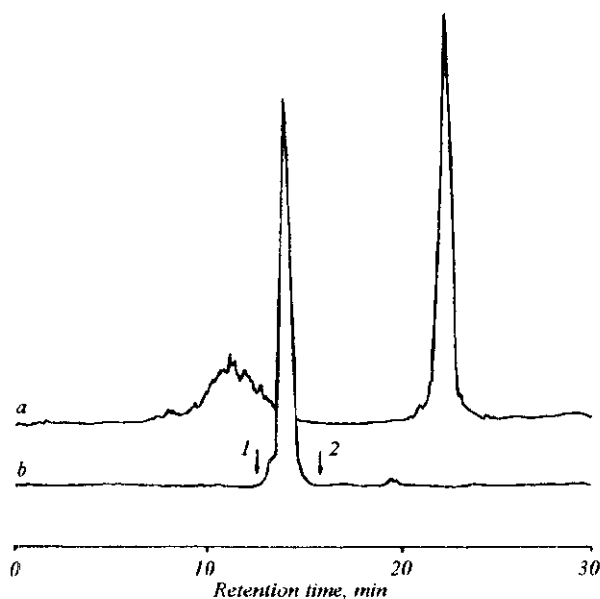

Fig. 1. Reverse phase HPLC analysis of oligonucleotides: a — crude reaction mixture containing 5'-DMTr-protected T_{15}-3'-disulfide; b — T_{15}-3'-SH after Oligo-Pak purification of product from a and disulfide bond cleavage. Arrows indicate the positions of normal T_{15} (1) and T_{15}-3'-disulfide (2) on the chromatogram

carried out at room temperature overnight under argon to avoid possible yield decrease due to oxidative dimerization of thiol oligonucleotides. As some DTT is expected to be present in thiol oligonucleotide even after desalting, the use of sufficient excess of labelling reagent is recommended. In the presence of 20 eq of fluorescein reagent, 3'-thiol oligomer was efficiently labelled with dye during the reported coupling period. At the same time, in the case of 3',5'-dithiol labelling starting oligomer disappeared too, but about 30 % of oligonucleotide material remained monolabelled under these reaction conditions. However, in the presence of 30 eq of IAF almost complete transformation into bis-labelled product was observed. Excess dye was removed by gel filtration passing the reaction mixtures through PD-10 desalting cartridge. Further purification of conjugates by preparative gel electrophoresis or HPLC is usually necessary to separate the conjugation product from unlabelled sequence. In our case, purification by Oligo-Pak cartridge seemed to be sufficient, as fluorescein residue, like trityl group, is enough hydrophobic to retain the conjugate on the column while impurities are separated. Indeed, this technique allowed the full separation of some amount

Scheme 3

O
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RO—P—OCH$_2$CH$_2$CH$_2$SH + I-CH$_2$CONH-Flu ⟶
|
O⁻

O
‖
⟶ RO—P—OCH$_2$CH$_2$CH$_2$SCH$_2$CONH-Flu
|
O⁻
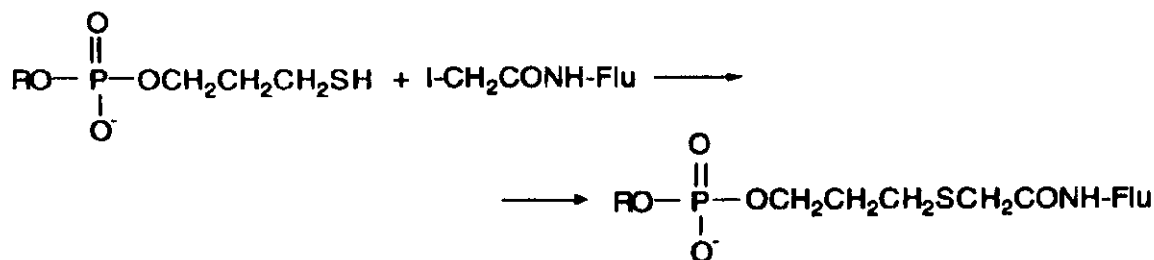
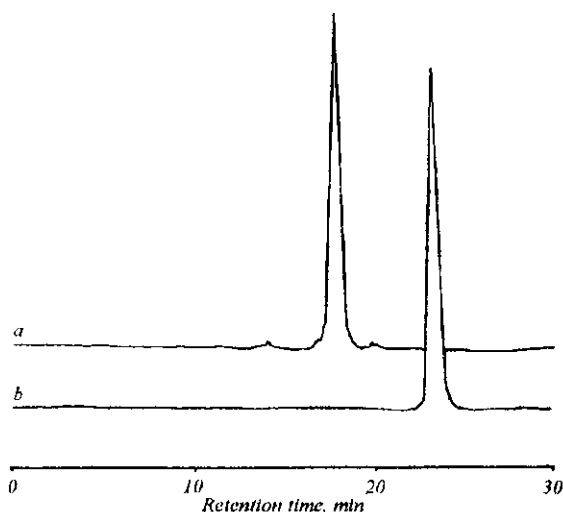### R - oligonucleotide, Flu - fluorescein residue

