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Bioorganic & Medicinal Chemistry 13 (2005) 4912–4920
Bioorganic & Medicinal Chemistry

Oligonucleotides containing 2′-*O*-[2-(2,3-dihydroxypropyl)amino-2-oxoethyl]uridine as suitable precursors of 2′-aldehyde oligonucleotides for chemoselective ligation

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Received 17 February 2005; revised 16 May 2005; accepted 20 May 2005

Abstract—2′-*O*-[2-(2,3-Diacetoxypropyl)amino-2-oxoethyl]uridine 3′-phosphoramidite was prepared and used in solid-phase synthesis to obtain oligonucleotides containing a 1,2-diol group, which may then be converted into a 2′-aldehyde group. The oligonucleotides were conjugated efficiently to various molecules by chemoselective ligation that involves an addition–elimination reaction between the 2′-aldehyde group and a suitable nucleophile, such as a hydrazine, a *O*-alkylhydroxylamine or an 1,2-aminothiol. The method was applied successfully to the conjugation of peptides to oligonucleotides at the 2′-position.

1. Introduction

Oligonucleotides and their analogues have been studied as sequence-specific inhibitors of gene expression for the last three decades.[1] However, their use as gene regulation agents within cells in culture has been plagued by poor cellular uptake, which has prompted the search for special transfection reagents.[2] One approach that promises to overcome this obstacle is conjugation of the oligonucleotide to a peptide that possesses cell penetration properties to facilitate trans-membrane delivery and/or alter internal localisation. Peptide conjugates of oligonucleotides are being studied extensively in an attempt to improve cytospecific targeting and delivery for various biological applications involving the inhibition of gene expression.[3–5] Numerous methods[6] of chemical synthesis of peptide–oligonucleotide conjugates have been developed so far, which may be divided into two general strategies. One is total stepwise solid-phase synthesis that requires an assembly of both peptide and oligonucleotide chains in a sequential fashion on a single solid support. The other is chemoselective ligation, which mainly refers to a solution-phase coupling of independently prepared and, if necessary, purified biomolecules mediated by chemical groups that react in a chemoselective manner. No single route is likely to be adequate for all applications and thus one has to test several strategies. Of the two conjugation strategies, the total solid-phase approach was recently applied to the preparation of a range of medium-sized 3′-peptide conjugates of antisense oligonucleotides and their structural analogues.[7,8] Solution-phase fragment coupling of peptides to oligonucleotides and analogues based either on a native ligation approach[9] or mediated by a water-soluble carbodiimide[10] has proven itself to be valuable in the case of longer sequences and for those peptides that contain certain amino acids, such as arginine, which at present cannot be introduced fully satisfactorily by a stepwise solid-phase route, although there are attempts to solve this problem by using a protected ornithine precursor[11,12] or by introducing a new protecting group for the guanidine moiety.[13] Another interesting method of chemoselective joining of unprotected peptide and oligonucleotide fragments is by an addition–elimination reaction of an electrophilic

Keywords: Oligonucleotide; Conjugate; Aldehyde; Oxime; Hydrazine.
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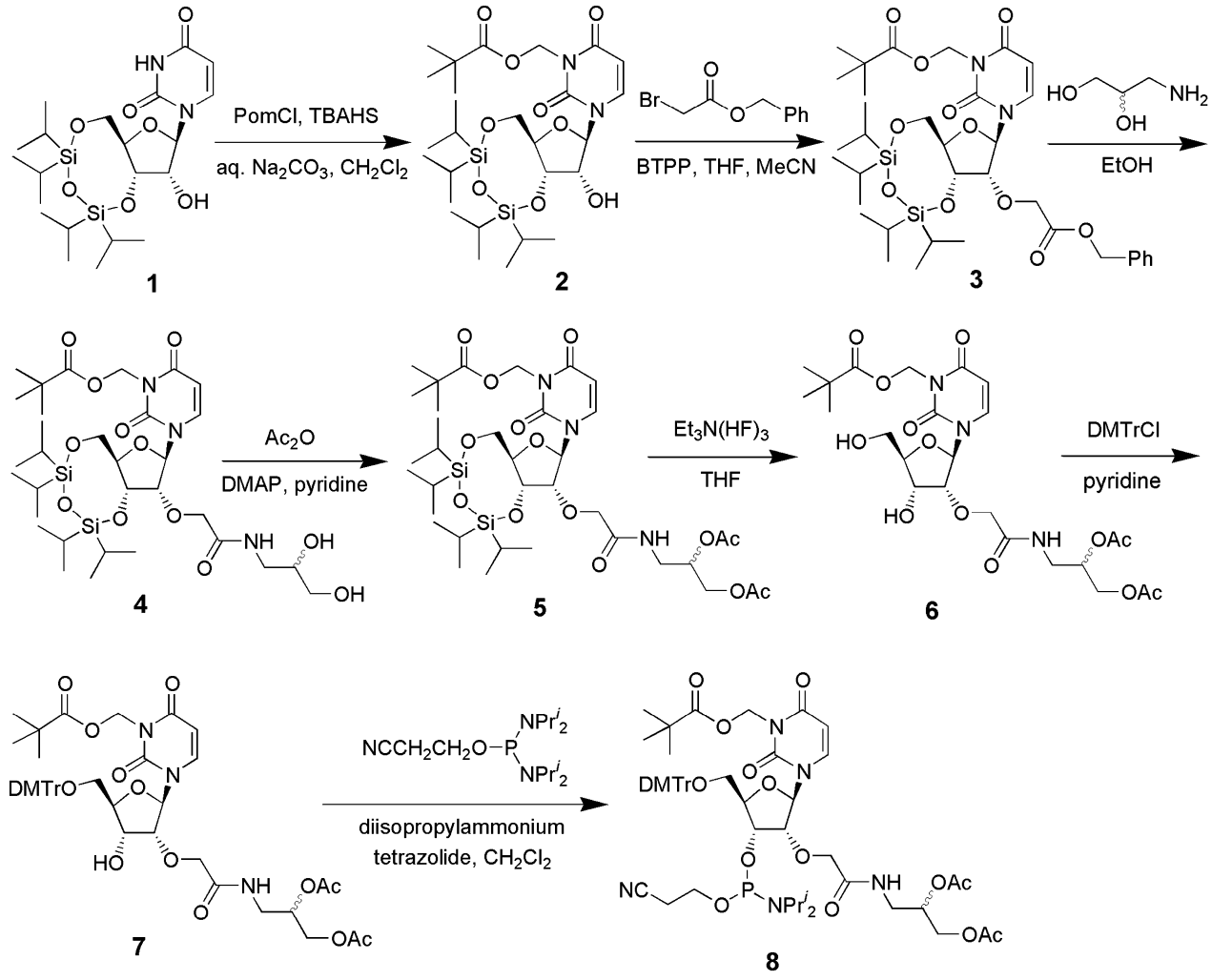
doi:10.1016/j.bmc.2005.05.024

