A New and Efficient Method for Synthesis of 5′-Conjugates of Oligonucleotides through Amide-Bond Formation on Solid Phase

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Dedicated to Prof. Dr. *Wolfgang Pfleiderer* on the occasion of his 75th birthday

An efficient method for synthesis of oligonucleotide 5′-conjugates through amide-bond formation on solid phase is described. Protected oligonucleotides containing a 5′-carboxylic acid function were obtained by use of a novel non-nucleosidic phosphoramidite building block, where the carboxylic acid moiety was protected by a 2-chlorotrityl group. The protecting group is stable to the phosphoramidite coupling conditions used in solid-phase oligonucleotide assembly, but is easily deprotected by mild acidic treatment. The protecting group may be removed also by ammonolysis. 5′-Carboxylate-modified oligonucleotides were efficiently conjugated on solid support under normal peptide-coupling conditions to various amines or to the N-termini of small peptides to yield products of high purity. The method is well-suited in principle for the synthesis of peptide-oligonucleotide conjugates containing an amide linkage between the 5′-end of an oligonucleotide and the N-terminus of a peptide.

1. Introduction. – Conjugation of antisense oligonucleotides to peptides and basic polymers (*e.g.*, polylysine) is aimed at enhancement of their cellular permeability, their targeting to specific tissues, or alteration of their intracellular locations (for reviews, see [1–4]). Conjugation of oligonucleotides to small molecules may also alter such parameters but is often used to attach specific labels (*e.g.*, fluorescent) or effector molecules, such as biotin (reviewed in [5][6]). In these ways, chemical functionalization of synthetic oligonucleotides has found wide application with the rapid growth of molecular biology and biotechnology.

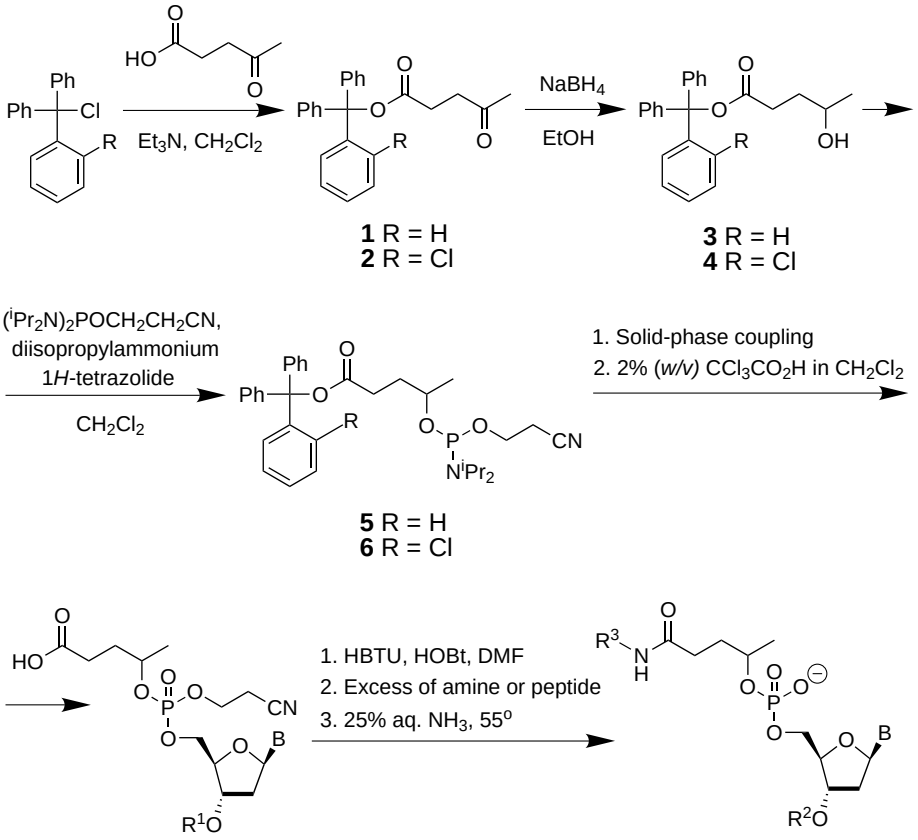
Most conjugation strategies involve prior attachment of a tether bearing a nucleophilic group to a modified nucleoside or to a special linker, which is then incorporated into DNA by synthesis of an appropriate phosphoramidite and subsequent oligonucleotide synthesis under standard conditions. Commonly used nucleophilic groups include primary amines and thiols [7–11]. For example, we reported a few years ago the solid-phase conjugation of small peptides to oligonucleotides that had been functionalized with an amino linker [12][13]. Electrophilic groups have been introduced into synthetic oligonucleotides relatively rarely, for example, at the 2′-position [14][15]. Modification of the 5′-terminus with an aldehyde or carboxylic acid has been reported previously by a number of authors [16–20]. In each case, the modifications were introduced into oligonucleotide chains after solid-phase oligonu-

