Incorporation of 2'-amido-nucleosides in oligodeoxynucleotides and oligoribonucleotides as a model for 2'-linked conjugates

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Received October 14, 1994; Revised and Accepted November 28, 1994

ABSTRACT

The functionalisation of oligodeoxynucleotides and oligoribonucleotides by incorporation of 2'-amido-2'-deoxyribonucleosides, possibly containing a reporter group via the 2'-amido bond, was examined. Therefore 2'-acetamido-ribonucleosides containing a small methyl group at the 2'-amido bond were synthesized as model compounds. In order to evaluate the influence of this 2'-modification on the hybridization capacities, 2'-acetamido-2'-deoxyuridine was incorporated in both RNA and DNA strands. The suitability of phosphoramidite chemistry for the introduction of this modified nucleoside was proven using laser desorption mass spectrometry of the final oligonucleotide. The presence of the 2'-modification destabilised both RNA-RNA, DNA-DNA and mixed duplexes. Therefore, it can be concluded that the 2'-acetamido group is not a good linker for attachment of reporter groups to oligonucleotides.

INTRODUCTION

The growing interest for antisense oligonucleotide-based therapeutics and nucleic acid probe-based diagnostics has encouraged the development of nucleosidic phosphoramidite derivatives for the functionalisation of specific oligonucleotides (1). In contrast to some small modifications at the base moieties (2,3), the derivatisation of oligonucleotides at the nucleobases may cause interference with base pairing and/or base stacking interactions. Derivatisation at the phosphodiester linkages may present solubility and/or chirality problems. Therefore the functionalisation of oligonucleotides at the carbohydrate moiety may render a suitable way for derivatisation with reporter groups. Reporter groups such as intercalating agents, DNA cleavage agents and fluorescent agents should be linked to the oligonucleotide so that the bond can not be cleaved under physiological conditions.

Therefore covalent linkages such as esters, thioesters and even disulfide bridges or phosphodiester bonds have to be avoided. The binding of reporter groups may be carried out after the assembly of the oligonucleotide or, alternatively, a nucleoside building block already carrying the reporter group may be used. Manoharan *et al.* (4) reported the synthesis of oligonucleotides containing 2'-O-aminoalkylated linkers which can react with compounds containing functional groups which possess inherent reactivity to an amino group.

The approach of the functionalisation of oligonucleotides by the incorporation of 2'-amido-ribonucleosides, already containing the reporter group via the 2'-amido bond, may provide an alternative. A 2'-amido bond may be expected to be metabolically and chemically stable and not interfering with oligonucleotide synthesis. In order to explore the feasibility of this approach we synthesized 2'-acetamido-ribonucleosides, thus containing a small alkyl group. The thermal stability of duplexes consisting of oligodeoxy- or oligoribonucleotides containing 2'-acetamido-2'-deoxyuridines in one strand and natural 2'-deoxy- or ribonucleotides in the other strand was examined.

MATERIALS AND METHODS

Synthesis of 2'-acetamido-2'-deoxynucleosides

UV spectra were recorded with a Philips PU 8700 UV-VIS spectrophotometer. The ¹H NMR and ¹³C NMR spectra were determined with a JEOL FX 90Q spectrometer with tetramethylsilane as internal standard for the ¹H NMR spectra and (CD₃)₂SO (δ_C 39.6) and CDCl₃ (δ_C 76.9) for the ¹³C NMR spectra. A Varian Gemini 200 spectrometer was used for 2'-acetamido-2'-deoxyguanosine and conformational NMR analysis was performed on a Bruker WH 360 spectrometer. Liquid secondary ion (LSIMS) and chemical ionisation (CIMS) mass spectra were obtained using a Kratos Concept IH mass spectrometer. Elemental analyses were performed at the university of Konstanz, Germany.

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2'-acetamido-2'-deoxyuridine (1). Crude 2'-amino-2'-deoxyuridine (16.5 g), obtained after catalytic hydrogenation of 19.1 g (70.9 mmol) of 2'-azido-2'-deoxyuridine (5), was coevaporated twice with dry pyridine and dissolved in 200 ml of a mixture of acetic anhydride and dry pyridine (1:4). The solution was stirred at room temperature overnight, whereafter the reaction mixture was quenched with ice, evaporated and coevaporated with toluene. The residue was treated with saturated methanolic ammonia at room temperature for 24 h. Evaporation and crystallisation from EtOH afforded 9.4 g (33 mmol, 47% starting from 2'-azido-2'-deoxyuridine) of **1**.

