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Synthesis of 2′-hydrazine oligonucleotides and their efficient conjugation with aldehydes and 1,3-diketones

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Abstract—Oligodeoxyribonucleotides that contain a novel nucleoside, 2′-*O*-(2-hydrazinoethyl)uridine, were synthesised by $NaBH_3CN$ reduction of hydrazones formed from 2′-*O*-(2-oxoethyl)oligonucleotides with $FmocNHNH_2$, followed by concd aq NH_3 deprotection. The 2′-hydrazine oligonucleotides obtained were then used to synthesise a number of conjugates with aldehydes via hydrazone formation and with1,3-diketones via pyrazole formation. The method was shown to be applicable for the preparation of oligonucleotide–peptide conjugates.

Since its extensive optimisation in the 1980s, automated oligonucleotide synthesis has now become almost routine. However, chemical modification of nucleic acids is still a challenge for the organic chemists. Rapid development of biotechnology, genomics and nucleic acid-based diagnostics over the recent years is largely a result of the widespread application of various chemically modified oligonucleotides. A number of papers describe the synthesis of reactive oligonucleotides, which possess nucleoside residues equipped with functionalised side-chains that could be used for conjugation with other molecules, notably peptides, to increase the uptake of the oligonucleotides by cells for in vitro and in vivo studies. Peptides seem to be particularly versatile 'carriers' as they can both improve transmembrane delivery and affect intracellular localisation.[1] Many methods for the preparation of oligonucleotide–peptide conjugates have been reviewed recently.[2–4] Amongst them, conjugation via addition–elimination reactions of the carbonyl group offers excellent yields and extraordinary regioselectivity, which are invaluable for the synthesis of oligonucleotide bioconjugates.[5–7]

For the modification of oligonucleotides, efforts have been focused mostly on their aldehyde and aminooxy derivatives and development of the oligonucleotide modification thereof. Very few examples of hydrazine-[8] and hydrazide-linked[9,10,11] oligonucleotides are found in the literature. Hydrazides appear to be less attractive than hydrazines since their hydrazones are more hydrolytically labile than those of hydrazines, and at least some hydrazides are unstable under typical conditions of oligonucleotide deprotection, for example, concd aq ammonia.[11]

Previously, we have shown that oligonucleotides containing 2′-*O*-(2-oxoethyl)uridine[12] and 2′-*O*-(2-oxoethyl)cytidine[13] are useful reagents for conjugation with structurally diverse hydrazines, hydrazides, *O*-alkylhydroxylamines and 1,2-aminothiols[14] both in solution[12,14] or on solid phase.[13] Here we report the conversion of the 2′-aldehyde group into a 2′-hydrazine and an efficient conjugation of the 2′-hydrazine oligonucleotides with aldehydes, including *N*-glyoxylylpeptides or 1,3-diketones. We demonstrate that pyrazoles formed in the case of conjugation with 1,3-diketones are stable over a wide pH range and remain unaffected by the standard oligonucleotide deprotection conditions.

The synthesis of oligonucleotides containing 2′-*O*-(2,3-dihydroxypropyl)uridine **1** was performed as described earlier.[12,14] The dihydroxypropyl group was then

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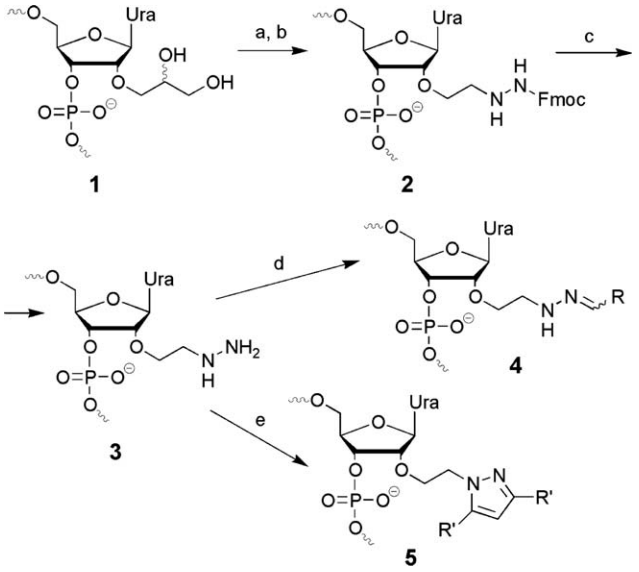
oxidised to a 2′-aldehyde group by sodium periodate treatment (Scheme 1). Subsequent addition of 9-fluorenylmethyl carbazate[15] to the 2′-aldehyde oligonucleotides was carried out in the presence of $NaBH_3CN$.[12] The resultant Fmoc-protected oligonucleotides **2** were purified easily by reverse-phase (RP) HPLC due to the strong hydrophobic properties of the Fmoc group (Fig. 1). Deprotection of **2** using concentrated aqueous ammonia led to 2′-hydrazine oligonucleotide **3**, which was then treated with aliphatic or aromatic aldehydes or 1,3-diketones[16] to give oligonucleotide derivatives of types **4** and **5**, respectively.

