A NEW LINKER FOR SOLID-PHASE SYNTHESIS OF OLIGONUCLEOTIDES WITH TERMINAL PHOSPHATE GROUP

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Synthesis of a novel cyanoethyl-type linker suitable for the solid-phase synthesis of oligodeoxynucleotides possessing terminal 3′-phosphate group is described. Since the linker is a 2-substituted 2-cyanoethanol, the release of the synthesized oligonucleotide from the solid support is accomplished by β-elimination in the ammonia deprotection step.

Keywords: 2-Substituted-2-cyanoethanol; β-Elimination; Modified LCAA-CPG; Solid-phase synthesis; Oligonucleotide 3′-phosphates; Solid support; Deprotection.

A variety of supports for the solid-phase synthesis of (deoxy)ribonucleotides have been introduced over past two decades, the (long-chain alkyl)-amino controlled-pore glass (LCAA-CPG) playing a prominent role among them for most purposes. However, with increasing number of cases in which oligomers containing terminal phosphate groups were needed for subsequent enzymatic[1,2] or chemical[3] ligations as well as for diagnostic[4] purposes, specific approaches were sought to provide appropriately adjusted supports and protocols.

There are several chemically different methods for the solid-phase synthesis of the terminally phosphorylated oligonucleotides which employ various linkers for the attachment of the first nucleotide unit. Thus, Horn et al.[5] described a solid support with the 2-[(2-hydroxyethyl)sulfonyl]-ethoxy group (Fig. 1, scaffold **A**). The desired 3′-phosphorylated oligonucleotide is released from the support by a β-elimination reaction (e.g., by treatment with ammonia). A chemically similar linker was also described later by Markiewicz et al.[6].

The method using an oximate-derived support was introduced by Häner et al.[7] (Fig. 1, scaffold **B**). The phosphodiester conjugate of oligonucleotide and oximate undergoes, under alkaline conditions, the elimination reaction resulting in the formation of terminally phosphorylated oligonucleotide.

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Another strategy for the preparation of terminally phosphorylated oligonucleotides made use of the oxidation of the *cis*-diol system of the terminal ribonucleoside moiety[8–11] with $NaIO_4$, followed by the β-elimination reaction (Fig. 1, scaffold **C**).

An interesting linker for the synthesis of oligonucleotides with 3′-terminal phosphates and phosphorothioates was described by Ravikumar et al.[12]. Their concept consists in the use of the 3-(2-hydroxyphenyl)propan-1-ol derivative anchored to the solid support via ester bond with phenolic hydroxyl (Fig. 1, scaffold **D**). The primary hydroxy group serves for the attachment of the first nucleotide unit. Upon ammonia treatment, the phenol ester group is cleaved and the following nucleophilic attack of phenolic OH on the carbon atom bearing phosphoester moiety releases the 3′-phosphorylated oligonucleotide.

The use of diethyl bis(hydroxymethyl)malonate, anchored to the solid support via one of the hydroxy groups, was reported by Guzaev et al.[13] (Fig. 1, scaffold **E**). Under alkaline conditions, two elimination reactions led to final release of the oligonucleotide from the solid support.

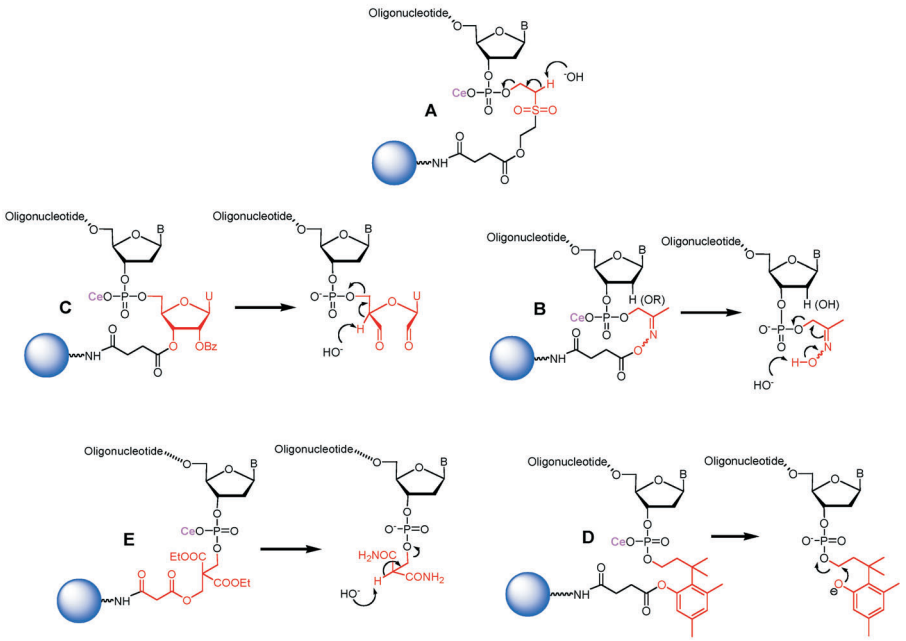


FIG. 1
Overview of linkers which employ β-elimination or related reactions in the preparation of terminally phosphorylated oligonucleotides

