ARTICLE

OBC
www.rsc.org/obc

Oligonucleotides with 2′-*O*-carboxymethyl group: synthesis and 2′-conjugation *via* amide bond formation on solid phase†

Anna Kachalova,[a] Eugeny Zubin,[a] Dmitry Stetsenko,*[b] Michael Gait[b] and Tatiana Oretskaya[a]

[a] *Chemistry Department, M. V. Lomonossov Moscow State University, Leninskie Gory, Moscow 119992, Russia. E-mail: oretskaya@genebee.msu.su; Fax: +7-095-9393181; Tel: +7-095-9395411*

[b] *MRC Laboratory of Molecular Biology, Hills Road, Cambridge UK CB2 2QH. E-mail: ds@mrc-lmb.cam.ac.uk; Fax: +44-1223-402070; Tel: +44-1223-248011*

Received 23rd June 2004, Accepted 30th July 2004
First published as an Advance Article on the web 3rd September 2004

An efficient method for synthesis of oligonucleotide 2′-conjugates *via* amide bond formation on solid phase is described. Protected oligonucleotides containing a 2′-*O*-carboxymethyl group were obtained by use of a novel uridine 3′-phosphoramidite, where the carboxylic acid moiety was introduced as its allyl ester. This protecting group is stable to the conditions used in solid-phase oligonucleotide assembly, but easily removed by Pd(0) and morpholine treatment. 2′-*O*-Carboxymethylated oligonucleotides were then efficiently conjugated on a solid support under normal peptide coupling conditions to various amines or to the N-termini of small peptides to give products of high purity in good yield. The method is well suited in principle for the preparation of peptide–oligonucleotide conjugates containing an amide linkage between the 2′-position of an oligonucleotide and the N-terminus of a peptide.

Introduction

There are many methodologies available for preparing natural oligonucleotides, as well as their analogues containing modified phosphate groups, nucleobases and sugars.[1] The advances in solid-phase synthesis inspired various strategies for preparation of oligonucleotide conjugates.[2,3] Oligonucleotide conjugation is predominantly carried out by use of a nucleophilic group on an oligonucleotide to react with an electrophilic group on a tag or a solid support. This strategy still predominates because the common oligonucleotide deprotection is performed by base treatment *e.g.* ammonia or methylamine which are inherently nucleophilic. Indeed, there are many methods of preparation of oligonucleotides modified with amino or thiol groups at a variety of positions.[1] On the other hand, there are many situations when researchers wish to introduce an electrophilic group into oligonucleotides.[4] In this vein, we developed a phosphoramidite incorporating a carboxylic acid function protected by a 2-chlorotrityl group.[5a] The reagent is suitable for solid-phase synthesis of 5′-carboxylated oligonucleotides that may be conjugated to various amines on solid supports after activation by a suitable peptide coupling reagent.[5] The monomer is now commercially available.[5b] Similarly, 5′-carboxy-modifier C10 is sold by Glen Research that contains a preformed carboxylic acid *N*-hydroxysuccinimide ester and can be used directly in solid-phase conjugation.[6]

In previous work in this field we[7] and others[8,9] described syntheses of 2′-*O*-[7,8] or 2′-*S*-carboxymethyloligonucleotides,[9] where an alkyl ester was chosen as a carboxylic acid protecting group. After completion of solid-phase oligonucleotide assembly, support-bound oligonucleotides containing methyl[7,8b] or ethyl[8a] ester were hydrolysed by treating with aq. NaOH or treated by an appropriate amine to afford either the carboxymethyl group or the corresponding amides, respectively, after final deprotection. 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.

Recently, we reported our preliminary studies[10,11] that show that oligonucleotides containing a 2′-carboxymethyl group can be conjugated to amines, amino acid derivatives and small peptides by amide bond formation on solid phase under peptide coupling conditions or in aqueous media by a water-soluble carbodiimide-mediated reaction.

We would like to report here the synthesis of the 2′-*O*-carboxymethylated oligonucleotides by use of a uridine 3′-phosphoramidite, where the carboxylic acid function carries an allyl protecting group, which is readily removed under mild and specific conditions of Pd(0)-catalysis. Subsequent conjugation of the 2′-carboxylic acid to a range of primary aliphatic and aromatic amines and peptides is achieved by peptide coupling reagent-promoted amide bond formation on a solid support. We demonstrate examples of the synthesis of 2′-*O*-methyl oligoribonucleotides containing a 2′-*O*-carboxymethyl group and their conjugation to a range of amines and peptides.