cleotide assembly, and the aldehyde or carboxylic acid function liberated either before or simultaneously with the final deblocking and release of the oligonucleotide from the support with ammonia. Thus, subsequent transformations could be achieved only by a solution-phase method. As a result, these methods are frequently plagued by low yields, long reaction times, the need for large excesses of reactant(s), and the formation of by-products that are difficult to separate. *Hovinen et al.* described reaction of a solid-supported oligonucleotide modified by a 5′-glycolic acid ester with several aliphatic diamines over 6–12 h [18]. Very recently, a method was described for synthesis of 5′-aldehydes *via* coupling of a protected diol-containing linker phosphoramidite in solid-phase oligonucleotide synthesis, where the acetal protecting group could be removed under mild acidic conditions [21]. We wish to report here the synthesis of a new type of modified oligonucleotide *via* coupling of a phosphoramidite linker reagent containing a 5′-carboxylic acid function carrying a 2-chlorotrityl (=(2-chlorophenyl)diphenylmethyl) protecting group, which may be removed under mild acidic conditions. Subsequent conjugation of the 5′-carboxylic acid function to a range of primary and secondary aliphatic amines is achieved through amide-bond formation on solid support. We show examples of synthesis of such carboxylate-modified oligonucleotides conjugated to various reporter groups (*e.g.*, fluorescent labels, biotin) or to small peptides.

2. Results and Discussion. – To obtain oligonucleotides modified at the 5′-end with a carboxylic group, we synthesized two novel non-nucleosidic phosphoramidite building-block linkers **5** and **6** (*Scheme*). For the selection of a phosphoramidite useful in the final coupling step in solid-phase oligonucleotide assembly, we investigated the utility of the trityl (=triphenylmethyl) family of protecting groups for the carboxylic acid moiety, since a 2-chlorotrityl ester had been found to be useful previously as a carboxylic acid protecting group in fragment peptide coupling [22]. Furthermore, 2-chlorotrityl ether has been used as a linker for polymer-supported peptide-fragment synthesis [23], which gained further popularity when it was found to be cleaved under extremely mild acidic conditions, for example, $(CF_3)_2CHOH/CH_2Cl_2$ 1:4 [24]. In the case of polymer-bound 2-chlorotrityl chloride, the $Et(i\text{-}Pr)_2NH$ salt of an *N*-α-Fmoc-protected amino acid is usually used for coupling [23]. In our case, levulinic acid (=4-oxopentanoic acid) was treated either with Ph_3CCl or with 2-chlorotrityl chloride in the presence of Et_3N in dry CH_2Cl_2 to give high yields of crystalline trityl or 2-chlorotrityl levulinate **1** and **2**, respectively. Each compound was easily reduced by $NaBH_4$ in EtOH to afford the corresponding hydroxy esters **3** and **4**, respectively, in good yield, but some decomposition of the unsubstituted trityl compound was observed during silica-gel column chromatography. The relative stabilities of the protecting groups under oligonucleotide synthesis conditions were then checked by TLC (*Table 1*). Whilst the trityl ester was only moderately stable to 0.5M 1*H*-tetrazole solution in MeCN, and noticeable cleavage was seen even after 15 min, the corresponding 2-chlorotrityl derivative remained unchanged after 1-h treatment. Both compounds were easily deprotected by 80% aq. AcOH, again the trityl removal being faster. Cleavage of both esters was almost instantaneous upon treatment with 2.5% (*v*/*v*) Cl_2CCOOH in CH_2Cl_2, a commonly used detritylating reagent in oligonucleotide synthesis.

Modified phosphoramidites were obtained by phosphitylation of the corresponding alcohols by (2-cyanoethoxy)bis(diisopropylamino)phosphine in the presence of

Scheme



R^1 = Support-bound protected oligonucleotide; R^2 = unprotected oligonucleotide; for the range of R^3 = see *Table 2*.