UV (MeOH) $\lambda_{\max}$ = 262 nm (ϵ = 10100)

^1H NMR [(CD₃)₂SO, TMS] δ 1.84 (s, CH₃CO-), 3.59 (m, H-5'), 3.94 (m, H-4'), 4.07 (m, H-3'), 4.48 (br m, H-2', OH-5'), 5.14 (br, OH-3'), 5.67 (d, J = 7.9 Hz, H-5), 5.90 (d, J = 8.8 Hz, H-1'), 7.79 (d, NH-COCH₃), 7.89 (d, J = 7.9 Hz, H-6), 11.10 (br, H-3)

^{13}C NMR [(CD₃)₂SO] δ 23.0 (CH₃), 55.3 (C-2'), 62.1 (C-5'), 71.0 (C-3'), 86.6, 87.3 (C-1', C-4'), 102.9 (C-5), 141.7 (C-6), 151.1 (C-2), 164.3 (C-4), 171.7 (-COCH₃)

CIMS (iC₄H₁₀): m/z 286 [M + H]⁺, 174 (sugar), 113 (base + 2 H)

El. anal. calcd. for C₁₁H₁₅N₃O₆: C 46.32%, H 5.30%, N 14.73%, found: C 45.86%, H 5.41%, N 14.55%.

1-(2-acetamido-2-deoxy-5-O-dimethoxytrityl- β -D-ribofuranosyl)uracil (2). Dimethoxytritylation of 1.17 g (4.1 mmol) of **1** with 1.74 g (5.13 mmol) of dimethoxytrityl chloride in pyridine (4 h at room temperature) followed by purification by column chromatography yielded 1.20 g (2.04 mmol, 50%) of the title compound.

UV (MeOH) $\lambda_{\max}$ = 235 nm (ϵ = 21300)

^{13}C NMR (CDCl₃) δ 22.4 (CH₃), 55.0 (2 $\times$ OCH₃), 56.0 (C-2'), 63.6 (C-5'), 71.1 (C-3'), 85.3, 85.8 (C-1', C-4'), 87.0 (Ph₃C), 102.7 (C-5), 140.1 (C-6), 151.6 (C-2), 163.4 (C-4), 171.5 (-COCH₃) + aromatic signals.

Rf (CH₂Cl₂-MeOH-Et₃N 90:10:0.5): 0.46.

[1-(2-acetamido-2-deoxy-5-O-dimethoxytrityl- β -D-ribofuranosyl)uracil]-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (3). Phosphitylation of 305 mg (0.52 mmol) of **2**, performed as described by Augustyns *et al.* (6), afforded 305 mg (0.39 mmol, 75%) of the title compound.

LSIMS (nitrobenzylalcohol): m/z 788 [M + H]⁺

Rf (hexane-acetone-Et₃N 38:60:2): 0.33 and 0.40.

2'-acetamido-2'-deoxycytidine (6). After coevaporation (twice) with dry pyridine, 2.2 g (7.7 mmol) of **1** was dissolved in 100 ml of dry pyridine. After addition of 3.5 ml (30.8 mmol) of benzoylchloride the mixture was stirred overnight at room temperature. The reaction mixture was quenched with MeOH, evaporated and coevaporated with toluene. The residue was dissolved in CH₂Cl₂ and washed with a sodium bicarbonate solution (5%). The organic layer was dried, evaporated and purified by column chromatography (CH₂Cl₂-MeOH 98:2), which afforded 2.4 g (4.86 mmol, 63%) of **4**.

^{13}C NMR [(CD₃)₂SO] δ 22.3 (CH₃), 52.3 (C-2'), 64.4 (C-5'), 72.3 (C-3'), 80.4 (C-4'), 86.1 (C-1'), 102.7 (C-5), 140.5 (C-6), 150.7 (C-2), 162.8 (C-4), 165.0, 165.5 (2 $\times$ PhCO), 170.1 (-COCH₃) + aromatic signals.