carbonyl group, for example, an aldehyde, with a suitable nucleophile, for example, hydrazine, *N*-alkoxyamine or 1,2-aminothiol. Oligonucleotide modification reagents that introduce an aldehyde group are attractive in providing electrophilic functionalisation of oligonucleotides. The main advantage of such functionalisation is that chemoselective reaction is possible with a range of nucleophiles, which (a) is carried out under mildly acidic aqueous conditions, (b) is reversible, that is, the bond is hydrolysable under strongly basic or acidic conditions, (c) is free from any additives, effectors or enhancers that may complicate the isolation of a final product and (d) does not require an additional pre-activation step, such as that required for a carboxylic acid group. Accordingly, Forget et al.[14] proposed the generation of an aldehyde group under mild conditions of periodate oxidation of a 1,2-diol moiety, which was introduced readily at the 5′-end of an oligonucleotide by automated solid-phase synthesis by use of a protected 1,2-diol phosphoramidite containing an acetal protecting group that could be removed under mildly acidic conditions. Chemoselective ligation of peptides to oligonucleotides at the 5′-end or alternatively at the 3′-end by use of an appropriate 1,2-diol-linked solid support[15] was accomplished by oxime or thiazolidine formation. On the other hand, functionalisation of the 2′-position of ribose moiety with a 1,2-diol group makes possible the introduction of the modification via a corresponding phosphoramidite into any preselected site of an oligonucleotide chain, whether at the 3′- or 5′-terminus or in the middle.[16] Thus, both ends remain free for labelling or for an enzymatic reaction, for example, 5′-phosphorylation or PCR. Therefore, by use of a previously synthesised 2′-*O*-(2,3-dibenzoyloxypropyl)uridine 3′-phosphoramidite,[17] a masked 1,2-diol group was introduced into the 2′-position of a sugar residue that is later deprotected and oxidised by periodate to afford the 2′-aldehyde oligonucleotide. Subsequent conjugation was accomplished by either oxime, thiazolidine or hydrazine chemistry and thus provided the possibility of introduction of a peptide moiety into any defined position within an oligonucleotide sequence.[18] An additional advantage of the 2′-conjugation is that it is possible to couple several peptide sequences to a single oligonucleotide if more than one 2′-aldehyde modification is introduced.[18] Recently, we improved upon the preparation of 2′-aldehyde oligonucleotides and briefly described another 2′-diol phosphoramidite that incorporates a new and longer diol side chain, which was obtained by the aminolysis of the corresponding 2′-carboxymethyl ester group.[19] It is also important that the related 2′-*O*-carboxamidomethyl modifications[20] were shown to increase thermal stability of corresponding duplexes with a complementary RNA, which is essential for antisense inhibition.[21] Here, we would like to report the full details of the preparation of 2′-*O*-[2-(2,3-diacetoxypropyl)amino-2-oxoethyl]uridine 3′-phosphoramidite and its incorporation into an oligonucleotide chain by machine-assisted solid-phase synthesis. Further versatility of the method is shown by efficient conjugation of a number of nucleophilic compounds, including several peptides, to oligodeoxynucleotides at the site of the new 2′-aldehyde modification.

2. Results and discussion

Previously, we had published the preparation of 2′-modified oligonucleotides containing 2′-*O*-(2,3-dihydroxypropyl)uridine.[17] This method involved a catalytic dihydroxylation of the protected 2′-*O*-allyluridine with osmium tetroxide and *N*-methylmorpholine *N*-oxide to afford the 1,2-diol function. The corresponding 2′-modified phosphoramidite was obtained in reasonable yield and was then successfully used in machine-assisted oligonucleotide synthesis. The 1,2-diol moiety was masked by benzoyl groups, which were removed under standard conditions of the final concd ammonia treatment. Our studies confirmed that the resultant 2′-alkoxy linkage does not interfere significantly with the duplex formation with the intended RNA target. However, to be able to increase duplex stability even further and to try a different linker length, another successful approach to the synthesis of 2′-electrophilic nucleosides was adopted later.[19] The chemical synthesis of the 2′-*O*-[2-(2,3-diacetoxypropyl)amino-2-oxoethyl]uridine building block **8** is outlined in Scheme 1. The starting 3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine **1** was obtained from uridine, as reported previously.[22,23] Protection of the 3-position of uracil is required to make the subsequent alkylation of the 2′-position regiospecific. This imido function was protected from any unwanted side reaction by use of an ammonia-labile pivaloyloxymethyl (Pom) group.[24] The Pom-protecting group was introduced regioselectively under conditions of phase-transfer catalysis.[25] When **1** was allowed to react with excess chloromethyl pivalate in a biphasic system aq sodium carbonate–dichloromethane in the presence of catalytic amounts of tetra-*n*-butylammonium hydrogen sulfate (TBAHS) for 48 h under vigorous stirring, N^3-pivaloyloxymethyl derivative **2** was isolated in good yield after column chromatography. Alkylation of compound **2** with benzyl bromoacetate and the phosphazene base P_1-*tert*-butyltris(tetramethylene) (BTPP) gave ester **3** in excellent yield. Ester **3** was converted to 2,3-dihydroxypropylamide **4** by reaction with excess 3-amino-1,2-propanediol in dry ethanol. The reaction was allowed to proceed at ambient temperature overnight. The crude nucleoside containing a 1,2-diol at the 2′-position of the sugar moiety was treated with acetic anhydride in pyridine with a catalytic amount of 4-dimethylaminopyridine (DMAP) to give the diacetate **5**. Compound **5** was then desilylated smoothly with triethylamine trihydrofluoride[23] in THF and then converted into the 5′-*O*-dimethoxytrityl (DMTr) ether **7** by the known procedure.[26] Subsequent phosphitylation of the 3′-hydroxy group in an inert atmosphere using bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine in dichloromethane in the presence of diisopropylammonium tetrazolide[27] afforded the title phosphoramidite **8**. The synthesis was much shorter, more straightforward and high yielding than in the case of that described previously.[17] In addition, the synthetic route avoids the use of highly toxic reagents such as osmium tetroxide.

Next, the phosphoramidite **8** was evaluated in machine-assisted solid-phase oligonucleotide synthesis by the standard 2-cyanoethyl phosphoramidite method. The



Scheme 1. Preparation of 2′-*O*-[2-(2,3-diacetoxypropyl)amino-2-oxoethyl]uridine phosphoramidite (**8**). Abbreviations: TIPS, 1,1,3,3-tetraisopropyldisiloxane-1,3-diyl; Pom, pivaloyloxymethyl; TBAHS, tetra-*n*-butylammonium hydrogen sulfate; BTPP, phosphazene base P_1-*tert*-butyltris(tetramethylene); DMTr, 4,4′-dimethoxytrityl.

average coupling efficiency of the novel monomer at a slightly higher concentration in dry acetonitrile and prolonged reaction time was found to be more than 97%. On completion of the synthesis, oligonucleotides were cleaved from their respective solid supports and deprotected by concd aq ammonia treatment at 55 °C overnight. Reaction mixtures obtained were analysed by reversed-phase HPLC in ion-pair mode and by MALDI-TOF mass spectroscopy. Both 15-mers **I** and **II** are oligodeoxyribonucleotides complementary to the HIV-1 TAR RNA apical stem-loop, the binding site for the HIV-1 trans-activator protein Tat[18] (Table 1). Oligonucleotide **III** is a 3′-fluorescently labelled 21-mer oligodeoxyribonucleotide containing several 2′-*O*-methyluridine insertions that enhance RNA binding and convey additional stability towards nucleases. Its sequence corresponds to the coding region 153–173 of GL3 firefly luciferase.[28]