Table 1. *Acid Stability*[a]) *of Trityl and 2-Chlorotrityl Esters*

Reagent	Trityl ester **3**	2-Chlorotrityl ester **4**
0.5M 1*H*-Tetrazole in MeCN	30 min	> 1 h
AcOH/H_2O 4 : 1 (*v*/*v*)	< 5 min	15 min
2.5% Cl_2CCOOH in CH_2Cl_2	< 1 min	< 1 min

[a]) Time needed for *ca.* 50% removal of the protecting group (TLC).

diisopropylammonium tetrazolide [25]. The compounds were purified by silica-gel column chromatography, the trityl phosphoramidite being obtained in lower yield and purity. However, both amidites were used successfully in machine-assisted solid-phase synthesis, demonstrating comparable coupling efficiencies (> 96%). However, we decided to use the more stable and easily obtainable phosphoramidite **6** for conjugation studies. This was utilized in the synthesis of a series of modified oligonucleotides (*Table 2*). Oligodeoxyribonucleotide **III** was synthesized as a model to select and

Table 2. *Properties of 5′-Carboxylated Oligonucleotides and Their Conjugates*

No.	Oligonucleotide sequence/ conjugated molecule[a])	MALDI-TOF-MS, calc./found[b])	Retention time [min][b])	Yield [%][c])	HPLC Purity [%][d])
I	5′-d(GCT CCC AGG CTC AAA)-3′	3502.8/3501.2	13.1	–	57
II	5′-d(AGC TCC CAG GCT CAA)-3′	4725.0/4724.9	12.8	–	67
III	5′-d(TTT TTT TTT T)-3′	3502.8/3501.2	13.0	–	–
I.7	2-(2-Hydroxyethoxy)ethylamine (**7**)	4789.2/4787.1	12.8	99	90
I.8	Tetrahydrofurfurylamine (**8**)	4785.3/4783.1, 4784.2	13.8, 14.6	99	89
I.9	2-Phenylethylamine (**9**)	4805.0/4801.6, 4803.2	18.7, 19.4	99	75
I.10	H-Leu-NH_2 (**10**)	4814.2/4812.2, 4811.7	14.7, 15.1	98	71
I.11	H-Phe-NH_2 (**11**)	4848.2/4847.8, 4848.7	15.4, 16.1	98	67
II.12	Spermine (**12**)	4887.4/4888.3	11.4	88	70
II.13	Octadecylamine (**13**)	4976.5/4974.1	–[e])	–	–
II.14	Histamine (**14**)	4818.2/4818.1	11.8, 12.0	81	60
II.15	Dansylcadaverine (**15**)	5042.5/5043.4	–[e])	–	–
II.16	Biotin-PEG-amine (**16**)	5081.5/5079.1	–[e])	–	–
II.17	3-Aminopropane-1,2-diol (**17**)	4773.9/4771.6	12.1	96	66
II.18	1-Pyrenemethylamine (**18**)	4916.3/4919.6	–[f])	–	–
I.19	H-Gly-Phe-NH_2 (**19**)	4905.3/4904.7	17.4	90	61
I.20	H-Gly-Leu-Met-NH_2 (**20**)	5003.5/5006.5	19.3	90	64
I.21	H-Pro-Leu-Gly-NH_2 (**21**)	4969.4/4973.0	15.5	80	66
II.22	H-Gly-Gly-Gly-Gly-Gly-NH_2 (**22**)	4984.9/4981.2	14.0	75	53

[a]) Amines **7**–**11** and peptides **19**–**21** were conjugated to oligonucleotide **I**; amines **12**–**18** and peptide **22** were conjugated to oligonucleotide **II**. [b]) Values separated by commas correspond to individual diastereoisomers. [c]) Conversion of oligonucleotide peak to the conjugate peak(s), calculated from HPLC trace. [d]) As integrated from HPLC traces. [e]) Purified by PAGE. [f]) Not purified.

optimize the specific conditions for 5′-deblocking and purification. Initially, we envisaged that the 2-chlorotrityl protecting group could be used in an analogous way to the 5′-dimethoxytrityl group as a purification handle on a reversed-phase (RP) HPLC column. However, the 2-chlorotrityl ester was cleaved after treatment with 25% aqueous NH_3 or ^{t}BuNH$_2$/MeOH/H_2O 1:2:1 (*v*/*v*/*v*) at room temperature for *ca.* 30 min. Therefore, the 5′-carboxylic group was routinely deprotected on column under standard detritylating conditions following oligonucleotide assembly (2% (*w*/*v*) Cl_3CCOOH in CH_2Cl_2).