Results and discussion

The route to the 2′-*O*-(allyloxycarbonyl)methyluridine building block **6** is illustrated in Scheme 1. The starting 3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl)uridine **1** was obtained from uridine as reported previously[12,13] and purified by column chromatography on silica gel in $CHCl_3$–EtOAc (9 : 1, then 2 : 1 v/v). Protection of the N^3 of uracil is required, since the subsequent alkylation of 3′,5′-*O*-protected uridine preferentially occurs at the base moiety. To protect the imido function from this side reaction, we chose to block the N^3-position with an ammonia-labile pivaloyloxymethyl (Pom) group. The group also prevents undesirable side reactions during oligonucleotide assembly. Furthermore, the greater lipophilicity of the Pom derivative is helpful for chromatographic separation of the target compound. Initially, Pom was reported as a protecting group for the lactam function of a uracil moiety.[14] This group was introduced by the transient protection procedure, which involved 2′-*O*-trimethylsilylation followed by N^3-protection.[14b] On the other hand, Sekine[15] showed earlier that various N^3-protected uridine derivatives may be obtained selectively in the presence of an unprotected 2′-OH group under conditions of phase-transfer catalysis. These results led us to consider a phase-transfer reaction to convert **1** into **2**. Indeed, when **1** was allowed to react with 10 equiv. of chloromethyl pivalate in a biphasic system 0.2 M Na_2CO_3–CH_2Cl_2 (2 : 1 v/v) in the presence of 0.2 equiv. of tetra-*n*-butylammonium hydrogen sulfate (TBAHS) as a phase-transfer catalyst at room temperature

† Electronic supplementary information (ESI) available: MALDI-TOF spectra. See http://www.rsc.org/suppdata/ob/b4/b409496d/

DOI: 10.1039/b409496d

Table 1 Properties of 2′-carboxy-modified 2′-O-methyloligonucleotides and their conjugates

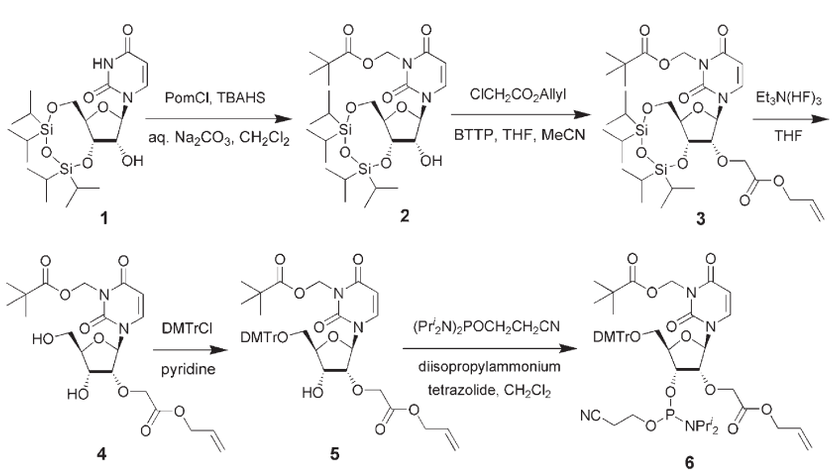
#	Oligonucleotide sequence, 5′-3′/conjugated molecule	MALDI-TOF MS calc./found (*m*/*z*)	Retention time[a]/min	Yield[b] (%)
I	UUUUUU*UUUU	3183.9/3182.6	15.5	—
II	CUCCCAGGCU*CA	3942.6/3942.7	15.7	—
I.7	*N*,*N*,*N*-Tris(aminoethyl)amine (**7**)	3311.6/3310.2	14.5	>90
I.8	Cyclohexylamine (**8**)	3264.6/3264.1	16.0	>90
I.9	3-Amino-1,2-propanediol (**9**)	3256.5/3254.8	15.0	>90
I.10	Histamine (**10**)	3276.5/3277.8	14.8	75
I.11	Spermine (**11**)	3367.7/3366.6	14.7	85
I.12	*N*,*N*,*N*′,*N*′-Tetrakis(3-aminopropyl)-1,4-butanediamine (**12**)	3481.8/3482.6	14.9	80
II.13	1-Aminopyrene (**13**)	4141.8/4138.4	27.1	65
II.14	1-Pyrenemethylamine (**14**)	4155.9/4153.4	27.5	85
II.15	H-Phe-NH_2 **15**)	4088.8/4084.3	17.2	>90
II.16	H-Leu-NH_2 (**16**)	4054.8/4051.0	16.2	>90
II.17	H-Phe-Phe-NH_2 (**17**)	4235.9/4230.4	21.6	>90
II.18	H-Leu-Phe-NH_2 (**18**)	4201.9/4199.4	16.9	>90
II.19	H-Gly-Leu-Met-NH_2 (**19**)	4243.0/4245.8	17.1	>90
II.20	H-Pro-Leu-Gly-NH_2 (**20**)	4208.9/4206.3	21.7	75
II.21	H-Asn-Arg-Asn-Phe-Leu-Arg-Phe-NH_2 (**21**)	4889.7/4894.0	21.5	80

[a] For conditions, see Experimental section. [b] Conversion of oligonucleotide peak to the conjugate peak, calculated from RP-HPLC traces.