In another approach, Letsinger et al.[14] described the formation of a phosphoramidate (P–N) bond between the first 3′-nucleotide unit and the amino group of the LCAA-CPG (Fig. 2, scaffold **F**). This bond remains stable during the whole oligonucleotide synthesis, including acid removal of the dimethoxytrityl group. On treatment with ammonia, the phosphoester protecting groups are removed, and the 3′-phosphorylated oligonucleotide attached to the solid support is easily cleaved off with 80% aqueous acetic acid. Similar method using benzidine as a ligand for attachment of the first nucleotide unit via the P–N linkage was reported by Markiewicz et al.[6], whereby the cleavage of the P–N linkage was accomplished using isopentylnitrite (Fig. 2, scaffold **G**).

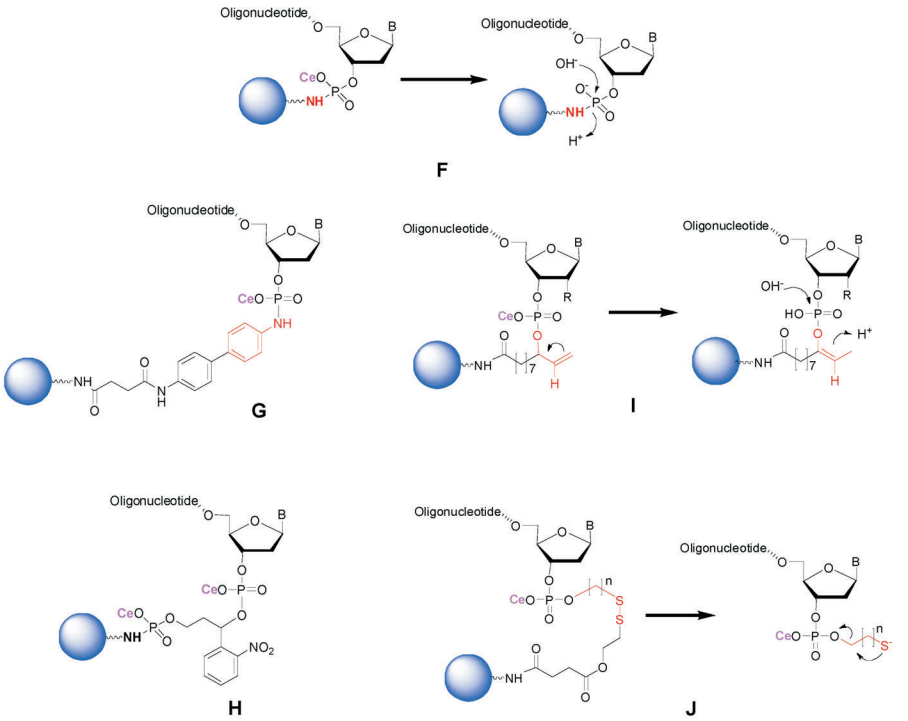


FIG. 2
Overview of linkers prepared by different chemistries for use in solid-phase synthesis of terminally phosphorylated oligonucleotides

Imbach et al.[15] developed an alternative solid support employing a photolabile 2-nitrobenzyl-alcohol-type linker for the attachment of the first nucleotide unit (Fig. 2, scaffold **H**). Irradiation of the solid support led to the elimination reaction under release of the attached phosphorylated oligonucleotide into solution. This approach seems to be suitable for the synthesis of base-sensitive oligonucleotides.

A universal allyl-type linker was reported by Zhang et al.[16]. The allyl ester group (Fig. 2, scaffold **I**) was isomerized under mild conditions (Pd(0) catalysis), and the formed vinyl phosphate moiety was subsequently hydrolyzed to release the oligonucleotide phosphate.

Several authors[17–19] reported the use of 2-sulfanylethan-1-ol attached to a solid support via disulfide bond (Fig. 2, scaffold **J**). After synthesis of the oligomer, the disulfide linkage was reduced with dithiotreitol under alkaline conditions. The free 2-sulfanylethyl phosphate underwent an intramolecular nucleophilic attack of the *S*-anion on the C1 carbon atom and released oligonucleotide phosphate and episulfide.

In this paper, we report the synthesis of a novel type of linker, 2-substituted-2-cyanoethan-1-ol attached to the LCAA-CPG, which is a promising alternative to anchoring of oligonucleotides in the solid-phase synthesis of terminally phosphorylated oligonucleotides (Fig. 3, scaffold **K**). The release of the synthesized oligonucleotide from the solid support is accomplished by β-elimination during the ammonia deprotection step.