Then, we investigated the H_2O-soluble carbodiimide-mediated reaction of 5′-modified fully deprotected oligonucleotide with aqueous ethylenediamine dihydrochloride. The yield of the reaction product was found to be almost quantitative, and product integrity was confirmed by RP-HPLC and by MALDI-TOF mass analysis. Further conjugations of 5′-carboxylic acid-modified oligonucleotides **I** and **II** were carried out on solid phase in organic solvent. The choice of amine was influenced by the expected application for the conjugate; for example, affinity labelling (biotin-PEG-amine (**16**)), fluorescent labelling (dansylcadaverine (**15**) and 1-pyrenemethylamine (**18**)), introduction of positive charge or nucleophile (spermine (**12**) and histamine (**14**)), addition of hydrophobicity (octadecylamine (**13**)) or chemical ligation [15][16] (3-aminopropane-1,2-diol (**17**)). To obtain a high yield of structurally diverse types of conjugates (*Table 2*), we used pre-activation of polymer-bound carboxylic oligonucleotide with HBTU/HOBT 1:1 at 35° in dry DMF for 35 min, followed by addition of

the amine or short peptide solution in DMF and incubation for a further 1 h. An equimolar amount of Et_3N was included also if the amine was present as its salt. After completion of the reaction, the polymer-bound oligonucleotide was cleaved from the support and deprotected by standard ammonia treatment [26], and the reaction mixtures were analyzed by RP-HPLC in ion-pair mode (*Fig.*). In some cases, HPLC traces showed the formation of two products, which were separated and found to have identical molecular masses by MALDI-TOF MS analysis (*Table 2*). Such twin products were expected and may be assigned to the formation of a mixture of diastereoisomeric conjugates that are resultant from the asymmetric C-atom coming from the alkyl side-chain of phosphoramidite **6** (see *Scheme*).

In conclusion, we have presented a new method for the synthesis of oligodeoxyribonucleotides containing a 5′-carboxylic acid derivative *via* coupling of a novel phosphoramidite derivative that contains a 2-chlorotrityl protecting group for the