For example, 2′-hydrazine oligonucleotides **III** and **IV** (Table 1) were reacted with a range of aldehydes or 1,3-diketones to give conjugates **IIIa–i** and **IVa–i**,



Scheme 1. Synthesis of the 2′-hydrazine oligonucleotides and their conjugates with aldehydes and 1,3-diketones. Reagents and conditions: (a) 0.1 M $NaIO_4$, 0.2 M NaOAc, pH 5.0, 1 h; (b) $FmocNHNH_2$, $NaBH_3CN$, 0.2 M NaOAc, pH 5.0, 1 h; (c) concd aq NH_3, 25 °C, 3 h; (d) RCHO, 0.2 M NaOAc, pH 5.0, 1 h; (e) $(R'CO)_2CH_2$, pH 6.0, 30 min.

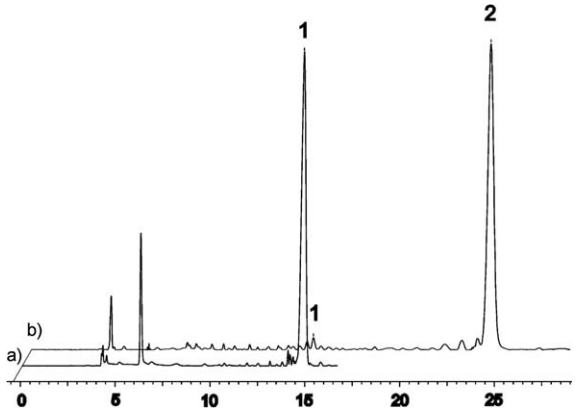


Figure 1. RP-HPLC of the reaction mixtures. Key: (a) crude oligonucleotide diol **I**; (b) conjugation of the 2′-aldehyde from **I** with $FmocNHNH_2$: peak 1—2′-aldehyde **I**, peak 2—Fmoc–oligonucleotide.

Table 1. Sequences and MALDI-TOF MS analysis of the 2′-modified oligonucleotides

	Oligonucleotide sequence, 5′-to-3′	MALDI-TOF MS calcd/found $[M+H]^+$
I	C**U**^{d}CCCAGGCTCA	3658.4/3660.1
II	C**U**^{d}CCCAGGC**U**^{d}CA	3734.5/3735.9
III	C**U**^{h}CCCAGGCTCA	3642.4/3645.2
IV	C**U**^{h}CCCAGGC**U**^{h}CA	3701.5/3702.1

U^d—2′-*O*-(2,3-dihydroxypropyl)uridine.
U^h—2′-*O*-(2-hydrazinoethyl)uridine.

respectively (Table 2). The conjugations proceeded smoothly, albeit an attempt to carry out a reaction with 2-(2-nitrophenyl)malondialdehyde led to the formation of a complex mixture (data not shown). The latter could be ascribed to the concomitant reaction of the 1,3-dialdehyde with nucleobases, for example, guanine, as has been shown for unmodified oligonucleotides.[17] Recently, Otteneder et al.[18] demonstrated that hydrazines react preferentially with dialdehydes and could disrupt the malondialdehyde–guanine adduct. However, additional treatment of the reaction mixture with 5 equiv of 2-hydrazinoethanol resulted in an increase in the number of products without any selective recovery. Surpisingly, no side reactions of the diketones with unmodified mixed-sequence oligonucleotides were observed under the reaction conditions except that 1,1,1,5,5,5-hexafluoro-2,4-pentanedione yielded less than 5% of side-products after 90 min of reaction (data not shown).