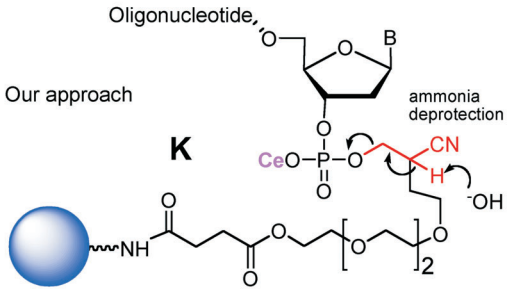
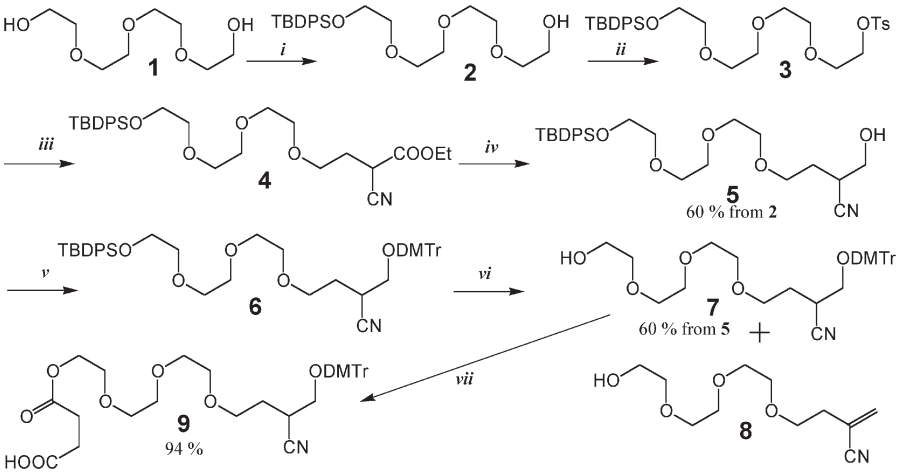


FIG. 3
Structure of novel linker

RESULTS AND DISCUSSION

Preparation of the key compound **9** (Scheme 1) to be used as the desired linking moiety started from the known monosilyl derivative of tetraethylene glycol **2** [20] which was tosylated in pyridine, and the obtained tosyl derivative **3** was treated with sodium salt of ethyl cyanoacetate. The nucleophilic displacement of the tosylate group by ethyl cyanoacetate *C*-anion proceeded smoothly to give compound **4**.



SCHEME 1
Synthesis of novel linker of 2-cyanoethyl type

When the *C*-alkylation was performed in ethanol in the presence of sodium ethoxide, a low yield of **4** (~15%) was obtained. In contrast to the previous reactions, the use of DBU in DMF to generate the ethyl cyanoacetate *C*-anion followed by the reaction with **3** provided a mixture of unidentified products. Reduction of the ester group of compound **4** with sodium borohydride in ethanol then proceeded smoothly to yield the desired hydroxy derivative **5** in a good yield (60% based on **2**). The subsequent dimethoxytritylation of **5** yielded compound **6**. Removal of the silyl protecting group of **6** with TBAF in THF under carefully controlled conditions afforded the dimethoxytrityl derivative **7** (60% based on **5**). The desilylation of **6** was found to proceed very fast with a freshly prepared 0.5 M TBAF·3H_2O in THF (ca. 1–2 h).

This was the crucial point of the synthesis since the prolonged time of the desilylation step (e.g., overnight) led to β-elimination reaction releasing acrylonitrile derivative **8** and dimethoxytrityl alcohol, the R_F of which on TLC is almost identical with that of compound **7**. The desilylated compound **7** was then acylated with succinic anhydride, and the obtained hydrogensuccinate **9** (94% on spectral characterization) was anchored via the amide bond to LCAA-CPG by the currently used procedure[21]. The achieved loading varied between 40 and 50 μmol/g.

Selected oligonucleotides prepared to provide the proof of suitability and efficiency of the designed solid support by means of furnishing 3′-phosphorylated oligomers were successfully synthesized from the respective 2′-deoxynucleoside 3′-phosphoramidites in conventional way in the trityl-off manner, as described in Experimental. The presence of the phosphoester group was proved unambiguously by the enzymatic dephosphorylation using alkaline phosphomonoesterase. The results were documented by the HPLC profiles (Fig. 4) and the MALDI-TOF data.