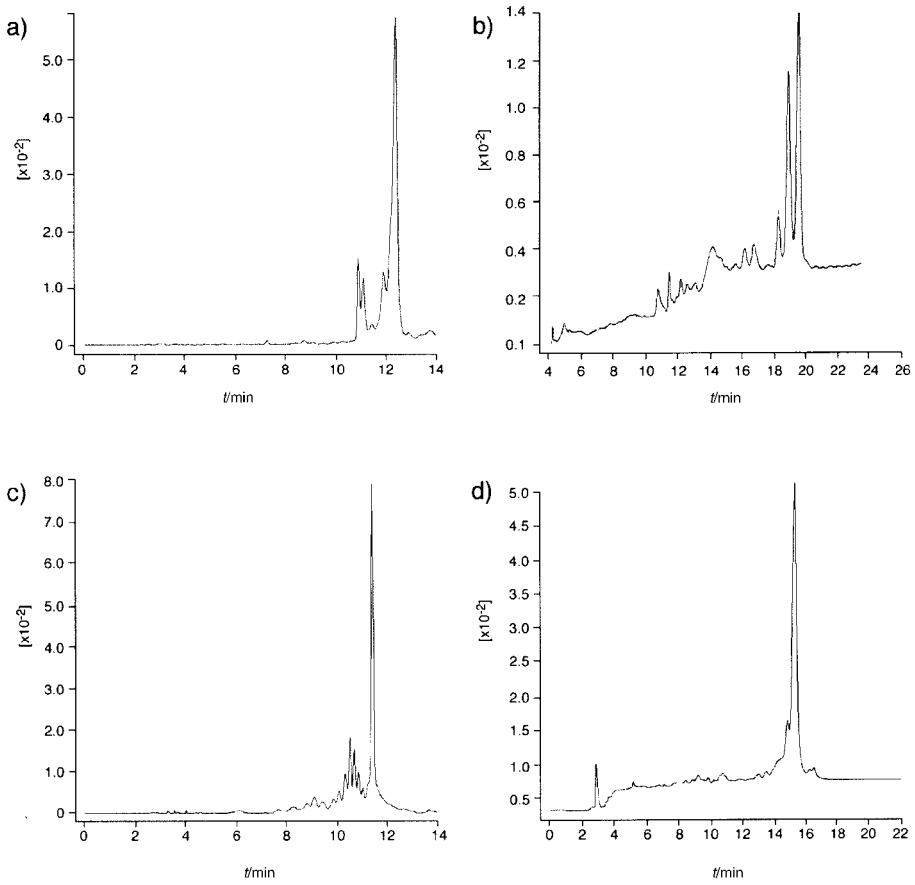


Figure. *HPLC Traces of crude reaction mixtures analyzed in ion-pair mode:* a) *5′-carboxy group-modified oligonucleotide* **II**; b)–d) *5′-conjugates with 2-phenylethylamine* **I.9** (b) (mixed diastereoisomers); *spermine* **II.12** (c); *and tripeptide H-Pro-Leu-Gly-NH*$_2$ **I.21** (d)

carboxylic acid moiety **6**. After deprotection under acidic conditions, such substituted oligonucleotides could be conjugated, whilst still attached to the solid support, with a range of small molecule amines or to the N-termini of short peptides. The methods described could prove useful in the synthesis of small molecule libraries of conjugates with oligonucleotides for diagnostic or therapeutic evaluation. In addition, the conjugation method can be explored as a useful route for attachment of the N-termini of cell penetrating peptides to the 5′-ends of oligonucleotides and their analogues for cell uptake studies.

We thank Dr. *Evgueni M. Zubin* for his helpful suggestions and *Donna Williams* for some initial oligonucleotide syntheses. This work was supported by *Wellcome Trust* grant No. 057361, and partially by *Universities of Russia – Fundamental Research* grant No. 05.03.010 and *Leading Scientific Schools* grant No. 00-15-97944.

Experimental Part

General. Materials were obtained from commercial suppliers and used without further purification unless otherwise noted. CH_2Cl_2 (*BDH*) was refluxed for 24 h over CaH_2, and then distilled. DMF was distilled *in vacuo* and used fresh. Et_3N, 1-hydroxybenzotriazole (HOBt), *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), dansylcadaverine, and DMF were from *Fluka*. *N*′-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide, ethylenediamine dihydrochloride, $LiClO_4$, and 2-(2-hydroxyethoxy)ethylamine were from *Sigma*. Levulinic acid, Ph_3CCl, 4-(dimethylamino)pyridine (DMAP), spermine, histamine, 1-pyrenemethylamine hydrochloride, 3-aminopropane-1,2-diol and $NaBH_4$ were from *Aldrich*. Tetrahydrofurfurylamine and 2-phenylethylamine were from *Reakhim* (USSR). Octadecylamine was from *Lancaster*. (+)-D-Biotin 8-amino-3,6-dioxaoctylamide (biotin-PEG-amine) was obtained from *Pierce*. 2-Chlorotrityl chloride was purchased from *Novabiochem*, amino acids and peptides were obtained from *Bachem*. TLC Plates (Kieselgel *60 F_{254}*, *Merck*) were developed in the appropriate system: AcOEt/hexane 2:3 (*v*/*v*) + 1% Et_3N (*A*); AcOEt/hexane 3:7 (*v*/*v*) + 1% Et_3N (*B*); AcOEt/hexane 1:9 + 1% Et_3N (*C*). Compounds were visualized by UV shadowing (254 nm) or by CF_3COOH vapor staining (yellow color).

Oligonucleotide Synthesis. Oligodeoxyribonucleotides were assembled on a *ABI 380B* DNA Synthesizer by the cyanoethyl phosphoramidite method [26] according to the manufacturer's recommendations. Protected 2′-deoxyribonucleoside phosphoramidites and *S*-ethylthiotetrazole were purchased from *Glen Research* (*via Cambio*). Prepacked 0.4-μmol functionalized columns of controlled-pore glass (*Glen*) were used throughout. For couplings with modified phosphoramidite **6**, a 0.15M concentration in anh. MeCN was used, and the coupling time was increased to 15 min. Oligonucleotides were cleaved from the support and deprotected with concentrated aq. NH_3 overnight at 55°.