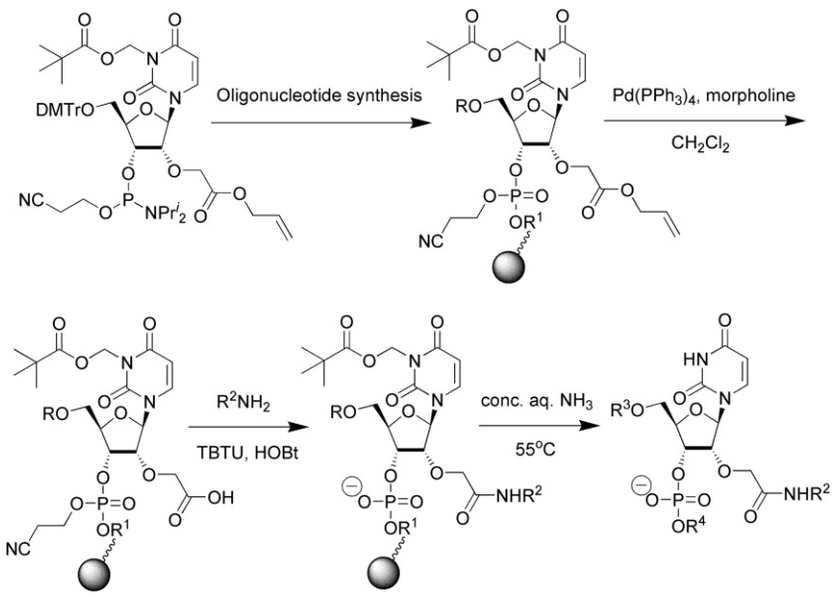
for 48 h under vigorous stirring, N^3-pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl)uridine **2** was isolated in 70% yield after column chromatography. General methods of 2′-*O*-alkylation of ribonucleosides have been reviewed by Zatsepin *et al.*[16] We previously adopted a convenient procedure[7] for 2′-*O*-alkylation by use of a strong sterically hindered organic base 2-*tert*-butylimino-2-diethylamino-1,3-dimethylperhydro-1,3,2-diazaphosphorine (BEMP) described in a number of publications.[17,18] Later we replaced BEMP by another phosphazene base P_1-*tert*-butyltris(tetramethylene) (BTPP), which is cheaper and even more basic, though less sterically hindered than BEMP.[19] Thus, we found that alkylation of compound **2** with 2.5 equiv. of allyl chloroacetate and 2.8 equiv. of BTPP gave **3** in 90% yield after 3–5 h. Compound **3** was then desilylated smoothly with TBAF[7] or, better, triethylamine trihydrofluoride[13] in THF and then converted into the 5′-*O*-dimethoxytrityl (DMTr) derivative **5** by the known procedure.[20] Subsequent phosphitylation of the 3′-hydroxy group in an inert atmosphere using bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine in CH_2Cl_2 in the presence of diisopropylammonium tetrazolide[21] afforded the phosphoramidite **6**. This was used successfully in machine-assisted solid-phase oligonucleotide synthesis. The average coupling efficiency of **6** at a 0.2 M concentration in dry MeCN and 30 min reaction time was found to be greater than 97%. The modified phosphoramidite was utilised in the synthesis of two 2′-*O*-methyloligoribonucleotides **I** and **II** (Table 1). Decamer **I** was synthesised as a model to select and optimise the specific conditions of deblocking, purification and conjugation. Oligonucleotide **II** is complementary to the HIV-1 TAR RNA apical stem-loop, the binding site for the HIV-1 *trans*-activator protein Tat.[22]

To remove the 2′-allyl protecting group on the solid phase, we used a mixture of tetrakis(triphenylphosphine)palladium(0), triphenylphosphine and morpholine[7] in dry CH_2Cl_2 for 50 min (Scheme 2). During the procedure, other protecting groups remain intact and the oligonucleotide is still linked to a polymer support.[23]

Further conjugations of 2′-*O*-carboxymethylated oligonucleotides **I** and **II** were carried out on solid phase in organic solvent (Scheme 2). The choice of amine was influenced by the expected application for the conjugate *e.g.* introduction of positive charge(s) or nucleophilic amino groups (*N*,*N*,*N*-tris(aminoethyl)amine **7**, histamine **10**, spermine **11** and *N*,*N*,*N*′,*N*′-tetrakis(3-aminopropyl)-1,4-butanediamine **12**), chemoselective ligation (3-amino-1,2-propanediol **9**)[22] or fluorescent labeling (1-aminopyrene **13** and 1-pyrenemethylamine **14**[13]). In the case of spermine that contains both primary and secondary amino groups, we expected selective reaction with a primary amino group facilitated by the excess of amine.[5a] For further conjugation experiments, we have chosen several amino acid derivatives **15** and **16** and short peptides **17**–**20** and FMRF amide-related peptide **21** demonstrated to be cardioactive neuropeptide.[24] To prevent the formation of byproducts both amino acids and peptides were utilised as *C*-terminal amides. To obtain a high yield of structurally diverse types of conjugates (Table 1) we used pre-activation of polymer-bound 2′-*O*-carboxymethyl-oligonucleotide with TBTU–HOBT (1 : 1) at 37 °C in dry DMF



Scheme 1 Preparation of 2′-*O*-(allyloxycarbonyl)methyluridine 3′-phosphoramidite (**7**). Abbreviations: TIPS - 1,1,3,3-tetraisopropyldisiloxan-1,3-diyl, Pom - pivaloyloxymethyl, TBAHS - tetra-*n*-butylammonium hydrogen sulfate, BTPP - phosphazene base P_1-*tert*-butyltris(tetramethylene), DMTr - 4,4′-dimethoxytrityl.