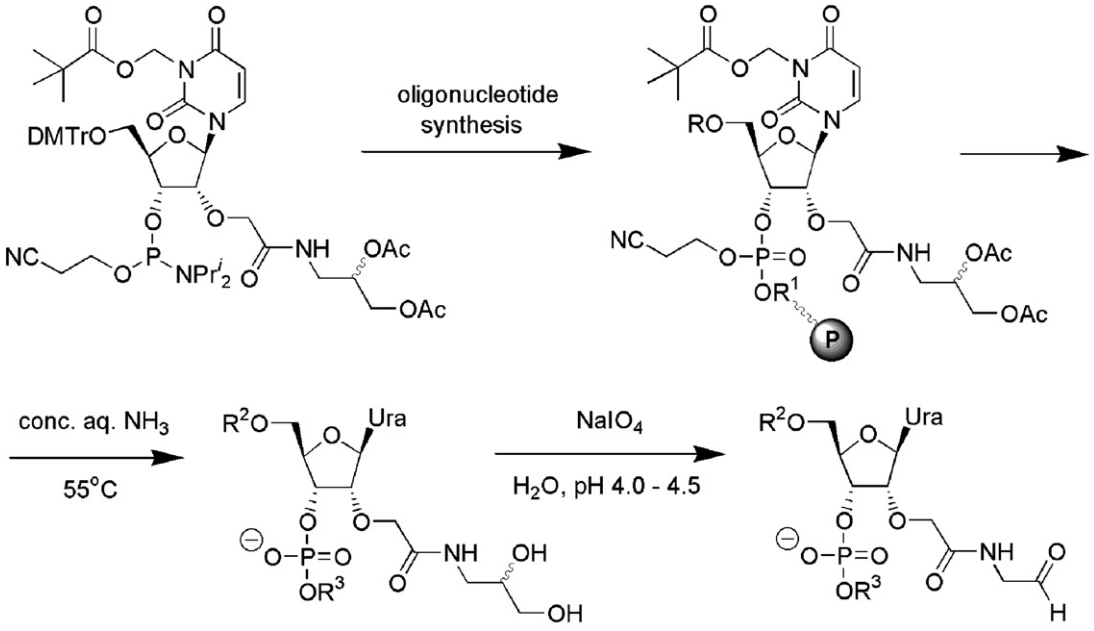
Oxidation of the 1,2-diol group was carried out by use of a 50-fold excess of sodium metaperiodate in acetate buffer (pH 4.0–4.5) at ambient temperature for 0.5–1 h (Scheme 2). Then excess ethylene glycol was added to the solution to quench the remaining periodate.

The resulting 2′-aldehyde oligonucleotides **I**–**III** were coupled successfully to various nucleophilic molecules via an addition–elimination reaction to furnish hydrazine, thiazolidine or oxime-linked conjugates (Table 1). The conjugated moieties include a fluorescent marker group and intercalator (acridine), succinic dihydrazide that can be used for oligonucleotide cross-linking and further chemoselective ligation, and several peptides: model cysteinylglycine dipeptide as well as *N*-aminooxyacetyl-Tat basic domain[29] and laminin[30] peptides (Scheme 3). Concentrations of small molecules and peptides were 50 and 10 mM, respectively. To obtain a high yield of structurally diverse types of conjugates, the reaction is best carried out in acetate buffer at a slightly acidic pH of 4.0–4.5 for hydrazone and oxime or 4.5–5.0 for thiazolidine at ambient temperature overnight in the presence of organic co-solvent, DMSO or acetonitrile. Note that the former is not suitable in the case of thiazolidine, as it may cause disulfide formation.[31] Use of

Table 1. Properties of the 2′-diol-modified oligonucleotides and their conjugates[a]

No.	Type[b]	Oligonucleotide sequence, 5′-3′ or conjugated molecule	MALDI-TOF, calcd/found	Retention time, min[c]	Yield, %[d]
I	—	CTCCCAGGC U* CAAAT	4628.8/4627.4	15.3	—
II	—	CU* CCCAGGCTCAAAT	4628.8/4628.4	15.7	—
III	—	CTuACGCuGAGuACTuCGAU* T[e]	7220.3/7219.9	23.4	—
I.9	H	9-Hydrazinoacridine (**9**)	4789.9/4787.9	20.2	74
I.10	H	Succinic dihydrazide (**10**)	4726.9/4723.4	14.9	72
II.10	H	Succinic dihydrazide (**10**)	4726.9/4727.2	15.0	82
II.11	T	H-Cys-Gly-OH (**11**)	4778.8/4777.1	16.2	79
II.12	O	H_2NOCH_2CO-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg-NH_2 (**12**)	5989.7/5988.7	13.9[f]	>90
III.13	O	H_2NOCH_2CO-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg-NH_2 (**13**)	8109.7/8112.0	—[g]	—

[a] 2′-Deoxyribonucleotides are in capital, 2′-*O*-methylribonucleotides in lowercase and U* is 2′-*O*-[2-(2,3-dihydroxypropyl)amino-2-oxoethyl]uridine.
[b] Type of linkage between oligonucleotide and conjugated moiety: H, hydrazine; T, thiazolidine and O, oxime.
[c] RP-HPLC in ion-pair mode, see Section 3.
[d] Conversion of oligonucleotide peak to the conjugate peak calculated from RP-HPLC traces.
[e] Oligonucleotide **III** was labelled with 6-carboxyfluorescein at the 3′-end during solid-phase synthesis.
[f] Conjugate **II.12** was purified by RP-HPLC, see Section 3.
[g] Purified by PAGE.



Scheme 2. Solid-phase synthesis and deprotection of 2′-diol oligonucleotides and preparation of 2′-aldehyde oligonucleotides. Abbreviations: R, R^1: protected oligonucleotide chain; R^2, R^3: unprotected oligonucleotide chain.

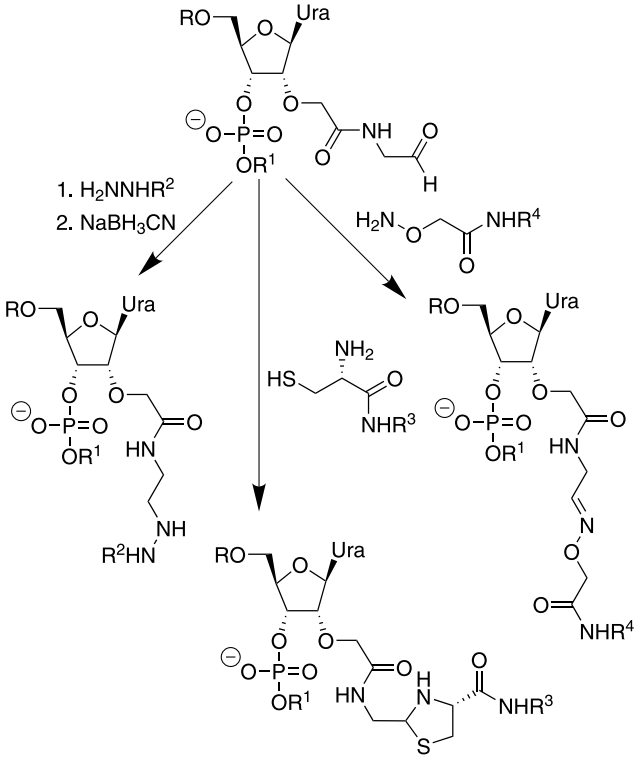
acetonitrile as a co-solvent that can be easily removed by evaporation usually provided the best results. The oxime and thiazolidine linkages are stable over a wide pH range, whilst hydrazones formed from acyl hydrazides, for example, succinyl dihydrazide are better to be reduced to hydrazines by $NaBH_3CN$ to add stability. Conversion yields of the conjugates were good, as may be seen from the corresponding HPLC pictures (Figs. 1 and 2). MALDI-TOF spectra showed the correct molecular masses for the peaks corresponding to target conjugates (Table 1). Products obtained were isolated by RP-HPLC or by denaturing polyacrylamide gel electrophoresis.