In the case of some of the 1,3-diketones two products were formed (Fig. 2). MALDI-TOF mass spectrometry showed the same molecular masses for both products. Such a difference in the retention time may be a result of the incomplete conversion of the intermediate mono-hydrazone **6** or hydroxypyrazoline **7** into pyrazole **8** during conjugation (Scheme 2). The water molecule could then be eliminated during MALDI-MS analysis. Such an explanation is supported by the ratio of the RP-HPLC signal areas and the gradual disappearance of peak **2** (Fig. 2). In the case of the 2,4-pentanedione reaction, only a single peak due to the pyrazole conjugate (**IIIf**) was observed (Table 2). Incorporation of one trifluoromethyl electron-withdrawing group led to the formation of two products (**IIIg**). The faster moving product seems to be the mono-hydrazone or hydroxypyrazoline (Fig. 2, peak **2**). The presence of two trifluoromethyl groups seems to decrease the rate of pyrazole formation, so the main part of the product is the pyrazoline or mono-hydrazone conjugate (**IIIh**, Table 2). Prolonged heating of the isolated faster-moving products in aqueous solution (55 °C overnight) led to the complete conversion into the appropriate slower-moving peak that is believed to be the pyrazole conjugate **5** (Scheme 1). Reaction with 4,6-dioxoheptanoic acid produced a broadened peak on RP-HPLC (data not shown), which could be explained by the formation of a mixture of isomeric 3,5-disubstituted pyrazoles.

We have also studied the hydrolytic stability of the conjugates[19] (Table 3). All the conjugates were sufficiently

Table 2. Yields and MALDI-TOF MS of the conjugates of the 2′-hydrazine oligonucleotides

	Conjugated molecule	Oligonucleotide	
		III	**IV**
a	Pentanal	82[a] (3694.5/3717.2)[b]	73[a] (3822.6/3826.2)[b]
b	4-Methoxybenzaldehyde	93[a] (3744.5/3769.1)[b]	78[a] (3922.7/3924.0)[b]
c	1-Pyrenecarboxaldehyde	89[a] (3842.7/3745.2)[b]	81[a] (4117.0/4117.1)[b]
d	OCHCO-LLK amide	85[a] (4035.9/4075.2)[b]	69[a] (4586.5/4626.3)[b]
e	OCHCO-LLGKV amide	82[a] (4206.1/4247.0)[b]	65[a] (4845.9/4887.3)[b]
f	$(MeCO)_2CH_2$	70[a] (3690.5/3693.5)[b]	60[a] (3814.6/3856.1)[b]
g	$MeCOCH_2COCF_3$	19 + 57[c] (3744.5/3740.4)[b]	67[d] (3922.6/3960.1)[b]
h	$(CF_3CO)_2CH_2$	67 + 5[c] (3798.4/3800.6)[b]	78[d] (4030.5/4033.1)[b]
i	$MeCOCH_2CO(CH_2)_2CO_2H$	69[d] (3741.5/3782.1)[b]	54[d] (3930.7/3932.4)[b]

[a] Yield of the conjugate is based on RP-HPLC signal areas.
[b] MALDI-TOF MS: calcd/found $[M+H]^+$.
[c] Yields of faster- and slower-moving conjugates are given.
[d] After overnight heating at 55 °C.

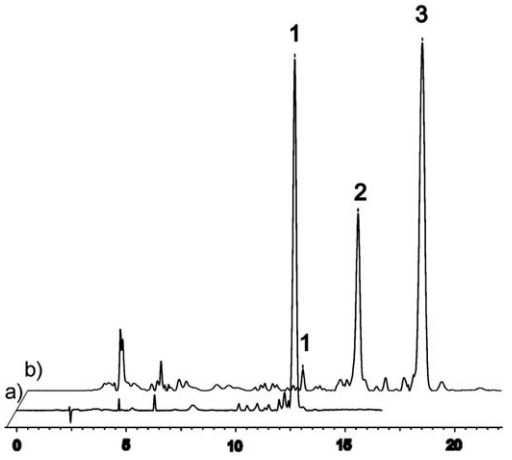
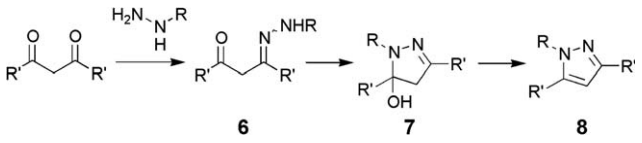