Scheme 2 Solid-phase synthesis, selective deprotection and conjugation of 2′-*O*-carboxymethylated oligonucleotides. Abbreviations: R, R^1 = protected oligonucleotide chain, R^2 = amine or peptide residue, R^3, R^4 = unprotected oligonucleotide chain; TBTU - *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate, HOBt - 1-hydroxybenzotriazole.

for 40 min, followed by addition of the amine, amino acid or peptide and further incubation.[25] Reaction times for the amines differed significantly from those for the amino acids and peptides. Whilst all amines reacted within 3 h, amino acids and peptides required overnight reaction. After completion of the reaction, polymer-bound conjugates were cleaved from their solid supports and deprotected by concentrated aqueous ammonia treatment at 55 °C overnight. Reaction mixtures obtained were analysed by reversed-phase HPLC and MALDI-TOF MS. Only a single product was observed in the case of spermine conjugate **I.11**. Examples of typical RP-HPLC traces are shown in Fig. 1. Noteworthy, the two unprotected arginine residues in peptide **21** did not interfere with its successful conjugation at the N-terminus. We ascribe this to the presence of an excess of HOBt that may protonate the guanidino group of arginine and thus protect it from acylation.

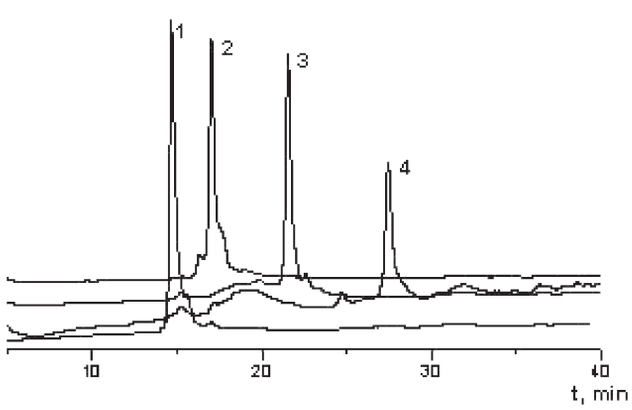


Fig. 1 Reversed-phase HPLC traces of crude oligonucleotide 2′-conjugates: (1) oligonucleotide **II**; (2) conjugate **II.20**; (3) conjugate **II.17**; (4) conjugate **II.14**. For HPLC conditions, see Experimental section.

In conclusion, we have described an efficient and reliable method for preparation of 2′-*O*-carboxymethyloligonucleotides using protected uridine 3′-phosphoramidite containing a 2′-carboxymethyl group protected as an allyl ester. After deprotection under conditions of Pd(0) catalysis, such modified oligonucleotides may be conjugated, while still attached to a solid support, to a range of primary or secondary aliphatic or aromatic amines or to the N-termini of short peptides. The method described could prove useful in synthesis of small molecule libraries of oligonucleotide conjugates for diagnostic or therapeutic evaluation. In addition, this conjugation method may be a useful route for attachment of membrane-penetrating peptides, particularly those containing multiple arginine residues, to the 2′-position of oligonucleotides and their analogues for cellular uptake studies.

Experimental

General

Chemicals were obtained from commercial suppliers and used without further purification unless otherwise noted. Chloromethyl pivalate, tetra-*n*-butylammonium hydrogen sulfate, allyl chloroacetate, triethylamine trihydrofluoride, tetrakis(triphenylphosphine)palladium(0), *N*,*N*,*N*-tris(aminoethyl)amine, 3-amino-1,2-propanediol, histamine, spermine, *N*,*N*,*N*′,*N*′-tetrakis(3-aminopropyl)-1,4-butanediamine, 1-aminopyrene and 1-pyrenemethylamine hydrochloride were purchased from Aldrich. Phosphazene base P_1-*tert*-butyltris(tetramethylene), bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine, triphenylphosphine, morpholine, 1-hydroxybenzotriazole, *O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate (TBTU) and cyclohexylamine were from Fluka. 4,4′-Dimethoxytrityl chloride (DMTrCl) was from Avocado. Amino acids and peptides were bought from Bachem. Diisopropylammonium tetrazolide was prepared from diisopropylamine (BDH) and 1*H*-tetrazole solution in acetonitrile (Glen Research).[21] 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, hexane, absolute ethanol and methanol were used as received.