Fig. 2. Reverse phase HPLC profiles of purified T$_{15}$-3'-Flu (a) and 5'-Flu-T$_{15}$-3'-Flu (b) oligonucleotide conjugates

of non-labelled sequence present in the reaction mixture from 3'-conjugate preparation. However, Oligo-Pak was not able to separate mono-labelled oligomer from bis-labelled oligonucleotide product in the case of 3',5'-bis-fluorescein conjugate preparation. In this case, preparative HPLC was used to isolate the desired product.

Oligonucleotide products purified by Oligo-Pak or reverse phase HPLC were analyzed by HPLC and PAGE. Fig. 2 showes the HPLC profiles of purified mono- and bis-labelled oligonucleotides T$_{15}$-3'-Flu

and 5'-Flu-T$_{15}$-3'-Flu. 3'-Fluorescein-labelled oligomer was retained longer than non-modified T$_{15}$, due to hydrophobic character of the dye; additional Flu residue further increased the retention time. PAGE analysis showed reasonable purity of oligonucleotides. Conjugate bands were easily identified in the gel by their green fluorescence under long wavelength UV light (365 nm). Substantial mobility shift was observed for fluorescein-labelled oligonucleotides. Each dye molecule attached to oligonucleotide decreased its mobility in the gel approximately as one additional nucleotide. Chromatographic and electrophoretic behaviour of prepared conjugates is in full agreement with data on oligonucleotides labelled with several dye residues by another methods [41—43].

Fluorescein conjugates were characterized by UV/Vis spectroscopy. The absorbance spectra of labelled oligonucleotides at pH 8 are showed in Fig. 3. The spectra of non-modified T$_{15}$ and IAF are included to illustrate the relative contribution of the dye and oligonucleotide to conjugate absorbance. Absorption pattern of dye residue in the conjugates is quite similar to that of IAF, with absorbance maximum at 494—495 nm. The extinction coefficient of fluorescein moiety in the conjugates at 260 nm can be estimated as ca. 25000 from the spectrum of IAF with known ε$_{495}$ = 75000 [10]. Taking into account dye extinction at these wavelengths, the observed absorbance ratios A$_{494}$/A$_{260}$ for fluorescein-labelled oligonucleotides are in good agreement with calculated values indicating clearly the presence of 1 and 2 dye residues per oligonucleotide, respectively. It is interesting to note that at pH 6 sharp intense peak of fluorescein absorbance at 494 nm almost disappeared having been converted into broad band of low intensity at 440—500 nm (λ$_{max}$ = 454 nm), due to the changes in dye structure; fluorescence at this pH is also known to be relatively low.