In conclusion, we have presented here an efficient and reliable method for the preparation of oligonucleotides containing a 1,2-diol as a masked aldehyde precursor at the 2′-position of a sugar residue using our newly developed modification reagent, 2′-*O*-[2-(2,3-diacetoxypropyl)amino-2-oxoethyl]uridine 3′-phosphoramidite. After standard ammonia deprotection and periodate oxidation, the modified oligonucleotides were conjugated efficiently to a number of target molecules. The method was shown to be a useful route for the attachment of peptides, particularly those containing several unprotected arginine residues, for example, Tat basic domain peptide, to the 2′-position of oligonucleotides and their analogues for cellular uptake studies.

3. Experimental

3.1. General

Chemicals were obtained from commercial suppliers and used without further purification unless otherwise noted.



Scheme 3. Preparation of oligonucleotide 2′-conjugates via hydrazine, thiazolidine or oxime formation. Abbreviations: R, R^1: unprotected oligonucleotide chain; R^2: 9-acridinyl or $–CO(CH_2)_2CONHNH_2$; R^3: $–CH_2CO_2H$; R^4: peptide moiety.

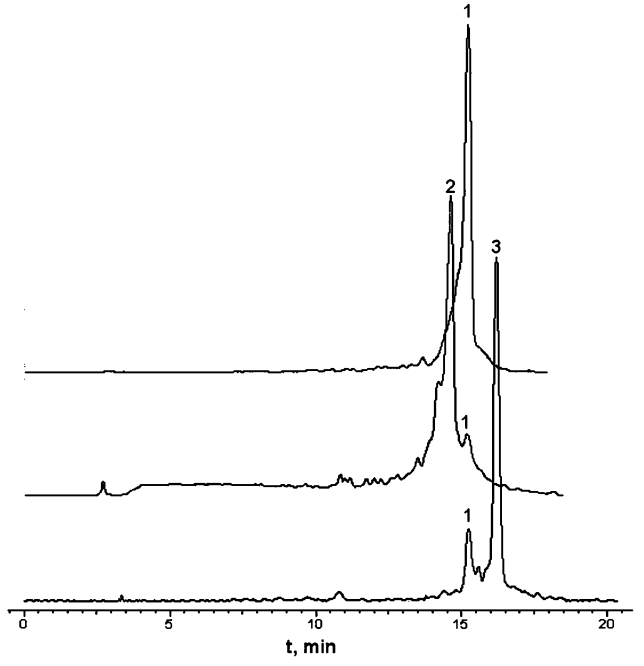


Figure 1. Reversed-phase HPLC traces (ion-pair mode) of crude oligonucleotide 2′-conjugates: (1) parent 2′-diol oligonucleotide **II**; (2) conjugate **II.10** with succinyl dihydrazide; (3) conjugate **II.11** with L-cysteinylglycine. For HPLC conditions, see Section 3.

Chloromethyl pivalate (Pom Cl) tetra-*n*-butylammonium hydrogen sulfate (TBAS) benzyl bromoacetate, 3-amino-1,2-propanediol, 4-dimethylaminopyridine

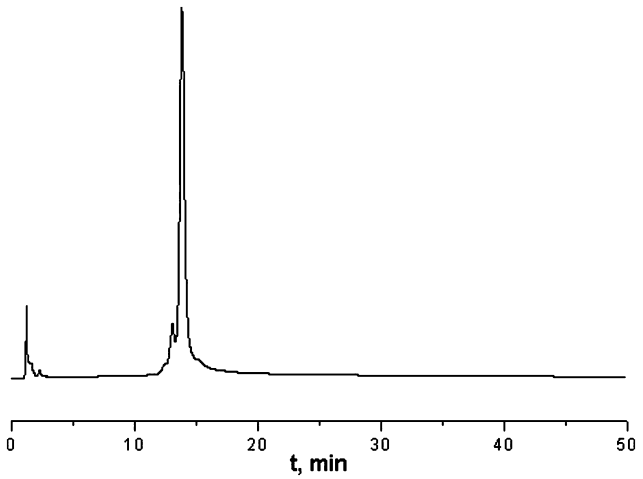


Figure 2. Reversed-phase HPLC of crude conjugate **II.12** of oligonucleotide **II** with Tat basic domain peptide (**12**). For HPLC conditions, see Section 3.

(DMAP), triethylamine trihydrofluoride and sodium cyanoborohydride were purchased from Aldrich. Phosphazene base P_1-*tert*-butyltris(tetramethylene), acetic anhydride, bis(*N*,*N*-diisopropylamino)-2-cyanoethoxy phosphine and sodium metaperiodate were from Fluka. 4,4′-Dimethoxytrityl chloride (DMTrCl) was purchased from Avocado. Cysteinylglycine was acquired from Bachem. Peptides **12** and **13** were prepared, as described previously.[18] Diisopropylammonium tetrazolide was obtained from diisopropylamine (BDH) and 1*H*-tetrazole solution in acetonitrile (Glen Research).[27] Dichloromethane (BDH) was used after refluxing over and distillation from CaH_2. DMF (Fisher) was distilled in vacuo and used fresh. Other solvents: benzene, chloroform, ethyl acetate, acetone, acetonitrile, THF, DMSO, hexane, absolute ethanol and methanol were used as received.

NMR spectra were recorded on Bruker AM-300, DRX-500 and WM-250 spectrometers (500.13, 300.13 MHz for ^1H, 75.47, 62.90 MHz for ^{13}C and 121.50 MHz for ^{31}P). Chemical shifts (δ, ppm) for ^1H, ^{13}C and ^{31}P are referenced to internal solvent resonances and reported relative to $SiMe_4$ and 85% aq H_3PO_4, respectively. 2D Spectra involved the use of adapted COSY and HSQC techniques. Chemical shifts are accurate to within 0.01 ppm for ^1H and ^{13}C, CSSI are accurate to within 0.5 Hz.

MALDI-TOF spectra were recorded on a Voyager DE system (Applied Biosystems) in positive-ion mode using the following matrices: a 1:1 (v/v) mixture of 2,6-dihydroxyacetophenone (2,6-DHAP) (40 mg/ml in MeOH) and aq diammonium hydrogen citrate (80 mg/ml) for all oligonucleotides, and either 2,5-dihydroxybenzoic acid (2,5-DHBA) (10 mg/ml in 50% aq MeOH) or 2,4,6-trihydroxyacetophenone (2,4,6-THAP) (10 mg/ml in 50% aq MeOH) for the low molecular weight compounds. TLC was carried out on Merck DC Kieselgel 60 F254 aluminium sheets. Compounds were visualised under short-wavelength UV and stained by

trifluoroacetic acid vapours for DMTr-containing species. Column chromatography was carried out on Kieselgel 60 0.040–0.063 mm (Merck).