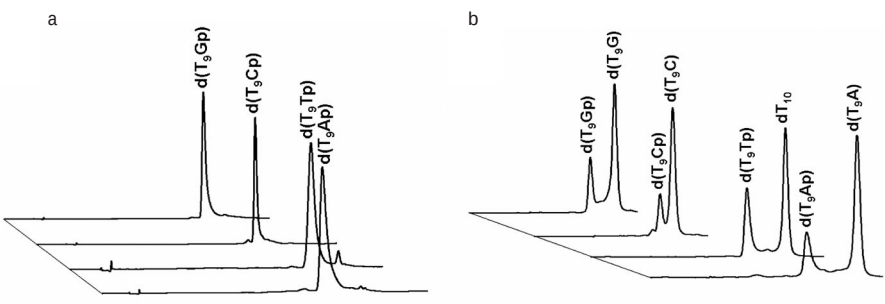


FIG. 4

HPLC analyses of: a crude oligonucleotide 3′-phosphates synthesized on the cyanoethyl-CPG (Luna C18 5 μm, 4.6 × 150 mm; 15→40% B/20 min, A = 10% acetonitrile in 0.1 M TEAA, B = 25% acetonitrile in 0.1 M TEAA, pH 7.5); b the mixtures of oligonucleotide 3′-phosphates and their dephosphorylated products

The cyanoethyl type of ligand presented here was already used, in the procedure refinement and without specific data, in our earlier work dealing with the synthesis of the 3′-phosphorylated (n = 0) and phosphonylated (n = 1) oligonucleotides (Fig. 5) as HIV integrase inhibitors[22], and with the attachment of the protected dA-PMEA phosphonate dinucleotide via internucleotide linkage followed by synthesis of the modified oligonucleotide primers useful for the study on eukaryotic DNA polymerases[23]. This proved compatibility of the novel linker use and the method of anchoring

for syntheses with both phosphate and phosphonate components adds to the versatility of the approach.

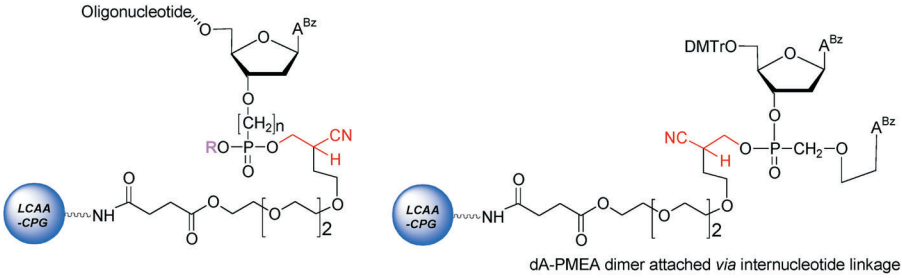


FIG. 5
Examples of use of novel cyanoethyl-type solid support

CONCLUSION

We succeeded in elaborating a synthetic route to a novel linker attached to LCAA-CPG for the preparation of 3′-phosphorylated oligonucleotides, utilizable in various conjugation reactions. The new linker was tested in synthesis of a set of deoxyoligonucleotides carrying the terminal 3′-phosphate group. The prepared oligomers were obtained, upon a convenient β-elimination release from the support, in a high yield and purity. The synthesis of the linker is simple, straightforward, and unambiguous and gives good yields. The subsequent anchoring to the LCAA-CPG proceeds with a high loading efficiency. The oligo(ethylene glycol) spacer arm of varying lengths may be used, thus enabling the tuning of physicochemical properties of the solid support surface to obtain a more convenient environment for the condensation. The used synthetic methodology offers the possibility of a simple three-step introduction of the 2-cyanoethyl-type linker onto polyethylene glycol-grafted polystyrene to obtain even more easily the cyanoethyl-type solid support for oligonucleotides carrying the terminal phosphate or phosphonate moieties (study in progress). In summary, the methodology presented here provides a technically easy, advantageous procedure without problematic steps offering an alternative worth consideration when searching for a reliable approach to oligonucleotides possessing terminal phosphate/phosphonate groups.