^{1}H NMR spectra were recorded on a Bruker DRX 500 spectrometer (500.13 MHz for ^{1}H and 202.4 MHz for ^{31}P) and 2–5 mM solutions. Chemical shifts (δ, ppm) for ^{1}H 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 use of adapted COSY and HMQC techniques. Chemical shifts are accurate to within 0.01 ppm for ^{1}H. CSSI are accurate to within 0.25 Hz. MALDI-TOF spectra were recorded on a Voyager DE system (Applied Biosystems) or Reflex IV (Bruker) in positive ion mode using either a 1 : 1 (v/v) mixture of 2,6-dihydroxyacetophenone (2,6-DHAP) (40 mg cm^{-3} in MeOH) and aq. diammonium hydrogen citrate (80 mg cm^{-3}) for all oligonucleotides, and 2,5-dihydroxybenzoic acid (2,5-DHBA) (10 mg cm^{-3} in 50% aq. MeOH) or 2,4,6-trihydroxyacetophenone (2,4,6-THAP)

(10 mg cm^{-3} in 50% aq. MeOH) for low molecular weight compounds as a matrix. TLC was carried out on Merck DC Kieselgel 60 F_{254} 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).

3′,5′-*O*-(Tetraisopropyldisiloxane-1,3-diyl)uridine (1). This was prepared as described previously.[13]

***N*³-Pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl)uridine (2).** To a two-phase solution of compound **1** (4.86 g, 10 mmol) in CH_2Cl_2 (200 cm^3)–0.2 M aq. Na_2CO_3 (400 cm^3) were added chloromethyl pivalate (14.3 cm^3, 99.2 mmol) and tetra-*n*-butylammonium hydrogen sulfate (0.74 g, 2.1 mmol). The resulting mixture was vigorously stirred at ambient temperature for 48 h. Then the organic phase was collected and washed with 5% $NaHCO_3$ (2 × 200 cm^3). The organic layers were combined, dried (Na_2SO_4) and filtered. The filtrate was evaporated, co-evaporated with benzene (3 × 25 cm^3) and the residue was chromatographed on silica gel (stepwise gradient of 0→2→4→6→8→10% EtOAc in benzene, v/v) to give compound **2** which was obtained as a white foam (4.2 g, 70%). R_f 0.85 ($CHCl_3$–EtOH, 9 : 1 v/v). MALDI-TOF (2,5-DHBA): $[M + Na]^+$ calc. *m*/*z* 623.85, found 624.05. ^1H NMR ($CDCl_3$): δ 7.81 (d, 1H, *H*-6, $J_{5,6}$ = 7.5), 5.92 (AB system, 2H, NCH_2OCOBu^t), 5.81 (s, 1H, *H*-1′), 5.76 (d, 1H, *H*-5, $J_{5,6}$ = 7.5), 4.67 (br d, 2H, *H*-3′, *H*-4′), 4.22 (br s, 2H, *H*-5′), 4.0 (d, 2H, *H*-2′), 1.19 (s, 9H, Bu^t), 1.1–0.9 (m, 28H, Pr^i).

2′-*O*-Allyloxycarbonylmethyl-*N*³-pivaloyloxymethyl-3′,5′-*O*-(tetraisopropyldisiloxan-1,3-diyl)uridine (3). Compound **2** (4.2 g, 7 mmol) was dried by co-evaporation with dry MeCN (3 × 20 cm^3) and was then dissolved in dry MeCN–THF (100 cm^3, 1 : 1 v/v). Phosphazene base P_1-*tert*-butyltris(tetramethylene) (6 cm^3, 19.6 mmol) followed immediately by allyl chloroacetate (2 cm^3, 17.5 mmol) were added to the stirred mixture at ambient temperature. TLC ($CHCl_3$–EtOH, 97 : 3 v/v) showed complete reaction after 3 h. The reaction mixture was evaporated to dryness *in vacuo*. The yellow oil was dissolved in $CHCl_3$ (100 cm^3) and washed with brine (1 × 100 cm^3) and water (2 × 100 cm^3). The combined organic layers were dried (Na_2SO_4), filtered, evaporated and co-evaporated with benzene (3 × 25 cm^3). The crude product was purified by column chromatography on silica gel (stepwise gradient of 0→2→4→6→8% EtOAc in benzene, v/v). Compound **3** was obtained as a pale yellow oil (4.4 g, 90%). R_f 0.58 ($CHCl_3$–EtOH, 97 : 3 v/v). MALDI-TOF (2,5-DHBA): $[M + H]^+$ calc. *m*/*z* 699.97, found 699.32. ^1H NMR ($CDCl_3$): δ 7.81 (d, 1H, *H*-6, $J_{5,6}$ = 7.5), 5.92 (AB system, 2H, NCH_2OCOBu^t), 5.89 (ddt, 1H, $CH{=}CH_2$), 5.81 (s, 1H, *H*-1′), 5.76 (d, 1H, *H*-5, $J_{5,6}$ = 7.5), 5.53 (d, 1H, $=CH_2$-(*Z*)), 5.24 (d, 1H, $=CH_2$-(*E*)), 4.67 (br d, 2H, *H*-3′, *H*-4′), 4.58, 4.43 (AB system, 2H, $CO_2CH_2CH{=}$), 4.25 (A part of AB system, 2H, $CO_2CH_2CH{=}$), 4.22 (br s, 2H, *H*-5′), 4.0 (overlap, 2H, *H*-2′, B part of AB system, $CO_2CH_2CH{=}$), 1.19 (s, 9H, Bu^t), 1.1–0.9 (m, 28H, Pr^i).