Figure 2. RP-HPLC profiles of reaction mixtures. Key: (a) purified 2′-hydrazine oligonucleotide **III** (peak 1); (b) conjugation of 2′-hydrazine oligonucleotide **III** with 1,1,1-trifluoro-2,4-pentanedione: peak 1—starting 2′-hydrazine **III**, peak 2—intermediate conjugate (mono-hydrazone or pyrazoline), peak 3—final conjugate **IIIg** (pyrazole).



Scheme 2. Reaction of a mono-substituted hydrazine with a 1,3-diketone.

Table 3. Stability of hydrazones **IIIa**, **IIIb** and pyrazole conjugates **IIIf**

pH	Hydrolysis (%)					
	3 h			24 h		
	IIIa	**IIIb**	**IIIf**	**IIIa**	**IIIb**	**IIIf**
3.0	<5	<1	<1	8	<5	<1
4.0	<1	<1	<1	<1	<1	<1
8.0	<5	<1	<1	11	<5	<1
9.0	15	<5	<1	35	9	<1
10.0	19	<5	<1	50	14	<1

stable at slightly acidic and neutral pH, but partial hydrolysis of hydrazones in basic conditions was observed. The pyrazole conjugates were unchanged at all the pH values studied.

In conclusion, we have developed an efficient method for the synthesis of 2′-*O*-(2-hydrazinoethyl)oligonucleotides from the corresponding 2′-aldehyde derivatives. The resulting 2′-hydrazine oligonucleotides were shown to react with various aliphatic and aromatic aldehydes, including side-chain functionalised, for example, *N*-glyoxylylpeptides and fluorescent, for example, pyrene, or 1,3-diketones. The 1,3-diketone reaction proceeds via an isolable intermediate that could be a mono-hydrazone of the 1,3-diketone or a hydroxypyrazoline, and is converted fully into a stable product (pyrazole) after further reaction. We have affirmed the stability of the hydrazone conjugates at neutral and mildly acidic pH, whilst the pyrazole conjugates are stable over the pH range studied. The method described could be employed for the preparation of oligonucleotide–peptide conjugates that are useful compounds for biological applications. The pyrazole conjugates appear to be the most interesting because of the stability of the linkage. Studies with the latter compounds are underway and will be published in due course.

Acknowledgements

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References and notes

1. (a) Gait, M. J. *Cell Mol. Life Sci.* **2003**, *60*, 844–853; (b) Juliano, R. L. *Curr. Opin. Mol. Ther.* **2005**, *7*, 132–136.
2. Stetsenko, D. A.; Arzumanov, A. A.; Korshun, V. A.; Gait, M. J. *Mol. Biol. (Russ.)* **2000**, *34*, 998–1006.
3. Zubin, E. M.; Romanova, E. A.; Oretskaya, T. S. *Russ. Chem. Rev.* **2002**, *71*, 239–264.
4. Zatsepin, T. S.; Turner, J. J.; Oretskaya, T. S.; Gait, M. J. *Curr. Pharm. Des.* **2005**, *11*, 3639–3654.
5. Tam, J. P.; Xu, J.; Eom, K. D. *Biopolymers* **2001**, *60*, 194–205.
6. Peri, F.; Nicotra, F. *Chem. Commun.* **2004**, 623–627.