EXPERIMENTAL

General

The solvents were evaporated at 40 °C and 2 kPa, and the products were dried over phosphorus pentoxide at room temperature and 13 Pa. The reaction course was checked using silica gel UV254 TLC sheets (Merck) whereby the products were detected by UV monitoring and by spraying with 1% ethanolic solution of 4-(4-nitrobenzyl)pyridine followed by gentle heating and subsequent exposing the sheets to ammonia vapour (blue colour of tosyl derivatives). For flash column chromatography, a 40–60 μm silica gel (Fluka) was used. The TLC was carried out in the following solvent systems (v/v): toluene (T), 5% EtOAc–toluene (T5), 10% ethyl acetate–toluene (T10), and 50% ethyl acetate–toluene (T50). Analytical HPLC was performed on a Luna C18 column (4.6 × 150 mm; Phenomenex) using a linear gradient of methanol and acetonitrile in 0.1 M TEAA at a flow rate of 1 ml/min and UV detection (260 nm). Preparative reversed-phase chromatography was carried out on an octadecyl silica column (25 × 250 mm, 20 μm, IOCB Prague); compounds were eluted with a linear gradient of methanol in water at 15 ml/min. UV spectra were taken on a Cary Bio 100 (Varian) spectrophotometer. High-resolution FAB mass spectra were recorded on a ZAB-EQ (VG Analytical) instrument with glycerol and thioglycerol as matrices. MALDI TOF spectra were performed on a Reflex IV (Bruker Daltonics). NMR spectra (δ, ppm; *J*, Hz) were measured on a Varian Unity-500 spectrometer (^{1}H at 500 MHz; ^{13}C at 125.7 MHz) in DMSO-d_6 at 20 °C. Chemical shifts were referenced to solvent signal (converted to δ-scale) using relations δ_H(DMSO) 2.50 ppm and δ_C(DMSO) 39.7 ppm. Proton 2D-COSY spectra were used for the structural assignment of coupled protons and 2D-ROESY spectra for detection of the NOE contacts. ^{13}C NMR chemical shifts and coupling constants *J*(C,P) were obtained from broadband proton-decoupled spectra using APT pulse sequence. IR spectra (ν, cm^{-1}) were performed on an IFS-55 (Bruker) instrument.

13-[(*tert*-Butyldiphenylsilyl)oxy]-2-hydroxymethyl-5,8,11-trioxatridecanenitrile (**5**)

The silyl derivative **2** (15 g, 38.6 mmol)[20] was treated with tosyl chloride (8.60 g, 45 mmol) in pyridine (100 ml) at room temperature for 16 h. The reaction mixture was cooled in an ice bath, quenched with water (20 ml) and, after 10 min, concentrated in vacuo. The residue was partitioned between ethyl acetate and water. The organic layer was washed several times with water, dried over anhydrous magnesium sulfate, filtered, and evaporated to dryness. The product was purified by silica gel chromatography using a linear gradient of acetone in toluene (0–10%). The obtained compound **3** (13.34 g, 64%, light yellow oil) was used in the next step without characterization.

Sodium hydride (1.73 g, 43.3 mmol) was added to a solution of ethyl cyanoacetate (6.6 g, 43.3 mmol) in dry DMF (150 ml) at 0 °C under argon atmosphere. Then, after 30 min of vigorous stirring, tosyl derivative **3** in DMF (50 ml) was added dropwise to the solution, and the reaction mixture was heated at 50 °C under argon for 15 h (TLC in T5). Several drops of acetic acid were added and the mixture was evaporated. The residue was partitioned between chloroform and water, the organic layer was washed several times with water, dried over anhydrous $MgSO_4$, filtered, and evaporated. The product was dissolved in ethanol (99%, 50 ml) and sodium borohydride (2.2 g, 43.3 mmol) was added at 0 °C. After stirring for 30 min, TLC in T5 indicated the absence of the starting material. Acetic acid (10.4 g, 173.2 mmol) was slowly added to destroy the excess of sodium borohydride, and the mix-

ture was stirred at room temperature for 30 min. The solvent was evaporated and the residue partitioned between chloroform and water. The organic layer was washed several times with water, dried over anhydrous $MgSO_4$, filtered, and evaporated. The product **5** was purified by silica gel chromatography using a linear gradient of acetone in toluene (0–20%). Yield 3.22 g (60%, light yellow oil). ^{1}H NMR (DMSO-d_6): 7.64 m, 4 H and 7.42 m, 6 H (arom. H); 5.28 t, 1 H, J = 5.6 (OH); 3.74 dd, 2 H, J = 4.9, 5.3 and 3.52 m, 14 H (OCH_2); 2.86 dddd, 1 H, J = 5.2, 5.6, 6.6 and 9.2 (CH-C); 1.76 m, 2 H (C-CH_2); 0.97 s, 9 H ($(CH_3)_3C$). ^{13}C NMR (DMSO-d_6): 133.31, 2 C (arom. C); 135.29, 4 C, 129. 96, 2 C and 128.00, 4 C (arom. C); 121.65, 1 C (CN); 71.81, 70.20, 70.03, 69.95, 69.95, 69.78, 67.50, 63.44 and 61.175, 8 C (OCH_2); 31.565, 1 C (CH); 28.31, 2 C (CH_2); 26.795 and 18.95, 4 C ($(CH_3)_3C$). FAB HR (glycerol, thioglycerol, methanol): For $C_{27}H_{39}NNaO_5Si$ calculated 508.2495; found 508.2448 [(M + Na].