2′-*O*-Allyloxycarbonylmethyl-*N*³-pivaloyloxymethyluridine (4). To a solution of **3** (4.4 g, 6.3 mmol) in THF (15 cm^3) in a 30 cm^3 screw-capped Teflon vial (Nalgene) was added triethylamine trihydrofluoride (2.6 cm^3, 15.7 mmol) and the mixture was left for 1.5 h at room temperature, the completion of deprotection was checked by TLC ($CHCl_3$–EtOH, 9 : 1 v/v), then diluted with EtOAc (50 cm^3), washed with 5% $NaHCO_3$ (2 × 50 cm^3), water (50 cm^3), 5% citric acid (2 × 50 cm^3), and brine (50 cm^3), then dried (Na_2SO_4), evaporated to dryness and co-evaporated with $CHCl_3$ (3 × 25 cm^3). The residue was chromatographed on a silica gel column (0→1→2→3→4% MeOH in $CHCl_3$, v/v). Yield (2.3 g, 80%). R_f 0.15 ($CHCl_3$–EtOH, 9 : 1 v/v). MALDI-TOF (2,5-DHBA): M^+ calc. *m*/*z* 456.45, found 456.26, $[M + Na]^+$ calc. *m*/*z* 479.44, found 479.11, $[M + K]^+$ calc. *m*/*z* 495.56, found 497.04. ^1H NMR ($CDCl_3$): δ 7.81 (d, 1H, *H*-6, $J_{5,6}$ = 7.5), 5.92 (AB system, 2H, NCH_2OCOBu^t), 5.89 (ddt, 1H, $CH{=}CH_2$), 5.78 (d, 1H, *H*-1′, $J_{1',2'}$ = 10), 5.76 (d, 1H, *H*-5, $J_{5,6}$ = 7.5), 5.53 (d, 1H,$=CH_2$-(*Z*)), 5.24 (d, 1H, $=CH_2$-(*E*)), 4.67 (br d, 2H, *H*-3′, *H*-4′), 4.61 (AB system, 2H, OCH_2CO), 4.58, 4.43 (AB system, 2H, $CO_2CH_2CH{=}$), 4.03, 4.0 (dd, 2H, *H*-3′, *H*-4′), 3.88 (dd, 1H, *H*-2′), 3.66 (dt, 1H, *H*-5′), 3.58 (dt, 1H, *H*-5′), 3.0 (s, 1H, O*H*), 1.19 (s, 9H, Bu^t).

2′-*O*-Allyloxycarbonylmethyl-5′-*O*-(4,4′-dimethoxytrityl)-*N*³-pivaloyloxymethyluridine (5). Compound **4** (2.3 g, 5 mmol) was co-evaporated with dry pyridine (3 × 20 cm^3), dissolved in dry pyridine (50 cm^3), cooled in an ice-bath, and DMTrCl (2.05 g, 6.05 mmol) was added in one portion. The reaction was monitored by TLC until the starting nucleoside disappears completely. Then the excess of DMTrCl was quenched with MeOH (2 cm^3), and after 10 min the mixture was partially evaporated, diluted with $CHCl_3$ (100 cm^3), washed with 5% $NaHCO_3$ (2 × 100 cm^3), and brine (100 cm^3), then dried (Na_2SO_4), evaporated, co-evaporated with benzene (3 × 25 cm^3) and the residue was chromatographed on a silica gel column (0→20→25→30→50→100% $CHCl_3$ in benzene and further 1→2→5% MeOH in $CHCl_3$ + 1% pyridine v/v/v). Yield (3.4 g, 90%). R_f 0.4 ($CHCl_3$–EtOH, 95 : 5 v/v). MALDI-TOF (2,4,6-THAP): $[M + Na]^+$ calc. *m*/*z* 780.81, found 780.57. ^1H NMR ($CDCl_3$): δ 7.8 (d, 1H, *H*-6, $J_{5,6}$ = 7.5), 7.38–7.17 (m, 10H, Ar), 6.85 (d, 4H, *o*-Ar), 5.92 (AB system, 2H, NCH_2OCOBu^t), 5.89 (m, 1H, $CH{=}CH_2$), 5.82 (d, 1H, *H*-1′), 5.58 (d, 1H, *H*-5, $J_{5,6}$ = 7.5), 5.22 (d, 2H, $CH_2CH{=}CH_2$), 4.69 (s, 2H, OCH_2CO), 4.64 (d, 2H, $CH_2CH{=}CH_2$), 4.39 (t, 1H, *H*-3′), 4.32 (t, 1H, *H*-2′), 4.22 (br s, 1H, *H*-4′), 3.8 (s, 6H, OCH_3), 3,52, 3.43 (dd, 2H, *H*-5′), 1.19 (s, 9H, Bu^t).