HPLC analysis and purification of 5′-DMTr-protected oligonucleotides were carried out on a Tracor instrument using 4 × 250 mm DIAKS-130-CETYL column; buffer A: 0.1 M ammonium acetate (pH 7); buffer B: 0.1 M ammonium acetate, 40% MeCN, pH 7; gradient of B from 0% to 100% in 60 min; flow rate 1 ml/min; temperature 45 °C. Oligonucleotides and conjugates were analysed by a reversed-phase HPLC in ion-pair mode on a Waters HPLC system using a DIAKS-130-CETYL column (4 × 250 mm) and a logarithmic gradient: 0–45.6% B (1 min); 45.6–48.1% B (1 min); 48.1–51.3% B (3 min); 51.3–53.7% B (5 min); 53.7–56.2% B (10 min); 56.2–57.6% B (10 min); 57.6–58.7% B (10 min). Separation of oligomers with a retention time step of 1 min/unit was carried out; mobile phase A: H_2O/MeCN (95:5 v/v), 2 mM tetra-*n*-butylammonium dihydrogen phosphate, 48 mM KH_2PO_4, pH 7; mobile phase B: H_2O/MeCN (60:40 v/v), 2 mM tetra-*n*-butylammonium dihydrogen phosphate, 48 mM KH_2PO_4, pH 7; flow rate 1 ml/min and temperature 45 °C. Conjugates were purified by RP-HPLC on a Gilson HPLC system using a Beckman Ultrasphere ODS column (4.6 × 250 mm) and dual-wavelength detection (215 and 254 nm); buffer A: 5% of MeCN (v/v) in 0.1 M triethylammonium acetate, buffer B: MeCN; flow rate 1 ml/min, gradient of B in A: 0–5%, 5 min, 5–15%, 10 min, 15–40%, 30 min, 40–80%, 10 min and 80–0%, 10 min. Denaturing gel electrophoresis of oligonucleotides was performed in 15% PAGE containing 2 M urea in Tris–borate buffer (50 mM Tris–HCl, 50 mM boric acid and 1 mM EDTA, pH 7.5).

3.2. 3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)uridine (1)

This was prepared, as described previously.[22,23]

3.3. N^3-Pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (2)

The compound was synthesised according to the previously published method.[32] Chromatography: stepwise gradient of 0 → 1 → 2 → 3 → 4 → 5 → 6 → 8 → 10 → 12% EtOAc (v/v) in benzene (caution: harmful!). Yield 1.1 g (70%). R_f 0.51 ($CHCl_3$/MeOH, 98:2 v/v). MALDI-TOF (2,5-DHBA): $[M+Na]^+$ calcd 623.84, found 624.05; $[M+K]^+$ calcd 639.95, found 641.77. ^{1}H NMR (500.13 MHz, $CDCl_3$): δ 7.82 (d, 1H, $J_{5,6}$ = 8.0, *H*-6), 5.95 (s, 2H, CH_2OCO^{t}Bu), 5.82 (d, 1H, $J_{5,6}$ = 8.0, *H*-5), 5.75 (app t, 1H, J = 2.9, *H*-1′), 4.33 (br s, 2H, *H*-2′, *H*-3′), 4.15 (m, 1H, *H*-4′), 3.98 (d, 1H, J_{gem} = 12.0, $J_{4',5'a}$ = 2.6, *H*-5′a), 3.82 (d, 1H, J_{gem} = 12.0, $J_{4',5'b}$ = 1.9, *H*-5′b), 1.20 (s, 9H, ^{t}Bu), 1.10–0.80 (m, 28H, ^{i}Pr). ^{13}C NMR (62.50 MHz, $CDCl_3$): δ 178.00 (CH$_2$OC*O*^{t}Bu), 161.75 (*C*-4), 151.50 (*C*-2), 140.02 (*C*-6), 101.85 (*C*-5), 92.58 (*C*-1′), 85.33 (*C*-4′), 74.97 (*C*-2′), 70.20 (*C*-3′), 64.58 (*C*H$_2$OCO^{t}Bu), 61.65 (*C*-5′), 38.92 (*C*(CH$_3$)$_3$), 27.01 (C(*C*H$_3$)$_3$), 17.09 (CH(*C*H$_3$)$_2$), 13.39, 13.07, 12.96, 12.85 (*C*H(CH$_3$)$_2$).

3.4. 2′-*O*-(2-Benzyloxy-2-oxoethyl)-N^3-pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyldisiloxane-1,3-diyl)uridine (3)

Compound **2** (1.1 g, 1.83 mmol) was dried by co-evaporation with dry MeCN (3× 20 ml) and was then dissolved in dry MeCN/THF (100 ml, 1:1 v/v). Phosphazene base P$_1$-*tert*-butyltris(tetramethylene) (1.57 ml, 5.12 mmol), followed immediately by benzyl bromoacetate (0.72 ml, 4.57 mmol), was added to the stirred mixture at ambient temperature. TLC ($CHCl_3$/MeOH, 99:1 v/v) showed complete reaction after 3 h. The reaction mixture was evaporated and co-evaporated with benzene (3× 25 ml). The crude product was purified by column chromatography on silica gel (stepwise gradient of 0 → 1 → 2 → 3 → 4 → 5 → 6% EtOAc in benzene, v/v). Compound **3** was obtained as a white oil (1.3 g, 95%). R_f 0.35 ($CHCl_3$/MeOH, 99:1 v/v). MALDI- TOF (2,4,6-THAP): $[M+H]^+$ calcd 750.02, found 750.17; $[M+Na]^+$ calcd 772.0, found 772.51; $[M+K]^+$ calcd 788.11, found 788.63. ^{1}H NMR (300.13 MHz, $CDCl_3$): δ 7.90 (d, 1H, $J_{5,6}$ = 8.0, *H*-6), 7.40–7.30 (br s, 5H, Ph), 5.97 (d, 1H, J_{gem} = 9.5, CH_2OCO^{t}Bu), 5.91 (d, 1H, J_{gem} = 9.5, CH_2OCO^{t}Bu), 5.81 (br s, 1H, *H*-1′), 5.71 (d, 1H, $J_{5,6}$ = 8.0, *H*-5), 5.21 (d, 1H, J_{gem} = 12.5, OCH_2Ph) 5.18 (d, 1H, J_{gem} = 12.5, OCH_2Ph,), 4.61 (d, 1H, J_{gem} = 16.5, OCH_2CO), 4.48 (d, 1H, J_{gem} = 16.5, OCH_2CO), 4.25 (d, 1H, J_{gem} = 13.3, *H*-5′a), 4.22 (br s, 2H, *H*-3′, *H*-4′), 4.05 (s, 1H, *H*-2′), 3.98 (d, 1H, J_{gem} = 13.3, *H*-5′b), 1.20 (s, 9H, ^{t}Bu), 1.10–0.80 (m, 28H, ^{i}Pr). ^{13}C NMR (62.90 MHz, $CDCl_3$): δ 177.36 (CH$_2$OC*O*^{t}Bu), 169.66 (OCH$_2$*C*O), 161.61 (*C*-4), 149.97 (*C*-2), 138.36 (*C*-6), 135.42 (*i*-Ph), 128.53, 128.35 (Ph), 101.10 (*C*-5), 89.13 (*C*-1′), 82.46 (*C*-2′), 81.60 (*C*-4′), 68.42 (*C*-3′), 67.53 (O*C*H$_2$CO), 66.56 (O*C*H$_2$Ph), 64.48 (*C*H$_2$OCO^{t}Bu), 59.36 (*C*-5′), 38.82 (*C*(CH$_3$)$_3$), 27.01 (C(*C*H$_3$)$_3$), 17.42, 17.31, 16.98, 16.76 (CH(*C*H$_3$)$_2$), 13.46, 13.14, 12.91, 12.37 (*C*H(CH$_3$)$_2$).