The data presented in this paper demonstrate the utility of proposed disulfide H-phosphonate reagent (5) for direct solid-phase oligonucleotide functio-
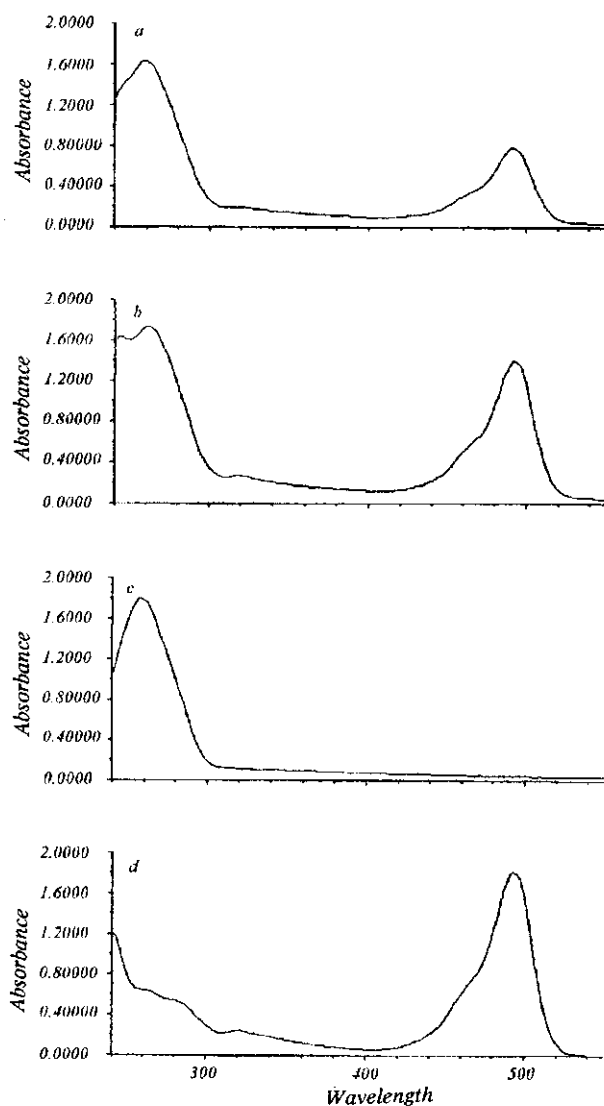
Fig. 3. UV/Vis spectra of oligonucleotides T_{15}-3'-Flu (a), 5'-Flu-T_{15}-3'-Flu (b), T_{15} (c), and IAF dye (d) in 25 mM Tris-HCl buffer (pH 8)

nalization at 3'- and/or 5'-end with one or two thiol groups. It is easily prepared, requires no changes of common H-phosphonate synthesis protocol, and provides a reliable and flexible way for oligonucleotide

labelling with fluorescent dyes or other molecules at any terminus.

І. Я. Дубей, Д. М. Федоряк

Синтез та використання дисульфідного Н-фосфонатного реагента для 3'- та/або 5'-мічення олігонуклеотидів через меркаптоалкільний лінкер

Резюме

Описано синтез нового дисульфідного Н-фосфонатного реагента для твердофазного введення в олігонуклеотиди меркаптопропільних груп по одному чи двох кінцях. З використанням цього реагента було синтезовано 3'- та 5'-дисульфідні похідні олігонуклеотидів. Дисульфідні зв'язки розщеплено дитіотреїтолом і тіольні групи, що утворилися, модифіковано йодацетамідофлюоресцеїном. З високим виходом одержано оліго-T_{15}, які несуть один чи два залишки флюоресцеїну на 3'- і 5'-кінцях.

И. Я. Дубей, Д. М. Федоряк

Синтез и использование дисульфидного Н-фосфонатного реагента для 3'- и/или 5'-мечения олигонуклеотидов через меркаптоалкильный линкер

Резюме

Описан синтез нового дисульфидного Н-фосфонатного реагента для твердофазного введения в олигонуклеотиды меркаптопропильных групп по одному или двум концам. С использованием этого реагента синтезированы 3',5'-дисульфидные производные олигонуклеотидов. Дисульфидные связи расщеплены дитиотреитолом и образовавшиеся тиольные группы модифицированы иодацетамидофлюоресцеином. С высоким выходом получены олиго-T_{15}, несущие один или два остатка флюоресцеина на 3' и 5'-концах.

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