2′-*O*-Allyloxycarbonylmethyl-3′-*O*-(*N*,*N*-diisopropylamino-2-cyanoethoxyphosphinyl)-5′-*O*-(4,4′-dimethoxytrityl)-*N*³-pivaloyloxymethyluridine (6). Compound **5** (3.4 g, 4.5 mmol) was co-evaporated with dry CH_2Cl_2 (3 × 20 cm^3), dissolved in dry CH_2Cl_2, diisopropylammonium tetrazolide (1.1 g, 6.7 mmol) and bis(*N*,*N*-diisopropylamino)-2-cyanoethoxyphosphine (2.3 cm^3, 7.3 mmol) were added, and the reaction mixture was stirred at 25 °C overnight. After TLC (CH_2Cl_2–Et_3N, 98 : 2 v/v) showed the completion of the reaction, the mixture was diluted with CH_2Cl_2 (50 cm^3), washed with brine (2 × 50 cm^3), dried (Na_2SO_4), and evaporated to dryness. The residue was dried *in vacuo* to afford **7** as a white foam (4.1 g, 95%). R_f 0.45 (CH_2Cl_2–Et_3N, 98 : 2 v/v). MALDI-TOF (2,6-DHAP–diammonium hydrogen citrate): $[M + H]^+$ calc. *m*/*z* 960.06, found 960.13. ^{31}P NMR (CD_3CN): δ 153.0, 153.5 (two diastereomers).

Oligonucleotide synthesis

2′-*O*-Methyloligoribonucleotides were assembled on an ABI 394B DNA Synthesizer by the phosphoramidite method according to the manufacturer's recommendations. Protected 2′-*O*-methylribonucleoside phosphoramidites and *S*-ethylthiotetrazole were purchased from Glen Research (*via* Cambio). Prepacked 0.4 μmol functionalised columns of controlled pore glass (Glen Research) were used throughout. For couplings with modified phosphoramidite **6**, 0.2 M concentration in dry MeCN was used, and the coupling time was increased to 30 min.

Deprotection of carboxylic acid function of oligonucleotides I, II

The 2′-allyl protecting group was removed from the support-bound modified oligonucleotides by treatment with a solution of morpholine (0.03 cm^3), tetrakis(triphenylphosphine)-palladium(0) (5 mg) and triphenylphosphine (5 mg) in dry CH_2Cl_2 (0.2 cm^3) for 40 min at ambient temperature. The supernatant was then decanted and the support was rinsed with CH_2Cl_2 (2 × 0.15 cm^3), EtOH (0.15 cm^3), water (0.15 cm^3) and CH_2Cl_2 (0.15 cm^3). The support was then dried on air.

Coupling of amines, amino acids and peptides to 2′-*O*-carboxymethyloligonucleotides I, II

After the completion of the 2′-carboxylic acid group deprotection, a solution of TBTU (5 mg) and HOBt (2 mg) in dry DMF (0.2 cm^3) was added. The slurry was incubated at 37 °C for 40 min with occasional swirling, then an amine, amino acid or peptide was added (100 equiv.). The reaction was carried out at 37 °C for 3 h in the case of amines or overnight for amino acid derivatives or peptides and shaking, then the supernatant was discarded, and glass beads washed successively with DMF (2×0.2 cm^3), water (2×0.2 cm^3) and EtOH (2×0.2 cm^3). Cleavage from the support and deprotection of phosphate and nucleobase residues were performed using conc. aq. ammonia overnight at 55 °C. Reaction mixtures were analysed by RP-HPLC, 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 cm^3 min^{-1}, gradient of B in A: 0–5%, 5 min, 5–15%, 10 min, 15–40, 30 min, 40–80%, 10 min, 80–0%, 10 min. Combined fractions containing the conjugate were evaporated, redissolved and precipitated by 4 M sodium acetate solution (0.2 cm^3) and ethanol (1.5 cm^3) or 2 M $LiClO_4$ (0.2 cm^3) and acetone (1.5 cm^3). Molecular masses of purified conjugates were then checked by MALDI-TOF MS.

Acknowledgements

This work was supported by the Wellcome Trust CRIG 069419 and AstraZeneca UK, Ltd. The authors thank Dr A. D. Malakhov and Mrs A. N. Muravieva for help with HPLC purification.