3.5. 2′-*O*-[2-(2,3-Diacetoxypropyl)amino-2-oxoethyl]-N^3-pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyl disiloxane-1,3-diyl)uridine (5)

To a solution of 3-amino-1,2-propanediol (1.58 g, 17.3 mmol) in dry ethanol (25 ml), compound **3** (1.3 g, 1.74 mmol) was added. The resulting mixture was stirred overnight at ambient temperature and then evaporated to dryness, and the residue was dissolved in EtOAc (50 ml) and washed with water (50 ml) and 20% NaCl (2× 50 ml). The organic layer was dried (Na_2SO_4), filtered and evaporated. The residue was co-evaporated with dry pyridine (3× 20 ml) and dissolved in dry THF (15 ml) and pyridine (0.7 ml, 8.65 mmol), acetic anhydride (0.82 ml, 8.65 mmol) and catalytic amounts of DMAP were added. The reaction was stirred at room temperature for 5 h and then quenched by the addition of MeOH (1 ml), and after 10 min the mixture was evaporated to dryness, dissolved in EtOAc (50 ml), washed with water (50 ml), 5% $NaHCO_3$ (50 ml) and 20% NaCl (2× 50 ml), dried (Na_2SO_4), filtered and evaporated to dryness. The residue was co-evaporated with benzene (3× 25 ml) and chromatographed on silica gel (stepwise gradient of 0 → 5 → 10 → 20 → 30 → 40 → 50 → 60 →

75 → 100% $CHCl_3$ in benzene, v/v) to give compound **5**, which was obtained as a white solid (1.24 g, 87.4%). R_f 0.64 ($CHCl_3$/MeOH, 98:2 v/v). MALDI-TOF (2,4,6-THAP): $[M+H]^+$ calcd 817.06, found 816.64; $[M+Na]^+$ calcd 839.04, found 838.38; $[M+K]^+$ calcd 855.15, found 854.53. ^1H NMR (500.13 MHz, $CDCl_3$): δ 7.82 (dd, 1H, $J_{5,6}$ = 8.2, *H*-6), 7.22 (dd, 1H, J_{NH,CH_2} = 13.0, 6.5, CON*H*CH_2), 5.96 (d, 1H, J_{gem} = 8.8, CH_2OCO^{t}Bu), 5.90 (d, 1H, J_{gem} = 8.8, CH_2OCO^{t}Bu), 5.79 (br s, 1H, *H*-1′), 5.72 (d, 1H, $J_{5,6}$ = 8.2, *H*-5), 5.18 (m, 1H, C*H*(OAc)CH_2(OAc)), 4.35 (d, 1H, J_{gem} = 16.5, OCH_2-CO), 4.30 (d, 1H, J_{gem} = 16.5, OCH_2CO), 4.28 (m, 2H, CH(OAc)CH_2(OAc), *H*-5′a), 4.22 (dd, 1H, $J_{2',3'}$ = 3.5, $J_{3',4'}$ = 8.5, *H*-3′), 4.15 (m, 2H, CH(OAc)CH_2(OAc), *H*-4′), 3.98 (d, 1H, J_{gem} = 13.0, $J_{4',5'b}$ = 2.3, *H*-5′b), 3.86 (app t, 1H, *J* = 4.0, *H*-2′), 3.63 (m, 1H, CONHCH_2), 3.50 (m, 1H, CONHCH_2), 2.10, 2.07 (2s, each 3H, CH_3CO), 1.20 (s, 9H, ^{t}Bu), 1.10–0.80 (m, 28H, ^{i}Pr). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 177.24 (CH_2OCO^tBu), 170.61, 169.72 (CH_3C=O), 161.41 (*C*-4), 150.28 (*C*-2), 137.57 (*C*-6), 101.44 (*C*-5), 89.43 (*C*-1′), 83.09 (*C*-2′), 81.64 (*C*-4′), 70.52 (*C*H(OAc)-CH_2(OAc)), 70.32 (O*C*H_2CO), 68.91 (*C*-3′), 64.33 (*C*H_2OCO^{t}Bu), 63.03 (CH(OAc)*C*H_2(OAc)), 59.12 (*C*-5′), 39.38 (*C*$(CH_3)_3$), 38.91 (CONH*C*H_2), 26.99 (C(*C*$H_3)_3$), 20.94, 20.72 (*C*H_3CO), 17.44, 17.37, 17.00, 16.86 (CH(*C*$H_3)_2$), 13.38, 13.02, 12.89, 12.71 (*C*H$(CH_3)_2$).

3.6. 2′-*O*-[2-(2,3-Diacetoxypropyl)amino-2-oxoethyl]-N^3-pivaloyloxymethyluridine (6)

To a solution of **5** (1.21 g, 1.48 mmol) in THF (5 ml) in a screw-top Teflon flask (Nalgene) equipped with a magnetic stirring bar was added triethylamine trihydrofluoride (0.48 ml, 2.96 mmol) and the mixture was left stirring for 1.5 h at ambient temperature. The completion of deprotection was checked by TLC ($CHCl_3$/MeOH, 96:4 v/v) and then the mixture was diluted with EtOAc (50 ml), washed with 5% $NaHCO_3$ (2× 50 ml), water (50 ml), 5% citric acid (2× 50 ml), and 20% NaCl (50 ml) and then dried (Na_2SO_4) and evaporated to dryness. The residue was co-evaporated with $CHCl_3$ (3× 25 ml) and then chromatographed on silica gel column (stepwise gradient of 0 → 1 → 2 → 3 → 4% MeOH in $CHCl_3$, v/v). Yield 0.81 g (95.3%). R_f 0.26 ($CHCl_3$/MeOH, 92:8 v/v). MALDI-TOF (2,4,6-THAP): $[M+H]^+$ calcd 574.55, found 574.75; $[M+Na]^+$ calcd 596.54, found 591.44; $[M+K]^+$ calcd 612.64, found 612.28. ^1H NMR (500.13 MHz, $CDCl_3$): δ 8.05 (dd, 1H, $J_{5,6}$ = 8.3, *H*-6), 7.40 (br s, 1H, CON*H*CH_2), 5.96 (d, 1H, J_{gem} = 9.5, CH_2OCO^{t}Bu), 5.90 (d, 1H, J_{gem} = 9.5, CH_2OCO^{t}Bu), 5.85 (app t, 1H, *J* = 2.8, *H*-1′), 5.80 (d, 1H, $J_{5,6}$ = 8.0, *H*-5), 5.10 (m, 1H, C*H*(OAc)CH_2(OAc)), 4.38 (d, 1H, J_{gem} = 16.6, OCH_2-CO), 4.32 (m, 1H, *H*-3′), 4.30 (d, 1H, J_{gem} = 16.6, OCH_2CO), 4.25 (m, 1H, *H*-4′), 4.20 (m, 1H, CH(OAc)-CH_2(OAc)), 4.10 (m, 1H, CH(OAc)CH_2(OAc)), 4.04 (d, 1H, J_{gem} = 12.0, *H*-5′a), 4.02 (m, 1H, *H*-2′), 3.90 (d, 1H, J_{gem} = 12.0, *H*-5′b), 3.6 (m, 1H, CONHCH_2), 3.45 (m, 1H, CONHCH_2), 3.25 (br s, 1H, O*H*), 2.10 (br s, 1H, O*H*), 2.05 (m, 6H, *C*H_3CO), 1.20 (s, 9H, ^{t}Bu). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 177.58 (CH_2OCO^tBu), 171.13, 171.03 (CH_3C=O), 169.99 (OCH_2*C*O), 161.66 (*C*-4), 150.47 (*C*-2), 139.29 (*C*-6), 101.62 (*C*-5), 89.39 (*C*-1′), 84.26, 83.84 (*C*-2′, *C*-4′), 70.45 (*C*H(OAc)-CH_2(OAc)), 69.91 (O*C*H_2CO), 68.35 (*C*-3′), 64.48 (*C*H_2OCO^{t}Bu), 62.94 (CH(OAc)*C*H_2(OAc)), 60.53 (*C*-5′), 39.31 (CONH*C*H_2), 38.87 (*C*$(CH_3)_3$), 27.00 (C(*C*$H_3)_3$), 20.99, 20.75 (*C*H_3CO).