2-[(4,4′-Dimethoxytrityl)oxy]-13-hydroxy-5,8,11-trioxatridecanenitrile (**7**)

The silyl derivative **5** (4.9 g, 10.4 mmol) was treated with 4,4′-dimethoxytrityl chloride (4.5 g, 13.11 mmol) in dry pyridine (50 ml) at room temperature overnight (TLC in T). Methanol (5 ml) was added and, after 1 h, the reaction mixture was concentrated to half volume in vacuo (30 °C bath temperature). The residue was partitioned between chloroform and water, the organic layer was washed several times with an ice-cold 10% aqueous citric acid to remove pyridine and then with water, dried over anhydrous $MgSO_4$, filtered, and evaporated. The residue was treated with 0.5 M TBAF·$3H_2O$ in THF (60 ml) for 1 h. TLC in T10 showed complete desilylation. Water (20 ml) was added and the mixture was concentrated in vacuo. The residue was partitioned between chloroform and water, the organic layer was washed several times with water, dried over anhydrous Na_2SO_4, filtered, and evaporated. The product **7** was purified by silica gel chromatography using a linear gradient of acetone in toluene (0–30%). Yield 3.22 g (60%, light yellow oil). ^{1}H NMR ($CDCl_3$): 7.45 m, 2 H; 7.34 m, 4 H; 7.30 m, 2 H; 7.22 m, 4 H; 6.84 m, 4 H (arom. H); 3.79 s, 6 H (CH_3-O-DMTr); 3.52–3.73 m, 14 H (H 4, 6, 7, 9, 10, 12, 13); 3.29 dd, 1 H, J = 9.0 and 5.0 (Ha-CHb-O); 3.21 dd, 1 H, J = 9.0 and 6.4 (Hb-CHa-O); 3.03 dddd, 1 H, $J_{2,3}$ = 9.5, 5.7, $J_{2,CH2O}$ = 6.4, 5.0 (H-2); 2.47 t, 1 H, $J_{OH,13}$ = 5.9 (OH); 1.9 ddt, 1 H, J_{gem} = 14.0, $J_{3a,2}$ = 9.5, $J_{3a,4}$ = 5.0 (H-3a); 1.86 ddt, 1 H, J_{gem} = 14.0, $J_{3b,4}$ = 8.2, 5.7, $J_{3b,2}$ = 5.7 (H-3b). ^{13}C NMR ($CDCl_3$): 158.56 (C-*p*-C_6H_4-DMTr); 144.34 (C-*i*-C_6H_5-DMTr); 135.44 and 135.48 (C-*o*-C_6H_4-DMTr); 129.99 (CH_3-*o*-C_6H_4-DMTr); 128.03 (C-*o*-C_6H_5-DMTr); 127.91 (C-*m*-C_6H_5-DMTr); 126.91 (C-*p*-C_6H_5-DMTr); 120.84 (CN); 113.20 (C-*m*-C_6H_4-DMTr); 86.37 (C-DMTr); 72.45, 70.58, 70.38, 70.30, 67.62, 67.07, 62.66, 61.71 (CH_2-13, 12, 10, 9, 8, 6, 4 + CH_2O); 55.21 (CH_3-O-DMTr); 29.51 (CH-2); 29.38 (CH_2-3). IR ($CHCl_3$): 3596, 3487, 2840, 2246, 1608, 1583, 1509, 1495, 1455, 1443–1447, 1254, 1176, 1153, 1121, 1096, 1083, 1015, 1036, 1003, 915, 830, 700–704. FAB (DS, CH_3CN, ($C_{32}H_{39}NO_7$, 549.27): 594.7 (M + 2 Na – H), 1143.3 (2 M + 2 Na – H). For $C_{32}H_{39}NO_7$ (549.3) calculated: 67.48% C, 7.15% H, 2.55% N; found: 68.23% C, 7.40% H, 2.15% N.

12-Cyano-13-[(4,4′-dimethoxytrityl)oxy]-3,6,9-trioxatridecanehydrogensuccinate (**9**)

The 4,4′-dimethoxytrityl derivative **7** (0.4 g, 0.79 mmol) in pyridine (5 ml) was treated with succinic anhydride (0.79 g, 7.9 mmol) in the presence of 4-dimethylaminopyridine (0.36 g, 2.9 mmol) at room temperature overnight. TLC in T50 showed complete esterification. The reaction was quenched with water (0.5 ml), and the solution was concentrated in vacuo. The residue was partitioned between chloroform and water. The organic layer was washed