References

1 *Current Protocols in Nucleic Acid Chemistry*, ed. S. L. Beaucage, D. E. Bergstrom, G. D. Glick and R. A. Jones, John Wiley & Sons, Inc., New York, 2000.
2 E. M. Zubin, E. A. Romanova and T. S. Oretskaya, *Russ. Chem. Rev.*, 2002, **71**, 239–264.
3 P. Virta, J. Katajisto, T. Niittymäki and H. Lönnberg, *Tetrahedron*, 2003, **59**, 5137–5174.
4 A. V. Kachalova, E. M. Zubin and T. S. Oretskaya, *Russ. Chem. Rev.*, 2002, **71**, 1041–1059.
5 (*a*) A. V. Kachalova, D. A. Stetsenko, E. A. Romanova, V. N. Tashlitsky, M. J. Gait and T. S. Oretskaya, *Helv. Chem. Acta*, 2002, **85**, 2409–2416; (*b*) further information on use of our reagent is available from Link Technologies, Ltd. on www.linktech.co.uk/Downloads/TIS-BC-02 v 1.3.pdf.
6 R. I. Hogrefe and M. M. Vaghefi, *US Pat.*, 6,320,041, 2001.
7 A. V. Kachalova, T. S. Zatsepin, E. A. Romanova, D. A. Stetsenko, M. J. Gait and T. S. Oretskaya, *Nucleosides, Nucleotides Nucleic Acids*, 2000, **19**, 1693–1707.
8 (*a*) C. A. Buhr and M. D. Matteucci, *Eur. Pat.*, EP 0942000A2, 1999; (*b*) T. P. Prakash, A. M. Kawasaki, E. A. Lesnik, S. R. Owens and M. Manoharan, *Org. Lett.*, 2003, **5**, 403–406.
9 H. Ozaki, S. Moriyama, K. Yokotsuka and H. Sawai, *Tetrahedron Lett.*, 2001, **42**, 677–680.
10 A. V. Kachalova, E. M. Zubin, T. S. Zatsepin, Yu. V. Agapkina, Yu. M. Ivanova, D. A. Stetsenko, M. J. Gait, T. S. Oretskaya, in *Innovation & Perspectives in Solid Phase Synthesis & Combinatorial Libraries 2004*, ed. R. Epton, Mayflower Worldwide, Kingswinford, in press.
11 A. V. Kachalova, D. A. Stetsenko, M. J. Gait and T. S. Oretskaya, *Bioorg. Med. Chem. Lett.*, 2004, **14**, 801–804.
12 B. Beijer, M. Grøtli, M. Douglas and B. Sproat, *Nucleosides Nucleotides*, 1994, **13**, 1905–1928.
13 V. A. Korshun, D. A. Stetsenko and M. J. Gait, *J. Chem. Soc., Perkin Trans. 1*, 2002, 1092–1104.
14 (*a*) M. Sekine and T. Hata, *J. Am. Chem. Soc.*, 1986, **108**, 4581–4586; (*b*) M. Grøtli, R. Eritja and B. Sproat, *Tetrahedron*, 1997, **53**, 11317–11346.
15 M. Sekine, *J. Org. Chem.*, 1989, **54**, 2321–2326.
16 T. S. Zatsepin, E. A. Romanova and T. S. Oretskaya, *Russ. Chem. Rev.*, 2002, **71**, 513–534.
17 R. Schwesinger, *Chimia*, 1985, **39**, 269–273.
18 M. Grøtli, M. Douglas, B. Beijer, R. G. Garsia, R. Eritja and B. Sproat, *J. Chem. Soc., Perkin Trans. 1*, 1997, 2779–2788.
19 For further information on phosphazene bases see, *Chem Files (Fluka)*, 2003, **3**, that can be downloaded from http://www.sigmaaldrich.com/Brands/Fluka__Riedel_Home/Literature/ChemFiles/Vol_3_No_1.html.
20 M. Smith, D. H. Rammler, I. H. Goldberg and H. G. Khorana, *J. Am. Chem. Soc.*, 1962, **84**, 430–440.
21 M. H. Caruthers, A. D. Barone, S. L. Beaucage, D. R. Dodds, E. F. Fisher, L. J. McBride, M. Matteucci, Z. Stabinsky and J.-Y. Tang, *Methods Enzymol.*, 1987, **154**, 287–313.
22 T. S. Zatsepin, D. A. Stetsenko, A. A. Arzumanov, E. A. Romanova, M. J. Gait and T. S. Oretskaya, *Bioconjugate Chem.*, 2002, **13**, 822–830.
23 E. M. Zubin, E. A. Romanova, E. M. Volkov, V. N. Tashlitsky, G. A. Korshunova, Z. A. Shabarova and T. S. Oretskaya, *FEBS Lett.*, 1999, **456**, 59–62.
24 A. J. Mercier, I. Orchard, V. TeBrugge and M. Skerrett, *Peptides*, 1993, **14**, 137–143.
25 P. D. Ribeiro, E. W. Alves and O. L. T. Machado, *Protein Peptide Lett.*, 1999, **6**, 203–208.