Purifications and Characterizations. HPLC Analysis and purification of deprotected oligonucleotides and conjugates were carried out on a *Tracor* instrument 4 × 250 mm *DIAKS-130-CETYL* column; buffer *A:* 0.1M ammonium acetate (pH 7); buffer *B:* 0.1M ammonium acetate, 40% MeCN (pH 7); gradient of *B* from 0 to 100% in 80 min; flow rate 1 ml/min at 45°. Oligonucleotides and conjugates were analyzed by a RP-HPLC (ion pair mode; *Waters*) on a *DIAKS-130-CETYL* column (3 × 250 mm) with a logarithmic gradient: 0–42.4% *B* (1 min); 42.4–45.7% *B* (1 min); 45.7–50.1% *B* (3 min); 50.1–53.5% *B* (5 min); 53.5–56.8% *B* (10 min); 56.8–58.7% *B* (10 min); 58.7–60.1% *B* (10 min). Separation of oligomers with a retention time step of 0.5 min/residue was carried out; mobile phase *A:* H_2O/MeCN 95:5 (*v*/*v*), 2 mM tetrabutylammonium dihydrogen phosphate, 48 mM KH_2PO_4, pH 7; mobile phase *B:* H_2O/MeCN 60:40 (*v*/*v*), 2 mM tetrabutylammonium dihydrogen phosphate, 48 mM KH_2PO_4, pH 7; flow rate 0.6 ml/min, and temp. 45°. MALDI-TOF-MS: on a *Voyager DE* workstation (*PE Biosystems*) in a freshly prepared 1:1 (*v*/*v*) mixture of 2,6-dihydroxyacetophenone (40 mg/ml in MeOH), and aq. diammonium hydrogen citrate (80 mg/ml) as a matrix. Compounds **1**–**4** were prepared in 2,4,6-trihydroxyacetophenone (10 mg/ml in 50% aq. MeOH). Denaturing gel electrophoresis of oligonucleotides was carried out on 15% PAGE containing 2M urea in *Tris*-borate buffer (50 mM Tris·HCl, 50 mM boric acid, 1 mM EDTA, pH 7.5).

Reaction of the Modified Oligonucleotide **I** *with Ethylenediamine Dihydrochloride.* 5′-Carboxylic group-modified oligonucleotide **I** (0.4 A_{260} units) was dissolved in 40 μl of deionized H_2O, ethylenediamine dihydrochloride (5.3 mg) and *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (3 mg) were

added, and the mixture was incubated for 3 h at r.t. The oligonucleotide was then precipitated by addition of 100 µl of 2M $LiClO_4$ and 1 ml of acetone, the pellet washed with 300 µl of acetone, redissolved in 100 µl of 2M $LiClO_4$ and precipitated again. Analysis of the products was carried out by RP-HPLC under conditions described above. Yield of ethylenediamine-modified oligonucleotide was 98% (HPLC).

Conjugate Synthesis. After completion of the oligonucleotide assembly and 5′-carboxylic group deprotection, a soln. of HBTU (100 equiv.) and HOBt (100 equiv.) in 150 µl of dry DMF was added to the support. The slurry was incubated at 35° for 35 min with occasional swirling, then an amine or peptide soln. was added (100 equiv.), with additional Et_3N (100 equiv.), if needed. The reaction was carried out for 1 h at 35° and shaking, the supernatant discarded, and glass beads washed successively with DMF (2 × 200 µl), EtOH (2 × 200 µl), and water (2 × 200 µl). Conjugate cleavage from the support and deprotection of phosphate and nucleobase residues were performed as usual. Reaction mixtures were analyzed by ion-pair RP-HPLC, conjugates were purified by RP-HPLC under conditions described above for 5′-carboxylic oligonucleotides, or by a denaturing PAGE. Their respective molecular masses were checked by MALDI-TOF-MS analysis.

Triphenylmethyl 4-Oxopentanoate (**1**). To a stirred soln. of 4-oxopentanoic acid (2.25 ml, 22 mmol) in 35 ml of CH_2Cl_2, containing EtN(^{i}Pr)$_2$ (5.14 ml, 30 mmol) and DMAP (122 mg, 1 mmol), a soln. of Ph_3CCl (5.58 g, 20 mmol) in 15 ml of CH_2Cl_2 was added dropwise over a period of 20 min. The mixture was stirred at 25° for 2 h. After TLC (system *A*, R_f 6.6) showed the completion of the reaction, the soln. was evaporated, dissolved in 50 ml of AcOEt, washed with 5% aq. $NaHCO_3$ soln. (2 × 30 ml) and brine (25 ml), dried (anh. Na_2SO_4), and evaporated to a small volume. Petroleum ether was then added, and the soln. was kept in a fridge overnight. The crystalline precipitate was collected by filtration, washed with AcOEt/petroleum ether 1:2 (*v*/*v*), and dried *in vacuo:* 6.68 g (93.1%) of **1**. White crystals. ^{1}H-NMR ((D$_6$)DMSO): 2.06 (*s*, Me); 2.65 (*q*, *J* = 5.1, 2 CH_2); 7.23, 7.29 (2*m*, 15 arom. H). MALDI-TOF-MS: 364.1 ($[M+H]^+$; calc. 359.4).