several times with an ice-cold, 10% aqueous citric acid to remove DAP, and finally with water. The organic layer was dried over anhydrous Na_2SO_4, filtered, and evaporated to dryness. The product 9 was afforded by chromatography on a C18 column using a linear gradient of methanol in water. Yield 0.45 g (94%, light yellow oil). ^{1}H NMR (DMSO-d_6): 7.40 m, 2 H (H-*o*-C_6H_5-DMTr); 7.33 m, 2 H (H-*m*-C_6H_5-DMTr); 7.26 m, 4 H (H-*o*-C_6H_4-DMTr); 7.24 m, 1 H (H-*p*-C_6H_5-DMTr); 6.91 m, 4 H (H-*m*-C_6H_4-DMTr); 4.08 m, 2 H (H-1); 3.74 s, 6 H (CH_3-O-DMTr); 3.38–3.61 m, 12 H (H-2, 4, 5, 7, 8, 10); 3.08, 3.22 m, 2 H (CH_2O); 3.05 m, 1 H (H-12); 2.43, 2.48 m, 4 H (H-2, 3 hydrogensuccinate); 1.74, 1.83 m, 2 H (H-11). ^{13}C NMR ($CDCl_3$): 172.41, 173.80 (CO); 158.40 (C-*p*-C_6H_4-DMTr); 144.71 (C-*i*-C_6H_5-DMTr); 135.30 and 135.37 (C-*i*-C_6H_4-DMTr); 129.82 (C-*o*-C_6H_4-DMTr); 128.14 (C-*m*-C_6H_5-DMTr); 127.75 (C-*o*-C_6H_5-DMTr); 127.05 (C-*p*-C_6H_5-DMTr); 121.41 (CN); 113.49 (C-*m*-C_6H_4-DMTr); 85.87 (C-DMTr); 69.95, 69.91, 69.81, 69.70, 68.44, 67.29, 66.55, 63.53, 62.51 (CH_2-1, 2, 4, 5, 7, 8, 10 + CH_2O); 55.25 (CH_3-O-DMTr); 29.15 (CH-2); 28.92, 29.02 (C-2, 3 hydrogensuccinate); 28.68 (CH_2-11). IR ($CHCl_3$): 3517, 2840, 2246, 1734, 1608, 1584, 1509, 1495, 1455, 1443–1447, 1254, 1121, 1099, 1083, 1076–1079, 1053, 1036, 1013, 1002, 915, 831, 700–704, 635. ES HR (NOBA, CH_3CN): For $C_{36}H_{43}NO_{10}$ calculated 648.2809; found 648.2809 [(M^-)]. For $C_{36}H_{43}NO_{10}$ (649.3) calculated: 66.55% C, 6.67% H, 2.16% N; found: 64.67% C, 7.66% H, 2.35% N.

Preparation of 2-Cyanoethyl-Modified LCAA-CPG

The LCAA-CPG 500 A, 80–120 mesh (1 g) was treated with a solution of 3% dichloroacetic acid in 1,2-dichloroethane (20 ml) at room temperature overnight. The suspension was filtered under argon, the CPG support was washed successively with 10% triethylamine in dichloromethane, dichloromethane, and ether, and finally dried in vacuo over phosphorus pentoxide. The support was treated with hydrogensuccinate of **7** (61 mg, 0.1 mmol), DAP (12 mg, 0.1 mmol), triethylamine (0.28 ml, 2 mmol), and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (382 mg, 2 mmol) in pyridine (10 ml) under gentle shaking at room temperature for 24 h. The modified CPG was washed with pyridine, and the unreacted amino groups were acetylated using a capping mixture (Table I). The loading of the ligand to CPG varied between 40 and 50 μmol/g (determined spectrophotometrically as the released dimethoxytrityl cation).

Solid-Phase Synthesis of Oligonucleotide 3′-Phosphates

The oligonucleotides were synthesized as trityl-off compounds on the cyanoethyl-modified LCAA-CPG at 0.5 μmol scale on the GenSyn V02 DNA/RNA synthesizer according to the protocol given in Table I.

MALDI-TOF: **d(T$_9$Gp)** ($C_{100}H_{131}N_{23}O_{70}P_{10}$, 3083.477): 3004.419 [M – HPO_3 + 2 H], 3084.384 [M + H], 3106.336 [M + Na – H], 3198.230 [M + 5 Na + H]; **d(T$_9$Cp)** ($C_{99}H_{131}N_{21}O_{70}P_{10}$, 3043.471): 2965.599 [M – HPO_3 + 2 H], 3044.585 [M + H], 3067.545 [M + Na + H], 3159.390 [M + 5 Na + H]; **d(T$_9$Ap)** ($C_{100}H_{131}N_{23}O_{69}P_{10}$, 3067.483): 2989.425 [M – HPO_3 + 2 H], 3068.421 [M + H], 3091.367 [M + Na]; **d(T$_9$Tp)** ($C_{100}H_{132}N_{20}O_{71}P_{10}$, 3058.471): 3059.273 [M + H], 3081.694 [M + Na], 3174.605 [M + 5 Na + H].