3.7. 2′-*O*-[2-(2,3-Diacetoxypropyl)amino-2-oxoethyl]-5′-*O*-(4,4′-dimethoxytrityl)-N^3-pivaloyloxymethyl uridine (7)

Compound **6** (0.77 g, 1.34 mmol) was co-evaporated with pyridine (3× 20 ml), dissolved in dry pyridine (25 ml) and cooled in an ice bath, and DMTrCl (0.73 g, 2.14 mmol) was added in one portion. The reaction was monitored by TLC until the starting nucleoside disappeared. After completion of the reaction, excess of DMTrCl was quenched with MeOH (1 ml), and after 10 min the mixture was evaporated two-thirds, diluted with $CHCl_3$ (50 ml), washed with 5% $NaHCO_3$ (2× 50 ml) and 20% NaCl (50 ml) and then dried (Na_2SO_4), evaporated and co-evaporated with toluene (3× 25 ml) and the residue was chromatographed on silica gel column (stepwise gradient of 0 → 20 → 25 → 30 → 50 → 100% $CHCl_3$ in toluene and further 1% MeOH in $CHCl_3$ + 1% pyridine v/v/v). Yield 0.99 g (84.3%). R_f 0.25 ($CHCl_3$/MeOH, 98:2 v/v). MALDI-TOF (2,4,6-THAP): $[M+Na]^+$ calcd 898.9, found 899.21; $[M+K]^+$ calcd 915.01, found 915.16. ^1H NMR (500.13 MHz, $CDCl_3$): δ 8.05 (dd, 1H, $J_{5,6}$ = 8.0, *H*-6), 7.40 (d, 2H, *J* = 7.8, *o*-Ph), 7.30 (d, 2H, *J* = 8.5, *o*-An), 7.25 (m, 4H, *m*,*p*-Ph, CON*H*CH_2), 6.80 (d, 2H, *J* = 8.5, *m*-An), 5.90 (m, 2H, CH_2OCO^{t}Bu), 5.85 (overlap, 1H, *H*-1′), 5.30 (d, 1H, $J_{5,6}$ = 8.0, *H*-5), 5.10 (m, 1H, C*H*(OAc)CH_2(OAc)), 4.50 (app q, 1H, *J* = 6.5, *H*-3′), 4.42 (d, 1H, J_{gem} = 15.8, OCH_2CO), 4.36 (d, 1H, J_{gem} = 15.8, OCH_2CO), 4.25 (m, 1H, CH(OAc)-CH_2(OAc)), 4.15 (m, 1H, CH(OAc)CH_2(OAc)), 4.10 (app q, 1H, *J* = 6.5, *H*-4′), 3.90 (dd, 1H, $J_{1',2'}$ = 4.5, $J_{2',3'}$ = 13.0, H-2′), 3.80 (s, 3H, OCH_3), 3.70 (m, 1H, 3′-O*H*), 3.65 (m, 1H, CONHCH_2), 3.55 (m, 2H, *H*-5′), 3.40 (m, 1H, CONHCH_2), 2.10 (s, 6H, *C*H_3CO), 1.20 (s, 9H, ^{t}Bu). ^{13}C NMR (62.90 MHz, $CDCl_3$): δ 177.80 (CH_2OCO^tBu), 171.10, 171.00 (CH_3C=O), 169.50 (OCH_2*C*O), 161.45 (*C*-4), 158.82 (*p*-An), 150.20 (*C*-2), 144.50 (*i*-Ph), 138.48 (*C*-6), 135.31, 135.11 (*i*-An), 130.16 (*o*-An), 128.17, 128.09 (*m*,*o*-Ph), 127.26 (*p*-Ph), 113.40 (*m*-An), 101.65 (*C*-5), 88.70 (*C*-1′), 87.10 (*C*(Ar)$_3$), 84.16 (*C*-2′), 82.80 (*C*-4′), 70.59 (*C*H(OAc)-CH_2(OAc)), 69.96 (O*C*H_2CO), 68.60 (*C*-3′), 64.00 (CH(OAc)*C*H_2(OAc)), 60.00 (*C*-5′), 55.30 (O*C*H_3), 39.49 (CONH*C*H_2), 39.40 (*C*$(CH_3)_3$), 27.05 (C(*C*$H_3)_3$), 21.01, 20.77 (*C*H_3CO).

3.8. 2′-*O*-[2-(2,3-Diacetoxypropyl)amino-2-oxoethyl]-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxy phosphinyl)-5′-*O*-(4,4′-dimethoxytrityl)-N^3-pivaloyloxymethyluridine (8)

Compound **7** (0.95 g, 1.09 mmol) was co-evaporated with dry CH_2Cl_2 (3× 20 ml), dissolved in dry CH_2Cl_2, diisopropylammonium tetrazolide (0.28 g, 1.63 mmol) and bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphos-