Conversion of 2.4 g (4.86 mmol) of **4** to the cytidine analogue **5** was carried out by means of 1.0 ml (10.8 mmol) of phosphorus

oxychloride and 3.0 g (43 mmol) of 1,2,4-triazole in 90 ml of dry pyridine (1 h at room temperature) followed by treatment with concentrated ammonia for 20 min at room temperature (7). After purification by column chromatography (CH₂Cl₂-MeOH 95:5) **5** was treated for 24 h with saturated methanolic ammonia. Evaporation, purification by column chromatography (CH₂Cl₂-MeOH 80:20) and crystallisation from 1 N HCl in MeOH afforded 634 mg (2.0 mmol, 41% calcd. from **4**) of the title compound as the hydrochloride salt.

UV (MeOH) $\lambda_{\max}$ = 279 nm (ϵ = 8900)

^1H NMR of the free base [(CD₃)₂SO, TMS] δ 1.82 (s, CH₃CO-), 3.56 (m, H-5'), 3.88 (m, H-3'), 4.05 (m, H-4'), 4.46 (m, H-2'), 5.10, 5.55 (br, OH-3', OH-5'), 5.77 (d, J = 7.4 Hz, H-5), 5.99 (d, J = 8.3 Hz, H-1'), 7.30 (br, NH₂), 7.72 (s, NH-COCH₃), 7.78 (d, J = 7.5 Hz, H-6)

^{13}C NMR of the free base [(CD₃)₂SO] δ 22.6 (CH₃), 54.7 (C-2'), 61.6 (C-5'), 70.4 (C-3'), 86.2 (C-1', C-4'), 94.5 (C-5), 141.8 (C-6), 155.4 (C-2), 165.2 (C-4), 169.6 (-COCH₃); LSIMS (glycerol): m/z 285 [M + H]⁺, 174 (sugar)

El. anal. calcd. for C₁₁H₁₆N₄O₅: C 41.19%, H 5.34%, N 17.47%, found: C 40.54%, H 5.41%, N 17.24%.

1-(2-acetamido-2-deoxy- β -D-ribofuranosyl)-N⁴-isobutyrylcytosine (7). Synthesis of **7** from 515 mg (1.6 mmol) of **6** was carried out following the general method described in reference 8. Yield: 436 mg (1.2 mmol, 77%).

UV (MeOH) $\lambda_{\max}$ = 250 nm (ϵ = 14800), $\lambda_{\max}$ = 302 nm (ϵ = 6900)

^{13}C NMR [(CD₃)₂SO] δ 19.3, 19.4 [(CH₃)₂CHCO-], 22.8 (CH₃CO), 35.5 [(CH₃)₂CHCO-], 56.2 (C-2'), 61.8 (C-5'), 70.7 (C-3'), 87.2, 87.7 (C-1', C-4'), 96.8 (C-5), 146.1 (C-6), 155.8 (C-2), 163.0 (C-4), 170.8 (-COCH₃), 178.5 [-COCH(CH₃)₂]

Rf (CH₂Cl₂-MeOH-Et₃N 90:10:0.5): 0.38.

[1-(2-acetamido-2-deoxy-5-O-dimethoxytrityl- β -D-ribofuranosyl)-N⁴-isobutyrylcytosine]-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (9). Dimethoxytritylation of 400 mg (1.13 mmol) of **7** with 479 mg (1.41 mmol) of dimethoxytrityl chloride in pyridine (24 h at room temperature), yielded 700 mg (1.06 mmol, 94%) of **8**.

UV (MeOH) $\lambda_{\max}$ = 237 nm (ϵ = 20400), $\lambda_{\max}$ = 304 nm (ϵ = 5300)

^{13}C NMR (CDCl₃) δ 18.8, 19.0 [(CH₃)₂CHCO-], 22.9 (CH₃CO), 36.4 [(CH₃)₂CHCO-], 55.1 (2 $\times$ OCH₃), 58.9 (C-2'), 63.8 (C-5'), 71.1 (C-3'), 86.0 (C-4'), 86.8 (Ph₃C), 87.0 (C-1'), 97.4 (C-5), 144.4 (C-6), 156.2 (C-2), 162.6 (C-4), 171.5 (-COCH₃), 177.1 [-COCH(CH₃)₂] + aromatic signals.

Rf (CH₂Cl₂-MeOH-Et₃N 90:10:0.5): 0.47.

Phosphitylation of 345 mg (0.52 mmol) of **8**, performed as described in reference 6, afforded 267 mg (0.31 mmol, 59%) of the title compound.