(2-Chlorophenyl)diphenylmethyl 4-Oxopentanoate (**2**). To a stirred soln. of 4-oxopentanoic acid (3.07 ml, 30 mmol) in 50 ml of CH_2Cl_2 containing EtN(^{i}Pr)$_2$ (8.56 ml, 50 mmol), a soln. of (2-chlorophenyl)diphenylmethyl chloride (7.86 g, 25 mmol) in 25 ml of CH_2Cl_2 was added dropwise over a period of 15 min. The mixture was stirred at 25° for 3 h. After TLC (system *B*, R_f 0.45) showed the completion of the reaction, the soln. was evaporated, dissolved in 60 ml of AcOEt, washed with 5% aq. $NaHCO_3$ soln. (2 × 35 ml) and brine (30 ml), dried (anh. Na_2SO_4), and evaporated to a small volume. Petroleum ether was then added, and the soln. was kept in a fridge overnight. The crystalline precipitate was collected by filtration, washed with AcOEt/petroleum ether 1:2 (*v*/*v*), and dried *in vacuo:* 9.60 g (97.5%) of **2**. White crystals. ^{1}H-NMR ((D$_6$)DMSO): 2.09 (*s*, CH_2COMe); 2.71 (*s*, Me); 3.31 (*s*, CH_2CO_2); 7.19, 7.30 (2*m*, 14 arom. H). MALDI-TOF-MS: 393.5 ($[M+H]^+$; calc. 393.9).

Triphenylmethyl 4-Hydroxypentanoate (**3**). To a stirred soln. of **1** (1.08 g, 3 mmol) in 10 ml of EtOH, solid $NaBH_4$ (0.17 g, 4.5 mmol) was added in small portions over 10 min. The mixture was stirred for another 1 h. After TLC (system *B*, R_f 0.29) showed disappearance of the starting material, the mixture was evaporated, dissolved in 20 ml of $CHCl_3$, washed by 5% aq. $NaHCO_3$ soln. (2 × 15 ml) and brine (10 ml), dried (anh. Na_2SO_4), and evaporated *in vacuo*. The residue was redissolved in $CHCl_3$ and chromatographed on a silica-gel column with a gradient of 1 to 5% MeOH in $CHCl_3$ (*v*/*v*) containing 0.5% Et_3N as an eluent. The appropriate fractions were pooled, evaporated to dryness, and dried in high vacuum: 0.77 g (71.5%) of **3**. Colorless oil. Triphenylmethanol (74 mg, 6.8%) was also isolated as a minor by-product. ^{1}H-NMR ((D$_6$)DMSO): 1.00 (*d*, *J* = 6.1, Me); 1.55 (*q*, *J* = 7.5, CH_2CHOH); 2.49 (*m*, CH_2CO); 3.31 (*d*, *J* = 7.3, OH); 3.52 (*m*, C*H*OH); 7.28 (*m*, 15 arom. H). MALDI-TOF-MS: 361.7 ($[M+H]^+$; calc. 360.5).

(2-Chlorophenyl)diphenylmethyl 4-Hydroxypentanoate (**4**). To a stirred soln. of **2** (3.94 g, 10 mmol) in 25 ml of EtOH, solid $NaBH_4$ (0.57 g, 15 mmol) was added in small portions over 10 min. The mixture was stirred for another 1 h. After TLC (system *B*, R_f 0.26) showed disappearance of the starting material, the mixture was evaporated, dissolved in 25 ml of AcOEt, washed with 5% aq. $NaHCO_3$ soln. (2 × 15 ml) and brine (10 ml), dried (anh. Na_2SO_4), and evaporated *in vacuo*. The residue was dissolved in $CHCl_3$ and chromatographed on a silica-gel column with a gradient of 1 to 5% MeOH in $CHCl_3$ (*v*/*v*) containing 0.5% Et_3N as an eluent. The appropriate fractions were pooled, evaporated to dryness, and dried in high vacuum: 3.48 g (87.9%) of **4**. Colorless oil. ^{1}H-NMR ((D$_6$)DMSO): 1.03 (*d*, *J* = 6.2, Me); 1.61 (*q*, *J* = 7.3, CH_2CHOH); 2.57 (*q*, *J* = 7.8, CH_2CO); 3.58 (*m*, C*H*OH); 4.51 (*d*, *J* = 4.8, OH); 7.29 (*m*, 14 arom. H). MALDI-TOF-MS: 393.4 ($[M+H]^+$; calc. 395.1).