phine (0.45 ml, 1.42 mmol) were added, and the mixture was stirred under an inert atmosphere for 5 h. After conversion of the starting compound complete by TLC ($CHCl_3/Et_3N$, 99:1 v/v), the mixture was evaporated, diluted with EtOAc (50 ml), washed with 5% $NaHCO_3$ (100 ml), 20% NaCl (100 ml), dried over Na_2SO_4, evaporated and co-evaporated with toluene (3× 25 ml) and the residue was chromatographed on silica gel column ($0 \rightarrow 50\%$ $CHCl_3$ in toluene + 1% Et_3N v/v/v). Yield 1.09 g (93.1%). R_f 0.27 ($CHCl_3/Et_3N$, 99:1 v/v). MALDI-TOF (2,6-DHAP–diammonium hydrogen citrate): $[M+H]^+$ calcd 1077.14, found 1077.48. ^1H NMR (300.13 MHz, $CDCl_3$): δ 8.04, 8.01 (2d overlapped, each 1H, *H*-6), 7.45–7.16 (m, 18H, Ph, *o*-An), 6.84 (m, 8H, *m*-An), 5.95 (s, 2H, CH_2OCO^tBu), 5.91 (br s, 4H, CH_2OCO^tBu and 2*H*-1′), 5.36 (d, 1H, $J_{5,6} = 8.8$, *H*-5), 5.28 (d, 1H, $J_{5,6} = 8.1$, *H*-5), 5.20, 5.12 (2m, each 1H, $CH(OAc)CH_2(OAc)$), 4.57 (m, 2H, *H*-3′), 4.45 (d, 1H, $J_{gem} = 15.8$, OCH_2CO), 4.35 (d, 1H, $J_{gem} = 15.8$, OCH_2CO), 4.35–4.06 (m, 12H, *H*-4′, $CH(OAc)CH_2(OAc)$, OCH_2CO, $NCH(CH_3)_2$), 3.93–3.37 (m, 14H, *H*-2′, *H*-5′, $CONHCH_2$, OCH_2CH_2CN), 3.81 (s, 12H, OCH_3), 2.80–2.40 (m, 4H, OCH_2CH_2CN), 2.07 (s, 12H, CH_3CO), 1.20 (s, 18H, ^{t}Bu), 1.29, 1.15, 1.03 (3d, 6H, 12H, 6H, respectively, $NCH(CH_3)_2$). ^{13}C NMR (75.47 MHz, $CDCl_3$): δ 177.32 (CH_2OCO^tBu), 170.57, 170.42, 170.28, 169.80, 169.10 ($CH_3C{=}O$, OCH_2CO), 161.54 (*C*-4), 158.75 (*p*-An), 150.45 (*C*-2), 144.20, 144.12 (*i*-Ph), 138.29, 138.21 (*C*-6), 135.16, 135.09, 134.95 (*i*-An), 130.26, 130.22 (*o*-An), 128.27, 127.97 (*o*,*m*-Ph), 127.19 (*p*-Ph), 113.27 (*m*-An), 101.73, 101.66 (*C*-5), 89.80, 88.90 (*C*-1′), 87.08 ($C(Ar)_3$), 82.80, 82.20, 81.65, 81.47 (*C*-2′, *C*-4′), 71.08, 70.90, 70.34, 70.24, 69.95, 69.80 ($CH(OAc)CH_2(OAc)$, CH_2CO, *C*-3′), 64.55 ($CH(OAc)CH_2(OAc)$), 62.97, 62.89 (*C*-5′), 58.14, 57.86 ($CH(CH_3)_2$), 55.25 (OCH_3), 43.40, 43.20 (OCH_2CH_2CN), 39.23, 39.08, 38.93, 38.83 ($C(CH_3)_3$, $CONHCH_2$), 27.02 ($C(CH_3)_3$), 24.67, 24.59 (OCH_2CH_2CN), 22.92, 20.93, 20.72, 20.49, 20.38, 20.29 ($CH(CH_3)_2$, CH_3CO). ^{31}P NMR (121.50 MHz, $CDCl_3$): 153.695, 151.351.

3.9. Oligonucleotide synthesis

Oligonucleotides were assembled on an ABI 394B DNA Synthesizer by the phosphoramidite method according to the manufacturer's recommendations. Protected 2′-deoxyribonucleoside 3′-phosphoramidites and *S*-ethylthio-1*H*-tetrazole were purchased from Glen Research (via Cambio). Prepacked 0.4 μmol functionalised columns of controlled pore glass (Glen Research) were used for the synthesis of oligonucleotides **I** and **II**. Oligonucleotide **III** was assembled on a solid support carrying 6-carboxyfluorescein that was prepared according to the published procedure.[7] For coupling with modified phosphoramidite **8**, 0.15 M concentration in dry MeCN was used and the coupling time was increased to 30 min. Oligonucleotides were cleaved from the support and deprotected using concd aq ammonia overnight at 55 °C.

3.9.1. Synthesis of 2′-aldehyde oligonucleotides. To a solution of 2′-diol oligonucleotide (1–5 A_{260} units) in 40 μl of 0.2 M sodium acetate, pH 4.0–4.5, was added 10 μl of 0.2 M $NaIO_4$, and the reaction mixture was incubated at ambient temperature for 0.5–1 h. To that, 10 μl of 2 M ethylene glycol was added and further incubation was carried out for 30 min. The 2′-aldehyde oligonucleotide was then precipitated with 4 M sodium acetate solution (10 μl) and ethanol (1.5 ml).

3.9.2. Coupling of hydrazines and hydrazides to 2′-aldehyde oligonucleotides. To a solution of 2′-aldehyde oligonucleotide (1–5 A_{260} units) in 50 μl of 0.4 M sodium acetate, pH 4.0–4.5, was added a solution of 9-hydrazinoacridine **9** or succinic dihydrazide **10** (ca. 1 mg) in 50 μl DMSO. The reaction mixture was incubated at ambient temperature overnight and then sodium cyanoborohydride (ca. 1 mg) was added. The reaction mixture was incubated for 1 h more, precipitated by the addition of 20 μl of 4 M sodium acetate and 1.5 ml ethanol, and analysed using RP-HPLC (ion-pair mode) and MALDI-TOF mass spectrometry.

3.9.3. Coupling of L-cysteinylglycine dipeptide (11) to 2′-aldehyde oligonucleotide. The dried oligonucleotide **II** (1–5 A_{260} units) was dissolved in 50 μl of 0.4 M sodium acetate, pH 4.5–5.0. Then, a solution of dipeptide **11** (ca. 1 mg) in 50 μl acetonitrile was added, and the reaction mixture was incubated at ambient temperature overnight, then evaporated in vacuo and analysed using RP-HPLC (ion-pair mode) and MALDI-TOF mass spectrometry.

3.9.4. Coupling of *N*-aminooxyacetyl peptides to 2′-aldehyde oligonucleotides

3.9.4.1. Coupling with peptide 12. The dried oligonucleotide **II** (1–3 A_{260} units) was dissolved in 50 μl of 0.4 M sodium acetate, pH 4.5–5.0. Then, a solution of Tat peptide **12** (ca. 1 mg) in 50 μl DMSO was added, and the reaction mixture was incubated at ambient temperature overnight, precipitated by the addition of 20 μl of 4 M sodium acetate and 1.5 ml ethanol, analysed and purified using RP-HPLC, desalted by ultrafiltration on Microcon YM-3 (Millipore Inc, USA) and analysed by MALDI-TOF mass spectrometry.

3.9.4.2. Coupling with peptide 13. A solution of laminin peptide **13** (60–70 nmol) in 50 μl of the mixture of 0.2 M sodium acetate, 3 M urea, pH 4.7, and DMSO (1:1 v/v) was added to the oligonucleotide **III** (5 A_{260} units). The reaction mixture was incubated at 37 °C for 3 h and purified by denaturing 15% PAGE. Product elution from gel was performed with 0.5 M sodium acetate for 24 h at 4 °C. Purified conjugate **III.13** was then desalted by ultrafiltration on Microcon YM-3 (Millipore Inc, USA) and analysed by MALDI-TOF mass spectrometry.

Acknowledgments

The work was supported by the Wellcome Trust CRIG 069419, AstraZeneca UK Ltd and partly by the Programme 'Universities of Russia' 05.02.547. The authors thank Dr. Anna V. Kachalova for her advice

on HPLC purification and help with the preparation of this manuscript.

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