LSIMS (nitrobenzylalcohol): m/z 857 [M + H]⁺

Rf (hexane-acetone-Et₃N 43:55:2): 0.35 and 0.43.

[9-(2-acetamido-2-deoxy-5-O-dimethoxytrityl- β -D-ribofuranosyl)-N⁶-benzoyladenine]-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (13). 2'-Acetamido-2'-deoxyadenosine **10** (9) (370 mg, 1.2 mmol) was dimethoxytritylated with 489 mg (1.44 mmol) of dimethoxytrityl chloride in pyridine (16 h at room temperature). After purification by column chromatography,

crude **11** was N⁶-benzoylated as described in reference 6, yielding 465 mg (0.65 mmol, 54% calcd. on **10**) of **12**.

LSIMS (thioglycerol—doped with NaCl): m/z 737 [M + Na]⁺.
Rf (CH₂Cl₂–MeOH–Et₃N 90:10:0.5): 0.55.

Phosphitylation of 460 mg (0.64 mmol) of **12** as described in reference 6, afforded 395 mg (0.43 mmol, 67%) of the title compound.

LSIMS (nitrobenzylalcohol): m/z 915 [M + H]⁺
Rf (hexane–acetone–Et₃N 49:49:2): 0.16 and 0.23.

N²-decanoylguanine. Preparation of the title compound from 22.7 g (150 mmol) of guanine and 93 ml (450 mmol) of decanoyl chloride was performed as described for the synthesis of N²-palmitoylguanine (**10**). Yield: 28.34 g (93 mmol, 62%).

LSIMS (nitrobenzylalcohol-trifluoroacetic acid): m/z 306 (M+H).

2'-acetamido-2'-deoxyguanosine (14). A suspension of 2.85 g (10 mmol) of **1** and 5.50 g (18 mmol) of N²-decanoylguanine in 60 ml of dry acetonitrile and 21.3 ml (86 mmol) of bis(trimethylsilyl)acetamide was heated at reflux temperature for 15 min. After addition of 2.5 ml (13 mmol) trimethylsilyl triflate the mixture was heated at reflux temperature for 2 h, whereafter 200 ml of saturated methanolic ammonia was added. After 24 h at room temperature, the solution was evaporated and partitioned between 1 l of 0.1 M aqueous Et₃N and 0.5 l of CH₂Cl₂. The water layer was evaporated and purified by column chromatography [1) CH₂Cl₂–MeOH 80:20, 2) CH₂Cl₂–MeOH 60:40], which afforded 1.50 g (4.6 mmol, 46%) of crude **14**. Crude **14** (250 mg) was further purified by high performance liquid chromatography on a column (250 × 25 mm) of Rogel. The product was eluted with a linear gradient of 14–31% MeOH in 40 min. The fractions containing **14** were combined and lyophilised (yield: 144 mg).

UV (MeOH) $\lambda_{\max}$ = 255 nm (ϵ = 14600)

¹H NMR [(CD₃)₂SO, TMS] δ 1.80 (s, CH₃CO-), 3.54 (m, H-5B'), 3.58 (m, H-5A'), 3.92 (m, H-4'), 4.14 (m, H-3'), 4.92 (m, H-2'), 5.12 (br, OH-5'), 5.64 (br, OH-3'), 5.72 (d, J = 8.74 Hz, H-1'), 6.45 (br, NH₂), 7.85 (s, H-8), 7.97 (d, J = 8.62 Hz, NH-COCH₃), 10.52 (br, ¹NH)

¹³C NMR [(CD₃)₂SO] δ 22.7 (CH₃), 54.3 (C-2'), 61.8 (C-5'), 70.3 (C-3'), 84.4 (C-1'), 86.9 (C-4'), 116.9 (C-5), 135.8 (C-8), 151.7 (C-4), 153.9 (C-2), 156.9 (C-6), 169.8 (-COCH₃)

LSIMS (glycerol): m/z 325 [M + H]⁺, 174 (sugar), 152 (base + 2 H)

El. anal. calcd. for C₁₂H₁₆N₆O₅·1½H₂O: C 41.03%, H 5.45%, N 23.92%, found: C 41.40%, H 5.29%, N 23.45%.