Triphenylmethyl 4-[(2-Cyanoethoxy)(diisopropylamino)phosphanyloxy]pentanoate (**5**). Compound **3** (0.75 g, 2.07 mmol), dried by repeated co-evaporations with dry MeCN, was dissolved in dry CH_2Cl_2 (10 ml), and diisopropylammonium tetrazolide (0.53 g, 3.11 mmol) and (2-cyanoethoxy)bis(diisopropylamino)phosphane (0.86 ml, 2.69 mmol) were added, and the mixture was stirred at 25° for 5 h. After TLC (system *C*, R_f 0.25) showed the completion of the reaction, the mixture was evaporated, dissolved in AcOEt (10 ml), washed with 5% aq. $NaHCO_3$ soln. (2 × 5 ml) and brine (5 ml), dried (anh. Na_2SO_4), and evaporated to dryness. The

residue was dissolved in CH_2Cl_2/hexane 3:1 (*v*/*v*) and applied on the top of silica-gel column eluted first by 20%, then by 15%, and finally by 10% hexane in CH_2Cl_2 containing 2% Et_3N (*v*/*v*). The appropriate fractions were pooled, evaporated to dryness, and dried in high vacuum: 0.44 g (38.2%) of **5**. Colorless oil. ^{1}H-NMR (CD_3CN): 1.20 (*m*, 5 Me); 1.79 (*m*, C*H*$_2$CHO); 2.61 (*m*, 2 CH_2); 3.71 (2*m*, 2 CHN, CH_2O); 3.99 (*m*, CHO); 7.31 (*m*, 15 arom. H). ^{31}P-NMR (CD_3CN): 147.37, 148.38 (two sets of diastereoisomers in *ca.* 2.5:1 ratio). MALDI-TOF-MS: 561.5 ($[M+H]^+$; calc. 561.3).

(2-Chlorophenyl)diphenylmethyl 4-[(2-Cyanoethoxy)(diisopropylamino)phosphanyloxy]pentanoate (**6**). Compound **4** (0.47 g, 1.2 mmol), dried by repeated co-evaporations with dry MeCN, was dissolved in dry CH_2Cl_2 (5 ml) and diisopropylammonium tetrazolide (0.31 g, 1.8 mmol) and (2-cyanoethoxy)bis(diisopropylamino)phosphane (0.49 ml, 1.5 mmol) were added, and the mixture was stirred at 25° for 4 h. After TLC (system *B*, R_f 0.71, 0.76, pair of diastereoisomers) showed the completion of the reaction, the mixture was evaporated, dissolved in AcOEt (10 ml), washed with 5% aq. $NaHCO_3$ soln. (2 × 5 ml) and brine (5 ml), dried (anh. Na_2SO_4), and evaporated to dryness. The residue was dissolved in $CHCl_3$/hexane 3:2 (*v*/*v*) and chromatographed on a silica-gel column eluted first by 40%, then by 30%, and finally by 20% hexane in $CHCl_3$ containing 2% Et_3N (*v*/*v*). The appropriate fractions were pooled, evaporated to dryness, and dried in high vacuum: 0.69 g (97.4%) of **6**. Colorless oil. ^{1}H-NMR ((D_6)DMSO): 1.14 (*m*, 5 Me); 1.72 (*m*, C*H*$_2$CHO); 2.65 (*m*, CH_2CH_2CN); 3.54, 3.69 (2*m*, 2 CHN, CH_2O); 3.95 (*m*, CHO); 7.18, 7.32 (2*m*, 14 arom. H). ^{31}P-NMR (CD_3CN): 147.35, 146.28 (two sets of diastereoisomers). MALDI-TOF-MS: 597.9 ($[M+H]^+$; calc. 596.1).

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Received May 6, 2002