TABLE I
Oligonucleotide synthetic protocol

Cyclus	Procedure	Reagent	Time, s
1.	Detritylation	3% CCl3COOH in 1,2-dichloroethane	180
2.	Phosphoramidite coupling	0.05 M phosphoramidite in CH_3CN (100 µl) 0.50 M tetrazolee in CH_3CN (100 µl)	180[c]
3.	Capping	20% acetic anhydride in CH_3CN (150 µl) DAP[a]/2,4,6-trimethylpyridine/CH_3CN (6/30/70; w/v/v (150 µl)	60[c]
4.	Oxidation	1.1 M *t*-BuOOH[b] in dichloromethane (300 µl)	60[c]
5.	Cleavage from CPG and deprotection	30% aq. ammonia	16 h/55 °C

[a] 4-Dimethylaminopyridine; [b] *tert*-butylhydroperoxide; [c] recycling of reagents through the column.

Enzymatic Dephosphorylation of Oligonucleotide 3′-Phosphates

The appropriate oligonucleotide 3′-phosphate (12 nmol) was treated with alkaline phosphatase (4.3×10^{-4} U) in 50 m M Tris pH 10.55 (80 µl) at 35 °C for 45 min. Then, an aliquot (10 µl) of the mixture was analyzed by HPLC on the reversed-phase column (Fig. 4a). The co-injection of the phosphorylated and dephosphorylated oligonucleotides resulted in two peaks (Fig. 4b).

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REFERENCES

1. Sambrook J., Fritsch E. F., Maniatis T. in: *Molecular Cloning: A Laboratory Manual*, 2nd ed. (C. Nolan, Ed.), pp. 561–572. Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.
2. Verma S., Eckstein F.: *Annu. Rev. Biochem.* **1998**, *67*, 99.
3. Shabarova Z. A., Merenkova I. N., Oretskaya T. S., Sokolova N. I., Skripkin E. A., Alexeyeva E. V., Balakin A. G., Bogdanov A. A.: *Nucleic Acids Res.* **1991**, *19*, 4247.
4. Jang G. Y., Steffens D. L.: *Nucleic Acids Res.* **1997**, *25*, 922.
5. Horn T., Urdea M.: *Tetrahedron Lett.* **1986**, *27*, 4705.
6. Markiewicz W. T., Wyrzykiewicz T. K.: *Nucleic Acids Res.* **1989**, *17*, 7149.
7. Langenegger S. M., Moesch L., Natt F., Hall J., Häner R.: *Helv. Chim. Acta* **2003**, *86*, 3476.

8. Adams S. P., Kavka K. S., Wykes E. J., Holder I. B., Galupi G. R.: *J. Am. Chem. Soc.* **1983**, *105*, 661.
9. Nadeau J. G., Singleton Ch. K., Kelly G. B., Wight H. L., Gough G. R.: *Biochemistry* **1984**, *23*, 6153.
10. Krynetskaya N. F., Zayakina G. V., Oretskaya T. S., Volkov F. M., Shabarova Z. A.: *Nucleosides Nucleotides* **1986**, *5*, 33.
11. Schwartz M. E., Breaker R. R., Asteriadis G. T., Gough G. R.: *Tetrahedron Lett.* **1995**, *36*, 27.
12. Cheruvallath Z. S., Cole D. L., Ravikumar V. T.: *Bioorg. Med. Chem. Lett.* **2003**, *13*, 281.
13. Guzaev A., Lönnberg H.: *Tetrahedron Lett.* **1997**, *38*, 3989.
14. Gryaznov S. M., Letsinger R. L.: *Tetrahedron Lett.* **1992**, *33*, 4127.
15. Dell'Aquila C., Imbach J. L., Rayner B.: *Tetrahedron Lett.* **1997**, *38*, 5289.
16. Zhang X., Jones R. A.: *Tetrahedron Lett.* **1996**, *37*, 3789.
17. Gupta K. C., Sharma P., Kumar P., Sathyanarayana S.: *Nucleic Acids Res.* **1991**, *19*, 3019.
18. Kumar P., Bose N. K., Gupta K. C.: *Tetrahedron Lett.* **1991**, *32*, 967.
19. Asseline U., Bonfils E., Kurfürst R., Chassignol M., Roig V., Thuong T. N.: *Tetrahedron* **1992**, *48*, 1233.
20. Matthew H. T., Engler M. M., Craig F. J.: *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2127.
21. Stetsenko D. A., Gait M. J.: *Bioconjugate Chem.* **2001**, *12*, 576.
22. Snášel J., Rejman D., Liboska R., Točík Z., Ruml T., Rosenberg I., Pichová I.: *Eur. J. Biochem.* **2001**, *268*, 980.
23. Birkus G., Rejman D., Otmar M., Votruba I., Rosenberg I., Holý A.: *Antiviral Chem. Chemother.* **2004**, *15*, 23.