[9-(2-acetamido-2-deoxy-5-O-dimethoxytrityl-β-D-ribofuranosyl)-N²-dimethylformamidineguanine]-3'-O-(2-cyanoethyl-N,N-diisopropylphosphoramidite) (**16**). 2.70 g (8.3 mmol) of **14** was coevaporated with dry MeOH and suspended in 35 ml of dry MeOH. After addition of 4.4 ml (33.3 mmol) of dimethylformamide dimethylacetal, the mixture was stirred at room temperature for 5 h. The solution was evaporated and the residue dried overnight in vacuo (**11**), after which it was dimethoxytritylated with 3.52 g (10.4 mmol) of dimethoxytrityl chloride in pyridine (5 h at room temperature). After purification by column chromatography, 3.79 g (5.6 mmol, 67%) of **15** was obtained.

¹³C NMR (CDCl₃) δ 23.5 (CH₃CO-), 35.5, 41.9 [-N(CH₃)₂], 55.6 (2 × OCH₃), 56.0 (C-2'), 64.6 (C-5'), 72.0 (C-3'), 85.7, 87.4 (C-1', C-4'), 87.0 (Ph₃C), 119.8 (C-5), 136.0 (C-8), 157.4, 158.5, 158.9 (C-2, C-6, N=CH-N), 172.1 (-COCH₃) + aromatic signals

LSIMS (thioglycerol—doped with NaCl): m/z 704 [M + Na]⁺.
Rf (CH₂Cl₂–MeOH–Et₃N 90:10:0.5): 0.50.

Phosphitylation of 800 mg (1.17 mmol) of **15** as described in reference 6, afforded 583 mg (0.66 mmol, 56%) of the title compound.

LSIMS (nitrobenzylalcohol): m/z 882 [M + H]⁺
Rf (hexane–acetone–Et₃N 10:88:2): 0.34.

Synthesis and purification of oligonucleotides

Oligodeoxynucleotides and oligoribonucleotides were prepared by automated chemical synthesis on a 1 μmol scale using the phosphoramidites from Applied Biosystems (deoxy) and Milligen Biosearch (ribo) following the standard protocols for DNA and RNA synthesis respectively. The modified phosphoramidites were used in a 0.12 M concentration with a coupling time of 10 min as for RNA. Oligodeoxynucleotides were deprotected and purified as described in reference 12. Oligoribonucleotides were base-deprotected by incubation in 2 M methanolic ammonia for 16 h at 55°C. After removal of the solvent the oligoribonucleotides were prepurified on a NAP-25® column (Sephadex G25—DNA grade, Pharmacia). The solution was lyophilised and the residue was treated with 1 ml of 1.0 M tetrabutylammonium fluoride/tetrahydrofuran (Janssen Chimica) for 24 h at room temperature. After addition of 1 ml buffer A (see below) the solution was desalted twice over a NAP-25® column. Purification was done on a Mono Q® HR 5/5 anion exchange column (Pharmacia) with the following gradient system [A: 0.03 M KH₂PO₄ pH 6.4, 20% CH₃CN; B: 0.67 M KH₂PO₄ pH 6.4, 0.6 M NaCl, 20% CH₃CN; gradient used depended on the oligo; flow rate 1 ml/min]. The purity of the isolated fractions was checked by means of the same chromatographic system (purity of at least 90%). The solution was then desalted on a NAP-25® column, followed by lyophilisation of the solvent.

Mass spectrometric analysis of the 7mer oligonucleotide

Mass spectrometric analysis of the oligonucleotide was performed following the method described by Pieles *et al.* (13). A 1 μl sample (80 pmol/μl in sterile water) was mixed with 10 μl of a 0.5 M solution of 2,4,6-trihydroxy acetophenone in ethanol and 5 μl of a 0.1 M solution of dibasic ammonium citrate in water. From this mixture, 1 μl was spotted on the metal target and allowed to dry by air.

The mass spectra were run on a VG Tofspec instrument equipped with a nitrogen laser (337 nm). The instrument was run in the linear as well as in the reflectron mode. Spectra were recorded in the negative ion mode using an acceleration voltage of 20 kV and, when used, a reflectron voltage of 22 kV. The spectra were obtained by overlaying 10–20 single laser pulses. The spectrometer was calibrated externally with the [M-H][−] mass peak of the matrix (2,4,6-trihydroxy acetophenone) and of dT₁₀ (Sigma) as reference.

Melting temperatures

Melting curves were measured and evaluated the same way as described in reference 6. Oligomer concentrations were determined as described in the same reference. An extinction coefficient of 10000 for uridine was determined at 80°C.