7. Zatsepin, T. S.; Stetsenko, D. A.; Gait, M. J.; Oretskaya, T. S. *Bioconjugate. Chem.* **2005**, *16*, 471–489.
8. Gao, K.; Orgel, L. E. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 14837–14842.
9. Ghosh, S. S.; Kao, M. P.; Kwoh, D. Y. *Anal. Biochem.* **1989**, *178*, 43–51.
10. Raddatz, S.; Mueller-Ibeler, J.; Kluge, J.; Burdinski, G.; Havens, J. R.; Onofrey, T. J.; Wang, D.; Schweitzer, M. *Nucleic Acids Res.* **2002**, *30*, 4793–4802.
11. Zubin, E.; Stetsenko, D.; Oretskaya, T. S.; Gait, M. J. *Nucleotides Nucleosides Nucleic Acids* **2003**, *22*, 1375–1378.
12. Kachalova, A. V.; Zatsepin, T. S.; Romanova, E. A.; Stetsenko, D. A.; Gait, M. J.; Oretskaya, T. S. *Nucleosides Nucleotides Nucleic Acids* **2000**, *19*, 1693–1707.
13. Zatsepin, T. S.; Stetsenko, D. A.; Gait, M. J.; Oretskaya, T. S. *Tetrahedron Lett.* **2005**, *46*, 3191–3195.
14. Zatsepin, T. S.; Stetsenko, D. A.; Arzumanov, A. A.; Romanova, E. A.; Gait, M. J.; Oretskaya, T. S. *Bioconjugate. Chem.* **2002**, *13*, 822–830.
15. Zhang, R. E.; Cao, Y. L.; Hearn, M. W. *Anal. Biochem.* **1991**, *195*, 160–167.
16. Oligodeoxynucleotides were assembled on an ABI 394A DNA Synthesiser by the 2-cyanoethyl phosphoramidite method following manufacturer recommendations. Deprotection and purification of the 1,2-diol-containing oligonucleotides were carried out using the conditions described.[12,14] MALDI-TOF mass spectra were recorded on a Voyager DE workstation (PE Biosystems) in a freshly prepared 1:1 (v/v) mixture of 2,6-dihydroxyacetophenone (40 mg/ml in MeOH), and aqueous diammonium hydrogen citrate (80 mg/ml) as a matrix. RP-HPLC: HPLC chromatograph (Tracor), DIAKS-130-CETYL column (4×250 mm); buffer A: 0.1 M ammonium acetate, pH 7; buffer B: 0.1 M ammonium acetate, pH 7, 40% MeCN (v/v); gradient of B from 0 to 100% in 60 min; flow rate 1 ml/min; temperature 45 °C. *Conversion of the dihydroxypropyl group of an oligonucleotide into the hydrazinoethyl group:* A dried oligonucleotide pellet (3.0 A_{260} units) was dissolved in 10 μl of 0.4 M sodium acetate buffer, pH 4.5. Then, 1 μl of 5 mM $NaIO_4$ solution was added and the reaction mixture was incubated at ambient temperature for 1 h. To the solution, 10 μl of 50 mM $FmocNHNH_2$ in DMSO was added and further incubation was carried out for 30 min, followed by treatment with 20 mM sodium cyanoborohydride (10 μl) for 1 h. The reaction was quenched by precipitation with 4 M sodium acetate solution (20 μl) and ethanol (1.5 ml) and then purified using RP-HPLC. Concd aq ammonia was added to the dried oligonucleotide pellet and after 1 h the solution was evaporated in vacuo. 2′-Hydrazino oligonucleotides were then analysed by RP-HPLC in the ion-pair mode. *Conjugation of 2′-O-(2-hydrazinoethyl)oligonucleotides with aldehydes or 1,3-diketones*: A 10 mM solution (10 μl) of either aldehyde in acetate buffer (pH 4.5) or 1,3-diketone in phosphate buffer (pH 6.0) was added to the dried pellet of the hydrazine oligonucleotide (0.5 A_{260} unit). The reaction mixtures were incubated for 30 min at ambient temperature, then precipitated by the addition of 2 M $LiClO_4$ solution (0.2 ml), followed by acetone (1.5 ml) and analysed using RP-HPLC ion-pair and MALDI-TOF mass spectroscopy.
17. Nair, V.; Turner, G. A.; Offerman, R. J. *J. Am. Chem. Soc.* **1984**, *106*, 3370–3371.
18. Otteneder, M.; Plastaras, J. P.; Marnett, L. J. *Chem. Res. Toxicol.* **2002**, *15*, 312–318.
19. *Study of the hydrolytic stability of the conjugates:* A conjugate (0.1 A_{260} unit) was dissolved in 100 mM acetate or phosphate buffer (10 μl) at the appropriate pH. After incubation for 3 or 24 h, the samples were analysed by RP-HPLC in the ion-pair mode.