Table 1. Overview of the synthesized products

	R	R'	B	
1	H	H	uracil-1-yl	
2	DMTr	H	uracil-1-yl	
3	DMTr	X	uracil-1-yl	
4	benzoyl	benzoyl	uracil-1-yl	
5	benzoyl	benzoyl	cytosin-1-yl	
6	H	H	cytosin-1-yl	
7	H	H	<i>N</i> ⁴ -isobutyrylcytosin-1-yl	
8	DMTr	H	<i>N</i> ⁴ -isobutyrylcytosin-1-yl	
9	DMTr	X	<i>N</i> ⁴ -isobutyrylcytosin-1-yl	
10	H	H	adenin-9-yl	
11	DMTr	H	adenin-9-yl	
12	DMTr	H	<i>N</i> ⁶ -benzoyladenin-9-yl	
13	DMTr	X	<i>N</i> ⁶ -benzoyladenin-9-yl	
14	H	H	guanin-9-yl	
15	DMTr	H	<i>N</i> ² -dimethylformamidinoguanin-9-yl	
16	DMTr	X	<i>N</i> ² -dimethylformamidinoguanin-9-yl	

RESULTS AND DISCUSSION

Synthesis of 2'-acetamido-2'-deoxynucleosides

Uridine was converted into 2,2'-anhydrouridine by means of diphenylcarbonate and sodium bicarbonate in hexamethylphosphoramide (HMPA) at 150°C (5). Reaction with sodium azide and benzoic acid in HMPA at 150°C gave 2'-azido-2'-deoxyuridine (5). The 2'-azido group was reduced by catalytic hydrogenation to an amino group (5). After acetylation of this group the desired 2'-acetamido-2'-deoxyuridine (1, see Table 1) was obtained. Protection of the primary hydroxyl group with a dimethoxytrityl function and conversion to the phosphoramidite afforded the building block (3) for RNA synthesis.

The synthesis of the cytidine analogue was started from 2'-acetamido-2'-deoxyuridine (1), which was benzoylated at the 3'- and 5'-positions. The uracil base was converted to the cytosine base by means of phosphorus oxychloride and triazole in pyridine, followed by treatment with ammonia (7). Removal of the 3'- and 5'-benzoyl groups by NH₃/MeOH afforded 2'-acetamido-2'-deoxycytidine (6), which was isolated as the hydrochloride salt. Transient protection of the sugar hydroxyl groups as their trimethylsilyl derivatives allowed protection of the exocyclic amino group of the base with an isobutyryl group (7) (8). Compound 7 was further converted to the reactive building block 9 as was done for 3.

2'-Acetamido-2'-deoxyadenosine (10) was obtained as described before (9). The exocyclic amino function of the base was protected with a benzoyl group and the product was dimethoxytritylated and phosphitylated (13).

For the synthesis of the guanosine analogue a similar chemical pathway as for adenosine (9) was followed first. This method included conversion of guanosine [protected with the Markiewicz reagent (14)] to its arabino analogue by oxidation [Dess–Martin reagent (15)] and stereoselective reduction [sodium triacetoxyborohydride (16)], followed by introduction of the 2'-azido function by means of trifluoromethanesulfonylchloride and lithium azide (17). However, this method was not followed for large scale synthesis because of numerous side reactions and low yields after purification of the final compound. Therefore the guanosine building block was synthesized following the transglycosylation method described by Imazawa *et al.* (18). They report the transglycosylation of 2'-trifluoroacetamido-2'-deoxyuridine with *N*²-palmitoylguanine (10). Here the transglycosylation was performed with 2'-acetamido-2'-deoxyuridine (1) and *N*²-decanoylguanine. The latter was prepared by reaction of guanine and decanoyl chloride in pyridine (10). The transglycosylation was followed by cleavage of the decanoyl group by NH₃/MeOH. Protection of the exocyclic amino function with a dimethylformamidine group (19), dimethoxytritylation at the 5'-position and phosphitylation at the 3'-position again afforded the nucleoside building block 16.

Incorporation of 2'-acetamido-2'-deoxyuridine into oligonucleotides

The synthesis of oligonucleotides on solid support was performed using the phosphoramidite chemistry (20,21) on an Applied Biosystems DNA synthesizer (model 392). In order to examine the stability of the 2'-acetamido group during the synthesis on

Figure 1. Mass spectrum of the 7mer of 2'-acetamido-2'-deoxyuridine.

solid phase, a short oligonucleotide (7mer) was synthesized using the 2'-acetamido-2'-deoxyuridine building block **3**. The oligonucleotide was identified by means of mass spectrometry [Maldi-TOF analysis (13,22)]. Spectra obtained in linear and reflectron mode showed a main peak of 2502.5 (not shown) and 2506.1 (Figure 1) respectively. Taking into account the, without internal calibration, routinely achievable accuracy of approximately 0.2 and 0.02% respectively, both values corresponded to the $[M-H]^-$ mass value of the synthesized oligonucleotide (calculated mass of $[M-H]^- = 2505.6$ Da). Therefore we could conclude that the molecular structure of the synthesized product corresponds to the intact 7mer of 2'-acetamido-2'-deoxyuridine. This proved that the building block could be incorporated without side reactions or degradation. Using the same building block **3** the oligonucleotides as indicated in Table 2 were synthesized.

In order to stabilize oligonucleotides from 3'-exonucleolytic attack, we previously used an acyclic nucleoside analogue at the 3'-end (23). However, over the last year a simple propanediol moiety has been used extensively in our laboratory. This was also reported by the group of Agrawal (24,25). 1-*O*-Dimethoxytrityl-1,3-propanediol functionalized LCAA-CPG was synthesized analogously to the reported synthesis (24) and was used for the synthesis of oligonucleotides **18** and **24** to **28**. The propanediol linker 1-*O*-dimethoxytrityl-3-*O*-(2-cyanoethyl-N,N-diisopropylphosphoramidite)-1,3-propanediol was prepared as described by Ono *et al.* (26) and was attached at the 5'-end of the oligonucleotides **24** to **28**.

Melting profiles

Melting curves of the synthesized oligo(ribo)nucleotides **19** to **28** (Table 2) annealed to unmodified 3'-d(CA₁₃G)-5' (**17**) or

3'-d(T₂)-r(CA₁₃G)-d(T₂)-5' (**18**) were recorded in the buffer indicated in Table 2 at a concentration of 3 μ M for each oligo(ribo)nucleotide. The melting curves were evaluated according to a bimolecular All-or-None mechanism (27). Fitting of a theoretical melting curve to the data resulted in the melting temperature and thermodynamic parameters as indicated in Table 2.

The incorporation of one 2'-acetamido-2'-deoxyuridine at the end of the oligonucleotide (**20** and **25**) leads to a decrease of the melting temperature going from 2.7°C for DNA*-DNA, 4.4°C and 5.3°C for RNA*-DNA and DNA*-RNA to 7.3°C for the RNA*-RNA duplexes. This decreasing effect is more pronounced when more 2'-acetamido-2'-deoxy-nucleosides are incorporated and when the modification is incorporated in the middle of the oligonucleotide (**23** and **28**), although in the last case the order of influence is inverted: the DNA*-DNA duplex shows the highest decrease in melting temperature (13.8°C for DNA*-DNA, 9.8°C for DNA*-RNA and 9.2°C for RNA*-RNA). These results are in agreement with those of Eckstein *et al.* (28), who reported the destabilising effect of the incorporation of 2'-amino-2'-deoxycytidines.

Conformational NMR analysis of one of the 2'-acetamido-2'-deoxynucleosides was carried out in order to determine the conformation of the carbohydrate moiety of the modified nucleoside. Additional coupling constants for 2'-acetamido-2'-deoxyguanosine (**14**) were obtained during this more profound analysis using a Bruker WH 360 apparatus operating at 360.03 MHz for protons: J (H-5A', OH) $\approx J$ (H-5B', OH) $\approx J$ (H-4', H-5A') $\approx J$ (H-4', H-5B') ≈ 5.2 Hz, J (H-5A', H-5B') ≈ -14 Hz, J (H-1', H-2') 8.74 Hz, J (H-2', H-3') 5.47 Hz, J (H-2', NH) 8.62 Hz.

Table 2. Melting temperatures and free energies for DNA*–DNA, RNA*–DNA, DNA*–RNA and RNA*–RNA duplexes of oligonucleotides containing 2'-acetamido-2'-deoxyuridine (U*)

	5→3'	T _m	17 3'-d(CA ₁₃ G)-5'			18 3'-d(T ₂)-r(CA ₁₃ G)-d(T ₂)-5'			
			–ΔG ^{25°C}	–ΔH	–ΔS	T _m	–ΔG ^{25°C}	–ΔH	–ΔS
19	d(GT ₁₃ C)	41.2	59.4	504	1491	40.2	55.8	460	1355
20	d(GT ₁₂ U* ₁ C)	38.5	53.5	463	1374	34.9	46.2	396	1172
21	d(GT ₁₁ U* ₂ C)	34.5	46.8	431	1288	31.1	40.4	347	1028
22	d(GT ₁₀ U* ₃ C)	30.1	40.1	397	1196	27.4	35.9	311	923
23	d(GT ₆ U* ₄ T ₆ C)	27.4	36.9	440	1352	30.4	40.3	383	1148
24	r(GU ₁₃ C)	13.0	10.9	540	1775	37.2	50.8	442	1311
25	r(GU ₁₂ U* ₁ C)	8.6	1.8	544	1820	29.9	39.0	370	1110
26	r(GU ₁₁ U* ₂ C)	5.2	7.0	372	1223	25.8	34.2	328	987
27	r(GU ₁₀ U* ₃ C)	< 5	n.d.	n.d.	n.d.	22.0	30.4	296	891
28	r(GU ₆ U* ₄ U ₆ C)	< 5	n.d.	n.d.	n.d.	28.0	36.9	407	1240

T_m = melting temperature in °C determined under the following conditions: 0.1 M NaCl, 0.02 M potassium phosphate pH 7.5, 0.1 mM EDTA, concentration = 3 μM for each oligonucleotide; ΔH in kJ/mol; ΔS in J/mol.K; ΔG^{25°C} in kJ/mol. n.d. = not determined.

Because of overlap of the patterns of H-5A' and H-5B', their coupling constants could only be assigned approximately. J (H-3', H-4') was not resolved but, according to the shape of the pattern, is very small.

The values for J (H-1', H-2'), J (H-2', H-3') and J (H-3', H-4') are very comparable to those previously obtained by S. Van Calenbergh *et al.* (9) for similar compounds. Following their arguments we can conclude that the ribose ring containing the 2'-acetamido group adopts the 2'-endo conformation. This conformation is typical for the B-helix of DNA and can thus explain the destabilisation of the A-helix of RNA, where the nucleotides are predominantly in the 3'-endo form (29). An explanation for the distortion (and subsequently destabilisation) of as well the DNA helix as the RNA helix, however, could be found in terms of steric hindrance of the 2'-acetamido group. The amido functionality has a planar geometry. The π -resonance system explains the absence of free rotation around the amido bond. The presence of such a bulky conformationally constrained substitution in the 2'-position may interfere sterically with normal duplex formation.

Because of the negative results obtained with the 2'-acetamido-2'-deoxyuridines we did not incorporate the other building blocks in order to measure their hybridization properties. Nevertheless all the building blocks were later successfully incorporated in the catalytic core of hammerhead ribozymes, where these modified nucleotides do not need to hybridize. This work will be reported elsewhere.

CONCLUSION

2'-Acetamido-2'-deoxyribonucleotides were synthesized in order to explore the feasibility of a 2'-amido bond as a way to attach reporter groups to the carbohydrate moiety. The 2'-acetamido-2'-deoxyuridine building block could be incorporated into oligonucleotides without side reactions or degradation. However, a considerable decrease in melting temperature is observed for oligodeoxynucleotides and oligoribonucleotides containing 2'-acetamido-2'-deoxyuridine, at the end as well as in

the middle of the strand. This means that the 2'-acetamido functionality can not be considered as a good linkage group for attaching reporter groups as well at the end as in the middle of oligodeoxynucleotides and oligoribonucleotides. Steric hindrance of the 2'-acetamido group is, most probably, the reason for the destabilisation of the helix.

ACKNOWLEDGEMENTS

This work was supported by a grant of the Flemish Institute for promotion of the scientific-technological research in industry (IWT) to Dr C. Hendrix and by a Concerted Research Action project to Dr J. Van Beeumen. Dr A. Van Aerschot is a research associate of the Belgian National Fund of Scientific Research. We are indebted to S. Van Calenbergh for his generous gift of 2'-acetamido-2'-deoxyadenosine. We thank G. Schepers for technical assistance.

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